



University of
Nottingham

UK | CHINA | MALAYSIA

Metabolism and Mechanism of Action of Cordycepin

Ammar Sabah Karim Khamis

Thesis submitted to the University of Nottingham
for the degree of **Doctor of Philosophy**

November 2025

Abstract

Cordycepin (3'-deoxyadenosine) is a nucleoside analogue with broad anti-inflammatory and antiproliferative effects whose therapeutic development has been limited by rapid deamination to 3'-deoxyinosine (3DI) and an incomplete understanding of how its active nucleotides couple cleavage and polyadenylation (CPA) to growth-factor and inflammatory signalling. This thesis investigates how cordycepin and its metabolites, particularly cordycepin triphosphate (CoTP), modulate mRNA 3'-end processing, poly(A)-tail homeostasis and PI3K/Akt/mTOR signalling, and explores the hypothesis that the effects on polyadenylation mediate the effects on signal transduction through one or more RNA targets of cordycepin (RToC).

Deamination of cordycepin to 3'-deoxyinosine (3DI) is known to occur rapidly and limit its potency. To explore the metabolism of cordycepin, siRNA knockdown of enzymes that could recycle 3DI to cordycepin were used. The data showed that, surprisingly, cordycepin activity in tissue culture is dependent on a previously unsuspected rescue pathway involving adenine phosphoribosyltransferase (APRT) and, in macrophages, the enzyme FAMIN/LACC1. These enzymes appear to channel adenine-linked 3'-deoxyribose metabolism, enabling generation of CoTP and repression of LPS-induced inflammatory transcripts. Data for 3DI suggested some differences between 3DI and cordycepin dependence on rescue pathways, but were difficult to interpret because contamination of the 3DI preparations with cordycepin was detected.

Second, a haploid piggyBac survival screen in eHAP cells identified three broad classes of genetic determinants of cordycepin resistance. Loss-of-function insertions in ADK were the most consistent and dose-dependent hits, confirming that phosphorylation to cordycepin mono-, di- and triphosphates is essential for cytotoxicity. A second class comprised genes involved in RNA 3'-end formation, deadenylation and RNA maturation

(including CNOT6, PARN and Integrator components), indicating that the state of mRNA 3'-end processing modulates sensitivity to CPA inhibition. A third class encompassed genes in AMPK-mTOR signalling, autophagy, mitophagy, ER-mitochondrial coupling and ubiquitin-dependent proteostasis, highlighting integrated stress-response and quality-control pathways that shape survival under cordycepin. Within this screen, the long non-coding RNA locus LINC03121 (Lnc-PLA2G4A-4) showed pronounced enrichment in one platform/dose combination; in light of independent evidence that LINC03121 promotes p-EGFR/p-AKT signalling via a miR-23b-3p/versican axis, this makes it a biologically credible candidate RTcC.

Third, comparative transcriptomics in LPS-stimulated RAW264.7 macrophages demonstrated that natural fermentation-derived and synthetic cordycepin produced highly similar gene-expression and pathway signatures. Both preparations repressed a broad panel of inflammatory transcripts and down-regulated pathways linked to NF- κ B, interferon signalling and PI3K/Akt/mTOR-dependent translation. The ProTide NUC-7738 reproduced these core features when used at exposures that generate comparable intracellular 3'-deoxyadenosine nucleotide levels, whereas 3DI showed a weaker but directionally concordant profile. These findings argue that the shared active species is intracellular CoTP rather than preparation-specific contaminants.

Fourth, Oxford Nanopore long-read sequencing revealed that cordycepin and NUC-7738 induced rapid, genome-wide poly(A)-tail shortening at 120 minutes (median shifts of ≈ 25 nt) with thousands of transcripts showing significant tail reduction and concomitant decreases in abundance, particularly among inflammatory and growth-factor-linked mRNAs. A smaller subset of transcripts exhibited tail lengthening and relative sparing of abundance, often enriched for mitochondrial and metabolic functions, suggesting selective stabilisation of messages that support bioenergetic adaptation under stress. Early-timepoint (15-minute) analyses showed that cordycepin already triggers coordinated shifts in poly(A)-tail length and expression in transcripts associated with translation control, signal transduction and growth-factor pathways, before wholesale

transcriptional re-programming is established.

Taken together, these strands support a model in which cordycepin acts principally as an intracellular nucleoside analogue that must be transported into cells, phosphorylated to CoTP, and then perturbs CPA and poly(A)-tail homeostasis to selectively restrain inflammatory and PI3K/Akt/mTOR outputs. Integrating these results with the literature on protein synthesis inhibition and Akt activation, the thesis advances the hypothesis that this signalling restraint is mediated by one or more non-coding RNAs whose polyadenylation-dependent function is cordycepin-sensitive—an RNA target of cordycepin (RToC). The convergence of strong loss-of-function enrichment for LINC03121 in the haploid screen, its established role as a positive regulator of Akt signalling, and the global evidence for cordycepin-induced CPA disruption leads this work to nominate LINC03121 as a leading candidate RToC, framed as a testable mechanistic hypothesis for future studies.

Acknowledgements

Foremost, I would like to express my sincere gratitude to my primary supervisor, Dr. Cornelia de Moor, for her continuous support, patience, and unwavering guidance throughout my PhD journey. Her immense knowledge, enthusiasm, and dedication to her students have been truly inspiring. I could not have written this thesis without her invaluable feedback and mentorship; she has shaped not only this work but also my growth as a researcher. I am profoundly grateful for her support during the most challenging times and consider myself fortunate to have had such an exceptional supervisor.

I would also like to thank my co-supervisor, Dr. Pavel Gershkovich, for his support and guidance throughout this project.

This research was made possible through the generous funding provided by the Higher Committee for Education Development in Iraq (HCED), to whom I extend my deepest appreciation.

I am greatly indebted to Dr. Keith Spriggs for his invaluable assistance and guidance in the execution of the haploid genetic screening analysis. The progression of this work would not have been feasible without his esteemed mentorship and insightful feedback. Special thanks are also due to Dr. Alexander Kondrashov, Dr. Emilie Daniel, and Davor Kresnik, who carried out the eHAP cell library preparation, puromycin selection, and Splinkerette PCR for amplification of insertion sites. I am also grateful to Dr. Alexander Kondrashov for his excellent advice in the lab throughout my PhD.

I am hugely appreciative of Steven Lawrence, who gave me excellent advice in the lab and trained me in cell culture techniques. My thanks also go to Dr. Rakesh Jha for his assistance with LC-MS, and to Dr. Abdul Hafeez, a good friend, who helped me in the lab and offered valuable advice on LC-MS. I would also like to acknowledge Dr. Angela Downie for her helpful advice and Sabesan Anandavijayan for his assistance with RAW cell culture.

I would like to thank Silvia Smith, Yixin Hu, Kelechi Okorie, and Gabriel Pietruczuk for allowing me to supervise them in the lab; it was a pleasure working with each of them.

Profound gratitude goes to Dr. Ian Withers, our wonderful technical specialist, for his support with HPC Facilities and to Dr. Hilary Collins for her dedication to organizing the lab.

I wish to extend my sincere thanks to Dr. Craig Doig and Dr. Awais Younis from Nottingham Trent University for their assistance with the Seahorse assay experiments.

Finally, but by no means least, I owe an immeasurable debt of gratitude to my family. To my lovely wife, Sarah, thank you for your tremendous and unbelievable patience and support throughout this PhD journey—I could not have accomplished this without you by my side. To my beloved children, Ridha and Ahmed, you have been my greatest source of joy and motivation. To my wonderful Mum and Dad, thank you for your incredible support and for believing in me always. To my brother and sister, many thanks for your encouragement throughout this experience.

Contents

Abstract	i
Acknowledgements	iv
1 Introduction	1
1.1 Cordycepin administration, absorption, distribution and excretion . . .	5
1.1.1 Routes of administration and solubility	5
1.1.2 ADA-mediated deamination and oral bioavailability	5
1.1.3 Intestinal absorption and nucleoside transport	6
1.1.4 Systemic distribution and first-pass metabolism	7
1.1.5 3'-Deoxyinosine as an orally bioavailable pro-drug	8
1.1.6 Cellular uptake and ADK-dependent activation	8
1.1.7 Excretion and downstream purine catabolism	9
1.1.8 Strategies to overcome metabolic bottlenecks: ADA inhibition and ProTides	10
1.2 Poly(A) tail synthesis and function in mRNA metabolism	12
1.2.1 Canonical cleavage and nuclear polyadenylation	12
1.2.2 Cytoplasmic poly(A) tails and poly(A)-binding proteins in trans- lation	14
1.2.3 Cytoplasmic polyadenylation as a translational switch.	15
1.2.4 Deadenylation pathways and mRNA turnover	15
1.2.5 Roles of poly(A) tails in stability, export and translation	16
1.3 Cordycepin triphosphate as an inhibitor of cleavage and polyadenylation	18
1.3.1 CoTP as a chain-terminating substrate for PAP α/γ	20

1.3.2	Trapping of CPSF4 and WDR33 on CoTP-terminated 3'-ends	20
1.3.3	Integration with CPSF4/WDR33 knockdown phenotypes	21
1.3.4	Consequences for PABPN1 loading, export competence and dead- enylation	22
1.3.5	Effects of cordycepin on alternative polyadenylation	23
1.3.6	Summary	24
1.4	Linking cleavage and polyadenylation to growth-factor signalling and inflammatory outputs	25
1.4.1	CPA factor knockdown recapitulates growth-factor signalling defects	25
1.4.2	Cordycepin as a pharmacological mimic of CPA-factor limitation	26
1.4.3	Inflammatory transcripts as CPA-sensitive outputs	27
1.4.4	Summary and working hypothesis	28
1.5	Growth-factor signalling and its modulation by cordycepin	31
1.5.1	Canonical growth-factor signalling: PI3K/Akt/mTOR and RAF/MEK/ERK	31
1.5.2	Cordycepin-mediated repression of growth-factor signalling	32
1.5.3	Protein synthesis inhibition and growth-factor signalling	35
1.6	Alternative proposed targets for cordycepin: adenosine receptors and other signalling nodes	38
1.6.1	Evidence for adenosine receptor-mediated actions of cordycepin	38
1.6.2	Transporter and kinase dependence in somatic cell models	39
1.6.3	Metabolic gating and the 3'-deoxyinosine rescue pathway	40
1.6.4	ProTide NUC-7738 as an adenosine receptor-independent tool	41
1.6.5	Integration with CPA-focused signalling models	42
1.7	In vivo models and integration.	43
1.7.1	Animal In Vivo Models	43
1.7.2	Human studies and early clinical trials	45
1.8	Biological activities of cordycepin	49
1.8.1	Anti-proliferative effects: cell death, survival and division	49
1.8.2	Effects of cordycepin on cell migration	50
1.8.3	Effects of cordycepin on the inflammatory response	52

1.9	Thesis aims and summary of findings	55
1.9.1	Thesis aims	55
1.9.2	Summary of findings	57
2	Methods	60
2.1	Material and Methods Overview	60
2.2	Cell Culture and Maintenance	63
2.2.1	Cell Lines and Culture Conditions	63
2.3	Treatment Conditions	65
2.4	Small Interfering RNA (siRNA) Transfection	66
2.4.1	siRNA Knockdown in RAW264.7 cells	66
2.4.2	siRNA Knockdown in A549 cells	68
2.5	RNA Extraction, cDNA Synthesis, and Quantitative Real-Time PCR (qPCR)	69
2.5.1	Designing qPCR primers for mature RNA (mRNA)	70
2.6	Liquid Chromatography – Mass Spectrometry (LC-MS)	73
2.6.1	Samples Preperation	73
2.6.2	Chromatography Conditions	74
2.6.3	Mass Spectrometry	77
2.6.4	Configurations and Procedures for Conducting an LC-MS Analysis – ZIC-pHILIC and Exactive MS	78
2.7	PrestoBlue™ HS Cell Viability Assay.	80
2.8	ONT long-read RNA-Seq and Bioinformatics Analysis	80
2.8.1	120-minute data methodology (Run DS1457) by Deep Seq staff, University of Nottingham	81
2.8.2	15-minute data methodology (Run DS1402) by Deep Seq staff, University of Nottingham	82
2.8.3	Quality Control of Fastq Files	84
2.8.4	Haploid Genetic Screening	85
2.8.5	Acquiring read counts and expression data for RNA sequencing	90
2.8.6	Converting Gene ID to Gene Name	91

2.8.7	Differential Gene Expression Analysis for Long-Read RNA Sequencing Data	91
2.8.8	Poly(A) Tail Length Analysis	91
3	Decoding 3'-Deoxyinosine Rescue Pathways: siRNA Knockdowns	
	Identify 3'-Deoxyribose-Salvage Requirements for Cordycepin Activity	97
3.1	Introduction	97
3.2	Results	102
3.2.1	Baseline anti-inflammatory activity of 3DI and cordycepin . . .	102
3.2.2	Testing the NT5C2/ADSS2 Phosphorylation-Amination Pathway	106
3.2.3	Evaluating the PNP-Mediated Salvage Pathway	110
3.2.4	Adenine Salvage Pathways Show Promise	113
3.2.5	APRT is Required for Cordycepin and 3DI Activity	117
3.2.6	FAMIN/LACC1 Shows Cell-Type-Specific Requirements	120
3.2.7	Additional Metabolic Nodes Show Variable Effects	123
3.2.8	Carbosynth Cordycepin Purity Analysis	126
3.2.9	Synthetic Cordycepin Purity Analysis	130
3.2.10	3'-Deoxyinosine Stock Contamination	133
3.3	Discussion	135
4	A Haploid Genetic Screen Identifies Three Classes of Resistance Mechanisms to Cordycepin	140
4.1	Introduction	140
4.2	Results	142
4.2.1	Loss-of-function logic of the haploid resistance screen	142
4.2.2	Screen design, mutagenesis and selection strategy	144
4.2.3	Mapping of transposon insertion sites	149
4.2.4	Computational analysis	151
4.3	Library Performance and Coverage	152
4.3.1	Insertion counts at different seeding densities	152
4.3.2	Chromosome-group distribution	152

4.4	Gene-Level Enrichment under Cordycepin	155
4.4.1	150 μ M cordycepin	156
4.4.2	200 μ M cordycepin	158
4.5	Cross-Condition Recurrence	160
4.6	Pathway-Level Analyses (IPA; LoF Interpretation)	163
4.7	Discussion	176
5	Transcriptomic analysis indicates cordycepin is the active ingredient of natural cordycepin preparations	185
5.1	Introduction	185
5.2	Platform overview (ONT long-read profiling)	189
5.3	Global transcriptional landscape across conditions	192
5.4	Source equivalence: natural vs synthetic cordycepin (20 μ M)	194
5.5	Comparator evaluation: NUC7738 vs. 3DI (200 μ M)	210
5.6	Discussion	224
6	Poly(A) tail profiling following cordycepin and NUC-7738 treatment of LPS-activated macrophages reveals genome-wide poly(A) tail shortening.	234
6.1	Introduction	234
6.2	LPS-Induced Poly(A) Remodeling (control behaviour)	237
6.3	Effects of compounds on poly(A) tail size	240
6.4	Relation between Gene Expression and Poly(A) Tail Size	247
6.5	Pathway analysis of poly(A) tail changes	252
6.5.1	Cordycepin effects on mitochondrial respiration (Seahorse assay)	266
6.6	Discussion	269
7	Cordycepin induces early poly(A)-tail changes in mRNAs related to growth-factor and innate immune signaling	285
7.1	Introduction	285
7.2	Results	288
7.2.1	Early RNA abundance changes at 15 minutes are modest	288

7.2.2	Canonical pathways from DGE: mTOR/EIF4/EIF2 and PI3K/ERK nodes emerge	290
7.2.3	Global poly(A) landscapes show minimal distributional shift at 15 min	296
7.2.4	Significant transcript-level tail changes are bidirectional and limited	298
7.2.5	Coupling between tail class and expression is weak at 15 min . .	300
7.2.6	IPA on tail-defined gene sets reveals translation / RNA-handling axes	303
7.3	Discussion	312
8	Discussion	319
8.1	Overview and Integration of Findings	319
8.2	Metabolic Activation and the 3'-Deoxyinosine Rescue Pathway	320
8.3	Genetic Determinants of Cordycepin Resistance: Three Mechanistic Classes	322
8.3.1	Class 1: Reduced Metabolic Activation via Nucleoside Salvage .	322
8.3.2	Class 2: mRNA 3'-End Processing, Deadenylation, and RNA Turnover	323
8.3.3	Class 3: mTOR Signalling, Autophagy, and Proteostasis	324
8.3.4	LINC03121 as a Candidate RNA Target of Cordycepin	324
8.3.5	SLC26A9 as a Genetic Modifier of Cordycepin Sensitivity	330
8.4	Transcriptomic Equivalence of Natural and Synthetic Cordycepin	331
8.5	Poly(A)-Tail Remodelling: Mechanism and Selectivity	332
8.5.1	Global Poly(A)-Tail Shortening at 120 Minutes	332
8.5.2	Pathway Selectivity of Tail Remodelling	333
8.5.3	Mechanistic Model for Tail Lengthening Under a Chain Terminator	334
8.5.4	The PP6C–GCN2–eIF2 α Axis: Linking mTORC1 Inhibition to the ISR	334
8.5.5	Early Temporal Dynamics at 15 Minutes	335
8.6	The RToC Hypothesis: Integration and Candidate Nomination	337
8.7	Limitations	344
8.7.1	Technical Limitations of siRNA and Genetic Approaches	344

8.7.2	Technical Limitations of Poly(A)-Tail Analysis	345
8.7.3	Dosing and Concentration Considerations	345
8.7.4	Limitations Related to eIF2 α Phosphorylation	346
8.7.5	Scope for Further Analysis	346
8.8	Future Directions	347
8.8.1	Validation of RToC Candidates	347
8.8.2	Metabolic Tracing and Pathway Validation	348
8.8.3	Temporal Resolution and RToC Identification	348
8.8.4	Phosphoproteomic Integration	349
8.8.5	Translational Applications	349
8.9	Conclusions	349
	Bibliography	353
	A List of Abbreviations	383
	B Bioinformatics Scripts and Code	387
B.1	Quality Control Scripts	387
B.2	Haploid Genetic Screening Scripts	389
B.3	RNA Sequencing Analysis Scripts	389
B.4	Poly(A) Tail Length Analysis Scripts	397

List of Tables

2.1	Reagents, chemicals and media used	60
2.2	Computer software used	63
2.3	Treatment compounds and concentrations	66
2.4	Transfection mixture composition	67
2.5	qPCR primer sequences	71
2.6	24-minute LC method, through the ZIC®-pHILIC column.	76
4.1	Prioritized IPA pathways from the suPB cordycepin-resistance screen (exact pathway names from IPA).	183
4.2	Prioritized IPA pathways from the hyPB cordycepin-resistance screen (exact pathway names from IPA).	184
5.1	Canonical pathways (IPA) for FDR-filtered DEGs at 120 min (20 μ M cordycepin 1 h $\rightarrow$ LPS 1 h) versus DMSO $\rightarrow$ LPS in RAW264.7. Em dash indicates z-score not provided by IPA.	204
5.2	Canonical pathways (IPA) for FDR-filtered DEGs at 120 min (20 μ M syn- thetic cordycepin 1 h $\rightarrow$ LPS 1 h) vs. DMSO $\rightarrow$ LPS in RAW264.7 (file: <code>z_- score_120min_Synth_CoR_LPS_vs_DMSO_LPS_FDR_filtered.xlsx</code>). Em dash indicates z-score not provided by IPA.	207
5.3	Selected canonical pathways (120 min NUC-7738 $\rightarrow$ LPS, RAW264.7). Genes are IPA "Molecules" from the dataset.	220

5.4	Selected IPA Canonical Pathways (120 min 3'deoxyinosine treatment, with LPS added at 60 min in the presence of the compound for a 1 h stimulation). Blank z-scores indicate that IPA did not report an activation z-score.	222
6.1	Median poly(A) tail length shifts (ECDF-based) for drug + LPS vs DMSO + LPS (<i>Control</i>).	240
6.2	IPA Canonical Pathways for Cordycepin-treated lengthened transcripts (120 min CoR/LPS vs DMSO/LPS, p-value filtered)	255
6.3	IPA Canonical Pathways for Cordycepin-treated shortened transcripts (120 min CoR/LPS vs DMSO/LPS, p-value filtered)	257
6.4	IPA Canonical Pathways for NUC-7738-treated lengthened transcripts (120 min NUC/LPS vs DMSO/LPS, p-value filtered)	261
6.5	IPA Canonical Pathways for NUC-7738-treated shortened transcripts (120 min NUC/LPS vs DMSO/LPS, p-value filtered)	263
7.1	IPA Canonical Pathways for Cordycepin treatment (15 min CoR/LPS vs DMSO/LPS, p-value filtered)	294
7.2	IPA Canonical Pathways for Cordycepin-treated lengthened transcripts (15 min CoR/LPS vs DMSO/LPS, p-value filtered)	308
7.3	IPA Canonical Pathways for Cordycepin-treated shortened transcripts (15 min CoR/LPS vs DMSO/LPS, p-value filtered)	310
8.1	Cordycepin-induced poly(A) tail changes for proposed PABPC1–LARP1–mTOR platform components at 120 min.	343

List of Figures

1.1	Overarching scheme of cordycepin's mechanisms of action	4
1.2	Metabolic pathways of cordycepin (3'-deoxyadenosine). Upper pathway: Intracellular phosphorylation by adenosine kinase (ADK) yields cordycepin monophosphate (CoMP), which is sequentially converted to cordycepin diphosphate (CoDP) and cordycepin triphosphate (CoTP), the principal active metabolite. Lower pathway: Adenosine deaminase (ADA)-mediated deamination produces 3'-deoxyinosine (3DI), which undergoes glycosidic cleavage by purine nucleoside phosphorylase (PNP) to release adenine and 3'-deoxyribose. Adenine enters standard purine catabolism via hypoxanthine, xanthine and uric acid. Adapted from Lee <i>et al.</i> [1].	9
1.3	Structural comparison of cordycepin (3'-deoxyadenosine, 3'dA; left) and the ProTide NUC-7738 (ProTide 7a; right). Cordycepin is susceptible to premature deamination by adenosine deaminase (ADA), requires adenosine kinase (ADK) for initial phosphorylation, and depends on equilibrative nucleoside transporter 1 (hENT1) for cellular uptake. NUC-7738 carries a 5'-aryloxy phosphoramidate cap that is cleaved intracellularly, thereby bypassing ADA-mediated degradation, the requirement for ADK-dependent first phosphorylation, and the dependence on nucleoside transporters for cellular entry. Note the absence of the 3'-hydroxyl group on the ribose in both compounds (3'-OH replaced by 3'-H), which is responsible for chain termination during poly(A) tail synthesis. Adapted from Serpi <i>et al.</i> [2].	11

1.4 Poly(A) tail synthesis, function, and metabolism in eukaryotic mRNA.

Schematic overview of the major steps governing poly(A) tail biogenesis and post-transcriptional regulation. (Top, nucleus): Cleavage and polyadenylation (CPA) of pre-mRNAs are initiated when the CPSF complex (CPSF1, CPSF4, WDR33) recognizes the polyadenylation signal (PAS; AAUAAA) embedded within the 3'UTR. Cooperative binding of CstF to a downstream GU/U-rich element positions the CPSF3/CPSF73 endonuclease to cleave the nascent transcript 10–30 nucleotides downstream of the PAS. Following cleavage, the canonical nuclear poly(A) polymerases PAP α/γ extend the 3' end to produce a $\approx$ 200–250-nt poly(A) tail, while PABPN1 binds the elongating tail to stimulate processivity and enforce a characteristic tail-length distribution. Mature mRNPs are assembled with export adaptors and transported to the cytoplasm. Alternative polyadenylation (APA), via selection of proximal or distal PASs within the 3'UTR, generates mRNA isoforms with different 3'UTR architectures and regulatory potential. (Bottom, cytoplasm): Cytoplasmic PABPC1 replaces PABPN1 and cooperates with eIF4G and eIF4E to form a cap–tail “closed-loop” mRNP that promotes efficient translation initiation and ribosome recycling. In specific contexts (e.g., oocytes, neurons), cytoplasmic polyadenylation factors such as CPEB recruit non-canonical poly(A) polymerases (GLD2/GLD4) to lengthen short poly(A) tails and activate translation. This process is counterbalanced by the deadenylase PARN, enabling rapid translational on/off switching. Bulk deadenylation during mRNA turnover proceeds through a two-step pathway: initial trimming by the PAN2–PAN3 complex, followed by processive shortening by the CCR4–NOT deadenylase to oligo(A) tails of < 15 nt. Loss of PABPC1 binding triggers decapping (DCP1–DCP2) and exonucleolytic decay by XRN1 (5' $\rightarrow$ 3') or the exosome (3' $\rightarrow$ 5'). Together, these pathways integrate transcriptional outputs with mRNA stability, translation, and decay dynamics across the mRNA life cycle.

1.5 Mechanism of cordycepin triphosphate (CoTP)-mediated inhibition of cleavage and polyadenylation and its downstream consequences. (1) CoTP as a chain-terminating substrate for PAP α/γ . Schematic illustrating incorporation of CoTP by nuclear poly(A) polymerases PAP α/γ during 3'-end extension. Substitution of ATP with CoTP results in premature chain termination, generating short, stalled poly(A) tails that fail to recruit sufficient PABPN1. Because PABPN1 normally stabilizes PAP α/γ processivity and defines canonical tail length, CoTP-terminated tails disrupt both tail growth and PABPN1 loading. (2) Trapping of CPSF4 and WDR33 on CoTP-terminated 3'-ends. CoTP-terminated transcripts retain the CPSF4–WDR33 module of the CPSF complex on the RNA, preventing normal recycling of these CPA factors to new transcripts. This functional sequestration reduces the pool of active CPSF4/WDR33 available for PAS recognition, thereby impairing processing of subsequent pre-mRNAs and amplifying CPA inhibition. (3) Convergence with CPSF4/WDR33 knockdown phenotypes. Both CoTP treatment and siRNA-mediated depletion of CPSF4/WDR33 lead to reduced functional CPA complexes, diminished CPA efficiency, defective 3'-end formation, and attenuated downstream pathways including PI3K/Akt/mTOR signaling and NF- κ B nuclear responses. This convergence supports a model in which CoTP exerts its primary effects through functional depletion of CPA factors. (4) Consequences for PABPN1 loading, nuclear export competence, and cytoplasmic stability. Short, CoTP-terminated poly(A) tails exhibit reduced PABPN1 occupancy, impairing formation of export-competent mRNPs and promoting nuclear retention and surveillance-mediated decay. Inefficiently exported transcripts that reach the cytoplasm display weakened cap–tail interactions and poor closed-loop formation (reduced eIF4E–eIF4G–PABPC1 engagement). These mRNAs undergo accelerated deadenylation via CCR4–NOT and are rapidly degraded, resulting in reduced stability. Together, these pathways explain how CoTP-induced CPA inhibition propagates to broad post-transcriptional repression of inflammatory and growth-factor-responsive gene expression. The figure was created with

1.6 Modulation of growth-factor signalling networks by cordycepin triphosphate (CoTP) via polyadenylation-dependent control. Schematic overview of how cordycepin perturbs growth-factor-responsive signalling in mammalian cells. Under basal conditions (left), receptor tyrosine kinases (RTKs) activated by growth factors such as EGF or IGF-1 stimulate class I PI3K, generating PIP_3 from PIP_2 and recruiting PDK1 and Akt to the plasma membrane. Akt is phosphorylated at Thr308 by PDK1 and at Ser473 by mTORC2, yielding fully active Akt. Activated Akt phosphorylates TSC1/2 and PRAS40 to relieve inhibition of Rheb and promote mTORC1 activation, which in turn phosphorylates S6K1 and 4E-BP1 to drive ribosome biogenesis, cap-dependent translation and anabolic growth, while repressing autophagy via ULK1. In parallel, RTK signalling through RAS–RAF–MEK–ERK culminates in activation of transcription factors such as ELK1 and AP-1, supporting proliferation and gene expression. Energy-stress signalling through LKB1– or CaMKK β –activated AMPK antagonises mTORC1 and favours catabolic responses, but AMPK activation is not required for cordycepin-induced repression of these growth-factor pathways. On the right, cordycepin is taken up by nucleoside transporters and phosphorylated to CoTP, which inhibits nuclear cleavage and polyadenylation (CPA) of specific transcripts. In this model, suppression of PI3K/Akt/mTOR signalling occurs via an unidentified RNA, referred to as the RNA target of cordycepin (RToC), whose function is influenced by alterations in polyadenylation. CoTP-mediated inhibition of cleavage and polyadenylation affects RToC, creating a cellular environment in which growth-factor signalling cannot be sustained, despite continued upstream RTK engagement. Incomplete 3'-end processing and altered poly(A) tail dynamics of RToC and related transcripts are proposed to impair the transcriptional and translational support required to maintain PI3K/Akt/mTOR and RAS/RAF/MEK/ERK activity, resulting in systemic collapse of anabolic signalling. The legend at the bottom of the figure indicates the colour-coding for growth-factor (anabolic) signalling, energy-stress (catabolic) pathways, CoTP actions, inhibitory interactions and phosphorylation events. The figure was cre-

xviii

2.1	24-minute LC method, through the ZIC®-pHILIC column.	76
2.2	Following chromatographic separation of the analytes using the ZIC-pHILIC® column, the eluent was introduced into the mass spectrometer. Ionisation was achieved via electrospray ionisation (ESI) in the Exactive MS system. Charged droplets generated by ESI were directed into a C-trap®, from which ions were transferred to the Orbitrap mass analyser in rapid pulses. The Orbitrap, a type of Fourier transform ion trap (FTIT) analyser, operates by confining ions in orbital motion around a central electrode. The resulting ion trajectories generate an image current that is detected by external electrodes and subsequently transformed into mass-to-charge (m/z) values.	77
2.3	Sequence of samples used in LC-MS runs	79
2.4	RNA ScreenTape results for 120-minute set of RAW264.7 RNA extraction samples. Each sample has a two clear bands representing the 28S:18S ratios for rRNA fragment.	83
2.5	RNA ScreenTape results for 15-minute set of RAW264.7 RNA extraction samples. Each sample has a two clear bands representing the 28S:18S ratios for rRNA fragment.	84
2.6	Schematic representation of the expected sequencing read structure (not to scale). The central red region denotes the human genomic insert of interest, flanked by the P5 adapter, Illumina barcode, and left/right inverted repeat (IR) sequences used for demultiplexing. Sixteen demultiplexing bins were defined: eight containing left IR (PB5) and eight containing right IR (PB3). The adapter, barcode, and IR sequences correspond to the oligonucleotides listed in the adaptor table [3], with an additional 3 guanine incorporated to complete the IR motifs [3]. Following adapter trimming using a customized script, all reads are expected to begin with the canonical TTAA sequence [3]. The figure was adapted from [3]	87
2.7	Caption for the image	93

2.8	Workflow schematic. Starting with two conditions, polyadenylated RNA is extracted and sequenced with nanopores. The electrical signal for the poly(A) tail is distinct from other nucleotides allowing the quantification of the poly(A) tail length. Following data processing, the poly(A) tail lengths for all replicates of two conditions are fed into NanopLen for differential length analysis adapted from [4] S. A. Dar, S. Malla, C. Belair, and M. Maragkakis, “Differential poly(A) tail length analysis using nanopore sequencing,” <i>Methods Mol. Biol.</i> , vol. 2723, pp. 267–283, 2024, doi: 10.1007/978-1-0716-3481-3_16.	96
3.1	Experimental time schemes for Chapter 3.	100
3.2	The fate of orally administered cordycepin. Cordycepin is rapidly deaminated by adenosine deaminase (ADA) in the gut brush border and systemic circulation, yielding 3′-deoxyinosine (3DI) as the main circulating species [1, 5, 6]. Several studies nevertheless report detection of cordycepin triphosphate in blood despite 3DI predominating, implying a metabolic rescue pathway that converts circulating 3DI back to cordycepin nucleotides.	101
3.3	3DI dose–response for CXCL1 and CXCL8 in A549 cells	103
3.4	3DI dose–response for Il1b and Acod1 in RAW264.7 cells	104
3.5	NUC-7738 dose–response for Il1b and Acod1 in RAW264.7 cells	105

- 3.6 Based on prior knowledge that the antiretroviral drug dideoxyinosine is phosphorylated by cytosolic 5'-nucleotidase and subsequently aminated by adenylosuccinate synthetase and lyase, we hypothesized that analogous enzymes may metabolize cordycepin and its derivative 3'-deoxyinosine. We propose that cytosolic 5'-nucleotidase can similarly phosphorylate 3'-deoxyinosine, followed by amination of the phosphorylated intermediate by adenylosuccinate synthetase and adenylosuccinate lyase to yield cordycepin monophosphate. This putative metabolic pathway connecting 3'-deoxyinosine to active cordycepin triphosphate was hypothesized by extrapolating known pathways. The chemical structures were drawn using ChemSketch from the ACD Lab, and the figure was created using ChemDraw. 107
- 3.7 Effect of NT5C2 knock-down on Cordycepin and 3'-Deoxyinosine responses (A, B, D) A549 epithelial cells were transfected with 5 nM siRNA targeting ADSS2 (siADSS2) or a non-targeting control (siCTRL) for 48 h. Cells were left untreated (No Tx) or exposed to Cordycepin 50 μ M (Cpn 50) or 3'-Deoxyinosine 200 μ M (3DI 200) for 2 h, with TNF (10 ng/ml) added 1 h after the start of compound treatment (i.e. in the presence of the compound) for a 1 h stimulation. qPCR data for NT5C2, CXCL1, and CXCL8 were normalised to GAPDH and expressed relative to the TNF + siCTRL condition (n = 3 biological replicates). Bars show mean $\pm$ SEM; dashed line indicates the TNF baseline. Significance: ordinary one-way ANOVA with Tukey post-test, $P < 0.05$ (*), < 0.01 (**), < 0.001 (***), ns = not significant. (C,E) Same experiments, but ratios were further normalised within each siRNA group to their corresponding DMSO + TNF value (Treated / [DMSO + TNF] in the same siRNA group), removing basal knock-down effects. Paired two-tailed t-tests compare siCTRL vs. siNT5C2 for each drug. Colour code: blue = siCTRL, red = siNT5C2. 108

3.8 Effect of ADSS2 knock-down on Cordycepin and 3'-Deoxyinosine responses (A, B, D) A549 epithelial cells were transfected with 5 nM siRNA targeting ADSS2 (siADSS2) or a non-targeting control (siCTRL) for 48 h. Cells were left untreated (No Tx) or exposed to Cordycepin 50 μ M (Cpn 50) or 3'-Deoxyinosine 200 μ M (3DI 200) for 2 h, with TNF (10 ng/ml) added 1 h after the start of compound treatment (i.e. in the presence of the compound) for a 1 h stimulation. qPCR data for ADSS2, CXCL1, and CXCL8 were normalised to GAPDH and expressed relative to the TNF + siCTRL condition (n = 3 biological replicates). Bars show mean $\pm$ SEM; dashed line indicates the TNF baseline. Significance: ordinary one-way ANOVA with Tukey post-test, $P < 0.05$ (*), < 0.01 (**), < 0.001 (***), ns = not significant. (C, E) Same experiments, but ratios were further normalised within each siRNA group to their corresponding DMSO + TNF value (Treated / [DMSO + TNF] in the same siRNA group), removing basal knock-down effects. Paired two-tailed t-tests compare siCTRL vs. siADSS2 for each drug. Colour code: blue = siCTRL, red = siADSS2. 109

3.9 Proposed metabolic pathway for 3'-deoxyinosine involving purine nucleoside phosphorylase (PNP). This hypothesized route entails PNP-mediated enzymatic cleavage of 3'-deoxyinosine into hypoxanthine and 3'-deoxyribose-1-phosphate. 3'-deoxyribose-1-phosphate can then be converted to 3'-deoxyribose-5-phosphate and combined with hypoxanthine by hypoxanthine-phosphoribosyl transferase to generate 3'-deoxyinosine monophosphate. To test this pathway, PNP was suppose to be stimulated upon glucose starvation in A549 cells; however, this did not affect the anti-inflammatory activity of 3'-deoxyinosine (In my experiment glucose starvation reduced the basal inflammatory response despite there is an article argued that glucose starvation trigger an inflammatory response and shifting the cells to use inosine as a carbon source), suggesting that this proposed route is not utilized for 3'-deoxyinosine metabolism. The chemical structures were drawn using ChemSketch from the ACD Lab, and the figure was created using ChemDraw. 111

3.10 A549 cells were cultured in glucose-free medium for 24 h before treatment with cordycepin (50 μ M) or 3'-deoxyinosine (200 μ M) for 2 h, with TNF (10 ng/ml) added 1 h after the start of compound treatment (i.e. in the presence of the compound) for a 1 h stimulation. Total RNA was extracted and cDNA was synthesized, followed by qPCR. Ct values of qPCR for A) the inflammatory genes CXCL1 and CXCL8, and B) PNP were analyzed by the $2^{-\Delta\Delta C_t}$ method using GAPDH as a reference gene. Relative mRNA expression of the tested genes is shown as a fold-change compared to TNF in glucose-containing medium (red dashed line). The results are presented as the mean $\pm$ SD of three independent experiments. 112

3.11	Proposed metabolic pathway for the conversion of 3'-deoxyinosine to cordycepin involving adenine and 3'-deoxyribose recycling. This hypothetical route entails an endogenous adenine reacting with the 3'-deoxyribose moiety released from 3'-deoxyinosine. Enzymes such as methylthioadenosine phosphorylase (MTAP) or FAMIN can combine adenine with 3'-deoxyribose to generate 3'-deoxyadenosine (cordycepin). The chemical structures were drawn using ChemSketch from the ACD Lab, and the figure was created using ChemDraw.	114
3.12	Proposed metabolic pathway for 3'-deoxyinosine conversion to cordycepin monophosphate involving adenine salvage. This hypothetical route entails 3'-deoxyribose-1- phosphate first being cleaved from 3'-deoxyinosine and then converted to 3'-deoxyribose-5- phosphate by phosphopentomutase (PGM2). 3'-deoxyribose-5-phosphate can then be converted to 5-phospho-3'-deoxyribosyl-1-pyrophosphate by phosphoribosyl pyrophosphate synthetase 1 (PRPS1). Finally, endogenous adenine reacts with 5-phospho-3'-deoxyribosyl- 1-pyrophosphate, catalyzed by adenine phosphoribosyltransferase (APRT) to generate 3'-deoxyadenosine monophosphate (cordycepin monophosphate).	115
3.13	Effects of cordycepin and 3'-deoxyinosine on inflammatory gene expression after adenine treatment. A549 cells were treated with adenine (800 μ M) for one hour then with 0.4% v/v DMSO, cordycepin (50 μ M), or 3'-deoxyinosine (200 μ M) for 2 h, with TNF (10 ng/ml) added 1 h after the start of compound treatment (i.e. in the presence of the compound) for a 1 h stimulation. The graph shows the mean $\pm$ standard deviation of the three biological replicates. Statistical significance was determined by unpaired t-tests at *P < 0.05, **P < 0.01, ***P < 0.005, and ****P < 0.0001****.	116

3.14 Effect of APRT knock-down on Cordycepin and 3'-Deoxyinosine responses

(A, B, D) A549 epithelial cells were transfected with 5 nM siRNA targeting APRT (siAPRT) or a non-targeting control (siCTRL) for 48 h. Cells were left untreated (No Tx) or exposed to Cordycepin 50 μ M (Cpn 50) or 3'-Deoxyinosine 200 μ M (3DI 200) for 2 h, with TNF (10 ng/ml) added 1 h after the start of compound treatment (i.e. in the presence of the compound) for a 1 h stimulation. qPCR data for APRT, CXCL1, and CXCL8 were normalised to GAPDH and expressed relative to the TNF + siCTRL condition (n = 3 biological replicates). Bars show mean $\pm$ SEM; dashed line indicates the TNF baseline. Significance: ordinary one-way ANOVA with Tukey post-test, $P < 0.05$ (*), < 0.01 (**), < 0.001 (***), ns = not significant. (C, E) Same experiments, but ratios were further normalised within each siRNA group to their corresponding DMSO + TNF value (Treated / [DMSO + TNF] in the same siRNA group), removing basal knock-down effects. Paired two-tailed t-tests compare siCTRL vs. siAPRT for each drug. Colour code: blue = siCTRL, red = siAPRT. 118

3.15 Effect of siAprt knock-down on Cordycepin and 3'-Deoxyinosine responses

(A, B, D) RAW264.7 macrophages were transfected with 5 nM siRNA targeting Aprt (siAprt) or a non-targeting control (siCTRL) for 48 h. Cells were left untreated (No Tx) or exposed to Cordycepin 20 μ M (Cpn 20) or 3'-Deoxyinosine 200 μ M (3DI 200) for 2 h, with LPS (1 μ g/ml) added 1 h after the start of compound treatment (i.e. in the presence of the compound) for a 1 h stimulation. qPCR data for Aprt, Acod1, and Il1B were normalised to Gapdh and expressed relative to the LPS + siCTRL condition (n = 3 biological replicates). Bars show mean $\pm$ SEM; dashed line indicates the LPS baseline. Significance: ordinary one-way ANOVA with Tukey post-test, $P < 0.05$ (*), < 0.01 (**), < 0.001 (***), ns = not significant. (C, E) Same experiments, but ratios were further normalised within each siRNA group to their corresponding DMSO + LPS value (Treated / [DMSO + LPS] in the same siRNA group), removing basal knock-down effects. Paired two-tailed t-tests compare siCTRL vs. siAprt for each drug. Colour code: blue = siCTRL, red = siAprt. 119

3.16 Effect of FAMIN knock-down on Cordycepin and 3'-Deoxyinosine responses (A, B, D) A549 epithelial cells were transfected with 5 nM siRNA targeting FAMIN (siFAMIN) or a non-targeting control (siCTRL) for 48 h. Cells were left untreated (No Tx) or exposed to Cordycepin 50 μ M (Cpn 50) or 3'-Deoxyinosine 200 μ M (3DI 200) for 2 h, with TNF (10 ng/ml) added 1 h after the start of compound treatment (i.e. in the presence of the compound) for a 1 h stimulation. qPCR data for FAMIN, CXCL1, and CXCL8 were normalised to GAPDH and expressed relative to the TNF + siCTRL condition ($n = 3$ biological replicates). Bars show mean $\pm$ SEM; dashed line indicates the TNF baseline. Significance: ordinary one-way ANOVA with Tukey post-test, $P < 0.05$ (*), < 0.01 (**), < 0.001 (***), ns = not significant. (C, E) Same experiments, but ratios were further normalised within each siRNA group to their corresponding DMSO + TNF value (Treated / [DMSO + TNF] in the same siRNA group), removing basal knock-down effects. Paired two-tailed t-tests compare siCTRL vs. siFAMIN for each drug. Colour code: blue = siCTRL, red = siFAMIN. 121

3.17 Effect of Lacc1 (Famin) knock-down on Cordycepin and 3'-Deoxyinosine responses (A, B, D) RAW264.7 macrophages were transfected with 5 nM siRNA targeting Lacc1 (siLacc1) or a non-targeting control (siCTRL) for 48 h. Cells were left untreated (No Tx) or exposed to Cordycepin 20 μ M (Cpn 20) or 3'-Deoxyinosine 200 μ M (3DI 200) for 2 h, with LPS (1 μ g/ml) added 1 h after the start of compound treatment (i.e. in the presence of the compound) for a 1 h stimulation. qPCR data for Lacc1 , Acod1, and Il1B were normalised to Gapdh and expressed relative to the LPS + siCTRL condition (n = 3 biological replicates). Bars show mean $\pm$ SEM; dashed line indicates the LPS baseline. Significance: ordinary one-way ANOVA with Tukey post-test, $P < 0.05$ (*), < 0.01 (**), < 0.001 (***), ns = not significant. (C, E) Same experiments, but ratios were further normalised within each siRNA group to their corresponding DMSO + LPS value (Treated / [DMSO + LPS] in the same siRNA group), removing basal knock-down effects. Paired two-tailed t-tests compare siCTRL vs. siLacc1 for each drug. Colour code: blue = siCTRL, red = siLacc1. 122

3.18 Effect of ADK knock-down on Cordycepin and 3'-Deoxyinosine responses

(A, B, D) A549 epithelial cells were transfected with 5 nM siRNA targeting ADK (siADK) or a non-targeting control (siCTRL) for 48 h. Cells were left untreated (No Tx) or exposed to Cordycepin 50 μ M (Cpn 50) or 3'-Deoxyinosine 200 μ M (3DI 200) for 2 h, with TNF (10 ng/ml) added 1 h after the start of compound treatment (i.e. in the presence of the compound) for a 1 h stimulation. qPCR data for ADK, CXCL1, and CXCL8 were normalised to GAPDH and expressed relative to the TNF + siCTRL condition (n = 3 biological replicates). Bars show mean $\pm$ SEM; dashed line indicates the TNF baseline. Significance: ordinary one-way ANOVA with Tukey post-test, $P < 0.05$ (*), < 0.01 (**), < 0.001 (***), ns = not significant. (C, E) Same experiments, but ratios were further normalised within each siRNA group to their corresponding DMSO + TNF value [(Treated / [DMSO + LPS] in the same siRNA group], removing basal knock-down effects. Paired two-tailed t-tests compare siCTRL vs. siADK for each drug. Colour code: blue = siCTRL, red = siADK. 124

3.19 Effect of siAdk knock-down on Cordycepin and 3'-Deoxyinosine responses

(A, B, D) RAW264.7 macrophages were transfected with 5 nM siRNA targeting Adk (siAdk) or a non-targeting control (siCTRL) for 48 h. Cells were left untreated (No Tx) or exposed to Cordycepin 20 μ M (Cpn 20) or 3'-Deoxyinosine 200 μ M (3DI 200) for 2 h, with LPS (1 μ g/ml) added 1 h after the start of compound treatment (i.e. in the presence of the compound) for a 1 h stimulation. qPCR data for Adk, Acod1, and Il1B were normalised to Gapdh and expressed relative to the LPS + siCTRL condition (n = 3 biological replicates). Bars show mean $\pm$ SEM; dashed line indicates the LPS baseline. Significance: ordinary one-way ANOVA with Tukey post-test, $P < 0.05$ (*), < 0.01 (**), < 0.001 (***), ns = not significant. (C, E) Same experiments, but ratios were further normalised within each siRNA group to their corresponding DMSO + LPS value (Treated / [DMSO + LPS] in the same siRNA group), removing basal knock-down effects. Paired two-tailed t-tests compare siCTRL vs. siAdk for each drug. Colour code: blue = siCTRL, red = siAdk. 125

3.20 A (top): Overlaid chromatograms: TIC (ESI⁺, red trace) and XIC at m/z 252.1 (green, shaded) across a 24-min run on a SeQuant ZIC-pHILIC column. A single, sharp XIC peak is centered at tR $\approx$ 7.28–7.31 min and is annotated “Cordycepin.” Minor features at the solvent front ($\approx$ 0.34–0.50 min) and late elution ($\approx$ 23.6–23.8 min) are visible in the TIC but do not appear in the XIC. Ion mode: ESI⁺ (labelled at top right). B (bottom): MS spectrum for the tR = 7.279 min peak with automatic component assignment in ACD/Labs Spectrus Processor. Base peak [M+H]⁺ = 252.109 (Mass(Amax) = 252.113), elemental formula C₁₀H₁₃N₅O₃, and Mass difference = 0.004 (Da). Additional labelled ions include 218.215 ([M+H–34]⁺), 246.243 ([M+H–6]⁺), 253.113, and a [M+Na]⁺ adduct (labelled). Software confidence tile reads “Excellent.” The component table shows tR = 7.279 min, neutral mass 251.102, [M–H][–] = 250.095 (reported), and Single XIC area = 100.00% for both ESI polarities; TIC area = 15.26% (ESI⁺) and 30.02% (ESI[–]) with All tRs(min) reported as TIC (ESI⁺) = 7.28, XIC = 7.28, TIC (ESI[–]) = 6.43, XIC = 7.29 (values as displayed in the figure). The chromatogram generated from the Thermo Exactive LC-MS employing the SeQuant ZIC-pHILIC column (Merck). The analysis was conducted over a 24-minute runtime. Visualization of the LC-MS/MS data was performed using the Spectrus Processor from ACD/Labs. 127

3.21 Mass spectrum for cordycepin. A (top): ESI⁺ MS spectrum at tR = 7.279 min, annotated with [M+H]⁺ and neutral-loss/adduct assignments. The base peak is [M+H]⁺ = 252.113/252.109, with visible signals at 218.215 ([M+H-34]⁺), 246.244 ([M+H-6]⁺), 219.215, 253.113, and a labelled [M+Na]⁺ adduct. B (bottom): Replicate/zoomed ESI⁺ MS view for the same tR, emphasizing the dominant [M+H]⁺ = 252.109 peak and nearby low-abundance ions (e.g., 118.090, 130.166, 136.071, 148.105, 212.095–212.097). Axes: m/z (x) and relative intensity (y). Ion mode: ESI⁺ (right-hand label). The chromatogram generated from the Thermo Exactive LC-MS employing the SeQuant ZIC-pHILIC column (Merck). The analysis was conducted over a 24-minute runtime. Visualization of the LC-MS/MS data was performed using the Spectrus Processor from ACD/Labs. 128

3.22 Cordycepin (Phyproof) calibration curve. Phyproof Cordycepin from Merck was used as standard to estimate the purity of cordycepin (Carbosynth) and the purity of synthetic cordycepin. The y-axis represents the concentration, expressed in nanomolar (nM), and the x-axis represents the area. The purity of cordycepin (Carbosynth), according to the calibration curve is 99.08% and the purity of synthetic cordycepin is 100%.129

3.23 A (top): TIC (ESI+, red trace) overlaid with the extracted-ion chromatogram (XIC, m/z 252.1; beige/filled) for synthetic cordycepin; apex at tR = 7.280 min. Below, centroid MS at the peak apex and the identification table. B (bottom): Replicate view of the TIC/XIC overlay with the integrated XIC peak at tR = 7.280 min and the same identification table. The chromatogram generated from the Thermo Exactive LC-MS employing the SeQuant ZIC-pHILIC column (Merck). The analysis was conducted over a 24-minute runtime. Visualization of the LC-MS/MS data was performed using the Spectrus Processor from ACD/Labs. . . 131

3.24 Mass spectrum for synthetic cordycepin. A (top): Centroid ESI+ mass spectrum at $t_R = 7.280$ min annotated with adduct/neutral-loss labels provided by the software. B (bottom): Replicate/zoomed view of the apex spectrum. The chromatogram generated from the Thermo Exactive LC-MS employing the SeQuant ZIC-pHILIC column (Merck). The analysis was conducted over a 24-minute runtime. Visualization of the LC-MS/MS data was performed using the Spectrus Processor from ACD/Labs. . . 132

3.25 A (top): Chromatogram + component table from ACD/Labs Spectrus Processor visualization showing two resolved components in the 3DI stock: cordycepin at $RT \approx 7.282$ min and 3DI at $RT \approx 8.008$ min. Accurate-mass matches are annotated in positive ESI: cordycepin $[M+H]^+$ calc 252.115, obs 252.109 ($\Delta \approx 5$ mDa); 3DI $[M+H]^+$ calc 253.094, obs 253.093 ($\Delta \approx 1$ mDa). B (bottom): Zoomed chromatogram view of the same injection highlighting the two peaks attributed to cordycepin and 3DI. The chromatogram generated from the Thermo Exactive LC-MS employing the SeQuant ZIC-pHILIC column (Merck). The analysis was conducted over a 24-minute runtime. Visualization of the LC-MS/MS data was performed using the Spectrus Processor from ACD/Labs. . . 134

3.26 Mass spectrum for 3DI. A (top): MS spectrum at RT 8.008 min in ESI+, showing precursor $[M+H]^+ 253.0935$ with characteristic fragments (e.g., m/z 214.094, 171.128, 118.090, 104.0735). B (bottom): Replicate/expanded MS view from the same retention-time window, again dominated by the 253.093 ion. The chromatogram generated from the Thermo Exactive LC-MS employing the SeQuant ZIC-pHILIC column (Merck). The analysis was conducted over a 24-minute runtime. Visualization of the LC-MS/MS data was performed using the Spectrus Processor from ACD/Labs. 135

4.1	PiggyBac (PB) transposon system. (a) Original PB elements contain the transposase gene (orange) and inverted terminal repeats (ITRs, yellow arrows). (b) Binary PB system used for gene delivery in plasmids: a transposase expression helper plasmid (HyPBase or SuPBase) driven by a promoter (gray arrow) and a donor plasmid containing the gene-trapping cassette PBDGTV (light blue) and a puromycin selection marker flanked by ITRs. (c) The transposon carrying the cassette is excised from the donor plasmid and integrated into the chromosome at TTAA sites. (d) Insertion of PBDGTV between exons (navy) can disable a gene by prematurely terminating transcription via the splice-acceptor-poly(A) element (SA-polyA), truncating transcript expression (exons 1–2). Figure created with BioRender.com.	143
4.2	Overview of haploid genetic screening. eHAP cells were transfected with the PiggyBac gene-trapping vector PBDGTV along with transposase expression plasmids (HyPBase or SuPBase), resulting in genomic integration and gene disruption across the genome. Cells were then selected with puromycin at 2 µg/mL for 3 days to enrich for cells with transposon insertions. The surviving mutant cell libraries were treated with cordycepin at 150 µM or 200 µM for 72 h (twice every 24 h) to select mutants resistant to metabolic inhibition by cordycepin. Dr. Alexander Kondrashov, Dr. Emilie Daniel and Davor Kresnik conducted the transfected library preparations and puromycin selection; I performed the cordycepin selection using the PrestoBlue™ HS Cell Viability Assay to monitor metabolic activity as the primary readout. Surviving resistant clones were then harvested for genomic DNA extraction, Splinkerette PCR amplification of transposon–genome junctions, and next-generation sequencing to identify the disrupted genes (see Figure 4.7). Figure created with BioRender.com.	145

4.3	Effect of different puromycin concentrations on eHAP cell viability, wild type vs. transfected. Wild-type eHAP and PBDGTV-transfected eHAP cells were treated with the indicated puromycin concentrations for 3 days. The 2 µg/mL condition eliminated wild-type cells while sparing most transfected cells, and was therefore used for library enrichment.	146
4.4	Metabolic inhibition of eHAP cells by single versus repeated cordycepin treatments. eHAP cells were treated with 25–400 µM cordycepin either as a single dose or with repeated dosing every 24 h for 3 days. Cell viability was measured using the PrestoBlue assay. Repeated doses produced stronger inhibition than a single dose, probably because cordycepin degrades over time with a single application. X indicates the total number of cordycepin doses applied across the specified time.	147
4.5	Crystal-violet clonogenic assay showing that multiple cordycepin doses reduce eHAP colony formation more than a single dose. Representative images show eHAP cells treated with a single dose of 150 or 200 µM cordycepin for 72 h (left) or with multiple doses of 150 or 200 µM cordycepin twice daily over 3 days (right). After treatment, cells were fixed and stained with crystal violet in 10-cm Petri dishes (bottom). Repeated dosing markedly reduced clonogenic growth compared with a single dose, supporting the choice of the repeated-dosing paradigm for the genetic screen.	148
4.6	Metabolic inhibition of HAP1 cells by 3′deoxyinosine. HAP1 cells were treated with 25–400 µM 3′deoxyinosine using repeated dosing every 24 h for 3 days. Cell viability was measured using the PrestoBlue assay. 3′Deoxyinosine achieved a maximal inhibition of approximately 20 % across the tested concentration range, indicating that it is a weak inhibitor under these conditions.	149

4.7	Overview of mapping piggyBac insertion sites in cordycepin-resistant cells. The surviving mutant cell library after puromycin selection, which contained transposon insertions, was treated with cordycepin to select mutants resistant to metabolic inhibition by cordycepin. Genomic DNA was extracted from cordycepin-resistant cells, fragmented and ligated to Splinkerette adapters [3, 7]. Two rounds of PCR (PB5 and PB3) were performed with transposon- and Splinkerette-specific primers to amplify regions flanking transposon insertion sites. These samples were sequenced to identify insertion sites and the disrupted genes. Dr. Alexander Kondrashov, Dr. Emilie Daniel and Davor Kresnik carried out the transfected library preparations and puromycin selection. Figure created with BioRender.com.	150
4.8	Total unique insertions by condition at 150K seeding density (T75, eHAP). Bar chart of mapped insertion sites for hyPB and suPB libraries under DMSO or cordycepin (150 μ M, 200 μ M), plus library-only controls. Each bar represents one library; bar height and the number above each bar indicate the total number of unique insertion sites in that condition (range 9,341–51,053).	153
4.9	Total unique insertions by condition at 300K seeding density (T75, eHAP). As in Figure 4.8, but for libraries generated at 300K. Each bar represents one library; bar height and the number above each bar indicate the total number of unique insertion sites (range 11,390–39,218).	154
4.10	Normalized insertion distribution by chromosome group at 150K. For each condition, the percentage of total insertions falling into Large (1–5), Medium (6–12), Small (13–22) and Sex (X, Y) chromosome groups is shown. Colours encode conditions, and the profiles across conditions overlap closely with the ordering Large > Medium > Small > Sex, indicating no gross chromosome-scale bias specific to hyPB or suPB at 150K.	155

4.11	Gene-level insertion enrichment under 150 μ M cordycepin. Each heatmap panel shows one transposase platform: suPB (a, left) or hyPB (b, right). Rows correspond to individual genes (labelled by HGNC symbols), columns to treatment or control, and each cell is annotated with the percentage of total insertions in that condition mapped to that gene. The colour scale encodes this percentage (lighter to darker indicating lower to higher values). Column labels use the abbreviations suPB and hyPB for the two transposases; “cordycepin_150” denotes repeated dosing with 150 μ M cordycepin and “Control_DMSO” denotes the vehicle-treated library. Abbreviations: suPB, SuPBase transposase; hyPB, hyperactive piggyBac transposase; DMSO, dimethyl sulfoxide vehicle control.	157
4.12	Gene-level insertion enrichment under 200 μ M cordycepin. Layout and colour coding as in Figure 4.11: each heatmap panel shows one transposase platform, suPB (a) or hyPB (b), with rows for genes and columns for treatment conditions. Each cell is annotated with the percentage of total insertions in that condition mapped to that gene; “cordycepin_200” denotes repeated dosing with 200 μ M cordycepin. Abbreviations as in Figure 4.11.	159
4.13	Top genes across conditions (normalised percentage of total insertions). Bar chart comparing the same set of enriched genes across all eight libraries (hyPB and suPB; DMSO, 150 μ M and 200 μ M cordycepin; library-only controls). Each coloured bar represents the percentage of total unique insertions in one condition mapped to the indicated gene; this normalisation allows direct comparison between libraries of different absolute size. The plot highlights the strong and dose-dependent enrichment of ADK, the pronounced spike of <i>LINC03121</i> in hyPB_cordycepin_200, and recurrent enrichment of genes such as <i>PSAT1</i> and <i>UBE2D1</i> in suPB cordycepin conditions.	161

4.14 Heatmap of normalised insertion percentages for recurrent genes across conditions. Each row corresponds to a gene and each column to one library (hyPB or suPB; DMSO, 150 μ M or 200 μ M cordycepin; library-only controls). Cells are coloured by, and annotated with, the percentage of total insertions in that condition mapped to the gene. Normalisation was performed by expressing the number of unique insertion sites in each gene as a percentage of the total unique insertions in that library, allowing direct comparison across libraries that differ in absolute insertion count. The “Library” columns represent the unselected mutant libraries (i.e. no cordycepin or DMSO treatment), which serve as a baseline for insertion frequency before any drug selection. The heatmap makes cross-condition patterns explicit, with ADK showing the most consistent enrichment under cordycepin and additional clusters of genes enriched preferentially in hyPB or suPB under higher-dose selection. 162

4.15 Ingenuity Pathway Analysis of suPB cordycepin-resistant clones (200 μ M vs DMSO). Horizontal bar plot of Ingenuity Canonical Pathways enriched in the suPB_CoR_200 population relative to the matched suPB DMSO control at 150K seeding density. Bar length represents the number of insertion-enriched genes (x-axis) mapped to each pathway (y-axis), and gene symbols inside each bar identify those genes as reported by IPA. eHAP libraries were selected with repeated 200 μ M cordycepin dosing for 72 h (twice daily) before mapping of insertion sites; pathway enrichment criteria and thresholds are described in the Methods. 165

4.16	Insertion support for IPA pathways in suPB cordycepin-resistant clones (200 μ M). Lollipop plot showing, for each IPA canonical pathway enriched in suPB_CoR_200 versus DMSO (y-axis), the total number of unique transposon insertion sites in genes belonging to that pathway (x-axis, log scale). Unlike the bar chart in Figure 4.15, which counts the number of distinct genes, this plot shows the cumulative insertion-site evidence supporting each pathway, reflecting both the number of contributing genes and the frequency of insertions in each gene.	166
4.17	Ingenuity Pathway Analysis of hyPB cordycepin-resistant clones (200 μ M vs DMSO). Horizontal bar plot of Ingenuity Canonical Pathways enriched in the hyPB_CoR_200 population relative to the matched hyPB DMSO control at 150K seeding density. Bar length represents the number of insertion-enriched genes (x-axis) mapped to each pathway (y-axis), and gene symbols inside each bar identify those genes as reported by IPA. Libraries were selected with repeated 200 μ M cordycepin dosing for 72 h (twice daily) before mapping of insertion sites; enrichment criteria and thresholds are described in the Methods.	169
4.18	Insertion support for IPA pathways in hyPB cordycepin-resistant clones (200 μ M). Lollipop plot showing, for each IPA canonical pathway enriched in hyPB_CoR_200 versus DMSO (y-axis), the total number of unique transposon insertion sites in genes belonging to that pathway (x-axis, log ₁₀ scale). As in Figure 4.16, this plot complements the bar chart (Figure 4.17) by showing cumulative insertion-site evidence rather than the number of distinct genes.	170
4.19	IPA graphical summary of suPB cordycepin-resistant clones.	173
4.20	IPA graphical summary of hyPB cordycepin-resistant clones.	174
4.21	IPA canonical pathway coverage (stacked bars): suPB cordycepin-resistant clones.	175
4.22	IPA canonical pathway coverage (stacked bars): hyPB cordycepin-resistant clones.	175

5.1	Experimental time scheme for Chapter 5.	188
5.2	Nanopore sequencing principle used to generate the RNA seq dataset. Schematic overview of Oxford Nanopore Technology (ONT) sequencing used to produce the transcriptome data analyzed in this chapter. A motor protein unwinds the nucleic acid and drives a single strand through a biological nanopore toward the positive side of the membrane; each base produces a characteristic change in ionic current that is decoded to se- quence. The graphic illustrates strand translocation, current modulation, and base calling at a high level; it is not drawn to scale. The figure was adapted from BioRender.	191
5.3	Heat map for differential gene expression across different conditions for RAW264.7 cells. Illustrating the top 50 variable genes in RAW264.7 murine macrophage cells under different treatment conditions. Cells were treated for 120 minutes with vehicle (DMSO), 3'deoxyinosine (3DI, 200 μM), cordycepin (CoR, 20 μM), synthetic cordycepin (Synth CoR, 20 μM), or NUC-7738 (NUC, 200 μM); lipopolysaccharide (LPS, 1 μg/mL) was added 60 minutes after the start of compound treatment (i.e. in the presence of the compound) and cells were stimulated for a further 60 minutes. RNA was isolated, converted to cDNA, and sequenced using Oxford Nanopore technology. Gene expression quantification and analysis were performed with IsoQuant and EdgeR using the pre-specified thresholds. Values shown represent normalised counts.	193

5.4	Cordycepin (natural or synthetic, 20 μ M) broadly represses LPS-induced transcription. Volcano plots show differential expression in RAW264.7 cells treated with cordycepin (20 μ M) or 0.02% DMSO for 2 h, with LPS (1 μ g/mL) added 1 h after the start of compound treatment (i.e. in the presence of the compound) and cells stimulated for a further 1 h. Data: ONT cDNA sequencing $\rightarrow$ IsoQuant $\rightarrow$ edgeR (TMM). Testing: edgeR GLM (glmFit) + LRT (glmLRT) with BH FDR; thresholds: $ \log_2 \text{FC} \geq 1$; $\text{FDR} < 0.05$; $n = 3$ per group. Panel A (natural cordycepin): 373 downregulated (green) and 29 upregulated (red) genes. Panel B (synthetic cordycepin): 352 down and 17 up. The x-axis shows the $\log_2$ fold change ($\log_2 \text{FC}$), defined as $\log_2(\text{compound}+\text{LPS}/\text{DMSO}+\text{LPS})$, so that negative values indicate genes repressed by the compound relative to LPS-only control. The y-axis shows $-\log_{10}(\text{FDR})$. Grey points denote genes not meeting significance thresholds.	195
5.5	IPA bubble chart of (A) natural cordycepin and (B) synthetic cordycepin. The bubble size represents the number of genes overlapping each pathway, while the color indicates the pathway activation z-score (blue for negative/inhibited, orange for positive/activated, grey for neutral/no significant activation).	197
5.6	IPA activation z-scores for canonical pathways: cordycepin vs. synthetic cordycepin.	199
5.7	Dual-cluster heatmap of IPA z-scores: cordycepin vs. synthetic cordycepin.	201
5.8	IPA canonical pathway coverage (stacked bars): natural vs. synthetic cordycepin.	203

5.9	NUC-7738 (200 μ M) shows a broader, predominantly negative DE profile than 3'-deoxyinosine (200 μ M). RAW264.7 macrophages were treated for 2 h with NUC-7738 (200 μ M), 3DI (200 μ M), or 0.02% DMSO, with LPS (1 μ g/mL) added 1 h after the start of compound treatment (i.e. in the presence of the compound) and cells stimulated for a further 1 h. ONT cDNA reads were quantified by IsoQuant and analysed in edgeR (TMM). DE testing used edgeR GLM (glmFit) + LRT (glmLRT) with BH FDR; significance thresholds: $ \log_2 \text{FC} \geq 1$; $\text{FDR} \leq 0.05$. Panel A (NUC-7738): 473 downregulated and 28 upregulated genes. Panel B (3DI): 86 down and 22 up. Each dot represents one gene (green = downregulated; red = upregulated; grey = not significant). The x-axis shows the $\log_2$ fold change ($\log_2 \text{FC}$), defined as $\log_2(\text{compound}+\text{LPS}/\text{DMSO}+\text{LPS})$; the y-axis shows $-\log_{10}(\text{FDR})$; $n = 3$ per group. Note: 3DI stock contained cordycepin contamination (purity assay, Chapter 3).	211
5.10	Canonical pathway analysis of (A) NUC-7738 and (B) 3' deoxyinosine. Bubble size represents the number of genes within each pathway, while color indicates pathway activation status: negative (blue, inhibited), positive (orange, activated), or neutral (grey).	213
5.11	IPA activation z-scores for canonical pathways: NUC-7738 vs. 3'-deoxyinosine.	215
5.12	Dual-cluster heatmap of IPA z-scores: NUC-7738 vs. 3'-deoxyinosine. .	217
5.13	IPA canonical pathway coverage (stacked bars): NUC-7738 vs. 3'-deoxyinosine.	219
5.14	IPA network overlay: "Role of PKR in Interferon Induction and Antiviral Response" (cordycepin RNA-seq).	229
5.15	IPA network overlay: "Role of PKR in Interferon Induction and Antiviral Response" (suPB haploid screen).	230
6.1	Experimental time scheme for Chapter 6.	236

- 6.2 RAW264.7 macrophages were treated with either vehicle (DMSO) for 120 min as control or vehicle (DMSO) for 60 min followed by LPS (1 $\mu\text{g}/\text{mL}$) for 60 min. cDNA long-read libraries were basecalled with Dorado and poly(A) tails quantified with NanopLen (modified to ingest Dorado poly(A) tags). `length.alt` = mean poly(A)-tail length (nt) for the treatment condition; NanopLen hard-codes “.alt” for treatment and uses the exact control name from metadata. (a) ECDFs of per-transcript mean poly(A)-tail length (nt); orange, DMSO; blue, LPS. (b) Per-transcript scatter vs DMSO. Axes: `mean_length.DMSO` (x) vs `mean_length.alt` (y; LPS). Statistics/thresholds used downstream: per-transcript tail change with an effect-size threshold of ± 10 nt and $\text{FDR} \leq 0.05$. Replicates: $n = 3$ biological replicates. 237
- 6.3 Same system as Fig. 6.2. (a) Volcano and (b) MA plots for LPS vs DMSO at 120 min. Transcripts surpassing ± 10 nt tail change and $\text{FDR} \leq 0.05$ are coloured red (longer) or blue (shorter). Although Fig. 6.2 shows a global distribution-level shortening, the thresholded significant set skews towards lengthening at the per-transcript level. Replicates: $n = 3$ 238
- 6.4 Cordycepin-induced poly(A) remodeling (ECDF and scatter). RAW264.7 cells were pre-treated with natural or synthetic cordycepin (20 μM , 1 h), then stimulated with LPS (1 $\mu\text{g}/\text{mL}$, 1 h); orange line. DMSO+LPS is the control; blue line. `length.alt` = mean poly(A)-tail length (nt) for the treatment condition; NanopLen hard-codes “.alt” for treatment and uses the exact control name from metadata. Panels: (A, C) natural cordycepin (ECDF, scatter); (B, D) synthetic cordycepin. Δ median ECDF shift (treatment – control): natural -25.4 nt (95%CI -26.0 to -24.8 ; $n = 10,446$), synthetic -25.1 nt (95%CI -25.7 to -24.6 ; $n = 10,491$). Downstream thresholds: ± 10 nt, $\text{FDR} \leq 0.05$. Replicates: $n = 3$ 242

- 6.5 Cordycepin-induced poly(A) remodeling (volcano and MA). Same samples as Fig. 6.4. (A, C) natural cordycepin; (B, D) synthetic. Volcano and MA show genes with ± 10 nt tail-change and $\text{FDR} \leq 0.05$; red = longer, blue = shorter. Approximately 3,000 genes show significant shortening under cordycepin. Replicates: $n = 3$ 243
- 6.6 NUC-7738 and 3DI (ECDF and scatter). RAW264.7 cells were treated with NUC-7738 (ProTide) or 3DI at 200 μM for 2 h, with LPS (1 $\mu\text{g/mL}$) added 1 h after the start of compound treatment (i.e. in the presence of the compound) for a 1 h stimulation; orange line. DMSO+LPS is the control; blue line. length.alt = mean poly(A)-tail length (nt) for the treatment condition; NanopLen hard-codes “.alt” for treatment and uses the exact control name from metadata. Panels: (A, C) NUC-7738; (B, D) Δ median ECDF shifts: NUC-7738 -26.3 nt (95%CI -26.9 to -25.6 ; $n = 10,414$); 3DI -6.9 nt (95%CI -7.5 to -6.4 ; $n = 10,505$).. Replicates: $n = 3$ 245
- 6.7 NUC-7738 and 3DI (volcano and MA). Same samples as Fig. 6.6. (A, C) NUC-7738; (B, D) 3DI. Thresholds: ± 10 nt, $\text{FDR} \leq 0.05$. More than 20% of expressed genes show significant tail shortening under NUC-7738, while less than 1% change under 3DI (as plotted). Replicates: $n = 3$. . 246
- 6.8 Gene-expression distributions stratified by poly(A)-tail class after exposure to natural versus synthetic cordycepin. (a) Natural cordycepin; (b) Synthetic cordycepin. Tail classes: Shortened (> 10 nt decrease), Unchanged (≤ 10 nt), Lengthened (> 10 nt increase). Violin plots show EdgeR $\log_2\text{FC}$ distributions (IsoQuant $\rightarrow$ EdgeR; $\text{FDR} \leq 0.05$). Statistics: pair-wise Dunn tests with FDR correction across classes; Red dots indicate the significantly differentially expressed genes. The figure revealed significant differences among all three classes for both compounds. 248

6.9	Poly(A)-tail class-specific gene-expression responses to NUC-7738 and 3'deoxyinosine in LPS-activated RAW264.7 macrophages. (A) NUC-7738. (B) 3'deoxyinosine (3DI). Tail classes: Shortened (> 10 nt decrease), Unchanged (≤ 10 nt), Lengthened (> 10 nt increase). Violin plots show EdgeR log2FC distributions (IsoQuant $\rightarrow$ EdgeR; FDR ≤ 0.05). Statistics: pair-wise Dunn tests with FDR correction across classes; Red dots indicate the significantly differentially expressed genes. all pair-wise differences significant for NUC-7738; all but one for 3DI.	251
6.10	Canonical-pathway z-score profiles after sequential cordycepin $\rightarrow$ LPS treatment. (A) Lengthened-tail transcripts. (B) Shortened-tail transcripts. The analysis was performed on genes whose tails became significantly longer or shorter, using the signed tail change as the log-fold-change metric.	254
6.11	IPA pathway molecule coverage: cordycepin poly(A) analysis.	259
6.12	Canonical-pathway z-score profiles after sequential NUC-7738 $\rightarrow$ LPS treatment. (A) Lengthened-tail transcripts. (B) Shortened-tail transcripts. The analysis was performed on genes whose tails became significantly longer or shorter, using the signed tail change as the log-fold-change metric.	260
6.13	IPA pathway molecule coverage: NUC-7738 poly(A) analysis.	265

6.14 (a) Time-resolved oxygen consumption rate (OCR) traces for NT (no treatment; black), DMSO vehicle (grey) and CPN (cordycepin; red). Standard Mito Stress Test injections were applied in sequence—oligomycin (ATP synthase inhibitor), FCCP (uncoupler), and rotenone/antimycin A (complex I/III inhibitors)—to derive bioenergetic parameters [8] [9] [10]. (b) Proton leak (OCR after oligomycin minus non-mitochondrial OCR) across conditions. Lower values indicate less oxygen consumption not coupled to ATP production [8] [9] [10]. (c) Spare respiratory capacity (SRC) (maximal OCR after FCCP minus basal OCR), a metric of the cell’s ability to respond to increased energy demand [8] [9] [10]. System: Human MCF-7 cells seeded at 300,000 cells/well; 50 μ M cordycepin for 2 h before the Seahorse assay. Instrument: Agilent Seahorse XF; Cell Mito Stress Test according to manufacturer guidance. NT = untreated; DMSO = vehicle; CPN = cordycepin. Acquisition and analysis: OCR is reported in pmol O₂/min. Derived parameters follow Agilent’s standard equations for the Cell Mito Stress Test (basal, ATP-linked, proton leak, maximal, SRC, and non-mitochondrial respiration). Statistics: One-way ANOVA across NT, DMSO, CPN with Tukey’s post-hoc test. 267

7.1 Early (15 min) cordycepin co-treatment modestly reshapes a subset of LPS-responsive transcripts in RAW264.7 macrophages. RAW264.7 cells were stimulated with LPS (1 μ g/mL) $\pm$ cordycepin (CoR, 20 μ M) for 15 min; three biological replicates per condition (columns DMSO_LPS_1–3, CoR_LPS_1–3). Heatmap of 51 DGE-prioritized genes (rows) with per-cell normalized counts overlaid (numbers) and row-wise scaled colors (blue = below-row mean; red = above-row mean; scale -1.5 to 2). Example features include Pik3c2a, Plcb4, Mapkbp1, Dhx29, Arrdc1, Rab29, Tifa, Cemip2 289

7.2	Canonical pathways significantly modulated by cordycepin At 15 min of simultaneous cordycepin (20 μ M) and LPS (1 μ g/mL) exposure in RAW264.7 cells. The activation z-scores, predicted by Ingenuity Pathway Analysis (IPA), indicate pathway activation (blue bars), inhibition (red bars), or no clear directional prediction (grey bars).	292
7.3	IPA canonical pathway coverage (stacked bars): cordycepin 15 min DGE.	293
7.4	RAW264.7 cells were exposed to cordycepin (20 μ M) together with LPS (1 μ g/mL) for 15 min; the treated library is labeled alt (CoR_-LPSalt) and the matched control is DMSO_-LPS. Transcript-wise mean poly(A)-tail lengths were inferred from Oxford Nanopore cDNA reads and compared with NanopLen's linear mixed-model (LMM). Panel A: Empirical cumulative distribution functions (ECDFs; linear scale) of mean tail length show near-complete overlap of alt (orange) with DMSO_-LPS (blue), indicating closely matched global distributions. Panel B: Transcript-wise scatter (linear scale) of mean tail length in alt versus control. Points cluster tightly along the identity line (red, $y = x$), with marginal box-plots summarizing each distribution, consistent with high concordance and only sparse gene-specific deviations. Method note: ONT cDNA 3'-end capture requires a free 3'-OH; transcripts terminating in 3'-deoxyadenosine would be under-represented, so absolute losses of such ends cannot be quantified here.	297

7.5 Early (15 min) cordycepin co-treatment elicits limited but detectable, bidirectional transcript-level poly(A)-tail remodeling in LPS-activated RAW264.7 macrophages (NanopLen LMM; $|\Delta\text{tail}|$ threshold = 10 nt). RAW264.7 cells were exposed simultaneously to cordycepin (20 μM) and LPS (1 $\mu\text{g}/\text{mL}$) for 15 min. The treated library is alt (CoR_LPS [alt]); the reference is DMSO + LPS. Transcript-wise mean poly(A)-tail lengths were inferred from Oxford Nanopore cDNA reads and compared with NanopLen’s linear mixed-model (LMM). (A) Volcano plot: absolute poly(A)-tail change ($\Delta = \text{alt} - \text{DMSO_LPS}$, nt) vs $-\log_{10}(p)$. Vertical dashes mark ± 10 nt; the horizontal dash marks $p = 0.05$. Significant shortening events are shown in blue ($n = 464$) and lengthening events in red ($n = 405$); non-significant transcripts are gray ($n = 22,026$). (B) MA plot: absolute Δtail (nt) vs $\log_{10}$ mean normalized counts. Colors as in (A). Larger $|\Delta\text{tail}|$ values are most frequent among low-to-moderate abundance transcripts, whereas highly expressed transcripts center near zero, consistent with modest, early remodeling rather than a global shift. 299

7.6	Early (15 min) gene-expression changes do not stratify by poly(A)-tail shift class under CoR+LPS. RAW264.7 murine macrophages; 20 μ M cordycepin (CoR) + 1 μ g/mL LPS applied simultaneously for 15 min versus DMSO+LPS control; RNA-Seq for DGE and NanopLen for transcript-level poly(A) analysis. Genes were partitioned by NanopLen LMM estimate of mean tail-length change (Δ polyA) using an absolute 10-nt threshold: Shortened (Δ polyA < -10 nt; n = 2,492; mean = -30.1 nt), Unchanged ($-10 \leq \Delta$ polyA ≤ 10 nt; n = 4,696; mean = -0.3 nt), Lengthened (Δ polyA > 10 nt; n = 2,319; mean = +30.9 nt). Y-axis: DGE log ₂ FC (CoR+LPS vs DMSO+LPS). Points: red = genes with nominal DGE p < 0.05 (edgeR; no FDR applied), grey = not significant. Violins show distribution; central bars denote medians. Pairwise comparisons annotated on the plot: Shortened vs Unchanged p = 0.465; Unchanged vs Lengthened p = 0.465; Shortened vs Lengthened p = 0.814. Poly(A) tails quantified by NanopLen (gene-level LMM; FDR set to 1.0 for inclusion—i.e., no FDR filtering of tail shifts). DGE by edgeR on bulk RNA-Seq (3 biological replicates per condition)	302
7.7	Canonical-pathway z-score profiles after simultaneous cordycepin and LPS treatment. (A) Lengthened-tail transcripts. (B) Shortened-tail transcripts. The analysis was performed on genes whose tails became significantly longer or shorter, using the signed tail change as the log-fold-change metric.	305
7.8	IPA canonical pathway coverage (stacked bars): cordycepin 15 min poly(A) lengthened and shortened gene sets.	307
8.1	Proposed LINC03121-miR-23b-3p-versican-EGFR-AKT axis and its disruption by cordycepin.	326

- 8.2 Proposed model for RToC-mediated PI3K/AKT/mTOR signalling platform assembly and its disruption by cordycepin. **(A) Normal signalling conditions.** The RNA Target of Cordycepin (RToC), potentially including LINC03121, undergoes polyadenylation resulting in a mature poly(A) tail. Poly(A)-binding protein cytoplasmic 1 (PABPC1) binds the poly(A) tail and recruits PABPC1-interacting protein 1 (PAIP1) and La-related protein 1 (LARP1). LARP1 in turn associates with mTOR complex 1 (mTORC1) via RAPTOR, forming a ribonucleoprotein signalling platform [11] [12]. This platform facilitates AKT activation and phosphorylation of downstream substrates, enabling PI3K/AKT/mTOR signal transduction. Critically, this mechanism does not require the RToC to be translated; rather, the polyadenylated RNA functions as a structural scaffold for signalling complex assembly. **(B) Cordycepin treatment.** Cordycepin is phosphorylated by adenosine kinase (ADK) to cordycepin triphosphate (CoTP), which acts as a chain-terminating substrate for poly(A) polymerases, resulting in premature termination and/or shortening of the RToC poly(A) tail. The truncated poly(A) tail cannot efficiently recruit or retain PABPC1, leading to dissociation of PAIP1, LARP1, and mTORC1 from the platform. This disassembly suppresses AKT activation and downstream PI3K/AKT/mTOR signalling independently of any requirement for ongoing protein synthesis. Abbreviations: RToC, RNA Target of Cordycepin; PABPC1, poly(A)-binding protein cytoplasmic 1; PAIP1, PABPC1-interacting protein 1; LARP1, La-related protein 1; mTORC1, mechanistic target of rapamycin complex 1; CoTP, cordycepin triphosphate; ADK, adenosine kinase. 329

8.3	Canonical and non-canonical mTORC1-mediated regulation of autophagy. Under conditions of nutrient deprivation, inhibition of mTORC1 permits assembly of the ULK1–ATG13 scaffold complex, thereby enabling the early nucleation steps required for autophagosome formation. In par- allel, mTORC1 inhibition also leads to activation of the phosphatase PP6C. Activated PP6C associates with GCN2 in a complex containing a PP6 regulatory subunit, promoting GCN2 dephosphorylation and con- sequent activation. Activated GCN2 then phosphorylates eIF2 α , which contributes to the induction of autophagy. Adapted from J. C. Wengrod and L. B. Gardner, “Cellular adaptation to nutrient deprivation: crosstalk between the mTORC1 and eIF2 α signaling pathways and implications for autophagy,” <i>Cell Cycle</i> , vol. 14, no. 16, pp. 2571–2577, Aug. 2015, doi: 10.1080/15384101.2015.1056947.	336
8.4	Cordycepin-induced poly(A) tail shortening of PABPC1–LARP1–mTOR platform components at 120 min in RAW264.7 macrophages.	342

Chapter 1

Introduction

Cordycepin (3'-deoxyadenosine) was first identified in *Cordyceps militaris* species and is structurally defined by the absence of a 3'-hydroxyl group on the ribose. This apparently minor modification is decisive for its biochemical behaviour: cellular enzymes recognise cordycepin as an adenosine analogue, and when its triphosphate form (cordycepin triphosphate, CoTP) is incorporated into a nascent poly(A) tail it cannot support further 3'-5' bond formation. After uptake via equilibrative nucleoside transporters, cordycepin is phosphorylated by adenosine kinase (ADK) and downstream nucleotide kinases to the mono-, di- and triphosphate forms, culminating in CoTP. This metabolic activation step is well established and underlies its inhibition of poly(A) synthesis, through which cordycepin can influence mRNA metabolism [13, 14]. Importantly, at the concentrations that modulate inflammation, survival and proliferation, cordycepin is not a general chain terminator of RNA polymerase II transcription: unspliced precursor levels and RNA polymerase II promoter occupancy remain largely preserved for many genes, whereas defects are detected at the level of 3'-end cleavage and poly(A) tail synthesis [15] [16] [17] [18] [19].

During canonical 3'-end processing, polyadenylation site (PAS) recognition is mediated by a multiprotein complex in which Wdr33 and Cpsf4 (also known as Cpsf30) cooperate with Cpsf1 to bind the AAUAAA motif and recruit the endonuclease Cpsf3 [20–22]. PAP α /PAP γ then extend the cleaved 3' end with adenosines. When CoTP is present, PAP α /PAP γ can mis-incorporate 3'-deoxyadenosine into the nascent tail. Be-

cause this residue lacks a 3'-OH group, incorporation causes obligatory chain termination and halts tail elongation on that molecule [13, 14]. Classic in vitro cleavage/polyadenylation assays have shown that cordycepin 5'-triphosphate inhibits both endonucleolytic cleavage and polyadenylation, consistent with observed stabilisation of the cleavage complex on the pre-mRNA [23] [24]. Consequently, increased levels of uncleaved RNA and readthrough transcripts can be detected in cordycepin-treated cells [19]. Together, these observations indicate that CoTP directly perturbs 3'-end processing by acting as a chain-terminating substrate for PAP α /PAP γ and by impairing efficient cleavage and transcription termination, rather than by globally blocking transcription. These direct effects on cleavage and polyadenylation appear to be independent of upstream receptor or kinase signalling and provide the best-characterised biochemical framework for interpreting cordycepin's downstream cellular phenotypes in this thesis.

In parallel with these CPA-centred effects, cordycepin produces highly reproducible repression of growth factor-activated signalling pathways. A systematic review of vertebrate cell studies concluded that cordycepin consistently reduces PI3K/Akt/mTOR and ERK signalling and often increases AMPK phosphorylation across diverse systems [13]. More recently, Lawrence *et al.* reported that in six cell lines, cordycepin rapidly (within ~ 30 min) attenuates PI3K/Akt/mTOR and/or RAF/MEK/ERK outputs, with marked decreases in Akt, S6K and 4E-BP1 phosphorylation [25]. In a macrophages cell line, Radhi *et al.* observed that cordycepin reduces Akt phosphorylation at both PDK1- and mTORC2-regulated sites within ~ 50 min, together with hypophosphorylation of 4E-BP1 and suppression of NF- κ B p65 nuclear localisation [26]. Although AMPK activation is frequently seen in these experiments, Lawrence *et al.* demonstrated that AMPK is not required for cordycepin to depress PI3K/Akt/mTOR signalling or to downregulate growth factor-inducible genes: the core signalling phenotype persists in AMPK-deficient cells [25].

In macrophages, depletion of the polyadenylation factor Wdr33 reduces PI3K/Akt/mTOR activity and NF- κ B p65 nuclear localisation, and CPSF4/WDR33 knockdown diminishes induction of inflammatory transcripts [26–28]. In RAW264.7 and MCF-7 cells, kinase inhibitor experiments revealed that the transcriptional response to cordycepin

most closely resembles PI3K-pathway blockade [27] [25]. Together, these observations support a working model in which intact CPA is necessary for sustained growth factor signalling and inflammatory gene expression, and in which CoTP-mediated CPA inhibition may contribute substantially to the observed repression of PI3K/Akt/mTOR and RAF/MEK/ERK pathways. At the same time, the precise direction and exclusivity of the causal relationship between CPA disruption and signalling changes remain unresolved.

Alternative explanations for cordycepin’s signalling effects have been proposed, most prominently adenosine receptor-mediated mechanisms arising from its structural similarity to adenosine. In some systems, particularly in the nervous system and certain tumour models, cordycepin or *Cordyceps* extracts appear to act, at least in part, via A₁-, A₂- or A₃-receptor activation. However, many such studies rely on extracellular cordycepin without assessing intracellular CoTP and do not address transporter or kinase dependence. In contrast, genetic and pharmacological work in macrophages and other somatic cells shows that equilibrative nucleoside transporter and ADK activity are required for the anti-inflammatory and signalling effects of cordycepin, pointing to an intracellular nucleotide mechanism rather than a surface receptor-driven one [13, 26]. The development of the ProTide NUC-7738 has provided a critical mechanistic tool. NUC-7738 delivers a masked 3′-deoxyadenosine monophosphate directly into cells, bypassing ADA-mediated deamination, nucleoside transport and the requirement for ADK, and does not act as an adenosine receptor agonist. Nevertheless, it reproduces the core antiproliferative and transcriptional effects of free cordycepin when matched for intracellular 3′-deoxyadenosine triphosphate exposure [2, 29]. These findings suggest that intracellular 3′-deoxyadenosine nucleotides (including CoTP) are sufficient to account for the relevant growth factor and NF- κ B signalling phenotypes in the systems studied here, and that adenosine receptors are unlikely to be the primary mechanism in these contexts, even if they may contribute elsewhere.

One report using purified AKT has suggested that unmodified cordycepin can directly inhibit AKT kinase activity [30]. However, most cellular and biochemical evidence in mammalian systems points to CoTP as the relevant active metabolite, and Lawrence’s

comparative transcriptional profiling found limited overlap between the effects of AKT inhibitors and cordycepin [25]. At present, we therefore favour the following hypotheses: (i) CoTP-mediated CPA inhibition is a well-established direct biochemical effect of cordycepin, (ii) repression of PI3K/Akt/mTOR and RAF/MEK/ERK signalling is a consistent and rapid cellular phenotype, and (iii) the exact molecular target(s) linking CoTP to these signalling changes remain to be identified. The experimental work in this thesis is designed to test how CPA inhibition and CoTP metabolism could cause these signalling and transcriptional effects Figure 1.1.

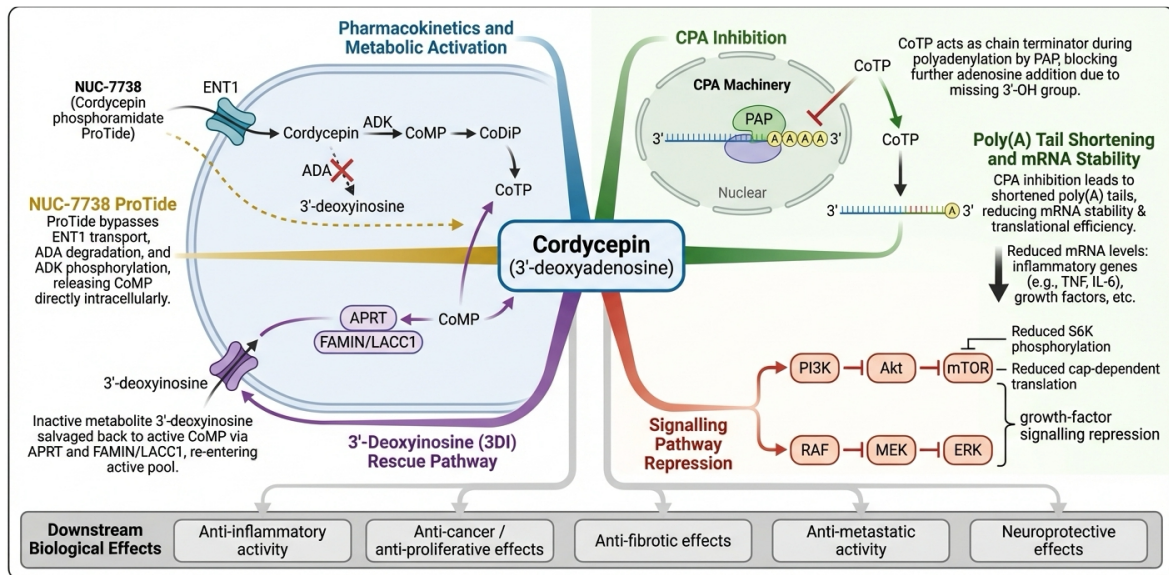


Figure 1.1: Overarching scheme of cordycepin's mechanisms of action. Cordycepin is taken up via equilibrative nucleoside transporters (ENT1) and phosphorylated by adenosine kinase (ADK) to cordycepin triphosphate (CoTP), which acts as a chain-terminating substrate for poly(A) polymerase during cleavage and polyadenylation (CPA). CPA inhibition leads to poly(A) tail shortening, altered mRNA stability and repression of PI3K/Akt/mTOR and RAF/MEK/ERK growth-factor signalling. Competing deamination by adenosine deaminase (ADA) converts cordycepin to 3'-deoxyinosine (3DI), which can be partially recycled via the FAMIN/LACC1-APRT salvage pathway. The ProTide NUC-7738 bypasses ENT1-dependent transport, ADA-mediated degradation and the ADK phosphorylation requirement, delivering the monophosphate directly to the intracellular nucleotide pool. Downstream biological consequences include anti-inflammatory, anti-cancer, anti-fibrotic, anti-metastatic and neuroprotective activities.

1.1 Cordycepin administration, absorption, distribution and excretion

Cordycepin (3'-deoxyadenosine) is a hydrophilic adenosine analogue with potent biological effects but unfavourable pharmacokinetics. Its in vivo disposition is dominated by rapid adenosine deaminase (ADA)-mediated deamination to 3'-deoxyinosine (3DI), dependence on equilibrative nucleoside transporters for cellular entry, and adenosine kinase (ADK)-dependent phosphorylation to the active triphosphate (cordycepin 5'-triphosphate, CoTP) [1] [31] [13]. These features constrain both oral bioavailability and tissue distribution of CoTP and are central to interpreting cordycepin's pharmacology.

1.1.1 Routes of administration and solubility

Cordycepin has been administered orally, intraperitoneally and intravenously in pre-clinical models and a small number of clinical studies [31] [13] [32]. Solubility is not the main barrier to exposure: Lee *et al.* showed that cordycepin is highly soluble in fasted-state simulated gastric fluid (FaSSGF), fasted-state simulated intestinal fluid (FaSSIF) and fed-state simulated intestinal fluid (FeSSIF), with the highest solubility in acidic FaSSGF [1]. Simple aqueous or co-solvent formulations are therefore sufficient to achieve complete dissolution at therapeutically relevant concentrations.

Parenteral pharmacokinetic studies indicate that cordycepin behaves as a high-clearance, short half-life compound. In rats, intravenous cordycepin displays rapid multi-exponential decline, high plasma clearance and a relatively large volume of distribution [1] [33]. Tsai *et al.* reported that after i.v. administration of 10 mg/kg cordycepin, blood levels decline rapidly within 30 min, with short elimination half-lives and high clearance comparable to adenosine [33]. These properties make sustained systemic exposure difficult to maintain with standard dosing regimens.

1.1.2 ADA-mediated deamination and oral bioavailability

The major metabolic liability of cordycepin is rapid deamination of its adenine base by ADA to yield 3DI. Cordycepin is an excellent substrate for ADA in plasma and liver. In

vitro, Lee *et al.* showed that cordycepin is rapidly degraded in rat and human plasma at 37 °C, whereas co-incubation with the ADA inhibitor pentostatin almost completely stabilises the nucleoside [1]. In liver microsomes, cordycepin exhibits high intrinsic clearance that is markedly reduced by ADA inhibition, while omission of NADPH has little effect, indicating that oxidative metabolism makes a minor contribution compared with deamination [1]. Reviews by Qin *et al.*, Radhi *et al.* and Chen *et al.* converge on the conclusion that rapid ADA-mediated deamination is the dominant clearance route limiting cordycepin’s systemic half-life and oral bioavailability [31] [13] [32].

In vivo, these metabolic properties translate into almost negligible oral bioavailability of the parent drug. In rats, oral administration of cordycepin at 8 or 80 mg/kg produced no detectable intact cordycepin in plasma at any time (limit of quantification 2 ng/mL), whereas substantial systemic levels of 3DI were observed [1]. Pharmacokinetic analysis at 8 mg/kg yielded an apparent oral bioavailability (F_{oral}) for 3DI of approximately 37%, indicating that the deaminated metabolite—not the parent—is efficiently absorbed [1]. Tsai *et al.* similarly found that cordycepin has a short half-life and high clearance in rats, and that the ADA inhibitor EHNA markedly increases the area under the curve (AUC), half-life and mean residence time of cordycepin [33]. These findings are consistent with more recent reviews emphasising that low oral bioavailability is due to a combination of low permeability, high hepatic first-pass extraction and rapid ADA-mediated deamination [31] [13] [32] [6].

1.1.3 Intestinal absorption and nucleoside transport

Caco-2 monolayer studies help to dissect intestinal handling of cordycepin and 3DI. In the presence of pentostatin, cordycepin shows low apparent permeability in both apical-to-basolateral and basolateral-to-apical directions, consistent with limited passive diffusion across the intestinal epithelium [1]. When cordycepin is applied without pentostatin, cordycepin itself is rapidly degraded and only 3DI is detected in the basolateral chamber, demonstrating active deamination by ADA expressed in or secreted from the Caco-2 cells [1]. Donor-chamber analyses confirm that cordycepin concentrations decline sharply in the absence of ADA inhibition, whereas they remain stable when pentostatin is present [1].

3DI exhibits modest Caco-2 permeability (P_{app} on the order of 0.2×10^{-6} cm/s) yet achieves moderate F_{oral} in vivo, implying the contribution of carrier-mediated uptake in the gut [1]. Together, these data support a model in which (i) cordycepin is poorly permeable and extensively deaminated during transit across the intestinal wall, and (ii) 3DI is the main species that crosses the epithelium and enters the portal circulation.

At the cellular level, cordycepin's entry into target cells such as macrophages and cancer cells depends on equilibrative nucleoside transporters (particularly hENT1). Qin *et al.* and Serpi *et al.* highlight that low hENT1 expression reduces cellular uptake of 3'-deoxyadenosine and contributes to resistance, and that transporter-independent Pro-Tides can circumvent this barrier [31] [2]. In macrophage-like RAW264.7 cells, inhibition of equilibrative nucleoside transporters with nitrobenzylthioinosine (NBTI) abolishes cordycepin's suppression of LPS-induced inflammatory gene expression, indicating that intracellular accumulation of the nucleoside is necessary for activity [1] [13].

1.1.4 Systemic distribution and first-pass metabolism

After intravenous dosing, cordycepin is rapidly distributed and cleared. In rats, Lee *et al.* reported unusual multi-peak plasma concentration–time profiles between 8 and 20 mg/kg i.v., suggesting complex distribution and elimination; clearance increased at the higher dose, and blood:plasma ratios < 1 excluded sequestration in red blood cells as the source of the multiple peaks [1]. Using intrinsic clearance data from rat and human liver microsomes, the same study predicted high hepatic extraction fractions for cordycepin, especially under parallel-tube assumptions, implying that any fraction absorbed from the intestine would be extensively removed on the first pass through the liver [1].

These conclusions are consistent with Tsai *et al.*, who observed short elimination half-lives and high clearance for cordycepin in rats, with ADA inhibition via EHNA substantially increasing exposure [33]. Reviews by Qin *et al.* and Chen *et al.* interpret these findings as evidence that high hepatic first-pass extraction, in combination with low permeability and gut-wall deamination, is sufficient to explain the absence of detectable intact cordycepin in plasma after oral dosing [31] [32].

1.1.5 3'-Deoxyinosine as an orally bioavailable pro-drug

A key result from Lee *et al.* is that 3DI, historically considered an inactive catabolite, is efficiently absorbed and achieves substantial systemic exposure after oral dosing of cordycepin [1]. In that study, oral cordycepin at 8 mg/kg yielded robust 3DI plasma levels with $F_{\text{oral}} \approx 37\%$, even though unchanged cordycepin remained below the limit of detection [1]. In RAW264.7 macrophage-like cells, both cordycepin and 3DI are converted to CoTP. Although 3DI is 10-fold less efficient than equimolar cordycepin at generating CoTP, it still suppresses LPS-induced inflammatory gene expression when present at higher concentrations, with lower maximal effect [1]. These observations led to the proposal of a “nucleoside rescue” pathway in which 3DI acts as an orally bioavailable pro-drug that is salvaged back to cordycepin nucleotides and CoTP in target tissues [1] [13].

Within this framework, orally administered cordycepin is deaminated to 3DI in the gut lumen, intestinal mucosa and liver; 3DI is then absorbed, distributed and, in susceptible cell types such as macrophages, reconverted to CoTP via purine salvage enzymes [1] [13]. This model reconciles the absence of measurable intact cordycepin in plasma with robust pharmacodynamic effects observed in rodent models after oral dosing, including anti-inflammatory activity and disease modification in osteoarthritis and other inflammatory settings [13] [6] [28].

1.1.6 Cellular uptake and ADK-dependent activation

Once inside the cell, cordycepin must be phosphorylated to mediate its principal biochemical actions. ADK catalyses the first and rate-limiting step, converting cordycepin to 3'-deoxyadenosine monophosphate (CoMP), which is then converted to the diphosphate and triphosphate forms by downstream kinases [31] [2]. Qin *et al.* summarise evidence that ADK is a key determinant of cellular sensitivity to cordycepin: cells with low ADK expression exhibit reduced CoTP formation and resistance to cordycepin, while ADK overexpression or activation enhances its cytotoxic and anti-inflammatory effects [31]. In RAW264.7 cells, ADK inhibition with 5-iodotubercidin prevents CoTP accumulation and abolishes cordycepin-mediated suppression of inflammatory markers,

confirming that phosphorylation is obligatory for activity in this system [1] [13].

Nucleoside transporter and ADK dependencies together define a strict gating mechanism: cordycepin must cross the plasma membrane via transporters such as hENT1 and be phosphorylated by ADK to initiate the CoTP cascade, whereas 3DI must enter cells and be salvaged into the cordycepin nucleotide pool via host purine metabolism [1] [31] [13] [2]. Tissues with low hENT1 or ADK expression are therefore expected to accumulate less CoTP and be relatively insensitive to cordycepin, a pattern consistent with resistance observed in some cancer cell lines [13] [2] [6] Figure 1.2.

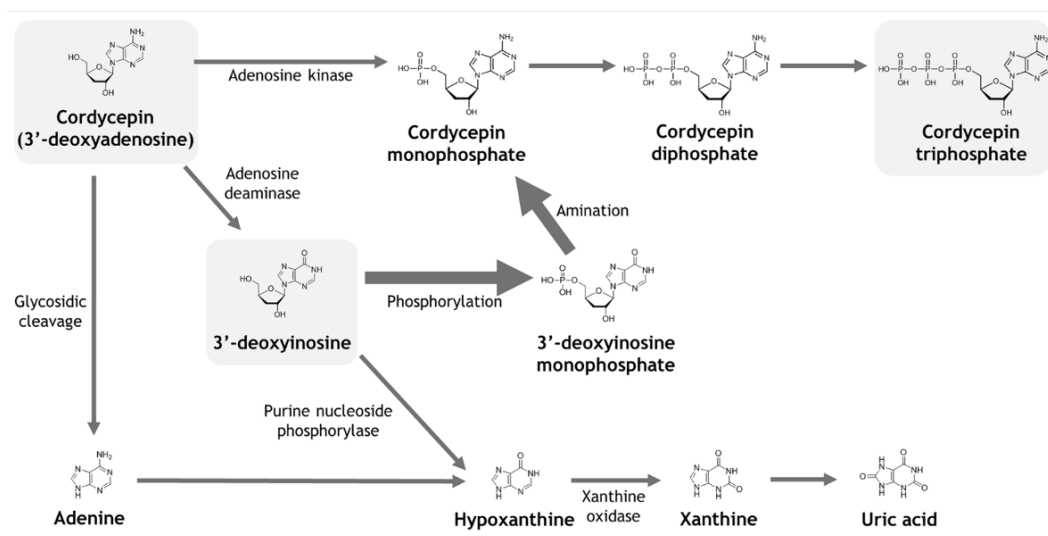


Figure 1.2: Metabolic pathways of cordycepin (3'-deoxyadenosine). **Upper pathway:** Intracellular phosphorylation by adenosine kinase (ADK) yields cordycepin monophosphate (CoMP), which is sequentially converted to cordycepin diphosphate (CoDP) and cordycepin triphosphate (CoTP), the principal active metabolite. **Lower pathway:** Adenosine deaminase (ADA)-mediated deamination produces 3'-deoxyinosine (3DI), which undergoes glycosidic cleavage by purine nucleoside phosphorylase (PNP) to release adenine and 3'-deoxyribose. Adenine enters standard purine catabolism via hypoxanthine, xanthine and uric acid. Adapted from Lee *et al.* [1].

1.1.7 Excretion and downstream purine catabolism

After deamination, 3DI enters canonical purine catabolic pathways. 3DI can be cleaved by purine nucleoside phosphorylase to produce 3'-deoxyhypoxanthine, which is further oxidised by xanthine oxidase to 3'-deoxyxanthine and ultimately to a uric acid analogue that is excreted renally [1] [31]. In the rat study by Lee *et al.*, 3DI exhibited moderate clearance and short half-lives after both intravenous and oral dosing, consistent with efficient renal elimination and/or subsequent metabolism [1]. Because cordycepin is

almost completely converted to 3DI prior to or during absorption—especially after oral administration—the majority of excreted material is expected to derive from 3DI and its downstream catabolites rather than from intact cordycepin [1] [31] [32].

1.1.8 Strategies to overcome metabolic bottlenecks: ADA inhibition and ProTides

Three principal strategies have been explored to address cordycepin’s metabolic liabilities: (i) ADA inhibition, (ii) structural modification (including ProTides), and (iii) novel delivery systems [31] [32].

Co-administration of ADA inhibitors such as pentostatin (deoxycoformycin) or EHNA can markedly prolong cordycepin stability and enhance efficacy in preclinical models. Tsai *et al.* showed that EHNA pretreatment significantly increased the AUC, half-life and mean residence time of cordycepin in rats [33]. In parasitology, Dalla Rosa *et al.* demonstrated that cordycepin–pentostatin combination therapy cures mice experimentally infected with *Trypanosoma evansi*, whereas either drug alone is less effective [34]. However, ADA inhibition also unmasks significant toxicity. In beagle dogs, Rodman *et al.* found that cordycepin combined with 2′-deoxycoformycin produced severe dose-limiting gastrointestinal and bone-marrow toxicity, while cordycepin alone was well tolerated [35]. In the mouse *T. evansi* model, the cordycepin–pentostatin combination caused hepatotoxicity and nephrotoxicity despite its efficacy [34]. Chen *et al.* therefore regard systemic ADA inhibition as a high-risk approach: it improves cordycepin exposure but at the cost of significant toxicity [32].

A more targeted pharmacological strategy is to use ProTide technology to mask the 5′-monophosphate and deliver it directly into cells. NUC-7738 is a 5′-aryloxy phosphoramidate prodrug of 3′-deoxyadenosine designed to bypass ADA, hENT1 and ADK dependence Figure 1.3 [29] [2]. The phosphoramidate cap is cleaved intracellularly by esterases and the phosphoramidase HINT1, releasing 3′-deoxyadenosine monophosphate (3′-dAMP), which is then converted to 3′-dADP and 3′-dATP (CoTP) [29] [2]. Schwenzer *et al.* showed that NUC-7738 is several-fold more potent than free 3′-deoxyadenosine across diverse cancer cell lines, and that its activity is largely unaffected by ADA

inhibition, hENT1 blockade or ADK knockout, whereas these manipulations strongly affect 3'-deoxyadenosine [29]. Serpi *et al.* confirmed that NUC-7738 is resistant to ADA, enters cells efficiently and produces sustained intracellular 3'-dATP accumulation, making it a useful tool for probing 3'-deoxyadenosine nucleotide mechanisms [2]. A phase I clinical trial reported predictable pharmacokinetics and evidence of antitumour activity, further supporting the ProTide concept [29].

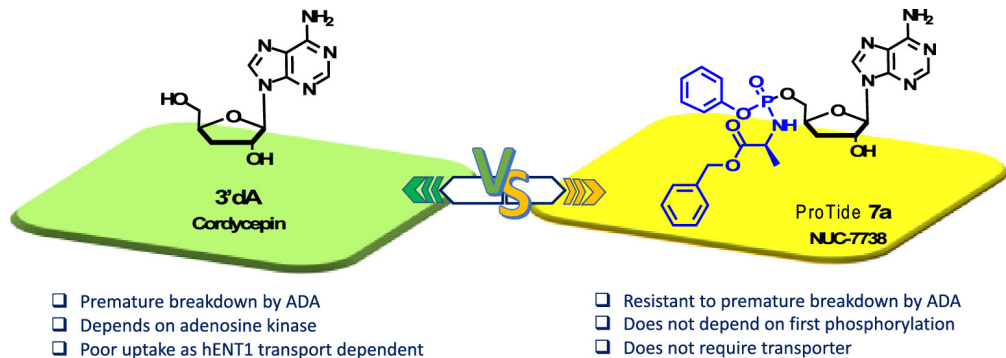


Figure 1.3: Structural comparison of cordycepin (3'-deoxyadenosine, 3'dA; left) and the ProTide NUC-7738 (ProTide 7a; right). Cordycepin is susceptible to premature deamination by adenosine deaminase (ADA), requires adenosine kinase (ADK) for initial phosphorylation, and depends on equilibrative nucleoside transporter 1 (hENT1) for cellular uptake. NUC-7738 carries a 5'-aryloxy phosphoramidate cap that is cleaved intracellularly, thereby bypassing ADA-mediated degradation, the requirement for ADK-dependent first phosphorylation, and the dependence on nucleoside transporters for cellular entry. Note the absence of the 3'-hydroxyl group on the ribose in both compounds (3'-OH replaced by 3'-H), which is responsible for chain termination during poly(A) tail synthesis. Adapted from Serpi *et al.* [2].

From the perspective of this thesis, NUC-7738 is primarily relevant as a mechanistic probe. By delivering a 3'-deoxyadenosine monophosphate moiety intracellularly without engaging ADA, hENT1 or ADK, it allows the contribution of intracellular 3'-deoxyadenosine nucleotides (CoTP/3'-dATP) to be evaluated independently of the parent nucleoside's pharmacokinetic limitations.

Overall, cordycepin's pharmacokinetic profile is characterised by (i) rapid ADA-mediated deamination to 3DI in plasma, liver and intestinal tissues, (ii) poor intrinsic oral bioavailability of the parent nucleoside, (iii) transporter- and ADK-dependent intracellular activation to CoTP, and (iv) substantial reliance on a "nucleoside rescue" pathway in which 3DI functions as an orally bioavailable pro-drug that is salvaged back to cordycepin nucleotides in target cells [1] [31] [13] [32]. These constraints are central to understanding when and where CoTP can accumulate sufficiently to inhibit cleavage

and polyadenylation and to modulate growth-factor signalling. They also motivate the use of ProTide derivatives such as NUC-7738 as mechanistic tools in the experimental work described in later chapters, rather than as primary therapeutic strategies in their own right.

The pharmacokinetic properties described above have practical implications for the concentrations of cordycepin and its derivatives used in cell-based experiments throughout this thesis. Cordycepin is typically effective at 20–50 μM in tissue culture, reflecting efficient uptake via ENT1 and ADK-dependent phosphorylation to CoTP. 3DI requires approximately 10-fold higher concentrations (200–400 μM) to achieve comparable anti-inflammatory effects, consistent with its lower efficiency of conversion to CoTP via the salvage pathway. NUC-7738, which bypasses ADA, ENT1 and ADK, achieves comparable intracellular CoTP levels at intermediate concentrations. These dose relationships are important for interpreting the comparative experiments in Chapters 3, 5, 6 and 7, where compound concentrations were chosen to give informative pharmacological dynamic range rather than strict equipotency.

1.2 Poly(A) tail synthesis and function in mRNA metabolism

Cleavage and polyadenylation at the 3'-end of pre-mRNAs are essential steps in eukaryotic mRNA biogenesis. After transcription of a polyadenylation signal (PAS) in the 3'-untranslated region (3'UTR), the nascent transcript is endonucleolytically cleaved and a poly(A) tail is added de novo to the new 3' terminus. This apparently simple modification underpins multiple layers of post-transcriptional regulation: it contributes to efficient transcription termination, licences nuclear export, and shapes mRNA stability and translational output in the cytoplasm [20] [36].

1.2.1 Canonical cleavage and nuclear polyadenylation

In the canonical pathway, PAS recognition is mediated by the cleavage and polyadenylation specificity factor (CPSF) complex, which includes CPSF1, CPSF4 and WDR33. CPSF1 directly contacts the conserved AAUAAA hexamer, while WDR33 and CPSF4

stabilise this interaction and help position the endonuclease subunit (often referred to as CPSF3/CPSF73 depending on the nomenclature) at a defined cleavage site 10–30 nucleotides downstream [20]. Cooperative interactions with the cleavage stimulation factor (CstF), which recognizes a GU/U-rich downstream sequence element, together with auxiliary multi-protein complexes, specifically cleavage factors I and II (CFIm and CFIIIm), ensure accurate definition of the 3' end of the vast majority of protein-coding genes [20].

Following cleavage, canonical nuclear poly(A) polymerases PAP α and PAP γ extend the new 3'-end with adenosine residues, typically towards lengths of $\approx$ 200–250 nucleotides in many metazoan systems [20] [36]. Poly(A) extension is tightly coupled to binding of the nuclear poly(A)-binding protein PABPN1. PABPN1 interacts with the nascent tail as it emerges from PAP α/γ , stimulates polymerase processivity and helps define a fairly uniform tail-length distribution by providing an allosteric brake when sufficient PABPN1 molecules are loaded [37]. In this way, the CPSF–PAP–PABPN1 module integrates sequence-specific PAS recognition with controlled tail synthesis.

The nuclear poly(A) tail serves several functions beyond simply protecting the 3' end. Correctly cleaved and polyadenylated mRNAs are preferentially assembled into export-competent messenger ribonucleoprotein particles (mRNPs), in part because PABPN1-occupied tails contribute to the recruitment of export adaptors and quality-control factors that discriminate mature transcripts from improperly processed RNA [36] [37]. Incomplete or aberrant polyadenylation instead promotes nuclear retention and degradation by the nuclear exosome. Thus, the presence of a PABPN1-coated poly(A) tail is an important component of the “maturity signature” that licences mRNAs for export.

A further layer of complexity arises from alternative polyadenylation (APA), where the same pre-mRNA uses multiple PASs to generate isoforms with different 3'UTR lengths. Shifts between proximal and distal PAS usage can remodel the repertoire of RNA-binding protein and miRNA binding sites in the 3'UTR and therefore alter mRNA stability and translational regulation without changing the encoded protein [20] [38]. APA patterns are sensitive to CPSF/CstF levels and kinetics, and are frequently

remodelled during proliferation, differentiation and stress. For the purposes of this thesis, it is sufficient to note that the CPA machinery not only decides whether a poly(A) tail is added, but also where in the transcript the tail is attached.

1.2.2 Cytoplasmic poly(A) tails and poly(A)-binding proteins in translation

Once export is complete, nuclear PABPN1 is replaced on the poly(A) tail by cytoplasmic poly(A)-binding proteins (PABPs), most prominently PABPC1. PABPC1 binds with high affinity to stretches of adenosines near the 3'-end, with each molecule requiring around a dozen nucleotides and covering approximately 25–30 nucleotides along the tail [36]. Multiple PABPC molecules can therefore coat a single tail.

Cytoplasmic PABPs have two central roles. First, they protect the poly(A) tail from rapid attack by 3'–5' exonucleases and deadenylase complexes. Second, they couple the 3'-end of the mRNA to the translation initiation machinery at the 5'-cap. PABPC1 interacts directly with the scaffold factor eIF4G, which also binds the cap-binding protein eIF4E. This eIF4E–eIF4G–PABPC1 interaction promotes formation of a “closed-loop” mRNP in which the 5' and 3' ends are brought into proximity [36]. Closed-loop formation enhances both the efficiency of translation initiation and the probability that terminating ribosomes will reinitiate on the same mRNA. Experiments in several systems have shown that mRNAs bearing both a cap and a poly(A) tail are translated more efficiently than those carrying only one of these features [36] [39]. However, genome-wide analyses in some tissues do not show a simple monotonic correlation between poly(A) tail length and translational efficiency, indicating that the relationship between tail size and translation is context-dependent and mechanistically complex [40] [41].

Different PABP family members (for example, PABPC1, PABPC4 and germ cell-specific PABPs) are expressed in distinct tissues, but they share the basic function of linking poly(A) tail integrity to translation and stability [36]. The number and occupancy of PABPC molecules on a given tail therefore act as a key readout of poly(A) tail length that can be interpreted by the translational machinery and decay systems.

1.2.3 Cytoplasmic polyadenylation as a translational switch.

In addition to nuclear tail synthesis, poly(A) tails can be extended in the cytoplasm. Cytoplasmic polyadenylation is particularly prominent in oocytes, early embryos and neurons, where many mRNAs are exported with short poly(A) tails and stored in a translationally repressed state. Upon appropriate stimuli, specific RNA-binding proteins, notably members of the cytoplasmic polyadenylation element-binding (CPEB) family, recognise sequence elements in the 3'UTR and recruit non-canonical cytoplasmic poly(A) polymerases such as GLD2/GLD4 (PAPD4/PAPD5) [39]. Tail extension increases PABPC occupancy, strengthens cap–tail synergy and thereby activates translation of previously silent mRNAs.

Cytoplasmic polyadenylation is opposed by deadenylases, especially PARN (poly(A)-specific ribonuclease), which can remove adenosines from the 3' end and thereby keep certain mRNAs in a short-tailed, translationally repressed state [37] [39]. Dynamic competition between CPEB–GLD2/GLD4-mediated tail extension and PARN-mediated deadenylation allows rapid on–off switching of translation in response to synaptic activity, developmental cues or other signalling events without requiring new transcription. Although most of the detailed work has been done in oocytes and neurons, the same logic applies in other cell types where regulated cytoplasmic polyadenylation has been implicated [42] [43].

1.2.4 Deadenylation pathways and mRNA turnover

For the majority of mRNAs, deadenylation is the first and often rate-limiting step in cytoplasmic mRNA decay. As long as the poly(A) tail is sufficiently long and densely coated with PABPC, the transcript is relatively protected from both 3'-5' and 5'-3' exonucleases. Progressive tail shortening reduces PABPC occupancy, exposes the 3' end to decay factors and eventually triggers decapping and body degradation [36] [39] [44].

Two major deadenylase systems mediate bulk tail shortening. PAN2–PAN3 usually initiates deadenylation on long tails by trimming distal adenosines, often while PABPC still occupies more proximal segments. This is followed by a more processive phase driven by the CCR4–NOT complex, which erodes the tail towards an oligo(A) remnant

of ≈ 10 – 15 nucleotides that can no longer support stable PABPC binding [36] [44]. Loss of PABPC opens the way for decapping by the DCP1–DCP2 complex and exposure of the 5' end to the 5'-3' exonuclease XRN1; in parallel, the exosome can degrade the RNA body from the 3' end [39] [44]. In this canonical deadenylation-dependent decay pathway, poly(A) tail shortening is the decisive checkpoint that commits mRNAs to degradation.

PARN is a specialised deadenylase with somewhat different properties. It is an allosterically regulated, processive enzyme that interacts with the mRNA cap and can act both in the nucleus and cytoplasm [37]. PARN contributes to nuclear surveillance of improperly polyadenylated transcripts and, in the cytoplasm, participates in regulated decay and translational repression of specific mRNAs, including those subject to cytoplasmic polyadenylation control [37] [39]. In CPEB-driven systems, PARN-mediated shortening of tails counterbalances GLD2/GLD4-mediated elongation, providing an additional lever to tune translation.

Deadenylation kinetics are further modulated by sequence elements and RNA-binding proteins in the 3'UTR. AU-rich and GU-rich elements, for example, recruit factors that accelerate recruitment of CCR4–NOT and other decay machineries, while stabilising elements can prolong tail lifetime [39] [44]. Ribosome occupancy also influences deadenylation: transcripts with long tails and high translational engagement tend to occupy heavy polysome fractions and are deadenylated more slowly than short-tailed, poorly translated isoforms [36] [12]. Overall, poly(A) tail length is both a determinant and a readout of mRNA stability and translation state.

1.2.5 Roles of poly(A) tails in stability, export and translation

Taken together, these nuclear and cytoplasmic processes make the poly(A) tail a central hub in mRNA metabolism. In the nucleus, proper cleavage and polyadenylation followed by PABPN1 loading are prerequisites for efficient transcription termination, mRNP assembly and export competence. Defective or incomplete tails are recognised by quality-control pathways and targeted for nuclear decay rather than export [20] [36] [37].

After export, the number and distribution of PABPC molecules on the tail influence

both translation and stability. Long, PABPC-rich tails favour formation of the cap–tail closed loop, efficient ribosome recruitment and reinitiation, and relative resistance to deadenylation. As deadenylation proceeds and the tail falls below the threshold required for stable PABPC binding, translation is dampened and the transcript is channelled into decapping and exonucleolytic decay [36] [39] [44]. In developmental systems with extensive cytoplasmic polyadenylation, changes in tail length directly encode changes in translational output; in many somatic contexts the relationship is more nuanced, but tail length still correlates with ribosome loading for specific classes of mRNAs, such as TOP mRNAs under mTOR/LARP1 control [36] [12].

From the perspective of this thesis, the key point is that poly(A) tail length and composition are not passive by-products of 3'-end processing, but actively shape which mRNAs are exported, how long they persist in the cytoplasm, and how efficiently they are translated. Any perturbation that alters the efficiency of canonical cleavage and polyadenylation, or the balance between cytoplasmic polyadenylation and deadenylation, will therefore reprogramme gene expression at the level of both mRNA abundance and protein synthesis. This canonical framework underlies the later sections, where cordycepin triphosphate will be considered as a chain-terminating substrate for poly(A) synthesis and as an entry point into poly(A)-tail-dependent control of inflammatory and growth-factor signalling transcripts. Figure 1.4 summarize Poly(A) tail synthesis, function, and metabolism in eukaryotic mRNA.

1.3 Cordycepin triphosphate as an inhibitor of cleavage and polyadenylation

The active metabolite of cordycepin, cordycepin triphosphate (cordyTP, hereafter CoTP), provides a well-defined biochemical entry point into the cleavage and polyadenylation (CPA) pathway. CoTP is an adenosine triphosphate (ATP) analogue: it can be used by nuclear poly(A) polymerases PAP α/γ in place of ATP, but the absence of a 3'-hydroxyl group on the ribose precludes formation of the next 3'-5' phosphodiester bond [27, 28]. Once incorporated into a nascent poly(A) tail, CoTP therefore acts as a true chain terminator for tail extension. Importantly, at the cordycepin concentrations that affect inflammation, survival and proliferation in mammalian cells, CoTP is *not* a general chain terminator of mRNA transcription: classic biochemical and cell-based studies in HeLa cells, nerve and glial cells, and more recent work in airway smooth muscle cells and yeast report that pre-mRNA synthesis, heterogeneous nuclear RNA levels and transcription of housekeeping genes are largely preserved, whereas poly(A)-dependent 3'-end processing and mRNA accumulation are selectively impaired [15] [16] [17] [18] [45] [19]

Critically, CoTP does not simply generate uniformly shorter tails. Biochemical work in nuclear extracts indicates that cordycepin-terminated tails reduce the efficiency of 3'-end cleavage, consistent with a model in which CoTP both terminates poly(A) synthesis and stabilises the PAS-bound CPA complex. In particular, CoTP-terminated 3' ends stall key CPA recognition subunits, notably CPSF4 (also known as CPSF30) and WDR33, on incomplete 3' ends and thereby reduce their availability for subsequent rounds of processing [23] [24] [28].

These two linked effects—poly(A)-tail chain termination and CPA-factor trapping—constitute a well-supported mechanism by which cordycepin nucleotides perturb RNA 3'-end formation and poly(A)-tail homeostasis.

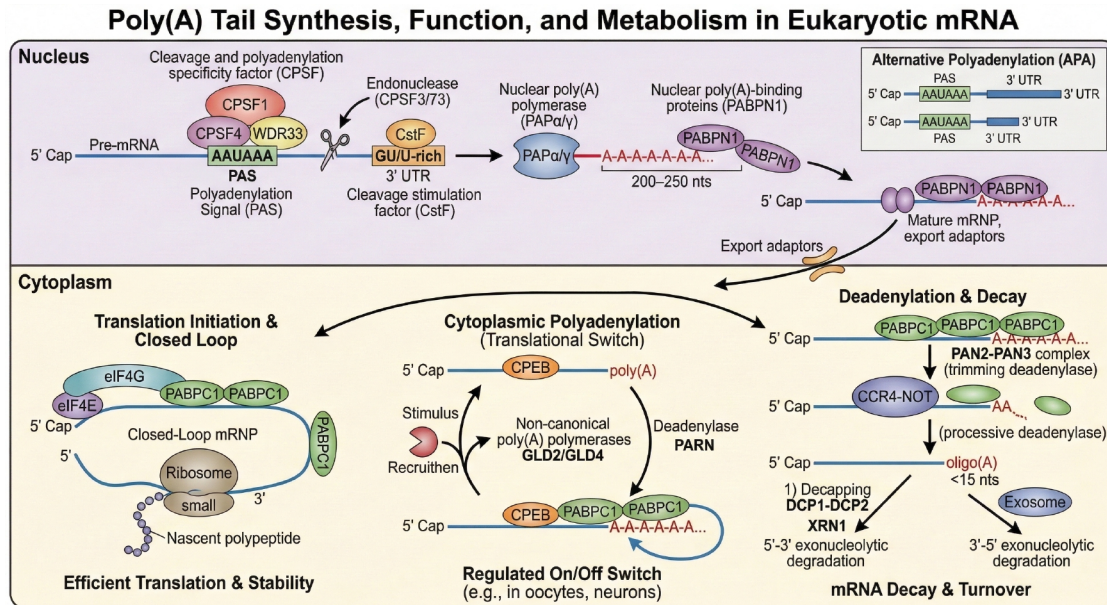


Figure 1.4: Poly(A) tail synthesis, function, and metabolism in eukaryotic mRNA. Schematic overview of the major steps governing poly(A) tail biogenesis and post-transcriptional regulation. (Top, nucleus): Cleavage and polyadenylation (CPA) of pre-mRNAs are initiated when the CPSF complex (CPSF1, CPSF4, WDR33) recognizes the polyadenylation signal (PAS; AAUAAA) embedded within the 3'UTR. Cooperative binding of CstF to a downstream GU/U-rich element positions the CPSF3/CPSF73 endonuclease to cleave the nascent transcript 10–30 nucleotides downstream of the PAS. Following cleavage, the canonical nuclear poly(A) polymerases PAP α / γ extend the 3' end to produce a $\approx$ 200–250-nt poly(A) tail, while PABPN1 binds the elongating tail to stimulate processivity and enforce a characteristic tail-length distribution. Mature mRNPs are assembled with export adaptors and transported to the cytoplasm. Alternative polyadenylation (APA), via selection of proximal or distal PASs within the 3'UTR, generates mRNA isoforms with different 3'UTR architectures and regulatory potential. (Bottom, cytoplasm): Cytoplasmic PABPC1 replaces PABPN1 and cooperates with eIF4G and eIF4E to form a cap–tail “closed-loop” mRNP that promotes efficient translation initiation and ribosome recycling. In specific contexts (e.g., oocytes, neurons), cytoplasmic polyadenylation factors such as CPEB recruit non-canonical poly(A) polymerases (GLD2/GLD4) to lengthen short poly(A) tails and activate translation. This process is counterbalanced by the deadenylase PARN, enabling rapid translational on/off switching. Bulk deadenylation during mRNA turnover proceeds through a two-step pathway: initial trimming by the PAN2–PAN3 complex, followed by processive shortening by the CCR4–NOT deadenylase to oligo(A) tails of < 15 nt. Loss of PABPC1 binding triggers decapping (DCP1–DCP2) and exonucleolytic decay by XRN1 (5' $\rightarrow$ 3') or the exosome (3' $\rightarrow$ 5'). Together, these pathways integrate transcriptional outputs with mRNA stability, translation, and decay dynamics across the mRNA life cycle. The figure was created with Sketch v 2025.3.1

1.3.1 CoTP as a chain-terminating substrate for PAP α/γ

Under normal conditions, CPSF1/CPSF4/WDR33 recognise the PAS and recruit CPSF3 for endonucleolytic cleavage, after which PAP α/γ extend the new 3' terminus with ATP in a PABPN1-stimulated reaction until tails reach ≈ 200 –250 nt and are saturated with PABPN1 [20] [36]. When CoTP is present at sufficient concentrations, PAP α/γ can mis-incorporate it into the growing poly(A) tail because it closely mimics ATP in the active site [46] [14] [47] [45]. However, incorporation of a 3'-deoxyribonucleotide immediately blocks further elongation, because no 3'-OH remains to support the next phosphodiester bond. Even rare CoTP incorporation events are thus catastrophic for completion of an individual tail: once a CoTP residue is added, PAP α/γ cannot resume extension on that molecule [46] [14] [47] [45].

