

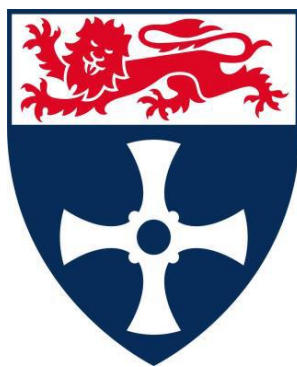
The role and regulation of ubiquitin-specific protease 4 in nuclear factor kappa B signalling

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Abstract

Deubiquitinating enzymes (DUBs) constitute a diverse family of regulators of ubiquitin signalling. Aberrations in this can underlie pathologies including osteoarthritis, cancers, inflammatory diseases, and even ageing. Understanding the biology of ubiquitin signalling components and how they regulate cell signalling pathways is therefore a widening area of research. The aim of this thesis was to further understanding on the function of the DUB, ubiquitin-specific protease 4 (USP4), and the mechanism of Usp4 regulation by its post-translational modification (PTM) at two serine residues, focusing on the nuclear factor of κ B (NF- κ B) pathway.

Initial observations in *Usp4* null (-/-) mice suggested a role for Usp4 in regulating circulating levels of specific inflammatory cytokines and, perhaps consequentially, a role in viral infection clearance. Thus, *in vitro*, the role of USP4 in IL-1- and TNF α -mediated NF- κ B signalling was examined. Overexpression of Usp4 significantly increased NF- κ B signalling, and using an RNAi approach to deplete USP4 resulted in suppressed NF- κ B activity and significantly reduced expression of NF- κ B-regulated genes (MMP13, IL-6 and IL-8). Depletion of USP4 also significantly reduced ubiquitination and degradation of I κ B α , and phosphorylation of p65 and IKK. Together, data were indicative of USP4 as a positive regulator of NF- κ B signalling by functioning upstream of the IKK complex. Importantly, aspects of these findings were confirmed in mouse embryonic fibroblasts (MEFs) isolated from the *Usp4*^{-/-} mice. Further, identification of potential USP4 substrates were assessed. Reductions in K48- and K63-linked polyubiquitination were observed with depletion of USP4.

To further understanding of the regulation of USP4, the PTM at serines 675 and 680 was examined. Lentiviral-mediated delivery of Usp4 phosphorylation variants revealed that a complex mechanism of phosphorylation/dephosphorylation of USP4 may regulate its function in NF- κ B signalling.

In conclusion, this thesis identifies USP4 as a positive regulator of IL-1-induced NF- κ B signalling, potentially regulating itself through its PTM.

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Contents

Abstract	i
Acknowledgements	ii
Contents	iii
List of figures	viii
List of tables	xi
Abbreviations	xii
Chapter 1 : Introduction	1
1.1 Ubiquitin signalling	1
1.1.1 Ubiquitin conjugation	1
1.1.2 E3 ligases	3
1.1.3 Deubiquitinating enzymes	4
1.1.4 The importance of proteolytic and non-proteolytic ubiquitin signalling in disease.....	8
1.2 Ubiquitin-specific protease 4 (USP4).....	10
1.2.1 Discovery of USP4	10
1.2.2 Structure and localisation of USP4.....	11
1.2.3 Substrates of USP4	12
1.2.4 Specificity of USP4	13
1.3 Cell signalling pathways	14
1.3.1 The p53 pathway.....	14
1.3.2 The Wnt/ β -catenin pathway.....	17
1.3.3 The TGF- β pathway.....	20
1.3.4 The RIG-I pathway	23
1.3.5 The NF- κ B pathway	25
1.3.6 The MAPK pathways	37
1.3.7 Implications of USP4 functions in cell signalling pathways on disease pathogenesis.....	39
1.4 Post-translational modifications (PTMs).....	41
1.4.1 Importance of PTMs in cell signalling pathways	42
1.4.2 Regulation of DUBs by PTMs.....	42
1.4.3 USP4 PTMs	43
1.5 Aims	44

Chapter 2 : Materials and Methods	45
2.1 Materials	45
2.1.1 Antibodies.....	45
2.1.2 Immunoblotting reagents	47
2.1.3 Cell lines	47
2.1.4 Cell culture reagents	48
2.1.5 Transfection reagents.....	48
2.1.6 Plasmids.....	48
2.1.7 Cytokines and other stimuli	49
2.1.8 Inhibitors.....	49
2.1.9 Immunohistochemistry reagents and antibodies.....	49
2.1.10 General molecular biology reagents	49
2.1.11 Commercially available kits	50
2.1.12 Culture vessels	51
2.2 Methods	51
2.2.1 Cell culture.....	51
2.2.2 Generation and use of expression vectors	53
2.2.3 Immunofluorescence.....	58
2.2.4 RNA interference (RNAi).....	58
2.2.5 Luciferase reporter assays.....	61
2.2.6 Protein analysis	64
2.2.7 Immunoprecipitation (IP)	66
2.2.8 RNA analysis	70
2.2.9 Genotyping mice.....	73
2.2.10 Cytokine array	75
2.2.11 Enzyme-linked immunosorbent assay (ELISA)	75
2.2.12 Immunohistochemistry	77
2.2.13 Lentivirus: inducible expression system.....	78
2.2.14 Lentivirus: second generation expression system	80
2.2.15 Computational modelling	84
2.2.16 Statistical analysis.....	84
Chapter 3 : USP4 in Inflammatory and Immune Pathways	85
3.1 Introduction	85
3.2 Aims	86

3.3 Results	87
3.3.1 Usp4 overexpression affects cells signalling pathways.....	87
3.3.2 Cytokine levels in <i>Usp4</i> ^{+/+} , <i>Usp4</i> ^{+/-} and <i>Usp4</i> ^{-/-} mice	89
3.3.3 Viral replication in <i>Usp4</i> ^{-/-} mouse embryonic fibroblasts	93
3.3.4 Viral replication in the brains of <i>Usp4</i> ^{-/-} mice	95
3.4 Discussion	97
3.4.1 Usp4 overexpression affected the activity of cell signalling pathways	97
3.4.2 Usp4 may affect cytokine levels.....	100
3.4.3 Increased viral replication in <i>Usp4</i> ^{-/-} mice	101
Chapter 4 : The Effect of USP4 Depletion on NF-κB Signalling	103
4.1 Introduction	103
4.1.1 Aims.....	104
4.2 Results	105
4.2.1 Depletion of <i>USP4</i> gene expression and USP4 protein levels by targeted siRNA	105
4.2.2 The effect of USP4 depletion on TNFα- and IL-1-induced NF-κB activity	107
4.2.3 The effect of USP4 depletion on TNFα- and IL-1-induced expression of NF-κB-regulated genes	108
4.2.4 The effect of USP4 depletion on levels of key NF-κB signalling pathway adaptor proteins	115
4.2.5 NF-κB signalling in <i>Usp4</i> ^{-/-} mouse embryonic fibroblasts.....	129
4.3 Discussion	134
4.3.1 IL-1- and TNFα-induced NF-κB-regulated gene expression	134
4.3.2 Depletion of USP4 altered IL-1α-induced NF-κB adaptor protein levels..	136
4.3.3 IL-1-induced phosphorylation of JNK was reduced with USP4 depletion	140
4.3.4 Altered levels of IL-1- and TNFα-induced NF-κB adaptor proteins were not cell-specific.....	142
4.3.5 IL-1-induced NF-κB activity and NF-κB-regulated gene expression were reduced in <i>Usp4</i> ^{-/-} MEFs.....	143
4.3.6 Summary.....	144
4.3.7 Conclusions	145
Chapter 5 : Target(s) of USP4 within the NF-κB Pathway	147
5.1 Introduction	147

Contents

5.1.1 Aims.....	149
5.2 Results	149
5.2.1 Computational modelling of USP4 in the IL-1-induced NF- κ B signalling pathway.....	149
5.2.2 IL-1-induced TAK1 and TRAF6 ubiquitination, K48- and K63-linked polyubiquitination.....	157
5.2.3 The effect of USP4 depletion on the IL-1-induced levels of proteins upstream of the IKK complex	167
5.3 Discussion	168
5.3.1 Computational modelling to determine potential USP4 substrate(s)	168
5.3.2 The effect of USP4 depletion on the IL-1-induced ubiquitination of TAK1 and TRAF6	169
5.3.3 Summary.....	177
5.3.4 Conclusions	178
Chapter 6 : USP4 Phosphorylation and NF-κB Signalling	179
6.1 Introduction	179
6.1.1 Aims.....	181
6.2 Results	182
6.2.1 Usp4 phosphorylation at Ser675/Ser680 does not affect subcellular localisation.....	182
6.2.2 The effect of Usp4 phosphorylation at Ser675/Ser680 on cell signalling pathways	184
6.2.3 The generation of Usp4 phosphorylation variants.....	188
6.2.4 The effect of Usp4 phosphorylation on IL-1-induced NF- κ B activity	194
6.2.5 The effect of depleting a USP4 kinase on NF- κ B signalling	202
6.3 Discussion	208
6.3.1 Overexpression of a non-phosphorylatable Usp4 variant, at Ser675 and Ser680, did not affect the subcellular localisation of Usp4	208
6.3.2 Usp4 PTM at Ser675/Ser680: effects on cell signalling pathways	209
6.3.3 The effect of Usp4 PTM on IL-1-induced NF- κ B activity	210
6.3.4 The effect of depletion of a USP4 kinase on NF- κ B signalling	216
6.3.5 Summary.....	218
6.3.6 Conclusions	219
Chapter 7 : General Discussion	220

Contents

7.1 USP4 in cell signalling pathways	221
7.2 Usp4 in immune and inflammatory pathways.....	221
7.3 USP4 in NF- κ B signalling.....	222
7.4 Targets of USP4 in the NF- κ B signalling pathway.....	224
7.5 The PTM of Usp4 at Ser675/Ser680	226
7.6 Future work	228
7.7 Summary	230
Appendix	231
References	238

List of figures

Figure 1.1. Schematic of ubiquitin conjugation	2
Figure 1.2. General functions of DUBs.....	5
Figure 1.3. Domain structure of USP4	11
Figure 1.4. A model of USP4 inhibition of p53 via interaction with ARF-BP1	16
Figure 1.5. The Wnt/ β -catenin pathway.....	19
Figure 1.6. The TGF- β signalling pathway	22
Figure 1.7. The RIG-I pathway.....	24
Figure 1.8. The NF- κ B signalling pathway	34
Figure 1.9. The non-canonical NF- κ B signalling pathway	36
Figure 1.10. The MAPK pathways	38
Figure 2.1. Schematic of the retroviral insertion in the <i>Usp4</i> gene.....	52
Figure 2.2. Optimisation of siRNA transfection time	60
Figure 2.3. Optimisation of stimulation concentration and time on luciferase activity	63
Figure 2.4. Optimisation of the transfection of FLAG-tagged proteins.....	68
Figure 2.5. Optimisation of IP conditions	69
Figure 2.6. <i>Usp4</i> mouse genotypes	74
Figure 2.7. Standard curves for ELISAs.....	76
Figure 3.1. Overexpression of <i>Usp4</i> affected the activity of cell signalling pathways	88
Figure 3.2. Cytokine levels in <i>Usp4</i> ^{+/+} (+/+), <i>Usp4</i> ^{+/-} (+/-) and <i>Usp4</i> ^{-/-} (-/-) mouse serum	90
Figure 3.3. Levels of Il-6 and Cxcl1 in the serum of mice	92
Figure 3.4. VSV viral replication in <i>Usp4</i> ^{-/-} MEFs.....	94
Figure 3.5. VSV viral replication in the brains of mice	96
Figure 4.1. USP4 protein levels and <i>USP4</i> gene expression were reduced following transfection with a USP4-targeting siRNA	106
Figure 4.2. Depletion of USP4 significantly reduced NF- κ B activity	107
Figure 4.3. USP4 regulated IL-1-induced NF- κ B-regulated gene expression	109
Figure 4.4. USP4 regulated TNF α -induced NF- κ B-regulated gene expression.....	110
Figure 4.5. USP4 regulated IL-1 α -induced NF- κ B-regulated gene expression	112
Figure 4.6. USP4 regulated TNF α -induced NF- κ B-regulated gene expression.....	114

