CORDYCEPIN AFFECTS GROWTH FACTOR-DEPENDENT GENE EXPRESSION

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Declaration

Except where acknowledged in the text, I declare that this dissertation is my own work and is based on research that was undertaken by me in the School of Pharmacy, Faculty of Science, University of Nottingham.

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Abstract

The natural compound cordycepin (3'-deoxyadenosine) causes a reduction in breast cancer cell viability. Microarray analysis showed that growth related genes are down-regulated by cordycepin. Indeed, mTOR, ERK and AMPK signalling was shown to be altered by cordycepin, but the effect was too fast to be mediated by transcriptional changes. It was hypothesised that cordycepin affected signal transduction through translation. However, polysome profiling did not identify clear candidates for the effects of cordycepin on signal transduction but unveiled that cordycepin leads to translation repression on 5' terminal oligopyrimidine (TOP) mRNAs. As TOP mRNAs are known to be regulated by mTOR signaling, this result consistently suggests mTOR signaling is inhibited by cordycepin treatment. To test if it is possible that cordycepin affects gene expression via signal transduction, we compared its effects to various signal transduction inhibitors and an activator. So far, Pictilisib, a pan-PI3K inhibitor, is the only inhibitor that mimics both the gene expression and signal transduction effects of cordycepin, indicating the PI3K-PDK1-AKT axis is affected by cordycepin.

The RNAs upregulated by cordycepin were highly enriched in a group of non-coding RNAs, which are also appeared to induce during serum withdrawal. Knockdown of poly(A) polymerases induced these RNAs, indicating that they probably are degraded by the PABPN1 and poly(A) polymerase dependent nuclear RNA decay pathway.

Thus the data suggest that cordycepin affects gene regulation by two distinct pathways, one affecting signal transduction and growth related mRNA expression and another affecting polyadenylation mediated decay of non-coding mRNAs.

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List of abbreviations

3' UTR	3' untranslated region
4E-BP1 or **PHAS-1**	eukaryotic translation initiation factor 4E-binding protein 1
ACC	acetyl coenzyme A carboxylase
ADP	adenosine diphosphate
AIF	apoptosis-inducing factor
AKT or **PKB**	protein kinase B
AMOTL2	angiomotin like 2
AMP	adenosine monophosphate
AMPK	AMP-activated protein kinase
AP-1	AP-1 transcription factor
ARAF	A-Rafproto-oncogene, serine/threonine kinase
ASK1	apoptosis signal-regulating kinase 1
ATF	activating transcription factor
ATF2	activating transcription factor 2
ATP	adenosine triphosphate
BAD	Bcl-2-associated death promoter
BARD1	BRCA1 associated RING domain 1
BCAR3	breast cancer anti-estrogen resistance 3
BRAF	B-Raf proto-oncogene, serine/threonine kinase
BRCA1	BRCA1, DNA repair associated
BRCA2	BRCA2, DNA repair associated
bZIP	basic region-leucine zipper
CAB39 or **MOD25**	calcium binding protein 39
CAMKK2 or **CAMKKβ**	calcium/calmodulin dependent protein kinase kinase 2
cAMP	cyclic adenosine monophosphate
CASP	caspase
CATSPER2	cation channel sperm associated 2
CCND1	cyclin D1
CCR4-NOT	C-C motif chemokine receptor 4- CCR4-NOT transcription complex subunit

CF	cleavage factor
CPSF	cleavage and polyadenylation factor
CPT1	carnitine palmitoyltransferase I
CREB	cAMP response element-binding protein
CstF	cleavage stimulation factor
C-TAK1 or **MARK3**	microtubule affinity regulating kinase 3
CTGF	connective tissue growth factor
CTSL	cathepsin L
DAVID	Database for Annotation, Visualization and Integrated Discovery
DEPTOR	DEP domain containing mTOR interacting protein
DMSO	Dimethyl sulfoxide
DNA	deoxyribonucleic acid
DSE	downstream element
DUSP1	dual specificity phosphatase 1
E17K	AKT1 mutation with glutamic acid (E) being substituted by lysine (K) at amino acid 17
E2F1	E2F transcription factor 1
ECM	extracellular matrix
eEF1A1	eukaryotic translation elongation factor 1 alpha 1
eEF1A2	eukaryotic translation elongation factor 1 alpha 2
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
EGR1	early growth response 1
eIF3	eukaryotic translation initiation Factor 3
eIF4B	eukaryotic translation initiation factor 4B
eIF4E	eukaryotic translation initiation factor 4E
eIF4F	eukaryotic initiation factor 4F
eIF4G	eukaryotic translation initiation factor 4 gamma
ELK1	ETS transcription factor
ER	estrogen receptor
ERBB2 or **HER2**	erb-b2 receptor tyrosine kinase 2

ERK5	mitogen-activated protein kinase 7
FAS	fatty acid synthase
FDA	Food and Drug Administration, the United States Department of Health and Human Services
FGFR1	fibroblast growth factor receptor 1
FIP1L1 or **hFip1**	factor interacting with PAP and CPSF1
FOS or **c-FOS**	Fos proto-oncogene, AP-1 transcription factor subunit
FOSL1	FOS like 1, AP-1 transcription factor subunit
FOX	forkhead box
GAP	GTP-bound TSC1/2 has GTPase-activating proteins
GATA3	GATA binding protein 3
GDP	guanosine diphosphate
GLD2 or **PAPD4**	poly(A) RNA polymerase D4, non-canonical
GO	Gene Ontology
GPCR	G-protein coupled receptor
GPD1	mitochondrial glycerophosphate dehydrogenase
GRB2	growth factor receptor binds protein 2
GSK3	glycogen synthase kinase-3
GTP	guanosine triphosphate
Gβγ	G beta-gamma complex
HGFR	hepatocyte growth factor receptor
HIF1	hypoxia-inducible factor 1
HRAS	HRas proto-oncogene, GTPase
IEG	immediate early gene
IER3	immediate early response 3
IKKβ or **IKBKB**	inhibitor of nuclear factor kappa B kinase subunit beta
IL6	interleukin 6
INPP5D or **SHIP1**	inositol polyphosphate-5-phosphatase D
Ir75a	Ionotropic receptor 75a
IRS1	insulin receptor substrate 1
ITu	5’-iodotubercidin

JNK or **SAPK**	c-Jun N-terminal kinase
JUN or **c-JUN**	Jun proto-oncogene, AP-1 transcription factor subunit
JUNB	JunB proto-oncogene, AP-1 transcription factor subunit
KRAS	KRAS proto-oncogene, GTPase
KSR	kinase suppressor of RAS
LARP1	La-related protein 1
LC-MS	liquid chromatography–mass spectrometry
LKB1 or **STK11**	serine/threonine kinase 11
lncRNA	long non-coding RNA
MAF	bZIP transcription factor
MALAT1	metastasis associated lung adenocarcinoma transcript 1
MAP3K1	mitogen-activated protein kinase kinase kinase 1
MAPK	mitogen-activated protein kinase
MAPKK or **MEK**	mitogen-activated protein-kinase kinase
MAPKKK or **MEKK**	mitogen-activated protein-kinase kinase kinase
MCL1	MCL1, BCL2 family apoptosis regulator
MDM2	MDM2 proto-oncogene
MEF2	myocyte enhancer factor-2
miRNA	microRNA
MK2 or **MAPKAPK2**	MAP kinase-activated protein kinase 2
MK3 or **MAPKAPK3**	MAP kinase-activated protein kinase 3
MKK4 or **JNKK1**	mitogen-activated protein kinase kinase 4
MKK7 or **JNKK2**	mitogen-activated protein kinase kinase 7
MKNK1 or **MNK1**	MAP kinase interacting serine/threonine kinase 1
MKNK2 or **MNK2**	MAP kinase interacting serine/threonine kinase 2
MKP	MAPK phosphatase
MLK	mixed-lineage kinase
MLST8	mTOR associated protein, LST8 homolog
MMP	matrix metalloproteinase
mSIN1 or **MAPKAP1**	mitogen-activated protein kinase associated protein 1
MSK	mitogen- and stress-activated kinases

mTOR	mechanistic target of rapamycin
mTORC1	mTOR complex 1
mTORC2	mTOR complex 2
MTPAP	mitochondrial poly(A) polymerase
mtRNA	mitochondrial RNA
MYC or **c-MYC**	MYC proto-oncogene, bHLH transcription factor
ncRNA	non-coding RNA
NEAT1	nuclear paraspeckle assembly transcript 1
NEK6	NIMA related kinase 6
NF90 or **ILF3**	interleukin enhancer binding factor 3
NFKBIA	NFKB inhibitor alpha
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells
NOS1	nitric oxide synthase
NR4A1	nuclear receptor subfamily 4 group A member 1
OPLS-DA	Orthogonal Projections to Latent Structures Discriminant Analysis
ORF	open reading frame
p38	p38 mitogen-activated protein kinases
PABPC	poly(A) binding protein cytoplasmic
PABPN1	poly(A) binding protein nuclear 1
PAIP	poly(A) binding protein interacting protein
PAP	poly(A) polymerase
PAPD5 or **TRF4-2**	poly(A) RNA polymerase D5, non-canonical
PAPD7	poly(A) RNA polymerase D7, non-canonical
PARP1	poly(ADP-ribose) polymerase 1
PAS	poly(A) signal
PCA	Principal Component Analysis
PDGFR	platelet-derived growth factor receptors
PDK1	3-phosphoinositide dependent protein kinase 1
PH or **PHD**	pleckstrin homology domain
PI3K	phosphatidylinositol 3-kinase

PIK3CA	phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha
PIKK	phosphatidylinositol 3-kinase-related kinases
PKA	protein kinase A
PKC	protein kinase C
PKCα	protein kinase C alpha
PLCβ2	phospholipase C beta 2
PLK2	polo like kinase 2
Pol II	RNA polymerase II
POLR2A	RNA polymerase II subunit A
PPARγ1	peroxisome proliferator-activated receptor γ1
PR	progesterone receptor
PRAK	p38 regulated/activated protein kinase
PRAS40	proline-rich AKT substrate of 40 kDa
pre-mRNA	precursor mRNA
PROMPT	PROMoter uPstream Transcript
PRR5 or **PROTOR1**	proline rich 5
PRR5L or **PROTOR2**	proline rich 5 like
PtdIns(3,4,5)P3 or **PIP3**	phosphatidylinositol 3,4,5-trisphosphate
PtdIns(4,5)P2 or **PIP2**	phosphatidylinositol 4,5-bisphosphate
PTEN	phosphatase and tensin homolog
PTENP1	phosphatase and tensin homolog pseudogene 1
PTP	protein tyrosine phosphatase
PTPA or **PP2A**	protein phosphatase 2 phosphatase activator
PTPRK	protein tyrosine phosphatase, receptor type K
PVDF	polyvinylidene fluoride
RAD51	RAD51 recombinase
RAF1 or **c-RAF**	Raf-1 proto-oncogene, serine/threonine kinase
RAG	RAS related GTP binding
RB1	RB transcriptional corepressor 1
RHEB	RAS homolog enriched in brain

RHO	RAS homolog family
RICTOR	RAPTOR independent companion of MTOR complex 2
RISC	RNA-induced silencing complex
RNA	ribonucleic acid
RNAi	RNA interference
RNU1-1 or **U1**	RNA U1 small nuclear
ROS	reactive oxygen species
RPL10A	ribosomal protein L10a
RPL23A	ribosomal protein L23a
RPL7	ribosomal protein L7
RPS15A	ribosomal protein S15a
RPS18	ribosomal protein S18
RPS6KA1 or **RSK**	ribosomal protein S6 kinase A1
RAPTOR	regulatory associated protein of mTOR complex 1
rRNA	ribosomal RNA
RTK	receptor tyrosine kinase
RT-qPCR	real-time quantitative polymerase chain reaction
S6 or **RPS6**	ribosomal protein S6
S6K1 or **P70S6K**	ribosomal protein S6 kinase beta-1
Ser-	serine-
SGK1	serum/glucocorticoid regulated kinase 1
SGK1	serum/glucocorticoid regulated kinase 1
SH2	SRC Homology 2
SH3	SRC Homology 3
SHG60	SNORD60-Host Gene
SKIV2L2 or **hMTR4**	Ski2 like RNA helicase 2
SLC25A25	Solute carrier family 25 member 25
SNAI1	snail family transcriptional repressor 1
SNAR	small NF90-associated RNA
snoRNA	small nucleolar RNA
snoRNP	small nucleolar ribonucleoprotein

snRNA	small nuclear RNA
snRNP	small nuclear ribonucleoprotein
SOS	son of sevenless
SPRY2	sprouty RTK signaling antagonist 2
SRE	serum response element
SRF	serum response factor
STRAD	STE20-like pseudokinase
TAK1	transforming growth factor-β-activated kinase 1
TCF	ternary complex factor
TELO2	telomere maintenance 2
Thr-	threonine-
TNBC	triple negative breast cancer
TNF	tumor necrosis factor
TOP	5’ terminal oligopyrimidine
TOS	conserved TOR signaling motif
TP53 or **P53**	tumor protein p53
TRAF2	TNF receptor associated factor 2
TRAMP	Trf4/5-Air1/2-Mtr4-polyadenylation
TRE	tetradecanoylphorbol-13-acetate response element
tRNA	transfer RNA
TSC1	TSC complex subunit 1
TSC2	TSC complex subunit 2
TTI1	TELO2 interacting protein 1
TUT1 or **PAPD2**	terminal uridylyl transferase 1, U6 snRNA-specific
Tyr-	tyrosine-
v/v	% volume per volume
V600E	BRAF mutation with valine (V) being substituted by glutamic acid (E) at amino acid 600
w/v	% weight per volume
WDR33	WD repeat domain 33
WEE1	WEE1 G2 checkpoint kinase

XIST	X inactive specific transcript
YMXM and **YXXM**	Tyr-Met-Xaa-Met, binding motif of p85 to RTK or adaptor proteins
YWHAQ or **14-3-3**	tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein theta
ZCCHC7 or **AIR1**	zinc finger CCHC-type containing 7
ZNF703	zinc finger protein 703

1 Introduction

1.1 Breast Cancer

Cancer has been one of the biggest threats to human health. According to the World Health Organisation, cancer is a large assembly of diseases with the ability to affect any part of a human body. In 2012, cancer was affecting 32.6 million people around the world according to the 2012 Globocan project (Ferlay et al. 2013). Cancer cells are characterised by sustained proliferative signaling, evasion of growth suppressors and metastasis, genome instability and mutations are thought to be the main cause of cancer (Hanahan & Weinberg 2011). Cancer is therefore a disease of the genome at the cellular level that is manifested by alterations in gene expression; an accurate understanding of its genetic mechanisms is required in order to develop effective cures for cancer (Stratton et al. 2009). Breast cancer topped the age-standardised incidence and mortality rates list in 2012, sharing 11.9% of the overall cancer types, making its way to the most frequent type of cancer within women (Ferlay et al. 2013). In the U.S., an estimated 231,840 women were diagnosed with breast cancer from 2015 to 2016 (American Cancer Society). This number is expected to climb up to 252,710 in 2017, among which 40,610 deaths are expected (Siegel et al. 2017). According to the 2017 report by World Cancer Research Fund International, breast cancer incidence has tight links with physical excise, weight, breast feeding and alcohol consumption (World Cancer Research Fund International).

Studies on immortalised breast cancer cell lines have contributed to the biological basis of breast cancer diagnosis and treatment. There are more than 40 breast cancer cell lines available for research use, but it is said that research on MCF-7, T47D and MDA-MB-231 comprises 2/3 of all submitted breast cancer-related abstracts (Neve et al. 2006; Hollestelle et al. 2010; Lacroix & Leclercq 2004). Depending on the gene expression pattern, a four-subtype classification of breast cancer is widely accepted for

its clinical consistency (Table 1.1), these are luminal A, luminal B, HER2 and basal (Sorlie et al. 2001; Perou et al. 2000; Sotiriou et al. 2003; Neve et al. 2006; Koboldt et al. 2012). Notably, because estrogen receptor, progesterone receptor and HER2 are not presented in the basal breast cancer subtype, this subtype is alternatively termed triple negative breast cancer (TNBC). The aggressiveness and resistance to hormone treatment of TNBC have caused poor clinical prognosis and outcome. The distribution of breast cancer cases by subtypes was reported by Carey et al., who analysed samples from 107 patients and found that luminal subtypes accounted for an overwhelming 58% of the overall cases, followed by basal (32%) and HER2 (10%) subtypes (Carey et al. 2007).

Subtype	Characteristics	Example cell lines
Luminal A	ER+, PR+/-, HER2-	MCF-7, T47D
Luminal B	ER+, PR+/-, HER+	BT474
HER2	ER-, PR-, HER2+	SKBR3, AU565
Basal	ER-, PR-, HER2-	MDA-MB-231, MDA-MB-468

Table 1.1 Breast Cancer Classification

ER: estrogen receptor, PR: progesterone receptor and HER2: receptor tyrosine-protein kinase erbB-2 (ERBB2, also known as HER2). Table adapted from Holliday & Speirs 2011; Neve et al. 2006.

1.1.2 Molecular Features of Breast Cancer

Based on their landmark paper from 2000, The Hallmarks of Cancer, Douglas Hanahan and Robert A. Weinberg optimised their hallmark system with the addition of four other cancer hallmarks, in the sequel paper, Hallmarks of Cancer: The Next Generation. Taken together, the ten hallmarks are: sustaining proliferative signaling, deregulating cellular energetics, resisting cell death, genome instability & mutation, inducing angiogenesis, activating invasion & metastasis, tumour-promoting inflammation, enabling replicative immortality, avoiding immune destruction and evading growth suppressors (Hanahan & Weinberg 2011; Hanahan & Weinberg 2000). In a way, cancer is a constantly evolving disease. Nowell and Peter proposed that mutations induced by carcinogens are under the selection of nature: mutations with physiological disadvantages die out and the

advantageous mutations are passed on to progeny cells (Nowell 1976). This implies that cancer, as the result of Darwinian evolution, progressively acquires more mutations to increase its survival and proliferative activity, indicating the importance of genome instability in tumorigenesis. However, unfortunately, this also means that cancer cells can evolve to switch off or avoid chemotherapy targets, leading to poor outcome in cancer relapse. As a result of mutation or induction by mutated upstream protein in signal transduction, some growth-promoting genes become hyperactive and accumulate in a deregulated fashion, these genes are termed oncogenes. In contrast, a tumour suppressor gene is a normal gene that exhibits anti-cancer properties, e.g. anti-proliferation, anti-angiogenesis and apoptosis-induction in tumour cells, protecting cells from becoming cancer cells.

As in most cancers, a high mutation rate is observed in breast cancer cells, maintaining invasiveness and unregulated growth. Clinical research shows that PIK3CA, an oncogene encoding the catalytic subunit of phosphatidylinositol kinase is mutated in 40% of breast cancer patients (Campbell et al. 2004), whereas in a group of 316 primary breast tumour patients, mutation of a key tumour suppressor gene, tumour protein p53 (TP53), was found in 22% of the samples (Sjögren et al. 1996). After whole genome analysis of 560 somatic breast cancer patients, Nik-Zanial et al. ranked the 10 most frequently mutated genes: TP53, PIK3CA, MYC, CCND1, PTEN, ERBB2, ZNF703/FGFR1, GATA3, RB1 and MAP3K1, in descending order. These account for 62% of mutation-driven breast cancers (Nik-Zainal et al. 2016). By understanding the mechanism through which mutations cause cancer, specific treatments can be developed for the improved therapy. Here, using the mutations as a guide, I will be introducing some of the most distinct molecular features of breast cancer, with exception of PIK3CA, which will be introduced in 1.2.2.3.

1.1.2.1 BRCA1 and BRCA2

BRCA1 and BRCA2 mutations represent examples of the most researched mutations in breast and ovarian cancer. Despite that only 10.3% of 1,008 breast cancer patients carry BRCA mutations, the chance of these BRCA mutation carriers to develop breast cancer is 82%, with risk increasing during aging (King et al. 2003). First reported in 1990, BRCA1 was identified as the gene located at chromosome 17q21 and was found responsible for familial breast cancer linkage (Hall et al. 1990; Miki et al. 1994). However, interestingly, the discovery of BRCA2 was driven by familial male breast cancer, when the linkage analysis failed to establish a link between BRCA1 and male hereditary breast cancer (Stratton et al. 1994). Following the eventual identification of BRCA2 by the Stratton lab (Wooster et al. 1994; Wooster et al. 1995), efforts were made to define the molecular mechanism of BRCA1 and BRCA2 in breast cancer cells. The breast cancer cell line MCF-7 harbours wild-type BRCA1. Using MCF-7 cells as a model, a complex composed of BRCA1, BRCA1 associated RING domain 1 (BARD1) and RAD51 was found being formed upon exposure to genotoxic insult, suggesting a role of BRCA1 in DNA-damage response (Scully, Chen, Ochs, et al. 1997), which is consistent with their follow-up investigation in vivo (Scully, Chen, Plug, et al. 1997). Besides, the interaction between BRCA1 and BARD1 was found to associate with cleavage stimulation factor (CstF), which is one of the core 3' end processing complex members in regulating cleavage and polyadenylation activity (see 1.5.1), therefore linking mRNA 3' processing and DNA damage in tumour suppression (Kleiman & Manley 2001; Kleiman & Manley 1999).

Whilst it has become clear that BRCA1 and BRCA2 participate in a DNA damage response pathway that constitutes homologous recombination and double-strand break repair (Chen et al. 1999), this cannot explain breast cancer tumorigenesis. Because in normal cells, unrepaired DNA damage would result in apoptosis or cell cycle arrest, Turner et al. proposed that cell cycle checkpoint should also be abrogated in BRCA

mutation breast cancer cells, which could be resulting from TP53 mutation (Turner et al. 2004). Indeed, TP53 has higher mutation rate in BRCA mutated breast cancer cells (Greenblatt et al. 2001; Crook et al. 1998), in agreement with the fact that BRCA1 mutation bearing breast cancer cell lines also bear TP53 mutation (Forbes et al. 2016; Elstrodt et al. 2006). Additionally, DNA repair dysfunction by BRCA mutation allows more genomic alterations to occur in tumours, which occur concurrently with other mutations in breast cancers.

1.1.2.2 HER2, PR and ER

As mentioned in 1.1 and Table 1.1, most breast cancers are distinguished by the presence/absence of three major receptors, receptor tyrosine-protein kinase erbB-2 (ERBB2 or HER2), progesterone receptor (PR) and estrogen receptor (ER), in breast cancer cells. Correct identification of these receptors on cell membrane guides specific treatment for breast cancer of different subtypes. Stimulated by estrogen, cellular ER was reported to be acting in two approaches, genomic and non-genomic, regulating transcription, cell growth and differentiation (Hall et al. 2001). Moreover, it has been suggested that estrogen controls the activity and synthesis of PR (Horwitz & McGuire 1978), which again was shown to induce proliferative responses and upregulate gene expression of signal transduction members (Lange & Yee 2008).

In a study of 189 human primary breast cancers, 30% were shown to bear alterations in the HER2 gene, with their expression ranging from 2- to 20-fold higher than control (Slamon et al. 1987). Although HER2 has a ligand binding domain, no specific ligands have been identified. As a receptor tyrosine kinase (RTK), HER2 activation is therefore through the formation of heterodimer with another ligand-bound RTK, which subsequently activates PI3K, MAPK, PKC and other signalling pathways to yield its effects, including proliferation, evasion of apoptosis and gene expression (see 1.2.2) (Keshamouni et al. 2002; Rusnak et al. 2001).

1.1.3 Treatment for Breast Cancer

In addition to surgical removal of cancer tissue and radiation therapy, the progressing understanding of breast cancer at the molecular level has a larger choice of therapies. As mentioned in 1.1.2, ER and PR are hormone receptors, from hormone production to receptors binding, medication that targets any step in this process is known as hormone or endocrine therapy. One of these treatments is tamoxifen, which itself has little affinity to ER (Fisher et al. 1998; Jordan 2006). However, when metabolised by liver cells, the resulting metabolites, including N-desmethyltamoxifen and 4-hydroxytamoxifen, competitively bind to ER with high affinity and therefore inhibit ER activity (Desta et al. 2004). Alternatively, drugs can be used to inhibit estrogen production, such as letrozole, which is widely used for breast cancer in postmenopausal women (Eiermann et al. 2001; Group 2005). In contrast, in pre-menopausal women, letrozole will trigger a feedback loop to eventually override the effect of letrozole and increase the production of estrogen, so the investigation of letrozole for pre-menopausal breast cancer has remained minimal (Wood et al. 2003). For HER2-positive breast cancer patients, another commonly used drug in treatment is Trastuzumab, a monoclonal HER2 antibody. Clinical research has successfully validated the safety and efficacy in using Trastuzumab, either as a single agent for $HER2^{+}$ breast cancer patients who have never been treated before (Vogel et al. 2003), or as combined adjuvant treatment with paclitaxel, a microtubule inhibitor (Romond et al. 2005). In TNBC cases, standard chemotherapy is commonly applied as no ER, PR or HER2 are expressed.

1.2 Signal Transduction

1.2.1 Growth Regulation and Energy Metabolism

Unlike the reliance on mitochondrial oxidative phosphorylation for ATP production in normal cells, the main energy source of most cancer cells is aerobic glycolysis. Because this phenomenon is originally reported by Otto Warburg, in 1924, this feature of cancer cells is termed the Warburg effect (Warburg 1924). But why do cancer cells act

differently in regard to energy metabolism? Introduced by Gatenby & Gillies, this aerobic glycolysis is a compromise, responding to the lack of oxygen supply in early tumourigenesis. As glycolysis continues, the resulting acidosis in the tumour microenvironment urges cancer cells to evolve, such that cells can become tolerant to acidosis while maintaining proliferative and invasive property (Gatenby & Gillies 2004), which again is consistent with the evolutionary view of cancer cells discussed in 1.1.2. However, this explanation is questioned by Heiden et al., who suggested that leukaemia cells, which reside in bloodstream with an unlimited supply of oxygen, still acquire a high aerobic glycolysis rate (Gottschalk et al. 2004; Heiden et al. 2009). Instead, they argued that, since aerobic glycolysis produces more carbon and nitrogen to support cell growth than oxidative phosphorylation, the need to metabolise nutrients for the benefit of cell growth is prioritised by cancer cells (Lunt & Vander Heiden 2011; Heiden et al. 2009). Moreover, Warburg effect was also observed in cancer stromal cells to generate energy-rich metabolites for cancer cells, facilitating cancer cell growth (Pavlides et al. 2009).

1.2.2 Signal Transduction in Cancer

1.2.2.1 mTOR Signaling

Since the discovery of the mammalian target of rapamycin (mTOR) in mid 90s (Sabatini et al. 1994; Sabers et al. 1995; Brown et al. 1994), this evolutionarily conserved mTOR kinase and its signaling has developed into one of the core regulators in cellular activities. Upstream, mTOR signaling receives extracellular stimulation from PI3K, MAPK, AMPK and other signaling cascades, and in turn regulates mainly cell growth, metabolism, autophagy, survival and proliferation via a series of downstream effectors (Laplante & Sabatini 2012a; Guertin & Sabatini 2007; Zoncu et al. 2011; Sabatini 2006). Given that high mutation rates were observed in PI3K and MAPK signaling in cancer cases (see 1.2.2.2 and 1.2.2.3), together with the energy sensing AMPK signaling, mTOR

signaling has therefore become recognised as one of the most important pathways in cancer biology. Not only growth factors, cytokines and hormones, but also energy crisis and cellular stress, like hypoxia, can activate mTOR signaling. Starting with the same mTOR kinase, two distinct complexes are formed through the interaction with: RAPTOR, PRAS40, DEPTOR, MLST8, TTI1 and TEL2 (mTORC1); RICTOR, MSIN1, PROTOR1/2, DEPTOR, MLST8, TTI1 and TEL2 (Figure 1.2.2.1)(mTORC2) (Laplante & Sabatini 2012a).

mTORC1 receives information signals mainly through the tuberous sclerosis (TSC)1/2 heterodimer mediator, which is in turn stimulated/inhibited by AKT, ERK, RSK, IKKβ, AMPK etc. (Shaw & Cantley 2006; Huang & Manning 2008). GTP-bound TSC1/2 has GTPase-activating proteins (GAP) activity for RAS homolog enriched in brain (RHEB), causing hydrolysis of GTP by RHEB, which converts RHEB to its inactive status (Manning & Cantley 2003; Li 2004). As RHEB directly binds and activates mTORC1 in a guanyl-nucleotide-independent manner, the role of TSC1/2 as a negative regulator of mTOR signaling was defined (Long et al. 2005). However, in addition to RHEB binding, the activation of mTORC1 requires more. RAS related GTP binding (RAG) is a family of small GTPases residing on lysosome membranes through the interaction with a regulator complex (Sancak et al. 2010), while intracellular distribution of mTOR concentrates on membrane systems throughout the cell (Drenan et al. 2004). Upon stimulation, mTORC1 is recruited to lysosome surface in a RAG-mediated manner. Although RAG proteins show no direct change to the kinase activity of mTORC1, this binding to lysosome localises mTORC1 to a compartment where RHEB is presented (Drenan et al. 2004; Durán et al. 2012; Sancak et al. 2010).

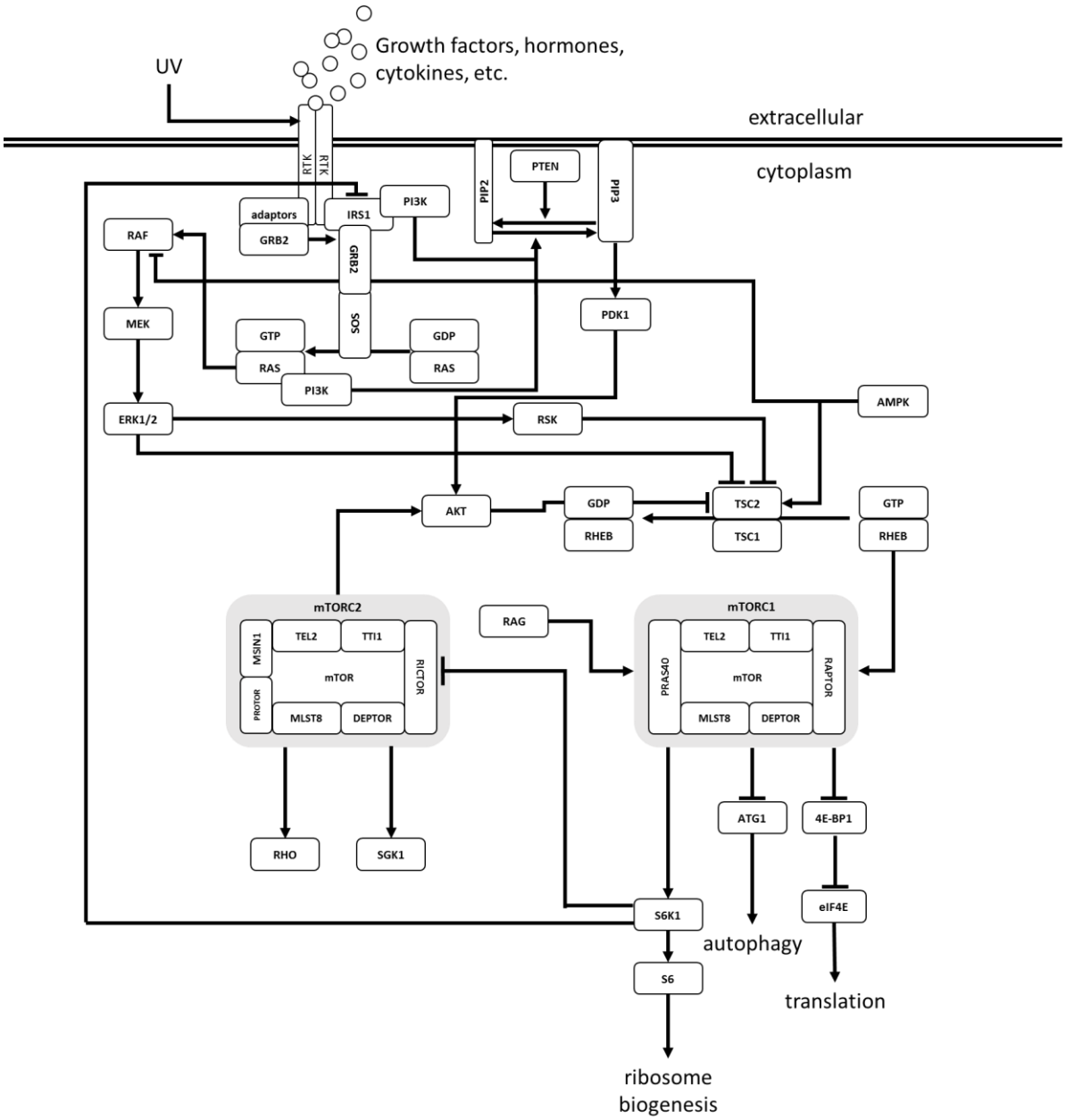


Figure 1.2.2.1 A Schematic Diagram of mTOR Pathway

(Kanehisa et al. 2016; Ogata et al. 1999; Kanehisa et al. 2017)

Processes ranging from lipid synthesis, apoptosis, cell survival, cell growth to cell proliferation are all affected by mTORC1 signaling through different downstream effectors, especially the best-characterised ribosomal protein S6 kinase beta-1 (S6K1, also known as P70S6K) and eukaryotic translation initiation factor 4E-binding protein 1 (4E-BP1, also known as PHAS-1) (Hoeffer & Klann 2010; Laplante & Sabatini 2012a; Neufeld 2010; Dowling et al. 2010; Zoncu et al. 2011; Dunlop & Tee 2009). 4E-BPs and eukaryotic translation initiation factor 4 gamma (eIF4G) share the same eukaryotic translation initiation factor 4E (eIF4E) binding sequence motif (Mader et al. 1995; Haghighat et al. 1995; A. C. Gingras et al. 2001; Mothe-Satney et al. 2000). When hypophosphorylated, 4E-BPs compete with eIF4Gs for the binding to eIF4E, which subsequently inhibits cap-dependent translation initiation via eukaryotic initiation factor 4F (eIF4F) (Haghighat et al. 1995; Lin et al. 1994; Mader et al. 1995; Pause et al. 1994; Sonenberg & Gingras 1998). This binding to eIF4G is inhibited when 4E-BP1 is phosphorylated by mTORC1 at Thr37 and Thr46 residues (Gingras et al. 1999). In parallel with 4E-BP1 as a downstream effector of mTORC1, S6K1 activity is also influenced by multiple phosphorylations (Dennis et al. 1996). Phosphorylation at Thr229 and Thr389 by mTORC1 and PDK1, respectively, are of essential to S6K1 activity (Pullen & Thomas 1997; von Manteuffel et al. 1997). With S6 being a component of the 40S ribosomal subunit, it is expected that, once stimulated, mTOR increases translation through S6K1-S6 signaling, which goes on to affect cell growth and metabolism (Magnuson et al. 2012; Dann et al. 2007; Meyuhas 2015). A more detailed theory was proposed by Holz et al., who suggested phosphorylation at Thr389 dissociates S6K1 from eIF3 complex. This dissociation allows the full activation of S6K1 via the phosphorylation at Thr229, which in turn phosphorylates eukaryotic translation initiation factor 4B (eIF4B) and regulates cap-dependent translation initiation (Holz et al. 2005). With the conserved TOR signaling (TOS) motif being discovered in the N

terminus of all S6 kinases and C terminus of all 4E-BPs, it is said that the specific binding of RAPTOR to this TOS motif is necessary for mTOR to efficiently phosphorylate S6K1 and 4E-BP1, so as to render their effects (Nojima et al. 2003; Schalm et al. 2003; Schalm & Blenis 2002). Taken together, when mTORC1 is stimulated, these two effectors work jointly to promote translation (Choo et al. 2008; Holz et al. 2005). Yet, this is not the only role of S6K1 in mTOR signaling, evidence has shown that S6K1 connects mTORC1 and mTORC2 through the phosphorylation of RAPTOR-independent companion of mTOR complex 2 (RICTOR) at Thr1135, one of the proteins that constitute mTORC2 (Dibble et al. 2009; Treins et al. 2010; Julien et al. 2010). Although phosphorylation at this site fails to alter mTORC2 assembly or activity, increased protein kinase B (PKB, also known as AKT) phosphorylation at Ser473 by mTORC2 was detected with mutated Thr1135A of RICTOR, indicating this phosphorylation of RICTOR by S6K1 represses mTORC2 and AKT signaling (Dibble et al. 2009; Julien et al. 2010).

As will be discussed in detail later in this chapter, the mTORC2 substrate AKT is a serine/threonine kinase downstream of PI3K signaling with a wide range of substrates, including TSC2 and proline-rich AKT substrate of 40 kDa (PRAS40), an mTORC1 subunit (Igor Vivanco & Sawyers 2002). In contrast to the positive regulation of mTOR signaling through TSC2/1 heterodimer, phosphorylation of PRAS40 by AKT facilitates the binding and phosphorylation of PRAS40 by mTOR, which eventually inhibits mTORC1 activity (Haar et al. 2007; Nascimento et al. 2010; Oshiro et al. 2007). Apart from AKT, researchers have identified three other major substrates of mTORC2, protein kinase C alpha (PKCα), serum/glucocorticoid regulated kinase 1 (SGK1) and RAS homolog family (RHO) GTPases (Guertin et al. 2006; Yin et al. 2016; García-Martínez & Alessi 2008; Jacinto et al. 2004; Gupta et al. 2013). Whilst PKCα and Rho GTPases play an important role in cytoskeleton regulation (Sarbassov et al. 2004; Gupta et al. 2013; Jacinto et al.

2004), cell survival and epithelial ion transport are reportedly affected by the mTORC2-activated SGK1 (Loffing et al. 2006).

1.2.2.2 MAPK Signaling

Mitogen-activated protein kinase (MAPK) signaling is another evolutionary conserved network of various kinases transmitting extracellular signals to regulate various cellular activities, e.g. proliferation, differentiation and apoptosis (Junttila et al. 2008; Murphy & Blenis 2006; Zhang et al. 2002). Kinases in this network are categorised clearly as MAP-kinase kinase kinase (MAPKKK, also known as MAP3K or MEKK), MAP-kinase kinase (MAPKK, also known as MAP2K or MEK) and MAP-kinase (MAPK). While phosphorylation of MAP2Ks and MAPKs is conducted by upper MAP3Ks and MAP2Ks, MAP3Ks are activated as a result of stimulation by different growth factors, cytokines, stress etc. (Murphy & Blenis 2006). There are, to date, three well-studied MAPK signaling pathways, ERK, p38 and JNK pathways, which are named after the MAPKs in each pathway (Junttila et al. 2008; Murphy & Blenis 2006; Zhang et al. 2002).

1.2.2.2.1 ERK Pathway

The MAPK/ERK pathway is the most researched one in all three MAPK pathways. Through various substrates, ERK regulates mainly cell growth and differentiation. More importantly, high mutation rate within ERK signal transduction, such as in KRAS, HRAS and BRAF, was observed in numerous cancers to acquire growth advantages, making the ERK pathway one the most important contributors to tumorigenesis (Steelman et al. 2011; Murphy & Blenis 2006). Because of this, targeting the ERK pathway has become a main topic in cancer treatment (Roberts & Der 2007).

MAPK3 and MAPK1 are also known as ERK1 and ERK2, respectively, short for extracellular signal–regulated kinases. At the top of ERK signaling pathway, RAF kinases serve as the MAP3K and interacts with GTP-RAS, a group of three small GTPases (Figure 1.2.2.2.1)(Vojtek et al. 1993; Koide et al. 1993; Moodie et al. 1993; Warne et al. 1993).

RAF kinases serve as the MAP3K and interact with GTP-RAS, a group of three small GTPases (Figure 1.2.2.2.1)(Vojtek et al. 1993; Koide et al. 1993; Moodie et al. 1993; Warne et al. 1993). RAS is inactive when bound to GDP, leading to the dissociation of the RAS-RAF dimer, suggesting a binary switch role for RAS in ERK signaling (Moodie et al. 1993; Koide et al. 1993). To activate RAS, stimulating signals start from either integrins or receptor tyrosine kinases (RTKs), the transmembrane enzymes that receive stimulation of various extracellular growth factors, hormones and other ligands (Schlessinger 2000; Hubbard & Miller 2008). Upon ligand stimulation, bound RTKs dimerise with adjacent RTKs, which activates their intracellular kinase domain by autophosphorylation of relative residues (Cadena & Gill 1992; Hubbard & Miller 2008). Binding of ligands to RTK leads to the autophosphorylation of the receptor or neighbouring substrates. Substrates with SRC Homology 2 (SH2) domain, for example, growth factor receptor binds protein 2 (GRB2), will then bind to the phosphotyrosine residue of RTKs though the SH2 domain with high affinity (Gale et al. 1993; Rozakis-Adcock et al. 1993). GRB2 also presents a SH3 domain for the binding of Son of Sevenless (SOS), while the latter then act as a guanine nucleotide exchange factor to activate RAS (Li et al. 1993; Rozakis-Adcock et al. 1993; Chardin et al. 1993; Egan et al. 1993; Simon et al. 1993). Because of this role of proteins like GRB2 in connecting SOS and RTKs, they are called signaling adaptors (Chardin et al. 1995). In addition to RAS-GTP binding, the highly complicated activation mechanisms for RAF involve localisation, phosphorylation and dephosphorylation (reviewed in McKay & Morrison 2007).

MEK1 and MEK2 serve as the two classic intermediate kinases that convey the signals from RAF to ERK in ERK signaling (Zheng & Guan 1993). The MEK activation mechanism was discovered in 1994, when two independent groups found that phosphorylation of MEK at Ser217 and Ser221 by RAF1 were needed for its activation (Alessi et al. 1994; Zheng & Guan 1994). The other two members of the RAF family, BRAF and ARAF, were

also verified as MEK activators by yeast two-hybrid, *in vivo* assay, pharmacological and genetic inhibition (Papin et al. 1996; Mercer et al. 2005; Solit et al. 2006; Yin et al. 2002). Next, as a dual specific protein kinases, MEK activates ERK1/2 by dual-phosphorylating Thr202/Tyr204 and Thr185/Tyr187 in their Thr-Glu-Tyr (TEY) motif (Roskoski 2012; Butch & Guan 1996; Rossomando et al. 1992).