Biochemically, this yields nuclear poly(A) tails that are markedly shorter than canonical lengths and that cannot accommodate the normal complement of PABPN1 molecules [45]. Steady-state analyses in cordycepin-treated systems report a shift in median tail lengths from ≈ 200 nt towards much shorter values, consistent with frequent premature termination during extension [45] [27].

1.3.2 Trapping of CPSF4 and WDR33 on CoTP-terminated 3'-ends

The second consequence of CoTP incorporation is altered CPA-factor dynamics. In a normal CPA cycle, CPSF4 and WDR33 bind the PAS together with CPSF1, coordinate recruitment of CPSF3 and PAP α/γ , and dissociate from the transcript once tail synthesis is completed and the mRNP is handed off to downstream nuclear export pathways [20] [36]. CoTP-terminated tails disrupt this cycle. Structural and functional work by Ryner and Zarkower indicates that when PAP α/γ stall at a CoTP residue, CPSF4 and WDR33 remain abnormally associated with the truncated 3' ends instead of being released efficiently [23] [24]. The modified tail terminus appears to stabilise protein–RNA contacts, producing long-lived complexes in which recognition subunits are effectively “locked” on incomplete mRNAs [27] [26].

Because CPSF4 and WDR33 are limiting for PAS recognition at many genes, their

sequestration on stalled substrates would lower the pool of functional CPA complexes available elsewhere in the nucleus [46] [14] [47] [26]. In contexts where CPA throughput must be increased rapidly—such as LPS-stimulated RAW264.7 macrophages undergoing strong cytokine induction—this reduction in free CPSF4/WDR33 creates a bottleneck in 3'-end processing. Transcriptome-wide, CPA could behave as though CPSF4/WDR33 had been partially knocked down, even though protein levels are unchanged: effective activity is reduced because complexes are immobilised on CoTP-terminated tails rather than redeployed to new PAS sites [26] [27]. Moreover, transcriptional readthrough beyond the canonical cleavage site is observed in numerous genes—including, but not limited to, those involved in inflammatory responses—in cordycepin-treated cells [19].

1.3.3 Integration with CPSF4/WDR33 knockdown phenotypes

The functional relevance of this trapping mechanism is supported by siRNA experiments targeting CPSF4 and WDR33. In LPS-stimulated RAW264.7 macrophages, depletion of either subunit reduces induction of inflammatory cytokine mRNAs such as *Tnf* and *Il1b* and lowers nuclear NF- κ B p65 localisation [26] [28].

WDR33 knockdown impairs both transcripts, whereas CPSF4 depletion exerts a stronger effect on *Il1b*, pointing to locus-specific sensitivity to CPA factor availability and recycling kinetics [26]. These perturbations also diminish PI3K/Akt/mTOR signalling outputs, with reduced phosphorylation of Akt and 4E-BP1 and increased phospho-AMPK [27] [26].

CoTP reproduces many of these phenotypes without changing CPSF4/WDR33 abundance. Instead, it functionally depletes them by trapping complexes on cordycepin-terminated tails. Radhi and colleagues showed that cordycepin treatment in a primary cells reduces NF- κ B p65 nuclear localisation, suppresses *Tnf* and *Il1b* induction and lowers p-Akt/4E-BP1 in a manner closely resembling CPSF4/WDR33 knockdown [26] [28]. Lawrence's analysis of these datasets and additional experiments supports a model in which CPA factor function is upstream of both inflammatory transcription and growth-factor signalling readouts [27]. In this framework, CoTP's primary action is on the CPA machinery; the signalling defects arise because key transcripts fail to be properly processed and exported.

1.3.4 Consequences for PABPN1 loading, export competence and deadenylation

At the level of individual transcripts, CoTP-induced chain termination and factor trapping could lead to defects in PABPN1 loading, export competence and cytoplasmic lifespan. Shortened poly(A) tails cannot bind the full complement of PABPN1 required for stable nuclear mRNP assembly, making such transcripts more likely to be retained and degraded by nuclear surveillance pathways rather than exported efficiently [20] [36]. Those that do escape to the cytoplasm arrive with pre-truncated tails that support fewer PABPC molecules and weaker cap–tail “closed-loop” interactions with eIF4E and eIF4G [36] [39]. This configuration could both reduce translation initiation efficiency and accelerates recruitment of CCR4–NOT, which can act directly on short tails without the usual PAN2–PAN3 pre-trimming step [36] [39] [44]. In effect, CoTP may shifts many transcripts into a “short-tailed, decay-prone” state immediately upon export, bypassing the longer stability window normally provided by gradual deadenylation.

These nuclear and cytoplasmic effects are tightly coupled. Loss of PABPN1 coverage and impaired CPA factor recycling in the nucleus could reduces the number of export-competent mRNPs produced for CPA-dependent genes. In the cytoplasm, the same transcripts may be degraded more quickly because their tails are too short to sustain prolonged translation. For inflammatory and growth-factor signalling transcripts that require high CPA throughput and sustained translation over time, this combination could be damaging. Ashraf and co-workers showed that cordycepin reduces expression of multiple inflammatory mediators in macrophages and attenuates NF- κ B-dependent outputs in rodent models of osteoarthritis [28]. Radhi’s work connects these phenotypes directly to CPA inhibition and CPA factor dependency [26]. Together, these data support the view that polyadenylation is not a passive downstream effect: intact CPA and adequate tail length are necessary for sustained inflammatory gene expression and for maintenance of PI3K/Akt/mTOR signalling.

1.3.5 Effects of cordycepin on alternative polyadenylation

Cordycepin has also been shown to influence alternative polyadenylation (APA). In budding yeast, cordycepin treatment induces a global shift towards usage of more distal poly(A) sites, resulting in the production of mRNA isoforms with longer 3' untranslated regions (3'UTRs) [38]. Genome-wide analyses demonstrated that thousands of transcripts display distal-site bias under cordycepin, and earlier studies on individual genes such as *ACT1* and *ASC1* similarly reported a redistribution of cleavage from proximal to distal sites, yielding longer 3'UTRs [45].

Mechanistically, this APA phenotype is not readily explained by simple RNA chain termination. Turner and colleagues instead proposed a kinetic control model in which cordycepin perturbs nucleotide metabolism and thereby alters the transcriptional elongation rate of RNA polymerase II (RNAPII) [38]. In their yeast system, metabolomic measurements indicated that cordycepin exposure is accompanied by accumulation of nucleotides and their biosynthetic intermediates; these changes were interpreted as more consistent with actions of cordycepin monophosphate (CoMP) on metabolic enzymes than with direct effects of cordycepin triphosphate (CoTP) at the polymerase active site [38]. Elevated nucleotide availability was proposed to increase the RNAPII elongation rate, effectively allowing polymerase to traverse 3'UTRs more quickly [38]. However, such accumulation could also reflect reduced nucleotide consumption, for example through decreased ribosomal RNA synthesis, and an alternative, not mutually exclusive explanation is that cordycepin functionally limits the availability of polyadenylation factors, which can also favour distal PAS usage when recruitment to proximal sites becomes rate-limiting [45].

In a kinetic competition framework, faster transcription would reduce the time window during which the 3'-end processing machinery can recognise and cleave at proximal poly(A) sites. Proximal PASs, which normally compete successfully when RNAPII dwells longer over the region, are less frequently engaged when elongation is accelerated, whereas stronger, more distal PASs remain accessible further downstream.

Several lines of evidence in yeast are consistent with such kinetic and CPA-capacity effects. The APA changes induced by cordycepin are reversed by mycophenolic acid

(MPA), a drug that reduces GTP levels and slows RNAPII elongation, and a slow RNAPII mutant counteracts cordycepin’s effects on 3’-end processing [38]. Moreover, the APA profile observed under cordycepin does not match that of any single 3’-end formation factor mutant, arguing against a simple direct inhibition of one specific polyadenylation factor [38], and the predominance of longer, rather than shorter, transcripts is difficult to reconcile with a model in which chain termination at the RNA 3’ end is the primary driver of the APA phenotype [45].

Collectively, these yeast data are consistent with a model in which cordycepin influences APA through coupled changes in nucleotide pools, RNAPII dynamics and the functional availability of 3’-end processing factors, leading to a shift from proximal to distal poly(A) site usage. It is important to note that these experiments were conducted in budding yeast, and no genome-wide analyses of cordycepin-induced APA or termination defects have yet been reported in mammalian cells. Nevertheless, they illustrate that cordycepin can reshape 3’UTR architecture not only by directly interfering with poly(A) tail synthesis via CoTP, but also indirectly by modulating the temporal window available for poly(A) site recognition and cleavage.

1.3.6 Summary

In summary, CoTP acts on the CPA machinery via two tightly linked mechanisms: it functions as a chain-terminating substrate for PAP α/γ , generating abnormally short poly(A) tails, and it traps CPSF4 and WDR33 on incomplete 3’ ends, reducing CPA factor recycling and global processing throughput. These nuclear events may compromise PABPN1 loading, export competence and cytoplasmic tail protection, thereby accelerating deadenylation and decay of affected transcripts. The available evidence from CPSF4/WDR33 knockdowns, cordycepin treatment in macrophages and animal models indicates that inflammatory and growth-factor signalling genes are particularly dependent on intact CPA under stimulation [27] [26] [28].

Taken together, the data summarised in this section establish CoTP-mediated CPA inhibition as the best-characterised direct biochemical effect of cordycepin relevant to this thesis. Subsequent sections will consider how these CPA-centred effects intersect with cordycepin’s robust but mechanistically unresolved repression of PI3K/Akt/mTOR

and MEK/ERK signalling, and how metabolic gating to CoTP determines the extent of CPA inhibition in different cellular contexts.

1.4 Linking cleavage and polyadenylation to growth-factor signalling and inflammatory outputs

The previous subsections established that cleavage and polyadenylation (CPA) generate the 3' ends and poly(A) tails required for mRNA stability, export and translation, and that cordycepin triphosphate (CoTP) can inhibit this process via chain termination and CPA-factor trapping. The same signalling modules that are consistently repressed by cordycepin — PI3K/Akt/mTOR and MEK/ERK — are also sensitive to genetic perturbation of CPA components, suggesting that intact CPA is a prerequisite for sustained growth-factor signalling and inflammatory gene induction. At the same time, the direction and exclusivity of this relationship remain unresolved: reduced signalling can plausibly be a downstream consequence of CPA impairment, while signalling pathways may also feed back onto CPA and other RNA-processing steps [48] [49] [50]. This section summarises the available linkage data and formulates a cautious working model

1.4.1 CPA factor knockdown recapitulates growth-factor signalling defects

Knockdown of individual CPA subunits provides a test of whether 3'-end processing capacity is required to maintain growth-factor signalling. Several studies have targeted CPSF subunits that are also implicated in cordycepin responses: CPSF4, CPSF7 and CPSF3 [48] [49] [51].

In lung adenocarcinoma cells, siRNA-mediated depletion of CPSF4 reduces proliferation and clonogenic survival and induces apoptosis, accompanied by loss of PI3K/Akt and MAPK signalling output [51] [53]. Akt phosphorylation decreases together with downstream mTOR substrates such as S6K and 4E-BP1, and ERK phosphorylation is similarly reduced. CPSF7 knockdown in the same disease setting produces a related phenotype, with impaired AKT/mTOR signalling, reduced viability and diminished

migration and invasion; pharmacological activation of Akt only partially rescues these defects [49]. In hepatocellular carcinoma models, CPSF3 depletion leads to cell-cycle arrest and repression of PI3K/Akt signalling, partly via effects on PTEN, again resulting in reduced mTOR activity and proliferation [48].

Although the exact transcript-level consequences vary between systems, a consistent pattern emerges: reducing the functional availability of CPA factors is sufficient to attenuate PI3K/Akt/mTOR and MEK/ERK signalling [48] [49] [51]. These knockdowns also alter alternative polyadenylation (APA) patterns in ways that resemble low-dose cordycepin treatment, supporting the idea that changes in 3'-end processing can propagate into signalling phenotypes via effects on mRNA abundance and isoform structure [38] [20] [50].

From the perspective of this thesis, these data are important for two reasons. First, they show that growth-factor signalling can be compromised by CPA impairment alone, consistent with the idea that signal transduction depends on adequate production and translation of CPA-dependent transcripts. Second, they provide a genetic “mirror” for cordycepin/CoTP effects: cordycepin functionally depletes CPSF4/WDR33 through chain termination and factor trapping, whereas RNAi physically depletes the proteins; in both cases, signalling outputs decline within a comparable timescale [48] [49] [51].

1.4.2 Cordycepin as a pharmacological mimic of CPA-factor limitation

CoTP incorporation into poly(A) tails and the resulting trapping of CPSF4/WDR33 on incompletely processed 3' ends provide a reasonable biochemical mechanism by which cordycepin could mimic CPA factor knockdown [45] [28] [15]. In this scenario, CPA complexes are not absent but become sequestered on truncated transcripts and recycle more slowly, effectively reducing the nuclear CPA capacity available for new pre-mRNAs. The net effect would be similar to that seen with CPSF4/CPSF7/CPSF3 knockdown: fewer transcripts are fully cleaved and polyadenylated, tail lengths are shortened, and APA patterns are shifted.

Consistent with this, cordycepin produces signalling and transcriptional phenotypes

that overlap with CPA-factor knockdown. In LPS-stimulated RAW 264.7 macrophages, cordycepin treatment reduces Akt and 4E-BP1 phosphorylation and lowers ERK, JNK and p38 activation within 15–30 minutes, while simultaneously decreasing induction of inflammatory cytokines such as *Tnf* and *Il1b* and reducing nuclear NF- κ B p65 levels [28] [52] [53] [54]. These outcomes resemble the combined effects of CPSF4/WDR33 depletion and polyadenylation inhibition in other systems [19] [48] [49] [51]. Importantly, in several models cordycepin-induced suppression of PI3K/Akt/mTOR signalling is not prevented by pharmacological AMPK inhibition or by knockout of AMPK, indicating that CPA-dependent translation bottlenecks can depress signalling independently of the canonical AMPK–mTOR axis [25] [46].

Taken together, the genetic and pharmacological data support a model in which CoTP-mediated CPA impairment and CPA-factor dependency contribute substantially to the observed repression of growth-factor signalling. However, they do not yet prove that CPA inhibition is the only mechanism, or that it is always strictly upstream of signalling changes. Temporal resolution of CPA defects versus signalling readouts remains limited in many studies, and isolated reports of direct effects of cordycepin on Akt phosphorylation have not been reproduced consistently across systems [55] [56] [30].

1.4.3 Inflammatory transcripts as CPA-sensitive outputs

Inflammatory gene expression provides a second line of evidence linking CPA to signalling. Many pro-inflammatory mediators, including TNF- α , IL-1 β , IL-6, iNOS and COX-2, are encoded by mRNAs with short half-lives and strong dependence on rapid CPA and poly(A)-tail turnover during stimulation [52] [19] [57]. Kondrashov and colleagues showed that pharmacological inhibition of polyadenylation reduces induction of inflammatory genes in response to TNF, pointing to a general requirement for efficient 3'-end processing in inflammatory programmes [19].

Cordycepin fits into this framework. In LPS-stimulated macrophages and microglia, cordycepin attenuates Akt and MAPK activation, and reduces production of NO, PGE₂, TNF- α and IL-1 β within 30–60 minutes [52] [54] [53]. The timing is again compatible with CPA-dependent translational restriction: CoTP incorporation and CPA-factor

trapping would be expected to limit the supply of fully processed cytokine transcripts and reduce their translational competence, while concurrent loss of PI3K/Akt and ERK signalling diminishes the transcriptional drive required to sustain maximal protein synthesis capacity in general.

In vivo, oral cordycepin treatment in rodent models of osteoarthritis decreases synovial *Il1b* and macrophage marker *Cd68* mRNAs, reduces angiogenic markers such as VEGF and nestin, and improves pain and joint pathology without affecting proliferation markers (PCNA, MYC) [28]. Other inflammatory models (cardiac transplantation, liver fibrosis, renal injury) show similar reductions in pro-inflammatory transcripts and signalling activity after cordycepin administration [28] [58] [13] [57]. These findings are compatible with a scenario in which CPA-dependent inflammatory transcripts are selectively vulnerable when CPA capacity is reduced, whether by polyadenylation factor depletion or by CoTP-mediated chain termination.

1.4.4 Summary and working hypothesis

The experiments summarised above support three conclusions:

1. Intact CPA is required for normal PI3K/Akt/mTOR and MEK/ERK signalling. Knockdown of CPSF subunits reduces these pathway outputs and proliferation in cancer models, indicating that CPA factor availability is a limiting determinant of growth-factor signalling capacity [48] [49] [51].
2. Inflammatory gene induction is CPA-sensitive. Both polyadenylation factor depletion and cordycepin reduce the abundance and translation of key inflammatory transcripts and dampen NF- κ B/MAPK signalling in macrophages and in vivo inflammatory models [28] [52] [19] [54] [57].
3. Cordycepin/CoTP produces signalling and inflammatory phenotypes that are consistent with CPA-factor limitation, but the molecular target(s) within the growth-factor pathways remain undefined, and no direct kinase target for CoTP has been demonstrated [25] [46] [56] [58] [13].

In this thesis, these observations motivate a working hypothesis in which CPA in-

tegrity and poly(A) tail homeostasis are necessary for sustained growth-factor signalling and inflammatory gene expression. CoTP-mediated inhibition of CPA and CPA-factor trapping are treated as potential mechanisms that can contribute to signaling repression. Figure 1.5 summarizes mechanism of cordycepin triphosphate (CoTP)-mediated inhibition of cleavage and polyadenylation and its downstream consequences

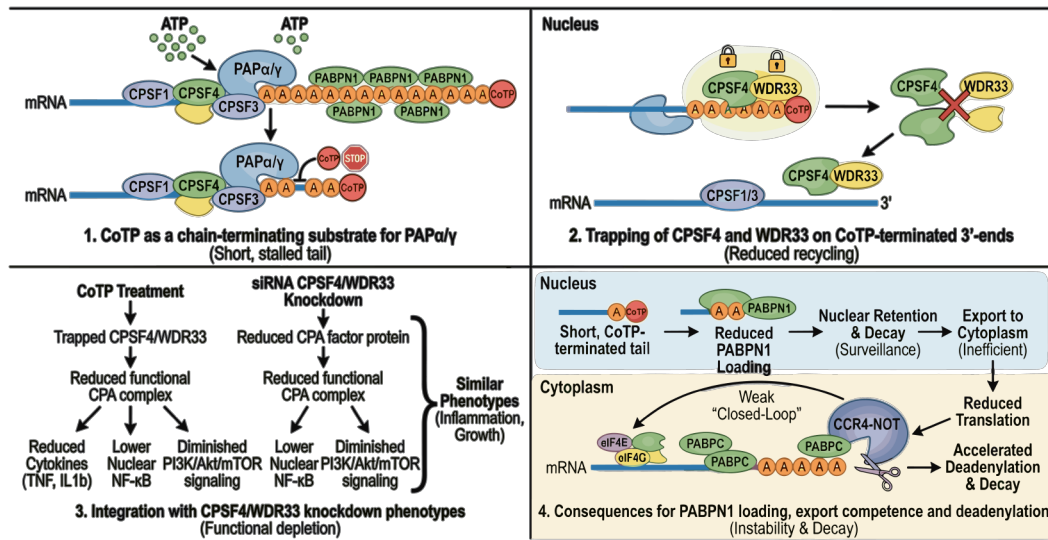


Figure 1.5: Mechanism of cordycepin triphosphate (CoTP)-mediated inhibition of cleavage and polyadenylation and its downstream consequences. (1) CoTP as a chain-terminating substrate for PAP α/γ . Schematic illustrating incorporation of CoTP by nuclear poly(A) polymerases PAP α/γ during 3'-end extension. Substitution of ATP with CoTP results in premature chain termination, generating short, stalled poly(A) tails that fail to recruit sufficient PABPN1. Because PABPN1 normally stabilizes PAP α/γ processivity and defines canonical tail length, CoTP-terminated tails disrupt both tail growth and PABPN1 loading. (2) Trapping of CPSF4 and WDR33 on CoTP-terminated 3'-ends. CoTP-terminated transcripts retain the CPSF4–WDR33 module of the CPSF complex on the RNA, preventing normal recycling of these CPA factors to new transcripts. This functional sequestration reduces the pool of active CPSF4/WDR33 available for PAS recognition, thereby impairing processing of subsequent pre-mRNAs and amplifying CPA inhibition. (3) Convergence with CPSF4/WDR33 knockdown phenotypes. Both CoTP treatment and siRNA-mediated depletion of CPSF4/WDR33 lead to reduced functional CPA complexes, diminished CPA efficiency, defective 3'-end formation, and attenuated downstream pathways including PI3K/Akt/mTOR signaling and NF- κ B nuclear responses. This convergence supports a model in which CoTP exerts its primary effects through functional depletion of CPA factors. (4) Consequences for PABPN1 loading, nuclear export competence, and cytoplasmic stability. Short, CoTP-terminated poly(A) tails exhibit reduced PABPN1 occupancy, impairing formation of export-competent mRNPs and promoting nuclear retention and surveillance-mediated decay. Inefficiently exported transcripts that reach the cytoplasm display weakened cap–tail interactions and poor closed-loop formation (reduced eIF4E–eIF4G–PABPC1 engagement). These mRNAs undergo accelerated deadenylation via CCR4–NOT and are rapidly degraded, resulting in reduced stability. Together, these pathways explain how CoTP-induced CPA inhibition propagates to broad post-transcriptional repression of inflammatory and growth-factor-responsive gene expression. The figure was created with Sketch v 2025.3.1

1.5 Growth-factor signalling and its modulation by cordycepin

1.5.1 Canonical growth-factor signalling: PI3K/Akt/mTOR and RAF/MEK/ERK

Growth factors such as EGF, IGF-1 and PDGF signal primarily through two interconnected kinase cascades: the phosphoinositide 3-kinase (PI3K)/Akt/mTOR axis and the RAF/MEK/ERK mitogen-activated protein kinase (MAPK) pathway [59] [60]. Together these networks integrate extracellular cues with intracellular programmes controlling cell growth, proliferation, metabolism, survival and motility, and are frequently dysregulated in cancer and chronic inflammatory disease [59] [61].

Upon ligand binding, receptor tyrosine kinases (RTKs) undergo autophosphorylation on cytoplasmic tyrosine residues, creating docking sites for adaptor proteins and class I PI3Ks. Activated PI3K converts phosphatidylinositol-4,5-bisphosphate (PIP₂) into phosphatidylinositol-3,4,5-trisphosphate (PIP₃) at the inner leaflet of the plasma membrane. PIP₃ then recruits pleckstrin-homology (PH) domain-containing proteins, including phosphoinositide-dependent kinase-1 (PDK1) and Akt (protein kinase B), to the membrane. PDK1 phosphorylates Akt at Thr308 in the activation loop, while mTOR complex 2 (mTORC2) phosphorylates Ser473 in the hydrophobic motif; together these two modifications fully activate Akt and expand its substrate range [60] [61].

Activated Akt phosphorylates multiple targets controlling metabolism and survival, notably the TSC1/2 complex and PRAS40. Phosphorylation of TSC2 and PRAS40 relieves inhibition of the small GTPase Rheb, thereby activating mTOR complex 1 (mTORC1). mTORC1 in turn phosphorylates downstream effectors such as p70 S6 kinase (S6K1) and the eIF4E-binding proteins (4E-BPs). Phosphorylated S6K1 promotes ribosome biogenesis and translation, while phosphorylation of 4E-BP1 causes its dissociation from eIF4E, permitting assembly of the eIF4F cap-binding complex and efficient cap-dependent translation initiation [61] [13]. Through these substrates, growth-factor signalling is tightly coupled to translational output and anabolic metabolism.

mTORC1 also suppresses autophagy by phosphorylating ULK1 and integrates amino acid signals via the Ragulator–Rag GTPase module at the lysosome, while mTORC2 acts upstream of Akt by providing Ser473 phosphorylation and regulates aspects of cytoskeletal organisation [61]. In parallel, many of the same RTKs activate the RAF/MEK/ERK cascade, in which RAS–RAF activation leads to MEK1/2- and ERK1/2-mediated phosphorylation of transcription factors (e.g. ELK1, AP-1) and regulators of proliferation and survival. PI3K/Akt/mTOR and RAF/MEK/ERK thus form a densely interconnected signalling module that governs growth-factor responses in most mammalian cells [59] [60].

AMP-activated protein kinase (AMPK) provides an important energetic counterweight to these growth-promoting pathways. As a heterotrimeric kinase that senses increases in AMP:ATP and ADP:ATP ratios, AMPK is activated by phosphorylation of Thr172 in its catalytic α subunit by upstream kinases such as LKB1 and CaMKK β when cellular energy is limited [62] [63]. Once activated, AMPK inhibits mTORC1 through phosphorylation of Raptor and TSC2, promotes autophagy, and broadly suppresses ATP-consuming anabolic processes [62] [63]. Under nutrient-replete conditions, PI3K/Akt/mTOR signalling dominates; under energy stress, AMPK constrains growth-factor-driven anabolism by re-imposing TSC2-mediated inhibition of mTORC1. The PI3K/Akt/mTOR module and AMPK therefore form an antagonistic pair that integrates growth-factor and nutrient/energy status.

1.5.2 Cordycepin-mediated repression of growth-factor signalling

Cordycepin (3'-deoxyadenosine) is widely reported to inhibit growth-factor signalling and reduce cell proliferation and survival across many cell types. A systematic review of more than 100 vertebrate cell-culture studies concluded that cordycepin has reproducible anti-proliferative and anti-inflammatory effects, typically associated with activation of AMPK and reduced phosphorylation of Akt and ERK [13]. However, most studies used the parent nucleoside and focused on pathway readouts rather than mechanism, so the precise molecular target(s) within the growth-factor network remained unclear.

In NIH3T3 fibroblasts, Wong and colleagues found that 50 μ M cordycepin strongly reduced Akt1 phosphorylation, decreased total Akt1 levels and induced dephosphoryla-

tion of 4E-BP1, coinciding with inhibition of protein synthesis and cell adhesion within 1–2 h [46]. Pharmacological experiments in this system implicated AMPK: inhibition of AMPK attenuated cordycepin-induced 4E-BP1 dephosphorylation and translational repression [46]. Similar observations were made in MCF-7 breast cancer cells, where cordycepin decreased Akt and 4E-BP1 phosphorylation and was initially proposed to inhibit both mTORC1 and mTORC2 via AMPK activation [64]. These data established that cordycepin impacts the PI3K/Akt/mTOR axis at the level of Akt phosphorylation and translational control nodes, but still left open whether AMPK activation is required and whether the parent nucleoside or its nucleotides are responsible.

More recent systems-pharmacology work has clarified several of these issues. Using six cell lines stimulated with various growth factors, Lawrence et al. showed that cordycepin consistently represses PI3K/Akt/mTOR and/or RAF/MEK/ERK signalling, with reduced phosphorylation of Akt, 4E-BP1 and S6K detectable within 30 min of treatment [25]. Transcriptional profiling and reference-compound comparisons revealed that cordycepin’s gene-expression footprint most closely resembled that of PI3K/mTOR pathway inhibition. Metabolic measurements and genetic perturbations implicated cordycepin triphosphate (CoTP) as the active metabolite and demonstrated that AMPK activation is not required for growth-factor signalling repression: in AMPK-deficient cells, cordycepin still reduced PI3K activity, suppressed growth-factor-inducible gene expression and dephosphorylated mTOR targets [25]. Together with the nucleoside-rescue data discussed elsewhere, these findings support a model in which intracellular nucleotides derived from cordycepin drive the growth-factor phenotype and in which repression of PI3K/Akt/mTOR signalling does not require AMPK.

Additional support for cordycepin’s action on PI3K/Akt/mTOR comes from work in adipogenic and inflammatory models. In 3T3-L1 preadipocytes, cordycepin blocks adipocyte differentiation and lipid accumulation. Takahashi et al. showed that cordycepin suppresses early induction of the C/EBP β –PPAR γ adipogenic programme, reduces Akt phosphorylation and inhibits mTORC1 activity, as judged by loss of p70S6K phosphorylation [65]. Importantly, they found that this anti-adipogenic effect requires nucleoside transporter activity but is not reversed by A₁, A₂ or A₃ adenosine receptor

antagonists, indicating that intracellular uptake of cordycepin is essential whereas adenosine receptors are dispensable in this context [65]. These data agree with macrophage studies in which equilibrative nucleoside transporter and adenosine kinase inhibitors abrogate cordycepin's effects on inflammatory gene expression and signalling, pointing to an intracellular nucleotide-based mechanism rather than a surface-receptor mechanism [13, 46].

In inflammatory systems, cordycepin's growth-factor signalling effects are tightly intertwined with its role as a polyadenylation inhibitor. Kondrashov et al. showed that knockdown of a poly(A) polymerase reduces induction of inflammatory mRNAs in TNF- α -stimulated A549 and airway smooth muscle (ASM) cells., identifying CPA as a potential anti-inflammatory target [19]. Ashraf et al. extended this concept in vivo, demonstrating that cordycepin reduces pain behaviour, synovial inflammation and joint pathology in rat osteoarthritis models while, in parallel, suppressing NF- κ B p65 nuclear localisation and inflammatory gene expression in macrophages [28]. In those primary cells experiments, knockdown of CPA factors CPSF4 and WDR33 recapitulated many of cordycepin's effects on NF- κ B localisation and inflammatory transcripts, and also reduced Akt and 4E-BP1 phosphorylation [26, 28]. These observations support the idea that efficient cleavage and polyadenylation are required for full growth-factor and inflammatory signalling, and that CPA-factor limitation—whether by genetic depletion or CoTP-mediated trapping—can depress PI3K/Akt/mTOR outputs.

Collectively, the available data support the following model for cordycepin action. First, cordycepin reproducibly represses PI3K/Akt/mTOR and, in many systems, RAF/MEK/ERK signalling, with decreased phosphorylation of Akt, 4E-BP1 and S6K occurring within 30–60 min of treatment [13, 25, 46]. Second, AMPK activation is a frequent correlate of cordycepin exposure but is not required for repression of growth-factor signalling or for downregulation of growth-factor-inducible genes [25, 26]. Third, transporter- and kinase-dependence, nucleoside-rescue experiments and ProTide studies with NUC-7738 together suggest that intracellular 3'-deoxyadenosine nucleotides (CoTP/3'-dATP) are sufficient to reproduce the core signalling phenotype, without any obligatory contribution from adenosine receptors in these systems [2, 25, 26, 46]. Finally,

CPA-factor knockdowns and polyadenylation inhibition phenocopy many of cordycepin’s signalling and inflammatory effects, suggesting that cleavage and polyadenylation are upstream requirements for sustained growth-factor signalling in macrophages and other cell types [19, 26, 28].

Despite this progress, the direct molecular target(s) linking CoTP to rapid alterations in kinase phosphorylation remain unresolved. No specific kinase, phosphatase or adaptor protein has yet been identified whose inhibition by CoTP fully explains the breadth and kinetics of PI3K/Akt/mTOR and RAF/MEK/ERK repression. The working hypothesis adopted in this thesis is therefore that cordycepin’s best-established biochemical action—CoTP-mediated inhibition of cleavage and polyadenylation—creates a cellular environment in which growth-factor signalling cannot be sustained.

1.5.3 Protein synthesis inhibition and growth-factor signalling

Classical work on immediate-early gene regulation established that growth-factor signal transduction and the first wave of transcriptional responses do not require ongoing protein synthesis. In quiescent fibroblasts, platelet-derived growth factor (PDGF) or other growth factors rapidly induce *c-fos* mRNA and protein within minutes; this *c-fos* burst precedes and is mechanistically separable from later induction of *c-myc* and other delayed-response genes [66] [67]. Strikingly, when protein synthesis is blocked with cycloheximide or anisomycin, the *c-fos* response is not lost but instead “superinduced”: PDGF plus cycloheximide produces a several-fold higher accumulation of *c-fos* transcripts than PDGF alone [66]. Nuclear run-on assays in 3T3 fibroblasts demonstrate that protein synthesis inhibitors potentiate *c-fos*, *c-myc* and actin transcription, consistent with relief of a labile repressor and delayed transcriptional shut-off rather than a requirement for new protein synthesis to initiate signalling [68]. Together, these data show that growth-factor-induced immediate-early gene transcription is a primary, protein-synthesis-independent output of receptor signalling, and that acute translational arrest can enhance rather than block this response.

More recent studies have extended this principle to upstream kinase pathways. In HEK-293FT and N2a neuroblastoma cells, cycloheximide or a distinct translation inhibitor (NSC119889) rapidly increases AKT phosphorylation on Thr308 and Ser473

and activates canonical AKT substrates including FoxO1, GSK3 α/β , p70S6K, AS160 and the E3 ligase MDM2 [69]. This AKT activation is blocked by the PI3K inhibitor LY294002, indicating that acute inhibition of protein biosynthesis can engage PI3K–AKT signalling rather than uniformly shutting it down [69]. In retinal neurons, cycloheximide or anisomycin similarly triggers a robust rise in nitric oxide production by mobilising intracellular L-arginine pools, leading to soluble guanylyl cyclase and cGMP-dependent protein kinase II (cGKII) activation; this nitric oxide–cGMP–cGKII axis is tightly coupled to phosphorylation and nuclear accumulation of AKT and to enhanced ERK phosphorylation [70]. Genetic deletion or knockdown of cGKII, or pharmacological inhibition of nitric oxide synthase, prevents cycloheximide-induced AKT and ERK activation in these systems [70].

Taken together, these findings make it clear that acute signal transduction does not require ongoing protein synthesis and can even be potentiated when translation is blocked: for example, cycloheximide-mediated inhibition of protein synthesis is associated with increased Akt phosphorylation and robust induction of immediate-early genes. Against this backdrop, the ability of cordycepin to suppress PI3K/Akt/mTOR signalling is unlikely to depend on reduced synthesis of canonical pathway components or on translation of a specific effector protein. Instead, this thesis adopts the working hypothesis that cordycepin acts through an as-yet-unidentified RNA whose 3'-end processing and polyadenylation are cordycepin-sensitive (the RNA target of cordycepin, RToC). In this model, altered processing of the RToC transcript is sufficient to reshape PI3K/Akt/mTOR outputs, and the RToC itself need not be efficiently translated and may even be a non-translatable RNA species. Figure 1.6 summarizes the modulation of growth-factor signalling networks by cordycepin triphosphate (CoTP) via polyadenylation-dependent control

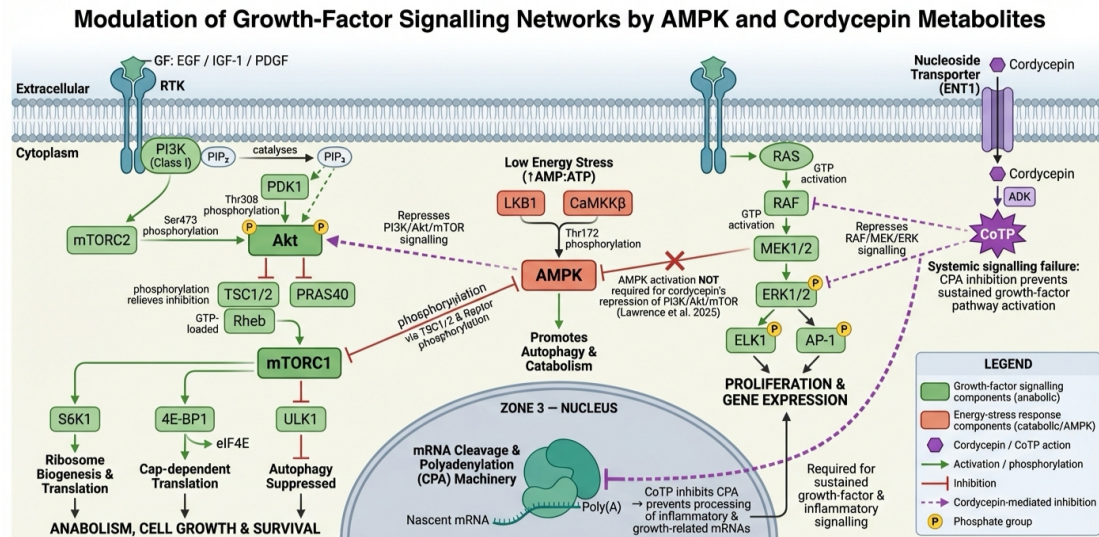


Figure 1.6: Modulation of growth-factor signalling networks by cordycepin triphosphate (CoTP) via polyadenylation-dependent control. Schematic overview of how cordycepin perturbs growth-factor-responsive signalling in mammalian cells. Under basal conditions (left), receptor tyrosine kinases (RTKs) activated by growth factors such as EGF or IGF-1 stimulate class I PI3K, generating *PIP*₃ from *PIP*₂ and recruiting PDK1 and Akt to the plasma membrane. Akt is phosphorylated at Thr308 by PDK1 and at Ser473 by mTORC2, yielding fully active Akt. Activated Akt phosphorylates TSC1/2 and PRAS40 to relieve inhibition of Rheb and promote mTORC1 activation, which in turn phosphorylates S6K1 and 4E-BP1 to drive ribosome biogenesis, cap-dependent translation and anabolic growth, while repressing autophagy via ULK1. In parallel, RTK signalling through RAS–RAF–MEK–ERK culminates in activation of transcription factors such as ELK1 and AP-1, supporting proliferation and gene expression. Energy-stress signalling through LKB1– or CaMKKβ-activated AMPK antagonises mTORC1 and favours catabolic responses, but AMPK activation is not required for cordycepin-induced repression of these growth-factor pathways. On the right, cordycepin is taken up by nucleoside transporters and phosphorylated to CoTP, which inhibits nuclear cleavage and polyadenylation (CPA) of specific transcripts. In this model, suppression of PI3K/Akt/mTOR signalling occurs via an unidentified RNA, referred to as the RNA target of cordycepin (RToC), whose function is influenced by alterations in polyadenylation. CoTP-mediated inhibition of cleavage and polyadenylation affects RToC, creating a cellular environment in which growth-factor signalling cannot be sustained, despite continued upstream RTK engagement. Incomplete 3'-end processing and altered poly(A) tail dynamics of RToC and related transcripts are proposed to impair the transcriptional and translational support required to maintain PI3K/Akt/mTOR and RAS/RAF/MEK/ERK activity, resulting in systemic collapse of anabolic signalling. The legend at the bottom of the figure indicates the colour-coding for growth-factor (anabolic) signalling, energy-stress (catabolic) pathways, CoTP actions, inhibitory interactions and phosphorylation events. The figure was created with Sketch v 2025.3.1

1.6 Alternative proposed targets for cordycepin: adenosine receptors and other signalling nodes

The preceding sections have focused on cleavage and polyadenylation (CPA) inhibition by cordycepin triphosphate (CoTP) and the associated repression of PI3K/Akt/mTOR and MEK/ERK signalling as the principal mechanistic axis relevant to this thesis. However, the wider literature has proposed several alternative or additional targets, most prominently adenosine receptors (A_1 , A_{2A} , A_{2B} , A_3), along with various kinases and ATP-binding proteins [13] [71]. A balanced account therefore requires (i) summarising the evidence that cordycepin can signal through adenosine receptors in particular contexts, and (ii) explaining why, in the growth-factor and inflammatory settings central to this thesis, adenosine receptors are unlikely to constitute the primary mechanism of action once intracellular CoTP and CPA inhibition are taken into account.

1.6.1 Evidence for adenosine receptor-mediated actions of cordycepin

Because cordycepin is a 3'-deoxyadenosine analogue, it is a credible ligand for adenosine receptors. Several studies demonstrate receptor-dependent effects in specific tissues. In the nervous system, cordycepin modulates synaptic transmission and plasticity in hippocampal CA1 neurons. Wang and colleagues showed that cordycepin suppresses both glutamatergic and GABAergic synaptic currents in rat CA1 pyramidal neurons, and that these effects are prevented by A_1 receptor antagonists, consistent with an A_1 -dependent mechanism [71, 72]. Other studies report cordycepin-induced neuroprotection and metaplasticity that are similarly sensitive to A_1 antagonism [71].

In dermal fibroblasts, Kim et al. found that adenosine and cordycepin stimulate Wnt/ β -catenin signalling and promote tissue-remodelling behaviours via adenosine receptors. Pharmacological blockade with A_{2A} and A_{2B} antagonists (ZM241385 and PSB603) attenuated cordycepin-induced β -catenin activation and fibroblast migration, implicating these receptor subtypes in the response [73]. In cancer models, cordycepin- or *Cordyceps* extract-induced apoptosis has been linked to A_3 receptor activation. In

human T24 bladder carcinoma cells, *Cordyceps militaris* extracts containing cordycepin decrease cell survival in an A_3 -dependent fashion: overexpression of A_3 mimics the extract's effects, while A_3 knockdown abolishes them [74]. Earlier work in mouse melanoma and lung carcinoma cells similarly implicated A_3 in cordycepin's anti-tumour effects [71].

These and related observations are collated in recent mechanistic reviews, which highlight adenosine receptors, death receptors and EGFR as candidate cell-surface mediators for some anticancer actions of cordycepin [71]. Together, they establish that cordycepin can act as a functional adenosine receptor agonist in particular systems, especially in neuronal, fibroblast and some tumour contexts. However, as discussed further in the metabolic-gating section below, cordycepin is rapidly deaminated to 3'-deoxyinosine (3DI), which appears to be the predominant extracellular metabolite in tissues [1], and 3DI itself has not been systematically tested in these receptor assays; any physiologically relevant receptor-mediated mechanism therefore ultimately needs either to demonstrate activity of 3DI at the relevant adenosine receptors or to invoke efficient extracellular regeneration of cordycepin from 3DI under the experimental conditions used.

1.6.2 Transporter and kinase dependence in somatic cell models

In contrast to these receptor-centric models, multiple lines of evidence in macrophages and other somatic cells indicate that cordycepin's anti-inflammatory and growth-factor signalling effects require transporter-mediated uptake and intracellular phosphorylation, rather than extracellular engagement of adenosine receptors. A systematic review by Radhi et al. concluded that cordycepin's anti-inflammatory activity in LPS-stimulated RAW264.7 macrophages is abolished by the equilibrative nucleoside transporter inhibitor NBTI and by adenosine kinase (ADK) inhibitors, supporting a model in which cordycepin must enter the cell and be phosphorylated to exert its effects [13]. In the same experimental system, both cordycepin and its deaminated metabolite 3'-deoxyinosine (3DI) generate CoTP and suppress inflammatory gene expression, albeit with different potencies and kinetics, further reinforcing the central role of intracellular nucleotides rather than extracellular receptor activation [13] [1].

The requirement for transporter-mediated uptake is particularly clear in adipogenic models. In 3T3-L1 preadipocytes, insulin-dependent PI3K/Akt activation drives mTORC1 signalling and adipocyte differentiation. Takahashi et al. showed that cordycepin blocks adipogenesis by inhibiting Akt phosphorylation, suppressing mTORC1 activity (loss of p70S6K phosphorylation) and down-regulating C/EBP β and PPAR γ expression [65]. Crucially, they demonstrated that this anti-adipogenic effect is “mediated by an adenosine transporter, but not A₁, A₂ or A₃ adenosine receptors”; adenosine receptor antagonists failed to reverse cordycepin’s effects, whereas the nucleoside transporter inhibitor NBTI restored adipocyte differentiation and mTORC1 signalling [65]. Cordycepin’s inhibition of adipogenesis was also not reproduced by ATP, ADP or adenosine [65]. These data argue that intracellular accumulation of cordycepin, not activation of surface adenosine receptors, is necessary for the observed blockade of PI3K/Akt/mTOR signalling in this setting.

Taken together, the macrophage and adipocyte data suggest that in at least some somatic contexts—particularly those involving sustained transcriptional responses and growth-factor signalling—cordycepin behaves as an intracellular nucleoside analogue whose activity depends on nucleoside transporters and ADK, rather than as a classical adenosine receptor agonist [13] [65] [1] [25].

1.6.3 Metabolic gating and the 3'-deoxyinosine rescue pathway

Pharmacokinetic studies provide additional mechanistic constraints. Lee et al. showed that after oral dosing in rats, unchanged cordycepin is essentially undetectable in plasma due to rapid adenosine deaminase-mediated conversion to 3DI, whereas 3DI itself is orally bioavailable ($F_{\text{oral}} \approx 37\%$) [1]. In RAW264.7 macrophage-like cells, both cordycepin and 3DI are converted to CoTP, and 3DI reproduces cordycepin’s suppression of LPS-induced inflammatory markers when present at higher concentrations, albeit with lower potency [1]. These findings led to the proposal of a “nucleoside rescue” pathway in which 3DI acts as an orally bioavailable pro-drug that is salvaged back to cordycepin nucleotides and ultimately CoTP in target tissues [1].

From the perspective of adenosine receptor involvement, these data are problematic for a receptor-first model of anti-inflammatory and growth-factor signalling effects. If

adenosine receptors were the primary mediators, the absence of measurable cordycepin in plasma would be expected to attenuate pharmacodynamic responses *in vivo*. Instead, robust anti-inflammatory and disease-modifying effects are observed in several rodent models, including osteoarthritis, where cordycepin lowers inflammatory gene expression and joint pathology despite its rapid deamination [26] [28]. The ability of 3DI to act as an upstream precursor of CoTP in macrophages therefore favours an intracellular nucleotide-centric mechanism over one that relies on extracellular adenosine receptor activation.

1.6.4 ProTide NUC-7738 as an adenosine receptor-independent tool

The ProTide NUC-7738 provides a more stringent test of whether adenosine receptors are required for cordycepin-like signalling effects. NUC-7738 is a 5'-O-aryl phosphoramidate prodrug of 3'-deoxyadenosine (3'-dA) designed to overcome cordycepin's dependence on ADA, hENT1 and ADK [29] [2]. The phosphoramidate cap masks the ribose 5'-phosphate and is cleaved intracellularly by esterases and the phosphoramidase HINT1 to release 3'-dAMP, which can then be converted to 3'-dADP and 3'-dATP (CoTP) [29] [2]. Because of its bulky 5'-substitution and intracellular activation pathway, NUC-7738 is not expected to function as an agonist at cell-surface adenosine receptors, and manufacturer profiling has not detected adenosine receptor activation by NUC-7738 under standard assay conditions (David Harrison and Hugh Griffiths, Nucana Pharmaceuticals, personal communication).

Schwenzer et al. showed that NUC-7738 is substantially more potent than 3'-dA across a panel of cancer cell lines and that it overcomes resistance mechanisms that limit 3'-dA activity [29]. In haploid genetic screens and chemical perturbation assays, ADA inhibition, hENT1 blockade and ADK inhibition markedly shifted the IC₅₀ for 3'-dA but had minimal effects on NUC-7738 potency, whereas disruption of HINT1 strongly reduced NUC-7738 activity [29]. These data indicate that NUC-7738 enters cells and is activated largely independently of adenosine receptors, nucleoside transporters and ADK, relying instead on ProTide cleavage [29] [2]. Transcriptomic analyses revealed that 3'-dA

and NUC-7738 induce largely overlapping, though not identical, patterns of pathway perturbation, prominently including repression of NF- κ B-associated transcriptional programs. Notably, the incomplete overlap likely reflects differences in treatment dynamics: 3'-dA-treated cells were profiled only after prolonged single-dose exposure, a condition under which declining cordycepin levels permit partial re-expression of previously suppressed transcripts [29].

The implication for adenosine receptor hypotheses is that a ProTide which cannot act as an adenosine receptor agonist may reproduce key antiproliferative, transcriptional and signalling effects of 3'-dA when intracellular triphosphate exposure is matched [29] [2]. These findings are therefore consistent with intracellular 3'-deoxyadenosine nucleotides (including CoTP/3'-dATP) making a major contribution to the relevant growth-factor and NF- κ B signalling phenotypes in these models, even though additional effects mediated by adenosine receptors or other targets cannot be excluded and are likely to be context dependent, particularly in vivo where extracellular cordycepin levels are expected to be low because of its unfavourable pharmacokinetics. From the perspective of this thesis, NUC-7738 is primarily relevant as a mechanistic probe: by delivering a 3'-deoxyadenosine monophosphate moiety intracellularly without engaging ADA, hENT1 or ADK, it allows the contribution of intracellular 3'-deoxyadenosine nucleotides (CoTP/3'-dATP) to be evaluated independently of the parent nucleoside's pharmacokinetic limitations [29] [2].

1.6.5 Integration with CPA-focused signalling models

Lawrence et al. recently used a systems-pharmacology approach to show that cordycepin consistently represses PI3K/Akt/mTOR and/or MEK/ERK signalling across six cell lines, with effects on mTOR-dependent translation detectable within 30 min and largely independent of AMPK activation [25]. Comparing cordycepin's transcriptomic footprint with reference perturbations indicated that its action converges on growth-factor signalling pathways, and metabolic measurements confirmed that CoTP is the likely active metabolite [25]. When these data are considered alongside the CPA-factor dependence of inflammatory and signalling responses in macrophages and other cells [26] [28], a coherent picture emerges in which cordycepin acts primarily as an

intracellular nucleoside analogue: it must enter cells via nucleoside transporters (or prodrug mechanisms), be phosphorylated to CoTP (or delivered as a monophosphate), and then interfere with CPA and/or another CoTP-sensitive component of growth-factor signal transduction.

Within this framework, adenosine receptors remain relevant as context-dependent targets in systems where receptor pharmacology is clearly demonstrated. For example, hippocampal neurons, dermal fibroblasts, and selected tumour models, with the important caveat that the extracellular presence of cordycepin *in vivo* is unlikely given its pharmacokinetic properties [71] [74]. However, in the macrophage and growth-factor signalling contexts that dominate this thesis, the combined evidence from transporter and kinase dependence, 3DI rescue, NUC-7738 ProTide behaviour and CPA-factor knockdown argues strongly against a primary role for adenosine receptors. Subsequent chapters will therefore treat adenosine receptors as potential modulators in specific tissues rather than as the main mechanism for cordycepin's inhibition of PI3K/Akt/mTOR and MEK/ERK signalling and its anti-inflammatory actions.

1.7 In vivo models and integration.

1.7.1 Animal In Vivo Models

In vivo, the best-characterised inflammatory context for cordycepin is osteoarthritis. Ashraf *et al.* showed that oral cordycepin (8–16 mg/kg) reduces pain behaviour, synovial macrophage infiltration (CD68), synovial proliferation (PCNA), angiogenesis (nestin/VEGF) and histological synovitis in rat MIA and mouse DMM models of knee osteoarthritis [28]. Cordycepin also diminishes IL-1 β and CD68 mRNA in synovial tissue, reduces NF- κ B p65 intensity in synovial macrophages and lowers osteoclast markers and cartilage/subchondral bone damage [28]. Immunofluorescence in human OA synovium and *in vitro* macrophage models links these *in vivo* effects to CPA: CPSF4 and NF- κ B are upregulated and co-localise in inflamed synovium, and cordycepin prevents LPS-induced nuclear accumulation of CPSF4, WDR33 and NF- κ B in human and murine macrophages [28] [26]. These findings support the idea that polyadenylation factors are required for inflammatory gene expression *in vivo* and that pharmacological

CPA inhibition by CoTP can simultaneously reduce pain, inflammatory gene expression and joint pathology.

Across other animal models—acute lung injury, asthma, hepatic and renal inflammation, neuroinflammation—cordycepin likewise reduces inflammatory markers and tissue damage, usually in association with reduced NF- κ B and MAPK signalling and, in some cases, increased AMPK activation [13] [31] [54] [52] [53] [75]. Qin *et al.* summarise these as evidence that cordycepin interacts with multiple inflammatory targets, but emphasise consistent suppression of PI3K/Akt/mTOR and NF- κ B pathways as a recurrent theme [31].

Taken together, the anti-inflammatory literature supports three conclusions. First, cordycepin has robust anti-inflammatory activity in vitro and in vivo across diverse systems, with especially strong evidence in LPS-stimulated RAW264.7 macrophages, airway smooth muscle and synovial macrophage-driven osteoarthritis models [13] [54] [52] [53] [19] [28] [26]. Second, in macrophages and airway cells, these effects are tightly linked to CoTP-dependent CPA inhibition: inflammatory transcripts show selective sensitivity to partial polyadenylation blockade, and genetic depletion of CPA factors (CPSF4/WDR33) phenocopies cordycepin’s impact on NF- κ B localisation, inflammatory gene expression and PI3K/Akt/mTOR readouts [19] [28] [26] [27]. Third, while cordycepin also suppresses PI3K/Akt/mTOR and ERK signalling, often with concurrent AMPK activation, current data do not yet identify a unique direct kinase target; instead, they are compatible with a model in which CPA integrity is necessary for sustained inflammatory and growth-factor signalling, and CoTP-mediated CPA inhibition destabilises both cytokine and signalling transcripts.

In the context of this thesis, RAW264.7 and A549 models sit squarely within this evidence base. In these cells, cordycepin can be treated as an intracellular nucleoside analogue whose anti-inflammatory effects are mediated by CoTP formation, CPA inhibition and secondary signalling repression rather than by extracellular adenosine receptor agonists.

1.7.2 Human studies and early clinical trials

Although the biological activities of cordycepin are best characterised in cell and animal models, a small but growing body of human data now supports anti-inflammatory, organ-protective and anticancer effects of cordyceps-derived preparations and cordycepin-based drugs. These studies are heterogeneous in design and formulation (whole *Cordyceps* extracts, cordycepin-enriched capsules, and the ProTide NUC-7738), but together they show that targeting cordycepin/CoTP biology is feasible and biologically active in patients.

Chronic kidney disease and systemic inflammation.

The most informative cordyceps-based clinical trial for inflammatory disease is a randomised, placebo-controlled study in chronic kidney disease (CKD). Sun *et al.* enrolled 98 adults with stage 3–4 CKD and randomised them to three months of *Cordyceps militaris* capsules (100 mg/day) or placebo [76]. HPLC and MS analysis of the extract showed that cordycepin was the most abundant nucleoside component (37 mg/100 g dried material), alongside adenosine, uridine and other constituents [76].

Compared with placebo, *C. militaris* significantly improved renal function markers: 24 h urinary protein fell by ~37%, blood urea nitrogen by ~13% and serum creatinine by ~18%, with a corresponding improvement in estimated glomerular filtration rate [76]. Histological analysis of kidney biopsies showed reduced interstitial inflammatory infiltrates and normalisation of the glomerular basement membrane in the treated group, whereas placebo patients retained thickened, sclerotic glomeruli [76]. In parallel, the cordyceps group exhibited favourable changes in lipid and redox profiles (reductions in triglycerides, total and LDL cholesterol, cystatin C, myeloperoxidase and malondialdehyde; increases in HDL cholesterol, nitric oxide and superoxide dismutase) compared with placebo [76]. These systemic effects indicate that cordyceps-derived therapy can modify both inflammatory and metabolic risk factors in CKD over a relatively short time frame.

Mechanistically, Sun *et al.* measured TLR4/NF- κ B pathway markers in renal biopsies and used HEK293T cells to dissect the contribution of individual nucleosides.

In patients, *C. militaris* reduced TLR4, NF- κ B p65, COX-2, TNF- α and IL-1 β mRNA and protein levels in kidney tissue relative to placebo [1]. In HEK293T cells, purified cordycepin recapitulated these changes more strongly than adenosine, uridine or other components and lost its effect when TLR4 was knocked down, implicating cordycepin as a key active constituent acting via TLR4/NF- κ B signalling [76]. While the multi-component nature of *C. militaris* capsules and the absence of direct CoTP measurements preclude a purely cordycepin-centric interpretation, this trial provides proof of concept that cordyceps therapy can improve renal function and inflammatory biomarkers in human CKD and that cordycepin likely contributes to these effects via suppression of TLR4/NF- κ B and oxidative stress.

Immunomodulation in healthy volunteers.

Several placebo-controlled trials in healthy adults have assessed immunomodulatory effects of *Cordyceps* preparations, which are relevant as systemic readouts of cordycepin exposure. In a 12-week randomised, double-blind trial in 100 Korean adults, encapsulated *C. militaris* (1.5 g/day) increased NK-cell cytotoxicity and salivary IgA compared with placebo, without significant adverse events or major changes in standard biochemical safety parameters [77]. A related study using a mycelial extract of *Paecilomyces hepiali* (CBG-CS-2), a cordyceps-type fungus, reported increased NK-cell activity and improved CD4:CD8 ratios after eight weeks in healthy volunteers [78]. More recently, Ontawong *et al.* performed a randomised controlled trial of a fermented *C. militaris* beverage in healthy adults; an 8-week intervention delivering 2.85 mg cordycepin per day enhanced NK-cell activity in both sexes and reduced circulating IL-1 β (males) and IL-6 (females) compared with placebo, again with good tolerability [79].

These trials use whole-fungus preparations with relatively low cordycepin content and do not directly measure CoTP, but they consistently show that cordyceps-based products can modulate innate immune function and pro-inflammatory cytokines in humans without major toxicity. Together with the CKD study, they support the translational relevance of the anti-inflammatory and immunomodulatory effects seen in macrophages and animal models, while also emphasising that in the clinic cordycepin will often be delivered as part of a complex mixture.

Oncology: Cordycepin combinations in early clinical trials

Earlier work explored direct cordycepin administration in haematological malignancies in combination with ADA inhibition. A pilot study in terminal deoxynucleotidyl transferase (TdT)-positive acute lymphoblastic leukaemia combined pentostatin (2–3 mg/m²) with daily cordycepin (6–24 mg/m² for three days) to inhibit ADA and allow systemic cordycepin exposure [80]. Pharmacodynamic monitoring showed that 3 mg/m² pentostatin achieved 90% ADA inhibition in plasma and blood cells, and dose-escalated cordycepin produced transient reductions in circulating blasts and partial restoration of normal haematopoiesis in some patients, with only mild non-haematological toxicity [80]. However, the small sample size, short treatment duration and lack of long-term follow-up preclude firm conclusions about efficacy, and subsequent development has shifted towards ProTides such as NUC-7738 rather than further trials of free cordycepin with systemic ADA inhibition.

Oncology: ProTide NUC-7738

Direct clinical experience with pure cordycepin in cancer is very limited, but its ProTide derivative NUC-7738 has now entered multi-centre trials in patients with advanced solid tumours. NUC-7738 is a 5'-aryloxy phosphoramidate of 3'-deoxyadenosine designed to bypass three key resistance mechanisms that limit cordycepin: extracellular ADA-mediated deamination, dependence on equilibrative nucleoside transporters and ADK-dependent first phosphorylation [29] [2]. In the first-in-human Phase I part of the NuTide:701 trial, 38 patients with heavily pre-treated advanced solid tumours received weekly NUC-7738 infusions (14–2000 mg/m²) in a dose-escalation design. NUC-7738 was well tolerated, with no grade 4 toxicities, low rates of grade 3 events and no dose-limiting myelosuppression; the recommended Phase II dose was established at 1350 mg/m² [29] [81] [82]. Pharmacokinetic and pharmacodynamic analyses demonstrated efficient intracellular generation of 3'-dATP with a prolonged intracellular half-life (42 h) and evidence of NF- κ B pathway modulation in tumour biopsies [2] [81]. Signals of anti-tumour activity were observed, including durable partial responses and disease stabilisation in melanoma and other solid tumours refractory to standard therapy [29] [81] [82].

Subsequent expansion cohorts and combination arms (NUC-7738 plus pembrolizumab) have reported encouraging disease control rates and prolonged progression-free survival in PD-1-refractory melanoma and other indications, with a safety profile dominated by low-grade fatigue, gastrointestinal symptoms and manageable haematological toxicity [81] [82]. Mechanistically, translational analyses from these trials show that NUC-7738 disrupts RNA polyadenylation and alters gene expression programmes in ways consistent with the polyadenylation inhibition and growth-factor pathway suppression described for cordycepin in preclinical models [29] [81]. These human data therefore support the central premise of this thesis: that intracellular 3'-deoxyadenosine nucleotides can be delivered safely to patients, achieve pharmacologically relevant exposure, and perturb polyadenylation and downstream signalling in human tumours.

In summary, human data on cordycepin and cordycepin-derived therapies remain limited but are consistent with three key themes emerging from preclinical work. First, *Cordyceps* preparations enriched for cordycepin can safely modulate inflammatory pathways, redox status and clinical biomarkers in chronic inflammatory disease (CKD) and in healthy volunteers, with evidence for TLR4/NF- κ B suppression and improved lipid and oxidative profiles [76] [77] [78] [79]. Second, ProTide chemistry allows direct delivery of 3'-deoxyadenosine nucleotides to tumours in humans, recapitulating growth-factor signalling inhibition and CPA disruption with manageable toxicity and early signs of anti-tumour activity [29] [2] [81] [82]. Third, early cordycepin-pentostatin combination studies demonstrate proof of concept that ADA protection can unmask cordycepin's cytotoxic potential in leukaemia, but also highlight the practical challenges and toxicity risks of systemic ADA inhibition [80]. These human findings provide important context and constraints for interpreting the *in vivo* models discussed above and motivate the mechanistic studies in RAW264.7 and A549 cells presented later in this thesis.

1.8 Biological activities of cordycepin

1.8.1 Anti-proliferative effects: cell death, survival and division

A large body of in vitro work demonstrates that cordycepin exerts robust anti-proliferative effects in diverse vertebrate cell types, although the effective concentration and mode of death are strongly cell-type and context dependent. A systematic review of 192 vertebrate cell-culture papers identified 103 datasets with usable IC_{50} values and reported a median IC_{50} of 135 μ M (range 15 μ M–2 mM) for cordycepin over 24–48 h, with most values clustering around the median and a minority of almost resistant lines skewing the distribution [13]. In the same analysis, 65 studies reported apoptosis and eight reported combined apoptosis and autophagy as the dominant modes of death, whereas 45 studies described little or no cytotoxicity at concentrations that still produced other biological effects, often around 50 μ M [13]. These data already imply that cordycepin is not purely cytotoxic but can be cytoprotective or neutral at lower doses, depending on cell state.

Mechanistically, the anti-proliferative effects are largely attributed to induction of apoptosis and, in some models, autophagic cell death. In breast cancer cells, cordycepin triggers caspase activation, mitochondrial depolarisation and modulation of BCL-2 family proteins, with apoptosis and autophagy contributing in parallel to loss of viability in ER-positive and ER-negative settings [71] [83]. In MCF-7 cells, both purified cordycepin and *Cordyceps militaris* extracts increase cleavage of caspases-7/-8/-9, raise the Bax:Bcl-2 ratio and reduce XIAP expression, consistent with activation of the intrinsic mitochondrial apoptosis pathway [83]. In colon cancer models, cordycepin inhibits proliferation via Bax-dependent mitochondrial apoptosis, again supporting a canonical pro-apoptotic mechanism in transformed cells [84] [5].

Cordycepin also represses proliferation in lung adenocarcinoma lines. In A549 and PC9 cells, 50–200 μ M cordycepin reduces viability in a concentration- and time-dependent manner, concomitant with suppression of ERK/Slug signalling, downregulation of anti-apoptotic proteins and induction of caspase-dependent apoptosis [85]. Similar concentrations reduce cell numbers below the seeding density in MCF-7 breast

cancer cells over 72 h and synergise with the ADA inhibitor pentostatin, consistent with CoTP as the active metabolite and ADA-mediated deamination as a resistance mechanism [25]. In xenograft models, twice-weekly intravenous cordycepin (22 mg/kg) slows MCF-7 tumour growth in nude mice, confirming that the anti-proliferative activity can be realised in vivo when pharmacokinetic barriers are managed [25].

Cell-cycle analyses across 20 flow-cytometry datasets show that cordycepin can arrest cells in G_0/G_1 , S or G_2/M , with no single “signature” phase predominating in all systems; G_2/M arrest is slightly more common but S-phase and mixed S+ G_2/M accumulations are also frequent [13]. Doses affecting the cell cycle (median 80 μ M) are similar to those that reduce cell numbers (median IC_{50} 135 μ M), but the precise checkpoint affected clearly depends on cell type, dose and timing. This contrasts with microtubule poisons, which show characteristic, pathway-specific arrests, and reinforces the view that cordycepin acts primarily through RNA metabolism and growth-factor signalling rather than a single classical cell-cycle target.

Complementing these mechanistic studies, high-throughput transcriptomic and signalling analyses show that cordycepin globally represses growth-promoting programmes. In six cell lines, cordycepin rapidly (≤ 30 min) dephosphorylates Akt, S6K and 4E-BP1 and reduces translation, with pathway analysis revealing convergence on PI3K/Akt/mTOR and ERK cascades and a transcriptional footprint resembling PI3K/mTOR inhibition [25] [27]. Importantly, AMPK activation is common but not required for the anti-proliferative phenotype; cordycepin still suppresses growth-factor-driven gene expression and translation in AMPK-deficient cells [25] [27]. Together, these data support a model in which intracellular cordycepin nucleotides, particularly CoTP, impose an anti-proliferative state by combining CPA inhibition, translational repression and sustained growth-factor signalling blockade, while allowing low-dose, non-cytotoxic exposures to modulate stress responses and survival in some non-malignant cells [13] [31] [71].

1.8.2 Effects of cordycepin on cell migration

Cordycepin has a remarkably consistent anti-migratory effect across a wide panel of vertebrate cells. The systematic review identified 27 vertebrate studies of migration;

26 reported reduced migration or invasion after cordycepin, in cell types including carcinoma lines, macrophages, vascular smooth muscle cells and endothelial cells, over a dose range of 0.4–400 μM (median lowest effective concentration 100 μM) [13]. Only one study reported no effect, and even there the migration data were incomplete [13]. In many experiments, cordycepin was used at concentrations that did not yet reduce cell viability, indicating that impairment of motility is not simply a secondary consequence of cell death.

In hormone-sensitive LNCaP prostate carcinoma cells, 10–50 μM cordycepin markedly inhibits migration and invasion in scratch and Matrigel assays. This is accompanied by reduced expression and activity of MMP-2 and MMP-9, increased TIMP-1/-2, downregulation of tight-junction proteins and inactivation of PI3K/Akt signalling; pharmacological inhibition of PI3K phenocopies much of the anti-migratory phenotype [86]. Similar patterns are seen in other epithelial cancers. In HSC-4 oral squamous carcinoma cells, cordycepin (10–20 μM) reduces migration and invasion, reverses epithelial–mesenchymal transition (increased E-cadherin, decreased N-cadherin and vimentin) and impairs FAK/Akt/mTOR signalling; these effects are enhanced by radiotherapy, consistent with interference in motility and stress-response pathways [87].

Our group’s work extends these observations to growth-factor-driven breast cancer models. In MCF-7 cells, 10 μM cordycepin significantly slows wound closure over 16 h in a scratch assay performed in 1% serum, at a concentration that does not yet reduce cell number, demonstrating a direct anti-migratory effect in the absence of overt cytotoxicity [25]. In vivo, cordycepin reduces metastatic dissemination in several tumour models, often in association with reduced MMP expression, lower FAK phosphorylation and partial reversal of EMT markers, although these studies are more heterogeneous in design [13] [71].

Mechanistically, the anti-migratory effects converge on known regulators of invasion and motility. Across multiple systems, cordycepin reduces phosphorylation of FAK and other focal adhesion components, downregulates MMP-2/-9 and uPA, increases protease inhibitors (TIMP-1/-2) and strengthens epithelial adhesion markers such as E-cadherin [13] [71] [86] [87]. In many of these studies, PI3K/Akt and, to a lesser extent,

ERK signalling are attenuated, and PI3K inhibitors partially mimic the anti-migratory phenotype [25] [86] [87]. Taken together, the migration data support viewing cordycepin as an anti-metastatic agent at sub-cytotoxic concentrations, with motility control tightly coupled to its broader effects on growth-factor signalling and, as discussed elsewhere, CPA-dependent mRNA maturation.

1.8.3 Effects of cordycepin on the inflammatory response

Overview and scope.

Inflammation is one of the biological activity of cordycepin. The systematic review identified 54 cell-culture studies and 28 animal studies examining inflammatory readouts; 36 of the cell studies reported broad reductions of inflammatory mediators, one described selective iNOS inhibition with TNF α induction, and only one reported a robust pro-inflammatory profile [13]. The most frequently examined outputs included TNF α , IL-1 β , IL-6, TGF β , NOS2/iNOS, PTGS2/COX-2, PTGES, VCAM-1 and MMP-3/-9 [13]. Macrophages and microglia are the dominant cellular systems, but airway smooth muscle, epithelial cells (including A549) and other cell types also show consistent anti-inflammatory responses [13] [31] [54] [52] [53] [19] [28]. At the tissue level, cordycepin reduces synovitis, lung injury, hepatic inflammation and other pathological inflammatory processes in multiple rodent models [31] [28] [75].

Because this thesis uses TNF-stimulated A549 cells and LPS-stimulated RAW264.7 macrophages, the focus here is on those models and related systems.

Airway smooth muscle and epithelial models: A549 and related systems.

Kondrashov *et al.* provided direct evidence that polyadenylation is a selective vulnerability of inflammatory transcripts in human airway cells [19]. In primary human airway smooth muscle cells, 30–50 μ M cordycepin strongly suppresses TNF α /IL-1 β -induced CCL2, CXCL1 and IL8 mRNA while leaving housekeeping mRNAs (e.g. GAPDH, RPL10A, ACTB) largely unchanged [19]. Cordycepin shortens nuclear poly(A) tails and specifically affects newly synthesised RNAs, consistent with inhibition of transcription-coupled polyadenylation [19]. Importantly, NF- κ B p65 nuclear translocation and RNA polymerase II recruitment to inflammatory promoters are not impaired; instead,

cordycepin causes defects in 3'-end cleavage and transcription termination, leading to accumulation of uncleaved and readthrough transcripts and loss of mature polyadenylated mRNA [19]. A non-polyadenylated histone reporter is insensitive to cordycepin, whereas reporters that depend on poly(A) signals from CXCL1 become cordycepin-sensitive, and another chain-terminating polyadenylation inhibitor (8-aminoadenosine) and PAP α knockdown both replicate the selective repression of inflammatory genes [19]. These findings establish polyadenylation as an authentic targetable node in TNF-driven airway inflammation and are highly relevant to TNF-treated A549 experiments.

In the same study, TNF-induced chemokine expression (e.g. CXCL1, IL8) in A549 alveolar carcinoma cells is also reduced by cordycepin, and PAP α knockdown in A549 lowers TNF-induced inflammatory transcripts without major effects on total poly(A) tail length, again indicating a specific sensitivity of inflammatory genes to partial CPA inhibition [19]. Independent work in lung epithelial systems complements this. In a mouse model of LPS-induced acute lung injury, cordycepin reduces leukocyte infiltration, inflammatory cytokines and tissue damage; in vitro, 40–80 μ M cordycepin suppresses LPS-induced, but not TNF α -induced, VCAM-1 expression in A549 cells, pointing to TLR4-specific anti-inflammatory effects at the epithelial surface [75]. These dual observations—strong suppression of TNF-driven chemokines via CPA inhibition and selective inhibition of TLR4-driven VCAM-1—indicate that cordycepin can modulate both airway-resident structural cells and recruited immune cells via overlapping but not identical mechanisms.

Macrophages and microglia: RAW264.7 and related models.

LPS-stimulated RAW264.7 macrophages have been the main workhorse system for defining cordycepin's anti-inflammatory mechanisms. Early work showed that 10–40 μ M cordycepin significantly reduces LPS-induced production of NO, PGE₂, TNF α and IL-1 β , with concordant decreases in NOS2 and PTGS2 protein expression [54]. These changes are accompanied by suppression of I κ B α degradation, reduced phosphorylation of Akt (Ser473) and p38, and impaired nuclear translocation of NF- κ B p65, leading the authors to conclude that cordycepin inhibits NF- κ B activation via the Akt/p38 axis downstream of TLR4 [54]. Subsequent studies extended this to broader signalling

cascades: in LPS-stimulated RAW264.7 cells, exposure to cordycepin for a duration of 24 hours resulted in a decrease TLR4 expression, blocked phosphorylation of ERK, JNK and p38, inhibited I κ B α and p65 phosphorylation, and reduced DNA binding of NF- κ B, resulting in attenuated expression of NOS2, PTGS2, TNF α and IL-1 β [52]. Similar signalling patterns—suppression of MAPKs and NF- κ B—have been observed in BV2 microglia, where cordycepin decreases production of TNF α , IL-1 β and NO in response to LPS [53].

Our group’s work in RAW264.7 macrophages adds an additional mechanistic layer by connecting these inflammatory phenotypes to cleavage and polyadenylation. Microarray and RNA-Seq analyses show that 20 μ M cordycepin markedly represses LPS-induced expression of inflammatory “master regulators” (e.g. Tnf, Il1b, Acod1) and NF- κ B/MAPK pathway genes while inducing a distinct subset of stress-response transcripts [26] [27]. Immunohistochemistry demonstrates that cordycepin prevents LPS-induced nuclear accumulation of NF- κ B p65, CPSF4 and WDR33 and reduces the nuclear:cytoplasmic ratio of these factors, consistent with interference in both signalling and CPA-factor recruitment [28] [27]. Crucially, siRNA knockdown of WDR33 or CPSF4 in RAW264.7 cells, in the absence of cordycepin, recapitulates many of these inflammatory and signalling phenotypes: reduced induction of Tnf and Il1b mRNAs, decreased NF- κ B p65 nuclear localisation, and suppression of PI3K/Akt/mTOR readouts such as p-Akt and p-4E-BP1 [26] [27]. These data strongly suggest that CPA factors are required for full LPS/TLR4-driven inflammatory responses and that CoTP-mediated CPA inhibition can phenocopy genetic depletion of CPSF4/WDR33.

At the level of RNA processing, cordycepin shortens nuclear poly(A) tails and reduces the efficiency of 3'-end cleavage and transcription termination for inflammatory transcripts, leading to loss of export-competent mRNPs and the production of mRNAs with truncated tails that are rapidly deadenylated and degraded in the cytoplasm [19] [26]. Mechanistic synthesis work from our group proposes that CoTP terminates poly(A) extension and traps CPSF4/WDR33 on incompletely processed 3' ends, thereby creating a rate-limiting constraint in CPA-factor availability that is particularly detrimental to rapidly induced NF- κ B targets such as Tnf and Il1b [26] [27]. In this view, the anti-

inflammatory phenotype arises from the intersection of (i) reduced nuclear throughput of cytokine transcripts, (ii) shortened cytoplasmic poly(A) tails that accelerate decay and impair closed-loop formation, and (iii) suppression of PI3K/Akt/mTOR signaling occurs via an unidentified RNA, referred to as the RNA target of cordycepin (RToC), whose function is influenced by alterations in polyadenylation, as described in section 1.5.3. This mechanism is unlikely to depend on the synthesis of a protein from this RNA. [54] [52] [26] [27] [69] [70].

1.9 Thesis aims and summary of findings

1.9.1 Thesis aims

The overarching aim of this thesis is to elucidate how cordycepin (3'-deoxyadenosine) and its metabolites, particularly 3'-deoxyinosine (3DI) and 3'-deoxyadenosine triphosphate (CoTP/3'-dATP), modulate inflammatory gene expression and growth-factor signalling through effects on mRNA 3'-end processing and polyadenylation. A central working model is that cordycepin does not suppress PI3K/Akt/mTOR signalling through direct kinase inhibition, but instead acts by altering the polyadenylation of one or more RNA species whose polyadenylation-dependent functions are sensitive to cordycepin. In this framework, an "RNA target of cordycepin" (RToC) is defined as an RNA whose disrupted 3'-end processing is sufficient to reshape growth-factor signalling outputs and which may act without encoding a protein product.

This model is motivated by several lines of evidence. Mechanistically, CoTP is known to interfere with cleavage and polyadenylation, trapping CPSF components at 3' ends and altering poly(A)-tail homeostasis. In parallel, independent studies show that direct inhibition of protein synthesis with cycloheximide or anisomycin paradoxically activates Akt via PI3K/PDK1- or NO-sGC-cGKII-dependent pathways, rather than suppressing it [69] [70]. In contrast, cordycepin consistently represses PI3K/Akt/mTOR signalling. Together, these observations argue against a simple "translation block = signalling suppression" model and instead support an RNA-centred mechanism in which CPA-sensitive transcripts act upstream of PI3K/Akt/mTOR.

Against this background, the specific objectives of the thesis are:

1. To define the metabolic “rescue” pathways that link 3'-deoxyinosine (3DI) to active cordycepin nucleotides.

This objective addresses how 3DI, the major circulating metabolite of cordycepin *in vivo*, is salvaged back to CoTP/3'-dATP in epithelial cells and macrophages, with particular emphasis on the roles of adenine phosphoribosyltransferase (APRT) and the macrophage-restricted enzyme FAMIN/LACC1.

2. To identify genetic determinants of cordycepin resistance using haploid insertional mutagenesis.

Using piggyBac gene-trap libraries in eHAP cells, this objective seeks to map genes and pathways whose loss-of-function confers a survival advantage in cordycepin, including nucleoside transport and salvage, RNA 3'-end processing, and AMPK–mTOR–autophagy modules. Within this framework, a particular goal is to highlight loci whose disruption could implicate candidate RNA targets of cordycepin (RToC).

3. To compare the anti-inflammatory and signalling signatures of natural and synthetic cordycepin and the ProTide NUC-7738.

Here, bulk RNA-seq is used to test whether fermentation-derived cordycepin, chemically synthesised cordycepin, 3DI and NUC-7738 produce equivalent transcriptional and pathway-level responses in LPS-stimulated RAW264.7 macrophages, and to exclude preparation-specific contaminants as the primary drivers of activity.

4. To quantify poly(A)-tail remodelling induced by cordycepin and NUC-7738 at later time points. Using Oxford Nanopore long-read sequencing, this objective examines how 120-minute exposure to cordycepin or NUC-7738 reshapes genome-wide poly(A)-tail length distributions and how these changes relate to altered transcript abundance and pathway-level outputs.
5. To resolve the early temporal relationship between poly(A)-tail dynamics, transcript abundance and pathway repression.

By profiling poly(A)-tail length and gene expression at an early 15-minute time

point, this objective aims to capture the proximal consequences of CPA inhibition, distinguish primary from secondary responses, and narrow down a set of cordycepin-sensitive transcripts that could serve as candidate RToC species linking CPA disruption to PI3K/Akt/mTOR restraint.

Collectively, these aims are designed to connect the pharmacokinetics and metabolism of cordycepin and 3DI to their effects on CPA, poly(A)-tail dynamics, inflammatory gene expression, and growth-factor signalling, and to generate concrete, testable hypotheses about RNA intermediates that mediate these effects.

1.9.2 Summary of findings

The experimental chapters converge on a coherent model in which cordycepin acts primarily as an intracellular nucleoside analogue: it must be taken up, phosphorylated to its nucleotide forms, and then perturbs CPA and downstream signalling in a way that selectively restrains inflammatory and growth-factor pathways.

First, Cordycepin undergoes rapid deamination to 3'-deoxyinosine (3DI), a process known to substantially constrain its biological efficacy. To delineate the metabolic fate of cordycepin, we employed siRNA-mediated knockdown and pharmacological inhibition of candidate enzymes that might reconvert 3DI to cordycepin. The resulting data demonstrated that, unexpectedly, cordycepin activity in tissue culture is contingent upon a previously unrecognized salvage pathway involving adenine phosphoribosyltransferase (APRT) and, in macrophages, the enzyme FAMIN/LACC1. These enzymes appear to direct the metabolism of adenine-conjugated 3'-deoxyribose, thereby facilitating the production of CoTP and the suppression of LPS-induced inflammatory gene expression. Results obtained with 3DI indicated potential distinctions between the dependence of 3DI and cordycepin on these salvage pathways; however, definitive interpretation was impeded by the detection of cordycepin contamination in the 3DI preparations.

Second, a haploid piggyBac survival screen in eHAP cells identifies three broad classes of resistance mechanisms. Loss-of-function insertions in ADK are the most

robust and recurrent hits across doses and platforms, confirming that phosphorylation to cordycepin mono/di/triphosphates is required for cytotoxicity. A second class comprises genes involved in RNA 3'-end formation, deadenylation and RNA maturation (e.g. CNOT6, PARN, Integrator components), consistent with the role of cordycepin as a polyadenylation inhibitor and indicating that the state of mRNA 3'-end processing modulates sensitivity. A third class includes genes in AMPK-mTOR signalling, autophagy, mitophagy, ER-mitochondrial coupling and ubiquitin-dependent proteostasis (e.g. PPP2R2B, ATG5, VPS41, UBE2D1, CASP8), pointing to integrated stress-response and quality-control pathways that shape survival under cordycepin.

Within this screen, the long non-coding RNA LINC03121 (Lnc-PLA2G4A-4) emerges as a striking, though platform-specific, hit: under hyPB at 200 μ M cordycepin, LINC03121 accounts for ≈ 10.86 % of insertions versus 0.21 % in DMSO controls. This non-coding RNA has been documented to be involved in AKT signalling [88].

Third, comparative transcriptomics in LPS-stimulated RAW264.7 macrophages show that natural and synthetic cordycepin induce highly similar gene-expression and pathway signatures. Both preparations repress a broad panel of inflammatory transcripts and down-regulate pathways linked to NF- κ B, interferon signalling, PI3K/Akt/mTOR and mTOR-dependent translation. The ProTide NUC-7738 reproduces these core features when used at concentrations that generate comparable 3'-deoxyadenosine nucleotide exposure, supporting the view that the shared active species is intracellular CoTP/3'-dATP rather than preparation-specific contaminants, ruling out contaminants as the primary drivers of activity.

Fourth, long-read nanopore sequencing reveals that cordycepin and NUC-7738 induce rapid, genome-wide poly(A)-tail shortening at 120 minutes, with concomitant reductions in the abundance of many inflammatory and growth-factor-linked transcripts. A smaller subset of transcripts shows tail lengthening and relative sparing of abundance, often enriched for mitochondrial and metabolic functions, suggesting selective stabilisation of mRNAs that support cellular bioenergetics under stress. These data link CPA inhibition directly to altered mRNA stability and provide a transcriptome-wide view of

how cordycepin re-weights the post-transcriptional landscape.

Fifth, early-timepoint (15-minute) analyses demonstrate that cordycepin triggers detectable poly(A)-tail remodelling and expression changes before wholesale transcriptional re-programming occurs. Even at this short exposure, transcripts associated with translation control, signal transduction and growth-factor signalling exhibit coordinated shifts in tail length and abundance. These early changes support a model in which interference with CPA and poly(A)-tail homeostasis is a proximal event that precedes, and likely contributes to, the downstream repression of PI3K/Akt/mTOR signalling and inflammatory gene induction.

Integrating these findings with the literature on translational inhibitors and Akt activation, the thesis advances the hypothesis that suppression of PI3K/Akt/mTOR by cordycepin is mediated by one or more non-coding RNAs whose polyadenylation-dependent function is disrupted by CoTP. In particular, the convergence of (i) strong loss-of-function enrichment of LINC03121 (Lnc-PLA2G4A-4) in the haploid screen, (ii) external evidence that LINC03121 (Lnc-PLA2G4A-4) promotes p-EGFR/p-AKT signalling via a miR-23b-3p/versican axis [88], and (iii) global evidence that cordycepin perturbs CPA and poly(A)-tail dynamics, leads this thesis to propose LINC03121 (Lnc-PLA2G4A-4) as a leading candidate RToC. This model—whereby cordycepin-induced disruption of LINC03121’s polyadenylation and ceRNA function would relieve versican expression and dampen EGFR → Akt signalling—is explicitly framed as a testable hypothesis in the Conclusion and proposed as a focus for future mechanistic work rather than as a mechanism already proven here.

In summary, the thesis links cordycepin’s pharmacokinetics and nucleoside rescue metabolism to CPA inhibition, poly(A)-tail remodelling, and restrained PI3K/Akt/mTOR signalling, and closes by nominating a specific non-coding RNA, LINC03121, as a plausible RNA intermediate that could connect these layers.

Chapter 2

Methods

2.1 Material and Methods Overview

This PhD project covers multiple fields, such as analytical chemistry, molecular biology, and bioinformatics. As a result, a diverse set of methodologies have been employed, including molecular biological techniques, cell culture experiments, haploid genetic screening, RNA-Seq using Oxford Nanopore technology long reads, Dorado basecalling to estimate and analyse Poly(A) tail lengths with NanopLen, and integrated chromatography and mass spectrometry protocols with targeted metabolomics analyses. Reagents, chemicals, and media used throughout the project are detailed in table 2.1, while computer software is documented in table 2.2.

Table 2.1: Reagents, chemicals and media used

Reagent/Material	Use(s)	Supplier
Reliaprep® RNA Cell Miniprep System	RNA extraction - caterpillars	Promega
- BL + TG buffer		
- RNA wash solution		
- DNase I		
- Yellow core buffer		
- MnCl ₂ (0.09M)		

Continued on next page

Table 2.1 – *Continued from previous page*

Reagent/Material	Use(s)	Supplier
- Column wash solution		
SuperScript® III First-Strand Synthesis System	Reverse transcription	ThermoFisher
- Random hexamers		
- dNTPs		
- First Strand Buffer		
- Dithiothreitol (DTT)		
- Superscript III		
GoTaq® qPCR Master Mix	Quantitative polymerase chain reaction (qPCR)	Promega
Distilled, deionised water (ddH ₂ O)	General use	
Isopropanol (HPLC grade)	RNA Extraction	VWR
Methanol (LC-MS grade)	LC-MS (sample preparation; solvent)	VWR
Acetonitrile (LC-MS grade)	LC-MS (phases)	VWR
Ammonium Carbonate	LC-MS (phases)	VWR
Trypan Blue (0.4%)	Cell counting	ThermoFisher
Dimethyl sulfoxide (DMSO)	Vehicle control	Sigma-Aldrich
Foetal Bovine Serum (FBS)	Cell culture	Gibco
Phosphate-buffered saline (PBS)	Cell culture	ThermoFisher
Oligonucleotides/Primers	qPCR	SigmaAldrich / Merck
DMEM	Medium for RAW264.7 cells	SigmaAldrich

Continued on next page

Table 2.1 – *Continued from previous page*

Reagent/Material			Use(s)	Supplier
DMEM/F12			Medium for A549 cells	SigmaAldrich
Iscove's medium	Modified Dulbecco's		Medium for eHAP cells	SigmaAldrich
PrestoBlue Cell Viability Reagent			Cell viability assays	Invitrogen
Dharmacon specific siRNA oligonucleotides			Gene knockdown	Horizon Discovery
Lipofectamine transfection Reagent	RNAiMAX Trans-		Transfection Reagent - In Vitro Delivery of siRNA	Invitrogen / ThermoFisher
INTERFERin Reagent	Transfection		Transfection Reagent - In Vitro Delivery of siRNA	Polyplus
Cordycepin (3'-deoxyadenosine)			Cell treatments	Carbosynth (Biosynth since 09 - 2022)
3'-Deoxyinosine (3DI)			Cell treatments	Santa Cruze Biotechnology
NUC-7738			Cell treatments	NuCana (a gift)
Lipopolysaccharide (LPS)			Inflammatory stimulus	Sigma-Aldrich
Tumor Necrosis Factor- α (TNF- α)			Inflammatory stimulus	ThermoFisher

Table 2.2: Computer software used

Software name	Use
Microsoft Office Excel® 365	General data analysis and calculations
GraphPad Prism® 10	Visualization and statistical evaluation for qPCR and siRNA data
R version 4.5.1	Advanced data statistics and figures (e.g. DGE and heatmaps)
Python version 3.13.0	Advanced data processing, statistics, and figures (e.g. NanopLen and volcano plots)
edgeR from Bioconductor	Differential gene expression
IsoQuant	ONT isoform and genes quantification
Dorado	ONT basecalling
NanopLen	Poly(A) tail analysis
pod5	Converting fast5 to pod5 for poly(A) estimation
QIAGEN IPA	Pathway analysis
ACD/Labs Spectrus Processor	LC-MS/MS visualization
Thermo XCalibur®	Overview of LC-MS on the (Q-)Exactive mass spectrometer; operation of liquid chromatography method

2.2 Cell Culture and Maintenance

2.2.1 Cell Lines and Culture Conditions

The RAW264.7 macrophages were cultured in Dulbecco's Modified Eagle Medium (DMEM; Sigma; 6429) supplemented with 10% foetal bovine serum (FBS; Gibco; 11550356). Cells were passaged when confluency reached ≈ 70 -80% in culture flasks

at ratios of 1:12. For passaging; RAW264.7 macrophages were suspended in culture media by scraping with sterile cell scrapers (Fisher Scientific; 11597692). RAW264.7 macrophages were used at passages ranging from 10 to 30 throughout this study. Prior to treatments, RAW264.7 macrophages were incubated for 24 hours in DMEM media supplemented with 0.5% FBS.

A549 cells were cultured in DMEM/F12 medium supplemented with 10% fetal bovine serum (FBS; SigmaAldrich). Cells were passaged when they reached approximately 70-80% confluency in culture flasks, using a 1:5 ratio. For passaging, the A549 cells were rinsed with sterile PBS before being detached from the flask by incubating for 5 minutes at 37°C with 5% CO₂ in 1x Trypsin-EDTA in PBS (FisherScientific; 10779413). Fresh culture medium was used to collect cells from the flask for passaging. A549 cells used in this study were at passages between 30 and 40. A549 cells were maintained in serum-free DMEM/F12 medium overnight prior to being treated with TNF.

eHAP cells were cultured in Iscove's Modified Dulbecco's medium supplemented with 10% FBS and 1% penicillin/streptomycin. Cells were maintained at 37 °C in a humidified environment with 5% CO₂. The cells were split when they were 65-70% confluent, with all experimental passages ranging from 6-30 in culture flasks.

The human breast adenocarcinoma cell line MCF-7 was maintained in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% % fetal bovine serum (FBS), 1% L-glutamine (Sigma; G7513) and 1% Penicillin/Streptomycin (Sigma; P4333). Subculture was initiated when cell confluence reached approximately 70-80% within the culture flasks, employing a split ratio of 1:3. For the passaging process, MCF-7 cells were washed with sterile phosphate-buffered saline (PBS) prior to the detachment procedure, which involved a 5-minute incubation at 37°C with 5% CO₂ using 1x Trypsin-EDTA in PBS (FisherScientific; 10779413). The cells were then harvested with fresh culture medium to facilitate passaging. Throughout this study, MCF-7 cells were utilized in passages ranging from 4 to 25.

Cells were incubated in serumfree media to synchronize in a quiescent state prior to activation of inflammatory signaling cascades. Serum starvation stops cellular proliferation and metabolism, allowing for a more controlled evaluation of the signaling

responses induced by subsequent inflammatory triggers. This synchronization step helps reduce the basal variability in inflammatory gene expression between samples.

2.3 Treatment Conditions

In this study, various bioactive compounds were used in differing concentrations based on the cell line used. RAW264.7 macrophages and A549 cells were initially seeded at predetermined cell densities using a hemocytometer. After a 24-hour period, the initial culture medium was replaced, as detailed in section 2.2.1. The cells were then rinsed with PBS and cultured for another 24 hours in a fresh medium containing reduced FBS or no FBS (0.5% FBS for RAW264.7 macrophages and no FBS for A549 cells) before undergoing treatment. To provoke an inflammatory response in RAW264.7 macrophages, bacterial lipopolysaccharide (LPS; Sigma; L4774) was used as outlined in the figure captions. For A549 cells, the inflammatory response was stimulated using Human Tumor Necrosis Factor - α (TNF; Sigma-Aldrich; H8916). MCF-7 cells were also seeded at specific densities using a hemocytometer 24 hours before treatment, without undergoing starvation.

The treatment concentrations used in this study are summarized in Table 2.3 and detailed within the corresponding figure descriptions in the Results section. These concentrations were validated through independent experiments that I performed (described in Chapter 3) or confirmed based on established protocols from previous research conducted in our laboratory. The subsequent sections specify the seeding densities applied for each experimental condition.

Table 2.3: Treatment compounds and concentrations

Compound	Concentration Range	Cell Line	Treatment Duration	Purpose
Cordycepin	20 μ M (RAW264.7), 50–200 μ M (A549, eHAP)	RAW264.7, A549, eHAP, MCF-7	15 min–72 h	Active compound
3'- Deoxyinosine	200 μ M	A549, RAW264.7	1 h	Metabolite rescue
NUC-7738	200 μ M	RAW264.7	1 h	ProTide com- parator
LPS	1 μ g/mL	RAW264.7	1 h	Inflammatory stimulus
TNF- α	10 ng/mL	A549	1 h	Inflammatory stimulus
DMSO	0.05–0.1% (v/v)	RAW264.7, A549, eHAP, MCF-7	Matched to compound	Vehicle control

2.4 Small Interfering RNA (siRNA) Transfection

2.4.1 siRNA Knockdown in RAW264.7 cells

The RAW264.7 macrophages were seeded in 6-wells at a density of 150000 cells per well to restrict over-confluency, as this procedure requires an extra 24 hours of incubation compared to other procedures. The cells were allowed to adhere for 24 hours. Subsequently, the siRNA transfection complexes were prepared in a total volume of 300 μ L of Opti-MEM medium for each siRNA condition. siRNA stock solutions were prepared at a concentration of 100 μ M and subsequently diluted to 10 μ M by mixing 10 μ L of the 100 μ M siRNA stock with 90 μ L of RNase-free water. Lipofectamine

RNAiMAX (Thermo Fisher Scientific) was used as transfection reagent for all RAW264.7 experiments, following the manufacturer’s recommended protocol as follows Table 2.4.

Table 2.4: Transfection mixture composition

siRNA mixture		Lipofectamine mixture	
Optimem	145 μ l	Optimem	142.5 μ l
siRNA	5 μ l	Lipofectamine	7.5 μ l

The components were gently combined using slow vortexing for approximately 10 seconds, followed by incubation at room temperature for 5–10 minutes to facilitate the formation of transfection complexes between siRNA and Lipofectamine. Meanwhile, the culture medium in each well was replaced with 1.7 mL of fresh pre-warmed medium containing 10% FBS. Subsequently, 300 μ L of the prepared transfection mixture was carefully added to each well (to give a final concentration of 25 nM siRNA in a medium volume of 2 ml) and mixed with gentle swirling to ensure an even distribution. The plate was then incubated for an additional 24 hours. On the third day, a new siRNA mixture was prepared and incubated for 10 minutes as described above. During this time, the medium was removed and the cells were rinsed with PBS before adding 1.7 mL of medium containing 0.5% FBS to each well. A further 300 μ L of the siRNA mixture was added and the cells were incubated for another 24 hours. The following day, cells were treated with either cordycepin (20 μ M) or 3DI (200 μ M) for a total of 120 minutes; LPS was added 60 minutes after the start of compound treatment (i.e. in the presence of the compound) and cells were stimulated for a further 60 minutes before being harvested for RNA isolation.

The lipofectamine RNAiMAX transfection reagent (cat no 13778150) was purchased from Thermo Fisher Scientific. SMARTpool: ON-TARGETplus Mouse Lacc1 siRNA (cat no L-052159-01-0005), SMARTpool: ON-TARGETplus Aprt Mouse siRNA (cat no L-042945-02-0005), and SMARTpool: ON-TARGETplus Adk Mouse siRNA (cat no L-062728-00-0005) were purchased from Dharmacon.

2.4.2 siRNA Knockdown in A549 cells

A549 lung carcinoma cells were seeded in 6-well plates at a density of 60000 cells per well to restrict over-confluency, as this procedure requires an extra 24 hours of incubation compared to other procedures. The cells were allowed to adhere for 24 hours. Subsequently, the siRNA transfection complexes were prepared in a total volume of 208 μ L of Opti-MEM medium for each siRNA condition. siRNA stock solutions were prepared at a concentration of 100 μ M and subsequently diluted to 2 μ M by mixing 2 μ L of the 100 μ M siRNA stock with 98 μ L of RNase-free water. INTERFERin (Polyplus) was used as a transfection reagent for all A549 experiments, following the manufacturer's recommended protocol as follows:

siRNA mix per well:

- 194.5 μ L Opti-MEM
- 5.5 μ L siRNA at 2 μ M concentration
- 8 μ L INTERFERin (Polyplus) vortex it for 5 seconds and spin it down

The components were gently combined using slow vortexing for approximately 10 seconds, followed by incubation at room temperature for 10 minutes, not exceeding 30 minutes, to facilitate the formation of transfection complexes between siRNA and INTERFERin. Meanwhile, the culture medium in each well was replaced with 2 mL of fresh pre-warmed medium containing 10% FBS. Subsequently, 200 μ L of the prepared transfection mixture was carefully added to each well (to give a final concentration of 5 nM siRNA in 2.2 mL medium volume) and mixed by gentle swirling to ensure an even distribution. The plate was then incubated for an additional 24 hours. On the third day, a new siRNA mixture was prepared and incubated for 10 minutes as described above. During this time, the medium was removed and the cells were rinsed with PBS before adding 2 mL of serum-free medium to each well. A further 200 μ L of the siRNA mixture was added and the cells were incubated for another 24 hours. The following day, cells were treated with either cordycepin (50 μ M) or 3DI (200 μ M) for a total of 120 minutes; TNF was added 60 minutes after the start of compound treatment (i.e.

in the presence of the compound) and cells were stimulated for a further 60 minutes before being harvested for RNA isolation.

The INTERFERin transfection reagent (cat no 101000028) was purchased from Polyplus. SMARTpool: ON-TARGETplus Human FAMIN siRNA (cat no L-015653-02-0005), SMARTpool: ON-TARGETplus APRT Human siRNA (cat no L-006328-00-0005), SMARTpool: ON-TARGETplus ADK Human siRNA (cat no L-004733-00-0005), SMARTpool: ON-TARGETplus ADSS2 Human siRNA (cat no L-009289-00-0005), and ON-TARGETplus NT5C2 Human siRNA (cat no L-009420-01-0005) were purchased from Dharmacon.

2.5 RNA Extraction, cDNA Synthesis, and Quantitative Real-Time PCR (qPCR)

For the RAW264.7 cell line, 300,000 cells, and for the A549.7 cell line, 150,000 cells were seeded in 6-well tissue culture plates, each in a maximum of 3 ml of cell culture media. Total RNA was isolated from the cells using the ReliaPrepTM RNA Cell Miniprep System (Cat. No. Z6012; Promega) according to the manufacturer's protocol, with the modification of extending the DNase treatment to 1 hour instead of 15 minutes. The RNA was eluted in 15–20 μ L of nuclease-free water into non-stick RNase-free tubes supplied with the kit. RNA quantity and purity were assessed using a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific). Samples exhibiting 260/280 ratios between 2.00 and 2.20 were considered of acceptable purity and suitable for cDNA synthesis. Although the 260/230 ratio was documented to account for potential downstream variability, it was not used as an exclusion criterion. Isolated RNA was stored at -20 °C for short-term use and at -80 °C for long-term preservation to minimize degradation.

cDNA was synthesised using the SuperScript III Reverse Transcriptase kit (Invitrogen, Cat. No. 10368252) following the manufacturer's protocol, starting from 500 ng of RNA. For each 500 ng RNA reaction, RNA was first incubated at 65–70 °C for 5 minutes with 2 μ L of random primers (30 ng/ μ L; Fisher Scientific Ltd., Cat. No.

10646313), 1 μ L of dNTP mix (10 mM; Fisher Scientific Ltd., Cat. No. 11863933), and nuclease-free water to a final volume of 14.5 μ L. The mixture was then cooled on ice for 5 minutes before adding 4 μ L of 5 $\times$ First-Strand Buffer, 1 μ L of DTT (100 mM), and 0.5 μ L of SuperScript III enzyme to a final volume of 20 μ L. The reverse transcription reaction was carried out at 50 °C for 1 hour, followed by enzyme inactivation at 70 °C for 15 minutes. The resulting cDNA was diluted 1:10 with nuclease-free water for subsequent qPCR analysis.

Relative mRNA levels were assessed using qPCR on a Qiagen Rotor-Gene Q qPCR cyclor, employing the GoTaq qPCR system (Promega, Cat. Number: A6002). The GoTaq qPCR master mix was prepared by combining 5 μ L of the GoTaq qPCR Mastermix (2x), 2 μ L of nuclease-free water, and 0.5 μ L of gene-specific primer pairs (Forward and Reverse primers at 20 μ M). The primer sequences are provided in table 2.5. For each target mRNA, a 10 μ L reaction mixture was prepared, consisting of 2 μ L of 1:10 diluted cDNA and 8 μ L of the GoTaq qPCR master mix, in triplicate.

The qPCR thermal cycling conditions on the Qiagen Rotor-Gene Q system began with an initial denaturation at 95 °C for 5 minutes, followed by 40 amplification cycles consisting of denaturation at 95 °C for 10 seconds, annealing at 60 °C for 20 seconds, and extension at 72 °C for 20 seconds. Relative mRNA expression levels were measured in triplicate, normalized to the GAPDH reference gene, and expressed as mean relative values per target gene. Quantitative data analysis was performed using the comparative fold-change method ($2^{-\Delta\Delta C_t}$).

2.5.1 Designing qPCR primers for mature RNA (mRNA)

Primers targeting mRNA were designed using NCBI Primer-Blast, based on the mRNA sequences retrieved from the NCBI database. The selected primer sets were subsequently validated in NCBI Primer-Blast to confirm target specificity for the intended mRNA. The expected amplicon length ranged between 150 and 250 nucleotides, with a maximum allowable melting temperature (T_m) difference of 2 °C between primer pairs. The guanine–cytosine (GC) content was maintained within 40–60%, and self-complementarity or 3′-complementarity was minimized to prevent the formation of primer-dimers and hairpin structures. The complete list of primer sequences employed in this study is

provided in Table 2.5.

Table 2.5: qPCR primer sequences

Mouse (<i>Mus musculus</i>) Primers			
Target (mature)	RNA	Strand Orientation	Primer Sequence (5'-3')
<i>Acod1</i> (<i>Irg1</i>)		Forward	ACTCCTGAGCCAGTTACCCT
		Reverse	CTGTGACAGACTTGAGCATCAT
<i>Aprt</i>		Forward	GGAGTATGGGAAGGCTGAG
		Reverse	CCAGGAGGTCATCCACAAT
<i>Lacc1</i>		Forward	ACAGAACATTCAAGACCAGAA
		Reverse	CCAGCCAAGAACCTCATT
<i>Adk</i>		Forward	GTGGCTCAGTGGTTGATT
		Reverse	GCTCGTTCTGCTCATAGTAAT
<i>Gapdh</i>		Forward	AAGAAGGTGGTGAAGCAGGC
		Reverse	ATCGAAGGTGGAAGAGTGGG
<i>Il1β</i>		Forward	AGATGAAGGGCTGCTTCCAAA
		Reverse	GGAAGGTCCACGGGAAAGAC
<i>Rpl28</i>		Forward	TACAGCACGGAGCCAAATAA
		Reverse	ACGGTCTTGCGGTGAATTAG
<i>Tnf</i>		Forward	CTATGGCCCAGACCCTCACA
		Reverse	CCACTTGGTGGTTTGCTACGA
Human (<i>Homo sapiens</i>) Primers			
Target (mature)	RNA	Strand Orientation	Primer Sequence (5'-3')
<i>CXCL1</i>		Forward	GATGCTGAACAGTGACAA

Continued on next page

Table 2.5 – *Continued from previous page*

Target (mature)	RNA	Strand Orientation	Primer Sequence (5'-3')
		Reverse	ACACATTAGGCACAATCC
<i>CXCL8</i>		Forward	TAGGACAAGAGCCAGGAA
		Reverse	AAACTGCACCTTCACACA
<i>NT5C2</i>		Forward	TGCGTCAGGTGGATACTAA
		Reverse	TCCCAACAGGTCACAGAT
<i>ADSS2</i>		Forward	GGAACAACAGAGACAAGAACAAG
		Reverse	GGTCGCACATCCTGAGTC
<i>ADK</i>		Forward	GTGGCTCAGTGGATGATTCA
		Reverse	CTGCTCATTCTGCTCGTAGTAAT
<i>FAMIN</i> (<i>LACC1</i>)		Forward	GAGAGGAGTCACAATAGCA
		Reverse	AGCATTCACTGTAGCCATA
<i>APRT</i>		Forward	GAGTACGGGAAGGCTGAG
		Reverse	ATCATCCACGACGACCAC
<i>GAPDH</i>		Forward	CCTCAAGATCATCAGCAATG
		Reverse	GAGTCCTTCCACGATACC
<i>PITPNC1</i>		Forward	GCCTATCATGTGCTCCTAC
		Reverse	GCCTGTCTATGTCCAATCA
<i>PNP</i>		Forward	TCCGGATTCTCTGCTCTC
		Reverse	CTCTGACCTTTGCCAGGAG
<i>RPL10A</i>		Forward	ATCAAGCAGATTCCACGAA
		Reverse	CCAGACATAACACCTTCTTCAT
<i>ADSL</i>		Forward	CCCACATTAGGTTTCACACATT

Continued on next page

Table 2.5 – *Continued from previous page*

Target (mature)	RNA Strand Orientation	Primer Sequence (5'-3')
	Reverse	ATCTCGGACACGCTTCAA

2.6 Liquid Chromatography – Mass Spectrometry (LC-MS)

LC-MS analyses were performed with the assistance of Rakesh Jha at the Centre for Analytical Bioscience (CAB), University of Nottingham, who operated the mass spectrometer and provided guidance on instrument calibration and data acquisition. Sample preparation, experimental design, data interpretation and all downstream analyses were performed by the candidate.

The method selected to chemically evaluate the purity of the treatments used in this thesis was liquid chromatography-mass spectrometry (LC-MS) analysis. Compared to UV detection, LC-MS offers a more selective approach for identifying small molecules and generally provides data with reduced background interference from solvents.

The instrumentation selected for this study consisted of a hydrophilic interaction liquid chromatography (HILIC) column in conjunction with Thermo Exactive® Orbitrap mass spectrometer, accessible through CAB. This selection was based on the high resolution and selectivity characteristics of this configuration, particularly for the analysis of polar compounds.

2.6.1 Samples Preparation

Cordycepin (Carbosynth), cordycepin (phyproof Reference Substance; cat. no. PHL82505; Merck) and 3'-deoxyinosine (Santa Cruz Biotechnology; cat. no. CAS 13146-72-0) were dissolved in 50% methanol (MeOH:ddH₂O, 1:1 v/v). Ten milligrams of cordycepin compounds were dissolved in 3.98 mL of 50% MeOH to obtain 10,000 µM (10 mM) stock solutions. The working standards were then prepared by serial dilution of the 10 mM stock solutions in 50% MeOH to yield concentrations of 1, 5, 10, 25, 50 and 100

μM ($\geq 500 \mu\text{L}$ per level to accommodate replicates and re-injections). Once prepared, the samples were stored at -80°C until LC-MS analysis.

Immediately prior to injection, all standards were diluted 1:10 with 50% MeOH (1 part standard + 9 parts solvent), resulting in final injected concentrations ranging from 0.1 to 10 μM (100 to 10,000 nM).

The Quality-control (QC) pool was prepared as follows: A 200 μL QC mixture was prepared by combining 40 μL from each of five 25 μM working solutions in 50% MeOH, comprising phyproof cordycepin, synthetic cordycepin, cordycepin-601, 3'-deoxyinosine (Santa Cruz) and 3'-deoxyinosine (Biosynth). This mixture contained 5 μM of each analyte prior to injection. Consistent with analytical standards, the QC mixture was further diluted 1:10 with 50% MeOH immediately before injection, producing 0.5 μM (500 nM) of each analyte on-column.

For 3'-Deoxyinosine (Santa Cruz) samples, working solutions of 3'-deoxyinosine (Santa Cruz) were prepared in 50% MeOH. For inclusion in the QC pool, a 25 μM working solution was prepared as described above. When 3'-deoxyinosine (Santa Cruz) was analysed as a standalone sample, it was prepared in the same solvent (50% MeOH) and subjected to the identical 1:10 pre-injection dilution applied to all materials (standards and QC), ensuring matrix consistency across injections.

2.6.2 Chromatography Conditions

Hydrophilic interaction liquid chromatography (HILIC) was utilized to separate compounds prior to mass spectrometric analysis. The use of an eluent containing a high proportion of organic solvent (100% acetonitrile) combined with a highly soluble aqueous buffer (20 mM ammonium carbonate) facilitated separation based on electrostatic interactions among polar analytes and hydrophilic partitioning mechanisms. These interactions occurred between the analytes and the stationary phase, which comprised a polymer functionalized with a sulfobetaine group within the ZIC-pHILIC® column (Sequant, Merck) [Merck: ZICpHILIC manual].

The chromatographic system was assembled in accordance with the manufacturer's specifications using an Accela® (Thermo Fisher) autosampler and LC pump. An isocratic elution was performed with a 20 mM ammonium carbonate buffer (pH 9.1)

(A) and 100% acetonitrile (B) at a ratio of 20:80. The flow rate was maintained at 300 $\mu\text{L}/\text{min}$, with the column oven temperature set to 45 $^{\circ}\text{C}$. A 24-minute per-sample run time was employed for each analysis, as previously described. [89] – as shown in table 2.6 and figure 2.1.

Table 2.6: 24-minute LC method, through the ZIC®-pHILIC column.

Time (minutes)	A (%)	B (%)
0	20	80
15	95	5
17	20	80
24	100	0

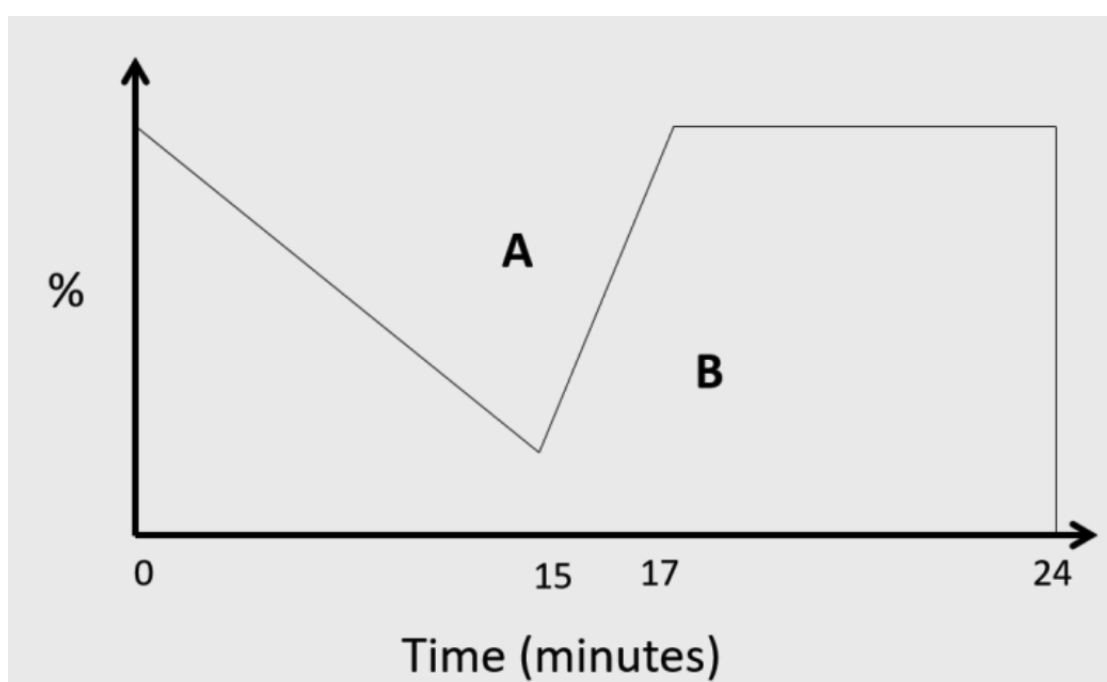


Figure 2.1: 24-minute LC method, through the ZIC®-pHILIC column.

2.6.3 Mass Spectrometry

The Thermo Exactive® Orbitrap mass spectrometer was used. Before use, mass spectrometers were calibrated with modified versions of the Pierce ESI positive and negative solutions (ThermoFisher). The mass spectrometers are detailed in 2.2

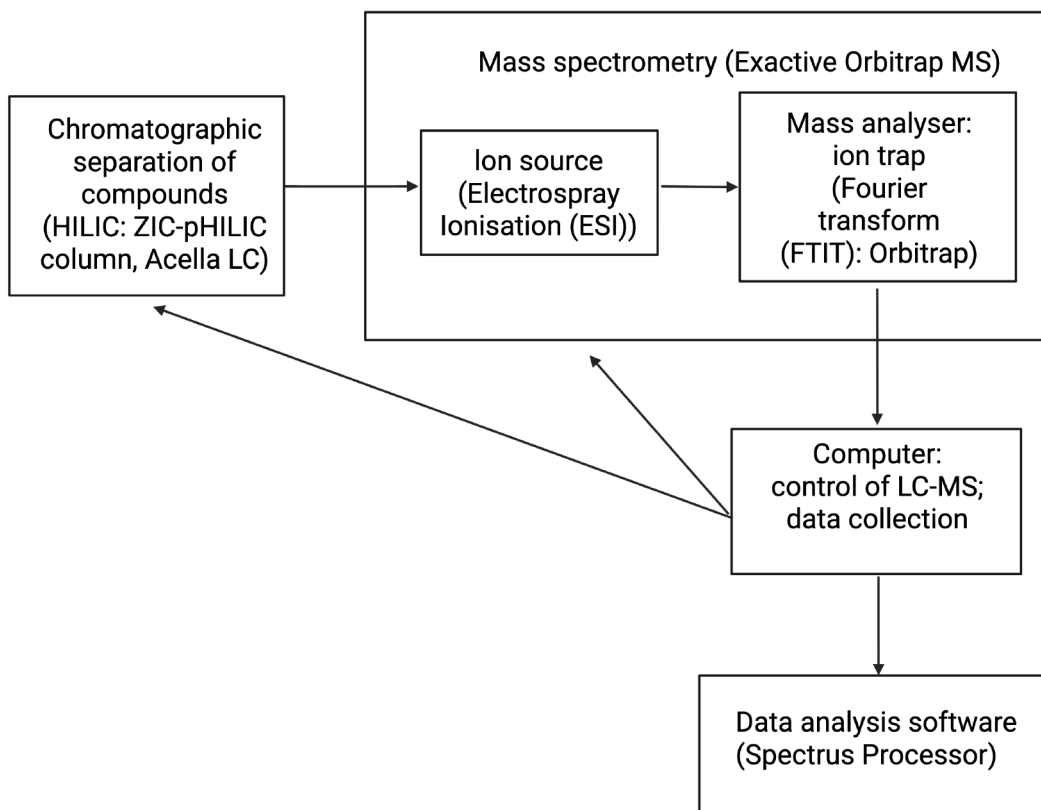


Figure 2.2: Following chromatographic separation of the analytes using the ZIC-pHILIC® column, the eluent was introduced into the mass spectrometer. Ionisation was achieved via electrospray ionisation (ESI) in the Exactive MS system. Charged droplets generated by ESI were directed into a C-trap®, from which ions were transferred to the Orbitrap mass analyser in rapid pulses. The Orbitrap, a type of Fourier transform ion trap (FTIT) analyser, operates by confining ions in orbital motion around a central electrode. The resulting ion trajectories generate an image current that is detected by external electrodes and subsequently transformed into mass-to-charge (m/z) values.

2.6.4 Configurations and Procedures for Conducting an LC-MS Analysis – ZIC-pHILIC and Exactive MS

The LC-MS system was electronically operated via a connected desktop computer, utilizing Thermo Tune® and Xcalibur® software (ThermoFisher). The experiments were conducted using the Accela HPLC and Exactive MS system.

Sample preparation prior to LC-MS run is described in section 2.6.1. Throughout the process, 50% LC-MS-grade methanol, diluted with ddH₂O, served as the sample solvent.

In general, the procedure for operating the LC-MS system, which includes the Thermo Accela® LC system with a SeQuant ZIC®-pHILIC (Merck) column and a Thermo Exactive® mass spectrometer, can be categorized into five primary steps. These steps are: (i) preparing samples and phases; (ii) calibrating the mass spectrometer; (iii) configuring HPLC settings and purging the system; (iv) conditioning the column; and (v) setting up the sequence and initiating the run.

(i) Preparation of samples and mobile phases. The detailed procedures for sample preparation have been outlined previously. All samples were maintained at -80 °C until analysis by LC-MS. The mobile phases used in the HPLC system consisted of 20 mM ammonium carbonate buffer (pH 9.1) as phase A and 100 % acetonitrile as phase B. The system wash solution corresponded to phase A, and all solvent lines were configured accordingly.

(ii) Calibration of the mass spectrometer was performed using modified Pierce ESI positive and negative calibration solutions (Thermo Fisher). To extend the calibration range and ensure accurate detection of low-mass metabolites, additional low-mass contaminants were incorporated into the calibration mixture—specifically C₂H₆NO₂ (m/z 76.0393) for the positive ion mode and C₃H₅O₃ (m/z 89.0244) for the negative ion mode. Calibration was conducted in both positive and negative ionization modes using the Thermo Tune® software. A 500 µL Hamilton syringe delivered 10–20 µL of calibration solution per minute into the mass spectrometer, and the calibration process was finalized once the total ion count (TIC) decreased below 5%.

(iii) HPLC configuration and system purging. The Accela HPLC parameters were configured via Xcalibur® software. Autosampler tray and column oven temperatures were maintained at 4 °C and 45 °C, respectively. Prior to each run, the injection needle and syringe were thoroughly washed, and the system was purged to eliminate any trapped air bubbles.

(iv) Column conditioning. Following purging, the ZIC®-pHILIC column was connected and equilibrated under standard chromatographic conditions. The mobile phase composition was maintained at 20% phase A and 80% phase B, with a constant flow rate of 300 µL/ min for a minimum of 30 minutes before initiating LC-MS acquisition.

(v) Sequence setup and initiation of LC-MS runs. The final stage of the standard operating procedure involved constructing the injection sequence within Xcalibur®. Targeted analyses incorporated calibration standards containing cordycepin at 100, 500, 1000, 2500, 5000, and 10000 nM. Sample injection order followed the guidelines of [90] and depicted in Figure 2.3, with QC samples interspersed after every five randomized treatment samples and reinjected at the beginning of each run.

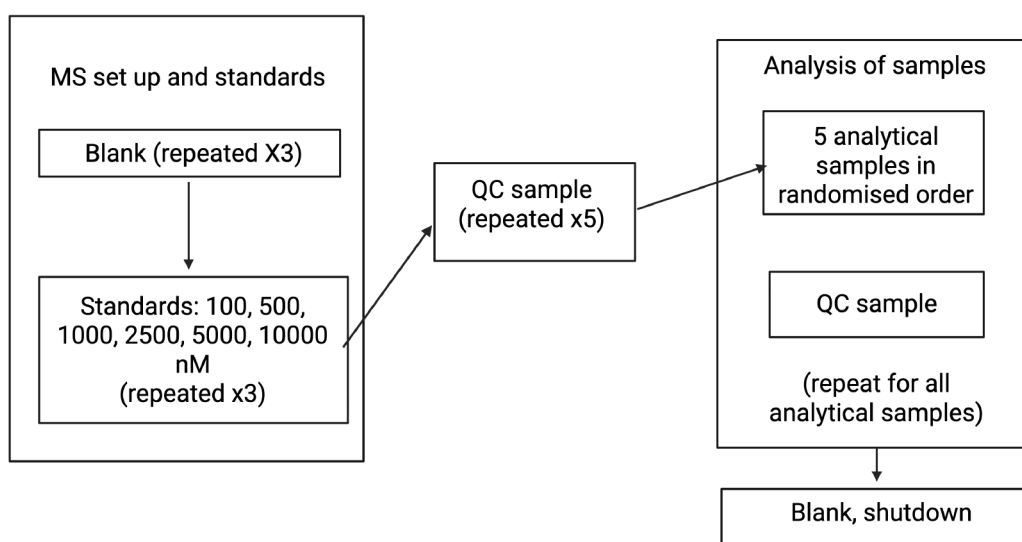


Figure 2.3: Sequence of samples used in LC-MS runs

2.7 PrestoBlue™ HS Cell Viability Assay.

The metabolic activity of eHAP cells was measured using the PrestoBlue HS assay (Invitrogen by ThermoFisher cat no P50200). Wild-type eHAP cells were seeded at 7,000 cells/well in a 96-well plate containing 100 μ L of culture medium. Cordycepin and 3'-deoxyinosine were administered over 24-72 hours at doses of 25-400 μ M, as a single dose over 24, 48 or 72 h or as multiple doses, twice every 24 h, over 24, 48 or 72 h to compensate for the degradation of cordycepin over time, as previously documented [91]. Three technical replicates were tested and 10 μ L of PrestoBlue was added to 100 μ L of medium according to the manufacturer's instructions. The PrestoBlue reagent contains the cell-permeable compound resazurin, which is blue in color and nonfluorescent. After addition to the cells, resazurin is reduced to resorufin by the reducing environment of the viable cells. Resorufin is red in color and is highly fluorescent. The conversion of resazurin to resorufin was detected using fluorescence or absorbance measurements. This reduction is proportional to the number of living cells and allows quantification of cell viability [92]. The fluorescence excitation/emission maxima for resorufin were approximately 535–560 nm/590–615 nm, respectively. The maximum absorption was approximately 570 nm. In our test, fluorescence was measured at an excitation wavelength of 535 nm and emission wavelength of 590 nm using a Synergy Plate Reader. The metabolic inhibition dose-response data informed the selection of appropriate cordycepin concentrations for subsequent haploid genetic screening.

2.8 ONT long-read RNA-Seq and Bioinformatics Analysis

All bioinformatics analyses were conducted using the Unix high-performance computing (HPC) facility at the University of Nottingham, within my user directory (/users/-paxak6/). Analyses were executed either by logging into available workstations via SSH or by submitting long-running jobs to the Condor job-scheduling system. For computational tasks requiring extended runtime or when Condor submission was incompatible (as observed during Dorado basecalling), commands were executed using 'nohup' in

combination with background execution ('&') to maintain process continuity. This approach ensured that long-running processes remained active and completed successfully even when the VPN connection was interrupted or temporarily disconnected.

Total RNA extracted using the ReliaPrep™ RNA Cell Miniprep System as previously described. The quantity and quality of total RNA was assessed using the NanoDrop 1000 (ThermoFisher). In addition to this, the quality of RNA samples was assessed by staff from the Deep Seq department at the University of Nottingham.

The RNA ScreenTape® System (Agilent) on the Agilent TapeStation® platform was used to assess the quality and integrity of RNA samples — specifically to evaluate how effective the RNA extraction method was. This analysis was carried out by Deep Seq staff (University of Nottingham).

The TapeStation generates an electropherogram and gel-like image showing two key ribosomal RNA (rRNA) peaks — the 28S and 18S rRNA. Ideally, high-quality eukaryotic RNA should have a 28S:18S ratio of 2:1, indicating minimal degradation.

The RNA Integrity Number (RIN) — a quantitative algorithmic score from 1 (degraded) to 10 (intact) — was carried out and was sufficiently high. According to Schroeder et al. 2006 [93], the RIN value integrates multiple electrophoretic features beyond the simple 28S:18S ratio, providing a more robust and objective measure of RNA quality.

2.8.1 120-minute data methodology (Run DS1457) by Deep Seq staff, University of Nottingham

RNA samples were diluted 1/10 with nuclease-free water and the concentrations of the diluted samples were measured using the Qubit 4 Fluorometer (Thermo Fisher Scientific) and the Qubit RNA BR Assay Kit (Thermo Fisher Scientific; Q10211). RNA integrities were assessed using the Agilent 4200 TapeStation and the RNA ScreenTape Assay (Agilent; 5067-5576 and 5067-5577) Figure 2.4.

Barcoded PCR-cDNA libraries were prepared using the Oxford Nanopore Technologies (ONT) PCR-cDNA Barcoding (ONT; SQK-PCB111.24) Library Prep Kit. The library prep protocol (Version: PCB-9155-v111-revG-18May2022) was followed and 14

cycles of PCR were used for cDNA amplification. The library prep input was 200 ng of total RNA. The total volume of each sample was made up to 10 ul with nuclease-free water. The first clean-up step was performed using Mag-Bind TotalPure NGS beads (Omega BIO-TEK; M1378-01). The second purification step was performed using AMPure XP beads (Beckman Coulter; A63882). Amplified cDNA samples were quantified using the Qubit 4 Fluorometer (Thermo Fisher Scientific) and the Qubit 1X dsDNA HS Assay Kit (Thermo Fisher Scientific; Q33231). Fragment-length distributions of the amplified cDNA products were assessed using the Agilent 4200 TapeStation and the High Sensitivity D5000 ScreenTape assay (Agilent; 5067-5592, 5067-5593). The eighteen libraries were pooled in equal amounts prior to the addition of the rapid sequencing adapter.

The final barcoded library pool was loaded onto an R9.4.1 PromethION flow cell (Oxford Nanopore Technologies; FLO-PRO002) and run on the PromethION 24.

All samples exhibited high quality, rendering them suitable for subsequent downstream analyses.

2.8.2 15-minute data methodology (Run DS1402) by Deep Seq staff, University of Nottingham

RNA concentrations and RNA integrities were assessed using the Agilent 4200 TapeStation and the RNA ScreenTape Assay (Agilent; 5067-5576 and 5067-5577) 2.5.

Barcoded amplified-cDNA libraries were prepared using the Oxford Nanopore Technologies (ONT) PCR-cDNA Barcoding Library Prep Kit (ONT; SQK-PCB114.24). The library prep protocol (Version: PCB-9201-v114-revC-6Dec2023) was followed and 12 cycles of PCR were used for amplification. The library prep input was 500 ng of total RNA.

The total volume of each sample was made up to 10 ul with nuclease-free water. The first clean-up step was performed using Mag-Bind TotalPure NGS beads (Omega BIO-TEK; M1378-01). The second purification step was performed using AMPure XP beads (Beckman Coulter; A63882). Amplified cDNA samples were quantified using the Qubit 4 Fluorometer (Thermo Fisher Scientific) and the Qubit 1X dsDNA HS

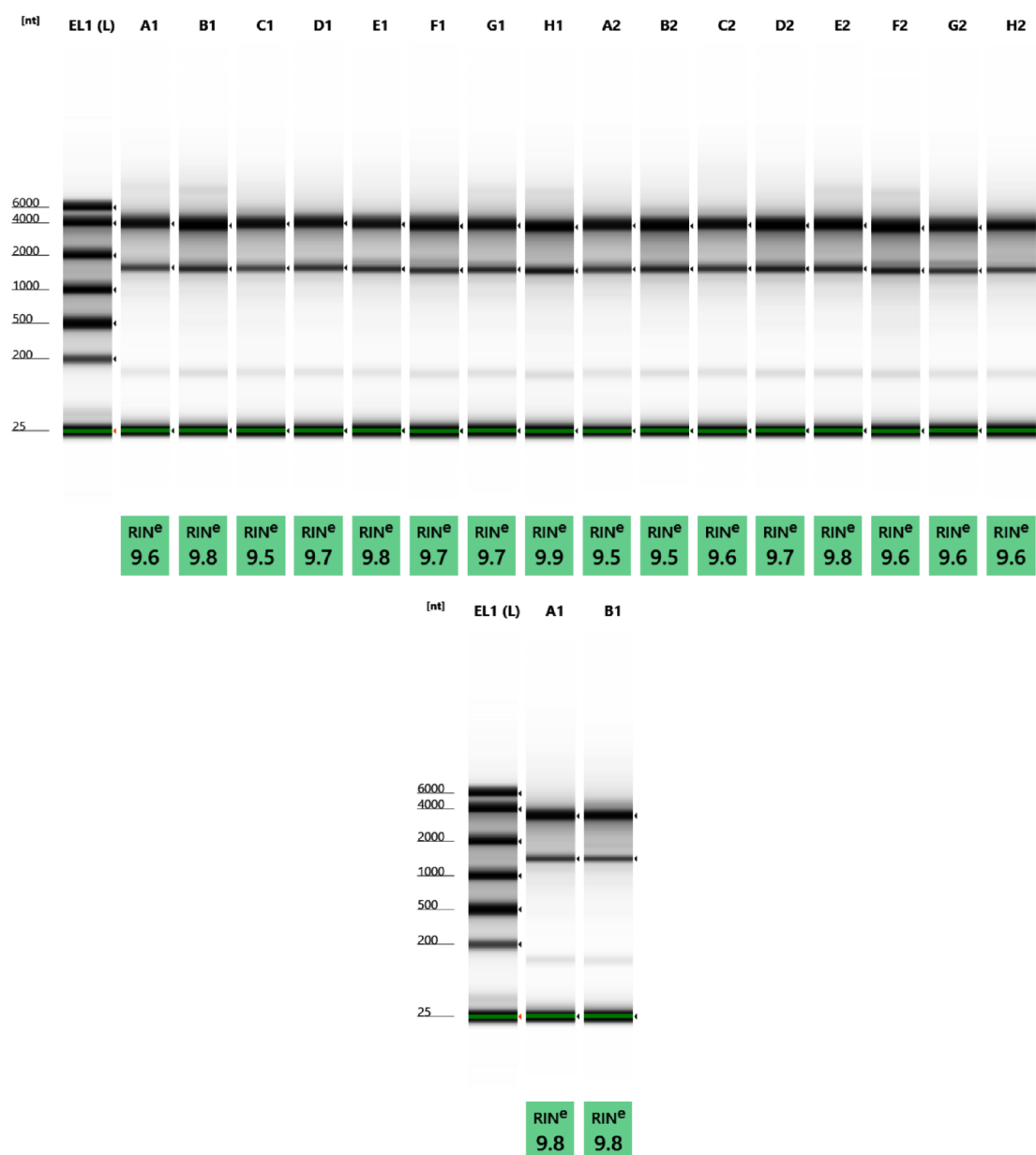


Figure 2.4: RNA ScreenTape results for 120-minute set of RAW264.7 RNA extraction samples. Each sample has a two clear bands representing the 28S:18S ratios for rRNA fragment.