Figure 4.7. USP4 depletion affected IκBα protein levels and <i>IκBα</i> gene expression	117
Figure 4.8. IκBα ubiquitination	119
Figure 4.9. USP4 depletion and the effect on the levels of phosphorylation of NF-κB subunit, p65	121
Figure 4.10. USP4 depletion and the effect on IKKα, IKKβ and phosphorylated IKKα/β protein levels	123
Figure 4.11. USP4 depletion and the effect on MAPK protein levels	125
Figure 4.12. USP4 depletion and the effect on TNFα-induced NF-κB adaptor protein levels	126
Figure 4.13. USP4 depletion and the effect on NF-κB adaptor protein levels in stimulated HeLa cells	128
Figure 4.14. IL-1-induced NF-κB activity in SW1353 cells	130
Figure 4.15. IL-1-induced NF-κB activity in MEFs	131
Figure 4.16. USP4 regulated IL-1-induced NF-κB-regulated gene expression in MEFs	133
Figure 5.1. IL-1-induced NF-κB signalling pathway model diagram	151
Figure 5.2. Simulation when USP4 deubiquitinates K48-linked polyubiquitinated IRAK1	153
Figure 5.3. Simulation when USP4 deubiquitinates K48-linked polyubiquitinated TAK1	154
Figure 5.4. Simulation when USP4 deubiquitinates K48-linked polyubiquitinated TRAF6	155
Figure 5.5. Simulation when USP4 deubiquitinates K63-linked polyubiquitinated TAK1 and TRAF6	156
Figure 5.6. FLAG immunoprecipitates were polyubiquitinated following IL-1 induction	158
Figure 5.7. IL-1-induced TAK1 ubiquitination with USP4 depletion	160
Figure 5.8. IL-1-induced TRAF6 ubiquitination with USP4 depletion	162
Figure 5.9. The effect of USP4 depletion on levels of NF-κB adaptor proteins in HEK 293T cells	164
Figure 5.10. IL-1-induced TAK1 ubiquitination with USP4 depletion in SW1353 cells	166

Figure 5.11. The effect of USP4 depletion on levels of NF-κB adaptor proteins upstream of IKK	167
Figure 6.1. USP4 modification sites	180
Figure 6.2. The subcellular localisation of Usp4 in NIH3T3 cells.....	183
Figure 6.3. Overexpression of Usp4 affects the activity of cell signalling pathways, potentially regulated by the phosphorylation of Usp4.....	185
Figure 6.4. Overexpression of Usp4 and Usp4 variants affected NF-κB activity..	187
Figure 6.5. Usp4 is serine phosphorylated	191
Figure 6.6. Overexpression of Usp4 and Usp4 phosphorylation variants on IL-1-induced NF-κB activity	193
Figure 6.7. Lentiviral-mediated expression of Usp4 and Usp4 phosphorylation mutants on IL-1-induced NF-κB activity in SW1353 cells with normalised amounts of viral preparations	196
Figure 6.8. USP4 expression with lentiviral Usp4 and Usp4 phosphorylation variants.....	197
Figure 6.9. Lentiviral-mediated expression of Usp4 and Usp4 phosphorylation mutants on IL-1-induced NF-κB activity in SW1353 cells with equal volumes of preparations.....	199
Figure 6.10. Lentiviral-mediated expression of Usp4 and Usp4 phosphorylation mutants on IL-1α-induced NF-κB activity in <i>Usp4</i>^{-/-} MEFs.....	201
Figure 6.11. A kinase screen identified GRK1, 2 and 6 as USP4 kinases	202
Figure 6.12. Depletion of GRK2 did not affect IL-1-induced NF-κB activity	204
Figure 6.13. GRK2 depletion reduced IL-1-induced expression of NF-κB-regulated genes	206
Figure 6.14. The effect of GRK2 depletion on levels of NF-κB pathway proteins	207
Appendix Figure 1. Full length blots of antibodies used in Chapter 4.....	237

List of tables

Table 2.1. Antibodies used for immunoblotting and immunoprecipitation experiments.....	45
Table 2.2. Sequences of primers	55
Table 2.3. The 4 sequences which make up the siGENOME SMARTpool siRNAs for USP4 and the non-targeting siRNA pool #2	58
Table 2.4. Pathways included in the Cancer 10-pathway Reporter Array.....	62
Table 2.5. Sequences of primers used for real-time qRT-PCR.....	72
Table 2.6. Sequences of primers used for genotyping mice.....	73
Table 2.7. Sequences of primers for infusion cloning.	81
Table 6.1. Usp4 phosphorylation variants generated by mutagenesis	189
Appendix Table 1. List of model species in the NF-κB computational model.....	231
Appendix Table 2. Reactions for the NF-κB computational model.....	233

Abbreviations

ABC	Avidin/Biotin complex
AD	Alzheimer's disease
Ala	Alanine
ANOVA	Analysis of variance
APC	Adenomatous polyposis coli
APS	Ammonium persulphate
ARF-BP1	ARF-binding protein 1
ASK1	Apoptosis signal-regulating kinase 1
Asp	Aspartic acid
ATCC	American Type Culture Collection
BAFF	B-cell activating factor belonging to the TNF family
bp	Base pairs
BSA	Bovine serum albumin
CARD	Caspase recruitment domain
CBP	CREB-binding protein
CD40L	CD40 ligand
cIAP	Cellular inhibitor of apoptosis protein
CK1	Casein kinase 1
COPASI	Complex Pathway Simulator
CTD	C-terminal domain
CXCL1	C-X-C motif ligand 1
CYLD	Cylindromatosis
Cys	Cysteine
DAB	Diaminobenzidine
DAPI	Diamidino-2-phenylindole
DF1	DharmaFECT transfection reagent
dH ₂ O	Distilled H ₂ O
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl sulphoxide
dNTP	Deoxyribonucleotide triphosphate
dsDNA	Double-stranded DNA
DUB	Deubiquitinating enzyme
DUSP	Domain present in USPs
ECL	Enhanced chemiluminescence
EGF	Epidermal growth factor
EGFR	EGF receptor
EMT	Epithelial-to-mesenchymal transition
ERK	Extracellular signal-related kinase
EtBr	Ethidium bromide
FBS	Foetal bovine serum
FrIDA	Framework for Image Dataset Analysis
Gly	Glycine

Abbreviations

GPCR	G-protein-coupled receptor
GRK	GPCR kinase
GS	Gly/Ser-rich
GSK3	Glycogen kinase 3
HAUSP	Herpesvirus-associated USP
HD	Huntington's disease
HECT	Homologous to E6-AP C terminus
His	Histidine
HOIL-1	Heme-oxidised IRP2 ubiquitin ligase-1
HOIP	HOIL-1 interacting protein
HRP	Horseshoe peroxidase
HUBL	USP7 C-terminal UBL domain
IB	Immunoblotted
IBM	IAP binding motif
IFN	Interferon
IKK	I κ B kinase
IL	Interleukin
IL-1R	IL-1 receptor
IL-1R AcP	IL-1 receptor accessory protein
IP	Immunoprecipitation
IRAK	IL-1R-associated kinase
IRF3	IFN regulatory factor 3
I κ B	Inhibitor of κ B
JAMM	JAB1/MPN/Mov34
JNK	c-Jun NH2-terminal kinase
LB	Luria-Broth
LEF	Lymphoid enhancer factor
LGB	Lower gel buffer
LPS	Bacterial lipopolysaccharide
LRP	Lipoprotein receptor related protein
LT- β	Lymphotoxin- β
LUBAC	Linear ubiquitin assembly complex
Lys	Lysine
MAPK	Mitogen-activation protein kinase
MAPKK	MAPK kinase
MAPKKK	MAPK kinase kinase
MAVS	Mitochondrial antiviral signalling protein
MCS	Multiple cloning site
MEF	Mouse embryonic fibroblast
MEKK1	MAP and ERK kinase kinase
MG132	Carbobenzoxy-Leu-Leu-leucinal
MJD	Machade-Joseph disease protease
M-MLV	Murine Leukaemia Virus
MMP	Matrix metalloproteinase

Abbreviations

MOI	Multiplicity of infection
MSD	Meso Scale Discovery
MyD88	Myeloid differentiation factor
NEM	N-ethylmaleimide
NEMO	NF- κ B essential modifier
NES	Nuclear export signal
NF- κ B	Nuclear factor κ B
NIK	NF- κ B-inducing kinase
Nik	Nemo-like kinase
NLS	Nuclear localisation signal
NZF	Npl4 zinc-finger domain
OTU	Ovarian tumour protease
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PD	Parkinson's disease
PK1	Phosphoinositide-dependent kinase 1
PFA	Paraformaldehyde
PKA	Protein kinase A
pRb	Retinoblastoma protein
PRR	Pattern-recognition receptor
PTM	Post-translational modification
PVDF	Polyvinylidene difluoride
qRT-PCR	Quantitative reverse transcriptase PCR
RHD	Rel homology domain
RIG-I	Retinoic acid-inducible gene I
RING	Really interesting new gene
RIP1	Receptor-interacting protein 1
RLR	RIG-I-like receptor
RLU	Relative light units
RNAi	RNA interference
R-Smad	Receptor-regulated Smad
S1P	Sphingosine-1-phosphate
SBML	Systems Biology Markup Language
SCF ^{β-TrCP}	Skp1, Cullin1, F-box containing complex, β -transducin repeat containing protein
SDS-PAGE	sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SEM	Standard error of the mean
Ser	Serine
SeV	Sendai virus
shRNA	Short hairpin RNA
SILAC	Stable isotope labelling by amino acids in cell culture
siRNA	Small interfering RNA
Smurf	Smad ubiquitin regulatory factor
snRNP	Small nuclear ribonucleoprotein

Abbreviations

SUMO	Small ubiquitin-related modifier
TAB	TAK1 binding protein
TAD	Transactivation domain
TAE	Tris-acetate-ethylenediamine tetraacetic acid
TAK1	Transforming growth factor- β -activated kinase 1
TBK1	TANK-binding kinase 1
TBS	Tris buffered saline
TBS-T	TBS-Tween
TCF	T-cell factor
TEMED	N,N,N',N'-Tetramethylethylenediamine
TGF- β	Transforming growth factor- β
Thr	Threonine
TIR	Toll/IL-1 receptor
TIRAP	TIR domain-containing adaptor protein
TLR	Toll-like receptor
TNF	Tumour necrosis factor
TNFR	TNF receptor
TRADD	TNF receptor-associated protein with a death domain
TRAF	TNF receptor-associated factor
TRAM	TRIF-related adaptor molecule
TRIM	Tripartite motif
TUBEs	Tandem-repeat ubiquitin-binding entities
Tween-20	Polyoxyethylene sorbitan
UAF1	USP1 associated factor 1
UBAN	Ubiquitin binding in ABIN and NEMO
UBD	Ubiquitin-binding domain
UBL	Ubiquitin-like domain
UCH	Ubiquitin C-terminal hydroxylase
UGB	Upper gel buffer
UIM	Ubiquitin-interaction motif
Unp	Ubiquitous nuclear protein
UPS	Ubiquitin-proteasome system
USP	Ubiquitin-specific protease
VSV	Vesicular stomatitis virus
WCL	Whole cell lysates
WWP1	WW domain containing E3 ubiquitin protein ligase 1
ZnF	Zinc-finger

Chapter 1 : Introduction

1.1 Ubiquitin signalling

Ubiquitin signalling is key in the regulation of many diverse cellular processes. The conjugation of the highly conserved, 76 residue, ubiquitin protein to a substrate is a key post-translational modification (PTM) event able to control the function, stability or localisation of many proteins. Previously, ubiquitin signalling was primarily known for targeting proteins for degradation by the ubiquitin-proteasome system (UPS) to control the turnover of cellular proteins and to degrade short-lived and abnormal proteins. Intracellular proteolysis is necessary in many cellular processes and is critical for cell survival, but additionally ubiquitin signalling is fast becoming recognised as crucial in regulating events independently of the proteasome. Rather than acting as a target for degradation, the conjugation of ubiquitin can serve to recruit other proteins via ubiquitin-binding domains (UBDs) and in this way regulate events including membrane trafficking, signal transduction, DNA damage repair and gene expression (Chen and Sun, 2009, Yao and Ndoja, 2012). Progress in the knowledge of the molecular mechanisms of ubiquitination in signalling pathways has led to increased understanding that aberrations in both proteolytic and non-proteolytic ubiquitin signalling underlie many pathologies including neurodegeneration, osteoarthritis, cancers and inflammatory diseases. Further understanding the biology of the components involved in ubiquitin signalling could provide key insight into these pathologies which could be exploited for new therapies (Popovic et al., 2014).