Depending on the scaffold protein, ERK signaling activation can take place in different locations (McKay & Morrison 2007). Of the four main scaffold proteins, kinase suppressor of RAS (KSR) remains the best studied one. Discovered in *Drosophila* and *Caenorhabditis elegans*, KSR is understood to interact with RAF1, MEK, ERK, C-TAK1 and 14-3-3 proteins (Cacace et al. 1999; Müller et al. 2000; Denouel-Galy et al. 1998; Nguyen et al. 2002; Roy et al. 2002; Therrien et al. 1996). These interactions facilitate the novel function of ERK signaling, as RNAi knockdown of KSR prevented the activation of MEK and ERK by insulin (Anselmo et al. 2002; Roy et al. 2002). In addition, KSR-deficient mice showed attenuated ERK activation despite that cell growth was not affected (Nguyen et al. 2002). Although it is still not known whether RAF1 binds to KSR before or after activation, it is proposed that, in the quiescent state, a complex associated with KSR, MEK, 14-3-3 and C-TAK1 is formed in cytosol. Upon activation, C-TAK1 is released due to dephosphorylation, resulting in the localisation of the remaining complex to plasma membrane, where RAF1 and KSR bind in a RAS-dependent manner (Morrison & Davis 2003; Müller et al. 2000; Cacace et al. 1999). The other three scaffold proteins are MP1, Sef and Paxillin, which direct ERK activation to late endosomes (Schaeffer 1998; Lunin et al. 2004; Teis et al. 2002; Mouchel-Vielh et al. 2008), Golgi apparatus (Torii et al. 2004) and focal adhesions (Ishibe et al. 2003), respectively (McKay & Morrison 2007).

The family of 90 kDa ribosomal S6 kinases (RSK) is located downstream of ERK1/2 signaling. Activation of RSK takes place at the plasma membrane where activated ERK1/2 phosphorylates the RSK (Richards et al. 2001). Similarly, this activated RSK exerts its regulating effects on cell proliferation, growth and motility through various phosphorylation events (Romeo et al. 2012). Activated ERK1/2 can also translocate to the nucleus (Lenormand et al. 1993; Gonzalez et al. 1993; Chen et al. 1992) where it activates mitogen- and stress-activated kinases (MSK), the two then cooperate to phosphorylate and regulate the expression of transcription factors, e.g. FOS and MYC (Murphy & Blenis 2006).

BRAF mutations are frequently found in melanomas (Maldonado et al. 2003) and colorectal cancer (Davies et al. 2002). V600E, the most identified BRAF mutation, accounts for an overwhelming 80% of all BRAF mutations (Davies et al. 2002). Consistent with the result from Davies et al., this V600E mutation activates the downstream RAF-MEK-ERK signaling in COS cells, the use of a PLX4720, a BRAF inhibitor, successfully inhibited ERK phosphorylation, induced cell cycle arrest and apoptosis in cell lines with V600E BRAF mutation exclusively (Tsai et al. 2008; Davies et al. 2002). Indeed, due to its high frequency in cancer, especially melanoma, the Food and Drug Administration (FDA) of the U.S.A. has approved the use of V600E BRAF inhibitor Vemurafenib (PLX4032, trade name Zelboraf) for the use of late stage melanoma, which was later followed by approval from Health Canada and European Commission. In a phase 2 clinical trial, more than 50% of V600E BRAF bearing melanoma patients responded to treatment with Vemurafenib (Sosman et al. 2012; Ribas et al. 2011). A phase 3 clinical trial compared the outcome of Vemurafenib with dacarbazine, a conventional chemotherapy medication for melanoma, and disclosed improved survival (Chapman et al. 2011). The combination of another BRAF inhibitor Dabrafenib with Trametinib, a MEK1/2 inhibitor, was also approved by FDA for the use against V600E-

positive melanoma. Because ERK is upstream the expression of transcription factors, this inhibition of RAF/MEK/ERK signaling thus induces growth repression through the negative regulation on transcription factors.

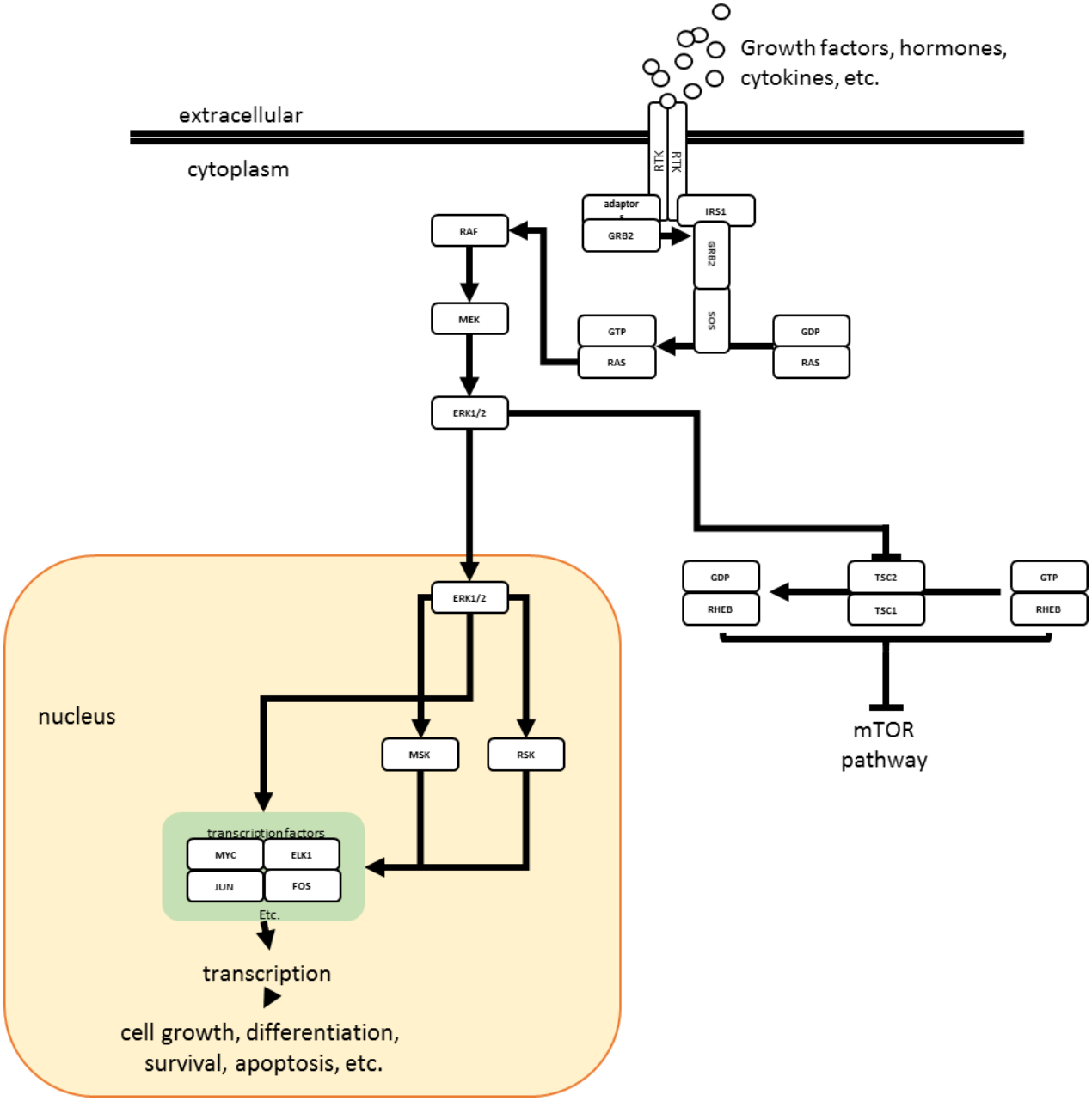


Figure 1.2.2.2.1 A Schematic Diagram of the ERK Pathway

1.2.2.2.2 JNK and p38 Pathway

Similarly, another two MAPK pathways, c-Jun N-terminal kinase (JNK, also known as stress-activated protein kinase, SAPK) and p38, integrate signals to regulate proliferation, differentiation, survival and migration. As the name suggests, JNK was named due to its ability to phosphorylate JUN at Ser63 and Ser73, of which the phosphorylation promotes the transactivating ability of JUN (Kyriakis et al. 1994; Pulverer et al. 1991). Progressing understanding of JUN has indicated the oncogenic property of JNK, as JUN is commonly overexpressed in cancers and forms AP-1 transcription factor (see 1.3.1.1), which causes transcription deregulation, a mechanism of oncogenic transformation (Vogt 2002; Vogt 2001). Additionally, because of its ability to be activated by UV stimulation, the JNK pathway has been determined to be of great importance in the formation of skin cancer (Bode & Dong 2000; Bachelor & Bowden 2004). Interestingly, the p38 pathway exhibits both pro- and anti-cancer activity (Wagner & Nebreda 2009). In various cancer cases, the increased level of p38α and p38δ correlated with cancer malignancy, highlighting the pro-cancerous effect of the p38 pathway (Junttila et al. 2007; Demuth et al. 2007; Elenitoba-Johnson et al. 2003; Pomérance et al. 2006; Greenberg et al. 2002; Esteva et al. 2004). On the other hand, p38 appears also to mediate cancer cell apoptosis, indicating the anti-cancer effect of p38 pathway (Olson & Hallahan 2004; Hallahan et al. 2003). Together, this evidence demonstrates the involvement of JNK and p38 pathways in human tumorigenesis.

The JNKs were first isolated from cyclohexamide-treated rats, with their sequences were found to be 40-45% identical to ERK1/2 (Kyriakis et al. 1994). Like the TEY motif in ERK1/2, the sequences of JNK embed a conserved Thr-Pro-Tyr motif in the kinase activation loop, of which a dual phosphorylation at Thr183 and Tyr 185 is required for the activation of JNKs (Lin et al. 1995). To date, two dual specific kinases were found to be responsible for the phosphorylation and activation of JNKs, mitogen-activated

protein kinase kinase 4 (MKK4, also known as JNKK1 or MEK4) and 7 (MKK7, also known as JNKK2 or MEK7) (Derijard et al. 1995; Lin et al. 1995; Sanchez et al. 1994; Tournier et al. 1997). Interestingly, although JNKs can be activated by each of the two MKKs, Thr183 phosphorylation is preferably performed by MKK7, while MKK4 shows higher selectivity for Tyr185 (Fleming et al. 2000; Lin et al. 1995). Furthermore, reports have suggested that activity of P38 mitogen-activated protein kinases (P38), another class of MAPK kinase, was potentiated by MKK4 (Fleming et al. 2000; Guan et al. 1998; Lin et al. 1995). Yet, MKK4 is not the only kinase the two pathways share in common. There is an extensive overlap between the MAP3Ks in JNK and P38 pathways that phosphorylate and activate downstream MKKs, for instance, apoptosis signal-regulating kinase 1 (ASK1) (Ichijo et al. 1997; Nishitoh et al. 1998), MEKKs (Guan et al. 1998; Sun et al. 2011; Abell et al. 2007), the mixed-lineage kinase (MLK) family (Gallo & Johnson 2002; Tibbles et al. 1996) and transforming growth factor-β-activated kinase 1 (TAK1, also known as MAP3K7 or MEKK7) (Shirakabe et al. 1997; Yamaguchi et al. 1995). Most MAP3Ks in JNK pathway are activated by RAC1 and CDC42 in response to stimulation by growth factors and cellular stress (Coso et al. 1995; Minden et al. 1995; Min & Pober 1997; Xu et al. 2001). Stimulated by inflammatory cytokines, like TNF-α, ASK1 is recruited to TNF receptor associated factor 2 (TRAF2) and therefore activated through this direct interaction (Liu et al. 2000; Nishitoh et al. 1998).

In fact, JUN is not the only transcription factor phosphorylated by JNK, other well-known substrates include activating transcription factor 2 (ATF2) (Gupta et al. 1995) and ETS transcription factor (ELK1) (Yang et al. 1998; Cavigelli et al. 1995). It is believed that, like ERK1/2, JNK affects transcription factors by translocating to nucleus, where it regulates transcription. In neuronal cells under hypoxic stress, JNK3 translocates to the nucleus with its fellow substrates (Zhang et al. 1998). In ischemic rat heart, increased translocation of JNK1 to nucleus was observed, where increased levels of JUN were in

turn phosphorylated by JNK1 (Mizukami et al. 1997). Besides, studies have seen phosphorylated MKK4 and JNK translocate to mitochondria, where JNK catalysed the phosphorylation of BCL2 family proteins to induce the release of cytochrome C, leading to programmed cell death (Chambers et al. 2013; Lei & Davis 2003; Putcha et al. 2003; Harris & Johnson 2001; B. J. Kim, Seung Wook Ryu & Song 2006; Fan et al. 2000; Schroeter et al. 2003; Yamamoto et al. 1999; Aoki et al. 2002).

As mentioned, consensus MAP3Ks between JNK and p38 pathways can as well activate p38 through the intermediate MKKs. It has already been shown that MKK4 can potentiate p38 phosphorylation (Derijard et al. 1995; Lin et al. 1995; Deacon & Blank 1997; Guan et al. 1998). However, interestingly, no marked change of p38 activation was seen in MKK4$^{-/-}$ fibroblasts *in vivo*, which implies that MKK4 has a redundant, or no role in p38 activity (Brancho et al. 2003). Later in that paper, Brancho et al. argued that this redundancy of MKK4 is due to the presence of MKK3 and MKK6, two major activators of p38, phosphorylating Thr180 and Tyr182 within its Thr-Gly-Tyr motif (Stein et al. 1996; Han et al. 1996; Derijard et al. 1995; Moriguchi et al. 1996; Doza et al. 1995; Brancho et al. 2003; Raingeaud et al. 1995).

In addition to transcription factors, protein kinases are reported to be the substrates of p38, some of which are MAP kinase-activated protein kinases 2 (MAPKAPK2, also known as MK2), 3 (MAPKAPK3, also known as MK3)(Rouse et al. 1994; McLaughlin et al. 1996; Freshney et al. 1994; Ono & Han 2000), MSK1 (Deak et al. 1998), MNK1/2 (Scheper et al. 2001; Fukunaga & Hunter 1997) and p38 regulated/activated protein kinase (PRAK)(New et al. 1998). In general, MKK3/6 knockout caused embryonic death mid gestation, suggesting the engagement of p38 in survival (Brancho et al. 2003). Since nuclear distribution of p38 was detected with or without UV irritation, it is believed that

p38 can also translocate to the nucleus, which also explains its phosphorylation of transcription factors (Raingeaud et al. 1995; Raingeaud et al. 1996).

1.2.2.2.3 ERK5 Pathway

Discovered in 1995, the mitogen-activated protein kinase 7 (ERK5, also known as big MAP kinase, BMK1) pathway remains as the least studied pathway in the MAPK signaling network (Zhou et al. 1995; Lee et al. 1995). ERK5 deficiency is lethal, as ERK5 knockout mice died during embryonic development due to cardiovascular defects and angiogenic failure (Hayashi & Lee 2004; Hayashi et al. 2004). As summarised by Hanahan and Weinberg, cancer cells induce angiogenesis to acquire nutrients, oxygen and evacuate metabolic wastes and carbon dioxide (Hanahan & Weinberg 2011; Hanahan & Weinberg 2000). Thus, the role of ERK5 in angiogenesis becomes important in cancer cells. This hypothesis is confirmed *in vivo*, where the volume of tumour xenografts was significantly reduced by ERK5 host gene depletion, due to decreased vascular density, confirming ERK5 is essential in cancer angiogenesis (Hayashi et al. 2005). Currently, ERK5 is known to affect cell survival, proliferation, differentiation, oncogene activation and epithelial-mesenchymal transition, a process where epithelial cells lose cell-cell adhesion and obtain migration activity, in addition to its angiogenic properties (Wang & Tournier 2006; Drew et al. 2012; Nithianandarajah-Jones et al. 2012; Lochhead et al. 2012).

It was not until the late 90s that MEKK2/3 were found to be the upstream activator of MEK5 (Chao et al. 1999; Sun et al. 2001). Later research unveiled that MEKK2/3 forms a complex with MEK5 through a PB1 domain within the N-terminal portion, which inhibits ERK5 activation (Nakamura & Johnson 2003). As MEKK2/3 phosphorylate and activate MEK5, this suggests the bidirectional regulation of ERK5 activity by MEKK2/3. The effects of ERK5 signaling span from cell migration (Spiering et al. 2009), proliferation (Kato et al. 1998) to transformation (Pearson et al. 2001). *In vivo*, similar to its

neighbouring p38 pathway, ERK5 knockout mice showed embryonic lethality due to placental, vascular and cardiovascular defects (Pearson et al. 2001; Regan et al. 2002; Sohn et al. 2002). Upon stimulation, the translocation to nucleus enables ERK5 to phosphorylate and regulate transcription factors, especially myocyte enhancer factor-2 (MEF2) family, peroxisome proliferator-activated receptor γ1 (PPARγ1), FOS proto-oncogene and FOSL1 (Kasler et al. 2000; Terasawa et al. 2003; Akaike et al. 2004; Kato et al. 1997; Esparís-ogando et al. 2002; Kondoh et al. 2006). Interestingly, Terasawa et al. found that FOS, which was previously known to be phosphorylated and activated by ERK1/2, was activated by MEK5-ERK5 signaling through phosphorylation on different sites than by ERK1/2. Moreover, phosphorylation by ERK1/2 only stabilised but failed to activate the activity of FOS, while ERK5 phosphorylation increased the transcription activating ability of FOS (Terasawa et al. 2003).

1.2.2.3 PI3K/AKT Signaling

Downstream of RTKs, the phosphatidylinositol 3-kinase (PI3K)/protein kinase B (PKB, also known as AKT) signaling works in parallel with ERK signaling to regulate various biological functions. The PI3K heterodimers, typically formed of a catalytic and a regulatory subunit, are categorised as class IA, IB, II and III based on their structures, substrates and products (Thorpe et al. 2014). Unlike the protein kinases mentioned above, PI3Ks phosphorylate lipids in its functioning. Essentially, Class IA PI3K heterodimer, p85-p110, can be activated in two ways: i) RTK activated RAS binds directly to the p110 catalytic subunit (Gupta et al. 2007; Pacold et al. 2000; Rodriguez-Viciana et al. 1996); ii) SH2 domain of p85 subunit specifically recognises and binds to the phosphotyrosine residues within the YMXM (Tyr-Met-Xaa-Met, Xaa indicates any amino acid) or YXXM (Tyr-Xaa-Xaa-Met) homologue motif of RTK or RTK substrates (Figure 1.2.2.3) (I Vivanco & Sawyers 2002). Although RAS activates p110, as introduced in 1.2.2.2.1, the activation of RAS requires the help from adaptor proteins such as GRB2

and SOS. Moreover, this activation of PI3K is regulated by its p85 subunit (Jiménez et al. 2002). Within signaling pathways, proteins harbouring YMXM or YXXM motifs range from RTKs like platelet-derived growth factor receptors (PDGFR) and hepatocyte growth factor receptor (HGFR, also known as MET) to RTK substrates, one of which is insulin receptor substrate 1 (IRS1) (Shoelson et al. 1992; Miralpeix et al. 1992; Sun et al. 1991). In the insulin response, the activated insulin receptor phosphorylates IRS1, the latter then recognises the SH2 domains and binds to the p85 regulatory subunit (Myers et al. 1992). Additionally, the SH2 domains of p85 can recognise the YXXM and YMXM motifs of RTKs, therefore interact directly with RTKs (Hu et al. 1992; Otsu et al. 1991; McGlade et al. 1992; Skolnik et al. 1991). In 1994, Stephens et al. reported that a novel PI3K, p110γ, was activated by G-protein coupled receptor (GPCR) through the Gβγ subunit in myeloid-derived cells (Stephens et al. 1994), which was later observed as well in other systems (Murga et al. 1998; Stoyanov et al. 1995). Instead of p85, p110γ was found to associate with p101 regulatory subunit to increase its PI3K activity (Stephens et al. 1997). Furthermore, there have been studies exploring the potential involvement of Class IA PI3K in GPCR-mediated activation. So far, it has been shown that p110β can also be activated by Gβγ (Guillermet-Guibert et al. 2008; Kurosu et al. 1997; Vanhaesebroeck et al. 2010). Interestingly, because GPCR can activate RAS through phospholipase Cβ2 (PLCβ2), it is thought that RAS activation may account for most of the PI3K activity (Suire et al. 2012; Vanhaesebroeck et al. 2010).

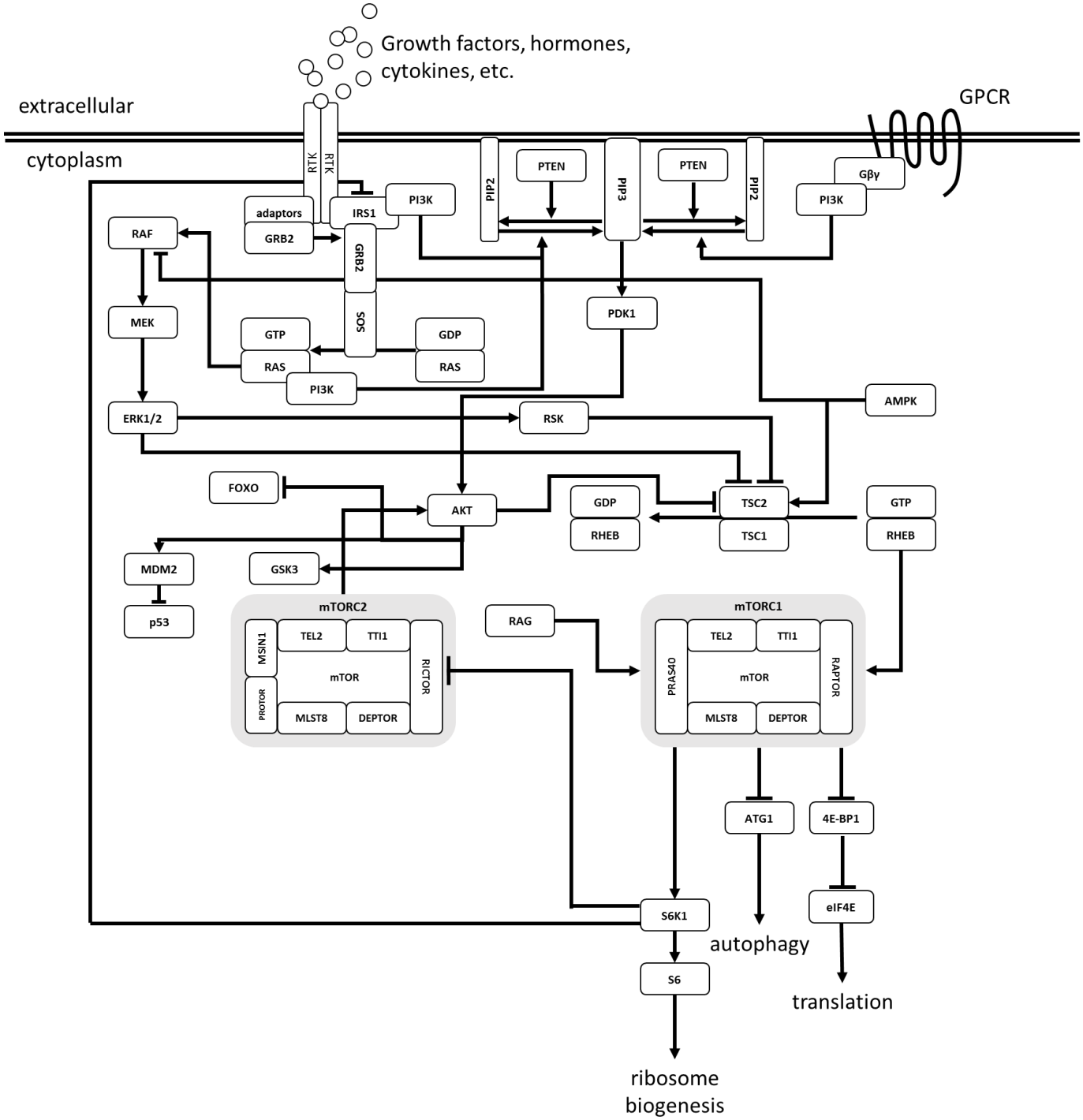


Figure 1.2.2.3 A Schematic Diagram of the PI3K Pathway

Upon the binding of ligands to relevant receptors, active RTKs/GPCRs activate PI3K, which in turn phosphorylates lipid PIP2, resulting in the production of PIP3. Association of PDK1 with membrane-attached PIP3 phosphorylates AKT at Thr308. This activation together with the phosphorylation at Ser473 by mTORC2, AKT is activated. Active AKT then yields its effect via phosphorylating TSC2, FOXO, MDM2, GSK3, etc.

Active Class I PI3K specifically phosphorylates the 3' position of the inositol ring in phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P2, also known as PIP2), producing phosphatidylinositol 3,4,5-trisphosphate (PtdIns(3,4,5)P3, also known as PIP3)(Auger et al. 1989). Studies have shown high affinity of AKT binding to PIP2 and PIP3 through its pleckstrin homology (PH) domain (James et al. 1996; Frech et al. 1997; Franke et al. 1997). Because an AKT mutant without this PH domain was not affected by PI3K activity, it has been suggested that the recruitment of AKT to plasma membrane by PIP2 and PIP3 is essential for the activation of AKT (Frech et al. 1997). However, addition of PIP2 or PIP3 failed to activate, or even inhibit, purified AKT activity *in vitro*, indicating that AKT activation needs more than binding to these phosphatidylinositol phosphates (James et al. 1996; Frech et al. 1997). The mechanism of AKT activation was not clear until Alessi et al. isolated 3-phosphoinositide-dependent protein kinase 1 (PDK1, also known as PDPK1), which phosphorylates AKT at Thr308 in a PIP3-dependent manner (Alessi et al. 1997). This finding also highlights the dual role of PIP3 in AKT activity, that is, its binding to the PH domain of AKT enables the phosphorylation by an PDK1, while PDK1 is again regulated by PIP3 (Stokoe et al. 1997; Alessi et al. 1997). This phosphorylation at Thr308 by PDK1 together with the phosphorylation at Ser473 by mTORC2 is required to achieve the full activation of AKT (Alessi et al. 1996; Bellacosa et al. 1998).

The phosphorylation of PIP2 by Class I PI3K can be reversed or inhibited by phosphatases, e.g. phosphatase and tensin homolog (PTEN) and inositol polyphosphate-5-phosphatase D (INPP5D, also known as SHIP1). PI3K signaling components are frequently mutated in tumour cells to maximise the effect of PI3K signaling on promoting cell growth (Engelman 2009). The well-known tumour suppressor PTEN dephosphorylates PIP3 at position 3 of the inositol ring, exactly the same position phosphorylated by PI3K (Maehama & Dixon 1998; Stambolic et al. 1998; Wu et al. 1998;

Myers et al. 1998). In a similar way, SHIP1 inhibits PI3K signaling by hydrolysing position 5 of the inositol ring of PIP3 to Ptdlns(3,4)P2 (Damen et al. 1996; Lioubin et al. 1996).

Since the successful cloning of the oncogene AKT1/2 from human cells (Staal 1987), the subsequent discovery of AKT substrates has led us to the understanding of the connection between PI3K/AKT signaling and its regulation on cell survival, growth, proliferation and glycolysis/gluconeogenesis (Osaki et al. 2004; Manning & Cantley 2007; Hers et al. 2011). I have discussed the positive regulation of mTOR signaling through AKT inhibiting TSC1/2 heterodimer in 1.2.2.1. It is through this interaction that PI3K/AKT signaling can affect protein synthesis. Notably, a negative feedback loop of mTOR inhibiting PI3K/AKT signaling was found when IRS1 was shown to be phosphorylated by activated S6K1 at Ser270 and mTORC1 at Ser636/639, which eventually leads to the degradation of IRS1 (Carracedo et al. 2008; Harrington et al. 2004; Zhang et al. 2008; Hsu et al. 2011). Another important substrate of AKT is glycogen synthase kinase-3 (GSK3), which is phosphorylated and therefore inhibited by AKT (Cross et al. 1995; Rommel et al. 2001). Apart from the well-identified role in glycolysis/glycogen metabolism, GSK3 has turned out to be involved in embryonic development, cell division, apoptosis, etc. (Cohen & Frame 2001). In terms of cell survival and proliferation, AKT regulates forkhead box (FOX) proteins (Brunet et al. 1999; Rena et al. 1999; Tang et al. 1999), IκB kinase (IKK) (Romashkova & Makarov 1999; Ozes et al. 1999), Bcl-2-associated death promoter (BAD) (Datta et al. 1997), cAMP response element-binding protein (CREB)(Pugazhenthi et al. 2000), caspase-9 (Cardone et al. 1998), ASK1 (Kim et al. 2001), mouse double minute 2 homolog (MDM2) and its human homolog HDM2 (Mayo & Donner 2001; Ashcroft et al. 2002).

PIK3CA is a frequently mutated gene in human cancer. Mutations occur within two small clusters of PIK3CA helical and kinase domains tend to increase PI3K activity

(Samuels et al. 2004). Breast cancer MCF-7 cells harbour a E545K phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha (PIK3CA) mutation, which encodes a permanently activated p110α subunit of the PI3K heterodimer (Hollestelle et al. 2010; A. Hollestelle et al. 2007). Phosphorylation of AKT and GSK3, the downstream targets of PI3K signaling, were shown to be up-regulated in cells with E545K PIK3CA mutation, it is therefore more likely that E545K PIK3CA contributes to sustainable growth signaling, resistance to cell death and other oncogenic effects (Kang et al. 2005; Ikenoue et al. 2005; Bader et al. 2006). In contrast to the predominant mutation of BRAF, V600E (see 1.2.2.2.1), mutations of PIK3CA exhibit diversity (Kang et al. 2005; Ikenoue et al. 2005; Bader et al. 2006; Hollestelle et al. 2010; A. Hollestelle et al. 2007). Therefore, the idea of using a single PIK3CA mutation-specific agent to target various PIK3CA mutation-bearing cancers becomes difficult to implement. Instead, PIK3CA or general PI3K inhibitors were identified as cancer chemotherapy candidates. For example, BYL719, which was shown to inhibit p110α at 5 nM, whereas the IC_{50} values for p110β, p110δ and p100γ were 1200, 290 and 250 nM, respectively, suggesting a high selectivity for PIK3CA (Fritsch et al. 2014; Juric et al. 2014; Furet et al. 2013). The efficiency and safety of BYL719 was also investigated in a Phase 1 clinical trial, where tumour regression and prolonged disease control were observed (Gonzalez-Angulo et al. 2013). It is to be noted that, because of the structural similarity between mTOR and PI3K subunits, PI3K inhibitors are always checked for selectivity over mTOR (reviewed in Bjornsti and Houghton 2004; Shuttleworth et al. 2011). As a result, dual inhibitory effects on mTOR and PI3K were seen with some inhibitors, such as NVP-BEZ235 and GDC-0980 (Serra et al. 2008; Wallin et al. 2011). Apart from PIK3CA, other common mutations in the PI3K pathway found in cancers are PTEN and AKT1 (Nik-Zainal et al. 2016; Li et al. 1997; Rudolph et al. 2016). Whilst most PTEN mutations negatively regulate PTEN tumour suppressor activity (Li et al. 1997), the E17K mutation of AKT1 leads to increased

association of AKT1 to plasma membrane, where constitutive activation of the enzyme can benefit cancer cell growth through downstream signaling (Rudolph et al. 2016; Carpten et al. 2007).

1.2.2.4 AMPK Signaling

The cellular energy sensor, AMP-activated protein kinase (AMPK), plays a significant role in maintaining energy homeostasis. In response to low energy status, AMPK is activated to promote ATP production and shut down ATP-consuming cellular synthetic processes (Hardie et al. 2012). AMPK regulates metabolism, cell growth and polarity controlled by its phosphorylation and subsequent activation by LKB1 tumour suppressor, as will be discussed later in this chapter, (Fogarty & Hardie 2010; Motoshima et al. 2006; Hemminki et al. 1998). These aspects of AMPK are mainly mediated through two axes, the AMPK-TSC2-mTOR and AMPK-p53 signaling (Motoshima et al. 2006). While, as discussed, mTOR regulates cell growth and proliferation, AMPK was found to also directly induce p53 activity, the famous tumour suppressor, by direct phosphorylation. Phosphorylating Ser15 of p53, AMPK activation was shown to cause cell cycle arrest in mouse embryonic fibroblasts cells (Jones et al. 2005).

Most commonly, mammalian AMPK is a heterotrimer composed of a catalytic α subunit and two regulatory subunits, β and γ (Mihaylova & Shaw 2011). Activation of AMPK requires phosphorylation of Thr172 on its α subunit (Hawley et al. 1996). So far, two main activators have been identified, liver kinase B1 (LKB1, also known as serine/threonine kinase 11 (STK11)(Lizcano et al. 2004) and calcium/calmodulin dependent protein kinase kinase 2 (CAMKKβ or CAMKK2) (Figure 1.2.2.4) (Hurley et al. 2005; Woods et al. 2005; Hawley et al. 2005). In the first case, upon energy crisis, inactive LKB1 forms a complex with STE20-like pseudokinase (STRAD) and calcium binding protein 39 (CAB39, also known as MO25) (Zeqiraj et al. 2009; Boudeau et al.

2003; Hawley et al. 2003). While STRAD binds and activates LKB1 by inducing autophosphorylation, it also mediates the localisation of LKB1 from nucleus to cytoplasm (Baas et al. 2003). This interaction between LKB1 and STRAD is enhanced by the addition of MO25, which acts as a scaffold protein in this complex (Lizcano et al. 2004). Apart from STRAD, Zhan et al. demonstrated that nuclear receptor subfamily 4 group A member 1 (NR4A1, also known as NUR77) binding to LKB1 also regulates the activation and localisation of LKB1 (Zhan et al. 2012). However, since STRAD was not investigated in their study, the question remains whether STRAD is affected by NR4A1. Further upstream, LKB1 is activated by protein kinase A (PKA), which in turn is activated by elevated cyclic adenosine monophosphate (cAMP) level (Collins et al. 2000). In cells that do not express LKB1, like HeLa cells, AMPK is activated alternatively by CAMKK2 (Hurley et al. 2005). As the name suggests, CAMKK2 is activated by calcium influx, with activation by calcium ionophore A23187 (Hawley et al. 2005).

In both situations, although the activation of AMPK is dependent on the phosphorylation at Thr172, the activity of AMPK is under regulation by adenosine phosphates. It is reported that there are 4 nucleotide binding sites on the regulatory γ subunit, of which site 1 and 3 binding with AMP can protect the Thr172 phosphorylation from being dephosphorylated and cause allosteric activation (Matthew J Sanders et al. 2007). Notably, protection against dephosphorylation of AMPK was found to be the major mechanism of activation for AMPK (Matthew J Sanders et al. 2007). ADP binding to these two sites, like AMP, can stimulate the phosphorylation of Thr172 but does not activate AMPK directly (Oakhill et al. 2011; Xiao et al. 2011). Affinity for these two sites of adenosine phosphates was determined, which revealed that ADP can bind tighter than ATP (Xiao et al. 2011). As ATP binding leads to dephosphorylation, this explains how AMPK is regulated under situations where ADP and AMP levels are relatively high (Xiao et al. 2011; Oakhill et al. 2011).

Downstream of RTK signaling, AMPK was found to exhibit repression of MAPK/ERK signaling. In HEK293 cells, Ser729 of BRAF was specifically targeted by AMPK, phosphorylation of which promotes the association between BRAF and 14-3-3, eventually disrupting its binding with KSR1 and repressing ERK signaling (Shen et al. 2013). Additionally, mTOR signaling is inhibited by AMPK in two aspects, negatively phosphorylating RAPTOR and enhancing TSC2 activity (Gwinn et al. 2008; Inoki et al. 2003). Since translation consumes large amount of ATP, this AMPK inhibitory effect via ERK/mTOR-dependent transcription and translation initiation will help conserve intercellular energy. Also conserving energy is the inhibition of AMPK on fatty acid synthesis key step, acetyl-CoA carboxylation (Henin et al. 1995). Acetyl-CoA carboxylase (ACC) catalyses acetyl-CoA carboxylation, which serves as a key step in fatty acid synthesis (Wakil et al. 1983). As one of the most-distinguished substrates of AMPK, ACC is inactivated by AMPK predominantly via the phosphorylation at Ser79 (Ha et al. 1994). Acetyl-CoA carboxylation produces malonyl-CoA, a potent inhibitor of fatty acid uptake by mitochondria, inhibiting the activity of carnitine palmitoyltransferase I (CPT1) and therefore inducing fatty acid oxidation (McGarry, Leatherman, et al. 1978; McGarry et al. 1977; McGarry, Takabayashi, et al. 1978; Yamauchi et al. 2002). In other words, AMPK activation not only shuts down energy consuming processes, including those necessary for cell proliferation, but promotes ATP production from fats (Hardie et al. 1998; D. G. Hardie 2002).

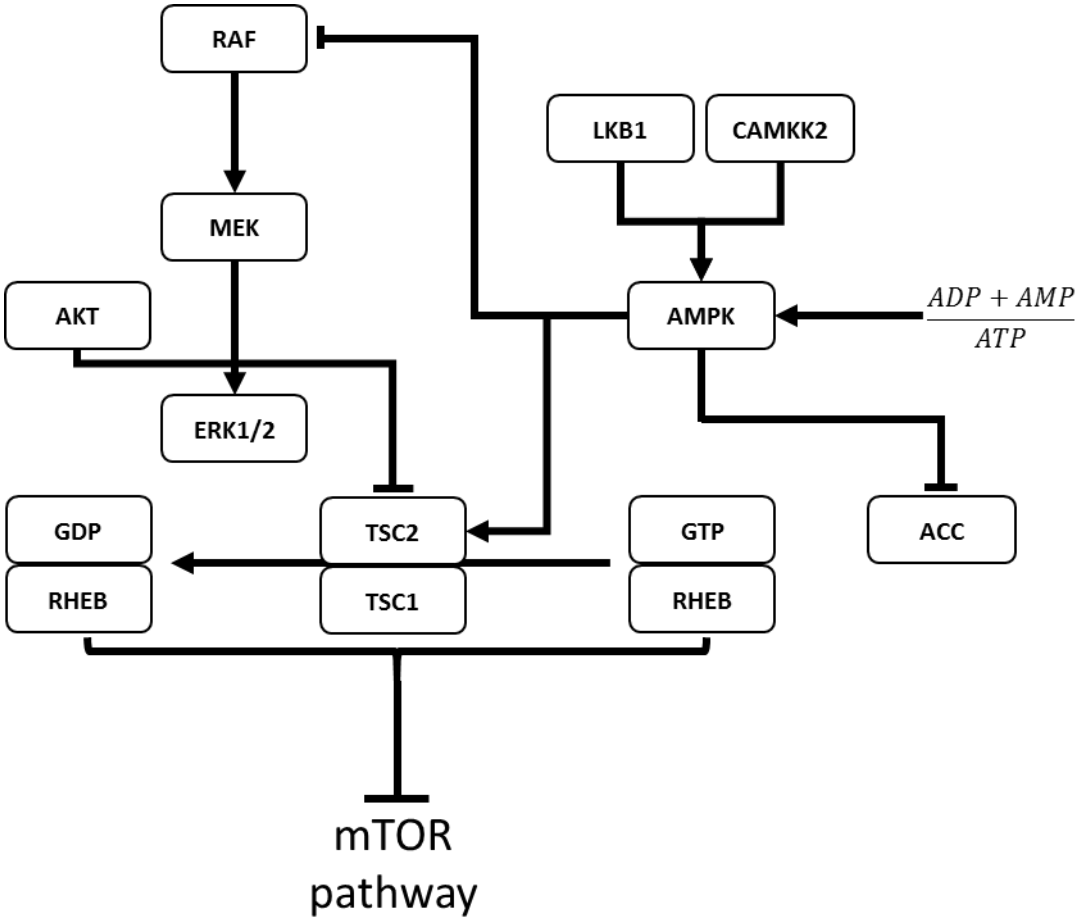


Figure 1.2.2.4 A Schematic Diagram of the AMPK Pathway

The ability of AMPK to repress mTOR and activate p53 has made AMPK a popular target for cancer therapy, especially in obesity- and diabetes-related cancer. One of the highly researched AMPK activators is metformin, which activates AMPK by inhibiting GPD1 redox shuttle enzyme, mitochondrial glycerophosphate dehydrogenase, negatively regulating the electron transport chain (Madiraju et al. 2014). Because of the involvement of AMPK in cell growth and metabolism, metformin is widely used in diabetes and obesity whilst clinical trials are investigating the use in various cancers (Yanovski & Yanovski 2002; Bailey & Turner 1996; Luo et al. 2005).

1.2.3 Problems with targeting signal transduction in cancer therapy

Indeed, the complex and significant involvement of signal transduction has led to the development and clinical introduction of drugs targeting signal transduction. A brief search on clinicaltrials.gov has returned large number of both ongoing and completed

clinical trials for the application of signal transduction-dependent drugs in various types of cancer (Table 1.2.4). As mentioned in 1.2.2.2.1, we have learnt that targeting BRAF V600E mutation resulted in improved survival. However, while ARAF was suggested to be a scaffold protein for the BRAF-RAF1 heterodimer (Rebocho & Marais 2013), with the existence of another functional member of the RAF kinase family, Raf-1 proto-oncogene serine/threonine kinase (RAF1, also known as c-RAF), the question arises that inhibiting BRAF may cause elevated activity of RAF1 as a means of displacement in cancers. Hence, developing a less specific drug that targets both RAF1 and BRAF could be the key to address the problem. This is also supported by the discussion above that cancer is constantly evolving to evade growth control and maintain its invasiveness. To date, Sorafenib remains one the most successful RAF inhibitors that inhibits RAF1 and both wild-type and mutated BRAF (Wilhelm et al. 2004; Roberts & Der 2007), making its way to clinical use against liver, kidney and thyroid cancers (Llovet et al. 2008; Escudier et al. 2007). However, the use of Sorafenib requires a precise drug delivery technique, as off target effects of Sorafenib on non-cancerous cells cause harm in normal tissue, which is another major problem with untargeted medication for signal transduction. Since most signaling systems are more of a network rather than a linear cascade, it is quite unlikely that a single inhibitor/activator could be used as a stand-alone cancer therapy. In addition, the use of a single drug in signal transduction inhibition is also limited by feedback loops. As mentioned in 1.2.2.3, mTORC1 negatively regulates IRS1 via direct phosphorylation. It has been reported consistently that mTOR inhibition activates PI3K and ERK signaling (Carracedo et al. 2008; O'Reilly et al. 2006). To achieve enhanced efficacy in blocking tumorigenesis related signal transduction, therapies that target multiple pathways are more promising.