Assay Kit (Thermo Fisher Scientific; Q33231). Fragment-length distributions of the amplified cDNA products were assessed using the Agilent 4200 TapeStation and the High Sensitivity D5000 ScreenTape assay (Agilent; 5067-5592, 5067-5593). The nine libraries were pooled in equal amounts prior to the addition of the rapid sequencing adapter.

The final barcoded library pool was loaded onto an R10.4.1M PromethION flow cell (Oxford Nanopore Technologies; FLO-PRO114M) and run on the PromethION 24.

All samples exhibited high quality, rendering them suitable for subsequent down-

stream analyses.

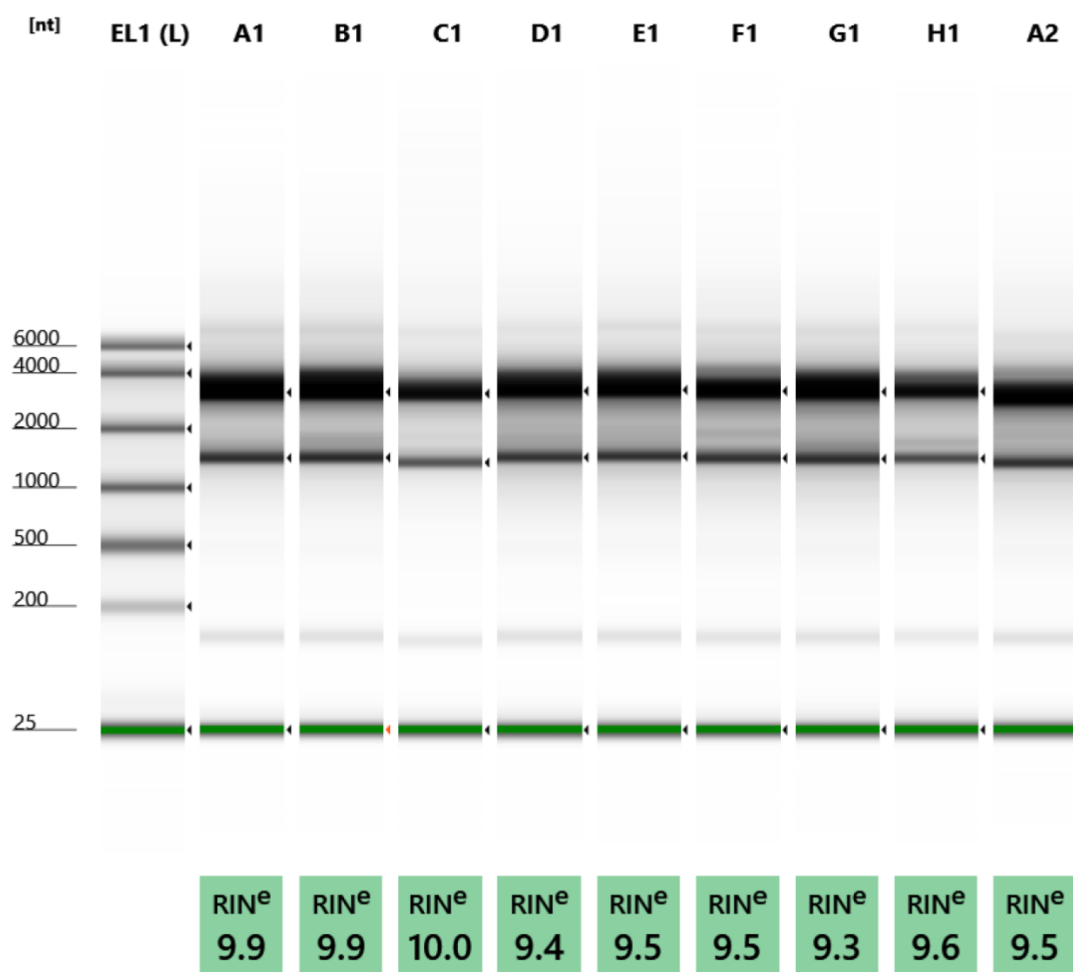


Figure 2.5: RNA ScreenTape results for 15-minute set of RAW264.7 RNA extraction samples. Each sample has a two clear bands representing the 28S:18S ratios for rRNA fragment.

2.8.3 Quality Control of Fastq Files

Before analyzing the RNA-Seq output, quality control of the data was necessary to ensure that the quality of the data of the replicates would not hinder the downstream output. The LongQC pipeline (version 1.2.1) from GitHub (<https://github.com/yfukasawa/LongQC>) [94] was used to perform quality control checks on the raw RNA-Seq sequence data in the Fastq.gz files. For every Fastq.gz file; reports the length, GC content in json and html summaries, coverage, and the fraction of reads which have no coverage are generated in html format. RNA-Seq Fastq.gz files were taken forward to downstream analysis based on these quality checks.

An example of the script used to run the LongQC pipeline is provided in Listing B.1

(Appendix B).

2.8.4 Haploid Genetic Screening

I wish to express my profound gratitude to Dr. Keith Spriggs for his invaluable assistance and guidance in the execution of this analysis. The progression of the genetic screening analysis of haploids would not have been feasible without his esteemed mentorship and insightful feedback.

A genome-wide haploid genetic screening was performed using eHAP cells harboring PiggyBac (PB) transposon insertions to identify genes involved in the cordycepin response. Two PiggyBac transposase systems were employed: hyperactive PiggyBac (hyPB) and super PiggyBac (suPB). Cells were treated with either DMSO (vehicle control), 150 μ M cordycepin, or 200 μ M cordycepin. The samples were processed and sequenced using Oxford Nanopore Technology (ONT) long-read sequencing. Illumina barcodes (01–08) were used to multiplex the experimental conditions, while the ONT barcodes differentiated the seeding densities: ONT bc07 corresponded to 150 K cells per well and ONT bc08 to 300 K cells per well. Therefore, each ONT barcode encompassed eight unique Illumina barcodes (01–08).

The comprehensive haploid genetic screening pipeline that I developed is accessible in my repository on GitHub at https://github.com/Ammar-Khamis/Haploid_screening. The pipeline involve the following steps:

1. Initial Data Preparation: Raw ONT sequencing data were organized by barcode (barcode07, barcode08, etc.) and concatenated for downstream processing.

```
cat barcode07/*fastq.gz >> reads_bc07.fastq | gzip
```

```
cat barcode08/*fastq.gz >> reads_bc08.fastq | gzip
```

The human reference genome (GRCh38 primary assembly) was obtained from Ensembl and indexed using SAMtools [95] within biotools conda environment:

```
conda activate biotools
```

```
samtools faidx Homo_sapiens.GRCh38.dna.primary_assembly.fa
```

Gene annotations were obtained from Ensembl release 113 (GTF format). FASTA file with barcode sequences was prepared `barcodes_and_adapters.fa`

2. Demultiplexing: Demultiplexing of ONT reads by Illumina barcode was performed

using the LAST aligner (<https://www.protocols.io/view/demultiplexing-nanopore-reads-with-last-14egnxw4zl5d/v8>). A custom training matrix was generated for each ONT barcode to optimize alignment parameters for the specific barcode and adapter sequences:

```
last-train -Q1 -P4 barcodes_and_adapters reads.fastq.gz > training_matrix.mat
```

Reads were aligned to the barcode/adapter reference using the trained matrix with split alignment enabled:

```
lastal --split -p training_matrix.mat -P4 barcodes_and_adapters reads.fastq.  
gz
```

Alignments were converted to tabular format using `maf-convert`, and barcode assignments were extracted. Reads were sorted and demultiplexed using the `fastx-fetch.pl` script, generating separate FASTQ files for each Illumina barcode condition (01-08) with directional information (left/right reads corresponding to 5' and 3' adapter orientations). In order to mitigate potential conflicts with pre-existing packages within a Unix operating system, a distinct computational environment, designated as `last_env`, was utilized for the installation and execution of LAST and associated Perl scripts. This environment can be activated through the use of the conda package management system, specifically via the command: `conda activate last_env`.

Figure 2.6 illustrates the architecture of the reads. Upon demultiplexing there will be sixteen demultiplexing bins: eight configured for left IR (PB5) and eight for right IR (PB3). The sequences for adapters, barcodes, and IRs align with the oligonucleotides outlined in the adaptor table [3], with a 3 guanine added to finalize the IR motifs [3]. After trimming adapters using a specific script, all reads are anticipated to commence with the standard TTAA sequence [3].



Figure 2.6: Schematic representation of the expected sequencing read structure (not to scale). The central red region denotes the human genomic insert of interest, flanked by the P5 adapter, Illumina barcode, and left/right inverted repeat (IR) sequences used for demultiplexing. Sixteen demultiplexing bins were defined: eight containing left IR (PB5) and eight containing right IR (PB3). The adapter, barcode, and IR sequences correspond to the oligonucleotides listed in the adaptor table [3], with an additional 3 guanine incorporated to complete the IR motifs [3]. Following adapter trimming using a customized script, all reads are expected to begin with the canonical TTAA sequence [3]. The figure was adapted from [3]

3. Adapter Trimming: For each Illumina barcode, left and right reads were merged into a single FASTQ file. Adapter sequences were identified by realigning reads to the barcode/adaptor reference using LAST with the same training parameters. The best alignment for each read was identified based on alignment score, using a multi-step filtering approach (Listing B.2, Appendix B).

Reads were trimmed using a custom Python script (`Improved_trim_fastq.py`) that removes adapter sequences based on alignment coordinates, `barcodes_and_adapters.fa`, while preserving genomic insert sequences. A minimum read length threshold of 50 bp was applied post-trimming to ensure adequate mappability. After trimming, all reads should be expected to start with TTAA motifs.

4. TTAA Motif Filtering: PiggyBac transposons specifically insert at TTAA tetranucleotide sites in the genome. To enrich for genuine transposon insertion reads, a custom Python script (`Filter_TTAA.py`) was applied to filter reads start with TTAA motifs after trimming in step 3. Reads passing the TTAA filter and minimum length requirement (50 bp) were retained for genomic mapping.

5. Genome Mapping: Filtered reads were mapped to the GRCh38 reference genome using minimap2 [96] v2.29 with ONT-specific presets (Listing B.3, Appendix B). Key mapping parameters included the `map-ont` preset optimised for Oxford Nanopore reads, suppression of secondary alignments (`-secondary=no`), and a minimum mapping quality threshold of 10.

Mapped reads were sorted and indexed using SAMtools. Alignment statistics were generated using samtools flagstat to assess mapping efficiency for each condition.

6. Insertion Site Extraction: Transposon insertion sites were extracted from aligned BAM files using a custom Python script, (`Extract_Insertion_Sites.py`). Only reads with MAPQ 10 were processed. Insertion sites within a 5 bp window were merged to account for ONT sequencing variability and represent unique insertion events. The merging was performed using BEDTools merge [97]

```
bedtools sort -i sites.bed -g genome.txt | \
bedtools merge -c 4,6 -o sum,distinct
```

7. Downstream data analysis: Genes were pre-filtered to include only those showing

higher normalized insertion frequencies in cordycepin-treated conditions compared to DMSO controls using `(filter_genes_fixed.py)`. This filtering step is biologically justified because, in haploid screens, increased insertion frequency indicates loss-of-function mutations that confer resistance to the selection pressure (cordycepin). Genes enriched in cordycepin-treated samples are therefore candidates for cordycepin resistance/response pathways.

For filtered gene sets, log2 fold changes (logFC) and statistical significance were calculated using a unified analysis script `(unified_logFC_for_IPA_v3.py)` with preset configurations for each experimental comparison

To account for differences in sequencing depth across conditions, library sizes (total insertions per condition) were calculated. Normalized insertion frequencies were calculated for each gene in each condition using a pseudocount approach

```
(Normalize_Matrix_Visualization_Enhanced_v2.py) :  
Normalized_frequency = (raw_count + pseudocount) / (library_size +  
pseudocount)
```

where pseudocount = 0.5 to avoid division by zero for genes with no insertions in a given condition.

Log Fold Change: Calculated with sign inversion to align with gene expression convention (more insertions = loss of function = down-regulation):

```
logFC = -log2(freq_treatment / freq_control)
```

For path-enrichment analysis, the filter gene lists with logFC values were uploaded to Ingenuity Pathway Analysis (IPA, QIAGEN) for canonical pathway enrichment analysis. IPA identifies significantly over-represented pathways using a right-tailed Fisher's exact test, comparing the proportion of genes from the input list in a given pathway to the expected proportion by chance. The results of the Canonical Pathways were exported from IPA as tab-delimited text files containing the path names, p-values, z-scores (activation state) and gene lists. These tab-delimited text files were visualized using customized scripts.

All analyses were performed on a Linux cluster with the following software:

- SAMtools

- BEDTools
- minimap2
- LAST aligner
- Python with libraries:
 - pandas (data manipulation)
 - numpy (numerical computing)
 - scipy (statistical tests)
 - statsmodels (FDR correction)
 - matplotlib (plotting)
 - seaborn (statistical visualization)
 - xlsxwriter (Excel output)

Conda environments were used for dependency management:

- biotools: SAMtools, BEDTools, minimap2
- last_env: LAST aligner and Perl scripts

All custom scripts and the complete analysis workflow are available at

https://github.com/Ammar-Khamis/Haploid_screening under MIT License © 2024

Ammar Sabah Khamis

2.8.5 Acquiring read counts and expression data for RNA sequencing

The typical RNA-Seq pipeline for analyzing differential gene expression from Fastq.gz files is divided into four main steps: mapping to a genome, estimating gene-level expression, normalizing the data, and comparing the normalized expression data [98]. For differential gene expression analysis primary genome assembly files were obtained from Gencode (Mouse genome, GRCm39) according to the recommendations [99].

IsoQuant pipeline from GitHub version 3.7.0 (<https://github.com/ablab/IsoQuant>) [98] was used for gene quantification. The script used to run IsoQuant with the recommended settings is provided in Listing B.4, together with the corresponding HTCondor submit file in Listing B.5 (Appendix B).

2.8.6 Converting Gene ID to Gene Name

The output generated by the IsoQuant pipeline is presented as a TXT file containing gene identifiers (IDs). For enhanced utility in subsequent analytical processes, it is more convenient to convert these gene IDs into gene names, thereby increasing the interpretability of the data. The Ensemble blast process was not efficient in mapping the gene ID to the gene name. A custom Python script was developed to parse gene names from the GTF file and map them to the corresponding identifiers in the IsoQuant output file (Listing B.6, Appendix B).

2.8.7 Differential Gene Expression Analysis for Long-Read RNA Sequencing Data

For differential gene expression (DGE) analysis, the edgeR package from the Bioconductor project was utilized [100]. Normalization is achieved by employing the trimmed mean of m values. To account for variability in library sizes and RNA composition across different samples [101]. The R script used for executing edgeR is provided in Listing B.7 (Appendix B).

2.8.8 Poly(A) Tail Length Analysis

A modified version of the NanopLen pipeline [4, 102] was employed for poly(A) tail length estimation. The original NanopLen workflow utilizes outputs from the Guppy basecaller and the Nanopolish software for poly(A) analysis [4]. In this study, the NanopLen pipeline was forked and adapted (<https://github.com/maragkakislabs/nanoplen>) to process data generated by the Dorado basecaller (<https://github.com/nanoporetech/dorado>), which provides enhanced accuracy in poly(A) length estimation compared with the legacy Guppy basecaller and Nanopolish-based implementation. Generally the following steps were used:

1. Sample Preparation and Library Construction: cDNA libraries were prepared using the Oxford Nanopore Technologies (ONT) PCR Barcoding Kit (SQK-PCB111-24) according to the manufacturer's protocol as described earlier in this chapter. Briefly, total RNA was reverse transcribed to generate first-strand cDNA, followed by PCR

amplification with barcoded primers to enable sample multiplexing. 18 samples were pooled and loaded onto a PromethION flow cell (R9.4.1 chemistry) for sequencing.

2. Raw Data Conversion: Raw sequencing data in FAST5 format was converted to POD5 format for efficient downstream processing using the pod5-file-format toolkit (version 0.3.23) (<https://github.com/nanoporetech/pod5-file-format>). The conversion maintained the original directory structure, with each barcode's FAST5 files (approximately 436 files per barcode across 18 barcodes) converted to corresponding POD5 files in separate subdirectories:

```
pod5 convert fast5 ./FAST5/*/*.fast5 --output ./POD5 --one-to-one ./FAST5/
```

3. Basecalling and Demultiplexing with Poly(A) Tail Estimation: POD5 files were basecalled using Dorado (version 0.9.6; Oxford Nanopore Technologies) with the high-accuracy DNA basecalling model (dna_r9.4.1_e8_hac@v3.3). Importantly, Dorado's integrated poly(A) tail estimation was enabled to computationally estimate poly(A) tail lengths directly from the raw nanopore signal (Listing B.8, Appendix B). Key basecalling parameters included: `-kit-name SQK-PCB111-24` (PCR barcoding kit for automatic demultiplexing), `-estimate-poly-a` (computational estimation of poly(A) tail lengths from raw signal), `-no-trim` (preserving adapter sequences to maintain poly(A) signal information), and `-reference` (alignment to the reference transcriptome index during basecalling).

This generated a single BAM file containing all multiplexed reads with poly(A) tail length information stored in the `pt:i` custom tag.

Following basecalling, reads were demultiplexed into individual samples using Dorado's demultiplexing module with a sample sheet mapping barcodes to sample identifiers (Listing B.9, Appendix B).

The sample sheet mapped 18 barcodes to sample identifiers encoding treatment condition and biological replicate (e.g., barcode01 → DMSO_Rep1, barcode02 → DMSO_LPS_Rep1). The demultiplexed BAM files were sorted by genomic coordinates and indexed using samtools (within the biotools conda environment):

```
samtools sort -@ 8 -o sorted.bam unsorted.bam
```

```
samtools index sorted.bam
```

The experiment included six treatment conditions with three biological replicates each (n=18) Figure 2.7:

Barcode	Sample	Condition
01, 07, 13	DMSO_Rep1-3	Vehicle control (unstimulated)
02, 08, 14	DMSO_LPS_Rep1-3	Vehicle control + LPS
03, 09, 15	3DI_LPS_Rep1-3	3'-Deoxyinosine + LPS
04, 10, 16	Synth_CoR_LPS_Rep1-3	Synthetic Cordycepin + LPS
05, 11, 17	NUC_LPS_Rep1-3	Nucleoside analog + LPS
06, 12, 18	CoR_LPS_Rep1-3	Cordycepin + LPS

Figure 2.7: Caption for the image

Pairwise comparisons were performed between each treatment condition and the DMSO_LPS control.

4. Read Alignment and Quality Control: Basecalled reads were aligned to the reference transcriptome (Esenble vM34 for mouse) using the minimap2 aligner integrated within Dorado basecaller. The minimap2 index was pre-built from the reference transcriptome FASTA file:

```
minimap2 -d reference_index.mmi reference_transcriptome.fa
```

5. Poly(A) Tail Length Extraction: Poly(A) tail lengths were extracted from aligned BAM files using a customized script, `bam2nanoplen_dorado_v2.py`. Quality filters were applied to ensure high-confidence measurements: only reads with mapping quality ≥ 20 (99% alignment confidence) were retained, and poly(A) tails exceeding 400 nucleotides were excluded as potential artifacts (Listing B.10, Appendix B).

This generated tab-separated files containing library ID (barcode identifier), transcript ID, and poly(A) tail length for each read.

6. Data Aggregation and Quality Control: Individual sample files from all 18 samples were combined into a single dataset while preserving the header:

```
awk 'FNR>1 || NR==1' *.tsv > all_samples.tsv
```

7. Experimental Design and Metadata: The experiment included six treatment conditions with three biological replicates each (n=18 total samples):

A. DMSO (vehicle control without LPS stimulation): DMSO_Rep1, DMSO_Rep2, DMSO_Rep3

B. DMSO_LPS (vehicle control with LPS stimulation): DMSO_LPS_Rep1, DMSO_LPS_Rep2, DMSO_LPS_Rep3

C. 3DI_LPS (3-Deazaadenosine + LPS): 3DI_LPS_Rep1, 3DI_LPS_Rep2, 3DI_LPS_Rep3

D. CoR_LPS (Cordycepin + LPS): CoR_LPS_Rep1, CoR_LPS_Rep2, CoR_LPS_Rep3

E. NUC_LPS (Nucleoside analog + LPS): NUC_LPS_Rep1, NUC_LPS_Rep2, NUC_LPS_Rep3

F. Synth_CoR_LPS (Synthetic Cordycepin + LPS): Synth_CoR_LPS_Rep1, Synth_CoR_LPS_Rep2, Synth_CoR_LPS_Rep3

For differential analysis, pairwise comparisons were performed between each treatment condition and the appropriate control (DMSO_LPS for all LPS-stimulated conditions). Metadata files were created for each comparison, specifying the sample identifiers and their corresponding condition labels (Listing B.11, Appendix B).

8. Statistical Analysis of Differential Poly(A) Tail Lengths: Differential poly(A) tail length analysis was performed using the NanopLen R package to identify transcripts with significant changes in absolute poly(A) tail length between conditions. For each transcript, mean poly(A) tail lengths (in nucleotides) were calculated by pooling all reads across biological replicates within each condition. The absolute difference in mean tail length between treatment and control conditions was computed as:

$$\text{Mean difference (L)} = \text{Mean tail length}_{\text{treatment}} - \text{Mean tail length}_{\text{control}}$$

where positive values indicate longer poly(A) tails in the treatment condition and negative values indicate shorter tails.

To account for replicate-level variation, a linear mixed-effects model (LMM) was applied. This model corrects for batch-related variability by incorporating both fixed effects (treatment condition) and random effects (library or replicate), thereby providing a robust statistical framework that controls for technical and biological variability across samples.

A linear mixed-effects model (LMM) was applied with $\log_2$ transformation of poly(A) tail lengths prior to statistical testing (Listing B.12, Appendix B).

The LMM accounts for both fixed effects (treatment condition) and random effects (biological replicate variation). The $\log_2$ transformation normalizes the skewed poly(A) tail length distribution, improving the validity of parametric statistical assumptions. A change in tail length of at least 10 nucleotides with a false discovery rate (FDR) below 0.05 was considered significant Figure 2.8

9. Data Visualization: Poly(A) tail length distributions were visualized using two-panel plots (ECDF and scatter) on linear scale. Genome-wide poly(A) tail length changes were visualized using volcano plots (effect size vs. statistical significance) and MA plots (effect size vs. mean expression). Plots were generated using absolute change thresholds to identify transcripts with biologically meaningful changes in poly(A) tail length (Listing B.13, Appendix B, summarises the command-line flags used in the customised visualisation scripts developed to plot poly(A) tail distributions and differential length changes across conditions.)

For the 15-minute dataset, poly(A) tail estimation followed the same analytical workflow, except that the updated sequencing chemistries natively generated high-efficiency POD5 files, eliminating the need for conversion from FAST5. A revised basecalling model, ‘dna_r10.4.1_e8.2_400bps_hac@v5.0.0’, was employed to accommodate the improved pore chemistry used in this experiment.

All custom scripts and the complete analysis workflow are available at (private access): https://github.com/Ammar-Khamis/PolyA_120min

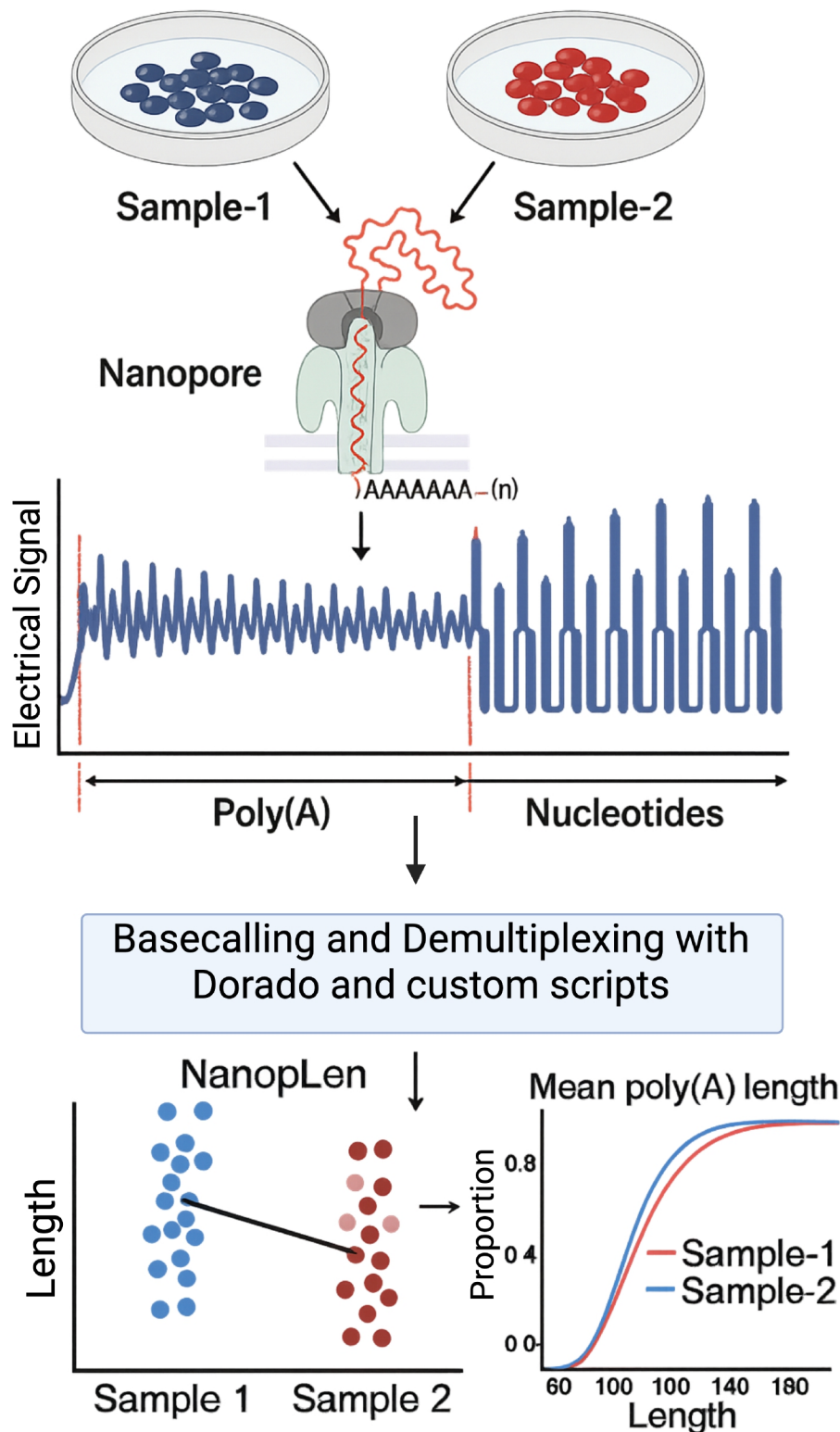


Figure 2.8: Workflow schematic. Starting with two conditions, polyadenylated RNA is extracted and sequenced with nanopores. The electrical signal for the poly(A) tail is distinct from other nucleotides allowing the quantification of the poly(A) tail length. Following data processing, the poly(A) tail lengths for all replicates of two conditions are fed into NanopLen for differential length analysis adapted from [4] S. A. Dar, S. Malla, C. Belair, and M. Maragkakis, “Differential poly(A) tail length analysis using nanopore sequencing,” *Methods Mol. Biol.*, vol. 2723, pp. 267–283, 2024, doi: 10.1007/978-1-0716-3481-3_16.

Chapter 3

Decoding 3'-Deoxyinosine Rescue

Pathways: siRNA Knockdowns

Identify 3'-Deoxyribose-Salvage

Requirements for Cordycepin Activity

3.1 Introduction

Prior work from the De Moor laboratory established that cordycepin represses LPS-induced inflammatory gene expression in RAW264.7 macrophages through a mechanism requiring nucleoside transport and adenosine kinase (ADK)-dependent phosphorylation to cordycepin triphosphate (CoTP) [26, 28]. Additionally, Lee *et al.* demonstrated that 3'-deoxyinosine (3DI), the major orally bioavailable metabolite of cordycepin, can be converted to CoTP in RAW264.7 cells and retains some anti-inflammatory activity at higher concentrations [1]. What remained unknown was which specific enzymatic pathway(s) mediate the intracellular conversion of 3DI back to cordycepin nucleotides—i.e. the identity of the “rescue” route—and whether the same pathway operates in non-macrophage cells. This chapter addresses that gap by systematically testing three candidate salvage routes using siRNA knockdown.

Cordycepin (3'-deoxyadenosine) shows broad anti-inflammatory and therapeutic

potential, yet its oral use is thought to be limited by rapid deamination by adenosine deaminase (ADA) to 3'-deoxyinosine (3DI) in blood and gut, which markedly lowers circulating cordycepin after dosing [1, 5, 6]. In several preclinical pharmacokinetic studies, cordycepin is often undetectable in plasma after oral administration, whereas 3DI appears systemically at high levels; co-treatment with ADA inhibitors such as pentostatin stabilises cordycepin in vitro and in vivo [1, 5, 6]. These pharmacokinetic observations have long raised the question of which enzymes mediate efficacy after oral dosing.

Recent work provides a mechanistic foothold. Macrophage-like RAW264.7 cells can generate cordycepin triphosphate (CoTP) from 3DI, and both equilibrative nucleoside transporters (ENT1/ENT2) and adenosine kinase (ADK) are required for cellular responses to cordycepin [1, 103]. Together with clinical and preclinical data on the cordycepin ProTide NUC-7738 [29], these findings support a model in which intracellular CoTP is the principal active metabolite of cordycepin, and in which nucleoside transport and phosphorylation are key control points.

Guided by that model, this chapter describes testing of three candidate 3'-deoxyribose-salvage routes for 3DI bioactivation (Figures 3.9, 3.11, 3.12):

1. **Phosphorylation/amination route (NT5C2/ADSS2 branch).** By analogy to the activation of 2',3'-dideoxyinosine (ddI) to ddATP, 3DI could in principle be phosphorylated to a monophosphate (e.g. 3dIMP) and then aminated to 3dAMP via adenylosuccinate synthetase (ADSS2) and adenylosuccinate lyase (ADSL)—canonical steps that convert IMP→AMP in purine metabolism. NT5C2 (cytosolic 5'-nucleotidase II) is included as a regulator of purine-monophosphate pools that can shape analogue activation [104–108].
2. **PNP-mediated 3'-deoxyribose salvage route.** Purine nucleoside phosphorylase (PNP) catalyses reversible phosphorolysis of inosine and deoxyinosine to base + (deoxy)ribose-1-phosphate. In this scenario, 3DI would be cleaved to hypoxanthine plus 3'-deoxyribose-1-phosphate, followed by salvage (e.g. via HGPRT/APRT) and re-attachment of adenine onto a 3'-deoxyribose phosphate intermediate to regenerate cordycepin or its nucleotides through the reversible

PNP reaction [109–111]. From a ribose-centric perspective, this and the third route below are both 3'-deoxyribose rescue pathways, differing mainly in the chemical form of the sugar at the point of coupling.

3. Adenine-linked 3'-deoxyribose salvage (APRT ± FAMIN/LACC1).

APRT converts adenine + PRPP to AMP in the purine salvage pathway, providing adenylylated nucleotides that can support re-adenylation of (3'-)deoxyribose pools. In macrophages, FAMIN/LACC1 enables a purine nucleotide cycle connecting adenosine, IMP and adenylosuccinate, positioning it to influence adenine-linked 3'-deoxyribose rescue [112, 113].

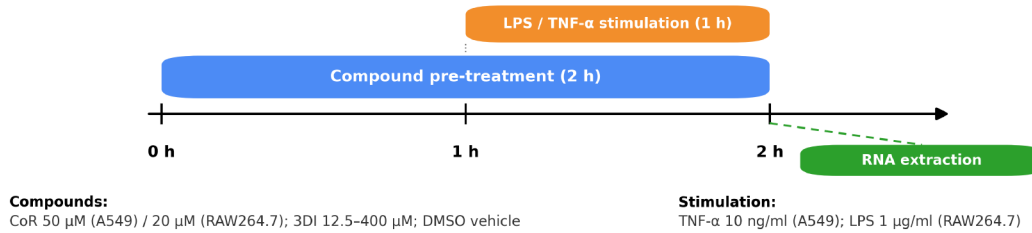
To evaluate these mechanisms, this chapter uses targeted siRNA knockdowns aligned to the above hypotheses in A549 lung epithelial cells and RAW264.7 macrophages, reading out TNF/LPS-induced inflammatory gene responses under cordycepin or 3DI treatment [28] [26] [27] [19]. late-stage LC–MS/MS analysis of nucleoside identity and purity is included to contextualise the knockdown data.

In this chapter, repression of TNF/LPS-induced inflammatory gene expression is used as a functional proxy for intracellular CoTP, because direct metabolomic quantification of CoTP was not technically feasible in these experiments. Overall, this chapter describes an investigation of whether the anti-inflammatory activity of 3DI *and* cordycepin can arise via specific cellular 3'-deoxyribose–salvage pathways, and which enzymes are necessary in epithelial versus macrophage contexts.

Experimental and analytical frameworks. A549 cells and RAW264.7 macrophages were treated with the test compounds for 2 h; TNF (for A549 cells) or LPS (for RAW264.7 macrophages) was added after 1 h of compound treatment (i.e. in the presence of the compound) and cells were stimulated for a further 1 h to induce a defined early inflammatory program. Subsequently, cells were lysed and total RNA was extracted. The experimental time schemes for both protocols are summarised in Figure 3.1.

(A) Standard treatment

A549 cells (TNF- α) / RAW264.7 macrophages (LPS)



(B) siRNA knockdown

A549 cells (TNF- α) / RAW264.7 macrophages (LPS)

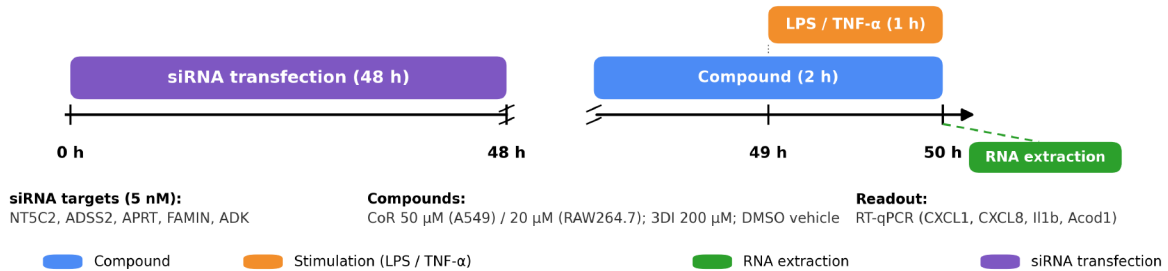


Figure 3.1: Experimental time schemes used in this chapter. **(A)** Standard treatment protocol: cells were treated with compound (cordycepin, 3DI, or DMSO vehicle) for a total of 2 h; TNF- α (A549) or LPS (RAW264.7) was added after 1 h of compound treatment (i.e. in the presence of the compound) and cells were stimulated for 1 h before harvesting for RNA extraction and RT-qPCR. **(B)** siRNA knockdown protocol: cells were transfected with 5 nM siRNA targeting the indicated gene for 48 h, then treated with compound for 2 h, with TNF- α or LPS added during the final hour (in the presence of compound) before RNA extraction. Total experimental duration: 2 h (standard) or 50 h (siRNA).

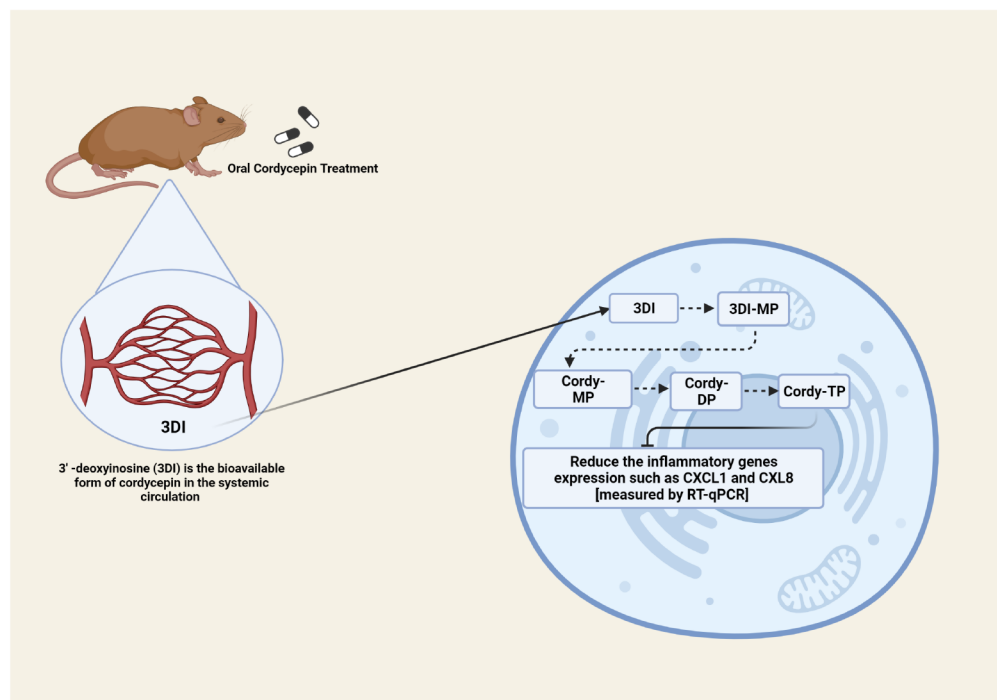


Figure 3.2: The fate of orally administered cordycepin. Cordycepin is rapidly deaminated by adenosine deaminase (ADA) in the gut brush border and systemic circulation, yielding 3'-deoxyinosine (3DI) as the main circulating species [1, 5, 6]. Several studies nevertheless report detection of cordycepin triphosphate in blood despite 3DI predominating, implying a metabolic rescue pathway that converts circulating 3DI back to cordycepin nucleotides.

3.2 Results

3.2.1 Baseline anti-inflammatory activity of 3DI and cordycepin

To establish the conditions for investigating the metabolism of 3DI and cordycepin, we first defined the concentration-dependent suppression of inflammatory gene expression in epithelial and macrophage models, and used this to select working doses. In A549 cells, CXCL1 and CXCL8 were chosen as readouts because previous work from the De Moor lab showed that these chemokines are robustly induced by TNF and selectively repressed by cordycepin in a polyadenylation-dependent manner [19]. In RAW264.7 macrophages, Acod1 and Il1b were used as sentinel genes for LPS-induced inflammatory activation based on a prior studies [26, 28].

A549 epithelial cells were treated for 2 h with DMSO (0.4% v/v vehicle control), cordycepin (50 μ M) as documented in previous work from the De Moor lab [19], or 3DI (12.5–400 μ M); TNF- α (10 ng/ml) was added after 1 h of compound treatment (i.e. in the presence of the compound) and cells were stimulated for a further 1 h. Both compounds dose-dependently suppressed TNF-induced CXCL8 and CXCL1 expression, with 3DI showing significant effects at concentrations ≥ 100 μ M (Figure 3.3). Cordycepin at 50 μ M achieved comparable suppression to 3DI at 400 μ M (**** $p < 0.0001$ for both CXCL8 and CXCL1, Dunnett’s test vs TNF alone, $n = 3$ biological replicates).

Similar anti-inflammatory activity was observed in RAW264.7 macrophages stimulated with LPS (1 μ g/ml). Cordycepin (20 μ M), as used previously in this system [26], and 3DI (50–400 μ M) significantly reduced Acod1 and Il1b induction (Figure 3.4). These dose–response patterns established working concentrations for subsequent knockdown experiments: 50 μ M cordycepin in A549, 20 μ M cordycepin in RAW264.7, and 200 μ M 3DI in both cell types.

As an additional control for nucleoside-analogue responsiveness, NUC-7738, a clinically advanced cordycepin ProTide, was tested in RAW264.7 macrophages under LPS stimulation. NUC-7738 showed potent suppression of Acod1 and Il1b over a similar concentration range (Figure 3.5), confirming the responsiveness of the experimental system to nucleoside analogues that generate intracellular cordycepin triphosphate [29].

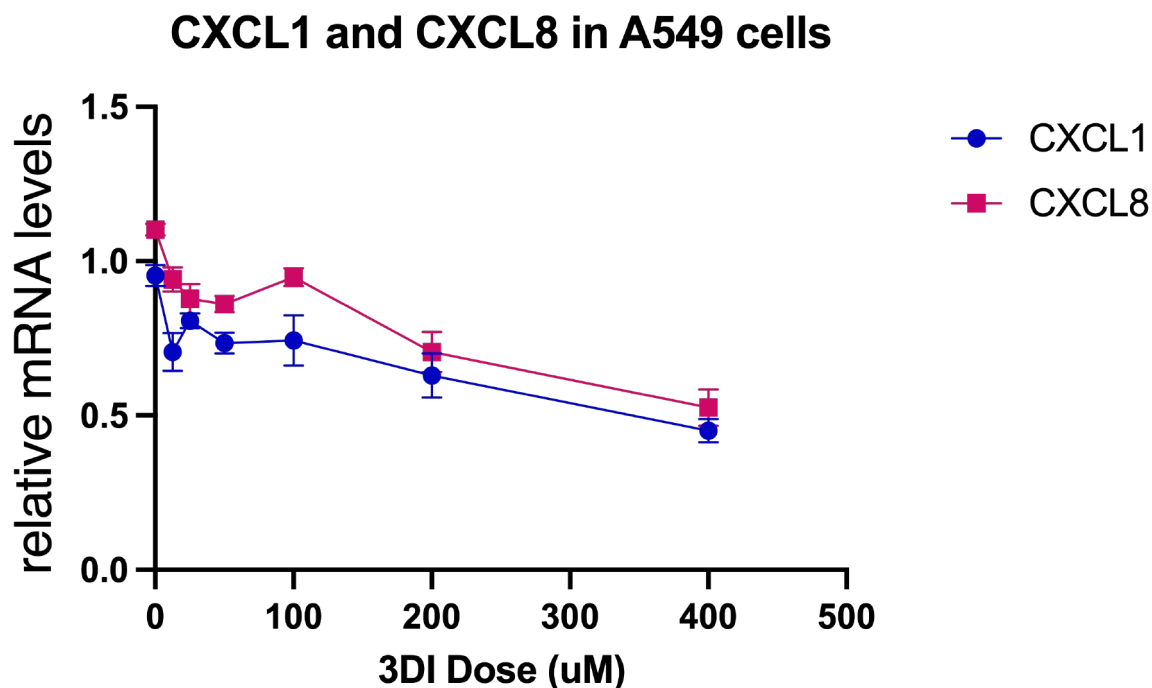


Figure 3.3: Selecting a working concentration of 3'-deoxyinosine (3DI) in A549 cells. Cells were stimulated with TNF (10 ng/ml) for 1 h in the presence of increasing concentrations of 3DI (0–200 μ M). CXCL1 (blue circles) and CXCL8 (pink squares) mRNA levels were measured by RT-qPCR and normalised to *GAPDH*. Both transcripts show a concentration-dependent decrease, with suppression plateauing above $\sim$ 100 μ M. A 3DI concentration of 200 μ M was selected for subsequent experiments as the dose that produced robust suppression of both inflammatory markers while remaining within the solubility and DMSO vehicle constraints (0.4% v/v maximum). Data are mean $\pm$ SD of three biological replicates.

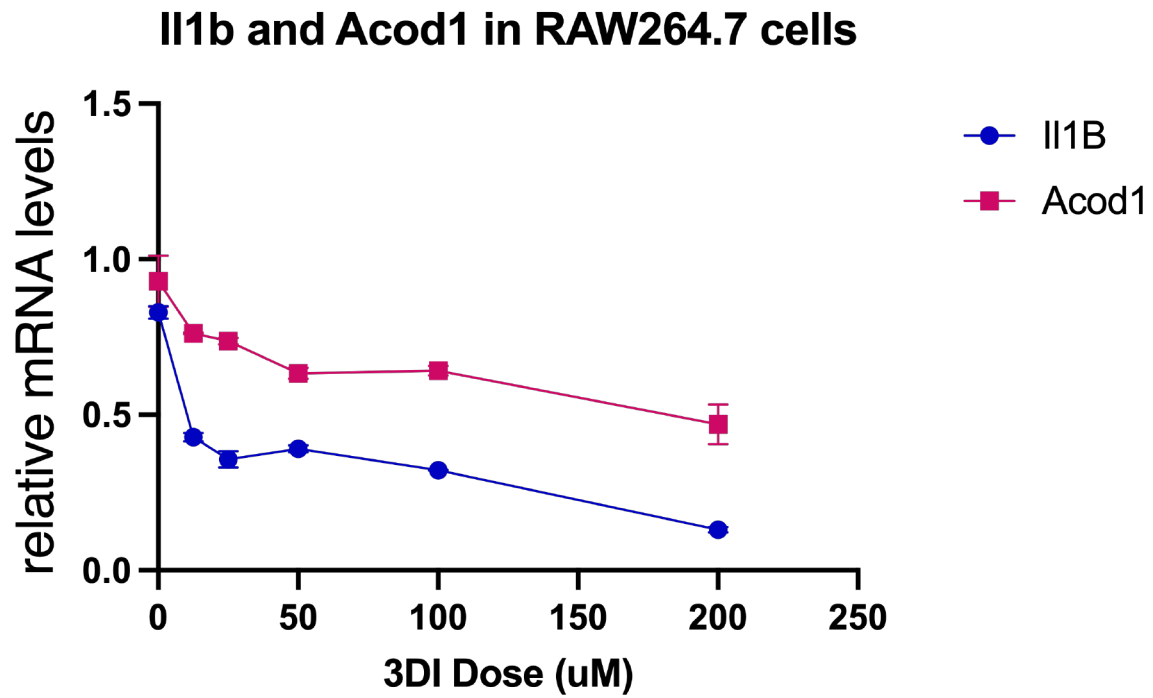


Figure 3.4: Selecting a working concentration of 3'-deoxyinosine (3DI) in RAW264.7 macrophages. Cells were stimulated with LPS (1 $\mu\text{g}/\text{ml}$) for 1 h in the presence of increasing concentrations of 3DI (0–200 μM). Il1b (blue circles) and Acod1 (pink squares) mRNA levels were measured by RT-qPCR and normalised to *Gapdh*. Il1b showed a steep decline that was already pronounced at 12.5 μM , whereas Acod1 decreased more gradually. A 3DI concentration of 200 μM was selected for subsequent experiments as the highest dose that produced an anti-inflammatory effect approaching that of cordycepin (20 μM) without exceeding the DMSO vehicle tolerance (0.4% v/v). Data are mean $\pm$ SD of three biological replicates.

Il1b and Acod1 in RAW264.7 cells

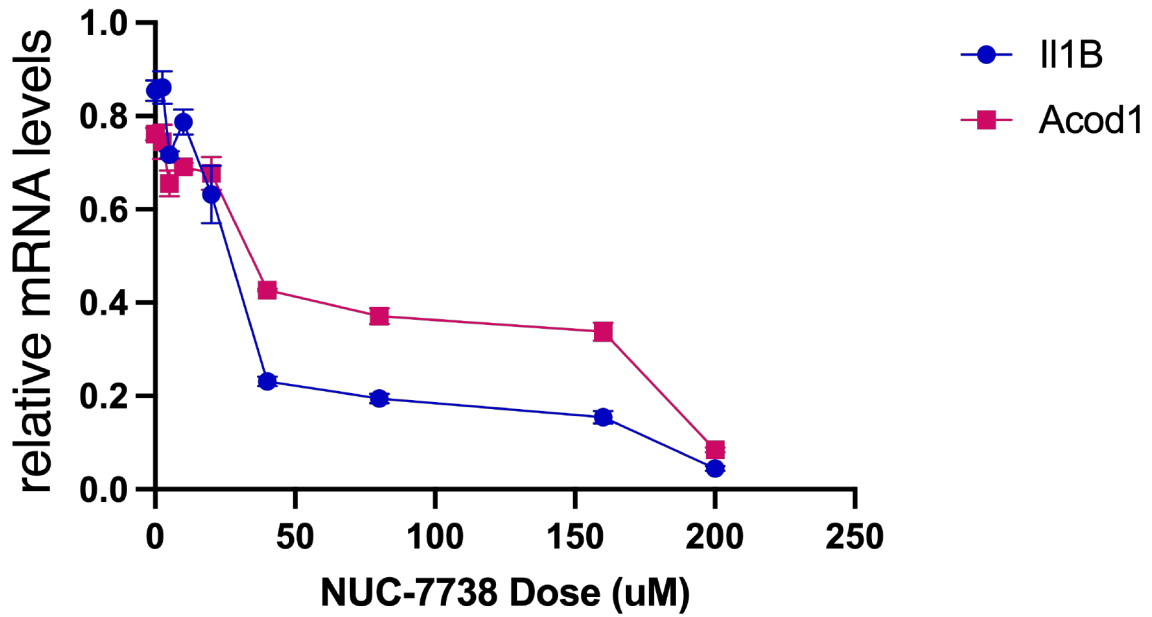


Figure 3.5: NUC-7738 dose-response in RAW264.7 macrophages. Cells were stimulated with LPS (1 $\mu\text{g}/\text{ml}$) for 1 h in the presence of increasing concentrations of NUC-7738 (0–200 μM). Il1b (blue circles) and Acod1 (pink squares) mRNA levels were measured by RT-qPCR and normalised to *Gapdh*. NUC-7738 produced potent, concentration-dependent suppression of both transcripts, with Il1b declining sharply by 5 μM and Acod1 following a similar but more gradual trend. These data confirm that the experimental system responds to a ProTide nucleoside analogue that generates intracellular cordycepin triphosphate independently of ENT1 transport, ADA-mediated degradation and ADK phosphorylation [29]. Data are mean $\pm$ SD of three biological replicates.

3.2.2 Testing the NT5C2/ADSS2 Phosphorylation-Amination Pathway

To Determine whether cytosolic 5'-nucleotidase (NT5C2) and adenylosuccinate synthetase (ADSS2) mediate 3DI conversion to cordycepin monophosphate, analogous to dideoxyinosine metabolism.

Based on the known metabolism of the antiretroviral drug dideoxyinosine [114] [115] [116] [117], we hypothesized that NT5C2 could phosphorylate 3DI, followed by amination via ADSS2 and ADSL to yield cordycepin monophosphate (Figure 3.6). To test this pathway, we performed siRNA knockdowns of NT5C2 and ADSS2 in A549 cells. NT5C2 knockdown achieved >80% reduction in target mRNA but did not significantly affect the anti-inflammatory efficacy of either cordycepin or 3DI (Figure 3.7 A-E). When normalized within each siRNA group to remove basal knockdown effects (Treated/[DMSO+TNF]), neither compound showed significant changes in efficacy (Figure 3.7 C,E; paired t-tests: all $p>0.05$, $n=3$). Similarly, ADSS2 knockdown (>90% target reduction) did not significantly impair the activity of either compound (Figure 3.8 A-E). The efficacy of cordycepin remained at 110.1% (CXCL1) and 107.3% (CXCL8) relative to control siRNA. For 3DI, efficacy was retained at 98.0% (CXCL1) and 77.4% (CXCL8) of control levels, with no significant differences (paired t-tests: all $p>0.05$).

The NT5C2/ADSS2 phosphorylation-amination pathway does not appear to be a major route for 3DI bioactivation under these experimental conditions. Although it is possible that sufficient protein persists under these conditions, it appears less likely that this is a key pathway for 3DI bioactivation.

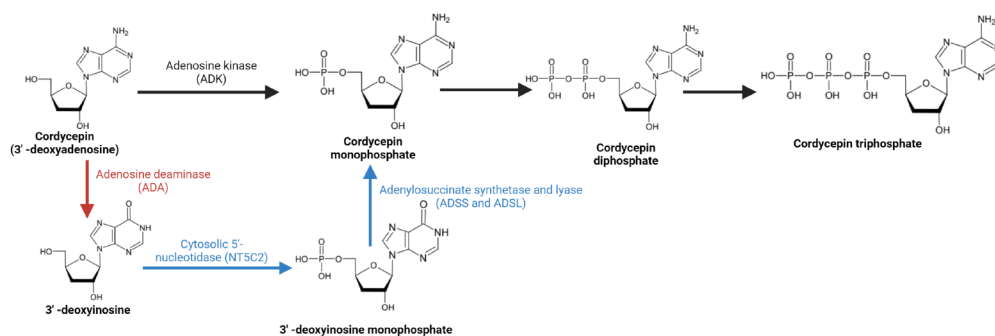


Figure 3.6: Based on prior knowledge that the antiretroviral drug dideoxyinosine is phosphorylated by cytosolic 5'-nucleotidase and subsequently aminated by adenylosuccinate synthetase and lyase, we hypothesized that analogous enzymes may metabolize cordycepin and its derivative 3'-deoxyinosine. We propose that cytosolic 5'-nucleotidase can similarly phosphorylate 3'-deoxyinosine, followed by amination of the phosphorylated intermediate by adenylosuccinate synthetase and adenylosuccinate lyase to yield cordycepin monophosphate. This putative metabolic pathway connecting 3'-deoxyinosine to active cordycepin triphosphate was hypothesized by extrapolating known pathways. The chemical structures were drawn using ChemSketch from the ACD Lab, and the figure was created using ChemDraw.

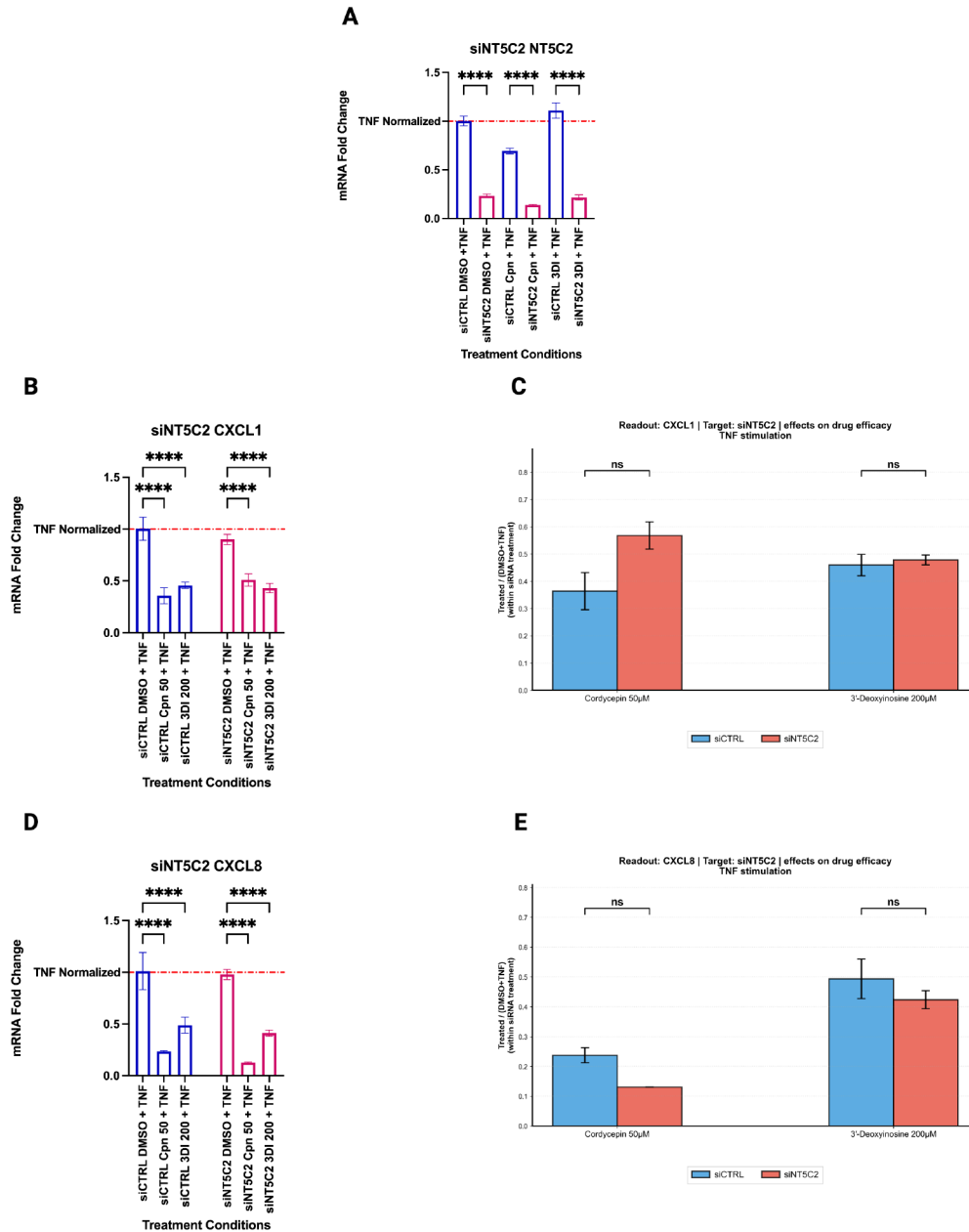


Figure 3.7: Effect of NT5C2 knock-down on Cordycepin and 3'-Deoxyinosine responses (A, B, D) A549 epithelial cells were transfected with 5 nM siRNA targeting ADSS2 (siADSS2) or a non-targeting control (siCTRL) for 48 h. Cells were left untreated (No Tx) or exposed to Cordycepin 50 µM (Cpn 50) or 3'-Deoxyinosine 200 µM (3DI 200) for 2 h, with TNF (10 ng/ml) added 1 h after the start of compound treatment (i.e. in the presence of the compound) for a 1 h stimulation. qPCR data for NT5C2, CXCL1, and CXCL8 were normalised to GAPDH and expressed relative to the TNF + siCTRL condition (n = 3 biological replicates). Bars show mean ± SEM; dashed line indicates the TNF baseline. Significance: ordinary one-way ANOVA with Tukey post-test, $P < 0.05$ (*), < 0.01 (**), < 0.001 (***), ns = not significant. (C,E) Same experiments, but ratios were further normalised within each siRNA group to their corresponding DMSO + TNF value (Treated / [DMSO + TNF] in the same siRNA group), removing basal knock-down effects. Paired two-tailed t-tests compare siCTRL vs. siNT5C2 for each drug. Colour code: blue = siCTRL, red = siNT5C2.

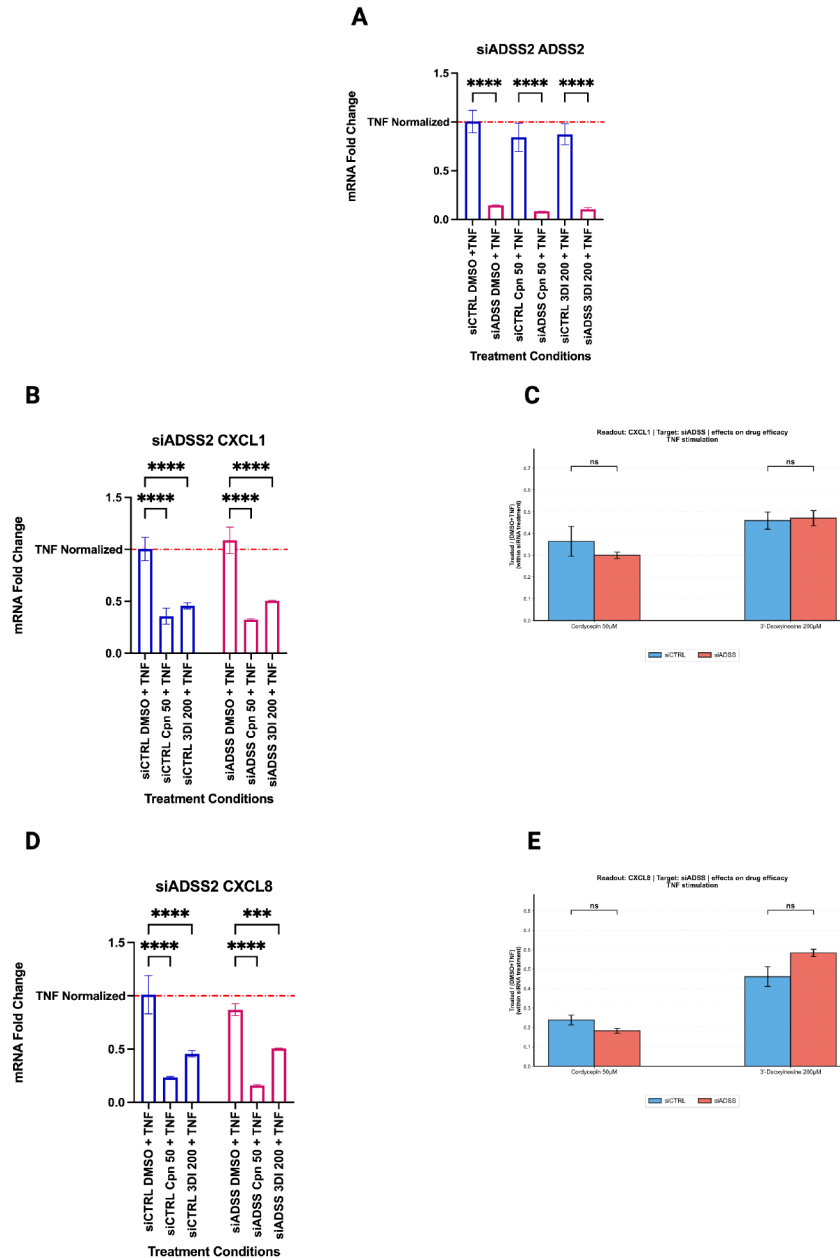


Figure 3.8: Effect of ADSS2 knock-down on Cordycepin and 3'-Deoxyinosine responses (A, B, D) A549 epithelial cells were transfected with 5 nM siRNA targeting ADSS2 (siADSS2) or a non-targeting control (siCTRL) for 48 h. Cells were left untreated (No Tx) or exposed to Cordycepin 50 μ M (Cpn 50) or 3'-Deoxyinosine 200 μ M (3DI 200) for 2 h, with TNF (10 ng/ml) added 1 h after the start of compound treatment (i.e. in the presence of the compound) for a 1 h stimulation. qPCR data for ADSS2, CXCL1, and CXCL8 were normalised to GAPDH and expressed relative to the TNF + siCTRL condition ($n = 3$ biological replicates). Bars show mean $\pm$ SEM; dashed line indicates the TNF baseline. Significance: ordinary one-way ANOVA with Tukey post-test, $P < 0.05$ (*), < 0.01 (**), < 0.001 (***), ns = not significant. (C, E) Same experiments, but ratios were further normalised within each siRNA group to their corresponding DMSO + TNF value (Treated / [DMSO + TNF] in the same siRNA group), removing basal knock-down effects. Paired two-tailed t-tests compare siCTRL vs. siADSS2 for each drug. Colour code: blue = siCTRL, red = siADSS2.

3.2.3 Evaluating the PNP-Mediated Salvage Pathway

To test whether purine nucleoside phosphorylase (PNP) could cleave 3DI into hypoxanthine and 3'-deoxyribose-1-phosphate, enabling salvage via hypoxanthine and 3'-deoxyribose recycling, we induced a metabolic state favouring inosine use as a carbon source and then examined 3DI responses under PNP upregulation [109–111, 118].

We proposed that PNP could cleave 3DI into hypoxanthine and 3'-deoxyribose-1-phosphate, which could then be converted to 3'-deoxyribose-5-phosphate and recombined via hypoxanthine-phosphoribosyltransferase (HPRT) to regenerate 3'-deoxyinosine monophosphate (Figure 3.9). To test whether upregulating PNP by glucose deprivation [118] would augment 3DI activity, we cultured A549 cells in glucose-free medium for 24 hours before drug treatment.

Although 50-100 μ M 3DI showed increased anti-inflammatory effects under glucose deprivation, the basal inflammatory response was itself dampened in glucose-free conditions (Figure 3.10 B-C). This global suppression of inflammatory markers made it impossible to determine whether any PNP-dependent mechanism specifically enhanced 3DI bioactivation. PNP expression levels were measured (Figure 9A) but the overall experimental design yielded inconclusive results.

No definitive inference on a PNP requirement for 3DI activation can be drawn under these conditions due to the confounding effect of globally reduced inflammatory responses in glucose-free media.

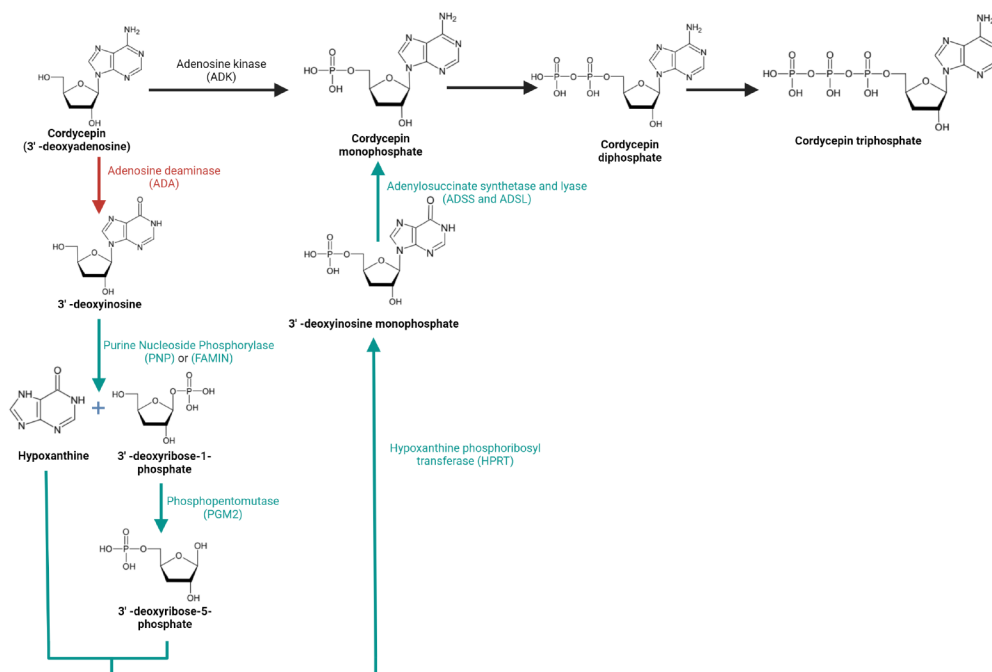


Figure 3.9: Proposed metabolic pathway for 3'-deoxyinosine involving purine nucleoside phosphorylase (PNP). This hypothesized route entails PNP-mediated enzymatic cleavage of 3'-deoxyinosine into hypoxanthine and 3'-deoxyribose-1-phosphate. 3'-deoxyribose-1-phosphate can then be converted to 3'-deoxyribose-5-phosphate and combined with hypoxanthine by hypoxanthine-phosphoribosyl transferase to generate 3'-deoxyinosine monophosphate. To test this pathway, PNP was supposed to be stimulated upon glucose starvation in A549 cells; however, this did not affect the anti-inflammatory activity of 3'-deoxyinosine (In my experiment glucose starvation reduced the basal inflammatory response despite there is an article argued that glucose starvation trigger an inflammatory response and shifting the cells to use inosine as a carbon source), suggesting that this proposed route is not utilized for 3'-deoxyinosine metabolism. The chemical structures were drawn using ChemSketch from the ACD Lab, and the figure was created using ChemDraw.

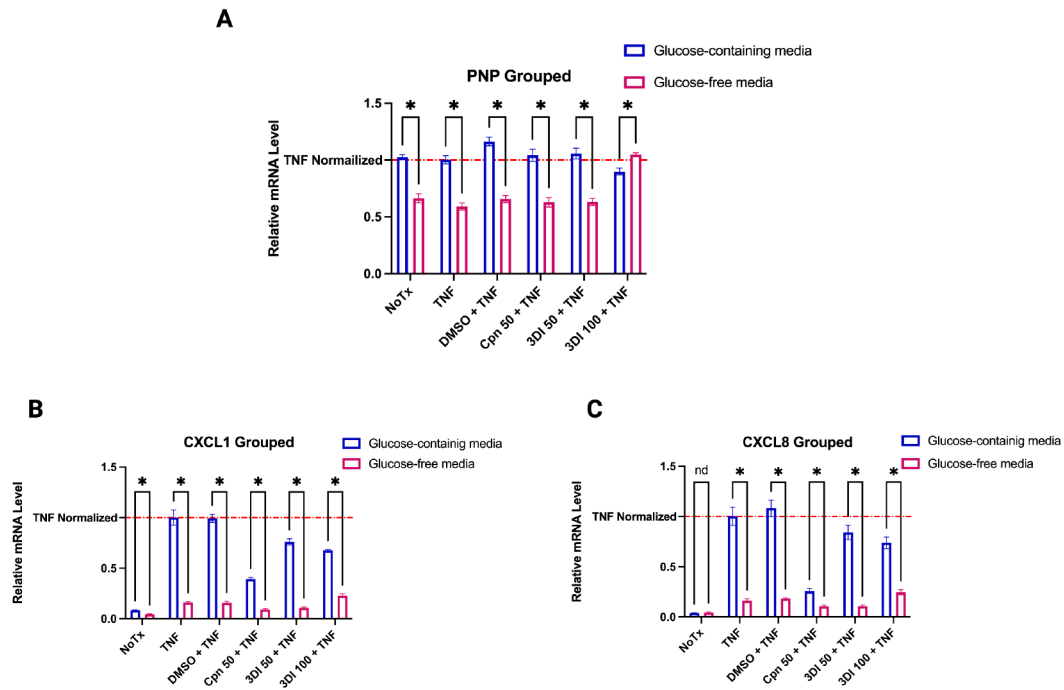


Figure 3.10: A549 cells were cultured in glucose-free medium for 24 h before treatment with cordycepin (50 μ M) or 3'-deoxyinosine (200 μ M) for 2 h, with TNF (10 ng/ml) added 1 h after the start of compound treatment (i.e. in the presence of the compound) for a 1 h stimulation. Total RNA was extracted and cDNA was synthesized, followed by qPCR. Ct values of qPCR for A) the inflammatory genes CXCL1 and CXCL8, and B) PNP were analyzed by the $2^{-\Delta\Delta C_t}$ method using GAPDH as a reference gene. Relative mRNA expression of the tested genes is shown as a fold-change compared to TNF in glucose-containing medium (red dashed line). The results are presented as the mean $\pm$ SD of three independent experiments.

3.2.4 Adenine Salvage Pathways Show Promise

To investigate whether endogenous adenine combines with 3'-deoxyribose moieties from 3DI to regenerate cordycepin via salvage enzymes. I hypothesized two related pathways involving adenine salvage (Figures 3.11 3.12):

1. Direct combination of adenine with 3'-deoxyribose via methylthioadenosine phosphorylase (MTAP) or FAMIN/LACC1
2. Phosphoribosyl transfer via APRT after conversion of 3'-deoxyribose to 5-phospho-3'-deoxyribosyl-1-pyrophosphate

To test whether adenine availability limits 3DI activity, A549 cells were co-treated with adenine (800 μ M) for 1 h before adding cordycepin or 3DI. Adenine supplementation significantly enhanced 3DI's suppression of CXCL1 (** $p < 0.001$) and showed a trend toward enhancement for CXCL8 (* $p < 0.05$) (Figure 3.13 A-B). However, interpretation is complicated by adenine's own anti-inflammatory properties [119], making it difficult to distinguish synergistic from additive effects.

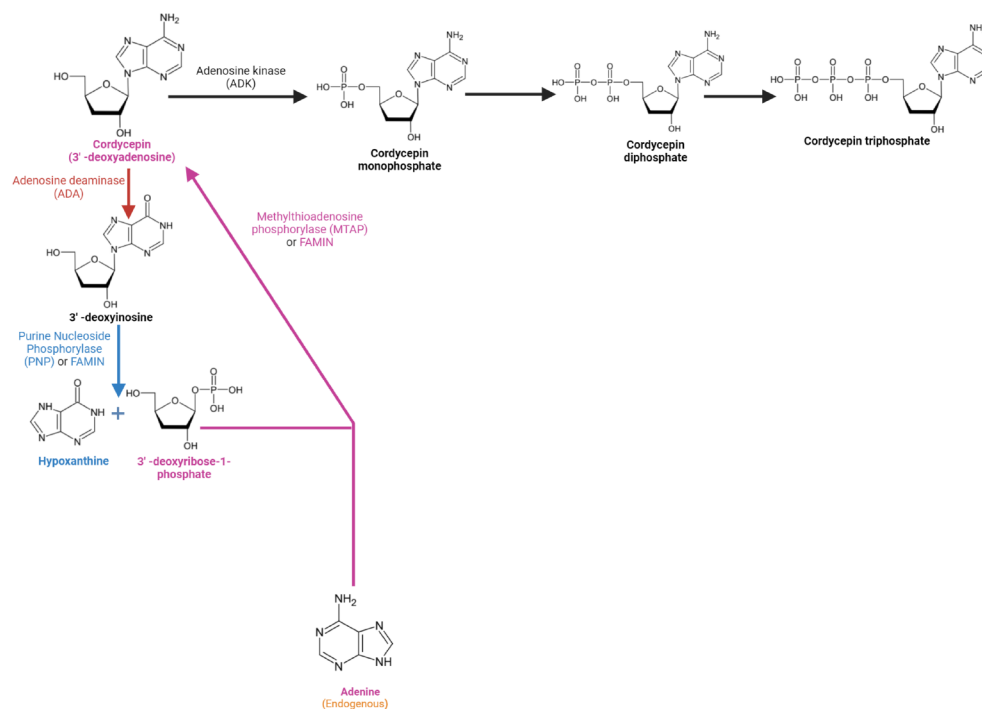


Figure 3.11: Proposed metabolic pathway for the conversion of 3'-deoxyinosine to cordycepin involving adenine and 3'-deoxyribose recycling. This hypothetical route entails an endogenous adenine reacting with the 3'-deoxyribose moiety released from 3'-deoxyinosine. Enzymes such as methylthioadenosine phosphorylase (MTAP) or FAMIN can combine adenine with 3'-deoxyribose to generate 3'-deoxyadenosine (cordycepin). The chemical structures were drawn using ChemSketch from the ACD Lab, and the figure was created using ChemDraw.

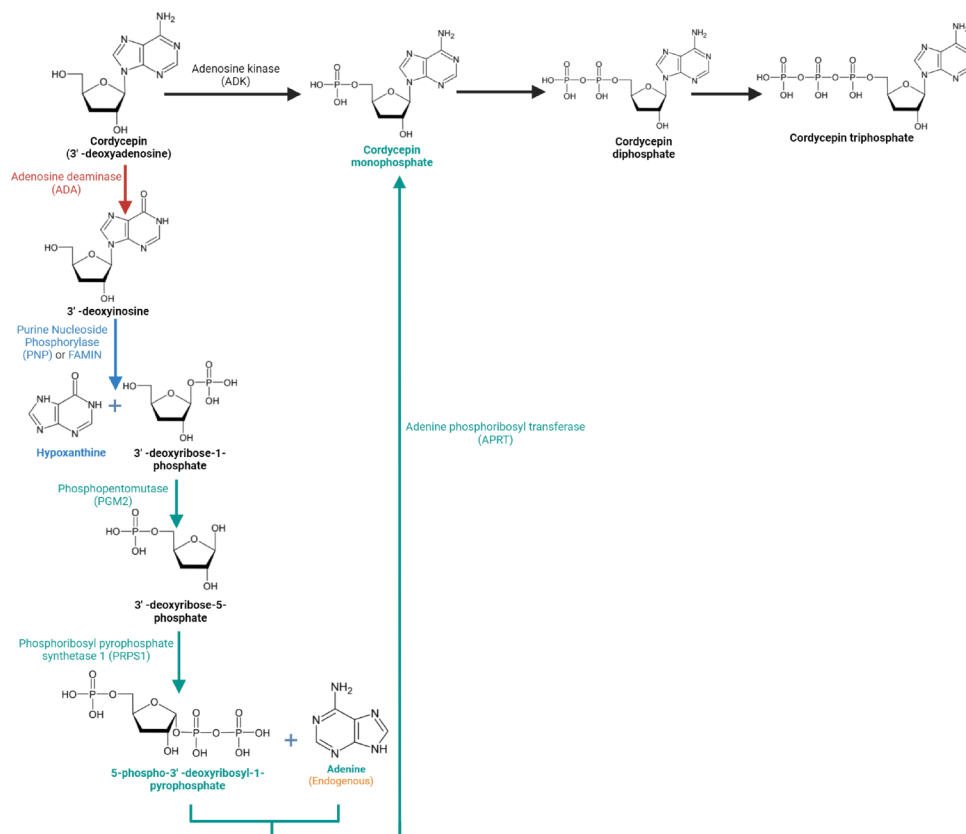


Figure 3.12: Proposed metabolic pathway for 3'-deoxyinosine conversion to cordycepin monophosphate involving adenine salvage. This hypothetical route entails 3'-deoxyribose-1-phosphate first being cleaved from 3'-deoxyinosine and then converted to 3'-deoxyribose-5-phosphate by phosphopentomutase (PGM2). 3'-deoxyribose-5-phosphate can then be converted to 5-phospho-3'-deoxyribosyl-1-pyrophosphate by phosphoribosyl pyrophosphate synthetase 1 (PRPS1). Finally, endogenous adenine reacts with 5-phospho-3'-deoxyribosyl-1-pyrophosphate, catalyzed by adenine phosphoribosyltransferase (APRT) to generate 3'-deoxyadenosine monophosphate (cordycepin monophosphate).

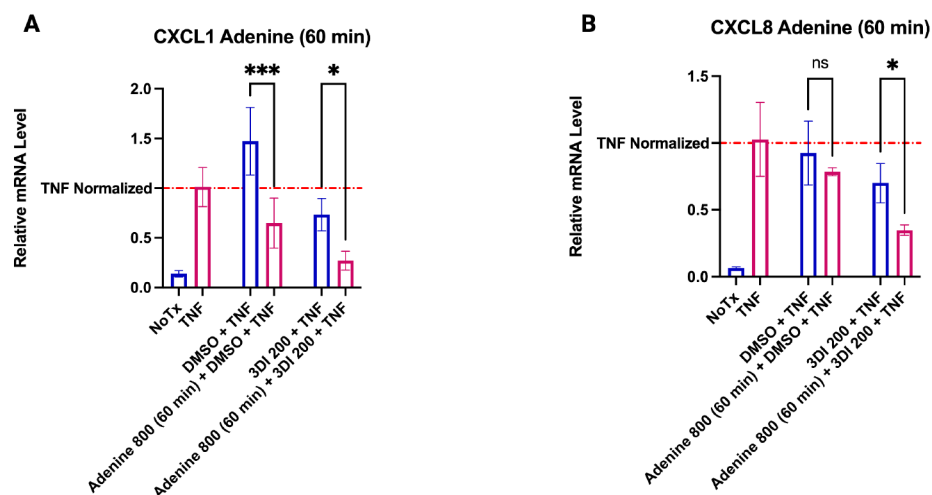


Figure 3.13: Effects of cordycepin and 3'-deoxyinosine on inflammatory gene expression after adenine treatment. A549 cells were treated with adenine (800 μ M) for one hour then with 0.4% v/v DMSO, cordycepin (50 μ M), or 3'-deoxyinosine (200 μ M) for 2 h, with TNF (10 ng/ml) added 1 h after the start of compound treatment (i.e. in the presence of the compound) for a 1 h stimulation. The graph shows the mean $\pm$ standard deviation of the three biological replicates. Statistical significance was determined by unpaired t-tests at * $P < 0.05$, ** $P < 0.01$, *** $P < 0.005$, and **** $P < 0.0001$ ****.

3.2.5 APRT is Required for Cordycepin and 3DI Activity

To determine whether adenine phosphoribosyltransferase (APRT) is necessary for the anti-inflammatory effects of cordycepin and 3DI, I conducted APRT knockdown

APRT knockdown in A549 cells (>95% reduction) did not significantly affect 3DI efficacy on CXCL1 (103.1% efficacy retained) or CXCL8 (68.2% retained). However, cordycepin's efficacy was significantly reduced for CXCL8 (63.2% retained, ** $p<0.01$), though not for CXCL1 (51.5% retained, $p=0.08$) (Figure 3.14 A-E). The requirement for APRT was more pronounced in RAW264.7 macrophages. Aprt knockdown significantly impaired both cordycepin and 3DI activity across both inflammatory readouts (Figure 3.15 A-E):

Cordycepin efficacy reduced to 66.0% (Acod1, * $p<0.05$) and 64.1% (Il1B, ** $p<0.01$)

3DI efficacy dramatically reduced to 23.9% (Acod1, ** $p<0.01$) and 44.2% (Il1B, ** $p<0.01$)

APRT is required for the anti-inflammatory activity of both compounds, with stronger dependency in macrophages than epithelial cells.

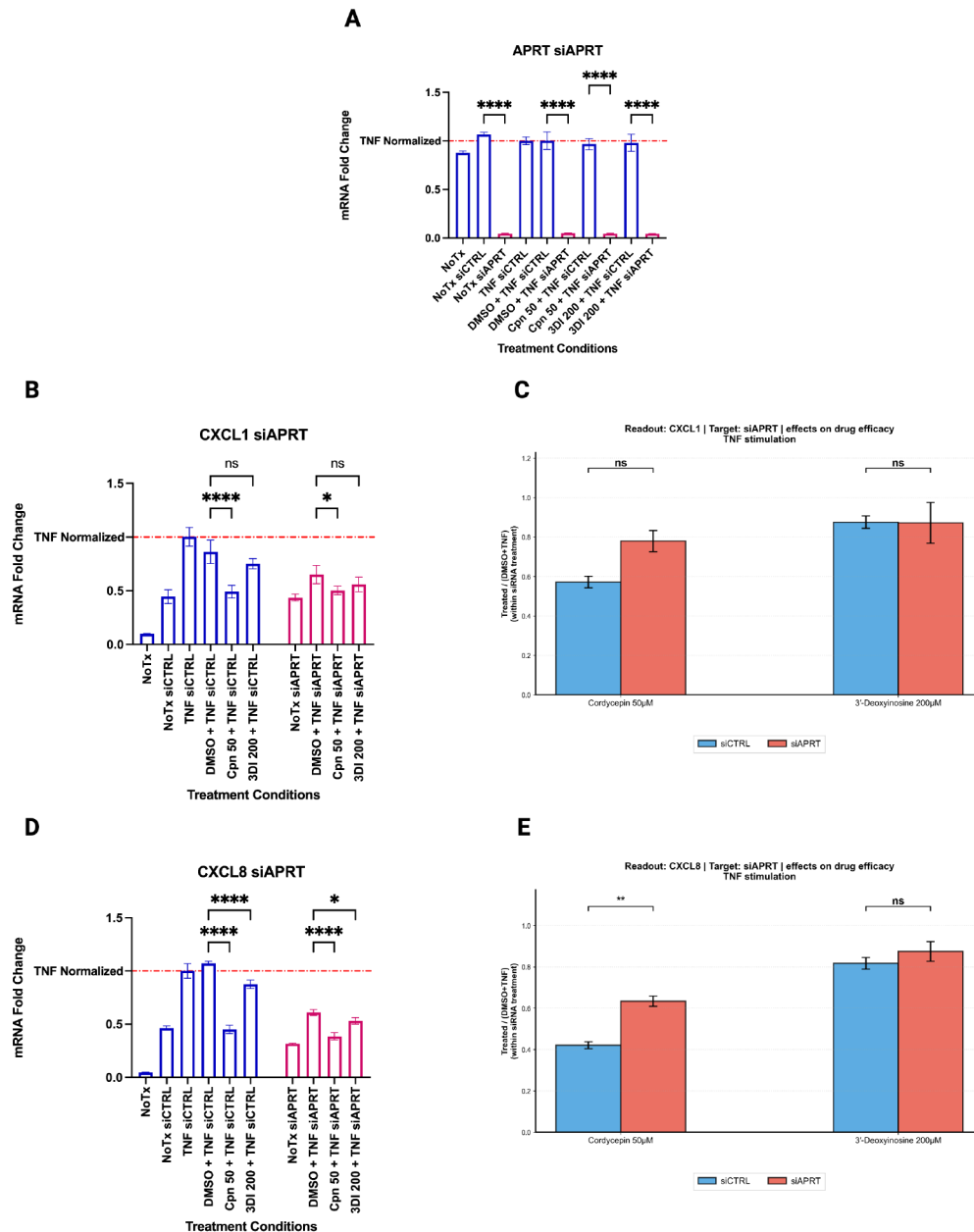


Figure 3.14: Effect of APRT knock-down on Cordycepin and 3'-Deoxyinosine responses (A, B, D) A549 epithelial cells were transfected with 5 nM siRNA targeting APRT (siAPRT) or a non-targeting control (siCTRL) for 48 h. Cells were left untreated (No Tx) or exposed to Cordycepin 50 µM (Cpn 50) or 3'-Deoxyinosine 200 µM (3DI 200) for 2 h, with TNF (10 ng/ml) added 1 h after the start of compound treatment (i.e. in the presence of the compound) for a 1 h stimulation. qPCR data for APRT, CXCL1, and CXCL8 were normalised to GAPDH and expressed relative to the TNF + siCTRL condition (n = 3 biological replicates). Bars show mean ± SEM; dashed line indicates the TNF baseline. Significance: ordinary one-way ANOVA with Tukey post-test, $P < 0.05$ (*), < 0.01 (**), < 0.001 (***), ns = not significant. (C, E) Same experiments, but ratios were further normalised within each siRNA group to their corresponding DMSO + TNF value (Treated / [DMSO + TNF] in the same siRNA group), removing basal knock-down effects. Paired two-tailed t-tests compare siCTRL vs. siAPRT for each drug. Colour code: blue = siCTRL, red = siAPRT.

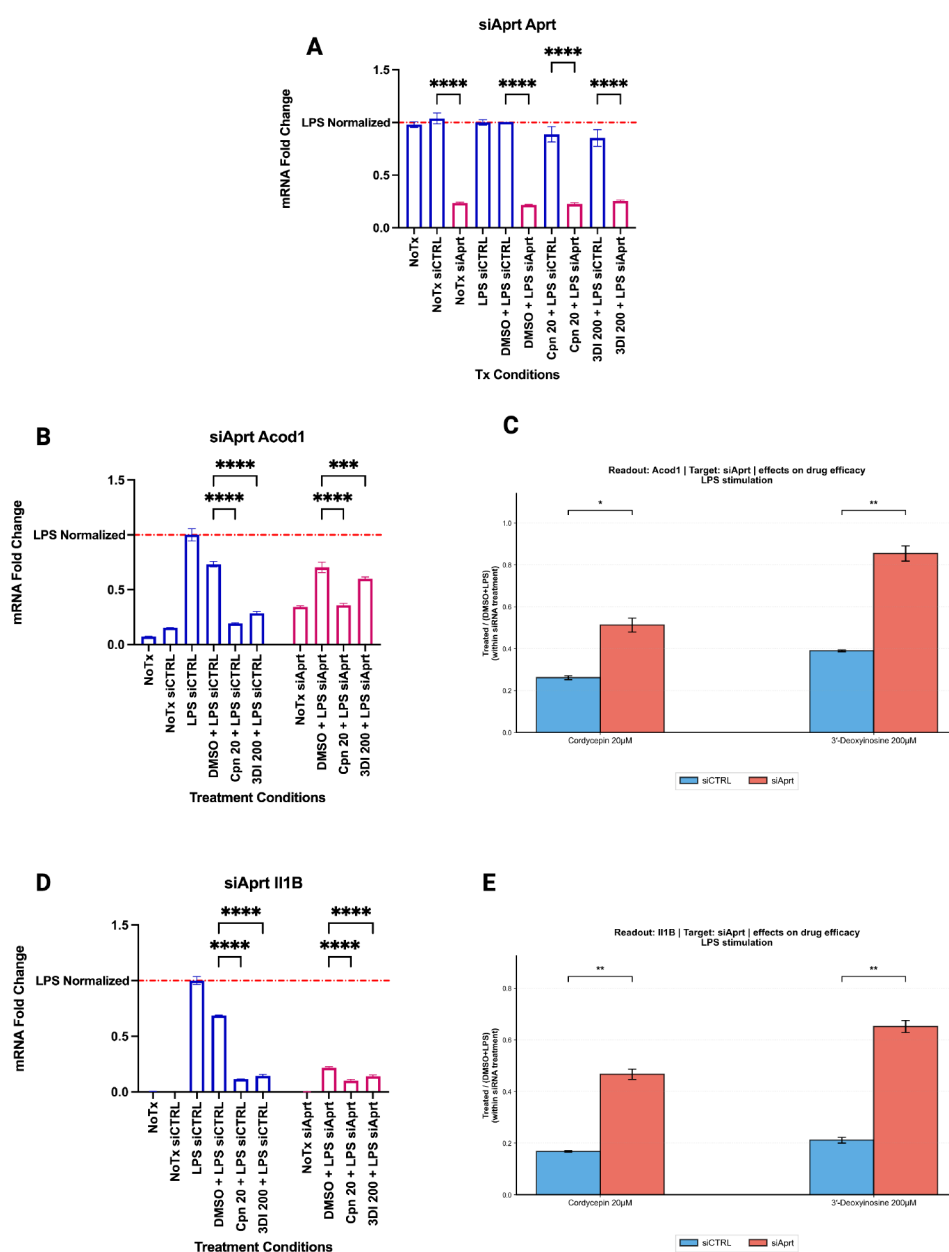


Figure 3.15: Effect of siApt knock-down on Cordycepin and 3'-Deoxyinosine responses (A, B, D) RAW264.7 macrophages were transfected with 5 nM siRNA targeting Appt (siApt) or a non-targeting control (siCTRL) for 48 h. Cells were left untreated (No Tx) or exposed to Cordycepin 20 µM (Cpn 20) or 3'-Deoxyinosine 200 µM (3DI 200) for 2 h, with LPS (1 µg/ml) added 1 h after the start of compound treatment (i.e. in the presence of the compound) for a 1 h stimulation. qPCR data for Appt, Acod1, and Il1B were normalised to Gapdh and expressed relative to the LPS + siCTRL condition (n = 3 biological replicates). Bars show mean ± SEM; dashed line indicates the LPS baseline. Significance: ordinary one-way ANOVA with Tukey post-test, P < 0.05 (*), < 0.01 (**), < 0.001 (***), ns = not significant. (C, E) Same experiments, but ratios were further normalised within each siRNA group to their corresponding DMSO + LPS value (Treated / [DMSO + LPS] in the same siRNA group), removing basal knock-down effects. Paired two-tailed t-tests compare siCTRL vs. siApt for each drug. Colour code: blue = siCTRL, red = siApt.

3.2.6 FAMIN/LACC1 Shows Cell-Type-Specific Requirements

To test whether FAMIN/LACC1, a purine 5'-nucleotidase implicated in inflammatory regulation, participates in 3DI bioactivation, I conducted a FAMIN/Lacc1 knockdown.

In A549 cells, FAMIN knockdown (>70% reduction) did not significantly affect the efficacy of either compound. Cordycepin retained 128.9% (CXCL1) and 107.7% (CXCL8) of control efficacy, while 3DI showed variable retention (Figure 3.16 A-E; all paired t-tests $p>0.05$).

In contrast, in RAW264.7 macrophages, Lacc1 knockdown significantly affected the activity of both compounds (Figure 3.17 A-E):

Cordycepin efficacy reduced to 80.5% (Acod1, $*p<0.05$) and 72.6% (Il1B, $**p<0.01$)

3DI efficacy reduced to 63.0% (Acod1, $*p<0.05$) and 53.1% (Il1B, $**p<0.01$)

FAMIN/LACC1 is required for cordycepin and 3DI activity specifically in macrophages, suggesting cell-type-specific metabolic requirements.

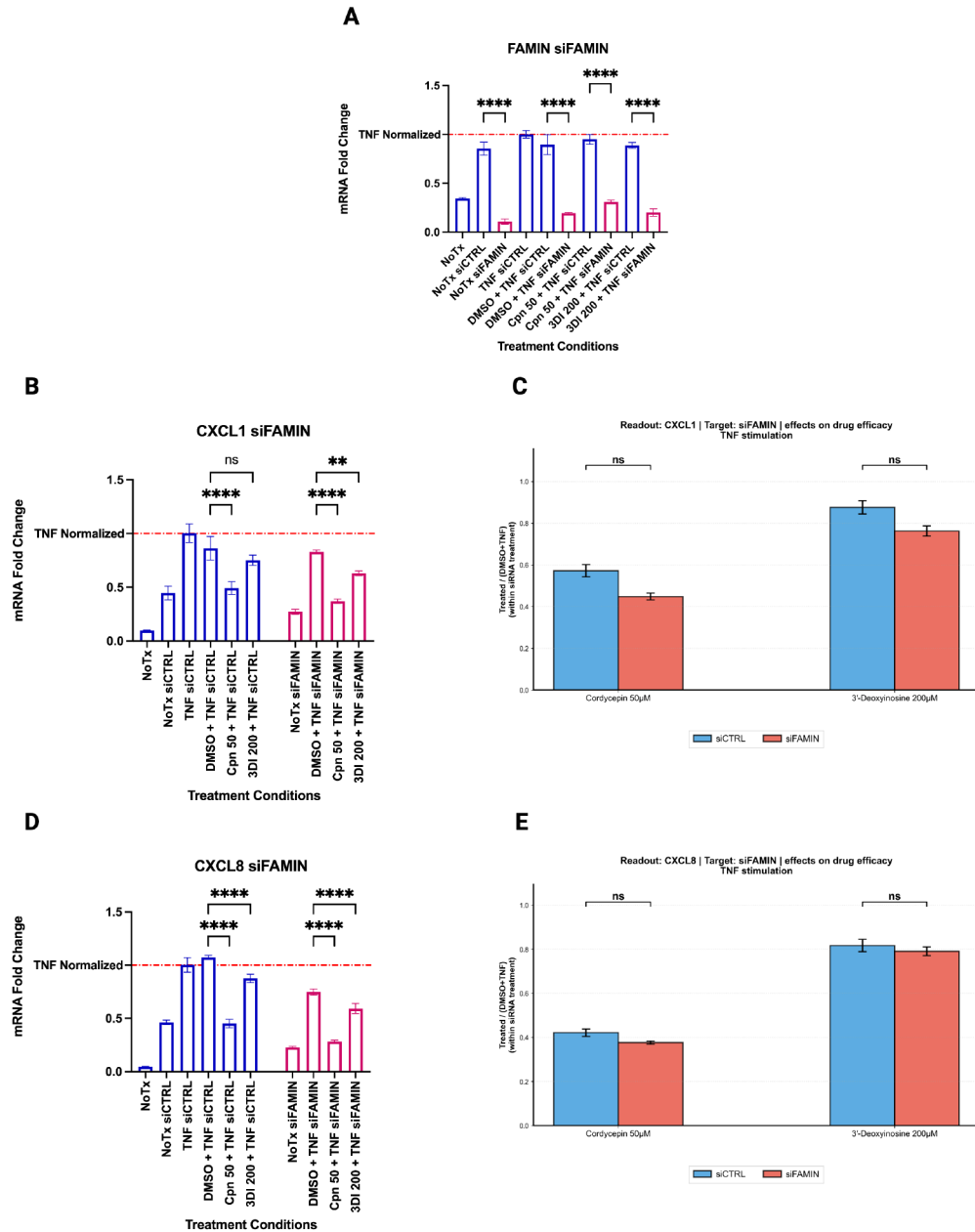


Figure 3.16: Effect of FAMIN knock-down on Cordycepin and 3'-Deoxyinosine responses (A, B, D) A549 epithelial cells were transfected with 5 nM siRNA targeting FAMIN (siFAMIN) or a non-targeting control (siCTRL) for 48 h. Cells were left untreated (No Tx) or exposed to Cordycepin 50 µM (Cpn 50) or 3'-Deoxyinosine 200 µM (3DI 200) for 2 h, with TNF (10 ng/ml) added 1 h after the start of compound treatment (i.e. in the presence of the compound) for a 1 h stimulation. qPCR data for FAMIN, CXCL1, and CXCL8 were normalised to GAPDH and expressed relative to the TNF + siCTRL condition (n = 3 biological replicates). Bars show mean ± SEM; dashed line indicates the TNF baseline. Significance: ordinary one-way ANOVA with Tukey post-test, $P < 0.05$ (*), < 0.01 (**), < 0.001 (***), ns = not significant. (C, E) Same experiments, but ratios were further normalised within each siRNA group to their corresponding DMSO + TNF value (Treated / [DMSO + TNF] in the same siRNA group), removing basal knock-down effects. Paired two-tailed t-tests compare siCTRL vs. siFAMIN for each drug. Colour code: blue = siCTRL, red = siFAMIN.

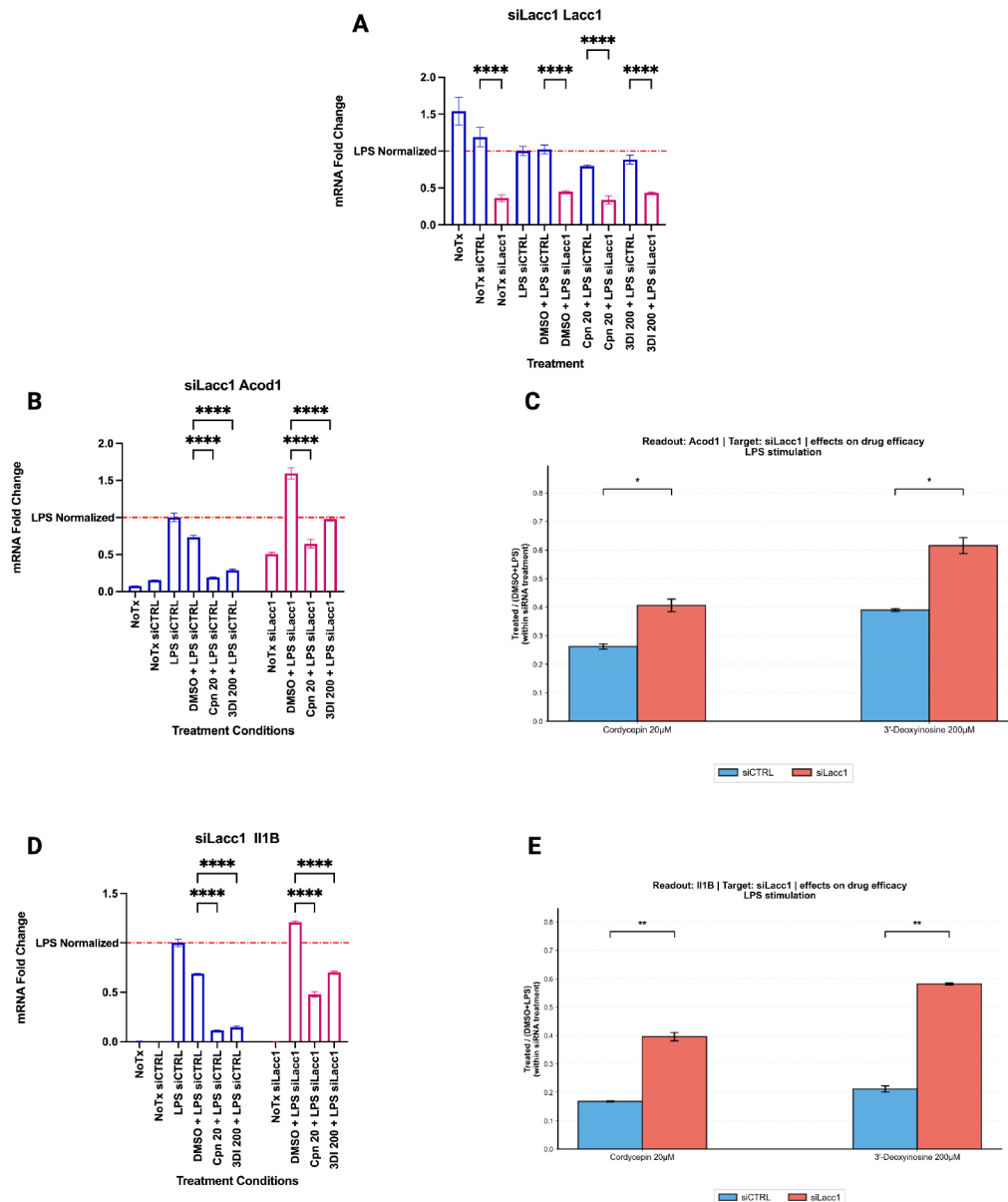


Figure 3.17: Effect of Lacc1 (Famin) knock-down on Cordycepin and 3'-Deoxyinosine responses (A, B, D) RAW264.7 macrophages were transfected with 5 nM siRNA targeting Lacc1 (siLacc1) or a non-targeting control (siCTRL) for 48 h. Cells were left untreated (No Tx) or exposed to Cordycepin 20 µM (Cpn 20) or 3'-Deoxyinosine 200 µM (3DI 200) for 2 h, with LPS (1 µg/ml) added 1 h after the start of compound treatment (i.e. in the presence of the compound) for a 1 h stimulation. qPCR data for Lacc1, Acod1, and Il1b were normalised to Gapdh and expressed relative to the LPS + siCTRL condition (n = 3 biological replicates). Bars show mean ± SEM; dashed line indicates the LPS baseline. Significance: ordinary one-way ANOVA with Tukey post-test, P < 0.05 (*), < 0.01 (**), < 0.001 (***), ns = not significant. (C, E) Same experiments, but ratios were further normalised within each siRNA group to their corresponding DMSO + LPS value (Treated / [DMSO + LPS] in the same siRNA group), removing basal knock-down effects. Paired two-tailed t-tests compare siCTRL vs. siLacc1 for each drug. Colour code: blue = siCTRL, red = siLacc1.

3.2.7 Additional Metabolic Nodes Show Variable Effects

Adenosine kinase (ADK) phosphorylates adenosine and related nucleosides to their monophosphate forms and is required for intracellular CoTP generation from cordycepin [2, 17, 29, 45, 120].

Remarkably, adenosine kinase (ADK) knockdown revealed opposite effects on the two compounds (Figure 3.18 A-E). In A549 cells:

Cordycepin efficacy was significantly reduced for both CXCL1 (27.4% retained, $*p < 0.05$) and CXCL8 (54.8% retained, $*p < 0.05$)

3DI efficacy was enhanced for CXCL8 (260% of control, $*p < 0.05$) with no significant effect on CXCL1

Similar divergent effects were observed in RAW264.7 cells (Figure 3.19 A-E), where Adk knockdown reduced cordycepin efficacy to 50.7% (Acod1, $**p < 0.01$) and essentially abolished it for Il1B (-1.8% efficacy, $**p < 0.01$), while partially reducing 3DI efficacy.

ADK is required for cordycepin activity but may limit 3DI bioactivation, suggesting distinct metabolic processing of these compounds.

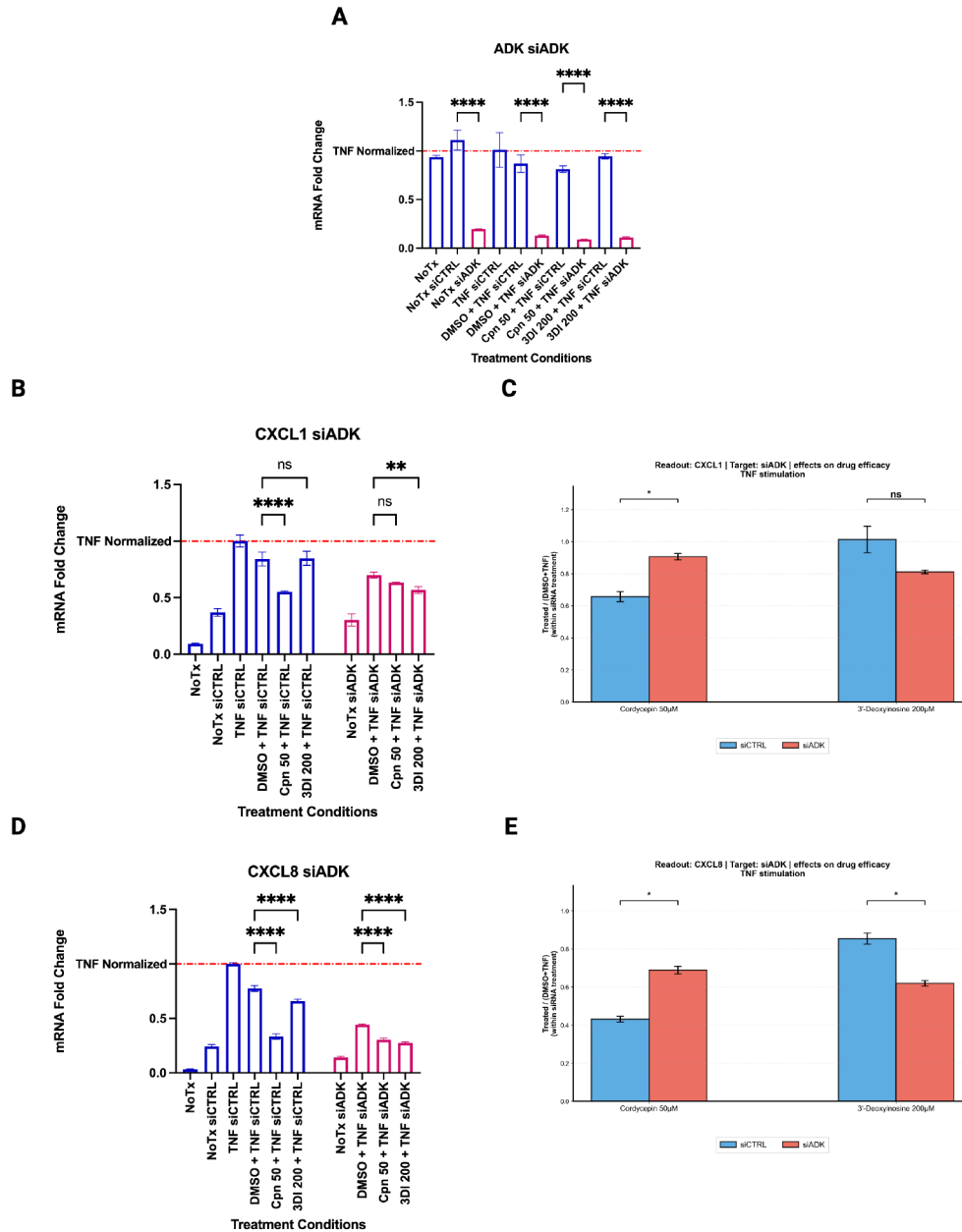


Figure 3.18: Effect of ADK knock-down on Cordycepin and 3'-Deoxyinosine responses (A, B, D) A549 epithelial cells were transfected with 5 nM siRNA targeting ADK (siADK) or a non-targeting control (siCTRL) for 48 h. Cells were left untreated (No Tx) or exposed to Cordycepin 50 µM (Cpn 50) or 3'-Deoxyinosine 200 µM (3DI 200) for 2 h, with TNF (10 ng/ml) added 1 h after the start of compound treatment (i.e. in the presence of the compound) for a 1 h stimulation. qPCR data for ADK, CXCL1, and CXCL8 were normalised to GAPDH and expressed relative to the TNF + siCTRL condition (n = 3 biological replicates). Bars show mean ± SEM; dashed line indicates the TNF baseline. Significance: ordinary one-way ANOVA with Tukey post-test, $P < 0.05$ (*), < 0.01 (**), < 0.001 (***), ns = not significant. (C, E) Same experiments, but ratios were further normalised within each siRNA group to their corresponding DMSO + TNF value [(Treated / [DMSO + LPS] in the same siRNA group)], removing basal knock-down effects. Paired two-tailed t-tests compare siCTRL vs. siADK for each drug. Colour code: blue = siCTRL, red = siADK.

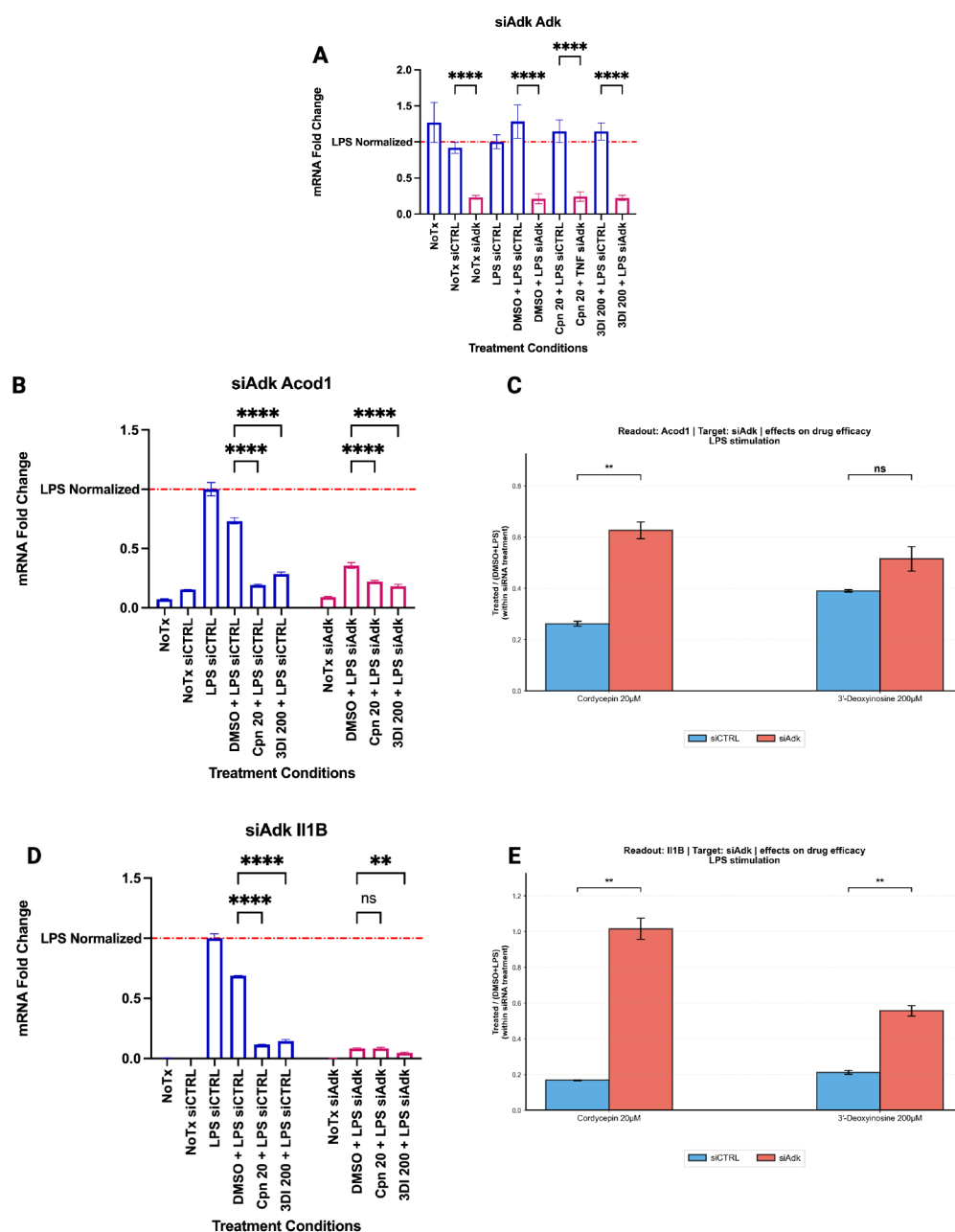


Figure 3.19: Effect of siAdk knock-down on Cordycepin and 3'-Deoxyinosine responses (A, B, D) RAW264.7 macrophages were transfected with 5 nM siRNA targeting Adk (siAdk) or a non-targeting control (siCTRL) for 48 h. Cells were left untreated (No Tx) or exposed to Cordycepin 20 µM (Cpn 20) or 3'-Deoxyinosine 200 µM (3DI 200) for 2 h, with LPS (1 µg/ml) added 1 h after the start of compound treatment (i.e. in the presence of the compound) for a 1 h stimulation. qPCR data for Adk, Acod1, and Il1B were normalised to Gapdh and expressed relative to the LPS + siCTRL condition (n = 3 biological replicates). Bars show mean ± SEM; dashed line indicates the LPS baseline. Significance: ordinary one-way ANOVA with Tukey post-test, P < 0.05 (*), < 0.01 (**), < 0.001 (***), ns = not significant. (C, E) Same experiments, but ratios were further normalised within each siRNA group to their corresponding DMSO + LPS value (Treated / [DMSO + LPS] in the same siRNA group), removing basal knock-down effects. Paired two-tailed t-tests compare siCTRL vs. siAdk for each drug. Colour code: blue = siCTRL, red = siAdk.

3.2.8 Carbosynth Cordycepin Purity Analysis

To ensure that the pathway dependencies observed in the knockdown experiments were not confounded by impurities in the cordycepin reagent, I next verified the identity and purity of the Carbosynth cordycepin stock by LC-MS on a Thermo Exactive with SeQuant[®] ZIC[®]-pHILIC separation, visualised in ACD/Labs Spectrus Processor.

The Carbosynth cordycepin ("cordycepin 601") eluted as a single dominant peak at $t_R \approx 7.28$ -7.31 min in the extracted ion chromatogram (XIC) at m/z 252.1, with software-reported Single XIC area = 100% (Figure 3.20 A). The MS spectrum at the chromatographic apex ($t_R = 7.279$ min) showed a base peak at $[M+H]^+ = 252.109$ (Mass(Amax) = 252.113) with $\Delta_{\text{mass}} = 0.004$ Da, consistent with the molecular formula $C_{10}H_{13}N_5O_3$. Additional labeled ions included m/z 218.215 ($[M+H-34]^+$), 246.243 ($[M+H-6]^+$), and a $[M+Na]^+$ adduct. The software confidence was reported as "Excellent" (Figure 3.20 B).

The positive-ion MS pattern at the chromatographic apex documented diagnostic ions supporting cordycepin identification (Figure 3.21). The base peak $[M+H]^+$ at m/z 252.109 (Mass(Amax) = 252.113) dominated the spectrum, with low-level adduct and neutral-loss ions annotated by the software including $[M+H-34]^+ = 218.215$, $[M+H-6]^+ = 246.244$, $[M+Na]^+$, and m/z 253.113. Minor ions at m/z 118.090, 130.166, 136.071, 148.105, and 212.095-212.097 were also visible in the zoomed view (Figure 3.21 B).

The calibration curve analysis using Phyproof cordycepin (Merck) as standard determined that the purity of Carbosynth cordycepin was 99.08% with $R^2 = 0.9813$ ($y = 1E-06x - 1004.4$) (Figure 3.22).



Figure 3.20: A (top): Overlaid chromatograms: TIC (ESI⁺, red trace) and XIC at m/z 252.1 (green, shaded) across a 24-min run on a SeQuant ZIC-pHILIC column. A single, sharp XIC peak is centered at $t_R \approx 7.28$ – 7.31 min and is annotated “Cordycepin.” Minor features at the solvent front (≈ 0.34 – 0.50 min) and late elution (≈ 23.6 – 23.8 min) are visible in the TIC but do not appear in the XIC. Ion mode: ESI⁺ (labelled at top right). B (bottom): MS spectrum for the $t_R = 7.279$ min peak with automatic component assignment in ACD/Labs Spectrus Processor. Base peak $[M+H]^+ = 252.109$ (Mass(Amax) = 252.113), elemental formula C₁₀H₁₃N₅O₃, and Mass difference = 0.004 (Da). Additional labelled ions include 218.215 ($[M+H-34]^+$), 246.243 ($[M+H-6]^+$), 253.113, and a $[M+Na]^+$ adduct (labelled). Software confidence tile reads “Excellent.” The component table shows $t_R = 7.279$ min, neutral mass 251.102, $[M-H]^- = 250.095$ (reported), and Single XIC area = 100.00% for both ESI polarities; TIC area = 15.26% (ESI⁺) and 30.02% (ESI⁻) with All t_R (min) reported as TIC (ESI⁺) = 7.28, XIC = 7.28, TIC (ESI⁻) = 6.43, XIC = 7.29 (values as displayed in the figure). The chromatogram generated from the Thermo Exactive LC-MS employing the SeQuant ZIC-pHILIC column (Merck). The analysis was conducted over a 24-minute runtime. Visualization of the LC-MS/MS data was performed using the Spectrus Processor from ACD/Labs.

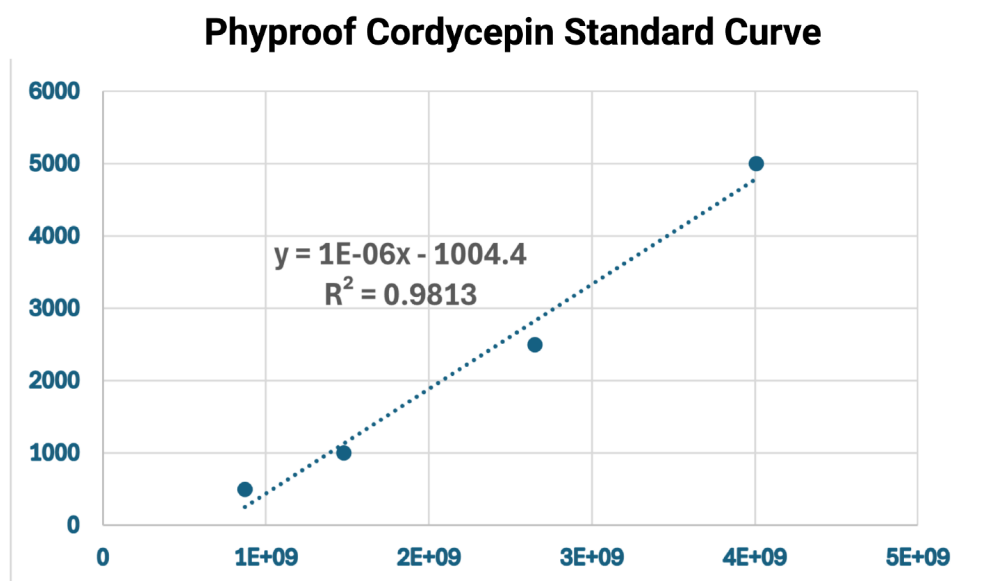


Figure 3.22: Cordycepin (Phyproof) calibration curve. Phyproof Cordycepin from Merck was used as standard to estimate the purity of cordycepin (Carbosynth) and the purity of synthetic cordycepin. The y-axis represents the concentration, expressed in nanomolar (nM), and the x-axis represents the area. The purity of cordycepin (Carbosynth), according to the calibration curve is 99.08% and the purity of synthetic cordycepin is 100%.

3.2.9 Synthetic Cordycepin Purity Analysis

Synthetic cordycepin produced identical chromatographic behaviour to Carbosynth cordycepin, with a single XIC peak at $t_R = 7.280$ min and $[M+H]^+ = 252.109$ (apex mass 252.115; $\Delta = 0.005$ Da), supporting 100% purity for the synthetic standard (Figure 3.23). The centroid ESI⁺ mass spectrum at the apex showed the base peak $[M+H]^+$ at m/z 252.115 with characteristic diagnostic ions including m/z 218.215, 212.100, 198.210, 219.215, 246.244, 253.113, 136.071, and 130.166, plus a sodium adduct $[M+Na]^+$ (Figure 3.24). The mass table confirmed Mass(A0) 251.102, $[M-H]^- = 250.095$, and molecular formula $C_{10}H_{13}N_5O_3$ with mass difference of 0.005 Da, supporting 100% purity for the synthetic standard.

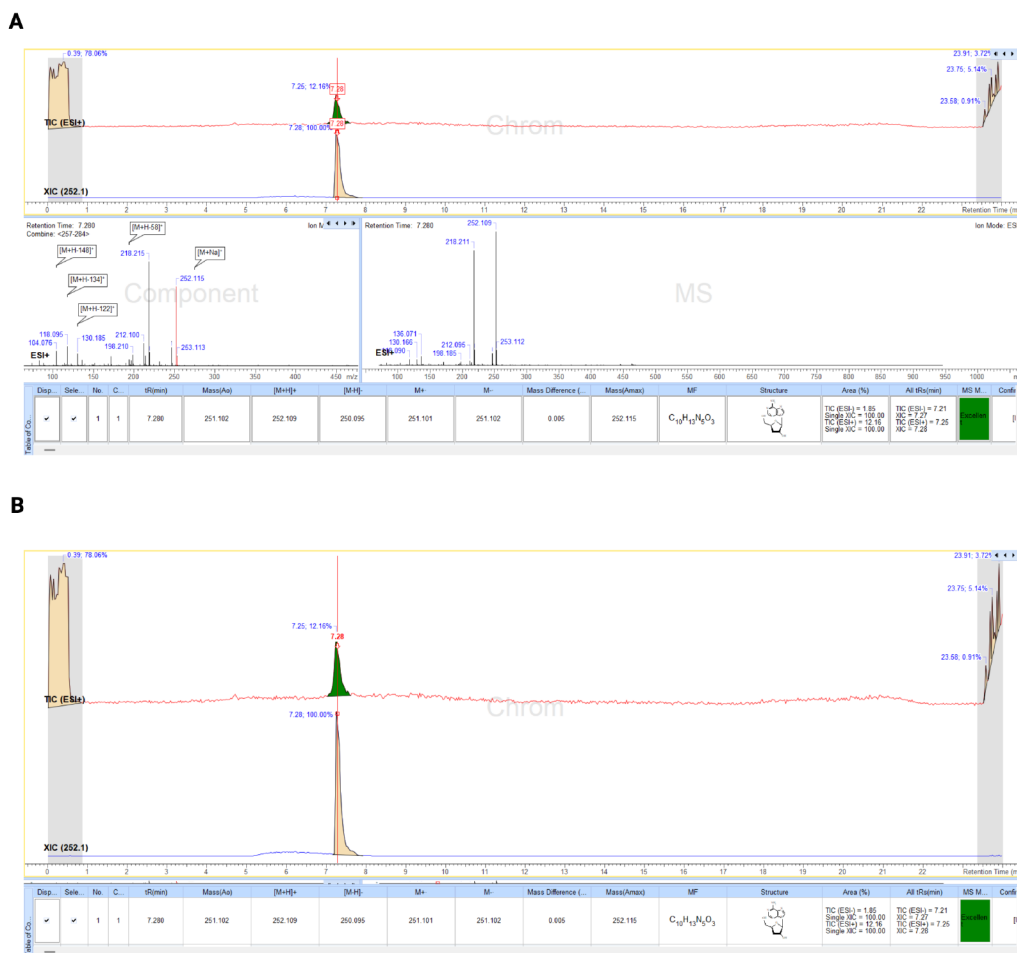


Figure 3.23: A (top): TIC (ESI+, red trace) overlaid with the extracted-ion chromatogram (XIC, m/z 252.1; beige/filled) for synthetic cordycepin; apex at tR = 7.280 min. Below, centroid MS at the peak apex and the identification table. B (bottom): Replicate view of the TIC/XIC overlay with the integrated XIC peak at tR = 7.280 min and the same identification table. The chromatogram generated from the Thermo Exactive LC-MS employing the SeQuant ZIC-pHILIC column (Merck). The analysis was conducted over a 24-minute runtime. Visualization of the LC-MS/MS data was performed using the Spectrus Processor from ACD/Labs.

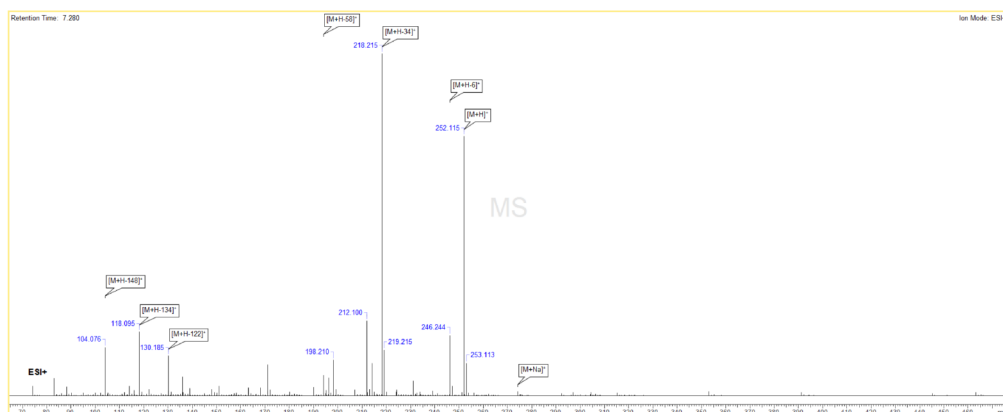
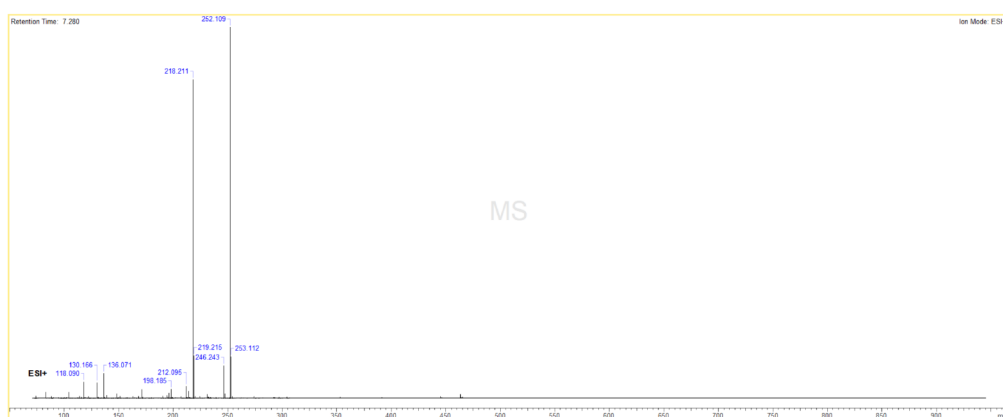
A**B**

Figure 3.24: Mass spectrum for synthetic cordycepin. A (top): Centroid ESI⁺ mass spectrum at $t_R = 7.280$ min annotated with adduct/neutral-loss labels provided by the software. B (bottom): Replicate/zoomed view of the apex spectrum. The chromatogram generated from the Thermo Exactive LC-MS employing the SeQuant ZIC-pHILIC column (Merck). The analysis was conducted over a 24-minute runtime. Visualization of the LC-MS/MS data was performed using the Spectrus Processor from ACD/Labs.

3.2.10 3'-Deoxyinosine Stock Contamination

Critical contamination was discovered in the 3'-deoxyinosine stock (Santa Cruz Biotechnology). LC-MS/MS analysis revealed two distinct peaks: cordycepin at RT $\approx$ 7.282 min ($[M+H]^+$ observed 252.109, $\Delta \approx$ 5 mDa) and 3DI at RT $\approx$ 8.008 min ($[M+H]^+$ observed 253.093, $\Delta \approx$ 1 mDa) (Figure 25A). The ACD/Labs component table listed both components with formulas consistent with cordycepin ($C_{10}H_{13}N_5O_3$) and 3DI ($C_{10}H_{12}N_4O_4$) with sub-10 mDa mass errors. The zoomed chromatogram confirmed baseline separation of these two peaks under the 24-minute HILIC method (Figure 3.25 B).

MS/MS analysis at RT 8.008 min confirmed 3'-deoxyinosine identity with the expected $[M+H]^+$ 253.0935 and characteristic fragments including m/z 214.094, 171.128, 118.090, and 104.0735 (Figure 3.26 A-B). While this confirmed the presence of 3DI in the stock, the cordycepin contamination represents a critical confounding factor.