1.1.1 Ubiquitin conjugation

Ubiquitin conjugation has been widely investigated and occurs in three steps involving ubiquitin-activating enzymes (E1), ubiquitin-conjugating enzymes (E2) and ubiquitin ligases (E3) which act together in an enzymatic cascade to covalently attach ubiquitin to a specific substrate protein. Firstly, E1 activates ubiquitin by the ATP-dependent formation of a thioester bond between the C-terminal of ubiquitin and an E1 catalytic cysteine. Secondly, the activated ubiquitin is transferred from the E1 cysteine to a cysteine residue of an E2 enzyme to form an E2-ubiquitin thioester intermediate. Thirdly, an E3 ligase catalyses the transfer of ubiquitin to the ϵ -amino group of a lysine (Lys) residue in the target protein substrate in a substrate-specific manner. This results in an isopeptide linkage via the ubiquitin C-terminal glycine (Gly) (Pickart and Eddins, 2004). Proteins

can remain conjugated to one ubiquitin (monoubiquitinated) or single ubiquitin moieties can link together via an isopeptide bond between the glycine on one ubiquitin moiety C-terminal and a lysine residue on the previous ubiquitin moiety to form a polyubiquitin chain. It is the ubiquitin-binding enzyme (E4), along with enzymes E1, E2 and E3, that in some cases is responsible for ubiquitin chain elongation (Koegl et al., 1999) (Figure 1.1).

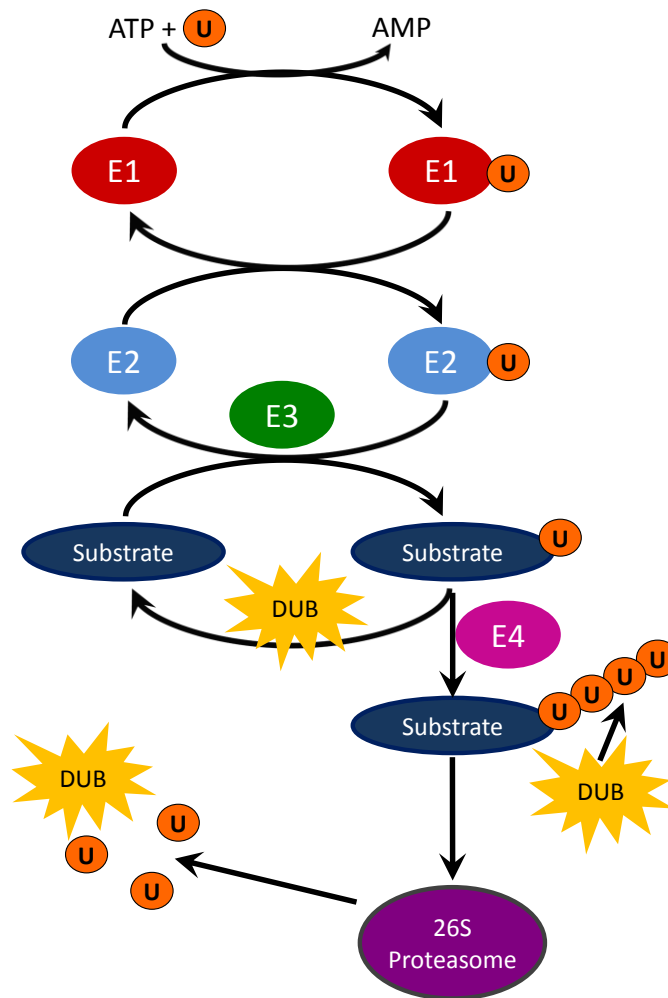


Figure 1.1. Schematic of ubiquitin conjugation. In an ATP-dependent reaction, E1 activates ubiquitin by forming a thioester with the ubiquitin C-terminal. The activated ubiquitin is then transferred to E2 before catalysis of the transfer to the target substrate protein by E3. Orange circles represent ubiquitin. Yellow stars represent deubiquitinating enzymes (DUBs).

1.1.2 E3 ligases

As with phosphorylation, a well-known PTM in the regulation of proteins carried out by a vast array of kinases, ubiquitination is carried out by a vast array of different families of ubiquitin enzymes. There are over 600 E3 ligases which target a broad array of substrates, confer specificity and diversify the ubiquitination of proteins (Li et al., 2008). Depending on their structural domains, E3 ligases can be categorised into different protein families. The vast majority of E3 ligases belong to the really interesting new gene (RING) and homologous to E6-AP C terminus (HECT) families. Different E3 ligase families adopt different mechanisms of ubiquitin transfer: RING domains act as a scaffold by binding directly to E2 enzymes to catalyse the transfer of ubiquitin to the target substrate, whereas HECT E3 ligases form a thioester intermediate with ubiquitin and the cysteine of the active site of the E3 (Berndsen and Wolberger, 2014). These RING and HECT domains generate a large number of E3 ligases which can dictate the spatial, temporal and substrate specificity of ubiquitin conjugation.

1.1.2.1 Polyubiquitin chain topology

Ubiquitin has seven intrinsic, internal lysines, all of which contribute to the linkages between ubiquitin moieties for the formation of polyubiquitin chains depending on which lysine (K) residue ubiquitin is accepted by: K6, K11, K27, K29, K33, K48 and K63 (Xu et al., 2009c). Polyubiquitin chains can be assembled from more than one linkage type (branched chains) or a single linkage type. These different topologies of ubiquitin moieties in a polyubiquitin chain can influence ubiquitin signalling, with physiological roles for each of them still emerging. The first two types of linkages to be characterised were the well-established K48- and K63-linked chains. It is generally thought that K48 polyubiquitin chains signal the targeting of substrates to the proteasome for degradation; whereas K63 polyubiquitin chains signal for a variety of non-proteolytic processes including kinase activation and signal transduction (Trempe, 2011). In addition to linkages involving the internal lysines of ubiquitin, linear ubiquitin chains can form when an isopeptide bond forms between the glycine of one ubiquitin moiety C-terminal and a methionine residue of the previous ubiquitin moiety N-terminal (Kirisako et al., 2006). Unusually, linear chains are catalysed by a specific E3 ligase complex made up of three proteins; Heme-oxidised IRP2 ubiquitin ligase1 (HOIL-1), HOIL-1 interacting protein (HOIP) and SHARPIN. These form the linear ubiquitin assembly complex (LUBAC), and play a role in IL-1- and TNF α -mediated signalling pathways (Walczak et al., 2012).

As well as ubiquitin chain topology, the length of the polyubiquitin chain also influences ubiquitin signalling. For proteasomal degradation the target protein needs to be sufficiently polyubiquitinated (a chain of at least four ubiquitins) (Pitrowski et al., 1997). Alternatively, ubiquitination can proceed with a single ubiquitin (monoubiquitination). This conjugation to a single lysine residue has been implicated in many non-proteolytic cellular processes including membrane trafficking, transcriptional regulation by histone modification and signal transduction pathways (Hicke, 2001, Sun and Allis, 2002, Wada et al., 2009). Ubiquitin chains are diverse structures with chains of different lysine linkage topology and lengths determining the fate of the substrate protein. The specificity of the lysine residue and the decision between mono- or polyubiquitination of the substrate protein occurs at the level of E3 ligases, explaining why there are such large and mechanistically different families of E3s, and making ligases attractive therapeutic targets (David et al., 2011).

1.1.3 Deubiquitinating enzymes

An additional level in the regulation of ubiquitin signalling is provided by a diverse family of key proteins: deubiquitinating enzymes (DUBs). The E3-mediated conjugation of ubiquitin to a substrate protein is a reversible process due to the enzymatic activity of DUBs which can attack the carbonyl group and thus cleave the isopeptide bond linking ubiquitin-substrate, ubiquitin-ubiquitin lysines, or N-terminals (Figure 1.1) (Komander et al., 2009, Reyes-Turcu et al., 2009). DUBs are less well understood than E3 ligases but may be as functionally important. In many cases E3 ligases and DUBs associate with the same complexes thereby highly regulating the ubiquitination/deubiquitination of the substrate as well as regulating the available pool of free ubiquitin to maintain ubiquitin homeostasis (Ventii and Wilkinson, 2008). This counter-regulation is a crucial mechanism in ubiquitin signalling and allows several functional roles of DUBs including cleaving ubiquitin precursor proteins, recycling ubiquitin, preventing proteins from degradation (e.g. by the removal of K48-linked chains), or modulating signalling through the editing or removal of ubiquitin chains (Figure 1.2) (Komander et al., 2009, Reyes-Turcu et al., 2009).

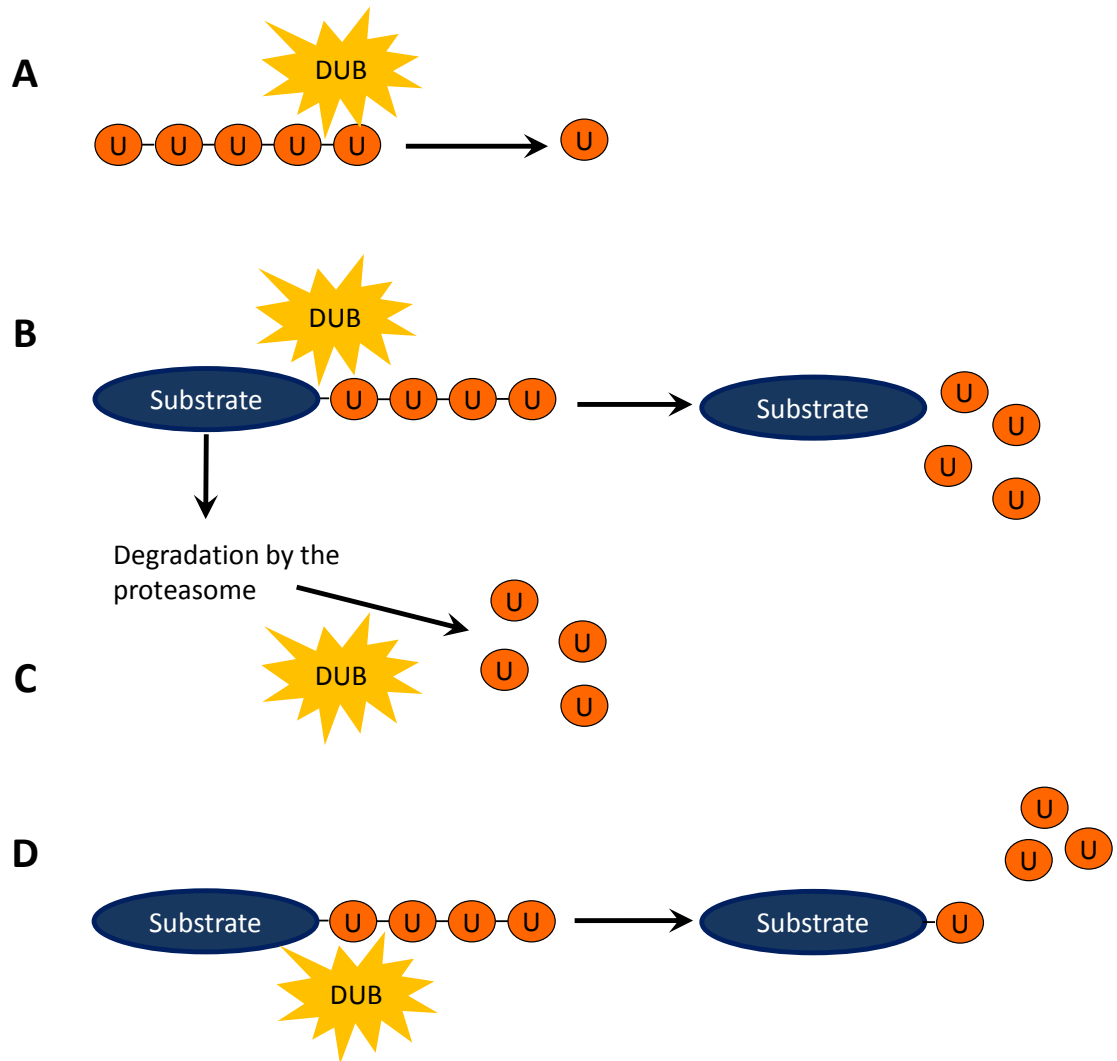


Figure 1.2. General functions of DUBs. A) Cleavage of ubiquitin precursors. B) Removal of degradative ubiquitin (most commonly K48-linked chains) to prevent degradation by the proteasome. C) Recycling of ubiquitin. D) Editing of non-degradative chains (e.g. K63-linked chains) in signal transduction pathways. The orange circles represent ubiquitin. Yellow stars represents DUBs. Adapted from Komander et al. (2009).

