

Molecular Characterisation of the Ubiquitin-Specific Protease 15 Catalytic Domain.

Stephanie Ward

Thesis submitted to the University of Nottingham
for the Degree of Doctor of Philosophy

Abstract

Ubiquitin-specific protease (USP15) is an important regulator of cellular pathways including transforming growth factor β , innate immune responses, mitophagy and mRNA processing. USP15 has been identified a possible therapeutic target because of its involvement in oncogenic pathways and high levels of USP15 have been associated with tumorigenesis. USP15 belongs to the DU family of USPs, sharing its domain architecture with paralogue USP4, and the more distantly related USP11. No structural information was available for the catalytic domain of USP15. Mitoxantrone was previously shown to be a common inhibitor of USP11 and USP15, but the mechanism of action was unknown.

In this thesis, mitoxantrone was used to promote the crystallisation the catalytic domain, allowing the first structural characterisation of the USP15 catalytic core. The structure revealed the canonical USP fold consisting of a finger, palm and thumb fold with a misaligned catalytic triad. In this unbound structure, the catalytic cysteine is located 10 Å from the catalytic histidine resulting in a misaligned catalytic triad. This configuration is unusual and has only been observed in USP7 so far. Furthermore, the ubiquitin binding channel was found to be open, facilitated by flexible active site loops. Moreover, the crystal structure revealed mitoxantrone binds to the S1' binding site and biochemical analysis confirmed mitoxantrone competes with the proximal substrate resulting a decrease in USP15 deubiquitinating activity.

Isothermal titration calorimetry experiments revealed USP15 binds ubiquitin with a lower affinity and displayed a different thermodynamic profile to its paralogue USP4. Structural comparisons identified residue differences in the Blocking Loop 2 region and sequential mutations in this loop confirmed the BL2 has a role in ubiquitin binding.

Finally, a novel ubiquitin fusion with a C-terminal isopeptide bond mimic was developed, which can easily be produced recombinantly. USP15 was found to bind this ubiquitin variant with significantly greater affinity compared to ubiquitin and linear di-ubiquitin, providing binding data to support previous observations that USP15 prefers isopeptide bond linked ubiquitin chains.

Acknowledgments

Firstly, I would like to thank Dr Ingrid Dreveny for the support and guidance and for the opportunity to complete this PhD. I would also like to thank Dr Lodewijk Dekker (University of Nottingham) for his help with assay development over the years, some of which has not made it into this thesis. Additionally, I would like to thank Gemma Cooke (University of Nottingham) and Professor Neil Oldham (University of Nottingham) from the Department of Chemistry for the mass spectrometry experiments described in this thesis.

I would like to thank all members of the Dreveny and Emsley lab group for their continued support and friendship over the last four years, which has made this PhD such an enjoyable experience. I am particularly grateful to Dr. Simon Caulton (University of Nottingham) for all his help and advice in the lab, and boundless enthusiasm over the years.

Thank you to Rishov Mukhopadhyay (University of Nottingham), Georgina Armstrong (University of Nottingham) and Camille Michavila (University of Nottingham), for their contributions to this thesis and for being such a pleasure to work with and supervise.

Finally, I would like to thank the BBSRC for the funding I have received to complete this PhD.

Contents

CHAPTER 1: INTRODUCTION	1
1.1. THE UBIQUITIN SYSTEM	1
1.1.1. UBIQUITINATION	1
1.1.2. UBIQUITIN CHAINS	3
1.2. UBIQUITIN-LIKE PROTEINS	8
1.3. DEUBIQUITINATING ENZYMES	9
1.4. UBIQUITIN-SPECIFIC PROTEASES	10
1.4.1. REGULATION OF DUBS	16
1.4.1.1. Regulation of DUB localisation and abundance	16
1.4.1.2. Regulation of catalytic activity	17
1.4.2. SUBSTRATE SPECIFICITY AMONG THE DUB FAMILY	21
1.4.2.1. Isopeptide vs. peptide linkages	22
1.4.2.2. Endoproteolytic and exoproteolytic activity of DUBS	23
1.5. AVAILABLE TOOLS FOR STUDYING DUBS	24
1.6. THE DU SUBFAMILY	27
1.6.1. USP15	29
1.6.1.1. Implications of USP15 in disease pathogenesis	29
1.7. SMALL MOLECULE INHIBITION OF USPs	37
1.7.1. INHIBITION OF USP11 BY MITOXANTHONE.	39
1.8. BACKGROUND TO THIS STUDY	40
1.9. AIMS AND OBJECTIVES	41
1.10. USP15 CONSTRUCTS USED IN THIS STUDY	42
1.11. CONTRIBUTIONS TO THIS THESIS	43
 CHAPTER 2: MATERIALS AND METHODS	 45
2.1. CONSTRUCTS PREPARATION	45
2.1.1. LIST OF CONSTRUCTS USED	45
2.1.2. POLYMERASE CHAIN REACTION	47
POLYMERASE CHAIN REACTION (PCR) WAS USED FOR THE AMPLIFICATION OF THE DESIRED GENE FRAGMENT. EACH REACTION WAS COMPOSED OF THE FOLLOWING COMPONENTS:	47
2.1.3. DIGESTION AND ANTARCTIC PHOSPHATASE TREATMENT	48
2.1.4. LIGATION	48
2.1.5. TRANSFORMATION	48
2.1.6. COLONY CRACKING, ANALYTICAL DIGESTS AND SEQUENCING	49
2.1.7. SITE DIRECTED MUTAGENESIS:	49
2.1.8. GLYCEROL STOCK PREPARATION	51
2.2. PROTEIN EXPRESSION IN <i>E. COLI</i>	52
2.2.1. PROTEIN EXPRESSION IN BL21 (DE3) CODON PLUS <i>E. COLI</i> CELLS	52
2.2.2. PROTEIN EXPRESSION IN ELAD DEFICIENT CELLS	52
2.3. PROTEIN PURIFICATION	54
2.3.1. SAMPLE PREPARATION	54
2.3.2. NICKLE AFFINITY	55

2.3.3.	CATION EXCHANGE	55
2.3.4.	SIZE EXCLUSION CHROMATOGRAPHY	56
2.3.5.	STORAGE OF PURIFIED PROTEIN	56
2.3.6.	SDS-PAGE GEL ELECTROPHORESIS	56
2.3.7.	PROTEIN AND LIGAND CONCENTRATION DETERMINATION.	57
2.3.7.1.	Mitoxantrone concentration determination	57
2.4.	CRYSTALLOGRAPHY	58
2.4.1.	SPARSE MATRIX CRYSTALLISATION SCREENS	58
2.4.2.	CO-CRYSTALLISATION TRIALS WITH USP15-D1D2 MITOXANTRONE	58
2.4.3.	MICRO SEEDING CRYSTALLISATION EXPERIMENTS	59
2.4.3.1.	96 well micro seeding experiments	59
2.4.3.2.	Micro-seeding in optimisation screens.	59
2.4.4.	CRYSTAL GROWTH USING MITOXANTRONE SCREEN	60
2.4.5.	SOAKING EXPERIMENTS	60
2.4.5.1.	PR-619 soaks	60
2.4.5.2.	Mitoxantrone soaks	61
2.4.5.3.	β -mecaptoethanol soaks	61
2.4.5.4.	Hydrogen peroxide soaks	61
2.4.6.	CRYOPROTECTION OF CRYSTALS.	61
2.5.	DATA COLLECTION, STRUCTURE DETERMINATION AND REFINEMENT	62
2.6.	DEUBIQUITINATING ASSAYS	63
2.6.1.	UBIQUITIN-AMC ASSAY	63
2.6.1.1.	Mitoxantrone inhibition test in the Ub-AMC assay	64
2.6.2.	GEL BASED CLEAVAGE ASSAY	65
2.6.2.1.	General methodology	65
2.6.2.2.	Inhibition screening	65
2.6.2.3.	Linearity test	66
2.6.2.4.	IC50 determination	66
2.7.	ISOTHERMAL TITRATION CALORIMETRY	66
2.7.1.	USP INTERACTIONS WITH UBIQUITIN VARIANTS.	67
2.7.2.	USP15-D1D2 INTERACTIONS WITH MITOXANTRONE	67
2.8.	THERMOFLUOR ASSAY	67
2.9.	MASS SPECTROMETRY	68
2.9.1.	NATIVE MASS SPECTROMETRY	68
2.9.2.	LC-MS DISULPHIDE COUNTING MASS SPECTROMETRY	69

RESULTS AND DISCUSSION **70**

CHAPTER 3: CRYSTALLISATION AND STRUCTURAL CHARACTERISATION OF THE USP15 CATALYTIC DOMAIN **71**

3.1.	EXPRESSION AND PURIFICATION OF USP15-D1D2 FOR CRYSTALLISATION	71
3.1.1.	FINDING NEW CRYSTALLISATION CONDITIONS	73
3.1.2.	OPTIMISATION OF MEMGOLD E10 CRYSTALLISATION HIT	75
3.1.2.1.	Optimisation of mitoxantrone preparation	76
		78
3.1.3.	SEEDING EXPERIMENTS	78
3.1.3.1.	Optimisation of crystals generated by seeding experiments.	80
3.1.4.	PR-619 CO-CRYSTALLISATION TRIALS	81
3.1.5.	MITOXANTRONE SOAKING EXPERIMENTS	82

3.1.6.	SUMMARY OF CRYSTAL TRIALS	83
3.2.	USP15 D1D2 X-RAY CRYSTALLOGRAPHIC MODEL	84
3.2.1.	USP15 D1D2 WT STRUCTURE SOLUTION	84
3.2.2.	OVERALL STRUCTURAL ANALYSIS	87
3.2.2.1.	Ubiquitin accommodation	95

CHAPTER 4: MOLECULAR BASIS OF MITOXANTRONE INHIBITION **98**

4.1.	MITOXANTRONE BINDS IN THE S1' POCKET	98
4.2.	CONFIRMATION OF THE MITOXANTRONE BINDING SITE	102
4.2.1.	ISOTHERMAL TITRATION CALORIMETRY ANALYSIS OF USP15-D1D2 MITOXANTRONE INTERACTION.	102
4.2.2.	SCREENING MITOXANTRONE INHIBITION OF USP15 IN THE UBIQUITIN-AMC ASSAY.	103
4.2.3.	SCREENING MITOXANTRONE INHIBITION OF USP15 IN A GEL SHIFT CLEAVAGE ASSAY.	106
4.2.4.	IC ₅₀ DETERMINATION USING DI-UBIQUITIN GEL SHIFT CLEAVAGE ASSAY.	107
4.2.5.	INVESTIGATION INTO THE SELECTIVITY OF MITOXANTRONE	110
4.2.6.	INVESTIGATION INTO THE IMPORTANCE OF TYR855 IN MITOXANTRONE BINDING.	112
4.2.7.	DISCUSSION OF MITOXANTRONE BINDING	113

CHAPTER 5: CHARACTERISATION OF USP15-D1D2 UBIQUITIN BINDING BEHAVIOUR **117**

5.1.1.	USP15 BINDS UBIQUITIN WITH A DIFFERENT K _D TO USP4.	118
5.2.	STRUCTURAL COMPARISON WITH USP4	120
5.2.1.	THE USP4 ACTIVE SITE REGION ADOPTS A DIFFERENT CONFORMATION TO USP15.	121
5.2.2.	ITC ANALYSIS OF CONTRIBUTIONS OF BL2 TO UBIQUITIN BINDING.	126
5.2.2.1.	Investigation into differences in the SL using ITC.	130
5.2.3.	DISCUSSION OF USP15 AND USP4 UBIQUITIN BINDING BEHAVIOUR	131
5.3.	CHARACTERISATION OF USP15 INTERACTIONS WITH NOVEL UBIQUITIN VARIANT: UBGGG	133
5.3.1.	ITC ANALYSIS OF USP15-D1D2 INTERACTIONS WITH UBGGG	136
5.3.1.1.	Comparison with linear di-ubiquitin binding	139
5.4.	CRYSTALLISATION OF TRIALS OF THE USP15-D1D2_UBGGG COMPLEX.	142
5.4.1.	EXPRESSION AND PURIFICATION OF USP15-D1D2 IN COMPLEX UBGGG.	142
5.4.1.1.	Mass spectrometry confirmation of USP15-D1D2_Uboggg complex formation	143
5.4.2.	USP15-D1D2-CYS269SER-UBGGG COMPLEX CRYSTALLISATION TRIALS	145
5.4.2.1.	Optimisation of USP15-D1D2_Uboggg complex crystal	146
5.5.	USP15 AND LINEAR DI-UBIQUITIN INVESTIGATION	147
5.5.1.	EXPRESSION AND PURIFICATION OF THE USP15-D1D2 LINEAR DI-UBIQUITIN COMPLEX	147
5.5.1.1.	Analysis of USP15-D1D2_Di-ub using native mass spectrometry.	150
5.5.2.	USP15-D1D2 CYS269SER_DI-UB COMPLEX CRYSTALLISATION TRIALS.	152

CHAPTER 6: INVESTIGATION INTO POTENTIAL REDOX REGULATION/ DISULPHIDE **153**

6.1.1.	IDENTIFICATION OF POTENTIAL DISULPHIDE WITHIN THE STRUCTURE	153
6.1.2.	USP15 REQUIRES DTT FOR ACTIVITY	157

6.1.3.	INFLUENCE OF CYS352 ON PROTEIN STABILITY	159
6.1.4.	CRYSTAL SOAKS WITH REDUCING AND OXIDISING AGENTS.	161
6.1.5.	DISULPHIDE COUNTING USING LC-MS.	162
6.1.5.1.	Soaking of crystals with β -mercaptoethanol	164

CHAPTER 7: CONCLUSION AND FUTURE WORK **168**

REFERENCES **172**

APPENDIX **190**

APPENDIX 1: USP15-D1D2 SEQUENCE	190
APPENDIX 2: USP15-D1D2 AND USP4-D1D2 SEQUENCE ALIGNMENT	191
APPENDIX 3: CRYSTALLISATION EXPERIMENTS.	192
APPENDIX 3A: USP15-D1D2 MITOXANTRONE CO-CRYSTALLISATION OPTIMISATIONS OF MEMGOLD AND CLEARSTRATEGY CONDITIONS.	193
APPENDIX 3B: OPTIMISATION OF PROPLEX A4 CONDITION	196
APPENDIX 3C: ADDITIONAL CRYSTALLISATION SCREEN	198
APPENDIX 3D: USP15-D1D2 CYS269SER_UBGGG COMPLEX CRYSTALLISATION SCREENS:	202
APPENDIX 3E: USP15-D1D2 CYS269SER_UBGGG COMPLEX OPTIMISATION SCREENS:	204
APPENDIX 3F : USP15-D1D2 CYS269SER_DI-UB COMPLEX CRYSTALLISATION SCREENS:	205
APPENDIX 3G: 96 WELL MITOXANTRONE SCREEN	206
APPENDIX 4: USP2 CATALYTIC DOMAIN PURIFICATION	210

List of Figures

Chapter 1: Introduction

Figure 1-1: Schematic representation of different ubiquitin chain types.	3
Figure 1-2: Ubiquitin.....	4
Figure 1-3: Lys63 and Lys48 linked ubiquitin have very different overall chain architecture.....	5
Figure 1-4: Surface representation of the different chain linkages.	7
Figure 1-5: Cartoon representation of the different DUB catalytic domain architectures.....	10
Figure 1-6: Catalytic mechanism of USPs.)	12
Figure 1-7: The USP catalytic domain.	13
Figure 1-8: The misaligned catalytic triad of USP7.).....	14
Figure 1-9: Example of conformational changes in the USP catalytic domain upon ubiquitin binding.....	15
Figure 1-10: Redox regulation of deubiquitinating enzymes.....	20
Figure 1-11: Differences in chemical features between the isopeptide and peptide bond.....	22
Figure 1-12: Schematic representation of endoproteolytic and exoproteolytic cleavage by DUBs.....	23
Figure 1-13: Schematic representation of commonly used ubiquitin probes in deubiquitinating enzyme investigations.....	25
Figure 1-14: Schematic representation of E1 catalysed ubiquitin c-terminal amidation for synthesis of deubiquitinase substrates.....	26
Figure 1-15: USP15 regulation of the TGF- β signalling pathway.....	31
Figure 1-16: USP15 stabilises MDM2.....	33
Figure 1-17: A viral E6 protein evades the host immune system and can promote tumorigenesis.....	34
Figure 1-18: Schematic representation of the relationship between USP15 and Parkin.....	36
Figure 1-19: Examples of DUB inhibitors.....	37
Figure 1-20: Inhibition of USP2 with 6TG.....	39
Figure 1-21: Preliminary USP15-D1D2 mitoxantrone crystal obtained from the JSGC+ screen.....	41
Figure 1-22: Schematic representation of USP15-FL and USP15-D1D2 domain structure.....	42

Chapter 3: Crystallisation and structural characterisation of the USP15 catalytic domain

Figure 3-1: Purification of USP15 D1D2 WT.....	72
Figure 3-2: Examples of initial crystals obtained in the 96 well screening of USP15-D1D2 and mitoxantrone.....	74
Figure 3-3: Example crystals from USP15-D1D2-mitoxantrone co-crystallisation optimisations.....	77
Figure 3-4: Schematic representation of distribution of crystals in optimisation trays.....	78
Figure 3-5: Crystals of USP15-D1D2 in complex with mitoxantrone obtained from micro-seeding experiments in 96 well screens.....	79

Figure 3-6: Mitoxantrone 96 well screen varying seed stock dilution from undiluted to 1:1000..	81
Figure 3-7: Crystals grown in the 96 well mitoxantrone screen used for PR-619 soaking experiments	82
Figure 3-8: Example crystal used for mitoxantrone soaking experiments.....	83
Figure 3-9: Superposition of all molecules of USP15-D1D2.....	86
Figure 3-10: Cartoon representation of free USP15-D1D2.....	88
Figure 3-11: The catalytic triad in the free USP15-D1D2 structure is in a catalytically incompetent conformation.. ..	89
Figure 3-12: Putty representation showing variation in B-factors throughout the USP15-D1D2 model.. ..	91
Figure 3-13: Sequence alignment of USP15, USP4 and USP7 switching loops.	92
Figure 3-14: Structural comparison of the switching loop in USP15-free, USP7-free, bound USP7 and USP4.....	93
Figure 3-15: Electrostatic networks in the USP7 switching loop stabilises the inactive conformation.....	94
Figure 3-16: Superposition of ubiquitin with USP15-free.	96
Figure 3-17: Superposition of ubiquitin bound USP2 (PDB: 2HD5) highlights clashes between USP15 and ubiquitin residues.. ..	97

Chapter 4: Molecular basis of mitoxantrone inhibition

Figure 4-1: Cartoon representation of USP15-D1D2-mitoxantrone complex crystal structure.....	98
Figure 1-2: Molecular basis of the mitoxantrone- USP15-D1D2 interaction.....	99
Figure 1-3: Mitoxantrone binding is not influenced by additional crystal contacts... ..	100
Figure 1-4: Mitoxantrone competes with the proximal substrate.....	101
Figure 1-5: ITC titration of mitoxantrone into USP15-D1D2 WT	103
Figure 1-6: Screening for inhibition of USP15-FL and USP15-D1D2 activity in the presence of 100 μ M mitoxantrone.....	105
Figure 1-7: Time course experiment of USP15-D1D2, USP15-FL, cleavage of di-ubiquitin (17 kDa) to mono-ubiquitin (8.5 kDa) with 0 μ M and 100 μ M mitoxantrone.....	107
Figure 1-8: IC50 determination of USP15-FL inhibition in the di-ubiquitin gel-shift cleavage assay.....	109
Figure 1-9: Time course experiment of USP11-D1D2, USP4-D1D2, cleavage of di-ubiquitin (17 kDa) to mono-ubiquitin (8.5 kDa) with 0 μ M and 100 μ M mitoxantrone.....	111
Figure 1-10: Sequence alignment of BL2 region.....	112
Figure 1-11: Time course experiment of USP15-FL Tyr855Lys and USP2-D1D2, cleavage of di-ubiquitin (17 kDa) to mono-ubiquitin (8.5 kDa) with 0 μ M and 100 μ M mitoxantrone.....	113

Chapter 5: Characterisation of USP15-D1D2 ubiquitin binding behaviour

Figure 5-1: ITC titration of ubiquitin into USP15-D1D2 WT, USP15-D1D2 Cys269Ser and USP4-D1D2 Cys311Ser at 25°C. n.	119
Figure 5-2: The catalytic cysteine (Cys311) is USP4 is modified with β -mecaptoethanol.	120
Figure 5-3: USP15 adopts an open binding channel.....	123
Figure 5-4: Surface representation of USP15-D1D2 highlighting residue differences with USP4.. ..	124

Figure 5-5: Surface representation of USP15-D1D2 and USP4-D1D2 showing associated charge of dissimilar residues..	125
Figure 5-6: Raw ITC data and binding isotherms for USP15-D1D2 Gly860Val and USP15-D1D2bl2usp4 at 25°C.	127
Figure 5-7: Raw ITC data and binding isotherms, of USP15-D1D2, USP15-D1D2 Gly860Val, USP15-D1D2 bl2usp4 and USP4-D1D2 with ubiquitin at 37°C.	128
Figure 5-8: ITC titration of ubiquitin into USP15-D1D2 WT and Cys352Ser.	131
Figure 5-9: Schematic representation of a native isopeptide bond, Ubggg conjugated lysine backbone mimic and a peptide bond.	134
Figure 5-10: Purification of ubiquitin and Ubggg.	136
Figure 5-11: ITC titration of Ubggg into USP15-D1D2.	138
Figure 5-12: Purification of linear di-ubiquitin.	140
Figure 5-13: ITC titration of linear di-ubiquitin into USP15-D1D2 Cys269Ser.	141
Figure 5-14: Figure: Purification of USP15 D1D2 Cys269Ser in complex with Ubggg.	143
Figure 5-15: Native mass spectrometry results for USP15-D1D2 Cys269Ser_Ubggg.	144
Figure 5-16: Crystal of USP15-D1D2 Cys269Ser_Ubggg grown in A10 of structure screen 1 + 2.	146
Figure 5-17: Schematic representation of USP15-D1D2-Cys269Ser-Ubggg complex optimisation plates.	147
Figure 5-18: Purification of USP15 D1D2 Cys269Ser in complex with di-ubiquitin.	149
Figure 5-19: Possible orientations of di-ubiquitin in the USP catalytic site.	14950
Figure 5-20: Native mass spectrometry analysis of the USP15-D1D2 Cys269Ser_Di-ub complex.	1511
Figure 5-21: Example of needle clusters grown in several conditions in the Morpheus screen.	152

Chapter 6: Investigations into potential redox regulation of USP15

Figure 6-1: Alignment of USP15-D1D2, USP4-D1D2 and USP35.	154
Figure 6-2: Modelling of potential disulphide between Cys269 and Cys352.	155
Figure 6-3: Ball and stick representation of possible disulphide bridge between Cys352 and Cys269.	156
Figure 6-4: Activity of USP15-D1D2 WT and Cys352Ser in the Ub-AMC assay in oxidising and reducing conditions.	159
Figure 6-5: Investigation into importance of Cys352 in protein stability.	161
Figure 6-6: : Ubiquitin-AMC assay confirms oxidation of USP15-D1D2 sample prior to LC-MS disulphide counting.	162
Figure 6-7: Crystal soaking experiments with 5 mM β -mercaptoethanol.	166
Figure 6-8: Superposition of USP15-free with a β -mercaptoethanol soaked structure.	167

Appendix

Figure A1: Sequence alignment of USP15-D1D2 and USP-D1D2.	191
Figure A2: Purification of USP2 catalytic domain.	192
Figure A3: Ub-AMC assay for verification that USP2 was present and fully functional after purification.	213

List of Tables:

Chapter 1: Introduction

Table 1: Effect of DTT on activity of DUBs.	19
---	----

Chapter 2: Materials and Methods

Table 2: List of constructs used in this thesis.	46
Table 3: PCR protocol.	47
Table 4: Primers used for the generation of the Ubggg construct	48
Table 5: Mutagenesis PCR reaction protocol.....	50
Table 6: Summary of mutations generated in USP15-FL and USP15-D1D2.....	51
Table 7: Summary of the protein expression protocol for each construct.....	53
Table 8: Summary of purification techniques used and respective buffer for each construct.	54
Table 9: Components of SDS-PAGE electrophoresis gels.....	57

Chapter 3: Crystallisation and structural determination of the USP15 catalytic domain

Table 10: Summary of data collected for MemGold E10	74
Table 11: Summary of screens and drop composition in the initial seeding experiments of USP15-D1D2 and mitoxantrone.	79
Table 12: Crystallographic data collection and refinement statistics	85

Chapter 5: Characterisation of the USP15-D1D2 ubiquitin binding behaviour

Table 13: Predicted and calculated masses of USP15-D1D2 Cys269Ser_Ubggg complex and components. Expected masses were calculated using protparam prediction tool.	144
Table 14: Predicted and calculated masses of USP15-D1D2 Cys269Ser_Di-Ub complex..	151

Chapter 6: Investigation into potential redox regulation of USP15

Table 15: Table of predicted masses for reduced, unmodified USP15, reduced and alkylated USP15 and oxidised and alkylated USP15.	163
Table 16: Summary of LC-MS disulphide counting.	164
Table 17: Statistics of β -mercaptoethanol soaked crystal after the resolution was cut back to 3 Å and re-indexed using Aimless.....	165

Appendix

Table 18: Summary of USP15-D1D2 co-crystallisation optimisations of MemGold E10 and clear strategy E5.	195
Table 19: Table summarising optimisation plates of Proplex A4	197
Table 20: 96 well screen designed around conditions which have been favourable for USP15-D1D2 crystallisation with mitoxantrone in previous trials.	209

Abbreviations

2-Methyl-2,4-pentanediol	MPD	N-terminal domain in USP	DUSP
2- β -mecaptoethanol	B-MERCAPTOETHANOL	nuclear factor kappa-light-chain-enhancer of activated B cells	NF- κ B
6-Thioguanine	6TG	nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha	I κ B α
Adenosine Monophosphate	AMP	ovarian tumour proteases	OUT
Adenosine Triphosphate	ATP	Polyethylene glycol	PEG
Ammonium Persulfate	APS	Polymerase chain reaction	PCR
AMP activated protein kinases	AMPK	Reactive oxygen species	ROS
Blocking loop 1	BL1	Really interesting new gene	RING
Blocking loop 2	BL2	receptor regulated SMADS	R-SMADS
Catalytic cleft loop	CC loop	Size exclusion chromatography	SEC
Column volume	CV	Sodium Dodecyl Sulfate	SDS
constitutive photomorphogenesis 9	CSN	squamous cell carcinoma antigen recognized by T-Cell 3	SART3
Cullin-ring complexes	CRL	Sulphenic acid	SOH
Deubiquitinase	DUB	Sulphinic acid	SO ₂ H
Dimethyl sulfoxide	DMSO	Sulphonic acid	SO ₃ H
Dinucleoside triphosphates	dNTP	Switching Loop	SL
Dithiothreitol	DTT	Tetramethylethylenediamine	TEMED
Domain present in USPs	DUSP	TGF- β receptor 2	T β R-II
endoplasmic reticulum associated degradation	ERAD	TGF- β receptor 1	T β R-I
Epidermal growth factor	EGF	TNF receptor-associated factor 2	TRAF2
Ethylenediaminetetraacetic acid	EDTA	transforming growth factor β	TGF- β
Genetically encoded orthogonal protection and activated ligation	GOPAL	Tripartite motif-containing protein 25	TRIM25
homologous E6 associated protein C- terminus domains	HECT	Ubiquitin C-terminal hydrolases	UCHs
Human papillomavirus	HPV	Ubiquitin ligase	E3
Isopropyl β -D-1-thiogalactopyranoside	IPTG	Ubiquitin like	Ubl
Isothermal titration calorimetry	ITC	Ubiquitin propargyl	Ub-Prg
Linear Ub chain assembly-complex	LUBAC	Ubiquitin specific protease	USP
Lysogeny Broth	LB	Ubiquitin vinylmethylester	Ub-VME
Melting temperature	T _m	Ubiquitin vinylsulfone	Ub-VS
motif interacting with ubiquitin (MIU)-containing novel DUB family	MINDY	Ubiquitin-7-amido-4-methylcoumarin	Ub-AMC
mouse double minute 2 homolog	MDM2	Ubiquitin conjugating enzyme	E2
MPN/MOV34	JAMM	Ubiquitin activating enzyme	E1

CHAPTER 1: Introduction

1.1. The ubiquitin system

Ubiquitin (Ub) is a highly conserved 76 amino acid protein and is fundamental to the regulation of numerous cellular processes ^{8, 21}. The conjugation of ubiquitin onto target proteins serves as a reversible posttranslational modification most commonly associated with degradation of the target protein via the 26S proteasome system²². Other roles of ubiquitination include, but are not limited to, cell cycle progression, transcriptional regulation, and signal transduction ²³.

1.1.1. Ubiquitination

Ubiquitin is attached via its C-terminal glycine onto a target proteins lysine ϵ -amino group. The conjugation event is catalysed by the sequential action of E1, E2 and E3 enzymes. The reaction cascade is initiated by an E1 enzyme, which activates ubiquitin via its C-terminal glycine residue in an ATP dependent manner. Ubiquitin binds to the adenylation domain of the E1 enzyme. The activation step involves the formation of a ubiquitin adenylate intermediate, in which PP_i is released, followed by thioester linkage of ubiquitin to the active site cysteine residue within the E1 enzyme, and release of AMP ²⁴⁻²⁷. The activation step is complete upon the binding of a second ubiquitin molecule to the E1 adenylation domain.

During the second step of the reaction, ubiquitin is transferred onto the active site cysteine of an E2 enzyme, a ubiquitin carrier protein²⁸. There are more than 38 E2 genes within the human genome²⁹. The E2s involved exclusively in ubiquitin chain assembly can be grouped into three subclasses; monoubiquitination E2s which exclusively mono ubiquitinate substrates³⁰⁻³², priming E2s which adds the first few

ubiquitin in a chain ^{33, 34} while the remaining E2 enzymes are involved in polyubiquitin chain elongation in a linkage specific manner³⁵⁻³⁷. Chain selectivity amongst these E2 enzymes gives rise to the first level of specificity with the ubiquitin system.

The final stage in the conjugation pathway involves the E3 ligase family, which bind the E2-Ub complex and catalyse the transfer of ubiquitin onto the target protein. There are hundreds of genes in the human genome encoding E3 ligases³⁸. The large E3 family can be classified into two predominant sub groups: really interesting new gene (RING) E3 ligases and homologous E6 associated protein C-terminus domains (HECT) E3 ligases³⁹. RING E3 ligases mediate the transfer of ubiquitin to the substrate by acting as a scaffold, recruiting the E2-Ub complex to a substrate whilst simultaneously orientating the substrate to receive the ubiquitin. In contrast, HECT E3 ligases receive ubiquitin via a covalent interaction with its active site cysteine, and subsequently catalyses the transfer of ubiquitin to the lysine of the target protein⁴⁰. This mechanism allows HECT E3 ligases to bypass the chain selectivity of the E2 ligase, though many E3 enzymes assemble polyubiquitin chains in a linkage specific manner^{41, 42}. Structural analysis of HECT E3 ligases has shown interdomain flexibility in the proximity of the catalytic cysteine allowing flexibility in chain accommodation, with residues in this region also contributing to linkage specificity⁴³. The different combinations of E2 and E3 enzymes ensure specificity of ubiquitin conjugation onto a target protein.

1.1.2. Ubiquitin chains

Divergence of the ubiquitin system arises from the generation of different types of polyubiquitin chains. An ubiquitin monomer is conjugated via its C-terminal glycine (Gly76) onto one of seven intrinsic lysine residues (Lys6, Lys11, Lys27, Lys29, Lys33, Lys48 and Lys63) on a recipient ubiquitin, giving rise to 7 different types of polyubiquitin chains linked via an isopeptide bond⁴⁴. In addition, attachment of ubiquitin via its N-terminal amine group, termed Met1 linked linear chains, gives rise to peptide bond linked polyubiquitin chains.

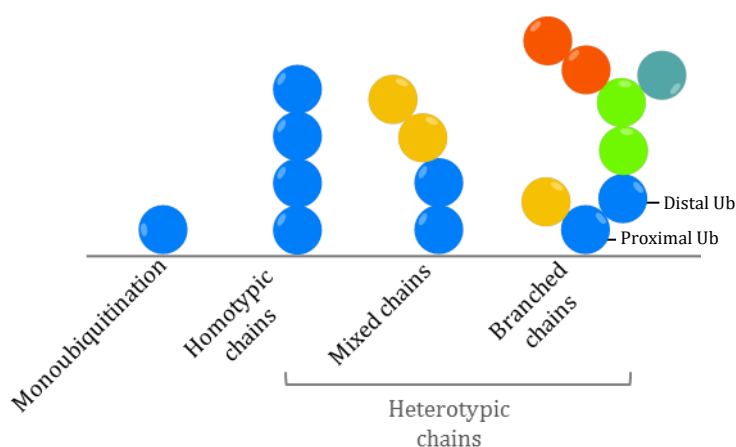


Figure 1-1: Schematic representation of different ubiquitin chain types. The different colours represent different chain linkages. The proximal and distal ubiquitin have been indicated.

It is common for the ubiquitin moiety closest to the point of conjugation via lysine or met1, which binds to the catalytic site of a deubiquitinating enzyme, to be referred as the proximal ubiquitin. The distal ubiquitin is the term used to describe the moiety immediately following the proximal ubiquitin in a polyubiquitin chain (see figure 1-1).

Polyubiquitin chains can consist of one chain type (homotypic) or multiple chain types (heterotypic) diversifying ubiquitin signalling even further. Heterotypic chains contain multiple chains types and can be unbranched or branched (also known as forked). Branched ubiquitin chains arise when one ubiquitin monomer

is ubiquitinated on several lysine residues⁴⁵ (see figure 1-2). The C-terminal RLRGG motif, along with the Ile44 hydrophobic patch (Leu8, Ile44, His68 and Val70) and the Ile36 hydrophobic patch (Leu8, Ile36, Leu71 and Leu73) are important features which distinguish ubiquitin from other ubiquitin like proteins^{18, 46}.

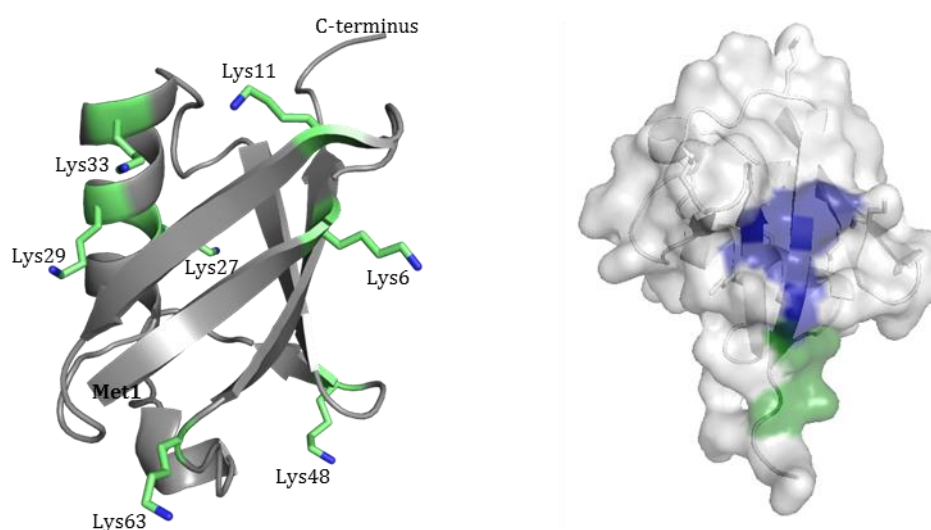


Figure 1-2: Ubiquitin. Left: Cartoon representation of ubiquitin highlighting the seven lysine residues (Lys6, Lys11, Lys 27, Lys29, Lys33, Lys 48 and Lys63). Lysine side chains are shown as green sticks. Right: Surface representation of ubiquitin. The Ile36 and Ile44 hydrophobic patches are highlighted in dark blue and green respectively PDB: 1UBQ⁸.

Met1 linked chains are generated during the translation of ubiquitin, whereby ubiquitin is transcribed as a polyubiquitin fusion and processed to yield mono-ubiquitin in the cell⁴⁷. An E3 ligase complex called linear Ub chain-assembly complex (LUBAC) specifically assembles this chain type⁴⁸ on components of nuclear factor- κ B (NF- κ B), and is critical for activation of this pathway^{49, 50}.

Presently, Lys63 and Lys48 linked chains are the best characterised chain types, mainly because of previous limitations in atypical linked chains synthesis. Previously, ubiquitin chain synthesis has relied on the use of linkage specific E2-

E3 pairs; however, incomplete knowledge of the ligase family limited this approach to Lys48 and Lys63 linked chains. More recently, atypical ubiquitin chains have been successfully engineered allowing great advancement in the understanding of their function within the cell⁵¹. The different linkage types give rise to different structural features and determine specific interactions with substrate proteins⁵².

Lys48 linked chains adopt a distinct compact globular structure (figure 1-3) with a buried Ile44 patch, and targets proteins to the 26S proteasome^{53, 54}. This enormous protein complex recognises and binds to predominantly Lys48 linked polyubiquitin chains of at least four ubiquitins, and subsequently disassembles

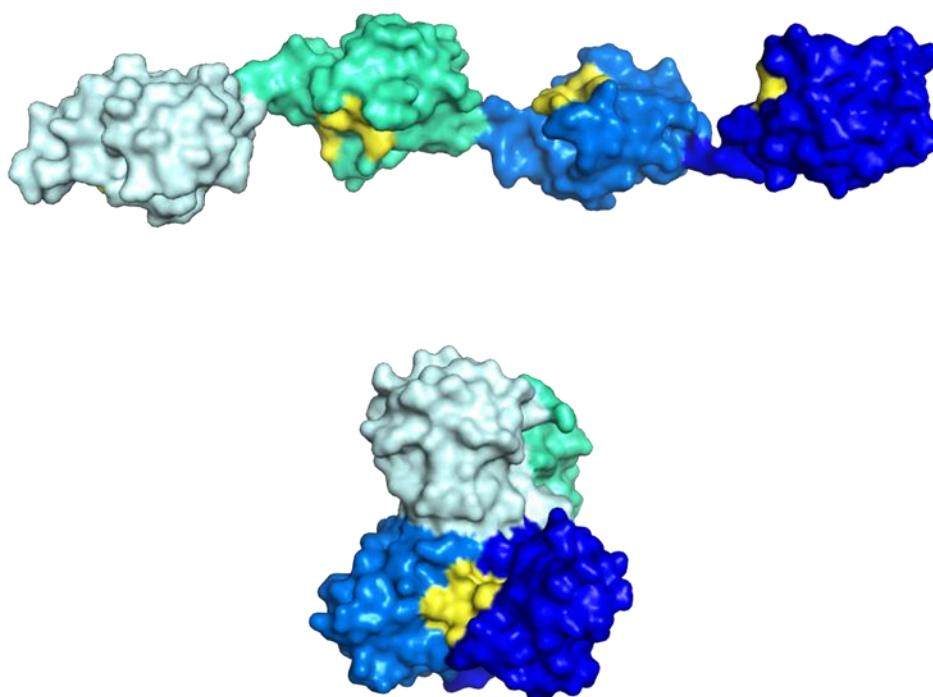


Figure 1-3: Lys63 and Lys48 linked ubiquitin have very different overall chain architecture. Lys63 linked tetra-ubiquitin (top) give a highly flexible linear architecture whilst Lys48 linked chains (bottom) are far more globular and compact (PDB codes: 2JF5 and 2O6V respectively). The Ile44 patch is shown in yellow.

and degrades the protein.⁵⁵ Conversely, Lys63-linked chains adopt a more extended, linear conformation (figure 1-3) and have been recognized in having roles in non-degradative cellular activities. For example, assembly of these chains on cytokine receptors is required for activation of downstream effectors in cytokine signalling pathways⁵⁶.

Lys11 linked chains are linked to several cellular processes including, regulation of the cell cycle, endoplasmic reticulum associated degradation (ERAD) membrane trafficking, TNF α signalling and it is thought to serve as a proteasomal degradation signal^{34, 57-60}. Lys6 linked chains are thought to be involved in non-proteolytic activities and have been shown to have roles in DNA damage response and mitophagy ^{61, 62}. Lys27 linkage types have been associated with mitochondrial biology, lysosomal localisation, autoimmunity and DNA damage response ⁶³⁻⁶⁶.

Lys33 linked polyubiquitin chains are believed to be a non-degradative signal ⁶⁷. Addition of Lys33 chains negatively regulates the activity of several proteins including AMP- activated protein kinases (AMPK) kinases ⁶⁸, and the T-cell antigen receptor⁶⁹. The Lys33 linkage type has also been linked to Golgi trafficking ⁷⁰.

Crystal structures of Lys33 linked di-ubiquitin and tri-ubiquitin revealed the capability of these chains to adopt both an extended and a compact conformation, distinct from the conformations observed for Lys6, Lys48, Lys63 and Met1 linked chains⁷¹. The compact conformation of these chains resembles that of Lys11 linked chains, which also adopts a compact conformation with a solvent exposed Ile44 patch¹⁶ (figure 1-4).

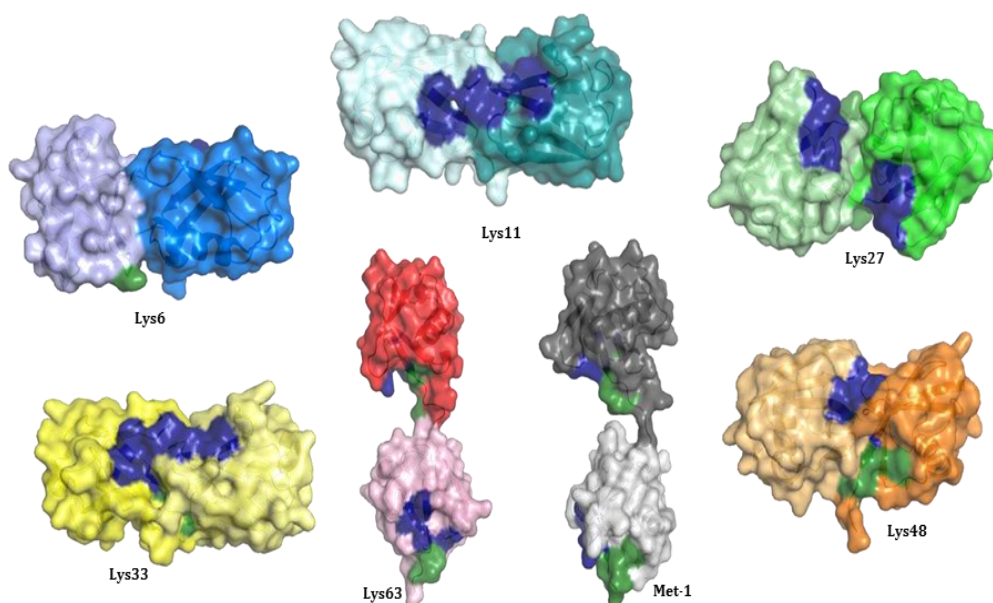


Figure 1-4: Surface representation of the different chain linkages. The Ile44 patch is shown in dark blue and Ile36 patch in dark green on all structures. PDB codes: Lys6, 5GOB¹³; Lys11, 2XEW¹⁶ Lys27; 5GOG¹⁷, Lys33¹⁸, 5AF4; Lys48 3ALB¹⁹; Lys63, 2JF5; Met1 2W9N²⁰.

High flexibility between monomers in these chains and different observed orientations of the ubiquitin moieties suggest these chains can freely assume different conformations in solution^{20, 72}. In solution, a Lys48 linked chain adopts a compact conformation⁵³ but extended conformations have been reported at lower pH's⁷³.

Similarly, Met1 linked di-ubiquitin and Lys63 linked can exist in both open and compact conformations^{13, 20, 53, 72, 74}. The discovery of these varying chain confirmations reveals just how complex this dynamic system is.

1.2. Ubiquitin-like proteins

Ubiquitin-like protein (Ubl) is the term used to describe a protein that shares similarities in both sequence and three-dimensional structure to ubiquitin. In particular, a distinctive fold originally discovered in ubiquitin termed the β -grasp fold, was found in several other proteins such as the small-ubiquitin-related modifier (SUMO), NEDD8, and interferon-stimulated gene 15 (ISG15)^{75, 76}. Similar to ubiquitination, Ubls are conjugated onto target proteins via an enzyme cascade involving E2-type conjugation enzymes and E3-type ligases, which is rapidly reversed by specific proteases⁷⁷. SUMO modification of proteins has many functions including but not limited to nuclear transportation, DNA repair and transcriptional regulation whilst NEDD8 has been implicated in cytoskeletal regulation and cell cycle progression⁷⁸⁻⁸⁰.

Of all the Ubls discovered so far, NEDD8 most closely resembles ubiquitin, sharing a ~60 % sequence similarity⁸¹. Despite this, enzymes exist which are specific for each homologue, permitting their distinct functions within the cell^{82, 83}. Structural analysis of NEDD8 in complex with a NEDD8 specific cysteine protease, NEDP1⁸⁴, revealed the importance of the C-terminus in NEDD8 and ubiquitin discrimination⁸⁵. Alanine 72, which is replaced by an arginine in ubiquitin, was found to be critical in allowing NEDP1 to discriminate between homologues. Replacing Ala72 with an arginine was shown to disrupt the binding interface between NEDP1 and NEDD8 and prevented NEDP1 deNEDDylation activity. Further, Arg72 in ubiquitin has been shown to form a salt bridge with residues in deubiquitinases, which is critical to the binding and processing of ubiquitin².

1.3. Deubiquitinating enzymes

A large family known as deubiquitinases (DUBs) catalyse the removal of ubiquitin and antagonise the activities of the E1/E2/E3 system. DUBs are essential for ubiquitin homeostasis in the cell and can be grouped according to three major functions. Firstly; chain removal by DUBs can promote protein stabilization by rescuing substrates from proteasomal or lysosomal degradation. Once the protein is already committed to the degradation pathway, chain removal prevents ubiquitin degradation and recycles the ubiquitin, helping sustain free ubiquitin levels in the cell. Furthermore, non-degradative ubiquitin signalling events can be attenuated and reversed by removal of ubiquitin chains.

Secondly, modification of ubiquitin chains by DUBs can alter the type of ubiquitin signal, allowing rapid interchange between different types of signals.

Finally, ubiquitin is expressed as linear fusions and DUB activity is required to process these linear fusions into monoubiquitin and remove any C-terminal modifications, sustaining the free ubiquitin pool¹⁰.

The DUB family comprises ~100 enzymes categorized into six structurally distinct families with respect to their catalytic fold ⁸⁶ (figure 1-5). Five of these sub-families are cysteine proteases and include ubiquitin C-terminal hydrolases (UCHs), ubiquitin-specific proteases (USPs), ovarian tumour proteases (OTUs) the Machado-Josephin (MJD) family and the more recently discovered motif interacting with ubiquitin (MIU)-containing novel DUB family (MINDY). Zinc dependent JAB1/MPN/MOV34 metalloprotease DUBs (JAMMs) constitute the only metalloprotease DUB family^{5, 86, 87}.

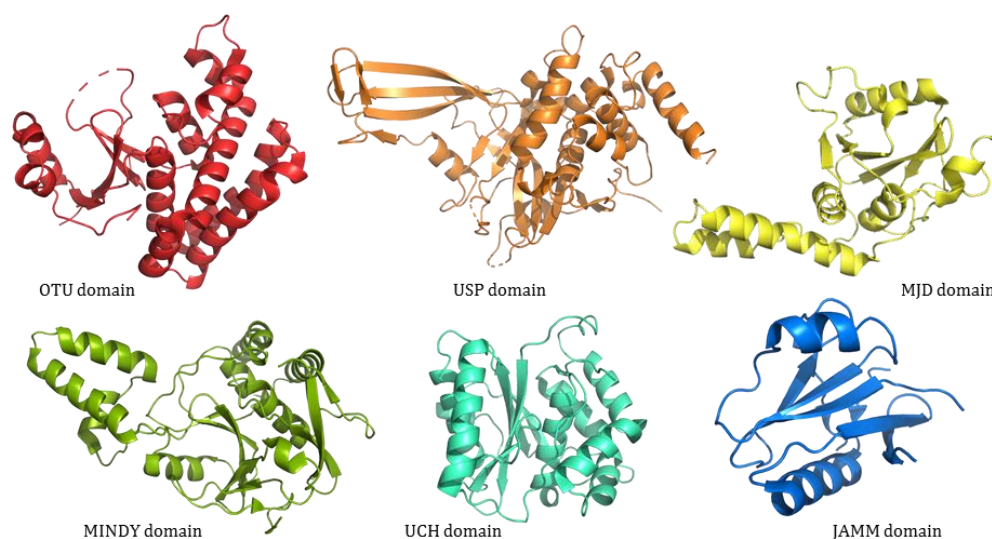


Figure 1-5: Cartoon representation of the different DUB catalytic domain architectures. PDB codes: OTU domain, Otubain-2, 1TFF¹ ; USP domain, USP7, 1NB8²; MJD domain, Ataxin-3, 1YZB³; MINDY domain, MINDY-1, 5JKN⁵; UCH domain, UCHL3 1XD3⁶; JAMM domain, AfJAMM, 1R5X¹¹.

1.4. Ubiquitin-specific proteases

The largest DUB family are the USPs with 56 individual members identified so far in the human genome. These enzymes vary significantly in size, ranging from 300-3500 amino acids in length. The USP family is highly divergent with multiple different domains existing within the enzyme which mediate protein interactions and substrate binding, subcellular location and regulation of enzyme activity. The architecture of the catalytic core defines the USP family, with all USPs containing a homologous papain like catalytic domain sharing an overall sequence similarity of approximately 30%⁸⁸.

A catalytic triad consisting of Cys-His-Asp/Asn is fundamental to the functionality of the USP family. The plant protease papain provided insight into the catalytic mechanism of these USP enzymes⁸⁹ (see figure 1-6). The catalytic histidine is

aligned and polarised by the catalytic Asn/Asp, allowing deprotonation of the thiol group of the catalytic cysteine. The deprotonated cysteine is able to act as a nucleophile, attacking the carbonyl group of the isopeptide/peptide bond. An oxyanion hole, commonly formed by nearby Asp/Asn residues in the USP active site, serves to stabilize the unstable tetrahedral intermediate formed after the nucleophilic attack. Collapse of the tetrahedral intermediate and consequential scission of the amine group releases the proximal ubiquitin/substrate, and leaves the distal ubiquitin covalently bound with the catalytic cysteine forming an acyl intermediate. Hydrolysis of this intermediate releases the distal ubiquitin and the enzyme returns to its original unbound state ^{2, 11}.

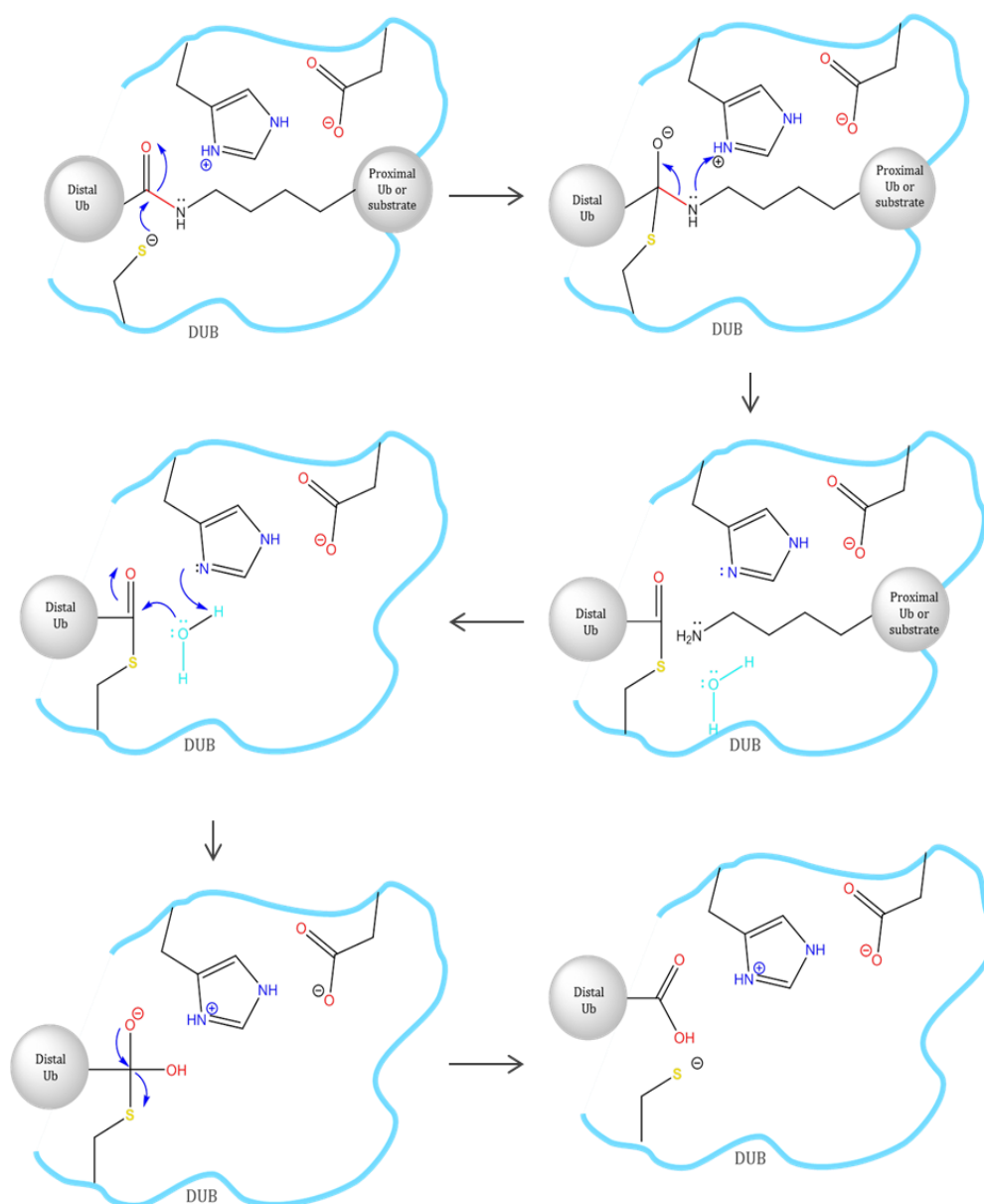


Figure 1-6: Catalytic mechanism of USPs. The catalytic cleft of the USP is outlined in blue and the substrate, di-ubiquitin, shown in grey. The deprotonated catalytic cysteine performs nucleophilic attack on the carbonyl group of the distal ubiquitin. A tetrahedral intermediate is formed which is stabilised by nearby asp/asn residues (not shown).

The proximal ubiquitin or substrate is released upon collapse of the tetrahedral intermediate leaving the distal ubiquitin bound to the catalytic cysteine via an acyl intermediate. Hydrolysis of the acyl group releases the distal ubiquitin and the catalytic centre resets. Adapted from Clague et al ¹⁵.

The overall structure of the catalytic domain of a USP was first characterized for USP7². The domain was described as having a resemblance to an open right hand conformation (see figure 1-7). Accordingly, three sub domains identified within the catalytic domain were referred to as the fingers, palm and thumb regions. Packing of the palm and thumb regions gives rise to a deep catalytic cleft where the catalytic triad resides. The finger region, comprising several β sheets, extends away from the catalytic site by over 30 Å. The extended fingers and thumb conformation creates a large binding interface (S1 pocket) which interacts with as much as 40 % of the ubiquitin surface. The S1 pocket is typically the binding site for the proximal ubiquitin. A second less well defined pocket (S1') binds the distal ubiquitin during proteolysis. Some USPs harbour S2 and S3 pockets which facilitate the binding of ubiquitin moieties preceding the proximal ubiquitin bound to the S1 pocket^{90, 91}.

The domain architecture observed in USP7 was thought to be conserved throughout the USP family, and has been confirmed by the subsequent successful crystallization of other USP catalytic domains.