The contamination level could not be quantified because integrated peak areas and calibration-based response factors for both compounds in the same run were not determined. Therefore, we report presence of cordycepin contamination in the 3DI stock but cannot specify the exact percentage.

While cordycepin stocks (both Carbosynth and synthetic) showed high purity (99-100%), the Santa Cruz 3DI stock contained measurable cordycepin contamination. This finding constrains interpretation of all 3DI-specific effects in this study and highlights the critical importance of comprehensive purity analysis for all reagents in metabolic pathway studies.

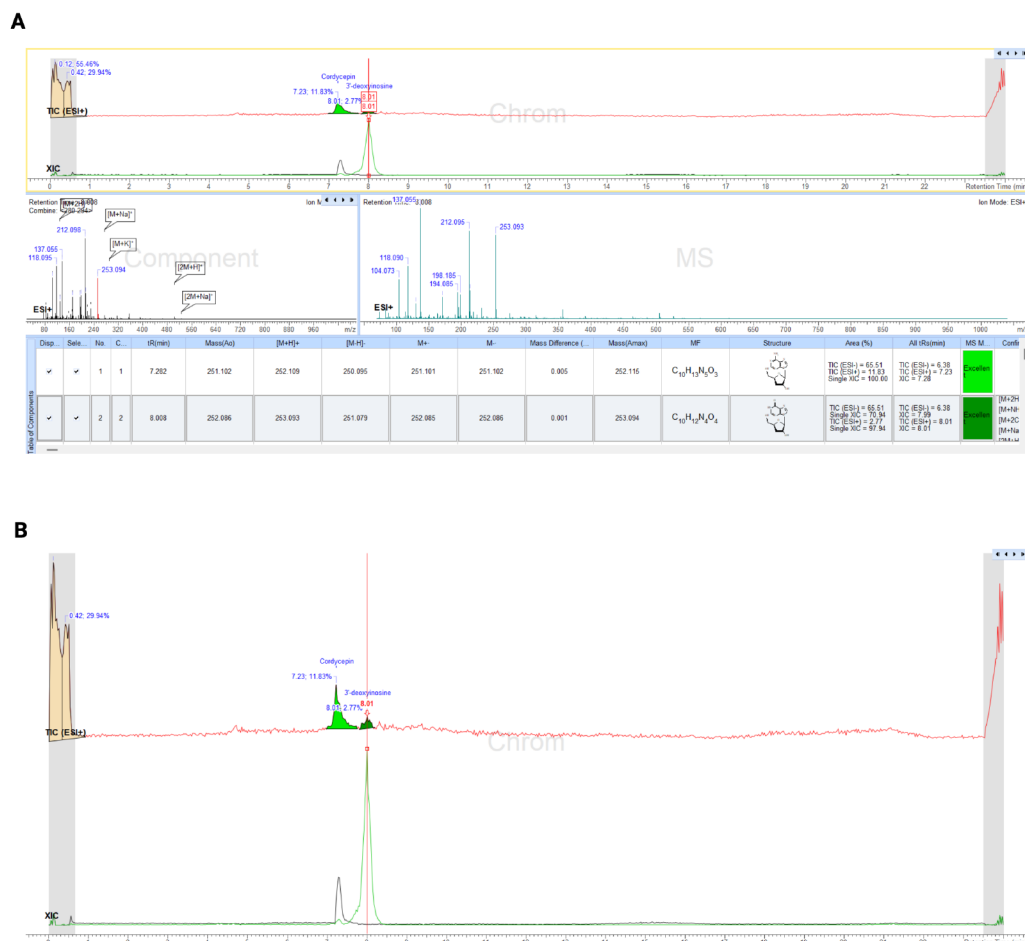
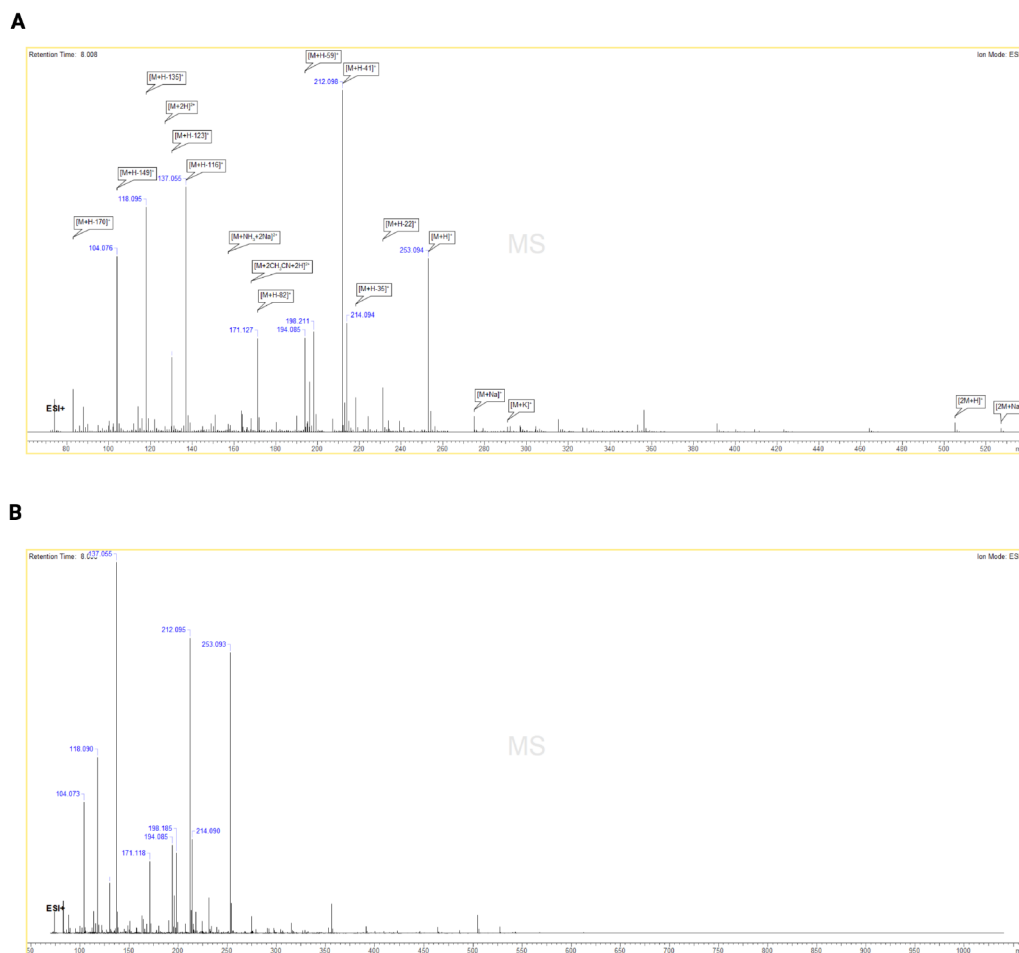


Figure 3.25: A (top): Chromatogram + component table from ACD/Labs Spectrus Processor visualization showing two resolved components in the 3DI stock: cordycepin at RT $\approx$ 7.282 min and 3DI at RT $\approx$ 8.008 min. Accurate-mass matches are annotated in positive ESI: cordycepin $[M+H]^+$ calc 252.115, obs 252.109 ($\Delta \approx 5$ mDa); 3DI $[M+H]^+$ calc 253.094, obs 253.093 ($\Delta \approx 1$ mDa). B (bottom): Zoomed chromatogram view of the same injection highlighting the two peaks attributed to cordycepin and 3DI. The chromatogram generated from the Thermo Exactive LC-MS employing the SeQuant ZIC-pHILIC column (Merck). The analysis was conducted over a 24-minute runtime. Visualization of the LC-MS/MS data was performed using the Spectrus Processor from ACD/Labs.



salvage-coupled routes rather than a direct NT5C2/ADSS2 phosphorylation-amination mechanism or a clearly demonstrable PNP-mediated pathway.

In RAW264.7 macrophages, Aprt knockdown reduced the anti-inflammatory activity of both cordycepin and 3DI (Acod1 and Il1b readouts), indicating a requirement for adenine phosphoribosyltransferase in the active-metabolite supply chain (Figure 3.15). In A549 epithelial cells, APRT loss primarily blunted cordycepin efficacy (CXCL8), with more modest effects on 3DI (Figure 3.14). Famin/Lacc1 knockdown reduced the efficacy of both nucleosides in RAW264.7 but had little impact in A549 (Figures 3.16, 3.17). Together with the enhancement of 3DI responses by adenine co-supplementation in A549 (Figure 3.13), these data support a model in which adenine-dependent 3'-deoxyribose salvage is required for full cordycepin activity, and in which macrophage-specific FAM-IN/LACC1 activity boosts this salvage throughput [112, 113].

Mechanistically, this fits canonical salvage biochemistry: APRT converts adenine to AMP and HPRT converts hypoxanthine to IMP—two major entry points that feed adenylylate pools and downstream nucleotide formation [121]. In macrophages, FAMIN/LACC1 has been shown to integrate adenosine, inosine and adenylosuccinate interconversions into a purine nucleotide cycle that couples purine flux to cellular metabolism [113, 122]. Under this framework, 3'-deoxyribose released from 3DI (via PNP or related enzymes) can be re-adenylylated through APRT- and FAMIN/LACC1-dependent pathways, regenerating cordycepin nucleotides. External systems-level work showing that adenine and inosine are particularly effective precursors for tissue nucleotide repletion is consistent with such adenine-linked rescue routes in vivo [123].

In contrast, the NT5C2/ADSS2 “didanosine-analogue” route, which had been proposed in a previous publication by Lee et al. [1], found little support. Knockdown of NT5C2 or ADSS2 did not measurably affect cordycepin or 3DI responses in A549 cells under the conditions used here, and NT5C2 is biochemically a 5'-nucleotidase rather than a kinase [108, 124]. Although it is possible that sufficient NT5C2/ADSS2 protein persists under these knockdown conditions, making a complete exclusion impossible, the combination of these negative data and the known enzymology makes it unlikely that direct phosphorylation-amination of 3DI to 3'-deoxyadenosine monophosphate is a

major activation route in this system.

The PNP-mediated pathway could not be evaluated definitively. Glucose restriction was used to increase reliance on inosine as an alternative carbon source [118], thereby increasing flux through PNP-dependent inosine metabolism, with the aim of testing whether 3DI bioactivation is enhanced when PNP-mediated cleavage to hypoxanthine and 3'-deoxyribose-1-phosphate is favoured [125, 126]. To maximise sensitivity to such an effect, lower 3DI concentrations were used under glucose restriction; based on the dose-response relationship under normal glucose, these doses were expected to produce anti-inflammatory responses comparable to the higher concentrations used in earlier experiments. However, the combined effects of glucose restriction and the associated changes in PNP-dependent inosine metabolism dampened basal inflammatory responses and did not yield an interpretable, PNP-specific enhancement or loss of 3DI activity. Under these conditions (glucose restriction to enhance PNP-dependent inosine metabolism), the experiments were therefore inconclusive with respect to a requirement for PNP-mediated 3'-deoxyribose re-attachment, and the present data do not support a firm conclusion on this route.

ADK knockdown revealed a useful contrast between cordycepin and 3DI. In both cell types, ADK was required for full cordycepin activity, consistent with the need to phosphorylate cordycepin to 3'-deoxy-AMP en route to CoTP [2, 29, 45]. By contrast, partial ADK loss in A549 cells actually enhanced 3DI responses in some readouts, suggesting that ADK can limit 3DI bioactivation while remaining essential for cordycepin. This “ADK paradox” argues that not all 3DI effects are due solely to cordycepin contamination in the 3DI stock, and supports the idea that 3DI and cordycepin experience distinct, pathway-specific bottlenecks even when they converge on shared adenine/3'-deoxyribose salvage nodes [1, 25, 28].

LC-MS characterisation at the end of the project established high purity for both the Carbosynth and synthetic cordycepin stocks but revealed measurable cordycepin contamination in the Santa Cruz 3DI preparation. This contamination confounds absolute estimates of 3DI potency and dose-response, and means that the present data cannot cleanly separate “pure 3DI” effects from those of co-delivered cordycepin. However, the

patterns of pathway dependence remain informative, particularly where siRNA effects are similar for cordycepin and 3DI (e.g. APRT and FAMIN/LACC1 in RAW264.7) or diverge between them (e.g. ADK). These contrasts, together with external evidence for cordycepin's triphosphate-dependent signalling repression and anti-inflammatory activity in vivo [25, 26, 28], support a central conclusion: intracellular CoTP levels—and thus cordycepin's pharmacology—are dependent on 3'-deoxyribose-salvage capacity.

Conclusions

1. **NT5C2/ADSS2 phosphorylation-amination route.** Under the knockdown conditions used here, NT5C2 and ADSS2 did not measurably affect cordycepin or 3DI responses in A549 cells, and NT5C2 is a nucleotidase rather than a kinase [108, 124]. While residual protein may persist, these data make it unlikely that direct phosphorylation-amination of 3DI is a major contributor to bioactivation in this system.
2. **PNP-mediated 3'-deoxyribose salvage.** Experiments performed under glucose restriction did not yield interpretable PNP-specific effects on 3DI activity, and physiological PNP activity is generally biased toward nucleoside breakdown to base + sugar-1-phosphate [125, 126]. The present data therefore do not allow a firm conclusion on PNP-mediated 3'-deoxyribose re-attachment.
3. **APRT ± FAMIN/LACC1 3'-deoxyribose salvage.** APRT and FAMIN/LACC1 knockdown markedly reduced the anti-inflammatory activity of both cordycepin and 3DI in RAW264.7 macrophages, with more modest but consistent effects in A549 cells, and adenine co-supplementation enhanced 3DI activity in A549. Combined with external evidence that adenine/inosine are privileged precursors for nucleotide repletion [103, 123], these findings support a novel model in which adenine-linked 3'-deoxyribose salvage is required for full cordycepin activity and for efficient rescue of 3DI to cordycepin nucleotides.

Future work

- Confirm the extent of protein depletion for each siRNA target (NT5C2, ADSS2,

APRT, FAMIN/LACC1, ADK) by immunoblotting or activity assays, to ensure that negative or partial phenotypes are not due to residual enzyme.

- Re-run key functional datasets with verified pure 3DI and internal LC–MS standards to remove the contamination confound and quantify 3DI→cordycepin conversion.
- Perform tracer metabolomics with uniformly-labeled [U-¹³C]adenine or suitably labelled 3'-deoxyribose/3DI to quantify adenine-coupled rescue into 3'-dAMP/3'-dATP/CoTP pools.
- Conduct orthogonal validation of the FAMIN/APRT axis (e.g. rescue by cDNA re-expression, enzyme-activity assays) to test whether restoring these nodes can normalise cordycepin/3DI responses.
- Implement time-resolved purine-pool profiling under defined knockdown conditions (APRT, FAMIN/LACC1, ADK), measuring AMP/IMP/ATP and 3'-deoxy-nucleotides including CoTP, to directly connect genetic nodes to 3'-deoxyribose–salvage efficiency.

Overall, these data suggest that the efficacy of cordycepin in both epithelial and macrophage models is constrained not only by transport and phosphorylation, but also by the capacity of adenine-linked 3'-deoxyribose salvage pathways to sustain intracellular CoTP levels.

Chapter 4

A Haploid Genetic Screen Identifies Three Classes of Resistance Mechanisms to Cordycepin

4.1 Introduction

Cordycepin (3′deoxyadenosine) is a fungal nucleoside analog whose cytotoxic and signalling effects in mammalian cells depend on intracellular phosphorylation to cordycepin mono-, di- and triphosphate, a step predominantly catalysed by adenosine kinase (ADK) [6, 103]. The major phosphorylated species detected in cells is cordycepin triphosphate, placing nucleoside salvage and phosphorylation at the centre of cordycepin metabolism [6, 103]. Cordycepin triphosphate inhibits mRNA 3′-end formation by interfering with canonical cleavage and polyadenylation and reduces cap-dependent translation via repression of PI3K/Akt/mTOR signalling and 4E-BP phosphorylation, with secondary consequences for global protein synthesis and cellular energy balance [13, 19, 46, 127–129]. In different experimental systems these changes are accompanied by altered expression of endoplasmic reticulum stress and integrated stress response genes, autophagy and mitophagy regulators, and innate immune sensors [130–135].

Despite decades of study, the way in which cordycepin metabolism, poly(A)-tail control and growth-factor signalling are wired together to determine whether a given cell dies or survives remains incompletely defined. Current data indicate that cordycepin

acts as a nucleotide analogue whose toxicity depends on intracellular metabolism and on its effects on poly(A) tail formation and signalling, but a systematic map of the genes and pathways that modulate these processes in human cells is still lacking [6, 13, 46, 103]. Defining the genetic determinants that control cordycepin activation, mRNA 3'-end processing and PI3K/mTOR–AMPK signalling is important both for clarifying its mechanism of action and for guiding the development of cordycepin analogues with improved pharmacological properties.

Haploid insertional genetics to map resistance

Despite initial expectations that haploidy would be incompatible with normal mammalian cell function, haploid cell lines have proven surprisingly viable and phenotypically normal. Unlike the genomic instability typically observed in aneuploid tumour cells, haploid embryonic stem cells (haESCs) can be expanded in vitro for extended periods while maintaining stable karyotypes and genomic integrity [136] [137]. Haploid human cell lines (HAP1 / eHAP) allow recessive loss-of-function phenotypes to emerge from a single, easily detected insertion event, increasing power and interpretability for chemogenetic screens [138, 139]. The *piggyBac* transposon system provides high-efficiency integration at TTAA sites with a reproducible bias toward accessible chromatin, yielding broad genomic coverage in practice [140–142]. Two engineered transposase variants are commonly used: hyPBase, a hyperactive mutant carrying seven amino-acid substitutions that increase transposition efficiency approximately tenfold over the wild-type enzyme [142], and suPBase (suPB), a codon-optimised variant that also achieves high transposition rates but through a distinct set of sequence modifications [139]. Both recognise the same TTAA target sites and inverted terminal repeats, and both support the gene-trap configuration described below, but their differing mutational spectra mean that each may favour a partly different subset of genomic loci and thereby provide complementary coverage of the insertion landscape. In a gene-trap configuration (PBDGTV), insertion into an expressed gene introduces a splice-acceptor–poly(A) cassette that prematurely terminates transcription and disrupts gene function, as illustrated in Figure 4.1. These properties make *piggyBac*-based mutagenesis an attractive approach for identifying genes whose loss confers resistance to small-molecule inhibitors in an unbiased fashion.

We performed parallel mutagenesis with the PBDGTV *piggyBac* gene trap and either hyPBase or suPBase transposases (hyPB and suPB) in eHAP cells and applied two cordycepin doses (150 and 200 μ M). These concentrations were chosen on the basis of dose-response experiments (see Section 4.2.2 and Figure 4.4): 150 μ M produced a moderate but sustained reduction in metabolic activity under repeated dosing, while 200 μ M imposed a stronger selection bottleneck, together spanning the range in which resistant mutants could plausibly survive while sensitive wild-type cells were eliminated. Seeding density was optimised to maximise insertional complexity while maintaining the haploid state of the culture; we ultimately used 150,000 cells per T75 flask, as supported by the library performance analyses presented later in this chapter [139]. Because *piggyBac*'s open-chromatin preference can subtly weight gene discovery, we later compared platforms (hyPB vs. suPB) and dose conditions and assessed chromosome-group distributions to rule out gross chromosomal bias [140, 141].

Study aims

This haploid screen was designed to define genetic determinants of resistance to cordycepin. I asked which hypotheses on the metabolism and mechanism of action of cordycepin are suggested by a genetic screen and if the target of cordycepin can be identified.

4.2 Results

4.2.1 Loss-of-function logic of the haploid resistance screen

The interpretation of the haploid screen is based on a simple loss-of-function logic. In a resistance screen, enrichment of disruptive transposon insertions in a gene among survivors indicates that loss of that gene's function confers a selective advantage in the presence of the drug. In other words, disruption of the gene helps cells survive cordycepin treatment. This is distinct from expression-based analyses: we are not assessing whether a pathway is transcriptionally up- or down-regulated, but rather which gene knockouts improve survival.

Resistance can arise at multiple levels of the drug's action, from cordycepin uptake

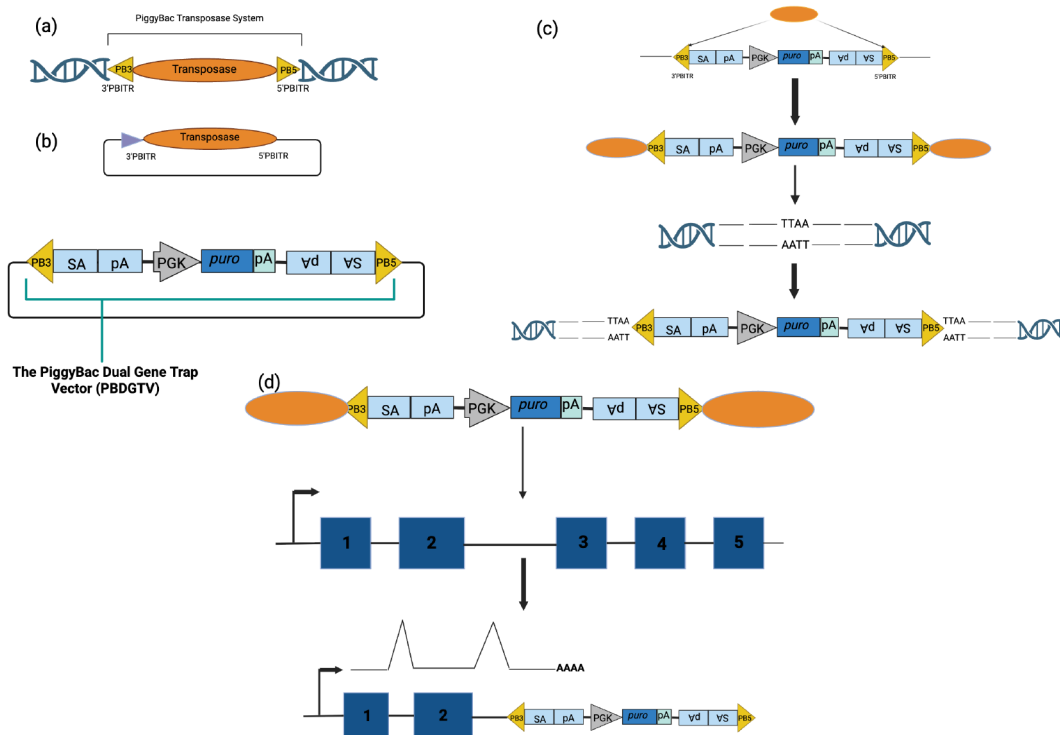


Figure 4.1: PiggyBac (PB) transposon system. (a) Original PB elements contain the transposase gene (orange) and inverted terminal repeats (ITRs, yellow arrows). (b) Binary PB system used for gene delivery in plasmids: a transposase expression helper plasmid (HyPBase or SuPBase) driven by a promoter (gray arrow) and a donor plasmid containing the gene-trapping cassette PBDGTV (light blue) and a puromycin selection marker flanked by ITRs. (c) The transposon carrying the cassette is excised from the donor plasmid and integrated into the chromosome at TTA/TAA sites. (d) Insertion of PBDGTV between exons (navy) can disable a gene by prematurely terminating transcription via the splice-acceptor-poly(A) element (SA-polyA), truncating transcript expression (exons 1-2). Figure created with BioRender.com.

and metabolism, through the direct molecular targets, to signalling pathways that mediate toxicity and pathways that buffer downstream consequences for proteostasis and metabolism. ADK serves as an internal conceptual control: as the primary kinase that phosphorylates cordycepin to active nucleotides, ADK loss-of-function is expected to reduce formation of cordycepin triphosphate and to confer resistance [6, 103]. For more complex pathways that include both positive and negative regulators, directionality is inferred gene by gene and then synthesised at the pathway level.

4.2.2 Screen design, mutagenesis and selection strategy

To systematically identify genetic determinants of cordycepin resistance, we performed a genome-wide haploid gene-trap screen in the eHAP cell line. This haploid human cell line provides a powerful platform for recessive loss-of-function screens, because a single, easily detected insertion can unmask a phenotype that would otherwise be buffered in a diploid background [138, 139]. We used the *piggyBac*-based gene-trap system (PBDGTV) described above (Figure 4.1), in which the transposase recognises inverted terminal repeats flanking the cassette and inserts it at genomic TTAA sites; the splice-acceptor–poly(A) module within PBDGTV can prematurely terminate transcription and disrupt gene expression when integrated into the genes [140–142].

The overall experimental workflow is summarised in Figure 4.2. eHAP cells were co-transfected with the PBDGTV vector and either hyPBase or suPBase transposase (hyPB and suPB), then selected with puromycin to enrich for cells carrying transposon insertions. The resulting mutant libraries were exposed to cordycepin at 150 or 200 μ M using a repeated dosing paradigm, and surviving cells were harvested. Genomic DNA from these survivors was extracted for mapping of transposon–genome junctions by Splinkerette PCR and next-generation sequencing (see below). By comparing insertion profiles between the cordycepin-resistant populations and unselected controls, I identified genes whose disruption confers resistance to cordycepin.

Optimisation of selection parameters

A critical step in the screen design was optimisation of the selection parameters to ensure efficient enrichment of cordycepin-resistant mutants. This involved determining

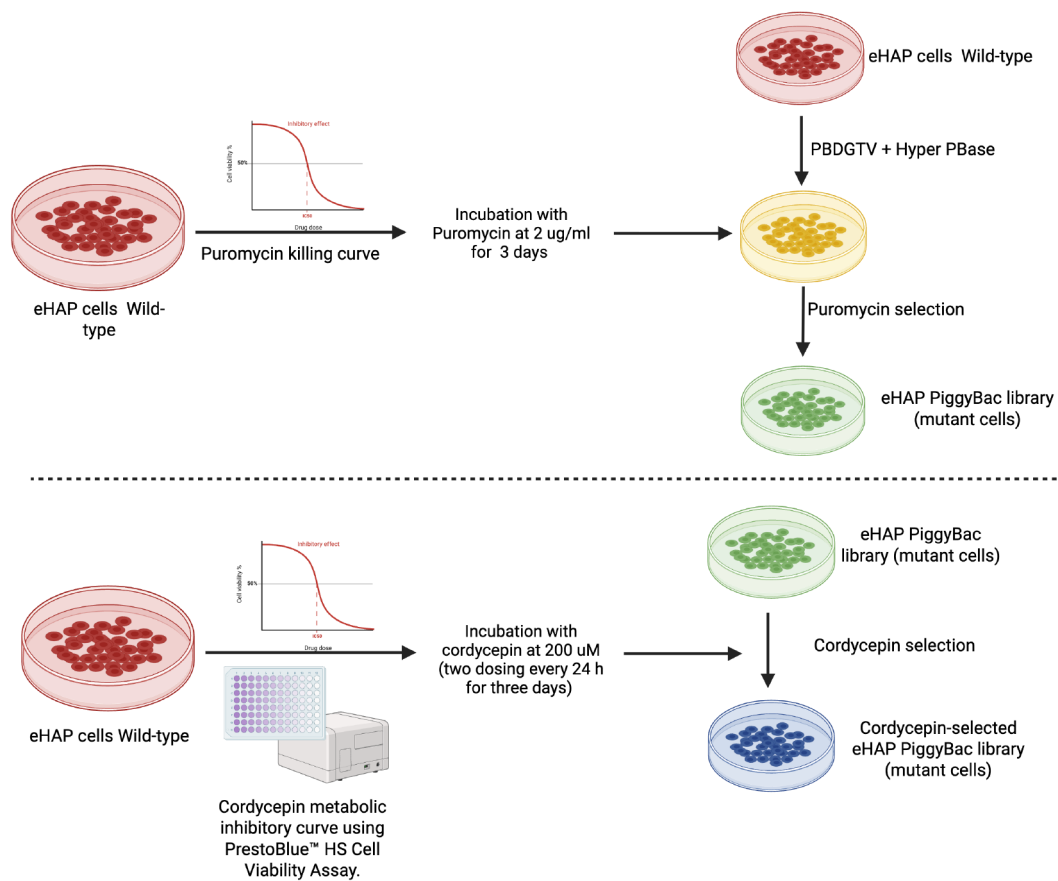


Figure 4.2: Overview of haploid genetic screening. eHAP cells were transfected with the PiggyBac gene-trapping vector PBDGTV along with transposase expression plasmids (HyPBase or SuPBase), resulting in genomic integration and gene disruption across the genome. Cells were then selected with puromycin at 2 $\mu\text{g}/\text{mL}$ for 3 days to enrich for cells with transposon insertions. The surviving mutant cell libraries were treated with cordycepin at 150 μM or 200 μM for 72 h (twice every 24 h) to select mutants resistant to metabolic inhibition by cordycepin. Dr. Alexander Kondrashov, Dr. Emilie Daniel and Davor Kresnik conducted the transfected library preparations and puromycin selection; I performed the cordycepin selection using the PrestoBlue™ HS Cell Viability Assay to monitor metabolic activity as the primary readout. Surviving resistant clones were then harvested for genomic DNA extraction, Splinkerette PCR amplification of transposon–genome junctions, and next-generation sequencing to identify the disrupted genes (see Figure 4.7). Figure created with BioRender.com.

the puromycin concentration for selecting transfected cells, establishing the cordycepin dose and dosing schedule, and evaluating 3'deoxyinosine (3DI) as a potential alternative selection agent. Puromycin selection, library preparation and Splinkerette PCR for amplification of insertion sites were carried out by Dr. Alexander Kondrashov, Dr. Emilie Daniel and Davor Kresnik; I performed the cordycepin and 3DI selection experiments and conducted the computational analyses.

Puromycin selection and library enrichment. To determine the puromycin concentration that efficiently eliminated untransfected eHAP cells while preserving PBDGTV-transfected cells, we performed a puromycin kill-curve on wild-type eHAP. A concentration of 2 $\mu\text{g}/\text{mL}$ puromycin resulted in near-complete death of wild-type cells while allowing survival of the transfected population (Figure 4.3). After transfection, cells were allowed to recover for 24 h and were then selected in 2 $\mu\text{g}/\text{mL}$ puromycin for 3 days. This regimen provided a mutant library that was highly enriched for PBDGTV insertions and of sufficient complexity for the subsequent cordycepin screen.

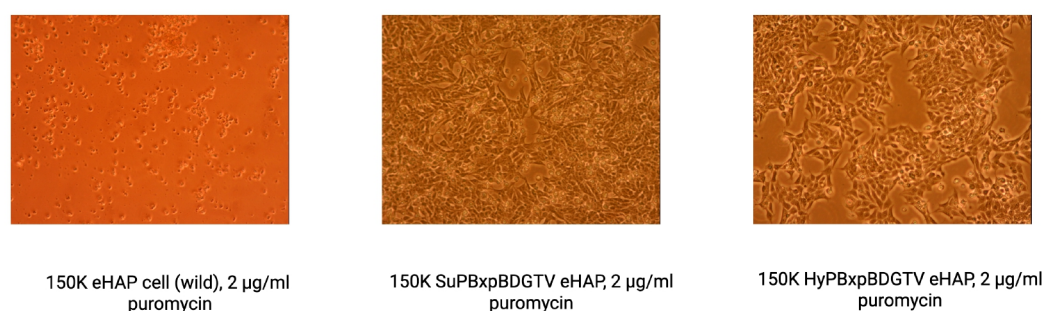


Figure 4.3: Effect of different puromycin concentrations on eHAP cell viability, wild type vs. transfected. Wild-type eHAP and PBDGTV-transfected eHAP cells were treated with the indicated puromycin concentrations for 3 days. The 2 $\mu\text{g}/\text{mL}$ condition eliminated wild-type cells while sparing most transfected cells, and was therefore used for library enrichment.

Cordycepin dosing paradigm. To define the cordycepin regimen for the screen, I performed dose-response experiments using PrestoBlue to monitor metabolic activity. eHAP cells were treated with 25–400 μM cordycepin for 24–72 h, comparing a single dose with repeated dosing every 24 h. Repeated dosing produced a stronger and more sustained reduction in metabolic activity than a single application, consistent with the known degradation of cordycepin over time [91], and these effects were mirrored

in a crystal-violet clonogenic assay (Figure 4.4 and Figure 4.5). On the basis of these optimisation experiments, we selected 150 μM and 200 μM cordycepin, administered twice daily for 3 days, as the selection conditions for the haploid screen.

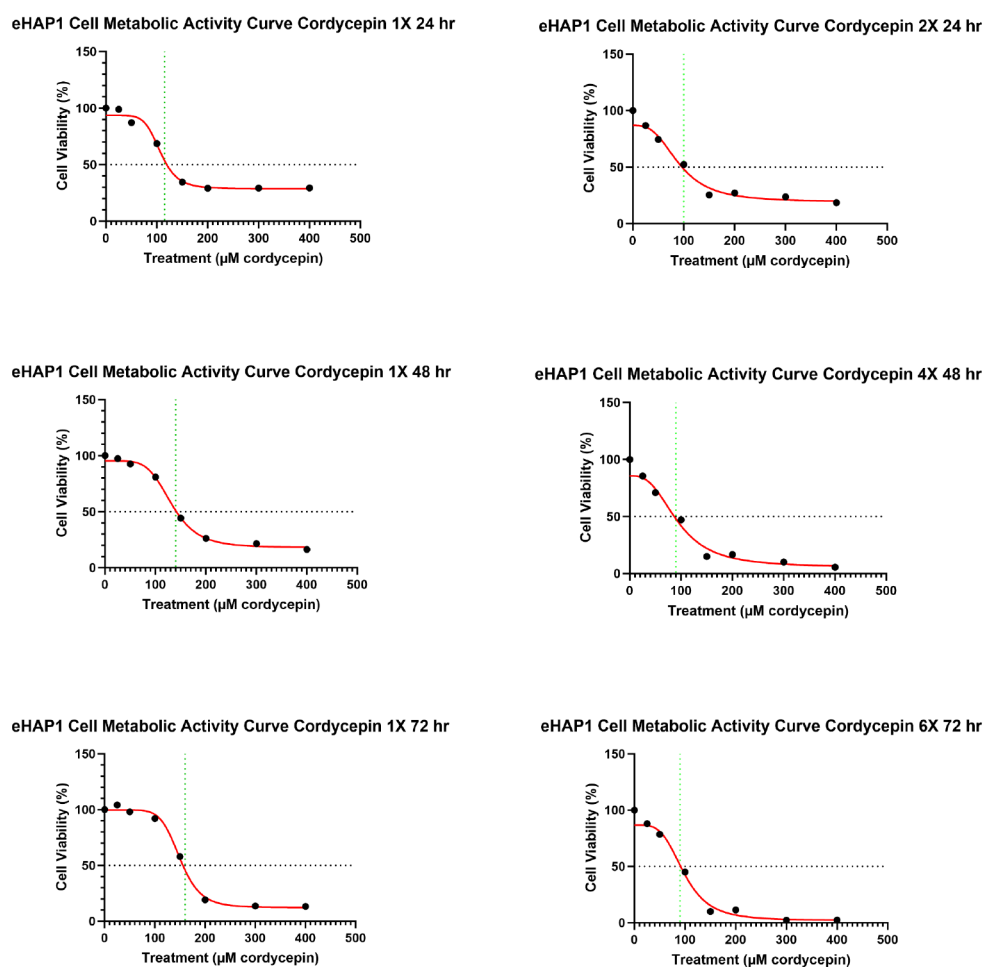


Figure 4.4: Metabolic inhibition of eHAP cells by single versus repeated cordycepin treatments. eHAP cells were treated with 25–400 μM cordycepin either as a single dose or with repeated dosing every 24 h for 3 days. Cell viability was measured using the PrestoBlue assay. Repeated doses produced stronger inhibition than a single dose, probably because cordycepin degrades over time with a single application. X indicates the total number of cordycepin doses applied across the specified time.

3'-Deoxyinosine dose-response as an alternative selection agent. We also evaluated the nucleoside analogue 3'deoxyinosine (3DI), a related metabolite of cordycepin, as a potential selection agent. eHAP cells were treated with 25–400 μM 3DI using the repeated dosing paradigm, and metabolic activity was measured with PrestoBlue. Even at the highest concentrations tested, 3DI produced only about 20 %

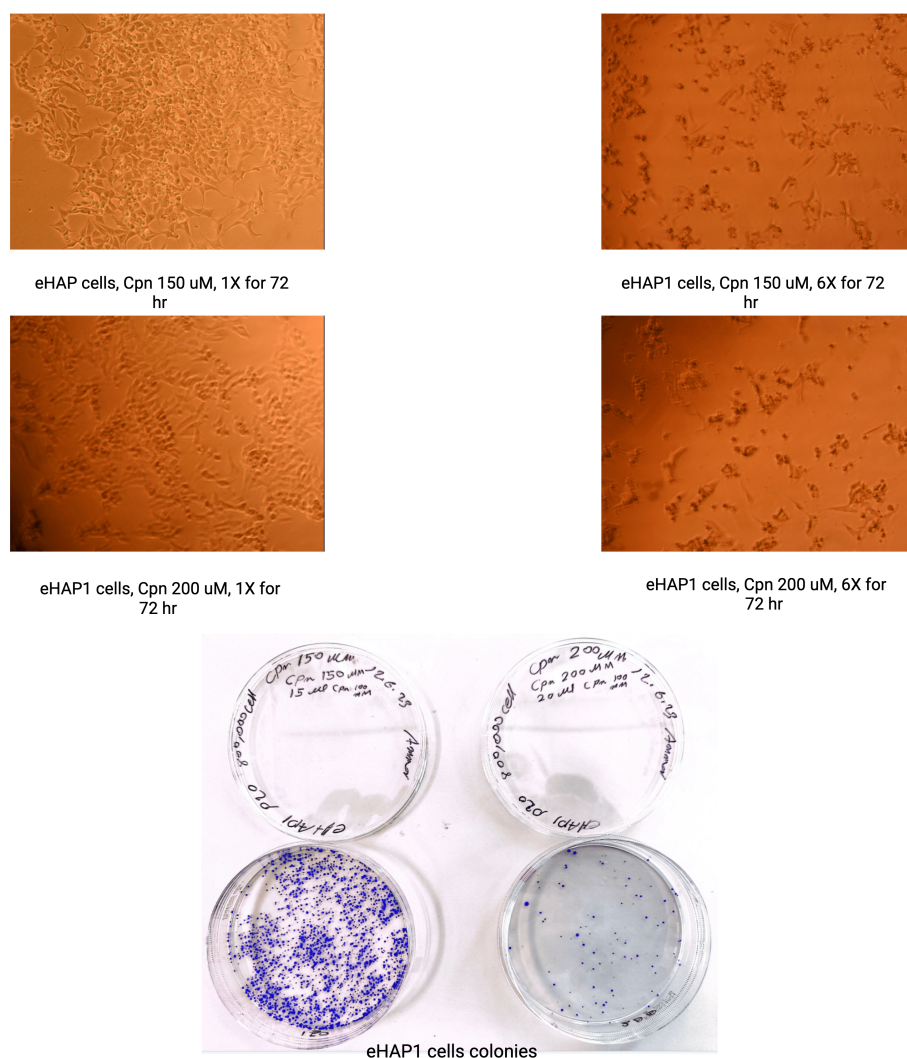


Figure 4.5: Crystal-violet clonogenic assay showing that multiple cordycepin doses reduce eHAP colony formation more than a single dose. Representative images show eHAP cells treated with a single dose of 150 or 200 μ M cordycepin for 72 h (left) or with multiple doses of 150 or 200 μ M cordycepin twice daily over 3 days (right). After treatment, cells were fixed and stained with crystal violet in 10-cm Petri dishes (bottom). Repeated dosing markedly reduced clonogenic growth compared with a single dose, supporting the choice of the repeated-dosing paradigm for the genetic screen.

maximal inhibition of metabolic activity (Figure 4.6). This limited potency indicated that 3DI could not impose a sufficiently strong selection pressure to enrich resistant mutants, so we did not pursue 3DI further and used cordycepin as the sole selection agent for the screen.

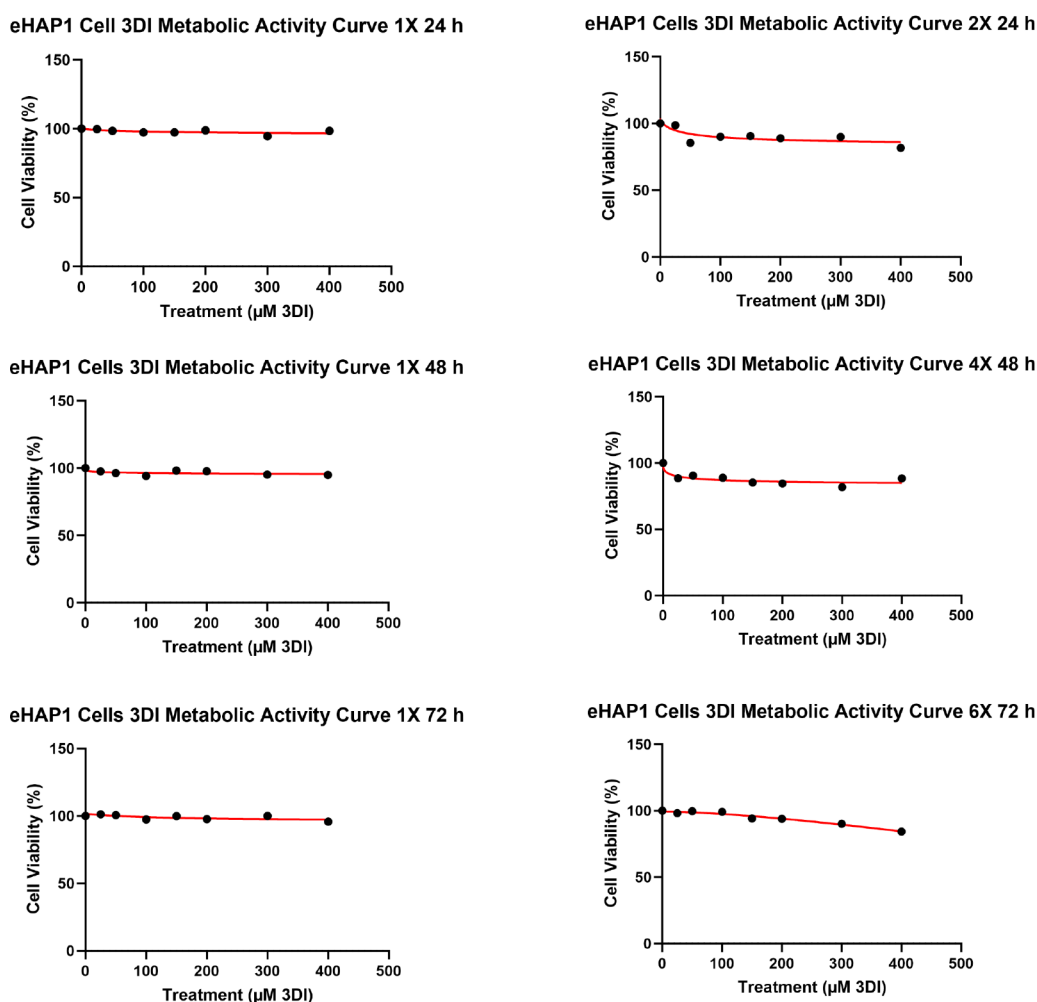


Figure 4.6: Metabolic inhibition of HAP1 cells by 3'-deoxyinosine. HAP1 cells were treated with 25–400 μM 3'-deoxyinosine using repeated dosing every 24 h for 3 days. Cell viability was measured using the PrestoBlue assay. 3'-Deoxyinosine achieved a maximal inhibition of approximately 20 % across the tested concentration range, indicating that it is a weak inhibitor under these conditions.

4.2.3 Mapping of transposon insertion sites

Following selection of cordycepin-resistant cells, the next step was to map the genomic locations of the *piggyBac* insertions. We used Splinkerette PCR, a ligation-mediated PCR method that allows specific amplification of genomic DNA sequences flanking

integrated transposons [3, 7]. Genomic DNA from resistant cell populations was fragmented, and a Splinkerette adapter was ligated to the fragment ends.

Separate PCR reactions were then performed for each end of the transposon (PB5 and PB3), using transposon-specific primers in combination with Splinkerette-specific primers. The design of the Splinkerette adapter and primers prevents amplification from Splinkerette to Splinkerette, thereby enriching specifically for fragments that contain a transposon–genome junction [3, 7]. The resulting PCR products, which carry the *piggyBac* inverted terminal repeat (ITR) and adjacent genomic sequence, were subjected to next-generation sequencing. Sequencing reads were mapped to the human genome to identify precise insertion sites. This mapping workflow, summarised in Figure 4.7, yielded a genome-wide map of transposon insertions in the cordycepin-resistant populations.

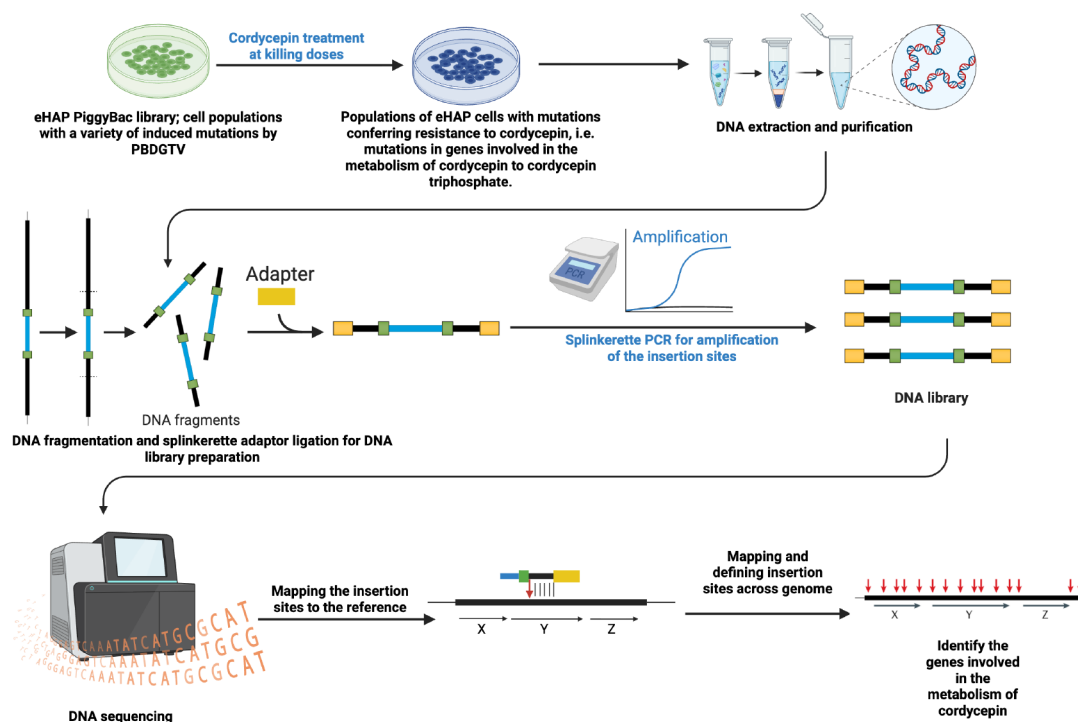


Figure 4.7: Overview of mapping *piggyBac* insertion sites in cordycepin-resistant cells. The surviving mutant cell library after puromycin selection, which contained transposon insertions, was treated with cordycepin to select mutants resistant to metabolic inhibition by cordycepin. Genomic DNA was extracted from cordycepin-resistant cells, fragmented and ligated to Splinkerette adapters [3, 7]. Two rounds of PCR (PB5 and PB3) were performed with transposon- and Splinkerette-specific primers to amplify regions flanking transposon insertion sites. These samples were sequenced to identify insertion sites and the disrupted genes. Dr. Alexander Kondrashov, Dr. Emilie Daniel and Davor Kresnik carried out the transfected library preparations and puromycin selection. Figure created with BioRender.com.

4.2.4 Computational analysis

I analysed the sequencing data from Splinkerette PCR libraries using a custom pipeline (GitHub: https://github.com/Ammar-Khamis/Haploid_screening). Briefly, reads were adapter-trimmed, mapped to the human genome, and used to call transposon insertion sites, which were then annotated to nearby genes. The number of independent insertion events per gene was counted and compared between cordycepin-treated and control samples to identify genes enriched for insertions in the cordycepin-resistant populations.

4.3 Library Performance and Coverage

The success of a genetic screen depends on the complexity and evenness of the insertion library. To assess whether the piggyBac-mutagenised eHAP libraries were suitable for mapping recessive resistance phenotypes, I compared the total number of unique insertions across seeding densities and examined their distribution across chromosome groups.

4.3.1 Insertion counts at different seeding densities

Mutant libraries were generated at two seeding densities, 150,000 (150K) and 300,000 (300K) cells per T75 flask, for both hyPB and suPB transposases under DMSO control and 150 μ M / 200 μ M cordycepin selection, as well as library-only controls. I then counted the number of unique mapped insertion sites in each library (Figures 4.8 and 4.9).

At 150K, insertion counts ranged from 9,341 (hyPB_Library_1) to 51,053 (hyPB_Cordycepin_200 μ M), with cordycepin-treated libraries generally showing higher insertion numbers than their DMSO counterparts. At 300K, counts ranged from 11,390 (hyPB_Library_1) to 39,218 (hyPB_Cordycepin_150 μ M). Thus, for the strongest selection condition (hyPB, 200 μ M cordycepin), the 150K seeding density produced substantially more unique insertions than 300K.

Because 150K consistently delivered equal or higher insertion totals in the cordycepin-treated conditions while using fewer starting cells, I used 150K libraries for all downstream analyses (chromosome-group coverage, gene-level enrichment and pathway analysis). A lower seeding density helps maintain a high haploid fraction and reduces diploidisation-related confounds in HAP1/eHAP cells [139, 143], while robust insertion counts satisfy the coverage requirements for piggyBac-based insertional mutagenesis screens [140, 142, 144, 145].

4.3.2 Chromosome-group distribution

To test whether piggyBac insertions were broadly distributed across the genome and to exclude large-scale chromosomal biases, I grouped the autosomes and sex chromosomes

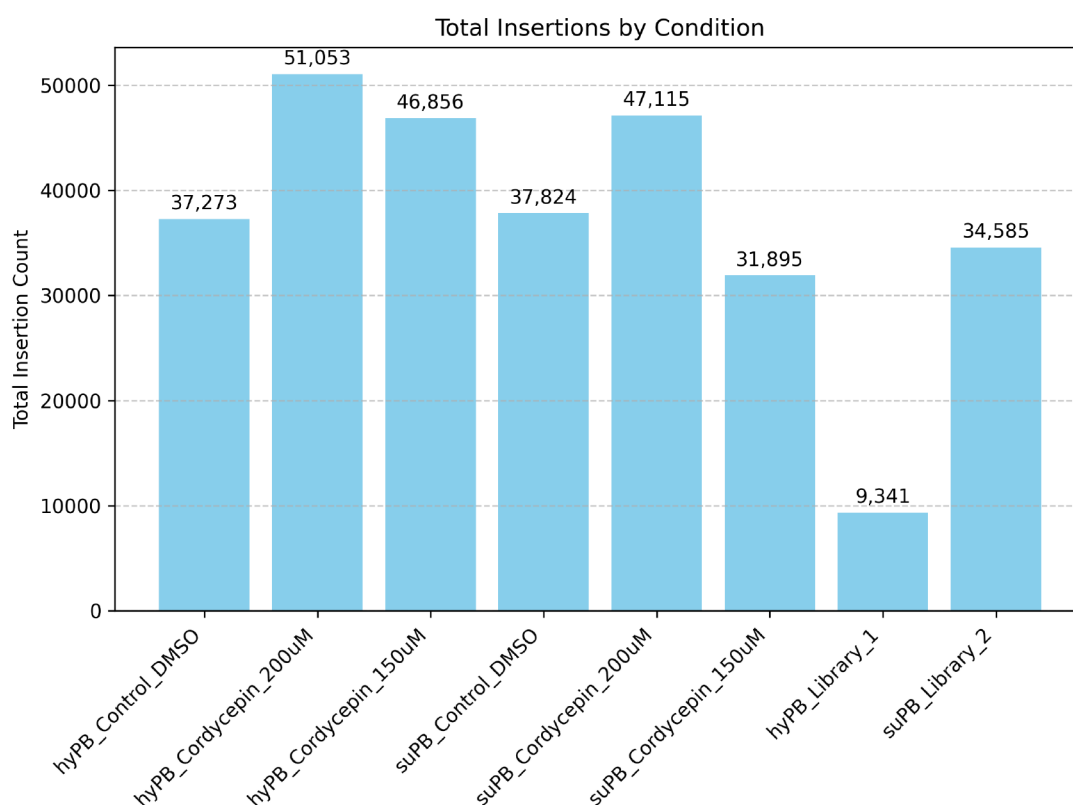


Figure 4.8: Total unique insertions by condition at 150K seeding density (T75, eHAP). Bar chart of mapped insertion sites for hyPB and suPB libraries under DMSO or cordycepin (150 μ M, 200 μ M), plus library-only controls. Each bar represents one library; bar height and the number above each bar indicate the total number of unique insertion sites in that condition (range 9,341–51,053).

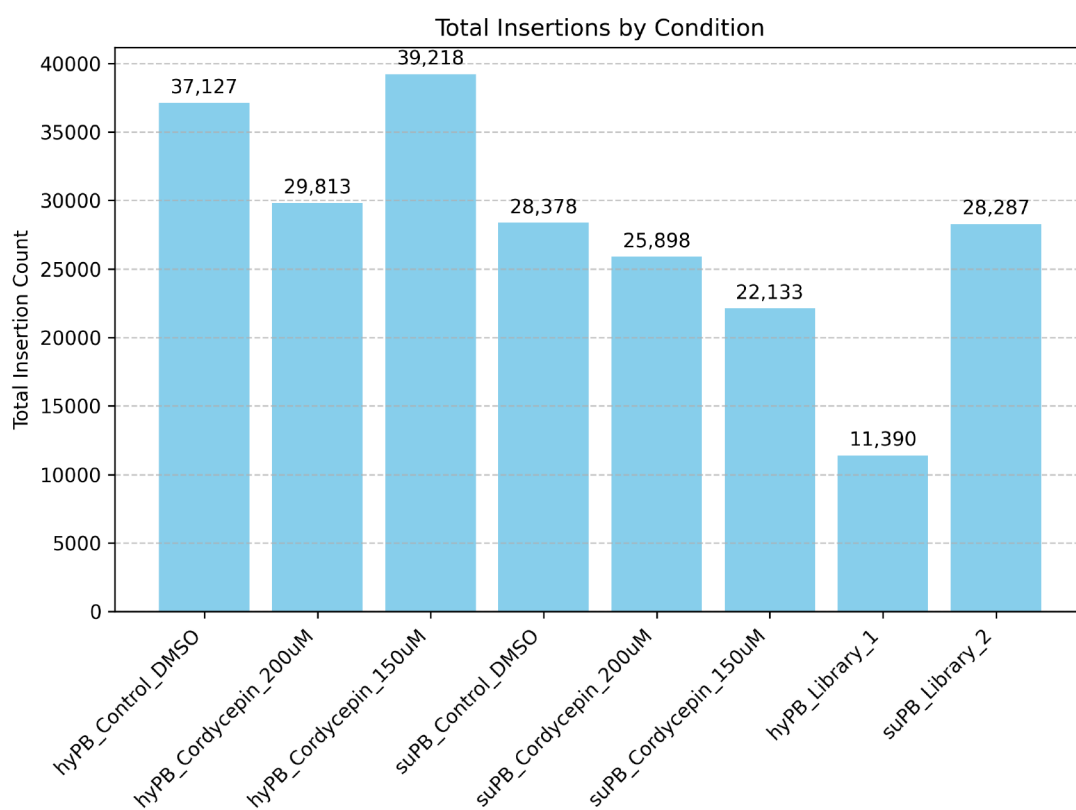


Figure 4.9: Total unique insertions by condition at 300K seeding density (T75, eHAP). As in Figure 4.8, but for libraries generated at 300K. Each bar represents one library; bar height and the number above each bar indicate the total number of unique insertion sites (range 11,390–39,218).

into four categories (Large: 1–5; Medium: 6–12; Small: 13–22; Sex: X, Y) and calculated, for each condition at 150K, the percentage of total insertions falling into each group. Figure 4.10 shows these percentages for hyPB and suPB libraries under DMSO and cordycepin selection.

Across all conditions, the profiles overlapped closely and shared the ordering Large > Medium > Small > Sex. Cordycepin treatment did not introduce new biases relative to DMSO controls. This pattern is consistent with the known integration behaviour of piggyBac, which targets TTAA sites and shows a preference for open, gene-dense chromatin rather than specific chromosomes [140, 141, 146]. The absence of chromosome-scale distortion supports the use of these libraries for genome-wide identification of resistance loci.

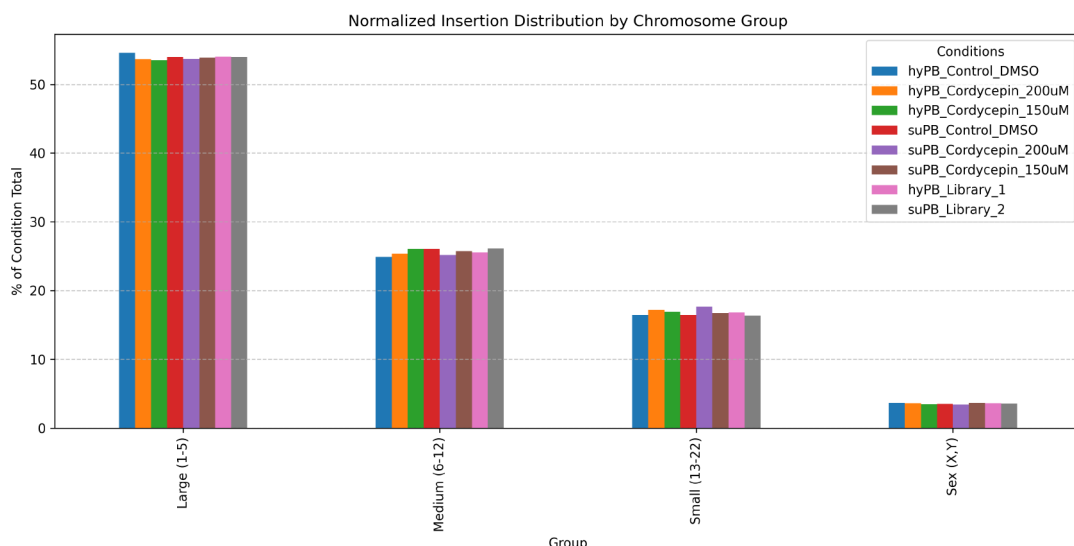


Figure 4.10: Normalized insertion distribution by chromosome group at 150K. For each condition, the percentage of total insertions falling into Large (1–5), Medium (6–12), Small (13–22) and Sex (X, Y) chromosome groups is shown. Colours encode conditions, and the profiles across conditions overlap closely with the ordering Large > Medium > Small > Sex, indicating no gross chromosome-scale bias specific to hyPB or suPB at 150K.

4.4 Gene-Level Enrichment under Cordycepin

With high-quality libraries established, I next asked which gene knockouts conferred resistance to cordycepin. For each platform (hyPB, suPB) and dose (150 μ M, 200 μ M), I compared the frequency of insertions per gene between cordycepin-treated and DMSO control populations and expressed this as the percentage of total insertions in that

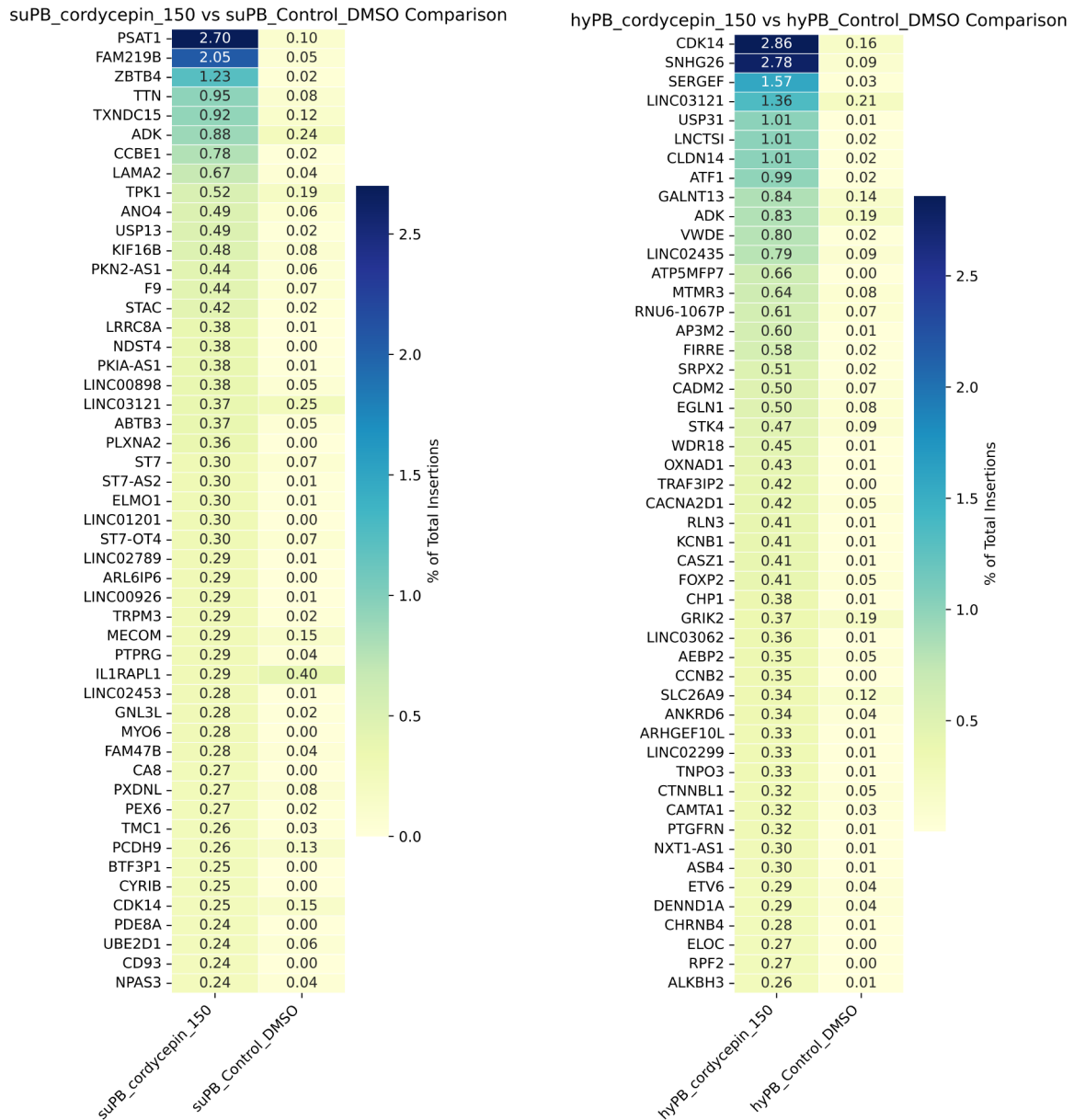
condition. The most strongly enriched genes are visualised as heatmaps (Figures 4.11 and 4.12).

4.4.1 150 μ M cordycepin

At 150 μ M cordycepin (Figure 4.11), both suPB and hyPB screens showed clear enrichment of ADK insertions in the cordycepin-treated libraries relative to DMSO controls. In the suPB panel, ADK accounted for 0.88 % of total insertions under cordycepin versus 0.24 % in the DMSO control; in the hyPB panel, ADK represented 0.83 % versus 0.19 %. These changes indicate that loss of ADK function provides a strong survival advantage under cordycepin at this dose, consistent with its role as the primary kinase that phosphorylates cordycepin to active nucleotides [6, 103].

Beyond ADK, hyPB at 150 μ M highlighted genes such as *EGLN1* and *MTMR3*, with increased insertion percentages in cordycepin-treated cells relative to DMSO. *EGLN1* encodes a prolyl hydroxylase that regulates HIF1 α stability and links to hypoxia and energy signalling, while *MTMR3* encodes a phosphatase implicated in PI(3)P turnover and autophagy regulation [127–129]. In the suPB panel, *PSAT1* (phosphoserine aminotransferase 1), a component of serine/one-carbon metabolism, showed marked enrichment (2.70 % vs 0.10 %). Several additional entries are long non-coding RNAs or poorly characterised loci; given piggyBac’s TTAA/open-chromatin preferences [140, 141, 146], these are interpreted cautiously unless they recur across conditions.

Overall, at 150 μ M cordycepin, the strongest and most consistent signal across both platforms is ADK, with additional platform-specific hits pointing toward hypoxia/energy signalling (*EGLN1*), autophagy-related lipid turnover (*MTMR3*), and amino-acid metabolism (*PSAT1*).



(a) suPB, 150 μ M cordycepin vs. DMSO control. (b) hyPB, 150 μ M cordycepin vs. DMSO control.

Figure 4.11: Gene-level insertion enrichment under 150 μ M cordycepin. Each heatmap panel shows one transposase platform: suPB (a, left) or hyPB (b, right). Rows correspond to individual genes (labelled by HGNC symbols), columns to treatment or control, and each cell is annotated with the percentage of total insertions in that condition mapped to that gene. The colour scale encodes this percentage (lighter to darker indicating lower to higher values). Column labels use the abbreviations suPB and hyPB for the two transposases; “cordycepin_150” denotes repeated dosing with 150 μ M cordycepin and “Control_DMSO” denotes the vehicle-treated library. Abbreviations: suPB, SuPBase transposase; hyPB, hyperactive piggyBac transposase; DMSO, dimethyl sulfoxide vehicle control.

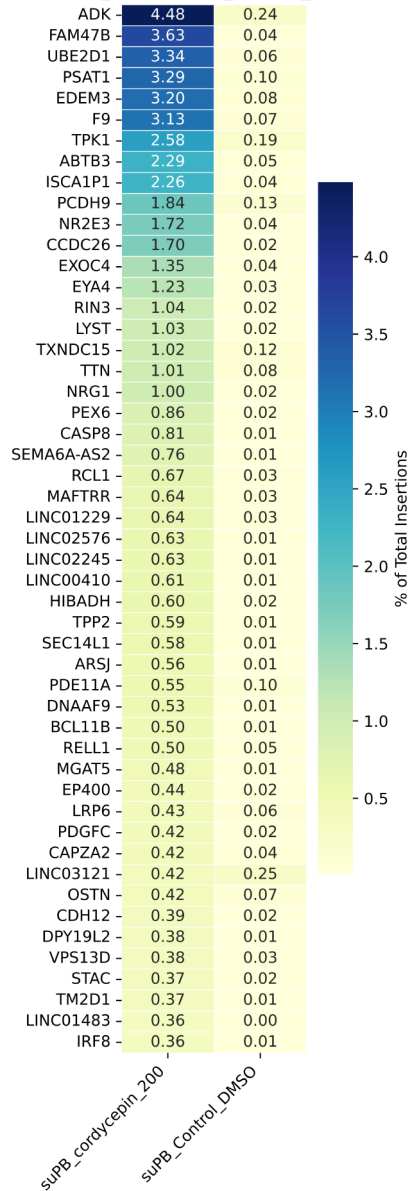
4.4.2 200 μ M cordycepin

At 200 μ M cordycepin (Figure 4.12), ADK enrichment became even more pronounced, especially in the suPB screen. In suPB, ADK accounted for 4.48 % of total insertions under cordycepin compared with 0.24 % in the DMSO control; in hyPB, ADK rose to 1.28 % vs 0.19 %. The dose-dependent increase in ADK enrichment across both platforms reinforces the conclusion that ADK-dependent phosphorylation of cordycepin is required for its cytotoxicity [6, 103].

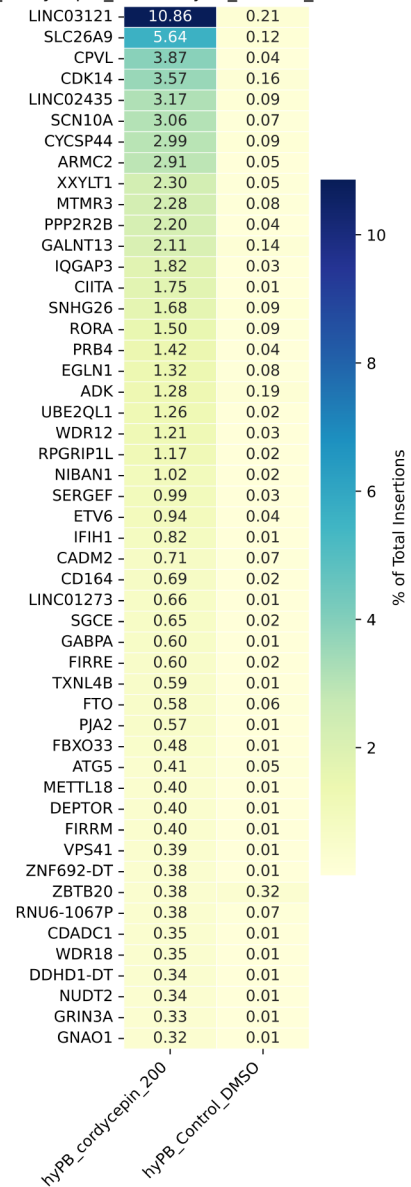
The hyPB panel at 200 μ M also contained several genes linked to AMPK–mTOR signalling and autophagy, including *PPP2R2B* (PP2A B55 regulatory subunit), *ATG5* and *VPS41*, as well as *EGLN1* [13, 127–129, 147, 148]. In the suPB panel, enriched genes included *UBE2D1* (ubiquitin-conjugating enzyme E2), *EDEM3* (ER-associated degradation), *PSAT1*, *TPK1* (thiamine pyrophosphokinase 1) and *CASP8*. Enrichment of *CASP8*, a key initiator caspase, is consistent with cordycepin inducing *CASP8*-dependent apoptosis in eHAP cells, because loss of *CASP8* function increases survival under treatment [130, 133, 134].

Notably, the lncRNA locus *LINC03121* was highly represented in hyPB at 200 μ M (10.86 % vs 0.21 % in DMSO). Given piggyBac’s strong bias toward TTAA sites in accessible chromatin and the architecture of some lncRNA loci, this spike is treated with caution; its potential biological significance is considered in the Discussion rather than inferred directly from the screen.

suPB_cordycepin_200 vs suPB_Control_DMSO Comparison



hyPB_cordycepin_200 vs hyPB_Control_DMSO Comparison



(a) suPB, 200 μ M cordycepin vs. DMSO control. (b) hyPB, 200 μ M cordycepin vs. DMSO control.

Figure 4.12: Gene-level insertion enrichment under 200 μ M cordycepin. Layout and colour coding as in Figure 4.11: each heatmap panel shows one transposase platform, suPB (a) or hyPB (b), with rows for genes and columns for treatment conditions. Each cell is annotated with the percentage of total insertions in that condition mapped to that gene; “cordycepin_200” denotes repeated dosing with 200 μ M cordycepin. Abbreviations as in Figure 4.11.

4.5 Cross-Condition Recurrence

To identify resistance determinants that were robust across dose and platform, I compared normalized insertion percentages for a common panel of genes across all eight libraries (suPB and hyPB; DMSO, 150 μ M and 200 μ M cordycepin; library-only controls). Figure 4.13 summarises these data as a bar chart, and Figure 4.14 shows the same values as a heatmap.

Across conditions, ADK showed the most consistent and largest increases in insertion percentage under cordycepin relative to DMSO. For example, ADK rose from 0.24 % in the suPB DMSO control to 0.88 % at 150 μ M and 4.48 % at 200 μ M; in hyPB, it increased from 0.19 % in DMSO to 0.83 % (150 μ M) and 1.28 % (200 μ M). These cross-condition patterns firmly establish ADK loss-of-function as a major resistance mechanism and support the conclusion that ADK-dependent phosphorylation of cordycepin to its nucleotide forms is required for toxicity [6, 103].

Other genes also showed recurrent enrichment, though with lower effect sizes than ADK. In hyPB, *PPP2R2B*, *ATG5*, *VPS41* and *EGLN1* were preferentially enriched at 200 μ M cordycepin, consistent with roles for PP2A regulatory subunits, autophagy machinery and hypoxia signalling in determining survival under treatment [13, 127–129, 147, 148]. In suPB, *UBE2D1*, *EDEM3*, *PSAT1*, *TPK1* and *CASP8* were recurrently enriched under cordycepin, pointing to contributions from ubiquitin–proteasome function, ER-associated degradation, amino-acid and cofactor metabolism, and apoptosis [130, 133, 134]. The screen itself does not specify whether these genes act “downstream” of ADK or in parallel; their smaller effect sizes may reflect partial redundancy or fewer potential TTAA intergration sites rather than a lesser mechanistic importance.

Because piggyBac preferentially inserts at TTAA sites in open chromatin, local TTAA density and chromatin accessibility can inflate insertion counts at specific loci, especially long non-coding RNAs such as *LINC03121* [140, 141, 146]. For this reason, interpretation focuses on genes that are enriched across multiple independent conditions (e.g., ADK) rather than on single-condition spikes, which may be more susceptible to technical artefacts.

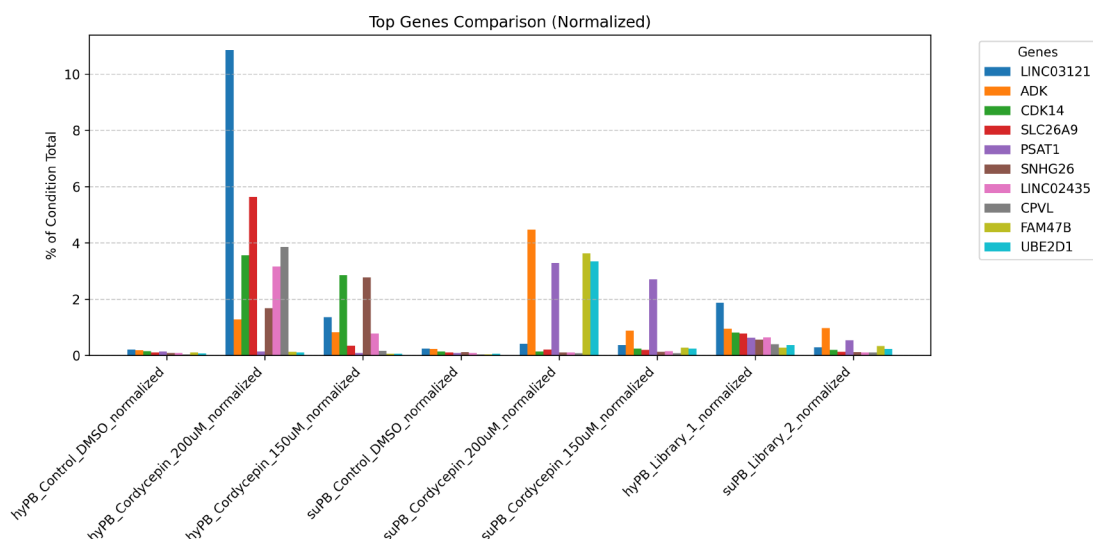


Figure 4.13: Top genes across conditions (normalised percentage of total insertions). Bar chart comparing the same set of enriched genes across all eight libraries (hyPB and suPB; DMSO, 150 μ M and 200 μ M cordycepin; library-only controls). Each coloured bar represents the percentage of total unique insertions in one condition mapped to the indicated gene; this normalisation allows direct comparison between libraries of different absolute size. The plot highlights the strong and dose-dependent enrichment of ADK, the pronounced spike of *LINC03121* in hyPB_cordycepin_200, and recurrent enrichment of genes such as *PSAT1* and *UBE2D1* in suPB cordycepin conditions.

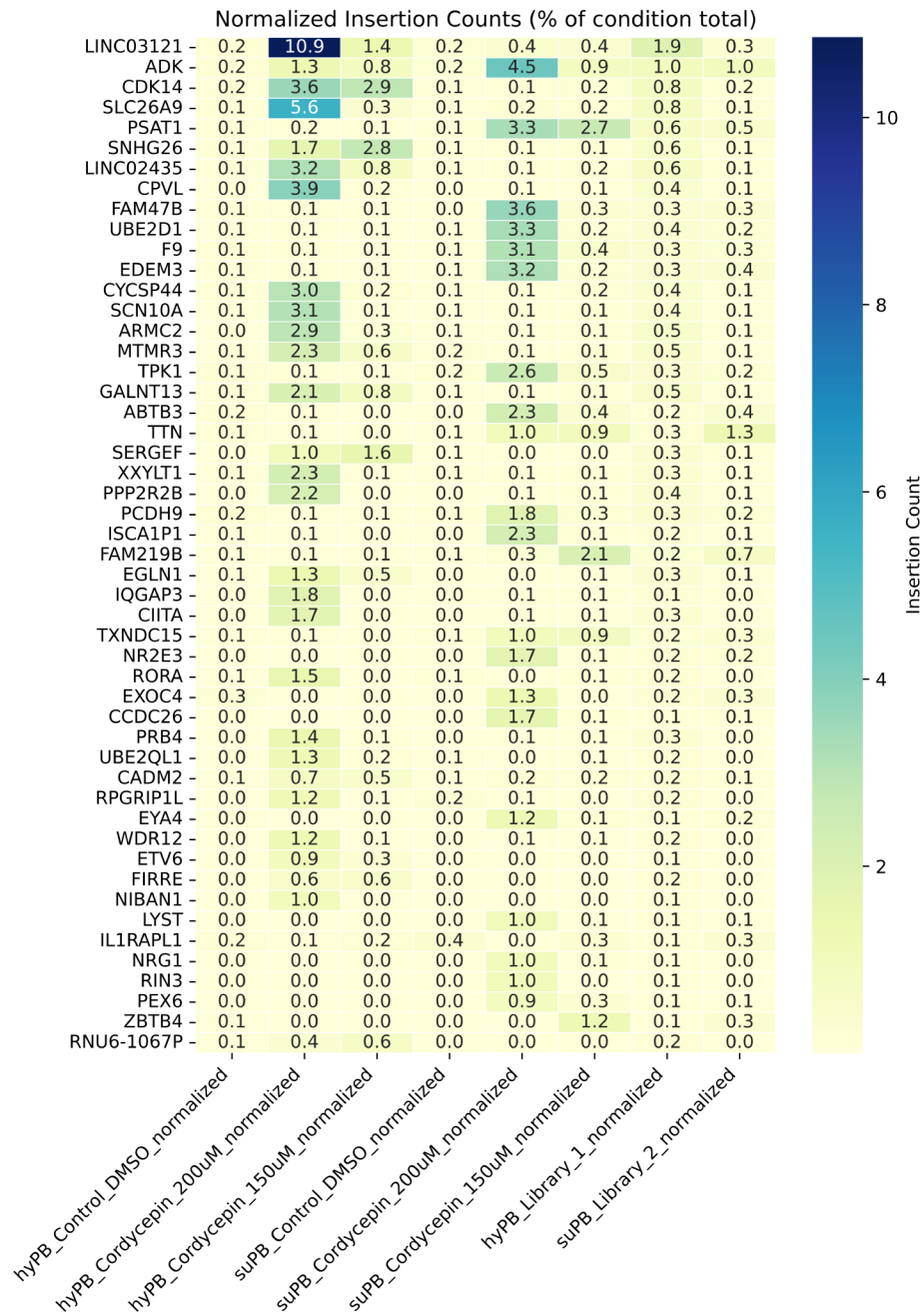


Figure 4.14: Heatmap of normalised insertion percentages for recurrent genes across conditions. Each row corresponds to a gene and each column to one library (hyPB or suPB; DMSO, 150 μ M or 200 μ M cordycepin; library-only controls). Cells are coloured by, and annotated with, the percentage of total insertions in that condition mapped to the gene. Normalisation was performed by expressing the number of unique insertion sites in each gene as a percentage of the total unique insertions in that library, allowing direct comparison across libraries that differ in absolute insertion count. The “Library” columns represent the unselected mutant libraries (i.e. no cordycepin or DMSO treatment), which serve as a baseline for insertion frequency before any drug selection. The heatmap makes cross-condition patterns explicit, with ADK showing the most consistent enrichment under cordycepin and additional clusters of genes enriched preferentially in hyPB or suPB under higher-dose selection.

4.6 Pathway-Level Analyses (IPA; LoF Interpretation)

To organise the gene-level resistance hits into broader processes, I used Ingenuity Pathway Analysis (IPA) on the insertion-enriched genes from the 200 μ M cordycepin conditions. Within the loss-of-function (LoF) framework established above, enrichment of a canonical pathway means that disruptive insertions in one or more of its components increase survival in cordycepin, i.e. the intact pathway normally contributes to cordycepin sensitivity. Here I focus on the suPB and hyPB 200 μ M datasets, which provide the strongest selection and clearest pathway structure (Figs. 4.15–4.18).

suPB LoF pathways at 200 μ M

In the suPB_CoR_200 versus suPB_DMSO comparison, IPA identifies a set of nucleoside/nucleotide salvage pathways (“Adenine and Adenosine Salvage VI”, “Nucleotide salvage”, “Ribavirin ADME”) that all contain ADK, consistent with the requirement for ADK-mediated phosphorylation of cordycepin for toxicity [6, 103]. LoF insertions in ADK therefore act as a resistance mechanism by reducing formation of cordycepin mono/di/triphosphate.

Beyond ADK, the most molecule-rich pathways in the suPB dataset map to autophagy, ER-mitochondria contacts, mitochondrial quality control, ubiquitin-dependent proteostasis, innate RNA sensing, and RNA processing (Figs. 4.15, 4.16). Autophagy and mitophagy modules (“Autophagy”, “Macroautophagy”, “Mitophagy”) are supported by LoF insertions in ATG12 and ATG5. ER-mitochondrial communication pathways (“ER-Mitochondrial Communication Signaling Pathway”, “Cellular response to mitochondrial stress”, “Mitochondrial biogenesis”) are backed by hits in VPS13D, YME1L1 and NCOA1, implicating mitochondrial turnover and biogenesis in shaping cordycepin sensitivity [147, 149–151].

Proteostasis- and innate-immunity-related terms are also prominent. “Protein Ubiquitination Pathway” and “Deubiquitination” contain UBE2D1, PSMB2, UBR2, TRRAP and USP3, while “DDX58/IFIH1-mediated induction of interferon-alpha/beta” and “Role of PKR in interferon induction and antiviral response” include ATG12, ATG5, CASP8

and UBE2D1. LoF of these components indicates that intact ubiquitin-coupled stress signalling, RIG-I-like receptor pathways and PKR/ISR outputs promote cordycepin toxicity in this system [130–135].

Finally, several enriched pathways involve RNA maturation and translation capacity. These include “Processing of Capped Intron-Containing Pre-mRNA”, “Regulation of mRNA stability by proteins that bind AU-rich elements”, “rRNA modification in the nucleus and cytosol”, and “tRNA splicing”, with hits in NUP160, TCERG1, PSMB2, RCL1 and PDE11A. LoF of these factors indicates that normal co-transcriptional processing, rRNA/tRNA maturation and ARE-mediated decay contribute to cordycepin sensitivity, and that reducing RNA processing and ribosome production can be protective under drug exposure [20, 44, 152–156].

Taken together, the suPB 200 μ M IPA results show that resistance arises from LoF in (i) nucleoside/nucleotide salvage containing ADK, (ii) autophagy/mitophagy and ER–mitochondrial communication, (iii) ubiquitin-dependent proteostasis and innate RNA sensing, and (iv) RNA processing and translation-related pathways.

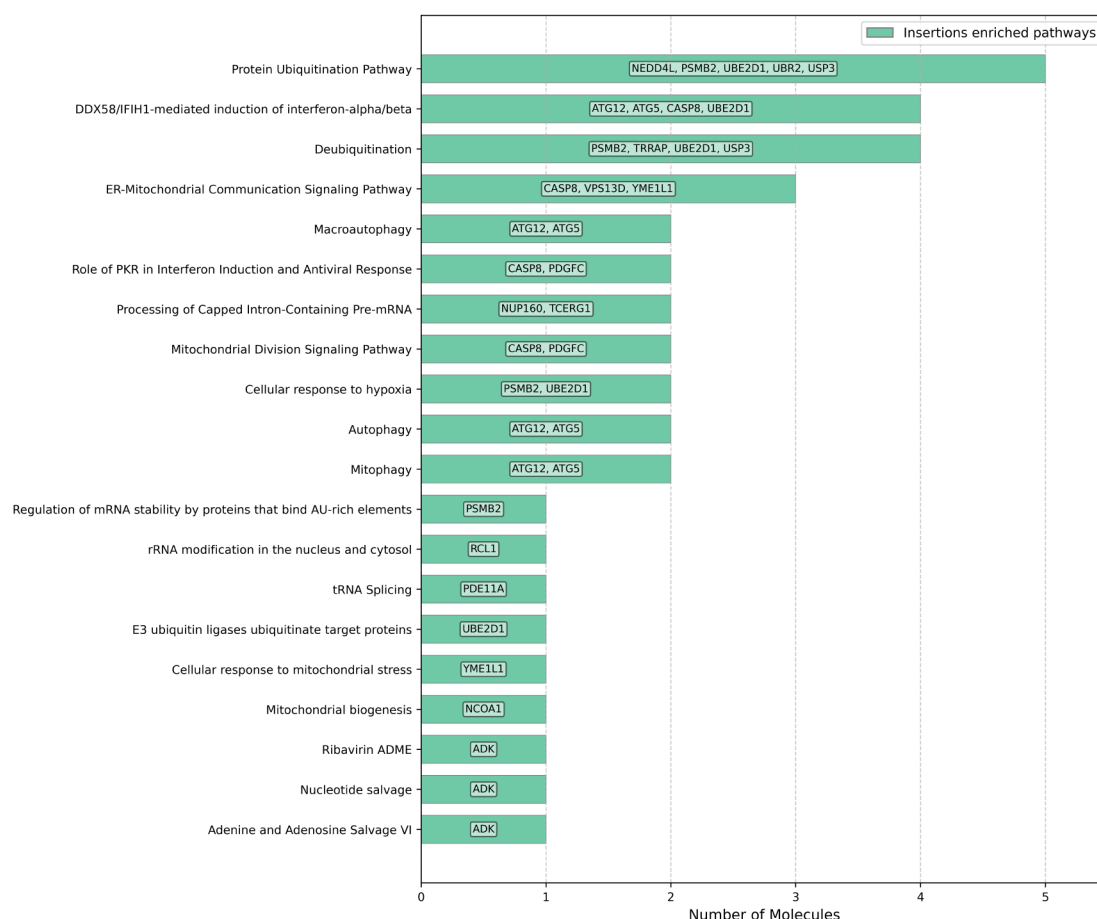


Figure 4.15: Ingenuity Pathway Analysis of suPB cordycepin-resistant clones (200 μ M vs DMSO). Horizontal bar plot of Ingenuity Canonical Pathways enriched in the suPB_CoR_200 population relative to the matched suPB DMSO control at 150K seeding density. Bar length represents the number of insertion-enriched genes (x-axis) mapped to each pathway (y-axis), and gene symbols inside each bar identify those genes as reported by IPA. eHAP libraries were selected with repeated 200 μ M cordycepin dosing for 72 h (twice daily) before mapping of insertion sites; pathway enrichment criteria and thresholds are described in the Methods.

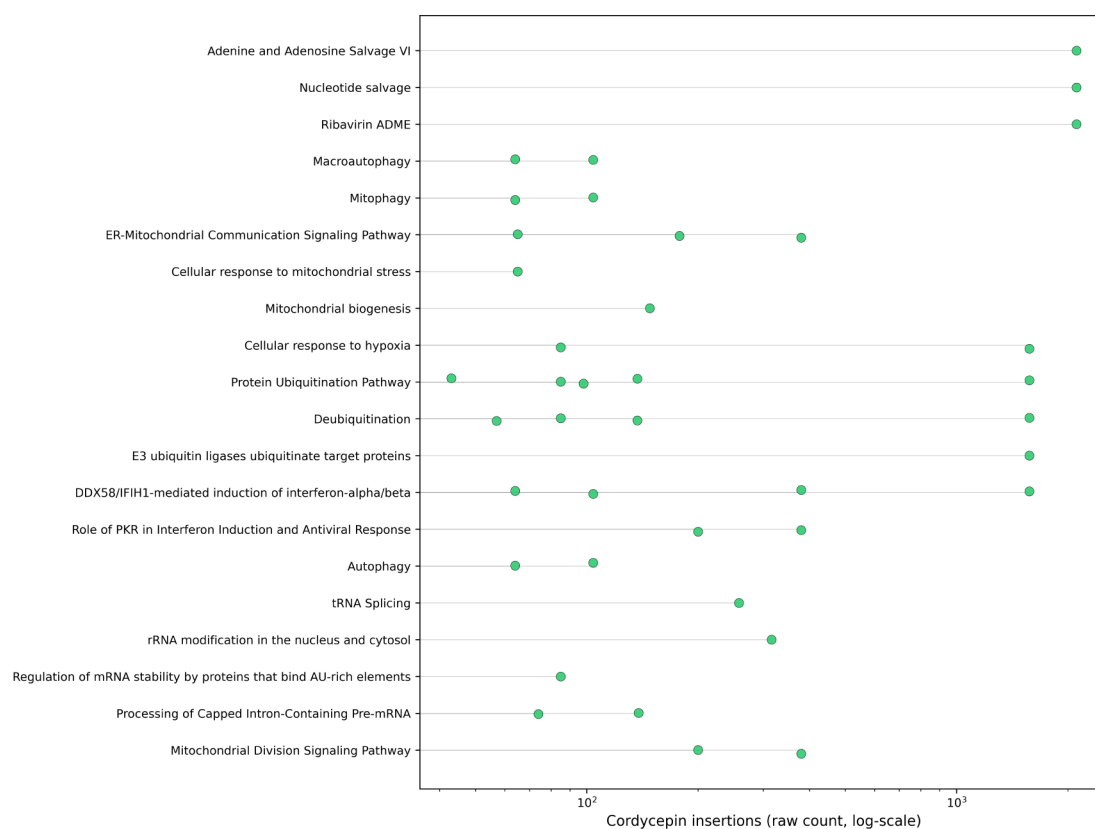


Figure 4.16: Insertion support for IPA pathways in suPB cordycepin-resistant clones (200 μ M). Lollipop plot showing, for each IPA canonical pathway enriched in suPB_CoR_200 versus DMSO (y-axis), the total number of unique transposon insertion sites in genes belonging to that pathway (x-axis, log scale). Unlike the bar chart in Figure 4.15, which counts the number of distinct genes, this plot shows the cumulative insertion-site evidence supporting each pathway, reflecting both the number of contributing genes and the frequency of insertions in each gene.

hyPB LoF pathways at 200 μ M

Cordycepin requires ADK-dependent phosphorylation for activity and has been shown to inhibit polyadenylation and alter mRNA 3'-end formation, with downstream consequences for AMPK–mTOR signalling [6, 19, 46, 103, 127]. In the hyPB_CoR_200 versus hyPB_DMSO dataset, IPA highlights three major groups of resistance pathways (Figs. 4.17, 4.18).

First, multiple enriched pathways centre on RNA 3'-end formation and mRNA stability. “Deadenylation-dependent mRNA decay”, “mRNA 3 Prime End Processing Signaling Pathway” and “Inhibition of ARE-Mediated mRNA Degradation Pathway” contain CNOT6, PARN and LSM3. The LoF of these deadenylase and 3'-end processing components is consistent with reduced poly(A) removal and altered 3'-end processing, which could compensate for maintaining poly(A) tail length and mRNA stability when cordycepin inhibits polyadenylation [19, 20, 44, 46, 152]. Additional RNA-related pathways (“RNA polymerase II transcribes snRNA genes”, “Processing of Capped Intron-Containing Pre-mRNA”, “Major pathway of rRNA processing in the nucleolus and cytosol”, “tRNA splicing”) include INTS3, LSM3, NUP35, RCL1, WDR12, WDR18, PDE3B and PDE7A, indicating that LoF in snRNA 3'-end formation, pre-mRNA processing, ribosome biogenesis and tRNA maturation can also increase resistance [155–158].

Second, a set of enriched pathways implicates energy-sensing and autophagy. “AMPK Signaling”, “Energy dependent regulation of mTOR by LKB1–AMPK” and “mTOR Signaling” include PPM1A and PPP2R2B, phosphatase regulators of AMPK–mTOR crosstalk [13, 103, 127–129]. “Macroautophagy”, “Microautophagy Signaling Pathway”, “Mitochondrial biogenesis” and “Cellular response to hypoxia” include ATG5, MTMR3, SEC24D, VPS41, GABPA, NCOA6, EGLN1 and UBE2D1 [147, 148, 150, 151, 159]. LoF of these nodes indicates that intact AMPK–mTOR checkpointing, autophagy/mitophagy, mitochondrial biogenesis and hypoxia signalling contribute to cordycepin-induced loss of viability in hyPB cells.

Third, hyPB survivors show LoF enrichment in proteostasis and innate-signalling pathways. “Unfolded protein response”, “ATF4 activates genes in response to endoplas-

mic reticulum stress”, “Deubiquitination” and “DDX58/IFIH1-mediated induction of interferon-alpha/beta” include PDIA6, PARN, USP14, USP16, IFIH1 and UBE2D1 [130–135]. LoF in these components indicates that the unfolded protein response, integrated stress response and ubiquitin-linked RIG-I-like receptor signalling all modulate cordycepin sensitivity, and that attenuating these pathways can improve survival when protein synthesis is reduced by cordycepin [46].

As in the suPB screen, ADK appears in “Adenine and Adenosine Salvage VI” and “Nucleotide salvage”, providing a positive control that confirms the requirement for phosphorylation of cordycepin to active nucleotides [6, 103]. Overall, the hyPB IPA results define a resistance landscape built from LoF in (i) 3'-end processing and deadenylation, (ii) AMPK–mTOR/autophagy and mitochondrial pathways, and (iii) UPR/ISR and ubiquitin-dependent innate signalling.

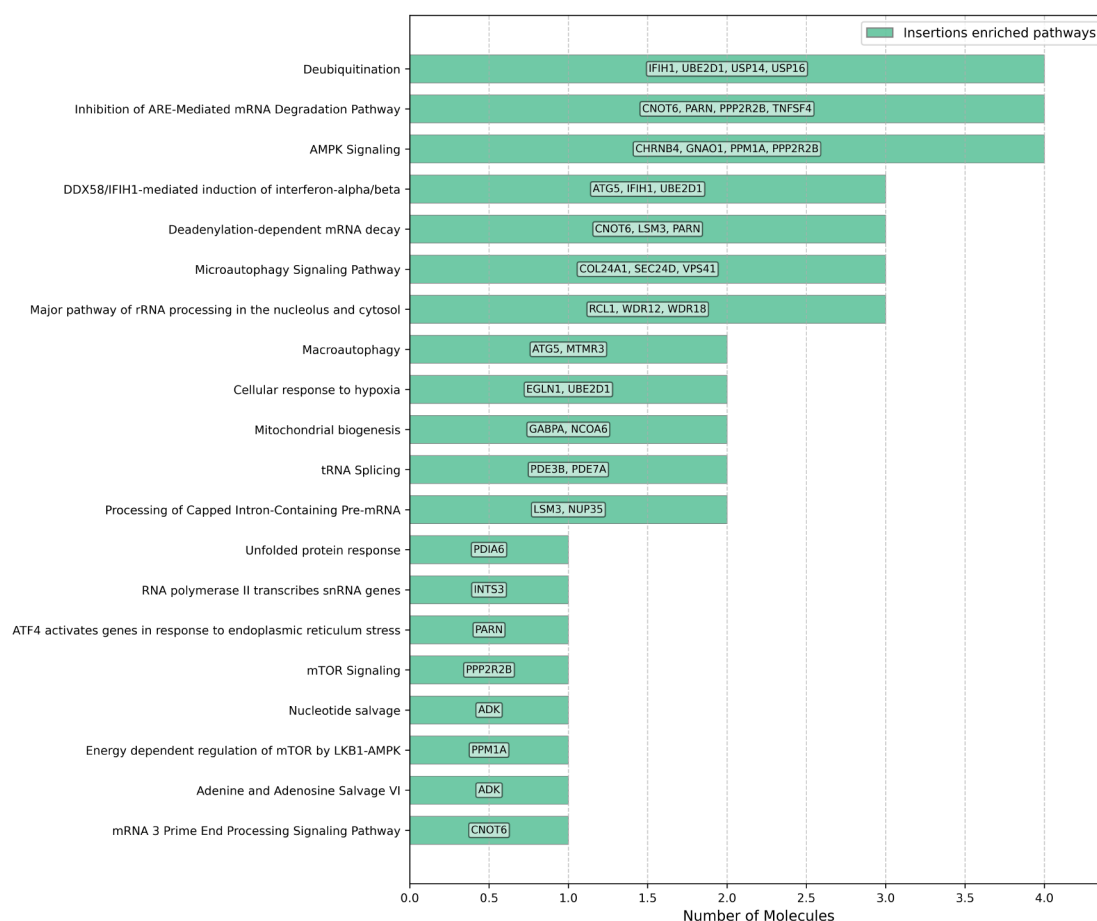


Figure 4.17: Ingenuity Pathway Analysis of hyPB cordycepin-resistant clones (200 μ M vs DMSO). Horizontal bar plot of Ingenuity Canonical Pathways enriched in the hyPB_CoR_200 population relative to the matched hyPB DMSO control at 150K seeding density. Bar length represents the number of insertion-enriched genes (x-axis) mapped to each pathway (y-axis), and gene symbols inside each bar identify those genes as reported by IPA. Libraries were selected with repeated 200 μ M cordycepin dosing for 72 h (twice daily) before mapping of insertion sites; enrichment criteria and thresholds are described in the Methods.

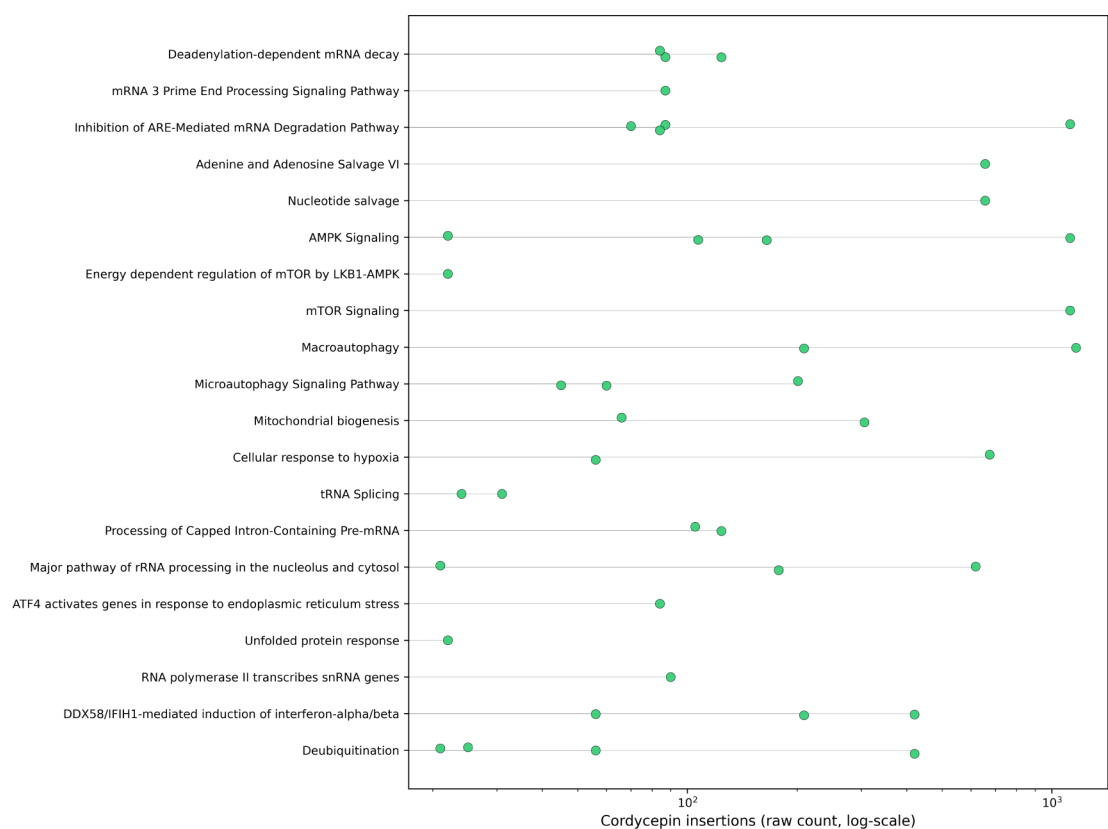


Figure 4.18: Insertion support for IPA pathways in hyPB cordycepin-resistant clones (200 μ M). Lollipop plot showing, for each IPA canonical pathway enriched in hyPB_CoR_200 versus DMSO (y-axis), the total number of unique transposon insertion sites in genes belonging to that pathway (x-axis, log₁₀ scale). As in Figure 4.16, this plot complements the bar chart (Figure 4.17) by showing cumulative insertion-site evidence rather than the number of distinct genes.

Section summary

Across the 200 μ M datasets, IPA of the LoF hits converges on three broad classes of resistance mechanisms. First, nucleoside/nucleotide salvage pathways containing ADK are repeatedly enriched, confirming that phosphorylation of cordycepin to its nucleotide forms is required for toxicity and that reducing ADK activity is a robust route to resistance [6, 103]. Second, hyPB in particular highlights RNA 3'-end processing, deadenylation and RNA maturation pathways (CNOT/PARN complexes, Integrator, rRNA and tRNA processing), indicating that components of mRNA processing and decay modulate cordycepin sensitivity in line with its established role as a polyadenylation inhibitor [13, 19, 46]. Third, both platforms implicate metabolic and quality-control pathways regulated by AMPK and mTOR—autophagy and mitophagy, mitochondrial biogenesis and hypoxia signalling, ubiquitin-dependent proteostasis, ER stress and innate RNA-sensing—consistent with a drug response in which changes in protein synthesis, protein degradation and mitochondrial function influence survival [13, 127–130, 133, 134].

Platform-specific patterns are superimposed on this shared architecture. hyPB emphasises RNA 3'-end and AMPK–mTOR/autophagy modules, with recurrent LoF in CNOT6, PARN, ATG5, PPP2R2B, VPS41 and EGLN1, whereas suPB emphasises ubiquitin/proteasome, ER–mitochondrial communication and innate sensors, with LoF in UBE2D1, EDEM3, CASP8, NEDD4L and VPS13D. Together with the gene-level enrichment analyses (Figs. 4.11–4.12) and cross-condition recurrence (Figs. 4.13, 4.14), these pathway-level data show that cordycepin resistance in eHAP is shaped by (i) conversion of cordycepin to its nucleotide forms via ADK, (ii) the state of mRNA 3'-end formation and poly(A) tail metabolism, and (iii) metabolic and proteostasis pathways regulated by AMPK/mTOR, autophagy and ER/mitochondrial crosstalk. Subsequent chapters return to these three classes of pathways using independent approaches.

To visualise how the enriched pathways and their constituent molecules are connected within each platform, IPA graphical summaries were generated for the suPB (Figure 4.19) and hyPB (Figure 4.20) 200 μ M cordycepin datasets. These network diagrams illustrate the predicted activation or inhibition states and the causal relationships among the

top-level biological functions and upstream regulators identified by IPA, providing an integrated overview of the resistance landscape for each transposase platform.

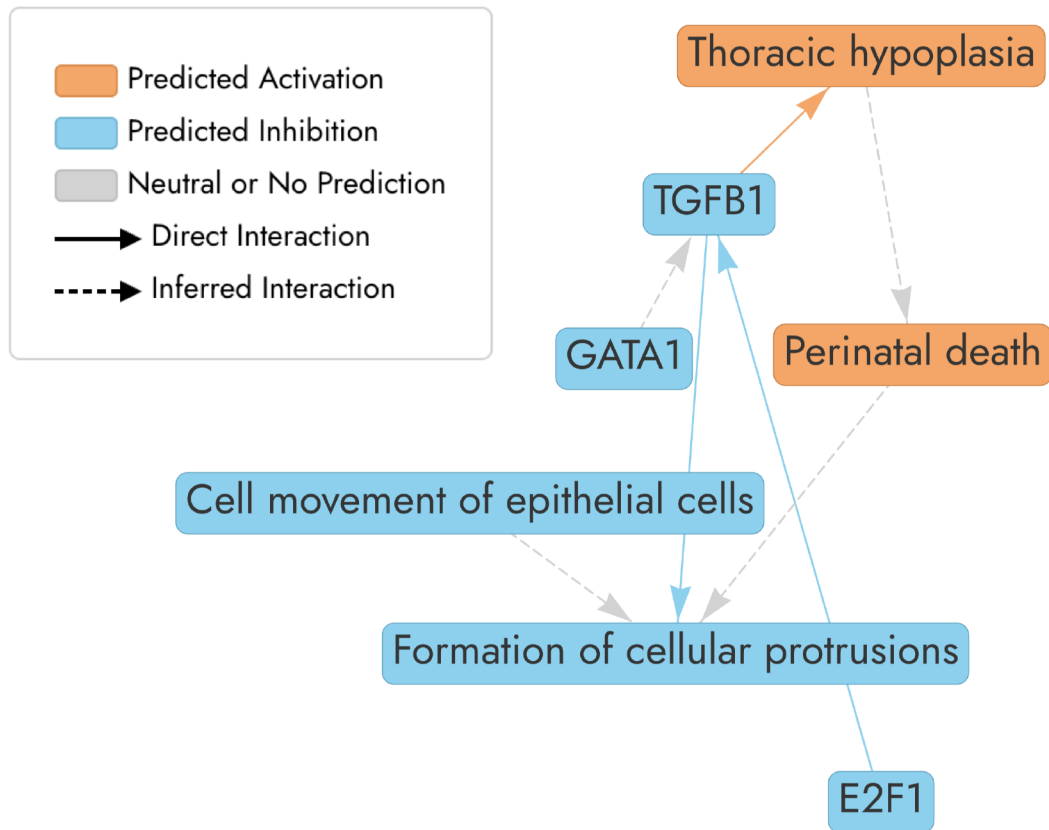


Figure 4.19: IPA graphical summary of the suPB_CoR_200 versus suPB_DMSO dataset (200 μ M cordycepin, 150K seeding density). The network shows predicted relationships among key biological functions and upstream regulators. Node colours and arrow styles as in Figure 4.20. The suPB network centres on TGFB1 signalling, with transcriptional regulation by E2F1 and GATA1 and downstream effects on cell movement, formation of cellular protrusions and developmental viability (thoracic hypoplasia, perinatal death). The predicted inhibition of TGFB1 and cell motility function is consistent with the suPB platform's enrichment of ubiquitin–proteasome, ER–mitochondrial communication and innate immune sensing pathways. Generated using the QIAGEN IPA Graphical Summary tool.

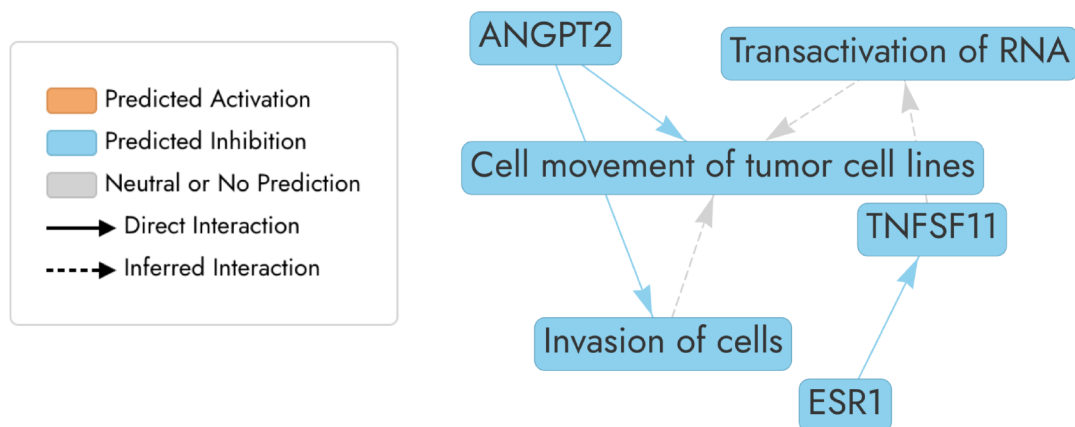


Figure 4.20: IPA graphical summary of the hyPB_CoR_200 versus hyPB_DMSO dataset (200 μ M cordycepin, 150K seeding density). The network shows predicted relationships among key biological functions and upstream regulators identified by Ingenuity Pathway Analysis. Nodes coloured blue indicate predicted inhibition, orange indicates predicted activation, and grey indicates neutral or no prediction. Solid arrows represent direct interactions and dashed arrows represent inferred interactions. The hyPB network centres on cell movement and invasion of tumour cell lines, with predicted inhibition of ANGPT2 (angiogenesis), TNFSF11 (RANKL; transcriptional activation) and ESR1 (oestrogen receptor signalling), consistent with the loss-of-function enrichment of genes in AMPK–mTOR, autophagy and RNA 3'-end processing pathways described above. Generated using the QIAGEN IPA Graphical Summary tool.

To complement the network-level view provided by the IPA graphical summaries and quantify the coverage of each enriched canonical pathway in the screen datasets, horizontal stacked bar charts were generated in QIAGEN IPA for the suPB and hyPB cordycepin-resistant libraries (Figures 4.21 and 4.22). Each bar represents an IPA canonical pathway, with the bar length reflecting the proportion of the pathway's total molecules that were recovered from the dataset. Within each bar, red segments indicate upregulated genes and green segments indicate downregulated genes, while the numeric label to the right of each bar gives the total number of molecules annotated to that pathway in the IPA knowledge base. These stacked bar views provide a direct, quantitative complement to the z-score and lollipop plots presented earlier in this section.

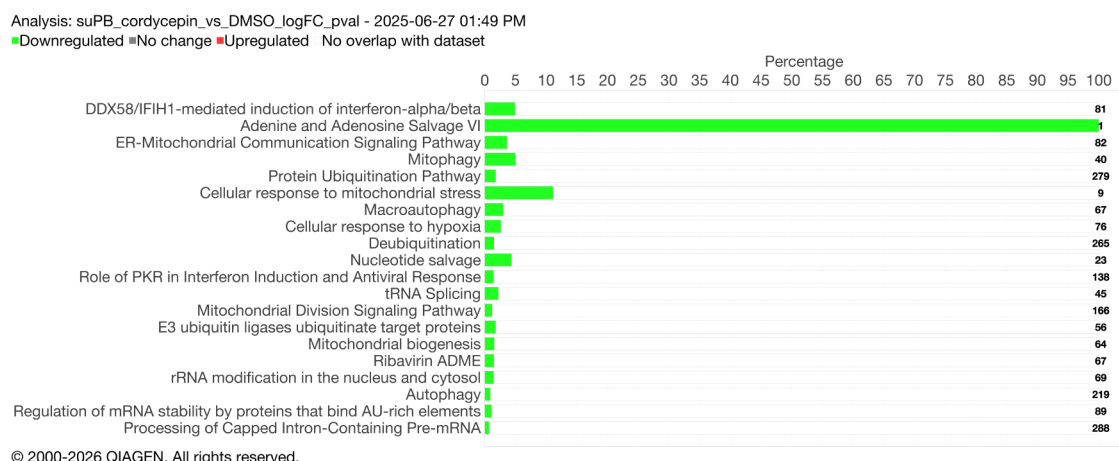


Figure 4.21: IPA canonical pathway coverage for the suPB_CoR_200 versus suPB_DMSO dataset (200 μ M cordycepin, 150K seeding density). Horizontal stacked bars show, for each enriched canonical pathway, the proportion of pathway molecules recovered in the suPB dataset; the number to the right of each bar is the total number of molecules annotated to that pathway in the IPA knowledge base. Red segments indicate upregulated genes and green segments indicate downregulated genes within each pathway. Generated using the QIAGEN IPA Stacked Bar Chart tool.

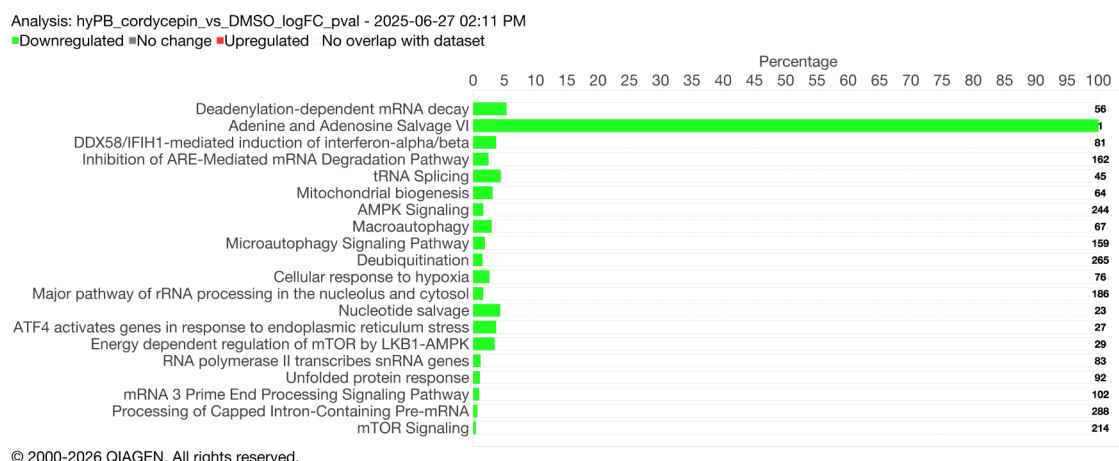


Figure 4.22: IPA canonical pathway coverage for the hyPB_CoR_200 versus hyPB_DMSO dataset (200 μ M cordycepin, 150K seeding density). Horizontal stacked bars show, for each enriched canonical pathway, the proportion of pathway molecules recovered in the hyPB dataset; the number to the right of each bar is the total number of molecules annotated to that pathway in the IPA knowledge base. Red segments indicate upregulated genes and green segments indicate downregulated genes within each pathway. Generated using the QIAGEN IPA Stacked Bar Chart tool.

4.7 Discussion

The experiments in this chapter were designed as a pilot haploid genetic screen to establish the piggyBac gene-trap system, seeding density, and cordycepin selection conditions in eHAP cells. Although we recovered tens of thousands of unique insertions per condition, the libraries were far from saturation: piggyBac recognises TTAA motifs, of which there are on the order of tens of millions (approximately 20 million) in the human genome, and any given insertion set will contain duplicated sites due to cell proliferation and PCR amplification. The data therefore provide a structured, but incomplete, view of the resistance landscape.

We used haploid eHAP cells with piggyBac gene-trap mutagenesis to expose recessive loss-of-function phenotypes and selected survivors under repeated cordycepin dosing. In this framework, enrichment of disruptive insertions in a gene among survivors indicates that loss of that gene improves viability under cordycepin; it does not imply transcriptional up-regulation of the corresponding pathway. ADK, the kinase that phosphorylates cordycepin to its mono- and triphosphate forms, serves as an internal positive control for this interpretation: disruptive insertions in ADK are reproducibly enriched in both hyPB and suPB libraries at 150 μ M and 200 μ M cordycepin (Figures 4.11–4.12, 4.13, 4.14). This is exactly what is expected if ADK-dependent phosphorylation is required for toxicity [6, 103].

The dose and selection paradigm were optimised to provide a strong but interpretable bottleneck. Repeated dosing (twice daily for three days) produced deeper and more sustained metabolic inhibition than a single dose (Figure 4.4) and yielded a clear reduction in clonogenic survival (Figure 4.5). By contrast, 3'-deoxyinosine (3DI), a deamination product of cordycepin, reached only approximately 20 % inhibition of metabolic activity even at 400 μ M under the same repeated-dosing paradigm (Figure 4.6). This limited potency is consistent with the observation that 3DI is not efficiently phosphorylated by ADK and therefore cannot generate the triphosphate species responsible for cordycepin's cytotoxic effects. Because a resistance screen requires a strong selection bottleneck that eliminates wild-type cells while permitting survival of resistant mutants, the shallow

dose–response of 3DI made it unsuitable as a selection agent; accordingly, cordycepin was used as the sole selection compound for the screen. Insertions were mapped by splinkerette PCR and next-generation sequencing (Figure 4.7). Library-quality metrics favoured the 150 K seeding condition, which produced higher insertion counts (e.g. 51,053 insertions in hyPB 200 μ M cordycepin) than 300 K and showed a broadly uniform distribution across chromosome groups (Large > Medium > Small > Sex; Figures 4.8, 4.9, 4.10).

Taken together, the gene-level and pathway-level analyses support three main classes of resistance mechanisms: (i) reduced metabolic activation of cordycepin via nucleoside/nucleotide salvage, centred on ADK; (ii) modulation of mRNA 3'-end processing and decay, in line with cordycepin as a polyadenylation inhibitor; and (iii) re-wiring of cellular metabolism, protein synthesis and degradation, and mitochondrial function through mTOR–AMPK signalling and organelle quality-control pathways. Below we discuss each class and how it connects to published models of cordycepin action.

Class 1: Reduced metabolic activation of cordycepin

Across all cordycepin doses and both transposase platforms, ADK is the most consistent resistance determinant. In suPB at 200 μ M, ADK accounts for 4.48 % of all insertions in survivors versus 0.24 % in DMSO controls; in hyPB at 200 μ M the corresponding values are 1.28 % versus 0.19 % (Figures 4.11–4.12, 4.13, 4.14). IPA groups these insertions into nucleoside/nucleotide salvage pathways such as “Adenine and Adenosine Salvage VI”, “Nucleotide salvage” and “Ribavirin ADME” (Tables 4.1, 4.2). Within our loss-of-function framework, this pattern confirms that cordycepin must be phosphorylated to kill cells and strengthens the case that cordycepin triphosphate (CoTP) is the key toxic metabolite [6, 103]. Rather than treating ADK simply as a technical control, these data place ADK-dependent metabolism at the top of the resistance hierarchy and anchor the subsequent pathway interpretation.

Class 2: mRNA 3'-end processing, deadenylation and RNA turnover

The most mechanistically informative hits in the hyPB screen are in RNA 3'-end processing and deadenylation. IPA terms such as “Deadenylation-dependent mRNA decay”, “mRNA 3 Prime End Processing Signaling Pathway”, “Inhibition of ARE-Mediated

mRNA Degradation Pathway”, “Processing of Capped Intron-Containing Pre-mRNA”, “Major pathway of rRNA processing in the nucleolus and cytosol”, “tRNA Splicing” and “RNA polymerase II transcribes snRNA genes” are all driven by genes that encode components of the deadenylase machinery or closely linked RNA-processing factors (CNOT6, PARN, LSM3, RCL1, WDR12, WDR18, INTS3, NUP35, NUP160) (Table 4.2). These genes converge on control of poly(A) tail length, 3'-end formation and ribosome/tRNA production [20, 44, 155–158].

In a loss-of-function screen, enrichment of CNOT6 and PARN strongly suggests that reducing deadenylation and ARE-mediated mRNA decay can buffer cells against cordycepin-induced inhibition of polyadenylation. Stabilising short-lived, stress-responsive mRNAs when poly(A) addition is compromised is a plausible way to maintain essential gene expression programmes [152–154]. The appearance of nucleoporins (NUP35, NUP160, and in the suPB pathways NUP160/TCERG1) implies that altering nuclear export offers a parallel adaptation: most mRNAs require a poly(A) tail for efficient export, so tuning nuclear pore components may partially compensate when tails are shortened or absent.

Several proteasome-linked factors, including PSMB2, are annotated in RNA stability pathways such as “Regulation of mRNA stability by proteins that bind AU-rich elements” in the suPB dataset (Table 4.1). This is consistent with reports linking components of the proteasome and deadenylation machinery, and raises the possibility that such factors may have dual roles in proteostasis and RNA decay [44, 155].

A note on terminology is important here. Throughout this thesis, the term “**RNA target of cordycepin**” (**RToC**) refers to an mRNA (or other polyadenylated RNA) whose stability, processing or translational efficiency is altered as a downstream consequence of cordycepin triphosphate (CoTP) inhibiting the cleavage and polyadenylation (CPA) machinery. An RToC is therefore not an RNA that is directly bound by the cordycepin molecule in the way that a small-molecule ligand binds a riboswitch; rather, it is an RNA whose poly(A)-dependent fate—export, translation, or turnover—is disrupted because CPA is compromised. This distinction is important because the screen identifies genes involved in poly(A)-tail metabolism (CNOT6, PARN, NUP35/160) whose

loss-of-function buffers cells against CPA inhibition, implying that the downstream RNA targets of this inhibition are central to cordycepin toxicity.

Overall, the enrichment of deadenylases and associated processing factors is one of the most convincing signals in the screen and fits well with the established role of cordycepin as a polyadenylation inhibitor [13, 19].

Class 3: Metabolic re-wiring, protein synthesis/degradation and mitochondrial control

A third set of resistance mechanisms involves metabolic and proteostasis pathways controlled by mTOR and AMPK. In hyPB, IPAs such as “AMPK Signaling”, “Energy-dependent regulation of mTOR by LKB1–AMPK”, “mTOR Signaling”, “Macroautophagy”, “Macroautophagy Signaling Pathway”, “Mitochondrial biogenesis” and “Cellular response to hypoxia” are driven by PPM1A, PPP2R2B, ATG5, MTMR3, SEC24D, VPS41, GABPA, NCOA6, EGLN1 and UBE2D1 (Table 4.2). In suPB, related terms such as “Macroautophagy”, “Mitophagy”, “Cellular response to mitochondrial stress”, “Mitochondrial biogenesis”, “Cellular response to hypoxia”, “Protein Ubiquitination Pathway”, “Deubiquitination”, “E3 ubiquitin ligases ubiquitinate target proteins” and “DDX58/IFIH1-mediated induction of interferon-alpha/beta” are supported by ATG12, ATG5, YME1L1, NCOA1, UBE2D1, NEDD4L, USP3 and CASP8 (Table 4.1).

Taken together, these patterns suggest that cordycepin-treated cells are under combined energy and proteotoxic stress and that resistance can be achieved by dampening both autophagic and ubiquitin-dependent degradation. ATG5/ATG12 and ATG5/MTMR3 insertions indicate that macroautophagy and mitophagy contribute to cell death under the chosen treatment, because disrupting these pathways improves survival [127–129, 147]. This is consistent with the idea that, when protein synthesis is strongly reduced, further degradation of existing proteins by autophagy can be maladaptive. Protein synthesis is one of the largest ATP consumers in proliferating cells; the combination of reduced translation (via mTOR/4E-BP signalling [46]) and reduced degradation (via attenuated autophagy and ubiquitin-proteasome pathways) would stabilise the remaining proteome and limit energy expenditure.

The mitochondrial hits (YME1L1, NCOA1, GABPA, EGLN1) point to altered

mitochondrial biogenesis and quality control [147, 148, 150, 151]. Loss of NCOA1 or NCOA6 would be expected to decrease PGC-1–driven biogenesis, lowering ATP demand, while YME1L1 loss may shift mitochondrial proteostasis and apoptotic thresholds. EGLN1 loss stabilises HIF-1 α and favours glycolytic metabolism, another way to adapt to cordycepin-induced energetic stress [127, 148]. Our data do not distinguish whether mitochondria are a primary target of cordycepin or whether these changes are secondary to AMPK–mTOR signalling; both possibilities are compatible with the observed resistance profile.

Proteostasis and innate immune signalling are tightly interwoven in the suPB dataset. Hits in UBE2D1, NEDD4L, USP3, USP14, USP16 and PSMB2 underlie enrichment of “Protein Ubiquitination Pathway”, “Deubiquitination” and related IPA terms, while IFIH1, ATG5, CASP8 and UBE2D1 support “DDX58/IFIH1-mediated induction of interferon-alpha/beta” and “Role of PKR in Interferon Induction and Antiviral Response” [130–135, 149]. A reasonable interpretation is that dampening RIG-I-like receptor and PKR signalling, and loosening the coupling between misfolded proteins and their degradation, helps cells avoid excessive ISR activation and apoptosis when RNA metabolism and translation are already compromised. Some individual annotations (for example, PDGFC in mitochondrial biogenesis or CASP8 in multiple pathways) are clearly pleiotropic and should not be over-interpreted on their own; they are best viewed as components of broader proteostasis and stress-signalling modules.

UPR/ISR-related hits (e.g. PDIA6; PARN in “ATF4 activates genes in response to endoplasmic reticulum stress”) are more difficult to interpret, because cordycepin has been reported to inhibit aspects of the UPR [130, 135]. Loss of these nodes could either blunt a pro-death arm of the UPR or perturb negative feedback. At present the screen cannot resolve these alternatives, and these genes remain candidates for follow-up rather than firm mechanistic anchors.

Platform-specific features and the LINC03121 signal

Although hyPB and suPB recover a shared core of resistance mechanisms, each platform also highlights distinct modules. HyPB at 200 μ M cordycepin shows pronounced enrichment of LINC03121 (10.86 % vs 0.21 % in control), together with PPP2R2B,

ATG5, VPS41 and EGLN1. The long noncoding RNA hit is noteworthy: lincRNAs are increasingly recognised as regulators of transcription, nuclear architecture and post-transcriptional control [160], and prior work from the de Moor laboratory showed that cordycepin treatment can induce a broad set of noncoding RNAs in breast cancer cell lines [161]. Given piggyBac’s TTAA/open-chromatin bias [141], we cannot exclude technical inflation of counts at a single lincRNA locus, but the magnitude of the LINC03121 signal makes it an interesting candidate for targeted follow-up rather than something to be dismissed outright.

In contrast, suPB emphasises ubiquitin–proteasome, ER stress and apoptosis modules (UBE2D1, EDEM3, CASP8, PSAT1, TPK1), pointing towards proteostasis adjustment and metabolic rewiring. These differences are best viewed as complementary slices through the same stress-response network, shaped by coverage differences between transposase variants, rather than as conflicting mechanisms.

Screen limitations and strength of the pathway assignments

Several caveats temper the interpretation of the pathway-level analyses. First, although we obtained up to 50,000 mapped insertions in the richest condition, this is still orders of magnitude below the total number of potential TTAA sites in the genome, and some insertions will be clonally related or PCR duplicates. Many IPA pathways in Tables 4.1 and 4.2 are driven by one or two genes; such categories are inherently fragile and should be treated as labels for recurrent genes rather than as fully validated pathways. The deadenylases (CNOT6, PARN) and core RNA-processing factors are supported by multiple, independent pathways and by strong prior biology [13, 19, 20, 44], and we consider these the most robust findings. Other entries, especially those based on a single pleiotropic gene (e.g. PDGFC), are better regarded as hypotheses.

Second, the screen was performed under a single cell line and dosing regime; mechanisms of resistance in other genetic backgrounds or under different schedules may differ. Notably, some proposed mechanisms of cordycepin action from the literature—stress kinase cascades, adenosine receptors, PARP/DNA damage signalling [13]—do not emerge as prominent hits here, suggesting that they are not major determinants of survival in eHAP cells under our conditions. Conversely, genetic deletion of AMPK has

been shown to reduce sensitivity to cordycepin [103], but AMPK itself does not appear as a strong hit; instead, our screen recovers phosphatases and upstream/downstream modulators (PPM1A, PPP2R2B, mitochondrial and proteostasis genes), implying that AMPK–mTOR signalling is adjusted indirectly rather than ablated.

Finally, because piggyBac integrates only at TTAA sites and favours open chromatin, some genes will be effectively invisible to this approach, and others (including lncRNA loci) may be over-represented. These biases reinforce the need to combine haploid genetics with orthogonal approaches such as CRISPR knock-out/knock-down, phosphoproteomics and direct metabolite measurements.

Implications for cordycepin mechanism of action

Despite these limitations, the three classes of resistance discussed above can be integrated into a mechanistic cascade: ADK-dependent phosphorylation generates cordycepin triphosphate, which inhibits polyadenylation and alters mRNA 3'-end processing; these RNA-level changes propagate through mTOR–AMPK signalling to reduce protein synthesis, engage autophagy and remodel mitochondrial function [6, 19, 46, 103, 127, 128]. The resistance mechanisms uncovered here can therefore be understood as ways of intercepting this cascade at different levels: loss of ADK prevents formation of toxic nucleotides, loss of deadenylases and related processing factors stabilises critical transcripts when poly(A) addition is compromised, and loss of selected autophagy, ubiquitin-proteasome and mitochondrial regulators reduces the energetic and proteostatic burden when protein synthesis is curtailed. Some signals, particularly in UPR-related pathways, remain difficult to reconcile with current models and require targeted follow-up. Subsequent chapters return to these findings using independent transcriptomic and metabolomic approaches.