Target Protein	Drug	Target Cancer	Phase	Identifier
MEK	AZD6244	Non-Small Cell Lung Cancer	1	NCT01146756
MEK	Binimetinib	Leukaemia	1	NCT02049801
ERK	BVD-523	Acute Myelogenous Leukaemia	1 and 2	NCT02296242
ERK	BVD-523	Pancreatic Cancer	1	NCT02608229
ERK	LTT462	Solid Tumour	1	NCT02711345
ERK	LY3214996	Advanced Cancer	1	NCT02857270
p38	LY3214996	Advanced Cancer	1	NCT02857270
p38	LY2228820	Glioblastoma	1 and 2	NCT02364206
mTOR	RAD001	Metastatic Breast Cancer	2	NCT02313051
mTOR	RAD001	Thyroid Cancer	2	NCT00936858
mTOR	Temsirolimus	Relapsed Bladder Cancer	2	NCT01827943
mTOR	rapamycin	Advanced Cancer	1	NCT01266057
AMPK	Metformin*	Relapsed Chronic Lymphocytic Leukaemia	2	NCT01750567
PI3K	BYl719	Metastatic Breast Cancer	2	NCT02506556
PI3K	BKM120	Thyroid Cancer	2	NCT01830504
PI3K	PQR309	Advanced Solid Tumour	1	NCT02483858
AKT	ARQ751	Solid Tumour	1	NCT02761694
AKT	GSK2110183	Solid Tumour	2	NCT01531894
AKT	GSK2141795	Advanced Triple Negative Breast Cancer	2	NCT01964924
AKT	MK2206	Metastatic Colorectal Cancer	2	NCT01802320
AKT	TAS-117	Solid Tumour	2	NCT03017521
EGFR	AC0010	Non-Small Cell Lung Cancer	1 and 2	NCT02274337
receptor tyrosine kinase	AL3818	Endometrial, Ovarian or Cervical Cancer	1 and 2	NCT02558348
receptor tyrosine kinase	MGCD265	Non-Small Cell Lung Cancer	2	NCT02544633
receptor tyrosine kinase	MGCD516	Advanced Cancer	1	NCT02219711

Table 1.2.3 List of Selected Clinical Trials

Unless specified, drugs mentioned in this table are inhibitors for the indicated proteins.
*activator of the indicated protein. Clinical trial data based on clinicaltrials.gov.

1.3 Gene Expression

The process of transforming information of the genome into gene products is called gene expression. The most well-known of these gene products are proteins, which are encoded by an intermediate gene product messenger RNA (mRNA). As opposed to the protein-coding mRNAs, some RNAs lack the open reading frame (ORF) and thus are not translated into proteins. These RNAs are termed non-coding RNAs (ncRNAs), which encompass RNAs like transfer RNA (tRNA), ribosomal RNA (rRNA), long non-coding RNA (lncRNA), microRNA (miRNA), small nuclear RNA (snRNA), small nucleolar RNA (snoRNA) and pseudogenes. Published in 2001, the human genome project disclosed that only about 1.5% of the human genome is consisted of protein-coding sequences, and only about one third of the genome would be transcribed into genes (Lander et al. 2001). In other words, this suggests there is a large number of genes that are transcribed but not translated. In 1.3, I will be focusing on mRNA and the regulation of mRNA expression that cordycepin could be affecting. Also introduced is the non-coding RNA which we hypothesise is involved in cordycepin mechanism of action.

1.3.1 Examples of the Regulation of Gene Expression and Signal Transduction

1.3.1.1 Growth factor Regulated mRNA Expression

In response to growth factors binding to RTKs, downstream signaling transforms this stimulation to regulation of various cellular activities, including gene expression. For cancer cells, where mutations within different pathways enable malignant growth, the concurrent deregulation of gene expression, particularly, at transcription stage is regarded as a key phenomenon in cancer development. As one of the early transcription factors being discovered, activator protein-1 (AP-1) is a dimeric complex made up of members of the JUN, FOS, basic region-leucine zipper (bZIP), ATF and MAF subfamilies. Most of these AP-1 components are also known as immediate early genes, because their mRNA is quickly induced after stimulation (Fambrough et al. 1999;

Herschman 1991). Among these IEGs/transcription factors, the regulation of the oncogenes FOS and JUN are well-understood. One of the three cis elements found in the promoter area of FOS is serum response element (SRE), which recruits ternary complex factors (TCFs) through serum response factors (SRFs) to form a ternary complex (Treisman 1992). ELK1 was identified as a TCF candidate in this complex (Shore & Sharrocks 1994). ELK1, a transcription factor that responds to MAPK pathways, is activated through phosphorylation by ERK, JNK or p38 (Janknecht et al. 1993; Raingeaud et al. 1996; Cavigelli et al. 1995) (Whitmarsh et al. 1995). ELK1 is involved in forming the ternary complex at SRE of FOS explaining FOS induction in response to growth factor stimulation (Müller et al. 1984; Kruijer et al. 1984). In addition to the regulation through ELK1, reports have demonstrated that ERK and its effector RSK co-ordinately phosphorylate FOS at Ser374 and Ser362 respectively (Chen et al. 1993; Okazaki & Sagata 1995). While these two phosphorylations stabilise the FOS protein, they also expose a FXFP (DEF) domain of FOS for additional phosphorylation by ERK, mutation of which reduced AP-1 activity and cellular transformation (Murphy et al. 2002). Similar to the SRE domain in FOS, the sequence of JUN embeds a tumour promoter 12-O-tetradecanoylphorbol-13-acetate response element (TRE) domain that is preferably targeted by the JUN-ATF2 AP-1 heterodimer (van Dam et al. 1993). induction of JUN-ATF2 by JNK phosphorylation promotes the transcription activation ability of the heterodimer, and subsequently induce JUN production (Gupta et al. 1995; Kyriakis et al. 1994). Taken together, all this evidence demonstrates that mRNA expression is under the regulation of signal transduction.

1.3.1.1.1 TOP mRNA Regulation by mTOR Signaling

TOP mRNA features a tract of pyrimidine at the 5' end of the sequence, therefore it is termed 5' terminal oligopyrimidine (TOP) mRNA. Most ribosomal protein mRNAs and elongation factors are TOP mRNAs. Over the years, there have been various opinions on

the mechanism of how TOP mRNAs are regulated. Early reports have proposed that S6K1 plays an important role in TOP mRNA expression (Jefferies et al. 1997), while later studies revealed that S6K1 inhibition failed to alter TOP mRNA levels, indicating that S6K1 is not essential to TOP mRNA expression (Stolovich et al. 2002; Tang et al. 2001). Instead, PI3K signaling was found to be the key pathway, as TOP mRNA translation was significantly inhibited by LY294002, a pan-PI3K inhibitor (Stolovich et al. 2002). As discussed in 1.2.2.3, PI3K-PDK1-AKT can target TSC2 and mTORC1, therefore mTOR signaling could be linking PI3K to TOP mRNA. A downstream target of mTORC1, La-related protein 1 (LARP1) was recently identified as the intermediate between mTORC1 and TOP mRNA (Tcherkezian et al. 2014). Deep-sequencing data unveiled that inactive LARP1 binds to both 5' and 3' UTRs of ribosomal protein mRNAs, while the binding on 5' was released when LARP1 is phosphorylated by mTORC1 and AKT/S6K1 (Lahr et al. 2017). Since the binding of LARP1 to 5' UTR of ribosomal protein RNAs exhibits inhibitory effect on their translation, this finding, consistently with others, suggests that LARP1 is the key to mTORC1 regulated TOP mRNA translation (Hong et al. 2017; Tcherkezian et al. 2014; Lahr et al. 2017; Fonseca et al. 2015).

1.3.2 Non-Coding RNA

With the overwhelming number of genes in human genome being ncRNAs, the question arises what these genes do in cells. Although not translated into protein, ncRNAs also participate in vital biological processes. For example, tRNA and rRNA, two most characterised ncRNAs, orchestrate translation in most cells across all species. This discovery was followed by snRNA, snoRNA and miRNA, which are involved in splicing, rRNA maturation and mRNA expression, as will be discussed later in this section. Besides, more and more long ncRNAs are being found as regulators in tumour-suppressor and oncogenic pathways, giving rise to the hypothesis that ncRNAs can be used as cancer biomarkers or potential therapeutic targets in the future (Gibb et al.

2011; Mattick & Makunin 2006; Esteller 2011; Huarte & Rinn 2010; Gutschner & Diederichs 2012).

1.3.2.1 snRNA

snRNAs are usually 60-300 nt RNAs that mostly exist in the form of small nuclear ribonucleoprotein (snRNP)(Y. Yu et al. 1999). So far, it has been generally accepted that snRNAs can be divided into two subclasses, Sm snRNA and Lsm snRNA, based on their structures (Y. Yu et al. 1999; Matera et al. 2007). Sm snRNA comprises U1, U2, U4, U4atac, U5, U7, U11 and U12, whereas only U6 and U6atac are the only two members in Lsm snRNA. According to Stark and Lührmann, U1 is the most abundant snRNA in cells (Stark & Lührmann 2006). U1, together with U2, U4/U6, U5 and more than 150 proteins, constitute the spliceosome to facilitate accurate pre-mRNA splicing (Wahl et al. 2009; Nagai & Muto 2001; Stark et al. 2001). In addition to its role in splicing, knockdown of U1 caused premature cleavage and polyadenylation at cryptic poly(A) signals (Kaida et al. 2010), which is consistent with the observation that U1 interacts with U1 70K protein and PAP to repress polyadenylation (Gunderson et al. 1998). As opposed to the spliceosome described above, U11 and U12, two relatively low abundant snRNAs, contribution to the formation of a minor spliceosome, which originally was known for splicing AT-AC introns but this range is expanding as research progresses (Hall & Padgett 1996; Yu & Steitz 1997; Incorvaia & Padgett 1998; Montzka & Steitz 1988; Kolossova & Padgett 1997; Wassarman & Steitz 1992; Tarn & Steitz 1996). Depending on which spliceosome is used, introns are therefore divided into U2-dependent and U12-dependent type (Sharp & Burge 1997). Unlike the effect of other snRNAs on mRNA regulation, U7 plays an important role in 3’ end processing of histone RNAs (Mowry & Steitz 1987; Spycher et al. 1994).

1.3.2.2 snoRNA

Classification of different small nucleolar RNAs (snoRNAs) is predominantly decided by the motifs embedded in their sequences, C/D or H/ACA box (Kiss 2002; Henras et al. 2004). Here, while C, D, H and ACA are all different yet conserved consensus motifs in these two types of snoRNAs, C/D and H/ACA snoRNAs also feature distinct structures to facilitate recruitment and positioning for partner enzymes (Ganot, Caizergues-Ferrer, et al. 1997; Maxwell & Fournier 1995). The most recognised function of snoRNAs is their ability to regulate rRNA biogenesis (Matera et al. 2007). As studies in HeLa cells have revealed that pre-rRNA processing efficiency was significantly reduced by inhibition of methylation (Wolf & Schlessinger 1977; Vaughan et al. 1967), the fact that C/D snoRNAs were shown to direct site-specific 2'-O-methylation suggests their effects in pre-rRNA processing (Nicoloso et al. 1996; Kiss-László et al. 1996). Similarly, site-specific pseudouridylation of pre-rRNA is understood to be mediated by H/ACA snoRNAs in a base complementary manner, suggesting that C/D and H/ACA snoRNAs function in parallel to modulate rRNA synthesis (Lafontaine 2015; Ni et al. 1997; Ganot, Bortolin, et al. 1997; Kiss et al. 2004). However, the effect of snoRNAs is not restricted in rRNA processing, further research has unveiled that tRNA, snRNA and pre-mRNA are all targets of snoRNAs modification (reviewed in Matera et al. 2007; Y.-T. Yu et al. 1999). Smith and Steitz have reported that mouse Gas5, an lncRNA encoding 9 C/D snoRNAs, possesses a tract of oligopyrimidine towards the 5' end of the sequence, and it is therefore considered to be a TOP RNA. This led to the mapping of all known snoRNA host gene 5' end sequences, from which uncovered 5' TOP features across all genes tested (Smith & Steitz 1998). As mentioned in 1.3.1.1.1, TOP mRNAs are regulated in response to growth stimulation, it is therefore possible that snoRNA and/or snoRNA host genes are coordinated with ribosome biogenesis through mTOR pathway (Y. Yu et al. 1999).

1.3.2.3 miRNA

The biogenesis of miRNA can be summarised in four steps: i) RNA polymerases synthesise initial transcripts pri-miRNA; ii) a ribonuclease III endonuclease, Drosha, performs nuclear cleavage on pri-miRNA to generate pre-miRNA; iii) Dicer, another ribonuclease, cuts off the stem loop of pre-miRNA, resulting in miRNA:miRNA* duplex; iv) helicase separation of the duplex, generating the mature miRNA (Bartel 2004; Lee et al. 2002; Du & Zamore 2005). By forming RNA-induced silencing complexes (RISC), which also include proteins, miRNA mediates effects on gene through mRNA decay and translation repression. While early work on miRNA suggested that cleavage was induced by RISC, which was in turn directed by miRNA (Valencia-Sanchez et al. 2006; Hutvágner & Zamore 2002; Doench et al. 2003; Gregory et al. 2005; Maniataki & Mourelatos 2005), recent studies demonstrated that deadenylase complexes, like the CCR4-NOT complex, are recruited to targeted mRNA by miRNA-mediated RISC, for mRNA degradation (Piao et al. 2010; Gregory et al. 2005; Behm-Ansmant et al. 2006; Braun et al. 2011; Lim et al. 2005; Chekulaeva et al. 2011). Although the repression of translation by miRNA is mainly considered to be at the initiation stage (Iwakawa & Tomari 2015), with some suggesting PABP is responsible (Moretti et al. 2012; Zekri et al. 2013), others argue that cap binding complex is regulated (Nishimura et al. 2015; Rouya et al. 2014; Meijer et al. 2013), a unified model is yet to be discovered.

1.3.2.4 lncRNA

Long non-coding RNAs (lncRNAs) are defined as ncRNAs of more than 200 nucleotides. In human, the understanding of lncRNA started with the discovery of X inactive specific transcript (XIST), which serves a major role in X chromosome inactivation (Brown et al. 1991). This is followed by the advent of metastasis associated lung adenocarcinoma transcript 1 (MALAT1) and nuclear paraspeckle assembly transcript 1 (NEAT1). While alternative splicing and cancer cell metastasis was shown to be regulated by MALAT1

(Tripathi et al. 2010; Gutschner et al. 2013; Zhe & Lin 2012), NEAT1 acts as an essential structural determinant in the formation of paraspeckles, depletion of which leads to the demolition of paraspeckles (Clemson et al. 2009). MALAT1 overexpression has also been observed in hepatocellular carcinoma cell lines and patient tissues comparing to non-tumour cell line/tissue, suggesting the potential of MALAT1 as a biomarker for hepatocellular carcinoma (Lai et al. 2012).

As provided by a global transcriptome analysis, antisense/sense pairs is a common yet consistent phenomenon in mammals (Katayama et al. 2005; Røsok & Sioud 2004). It is therefore suggested that antisense RNA, as another class of lncRNA, is widely involved in various biological activities. Indeed, in the mouse brain for instance, the expression of neuronal isoform of nitric oxide synthase (Nos1) was negatively regulated by its relative antisense, which is observed in neuroblastoma Neuro2a cells (Korneev et al. 2015). Also, antisense of p15 tumour suppressor, p15AS, silences the *cis* and *trans* elements of normal p15 in leukaemia by inducing heterochromatin formation, implicating the possible contribution of antisense RNA in tumour suppressor gene silencing (Yu et al. 2008).

The 21st century has seen emerging progress in lncRNA research (Gibb et al. 2011). However, this is just the tip of the iceberg, as 5,481 non-coding genes were predicted by full-length analysis of the human genome, meaning that more lncRNAs are awaiting discovery and validation (Ota et al. 2004).

1.3.2.5 Pseudogenes

A pseudogene is defined as a piece of apparently non-functional DNA sequence presented in the genome with high homology to a functional gene. In the human genome, ribosomal protein genes are the gene family where most pseudogenes are derived from, accounting for 67% of the estimated pseudogene pool (Venter et al.

2001). Pseudogenes generated by retrotransposon are known as processed pseudogenes, as opposed to unprocessed pseudogenes, which originate from segmental duplication (Vanin 1985; Maestre et al. 1995). Because of the high homology with original genes, the detection of pseudogenes relies on fast-developing DNA sequencing techniques.

Proposed by Kimura and Ohta more than 40 years ago, the neutral mutation hypothesis emphasises that nucleotide substitution is expected to be more frequent in biologically less important genes than biologically important genes, because the latter are subjected to relatively stronger natural selection pressures (Kimura & Ota 1974; King & Jukes 1969). Hence, Li et al. suggested that the existence of pseudogenes is a paradigm of neutral evolution (Li et al. 1981). This idea is however challenged by a series of recent discoveries. Firstly, derived from PTEN, PTENP1 is a pseudogene that encodes a shorter 3' UTR than the PTEN mRNA, which, as mentioned in 1.3.2.3, could be targeted by miRNA and degraded. Poliseno et al. showed that PTENP1 abundance is indeed significantly reduced by miR-19b and miR-20a, and that the presence of PTENP1 caused growth inhibition in human prostate cancer DU145 cells. This suggests a tumour suppressive activity of PTENP1 conveyed by titrating the miRNA-mediated degradation machinery to protect normal PTEN function (Poliseno et al. 2010). In addition, bioinformatics analysis of a ribosome profiling experiment has found that 40% of lncRNAs and pseudogenes are ribosome associated, providing further evidence that pseudogenes could be functioning in human cells, and may even encode proteins (Ji et al. 2015). This was shown to be true with the discovery of protein-coding pseudogene, ionotropic receptor 75a (Ir75a), in *Drosophila sechellia* (Prieto-Godino et al. 2016). With the increasing understanding of different regulatory RNAs and modern high throughput techniques, more controversial findings on pseudogenes are to be expected.

1.4 Cordycepin

1.4.1 Introduction to Cordycepin

Cordycepin, also known as 3'-deoxyadenosine, was originally discovered in extracts from the caterpillar fungus *Cordyceps militaris*. Caterpillar fungi have been used as a tonic to strengthen the human immune system, or provide energy after serious illness in traditional Chinese pharmacy for more than 500 years (Cunningham et al. 1950; Pegler et al. 1994). In modern medicine, cordycepin has been proposed to have a wide spectrum of biological properties from anti-polyadenylation, anti-tumour, anti-proliferation, anti-metastasis to anti-inflammation (Paterson 2008; Yoshikawa et al. 2004; Müller, Seibert, Beyer, Muller, et al. 1977; Ng & Wang 2005; Kondrashov et al. 2012). Cordycepin is a derivate of adenosine, which differs from cordycepin by the addition of a 3'-hydroxyl group (Figure 1.4.1). This structural similarity indicates the possibility of cordycepin interfering with any of the many processes that require adenosine or phosphorylated adenosine (adenosine monophosphate, diphosphate and triphosphate), including polyadenylation, the well-known target of cordycepin. Moreover, cordycepin has been found to inhibit protein synthesis and cell adhesion via mTOR pathway inhibition in fibroblast NIH3T3 cells (Wong et al. 2010), suggesting the potential use of cordycepin in mTOR pathway inhibition and mTOR related cancers.

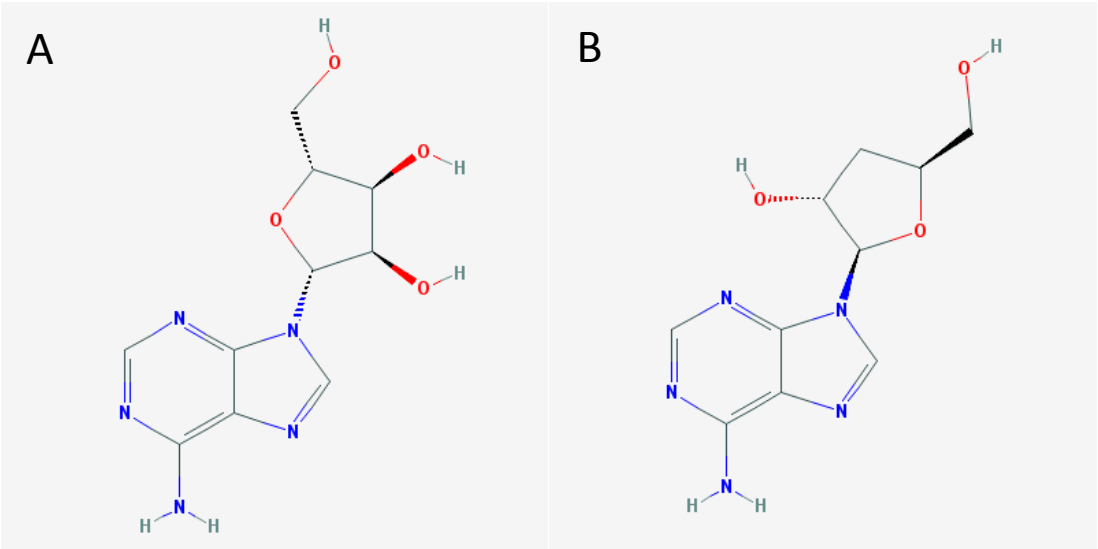


Figure 1.4.1 The Chemical Structure of Cordycepin and Adenosine

A. Adenosine. B. Cordycepin. Pictures adapted from PubChem (chemical structure identifier CID: 60961 and 6303 respectively)(Kim et al. 2016).

1.4.2 Cordycepin Metabolism

With the structural similarity between cordycepin and adenosine, cordycepin is believed to be transported via adenosine transporters, e.g. equilibrative nucleoside transporter 1 (ENT1), as nitrobenzylthioinosine rescued the effect of cordycepin in different cells (Griffith & Jarvis 1996; Plagemann et al. 1988; Wong et al. 2010; Kondrashov et al. 2012). In spite of this observation, there are published data suggesting the adenosine receptor as a means of cordycepin metabolism. For example, in mouse melanoma B16-BL6 cells, binding of CI-IB-MECA, a specific adenosine A_3 receptor agonist, to A_3 was inhibited by cordycepin in a dose-dependent manner, and that inhibition of this receptor also resulted in tumour growth defects (Nakamura et al. 2006; Yoshikawa et al. 2008). Therefore, cordycepin metabolism may be cell type specific. However, given that adenosine and cordycepin share structural similarity and is claimed to be an activator of adenosine receptors, it is to be expected that adenosine would have similar effects as cordycepin on cancer cells. Yet, the effect of adenosine on the adenosine receptor was not tested in the two studies cited above.

In collaboration with David Barrett's lab in the School of Pharmacy, University of Nottingham, Wahyu Utami has examined the levels of cordycepin, cordycepin triphosphate and deoxyinosine, a metabolite of deoxyadenosine deamination, with liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS). Two major findings were observed: i) cordycepin was stabilised by pentostatin (2'deoxycoformycin); ii) cordycepin was unstable in serum, plasma and cells and transformed into stable cordycepin triphosphate rapidly inside the cell (Figure 1.4.2). On one hand, it is within expectation that pentostatin, a potent inhibitor of adenosine deamination, stabilised cordycepin (Agarwal et al. 1977; Brogden & Sorkin 1993; Johns & Adamson 1976); on the other, the quick accumulation of cordycepin triphosphate agrees with the observation in mouse lymphoma L5178Y and leukaemia cells (Kodama et al. 2000; Müller, Seibert, Beyer, Breter, et al. 1977). Consequently the question arises: which is the active form, cordycepin, deoxyinosine or cordycepin triphosphate? In cells, the phosphorylation of adenosine into its monophosphate is catalysed by adenosine kinase (Mimouni et al. 1994; Anderson 1973) and inhibited by 5'-iodotubercidin (ITu) (Parkinson & Geiger 1996; Hinshaw et al. 1969). Treatment with ITu in NIH3T3 cells completely abrogated the inhibitory effect of cordycepin on protein synthesis and inflammatory gene expression (Wong et al. 2010; Kondrashov et al. 2012), which suggests the phosphorylation of cordycepin is needed for these effects. In other words, cordycepin triphosphate is most likely to be the active form of cordycepin.

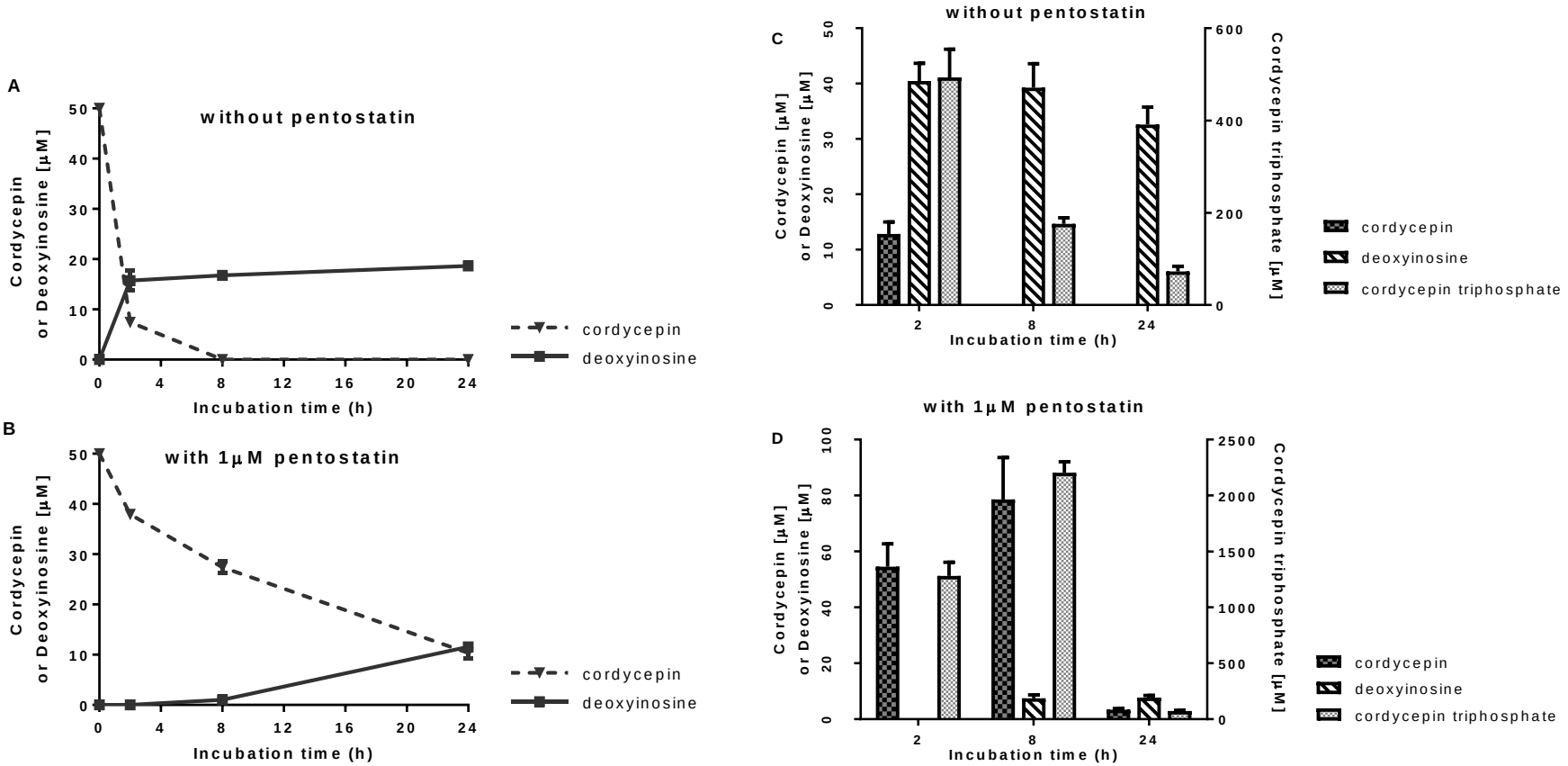


Figure 1.4.2 Cordycepin Metabolism

MCF-7 cells were exposed to either 50 µM cordycepin (A and C) or the combination of 50 µM cordycepin with 1 µM pentostatin (B and D) for indicated times. The level of cordycepin, deoxyinosine and cordycepin triphosphate was measured in these samples by LC-MS/MS method. All graphs here are adapted from the thesis of Wahyu Utami submitted for the degree of Doctor of Philosophy, 2015. (Wahyu Utami, unpublished data)

1.4.3 Cordycepin in Signal Transduction

1.4.3.1 MAPK Signaling

The mitogen-activated protein kinase (MAPK) signaling pathways are made up of an extensive kinase network, regulating different physiological processes. So far, three parallel MAPK pathways have been described in detail (Figure 1.2.2.2). Various reports have shown that the activity of ERK1/2 (Liao et al. 2015; Pao et al. 2012; Jin et al. 2014), p38 mitogen-activated protein kinases (p38)(H. G. Kim, Shrestha, Lim, Yoon, Chang, D. J. Shin, et al. 2006; Lee et al. 2009; Noh et al. 2009; K.-J. et al. 2009) and JNK signaling (Lee, Jeong, et al. 2013; Lee et al. 2009; Noh et al. 2009; Pao et al. 2012) were repressed by cordycepin in different cells. Additionally, as will be discussed in Chapter 3, a microarray based on cordycepin treated breast cancer MCF-7 cells was done in the De Moor lab, data analysis of which revealed that a large number of genes significantly down-regulated by cordycepin were listed as transcription-related genes (97/289, 33.56%, Table 1.4.3.1). As these included immediate early genes (IEG) like JUN, JUNB and MYC, it is therefore hypothesised that MAPK signaling, especially ERK1/2 signaling, was repressed by cordycepin in these cells (Murphy & Blenis 2006; Murphy et al. 2004).

1.4.3.2 AMPK Signaling

The activation of AMPK is possibly one of the most consistent effects of cordycepin, which has been observed in many different cell lines (Zhang et al. 2014; Guo et al. 2010; Wang et al. 2010; Wong et al. 2010; W.-D. Wu et al. 2014). Since its discovery, efforts were made to understand the mechanism, but no unified explanation has been reached. Wu et al. claim that, in HepG2 cells, the activation of AMPK is due to the direct interaction of cordycepin with the AMPK γ1 subunit, whilst Wang et al suggest the binding of cordycepin monophosphate to AMPK is responsible (C. Wu et al. 2014; Wang et al. 2010). In the first case, as cordycepin and adenosine share a similar chemical structure, there is a need to compare the affinity of the two compounds in reacting with AMPKγ1. Both cases highlighted the binding of cordycepin/cordycepin monophosphate

to AMPK, we have however introduced above that intracellular cordycepin was predominantly transformed into cordycepin triphosphate, while the levels of other metabolites except deoxyinosine are low, which suggests very high affinity of cordycepin or cordycepin monophosphate is needed to trigger AMPK activation in this model. Further research by Wahyu Utami revealed that, while ATP was surprisingly upregulated, ADP or AMP levels were not altered 2 hrs after cordycepin treatment (Wahyu Utami, unpublished data). Theoretically, the ensuing lowered $\left[\frac{AMP+ADP}{ATP}\right]$ ratio should result in dephosphorylation of AMPK, the very opposite of observations in NIH3T3 cells. Therefore, the mechanism behind cordycepin activation of AMPK remains elusive.

GO ID	Functional cluster	Number	Genes	*P* value
GO:0006355	Regulation of transcription	97	*ATF3, ADRB2, ARID5B, PRR15L, BRF2, ABL1, CITED2, CEBPB, CD86, CBX2, CBX8, CRY2, CSRNP1, DDX20, DLX1, DLX2, E2F6, EGR1, EPC2, FOXA1, FOXC1, FOXL2, FOXQ1, , FOSL1, GATA6, GLIS2, HSF2, CENPBD1, ID1, ID3, ING1, IL11, IL6, JUNB, JUN, KLF5, KLF6, LIF, MED13L, MED9, MEIS1, MIER2, MIER3, MLL5, MYNN, NFIL3, NR4A3, PPRC1, LOC152845, PLAGL2, RHEBL1, RLF, RARA, RUNX1, SERTAD1, SERTAD2, SETD1A, SMAD6, SMAD7, TGIF1, TIGD5, TICAM1, TLE4, TRIB1, TRIM27, MAFF, MAFG, MYC, ZBTB3, ZBTB43, ZBTB45, BTB5, ZNF107, ZNF165, ZNF189, ZNF200, ZNF263, ZNF283, ZNF317, ZNF394, ZNF433, ZNF48, ZNF511, ZNF555, ZNF562, ZNF574, ZNF581, ZNF584, ZNF613, ZNF628, ZNF629, ZNF646, ZNF668, ZNF689, ZNF7, ZNF784, ZNF8, ZKSCAN2*	1.9E-19
GO:0006915	Apoptosis	22	*AEN, AIFM2, C8ORF4, F3, CSRNP1, DDIT4, FADD, FOXL2, GATA6, IER3, IL6, JUN, NUAK2, PLEKHF1, PPP1R15A, RHOB, SGK1, SIAH1, SIAH2, SHB, TRAF2, MYC*	3.50E-04
GO:0042981	Regulation of apoptosis	29	*ABL1, ADRB2, FOXL2, CEBPB, DLX1, DUSP1, F3, FOXC1, ID3, IL6, JUN, SMAD6, MYC, TRAF2, FOSL1, FADD, IER3, SOCS3, HERPUD1, CITED2, DDX20, CLCF1, SH3RF1, AEN, NUAK2, AIFM2, TICAM1, PIM3, LOC152845, PLAGL2*	3.50E-05
GO:0001525	Angiogenesis	7	*CTGF, CYR61, ID1, JUN, KLF5, RHOB, SHB*	2.60E-02
GO:0045765	Regulation of angiogenesis	5	*F3, ID1, IL6, RHOB, RUNX1*	1.50E-02
GO:0042127	Regulation of cell proliferation	22	*ATF3, ADRB2, ADM, CD86, F3, FOSL1, HBEGF, ING1, IRS2, IL11, IL6, JUN, KLF5, LIF, PPM1D, SERTAD1, TGIF1, TICAM1, TOB2, TRIB1, MAFG, MYC*	8.80E-03
GO:0007049	Cell cycle	21	*ABL1, CDC25A, CDT1, DUSP1, E2F6, HJURP, ING1, MLL5, PELO, PLK2, PLK3, PPP1R15A, PPM1D, RASSF1, SESN2, SIAH1, SIAH2, SH3BP4, MYC, WEE1, ZNF830*	1.50E-02
GO:0051726	Regulation of cell cycle	17	*CITED2, CDC25A, CDT1, CYP1A1, FOXC1, FOSL1, ID3, JUNB, JUN, LIF, MAD2L1BP, PIM3, RHOB, SERTAD1, SMAD6, MYC, OBFC2A*	4.80E-05
GO:0030334	Regulation of cell migration	7	*CITED2, F3, HBEGF, IRS2, IL6, SMAD7, TRIB1*	4.50E-02
GO:0045597	Positive regulation of cell differentiation	11	*CD84, GATA6, TGIF1, FOXA1, IL6, JUNB, JUN, LIF, RARA, RUNX1, SOCS3*	2.70E-03

Table 1.4.3.1 Gene Ontology Analysis of the Most Significantly Down-regulated Genes in MCF-7 Microarray

Table taken from the thesis of Rocky submitted for the degree of Master of Research Advanced genomic and proteomic Sciences, 2013 (Irengbam Rocky Mangangcha, unpublished data).

1.4.3.3 PI3K/AKT and mTOR Signaling

Originating from the receptor tyrosine kinase (RTK), signals from growth factors are transmitted through RAS-RAF-MEK-ERK and/or PI3K/AKT signaling (Gschwind et al. 2004; Lemmon & Schlessinger 2010; Cantley 2002). AKT, the core effector of PI3K/AKT signaling, exerts regulatory effects on a wide range of biological processes, namely cell survival, proliferation, metabolism, growth and others (Hers et al. 2011; Manning & Toker 2017; Nicholson & Anderson 2002; Vara et al. 2004). With studies showing AKT phosphorylation being inhibited by cordycepin administration in many different experiments by different groups, it is suggested that PI3K/AKT signaling is affected by cordycepin (Pan et al. 2015; Takahashi et al. 2012; Jang et al. 2015; Wong et al. 2010; H. G. Kim, Shrestha, Lim, Yoon, Chang, D.-J. Shin, et al. 2006). By applying LY294002, a classic PI3K inhibitor, Kim et al. witnessed the similar reduction in p-IκB levels as in the cordycepin treatment group (H. G. Kim, Shrestha, Lim, Yoon, Chang, D.-J. Shin, et al. 2006). In another experiment, treatment with LY294002 mimicked the repression on MMP-2 and -9 level by cordycepin in human prostate carcinoma LNCaP cells (Jeong et al. 2012). Most of these reports only focused on the phosphorylation at Ser473 instead of Thr308. This, however, is not the direct way to study the effect of cordycepin on PI3K signaling. As has been (see 1.2.2.3) and will be discussed later in this section, Ser473 is an mTORC2 phosphorylation site, while Thr308 is phosphorylated by PDK1, an upstream kinase in the PI3K signaling pathway. Despite all these studies, Yoon et al. examined the effect of cordycepin on *in vitro* immunoprecipitated AKT and found the enzyme activity is reduced by 85%, suggesting a the direct inhibition of cordycepin over AKT (Ju Young Yoon et al. 2015).

Notably, AMPK signaling, RAF-MEK-ERK and PI3K-PDK1-AKT axes all point to Tuberous Sclerosis Complex 2 (TSC2), one of the two subunits that form the TSC1/2 heterodimer, which in turn regulates mTORC1 activity via RAS homolog enriched in brain (RHEB)

(Laplante & Sabatini 2012b; Nishimoto 2000; Guertin & Sabatini 2007). Due to the role of mTOR signaling in protein synthesis, lipid synthesis, autophagy, energy metabolism and lysosome biogenesis (Laplante & Sabatini 2012a), the effects of cordycepin on mTOR have been widely researched. Our laboratory has previously reported that protein synthesis in NIH3T3 cells was inhibited by cordycepin in a 4E-BP1 dependent manner (Wong et al. 2010), which agrees with the findings in non-small cell lung cancer cells (Yu et al. 2017). As the phosphorylation of ribosomal protein S6 Kinase Beta-1 (S6K1) was also down-regulated by cordycepin (W.-D. Wu et al. 2014; Yu et al. 2017), results concur to suggest that cordycepin inhibits mTORC1 activity. Wong et al. detected down-regulation of AKT phosphorylation at Ser473 AKT, which is the phosphorylation site of mTORC2 (Wong et al. 2010; Sarbassov et al. 2005). In other words, it seems that not only the activity of mTORC1, but mTORC2 was also repressed by cordycepin in NIH3T3 cells. There have been some studies focusing on the interaction between S6K1 and mTORC2, suggesting the mTORC1-activated S6K1 inhibits mTORC2 activity through phosphorylation of the rapamycin-insensitive companion of mammalian target of rapamycin (RICTOR) (Tato et al. 2011; Julien et al. 2010). However, if only mTORC1 is inhibited by cordycepin, this feedback loop will, in contrast to the observation in NIH3T3 cells, lead to up-regulation of mTORC2 activity, suggesting that both mTOR complexes are repressed by cordycepin.

1.4.3.4 NF-κB Signaling

Including our previous study, the suppression of cordycepin on NF-κB signaling is one of the most promising effects in various cell lines, contributing to the anti-tumour and anti-inflammatory properties of cordycepin (Kadomatsu et al. 2012; Jeong et al. 2010; Tuli et al. 2013; Kondrashov et al. 2012; H. G. Kim, Shrestha, Lim, Yoon, Chang, D.-J. Shin, et al. 2006). The activity of NF-κB is tightly regulated by NF-κB inhibitors (IκB), the degradation of which will release NF-κB for its translocation to the nucleus and

transcription regulation (Hayden & Ghosh 2008; Gilmore 2006; Perkins 2007). In mouse microglial BV2 cells, Jeong et al. demonstrated that the LPS-induced translocation of P65, one of the subunits that form the NF-κB heterodimer, was reversed by cordycepin, along with the increased cytosolic amount of IκBα (Jeong et al. 2010). This effect of cordycepin was also seen in LPS-treated mouse macrophage RAW264.7 cells, where P65 mostly remained in cytoplasm after cordycepin addition (Masar Radhi, unpublished data). Downstream, the expression of matrix metalloproteinase (MMP) is regulated by NF-κB signaling (Bond et al. 2001; S.-J. Lee et al. 2008; S. Lee et al. 2008; Kao et al. 2012; Bond et al. 1998). With cordycepin, the induction of MMPs by Interleukin 1 Beta (IL-1β) or tumour necrosis factor alpha (TNF-α) was inhibited, which indicates that NF-κB activity is repressed by cordycepin (Noh et al. 2009; Lee et al. 2010). However, in airway smooth muscle cells, expression of NF-κB targeted inflammatory mRNAs was inhibited by cordycepin in a NF-κB-independent manner, as cordycepin did not affect NF-κB nuclear translocation, whereas polyadenylation-mediated mRNA stability was suggested to be responsible instead (Kondrashov et al. 2012).