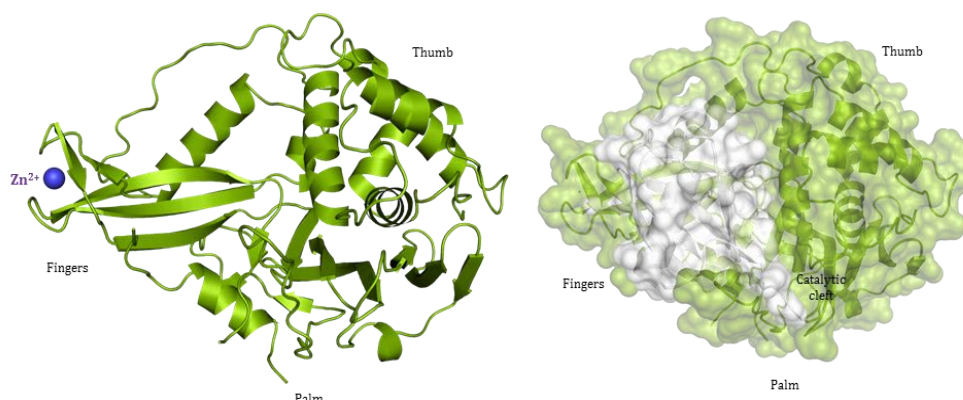


Figure 1-7: The USP catalytic domain. Left: Cartoon representation of canonical USP fold showing the fingers palm and thumb region. A single Zn^{2+} ion is co-ordinated by the zinc-binding motif in the finger region (purple sphere). Right: Surface representation of the USP4 catalytic domain (green) superimposed with ubiquitin (white). Ubiquitin binds to a large interface, termed the S1 pocket, formed by the finger region. The C-terminus binds to the catalytic cleft formed between the thumb and palm region. PDB codes: USP4, 2Y6E¹⁴; Ub, 1UBQ⁸

Structures of the free catalytic domain are available for USP7 (PDB: 1NB8) USP8 (PDB: 2GF0), USP14 (PDB: 2AYN) USP12 (PDB: 5K16) USP18 (PDB: 5CHT) and USP4 (PDB: 2Y6E) ^{2, 4, 14, 92-95}. Whilst the overall architecture is consistent, USP7 is unique in having a misaligned catalytic core. The catalytic cysteine is located 10 Å away from the catalytic histidine indicating the need for significant structural rearrangement upon ubiquitin binding for catalysis to occur (figure 1-8). In other USP catalytic domains, the catalytic triad is aligned and the enzymes are poised for catalysis.

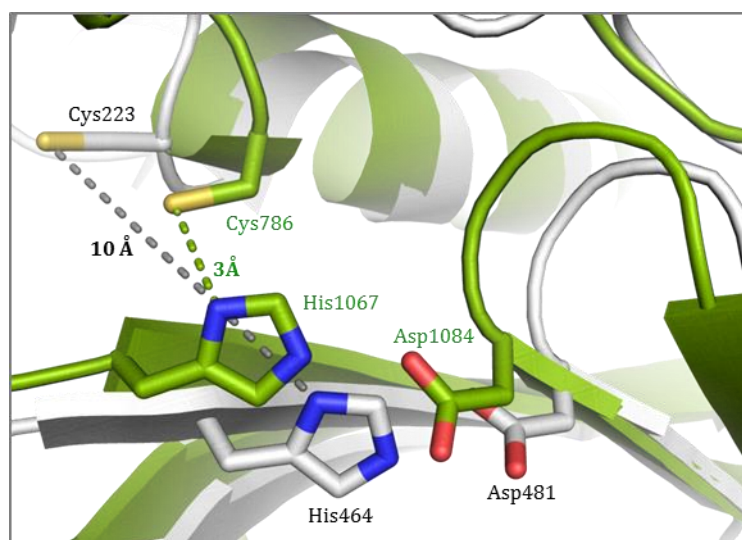


Figure 1-8: The misaligned catalytic triad of USP7. The catalytic triad is shown in stick representation. The catalytic cysteine of USP7 (white) is located 10 Å away from the catalytic histidine (grey dashed line). The pre-aligned aligned catalytic triad of USP8 (green) is shown for comparison. PDB codes: USP7 1NB8²; USP8, 2GF0⁴.

Crystallization of USPs in complex with ubiquitin has helped shed light on the conformational changes occurring within the catalytic domain upon ubiquitin binding. Ubiquitin-aldehyde bound structures helped our understanding of the stabilization of the usually unstable oxyanion tetrahedral intermediate formed during the catalysis of an ubiquitin conjugate. In these structures, the oxyanion intermediate is mimicked by the additional oxygen on Gly76 of the ubiquitin-aldehyde. The negative charge is stabilized via hydrogen bonding to the amide

backbone of the catalytic cysteine and neighbouring Asp and Asn residues in the catalytic cleft^{2,11}.

In USP7 ubiquitin bound structure, the catalytic triad was in a productive conformation, with the cysteine in close proximity to the catalytic histidine. The rearrangement of the catalytic residues coincided with large changes in the active site region and a nearby loop termed the Switching Loop (SL)⁹⁶. Furthermore, the catalytic cleft of unbound USP14 was found to be partially obstructed by two loop regions termed Blocking Loop 1 (BL1) and Blocking Loop 2 (BL2). These flexible loops are highlighted in figure 1-9. When bound to ubiquitin, these loops translocated over 3-5 Å and facilitated accommodation of the ubiquitin C-terminal tail into the catalytic cleft⁹³. Unbound USP8 revealed a closed S1 pocket, caused by the finger region appearing to be tightened in towards the catalytic cleft.

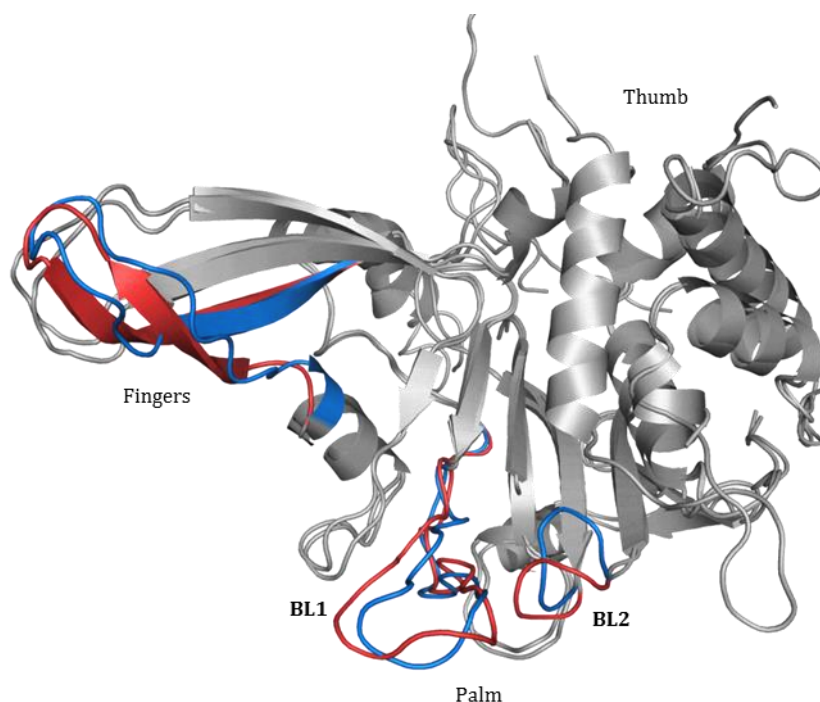


Figure 1-9: Example of conformational changes in the USP catalytic domain upon ubiquitin binding. The free and bound structures of USP14 demonstrate the significant rearrangement required in the BL1 BL2 and finger region to accommodate ubiquitin in the S1 pocket. Mobile regions are shown in blue (free) and red (bound). PDB codes: Free USP14, 2AYN; bound USP14 2AYO.

Ubiquitin binding is thought to induce a large structural shift in the finger region, and may serve as additional layer of regulation for USP8⁴. Similar conformational changes are thought to occur in USP4. In the free form, the S1 pocket is blocked by a closed finger region, and the BL1, and BL2¹⁴ impinge on the catalytic cleft.

Despite the inactive conformations observed for these enzymes in crystal structures, they have been shown to be active against ubiquitin substrates in solution. Cooperative binding is suggested to be the most likely mechanism, whereby initial substrate binding induces conformational changes. Inability of USPs to bind C-terminal peptides of ubiquitin and ubiquitin truncations corroborates this theory^{4, 97, 98}. However, this may not universally apply to the USP family, as USP5 has been shown to cleave LRGG peptides⁹⁹. In addition, intramolecular domains can instigate conformational rearrangements and enhance catalytic activity in some USPs^{14, 46, 96, 98}.

1.4.1. Regulation of DUBs

1.4.1.1. Regulation of DUB localisation and abundance

Appropriate DUB responses are regulated by modifying catalytic activity, localization and cellular quantity. In some cases, the DUB is only expressed when required. For example, USP1 is associated with DNA repair and expression is cell cycle dependent¹⁰⁰.

Subcellular localization of DUBs is important in determining the access to target proteins and subsequent activity. USP30 harbours a transmembrane domain and has been shown to localise to the outer mitochondrial membrane^{101, 102}. Other DUBs localise to specific protein complexes, a well characterised example is USP14 which is bound to the proteasome¹⁰³. Some interaction partners of DUBs govern cellular localisation. Nuclear import of USP15 and USP4 is facilitated by squamous

cell carcinoma antigen recognized by T-Cell 3 (SART3) allowing access to their respective substrates within the nucleus¹⁰⁴⁻¹⁰⁶. USP8 association with epidermal growth factor (EGF) mediates its translocation to endosomes¹⁰⁷. Localisation can be directed by posttranslational modifications. Phosphorylation of USP10 results in nuclear import, while phosphorylation of USP4 leads to nuclear export^{108, 109}.

1.4.1.2. Regulation of catalytic activity

Posttranslational modifications are a very effective and dynamic method of regulation DUB activity.

Phosphorylation of deubiquitinating enzymes can result in both activation and deactivation of DUB activity. USP8 associates with 14-3-3 proteins in a phosphorylation dependent manner, inhibiting its DUB activity¹¹⁰. CYLD is prevented from deubiquitinating TNF receptor-associated factor 2 (TRAF2) through phosphorylation mediated regulation¹¹¹. Phosphorylation mediated activation has been observed for USP37, A20, and USP7, prolonging substrate deubiquitination¹¹²⁻¹¹⁴. Indirect activation of DUB activity arises from phosphorylation sites permitting interactions with activator proteins¹¹⁵.

Further posttranslational modifications such as sumoylation, ubiquitination and proteolytic processing can regulate DUB activity^{116, 117}. USP30 has been found to be ubiquitinated by the E3 ligase parkin in the fingers region, which may compete with substrate binding to the distal pocket and reduce USP30 activity¹¹⁸. USP1 undergoes auto-proteolysis after exposure to U.V ¹¹⁹, and proteolytic cleavage of CYLD leads to protein destabilisation ¹²⁰.

DUB activity may be instigated or enhanced via allosteric interactions with their ancillary domains. This has been particularly well studied in USP7, whereby the

catalytic domain shows very little DUB activity in comparison to the full-length protein. The two C-terminal ubiquitin like (Ubl) domains in cooperation with a C-terminal activation peptide dramatically enhance protein activity by stabilising the bound conformation of the enzyme and making this conformation more favourable. The N-terminal domain in USP (DUSP) and Ubl domains have been shown to enhance USP4 activity by reducing ubiquitin retention in the catalytic domain ^{14, 46}.

Ubiquitin can be subject to phosphorylation on several serine residues ¹²¹⁻¹²³. DUBs have been found to be less active against this modified ubiquitin, but the physiological relevance of this modified ubiquitin is still under investigation ¹²⁴.

Reversible oxidation has been shown to be a mechanism of regulation of DUB family members. The different oxidation states of Dub enzymes are summarised in figure 1-10. High levels of reactive oxygen species (ROS) in cells lead to oxidative stress, a common denominator of diseases such as ageing, cancer and Alzheimer's disease ¹²⁵⁻¹²⁷. More recently, ROS has been implicated in having crucial cell regulatory roles ¹²⁸. Lee et al tested 34 different DUBs encompassing several subfamilies and were able to group them into three categories (see table 1), highlighting the variation of oxidation susceptibility within the DUB family.

Group 1 No activity detected		Group 2 Activated by DTT		Group 3 Enhanced by DTT
USP1	USP52	USP3	USP19	USP5
USP13	USP53	USP4	USP21	USP7
USP14	JOSD2	USP8	USP25	CYLD
USP22	YOD1	USP10	USP26	UCHL1
USP28	OTUD7B	USP11	USP29	UCHL3
USP44	A20	USP15	USP37	UCHL5
USP48	VCPIP1	USP16		BAP1

Table 1: Effect of DTT on activity of DUBs. DUBs were categorised into three groups based on their activity in the presence of DTT. Group 1 showed no activity even in the presence of DTT. Group 2 showed very weak activity which improved significantly by the addition of DTT. Group 3 were active even in the absence of DTT but addition of the reducing agent enhanced activity¹²⁹.

DUBs are susceptible because of the low pKa associated with the active site cysteine. The cysteine can be oxidised into several different oxidation states. Modification to a sulphenic acid intermediate (SOH) is reversible with reducing agents whilst higher oxidation states, such as sulphinic (SO₂H) or sulphonic acid (SO₃H), are irreversible. Disulphide bridges and sulphenyl-amide formation are another form of reversible oxidation and can serve to protect the cysteine from undergoing irreversible oxidation¹³⁰.

Kulathu et al identified a possible mechanism of action for a reversible regulation of DUBs¹³⁰. They conducted a structural investigation into the mechanism of reversible oxidation observed for the DUB A20. They were able to show the reversible intermediate, Cys-SOH, was stabilised via a hydrogen bond between the sulphinic acid group and the backbone of the preceding loop. They demonstrated that cysteine modified with higher oxidative states formed an additional hydrogen bond with a neighbouring histidine. They postulated the stability of the Cys-SOH intermediate likely protected the cysteine from further oxidation. Furthermore, the authors highlighted the concentration range of H_2O_2 which reversible oxidation occurred (10-100 μ M) coincides with the physiological range of H_2O_2 in the cell.

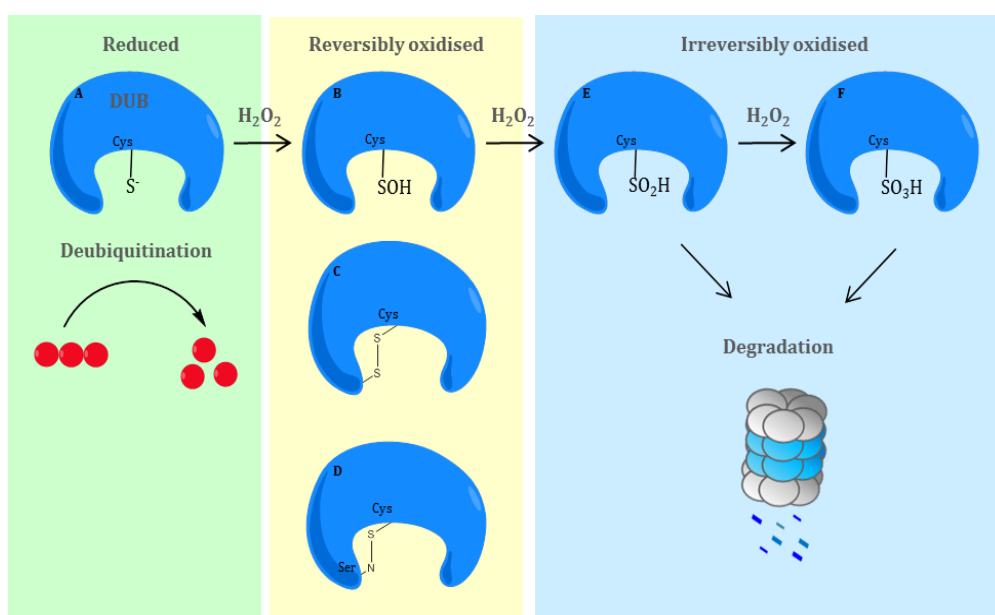


Figure1-10: Redox regulation of deubiquitinating enzymes. Schematic representation of the different oxidation states upon exposure to H_2O_2 . The reduced DUB has deubiquitinating activity against polyubiquitin chains (A). Oxidation by a reactive oxygen species such as H_2O_2 can lead to the formation of sulphenic acid (B) a disulphide bridge (C) or a sulphenylamide (D) on the catalytic cysteine. These oxidative states inhibit the enzyme but can be reversed by the presence of a reducing agent. Further oxidation yields sulfinic acid (E) or sulphonic acid (F) which are both irreversible modifications. Adapted from Clague ⁷.

Furthermore, Lee et al used USP19 as a model DUB for probing into the mechanism of redox regulations in DUBs and discovered an alternative mechanism. They revealed the catalytic cysteine may form a sulphenyl-amide intermediate with a neighbouring serine residue rendering the enzyme reversibly inactive. Serine residues in this position are reasonably well conserved throughout the USP family⁸⁸ and the authors propose that mechanism of redox regulation exploited by USP19 may occur in other USP family members ¹²⁹.

Several diseases such as cancer diabetes and chronic inflammation have been associated with high levels of ROS within the cell. Regulation of DUBs through oxidation may be a fundamental part of cell signalling and disease progression. The disordered architecture of USP7 catalytic triad may serve to protect against irreversible oxidation.

1.4.2. Substrate specificity among the DUB family

As discussed in section 1.2. DUBS specificity for ubiquitin chains over other Ubbs arises from recognition of the RLRGG motif at the C-terminus of ubiquitin. Moreover, the hydrophobic patches on the ubiquitin surface, Ile36 patch and Ile44 patch, differentiate ubiquitin from ubiquitin-like modifiers and mediate DUB specificity for ubiquitin ⁹¹. DUBs exert their regulatory effect on ubiquitination by binding directly to a target protein or a ubiquitin chain where they exert their deubiquitinating activity.

Overall, the USP family is very promiscuous and can cleave most chains types, and chain specificity is uncommon. The specificity displayed by some USPs for certain chain linkages has only been understood on a molecular level for USP30 and CYLD. USP30 has been found to be anchored in the mitochondrial membrane ¹⁰² and preferentially cleaves Lys6 linked polyubiquitin chains ¹⁰¹. Biochemical and structural analysis of the catalytic domain revealed a weakened affinity for the

distal ubiquitin in comparison to other USP catalytic domains. USP30 is also unusual with respect to its catalytic triad, harbouring a serine in place of an Asn/Asp seen in the majority of USPs. The catalytic serine, along with other residues unique to USP30, mediates chain selectivity through interactions with the proximal ubiquitin¹³¹. Similarly, CYLD chain specificity is mediated by β -hairpin loop found in the vicinity of the catalytic site which dictates the orientation of the proximal ubiquitin^{132, 133}. A high affinity distal ubiquitin binding site and lack of a proximal binding site is a likely rationale for lack of distinct chain selectivity amongst other USPs^{46, 91, 134}.

1.4.2.1. Isopeptide vs. peptide linkages

Isopeptide bonds are fundamental to the deubiquitinating system and arise through the conjugation of the carboxyl group on the C-terminal glycine to the lysine ϵ -amino group in the target protein. Overall, Met1 linked di-ubiquitin and Lys63 linked di-ubiquitin adopt a similar conformation and both display the same degree of flexibility in orientation of the ubiquitin monomers, however DUBs could still distinguish between chain types²⁰. Studies have shown USPs were able to cleave linear di-ubiquitin chains but showed much higher efficiency towards Lys63 linked chains^{20, 46, 134}. The different bond types have different chemical features (see figure 1-11). For example, the isopeptide bond has 5 freely rotatable bonds and therefore no conformational restraints in contrast to the peptide bond

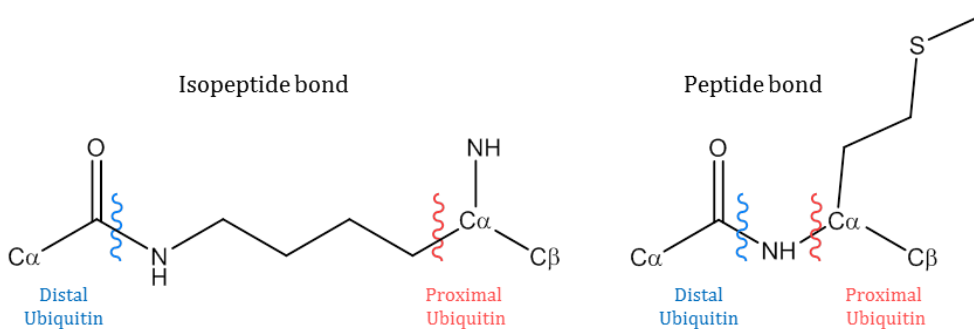


Figure 1-11: Differences in chemical features between the isopeptide and peptide bond.

which is much more restricted. Additionally, the following side and carboxyl group in the Met1 linked chain require more space in the active site²⁰.

1.4.2.2. Endoproteolytic and exoproteolytic activity of DUBs

DUBs can cleave ubiquitin from the end of a chain (exo) releasing monoubiquitin or within a polyubiquitin chain (endo) yielding free polyubiquitin chains (figure 1-12). USP5 has been shown to process free polyubiquitin chains from the proximal end⁹⁰. For endo-deubiquitination to occur, the DUB must be able to accommodate both a distal and proximal ubiquitin whilst exoproteolytic cleavage only involves the binding of one ubiquitin.

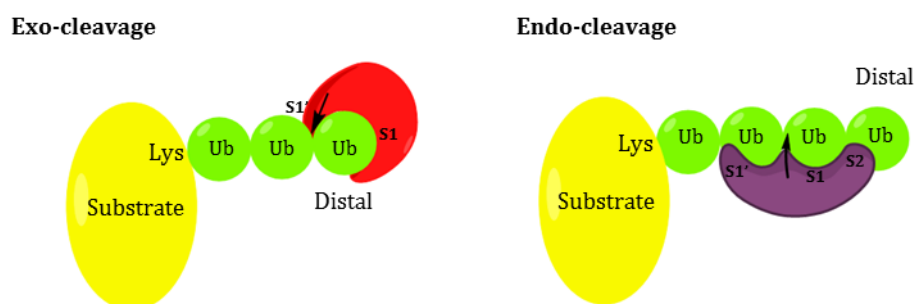


Figure 1-12: Schematic representation of endoproteolytic and exoproteolytic cleavage by DUBs. Ubiquitin molecules either side of the cleavage site must be accommodated and additional ubiquitin binding pockets such as an S2 pocket may permit this type of cleavage. Adapted from Komander et al¹⁰.

The type of cleavage, in part, is dictated by chain type. USP21 processes Lys6-linked polyubiquitin via exoproteolytic activity and Lys63 linked chains via endoproteolytic activity^{91, 135}. When ubiquitin is bound within the S1 pocket of the USP catalytic domain, Lys63 is unobstructed, additional ubiquitin molecules within the chain would be accommodated, allowing endoproteolytic cleavage to occur. Conversely, Lys6 is buried, and this linkage type lends its self to exoproteolytic cleavage^{91, 136}. USP14 exhibits exoproteolytic activity only. The finger region present in most USPs, blocks access to other Lys48 linked ubiquitin, preventing endoproteolytic cleavage of these chain types. In contrast, CYLD lacks the fingers domain and is capable of cleaving in the middle of polyubiquitin chains^{132, 133}.

Surprisingly, DUBs have been shown to be capable of cleaving branched ubiquitin chains, despite the multiple positions of ubiquitin modifications⁴⁵. Furthermore, additional ubiquitin binding pockets have been shown for some DUBs. USP21 harbours an S2 binding site located on the back of the fingers domain⁹¹, and S2/S3 sites have been determined in USP5⁹⁰. As polyubiquitin probe tools develop endoproteolytic and exoproteolytic processing selectivity of deubiquitinases will be become better characterised.

1.5. Available Tools for studying DUBs

Modified ubiquitins have been instrumental in characterising the binding mechanism and enzymatic activity of DUBs. The fluorogenic substrate ubiquitin-AMC, whereby the C-terminus of ubiquitin has been modified with fluorophore 7-amino-4-methylcoumarin, was one of the first fluorogenic substrates developed, and is still widely used for DUB activity assays (figure 1-13 A). Prior to this the gly76 ethyl ester of ubiquitin (Ub-OEt) (figure 1-13 B) was used however it was very limiting due to a lack of sensitivity and the inability for continuous monitoring of DUB activity ^{137, 138}.

Ubiquitin based activity probes are equipped with C-terminal chemical warheads, such as aldehyde (figure 1-13 C)¹³⁹, vinylmethylester (Ub-VME) (figure 1-13 D)¹⁴⁰, vinylsulfone (Ub-VS)¹⁴¹ (figure 1-13 E) and propargyl (Ub-prg)¹⁴² (figure 1-13 F). These reagents, have allowed extensive biochemical characterisation of DUB activity and mechanisms. The probes covalently modify the catalytic cysteine forming a covalently linked DUB-Ub complex. USP14 is specifically inhibited by Ub-VS, pivotal in the discovery of USP14 as a 26 proteasome associated DUB ¹⁴¹.

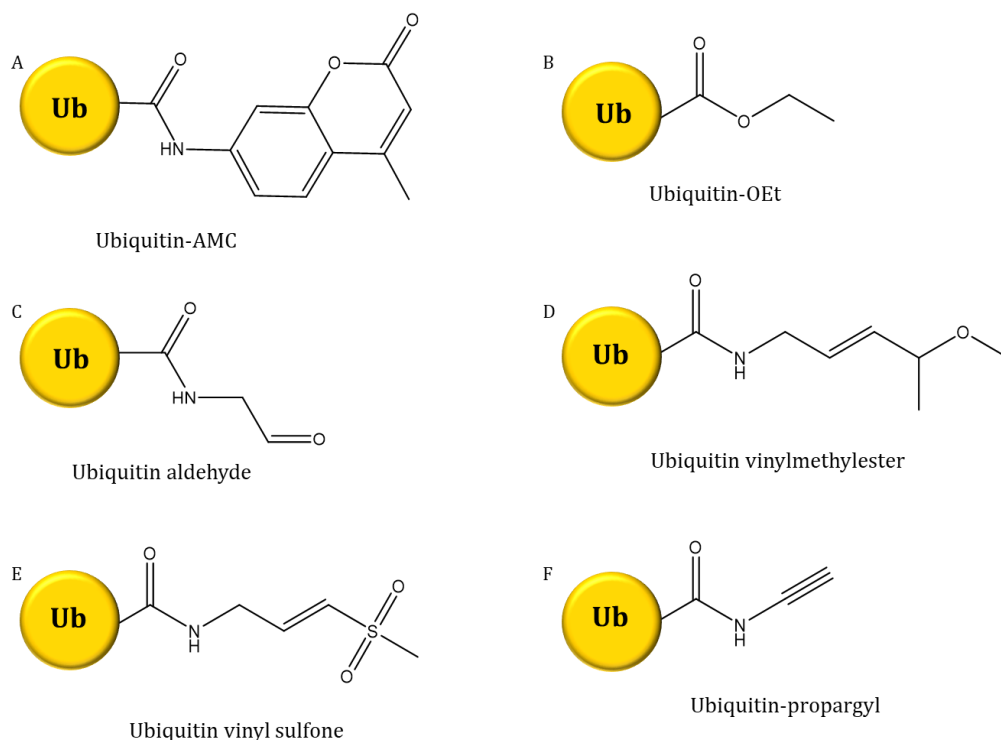


Figure 1-13: Schematic representation of commonly used ubiquitin probes in deubiquitinating enzyme investigations. A: Ubiquitin 7-amino-4-methylcoumarin, (Ub-AMC). B: Gly76 ubiquitin ethyl ester, (Ub-OEt). C: Ubiquitin aldehyde. D: Ubiquitin vinylmethylester, (Ub-VME). E: Ubiquitin vinylsulfone (Ub-VS). F: Ubiquitin propargyl

Whilst these activity based probes have been critical for enhancing our understanding of the DUB family, they are limited in the information they can provide with respect to chain linkage and target specificity because of the lack of a proximal substrate. More recently, di-ubiquitin activity probes have been developed with an electrophilic group located on the linker between the proximal and distal ubiquitin which serves to trap the DUB in the S1-S1' bound orientation¹⁴³⁻¹⁴⁵. However even these probes are limited because they do not provide information on additional ubiquitin binding sites in DUBs related to chain processing. At present, knowledge of S2 and further ubiquitin binding sites in USP catalytic domains is very limited, with USP21 and USP5 the only enzymes with an identified S2 binding site so far^{90,91}. C-terminal propargyl di-ubiquitin probes have since been developed to try and address this¹⁴⁶.

As briefly mentioned in section 1.1.2, synthesis of atypical ubiquitin chains has only become available very recently. The chain types can be made both fully synthetically and semi-synthetically ¹⁴⁷. Synthetic methods of ubiquitin and ubiquitin conjugates has opened many doors in the field of ubiquitin study however is it beyond the scope of this thesis and will not be discussed any further.

Semi-synthetic strategies utilise expressed protein as the starting material. Cysteine residues do not naturally occur in ubiquitin and addition of a cysteine to ubiquitin, either in place of a lysine or at the C-terminus, can be exploited for site-specific addition of labels, chemical reactive groups, a fluorogenic dye, and other ubiquitin moieties.

Wang et al, who used ubiquitin activation reaction catalysed by the universal E1 ligase to modify ubiquitin, demonstrate a very simple example of this. This method uses aminolysis of the E1-Ub thioester linkage (figure 1-14) to generate C-terminally modified fluorescent based ubiquitin substrates and di-ubiquitin mimics.⁵¹ Ubiquitin can be linked to fluorophores via an isopeptide bond mimic using this methods and may be better hydrolysed by some DUBs ⁹⁸. Furthermore, diubiquitin substrate mimics were generated via thiol-ene photo-click chemistry

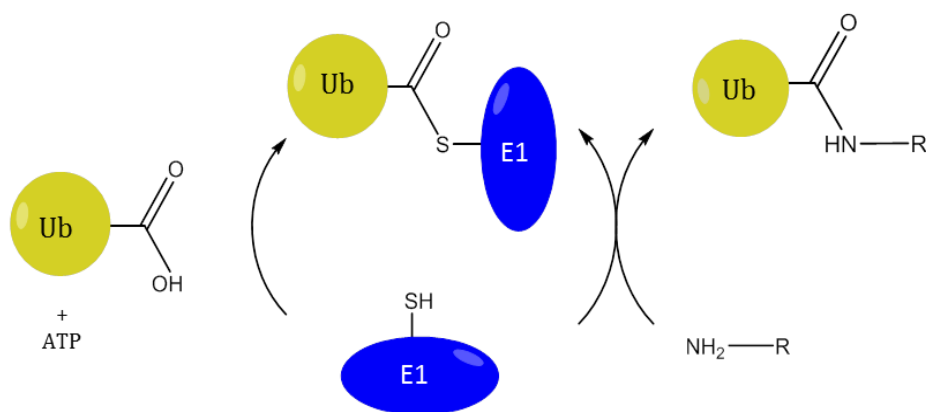


Figure 1-14: Schematic representation of E1 catalysed ubiquitin c-terminal amidation for synthesis of deubiquitinase substrates. Ubiquitin is activated using the E1 enzyme, and aminolysis is utilised to incorporate different functional groups indicated by R.

using a Lys-Cys ubiquitin mutant and a ubiquitin variant product of the E1 amidation reaction ^{51, 148}.

Genetically encoded orthogonal protection and activated ligation (GOPAL) has been an instrumental technique in the synthesis of atypical chains. This method utilises cellular machinery to incorporate site specific modified lysine residues (such as δ -thiol-L-lysine) which can undergo native chemical ligation and desulfurization with a ubiquitin thioester to yield ubiquitin conjugates linked via a native isopeptide bond¹⁴⁹.

Collectively these methods have allowed development of linkage specific di-ubiquitin FRET molecules, allowing for further characterisation of the processing of different chain types ¹³⁸.

1.6. The DU subfamily

The DU family of USPs comprises of USP15, USP11 and USP4, which share similar structure and functions and are evolutionarily related. Within the DU family, USP4 and USP15 are closely related, sharing an overall sequence similarity of 56.9 % whilst USP11 is more distantly related with a sequence similarity of 48%. This sub family share the same overall domain architecture, and is distinguished by their DUSP followed by a ubiquitin-like (Ubl) domain. The DUSP-Ubl region is termed the DU domain and is connected to the catalytic core via a short linker known as the DU finger¹⁵⁰. The catalytic core consists of two catalytic halves with a large insertion (~300 residues) between residues 440-756 comprising a second Ubl (Ubl2).

A specific function of the DU domain in this family of enzymes has yet to be described; however, its importance in mediating protein interactions is becoming

clearer. The USP4 DUSP-Ubl domains form a dimer in the crystal structure but it is thought USP4, USP15 and USP11 are monomeric *in vivo* ¹⁵¹.

USP15 and USP4 share some functional overlap however; shared substrates amongst these enzymes are uncommon. Interestingly, viability in mice is dependent on a functional copy of USP15 or USP4 ¹⁵⁰. TGF- β type 1 receptor activity is regulated by all three DU members albeit on different substrates. USP15 antagonises E3 ligase activity promoting TGF- β receptor stability, as well has deubiquitinating downstream effectors, prolonging TGF- β mediated signalling events ^{152, 153}. USP4 and USP11 also regulate TGF- β signalling interacting with different components within the pathway ¹⁵⁴. USP4 and USP15 are both implicated in mRNA splicing, interacting with different components of the spliceosome ^{155, 156} as well as innate immune signalling ^{157, 158}.

Unusually, USP4 and USP15 were both shown to bind to SART3, an interaction that is mediated by the DU domains. Differences in residue composition in the DU finger means that USP11 does not bind SART3, and the interaction is specific to USP4 and USP15 ^{105, 106}. SART3 serves to shuttle USP4 and USP15 from the cytoplasm into the nucleus. Interestingly, once in the nucleus the paralogues target different substrates, with USP15 deubiquitinating free ubiquitinated histone H2B ¹⁰⁴ whilst USP4 targets components of the spliceosome ¹⁵⁶.

USP15 and USP4 display similar sigmoidal kinetic behaviour *in vitro*. The DUSP-UB and catalytic insert are required for full USP4 catalytic activity and removal of these domains leads to high product retention and reduced catalytic turnover. Interestingly the USP15 catalytic domain is less hampered by slow product discharge, and only showed a slight reduction in catalytic activity upon removal of the DUSP-Ubl domains ¹⁴.

1.6.1. USP15

USP15 is a 109.3 kDa protein (UniProt code: Q9Y4E8 (isoform2)) involved in a variety of cellular signalling events. As described above USP15 harbours an N-terminal DUSP-Ubl domain and a second Ubl domain inserted between the two catalytic sub-domain halves. The N-terminal DUSP-Ubl has been solved via X-ray crystallography (PDB: 3T9L, 3PPA) but structural information for the catalytic core is unavailable^{151, 159}. Studies of USP15 behaviour in solution using techniques such as analytical ultracentrifugation and SAXS have confirmed USP15 is a monomer in solution.

1.6.1.1. Implications of USP15 in disease pathogenesis

More than 30 USPs have been associated with cancer. A table summarizing the USPs associated with different cancers can be found in a review by Pal et al¹⁶⁰.

USP15 has been shown to be involved in signalling pathways with reported oncogenic activity. Gene amplification of USP15 has been identified in glioblastoma, ovarian and breast cancer and is correlated with poor prognosis in glioblastoma patients. It was hypothesised that USP15 is required for the invasiveness and proliferation of glioblastoma multiform cells.^{152, 153, 161, 162}

Inui et al discovered USP15 to be an important DUB associated with the regulation of Transforming growth factor β (TGF- β) signalling¹⁵³, summarised is in figure 1-15. TGF- β is a secreted cytokine and a key regulator of tissue homeostasis. TGF- β and its associated signalling pathway has been identified to have oncogenic properties, inducing cell invasion and metastasis, and high levels of TGF- β have been associated with aggressive glioblastomas in the brain^{162, 163}. TGF- β binds to a heterodimeric complex consisting of TGF- β receptor 1 and 2 (T β R-I and T β R-II) on the cell membrane, initiating the intracellular cascade of phosphorylation and

subsequent heterodimerization of receptor regulated SMADS (R-SMADS) with SMAD4 within the cytoplasm, permitting their translocation into the nucleus where they alter gene activation and transcription¹⁶⁴.

Ubiquitination of R-SMADS serves as another layer of regulation of SMAD activity in addition to phosphorylation. Monoubiquitination of SMAD3 is catalysed by the E3 ligase SMURF2, inhibiting dimerization and hampering its ability to interact with DNA and acts as a regulatory step in the signalling of the pathway. USP15 was shown to reverse this ubiquitination allowing further R-SMAD activity ¹⁵³. It should however be noted that Al-Salihi et al, who investigated the role of USP11 in TGF- β signalling, were unable to detect USP15 interactions with any SMADs¹⁵⁴.

Additional negative feedback loops mediate the activity of the TGF- β pathway. Activation of the TGF- β pathway induces the transcription and translation of SMAD7. SMAD7 recruits the E3 ubiquitin ligase SMURF2 to the TGF- β receptor complex on the cell membrane, instigating ubiquitination and proteasomal degradation of the complex ^{23, 165, 166}. Here, USP15 counteracts the activity of SMURF2 by removing the ubiquitin chains and prolonging the TGF- β signalling event ¹⁵². A high level of USP15 activity gives rise to prolonged TGF- β signalling and is thought to induce oncogenesis in some tumours through the upregulation of the TGF- β pathway¹⁵².

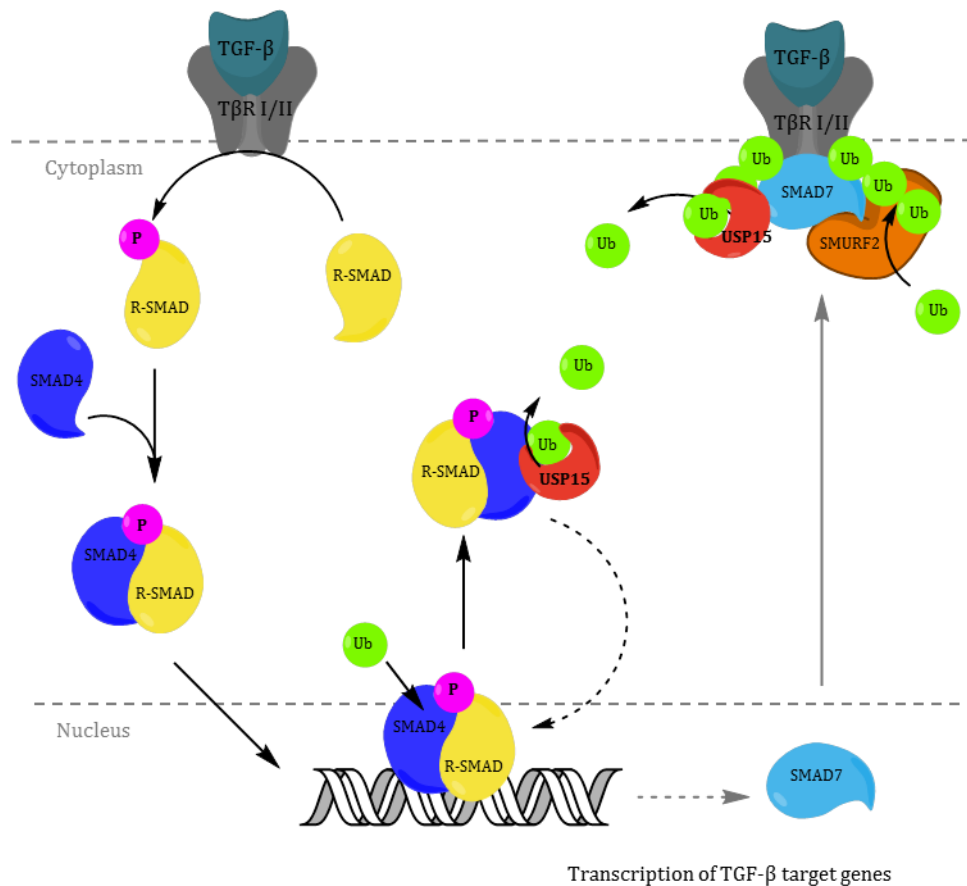


Figure 1-15: USP15 regulation of the TGF- β signalling pathway. TGF- β binds to receptors on the cell surface initiating phosphorylation of cytosolic R-SMADS. Phosphorylated SMADs dimerize with SMAD4 and translocate into the nucleus where they bind to DNA initiating activation or repression of gene transcription. Binding of R-SMADS to DNA leads to monoubiquitination of the SMAD complex which is reversed by USP15 prolonging the TGF- β signal. Activation of the TGF- β pathway leads to production of SMAD7, which recruits the E3 ligase SMURF2 to the TGF- β receptor resulting in ubiquitination and proteasomal degradation of the receptor. USP15 counteracts SMURF2 by removing ubiquitin chains from the receptor, again prolonging the TGF- β signal.

However, more recently USP15 has been shown to mediate cross talk between TGF- β pathway and p53 activity^{167, 168}. The p53 pathway is pivotal in controlling cell proliferation, and activation of the pathway upon oncogenic stress induces cell cycle arrest and apoptosis. Careful regulation of p53 activity is critical in ensuring the correct amount of cell growth and proliferation occurs. Cellular levels of p53 are tightly regulated by ubiquitination/deubiquitination¹⁶⁹.

Lui et al showed USP15 could directly form a complex with p53, stabilising the tumour suppressor via its deubiquitinating activity. They also established an increase in USP15 expression was induced by the TGF- β signal, promoting the stability of p53 ensuring TGF- β induced tumour suppression ¹⁶⁷. On the contrary, a very recent study by Padmanabhan et al found no regulatory role of USP15 on WT p53 but identified a role for the DUB in the stability of p53-R175H mutant commonly found in ovarian cancers ¹⁷⁰. The role of USP15 in mediating TGF- β signalling is complex and may serve as switch between TGF- β induced tumour suppression and tumour promotion.

One of the key regulators of p53 activity is mouse double minute 2 homolog (MDM2). MDM2 exhibits E3 ligase activity, ubiquitinating p53 predominantly on six lysine residues located at the C-terminus of p53 targeting it for degradation by the proteasome ¹⁷¹. MDM2 can also target p53 for nuclear export through monoubiquitination, sequestering its transcriptional activity and releasing it for further modification and degradation¹⁷².

Several USPs have been found to regulate p53 through a variety of both direct and indirect mechanisms. USP7 ^{173, 174}, USP2a ¹⁷⁵ and more recently USP15 has been found to deubiquitinate MDM2 thus increasing its stability and the rate of p53 degradation ¹⁷⁶ (figure 1-16).

Zou et al demonstrated USP15 mediated stability of MDM2 in both T-Cells and cancer cells. Loss of USP15 lead to the degradation of MDM2 and thus an increase in p53 activity. Furthermore, they demonstrated that NFATc2, a transcription factor involved in T-Cell activation, is negatively regulated through ubiquitination by MDM2. Consequently, an increased degradation of MDM2 was coupled with an increase in T-Cell activation. Based on these observations it is likely that USP15 inhibition would result in cellular apoptosis and T-Cell responses. This study

highlighted the potential of USP15 as both an anticancer target and immunotherapy target, of which a combination could be particularly effective for improved treatment outcomes.¹⁷⁶

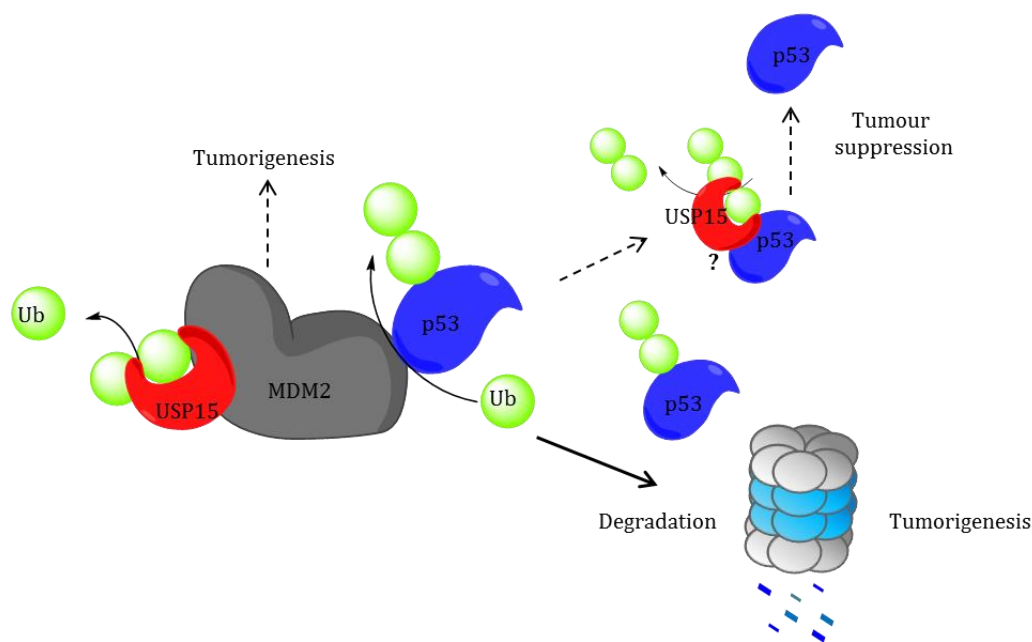


Figure 1-16: USP15 stabilises MDM2. MDM2 ubiquitinates p53 targeting it to the proteasome for degradation. USP15 interacts with MDM2 and promotes its stabilisation through its deubiquitinating activity. USP15 may also bind directly to p53 and protect it from degradation through deubiquitination; however, this is still unclear (indicated by the question mark). Figure was created using ChemBioDraw software.

The constitutive photomorphogenesis 9 signalosome (CSN) is a large multi subunit protease and a key player in the ubiquitin-proteasome-system, which has multiple roles in the regulation cancer associated pathways¹⁷⁷. The CSN regulates the activity of cullin-ring complexes (CRLs)¹⁷⁸. USP15 was shown to associate with CSN5, a component of the CSN, and reverse auto-ubiquitination of components of the CRLs, promoting the stability of the CRL complex^{179, 180}. In addition, the cooperation of USP15 with the CSN has been shown to target USP15 activity towards I κ B α with possible role in preventing its degradation¹⁸¹.

USP15 has shown further oncogenic activity through its regulation of the stability of the human papillomavirus (HPV) E6 protein 9 (figure 1-17) . The E6 protein is prevalent in HPV positive cancers such as human cervical head, neck and cutaneous cancers¹⁸². Degradation of the tumour suppressor p53 is fundamental to the life cycle of the HPV and is achieved through cooperation with cellular E6-AP, an E3 ubiquitin ligase ^{183, 184}. The E6 protein associates with E6-AP¹⁸⁵, targeting it to p53 inducing ubiquitination and proteasomal degradation of the tumour

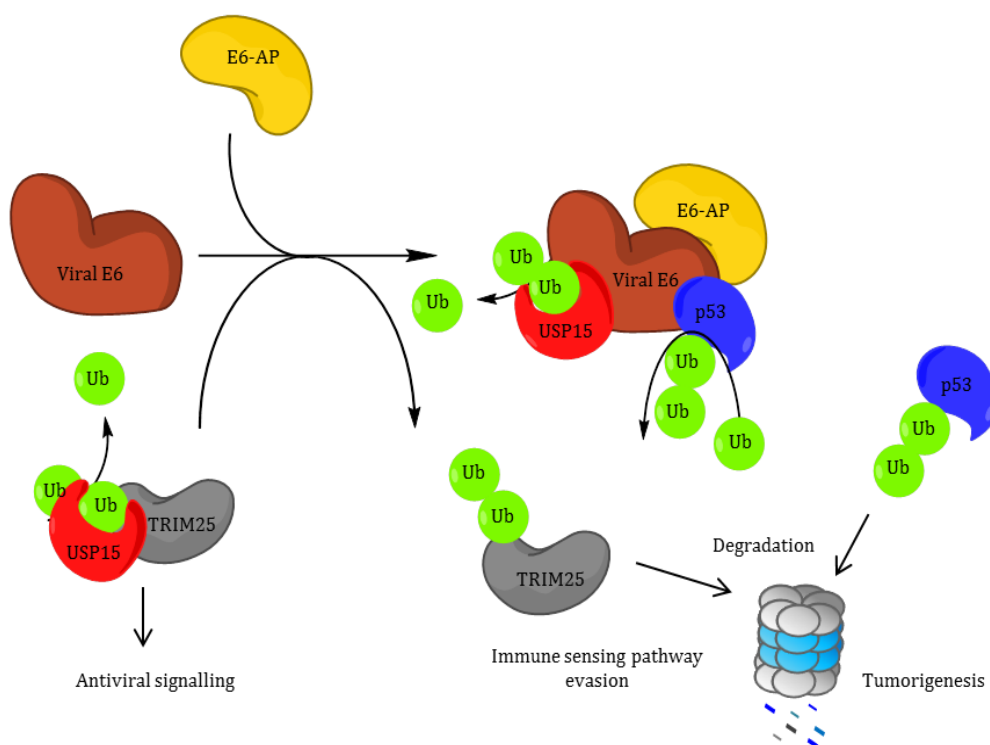


Figure 1-17: A viral E6 protein evades the host immune system and can promote tumorigenesis. USP15 stabilises TRIM25 and promotes antiviral activity. The HPV E6 protein associates with USP15 and E6-AP, disrupting the USP15-TRIM25 complex leading to degradation of TRIM25. Consequently the viral E6 remains hidden from the hosts immune system and is stabilised by interactions with USP15. The E6 protein targets p53, leading to its degradation and tumorigenesis. Figure was created using Chemdrawbio.

suppressor¹⁸⁶. Consequently, the presence of the E6 protein disrupts the cell cycle and apoptosis control promoting extended and uncontrolled proliferation and eventual transformation of cancerous cells¹⁸⁷.

USP15 was shown to stabilise the E6 oncoprotein in the cell, whereby silencing of USP15 lead to a significant reduction of E6 protein levels and overexpression of

the DUB lead to an increase in E6 protein¹⁸⁸. Furthermore, USP15 interacts with TRIM25 during viral infections permitting a cascade of events, which contribute to initiation of the host antiviral immune response ¹⁸⁹. Targeting of the E6 oncoprotein to the USP15-TRIM25 complex, is believed to disrupt this pathway and play a role in the evasion of detection by immune-sensing pathways¹⁵⁷.

USP15 expression levels have been shown to correlate positively with sensitivity to the anti-tumour agent paclitaxel, with the implication that cellular USP15 levels could be used as an indicator for the potential occurrence of chemoresistance cancer cells and treatment success¹⁹⁰.

Collectively the information available points towards USP15 being a novel therapeutic target in the treatment of cancer, however it is evident USP15s role in tumour suppression and oncogenesis is still unclear and needs further clarification.

USP15 has been implicated in having a role in Parkinson's disease. Parkin, an E3 ligase, ubiquitinates the outer mitochondrial membrane proteins and is a key player in the induction of mitophagy ^{64, 191}. A common cause of recessive Parkinson's disease is loss of Parkin resulting in the accumulation of damaged mitochondria ¹⁹². USP15 has been shown to antagonise Parkin activity and inhibition of USP15 in cells where Parkin activity is deficient appears to restore Parkin-mediated mitophagy ¹⁹³. Interestingly, decreased Parkin activity has been found in glioblastomas and the USP15-Parkin relationship may also be relevant in oncogenesis ^{194, 195}.

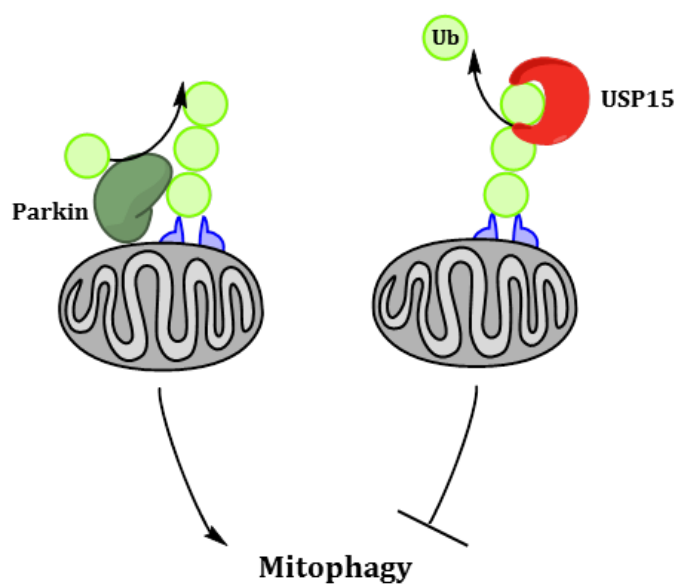


Figure 1-18: Schematic representation of the relationship between USP15 and Parkin. Drawn using Chembiodraw software.

1.7. Small molecule inhibition of USPs

Targeting USPs for therapy requires small molecule inhibitors to reduce or abolish their activity. As such, there is limited availability, particularly of potent selective USP compounds and there is currently no selective USP15 compound available.

Early compounds found to have inhibitory activity were often not very potent and often exhibited poor stability, low selectivity and undesirable physicochemical properties. Examples of broad spectrum USP inhibitors are shown in figure 1-19, and include PR-619, which was found to be selective for DUBs over other cysteine proteases but showed no selectivity amongst enzymes of the DUB family¹⁹⁶. Small molecule inhibitor HBX 41,108 forms covalent interactions with the catalytic cysteine and was also found to be a potent but non-selective amongst the USP family¹⁹⁷. Selective inhibition of USP14 and UCHL5 was achieved using b-AP15, a proteasome inhibitor, which lead to an increase in cell apoptosis^{87, 198}. Selective inhibition of USP7 was achieved using the compound P5019¹⁹⁹.

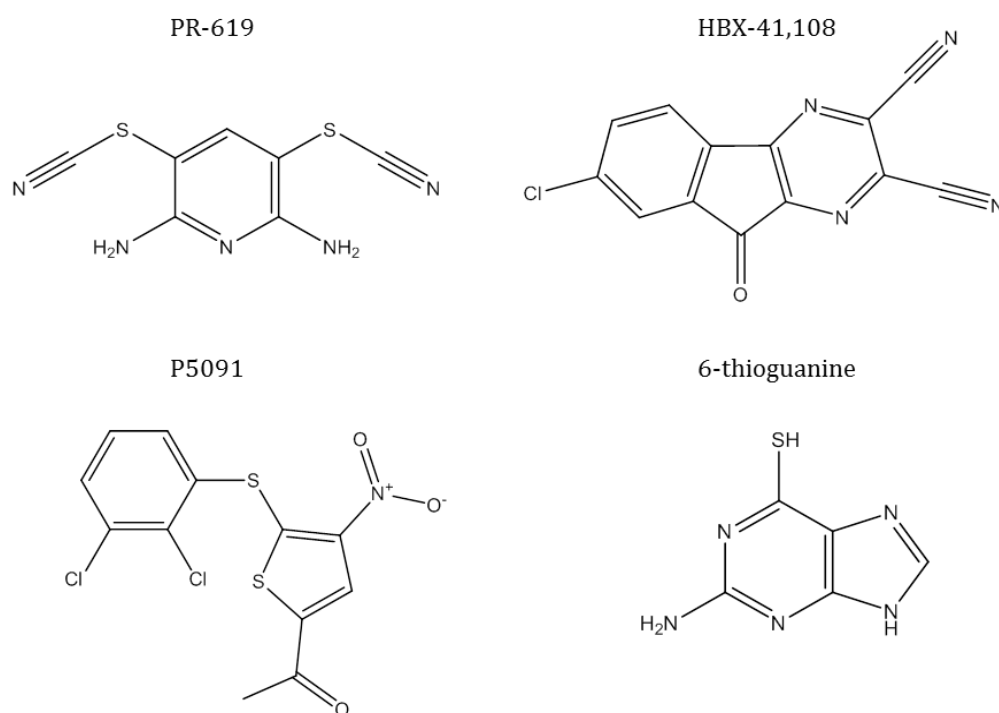


Figure 1-19: Examples of DUB inhibitors. PR-619 and HBX 41,109 are non-specific inhibitors whilst P5091 and 6-thioguanine are selective for USP7 and USP2. respectively⁹

Gavory et al²⁰⁰, obtained structures of small molecule inhibitors bound to USP7 and showed the compounds were both potent and highly selective *in vitro*. These inhibitors were found to be non-competitive working through a novel allosteric binding site. The co-crystal structures revealed conformational changes within the vicinity of the active site, which were postulated to stabilise the inactive conformation. The inhibitors also protruded into the ubiquitin C-terminal tail binding channel, possibly interfering with the alignment of the c-terminal tail and thus preventing catalysis. Selective inhibitors for USP30 have been developed (selective for USP30 over USP1 USP8 and USP9) although the authors did not suggest a potential mechanism of action for these compounds²⁰¹.

Chuang et al identified the FDA approved drug 6-thioguanine (6TG) as an inhibitor of USP2 with an IC₅₀ of $40 \pm 2 \mu\text{M}$. Similar to the USP7 inhibitors, 6TG was found to be a non-competitive inhibitor. By obtaining crystal structures of USP2-Ub-6TG, they were able to show a novel mechanism of USP inhibition. By comparing with the previously published USP2-Ub structure (PDB: 2HD5) the authors identified inhibitor induced conformational changes in the active site region. Asp575, thought to be important in oxyanion hole formation and aligning the catalytic histidine²⁰², was found to have shifted by $3.2 \text{ \AA}$. The catalytic cysteine was found to be engaged in a disulfide bridge with 6TG (figure 1-20) and this covalent modification of the catalytic cysteine was shown to be fundamental in the inhibition activity of 6TG. Chuang et al also tested 6MP, another anti-leukemia drug, and found it was ineffective against USP2 activity. They suggested the difference in activity arose from the additional amine group in 6TG, which formed a hydrogen bond with a glutamine side chain. Their data sheds light on a second non-competitive mechanism for inhibition of USPs by small molecule inhibitors,

and provides a strong argument for trialing 6TG as a therapeutic agent against cancers displaying upregulation of USP2. However, there is no evidence yet for the specificity of 6TG for USP2 over other USPs.