Table 4.1: Prioritized IPA pathways from the suPB cordycepin-resistance screen (exact pathway names from IPA).

Ingenuity Canonical Pathways	Molecules
Adenine and Adenosine Salvage VI	ADK
Nucleotide salvage	ADK
Ribavirin ADME	ADK
Macroautophagy	ATG12, ATG5
Mitophagy	ATG12, ATG5
ER-Mitochondrial Communication Signaling Pathway	CASP8, VPS13D, YME1L1
Cellular response to mitochondrial stress	YME1L1
Mitochondrial biogenesis	NCOA1
Cellular response to hypoxia	PSMB2, UBE2D1
Protein Ubiquitination Pathway	NEDD4L, PSMB2, UBE2D1, UBR2, USP3
Deubiquitination	PSMB2, TRRAP, UBE2D1, USP3
E3 ubiquitin ligases ubiquitinate target proteins	UBE2D1
DDX58/IFIH1-mediated induction of interferon-alpha/beta	ATG12, ATG5, CASP8, UBE2D1
Role of PKR in Interferon Induction and Antiviral Response	CASP8, PDGFC
Autophagy	ATG12, ATG5
tRNA Splicing	PDE11A
rRNA modification in the nucleus and cytosol	RCL1
Regulation of mRNA stability by proteins that bind AU-rich elements	PSMB2
Processing of Capped Intron-Containing Pre-mRNA	NUP160, TCERG1
Mitochondrial Division Signaling Pathway	CASP8, PDGFC

Table 4.2: Prioritized IPA pathways from the hyPB cordycepin-resistance screen (exact pathway names from IPA).

Ingenuity Canonical Pathways	Molecules
Deadenylation-dependent mRNA decay	CNOT6, LSM3, PARN
mRNA 3 Prime End Processing Signaling Pathway	CNOT6
Inhibition of ARE-Mediated mRNA Degradation Pathway	CNOT6, PARN, PPP2R2B, TNFSF4
Adenine and Adenosine Salvage VI	ADK
Nucleotide salvage	ADK
AMPK Signaling	CHRNA4, GNAO1, PPM1A, PPP2R2B
Energy-dependent regulation of mTOR by LKB1-AMPK	PPM1A
mTOR Signaling	PPP2R2B
Macroautophagy	ATG5, MTMR3
Microautophagy Signaling Pathway	COL24A1, SEC24D, VPS41
Mitochondrial biogenesis	GABPA, NCOA6
Cellular response to hypoxia	EGLN1, UBE2D1
tRNA Splicing	PDE3B, PDE7A
Processing of Capped Intron-Containing Pre-mRNA	LSM3, NUP35
Major pathway of rRNA processing in the nucleolus and cytosol	RCL1, WDR12, WDR18
ATF4 activates genes in response to endoplasmic reticulum stress	PARN
Unfolded protein response	PDIA6
RNA polymerase II transcribes snRNA genes	INTS3
DDX58/IFIH1-mediated induction of interferon-alpha/beta	ATG5, IFIH1, UBE2D1
Deubiquitination	IFIH1, UBE2D1, USP14, USP16

Chapter 5

Transcriptomic analysis indicates cordycepin is the active ingredient of natural cordycepin preparations

5.1 Introduction

Cordycepin (3′deoxyadenosine) is the main bioactive nucleoside associated with the species *Cordyceps* and has long been recognised for its broad anti-inflammatory and antiproliferative effects. Mechanistically, cordycepin and other adenosine analogue triphosphates can interfere with 3′ end formation and poly(A) tail synthesis, a control point that selectively constrains the induction of inflammatory transcripts, placing mRNA polyadenylation as a tractable anti-inflammatory target [19] [45]. Furthermore, unpublished data in the De Moor lab indicate that the knockdown of polyadenylation factors produces similar effects to cordycepin on inflammatory markers [26] [27]. Complementing these cellular findings, in vivo work has shown the analgesic and disease-modifying activity of cordycepin in rodent osteoarthritis models, with macrophage-linked mechanisms [28]. A systematic review also concludes that cordycepin consistently dampens PI3K / AKT / mTOR and ERK signaling while repressing inflammatory outputs in various models [13]. Together, these observations motivate a pathway-anchored, transcriptome-level dissection of cordycepin’s actions in macrophage inflammation.

Despite extensive study, two practical uncertainties remain central for translation

and for interpreting the literature. First, the biological effects reported for natural (fermentation-derived) preparations could be confounded by co-purified contaminants, which are remarkably similar across commercial preparations, probably because the protocols are similar [162]; this underpins the need to demonstrate the equivalence between natural and chemically synthesised cordycepin. Second, the behaviour of clinically orientated cordycepin comparators in acute innate immune settings is incompletely defined, including whether they recapitulate cordycepin’s suppression of inflammatory responses. NUC7738, a ProTide 5’aryloxy phosphoramidate of 3’deoxyadenosine, was designed to bypass extracellular deamination by adenosine deaminase (ADA) and the requirement for equilibrative nucleoside transporters, thus improving intracellular monophosphate delivery and subsequent formation of 3’dATP [29] [2]. In addition, pharmacokinetic studies show that orally administered cordycepin is rapidly deaminated to 3’deoxyinosine (3DI), which is systemically bioavailable and can be reconverted intracellularly to cordycepin triphosphate, suggesting a “nucleoside rescue” route for oral efficacy [1]. These developments make NUC7738 and 3DI mechanistically relevant comparators when comparing cordycepin macrophage actions.

This chapter addresses both uncertainties by (i) establishing the functional equivalence of natural versus synthetic cordycepin in an acute macrophage inflammation model and (ii) comparing cordycepin with NUC7738 and 3DI under matched LPS challenge conditions. Global differential expression (DE) profiling and pathway-level interpretation are applied throughout. Oxford Nanopore cDNA sequencing provides whole-transcriptome coverage; gene-level quantification uses IsoQuant with Trimmed Mean of M-values (TMM) normalisation, and DE is tested using edgeR generalised linear models with Benjamini–Hochberg (BH) FDR control (prespecified thresholds $|\log_2 \text{FC}| \geq 1$; $\text{FDR} \leq 0.05$; $n = 3$ per group). Compound exposures were selected to give broadly comparable suppression of a small panel of LPS-induced inflammatory genes measured by RT-qPCR: cordycepin was applied at 20 μM , as informed by previous studies [26] [1], while NUC7738 and 3DI were used at 200 μM , based on earlier potency assessments for 3DI [26] and preliminary experimental trials for NUC7738 in this macrophage LPS model (Chapter 3).

Rationale for the set of molecules Cordycepin is used as the reference entity because it has been extensively researched, with many different preparations showing similar biological effects over decades, and because it directly probes the RNA-centric control of inflammation (polyadenylation / 3' end processing) [19] [13]. At pharmacologically relevant exposures, cordycepin also modulates growth factor signaling programmes that interface with innate immune amplification [25]. NUC7738 represents a clinically advanced and metabolically protected form of cordycepin that overcomes ADA-mediated deamination and transporter bottlenecks; it is active in vitro and in a first study in humans, with evidence of conversion to 3'dATP in patients [29]. Finally, 3DI is included because it is the predominant bioavailable species after oral cordycepin and can be an intracellular source of active 3'deoxyadenosine triphosphate in macrophages [1]. Comparing these three molecules under identical stimulation and analysis conditions decouples issues of source purity (natural versus synthetic) from delivery- and metabolism-driven pharmacology (cordycepin vs. NUC7738 vs. 3DI).

Important methodological caveat. A limitation of the Oxford Nanopore cDNA sequencing approach used here and in subsequent chapters (Chapters 6–7) should be noted at the outset. The library preparation relies on oligo(dT)-primed reverse transcription, which requires a poly(A) tail for capture, and the nanopore poly(A)-tail measurement depends on a free 3'-OH for adapter ligation [163]. Because cordycepin (3'-deoxyadenosine) lacks a 3'-OH group, transcripts whose poly(A) tails terminate in a cordycepin residue may be under-represented in the sequencing library and refractory to tail-length estimation. This chemistry-dependent bias means that the most severely affected transcripts—those with cordycepin-terminated tails—could be missed in the poly(A) analysis, although their altered abundance would still be detectable through read counting. However, this artefact is unlikely to be a major driver of the observed patterns for two reasons. First, Kondrashov et al. demonstrated that cordycepin-terminated tails are transient and rare at steady state, as evidenced by similar tail-length distributions obtained from both 3'- and 5'-end labelling approaches [19]. Second, the overall transcriptional signatures reported here closely recapitulate those detected by microarray [26, 164] and Illumina RNA-seq [27] in nearly identical RAW264.7/LPS experimental

systems—platforms whose library chemistries do not share the same poly(A) 3'-OH dependency. This cross-platform concordance supports the interpretation that the differential expression patterns primarily reflect true changes in RNA abundance. Nonetheless, this limitation should be borne in mind when interpreting both the differential expression and the poly(A)-tail analyses presented in this and the following chapters.

Experimental and analytical frameworks. RAW264.7 macrophages are treated with test compounds for 2 h; LPS is added 1 h after the start of compound treatment (i.e. in the presence of the compound) and cells are challenged for a further 1 h to trigger a defined early inflammatory programme. Transcriptomes are profiled by ONT cDNA sequencing and analysed as described above. The experimental time scheme is summarised in Figure 5.1.

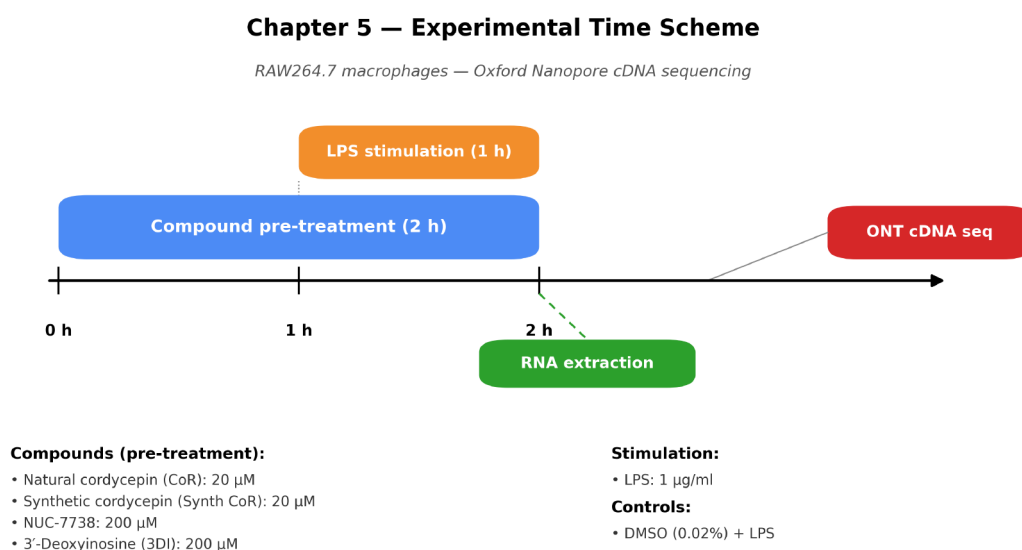


Figure 5.1: Experimental time scheme for the transcriptomic analysis. RAW264.7 macrophages were treated for 2 h with natural cordycepin (20 µM), synthetic cordycepin (20 µM), NUC-7738 (200 µM), 3'-deoxyinosine (200 µM), or DMSO vehicle (0.02%). LPS (1 µg/ml) was added after 1 h of compound treatment (i.e. in the presence of the compound) and cells were stimulated for 1 h. Total RNA was extracted at 2 h and processed for Oxford Nanopore cDNA sequencing.

5.2 Platform overview (ONT long-read profiling)

To establish a robust methodological framework for differential expression and pathway analyses, Oxford Nanopore Technologies (ONT) sequencing was employed as the primary approach for transcriptomic profiling. The objective of this section was to confirm the analytical suitability and reliability of the ONT sequencing platform for downstream molecular analyses. ONT sequencing detects transcript sequences by monitoring ionic current fluctuations as nucleic acids pass through nanopores embedded in a synthetic membrane (Figure 5.2). Briefly, this method utilizes a motor protein to regulate the translocation speed of nucleic acid strands through a nanopore, guided by an applied positive electrode. As successive k-mers traverse the nanopore’s constriction zone, they generate distinct ionic current signatures continuously captured and recorded. Computational decoding of these raw current signals via basecalling algorithms converts electrical patterns into nucleotide sequences, which serve as the foundational data for all analyses presented herein [165] (Figure 5.2).

The key advantage of the ONT platform—its capacity to produce exceptionally long reads—enabled isoform-level transcript quantification, which is challenging to achieve using short-read sequencing technologies [165]. Sequencing reads were processed using IsoQuant to perform transcript-level quantification, providing sufficient resolution to discriminate closely related transcript isoforms accurately. These isoform-specific quantifications subsequently underwent differential expression testing using edgeR, enabling precise identification of expression changes across different experimental conditions. Collectively, these results validated ONT sequencing as a reliable method for isoform-level expression analysis and established a rigorous analytical foundation for subsequent differential expression studies. It should be noted that ONT sequencing has a higher per-read error rate and lower per-run throughput than short-read platforms such as Illumina, which can reduce sensitivity for lowly expressed transcripts and introduce basecalling noise [165]. To mitigate these limitations, gene-level quantification (rather than single-nucleotide resolution) was used throughout, and the differential expression signatures reported here are consistent with those obtained from microarray [26, 164]

and Illumina RNA-seq [27] studies using nearly identical RAW264.7/LPS experimental systems, providing cross-platform validation of the main findings. With this sequencing methodology validated, the next section characterises the global expression landscape through hierarchical clustering, offering insights into overall transcriptomic structure and reproducibility across experimental replicates.

Nanopore Sequencing

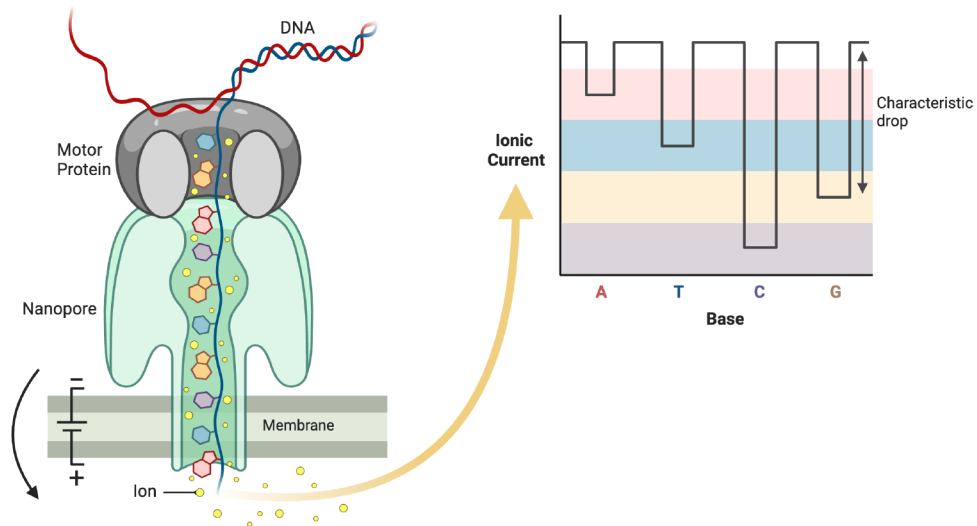


Figure 5.2: Nanopore sequencing principle used to generate the RNA seq dataset. Schematic overview of Oxford Nanopore Technology (ONT) sequencing used to produce the transcriptome data analyzed in this chapter. A motor protein unwinds the nucleic acid and drives a single strand through a biological nanopore toward the positive side of the membrane; each base produces a characteristic change in ionic current that is decoded to sequence. The graphic illustrates strand translocation, current modulation, and base calling at a high level; it is not drawn to scale. The figure was adapted from BioRender.

5.3 Global transcriptional landscape across conditions

To provide a comprehensive replicate-level overview of transcriptional responses across all experimental conditions, hierarchical clustering was performed on the top 50 most variable genes identified by Oxford Nanopore cDNA sequencing. Expression counts were derived using IsoQuant and TMM-normalised in edgeR prior to heat-map generation (Methods). The resulting heat map showed that LPS stimulation (DMSO+LPS) induced a broad increase in inflammatory gene expression relative to DMSO controls, visible as elevated normalised counts (high-intensity shading) across DMSO+LPS replicates (Figure 5.3). This LPS-driven pattern was largely preserved in the presence of 3′deoxyinosine (3DI, 200 μ M). In contrast, natural cordycepin (20 μ M), synthetic cordycepin (20 μ M), and the prodrug NUC-7738 (200 μ M) all produced reduced expression values (low-intensity shading) across the LPS-induced inflammatory gene set, consistent with attenuated transcriptional activation (Figure 5.3).

The hierarchical clustering further resolved these patterns at the sample level. Replicates treated with natural cordycepin, synthetic cordycepin, or NUC-7738 clustered together and away from the DMSO+LPS and 3DI+LPS groups, indicating a reproducible suppression of LPS-induced inflammatory gene expression by all three cordycepin-derived entities. By contrast, the transcriptional profile of 3DI-treated samples clustered more closely with DMSO+LPS than with any cordycepin-containing condition, supporting only modest anti-inflammatory activity at the tested dose. Overall, the top-50 heat map indicates that both natural and synthetic cordycepin, and NUC-7738 at higher exposure, consistently dampen LPS-induced transcriptional programmes across biological replicates, whereas 3DI largely tracks the DMSO+LPS profile. These global patterns motivated more granular gene-level comparisons using volcano plots for natural versus synthetic cordycepin (Figure 5.4) and NUC-7738 versus 3′deoxyinosine (Figure 5.9).

5.4 Source equivalence: natural vs synthetic cordycepin (20 μ M)

Gene-level differential expression (volcano)

To test whether natural and synthetic cordycepin preparations exert equivalent anti-inflammatory effects, we performed differential expression analysis in RAW264.7 macrophages treated with either compound at 20 μ M for 2 h, with LPS (1 μ g/mL) added after 1 h of compound treatment (i.e. in the presence of the compound) and cells stimulated for a further 1 h. Both preparations were processed through the same analytical workflow: RNA was profiled by Oxford Nanopore cDNA sequencing, quantified with IsoQuant, and normalised using TMM in edgeR, with differential expression called using the prespecified thresholds ($|\log_2\text{FC}| \geq 1$; $\text{FDR} \leq 0.05$). Natural cordycepin downregulated 373 genes and upregulated 29, while synthetic cordycepin downregulated 352 genes and upregulated 17 (Figure 5.4).

In both volcano plots, differentially expressed genes clustered predominantly on the negative $\log_2\text{FC}$ axis, with many transcripts exceeding the FDR threshold and encoded as green (downregulated) points, whereas relatively few red (upregulated) genes were detected. The close match in the number of DE genes, the magnitude of fold changes and the strong bias towards repression in both treatments support functional equivalence between natural and synthetic cordycepin at 20 μ M. Having established this equivalence at the gene level, we next examined whether the same convergence is observed at the level of biological pathways using IPA category enrichment analysis.

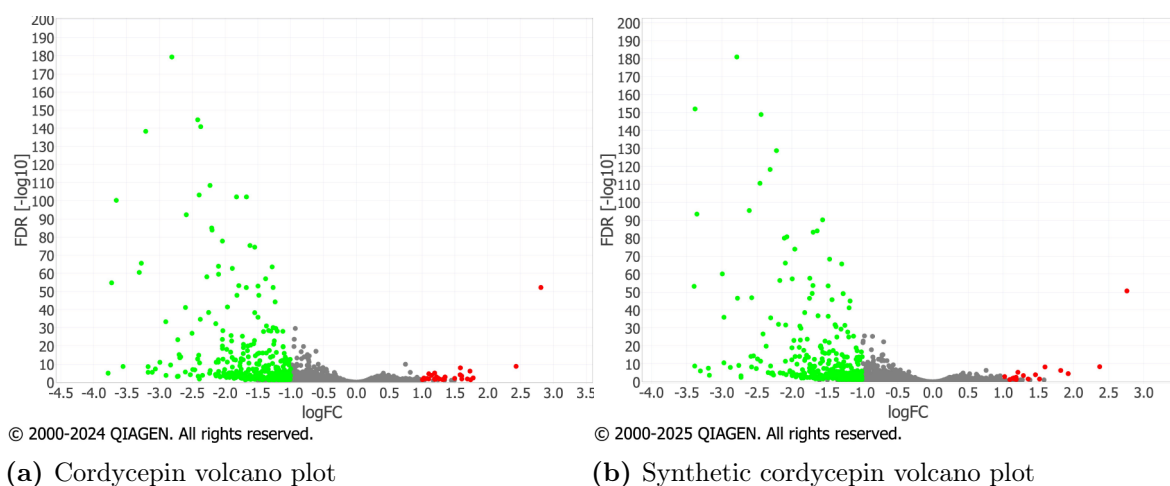
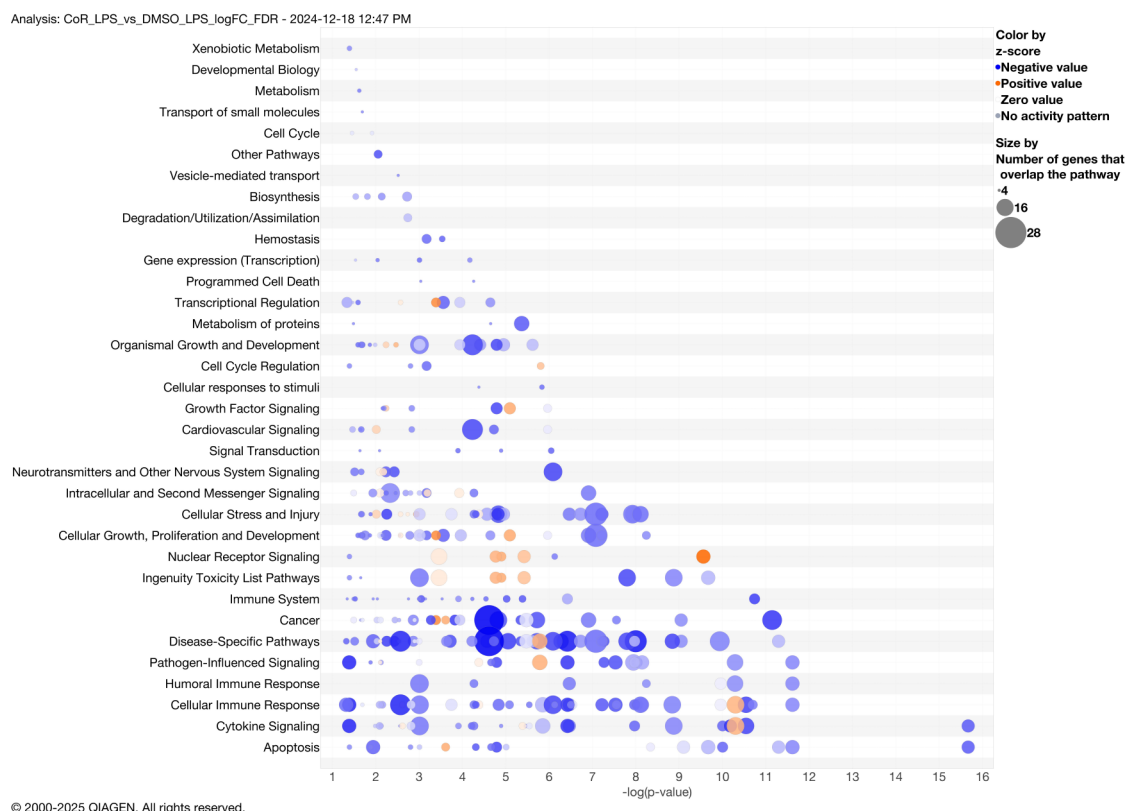


Figure 5.4: Cordycepin (natural or synthetic, 20 μM) broadly represses LPS-induced transcription. Volcano plots show differential expression in RAW264.7 cells treated with cordycepin (20 μM) or 0.02% DMSO for 2 h, with LPS (1 $\mu\text{g}/\text{mL}$) added 1 h after the start of compound treatment (i.e. in the presence of the compound) and cells stimulated for a further 1 h. Data: ONT cDNA sequencing $\rightarrow$ IsoQuant $\rightarrow$ edgeR (TMM). Testing: edgeR GLM (glmFit) + LRT (glmLRT) with BH FDR; thresholds: $|\log_2 \text{FC}| \geq 1$; $\text{FDR} < 0.05$; $n = 3$ per group. Panel A (natural cordycepin): 373 downregulated (green) and 29 upregulated (red) genes. Panel B (synthetic cordycepin): 352 down and 17 up. The x-axis shows the $\log_2$ fold change ($\log_2 \text{FC}$), defined as $\log_2(\text{compound} + \text{LPS} / \text{DMSO} + \text{LPS})$, so that negative values indicate genes repressed by the compound relative to LPS-only control. The y-axis shows $-\log_{10}(\text{FDR})$. Grey points denote genes not meeting significance thresholds.

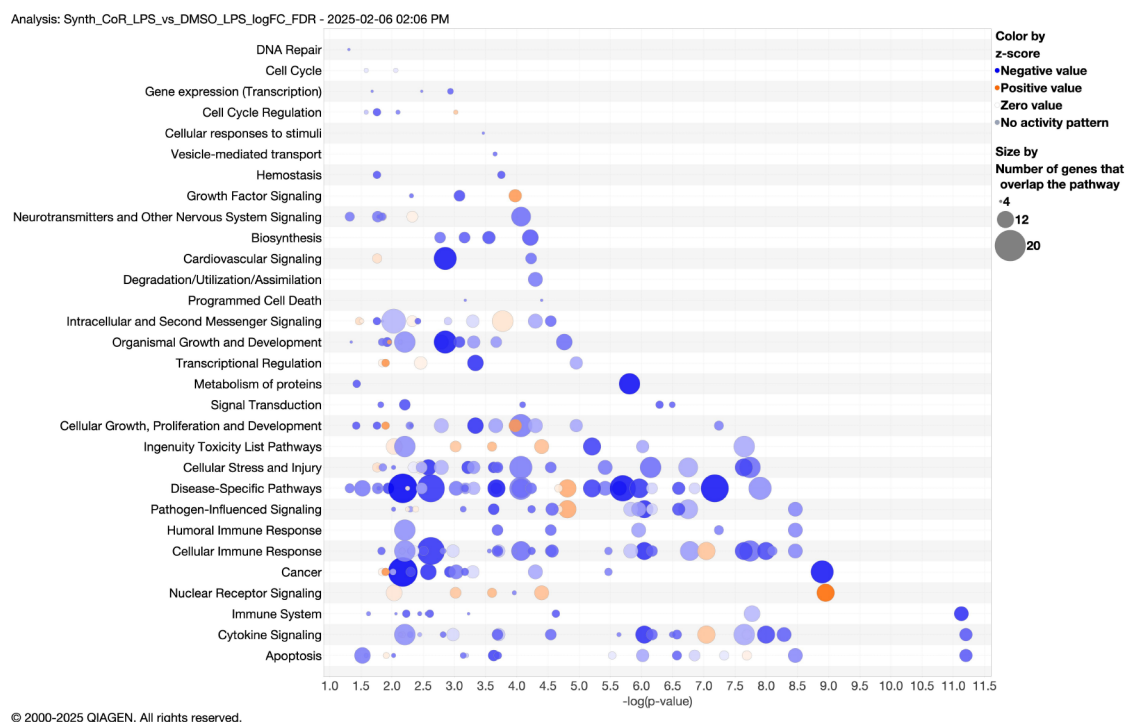
Category enrichment

To compare the effects of natural and synthetic cordycepin on biological pathways, Ingenuity Pathway Analysis (IPA) [166] category enrichment was performed on the differentially expressed gene lists derived from Figure 5.4. Both datasets were analysed with identical significance thresholds and plotting parameters to allow direct comparison. In each case, immune and cytokine-related categories were strongly enriched and exhibited predominantly negative z-scores, consistent with broad suppression of inflammatory programmes in LPS-stimulated macrophages.

The stacked bubble charts in Figure 5.5 illustrate this convergence. For natural cordycepin (panel A), immune system categories cluster at high $\log_{10}(\text{p-value})$ with large blue bubbles, indicating strong negative regulation. Synthetic cordycepin (panel B) shows a closely matching pattern, with similar immune, cytokine and related categories enriched to comparable significance and direction. Both treatments also enriched signal transduction and growth factor signalling pathways under the same colour scale and axis limits, facilitating like-for-like comparison of effect size and direction. These observations prompted a more explicit assessment of pathway directionality using IPA activation z-scores.



(a) Natural cordycepin



(b) Synthetic cordycepin

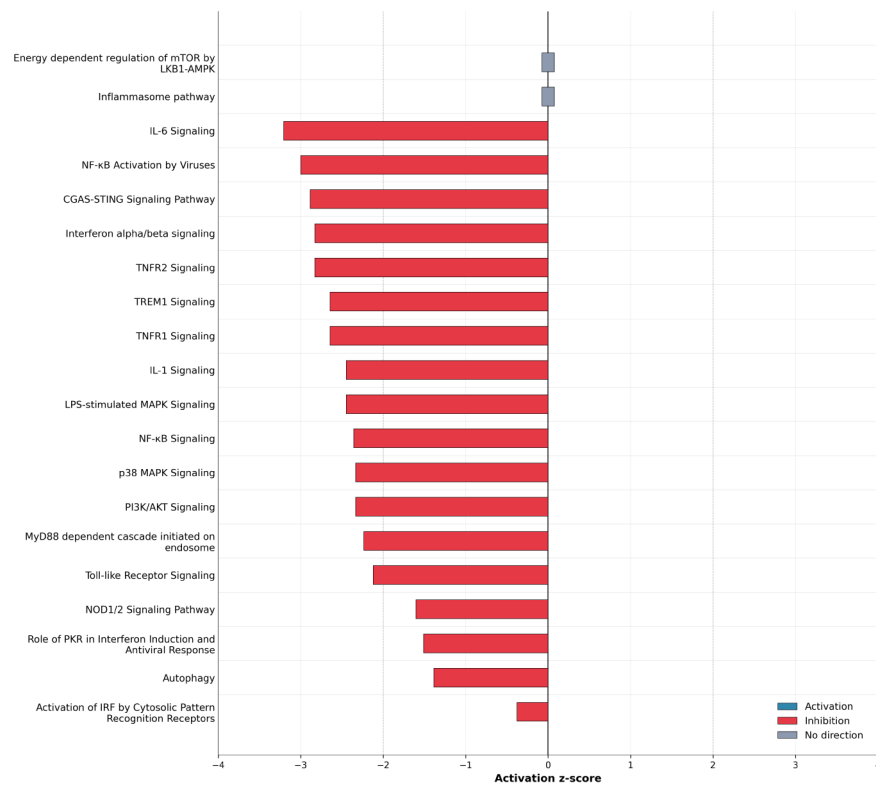
Figure 5.5: IPA bubble chart of (A) natural cordycepin and (B) synthetic cordycepin. The bubble size represents the number of genes overlapping each pathway, while the color indicates the pathway activation z-score (blue for negative/inhibited, orange for positive/activated, grey for neutral/no significant activation).

Pathway directionality (IPA z-scores)

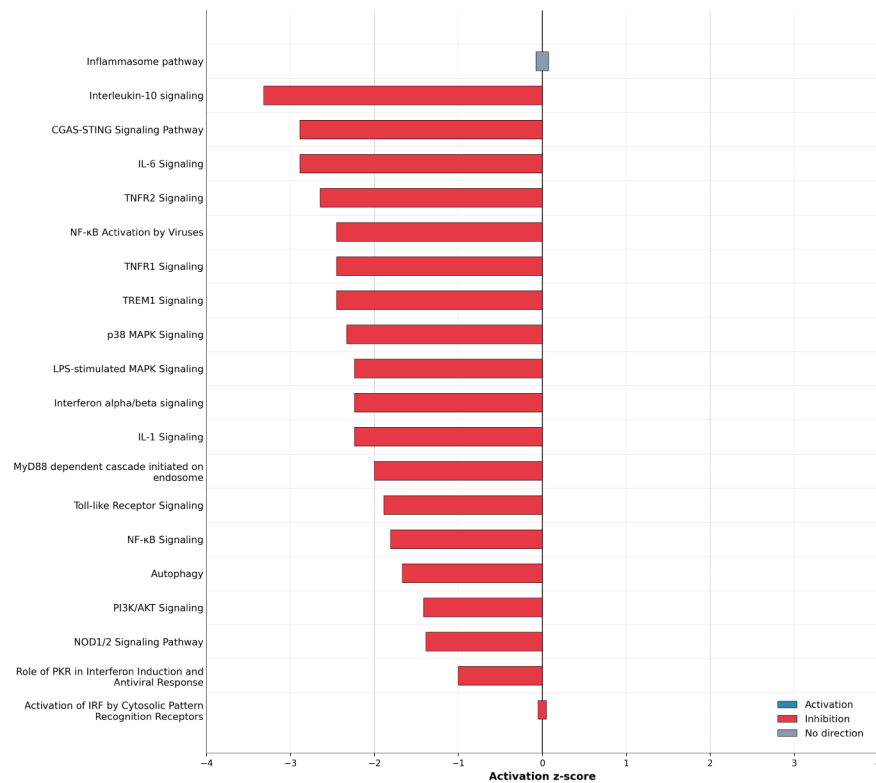
To assess the directionality and consistency of pathway-level effects between natural and synthetic cordycepin, IPA activation z-scores were computed from the differentially expressed gene lists using an FDR threshold of 0.05 and the predefined fold-change cut-off. This provided a quantitative summary of predicted pathway activation or inhibition under identical experimental and analytical conditions. Both preparations produced highly coherent negative z-scores across multiple inflammatory signalling pathways, with closely aligned pathway rankings and effect sizes, indicating functional equivalence at the pathway direction level.

The stacked bar charts in Figure 5.6 illustrate these inhibitory patterns. For natural cordycepin (panel A), key inflammatory modules, including the Inflammasome pathway, IL-6 signalling, cGAS–STING signalling, type I interferon (IFN- α/β) signalling, NF- κ B activation by viruses, and TNFR1/TNFR2 signalling, show prominent red bars extending to the left, indicating predicted inhibition. Synthetic cordycepin (panel B) closely mirrors these profiles, with similar inhibitory z-scores across the same pathways. Grey bars near zero indicate pathways that are significantly enriched but lack sufficient directional evidence for a reliable activation state prediction. Tables 5.1 and 5.2 list these pathways and the contributing genes. Taken together with the category-level enrichment in Figure 5.5, these z-score data show that natural and synthetic cordycepin are functionally equivalent in their ability to inhibit central inflammatory signalling modules.

Overall, these analyses indicate that cordycepin, irrespective of source, consistently suppresses immune and cytokine response categories alongside key signal transduction pathways in LPS-stimulated RAW264.7 macrophages. Given this close match, any residual contaminants in the natural preparation [162] are unlikely to make a major contribution to the observed biological effects. Having established this source-independent effect profile, we next applied the same pathway analysis framework to NUC-7738 and 3'-deoxyinosine (3DI) (Figures 5.9, 5.10 and 5.11) to compare the breadth and directionality of their biological effects.



(a) Natural cordycepin



(b) Synthetic cordycepin

Figure 5.6: IPA canonical pathway activation z-scores for (A) natural cordycepin and (B) synthetic cordycepin (both 20 μ M) in LPS-stimulated RAW264.7 macrophages. Each horizontal bar represents one IPA canonical pathway; bar direction and colour encode the predicted activation state: blue/rightward = predicted activation (+z), red/leftward = predicted inhibition (−z), grey = significantly enriched but lacking directional evidence. Pathways were selected by IPA based on significant overlap ($p < 0.05$, Fisher’s exact test) between the FDR-filtered DE gene list and the IPA canonical pathway gene sets.

To facilitate direct comparison, the z-scores from both preparations were combined into a single dual-cluster heatmap (Figure 5.7). The clustered layout confirms the close correspondence between natural and synthetic cordycepin: nearly all pathways cluster together with similar inhibitory z-scores, reinforcing the conclusion of functional equivalence at the pathway level.

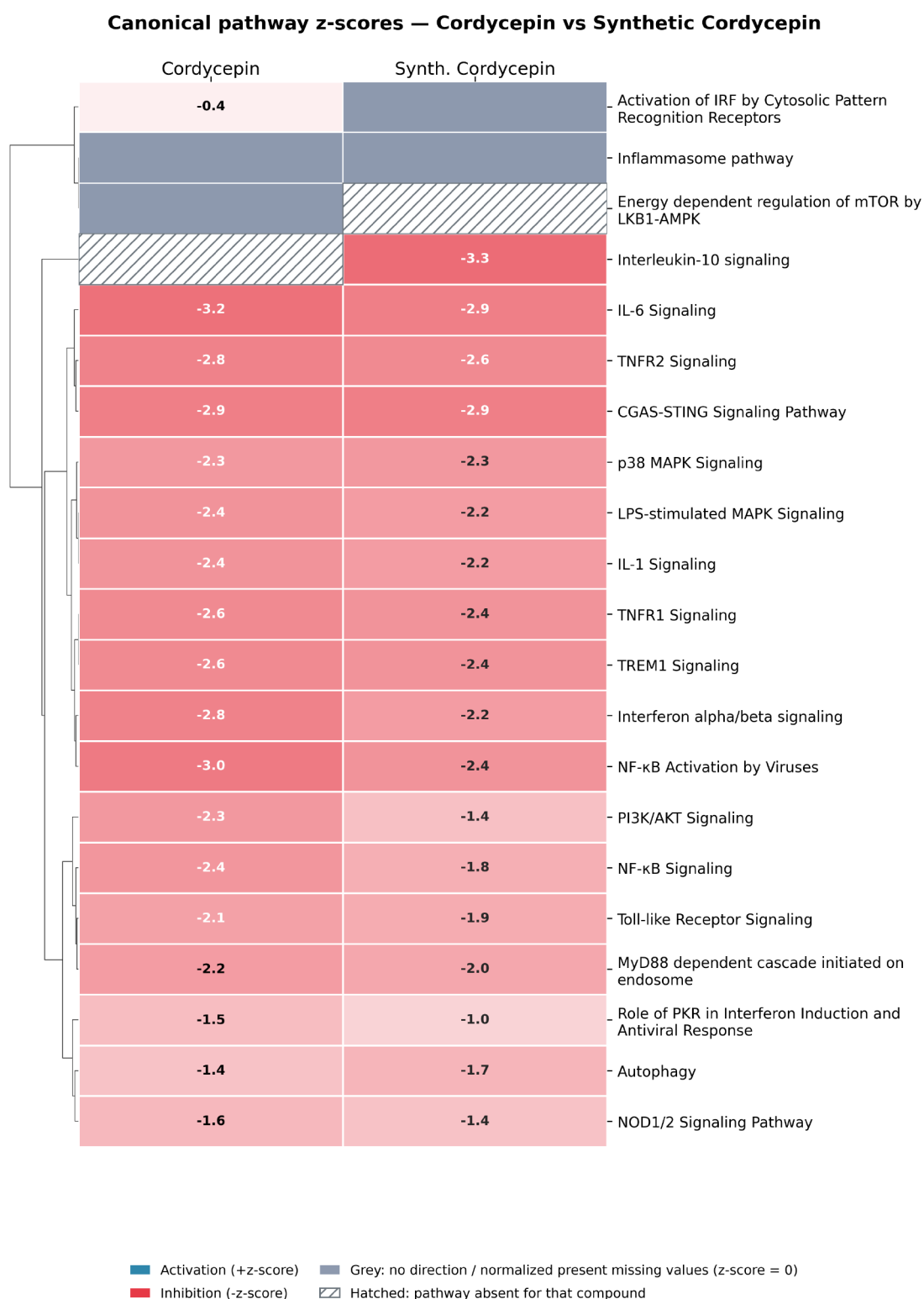
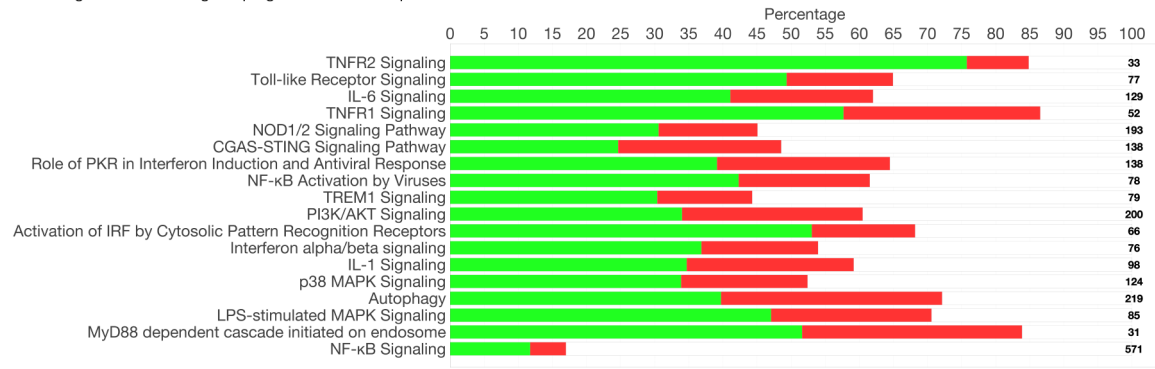


Figure 5.7: Dual-cluster heatmap of IPA canonical pathway activation z-scores for LPS-stimulated RAW264.7 macrophages following treatment with natural cordycepin or synthetic cordycepin (20 μ M, 2 h treatment; LPS added at 1 h, in the presence of compound, for a 1 h stimulation). Rows represent the union of pathways identified in the paired analyses shown in Figure 5.6, and columns represent the two compounds. Cell colour encodes the IPA activation z-score: blue indicates predicted activation (+z), red indicates predicted inhibition (-z). Grey cells denote pathways present in the IPA output but without a returned z-score; hatched cells denote pathways not reported for that compound. Hierarchical clustering was applied across pathways (dendrogram, left) to group similar response patterns and facilitate identification of commonalities and differences between the two preparations.

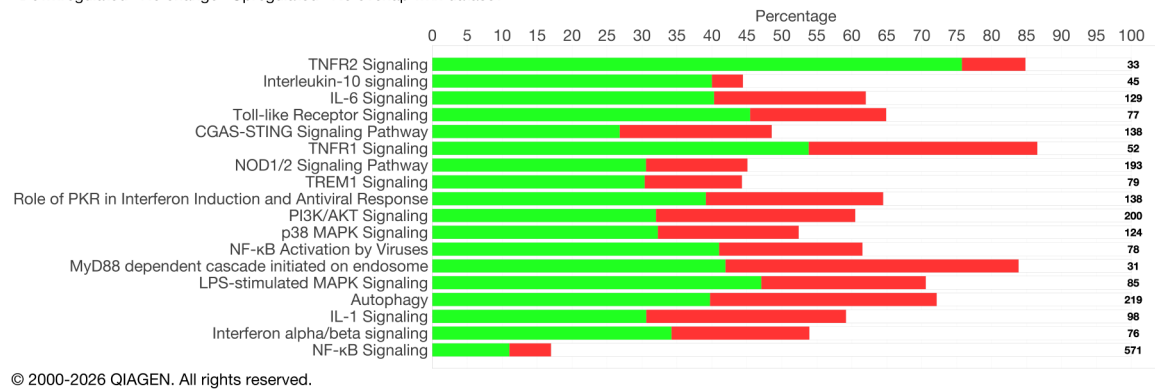
To further quantify the overlap between each enriched canonical pathway and the differentially expressed gene sets, horizontal stacked bar charts were generated in QIAGEN IPA for both natural and synthetic cordycepin (Figure 5.8). Each bar represents an IPA canonical pathway, with bar length reflecting the proportion of the pathway's total molecules recovered in the dataset and the numeric label to the right of each bar indicating the total number of molecules annotated to that pathway in the IPA knowledge base. Red segments indicate upregulated genes and green segments indicate downregulated genes within each pathway. The two preparations display highly similar pathway coverage patterns, further supporting the functional equivalence of natural and synthetic cordycepin at the transcriptional level.

Analysis: CoR_LPS_vs_DMSO_LPS_logFC_Pvalue - 2025-02-06 01:34 PM
 ■Downregulated ■No change ■Upregulated No overlap with dataset



(a) Natural cordycepin

Analysis: Synth_CoR_LPS_vs_DMSO_LPS_logFC_Pvalue - 2025-02-06 03:11 PM
 ■Downregulated ■No change ■Upregulated No overlap with dataset



(b) Synthetic cordycepin

Figure 5.8: IPA canonical pathway coverage for DEGs at 120 min following (A) natural cordycepin or (B) synthetic cordycepin treatment (20 μ M, 2 h treatment; LPS added at 1 h, in the presence of compound, for a 1 h stimulation) in RAW264.7 macrophages. Horizontal stacked bars show, for each enriched canonical pathway, the proportion of pathway molecules recovered in the dataset; the number to the right of each bar is the total number of molecules annotated to that pathway in the IPA knowledge base. Red segments indicate upregulated genes and green segments indicate downregulated genes within each pathway. Generated using the QIAGEN IPA Stacked Bar Chart tool.

Table 5.1: Canonical pathways (IPA) for FDR-filtered DEGs at 120 min (20 μ M cordycepin 1 h $\rightarrow$ LPS 1 h) versus DMSO $\rightarrow$ LPS in RAW264.7. Em dash indicates z-score not provided by IPA.

Pathway	z-score	Genes from the dataset
TNFR2 Signaling	-2.828	Birc3; MAP3K14; NFKB2; NFKBIA; NFKBID; NFKBIE; REL; RELA; RELB; TNFAIP3; TNFRSF1B; TNIP1; TRAF1
Toll-like Receptor Signaling	-2.121	EIF2AK2; IL1B; IRAK2; MAP3K14; NFKB2; NFKBIA; REL; RELA; RELB; TNFAIP3; TNFSF10; TRAF1; TRAF2; TRIM25
TNFR1 Signaling	-2.646	Birc3; MAP3K14; NFKB2; NFKBIA; NFKBID; NFKBIE; REL; RELA; RELB; TNFAIP3; TRAF1
IL-6 Signaling	-2.449	CEBPD; CHUK; FOS; GADD45B; HSP90AB1; HSP90B1; HSP90AA1; IKBKB; JAK2; MAPK14; MAPKAPK2; NFKB2; NFKBIA; RELA; RELB; RICTOR; SOCS3; STAT3; TNFAIP3
IL-1 Signaling	-2.449	FOS; HSP90AB1; HSP90B1; HSP90AA1; MAPK14; MAPKAPK2; RELA; RELB
NF- κ B Signaling	-2.000	BCL3; CHUK; DUSP2; GADD45B; IKBKB; MAP3K14; NFKB2; NFKBIA; NFKBID; NFKBIE; REL; RELA; RELB; RIPK2; TNFAIP3; TNFRSF1B; TNIP1; TRAF1

Continued on next page

Table 5.1 – *Continued from previous page*

Pathway	z-score	Genes from the dataset
NF- κ B Activation by Viruses	-1.897	CHUK; IKBKB; NFKB2; NFKBIA; RELA; RELB; TRAF2
TREM1 Signaling	-1.941	BCL3; CHUK; IKBKB; NFKB2; NFKBIA; RELA; RELB; TNFAIP3; TNFRSF1B; TRAF1
PI3K/AKT Signaling	-1.890	ARAF; CRKL; FOS; GNB4; GNG5; HSP90AB1; HSP90B1; HSP90AA1; IKBKB; JAK2; MAPK14; RELA; STAT3; TNFRSF1B
LPS-stimulated MAPK Signaling	-1.667	DUSP2; FOS; MAP3K14; MAPK14; MAPKAPK2; RELA; RELB
p38 MAPK Signaling	-1.414	FOS; HSP90AB1; HSP90B1; HSP90AA1; MAPKAPK2
Role of PKR in Interferon Induction and Antiviral Response	—	EIF2AK2; MAP3K14; NFKB2; NFKBIA; RELA; RELB
Interferon alpha/beta signaling	—	EIF2AK2; JAK2; NFKB2; NFKBIA; RELA; RELB; STAT3
Activation of IRF by Cytosolic Pattern Recognition Receptors	—	DDX58; EIF2AK2; HSP90AB1; HSP90B1; HSP90AA1; IRF3; NFKB2; NFKBIA; RELA
NOD1/2 Signaling Pathway	—	MAPK14; NFKB2; NFKBIA; RELA
CGAS-STING Signaling Pathway	—	DDX58; NFKB2; NFKBIA; RELA; TRIM25
Autophagy	—	HSP90AB1; HSP90B1; HSP90AA1; RICTOR
Energy dependent regulation of mTOR by LKB1-AMPK	—	HSP90AB1; HSP90B1; HSP90AA1; RICTOR

Continued on next page

Table 5.1 – *Continued from previous page*

Pathway	z-score	Genes from the dataset
Inflammasome pathway	—	HSP90AB1; HSP90B1; HSP90AA1
MyD88 dependent cascade initiated on endosome	—	IRAK2; NFKB2; NFKBIA; RELA

Table 5.2: Canonical pathways (IPA) for FDR-filtered DEGs at 120 min (20 μ M synthetic cordycepin 1 h $\rightarrow$ LPS 1 h) vs. DMSO $\rightarrow$ LPS in RAW264.7 (file: `z_score_120min_Synth-CoR_LPS_vs_DMSO_LPS_FDR_filtered.xlsx`). Em dash indicates z-score not provided by IPA.

Pathway	z-score	Genes from the dataset
Toll-like Receptor Signaling	-1.89	IL1A; IL1B; MAP3K14; NFKBIA; REL; TICAM1; TICAM2; TNF; TNFAIP3; TRAF1; UBC
TNFR2 Signaling	-2.65	MAP3K14; NFKBIA; NFKBID; NFKBIE; REL; TNF; TNFAIP3; TNFRSF1B; TRAF1; TRAF2
TNFR1 Signaling	-2.45	MAP3K14; NFKBIA; NFKBID; NFKBIE; REL; TNF; TNFAIP3; TRAF1
NF- κ B Signaling	-1.81	BAX; CCL2; CCL5; CD40; CD83; CXCL10; HSP90AA1; ICAM1; IL1A; IL1B; IL6; NFKBIA; PTGS2; SELE; TNF
IL-6 Signaling	-2.89	CCL2; CCL5; IFNAR2; IFNGR2; IL1A; IL1B; IL6; JAK2; NFKBIA; OAS1; PIK3CD; PTGS2; TNF
IL-1 Signaling	-2.24	IL1A; IL1B; IL1R1; IRAK2; MAP3K14; TNF
p38 MAPK Signaling	-2.33	GNA12; GNA13; IL1A; IL1B; MAP3K14; PTGS2; REL; TNF; TRAF1
LPS-stimulated MAPK Signaling	-2.24	ELK1; GNA12; GNA13; MAP3K14; REL; TNF

Continued on next page

Table 5.2 – *Continued from previous page*

Pathway	z-score	Genes from the dataset
PI3K/AKT Signaling	-1.41	BAX; CD40; CSF1; IFNGR2; IL6; JAK2; PIK3CD; PTGS2; REL; TNF; TRAF1
Interleukin-10 signaling	-3.32	CCL2; CCL5; CSF1; CSF3; IL10RA; IL1A; IL1B; PTAFR; PTGES; PTGIS; REL
TREM1 Signaling	-2.45	BAX; CCL2; CCL5; ELANE; IL1A; IL1B; NFKBIA; REL; TNF
NOD1/2 Signaling Pathway	-1.39	BIRC3; BMX; CARD9; HSP90AA1; IL1A; IL1B; IRAK2; MAP3K14; NFKBIA; NFKBID; NFKBIE; REL; RIPK2; TNF
Role of PKR in Interferon Induction and Antiviral Response	-1.00	DDX58; EIF2AK2; HSP90AA1; IFNGR2; IL6; IRAK2; OAS1; REL; RIPK2; TNF; TRAF1
Activation of IRF by Cytosolic Pattern Recognition Receptors	0.00	DDX58; HSP90AA1; IFNGR2; IRAK2; OAS1; REL; RIPK2
CGAS-STING Signaling Pathway	-2.89	CCL2; CCL5; CD40; CXCL10; IFNGR2; IL1A; IL1B; IL6; NFKBIA; PIK3CD; REL; TNF; TRAF1
Interferon alpha/beta signaling	-2.24	IFNAR2; JAK2; OAS1; PIK3CD; STAT1
MyD88 dependent cascade initiated on endosome	-2.00	IRAK2; NFKBIA; TICAM2; TNF

Continued on next page

Table 5.2 – *Continued from previous page*

Pathway	z-score	Genes from the dataset
Autophagy	-1.67	ATG7; BAX; CD40; HSP90AA1; IFNGR2; IL1A; IL1B; MAP3K14; NFKBIA; REL
Inflammasome pathway	—	IL1B; TNF
NF- κ B Activation by Viruses	-2.45	BAX; HSP90AA1; NFKBIA; REL; SELE; TNF; TRAF1

5.5 Comparator evaluation: NUC7738 vs. 3DI (200 μ M)

Gene-level differential expression (volcano)

To compare the breadth and direction of transcriptomic responses between NUC-7738 and 3'deoxyinosine (3DI), both compounds were tested at 200 μ M under identical LPS challenge conditions. Dosing, timing and analytical thresholds were matched, and differential expression was assessed using edgeR generalised linear model likelihood ratio tests with Benjamini–Hochberg FDR correction. NUC-7738 modulated substantially more genes than 3DI, with 501 DE genes (473 downregulated and 28 upregulated) versus 108 DE genes for 3DI (86 downregulated and 22 upregulated). Both compounds showed a predominantly repressive effect on LPS-induced genes (Figure 5.9).

The volcano plots visualise this difference in potency. Panel A (NUC-7738) shows a dense cluster of green (downregulated) genes to the left of the $-1 \log_2\text{FC}$ threshold, many with high $-\log_{10}(\text{FDR})$ values, whereas panel B (3DI) is noticeably sparser, with fewer genes reaching significance and lower overall $-\log_{10}(\text{FDR})$ values. In both panels, relatively few red (upregulated) genes are detected, indicating shared overall directionality despite the difference in magnitude. These data indicate that, at 200 μ M, NUC-7738 drives broader and deeper repression of LPS-induced transcriptional programmes than 3DI. To identify which biological programmes underlie this divergence, we next examined pathway-level enrichment using IPA bubble charts.

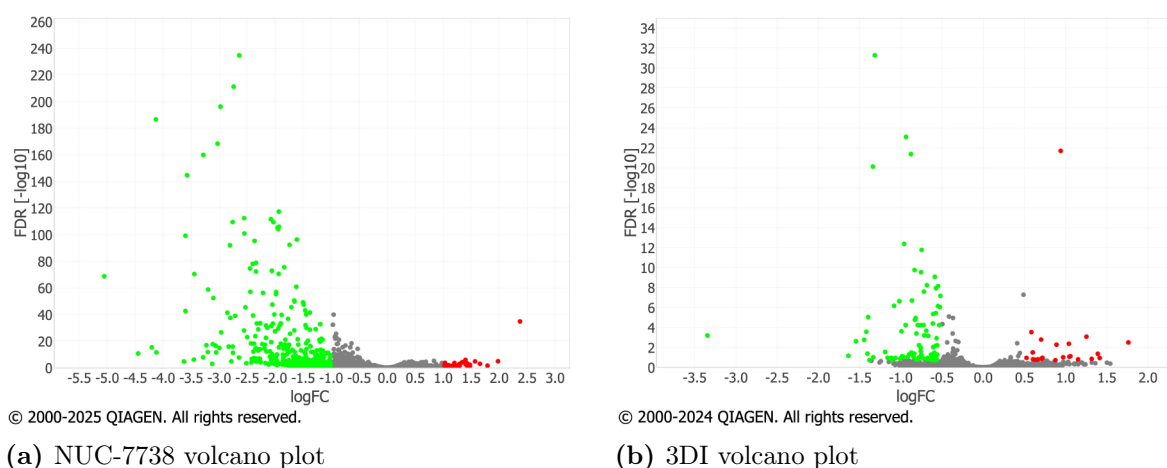
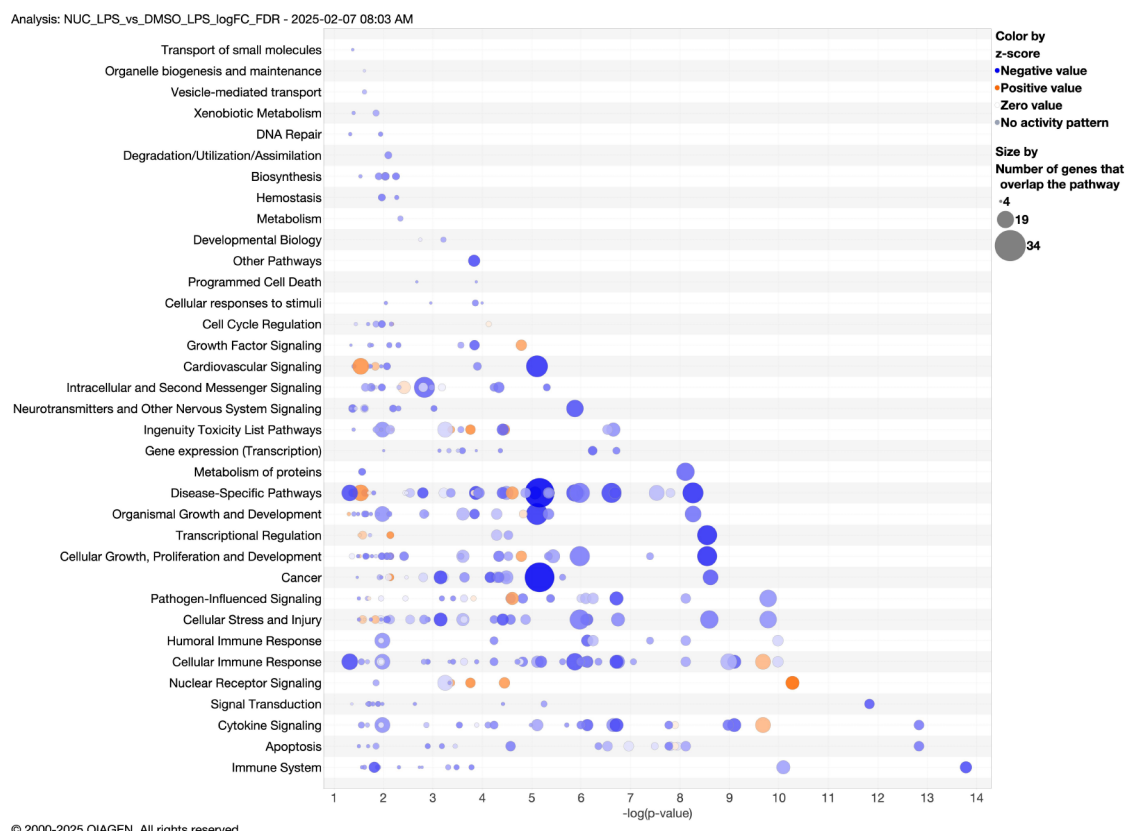


Figure 5.9: NUC-7738 (200 μ M) shows a broader, predominantly negative DE profile than 3'-deoxyinosine (200 μ M). RAW264.7 macrophages were treated for 2 h with NUC-7738 (200 μ M), 3DI (200 μ M), or 0.02% DMSO, with LPS (1 μ g/mL) added 1 h after the start of compound treatment (i.e. in the presence of the compound) and cells stimulated for a further 1 h. ONT cDNA reads were quantified by IsoQuant and analysed in edgeR (TMM). DE testing used edgeR GLM (glmFit) + LRT (glmLRT) with BH FDR; significance thresholds: $|\log_2 \text{FC}| \geq 1$; $\text{FDR} \leq 0.05$. Panel A (NUC-7738): 473 downregulated and 28 upregulated genes. Panel B (3DI): 86 down and 22 up. Each dot represents one gene (green = downregulated; red = upregulated; grey = not significant). The x-axis shows the $\log_2$ fold change ($\log_2 \text{FC}$), defined as $\log_2(\text{compound} + \text{LPS} / \text{DMSO} + \text{LPS})$; the y-axis shows $-\log_{10}(\text{FDR})$; $n = 3$ per group. Note: 3DI stock contained cordycepin contamination (purity assay, Chapter 3).

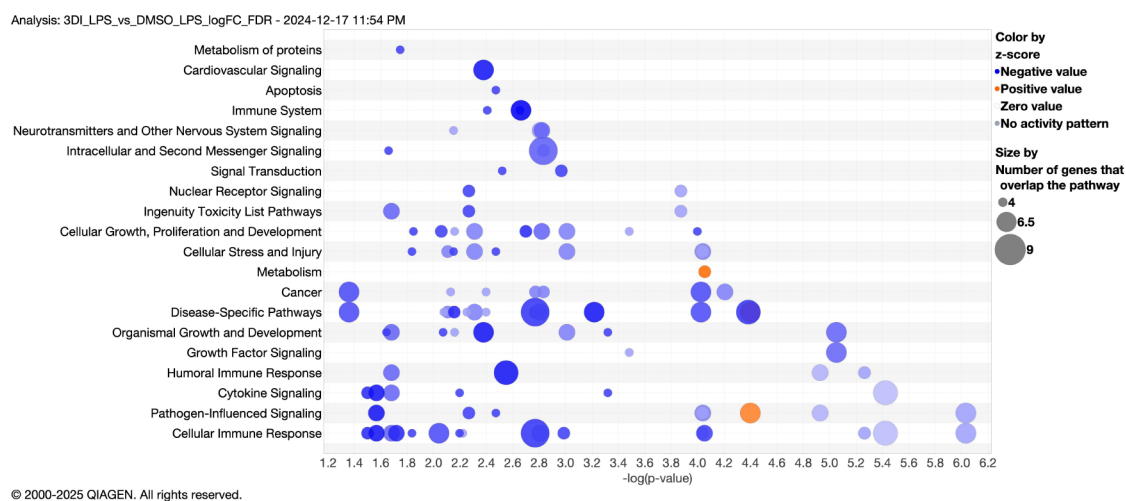
Category enrichment

To compare the pathways affected by NUC-7738 and cordycepin's bioavailable metabolite 3DI, and to assess their relative enrichment strength, IPA category enrichment was performed on the DE gene lists shown in Figure 5.9. Identical plotting parameters and significance thresholds were used to allow direct comparison. Both compounds enriched immune and cytokine-related categories with negative z-scores, consistent with suppression of inflammatory pathways. However, NUC-7738 showed more extensive and stronger enrichment, with more significant categories and larger bubbles at higher $\log_{10}(\text{p-value})$ values.

The stacked bubble charts in Figure 5.10 highlight these differences. For NUC-7738 (panel A), immune-related categories such as Immune System, Cytokine Signaling, Cellular and Humoral Immune Response, and Pathogen-Influenced Signaling are represented by large blue bubbles, indicating strong predicted inhibition. Additional enrichment is seen in Signal Transduction, Growth Factor Signaling, and Intracellular and Second Messenger Signaling pathways. In contrast, 3DI (panel B) engages similar categories but with consistently smaller bubbles and lower $-\log_{10}(\text{p-value})$ values, in line with the smaller DE gene set observed for 3DI in Figure 4. Thus, while 3DI retains directional concordance with NUC-7738 at the category level, NUC-7738 clearly shows greater pathway breadth and enrichment strength. To capture pathway directionality and magnitude more explicitly, we next examined IPA activation z-score bar charts.



(a) NUC-7738



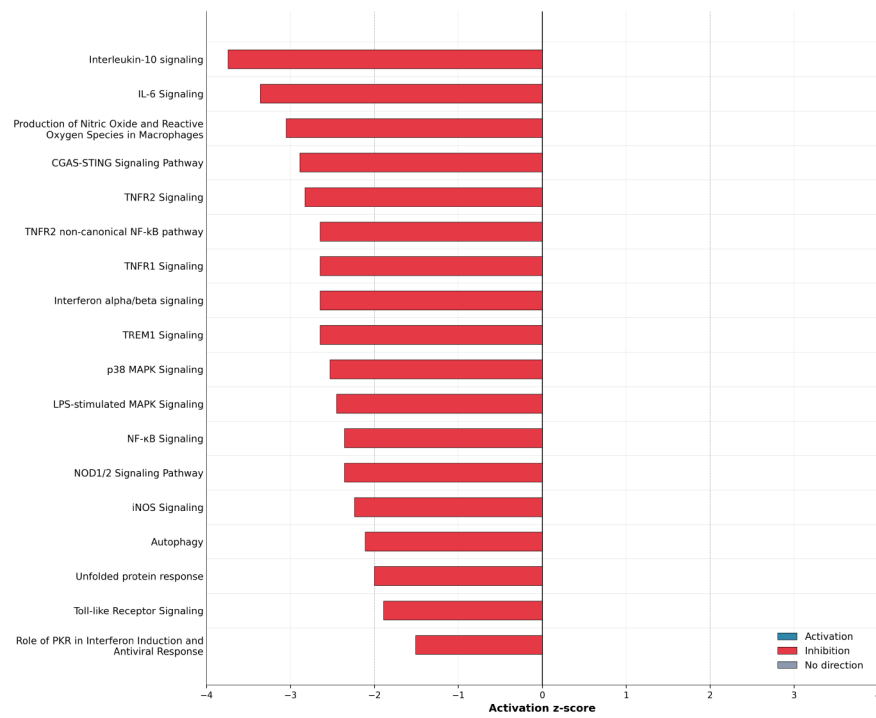
(b) 3DI

Figure 5.10: Canonical pathway analysis of (A) NUC-7738 and (B) 3' deoxyinosine. Bubble size represents the number of genes within each pathway, while color indicates pathway activation status: negative (blue, inhibited), positive (orange, activated), or neutral (grey).

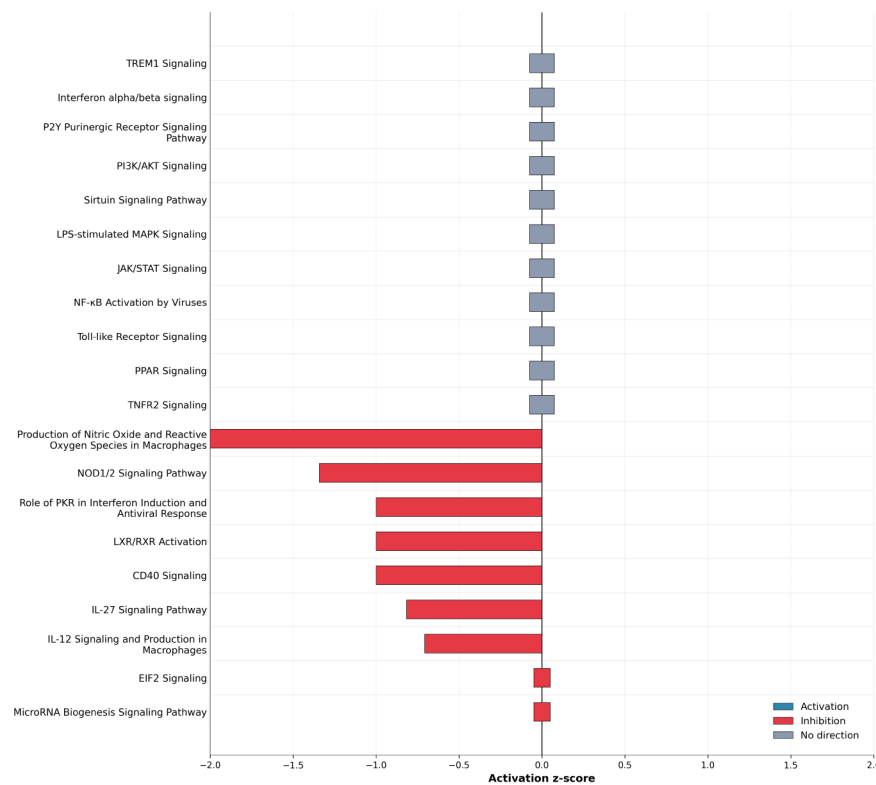
Pathway directionality (IPA z-scores)

To compare pathway-level directionality and effect size between NUC-7738 and 3DI, IPA activation z-scores were calculated from DE gene lists filtered by FDR and fold-change thresholds under identical experimental conditions. These z-scores were visualised as stacked bar charts on a common scale to enable direct quantitative comparison. NUC-7738 displayed a coherent inhibitory profile across multiple inflammatory signalling cascades, whereas 3DI produced a weaker and more mixed response.

As shown in Figure 5.11, NUC-7738 (panel A) yields prominent leftward red bars (inhibition) across key immune modules, including IL-10 signalling, IL-6 signalling, the cGAS–STING pathway, interferon signalling, TNFR2 non-canonical NF- κ B signalling, and LPS-stimulated MAPK signalling. In contrast, 3DI (panel B) shows a much sparser pattern: some pathways, such as Production of nitric oxide and reactive oxygen species in macrophages and NOD-like receptor signalling, retain negative z-scores, but many others sit near zero or show slightly positive (blue) values, indicating minimal or potentially weak activating effects. Tables 5.3 and 5.4 summarise these pathways and their associated genes. Overall, NUC-7738 engages a broader and more potent inhibitory programme than 3DI at the pathway level. In the context of Chapter 3, where cordycepin contamination was detected in the 3DI stock, these results are consistent with the interpretation that much of the measured biological activity under 3DI treatment may be driven by trace cordycepin rather than by 3DI itself.



(a) NUC-7738



(b) 3DI

Figure 5.11: IPA canonical pathway activation z-scores for (A) NUC-7738 (200 μ M) and (B) 3'-deoxyinosine (3DI, 200 μ M) in LPS-stimulated RAW264.7 macrophages. Bar direction and colour encode the predicted activation state: blue/rightward = activation (+z), red/leftward = inhibition (−z), grey = significantly enriched but lacking directional evidence. Pathways were selected by IPA based on significant gene overlap ($p < 0.05$, Fisher's exact test; $|\log_2 \text{FC}| \geq 1$; $\text{FDR} \leq 0.05$). NUC-7738 shows broad inhibition across inflammatory and innate-immune modules (IL-6, cGAS–STING, Interferon, TNFR, NF- κ B), whereas 3DI displays a sparser, weaker pattern with many pathways absent or lacking directional prediction.

As for the cordycepin comparison above, the z-scores for NUC-7738 and 3DI were combined into a single dual-cluster heatmap (Figure 5.12). The contrast between the two compounds is striking: NUC-7738 shows deep inhibitory z-scores across a broad set of inflammatory and innate-immune pathways, whereas 3DI columns are dominated by grey (no z-score returned) and hatched (pathway absent) cells, consistent with its minimal transcriptional impact. The few pathways that do show z-scores under 3DI cluster with the weaker end of the NUC-7738 response, consistent with residual cordycepin contamination in the 3DI stock rather than intrinsic 3DI activity.

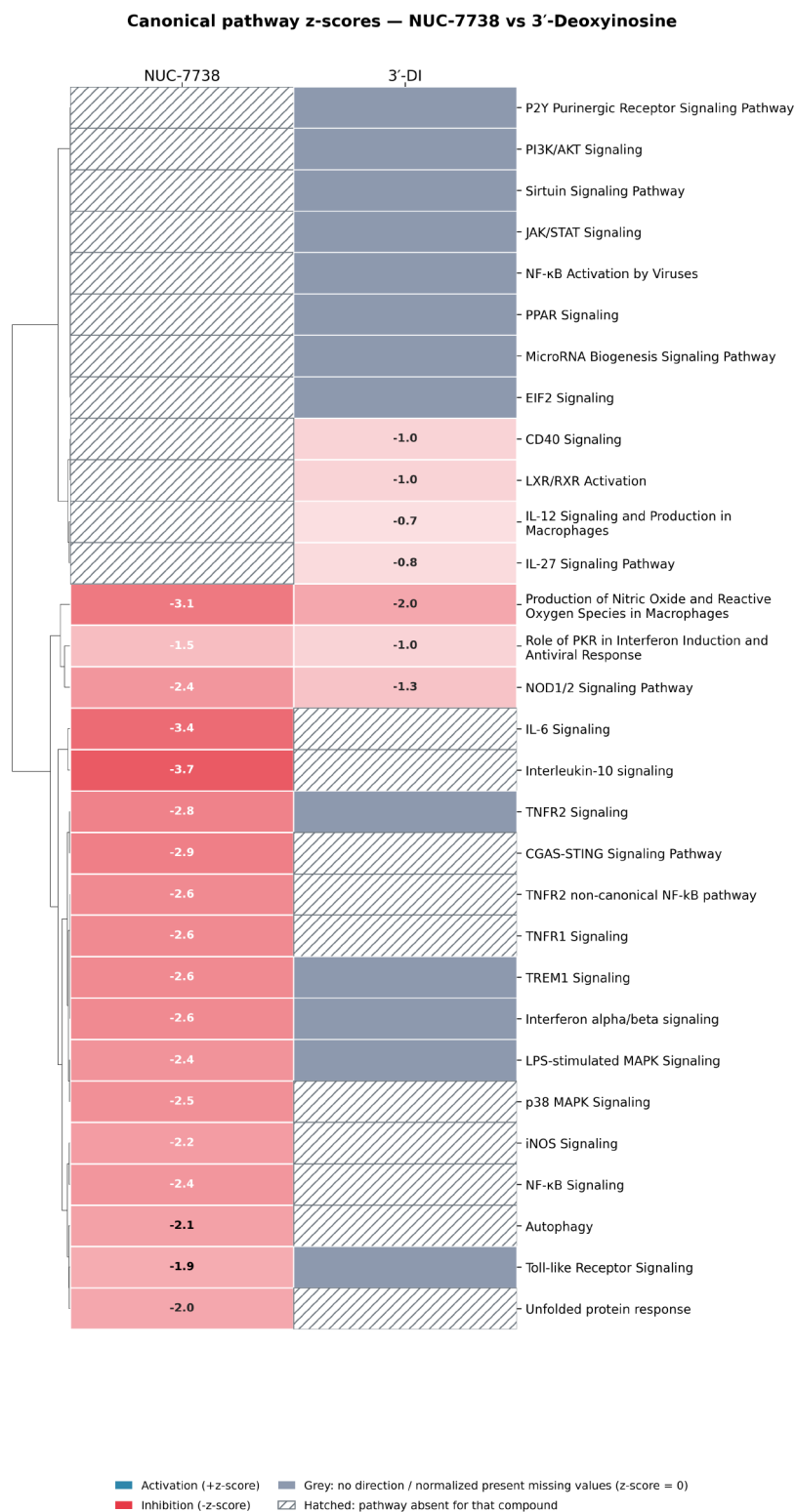
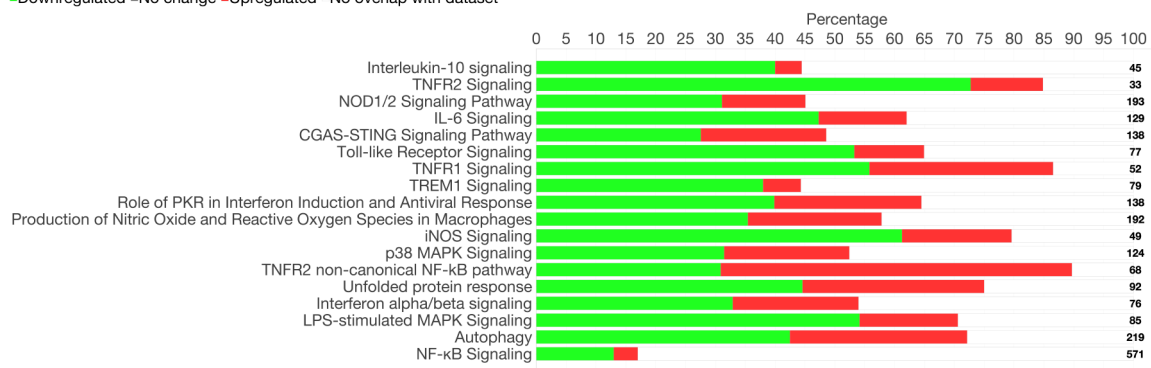


Figure 5.12: Dual-cluster heatmap of IPA canonical pathway activation z-scores for LPS-stimulated RAW264.7 macrophages treated with NUC-7738 or 3'-deoxyinosine (3DI) (200 μM, 2 h treatment; LPS added at 1 h, in the presence of compound, for a 1 h stimulation). Rows represent the union of pathways from the paired analyses in Figure 5.11; columns represent the two compounds. Cell colour encodes the activation z-score: blue = activation (+z), red = inhibition (-z). Grey cells denote pathways without a returned z-score; hatched cells denote pathways not reported for that compound. Hierarchical clustering (dendrogram, left) groups similar response patterns. The heatmap highlights the broad inhibitory profile of NUC-7738 across inflammatory and innate-immune modules, in contrast to the sparse and weak response under 3DI.

As with the natural versus synthetic cordycepin comparison, horizontal stacked bar charts were generated in QIAGEN IPA to quantify the pathway coverage of NUC-7738 and 3DI at 120 min (Figure 5.13). Each bar represents an IPA canonical pathway, with bar length reflecting the proportion of the pathway's molecules recovered in the dataset and the numeric label to the right of each bar indicating the total number of molecules annotated to that pathway in the IPA knowledge base. Red segments indicate upregulated genes and green segments indicate downregulated genes within each pathway. NUC-7738 returns broad, well-populated pathway coverage across inflammatory and innate-immune modules, whereas 3DI returns a much sparser set of pathways with lower molecule coverage, consistent with its minimal transcriptional impact at this dose.

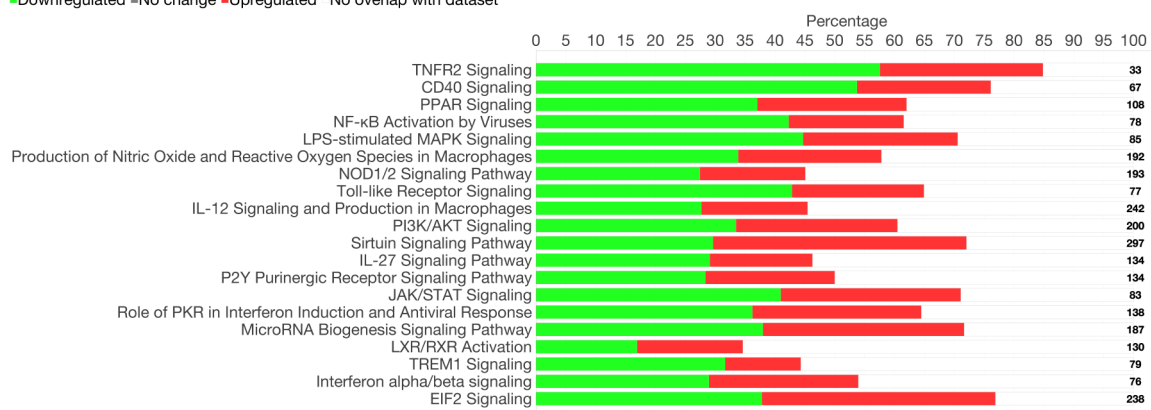
Analysis: NUC_LPS_vs_DMSO_LPS_logFC_Pvalue - 2025-02-07 09:05 AM
 ■Downregulated ■No change ■Upregulated No overlap with dataset



© 2000-2026 QIAGEN. All rights reserved.

(a) NUC-7738

Analysis: 120min 3DI_LPS vs DMSO_LPS_logFC_Pvalue - 2024-12-17 09:51 PM
 ■Downregulated ■No change ■Upregulated No overlap with dataset



© 2000-2026 QIAGEN. All rights reserved.

(b) 3DI

Figure 5.13: IPA canonical pathway coverage for DEGs at 120 min following (A) NUC-7738 or (B) 3'-deoxyinosine (3DI) treatment (200 μM, 2 h treatment; LPS added at 1 h, in the presence of compound, for a 1 h stimulation) in RAW264.7 macrophages. Horizontal stacked bars show, for each enriched canonical pathway, the proportion of pathway molecules recovered in the dataset; the number to the right of each bar is the total number of molecules annotated to that pathway in the IPA knowledge base. Red segments indicate upregulated genes and green segments indicate downregulated genes within each pathway. Generated using the QIAGEN IPA Stacked Bar Chart tool.

Table 5.3: Selected canonical pathways (120 min NUC-7738 $\rightarrow$ LPS, RAW264.7). Genes are IPA "Molecules" from the dataset.

Pathway	z-score	Genes from the dataset
Interleukin-10 signaling	-3.742	CCL2; CCL5; CCR1; CSF1; CSF3; ICAM1; IL10RA; IL1A; IL1B; LIF; PTAFR; PTGS2; TNF; TNFRSF1B
TNFR2 Signaling	-2.828	BIRC3; FOS; MAP3K14; NFKBIA; NFKBID; NFKBIE; REL; TNF; TNFAIP3; TNFRSF1B; TRAF1; TRAF2
TNFR1 Signaling	-2.646	BIRC3; FOS; ICAM1; IRF1; MAP3K14; NFKBIA; NFKBID; NFKBIE; TNFAIP3; TRAF1
Toll-like Receptor Signaling	-1.890	FOS; IL1A; IL1B; MAP3K14; NFKBIA; REL; TICAM1; TICAM2; TNF; TNFAIP3; TRAF1; UBC
TREM1 Signaling	-2.646	CCL2; CD40; CD83; CXCL2; ICAM1; IL1B; ITGA5; NLRP3; REL; TNF
NOD1/2 Signaling Pathway	-2.357	ATG5; BCL10; BIRC3; CXCL10; CXCL3; FOS; ICAM1; IRF1; NFKBIA; NOD1; REL; TNF; TRAF1
IL-6 Signaling	-2.000	IL1A; IL1B; LIFR; MAP3K14; NFKBIA; TNF; TNFRSF1B
NF- κ B Signaling	-2.357	NFKBIA; REL; TNF; TNFAIP3
TNFR2 non-canonical NF- κ B pathway	-2.000	BIRC3; MAP3K14; NFKBIA; TNFAIP3; TRAF1; TRAF2
LPS-stimulated MAPK Signaling	-2.449	FOS; MAP3K14; NFKBIA; TNF

Continued on next page

Table 5.3 – *Continued from previous page*

Pathway	z-score	Genes from the dataset
p38 MAPK Signaling	-2.530	ATF3; ATF4; DUSP1; DUSP2; HSPB1; HSPA5; IL1B; MAP3K14; NFKBIA; TNF; TRAF1
Production of Nitric Oxide and Reactive Oxygen Species in Macrophages	-1.342	AKT3; ATG5; CCL2; ICAM1; NOS2; REL; SOD2; TNF; UBC
iNOS Signaling	-2.000	GCH1; NOS2; TNF
Role of PKR in Interferon Induction and Antiviral Response	-1.508	ATF3; DDX58; EIF2AK2; FOS; IFIT2; IRF1; NFKBIA; TNF; TRAF1; TRAF2
Interferon alpha/beta signaling	-2.646	DDX58; IFIT2; IRF1; STAT1; STAT2
CGAS-STING Signaling Pathway	-2.887	DDX58; NFKBIA; REL; TMEM173; TNF; UBC
Autophagy	-2.111	ATG10; ATG12; ATG5; ATG7; BCL2; FOS; HSP90AA1; HSPA1A/HSPA1B; IRF1; RPS6KB1
Unfolded protein response	-2.000	ATF4; HSP90AA1; HSPA5

Table 5.4: Selected IPA Canonical Pathways (120 min 3'deoxyinosine treatment, with LPS added at 60 min in the presence of the compound for a 1 h stimulation). Blank z-scores indicate that IPA did not report an activation z-score.

Pathway	z-score	Genes from the dataset
Role of PKR in Interferon Induction and Antiviral Re- sponse	-1	ATF3; ELP1; IFNB1; IRF1; NLRP3; PDGFA; REL
Production of Nitric Oxide and Reactive Oxygen Species in Macrophages	-2	ELP1; IRF1; MAP3K8; PIK3C2A; REL; TNFRSF1B
IL-12 Signaling and Produc- tion in Macrophages TNFR2 Signaling	-0.707	ELP1; IFNB1; IRF1; MAP3K8; NFATC1; PIAS2; PIK3C2A; REL REL; TNFAIP3; TNFRSF1B; TRAF1
CD40 Signaling	-1	ELP1; PIK3C2A; REL; TNFAIP3; TRAF1
Toll-like Receptor Signaling LPS-stimulated MAPK Signal- ing		REL; TNFAIP3; TRAF1; UBC ELP1; PIK3C2A; RAP2B; REL
IL-27 Signaling Pathway	-0.816	IFNB1; IRF1; NLRP3; PIK3C2A; REL; TNFRSF1B
NF- κ B Activation by Viruses		ELP1; PIK3C2A; RAP2B; REL
NOD1/2 Signaling Pathway	-1.34	ELP1; IFNB1; MAP3K8; NLRP3; SLC15A4; TNFAIP3
LXR/RXR Activation	-1	ABCA1; ABCG1; LDLR; REL; TN- FRSF1B
PPAR Signaling		ELP1; PDGFA; RAP2B; REL; TN- FRSF1B
JAK/STAT Signaling		PIAS2; PIK3C2A; RAP2B; REL

Continued on next page

Table 5.4 – *Continued from previous page*

Pathway	z-score	Genes from the dataset
PI3K/AKT Signaling		ELP1; GAB2; MAP3K8; RAP2B; REL
TREM1 Signaling		CCL2; PIK3C2A; REL
Sirtuin Signaling Pathway		ABCA1; BCL2L11; CPT1A; MYC; NDRG1; REL
EIF2 Signaling	0	ATF3; MYC; PIK3C2A; RAP2B
Interferon alpha/beta signaling		IFNB1; IRF1; UBC
P2Y Purinergic Receptor Signaling Pathway		MYC; PIK3C2A; RAP2B; REL
MicroRNA Biogenesis Signaling Pathway	0	IFNB1; MYC; PIK3C2A; RAP2B

5.6 Discussion

Across all conditions, the transcriptomic data indicate that natural and synthetic cordycepin (20 μ M) behaved very similarly in LPS-stimulated RAW264.7 macrophages, producing broad repression of LPS-induced transcripts and consistent inhibition of immune and cytokine pathways in IPA (Figs. 5.3, 5.4, 5.5, and 5.6). At 200 μ M, NUC-7738 generated a similar but more pronounced inhibitory signature than 3'-deoxyinosine (3DI, 200 μ M), whereas 3DI showed weaker and partially mixed effects (Figs. 5.3, 5.9, 5.10, and 5.11). Across the active treatments, IPA consistently returned negative activation z -scores in PI3K/AKT/mTOR-, autophagy- and NF- κ B-linked pathways (Fig. 5.6), in line with earlier studies showing that cordycepin dampens growth-factor and inflammatory signalling [25, 28]. IPA also highlighted the "Role of PKR in Interferon Induction and Antiviral Response" module with negative directionality for several cordycepin analogues (Figs. 5.6 and 5.11), suggesting that stress- and interferon-associated nodes sit within the broader cordycepin response network.

Natural and synthetic cordycepin produced closely matched volcano plots dominated by down-regulation (natural: 373 down/29 up; synthetic: 352 down/17 up at $|\log_2 \text{FC}| \geq 1$, $\text{FDR} < 0.05$), indicating that the anti-inflammatory action is intrinsic to cordycepin rather than to co-purified constituents (Fig. 5.4). IPA category enrichment reinforced this: both sources generated large, right-shifted immune and cytokine categories with negative activity direction (Signal Transduction, Cytokine Signaling, Cellular Immune Response, Humoral Immune Response, Immune System, Pathogen-Influenced Signaling), and ranked z -score panels dominated by negative bars across the Inflammasome pathway, IL-6 Signaling, cGAS-STING, Interferon Signaling, NF- κ B Activation by Viruses, and TNFR1/TNFR2 (Figs. 5.5 and 5.6). Together with the replicate-level heat map, where both cordycepin preparations cluster away from DMSO+LPS and 3DI (Fig. 5.3), these observations support source-independent suppression of early inflammatory programmes, with the biological activity attributable to cordycepin itself rather than to co-purified contaminants previously detected in commercial preparations [162].

NUC-7738 at 200 μ M down-regulated 473 genes and up-regulated 28, giving a broader

negative DE profile than 3DI at the same concentration (86 down/22 up) (Fig. 5.9). Consistently, IPA showed more numerous and more strongly enriched immune and cytokine categories with negative z -scores for NUC-7738 (Immune System, Cytokine Signaling, Cellular and Humoral Immune Response, Pathogen-Influenced Signaling and related signal-transduction modules), whereas 3DI produced fewer, smaller bubbles and more categories with near-zero directionality (Fig. 5.10). Activation z -score panels again favoured NUC-7738, with clear predicted inhibition across IL-10 Signaling, IL-6 Signaling, Production of Nitric Oxide and ROS in Macrophages, cGAS-STING, Interferon Signaling and TNFR2 non-canonical NF- κ B, while 3DI showed a weaker and partly mixed pattern (Fig. 5.11). These data indicate that, in this macrophage model, NUC-7738 has a stronger and more coherent inhibitory impact on inflammatory pathways than 3DI at matched nominal doses.

NUC-7738 and 3DI reproduced the core transcriptional pattern seen with cordycepin but at 200 μ M rather than 20 μ M in this system (Figs. 5.9 and 5.10). This rank order is compatible with their biochemistry. 3DI is the major circulating species after oral cordycepin and can be reconverted intracellularly to cordycepin triphosphate (3'dATP) via nucleoside salvage, albeit less efficiently than direct cordycepin phosphorylation [1]. NUC-7738 is a ProTide in which a 5' aryloxy phosphoramidate masks the monophosphate, designed to bypass ADA-mediated deamination and nucleoside transporter and kinase bottlenecks, with clinical data confirming intracellular 3'dATP formation and NF- κ B pathway modulation [2, 29]. Within this framework, the simplest interpretation of the convergent pathway signatures is that all four treatments act via the same downstream metabolite, cordycepin triphosphate, with 3DI and NUC-7738 serving as delivery variants.

The 3DI data nonetheless require caution. Purity analyses in Chapter 3 demonstrated cordycepin contamination in the 3DI stock, so at least part of the observed activity is likely mediated by this contaminant rather than by 3DI itself. In contrast, the stronger and broader inhibitory signature with NUC-7738 compared with 3DI at 200 μ M is in keeping with medicinal-chemistry expectations for a ProTide that improves intracellular 3'deoxyadenosine nucleotide delivery [2, 29]. The higher concentrations

required for NUC-7738 in this RAW264.7 cell line system do not contradict its in vivo pharmacokinetic advantages (enhanced exposure, distribution and ADA bypass); the present experiments primarily report on macrophage pharmacodynamics at a fixed time point rather than on comparative potency in patients. The key conclusion is the directional alignment of NUC-7738 with cordycepin at the transcriptomic and pathway levels, not a definitive potency ranking across all four molecules.

Although the present data are transcriptomic, several contrasts returned negative directionality in growth-factor-linked categories such as Growth Factor Signaling and Signal Transduction. In the cordycepin conditions, negative z -scores in PI3K/AKT Signaling, Energy-dependent regulation of mTOR by LKB1-AMPK, and Autophagy (Fig. 5.6) mirror systematic work showing that cordycepin generally attenuates PI3K/AKT/mTOR signalling and generates gene-expression profiles similar to those elicited by the PI3K inhibitor LY294002 [25]. In this context, the broad reduction in immediate inflammatory transcripts is compatible with upstream restraint of PI3K-mTOR-driven translation and biogenesis processes that support cytokine induction. However, these data do not themselves distinguish direct inhibition of PI3K from upstream events that secondarily dampen PI3K-mTOR output, and phospho-protein readouts remain essential for mechanistic dissection.

PKR-IFN-eIF2 α module within the cordycepin response Ingenuity Pathway Analysis (IPA) repeatedly flagged the “Role of PKR in Interferon Induction and Antiviral Response” as significantly affected across the cordycepin analogues, with negative activation z -scores for synthetic cordycepin, NUC-7738 and 3DI, and a more neutral call for natural cordycepin (Figs. 5.6 and 5.11; Tables 5.1–5.4). In the IPA network overlays, the dominant changes lie along TRAF-IKK $\rightarrow$ NF- κ B and IFN/JAK/S-TAT/ISG branches (Fig. 5.14), consistent with well-described effects of cordycepin on proximal TLR4 signalling, AKT and p38 phosphorylation, and p65 nuclear translocation in RAW264.7 macrophages [27, 28, 52, 54]. Most of the responsive nodes therefore reflect general inflammatory and interferon signalling rather than PKR-specific outputs, and the pathway label should be interpreted in that broader sense.

A related pattern emerged in an independent suPB haploid survival screen in eHAP

cells, where resistance-enriched insertions mapped to genes within the same IPA module, including CASP8 and PDGFC (Figs. 5.15, 4.15, 4.16, and 4.1). Loss of CASP8 is expected to blunt apoptotic execution in this network, whereas loss of PDGFC is predicted to dampen PDGFR→PI3K–AKT–mTOR signalling that cordycepin also represses [25, 52, 166–173]. Together with the RNA-seq→IPA predictions, these findings suggest that cordycepin and its analogues repeatedly impact a PKR/IFN/eIF2 α -centred stress and antiviral module while suppressing NF- κ B-driven outputs. Because PKR activity and eIF2 α phosphorylation are post-translational, these inferences remain predictive rather than causal; they will need to be tested against direct measurements of pathway activation and targeted perturbations of the integrated stress response [135, 174]. For this reason, the PKR–IFN–eIF2 α module is treated here as a candidate node that complements, rather than replaces, the primary PI3K–mTOR and NF- κ B interpretations.

Signals from distinct visualisations converged. The top-50 heat map showed that cordycepin (both sources) and NUC-7738 reproducibly reduced expression of LPS-induced genes across triplicates, whereas 3DI largely tracked the DMSO+LPS profile (Fig. 5.3). Volcano plots quantified this skew towards repression for each condition (Figs. 5.4 and 5.9). Pathway-level IPA summaries then translated these gene-level effects into consistent negative predictions across immune and cytokine modules (Figs. 5.5, 5.6, 5.10, and 5.11). The alignment between the global gene-level shift (volcano and heat map) and the IPA-level direction (bubble and z -score analyses) supports the internal consistency of the dataset and of the pre-specified thresholds. Importantly, both the category enrichment (Figs. 5.5 and 5.10) and activation z -score (Figs. 5.6 and 5.11) readouts independently recapitulated the negative direction for IL-6 Signaling, Interferon Signaling, TNFR1/TNFR2 and the Inflammasome pathway, indicating that these features are robust across analytical views rather than artefacts of any single analysis.

Across conditions, the qualitative pattern persisted. Natural versus synthetic cordycepin produced almost superimposable outcomes at both gene and pathway levels (Figs. 5.4, 5.5 and 5.6), and NUC-7738 at 200 μ M gave a similar but more pronounced pattern (Figs. 5.9, 5.10 and 5.11). Where divergences occurred (for example, smaller or near-zero z -scores with 3DI), they tracked with weaker DE magnitude (Fig. 5.9), indicating a quantitative rather than qualitative difference.

Library-capture caveat for cordycepin-terminated RNAs. A technical consideration specific to poly(A)-anchored sequencing methods is that cordycepin-terminated transcripts may be under-represented during library synthesis. The ONT PCR-cDNA protocol relies on oligo-dT priming of the poly(A) tail, followed by reverse transcription that requires a free 3'-hydroxyl for strand extension. RNAs that have incorporated cordycepin at the 3' terminus of their poly(A) tail will carry a 3'-deoxyadenosine residue lacking this hydroxyl group, and such molecules would be inefficiently captured and depleted from the sequencing library [163]. Consequently, some proportion of the apparent decreases in transcript abundance observed under cordycepin treatment could, in principle, reflect efficient and stable cordycepin incorporation rather than true

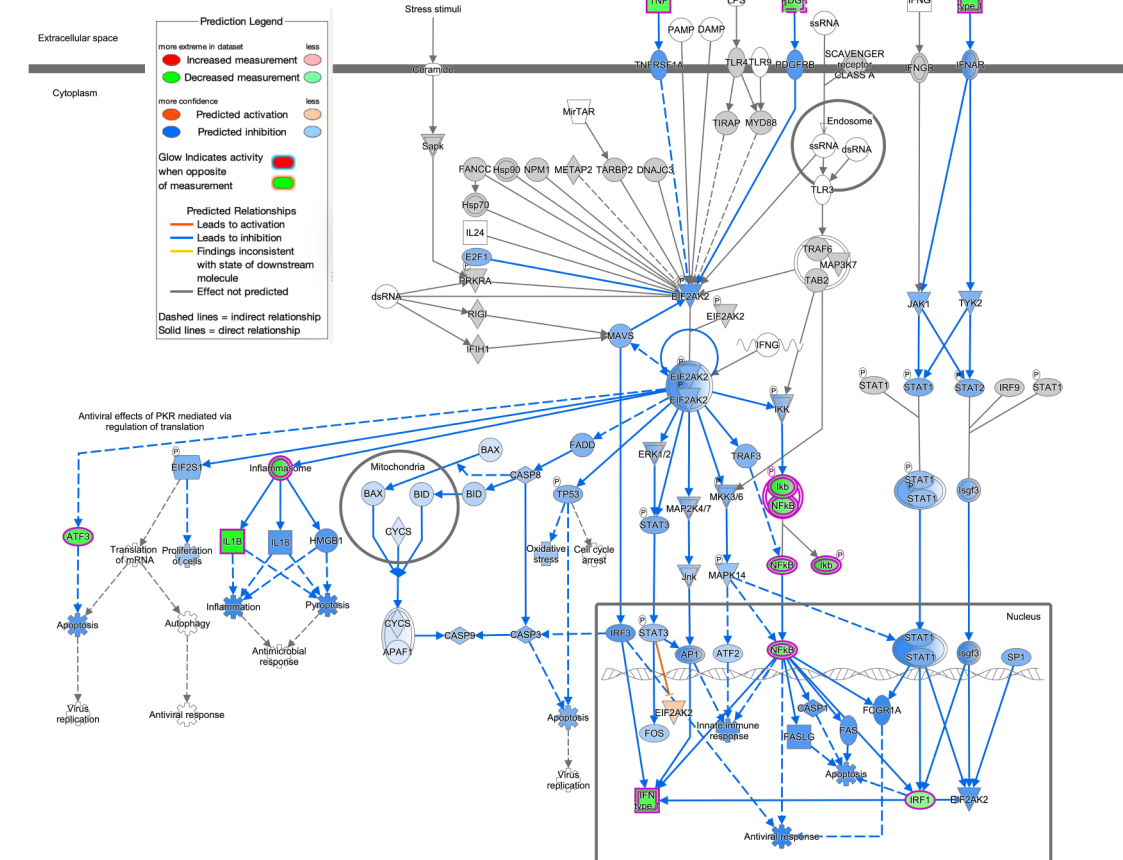


Figure 5.14: IPA mechanistic network for the canonical pathway “Role of PKR in Interferon Induction and Antiviral Response,” overlaid with differential gene expression data from RAW264.7 macrophages treated with natural cordycepin (20 μ M) versus vehicle (0.02% DMSO) for 2 h, with LPS (1 μ g/mL) added 1 h after the start of compound treatment (i.e. in the presence of the compound) and cells stimulated for a further 1 h. RNA was profiled by ONT cDNA sequencing and analysed with IsoQuant/edgeR (TMM; $|\log_2 \text{FC}| \geq 1$; FDR ≤ 0.05).

How to read the network: each node represents a gene or functional category. Node fill colour encodes the measured expression change (green = downregulated; red = upregulated; white = not differentially expressed). Coloured halos around nodes encode the IPA-predicted activation state (blue halo = predicted inhibition; orange halo = predicted activation). Edges (connecting lines) encode regulatory relationships: solid lines denote direct interactions and dashed lines denote indirect (inferred) interactions; blue edges indicate predicted inhibitory relationships and orange edges indicate predicted activating relationships.

Key observation: the overlay shows coordinated downregulation at multiple nodes along two principal signalling axes—TRAF $\rightarrow$ IKK $\rightarrow$ NF- κ B and IFN/JAK/STAT/IRF—with IPA predicting inhibitory flow through both branches. This pattern is consistent with the negative z -scores for inflammatory and interferon pathways reported in the IPA bar charts (Figures 5.6 and 5.7). Note that most responsive nodes reflect general inflammatory and interferon signalling rather than PKR-specific outputs; the IPA pathway label should be interpreted in this broader sense.

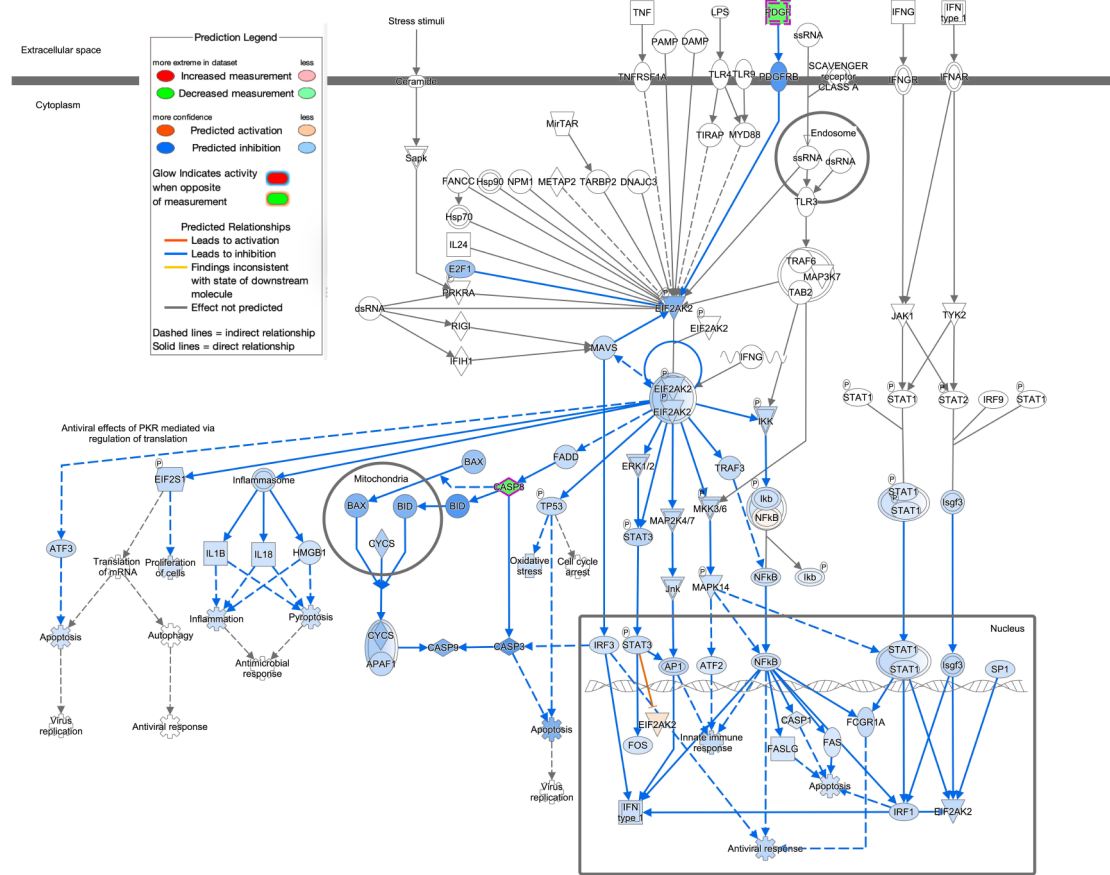


Figure 5.15: The same IPA mechanistic network as in Figure 5.14, now overlaid with gene-level signals from the suPB haploid *piggyBac* gene-trap screen in eHAP cells (cordycepin 200 μ M vs. DMSO selection; Chapter 4). **How to read the network:** node fill, halos and edge colours follow the same conventions as Figure 5.14—green fill = decreased signal, red fill = increased signal, blue halo = predicted inhibition, orange halo = predicted activation, solid edges = direct interactions, dashed edges = indirect interactions. Outlined (highlighted) nodes indicate genes that were mapped as resistance-enriched insertion sites in the screen. **Key observation:** transposon insertions enriched under cordycepin selection localise to CASP8 and PDGFC within this module. Loss of CASP8 function is predicted to attenuate apoptotic execution downstream of the network, while loss of PDGFC is predicted to reduce PDGFR→PI3K–AKT–mTOR signalling. Together with the transcriptomic overlay in Figure 5.14, these data suggest that both gene expression changes (RNA-seq) and genetic resistance mechanisms (haploid screen) converge on the same PKR/IFN/NF- κ B signalling architecture. IPA overlay label: “suPB_cordycepin_vs_DMSO_logFC_pval_without_qual.”

reductions in RNA levels. However, this artifact is unlikely to be a major driver of the observed patterns for two reasons. First, Kondrashov et al. demonstrated that cordycepin-terminated tails are transient and rare at steady state, as evidenced by similar tail-length distributions obtained from both 3'- and 5'-end labelling approaches [19]. Second, the overall transcriptional signatures reported here closely recapitulate those detected by microarray [26] [164] and Illumina RNA-seq [27] in nearly identical RAW264.7/LPS experimental systems—platforms whose library chemistries do not share the same poly(A) 3'-OH dependency. This cross-platform concordance supports the interpretation that the differential expression patterns primarily reflect true changes in RNA abundance. A formal quantitative comparison of the present ONT dataset with the existing microarray and Illumina RNA-seq datasets would provide a direct test of this assumption.

Internal consistency and design boundaries Three features increase confidence in the main conclusions of this chapter. First, multiple readouts converge: the replicate-resolved heat map, gene-level DE (volcano plots) and pathway-level enrichment/direction (IPA) all indicate broad suppression of LPS-induced transcription, with concordant direction across figures and across the active compounds (Figs. 5.3, 5.4, 5.5, 5.6, 5.9, 5.10, and 5.11). Second, source comparison shows that natural and synthetic cordycepin give near-identical outcomes at gene and pathway levels, directly addressing the concern that activity might be driven by co-purified contaminants in natural preparations [162]. Third, pathway-level coherence is high: the recurrent negative direction across IL-6 Signaling, Interferon Signaling, TNFR1/TNFR2 and the Inflammasome pathway, together with mTOR-adjacent categories, yields a biologically consistent picture that agrees with independent evidence for cordycepin's anti-inflammatory and PI3K-mTOR-modulating actions [13, 19, 25, 28].

The work also has defined boundaries that shape interpretation. All experiments were performed in a single macrophage cell line (RAW264.7) and at an early 1 h LPS time point chosen to capture proximal, likely causative events in the inflammatory programme and to minimise confounding from cordycepin degradation or delayed compensatory responses. This design is appropriate for comparing mechanisms of action between

compounds, but later transcriptional or secretory phases and other cell types remain outside the scope of this chapter. Inferences are based on transcriptomics and IPA predictions; while activation *z*-scores provide useful hypotheses, causal statements about PKR, the integrated stress response or PI3K–mTOR engagement require integration with phospho-proteomics and functional perturbation data, some of which are considered elsewhere in this thesis and in the literature [135, 174]. Dosing was intentionally non-equipotent: cordycepin was examined at 20 μ M, whereas NUC-7738 and 3DI were used at 200 μ M. These choices were guided by prior RT-qPCR and pharmacological data to give informative dynamic range rather than to allow strict potency ranking across all four molecules. Finally, interpretation of 3DI is constrained by the documented cordycepin contamination of the stock (Chapter 3), so 3DI-specific mechanistic claims are kept deliberately limited.

Biological and therapeutic implications The data strengthen the rationale that cordycepin itself, independent of source, can acutely dampen macrophage inflammatory programmes and growth-factor-linked signalling. This is consistent with *in vivo* anti-inflammatory activity and supports polyadenylation/signalling cross-talk as a therapeutically exploitable axis [13, 19, 28]. The stronger and broader signature with NUC7738 compared with 3DI suggests that delivery-optimised 3′deoxyadenosine variants can achieve more complete early pathway control, with potential relevance for growth factor-driven and inflammatory pathologies [2, 29]. Given the RNA-centric rationale for cordycepin’s action, the subsequent chapter focuses on poly(A)-tail assays to connect these transcriptomic signatures to changes in 3′-end processing and to define the scope of cordycepin’s effects on mRNA maturation.

In summary, the RAW264.7 data show that natural and synthetic cordycepin are functionally very similar at 20 μ M, converging on broad repression of LPS-induced transcripts and coherent inhibition of immune and cytokine pathways in IPA. NUC-7738 has a very similar effect at the higher 200 μ M exposure, whereas 3DI is weaker and confounded by purity, reinforcing the conclusion that cordycepin triphosphate is most likely the common active driver generated by all four treatments. The recurrent negative direction on growth-factor-linked pathways and the appearance of PI3K/AKT/mTOR-

and PKR/IFN/ $\text{eIF2}\alpha$ -annotated modules support a working model in which restraint of PI3K–mTOR signalling, coupled with stress-responsive translational control, contributes to early dampening of inflammatory programmes. In light of this RNA-centric mechanism, the next chapter tests this model directly by measuring poly(A)-tail dynamics on LPS-induced transcripts.

Chapter 6

Poly(A) tail profiling following cordycepin and NUC-7738 treatment of LPS-activated macrophages reveals genome-wide poly(A) tail shortening.

6.1 Introduction

Poly(A) tails regulate mRNA translation and stability through interactions with PABP and by gating access to the major cytoplasmic deadenylases (predominantly the CCR4-NOT complex) that initiate decay. These processes rapidly reshape gene expression during immune activation [36, 152]. Cordycepin (3'-deoxyadenosine) nucleotides are accepted by poly(A) polymerases; incorporation at a tail terminus removes the 3'-OH, lowers 3'-end formation efficiency, and alters polyadenylation dynamics in vivo [45]. Complementary genetic and pharmacological work also shows that cordycepin changes alternative poly(A) site choice through kinetic coupling with RNAPII elongation [38]. Together, these observations suggest that adenosine analogues can alter both tail length and 3'-UTR architecture in ways expected to dampen inflammatory programmes.

This chapter extends the transcriptomic analysis presented in Chapter 5 by exploiting the same Oxford Nanopore long-read cDNA sequencing dataset to quantify

how adenosine analogues that affect mRNA 3'-end formation modulate poly(A) tail length and mRNA abundance during acute macrophage activation, and relates these changes to pathway-level programmes. We focus on cordycepin (3'-deoxyadenosine), its ProTide derivative NUC-7738, and 3'-deoxyinosine (3DI), using an LPS-primed RAW264.7 model and a joint poly(A)-tail / differential-expression analysis framework.

NUC-7738 was designed to bypass cordycepin pharmacological liabilities (e.g. ADA-mediated deamination and transporter dependence) and to deliver high intracellular triphosphate levels through ProTide chemistry. First-in-human data and genome-wide analyses indicate on-target activity with effects converging on NF- κ B-linked programmes, motivating its inclusion here as a mechanistically related comparator [2, 29].

Across all conditions, RAW264.7 macrophages were treated for 2 h with cordycepin 20 μ M (natural or synthetic), NUC-7738 200 μ M, 3DI 200 μ M, or DMSO; LPS (1 μ g/mL) was added 1 h after the start of compound treatment (i.e. in the presence of the compound) and cells were stimulated for a further 1 h. Long-read cDNA sequencing was then used to obtain per-transcript poly(A)-tail estimates alongside gene-level counts, enabling integrated analysis of tail remodeling and mRNA abundance under LPS alone and in the presence of each analogue. The primary aim is to identify RNAs whose behaviour under these conditions indicates sensitivity to perturbed 3'-end formation—either through altered poly(A) tail size or through loss from the library consistent with a blocked 3'-end—and to organise these responses at transcript and pathway level. The experimental time scheme is summarised in Figure 6.1.

Chapter 6 — Experimental Time Scheme

RAW264.7 macrophages — Oxford Nanopore poly(A) tail analysis

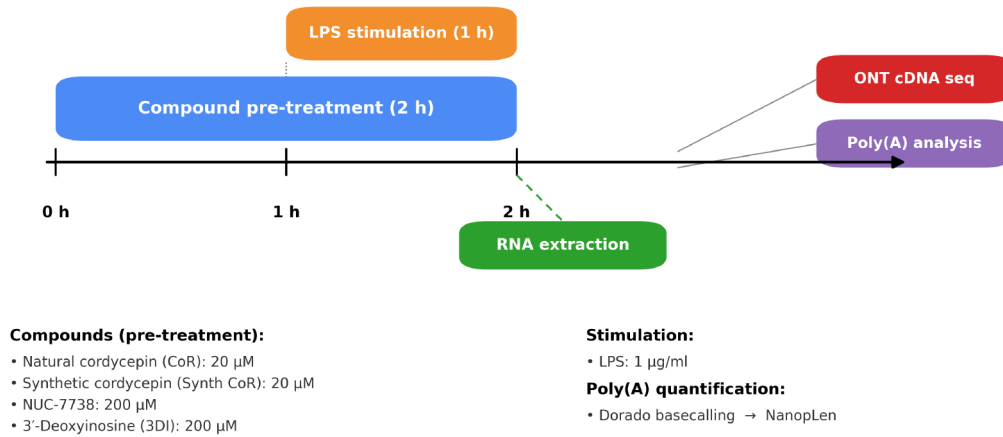


Figure 6.1: Experimental time scheme for the poly(A) tail analysis. RAW264.7 macrophages were treated for 2 h with cordycepin (20 μ M, natural or synthetic), NUC-7738 (200 μ M), 3'-deoxyinosine (200 μ M), or DMSO vehicle. LPS (1 μ g/ml) was added after 1 h of compound treatment (i.e. in the presence of the compound) and cells were stimulated for 1 h. Total RNA was extracted at 2 h and processed for Oxford Nanopore long-read cDNA sequencing. Poly(A) tail lengths were estimated from Dorado basecalling tags using NanopLen.

6.2 LPS-Induced Poly(A) Remodeling (control behaviour)

To determine how inflammatory stimulation alone affects poly(A) tails, we first examined the changes in tail length induced by LPS in the absence of adenosine analogues. The empirical cumulative distribution function (ECDF) in panel A of Figure 6.2 shows a clear leftward shift in the distribution of poly(A)-tail lengths in the LPS-treated group compared with the DMSO control, indicating a global trend towards shorter poly(A) tails after LPS stimulation. The scatter plot in panel B of Figure 6.2 confirms this observation, with most transcripts lying below the identity line and showing a decrease in mean poly(A)-tail length after LPS treatment.

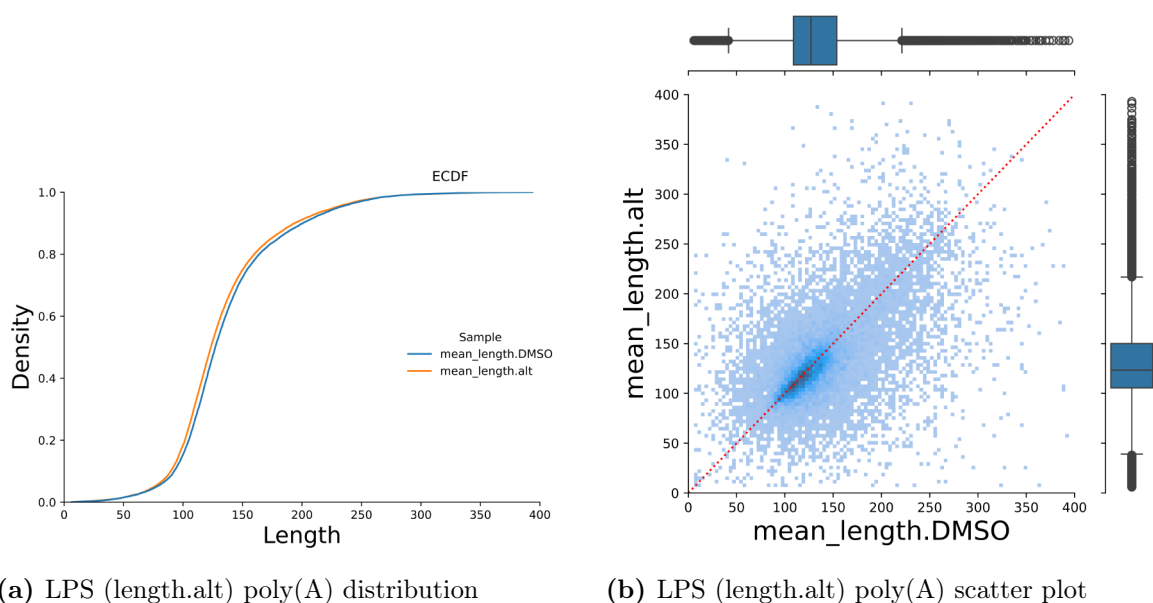


Figure 6.2: RAW264.7 macrophages were treated with either vehicle (DMSO) for 120 min as control or vehicle (DMSO) for 60 min followed by LPS (1 μ g/mL) for 60 min. cDNA long-read libraries were basecalled with Dorado and poly(A) tails quantified with NanopLen (modified to ingest Dorado poly(A) tags). `length.alt` = mean poly(A)-tail length (nt) for the treatment condition; NanopLen hard-codes “.alt” for treatment and uses the exact control name from metadata. (a) ECDFs of per-transcript mean poly(A)-tail length (nt); orange, DMSO; blue, LPS. (b) Per-transcript scatter vs DMSO. Axes: `mean_length.DMSO` (x) vs `mean_length.alt` (y; LPS). Statistics/thresholds used downstream: per-transcript tail change with an effect-size threshold of ± 10 nt and $\text{FDR} \leq 0.05$. Replicates: $n = 3$ biological replicates.

When we restricted the analysis to statistically significant per-transcript changes, a more complex pattern emerged. The volcano plot and MA plot in Figure 6.3 show that, despite the global shortening trend, the significant set is skewed towards lengthening:

most transcripts passing the ± 10 nt and $\text{FDR} \leq 0.05$ thresholds have longer tails under LPS. This apparent discrepancy reflects the fact that many transcripts undergo small but widespread tail shortening that does not exceed the per-transcript effect-size cut-off, whereas a smaller subset shows larger, often lengthening, shifts in poly(A)-tail length. Long-read tail estimates therefore reveal a global left-shift in the distribution after LPS (shorter tails overall), with the volcano and MA plots highlighting a discrete, significantly remodeled subset ($\approx 0.7\%$ of transcripts) biased towards lengthening under the chosen thresholds. This illustrates how distribution-level summaries and thresholded per-transcript calls emphasise complementary facets of the same data.

Standard 3'-end-anchored tail-calling workflows use ligation or capture steps that require a free 3'-OH on the RNA. T4 RNA ligase efficiently joins 3'-OH/5'-phosphate nicks, whereas a terminal 3'-deoxyadenosine (3'-dA) lacks the 3'-OH and is resistant to ligation, so such molecules are under-represented in these libraries [163]. As a result, the ONT/NanopLen readouts used here primarily capture RNAs without a terminal 3'-dA; this design constraint is carried forward when interpreting compound-treated samples in subsequent sections.

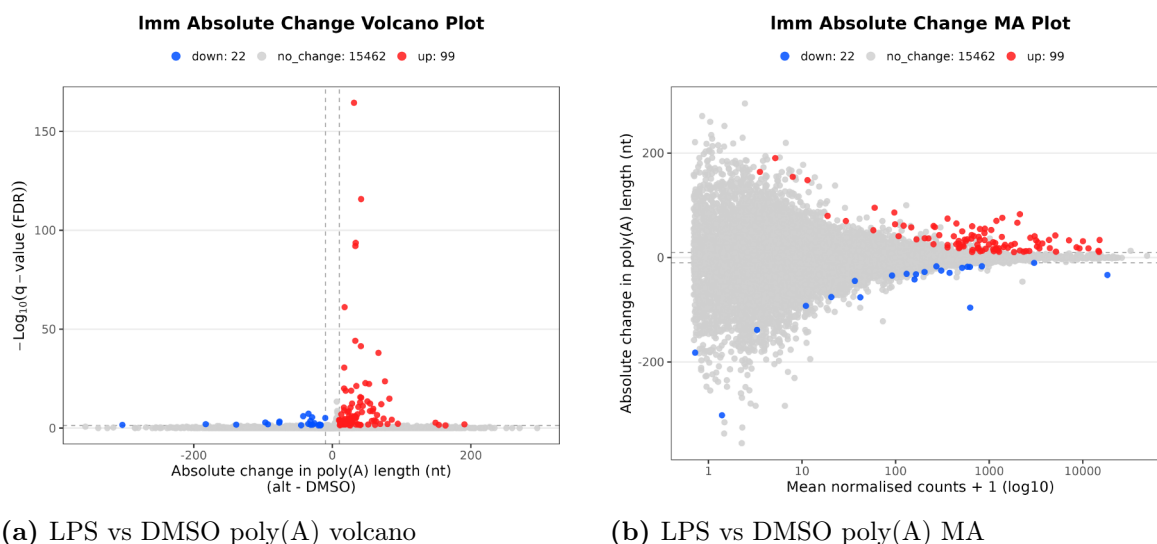


Figure 6.3: Same system as Fig. 6.2. (a) Volcano and (b) MA plots for LPS vs DMSO at 120 min. Transcripts surpassing ± 10 nt tail change and $\text{FDR} \leq 0.05$ are coloured red (longer) or blue (shorter). Although Fig. 6.2 shows a global distribution-level shortening, the thresholded significant set skews towards lengthening at the per-transcript level. Replicates: $n = 3$.

Having established the pattern of LPS-induced poly(A) remodeling in the absence of adenosine analogues, we next compared the poly(A)-tail length profiles of

compound-treated groups with those of the DMSO+LPS control.

6.3 Effects of compounds on poly(A) tail size

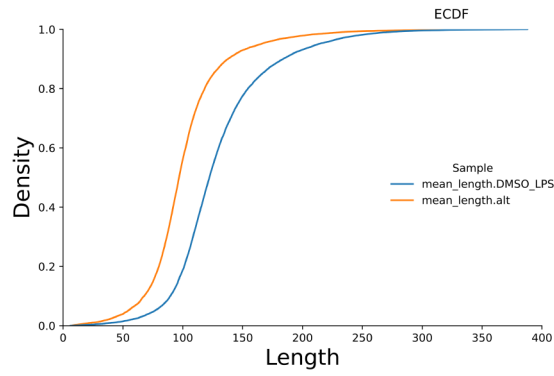
To study the impact of adenosine analogues on the size of the poly(A) tail in the context of inflammation induced by LPS, poly(A) tail length profiles of the groups treated with the compounds were compared to those of the DMSO+LPS control group. The effects of the adenosine analogues tested on the poly(A) size are summarized in Table 6.1.

Table 6.1: Median poly(A) tail length shifts (ECDF-based) for drug + LPS vs DMSO + LPS (*Control*).

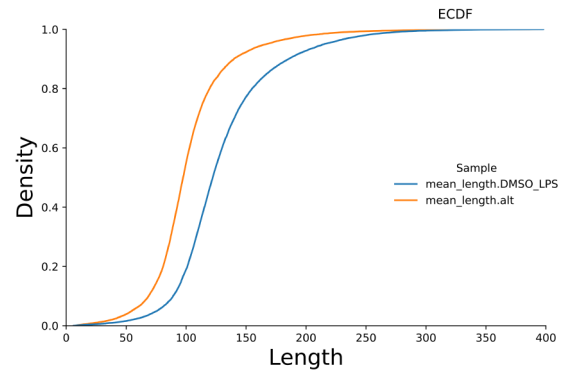
Comparison	n (genes)	Median control (nt)	Median treatment (nt)	Δ median (nt)
CoR_LPS vs DMSO_LPS	10446	119.6	94.2	-25.4
NUC_LPS vs DMSO_LPS	10414	119.6	93.3	-26.3
3DI_LPS vs DMSO_LPS	10505	119.7	112.8	-6.9
Synth_CoR_LPS vs DMSO_LPS	10491	119.6	94.5	-25.1

Cordycepin and Synthetic Cordycepin

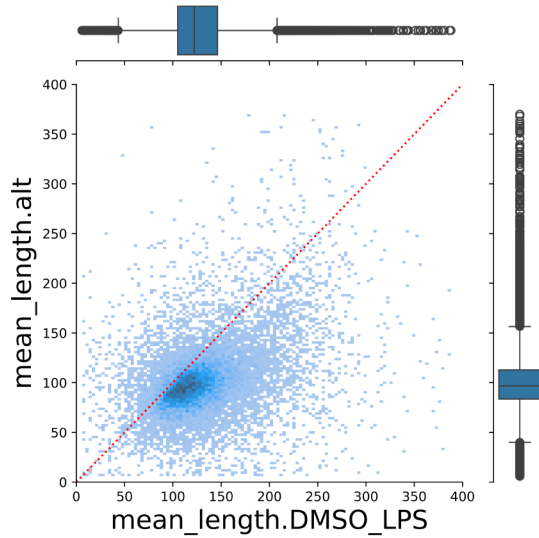
Natural and synthetic cordycepin induced a genomewide reduction of approximately 25 nucleotides in the length of poly(A) tails (Figure 6.4). The scatter plots confirm this observation, and the vast majority of transcripts exhibit a substantial decrease in poly(A) tail length upon cordycepin treatment. The volcano and MA plots in Figure 6.5 further highlight the magnitude of this effect, with approximately 3,000 genes showing a significant shortening of their poly(A) tails by at least 10 nucleotides. This widespread deadenylation is a hallmark of cordycepin's mechanism of action and is consistent with its known ability to inhibit polyadenylation.