1.4.5 Anti-Apoptosis

The evolutionary conserved family of cysteine aspartic acid proteases (caspase) plays the central role in mammalian cell apoptosis, activation of which causes apoptotic activities, e.g. DNA fragmentation, mainly via the cleavage of caspase substrates (Hengartner 2000; Elmore 2007). Because of this, efforts have been made to investigate whether the apoptotic effect of cordycepin comprises the regulation of caspase signaling. Indeed, in human prostate carcinoma, Lee et al. have shown that caspases were activated by cordycepin through reactive oxygen species (ROS), yielding pro-apoptotic effect via mitochondria-mediated mitochondrial death pathway (Lee, Park, et al. 2013). Apoptosis was also witnessed in cordycepin treated breast cancer MDA-MB-231 cells, where cytosolic cytochrome c levels were elevated coupled with caspase-3

and -9 activation, as a result of the induction of Bax in response to high concentrations of cordycepin (Choi et al. 2011). In addition, cordycepin increased levels of BCL2 pro-apoptotic molecules (Bax, Bid, Bim and Puma) in human colorectal cancer SW480 and SW620 cells (He et al. 2010). All these cases indicate that the intrinsic apoptotic pathway is activated by cordycepin. Yet, in human liver cancer HepG2 and multiple myeloma cells, cordycepin induced the cleavage and activation of caspase-8 (Shao et al. 2016; Chen et al. 2008). Consequently, it has now been shown that not only intrinsic but extrinsic apoptosis pathways are among the mechanisms through which cordycepin induces apoptosis in cells (Hengartner 2000; Lawton 2016; Elmore 2007; Tuli et al. 2013).

Poly(ADP-ribose) polymerase (PARP) is a family of proteins involved in DNA damage repair and genome stability (Herceg & Wang 2001). In a cell-free system, Kim et al. have shown attenuated activity of recombinant PARP1 in response to cordycepin, indicating a direct regulation on PARP-mediated DNA repair, which has as yet not been confirmed (Kim et al. 2011). Mediated by the activation of PARP1, translocation of apoptosis-inducing factor (AIF) from mitochondria to nucleus facilitates cell death (Yu et al. 2002). In addition, PARP cleavage by caspases abolishes its catalytic activity and it is referred as one of the universal biomarkers of apoptosis (Nicholson et al. 1995; Tewari et al. 1995; Lazebnik et al. 1994; Herceg & Wang 2001). In agreement with the introduction of cordycepin inducing caspase activity, caspase-cleaved PARP1 was induced by cordycepin in different cell lines (Chen et al. 2014; Chen et al. 2010; Jeong et al. 2011). All this evidence demonstrates that PARP1 is a potential target of cordycepin, and its inhibition contributes to the effect of cordycepin-induced cell death.

1.4.6 Anti-Metastasis

Both *in vitro* and *in vivo* studies have demonstrated the anti-metastatic property of cordycepin (Nakamura et al. 2005; Yoshikawa et al. 2009; Sato et al. 2013; Zhang et al.

2015; Wong et al. 2010; Jeong et al. 2012). Interaction between extracellular matrix (ECM) and cells provides cells with information essential to diverse cellular activities (Hynes 2009; Juliano & Haskill 1993). Given that MMPs are responsible for the degradation of ECM and cell surface proteins, it is safe to hypothesise that the anti-metastatic effect of cordycepin could occur via the inhibition on NF-κB or MAPK signaling (Tuli et al. 2013). In melanoma cells, Zhang et al. found that miR-33b directly binds and suppresses the expression of HMGA2, TWIST1 and ZEB, while increases expression (of HMGA2, TWIST1 and ZEB) positively regulates tumour migration and invasion. Cordycepin treatment elevated the level of miR-33b, which therefore inhibited metastasis in melanoma (Zhang et al. 2015).

1.4.7 Anti-angiogenesis

Most papers emphasising the anti-angiogenic effect of cordycepin cited the paper from Yoo et al., where *Cordyceps militaris* extract was used instead of cordycepin (Yoo et al. 2004). Indeed, cordycepin is one of the compounds found within this extract (Cunningham et al. 1950; Pegler et al. 1994), but more evidence is needed to confirm this observation. In HepG2 and EA.hy926 cells, Lu et al. assessed the anti-angiogenic effect of cordycepin using tube formation assay, with which they found the length of the tube was reduced significantly by cordycepin, indicating an anti-angiogenic activity of cordycepin (Lu et al. 2014). As another major contributor to angiogenesis, MMPs promote pro-angiogenic properties via degradation of ECM, which could also be inhibited by cordycepin (Haas et al. 2000; Rundhaug 2005).

1.5 The Polyadenylation Inhibitory Effect of Cordycepin

After the nascent mRNA is transcribed, post-transcriptional modification will be performed by cells to yield functional mRNA. RNA processing procedures include the addition of the 5’ cap, 3’ cleavage, polyadenylation and splicing. The observation of cordycepin inhibiting mRNA polyadenylation was first obtained > 40 years ago (Rose et

al. 1977). In 1.5, I will be introducing the machinery of cleavage and polyadenylation, before going into detail about the effect of cordycepin on polyadenylation.

1.5.1 Cleavage and Polyadenylation

While cleavage is the endonucleolytic removal of the 3' redundant sequence of precursor mRNA (pre-mRNA), polyadenylation is the addition of a poly(A) tail after the cleavage site (Wahle 1995). These two steps are tightly coupled as they take place one after the other and share the same 3'-end processing complex, which is composed of cleavage and polyadenylation factor (CPSF)s, cleavage stimulation factor (CstF)s, cleavage factor I (CFI), CFII, RNA polymerase II (Pol II) (Hirose & Manley 1998) and poly(A) polymerase (PAP) (Takagaki et al. 1989). Although later studies using high-throughput proteomic assays have characterised ~85 proteins in this complex, including WDR33, FIP1L1 and PSF, the six complexes described above remain the founding members of the 3'end processing complex (Shi et al. 2009).

The sequence AAUAAA is a highly conserved consensus sequence towards the 3' end of most mRNAs, which serves as a polyadenylation signal (PAS) found in >90% of all genes (Proudfoot & Brownlee 1976; Wahle 1995). Functional analysis of the point mutated AAUAAA sequence has observed failed cleavage or polyadenylation in all variants tested except in mRNAs with AUUAAA or AGUAAA signal, where polyadenylation of lower efficiency was detected (Wickens & Stephenson 1984; Wilus et al. 1989; Sheets et al. 1990). Notably, AGUAAA is not usually found in natural mRNA, this makes AUUAAA the most dominant natural AAUAAA variant, accounting for 10% of the total mRNA (Sheets et al. 1990). Among messenger RNAs, only histone precursor mRNA, which doesn't use a AAUAAA signal and ends with a stem loop instead of a poly(A) tail (Pandey & Marzluff 1987; López & Samuelsson 2008; Marzluff et al. 2008) All other mammalian pre-mRNA is thought to contain a form of the AAUAAA signal, which will be recognised by CPSF4 and WD repeat domain 33 (WDR33) (Schönemann et al. 2014; Chan et al. 2014).

Downstream of the PAS, a stretch of U-rich or GU-rich sequence is termed the downstream element (DSE), which is responsible for the specific binding of CstF2 (Gil & Proudfoot 1987; Chou et al. 1994; Takagaki & Manley 1997). Compared with PAS, DSE is not as conserved as PAS (Mandel et al. 2008). Interacting with CPSFs, CstF3 links CPSFs with the other two subunits of CstF, CstF1 and CstF2 (Takagaki & Manley 1994; Takagaki et al. 1990). Lying between PAS and DSE is the cleavage site, also referred to as the poly(A) site because poly(A) are added after the cleavage site. CA is the most frequent sequence of a cleavage site (Sheets et al. 1990). Nevertheless, with the help of SV40 late polyadenylation signal, Chen et al. discovered that 3' cleavage has a preference for the second residue of this sequence, in the order of: A>U>C>>G (Chen et al. 1995). This cleavage process is carried out by CPSF3 as purified recombinant CPSF3 exhibited endonuclease activity (Mandel et al. 2006). An *in vitro* study has suggested that, surprisingly, successful cleavage requires no ATP but creatine phosphate (Hirose & Manley 1997). A factor interacting with PAP and CPSF1 (FIP1L1, also known as hFip1) is a human CPSF subunit homologous to Fip1 in *Saccharomyces cerevisiae*, which was found to be essential in yeast polyadenylation (Preker et al. 1995; Preker et al. 1997; Kaufmann et al. 2004). It has been reported that human FIP1L1, on one hand, binds to the U-rich sequence towards the 3' end of pre-mRNAs; on the other, it binds PAP and forms a ternary complex with PAP and CPSF1, to ensure efficient polyadenylation (Kaufmann et al. 2004).

Despite their intimate bound in the 3' end processing complex, our understanding of CFI and CFII remains at the very preliminary stage. Whereas CFI was shown to play a role in alternative polyadenylation and the regulation of 3' UTR length and bind the upstream element (USE) (Hardy & Norbury 2016; Kubo et al. 2006; Martin et al. 2012; Sartini et al. 2008), we are hoping for more research regarding CFII. In human cells, the canonical poly(A) polymerases are PAPα, PAPβ and PAPγ, encoded by PAPOLA, PAPOLB and

PAPOLG respectively (Zhao & Manley 1996; Kyriakopoulou et al. 2001; Wahle et al. 1991). However, the majority of polyadenylation is carried out by PAPα and PAPγ (Bardwell et al. 1990; Topalian et al. 2001). PAPβ was found expressed exclusively in testis (Le et al. 2001; Lee et al. 2000). Other non-canonical poly(A) polymerase includeTUT1 (also known as Star-PAP or PAPD2) (Mellman et al. 2008), GLD2 (PAPD4) (Kwak et al. 2004), mitochondrial poly(A) polymerase MTPAP or PAPD1 (Tomecki et al. 2004), PAPD5 (Rammelt et al. 2011) and PAPD7 (Ogami et al. 2013).

Sometimes, a pre-mRNA harbours more than one AAUAAA element in the sequence. If these elements are also functional PASs with USE and/or DSE elements, cleavage and polyadenylation could occur at these sites, leading to the production of transcripts in different sizes. This phenomenon of polyadenylation happening in multiple sites is termed alternative polyadenylation. In fact, a genome-wide survey for poly(A) site in human genes disclosed that ~54% of the genes exhibited alternative polyadenylation (Tian et al. 2005). They also proposed that, depending on the location of PAS, 8 types of alternative polyadenylation can be classified, which they were able to validate in human tissues (Tian et al. 2005; Zhang et al. 2005).

1.5.2 Other Types of Polyadenylation

After exportation from nucleus, the poly(A) tail can still be elongated through cytoplasmic polyadenylation (Charlesworth et al. 2013). An example of cytoplasmic polyadenylation is the mRNAs in oocytes, where these dormant or masked mRNAs accumulate before fertilization. During oocyte maturation, the poly(A) tail of these mRNAs, originally about 20 nt long, will be elongated to roughly 150 nt, enabling efficient translation of these mRNAs (Radford et al. 2008; Richter 1999). Also, cytoplasmic polyadenylation has been observed in embryos (Charlesworth et al. 2013).

Polyadenylation plays an important role in mitochondrial RNA (mtRNA) stability, as significant reduction of steady state mtRNA and mitochondrial dysfunction was seen following MTPAP knockdown, even though protein levels were only slightly affected (Nagaike et al. 2005; Wilson et al. 2014), which is consistent with a previous study (Chrzanowska-Lightowlers et al. 2004). In addition, mitochondrial polyadenylation appears to be involved in generating mtDNA UAA stop codons (Temperley et al. 2003) and tRNA maturation (Yokobori & Pa 1997; Tomita et al. 1996).

In yeast, the Trf4/5-Air1/2-Mtr4-polyadenylation (TRAMP) complex exerts RNA quality control activity by recruiting exosomes to target RNAs in a polyadenylation/poly(A) tail-dependent manner (Wyers et al. 2005; Vaňáčová et al. 2005; LaCava et al. 2005; Bresson et al. 2017), demonstrating that the poly(A) tail is needed for regulation of certain yeast RNAs. Since the discovery of this exosome-dependent RNA degradation complex, efforts have been made to identify the homologues in human. Lubas et al. used exosome immunoprecipitation, followed by stable isotope labelling in cell culture (SLIAC), to uncover PAPD5, ZCCHC7 and SKIV2L2 as the human counterparts of Trf4p, Air2p and Mtr4 respectively (Lubas et al. 2011). In discovering PAPD5, Rammelt et al. have identified pre-rRNA as the preferred substrate of PAPD5 *in vivo* (Rammelt et al. 2011). Together with the 5.8S rRNA accumulation seen after PAPD5 knockdown (Lubas et al. 2011), it seems likely that the poly(A) tail mediates human TRAMP complex-dependent degradation of rRNA. Besides, further analysis suggests that the human TRAMP complex specifically targets PROMoter uPstream Transcripts (PROMPTs), which are unstable RNA transcripts with 5’ cap and 3’ adenosine tail structure (Preker et al. 2011; Preker et al. 2008). With PROMPTs being found in RNAs produced by all three RNA polymerases (Preker et al. 2011), the stability of PROMPT-embedding RNAs, including mRNA, is likely to be subjected to regulation by human TRAMP complex (Andersen et al. 2013; Lubas et al. 2011; Schneider & Tollervey 2013).

1.5.3 Poly(A) Tail Length

It is generally believed that the initial mRNA poly(A) tail size is ~150-250 nt in mammalian cells (Darnell et al. 1971). Chang et al. used a genome-wide poly(A) tail determination method, Tail-seq, and found that some mRNAs have poly(A) tails of ~20 nt, which is significantly different from the commonly referred 150-250 nt (Chang et al. 2014). However, like many other similar tests, this experiment did not specifically analyse poly(A) tail size at the time of polyadenylation (Yang et al. 2011; Meijer et al. 2007; Choi & Hagedorn 2003; Chang et al. 2014). In the De Moor lab, by employing 4-thiouridine labelling, which specially labels newly synthesised mRNA incorporated into the sequence, Singhania et al. were able to determine initial poly(A) tail sizes of different mRNAs (Singhania et al., manuscript in preparation). With the help of the poly(A) tail test (PAT), they found that poly(A) tails of serum response mRNAs were immensely shortened. Also shown was that initial poly(A) tails of housekeeping genes, e.g. Actb and Actg1, were in fact much shorter than the canonical 200 nt (Singhania et al., manuscript in preparation). As gene ontology analysis by Chang et al. has disclosed that most of the mRNAs with short poly(A) tails are transcription factors, cell-cycle regulators, embryonic morphogenesis and protein modification (Chang et al. 2014), our findings consistently suggest that initial poly(A) tail size varies between mRNAs.

1.5.4 Poly(A) Tail-Mediated mRNA Regulation

Poly(A) tail regulates mRNA expression mainly through the interaction with different proteins/complex. In eukaryotic cells, most mRNA degradation is mediated by exonucleases in a deadenylation-dependent manner (Wu & Brewer 2012; Balagopal et al. 2012; Schoenberg & Maquat 2012). Poly(A) binding proteins, including PABPCs and PABPN1, mediate mRNA stability and translation efficiency in a poly(A) tail-dependent manner. The poly(A) tail bound PABPs were incorporated into the cap-dependent translation initiation complex to facilitate protein translation, while forming a closed

loop with the mRNA. As opposed to the degradation caused by shortened poly(A) tail, the formation of this complex protects 3' nucleolytic attack and therefore deadenylation (Peltz & Jacobson 1992; Bernstein & Ross 1989; Bernstein et al. 1989; Ross 1995). Besides, there are indications that translation efficiency is in positive correlation with poly(A) tail length via PABP, suggesting not only mRNA stability, but translation can be affected by PABPs (Wilkie et al. 2003; Gray et al. 2000; Craig et al. 1998; Charlesworth et al. 2013).

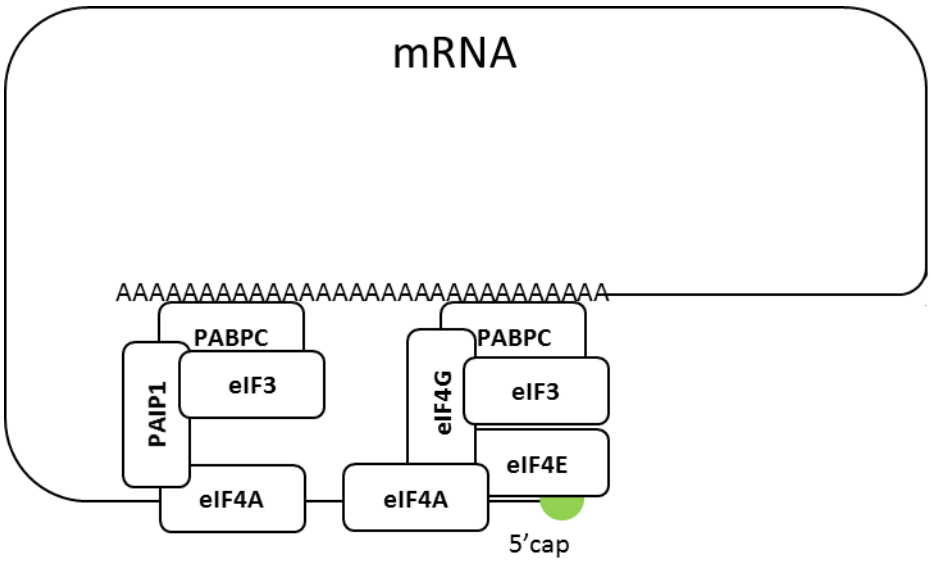


Figure 1.5.4 A Schematic Diagram of the Translation Initiation Complex

Diagram adapted from Charlesworth et al. 2013.

Several RNA-binding motifs in PABP have been characterised so far, with RNA recognition motif (RRM) being the most widely found motif in RNAs (Burd & Dreyfuss 1994). Poly(A) binding protein cytoplasmic 1 (PABPC1, also known as PABP1) was discovered in 1987, which was later found to be locating in cytoplasm with ability to be transported to nucleus (Görlach et al. 1994; Grange et al. 1987; Afonina et al. 1998). This is consistent with the observation that PABPC1 binds to poly(A) tails of nuclear pre-mRNA (Hosoda et al. 2006). When bound with RNA, PABPC1 interacts with eIF4G (Imataka et al. 1998), poly(A) binding protein interacting proteins (PAIPs)(Craig et al. 1998; Khaleghpour et al. 2001) and eIF4B (Bushell et al. 2001) of the cap-dependent

translation initiation eIF4F complex to regulate translation (Figure 1.5.4). The bond between PABPC1 and these initiation factors forms a cap-dependent translation initiation complex, which facilitates the translation process (Mangus et al. 2003), whereas disruption of the PABPC1-mRNA bond labels mRNA for deadenylation and/or 3'-5' decay (Schoenberg & Maquat 2012). This role of PABPC1 is consistent with the dramatic reduction of translation after PABPC1 depletion (Kahvejian et al. 2005). Another PABP, poly(A) binding protein nuclear 1 (PABPN1, also known as PABP2), was found to be in tight connection with alternative polyadenylation, that loss of PABPN1 enhanced cleavage at proximal PAS sites and shortened 3' UTR (De Klerk et al. 2012; Jenal et al. 2012). In addition, whilst it is evident that PABPN1 controls poly(A) length by interaction with PAP and CPSF (Kühn et al. 2009; Apponi et al. 2010), depletion of PABPN1 in myoblast caused nuclear accumulation of poly(A) RNA , suggesting that i) poly(A) tail is needed for efficient RNA nuclear export and ii) PABPN1 regulates mRNA biogenesis through regulation of the poly(A) tail (Fuke & Ohno 2007). Other PABPs include PABPC1L, PABPC3 and PABPC4, but research into these PABPs remains at the very early stage (Wilkie et al. 2005; Yang et al. 1995; Houng et al. 1997; Guzeloglu-Kayisli et al. 2008; Féral et al. 2001; Gorgoni et al. 2011).

1.5.5 The Cleavage and Polyadenylation Defect of Cordycepin

As mentioned, cordycepin triphosphate was found being a chain terminator in poly(A) tail extension, presumably because it is mistaken for adenosine triphosphate (Rose et al. 1977). Having known that cordycepin is quickly transformed into triphosphate when in cells, since the discovery, cordycepin and cordycepin triphosphate have been widely used to study polyadenylation *in vitro* (Zarkower & Wickens 1987; Rose et al. 1977; Ryner & Manley 1987; Holbein et al. 2009). In addition to terminating polyadenylation, cordycepin triphosphate arrested a complex, which includes all polyadenylation factors, on the processed mRNA in *in vitro* polyadenylation reactions (Zarkower & Wickens

1987; Shi et al. 2009). Utilising luciferase assays and poly(A) tests, the De Moor group has successfully detected that polyadenylation was specifically targeted and the subsequent poly(A) tail was shortened by cordycepin (Wong et al. 2010; Kondrashov et al. 2012). Moreover, RT-qPCR in airway smooth muscle cells using primers that span the cleavage site has demonstrated increased read through levels after cordycepin treatment, suggesting that precursor mRNA cleavage was affected by cordycepin (Kondrashov et al. 2012). This result is in agreement with Ryner et al., who showed that cordycepin triphosphate can inhibit cleavage as well as polyadenylation (Ryner & Manley 1987). Lastly, it is to be noted that, because of the importance of the poly(A) tail in gene expression, there is concern as to whether cordycepin could be affecting the poly(A) tails of all the genes. In NIH3T3 cells, the addition of cordycepin failed to affect the poly(A) tail size of Rpl28, which serves as a control gene in the experiment (Wong et al. 2010).

1.6 Aims and Summary of the Study

In short, the aims of this study are:

- Characterise the documented cell viability effects of cordycepin in breast cancer cell lines, with focus on interrogation of signal transduction, gene expression, cleavage and polyadenylation
- Characterise the effect of cordycepin on growth-factor-dependent gene expression in breast cancer cell lines
- Investigate the effect of cordycepin on ncRNAs
- Investigate the link between growth factor-dependent gene expression and signal transduction in response to cordycepin
- Determine whether inhibitors/activators of the known cordycepin effectors can mimic the effect of cordycepin on gene expression

Based on the pilot experiments by another student in the De Moor lab, the aim of my project is to first validate the documented effects of cordycepin, as mentioned above, on breast cancer MCF-7, MDA-MB-468 and MDA-MB-231 cells, before trying to explore the mechanisms involved. In terms of cell survival, cordycepin exhibits concentration-dependent anti-survival effects in all cell lines tested, and the effect was enhanced by the addition of pentostatin, an inhibitor of cordycepin degradation pathway. Secondly, growth factor response was inhibited by cordycepin: i) negative regulating effect of cordycepin on mTOR, ERK/MAPK and PI3K signaling was shown in MCF-7 cells, while AMPK was predominantly phosphorylated on the activation site; ii) cordycepin significantly inhibited EGF response in EGFR overexpressing MDA-MB-468 cells. Selected from the microarray data from a previous project, genes down-regulated by cordycepin included a large number of transcription factors. Consistently, the same group of genes was also down-regulated by cordycepin in MDA-MB-231 Fluc cells deep sequencing, suggesting that this phenomenon is not cell type-specific. Since the effects on both gene expression and signal transduction initiated at around the same time, polysome profiling was conducted to see if cordycepin affects translation efficiency of signal transduction proteins, perhaps through effects on cytoplasmic polyadenylation. Disappointingly, the ribosome association rates for key signal transduction protein genes were not significantly changed by cordycepin, which could have explained the effect of cordycepin on signal transduction. However, the number of ribosomes associated with TOP mRNA were greatly reduced. Since TOP mRNAs are under established regulation of mTOR pathway, this finding suggests that the mTOR pathway is inhibited by cordycepin. In searching for the primary target(s) of cordycepin, different inhibitors for AKT, mTOR, PI3K, ERK, PARP and an activator for AMPK were applied to MCF-7 to mimic the effect of cordycepin. Pictilisib, a PI3K inhibitor, is so far the only compound that exhibited similar regulating effects on signal transduction and gene

expression, demonstrating the possibility that the PI3K inhibition is important in cordycepin mechanism of action. As opposed to the mRNAs down-regulated by cordycepin, up-regulated by cordycepin were predominantly lncRNAs and pseudogenes. Moreover, in agreement with a recent paper, these lncRNAs and pseudogenes were subjected to polyadenylation-dependent degradation, again confirming the effect of cordycepin in inhibiting polyadenylation. Taken together, the present study has investigated two aspects of cordycepin effect, signal transduction and gene expression, and found that the growth factor response genes is repressed by cordycepin.

2 Materials and Methods

2.1 General Reagents and Bioactive Compounds

Cordycepin was purchased from Sigma (C3394) and Carbosynth (ND02930). Agarose (BP1356500) and Tris Base (BP152-1) were purchased from Fisher Scientific. 30% Acrylamide/Bis-acrylamide (A3699) and PI3K inhibitor LY294002 (L9908) were purchased from Sigma. Phosphate-buffered saline (PBS, BR0014G) was purchased from Thermo Fisher Scientific.

Pictilisib was kindly given by Tracey Bradshaw, originally synthesised by Michael Stocks from School of Pharmacy, University of Nottingham. MEK inhibitor PD98059 was purchased from Cell Signalling Technology (#9900). mTOR inhibitor Torin1 (#4247) and AMPK activator A769662 (#3336) was purchased from Tocris Bioscience. AKT inhibitor MK2206 was purchased from Source BioScience (ABE4221). PARP inhibitor NU1025 was purchased from Calbiochem (#493800).

2.2 Cell Culture

In this study, MCF-7 cells were kindly provided by Sebastiaan Winkler from the School of Pharmacy, University of Nottingham, who acquired the cells originally from the Netherlands Cancer Institute. These cells were verified as MCF-7 cells by short tandem repeat analysis to check the relative genotypes (DNA Diagnostics Centre). The cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM, Sigma), supplemented with 10% (v/v) fetal bovine serum, 1% (v/v) L-glutamine and 1% (v/v) penicillin/streptomycin. MDA-MB-468 cells were kindly provided by Tracey Bradshaw (School of Pharmacy, University of Nottingham) and cultured in RPMI-1640 (Sigma) with 10% (v/v) fetal bovine serum. MDA-MB-231 Fluc and Dlux were originally derived from MDA-MB-231 cells. Genetic modification of these cells enables bioluminescence imaging of the cells once transplanted *in vivo* (Priddle et al. 2009; Kumari et al. 2007). These cells were kindly provided by Anna Grabowska and Philip Clarke from the Cancer Biology Unit,

School of Medicine, University of Nottingham. These cells were cultured in RPMI-1640 with 10% (v/v) fetal bovine serum. All the cells mentioned here were incubated at 37°C in 5% CO2.

For harvesting, medium was removed from the cell container, the cells were washed with warm PBS (37°C) and incubated with trypsin (Sigma) for 5 mins at 37°C. PBS was added to wash the cells off the flasks. This cell mixture was then transferred to a Falcon tube to centrifuge for 5 mins at 1,100 g, following which the supernatant was removed from the tube and the cell pellet re-suspended in medium. If the cells were to be seeded for experiments, this suspension was subjected to cell counting with haemocytometer to obtain a precise seeding density. For passaging, a proportion (≥1:10) of this suspension was seeded into a new flask.

In case of freezing cells, cells were detached by trypsinization before mixing with freezing mix (20% (v/v) DMSO in serum). For a T75 flask, a dilution of 1:3 or 1:5 was used, whereas 1:6 was usually the ratio used for a T150. Aliquots of the cell mixture were transferred to -80°C immediately and kept overnight before transferring to liquid nitrogen for long-term storage. To defrost cells, cells were taken out from liquid nitrogen to defrost. Once defrosted, cells were mixed with pre-warmed medium at 1:10 to avoid toxicity of DMSO. The mix was then centrifuged for 5 mins at 1,500 g and cell pellets were re-suspended in fresh medium. Cells could then be seeded in flasks or plates for use in experiment.

2.3 Cell Counting

Cells were seeded in 6-well plates at a density of 0.3×10^6 cells/well (0.15×10^6 cells/well for MDA-MB-231 Dlux cells) in triplicate. After 24 hrs incubation, cells were treated with cordycepin and/or pentostatin as indicated. At 72 hrs, medium was collected separately to conserve the dead cells. Cells were washed twice with PBS. To collect the cells, 1 mL

of trypsin was added to each well. The plates were then swirled to mix the trypsin before the trypsin was removed. This step was finished within a few seconds to ensure the cells were not killed by the intense exposure to trypsin. The cells were then incubated at 37°C for 5 mins. Medium collected before was used to re-suspend the cells. The cell suspension was then centrifuged at 1,500 g for 5 minutes. The supernatant was then discarded and 200 µL medium collected before was used to resuspend the cells. 50 µL of 0.4% trypan blue (15250-06, Gibco) was mixed with 50 µL of the suspension to enable counting of live and dead cells using a dual-chamber haemocytometer.

2.4 Immunoblotting

3×10^6 MCF-7 cells were seeded in a 15 cm petri dish (TPP) 24 hrs before use. For serum deprivation, the original medium was replaced by serum-free DMEM medium 4 hrs before collection. For other groups, treatments were added 2 hrs before harvest. Medium in petri dishes was removed; cells were washed with PBS and collected by scraping (Greiner Bio-One, cat no 541070). Cell pellets were acquired by centrifugation at 1,500 for 5 mins. Cell lysates were prepared with RIPA buffer (PBS containing 0.5 % (w/v) Igepal, 0.5% (w/v) deoxycholate, 0.05% SDS, 1 mM β-glycerophosphate, and 1 mM Na3VO4, 1 mM Phenylmethane Sulfonyl Fluoride (PMSF) in PBS) and SDS loading buffer (65 mM Tris/HCl pH 6.8, 3% (w/v) sodium dodecyl sulfate, 10% (v/v) glycerol, 5% (v/v) β- mercaptoethanol and 0.004% (w/v) bromophenol blue in deionised water). Bradford assay (Zor & Selinger 1996; Bradford 1976) was performed to determine protein concentrations, using Pierce Coomassie (Bradford) Protein Assay Kit (ThermoFisher Scientific #23200). Lysates were boiled at 95°C for 5 mins after lysing and again before loading. Equal amounts of protein were resolved by SDS-PAGE gel electrophoresis, transferred onto Polyvinylidene fluoride (PVDF) membrane (Thermo) with a semi-dry blotting system (Scie-Plas). For a mini gel, this step was carried out at

constant-current 0.8 mA/cm^2 for 2 hrs, with the supplement of blotting buffer (0.3% (w/v) Tris-base, 1.44% (w/v) glycine, 20% (v/v) methanol and make up with deionised water) to prevent drying out. For large gels, this step was extended to 4 hrs. Antibodies against phosphorylated AKT (Ser473: #4060, Thr308: #13038), total AKT (#9272), phosphorylated ERK (#4370), total ERK (#4695), phosphorylated 4E-BP1 (#9459), total 4E-BP1 (#9452), phosphorylated MEK1/2 (#9154), total MEK (#9122), phosphorylated AMPKα (#2535) and total AMPKα (#5831) were purchased from Cell Signalling Technology. Vinculin antibody (#V9131) was purchased from Sigma. Anti-Rabbit Immunoglobulins/HRP (P0217) and Anti-Mouse Immunoglobulins (P0447) were purchased from DAKO. Depending on the antibody, primary antibodies were either diluted in TBST (10 mM (v/v) Tris-HCl pH 8, 150 mM sodium chloride, 0.05% (v/v) Tween 20 and in deionised water) with 5% bovine serum albumin (antibodies against phosphorylated proteins) or TBST with 5% non-fat milk (antibodies against total proteins). Membranes from above were blocked with 5% non-fat milk for 1 hr and incubated with primary antibodies overnight at 4°C. Alternatively, if incubated with antibodies that are not specific for phosphorylation sites, the primary incubation were performed at room temperature for 2 hrs. Membranes were washed three times with TBST for 5 mins before being incubated with the appropriate secondary antibody. The membranes were again washed three times, before developing with chemiluminescence reagent (RPN2232, GE Health Life Sciences).

Pictures were taken with ImageQuant LAS4000 (GE Healthcare Life Sciences) and analysed with Image J (v1.51j, https://imagej.nih.gov/ij/index.html). To quantify the result of the blots, the density of the phosphorylated and total proteins was first quantified. With background subtracted, this density of the bands was then compared to individual loading control. The resulting ratio was then normalised to the ratio of control lane.

If needed, stripping was performed by incubating the membrane with stripping buffer (100 mM beta-mercaptoethanol, 2% (w/v) sodium dodecyl sulfate, 62.5 mM Tris-HCl pH 6.7 and make up with deionised water) at 50°C for 30 mins. Afterwards, the membrane was blocked and probed as described above again.

2.5 RNA isolation and RT-qPCR

Upon the end of incubation, 6-well plates with cells were removed from incubator and placed on ice. Medium in the plates was removed. Ice-cold PBS was used to wash the cells. Total RNA was isolated using the Macherey-Nagel NucleoSpin RNA kit (cat NO. 740955) or the ReliaPrep RNA Miniprep System (Z6012, Promega), both with increased 1 hr DNase incubation. For Macherey-Nagel NucleoSpin RNA kit, cells were detached by trypsinisation before centrifugation and subsequent lysis using the included lysis buffer. For ReliaPrep RNA Miniprep System, cells were directly lysed on the plate with the included BL+TG buffer before being processed. RNA concentration was determined with NanoDrop 1000 (Thermo Scientific). In case of low concentrations, 260/280 or 260/230 ratios, which indicates low purity of the RNA samples, ethanol precipitation was conducted to purify the RNA (Wilfinger et al. 1997; Desjardins & Conklin 2010).

In case of precious RNA samples or high demand for RNA quality, RNA isolated from above was analysed with an agarose gel to check relative integrity. To make up the gel, agarose was dissolved in 1x TBE buffer (1.08% (w/v) Tris-base, 0.55% (w/v) boric acid, 2% (v/v) EDTA pH 8.0 and make up with deionised water) to maintain a concentration of 1% (w/v). This gel solution was then autoclaved to reduce RNase activity. SYBR safe (Invitrogen, S33102) was added to autoclaved gel solution at the ratio of 1:10,000 and mixed well. Warm gel solution was then added to the gel tray and bubbles were removed with a pipette tip. Up to 2 µL of RNA sample was mixed with 10 µL loading buffer (0.25% (w/v) bromophenol blue dye and xylene cyanol dye, 10% (v/v) 1M Tris-HCl pH 7.5-8.0, 90% (v/v) deionised formamide), followed by heating at 70°C for 3 mins.

Heated RNA samples were loaded on the gel and run at 4V/cm^2 till around 3 cm from the end. Pictures were taken using ImageQuant LAS4000 (GE Healthcare Life Sciences) or BIO-RAD Gel Doc Universal Hood II.

After RNA isolation, 0.1 volume of 3 M sodium acetate pH 5.2 was added to the RNA sample, as well as 2 volumes of 100 % ethanol (Chirgwin et al. 1979; Cox 1967; Volkin & Carter 1951; Sela et al. 1957). 10 - 20 µg glycogen blue was added to highlight the pellet. The mixture was mixed well before incubating at -20°C for at least 30 mins, followed by centrifugation at 4°C for 30 mins at 19,000 g. Supernatant from above was removed, with the pellet washed three times with 200 µL 70 % ethanol. Air dried pellet was then dissolved in RNase-free water for concentration determination.

With SuperScript III/IV Reverse Transcriptase (Invitrogen, cat NO. 18080-044 and 18090050), cDNA was synthesised using the protocol recommended by the manufacturer. Typically, 500 ng RNA and 120 ng random hexamers were used in one reaction. For SNAR-A1 detection, instead of random primers, which were used in non-SNAR detection, a specific set of primers that are complementary to the sequence of SNAR-A1 was used to increase the efficiency of cDNA synthesis (Table 2.5).

All cDNA obtained was diluted 1:10 with RNase-free water. SNAR-A1 cDNA can only be used for SNAR-A1 RT-qPCR. RT-qPCR was performed with GoTaq qPCR Master Mix (Promega, A6002). Stratagene Mx3005P and Qiagen Rotor-Gene Q were used to run the RT-qPCR. Thermal profile setup for these two machines is indicated in Figure 2.5. Unless otherwise indicated, all RT-qPCR data was analysed using the $2^{-\Delta\Delta CT}$ method (Livak & Schmittgen 2001; Schmittgen & Livak 2008) and corrected with the mRNA levels of GAPDH. Primers used in RT-qPCR were purchased from Sigma sequences are listed in Table 2.5.

To design primers, different strategies were used for different types of primer. For primers designed for mature mRNAs, NCBI Primer-Blast was used to choose primers based on the mRNA sequence from NCBI. To specifically detect mature mRNAs, one of the primers must span an exon-exon junction. For primers designed for pre-mRNAs, relative sequences were based on UCSC Genome Browser. Primer3 (give hyperlink) was used to select suitable primers from sequences at exon-intron junctions. Pre-mRNA primers were chosen according to the principle that the forward primer is located within the exon and the reverse primer is located within the intron, or vice versa. Candidate primers were then examined again by NCBI Primer-Blast, to make sure no off-target products are predicted. For primers designed for uncleaved mRNAs, the 3' end sequence of the mRNA was first found in NCBI. The 3' end segment of the sequence was chosen, together with sequence about 200 nt outside the cleavage site, for Primer3 blast. Similar to the design for pre-mRNA primers, primers for uncleaved mRNAs were selected according to the principle that the forward primer is located within the cleavage site and the reverse primer is located outside the cleavage site. For all primers designing, product size was restricted to between 150 nt and 250 nt, with melting temperatures higher than 58°C but lower than 60°C. GC% was optimised to be more than 40% and less than 60%. Scores for self-complementarity and self-3'-complementarity should be as low as possible.

Primer specificity was determined by analysing the melt curve and running PCR products on an agarose gel. Presence of multiple peaks on the melt curve or multiple bands when running the PCR products on an agarose gel would indicate that the primers are non-specific and unsuitable for use, and would need to be redesigned. For analysis, all threshold cycle (Ct) values were analysed using the $2^{-\Delta\Delta Ct}$ method (Schmittgen & Livak 2008). Unless specified, all analysis was done by normalising to the Ct value of GAPDH.

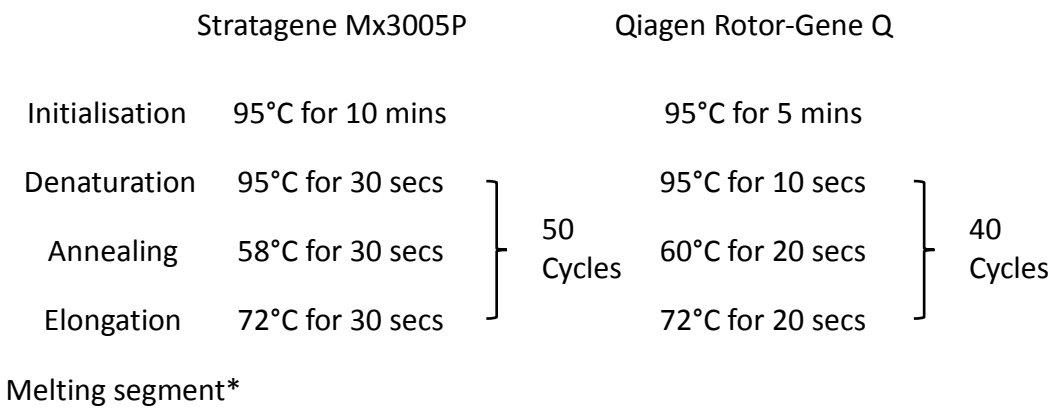


Figure 2.5 RT-qPCR Thermal Profile Setup

*To collect melting curves, after each run, RT-qPCR products were heated to 50°C, with the temperature gradually increased to 99°C.

Gene	Forward Primer 5'-3'	Reverse Primer 5'-3'
MYC	TCCTCGGATTCTCTGCTCTC	CTCTGACCTTTTGCCAGGAG
PLK2	GGTGCATAAGGGAAGCAAG	AGTCTGTCCGGAGTGAAGC
SGK1	ATGAAGCAGAGGAGGATGGG	GGCCAAGGTTGATTTGCTGA
DUSP1	GAGCTGTGCAGCAAACAGT	CAGGTACAGAAAGGGCAGGA
JUNB	AAGGGACACGCCTTCTGAA	AAACGTCGAGGTGGAAGGA
BCAR3	GGCACCAGTACACCCAAACT	CACATGTCGGTTCCTTCAAA
AMOTL2	CCATGAGAGCCTGACCAGAG	GCCTCCTGTGTCTTGCTTG
CTGF	CAGAGCAGCTGCAAGTACCA	GCCAAACGTGTCTTCCAGTC
WEE1	GGGAATTGATTCCAGCTCT	CACTGGCTTCCATGTCTTCA
MCL1	AGAAAGCTGCATCGAACCA	CCAGCTCCTACTCCAGCAA
RPL10A	TCTCTCGCGACACCCTGT	TTAGCCTCGTCACAGTGCTG
IL6	AGCTCTGGCTTGTTCCTA	AAAGAGGCACTGGCAGAAAA
EGR1	TTCACGTCTTGGTGCCTTTT	AGCGGCCAGTATAGGTGAT
ABL2	GCTGCAGTAATGAAGGAAATCA	CTGAGTGGCCATGTAGAGCA
DUSP2	CCACTGCCGTGTACTTCCTG	GGTAGGGCAAGATCTCCACA
TP53	GTGGAAGGAAATTTGCGTGT	CCAGTGTGATGATGGTGAG
NFKBIA (1st set)	CTACACCTTGCCTGTGAGCA	TCCTGAGCATTGACATCAG
NFKBIA (2nd set)	GCTCCGAGACTTTCGAGGAA	CAGGGCTCCTGAGCATTGA

JUN	ACAGAGCATGACCCTGAAAC	CCGTTGCTGGACTGGATTAT
NEK6	ATCCCAACACGCTGTCTTTT	GCCGATCTCCTTGACACAGT
CSTF1	GATCCTGGCTTCTGGTTCAA	AGGCGAAGAGTAGGATGCTG
BTG1	CCATGCATCCCTTCTACACC	GCTTTTCTGGGAACCAGTGA
PTPRK	AGGGATGCTACGATATGGC	CTCGATGAGAAGCCCATCCT
CTSL1	ACAGTGGACCAAGTGGAAGG	TGGGCTTACGGTTTTGAAA
CATSPER2	ATCCTTTGGTTGTTGCTTGG	TGACGTCAGTGCTTCATGTG
AREG	TTGATACTCGGCTCAGGCCA	GTAGTCATAGTCGGCTCCCG
IER3	GGCTTCTCTTTCTGCTGCTC	ACACCCTCTTCAGCCATCAG
SPRY2	GCGCTTGTAGAAGGGGAGTC	ACACATCTGAACTCCGTGATCG
SNAR-A1	ATTGTGGCTCAGGCCGGTT	TTTTTCCGACCCATGTGGACC
SNAR-A1 reverse transcription	AAAAAAAAGTCCT	TTTTTCCGACCCATGTGGACC
RPL7	GAATGGCGAGGATGGCAAGA	GGGCTCACTCCATTGATACCTCT
EEF1A2	GTCAAGGAAGTCAGCGCCTA	CTTGAACCACGGCATGTTGG
RPL23A	CCTGATTCGGCCTGATGGAG	CTCCCAGCCCAACCAGAAAT
RPS18	CACTGAGGATGAGGTGGAACG	GAAGTGACGCAGCCCTCTAT
RPS15A	CGCGCCGCCACAATG	CGGACGATGACTTTGGAGCA
GAPDH	CATCGCTCAGACACCATGGG	CGTTCTCAGCCTTGACGGTG
pre-CTGF	CAAGCTGCCCGGGAAATG	GTTCGGTCGGCACAGTTAG
pre-DUSP1	TCCCCTGAGTACTAGCGTCC	CCTGTGTCCCCTCCGTTATA
pre-AMOTL2	ACTCCACCTTGAATCCCCAG	CATCTCTGCTCCCGTGTTTG
pre-WEE1	ATCGGCTCTGGAGAATTTG	CCATAGGGGACAAAAGAGC
pre-BCAR3	AGCTACCGGATGAATGCTGA	CACAAGGAGAAACAGGGCAC
pre-IL6	GTCCCAAAACATGCTGCCTA	TGGCTTGTTCCTCACTACTCT
pre-MYC	CGGATTCTCTGCTCTCCTCG	AGTGGCCCGTTAAATAAGCTG
pre-PLK2	TCTTGCTTTTGAATGGGCGA	GTGTTCCAAGGGTTCTAGCC
HNRNPA3P2	TGCTGGATGGTAAGTAGTCGTG	ATCTCCACCACCACAGTCTTC
U73166.2	TATAGCCCTTGCTGCTTCCC	AAGACTTTGCTTGCGCTGTG
CTD-2619J13.13	CCTGCCTATCCACTGCTTTCA	GATAGGAGGGCACAACTCCA

RP11-873E20.1	TGTCAGATGCCTCACTCCAA	ACACAATTCGACTCACTGCG
SPACA6P-AS	TGTTGAAAGCCCAGCACCAT	CTACAACTCCCTGAGTCTGGC
RP11-188D8.1	AGACCCCTTTTCCTTTGCTTG	TCATTGCTGGTGTAGGAGTGT
uncleaved PLK2	CACTGCTTGGCACGATCTTATC	ATTACAGCCCAAAGAGCAGTAT
uncleaved SGK1	AAACTAACCCATGATTACCCCCT	GTACAATCCTTCCCTCATCCCAT
uncleaved SNAI1	GGCCTGGGAGGAAGATGTTTAC	ACCCACTCCTCTATGACACCA
uncleaved WEE1	TGTCTAAATCCTTGTGATGCCTGT	TTCAATCAAAATGTGCCCTCTGC
uncleaved AMOTL2	CACATGCTGACTGGGAACCT	ATGTTCTTAGCCACCGCCTC
yeast Act1	CCGGACTCGAGAAGAAACAT	AACCACCTTTTTCCGCTCTT

Table 2.5 Primer List

Unless specified, these primers were designed for *Homo sapiens*.