1.1.3.1 DUB families

There are five families of DUBs categorised by their catalytic domain architecture: one metalloprotease JAB1/MPN/Mov34 (JAMM) family, and four cysteine proteases: the ubiquitin C-terminal hydroxylase (UCH), Ovarian Tumour protease (OTU), Machado-Joseph disease protease (MJD), and ubiquitin-specific protease (USP) families. The papain-like cysteine protease families are characterised by a catalytic triad: usually an aspartic acid, histidine and cysteine residue. In most cases, the aspartic acid polarises the histidine which lowers the pKa of the catalytic cysteine facilitating a nucleophilic attack on the carbonyl group for the hydrolysis of the isopeptide bond between ubiquitins or ubiquitin-substrate (Komander et al., 2009). In contrast to this, the JAMM family are a group of zinc metalloproteases in which a catalytic zinc atom is attached to two histidine, one aspartate and one polarised water molecule for activity in carrying out a nucleophilic attack on the isopeptide bond (Ambroggio et al., 2004).

1.1.3.2 The USP family and their regulation

DUBs play a key role in diversifying ubiquitin signalling by displaying selectivity for substrates and types of ubiquitin chains. As such, DUBs themselves use multiple mechanisms for the regulation of their specificity and activity.

The USPs are the largest family of DUBs, with over 60 USPs identified. These are characterised by the conserved catalytic core containing cysteine and histidine (Cys and His) boxes necessary for the enzymatic activity of USPs. Besides the catalytic domain, DUBs contain a unique modular combination of other internal domains which vary between DUBs and may regulate their activity. Out of all DUBs, USPs contain the biggest array of additional domains that frequently include: ubiquitin-binding domains (UBDs); ubiquitin-like (UBL) domains, which are involved in recruitment to the proteasome and both inhibition and activation of USP catalysis; domain present in USPs (DUSPs), which contribute to substrate recognition; and zinc-finger (ZnF) domains, which can regulate USP catalytic activity (Faesen et al., 2012, Bonnet et al., 2008, Clague et al., 2013).

Although the cysteine protease families of DUBs share a mechanism for catalytic activity, individual DUBs within these families may require rearrangement of the catalytic site, action of other DUB domains, or interactions with non-substrate proteins to become fully active. USP7 (also known as herpesvirus-associated USP, HAUSP) has several identified substrates involved in tumour suppression, DNA damage and immune responses. For the hydrolysis of the isopeptide bond between ubiquitin and the substrates of USP7, the

residues in the catalytic site triad must first be realigned upon ubiquitin binding (Hu et al., 2002). This conformational change is achieved through USP7 C-terminal UBL domains (HUBLs). In its active state, USP7 UBLs interact with the catalytic domain to promote affinity for ubiquitin binding and increase activity (Faesen et al., 2011). However, not all USPs possess inactive conformations; they generally feature accessible and active catalytic sites and are regulated by other mechanisms.

Another mechanism of regulation for USP DUBs can be through protein-protein interactions. USP1, a DUB involved in the deubiquitination of monoubiquitinated enzymes in the DNA damage response, directly associates with the WD40-containing protein, USP1 associated factor 1 (UAF1). UAF1 forms a complex with USP1 which is essential for the catalytic activity of USP1 and additionally functions to maintain its stability (Cohn et al., 2007). UAF1 and other WD40-containing proteins have also been documented to interact with many other USPs, likely to represent a mechanism for their regulation (Villamil et al., 2013).

Like a wide number of proteins, the function of DUBs can also be regulated by PTMs. Monoubiquitination, sumoylation, phosphorylation, methylation and acetylation can all be involved in the regulation of proteins. The phosphorylation of USP1 on serine (Ser) 313 allows the interaction of UAF1 with USP1 for the stimulation of its catalytic activity (Villamil et al., 2012). Conversely, USP25 is sumoylated with small ubiquitin-related modifiers 2/3 (SUMO2/3) within its ubiquitin interaction motifs (UIMs) to inhibit the affinity of USP25 for binding to ubiquitin and subsequently its activity, demonstrating that PTMs can impair or enhance the activity of USPs (Meulmeester et al., 2008).

The regulation of USP DUB activity has been characterised in multiple studies, a fraction of which have been touched upon as examples here. It appears that USPs can be regulated through their subset of domains, protein-protein interactions and PTMs (or through a combination of PTMs), though these have been ascertained for only a few well-studied DUBs. The mechanism of regulation for the majority of DUBs still has a fairly limited understanding and the full spectrum of DUB substrates remain unidentified. However, it is clear the regulation of these proteins is extensive and there is most likely cross-talk between the different mechanisms to keep the tight regulation necessary for ubiquitin signalling.

1.1.3.3 Specificity of DUBs

There are many levels to the specificity of DUBs which allow them to recognise substrates and individual polyubiquitin chain types.

One feature that is shared between all DUBs is the binding of ubiquitin and the cleavage of the isopeptide bond by the DUBs catalytic site. DUBs are able to cleave ubiquitin chains from the proximal or distal ends of the chain, or in the middle. They work by binding ubiquitin to their ubiquitin-binding sites and it is the conserved ubiquitin C-terminal sequence, Leu-Arg-Leu-Arg-Gly-Gly, which DUBs are able to recognise and distinguish from molecules that are ubiquitin-like, including SUMO. (Drag et al., 2008).

The versatility of ubiquitin's ability to form topologically different polyubiquitin chains via its different lysines suggests DUBs assume specificity in recognising these different polyubiquitin chains. Studies have profiled DUB specificity for polyubiquitin chain topology and have identified that most DUBs can discriminate particular linkages. Although some show broad specificity for recognising different polyubiquitin linkages, others show high specificity preferences for certain types of linkages (McGouran et al., 2013). DUBs are also able to cleave ubiquitin from monoubiquitinated substrates and cleave unanchored ubiquitin chains. USP5 is able to disassemble unanchored ubiquitin chains by cleavage of one ubiquitin at a time from the proximal end. It works through recognition of a C-terminal Gly-Gly motif of the unanchored ubiquitin chains by its ZnF-UBP domain as part of maintaining ubiquitin pools (Reyes-Turcu et al., 2006). DUBs also show specificity towards specific protein substrates and may exert their specificity in part by acting at distinct subcellular locations (Komander et al., 2009).

1.1.4 The importance of proteolytic and non-proteolytic ubiquitin signalling in disease

The specificity, diversity and tight regulation in ubiquitin signalling mean that any aberrations or dysregulation of ubiquitin signalling, or the vast array of proteins involved in ubiquitin signalling, can be highly detrimental to cellular functioning and is consequently implicated in the development of many diseases and pathologies (Kessler, 2013, Popovic et al., 2014).

1.1.4.1 Ubiquitin signalling in neurodegenerative diseases

Ubiquitination was initially discovered as targeting proteins for degradation by the proteasome. Although it is now known that ubiquitination is also involved in many non-

proteolytic processes, its role in the degradation of proteins is still significant. The UPS controls the degradation and turnover of abnormal or short-lived proteins and changes in its function can lead to the accumulation of protein aggregates, a characteristic of neurodegenerative diseases including Huntington's (HD), Parkinson's (PD) and Alzheimer's diseases (AD), which can disrupt homeostasis within the cells and the function of neurons (Sherman and Goldberg, 2001). For example, HD is a polyglutamine disorder which feature an expanded polyglutamine tract in the *HTT* gene leading to aggregation of misfolded proteins forming inclusion bodies which result in the progressive pathogenesis of HD. As the UPS is responsible for the removal of misfolded proteins, aggregation of these as in HD suggest UPS impairment and disruption of ubiquitin homeostasis (Ortega and Lucas, 2014).

1.1.4.2 Ubiquitin signalling in cancer

Additionally, aberrations within the UPS can underlie the pathology of many cancers through changes in the turnover of oncoproteins or tumour suppressors (Satija et al., 2013). Many oncoproteins and tumour suppressors can be subject to ubiquitination by E3 ligases to alter their stability. This can be emphasised using the example of the tumour suppressor p53, mutations of which are common in many cancers. UPS-mediated p53 regulation includes several E3 ligases: the first E3 identified to directly ubiquitinate p53 and promote its subsequent degradation was the RING finger protein Mdm2 (Haupt et al., 1997). In counterplay with this, USP7 deubiquitinates p53, preventing Mdm2-mediated degradation and stabilising the protein, crucial for its tumour suppressing functions on apoptosis and inhibition of cell proliferation (Li et al., 2002). Imbalance in this regulation can lead to the progression of tumour development with overexpression of Mdm2 reported in many cancers.

Non-proteolytic functions of ubiquitin conjugation can also become aberrant leading to the disruption of many cellular functions including abnormal activation and inactivation of cell signalling pathways (Satija et al., 2013). Pathways which are oncogenic or tumour suppressing can both be regulated by ubiquitin signalling. The nuclear factor κ B (NF- κ B) signalling pathway is predominantly associated with inflammation but the transcription factor NF- κ B not only induces many inflammatory genes but also many pro-survival and pro-proliferative genes all of which can contribute to the development of tumours. Constitutive activation of NF- κ B can lead to chronic inflammation and drive the expression of these NF- κ B target genes to promote the progression of tumours (Pal et al.,

2014). Ubiquitination in NF- κ B signalling has been the subject of many studies as it plays a crucial part in its activation (Napetschnig and Wu, 2013, Chen, 2012). In addition to altering the stability of proteins involved in signalling, polyubiquitination is key in recruiting proteins at various stages of the NF- κ B signalling cascade (Iwai, 2014). As with proteolytic ubiquitination, the interplay between E3 ligases and DUBs in this pathway play a critical part in its tight regulation and disruption to this may lead to development of cancers. Amongst others, members of the TNF receptor-associated factor (TRAF) family, which have an E3 ligase RING domain, are involved in NF- κ B signalling and mutations in at least two of these (which cause constitutive activation of the NF- κ B pathway) have been identified in multiple myeloma, an example highlighting the importance of ubiquitin signalling in the NF- κ B pathway in the pathogenesis of cancer (Keats et al., 2007).

The examples given here emphasise the tight regulation involved in ubiquitin signalling and the critical importance of this in maintaining cellular homeostasis to avoid aberrations preceding many disease pathologies. Although components of ubiquitin signalling, particularly many E3 ligases and DUBs, have become increasingly studied in recent years full knowledge of the regulation and function of these enzymes has not yet been established. Further understanding into the molecular mechanisms of ubiquitin signalling could provide openings for potential therapies for these diseases.

1.2 Ubiquitin-specific protease 4 (USP4)

1.2.1 Discovery of USP4

The DUB USP4 was first identified during a survey of mouse genes and, due to detection of a protein product in fractionated nuclei and a putative nuclear localisation signal (NLS), it was originally designated ubiquitous nuclear protein (Unp). It was described as a proto-oncogene with similarities to the human oncogene *tre-2/TRE17* (USP6) and localised to mouse chromosome 9 (Gupta et al., 1993, Gupta et al., 1994). USP4 has been shown to have oncogenic properties as consistently elevated expression levels of the human *USP4* were observed in primary lung tumour cell lines and lung adenocarcinomas (Gray et al., 1995). Later, reduced *USP4* expression was reported, however this was in small cell lung carcinoma cells (Frederick et al., 1998). Further, the human *USP4* gene was mapped to chromosome 3p21.3, a locus previously implicated in lung cancer, and associations of USP4 with the retinoblastoma protein (pRb), a tumour suppressor central in cell cycle

regulation, were established (Blanchette et al., 2001, Desalle et al., 2001). Later, transcriptional profiling studies found increased *USP4* expression in adrenocortical carcinomas, further implicating *USP4* in cancers (Velázquez-Fernández et al., 2005, Laurell et al., 2009).

1.2.2 Structure and localisation of USP4

USP4 is a cysteine protease USP containing the characteristic Cys and His residues within the catalytic domain essential for its deubiquitinating activity. Additionally, a number of other functional domains within *USP4* have been delineated as depicted in Figure 1.3. *USP4* contains two UBL domains, one N-terminal to and one within the catalytic domain. The function of the catalytic UBL domain is incompletely defined but the N-terminal UBL domain binds to the S9 subunit of the proteasome meaning this domain can be involved in mediating the interactions of *USP4* (Zhao et al., 2012a). This UBL domain is found juxtaposed with the DUSP domain and is essential for the catalytic activity of *USP4*. Through binding to the catalytic site, DUSP-UBL enhances ubiquitin dissociation, preventing retention of ubiquitin after hydrolysis of the isopeptide bond with the substrate and thus allowing full catalytic turnover of *USP4* (Clerici et al., 2014). The most closely related USP to *USP4* is *USP15*, the DUSP-UBL domain tandem of which is conserved between both of these USPs (Harper et al., 2011). In addition, the *USP4* DUSP domain has been implicated in substrate interactions. It is required for direct interaction with Sart3, a recycling factor of components of the spliceosome (Song et al., 2010). Although the function of the domains of *USP4* are not well understood they are likely involved in mediating protein interactions, as with S9 and Sart3, and in regulating the activity of *USP4*, as appears to be the case in the novel method of DUSP-UBL-mediated catalytic activity.