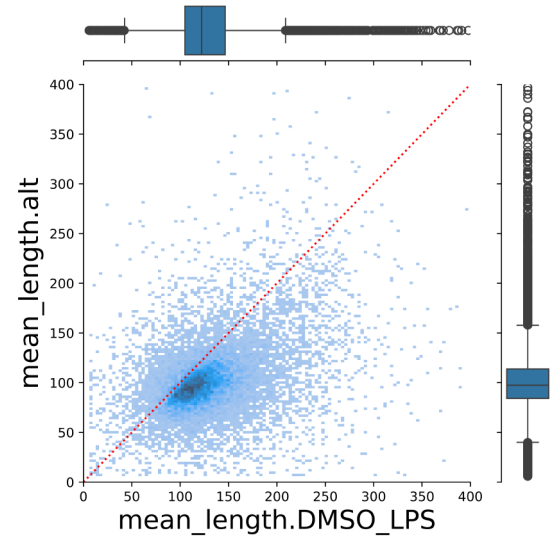
(a) Natural cordycepin



(b) Synth cordycepin

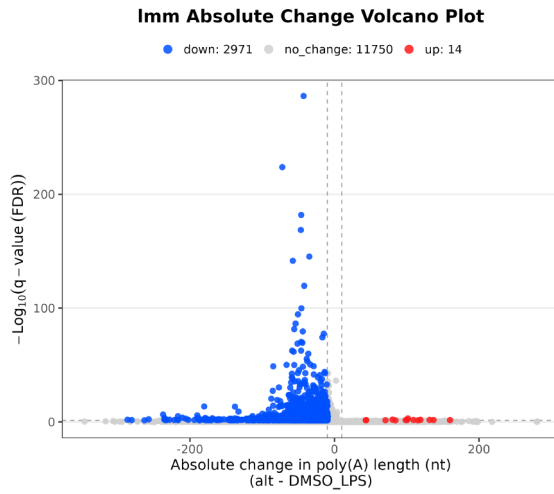


(c) Natural cordycepin

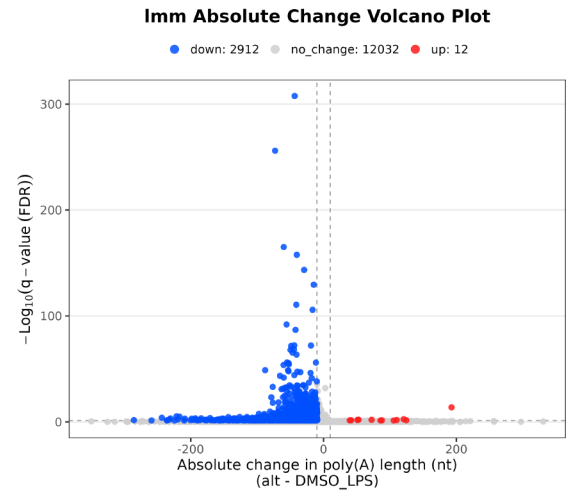


(d) Synth cordycepin

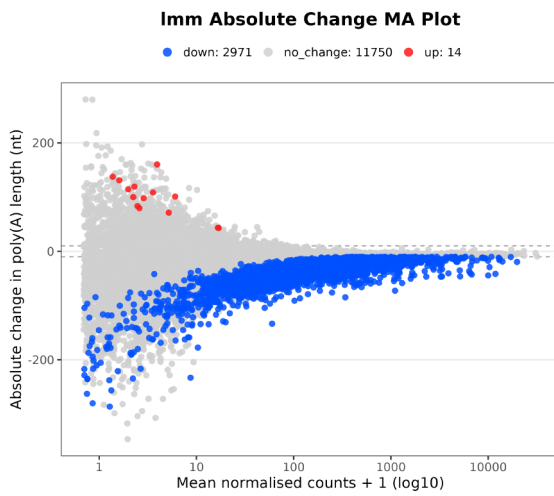
Figure 6.4: Cordycepin-induced poly(A) remodeling (ECDF and scatter). RAW264.7 cells were pre-treated with natural or synthetic cordycepin (20 μ M, 1 h), then stimulated with LPS (1 μ g/mL, 1 h); orange line. DMSO+LPS is the control; blue line. length.alt = mean poly(A)-tail length (nt) for the treatment condition; NanopLen hard-codes “.alt” for treatment and uses the exact control name from metadata. Panels: (A, C) natural cordycepin (ECDF, scatter); (B, D) synthetic cordycepin. Δ median ECDF shift (treatment – control): natural –25.4 nt (95%CI –26.0 to –24.8; n = 10,446), synthetic –25.1 nt (95%CI –25.7 to –24.6; n = 10,491). Downstream thresholds: ± 10 nt, FDR ≤ 0.05 . Replicates: n = 3



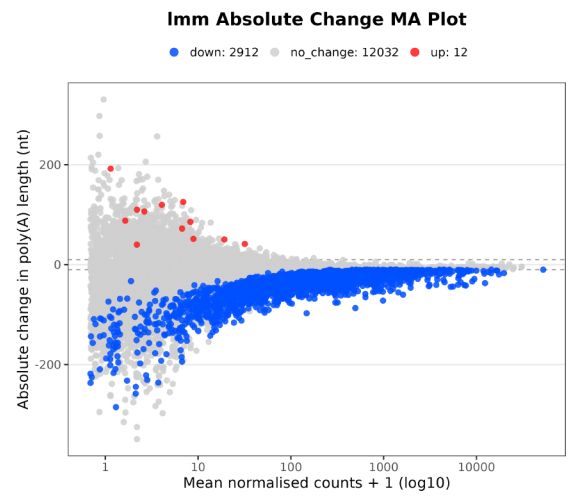
(a) Natural cordycepin



(b) Synth cordycepin



(c) Natural cordycepin



(d) Synth cordycepin

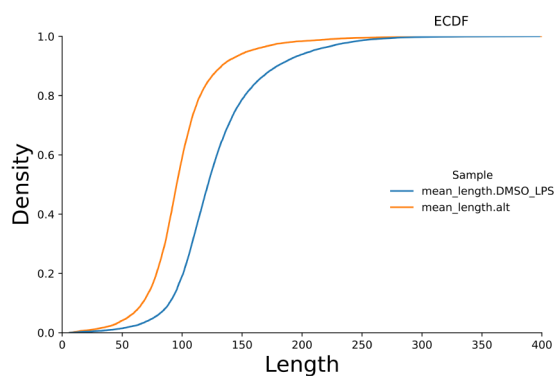
Figure 6.5: Cordycepin-induced poly(A) remodeling (volcano and MA). Same samples as Fig. 6.4. (A, C) natural cordycepin; (B, D) synthetic. Volcano and MA show genes with ± 10 nt tail-change and $FDR \leq 0.05$; red = longer, blue = shorter. Approximately 3,000 genes show significant shortening under cordycepin. Replicates: $n = 3$.

NUC-7738

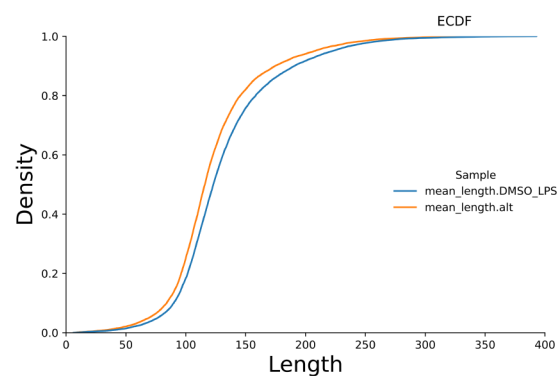
NUC-7738, the ProTide derivative of cordycepin, induced a genomewide reduction of approximately 26 nucleotides in the length of poly(A) tails (Figure 6.6). The ECDF plot shows a similar leftward shift to that observed for cordycepin, although at a higher concentration, with NUC-7738 at 200 μ M compared to 20 μ M for cordycepin, indicating a global deadenylation effect. The volcano and MA plots in Figure 6.7 reveal that more than 20% of all expressed genes underwent a significant shortening of their poly(A) tails.

3'deoxyinosine

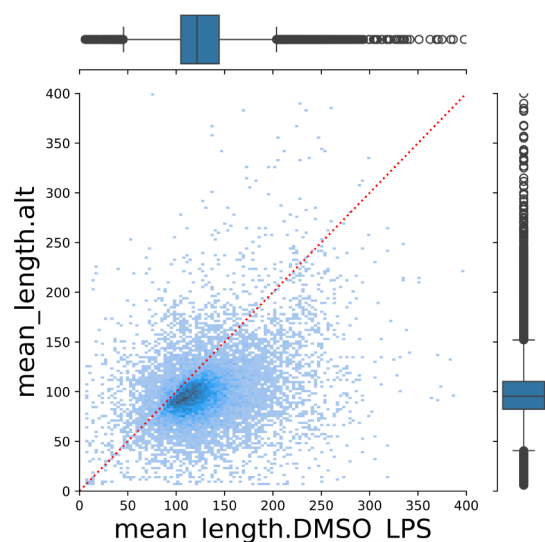
In contrast to the other analogues, 3'deoxyinosine had a much more modest effect on the length of the poly (A) tail (Figure 6.6). The ECDF plot shows only a slight shift to the left, and the volcano and MA plots in Figure 6.7 indicate that less than 1% of the genes exhibited a significant change in poly(A) tail length. This decreased effect aligns with the reduced potency of 3DI observed in gene expression analyses and may be attributed to its indirect mechanism of action, which requires intracellular conversion to cordycepin. Furthermore, the purity analysis presented in Chapter 1 revealed that the 3DI stock is contaminated with cordycepin, suggesting that the observed effect may be attributable to the cordycepin contaminant rather than to 3DI itself.



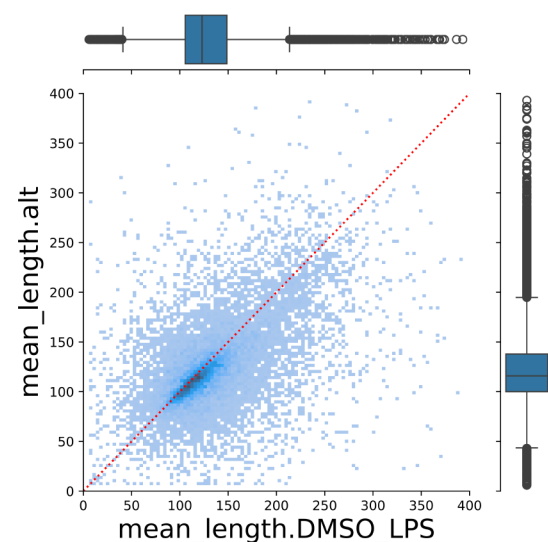
(a) NUC-7738



(b) 3DI

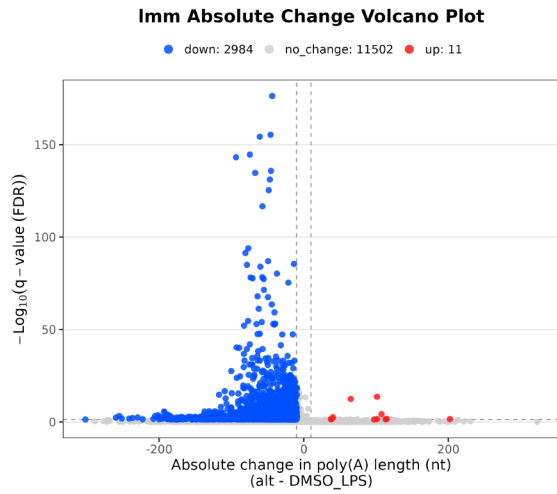


(c) NUC-7738

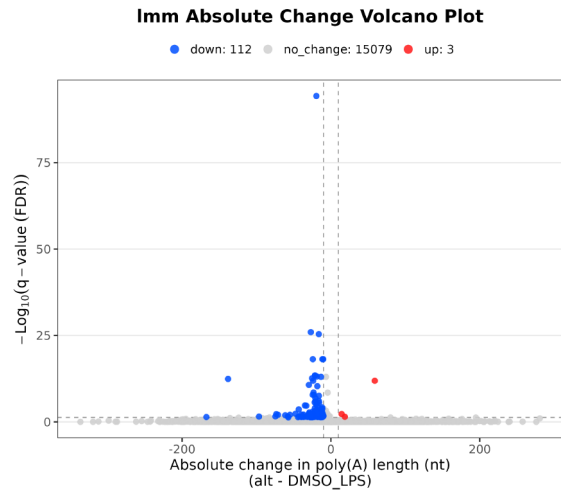


(d) 3DI

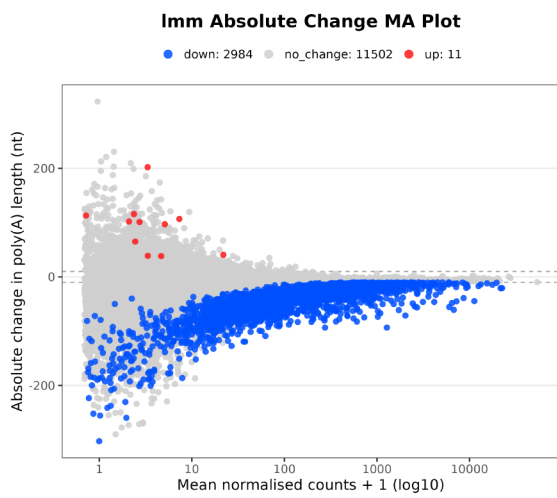
Figure 6.6: NUC-7738 and 3DI (ECDF and scatter). RAW264.7 cells were treated with NUC-7738 (ProTide) or 3DI at 200 μ M for 2 h, with LPS (1 μ g/mL) added 1 h after the start of compound treatment (i.e. in the presence of the compound) for a 1 h stimulation; orange line. DMSO+LPS is the control; blue line. length.alt = mean poly(A)-tail length (nt) for the treatment condition; NanopLen hard-codes “.alt” for treatment and uses the exact control name from metadata. Panels: (A, C) NUC-7738; (B, D) Δ median ECDF shifts: NUC-7738 -26.3 nt (95%CI -26.9 to -25.6 ; $n = 10,414$); 3DI -6.9 nt (95%CI -7.5 to -6.4 ; $n = 10,505$).. Replicates: $n = 3$



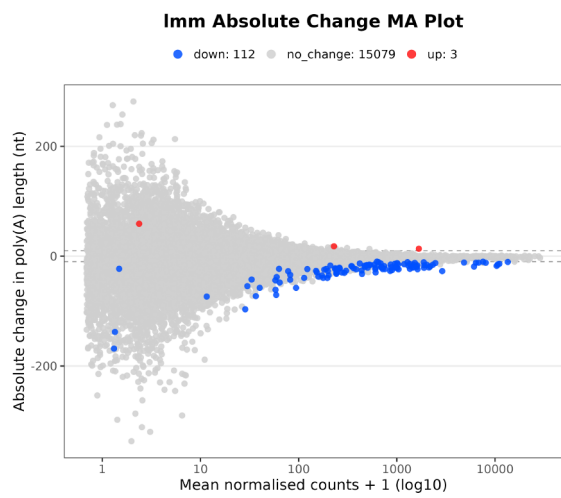
(a) NUC-7738



(b) 3DI



(c) NUC-7738



(d) 3DI

Figure 6.7: NUC-7738 and 3DI (volcano and MA). Same samples as Fig. 6.6. (A, C) NUC-7738; (B, D) 3DI. Thresholds: ± 10 nt, $FDR \leq 0.05$. More than 20% of expressed genes show significant tail shortening under NUC-7738, while less than 1% change under 3DI (as plotted). Replicates: $n = 3$.

6.4 Relation between Gene Expression and Poly(A)

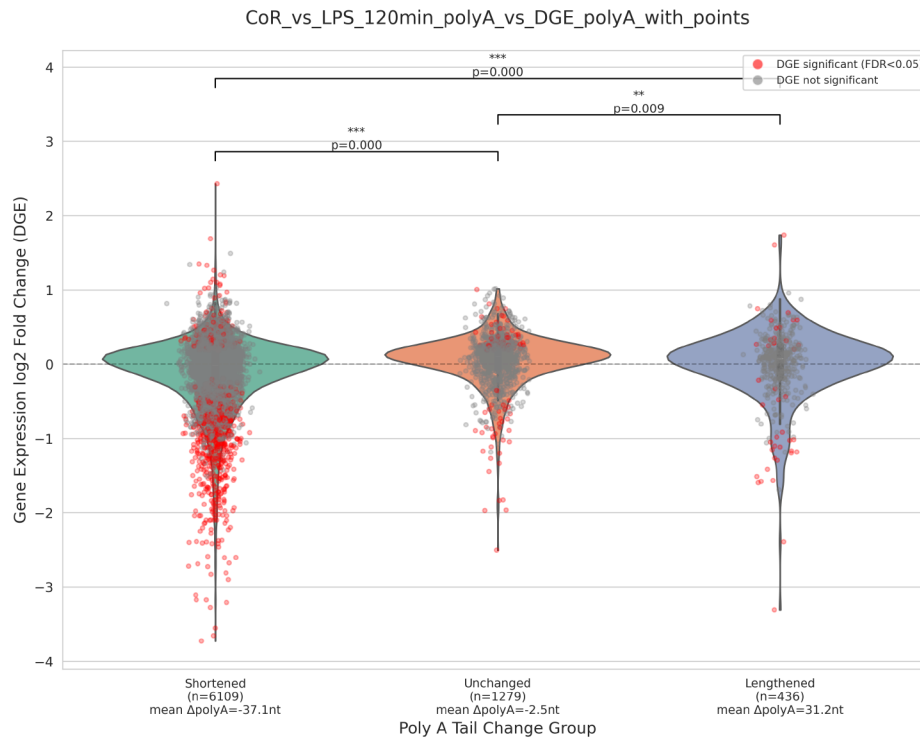
Tail Size

To directly investigate the relationship between the dynamics of the poly(A) tail and gene expression, we stratified the genes into three classes based on their change in poly(A) tail length: "shortened" (>10 nt decrease), "Unchanged" (≤ 10 nt change), and "lengthened" (>10 nt increase). We then examined the distribution of log2-fold changes in mRNA abundance for each of these classes.

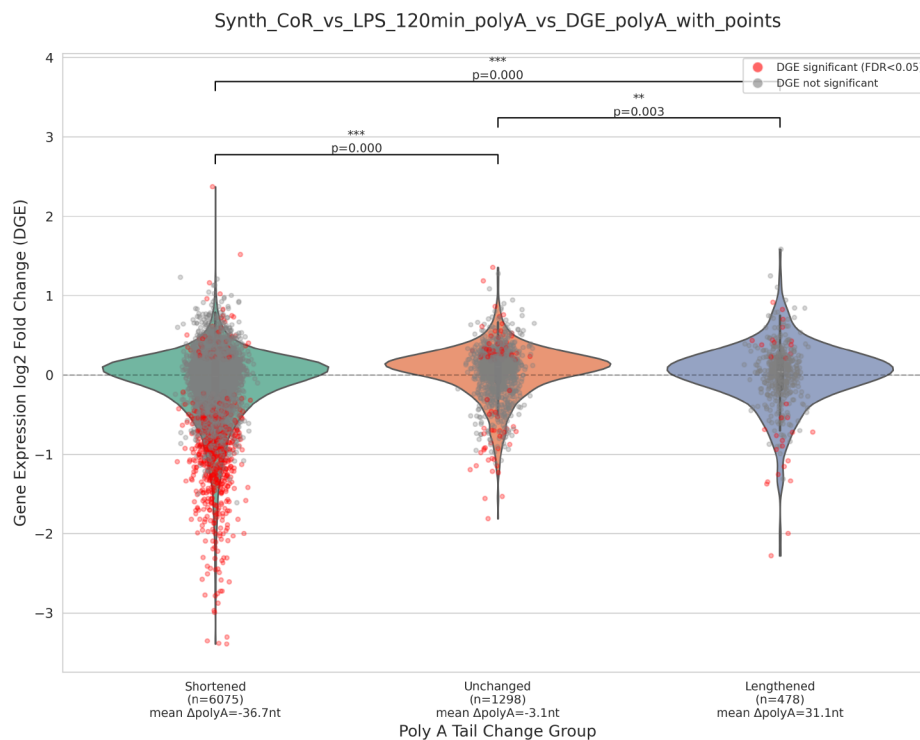
For each compound, stratifying genes by tail class (Shortened/Unchanged/Lengthened) revealed consistent ordering of log2 fold-changes: Shortened $<$ Unchanged $<$ Lengthened (i.e., shorter tails associate with lower abundance). This provides evidence for correlation, not proof of mechanism, especially under a 2 h design where steady state is not guaranteed.

Cordycepin Natural and Synthetic

For natural and synthetic cordycepin, we observed a clear and consistent relationship between poly(A) tail length and gene expression (Figure 6.8). The "Shortened" class of genes exhibited a significant decrease in mRNA abundance compared to the "Unchanged" class. Conversely, the "Lengthened" class showed a slight but significant increase in expression. This graded pattern of Shortened $<$ Unchanged $<$ Lengthened provides evidence that there is a correlation between mRNA abundance and poly(A) tail length. This correlation may be attributed either to variations in mRNA stability or to alterations in the poly(A) tail length reflecting changes in transcript abundance; because the nanopore chemistry requires a free 3'-OH for ligation, transcripts whose tails terminate in cordycepin (3'-deoxyadenosine) may be under-represented in the poly(A) measurement, and changes in steady-state abundance cannot be equated directly with changes in transcription rate without additional evidence (e.g. from unspliced RNA levels).



(a) Gene-expression distributions stratified by poly(A) tail class for natural cordycepin



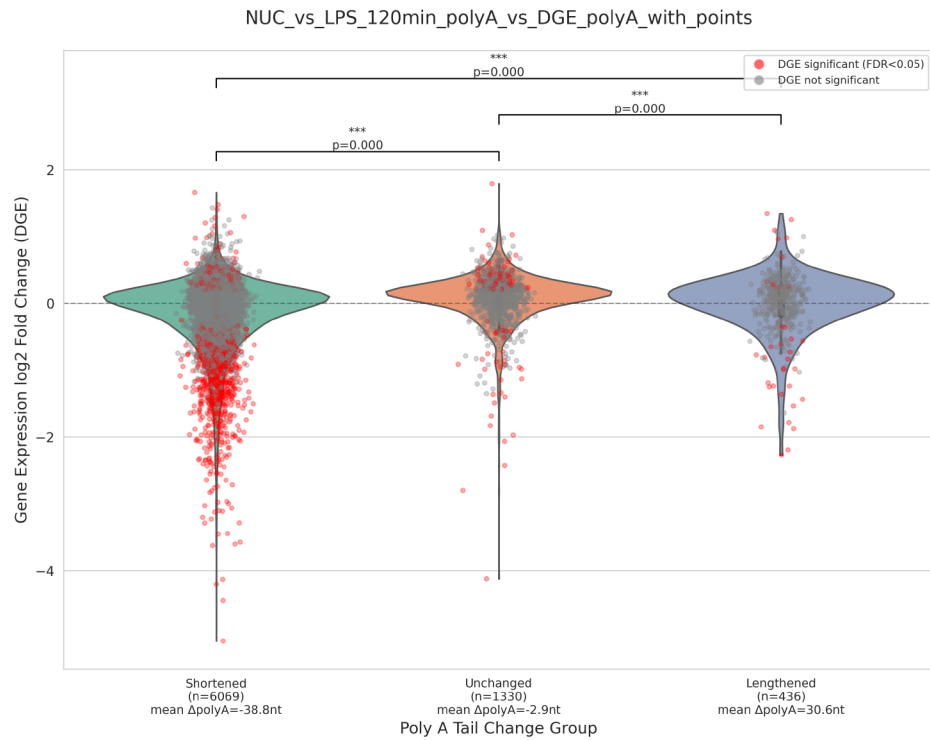
(b) Gene-expression distributions stratified by poly(A) tail class for synthetic cordycepin

Figure 6.8: Gene-expression distributions stratified by poly(A)-tail class after exposure to natural versus synthetic cordycepin. (a) Natural cordycepin; (b) Synthetic cordycepin. Tail classes: Shortened (> 10 nt decrease), Unchanged (≤ 10 nt), Lengthened (> 10 nt increase). Violin plots show EdgeR log2FC distributions (IsoQuant $\rightarrow$ EdgeR; FDR ≤ 0.05). Statistics: pair-wise Dunn tests with FDR correction across classes; Red dots indicate the significantly differentially expressed genes. The figure revealed significant differences among all three classes for both compounds.

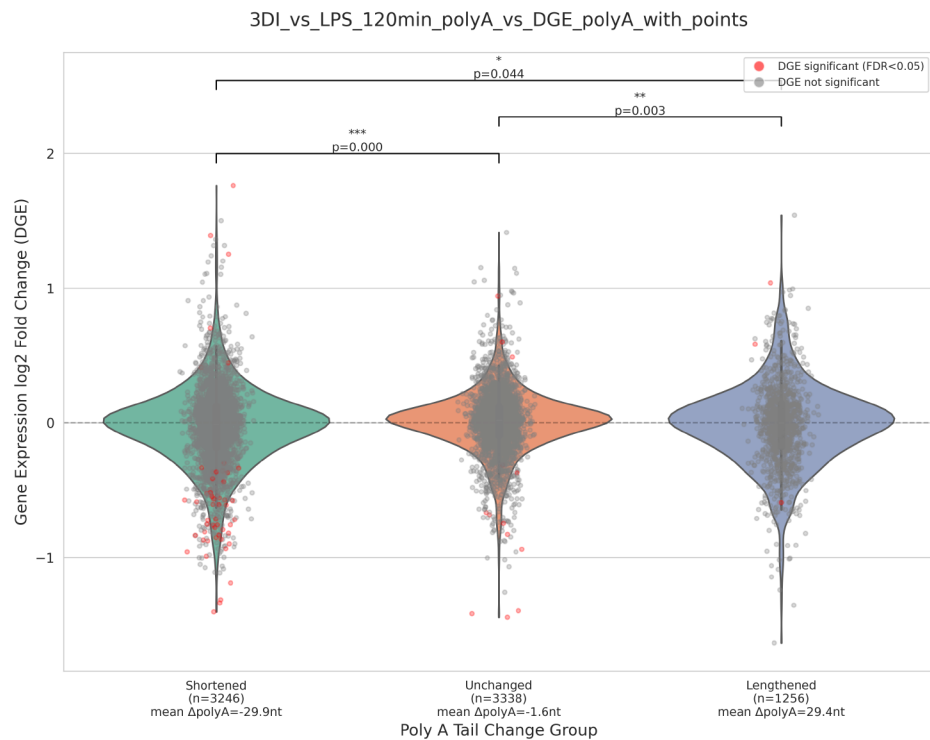
Both natural and synthetic cordycepin induce transcriptome-wide deadenylation that correlates with modest but significant shifts in mRNA abundance. Transcripts whose poly(A) tails shorten (> 10 nt) show a significant decrease in expression relative to unchanged tails.

NUC-7738 and 3'deoxyinosine

A similar trend was observed for NUC-7738 and 3'deoxyinosine, although with some quantitative differences (Figure 5.17). NUC-7738 showed the most pronounced separation relative to 3ID, with the "Shortened" group showing a strong and highly significant decrease in expression. For 3'deoxyinosine, the differences between the classes were smaller but still statistically significant, consistent with its weaker overall effect.



(a) Gene-expression distributions stratified by poly(A) tail class for NUC-7738



(b) Gene-expression distributions stratified by poly(A) tail class for 3DI

Figure 6.9: Poly(A)-tail class-specific gene-expression responses to NUC-7738 and 3'deoxyinosine in LPS-activated RAW264.7 macrophages. (A) NUC-7738. (B) 3'deoxyinosine (3DI). Tail classes: Shortened (> 10 nt decrease), Unchanged (≤ 10 nt), Lengthened (> 10 nt increase). Violin plots show EdgeR log₂FC distributions (IsoQuant $\rightarrow$ EdgeR; FDR ≤ 0.05). Statistics: pair-wise Dunn tests with FDR correction across classes; Red dots indicate the significantly differentially expressed genes. all pair-wise differences significant for NUC-7738; all but one for 3DI.

6.5 Pathway analysis of poly(A) tail changes

To identify the specific biological processes that are associated with tail shortening and lengthening and to gain deeper mechanistic insights into the interplay between poly(A) tail dynamics and transcriptional regulation, we performed a pathway analysis, this time stratifying the genes based on their poly(A) tail changes. The results provided a clear and coherent picture, further strengthening the link between poly(A) tail metabolism and the control of inflammation.

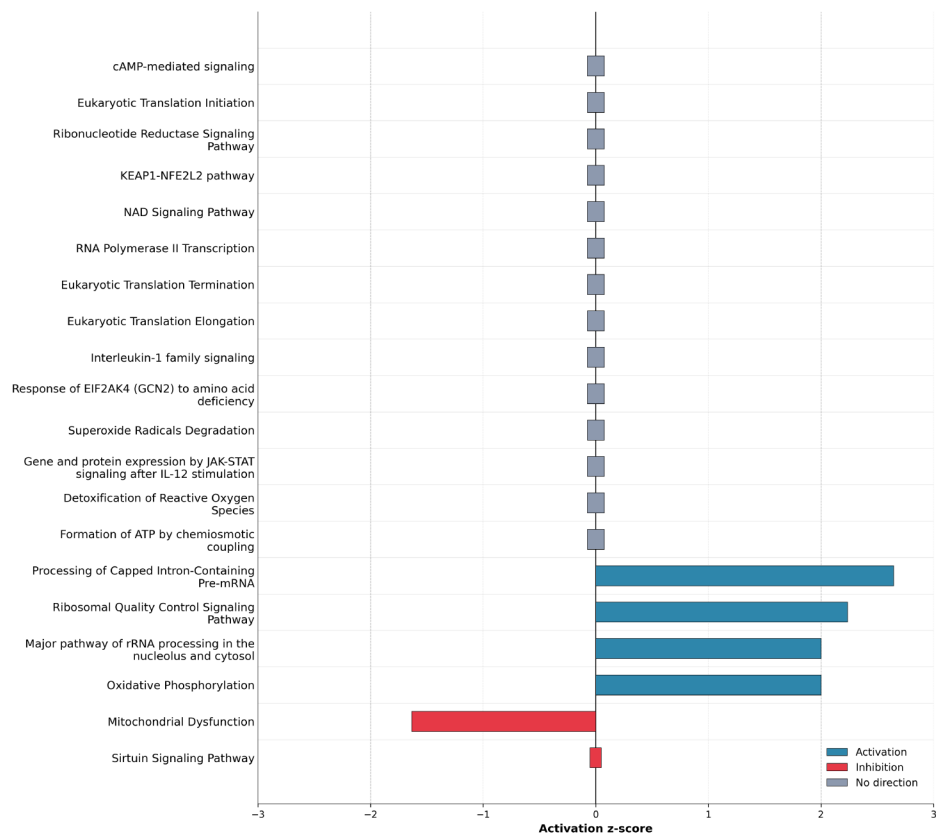
For cordycepin, the analysis of the set of shortened tail genes revealed a strong enrichment for pathways involved in the innate immune response, including TLR4/NF- κ B/MAPK signaling, as well as pathways related to the inflammasome, ROS production, and antiviral responses (Figure 6.10). The effects on pA tails of inflammatory mRNAs were expected, as NF κ B does not translocate [27]. The strong down-regulation of inflammatory mRNAs is expected given prior work in de Moor's lab showing cordycepin blocks NF κ B nuclear accumulation in RAW264.7 cells and in primary human macrophages within 1 h, with concordant repression of LPS-induced genes [27] [28].

Among the shortened-tail cordycepin-treated transcripts, several encode components that lie upstream of, or within, the PI3K/AKT signalling cascade. Notably, PIP5K1A—which encodes the kinase that generates phosphatidylinositol 4,5-bisphosphate (PIP2), the obligate substrate for Class I PI3K—appeared in both the "Fc γ Receptor-mediated Phagocytosis" and "mRNA 3' End Processing Signaling" pathways (Table 6.3). Poly(A) tail shortening of PIP5K1A could reduce PIP2 availability and thereby limit PI3K-dependent PIP3 production, providing a potential upstream mechanism for cordycepin's suppression of AKT activation that is distinct from direct kinase inhibition [175].

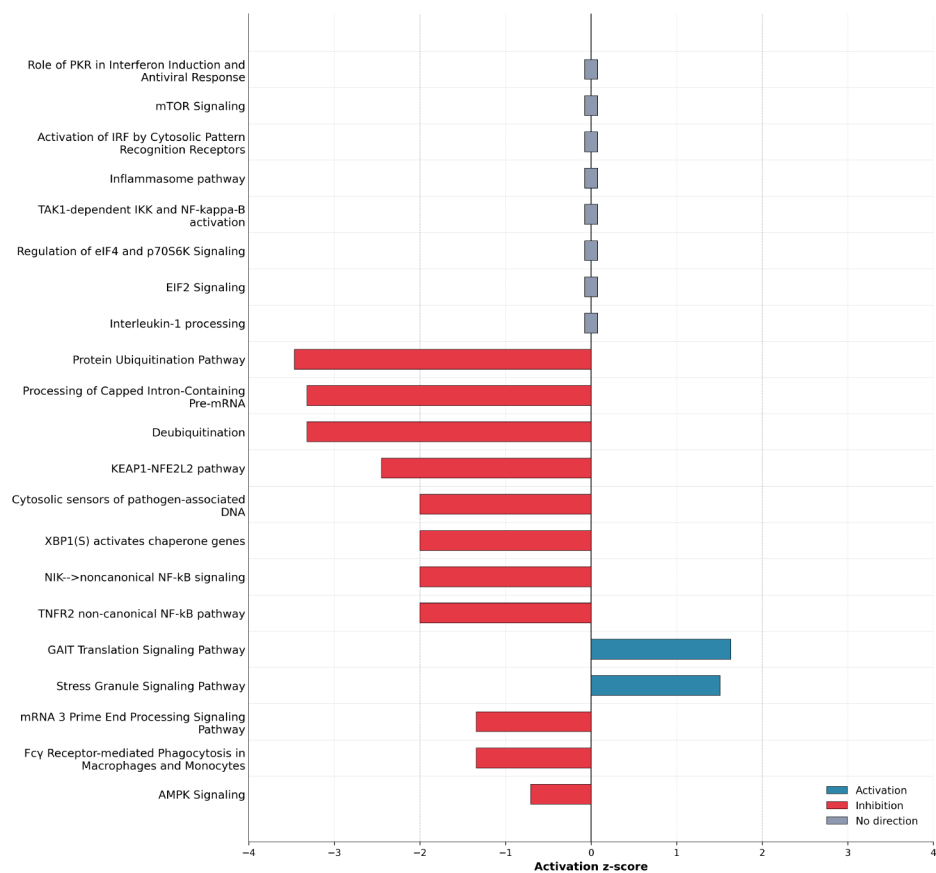
NUC-7738 treatment resulted in the shortening of poly(A) tails across a distinct range of pathways (Figure 6.12), with inflammatory and immune pathways accounting for approximately 35.0% of the enriched categories. Among these were CD40 signaling, TREM1 signaling, interleukin-10 signaling, and toll-like receptor signaling, as well as interferon-related pathways such as the DDX58/IFIH1-mediated induction of interferon-

α/β and the activation of IRF by cytosolic pattern recognition receptors, all of which provide substantial evidence supporting the assertion that NUC-7738 exerts anti-inflammatory effects.

Beyond the expected inflammatory pathway modulation, the IPA analyses revealed significant enrichment of RNA-processing and translation control pathways in both lengthened and shortened poly(A) tail lists, suggesting cordycepin having a post-transcriptional effect . Also, IPA analysis reveals pathways for integrated stress response (ISR) activation through eIF2 α signaling cascades and concurrent restraint of growth factor-mediated signal transduction. The involvement of growth factor pathway inhibition was independently validated in a recent systems pharmacology investigation that demonstrated broad suppression of growth factor signal transduction networks by cordycepin treatment [25]. The table delineates these pathways along with the genes from the dataset that are associated with these pathways.



(a) Canonical pathway for lengthened-tail transcripts after cordycepin treatment



(b) Canonical pathway for shortened-tail transcripts after cordycepin treatment

Figure 6.10: Canonical-pathway z-score profiles after sequential cordycepin → LPS treatment. (A) Lengthened-tail transcripts. (B) Shortened-tail transcripts. The analysis was performed on genes whose tails became significantly longer or shorter, using the signed tail change as the log-fold-change metric.

Table 6.2: IPA Canonical Pathways for Cordycepin-treated lengthened transcripts (120 min CoR/LPS vs DMSO/LPS, p-value filtered)

Pathways	z-score	Genes from the dataset
Sirtuin Signaling Pathway	0.0	ATP5F1A; ATP5F1C; LDHA; ND- UFA4; SOD1; SOD2
Mitochondrial Dysfunction	-1.633	ATP5F1A; ATP5F1C; ATP5MF; NDUFA4; SOD1; SOD2; UQCRB
Oxidative Phosphorylation	2.0	ATP5F1A; ATP5F1C; ATP5MF; NDUFA4; UQCRB
Major pathway of rRNA process- ing in the nucleolus and cytosol	2.0	FCF1; KRR1; RPL23A; RPL37; RPS26
Ribosomal Quality Control Sig- naling Pathway	2.236	ABCE1; PSMD5; RPL23A; RPL37; RPS26
Processing of Capped Intron- Containing Pre-mRNA	2.646	CWC25; PHF5A; SAP18; SF3B1; SNRPD2; SYMPK; YBX1
Formation of ATP by chemios- motic coupling		ATP5F1A; ATP5F1C; ATP5MF
Detoxification of Reactive Oxygen Species		ATOX1; SOD1; SOD2
Gene and protein expression by JAK-STAT signaling after IL-12 stimulation		LCP1; SOD1; SOD2
Superoxide Radicals Degradation		SOD1; SOD2
Response of EIF2AK4 (GCN2) to amino acid deficiency		RPL23A; RPL37; RPS26
Interleukin-1 family signaling		MYD88; PSMD5; SKP1
Eukaryotic Translation Elonga- tion		RPL23A; RPL37; RPS26

Continued on next page

Table 6.2 – *Continued from previous page*

Pathways	z-score	Genes from the dataset
Eukaryotic Translation Termination		RPL23A; RPL37; RPS26
RNA Polymerase II Transcription		CCNH; SYMPK; TAF6
NAD Signaling Pathway		LDHA; PARP9; SOD2
KEAP1-NFE2L2 pathway		DPP3; PSMD5; SKP1
Ribonucleotide Reductase Signaling Pathway		PARP9; SKP1; YBX1
Eukaryotic Translation Initiation		RPL23A; RPL37; RPS26
cAMP-mediated signaling		ADCY7; GRK2; PDE8B

Table 6.3: IPA Canonical Pathways for Cordycepin-treated shortened transcripts (120 min CoR/LPS vs DMSO/LPS, p-value filtered)

Pathways	z-score	Genes from the dataset
AMPK Signaling	-0.707	ELAVL1; EP300; GNG5; HMGCR; ILKAP; KAT2B; PFKP; PIK3CD; RPS6KB1
Fcγ Receptor-mediated Phagocytosis in Macrophages and Monocytes	-1.342	CBL; CRK; PIP5K1A; PLA2G6; RPS6KB1
mRNA 3 Prime End Processing Signaling Pathway	-1.342	CIRBP; CPSF7; EIF4G3; ELAVL1; PIP5K1A
Stress Granule Signaling Pathway	1.508	EIF4G3; ELP6; EP300; NFKB2; Oas2; PIK3CD; PPP1R15A; RPS4X; RPS6KB1; RPSA; TP53
GAIT Translation Signaling Pathway	1.633	EIF4G3; ELP6; RPL23; RPS4X; RPS6KB1; RPSA; VEGFA
TNFR2 non-canonical NF-kB pathway	-2.0	CD40; PSMA7; PSMC2; TNFRSF12A
NIK→noncanonical NF-kB signaling	-2.0	NFKB2; PSMA7; PSMC2; UBE2M
XBP1(S) activates chaperone genes	-2.0	DNAJB11; SRPRB; SYVN1; ZBTB17
Cytosolic sensors of pathogen-associated DNA	-2.0	CGAS; EP300; NFKB2; NKIRAS1
KEAP1-NFE2L2 pathway	-2.449	EP300; MUL1; PIK3CD; PSMA7; PSMC2; RPS6KB1

Continued on next page

Table 6.3 – *Continued from previous page*

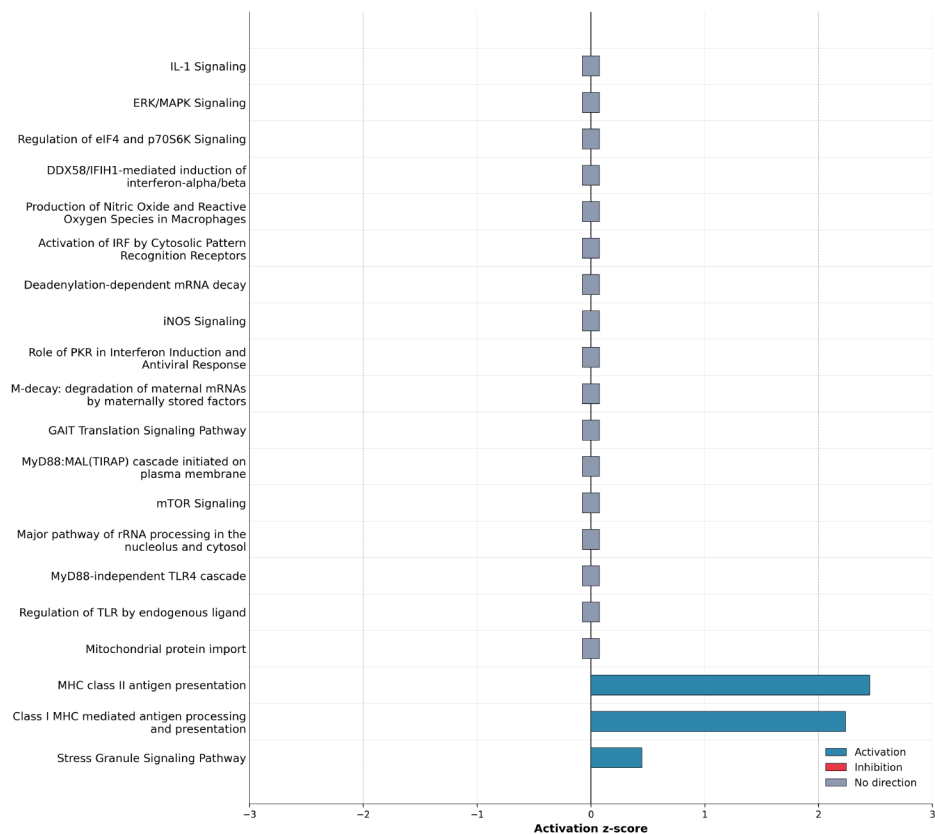
Pathways	z-score	Genes from the dataset
Deubiquitination	-3.317	BAP1; EP300; KAT2B; MUL1; PSMA7; PSMC2; TNKS; TP53; USP16; USP48; USP8
Processing of Capped Intron- Containing Pre-mRNA	-3.317	CHTOP; CPSF7; CWC22; DHX15; RBM7; RBMX; SNRNP40; TCERG1; TRA2B; U2SURP; ZCRB1
Protein Ubiquitination Pathway	-3.464	ANAPC4; BAP1; CBL; DNAJB11; DNAJC27; PSMA7; PSMC2; UBE2M; UBE3A; USP16; USP48; USP8
Interleukin-1 processing		IL1B; NFKB2
EIF2 Signaling		EIF4G3; ELP6; PIK3CD; PPP1R15A; RPL23; RPS4X; RPSA; VEGFA
Regulation of eIF4 and p70S6K Signaling		EIF4G3; ELP6; ITGB7; PIK3CD; RPS4X; RPS6KB1; RPSA
TAK1-dependent IKK and NF- kappa-B activation		NFKB2; NKIRAS1; TP53
Inflammasome pathway		IL1B; NFKB2
Activation of IRF by Cytosolic Pattern Recognition Receptors		CD40; IFIT2; NFKB2
mTOR Signaling		EIF4G3; PIK3CD; RPS4X; RPS6KB1; RPSA; VEGFA
Role of PKR in Interferon Induc- tion and Antiviral Response		CYC1; IL1B; NFKB2; TP53

To assess the depth of pathway coverage underlying the z-score predictions, Fig-

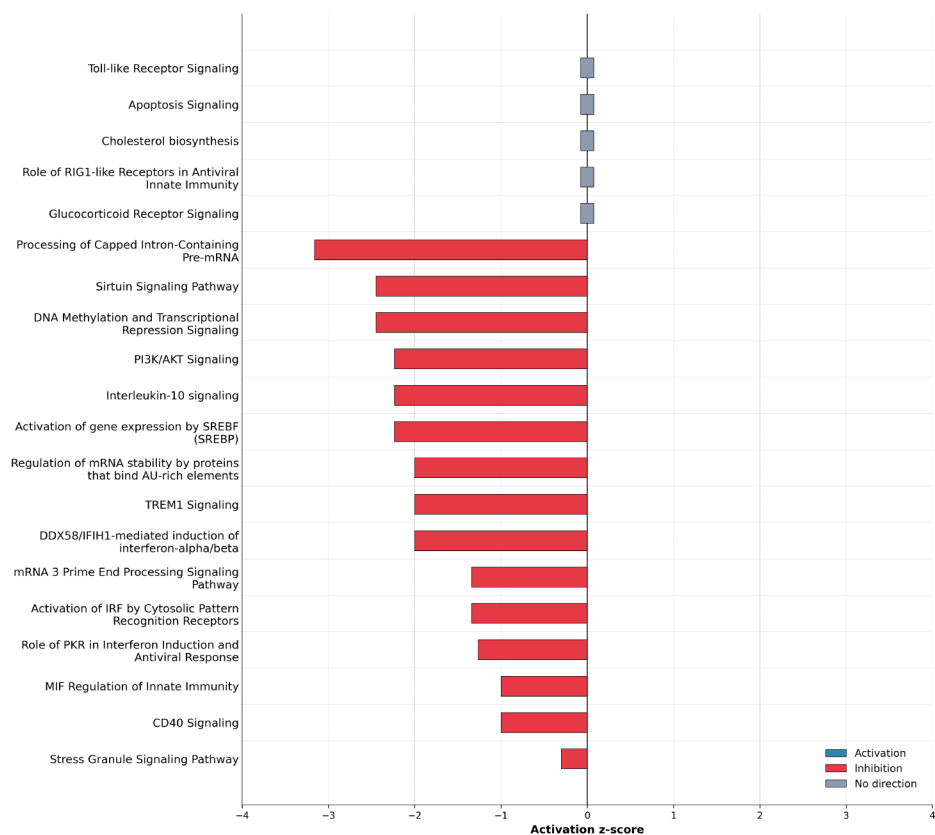
ure 6.11 shows the proportion of molecules in each IPA canonical pathway that were represented in the cordycepin poly(A) dataset. For each pathway, the horizontal bar indicates the percentage of the total pathway molecules (number shown at right) that overlap with genes in the lengthened- or shortened-tail lists. High coverage strengthens confidence in the z-score directionality, whereas low coverage flags pathways whose predictions rest on a small gene subset.



Figure 6.11: IPA canonical pathway molecule coverage for the cordycepin poly(A) tail analysis (120 min CoR+LPS vs DMSO+LPS). For each pathway, the horizontal bar shows the percentage of total pathway molecules (number at right) that were present in the dataset. Red segments indicate upregulated genes and green segments indicate downregulated genes within each pathway. **(A)** Pathways enriched among lengthened-tail transcripts. **(B)** Pathways enriched among shortened-tail transcripts.



(a) Canonical pathway for lengthened-tail transcripts after NUC-7738 treatment



(b) Canonical pathway for shortened-tail transcripts after NUC-7738 treatment

Figure 6.12: Canonical-pathway z-score profiles after sequential NUC-7738 → LPS treatment. (A) Lengthened-tail transcripts. (B) Shortened-tail transcripts. The analysis was performed on genes whose tails became significantly longer or shorter, using the signed tail change as the log-fold-change metric.

Table 6.4: IPA Canonical Pathways for NUC-7738-treated lengthened transcripts (120 min NUC/LPS vs DMSO/LPS, p-value filtered)

Pathways	z-score	Genes from the dataset
Stress Granule Signaling Pathway	0.447	EIF4A3; HDAC5; MRPS18B; RPS6KA1; RPS8
Class I MHC mediated antigen processing and presentation	2.236	CYBA; HMGB1; LY96; RCHY1; SEC24A
MHC class II antigen presentation	2.449	AP1S1; CAPZA2; DCTN3; DCTN6; RAB7A; SEC24A
Mitochondrial protein import		ATP5F1A; FXN; SLC25A12
Regulation of TLR by endogenous ligand		HMGB1; LY96
MyD88-independent TLR4 cascade		IKBKE; LY96
Major pathway of rRNA processing in the nucleolus and cytosol		BUD23; FCF1; MRPS18B; RPS8
mTOR Signaling		EIF4A3; MRPS18B; RPS6KA1; RPS8
MyD88:MAL(TIRAP) cascade initiated on plasma membrane		HMGB1; LY96
GAIT Translation Signaling Pathway		EIF4A3; MRPS18B; RPS6KA1; RPS8
M-decay: degradation of maternal mRNAs by maternally stored factors		CNOT10; EIF4A3
Role of PKR in Interferon Induction and Antiviral Response		HMGB1; IFNAR1; IKBKE
iNOS Signaling		IKBKE; LY96

Continued on next page

Table 6.4 – *Continued from previous page*

Pathways	z-score	Genes from the dataset
Deadenylation-dependent mRNA decay		CNOT10; EIF4A3
Activation of IRF by Cytosolic Pattern Recognition Receptors		IFNAR1; IKBKE
Production of Nitric Oxide and Reactive Oxygen Species in Macrophages		CYBA; IKBKE; PPP1R11
DDX58/IFIH1-mediated induction of interferon-alpha/beta		HMGB1; IKBKE
Regulation of eIF4 and p70S6K Signaling		EIF4A3; MRPS18B; RPS8
ERK/MAPK Signaling		ARAF; PPP1R11; RPS6KA1
IL-1 Signaling		ADCY7; IKBKE

Table 6.5: IPA Canonical Pathways for NUC-7738-treated shortened transcripts (120 min NUC/LPS vs DMSO/LPS, p-value filtered)

Pathways	z-score	Genes from the dataset
Stress Granule Signaling Pathway	-0.302	HSPA4; JMJD6; MRPS18A; Oas2; PPP1R15A; PTGS2; RPS29; RPS6KB1; RPS6KC1; RPSA; TP53
CD40 Signaling	-1.0	CD40; ICAM1; NFKBIB; PTGS2; REL; TRAF1
MIF Regulation of Innate Immunity	-1.0	NFKBIB; PLA2G6; PTGS2; REL; TP53
Role of PKR in Interferon Induction and Antiviral Response	-1.265	APAF1; CYC1; FANCC; HSPA4; IFIH1; IFNB1; MYD88; NFKBIB; PDGFB; REL; SP1; TP53
Activation of IRF by Cytosolic Pattern Recognition Receptors	-1.342	CD40; IFIH1; IFIT2; IFNB1; NFKBIB; REL
mRNA 3 Prime End Processing Signaling Pathway	-1.342	CNOT2; E2F8; ELAVL1; EXOSC9; H3-3B; PCF11
DDX58/IFIH1-mediated induction of interferon-alpha/beta	-2.0	IFIH1; IFNB1; NFKBIB; TRIM25
TREM1 Signaling	-2.0	CCL2; CD40; ICAM1; ITGA5; MYD88; REL
Regulation of mRNA stability by proteins that bind AU-rich elements	-2.0	ELAVL1; EXOSC9; PSMA7; PSMD8
Activation of gene expression by SREBF (SREBP)	-2.236	GGPS1; HMGCR; HMGCS1; NCOA2; SP1

Continued on next page

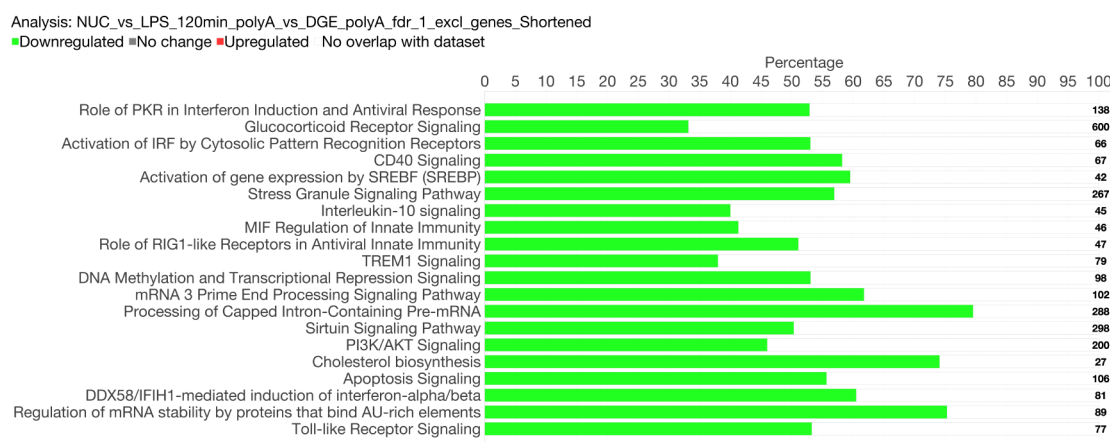
Table 6.5 – *Continued from previous page*

Pathways	z-score	Genes from the dataset
Interleukin-10 signaling	-2.236	CCL2; CCL5; CSF3; ICAM1; PTGS2
PI3K/AKT Signaling	-2.236	IL6R; IL7R; ITGA5; NFKBIB; PTGS2; REL; RPS6KB1; TP53
DNA Methylation and Transcriptional Repression Signaling	-2.449	CDK6; E2F8; GADD45G; SP1; TET3; TP53
Sirtuin Signaling Pathway	-2.449	ABCA1; ARG2; BECN1; CYC1; GABPB2; GADD45G; H3-3B; REL; SP1; TP53
Processing of Capped Intron-Containing Pre-mRNA	-3.162	CHTOP; PCF11; PRPF3; RBM25; RBM39; RBMX; SNRNP70; TCERG1; THOC1; ZCRB1
Glucocorticoid Receptor Signaling		ARG2; CCL2; CCL5; CYC1; GABPB2; H2-K2/H2-Q9; H3-3B; HSPA4; ICAM1; IFNB1; IL6R; IL7R; NCOA2; NFKBIB; PLA2G6; PPP3CB; PTGS2; TAF6L
Role of RIG1-like Receptors in Antiviral Innate Immunity		IFIH1; IFNB1; NFKBIB; REL; TRIM25
Cholesterol biosynthesis		GGPS1; HMGCR; HMGCS1
Apoptosis Signaling		APAF1; CYC1; NFKBIB; REL; TP53
Toll-like Receptor Signaling		MYD88; REL; TRAF1

As for cordycepin, the pathway molecule coverage for NUC-7738 is shown in Figure 6.13. The stacked bars indicate the fraction of each pathway’s total molecule count that overlapped with the NUC-7738 poly(A) dataset, providing context for interpreting the z-score predictions in Figure 6.12.



(a) Lengthened-tail transcripts



(b) Shortened-tail transcripts

Figure 6.13: IPA canonical pathway molecule coverage for the NUC-7738 poly(A) tail analysis (120 min NUC+LPS vs DMSO+LPS). For each pathway, the horizontal bar shows the percentage of total pathway molecules (number at right) that were present in the dataset. Red segments indicate upregulated genes and green segments indicate downregulated genes within each pathway. **(A)** Pathways enriched among lengthened-tail transcripts. **(B)** Pathways enriched among shortened-tail transcripts.

6.5.1 Cordycepin effects on mitochondrial respiration (Seahorse assay)

The enrichment of oxidative phosphorylation and the decrease in mitochondrial dysfunction within the poly(A)-lengthened gene set under cordycepin $\rightarrow$ LPS conditions suggested improved mitochondrial function at the pathway level. To test whether cordycepin influences mitochondrial respiration, a Seahorse XF Cell Mito Stress Test (Agilent) was performed in MCF-7 cells. MCF-7 cells were chosen for this assay for two reasons: first, RAW264.7 macrophages require a sequential compound-then-LPS protocol that is difficult to implement in the Seahorse plate format within the required timing constraints; second, MCF-7 cells are well characterised in the De Moor laboratory and respond to cordycepin by altering oncogene-related readouts without the need for an induction stimulus other than cordycepin itself, making them a technically tractable system for assessing acute cordycepin effects on respiration. (Fig. 6.14). The results should be interpreted as supportive evidence rather than a direct demonstration of mitochondrial effects in macrophages.

Cordycepin reduced mitochondrial proton leak in MCF-7 cells (50 μ M, 2 h), with a significant fall in leak OCR versus DMSO (Tukey, mean difference $\approx$ 48.9 pmol/min, $p = 0.0039$). In the same assay, maximal FCCP-driven OCR and spare respiratory capacity were lower with cordycepin by one-way ANOVA, whereas basal OCR was only modestly changed (Fig. 6.14). These functional data indicate tighter coupling (lower leak) but reduced respiratory capacity during acute cordycepin exposure [8] [9] [10].

Compared with the RAW264.7+LPS data, the IPA results for the poly(A)-lengthened gene list included mitochondria-related canonical pathways (increased Oxidative Phosphorylation and decreased Mitochondrial Dysfunction). The lower proton leak in MCF-7 is directionally consistent with reduced dysfunction (improved coupling), whereas the lower SRC does not fit a simple interpretation of increased oxidative phosphorylation that might be inferred from gene-set directionality. Overall, the transcriptomic and functional readouts show partial alignment: coupling appears improved, but maximal respiratory capacity is reduced.

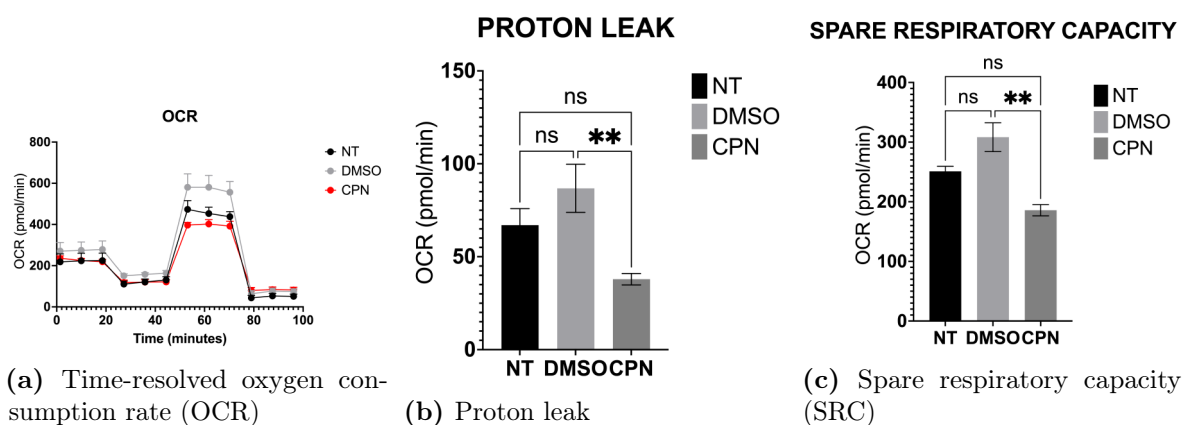


Figure 6.14: (a) Time-resolved oxygen consumption rate (OCR) traces for NT (no treatment; black), DMSO vehicle (grey) and CPN (cordycepin; red). Standard Mito Stress Test injections were applied in sequence—oligomycin (ATP synthase inhibitor), FCCP (uncoupler), and rotenone/antimycin A (complex I/III inhibitors)—to derive bioenergetic parameters [8] [9] [10]. (b) Proton leak (OCR after oligomycin minus non-mitochondrial OCR) across conditions. Lower values indicate less oxygen consumption not coupled to ATP production [8] [9] [10]. (c) Spare respiratory capacity (SRC) (maximal OCR after FCCP minus basal OCR), a metric of the cell's ability to respond to increased energy demand [8] [9] [10]. System: Human MCF-7 cells seeded at 300,000 cells/well; 50 μ M cordycepin for 2 h before the Seahorse assay. Instrument: Agilent Seahorse XF; Cell Mito Stress Test according to manufacturer guidance. NT = untreated; DMSO = vehicle; CPN = cordycepin. Acquisition and analysis: OCR is reported in pmol O_2 /min. Derived parameters follow Agilent's standard equations for the Cell Mito Stress Test (basal, ATP-linked, proton leak, maximal, SRC, and non-mitochondrial respiration). Statistics: One-way ANOVA across NT, DMSO, CPN with Tukey's post-hoc test.

Two contextual factors limit direct reconciliation. First, the systems differ: IPA directions were inferred from transcript sets in LPS-activated murine macrophages, whereas the Seahorse assay used unstimulated human MCF-7 cells; cell type and stimulus alter both mitochondrial control points and bioenergetic demand. Second, there is an endpoint mismatch: IPA z-scores report gene-level directionality, not flux. At 2 h, many respiratory-chain proteins may not yet have changed in abundance, while cordycepin is already suppressing growth-factor/PI3K–AKT–mTOR signaling and modulating AMPK, both of which are tightly integrated with mitochondrial metabolism [103] [25]. The Seahorse data therefore support an effect of cordycepin on mitochondrial function but do not allow a one-to-one mapping from pathway z-scores to respiratory capacity.

6.6 Discussion

This chapter provides, to our knowledge, the first genome-wide measurement of poly(A)-tail lengths following cordycepin and NUC-7738 treatment, and the first integration of tail-length changes, gene expression, pathway analysis and mitochondrial phenotyping in this context. Across nanopore-based poly(A) tail profiling in RAW264.7 macrophages, LPS alone produced a left-shift in the empirical cumulative distribution function (ECDF) of poly(A)-tail lengths (Fig. 6.2 6.1) and a predominance of gene-wise tail shortening (Fig. 6.3 6.2). Cordycepin pretreatment (20 μ M) accentuated these patterns with stronger ECDF shifts and widespread gene-level shortening (Figs. 6.4 6.5 6.3–6.4). NUC-7738 (200 μ M) showed similar directionality but with greater dispersion, while 3'-deoxyinosine (3DI, 200 μ M) had comparatively modest effects (Figs. 6.6 6.7 6.5–6.6; Table 6.1 6.1).

Gene-abundance analyses stratified by NanopLen tail classes revealed a consistent association between tail length and mRNA levels: transcripts with shortened tails tended to have lower abundance, whereas lengthened-tail classes showed increased abundance under the same stimuli (Figs. 6.8 6.9 6.7–6.8). QIAGEN IPA applied to NanopLen-defined gene sets highlighted inflammatory signaling and mRNA processing among the top canonical pathways: shortened-tail genes were enriched for TLR/NF- κ B/MAPK, mRNA 3'-end processing, eIF2/translation-control and ubiquitin pathways, whereas lengthened-tail genes were enriched for mitochondrial respiration (Oxidative Phosphorylation/Mitochondrial Dysfunction), sirtuin/NAD signaling and ribosome/translation terms (Figs. 6.10 6.12 6.2 6.3 6.4 6.5 6.9–6.10; Tables 6.2–6.3). The Seahorse assay in MCF-7 cells (reduced proton leak and spare respiratory capacity; Fig. 6.14 6.11) supports the conclusion that cordycepin modulates mitochondrial function, in line with the mitochondrial pathway signatures and its known effects on PI3K/AKT/mTOR and AMPK signaling [103] [25].

Poly(A) tail metrics. LPS alone shortened tails transcriptome-wide (ECDF left-shift; Fig. 6.2 6.1), with numerous genes falling below the equality line in gene-wise length scatter (Fig. 6.3 6.2). Cordycepin deepened this left-shift and increased the

fraction of significantly shortened genes (Figs. 6.4 6.5 6.3–6.4; Table 6.1 6.1, Δ median values). NUC-7738 reproduced the shortening trend at 200 μ M, whereas 3DI had the smallest effect at the same concentration (Figs. 6.6 6.7 6.5–6.6; Table 6.1 6.1). Because NUC-7738 and 3DI were profiled at 10-fold higher dose than cordycepin, no direct potency ranking is inferred. Together, these data show that cordycepin and NUC-7738 drive robust genome-wide deadenylation during acute LPS activation, while 3DI produces only a modest shift.

Tail–abundance association and lack of steady state. Within the 2 h total exposure (2 h compound, with LPS added at 1 h and therefore present for the final 1 h alongside the compound), shortened-tail classes generally showed lower mRNA abundance and lengthened-tail classes higher abundance (Figs. 6.8 6.9 6.7–6.8). Median mammalian mRNA half-lives are typically several hours, so 2 h lies within a transient regime where transcriptional induction and repression strongly influence abundance [176]. Many transcripts are therefore unlikely to have reached steady state. Tail shortening can occur in parallel with transcriptional repression of highly transcribed genes, so that existing molecules age and are deadenylated as transcription is curtailed. Conversely, some lengthened-tail transcripts may represent newly induced RNAs that have not yet been substantially deadenylated. For these reasons, the observed ordering (Shortened < Unchanged < Lengthened) provides evidence for correlation between tail length and abundance rather than proof that tail changes are driving expression changes. However, a further analysis of the dataset for changes in unspliced transcript levels, which are likely to follow changes in transcription rate, might allow us to at least partially untangle this relationship.

Pathway-level signals. Shortened-tail sets were enriched for inflammatory and innate immune modules (e.g., TLR/NF- κ B/MAPK), for mRNA 3'-end processing and for stress-adaptive translation control (eIF2 signaling; Regulation of eIF4 and p70S6K), together with protein ubiquitination pathways (Figs. 6.10 6.12; Table 6.3). Lengthened-tail sets were enriched for oxidative phosphorylation, mitochondrial dysfunction, sirtuin/NAD signaling and ribosome/translation pathways (Table 6.2). These pathway signatures show that the tail-length changes we detect map onto known

nodes of inflammatory signaling, 3'-end processing and translation control, and, more unexpectedly, onto mitochondrial and metabolic programs. The Seahorse data are consistent with this picture but also illustrate that transcriptional signatures (oxidative phosphorylation, mitochondrial dysfunction) and functional respiration (coupling vs spare capacity) need not align in a simple way within a 2 h window.

Signals involving PIP5K1A. The observation that PIP5K1A undergoes poly(A) tail shortening under cordycepin treatment -appeared in both the "Fc γ Receptor-mediated Phagocytosis" and "mRNA 3' End Processing Signaling" pathways (Table 6.3)- offers a mechanistically coherent link between CPA inhibition and suppression of the PI3K/AKT pathway. PIP5K1A generates PIP2, the lipid substrate that Class I PI3Ks convert to PIP3; without sufficient PIP2, even fully active PI3K cannot efficiently produce the PIP3 required for membrane recruitment and activation of AKT [175]. Reduced PIP5K1A mRNA stability or translation would therefore act as an upstream brake on the pathway, consistent with the rapid attenuation of AKT phosphorylation observed within 30 minutes of cordycepin treatment [25]. Importantly, this mechanism is independent of AMPK, aligning with Lawrence et al.'s demonstration that cordycepin suppresses PI3K/AKT signalling even when AMPK is knocked down [25]. Whether the poly(A) tail shortening of PIP5K1A directly reduces protein output, or serves primarily as a marker of broader translational restraint, remains to be determined.

Dynamic tail regulation under cordycepin. Many 3'-end-anchored RNA-seq workflows, including our ONT cDNA tail-calling, rely on 3'-OH-dependent ligation steps. T4 RNA ligase joins are efficient at 3'-OH/5'-phosphate nicks [163], so RNAs terminating in cordycepin [3'-deoxyadenosine (3'-dA)] are expected to be refractory to ligation and under-represented. Despite this detection bias, our nanopore tail measurements closely track patterns seen in independent short-read datasets from de Moor's lab [25] [27], arguing that RNAs with terminal cordycepin are rare at 120 min. This agrees with prior work showing that cordycepin-terminated poly(A) tails do not substantially accumulate over a 2 h window [19] and that cordycepin interferes with 3'-end formation and polyadenylation in vivo [45]. Together, the data support a model in which cordycepin labelling of 3' ends is transient and shifts the balance between

polyadenylation and deadenylation, rather than causing persistent chain termination.

PARN-sensitive transcripts among the lengthened-tail subsets. Poly(A)-specific ribonuclease (PARN) is a $3' \rightarrow 5'$ deadenylase whose nuclease activity requires a free 3'-OH on the RNA substrate. Classic work and subsequent studies indicate that the presence of cordycepin at the 3' end of a poly(A) tail inhibits PARN activity [177] [19]. Consequently, for transcripts that are normally PARN substrates, transient incorporation of cordycepin could reduce PARN-mediated shortening while other deadenylases (e.g., CCR4-NOT) remain active, yielding an apparent lengthening signature for those mRNAs. This provides a plausible contribution to the subset of lengthened-tail genes under cordycepin/NUC-7738 (Tables 6.2 6.4 6.2), although transcriptional induction of new RNAs in this 2 h window is an alternative explanation that is explored further in Chapter 7.

Transcription versus decay within 2 h. The co-occurrence of tail shortening and reduced abundance is compatible with transcriptional repression of rapidly induced genes together with decreased tail addition. Cordycepin is known to perturb 3' processing and to promote usage of distal poly(A) sites via kinetic competition, generating transcripts with extended 3'UTRs in budding yeast and mammalian systems [19] [38]. Under our 2 h design, these effects can blunt accumulation of LPS-induced cytokine and chemokine mRNAs after RNAPII recruitment without allowing steady-state feedback loops to develop. Accordingly, we interpret the alignment between tail classes and abundance as supportive correlation, not as definitive evidence that tail changes are the primary cause of the observed transcriptional phenotypes. Further analysis of this dataset for changes in poly(A) site choice and transcription termination may shed further light on this subject.

Inflammation versus RNA-processing/translation control. Enrichment of inflammatory pathways among shortened-tail genes is expected under adenosine-analogue treatment that impairs NF- κ B program induction by reducing PI3K/AKT signaling [25]. More informative in this dataset are the coherent RNA-processing and translation-control terms—eIF2 signaling and eIF4/p70S6K regulation among shortened-tail genes (Tables 6.3, 6.5) and ribosome/elongation/termination pathways

among lengthened-tail genes (Table 6.2)—which together suggest an integrated-stress-response-like restraint of initiation and a broader recalibration of protein synthesis [178] [135]. Mechanistically, Wong et al. demonstrated that cordycepin induces both eIF2 α phosphorylation and 4E-BP1 dephosphorylation in NIH3T3 fibroblasts; using mutant cell lines, they showed that 4E-BP1 dephosphorylation (which sequesters eIF4E and blocks cap-dependent initiation) is the dominant effector of translational repression, although eIF2 α phosphorylation also contributes [46]. The enrichment of both eIF2- and eIF4-related pathway terms in the present poly(A)-tail data is therefore consistent with engagement of these dual translational checkpoints. These signatures align with cordycepin’s ability to modulate PI3K/AKT/mTOR and MEK/ERK signaling and to alter translation within 30–120 min [25].

A mechanistic link to LPS-induced deadenylation. LPS transiently represses and then induces several HDACs in murine macrophages (HDAC4/5/7 and HDAC1) [177]. In parallel, inhibition of HDAC1/2 allows CBP/p300-mediated acetylation of the CCR4–CAF1–NOT deadenylase subunit CAF1a (CNOT7), which triggers widespread degradation of normally stable poly(A)+ RNA [179]. An early LPS window of reduced HDAC activity could therefore enable CBP/p300-dependent acetylation of CAF1a (CNOT7), transiently accelerating deadenylation and contributing to the observed left-shifts under LPS (Fig. 6.2). This is proposed as a mechanistic hypothesis grounded in the literature and compatible with the tail signatures observed here.

IPA pathways associated with poly(A) lengthening induced by cordycepin treatment

Table 6.2 and Figure 6.10 show the IPA pathways associated with the lengthening of poly(A) tails after cordycepin treatment and the key genes contributing to each pathway. Cordycepin (3'-deoxyadenosine) is rapidly converted to nucleotide metabolites, including cordycepin monophosphate (CoMP) and cordycepin triphosphate (CoTP). Hawley et al. showed that CoMP accumulates to levels comparable to AMP and is not more potent than AMP as an AMPK agonist, even though AMPK becomes activated within ~ 1 h [103]. More recent systems-pharmacology work indicates that CoTP is likely to be the active metabolite that represses growth-factor-induced gene expression

and that cordycepin consistently blocks PI3K/AKT/mTOR and/or MEK/ERK outputs in multiple cell types, with AMPK activation occurring in parallel but not required for these effects [25]. AMPK knockdown does not prevent the transcriptional response to cordycepin [25]. These observations together argue that growth-factor pathway suppression is primarily driven by cordycepin nucleotides themselves, rather than being secondary to AMPK activation.

In RAW264.7 macrophages subsequently challenged with LPS, these signaling effects cohere with the strong enrichment of oxidative phosphorylation and ATP formation by chemiosmotic coupling among lengthened-tail genes, reflecting rapid remodeling of ETC/ATP synthase subunits to rebalance ATP production and redox state [148] [103]. Complementary enrichments—mitochondrial dysfunction, detoxification of reactive oxygen species, degradation of superoxide radicals and the KEAP1–NFE2L2 pathway—indicate early AMPK/PI3K–AKT–mTOR–NRF2 crosstalk that both dampens ROS and strengthens antioxidant defenses (e.g. SOD1/2), consistent with cordycepin’s documented suppression of LPS-induced oxidative stress and inflammasome activation [52] [148] [180].

In the context of RNA, the best-established mechanism of cordycepin is the inhibition of the formation of 3′-end and polyadenylation, which selectively reduces the accumulation of inflammatory transcripts by preventing NFκB recruitment to the nucleus [28] [26]. The enrichment of “Processing of capped intron-containing pre-mRNA” and “RNA Polymerase II Transcription” among lengthened-tail genes highlights perturbation of transcription–3′-end coupling. In a mixed stimulus (cordycepin then LPS), transcripts that evade or compensate for 3′-end inhibition can nevertheless display longer poly(A) tails, consistent with the coexistence of APA/CPA effects and dynamic deadenylation [19] [45].

Energy/redox stress feeds directly into translation control. PI3K/AKT/mTOR inhibition and ISR activation limit ATP consumption by reducing initiation and elongation, aligning with enrichments for initiation/elongation/termination of eukaryotic translation and ribosomal quality-control signaling. Although direct evidence that cordycepin targets elongation or termination per se is limited, its combined effects on

mRNA maturation and growth-factor signaling may increase stalled ribosomes and activate RQC pathways that remove defective nascent chains [103] [181] [182] [135].

The "Response of EIF2AK4 (GCN2) to amino acid deficiency" pathway offers a mechanistic link: GCN2 senses uncharged tRNAs and ribosome collisions, phosphorylates eIF2 α and reduces global translation, a programme reported to temper inflammatory signalling in macrophages [135] [183] [174]. In the classical model, uncharged tRNAs accumulate when amino acid pools are depleted. However, an alternative hypothesis is that cordycepin may directly impair tRNA maturation. All functional tRNAs require post-transcriptional addition of a 3'-terminal CCA sequence by CCA-adding enzymes (tRNA nucleotidyltransferases), and the 3'-hydroxyl of this terminal adenosine is the obligate attachment site for aminoacylation [184]. If CCA-adding enzymes mis-incorporate cordycepin triphosphate in place of ATP, the resulting tRNAs would terminate in 3'-deoxyadenosine and lack the 3'-OH required for amino acid attachment. Such tRNAs would be permanently uncharged regardless of amino acid availability, potentially activating GCN2 through a mechanism that is independent of metabolic stress and consistent with the AMPK-independent nature of cordycepin's effects on PI3K/AKT/mTOR signalling [25]. This hypothesis remains to be tested directly but would provide an elegant link between cordycepin's established chain-terminating activity and its engagement of the integrated stress response.

On the immune axis, enrichment of interleukin-1 family signaling is expected under LPS. Cordycepin attenuates IL-1 β and NLRP3 activation while suppressing TLR4-MAPK/NF- κ B signaling, coherently explaining representation of the IL-1 pathway among lengthened-tail transcripts [52] [180]. "Gene and protein expression by JAK-STAT signaling after IL-12 stimulation" (although IL-12 was not added) likely reports early STAT-dependent crosstalk triggered by LPS-TLR4-IFN signaling (e.g. STAT3); cordycepin can inhibit STAT3 in other systems, but direct cordycepin-IL-12/JAK-STAT coupling in macrophages has not yet been demonstrated [185].

Finally, metabolic signaling nodes such as Sirtuin Signaling and NAD Signaling connect cordycepin to NAD⁺/SIRT activity. Cordycepin has been shown to elevate SIRT1 via AMPK and to modulate NAD-dependent programs, linking mitochondrial, antiox-

ident and transcriptional adaptations in these pathways [186] [187]. Ribonucleotide reductase signaling plausibly reflects nucleotide-pool stress: cordycepin nucleotides can partially replace adenosine nucleotides, although direct targeting of RNR by cordycepin has not been reported [103]. Overall, the lengthened-tail IPA pathways support a coherent early-acting network in which cordycepin imposes energy/redox and RNA-processing stress that gates transcription and translation and modulates inflammasome-centered cytokine programs during the first 2 h of cordycepin → LPS exposure.

IPA pathways associated with poly(A) shortening induced by cordycepin treatment

Table 6.3 and Figure 6.10 show the IPA pathways associated with shortening of poly(A) tails after cordycepin treatment and the key genes in each pathway. IPA enrichment for RAW264.7 cells with shortened poly(A) tails after 2 h cordycepin treatment (with LPS added at 1 h, in the presence of the compound, for a 1 h stimulation) converges on early-acting nodes that cordycepin perturbs directly, alongside innate immune modules that are likely second-order consequences of altered RNA metabolism and energy signaling.

First, cordycepin is converted intracellularly to nucleotide metabolites that suppress PI3K/AKT and thereby reduce mTORC1/2 signalling, p70S6K activity and cap-dependent initiation [25] [46]. Lawrence et al. showed that cordycepin triphosphate represses growth-factor signalling and that these effects are maintained when AMPK is knocked down, indicating that growth-factor pathway inhibition is AMPK-independent [25]. AMPK is nevertheless activated by cordycepin, although this may be a consequence of PI3K/AKT/mTOR inhibition rather than a parallel event, given the extensive crosstalk between these pathways—for example, via LKB1 [188]. This aligns with the enrichment for eIF2 signalling, regulation of eIF4 and p70S6K, and stress-adaptive modules (e.g. stress granule/mTOR) and explains how a translation-centric shift can emerge within 30–60 min and remain evident during the subsequent LPS challenge [46] [103]. Notably, cordycepin retains its ability to suppress inflammatory gene expression even when administered simultaneously with LPS rather than as a pre-treatment, underscoring the rapidity of its effects on this axis [164].

Second, rapid inhibition of mRNA 3'-end processing/polyadenylation is a hallmark of cordycepin, producing immediate poly(A) tail shortening with gene-selective sensitivity. This mechanism aligns with pathways such as "mRNA 3'-end processing" and "Processing of capped intron-containing pre-mRNA" and predicts attenuated induction of rapidly turned-over inflammatory transcripts after RNAPII recruitment, as observed when PAP α is genetically depleted [19] [189]. Given the 2 h timeline, such 3'-end effects can blunt LPS-driven cytokine/chemokine mRNAs before steady-state feedback loops dominate [164].

Third, macrophage TLR4–NF- κ B modules feature prominently. Cordycepin consistently reduces NF- κ B signaling, lowering iNOS/COX-2, TNF- α /IL-1 β and downstream transcriptional arms in RAW264.7 and related models [52] [15]. Enrichment of canonical and non-canonical NF- κ B branches is therefore mechanistically credible, although the non-canonical branch typically evolves more slowly; here, early transcript shortening and translational restraint would be expected to bias effects toward the fast MyD88/TRAF6–IKK axis while muting sustained NF- κ B feed-forward loops [190]. Ubiquitin-pathway components (E3 ligases, deubiquitinases, proteasome subunits) intersect these axes and may be secondarily affected by translation and RNA-processing constraints [190] [191].

Fourth, inflammasome-linked pathways (e.g. NLRP3/caspase-1/GSDMD) are plausible in this timeframe. Recent work shows that cordycepin restricts NLRP3 activation and macrophage pyroptosis, partly via suppression of ROS/xanthine oxidase and promotion of autophagy; this is consistent with IPA detection of inflammasome–autophagy crosstalk and would manifest as reduced IL-1 β /IL-18 maturation during LPS priming [192] [193]. The 2 h window permits priming signals but is compatible with attenuated processing when poly(A) dynamics and ROS control are perturbed. It remains important to distinguish pathways primarily impacted at the level of poly(A) tail modulation from those driven mainly by changes in mRNA abundance; this will require additional experiments.

Fifth, KEAP1–NRF2 antioxidant signaling fits both cordycepin's pharmacology and early timing. Cordycepin activates the NRF2–HO1 axis in macrophages, limiting

oxidative stress that fuels TLR4–NF- κ B and NLRP3 activation [194]. This provides a unifying axis linking redox control, translational restraint and reduced inflammatory amplifier loops during the LPS challenge.

Finally, several enriched modules warrant consideration in light of recent evidence that macrophage activation involves dynamic and widespread regulation of poly(A) tail length. Kwak et al. demonstrated that LPS stimulation of macrophages triggers extensive remodelling of poly(A) tails through the coordinated action of cytoplasmic polyadenylation (CPEB-dependent) and deadenylation (CCR4–NOT-mediated) pathways, with hundreds of transcripts undergoing tail lengthening or shortening independently of changes in mRNA abundance [195]. This provides a mechanistic framework for interpreting the poly(A) tail signatures observed here: inflammatory and signalling transcripts may be direct targets of regulated cytoplasmic polyadenylation or deadenylation during LPS responses, and cordycepin’s inhibition of poly(A) synthesis could perturb this dynamic equilibrium. Pathways such as GAIT-mediated translational silencing (EPRS/L13a), which post-transcriptionally represses cohorts of inflammatory mRNAs [196], and cGAS–STING cytosolic DNA sensing, which crosstalks with TLR4 signalling [197] [198], also appeared in the IPA enrichment. However, the direct mechanistic links between these pathways and poly(A) tail regulation remain to be established; their appearance here may reflect broader stress-signalling convergence rather than specific targeting by polyadenylation or deadenylation machinery.

Fc γ R-mediated phagocytosis likely reflects LPS-licensed uptake programs; class I PI3K is required for ingestion of larger particles, and there is emerging evidence that cordycepin can enhance macrophage phagocytosis in tumour contexts by lowering CD47 “don’t-eat-me” signaling, although a direct Fc γ R-specific link to cordycepin has not been established [199].

IPA pathways associated with poly(A) lengthening induced by NUC-7738 treatment

Table 6.4 and Figure 6.12 show the IPA pathways associated with poly(A) tail lengthening after treatment with NUC-7738 and the corresponding genes. NUC-7738 is a cordycepin (3′-deoxyadenosine) ProTide designed to deliver intracellular 3′-dATP and

overcome adenosine-deaminase inactivation and transport limitations. In first-in-human studies and preclinical models, NUC-7738 reduces NF- κ B activity and broadly perturbs RNA metabolism and protein synthesis, consistent with the core cordycepin mechanisms of CPA inhibition and PI3K/AKT/mTOR/MEK/ERK suppression [19] [29] [2] [25] [46]. AMPK activation is observed but is not required for growth-factor pathway repression [25]. Under the short (2 h) NUC-7738 $\rightarrow$ LPS protocol, immediate-early signaling and translational control dominate, and the lengthened-tail IPA profiles largely mirror those seen with cordycepin.

The lengthened-poly(A) gene set shows strong innate-immunity signatures. TLR4 modules (MyD88:Mal and TRIF/IKK ϵ) and IL-1/MAPK pathways are enriched, matching LPS stimulation and the known ability of cordycepin to reduce TLR4–NF- κ B/MAPK activation in RAW264.7 macrophages; NUC-7738 likewise lowers NF- κ B activity in patient tumours [29] [52] [54] [200] [201]. Tail lengthening of adaptors (LY96/MD-2) and DAMP mediators (HMGB1) may reflect compensatory stabilization when upstream signaling is pharmacologically damped. These nodes are engaged within minutes of LPS, fitting the 2 h window.

Interferon-axis pathways (IKK ϵ /PKR; RIG-I/MDA5 $\rightarrow$ IRF) indicate potential crosstalk between TLR4 endosomal signaling and cytosolic RNA detection. Although no direct effect of NUC-7738 on RLR/PPR signaling is reported, cordycepin’s translational blockade intersects with ISR logic (eIF2/eIF4), plausibly biasing IRF outputs while attenuating global initiation; this can arise rapidly after TRIF engagement and is considered mechanistically plausible but novel for NUC-7738 specifically [152] [131] [46] [202] [203] [204].

Consistent with cordycepin’s primary impact on 3′-end metabolism, deadenylation-coupled decay pathways (e.g. canonical Deadenylation-dependent mRNA decay) and related modules are enriched. Cordycepin and NUC-7738 block polyadenylation and shorten poly(A) tails on inflammatory mRNAs, restricting their accumulation without preventing transcription-factor recruitment—exactly the behaviour expected under a 2 h exposure [152] [19]. At the translation level, regulation of mTOR/eIF4 and p70S6K is prominent, consistent with PI3K/AKT/mTOR suppression [25] [46]. Reduced 4E-BP

phosphorylation limits cap-dependent initiation, and changes in S6K signaling further curtail protein synthesis. Stress-granule signaling aligns with global initiation slowdown, which partitions mRNAs into storage granules while allowing a subset of immediate-early messages to remain engaged [46] [203] [202] [29].

Antigen-presentation pathways (MHC II and class I) and organelle/biogenesis modules (rRNA processing, mitochondrial protein import) probably represent early compensatory responses to translational stress and altered trafficking. LPS up-regulates MHC programs; lengthened tails on vesicle/ER–Golgi genes (e.g. SEC24A, RAB7A) could help prepare the system for antigen handling even as NUC-7738 constrains inflammatory transcription and translation [19] [29] [2] [200] [201]. Overall, the NUC-7738 lengthened-tail pathways reinforce the same two-pronged mechanism inferred for cordycepin: 3'-end/translation control plus innate-immune rewiring.

IPA pathways associated with poly(A) shortening induced by NUC-7738 treatment

Table 6.5 and Figure 6.12 show the IPA pathways associated with shortened poly(A) tails after NUC-7738 treatment and the corresponding genes. NUC-7738 is a cordycepin ProTide designed to bypass adenosine metabolism and deliver active 3'-deoxyadenosine nucleotides to cells. Cordycepin triphosphate is a potent inhibitor of 3'-end RNA processing, causing CPA defects that shorten poly(A) tails and destabilize transcripts, particularly those with high CPA demand such as rapidly induced inflammatory mRNAs [19] [45] [29]. In RAW264.7 macrophages subjected to 2 h NUC-7738 treatment (with LPS added at 1 h, in the presence of the compound, for a 1 h stimulation), pathways enriched among poly(A)-shortened genes match this mechanism and the short timeframe: RNA-processing modules, innate immune sensors, translational stress mediators and early metabolic rewiring.

Processing of the 3' end of mRNA and processing of capped intron-containing pre-mRNA indicate direct engagement of CPA/splicing coupling. Tail shortening of CPA regulators (e.g. PCF11, ELAVL1, CNOT2) is expected when CPA complex assembly or availability is perturbed by 3'-deoxynucleotides [205] [45] [29]. These changes couple with stress-granule signaling, reflecting rapid eIF2 α -dependent translational pausing

and RNP sequestration under RNA stress [206] [207]. Together, these RNA-centric events can suppress production of labile LPS-induced transcripts.

Early pattern-recognition pathways (PKR, RIG-I/MDA5 $\rightarrow$ IRF3, IFN- α/β induction) among shortened genes are consistent with the idea that defective RNA maturation alters endogenous RNA ligands and the set point of antiviral sensors [131] [205]. Convergence on NF- κ B/IRF axes provides a mechanistic bridge to the reported activity of NUC-7738, which reduces LPS-induced NF- κ B nuclear accumulation and dampens inflammatory transcription within an hour in human and murine macrophage systems [29] [208].

Metabolic rewiring is signaled by the PI3K/AKT, Sirtuin, and SREBF/SREBP - cholesterol - biosynthesis pathways. Cordycepin inhibits PI3K/AKT [25], which antagonizes mTORC1 and SREBP-driven lipogenesis [209] [61]. Within 2 h, full transcriptional remodeling of lipid pathways is unlikely, but poly(A) tail shortening of mRNAs encoding sterol/fatty-acid enzymes (e.g. HMGCR, HMGCS1) is plausible as an immediate post-transcriptional brake. The sirtuin axis (e.g. SIRT1) offers additional rapid control of inflammatory transcription through deacetylation of NF- κ B p65 [210], complementing NUC-7738's attenuation of nuclear NF- κ B after LPS [29].

ARE-mediated mRNA stability emerges as a post-transcriptional checkpoint for cytokines and immediate-early genes; CPA defects often synergize with ARE-dependent decay to reduce inflammatory mRNAs [211] [19] [212]. Apoptosis signaling appears as a downstream consequence: NUC-7738 is pro-apoptotic in multiple models [29] [213], although within 2 h we expect primarily priming rather than full execution. DNA-methylation and transcriptional-repression pathways likely reflect early recruitment of repressive complexes interfacing with CPA/termination, rather than de novo methylation changes over this timescale [205].

Collectively, the pathways enriched among poly(A)-shortened transcripts suggest an early-phase mechanistic footprint for NUC-7738/cordycepin in LPS-activated macrophages: (i) primary disruption of 3'-end RNA processing and translation control; (ii) tuned innate-immune sensor thresholds with reduced NF- κ B/IRF outputs; and (iii) rapid metabolic anti-anabolism via PI3K/AKT $\rightarrow$ mTORC1/SREBP antagonism, together

limiting the inflammatory burst [2, 13, 19, 25, 29, 45, 61, 131, 205, 210–218].

Mechanistic model: three linked processes on the 2 h timescale

Three processes emerge from these data as key contributors to cordycepin/NUC-7738 action within 2 h.

First, 3'-end formation and deadenylation balance. Cordycepin triphosphate compromises CPA, which is sufficient to shorten poly(A) tails and limit accumulation of rapidly induced messages such as LPS-responsive cytokines [45]. In budding yeast and mammalian systems, CPA interference and kinetic competition at alternative poly(A) sites are well established [45] [38]. The requirement of PARN for a free 3'-OH at RNA termini, together with the potential for terminal 3'-dA to inhibit PARN, offers a plausible explanation for a subset of lengthened-tail transcripts, although our 3'-OH-dependent library chemistry under-captures 3'-dA-ended RNAs and therefore cannot by itself prove their existence in vivo [219]. The present data are best interpreted as consistent with transient cordycepin incorporation that perturbs the balance between polyadenylation and deadenylation.

Second, translation initiation restraint. IPA results repeatedly implicate eIF2/ISR and mTOR/eIF4E–4E-BP in the “shortened” class (Figs. 6.10 6.12 6.9–6.10), matching reports that cordycepin reduces PI3K/AKT/mTOR and MEK/ERK signaling within 30–120 min and lowers cap-dependent initiation [25]. The stress-granule signature that emerges in the shortened set is consistent with canonical initiation slowdown and selective message triage [178] [135].

Third, early metabolic resetting. Enrichment for oxidative phosphorylation and mitochondrial quality-control pathways among the lengthened-tail genes, together with reduced proton leak and spare respiratory capacity in the Seahorse assay, suggests a rapid energy-conservation response accompanying translation restraint (Fig. 6.14 6.11; [220]). Mitochondrial activity is tightly integrated with PI3K/AKT/mTOR and AMPK signaling [103] [25], providing a mechanistic link between the transcriptional and functional observations.

Under the brief (2 h) LPS $\pm$ adenosine-analogue exposures used here, the data therefore support a two-pronged mechanism: (a) CPA inhibition plus selective deadeny-

lase effects leading to shorter tails and reduced accumulation of inflammatory mRNAs; and (b) rapid translation suppression via PI3K/mTOR/ISR nodes, with mitochondrial changes consistent with an energy-saving state [45] [38] [25] [178] [135] [220].

The tail-based pathway shifts cohere with orthogonal gene- and protein-level readouts reported for cordycepin/NUC-7738. A systems-pharmacology study across multiple cell types shows broad repression of PI3K/AKT/mTOR and MEK/ERK outputs within 30–120 min, polysome-level effects within 30 min and AMPK independence of the core response [25]. Cordycepin reduces NF- κ B nuclear accumulation in RAW264.7 and primary human macrophages and attenuates pain/synovitis in osteoarthritis models [28]; aggregate evidence confirms net anti-inflammatory trends with cell-type variability [13]. Together with classic CPA interference by cordycepin [45] and the principles of APA kinetic competition [38], these findings align closely with the “shorten and diminish” inflammatory axis and the rebalancing of translation and mitochondrial metabolism inferred here (Figs. 6.8 6.9 6.10 6.12 6.14 6.7–6.11).

Limitations and future directions. A key design feature of our nanopore tail-calling workflow is its reliance on 3'-OH-dependent ligation. RNAs terminating in cordycepin (3'-deoxyadenosine) are therefore under-represented, meaning that molecules with the most severely affected tails may be missed [219]. This is inherent to the chemistry rather than a flaw, but it highlights the value of complementary comparisons with Illumina RNA-seq datasets for cordycepin in similar conditions, which could reveal transcripts that disappear from poly(A)-selected libraries because of blocked tails. While there is no reason to think that the method fails to measure poly(A) tails in general, it will be important to validate at least some shortened and lengthened candidates with independent assays (e.g. PCR-based tail measurements) in future work.

A formal analysis of alternative polyadenylation (APA)—i.e. shifts between proximal and distal poly(A) sites—was not conducted in this study, despite the well-established role of cordycepin in altering poly(A) site choice [38]. Nanopore long reads are in principle well suited for APA quantification, but the computational pipeline used here (IsoQuant $\rightarrow$ edgeR), as well as NanopLen, was primarily optimized for gene-level differential expression analysis and for estimating median poly(A)-tail lengths

rather than isoform-level 3'-end mapping. A dedicated APA analysis would require transcript-level 3'-end clustering and quantification tools such as LAPA [221], TAPAS, or polyApipe, and constitutes an important future direction that could reveal whether cordycepin shifts poly(A) site usage in addition to shortening tails.

IPA z-scores provide directional predictions rather than quantitative effect sizes; pathway calls (e.g. stress-granule/ISR) are therefore treated here as signatures that require direct experimental validation. The Seahorse validation used MCF-7 rather than macrophages, reflecting availability of optimized assays; mitochondrial conclusions are therefore supportive rather than definitive for RAW264.7. Finally, the 2 h timeframe makes it difficult to separate tail changes caused directly by cordycepin incorporation from those secondary to changes in transcription rates. The shorter-timelapse experiments in Chapter 7 are designed to enrich for more direct effects by further reducing the contribution of transcriptional feedback.

Within 2 h of exposure, cordycepin and NUC-7738 shorten poly(A) tails genome-wide and reduce inflammatory mRNA accumulation, while a defined subset of transcripts shows lengthening, plausibly reflecting PARN-sensitive or newly synthesized RNAs. These tail dynamics are coupled to suppressed initiation (PI3K/mTOR/ISR), stress-granule signatures and mitochondrial adjustments, providing a coherent, rapid-acting axis for reshaping the early macrophage response to LPS and setting the stage for the more fine-grained kinetic analysis in Chapter 7.

Chapter 7

Cordycepin induces early poly(A)-tail changes in mRNAs related to growth-factor and innate immune signaling

7.1 Introduction

Work in this thesis, together with the wider literature, suggests that cordycepin (3'-deoxyadenosine) inhibits PI3K/AKT/mTOR and MEK/ERK signal transduction and affects mRNA polyadenylation in multiple systems [13, 25, 28]. Although inhibition of polyadenylation can influence signal transduction, it remains unclear how mRNA 3'-end processing is mechanistically linked to growth-factor and innate immune signaling.

In biochemical and genetic systems, cordycepin triphosphate perturbs pre-mRNA 3'-end formation and alters poly(A)-tail length [28]. The literature indicates two-way communication between 3'-end processing and PI3K/AKT/mTOR signaling: polyadenylation factors (e.g., CPSF/CstF/CFIm components) can tune PI3K/AKT outputs, while mTOR activity and its effectors feed back on alternative polyadenylation choices and polyadenylation-factor localization [222–226]. Together, these observations provide a conceptual bridge between cordycepin's canonical action at the mRNA 3'-end and its

impact on signaling pathways.

The PI3K/AKT/mTOR pathway is a principal regulator of cap-dependent translation through phosphorylation of 4E-BP1 and activation of p70S6K, thereby controlling eIF4F assembly and ribosome engagement. In innate immune cells, mTOR integrates inflammatory cues with metabolic state to shape effector programs, making this axis a plausible and temporally compatible target for the rapid effects of cordycepin treatment under LPS [227–230]. Systems-level work shows that cordycepin suppresses growth-factor signal transduction converging on PI3K/AKT/mTOR, with characteristic repression of translation-initiation outputs; notably, polysome profiling revealed redistribution of TOP mRNAs within ≈ 30 min of exposure, an early hallmark of mTOR pathway inhibition that precedes broad transcriptional remodeling [25].

Cytoplasmic polyadenylation provides a mechanism for tail remodeling on pre-existing mRNAs to control translation; CPEB family proteins can recruit both poly(A) polymerases and deadenylases, linking tail length to translational activation or repression in somatic cells [39, 42]. Based on previous chapters and these studies, I hypothesised that mRNAs related to growth-factor and inflammatory (innate immune) signaling would be particularly sensitive to cordycepin, showing early changes in RNA abundance or poly(A)-tail length. I further considered the possibility of one or more RNA targets of cordycepin (RToCs): RNAs whose polyadenylation is required for PI3K/mTOR and inflammatory signaling to proceed, in the context of evidence that general inhibition of translation can superinduce immediate-early transcription and activate AKT [69] [70].

This chapter builds directly on the transcriptomic (Chapter 5) and poly(A)-tail (Chapter 6) analyses performed at 120 minutes, and is further motivated by the independent haploid resistance screen in Chapter 4. Together, those earlier chapters showed that (i) loss-of-function disruptions in mRNA 3'-end processing and deadenylation machinery (CNOT6, PARN, Integrator subunits, nucleoporins) confer resistance to cordycepin in a genetic screen (Chapter 4), (ii) cordycepin and NUC-7738 converge on inhibition of PI3K/AKT/mTOR and inflammatory signalling modules at the gene-expression level (Chapter 5), and (iii) cordycepin produces genome-wide poly(A)-tail shortening with selective remodelling of inflammatory transcripts (Chapter 6). The

convergence of genetic, transcriptomic and poly(A)-tail evidence supports a model in which cordycepin's impact on mRNA 3'-end formation is mechanistically linked to its suppression of growth-factor and innate immune signalling.

However, the 2-hour timeframe used in Chapters 5 and 6 made it difficult to separate direct CPA-related effects from secondary transcriptional remodelling — an especially important concern given that cordycepin has been reported to suppress PI3K/AKT/mTOR outputs and redistribute TOP mRNAs from polysomes within approximately 20–30 minutes of exposure [25], well before the 2-hour sampling window used in those chapters. The present chapter therefore examines a much earlier time point (15 minutes) to capture changes that are more likely to reflect the proximal biochemical consequences of cordycepin treatment, before wholesale transcriptional re-programming is established, and to test whether the pathway signatures identified at 120 min are already detectable when transcription is not yet the dominant contributor.

To probe such early events, I focused on a 15-min window after LPS stimulation, during which immediate-early mRNAs have not yet accumulated: transcriptional pulses typically begin at ≥ 20 min and TNF/IL-1 peaks are ≈ 60 min [231–233]. In this regime, any RNA-level differences primarily reflect changes in the abundance or tail state of pre-existing transcripts, rather than de novo transcription programs.

Here, I set out to define the earliest, genome-wide consequences of simultaneous cordycepin + LPS exposure in RAW264.7 macrophages. Using Oxford Nanopore-based analyses of poly(A)-tail lengths (Dorado basecalling and NanopLen linear mixed models at the transcript level) alongside edgeR differential gene-expression analysis, I quantify RNA-abundance and poly(A)-tail changes after 15 min of co-treatment. I then leverage QIAGEN Ingenuity Pathway Analysis (IPA) to map differentially expressed genes and transcripts with shortened or lengthened poly(A) tails onto Canonical Pathways and Upstream Regulators. Throughout, I evaluate these data in the context of inflammation and the emerging model of bidirectional coupling between 3'-end processing and PI3K/AKT/mTOR signaling in shaping the early inflammatory response [13, 28, 38, 222–226].

7.2 Results

7.2.1 Early RNA abundance changes at 15 minutes are modest

I first asked whether a 15-minute co-treatment with cordycepin under LPS is sufficient to produce detectable changes in steady-state mRNA abundance. RAW264.7 macrophages were treated with LPS (1 $\mu\text{g/mL}$) in the presence or absence of cordycepin (20 μM) for 15 minutes, with three biological replicates per condition. Differential gene expression analysis identified a small set of transcripts with the most pronounced changes between CoR+LPS and DMSO+LPS, which were visualised as a hierarchically clustered, row-wise scaled heatmap of 51 DGE-prioritised genes with overlaid normalised counts (Figure 7.1).

Despite the brief exposure period, cordycepin co-treatment induced reproducible, directionally consistent alterations in specific transcript subsets across all three replicates. PI3K/MAPK-associated transcripts demonstrated increased expression under cordycepin co-treatment, with *Pik3c2a* (encoding PI3K class II α) rising from 95/92/77 to 124/117/101 normalised counts and *Plcb4* increasing from 170/184/159 to 232/205/191 across the three replicates. Similarly elevated were *Mapkbp1* (MAPK binding protein 1) and *Dhx29* (a translation-associated helicase), while transcripts involved in vesicular trafficking and innate immune signalling showed decreased expression, including *Arrdc1* (arrestin-domain containing protein), *Rab29* (vesicular trafficking regulator), *Tifa* (innate immune adaptor), and *Cemip2*. The heatmap was used to assess the directionality of these early changes relative to the LPS-only condition, and to determine whether initial alterations contradict or align with known LPS-induced responses, such as a potential elevation of *Pik3c2a* under LPS alone.

Overall, only a limited subset of LPS-responsive transcripts showed measurable abundance changes at 15 minutes, indicating that cordycepin's early impact on RNA levels is modest. These DGE-prioritised genes then served as the input for pathway-level analysis using Ingenuity Pathway Analysis (IPA) in the next subsection.

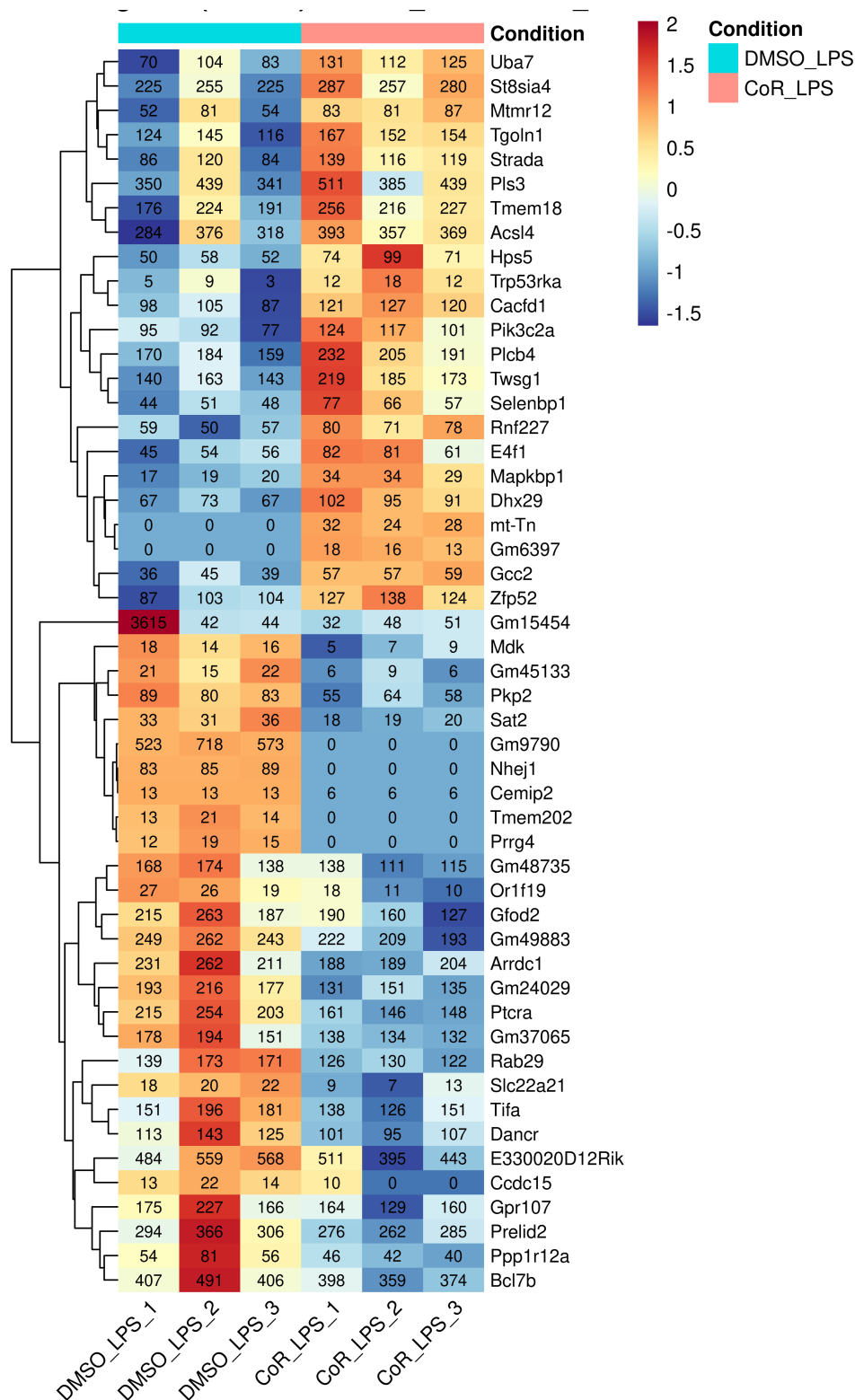


Figure 7.1: Early (15 min) cordycepin co-treatment modestly reshapes a subset of LPS-responsive transcripts in RAW264.7 macrophages. RAW264.7 cells were stimulated with LPS (1 $\mu\text{g/mL}$) $\pm$ cordycepin (CoR, 20 μM) for 15 min; three biological replicates per condition (columns DMSO_LPS_1–3, CoR_LPS_1–3). Heatmap of 51 DGE-prioritized genes (rows) with per-cell normalized counts overlaid (numbers) and row-wise scaled colors (blue = below-row mean; red = above-row mean; scale -1.5 to 2). Example features include Pik3c2a, Plcb4, Mapkbp1, Dhx29, Arrdc1, Rab29, Tifa, Cemip2

7.2.2 Canonical pathways from DGE: mTOR/EIF4/EIF2 and PI3K/ERK nodes emerge

To place the modest early DGE signature into a pathway context, Ingenuity Pathway Analysis (IPA) was applied to the edgeR-derived differentially expressed gene set from RAW264.7 macrophages exposed to cordycepin (20 μ M) and LPS (1 μ g/mL) for 15 minutes (Figure 7.2 and Table 7.1). IPA revealed enrichment of canonical pathways converging on cap-dependent translation and growth-factor signaling. The most prominently enriched pathways included p70S6K Signaling, mTOR Signaling, Regulation of eIF4 and p70S6K, and PI3K/AKT Signaling, forming a coherent module around PI3K/AKT/mTOR and translation-initiation control. Activation z-scores for these pathways were close to zero, with predominantly grey bars indicating no clear directional prediction at 15 minutes, but the pathway identities were consistent with previous work showing that cordycepin treatment represses PI3K/mTOR outputs and rapidly displaces TOP mRNAs from polysomes [25]. Overall, the IPA results point to an early focus on translational control rather than broad transcriptional reprogramming at this time point.

Alongside these translation-related modules, IPA also identified enrichment of innate immune and stress-responsive pathways, including ERK/MAPK Signaling, TAK1-dependent IKK and NF- κ B activation, and NF- κ B Activation by Viruses, consistent with engagement of the TLR4 signaling axis by LPS. At 15 minutes, however, activation z-scores for these inflammatory pathways remained direction-neutral, so the analysis does not yet provide evidence for NF- κ B pathway activation or repression at this time point, although previous work has shown that cordycepin can repress inflammatory gene expression with longer exposure [28]. Multiple trafficking and cytoskeletal regulatory pathways were also enriched, including Clathrin-mediated Endocytosis, Macropinocytosis, Integrin Signaling, Signaling by Rho Family GTPases, Actin Cytoskeleton Signaling, and PAK Signaling. Finally, enrichment of PDGF Signaling, Sphingosine-1-phosphate Signaling, and energy-dependent regulation of mTOR by LKB1-AMPK/AMPK Signaling is compatible with a response organised around growth-factor pathway inhibition

and energetic checkpoint control, in line with systems-pharmacology analyses that characterise cordycepin as a general inhibitor of growth-factor signal transduction rather than a selective AMPK agonist [25].

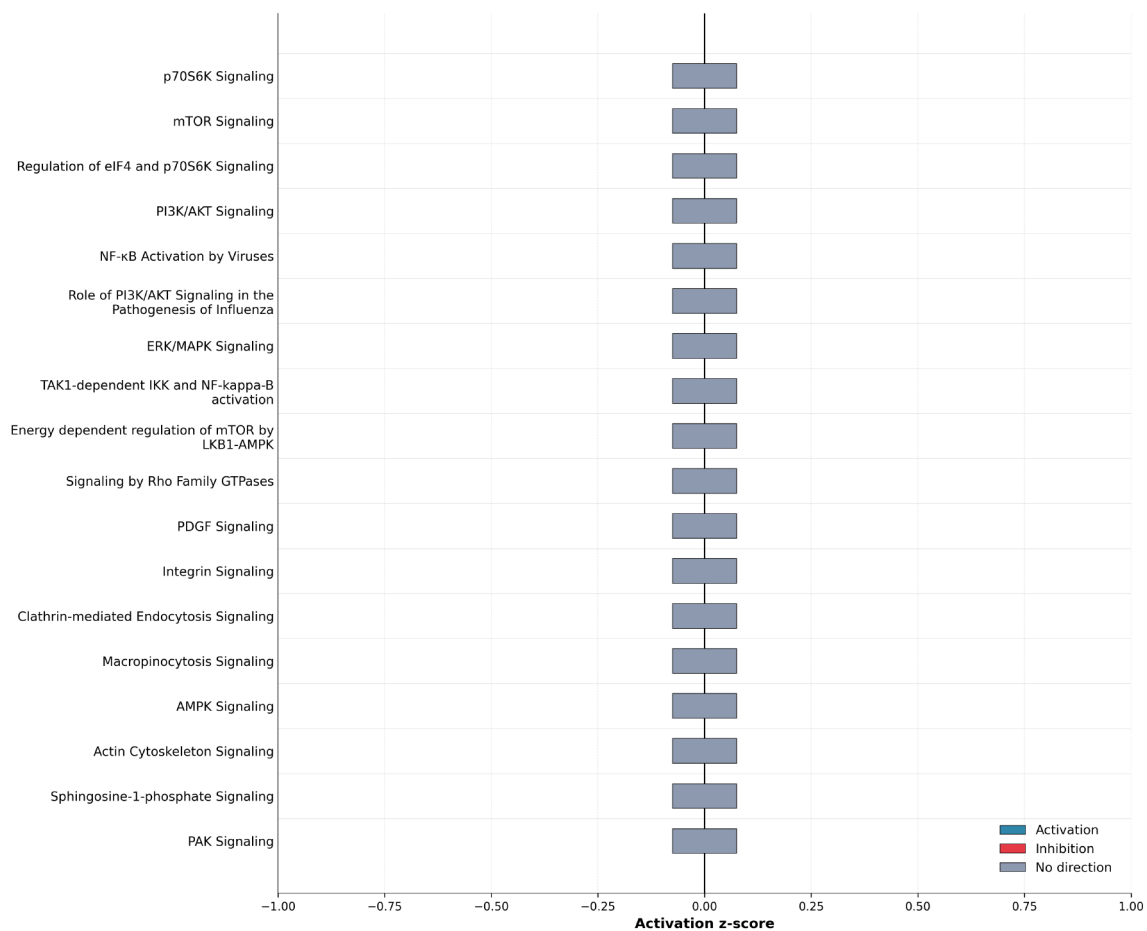


Figure 7.2: Canonical pathways significantly modulated by cordycepin At 15 min of simultaneous cordycepin (20 μ M) and LPS (1 μ g/mL) exposure in RAW264.7 cells. The activation z-scores, predicted by Ingenuity Pathway Analysis (IPA), indicate pathway activation (blue bars), inhibition (red bars), or no clear directional prediction (grey bars).

To quantify how much of each enriched canonical pathway was actually represented in the 15-min cordycepin DGE dataset, a horizontal stacked bar chart was generated in QIAGEN IPA (Figure 7.3). Each bar corresponds to one IPA canonical pathway; bar length reflects the proportion of the pathway's total molecules recovered in the dataset, and the numeric label to the right of each bar gives the total number of molecules annotated to that pathway in the IPA knowledge base. Red segments indicate upregulated genes and green segments indicate downregulated genes within each pathway. The chart shows that most of the pathways identified at 15 min (PAK Signaling, AMPK Signaling, ERK/MAPK Signaling, PI3K/AKT Signaling, Regulation of eIF4 and p70S6K, mTOR Signaling and related modules) are represented by only a small fraction of their total annotated molecules, consistent with the modest early DGE signature at this time point.

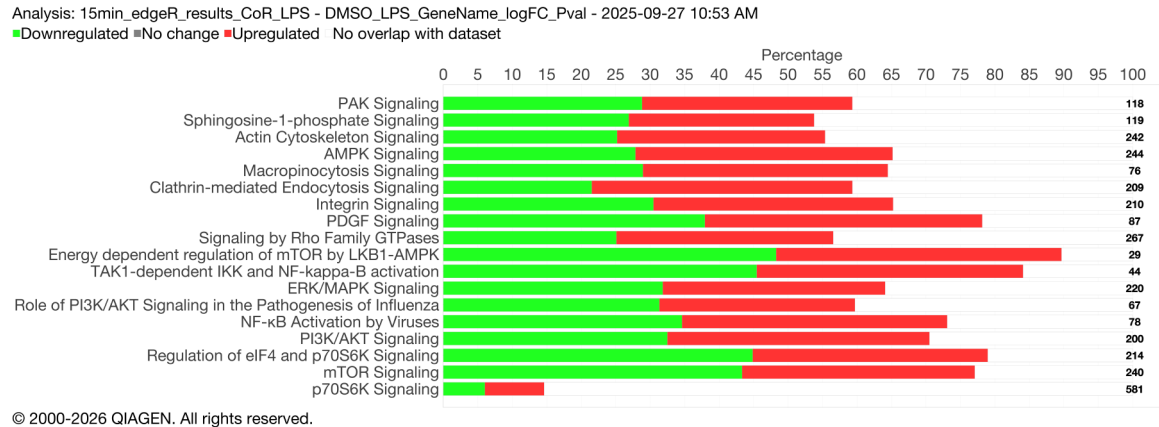


Figure 7.3: IPA canonical pathway coverage for DEGs at 15 min following co-treatment of RAW264.7 macrophages with cordycepin (20 μ M) and LPS (1 μ g/mL) versus DMSO + LPS. Horizontal stacked bars show, for each enriched canonical pathway, the proportion of pathway molecules recovered in the dataset; the number to the right of each bar is the total number of molecules annotated to that pathway in the IPA knowledge base. Red segments indicate upregulated genes and green segments indicate downregulated genes within each pathway. Generated using the QIAGEN IPA Stacked Bar Chart tool.

Table 7.1: IPA Canonical Pathways for Cordycepin treatment (15 min CoR/LPS vs DM-SO/LPS, p-value filtered)

Pathways	z-score	Genes from the dataset
PAK Signaling	—	PDGFA; PIK3C2A; WASL
Sphingosine-1-phosphate Signaling	—	PDGFA; PIK3C2A; PLCB4
Actin Cytoskeleton Signaling	—	PDGFA; PIK3C2A; PPP1R12A; WASL
AMPK Signaling	—	CRTC2; ORAI2; PIK3C2A; STRADA
Macropinocytosis Signaling	—	PDGFA; PIK3C2A
Clathrin-mediated Endocytosis Signaling	—	PDGFA; PIK3C2A; WASL
Integrin Signaling	—	PIK3C2A; PPP1R12A; WASL
PDGF Signaling	—	PDGFA; PIK3C2A
Signaling by Rho Family GTPases	—	PIK3C2A; PPP1R12A; WASL
Energy dependent regulation of mTOR by LKB1-AMPK	—	STRADA
TAK1-dependent IKK and NF-kappa-B activation	—	TIFA
ERK/MAPK Signaling	—	PIK3C2A; PPP1R12A
Role of PI3K/AKT Signaling in the Pathogenesis of Influenza	—	PIK3C2A
NF-κB Activation by Viruses	—	PIK3C2A
PI3K/AKT Signaling	—	THEM4
Regulation of eIF4 and p70S6K Signaling	—	PIK3C2A

Continued on next page

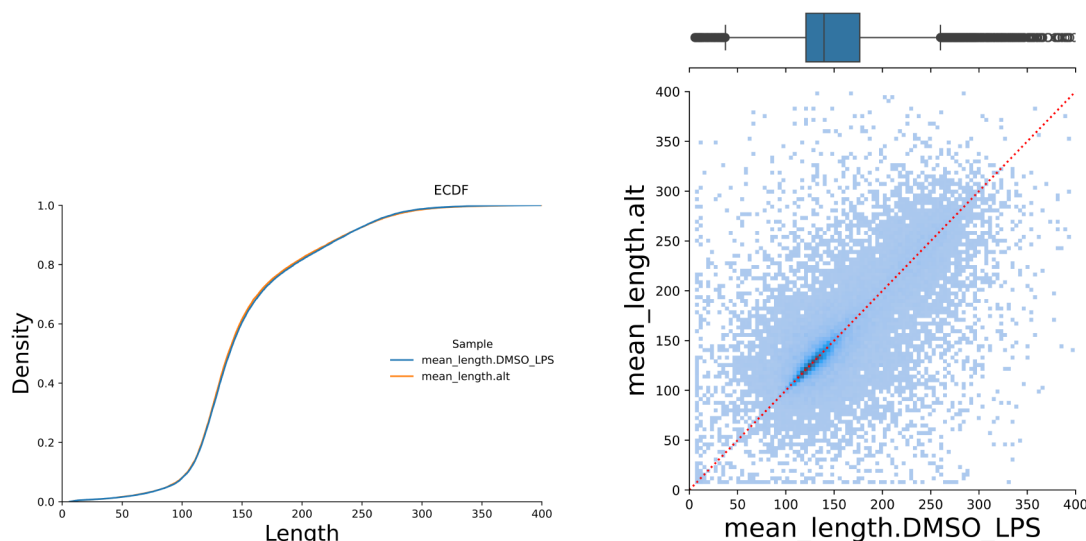
Table 7.1 – *Continued from previous page*

Pathways	z-score	Genes from the dataset
mTOR Signaling	—	PIK3C2A
p70S6K Signaling	—	PIK3C2A; PLCB4

7.2.3 Global poly(A) landscapes show minimal distributional shift at 15 min

To determine whether cordycepin induces early, transcriptome-wide alterations in poly(A)-tail length during inflammatory activation, RAW264.7 macrophages were co-treated with cordycepin (20 μ M) and LPS (1 μ g/mL) for 15 minutes, followed by poly(A)-tail length profiling using Oxford Nanopore cDNA sequencing with poly(A)-tail calling via Dorado. Transcript-wise mean poly(A)-tail lengths were inferred from the sequencing reads and statistically compared between cordycepin-treated (alt) and control (DMSO-LPS) conditions using NanopLen's linear mixed-model framework. The empirical cumulative distribution functions of mean tail lengths demonstrated near-complete overlap between cordycepin-treated and control samples, with the distributions appearing virtually superimposable (Figure 7.4, Panel A). Transcript-wise comparison revealed tight clustering along the identity line ($y = x$) in the scatter plot analysis, with marginal box-plots confirming highly concordant distributions between conditions (Panel B). While sparse outliers were detectable, suggesting potential gene-specific responses, the overwhelming majority of transcripts maintained comparable poly(A)-tail lengths between treatments.

Taken together, these global analyses indicate that cordycepin does not measurably shift the overall distribution of poly(A)-tail lengths after 15 minutes of co-treatment with LPS, as would be expected for such a short incubation. Any cordycepin-induced changes in tail length at this early time point are therefore likely to be modest and transcript-specific, motivating focused transcript-level analyses of significant tail remodeling in the subsequent section.



(a) Panel A

(b) Panel B

Figure 7.4: RAW264.7 cells were exposed to cordycepin (20 μ M) together with LPS (1 μ g/mL) for 15 min; the treated library is labeled alt (CoR_LPSalt) and the matched control is DMSO_LPS. Transcript-wise mean poly(A)-tail lengths were inferred from Oxford Nanopore cDNA reads and compared with NanopLen's linear mixed-model (LMM). Panel A: Empirical cumulative distribution functions (ECDFs; linear scale) of mean tail length show near-complete overlap of alt (orange) with DMSO_LPS (blue), indicating closely matched global distributions. Panel B: Transcript-wise scatter (linear scale) of mean tail length in alt versus control. Points cluster tightly along the identity line (red, $y = x$), with marginal box-plots summarizing each distribution, consistent with high concordance and only sparse gene-specific deviations. Method note: ONT cDNA 3'-end capture requires a free 3'-OH; transcripts terminating in 3'-deoxyadenosine would be under-represented, so absolute losses of such ends cannot be quantified here.

7.2.4 Significant transcript-level tail changes are bidirectional and limited

To pinpoint individual transcripts with early poly(A)-tail changes at 15 minutes, I analysed poly(A)-tail dynamics in RAW264.7 macrophages co-treated with cordycepin (20 μ M) and LPS (1 μ g/mL) using NanopLen's linear mixed-model framework. Applying a significance threshold of $p < 0.05$ and an absolute change in tail length of $|\Delta\text{tail}| \geq 10$ nucleotides, the volcano plot identified 869 transcripts with significant poly(A)-tail alterations, comprising 464 transcripts with tail shortening and 405 with tail lengthening relative to DMSO + LPS controls (Figure 7.5, panel A). Most significant changes clustered just beyond the ± 10 -nucleotide threshold, although a minority of transcripts showed larger shifts of approximately 100–250 nucleotides. The MA plot showed a characteristic funnel pattern in which larger absolute tail-length changes were concentrated among transcripts with low-to-moderate expression levels, whereas highly abundant transcripts exhibited small, symmetric changes centered around zero (panel B).

These transcript-level results complement the global distribution analyses, which showed minimal overall shift in poly(A)-tail lengths, by highlighting a restricted subset of transcripts that already display altered tails at 15 minutes. The near-equal numbers of shortening and lengthening events indicate that cordycepin treatment does not simply shorten or lengthen tails globally, but instead produces selective, transcript-specific changes in both directions. Overall, the data point to modest, early remodeling of poly(A) tails rather than wholesale redistribution across the transcriptome, consistent with previous evidence that cordycepin can influence cytoplasmic poly(A)-tail size through effects on cytoplasmic polyadenylation and deadenylation [19]. The concentration of larger tail-length changes among lower-abundance transcripts further suggests that genes with more dynamic expression or regulatory functions may be more sensitive to these early perturbations, whereas highly expressed housekeeping transcripts retain relatively stable tail lengths over this short exposure. I next asked whether these tail shifts are associated with changes in mRNA abundance at 15 minutes.

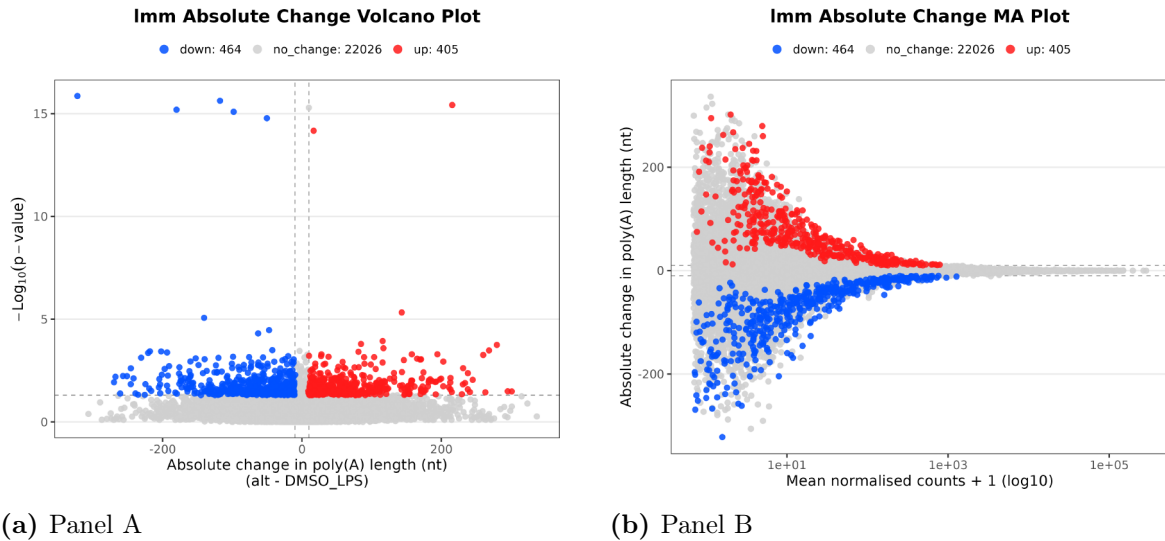


Figure 7.5: Early (15 min) cordycepin co-treatment elicits limited but detectable, bidirectional transcript-level poly(A)-tail remodeling in LPS-activated RAW264.7 macrophages (NanopLen LMM; $|\Delta\text{tail}|$ threshold = 10 nt). RAW264.7 cells were exposed simultaneously to cordycepin (20 μM) and LPS (1 $\mu\text{g}/\text{mL}$) for 15 min. The treated library is alt (CoR_LPS [alt]); the reference is DMSO + LPS. Transcript-wise mean poly(A)-tail lengths were inferred from Oxford Nanopore cDNA reads and compared with NanopLen's linear mixed-model (LMM). (A) Volcano plot: absolute poly(A)-tail change ($\Delta = \text{alt} - \text{DMSO_LPS}$, nt) vs $-\log_{10}(p)$. Vertical dashes mark ± 10 nt; the horizontal dash marks $p = 0.05$. Significant shortening events are shown in blue ($n = 464$) and lengthening events in red ($n = 405$); non-significant transcripts are gray ($n = 22,026$). (B) MA plot: absolute Δtail (nt) vs $\log_{10}$ mean normalized counts. Colors as in (A). Larger $|\Delta\text{tail}|$ values are most frequent among low-to-moderate abundance transcripts, whereas highly expressed transcripts center near zero, consistent with modest, early remodeling rather than a global shift.

7.2.5 Coupling between tail class and expression is weak at 15 min

To test whether early cordycepin-induced gene-expression changes correlate with poly(A)-tail remodeling, genes were stratified according to their mean tail-length alterations and analysed for differential expression patterns 15 minutes after simultaneous CoR+LPS exposure. Using NanopLen linear mixed-model (LMM) estimates of mean tail-length change (ΔpolyA), genes were partitioned into three mutually exclusive categories based on an absolute 10-nucleotide threshold: shortened ($\Delta\text{polyA} < -10$ nt; $n = 2,492$; mean = -30.1 nt), unchanged ($-10 \leq \Delta\text{polyA} \leq 10$ nt; $n = 4,696$; mean = -0.3 nt), and lengthened ($\Delta\text{polyA} > 10$ nt; $n = 2,319$; mean = $+30.9$ nt). No FDR filtering was applied to tail shifts (FDR set to 1.0) to maximise inclusion. edgeR-derived differential gene-expression $\log_2$ fold-changes (CoR+LPS vs DMSO+LPS) were then compared across these tail-shift classes using violin-plot representations (Figure 7.6).

Despite clear separation in mean poly(A)-tail change of approximately ± 31 nucleotides between the shortened and lengthened classes, the gene-expression distributions overlapped closely. Median $\log_2\text{FC}$ values were centred near zero for all three tail categories, and pairwise statistical comparisons revealed no significant differences between shortened versus unchanged ($p = 0.465$), unchanged versus lengthened ($p = 0.465$), or shortened versus lengthened ($p = 0.814$). Consistently, most genes in all three classes were not differentially expressed at the nominal level (grey points; $p > 0.05$), with only a minority showing nominal DGE (red points).

These data show that, at 15 minutes, early poly(A)-tail shifts do not stratify gene-expression responses: transcripts with shortened, unchanged, or lengthened tails exhibit similar $\log_2\text{FC}$ distributions. Taken together with systems-level evidence that cordycepin rapidly represses PI3K/AKT/mTOR- and MEK/ERK-linked translational outputs within approximately 30 min across multiple cell types [25], these 15-minute data are consistent with early poly(A)-tail remodelling acting upstream of widespread changes in mRNA abundance.

The disconnection between detectable changes in poly(A) tail length and transcript-

level expression at this early time point suggests that poly(A) tail remodelling constitutes a primary molecular event that potentially connects alterations in cytoplasmic poly(A) tails to modulation of signal transduction pathways.