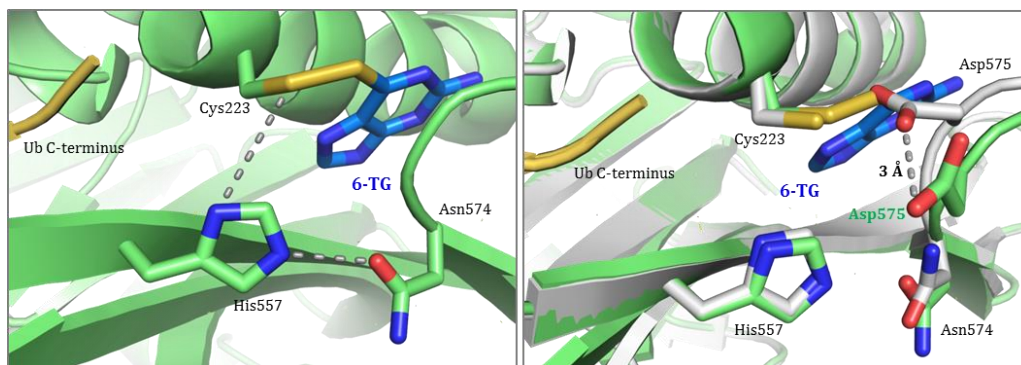


Figure 1-20: Inhibition of USP2 with 6TG. Left: 6-TG (blue) forms a covalent interaction with the catalytic cysteine. The catalytic triad is shown in stick representation. The C-terminus of ubiquitin (yellow) is visible on the left. Right: Same as before, super-imposed with uninhibited USP2 (white). The superposition reveals the movement of Asp575, a crucial residue for stabilising the oxanion intermediate during deubiquitination, caused by 6-TG. PDB codes: USP2-6TG, 5XU8; USP2, 2HD5.

1.7.1. Inhibition of USP11 by mitoxantrone.

Burkhart et al, adopted the approach of screening the use of FDA approved drugs to identify new inhibitors for members of the USP family. USP11, for which no selective inhibitor are available, was selected for the study because of its importance in the DNA damage repair pathway and is implicated in having a role in cancer cell survival. The authors tested 2000 compounds for inhibitor activity against USP11 and identified mitoxantrone to be a robust inhibitor of USP11 *in vitro* (IC₅₀: 3 μ M). They were able to further characterize mitoxantrone potency using pancreatic ductal adenocarcinoma cell lines and deduce that mitoxantrone induced cell death was, in part, due to inhibition of USP11. Their study emphasized the importance of finding novel therapeutic targets for current FDA approved compounds. This approach may help tackle the problem of drug resistance in cancers, allow rapid progression of a compound through phase II trials and open new avenues for treatment of diseases ²⁰³.

Mitoxantrone is clinically used for the treatment of leukemia and multiple sclerosis²⁰⁴, however the exact mechanism of action is unknown. It is believed to intercalate with DNA leading to DNA double stranded breaks and the stalling of Topoisomerase II ²⁰⁵⁻²⁰⁷.

1.8. Background to this study

The importance of USP15 in the regulation of numerous cellular events, and its implications in several diseases is rapidly emerging, thus it has become an attractive druggable target. Structural characterizations of USPs has greatly enhanced our knowledge but at present no structural information is available for the catalytic domain of USP15. At the time of beginning this work, there were 15 crystal structures available of the catalytic domains of the USP family. The majority of the structures were obtained using modified substrates such as UB-VME ^{91, 93, 94, 97, 133} or bound to small molecules^{9, 200}. As technology and knowledge has advanced, more structures of USP catalytic domains have become available but the USP15 catalytic domain has remained elusive to crystallization.

USP11 was shown to be inhibited by the FDA approved drug mitoxantrone in an Ubiquitin-AMC assay, with an IC₅₀ of 3 μ M. USP11 is closely related to USP15 and investigations into the inhibitory effect of mitoxantrone on USP15 would help shed light on the compounds specificity amongst the USP family.

Preliminary data obtained prior to the start of this PhD showed that 100 μ M mitoxantrone was able to reduce USP15-D1D2 activity by 50% in the Ub-AMC assay. Co-crystallization experiments of USP15-D1D2 with mitoxantrone using vapour diffusion 96 well screening methods were performed and a single blue crystal was obtained in one well of the JCSG+ screen (figure 1-21). The crystal was taken to Diamond light source for data collection and diffracted to 4 Å, however

the overlapping nature of the crystal gave a poor data set which was not suitable for further processing.²⁰⁸.



Figure 1-21: Preliminary USP15-D1D2 mitoxantrone crystal obtained from the JSGC+ screen. The crystal grew at 20°C in the condition containing 0.2 M tri-methylamine N-oxide, 0.1 M Tris pH7.520 % w/v PEG 2000 MME. USP15-D1D2 and mitoxantrone concentrations were 3 mg/ml and 0.75 mg/ml respectively.

1.9. Aims and Objectives

USP15 is emerging as potential therapeutic target, in diseases such as Parkinson's and some forms of cancer. When beginning this project the crystal structure of the USP15 catalytic domain was unknown and there was limited structural data available for the interaction of small molecules with USPs. One of the aims of this project were to obtain and analyse the structure of the USP15 catalytic domain, and to deduce a mechanism of action for the inhibitory effect of mitoxantrone observed in previous experiments. Secondly, this project sought to develop a novel ubiquitin variant, based on the structure lysine backbone present in the isopeptide bond, which could be produced cheaply and efficiently using recombinant protein techniques. Thirdly, due to the unavailability of structural data for USP15, a detailed structural comparison between the paralogue USP4 has not been completed. Thus this project aimed to further probe differences between USP4 and USP15 using both structural and biochemical data.

Research Objectives:

- Structural determination and characterisation of the USP15 catalytic domain (Chapter 3 and 6)
- Characterisation of USP15 catalytic domain interactions with ubiquitin, linear di-ubiquitin and a new ubiquitin variant (Chapter 5)
- Investigation of inhibition of USP15 by mitoxantrone and identification of a mechanism of action (Chapter 4)

1.10. USP15 constructs used in this study

The USP15-D1D2 construct was designed and cloned by Dr Hayley Gratton²⁰⁸. Domain boundaries were selected based on sequence alignment with the published USP4-D1D2, which had been designed using limited proteolysis and was used for the successful crystallisation of the catalytic core¹⁴. The construct spans residues 256-919 with the catalytic insert (440-756) removed. A short linker found in USP8 with the residues ASTSK was selected to replace the catalytic insert and link the two halves of the domain together. The C-terminus, which is predicted to be disordered, was truncated to facilitate crystallisation. This construct, named USP15-D1D2, is shown in figure 1-22 and formed the basis for all further catalytic core experiments described in this thesis.

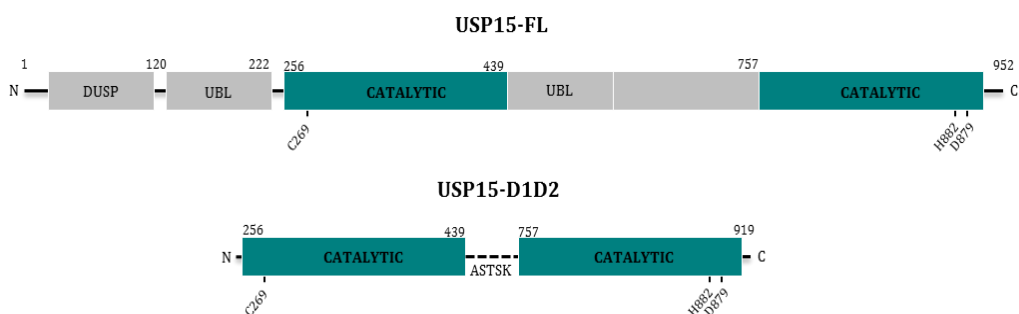


Figure 1-22: Schematic representation of USP15-FL and USP15-D1D2 domain structure. The catalytic sub domain halves are shown in teal and additional DUSP and Ubl domains labelled accordingly. USP-D1D2 comprises the catalytic sub domain halves with the Ubl insertion substituted with the USP8 linker (ASTSK).

1.11. Contributions to this thesis

I have summarised below contributions of others to this thesis. Experiments conducted by Georgina Armstrong, Rishov Mukhopadhyay, and Camille Michavila were performed under my supervision.

Dr Hayley Gratton had developed the constructs and optimised the purification methodology used in this thesis for both USP15-FL, USP15-D1D2 and USP15-D1D2 Cys269Ser. Furthermore, Dr Hayley Gratton had optimised the ubiquitin-AMC assay, and performed the preliminary investigations, described in section 1.8, into the interaction of mitoxantrone with USP15²⁰⁸. Under my supervision, Georgina Armstrong and Rishov Mukhopadhyay generated the USP15-D1D2 Cys352Ser, USP15-D1D2 Tyr855Lys constructs respectively, and Camille Michavila created the, USP15-D1D2 Gly860Val and USP15-D1D2bl2USP4 constructs. All these constructs were expressed and purified by me or under my supervision.

Chapter (5) describes the comparison of USP15 with paralogue USP4 using structural analysis and isothermal titration calorimetry (ITC). The work described in this chapter compliments the ITC experiments performed by Dr Peni Indrayudha²⁰⁹. Ubiquitin titrations into USP4-D1D2 WT and USP4 Cys311Ser were performed by Dr Peni Indrayudha and have been included for the purpose of comparison (figure 5-1) Camilla Michavila also performed ubiquitin titrations into USP15-D1D2 Gly860Val and USP15-D1D2bl2USP4 at different temperatures (figures 5-6 and 5-7).

Finally, Georgina Armstrong purified and tested USP15-D1D2 Cys352Ser in the Ub-AMC assay (figure 6-4) and the Thermofluor assay (figure 6-5) described in chapter 6.

CHAPTER 2: Materials and Methods

2.1. Constructs preparation

2.1.1. List of constructs used

Several constructs were designed, expressed and purified for experiments described later in this thesis. The table 2 summaries all constructs used. Most constructs were made by previous members of the Dreveny group, I have indicated who they were made by and any additional modifications where appropriate. These constructs were used as templates for the generation of new variations of these constructs.

The human USP15 constructs used were based on a C-terminal truncated USP15 (Δ 920-952) USP15-FL (UniProtKB identifier Q9Y4E8-2) termed USP15-FL, and a USP15 catalytic core domain construct. The Ubl2 domain (440-756) was predicted as being a highly disordered region, and was replaced with a small linker (ASTSK), based on the USP8 sequence, to link the two catalytic sub domain halves and form that catalytic core construct termed USP15-D1D2.

Human USP2-D1D2 construct was gifted by Cheryl Arrowsmith. Ubiquitin and linear di-ubiquitin constructs were cloned by Dr Ingrid Dreveny and Dr Simon Caulton respectively.

Construct	Residue range	Purification Tag	Vector	Restriction sites	Cloned by
USP15-FL	1-919	N-term His Tag	pCold-I	SacI, HindIII	Dr Hayley Gratton <i>University of Nottingham</i>
hUSP15-D1D2	256-919 Δ440-756	N-Term His Tag	pET21d(+)	NcoI NotI	Dr Hayley Gratton <i>University of Nottingham</i>
hUSP2-D1D2	258-605	N-Term His Tag	pET28a-LIC	N/A	Gift from Cheryl Arrowsmith <i>Addgene plasmid #36894</i>
Ubiquitin	1-76	Untagged	pRSf13	NcoI HindIII	Dr Ingrid Dreveny <i>University of Nottingham</i>
Ubiquitin-ggg	1-76-GGG	Untagged	pCDFDuet-1	NdeI KpnI	Stephanie Ward <i>University of Nottingham</i>
Linear Di-ubiquitin	1-152	Untagged	pCDF-Duet-1	NdeI KpnI	Dr Simon Caulton <i>University of Nottingham</i>

Table 2: List of constructs used in this thesis.

2.1.2. Polymerase chain reaction

Polymerase chain reaction (PCR) was used for the amplification of the desired gene fragment. Each reaction was composed of the following components:

20-50 ng DNA template
0.8 μ M forward and reverse primers
0.2 mM dNTPs (Thermo Fisher Scientific)
2% DMSO
1 x HF buffer (NEB)
1 unit of Phusion enzyme (NEB)
Topped up to 50 μ l with molecular grade H₂O.

The PCR was carried out using the protocol table 3, in a PTC-200 Peltier Thermocycler.

Stage	Temperature	Time	
Initial Denaturation	95°C	5 minutes	
Denaturation	95°C	30 seconds	] X 24
Annealing	55-65°C	30 seconds	
Extension	72°C	1 minute per Kb	
Final extension	72°C	10 minutes	

Table 3: PCR protocol.

Reactions were set up in duplicate and either run at 55°C and 65°C. The result of the PCR protocol was assessed using agarose gel electrophoresis. Samples were combined with 6x Purple Loading Dye (NEB) in a 5:1 ratio and loaded onto a 1% agarose gel. 5 μ L of 500 x NANCY-520 (Sigma Aldrich) was combined with agarose solution during gel preparation. Gels were run at 100 V for 30 minutes visualized using Dark Reader Transilluminator (Clare Chemical) under blue light. 100 bp or 1 kb DNA markers (NEB) were used to confirm PCR product size and a single band indicated a pure PCR product. PCR products were cleaned up using GenElute PCR Clean-up kit (Sigma Aldrich).

Construct	Residue range	Forward Primer	Reverse Primer
Ubiquitin-GGG	1-76-GGG	GGAATTCCATATGCAGATC TTCGTGAAGACTC	CGGGGTACCTCAACCACCAC CCCCACCTCTGAGACGGAG

Table 4: Primers used for the generation of the Ub_{ggg} construct

2.1.3. Digestion and Antarctic Phosphatase treatment

The purified PCR product and corresponding vectors were subjected to digestion by suitable restriction enzymes. The digestion reaction contained 16 µl of PCR product or vector, and 5 µL of each restriction enzyme (20000 U/ml) (NEB) and 2 µl of 10 x CutSmart Buffer (NEB). The reactions were incubated for 3-4 hours at 37°C. 2 µL of 10 x Antarctic phosphatase buffer (NEB) and 1 µl of Antarctic phosphatase enzyme (500 U/ml) (NEB) were added to the digested vector sample and allowed to incubate at 37°C for 2-3 hours. The PCR product and vector digestion mixtures were purified using GenElute PCR Clean-Up kit (Sigma-Aldrich).

2.1.4. Ligation

Ligation was carried out by T4 DNA ligase (NEB). Reactions were performed using 3:1 and 6:1 ratios of DNA and insert respectively. To determine the amount of DNA required for the correct ratios in each reaction, the following equation was used:

$$\frac{\text{Size of insert} \times \text{Concentration of vector}}{\frac{\text{Size of vector}}{\text{Concentration of insert}}} = \text{Volume of insert required for 1:1 ratio}$$

1 µl of 10 x T4 DNA ligase Buffer and 1 µl of T4 DNA ligase (400,000 U/ml) was added to 8 µl of vector/insert DNA mix and left overnight at 16°C. 2 µl of these reactions were then transformed into Nova Blue Cells.

2.1.5. Transformation

2 µl of DNA sample was added to NovaBlue (DE3) *E. coli* competent cells (Novagen) and incubated on ice for 10 minutes. The samples were then heat shocked at 42°C

for 1 minute before a further 5 minute incubation on ice. 800 µl, (500 µl for a mutagenesis transformation) of Lysogeny Broth (LB) were added to the mixture and incubated at 37°C and 180 rpm for 45-60 minutes. 240 µl of the sample mixture are then spread onto an LB agar plate containing appropriate antibiotics and incubated at 37°C overnight in a static incubator.

2.1.6. Colony cracking, analytical digests and sequencing

Colonies were screened for the presence of the desired plasmid using analytical digest and colony cracking. Colonies were picked and small cultures were with 5 ml of LB containing a suitable antibiotic and were incubated in shaking incubator over night at 37°C. Cracking buffer contains (50% sucrose, 0.5% SDS, 0.5 M NaOH, bromophenol blue in H₂O) was mixed with 20 µL of each culture and analysed using agarose gel electrophoresis. Samples containing the desired insert were identified and purified using GenElute PCR clean-up kit (Sigma Aldrich). Test restriction digests were performed on the purified plasmids to confirm the presence of an insert at the correct size. Reagent volumes were halved for the analytical digests and incubated at 37°C for 2 hours. Agarose gel electrophoresis was used visualize digested plasmids and those found to have an insert were sequenced using the Sanger sequencing method (Source Bioscience).

2.1.7. Site Directed mutagenesis:

Mutagenesis was used to introduce single point mutation into constructs following the QuickChange mutagenesis protocol. Table 6 summaries each construct and any mutations introduced. Typically two PCR reactions were set up containing different amounts of DNA.

The reaction mixes contained the following:

10-50 ng of DNA,
 5 μ L of 10 x PfuUltra reaction buffer (Aligent),
 2 μ L of 20 μ M forward and reverse primer,
 1 μ L of 10 mM dNTP mix (Thermofisher Scientific),
 1 μ L of 2.5 U/ μ L PfuUltra II polymerase (Aligent)
 and were made up to 50 μ L with molecular grade H₂O.

The PCR reaction protocol is given in table 5. for the PCR reaction.

Stage	Temperature	Time	
Initial Denaturation	95°C	5 minutes	
Denaturation	95°C	30 seconds	X 24
Annealing	55°C	1 minute	
Extension	68°C	1 minute per Kb	
Final extension	68°C	10 minutes	

Table 5: Mutagenesis PCR reaction protocol.

1 μ L of DpnI enzyme was added to the PCR reactions and incubated for 1 hour at 37°. 2 μ L of the samples were then transformed into Nova blue (DE3) cells. Single colonies were picked and inoculated with 5 ml of LB containing suitable antibiotics and grown overnight at 37°C. The plasmid was extracted from the culture using a GeneElute plasmid miniprep kit and sent for sequencing (Source Bioscience) to confirm the mutation had been successfully incorporated.

Construct	Forward primer	Reverse primer	Created by	Mutation type
FL USP15 Cys29Ser	GTAACCTGGGAAATACGTCTT TCATGAACTCAGCTATTCAG	CTGAATAGCTGAGTTCATGA AAGACGTATTTCCCAAGTTAC	Dr Hayley Gratton <i>University of Nottingham</i>	Active site mutation
FL USP15 Try855Lys	CTCCTCCCATCCCTCCC TTGTGGTTGGAAACAGC	GCTGTTTCCAACCACAAG GGAGGGATGGGAGGAG	Stephanie Ward <i>University of Nottingham</i>	
USP15-D1D2 Cys269	GTAACCTGGGAAATACGTCTT TCATGAACTCAGCTATTCAG	CTGAATAGCTGAGTTCATGA AAGACGTATTTCCCAAGTTAC	Dr Hayley Gratton <i>University of Nottingham</i>	Active site mutation
USP15-D1D2 Tyr855Lys	CTCCTCCCATCCCTCCC CTTGTGGTTGGAAACAGC	GCTGTTTCCAACCACAAG GGAGGGATGGGAGGAG	Rishov Mukhopadhyay <i>University of Nottingham</i>	
USP15-D1D2 Cys352Ser	AAGCTAACAGTTCTTGACTGT CT TGCTGCTGATATCC	GGATATCAGCAGCAAGACAGTC AAGAACTGTTAGCTT	Georgina Armstrong <i>University of Nottingham</i>	
USP15-D1D2 Gly860Val	CTATGGAGGGATGGGAGTA GGACACTATACTGCTTTTG	CAAAAGCAGTATAGTGCCT ACTCCCATCCCTCCATAG	Camille Michavila <i>University of Nottingham</i>	

Table 6: Summary of mutations generated in USP15-FL and USP15-D1D2

2.1.8. Glycerol stock preparation

Glycerol stocks were made for each new construct the stocks were prepared by picking a single colony and inoculated with 100 ml LB containing the appropriate antibiotics and incubated overnight at 37°C. 100 µL of the resulting culture was mixed with 100 µl of autoclaved 60 % glycerol and stored at -80°C until further use.

2.2. Protein expression in *E.coli*

Constructs that had been successfully cloned were transformed into either BL21 (DE3) codon plus (BL21 (DE3)-CP) cells (Aligent) or ElaD deficient cells (ElaD – ve) as indicated for expression. The ElaD deficient cells used in this thesis are an engineered ElaD knockout C41 strain and were a gift from Dr Jed Long. Table 7 summarises the expression protocol used for each construct.

2.2.1. Protein expression in BL21 (DE3) codon plus *E.coli* cells

The BL21 (DE3)-CP cells harbour extra t-RNA genes to prevent the stalling of translation within the *E.coli* cells due to depletion of rare t-RNA codons. Construct expression was carried out in large scale expression using an appropriate media and time scale, summarized in table 7. The cells were grown at 37°C to a suitable OD₆₀₀ (see table 7), monitored by measuring the culture at 600nm. Protein expression was then induced following the addition of 0.5 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) to the flasks. The temperature was adjusted according to the construct being expressed (see table 7). Cells were harvested following 4-96 hours of incubation via centrifugation for 30 minutes at 2360 rcf, and pellets stored at -20°C until further use.

2.2.2. Protein expression in ElaD deficient cells

Some constructs were expressed in ElaD deficient cells. ElaD is a bacterial deubiquitinase which can cleave modifications on the C-terminal tail of ubiquitin. To avoid any intracellular cleavage of ubiquitin constructs, ubiquitin variants and any USP-ubiquitin complexes were expressed in ElaD deficient cells. Expression of proteins in ElaD deficient cells follows the same protocol as for BL21 (DE3)-CP however, the use of chloramphenicol is omitted due the strain not resistant to the antibiotic.

Construct	Media	OD ₆₀₀	Temperature °C	Time (hours)	Cell type
Single constructs					
FL USP15	2YT	1.5-1.8	10	72	BL21 (DE3)-CP
USP15-D1D2	2YT	0.55	25	20	BL21 (DE3)-CP
Ubiquitin	LB	0.55	37	4	BL21 (DE3)-CP
Ubggg	LB	0.55	37	4	ElaD-ve
Di-ubiquitin	LB	0.55	37	4	ElaD-ve
Ubgggc	LB	0.55	37	4	ElaD-ve
Complexes					
USP15-D1D2 C269S +Ubggg	2YT	0.55	25	20	BL21 (DE3)-CP/ ElaD-ve
USP15-D1D2 C269S + Di-ubiquitin	2YT	0.55	25	20	BL21 (DE3)-CP/ ElaD-ve

Table 7: Summary of the protein expression protocol for each construct.

2.3. Protein Purification

The buffers used for the different purifications of the constructs is summarized below:

Construct	Purification techniques	Buffer A	Buffer B	Gel filtration buffer:
Single constructs				
FL USP15	Nickel/Gel filtration	50 mM tris pH 7.5, 20 mM Imidazole, 300 mM NaCl, 10% glycerol	50 mM tris pH 7.5, 500 mM Imidazole, 300 mM NaCl, 10% glycerol	20 mM tris pH7.5, 300 mM NaCl, 1% glycerol
USP15-D1D2	Nickel/Gel filtration	50 mM tris pH 7.5, 20 mM Imidazole, 300 mM NaCl, 1% glycerol	50 mM tris pH 7.5, 500 mM Imidazole, 300 mM NaCl, 1% glycerol	20 mM tris pH 7.5, 300 mM NaCl, 1% glycerol
USP2-D1D2	Nickel/Gel filtration	50 mM tris pH 7.5, 20 mM Imidazole, 300 mM NaCl, 1% glycerol	50 mM tris pH 7.5, 500 mM Imidazole, 300 mM NaCl, 1% glycerol	20 mM tris pH 7.5, 300 mM NaCl, 1% glycerol
Ubiquitin and ubiquitin variants	Ion exchange/gel filtration	20 mM ammonium acetate	500 mM ammonium acetate	20 mM tris pH7.5, 150 mM NaCl, 1% glycerol
Complexes				
USP15-D1D2 Cvs269Ser + Ubggg	Nickel/Gel filtration	50 mM tris pH 7.5, 20 mM Imidazole, 150 mM NaCl, 1% glycerol	50 mM tris pH 7.5, 500 mM Imidazole, 150 mM NaCl, 1% glycerol	20 mM tris pH7.5, 150 mM NaCl, 1% glycerol
USP15-D1D2 Cys269Ser + Di-ub	Nickel/Gel filtration	50 mM tris pH 7.5, 20 mM Imidazole, 150 mM NaCl, 1% glycerol	50 mM tris pH 7.5, 500 mM Imidazole, 150 mM NaCl, 1% glycerol	20 mM tris pH7.5, 150 mM NaCl, 1% glycerol

Table 8: Summary of purification techniques used and respective buffer for each construct.

2.3.1. Sample preparation

Cells were thawed on ice and re-suspended in buffer A, using a ratio of 1 g cell pellet to 10 ml of buffer A. Protease inhibitors (cOmplete, Sigma-Aldrich) were added at this point during the preparation of Ubggg and linear di-ubiquitin constructs, and solubilized during the resuspension of the pellet. Re-suspended

cells were kept on ice and subjected to sonication for a total time of 4 minutes in 20 second intervals at 14 microns. Sonicated cells were centrifuged at 24104 rcf for 1 hour to pellets the cell debris. The cleared lysate was then syringe filtered using 45 µm filters.

2.3.2. Nickle affinity

A 5 ml HisTrap FF Crude Nickle column (GE Healthcare Life Sciences) was used for all nickle affinity purifications. All proteins purified by this method require a 6xHis tag either at the C-terminus or the N-terminus of the protein.

The column was prepared prior to loading of the sample in the following order: 3 column volumes of water, 5 column volumes of 100 mM EDTA, 1 column volume of water, 5 column volumes of 100 mM nickel sulphate and 5 column volumes of buffer A. The lysate was then loaded onto the column and washed with buffer A to remove non-specifically bound proteins. The protein was eluted using a linear concentration gradient (0-80%) of buffer B over 100 mL. The flow through was collected in 2.5 ml fractions and checked for protein content via SDS-PAGE gel electrophoresis.

2.3.3. Cation Exchange

A High-Trap SP-FF cation ion exchange column was used for the purification of all ubiquitin based constructs. The cell lysate was prepared as in section 2.3.1 however, following the centrifugation at 24104 rcf. Acetic acid (10 %) was used to drop the pH of the lysate to 4.5, causing precipitation of many proteins in the sample. The pH was returned to 5.1 using sodium hydroxide to prevent further precipitation from occurring on the column, and centrifuged for a further 10 minutes at 24104 rcf to remove the precipitated protein. The lysate was then filtered and loaded onto the column using a peristaltic pump. The protein was

eluted using a linear concentration gradient (0-80%) of buffer B over 100 mL. The flow through was collected in 2.5 ml fractions and checked for protein content via SDS-PAGE gel electrophoresis.

2.3.4. Size exclusion chromatography

The protein was then further purified by size exclusion chromatography (SEC). The column was equilibrated with 1 column volume of appropriate gel filtration buffer (see table 8) in preparation for loading of the protein. The protein was concentrated in the presence of 1 mM DTT (unless stated otherwise), using a Vivaspın® 20 concentrator (GE Healthcare), with a suitable molecular weight cut off, until the volume of the sample was 2.5 ml. The protein was loaded onto a 5 ml loop and injected on to a HiLoad™ Superdex™ 200 column (GE Healthcare) (USP15 constructs and complexes) or HiLoad™ Superdex™ 75 column (GE Healthcare) (ubiquitin constructs). Eluate from the column was collected in 2.5 ml fractions were collected and analyzed for protein content by SDS page.

2.3.5. Storage of purified protein

Protein which was not used immediately or was left over from experiments, was snap frozen using liquid nitrogen and stored at -80°C.

2.3.6. SDS-PAGE gel electrophoresis

SDS page gels were used to visualize the protein content in samples. The gels are composed of a resolving gel and a stacking gel. Resolving gels comprised of 12-20% acrylamide depending on the size of the protein being resolved. Typically, 18-20% gels were used to visualize ubiquitin, 15% for USP catalytic domains and 12% for FL USP constructs. The stacking gel consisted of 6% acrylamide in all cases. The gel compositions are shown in table 9. Protein samples were mixed

with 3 x SDS-PAGE loading buffer at a 2:1 ratio respectively. Samples were boiled for 5 minutes before being loaded onto the gel.

Component	Resolving gel (ml)				Stacking gel
	12%	15%	18%	20%	
30% Acrylamide	5.7	7.3	9	10.6	2
H ₂ O	5.9	4.3	2.7	1.1	5.3
1.5 M Tris-Cl pH 8.8	4	4	4	4	-
0.5 M Tris-Cl pH 6.8	-	-	-	-	2.5
10 % SDS	0.16	0.16	0.16	0.16	0.1
10 % APS	0.16	0.16	0.16	0.16	0.1
TEMED	0.016	0.016	0.016	0.016	0.01

Table 9: Components of SDS-PAGE electrophoresis gels.

Gels were ran at 200 volts, with the exception of assay gels, which were run at 160 volts, until the band front reached the bottom of the gel. Gels were stained with 0.1% Coomassie Brilliant Blue R-250, 50% isopropanol 10%, acetic acid and de-stained with 15 % isopropanol and 15 % glacial acetic acid.

2.3.7. Protein and ligand concentration determination.

Protein concentration was determined using a Nanodrop 1000 (ThermoFisher Scientific). The NanoDrop was blanked accordingly prior to concentration measurement. Protein concentration was determined by measuring UV absorption at 280 nm protein using extinction co-efficient determined using Exapsy Protparam prediction tool.

2.3.7.1. Mitoxantrone concentration determination

Mitoxantrone (Sigma Aldrich) concentration was measured using the Nanodrop 1000 (ThermoFisher scientific). Concentrations were measured at $\lambda=682$ using the extinction coefficient $\epsilon=8360 \text{ M}^{-1}\text{cm}^{-1}$. Mitoxantrone forms dimers at higher

concentrations and the wavelengths were chosen as the most appropriate to account for variations in oligomeric state of mitoxantrone in solution. Stock solutions were usually too concentrated, and 1:10 dilutions were performed to increase the accuracy of concentration determination.

2.4. Crystallography

2.4.1. Sparse matrix crystallisation screens

Samples containing protein of the highest quality and purity were selected for crystallization trials. The protein was centrifuged for 5 minutes at 16000 rcf at 6°C to remove any remaining precipitant or debris.

Crystal conditions were screened using sitting drop vapour diffusion method in 96-well MRC crystallisation plates. Various commercial screens were used which were purchased from Hampton and Molecular Dimensions. The exact commercial screen used in each sparse matrix crystallisation experiment is detailed in appendix 3c,3d and 3f.

A multi-channel pipette was used to load 80 µl of the screen solutions in the reservoir well. The Mosquito Crystal (TTP Labtech) robot was used to dispense 200 nl of protein and 200 nl of reservoir solution sample into the smaller wells of the 96 well screen. The plates were immediately sealed and incubated at either 10°C, 15°C or 20°C.

2.4.2. Co-crystallisation trials with USP15-D1D2 mitoxantrone

Stocks of mitoxantrone (Sigma-Aldrich) were prepared by solubilizing small amounts of the powder in either 100 % ethanol or 50 % DMSO. Initial screens utilised mitoxantrone solubilized in EtOH and later screens used 50 % DMSO solubilized mitoxantrone. DMSO is a less suitable solvent for long-term storage of mitoxantrone, thus fresh stocks of 50 % DMSO mitoxantrone solutions were

prepared for each experiment. Mitoxantrone stocks solubilised in ethanol were stored at -20°C.

2.4.3. Micro seeding crystallisation experiments

Micro seeding experiments utilized initial crystal hits to improve overall morphology and size of the crystal form. The crystals and remaining reservoir solution (~80 µL) were removed from the drop and placed in a 1.5 ml Eppendorf. A single glass seed bead (Hampton research) was added and the sample vortexed thoroughly for 5 minutes to produce a concentrated seed stock. Dilutions of the seed stock were made using the same reservoir solution. Seed stocks were checked under a microscope prior to use to ensure the seeding crystal had been sufficiently distributed.

2.4.3.1. 96 well micro seeding experiments

The reservoir solutions were prepared as before. The mosquito robot was used to dispense the protein, reservoir and seed stock solutions. The protein concentration was reduced by 20-40 % of the original crystallisation condition in preliminary seeding experiments. Larger volumes were used for seeding experiments, with the drops consisting of 400 nl protein solution, 500 nl reservoir solution, and 100 nl of seed stock. Trays were sealed and allowed to equilibrate for 48 hours prior to the addition of the seed stock.

2.4.3.2. Micro-seeding in optimisation screens.

Some conditions were optimized in 24 well plates to improve crystal size. The protein concentration was reduced by 20-30% in preliminary screen to account for the addition of the seed stock. The drop composition of these trays was as follows: 1.5 µl of protein sample, 2 µl of reservoir solution and 0.5 µL seed stock.

Similar to the 96 well screens, trays were sealed and allowed to equilibrate for 48 hours prior to the addition of the seed stock.

2.4.4. Crystal growth using mitoxantrone screen

A 96-well screen was developed based on the crystallisation hits obtained for USP15-mitoxantrone co-crystals in the various 96 well sparse matrix commercial screens (Hampton Research and Molecular Dimensions) and optimisation screens. Details of the 96 well screen components can be found in appendix 3g. Stocks of reservoir solutions were made to final volume of 2 ml and stored in a block similar to the commercial screens at 4°C. The reservoir solutions could be dispensed into 96 well screens as described in section 2.4.3.1 .

2.4.5. Soaking experiments

USP15-D1D2-mitoxantrone crystals grown in 96 well screens and optimization screens were subjected to a variety of soaks. The different soaking experiments are summarized below.

2.4.5.1. PR-619 soaks

PR-619 was solubilized in 100% DMSO to 5 mg/ml and stored at -20°C. For increased solubility during soaking experiments PR-619 was mixed with a multicomponent cryo-protectant mix (17.5% Dioxane, 25% glycerol, 25% ethylene glycol) in a 1:4 ratio respectively. The resulting solution was mixed with 2 x the precipitant and 1 x crystallisation buffer in a 5:4:1 ratio respectively. Consequently the final concentrations of the components were: 2.2 mM PR-619, 7 % Dioxane, 10 % glycerol, 10% ethylene glycol and 10% DMSO.

1 µL of the soak solution was added to drops containing crystals in 96 well screens used in seeding experiments, and 2 µl was added to optimisation plates. Soaks

were carried out 24 hours or 7 days prior to data collection. No cryoprotectant of the crystals was required due to the presence of cryoprotecting components in the soaking solution.

2.4.5.2. Mitoxantrone soaks

USP15-D1D2- mitoxantrone crystals were soaked with 1.5 mM mitoxantrone for 1 hour. Crystal were fished and cryoprotected in a cryoprotectant supplemented with 1.5 mM mitoxantrone.

2.4.5.3. β -mecaptoethanol soaks

Crystals grown in 24 well optimisations plates and the 96 well mitoxantrone screen were used for these soaking experiments. Crystals that were single and of a reasonable thickness and size were used. β -mecaptoethanol was diluted in the reservoir solution to a final concentration of 5 mM and added to the drop in a 1:1 ratio. Crystals were soaked for either 4 hours or 24 hours prior to data collection. The crystals were cryoprotected in a solution supplemented with 5 mM β -mecaptoethanol.

2.4.5.4. Hydrogen peroxide soaks

Crystals grown using the mitoxantrone screen were used for soaks with hydrogen peroxide (H_2O_2). Crystals that were of a suitable size and thickness were used. H_2O_2 was diluted in the reservoir solution to a final concentration of 100 μM and added to the drop in 1:1 ratio. Crystals were soaked for either 1 hour or 6 hours prior to data collection.

2.4.6. Cryoprotection of crystals.

Crystals were cryoprotected by immersion in the reservoir solution supplemented with the cryoprotectant before being cryocooled. Appropriate cryoprotectant were selected based on the composition of the reservoir solution.

30% MPD was used to cryoprotect crystals grown in MPD containing conditions. Crystals grown in various PEG conditions were protected using 20-30 % glycerol. The introduction of the cryoprotectant was done in a step wise manner to preserve the crystal quality.

2.5. Data collection, structure determination and refinement

Data was collected at Diamond Light Source, Harwell, Oxford and ESRF, Grenoble. Data sets were indexed using either an automated pipelines Autoproc²¹⁰ or FastDP or manually using Imosfilm²¹¹. XDS and aimless were used for further data processing. In some cases, several data sets from one crystal were merged and re-indexed using Imosfilm, Aimless and Pointless to improve overall data quality²¹². Crystals belonged to the space group P 2₁ and contained one or two copies in the asymmetric unit.

Molecular replacement (MR) was used to obtain the phases for each new structure. MR utilises the phases from a model protein, which, and has already been solved. Typically, the model has greater than 30 % sequence identity with the target protein and any flexible loop regions and additional atoms (such as water) have been removed.

Further, for molecular replacement to succeed, information on the number of copies of protein within the asymmetric unit must be provided. The Mathews coefficient was used to estimate the number of molecules in the asymmetric unit

and is calculated using the following equation:
$$\frac{\text{Volume of unit cell}}{\text{Molecular weight of protein} \times Z \times Y}$$

where Z is the number of asymmetric units and X represents the number of unknown molecules in the unit cell.

Phaser MR ²¹³ was used to solve the first USP15-D1D2 structure using USP4 (2Y6E)¹⁴ as a search model. This method estimates the phases for the new structure by rotating and translating the model within the asymmetric unit, and uses maximum likelihood methods for determining the probability of obtaining the observed experimental data for each function tested. All structures were solved using the automated pipeline in Phaser, which consisted of the following 6 steps: anisotropy correction, model generation, rotational searches, translational searches, assessment of packing, and refinement. The success of a molecular replacement experiment was determined using the score provided by Phaser for guidance, and a detailed inspection of the electron density map.

Model building and refinement was completed using Coot²¹⁴, Phenix refine²¹⁵, and refmac5²¹⁶. This first USP15-D1D2 structure was used as search model for subsequent data sets of USP15-D1D2 collected.

2.6. Deubiquitinating assays

2.6.1. Ubiquitin-AMC assay

The ubiquitin-AMC (Ub-AMC; Enzo Life Sciences) assay was used to assess deubiquitinating activity of USP enzymes. Fluorescence of the AMC fluorophore can be detected at 460 nm when excited at 380 nm. The AMC only fluoresces once it has been cleaved from ubiquitin and therefore enzyme activity can be monitored by measuring the change in fluorescence over time. Assays were performed in white 384-well black plates (Nunc) at a final volume of 30 μ L. Enzymes were diluted in assay buffer to a concentration of 42 μ M. Stock concentration of Ub-AMC were 114 μ M and diluted to 4.5 μ M in H₂O. Samples were incubated at room temperature for at least 30 minutes prior to the assay. Reaction were initiated by the addition of 25 μ l of enzyme-buffer mix to 5 μ l of 4.5 μ M Ub-AMC and immediately transferred to the plate reader. The assay was followed using an

EnVision 2104 multilabel plate reader. Fluorescence was measured using $\lambda_{\text{Ex}} = 380\text{nm}$ (bandwidth 19) and $\lambda_{\text{Em}} = 460$ (bandwidth 25) excitation light 10%, detector gain 10, and 3 flashes. This method previously optimized by Dr Hayley Gratton. Measurements were taken every 4 seconds or every minute. Background fluorescence was controlled for by wells containing Buffer only, Buffer + Ub-AMC, and Buffer + protein.

2.6.1.1. Mitoxantrone inhibition test in the Ub-AMC assay

The Ub-AMC assay was used to evaluate the extent of USP15 inhibition in the presence of 100 μM mitoxantrone. The assay was set up using the same concentrations and parameters described in section 2.6.1. Mitoxantrone was at a final concentration of 100 μM in the assay. The protein was incubated for half an hour at 25°C prior to starting the assay. Additional controls were used in the assay: Buffer + USP15 + mitoxantrone, Buffer + mitoxantrone, Buffer + Ub-AMC + mitoxantrone.

Free AMC standard curves were generated to account for the quenching effect of mitoxantrone. A 30 μM stock of free AMC was diluted to 3 μM and serial dilutions performed to yield a concentration of 1.4 nM in assay buffer supplemented with 100 μM mitoxantrone. A standard curve containing no mitoxantrone was also generated. Standard curves were normalised using the appropriate buffer which contained 0 μM AMC. Measurements were made using the EnVision 2104 multilabel plate reader utilising the same settings as described in section 2.6.1. Both standard curves were linear and fit using linear regression to equation $y = mx + C$. The resulting equation was used to convert fluorescence into nM AMC, which was plotted over time. All analysis was completed using GraphPad prism.

2.6.2. Gel Based cleavage assay

2.6.2.1. General methodology

Activity of USPs was monitored using a gel based cleavage assay following the cleavage of linear di-ubiquitin over time. The reactions were performed in triplicate and carried out in 50 mM Tris pH 7.5, 300 mM NaCl, 1% Glycerol and 1 mM DTT at 25°C. Samples were allowed to equilibrate to 25°C for 30 minutes before starting the assay. Reactions were initiated by the addition of linear di-ubiquitin to USP15 resulting in final concentration of 5 μ M and 400 nM respectively. Reactions were stopped by the addition of 3 x SDS PAGE loading buffer to samples in a 2:1 ratio respectively. Cleavage of the di-ubiquitin to ubiquitin was visualized using SDS PAGE gel electrophoresis as described in section 2.3.6.

ImageJ was used to analyze the gels via densitometry using the gel plotting macro function.²¹⁷ Using the rectangle tool in ImageJ individual lanes could be assigned and the intensity of protein within each lane could be calculated. Each protein band produced a corresponding peak and calculating the area under the peaks determines the amount of ubiquitin and di-ubiquitin present in each lane. Percentage ubiquitin or di-ubiquitin were plotted over time and fit using nonlinear regression to a one phase decay (% di-ubiquitin) or one phase association (% mono-ubiquitin) model.

2.6.2.2. Inhibition screening

The linear di-ubiquitin assay was used to test for a change in USP activity in the presence of 100 μ M mitoxantrone. The same concentrations were used as described in section 2.6.2.1. Reactions containing 0 μ M mitoxantrone served as a positive control. Mitoxantrone was added to the samples prior and incubated for 30 minutes prior to beginning the assay. The reactions were stopped every 6

minutes for 60 minutes and at 90 and 120 minutes thereafter. Gels were analysed as described in section 2.6.2.1. Comparisons of the rate constant K were used to determine the significance of the differences between the curves.

2.6.2.3. Linearity test

Assays were set up following the protocol described in section (2.6.2.1). The reaction was stopped every 3 minutes for 45 minutes to ensure the linear region of reaction was captured. Gels were analysed using ImageJ software and mono-ubiquitin (%) was plotted over time. The data was fit using linear regression. The linear range was determined to finish at 36 minutes.

2.6.2.4. IC50 determination

Experiments were conducted in triplicate and 12 concentrations were used for IC50 determination. Mitoxantrone was solubilized in assay buffer and various concentrations (0-800 μM) were incubated with USP15-FL for 30 minutes prior to initiation of the reaction. Reactions were stopped after 30 minutes and SDS PAGE gels were using ImageJ. Percentage di-ubiquitin was plotted as function of $\text{Log}_{10}[\text{mitoxantrone}]$ and a curve fit using non-linear regression using the $\log(\text{inhibitor})$ vs. response—variable 4 slope parameters function. All data points were included in the fitting and error bars represent the standard error mean associated with each concentration.

2.7. Isothermal titration calorimetry

ITC experiments were carried out using an ITC200 instrument (Microcal USA) and ITC Pea-Q (updated version of ITC200). Data analysis was performed using the MicroCal PEAQ-ITC Analysis Software. Control titrations were performed to ensure the heat of dilution was negligible and did not generate a sigmoidal curve. Data were fit to a one site binding model.

2.7.1. USP interactions with ubiquitin variants.

All proteins used in the titrations were dialyzed into a buffer containing 50 mM tris pH7.5, 150 mM NaCl, 1% glycerol overnight prior to experiments to avoid any buffer mis-matches. The ubiquitin variant was titrated into the cell containing the USP protein in question. Titrations were performed in 180-second intervals, and injection volumes were between 1.7 μ l – 2.4 μ l with the number of injections (19-24) varied accordingly. Concentration of the USPs were between 20 μ M to 60 μ M and ubiquitin variant concentrations were around 200 μ M-600 μ M. Titrations were performed at 25°C, 30°C and 37°C. A stirring speed of 750 rpm was used for all experiments.

2.7.2. USP15-D1D2 interactions with mitoxantrone

For these titrations mitoxantrone was solubilised in USP15-D1D2 gel filtration buffer. Control titrations of mitoxantrone into buffer were found to give an apparent binding curve, attributed to the disassociation of the dimers. Therefore 2.4 μ l titrations of USP15-D1D2 into mitoxantrone were performed. USP15-D1D2 was titrated at a concentration of 230 μ M into 23 μ M mitoxantrone at 25°C. Control titrations showed minimal heats of dilution and were consistent throughout the experiment.

2.8. Thermofluor assay

A thermofluor assay²¹⁸ was used to assess the effect oxidation and reduction has on the stability of USP15-D1D2 WT and USP15-D1D2 Cys352Ser. The SPYRO orange dye (stock concentration 5000x) (Sigma Aldrich) was prepared by diluting 1:500 in H₂O resulting in a 10x solution. Protein was diluted to final concentration 1 mg/ml. 5 μ g of the protein was mixed with 5 μ l of the 10x SPYRO orange dye and 40 μ l of buffer (300 mM NaCl, 20 mM Tris pH7.5, 1% Glycerol $\pm$ 1 mM DTT) into clear capped PCR tubes and thoroughly mixed. Each protein was tested in

triplicate in the presence and absence of DTT. Neither proteins had been exposed to a reducing agent prior to the assay. A Stratagene Mx3005p RT-PCR machine was used for the assay. Fluorescence of the SPYRO orange dye was continually monitored throughout the assay. The machine performed 70 cycles increasing the temperature from 25°C to 95°C in 1°C increments.

The output from the RT-PCR machine were converted using Excel and GraphPad prism into a usable format using templates sourced from: <ftp://ftp.sgc.ox.ac.uk/pub/biophysics> Fluorescence was plotted over time temperature (°C) and fit to Boltzmann sigmoidal equation using GraphPad prism²¹⁸. The inflection point of the curve corresponds to the melting temperature of the sample (T_m). Statistical tests were completed in GraphPad.

2.9. Mass spectrometry

Protein samples were prepared by myself following the standard purification routes, but the mass spectrometry experiments and analysis was performed by Gemma Cooke in the school of Chemistry and the University of York, Mass spectrometry centre of excellence.

2.9.1. Native mass spectrometry

Native mass spectrometry was used to assess the quality of the USP15-D1D2 Ub and linear di-ubiquitin complexes. Purified samples (section 2.3) were concentrated and dialysed overnight into low salt buffers or buffer exchanged using the PD-10 columns (GE Healthcare). The mass spectrometry experiments were conducted by Gemma Cooke as part of her masters' project in the school of chemistry under the supervision of Professor Neil Oldham.

2.9.2. LC-MS disulphide counting mass spectrometry

USP15-D1D2 was purified as in section 2.3, however DTT was excluded during the purification. The purest fractions from SEC were concentrated to 4 mg/ml and dialysed over night at 4°C into 50 mM ammonium acetate. Samples were snap frozen and stored in the -80°C. The York Centre of Excellence in Mass Spectrometry, University of York, conducted mass spectrometry analysis.

Results and Discussion

CHAPTER 3: Crystallisation and structural characterisation of the USP15 catalytic domain

3.1. Expression and purification of USP15-D1D2 for crystallisation

The expression and purification of C-terminal His-tagged USP15-D1D2 WT had previously been optimised²⁰⁸. This catalytic core construct expresses well in *E.coli* cells, and 3 litres of culture grown over night at 25°C typically yields around 4 mg of pure protein. Nickel affinity chromatography was sufficient for the separation of most impurities. The reducing agent dithiothreitol (DTT) is commonly used to prevent disulphide bridge formation between proteins and was found to greatly improve the peak resolution during size exclusion chromatography (SEC). Furthermore USP15 has been established as a protein that is sensitive to oxidation and requires reduction in order to be active, thus DTT was added to the sample prior to SEC.

USP15-D1D2 WT eluted as a single peak at 85 ml from gel filtration with a slight shoulder preceding the main peak. SDS PAGE gel electrophoresis showed this shoulder was likely due to the presence of some contaminants (figure 3-1). The latter half of the peak (90-97.5 ml) contained the purest fractions. Crystallography requires very pure monodispersed samples and the presence of contaminants may be detrimental to the crystallisation of the protein. Therefore, only the purest fractions were taken for further experiments.

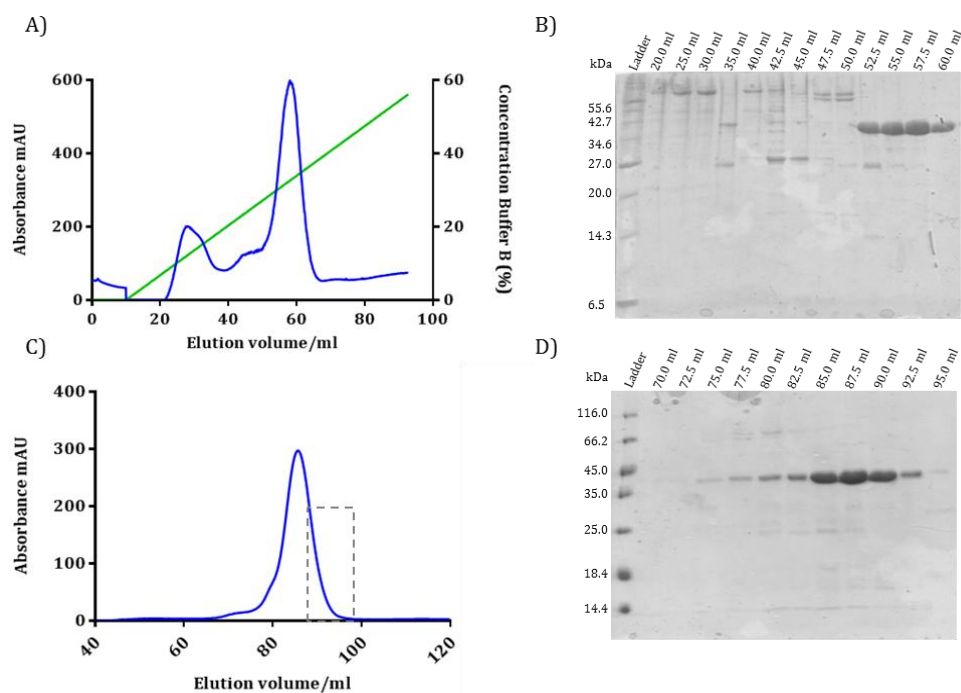


Figure 3-1: Purification of USP15 D1D2 WT. (A) Chromatogram of the NT-His Tag purification. The UV trace is shown in blue and concentration of buffer B (%) in green. (B) SDS-PAGE gel showing corresponding fractions from the NT-His tag purification. USP15-D1D2 has a molecular weight of 41 kDa. (C) Chromatogram of the SEC. USP15-D1D2 WT elutes at 85 ml as a single peak with a slight shoulder. The grey dashed box encompasses the fractions which were used for further experiments. (D) SDS PAGE gel of fractions corresponding to the SEC.

3.1.1. Finding new crystallisation conditions

USP15-D1D2 WT + mitoxantrone co-crystallisation trials conducted prior to beginning of this PhD (see section 1.8) yielded a single blue crystal which diffracted to 7 Å. Following on from this initial success, one the objectives of this project was to reproduce and optimize the crystal in the hope of improving the morphology and obtaining a higher quality data set.

A stock solution of mitoxantrone at a concentration of 1 mg/ml mitoxantrone solubilized in 100 % EtOH was prepared and used for subsequent experiments. Pure USP15-D1D2 WT was incubated with mitoxantrone at final concentrations of 97 µM and 560 µM respectively. The mixture was allowed to incubate for 30 minutes prior to use in crystallization screens. 24-well optimization plates containing variations in pH and PEG 2000 MME concentrations were tested (appendix 8.2.1). The trays contained mostly precipitate after 1 month incubation time at 20°C and crystal growth was absent in these optimizations.

Subsequently, sparse matrix screening methods were used to identify other conditions which might be favourable for USP15-D1D2 crystallization. The sample preparation was identical to before except a lower concentration of USP15-D1D2 WT (55 µM) incubated with 280 µM mitoxantrone was also included in the screens. All USP15-D1D2 WT + mitoxantrone 96 well co-crystallisation screens are summarized in appendix 3a.

Several conditions in the Memgold (I) screen and a couple of conditions in the Clearstrategy screen yielded crystals with a large fine needle cluster morphology, (figure 3-2). These crystals had grown in different conditions that were different to the original crystal and exhibited a new morphology. Crystals with a needle

cluster morphology are notoriously thin and fragile rendering them difficult to use for data collection, but can still be useful for providing insight into whether or not the crystal is a salt or protein.

The clusters were broken apart into single fragments, fished, cryoprotected and taken to Diamond Light Source for data collection. Dr Chan Li, University of Nottingham, collected the data. Most of the samples did not diffract at all, likely due to the fragments being too thin. The best crystal was obtained in 0.1 M bicine pH 8.9, 31% PEG 2000 (Memgold E10) and diffracted to 6.5 Å (see figure 3-2) and table 10). The low resolution meant structure determination was difficult, but it confirmed the crystal was protein and not salt.

Space group	Resolution	Unit cell parameters						I/sig(i)	Rmerge	Completeness
		A	B	C	α	β	γ			
P1	6.5 Å	42.66	42.83	92.73	79.96	87.88	89.60	2.1	0.33	97.4

Table 10: Summary of data collected for crystal fragment grown in MemGold E10.

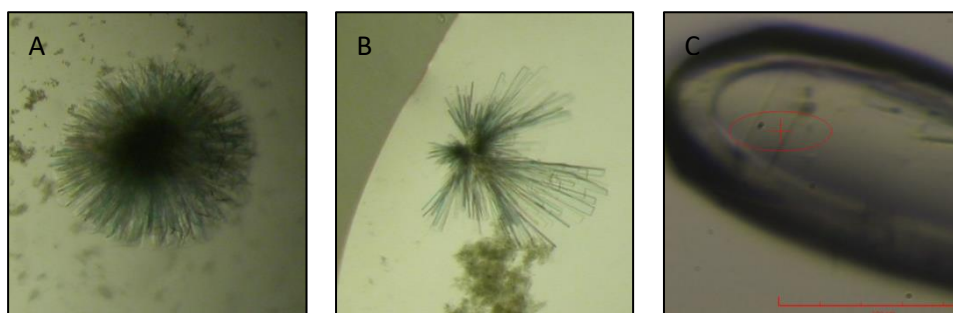


Figure 3-2: Examples of initial crystals obtained in the 96 well screening of USP15-D1D2 and mitoxantrone. (A) Very large needle cluster obtained in Clearstrategy E5. (B) Needle cluster grown in Memgold E10 (0.1 M bicine pH 8.9, 31% PEG 2000), several crystals in this screen shared this morphology. (C) Fragment taken from cluster in (B) and taken to the Diamond Light Source for testing which diffracted to 6.5 Å.

3.1.2. Optimisation of MemGold E10 crystallisation hit

Memgold E10 and Clearstrategy E5 yielded the biggest crystals. The Clearstrategy condition was included in the initial optimizations despite no diffraction being detected because the needles were likely to thin. Optimizations varying precipitant concentration and pH around these two conditions were tested. The optimization screens based around the Memgold condition yielded several crystallisation hits in contrast to Clearstrategy optimizations, where the crystal was not reproducible. Consequently, further optimizations concentrated on the Memgold E10 condition.

Further optimizations (appendix 3a) varying the precipitant composition and concentration, salt concentration buffer, pH, drop size, drop ratio, temperature and protein concentration, were able to consistently reproduce the needle clusters but not improve the overall morphology. The clusters had a light blue tinge which implied the mitoxantrone was present within the crystal.

Protein concentration was trialled at 2, 4, 6 and 8 mg/ml, with 4 mg/ml being deemed the optimum concentration due to excessive protein precipitation at higher concentrations and reduced crystal formation at lower concentrations. No trend in crystal formation with respect to variation in precipitant concentration, buffer components, pH, and salt concentration was identified. Equally, variation in drop size and drop ratio had no influence over the crystallization behaviour. Trays were set up at 10°C, 15°C, 20°C and 25°C (trays 10-16, appendix 3a) with the aim of reducing the rate of crystal nucleation and increasing crystal size. The temperature had minimal effect on the crystal morphology and size but resulted in greater numbers of crystals per drop at 25°C and fewer crystals at 10°C and 15°C.