2.6 EGF Stimulation

MDA-MB-468 cells were seeded in a 6-well plate at the density of 0.2×10^6 cells/well. 24 hrs after seeding, medium in all wells was replaced by fresh serum-free RPMI-1640 medium and then replaced again immediately before treatments were made. Recombinant Human Protein EGF (Gibco, PHG0314) was dissolved in DMSO to obtain a stock concentration at 10 ng/mL. The cells from above were then treated with different concentrations of EGF at different time points as indicated in Figure 4.2. At the end of the incubation, cells were collected. RNA isolation and RT-qPCR was conducted as described in 2.5.

2.7 Polysome Profiling and Preparation for Microarray

3×10^6 MCF-7 cells were seeded in each 15 cm petri dish 24 hrs before collection. Three dishes were assigned to each condition. Cells in the cordycepin group were treated with 50 µM cordycepin 30 mins before harvest, while the same volume of DMSO was added to the control group. By the end of the incubation, cells were washed with ice-cold PBS, harvested by scrapping and pelleted by centrifugation (4 mins at 1600 g, 4°C). Subsequent pellets were suspended with 500 µL lysis buffer (0.3 M NaCl, 15mM $MgCl_2$, 15 mM Tris/HCl pH7.5, 0.1 mg/mL cycloheximide, 1 mg/mL Heparin and 1% (v/v) Triton

X in RNase-free water), followed by centrifuging at 13, 000 g for 1 min at 4°C. Supernatants from above were loaded onto 10–50% (w/v) sucrose gradients and centrifuged at 38, 000 rpm for 2 hrs at 4°C in a Sorvall Discovery 100SE ultracentrifuge.

Prior to the assembly of the gradients, sucrose solutions were prepared at the concentration of 10%, 18%, 26%, 34%, 42%, 50% and 60% (w/v). In a Sorvall PA 12ml tube, 0.5 mL of the 60% sucrose solution was added to the bottom before freezing in the freezer. Repeat this load-freeze cycle for other sucrose solutions at the volume of 1.6 mL, in the order of high to low concentration. After the final layer, the gradients can be stored in the freezer and thawed before use.

The gradients were then fractionated by upward displacement into a Type 11 Optical Unit (Teledyne ISCO) with 65% (w/v) sucrose at the speed of 1 mL/min using a dispenser. To collect fractions, run-throughs were mixed with 3 mL 7.7 M guanidine HCl and 4 mL 100% ethanol. Collector was set to change to another tube every 1 min. Different fractions were then stored at -20°C for further use. Pooled subpolysomal (1-5) and polysomal (6-10) fractions were mixed with 500 µL 5 M LiCl before storing at -20°C overnight. The next day, these fractions were centrifuged in a pre-cooled bench top centrifuge at full speed. The supernatants were removed and RNA precipitation was then performed using 100% ethanol and 3 M NaAc pH5.2. Pellets from above were suspended with 50 µL RNase-free water and RNA concentration was determined by NanoDrop (Spriggs et al. 2008; Moreno et al. 2012).

For validation, LiCl purification and RNA precipitation was performed for each individual fraction to eliminate heparin, followed by reverse transcription and RT-qPCR analysis as described in 2.5. However, while usual RT-qPCR uses endogenous house-keeping genes as reference, the level of these house-keeping genes will vary across different fractions, making it unsuitable for the use of reference gene. Hence, for validation of the

polysome data, we spiked in 2.5 µL of RNA from each fraction with 2 µL yeast *Schizosaccharomyces pombe* RNA. This yeast RNA was extracted from a *Schizosaccharomyces pombe* cell pellet using the acid guanidinium thiocyanate-phenol-chloroform method (Chomczynski & Sacchi 2006; Chomczynski & Sacchi 1987). The yeast pellet was kindly provided by Caia Duncan and Juan Mata from Department of Biochemistry, University of Cambridge. This mixed RNA was then subjected to cDNA synthesis for further RT-qPCR determination as mentioned in 2.5. Yeast Act1 was used as the house-keeping gene in this polysome profiling RT-qPCR validation.

2.8 Microarray Assay

MCF-7 cells were seeded at the density of 2.3×10^6 per 15 cm petri dish 24 hrs before collection. 2 hrs before collection, the cells were treated with DMSO and 50 µM cordycepin, or medium without serum 4 hrs before collection. The cells were then lysed, with relative RNA isolated according to the method mentioned in 2.5. The RNA was then reverse transcribed into cDNA and probed against a Human Gene Expression 8×60K v2 microarray (Agilent Technologies, G4851B). This microarray was done in one colour with three biological replicates for each condition. A DNA microarray scanner (G2505C, Agilent Technologies) was used to obtain the raw data.

For the polysome profiling microarray, RNA from control and cordycepin treated groups was isolated from the pooled subpolysomal and polysomal fractions as described in 2.6. The microarray was done in a dye-swap method, which labelled, i.e., subpolysomal and polysomal samples respectively in Cy5 and Cy3 in the first group and swapped the dyes in the second group. Two biological replicates were used in this microarray, which ended up with 8 samples in total. The cDNA synthesis with these RNA samples was also probed against a Human Gene Expression 8×60K v2 microarray (Agilent Technologies, G4851B), with the raw data obtained from a DNA microarray scanner (G2505C, Agilent Technologies).

While samples for the first two sets of microarray were prepared by Asma Khurshid and Richa Singhania, and initially analysed by Irengbam Rocky Mangangcha, the majority of the raw data analysis for both arrays was finished by Graeme Thorn and Cornelia H de Moor. All three microarray experiments mentioned above were performed by Kate Dudek and Carolyn Jones, in collaboration with Anne E. Willis at the Medical Research Council Toxicology Unit, University of Leicester.

2.9 RNA sequencing

MCF-7 cells were seeded 24 hrs before collection at the density of 0.3×10^6 cells/well in each well of a 6-well plate, three of which were assigned to control and the others to cordycepin. 2 hrs before collection, cells in the cordycepin and control groups were treated with 50 μM cordycepin and the same volume of DMSO respectively. After harvest, cells were lysed with relative RNA isolated as described in 2.5. The subsequent RNA was then sent to the Deep Seq department, School of Life Sciences, University of Nottingham, where the actual sequencing and analysis was conducted.

A next-generation whole transcriptome shotgun sequencing (NextSeq) was conducted in duplicate with the RNA isolated from DMSO control and cordycepin treated cells, from which around 120 million 75 bp paired-end reads were obtained, with 115-126 million (86%-90%) reads being mapped correctly to human genome Ensembl 86, hg38. In total, 197898 transcripts were annotated reference transcripts.

Raw reads were trimmed against adaptors using Scythe (https://github.com/vsbuffalo/scythe), followed by quality trimming using Sickle (https://github.com/najoshi/sickle). Tophat maping tool was used to map the reads onto the human genome GRCh38. Ensembl v86 (http://www.ensembl.org) was used as the annotation source. Read counts per gene was calculated using htseq-count (http://www-huber.embl.de/users/anders/HTSeq/doc/count.html). Principal

component analysis (PCA) was introduced to compare how close the two replicates were to each other. Differentially expressed genes were determined by DEseq (Anders et al. 2010), with thresholds set at Fragments Per Kilobase of transcript per Million mapped reads (FPKM) < 1, or alternatively, P value < 0.5 and foldchange > 2. For the discovery of new transcripts, the transcriptome in MDA-MB-231 Fluc cells was created by means of *de novo* transcriptome assembly via Cufflinks (Roberts et al. 2011). Transcripts with FPKM < 1 were filtered out to reduce background noise. Cuffcompare compared the transcript assembly and genome annotation to identified novel and other types of splicing.

The actual sequencing and mapping was performed and analysed by Sunir Malla, Sang Fei and Victoria Wright from the Deep Seq department, School of Life Sciences, University of Nottingham (Amer et al. 2014; Daly et al. 2017).

2.10 siRNA Knockdown of Poly(A) Polymerases by Asma Khurshid

7.5×10^4 MCF-7 cells were seeded in each well of a 12-well plate. siRNA (Dharmacon) of different polymerases was prepared by incubating in INTERFERin (Polyplus) for 10 mins. A double knockdown method was used in this experiment with the two siRNA transfections being done at 24 and 48 hrs after seeding. Serum-containing medium was used to replace medium before the first transfection. Serum-free medium was used to replace the medium before the second transfection. siRNA was added in both transfections to obtain a concentration of 5 nM in medium. 24 hrs after the second transfection, the cells were lysed with RNA isolated as mentioned in 2.5.

2.11 Statistics

All primary analysis was finished using Excel 2013 (Microsoft Office). Graphs were designed using GraphPad Prism 6 and 7. Statistical analysis was conducted using SPSS 22 (IBM) (Table 2.11). For RT-qPCR, ΔCt values were used for statistical analysis. All statistical analyses in this thesis were performed in a 2-tail manner. Microarray data

was processed using R studio (V2.13.2). Gene ontology analysis was performed using the Database for Annotation, Visualization and Integrated Discovery (DAVID) v6.8 (https://david.ncifcrf.gov/) (Huang, Sherman, et al. 2009; Huang, Lempicki, et al. 2009).

Test	Reference Graphs
Independent sample student's T Test	Figure 3.4B, 3.4C, 4.1A, 5.1, 5.3 and 7.1B
Dunnett's T Test	Figure 3.1A, 3.3, 4.2B, 5.2, 7.2B, 7.2C, 7.2D, 7.3B, 7.3C and 7.3D

Table 2.11 Statistical Analysis Used

Listed are the tests used for statistical analysis for every set of data in this project, with reference for specific graphs.

3 Previously Reported Effects of Cordycepin in Breast Cancer Cells

In first instance, previously reported effects of cordycepin were investigated, including its effects on cell survival, signal transduction, gene expression and pre-mRNA cleavage. In a previous project in the De Moor lab, a microarray was performed with cordycepin treated MCF-7 samples to determine the genome-wide regulation on gene expression, followed by gene ontology analysis (Asma Khurshid, Irengbam Rocky Mangangcha, Graeme Thorn and Cornelia De Moor, data unpublished). 97 of the 289 most down-regulated genes were categorised as transcription-related. Among the most significantly down-regulated genes from the microarray, genes like EGR1, FOSL1, JUN, JUNB and MYC are known as immediate early genes (IEG) that are located downstream and consequently regulated by the ERK1/2 signalling (Murphy & Blenis 2006; Murphy et al. 2004). This led to the hypothesis that the signal transduction through ERK1/2 is inhibited by cordycepin. To investigate inhibition of signal transduction, MCF-7 cells were incubated in serum free medium for 4 hours and analysed with RNA microarray. Comparison between serum-withdrawal and cordycepin treated MCF-7 cell microarrays unveiled an extensive overlap of the changes in gene expression (Richa Singhania, Graeme Thorn and Cornelia De Moor, data unpublished). All these data indicate the possible involvement of cordycepin in regulating signal transduction and growth factor dependent gene expression.

3.1 Cordycepin Affects Cell Viability of Breast Cancer Cell Lines

To assess the effects of cordycepin on cell viability of breast cancer cell lines, cell counting was performed for MCF-7, MDA-MB-468, MDA-MB-231 Dlux and Fluc cells. MCF-7 is a luminal A breast cancer cell line, whilst MDA-MB-468, MDA-MB-231 Dlux and Fluc cells are basal/triple negative breast cancer cells (see 1.1 and Table 1.1). The

luciferase bearing version of MDA-MB-231 were selected because these will be used in xenograft experiments at a later stage.

MCF-7, MDA-MB-468 and MDA-MB-231 Fluc cells were treated with 10 and 50 μM cordycepin for 48 hrs, followed by cell counting. In cells, adenosine deaminase is responsible for the degradation of adenosine and deoxyadenosine (Cristalli et al. 2001; Clarke et al. 1952). As this reaction is thought to be irreversible, the deamination of cordycepin causes a decrease in the intracellular level of cordycepin. Hence we introduced pentostatin (2'-deoxycoformycin), a potent inhibitor of adenosine deaminase (Agarwal et al. 1977; Brogden & Sorkin 1993; Johns & Adamson 1976), to explore the role of degradation in cordycepin activity. With MCF-7 cells, treatment with 10 μM cordycepin produced a significant reduction in live cell number and this effect enhanced by the application of a higher concentration (50 μM). Addition of cordycepin induced a decrease of cell survival in MDA-MB-468 cells as well, but a moderate effect with 10 μM cordycepin indicates more resistance to cordycepin than for MCF-7 cells. In MDA-MB-231 Fluc cells, the cell viability dropped to a significant level with pentostatin solely. Consequently, it is not certain whether, in MDA-MB-231 Fluc cells, the defect on survival with 10 and 50 μM cordycepin is caused by pentostatin or cordycepin.

Similarly, in the two luciferase-bearing breast cancer cell lines, cell viability was reduced by cordycepin in a dose-dependent fashion (Figure 3.1B). The LD_{50} of cordycepin for MDA-MB-231 Dlux is between 50 μM and 75 μM, and 50 μM for the Fluc cells, which will be used in planned animal experiments in the future.

In all three cell lines tested, inhibition of adenosine deaminase by pentostatin augmented the effect of cordycepin, which suggests that deamination plays an important role in the activity of cordycepin. As MDA-MB-468 cells are still more

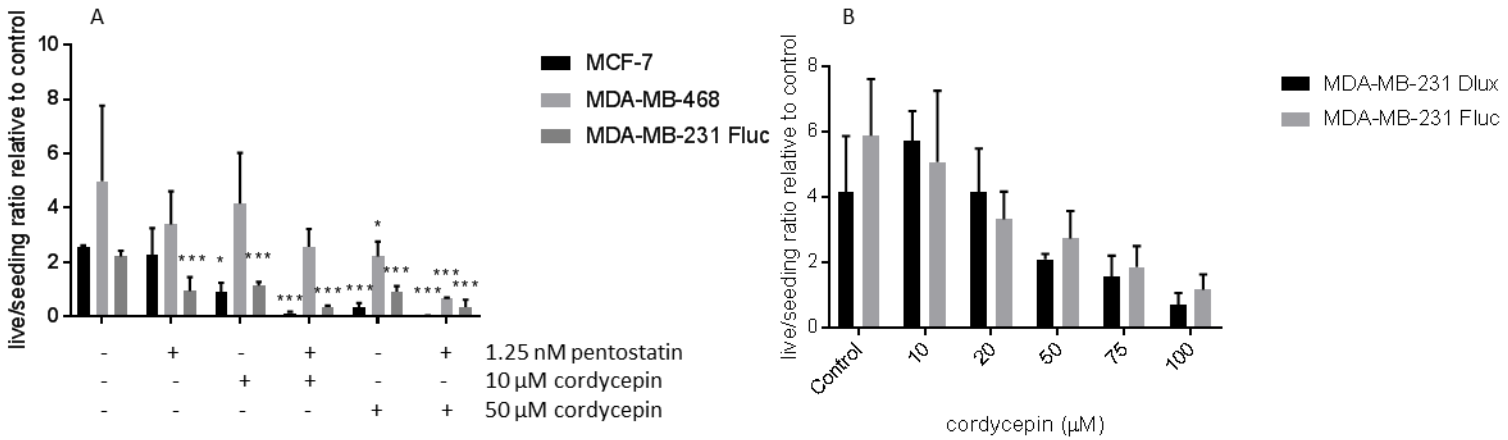


Figure 3.1 Cordycepin Affects Cell Viability of Breast Cancer Cell Lines

A. 48 hrs before harvest, cells were treated with 10 µM cordycepin, 50 µM cordycepin and 1.25 nM pentostatin as indicated.
B. 48 hrs before harvest, MDA-MB-231 Dlux (n=3) and Fluc (n=2) cells were treated with cordycepin at indicated concentrations.
Cells from above were harvested and processed as indicated in 2.4. Live cell number was counted using haemocytometer. Cell viability is presented as the ratio of live amount over seeding amount. (mean ± SD, where applicable *P<0.05, **P<0.01, ***P<0.001 compared with untreated control; p values were obtained using Dunnett's test).

resistant to cordycepin than MCF-7 cells in the presence of pentostatin, it appears that this increased resistance is not due to a difference in adenosine deaminase activity.

3.2 Cordycepin Affects Signal Transduction

Wong et al. have reported that, in mouse fibroblast NIH3T3 cells, cordycepin inhibits protein synthesis via the inhibition of the mTOR dependent phosphorylation of eukaryotic translation initiation factor 4E-binding protein 1 (4E-BP1) and activation of AMPKα (Wong et al. 2010). To explore this phenomenon in MCF-7 cells, both phosphorylated and total levels of MEK, 4E-BP1, AKT and AMPKα in cordycepin treated MCF-7 samples were determined with western blot. AMPKα phosphorylation at Thr172 was sharply increased as early as 15 mins after adding cordycepin, while AKT and 4E-BP1 were being dephosphorylated throughout the time course (Figure 3.2). Although no obvious reduction was seen with the ratio of phosphorylation and total 4E-BP1 until 60 mins, the level of phosphorylated 4E-BP1 has started changing at 15 mins. Cordycepin caused a dual decrease in 4E-BP1 phosphorylation and total level, resulting in relatively little changes in the P-4E-BP1/4E-BP1 ratio at early time points. Yet, since the more highly phosphorylated the slower 4E-BP1 migrates (Lin et al. 1994; Brunn et al. 2012), the reduction in upper band and intensified lower band in total 4E-BP1 suggests the phosphorylation of 4E-BP1 is reduced after cordycepin treatment, which coincides with the observation from the P-4E-BP1 result. This effect can be seen across all three replicates of the experiment.

In the famous RAF-MEK-ERK axis, Mitogen-activated protein kinase kinase (MAPKK, also termed MEK) activates ERK1/2 by dual-phosphorylating Thr202/Tyr204 and Thr185/Tyr187 in the Thr-Glu-Tyr (TEY) motif (Roskoski 2012; Butch & Guan 1996; Rossomando et al. 1992). Considering genes regulated by ERK1/2 are being down-

regulated by cordycepin, it is striking to see that MEK phosphorylation is being induced, which suggests that cordycepin either affects ERK activation despite increased upstream signal or that there is an ERK1/2 signalling-independent cause for the changes in gene expression. However, with all these western blot results, it is clear that cordycepin affects signal transduction in MCF-7 cells, especially mTOR and AMPK signalling.

3.3 Cordycepin Regulates Gene Expression

Genes down-regulated by cordycepin participate in important cellular activities, e.g. transcription, apoptosis, cell proliferation and cell cycle (Asma Khurshid, Irengbam Rocky Mangangcha, Graeme Thorne and Cornelia de Moor, unpublished results). Of these down-regulated genes, we focused on the expression of the cancer related genes MYC, PLK2, SGK1, DUSP1, JUNB, BCAR3, AMOTL2, CTGF, WEE1 and MCL1 overtime after the administration of 50 μM cordycepin.

RPL10A and POLR2A were used as control mRNAs, of which the level showed little sensitivity to cordycepin (Figure 3.3). All the mRNAs tested showed the lowest level of mRNA at the end of the time course, which coincides with the microarray data that the expression of these genes are indeed repressed by cordycepin. For MYC, JUNB, PLK2, AMOTL2 and CTGF, the down-regulating effect starts as early as 30 mins after the addition of cordycepin, whereas other genes take slightly longer time (60 mins) to show decrease (Figure 3.3).

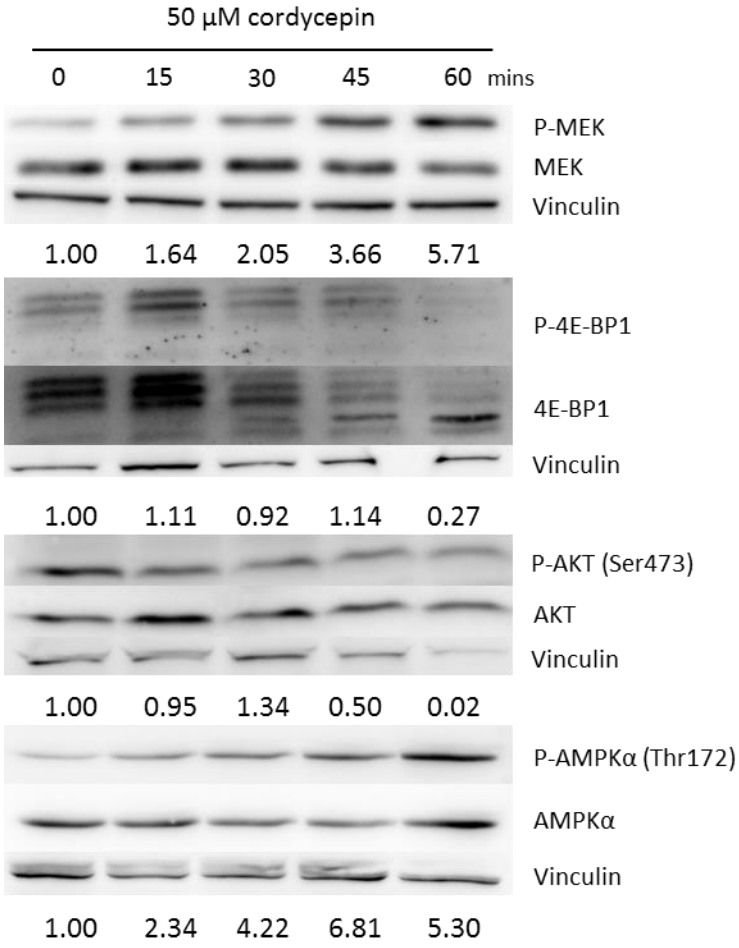


Figure 3.2 Cordycepin Affects Signal Transduction

MCF-7 cells were treated with 50 µM cordycepin at indicated times before harvest. Cells collected were lysed and analysed with western blot to measure the level of phosphorylated (P-) and total MEK, 4E-BP1, AKT (Ser473) and AMPK. Vinculin was used as loading control. Quantification of the blots was performed as described in 2.4. The numbers below each panel represent the ratio of phosphorylated over total protein level, normalised to control. The picture shown represents 1 of the 3 biological replicates generated.

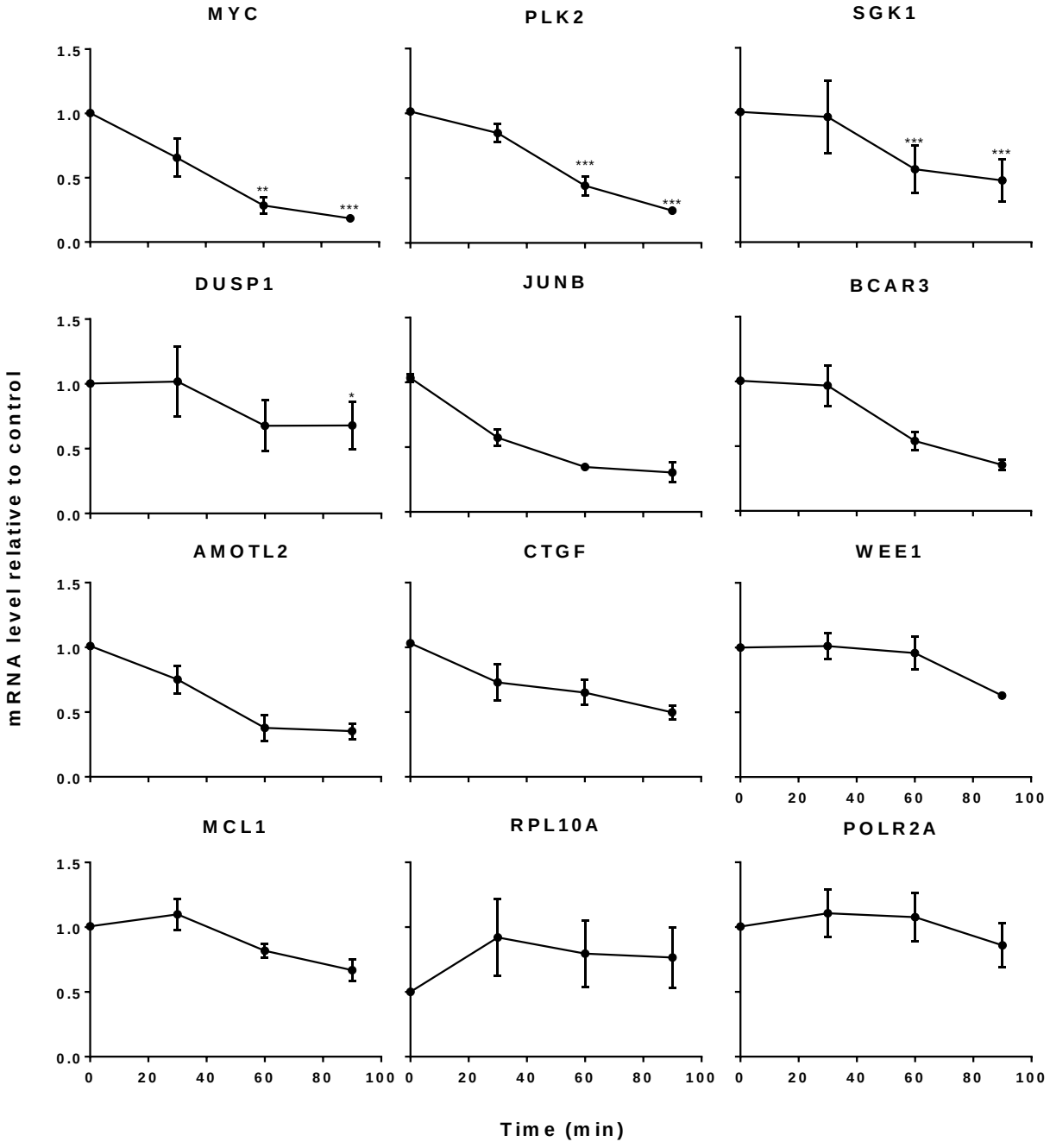


Figure 3.3 Cordycepin Regulates Gene Expression

MCF-7 cells were treated with 50 µM cordycepin 30, 45 and 90 mins before harvest, lysed. Relative RNA was extracted and reverse transcribed into cDNA. mRNA levels for selected genes were determined by RT-qPCR and presented as the ratio to the levels in 0 min timepoint. All Ct values were analysed using $2^{-\Delta\Delta Ct}$ and normalised to the Ct values of GAPDH. 3 replicates in this experiment was prepared by Irengbam Rocky Mangangcha. (mean ± SEM, n = 6 independent experiments; *P<0.05, **P<0.01 and ***P<0.005 compared with untreated control; p values were obtained using Dunnett's tests).

3.4 Cordycepin Causes a Cleavage Defect for Some pre-mRNAs

Kondrashov et al. demonstrated the defect of cordycepin on pre-mRNA cleavage in human airway smooth muscle cells, in which the cleavage complex was arrested and RNA polymerase II (Pol II) was accumulating towards 3' end of precursor mRNA (Kondrashov et al. 2012). To see if this effect also happens in cordycepin treated MCF-7 cells, primers were designed to pick up the readthrough across the end of the mRNA sequence (Figure 3.4A), followed by RT-qPCR to determine the uncleaved mRNA level. The five genes tested, PLK2, SGK1, SNAI1, WEE1 and AMOTL2, are all down-regulated by cordycepin in mature mRNA level. As shown, cordycepin did not alter the level of SGK1 or SNAI1 readthrough (Figure 3.4B). In agreement with the Kondrashov et al. result, readthrough levels for WEE1 and AMOTL2 were increased following the treatment with cordycepin, suggesting a cleavage defect caused by cordycepin. In contrast, uncleaved PLK2 level was reduced by cordycepin, suggesting effects at the transcriptional level as previously observed for IL8 (Kondrashov et al. 2012).

As part of transcription termination, the changing mRNA readthrough level could be the result of altered precursor mRNA (pre-mRNA) level (Richard & Manley 2009; West et al. 2008). In addition, besides the defect on cleavage, Kondrashov et al. also stated that pre-mRNA level was not sensitive to cordycepin treatment in human airway smooth muscle cells, indicating a post-transcriptional regulation by cordycepin (Kondrashov et al. 2012). This would agree with the long-known role of cordycepin as a polyadenylation inhibitor (Wong et al. 2010; Kondrashov et al. 2012; Müller, Seibert, Beyer, Muller, et al. 1977). In MCF-7 cells, the pre-mRNA level of PLK2, SGK1, SNAI1, WEE1 and AMOTL2 has been determined after adding cordycepin. Consistently, in a set of three biological replicates for the test, the pre-mRNA level of these genes showed little sensitivity to cordycepin as compared to the corresponding mature level (Figure 3.4C left). This effect

was later lost in another set of three biological replicates (Figure 3.4C right), making it hard to draw definitive conclusions.

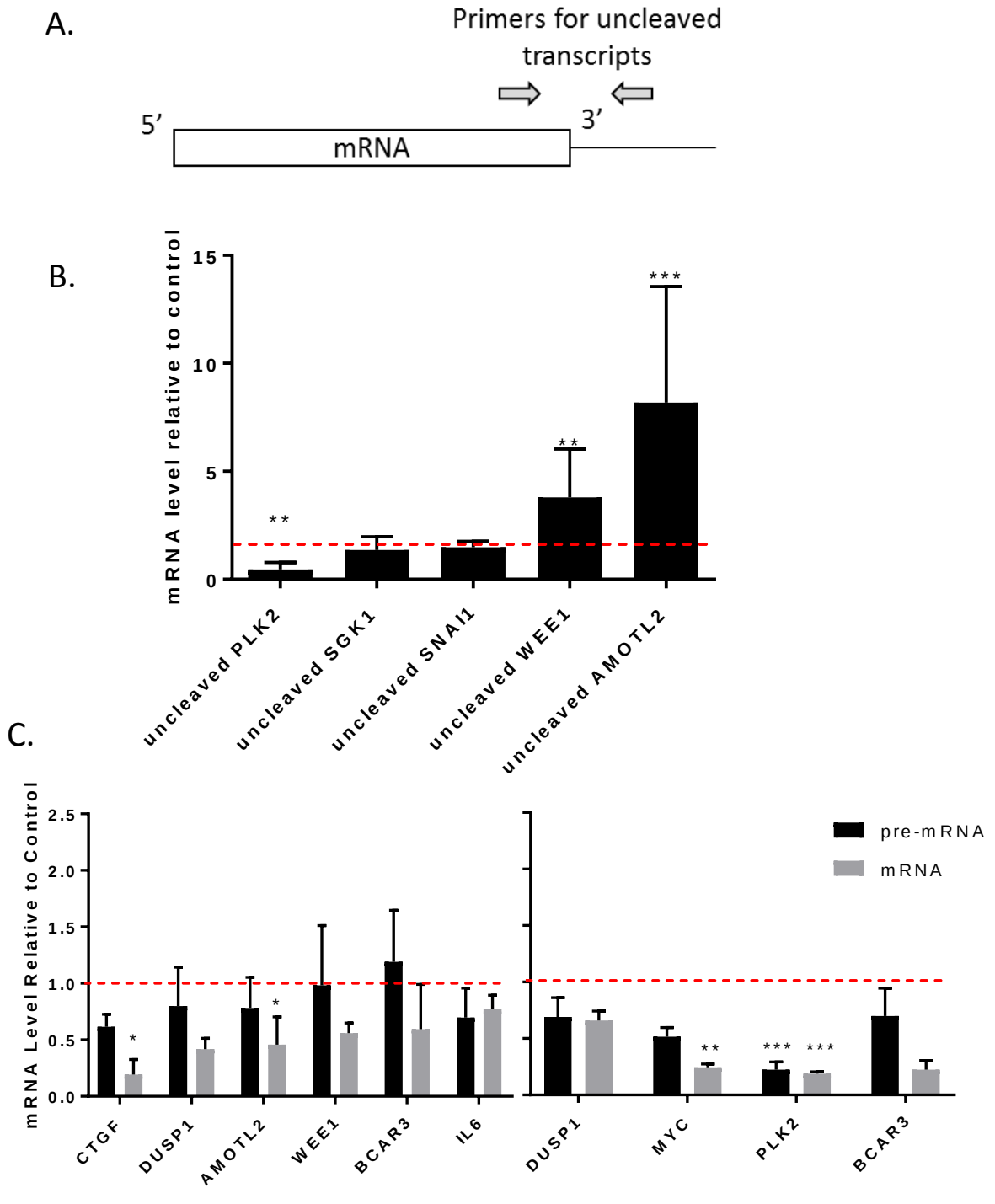


Figure 3.4 Cordycepin regulates cleavage efficiency in MCF-7 cells

A. A schematic view for the design of uncleaved primers.

B. MCF-7 cells were treated with 50 μM cordycepin 2 hrs before harvest, lysed. Relative RNA was extracted and reverse transcribed into cDNA.

C. MCF-7 cells were treated and processed as described in *B*. Left and Right were both independent triplicates. Left was finished prior to Right. mRNA levels for selected genes were

determined by RT-qPCR and presented as the ratio to the levels in untreated control (red dashed line). All Ct values were analysed using $2^{-\Delta\Delta Ct}$ and normalised to the Ct values of GAPDH. (mean ± SD, n = 3 independent experiments; *P<0.05, **P<0.01 and ***P<0.005 compared with untreated control; p values were obtained using student t tests.)

Discussion

In this chapter, total 4E-BP1 level was reduced by cordycepin, in addition to its effect on 4E-BP1 phosphorylation. While the mechanism behind this reduction remains unknown, there is evidence that 4E-BP1 degradation can be regulated by protein phosphatase 2 phosphatase activator (PTPA, also known as PP2A) (A. Gingras et al. 2001). In human lung fibroblast cells, increased expression of SRC (SRC proto-oncogene, non-receptor tyrosine kinase) non-receptor tyrosine kinase inactivated PP2A by phosphorylating Tyr307, which in turn caused 4E-BP1 degradation (Nho & Peterson 2011). Furthermore, an inhibitory role of PP2A in AMPK activity was also suggested (Gimeno-Alcañiz & Sanz 2003). Therefore, this provides a hypothesis that cordycepin could be interfering the SRC-PP2A-4E-BP1 axis to regulate total 4E-BP1 level and AMPK phosphorylation.

Phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha (PI3KCA) is a frequently mutated gene in breast cancer cases (~36%) (Koboldt et al. 2012). MCF-7 has a PI3KCA mutation at E545K in exon 9, encoding the catalytic subunit p110α of PI3K, which causes constitutive phosphorylation and subsequent activation of AKT (Antoinette Hollestelle et al. 2007). Therefore, it is surprising to see the repression of P-AKT in cordycepin administered MCF-7 samples, suggesting that cordycepin represses the programmed elevation of AKT activity. AKT has been reported to inhibit AMPK activity by phosphorylation at Ser485/491 of the AMPKα activating subunit in different tissues (Horman et al. 2006; Hahn-Windgassen et al. 2005; Valentine et al. 2014). This phosphorylation in turn decreases the phosphorylation of AMPK by LKB1 and hence AMPK activity. In addition, translocation to the plasma membrane and a dual phosphorylation on residue Ser473 and Thr308 is required to fully activate AKT

(Bellacosa et al. 1998; Andjelkovic et al. 1997; I Vivanco & Sawyers 2002). Therefore the lack of activated AKT could explain the increasing phosphorylation of AMPKα.

Unlike the eventual dephosphorylation of ERK (discussed later in 7.1A), MEK phosphorylation was up-regulated in response to cordycepin treatment, which cannot be explained by the established RAF-MEK-ERK signaling. Indeed, ERK phosphorylation is mainly mediated by MEK, but this phosphorylation is also under the negative control of MAPK phosphatase (MKP, also known as Dual-specificity Protein-tyrosine Phosphatase (DUSP)) and protein tyrosine phosphatase (PTP)(Keyse 2008; Todd et al. 1999; Alonso et al. 2004; Sun et al. 1993). For example, Kwak and Dixon have found that active ERK1 was dephosphorylated by DUSP5, whereas this dephosphorylation was blocked by phosphatase inhibitor Na_3VO_4 (Kwak & Dixon 1995). However, even though this potentially separates ERK dephosphorylation from MEK phosphorylation, the link between cordycepin and MKPs and/or PTPs is still elusive.

In Figure 3.4C, the two sets of triplicate were generated with the cells from the same string of MCF-7 under the same conditions, passage number was thought to be the suspect factor. As similar mRNA level was detected with another replicate generated with cells from an early passage, this hypothesis was rejected. Notably, the first set of replicates were processed using Macherey-Nagel NucleoSpin RNA kit, whereas ReliaPrep RNA Miniprep System (Promega) was used in the second set. It is therefore assumed that samples from the second set of replicates were contaminated with undigested endogenous DNA. There are a number of potential mechanisms for cordycepin acting transcriptionally on gene expression: i) the cordycepin dephosphorylated ERK is known to regulate gene expression transcriptionally in a transcription factor-dependent manner (see 1.3.1.1); ii) cordycepin caused cleavage defect by arresting cleavage complex, which includes transcriptional regulators ; iii)

defective polyadenylation decreases transcription initiation of the same gene (Mapendano et al. 2010).

While many mRNAs that are sensitive to cordycepin encode transcription factors, the RAF-MEK-ERK signalling hereby becomes a putative mechanism, via which the effect of cordycepin on gene expression is mediated. Yet, the timecourse experiments on both effects suggests the two start around the same time (15-30 mins, Figure 3.2 & 3.3). 15 mins is generally considered too short for alterations in transcription to affect protein levels (Lau & Nathans 1987; Greenberg & Ziff 1984; Greenberg et al. 1986), therefore indirect effects of cordycepin on transcription by initial post-transcriptional suppression of the synthesis of one or a few transcription factors is unlikely in the time frame studied.

In summary, cordycepin caused viability defect on breast cancer cell lines, MCF-7, MDA-MB-468, MDA-MB-231 Dlux and Fluc. At the molecular level, growth-factor dependent gene expression was down-regulated by cordycepin, together with genes involved in other cellular activities. Like in NIH3T3 cells, cordycepin inhibits mTOR pathway by activating AMPKα and dephosphorylating 4E-BP1 and AKT. Curiously, MEK was activated, probably indicating that a feedback loop controlling ERK activity is activated. An effect of cordycepin on pre-mRNA cleavage was detected, but it is unlikely to be the reason that drives the regulation in mRNA level for all the genes. Overall, MCF-7 cells are responding similarly to cordycepin to most cell types described in the literature, with growth and survival signalling and gene expression being inhibited (Pan et al. 2015; Ioannidis et al. 1999; Wong et al. 2010; W.-D. Wu et al. 2014; H. G. Kim, Shrestha, Lim, Yoon, Chang, D.-J. Shin, et al. 2006; Zhang et al. 2014; Chen et al. 2008; Kondrashov et al. 2012; Zarkower & Wickens 1987).

4 Effects of Cordycepin in other Breast Cancer Cell Lines

In the previous chapter, I have investigated the reported effects in breast cancer cells. However, the majority of the analysis was based on MCF-7 cells. Unlike MCF-7, MDA-MB-468 and MDA-MB-231 are two triple negative breast cell lines, specified as estrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor 2 (HER2) negative, resulting in poor outcome and restricted therapy options (Neve et al. 2006; Foulkes et al. 2010; Lehmann et al. 2011). In order to explore the effects in MDA-MB-231 Fluc and MDA-MB-468 cells, mRNA levels in cordycepin administered MDA-MB-468 and MDA-MB-231 Fluc samples were measured with RT-qPCR.