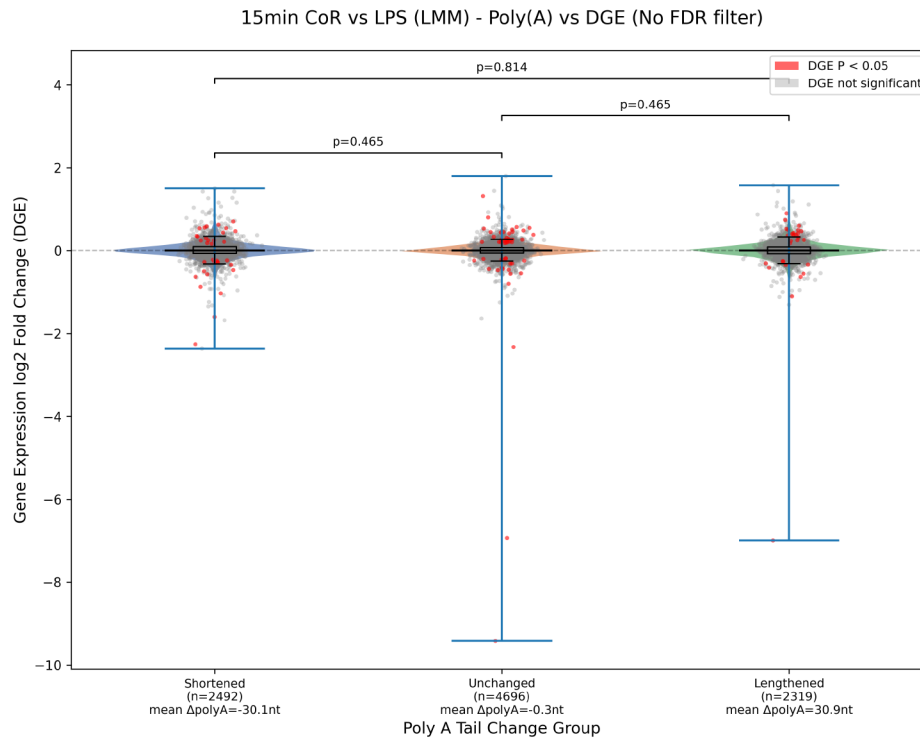


Figure 7.6: Early (15 min) gene-expression changes do not stratify by poly(A)-tail shift class under CoR+LPS. RAW264.7 murine macrophages; 20 μ M cordycepin (CoR) + 1 μ g/mL LPS applied simultaneously for 15 min versus DMSO+LPS control; RNA-Seq for DGE and NanopLen for transcript-level poly(A) analysis. Genes were partitioned by NanopLen LMM estimate of mean tail-length change (Δ polyA) using an absolute 10-nt threshold: Shortened (Δ polyA < -10 nt; n = 2,492; mean = -30.1 nt), Unchanged ($-10 \leq \Delta$ polyA ≤ 10 nt; n = 4,696; mean = -0.3 nt), Lengthened (Δ polyA > 10 nt; n = 2,319; mean = +30.9 nt). Y-axis: DGE log₂FC (CoR+LPS vs DMSO+LPS). Points: red = genes with nominal DGE p < 0.05 (edgeR; no FDR applied), grey = not significant. Violins show distribution; central bars denote medians. Pairwise comparisons annotated on the plot: Shortened vs Unchanged p = 0.465; Unchanged vs Lengthened p = 0.465; Shortened vs Lengthened p = 0.814. Poly(A) tails quantified by NanopLen (gene-level LMM; FDR set to 1.0 for inclusion—i.e., no FDR filtering of tail shifts). DGE by edgeR on bulk RNA-Seq (3 biological replicates per condition)

7.2.6 IPA on tail-defined gene sets reveals translation / RNA-handling axes

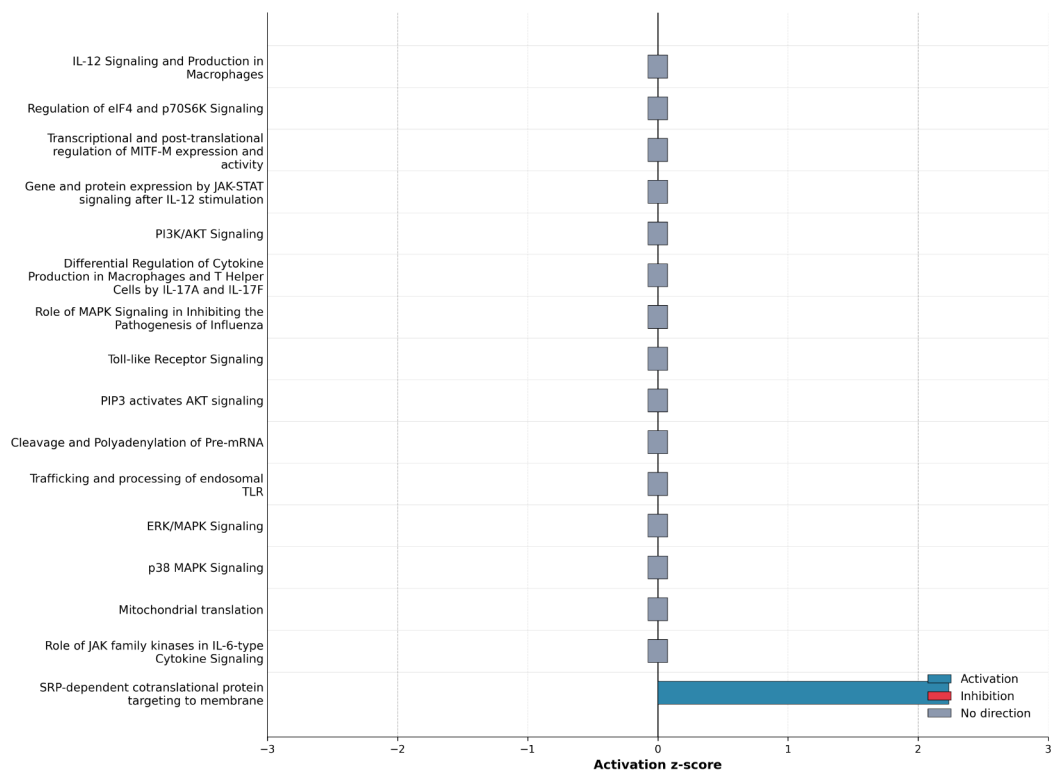
To test whether transcripts undergoing early poly(A)-tail remodeling cluster into specific functional pathways, Ingenuity Pathway Analysis (IPA) was applied to transcript sets stratified by NanopLen-estimated tail changes after 15-minute co-treatment with cordycepin (20 μ M) and LPS (1 μ g/mL) versus DMSO + LPS. Using the signed poly(A)-tail change (Δ tail, nucleotides) as the effect-size metric for z-score computation, pathway enrichment and directional predictions were assessed separately for transcripts with significantly lengthened or shortened tails.

Among transcripts with lengthened poly(A) tails, only “SRP-dependent cotranslational protein targeting to membrane” showed a positive activation z-score (Figure 7.7, Panel A, and Table 7.2), indicating selective engagement of co-translational targeting and endoplasmic reticulum entry machinery during this early response window. Multiple growth-factor and innate immune pathways were also enriched in the lengthened-tail set—including Regulation of eIF4 and p70S6K Signaling, PI3K/AKT Signaling, PIP3 activates AKT signaling, ERK/MAPK Signaling, Toll-like Receptor Signaling, and IL-12-linked JAK-STAT programs—but these carried grey z-scores, indicating representation of constituent transcripts without coherent activation or inhibition patterns at 15 minutes. The enrichment of “Cleavage and Polyadenylation of Pre-mRNA” among lengthened-tail transcripts further implicated 3'-end processing components. PAPOLA, the primary poly(A) polymerase, and NUDT21 (CFIm25) were among the affected transcripts, consistent with an early impact on mRNA 3'-end processing; this pattern suggests that cellular homeostasis mechanisms may begin to adjust core polyadenylation machinery in the presence of cordycepin.

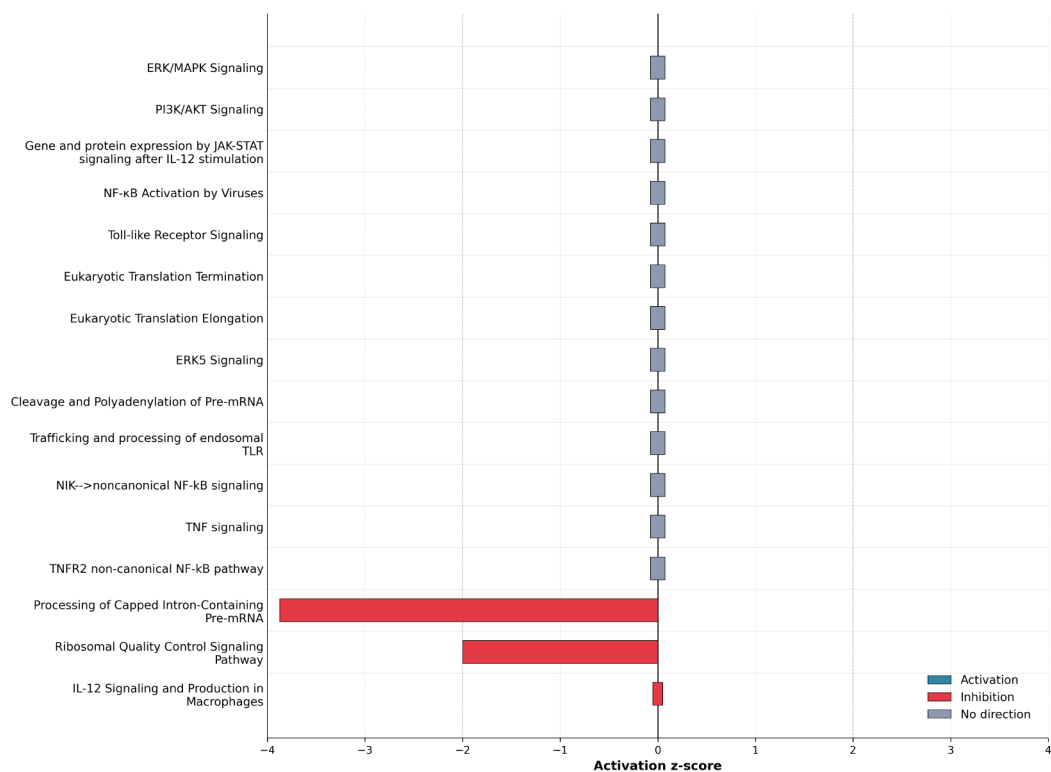
Transcripts exhibiting poly(A)-tail shortening showed directional inhibition of “Processing of Capped Intron-Containing Pre-mRNA” and “Ribosomal Quality Control Signaling Pathway” (Figure 7.7, Panel B, and Table 7.3), consistent with acute perturbation of nuclear mRNA maturation and translation-surveillance circuits in a subset of RNAs. As in the lengthened-tail set, numerous immune and signaling pathways

were enriched but without directional predictions, including ERK/MAPK, PI3K/AKT, NF- κ B Activation by Viruses, Toll-like Receptor Signaling, TNF/TNFR2 non-canonical NF- κ B, and translation-associated modules such as Eukaryotic Translation Elongation / Termination. The concurrent enrichment of “Cleavage and Polyadenylation of Pre-mRNA” alongside negative z-scores for general mRNA-processing and ribosome-quality pathways is consistent with cordycepin primarily perturbing mRNA 3'-end formation and related RNA-processing homeostasis mechanisms at this early timepoint.

Collectively, these z-score profiles indicate that, at 15 minutes, cordycepin most clearly intersects RNA processing and translation-coupled pathways, whereas growth-factor (PI3K/AKT/mTOR, eIF4/p70S6K, ERK/MAPK) and innate immune (TLR/NF- κ B, IL-12/JAK-STAT) nodes are represented but remain largely unpolarized. This selective pattern, with directional calls confined to co-translational targeting, mRNA processing, and ribosome-quality control, is consistent with previous work showing that the earliest cellular effects of cordycepin converge on mTOR-regulated translational control within approximately 30 minutes, before wider pathway activation/inhibition and transcriptional reprogramming become evident [25] [26]. The functional analysis of the poly(A)-tail-defined gene sets therefore supports the view that cordycepin initially affects mRNAs linked to signal transduction and RNA processing, in line with the proposed mechanism of action.



(a) Canonical pathway for lengthened-tail transcripts after 15-min cordycepin treatment



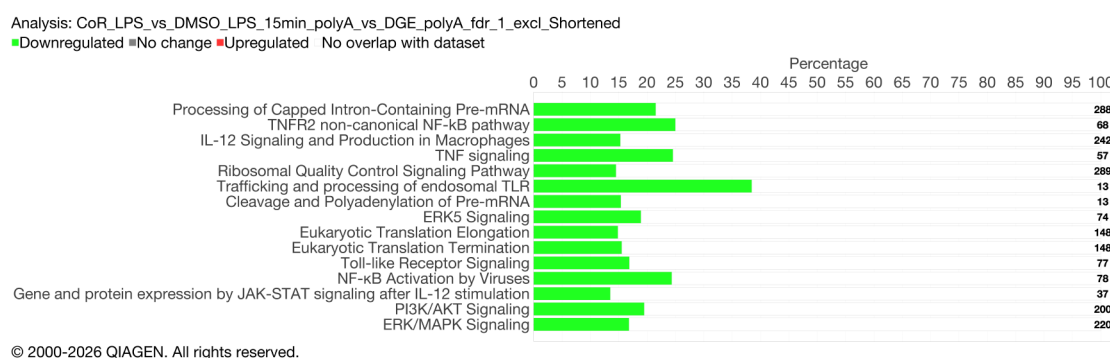
(b) Canonical pathway for shortened-tail transcripts after 15-min cordycepin treatment

Figure 7.7: Canonical-pathway z-score profiles after simultaneous cordycepin and LPS treatment. (A) Lengthened-tail transcripts. (B) Shortened-tail transcripts. The analysis was performed on genes whose tails became significantly longer or shorter, using the signed tail change as the log-fold-change metric.

To complement the z-score view of the tail-defined pathway analyses and show how much of each enriched canonical pathway is populated by transcripts with lengthened or shortened poly(A) tails, horizontal stacked bar charts were generated in QIAGEN IPA for both tail classes (Figure 7.8). As in the other IPA stacked bar views, bar length reflects the proportion of the pathway's total molecules recovered in the dataset, the numeric label to the right of each bar gives the total number of molecules annotated to that pathway in the IPA knowledge base, and red/green segments indicate upregulated and downregulated genes, respectively, within each pathway. Both the lengthened- and shortened-tail gene sets populate translation (eIF4/p70S6K, mTOR, EIF2), growth-factor (PI3K/AKT, ERK/MAPK), and innate-immune (NF- κ B, TLR, IL-12/JAK-STAT) modules, with the shortened-tail set additionally showing broader coverage of mRNA processing and ribosome quality-control pathways, consistent with the z-score profile in Figure 7.7.



(a) Lengthened poly(A) tails



(b) Shortened poly(A) tails

Figure 7.8: IPA canonical pathway coverage for transcripts with (A) significantly lengthened or (B) significantly shortened poly(A) tails at 15 min following co-treatment of RAW264.7 macrophages with cordycepin (20 μ M) and LPS (1 μ g/mL) versus DMSO + LPS. Horizontal stacked bars show, for each enriched canonical pathway, the proportion of pathway molecules recovered in the dataset; the number to the right of each bar is the total number of molecules annotated to that pathway in the IPA knowledge base. Red segments indicate upregulated genes and green segments indicate downregulated genes within each pathway. Generated using the QIAGEN IPA Stacked Bar Chart tool.

Table 7.2: IPA Canonical Pathways for Cordycepin-treated lengthened transcripts (15 min CoR/LPS vs DMSO/LPS, p-value filtered)

Pathways	z-score	Genes from the dataset
SRP-dependent cotranslational protein targeting to membrane	2.236	RPL23A; SEC11C; SPCS2; SRP14; SSR4
Role of JAK family kinases in IL-6-type Cytokine Signaling	—	MYC; OSM; STAT5B
Mitochondrial translation	—	GFM2; MRPL15; MTIF2
p38 MAPK Signaling	—	IRAK2; MYC; Pla2g5
ERK/MAPK Signaling	—	MYC; Pla2g5; PPP2R5B; PRKCD
Trafficking and processing of endosomal TLR	—	TLR3
Cleavage and Polyadenylation of Pre-mRNA	—	NUDT21
PIP3 activates AKT signaling	—	AKT1S1; CDKN1B; PPP2R5B
Toll-like Receptor Signaling	—	IRAK2; TLR3
Role of MAPK Signaling in Inhibiting the Pathogenesis of Influenza	—	CCL2; Pla2g5
Differential Regulation of Cytokine Production in Macrophages and T Helper Cells by IL-17A and IL-17F	—	CCL2
PI3K/AKT Signaling	—	CDKN1B; GDF15; PPP2R5B
Gene and protein expression by JAK-STAT signaling after IL-12 stimulation	—	LCP1

Continued on next page

Table 7.2 – *Continued from previous page*

Pathways	z-score	Genes from the dataset
Transcriptional and post-translational regulation of MITF-M expression and activity	—	HINT1
Regulation of eIF4 and p70S6K Signaling	—	EIF3M; PPP2R5B
IL-12 Signaling and Production in Macrophages	—	PRKCD; TLR3

Table 7.3: IPA Canonical Pathways for Cordycepin-treated shortened transcripts (15 min CoR/LPS vs DMSO/LPS, p-value filtered)

Pathways	z-score	Genes from the dataset
IL-12 Signaling and Production in Macrophages	0.0	Fcgr2b; IKBKB; JMJD6; LCK; MAP3K8
Ribosomal Quality Control Signaling Pathway	-2.0	MRPS18A; PSME2; RPL15; RPL24; UFD1
Processing of Capped Intron-Containing Pre-mRNA	-3.873	ACIN1; DDX5; DHX15; LSM6; PAPOLA; POLR2C; POLR2G; POLR2J; PRP4K; RANBP2; RBM7; RNPS1; SF3B1; SNRPA; WTAP
TNFR2 non-canonical NF-kB pathway	—	PSME2; TNFRSF12A; TNFSF12
TNF signaling	—	IKBKB; OTULIN
NIK→noncanonical NF-kB signaling	—	BTRC; PSME2
Trafficking and processing of endosomal TLR	—	CNPY3
Cleavage and Polyadenylation of Pre-mRNA	—	PAPOLA
ERK5 Signaling	—	MAP3K8; RPS6KA5
Eukaryotic Translation Elongation	—	MRPS18A; RPL15; RPL24
Eukaryotic Translation Termination	—	MRPS18A; RPL15; RPL24
Toll-like Receptor Signaling	—	IKBKB; MAP4K4
NF-κB Activation by Viruses	—	IKBKB; LCK

Continued on next page

Table 7.3 – *Continued from previous page*

Pathways	z-score	Genes from the dataset
Gene and protein expression by JAK-STAT signaling after IL-12 stimulation	—	PSME2
PI3K/AKT Signaling	—	IKBKB; MAP3K8
ERK/MAPK Signaling	—	MAPKAPK5; RPS6KA5

7.3 Discussion

At 15 minutes post-treatment, a small number of transcripts showed statistically significant differences in mRNA abundance between cordycepin+LPS and DMSO+LPS conditions. Transcripts with relatively higher abundance in cordycepin-treated cells included *Pik3c2a* and *Mapkbp1*, which encode components of the PI3K and MAPK signalling networks, as well as *Dhx29*, which encodes a translation-associated DEAH-box helicase involved in ribosomal scanning through structured 5' UTRs [234]. Transcripts with relatively lower abundance included the innate-signalling adaptor *Tifa* and vesicular-trafficking components *Arrdc1* and *Rab29*. Notably, the major inflammatory transcripts induced by LPS at 1 hour—such as *Il1b* and *Tnf*—were not yet detectably elevated at this timepoint, confirming that the inflammatory transcriptional programme had not yet commenced.

The timing of these changes—15 minutes post-treatment—places them within the window of established rapid-response effects of cordycepin on PI3K/AKT/mTOR signalling, which are detectable within approximately 30 minutes [25]. However, the increased abundance of transcripts encoding PI3K- and MAPK-pathway components (*Pik3c2a*, *Mapkbp1*) presents an apparent paradox: if cordycepin inhibits these pathways, why would their component mRNAs accumulate? True transcriptional induction at 15 minutes is unlikely, as insufficient time has elapsed for the full sequence of signalling, chromatin remodelling, transcription, RNA processing, and export. A more parsimonious explanation is that these changes reflect post-transcriptional effects on mRNA stability. Many signalling-component mRNAs are subject to rapid turnover via translation-coupled decay mechanisms; when translation is inhibited, such transcripts can be stabilised because ribosome-associated quality-control pathways and co-translational decay are attenuated [235] [236]. The increased abundance of *Dhx29*, which facilitates ribosomal scanning through structured 5' UTRs, may similarly reflect stabilisation of a transcript normally turned over in association with translational activity [234].

Conversely, the selective reductions in *Tifa* and vesicular-trafficking transcripts (*Ar-*

rdc1, Rab29) suggest early perturbation at receptor-proximal innate-signalling and trafficking nodes. TIFA is an adaptor protein that functions in the ALPK1–TIFA–TRAF6 axis to activate NF- κ B in response to bacterial metabolites and can amplify innate immune signalling [237]. ARRDC1 and RAB29 function in endosomal trafficking pathways that regulate receptor internalisation and signal termination [200]. Reductions in these transcripts at 15 minutes could reflect either rapid destabilisation or, alternatively, their sequestration into translationally repressed complexes such as stress granules or P-bodies.

At 15 minutes post-LPS, the inflammatory gene-expression programme is already committed: NF- κ B translocates to the nucleus within minutes of TLR4 engagement, and the downstream transcriptional response is therefore determined well before it becomes manifest at the mRNA level [238]. The delay between signalling commitment and detectable transcriptional output reflects the time required for chromatin remodelling at inflammatory gene promoters, recruitment of transcriptional machinery, and synthesis and processing of nascent transcripts. Accordingly, the mRNA abundance changes observed here are best interpreted as very early post-transcriptional alterations—likely involving differential mRNA stability—rather than products of *de novo* transcription. This interpretation could be tested by examining unspliced (nascent) transcript levels using intronic reads, although the depth of the present dataset may limit statistical power for individual genes.

The analyses shown above indicate that 15-min co-treatment with cordycepin under LPS elicits a coherent signaling-led, RNA-centric response that affects a specific group of RNAs at the levels of abundance and polyadenylation. DGE-anchored pathways (Fig. 7.2) emphasize PI3K/AKT/mTOR $\rightarrow$ eIF4/p70S6K and ERK/MAPK nodes, with innate modules present but largely direction-neutral at this early window. Global poly(A)-tail distributions are near-identical between conditions (Fig. 7.4), yet NanopLen detects statistically significant transcript-level tail changes, both shortening and lengthening (Fig. 7.5). Crucially, DGE does not stratify by tail class at 15 min (Fig. 7.6), and IPA on tail-defined sets highlights translation and RNA-handling pathways (SRP-dependent co-translational targeting for lengthened tails; inhibition

of capped-pre-mRNA processing and ribosome-quality control for shortened tails; Fig. 7.7). Together, these observations are compatible with a model in which cordycepin rapidly reduces growth-factor/PI3K-convergent signaling and cap-dependent initiation through early effects on RNA abundance and cytoplasmic poly(A) tails, with changes in nuclear polyadenylation becoming more evident at later times [25] [28] [26].

Early translation-gatekeeper signature and macrophage context. The tight clustering of Regulation of eIF4 & p70S6K, p70S6K, and mTOR signaling (Fig. 7.2) matches systems-level evidence that cordycepin rapidly represses mTORC1 outputs and reduces TOP-mRNA loading within ≈ 30 min—well inside the sampling window used here [25]. In macrophages, cordycepin also diminishes NF- κ B (p65) nuclear accumulation and LPS-induced transcription [28] [26], consistent with the presence (but weak directional predictions) of TAK1/IKK $\rightarrow$ NF- κ B-related terms at 15 min. The heatmap (Fig. 7.1) places several early movers at the signaling/trafficking interface (e.g., Pik3c2a, Plcb4, Mapkbp1, Arrdc1, Rab29, Tifa), aligning with receptor-proximal and PI-lipid control points that feed into PI3K/mTOR and ERK pathways.

Poly(A)-tail behavior at 15 min: global stability with selective shifts. The ECDF and scatter analyses (Fig. 7.4) show that there is no global shift in tail lengths at 15 min. Nonetheless, the volcano and MA plots (Fig. 7.5) reveal hundreds of significant transcript-level tail changes (shortening and lengthening), concentrated among lower-to-moderate abundance transcripts, which is typical of early, heterogeneous remodeling. The lack of DGE separation by tail class (Fig. 7.6) indicates that abundance changes have not yet propagated in a tail-class-dependent fashion, as would be expected if the dominant proximal effect is on translation initiation and signaling rather than on immediate changes in RNA levels.

What the tail-class IPA adds. Using the signed Δ tail as the effect metric, IPA assigns biologically specific, time-compatible pathways. For lengthened tails, SRP-dependent co-translational protein targeting to membrane is the only pathway with a positive activation z-score, suggesting that a subset of ER-targeted transcripts are already registering detectable tail-length changes, while other translation-gatekeeper and innate pathways are present but undirected (Fig. 7.7A). For shortened tails, the predicted inhibition of processing of capped intron-containing pre-mRNA and of the ribosomal quality-control pathway (Fig. 7.7B) points to acute pressure on nuclear mRNA processing and translation-surveillance functions in a subset of transcripts, consistent with a state in which signaling and translation are being rapidly reset rather than with established immune transcriptional rewiring.

Mechanistic integration with cordycepin’s 3’-end biochemistry: an RToC hypothesis and distinction from general translation inhibitors. The convergence between the pathways enriched among the earliest RNA changes and the known pharmacology of cordycepin suggests that an RNA target of cordycepin (RToC) could lie within this affected transcript set. In this working model, polyadenylation of a particular RNA is required for efficient signal transduction, such that interference with tail dynamics reduces pathway output. Importantly, general translation inhibitors such as cycloheximide typically superinduce immediate-early transcripts and activate rather than suppress AKT signalling [69] [70] [239]. This demonstrates that ongoing protein synthesis is not required for signal transduction to proceed. Because cordycepin nevertheless suppresses PI3K/AKT/mTOR signalling, its mechanism of action is unlikely to involve blocking translation of any mRNA—whether globally or of a specific target. Rather, an RToC would function through a non-translational mechanism: it could serve as a ribonucleoprotein assembly platform that requires poly(A)-binding protein (PABP) engagement to scaffold signal-transduction complexes, with cordycepin altering the poly(A) tail and thereby disrupting PABP-dependent assembly [69] [70] [240]. This model is consistent with the AMPK-independent nature of cordycepin’s effects on growth-factor signalling [25].

Dynamic incorporation/removal model and library-capture caveat. A technical constraint of the 15-min libraries is that 3’-end-anchored ONT cDNA workflows favour RNAs with a free 3’-hydroxyl [163]. RNAs that happen to carry a terminal 3’-deoxyadenosine from cordycepin incorporation will be under-represented at the cDNA synthesis step and would primarily register as reduced read counts. Biochemically, cordycepin triphosphate is a potent chain terminator of polyadenylation in biochemical systems and interferes with 3’-end formation in cells [45]. Kondrashov et al. showed that cordycepin treatment shortens nuclear and cytoplasmic poly(A) tails and, critically, that 3’- and 5’-end labelling yield similar tail-length distributions, implying that cordycepin-terminated tails are transient and rare at steady state [19]. In combination with the present data, the near-equal numbers of tail-shortening and tail-lengthening events are consistent with a dynamic balance in which cordycepin incorporation and removal shift

the equilibrium between polyadenylation and deadenylation rather than imposing a permanent block to either process. PARN, a cap-interacting deadenylase, is inhibited by terminal cordycepin residues [37]; transient incorporation at the 3'-end could therefore both protect some tails from PARN-mediated deadenylation and, indirectly, permit tail lengthening. In this context, the identification of PARN mutation as a cordycepin-resistance mechanism in Chapter 4 is particularly notable, because it links 3'-end chemistry directly to cordycepin sensitivity.

Together with the tail-class pathway analyses, which highlight RNA-processing and signaling components—including PAPOLA (the main poly(A) polymerase) and NUDT21 (CFIm)—these observations are consistent with early effects of cordycepin on mRNA 3'-end processing. A parsimonious interpretation is that the earliest anti-inflammatory effects of cordycepin arise from RNA-mediated, PABP-dependent events coupled to dynamic tail remodeling [69] [70] [19] [37] [45] [240] [239].

It is likely that the 15-minute incubation used here is not the single optimal timepoint at which to capture all functionally important changes in RNA abundance and polyadenylation. The most informative changes may occur earlier or later, and this experiment samples only one point along that trajectory. Now that candidate RNAs have been identified, a finer time course around this window should help pinpoint when the largest abundance and tail-length changes emerge, which will aid both validation and mechanistic studies. Moreover, because Nanopore-based tail measurements are noisy for low-abundance RNAs, some genuine poly(A)-tail changes are likely to have been missed; in particular, RNAs that show strong abundance changes may also undergo tail remodeling that lies below current detection thresholds.

Although this study nominates RNAs that represent RToC candidates, further work is required to hone in on the most promising ones. Within the existing dataset, an obvious next step would be to identify RNAs that change upon LPS treatment but whose responses are attenuated or reversed by cordycepin, and then to examine whether these RNAs show distinctive poly(A)-tail behaviour. Motif analysis of their 3'-UTRs could suggest which RNA-binding proteins participate in their regulation, providing clues to the underlying mechanism. Given the known effects of cordycepin on signal

transduction, mRNAs encoding signaling components are among the first candidates for further investigation, including PIK3C2A, PLCB4, PRKCD, AKT1S1, PPP2R5B, LCK, MAP3K8, MAP4K4, RPS6KA5, MAP5K4 and MAPKAP5. If some of these mRNAs contribute to the assembly of signal-transduction complexes, immunoprecipitation of key signaling machinery could be used to test whether they are physically present in those complexes in a cordycepin-sensitive manner.

Together, these data support a model in which early cytoplasmic poly(A)-tail modulation is upstream of, and mechanistically linked to, the restraint of growth-factor and inflammatory signaling.

Chapter 8

Discussion

8.1 Overview and Integration of Findings

This thesis set out to elucidate the molecular pharmacokinetics and cellular mechanism of action of cordycepin (3'-deoxyadenosine), with a particular focus on how this nucleoside analogue and its metabolites modulate inflammatory gene expression and growth-factor signalling through effects on mRNA 3'-end processing and polyadenylation. The experimental work spans five interconnected strands: (i) characterisation of 3'-deoxyinosine (3DI) "rescue" pathways that underwrite oral bioavailability (Chapter 3); (ii) haploid piggyBac screening to define genetic determinants of resistance (Chapter 4); (iii) comparative transcriptomics of natural versus synthetic cordycepin preparations and the ProTide NUC-7738 (Chapter 5); (iv) genome-wide poly(A)-tail remodelling at 120 minutes (Chapter 6); and (v) early temporal poly(A) dynamics at 15 minutes (Chapter 7).

Collectively, these datasets converge on a coherent mechanistic model in which cordycepin acts primarily as an intracellular nucleoside analogue that must be phosphorylated by adenosine kinase (ADK) to cordycepin triphosphate (CoTP/3'-dATP) in order to inhibit cleavage and polyadenylation (CPA). This CPA inhibition selectively restrains inflammatory and growth-factor signalling. The strong enrichment of ADK loss-of-function insertions in the haploid screen (Figures 4.13–4.14) provides genetic validation that ADK-dependent phosphorylation is required for cordycepin's cellular effects.

The central conceptual framework emerging from the work is the "RNA target of cordycepin" (RToC) hypothesis: suppression of PI3K/Akt/mTOR signalling by cordycepin is mediated by one or more RNA species whose polyadenylation-dependent function is cordycepin-sensitive. The RToC is unlikely to require translation to facilitate signal transduction, because signal transduction is activated, rather than repressed, by the inhibition of protein synthesis, indicating that signal transduction does not require ongoing protein synthesis. This distinguishes cordycepin from classical translation inhibitors such as cycloheximide and anisomycin, which paradoxically activate Akt via PI3K/PDK1- or NO-sGC-cGKII-dependent pathways rather than suppressing it [69] [70]. Recent systems pharmacology evidence that cordycepin blocks PI3K/AKT/mTOR and/or MEK/ERK signalling across diverse cell types without requiring AMPK activation [25] is particularly constraining: it places the key locus of drug action upstream of AMPK and favours an RNA-centred mechanism that operates at the level of CPA-sensitive intermediates.

The sections that follow integrate the main experimental strands around this RToC framework: first, metabolic activation and 3DI rescue (Section 8.2; Chapter 3); second, genetic determinants of resistance (Section 8.3; Chapter 4); third, transcriptomic equivalence of natural and synthetic cordycepin and NUC-7738 (Section 8.4; Chapter 5); fourth, poly(A)-tail remodelling and its pathway selectivity (Section 8.5; Chapters 6–7); and finally, the RToC hypothesis integration and candidate nomination (Section 8.6).

8.2 Metabolic Activation and the 3'-Deoxyinosine Rescue Pathway

A longstanding pharmacokinetic limitation of cordycepin is its rapid deamination by adenosine deaminase (ADA) to 3'-deoxyinosine (3DI), particularly after oral dosing [1] [5] [6]. The work presented in Chapter 3 directly addresses this challenge by suggesting that 3DI is not metabolically inert but can support anti-inflammatory activity through a nucleoside "rescue" pathway that feeds into the active cordycepin nucleotide pool.

Using siRNA knockdown in A549 epithelial cells and RAW264.7 macrophages, three candidate routes were tested: (i) direct phosphorylation–amination via NT5C2/ADSS2, (ii) PNP-mediated 3'-deoxyribose salvage, and (iii) adenine-linked 3'-deoxyribose salvage via APRT with or without FAMIN/LACC1.

The data support a clear winner among these pathways. APRT knockdown reduced the antiinflammatory effects of both cordycepin and 3DI in RAW264.7 cells across *Acod1* and *Il1b* readouts (Figure 3.15), demonstrating a requirement for adenine phosphoribosyltransferase in generating active 3'-deoxyadenosine nucleotides. FAMIN/LACC1 knockdown produced parallel reductions in macrophages but minimal effects in A549 cells (Figure 3.16), consistent with the function of this enzyme in purine nucleotide cycling [112] [113]. Enhancement of 3DI responses by adenine co-supplementation in A549 cells further supports adenine-linked salvage (Figure 3.13), although interpretation is partly confounded by adenine's intrinsic antiinflammatory properties [119].

The "ADK paradox" uncovered in these experiments provides particularly informative mechanistic insight (Figure 3.18 and Figure 3.19). As expected, ADK loss reduced cordycepin efficacy, reflecting the requirement for phosphorylation to CoTP. However, partial ADK knockdown enhanced 3DI responses in some readouts. This divergence suggests that 3DI and cordycepin encounter distinct bottlenecks even when they converge on shared adenine/3'-deoxyribose salvage nodes, and crucially implies that 3DI's biological effects cannot be dismissed as simple artefacts of cordycepin contamination in commercial 3DI stocks. A plausible mechanistic explanation for the increased efficacy of 3DI observed in certain readouts following ADK knockdown is that the reduction in adenosine monophosphate (AMP) synthesis caused by decreased ADK activity leads to an accumulation of AMP precursors, such as adenine. This accumulation may, in turn, enhance adenine-dependent salvage pathways.

LC–MS characterisation confirmed high purity of both Carbosynth and synthetic cordycepin stocks but revealed measurable cordycepin contamination in the Santa Cruz 3DI preparation (Chapter 3, Sections 3.2.8 - 3.2.10). While this contamination precludes clean potency estimates for "pure 3DI", the knockdown patterns remain mechanistically informative: ADK effects diverge between cordycepin and 3DI, whereas

APRT and FAMIN/LACC1 affect both similarly in macrophages. Together, these data establish that intracellular CoTP levels, and hence cordycepin pharmacology, are strongly shaped by 3'-deoxyribose-salvage capacity. Interindividual variation in APRT and FAMIN/LACC1 function is therefore a plausible contributor to variability in therapeutic responses (pharmacogenomic) and a rational target for optimisation of cordycepin analogues.

8.3 Genetic Determinants of Cordycepin Resistance: Three Mechanistic Classes

The haploid piggyBac gene-trap screen in eHAP cells (Chapter 4) provides an unbiased, genome-wide view of the genetic architecture underlying cordycepin sensitivity. Exploiting the haploid state to expose recessive loss-of-function phenotypes, the screen infers resistance drivers from enrichment of disruptive insertions in survivors relative to DMSO controls [138] [139]. The experimental design, including mutagenesis with hyPBase and suPBase transposases and selection with repeated cordycepin dosing, is summarised in Figures 4.1–4.5. The resulting data resolve three broad classes of resistance mechanisms: (i) reduced metabolic activation, (ii) modulation of RNA 3'-end processing and deadenylation, and (iii) rewiring of mTOR/autophagy/proteostasis networks.

8.3.1 Class 1: Reduced Metabolic Activation via Nucleoside Salvage

ADK emerged as the most robust and recurrent determinant of resistance across all cordycepin doses and both transposase platforms (Figures 4.11–4.14). At 200 μ M cordycepin, ADK accounted for 4.48% of all suPB insertions versus 0.24% in DMSO controls (Figure 4.12aa), with hyPB showing parallel enrichment (1.28% versus 0.19%; Figure 4.12bb). The cross-condition heatmap (Figure 4.14) confirms that ADK shows the most consistent enrichment under cordycepin selection across all experimental conditions.

These insertions map to nucleoside/nucleotide salvage pathways including "Adenine

and Adenosine Salvage VI", "Nucleotide salvage", and "Ribavirin ADME" in Ingenuity Pathway Analysis (Figure 4.15). Within the loss-of-function framework of the screen, this pattern confirms that cordycepin must be phosphorylated to its nucleotide forms to exert toxicity and places ADK-dependent metabolism at the apex of the resistance hierarchy [6] [105]. In addition to serving as a positive control that has been found in cordycepin resistance screens before [29], these data also strengthen the case that CoTP is the key active metabolite and anchor the interpretation of downstream pathway effects.

8.3.2 Class 2: mRNA 3'-End Processing, Deadenylation, and RNA Turnover

The most mechanistically informative hits beyond ADK involve RNA 3'-end processing and deadenylation. IPA terms such as "Deadenylation-dependent mRNA decay", "mRNA 3 Prime End Processing Signaling Pathway", "Inhibition of ARE-Mediated mRNA Degradation Pathway", and "Processing of Capped Intron-Containing Pre-mRNA" are driven by genes encoding components of the deadenylase machinery or closely linked RNA-processing factors (Figures 4.15–4.18), including CNOT6, PARN, LSM3, RCL1, WDR12, WDR18, INTS3, NUP35, and NUP160. These genes converge on control of poly(A) tail length, 3'-end formation, and ribosome/tRNA production [20] [44] [157] [158] [156] [155].

The enrichment of CNOT6 and PARN in the loss-of-function screen is particularly significant. PARN is a 3' → 5' deadenylase whose nuclease activity requires a free 3'-OH on the RNA substrate; terminal cordycepin residues inhibit PARN activity [37]. The emergence of PARN as a resistance determinant thus provides a direct genetic link between 3'-end chemistry and cordycepin sensitivity. In the loss-of-function framework, reducing deadenylation and ARE-mediated mRNA decay may buffer cells against cordycepin-induced inhibition of polyadenylation by stabilising short-lived, stress-responsive mRNAs when poly(A) addition is compromised [152] [153] [154]. This interpretation aligns precisely with cordycepin's established role as a polyadenylation inhibitor and indicates that the state of mRNA 3'-end processing modulates drug

sensitivity.

8.3.3 Class 3: mTOR Signalling, Autophagy, and Proteostasis

The third class of resistance mechanisms involves genes in mTOR signalling, autophagy, mitophagy, ER-mitochondrial coupling, and ubiquitin-dependent proteostasis. Recurrent hits include PPP2R2B, ATG5, VPS41, UBE2D1, CASP8, EDEM3, NEDD4L, and VPS13D (Figures 4.13–4.14), which collectively point to integrated stress-response and quality-control pathways that shape survival under cordycepin [127] [128] [129] [130] [133] [134]. IPA enrichment of terms such as "Macroautophagy", "Mitophagy", "ER-Mitochondrial Communication Signaling Pathway", "Cellular response to hypoxia", and "Protein Ubiquitination Pathway" (Figures 4.17–4.18) indicates that changes in protein synthesis, protein degradation, and mitochondrial function influence survival when cordycepin inhibits polyadenylation.

It is important to note that while AMPK-related IPA terms appear in the screen output, the Lawrence et al. (2025) finding that cordycepin blocks PI3K/AKT/mTOR signalling independently of AMPK activation fundamentally constrains mechanistic interpretation [25]. The resistance genes in this class are best understood not as components of an AMPK-driven pathway, but rather as downstream effectors of mTORC1 inhibition that occurs through cordycepin's AMPK-independent mechanism. Loss of autophagy machinery (ATG5), vesicular trafficking components (VPS41, VPS13D), or proteostasis regulators (UBE2D1, NEDD4L) would reduce the adaptive autophagic response to mTORC1 inhibition, thereby conferring relative resistance.

Within this framework, the resistance architecture can be viewed as tiered: loss of ADK prevents formation of toxic nucleotides; loss of deadenylases and processing factors stabilises key transcripts when poly(A) addition is impaired; and loss of autophagy/mitochondrial regulators reduces the energetic and proteostatic burden when protein synthesis is curtailed.

8.3.4 LINC03121 as a Candidate RNA Target of Cordycepin

Within the hyPB arm of the screen, the long non-coding RNA LINC03121 (Lnc-PLA2G4A4) stands out as a striking hit that was not observed in the suPB arm of

the screen; this apparent specificity may reflect the limited size of the libraries rather than a true platform-dependent effect. At 200 μ M cordycepin, LINC03121 accounts for approximately 10.86% of insertions versus 0.21% in DMSO controls (Figure 4.12bb). This marked enrichment indicates that loss-of-function events at this locus confer a strong survival advantage under drug selection and nominates LINC03121 as an attractive candidate for the RToC hypothesis.

Given piggyBac's strong preference for TTAA sites in open chromatin and the propensity of some lncRNA loci to accumulate insertions, it was important to examine whether the LINC03121 signal could be explained by trivial structural or genomic factors [140] [141] [146]. Public RefSeq/NCBI annotation indicates that LINC03121 is a single-exon lncRNA at chromosome 1q31.1 with a genomic span of $\approx$ 2.3 kb. This footprint is modest relative to many protein-coding genes and large lncRNA loci, making it unlikely that the extreme insertion burden simply reflects an unusually large exonic target for piggyBac. The gene does not reside within a dense cluster of annotated transposable elements, and 1q31.1 is not a canonical common fragile site. Taken together, these structural considerations argue against simple explanations as the primary cause of the high insertion burden at LINC03121.

Independent functional data from the literature strengthen LINC03121's candidacy. Xiong and colleagues demonstrated that LINC03121 functions as a competing endogenous RNA (ceRNA) that sponges miR-23b-3p, thereby relieving repression of versican (VCAN) and promoting p-EGFR/p-AKT signalling in hepatocellular carcinoma [88]. This ceRNA activity places LINC03121 at a mechanistically plausible nodal point to influence growth-factor signalling in a polyadenylation-dependent manner, and provides a basis for proposing a point at which cordycepin could disrupt the axis (Figure 8.1).

If cordycepin disrupts LINC03121 polyadenylation or stability, its capacity to support PI3K/AKT/mTOR signalling would be impaired. Although LINC03121 has been proposed to act as a competing endogenous RNA (ceRNA) that sponges miR-23b-3p to promote versican expression and EGFR $\rightarrow$ AKT signalling [88], this mechanism is unlikely to account for cordycepin's effects on signal transduction for two reasons. First, versican is a large, glycosylated extracellular protein whose synthesis and deposition would be too

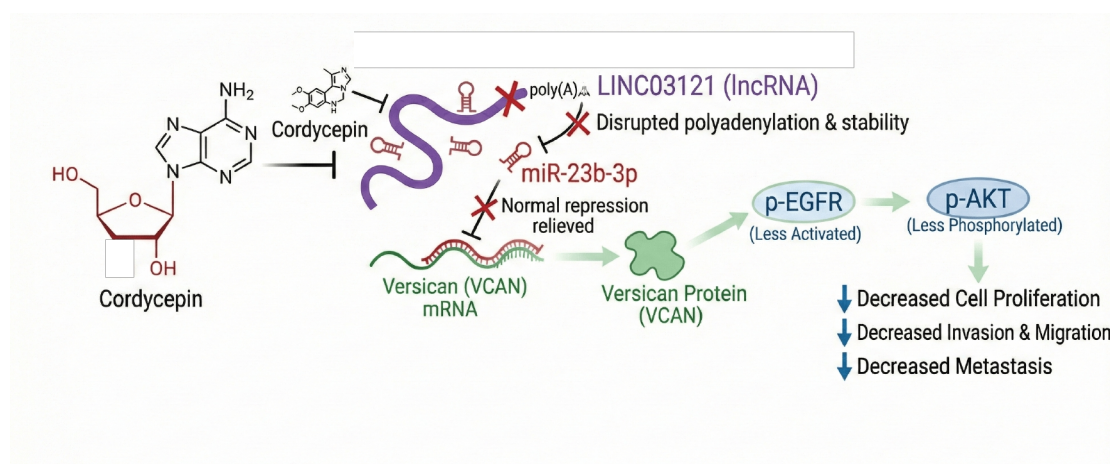


Figure 8.1: Proposed mechanism by which the long non-coding RNA LINC03121 (Lnc-PLA2G4A-4) supports PI3K/AKT/mTOR signalling, and the proposed point of cordycepin action. Under normal conditions, polyadenylated and stable LINC03121 acts as a competing endogenous RNA (ceRNA) that sequesters miR-23b-3p and so relieves miRNA-mediated repression of versican (VCAN) mRNA. The resulting versican protein activates EGFR signalling, leading to phosphorylation of AKT and downstream proliferative, migratory and invasive responses, as demonstrated in hepatocellular carcinoma by Xiong and colleagues [88]. The proposed cordycepin disruption point (this thesis) is shown at the top of the schematic: cordycepin (via its triphosphate metabolite CoTP) acts as a chain-terminating substrate for poly(A) polymerases and disrupts LINC03121 polyadenylation and stability; the resulting release of miR-23b-3p reimposes repression of *VCAN* mRNA, lowering versican protein, p-EGFR and p-AKT, and reducing the associated proliferative, invasive and metastatic outputs. As discussed in the main text, this ceRNA-based mechanism is unlikely on its own to account for cordycepin's rapid effects on signal transduction (because of the slow kinetics of versican synthesis and the cell-type-restricted expression of versican); a more parsimonious, polyadenylation-dependent ribonucleoprotein platform model that does not require ongoing protein synthesis is presented in Figure 8.2. Mechanism schematic adapted from Xiong et al. [88].

slow to explain the rapid signalling changes observed [25]. Second, versican expression is cell-type restricted, whereas cordycepin inhibits PI3K/AKT/mTOR signalling across all cell types examined.

A more parsimonious model is that LINC03121 functions as a ribonucleoprotein platform for signal transduction complex assembly. Recent work has established that poly(A)-binding protein cytoplasmic 1 (PABPC1) can recruit La-related protein 1 (LARP1), which in turn associates with mTORC1 via RAPTOR [11] [12]. Importantly, the LARP1–PABP interaction is maintained independently of mTORC1 activity, suggesting that PABP serves as a stable anchor for this signalling complex [11]. In this model, polyadenylation of the RToC enables recruitment of PABPC1, which brings in LARP1 and mTOR, thereby facilitating AKT activation and phosphorylation of downstream substrates. Disruption of RToC polyadenylation by cordycepin would disassemble this signalling platform, leading to reduced PI3K/AKT/mTOR activity without requiring changes in protein synthesis (Figure 8.2). LINC03121 remains an excellent candidate RToC under this revised framework, though it may not operate through the ceRNA mechanism originally proposed.

At first sight, this wiring appears paradoxical in the context of the screen: loss-of-function in LINC03121 would be expected to reduce VCAN, attenuate EGFR activation and lower basal p-AKT signalling, which might intuitively be predicted to sensitise cells to further inhibition by cordycepin rather than to confer resistance. However, this interpretation neglects pathway dependence and relative fitness under selection. Wild-type cells with intact LINC03121 are highly reliant on EGFR→AKT→mTOR activity for growth; acute suppression of this axis by cordycepin therefore represents a large functional drop and is associated with substantial loss of viability. By contrast, LINC03121-deficient cells are chronically adapted to a "low EGFR/AKT/mTOR" state. Their proliferation is already uncoupled, at least in part, from this pathway. In a pooled competition assay, such clones therefore appear relatively cordycepin resistant, not because they are drug-insensitive in an absolute sense, but because they lose less fitness than their high-signalling competitors under identical drug pressure.

Thus, the convergence of (i) strong loss-of-function enrichment of LINC03121 in

the haploid screen (Figure 4.12bb), (ii) its established role as a positive regulator of EGFR/AKT/mTOR signalling via a VCAN–EGFR axis, and (iii) genome-wide evidence that cordycepin perturbs CPA and poly(A)-tail dynamics (Chapters 6–7) provides a biologically coherent, testable model in which LINC03121 is a leading candidate RToC.

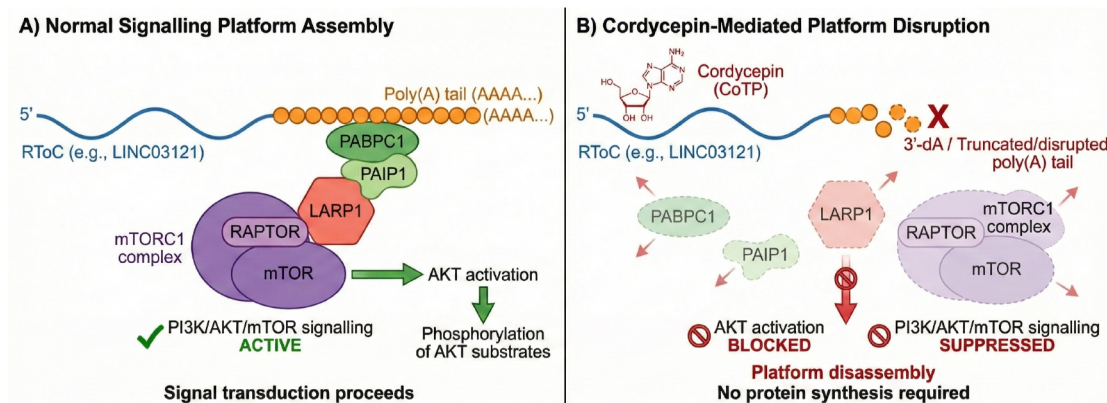


Figure 8.2: Proposed model for RToC-mediated PI3K/AKT/mTOR signalling platform assembly and its disruption by cordycepin. **(A) Normal signalling conditions.** The RNA Target of Cordycepin (RToC), potentially including LINC03121, undergoes polyadenylation resulting in a mature poly(A) tail. Poly(A)-binding protein cytoplasmic 1 (PABPC1) binds the poly(A) tail and recruits PABPC1-interacting protein 1 (PAIP1) and La-related protein 1 (LARP1). LARP1 in turn associates with mTOR complex 1 (mTORC1) via RAPTOR, forming a ribonucleoprotein signalling platform [11] [12]. This platform facilitates AKT activation and phosphorylation of downstream substrates, enabling PI3K/AKT/mTOR signal transduction. Critically, this mechanism does not require the RToC to be translated; rather, the polyadenylated RNA functions as a structural scaffold for signalling complex assembly. **(B) Cordycepin treatment.** Cordycepin is phosphorylated by adenosine kinase (ADK) to cordycepin triphosphate (CoTP), which acts as a chain-terminating substrate for poly(A) polymerases, resulting in premature termination and/or shortening of the RToC poly(A) tail. The truncated poly(A) tail cannot efficiently recruit or retain PABPC1, leading to dissociation of PAIP1, LARP1, and mTORC1 from the platform. This disassembly suppresses AKT activation and downstream PI3K/AKT/mTOR signalling independently of any requirement for ongoing protein synthesis. Abbreviations: RToC, RNA Target of Cordycepin; PABPC1, poly(A)-binding protein cytoplasmic 1; PAIP1, PABPC1-interacting protein 1; LARP1, La-related protein 1; mTORC1, mechanistic target of rapamycin complex 1; CoTP, cordycepin triphosphate; ADK, adenosine kinase.

8.3.5 SLC26A9 as a Genetic Modifier of Cordycepin Sensitivity

Beyond LINC03121, another striking hit in the hyPB arm of the haploid screen was SLC26A9, encoding solute carrier family 26 member 9. At 200 μ M cordycepin, SLC26A9 accounted for approximately 5.6% of insertions compared with $\approx$ 0.1% in DMSO controls, making it the second-strongest loss-of-function hit after LINC03121 (Figure 4.12bb). SLC26A9 is a multifunctional anion transporter that functions as a Cl^- channel, $\text{Cl}^-/\text{HCO}_3^-$ exchanger, or sodium transporter, with high expression in the stomach, lungs, and kidneys but low expression in normal mammary epithelium [241] [242].

The emergence of SLC26A9 as a resistance determinant acquires particular mechanistic significance in light of recent work by Ma and colleagues, who demonstrated that SLC26A9 functions as an oncogene in breast cancer by activating the PI3K/AKT/mTOR signalling pathway [243]. In their study, SLC26A9 knockdown in MDA-MB-231, MDA-MB-468, and SKBR3 breast cancer cells significantly reduced phosphorylation of PI3K, AKT, and mTOR, while SLC26A9 overexpression produced the converse effect. Critically, the PI3K-specific agonist 740Y-P rescued the proliferative, migratory, and invasive phenotypes suppressed by SLC26A9 knockdown, establishing a causal link between SLC26A9 and PI3K/AKT pathway activation [243].

This finding provides a coherent explanation for the enrichment of SLC26A9 loss-of-function insertions among cordycepin-resistant clones. If SLC26A9 normally promotes PI3K/AKT/mTOR signalling, then cells lacking functional SLC26A9 would exhibit constitutively reduced pathway activity—a state that partially mimics, or "pre-adapts" the cell to, cordycepin's effects. Under cordycepin treatment, wild-type cells experience acute suppression of PI3K/AKT/mTOR signalling (whether through the RToC mechanism or other CPA-dependent routes), leading to growth arrest or death. In contrast, SLC26A9-deficient cells already operate with dampened PI3K/AKT/mTOR output, reducing the marginal impact of cordycepin-induced signalling suppression and thereby conferring relative resistance.

It is notable that both SLC26A9 and LINC03121—the two strongest non-ADK hits in the hyPB screen (Figure 4.12bb)—converge on PI3K/AKT signalling. This

convergence strengthens the case that suppression of PI3K/AKT/mTOR signalling is a central, therapeutically relevant consequence of cordycepin action, and supports the RToC hypothesis by demonstrating that genetic perturbations mimicking cordycepin's signalling effects confer resistance to the drug itself.

8.4 Transcriptomic Equivalence of Natural and Synthetic Cordycepin

A persistent concern in cordycepin research has been whether the biological effects reported for natural (fermentation-derived) preparations could be confounded by co-purified contaminants, which are remarkably similar across commercial preparations [162]. The comparative transcriptomics presented in Chapter 5 directly addresses this concern by demonstrating that natural and synthetic cordycepin produce nearly identical gene-expression and pathway-level signatures in LPS-stimulated RAW264.7 macrophages.

At 20 μ M, both natural and synthetic cordycepin produced closely matched volcano plots dominated by down-regulation (natural: 373 down/29 up; synthetic: 352 down/17 up at $|\log_2\text{FC}| \geq 1$, $\text{FDR} < 0.05$) (Figure 5.4), indicating that the anti-inflammatory action is intrinsic to cordycepin rather than to co-purified constituents. Hierarchical clustering on the top 50 most variable genes showed that LPS stimulation induced a broad increase in inflammatory gene expression, which was largely preserved in the presence of 3DI (200 μ M) but markedly attenuated by natural cordycepin, synthetic cordycepin, and NUC-7738 (Figure 5.3). The ProTide NUC-7738 at 200 μ M generated a similar but more pronounced inhibitory signature than 3DI (Figure 5.9), consistent with its design to bypass ADA-mediated deamination and deliver higher intracellular triphosphate levels [29] [2].

IPA consistently returned negative activation z-scores for PI3K/AKT/mTOR-, autophagy-, and NF- κ B-linked pathways across the active treatments (Figures 5.5–5.6, 5.10–5.11), aligning with earlier studies showing that cordycepin dampens growth-factor and inflammatory signalling [25] [28]. The recurrent negative direction on pathways

including IL-6 Signaling, Interferon Signaling, TNFR1/TNFR2, and the Inflammasome pathway indicates that these features are robust across analytical views rather than artefacts of any single analysis. The highlighting of "Role of PKR in Interferon Induction and Antiviral Response" with negative directionality (Figures 5.14–5.15) suggests that stress- and interferon-associated nodes are integrated within a broader cordycepin response network, consistent with engagement of translational control mechanisms.

These data resolve a long-standing ambiguity: natural and synthetic cordycepin are functionally equivalent at the transcriptome level, and the ProTide NUC-7738 reproduces and extends their effects. This strongly supports a model in which intracellular CoTP/3'-dATP is the common active species and rules out fermentation contaminants as dominant drivers of biological activity. The more extensive pathway modulation observed with NUC-7738 suggests that delivery-optimised 3'-deoxyadenosine variants could achieve deeper and earlier pathway control in growth-factor-driven and inflammatory settings.

8.5 Poly(A)-Tail Remodelling: Mechanism and Selectivity

Chapters 6 and 7 provide a genome-wide view of how cordycepin reshapes the post-transcriptional landscape via CPA inhibition. Using Oxford Nanopore sequencing with Dorado basecalling and NanopLen differential length analysis, poly(A)-tail dynamics were quantified at 120 minutes (Chapter 6) and 15 minutes (Chapter 7) in LPS-treated RAW264.7 macrophages exposed to cordycepin, NUC-7738 or 3DI.

8.5.1 Global Poly(A)-Tail Shortening at 120 Minutes

At 120 minutes, both cordycepin and NUC-7738 induced rapid, genome-wide shortening of poly(A) tails. ECDF analysis showed a median shift of -26 nucleotides for NUC-7738 (95% CI -26.9 to -25.6 nt; $n = 10,414$ genes), with comparable shortening for cordycepin (Figure 6.4). More than 20% of expressed genes exhibited statistically significant tail shortening under NUC-7738, whereas 3DI produced a smaller median shift (-6.9 nt; 95% CI -7.5 to -6.4 nt) with <1% of genes significantly affected (Figure 6.6), in line

with its indirect reliance on intracellular conversion to cordycepin and the confounding cordycepin contamination in the 3DI stock.

Across treatments, a graded relationship between tail class and expression emerged: Shortened < Unchanged < Lengthened for $\log_2$ fold-change in mRNA abundance (Figures 6.8–6.9). Genes with shortened tails showed reduced abundance relative to those with unchanged tails, whereas the lengthened class showed modest but significant increases in expression. This pattern, observed for natural cordycepin, synthetic cordycepin, NUC-7738 and even 3DI, is consistent with a general link between tail dynamics and transcript fate, even though the 2-hour design cannot fully separate altered stability from transcriptional effects.

8.5.2 Pathway Selectivity of Tail Remodelling

Stratifying transcripts by tail class revealed striking pathway selectivity (Figures 6.10–6.12). Shortened-tail transcripts were enriched for innate immune pathways including TLR4/NF κ B/MAPK signalling, inflammasome components, ROS production and antiviral responses. This matches earlier work showing that cordycepin blocks NF- κ B nuclear accumulation and represses LPS-induced genes in RAW264.7 cells and primary human macrophages within 1 hour [26] [28]. Reduced nuclear throughput of cytokine transcripts (due to impaired CPA) combined with shortened cytoplasmic tails that accelerate decay and impair closed-loop formation provides a coherent mechanistic basis for this anti-inflammatory action.

By contrast, lengthened-tail transcripts were enriched for mitochondrial and metabolic functions (Figures 6.10–6.12), suggesting selective stabilisation of mRNAs that support bioenergetics under stress. Seahorse assays in MCF-7 cells (Figure 6.14) confirmed reduced proton leak and spare respiratory capacity under cordycepin (Chapter 6, Section 6.5), consistent with an energy-conservation response accompanying translation restraint. This dichotomy—shortened, depleted inflammatory transcripts versus lengthened, relatively spared bioenergetic transcripts—represents a coordinated reprogramming that is not readily explained by a simple global inhibition of polyadenylation.

8.5.3 Mechanistic Model for Tail Lengthening Under a Chain Terminator

At first glance, tail lengthening under a chain-terminating substrate such as CoTP seems paradoxical. Several mechanisms help reconcile this. First, poly(A) polymerases are highly biased towards ATP, so most incorporated residues remain standard adenosines; rare CoTP incorporations can be superimposed on net tail growth. Second, terminal cordycepin residues block PARN-mediated deadenylation by removing the 3'-OH required for exonucleolytic attack [37] [177]. For transcripts that are normal PARN substrates, transient cordycepin incorporation could selectively suppress PARN-mediated shortening while CCR4-NOT and other deadenylases remain active, yielding apparent lengthening for transcripts that are predominantly deadenylated by PARN. Third, cytoplasmic polyadenylation via CPEB-family proteins and TENT polymerases can add tails to preexisting mRNAs during immune activation [39] [42] [244] [245]; under LPS stimulation, both nuclear and cytoplasmic polyadenylation proceed, and occasional CoTP incorporation in either compartment can position a 3'-dA at the terminus while allowing overall tail extension.

That cordycepin-terminated tails are rare at steady state, as shown by Kondrashov et al. [19], supports a dynamic balance model in which cordycepin incorporation and removal shift the equilibrium between polyadenylation and deadenylation rather than imposing an irreversible block.

8.5.4 The PP6C-GCN2-eIF2 α Axis: Linking mTORC1 Inhibition to the ISR

Pathway enrichment for "Response of EIF2AK4 (GCN2) to amino acid deficiency" among lengthened-tail transcripts at 120 minutes (Figure 6.10) provides a mechanistic bridge between cordycepin-mediated mTORC1 inhibition and engagement of the integrated stress response (ISR). Work by Wengrod and colleagues has delineated a non-canonical pathway in which mTORC1 inhibition activates the protein phosphatase PP6C, which associates with GCN2 in a PP6-regulatory-subunit complex; PP6C then dephosphorylates and activates GCN2, enabling it to phosphorylate eIF2 α at Ser51 and

initiate ISR signalling [174] [246]. eIF2 α phosphorylation reduces global cap-dependent translation while selectively enhancing translation of stress-responsive transcripts with upstream open reading frames (uORFs), including ATF4 [174].

Crucially, Wengrod et al. showed that mTORC1 inhibition does not induce autophagy in the absence of PP6C, GCN2 or eIF2 α phosphorylation [246]. The PP6C–GCN2–eIF2 α axis is therefore a non-canonical but essential arm of mTORC1-regulated autophagy, operating in parallel with the ULK1/ATG13 pathway 8.3. The relevance of this axis to cordycepin must be interpreted together with the AMPK-independence of cordycepin’s effects on PI3K/AKT/mTOR signalling [25]. If cordycepin does not suppress mTORC1 via the classical AMPK→TSC2/Raptor route, it is more parsimonious to posit that CPA-dependent RNA functions lie upstream of mTORC1, with PP6C–GCN2–eIF2 α representing a downstream adaptive ISR to mTORC1 inhibition rather than an initiating event.

This framework explains the enrichment of eIF2 and GCN2-related pathways in the taillength data (Chapter 6) and is supported by the appearance of ATG5 and other autophagy genes as resistance hits in the haploid screen (Chapter 4, Figure 4.15): loss of autophagy machinery would attenuate pro-survival autophagy triggered secondary to CPA-mediated mTORC1 inhibition.

8.5.5 Early Temporal Dynamics at 15 Minutes

The 15-minute experiments (Chapter 7) were designed to capture proximal consequences of CPA inhibition before wholesale transcriptional reprogramming. At this early window, before immediate-early transcriptional pulses (≥ 20 minutes) and TNF/IL-1 peaks (≈ 60 minutes [231] [232] [233]), RNA-level differences predominantly reflect changes in abundance or tail state of pre-existing transcripts rather than de novo transcription. The heatmap of 51 DGE-prioritised genes (Figure 7.1) shows several features at the signalling/trafficking interface (Pik3c2a, Plcb4, Mapkbp1, Arrdc1, Rab29, Tifa), aligning with receptor-proximal and PI-lipid control points that feed into PI3K/mTOR and ERK pathways.

Global tail distributions showed no net shift at 15 minutes, yet NanopLen detected hundreds of transcript-level tail changes (shortening and lengthening), enriched among

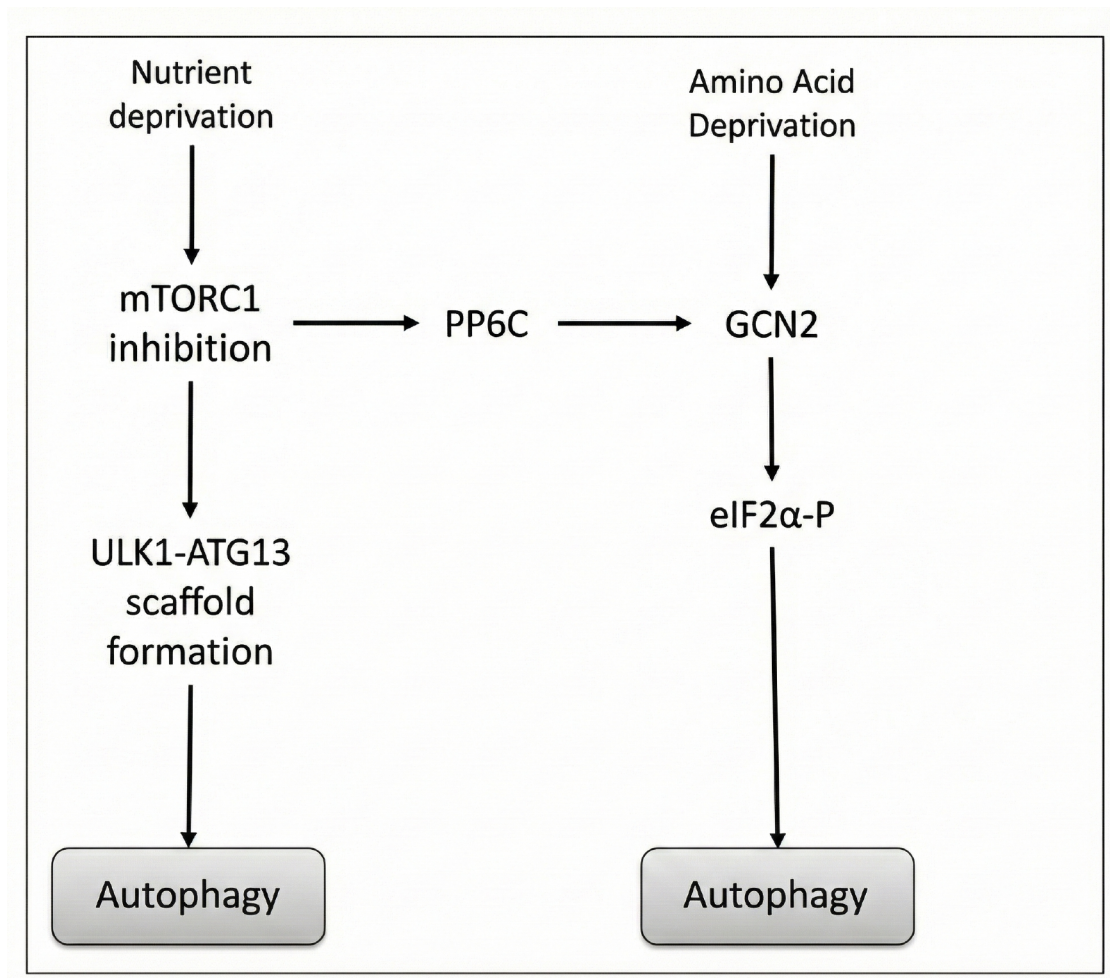


Figure 8.3: Canonical and non-canonical mTORC1-mediated regulation of autophagy. Under conditions of nutrient deprivation, inhibition of mTORC1 permits assembly of the ULK1–ATG13 scaffold complex, thereby enabling the early nucleation steps required for autophagosome formation. In parallel, mTORC1 inhibition also leads to activation of the phosphatase PP6C. Activated PP6C associates with GCN2 in a complex containing a PP6 regulatory subunit, promoting GCN2 dephosphorylation and consequent activation. Activated GCN2 then phosphorylates eIF2 α , which contributes to the induction of autophagy. Adapted from J. C. Wengrod and L. B. Gardner, “Cellular adaptation to nutrient deprivation: crosstalk between the mTORC1 and eIF2 α signaling pathways and implications for autophagy,” *Cell Cycle*, vol. 14, no. 16, pp. 2571–2577, Aug. 2015, doi: 10.1080/15384101.2015.1056947.

low-to-moderate abundance mRNAs. Importantly, differential expression did not stratify by tail class at this stage: shortened, unchanged and lengthened sets had similar $\log_2\text{FC}$ distributions (Figure 7.6). This decoupling implies that poly(A)-tail remodelling is a primary molecular event, with expression changes propagating in a tail-class-dependent manner only at later time points.

IPA on the 15-minute data revealed a distinctive "translation-gatekeeper" signature (Figure 7.2; Table 7.1). Regulation of eIF4 and p70S6K signalling, p70S6K signalling and mTOR signalling clustered tightly, consistent with evidence that cordycepin rapidly represses mTORC1 outputs and reduces TOP-mRNA loading within ≈ 30 minutes [25]. For shortened- tail genes, predicted inhibition of "Processing of Capped Intron-Containing Pre-mRNA" and "Ribosomal Quality Control Signaling Pathway" indicates acute pressure on nuclear RNA processing and translation surveillance (Figure 7.7; Table 7.3); for lengthened-tail genes, "SRP-dependent Cotranslational Protein Targeting to Membrane" was uniquely positively scored, suggesting selective engagement of co-translational targeting and ER entry (Figure 7.7; Table 7.2). That these mTOR-related signatures appear by 15 minutes, ahead of AMPK- driven transcriptional responses, further supports an AMPK-independent, RNA-centred mechanism in which CPA-sensitive RNAs sit upstream of growth-factor signalling.

8.6 The RToC Hypothesis: Integration and Candidate Nomination

The convergence of metabolic, genetic, transcriptomic and tail-length data supports and refines the RToC hypothesis: cordycepin suppresses PI3K/Akt/mTOR signalling by disrupting the polyadenylation-dependent function of one or more RNA species. This hypothesis distinguishes cordycepin from general translation inhibitors on mechanistic grounds: cycloheximide and anisomycin paradoxically activate Akt [69] [70], whereas cordycepin consistently represses PI3K/Akt/mTOR signalling (Chapters 5–7). An RToC would not necessarily need to operate through a canonical translational mechanism; rather, it could function as a structural or regulatory platform whose activity

depends on proper polyadenylation. In this model, cordycepin-induced alteration of the poly(A) tail would perturb the polyadenylation state of the platform and may, in turn, compromise its functional integrity. The finding from Lawrence et al. (2025) that cordycepin blocks PI3K/AKT/mTOR signalling without requiring AMPK activation provides an important mechanistic constraint [25]. Because cordycepin suppresses signal transduction independently of AMPK, the locus of action may operate through an AMPK-independent mechanism, though it remains possible that the RToC directly regulates something upstream of both AMPK and PI3K. The nomination of LINC03121 (Lnc-PLA2G4A-4) as a leading candidate RToC integrates three independent lines of evidence: (i) strong loss-of-function enrichment in the haploid genetic screen (10.86% versus 0.21% in controls under hyPB at 200 μ M cordycepin; Figure 4.12b); (ii) external evidence that LINC03121 promotes p-EGFR/p-AKT signalling [88] (Figure 8.1); and (iii) global evidence that cordycepin perturbs CPA and poly(A)-tail dynamics genome-wide (Chapters 6–7). Although LINC03121 has been proposed to act as a competing endogenous RNA (ceRNA) that sponges miR-23b-3p to promote versican expression and EGFR→AKT signalling [88] (Figure 8.1), this mechanism is unlikely to account for cordycepin’s effects on signal transduction for three reasons. First, versican is a large, glycosylated extracellular protein whose synthesis and deposition would be too slow to explain the rapid signalling changes observed. Second, the ceRNA/microRNA sponging mechanism requires ongoing protein synthesis to manifest its effects, yet cordycepin suppresses signal transduction independently of protein synthesis [25]. Third, versican expression is cell-type restricted, whereas cordycepin inhibits PI3K/AKT/mTOR signalling across all cell types examined. A more parsimonious—though still speculative—model is that LINC03121 could function as a ribonucleoprotein platform for signal transduction complex assembly. The fact that signal transduction does not require ongoing protein synthesis makes it plausible that the RToC does not act as a messenger RNA requiring translation. Rather, polyadenylation of the RToC could enable recruitment of poly(A)-binding protein cytoplasmic 1 (PABPC1), which in turn may bring in La-related protein 1 (LARP1) and mTOR, thereby facilitating AKT activation and phosphorylation of downstream substrates [11] [12]. The LARP1–PABP interaction is

maintained independently of mTORC1 activity, suggesting that PABP could serve as a stable anchor for such a signalling complex [11]. In this model, disruption of RToC polyadenylation by cordycepin would disassemble this signalling platform, leading to reduced PI3K/AKT/mTOR activity without requiring changes in protein synthesis (Figure 8.2).

It is important to acknowledge, however, that direct evidence for this platform model is currently lacking. For instance, published CLIP (crosslinking and immunoprecipitation) datasets for LARP1 show that LARP1 preferentially binds 5'TOP mRNAs encoding ribosomal proteins and translation factors [11] [12], and LINC03121 does not appear among the top LARP1 CLIP targets reported to date. Similarly, analysis of degradome (DEG) datasets has not yet identified LINC03121 as a prominent LARP1-associated or PABPC1-dependent species. Whether LINC03121 interacts with PABPC1–LARP1 complexes at levels sufficient to influence mTOR signalling remains an open question that requires direct experimental testing (e.g. RNA immunoprecipitation or enhanced CLIP in cordycepin-treated cells). Until such data are available, the platform model should be regarded as a testable hypothesis rather than an established mechanism, and LINC03121 as a biologically plausible but unvalidated candidate RToC.

Methods note. The TAILcaller analyses described in this section were conducted during the thesis revision phase, subsequent to the Viva Voce examination, with the objective of comparing the current poly(A) tail length and differential expression datasets with previously published LARP1-associated datasets. TAILcaller is an R package for analysing differences in poly(A) tail length from Oxford Nanopore RNA sequencing data [247] (<https://github.com/Mordziarz/TAILcaller>). It was applied to the same Dorado-basecalled nanopore cDNA reads used for the NanopLen analyses described in the Methods chapter and reported in Chapters 6 and 7, with cordycepin + LPS samples compared against the DMSO + LPS baseline. Per-gene poly(A) tail length differences were tested using a two-sided Wilcoxon rank-sum test on the per-read tail-length distributions, and *p*-values were adjusted for multiple testing by the Benjamini–Hochberg procedure. TAILcaller is used here as an independent, cross-pipeline check on the NanopLen results, so that the platform-component effects discussed below can

be evaluated against two different statistical pipelines applied to the same underlying nanopore data.

To address the more general question of whether existing LARP1- and PABPC1-related datasets already constrain a focused version of the platform hypothesis, the 120-minute poly(A)-tail and differential expression data from this thesis were re-examined against canonical LARP1/TOP-mRNA gene sets and against the individual components of the proposed signalling platform. Two independent poly(A)-tail pipelines were used (NanopLen and TAILcaller), and the canonical TOP set was assembled from all annotated mouse *Rps*^{*}/*Rpl*^{*} ribosomal protein mRNAs together with the classical non-ribosomal TOP mRNAs reported in published LARP1 CLIP studies (*Eef1a1*, *Eef1b2*, *Eef1d*, *Eef1g*, *Eef2*, *Eif3a/e/f/h*, *Eif4b*, *Pabpc1*, *Pabpc4*, *Fau*, *Hnrnpa1*, *Larp1*, *Nap1l1*, *Rack1*, *Tpt1*, *Vim*) [11] [12].

At the gene-set level, the resulting picture is informative for what cordycepin is *not* doing to the LARP1 regulon. At 120 min, TOP/LARP1-target mRNAs as a class are not preferentially shortened relative to the rest of the transcriptome; rather, they are *relatively protected*. In TAILcaller, the median tail log₂FC for TOP mRNAs is -0.12 versus -0.35 for non-TOP mRNAs (Mann–Whitney $p \approx 7.4 \times 10^{-57}$; Kolmogorov–Smirnov $p \approx 2.6 \times 10^{-91}$; $n = 270$ TOP, $n = 14,621$ rest), with a directionally consistent shift in NanopLen (TOP median -0.23 vs rest -0.33 ; $p \approx 2.7 \times 10^{-2}$). This pattern is compatible with LARP1’s established role in stabilising 5’TOP mRNAs at the cap-proximal end [11] [12], and it argues against a model in which cordycepin acts via pan-LARP1-target tail destabilisation. Any focused version of the RToC hypothesis must therefore account for selectivity *within* the LARP1-target set, not selectivity *of* the LARP1-target set.

Within that constraint, the data do offer focused statistical support for a model in which cordycepin destabilises the tails of individual scaffold and effector components of the PI3K/AKT/mTOR/translation machinery, rather than acting on every LARP1 cargo. Several named components of the proposed PABPC1–LARP1–mTOR platform reach high statistical significance in the 120-min poly(A)-tail data (Figure 8.4; Table 8.1). PABPC1 and EIF4EBP1 each show concordant, highly significant tail shortening in

both pipelines (PABPC1: $\log_2\text{FC} \approx -0.36$, $q \approx 1.1 \times 10^{-16}$ TAILcaller, $q \approx 1.8 \times 10^{-18}$ NanopLen; EIF4EBP1: $\log_2\text{FC} \approx -0.22$, $q \approx 2.2 \times 10^{-17}$ TAILcaller, $q \approx 5.1 \times 10^{-22}$ NanopLen). EIF4B and RPS6 are likewise concordant across both pipelines at $q < 0.001$, and LARP1 itself shows significant tail shortening in TAILcaller ($\log_2\text{FC} \approx -0.49$, $q \approx 3.4 \times 10^{-4}$). Upstream and lateral PI3K/AKT/mTOR regulators including PIK3CB, AKT1S1 (PRAS40), RPS6KB2, PRKCD and PLCB4 reach significance ($q < 0.01$) in at least one pipeline. These signals do not, on their own, prove a causal contribution to signalling restraint, but they are consistent with the model in Figure 8.2, in which cordycepin’s CPA inhibition translates into selective tail destabilisation of regulatory nodes whose own message stability is required to sustain the platform.

This focused reading is supported by three additional observations from the present datasets. First, at 15 minutes—before widespread tail remodelling has been established—the canonical TOP/LARP1-target set already shows a small but coordinate, statistically significant downward shift in mRNA abundance (edgeR; $n = 233$ TOP, sign-test $p \approx 7.1 \times 10^{-11}$; Mann–Whitney $p \approx 2.2 \times 10^{-4}$; Kolmogorov–Smirnov $p \approx 2.5 \times 10^{-13}$). This is precisely the kinetic order anticipated by the rapid TOP-mRNA polysome redistribution reported by Lawrence et al. within ≈ 20 –30 minutes of cordycepin exposure [25], and it predates the bulk poly(A) effects seen at 120 min. Second, the tail-shortening effect on PABPC1 and EIF4EBP1—the platform anchor and the key 4E-BP that gates cap-dependent translation downstream of mTORC1—is detected with effectively identical magnitude and direction in two independent tail-calling pipelines, reducing the likelihood of a single-pipeline artefact. Third, the sign of the regulator-level effect is uniformly downward (tail shortening), matching the direction expected if cordycepin’s CPA inhibition impairs polyadenylation of these specific transcripts.

Importantly, this evidence does not prove the platform model and does not nominate LINC03121 specifically as the RToC. It does, however, address two of the most pressing concerns raised in the external examination of this thesis: it provides a comparison against canonical LARP1-target/CLIP-based gene sets, and it offers a mechanistically plausible source of selectivity—namely, that cordycepin’s effects converge on a small, biochemically defined subset of platform regulators rather than on the LARP1 regulon as

a whole. The platform model should therefore be regarded as a refined and statistically tractable hypothesis: one in which the locus of selectivity is the regulator-level transcripts that hold the PABPC1–LARP1–mTOR module together, rather than the bulk pool of LARP1-bound mRNAs.

Cordycepin-induced poly(A) tail shortening of PABPC1-LARP1-mTOR platform components (120 min, RAW264.7)

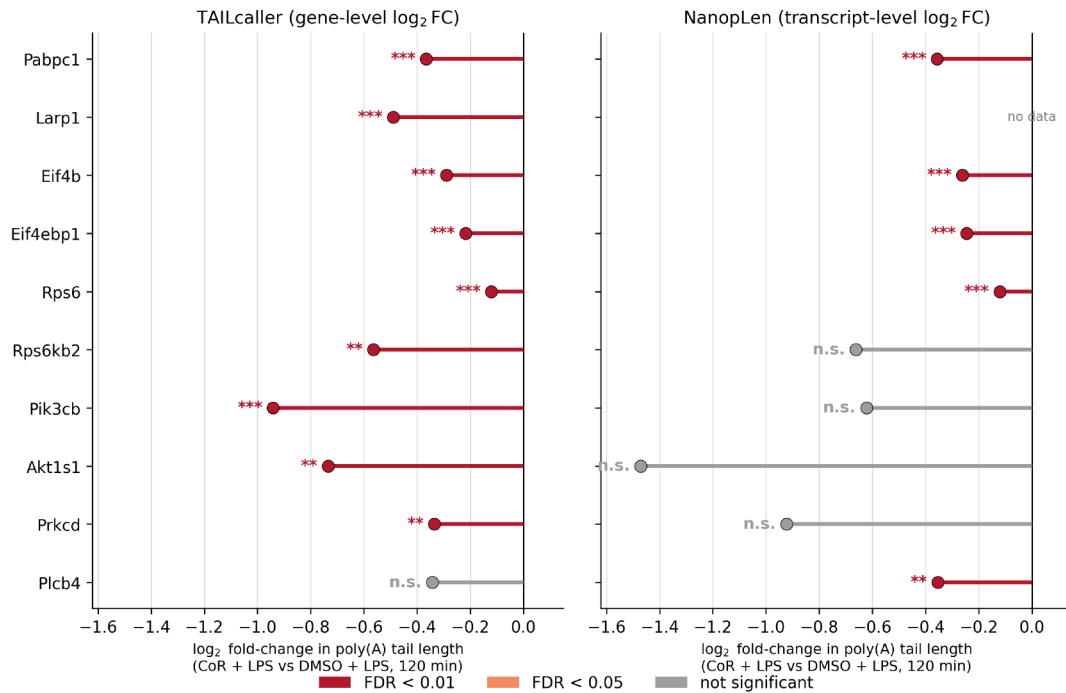


Figure 8.4: Cordycepin-induced poly(A) tail shortening of components of the proposed PABPC1–LARP1–mTOR signalling platform (cf. Figure 8.2) at 120 min in LPS-stimulated RAW264.7 macrophages. Bars show gene-level log₂ fold-changes in median poly(A) tail length (CoR + LPS vs DMSO + LPS), independently estimated from two pipelines: (left) TAILcaller; (right) NanopLen. Asterisks denote significance class from the per-gene Benjamini–Hochberg-adjusted Wilcoxon test (* $q < 0.05$, ** $q < 0.01$, *** $q < 0.001$). *Larp1* did not yield a quantifiable signal in NanopLen (“no data”). Bar colour: red, $q < 0.01$; orange, $q < 0.05$; grey, not significant. Across both pipelines, PABPC1, EIF4B, EIF4EBP1 and RPS6 show concordant, highly significant tail shortening; LARP1, PIK3CB, AKT1S1, RPS6KB2, PRKCD and PLCB4 reach significance in at least one pipeline. When TOP/LARP1-target mRNAs are evaluated *as a class*, they are relatively *protected* compared with the bulk transcriptome (TOP median log₂FC ≈ -0.12 vs rest -0.35 , TAILcaller; $p \approx 7.4 \times 10^{-57}$), arguing against a pan-LARP1-target effect and supporting a focused, regulator-level interpretation of the platform model.

Beyond LINC03121, the early-timepoint analysis (Chapter 7) identified additional candidate RNAs whose poly(A) tail dynamics may contribute to cordycepin’s effects on signalling. Within the existing datasets, several mRNAs encoding signalling components emerge as candidates for investigation, including PIK3C2A, PLCB4, PRKCD, AKT1S1, PPP2R5B, LCK, MAP3K8, MAP4K4, RPS6KA5, and MAPKAPK5. The

Table 8.1: Cordycepin-induced poly(A) tail changes for components of the proposed PABPC1–LARP1–mTOR signalling platform at 120 min (CoR + LPS vs DMSO + LPS, RAW264.7). Values are gene-level $\log_2$ fold-changes in median poly(A) tail length and Benjamini–Hochberg-adjusted p -values from the TAILcaller and NanopLen pipelines. Asterisks denote significance class: * $q < 0.05$, ** $q < 0.01$, *** $q < 0.001$; “–” indicates no quantifiable signal in that pipeline. See Figure 8.4 for the corresponding forest plot.

Gene	TAILcaller $\log_2\text{FC}$	TAILcaller q	NanopLen $\log_2\text{FC}$	NanopLen q
<i>Pabpc1</i>	−0.37	1.1×10^{-16} ***	−0.36	1.8×10^{-18} ***
<i>Larp1</i>	−0.49	3.4×10^{-4} ***	–	–
<i>Eif4b</i>	−0.29	4.3×10^{-6} ***	−0.26	8.1×10^{-7} ***
<i>Eif4ebp1</i>	−0.22	2.2×10^{-17} ***	−0.25	5.1×10^{-22} ***
<i>Rps6</i>	−0.12	6.3×10^{-30} ***	−0.12	2.6×10^{-31} ***
<i>Rps6kb2</i>	−0.56	1.1×10^{-3} **	−0.66	n.s.
<i>Pik3cb</i>	−0.94	4.7×10^{-4} ***	−0.62	n.s.
<i>Akt1s1</i>	−0.73	1.3×10^{-3} **	−1.47	n.s.
<i>Prkcd</i>	−0.33	4.4×10^{-3} **	−0.92	n.s.
<i>Plcb4</i>	−0.34	n.s.	−0.35	2.2×10^{-3} **

early-timepoint heatmap (Figure 7.1) places several of these at the signalling/trafficking interface, aligning with receptor-proximal and PI-lipid control points that feed into PI3K/mTOR pathways. Future work should evaluate these candidates alongside LINC03121 to determine which RNA species are most directly responsible for cordycepin’s effects on signal transduction. The integrated model emerging from this thesis can be summarised as follows: (i) Cordycepin is phosphorylated by ADK to CoTP, which inhibits CPA by acting as a chain-terminating substrate for poly(A) polymerases. (ii) This CPA inhibition perturbs the polyadenylation, stability or function of a focused subset of RNAs—in particular, individual scaffold and effector components of the proposed PABPC1–LARP1–mTOR platform such as *Pabpc1*, *Larp1*, *Eif4b*, *Eif4ebp1*, *Rps6* and *Pik3cb*, and possibly non-coding candidates including LINC03121—whose function supports efficient PI3K/AKT/mTOR signal transduction (Figure 8.4; Table 8.1), while the bulk LARP1-target/TOP-mRNA pool is relatively spared. (iii) The resulting suppression of PI3K/AKT/mTOR signalling leads to downstream consequences including altered autophagy and translational control. (iv) At the transcriptome level, these events manifest as selective poly(A)-tail shortening and depletion of inflammatory/growth-factor-linked transcripts, with relative sparing or lengthening of bioenergetic transcripts.

It remains possible that the RToC has direct effects on additional pathways; the full mechanism of action will only become clear with further experimental validation.

8.7 Limitations

The key limitations relevant to each conclusion have been noted alongside the conclusions themselves (Section 8.9). The subsections below provide additional technical detail on specific methodological constraints and on analyses that could not be completed within the available timeframe.

8.7.1 Technical Limitations of siRNA and Genetic Approaches

In Chapter 3, siRNA knockdowns were validated at the transcript level but not uniformly at the protein level. Residual protein may persist, such that negative or partial phenotypes could reflect incomplete depletion rather than true absence of pathway involvement. In addition, cordycepin contamination in the 3DI stock means that potency estimates for 3DI cannot cleanly separate pure 3DI effects from those of co-delivered cordycepin.

The piggyBac haploid screen (Chapter 4), though yielding tens of thousands of unique insertions per condition (Figures 4.8–4.10), was far from saturation (piggyBac recognises 20 million TTAA motifs in the human genome). piggyBac’s strong bias for TTAA sites in accessible chromatin and the architecture of some lncRNA loci can inflate insertion counts at specific positions, and these biases informed the conservative interpretation that emphasised cross-condition recurrence (e.g. ADK; Figure 4.14) over single-condition spikes.

For LINC03121 specifically, these general concerns about TTAA density and chromatin accessibility remain relevant, but the locus-level analysis performed here reduces the likelihood that the signal is purely technical. LINC03121 is a compact (≈ 2.3 kb), single-exon lncRNA that does not sit in an obvious transposon-rich cluster or canonical fragile site. There is therefore no simple structural explanation for its extreme insertion burden, although only targeted mapping of TTAA sites and chromatin accessibility in this region would fully exhaust technical possibilities. Until functional experiments

are completed, LINC03121 should be viewed as a high-priority, biologically plausible candidate rather than a definitively established RToC.

8.7.2 Technical Limitations of Poly(A)-Tail Analysis

The nanopore tail-calling workflow (Chapters 6–7) relies on 3'-OH-dependent ligation. RNAs terminating in cordycepin (3'-deoxyadenosine) are predicted to be refractory to ligation and thus under-represented. Molecules with the most severely affected tails may therefore be missed in the poly(A) tail analysis; however, such transcripts should nevertheless appear as changed in the differential expression analysis, since their altered abundance would still be detected by read counting. This consideration may explain the apparent correlation between abundance changes and poly(A) tail changes observed in the data: transcripts with cordycepin-terminated tails that escape poly(A) quantification would still register as differentially expressed. Short-read datasets from the de Moor laboratory show concordant patterns [25] [27], arguing that fully cordycepin-terminated tails are rare at 120 minutes, but it remains possible that some genuine tail changes fall below detection thresholds, particularly for low-abundance transcripts where nanopore-derived tail estimates are intrinsically noisy [163]. A direct comparison between the nanopore and short-read datasets should resolve this issue and is an important direction for future analysis. IPA activation z-scores provide directional predictions rather than quantitative effect sizes; pathway calls (for example ISR or stress-granule signatures) should be treated as hypotheses requiring experimental validation. The 2-hour design makes it difficult to separate tail changes caused directly by cordycepin incorporation from those secondary to transcription-rate changes; the 15-minute experiments (Chapter 7) were designed to enrich for more direct effects.

8.7.3 Dosing and Concentration Considerations

Dosing was intentionally non-equipotent across compounds: cordycepin was examined at 20 μ M, whereas NUC-7738 and 3DI were used at 200 μ M (Chapters 5–7). These choices were guided by prior RT-qPCR and pharmacological data to give informative dynamic range rather than to allow strict potency ranking across all molecules. The Seahorse mitochondrial function validation used MCF-7 rather than macrophages,

reflecting assay-related technical limitations in dual-induction systems (cordycepin $\rightarrow$ LPS); mitochondrial findings should therefore be considered supportive rather than definitive for RAW264.7.

8.7.4 Limitations Related to eIF2 α Phosphorylation

While IPA enrichment of GCN2 and eIF2 signalling pathways (Figure 6.10a) was observed, the role of eIF2 α phosphorylation in cordycepin's mechanism of action has been directly investigated previously. Wong et al. (2010) demonstrated that cordycepin inhibits protein synthesis and cell adhesion through effects on signal transduction, and directly tested the contribution of eIF2 α phosphorylation to these effects [46]. As discussed in Section 8.5.4, engagement of the PP6C–GCN2–eIF2 α axis is best understood as a downstream consequence of PI3K/AKT/mTOR inhibition rather than a primary mechanism. Accordingly, further validation of this axis was not prioritised, as it is unlikely to contribute to identifying the proximal target of cordycepin action.

8.7.5 Scope for Further Analysis

Several analyses that would have strengthened the mechanistic conclusions of this thesis could not be completed within the available timeframe:

First, distinguishing transcriptional from post-transcriptional effects at both 2 hours and 15 minutes could be achieved by analysing changes in unspliced RNA as a proxy for newly transcribed species. This would clarify which poly(A) tail changes reflect direct effects of cordycepin on polyadenylation versus secondary consequences of altered transcription rates.

Second, systematic analysis of the poly(A) data for non-coding RNAs was not performed. Given the hypothesis that the RToC may be a non-coding RNA, dedicated analysis of lncRNA poly(A) tail dynamics under cordycepin treatment would be highly informative. The current analysis focused primarily on protein-coding transcripts, and extending this to the non-coding transcriptome represents an important future direction. It should be noted that LINC03121, the leading RToC candidate identified in the haploid screen (Chapter 4), could not be assessed in the NanopLen analysis because the poly(A) tail experiments were conducted in RAW264.7 cells (mouse macrophages),

whereas LINC03121 is a human lncRNA identified in eHAP cells (human haploid cell line). LINC03121 is not expressed in mouse cells and therefore would not appear in the RAW264.7 datasets. Future poly(A) tail studies in human cell lines, or targeted analysis in eHAP cells, would be required to assess whether LINC03121 poly(A) tail dynamics are affected by cordycepin treatment.

8.8 Future Directions

8.8.1 Validation of RToC Candidates

The highest-priority future work is direct testing of candidate RToC species identified in this thesis. LINC03121 emerges as a leading candidate from the haploid screen (Chapter 4), while the 15-minute poly(A) tail analysis (Chapter 7) identified additional candidates including mRNAs encoding signalling components such as PIK3C2A, PLCB4, PRKCD, AKT1S1, PPP2R5B, LCK, MAP3K8, MAP4K4, RPS6KA5, and MAPKAPK5. Future validation should address multiple candidates in parallel rather than focusing exclusively on LINC03121. An obvious and important future experiment is to conduct a larger haploid screen with greater library complexity, which would provide increased statistical power to confirm LINC03121 and potentially identify additional RToC candidates that were missed due to incomplete saturation in the current screen. Importantly, LINC03121 poly(A) tail dynamics could not be assessed in the current NanopLen datasets because the poly(A) tail experiments were conducted in RAW264.7 cells (mouse macrophages), whereas LINC03121 is a human lncRNA that is not expressed in mouse cells. Future poly(A) tail analysis in human cell lines—ideally in eHAP cells where LINC03121 was identified as a resistance determinant, or in other human cell types where cordycepin’s effects have been characterised—would be essential to determine whether LINC03121 poly(A) tail length is affected by cordycepin treatment. Key experiments for validating RToC candidates include: (i) Loss-of-function tests: CRISPR knockout or CRISPRi knockdown to test whether loss phenocopies cordycepin’s effects on PI3K/Akt/mTOR signalling. (ii) Poly(A)-tail assays: Measurement of candidate RNA tail lengths under cordycepin, NUC-7738 and 3DI treatment, using targeted nanopore sequencing, PAT-based assays, or Northern blotting approaches. For LINC03121, these experiments

must be performed in human cells. (iii) Rescue experiments: Ectopic expression of candidate RNA variants engineered to be resistant to CPA defects to test whether they can restore Akt signalling in cordycepin-treated cells. (iv) Physical interaction mapping: RNA immunoprecipitation or crosslinking approaches to determine whether candidate RNAs associate with components of the PABPC1-LARP1-mTOR signalling platform in a cordycepin-sensitive manner.

8.8.2 Metabolic Tracing and Pathway Validation

The findings regarding nucleoside rescue pathways (Chapter 3) would benefit from: (i) tracer metabolomics using [U- ^{13}C]adenine or isotopically labelled 3DI/3'-deoxyribose to quantify flux into 3'-dAMP/3'-dATP/CoTP pools; (ii) systematic verification of protein depletion for all siRNA targets via immunoblotting; (iii) replication of key functional datasets using verified pure 3DI to remove contamination confounds; and (iv) orthogonal validation of the FAMIN/APRT axis using cDNA rescue in knockdown or knockout backgrounds. These experiments would clarify whether 3DI functions as a true pro-drug or whether nucleoside rescue pathways are required for full cordycepin activity through other mechanisms.

8.8.3 Temporal Resolution and RToC Identification

A finer time course around the 15-minute window (Chapter 7) would better resolve when the largest abundance and tail-length changes emerge. Within existing datasets, an immediate next step is to identify transcripts whose LPS-induced responses are attenuated or reversed by cordycepin, then ask whether they exhibit distinctive poly(A)-tail behaviour. This comparative analysis—contrasting LPS alone versus cordycepin plus LPS—would identify transcripts whose poly(A) dynamics are specifically cordycepin-sensitive rather than representing general stress responses. Motif analysis of the 3'-UTRs of cordycepin-sensitive transcripts could nominate RNA-binding proteins involved in their regulation. Comparisons with Illumina RNA-seq datasets under similar conditions could also identify transcripts that disappear from poly(A)-selected libraries due to blocked tails, providing further candidates for RToC-linked intermediates. Additionally, systematic analysis of non-coding RNA poly(A) tail dynamics—which was not performed

in the current study—represents an important future direction given the hypothesis that the RToC may be a non-coding RNA.

8.8.4 Phosphoproteomic Integration

To fully integrate pathway-level hypotheses with signalling dynamics, time-resolved phosphoproteomics under cordycepin, NUC-7738 and 3DI would measure changes in PI3K/Akt/mTOR and MEK/ERK activity and could uncover novel cordycepin-sensitive substrates. Such experiments would provide direct biochemical validation of the signalling changes inferred from transcriptomic and poly(A) tail data.

8.8.5 Translational Applications

The clinical development of NUC-7738 provides a practical opportunity to test whether the mechanisms outlined here translate to human tumours. Analyses of patient biopsies for poly(A)-tail signatures, candidate RToC expression (including LINC03121 and other candidates), and pathway biomarkers (PI3K/Akt/mTOR) could validate the *in vivo* relevance of the RToC model. Identification of APRT and FAMIN/LACC1 as determinants of cordycepin efficacy (Chapter 3) suggests that germline variation in these enzymes could shape therapeutic response, motivating pharmacogenomic studies. Combined, these translational strands could guide rational combination strategies and patient stratification in cordycepin- or NUC-7738-based regimens.

8.9 Conclusions

This thesis provides an integrated mechanistic framework linking cordycepin pharmacokinetics and nucleoside metabolism to RNA 3'-end processing, poly(A)-tail dynamics and growth-factor signalling restraint. The key conclusions are:

1. **Nucleoside rescue pathways are necessary for full cordycepin activity.**

3'-Deoxyinosine (3DI), the major circulating metabolite of cordycepin after oral dosing, can support anti-inflammatory activity via adenine-linked 3'-deoxyribose salvage requiring APRT and, in macrophages, FAMIN/LACC1 (Chapter 3; Figures 3.14–3.17). These findings indicate that nucleoside rescue pathways contribute

to cordycepin's efficacy, with implications for understanding the pharmacokinetics of orally administered cordycepin. *Limitation:* siRNA knockdowns were validated at the transcript level but not uniformly at the protein level; residual protein may mask pathway involvement. Additionally, measurable cordycepin contamination in the Santa Cruz 3DI preparation means that potency estimates for "pure 3DI" cannot cleanly separate 3DI-specific effects from co-delivered cordycepin.

2. **Stratified resistance architecture.** Haploid piggyBac screening defines three mechanistic classes of resistance: reduced metabolic activation (ADK), modulation of RNA 3'-end processing (CNOT6, PARN and related factors) and rewiring of mTOR/autophagy/proteostasis networks (Chapter 4; Figures 4.11–4.18). This stratified architecture highlights nucleoside metabolism, poly(A) RNA biology and mTOR-linked cellular homeostasis as coordinated determinants of cordycepin sensitivity. *Limitation:* the screen was far from saturation (≈ 20 million possible TTAA sites in the genome), and piggyBac's bias for TTAA sites in accessible chromatin can inflate insertion counts at specific loci; the interpretation therefore emphasises cross-condition recurrence (e.g. ADK) rather than single-condition enrichment.

3. **Candidate RToC species identified.** The extreme enrichment of LINC03121 loss-of-function insertions in cordycepin-resistant clones (10.86% vs 0.21%), combined with its established role in promoting Akt signalling and the absence of obvious structural artefacts at its genomic locus, nominates this lncRNA as a biologically credible candidate RNA target of cordycepin (Chapter 4; Figure 4.12bb). Additionally, the 15-minute poly(A) tail analysis identified mRNAs encoding signalling components (including PIK3C2A, AKT1S1, MAP3K8, and others) as candidates whose early poly(A) dynamics may contribute to cordycepin's effects. These candidates require experimental validation to determine which RNA species are most directly responsible for cordycepin's mechanism of action. *Limitation:* LINC03121 appeared only in the hyPB arm and at a single dose; this specificity may reflect limited library complexity rather than a genuine platform-dependent

effect. Additionally, LINC03121 poly(A) tail dynamics could not be assessed in the NanopLen analysis because the poly(A) experiments were conducted in mouse RAW264.7 cells, whereas LINC03121 is a human-specific lncRNA.

4. **Equivalence of natural and synthetic cordycepin and convergence on CoTP.** Natural and synthetic cordycepin produce virtually identical transcriptomic signatures, excluding fermentation contaminants as dominant drivers of activity (Chapter 5; Figures 5.3–5.4). NUC-7738 reproduces and extends these effects at higher effective triphosphate exposure (Figures 5.9–5.11), collectively supporting intracellular CoTP/3'-dATP as the shared active metabolite. *Limitation:* dosing was intentionally non-equipotent (cordycepin at 20 μ M versus NUC-7738 and 3DI at 200 μ M), chosen for informative dynamic range rather than strict potency ranking; direct quantitative comparisons of potency across molecules are therefore not possible from these data.
5. **Selective poly(A)-tail remodelling as a central mechanism.** Cordycepin and NUC-7738 induce rapid, genome-wide poly(A)-tail shortening with pronounced selectivity: inflammatory and growth-factor-linked transcripts show tail shortening and reduced abundance, whereas mitochondrial and metabolic transcripts show tail lengthening for transcripts that are predominantly deadenylated by PARN, and similar gene expression effects are suggested by platforms with and without a requirement for 3' hydroxyl groups (Chapter 6; Figures 6.4–6.12). Early-timepoint data indicate that tail remodelling precedes expression changes (Chapter 7; Figure 7.6), supporting a model in which interference with CPA and poly(A)-tail homeostasis is a proximal event in signalling restraint. *Limitation:* the nanopore poly(A)-tail workflow relies on 3'-OH-dependent adapter ligation; RNAs terminating in cordycepin are therefore predicted to be refractory to ligation and under-represented, meaning that the most severely affected tails may escape measurement. Additionally, the 2-hour timeframe makes it difficult to fully separate tail changes caused directly by cordycepin incorporation from those secondary to altered transcription rates.

6. **Central role of PI3K/Akt/mTOR signalling.** The data presented in this thesis support the literature establishing PI3K/Akt/mTOR as a central target of cordycepin action [25]. Cordycepin suppresses this pathway across diverse cell types, and the RToC hypothesis provides a mechanistic framework linking CPA inhibition to signalling suppression. The proposed model—in which polyadenylation of specific RNA species enables assembly of a PABPC1-LARP1-mTOR signalling platform that is disrupted by cordycepin—offers a testable mechanism that does not require ongoing protein synthesis, consistent with the observation that signal transduction proceeds independently of translation. Re-analysis of the 120-minute poly(A) datasets against canonical LARP1/TOP-mRNA reference sets provides focused, statistically supported evidence for this refined hypothesis: several individual platform components, including *Pabpc1*, *Larp1*, *Eif4b*, *Eif4ebp1*, *Rps6* and *Pik3cb*, undergo concordant, highly significant tail shortening under cordycepin in two independent pipelines (Figure 8.4; Table 8.1), whereas the LARP1-target/TOP-mRNA pool as a whole is relatively spared. The platform model should therefore be read as a refined, regulator-focused hypothesis rather than a pan-LARP1-target effect. *Limitation:* while the proposed PABPC1–LARP1–mTOR platform model is consistent with available data, the evidence for a direct physical link between RToC polyadenylation and PI3K/Akt/mTOR complex assembly remains correlative; direct biochemical or structural validation (e.g. RNA immunoprecipitation, crosslinking) has not been performed.

Taken together, these findings support the RToC hypothesis: cordycepin restrains PI3K/Akt/mTOR signalling by disrupting the polyadenylation-dependent function of specific RNA species, with LINC03121 and several signalling-related mRNAs emerging as candidates for future validation. This work provides a mechanistic bridge from cordycepin’s pharmacokinetics and metabolic activation to its effects on mRNA processing and growth-factor signalling, while nominating RNA targets for future experimental validation and translational exploitation. It remains possible that the RToC has direct effects on additional pathways; the full mechanism of action will only become clear with further experimental work.

Bibliography

- [1] Jong Bong Lee, Masar Radhi, Elena Cipolla, Raj D Gandhi, Sarir Sarmad, Atheer Zgair, Tae Hwan Kim, Wanshan Feng, Chaolong Qin, Cecilia Adrower, Catherine A Ortori, David A Barrett, Leonid Kagan, Peter M Fischer, Cornelia H de Moor, and Pavel Gershkovich. A novel nucleoside rescue metabolic pathway may be responsible for therapeutic effect of orally administered cordycepin. *Sci. Rep.*, 9(1):15760, October 2019. ISSN 2045-2322. doi: 10.1038/s41598-019-52254-x. URL <http://dx.doi.org/10.1038/s41598-019-52254-x>.
- [2] Michaela Serpi, Valentina Ferrari, Christopher McGuigan, Essam Ghazaly, and Chris Pepper. Synthesis and characterization of NUC-7738, an aryloxy phosphoramidate of 3'-deoxyadenosine, as a potential anticancer agent. *J. Med. Chem.*, 65(23):15789–15804, December 2022. ISSN 1520-4804. doi: 10.1021/acs.jmedchem.2c01348. URL <http://dx.doi.org/10.1021/acs.jmedchem.2c01348>. Publisher: American Chemical Society (ACS).
- [3] Roland H Friedel, Caroline C Friedel, Thomas Bonfert, Ruijin Shi, Roland Rad, and Philippe Soriano. Clonal expansion analysis of transposon insertions by high-throughput sequencing identifies candidate cancer genes in a PiggyBac mutagenesis screen. *PLoS One*, 8(8):e72338, August 2013. ISSN 1932-6203. doi: 10.1371/journal.pone.0072338. URL <http://dx.doi.org/10.1371/journal.pone.0072338>.
- [4] Showkat A Dar, Sulochan Malla, Cedric Belair, and Manolis Maragkakis. Differential poly(A) tail length analysis using nanopore sequencing. *Methods Mol. Biol.*, 2723:267–283, 2024. ISSN 1940-6029. doi: 10.1007/978-1-0716-3481-3_16. URL http://dx.doi.org/10.1007/978-1-0716-3481-3_16. Publisher: Methods Mol Biol.
- [5] Shenglan Qi, Huida Guan, Yongli Wang, Qinqin Fang, Xuemei Cheng, Ping Liu, Hai Wei, Wei Liu, and Changhong Wang. Simultaneous determination of cordycepin and its metabolite 3'-deoxyinosine in rat whole blood by ultra-high-performance liquid chromatography coupled with Q Exactive hybrid quadrupole orbitrap high-resolution accurate mass spectrometry and its application to accurate pharmacokinetic studies. *J. Sep. Sci.*, November 2022. ISSN 1615-9306. doi: 10.1002/jssc.202200602. URL <http://dx.doi.org/10.1002/jssc.202200602>.
- [6] Jinming Zhang, Ziling Yang, Zhuo Zhao, and Nan Zhang. Structural and pharmacological insights into cordycepin for neoplasms and metabolic disorders. *Frontiers in Pharmacology*, 15:1367820, June 2024. ISSN 1663-9812. doi: 10.3389/fphar.2024.1367820. URL <https://www.ncbi.nlm.nih.gov/pmc/articles/PMC11215060/>.
- [7] Guang Liu, Xue Wang, Yufang Liu, Meili Zhang, Tao Cai, Zhirong Shen, Yuyan Jia, and Yue Huang. Arrayed mutant haploid embryonic stem cell libraries

- facilitate phenotype-driven genetic screens. *Nucleic Acids Res.*, 45(22):e180, December 2017. ISSN 0305-1048. doi: 10.1093/nar/gkx857. URL <http://dx.doi.org/10.1093/nar/gkx857>.
- [8] Seahorse XF Cell Mito Stress Test Kit User Guide.
- [9] Benjamin Paradis and Agilent Technologies. Report Generator User Guide - Agilent Seahorse XF Cell Mito Stress Test.
- [10] Ajit S. Divakaruni and Martin Jastroch. A practical guide for the analysis, standardization and interpretation of oxygen consumption measurements. *Nature Metabolism*, 4(8):978–994, August 2022. ISSN 2522-5812. doi: 10.1038/s42255-022-00619-4. URL <https://www.nature.com/articles/s42255-022-00619-4>. Publisher: Nature Publishing Group.
- [11] Bruno D. Fonseca, Chadi Zakaria, Jian-Jun Jia, Tyson E. Graber, Yuri Svitkin, Soroush Tahmasebi, Danielle Healy, Huy-Dung Hoang, Jacob M. Jensen, Ilo T. Diao, Alexandre Lussier, Christopher Dajadian, Niranjana Padmanabhan, Walter Wang, Edna Matta-Camacho, Jaclyn Hearnden, Ewan M. Smith, Yoshinori Tsukumo, Akiko Yanagiya, Masahiro Morita, Emmanuel Petroulakis, Jose L. González, Greco Hernández, Tommy Alain, and Christian K. Damgaard. Lar-related Protein 1 (LARP1) Represses Terminal Oligopyrimidine (TOP) mRNA Translation Downstream of mTOR Complex 1 (mTORC1). *The Journal of Biological Chemistry*, 290(26):15996–16020, June 2015. ISSN 1083-351X. doi: 10.1074/jbc.M114.621730.
- [12] Koichi Ogami, Yuka Oishi, Kentaro Sakamoto, Mayu Okumura, Ryota Yamagishi, Takumi Inoue, Masaya Hibino, Takuto Nogimori, Natsumi Yamaguchi, Kazuya Furutachi, Nao Hosoda, Hiroto Inagaki, and Shin-ichi Hoshino. mTOR- and LARP1-dependent regulation of TOP mRNA poly(A) tail and ribosome loading. *Cell Reports*, 41(4), October 2022. ISSN 2211-1247. doi: 10.1016/j.celrep.2022.111548. URL [https://www.cell.com/cell-reports/abstract/S2211-1247\(22\)01404-8](https://www.cell.com/cell-reports/abstract/S2211-1247(22)01404-8). Publisher: Elsevier.
- [13] Masar Radhi, Sadaf Ashraf, Steven Lawrence, Asta Arendt Tranholm, Peter Arthur David Wellham, Abdul Hafeez, Ammar Sabah Khamis, Robert Thomas, Daniel McWilliams, and Cornelia Huiberdina de Moor. A systematic review of the biological effects of cordycepin. *Molecules*, 26(19):5886, September 2021. ISSN 1420-3049. doi: 10.3390/molecules26195886. URL <https://www.mdpi.com/1420-3049/26/19/5886>. Publisher: MDPI AG.
- [14] H. Klenow. FORMATION OF THE MONO-, DI- AND TRIPHOSPHATE OF CORDYCEPIN IN EHRlich ASCITES-TUMOR CELLS IN VITRO. *Biochimica Et Biophysica Acta*, 76:347–353, November 1963. ISSN 0006-3002.
- [15] K M Rose, L E Bell, and S T Jacob. Specific inhibition of chromatin-associated poly(A) synthesis in vitro by cordycepin 5'-triphosphate. *Nature*, 267(5607):178–180, May 1977. ISSN 0028-0836. doi: 10.1038/267178a0. URL <http://dx.doi.org/10.1038/267178a0>.
- [16] S Penman, M Rosbash, and M Penman. Messenger and heterogeneous nuclear RNA in HeLa cells: differential inhibition by cordycepin. *Proc. Natl. Acad. Sci. U. S. A.*,

- 67(4):1878–1885, December 1970. ISSN 0027-8424. doi: 10.1073/pnas.67.4.1878. URL <http://dx.doi.org/10.1073/pnas.67.4.1878>.
- [17] Peter G W Plagemann and Robert M Wohlhueter. 5'-Deoxyadenosine metabolism in various mammalian cell lines. *Biochem. Pharmacol.*, 32(8):1433–1440, April 1983. ISSN 0006-2952. doi: 10.1016/0006-2952(83)90458-6. URL [http://dx.doi.org/10.1016/0006-2952\(83\)90458-6](http://dx.doi.org/10.1016/0006-2952(83)90458-6).
 - [18] B. Grahm and H. Lovtrup-Rein. The Effect of Cordycepin on Nuclear RNA Synthesis in Nerve and Glial Cells. *Acta Physiologica Scandinavica*, 82(1):28–34, 1971. ISSN 1365-201X. doi: 10.1111/j.1748-1716.1971.tb04939.x. URL <https://onlinelibrary.wiley.com/doi/abs/10.1111/j.1748-1716.1971.tb04939.x>. _eprint: <https://onlinelibrary.wiley.com/doi/pdf/10.1111/j.1748-1716.1971.tb04939.x>.
 - [19] Alexander Kondrashov, Hedda A Meijer, Adeline Barthet-Barateig, Hannah N Parker, Asma Khurshid, Sarah Tessier, Marie Sicard, Alan J Knox, Linhua Pang, and Cornelia H De Moor. Inhibition of polyadenylation reduces inflammatory gene induction. *RNA*, 18(12):2236–2250, December 2012. ISSN 1355-8382. doi: 10.1261/rna.032391.112. URL <http://dx.doi.org/10.1261/rna.032391.112>. Publisher: Cold Spring Harbor Laboratory.
 - [20] Ina Hollerer, Kerstin Grund, Matthias W. Hentze, and Andreas E. Kulozik. mRNA 3'end processing: A tale of the tail reaches the clinic. *EMBO molecular medicine*, 6(1):16–26, January 2014. ISSN 1757-4684. doi: 10.1002/emmm.201303300.
 - [21] Junjie Liu, Xubing Lu, Siyu Zhang, Ling Yuan, and Yadong Sun. Molecular Insights into mRNA Polyadenylation and Deadenylation. *International Journal of Molecular Sciences*, 23(19):10985, January 2022. ISSN 1422-0067. doi: 10.3390/ijms231910985. URL <https://www.mdpi.com/1422-0067/23/19/10985>. Publisher: Multidisciplinary Digital Publishing Institute.
 - [22] Jing Zhao, Linda Hyman, and Claire Moore. Formation of mRNA 3 Ends in Eukaryotes: Mechanism, Regulation, and Interrelationships with Other Steps in mRNA Synthesis. *Microbiology and Molecular Biology Reviews*, June 1999. doi: 10.1128/mmbr.63.2.405-445.1999. URL <https://journals.asm.org/doi/10.1128/mmbr.63.2.405-445.1999>. Publisher: American Society for Microbiology.
 - [23] L. C. Ryner and J. L. Manley. Requirements for accurate and efficient mRNA 3' end cleavage and polyadenylation of a simian virus 40 early pre-RNA in vitro. *Molecular and Cellular Biology*, 7(1):495–503, January 1987. ISSN 0270-7306. doi: 10.1128/mcb.7.1.495-503.1987.
 - [24] D Zarkower and M Wickens. Formation of mRNA 3' termini: stability and dissociation of a complex involving the AAUAAA sequence. *The EMBO Journal*, 6(1):177–186, January 1987. ISSN 0261-4189. doi: 10.1002/j.1460-2075.1987.tb04736.x. URL <https://pmc.ncbi.nlm.nih.gov/articles/PMC553374/>.
 - [25] Steven Lawrence, Jialiang Lin, Asma Khurshid, Wahyu Utami, Richa Singhania, Sadaf Ashraf, Graeme J Thorn, Irengbam Rocky Mangangcha, Keith Spriggs, Dong-Hyun Kim, David Barrett, and Cornelia H de Moor. Cordycepin generally inhibits growth factor signal transduction in a systems pharmacology study. *FEBS Lett.*, 599(3):415–435, February 2025. ISSN 1873-3468. doi: 10.1002/1873-3468.15046. URL <http://dx.doi.org/10.1002/1873-3468.15046>. Publisher: FEBS Lett.

- [26] Masar Radhi. *Polyadenylation as a target for anti-inflammatory drugs*. Ph.D. Thesis, University of Nottingham, April 2019. Publication Title: School of Pharmacy Volume: Phd.
- [27] Steven Lawrence. *A POTENTIAL EFFECT OF CORDYCEPIN ON THE FEEDBACK BETWEEN POLYADENYLATION AND PI3K/AKT/MTOR SIGNALING*. PhD thesis, University of Nottingham.
- [28] Sadaf Ashraf, Masar Radhi, Peter Gowler, James J Burston, Raj D Gandhi, Graeme J Thorn, Anna M Piccinini, David A Walsh, Victoria Chapman, and Cornelia H de Moor. The polyadenylation inhibitor cordycepin reduces pain, inflammation and joint pathology in rodent models of osteoarthritis. *Sci. Rep.*, 9(1):4696, March 2019. ISSN 2045-2322. doi: 10.1038/s41598-019-41140-1. URL <http://dx.doi.org/10.1038/s41598-019-41140-1>.
- [29] Hagen Schwenzer, Erica De Zan, Mustafa Elshani, Ruud van Stiphout, Mary Kudsy, Josephine Morris, Valentina Ferrari, In Hwa Um, James Chettle, Farasat Kazmi, Leticia Campo, Alistair Easton, Sebastian Nijman, Michaela Serpi, Stefan Symeonides, Ruth Plummer, David J Harrison, Gareth Bond, and Sarah P Blagden. The Novel Nucleoside Analogue ProTide NUC-7738 Overcomes Cancer Resistance Mechanisms In Vitro and in a First-In-Human Phase I Clinical Trial. *Clin. Cancer Res.*, 27(23):6500–6513, December 2021. ISSN 1078-0432. doi: 10.1158/1078-0432.CCR-21-1652. URL <http://dx.doi.org/10.1158/1078-0432.CCR-21-1652>.
- [30] JaeYoul Cho, HyungJun Noh, JuYoung Yoon, JiHye Kim, Kwang-Soo Baek, GeumSoog Kim, SeungEun Lee, DaeYoung Lee, JeHun Choi, SeungYu Kim, HyunBong Park, Gi-Ho Sung, and KangRo Lee. A direct protein kinase B-targeted anti inflammatory activity of cordycepin from artificially cultured fruit body of *Cordyceps militaris*. *Pharmacognosy Magazine*, 11(43):477, 2015. ISSN 0973-1296. doi: 10.4103/0973-1296.160454. URL <https://phcog.com/article/view/2015/11/43/477-485>.
- [31] Peng Qin, XiangKai Li, Hui Yang, Zhi-Ye Wang, and DengXue Lu. Therapeutic Potential and Biological Applications of Cordycepin and Metabolic Mechanisms in Cordycepin-Producing Fungi. *Molecules*, 24(12):2231, January 2019. ISSN 1420-3049. doi: 10.3390/molecules24122231. URL <https://www.mdpi.com/1420-3049/24/12/2231>. Publisher: Multidisciplinary Digital Publishing Institute.
- [32] Min Chen, Jiahao Luo, Wenming Jiang, Lijing Chen, Longxing Miao, and Chun-chao Han. Cordycepin: A review of strategies to improve the bioavailability and efficacy. *Phytotherapy research: PTR*, 37(9):3839–3858, September 2023. ISSN 1099-1573. doi: 10.1002/ptr.7921.
- [33] Yung-Jen Tsai, Lie-Chwen Lin, and Tung-Hu Tsai. Pharmacokinetics of adenosine and cordycepin, a bioactive constituent of *Cordyceps sinensis* in rat. *J. Agric. Food Chem.*, 58(8):4638–4643, April 2010. ISSN 0021-8561. doi: 10.1021/jf100269g. URL <http://dx.doi.org/10.1021/jf100269g>.
- [34] Luciana Dalla Rosa, Aleksandro S. Da Silva, Lucas T. Gressler, Camila B. Oliveira, Maria G. C. Dambrós, Luiz C. Miletto, Raqueli T. França, Sonia T. A. Lopes, Yasmin N. Samara, Marcelo L. Da Veiga, and Silvia G. Monteiro. Cordycepin (3-deoxyadenosine) pentostatin (deoxycoformycin) combination treatment of mice

- experimentally infected with *Trypanosoma evansi*. *Parasitology*, 140(5):663–671, April 2013. ISSN 0031-1820, 1469-8161. doi: 10.1017/S0031182012001990. URL https://www.cambridge.org/core/journals/parasitology/article/abs/cordycepin-3deoxyadenosine-pentostatin-deoxycoformycin-combination-treatment-of-mice-exper/CE22CC6932AFBE3F900FC476BC6E8BD8?utm_source=chatgpt.com.
- [35] L. E. Rodman, D. R. Farnell, J. M. Coyne, P. W. Allan, D. L. Hill, K. L. Duncan, J. E. Tomaszewski, A. C. Smith, and J. G. Page. Toxicity of cordycepin in combination with the adenosine deaminase inhibitor 2'-deoxycoformycin in beagle dogs. *Toxicology and Applied Pharmacology*, 147(1):39–45, November 1997. ISSN 0041-008X. doi: 10.1006/taap.1997.8264.
- [36] Lori A. Passmore and Jeff Collier. Roles of mRNA poly(A) tails in regulation of eukaryotic gene expression. *Nature Reviews Molecular Cell Biology*, 23(2): 93–106, February 2022. ISSN 1471-0080. doi: 10.1038/s41580-021-00417-y. URL <https://www.nature.com/articles/s41580-021-00417-y>. Publisher: Nature Publishing Group.
- [37] Anders Virtanen, Niklas Henriksson, Per Nilsson, and Mikael Nissbeck. Poly(A)-specific ribonuclease (PARN): An allosterically regulated, processive and mRNA cap-interacting deadenylase. *Critical Reviews in Biochemistry and Molecular Biology*, 48(2):192–209, March 2013. ISSN 1040-9238. doi: 10.3109/10409238.2013.771132. URL <https://doi.org/10.3109/10409238.2013.771132>. Publisher: Taylor & Francis _eprint: <https://doi.org/10.3109/10409238.2013.771132>.
- [38] Rachael Emily Turner, Paul F Harrison, Angavai Swaminathan, Calvin A Kraupner-Taylor, Belinda J Goldie, Michael See, Amanda L Peterson, Ralf B Schittenhelm, David R Powell, Darren J Creek, Bernhard Dichtl, and Traude H Beilharz. Genetic and pharmacological evidence for kinetic competition between alternative poly(A) sites in yeast. *eLife*, 10:e65331, July 2021. ISSN 2050-084X. doi: 10.7554/eLife.65331. URL <https://doi.org/10.7554/eLife.65331>. Publisher: eLife Sciences Publications, Ltd.
- [39] Amanda Charlesworth, Hedda A Meijer, and Cornelia H de Moor. Specificity factors in cytoplasmic polyadenylation: Factors in cytoplasmic polyadenylation. *Wiley Interdiscip. Rev. RNA*, 4(4):437–461, July 2013. ISSN 1757-7004. doi: 10.1002/wrna.1171. URL <http://dx.doi.org/10.1002/wrna.1171>.
- [40] Kehui Xiang, Jimmy Ly, and David P. Bartel. Control of poly(A)-tail length and translation in vertebrate oocytes and early embryos. *Developmental Cell*, 59(8): 1058–1074.e11, April 2024. ISSN 15345807. doi: 10.1016/j.devcel.2024.02.007. URL <https://linkinghub.elsevier.com/retrieve/pii/S1534580724001059>.
- [41] Alexander O. Subtelny, Stephen W. Eichhorn, Grace R. Chen, Hazel Sive, and David P. Bartel. Poly(A)-tail profiling reveals an embryonic switch in translational control. *Nature*, 508(7494):66–71, April 2014. ISSN 1476-4687. doi: 10.1038/nature13007. URL <https://www.nature.com/articles/nature13007>. Publisher: Nature Publishing Group.
- [42] Helois E. Radford, Hedda A. Meijer, and Cornelia H. de Moor. Translational control by cytoplasmic polyadenylation in *Xenopus* oocytes. *Biochimica et Biophysica Acta (BBA) - Gene Regulatory Mechanisms*, 1779(4):217–

- 229, April 2008. ISSN 1874-9399. doi: 10.1016/j.bbagra.2008.02.002. URL <https://www.sciencedirect.com/science/article/pii/S1874939908000436>.
- [43] Yi-Shui Huang, Raul Mendez, Mercedes Fernandez, and Joel D. Richter. CPEB and translational control by cytoplasmic polyadenylation: impact on synaptic plasticity, learning, and memory. *Molecular Psychiatry*, 28(7):2728–2736, July 2023. ISSN 1476-5578. doi: 10.1038/s41380-023-02088-x. URL <https://www.nature.com/articles/s41380-023-02088-x>. Publisher: Nature Publishing Group.
- [44] Chyi-Ying A. Chen and Ann-Bin Shyu. Mechanisms of deadenylation-dependent decay. *Wiley interdisciplinary reviews. RNA*, 2(2):167–183, 2011. ISSN 1757-7004. doi: 10.1002/wrna.40. URL <https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3624885/>.
- [45] Sandra Holbein, Agnieszka Wengi, Laurence Decourty, Florian M Freimoser, Alain Jacquier, and Bernhard Dichtl. Cordycepin interferes with 3' end formation in yeast independently of its potential to terminate RNA chain elongation. *RNA*, 15(5):837–849, May 2009. ISSN 1355-8382. doi: 10.1261/rna.1458909. URL <http://dx.doi.org/10.1261/rna.1458909>.
- [46] Ying Ying Wong, Alice Moon, Ruth Duffin, Adeline Barthet-Barateig, Hedda A Meijer, Michael J Clemens, and Cornelia H de Moor. Cordycepin inhibits protein synthesis and cell adhesion through effects on signal transduction. *Journal of Biological Chemistry*, 285(4):2610–2621, January 2010. ISSN 1083-351X. doi: 10.1074/jbc.M109.071159. URL <http://dx.doi.org/10.1074/jbc.M109.071159>. tex.journaltitle: J. Biol. Chem.
- [47] Werner E. G. Müller, Gerhard Seibert, Rudolf Beyer, Hans J. Breter, Armin Maidhof, and Rudolf K. Zahn. Effect of Cordycepin on Nucleic Acid Metabolism in L5178Y Cells and on Nucleic Acid-synthesizing Enzyme Systems1. *Cancer Research*, 37(10):3824–3833, October 1977. ISSN 0008-5472.
- [48] Ning Li, Shaotao Jiang, Rongdang Fu, Jin Lv, Jiyao Yao, Jialuo Mai, Xuefeng Hua, Huan Chen, Jie Liu, and Minqiang Lu. Cleavage and Polyadenylation-Specific Factor 3 Induces Cell Cycle Arrest Via Pi3K/Akt/Gsk-3 β Signaling Pathways and Predicts A Negative Prognosis in Hepatocellular Carcinoma. *Biomarkers in Medicine*, 15(5):347–358, April 2021. ISSN 1752-0363. doi: 10.2217/bmm-2021-0039. URL <https://doi.org/10.2217/bmm-2021-0039>. Publisher: Taylor & Francis _eprint: <https://doi.org/10.2217/bmm-2021-0039>.
- [49] Weishao An and Fang Yu. Silencing of CPSF7 inhibits the proliferation, migration, and invasion of lung adenocarcinoma cells by blocking the AKT/mTOR signaling pathway. *Open Medicine*, 17(1):1655–1663, January 2022. ISSN 2391-5463. doi: 10.1515/med-2022-0570. URL <https://www.degruyterbrill.com/document/doi/10.1515/med-2022-0570/html>. Publisher: De Gruyter Open Access.
- [50] Veronica Ruta, Vittoria Pagliarini, and Claudio Sette. Coordination of RNA Processing Regulation by Signal Transduction Pathways. *Biomolecules*, 11(10):1475, October 2021. ISSN 2218-273X. doi: 10.3390/biom11101475. URL <https://www.mdpi.com/2218-273X/11/10/1475>. Publisher: Multidisciplinary Digital Publishing Institute.