3.1.2.1. Optimisation of mitoxantrone preparation

A substantial amount of precipitant was observed in the protein-mitoxantrone sample after the 30 minute incubation period which may have been detrimental to crystal formation. In addition mitoxantrone was not fully soluble in 100 % ethanol. Consequently, use of an alternative solvent to solubilize the mitoxantrone was explored, as mitoxantrone is readily soluble to high concentrations in DMSO. Since high concentrations of DMSO can be damaging to proteins, 50 % DMSO was chosen to solubilize mitoxantrone and the final concentration of DMSO never exceeded 20 %. Optimization plates containing conditions which had previously yielded high numbers of crystals were used to test the effect of solubilizing mitoxantrone in 50 % DMSO. USP15-D1D2 was incubated with mitoxantrone solubilized in both 50 % DMSO and 100% ethanol for 30 minutes. Samples containing the DMSO solubilized mitoxantrone had significantly less precipitant after 30 minute incubation at room temperature, and less precipitation was observed in the drops after 1 week incubation time. Four trays were trialled varying protein concentration from 2.5-4 mg/ml and PEG 2000 concentration from 25-35 % w/v in 0.1 M bicine pH 8.9, and incubated at 15°C or 25°C (see appendix 3b).

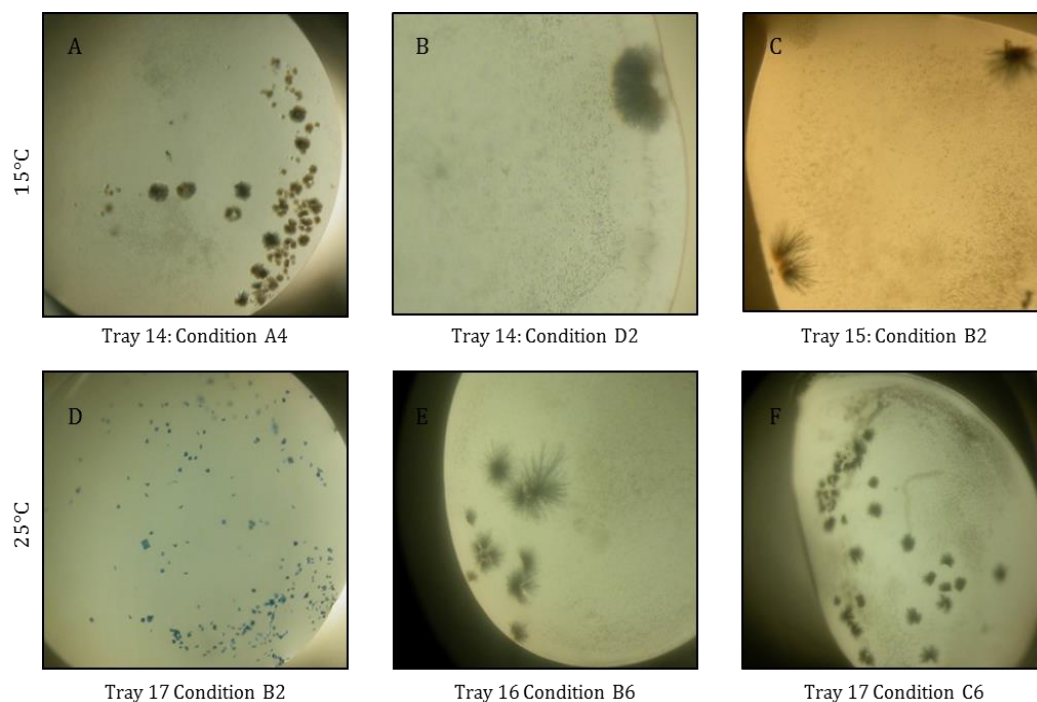


Figure 3-3: Example crystals from USP15-D1D2-mitoxantrone co-crystallisation optimisation trays. (A-C) contain needle clusters grown at 15°C in the presence of mitoxantrone solubilised in 100 % ethanol and 50 % DMSO. An example of the small blue crystals found in several conditions at both temperatures containing mitoxantrone solubilised in 50 % DMSO is shown in (D). Needle clusters grown at 25°C using 100% ethanol and 50 % DMSO are shown in (E-F)

Trays containing 50 % DMSO solubilized mitoxantrone resulted in several conditions containing needle clusters of similar morphology to previously obtained crystals. In addition several drops comprising of showers of bright blue, small quadratic shaped crystals were present in trays grown at both 15°C and 25°C (figure 3-3, D) . These blue crystals were unfortunately too small to be fished and taken to Diamond Light Source to be tested to confirm whether these were protein crystals or crystals of mitoxantrone. All further crystallizations experiments utilized 50 % DMSO for solubilisation of the mitoxantrone.

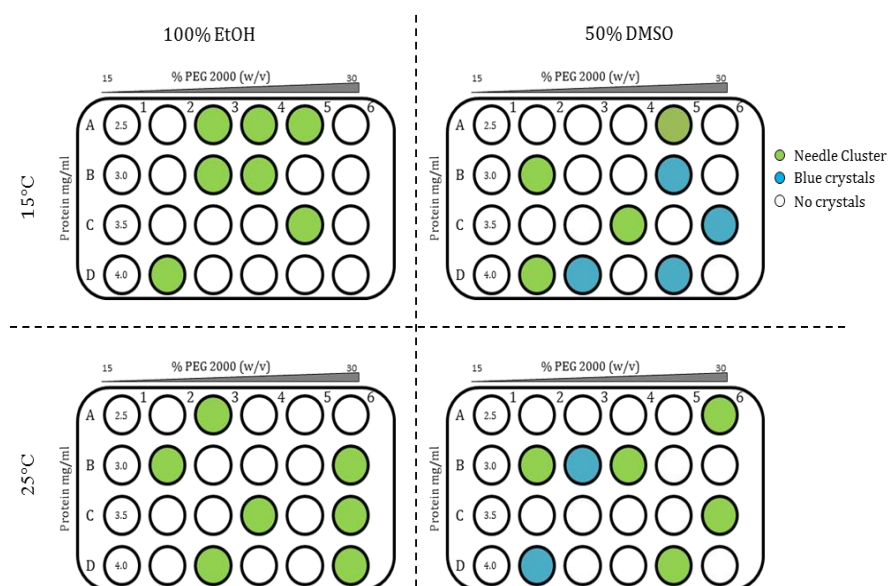


Figure 3-4: Schematic representation of distribution of crystals in optimisation trays. Trays tested the effect of varying protein concentration, % PEG 2000 and temperature with mitoxantrone either solubilised in 100% ethanol or 50 % DMSO. White wells represent clear drops or drops containing precipitate, green wells contained needle clusters in varying numbers and sizes and blue wells contained showers of tiny blue crystals.

3.1.3. Seeding experiments

Once an initial crystal has been obtained, micro and macro seeding can be very powerful techniques for promoting crystal growth in different conditions and can result in the formation of crystals in alternative morphologies. A seed stock was prepared as described in methods section 2.4.3 using the needle clusters obtained in the optimization plates described above.

Protein was prepared as before to a final concentration of 97 μM (4 mg/ml) and 48 μM (2 mg/ml), and mitoxantrone was solubilized in 50 % DMSO and incubated with the protein for 30 minutes at a final concentration of 970 μM and 480 μM respectively. Six screens were trialled and are summarized in table 11. The trays were allowed to equilibrate at 20°C following addition of the protein-

mitoxantrone to the mother liquor for 2 days, before the seed stock was introduced.

Screens	Drop size	Mother liquor	Protein	Seed stock
PEGs Procomplex Index The cations Classics MPD	1 μ L	500 nL	400 nL	100 nL

Table 11: Summary of screens and drop composition in the initial seeding experiments of USP15-D1D2 and mitoxantrone. Needle clusters from figure 3-3 were used to create the seed stock

After 1 week, several large blue crystals were obtained in several conditions in screens: MPD, Procomplex and Index. In general, the crystals were a rich blue colour and exhibited an elongated irregular pentagon shape and were a mix of overlapping and individual crystals (figure 3-5, A-E). In addition, several drops contained showers of smaller, thinner crystals (figure 3-5, F), similar to those observed in the DMSO optimization attempts.

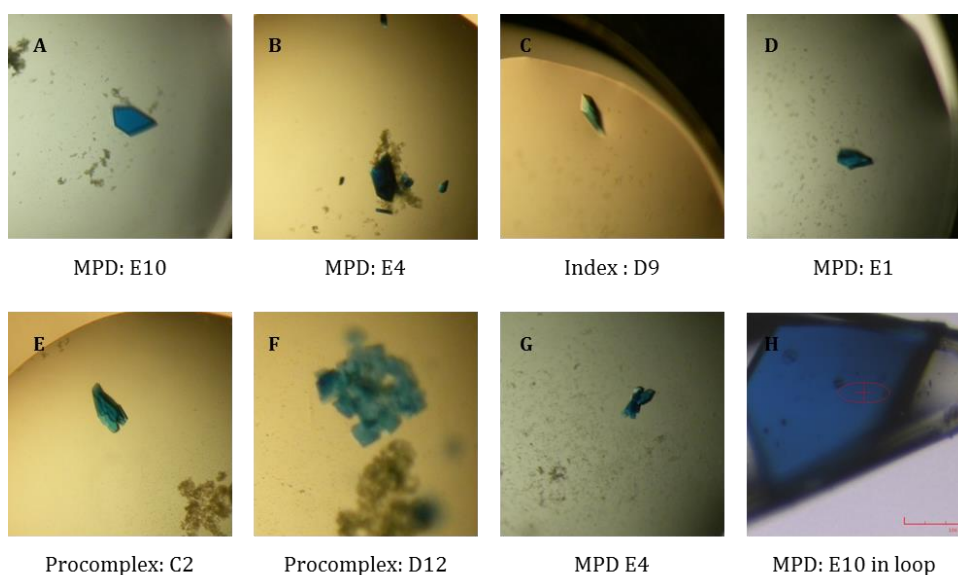


Figure 3-5: Crystals of USP15-D1D2 in complex with mitoxantrone obtained from micro-seeding experiments in 96 well screens.

The most promising crystals were cryocooled and taken for data collection at the synchrotron. The best crystal was a large blue single crystal obtained in the condition MPD E10 (0.1 M Hepes pH 7.0, 10% MPD) at 4 mg/ml protein concentration which diffracted to 2.3 Å (figure 3-5, A and H).

3.1.3.1. Optimisation of crystals generated by seeding experiments.

These crystals were easily reproducible in a variety of conditions based around the MPD E10 components in 24 well optimization screens; however, crystals that were single and of suitable size thickness were harder to obtain. Further 96 well screens identified additional conditions which promoted the co-crystallization of USP15-D1D2 WT with mitoxantrone using seed-stocks to initiate crystals formation. Subsequent rounds of optimizations (screens 34-39) deduced a series of conditions which could reliably produce crystals of a high quality. These conditions were used to design a 96 well screen for further crystallization experiments with USP15-D1D2 and mitoxantrone. Seeds stocks were used in the 96 well screens to maximize crystallization potential.

Undiluted seed stocks produced hundreds of minute crystals in every drop and each ten fold dilution of the seedstock resulted in crystals in more practical size and number (figure 3-6). This mitoxantrone screen provided a robust foundation for additional crystallization experiments such as soaking. Crystallization of USP15-D1D2 WT with no mitoxantrone was trialled on multiple occasions in parallel with co-crystallization experiments with mitoxantrone however most drops contained precipitant after 1 week incubation time, even at concentrations as low as 1 mg/ml.

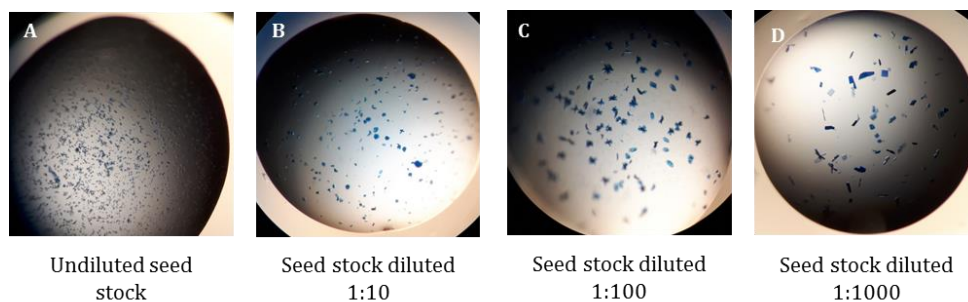


Figure 3-6: Mitoxantrone 96 well screen varying seed stock dilution from undiluted to 1:1000. Screens were set up with 4 mg/ml of USP15-D1D2 and 0.43 mg/ml mitoxantrone and incubated 20°C. Crystals grew within 48 hours. Undiluted seed stock resulted in thousands of micro crystals (A) and each ten fold dilution of the seed stock (B-D) resulted in larger crystals.

3.1.4. PR-619 co-crystallisation trials

PR-619 is a broad-spectrum DUB inhibitor, which forms an irreversible covalent interaction with the catalytic cysteine. As the presence of a new ligand may alter crystal packing and crystallisation behaviour, new 96 well screens in addition to the mitoxantrone screen, were trialled to probe the co-crystallisation of USP15-D1D2 with PR-619. The first trays contained predominantly precipitate. Trials were also performed with mitoxantrone present as an additive but the screen still contained large amounts of precipitant. The solubility of PR-619 was identified as an issue with heavy precipitation of the compound occurring during sample preparation.

Multi-component solutions were explored in an attempt to improve the solubility of PR-619²¹⁹. The use of high concentrations of glycerol, ethyleneglycol and dioxane improved solubility of the compound but the presence of glycerol could hamper crystal nucleation, thus crystal soaking was selected as a more suitable method.

Crystals grown in the 96 well mitoxantrone screen were subjected to a 1 week soak with 2.2 mM PR-619 solubilised in the multicomponent solution. Crystals were fished, cryocooled and sent to Diamond Light Source for data collection. The crystal, shown in figure 3-7, diffracted to 2 Å and yielded a high quality data set.

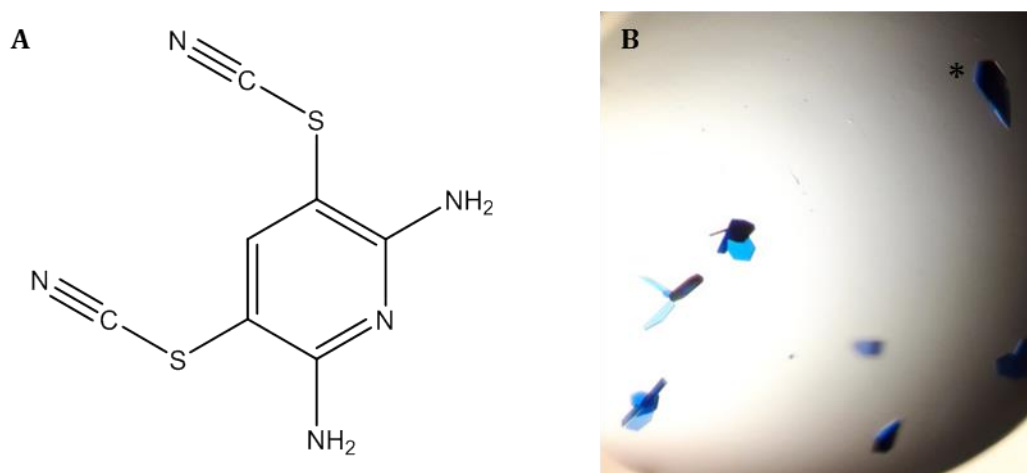


Figure 3-7: Crystals grown in the 96 well mitoxantrone screen used for PR-619 soaking experiments. (A) shows a schematic of PR-619 drawn in ChemDraw. (B) Crystals grown in the 96 well mitoxantrone screen and soaked for 1 week with 2.2 mM PR-619 solubilised in 50% glycerol, 25% ethylene glycol and 8.75% dioxane and 10% DMSO. The star indicates the crystal, which gave a 2 Å data set.

3.1.5. Mitoxantrone soaking experiments

Mitoxantrone soaking experiments were performed to maximise the chance of locating a possible binding site for the inhibitor. The blue colour of the crystals lead to the assumption that mitoxantrone had been incorporated into the crystal lattice (see figure 3-8 for an example crystal used in soaking experiments). It was possible that the mitoxantrone was disassociating upon cryoprotection as there was no mitoxantrone present in the cryoprotectant solution. USP15-D1D2 WT was co-crystallized in the presence of 1.5 mM mitoxantrone, a 1:15 ratio respectively. The crystals were allowed to soak for 3 hours before being fished and cryoprotected for data collection. A further 1.5 mM mitoxantrone was added to the cryoprotectant to reduce the chance of disassociation of the mitoxantrone during

the cryoprotection step. A 2.1 Å data set was collected at the Diamond Light Source and the data was auto-processed using the pipelines available.



Figure 3-8: Example crystal used for mitoxantrone soaking experiments. Crystals were grown in the presence of 1.5 mM mitoxantrone.

3.1.6. Summary of crystal trials

Mitoxantrone was found to be integral for the crystallisation of USP15-D1D2 WT. Crystals were initially obtained in a needle cluster morphology and optimisations and micro seeding experiments successfully improved the crystals allowing a 2.3 Å data set to be collected. The crystal was reproducible and optimised further to deduce a series of conditions which formed the basis of a new 96 well screen.

Soaking experiments were performed with the broad-spectrum DUB inhibitor PR-619 on crystals grown in the mitoxantrone 96 well screen, however despite the crystal giving a high resolution data set of 2 Å and different cell parameters (see section 3.2.1, table 12) PR-619 was not present.

3.2. USP15 D1D2 x-ray crystallographic model

3.2.1. USP15-D1D2 WT structure solution

Two high quality data sets were obtained from the crystallization experiments, with the highest resolution of 2 Å achieved during the PR-619 soaking experiment (referred to as USP15-free henceforth). The crystals belonged to space group $P 2_1$ and were indexed through an automated pipeline such as Autoproc²¹⁰ and DP, or manually indexed using imosfilm²¹¹. The structure was initially solved using the published USP4-D1D2 structure (2Y6E)¹⁴ as a search model and subsequent structures used the newly solved USP15-D1D2 structure for molecular replacement. Single solutions were found and Refmac5²¹⁶ and Phenix refine²¹⁵ were utilized for subsequent rounds of model refinement intercepted by model adjustments. Data collection and refinement statistics are shown in table 12.

	USP15-D1D2 (USP15-free)	USP15-D1D2- mitoxantrone
Data collection		
Space group	P2 ₁	P2 ₁
Cell dimensions		
<i>a</i> , <i>b</i> , <i>c</i> (Å)	48.51, 62.62, 62.04	62.07, 94.39, 63.29,
β (°)	104.97	90.08
Resolution (Å)	1.98	2.09
<i>R</i> _{merge}	0.111 (1.060)	0.102 (1.619)
<i>R</i> _{pim}	0.074 (0.711)	0.052 (0.87)
<i>I</i> / σ <i>I</i>	4.7 (1.7)	9.9 (1.57)
CC1/2	0.991(0.527)	0.998 (0.668)
Completeness (%)	97.7 (86.3)	98.8 (98.5)
Redundancy	3.0 (3.0)	4.8 (4.9)
Wilson B-factor (Å ²)	33.0	38.1
Refinement		
Resolution range (Å)	46.54-1.98	62.07-2.09
No. of reflections	24825	42810
<i>R</i> _{work} / <i>R</i> _{free}	0.198/0.234	0.203/0.251
No. atoms	2734	5319
Protein	2602	5129
Other	1	37
Water	131	153
<i>B</i> -factors (Å ²)		
Protein	48.1	59.0
Ligand	-	88.3
Water	50.5	50.5
R.m.s. deviations		
Bond lengths (Å)	0.007	0.007
Bond angles (°)	0.794	0.904

*Values in parentheses are for highest-resolution shell.

Table 12: Crystallographic data collection and refinement statistics

The USP15-free data set (PDB code: 6GHA) contained one molecule of USP15-D1D2 in the asymmetric unit. No density corresponding to PR-619 or mitoxantrone was observed in the PR-619 soaked crystal electron density. In contrast the mitoxantrone soaked crystal (termed USP15-D1D2-mitoxantrone) contained two copies in the asymmetric unit (PDB: 6GH9).

The structures showed the same overall architecture with differences arising in the modelling of the flexible regions (figure 3-9). All the individual USP15-D1D2 molecules could be superimposed with an RMSD of 0.635 Å.

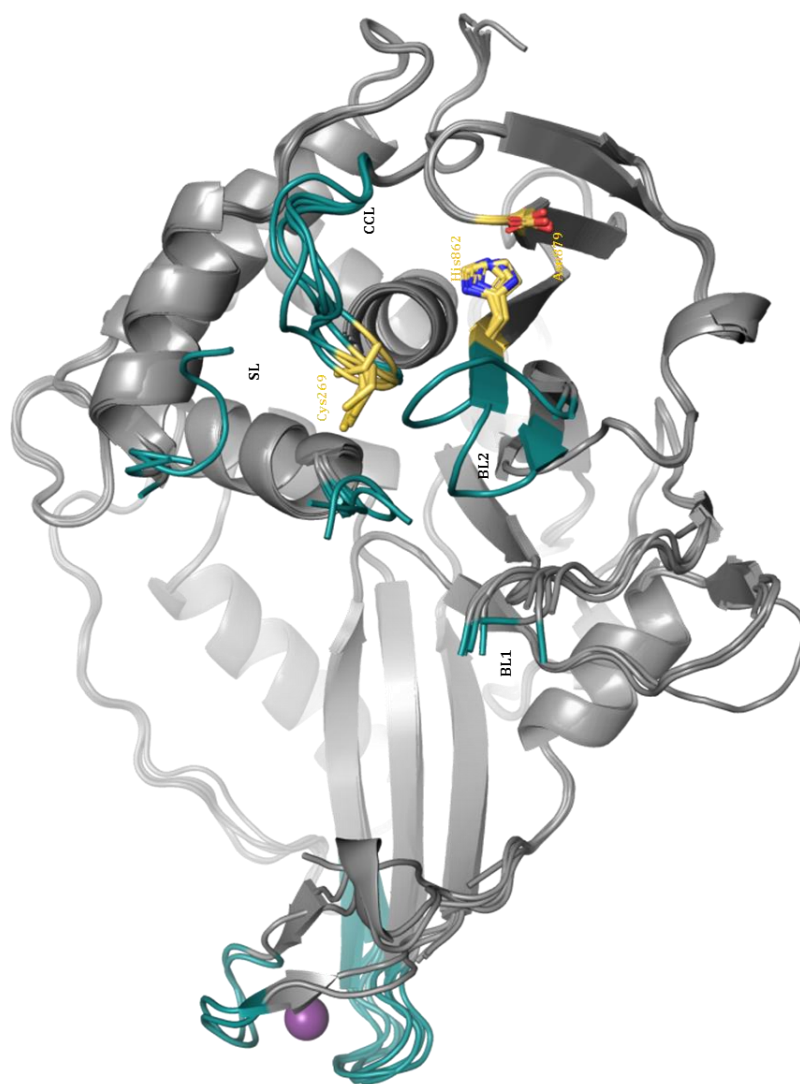


Figure 3-9: Superposition of all molecules of USP15-D1D2. The variable regions are highlighted in (teal) between each structure. Variable regions map to the BL1, BL2 and SL as well as the zinc finger region. The catalytic triad is shown as yellow sticks.

3.2.2. Overall structural analysis

Of the available structures for USP catalytic domains, two thirds are bound to an ubiquitin variant or a covalent inhibitor with the remainder of the structures in the unbound form. Unbound USP structures are available for USP7 (PDB: 1NB8) USP14 (PDB: 2AYN), USP12 (PDB: 5K16), USP8 (2GFO), USP18 (5CHT), USP4 (2Y6E) and CYLD (2VHF)^{2, 4, 14, 93-95}.

The USP15-D1D2 structure encompasses 18 β -sheets and 9 α -helices. The model displays the classic USP catalytic domain fold with a finger thumb and palm region (figure 3-10). USP15 harbours the zinc binding motif Cys-X-X-Cys⁹⁷ at the tip of the fingers region and is fully resolved in USP15-free and USP15-mitoxantrone. A single Zn^{2+} ion is coordinated by four cysteine residues at the tip of the finger region and has been shown to be integral for the deubiquitinating activity of USP15 against poly-ubiquitin chains¹⁸⁰. Functional zinc binding motifs are found in other USPs containing similar large insertions in the catalytic core and it is presumed the co-ordination of zinc plays a role in protein stabilization⁸⁸.

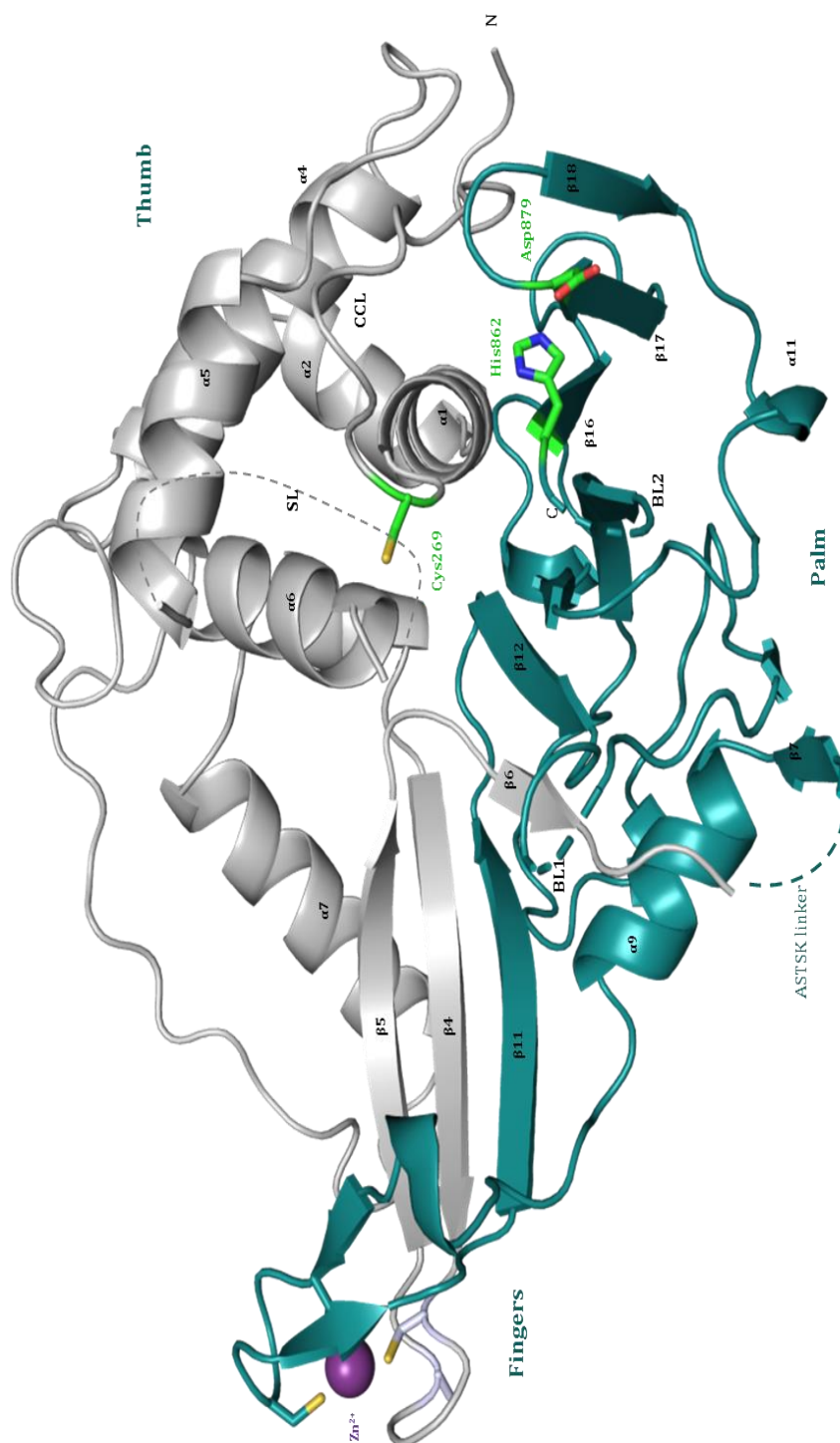


Figure 3-10: Cartoon representation of free USP15-DID2 The two halves of the catalytic core are shown in light grey and teal and the ASTSK linker shown as dotted line. The catalytic triad are depicted as sticks (green). The four cysteine residues, represented as sticks, are co-ordinating a single zinc ion (purple sphere). Missing residues in the BL1, BL2 and SL loops are indicated with dashed lines.

The catalytic cleft loop (referred to as the CC loop or CCL) comprised of residues Ser263-Phe270 (SNLGNTCF) is located between $\beta 1$ and $\alpha 1$ and houses the catalytic cysteine (Cys269). The other catalytic triad residues His862 and Asp879 are found at the end of $\beta 16$ and $\beta 17$ respectively. Importantly, Cys269 is located 10 Å from the catalytic histidine, too far for any meaningful interactions to occur (figure 3-11, A). The catalytic histidine and aspartate are pre-aligned and stabilized via a hydrogen bond. In this misaligned configuration, USP15 would be

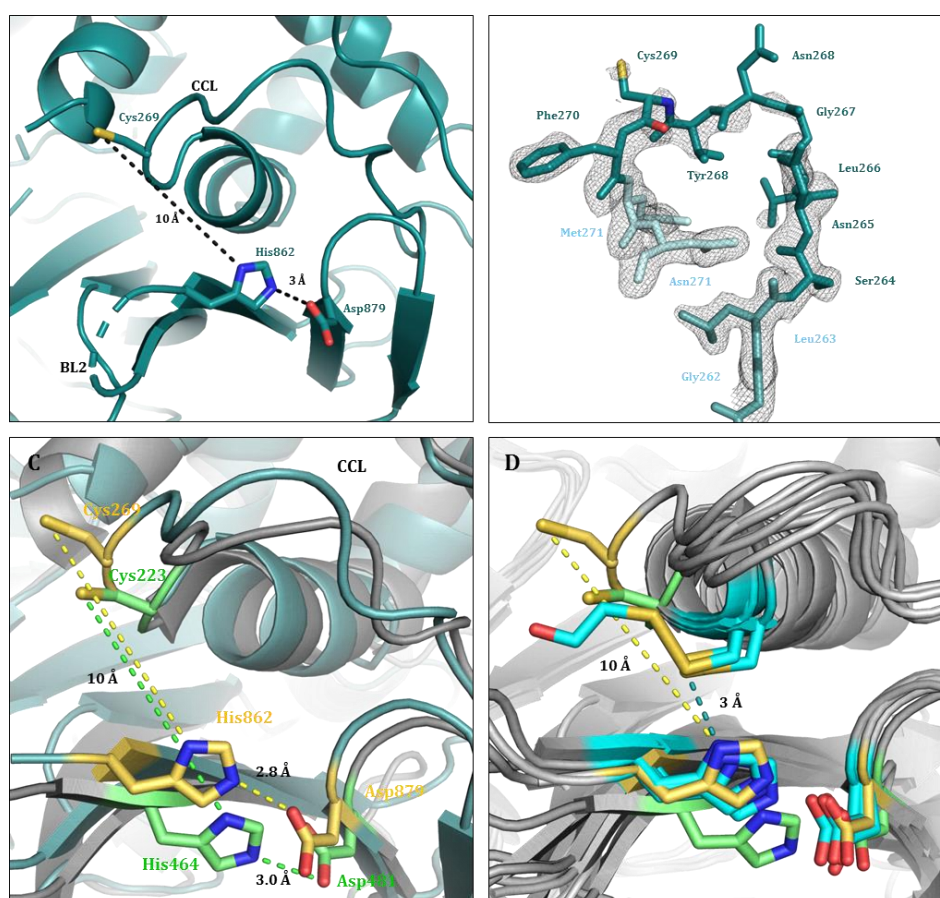


Figure 3-11: The catalytic triad in the free USP15-D1D2 structure is in a catalytically incompetent conformation. (A) The catalytic triad is shown as sticks with Cys269 located 10 Å from His862. (B) The CC loop (SNLGNTCF) shown in dark teal and local residues (light teal) are shown as sticks along with the 2mF_o-F_c map. (C) Superposition of USP15-D1D2 catalytic triad (Yellow) with unbound USP7 (green) (PDB: 1NB8) showing similarities in catalytic triad arrangement (D) Superposition of free USP4 (PDB: 2Y6E), USP14 (PDB: 2AYN), USP8 (PDB: 2GFO) USP7 and USP15 catalytic triads (blue).

unable to cleave the C-terminal tail of ubiquitin and would require rearrangement of the catalytic triad.

USP7 is the only other USP to display similar misalignment of the catalytic cysteine amongst the free USP structures available, whereby the catalytic cysteine is also located 10 Å away from the catalytic histidine (figure 3-11, C). Subsequent structures of the USP7 catalytic domain in complex with ubiquitin revealed a large conformational change in the CC loop and switching loop (SL) region^{2, 92, 220}. Alignment of all the free USP catalytic domains available reveals the catalytic histidine and aspartate in USP15 adopt similar conformations and are pre-aligned for catalysis (figure 3-11, D). Similar to USP7 it is likely that the rearrangement of the CC loop and SL bring the catalytic cysteine to close proximity of the His862 permitting cleavage of the ubiquitin C-terminus.

B-factors are an indicator of uncertainty for atom localization and positioning within a crystal structure. High B-factors are associated with low electron density and are usually indicative of a flexible region within the crystal lattice. B-factor 'putty' representation of the structure, whereby thick darker regions are associated with high B-factors helps to visualize the flexible regions within the model (figure 3-12).

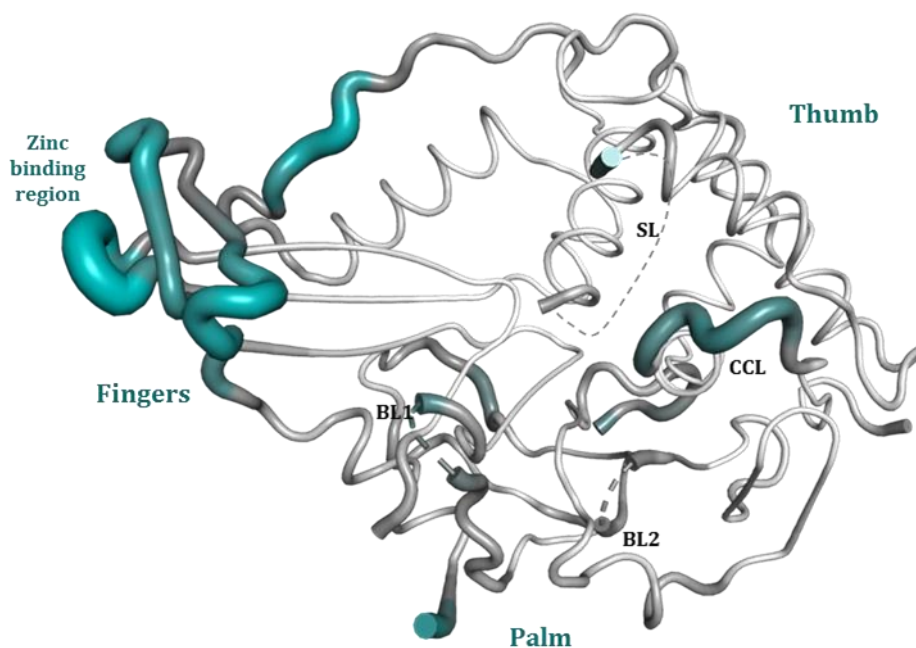


Figure 3-12: Putty representation showing variation in B-factors throughout the USP15-D1D2 model. Thickness of the putty is directly proportional to the B-factor and is indicative flexibility in that region, and the highest b-factors are highlighted in teal and the lowest in pale grey.

The CC loop is associated with high B-factors and is highlighted in figure 3-12 as a region of flexibility. The density in this region was also weaker in comparison to other areas of the structure (figure 3-11, B), supporting the notion that the area is flexible and able to undergo conformational changes upon ubiquitin binding.

In USP7, conformational changes arising from the accommodation of ubiquitin predominantly map to the active site. The SL was highlighted as an important contributor to the catalytic triad realignment and was subject to rearrangement

upon ubiquitin binding. The SL is defined well in both free and bound structures of USP7, which has been attributed to possible local crystal contacts stabilizing the loop.

USP15 residues conferring to the SL (Pro341-Glu353; PQFSGYQQQDCQ) are found between helices $\alpha 5$ and $\alpha 6$ and include the highly conserved QQD box. In all USP15 structures, the switching loop is flexible and was unable to be fully modelled due to poor electron density.

USP15	P-QFSGYQQQDCQ
USP4	P-QFSGYQQQDSQ
USP7	WETLDSFMQHADVQ

Figure 3-13: Sequence alignment of USP15, USP4 and USP7 switching loops. Conserved residues are highlighted in black and Cys352 is highlighted in green. The alignment shows the single residue difference between USP15 and USP4 with much greater variation for USP7

Structural alignment with both the bound and unbound USP7 structures and USP4-D1D2, in which the SL was resolved helps shed light on the necessity for the rearrangement of this loop (figure 3-14). The neighbouring tyrosine (Tyr224) to the catalytic cysteine in USP7 flips when bound to ubiquitin resulting in a shift of 6 Å and is thought to dictate the conformation of the catalytic cysteine. The position of the equivalent residue in USP4, Phe312, reflects that of the ubiquitin bound form of USP7 whereas the USP15 equivalent, Phe270, is rotated by over 8 Å and resembles the conformation of the Tyr224 in the free USP7. In addition, the current conformation of USP15 Phe270 clashes with Ser394 in the SL of USP4 and Val296 in free USP7, indicative of an alternate SL conformation for USP15 (figure 3-14, B). Comparison of all the USP structures (free and bound) shows common SL conformations with exception of free USP7.

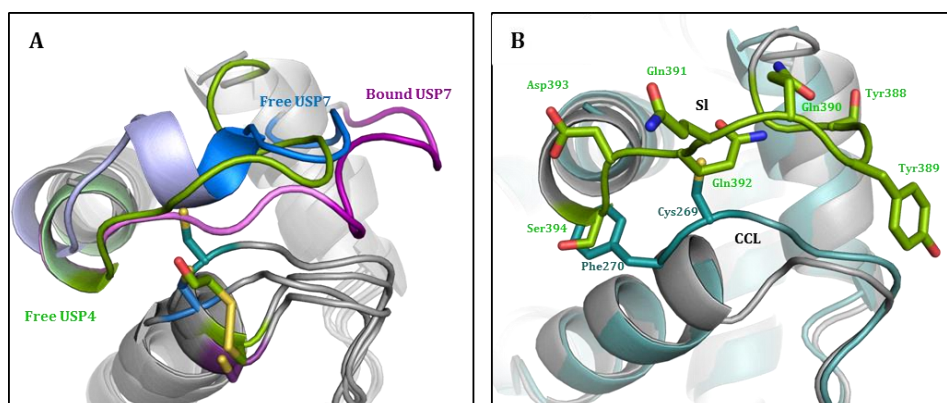


Figure 3-14: Structural comparison of the switching loop in USP15-free, USP7-free, bound USP7 and USP4. (A) Cartoon representation of the different conformations of USP7 free (blue) USP7 bound to ubiquitin (purple) and USP4 (green) the catalytic cysteine are shown as sticks in the respective colours. Darker colours indicate SL residues. (B) The SL of USP4 (green) is depicted as sticks and highlights the clashes with USP15-D1D2 (teal) Cys269 and Phe270 and USP4 Gln392 and Ser394 respectively.

The misaligned catalytic triad has been associated with poor activity of the isolated USP7 catalytic domain. Electrostatic interactions in the SL of the unbound form of USP7 and unfavourable hydrophobic packing upon ubiquitin binding in the active sight region was identified as a reason for the USP7 catalytic domain favouring the inactive conformation. His294 in the SL, which is protonated in the unbound form, was identified to form an electrostatic network with Glu298 and Trp285. Deprotonation of this histidine occurs upon ubiquitin binding, and the network is disrupted (figure 3-15). In addition, several leucine residues, which are replaced by larger aromatic residues in many USPs, was found to contribute to unfavourable hydrophobic packing upon ubiquitin binding⁹⁶.

In contrast, USP15 shows high catalytic activity irrespective of the presence of its ancillary domains suggesting the disrupted catalytic triad easily rearranges to accommodate ubiquitin^{14, 208}. Due to the SL not being fully modelled conclusions cannot be made with regards to bonding networks within the SL influencing conformational changes. However, comparison of the position of Glu354 and Pro342 with the equivalent USP7 residues Glu298 and Trp285 reveals the residues adopt different conformations and are not in positions favourable for forming meaningful interactions. Furthermore, USP7 Tyr224 is able to contribute more to hydrogen bonding than its phenylalanine counterpart in USP15, which also clashes with Val296 in the SL of USP7. USP15 contains a high proportion of aromatic residues (namely Tyr313, Phe333 and Tyr347) which replace the leucine residues in USP7, and are likely to contribute to favourable hydrophobic packing during the active site rearrangement, stabilizing the active conformation.

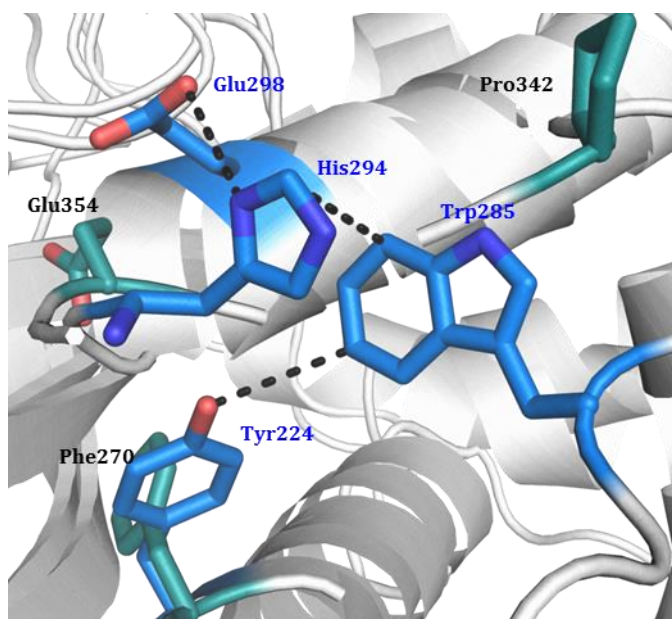


Figure 3-15: Electrostatic networks in the USP7 switching loop stabilises the inactive conformation. USP7 (PDB: 1NB8) residues are represented as blue sticks and USP15 and in teal. Interactions between Glu298, His294, Trp285 and Tyr224 in the SL are indicated with the dashed line and contribute to the stabilisation of the inactive conformation. Equivalent USP15 residues adopt different conformations.

Collectively these observations suggest an alternative new flexible conformation of the SL in unbound USP15 to accommodate the inactive catalytic triad conformation. It is predicted that USP15 will undergo similar conformational changes to USP7 in order to accommodate ubiquitin into catalytic cleft. Differences between USP7 and USP15 in the SL region and different in catalytic domain activity suggests these rearrangements will be more favourable in USP15.

3.2.2.1. Ubiquitin accommodation

Superposition of ubiquitin onto the USP15-free structure reveals steric clashes between several residues in ubiquitin (figure 3-16) with the SL, CC loop, Blocking Loop 1 (BL1), Blocking Loop 2 (BL2) and the finger region, indicating movement in these regions to accommodate ubiquitin into the active site. These observations coincide with flexible regions highlighted in figures and 3-9 and 3-11.

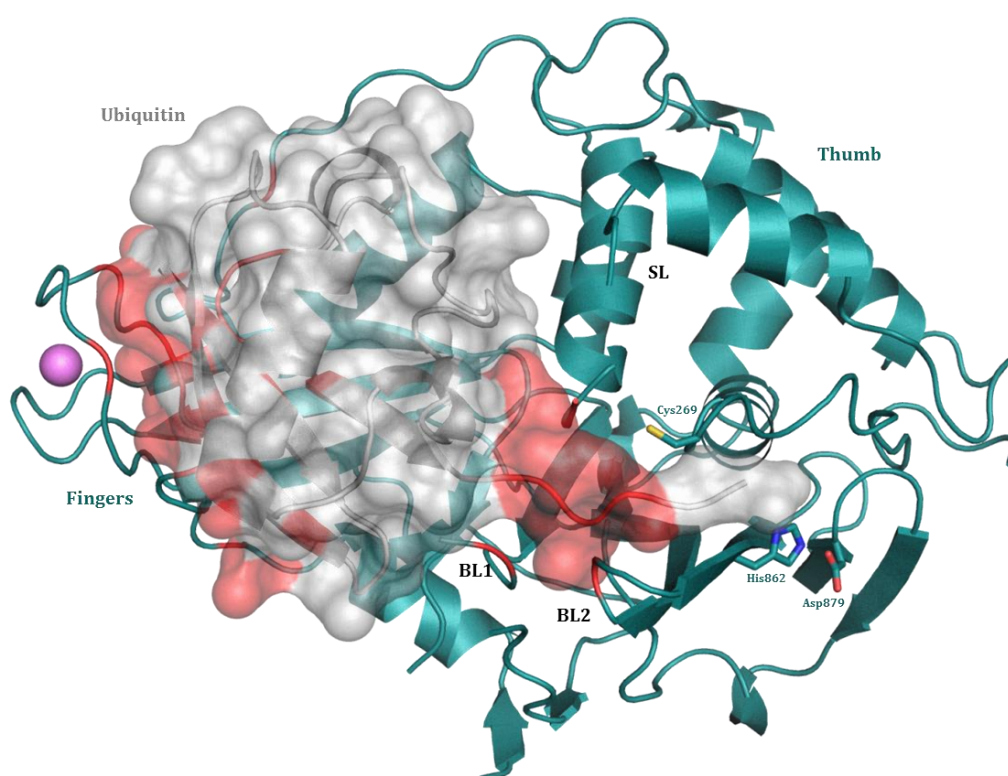


Figure 3-16: Superposition of ubiquitin with USP15-free. The surface structure of ubiquitin is modelled on to USP15-free shown in teal cartoon representation. Regions in red highlight clashes between ubiquitin residues and USP15-free in the superimposed structure. Most clashes map to the BL1, BL2, SL and finger region of USP15. The catalytic triad is shown in sticks representation and the Zn^{2+} as a purple sphere. (USP2-ub structure PDB: 2HD5)

Closer examination of the C-terminal tail of ubiquitin highlights residue clashes within the catalytic cleft. Residues in the SL, Cys352 and Gln353, clash with the side chain of Arg72 and the $C\alpha$ of Leu73 respectively (figure 3-17, A and B). Phe809, the preceding residue to the BL1 interferes with the side chain of Leu73 (figure 3-17, C). In addition, Met858 located at the tip of BL2 clashes with ubiquitin Arg74 (figure 3-17, D). Realignment of the catalytic triad upon ubiquitin binding will likely coincide with movement of the BL2 and the SL and rearrangement of the side chains as observed for USP14⁹³.

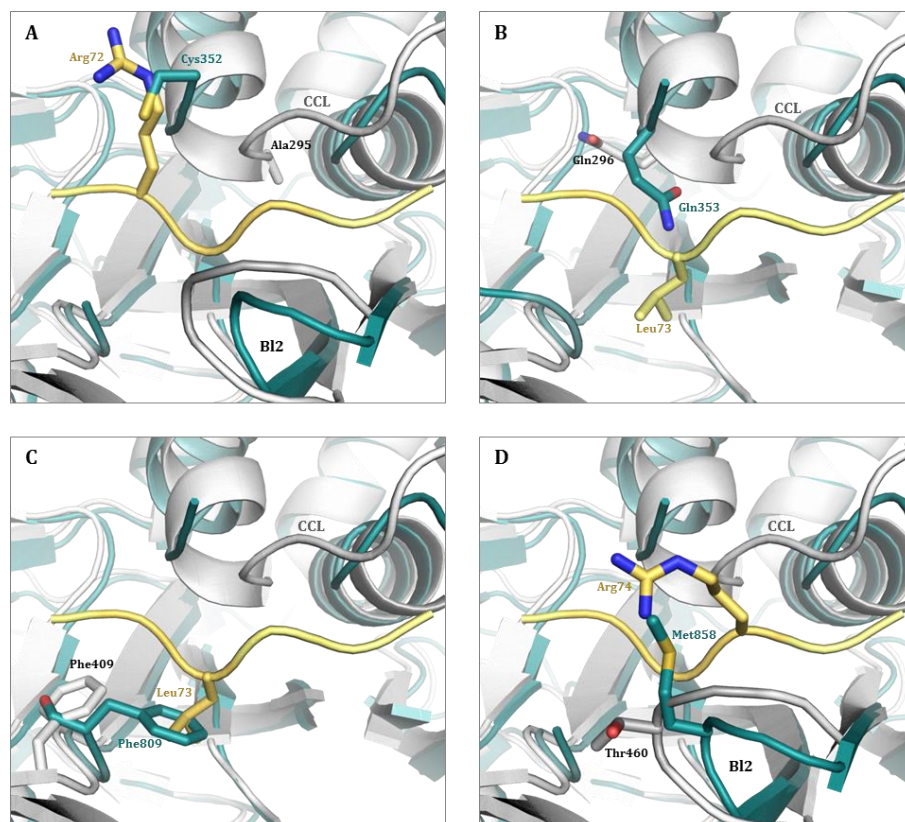


Figure 3-17: Superposition of ubiquitin bound USP2 (PDB: 2HD5) highlights clashes between USP15 and ubiquitin residues. Ubiquitin residues are portrayed in pale yellow, USP15 in teal and USP2 in white. Clashes between USP15 and the C-terminal tail are shown with the equivalent USP2 residue shown in a different conformation.

CHAPTER 4: Molecular basis of mitoxantrone inhibition

4.1. Mitoxantrone binds in the S1' pocket

Excitingly, in the USP15-mitoxantrone structure, an additional planar shape of electron density could be seen in close proximity to His862 in the BL2 region (figure 4-1). The anthracene rings of mitoxantrone fitted well into the density with weaker density observed for the flexible side arms of mitoxantrone (figure 4-2, A).

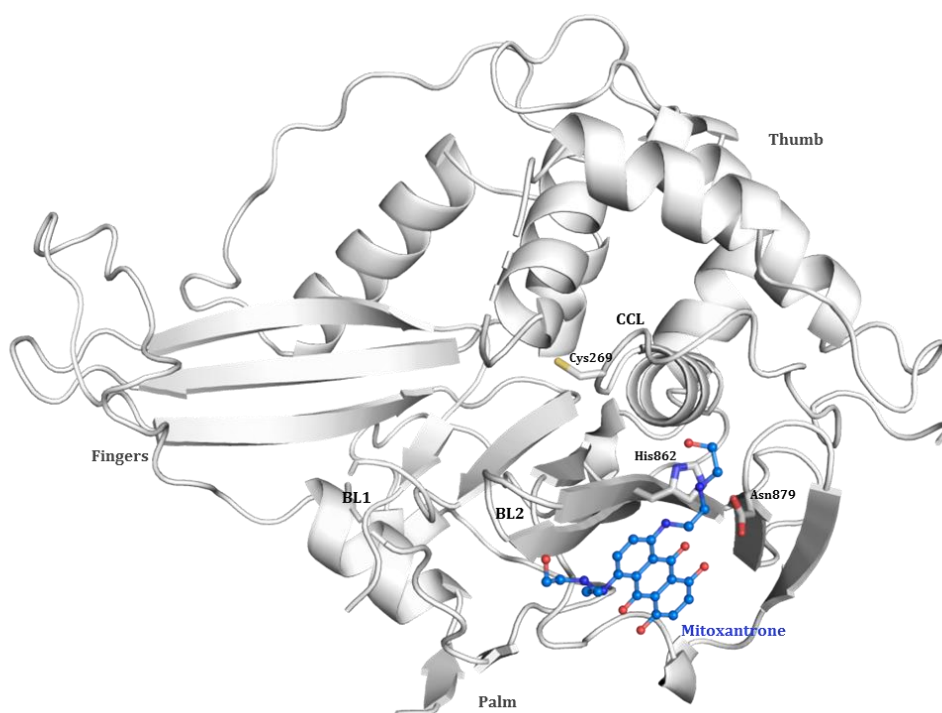


Figure 4-1: Cartoon representation of USP15-D1D2-mitoxantrone complex crystal structure. The USP15 is depicted in white and mitoxantrone is represented as ball and stick structure (blue). The catalytic triad residues are shown in stick representation.

Hydrophobic contacts between mitoxantrone and Tyr855, Gly856, Gly860, and His862 in the BL2 dominate the interaction (figure 4-2). One of the side arms contacts the amine group of Asn264 in the CC loop, forming a weak hydrogen bond. The presence of mitoxantrone in the structure induces few conformational changes and has no influence over the conformation of the catalytic triad, with the catalytic cysteine

remaining 10 Å from the catalytic histidine. The density for the BL2 was better defined in the presence of mitoxantrone, suggesting stabilization of the loop. Finally, generation of symmetry mates in Pymol confirms the binding of mitoxantrone at this site is not aided by crystal contacts, figure 4-3.

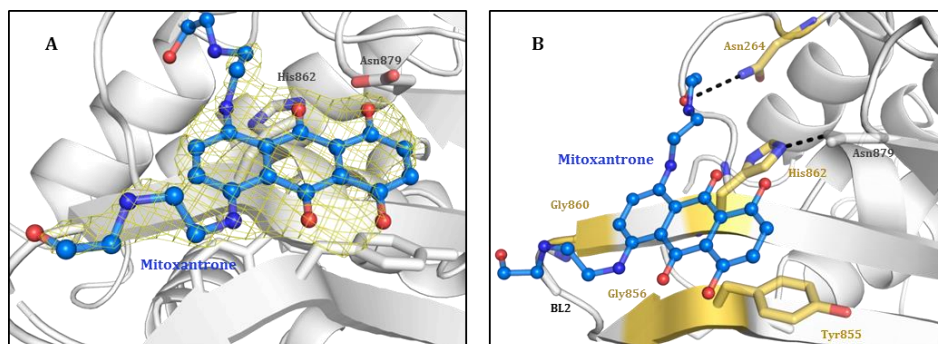


Figure 4-2: Molecular basis of the mitoxantrone- USP15-D1D2 interaction.

Mitoxantrone is shown in blue as a ball and stick representation in both (A) and (B). The $2mF_o - F_c$ electron density map contoured to a sigma level of 1 is shown in yellow (A). Residues thought to be important for the interaction are highlighted in yellow and shown in stick representation (B).

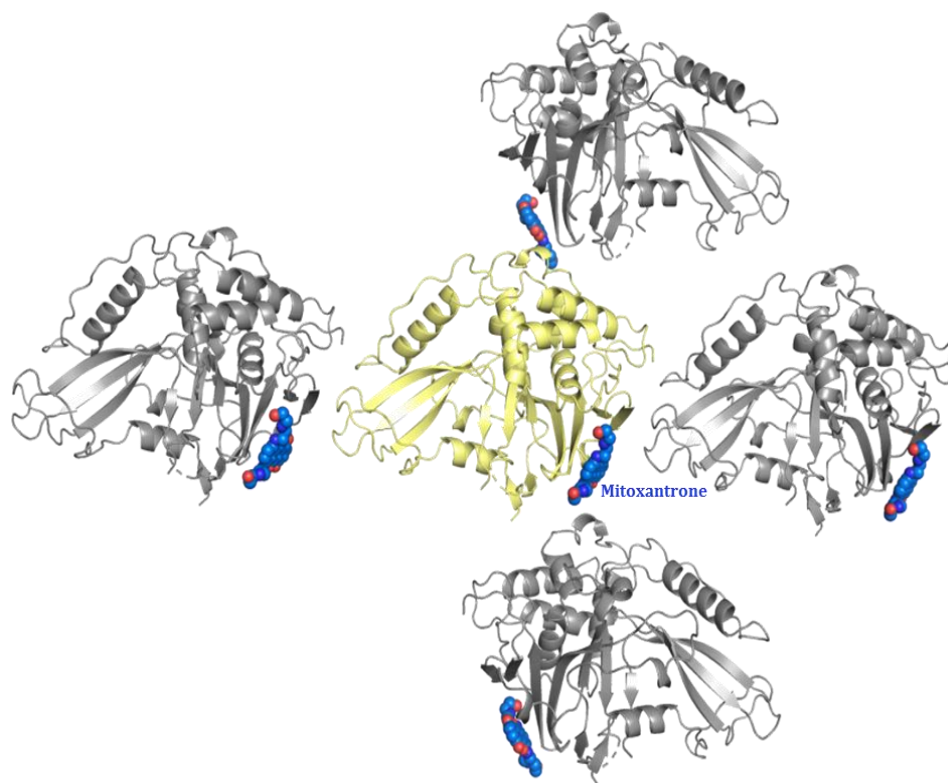


Figure 4-3: Mitoxantrone binding is not influenced by additional crystal contacts. Generation of symmetry mates Pymol, shown in (grey), confirms the interaction of mitoxantrone with the BL2 is independent of additional crystal contacts. Mitoxantrone (blue) is shown in space fill representation in each molecule. For the simplicity of this figure, the second chain of USP15-D1D2 is not shown.

Importantly, the BL2 region, which is the main mitoxantrone binding site, corresponds to the S1' binding region within the catalytic domain, the site which accommodates the proximal ubiquitin/substrate. Superposition of K6 linked di-ubiquitin co-crystallized with USP30¹³¹, shows clear overlap between the proximal ubiquitin and mitoxantrone in the S1' binding site (figure 4-4). Therefore it was hypothesized that mitoxantrone would compete with the proximal substrate in the S1' pocket, resulting in reduced binding and therefore cleavage of the substrate.