4.1 Cordycepin Sensitive Genes Are Regulated Similarly in Breast Cancer Cells

To validate the MCF-7 microarray, Asma Khurshid and Irengbam Rocky Mangancha examined the mRNA level with RT-qPCR using the same set of samples. Notably, in MCF-7, PTPRK, CTSL1 and CATSPER2 were up-regulated by cordycepin whereas other genes tested were down-regulated. These candidate genes were chosen either because they showed high sensitivity to cordycepin or due to their contribution to significant cell activities. I have found that, in MDA-MB-468 cells, most genes down-regulated in MCF-7 cells were consistently down-regulated, with some exceptions from DUSP1, SNAI1 and IL6, of which the mRNA level was induced by contrast (Figure 4.1A top panel). Instead of induction, PTPRK, CTSL1 and CATSPER2 remained unchanged in MDA-MB-468.

Similar tests were conducted with cordycepin treated MDA-MB-231 Fluc cells. RPL10A was used as a control (Figure 4.1A lower panel). The remaining 11 genes were down-regulated in MCF-7 after treatment. DUSP1 and IL6 were again induced in MDA-MB-231 Fluc cells despite the reduction in MCF-7, while mRNA levels of other genes were repressed by cordycepin as they were in MCF-7.

To gain a more straightforward view, we calculated the log 2 fold change of the results from the two cell lines and plotted them against the data from MCF-7. Most genes repressed in MCF-7 cells were as well repressed in MDA-MB-468 (Figure 4.1B left). Yet, genes induced by cordycepin in MCF-7 cells were down-regulated or unchanged in MDA-MB-468. When plotted against MCF-7, except for two spots which represent the induced DUSP1 and IL6 levels, an overlap can be seen with the mRNAs down-regulated in both MDA-MB-231 FLuc and MCF-7 cells. To better understand the effect of cordycepin in MDA-MB-231 Fluc cells, a next-generation whole transcriptome shotgun sequencing (Nextseq) was conducted in duplicate with the RNA isolated from DMSO control and cordycepin treated cells, from which we obtained around 120 million 75 bp paired-end reads. Around 60 million reads per sample were obtained from the experiment, with 115-126 million (86%-90%) reads being mapped correctly to human genome Ensembl 86, hg38 (Table 4.1). In total, 197898 transcripts were annotated reference transcripts.

A full bioinformatic analysis was not carried out due to the limited time for this project. As a primary comparison, we selected genes based on the same principles as mentioned above, genes that are highly sensitive to cordycepin in MCF-7 cells or participate in major cellular functions, and compare the expression of these genes in MCF-7 microarray and MDA-MB-231 Fluc RNA-seq. The log 2 fold change of these genes was calculated and plotted against that in MDA-MB-231 Fluc RNA-seq data. As shown, both up- and down-regulated genes were regulated in the same fashion in the two cell lines (Figure 4.1C). Furthermore, gene ontology (GO) analysis using Database for Annotation, Visualization and Integrated Discovery (DAVID) (Huang, Sherman, et al. 2009; Huang, Lempicki, et al. 2009) for the RNA-seq data found a large amount (261 out of 747 genes, 37.61%) of the most significantly down-regulated genes in cordycepin treated MDA-MB-231 Fluc cells were annotated as transcription related (Table 4.2), which is consistent

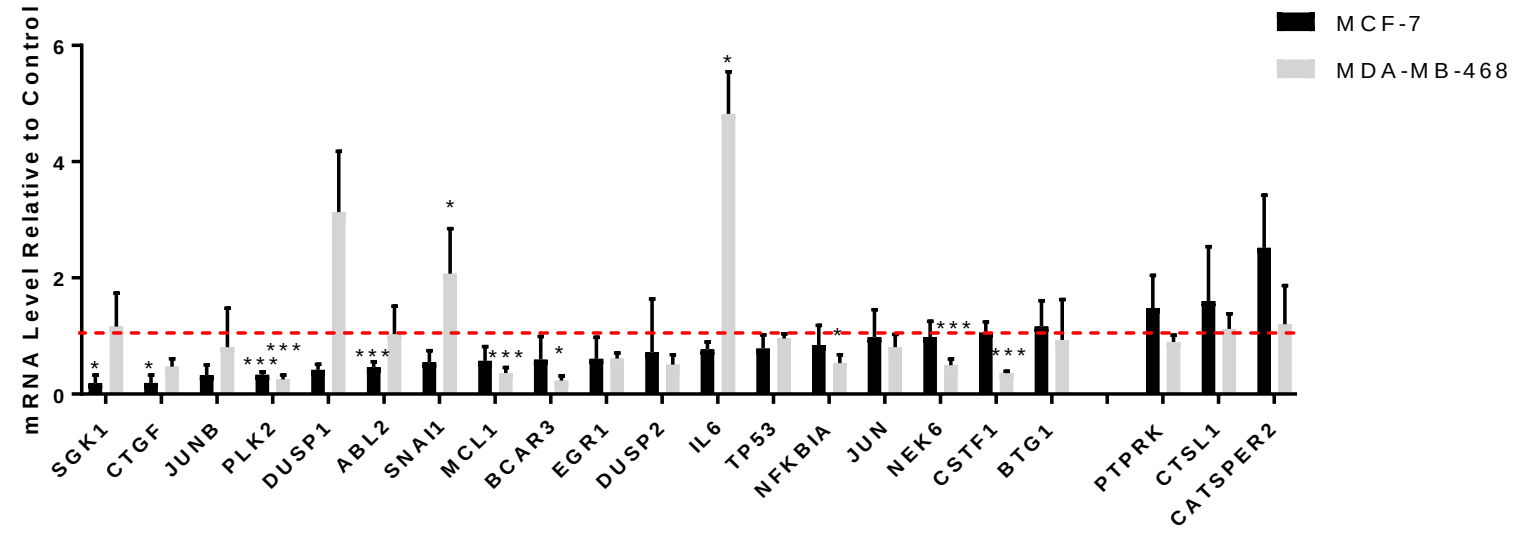
with the GO analysis result for MCF-7 (Asma Khurshid, Irengbam Rocky Mangangcha, Graeme Thorn and Cornelia De Moor, data unpublished). In addition to transcription related genes, genes involved in protein serine/threonine kinase activity, protein polyubiquitination, circadian rhythm and I-kappaB kinase/NF-kappaB signaling (Table 4.2). In particular, the fact, that genes participate in protein serine/threonine kinase activity are significantly down-regulated by cordycepin, overlaps with the analysis of the microarray in cordycepin treated MCF-7 cells, where a group of significantly down-regulated genes were part of the MAPK signaling (Irengbam Rocky Mangangcha, Asma Khurshid, Graeme Thorn and Cornelia H de Moor, unpublished data). Although with a few exceptions, all these results reveal the same group of genes is being modified similarly in breast cancer cells, suggesting that gene expression in these cells was regulated by the same mechanism in response to cordycepin.

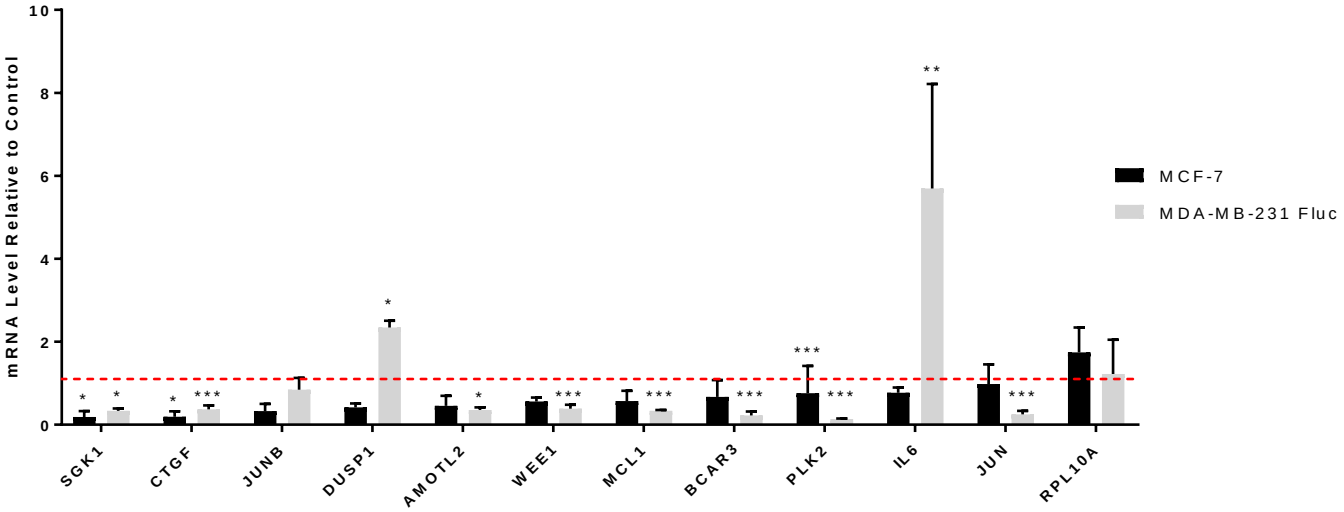
Sample	TrimmedReads	FilteredReads	MappedReads		Uniquely and Correctly Mapped Reads	
			Count	Percentage	Count	Percentage
E1 Control	119866030	119,038,890	114,534,304	96.22%	106,647,410	89.59%
E1 Cordycepin	132080232	131,193,026	126,252,452	96.23%	118,977,764	90.69%
E2 Control	131721378	130,695,036	125,162,842	95.77%	115,416,782	88.31%
E2 Cordycepin	124724952	123,478,236	119,298,438	96.61%	106,616,692	86.34%

Table 4.1 Mapping Profile of the MDA-MB-231 Fluc RNA-Seq Data

Listed are the details for mapping the MDA-MB-231 Fluc RNA-seq raw data to human genome GRCh38. Raw reads were first trimmed with Scythe and Sickle. The Filtering Pipeline was then used to filter reads with low sequencing scores. Mapping was performed using the tophat mapping tool in reference to the known gene exon coordinates. Reads mapped to only a single location with no significant hits to other locations are termed uniquely mapped reads. This analysis was performed by Sang Fei from the Victoria Wright lab, School of Medicine, University of Nottingham.

A.





(legend on next page)

Figure 4.1 Cordycepin Regulates Gene Expression Similarly in Breast Cancer Cells

A. MCF-7, MDA-MB-468 and MDA-MB-231 Fluc cells were treated with 50 µM cordycepin for 2h before collection. Cells were lysed, RNA was extracted and reverse transcribed into cDNA. mRNA level for selected genes were determined by RT-qPCR and the Ct values were analysed using the $2^{-\Delta\Delta Ct}$ method, normalising to the Ct values of GAPDH. The RT-qPCR result is presented as the ratio of fold change relative to control (dash line) (mean ± SD, n = 3 independent experiments, *P<0.05, **P<0.01 and ***P<0.005 compared with untreated control; p values were obtained using Student's t test).
B. MCF-7, MDA-MB-468 and MDA-MB-231 Fluc cells were processed as described in A. Log 2 fold changes for selected mRNAs in MDA-MB-468 (from RT-qPCR, left) and MDA-MB-231 Fluc (from RNA-seq, right) cell lines were plotted against the log 2 fold changes in MCF-7 (from microarray). Each spot represents the mean (For MCF-7 and MDA-MB-231 Fluc, n = 2 independent experiments; for MDA-MB-468, n=3 independent experiments).
C. Log 2 fold change for selected mRNAs in MDA-MB-231 Fluc RNA-seq were plotted against the log 2 fold change in MCF-7 microarray. Each spot represents the mean (n = 2 independent experiments).

Term	Count	Genes	P Value
GO:0006351~transcription, DNA-templated	261	ZNF57, ZNF253, ZNF254, CITED2, EPC1, ZNF778, EPC2, ZNF777, BRPF1, CRY2, MAP3K9, MED26, ZNF772, RARA, PATZ1, ZNF391, ZNF100, ZNF644, ZNF646, ZNF507, ZNF48, ZHX2, ZNF501, ZNF791, ZNF502, ZNF649, ZNF792, ZNF503, MECOM, HES1, ZFP82, ZNF784, MCIDAS, ZNF383, TGIF1, PRDM1, ZNF613, ZNF614, ZNF79, TADA1, ZNF230, ZNF615, ZNF616, ZNF225, ZNF799, ZNF222, ZNF71, ZNF124, BCOR, NKRF, ZBTB49, ZNF398, KLF6, ZNF624, SMAD7, ZNF121, KLF10, ZBTB45, ZNF627, ZNF585A, ZNF628 …	1.86E-91
GO:0006355~regulation of transcription, DNA-templated	209	SNIP1, ZNF57, ZNF253, ZNF254, CITED2, ZNF778, ZNF777, MAP3K9, ZNF772, PATZ1, ZNF391, ZNF100, ZNF644, RREB1, ZNF646, ZNF507, ZNF48, ZNF501, ZNF791, ZNF649, ZNF502, ZNF792, ZNF503, MECOM, PPARGC1B, HES1, ZFP82, ZNF784, ZNF383, TGIF1, ZNF613, ZNF614, ZNF79, TADA1, ZNF230, ZNF615, ZNF616, ZNF225, ZNF799, ZNF222, ZNF71 …	3.59E-73
GO:0004674~protein serine/threonine kinase activity	23	CCNT2, SGK1, NUAK2, SNX15, NUAK1, CCNT1, STK35, STK17B, PIM1, PIM3, CLK1, PDIK1L, MAST4, ACVR2A, OSR1, PLK2, SNRK, MAP3K1, MAP3K9, DYRK1A, CLK4, DYRK2, RIPK4	0.019563
GO:0000209~protein polyubiquitination	14	RNF144B, CBLB, SPSB1, RNF19A, TRIM32, SIAH1, TOPORS, NHLRC1, SIAH2, UBOX5, MYLIP, RNF19B, TRAF6, RNF111	0.013606
GO:0007623~circadian rhythm	10	CRY2, ID1, JUN, KLF10, DYRK1A, PER2, ID3, NFIL3, NOCT, NRIP1	0.001251
GO:0007249~I-kappaB kinase/NF-kappaB signaling	7	BCL10, REL, SNIP1, TICAM1, ZNF675, TRAF6, TIAF1	0.018725

Table 4.2 GO Analysis for the Significantly Down-regulated Genes in Cordycepin Treated MDA-MB-231 Fluc Cells

According to the RNA-seq result, 747 most significantly down-regulated (log2foldchange<-2, P<0.005) genes were put into DAVID (V6.8) for functional annotation bioinformatics microarray analysis, of which 694 genes were recognised and annotated. These genes were categorised into different terms based on their cellular functions. The terms presented here were pick because they show the low P values and/or the same term was seen being affected by cordycepin in previous analysis.

4.2 Cordycepin Interferes EGF Response in MDA-MB-468 Cells

So far, we have seen growth factor dependent gene expression being regulated by cordycepin in MCF-7 cells. Given the inference that the same group of genes are regulated by cordycepin in different breast cancer cell lines, it is therefore expected and possibly that growth factor response would be inhibited by cordycepin in other breast cancer cell lines as well. A distinct feature of MDA-MB-468 cells is the over-expression of epidermal growth factor receptor (EGFR) (Filmus et al. 1985). As one of the responsive pathways downstream receptor tyrosine kinase, RAF-MEK-ERK signalling is activated and manipulates the expression of transcription factors (Avraham & Yarden 2011; Yarden & Shilo 2007; Wells 1999).

A MDA-MB-468 model was therefore used to assess if cordycepin interferes with growth factor response, in which cells were treated with human recombinant epidermal growth factor (EGF) to induce downstream mRNA expression. Indeed, elevated level of EGR1 and FOS was observed after the stimulation of EGF (Figure 4.2A), validating the responsiveness of the model. Next we examined how mRNA level of cordycepin down-regulated AMOTL2, PLK2, DUSP1, AREG, EGR1, JUN, IER3, SPRY2 and MYC reacts to EGF and/or cordycepin. Although not sensitive to EGF stimulation, AMOTL2 and PLK2 are regulated by cordycepin (Figure 4.2B). Two growth factor response genes, DUSP1 and AREG, were unaffected by cordycepin treatment. In contrast, the induction of EGR1, JUN, IER3, SPRY2 and MYC by EGF was reduced by cordycepin. Note that these five genes are also known as immediate early genes, presumably modulated via RAF-MEK-ERK signalling. The inhibitory effect of cordycepin on these mRNA suggests the interruption of a key part of the EGF response by cordycepin.

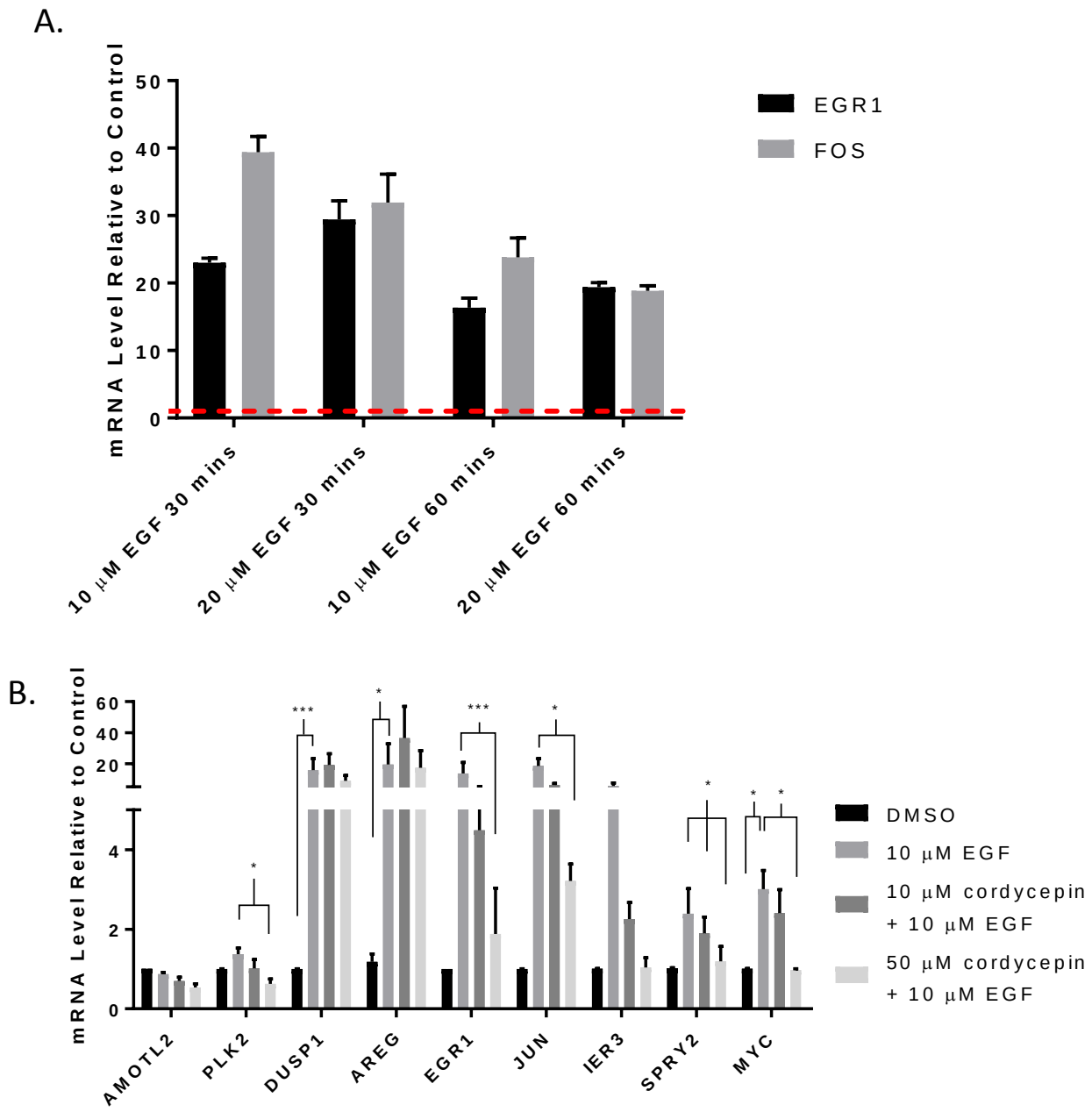


Figure 4.2 Cordycepin Interferes EGF Response in MDA-MB-468 Cells

A. MDA-MB-468 cells were treated with EGF as indicated. Cells were harvested, lysed. Relative RNA was extracted and reverse transcribed into cDNA. mRNA levels were analysed with RT-qPCR and the Ct values were analysed using the $2^{-\Delta\Delta Ct}$ and normalised to the Ct values of GAPDH. The result of RT-qPCR is presented as the ratio of fold change level relative to DMSO control (red dash line). (mean ± SD, n = 1 independent experiments).

B. MDA-MB-468 cells were treated with EGF 1 hr before collection. Cordycepin of different concentrations were administered 30 mins before collection. Cells were harvested, lysed. Relative RNA was extracted and reverse transcribed into cDNA. mRNA levels were determined with RT-qPCR and Ct values were analysed using the $2^{-\Delta\Delta Ct}$ method, normalising to the Ct values of GAPDH. The result is presented as the ratio of fold change level relative to DMSO control (dash line). (mean ± SD, n = 3 independent experiments, *P<0.05, **P<0.01 and ***P<0.005 compared with cells with only EGF treatment; p values were obtained using Dunnett's test).

Discussion

While MDA-MB-468 harbours a phosphatase and tensin homolog (PTEN) mutation, V-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog (KRAS) and v-Raf murine sarcoma viral oncogene homolog B (BRAF) mutations are expressed in MDA-MB-231 cells (A. Hollestelle et al. 2007; Lehmann et al. 2011; Forbes et al. 2016). In MCF-7 cells, a PIK3CA mutation has been found (A. Hollestelle et al. 2007). The mutations in all three breast cancer cell lines exhibit oncogenic properties (A. Hollestelle et al. 2007). In the data presented, gene expression in these cell lines were regulated in a similar manner by cordycepin. Transcription factors can be altered by RAF-MEK-ERK signalling, whereas the effect on PI3K-PDK1-AKT could affect gene expression through the mTOR pathway, suggesting that this change in gene expression could be resulted from the effect of cordycepin in signal transduction.

In summary, we have found the same group of genes down-regulated by cordycepin in not only MCF-7 cells, but also MDA-MB-468 and MDA-MB-231 Fluc cells. Rather than a cell-type specific effect, this result suggests the same mechanism was utilised by cordycepin in regulating these genes.

5 The Effect of Cordycepin on Non-coding RNA

Introduction

As mentioned previously, cordycepin mimics the effect of serum deprivation on gene expression (Figure 5A, Khurshid, Mangangcha, Thorn and De Moor, unpublished data). Gene ontology analysis of the cordycepin microarray data revealed that most down-regulated genes were angiogenesis, apoptosis, cell cycle, differentiation, proliferation and transcription related (Figure 5B, Khurshid, Mangangcha, Thorn and De Moor, unpublished data). Remarkably, most genes up-regulated by cordycepin are non-coding RNAs (ncRNA). According to the human genome project, only around 1.5% of the human genome are translated into functional proteins, while non-coding transcripts account for the rest 98.5 % (Lander et al. 2001). Considering the amount of ncRNAs and that the function of most ncRNAs remains elusive, one could hypothesise that cordycepin induced ncRNAs are defective transcripts without function that just escape quality control because of this artificial treatment. Yet, the fact that these ncRNAs are also up-regulated by serum deprivation suggests their potential involvement in growth regulation. Apart from the well-known tRNA and rRNA, ncRNAs include small nucleolar RNA (snoRNA), small nuclear RNA (snRNA), pseudogene (ncRNA with high homology to a functional gene), long non-coding RNA (lncRNA) etc. (Mattick & Makunin 2006).

In contrast to the established stabilising effect of poly(A) tails on RNAs, it has been reported that polyadenylation could be promoting the exosome-mediated RNA degradation of non-coding RNAs (Drummond et al. 1985; West et al. 2006; Kuai et al. 2004; Kadaba et al. 2004; van Hoof et al. 2000; Houseley et al. 2006). This is well-studied in yeast, where the Trf4/5-Air1/2-Mtr4-polyadenylation (TRAMP) complex recruits exosomes to degrade the host RNA in a poly(A) tail-dependent manner (Wyers et al. 2005; Vaňáčová et al. 2005; LaCava et al. 2005; Bresson et al. 2017). In mammalian cells, an equivalent complex was found, being composed of PAPD5, ZCCHC7 and

SKIV2L2,which mediates the degradation of PROMPTs, PROMoter uPstream Transcripts, in a poly(A) tail-dependent manner (see 1.4.4.2)(Preker et al. 2011; Preker et al. 2008).

As opposed to the TRAMP-mediated nuclear RNA decay, a canonical mammalian nuclear RNA decay model was proposed by the Nicholas Conrad lab. Using RNA-seq, they have witnessed the elevation of ncRNAs in response to poly(A) polymerase inactivation and PABPN1 knockdown, which is consistent to another report (Bresson et al. 2015; Beaulieu et al. 2012). Moreover, PABPN1 interacts directly with exosome, indicating its regulation in exosome-mediated RNA degradation (Lemay et al. 2010; Beaulieu et al. 2012). Together, these studies suggest that ncRNAs are subjected to nuclear RNA decay in a polyadenylation-dependent manner. As cordycepin inhibits polyadenylation, this would explain the increasing ncRNA level upon cordycepin treatment.

At top of the most down-regulated genes in the microarray of cordycepin treated MCF-7, as well as in serum-deprived MCF-7, surprisingly, are the small NF90-associated RNA (SNAR), a class of small non-coding RNAs (Irengbam Rocky Mangangcha, Asma Khurshid, Graeme Thorn and Cornelia H de Moor, unpublished data). SNARs were first discovered by immunoprecipitating UV-crosslinked NF90 protein in HEK293 cells, with one of the SNARs, SNAR-A, being highly structured (Parrott & Mathews 2007). Although SNARs is still a relatively fresh topic with very limited understanding, pilot study has revealed a tight bound between SNAR-A and ribosomes, suggesting a potential role in translation regulation (Parrott et al. 2011). Given that NF90 directly targets HIV-1 RNA transcription (Agbottah et al. 2007), validation of SNAR functions in NF90 will also be beneficial for HIV research.

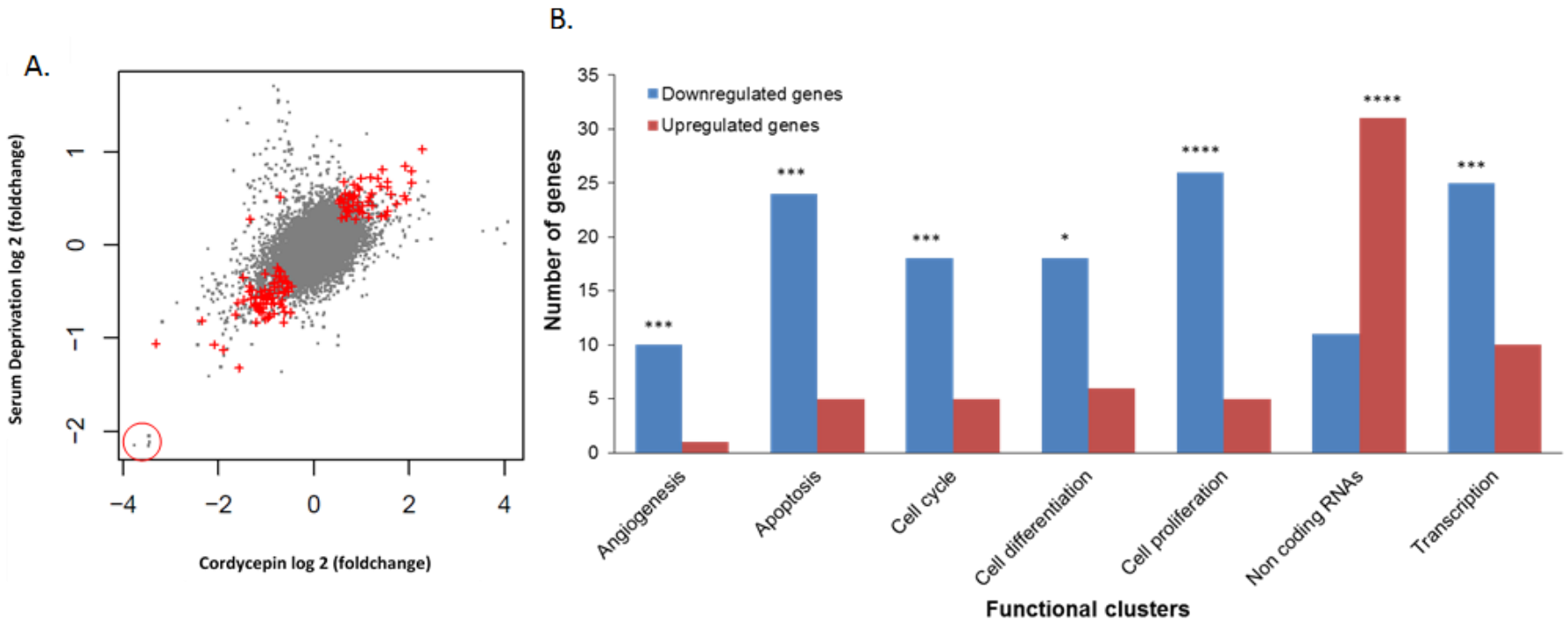


Figure 5 GO Analysis for the Significantly Down-regulated Genes in Cordycepin Treated MDA-MB-231 Fluc Cells

A. The resemblance of gene expression between serum-withdrawal and 50 µM cordycepin treated samples from MCF-7. Red dots stand for the genes significantly regulated in both conditions; grey dots stand for genes not significantly regulated in one or both conditions. SNARs were indicated within the red circle. (adapted from Khurshid, Mangangcha, Thorn and De Moor, unpublished data)

B. Functional association of the most regulated genes in cordycepin treated MCF-7 cells. Data presented as number of genes in related biological activities. Fisher's Exact test p values are indicated as *p<0.05, **p<0.01, ***p<0.001, ****p< 0.0001. (adapted from Khurshid, Mangangcha, Thorn and De Moor, unpublished data).

5.1 Cordycepin Down-regulates SNAR Level

As mentioned, SNARs are significantly down-regulated in cordycepin treated and serum-deprived MCF-7 cells, according to microarray result (Irengbam Rocky Mangangcha, Asma Khurshid, Graeme Thorn and Cornelia H de Moor, unpublished data). To validate this observation, RT-qPCR was performed. However, due to the small size of the SNARs (~117 nt)(Parrott & Mathews 2007), general reverse transcription was found not suitable for SNAR detection, as it leads to high and therefore error-prone cycle numbers. To improve the reverse transcription efficiency for SNARs, a specific reverse transcription was developed using primers designed for SNARs (see 2.5).

In agreement with the microarrays, SNAR-A1 level in MCF-7 cells was repressed remarkably by cordycepin, as well as in serum deprivation sample (Figure 5.1). With transcription factors being regulated by cordycepin, it is suspected that transcription could play a role in altering the level of SNARs. Actinomycin D blocks transcription both by interfering with the transcription complex and inhibiting RNA elongation (Trask & Muller 1988; Koba & Konopa 2005; Sobell 1985). By treating with actinomycin D, the level of SNAR-A1 was again remarkably reduced (Figure 5.1), suggesting that SNAR-A1 was very unstable and transcriptionally repressed. Additionally, SNAR-A1 level was also decreased in cordycepin treated MDA-MB-468 cells (Figure 5.1), which implicates this is not a cell type-specific observation.

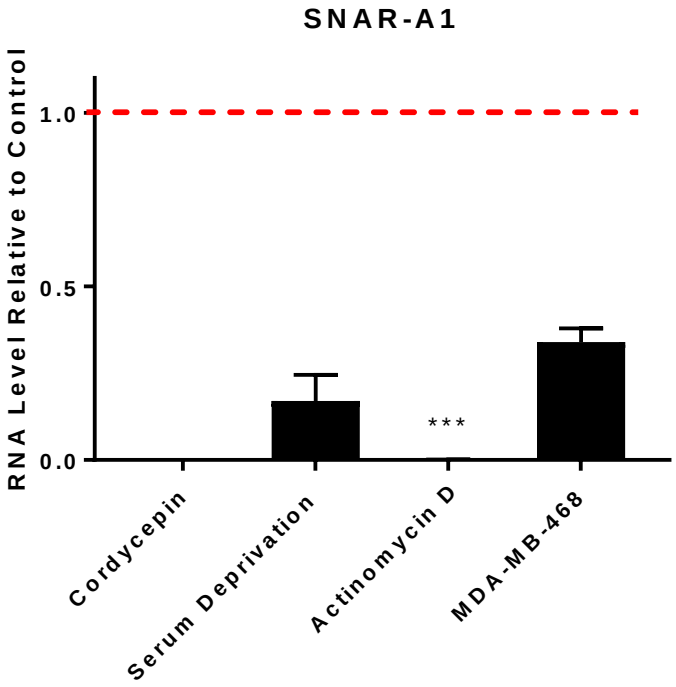


Figure 5.1 Cordycepin Down-Regulates SNAR-A1 Level

MCF-7 cells from 50 µM cordycepin (n=1), serum deprived (n=1), Actinomycin D (n=3) treated group and MDA-MB-468 cells from 50 µM cordycepin (n=1) were collected and lysed. Relative RNA was extracted and reverse transcribed into cDNA. RNA levels for selected genes were determined by RT-qPCR. Ct values were analysed using the $2^{-\Delta\Delta Ct}$ method and normalised to the Ct values of GAPDH. The result is presented as the ratio of fold change relative to control (red dashed line). (mean ± SD; *P<0.05, **P<0.01 and ***P<0.005 compared with untreated control; p values were obtained using Student's t test).

5.2 Non-coding RNAs Are Up-regulated by Cordycepin in MCF-7 and MDA-MB-231 Fluc Cells

64 significantly up-regulated genes were sorted (log2(foldchange)>1, P<0.05) from the RNA-seq data of cordycepin treated MDA-MB-231 Fluc sample (Table 5.2), of which 31 (48.44%) genes were listed as non-coding RNAs according to Ensembl (Release 88) (Kersey et al. 2016). This includes long non-coding RNAs (lncRNA), pseudogenes, small nuclear RNA (snRNA) and small nucleolar RNA (snoRNA). To validate the RNA-seq data, RT-qPCR was used to determine the level of the non-coding RNAs chosen from the list. Consistently, induction of the 6 ncRNAs picked, SPACA6P-AS, RP11-188D8.1, RP11-873E20.1, U73166.2, HNRNPA3PS and CTD-2619J13.13, were seen in cordycepin treated MDA-MB-231 Fluc samples (Figure 5.2). Except SPACA6P-AS, the rest of the ncRNAs tested above exhibited similar regulation upon cordycepin treatment (Figure 5.2), this again proves the regulation of non-coding RNAs is not a cell type-specific effect of cordycepin.

GeneID	GeneName	log2FoldChange	pvalue	
ENSG00000227688	HNRNPA3P2	3.85	4.43E-11	processed pseudogene
ENSG00000105523	FAM83E	3.31	4.81E-11	
ENSG00000172426	RSPH9	2.47	3.79E-05	
ENSG00000230454	U73166.2	2.38	7.40E-07	lncRNA
ENSG00000268307	CTD-2619J13.13	2.28	1.93E-04	lncRNA
ENSG00000261857	MIA	1.90	1.63E-03	
ENSG00000068976	PYGM	1.88	3.74E-06	
ENSG00000234618	RPSAP9	1.80	1.04E-03	processed pseudogene
ENSG00000180043	FAM71E2	1.78	3.08E-03	
ENSG00000130518	KIAA1683	1.72	2.04E-03	
ENSG00000205663	RP11-706O15.5	1.65	1.99E-03	lncRNA
ENSG00000264281	CTD-2031P19.4	1.63	7.42E-13	processed pseudogene
ENSG00000273416	RP4-597N16.4	1.54	1.14E-02	lncRNA
ENSG00000081041	CXCL2	1.53	5.18E-07	
ENSG00000262096	PCDHB19P	1.51	1.35E-02	processed pseudogene
ENSG00000280087	CTB-129P6.7	1.50	1.20E-02	To be Experimentally Confirmed
ENSG00000163395	IGFN1	1.48	1.51E-02	
ENSG00000240457	RN7SL472P	1.42	1.98E-02	miscRNA
ENSG00000267397	RP11-873E20.1	1.41	2.08E-02	lncRNA
ENSG00000206652	RNU1-1	1.41	1.73E-02	snRNA
ENSG00000231841	RP11-206F17.2	1.38	1.50E-02	processed pseudogene
ENSG00000273597	RP11-392A14.9	1.35	2.30E-02	To be Experimentally Confirmed
ENSG00000256615	RP11-59N23.3	1.34	2.56E-02	antisense
ENSG00000144063	MALL	1.31	2.61E-02	
ENSG00000240038	AMY2B	1.30	1.55E-02	
ENSG00000232448	RP11-416N4.1	1.30	2.77E-02	lncRNA
ENSG00000235959	AC009237.17	1.27	3.11E-02	processed Pseudogene
ENSG00000119508	NR4A3	1.26	5.59E-03	
ENSG00000215861	WI2-1896O14.1	1.25	6.72E-03	unprocessed pseudogene
ENSG00000214810	CYCSP55	1.24	4.03E-02	processed pseudogene
ENSG00000213640	EEF1DP4	1.23	4.23E-02	processed pseudogene
ENSG00000261371	PECAM1	1.22	4.58E-02	
ENSG00000230626	RP11-286H14.4	1.22	4.02E-02	
ENSG00000272843	RP11-313P13.5	1.22	3.76E-02	antisense

ENSG00000271644	RP3-365I19.2	1.22	3.10E-02	processed pseudogene
ENSG00000225850	RP11-452K12.4	1.21	4.36E-02	antisense
ENSG00000232742	RHOQP2	1.21	4.78E-02	processed pseudogene
ENSG00000009724	MASP2	1.21	2.58E-02	
ENSG00000213621	RPSAP54	1.21	2.78E-04	processed pseudogene
ENSG00000101203	COL20A1	1.19	1.89E-03	
ENSG00000161860	SYCE2	1.19	1.37E-02	
ENSG00000213939	RP11-314A20.1	1.19	4.54E-03	processed pseudogene
ENSG00000270953	RP11-2E11.9	1.19	2.80E-02	antisense
ENSG00000141404	GNAL	1.18	4.25E-02	
ENSG00000256338	RPL41P2	1.16	2.74E-06	processed pseudogene
ENSG00000172020	GAP43	1.16	5.70E-02	
ENSG00000242147	RP13-463N16.6	1.15	2.38E-02	lncRNA
ENSG00000229927	RHEBP1	1.15	4.56E-02	processed pseudogene
ENSG00000140009	ESR2	1.15	3.94E-02	
ENSG00000241680	RPL31P49	1.15	5.79E-02	processed pseudogene
ENSG00000231993	EP300-AS1	1.15	5.15E-02	antisense
ENSG00000087589	CASS4	1.15	5.67E-02	
ENSG00000279722	RP11-44F14.6	1.14	5.03E-02	To be Experimentally Confirmed
ENSG00000279145	RP11-547D13.1	1.14	3.61E-02	To be Experimentally Confirmed
ENSG00000136244	IL6	1.14	1.74E-03	
ENSG00000213453	FTH1P3	1.13	1.36E-03	processed pseudogene
ENSG00000231351	AC111200.7	1.13	2.06E-02	processed pseudogene
ENSG00000178776	C5orf46	1.12	5.13E-02	
ENSG00000271225	BNIP3P4	1.12	6.38E-02	processed pseudogene
ENSG00000232134	RPS15AP12	1.11	5.51E-02	processed pseudogene
ENSG00000240963	RP11-518L10.5	1.11	4.58E-02	antisense
ENSG00000140506	LMAN1L	1.10	4.69E-02	
ENSG00000230516	RP11-555J4.3	1.10	4.69E-02	lncRNA
ENSG00000237017	AC012314.8	1.09	5.72E-02	antisense
ENSG00000206878	SNORA51	1.09	7.40E-02	snoRNA
ENSG00000241511	RPS15AP24	1.09	7.46E-02	processed pseudogene
ENSG00000184110	EIF3C	1.09	4.83E-02	
ENSG00000196604	POTEF	1.08	7.66E-02	

ENSG00000226102	SEPT7P3	1.08	5.58E-02	unprocessed pseudogene
ENSG00000269959	SPACA6P-AS	1.07	3.48E-02	lncRNA
ENSG00000125246	CLYBL	1.07	1.67E-02	
ENSG00000230397	SPTLC1P1	1.07	6.06E-02	transcribed processed pseudogene
ENSG00000236937	PTGES3P4	1.07	7.44E-02	processed pseudogene
ENSG00000274460	CTD-2649C14.2	1.06	2.62E-03	processed transcript
ENSG00000175886	RPL7AP66	1.06	2.76E-02	processed pseudogene
ENSG00000036448	MYOM2	1.06	5.04E-02	
ENSG00000261744	RP11-21B21.4	1.06	8.32E-02	antisense
ENSG00000260265	RP11-44F21.5	1.06	8.38E-02	lncRNA
ENSG00000185829	ARL17A	1.06	2.41E-03	
ENSG00000258818	RNASE4	1.06	5.44E-02	
ENSG00000172771	EFCAB12	1.06	6.61E-02	
ENSG00000166257	SCN3B	1.05	6.44E-02	
ENSG00000120251	GRIA2	1.05	7.26E-02	
ENSG00000216285	RP11-490H24.5	1.05	8.46E-02	processed pseudogene
ENSG00000274922	RP11-88E10.5	1.05	8.59E-02	lncRNA
ENSG00000186340	THBS2	1.04	7.91E-02	
ENSG00000104888	SLC17A7	1.04	7.41E-02	
ENSG00000219391	AC019129.1	1.04	8.42E-02	processed pseudogene
ENSG00000233766	AC098617.1	1.04	8.57E-02	antisense
ENSG00000154277	UCHL1	1.03	5.82E-02	
ENSG00000271427	RP11-188D8.1	1.03	7.79E-02	lncRNA
ENSG00000240914	RPL15P2	1.03	7.24E-02	processed pseudogene
ENSG00000218305	CDC14C	1.03	2.49E-02	processed pseudogene
ENSG00000137699	TRIM29	1.02	6.43E-02	
ENSG00000261888	AC144831.1	1.02	9.52E-02	lncRNA
ENSG00000226856	AC093901.1	1.02	7.95E-02	lncRNA
ENSG00000277452	RN7SL473P	1.02	9.63E-02	miscRNA
ENSG00000154096	THY1	1.02	9.45E-02	
ENSG00000127324	TSPAN8	1.02	9.44E-02	
ENSG00000248079	DPH6-AS1	1.02	9.38E-02	lncRNA
ENSG00000196205	EEF1A1P5	1.01	1.41E-11	processed pseudogene
ENSG00000207142	Y_RNA	1.01	5.56E-02	miscRNA
ENSG00000004838	ZMYND10	1.00	4.94E-02	
ENSG00000255909	PDCD5P1	1.00	1.02E-01	processed pseudogene

ENSG00000235084	CHCHD2P6	1.00	1.03E-01	processed pseudogene
ENSG00000199683	RN7SKP185	1.00	6.56E-02	miscRNA

Table 5.2 Genes Significantly Up-regulated by Cordycepin in MDA-MB-231 Fluc Cells

64 genes significantly up-regulated (log2(foldchange)>1, P<0.05) by cordycepin in MDA-MB-231 Fluc cells.