- [51] Wangbing Chen, Wei Guo, Mei Li, Dingbo Shi, Yun Tian, Zhenlin Li, Jingshu Wang, Lingyi Fu, Xiangsheng Xiao, Quentin Qiang Liu, Shusen Wang, Wenlin Huang, and Wuguo Deng. Upregulation of Cleavage and Polyadenylation Specific Factor 4 in Lung Adenocarcinoma and Its Critical Role for Cancer Cell Survival and Proliferation. *PLoS ONE*, 8(12):e82728, December 2013. ISSN 1932-6203. doi: 10.1371/journal.pone.0082728. URL <https://dx.plos.org/10.1371/journal.pone.0082728>.
- [52] Yung Hyun Choi, Gi-Young Kim, and Hye Hyeon Lee. Anti-inflammatory effects of cordycepin in lipopolysaccharide-stimulated RAW 264.7 macrophages through Toll-like receptor 4-mediated suppression of mitogen-activated protein kinases and NF- κ B signaling pathways. *Drug Design, Development and Therapy*, 8: 1941–1953, October 2014. ISSN 1177-8881. doi: 10.2147/DDDT.S71957. URL <https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4206205/>.
- [53] Jin-Woo Jeong, Cheng-Yun Jin, Gi-Young Kim, Jae-Dong Lee, Cheol Park, Gun-Do Kim, Wun-Jae Kim, Won-Kyo Jung, Su Kil Seo, Il-Whan Choi, and Yung Hyun Choi. Anti-inflammatory effects of cordycepin via suppression of inflammatory mediators in BV2 microglial cells. *International Immunopharmacology*, 10(12): 1580–1586, December 2010. ISSN 1567-5769. doi: 10.1016/j.intimp.2010.09.011. URL <https://www.sciencedirect.com/science/article/pii/S1567576910002924>.
- [54] Ho Gyoung Kim, Bhushan Shrestha, So Yeon Lim, Deok Hyo Yoon, Woo Chul Chang, Dong-Jik Shin, Sang Kuk Han, Sang Min Park, Jung Hee Park, Hae Il Park, Jae-Mo Sung, Yangsoo Jang, Namsik Chung, Ki-Chul Hwang, and Tae Woong Kim. Cordycepin inhibits lipopolysaccharide-induced inflammation by the suppression of NF-kappaB through Akt and p38 inhibition in RAW 264.7 macrophage cells. *European Journal of Pharmacology*, 545(2-3):192–199, September 2006. ISSN 0014-2999. doi: 10.1016/j.ejphar.2006.06.047.
- [55] Bo-Syong Pan, Yang-Kao Wang, Meng-Shao Lai, Yi-Fen Mu, and Bu-Miin Huang. Cordycepin induced MA-10 mouse Leydig tumor cell apoptosis by regulating p38 MAPKs and PI3K/AKT signaling pathways. *Scientific Reports*, 5(1):13372, August 2015. ISSN 2045-2322. doi: 10.1038/srep13372. URL <https://www.nature.com/articles/srep13372>. Publisher: Nature Publishing Group.
- [56] Md Asaduzzaman Khan and Mousumi Tania. Cordycepin and kinase inhibition in cancer. *Drug Discovery Today*, 28(3):103481, March 2023. ISSN 1359-6446. doi: 10.1016/j.drudis.2022.103481. URL <https://www.sciencedirect.com/science/article/pii/S1359644622004743>.
- [57] Lu Tan, Xiaominting Song, Yali Ren, Miao Wang, Chuanjie Guo, Dale Guo, Yucheng Gu, Yuzhi Li, Zhixing Cao, and Yun Deng. Anti-inflammatory effects of cordycepin: A review. *Phytother. Res.*, October 2020. ISSN 0951-418X. doi: 10.1002/ptr.6890. URL <http://dx.doi.org/10.1002/ptr.6890>.
- [58] Linhai Wang, Shu Tian, Sisi Ruan, Jingjing Wei, Sijia Wei, Weiwei Chen, Hangcui Hu, Weiwei Qin, Yan Li, Hang Yuan, Jian Mao, Yan Xu, and Jianping Xie. Neuroprotective effects of cordycepin on MPTP-induced Parkinson’s disease mice via suppressing PI3K/AKT/mTOR and MAPK-mediated neuroinflammation. *Free Radical Biology and Medicine*, 216:60–77, April 2024. ISSN 0891-5849.

doi: 10.1016/j.freeradbiomed.2024.02.023. URL <https://www.sciencedirect.com/science/article/pii/S089158492400114X>.

- [59] Antonino Glaviano, Aaron S. C. Foo, Hiu Y. Lam, Kenneth C. H. Yap, William Jacot, Robert H. Jones, Huiyan Eng, Madhumathy G. Nair, Pooyan Makvandi, Birgit Geoerger, Matthew H. Kulke, Richard D. Baird, Jyothi S. Prabhu, Daniela Carbone, Camilla Pecoraro, Daniel B. L. Teh, Gautam Sethi, Vincenzo Cavalieri, Kevin H. Lin, Nathalie R. Javidi-Sharifi, Eneda Toska, Matthew S. Davids, Jennifer R. Brown, Patrizia Diana, Justin Stebbing, David A. Fruman, and Alan P. Kumar. PI3K/AKT/mTOR signaling transduction pathway and targeted therapies in cancer. *Molecular Cancer*, 22(1):138, August 2023. ISSN 1476-4598. doi: 10.1186/s12943-023-01827-6. URL <https://doi.org/10.1186/s12943-023-01827-6>.
- [60] Alex Toker and Sandra Marmiroli. Signaling specificity in the Akt pathway in biology and disease. *Advances in Biological Regulation*, 55:28–38, May 2014. ISSN 2212-4926. doi: 10.1016/j.jbior.2014.04.001. URL <https://www.sciencedirect.com/science/article/pii/S2212492614000037>.
- [61] Robert A Saxton and David M Sabatini. mTOR signaling in growth, metabolism, and disease. *Cell*, 168(6):960–976, March 2017. ISSN 0092-8674. doi: 10.1016/j.cell.2017.02.004. URL <http://dx.doi.org/10.1016/j.cell.2017.02.004>.
- [62] D. Grahame Hardie, Fiona A. Ross, and Simon A. Hawley. AMPK: a nutrient and energy sensor that maintains energy homeostasis. *Nature Reviews Molecular Cell Biology*, 13(4):251–262, April 2012. ISSN 1471-0080. doi: 10.1038/nrm3311. URL <https://www.nature.com/articles/nrm3311>. Publisher: Nature Publishing Group.
- [63] Sheng-Cai Lin and D. Grahame Hardie. AMPK: Sensing Glucose as well as Cellular Energy Status. *Cell Metabolism*, 27(2):299–313, February 2018. ISSN 1550-4131. doi: 10.1016/j.cmet.2017.10.009. URL <https://www.sciencedirect.com/science/article/pii/S1550413117306228>.
- [64] Asma Khurshid. *The effect of the polyadenylation inhibitor Cordycepin on MCF-7 cells*. Ph.D. Thesis, University of Nottingham, July 2015. Publication Title: School of Pharmacy Volume: Phd.
- [65] Shuhei Takahashi, Minori Tamai, Shotaro Nakajima, Hironori Kato, Hisashi Johno, Tomoyuki Nakamura, and Masanori Kitamura. Blockade of adipocyte differentiation by cordycepin. *British Journal of Pharmacology*, 167(3): 561–575, 2012. ISSN 1476-5381. doi: 10.1111/j.1476-5381.2012.02005.x. URL <https://onlinelibrary.wiley.com/doi/abs/10.1111/j.1476-5381.2012.02005.x>. _eprint: <https://bpspubs.onlinelibrary.wiley.com/doi/pdf/10.1111/j.1476-5381.2012.02005.x>.
- [66] Brent H. Cochran, John Zullo, Inder M. Verma, and Charles D. Stiles. Expression of the c-fos Gene and of an fos-Related Gene Is Stimulated by Platelet-Derived Growth Factor. *Science*, 226(4678):1080–1082, November 1984. doi: 10.1126/science.6093261. URL <https://www.science.org/doi/10.1126/science.6093261>. Publisher: American Association for the Advancement of Science.
- [67] Rolf Müller, Rodrigo Bravo, Jean Burckhardt, and Tom Curran. Induction of c-fos gene and protein by growth factors precedes activation of c-myc. *Nature*,

- 312(5996):716–720, December 1984. ISSN 1476-4687. doi: 10.1038/312716a0. URL <https://www.nature.com/articles/312716a0>. Publisher: Nature Publishing Group.
- [68] M E Greenberg, A L Hermanowski, and E B Ziff. Effect of protein synthesis inhibitors on growth factor activation of c-fos, c-myc, and actin gene transcription. *Molecular and Cellular Biology*, 6(4):1050–1057, April 1986. ISSN 0270-7306. doi: 10.1128/mcb.6.4.1050. URL <https://pmc.ncbi.nlm.nih.gov/articles/PMC367614/>.
- [69] Chun-Ling Dai, Jianhua Shi, Yanxing Chen, Khalid Iqbal, Fei Liu, and Cheng-Xin Gong. Inhibition of Protein Synthesis Alters Protein Degradation through Activation of Protein Kinase B (AKT) *. *Journal of Biological Chemistry*, 288(33):23875–23883, August 2013. ISSN 0021-9258, 1083-351X. doi: 10.1074/jbc.M112.445148. URL [https://www.jbc.org/article/S0021-9258\(20\)45278-0/abstract](https://www.jbc.org/article/S0021-9258(20)45278-0/abstract). Publisher: Elsevier.
- [70] Marcelo Cossenza, Renato Socodato, Telmo A. Mejía-García, Ivan Domith, Camila C. Portugal, Luis F. H. Gladulich, Aline T. Duarte-Silva, Latika Khatri, Shannon Antoine, Franz Hofmann, Edward B. Ziff, and Roberto Paes-de Carvalho. Protein synthesis inhibition promotes nitric oxide generation and activation of CGKII-dependent downstream signaling pathways in the retina. *Biochimica et Biophysica Acta (BBA) - Molecular Cell Research*, 1867(8):118732, August 2020. ISSN 0167-4889. doi: 10.1016/j.bbamcr.2020.118732. URL <https://www.sciencedirect.com/science/article/pii/S0167488920300902>.
- [71] So Young Yoon, Soo Jung Park, and Yoon Jung Park. The Anticancer Properties of Cordycepin and Their Underlying Mechanisms. *Int. J. Mol. Sci.*, 19(10), October 2018. ISSN 1422-0067. doi: 10.3390/ijms19103027. URL <http://dx.doi.org/10.3390/ijms19103027>.
- [72] Jinxiu Wang, Yanchun Gong, Haoyuan Tan, Wenxi Li, Baiyi Yan, Chunfang Cheng, Juan Wan, Wei Sun, Chunhua Yuan, and Li-Hua Yao. Cordycepin suppresses glutamatergic and GABAergic synaptic transmission through activation of A1 adenosine receptor in rat hippocampal CA1 pyramidal neurons. *Biomedicine & Pharmacotherapy*, 145:112446, January 2022. ISSN 07533322. doi: 10.1016/j.biopha.2021.112446. URL <https://linkinghub.elsevier.com/retrieve/pii/S0753332221012324>.
- [73] Jaeyoon Kim, Jae Young Shin, Yun-Ho Choi, So Young Lee, Mu Hyun Jin, Chang Deok Kim, Nae-Gyu Kang, and Sanghwa Lee. Adenosine and Cordycepin Accelerate Tissue Remodeling Process through Adenosine Receptor Mediated Wnt/ β -Catenin Pathway Stimulation by Regulating GSK3b Activity. *Int. J. Mol. Sci.*, 22(11), May 2021. ISSN 1422-0067. doi: 10.3390/ijms22115571. URL <http://dx.doi.org/10.3390/ijms22115571>.
- [74] Hong-Li Cao, Zi-Jin Liu, and Zheng Chang. Cordycepin induces apoptosis in human bladder cancer cells via activation of A3 adenosine receptors. *Tumour Biology: The Journal of the International Society for Oncodevelopmental Biology and Medicine*, 39(7):1010428317706915, July 2017. ISSN 1423-0380. doi: 10.1177/1010428317706915.
- [75] Hogyoung Kim, Amarjit S Naura, Youssef Errami, Jihang Ju, and A Hamid Boulares. Cordycepin Blocks Lung Injury-Associated Inflammation and Promotes

- BRCA1-Deficient Breast Cancer Cell Killing by Effectively Inhibiting PARP. *Molecular Medicine*, 17(9-10):893–900, 2011. ISSN 1076-1551. doi: 10.2119/molmed.2011.00032. URL <https://pmc.ncbi.nlm.nih.gov/articles/PMC3188885/>.
- [76] Tingli Sun, Wenpeng Dong, Guohong Jiang, Jingbo Yang, Jizhang Liu, Lijie Zhao, and Peilong Ma. Cordyceps militaris Improves Chronic Kidney Disease by Affecting TLR4/NF- κ B Redox Signaling Pathway. *Oxidative Medicine and Cellular Longevity*, 2019(1):7850863, 2019. ISSN 1942-0994. doi: 10.1155/2019/7850863. URL <https://onlinelibrary.wiley.com/doi/abs/10.1155/2019/7850863>. _eprint: <https://onlinelibrary.wiley.com/doi/pdf/10.1155/2019/7850863>.
- [77] Su Jin Jung, Ji Hyun Hwang, Mi Ra Oh, and Soo Wan Chae. Effects of Cordyceps militaris supplementation on the immune response and upper respiratory infection in healthy adults: a randomized, double-blind, placebo-controlled study. *Journal of Nutrition and Health*, 52(3):258–267, June 2019. doi: 10.4163/jnh.2019.52.3.258. URL <https://synapse.koreamed.org/articles/1128185>. Publisher: The Korean Nutrition Society.
- [78] Su-Jin Jung, Eun-Soo Jung, Eun-Kyung Choi, Hong-Sig Sin, Ki-Chan Ha, and Soo-Wan Chae. Immunomodulatory effects of a mycelium extract of Cordyceps (Paecilomyces hepiali; CBG-CS-2): a randomized and double-blind clinical trial. *BMC Complementary and Alternative Medicine*, 19(1):77, March 2019. ISSN 1472-6882. doi: 10.1186/s12906-019-2483-y. URL <https://doi.org/10.1186/s12906-019-2483-y>.
- [79] Atcharaporn Ontawong, Sirinat Pengnet, Arthid Thim-Uam, Narongsuk Munkong, Nukrob Narkprasom, Kanjana Narkprasom, Kullanat Kuntakhut, Natakorn Kamkeaw, and Doungporn Amornlerdpison. A randomized controlled clinical trial examining the effects of Cordyceps militaris beverage on the immune response in healthy adults. *Scientific Reports*, 14(1):7994, April 2024. ISSN 2045-2322. doi: 10.1038/s41598-024-58742-z. URL <https://www.nature.com/articles/s41598-024-58742-z>. Publisher: Nature Publishing Group.
- [80] Francine M. Foss MD. Combination Therapy With Purine Nucleoside Analogs | CancerNetwork. October 2025. URL <https://www.cancernetwork.com/view/combination-therapy-purine-nucleoside-analogs>.
- [81] S. N. Symeonides, A. Skolariki, N. R. M. Haris, Z. Boh, M. Myers, E. M. Oelmann, J. D. Bloss, R. Plummer, and S. P. Blagden. 455MO NUC-7738 in patients with advanced solid tumours: Phase I results from the NuTide:701 phase I/II study. *Annals of Oncology*, 33:S745–S746, September 2022. ISSN 0923-7534, 1569-8041. doi: 10.1016/j.annonc.2022.07.584. URL [https://www.annalsofoncology.org/article/S0923-7534\(22\)02435-8/fulltext](https://www.annalsofoncology.org/article/S0923-7534(22)02435-8/fulltext). Publisher: Elsevier.
- [82] S. Blagden, M. Elshani, S. N. Symeonides, A. Skolariki, N. R. Haris, Z. Y. Boh, A. Dickson, I. H. Um, Y. Zhang, F. G. McKissock, E. Oelmann, J. Bloss, N. Cook, T. R. J. Evans, R. Plummer, and D. J. Harrison. 666P Final results of phase I/II study of NUC-7738 as monotherapy and in combination with pembrolizumab: Anti-tumor immune response in PD-1 inhibitor-resistant patients. *Annals of Oncology*, 35:S523–S524, September 2024. ISSN 0923-7534, 1569-8041. doi: 10.1016/j.annonc.2024.08.732. URL [https://www.annalsofoncology.org/article/S0923-7534\(24\)02251-8/fulltext](https://www.annalsofoncology.org/article/S0923-7534(24)02251-8/fulltext). Publisher: Elsevier.

- [83] Dahae Lee, Won-Yung Lee, Kiwon Jung, Yong Sam Kwon, Daeyoung Kim, Gwi Seo Hwang, Chang-Eop Kim, Sullim Lee, and Ki Sung Kang. The Inhibitory Effect of Cordycepin on the Proliferation of MCF-7 Breast Cancer Cells, and its Mechanism: An Investigation Using Network Pharmacology-Based Analysis. *Biomolecules*, 9(9), August 2019. ISSN 2218-273X. doi: 10.3390/biom9090414. URL <http://dx.doi.org/10.3390/biom9090414>.
- [84] Shang-Ze Li, Jian-Wei Ren, Jing Fei, Xiao-Dong Zhang, and Run-Lei Du. Cordycepin induces Bax-dependent apoptosis in colorectal cancer cells. *Molecular Medicine Reports*, 19(2):901–908, February 2019. ISSN 1791-3004. doi: 10.3892/mmr.2018.9717.
- [85] Jung Hoo Hwang, Soo Jung Park, Won Gyu Ko, Seong-Mun Kang, Da Bin Lee, Junho Bang, Byung-Joo Park, Chung-Beum Wee, Dae Joon Kim, Ik-Soon Jang, and Jae-Hong Ko. Cordycepin induces human lung cancer cell apoptosis by inhibiting nitric oxide mediated ERK/Slug signaling pathway. *American Journal of Cancer Research*, 7(3):417–432, March 2017. ISSN 2156-6976. URL <https://pmc.ncbi.nlm.nih.gov/articles/PMC5385633/>.
- [86] Jin-Woo Jeong, Cheng-Yun Jin, Cheol Park, Min Ho Han, Gi-Young Kim, Sung-Kwon Moon, Chan Gil Kim, Yong Kee Jeong, Wun-Jae Kim, Jae-Dong Lee, and Yung Hyun Choi. Inhibition of migration and invasion of LNCaP human prostate carcinoma cells by cordycepin through inactivation of Akt. *International Journal of Oncology*, 40(5):1697–1704, May 2012. ISSN 1019-6439. doi: 10.3892/ijo.2012.1332. URL <https://www.spandidos-publications.com/ijo>. Publisher: Spandidos Publications.
- [87] Thunwa Binlath, Nawapol Uppatcha, Jutamast Thepchai, Yanaphat Pleungtuk, Parinya Noisa, Pilaiwanwadee Hutamekalin, and Paiboon Jitprasertwong. Cordycepin attenuates migration and invasion of HSC-4 oral squamous carcinoma cells through autophagy-dependent FAK/Akt and MMP2/MMP9 suppression. *Journal of Dental Sciences*, 17(4):1677–1688, October 2022. ISSN 2213-8862. doi: 10.1016/j.jds.2022.03.002.
- [88] Jiahui Xiong, Yongping Lai, Niangmei Cheng, Jizhe Liu, Fei Wang, Xiaoyuan Zheng, Yingchao Wang, Qiuyu Zhuang, Yantin Lin, Jingfeng Liu, Yixuan Yang, Bixing Zhao, and Xiaoyu Yang. Lnc-PLA2G4A-4 facilitates the progression of hepatocellular carcinoma by inducing versican expression via sponging miR-23b-3p. *Heliyon*, 9(8), August 2023. ISSN 2405-8440. doi: 10.1016/j.heliyon.2023.e18698. URL [https://www.cell.com/heliyon/abstract/S2405-8440\(23\)05906-6](https://www.cell.com/heliyon/abstract/S2405-8440(23)05906-6). Publisher: Elsevier.
- [89] Sarah Schatschneider, Salah Abdelrazig, Laudina Safo, Anne M Henstra, Thomas Millat, Dong-Hyun Kim, Klaus Winzer, Nigel P Minton, and David A Barrett. Quantitative isotope-dilution high-resolution-mass-spectrometry analysis of multiple intracellular metabolites in *Clostridium autoethanogenum* with uniformly ¹³C-labeled standards derived from *Spirulina*. *Anal. Chem.*, 90(7):4470–4477, April 2018. ISSN 1520-6882. doi: 10.1021/acs.analchem.7b04758. URL <https://pubmed.ncbi.nlm.nih.gov/29533656/>. Publisher: Anal Chem.
- [90] Elizabeth J Want, Ian D Wilson, Helen Gika, Georgios Theodoridis, Robert S Plumb, John Shockcor, Elaine Holmes, and Jeremy K Nicholson. Global metabolic

- profiling procedures for urine using UPLC-MS. *Nat. Protoc.*, 5(6):1005–1018, June 2010. ISSN 1750-2799. doi: 10.1038/nprot.2010.50. URL <https://pubmed.ncbi.nlm.nih.gov/20448546/>. Publisher: Springer Science and Business Media LLC.
- [91] Wahyu Utami. *Ion pairing LC-MS/MS method for analysis of intracellular phosphorylated metabolites*. PhD thesis, University of Nottingham, December 2015. URL <http://eprints.nottingham.ac.uk/30566/>. Volume: PhD.
 - [92] Bogusława Luzak, Przemysław Siarkiewicz, and Magdalena Boncler. An evaluation of a new high-sensitivity PrestoBlue assay for measuring cell viability and drug cytotoxicity using EA.hy926 endothelial cells. *Toxicol. In Vitro*, 83:105407, September 2022. ISSN 0887-2333. doi: 10.1016/j.tiv.2022.105407. URL <http://dx.doi.org/10.1016/j.tiv.2022.105407>.
 - [93] Andreas Schroeder, Odilo Mueller, Susanne Stocker, Ruediger Salowsky, Michael Leiber, Marcus Gassmann, Samar Lightfoot, Wolfram Menzel, Martin Granzow, and Thomas Ragg. The RIN: an RNA integrity number for assigning integrity values to RNA measurements. *BMC Molecular Biology*, 7(1):3, January 2006. ISSN 1471-2199. doi: 10.1186/1471-2199-7-3. URL <https://doi.org/10.1186/1471-2199-7-3>.
 - [94] Yoshinori Fukasawa, Luca Ermini, Hai Wang, Karen Carty, and Min-Sin Cheung. LongQC: A Quality Control Tool for Third Generation Sequencing Long Read Data. *G3 Genes/Genomes/Genetics*, 10(4):1193–1196, April 2020. ISSN 2160-1836. doi: 10.1534/g3.119.400864. URL <https://doi.org/10.1534/g3.119.400864>.
 - [95] Heng Li, Bob Handsaker, Alec Wysoker, Tim Fennell, Jue Ruan, Nils Homer, Gabor Marth, Goncalo Abecasis, Richard Durbin, and 1000 Genome Project Data Processing Subgroup. The Sequence Alignment/Map format and SAMtools. *Bioinformatics*, 25(16):2078–2079, August 2009. ISSN 1367-4803. doi: 10.1093/bioinformatics/btp352. URL <https://doi.org/10.1093/bioinformatics/btp352>.
 - [96] Heng Li. Minimap2: pairwise alignment for nucleotide sequences. *Bioinformatics*, 34(18):3094–3100, September 2018. ISSN 1367-4803. doi: 10.1093/bioinformatics/bty191. URL <https://doi.org/10.1093/bioinformatics/bty191>.
 - [97] Aaron R. Quinlan and Ira M. Hall. BEDTools: a flexible suite of utilities for comparing genomic features. *Bioinformatics*, 26(6):841–842, March 2010. ISSN 1367-4803. doi: 10.1093/bioinformatics/btq033. URL <https://doi.org/10.1093/bioinformatics/btq033>.
 - [98] Andrey D Prjibelski, Alla Mikheenko, Anoushka Joglekar, Alexander Smetanin, Julien Jarroux, Alla L Lapidus, and Hagen U Tilgner. Accurate isoform discovery with IsoQuant using long reads. *Nat. Biotechnol.*, 41(7):915–918, July 2023. ISSN 1546-1696. doi: 10.1038/s41587-022-01565-y. URL <https://pubmed.ncbi.nlm.nih.gov/36593406/>. Publisher: Springer Science and Business Media LLC.
 - [99] Yu Hamaguchi, Chao Zeng, and Michiaki Hamada. Impact of human gene annotations on RNA-seq differential expression analysis. *BMC Genomics*, 22(1):730, October 2021. ISSN 1471-2164. doi: 10.1186/s12864-021-08038-7. URL <https://bmcbgenomics.biomedcentral.com/articles/10.1186/s12864-021-08038-7>. Publisher: Springer Science and Business Media LLC.

- [100] Mark D. Robinson, Davis J. McCarthy, and Gordon K. Smyth. edgeR: a Bioconductor package for differential expression analysis of digital gene expression data. *Bioinformatics*, 26(1):139–140, January 2010. ISSN 1367-4803. doi: 10.1093/bioinformatics/btp616. URL <https://pmc.ncbi.nlm.nih.gov/articles/PMC2796818/>.
- [101] Mark D. Robinson and Alicia Oshlack. A scaling normalization method for differential expression analysis of RNA-seq data. *Genome Biology*, 11(3):R25, March 2010. ISSN 1474-760X. doi: 10.1186/gb-2010-11-3-r25. URL <https://doi.org/10.1186/gb-2010-11-3-r25>.
- [102] Showkat Ahmad Dar, Sulochan Malla, Vlastimil Martinek, Matthew John Payea, Christopher Tai-Yi Lee, Jessica Martin, Aditya Jignesh Khandeshi, Jennifer L Martindale, Cedric Belair, and Manolis Maragkakis. Full-length direct RNA sequencing uncovers stress granule-dependent RNA decay upon cellular stress. *Elife*, 13, December 2024. ISSN 2050-084X. doi: 10.7554/eLife.96284. URL <http://dx.doi.org/10.7554/eLife.96284>. Publisher: eLife Sciences Publications Limited.
- [103] Simon A Hawley, Fiona A Ross, Fiona M Russell, Abdelmadjid Atrih, Douglas J Lamont, and D Grahame Hardie. Mechanism of Activation of AMPK by Cordycepin. *Cell Chem Biol*, 27(2):214–222.e4, February 2020. ISSN 2451-9448. doi: 10.1016/j.chembiol.2020.01.004. URL <http://dx.doi.org/10.1016/j.chembiol.2020.01.004>.
- [104] M. D. Hatch. Adenylosuccinate synthetase and adenylosuccinate lyase from plant tissues. *Biochemical Journal*, 98(1):198–203, January 1966. ISSN 0264-6021. doi: 10.1042/bj0980198. URL <https://pmc.ncbi.nlm.nih.gov/articles/PMC1264815/>.
- [105] Kenta Imamura, Momoko Asai, Kazuhiro Sugamoto, Tomoko Matsumoto, Yumi Yamasaki, Ichiro Kamei, Takahiro Hattori, Masaoki Kishimoto, Seiji Niisaka, Masaomi Kubo, Kazuo Nishiyama, and Masao Yamasaki. Suppressing effect of cordycepin on the lipopolysaccharide-induced nitric oxide production in RAW 264.7 cells. *Biosci. Biotechnol. Biochem.*, 79(6):1021–1025, February 2015. ISSN 0916-8451. doi: 10.1080/09168451.2015.1008977. URL <http://dx.doi.org/10.1080/09168451.2015.1008977>.
- [106] M A Johnson, G Ahluwalia, M C Connelly, D A Cooney, S Broder, D G Johns, and A Fridland. Metabolic pathways for the activation of the antiretroviral agent 2',3'-dideoxyadenosine in human lymphoid cells. *J. Biol. Chem.*, 263(30):15354–15357, October 1988. ISSN 0021-9258. URL <https://www.ncbi.nlm.nih.gov/pubmed/3262616>.
- [107] Gannie Tzoneva, Arianne Perez Garcia, Zachary Carpenter, Hossein Khiabani, Valeria Tosello, Maddalena Allegretta, Elisabeth Paietta, Janis Racevskis, Jacob M. Rowe, Martin S. Tallman, Maddalena Paganin, Giuseppe Basso, Jana Hof, Renate Kirschner-Schwabe, Teresa Palomero, Raul Rabadan, and Adolfo Ferrando. Activating mutations in the NT5C2 nucleotidase gene drive chemotherapy resistance in relapsed ALL. *Nature medicine*, 19(3):368–371, March 2013. ISSN 1078-8956. doi: 10.1038/nm.3078. URL <https://pmc.ncbi.nlm.nih.gov/articles/PMC3594483/>.

- [108] Marcella Camici, Mercedes Garcia-Gil, Simone Allegrini, Rossana Pesì, and Maria Grazia Tozzi. Evidence for a Cross-Talk Between Cytosolic 5-Nucleotidases and AMP-Activated Protein Kinase. *Frontiers in Pharmacology*, 11, December 2020. ISSN 1663-9812. doi: 10.3389/fphar.2020.609849. URL <https://www.frontiersin.org/journals/pharmacology/articles/10.3389/fphar.2020.609849/full>. Publisher: Frontiers.
- [109] Andrey Kropotov, Veronika Kulikova, Ljudmila Solovjeva, Alexander Yakimov, Kirill Nerinovski, Maria Svetlova, Julia Sudnitsyna, Alena Plusnina, Maria Antipova, Mikhail Khodorkovskiy, Marie E. Migaud, Stepan Gambaryan, Mathias Ziegler, and Andrey Nikiforov. Purine nucleoside phosphorylase controls nicotinamide riboside metabolism in mammalian cells. *Journal of Biological Chemistry*, 298(12), December 2022. ISSN 0021-9258, 1083-351X. doi: 10.1016/j.jbc.2022.102615. URL [https://www.jbc.org/article/S0021-9258\(22\)01058-4/abstract](https://www.jbc.org/article/S0021-9258(22)01058-4/abstract). Publisher: Elsevier.
- [110] Mark D. Erion, Johanna D. Stoeckler, Wayne C. Guida, and Richard L. Walter. Purine Nucleoside Phosphorylase. 2. Catalytic Mechanism. *Biochemistry*, 36(39): 11735–11748, September 1997. ISSN 0006-2960. doi: 10.1021/bi961970v. URL <https://doi.org/10.1021/bi961970v>. Publisher: American Chemical Society.
- [111] Joel M. Linden. Purine Release and Metabolism. In *Basic Neurochemistry: Molecular, Cellular and Medical Aspects. 6th edition*. Lippincott-Raven, 1999. URL <https://www.ncbi.nlm.nih.gov/books/NBK28118/>.
- [112] Guang-Huar Young, Jiun-Tsai Lin, Yi-Fang Cheng, Chia-Fang Ho, Qian-Yu Kuok, Ru-Chun Hsu, Wan-Rou Liao, Chin-Chen Chen, and Han-Min Chen. Modulation of adenine phosphoribosyltransferase-mediated salvage pathway to accelerate diabetic wound healing. *FASEB journal: official publication of the Federation of American Societies for Experimental Biology*, 35(3):e21296, March 2021. ISSN 1530-6860. doi: 10.1096/fj.202001736RR.
- [113] M Zaeem Cader, Rodrigo Pereira de Almeida Rodrigues, James A West, Gavin W Sewell, Muhammad N Md-Ibrahim, Stephanie Reikine, Giuseppe Sirago, Lukas W Unger, Ana Belén Iglesias-Romero, Katharina Ramshorn, Lea-Maxie Haag, Svetlana Saveljeva, Jana-Fabienne Ebel, Philip Rosenstiel, Nicole C Kaneider, James C Lee, Trevor D Lawley, Allan Bradley, Gordon Dougan, Yorgo Modis, Julian L Griffin, and Arthur Kaser. FAMIN Is a Multifunctional Purine Enzyme Enabling the Purine Nucleotide Cycle. *Cell*, 180(2):278–295.e23, January 2020. ISSN 0092-8674. doi: 10.1016/j.cell.2019.12.017. URL <http://dx.doi.org/10.1016/j.cell.2019.12.017>.
- [114] Jean-Francois Navé, Anne Eschbach, Dominique Wolff-Kugel, Serge Halazy, and Jan Balzarini. Enzymatic phosphorylation and pyrophosphorylation of 2',3'-dideoxyadenosine-5'-monophosphate, a key metabolite in the pathway for activation of the anti-HIV (human immunodeficiency virus) agent 2',3'-dideoxyinosine. *Biochem. Pharmacol.*, 48(6):1105–1112, September 1994. ISSN 0006-2952. doi: 10.1016/0006-2952(94)90146-5. URL [http://dx.doi.org/10.1016/0006-2952\(94\)90146-5](http://dx.doi.org/10.1016/0006-2952(94)90146-5).
- [115] A S Bretonnet, L P Jordheim, C Dumontet, and J M Lancelin. Regulation and activity of cytosolic 5'-nucleotidase II. A bifunctional allosteric enzyme of the Haloacid Dehalogenase superfamily involved in cellular metabolism. *FEBS Lett.*,

579(16):3363–3368, June 2005. ISSN 0014-5793. doi: 10.1016/j.febslet.2005.05.014. URL <http://dx.doi.org/10.1016/j.febslet.2005.05.014>.

- [116] Carolina Horta Andrade, Lenis Medeiros de Freitas, and Valéria de Oliveira. Twenty-six years of HIV science: An overview of anti-HIV drugs metabolism. *Brazilian Journal of Pharmaceutical Science*, 47(2):209–230, June 2011. ISSN 1984-8250. doi: 10.1590/S1984-82502011000200003. URL https://www.researchgate.net/publication/262460198_Twenty-six_years_of_HIV_science_An_overview_of_anti-HIV_drugs_metabolism. Publisher: unknown.
- [117] M G Tozzi, M Camici, R Pesì, S Allegrini, F Sgarrella, and P L Ipata. Nucleoside phosphotransferase activity of human colon carcinoma cytosolic 5'-nucleotidase. *Arch. Biochem. Biophys.*, 291(2):212–217, December 1991. ISSN 0003-9861. doi: 10.1016/0003-9861(91)90125-3. URL [http://dx.doi.org/10.1016/0003-9861\(91\)90125-3](http://dx.doi.org/10.1016/0003-9861(91)90125-3).
- [118] Tingting Wang, J N Rashida Gnanaprakasam, Xuyong Chen, Siwen Kang, Xuequn Xu, Hua Sun, Lingling Liu, Hayley Rodgers, Ethan Miller, Teresa A Cassel, Qiushi Sun, Sara Vicente-Muñoz, Marc O Warmoes, Penghui Lin, Zayda Lizbeth Piedra-Quintero, Mireia Guerau-de Arellano, Kevin A Cassady, Song Guo Zheng, Jun Yang, Andrew N Lane, Xiaotong Song, Teresa W-M Fan, and Ruoning Wang. Inosine is an alternative carbon source for CD8+ T-cell function under glucose restriction. *Nat Metab*, 2(7):635–647, July 2020. ISSN 2522-5812. doi: 10.1038/s42255-020-0219-4. URL <http://dx.doi.org/10.1038/s42255-020-0219-4>.
- [119] Toshihiko Fukuda, Kaustav Majumder, Hua Zhang, Toshiro Matsui, and Yoshinori Mine. Adenine has an anti-inflammatory effect through the activation of adenine receptor signaling in mouse macrophage. *J. Funct. Foods*, 28:235–239, January 2017. ISSN 1756-4646. doi: 10.1016/j.jff.2016.11.013. URL <https://www.sciencedirect.com/science/article/pii/S1756464616303553>.
- [120] E Szabados, R G Duggleby, and R I Christopherson. Metabolism of adenosine and deoxyadenosine by human erythrocytes and CCRF-CEM leukemia cells. *Int. J. Biochem. Cell Biol.*, 28(12):1405–1415, December 1996. ISSN 1357-2725. doi: 10.1016/s1357-2725(96)00080-5. URL [http://dx.doi.org/10.1016/s1357-2725\(96\)00080-5](http://dx.doi.org/10.1016/s1357-2725(96)00080-5).
- [121] Navdeep S. Chandel. Nucleotide Metabolism. *Cold Spring Harbor Perspectives in Biology*, 13(7):a040592, July 2021. ISSN 1943-0264. doi: 10.1101/cshperspect.a040592. URL <https://pmc.ncbi.nlm.nih.gov/articles/PMC8247561/>.
- [122] Svetlana Saveljeva, Gavin W Sewell, Katharina Ramshorn, M Zaeem Cader, James A West, Simon Clare, Lea-Maxie Haag, Rodrigo Pereira de Almeida Rodrigues, Lukas W Unger, Ana Belén Iglesias-Romero, Lorraine M Holland, Christophe Bourges, Muhammad N Md-Ibrahim, James O Jones, Richard S Blumberg, James C Lee, Nicole C Kaneider, Trevor D Lawley, Allan Bradley, Gordon Dougan, and Arthur Kaser. A purine metabolic checkpoint that prevents autoimmunity and autoinflammation. *Cell Metab.*, 34(1):106–124.e10, January 2022. ISSN 1550-4131. doi: 10.1016/j.cmet.2021.12.009. URL <http://dx.doi.org/10.1016/j.cmet.2021.12.009>.

- [123] Diem H. Tran, Dohun Kim, Rushendhiran Kesavan, Harrison Brown, Trishna Dey, Mona Hoseini Soflaee, Hieu S. Vu, Alpaslan Tasdogan, Jason Guo, Divya Bezwada, Houssam Al Saad, Feng Cai, Ashley Solmonson, Halie Rion, Rawand Chabatya, Salma Merchant, Nathan J. Manales, Vanina T. Tcheuyap, Megan Mulkey, Thomas P. Mathews, James Brugarolas, Sean J. Morrison, Hao Zhu, Ralph J. DeBerardinis, and Gerta Hoxhaj. *De novo* and salvage purine synthesis pathways across tissues and tumors. *Cell*, 187(14):3602–3618.e20, July 2024. ISSN 0092-8674. doi: 10.1016/j.cell.2024.05.011. URL <https://www.sciencedirect.com/science/article/pii/S0092867424005208>.
- [124] Rossana Pesi, Simone Allegrini, Francesco Balestri, Mercedes Garcia-Gil, Federico Cividini, Laura Colombaioni, Lars Petter Jordheim, Marcella Camici, and Maria Grazia Tozzi. Cytosolic 5-Nucleotidase II Is a Sensor of Energy Charge and Oxidative Stress: A Possible Function as Metabolic Regulator. *Cells*, 10(1):182, January 2021. ISSN 2073-4409. doi: 10.3390/cells10010182. URL <https://pmc.ncbi.nlm.nih.gov/articles/PMC7831490/>.
- [125] Mahmoud Ghanem, Andrew S. Murkin, and Vern L. Schramm. Ribocation Transition State Capture and Rebound in Human Purine Nucleoside Phosphorylase. *Chemistry & biology*, 16(9):971–979, September 2009. ISSN 1074-5521. doi: 10.1016/j.chembiol.2009.07.012. URL <https://pmc.ncbi.nlm.nih.gov/articles/PMC2752651/>.
- [126] Akihiko Hatano, Hiroyuki Wakana, Nanae Terado, Aoi Kojima, Chisato Nishioka, Yu Iizuka, Takuya Imaizumi, and Sanae Uehara. Bio-catalytic synthesis of unnatural nucleosides possessing a large functional group such as a fluorescent molecule by purine nucleoside phosphorylase. *Catalysis Science & Technology*, 9(18):5122–5129, September 2019. ISSN 2044-4761. doi: 10.1039/C9CY01063G. URL <https://pubs.rsc.org/en/content/articlelanding/2019/cy/c9cy01063g>. Publisher: The Royal Society of Chemistry.
- [127] Wei-Ding Wu, Zhi-Ming Hu, Min-Jie Shang, Da-Jian Zhao, Cheng-Wu Zhang, De-Fei Hong, and Dong-Sheng Huang. Cordycepin Down-Regulates Multiple Drug Resistant (MDR)/HIF-1 α through Regulating AMPK/mTORC1 Signaling in GBC-SD Gallbladder Cancer Cells. *International Journal of Molecular Sciences*, 15(7):12778–12790, July 2014. ISSN 1422-0067. doi: 10.3390/ijms150712778. URL <https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4139874/>.
- [128] Adriana Marcelo, Filipa Brito, Sara Carmo-Silva, Carlos A Matos, João Alves-Cruzeiro, Ana Vasconcelos-Ferreira, Rebekah Koppenol, Liliana Mendonça, Luís Pereira de Almeida, and Clévio Nóbrega. Cordycepin activates autophagy through AMPK phosphorylation to reduce abnormalities in Machado–Joseph disease models. *Human Molecular Genetics*, 28(1):51–63, January 2019. ISSN 0964-6906. doi: 10.1093/hmg/ddy328. URL <https://doi.org/10.1093/hmg/ddy328>.
- [129] Xin Li, Yue Zhou, Xue Zhang, Xiaoxue Cao, Chongming Wu, and Peng Guo. Cordycepin stimulates autophagy in macrophages and prevents atherosclerotic plaque formation in ApoE $^{-/-}$ mice. *Oncotarget*, 8(55):94726–94737, October 2017. ISSN 1949-2553. doi: 10.18632/oncotarget.21886. URL <https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5706907/>.

- [130] Ming-Min Chang, Bo-Syong Pan, Chia-Yih Wang, and Bu-Miin Huang. Cordycepin-induced unfolded protein response-dependent cell death, and AKT/MAPK-mediated drug resistance in mouse testicular tumor cells. *Cancer Med.*, 8(8):3949–3964, July 2019. ISSN 2045-7634. doi: 10.1002/cam4.2285. URL <http://dx.doi.org/10.1002/cam4.2285>. Publisher: Wiley.
- [131] Jan Rehwinkel and Michaela U. Gack. RIG-I-like receptors: their regulation and roles in RNA sensing. *Nature Reviews Immunology*, 20(9):537–551, September 2020. ISSN 1474-1741. doi: 10.1038/s41577-020-0288-3. URL <https://www.nature.com/articles/s41577-020-0288-3>. Publisher: Nature Publishing Group.
- [132] Yueh-Ming Loo and Michael Gale. Immune signaling by RIG-I-like receptors. *Immunity*, 34(5):680–692, May 2011. ISSN 1074-7613. doi: 10.1016/j.immuni.2011.05.003. URL <https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3177755/>.
- [133] Hongrui Li, Zizhao Zhao, Jing Ling, Linhui Pan, Xibao Zhao, Huihui Zhu, Juan Yu, Bin Xie, Jianzhong Shen, and Weilin Chen. USP14 promotes K63-linked RIG-I deubiquitination and suppresses antiviral immune responses. *European Journal of Immunology*, 49(1):42–53, 2019. ISSN 1521-4141. doi: 10.1002/eji.201847603. URL <https://onlinelibrary.wiley.com/doi/abs/10.1002/eji.201847603>. _eprint: <https://onlinelibrary.wiley.com/doi/pdf/10.1002/eji.201847603>.
- [134] Christian Montellese, Jasmin van den Heuvel, Caroline Ashiono, Kerstin Dörner, André Melnik, Stefanie Jonas, Ivo Zemp, Paola Picotti, Ludovic C Gillet, and Ulrike Kutay. USP16 counteracts mono-ubiquitination of RPS27a and promotes maturation of the 40S ribosomal subunit. *eLife*, 9:e54435, March 2020. ISSN 2050-084X. doi: 10.7554/eLife.54435. URL <https://doi.org/10.7554/eLife.54435>. Publisher: eLife Sciences Publications, Ltd.
- [135] Karolina Pakos-Zebrucka, Izabela Koryga, Katarzyna Mnich, Mila Ljubic, Afshin Samali, and Adrienne M Gorman. The integrated stress response. *EMBO Rep.*, 17(10):1374–1395, October 2016. ISSN 1469-221X. doi: 10.15252/embr.201642195. URL <http://dx.doi.org/10.15252/embr.201642195>.
- [136] Martin Leeb and Anton Wutz. Derivation of haploid embryonic stem cells from mouse embryos. *Nature*, 479(7371):131–134, November 2011. ISSN 1476-4687. doi: 10.1038/nature10448. URL <https://www.nature.com/articles/nature10448>. Publisher: Nature Publishing Group.
- [137] M. Bai, Y. Wu, and J. Li. Generation and application of mammalian haploid embryonic stem cells. *Journal of Internal Medicine*, 280(3):236–245, 2016. ISSN 1365-2796. doi: 10.1111/joim.12503. URL <https://onlinelibrary.wiley.com/doi/abs/10.1111/joim.12503>. _eprint: <https://onlinelibrary.wiley.com/doi/pdf/10.1111/joim.12503>.
- [138] Stephen J Pettitt, Farah L Rehman, Ilirjana Bajrami, Rachel Brough, Fredrik Wallberg, Iwanka Kozarewa, Kerry Fenwick, Ioannis Assiotis, Lina Chen, James Campbell, Christopher J Lord, and Alan Ashworth. A genetic screen using the PiggyBac transposon in haploid cells identifies Parp1 as a mediator of olaparib toxicity. *PLoS One*, 8(4):e61520, April 2013. ISSN 1932-6203. doi: 10.1371/journal.pone.0061520. URL <http://dx.doi.org/10.1371/journal.pone.0061520>.

- [139] Tobias B. Beigl, Ine Kjosås, Emilie Seljeseth, Nina Glomnes, and Henriette Aksnes. Efficient and crucial quality control of HAP1 cell ploidy status. *Biology Open*, 9 (11):bio057174, November 2020. ISSN 2046-6390. doi: 10.1242/bio.057174. URL <https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7673356/>.
- [140] Meng Amy Li, Stephen J. Pettitt, Sabine Eckert, Zemin Ning, Stephen Rice, Juan Cadiñanos, Kosuke Yusa, Nathalie Conte, and Allan Bradley. The piggyBac Transposon Displays Local and Distant Reintegration Preferences and Can Cause Mutations at Noncanonical Integration Sites. *Molecular and Cellular Biology*, 33 (7):1317–1330, April 2013. ISSN 0270-7306. doi: 10.1128/MCB.00670-12. URL <https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3624274/>.
- [141] Junko Yoshida, Keiko Akagi, Ryo Misawa, Chikara Kokubu, Junji Takeda, and Kyoji Horie. Chromatin states shape insertion profiles of the piggyBac, Tol2 and Sleeping Beauty transposons and murine leukemia virus. *Scientific Reports*, 7 (1):43613, March 2017. ISSN 2045-2322. doi: 10.1038/srep43613. URL <https://www.nature.com/articles/srep43613>. Publisher: Nature Publishing Group.
- [142] Kosuke Yusa, Liqin Zhou, Meng Amy Li, Allan Bradley, and Nancy L. Craig. A hyperactive piggyBac transposase for mammalian applications. *Proceedings of the National Academy of Sciences*, 108(4):1531–1536, January 2011. doi: 10.1073/pnas.1008322108. URL <https://www.pnas.org/doi/10.1073/pnas.1008322108>. Publisher: Proceedings of the National Academy of Sciences.
- [143] Gemma Llargués-Sistac, Laia Bonjoch, and Sergi Castellvi-Bel. HAP1, a new revolutionary cell model for gene editing using CRISPR-Cas9. *Frontiers in Cell and Developmental Biology*, 11, March 2023. ISSN 2296-634X. doi: 10.3389/fcell.2023.1111488. URL <https://www.frontiersin.org/journals/cell-and-developmental-biology/articles/10.3389/fcell.2023.1111488/full>. Publisher: Frontiers.
- [144] Stephen J Pettitt, E-Pien Tan, and Kosuke Yusa. piggyBac transposon-based insertional mutagenesis in mouse haploid embryonic stem cells. *Methods Mol. Biol.*, 1239:15–28, 2015. ISSN 1064-3745. doi: 10.1007/978-1-4939-1862-1_2. URL http://dx.doi.org/10.1007/978-1-4939-1862-1_2. Place: New York, NY Publisher: Springer New York.
- [145] Juan Cadiñanos and Allan Bradley. Generation of an inducible and optimized piggyBac transposon system†. *Nucleic Acids Research*, 35(12):e87, June 2007. ISSN 0305-1048. doi: 10.1093/nar/gkm446. URL <https://www.ncbi.nlm.nih.gov/pmc/articles/PMC1919496/>.
- [146] Yaa-Jyuhn J. Meir, Matthew T. Weirauch, Herng-Shing Yang, Pei-Cheng Chung, Robert K. Yu, and Sareina C-Y Wu. Genome-wide target profiling of piggyBac and Tol2in HEK 293: pros and cons for gene discovery and gene therapy. *BMC Biotechnology*, 11(1):28, March 2011. ISSN 1472-6750. doi: 10.1186/1472-6750-11-28. URL <https://doi.org/10.1186/1472-6750-11-28>.
- [147] Reini E N van der Welle, Rebekah Jobling, Christian Burns, Paolo Sanza, Jan A van der Beek, Alfonso Fasano, Lan Chen, Fried J Zwartkruis, Susan Zwakenberg, Edward F Griffin, Corlinda ten Brink, Tineke Veenendaal, Nalan Liv, Conny M A van Ravenswaaij-Arts, Henny H Lemmink, Rolph Pfundt, Susan

- Blaser, Carolina Sepulveda, Andres M Lozano, Grace Yoon, Teresa Santiago-Sim, Cedric S Asensio, Guy A Caldwell, Kim A Caldwell, David Chitayat, and Judith Klumperman. Neurodegenerative VPS41 variants inhibit HOPS function and mTORC1-dependent TFEB/TFE3 regulation. *EMBO Molecular Medicine*, 13 (5):e13258, May 2021. ISSN 1757-4676. doi: 10.15252/emmm.202013258. URL <https://www.ncbi.nlm.nih.gov/pmc/articles/PMC8103106/>.
- [148] Ziwen Wang, Zelin Chen, Zhongyong Jiang, Peng Luo, Lang Liu, Yu Huang, Huilan Wang, Yu Wang, Lei Long, Xu Tan, Dengqun Liu, Taotao Jin, Yawei Wang, Yang Wang, Fengying Liao, Chi Zhang, Long Chen, Yibo Gan, Yunsheng Liu, Fan Yang, Chunji Huang, Hongming Miao, Jieping Chen, Tianmin Cheng, Xiaobing Fu, and Chunmeng Shi. Cordycepin prevents radiation ulcer by inhibiting cell senescence via NRF2 and AMPK in rodents. *Nature Communications*, 10 (1):2538, June 2019. ISSN 2041-1723. doi: 10.1038/s41467-019-10386-8. URL <http://dx.doi.org/10.1038/s41467-019-10386-8>. tex.journaltitle: Nat. Commun.
- [149] György Csordás, David Weaver, and György Hajnóczky. Endoplasmic Reticulum–Mitochondrial Contactology: Structure and Signaling Functions. *Trends in Cell Biology*, 28(7):523–540, July 2018. ISSN 0962-8924, 1879-3088. doi: 10.1016/j.tcb.2018.02.009. URL [https://www.cell.com/trends/cell-biology/abstract/S0962-8924\(18\)30033-3](https://www.cell.com/trends/cell-biology/abstract/S0962-8924(18)30033-3). Publisher: Elsevier.
- [150] Othman Abu Shelbayeh, Tasnim Arroum, Silke Morris, and Karin B. Busch. PGC-1 α Is a Master Regulator of Mitochondrial Lifecycle and ROS Stress Response. *Antioxidants*, 12(5):1075, May 2023. ISSN 2076-3921. doi: 10.3390/antiox12051075. URL <https://www.mdpi.com/2076-3921/12/5/1075>. Publisher: Multidisciplinary Digital Publishing Institute.
- [151] Houyou Yu, Xin Hong, Lihua Liu, Yangpeng Wu, Xuemei Xie, Guoxiang Fang, and Shaomin Zhi. Cordycepin Decreases Ischemia/Reperfusion Injury in Diabetic Hearts via Upregulating AMPK/Mfn2-dependent Mitochondrial Fusion. *Frontiers in Pharmacology*, 12:754005, October 2021. ISSN 1663-9812. doi: 10.3389/fphar.2021.754005. URL <https://www.ncbi.nlm.nih.gov/pmc/articles/PMC8563605/>.
- [152] Lorenzo Pavanello, Michael Hall, and Gerlof Sebastiaan Winkler. Regulation of eukaryotic mRNA deadenylation and degradation by the Ccr4-Not complex. *Front. Cell Dev. Biol.*, 11:1153624, April 2023. ISSN 2296-634X. doi: 10.3389/fcell.2023.1153624. URL <http://dx.doi.org/10.3389/fcell.2023.1153624>. Publisher: Frontiers.
- [153] Jens Lykke-Andersen and Eileen Wagner. Recruitment and activation of mRNA decay enzymes by two ARE-mediated decay activation domains in the proteins TTP and BRF-1. *Genes & Development*, 19(3):351–361, February 2005. ISSN 0890-9369, 1549-5477. doi: 10.1101/gad.1282305. URL <http://genesdev.cshlp.org/content/19/3/351>. Company: Cold Spring Harbor Laboratory Press Distributor: Cold Spring Harbor Laboratory Press Institution: Cold Spring Harbor Laboratory Press Label: Cold Spring Harbor Laboratory Press Publisher: Cold Spring Harbor Lab.
- [154] Bérengère de Toeuf, Romuald Soin, Abdelkarim Nazih, Marija Dragojevic, Dukas Jurénas, Nadège Delacourt, Long Vo Ngoc, Abel Garcia-Pino, Véronique Kruys, and Cyril Gueydan. ARE-mediated decay controls gene expression and cellular

- metabolism upon oxygen variations. *Scientific Reports*, 8(1):5211, March 2018. ISSN 2045-2322. doi: 10.1038/s41598-018-23551-8. URL <https://www.nature.com/articles/s41598-018-23551-8>. Publisher: Nature Publishing Group.
- [155] Anthony K. Henras, Célia Plisson-Chastang, Marie-Françoise O'Donohue, Anirban Chakraborty, and Pierre-Emmanuel Gleizes. An overview of pre-ribosomal RNA processing in eukaryotes. *WIREs RNA*, 6(2):225–242, 2015. ISSN 1757-7012. doi: 10.1002/wrna.1269. URL <https://onlinelibrary.wiley.com/doi/abs/10.1002/wrna.1269>. _eprint: <https://wires.onlinelibrary.wiley.com/doi/pdf/10.1002/wrna.1269>.
- [156] Johannes Popow, Markus Englert, Stefan Weitzer, Alexander Schleiffer, Beata Mierzwa, Karl Mechtler, Simon Trowitzsch, Cindy L. Will, Reinhard Lührmann, Dieter Söll, and Javier Martinez. HSPC117 Is the Essential Subunit of a Human tRNA Splicing Ligase Complex. *Science*, 331(6018):760–764, February 2011. doi: 10.1126/science.1197847. URL <https://www.science.org/doi/10.1126/science.1197847>. Publisher: American Association for the Advancement of Science.
- [157] Jiandong Chen and Eric J. Wagner. snRNA 3 end formation: the dawn of the Integrator complex. *Biochemical Society transactions*, 38(4):1082–1087, August 2010. ISSN 0300-5127. doi: 10.1042/BST0381082. URL <https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3969742/>.
- [158] María Sarai Mendoza-Figueroa, Deirdre C. Tatomer, and Jeremy E. Wilusz. The Integrator complex in transcription and development. *Trends in biochemical sciences*, 45(11):923–934, November 2020. ISSN 0968-0004. doi: 10.1016/j.tibs.2020.07.004. URL <https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7572659/>.
- [159] Dalibor Mijaljica, Mark Prescott, and Rodney J. Devenish. Microautophagy in mammalian cells: revisiting a 40-year-old conundrum. *Autophagy*, 7(7):673–682, July 2011. ISSN 1554-8635. doi: 10.4161/auto.7.7.14733.
- [160] Xiaopei Zhang, Wei Wang, Weidong Zhu, Jie Dong, Yingying Cheng, Zujun Yin, and Fafu Shen. Mechanisms and Functions of Long Non-Coding RNAs at Multiple Regulatory Levels. *International Journal of Molecular Sciences*, 20(22):5573, January 2019. ISSN 1422-0067. doi: 10.3390/ijms20225573. URL <https://www.mdpi.com/1422-0067/20/22/5573>. Publisher: Multidisciplinary Digital Publishing Institute.
- [161] Jialiang Lin. *CORDYCEPIN AFFECTS GROWTH FACTOR-DEPENDENT GENE EXPRESSION*. PhD thesis, University of Nottingham, September 2017. Publication Title: School of Pharmacy Volume: Phd.
- [162] Abdul Hafeez. *Activity and composition of commercial cordycepin and Cordyceps militaris*. PhD thesis, University of Nottingham, September 2022.
- [163] Mathieu Chauleau and Stewart Shuman. Kinetic mechanism of nick sealing by T4 RNA ligase 2 and effects of 3-OH base mispairs and damaged base lesions. *RNA*, 19(12):1840–1847, December 2013. ISSN 1355-8382, 1469-9001. doi: 10.1261/rna.041731.113. URL <http://rnajournal.cshlp.org/content/19/12/1840>. Company: Cold Spring Harbor Laboratory Press Distributor: Cold Spring Harbor Laboratory Press Institution: Cold Spring Harbor Laboratory Press Label: Cold Spring Harbor Laboratory Press Publisher: Cold Spring Harbor Lab.

- [164] Raj D. Gandhi. *The role of polyadenylation in the induction of inflammatory genes*. Thesis (University of Nottingham only), University of Nottingham, July 2017. URL <https://eprints.nottingham.ac.uk/41038/>.
- [165] Yunhao Wang, Yue Zhao, Audrey Bollas, Yuru Wang, and Kin Fai Au. Nanopore sequencing technology, bioinformatics and applications. *Nature Biotechnology*, 39(11):1348–1365, November 2021. ISSN 1087-0156, 1546-1696. doi: 10.1038/s41587-021-01108-x. URL <https://www.nature.com/articles/s41587-021-01108-x>.
- [166] Andreas Krämer, Jeff Green, Jack Pollard, Jr, and Stuart Tugendreich. Causal analysis approaches in Ingenuity Pathway Analysis. *Bioinformatics*, 30(4):523–530, February 2014. ISSN 1367-4803. doi: 10.1093/bioinformatics/btt703. URL <https://doi.org/10.1093/bioinformatics/btt703>.
- [167] Jesús Gil, Maria Angel García, Paulino Gomez-Puertas, Susana Guerra, Joaquín Rullas, Hiroyasu Nakano, José Alcamí, and Mariano Esteban. TRAF Family Proteins Link PKR with NF- κ B Activation. *Molecular and Cellular Biology*, 24(10):4502–4512, May 2004. ISSN 0270-7306. doi: 10.1128/MCB.24.10.4502-4512.2004. URL <https://pmc.ncbi.nlm.nih.gov/articles/PMC400457/>.
- [168] Marion C. Bonnet, Robert Weil, Elisabeth Dam, Ara G. Hovanessian, and Eliane F. Meurs. PKR Stimulates NF- κ B Irrespective of Its Kinase Function by Interacting with the I κ B Kinase Complex. *Molecular and Cellular Biology*, 20(13):4532–4542, July 2000. ISSN 0270-7306. doi: 10.1128/mcb.20.13.4532-4542.2000. URL <https://pmc.ncbi.nlm.nih.gov/articles/PMC85837/>.
- [169] Johanna Andrae, Radosa Gallini, and Christer Betsholtz. Role of platelet-derived growth factors in physiology and medicine. *Genes & Development*, 22(10):1276–1312, May 2008. ISSN 0890-9369, 1549-5477. doi: 10.1101/gad.1653708. URL <http://genesdev.cshlp.org/content/22/10/1276>. Company: Cold Spring Harbor Laboratory Press Distributor: Cold Spring Harbor Laboratory Press Institution: Cold Spring Harbor Laboratory Press Label: Cold Spring Harbor Laboratory Press Publisher: Cold Spring Harbor Lab.
- [170] Nina Noskovičová, Martin Petřek, Oliver Eickelberg, and Katharina Heinzelmann. Platelet-Derived Growth Factor Signaling in the Lung. From Lung Development and Disease to Clinical Studies. *American Journal of Respiratory Cell and Molecular Biology*, 52(3):263–284, March 2015. ISSN 1044-1549. doi: 10.1165/rcmb.2014-0294TR. URL <https://www.atsjournals.org/doi/full/10.1165/rcmb.2014-0294TR>. Publisher: American Thoracic Society - AJRCMB.
- [171] Chun-Chao Wang, Murat Cirit, and Jason M Haugh. PI3K-dependent cross-talk interactions converge with Ras as quantifiable inputs integrated by Erk. *Molecular Systems Biology*, 5:246, February 2009. ISSN 1744-4292. doi: 10.1038/msb.2009.4. URL <https://pmc.ncbi.nlm.nih.gov/articles/PMC2657535/>.
- [172] Hongbing Zhang, Natalia Bajraszewski, Erxi Wu, Hongwei Wang, Annie P. Moseman, Sandra L. Dabora, James D. Griffin, and David J. Kwiatkowski. PDGFRs are critical for PI3K/Akt activation and negatively regulated by mTOR. *The Journal of Clinical Investigation*, 117(3):730–738, March 2007. ISSN 0021-9738. doi: 10.1172/JCI28984. URL https://www.jci.org/articles/view/28984?utm_source=chatgpt.com. Publisher: American Society for Clinical Investigation.

- [173] Hua-Zhong Ying, Qin Chen, Wen-You Zhang, Huan-Huan Zhang, Yue Ma, Song-Zhao Zhang, Jie Fang, and Chen-Huan Yu. PDGF signaling pathway in hepatic fibrosis pathogenesis and therapeutics. *Molecular Medicine Reports*, 16(6):7879–7889, December 2017. ISSN 1791-2997. doi: 10.3892/mmr.2017.7641. URL <https://pmc.ncbi.nlm.nih.gov/articles/PMC5779870/>.
- [174] Jordan C Wengrod and Lawrence B Gardner. Cellular adaptation to nutrient deprivation: crosstalk between the mTORC1 and eIF2 α signaling pathways and implications for autophagy. *Cell Cycle*, 14(16):2571–2577, August 2015. ISSN 1538-4101, 1551-4005. doi: 10.1080/15384101.2015.1056947. URL <https://www.tandfonline.com/doi/full/10.1080/15384101.2015.1056947>.
- [175] Man Yin and Yunfei Wang. The role of PIP5K1A in cancer development and progression. *Medical Oncology*, 39(10):151, July 2022. ISSN 1559-131X. doi: 10.1007/s12032-022-01753-5. URL <https://doi.org/10.1007/s12032-022-01753-5>.
- [176] Vikram Agarwal and David R. Kelley. The genetic and biochemical determinants of mRNA degradation rates in mammals. *Genome Biology*, 23(1):245, November 2022. ISSN 1474-760X. doi: 10.1186/s13059-022-02811-x. URL <https://doi.org/10.1186/s13059-022-02811-x>.
- [177] Hnin Thanda Aung, Kate Schroder, Stewart R. Himes, Kristian Brion, Wendy Van Zuylen, Angela Trieu, Harukazu Suzuki, Yoshihide Hayashizaki, David A. Hume, Matthew J. Sweet, and Timothy Ravasi. LPS regulates proinflammatory gene expression in macrophages by altering histone deacetylase expression. *The FASEB Journal*, 20(9):1315–1327, 2006. ISSN 1530-6860. doi: 10.1096/fj.05-5360com. URL <https://onlinelibrary.wiley.com/doi/abs/10.1096/fj.05-5360com>. _eprint: <https://faseb.onlinelibrary.wiley.com/doi/pdf/10.1096/fj.05-5360com>.
- [178] David S. W. Protter and Roy Parker. Principles and Properties of Stress Granules. *Trends in Cell Biology*, 26(9):668–679, September 2016. ISSN 0962-8924, 1879-3088. doi: 10.1016/j.tcb.2016.05.004. URL [https://www.cell.com/trends/cell-biology/abstract/S0962-8924\(16\)30047-2](https://www.cell.com/trends/cell-biology/abstract/S0962-8924(16)30047-2). Publisher: Elsevier.
- [179] Sahil Sharma, Fabian Poetz, Marius Bruer, Thi Bach Nga Ly-Hartig, Johanna Schott, Bertrand Séraphin, and Georg Stoecklin. Acetylation-Dependent Control of Global Poly(A) RNA Degradation by CBP/p300 and HDAC1/2. *Molecular Cell*, 63(6):927–938, September 2016. ISSN 1097-2765. doi: 10.1016/j.molcel.2016.08.030. URL <https://www.sciencedirect.com/science/article/pii/S1097276516304774>.
- [180] Jing Yang, Yun-Zhou Li, Phillip B. Hylemon, Lu-Yong Zhang, and Hui-Ping Zhou. Cordycepin inhibits LPS-induced inflammatory responses by modulating NOD-Like Receptor Protein 3 inflammasome activation. *Biomedicine & Pharmacotherapy = Biomedecine & Pharmacotherapie*, 95:1777–1788, November 2017. ISSN 1950-6007. doi: 10.1016/j.biopha.2017.09.103.
- [181] Onn Brandman and Ramanujan S Hegde. Ribosome-associated protein quality control. *Nature Structural & Molecular Biology*, 23(1):7–15, 2016. ISSN 1545-9985. doi: 10.1038/nsmb.3147. URL <http://dx.doi.org/10.1038/nsmb.3147>. tex.journaltitle: Nat. Struct. Mol. Biol.
- [182] Joazeiro and A P Claudio. Mechanisms and functions of ribosome-associated protein quality control. *Nat. Rev. Mol. Cell Biol.*, 20(6):368–383, June 2019. ISSN

1471-0072. doi: 10.1038/s41580-019-0118-2. URL <http://dx.doi.org/10.1038/s41580-019-0118-2>.

- [183] Buvana Ravishankar, Haiyun Liu, Rahul Shinde, Kapil Chaudhary, Wei Xiao, Jillian Bradley, Marianne Koritzinsky, Michael P Madaio, and Tracy L McGaha. The amino acid sensor GCN2 inhibits inflammatory responses to apoptotic cells promoting tolerance and suppressing systemic autoimmunity. *Proceedings of the National Academy of Sciences of the United States of America*, 112(34):10774–10779, August 2015. ISSN 0027-8424. doi: 10.1073/pnas.1504276112. URL <http://dx.doi.org/10.1073/pnas.1504276112>. tex.journaltitle: Proc. Natl. Acad. Sci. U. S. A.
- [184] Yong Xiong and Thomas A Steitz. A story with a good ending: tRNA 3-end maturation by CCA-adding enzymes. *Current Opinion in Structural Biology*, 16(1):12–17, February 2006. ISSN 0959-440X. doi: 10.1016/j.sbi.2005.12.001. URL <https://www.sciencedirect.com/science/article/pii/S0959440X05002204>.
- [185] Jiping Zhao, Hao Yu, Yudong Liu, Sara A Gibson, Zhaoqi Yan, Xin Xu, Amit Gaggar, Pui-Kai Li, Chenglong Li, Shi Wei, Etty N Benveniste, and Hongwei Qin. Protective effect of suppressing STAT3 activity in LPS-induced acute lung injury. *Am. J. Physiol. Lung Cell. Mol. Physiol.*, 311(5):L868–L880, November 2016. ISSN 1040-0605. doi: 10.1152/ajplung.00281.2016. URL <http://dx.doi.org/10.1152/ajplung.00281.2016>.
- [186] Phongsakorn Chueaphromsri, Phongsakorn Kunhorm, Ruchee Phonchai, Nipha Chaicharoenaudomrung, and Parinya Noisa. Cordycepin enhances SIRT1 expression and maintains stemness of human mesenchymal stem cells. *In Vivo*, 37(2):596–610, March 2023. ISSN 0258-851X. doi: 10.21873/invivo.13118. URL <http://dx.doi.org/10.21873/invivo.13118>.
- [187] Xiao Sun, Zhonghua Dong, Nan Li, Xiuli Feng, Yan Liu, Ang Li, Xiaosong Zhu, Chunyan Li, and Zhongxi Zhao. Nucleosides isolated from *Ophiocordyceps sinensis* inhibit cigarette smoke extract-induced inflammation via the SIRT1-nuclear factor- κ B/p65 pathway in RAW264.7 macrophages and in COPD mice. 13:2821–2832, September 2018. ISSN 1178-2005. doi: 10.2147/copd.s172579. URL <http://dx.doi.org/10.2147/COPD.S172579>. tex.journaltitle: Int. J. Chron. Obstruct. Pulmon. Dis.
- [188] Reuben J. Shaw, Monica Kosmatka, Nabeel Bardeesy, Rebecca L. Hurley, Lee A. Witters, Ronald A. DePinho, and Lewis C. Cantley. The tumor suppressor LKB1 kinase directly activates AMP-activated kinase and regulates apoptosis in response to energy stress. *Proceedings of the National Academy of Sciences*, 101(10):3329–3335, March 2004. doi: 10.1073/pnas.0308061100. URL <https://www.pnas.org/doi/full/10.1073/pnas.0308061100>. Publisher: Proceedings of the National Academy of Sciences.
- [189] Patrick Imesch, René Hornung, Daniel Fink, and André Fedier. Cordycepin (3'-deoxyadenosine), an inhibitor of mRNA polyadenylation, suppresses proliferation and activates apoptosis in human epithelial endometriotic cells in vitro. *Gynecologic and Obstetric Investigation*, 72(1):43–49, 2011. ISSN 1423-002X. doi: 10.1159/000322395. URL <http://dx.doi.org/10.1159/000322395>. tex.journaltitle: Gynecol. Obstet. Invest.

- [190] Hui Yu, Liangbin Lin, Zhiqiang Zhang, Huiyuan Zhang, and Hongbo Hu. Targeting NF- κ B pathway for the therapy of diseases: mechanism and clinical study. *Signal Transduct. Target. Ther.*, 5(1):209, September 2020. ISSN 2095-9907. doi: 10.1038/s41392-020-00312-6. URL <http://dx.doi.org/10.1038/s41392-020-00312-6>.
- [191] Ting Liu, Lingyun Zhang, Donghyun Joo, and Shao-Cong Sun. NF- κ B signaling in inflammation. *Signal Transduct. Target. Ther.*, 2(1):1–9, July 2017. ISSN 2095-9907. doi: 10.1038/sigtrans.2017.23. URL <http://dx.doi.org/10.1038/sigtrans.2017.23>.
- [192] Zige Liu, Li Lv, Jiao Wei, Yuli Xie, Mujia Jili, Yian Huang, Rirong Yang, and Yu Luo. Cordycepin attenuates NLRP3/Caspase-1/GSDMD-mediated LPS-induced macrophage pyroptosis. *Frontiers in Pharmacology*, 16:1526616, February 2025. ISSN 1663-9812. doi: 10.3389/fphar.2025.1526616. URL <http://dx.doi.org/10.3389/fphar.2025.1526616>. tex.journaltitle: Front. Pharmacol.
- [193] Monika Biasizzo and Nataša Kopitar-Jerala. Interplay between NLRP3 inflammasome and autophagy. *Frontiers in Immunology*, 11:591803, October 2020. ISSN 1664-3224. doi: 10.3389/fimmu.2020.591803. URL <http://dx.doi.org/10.3389/fimmu.2020.591803>. tex.journaltitle: Front. Immunol.
- [194] Rui Qing, Zezhi Huang, Yufei Tang, Qingke Xiang, and Fan Yang. Cordycepin alleviates lipopolysaccharide-induced acute lung injury via Nrf2/HO-1 pathway. *International Immunopharmacology*, 60:18–25, July 2018. ISSN 1567-5769. doi: 10.1016/j.intimp.2018.04.032. URL <http://dx.doi.org/10.1016/j.intimp.2018.04.032>. tex.journaltitle: Int. Immunopharmacol.
- [195] Yeonui Kwak, Ciarán W. P. Daly, Elizabeth A. Fogarty, Andrew Grimson, and Hojoong Kwak. Dynamic and widespread control of poly(A) tail length during macrophage activation. *RNA (New York, N.Y.)*, 28(7):947–971, July 2022. ISSN 1469-9001. doi: 10.1261/rna.078918.121.
- [196] Abul Arif, Peng Yao, Fulvia Terenzi, Jie Jia, Partho Sarothi Ray, and Paul L Fox. The GAIT translational control system. *Wiley Interdiscip. Rev. RNA*, 9(2):e1441, March 2018. ISSN 1757-7012. doi: 10.1002/wrna.1441. URL <http://dx.doi.org/10.1002/wrna.1441>.
- [197] Dongshan Wan, Wei Jiang, and Junwei Hao. Research advances in how the cGAS-STING pathway controls the cellular inflammatory response. *Frontiers in Immunology*, 11:615, April 2020. ISSN 1664-3224. doi: 10.3389/fimmu.2020.00615. URL <http://dx.doi.org/10.3389/fimmu.2020.00615>. tex.journaltitle: Front. Immunol.
- [198] Steve Dvorkin, Stephanie Cambier, Hannah E Volkman, and Daniel B Stetson. New frontiers in the cGAS-STING intracellular DNA-sensing pathway. *Immunity*, 57(4):718–730, April 2024. ISSN 1074-7613. doi: 10.1016/j.immuni.2024.02.019. URL <http://dx.doi.org/10.1016/j.immuni.2024.02.019>.
- [199] Daniel Schlam, Richard D Bagshaw, Spencer A Freeman, Richard F Collins, Tony Pawson, Gregory D Fairn, and Sergio Grinstein. Phosphoinositide 3-kinase enables phagocytosis of large particles by terminating actin assembly through Rac/Cdc42 GTPase-activating proteins. *Nature Communications*, 6(1):8623, October 2015.

ISSN 2041-1723. doi: 10.1038/ncomms9623. URL <http://dx.doi.org/10.1038/ncomms9623>. tex.journaltitle: Nat. Commun.

- [200] Anna Ciesielska, Marta Matyjek, and Katarzyna Kwiatkowska. TLR4 and CD14 trafficking and its influence on LPS-induced pro-inflammatory signaling. *Cell. Mol. Life Sci.*, 78(4):1233–1261, 2021. ISSN 1420-682X. doi: 10.1007/s00018-020-03656-y. URL <http://dx.doi.org/10.1007/s00018-020-03656-y>.
- [201] Ivan Zanoni, Renato Ostuni, Lorri R Marek, Simona Barresi, Roman Barbalat, Gregory M Barton, Francesca Granucci, and Jonathan C Kagan. CD14 controls the LPS-induced endocytosis of Toll-like receptor 4. *Cell*, 147(4):868–880, November 2011. ISSN 0092-8674. doi: 10.1016/j.cell.2011.09.051. URL <http://dx.doi.org/10.1016/j.cell.2011.09.051>.
- [202] P Anderson and N Kedersha. Visibly stressed: the role of eIF2, TIA-1, and stress granules in protein translation. *Cell Stress & Chaperones*, 7(2):213–221, April 2002. ISSN 1466-1268. doi: 10.1379/1466-1268(2002)007<0213:vstroe>2.0.co;2. URL [http://dx.doi.org/10.1379/1466-1268\(2002\)007<0213:VSTROE>2.0.CO;2](http://dx.doi.org/10.1379/1466-1268(2002)007<0213:VSTROE>2.0.CO;2).
- [203] Carmela Sidrauski, Anna M McGeachy, Nicholas T Ingolia, and Peter Walter. The small molecule ISRIB reverses the effects of eIF2 α phosphorylation on translation and stress granule assembly. *Elife*, 4, February 2015. ISSN 2050-084X. doi: 10.7554/elife.05033. URL <http://dx.doi.org/10.7554/eLife.05033>.
- [204] Koji Onomoto, Kazuhide Onoguchi, and Mitsutoshi Yoneyama. Regulation of RIG-I-like receptor-mediated signaling: interaction between host and viral factors. *Cellular & Molecular Immunology*, 18(3):539–555, 2021. ISSN 1672-7681. doi: 10.1038/s41423-020-00602-7. URL <http://dx.doi.org/10.1038/s41423-020-00602-7>. tex.journaltitle: Cell. Mol. Immunol.
- [205] Jamie Nourse, Stefano Spada, and Sven Danckwardt. Emerging roles of RNA 3'-end cleavage and polyadenylation in pathogenesis, diagnosis and therapy of human disorders. *Biomolecules*, 10(6):915, June 2020. ISSN 2218-273X. doi: 10.3390/biom10060915. URL <http://dx.doi.org/10.3390/biom10060915>.
- [206] Wenyan Li and Yao Wang. Stress granules: potential therapeutic targets for infectious and inflammatory diseases. *Frontiers in Immunology*, 14:1145346, May 2023. ISSN 1664-3224. doi: 10.3389/fimmu.2023.1145346. URL <http://dx.doi.org/10.3389/fimmu.2023.1145346>. tex.journaltitle: Front. Immunol.
- [207] Lavanya Manjunath, Sunwoo Oh, Pedro Ortega, Alexis Bouin, Elodie Bournique, Ambrocio Sanchez, Pia Møller Martensen, Ashley A Auerbach, Jordan T Becker, Marcus Seldin, Reuben S Harris, Bert L Semler, and Rémi Buisson. APOBEC3B drives PKR-mediated translation shutdown and protects stress granules in response to viral infection. *Nat. Commun.*, 14(1):820, February 2023. ISSN 2041-1723. doi: 10.1038/s41467-023-36445-9. URL <http://dx.doi.org/10.1038/s41467-023-36445-9>. Publisher: Nature Publishing Group.
- [208] Pucharee Songprakhon, Aussara Panya, Kornkan Choomee, Thawornchai Limjindaporn, Sansanee Noisakran, Mayuri Tarasuk, and Pa-Thai Yenchitsomanus. Cordycepin exhibits both antiviral and anti-inflammatory effects against dengue virus infection. *iScience*, 27(9):110711, September 2024. ISSN 2589-0042. doi: 10.1016/j.isci.2024.110711.

- [209] Inan Bakan and Mathieu Laplante. Connecting mTORC1 signaling to SREBP-1 activation. *Current Opinion in Lipidology*, 23(3):226–234, 2012. ISSN 0957-9672. doi: 10.1097/mol.0b013e328352dd03. URL <http://dx.doi.org/10.1097/MOL.0b013e328352dd03>. tex.journaltitle: Curr. Opin. Lipidol.
- [210] Hongying Yang, Wei Zhang, Heng Pan, Heidi G Feldser, Elden Lainez, Christine Miller, Stewart Leung, Zhong Zhong, Huizhen Zhao, Sharon Sweitzer, Thomas Considine, Thomas Riera, Vipin Suri, Brian White, James L Ellis, George P Vlasuk, and Christine Loh. SIRT1 activators suppress inflammatory responses through promotion of p65 deacetylation and inhibition of NF- κ B activity. *PLoS One*, 7(9):e46364, September 2012. ISSN 1932-6203. doi: 10.1371/journal.pone.0046364. URL <http://dx.doi.org/10.1371/journal.pone.0046364>.
- [211] Shengli Hao and David Baltimore. The stability of mRNA influences the temporal order of the induction of genes encoding inflammatory molecules. *Nature Immunology*, 10(3):281–288, March 2009. ISSN 1529-2908. doi: 10.1038/ni.1699. URL <http://dx.doi.org/10.1038/ni.1699>. tex.journaltitle: Nat. Immunol.
- [212] Dirk H Ostareck and Antje Ostareck-Lederer. RNA-binding proteins in the control of LPS-induced macrophage response. *Front. Genet.*, 10:31, February 2019. ISSN 1664-8021. doi: 10.3389/fgene.2019.00031. URL <http://dx.doi.org/10.3389/fgene.2019.00031>. Publisher: Frontiers Media SA.
- [213] Zhen Yang Cui, Soo Jung Park, Eunbi Jo, In-Hu Hwang, Kyung-Bok Lee, Sung-Woo Kim, Dae Joon Kim, Jong Chun Joo, Seok Hoon Hong, Min-Goo Lee, and Ik-Soon Jang. Cordycepin induces apoptosis of human ovarian cancer cells by inhibiting CCL5-mediated Akt/NF- κ B signaling pathway. *Cell Death Discov.*, 4(1):62, May 2018. ISSN 2058-7716. doi: 10.1038/s41420-018-0063-4. URL <http://dx.doi.org/10.1038/s41420-018-0063-4>.
- [214] Francisco Sanchez-Vega, Marco Mina, Joshua Armenia, Walid K Chatila, Augustin Luna, Konnor C La, Sofia Dimitriadoy, David L Liu, Havish S Kantheti, and Saghafinia. Oncogenic Signaling Pathways in The Cancer Genome Atlas. *Cell*, 173(2):321–337.e10, April 2018. ISSN 0092-8674. doi: 10.1016/j.cell.2018.03.035. URL <https://www.sciencedirect.com/science/article/pii/S0092867418303593>.
- [215] Bruce S Hostager and Gail A Bishop. CD40-mediated activation of the NF- κ B2 pathway. *Frontiers in Immunology*, 4:376, November 2013. ISSN 1664-3224. doi: 10.3389/fimmu.2013.00376. URL <http://dx.doi.org/10.3389/fimmu.2013.00376>. tex.journaltitle: Front. Immunol.
- [216] Lee Swanson, Gajanan D Katkar, Julian Tam, Rama F Pranadinata, Yogitha Chareddy, Jane Coates, Mahitha Shree Anandachar, Vanessa Castillo, Joshua Olson, Victor Nizet, Irina Kufareva, Soumita Das, and Pradipta Ghosh. TLR4 signaling and macrophage inflammatory responses are dampened by GIV/Girdin. *Proceedings of the National Academy of Sciences of the United States of America*, 117(43):26895–26906, October 2020. ISSN 0027-8424. doi: 10.1073/pnas.2011667117. URL <http://dx.doi.org/10.1073/pnas.2011667117>. tex.journaltitle: Proc. Natl. Acad. Sci. U. S. A.
- [217] Valentina Carlini, Douglas M Noonan, Eslam Abdalalem, Delia Goletti, Clementina Sansone, Luana Calabrone, and Adriana Albini. The multifaceted

- nature of IL-10: regulation, role in immunological homeostasis and its relevance to cancer, COVID-19 and post-COVID conditions. *Front. Immunol.*, 14: 1161067, June 2023. ISSN 1664-3224. doi: 10.3389/fimmu.2023.1161067. URL <http://dx.doi.org/10.3389/fimmu.2023.1161067>. Publisher: Frontiers.
- [218] Mathieu Laplante and David M Sabatini. An emerging role of mTOR in lipid biosynthesis. *Curr. Biol.*, 19(22):R1046–52, December 2009. ISSN 0960-9822. doi: 10.1016/j.cub.2009.09.058. URL <http://dx.doi.org/10.1016/j.cub.2009.09.058>. Publisher: Elsevier BV.
- [219] Mousheng Wu, Michael Reuter, Hauke Lilie, Yuying Liu, Elmar Wahle, and Haiwei Song. Structural insight into poly(A) binding and catalytic mechanism of human PARN: Crystal structure of PARN. *EMBO J.*, 24(23):4082–4093, December 2005. ISSN 0261-4189. doi: 10.1038/sj.emboj.7600869. URL <http://dx.doi.org/10.1038/sj.emboj.7600869>.
- [220] Martin D. Brand and David G. Nicholls. Assessing mitochondrial dysfunction in cells. *The Biochemical Journal*, 435(2):297–312, April 2011. ISSN 1470-8728. doi: 10.1042/BJ20110162.
- [221] Muhammed Hasan Çelik and Ali Mortazavi. Analysis of alternative polyadenylation from long-read or short-read RNA-seq with LAPA, November 2022. URL <https://www.biorxiv.org/content/10.1101/2022.11.08.515683v1>. Pages: 2022.11.08.515683 Section: New Results.
- [222] Hsin-Wei Tseng, Anthony Mota-Sydor, Rania Leventis, Predrag Jovanovic, Ivan Topisirovic, and Thomas F Duchaine. Distinct, opposing functions for CFIm59 and CFIm68 in mRNA alternative polyadenylation of Pten and in the PI3K/Akt signalling cascade. *Nucleic Acids Research*, 50(16):9397–9412, September 2022. ISSN 0305-1048. doi: 10.1093/nar/gkac704. URL <https://doi.org/10.1093/nar/gkac704>.
- [223] Ting Shen, Huan Li, Yifang Song, Li Li, Jinzhong Lin, Gang Wei, and Ting Ni. Alternative polyadenylation dependent function of splicing factor SRSF3 contributes to cellular senescence. *Aging*, 11(5):1356–1388, March 2019. ISSN 1945-4589. doi: 10.18632/aging.101836. URL <https://www.aging-us.com/article/101836/text>.
- [224] Shirong Zhang, Xiaochen Zhang, Wei Lei, Jiafeng Liang, Yasi Xu, Hailiang Liu, and Shenglin Ma. Genome-wide profiling reveals alternative polyadenylation of mRNA in human non-small cell lung cancer. *Journal of Translational Medicine*, 17(1):257, August 2019. ISSN 1479-5876. doi: 10.1186/s12967-019-1986-0. URL <https://doi.org/10.1186/s12967-019-1986-0>.
- [225] Jae-Woong Chang, Wei Zhang, Hsin-Sung Yeh, Meeyeon Park, Chengguo Yao, Yongsheng Shi, Rui Kuang, and Jeongsik Yong. An integrative model for alternative polyadenylation, IntMAP, delineates mTOR-modulated endoplasmic reticulum stress response. *Nucleic Acids Research*, 46(12):5996–6008, July 2018. ISSN 0305-1048. doi: 10.1093/nar/gky340. URL <https://doi.org/10.1093/nar/gky340>.
- [226] Richard S. Herron, Alexander Kunisky, and Hun-Way Hwang. Identification of a direct role of CPSF6 and a key cis-acting motif in promoting TRIM9 proximal polyA site usage. *The FASEB Journal*, 36(S1),

2022. ISSN 1530-6860. doi: 10.1096/fasebj.2022.36.S1.R5796. URL <https://onlinelibrary.wiley.com/doi/abs/10.1096/fasebj.2022.36.S1.R5796>. _eprint: <https://faseb.onlinelibrary.wiley.com/doi/pdf/10.1096/fasebj.2022.36.S1.R5796>.
- [227] Ali Hassan and Corinne Aubel. The PI3K/Akt/mTOR Signaling Pathway in Triple-Negative Breast Cancer: A Resistance Pathway and a Prime Target for Targeted Therapies. *Cancers*, 17(13):2232, July 2025. ISSN 2072-6694. doi: 10.3390/cancers17132232. URL <https://pmc.ncbi.nlm.nih.gov/articles/PMC12248877/>.
- [228] Sabrina D’Agostino, Caitlin Davies, Marissa V Powers, and Paul A Clarke. Advances in understanding and targeting eIF4E activity. *Biochemical Society Transactions*, page BST20241045, July 2025. ISSN 0300-5127. doi: 10.1042/BST20241045. URL <https://pmc.ncbi.nlm.nih.gov/articles/PMC12410004/>.
- [229] Angelika Fedor, Krzysztof Bryniarski, and Katarzyna Nazimek. mTOR Signaling in Macrophages: All Depends on the Context. *International Journal of Molecular Sciences*, 26(15):7598, August 2025. ISSN 1422-0067. doi: 10.3390/ijms26157598. URL <https://pmc.ncbi.nlm.nih.gov/articles/PMC12347599/>.
- [230] Ran Huang, Qiong Dai, Ruixue Yang, Yi Duan, Qi Zhao, Johannes Haybaeck, and Zhihui Yang. A Review: PI3K/AKT/mTOR Signaling Pathway and Its Regulated Eukaryotic Translation Initiation Factors May Be a Potential Therapeutic Target in Esophageal Squamous Cell Carcinoma. *Frontiers in Oncology*, 12, April 2022. ISSN 2234-943X. doi: 10.3389/fonc.2022.817916. URL <https://www.frontiersin.org/journals/oncology/articles/10.3389/fonc.2022.817916/full>. Publisher: Frontiers.
- [231] Katrin Eichelbaum and Jeroen Krijgsveld. Rapid Temporal Dynamics of Transcription, Protein Synthesis, and Secretion during Macrophage Activation *. *Molecular & Cellular Proteomics*, 13(3):792–810, March 2014. ISSN 1535-9476, 1535-9484. doi: 10.1074/mcp.M113.030916. URL [https://www.mcponline.org/article/S1535-9476\(20\)33391-0/abstract](https://www.mcponline.org/article/S1535-9476(20)33391-0/abstract). Publisher: Elsevier.
- [232] Marcia R. Saban, Helen Hellmich, Ngoc-Bich Nguyen, John Winston, Timothy G. Hammond, and Ricardo Saban. Time course of LPS-induced gene expression in a mouse model of genitourinary inflammation. *Physiological Genomics*, 5(3):147–160, April 2001. ISSN 1094-8341. doi: 10.1152/physiolgenomics.2001.5.3.147. URL <https://journals.physiology.org/doi/full/10.1152/physiolgenomics.2001.5.3.147>. Publisher: American Physiological Society.
- [233] Yiqing Pan, Chen Xu, and Zhixing K. Pan. MKP-1 negative regulates Staphylococcus aureus induced inflammatory responses in Raw264.7 cells: roles of PKA-MKP-1 pathway and enhanced by rolipram. *Scientific Reports*, 7(1):12366, September 2017. ISSN 2045-2322. doi: 10.1038/s41598-017-10187-3. URL <https://www.nature.com/articles/s41598-017-10187-3>. Publisher: Nature Publishing Group.
- [234] Vera P. Pisareva, Andrey V. Pisarev, Anton A. Komar, Christopher U. T. Hellen, and Tatyana V. Pestova. Translation Initiation on Mammalian mRNAs with Structured 5UTRs Requires DExH-Box Protein DHX29. *Cell*, 135(7):1237–1250, December 2008. ISSN 0092-8674, 1097-4172. doi: 10.1016/j.cell.2008.10.037. URL [https://www.cell.com/cell/abstract/S0092-8674\(08\)01374-3](https://www.cell.com/cell/abstract/S0092-8674(08)01374-3). Publisher: Elsevier.

- [235] Adam M. Heck and Jeffrey Wilusz. The Interplay between the RNA Decay and Translation Machinery in Eukaryotes. *Cold Spring Harbor Perspectives in Biology*, 10(5):a032839, May 2018. ISSN , 1943-0264. doi: 10.1101/cshperspect.a032839. URL <http://cshperspectives.cshlp.org/content/10/5/a032839>. Company: Cold Spring Harbor Laboratory Press Distributor: Cold Spring Harbor Laboratory Press Institution: Cold Spring Harbor Laboratory Press Label: Cold Spring Harbor Laboratory Press Publisher: Cold Spring Harbor Lab.
- [236] Ramona Weber, Min-Yi Chung, Csilla Keskeny, Ulrike Zinnall, Markus Landthaler, Eugene Valkov, Elisa Izaurralde, and Cátia Igreja. 4EHP and GIGYF1/2 Mediate Translation-Coupled Messenger RNA Decay. *Cell Reports*, 33(2), October 2020. ISSN 2211-1247. doi: 10.1016/j.celrep.2020.108262. URL [https://www.cell.com/cell-reports/abstract/S2211-1247\(20\)31251-1](https://www.cell.com/cell-reports/abstract/S2211-1247(20)31251-1). Publisher: Elsevier.
- [237] Diego García-Weber and Cécile Arrieumerlou. ADP-heptose: a bacterial PAMP detected by the host sensor ALPK1. *Cellular and Molecular Life Sciences*, 78(1): 17–29, January 2021. ISSN 1420-9071. doi: 10.1007/s00018-020-03577-w. URL <https://doi.org/10.1007/s00018-020-03577-w>.
- [238] Alexander Hoffmann, Andre Levchenko, Martin L. Scott, and David Baltimore. The I κ B-NF- κ B Signaling Module: Temporal Control and Selective Gene Activation. *Science*, 298(5596):1241–1245, November 2002. doi: 10.1126/science.1071914. URL <https://www.science.org/doi/10.1126/science.1071914>. Publisher: American Association for the Advancement of Science.
- [239] L F Lau and D Nathans. Expression of a set of growth-related immediate early genes in BALB/c 3T3 cells: coordinate regulation with c-fos or c-myc. *Proceedings of the National Academy of Sciences*, 84(5):1182–1186, March 1987. ISSN 0027-8424, 1091-6490. doi: 10.1073/pnas.84.5.1182. URL <https://pnas.org/doi/full/10.1073/pnas.84.5.1182>.
- [240] Maria Ivshina, Ilya M. Alexandrov, Anastassiia Vertii, Stephen Doxsey, and Joel D. Richter. CPEB regulation of TAK1 synthesis mediates cytokine production and the inflammatory immune response. *Molecular and Cellular Biology*, 35(3):610–618, February 2015. ISSN 1098-5549. doi: 10.1128/MCB.00800-14.
- [241] Xuemei Liu, Taolang Li, Brigitte Riederer, Henrike Lenzen, Lisa Ludolph, Sunil Yeruva, Biguang Tuo, Manoocher Soleimani, and Ursula Seidler. Loss of Slc26a9 anion transporter alters intestinal electrolyte and HCO₃⁻ transport and reduces survival in CFTR-deficient mice. *Pflügers Archiv: European Journal of Physiology*, 467(6):1261–1275, June 2015. ISSN 1432-2013. doi: 10.1007/s00424-014-1543-x.
- [242] Hannes Lohi, Minna Kujala, Siru Mäkelä, Eero Lehtonen, Marjo Kestilä, Ulpu Saarialho-Kere, Daniel Markovich, and Juha Kere. Functional Characterization of Three Novel Tissue-specific Anion Exchangers SLC26A7, -A8, and -A9*. *Journal of Biological Chemistry*, 277(16):14246–14254, April 2002. ISSN 0021-9258. doi: 10.1074/jbc.M111802200. URL <https://www.sciencedirect.com/science/article/pii/S0021925819610331>.
- [243] Zhiyuan Ma, Hu Wang, Zhengxing Zhou, Chengli Lu, Minglin Zhang, Renmin Mu, Chengmin Zhang, Zhiqiang Yi, Zilin Deng, Yingying Zhao, Jiaying Zhu, Guorong Wen, Hai Jin, Jiaying An, Biguang Tuo, Peng Yuan, Xuemei Liu, and

- Taolang Li. SLC26A9 promotes the initiation and progression of breast cancer by activating the PI3K/AKT signaling pathway. *Biochimica et Biophysica Acta (BBA) - Molecular Cell Research*, 1872(3):119912, March 2025. ISSN 0167-4889. doi: 10.1016/j.bbamcr.2025.119912. URL <https://www.sciencedirect.com/science/article/pii/S0167488925000175>.
- [244] Clara Suñer, Annarita Sibilio, Judit Martín, Chiara Lara Castellazzi, Oscar Reina, Ivan Dotu, Adrià Caballé, Elisa Rivas, Vittorio Calderone, Juana Díez, Angel R. Nebreda, and Raúl Méndez. Macrophage inflammation resolution requires CPEB4-directed offsetting of mRNA degradation. *eLife*, 11:e75873, April 2022. ISSN 2050-084X. doi: 10.7554/eLife.75873.
- [245] Vladyslava Liudkovska, Paweł S Krawczyk, Aleksandra Brouze, Natalia Gumińska, Tomasz Wegierski, Dominik Cysewski, Zuzanna Mackiewicz, Jonathan J Ewbank, Krzysztof Drabikowski, Seweryn Mroczek, and Andrzej Dziembowski. TENT5 cytoplasmic noncanonical poly(A) polymerases regulate the innate immune response in animals. *Science Advances*, 8(46):eadd9468, November 2022. ISSN 2375-2548. doi: 10.1126/sciadv.add9468. URL <http://dx.doi.org/10.1126/sciadv.add9468>. tex.journaltitle: Sci. Adv.
- [246] Jordan Wengrod, Ding Wang, Sarah Weiss, Hua Zhong, Iman Osman, and Lawrence B. Gardner. Phosphorylation of eIF2 α by mTORC1 inhibition and PP6C activation is required for autophagy and is aberrant in PP6C-mutated melanoma. *Science signaling*, 8(367):ra27, March 2015. ISSN 1945-0877. doi: 10.1126/scisignal.aaa0899. URL <https://pmc.ncbi.nlm.nih.gov/articles/PMC4580977/>.
- [247] Mateusz Maździarz, Łukasz Paukszto, and Jakub Sawicki. TAILcaller: an R package for analyzing differences in poly(A) tail length for Oxford Nanopore RNA sequencing. *Bioinformatics Advances*, 5(1):vbaf235, January 2025. ISSN 2635-0041. doi: 10.1093/bioadv/vbaf235. URL <https://doi.org/10.1093/bioadv/vbaf235>.

Appendix A

List of Abbreviations

The table below lists the recurring abbreviations used throughout Chapters 1–8.

Abbreviation	Meaning
3DI	3'-deoxyinosine
3'-dAMP	3'-deoxyadenosine monophosphate
3'-dATP	3'-deoxyadenosine triphosphate
4E-BP1	eukaryotic translation initiation factor 4E-binding protein 1
A549	Human lung adenocarcinoma epithelial cell line
ADA	Adenosine deaminase
ADK	Adenosine kinase
ADME	Absorption, distribution, metabolism and excretion
ADSL	Adenylosuccinate lyase
ADSS2	Adenylosuccinate synthetase 2
AKT	Protein kinase B
AMP	Adenosine monophosphate
AMPK	AMP-activated protein kinase
APA	Alternative polyadenylation
APRT	Adenine phosphoribosyltransferase
ARE	AU-rich element
ASM	Airway smooth muscle
ATP	Adenosine triphosphate
AUC	Area under the curve
BAM	Binary Alignment/Map format
BH	Benjamini-Hochberg
CAB	Centre for Analytical Bioscience
Caco-2	Human colorectal adenocarcinoma epithelial cell line
cDNA	Complementary DNA
CCR4-NOT	Major cytoplasmic deadenylase complex
CFIm	Cleavage factor I mammalian
cGKII	cGMP-dependent protein kinase II
cGMP	Cyclic guanosine monophosphate
CoMP	Cordycepin monophosphate
CoR	Cordycepin
CoTP	Cordycepin triphosphate
CPA	Cleavage and polyadenylation
CPEB	Cytoplasmic polyadenylation element-binding protein

Continued on next page

Abbreviation	Meaning
CPSF	Cleavage and polyadenylation specificity factor
CstF	Cleavage stimulation factor
DCP1/DCP2	Decapping proteins 1 and 2
DE	Differential expression
DEG	Differentially expressed gene
DGE	Differential gene expression
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
ECDF	Empirical cumulative distribution function
eHAP	Engineered haploid human cell line derived from HAP1
EGF	Epidermal growth factor
EHNA	Erythro-9-(2-hydroxy-3-nonyl)adenine
ENT1	Equilibrative nucleoside transporter 1
ENT2	Equilibrative nucleoside transporter 2
ER	Endoplasmic reticulum
ERK	Extracellular signal-regulated kinase
FaSSGF	Fasted-state simulated gastric fluid
FaSSIF	Fasted-state simulated intestinal fluid
FAMIN/LACC1	Fatty acid metabolism-immunity nexus / laccase domain-containing 1
FBS	Foetal bovine serum
FDR	False discovery rate
FeSSIF	Fed-state simulated intestinal fluid
GC	Guanine-cytosine
GCN2	General control nonderepressible 2 kinase
GLM	Generalized linear model
GTF	Gene Transfer Format
HAP1	Human haploid cell line
hENT1	Human equilibrative nucleoside transporter 1
HILIC	Hydrophilic interaction liquid chromatography
HINT1	Histidine triad nucleotide-binding protein 1
HPC	High-performance computing
HPLC	High-performance liquid chromatography
HPRT	Hypoxanthine phosphoribosyltransferase
HTCondor	High-throughput computing workload manager
hyPB	Hyperactive piggyBac transposase
IFN	Interferon
IGF-1	Insulin-like growth factor 1
IL	Interleukin
IPA	Ingenuity Pathway Analysis
IR	Inverted repeat
ISR	Integrated stress response
ITR	Inverted terminal repeat
LARP1	La-related protein 1
LAST	Local Alignment Search Tool
LC-MS	Liquid chromatography-mass spectrometry
LC-MS/MS	Liquid chromatography-tandem mass spectrometry

Continued on next page

Abbreviation	Meaning
LMM	Linear mixed-effects model
lncRNA	Long non-coding RNA
LoF	Loss of function
LPS	Lipopolysaccharide
MA	Log ratio versus mean average abundance plot
MAPK	Mitogen-activated protein kinase
MAPQ	Mapping quality
MCF-7	Human breast adenocarcinoma cell line
MEK	Mitogen-activated protein kinase kinase
mRNA	Messenger RNA
mRNP	Messenger ribonucleoprotein
MS	Mass spectrometry
MTAP	Methylthioadenosine phosphorylase
mTOR	Mechanistic target of rapamycin
mTORC1	Mechanistic target of rapamycin complex 1
mTORC2	Mechanistic target of rapamycin complex 2
NBTI	Nitrobenzylthioinosine
NF- κ B	Nuclear factor kappa B
NO	Nitric oxide
NT5C2	Cytosolic 5'-nucleotidase II
NUC	NUC-7738
NUC-7738	Phosphoramidate ProTide form of cordycepin
OCR	Oxygen consumption rate
ONT	Oxford Nanopore Technologies
PABP	Poly(A)-binding protein
PABPC1	Poly(A)-binding protein cytoplasmic 1
PABPN1	Poly(A)-binding protein nuclear 1
PAIP1	PABP-interacting protein 1
PAP	Poly(A) polymerase
PARN	Poly(A)-specific ribonuclease
PAS	Polyadenylation site
PB	piggyBac transposon system
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PDGF	Platelet-derived growth factor
PDK1	Phosphoinositide-dependent kinase 1
PGM2	Phosphopentomutase 2
PH	Pleckstrin homology
PI3K	Phosphoinositide 3-kinase
PIP2	Phosphatidylinositol 4,5-bisphosphate
PIP3	Phosphatidylinositol 3,4,5-trisphosphate
PKR	Protein kinase R
PNP	Purine nucleoside phosphorylase
POD5	Oxford Nanopore raw signal file format
poly(A)	Polyadenosine
PRPP	Phosphoribosyl pyrophosphate
PRPS1	Phosphoribosyl pyrophosphate synthetase 1
ProTide	Phosphoramidate prodrug technology

Continued on next page

Abbreviation	Meaning
QC	Quality control
qPCR	Quantitative polymerase chain reaction
Q-Exactive	Thermo Q-Exactive Orbitrap mass spectrometer
RAF	RAF kinase family
RAW264.7	Murine macrophage cell line
RIN	RNA integrity number
RNA	Ribonucleic acid
RNA-Seq	RNA sequencing
RNAPII	RNA polymerase II
RTcC	RNA target of cordycepin
RTK	Receptor tyrosine kinase
RT-qPCR	Reverse transcription quantitative polymerase chain reaction
SEM	Standard error of the mean
siRNA	Small interfering RNA
suPB	Super piggyBac transposase
Synth CoR	Synthetic cordycepin
T75	75 cm ² tissue culture flask
TLR4	Toll-like receptor 4
TMM	Trimmed mean of M-values
TNF	Tumour necrosis factor
TNFR1	Tumour necrosis factor receptor 1
TNFR2	Tumour necrosis factor receptor 2
TOP	Terminal oligopyrimidine
TTAA	piggyBac target tetranucleotide insertion site
uORF	Upstream open reading frame
UTR	Untranslated region
XIC	Extracted ion chromatogram
ZIC-pHILIC	Zwitterionic hydrophilic interaction liquid chromatography

Appendix B

Bioinformatics Scripts and Code

B.1 Quality Control Scripts

Listing B.1 shows the LongQC execution script used for quality control of FASTQ files from the 18-sample sequencing experiment.

```
1 #!/bin/bash
2
3 # Activate conda environment
4 eval "$(conda shell.bash hook)"
5 source ~/.bashrc
6 conda activate longqc
7
8 # Define input files and output directory
9 # Array of input FASTQ files for 18 samples (6 conditions x 3
   replicates)
10 INPUT_FILES=(
11     "/users/paxak6/RAW264_18samples_120min/
   P1_and_P2_Pooled_Concatenated_Fastq/barcode02/DMSO_LPS_1.fastq
   .gz"
12     "/users/paxak6/RAW264_18samples_120min/
   P1_and_P2_Pooled_Concatenated_Fastq/barcode08/DMSO_LPS_2.fastq
   .gz"
13     "/users/paxak6/RAW264_18samples_120min/
   P1_and_P2_Pooled_Concatenated_Fastq/barcode14/DMSO_LPS_3.fastq
   .gz"
14     "/users/paxak6/RAW264_18samples_120min/
   P1_and_P2_Pooled_Concatenated_Fastq/barcode03/3DI_LPS_1.fastq.
   gz"
15     "/users/paxak6/RAW264_18samples_120min/
   P1_and_P2_Pooled_Concatenated_Fastq/barcode09/3DI_LPS_2.fastq.
   gz"
16     "/users/paxak6/RAW264_18samples_120min/
   P1_and_P2_Pooled_Concatenated_Fastq/barcode15/3DI_LPS_3.fastq.
   gz"
17     "/users/paxak6/RAW264_18samples_120min/
   P1_and_P2_Pooled_Concatenated_Fastq/barcode04/Synth_CoR_LPS_1.
   fastq.gz"
18     "/users/paxak6/RAW264_18samples_120min/
   P1_and_P2_Pooled_Concatenated_Fastq/barcode10/Synth_CoR_LPS_2.
   fastq.gz"
```

```

19     "/users/paxak6/RAW264_18samples_120min/
    P1_and_P2_Pooled_Concatenated_Fastq/barcode16/Synth_CoR_LPS_3.
    fastq.gz"
20     "/users/paxak6/RAW264_18samples_120min/
    P1_and_P2_Pooled_Concatenated_Fastq/barcode05/NUC_LPS_1.fastq.
    gz"
21     "/users/paxak6/RAW264_18samples_120min/
    P1_and_P2_Pooled_Concatenated_Fastq/barcode11/NUC_LPS_2.fastq.
    gz"
22     "/users/paxak6/RAW264_18samples_120min/
    P1_and_P2_Pooled_Concatenated_Fastq/barcode17/NUC_LPS_3.fastq.
    gz"
23     "/users/paxak6/RAW264_18samples_120min/
    P1_and_P2_Pooled_Concatenated_Fastq/barcode06/CoR_LPS_1.fastq.
    gz"
24     "/users/paxak6/RAW264_18samples_120min/
    P1_and_P2_Pooled_Concatenated_Fastq/barcode12/CoR_LPS_2.fastq.
    gz"
25     "/users/paxak6/RAW264_18samples_120min/
    P1_and_P2_Pooled_Concatenated_Fastq/barcode18/CoR_LPS_3.fastq.
    gz"
26 )
27 # Directory to store LongQC output
28 OUTPUT_DIR="/users/paxak6/RAW264_18samples_120min/
    LongQC_output_18samples_120min"
29
30 # Create output directory if it doesn't exist
31 mkdir -p "$OUTPUT_DIR"
32
33 # Check if all input files are readable
34 for input_file in "${INPUT_FILES[@]"; do
35     if [ ! -r "$input_file" ]; then
36         echo "Error: Cannot read input file $input_file. Please
        check file permissions."
37         exit 1
38     fi
39 done
40
41 # Run LongQC for each input file
42 for input_file in "${INPUT_FILES[@]"; do
43     # Extract sample name from input file path
44     sample_name=$(basename "$input_file" .fastq.gz)
45     echo "Processing $sample_name"
46
47     # Run LongQC command
48     python /users/paxak6/LongQC/longQC.py sampleqc \
49         -x ont-rapid \
50         -o "$OUTPUT_DIR/$sample_name" \
51         "$input_file"
52
53     # Check if LongQC ran successfully
54     if [ $? -eq 0 ]; then
55         echo "LongQC completed successfully for $sample_name"

```

```

56     else
57         echo "LongQC encountered an error for $sample_name"
58         exit 1 # Exit the script if an error occurs
59     fi
60 done
61
62 echo "All samples processed successfully"

```

Listing B.1: LongQC Execution Script

B.2 Haploid Genetic Screening Scripts

Listing B.2 demonstrates the multi-step filtering approach used to identify the best alignment hit for each read during the haploid genetic screening analysis.

```

1 # Extract alignment information and identify best hit per read
2 cut -f7,8,9,10,13 alignments.tsv | \
3     awk 'BEGIN{FS="\t";OFS="\t"}{print $5,$2,$3,$4,$1}' | \
4     sort -g -k1,1 | nl | sort -k6,6 -k1,1n | uniq -f5

```

Listing B.2: multi-step filtering approach for alignments.tsv file

Listing B.3 shows the minimap2 configuration and parameters used for mapping filtered reads to the GRCh38 reference genome.

```

1 minimap2 -ax map-ont --secondary=no -t 4 reference.fa reads.fq.
   gz | \
2     samtools view -h -b -q 10 | \
3     samtools sort > mapped.bam
4
5 # Key mapping parameters included:
6 # - Preset: -ax map-ont (optimized for Oxford Nanopore reads)
7 # - Secondary alignments: suppressed (--secondary=no)
8 # - Minimum mapping quality (MAPQ): 10
9 # - Threads: 4

```

Listing B.3: Minimap2 setting

B.3 RNA Sequencing Analysis Scripts

Listing B.4 presents the IsoQuant script used for gene quantification from long-read RNA sequencing data across all 18 samples.

```

1 #!/bin/bash
2
3 # Initialize conda
4 eval "$(conda shell.bash hook)"
5
6 # Activate conda environment
7 source ~/.bashrc
8 conda activate isoquant
9
10 # Check IsoQuant version
11 isoquant.py --version
12
13 # Set the number of threads based on Condor allocation

```

```

14 # If CONDOR_CPUS is not set, default to 16
15 THREADS=${CONDOR_CPUS:-$(nproc)}
16
17 # Define the output directory
18 OUTPUT_DIR="/users/paxak6/RAW264_18samples_120min/IsoQuant/
    IsoQuant_Results_Without_Illumina_BAM_24042025"
19
20 echo "Starting IsoQuant run with $THREADS threads..."
21 # Ensure the output directory exists
22 mkdir -p "$OUTPUT_DIR"
23
24 isoquant.py \
25 --reference /users/paxak6/RAW264_15min/ref_gtf/GRCm39.
    primary_assembly_genome.fa.gz \
26 --genedb /users/paxak6/RAW264_15min/ref_gtf/gencode.vM34.
    primary_assembly.basic_annotation.gtf --complete_genedb \
27 --fastq \
28 /users/paxak6/RAW264_18samples_120min/
    P1_and_P2_Pooled_Concatenated_Fastq/barcode01/DMSO_1.fastq.gz
    \
29 /users/paxak6/RAW264_18samples_120min/
    P1_and_P2_Pooled_Concatenated_Fastq/barcode07/DMSO_2.fastq.gz
    \
30 /users/paxak6/RAW264_18samples_120min/
    P1_and_P2_Pooled_Concatenated_Fastq/barcode13/DMSO_3.fastq.gz
    \
31 /users/paxak6/RAW264_18samples_120min/
    P1_and_P2_Pooled_Concatenated_Fastq/barcode02/DMSO_LPS_1.fastq
    .gz \
32 /users/paxak6/RAW264_18samples_120min/
    P1_and_P2_Pooled_Concatenated_Fastq/barcode08/DMSO_LPS_2.fastq
    .gz \
33 /users/paxak6/RAW264_18samples_120min/
    P1_and_P2_Pooled_Concatenated_Fastq/barcode14/DMSO_LPS_3.fastq
    .gz \
34 /users/paxak6/RAW264_18samples_120min/
    P1_and_P2_Pooled_Concatenated_Fastq/barcode03/3DI_LPS_1.fastq.
    gz \
35 /users/paxak6/RAW264_18samples_120min/
    P1_and_P2_Pooled_Concatenated_Fastq/barcode09/3DI_LPS_2.fastq.
    gz \
36 /users/paxak6/RAW264_18samples_120min/
    P1_and_P2_Pooled_Concatenated_Fastq/barcode15/3DI_LPS_3.fastq.
    gz \
37 /users/paxak6/RAW264_18samples_120min/
    P1_and_P2_Pooled_Concatenated_Fastq/barcode04/Synth_CoR_LPS_1.
    fastq.gz \
38 /users/paxak6/RAW264_18samples_120min/
    P1_and_P2_Pooled_Concatenated_Fastq/barcode10/Synth_CoR_LPS_2.
    fastq.gz \
39 /users/paxak6/RAW264_18samples_120min/
    P1_and_P2_Pooled_Concatenated_Fastq/barcode16/Synth_CoR_LPS_3.
    fastq.gz \

```



```

40 /users/paxak6/RAW264_18samples_120min/
    P1_and_P2_Pooled_Concatenated_Fastq/barcode05/NUC_LPS_1.fastq.
    gz \
41 /users/paxak6/RAW264_18samples_120min/
    P1_and_P2_Pooled_Concatenated_Fastq/barcode11/NUC_LPS_2.fastq.
    gz \
42 /users/paxak6/RAW264_18samples_120min/
    P1_and_P2_Pooled_Concatenated_Fastq/barcode17/NUC_LPS_3.fastq.
    gz \
43 /users/paxak6/RAW264_18samples_120min/
    P1_and_P2_Pooled_Concatenated_Fastq/barcode06/CoR_LPS_1.fastq.
    gz \
44 /users/paxak6/RAW264_18samples_120min/
    P1_and_P2_Pooled_Concatenated_Fastq/barcode12/CoR_LPS_2.fastq.
    gz \
45 /users/paxak6/RAW264_18samples_120min/
    P1_and_P2_Pooled_Concatenated_Fastq/barcode18/CoR_LPS_3.fastq.
    gz \
46 --stranded none \
47 --data_type ont \
48 --genedb_output /users/paxak6/RAW264_18samples_120min/IsoQuant/
    IsoQuant_gene_db_Without_Illumina_BAM \
49 --no_model_construction \
50 --gene_quantification unique_splicing_consistent \
51 --threads $THREADS \
52 --label DMSO_1 DMSO_2 DMSO_3 DMSO_LPS_1 DMSO_LPS_2 DMSO_LPS_3 3
    DI_LPS_1 3DI_LPS_2 3DI_LPS_3 Synth_CoR_LPS_1 Synth_CoR_LPS_2
    Synth_CoR_LPS_3 NUC_LPS_1 NUC_LPS_2 NUC_LPS_3 CoR_LPS_1
    CoR_LPS_2 CoR_LPS_3 \
53 --keep_tmp \
54 --output "$OUTPUT_DIR"
55
56 # Check if IsoQuant ran successfully
57 if [ $? -eq 0 ]; then
58     echo "IsoQuant completed successfully."
59 else
60     echo "IsoQuant encountered an error."
61     exit 1
62 fi

```

Listing B.4: IsoQuant Script

Listing B.5 provides the Condor submit file configuration for running IsoQuant on a high-performance computing cluster.

```

1 # Condor submit file for running or resuming IsoQuant analysis
2
3 # Including Path to Condor Common Template
4 FNAME = isoquant_run_Without_Illumina_BAM
5
6 include : /users/paxak6/condor_common.tmpl
7
8 # Job description
9 universe = vanilla
10 getenv = True

```

```

11
12 executable = /users/paxak6/RAW264_18samples_120min/IsoQuant/
    run_isoquant_Without_Illumina_BAM_18samples_120min_v3.sh
13
14 # Resources Requirements at Least 50 GB RAM and all the
    available CPUs cores in a workstations
15 request_memory = 50 GB
16 Request_CPUs = Target.TotalCPUs
17 rank = KFlops
18
19 queue 1

```

Listing B.5: condor submit file for IsoQuant to choose an HPC workstation with highest configuration

Listing B.6 presents the Python script used to convert gene IDs from the IsoQuant output to gene names by parsing the GTF annotation file.

```

1 #!/usr/bin/env python3
2
3 import pandas as pd
4
5 def parse_gtf(gtf_file):
6     """
7     Parses a GTF file to extract gene_id (with version) to
    gene_name mapping.
8
9     Parameters:
10    - gtf_file: path to the GTF file
11
12    Returns:
13    - gene_id_to_name: dictionary mapping gene_id (with version)
    to gene_name
14    """
15    gene_id_to_name = {}
16    with open(gtf_file, 'r') as f:
17        for line in f:
18            if line.startswith('#'):
19                continue # Skip header lines
20            fields = line.strip().split('\t')
21            if len(fields) < 9:
22                continue # Skip incomplete lines
23            if fields[2] != 'gene':
24                continue # Process only 'gene' entries
25            # Parse attributes field
26            attributes_field = fields[8]
27            attributes = {}
28            for attr in attributes_field.strip().split(';'):
29                if attr.strip() == '':
30                    continue
31                key_value = attr.strip().split(' ', 1)
32                if len(key_value) == 2:
33                    key = key_value[0]
34                    value = key_value[1].strip('"')
35                    attributes[key] = value

```

```

36         gene_id = attributes.get('gene_id', '')
37         gene_name = attributes.get('gene_name', '')
38         if gene_id and gene_name:
39             # Use gene_id with version as key
40             gene_id_to_name[gene_id] = gene_name
41     return gene_id_to_name
42
43 def main():
44     # Paths to your files
45     counts_file = 'OUT.gene_grouped_counts.tsv'
46     gtf_file = 'gencode.vM34.primary_assembly.basic_annotation.
47     gtf'
48     output_file = 'OUT.gene_grouped_counts_with_gene_names.tsv'
49
50     # Step 1: Parse GTF file to get gene_id to gene_name mapping
51     print("Parsing GTF file to extract gene_id to gene_name
52     mapping...")
53     gene_id_to_name = parse_gtf(gtf_file)
54     print(f"Total gene_id to gene_name mappings: {len(
55     gene_id_to_name)}")
56
57     # Step 2: Read counts file
58     print("Reading counts file...")
59     # Read the header line (which starts with '#')
60     with open(counts_file, 'r') as f:
61         for line in f:
62             if line.startswith('#'):
63                 header = line.strip().lstrip('#').split('\t')
64                 break
65
66     # Read the data, skipping lines starting with '#'
67     df_counts = pd.read_csv(counts_file, sep='\t', comment='#',
68     header=None, names=header)
69     print(f"Total genes in counts file: {len(df_counts)}")
70
71     # Step 3: Map gene IDs to gene names using the mapping from
72     GTF file
73     print("Mapping gene IDs to gene names...")
74     df_counts['Gene_name_mapped'] = df_counts['feature_id'].map(
75     gene_id_to_name)
76
77     # Step 4: For gene IDs not found in mapping, keep original
78     gene ID
79     df_counts['Gene_name_final'] = df_counts['Gene_name_mapped']
80     .fillna(df_counts['feature_id'])
81
82     # Step 5: Drop unnecessary columns
83     df_counts.drop(['feature_id', 'Gene_name_mapped'], axis=1,
84     inplace=True)
85
86     # Step 6: Group counts by gene name, summing counts for
87     genes with the same gene name
88     print("Grouping counts by gene name...")
89     df_counts_grouped = df_counts.groupby('Gene_name_final',

```

```

79     as_index=False).sum()
80
81     # Step 7: Save the result to a new file
82     print("Saving the grouped counts to output file...")
83     df_counts_grouped.rename(columns={'Gene_name_final': 'Gene
84     name'}, inplace=True)
85     df_counts_grouped.to_csv(output_file, sep='\t', index=False,
86     float_format='%.2f')
87     print(f"Output file created: {output_file}")
88     print(f"Total genes after grouping: {len(df_counts_grouped)}
89     ")
90
91     # Optional: Print first few rows of the final DataFrame
92     print("\nFirst few rows of the final DataFrame:")
93     print(df_counts_grouped.head().to_string())
94
95 if __name__ == "__main__":
96     main()

```

Listing B.6: Script to convert genes ID to gene name

Listing B.7 shows the R script used to perform differential gene expression analysis on the normalized count data using the edgeR package.

```

1 # Load required libraries
2 library(edgeR)      # For differential expression analysis
3 library(writexl)    # For writing Excel files
4
5 # Configurable parameters
6 input_file <- "OUT.gene_grouped_counts_with_gene_names.tsv" #
7   Input file with DMSO data
8 output_prefix <- "edgeR_results"
9 fdr_cutoff <- 0.05
10
11 # Load the raw counts file
12 raw_counts <- read.delim(input_file, header=TRUE, sep="\t", row.
13   names=1, check.names=TRUE)
14
15 # Print column names to verify column order
16 cat("Column names in data file:\n")
17 print(colnames(raw_counts))
18
19 # Define groups in the EXACT order they appear in the columns
20 # Using the column order from the print output
21 group <- factor(c(rep("X3DI_LPS",3),
22   rep("CoR_LPS",3),
23   rep("DMSO",3),
24   rep("DMSO_LPS",3),
25   rep("NUC_LPS",3),
26   rep("Synth_CoR_LPS",3)))
27
28 # Verify group assignment is correct
29 cat("\nGroup assignments:\n")
30 print(data.frame(Sample=colnames(raw_counts), Group=group))

```

```

30 # Create a DGEList object from the raw counts and groups
31 y <- DGEList(counts=raw_counts, group=group)
32
33 # Filter out low-count genes (optional, but recommended)
34 keep <- filterByExpr(y, group=group)
35 y <- y[keep,,keep.lib.sizes=FALSE]
36
37 # Normalize using the TMM method
38 y <- calcNormFactors(y, method="TMM")
39
40 # Create a design matrix without an intercept, one column per
  group
41 design <- model.matrix(~0+group)
42 colnames(design) <- levels(group)
43
44 # Print design matrix information for debugging
45 cat("\nDesign matrix column names:\n")
46 print(colnames(design))
47
48 # Estimate dispersions
49 y <- estimateDisp(y, design)
50 fit <- glmFit(y, design)
51
52 #####
53 # Define all the requested comparisons
54 #####
55
56 # Create all contrasts as requested
57 cat("\nCreating contrasts...\n")
58 # Use the exact column names from the design matrix (without '
  group' prefix)
59 my_contrasts <- makeContrasts(
60   # DMSO_LPS vs DMSO (DMSO as control)
61   DMSO_LPS_vs_DMSO = DMSO_LPS - DMSO,
62
63   # Other treatments vs DMSO_LPS (DMSO_LPS as control)
64   X3DI_LPS_vs_DMSO_LPS = X3DI_LPS - DMSO_LPS,
65   CoR_LPS_vs_DMSO_LPS = CoR_LPS - DMSO_LPS,
66   Synth_CoR_LPS_vs_DMSO_LPS = Synth_CoR_LPS - DMSO_LPS,
67   NUC_LPS_vs_DMSO_LPS = NUC_LPS - DMSO_LPS,
68
69   levels = design
70 )
71
72 cat("Contrast matrix created with columns:\n")
73 print(colnames(my_contrasts))
74
75 # Loop over each contrast, perform LRT, and write outputs
76 for (contrast_name in colnames(my_contrasts)) {
77   cat("\nProcessing contrast:", contrast_name, "\n")
78
79   # Perform likelihood ratio test for the given contrast
80   lrt <- glmLRT(fit, contrast=my_contrasts[,contrast_name])

```

```

81
82 # Get all results (no filtering yet)
83 top <- topTags(lrt, n=Inf, p.value=1)
84
85 # Subset significant results based on FDR cutoff
86 sig <- top$table[top$table$FDR < fdr_cutoff, ]
87 cat("Significant genes at FDR <", fdr_cutoff, ":", nrow(sig)
88 , "\n")
89
90 # Move GeneName from rownames to a new column
91 res_full <- top$table
92 res_full <- cbind(GeneName=rownames(res_full), res_full)
93 rownames(res_full) <- NULL
94
95 # Write the full results to a tab-delimited file
96 write.table(res_full,
97             file=paste0(output_prefix, "_", contrast_name, "_
98 full.txt"),
99             sep="\t", quote=FALSE, row.names=FALSE)
100
101 # If we have any significant results, write them to a
102 separate file
103 if (nrow(sig) > 0) {
104     sig_res <- cbind(GeneName=rownames(sig), sig)
105     rownames(sig_res) <- NULL
106     write.table(sig_res,
107                 file=paste0(output_prefix, "_", contrast_name,
108 "_sigFDR_", fdr_cutoff, ".txt"),
109                 sep="\t", quote=FALSE, row.names=FALSE)
110 }
111
112 # Create two additional output files for QIAGEN IPA:
113 # 1. GeneName, logFC, and PValue
114 # 2. GeneName, logFC, and FDR
115 #
116 # These are written as Excel files using the writexl package
117 .
118 #
119 # res_full contains all results; just subset the columns
120 needed.
121 res_for_pval_excel <- res_full[, c("GeneName", "logFC", "
122 PValue")]
123 res_for_fdr_excel <- res_full[, c("GeneName", "logFC", "FDR")
124 ]
125
126 # Write them to Excel files
127 write_xlsx(res_for_pval_excel, paste0(output_prefix, "_",
128 contrast_name, "_GeneName_logFC_Pvalue.xlsx"))
129 write_xlsx(res_for_fdr_excel, paste0(output_prefix, "_",
130 contrast_name, "_GeneName_logFC_FDR.xlsx"))
131 }
132
133 # Print a summary of what was done

```

```

124 cat("\nCompleted analysis with the following comparisons:\n")
125 for (cn in colnames(my_contrasts)) {
126     cat("    - ", cn, "\n", sep="")
127 }
128 cat("\nResults have been saved to files with prefix: ", output_
    prefix, "\n", sep="")

```

Listing B.7: Running edgeR for DGE

B.4 Poly(A) Tail Length Analysis Scripts

Listing B.8 demonstrates the Dorado basecalling command with integrated poly(A) tail estimation used to process POD5 files.

```

1  #!/usr/bin/env bash
2  set -euo pipefail
3
4  POD5="/users/paxak6/RAW264_18samples_120min/POD5"
5  MODEL="/users/paxak6/software/dorado/models/dna_r9.4.1_e8_hac@v3
6  .3" # Update to latest version if available
7  IDX="/users/paxak6/RAW264_15min/ref_gtf/mmus_r114_cdna.mmi" #
    minimap2 index
8
9  OUT="/users/paxak6/RAW264_18samples_120min/NanopLen_120min/
    called_bam_120min"
10
11 mkdir -p "$OUT"
12 # Use Dorado 0.9.6 for R9.4.1 support
13 export LD_LIBRARY_PATH=/users/paxak6/software/dorado/dorado
14 -0.9.6-linux-x64/lib:${LD_LIBRARY_PATH:-}
15 unset CUDA_VISIBLE_DEVICES # lets Condor set the GPU
16
17 /users/paxak6/software/dorado/dorado-0.9.6-linux-x64/bin/dorado
18 basecaller \
19     "$MODEL" "$POD5" \
20     --recursive \
21     --device cuda:0 \
22     --no-trim \
23     --kit-name SQK-PCB111-24 \
24     --estimate-poly-a \
25     --reference "$IDX" \
26     --output-dir "$OUT"
27
28 # Produces $OUT/calls_*.bam; each read is already aligned and
29 # carries the pt:i tag
30
31 Key parameters included:
32 - --kit-name SQK-PCB111-24: Specified the PCR barcoding kit
33   for automatic demultiplexing
34 - --estimate-poly-a: Enabled computational estimation of poly(A)
35   tail lengths from raw signal
36 - --no-trim: Preserved adapter sequences during basecalling to
37   maintain poly(A) signal information

```

```

30 - --reference: Aligned reads to the reference transcriptome
    index (minimap2 format) during basecalling

```

Listing B.8: dorado_basecalling

Listing B.9 shows the Dorado demultiplexing command used to separate multiplexed reads into individual samples using a sample sheet.

```

1 dorado demux \
2   --output-dir /path/to/demux_output \
3   --sample-sheet sample_sheet.csv \
4   --no-classify \
5   --no-trim \
6   --emit-summary \
7   --threads 8 \
8   basecalled.bam

```

Listing B.9: Reads Demultiplexing

Listing B.10 presents the command used to extract poly(A) tail lengths from aligned BAM files with quality filtering criteria.

```

1 python bam2nanoplen_dorado_v2.py \
2   --mapq 20 \
3   --tail-max 400 \
4   input.bam > output.tsv

```

Listing B.10: extract_polyA_data

Listing B.11 demonstrates the metadata file format specifying sample identifiers and their corresponding treatment condition labels.

library_id	condition
DMSO_LPS_Rep1	DMSO_LPS
DMSO_LPS_Rep2	DMSO_LPS
DMSO_LPS_Rep3	DMSO_LPS
CoR_LPS_Rep1	CoR_LPS
CoR_LPS_Rep2	CoR_LPS
CoR_LPS_Rep3	CoR_LPS

Listing B.11: metadata

Listing B.12 shows the command-line flags used for differential poly(A) tail length analysis with linear mixed-effects modeling.

```

1 Rscript diff_length.R \
2   --data_path all_samples.tsv \
3   --metadata_path metadata.tsv \
4   --test m \
5   --baseline DMSO_LPS \
6   --logscale \
7   --ofile results_lmm.tsv

```

Listing B.12: lmm_flg

Listing B.13 summarizes the command-line flags used in customized visualization scripts for plotting poly(A) tail distributions and differential length changes.

```

1 # Linear scale (0-400 nucleotides)
2 python nanoplen_two_col_plots_final.py \
3   -i results.tsv \

```



```

4     -c mean_length.DMSO_LPS \
5     -t mean_length.alt \
6     -l 0 -u 400 \
7     -f plots_linear.pdf
8
9 # Volcano plot with absolute change threshold
10 Rscript ma_or_volcano_nanoplen_ABSOLUTE.R \
11     -t results.tsv \
12     -o volcano_absolute.png \
13     --type volcano \
14     --abs-threshold 10 \
15     --stat padj \
16     --th 0.05 \
17     --jitter-width -1
18
19 # MA plot with absolute change threshold
20 Rscript ma_or_volcano_nanoplen_ABSOLUTE.R \
21     -t results.tsv \
22     -o ma_absolute.png \
23     --type ma \
24     --count-col n.alt \
25     --abs-threshold 10 \
26     --stat padj \
27     --th 0.05 \
28     --jitter-width -1

```

Listing B.13: polyA_Data_Visualization