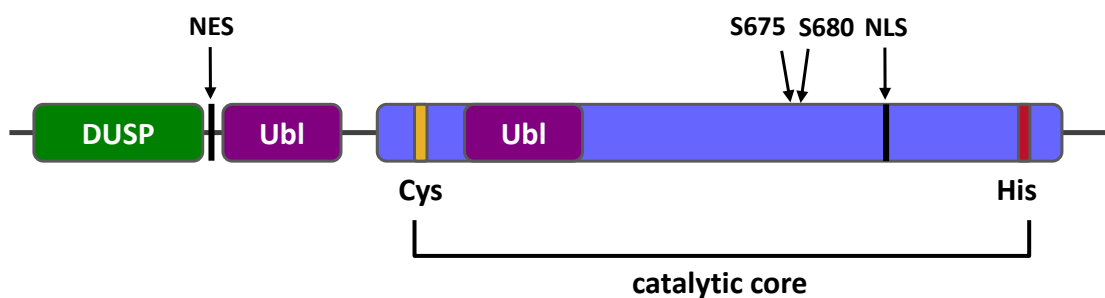


Figure 1.3. Domain structure of *USP4*. Schematic of the Cys and His domains comprising the catalytic core and DUSP and UBL domains. Also shown are nuclear import and export signals, NLS and NES respectively. Figure adapted from Gray, unpublished.

Although originally designated a nuclear protein, USP4 was also found located to the cytoplasm (Frederick et al., 1998). Later, nuclear export and nuclear import signals (NES and NLS, respectively) were identified and it was demonstrated to exhibit nucleocytoplasmic shuttling properties (Frederick et al., 1998, Soboleva et al., 2005). The equilibrium between nuclear and cytoplasmic USP4 appears to differ depending on the level of expression and also between cell types, possibly explaining why initial findings identified nuclear USP4 and later identified USP4 localised cytoplasmically. Shuttling between the nucleus and cytoplasm is essential for the correct and efficient functioning of many proteins. It is a highly regulated process requiring the active transport of proteins through the nuclear envelope aided by interactions with the karyopherin- β family of transporters: importins recognise specific NLSs for nuclear import and exportins recognise specific NESs for nuclear export (Sorokin et al., 2007). The physiological relevance of USP4 subcellular localisation is not fully understood but the nucleocytoplasmic shuttling of USP4 may be central to its function and studies have identified instances for which this appears to be the case. Protein interactions can influence relocation within the cell and it has been documented that complex formation of USP4 with Sart3 is sufficient to trigger the translocation of USP4 from the cytoplasm to the nucleus where it can deubiquitinate Prp3, a component of the spliceosomal U4 small nuclear ribonucleoprotein (snRNP) (Song et al., 2010).

1.2.3 Substrates of USP4

The discovery of USP4 and its potential link to some cancers led to increased interest in the enzyme and reports identifying substrates for USP4 and of USP4 acting in multiple cellular systems have since emerged. Through its catalytic activity USP4 deubiquitinates many of its substrates, but for some the catalytic activity is not involved and their interaction must have other functions. Amongst its substrates are the spliceosome U4 component Prp3 which is deubiquitinated by USP4 to regulate the spliceosome by controlling the stability of spliceosome components, snRNPs U4/U6.5, upon complex formation with Sart3 (as discussed above) (Song et al., 2010); the G protein-coupled receptor A_{2A} adenosine receptor which becomes enhanced at the cell surface after deubiquitination by USP4 (Milojević et al., 2006); phosphoinositide-dependent kinase 1 (PDK1) whose monoubiquitination is modulated by USP4 (Uras et al., 2012); and the S9/Rpn6 subunit of the proteasome, potentially to regulate protein turnover or the stability or function of the proteasome (Zhao et al., 2012a).

Interestingly, as well as deubiquitinating some of the substrates mentioned above, USP4 has also been reported to deubiquitinate itself. Wada and Kamitani (2006b) reported ubiquitination, possibly monoubiquitination, of USP4 by the autoantigen E3 ligase Ro52 which is reversed by deubiquitination of itself. Conversely, Ro52 can ubiquitinate itself which can be reversed by deubiquitination by USP4 (Wada and Kamitani, 2006a, Wada et al., 2006). The functional consequence of this auto-ubiquitinating/deubiquitinating complex is not clear but many E3 ligases and DUBs are found in complexes, presumably for a higher level of regulation, and so this heterodimeric complex may be involved in the ubiquitination/deubiquitination of other substrates.

Recently, a study has explored the role of ubiquitination, particularly USPs, involved in neuronal morphogenesis and connectivity in the brain. This is an area for which regulation by ubiquitin signalling mechanisms is only just beginning to be elucidated. Using an USP4 RNAi plasmid generated using short hairpin RNA (shRNA), this study determined an interesting role for USP4 in granule neuron axon and dendrite development: USP4 promoted growth of axons whilst the growth of dendrites was restricted. An *in vivo* RNAi approach revealed that indeed USP4 is involved in proper granule neuron dendrite development in the cerebellum. Although a role for USP4 in neuronal morphogenesis has been described, this is one of the first studies exploring USPs in this field and as such the mechanism of action or possible substrate targets for USP4 have not yet been ascertained (Anckar and Bonni, 2015).

1.2.4 Specificity of USP4

Ubiquitin is synthesised as a fusion protein, either to other ubiquitins in a polyubiquitin chain or the N-terminus of one of two ribosomal proteins. It is encoded by four genes: *UBC* and *UBB*, encoding polyubiquitin precursors; *UBA52*, encoding a fusion protein of ubiquitin and ribosomal protein L40; *UBA80*, encoding a fusion protein of ubiquitin and ribosomal protein S27a (Shabek and Ciechanover, 2010). A key function of DUBs is the post-translational cleavage of these precursor proteins to process them for the generation of free ubiquitin to allow conjugation to substrate proteins or ubiquitin (Komander et al., 2009). Biological techniques have utilised ubiquitin fusion proteins as it allows the synthesis of a protein with any residue at its N-terminus. USP4 is able to cleave ubiquitin-proline fusions, a fusion which is usually inefficiently cleaved and does not occur naturally. This gives rise to the possibility that USP4 may be able to cleave ubiquitin

precursors or ubiquitin-like proteins and suggests some flexibility in the USP4 catalytic domain, consistent with its broad cleavage activity (Gilchrist et al., 1997).

Many DUBs show a broad specificity for recognising different ubiquitin linkage topologies whereas others have a greater affinity for specific linkages. In a profiling study to examine DUB selectivity for recognising different ubiquitin linkage Di-ubiquitin probes were engineered to mimic linkages for all seven lysines and for linear ubiquitination. USP4 was a DUB identified by mass spectrometry from immunoprecipitates of cell lysates labelled with Di-ubiquitin probes. It was found to show reactivity for all seven types of ubiquitin linkage, most notably for the linear linkage of which 20% of total USP4 bound to out of all the different linkage topologies (McGouran et al., 2013). The most studied ubiquitin linkages topologies are for K48 and K63 linkages. Through incubation of USP4 with K48 and K63 polyubiquitin chains it has been shown that USP4 can hydrolyse both types of these linkages with equal efficiency. This may be important for its functions in cell signalling pathways where these types of linkages are commonly found to regulate pathways by affecting the stability and recruitment of proteins (Zhao et al., 2009).

1.3 Cell signalling pathways

USP4 has been reported to have substrates in many cell signalling pathways including the p53 pathway, Wnt/ β -catenin pathway, transforming growth factor- β (TGF- β) pathway, retinoic acid-inducible gene I (RIG-I) pathway and NF- κ B pathway, all of which will be discussed further in this section (Zhang et al., 2011, Zhao et al., 2009, Fan et al., 2011b, Zhang et al., 2012, Wang et al., 2013).

1.3.1 The p53 pathway

The transcription factor p53 is critical in cellular regulation; activation in response to stress induces the expression of genes that prevent cell proliferation or induce DNA repair, senescence or apoptosis. Mutations in the p53 tumour suppressor gene, and its regulatory genes which inactivate p53, are widely found in human cancers (Toledo and Wahl, 2006). p53 is modulated by a number of PTMs to regulate its stability, subcellular localisation or transcriptional regulation and ubiquitination plays a large part in this mechanism of p53 control. Under normal conditions, p53 is subject to constant proteasomal degradation to keep the tumour suppressor at low levels but constitutively expressed. Under stressed conditions, this is inhibited and p53 is stabilised. Many E3

ligases are associated with the regulation of p53 through its proteasomal degradation including Mdm2, ARF-binding protein 1 (ARF-BP1), COP1 and Pirh2 (Chao, 2015). The predominant E3 ligase and the first to be identified is the RING finger E3 ligase, Mdm2, which directly ubiquitinates p53 for nuclear export and subsequent degradation (Haupt et al., 1997, Lohrum et al., 2001). Mdm2 is also a transcriptional target of p53, establishing a feedback loop in the negative regulation of p53 function (Barak et al., 1993). The degradation of p53 can be reversed by a number of DUBs including USP7 which directly deubiquitinates p53, preventing degradation and stabilising the protein (Li et al., 2002). Further, USP7 also interacts with and deubiquitinates Mdm2 resulting in an interesting feedback loop. Under normal conditions, USP7 has a higher affinity for and stabilises Mdm2, but under stressed conditions the DNA damage-induced ATM-mediated phosphorylation of Mdm2 causes an affinity switch. The result of this is the destabilisation of Mdm2 and therefore stabilisation of p53 which enables p53-mediated apoptosis, inhibition of cell proliferation or DNA repair (Li et al., 2004, Meulmeester et al., 2005).

Other DUBs can also regulate p53 activity directly by interacting with p53, or indirectly without any direct interaction or deubiquitination of p53. USP4 is an important regulator of p53 through interactions with the HECT domain-containing p53 E3 ligase, ARF-BP1 (Zhang et al., 2011). ARF-BP1 is a critical mediator in tumour suppression: it directly induces the ubiquitination of p53 but its ubiquitin ligase activity can be inhibited by the binding of the ARF tumour suppressor to ARF-BP1 thereby stabilising p53 (Chen et al., 2005). USP4 deubiquitinates ARF-BP1 thereby stabilising the ligase and consequently leading to the ubiquitination and degradation of p53 (Figure 1.4). Moreover, mouse embryonic fibroblasts (MEFs) from *Usp4* null mice exhibit enhanced apoptosis as well as a reduced cell growth and enhanced premature senescence, consistent with increased levels of p53 (Zhang et al., 2011).

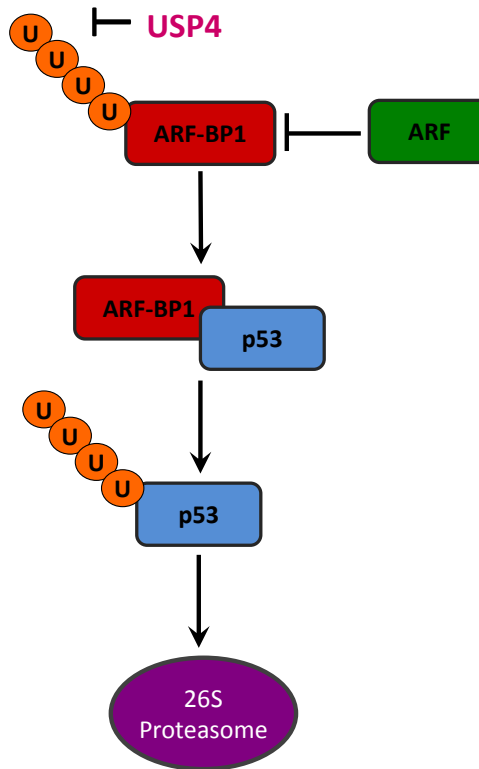


Figure 1.4. A model of USP4 inhibition of p53 via interaction with ARF-BP1. USP4 deubiquitinates ARF-BP1 leading to its stabilisation and subsequent ubiquitination and proteasomal degradation of p53.