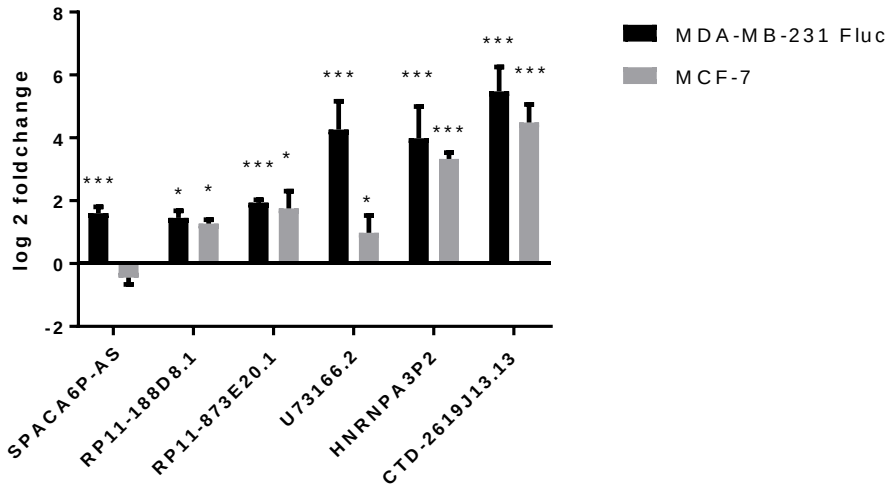


Figure 5.2 Non-coding RNAs are up-regulated by cordycepin in MCF-7 and MDA-MB-231 Fluc cells

MCF-7 and MDA-MB-231 Fluc cells were treated with 50 µM cordycepin 2 hrs before harvest. Cells collected were lysed. Relative RNA was isolated and reversed transcribed into cDNA for RT-qPCR determination. Ct values were analysed using the $2^{-\Delta\Delta Ct}$ method and normalised to the Ct values of GAPDH. RNA levels were presented as the log2(foldchange) relative to untreated control. (mean ± SD; n=3 independent experiments; *P<0.05, **P<0.01 and ***P<0.005 compared with untreated control; p values were obtained using Student's t test).

5.3 Non-coding RNAs Induced by Cordycepin Are Subjected to Polyadenylation Dependent Decay

To investigate if polyadenylation is required for the decay of these cordycepin induced ncRNAs, MCF-7 cells were transfected with small interfering RNA (siRNA) to knockdown different poly(A) polymerases (PAP). Samples used here were kindly provided by Asma Khurshid and Cornelia H de Moor. While the levels of lncRNAs, RP11-873E20.1, RP11-188D8.1, CTD-2619J13.13 and U73166.2, were significantly up-regulated by cordycepin in both MDA-MB-231 Fluc and MCF-7 cells, elevated level of these two genes was also

observed in PAP knockdown samples (Figure 5.3). Combined knockdown of PAPα and γ exhibited the strongest induction throughout the experiment. Three replicates were

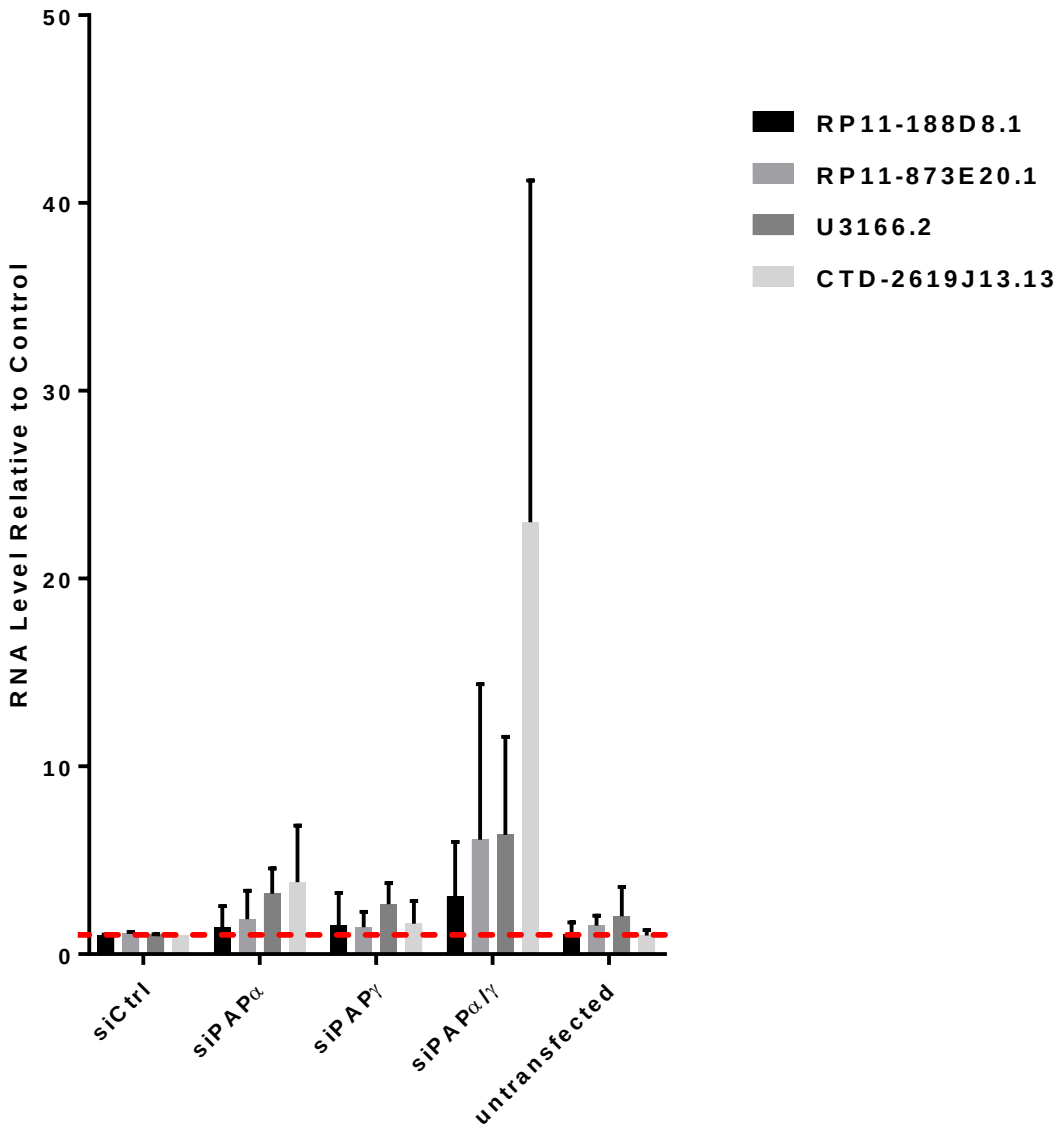


Figure 5.3 Non-coding RNAs Induced by Cordycepin Are Subjected to Polyadenylation Dependent Decay

MCF-7 cells were transfected with siRNA to knockdown different poly(A) polymerases as shown. Cells collected were lysed. Relative RNA was isolated and reversed transcribed into cDNA for RT-qPCR determination. Ct values were analysed using the $2^{-\Delta\Delta Ct}$ method and normalised to the Ct values of GAPDH. RNA levels were presented as the ratios relative to untreated control (red dashed line). (mean ± SD; n=3 independent experiments).

used in this experiment. However, one of the replicates was not prepared by the same person as the other two due to technical problem. Although the same reagents and cells from the same strand of MCF-7 were used, the result varies. It is presumed that this is what caused the high standard deviation and thus lack of significance. Despite this, it is clearly worth repeating this experiment as the result indicates that PAP knockdowns have positive regulation on lncRNA levels tested. This result also agrees with the findings of Nicholas Conrad's laboratory (Bresson et al. 2015), suggesting that ncRNAs induced by cordycepin are subjected to polyadenylation-dependent decay.

Discussion

For the well-established polyadenylation inhibitory effect of cordycepin, Bresson et al. did test the canonical nuclear RNA decay model with cordycepin and found the same group of RNAs was induced (Bresson et al. 2015). Moreover, Bresson et al. is not the only group to report this observation. Beaulieu et al. found lncRNA SNORD60-Host Gene (SHG60) was significantly stabilised by cordycepin (Beaulieu et al. 2012). Additionally, knockdown of poly(A) binding protein nuclear 1 (PABPN1) mimics this stabilisation of these endogenous long noncoding RNAs (Beaulieu et al. 2012). Including our result, it is consistently implied that polyadenylation is required for lncRNA turnover, which could be repressed by cordycepin. On the other hand, Parrott et al. proposed that SNARs are not polyadenylated in cells (Parrott et al. 2007), which suggests that the machinery behind the down-regulation of SNARs by cordycepin is not the same as for the upregulation of lncRNAs.

In this chapter, we identified the ncRNAs up- and down-regulated in response to cordycepin. Small non-coding RNA SNARs are down-regulated by cordycepin but their function remains elusive. Interestingly, SNARs are also down-regulated by serum deprivation, indicating a potential role of SNARs in growth factor regulation (data not shown). Although the function for most of the lncRNAs and pseudogenes are not yet

understood, there are a few examples which demonstrate their involvement in cellular activity. One of them is nuclear paraspeckle assembly transcript 1 (NEAT1), which has an essential role in the formation of paraspeckles (Clemson et al. 2009). PTENP1, a pseudogene of PTEN, acts as a decoy to attract miRNA-mediated degradation, so as to promote the normal activity of tumour suppressor PTEN (Poliseno et al. 2010). It is hereby an open question whether these up-regulated lncRNAs and/or pseudogenes are intermediates that cordycepin regulates to exert its anti-cancer property.

Microarray and high throughput sequencing data unveiled that the majority of the up-regulated genes are ncRNAs, was validated by RT-qPCR in both MDA-MB-231 Fluc and MCF-7. The same lncRNA stabilising effect was also observed in PAP knockdown samples in MCF-7, which indicates that these cordycepin up-regulated lncRNAs are subjected to polyadenylation-dependent decay. Finally, the fact that serum deprivation mimicked this regulation of ncRNAs by cordycepin suggests the potential involvement of these genes in important biological activities.

6 Cordycepin Inhibits the Translation of Ribosomal Proteins

Introduction

In agreement with cordycepin being reported as a polyadenylation inhibitor, the De Moor lab has reported that nuclear polyadenylation is inhibited by cordycepin. After cordycepin treatment, shortened poly(A) tail was observed in different genes, of which the mRNA level was repressed by cordycepin (Wong et al. 2010). Although it was later suggested that the polyadenylation defect of cordycepin does not correlate with mRNA stability (Kondrashov et al. 2012), the role of poly(A) tail as an enhancer of translation suggests a possibility of cordycepin affecting translation efficiency (Munroe & Jacobson 1990; Jackson 1993; Richter 1999). In addition, we have seen the effect of cordycepin on signal transduction start as early as 15 mins (Figure 3.2), which presumably is too soon for the effect on mRNA expression to affect protein level. Hence we wondered if cordycepin directly alters translation efficiency of some key signal transduction molecules leading to a rapid change in protein level that can explain the effects on signal transduction.

The polyribosome structure was first documented in 1962 by separating mRNAs according to the amount of ribosome/ribosome subunit associated using sucrose gradient (Warner et al. 1963). Polysome profiling combines the separation of ribosome associated mRNAs and a cDNA microarray to procure translation efficiency level in a genome-wide perspective (Figure 6.1A) (Johannes et al. 1999; Piccirillo et al. 2014).

Results

As translation is thought to be predominantly regulated at the translation initiation stage (Jackson et al. 2010; Sonenberg & Hinnebusch 2009), the number of ribosomes bound to an mRNA is thought to usually correlate with translation efficiency. mRNPs are separated over 10 fractions of a sucrose gradient. While the first 5 fractions contain mRNAs with one ribosome, ribosome subunit(s) or no ribosome at all, the next 5

fractions comprise mRNA with two or more ribosomes. Based on this principle, the first 5 factions were pooled together as the subpolysomal fraction and the next 5 together as the polysomal fraction. The two pooled fractions were then labelled with different colour dyes respectively and determined by microarray. After the first run, the two pooled fractions were labelled with the dye used for the other fraction in the first run, followed by another microarray. Therefore, by analysing the ratio of polysomal reads over subpolysomal reads, a view on the gene specific translational effect by cordycepin with improved accuracy was generated.

With cordycepin treated MCF-7 samples, there is a decrease in polysomal mRNA levels and increase in 40S, 60S and 80S (Figure 6.1B), which is consistent with the result in NIH3T3 cells (Wong et al. 2010). Gene ontology (GO) analysis of the microarray data uncovered that most of the genes translationally down-regulated by cordycepin were annotated as translation related genes (23.83%)(Table 6A). Also down-regulated are genes related to cell-cell adhesion and ATP-binding.

Ribosomal protein mRNAs have a tract of pyrimidine at the 5' end, typically starting with a cytosine (C), such mRNAs are known as 5' terminal oligopyrimidine (TOP) mRNAs (Meyuhas 2000; Thoreen et al. 2012). TOP mRNAs are regulated by mTOR signal transduction pathway. Although it was once suspected that ribosomal protein S6 was the key factor in this fashion (Pende et al. 2004; Jefferies et al. 1997), recent reports have shown direct interaction between LARP1, a mTORC1 substrate, which releases the 5' of the TOP mRNA for translation initiation when phosphorylated by mTORC1 (Tcherkezian et al. 2014; Lahr et al. 2017). Noticeably, most ribosomal protein mRNAs are TOP mRNA (Meyuhas 2000). As TOP mRNAs are listed as some of the most translationally down-regulated genes, coupling with the observation that both total and phosphorylated 4E-BP1 levels were down-regulated by cordycepin (Figure 3.2), this

confirms the inhibitory effect of cordycepin on mTOR pathway and demonstrates that it has major effects within 30 minutes of administration.

Now that the effect on TOP genes is very clear, we then started investigating the effect of the rest of the gene on the list. Because most TOP genes are, as mentioned, ribosomal protein genes and elongation factors (eEF1A1 and eEF1A2) (Meyuhas 2000), these genes were filtered out according to the relative GoTerm lists in Gene Ontology Consortium (Carbon et al. 2009). A separate GO analysis was performed for the remaining genes, which shows significant ($P<0.005$) involvement of these genes in mitochondrial inner membrane activity, cell-cell adhesion, translation initiation and gluconeogenesis, implicating a potential role of cordycepin in these activities (Table 6B). Significantly up-regulated are DNA-binding related genes (Table 6C). Transcription related genes are listed in both down- and up-regulated GO analysis, though it is unlikely that a full story can be told due to the high P value. Also, due to limited chip capacity, only two biological replicates were used in this experiment, making it more difficult to show significance for subtle changes.

A.

Term	Count	Genes	P Value
GO:0006412~translation	65	RPL17, RPL36A, RPL14, RPL13, RPL22L1, RPS2, RPS3, EIF4EBP2, RPS3A, RPLP0, RPLP1, SLC25A3, RPL10, RPL11, RPL12, SLC25A5, SLC25A6, RPS4X, RPS18, RPS19, RPS16, RPS17, RPS14, RPS12, RPS13, UBA52, RPS15A, RPL36, RPL39, RPL13AP3, RPS25, RPL10L, RPS27, RPL30, RPL32, RPL7, RPL6, RPL31, RPL34, RPL9, RPL8, RPL3, RPL5, RPS20, RPL7A, RPL10A, RPS24, RPSA, RPL26, RPS9, RPL27, RPL23A, RPL24, RPS6, RPS5, ZNF525, RPL28, RPS8, ANKRD42, RPS7, RPL23, RPL13A, RPL22, RPL21, RPL37A	2.36E-61
GO:0005840~ribosome	53	RPL36A, RPL14, RPL13, RPL22L1, RPS2, RPS3, RPS3A, RPLP0, RPLP1, RPL10, RPL11, RPS4X, RPS18, RPS19, RPS16, RPS17, RPS14, RPS13, UBA52, RPL36, RPS15A, RPL13AP3, RPS25, RPL30, RPS27, RPL32, RPL7, RPL6, RPL31, RPL34, RPL9, RPL8, RPL3, RPL5, RPL7A, RPL10A, RPS20, RPS24, RPS9, RPL27, RPL23A, RPL24, RPS6, RPS8, ANKRD42, RPL28, ZNF525, RPS7, RPL23, RPL22, RPL13A, RPL21, RPL37A	1.88E-56
GO:0098609~cell-cell adhesion	24	ALDOA, HSP90AB1, RPL14, RAN, PFKP, OLA1, RPL24, RPL23A, HSPA1A, EEF2, RPS2, KRT18, PRDX6, RPL6, SH3GLB1, UBFD1, SND1, RARS, RPL34, EEF1G, CNN2, RPL7A, HSPA8, SEPT9	1.91E-11
GO:0005524~ATP binding	26	HSP90AB1, HSP90AA1, ASS1, MKNK2, PFKP, OLA1, CCT2, HSPA1A, ASNS, QARS, ATP1A1, TRIB1, KIF1C, HSPH1, NME2, DDX28, CCT4, CSNK1E, RARS, EIF4A1, LARS, PEBP1, MYO19, HSPD1, PGK1, HSPA8	0.304992
GO:0006355~regulation of transcription, DNA-templated	16	E2F1, EEF1A1, EFEMP1, YBX3, ZNF525, RPS3, ZFP36L2, NME2, HDAC1, RPL6, SND1, PQBP1, CSDE1, TCF3, MCTS1, ZNF768	0.968299
GO:0006351~transcription, DNA-templated	19	E2F1, EEF1A1, NACC2, YBX3, FHL2, ZNF525, RPS3, NME2, PA2G4, AES, HDAC1, NCOA4, SND1, PQBP1, TDG, KLF2, TCF3, MCTS1, HSPA8	0.993057

B.

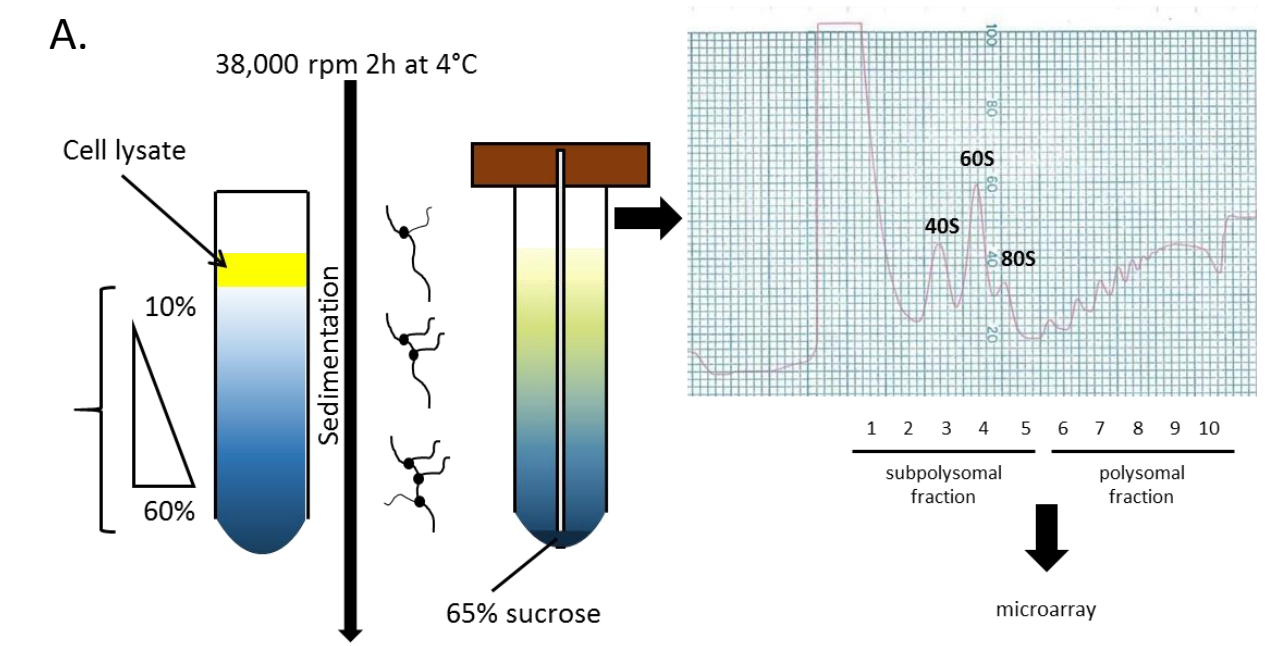
Term	Count	Genes	P Value
GO:0005743~mitochondrial inner membrane	19	UQCRC2, SQRDL, SHMT2, LGALS3, SLC25A5, SLC25A6, ATP5F1, COX5A, NDUFA10, VDAC2, CPT1A, TST, GOT2, GHITM, MTCH1, CSDE1, SLC25A3, HSPD1, HADH	0.000001
GO:0098609~cell-cell adhesion	14	HSP90AB1, ALDOA, PFKP, OLA1, HSPA1A, KRT18, SH3GLB1, UBFD1, PRDX6, RARS, SND1, EEF1G, CNN2, HSPA8	0.000009
GO:0003743~translation initiation factor activity	7	EIF3C, EIF3D, EIF4EBP2, EIF3H, EIF4A1, EIF1, EIF3M	0.000052
GO:0006094~gluconeogenesis	6	ALDOA, GOT2, PGAM4, PGK1, GAPDH, MDH1	0.000112

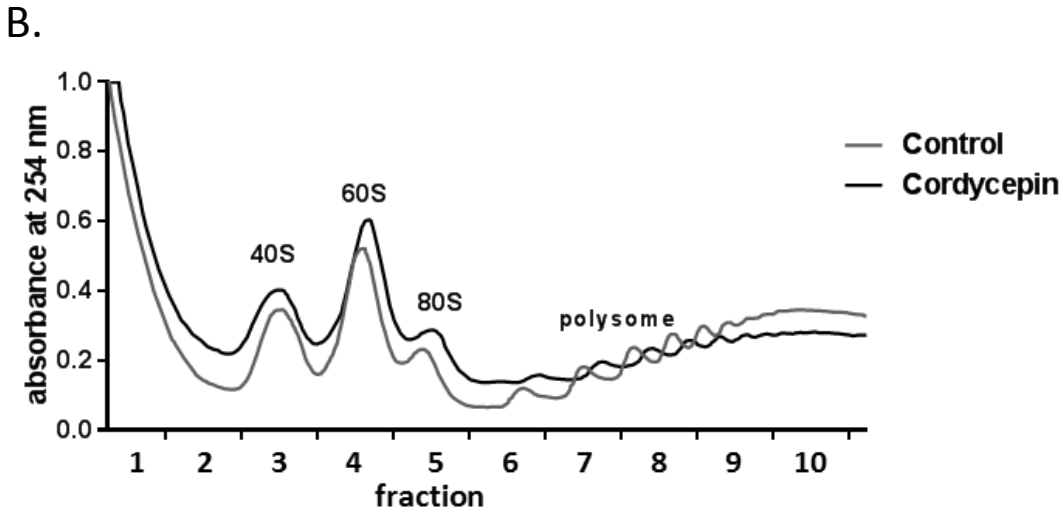
C.

Term	Count	Genes	P Value
GO:0003677~DNA binding	16	POLR2F, HIST1H2BC, CCNT1, TRIM26, RAD54L, ZNF2, ZNF157, TAF13, APP, CDKN2A, ZNF326, HIST2H2BE, HIST1H2BJ, TOP2B, HIST1H2AL, NCOR1	0.001027
GO:0006351~transcription, DNA-templated	9	ZNF157, POLR2F, CDKN2A, ZNF326, PRMT6, CCNT1, AGO2, URI1, ZNF2	0.398755

Table 6 GO Analysis for the Significantly Down-regulated Genes in Cordycepin Treated MDA-MB-231 Fluc Cells

According to the polysome profiling microarray result, significantly regulated genes were put into DAVID (V6.8) for functional annotation bioinformatics microarray analysis. A. Genes with translation efficiency down-regulated by cordycepin were sorted by the level of change in an ascending order, from which 534 genes were chosen ($P<0.01$). 278 of them were recognised and annotated by the system. B. 297 non-TOP genes were filtered out from the A list, with 210 genes being recognised and annotated by the system. C. Genes with translation efficiency up-regulation by cordycepin were sorted by the level of change in a descending order, from which 105 genes were chosen ($P<0.01$). 84 genes were recognised and annotated by the system. The terms shown here were chosen because they have the lowest P values and/or were shown affected by cordycepin in previous analysis.





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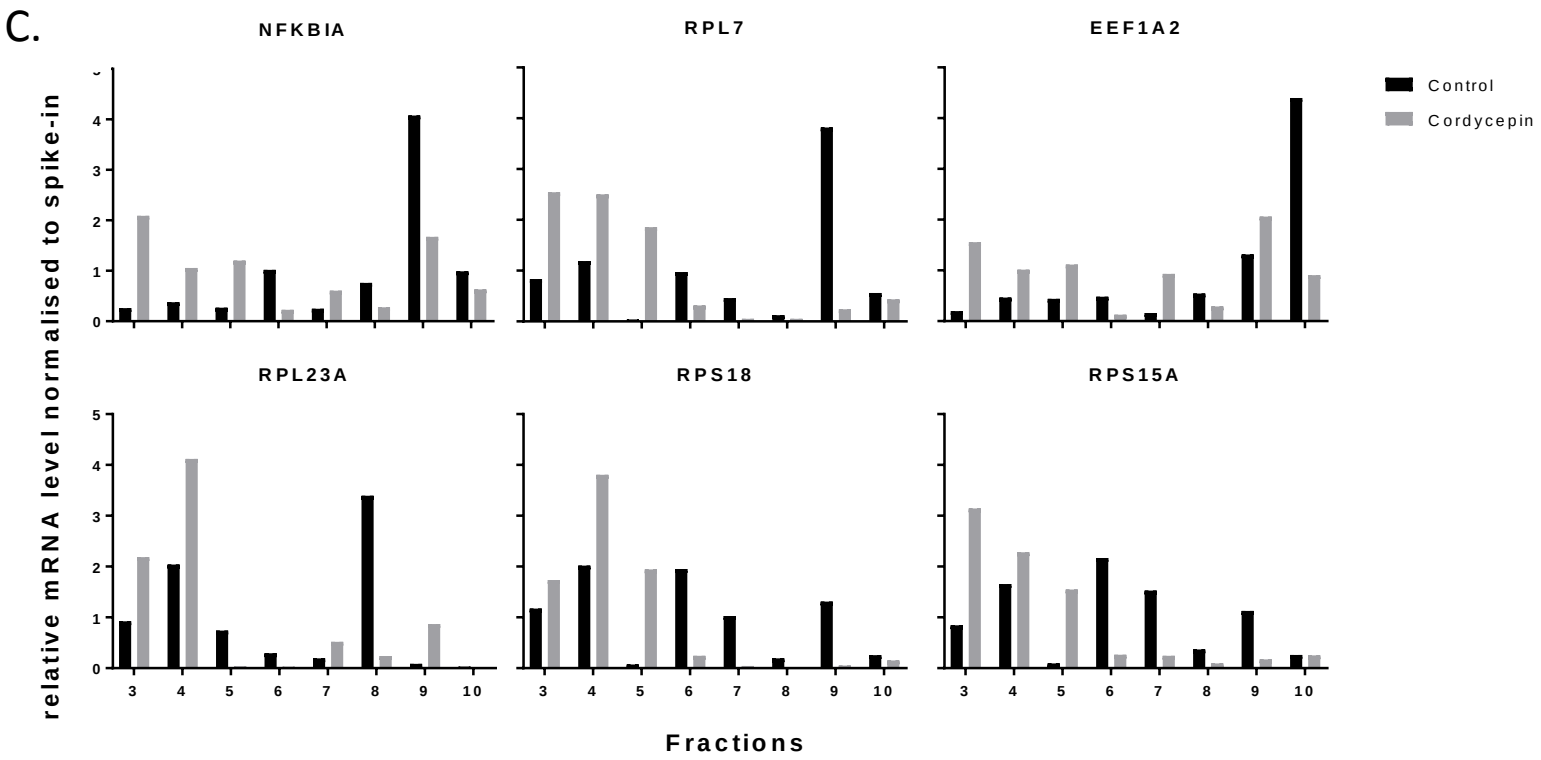

Figure 6.1 Cordycepin Inhibits the Translation of Ribosomal Proteins

A. A schematic view for the design of polysome profiling.

B. Polysome profiles of control cells and cells treated with 50 µM cordycepin for 30 min. *40S* and *60S* indicate the dissociated free ribosomal subunit peaks; *80S* indicates the RNA with only one ribosome binding; *polysome* indicates mRNA with two or more than two ribosomes binding. The presented graph represents 1 of the 3 biological replicates.

C. Relative mRNA level of selected genes in different fractions from the first replicate were measured with RT-qPCR and presented as the ratio to the level in DMSO control. Ct values were analysed using the $2^{-\Delta\Delta Ct}$ method and normalised to the Ct values of yeast Act1. Control level is coloured in black and cordycepin treated in grey. The presented graphs are from the analysis of one of the 2 biological replicates.

RPL7, RPL23A, RPS18, RPS15A, EFF1A2 and NFKBIA were down-regulated by cordycepin according to the microarray result, of which NFKBIA is the most down-regulated gene and the rest are TOP mRNAs. RT-qPCR was performed to measure the relative mRNA level of these genes in each fraction. Because the level of mRNA associated with different number of ribosomes is unknown, even the level of common reference genes varies. For this reason, we spiked in each fraction the same volume of *Schizosaccharomyces pombe* RNA before reverse transcription, so that a relatively stable level of yeast Act1 can be used for normalisation. As expected, the administration of cordycepin caused repression to the level of polysomal mRNA, while mRNA level in subpolysomal fractions was induced (Figure 6.1C). This shift confirms the translational down-regulating effect as shown in the microarray result.

The MYC oncogene has been a gene of our interest, not only because it is involvement in many aspects of cellular biology, but the expression of MYC mRNA was consistently inhibited by cordycepin in experiments in many different cell lines (Figure 3.3, 3.4C and Table 4.2). In HeLa cells, the decline in MYC mRNA level did not result in relative protein level change either (Ioannidis et al. 1999). This observation was then validated by MYC western blotting, in which no obvious change was revealed by compared to Vinculin loading control (Figure 6.2). While the effect of mTOR inhibition potentially affects cap-dependent translation initiation via 4E-BP1, the existence of an internal ribosome entry site suggests an evading mechanism for the binding of ribosome to MYC mRNA and eventually translation (Evans et al. 2003).

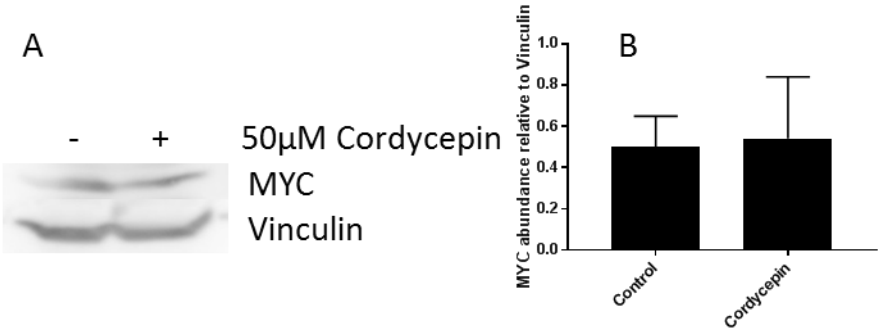

Figure 6.2 MYC Protein Level Is Not Sensitive to Cordycepin

A. MCF-7 cells were treated with 50 μM cordycepin for 2 hrs before harvest. Cells collected were lysed and the lysate was analysed with western blot to measure the level of MYC. Vinculin was used as loading control. The picture shown here represents 1 of the 3 biological replicates generated. B. Quantification of the blots was performed as described in 2.4. The graph represents the average MYC/Vinculin ratio of the triplicate. (mean ± SD, n=3 independent experiments)

Discussion

In this chapter, a high salt extraction and gradients were used for this polysome analysis, which would cause non-translating ribosomes to fall apart. Therefore, all mono-ribosomes in this profile represent mRNAs with one active translating ribosome (Spriggs et al. 2008; Moreno et al. 2012). Due to limited capacity of the chip, this polysome profiling was done only in duplicate. Because of this, it is possible that some of the results are false positive, as well as that some relatively smaller changes are not statistically significant when tested more stringently in an increased number of replicates.

Previous research into the effect of poly(A) tail indicates that translation initiation is the step that the poly(A) tail is most likely to be modulating (reviewed in Jackson & Standart 1990). Having seen signal transduction being regulated by cordycepin, it is thus within expectation that the translation efficiency of signal transduction members is changed in this polysome result. However, major signal transduction effectors were not listed as significantly regulated in the level of ribosomes associated, suggesting their translation efficiency was not altered.

Collectively, in this chapter, I investigated the translation efficiency in MCF-7 cells in response to the addition of cordycepin using polysome profiling. Most of the genes being translationally down-regulated by cordycepin are TOP genes. It appears that rather than causing the effects on signal transduction, the predominant changes in translation are due to early effects on signal transduction. The TOP mRNA translation, which is regulated by mTOR pathway, was significantly inhibited by cordycepin. This again indicates that mTOR pathway is affected by cordycepin within half an hour of treatment.

7 Comparison with Signal Transduction Inhibitors and Activators

Introduction

Over the years, various reports have proposed cordycepin could work as an AKT inhibitor (J Y Yoon et al. 2015), PARP inhibitor (Kim et al. 2011) or AMPK activator (C. Wu et al. 2014). From the western blotting data, we have observed increases in P-AMPKα level following cordycepin treatment in MCF-7 cells, as well as the dephosphorylation of P-AKT(Ser473) (Figure 3.2), which is consistent to the papers above. In a previous project from the De Moor lab, rapamycin was used to compare the mTOR inhibition with cordycepin, which suggests the role of mTOR as the key mediator in the protein synthesis repression by cordycepin (Wong et al. 2010). Having discussed in chapter 6, the advent of TOP mRNA as the most translationally down-regulated genes by cordycepin, once again, cast a strong indication that mTOR signaling is inhibited by cordycepin. With MCF-7 cells, AMPK phosphorylation and AKT dephosphorylation remain two of the most promising and consistent effects of cordycepin in signal transduction. PIK3CA is mutated to be, presumably, constitutively active in MCF-7 cells leading to continuous downstream signalling (Crowder et al. 2009; Hollestelle et al. 2010). Cordycepin represses this programmed activation to dephosphorylate AKT, leading us to the hypothesis that PI3K-PDK1-AKT signalling is targeted by cordycepin.

In an attempt to address all the reports from the literature and our western observations, different inhibitors and activators were introduced to obtain the inhibition on AKT, PARP, ERK, mTOR, PI3K and activation in AMPK, such that the effect of cordycepin on signal transduction and gene expression can be compared.

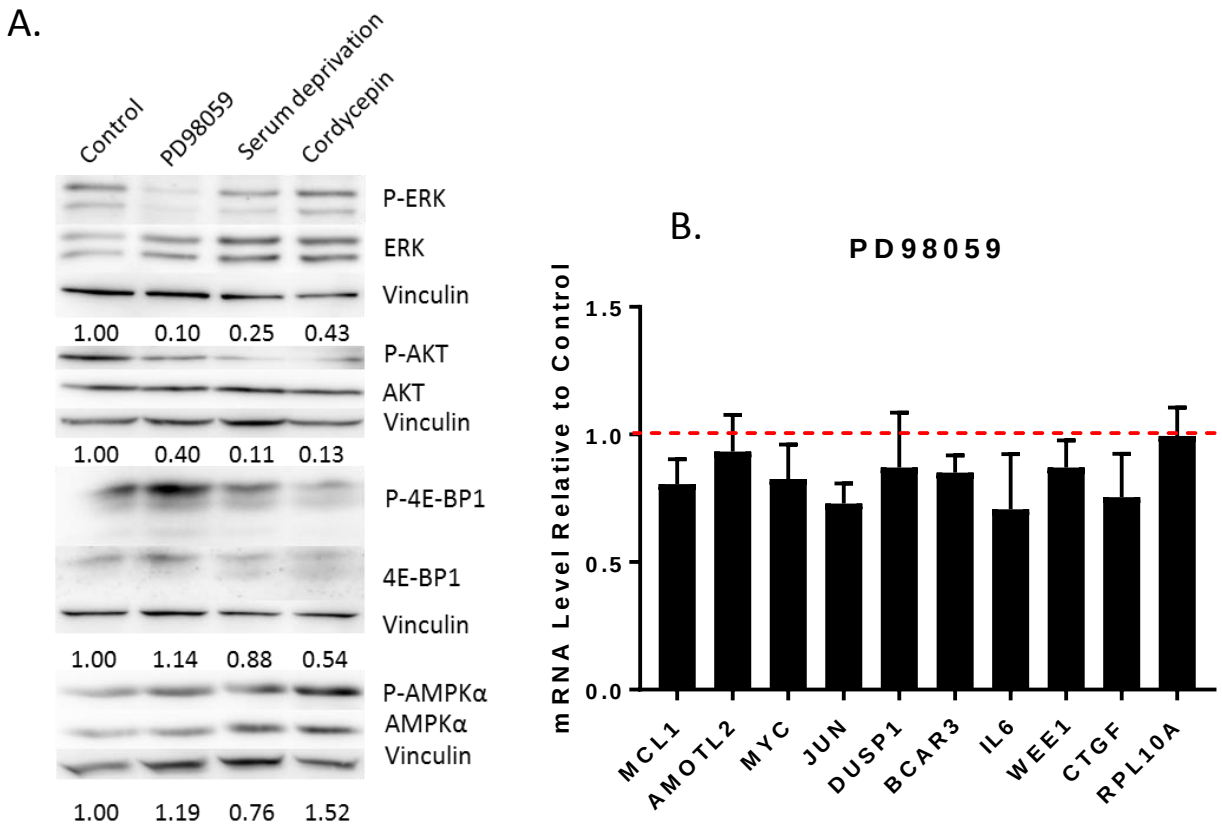

Figure 7.1 Comparison with PD98059

A. 4 hrs before collection, medium in serum deprivation sample was replaced with serum withdrawal medium. 2 hrs before collection, 15 µM PD98059 (MEK inhibitor) or 50 µM cordycepin was added to relative samples respectively. Cells collected were lysed and analysed with western blotting for indicated proteins. Vinculin was used as loading control. Quantification of the blots was performed as described in 2.4. The numbers below each panel represent the ratio of phosphorylated over total protein level, normalised to control. The picture shown here represents 1 of the 3 biological replicates generated.

B. MCF-7 cells were treated with PD98059 (MEK inhibitor) 2 hrs before harvest and lysed. Relative RNA was extracted and reverse transcribed into cDNA. mRNA levels for selected genes were determined by RT-qPCR and analysed using the $2^{-\Delta\Delta Ct}$ method, normalising to the Ct values of GAPDH. The result is presented as the ratio to the levels in control (red dashed line). (mean ± SD, $3 \leq n \geq 8$ independent experiments; *P<0.05, **P<0.01 and ***P<0.005 compared with untreated control; p values were obtained using Student's t test).

7.1 Comparison with PD98059

Both *in vivo* and *in vitro* study has provided evidence that dephosphorylated MEK1 and the activation by RAF or MEKK are inhibited by PD98059 with high selectivity (Alessi et al. 1995; Dudley et al. 1995). To confirm if PD98059 is inhibiting MEK activity in MCF-7, western blotting was performed for the phosphorylation of the downstream target ERK. Indeed, hypophosphorylation of ERK was observed upon PD98059 addition, which is similar to serum deprivation and cordycepin (Figure 7.1A). Despite the fact that dephosphorylated AKT was seen with both PD98059 and cordycepin, the ratio of P-4E-BP1/4E-BP1 and P-AMPKα/AMPKα with PD98059 did not change much in PD95059 group, while cordycepin consistently caused 4E-BP1 dephosphorylation and AMPKα phosphorylation.

Together with RPL10A, which serves as a control gene, mRNAs that showed repression following cordycepin treatment were determined with RT-qPCR to compare the effect on gene expression between samples. While it is within expectation for the level of IEGs, MYC and JUN, to be restricted as a result of the inhibition on the RAF-MEK-ERK signalling, the rest of the cordycepin sensitive genes were all down-regulated by PD98059 to a relatively mild level, comparing to cordycepin (Figure 7.1B).

7.2 Comparison with MK2206, Torin1 and NU1025

MK2206, Torin1 and NU1025 were employed to suppress the activity of AKT, mTOR and PARP respectively. MK2206 exhibits pan-AKT inhibitory effect on all three homologous AKT isoforms allosterically, and this requires the presence of Pleckstrin homology domain (PHD) (Hirai et al. 2010; Yan 2009). As mentioned, rapamycin was used in a previous project to obtain mTOR inhibition. Although prolonged exposure to rapamycin leads to the inhibition of mTORC2 assembly (Sarbassov et al. 2006), short-term treatment with rapamycin has higher selectivity for mTORC1 (Jacinto et al. 2004). Hence we used Torin1, which has been reported to inhibit phosphorylation of substrates of

mTORC1 and mTORC2 at single-digit nanomolar concentrations, 2 nM and 10 nM respectively, with high selectivity (Thoreen et al. 2009; Liu et al. 2010). PARP is inhibited by NU1025, which shows potentiation in the cytotoxicity of anti-cancer agents (White et al. 2000; Boulton et al. 1995; Delaney et al. 2000).