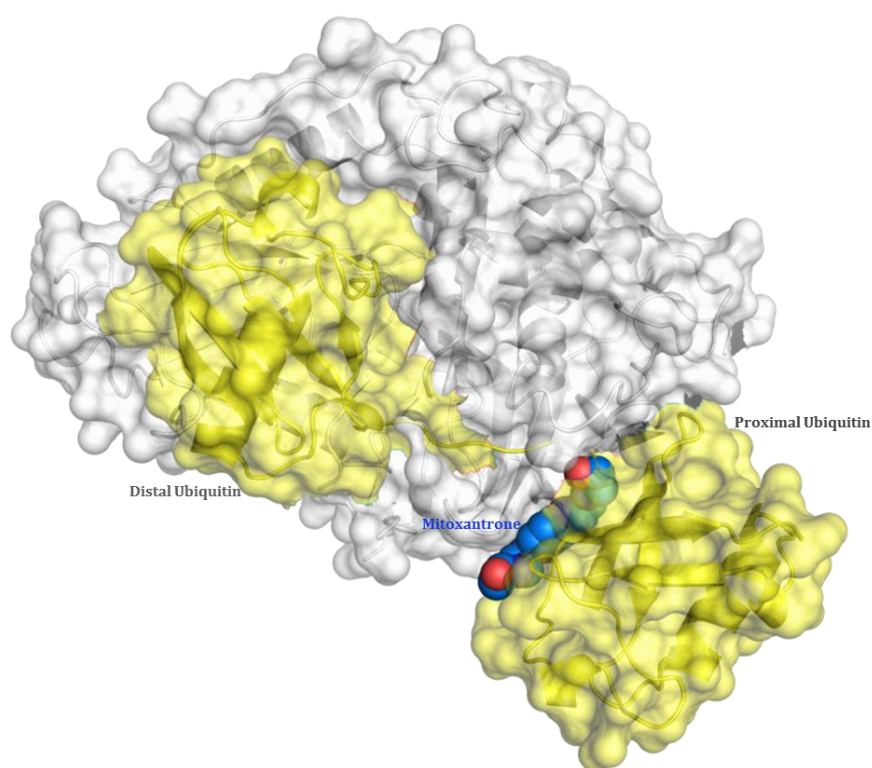


Figure 4-4: Mitoxantrone competes with the proximal substrate. Semi-transparent surface representation of and K6 linked di-ubiquitin, from the USP30-Ly6-di-ubiquitin structure (PDB ID: 5OHP), superimposed with USP15-D1D2 (white). Mitoxantrone is shown in space-fill representation (blue) and occupies S1' binding pocket, clashing with the proximal ubiquitin.

4.2. Confirmation of the mitoxantrone binding site

Having identified mitoxantrone binding to the S1' pocket of USP15-D1D2 we sought to test the theory that USP15 would compete with the proximal substrate. To test this hypothesis, we first established that mitoxantrone was binding specifically to USP15. We then tested USP15 activity in the presence of 100 μM mitoxantrone in a ubiquitin-AMC assay and in a gel shift cleavage assay using linear di-ubiquitin, a substrate of USP15 which binds to both the S1 and S1' pocket. The assays were performed using both USP15-D1D2 and USP15-FL.

4.2.1. Isothermal titration calorimetry analysis of USP15-D1D2 mitoxantrone interaction.

Isothermal titration calorimetry (ITC) was used to probe the USP15-D1D2 mitoxantrone interaction. ITC is a powerful tool for calculating thermodynamic parameters and dissociation constants associated with the binding of a protein and ligand. In addition the stoichiometry (N) of the interaction can be determined. Mitoxantrone forms dimers and oligomers at concentrations above 100 μM , which were found to dissociate upon titration into the cell. ITC machines are sensitive enough to be able to detect the disassociation event meaning a false positive result could be obtained. Therefore, 230 μM of USP15-D1D2 was titrated into 23 μM mitoxantrone, in which monomeric mitoxantrone will be the predominant species. The titrations were performed at 25 °C. A good sigmoidal curve was obtained and fitted to a one-site binding model (figure 4-5). A dissociation constant of 2.56 μM and a stoichiometry of 0.869 were calculated. Therefore, the mitoxantrone-USP15 interaction is a specific but weak interaction. Favourable enthalpy changes drive the interaction, indicated by analysis of the ΔH and $-T\Delta S$. The observed decrease in entropy could be reflective of the increased order around the BL2 observed in the USP15-mitoxantrone structure.

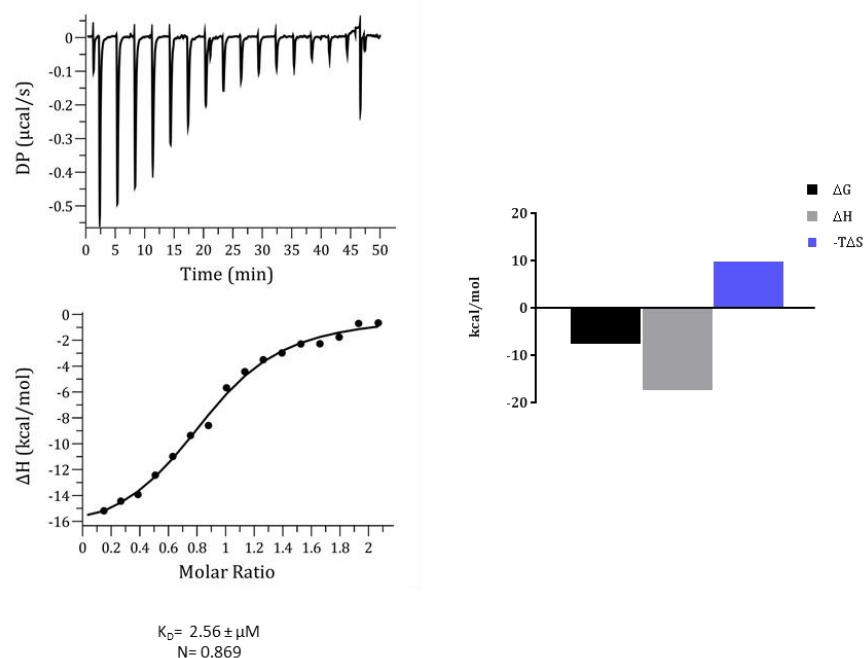


Figure 4-5: ITC titration of mitoxantrone into USP15-D1D2 WT. Top panels show the raw data and the bottom panel show the integrated thermograms, with the respective K_D and stoichiometry (N) given underneath. (B) Representative bar chart of ΔG (black) ΔH (Grey) and $-T\Delta S$ (Blue) for each titration. The data presented is representative of two repeat titration experiments.

4.2.2. Screening mitoxantrone inhibition of USP15 in the Ubiquitin-AMC assay.

Following a similar method to Burkhart et al²⁰³ the Ubiquitin-AMC assay was used to test the effect of 100 μM of mitoxantrone on USP15 activity. Each well contained 750 nM Ub-AMC, 35 nM USP15-FL and 100 μM mitoxantrone. Reactions containing 0 μM mitoxantrone were used as a positive control. Wells containing, buffer, mitoxantrone and free AMC were used to monitor background fluoresce of the assay. Measurements were taken every 4 seconds for 10 minutes to ensure the linear portion of the reaction was captured. Background wells showed no change in fluorescence over the course of the experiment.

Mitoxantrone is a quinone-based compound. Quinones are notorious for their interference in fluorescence-based assays because of their conjugated electron system and tendency to behave like fluorophores, and it is critical to account for this within the assay. Mitoxantrone excites at 610 nm with an emission at 680 nm and, as expected, no background fluorescence of the mitoxantrone was detected using the ubiquitin-AMC wavelengths. However, the background signal was reduced by over a third in control wells containing 100 μ M mitoxantrone and it was unknown how the compound would interfere with the fluorescence signal generated by different concentrations of free-AMC.

To account for the possible quenching effect of mitoxantrone on the AMC fluorophore, a free-AMC standard curve using concentrations ranging from 0-150 nM, was generated in absence and presence of 100 μ M mitoxantrone. The standard curves showed mitoxantrone obstructs the fluorescence of free-AMC with the difference becoming more apparent as the concentration of AMC increases (figure 4-6). The relationship is linear and could be fitted using linear regression to the equation $y = mx + c$. The fluorescence signal never exceeded 50,000 RFU in the assay which is within the linear range of the standard curve, meaning the standard curves was a suitable method for accounting for mitoxantrone interference. The equations generated from the standard curve were used to convert the fluorescence signal in the assay into AMC concentration in nM. The normalized data was plotted as a function of time and fit to a one-phase association curve. To determine whether the presence of mitoxantrone had any effect on USP15 activity in the ubiquitin-AMC assay, the initial rate of reaction was calculated for the linear portion of the reaction in the first 100 seconds (figure 4-6).

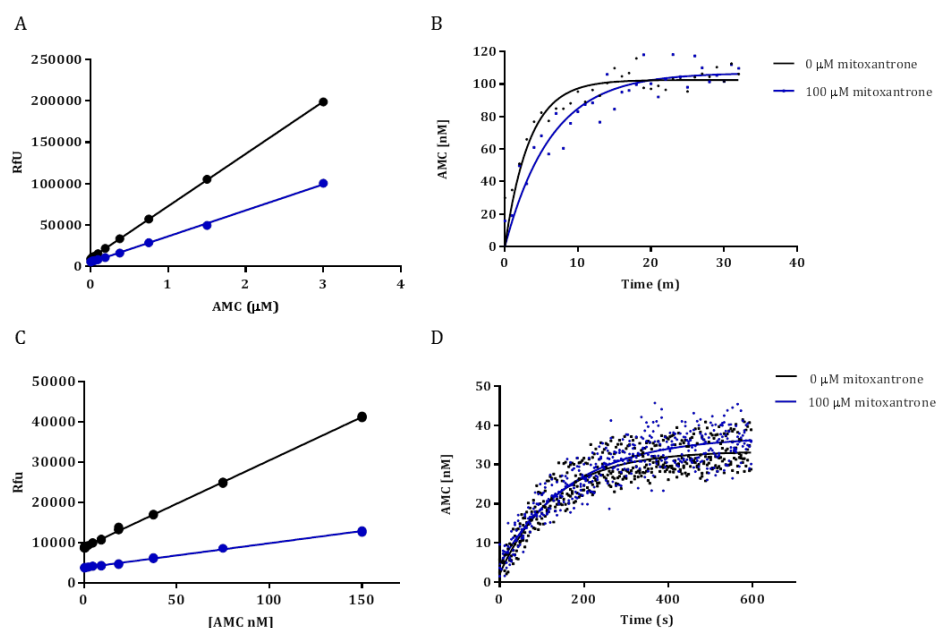


Figure 4-6: Screening for inhibition of USP15-FL and USP15-D1D2 activity in the presence of 100 μM mitoxantrone. Standard curves were generated using free AMC with 0 μM and 100 μM mitoxantrone to account for the quenching effect of mitoxantrone (A and C). Standard curve measurements were made in triplicate, error (SEM) bars are very small and hidden by the marker. USP15 D1D2 (B), USP15-FL (D) and Ub-AMC concentrations were 35 nM and 750 nM respectively and compared with a control sample containing 0 μM mitoxantrone. Experiments were conducted in triplicate.

For both FL USP15 and USP15-D1D2, the curves looked identical irrespective of mitoxantrone, and statistical analysis of the initial rate of reaction revealed there was no significant difference ($P=0.3146$) between them and therefore mitoxantrone does not inhibit USP15's activity against ubiquitin-AMC.

4.2.3. Screening mitoxantrone inhibition of USP15 in a gel shift cleavage assay.

Mitoxantrone was also tested in a gel based cleavage assay, utilizing linear di-ubiquitin as a substrate. Linear di-ubiquitin is a substrate of USP15 and is cleaved into two mono-ubiquitin molecules. Di-ubiquitin occupies both the S1 and S1' pocket during the reaction and will compete with mitoxantrone for the S1' pocket. Experiments were set up in triplicate, and samples taken every 6 minutes for 60 minutes, and then at 90 and 120 minutes. The reaction was analyzed using SDS-PAGE gel electrophoresis to observe the cleavage of linear di-ubiquitin (17.1 kDa) to mono ubiquitin (8.5 kDa) over time. Each lane of the gels were analyzed via densitometry using ImageJ²¹⁷ as described in section 2.6.2, and the percentage of di-ubiquitin and mono-ubiquitin in each lane determined.

Subsequent graphs of percentage di-ubiquitin loss over time with 0 μ M and 100 μ M mitoxantrone were plotted using a one phase decay model in graph pad prism (figure 4-7). As no cleavage had occurred at X=0, Y0 was constrained to 100 %. The rate constant K, was used to compare the two curves and determine if the difference is significant.

Both USP15-D1D2 and USP15-FL showed reduced activity with the addition of 100 μ M to the reaction. USP15-FL was able to cleave 88 % of the di-ubiquitin in comparison to 33 % in the presence of mitoxantrone. Comparison of the rate constant K, confirmed there was a significant difference in USP15 activity in the presence of mitoxantrone ($p=0.0008$). Therefore, mitoxantrone reduces USP15-FL cleavage of linear di-ubiquitin. Similarly, USP15-D1D2 had cleaved 95 % of the di-ubiquitin within 24 minutes in contrast to reactions containing mitoxantrone, whereby 95% cleavage was not attained until 60 minutes.

The absence of inhibition in the Ub-AMC assay in comparison to the significant change in activity observed in the gel shift cleavage assay strongly suggests mitoxantrone is competing with the proximal ubiquitin, and the binding site observed in the crystal structure is a true reflection of where mitoxantrone binds in solution.

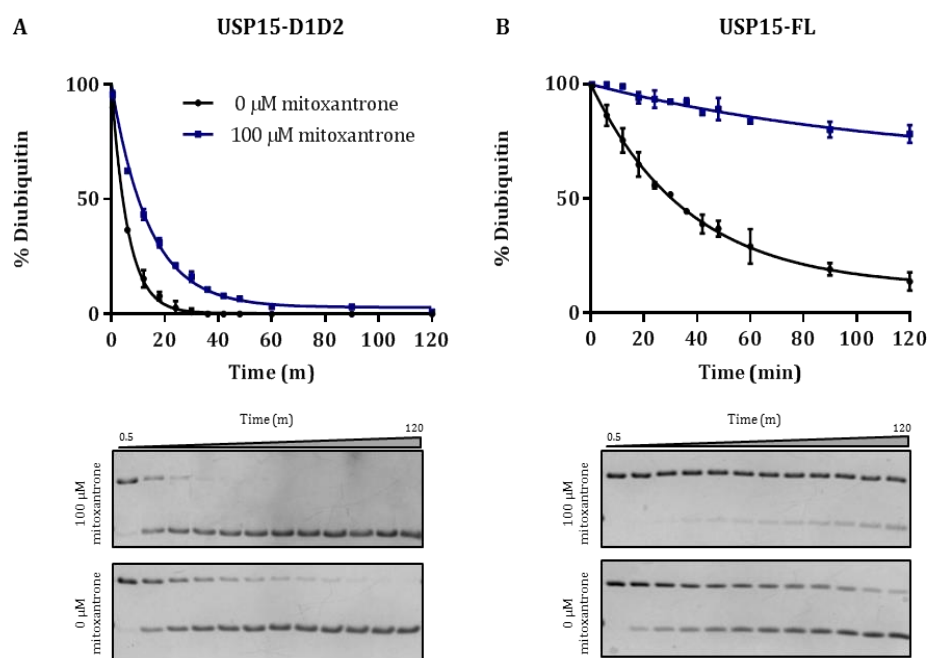


Figure 4-7: Time course experiment of USP15-D1D2, USP15-FL, cleavage of di-ubiquitin (17 kDa) to mono-ubiquitin (8.5 kDa) with 0 μM and 100 μM mitoxantrone. Curves were fit using a one phase decay model. Each experiment was conducted in triplicate and error bars (SEM) are shown.

4.2.4. IC₅₀ determination using di-ubiquitin gel shift cleavage assay.

To deduce the extent of the inhibition of USP15-FL activity in the linear di-ubiquitin gel shift assay, an IC₅₀ value was calculated in the linear range of the reaction (figure 4-8). A time course experiment, conducted in triplicate, was used to identify the linear region and select a suitable end-point for the IC₅₀ experiments. USP15-FL at a final concentration of 400 nM was incubated with 5 μM linear di-ubiquitin at 25°C for 45 minutes. Samples were taken at 5 minutes

and every 3 minutes thereafter for 45 minutes. SDS-PAGE gels were used to detect cleavage of di-ubiquitin to mono-ubiquitin over time. The amount of mono-ubiquitin (%) in each sample was determined via densitometrical analysis of the gels. Percentage mono-ubiquitin was plotted over time and the data fit using linear regression. Using the R^2 value as an indicator of the quality of the fit of the trend line, the data was gradually truncated until an acceptable fit was found. Consequently 30 minutes was determined to be the most appropriate end point for the assay, providing a good signal to noise whilst remaining within the linear range (figure 4-8).

Reactions were set up using a mitoxantrone concentration of 0.5-800 μ M. USP15 was incubated with mitoxantrone at 25°C for half an hour prior to the addition of di-ubiquitin. The reaction was initiated by the addition of di-ubiquitin and stopped after 30 minutes. Samples were analyzed via SDS PAGE gel electrophoresis, as described previously and the percentage mono-ubiquitin at 30 minutes was determined for each mitoxantrone concentration. These values were plotted against log₁₀ mitoxantrone concentrations and a curve fitted using the model:

$$Y = \frac{Bottom + (Top - Bottom)}{1 + \left(\frac{x^{Hillslope}}{IC_{50}^{Hillslope}}\right)}$$

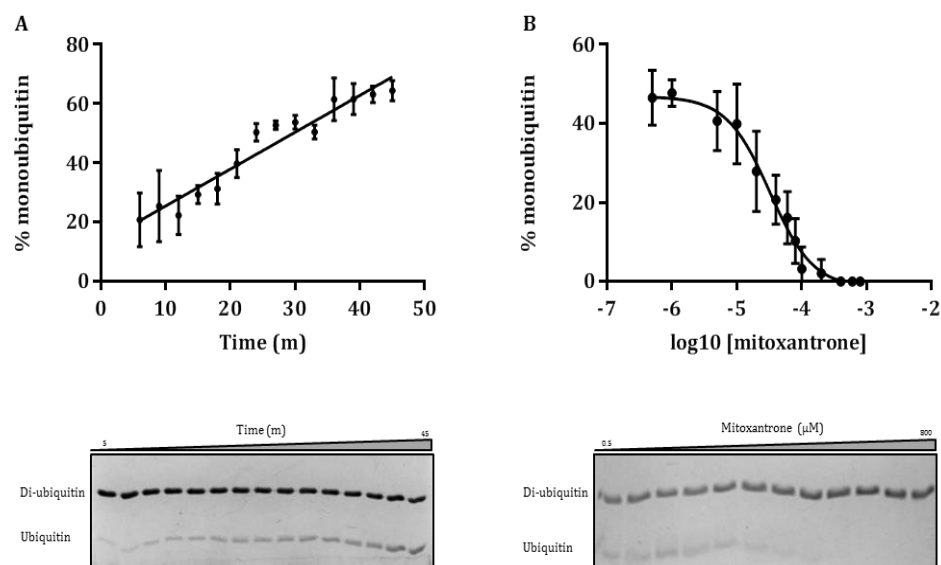


Figure 4-8: IC₅₀ determination of USP15-FL inhibition in the di-ubiquitin gel-shift cleavage assay. (A) Assays were performed to determine the linear region of the reaction. The reaction was stopped every 3 minutes for 45 minutes and a line of best fit determined in graph pad prism using linear regression. (B) The cleavage of di-ubiquitin was measured after 30 minutes in the presence of mitoxantrone concentrations ranging from 0 μ M - 800 μ M. Mitoxantrone is shown as log[concentration] and the data fit using nonlinear regression (*log(inhibitor) vs. response variable slope (four parameters)*) to give an IC₅₀ of 33 ± 11 μ M. Assays were conducted in triplicate with representative gels shown for both. Error bars represent SEM

The data showed that mitoxantrone weakly inhibits USP15-FL cleavage of di-ubiquitin with an IC₅₀ of 33 ± 11 μ M. The full inhibition of USP15-FL (indicated by the plateau at 0 % mono-ubiquitin suggests that mitoxantrone is not a partial inhibitor and is capable of completely blocking USP15 activity. However due to limitations in detection of low concentrations of protein, it should be noted that this assay is not sensitive to very subtle changes in activity, and therefore the IC₅₀ should be taken as an indicative value rather than an absolute value.

4.2.5. Investigation into the selectivity of mitoxantrone

We decided to investigate whether paralogues USP4 and USP11 were inhibited to the same extent in the gel shift cleavage assay. The paralogues harbour some variations in the BL2 region (figure 4-9) and may cause mitoxantrone to behave differently in the assay. USP11 activity was reduced in the Ub-AMC assay in the presence of mitoxantrone²⁰³ although a different method was used to account for mitoxantrone quenching the AMC fluorophore. It was however still anticipated that USP11 would also reduce activity in the gel shift cleavage assay. Mitoxantrone has never been tested against USP4.

Assays testing USP11-D1D2 and USP4-D1D2 activity against linear di-ubiquitin in the presence of 100 μ M mitoxantrone were set up as described for USP15. The USP11-D1D2 assay was performed by Sigrun Maurer. Linear di-ubiquitin is a poor substrate for USP11 and the assay was required to run over 22 hours. Unfortunately, due to the sigmoidal nature of the USP11 curve, it be would inappropriate to directly compare activity with USP15. It was however very clear that mitoxantrone was able to significantly reduce activity of USP11 and USP4 displaying promiscuous inhibition activity amongst the DU family of USPs.

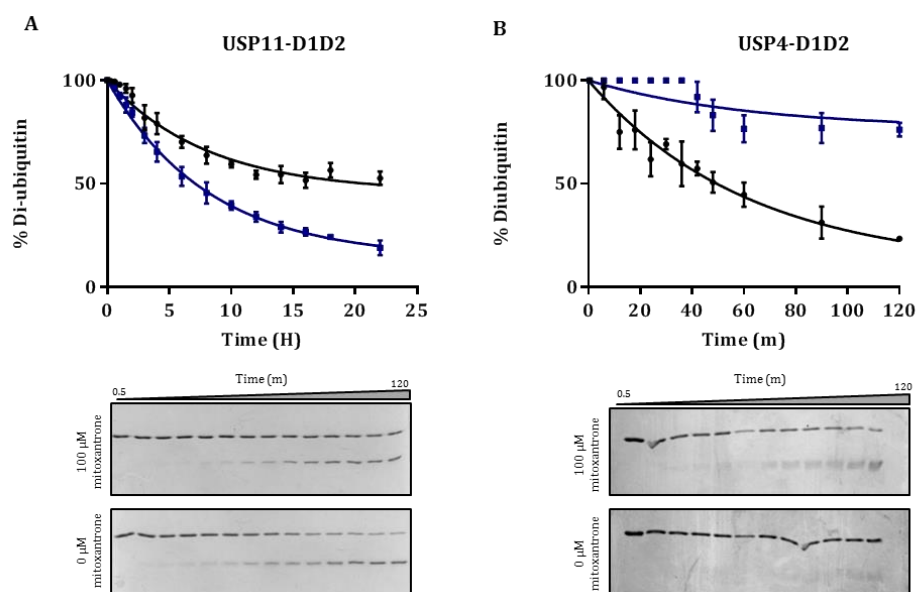


Figure 4-9 Time course experiment of USP11-D1D2, USP4-D1D2, cleavage of di-ubiquitin (17 kDa) to mono-ubiquitin (8.5 kDa) with 0 μ M and 100 μ M mitoxantrone. Curves were fit using a one phase decay model. Experiments were performed in triplicate and the error bars represent SEM.

4.2.6. Investigation into the importance of Tyr855 in mitoxantrone binding.

Tyrosine 855 was one of the residues identified to be interacting with mitoxantrone. It is conserved between the USP15, USP11 and USP4 and could provide a rationale for the lack of selectivity observed in the gel shift cleavage assay (figure 4-10)



Figure 4-10: Sequence alignment of BL2 region. Residues contributing to the BL2 are indicated and residues thought to be making interactions with mitoxantrone are marked with an asterisk (*). The catalytic histidine is highlighted in red and conserved residues are highlighted in black. Sequences were aligned using T-coffee ¹².

To determine the importance of this residue in mitoxantrone binding, Tyr855 was substituted for a lysine in USP15-FL. Lysine is also found in this position in USP40. The mutant behaved similarly to USP15 WT during over expression and purification and was active in the Ub-AMC assay. The mutant was tested in the gel shift cleavage assay as described previously. Analysis of the results revealed mitoxantrone was just as potent against USP15-FL Tyr855Lys as USP15-FL WT. However, it should be considered that the lysine side chain may also be able to contribute to mitoxantrone binding through hydrophobic interactions.

USP2 is more distantly related to USP15 and harbours a serine in the equivalent position of Tyr855. We decided to test inhibition of USP2 with mitoxantrone in the gel shift cleavage assay to further probe the specificity of mitoxantrone interaction with the BL2. Similar to USP11, linear di-ubiquitin is a poor substrate of USP2 and

the assay conducted over 10 hours with samples taken every 1-2 hours. Mitoxantrone also reduced USP2 activity in the gel-shift cleavage assay. This confirms Y855 alone is not critical for interactions of the BL2 with mitoxantrone and a rather flat surface in this region, probably maintained by these substitutions, may be sufficient to mediate mitoxantrone binding.

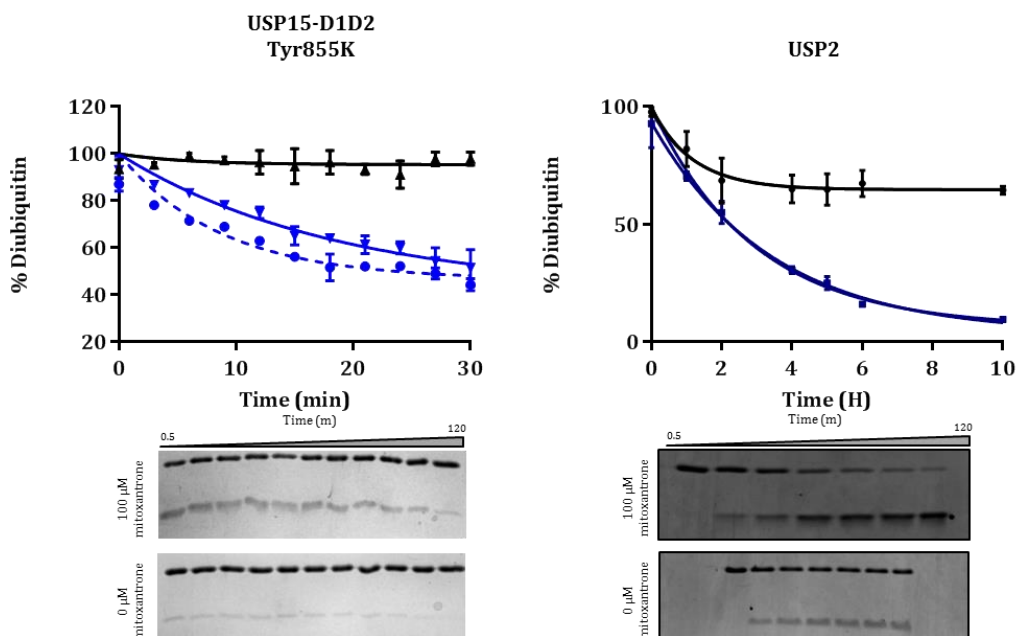


Figure 4-11: Time course experiment of USP15-FL Tyr855Lys and USP2-D1D2, cleavage of di-ubiquitin (17 kDa) to mono-ubiquitin (8.5 kDa) with 0 μ M and 100 μ M mitoxantrone. Curves were fit using a one phase decay model. Experiments were performed in triplicate and error bars represent SEM. USP15-FL WT was included in the USP15-FL Tyr855Lys assay and is represented by the dashed line. Due to the similarity of the curves, the sample containing 100 μ M mitoxantrone is hidden behind the USP15-FL Tyr855Lys curve.

4.2.7. Discussion of mitoxantrone binding

The work in this chapter has revealed that mitoxantrone is a weak inhibitor of USP15 ($IC_{50} = 33 \mu M$) and competes with the proximal substrate in the S1' binding site.

Mitoxantrone was found to bind to the S1' pocket in the BL2 region in the crystal structure. The interaction was found to be unaided by crystal contacts and appears

to be driven by hydrophobic interactions. Differences in inhibition of USP15 activity between the Ub-AMC and the di-ubiquitin gel shift cleavage assay reinforces the binding site observed in the structure is representative of its behaviour in solution. Furthermore the stoichiometry obtained from the ITC experiments is consistent with the observation of only one molecule of mitoxantrone bound to USP15 in the crystal structure and supports the theory that mitoxantrone competes with the proximal substrate by binding to the S1' pocket.

Comparison of mitoxantrone's inhibitory activity against other USPs from the DU subfamily, showed variation in the BL2 region has no significant impact on the ability of mitoxantrone to inhibit the enzymes. Further, testing of Tyr855Lys and USP2 in the linear di-ubiquitin gel shift cleavage assay confirms that Tyr855 is not critical for the interaction of USP15 with mitoxantrone. We were unable to pinpoint the crucial residues for mitoxantrone binding through these limited mutagenesis and comparison with other USP studies. A relatively flat hydrophobic surface in this region and the presence of the histidine may be sufficient for mitoxantrone binding. However, it cannot be ruled out that other mechanisms may contribute.

Mitoxantrone is a quinone based compound and has been commonly excluded from high-throughput screens after being identified as artefact and a pan-assay interference compound (PAIN). Quinone groups are commonly found in anticancer agents and are notoriously promiscuous amongst protein families, although an exact mechanism of action (MoA) has yet to be established. Some suggested mechanisms for their occurrence as artefacts in screens, include but are not limited to; chemical reactivity, high molecular flexibility, redox activity, aggregation and hydrophobicity. Hydrophobicity can be excluded as possible mechanism because mitoxantrone is highly soluble in H₂O (83 ml/ml according to

Sigma-Aldrich). The compound is known to form aggregates through pi-pi stacking of the anthracene ring, typically at concentration higher than 100-200 μ M, which could lead to protein destabilisation. However, USP15 activity was unimpeded in the Ub-AMC assay, despite the presence of 100 μ M mitoxantrone which strongly suggests that the decrease in enzyme activity was not a result of non-specific interference through mechanisms such as protein aggregation or chemical reactivity of the compound.

Increased flexibility within a protein has been linked to increased promiscuity in ligand binding. With this in mind, the non-specificity observed between DU USP members and the more distant relative USP2 could perhaps be accounted for by the flexibility of the BL2, which was identified in the crystal structure as the binding site of mitoxantrone. More rigorous testing of mitoxantrone against other members of the USP family would be required to confirm this conjecture. Equally the shallow nature and flexibility of the S1' pocket, means mutagenesis studies within this area are challenging.

Collectively the data above shed light on a possible new MoA and binding site for small molecule inhibitors of the USP family; whereby mitoxantrone competes with the proximal part of a substrate. It would be interesting to further explore this interaction further using different proximal substrates of USP15.

CHAPTER 5: **Characterisation of USP15-D1D2**

ubiquitin binding behaviour

This chapter will describe experiments conducted with the aim of further understanding specificity within the USP family. Comparisons are made between the structures of USP15 and USP4 to investigate specificity arising from differences within the catalytic core and complemented with ITC investigations into ubiquitin binding. Ubiquitin variants are used to elucidate the importance of the isopeptide bond in deubiquitinating enzymes. The work in this chapter complements work started by Dr Peni Indrayudha, who primarily focused on USP4²⁰⁹. Until now, a direct comparison of the two paralogues interaction with the different forms of ubiquitin has not completed. In this chapter, I will discuss a detailed structural comparison of USP15-D1D2 and USP4-D1D2 and use this to inform other experiments. I will compare data generated from my own investigations of USP15 binding behaviours with ubiquitin variants to the USP4 data collected by Dr Peni Indrayudha, Sigrun Maurer and Camille Michavila.

5.1.1. USP15 binds ubiquitin with a different K_D to USP4.

Initial titrations were performed to determine the effect of mutating the catalytic Cys269 in USP15-D1D2 on ubiquitin binding, as this construct was paramount to future binding experiments. Cell concentrations between 20 μ M and 30 μ M were found to be suitable for giving an acceptable signal relative to noise, with a ubiquitin concentration 10 x that of the cell. Ubiquitin was titrated into USP15-D1D2 WT and USP15-D1D2 Cys269Ser at 25°C. Both titrations yielded good sigmoidal curves with both proteins exhibiting the same overall binding pattern and K_D in the high nanomolar range (Figure 5-1).

Ubiquitin was titrated into USP4-D1D2 Cys311Ser under similar conditions, with each result confirmed by at least one repeat titration. Titrating ubiquitin into USP4-D1D2 Cys311Ser resulted in a very steep curve. High C-value curves such as the USP4 curve reduce the accuracy in which the K_D can be determined. Nevertheless, there was a clear difference in binding behaviour between USP15 and USP4.

Intriguingly at 25°C, USP15-D1D2 binding to ubiquitin gives an endothermic thermogram in contrast to the exothermic profile of USP4-D1D2. Of particular note is the significant change in the K_D with USP4-D1D2 binding ubiquitin over one hundred fold tighter. The stoichiometry (N) was lower than expected and varied between 0.5-0.9 between the different titration experiments. One explanation for the unexpected stoichiometry is that a fraction of the protein in the cell is misfolded and therefore unavailable for binding. A new batch of protein was prepared for each experiment and therefore the proportion of misfolded protein may vary each time, which would account for the variation in stoichiometry between each run.

Analysis of parameters ΔH , ΔG and $-\Delta S$ provide further insight into the binding events. USP4 binding to ubiquitin is predominantly enthalpy driven with minor entropic contributions, indicated by the negative ΔH and $-\Delta S$. In contrast, USP15 binding to ubiquitin is predominantly driven by favourable entropy changes, which counteract the unfavourable changes in enthalpy.

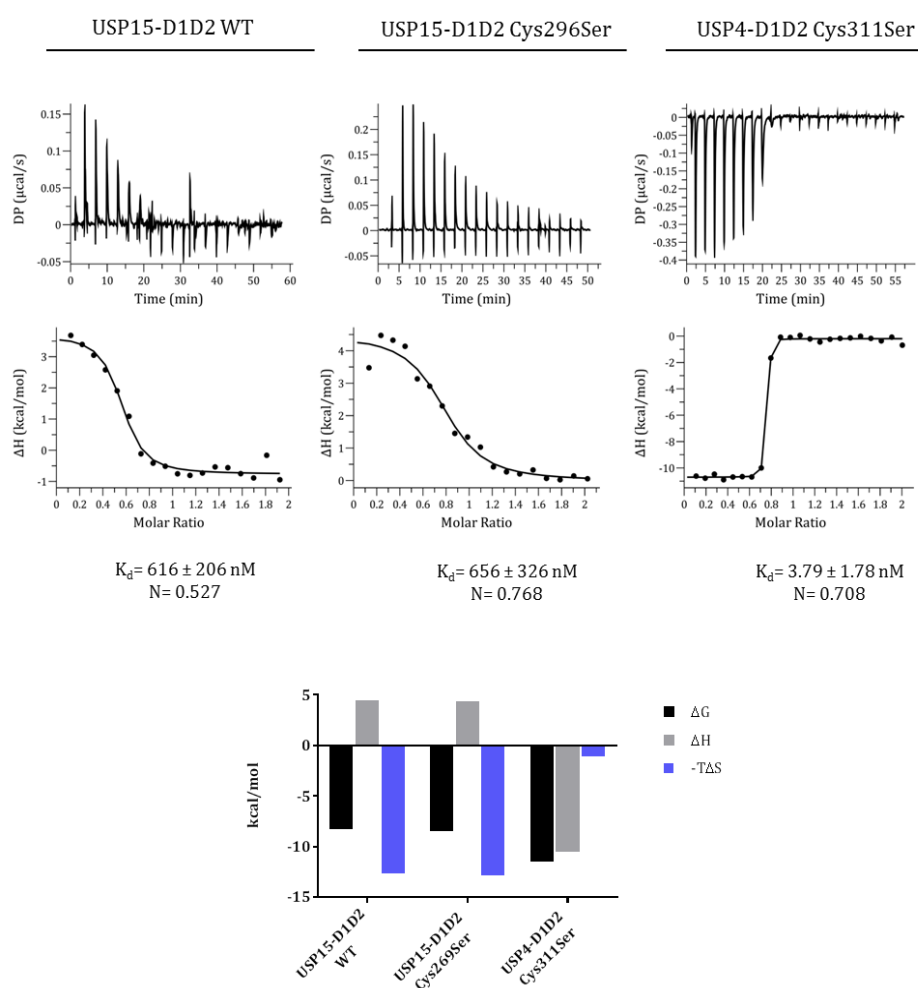


Figure 5-1: ITC titration of ubiquitin into USP15-D1D2 WT, USP15-D1D2 Cys296Ser and USP4-D1D2 Cys311Ser at 25°C. (A) Top panels show the raw data and the bottom panel show the integrated thermograms, with the respective K_D and stoichiometry (N) given underneath. (B) Representative bar chart of ΔG (black) ΔH (Grey) and $-\Delta S$ (Blue) for each titration.

5.2. Structural comparison with USP4

USP15 and USP4 share 56.9 % sequence identity over the full length of the protein with 77.4 % sequence similarity residing in the catalytic domain¹⁵⁰. Therefore, comparison of the two structures may highlight subtle structural differences, which could account for the observed differences in ubiquitin binding behaviour, and may provide more clues as to how substrate specificity is achieved amongst the USP family. Structural alignment of USP15-D1D2 and USP4-D1D2 (2Y6E) (RMSD of 0.916 Å over 315 equivalent Cα positions), highlights regions of differences between the two homologues. It should be noted that in the USP4-D1D2 model, the catalytic cysteine (Cys311) is covalently modified by a β-mercaptoethanol molecule and may mimic the C-terminal tail of ubiquitin (figure 5-2).

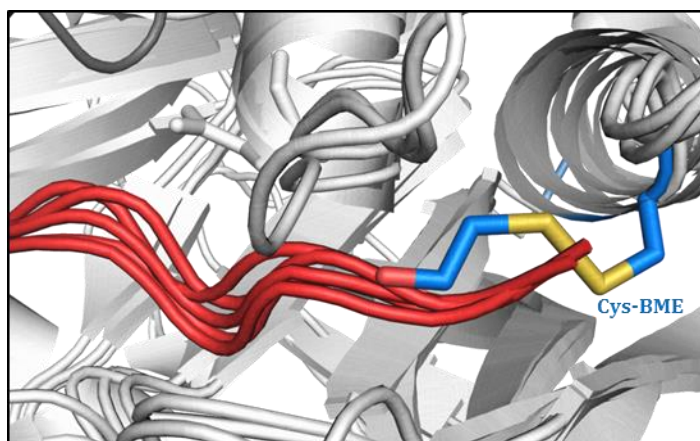


Figure 5-2: The catalytic cysteine (Cys311) is USP4 is modified with β-mercaptoethanol. Ubiquitin (red) from several Ub bound USP structures were superimposed onto USP4 showing how the β-mercaptoethanol may act as a mimic of the ubiquitin C-terminal tail.

5.2.1. The USP4 active site region adopts a different conformation to USP15.

As mentioned previously, the catalytic triad in USP15 is in a catalytically incompetent conformation. Intriguingly, despite both structures being solved in the absence of ubiquitin, the two proteins adopt different catalytic conformations in the unbound form. In contrast to the misalignment of the USP15 triad, the USP4 catalytic triad is in a catalytically competent conformation. The most notable structural difference arises within the active site region and coincides with the different conformations of the catalytic triad. As described in section 3.2.2, the USP4 SL is ordered and superposition with USP15 reveals clashes between residues in the USP15 CC loop with USP4 SL residues, indicating the SL must adopt an alternative conformation to accommodate the misaligned catalytic cysteine.

Notable conformational differences arise at the N-terminal end of the SL, whereby Phe382 and Phe340 at the end helix $\alpha 5$ adopt different conformations in USP4 and USP15 respectively. In USP4 Phe382 packs against the side chain of Met347 whereas in USP15 Met305 adopts a different conformation and Phe340 appears to pack against the backbone of helix $\alpha 6$. USP15 Pro342, the last residue modelled in the N-terminal region of the SL adopts a similar conformation to USP4 Pro384 (figure 5-3, B). Further conformational differences arise in helix $\alpha 6$ with Phe358 in USP15 adopting an outwards position in contrast to USP4 Phe400 which face inwards (figure 5-3, D). Furthermore, in line with differences in the catalytic cysteine positioning the neighbouring phenylalanine adopts very different conformations. The differences highlighted above are collectively likely to contribute to the stability of the USP4 SL and aligned catalytic triad whereby Phe386 packs against Phe400, Leu397 and Phe312 (figure 5-3, D). The residues in this region are highly conserved and the only residue difference arising from a Cys-

Ser substitution in USP4. The density for Cys352 was ambiguous and the exact conformation of this residue was difficult to discern (discussed further in Chapter 6) but it highly likely to adopt a different conformation in USP15 due to the positioning of the catalytic triad.

The SL, and in particular Phe386, is thought to be responsible for ubiquitin retention in USP4 with Phe386 postulated to serve as a kind of relay between the DUSP-Ubl and the catalytic domain in USP4 promoting ubiquitin discharge¹⁴. The exact mechanism of USP4 activation by the DUSP-Ubl domain is not yet known. It would be interesting to see if 1) the DUSP-Ubl residues involved in the activation are conserved in USP15 and USP11 and 2) if the misaligned catalytic triad in USP15 plays a role in ubiquitin dissociation.

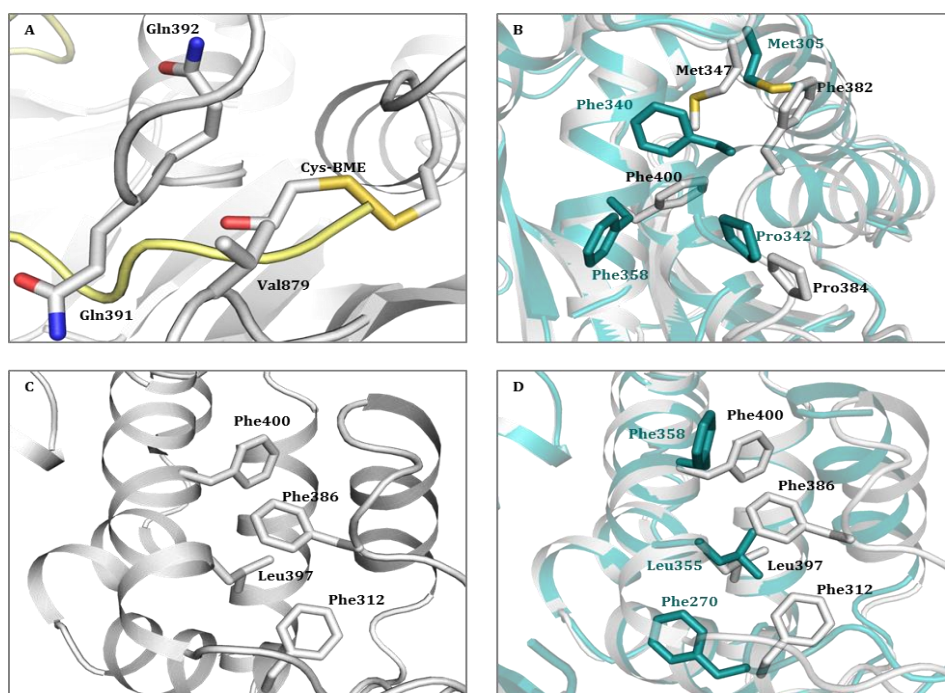


Figure 5-3: USP15 adopts an open binding channel. A) Stick representation of residues contributing to the more closed ubiquitin binding cleft. USP4 residues are shown in white, with the Ub C-terminus shown in yellow. (B) Stick representation of different conformations of residues found in the N-terminal region of the SL. USP15 residues are shown in teal and USP4 in white. (C) Stick representation of USP4 residues contributing the packing in the SL region. (D) Same colour scheme as in B, stick representation highlighting differences in residue conformations in the regions between USP4 and USP15.

It is possible that the presence of the β -mercaptoethanol adduct on the catalytic cysteine in USP4 mimics the ubiquitin C-terminal tail and influences some of the conformational differences observed. The binding channel in USP4 is more closed relative to USP15 with Gln391 reaching across the binding channel, which may be stabilized by interactions of the NH and carbonyl groups in the peptide backbone of Gln391, Gln392 and Val879 with the β -mercaptoethanol adduct (figure 5-3, A). Conversely, Gln349 in USP15 is flexible and consequently the ubiquitin binding channel is more open in comparison to USP4.

Sequence alignment of the catalytic cores highlighted residue differences between the two paralogues (appendix 2). The differences were mapped onto the USP15

surface structure (figure 5-4), which revealed high sequence conservation in the S1 ubiquitin binding pocket. Notable differences predominantly mapped to the S1' proximal ubiquitin binding region and back of the catalytic domain. In particular, a cluster of changes occurs between $\alpha 5$ and $\alpha 6$ helices and within close proximity to the linker region connecting the N-terminal Ubl domain to the catalytic domain. Here, USP15 Phe325, Ser326, Tyr327, Lys324 and Glu255 are replaced with Asp367, Ala368, His369, Arg366 and Ile297 in USP4. Additional changes in the S1' pocket are Thr885. Residues, which were dissimilar, were then mapped onto USP15 and USP4 with respect to charge and showed contributions the residues made to localized charge differences between USP4 and USP15 (Figure 5-5)

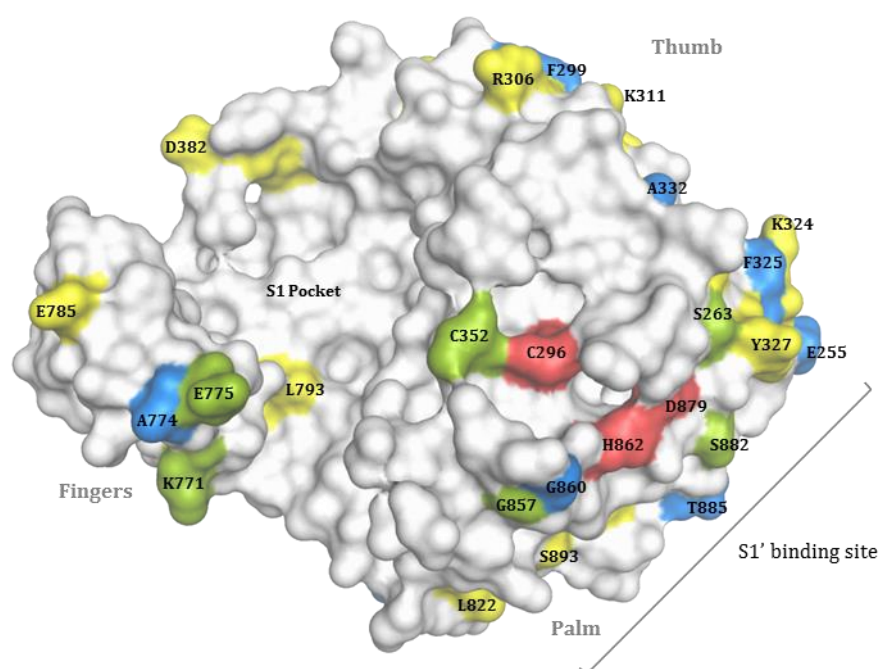


Figure 5-4: Surface representation of USP15-D1D2 highlighting residue differences with USP4. Conserved residues are shown in white, residues with similar properties are shown in yellow, weakly similar residues are shown in green and dissimilar residues are shown in blue. The catalytic triad is shown in red.

The mapping of residues highlighted differences in the BL2 region (GGMGG) with Gly857 and Gly860 substituted for Ala876 and Val879 in USP4 (GAMGV) respectively. In addition, the BL2 adopts different conformations and Val879 is located on the rim of the ubiquitin binding channel (figure 5-3 A). Furthermore, the BL2 of USP15 may be more flexible owing to the additional glycine residues and may contribute to the observation of a more open ubiquitin binding channel. However, the only structure in which the BL2 was able to be modelled was USP15-mitoxantrone, whereby mitoxantrone was identified to be bound to the BL2 modelled may influence the positioning of the BL2.

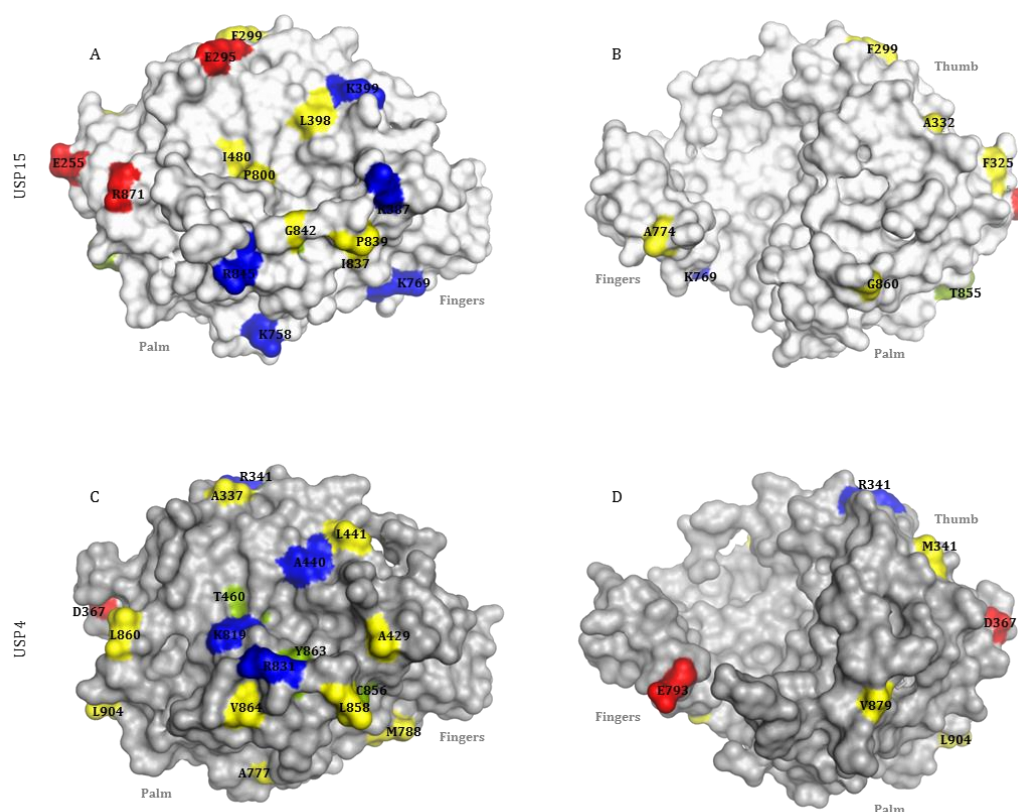


Figure 5-5: Surface representation of USP15-D1D2 and USP4-D1D2 showing associated charge of dissimilar residues. USP15-D1D2 back (A) and front (B) are shown on top and USP4-D1D2 back (C) and front (D) are shown on the bottom. Hydrophobic residues are depicted in yellow, basic residues in blue, acidic residues in red and OH or sulphydryl containing residues in green.

5.2.2. ITC analysis of contributions of BL2 to ubiquitin binding.

Structural analysis of the catalytic domains of USP15 and USP4 highlighted residue differences in BL2, which may contribute to the observed differences in ubiquitin binding. We hypothesized that substituting USP15 BL2 residues (GGMGG) with the equivalent USP4 residues (GAMGV) would cause the USP15 interaction with ubiquitin to resemble that of USP4.

Further explorations of the contributions the BL2 to ubiquitin binding were conducted using a USP15-D1D2 Gly860Val mutant (GGMGV) and USP15-D1D2bl2usp4 (GAMGV).

USP15-D1D2 Gly860Val interactions with ubiquitin were tested using ITC. Concentrations of USP15-D1D2 and mono-ubiquitin were 30 μ M and 300 μ M respectively and the experiment was set up using similar parameters to previous ITC experiments (section 5.1.1).

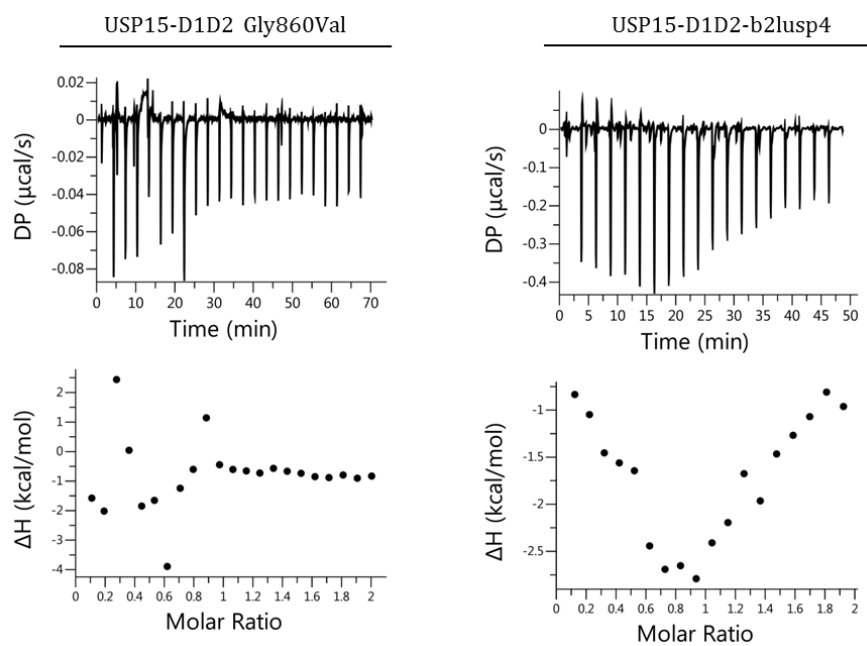


Figure 5-6: Raw ITC data and binding isotherms for USP15-D1D2 Gly860Val and USP15-D1D2-b2lusp4 at 25°C. Binding curves were unable to be generated at this temperature.

Interestingly, little heat change was observed when the ITC experiment was conducted at 25°C (figure 5-6), despite the constructs being viable in the Ub-AMC assay and behaving the same as USP15-D1D2 WT during expression and purification. This pointed towards the experiment becoming more exothermic, with a shift in the ΔH towards zero making the binding difficult to detect. To confirm this, ITC experiments were repeated at 37°C alongside USP15-D1D2 WT and USP4-D1D2 WT for comparison (figure 5-7).

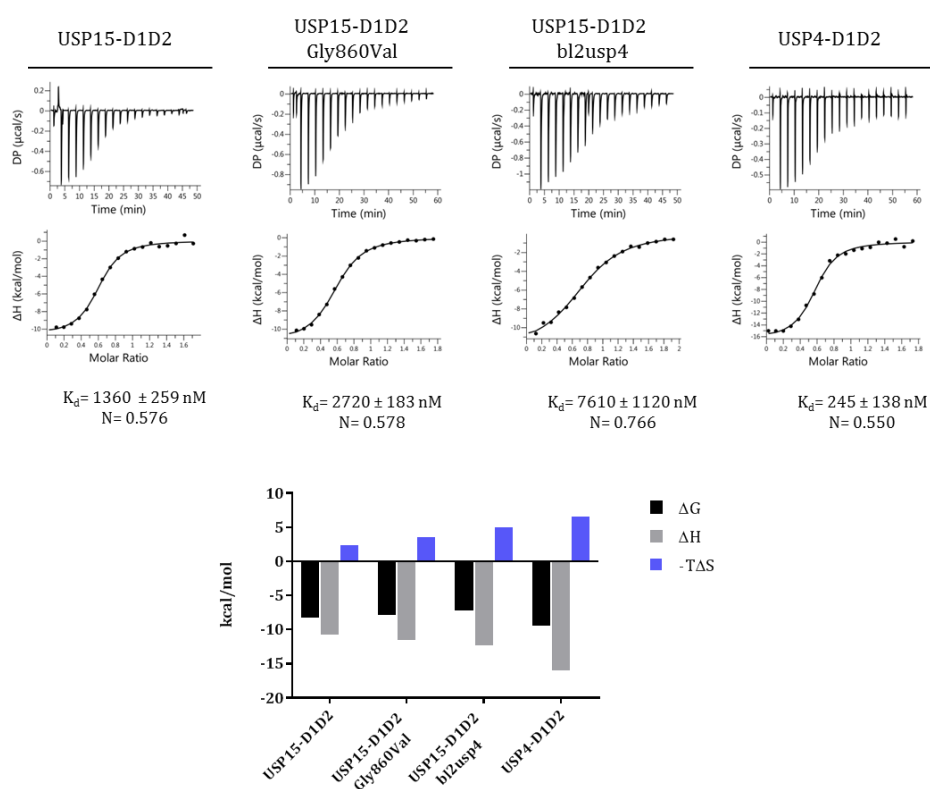


Figure 5-7: Raw ITC data and binding isotherms, of USP15-D1D2, USP15-D1D2 Gly860Val, USP15-D1D2 bl2usp4 and USP4-D1D2 with ubiquitin at 37°C. The data was fit to a one-site binding model. The K_D and stoichiometry (N) are given underneath the curve and the associated ΔG , ΔH and $-T\Delta S$ values are graphically depicted beneath.