1.3.2 The Wnt/ β -catenin pathway

The Wnt signalling pathway is involved in embryonic developmental processes and tissue homeostasis and dysregulation of this pathway has been linked to diseases including osteoporosis and cancers (MacDonald et al., 2009). Members of the Wnt family are capable of activating non-canonical and canonical Wnt signalling pathways and it is the latter, the Wnt/ β -catenin pathway, which is the most studied and which USP4 is reported to regulate (Zhao et al., 2009).

Wnt can bind to the seven transmembrane cell surface receptors known as Frizzled proteins. This, and interactions with the co-receptor, low density lipoprotein receptor related protein 5 or 6 (LRP5/6), can induce the canonical Wnt/ β -catenin signalling pathway (He et al., 2004). Upon binding of a ligand to a Wnt receptor, the central player in the canonical pathway, β -catenin, accumulates and translocates to the nucleus to exert transcriptional effects through various interactions. There are many components of the Wnt cascade which regulate the stability of this central player: components which form the destruction complex are able to cause the degradation of β -catenin. The tumour suppressor adenomatous polyposis coli (APC), the scaffolding protein Axin, and two Ser/threonine (Thr) kinases: casein kinase 1 (CK1) and glycogen kinase 3 (GSK3) all comprise the destruction complex. In the absence of a ligand bound to Wnt receptors, APC and Axin adhere to both β -catenin and CK1 and GSK3 β of the destruction complex (Kim et al., 2013). These kinases promote the phosphorylation of specific Ser/Thr residues at the N-terminus of β -catenin allowing recognition by the E3 ligase complex known as Skp1, Cullin1, F-box containing complex, β -transducin repeat containing protein (SCF $^{\beta$ -TrCP) (Latres et al., 1999). Consequently, β -catenin is polyubiquitinated by SCF $^{\beta$ -TrCP and targeted for proteasomal degradation (Figure 1.5) (Aberle et al., 1997).

Induction of the Wnt signalling pathway through binding of a ligand induces CK1 and GSK β -mediated phosphorylation of the co-receptor LRP6. This recruits Axin to the LRP5/6 receptor complex thereby disassembling the destruction complex and causing its dissociation from β -catenin. Subsequently, this leads to the accumulation of β -catenin and its translocation to the nucleus to enable binding to transcription factors, including T-cell factor (TCF) and lymphoid enhancer factor (LEF), to trigger transcriptional activation of an array of Wnt target genes (Kim et al., 2013). Additional factors are involved in this signalling pathway to keep β -catenin highly regulated, including the MAP kinase-related Nemo-like kinase (Nlk) which acts as a negative regulator of canonical Wnt signalling.

Nlk interacts with a TCF/LEF complex bound to β -catenin to suppress transcriptional activity through Nlk-mediated phosphorylation of Ser/Thr residues on the TCF/LEF complex (Figure 1.5) (Ishitani et al., 2003).

Although ubiquitin signalling has not been widely studied in terms of the Wnt signalling pathway, it does play an important role in the control of β -catenin which, as well as being targeted for degradation, can be ubiquitinated by K11 or K29 polyubiquitin chains for the promotion of nuclear localisation and the upregulation of β -catenin levels (Hay-Koren et al., 2011). USP4 has been reported to be a negative regulator of canonical Wnt signalling (Zhao et al., 2009). Downstream of the destruction complex, USP4 interacts with Nlk and translocates to the nucleus to allow USP4 interaction with, and deubiquitination of, the predominantly nuclear transcription factor TCF4. Overall, loss of USP4 results in an increase in Wnt signalling but the consequences of TCF4 ubiquitination/deubiquitination and how USP4 may regulate Wnt signalling in this way are not clear. TCF4 can be subjected to ubiquitin-mediated degradation, but USP4 does not affect TCF4 stability and prevention of the ubiquitin-mediated degradation of TCF4 would be expected to enhance Wnt target gene activation, not consistent with downregulation of Wnt by USP4. USP4 may deubiquitinate non-degradative polyubiquitin chains on TCF but there are currently no studies to determine this (Zhao et al., 2009).

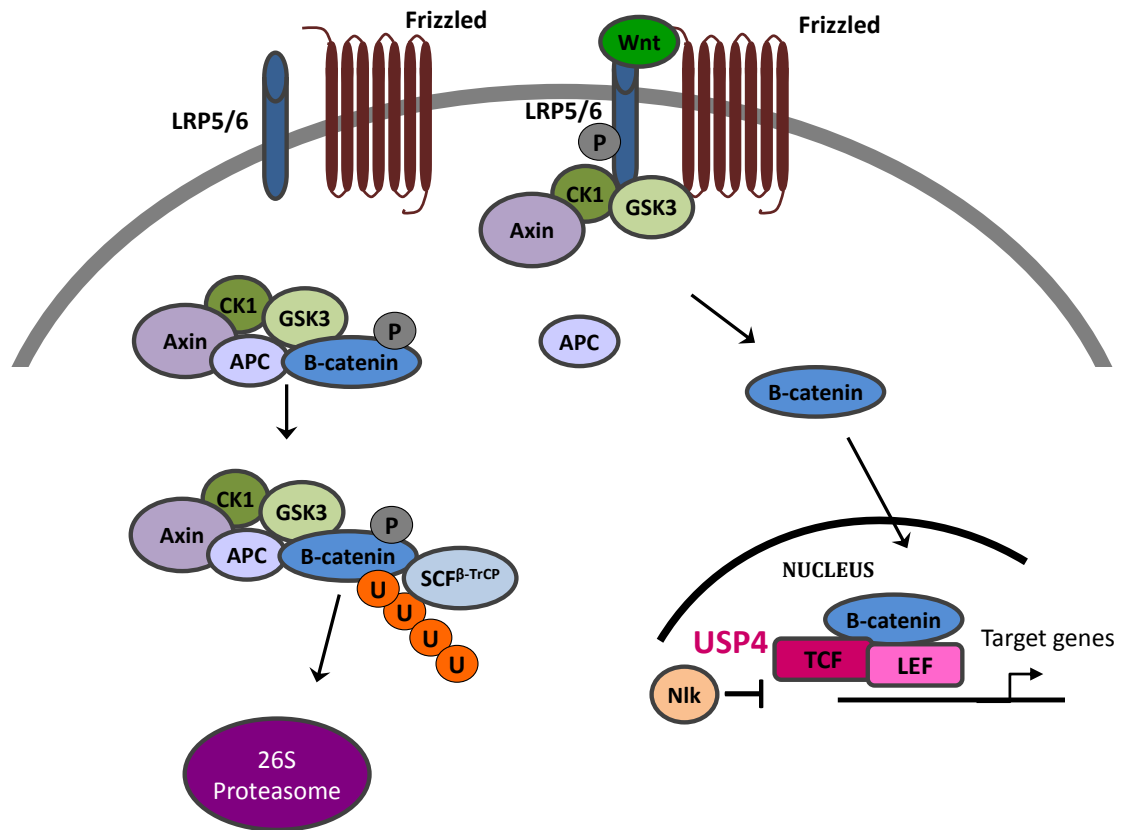


Figure 1.5. The Wnt/ β -catenin pathway. With absence of Wnt (left): Axin, APC, CK1 and GSK3 form the destruction complex which binds to and phosphorylates β -catenin for ubiquitination and degradation. Binding of Wnt to the Frizzled receptor (right) initiates the dissociation of the destruction complex. β -catenin is then free to translocate to the nucleus where it accumulates and binds to transcription factors TCF and LEF for the transcription of target genes. Nlk inhibits this transcription and recruits USP4 (highlighted in pink) for deubiquitination of TCF4. Grey circles represent phosphorylation. Orange circles represent ubiquitin.

1.3.3 The TGF- β pathway

The TGF- β signalling pathway regulates biological processes including proliferation, differentiation, apoptosis, and migration, invasion and metastasis in cancers (Katz et al., 2013). The TGF- β pathway involves type I and type II transmembrane receptors (T β R-I and T β R-II) which exist as homodimers on the cell surface but form heterotetramers upon binding of TGF- β . Both receptors have an N-terminal domain which binds the ligand and a C-terminal Ser/Thr kinase domain. Additionally, T β R-Is have a Gly/Ser-rich (GS) domain which becomes phosphorylated by the constitutively active T β R-II Ser/Thr kinase activity to activate the type I receptors (Wrana et al., 1994). A family of Smad proteins mediate TGF- β signalling. Upon T β R-I activation receptor-regulated Smads (R-Smads) including Smads 1, 2, 3, 5 and 8, themselves become activated by phosphorylation. The phosphorylated R-Smads dissociate from the receptor complex and form a heterooligomer complex with a common partner for all R-Smads, Smad4 (Co-Smad) (Feng and Derynck, 2005). This allows translocation of the complex and accumulation in the nucleus for the regulation of the transcription of an array of target genes (Figure 1.6). Factors induced by TGF- β -activated transcriptional regulation include Smad6 and Smad7 (I-Smads) which are inhibitory in TGF- β signalling (Imamura et al., 2013).

Like many cell signalling pathways, TGF- β signalling is also under tight regulation by ubiquitination. Smad ubiquitin regulatory factor 1 and 2 (Smurf1 and 2) are HECT E3 ligases involved in TGF- β signalling. Smurf1 and Smurf2 interact with I-Smads to induce their nuclear export to the TGF- β receptors. This targets the receptors and I-Smads for ubiquitin-dependent proteasomal degradation. In addition, Smurf1 and Smurf2 ubiquitinate R-Smads to mediate their proteasomal degradation. The phosphorylation of R-Smads allows recognition of Smad1 and Smad5 by Smurf1 and Smad1 and Smad2 by Smurf2 for their polyubiquitination (Zhang et al., 2001). Non-degradative ubiquitination is also implicated in TGF- β regulation. Smad4 is monoubiquitinated by the E3 ligase Ectoderm/Tif1 γ , preventing complex formation with Smad2 and thereby inhibiting the TGF- β pathway (Dupont et al., 2009).

Equally, deubiquitination also regulates TGF- β signalling and some of the DUBs involved in this have been identified. It was actually the discovery of the FAM/USP9x as a DUB able to deubiquitinate Smad4 in TGF- β signalling which led to the knowledge that Smad4 monoubiquitination occurs in the control of the pathway (Dupont et al., 2009). USP15, a DUB sharing significant sequence similarity with USP4, regulates TGF- β

signalling through deubiquitination of both monoubiquitinated and Smurf1-induced polyubiquitinated R-Smads; Smad1 and Smad3. USP15 plays some role in regulating the stability of R-Smads but mainly enhances TGF- β signalling through the suppression of R-Smad monoubiquitination to enable R-Smad DNA-binding and promoter recognition required for full transcriptional activity in TGF- β signalling (Inui et al., 2011). Additionally, USP15 is recruited to the T β R-I complex by binding to Smad7 of the Smad7-Smurf2 complex. This allows USP15 to remove the Smurf2-mediated ubiquitination of T β R-I, thereby stabilising the receptor and resulting in enhanced phosphorylation and activation of R-Smads. At low concentrations of TGF- β stimulation USP15 counteracts the Smurf2-mediated degradation of T β R-I, but at higher TGF- β stimulation USP15 dissociates from Smad7 and therefore T β R-I, indicating there is a balance between USP15 and Smurf2 in regulating TGF- β signalling (Eichhorn et al., 2012). The stability of the T β R-I is also regulated by USP4 in the TGF- β signalling pathway, although it does this through utilisation of a different mechanism to USP15 (Aggarwal and Massagué, 2012). USP4 increases activation of TGF- β , Smad2 phosphorylation and Smad2-Smad4 complex formation. It directly interacts with the T β R-I intracellular domain to deubiquitinate and stabilise the receptor at the membrane, unlike USP15 which required interactions with Smad7 (Figure 1.6) (Zhang et al., 2012).