Western blotting was also conducted to validate the efficacy of MK2206 and Torin1. As mTORC2 phosphorylates AKT at Ser473 (Liu et al. 2010; Sarbassov et al. 2005), the down-regulated P-AKT(Ser473) level confirms the efficacy of Torin1 as a mTOR inhibitor (Figure 7.2A). Unlike the down-regulation in cordycepin group, a majority of the cordycepin repressed genes were elevated in mRNA level following MK2206 treatment, while MCL1, AMOTL2 and RPL10A remained relatively unaltered. Also, various mRNA expressions were seen with Torin1 and NU1025 administered MCF-7, in which predominant up-regulation occurred to these mRNAs rather than down-regulation as with cordycepin.

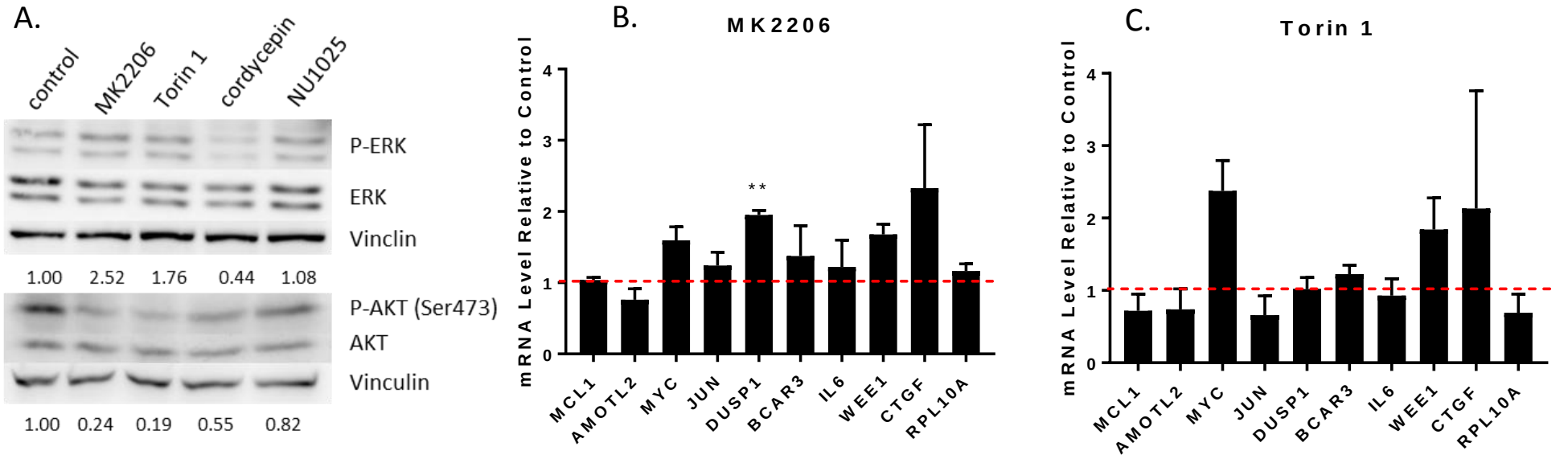

Figure 7.2 Comparison with MK2206, Torin1 and NU1025

A. 2 hrs before collection, MCF-7 cells were treated with 5 µM MEK inhibitor (MK2206), 250 nM mTOR inhibitor (Torin1), 10 µM PARP inhibitor (NU1025) and 50 µM cordycepin accordingly. Cells collected were lysed and analysed with western blotting for indicated proteins. Vinculin was used as loading control. Quantification of the blots was performed as described in 2.4. The numbers below each panel represent the ratio of phosphorylated over total protein level, normalised to control. The picture shown here represents 1 of the 3 biological replicates generated.

B. C. D. MCF-7 cells were treated as described in *A*. Cells were then collected and lysed. Relative RNA was extracted and transcribed into cDNA. mRNA levels for selected genes were determined by RT-qPCR and analysed using the $2^{-\Delta\Delta Ct}$ method, normalising to the Ct values of GAPDH. The result is presented as the ratio to the levels in control (red dashed line). (mean ± SD, n = 3 independent experiments; *P<0.05, **P<0.01 and ***P<0.005 compared with untreated control; p values were obtained using Dunnett's test).

7.3 Comparison with Pictilisib and A769662

Pictilisib was used to inhibit PI3K activity and A769662 was used to activate AMPK. Identified in 2008, Pictilisib is a potent pan-PI3K inhibitor with effects on all p110 isoforms (Folkes et al. 2008). Apart from the similarity with AMP in inhibiting the dephosphorylation of Thr172, A769662 directly activates AMPK allosterically in a β1 subunit-dependent manner (Matthew J. Sanders et al. 2007; Scott et al. 2008; Cool et al. 2006).

Consistent with our previous western blotting data (Figure 3.2), cordycepin up-regulated AMPK phosphorylation, which is also shown with A769662 (Figure 7.3A). After phosphorylation by PI3K, downstream 3-phosphoinositide dependent protein kinase 1 (PDK1) phosphorylates AKT at Thr308 (Stephens et al. 1998; Alessi et al. 1997), one of the two phosphorylations required for the complete activation of AKT (Andjelkovic et al. 1997; Bellacosa et al. 1998; I Vivanco & Sawyers 2002). With the phosphorylation of AKT at Thr308 being down-regulated, it is indicated that PI3K was indeed inhibited by Pictilisib (Figure 7.3A). Since no P-AKT(Thr308) detection was tried prior to this western blotting, we were surprised to see this phosphorylation was also repressed by cordycepin (Figure 7.3A), which suggests an effect upstream of the PI3K-PDK1-AKT axis. Moreover, the addition of Pictilisib mimicked all the effect of cordycepin observed on signal transduction, which down-regulates P-ERK, P-4E-BP1 and P-AKT (both Ser473 and Thr308) and promotes the phosphorylation of MEK and AMPK. Despite the activation of AMPK and deactivation of AKT (Ser473), A769662 caused ERK phosphorylation and slight dephosphorylation in MEK, separating itself from cordycepin in terms of signal transduction regulation.

Next we examined whether Pictilisib or A769662 could also mimic the alteration on gene expression by cordycepin with RT-qPCR. Unlike cordycepin, mRNA levels were mostly up-regulated by A769662, if not unchanged (Figure 7.3C). However, down-

regulation was seen with MCL1, AMOTL2, MYC, DUSP1 and CTGF in Pictilisib samples, which correlates with the effects of cordycepin (Figure 7.3B). Rather than the high selectivity for class IA PI3K, LY294002, another pan-PI3K inhibitor, was reported to have direct inhibitory effect on not only p110α and p110δ, but class III PI3K, phosphatidylinositol 4-kinase (PI4K) and other targets (Gharbi et al. 2007; Folkes et al. 2008). Application of LY294002 did down-regulate BCAR3 and PLK2, two mRNAs that down-regulated by cordycepin (Figure 7.3D), but failed to affect MYC level, which remains as one of the most important target of cordycepin for its oncological properties and immediate early response. While repressed in Pictilisib and cordycepin, DUSP1 was induced by LY294002.

Discussion

So far, Pictilisib is the only inhibitor to regulate signal transduction and gene expression in a similar manner to cordycepin. Both PD98059 and Pictilisib reduced the mRNA level of the cordycepin sensitive genes. For all the transcription factors regulated by the RAF-MEK-ERK signalling, it is believed that MEK inhibitor, in this case PD98059, act on the transcriptional stage of gene expression (reviewed in Whitmarsh 2007; Junttila et al. 2008; Chang and Karin 2001). For instance, the heterodimer of MYC-MAX (short for myc-associated factor X) has a high affinity against a specific CACGTG sequence of the target DNA, to which the subsequent binding of this heterodimer is required for transcription activation (Kretzner et al. 1992; Amati et al. 1993; Gu et al. 1993). As transcription factors were also significantly down-regulated upon cordycepin administration (Figure 3.3), we speculate whether cordycepin might regulate gene expression at the stage of transcription as well. While it is known that cleavage was affected by cordycepin (Figure 3.4), the fact that shortened poly(A) tail can affected mRNA stability correlates with the long-standing role of cordycepin as a polyadenylation inhibitor, suggesting that cordycepin can affects mRNA level at post-transcriptional

stage in a polyadenylation-dependent manner (see 1.4.4.4)(Holbein et al. 2008; Kondrashov et al. 2012). This distinguishes cordycepin from MEK inhibitors regarding gene expression.

As the carboxyl terminus of mTOR is homologous to the PI3K catalytic domain, mTOR is also a member of the phosphatidylinositol 3-kinase-related kinases (PIKK) family, in which p110α, p110β, p110γ and p110δ are known as Class I and mTOR as Class IV (reviewed in Bjornsti and Houghton 2004; Shuttleworth et al. 2011). For this reason, inhibitors targeting PI3K or mTOR would be tested against the other. In discovering Pictilisib as a PI3K inhibitor, Folkes et al. did test the effect against mTOR, where they found it needed 580 nM of Pictilisib to reach half maximal inhibitory effect (Folkes et al. 2008). However, in this project, mTOR activity was measured indirectly through the phosphorylation of 4E-BP1, which ended up with an apparent inhibitory constant (K_{iapp}) instead of an IC_{50} (Folkes et al. 2008). While a relative high concentration of Pictilisib, 500 nM, was used in our experiment, it is possible that Pictilisib is inhibiting PI3K and mTOR. Indeed, mTORC2 phosphorylation of AKT at Thr473 was strongly repressed by Pictilisib. Because mTORC2 is not a direct target of AKT, this suggests the mTOR inhibition by Pictilisib. In addition, Torin1 administration did not have the same effect as cordycepin or Pictilisib (Figure 7.2A and 7.3A), indicating the possible dual effect of cordycepin and Pictilisib on mTOR and PI3K signaling. Although it failed to down-regulated the cordycepin-sensitive genes, it would be of interest to see what LY294002, another PI3K inhibitor, does to signal transduction. For the first time, Thr308 phosphorylation of AKT was shown to be down-regulated by cordycepin, implicating the potential inhibitory effect of cordycepin in the PI3K-PDK1-PI3K signalling. If cordycepin, like PI3K inhibitor, inhibits PI3K activity, the possibility of cordycepin inhibiting mTORC2 will be able to explain the dephosphorylation at Ser473 of AKT appropriately.

In chapter 3, we learnt that cordycepin acts on signal transduction and gene expression almost concurrently, 15 mins after treated with cordycepin. While 15 mins is sufficient for the mTOR pathway or RAF-MEK-ERK signalling to regulate gene expression in response to direct stimuli, mRNAs whose level were affected by cordycepin would not be capable of producing significant protein levels in such a short time frame. Proto-oncogene FOS (also known for its old name c-FOS) is one of the IEGs in mammalian cells, mRNA induction of which was seen to reach the peak level as soon as 15 mins after quiescent NIH3T3 cells were stimulated with serum (Greenberg et al. 1986; Greenberg & Ziff 1984). Yet, mRNA has to be exported and translated (Fos needs to translocate to the nucleus to enable its transcriptional regulatory property), which makes it unlikely that cordycepin is regulating signal transduction in this way. This is why in this series of comparisons, western blotting was performed to investigate if small molecule modulators of ERK, AMPK, AKT, mTOR and PI3K signaling are altered by cordycepin, followed by RT-qPCR to testify the correlation in gene regulation. In conclusion, the effects of cordycepin are consistent with PI3K inhibition on signal transduction, perhaps in combination with mTOR inhibition.

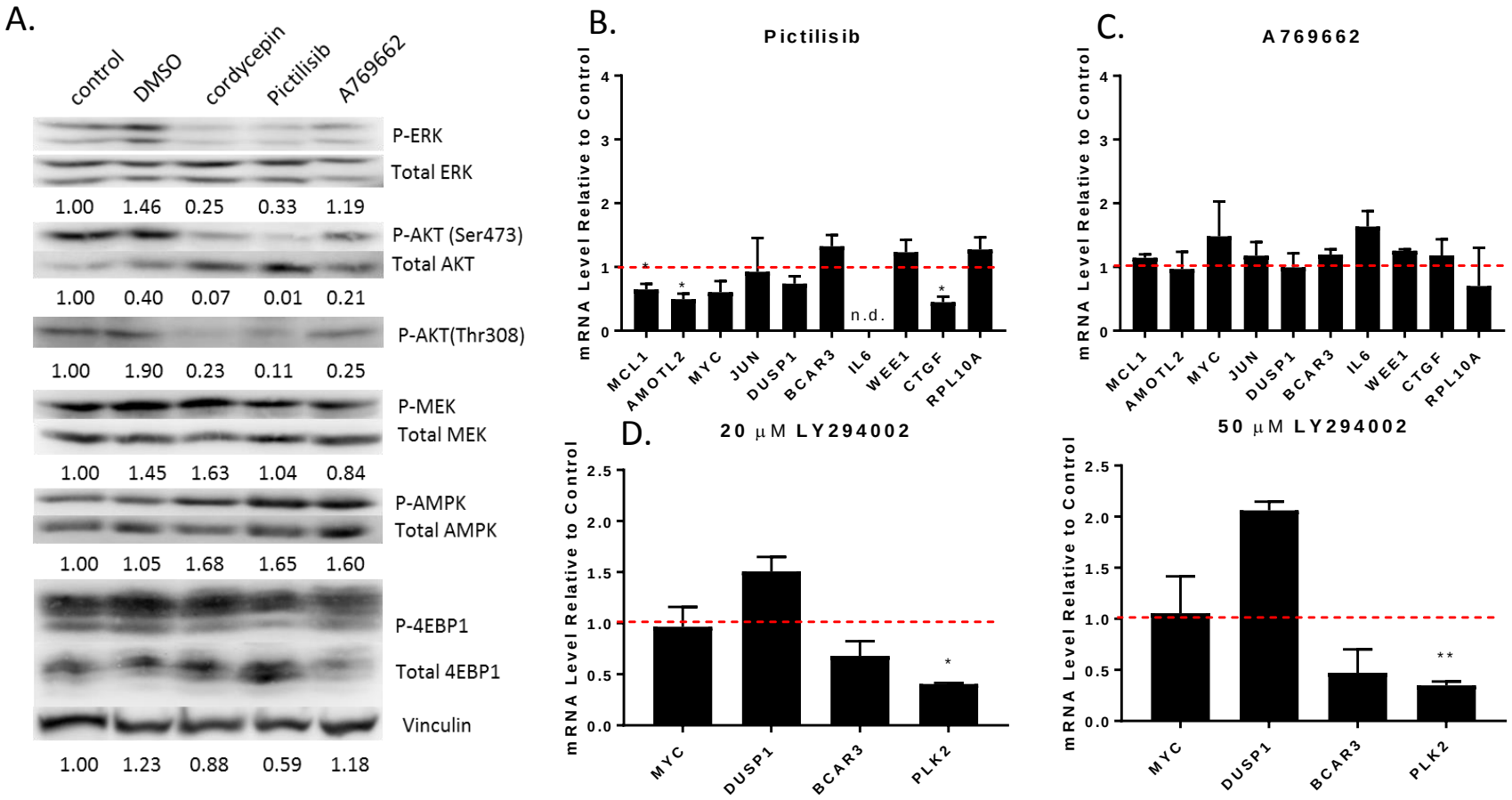

Figure 7.3 Comparison with Pictilisib and A769662

A. 2 hrs before collection, MCF-7 cells were treated with DSMO, 50 µM cordycepin, 500 nM Pictilisib and 10 µM A769662 accordingly. Cells collected were lysed and analysed with western blotting for indicated proteins. Vinculin was used as loading control. Quantification of the blots was performed as described in 2.4. The numbers below each panel represent the ratio of phosphorylated over total protein level, normalised to control. The picture shown here represents 1 of the 3 biological replicates generated.

B. C. MCF-7 cells were treated as described in *A*. D. MCF-7 cells were treated with 20 and 50 µM of LY294002. Cells were then collected and lysed Relative RNA was extracted and reverse transcribed into cDNA. mRNA levels for selected genes were determined by RT-qPCR and analysed using the $2^{-\Delta\Delta Ct}$ method, normalising to the Ct values of GAPDH. The result is presented as the ratio to the levels in control (red dashed line). (mean ± SD, n = 3 independent experiments; *P<0.05, **P<0.01 and ***P<0.005 compared with untreated control; p values were obtained using Dunnett's test).

8 Discussion

The work described here demonstrates that cordycepin affects cancer cell proliferation and survival in three breast cancer cell lines. Further investigation on molecular level uncovered that signal transduction, gene expression and translation efficiency are among the processes affected by cordycepin. Growth factor responses were repressed by cordycepin, including growth factor dependent signal transduction and gene expression. Microarray analysis of cordycepin-administrated MCF-7 cells by a previous lab member has revealed that down-regulated genes are predominantly growth-related transcription factors, which is consistent with the high-throughput sequencing data from cordycepin treated MDA-MB231 Fluc cells, indicating that this gene expression effect of cordycepin is not cell type-specific.

RT-qPCR analysis of cordycepin down-regulated genes showed that cordycepin induces a defect in 3' processing of the mRNAs. Non-coding RNAs, especially lncRNAs and pseudogenes, displayed increased mRNA expression in response to cordycepin. It appears likely that these ncRNAs are degraded by a poly(A) polymerase (PAP) dependent RNA decay pathway (Bresson et al. 2015). In agreement with this, ncRNAs up-regulated by cordycepin also appeared up-regulated in PAP knockdown samples, confirming: i) these ncRNAs are subjected to polyadenylation-dependent degradation; ii) the long-known role of cordycepin as a polyadenylation inhibitor.

Because both signal transduction and gene expression are affected by cordycepin at roughly the same time, polysome profiling was performed to determine if cordycepin directly affects translation efficiency. This polysome profiling, although failed to characterise a key coordinator between signal transduction and gene expression, characterised a large number of ribosomal protein/TOP mRNAs as the most translationally inhibited genes. Because TOP mRNAs are known to be regulated by

mTOR signaling (Hong et al. 2017; Tcherkezian et al. 2014; Lahr et al. 2017; Thoreen et al. 2012), this result once again indicates that mTOR signaling is inhibited upon cordycepin treatment. With the help of different inhibitors/activator, PI3K and AMPK signaling have been identified as the most upstream targets of cordycepin in signal transduction.

8.1 Signal Transduction or Gene Expression?

So far, we have seen effects of cordycepin in signal transduction, inhibiting on PI3K signaling, mTOR signaling, ERK phosphorylation and inducing phosphorylation on AMPK activation site (Figure 3.2), among which the inhibition on PI3K signaling and AMPK activation has remained the most upstream effects of cordycepin.

For AMPK, our lab has shown that its activation seems to be upstream of mTOR, because the application of compound C, a potent AMPK inhibitor, rescued the inhibition on mTOR (Wong et al. 2010). As mentioned in 1.4.3.3, a previous project has detected surprisingly up-regulated ATP level, steady level of ADP and AMP 2 hrs after cordycepin treatment (Wahyu Utami, unpublished data), which, by default, should have inactivated AMPK. A possibility is the potential crosstalk between PI3K signaling and AMPK activity. Whilst low ATP level can promote the activation of AMPK, negative regulation of AMPK was also found with glycogen synthase kinase 3 (GSK3) and E2F transcription factor 1 (E2F1), substrates of AKT (Hallstrom & Nevins 2003; Suzuki et al. 2013; Hallstrom et al. 2008). Hence, cordycepin could be activating AMPK via the repression on PI3K-PDK1-AKT axis, which can also explain the AMPK activating effect by pictilisib, a potent PI3K inhibitor (Figure 7.3A).

Solute carrier family 25 member 25 (SLC25A25/MCSC) encodes a Ca^{2+}-sensitive ATP-Mg^{2+}/Pi carrier, which is located on the inner mitochondrial membrane (Del Arco & Satrústegui 2004; Fiermonte et al. 2004; Mashima et al. 2003). By controlling a Ca^{2+}-

regulated shuttle of ATP-Mg^{2+} and Pi across the inner mitochondrial membrane, SLC25A25 was proposed to play an important role in ATP homeostasis, where its absence was shown to induce intracellular energy inefficiency (Anunciado-Koza et al. 2011). Interestingly, SLC25A25 is also listed as one of the most down-regulated mRNAs in both the MCF-7 microarray and the MDA-MB231 Fluc deep sequencing with cordycepin treatment (data not shown). If by all means, cordycepin regulates SLC25A25 mRNA and hence the protein expression very shortly after administration, the subsequent defect on ATP-Mg^{2+} influx could result in increased $\left[\frac{AMP+ADP}{ATP}\right]$ ratio, which in turn activates AMPK. However, in NIH3T3 cells, detection of the adenosine phosphates revealed elevation of ATP and stable level of AMP and ADP, which should, conversely, deactivate AMPK due to declined $\left[\frac{AMP+ADP}{ATP}\right]$ ratio (Wahyu Utami, unpublished data). Therefore, this hypothesis of cordycepin manipulating ATP-Mg^{2+} influx fails.

Phosphorylation at Ser308 of AKT is inhibited by cordycepin, which is consistent with the effect of pictilisib (Figure 7.3A), indicating that PI3K signaling is interrupted in response to cordycepin. While the potential of cordycepin directly regulating PI3K signaling remains, its repression on mTOR could be positively regulating PI3K signaling through the mTORC1/S6K1/IRS1 feedback loop, in which mTORC1 activates S6K1 and S6K1 negatively phosphorylates insulin receptor substrate 1 (IRS1), a PI3K adaptor, leading to IRS1 degradation (Carracedo et al. 2008; Harrington et al. 2004; Zhang et al. 2008; Hsu et al. 2011). Moreover, IRS1 interacts with GRB2 to form the adaptor complex, transferring the growth factor stimulation from RTKs to ERK signaling (Myers et al. 1994). As MCF-7 cells express IRS1 (Human Protein Atlas), if cordycepin is an mTOR inhibitor, the subsequent reduced S6K1 activity would increase IRS1 activity. This

cannot explain the dephosphorylation of AKT at Ser308, implicating that PI3K signaling interruption is more possible the path cordycepin is affecting.

It is now evident that, after breast cancer cells were treated with cordycepin, both signal transduction and the expression of growth factor-dependent genes are regulated. So, is it possible that the regulation of cordycepin on gene expression is due to the effect on signal transduction? A microarray performed by previous lab members has shown that transcription-related genes are among the most down-regulated ones by cordycepin (Table 1.4.3.2 and Figure 5B). Having known that cordycepin negatively regulates the phosphorylation of ERK, it is within expectation that these mRNAs are regulated transcriptionally, as ERK plays an important role in phosphorylating and subsequently activating members of the AP-1 transcription factor complex (see 1.3.1.1). However, with primers specifically designed, RT-qPCR result of the cordycepin-sensitive genes suggests that the down-regulation of these mRNAs are possibly due to the pre-mRNA cleavage defect caused by cordycepin (Figure 3.4B). Although, this result agrees with the observation that 3' processing complex was arrested by cordycepin triphosphate (Ryner & Manley 1987) and the well-known role of cordycepin as a polyadenylation inhibitor (Penman et al. 1970), there is also chance that polyadenylation can be targeted by signal transduction. A proteomic analysis of the 3' processing complex has unveiled that, aside from the conventional components such as CstF, CPSF and PAP, transcription factors are also incorporated within (Shi et al. 2009), connecting ERK signaling with mRNA 3' end processing. Moreover, research into TOP mRNA has identified a 5' TOP motif in PABPC1 (Hornstein et al. 1999; Tcherkezian et al. 2014), demonstrating that mTOR could be regulating the PABPC1-dependent mRNA stability, apart from its established effect on cap-dependent translation initiation. But if this is the case, inhibition of mTOR signaling with Torin1 should inhibit the same group of genes as cordycepin, instead of induction for some genes (Figure 7.2C). ERK and PI3K

inhibitor, PD98059 and pictilisib, are the only two inhibitors that generated declines to all cordycepin down-regulated mRNAs tested (Figure 7.1B and 7.3B). As discussed in chapter 7, ERK inactivation is more likely to affect mRNA level at transcriptional stage rather than post-transcriptional stage, which was observed with cordycepin, therefore distinguishing itself from cordycepin. Since no determination of pre-mRNA in pictilisib samples were done, it is still unknown whether these mRNAs are regulated by PI3K inhibition transcriptionally or post-transcriptionally. Taken together, it is possible that cordycepin-mediated gene expression results from its effect on signal transduction.

CPSF4, together with three other CPSFs, contributes to the formation of the 3' end processing complex. Unlike the conventional understanding in cleavage and polyadenylation, recently studies have discovered new aspects of CPSF4 in regulating tumour cell growth and signal transduction. Firstly, CPSF4 overexpression was found in different cancer cells and knockdown of CPSF4 inhibits cancer cell growth and proliferation (Yi et al. 2016; Tang et al. 2016; N. Zhang et al. 2016; Chen et al. 2013). Moreover, CPSF4 inhibition leads to attenuated PI3K, MAPK and NF-κB signaling (Chen et al. 2013; Yi et al. 2016). These findings open up the possibility that the sequestration of this 3'end processing is entirely responsible for the effects of cordycepin.

To examine the cordycepin inhibitory effect on polyadenylation, the poly(A) tail size of total RNA in cordycepin treated MCF-7 cells was measured by 3' end labelling, from which a clear shortened patent was seen comparing to control (Khurshid, Asma, unpublished data). As introduced in 1.4.4.4, a shortened poly(A) tail endangers mRNA stability and translation initiation in a PABP-dependent manner (Dreyfus & Régnier 2002; Wang et al. 1999; Körner & Wahle 1997; Ross 1995; Bernstein et al. 1989; Mangus et al. 2003). Following transcription, the mature mRNA can be translated into protein by ribosomes. While signal transduction is typically composed of different

proteins, it is an open question if the cordycepin regulated gene expression can conversely lead to changes in signal transduction. As discussed in chapter 7, cordycepin is unlikely to affect signal transduction through the transcription→mRNA processing→mRNA export→translation axis, because the demand of time will exceed the 15 mins needed for signal transduction to be affected (Figure 3.2). Consistently, mTOR, PI3K, MAPK or AMPK-related mRNAs were not among the genes significantly changed in polysome profiling data. In other words, translation efficiency of these mRNAs are not affected either.

8.2 Non-Coding RNA

It was observed in both MCF-7 microarray and MDA-MB-231 Fluc deep sequencing data that there is a widespread up-regulation of non-coding RNAs in response to cordycepin (Figure 5 and table 5.2). With high-throughput deep sequencing, we were able to identify these ncRNAs induced by cordycepin, which accounts for 48.44% of all the significantly induced genes (see Table 5.2). While most of these ncRNAs are lncRNAs (including antisense and pseudogene), the fact that they are both up-regulated in cordycepin treated and serum-deprived samples shows that they are regulated and probably have biological functions (Figure 5). Furthermore, it has been shown that at least some of these lncRNAs are subjected to polyadenylation-dependent decay (Figure 5.3), consistent with previous findings (Bresson et al. 2015; Beaulieu et al. 2012). This result demonstrates that the polyadenylation inhibitory effect of cordycepin is not restricted to mRNA, but also affects lncRNAs.

Compared to the established understanding of small RNAs, such as snRNA, snoRNA, siRNA and miRNA, investigation into lncRNAs is relatively minimal but growing at a fast pace. Perspectives about lncRNAs span from scaffolds for protein-protein and protein-DNA interaction, to oncogenic activity (Yan et al. 2015). A project by Y. Zhang et al. has successfully characterised an lncRNA, lncRNA in non-homologous end joining pathway 1

(LINP1), as a scaffold for repairing DNA double strand breaks in triple negative breast cancer cell lines (Y. Zhang et al. 2016). Similarly, HOX transcript antisense RNA (HOTAIR) links lysine specific demethylase 1 (LSD1) and polycomb repressive complex 2 (PRC2), whilst LSD1 and PRC2 coordinate to alter histone methylation/demethylation, which eventually promotes tumour invasiveness and metastasis (Y. Zhang et al. 2016; Gupta et al. 2010). Therefore, in the case of cordycepin mechanism, one hypothesis is that one/some of the induced lncRNAs can function as a scaffold to regulate signal transduction or specific protein translation.

It has been introduced that PTENP1, as a PTEN pseudogene, shares a piece of 3' UTR with PTEN and functions as a sponge to distract the miRNA-mediated translation regulation (see 1.3.2.5). In this way, the existence of PTENP1 assures the normal function of tumour suppressor PTEN (Poliseno et al. 2010). Following this logic, we speculate whether the lncRNAs/pseudogenes regulated by cordycepin can act as decoy to enhance the function of signal transduction-related protein, thus linking gene expression to protein regulation in a relatively quicker manner. Moreover, if these lncRNAs or pseudogenes are under the regulation of polyadenylation, the inhibitory effect of cordycepin on polyadenylation and subsequently the lncRNA/pseudogene stability could explain the change in signal transduction.

Interestingly, among all these ncRNAs induced by cordycepin, there is a snRNA, RNA U1 small nuclear 1 (RNU1-1, also known as U1), standing out as the only snRNA being significantly up-regulated (log2foldchange 1.41, Table 5.2). U1 contributes to spliceosome formation (see 1.3.2.1). However, the abundance of U1 far exceeds the need for spliceosomes, suggesting a splicing-independent function of U1 snRNA (Kaida et al. 2010). Investigations in different models have shown that U1, in the form of snRNP with U1 70K and/or U1A proteins, prevents premature cleavage, polyadenylation

and shortened 3' UTR (Wassarman & Steitz 1993; Vagner et al. 2000; Kaida et al. 2010; Berg et al. 2012). Considering that the widespread short 3' UTRs is a characteristic of cancer and proliferative cells, as a mechanism to avoid miRNA regulation and produce more protein (Sandberg et al. 2008; Mayr & Bartel 2009), the elevation of U1 snRNA by cordycepin therefore could have anti-tumour activity through this mechanism. In yeast, exposure to cordycepin stimulated RNA 3' end extension, which theoretically, should increase the chance of RNA being regulated through a 3' end-dependent manner, correlating with the U1 induction above (Holbein et al. 2009). Nevertheless, this mechanism would take some time to effect, as the U1-affected transcripts should be freshly transcribed, and the existing transcripts, presumably with shorter 3' UTRs, will need to be degraded. Furthermore, because of this elevation, it is expected that factors involved in U1 biogenesis were up-regulated in the microarray/deep sequencing/polysome profiling (Fischer et al. 2011). But in fact, most of them remain unchanged, or even down-regulated significantly (GTF2E1, GTF2B, TBP, POU2F1, ZNF143, SNAPCs, CDK9 and CCNT1, data not shown), leaving the mechanism behind this increase elusive.

Last but not least, according to the MDA-MB-231 Fluc deep sequencing data, about 5.35% (40 out of 747) of the most significantly decreased genes are listed as snoRNAs (data not shown). While there are, to date, only approximately 750 snoRNAs in 115 subfamilies in the human snoRNAome (Jorjani et al. 2016; Maden & Hughes 1997), 20,246 protein-coding genes were annotated in human genome reference assembly (GRCh38), suggesting the 40 snoRNAs regulated by cordycepin could be of significant biological value. Indeed, overexpression of snoRNAs and fibrillarin (FBL), a protein component of snoRNP, in breast cancer has been reported to be essential for tumorigenicity *in vitro* and *in vivo* (Su et al. 2014). To be precise, up-regulation of FBL inhibits TP53 activity and induces snoRNA production, whereas repression on FBL

causes ribosomal stress via snoRNA down-regulation, eventually activating TP53 by MDM2 pathway and elevated translation (Su et al. 2014; Marcel et al. 2013). It is yet, not clear how these snoRNAs are down-regulated by cordycepin, relative guesses include: i) ribosomal stress resulted from cordycepin-induced TOP mRNA translation inhibition activates a negative feedback loop in snoRNA biogenesis; ii) transcription factors inhibited by cordycepin lead to inhibited snoRNA or snoRNA host gene transcription; iii) polyadenylation defect of cordycepin leads to degradation of snoRNA host pre-mRNAs.

8.3 Cordycepin Metabonomic Analysis by Ngamratanapaiboon Surachai

In collaboration with the Barrett lab (School of Pharmacy, University of Nottingham), samples from cordycepin early timecourse in MCF-7 cells were sent for metabonomics analyses (Surachai, Kim and Barrett, unpublished data). Quantitative measurement of different metabolites of these samples was acquired from liquid chromatography–mass spectrometry (LC-MS). Principal Component Analysis (PCA) and Orthogonal Projections to Latent Structures Discriminant Analysis (OPLS-DA) were used to identify different metabolites, among which compounds participated in amino acid, purine and pyrimidine metabolism were shown to be significantly regulated. As cordycepin shares structural similarities with purine and pyrimidine, it is of no surprise that purine and pyrimidine metabolism was among the targets of cordycepin, which is consistent with previous findings (Rottman & Guarino 1964; Rottenberg et al. 2005). However, also revealed from the result is the sharp reduction of D-glyceraldehyde phosphate and phosphoenolpyruvic acid, two important compounds in glycolysis (Bolaños et al. 2010; Jang et al. 2013). In MCF-7, a cancer cell line with high aerobic glycolysis activity (Gatenby & Gillies 2004), down-regulation of these intermediates indicates lowered glycolysis activity caused by cordycepin. Moreover, researchers have uncovered the positive regulating effect of MYC, AKT and hypoxia-inducible factor 1 (HIF1), a

downstream target of mTOR, over glycolysis activity, consistently suggesting glycolysis in MCF-7 cells was interrupted by cordycepin (Elstrom et al. 2004; Osthus et al. 2000; Jang et al. 2013; J. W. Kim, Irina Tchernyshyov, Semenza, et al. 2006).

8.4 Cordycepin as a Potential Cancer Therapy Drug

The research of natural compounds in modern medical use has been minimal, mainly because many natural compounds are not patentable and their mechanisms of action are unclear. Hence, research has been more focussed upon synthetic compounds with specific targets, as relative function of the target would have been studied and validated beforehand. However, ironically, some of the most lethal toxins are from nature, such as cobratoxin of Naja cobras, latrotoxin of *Latrodectus* (widow spider) and CfTX-1 of *Chironex fleckeri* (box jellyfish). Having introduced in 1.1.2, in the view of Darwinian evolution, cancer is the product of natural selection, the question arises if the solution to cancer is also produced by selection of nature, which points to natural compounds. *Cordyceps militaris*, the original source of cordycepin (Cunningham et al. 1950), is an endoparasitoid which, according to Shrestha et al. infects 44 different species of insects (Shrestha et al. 2012). As a result, it is safe to assume the evolution of *Cordyceps militaris* to produce cordycepin is of beneficial effect for its invasiveness in insects. This hypothesis agrees with the observation of cordycepin inhibiting inflammatory gene expression and NF-κB activity (see 1.4.3.4)(J Y Yoon et al. 2015; Kondrashov et al. 2012), as fungal infection would be expected to attenuate inflammatory response of the host.

One successful example of natural compound in treating cancer is paclitaxel (also known as taxol), which is originally isolated from *Taxus brevifolia* (Wani et al. 1971). By targeting tubulin, paclitaxel bocks the assembly of microtubule, leading to mitotic and subsequently cell proliferative defect (Jordan et al. 1993; Jordan & Wilson 2004; Schiff et al. 1979; Wani et al. 1971). Undoubtedly, the cytotoxicity of cordycepin in cancer

cells has been documented in various literature reports, both *in vitro* and *in vivo* (Lee, Park, et al. 2013; Lee et al. 2010; Choi et al. 2011; Liao et al. 2015; Pao et al. 2012; Yoshikawa et al. 2009; Nakamura et al. 2006; Sato et al. 2013). These observations have eventually led to two clinical trials using cordycepin, together with pentostatin, in leukaemia, but unfortunately no updates/results have yet been published. It is generally accepted that signal transduction, polyadenylation and DNA repair are the main targets of cordycepin (Tuli et al. 2013). Yet how these targets are affected by cordycepin remains unknown, casting an obstacle that hinders further research and the use of cordycepin.

8.5 Future Work

The presented study investigated the mechanism of cordycepin-induced gene expression and signal transduction regulation in breast cancer cells. Despite lacking a unifying conclusion, it is possible that change in either gene expression or signal transduction can lead to the change in the other. As mentioned, CPSF4 deficiency could result in attenuation of PI3K, ERK and NF-κB signaling. In favour of this hypothesis, a pilot experiment by another member of the De Moor lab used siRNA knockdown of WDR33, a newly discovered CPSF subunit (Shi et al. 2009), and saw inhibited translocation of NF-κB and AMPK activation in RAW264.7 cells (Masar Radhi, unpublished data). In the long run, proteomics analysis for immunoprecipitated signal transduction proteins or 3' end processing complex can help understand if CPSF4 is involved in their regulation. To examine the potential use of lncRNAs as scaffold in facilitating protein functioning, western blotting can be done for proteins of interest in lncRNA knockdown samples using GAPmers. Once changes are seen in protein functioning, direct interaction can be studied by immunoprecipitation of the protein, followed by RNA isolation and RT-qPCR for lncRNAs. With the luminescent breast cancer MDA-MB-231 Fluc cells, *in vivo* studied can be planned. After the cells are injected into

mice, tumour size change can be monitored by luminescence imaging. Collected tumour tissue can be studied for signal transduction, gene expression and polyadenylation. Recently introduced cellular thermal shift assay (CTESA) relies on the thermodynamics stabilisation of proteins in reaction to ligand binding, serving possibility in detecting direct drug targets (Jafari et al. 2014; Molina et al. 2013; Franken et al. 2015; Savitski et al. 2014). With successful validation of use in detecting drug targets, CESTA provides a high-throughput candidate method for identifying cordycepin's primary targets (Molina & Nordlund 2015).

The discussion above has, from different perspectives, analysed the possible mechanisms of the anti-cancer properties of cordycepin. Our data here further investigated the documented effect of cordycepin on signal transduction and growth factor-dependent gene expression in breast cancer cells, unveiling the potentially interchangeable connection between the two. For cancer cells, especially, which maintain the ability to evolve and adapt, could switch off or evolve around signalling to evade further regulation by the drug. Cordycepin, on the contrary, appears to target multiple signalling nodes (Figure 8.5), therefore making it one of the most promising candidates for cancer therapy.

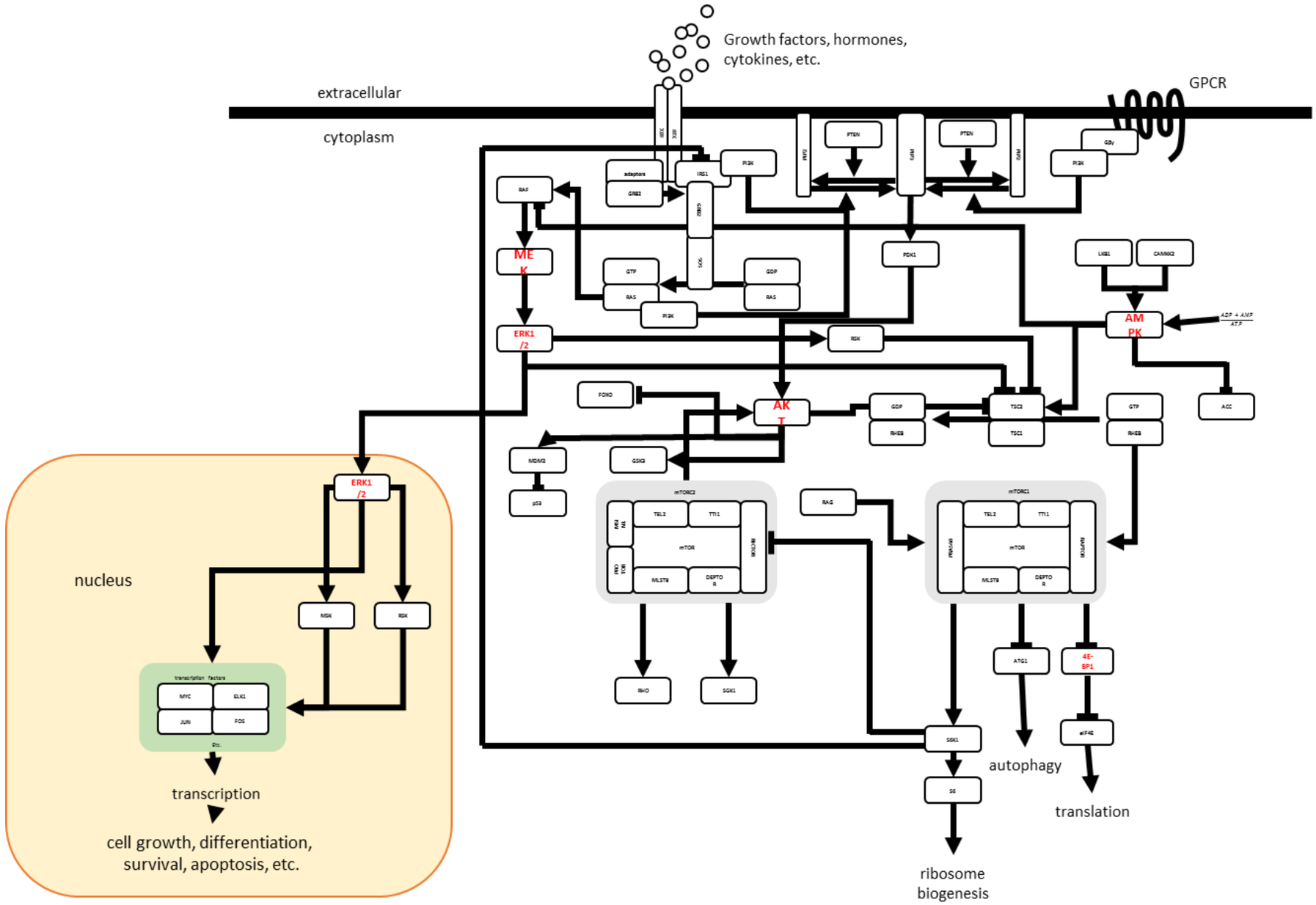

Figure 8.5 Regulated Signal Transduction by Cordycepin

An overall view of the signaling pathways potentially regulated by cordycepin. Highlighted in red are the proteins tested in western blotting.

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