Exothermic sigmoidal binding curves were observed for all constructs at 37°C and data were fit to a one site-binding model. At the higher temperature, the binding mode for both USP15 and USP4 was enthalpy driven with $-T\Delta S$ becoming positive for both. The bar chart depicting the binding parameters ΔG , ΔH and $-T\Delta S$ highlights the trend in the binding behaviour becoming more like USP4 with each residue change.. It also confirms the change in ΔH was certainly the cause of apparent lack of binding for USP15-D1D2 Gly860Val and USP15-D1D2usp4bl2 at 25°C. The substitution of valine and alanine in the Bl2 of USP4 contributes to the favourable enthalpy change upon ubiquitin binding, which may be counteracted unfavourable entropy changes. The change in binding parameters, however is not reflected in the K_D , with the dissociation constants for the mutant remaining in the low μM range.

Curiously, the difference in K_D is less prevalent at the higher temperature with USP4 binding ubiquitin with a K_D of 245 ± 138 nM compared to 1380 ± 259 nM for USP15. This difference may have arisen from USP4-D1D2 WT being used in replacement of USP4-D1D2 Cys311Ser. Mutations to the USP4 catalytic cysteine, particularly Cys-Ala mutations have been reported to give rise to very large changes in affinities for its substrates²²¹, thought to be due to absence of steric clashes between the C-terminal ubiquitin and the catalytic cysteine in the active site. The influence of the mutant on the affinity was later confirmed by repeating the ubiquitin titration into USP4D1D2 Cys311Ser at 37°C, which yielded a K_D of 1.69 ± 1.49 nM.

5.2.2.1. Investigation into differences in the SL using ITC.

Sequence alignment of the SL revealed one residue difference between USP4 and USP15. USP15 harbours a cysteine in place of serine in the equivalent position in USP4. As mentioned previously, the cysteine was unable to be modelled due to poor density; however, it is certainly in an alternative position to USP4 Ser394, which would clash with the USP15 catalytic cysteine. USP15 Cys352 was mutated to serine to assess the role of this residue with respect to ubiquitin binding. A sigmoidal curve was obtained for Cys352Ser and fitted to a one site binding model with a calculated K_D of 1340 ± 259 nM (figure 5-8). The thermodynamic properties of the interaction determined by ΔG , ΔH and $-T\Delta S$ remains unchanged, showing that the Cys352Ser mutation has no influence over the mechanism of ubiquitin binding within USP15-D1D2. Whilst this result suggests the Cys352 has no effect on Ub binding it should be considered that USP15-D1D2 WT and USP15-D1D2 Cys352Ser was exposed to DTT prior to these experiments and may therefore behave differently when oxidized. This will be discussed further in chapter 6. The results showed that Ser394 does not contribute to tighter binding in USP4 and its presence in USP15 has no influence over the mechanism of ubiquitin binding.

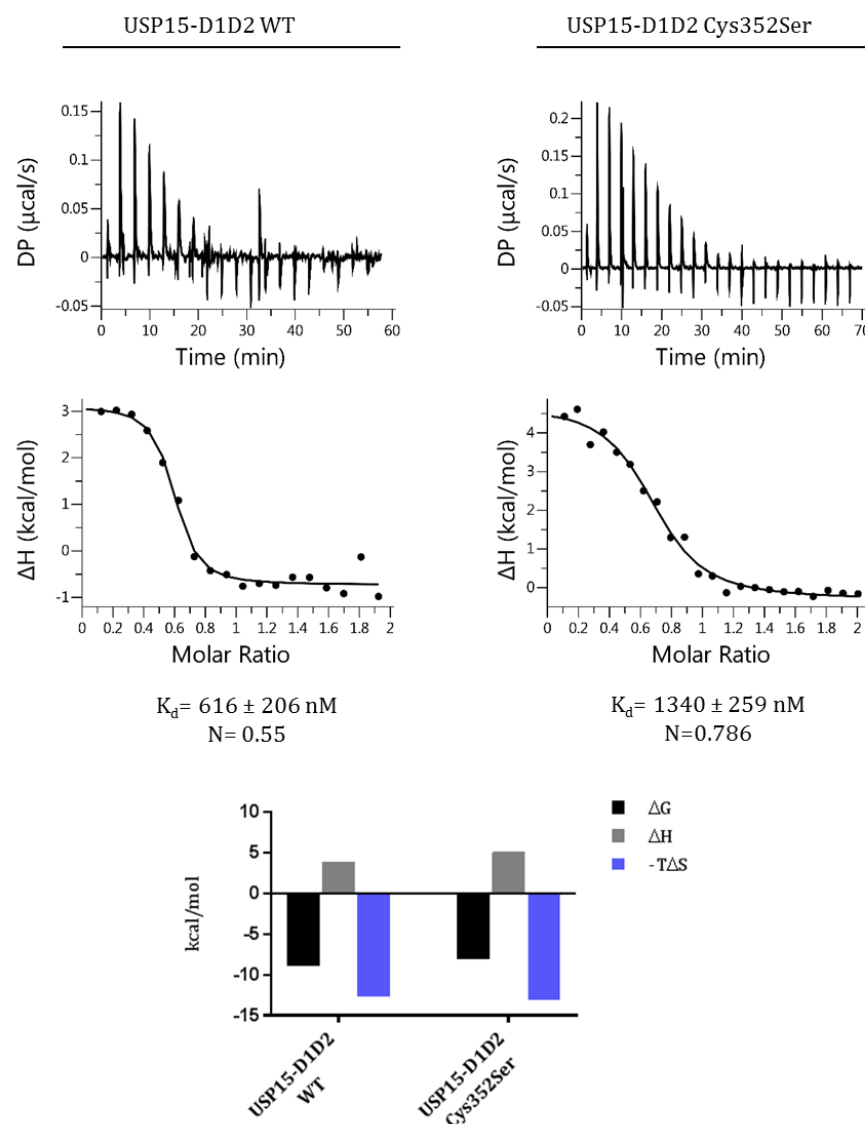


Figure 5-8: ITC titration of ubiquitin into USP15-D1D2 WT and Cys352Ser. From left to right: Titration of ubiquitin into USP15-D1D2 WT and into USP15-D1D2 Cys352Ser. Top panels show the raw data and the bottom panel shows the integrated thermograms, with the respective K_D and stoichiometry (N) given underneath. A representative bar chart of ΔG (black) ΔH (Grey) and $-T\Delta S$ (Blue) for each titration is shown.

5.2.3. Discussion of USP15 and USP4 ubiquitin binding behaviour

Collectively, the data presented above provides insight into a possible activation mechanism for USP15 and USP4. Key structural differences between the paralogues are around the active site region, with USP15 displaying a catalytically incompetent triad and open ubiquitin binding channel, in contrast to the USP4 open and pre-aligned conformation. A β -mercaptoethanol adduct on the catalytic

cysteine of USP4 may serve to mimic the C-terminal tail of ubiquitin and therefore the USP4 structure may represent the closed active conformation in contrast to USP15 which represents the open inactive conformation

Rearrangement of the USP15 catalytic triad and other residues in the active site region are necessary for the accommodation of ubiquitin and may coincide with the conformational differences observed between USP4 and USP15. Therefore it is possible that the following events will occur upon ubiquitin binding: (1) ordering of the flexible active site loops (BL1 and BL2). (2) closure of the ubiquitin binding channel, Glu349 would reach across the ubiquitin channel and form a salt bridge with ubiquitin Arg72 as seen in USP structures. (3) ordering of the SL and conformational change would lead to displacement of CC loop residue Phe270 by SL residue Cys352. Consequently, movement of Cys269 would be required to avoid steric clashes and would result in the alignment of the catalytic triad. Further information, such as bound USP15 structural data, and unbound USP4 data would help to confirm this mechanism.

Furthermore, ITC analysis of ubiquitin binding behaviour revealed USP4-D1D2 binds ubiquitin with a higher affinity and displayed a different thermodynamic profile to USP15. This is in line with previously reported data which demonstrated that ubiquitin retention in the active site was more prevalent in USP4 than USP15¹⁴. Differences in the BL2 were found to contribute to the differences in binding behaviour observed between the two paralogues. Entropy changes were found to be more favourable in USP15 than USP4 and may be a reflection of increased interactions and order in the USP4 BL2 with the ubiquitin C-terminus. Additional glycine residues in the USP15 BL2 likely confers to increased flexibility and an increase in entropy, and may play a role in ubiquitin discharge.

5.3. Characterisation of USP15 interactions with novel ubiquitin variant: Ubggg

USP15 is known to preferentially cleave isopeptide bond linked substrates over peptide bond linked substrates²⁰. Further, USPs have displayed higher affinity for substrates with isopeptide bond mimics, such as Ub-Lys-Tamra, which contains an isoamide bond, over substrates such as Ub-AMC⁹⁸. Ubiquitin probes with native C-terminal isopeptide bonds can be made using a combination of enzymatic reactions and synthetic chemistry techniques⁵¹, however these methods can be expensive and complex.

Ubiquitin expresses very well in *E.coli* expression systems and pure samples can be easily obtained via a two-step purification (see figure 5-10), thus it is a very efficient method for producing large yields of protein. We sought to develop a novel ubiquitin fusion with a minimal C-terminal mimic of the conjugated lysine backbone, present in a native isopeptide bond, which could be produced cheaply and easily by using recombinant bacterial protein expression. We termed our new ubiquitin variant Ubggg. Composed of three glycine residues fused to the C-terminal glycine of ubiquitin, it is designed to mimic the lysine backbone present in the isopeptide bond formed between ubiquitin and the target protein when the C-terminus of ubiquitin is conjugated to a lysine residue (figure 5-9). Ubggg would provide the starting material for the development of additional probes by attaching fluorophores, and as a substrate for co-crystallization experiments with USP15.

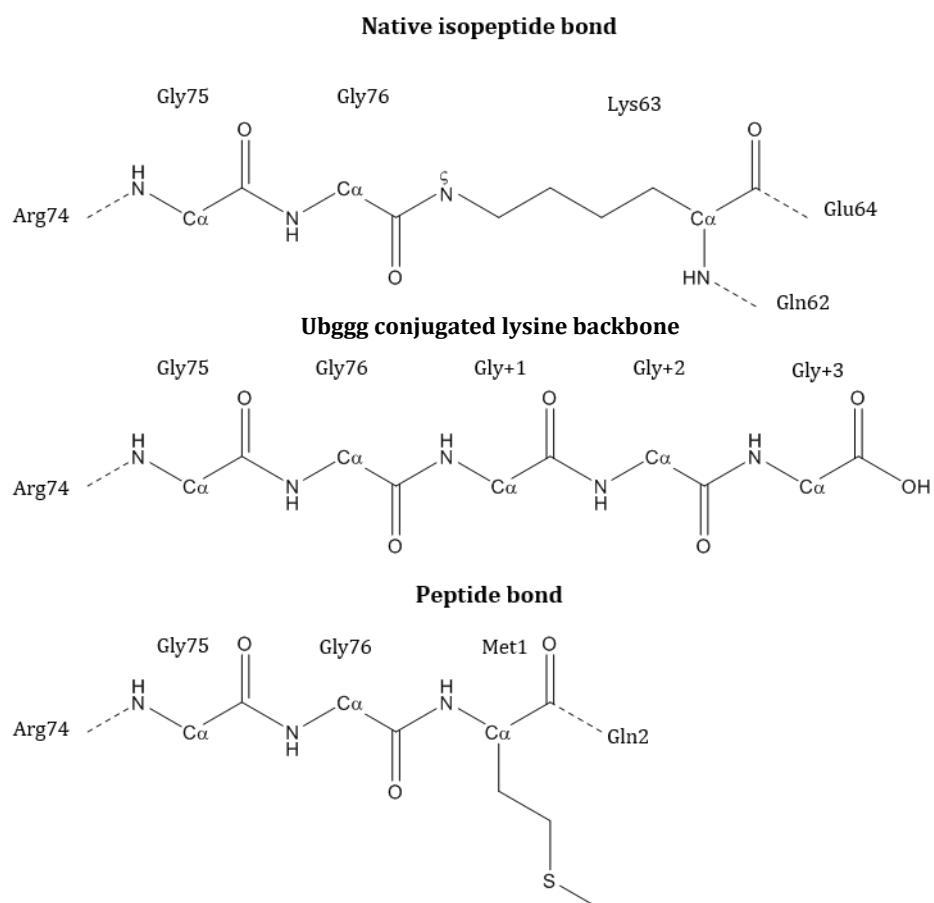


Figure 5-9: Schematic representation of a native isopeptide bond, Uboggg conjugated lysine backbone mimic and a peptide bond.

Ub_{ggg} was initially expressed in BL21 (DE3)-CP cells and later in ElaD deficient cells (discussed in section 5.4.1.1) and yielded high quantities of pure protein after a two-step purification. The ubiquitin variant behaved like ubiquitin during purification, and eluted of the gel filtration column as a single monodispersed peak at around ~85 ml. Large smears were visible under the bands on the SDS-PAGE gel, and these were attributed to clipping of the C-terminal glycine residues.

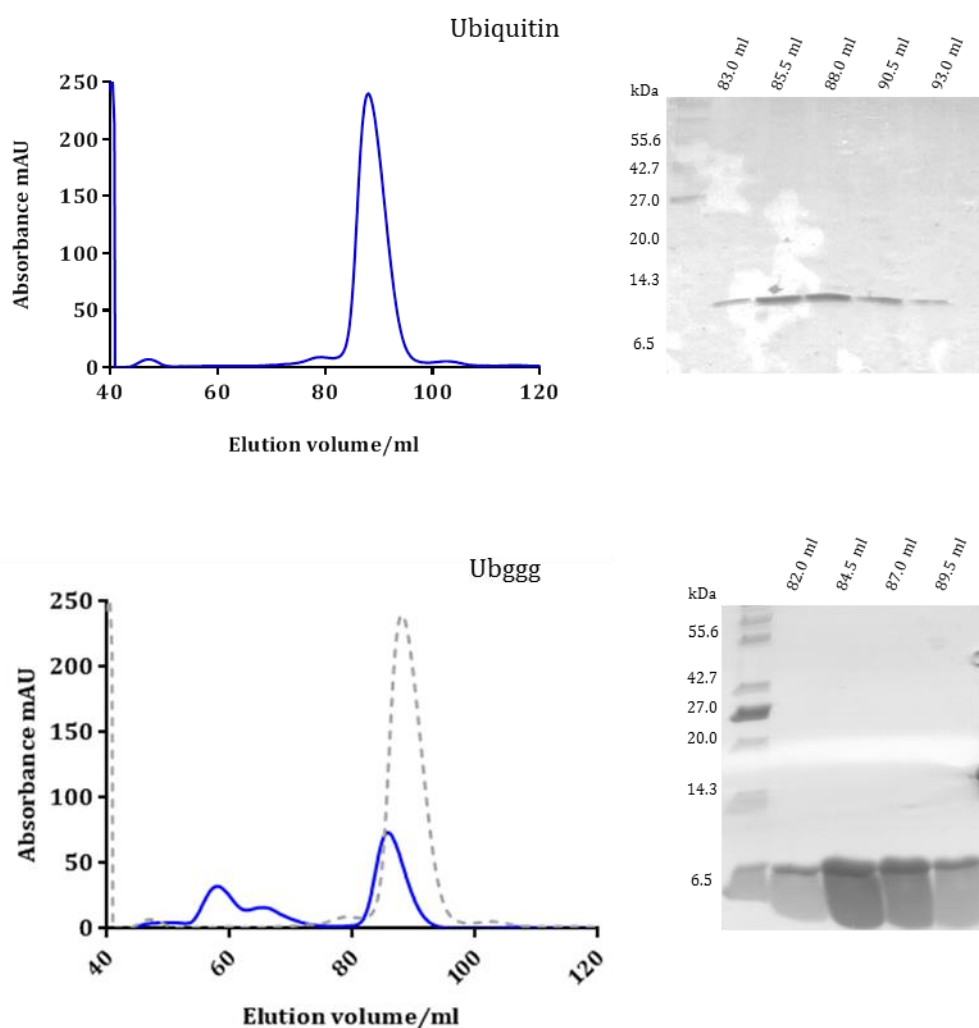


Figure 5-10: Purification of ubiquitin and Ubggg. Top left: Ubiquitin elutes from S75 SEC column at ~87 ml. The UV trace is shown in blue. The adjacent SDS-PAGE gel shows the purity of ubiquitin after gel filtration. Bottom: Ubggg elutes from the S75 SEC column at ~85ml. The UV trace of ubiquitin is represented by the dashed grey line, and UV trace of Ubggg is shown in the blue. The corresponding SDS-PAGE gel is shown on the right. The sample is pure, however a large smear present underneath the band.

5.3.1. ITC analysis of USP15-D1D2 interactions with Ubggg

USP15 is known to be a promiscuous enzyme which is able to cleave all ubiquitin chain types but has shown preference for lysine linked ubiquitin chains over linear ubiquitin chains. It is often assumed that this preference in selectivity arises because of the isopeptide bond, which is present when ubiquitin is linked to

another protein via a lysine; however there is little evidence to support this with binding data. Using ITC we sought to test this theory and hypothesised that USP15 would exhibit greater affinity for Ub_{ggg} over linear di-ubiquitin.

Ub_{ggg} was titrated into USP15-D1D2 Cys269Ser at 25°C. Several concentrations were tested in an effort to obtain a good sigmoidal curve. However, the interaction of USP15 with Ub_{ggg} was a high affinity interaction resulting in a 'high c-value' curve. Usually a reduction in cell concentrations would help resolve the curve. However, this was not possible due to the small ΔH , which meant at low concentrations poor signal to noise resulted in a loss of detection of binding.

Subsequently titrations were performed using 20 μM and 200 μM USP15-D1D2 Cys269Ser and Ub_{ggg} respectively. A sigmoidal curve was obtained and fit to a one site-binding model (figure 5-11). A K_D of $<1 \pm 11$ nM was calculated with a stoichiometry of 1.06. The affinity of USP15-D1D2 for Ub_{ggg} is 100 fold tighter than ubiquitin. Of course, this K_D value cannot be taken as an absolute value due to the nature of the curve. Nevertheless, it is very clear that a dramatic change in affinity is induced by the addition of the conjugated lysine backbone mimic on to the C-terminal tail of ubiquitin. Examination of the additional binding parameters ΔH and $-T\Delta S$ reveal the interaction is predominantly driven by favourable entropy changes with small enthalpy contributions. This reflects the interaction of USP15-D1D2 with ubiquitin, which is also entropy driven. The remarkable shift in affinity is attributed to the change ΔH (-5.65 kcal/mol) meaning the interaction has favourable enthalpy contributions in addition to entropy changes, in contrast to ubiquitin binding whereby the enthalpy contributions were unfavourable. This is indicated by the endothermic and exothermic curves.

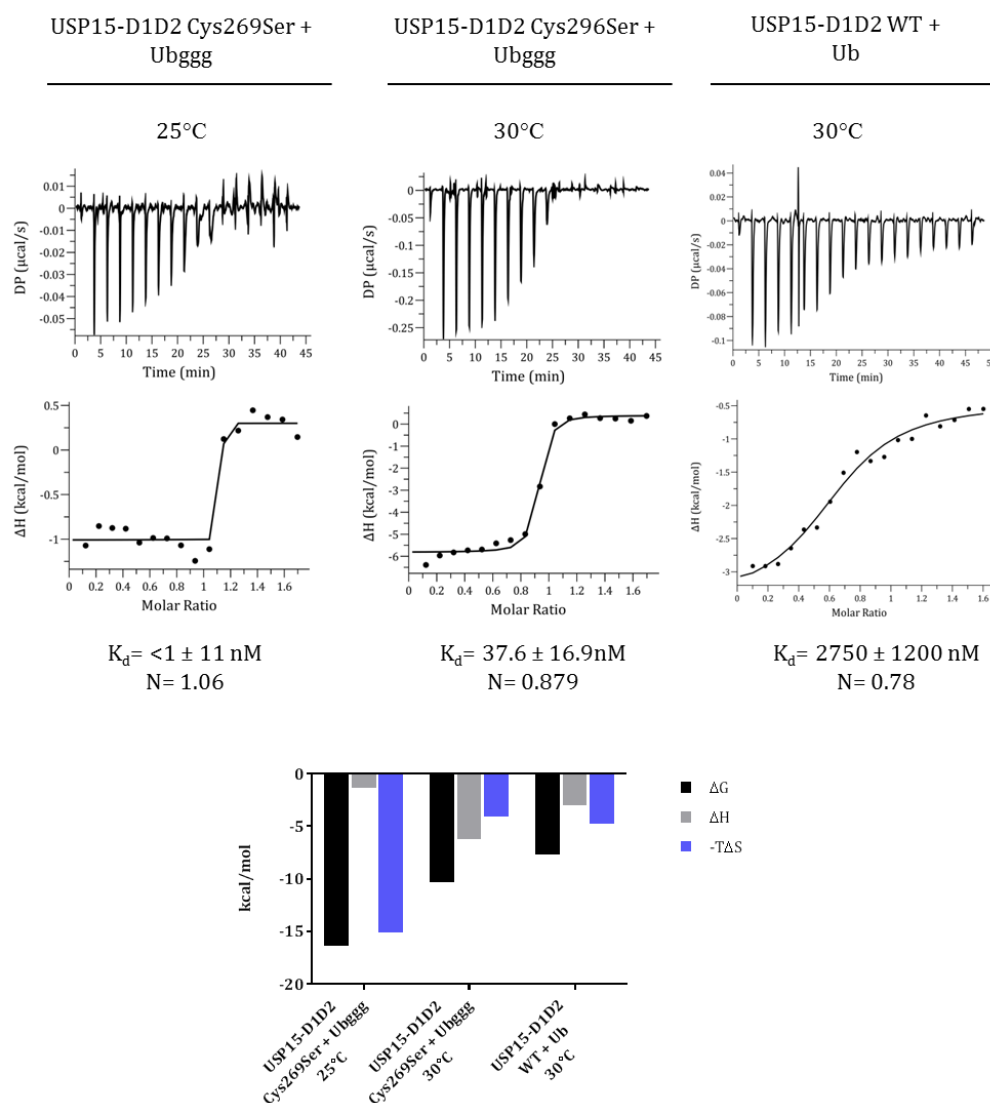


Figure 5-11: ITC titration of Ubggg into USP15-D1D2. From left to right: Titration on Ubggg in to USP-15-D1D2 Cys269Ser at 25°C, and at 30°C and titration of ubiquitin into USP15-D1D2 WT at 30°C. Top panels show the raw data and the bottom panel show the integrated thermograms, with the respective K_D and stoichiometry (N) given underneath. A representative bar chart of ΔG (black) ΔH (Grey) and $-T\Delta S$ (Blue) for each titration is shown.

ITC experiments were also performed at 30°C (figure 5-11). Ubggg at a concentration of 200 μM was titrated into 20 μM USP15-D1D2 Cys269Ser. As the ΔH for Ubggg binding to USP15 was small (-1.31 kcal/mol) at 25°C it was hoped that the temperature change would improve the signal to noise levels permitting calculation of a more accurate K_D value. Ubiquitin (200 μM) was also titrated in to

USP15-D1D2 WT (20 μ M) allowing direct comparison between Ub_{ggg} and ubiquitin binding at this temperature (figure 5-11).

The increase in temperature did improve the signal to noise however; the curve was still suboptimal for accurate K_D determination. This time the K_D was estimated to be 37 ± 16 nM. Ubiquitin titration into USP15-D1D2 at this temperature resulted in a low μ M K_D (2.7 ± 1.2 μ M) meaning there is still approximately a 100 fold difference in affinity. At this temperature, the interaction of USP15 with Ub_{ggg} is driven by both favourable entropy and enthalpy changes although interestingly the entropy changes are less prevalent than at 25°C with bigger changes in ΔH contributing to the tighter binding. Ubiquitin binding at 30°C is also both enthalpy and entropy driven, with entropy changes still contributing more to the interaction. This data fits with trends observed in section (5.2.2) whereby entropy changes become unfavourable at 37°C for USP15-D1D2 interacting with ubiquitin.

5.3.1.1. Comparison with linear di-ubiquitin binding

It was anticipated that USP15 would bind to linear di-ubiquitin with a lower affinity. Linear di-ubiquitin used in these experiments was generated recombinantly through over-expression in *E.coli* ElaD deficient cells, and purified via cation exchange and size exclusion chromatography. Linear di-ubiquitin elutes from a S75 SEC at around 78 ml as a single monodispersed peak (figure 5-12). A

small amount of higher molecular weight contaminants are present in early fractions (~70-75ml), these fractions were excluded from further experiments.

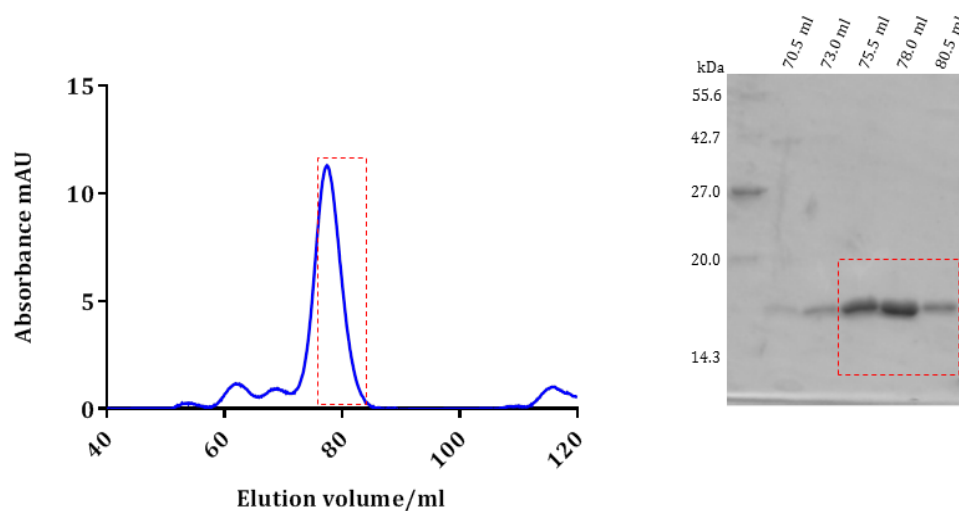


Figure 5-12: Purification of linear di-ubiquitin. Linear di-ubiquitin elutes at ~77 ml from the SEC column. The UV trace is shown blue. The corresponding SDS-PAGE is shown on the right. Higher molecular weight contaminants are visible in the earlier fractions, indicated by the red dashed box, and these fractions were omitted to ensure the high purity of the sample.

Experiments were set up as for mono-ubiquitin. At 25°C a good sigmoidal curve was obtained, using concentrations in the range of 30-60 μM and 300-600 μM in the cell and syringe respectively.

Interestingly, the binding of linear di-ubiquitin to USP15-D1D2 displayed similar binding behaviour to that of ubiquitin with same endothermic profile (figure 5-13). A K_D of 1.063 μM was calculated and the interaction driven by favourable entropy changes. The $-T\Delta S$ is larger than ubiquitin binding which counteracted by the large positive ΔH meaning the overall binding affinity was not notably different to ubiquitin binding.

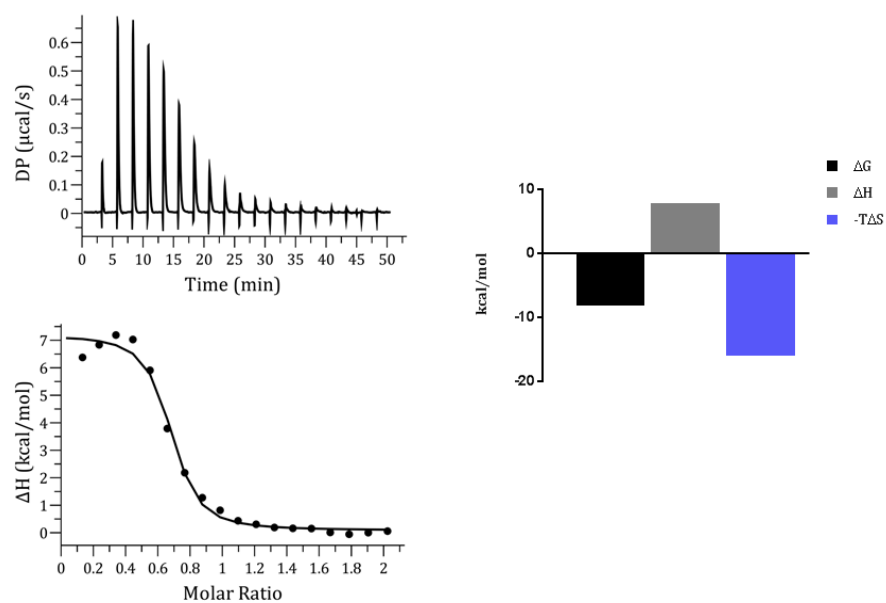


Figure 5-13: ITC titration of linear di-ubiquitin into USP15-D1D2 Cys269Ser. Top panel shows the raw data and the bottom panel show the integrated thermograms, with the respective K_D and stoichiometry (N) given underneath. A representative bar chart of ΔG (black) ΔH (Grey) and $-T\Delta S$ (Blue) for each titration is shown.

5.4. Crystallisation of trials of the USP15-D1D2_Ubggg complex.

5.4.1. Expression and purification of USP15-D1D2 in complex Ubggg.

By mutating the catalytic cysteine (Cys269) to serine, it was anticipated that the enzyme would no longer be able to cleave the C-terminal tail of Ub and the Ubggg will remain bound within the active site of the USP15. This would result in the formation of a complex, which could be used for further investigation of the Ubggg using crystallography and mass spectrometry

The USP15-D1D2_Ubggg complex was co-expressed. High salt buffers can disrupt electrostatic interactions, therefore a buffer containing 150 mM NaCl (as opposed to 300 mM) was chosen for the purification. Only USP15 contains a His-Tag, thus nickel affinity chromatography would separate out most bacterial contaminants and any unbound ubiquitin, leaving free USP15-D1D2 Cys269Ser and the USP15-D1D2_Ubggg complex. SDS PAGE gels show the complex elutes at around 25-35 % buffer B, with free USP15 eluting later at around 40 % B. Fractions containing the complex were concentrated in the presence of 1 mM DTT in preparation for SEC. The complex eluted from the S200 SEC column as a single peak at 87 ml and was clearly visible on the SDS-PAGE gels as the predominant species in the peak (figure 5-14). It is not clear whether the first and final fractions contain the complex or just free USP15-D1D2 as the Ubggg band may be too faint to be visible on the gel. Overlaying the trace with USP15-D1D2 WT shows no change in the elution profile. This however is not unexpected as gel filtration is dependent on the hydrodynamic radius of the molecule as opposed to its molecular weight, and it was unlikely that the binding of Ubggg would significantly alter the diameter of USP15-D1D2.

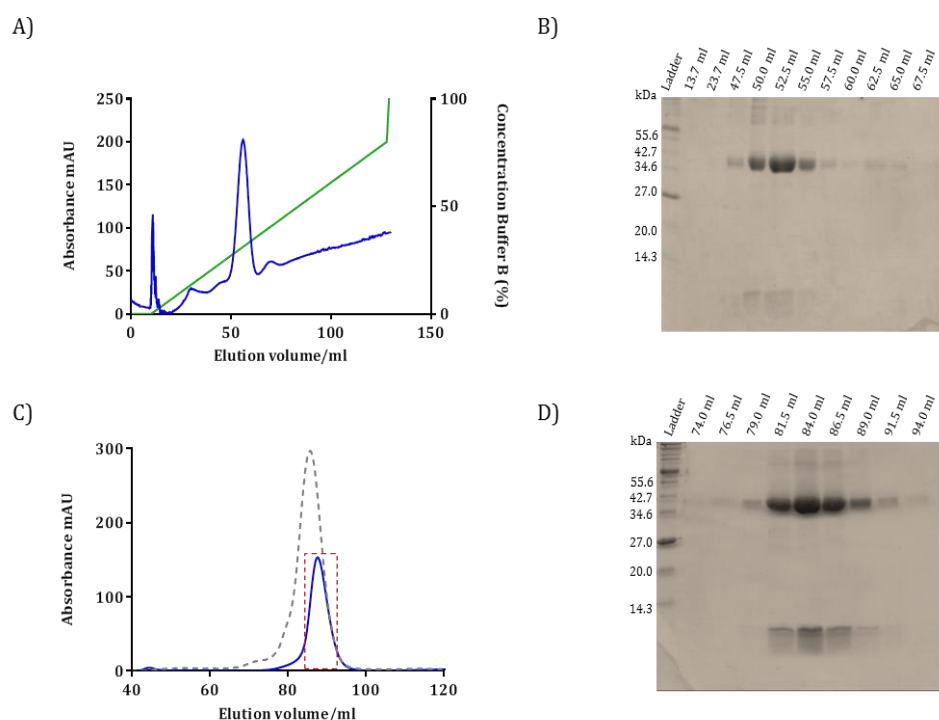


Figure 5-14: Figure: Purification of USP15 D1D2 Cys269Ser in complex with Ubggg. (A) Chromatogram of Ni-His trap purification step. The UV trace is shown in blue and the buffer B (%) gradient shown in green. (B) SDS-PAGE gel shows the purity corresponding NiHis-Trap fractions. (C) UV trace (blue) obtained from gel filtration step. The complex eluted as a single peak at 87 ml. The UV trace of free USP15-D1D2 (elution volume 85 ml) is shown in grey. The dashed red box indicates the fractions which taken. (D) SDS PAGE gel of fractions 73-94 ml from the gel filtration.

5.4.1.1. Mass spectrometry confirmation of USP15-D1D2_Ubggg complex formation

Native mass spectrometry was used to verify the USP15-D1D2_Ubggg complex formation and determine how much free USP15-D1D2 was present in the sample (figure 5-15). (Gemma Cooke, Neil Oldham group, University of Nottingham conducted the native mass spec experiments)

Protein	Predicted mass Da	Mass spectrometry mass Da	Error $\pm$
Ubggg	8736.0	8634.11	74.46
Ub	8678.1		
Ub	8621.90		
USP15-D1D2 Cys269Ser	41624.2	-	-
USP15-D1D2 Cys269Ser_Ub	50360.2	51657.28	44.36
		51807.29	77.90

Table 13: Predicted and calculated masses of USP15-D1D2 Cys269Ser_Ub complex and components. Expected masses were calculated using protparam prediction tool.

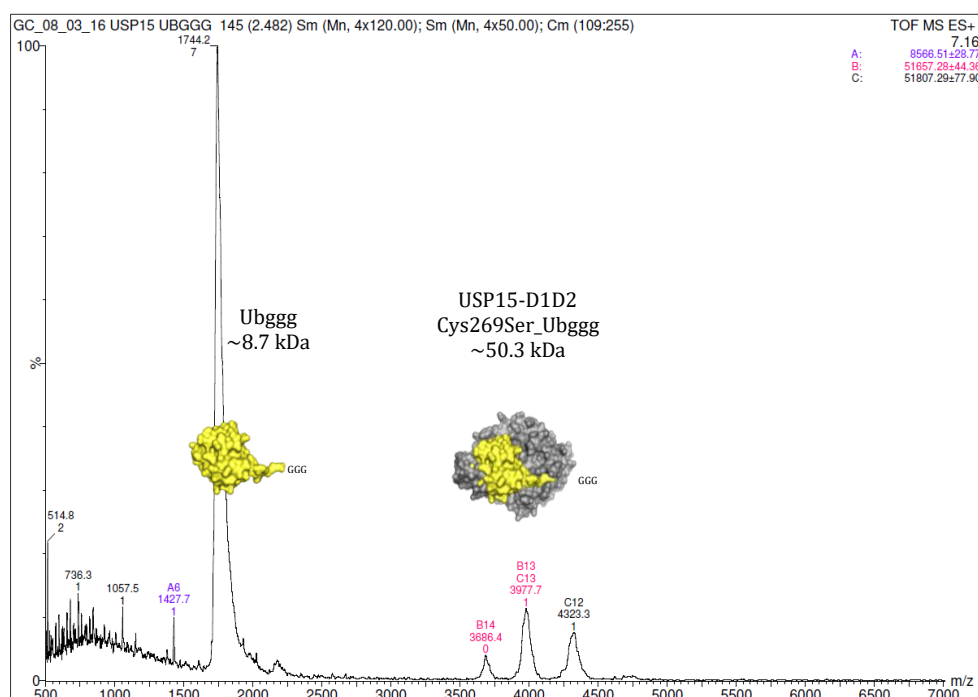


Figure 5-15: Native mass spectrometry results for USP15-D1D2 Cys269Ser_Ub. Calculated masses are shown in pink purple and black and are represented with a corresponding schematic. Ubggg is shown in yellow and USP15 is shown in grey. No free USP15 was detected by the mass spectrometry.

The mass spectrometry results indicated that the sample contained a mix of free Ubggg and complex. The masses dictated did not exactly match the predicted masses, likely a consequence of the C-terminal clipping of Ubggg. The mass observed corresponding to free Ubggg was 8634.11 ± 74.76 Da, and was closest to the predicted mass of Ub (8621.90 Da). Cleavage of the Ubggg C-terminus may

have contributed to the disassociation of the complex resulting in the large errors. Despite this, no free USP15 was detected and the results were indicative that the USP15-Ubggg complex is very tight, with complete saturation of the USP15 catalytic domain and binding with a stoichiometry of 1:1.

To combat unwanted cleavage of Ubggg during co-expression, a cell line deficient in ELaD, a bacterial protease, were used for all further expression of the Ubggg alone and linear di-ubiquitin and respective complexes.

5.4.2. USP15-D1D2-Cys269Ser-Ubggg complex crystallisation trials

The USP15-D1D2_Ubggg complex was subjected to crystallization trials, summarized in appendix 3d. The protein was initially trialled at 5 mg/ml and 10 mg/ml and screened at 10°C. After 1 week there was a good distribution of clear drops and drops containing protein precipitate, indicating the concentrations chosen were suitable for crystal screening. After 1 month, a single crystal was obtained in the condition: *1.0 M Ammonium phosphate monobasic, 0.1 M Sodium citrate pH 5.6* from the screen StructureScreen 1 + 2. The crystal was small and resembled a 3D rhomboid (figure 5-16). The crystal was fished, cryo-protected and cryo-cooled for data collection. The samples were fished by Dr Ingrid Dreveny, University of Nottingham, and were taken to the Diamond Light Source for testing. The crystal diffracted to 7 Å, indicating the crystal was a protein crystal.

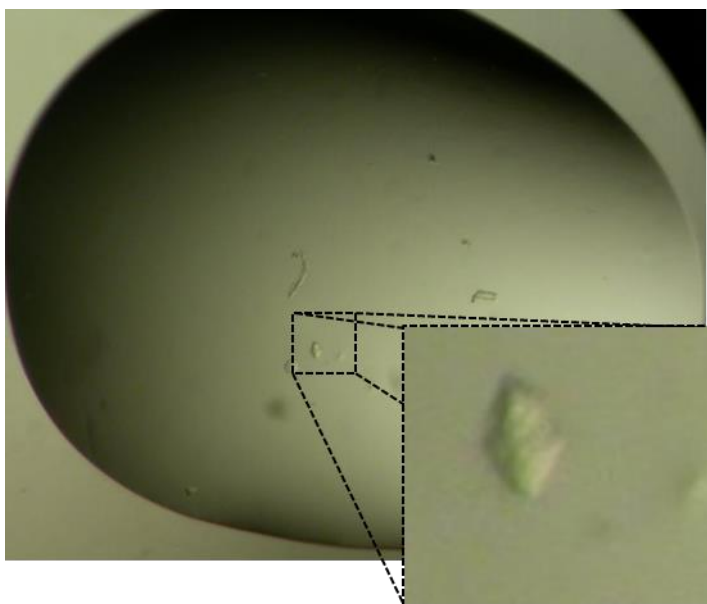


Figure 5-16: Crystal of USP15-D1D2 Cys269Ser_Ubggg grown in A10 of structure screen 1 + 2. The crystal is shown in the drop (400 nL) to give an indication size of the crystal and it has been magnified to show the morphology. The crystal diffracted to 7 Å.

5.4.2.1. Optimisation of USP15-D1D2_Ubggg complex crystal

Optimization plates were set up in which the pH and Ammonium phosphate concentration and temperature was varied (appendix 3e). A protein concentration of 5 mg/ml was used and trays were incubated at 10°C and 20°C. After a couple of weeks, 2 crystals were obtained in 2 conditions (B2 and B4) in optimization tray 55. The conditions are very similar to the original condition in which the crystal was obtained. The crystals were bigger and shared the same morphology as the original crystal. These were fished, cryoprotected and taken to diamond for testing. One crystal diffracted to 6 Å.

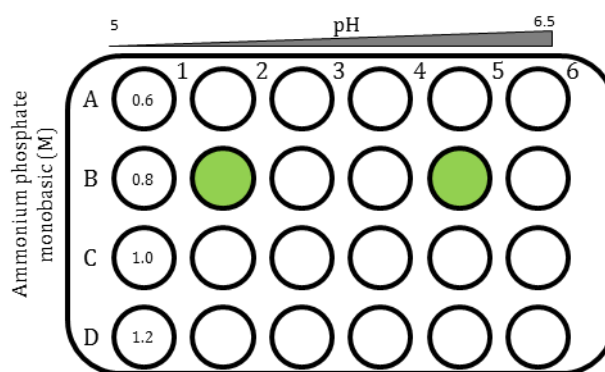


Figure 5-17: Schematic representation of USP15-D1D2-Cys269Ser-Ubggg complex optimisation plates. Green circles represent conditions in which hits were obtained.

Further optimization experiments were unable to reproduce these crystals. Following the success of using mitoxantrone as a crystallization agent for USP15-D1D2 WT, 96 well screens were set up as before with 500 μ M mitoxantrone present acting as an additive. Unfortunately, the screen yielded mostly clear drops and some salt crystals.

5.5. USP15 and linear di-ubiquitin investigation

As described above, the Cys269Ser mutant was used to allow the formation of a USP15-D1D2_Di-Ub complex, which could be purified for further experiments.

5.5.1. Expression and purification of the USP15-D1D2 linear di-ubiquitin complex

The USP15-D1D2_Di-ub complex was co-expressed in ELaD deficient cells. The complex expresses well and a 3-liter expression typically yields about 5 mg of protein. Similar to USP15-D1D2_Ubggg, the separation of USP15-D1D2_Di-Ub relies on the C-terminal His-tag of USP15. The complex elutes at around 40 % buffer B and eluted after free USP15-D1D2, in contrast to USP15-D1D2_Ubggg, which eluted before. The formation and purity of the complex was assessed on an SDS-PAGE gel with the complex clearly visible in several fractions.

Interestingly, the USP15-D1D2_Di-Ub complex eluted as a double peak at 78 ml and 82 ml. Some shift in elution volume can be accounted for by variation in SEC columns, or due to the overall dimension of the complex being slightly larger than free USP15-D1D2. The double peak suggests there are two species of the complex present in the sample given the fact the complex is visible in both peaks on an SDS-PAGE gel (figure 5-18).

The different species may have arisen from alternate conformations of the USP15-D1D2_Di-ub, with the distal ubiquitin binding to the S1 pocket and proximal ubiquitin to the S1', which would correspond to the peak at 82 ml. Conversely the proximal ubiquitin could bind to the distal ubiquitin pocket, as seen in the USP21_di-ub structure⁹¹ (figure 5-19), resulting in a larger diameter of the complex and could be attributed to the peak at 78 ml. However, an S2 binding site has not yet been identified in USP15. The complex is pure on SDS PAGE gel electrophoresis, and the shoulder was unable to be separated by an S75 column. One of the requirements for crystallization is a homogenous sample. Fractions 73-80 ml and 83-90 ml were combined separately to try and improve the quality of the sample.

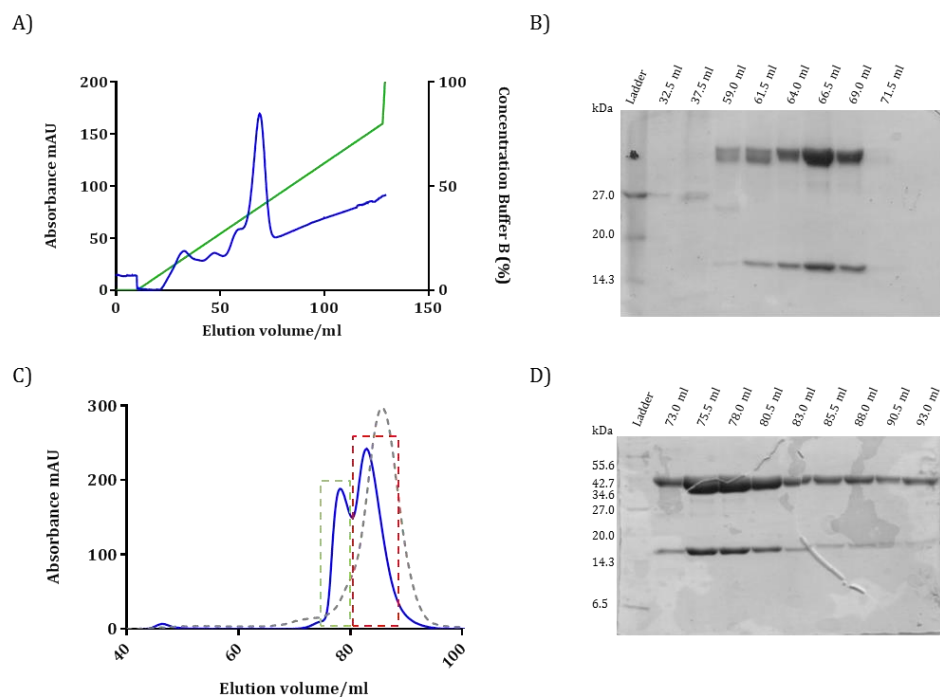


Figure 5-18: Purification of USP15 D1D2 Cys269Ser in complex with di-ubiquitin.

(A) Chromatogram of Ni-His trap purification step. The UV trace is shown in blue and the buffer B (%) gradient shown in green B) SDS-PAGE gel shows the purity corresponding Ni-His-Trap fractions. C) UV trace (blue) obtained from gel filtration step. The complex eluted as a double peak at 78 and 82 ml. The UV trace of free USP15-D1D2, which eluted at 85ml is shown in grey Dashed red and green boxes indicate the fractions taken. (D) SDS PAGE gel of fractions 73-93 ml from the gel filtration.

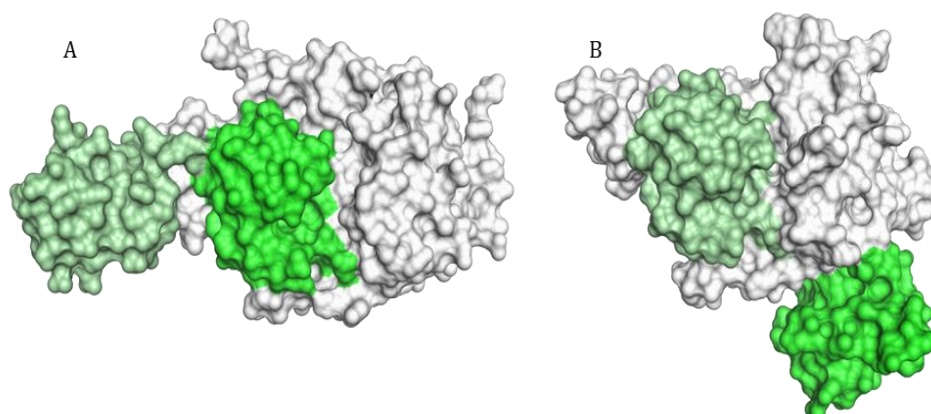


Figure 5-19: Possible orientations of di-ubiquitin in the USP catalytic site.

The proximal ubiquitin has been captured binding in the S1 pocket in USP21 (PDB: 2Y5B) (A) whilst in USP30 (PDB: 50HP) it is bound in the S1' pocket. The proximal ubiquitin is shown in bright green and the distal ubiquitin in pale green. USP21 and USP30 are shown in white.

5.5.1.1. Analysis of USP15-D1D2_Di-ub using native mass spectrometry.

Given the presence of multiple species following gel filtration, it was particularly important to assess the quality of the sample prior to crystallisation experiments. The mass spectrometry results revealed the sample contained USP15-D1D2_Di-ub and free linear di-ubiquitin confirming the formation of a tight complex whereby USP15 is saturated (figure 5-20). The data also confirms a 1:1 stoichiometry and endorses the presence of different conformations of USP15-D1D2_Di-Ub causing a change in the elution profile during SEC as opposed to a different stoichiometry. Finally, the absence of mono-ubiquitin in the sample confirms no residual DUB activity caused by the serine substitution which has been observed in other catalytic cysteine to serine mutations ²²².

Protein	Predicted mass kDa	Mass spectrometry mass kDa	Error $\pm$
Di-ubiquitin	17111.6	A:17111.94	0.15
Usp15 C1C2 C269S	41624.2	-	-
Usp15 C1C2 C269S + di-ubiquitin	58735.6	B:56514.19	1.89
		C:58856.57	0.47
		D:58956.66	0.52
		E: 57863.03	39.90
		F: 57475.27	2.40

Table 14: Predicted and calculated masses of USP15-D1D2 Cys269Ser_Di-Ub complex and components. Expected masses were calculated using protparam prediction tool.

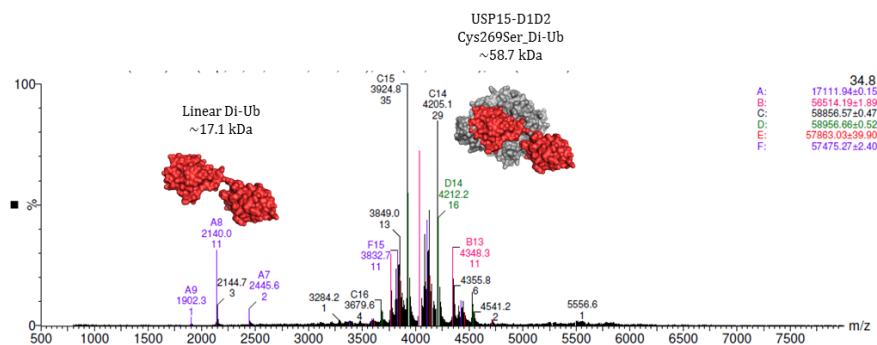


Figure 20: Native mass spectrometry analysis of the USP15-D1D2 Cys269Ser_Di-ub complex. Calculated masses are shown in pink, purple, green, red, blue and black. A schematic representation of the species detected is shown. Di-ubiquitin is shown in red and USP15 is shown in grey. No free USP15 was detected by the mass spectrometry.

5.5.2. USP15-D1D2 Cys269Ser_Di-Ub complex crystallisation trials.

Samples purified in section (5.5.1) were concentrated to 4 and 2 mg/ml for crystal trials. The complex was trialled in several 96 well screens summarized in appendix 3f. Small fine needle clusters were obtained in a few conditions from the Morpheus screen (figure 5-21) after 1 month. Crystals were fished, cryo-protected and taken to diamond for data collection. No diffraction could be detected for these



Figure 5-21: Example of needle clusters grown in several conditions in the Morpheus screen. Several clusters were present in each drop and were all very small and fine.

crystals, likely due to the size and thinness of the needles and the content of the crystals remained unknown. Crystal optimizations were set up to improve crystals size and morphology but the crystals were not reproducible in the optimization screens.

CHAPTER 6: Investigation into potential redox regulation of USP15

As discussed in section 5.2.2.1, USP15 contains a cysteine residue within the SL, which is within close proximity to the catalytic cysteine. This section will report on experimental attempts to understand the importance of this cysteine in terms of potential redox regulation.

6.1.1. Identification of potential disulphide within the structure

Disulphide bonds within proteins can be characterised into two categories, structural and reversible (redox-sensitive)²²³. USP's have been shown have varying degrees of sensitivity to oxidation and redox^{130, 224}.

Section (3.2) highlighted the SL as being a critical factor in the conformational rearrangements upon ubiquitin binding. Flexibility of the loop meant the density was poor and the majority of this region was unable to be modelled in all structures.

Cys352 was highlighted as the only difference in this region between USP15 and USP4. Cysteines are only found in this position in two other USPs; USP35 and USP38, however structural information is only available for USP35 in the bound form²²². Alignment of USP15-D1D2, USP4-D1D2 and ubiquitin bound USP35 revealed USP35 adopts the same conformation with respect to the SL and CC loop as USP4 (figure 6-1).

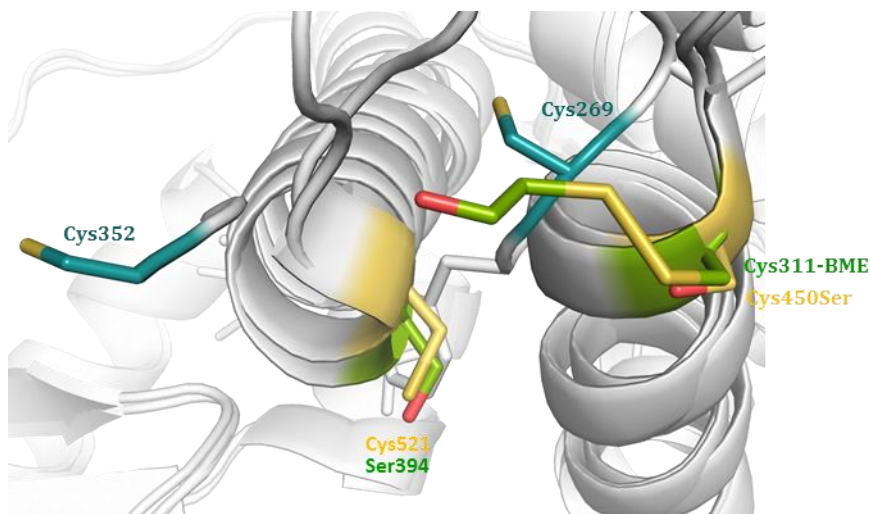


Figure 6-1: Alignment of USP15-D1D2, USP4-D1D2 and USP35. Catalytic cysteines (mutated to serine in USP35) and equivalent Cys352 residues are represented as sticks. USP15-D1D2 is shown in teal, USP4-D1D2 (2Y6E) is shown in green and USP35 (5TXK) is shown in yellow.

Alanine is the most prevalent residue in this position and is present in 62 % of the USP family. Serine and valine are present in 12 % and 10 % of USPs respectively with the remaining USPs harbouring a threonine (USP20), Isoleucine (USP52), methionine (USP34), Arginine (USP1) or glycine (USP39)⁸⁸.

Cys352 is in close proximity to the catalytic cysteine, which raised the question of the possibility of a disulphide bridge forming between the two residues. The density around the SL region was poor and varied greatly between molecules. In one molecule density was visible linking the CC loop to SL in the vicinity of the catalytic cysteine and Cys352, whereas in other models the density was clearly absent and Cys352 was most likely in an alternative conformation. A disulphide

bridge was modelled in one chain after the density remained over several refinements (figure 6-2).

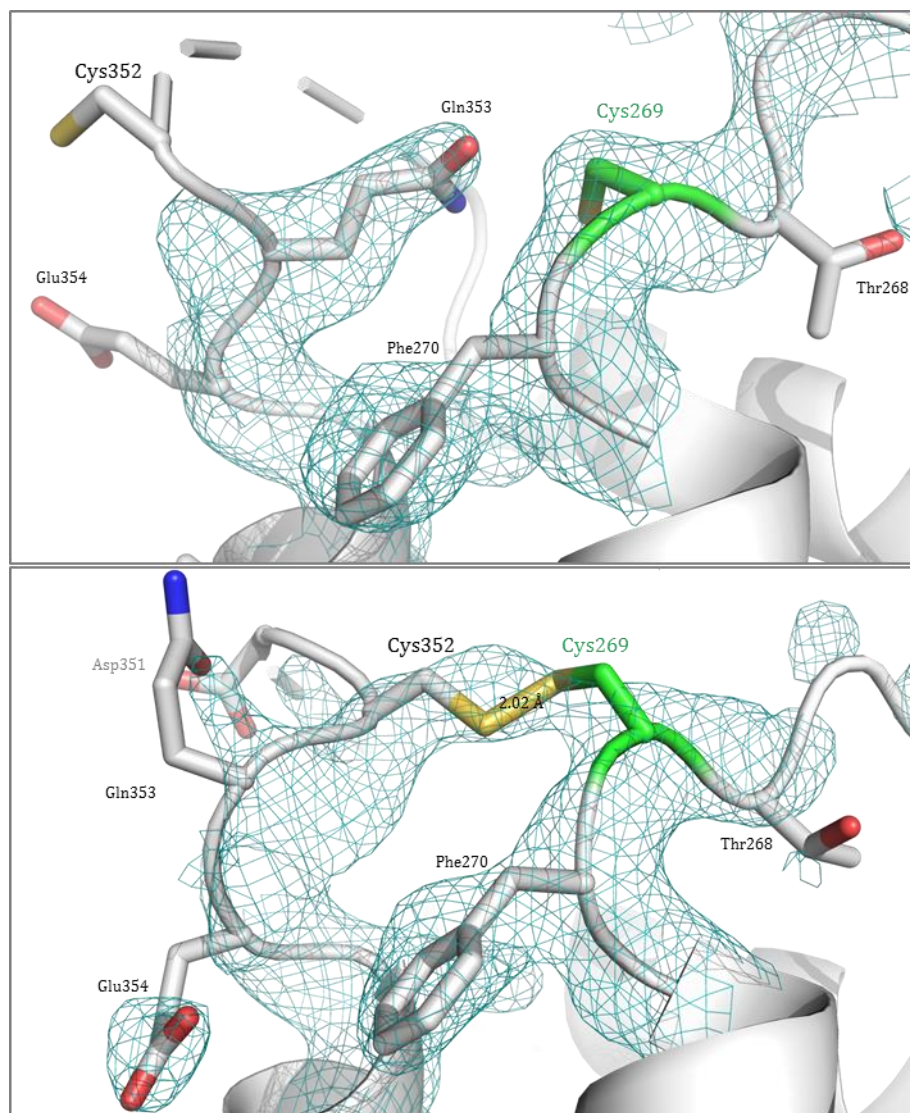


Figure 6-2: Modelling of potential disulphide between Cys269 and Cys352. Top panel shows a molecule where the density linking the SL and CC loop is absent. The bottom panel shows the modelling of a disulphide bridge between Cys269 and Cys352 after the density remained over several refinements. Cys269 is shown in green. The $2mF_o - F_c$ map is shown in blue.