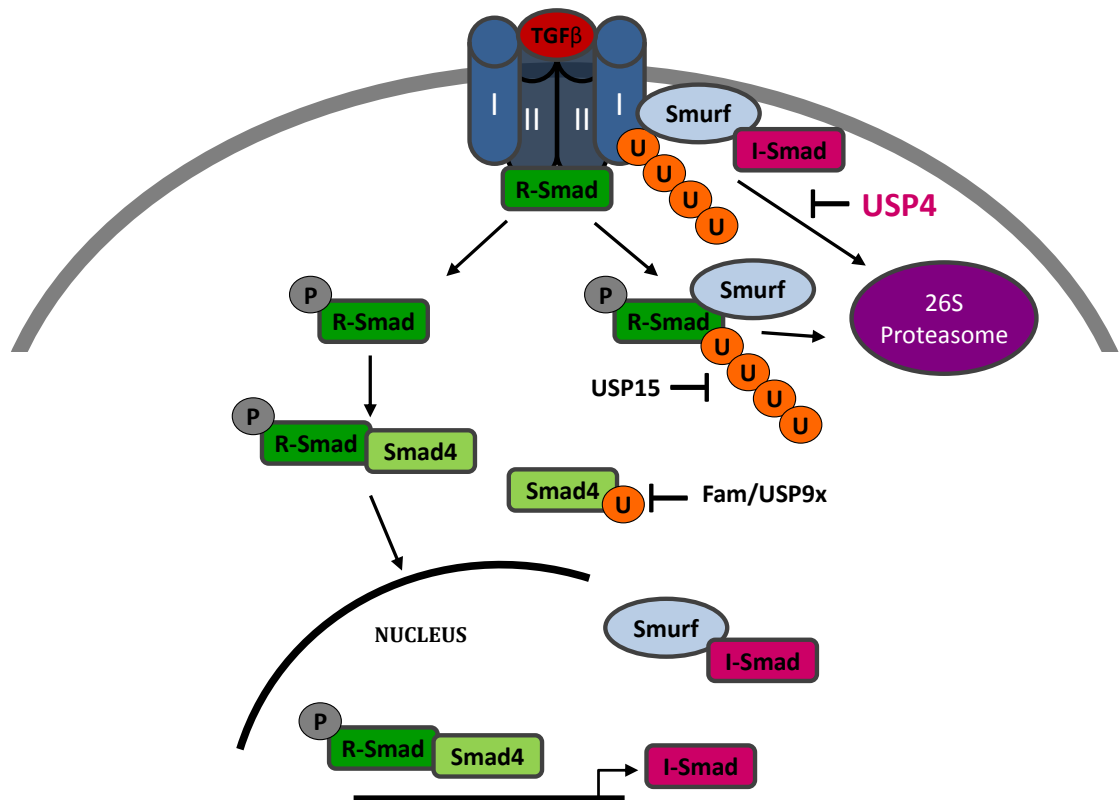


Figure 1.6. The TGF- β signalling pathway. Diagram of the cascade of events in the TGF- β pathway. Upon activation of the T β R-I, R-Smads (Smads 1, 2, 3, 5 and 8) are recruited and phosphorylated to trigger dissociation from the receptor complex allowing recruitment of Smad4 and translocation to the nucleus for the transcription of target genes, including I-Smads (Smad6 and Smad 7). For the negative regulation of the pathway, R-Smads are subject to K48-linked polyubiquitination by Smurf1 and Smurf 2 (Smurf) for degradation by the proteasome. Smurf also binds to I-Smads in the nucleus for transportation to the plasma membrane and the K48-linked polyubiquitination and subsequent degradation of T β R-I. This is reversed by USP4 (highlighted in pink). Grey circles represent phosphorylation. Orange circles represent ubiquitin.

1.3.4 The RIG-I pathway

Viral infection triggers the activation of innate immunity signalling pathways critical in the immune response. RIG-I-like receptors (RLRs) detect viral RNA in viral infection and initiate downstream signalling events to induce expression of cytokines and type I interferons (IFNs): IFN- α and IFN- β . RIG-I is a member of the RLR family, with domains including two N-terminal caspase recruitment domains (CARDs), a C-terminal domain (CTD) and a central DExD/H-box RNA helicase domain (Yoneyama et al., 2004). Upon detection of viral RNA by the receptor, such as Sendai virus (SeV) and vesicular stomatitis virus (VSV), the ATP-dependent conformational change of the CTD allows CARDs to interact with the adaptor protein mitochondrial antiviral signalling protein (MAVS or also known as IPS-I) (Yoneyama et al., 2015). The RING-finger E3 ligase tripartite motif 25 (TRIM25) catalyses the K63-linked polyubiquitination of RIG-I CARDs which is crucial in mediating the recruitment of MAVS and inducing RIG-I antiviral signal transduction (Gack et al., 2007). Interaction of MAVS with CARDs recruits signalling components including TANK-binding kinase 1 (TBK1) which results in the phosphorylation and dimerisation of the transcription factor IFN regulatory factor 3 (IRF3) (Seth et al., 2005). This leads to nuclear translocation of IRF3 for the transcriptional activation of IRF3-target genes including IFN- β and possibly matrix metalloproteinase 13 (MMP13) (Figure 1.7) (Radwan et al., 2013). Inhibitor of κ B (I κ B) kinase (IKK) complex components are also recruited to MAVS. The IKK complex is required for the activation of the transcription factor NF- κ B and so in this way RIG-I activation can also lead to the activation of NF- κ B target genes including many inflammatory cytokines (Figure 1.7) (Yoneyama et al., 2015, Seth et al., 2005). Ubiquitin signalling events are an important aspect in the tight regulation of RIG-I. As well as the K63-linked polyubiquitination of CARDs by TRIM25 to induce RIG-I activation, TRIM25 is itself regulated by K48-linked polyubiquitination to mediate its degradation to inhibit RIG-I activation. This can be reversed by USP15 (Pauli et al., 2014). RIG-I is also ubiquitinated with K48-linked polyubiquitin chains to mediate its proteasomal degradation in the negative regulation of this pathway. The RING-finger E3 ligase RNF125 interacts with RIG-I and, together with the E2 conjugating enzyme UbcH5c, catalyses its K48-linked polyubiquitination for the degradation of RIG-I and downstream suppression of IRF3 and NF- κ B (Arimoto et al., 2007). This K48-linked polyubiquitination of RIG-I can be reversed by the deubiquitinating activity of USP4

indicating that USP4 acts as a positive regulator of RIG-I signalling by stabilising RIG-I and enhancing RIG-I signalling (Wang et al., 2013).

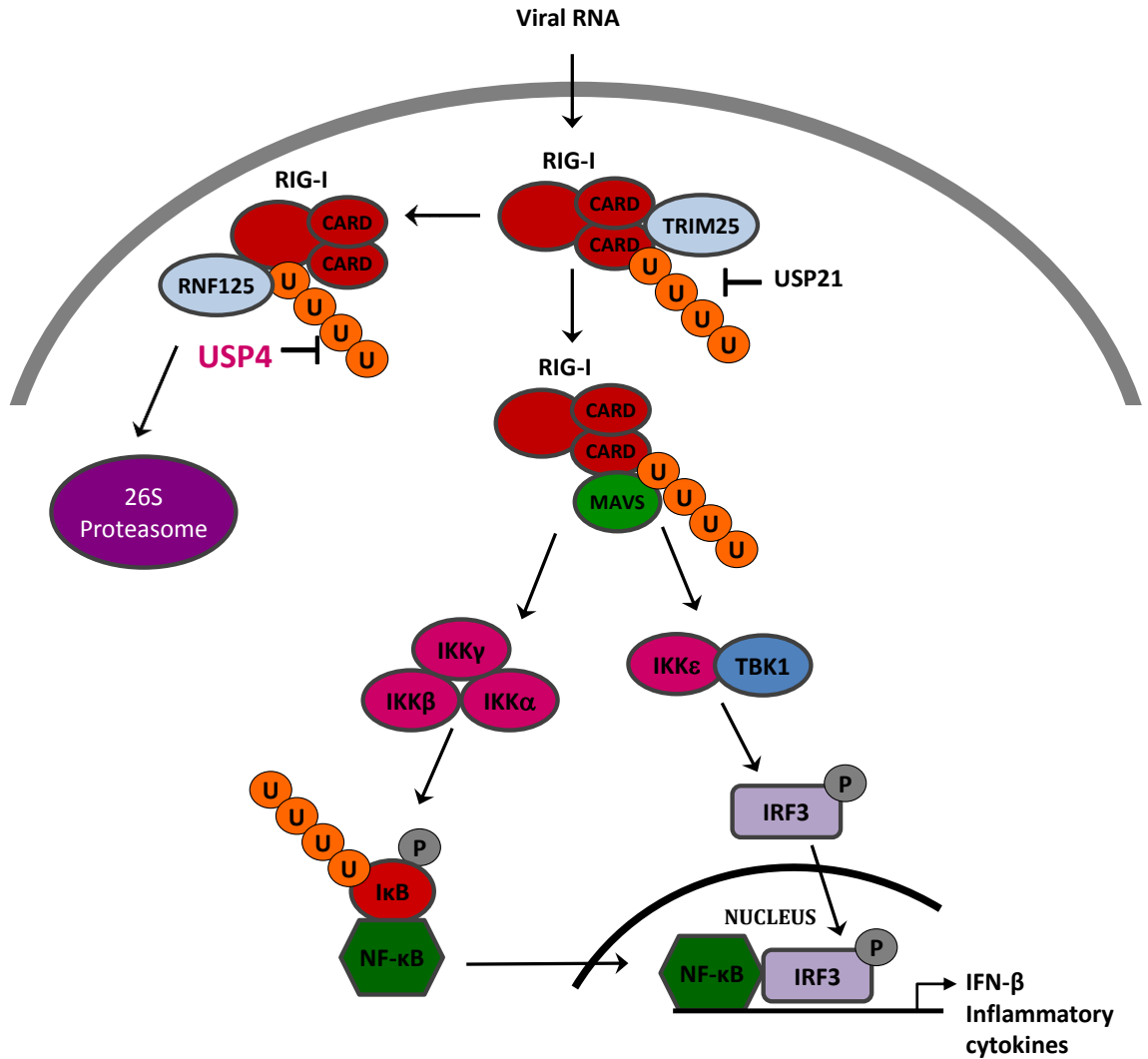


Figure 1.7. The RIG-I pathway. Detection of viral RNA by RIG-I leads to the TRIM25-mediated K63-linked polyubiquitination of RIG-I CARD domains and the recruitment of MAVS for the cascade of events leading downstream translocation of IRF3 and NF- κ B and the transcription of target genes. For the negative regulation of RIG-I signalling, RIG-I is polyubiquitinated with K48-linked chains to mediate its proteasomal degradation. USP4 is highlighted in pink. The phosphorylation of proteins is represented by grey circles. Ubiquitination of proteins is represented by orange circles.

1.3.5 The NF- κ B pathway

The NF- κ B pathway is immensely critical in inflammation, cell proliferation and apoptosis. Inappropriate activation of the NF- κ B signalling pathway is implicated in the pathogenesis of cancers and chronic inflammation (Pal et al., 2014). In unstimulated cells, I κ B proteins bind to a Rel-related transcription factor dimer of NF- κ B and sequester it to the cytoplasm by masking its NLS. Induction of NF- κ B leads to a cascade of events resulting in the phosphorylation, ubiquitination and degradation of I κ B. This exposes the NLS of NF- κ B and facilitates its translocation to the nucleus where the activation of NF- κ B induces the expression of a number of target genes including pro-survival and proinflammatory genes. Many adaptor proteins are involved in NF- κ B signalling which can interact to ensure tight regulation is maintained (Karin and Ben-Neriah, 2000). Ubiquitin signalling also plays a key part in this tight regulation: the determining step in NF- κ B activation is the phosphorylation of I κ B which is a prerequisite for its K48-linked polyubiquitination and degradation. This was the first ubiquitin-dependent proteolysis to be shown as an integral step in signal transduction but ubiquitination is prevalent throughout the pathway and non-proteolytic ubiquitination is important in the activation and recruitment of multiple NF- κ B adaptor proteins (Iwai, 2014).

1.3.5.1 The NF- κ B transcription factor

The highly conserved NF- κ B family of proteins share an N-terminal region Rel homology domain (RHD) and can form homo- or heterodimers of subunits p50, p52, p65 (also known as RelA), c-Rel or RelB. Both p50 and p52 are synthesised as precursor proteins, p105 and p100, which are proteolytically processed to p50 and p52, respectively (Karin and Ben-Neriah, 2000, Ghosh et al., 1998). Typically, non-canonical NF- κ B activation is mediated by RelB/p52 heterodimers and canonical NF- κ B activation, which will be the focus of this section, is mediated by p65/p50 heterodimers (Hayden and Ghosh, 2008). I κ Bs, including I κ B α , I κ B β and I κ B γ , are inhibitors of NF- κ B which bind to NF- κ B dimers to retain them in the cytoplasm. I κ Bs contain ankyrin repeats which interact with the RHD of NF- κ B to mask the NLS and prevent nuclear translocation in unstimulated cells (Karin and Ben-Neriah, 2000).