Reversible disulphide bonds are generally of a higher energy than their structural counterparts, with a slightly longer bond length of $<2.10 \text{ \AA}$ ²²⁵. Analysis of the restraints file generated by Refmac5 in CCP4 did not highlight any unfavourable bond angles induced by the modelling of the disulphide bridge. Further inspection of the geometry of Cys352Ser revealed that the $\text{C}\alpha\text{-C}\beta\text{-S}\gamma$ and $\text{C}\beta\text{-S}\gamma\text{-S}\gamma$ angles were 114° and 102° respectively, and the torsion angle defined by $\text{C}\beta\text{-S}\gamma\text{-S}\gamma\text{-C}\beta$ was $\sim 128^\circ$ and were within acceptable geometry^{226, 227} (figure 6-3). Additional examination found the torsion angles defined by $\text{N-C}\alpha\text{-C}\beta\text{-S}\gamma$ and $\text{C}\alpha\text{-C}\beta\text{-S}\gamma\text{-S}\gamma$ for Cys352 were $\sim 50^\circ$ and $\sim 84^\circ$ respectively and $\sim 178^\circ$ and $\sim 141^\circ$ for Cys269. The optimum torsion angles for the bonds are $\pm 60^\circ$ and $\pm 180^\circ$ with deviations from these values resulting in an increase in bond energy and consequently reduced stability of the disulphide bond. Higher energy bonds are associated with reversible disulphide bridge formation, which is more likely to contribute to redox sensitivity within a protein²²⁵.

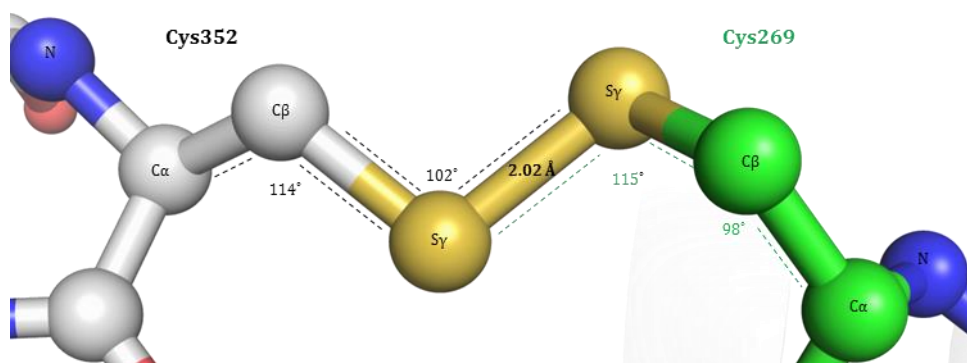


Figure 6-3: Ball and stick representation of possible disulphide bridge between Cys352 and Cys269. The bond length was found to be $2.02 \text{ \AA}$. $\text{C}\alpha\text{-C}\beta\text{-S}\gamma$ and $\text{C}\beta\text{-S}\gamma\text{-S}\gamma'$ angles are shown in white and green for Cys352 and Cys269 respectively. The torsion angle defined by $\text{C}\beta\text{-S}\gamma\text{-S}\gamma'\text{-C}\beta'$ was found to be 128° .

A possible explanation for the inconsistencies between structures could be the absence of any additional reducing agent following SEC. During purification, the protein will have only been exposed to a reducing agent once, unless stated otherwise, and it is possible that (1) the protein was never completely reduced and (2) the protein is slowly oxidised over time resulting in a heterogeneous solution containing protein of varying oxidation states. Moreover, X-ray induced disulphide-bond cleavage could occur during data collection. It is curious to note that the unbound USP15 structure, which only contained 1 copy of USP15 in the asymmetric unit unlike all other structures which contained two copies, was also the only sample to contain DTT in the crystallisation condition, and therefore may have resulted in consistent reduction throughout. Importantly the reduction of the sample in PR-619 implies the misalignment of the catalytic triad had not occurred due to oxidation of the catalytic cysteine.

The density is inconsistent between different molecules and the lack of density for the SL makes this region very difficult to model so it would be prudent to obtain evidence that is more conclusive before drawing conclusions about the importance of Cys352 and possible redox regulation of USP15.

Further, USP15 harbours a serine in the location thought to form a sulfanyl-amide interaction in USP19²²⁴, and it is possible that USP15 also exploits this mechanism for protection against oxidation.

6.1.2. USP15 requires DTT for activity

Disulphide bridges linking flexible regions within a protein generally confer an increase in protein stability and alter the unfolding behaviour of a protein by preventing early unfolding of some regions.

We utilised the Cys352Ser mutant to probe the role this cysteine may play in stability of USP15-D1D2. We hypothesised that replacement of the cysteine with a serine would disrupt the disulphide bridge formed between these residues which could be detected by changes in protein behaviour in different assays. We tested protein activity and stability and screened the mutant in crystal trials.

In line with Lee et al, who demonstrated that many of USP family require the presence of a reducing agent to be active *in vitro*²²⁴, we demonstrated that USP15 requires reduction with DTT following purification in order to be active in assays.

To allow the protein to become oxidised, the addition of DTT prior to gel filtration during the purification USP15-D1D2 and USP15-D1D2 Cys352Ser was omitted. Both samples were purified in tandem to ensure exposure to oxidation was consistent. The reducing agent DTT was added to a portion the sample whilst the rest remained in the oxidised state. Both oxidised and reduced samples were screened for activity using the Ub-AMC assay. In line with observations in the literature, our results showed that USP15 loses activity when oxidised, which is reversible with the addition of a reducing agent such as DTT (figure 6-4.) We were unable to further explore the importance of this cysteine and its involvement in the oxidation/reduction properties of USP15 using activity assays because of the requirement of a reducing environment for USP15 activity.

This result is consistent with the observations of USP15-D1D2 WT and USP15-D1D2 Cys352Ser displaying similar binding behaviours with ubiquitin during ITC experiments when exposed to DTT (section 5.2.2.1). However, Lee et al determined that oxidation of USP19 affects its ability to cleave the isopeptide bond but not its ability to ubiquitin²²⁴, and it is therefore it is possible that techniques such as ITC may not be that useful in exploring oxidative modifications in USPs.

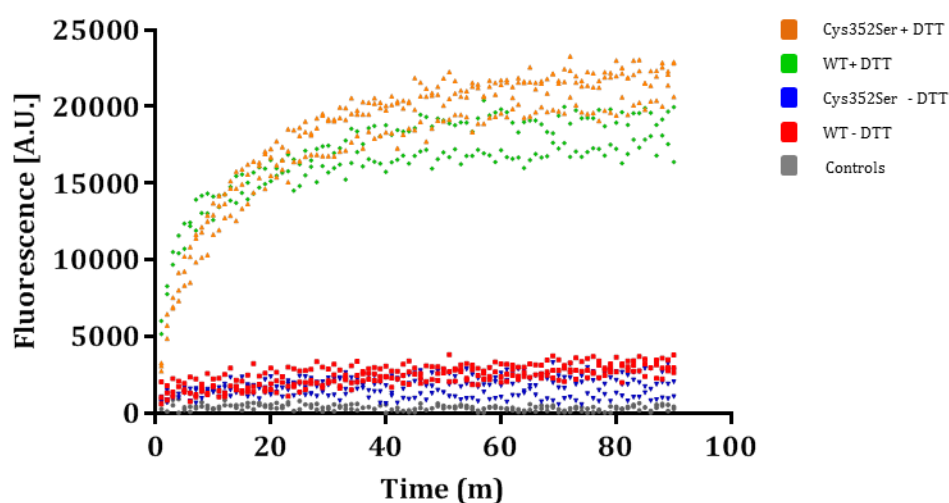


Figure 6-44: Activity of USP15-D1D2 WT and Cys352Ser in the Ub-AMC assay in oxidising and reducing conditions. Experiments were conducted in duplicate and 150 nM of enzymes was incubated with 1 μ M Ub-AMC and readings taken every minute. WT and Cys352Ser were tested in the presence of DTT (Green and orange curves) and with no DTT added to the buffer (Red and blue curves). Background signal is shown in grey.

6.1.3. Influence of Cys352 on protein stability

We used the thermofluor assay²¹⁸ to explore the effect that the Cys352 has on protein stability. The assay utilised the properties of the SYPRO orange dye, which undergoes a significant quantum yield once bound to hydrophobic regions in a protein. The dye excites at $\lambda=470$ nm and emits at $\lambda=570$ nm allowing detection in a qPCR machine. The assay set up is described in section (2.6.1). USP15-D1D2 WT was compared to USP15-D1D2 Cys352Ser in the presence and absence of DTT. Experiments comparing USP15-D1D2 WT and USP15-D1D2 Cys352Ser in the

presence of 0 mM and 2 mM DTT were conducted in triplicate. Analysis of the data using non-linear fitting to a Boltzman equation, yielded sigmoidal curves for all samples with the exception of USP15-D1D2 WT with no DTT, which displayed a biphasic melting profile (figure 6-5). This is intriguing, as in the presence of DTT both proteins showed no difference in unfolding behaviour (a change greater than 1°C is considered significant). Curiously, the presence of DTT increased the T_m USP15-D1D2 Cys352Ser by 3°C suggesting improved stability of the reduced protein.

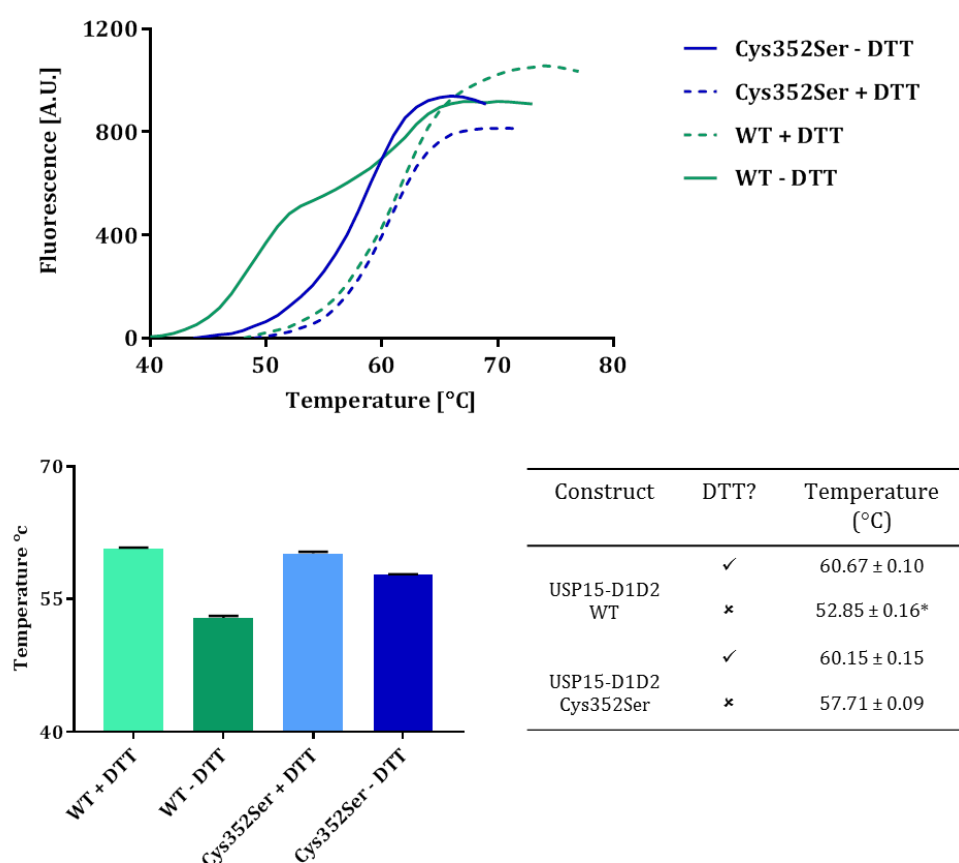


Figure 6-5: Investigation into importance of Cys352 in protein stability. The melting temperatures USP15-D1D2 Cys352Ser and USP15-D1D2 WT were measured in triplicate in the presence and absence of DTT, using the thermofluor. USP15-D1D2 in the absence of DTT yielded a bisphasic melting profile. The bar chart depicts the melting temperatures given in the table and errors are standard mean errors.

6.1.4. Crystal soaks with reducing and oxidising agents.

In light of the behaviour of WT USP15 in the thermofluor, we perused further crystallisation experiments in an attempt to reduce the ambiguity surrounding the Cys352 residue.

Crystals obtained from the 96 well mitoxantrone screen (see methods section 2.4.4) were subjected to soaks with 50 μM H_2O_2 over a period of 1 hour and 6 hours. Crystals were checked regularly during this time for evidence of cracking and discolouring of which none was observed. Crystals were fished and cryo-protected

using appropriate cryo-protectants supplemented with 50 μM H_2O_2 . Unfortunately, despite the crystal appearing to be of high quality, the best diffraction achieved was only 3.2 Å. At this resolution, the electron density was still ambiguous.

The reduced data quality may have been a consequence of the H_2O_2 soaking disrupting the crystal lattice. In attempt to improve diffraction quality, the protein was incubated with 50 μM H_2O_2 prior to crystallisation and 96 well screens were set up using the mitoxantrone screen. Despite a wealth of crystals growing in the screen none were of good enough quality of data collection.

6.1.5. Disulphide counting using LC-MS.

Finally, we tried LC-MS disulphide counting to try clarify the question surrounding the oxidation modifications of Cys269. The technique utilises an alkylation reaction of cysteine residues, with each Cys-alkylation contributing an additional 57 Da to the final mass. Samples were prepared as described in section 2.3, and DTT was omitted from the purification. To confirm oxidation of USP15 had occurred, the samples were tested in the Ub-AMC assay (figure 6-6).

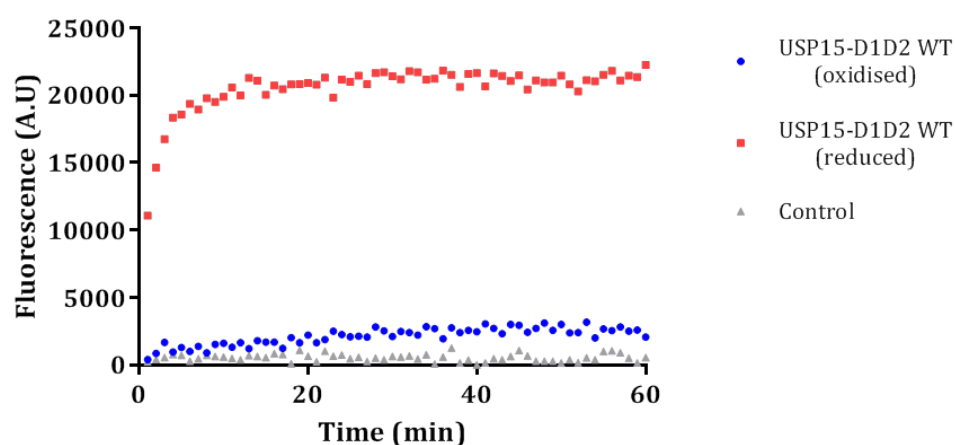


Figure 6-6: Ubiquitin-AMC assay confirms oxidation of USP15-D1D2 sample prior to LC-MS disulphide counting. USP15-D1D2 showed minimal activity against Ub-AMC (blue). Addition of 1 mM DTT to the assay buffer restores USP15s deubiquitinating activity resulting in rapid accumulation of free AMC.

Minimal activity was observed in the non-reduced sample, which was restored upon addition of 1 mM DTT to the reaction buffer, confirming oxidation of the catalytic cysteine had occurred.

There are 11 cysteines in USP15-D1D2, thus we were anticipating a ~627 Da change in mass in the reduced sample and a ~513 Da change in the oxidised sample (table 15). The samples tested were reduced, reduced and alkylated, oxidised and oxidised and alkylated. Ten cysteine residues were identified in the reduced sample following alkylation, whereas only two free cysteine were alkylated in the oxidised sample implying 8 of the 10 cysteine residues were forming disulphide bonds (table 16).

This was unexpected and may have occurred due to the cysteine residues being inaccessible for alkylation in the oxidised state.

Species (See appendix 1 for the full USP15-D1D2 sequence)	Predicted Mass
USP15-D1D2 WT	41640.26
USP15-D1D2 WT Reduced + alkylated	42267.26
USP15-D1D2 WT Oxidised + alkylated (Assuming a single disulphide bridge forms)	42153.26

Table 15: Table of predicted masses for reduced, unmodified US15, reduced and alkylated USP15 and oxidised and alkylated USP15.

Protein	Starting Material Mass	Reduced	Alkylated (oxidised)	Reduced then Alkylated	No. Alkylated Cys
#1	39353.43	39354.52			0
			39410.81		1
			39467.71		2
				39637.82	5
				39695.72	6
				39752.79	7
				39810.48	8
				39868.6	9
				39927.73	10
#2	40559.35	40560.42			0
			40617.02		1
				40793.28	4
#3	40743.73	40744.58			0
			40799.51		1
			40858.74		2
				41140.33	7
				41199.8	8
				41258.6	9
				41317.39	10
#4	41283.44	41285.08			0
			41340.03		1
				41564.05	5
				41681.87	7
#5	41693.41	41696.09			0
			41751.14		1
				42157.92	8
				42210.36	9

Table 16: Summary of LC-MS disulphide counting. Five different protein species were detected in the sample, indicated by #1-#5. Ten cysteines were detected in species #1 and #3, nine in species #5, 7 in sample #4 and 4 in sample #2. In the oxidised samples, the maximum number of cysteines detected was two, suggesting 8 cysteines are involved in a disulphide bridge.

6.1.5.1. Soaking of crystals with β -mercaptoethanol

During the structural investigation of the USP15 catalytic core, I highlighted the inactive conformation of the USP15 catalytic triad as being one of the key differences with its paralogue USP4, whose catalytic triad was in a catalytically competent configuration. It was suggested that the β -mercaptoethanol adduct may be a contributing factor, as it mimics the C-terminal tail of ubiquitin, inducing localized

	Inner shell	Outer shell	Overall
Resolution	8.79 Å	3.01 Å	3.00 Å
Rmerge	0.052	0.661	0.127
Rmeas	0.056	0.751	0.137
Rpim	0.023	0.272	0.052
I/ Φ I	31.5	3.9	14.7
CC1/2	0.999	0.943	0.998
Completeness	97.4	99.4	99.8

Table 17: Statistics of β -mercaptoethanol soaked crystal after the data was cut back to 3 Å and re-indexed using Aimless.

conformational changes corresponding to that of bound ubiquitin. Another reason could be the SL in USP4, is involved in crystal contacts which are absent in the USP15 crystals structures. Verification of this theory would require a structure of USP15-D1D2 with the same β -mercaptoethanol adduct, or a free USP4 structure. Crystals grown in optimization screens with mitoxantrone were used for soaking experiments. Crystals were exposed to 5 mM β -mercaptoethanol for 1 hour or 24 hours. As with the H₂O₂ soaks crystal integrity was monitored throughout the soaking experiment. Single crystals were fished, cryo-protected with the suitable cryo-protectant supplemented with 5 mM β -mercaptoethanol and taken to Diamond Light Source for data collection. Diffraction to 2.3Å was obtained for one

crystal. However due to poor completeness the data were truncated to 3 Å and re-indexed using aimless.

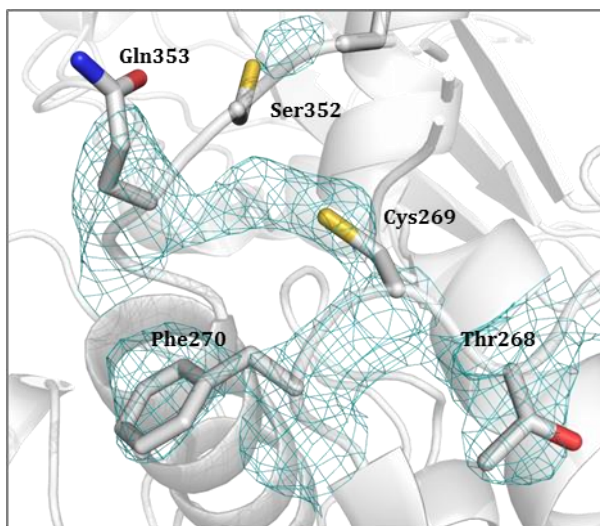


Figure 6-7: Crystal soaking experiments with 5 mM β -mercaptoethanol. Residues are represented as sticks and the corresponding $2mF_o - F_c$ map contoured to a sigma level of 1 is shown on blue. No modification of Cys269 was observed.

Poor density was observed in the CC loop region with both molecules in the asymmetric unit exhibiting the same disrupted catalytic conformation seen in previous structures (figure 6-7). The density around Cys269 was seen to bridge across and merge with density corresponding to parts of the C-terminal end of the SL. Once again, the SL was disordered and could not be fully modelled meaning the positing of Cys352 with respect to the catalytic cysteine could not be deduced. A β -mercaptoethanol adduct could not be modelled into the additional density observed around the catalytic cysteine due to clashes with the Gln353 and no other density corresponding to β -mercaptoethanol in this region was observed. Further efforts were made to obtain a USP15-D1D2- β -mercaptoethanol co-crystal by resorting back 96 well screens however, the trials were unsuccessful in yielding crystals of a high enough quality for data collection.

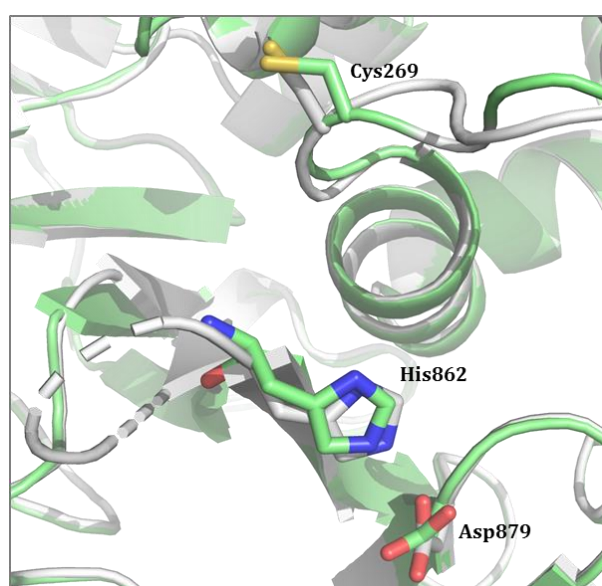


Figure 6-8: Superposition of USP15-free with a β -mercaptoethanol soaked structure. The catalytic residues are shown as sticks and USP15-free is shown in white and the β -mercaptoethanol soaked crystal.

CHAPTER 7: Conclusion and Future Work

Mitoxantrone promotes the crystallization of the USP15 catalytic domain allowing the first unbound USP15 catalytic domain and USP15-mitoxantrone complex structures to be solved. Structural characterization of the unbound catalytic domain and revealed USP15 harbours the canonical USP fold and a misaligned catalytic triad, which has only been seen in unbound USP7 before.

Comparison of USP15 with USP4, highlighted sequence differences between the two paralogues predominantly mapped to the S1' substrate binding region. Notable structural differences arose in the active site region with the USP4 catalytic triad adopting a catalytically competent conformation, as opposed to the disrupted conformation seen in USP15. In order to accommodate the misaligned catalytic triad, the flexible loop in USP15, is most likely in a different conformation to USP4, with a Cys-Ser substitution being the only residue difference in this region. . The catalytic cysteine of USP4 is modified with a β -mercaptoethanol adduct which mimics the C-terminal tail of ubiquitin. Furthermore the ubiquitin binding channel in USP15 is 'open' as opposed to the closed conformation observed in USP4. Residues in the USP4 BL2, namely Val879 which is substituted for a glycine in USP15, appeared to be contributing to the closed contribution of USP4.

The ubiquitin binding behaviour of USP15 and USP4 was compared using isothermal titration calorimetry. USP15 was found to bind ubiquitin with a weaker affinity in comparison to USP4 and displayed a different thermodynamic profile. Residue differences in the BL2 were found to possibly contribute to ubiquitin binding and sequential USP15-USP4 BL2 mutations shifted the thermodynamic profile to resemble USP4 but did not affect the overall affinity. These differences

in affinity likely correspond to the different ubiquitin discharge behaviour of the catalytic domains USP15 and USP4. Collectively this data provides the first insights into a possible activation mechanism for USP15. Bound USP15 and true unbound USP4 structures will help corroborate the mechanism identified in this work.

The substitution of the cysteine found in the USP15 SL with the equivalent USP4 residue (serine) did not alter the ubiquitin binding behaviour of USP15. This cysteine residue was within close proximity to the catalytic cysteine, and in some models could be modelled as a disulphide bridge, however density was ambiguous in this region. Attempts to clarify the existence of the disulphide using further crystallisation experiments and LC-MS were unsuccessful.

One of the limiting factors in using LC-MS and other disulphide counting assays is the availability of the cysteine residues within the native protein. Other approaches to Mass Spectrometry using techniques such as trypsin digest of the protein may provide a more conclusive answer.

Ubggg was demonstrated to be an effective minimal substrate for USP15 which could be easily produced recombinantly to yield large quantities of protein. This novel ubiquitin variant was demonstrated to bind to USP15 with remarkably higher affinity in comparison to ubiquitin and linear di-ubiquitin and displayed different thermodynamic binding parameters. This dramatic change in affinity coincides with observations of isopeptide bond linked substrate preference displayed by USP15. This Ubggg construct can easily be further modified for fluorogenic based activity assays and it would be interesting to compare the K_m and K_{cat} for this isopeptide bond mimic with other substrates such as Ub-AMC and a substrate containing a native isopeptide bond.

Finally, co-crystallisation with novel USP15 inhibitor mitoxantrone uncovered a new mechanism of USP inhibition. A single molecule of mitoxantrone was found to

bind to the BL2, via hydrophobic interactions, in the solvent exposed S1' pocket. Characterization using ITC, Ub-AMC activity assay and a linear di-ubiquitin cleavage assay confirmed the interaction was specific and mitoxantrone exerted its inhibitory activity by competing with the proximal substrate. Further investigations using USP11, USP4 and the more distantly related USP2 demonstrated mitoxantrone is likely a promiscuous inhibitor of the USP family. Determining the K_M and K_{cat} for USP15 activity against proximal substrates in the presence of different mitoxantrone concentrations would be valuable in further validating the competitive mechanism described in this thesis. Further studies using cell lines, particularly glioblastoma cell lines, would be useful in validating this mechanism in vivo, and may have important implications in new therapeutic uses of mitoxantrone.

References

- [1] Nanao, M. H., Tcherniuk, S. O., Chroboczek, J., Dideberg, O., Dessen, A., and Balakirev, M. Y. (2004) Crystal structure of human otubain 2, *EMBO reports* 5, 783-788.
- [2] Hu, M., Li, P., Li, M., Li, W., Yao, T., Wu, J. W., Gu, W., Cohen, R. E., and Shi, Y. (2002) Crystal structure of a UBP-family deubiquitinating enzyme in isolation and in complex with ubiquitin aldehyde, *Cell* 111, 1041-1054.
- [3] Nicastro, G., Menon, R. P., Masino, L., Knowles, P. P., McDonald, N. Q., and Pastore, A. (2005) The solution structure of the Josephin domain of ataxin-3: structural determinants for molecular recognition, *Proceedings of the National Academy of Sciences of the United States of America* 102, 10493-10498.
- [4] Avvakumov, G. V., Walker, J. R., Xue, S., Finerty, P. J., Jr., Mackenzie, F., Newman, E. M., and Dhe-Paganon, S. (2006) Amino-terminal dimerization, NRDP1-rhodanese interaction, and inhibited catalytic domain conformation of the ubiquitin-specific protease 8 (USP8), *The Journal of biological chemistry* 281, 38061-38070.
- [5] Abdul Rehman, S. A., Kristariyanto, Y. A., Choi, S. Y., Nkosi, P. J., Weidlich, S., Labib, K., Hofmann, K., and Kulathu, Y. (2016) MINDY-1 Is a Member of an Evolutionarily Conserved and Structurally Distinct New Family of Deubiquitinating Enzymes, *Mol Cell* 63, 146-155.
- [6] Ambroggio, X. I., Rees, D. C., and Deshaies, R. J. (2004) JAMM: a metalloprotease-like zinc site in the proteasome and signalosome, *PLoS biology* 2, E2.
- [7] Clague, M. J. (2013) Oxidation controls the DUB step, *Nature* 497, 49.
- [8] Vijay-Kumar, S., Bugg, C. E., and Cook, W. J. (1987) Structure of ubiquitin refined at 1.8 Å resolution, *J Mol Biol* 194, 531-544.
- [9] Chuang, S. J., Cheng, S. C., Tang, H. C., Sun, C. Y., and Chou, C. Y. (2018) 6-Thioguanine is a noncompetitive and slow binding inhibitor of human deubiquitinating protease USP2, *Sci Rep* 8, 3102.
- [10] Komander, D., Clague, M. J., and Urbe, S. (2009) Breaking the chains: structure and function of the deubiquitinases, *Nat Rev Mol Cell Biol* 10, 550-563.
- [11] Johnston, S. C., Larsen, C. N., Cook, W. J., Wilkinson, K. D., and Hill, C. P. (1997) Crystal structure of a deubiquitinating enzyme (human UCH-L3) at 1.8 Å resolution, *The EMBO Journal* 16, 3787-3796.
- [12] Notredame, C., Higgins, D. G., and Heringa, J. (2000) T-Coffee: A novel method for fast and accurate multiple sequence alignment, *J Mol Biol* 302, 205-217.
- [13] Gao, S., Pan, M., Zheng, Y., Huang, Y., Zheng, Q., Sun, D., Lu, L., Tan, X., Tan, X., Lan, H., Wang, J., Wang, T., Wang, J., and Liu, L. (2016) Monomer/Oligomer Quasi-Racemic Protein Crystallography, *J Am Chem Soc* 138, 14497-14502.
- [14] Clerici, M., Luna-Vargas, M. P., Faesen, A. C., and Sixma, T. K. (2014) The DUSP-Ubl domain of USP4 enhances its catalytic efficiency by promoting ubiquitin exchange, *Nat Commun* 5, 5399.

- [15] Clague, M. J., Barsukov, I., Coulson, J. M., Liu, H., Rigden, D. J., and Urbe, S. (2013) Deubiquitylases from genes to organism, *Physiol Rev* 93, 1289-1315.
- [16] Bremm, A., Freund, S. M., and Komander, D. (2010) Lys11-linked ubiquitin chains adopt compact conformations and are preferentially hydrolyzed by the deubiquitinase Cezanne, *Nat Struct Mol Biol* 17, 939-947.
- [17] Pan, M., Gao, S., Zheng, Y., Tan, X., Lan, H., Tan, X., Sun, D., Lu, L., Wang, T., Zheng, Q., Huang, Y., Wang, J., and Liu, L. (2016) Quasi-Racemic X-ray Structures of K27-Linked Ubiquitin Chains Prepared by Total Chemical Synthesis, *J Am Chem Soc* 138, 7429-7435.
- [18] Kulathu, Y., and Komander, D. (2012) Atypical ubiquitylation - the unexplored world of polyubiquitin beyond Lys48 and Lys63 linkages, *Nat Rev Mol Cell Biol* 13, 508-523.
- [19] Satoh, T., Sakata, E., Yamamoto, S., Yamaguchi, Y., Sumiyoshi, A., Wakatsuki, S., and Kato, K. (2010) Crystal structure of cyclic Lys48-linked tetraubiquitin, *Biochem Biophys Res Commun* 400, 329-333.
- [20] Komander, D., Reyes-Turcu, F., Licchesi, J. D., Odenwaelder, P., Wilkinson, K. D., and Barford, D. (2009) Molecular discrimination of structurally equivalent Lys 63-linked and linear polyubiquitin chains, *EMBO Rep* 10, 466-473.
- [21] Goldstein, G., Scheid, M., Hammerling, U., Schlesinger, D. H., Niall, H. D., and Boyse, E. A. (1975) Isolation of a polypeptide that has lymphocyte-differentiating properties and is probably represented universally in living cells, *Proc Natl Acad Sci U S A* 72, 11-15.
- [22] Hershko, A., and Ciechanover, A. (1992) The Ubiquitin System for Protein Degradation, *Annual Review of Biochemistry* 61, 761-807.
- [23] Kavsak, P., Rasmussen, R. K., Causing, C. G., Bonni, S., Zhu, H., Thomsen, G. H., and Wrana, J. L. (2000) Smad7 binds to Smurf2 to form an E3 ubiquitin ligase that targets the TGF beta receptor for degradation, *Mol Cell* 6, 1365-1375.
- [24] Schafer, A., Kuhn, M., and Schindelin, H. (2014) Structure of the ubiquitin-activating enzyme loaded with two ubiquitin molecules, *Acta crystallographica. Section D, Biological crystallography* 70, 1311-1320.
- [25] Lee, I., and Schindelin, H. (2008) Structural insights into E1-catalyzed ubiquitin activation and transfer to conjugating enzymes, *Cell* 134, 268-278.
- [26] Haas, A. L., Warmes, J. V., Hershko, A., and Rose, I. A. (1982) Ubiquitin-activating enzyme. Mechanism and role in protein-ubiquitin conjugation, *The Journal of biological chemistry* 257, 2543-2548.
- [27] Haas, A. L., and Rose, I. A. (1982) The mechanism of ubiquitin activating enzyme. A kinetic and equilibrium analysis, *The Journal of biological chemistry* 257, 10329-10337.
- [28] Scheffner, M., Nuber, U., and Huibregtse, J. M. (1995) Protein ubiquitination involving an E1-E2-E3 enzyme ubiquitin thioester cascade, *Nature* 373, 81-83.
- [29] Ye, Y., and Rape, M. (2009) Building ubiquitin chains: E2 enzymes at work, *Nat Rev Mol Cell Biol* 10, 755-764.

- [30] Alpi, A. F., Pace, P. E., Babu, M. M., and Patel, K. J. (2008) Mechanistic insight into site-restricted monoubiquitination of FANCD2 by Ube2t, FANCL, and FANCI, *Mol Cell* 32, 767-777.
- [31] Longerich, S., San Filippo, J., Liu, D., and Sung, P. (2009) FANCI binds branched DNA and is monoubiquitinated by UBE2T-FANCL, *The Journal of biological chemistry* 284, 23182-23186.
- [32] Scaglione, K. M., Zavodszky, E., Todi, S. V., Patury, S., Xu, P., Rodriguez-Lebron, E., Fischer, S., Konen, J., Djarmati, A., Peng, J., Gestwicki, J. E., and Paulson, H. L. (2011) Ube2w and ataxin-3 coordinately regulate the ubiquitin ligase CHIP, *Mol Cell* 43, 599-612.
- [33] Williamson, A., Banerjee, S., Zhu, X., Philipp, I., Iavarone, A. T., and Rape, M. (2011) Regulation of ubiquitin chain initiation to control the timing of substrate degradation, *Mol Cell* 42, 744-757.
- [34] Dynek, J. N., Goncharov, T., Dueber, E. C., Fedorova, A. V., Izrael-Tomasevic, A., Phu, L., Helgason, E., Fairbrother, W. J., Deshayes, K., Kirkpatrick, D. S., and Vucic, D. (2010) c-IAP1 and UbcH5 promote K11-linked polyubiquitination of RIP1 in TNF signalling, *EMBO J* 29, 4198-4209.
- [35] Moraes, T. F., Edwards, R. A., McKenna, S., Pastushok, L., Xiao, W., Glover, J. N., and Ellison, M. J. (2001) Crystal structure of the human ubiquitin conjugating enzyme complex, hMms2-hUbc13, *Nat Struct Biol* 8, 669-673.
- [36] Eddins, M. J., Carlile, C. M., Gomez, K. M., Pickart, C. M., and Wolberger, C. (2006) Mms2-Ubc13 covalently bound to ubiquitin reveals the structural basis of linkage-specific polyubiquitin chain formation, *Nat Struct Mol Biol* 13, 915-920.
- [37] Wickliffe, K. E., Lorenz, S., Wemmer, D. E., Kuriyan, J., and Rape, M. (2011) The mechanism of linkage-specific ubiquitin chain elongation by a single-subunit E2, *Cell* 144, 769-781.
- [38] Medvar, B., Raghuram, V., Pisitkun, T., Sarkar, A., and Knepper, M. A. (2016) Comprehensive database of human E3 ubiquitin ligases: application to aquaporin-2 regulation, *Physiological genomics* 48, 502-512.
- [39] Metzger, M. B., Hristova, V. A., and Weissman, A. M. (2012) HECT and RING finger families of E3 ubiquitin ligases at a glance, *J Cell Sci* 125, 531-537.
- [40] Rotin, D., and Kumar, S. (2009) Physiological functions of the HECT family of ubiquitin ligases, *Nat Rev Mol Cell Biol* 10, 398-409.
- [41] Kim, H. C., and Huibregtse, J. M. (2009) Polyubiquitination by HECT E3s and the determinants of chain type specificity, *Mol Cell Biol* 29, 3307-3318.
- [42] You, J., and Pickart, C. M. (2001) A HECT domain E3 enzyme assembles novel polyubiquitin chains, *The Journal of biological chemistry* 276, 19871-19878.
- [43] Huang, L., Kinnucan, E., Wang, G., Beaudenon, S., Howley, P. M., Huibregtse, J. M., and Pavletich, N. P. (1999) Structure of an E6AP-UbcH7 complex: insights into ubiquitination by the E2-E3 enzyme cascade, *Science* 286, 1321-1326.
- [44] Pickart, C. M. (2001) Mechanisms underlying ubiquitination, *Annu Rev Biochem* 70, 503-533.

- [45] Nakasone, M. A., Livnat-Levanon, N., Glickman, M. H., Cohen, R. E., and Fushman, D. (2013) Mixed-linkage ubiquitin chains send mixed messages, *Structure* 21, 727-740.
- [46] Faesen, A. C., Luna-Vargas, M. P., Geurink, P. P., Clerici, M., Merks, R., van Dijk, W. J., Hameed, D. S., El Oualid, F., Ovaa, H., and Sixma, T. K. (2011) The differential modulation of USP activity by internal regulatory domains, interactors and eight ubiquitin chain types, *Chem Biol* 18, 1550-1561.
- [47] Reyes-Turcu, F. E., Ventii, K. H., and Wilkinson, K. D. (2009) Regulation and cellular roles of ubiquitin-specific deubiquitinating enzymes, *Annu Rev Biochem* 78, 363-397.
- [48] Kirisako, T., Kamei, K., Murata, S., Kato, M., Fukumoto, H., Kanie, M., Sano, S., Tokunaga, F., Tanaka, K., and Iwai, K. (2006) A ubiquitin ligase complex assembles linear polyubiquitin chains, *The EMBO Journal* 25, 4877-4887.
- [49] Tokunaga, F., Sakata, S., Saeki, Y., Satomi, Y., Kirisako, T., Kamei, K., Nakagawa, T., Kato, M., Murata, S., Yamaoka, S., Yamamoto, M., Akira, S., Takao, T., Tanaka, K., and Iwai, K. (2009) Involvement of linear polyubiquitylation of NEMO in NF-kappaB activation, *Nat Cell Biol* 11, 123-132.
- [50] Rahighi, S., Ikeda, F., Kawasaki, M., Akutsu, M., Suzuki, N., Kato, R., Kensche, T., Uejima, T., Bloor, S., Komander, D., Randow, F., Wakatsuki, S., and Dikic, I. (2009) Specific recognition of linear ubiquitin chains by NEMO is important for NF-kappaB activation, *Cell* 136, 1098-1109.
- [51] Wang, X. A., Kurra, Y., Huang, Y., Lee, Y. J., and Liu, W. R. (2014) E1-catalyzed ubiquitin C-terminal amidation for the facile synthesis of deubiquitinase substrates, *ChemBiochem* 15, 37-41.
- [52] Thrower, J. S., Hoffman, L., Rechsteiner, M., and Pickart, C. M. (2000) Recognition of the polyubiquitin proteolytic signal, *EMBO J* 19, 94-102.
- [53] Tenno, T., Fujiwara, K., Tochio, H., Iwai, K., Morita, E. H., Hayashi, H., Murata, S., Hiroaki, H., Sato, M., Tanaka, K., and Shirakawa, M. (2004) Structural basis for distinct roles of Lys63- and Lys48-linked polyubiquitin chains, *Genes Cells* 9, 865-875.
- [54] Cook, W. J., Jeffrey, L. C., Kasperek, E., and Pickart, C. M. (1994) Structure of Tetraubiquitin Shows How Multiubiquitin Chains Can Be Formed, *Journal of Molecular Biology* 236, 601-609.
- [55] Chau, V., Tobias, J. W., Bachmair, A., Marriotti, D., Ecker, D. J., Gonda, D. K., and Varshavsky, A. (1989) A multiubiquitin chain is confined to specific lysine in a targeted short-lived protein, *Science* 243, 1576-1583.
- [56] Adhikari, A., Xu, M., and Chen, Z. J. (2007) Ubiquitin-mediated activation of TAK1 and IKK, *Oncogene* 26, 3214-3226.
- [57] Min, M., Mevissen, T. E., De Luca, M., Komander, D., and Lindon, C. (2015) Efficient APC/C substrate degradation in cells undergoing mitotic exit depends on K11 ubiquitin linkages, *Mol Biol Cell* 26, 4325-4332.
- [58] Wickliffe, K. E., Williamson, A., Meyer, H. J., Kelly, A., and Rape, M. (2011) K11-linked ubiquitin chains as novel regulators of cell division, *Trends Cell Biol* 21, 656-663.

- [59] Boname, J. M., Thomas, M., Stagg, H. R., Xu, P., Peng, J., and Lehner, P. J. (2010) Efficient internalization of MHC I requires lysine-11 and lysine-63 mixed linkage polyubiquitin chains, *Traffic* 11, 210-220.
- [60] Goto, E., Yamanaka, Y., Ishikawa, A., Aoki-Kawasumi, M., Mito-Yoshida, M., Ohmura-Hoshino, M., Matsuki, Y., Kajikawa, M., Hirano, H., and Ishido, S. (2010) Contribution of lysine 11-linked ubiquitination to MIR2-mediated major histocompatibility complex class I internalization, *The Journal of biological chemistry* 285, 35311-35319.
- [61] Ordureau, A., Heo, J.-M., Duda, D. M., Paulo, J. A., Olszewski, J. L., Yanishevski, D., Rinehart, J., Schulman, B. A., and Harper, J. W. (2015) Defining roles of PARKIN and ubiquitin phosphorylation by PINK1 in mitochondrial quality control using a ubiquitin replacement strategy, *Proceedings of the National Academy of Sciences* 112, 6637-6642.
- [62] Elia, A. E., Boardman, A. P., Wang, D. C., Huttlin, E. L., Everley, R. A., Dephoure, N., Zhou, C., Koren, I., Gygi, S. P., and Elledge, S. J. (2015) Quantitative Proteomic Atlas of Ubiquitination and Acetylation in the DNA Damage Response, *Mol Cell* 59, 867-881.
- [63] Ikeda, H., and Kerppola, T. K. (2008) Lysosomal localization of ubiquitinated Jun requires multiple determinants in a lysine-27-linked polyubiquitin conjugate, *Mol Biol Cell* 19, 4588-4601.
- [64] Geisler, S., Holmstrom, K. M., Skujat, D., Fiesel, F. C., Rothfuss, O. C., Kahle, P. J., and Springer, W. (2010) PINK1/Parkin-mediated mitophagy is dependent on VDAC1 and p62/SQSTM1, *Nat Cell Biol* 12, 119-131.
- [65] Liu, J., Han, C., Xie, B., Wu, Y., Liu, S., Chen, K., Xia, M., Zhang, Y., Song, L., Li, Z., Zhang, T., Ma, F., Wang, Q., Wang, J., Deng, K., Zhuang, Y., Wu, X., Yu, Y., Xu, T., and Cao, X. (2014) Rbddd3 controls autoimmunity by suppressing the production of IL-6 by dendritic cells via K27-linked ubiquitination of the regulator NEMO, *Nat Immunol* 15, 612-622.
- [66] Gatti, M., Pinato, S., Maiolica, A., Rocchio, F., Prato, M. G., Aebbersold, R., and Penengo, L. (2015) RNF168 promotes noncanonical K27 ubiquitination to signal DNA damage, *Cell Rep* 10, 226-238.
- [67] Xu, P., Duong, D. M., Seyfried, N. T., Cheng, D., Xie, Y., Robert, J., Rush, J., Hochstrasser, M., Finley, D., and Peng, J. (2009) Quantitative proteomics reveals the function of unconventional ubiquitin chains in proteasomal degradation, *Cell* 137, 133-145.
- [68] Al-Hakim, A. K., Zagorska, A., Chapman, L., Deak, M., Pegg, M., and Alessi, D. R. (2008) Control of AMPK-related kinases by USP9X and atypical Lys(29)/Lys(33)-linked polyubiquitin chains, *Biochem J* 411, 249-260.
- [69] Huang, H., Jeon, M. S., Liao, L., Yang, C., Elly, C., Yates, J. R., 3rd, and Liu, Y. C. (2010) K33-linked polyubiquitination of T cell receptor-zeta regulates proteolysis-independent T cell signaling, *Immunity* 33, 60-70.
- [70] Yuan, W. C., Lee, Y. R., Lin, S. Y., Chang, L. Y., Tan, Y. P., Hung, C. C., Kuo, J. C., Liu, C. H., Lin, M. Y., Xu, M., Chen, Z. J., and Chen, R. H. (2014) K33-Linked Polyubiquitination of Coronin 7 by Cul3-KLHL20 Ubiquitin E3 Ligase Regulates Protein Trafficking, *Mol Cell* 54, 586-600.
- [71] Kristariyanto Y, A., Choi, S. Y., Rehman, Sa A., Ritorto M, S., Campbell D, G., Morrice N, A., Toth, R., and Kulathu, Y. (2015) Assembly and

structure of Lys(33)-linked polyubiquitin reveals distinct conformations, *Biochem J* 467, 345-352.

[72] Varadan, R., Assfalg, M., Haririnia, A., Raasi, S., Pickart, C., and Fushman, D. (2004) Solution conformation of Lys63-linked di-ubiquitin chain provides clues to functional diversity of polyubiquitin signaling, *The Journal of biological chemistry* 279, 7055-7063.

[73] Hirano, T., Serve, O., Yagi-Utsumi, M., Takemoto, E., Hiromoto, T., Satoh, T., Mizushima, T., and Kato, K. (2011) Conformational dynamics of wild-type Lys-48-linked diubiquitin in solution, *The Journal of biological chemistry* 286, 37496-37502.

[74] Rohaim, A., Kawasaki, M., Kato, R., Dikic, I., and Wakatsuki, S. (2012) Structure of a compact conformation of linear diubiquitin, *Acta crystallographica. Section D, Biological crystallography* 68, 102-108.

[75] Burroughs, A. M., Balaji, S., Iyer, L. M., and Aravind, L. (2007) Small but versatile: the extraordinary functional and structural diversity of the beta-grasp fold, *Biology direct* 2, 18-18.

[76] Overington, J. P. (1992) Comparison of three-dimensional structures of homologous proteins, *Current Opinion in Structural Biology* 2, 394-401.

[77] Streich, F. C., Jr., and Lima, C. D. (2014) Structural and functional insights to ubiquitin-like protein conjugation, *Annual review of biophysics* 43, 357-379.

[78] Hilgarth, R. S., Murphy, L. A., Skaggs, H. S., Wilkerson, D. C., Xing, H., and Sarge, K. D. (2004) Regulation and function of SUMO modification, *The Journal of biological chemistry* 279, 53899-53902.

[79] Kamitani, T., Kito, K., Nguyen, H. P., and Yeh, E. T. (1997) Characterization of NEDD8, a developmentally down-regulated ubiquitin-like protein, *The Journal of biological chemistry* 272, 28557-28562.

[80] Soucy, T. A., Dick, L. R., Smith, P. G., Milhollen, M. A., and Brownell, J. E. (2010) The NEDD8 Conjugation Pathway and Its Relevance in Cancer Biology and Therapy, *Genes & cancer* 1, 708-716.

[81] Whitby, F. G., Xia, G., Pickart, C. M., and Hill, C. P. (1998) Crystal structure of the human ubiquitin-like protein NEDD8 and interactions with ubiquitin pathway enzymes, *The Journal of biological chemistry* 273, 34983-34991.

[82] Liakopoulos, D., Doenges, G., Matuschewski, K., and Jentsch, S. (1998) A novel protein modification pathway related to the ubiquitin system, *EMBO J* 17, 2208-2214.

[83] Osaka, F., Kawasaki, H., Aida, N., Saeki, M., Chiba, T., Kawashima, S., Tanaka, K., and Kato, S. (1998) A new NEDD8-ligating system for cullin-4A, *Genes & development* 12, 2263-2268.

[84] Mendoza, H. M., Shen, L. N., Botting, C., Lewis, A., Chen, J., Ink, B., and Hay, R. T. (2003) NEDP1, a highly conserved cysteine protease that deNEDDylates Cullins, *The Journal of biological chemistry* 278, 25637-25643.

[85] Shen, L.-n., Liu, H., Dong, C., Xirodimas, D., Naismith, J. H., and Hay, R. T. (2005) Structural basis of NEDD8 ubiquitin discrimination by the deNEDDylating enzyme NEDP1, *The EMBO journal* 24, 1341-1351.

- [86] Nijman, S. M., Luna-Vargas, M. P., Velds, A., Brummelkamp, T. R., Dirac, A. M., Sixma, T. K., and Bernards, R. (2005) A genomic and functional inventory of deubiquitinating enzymes, *Cell* 123, 773-786.
- [87] D'Arcy, P., Brnjic, S., Olofsson, M. H., Fryknas, M., Lindsten, K., De Cesare, M., Perego, P., Sadeghi, B., Hassan, M., Larsson, R., and Linder, S. (2011) Inhibition of proteasome deubiquitinating activity as a new cancer therapy, *Nat Med* 17, 1636-1640.
- [88] Ye, Y., Scheel, H., Hofmann, K., and Komander, D. (2009) Dissection of USP catalytic domains reveals five common insertion points, *Mol Biosyst* 5, 1797-1808.
- [89] Cstorer, A., and Ménard, R. (1994) [33] Catalytic mechanism in papain family of cysteine peptidases, In *Methods in Enzymology*, pp 486-500, Academic Press.
- [90] Reyes-Turcu, F. E., Shanks, J. R., Komander, D., and Wilkinson, K. D. (2008) Recognition of polyubiquitin isoforms by the multiple ubiquitin binding modules of isopeptidase T, *The Journal of biological chemistry* 283, 19581-19592.
- [91] Ye, Y., Akutsu, M., Reyes-Turcu, F., Enchev, R. I., Wilkinson, K. D., and Komander, D. (2011) Polyubiquitin binding and cross-reactivity in the USP domain deubiquitinase USP21, *EMBO Rep* 12, 350-357.
- [92] Kim, R. Q., van Dijk, W. J., and Sixma, T. K. (2016) Structure of USP7 catalytic domain and three Ubl-domains reveals a connector alpha-helix with regulatory role, *J Struct Biol* 195, 11-18.
- [93] Hu, M., Li, P., Song, L., Jeffrey, P. D., Chenova, T. A., Wilkinson, K. D., Cohen, R. E., and Shi, Y. (2005) Structure and mechanisms of the proteasome-associated deubiquitinating enzyme USP14, *EMBO J* 24, 3747-3756.
- [94] Dharadhar, S., Clerici, M., van Dijk, W. J., Fish, A., and Sixma, T. K. (2016) A conserved two-step binding for the UAF1 regulator to the USP12 deubiquitinating enzyme, *J Struct Biol* 196, 437-447.
- [95] Basters, A., Geurink, P. P., Rocker, A., Witting, K. F., Tadayon, R., Hess, S., Semrau, M. S., Storici, P., Ovaa, H., Knobeloch, K. P., and Fritz, G. (2017) Structural basis of the specificity of USP18 toward ISG15, *Nat Struct Mol Biol* 24, 270-278.
- [96] Faesen, A. C., Dirac, A. M., Shanmugham, A., Ovaa, H., Perrakis, A., and Sixma, T. K. (2011) Mechanism of USP7/HAUSP activation by its C-terminal ubiquitin-like domain and allosteric regulation by GMP-synthetase, *Mol Cell* 44, 147-159.
- [97] Renatus, M., Parrado, S. G., D'Arcy, A., Eidhoff, U., Gerhartz, B., Hassiepen, U., Pierrat, B., Riedl, R., Vinzenz, D., Worpenberg, S., and Kroemer, M. (2006) Structural basis of ubiquitin recognition by the deubiquitinating protease USP2, *Structure* 14, 1293-1302.
- [98] Fernandez-Montalvan, A., Bouwmeester, T., Joberty, G., Mader, R., Mahnke, M., Pierrat, B., Schlaeppi, J. M., Worpenberg, S., and Gerhartz, B. (2007) Biochemical characterization of USP7 reveals post-translational modification sites and structural requirements for substrate processing and subcellular localization, *FEBS J* 274, 4256-4270.

- [99] Stein, R. L., Chen, Z., and Melandri, F. (1995) Kinetic studies of isopeptidase T: modulation of peptidase activity by ubiquitin, *Biochemistry* 34, 12616-12623.
- [100] García-Santisteban, I., Peters, G. J., Giovannetti, E., and Rodríguez, J. A. (2013) USP1 deubiquitinase: cellular functions, regulatory mechanisms and emerging potential as target in cancer therapy, *Molecular Cancer* 12, 91.
- [101] Cunningham, C. N., Baughman, J. M., Phu, L., Tea, J. S., Yu, C., Coons, M., Kirkpatrick, D. S., Bingol, B., and Corn, J. E. (2015) USP30 and parkin homeostatically regulate atypical ubiquitin chains on mitochondria, *Nat Cell Biol* 17, 160-169.
- [102] Nakamura, N., and Hirose, S. (2008) Regulation of mitochondrial morphology by USP30, a deubiquitinating enzyme present in the mitochondrial outer membrane, *Mol Biol Cell* 19, 1903-1911.
- [103] Lee, M. J., Lee, B. H., Hanna, J., King, R. W., and Finley, D. (2011) Trimming of ubiquitin chains by proteasome-associated deubiquitinating enzymes, *Mol Cell Proteomics* 10, R110 003871.
- [104] Long, L., Thelen, J. P., Furgason, M., Haj-Yahya, M., Brik, A., Cheng, D., Peng, J., and Yao, T. (2014) The U4/U6 recycling factor SART3 has histone chaperone activity and associates with USP15 to regulate H2B deubiquitination, *The Journal of biological chemistry* 289, 8916-8930.
- [105] Park, J. K., Das, T., Song, E. J., and Kim, E. E. (2016) Structural basis for recruiting and shuttling of the spliceosomal deubiquitinase USP4 by SART3, *Nucleic Acids Res* 44, 5424-5437.
- [106] Zhang, Q., Harding, R., Hou, F., Dong, A., Walker, J. R., Bteich, J., and Tong, Y. (2016) Structural Basis of the Recruitment of Ubiquitin-specific Protease USP15 by Spliceosome Recycling Factor SART3, *The Journal of biological chemistry* 291, 17283-17292.
- [107] Row, P. E., Prior, I. A., McCullough, J., Clague, M. J., and Urbe, S. (2006) The ubiquitin isopeptidase UBPY regulates endosomal ubiquitin dynamics and is essential for receptor down-regulation, *The Journal of biological chemistry* 281, 12618-12624.
- [108] Yuan, J., Luo, K., Zhang, L., Cheville, J. C., and Lou, Z. (2010) USP10 regulates p53 localization and stability by deubiquitinating p53, *Cell* 140, 384-396.
- [109] Zhang, L., Zhou, F., Drabsch, Y., Gao, R., Snaar-Jagalska, B. E., Mickanin, C., Huang, H., Sheppard, K. A., Porter, J. A., Lu, C. X., and ten Dijke, P. (2012) USP4 is regulated by AKT phosphorylation and directly deubiquitylates TGF-beta type I receptor, *Nat Cell Biol* 14, 717-726.
- [110] Mizuno, E., Kitamura, N., and Komada, M. (2007) 14-3-3-dependent inhibition of the deubiquitinating activity of UBPY and its cancellation in the M phase, *Exp Cell Res* 313, 3624-3634.
- [111] Reiley, W., Zhang, M., Wu, X., Granger, E., and Sun, S. C. (2005) Regulation of the deubiquitinating enzyme CYLD by I κ B kinase gamma-dependent phosphorylation, *Mol Cell Biol* 25, 3886-3895.
- [112] Huang, X., Summers, M. K., Pham, V., Lill, J. R., Liu, J., Lee, G., Kirkpatrick, D. S., Jackson, P. K., Fang, G., and Dixit, V. M. (2011) Deubiquitinase USP37 is activated by CDK2 to antagonize APC(CDH1) and promote S phase entry, *Mol Cell* 42, 511-523.

- [113] Hutti, J. E., Turk, B. E., Asara, J. M., Ma, A., Cantley, L. C., and Abbott, D. W. (2007) IkkappaB kinase beta phosphorylates the K63 deubiquitinase A20 to cause feedback inhibition of the NF-kappaB pathway, *Mol Cell Biol* 27, 7451-7461.
- [114] Khoronenkova, S. V., Dianova, II, Ternette, N., Kessler, B. M., Parsons, J. L., and Dianov, G. L. (2012) ATM-dependent downregulation of USP7/HAUSP by PPM1G activates p53 response to DNA damage, *Mol Cell* 45, 801-813.
- [115] Villamil, M. A., Liang, Q., Chen, J., Choi, Y. S., Hou, S., Lee, K. H., and Zhuang, Z. (2012) Serine phosphorylation is critical for the activation of ubiquitin-specific protease 1 and its interaction with WD40-repeat protein UAF1, *Biochemistry* 51, 9112-9123.
- [116] Meulmeester, E., Kunze, M., Hsiao, H. H., Urlaub, H., and Melchior, F. (2008) Mechanism and consequences for paralog-specific sumoylation of ubiquitin-specific protease 25, *Mol Cell* 30, 610-619.
- [117] Zhen, Y., Knobel, P. A., Stracker, T. H., and Reverter, D. (2014) Regulation of USP28 deubiquitinating activity by SUMO conjugation, *The Journal of biological chemistry* 289, 34838-34850.
- [118] Bingol, B., Tea, J. S., Phu, L., Reichelt, M., Bakalarski, C. E., Song, Q., Foreman, O., Kirkpatrick, D. S., and Sheng, M. (2014) The mitochondrial deubiquitinase USP30 opposes parkin-mediated mitophagy, *Nature* 510, 370-375.
- [119] Huang, T. T., Nijman, S. M., Mirchandani, K. D., Galardy, P. J., Cohn, M. A., Haas, W., Gygi, S. P., Ploegh, H. L., Bernards, R., and D'Andrea, A. D. (2006) Regulation of monoubiquitinated PCNA by DUB autocleavage, *Nat Cell Biol* 8, 339-347.
- [120] Staal, J., Driege, Y., Bekaert, T., Demeyer, A., Muylleert, D., Van Damme, P., Gevaert, K., and Beyaert, R. (2011) T-cell receptor-induced JNK activation requires proteolytic inactivation of CYLD by MALT1, *EMBO J* 30, 1742-1752.
- [121] Koyano, F., Okatsu, K., Kosako, H., Tamura, Y., Go, E., Kimura, M., Kimura, Y., Tsuchiya, H., Yoshihara, H., Hirokawa, T., Endo, T., Fon, E. A., Trempe, J. F., Saeki, Y., Tanaka, K., and Matsuda, N. (2014) Ubiquitin is phosphorylated by PINK1 to activate parkin, *Nature* 510, 162-166.
- [122] Kane, L. A., Lazarou, M., Fogel, A. I., Li, Y., Yamano, K., Sarraf, S. A., Banerjee, S., and Youle, R. J. (2014) PINK1 phosphorylates ubiquitin to activate Parkin E3 ubiquitin ligase activity, *J Cell Biol* 205, 143-153.
- [123] Kazlauskaitė, A., Kondapalli, C., Gurlay, R., Campbell, D. G., Ritorto, M. S., Hofmann, K., Alessi, D. R., Knebel, A., Trost, M., and Muqit, M. M. (2014) Parkin is activated by PINK1-dependent phosphorylation of ubiquitin at Ser65, *Biochem J* 460, 127-139.
- [124] Wauer, T., Swatek, K. N., Wagstaff, J. L., Gladkova, C., Pruneda, J. N., Michel, M. A., Gersch, M., Johnson, C. M., Freund, S. M., and Komander, D. (2015) Ubiquitin Ser65 phosphorylation affects ubiquitin structure, chain assembly and hydrolysis, *EMBO J* 34, 307-325.
- [125] Butterfield, D. A., and Boyd-Kimball, D. (2004) Amyloid beta-peptide(1-42) contributes to the oxidative stress and neurodegeneration found in Alzheimer disease brain, *Brain Pathol* 14, 426-432.

- [126] Benhar, M., Engelberg, D., and Levitzki, A. (2002) ROS, stress-activated kinases and stress signaling in cancer, *EMBO Rep* 3, 420-425.
- [127] Hekimi, S., Lapointe, J., and Wen, Y. (2011) Taking a "good" look at free radicals in the aging process, *Trends Cell Biol* 21, 569-576.
- [128] Finkel, T. (2003) Oxidant signals and oxidative stress, *Curr Opin Cell Biol* 15, 247-254.
- [129] Lee, J.-G., Baek, K., Soetandyo, N., and Ye, Y. (2013) Reversible inactivation of deubiquitinases by reactive oxygen species in vitro and in cells, *Nature Communications* 4, 1568.
- [130] Kulathu, Y., Garcia, F. J., Mevissen, T. E., Busch, M., Arnaudo, N., Carroll, K. S., Barford, D., and Komander, D. (2013) Regulation of A20 and other OTU deubiquitinases by reversible oxidation, *Nat Commun* 4, 1569.
- [131] Gersch, M., Gladkova, C., Schubert, A. F., Michel, M. A., Maslen, S., and Komander, D. (2017) Mechanism and regulation of the Lys6-selective deubiquitinase USP30, *Nat Struct Mol Biol* 24, 920-930.
- [132] Komander, D., Lord, C. J., Scheel, H., Swift, S., Hofmann, K., Ashworth, A., and Barford, D. (2008) The structure of the CYLD USP domain explains its specificity for Lys63-linked polyubiquitin and reveals a B box module, *Mol Cell* 29, 451-464.
- [133] Sato, Y., Goto, E., Shibata, Y., Kubota, Y., Yamagata, A., Goto-Ito, S., Kubota, K., Inoue, J., Takekawa, M., Tokunaga, F., and Fukai, S. (2015) Structures of CYLD USP with Met1- or Lys63-linked diubiquitin reveal mechanisms for dual specificity, *Nat Struct Mol Biol* 22, 222-229.
- [134] Ritorto, M. S., Ewan, R., Perez-Oliva, A. B., Knebel, A., Buhrlage, S. J., Wightman, M., Kelly, S. M., Wood, N. T., Virdee, S., Gray, N. S., Morrice, N. A., Alessi, D. R., and Trost, M. (2014) Screening of DUB activity and specificity by MALDI-TOF mass spectrometry, *Nat Commun* 5, 4763.
- [135] Hospenthal, M. K., Freund, S. M. V., and Komander, D. (2013) Assembly, analysis and architecture of atypical ubiquitin chains, *Nature Structural & Molecular Biology* 20, 555.
- [136] Reyes-Turcu, F. E., Horton, J. R., Mullally, J. E., Heroux, A., Cheng, X., and Wilkinson, K. D. (2006) The ubiquitin binding domain ZnF UBP recognizes the C-terminal diglycine motif of unanchored ubiquitin, *Cell* 124, 1197-1208.
- [137] Piccinini, M., Merighi, A., Bruno, R., Cascio, P., Curto, M., Mioletti, S., Ceruti, C., and Rinaudo, M. T. (1996) Affinity purification and characterization of protein gene product 9.5 (PGP9.5) from retina, *Biochemical Journal* 318, 711-716.
- [138] Dang, L. C., Melandri, F. D., and Stein, R. L. (1998) Kinetic and mechanistic studies on the hydrolysis of ubiquitin C-terminal 7-amido-4-methylcoumarin by deubiquitinating enzymes, *Biochemistry* 37, 1868-1879.
- [139] Pickart, C. M., and Rose, I. A. (1986) Mechanism of ubiquitin carboxyl-terminal hydrolase. Borohydride and hydroxylamine inactivate in the presence of ubiquitin, *The Journal of biological chemistry* 261, 10210-10217.
- [140] Borodovsky, A., Ovaa, H., Kolli, N., Gan-Erdene, T., Wilkinson, K. D., Ploegh, H. L., and Kessler, B. M. (2002) Chemistry-based functional

proteomics reveals novel members of the deubiquitinating enzyme family, *Chem Biol* 9, 1149-1159.

[141] Borodovsky, A., Kessler, B. M., Casagrande, R., Overkleeft, H. S., Wilkinson, K. D., and Ploegh, H. L. (2001) A novel active site-directed probe specific for deubiquitylating enzymes reveals proteasome association of USP14, *The EMBO Journal* 20, 5187-5196.

[142] Ekkebus, R., van Kasteren, S. I., Kulathu, Y., Scholten, A., Berlin, I., Geurink, P. P., de Jong, A., Goerdal, S., Neefjes, J., Heck, A. J. R., Komander, D., and Ovaa, H. (2013) On Terminal Alkynes That Can React with Active-Site Cysteine Nucleophiles in Proteases, *Journal of the American Chemical Society* 135, 2867-2870.

[143] Li, G., Liang, Q., Gong, P., Tencer, A. H., and Zhuang, Z. (2014) Activity-based diubiquitin probes for elucidating the linkage specificity of deubiquitinating enzymes, *Chem Commun (Camb)* 50, 216-218.

[144] McGouran, J. F., Gaertner, S. R., Altun, M., Kramer, H. B., and Kessler, B. M. (2013) Deubiquitinating enzyme specificity for ubiquitin chain topology profiled by di-ubiquitin activity probes, *Chem Biol* 20, 1447-1455.

[145] Haj-Yahya, N., Hemantha, H. P., Meledin, R., Bondalapati, S., Seenaiiah, M., and Brik, A. (2014) Dehydroalanine-Based Diubiquitin Activity Probes, *Organic Letters* 16, 540-543.

[146] Flierman, D., van der Heden van Noort, Gerbrand J., Ekkebus, R., Geurink, Paul P., Mevissen, Tycho E., Hospenthal, Manuela K., Komander, D., and Ovaa, H. (2016) Non-hydrolyzable Diubiquitin Probes Reveal Linkage-Specific Reactivity of Deubiquitylating Enzymes Mediated by S2 Pockets, *Cell Chemical Biology* 23, 472-482.

[147] van Tilburg, G. B., Elhebieshy, A. F., and Ovaa, H. (2016) Synthetic and semi-synthetic strategies to study ubiquitin signaling, *Curr Opin Struct Biol* 38, 92-101.

[148] Valkevich, E. M., Guenette, R. G., Sanchez, N. A., Chen, Y. C., Ge, Y., and Strieter, E. R. (2012) Forging isopeptide bonds using thiol-ene chemistry: site-specific coupling of ubiquitin molecules for studying the activity of isopeptidases, *J Am Chem Soc* 134, 6916-6919.

[149] Virdee, S., Kapadnis, P. B., Elliott, T., Lang, K., Madrzak, J., Nguyen, D. P., Riechmann, L., and Chin, J. W. (2011) Traceless and site-specific ubiquitination of recombinant proteins, *J Am Chem Soc* 133, 10708-10711.

[150] Vlasschaert, C., Xia, X., Coulombe, J., and Gray, D. A. (2015) Evolution of the highly networked deubiquitinating enzymes USP4, USP15, and USP11, *BMC Evolutionary Biology* 15, 230.

[151] Elliott, P. R., Liu, H., Pastok, M. W., Grossmann, G. J., Rigden, D. J., Clague, M. J., Urbe, S., and Barsukov, I. L. (2011) Structural variability of the ubiquitin specific protease DUSP-UBL double domains, *FEBS Lett* 585, 3385-3390.

[152] Eichhorn, P. J., Rodon, L., Gonzalez-Junca, A., Dirac, A., Gili, M., Martinez-Saez, E., Aura, C., Barba, I., Peg, V., Prat, A., Cuartas, I., Jimenez, J., Garcia-Dorado, D., Sahuquillo, J., Bernards, R., Baselga, J., and Seoane, J. (2012) USP15 stabilizes TGF-beta receptor I and promotes oncogenesis through the activation of TGF-beta signaling in glioblastoma, *Nat Med* 18, 429-435.

- [153] Inui, M., Manfrin, A., Mamidi, A., Martello, G., Morsut, L., Soligo, S., Enzo, E., Moro, S., Polo, S., Dupont, S., Cordenonsi, M., and Piccolo, S. (2011) USP15 is a deubiquitylating enzyme for receptor-activated SMADs, *Nat Cell Biol* 13, 1368-1375.
- [154] Al-Salihi, M. A., Herhaus, L., Macartney, T., and Sapkota, G. P. (2012) USP11 augments TGFbeta signalling by deubiquitylating ALK5, *Open Biol* 2, 120063.
- [155] Das, T., Park, J. K., Park, J., Kim, E., Rape, M., Kim, E. E., and Song, E. J. (2017) USP15 regulates dynamic protein-protein interactions of the spliceosome through deubiquitination of PRP31, *Nucleic Acids Res* 45, 4866-4880.
- [156] Song, E. J., Werner, S. L., Neubauer, J., Stegmeier, F., Aspden, J., Rio, D., Harper, J. W., Elledge, S. J., Kirschner, M. W., and Rape, M. (2010) The Prp19 complex and the Usp4Sart3 deubiquitinating enzyme control reversible ubiquitination at the spliceosome, *Genes Dev* 24, 1434-1447.
- [157] Chiang, C., Pauli, E. K., Biryukov, J., Feister, K. F., Meng, M., White, E. A., Munger, K., Howley, P. M., Meyers, C., and Gack, M. U. (2018) The Human Papillomavirus E6 Oncoprotein Targets USP15 and TRIM25 To Suppress RIG-I-Mediated Innate Immune Signaling, *J Virol* 92.
- [158] Wang, L., Zhao, W., Zhang, M., Wang, P., Zhao, K., Zhao, X., Yang, S., and Gao, C. (2013) USP4 Positively Regulates RIG-I-Mediated Antiviral Response through Deubiquitination and Stabilization of RIG-I, *Journal of Virology* 87, 4507-4515.
- [159] Harper, S., Besong, T. M., Emsley, J., Scott, D. J., and Dreveny, I. (2011) Structure of the USP15 N-terminal domains: a beta-hairpin mediates close association between the DUSP and UBL domains, *Biochemistry* 50, 7995-8004.
- [160] Pal, A., Young, M. A., and Donato, N. J. (2014) Emerging potential of therapeutic targeting of ubiquitin-specific proteases in the treatment of cancer, *Cancer Res* 74, 4955-4966.
- [161] Xu, K., Pei, H., Zhang, Z., Wang, H., Li, L., and Xia, Q. (2018) Ubiquitin-specific protease 15 promotes tumor cell invasion and proliferation in glioblastoma, *Oncol Lett* 15, 3846-3851.
- [162] Bruna, A., Darken, R. S., Rojo, F., Ocana, A., Penuelas, S., Arias, A., Paris, R., Tortosa, A., Mora, J., Baselga, J., and Seoane, J. (2007) High TGFbeta-Smad activity confers poor prognosis in glioma patients and promotes cell proliferation depending on the methylation of the PDGF-B gene, *Cancer Cell* 11, 147-160.
- [163] Rich, J. N. (2003) The role of transforming growth factor-beta in primary brain tumors, *Front Biosci* 8, e245-260.
- [164] Massagué, J. (2012) TGFβ signalling in context, *Nature Reviews Molecular Cell Biology* 13, 616.
- [165] Wicks, S. J., Grocott, T., Haros, K., Maillard, M., ten Dijke, P., and Chantry, A. (2006) Reversible ubiquitination regulates the Smad/TGF-beta signalling pathway, *Biochem Soc Trans* 34, 761-763.
- [166] Ogunjimi, A. A., Briant, D. J., Pece-Barbara, N., Le Roy, C., Di Guglielmo, G. M., Kavsak, P., Rasmussen, R. K., Seet, B. T., Sicheri, F., and Wrana, J. L. (2005) Regulation of Smurf2 ubiquitin ligase activity by anchoring the E2 to the HECT domain, *Mol Cell* 19, 297-308.

- [167] Liu, W. T., Huang, K. Y., Lu, M. C., Huang, H. L., Chen, C. Y., Cheng, Y. L., Yu, H. C., Liu, S. Q., Lai, N. S., and Huang, H. B. (2017) TGF-beta upregulates the translation of USP15 via the PI3K/AKT pathway to promote p53 stability, *Oncogene* 36, 2715-2723.
- [168] Liu, W. T., Huang, K. Y., Lu, M. C., Huang, H. L., Chen, C. Y., Cheng, Y. L., Yu, H. C., Liu, S. Q., Lai, N. S., and Huang, H. B. (2017) TGF- β upregulates the translation of USP15 via the PI3K/AKT pathway to promote p53 stability, *Oncogene* 36, 2715-2723.
- [169] Harris, S. L., and Levine, A. J. (2005) The p53 pathway: positive and negative feedback loops, *Oncogene* 24, 2899.
- [170] Padmanabhan, A., Candelaria, N., Wong, K. K., Nikolai, B. C., Lonard, D. M., O'Malley, B. W., and Richards, J. S. (2018) USP15-dependent lysosomal pathway controls p53-R175H turnover in ovarian cancer cells, *Nat Commun* 9, 1270.
- [171] Momand, J., Zambetti, G. P., Olson, D. C., George, D., and Levine, A. J. (1992) The mdm-2 oncogene product forms a complex with the p53 protein and inhibits p53-mediated transactivation, *Cell* 69, 1237-1245.
- [172] Wade, M., Li, Y.-C., and Wahl, G. M. (2013) MDM2, MDMX and p53 in oncogenesis and cancer therapy, *Nature Reviews Cancer* 13, 83.
- [173] Li, M., Chen, D., Shiloh, A., Luo, J., Nikolaev, A. Y., Qin, J., and Gu, W. (2002) Deubiquitination of p53 by HAUSP is an important pathway for p53 stabilization, *Nature* 416, 648-653.
- [174] Kon, N., Kobayashi, Y., Li, M., Brooks, C. L., Ludwig, T., and Gu, W. (2010) Inactivation of HAUSP in vivo modulates p53 function, *Oncogene* 29, 1270-1279.
- [175] Kim, J., Keay, S. K., You, S., Loda, M., and Freeman, M. R. (2012) A synthetic form of frizzled 8-associated antiproliferative factor enhances p53 stability through USP2a and MDM2, *PloS one* 7, e50392.
- [176] Zou, Q., Jin, J., Hu, H., Li, H. S., Romano, S., Xiao, Y., Nakaya, M., Zhou, X., Cheng, X., Yang, P., Lozano, G., Zhu, C., Watowich, S. S., Ullrich, S. E., and Sun, S. C. (2014) USP15 stabilizes MDM2 to mediate cancer-cell survival and inhibit antitumor T cell responses, *Nat Immunol* 15, 562-570.
- [177] Lee, M. H., Zhao, R., Phan, L., and Yeung, S. C. (2011) Roles of COP9 signalosome in cancer, *Cell Cycle* 10, 3057-3066.
- [178] Wei, N., Serino, G., and Deng, X. W. (2008) The COP9 signalosome: more than a protease, *Trends Biochem Sci* 33, 592-600.
- [179] Cayli, S., Klug, J., Chapiro, J., Frohlich, S., Krasteva, G., Orel, L., and Meinhardt, A. (2009) COP9 signalosome interacts ATP-dependently with p97/valosin-containing protein (VCP) and controls the ubiquitination status of proteins bound to p97/VCP, *The Journal of biological chemistry* 284, 34944-34953.
- [180] Hetfeld, B. K., Helfrich, A., Kapelari, B., Scheel, H., Hofmann, K., Guterman, A., Glickman, M., Schade, R., Kloetzel, P. M., and Dubiel, W. (2005) The zinc finger of the CSN-associated deubiquitinating enzyme USP15 is essential to rescue the E3 ligase Rbx1, *Curr Biol* 15, 1217-1221.
- [181] Schweitzer, K., Bozko, P. M., Dubiel, W., and Naumann, M. (2007) CSN controls NF-kappaB by deubiquitylation of IkappaBalpha, *EMBO J* 26, 1532-1541.

- [182] zur Hausen, H. (2009) Papillomaviruses in the causation of human cancers - a brief historical account, *Virology* 384, 260-265.
- [183] Jones, D. L., Thompson, D. A., and Munger, K. (1997) Destabilization of the RB tumor suppressor protein and stabilization of p53 contribute to HPV type 16 E7-induced apoptosis, *Virology* 239, 97-107.
- [184] Park, R. B., and Androphy, E. J. (2002) Genetic Analysis of High-Risk E6 in Episomal Maintenance of Human Papillomavirus Genomes in Primary Human Keratinocytes, *Journal of Virology* 76, 11359-11364.
- [185] Huibregtse, J. M., Scheffner, M., and Howley, P. M. (1993) Cloning and expression of the cDNA for E6-AP, a protein that mediates the interaction of the human papillomavirus E6 oncoprotein with p53, *Molecular and Cellular Biology* 13, 775-784.
- [186] Scheffner, M., Huibregtse, J. M., Vierstra, R. D., and Howley, P. M. (1993) The HPV-16 E6 and E6-AP complex functions as a ubiquitin-protein ligase in the ubiquitination of p53, *Cell* 75, 495-505.
- [187] Thomas, M., Pim, D., and Banks, L. (1999) The role of the E6-p53 interaction in the molecular pathogenesis of HPV, *Oncogene* 18, 7690-7700.
- [188] Vos, R. M., Altreuter, J., White, E. A., and Howley, P. M. (2009) The ubiquitin-specific peptidase USP15 regulates human papillomavirus type 16 E6 protein stability, *J Virol* 83, 8885-8892.
- [189] Pauli, E. K., Chan, Y. K., Davis, M. E., Gableske, S., Wang, M. K., Feister, K. F., and Gack, M. U. (2014) The ubiquitin-specific protease USP15 promotes RIG-I-mediated antiviral signaling by deubiquitylating TRIM25, *Sci Signal* 7, ra3.
- [190] Xu, M., Takanashi, M., Oikawa, K., Tanaka, M., Nishi, H., Isaka, K., Kudo, M., and Kuroda, M. (2009) USP15 plays an essential role for caspase-3 activation during Paclitaxel-induced apoptosis, *Biochem Biophys Res Commun* 388, 366-371.
- [191] Shimura, H., Hattori, N., Kubo, S., Mizuno, Y., Asakawa, S., Minoshima, S., Shimizu, N., Iwai, K., Chiba, T., Tanaka, K., and Suzuki, T. (2000) Familial Parkinson disease gene product, parkin, is a ubiquitin-protein ligase, *Nat Genet* 25, 302-305.
- [192] Kitada, T., Asakawa, S., Hattori, N., Matsumine, H., Yamamura, Y., Minoshima, S., Yokochi, M., Mizuno, Y., and Shimizu, N. (1998) Mutations in the parkin gene cause autosomal recessive juvenile parkinsonism, *Nature* 392, 605-608.
- [193] Cornelissen, T., Haddad, D., Wauters, F., Van Humbeeck, C., Mandemakers, W., Koentjoro, B., Sue, C., Gevaert, K., De Strooper, B., Verstreken, P., and Vandenberghe, W. (2014) The deubiquitinase USP15 antagonizes Parkin-mediated mitochondrial ubiquitination and mitophagy, *Hum Mol Genet* 23, 5227-5242.
- [194] Veeriah, S., Taylor, B. S., Meng, S., Fang, F., Yilmaz, E., Vivanco, I., Janakiraman, M., Schultz, N., Hanrahan, A. J., Pao, W., Ladanyi, M., Sander, C., Heguy, A., Holland, E. C., Paty, P. B., Mischel, P. S., Liau, L., Cloughesy, T. F., Mellingshoff, I. K., Solit, D. B., and Chan, T. A. (2010) Somatic mutations of the Parkinson's disease-associated gene PARK2 in glioblastoma and other human malignancies, *Nat Genet* 42, 77-82.

- [195] Poulogiannis, G., McIntyre, R. E., Dimitriadi, M., Apps, J. R., Wilson, C. H., Ichimura, K., Luo, F., Cantley, L. C., Wyllie, A. H., Adams, D. J., and Arends, M. J. (2010) *PARK2* deletions occur frequently in sporadic colorectal cancer and accelerate adenoma development in *Apc* mutant mice, *Proceedings of the National Academy of Sciences* 107, 15145-15150.
- [196] Tian, X., Isamiddinova, N. S., Peroutka, R. J., Goldenberg, S. J., Mattern, M. R., Nicholson, B., and Leach, C. (2011) Characterization of Selective Ubiquitin and Ubiquitin-Like Protease Inhibitors Using a Fluorescence-Based Multiplex Assay Format, *Assay and Drug Development Technologies* 9, 165-173.
- [197] Reverdy, C., Conrath, S., Lopez, R., Planquette, C., Atmanene, C., Collura, V., Harpon, J., Battaglia, V., Vivat, V., Sippl, W., and Colland, F. (2012) Discovery of specific inhibitors of human USP7/HAUSP deubiquitinating enzyme, *Chem Biol* 19, 467-477.
- [198] Tian, Z., D'Arcy, P., Wang, X., Ray, A., Tai, Y. T., Hu, Y., Carrasco, R. D., Richardson, P., Linder, S., Chauhan, D., and Anderson, K. C. (2014) A novel small molecule inhibitor of deubiquitylating enzyme USP14 and UCHL5 induces apoptosis in multiple myeloma and overcomes bortezomib resistance, *Blood* 123, 706-716.
- [199] Chauhan, D., Tian, Z., Nicholson, B., Kumar, K. G., Zhou, B., Carrasco, R., McDermott, J. L., Leach, C. A., Fulciniti, M., Kodrasov, M. P., Weinstock, J., Kingsbury, W. D., Hideshima, T., Shah, P. K., Minvielle, S., Altun, M., Kessler, B. M., Orlowski, R., Richardson, P., Munshi, N., and Anderson, K. C. (2012) A small molecule inhibitor of ubiquitin-specific protease-7 induces apoptosis in multiple myeloma cells and overcomes bortezomib resistance, *Cancer Cell* 22, 345-358.
- [200] Gavory, G., O'Dowd, C. R., Helm, M. D., Flasz, J., Arkoudis, E., Dossang, A., Hughes, C., Cassidy, E., McClelland, K., Odrzywol, E., Page, N., Barker, O., Miel, H., and Harrison, T. (2018) Discovery and characterization of highly potent and selective allosteric USP7 inhibitors, *Nat Chem Biol* 14, 118-125.
- [201] Kluge, A. F., Lagu, B. R., Maiti, P., Jaleel, M., Webb, M., Malhotra, J., Mallat, A., Srinivas, P. A., and Thompson, J. E. (2018) Novel highly selective inhibitors of ubiquitin specific protease 30 (USP30) accelerate mitophagy, *Bioorg Med Chem Lett* 28, 2655-2659.
- [202] Zhang, W., Sulea, T., Tao, L., Cui, Q., Purisima, E. O., Vongsamphanh, R., Lachance, P., Lytvyn, V., Qi, H., Li, Y., and Ménard, R. (2011) Contribution of Active Site Residues to Substrate Hydrolysis by USP2: Insights into Catalysis by Ubiquitin Specific Proteases, *Biochemistry* 50, 4775-4785.
- [203] Burkhart, R. A., Peng, Y., Norris, Z. A., Tholey, R. M., Talbott, V. A., Liang, Q., Ai, Y., Miller, K., Lal, S., Cozzitorto, J. A., Witkiewicz, A. K., Yeo, C. J., Gehrmann, M., Napper, A., Winter, J. M., Sawicki, J. A., Zhuang, Z., and Brody, J. R. (2013) Mitoxantrone targets human ubiquitin-specific peptidase 11 (USP11) and is a potent inhibitor of pancreatic cancer cell survival, *Mol Cancer Res* 11, 901-911.
- [204] FDA. Mitoxantrone Available: www.fda.gov.

- [205] Bellosillo, B., Colomer, D., Pons, G., and Gil, J. (1998) Mitoxantrone, a topoisomerase II inhibitor, induces apoptosis of B-chronic lymphocytic leukaemia cells, *Br J Haematol* 100, 142-146.
- [206] Fox, E. J. (2004) Mechanism of action of mitoxantrone, *Neurology* 63, S15-18.
- [207] Huff, A. C., and Kreuzer, K. N. (1990) Evidence for a common mechanism of action for antitumor and antibacterial agents that inhibit type II DNA topoisomerases, *The Journal of biological chemistry* 265, 20496-20505.
- [208] Gratton, H. (2014) Investigating the structure and function of ubiquitin specific protease 11, a deubiquitinating enzyme implicated in cancer and DNA damage repair.
- [209] Rasulan, P. I. (2017) Characterisation of the Deubiquitinating enzyme USP4.
- [210] Vonrhein, C., Flensburg, C., Keller, P., Sharff, A., Smart, O., Paciorek, W., Womack, T., and Bricogne, G. (2011) Data processing and analysis with the autoPROC toolbox, *Acta crystallographica. Section D, Biological crystallography* 67, 293-302.
- [211] Powell, H. R., Johnson, O., and Leslie, A. G. (2013) Autoindexing diffraction images with iMosflm, *Acta crystallographica. Section D, Biological crystallography* 69, 1195-1203.
- [212] Winn, M. D., Ballard, C. C., Cowtan, K. D., Dodson, E. J., Emsley, P., Evans, P. R., Keegan, R. M., Krissinel, E. B., Leslie, A. G. W., McCoy, A., McNicholas, S. J., Murshudov, G. N., Pannu, N. S., Potterton, E. A., Powell, H. R., Read, R. J., Vagin, A., and Wilson, K. S. (2011) Overview of the CCP4 suite and current developments, *Acta Crystallographica Section D: Biological Crystallography* 67, 235-242.
- [213] McCoy, A. J., Grosse-Kunstleve, R. W., Adams, P. D., Winn, M. D., Storoni, L. C., and Read, R. J. (2007) Phaser crystallographic software, *Journal of applied crystallography* 40, 658-674.
- [214] Emsley, P., Lohkamp, B., Scott, W. G., and Cowtan, K. (2010) Features and development of Coot, *Acta crystallographica. Section D, Biological crystallography* 66, 486-501.
- [215] Adams, P. D., Afonine, P. V., Bunkoczi, G., Chen, V. B., Davis, I. W., Echols, N., Headd, J. J., Hung, L. W., Kapral, G. J., Grosse-Kunstleve, R. W., McCoy, A. J., Moriarty, N. W., Oeffner, R., Read, R. J., Richardson, D. C., Richardson, J. S., Terwilliger, T. C., and Zwart, P. H. (2010) PHENIX: a comprehensive Python-based system for macromolecular structure solution, *Acta crystallographica. Section D, Biological crystallography* 66, 213-221.
- [216] Kovalevskiy, O., Nicholls, R. A., Long, F., Carlon, A., and Murshudov, G. N. (2018) Overview of refinement procedures within REFMAC5: utilizing data from different sources, *Acta Crystallogr D Struct Biol* 74, 215-227.
- [217] Schneider, C. A., Rasband, W. S., and Eliceiri, K. W. (2012) NIH Image to ImageJ: 25 years of image analysis, *Nature methods* 9, 671-675.
- [218] Niesen, F. H., Berglund, H., and Vedadi, M. (2007) The use of differential scanning fluorimetry to detect ligand interactions that promote protein stability, *Nature protocols* 2, 2212-2221.

- [219] Ciccone, L., Vera, L., Tepshi, L., Rosalia, L., Rossello, A., and Stura, E. A. (2015) Multicomponent mixtures for cryoprotection and ligand solubilization, *Biotechnology Reports* 7, 120-127.
- [220] Molland, K., Zhou, Q., and Mesecar, A. D. (2014) A 2.2 Å resolution structure of the USP7 catalytic domain in a new space group elaborates upon structural rearrangements resulting from ubiquitin binding, *Acta Crystallogr F Struct Biol Commun* 70, 283-287.
- [221] Morrow, M., Morgan, M., Clerici, M., Growkova, K., Yan, M., Komander, D., Šimíček, M., Sixma, T., and Wolberger, C. (2017) Active site alanine substitutions can convert deubiquitinating enzymes into avid ubiquitin-binding domains, *bioRxiv*.
- [222] Leznicki, P., Natarajan, J., Bader, G., Spevak, W., Schlattl, A., Abdul Rehman, S. A., Pathak, D., Weidlich, S., Zoephel, A., Bordone, M. C., Barbosa-Morais, N. L., Boehmelt, G., and Kulathu, Y. (2018) Expansion of DUB functionality generated by alternative isoforms - USP35, a case study, *J Cell Sci* 131.
- [223] Cremers, C. M., and Jakob, U. (2013) Oxidant sensing by reversible disulfide bond formation, *The Journal of biological chemistry* 288.
- [224] Lee, J. G., Baek, K., Soetandyo, N., and Ye, Y. (2013) Reversible inactivation of deubiquitinases by reactive oxygen species in vitro and in cells, *Nat Commun* 4, 1568.
- [225] Sun, M. A., Wang, Y., Zhang, Q., Xia, Y., Ge, W., and Guo, D. (2017) Prediction of reversible disulfide based on features from local structural signatures, *BMC Genomics* 18, 279.
- [226] Dombkowski, A. A., Sultana, K. Z., and Craig, D. B. (2014) Protein disulfide engineering, *FEBS Lett* 588, 206-212.
- [227] Haworth, N. L., Gready, J. E., George, R. A., and Wouters, M. A. (2007) Evaluating the stability of disulfide bridges in proteins: a torsional potential energy surface for diethyl disulfide, *Mol Simul* 33, 475-485.

Appendix

Appendix 1: USP15-D1D2 sequence

The sequence for USP15-D1D2 is given below. The catalytic residues are shown in red and the ASTSK linker taken from USP8 is shown in green.

Human USP15 isoform 2 256-919 Δ 440-756:

MEQPGLCGLSNLGNT^CFMNSAIQCLSNTPLTEYFLNDKYQEELNFDNPLGMRGEIAKSYAELIKQMWSGKF
SYVTPRAFKTQVGRFAPQFSGYQQDCQELLAFLLDGLHEDLNIRKKPYIQLKDADGRPDKVVAEEAWEN
HLKRNDISIIVDIFHGLFKSTLVCPECAKISVTFDPFCYLTLP^{ASTSK}VKLKDCIELFTTKEKLGAE^{DPWYCPNC}
KEHQATKKLDLWSLPPVLVVHLKRFSYSRYMRDKLDTLVDFPINDLDMSEFLINPNAGPCRYNLI^{AVSNHY}
GGMGGG^{HYTAF}AKNKDDGK^{WYYF}^DDSSVSTASE^{DQIVSKAAYVLFYQRQDTFSGTGFFPLDRETA}AEDHH
HHHH

Appendix 2: USP15-D1D2 and USP4-D1D2 sequence alignment

```

USP15-D1D2  EQPGLCGLSNLGNTCFMNSAIQCLSNTPEPLTYFLNDKYQEEINFDNPLG
USP4-D1D2   EQPGLCGLSNLGNTCFMNSAIQCLSNTAPEPLTYFLNDKYQEEINFDNPLG
          *****:*****:*****:*****:*****:*****:*****
USP15-D1D2  MRGEIAKSYAELIKQMWSGKFSYVTPRAFKTQVGRFAPQFSGYQQQDCQE
USP4-D1D2   MRGEIAEAYAEIKQMWSGRDAFVAPRMFKTQVGRFAPQFSGYQQQDSQE
          *:*****:*****:*****:*****:*****:*****:*****
USP15-D1D2  LLAFLLDGLHEDLNRITKKPYIQLKDADGRPDKVVAAEAWENHLKRNDSI
USP4-D1D2   LLAFLLDGLHEDLNRVKKPYLELKDANGRPDAVVAEAWENHLKRNDSV
          *****:*****:*****:*****:*****:*****:*****
USP15-D1D2  IVDIFHGLFKSTLVCPECAKISVTFDPFCYLTPLPVLLKDCIELFTTKE
USP4-D1D2   IVDIFHGLFKSTLVCPECAKISVTFDPFCYLTPLPVLLKDCIELFTTKE
          ***:*****:*****:*****:*****:*****:*****
USP15-D1D2  KLGAEIDPWYCPNCKEHQQATKKLDLWSLPVLVVLHVKRFSYSRYNRDKLD
USP4-D1D2   TLGAEIDPWYCPNCKKHQQATKKLDLWSLPKLLVVLHVKRFSYSRYNRDKLD
          .**.:*****:*****:*****:*****:*****:*****
USP15-D1D2  TLVDFPINDLMSEFLINPNAGPCRYNLIAVSNHYGMCGGYTAAKKNK
USP4-D1D2   TLVDFPIRGLMSEFVGNLSARPYVDLIAVSNHYGMGVGYTAAKKNK
          .....:.....:.....:.....:.....:.....:.....

```

Figure A1: Sequence alignment of USP15-D1D2 and USP4-D1D2. Residue differences are highlighted in green, and the catalytic residues are shown in red. An * indicates conserved residues, residues with similar properties are indicated by a colon (:), and residues with weakly properties are represented with a (.) and non-conserved residues are marked by a blank space.

Appendix 3: Crystallisation experiments.

The following tables summarise the crystallisation trials described in this thesis. The components of the 96 well mitoxantrone screen are also given.

Appendix 3a: USP15-D1D2 mitoxantrone co-crystallisation optimisations of Memgold and Clearstrategy conditions.

The table below summarises all mitoxantrone optimisation screens based on initial hits obtained in clear strategy E5 and MemgoldE10

Tray No:	[Protein] mg/ml	[Mitoxantrone] mg/ml	Seeding	Mitoxantrone solvent	Drop size (μ L)	Drop ratio Protein:resevoir	Salt (M)	Buffer (M)	pH	Precipitant	Temperature °C
1	4	1		100% EtOH	4	1:1	N/A	0.1 Bicine	7.9-8.9	PEG 2000 26-38.5 % (w/v)	20
2	4	1		100% EtOH	4	1:1	0.1-0.25 Potassium thiocyanate	0.1 Tris	7.5	PEG 2000 MME 15-27.5% (w/v)	20
3	4	1		100% EtOH	4	1:1	N/A	0.1 Bicine	8.0-8.9	PEG 2000 25-35 % (w/v)	20
4	4-8	1		100% EtOH	4-8	1:1 2:1 1:2	N/A	0.1 Bicine	8.9	PEG 2000 31% (w/v)	20
5	4-8	1		100% EtOH	4	1:1	N/A	0.1 Bicine	8.9	PEG 2000 25-35% (w/v)	20
6	4-8	1		100% EtOH	4	1:1	N/A	0.1 Bicine	8.9	PEG 3300 25-35% (w/v)	20
7	4-8	1		100% EtOH	4	1:1	N/A	0.1 Bicine	8.9	PEG 1500 25-35% (w/v)	20
8	3.25-4	0.35-0.44		100% EtOH	2.5 (Hanging drop)	1:1	N/A	0.1 Bicine	8.9	PEG 2000 25-35% (w/v)	20
9	4	0.44		100% EtOH	2-6	1:1	N/A	0.1 Bicine	8.9	PEG 2000 25-35% (w/v)	20
10	4	0.44		100% EtOH	4	1:1	N/A	0.1 Bicine	7.9-8.9	PEG 2000 25-35% (w/v)	10
11	4	0.44		100% EtOH	4	1:1	N/A	0.1 Bicine	7.9-8.9	PEG 2000 25-35% (w/v)	15
12	4	0.44		100% EtOH	4	1:1	N/A	0.1 Bicine	7.9-8.9	PEG 2000 25-35% (w/v)	25

13	2.5-4	0.27-0.44		100% EtOH	4	1:1	N/A	0.1 Bicine	8.9	PEG 2000 25-35% (w/v)	15
14	2.5-4	0.27-0.44		50% DMSO	4	1:1	N/A	0.1 Bicine	8.9	PEG 2000 25-35% (w/v)	15
15	2.5-4	0.27-0.44		100% EtOH	4	1:1	N/A	0.1 Bicine	8.9	PEG 2000 25-35% (w/v)	25
16	2.5-4	0.27-0.44		100% DMSO	4	1:1	N/A	0.1 Bicine	8.9	PEG 2000 25-35% (w/v)	25

Table 18: Summary of USP15-D1D2 co-crystallisation optimisations of MemGold E10 and clear strategy E5.

Appendix 3b: Optimisation of Proplex A4 condition

Tray No:	[Protein] mg/ml	[Mitoxantrone] mg/ml	Seeding	Mitoxantrone solvent	Drop size (μ L)	Drop ratio Protein:resevoir	Salt (M)	Buffer (M)	pH	Precipitant	Temperature °C
17	4	0.44	No	50 % DMSO	4	2:2	N/A	Tris	8.5	PEG 2000 PEG 4000 PEG 6000 PEG 8000 8-26% w/v	20
18	4	0.44	No	50 % DMSO	4	2:2	N/A	Tris	8.5	PEG 400 15-40% w/v	20
19	4	0.28	Yes	1 mg/ml in 50% DMSO	4	2:1.5:0.5	N/A	0.1 NaCitrate 0.1 Mes 0.1 Hepes	4.5 6.0 7.0	MPD 6-26 % w/v	20
20	4	0.28	No	1 mg/ml in 50% DMSO	4	1:1	N/A	0.1 NaCitrate 0.1 Mes 0.1 Hepes	4.5 6.0 7.0	MPD 6-26 % w/v	20
21	4	0.14 0.28 0.42 0.56	Yes	3 mg/ml in 50% DMSO	4	2:1.5:0.5	N/A	0.1 NaCitrate 0.1 Mes 0.1 Hepes	4.5 6.0 7.0	MPD 6-26 % w/v	20

22	4	0.14 0.28 0.42 0.56	No	3 mg/ml in 50% DMSO	4	1:1	N/A	0.1 NaCitrate 0.1 Mes 0.1 Hepes	4.5 6.0 7.0	MPD 6-26 % w/v	20
23	4 and 2	0.28	Yes	1 mg/ml in 50% DMSO	4	1.5:2:0.5	N/A	0.1 Hepes	7.0	MPD 10-56 & W/V	20
24	4 and 2	0.28	Yes	1 mg/ml in 50% DMSO	4	1.5:2:0.5	N/A	0.1 MES	6.0	MPD 10-56 & W/V	20

Table 19 Table summarising optimisation plates of Proplex A4

Appendix 3c: Additional crystallisation screen

The following screens tested a variety of things such as USP15-D1D2 crystallisation in the absence of mitoxantrone and additional additives such as PR-619 and β -mercaptoethanol.

Screen no:	Screen	Protein mg/ml	Mioxastrone concentration	Solvent	Temperature	Additional additives?	Seeded?	Additional comments
25	PEG II	4	0.43	50% DMSO	20	1 mM B-MERCAPTOETHANOL	No	
26	PEG II	4	-	50% DMSO	20	1 mM B-MERCAPTOETHANOL	No	
27	PEG II	4	-	50% DMSO	20		No	
28	MPD	4	0.43	50% DMSO	20	1 mM B-MERCAPTOETHANOL	No	
29	MPD	4	-	50% DMSO	20	1 mM B-MERCAPTOETHANOL	No	
30	MPD	4	-	50% DMSO	20		No	
31	Proplex	4	0.43	50% DMSO	20	1 mM B-MERCAPTOETHANOL	No	
32	Proplex	4	-	50% DMSO	20	1 mM B-MERCAPTOETHANOL	No	

33	Proplex	4	-	50% DMSO	20		No	
34	Pactprem	4	0.43/none	50% DMSO	20	PR-619	Yes	Bottom drop contained no mitoxantrone
35	Proplex	4	0.43/none	50% DMSO	20	PR-619	Yes	Bottom drop contained no mitoxantrone
36	MPD	4	0.43/none	50% DMSO	20	PR-619	Yes	
37	Midas	4	0.43/none	50% DMSO	20	PR-619	Yes	
38	Proplex	4	0.43/none					
39	MPD	4	0.43/none	50% DMSO	20	PR-619	Yes	
40	AnSO ₄	4	0.43/none	50% DMOS	20	PR-619	No	Different solutions trialled for solubilising PR-619, no mitoxantrone in bottom drop
41	PEGII	4	0.43/none	50% DMSO	20	PR-619	No	Different solutions trialled for solubilising PR-619, Different solutions trialled for solubilising PR-619, no

								mitoxantrone in bottom drop
42	Mitoxantrone screen*	4	0.43/none	50% DMSO	20		Undiluted seedstock and 1:10 seedstock	
42	Mitoxantrone screen*	4	0.43/none	50% DMSO	20		1:50 and 1:100 seedstock	
43	Mitoxantrone screen*	2	0.18 (1:3 ratio)	50% DMSO	10		1:50 and 1:100 seedstock	
44	Mitoxantrone screen*	2	0.43 (1:10 ratio)	50% DMSO	10		1:50 and 1:100 seedstock	
45	Mitoxantrone screen*	2	0.43 (1:10 ratio)	50% DMSO	20		1:50 and 1:100 seedstock	

Appendix 3d: USP15-D1D2 Cys269Ser_Ubggg complex crystallisation screens:

Screen no:	Screen	Protein mg/ml	Temperature	Additional additives?	Additional comments
46	Proplex	3 mg/ml 5 mg/ml	10°C	No	
47	Structure Screen 1 + 2	3 mg/ml 5 mg/ml	10°C	No	
48	PEG suit	3 mg/ml 5 mg/ml	10°C	No	
49	Morpheus	5 mg/ml 10 mg/ml	20°C	No	

50	Midas	5 mg/ml 10 mg/ml	20°C	No	
51	Pact premier	5 mg/ml 10 mg/ml	20°C	No	
52	MPD	2 mg/ml	10°C	Yes: Mitoxantrone	Tray were set up with 500 μ M added to complex prior to crystallisation. One drop contained mitoxantrone and the other did not.
53	Porplex	2 mg/ml	10°C	Yes: Mitoxantrone	Tray were set up with 500 μ M added to complex prior to crystallisation. One drop contained mitoxantrone and the other did not.
54	Procomplex	2 mg/ml	10°C	Yes: Mitoxantrone	Tray were set up with 500 μ M added to complex prior to crystallisation. One drop contained mitoxantrone and the other did not.

Appendix 3e: USP15-D1D2 Cys269Ser_Ubggg complex optimisation screens:

Tray No:	[Protein] mg/ml	Drop size (μL)	Drop ratio Protein:resevoir	Salt (M)	Buffer (M)	pH	Precipitant	Temperature °C
55	5 mg/ml	2	1:1	0.6 M- 1.2 M Ammonium di- hydrogen phosphate	0.1 M Na- citrate	5.0- 6.5	-	10°C
56	5 mg/ml	2	1:1	0.6 M- 1.2 M Sodium dihydrogen phosphate	0.1 M Na- Hepes	7.0- 8.0	-	10°C
57	5 mg/ml	2	1:1	0.6 M- 1.2 M Potassium dihydrogen phosphate	0.1 M Na- Hepes	7.0- 8.0	-	10°C

Appendix 3f : USP15-D1D2 Cys269Ser_Di-ub complex crystallisation screens:

Screen no:	Screen	Protein mg/ml	Temperature	Additional additives?	Additional comments
58	Morpheus	4 mg/ml 2 mg/ml	20	No	
59	JCSG+	4 mg/ml 2 mg/ml	20	No	

Appendix 3g: 96 well mitoxantrone screen

The following 96 well screen was designed from optimisation screens, utilising conditions, which had consistently yielded crystals, with the intention of this screen being used for high throughput generation of USP15-D1D2 crystals for further experiments.

Well No:	Concentration (M)	Salt	Buffer	pH	Precipitant	Concentration (% w/v)
1			MES	6	PEG 400	15
2			MES	6	PEG 400	20
3			MES	6	PEG 400	25
4			MES	6	PEG 400	30
5			Hepes	7	PEG 400	15
6			Hepes	7	PEG 400	20
7			Hepes	7	PEG 400	25
8			Hepes	7	PEG 400	30
9			Hepes	7.5	PEG 400	15
10			Hepes	7.5	PEG 400	20
11			Hepes	7.5	PEG 400	25
12			Hepes	7.5	PEG 400	30
13			Tris	8	PEG 400	15
14			Tris	8	PEG 400	20
15			Tris	8	PEG 400	25
16			Tris	8	PEG 400	30
17			Tris	8.5	PEG 400	15
18			Tris	8.5	PEG 400	20
19			Tris	8.5	PEG 400	25
20			Tris	8.5	PEG 400	30
21			MES	6	MPD	10
22			MES	6	MPD	15
23			MES	6	MPD	20
24			MES	6	MPD	25
25			Hepes	7	MPD	10
26			Hepes	7	MPD	15
27			Hepes	7	MPD	20
28			Hepes	7	MPD	25
29			Hepes	7.5	MPD	10
30			Hepes	7.5	MPD	15
31			Hepes	7.5	MPD	20
32			Hepes	7.5	MPD	25
33			Tris	8	MPD	10
34			Tris	8	MPD	15
35			Tris	8	MPD	20
36			Tris	8	MPD	25
37			Tris	8.5	MPD	10
38			Tris	8.5	MPD	15
39			Tris	8.5	MPD	20
40			Tris	8.5	MPD	25
41			MES	6	PEG 2000	15
42			MES	6	PEG 2000	20
43			MES	6	PEG 2000	25
44			MES	6	PEG 2000	30

45			Hepes	7	PEG 2000	15
46			Hepes	7	PEG 2000	20
47			Hepes	7	PEG 2000	25
48			Hepes	7	PEG 2000	30
49			Hepes	7.5	PEG 2000	15
50			Hepes	7.5	PEG 2000	20
51			Hepes	7.5	PEG 2000	25
52			Hepes	7.5	PEG 2000	30
53			Tris	8	PEG 2000	15
54			Tris	8	PEG 2000	20
55			Tris	8	PEG 2000	25
56			Tris	8	PEG 2000	30
57			Tris	8.5	PEG 2000	15
58			Tris	8.5	PEG 2000	20
59			Tris	8.5	PEG 2000	25
60			Tris	8.5	PEG 2000	30
61			MES	6	PEG 4000	15
62			MES	6	PEG 4000	20
63			MES	6	PEG 4000	25
64			MES	6	PEG 4000	30
65			Hepes	7	PEG 4000	15
66			Hepes	7	PEG 4000	20
67			Hepes	7	PEG 4000	25
68			Hepes	7	PEG 4000	30
69			Hepes	7.5	PEG 4000	15
70			Hepes	7.5	PEG 4000	20
71			Hepes	7.5	PEG 4000	25
72			Hepes	7.5	PEG 4000	30
73			Tris	8	PEG 4000	15
74			Tris	8	PEG 4000	20
75			Tris	8	PEG 4000	25
76			Tris	8	PEG 4000	30
77			Tris	8.5	PEG 4000	15
78			Tris	8.5	PEG 4000	20
79			Tris	8.5	PEG 4000	25
80			Tris	8.5	PEG 4000	30
81	0.2	trimethylamine N-oxide dihydrate	Hepes	7	MPD	10
82	0.2	trimethylamine N-oxide dihydrate	Hepes	7	MPD	15
83	0.2	trimethylamine N-oxide dihydrate	Hepes	7	MPD	20
84	0.2	trimethylamine N-oxide dihydrate	Hepes	7	MPD	25
85	0.2	trimethylamine N-oxide dihydrate	Hepes	7.5	MPD	10
86	0.2	trimethylamine N-oxide dihydrate	Hepes	7.5	MPD	15
87	0.2	trimethylamine N-oxide dihydrate	Hepes	7.5	MPD	20

88	0.2	trimethylamine N-oxide dihydrate	Hepes	7.5	MPD	25
89	0.2	trimethylamine N-oxide dihydrate	Tris	8	MPD	10
90	0.2	trimethylamine N-oxide dihydrate	Tris	8	MPD	15
91	0.2	trimethylamine N-oxide dihydrate	Tris	8	MPD	20
92	0.2	trimethylamine N-oxide dihydrate	Tris	8	MPD	25
93	0.2	trimethylamine N-oxide dihydrate	Tris	8.5	MPD	10
94	0.2	trimethylamine N-oxide dihydrate	Tris	8.5	MPD	15
95	0.2	trimethylamine N-oxide dihydrate	Tris	8.5	MPD	20
96	0.2	trimethylamine N-oxide dihydrate	Tris	8.5	MPD	25

Table 20: 96 well screen designed around conditions which have been favourable for USP15-D1D2 crystallisation with mitoxantrone in previous trials.

Appendix 4: USP2 catalytic domain purification

The USP2 expression plasmid was gifted by Cheryl Arrowsmith. USP2 was purified following the same protocol as for USP15-D1D2. USP2 expressed exceptionally well in BL2- CP *E.coli* cells, and 3 litres of culture grown over night at 25°C yielded around 8 mg of pure protein. Nickel affinity chromatography was sufficient for the separation of most impurities. DTT was added to the protein during concentration for SEC.

SEC was performed on an S75 column. The protein eluted as a single mono dispersed peak at 72 ml. SDS PAGE gel electrophoresis was used to confirm the protein size and purity.

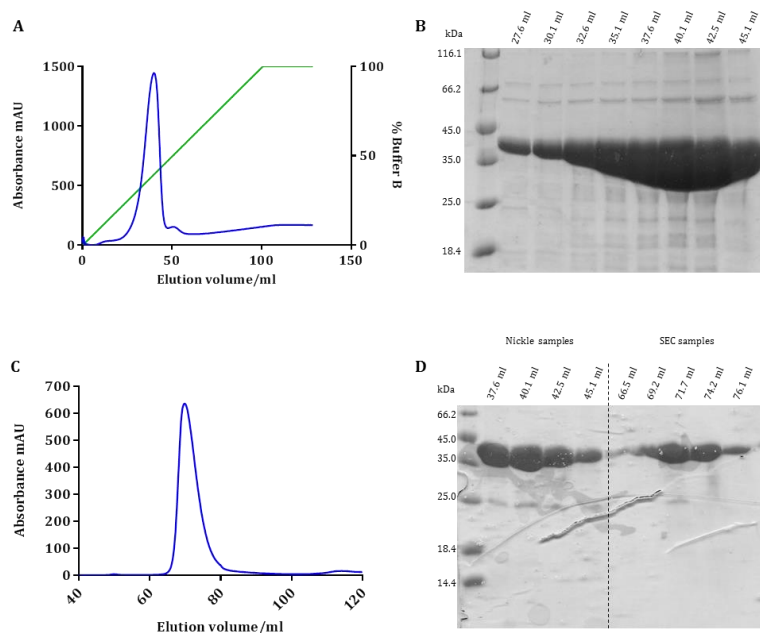


Figure A2: Purification of USP2 catalytic domain (A)

Chromatogram of the NT-His Tag purification. The UV trace is shown in blue and concentration of buffer B (%) in green. (B) SDS Page Gel showing corresponding fractions from the NT-His tag purification. Samples contain very high concentrations of protein resulting in over loading of the gel. USP2 has a molecular weight of 42 kDa. (C) Chromatogram of gel filtration. USP2 eluted at 72 ml as a single peak. (D) SDS PAGE gel of fractions from the gel filtration. Nickle affinity samples were diluted 1:10 and run alongside SEC samples.

The purified USP2 was tested in the Ub-AMC assay to confirm the protein was fully functional and the correct protein. USP15-D1D2 was included as positive control. USP2 was tested at 50 nM 500 nM and 1 μ M to ensure activity was detected. Activity increased with increasing concentrations of USP2 in the assay.

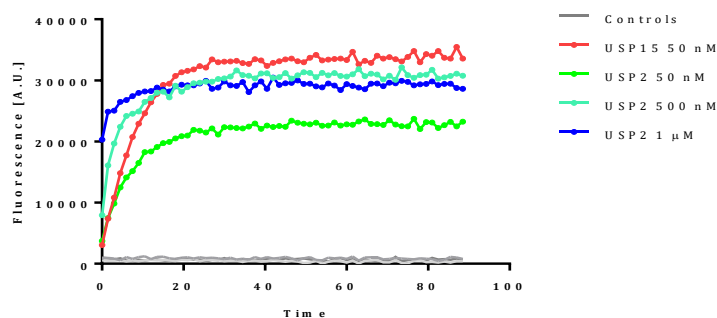


Figure A3: Ub-AMC assay for verification that USP2 was present and fully functional after purification. USP2 activity was tested at concentrations 50 nM (green) 500 nM (Aqua) and 1 μM (Blue) in the presence of 750 nM Ub-AMC. USP15-D1D2 (red) was included in the assay as a positive control. Wells containing buffer and Ub-AMC alone were used to control for back ground fluorescence (grey).