Induction of canonical NF- κ B activation can be achieved by a wide variety of stimuli including cytokines tumour necrosis factor α (TNF α), interleukin-1 (IL-1) or bacterial lipopolysaccharide (LPS) leading to a cascade of events involving the recruitment of numerous adaptor proteins (Ghosh et al., 1998). The different stimuli may lead to a

differing cascade of events but the key step in all canonical NF- κ B activation is the liberation of the NF- κ B dimer from its inhibitory proteins through the IKK-mediated phosphorylation and consequent ubiquitination and degradation of I κ B.

1.3.5.2 TNF α -mediated NF- κ B activation

TNF α can bind to and activate TNF receptor 1 (TNFR1) or TNFR2 to mediate effects on proliferation, inflammation and apoptosis. The activation of NF- κ B mediated by TNF α is primarily through TNFR1, with TNFR2 potentially suppressing NF- κ B activation (Puimège et al., 2014). The binding of TNF α to the TNFR1 promotes specific interaction with the adaptor protein TNF receptor-associated protein with a death domain (TRADD) which interacts with the death domain of TNFR1 to induce two distinct signalling cascades for either apoptosis or NF- κ B activation (Hsu et al., 1995). The recruitment of TRAF2 by TRADD then signals the cascade leading to NF- κ B activation rather than leading to apoptosis (Hsu et al., 1996b). In addition to TRAF2, the binding of TRADD to the TNFR1 also promotes the simultaneous recruitment of another death domain protein: the Ser/Thr kinase receptor-interacting protein 1 (RIP1) (Hsu et al., 1996a). TRAF2 forms complexes with cellular inhibitor of apoptosis protein 1 and 2 (cIAP1/2) thereby also recruiting them to the TNFR1 signalling complex. Many of the proteins within this TNFR1 complex possess ubiquitin ligase activity including the RING domain-containing cIAP1/2 which directly ubiquitinate RIP1 with K63 polyubiquitin chains, crucial for TNF α -induced NF- κ B activation (Varfolomeev et al., 2008). TRAF2 also possesses an E3 ligase RING domain and was initially assumed to be the ligase responsible for the K63-linked polyubiquitination of RIP1, however there is a lack of evidence that TRAF2 directly catalyses RIP1 ubiquitination. Moreover, TRAF2 lacks key amino acid residues in its RING domain required for binding Ubc13, part of the E2 complex specific for K63-linked polyubiquitination (Yin et al., 2009). It has been suggested that the E3 ligase activity of TRAF2 may be initiated by the pro-survival lipid mediator, sphingosine-1-phosphate (S1P), but the exact role of TRAF2 is still unclear and TRAF2 may only act as an aid in cIAP1/2-mediated RIP1 K63-linked polyubiquitination (Alvarez et al., 2010).

Throughout the NF- κ B pathway, K63 polyubiquitin chains and other polyubiquitin chains, including K11 and linear chains, have crucial functions in serving as a platform for recruiting the UBD-containing proteins required for the activation of NF- κ B (Husnjak and Dikic, 2012, Wertz, 2014). The cIAP1/2-mediated RIP1 (and also TRAF2) K63-linked polyubiquitination mediates the recruitment and activation of the transforming

growth factor- β -activated kinase 1 (TAK1) through interactions with TAK1 binding protein (TAB) 2 and 3. TAB2 and TAB3 act as adaptors by binding ubiquitin through their C-terminal Npl4 zinc-finger domains (NZFs) which specifically recognise the Ile 44 hydrophobic patch on ubiquitin in K63 polyubiquitin chains (Sato et al., 2009). This recruitment of TAK1 is significant in the NF- κ B signalling cascade as TAK1 is a key modulator of NF- κ B activation. It is a member of the mitogen-activation protein kinase (MAPK) kinase kinase (MAPKKK) family essential in the activation of not only NF- κ B but also JNK and p38 MAPK pathways (Ajibade et al., 2013). The activation of TAK1 through the TAB2- and TAB3-mediated binding to polyubiquitinated RIP1 leads to its own K63 polyubiquitination and consequently the recruitment of the IKK complex via the binding of the NF- κ B essential modifier (NEMO) subunit (also known as IKK γ) to the polyubiquitinated TAK1 (Fan et al., 2010). In addition to K63 polyubiquitination, RIP1 is also subjected to K11 polyubiquitination by cIAP1/2 and linear polyubiquitination by the LUBAC complex which is recruited to RIP1 by cIAP1/2 (Haas et al., 2009, Dynek et al., 2010, Gerlach et al., 2011). The NEMO subunit has a high affinity for specifically recognising and binding to linear ubiquitin chains through its ubiquitin binding in ABIN and NEMO (UBAN) motif and, as well as K63 polyubiquitin and K11 chains, linear ubiquitin chains are important in the recruitment of the IKK complex (Rahighi et al., 2009, Hadian et al., 2011). Additionally, NEMO is itself linearly ubiquitinated through recognition of NEMO by the NZF1 domain of HOIP of the LUBAC complex, which enhances interactions of the TNFR1 complex with the IKK complex (Fujita et al., 2014).

The IKK complex is another key modulator required in NF- κ B activation as it is responsible for the most crucial regulatory event in the activation of NF- κ B: the phosphorylation and subsequent degradation of I κ B (Hinz and Scheidereit, 2014). RIP1 serves as a docking platform for the recruitment of the IKK complex, particularly the NEMO subunit which can bind to the many ubiquitin chains topologies attached to RIP1. This ubiquitin-dependent concomitant recruitment of the IKK complex via RIP1 and TRAF2 brings TAK1 into close proximity with the IKK complex to allow the phosphorylation of IKK necessary for its activation (Wertz, 2014).

Along with the regulatory subunit NEMO, the IKK complex is made up of two other subunits: kinases IKK α and IKK β (Zandi et al., 1997). IKK β is the major IKK subunit for TNF α -induced NF- κ B activation and its phosphorylation at Ser177 and Ser181 in the IKK β activation loop is required for the activation of the IKK complex. There is much

evidence to indicate that TAK1 is essential in the direct phosphorylation and activation of the IKK complex and it was recently proposed that activated TAK1 phosphorylates IKK β at Ser177 which then enables the sequential autophosphorylation of IKK β at Ser 181 for the activation of IKK β (Zhang et al., 2014). Once activated, IKK β in turn phosphorylates I κ B, specifically Ser32 and Ser36 of I κ B α , which creates a specific phosphodegron motif to be recognised by the E3 ligase SCF $^{\beta$ -TrCP (Spencer et al., 1999). Similarly to the recognition of phosphorylated β -catenin, SCF $^{\beta$ -TrCP only recognises the phosphorylated form of I κ B α . The F-box component of SCF $^{\beta$ -TrCP, β -TrCP, is responsible for this recognition whilst Skp1 and Cull1 act as scaffolds and the RING domain acts as an E2 adaptor. Together this complex recognises phosphorylated I κ B α and catalyses its K48-linked polyubiquitination to tag it for degradation by the 26S proteasome (Spencer et al., 1999). Degradation of I κ B α releases dimeric NF- κ B, exposing the NLS of the p65 subunit of the dimer and allowing its translocation to the nucleus for the transcription of an array of NF- κ B target genes (Figure 1.8).

1.3.5.3 IL-1-mediated NF- κ B activation

Similarly to TNF α -induced NF- κ B activation, IL-1-induced NF- κ B activation requires the recruitment of a series of adaptor proteins leading to the activation of the IKK complex for the phosphorylation, ubiquitination and degradation of I κ B α . The adaptor proteins required for this differ to those involved in TNF α induction upstream of the IKK complex, but again this cascade of events is ubiquitin-dependent.

The IL-1 receptor (IL-1R), aided by the IL-1 receptor accessory protein (IL-1R AcP), can bind IL-1 α or IL-1 β to activate the NF- κ B signalling pathway (Martin and Wesche, 2002). Upon binding of IL-1 to IL-1R, the adaptor protein death domain-containing adaptor myeloid differentiation factor (MyD88) is recruited to the receptor through interactions with its Toll/IL-1 receptor (TIR) domain, a domain which also shares homology with Toll-like receptors (TLRs). This in turn leads to the recruitment of members of the IL-1R-associated kinase (IRAK) family including IRAK1, IRAK2 and IRAK4 to the IL-1R complex. MyD88 and IRAK1 both possess death domains which are able to interact with each other. The parallel recruitment of IRAK4 to the receptor complex scaffold allows it to phosphorylate IRAK1 on Thr209 and Thr387 which subsequently initiates the hyperphosphorylation of IRAK1, potentially through further autophosphorylation (Martin and Wesche, 2002, Verstrepen et al., 2008).

The phosphorylation of IRAK1 enables the recruitment of TRAF6 to the receptor complex and is thought to cause dissociation of IRAK1 and TRAF6 from the receptor complex (Cao et al., 1996). The self-ubiquitination of TRAF6 with K63-polyubiquitin chains and the formation of TRAF6-catalysed unanchored K63-polyubiquitin chains, together with E2 ubiquitin conjugating complex Ubc13/Uev1A or Ubc5, then leads to the recruitment of TAK1 through TAB2 and TAB3 interactions (Xia et al., 2009). The RING domain-containing TRAF6 also ubiquitinates IRAK1 with K63-linked polyubiquitin chains and in this way, as well as acting as an adaptor in the recruitment of TAK1, recruits the IKK complex through the binding of NEMO to K63 polyubiquitinated IRAK1 (Conze et al., 2008).

As with TNF α -induced NF- κ B signalling, IL-1 induces the formation of linear ubiquitin chains by LUBAC. It has been reported that the formation of K63-linked chains in response to IL-1 stimulation is a prerequisite for the formation of linear ubiquitin chains by the LUBAC complex which, through interactions of HOIP with K63-linked chains, covalently attach to the IRAK1 K63-linked polyubiquitin chains leading to both the recruitment of TAK1 through TAB2 binding of K63-linked chains required for IKK phosphorylation and activation and also to the recruitment of IKK itself through NEMO binding to linear chains (Emmerich et al., 2013). Again, linear and K63 polyubiquitin chains serve as a platform for recruiting TAK1 and IKK, essential for NF- κ B activation. This recruitment leads to autophosphorylation of TAK1 and direct activation of the kinase to induce the TAK1-mediated phosphorylation of IKK β , as in TNF α -induced signalling.

As well as K63-linked polyubiquitination and linear ubiquitination, IRAK1 later undergoes K48-linked polyubiquitination. The E3 ligase SCF $^{\beta}$ -TrCP recognises IRAK1 and eventually targets the protein for degradation allowing the TRAF6-TAK1 complex to dissociate from the membrane complex where it can activate IKK (Cui et al., 2012). A model for the ubiquitination of IRAK1 could involve initial K63-linked polyubiquitination of IRAK1 for the recruitment of IKK, later followed by removal of these chains and the addition of K48-linked polyubiquitination to for its degradation and to allow TAK1 to dissociate from the membrane to activate IKK in the cytosol (Figure 1.8).

1.3.5.4 Overview of ubiquitination in TNF α - and IL-1-induced NF- κ B activation

In addition to the key integral step in NF- κ B activation of the phosphorylation and subsequent K48-linked polyubiquitination for the degradation of I κ B α , other ubiquitin

chain topologies are required for the activation of the IKK complex which leads to this I κ B α degradation in both TNF α - and IL-1 α -induced NF- κ B signalling. TNF α stimulation causes the linear, K11- and K63-linked polyubiquitination of RIP1 which serves to recruit ubiquitin-binding proteins TAB2 and NEMO, which act as regulators of TAK1 and the IKK complex, respectively (Wertz, 2014). IL-1 stimulation also promotes linear and K63-linked polyubiquitination, possibly of TRAF6 and/or IRAK1, for the recruitment of TAK1 and the IKK complex (Verstrepen et al., 2008). This ubiquitin-dependent recruitment mechanism of these proteins initiates the activation of TAK1 and may be required to co-localise TAK1 and the IKK complex for the TAK1-mediated phosphorylation of IKK β (Figure 1.8).

1.3.5.5 p65 activation

The release of NF- κ B from its inhibitory proteins unmasks the NLS on NF- κ B of the most abundant and ubiquitously expressed NF- κ B dimer, p65/p50, allowing its nuclear translocation and the expression of many NF- κ B target genes. The mechanism of efficient transactivation of NF- κ B is itself regulated, for example the p65 NF- κ B subunit undergoes phosphorylations to regulate its activity. The family of NF- κ B proteins all share a RHD which is responsible for dimerisation and DNA binding activity. p65 is phosphorylated at Ser276 of its N-terminal RHD by protein kinase A (PKA) which promotes interactions with transcriptional activator CREB-binding protein (CBP)/p300, critical for p65 activation and NF- κ B-dependent transcription (Zhong et al., 1998). The p65 subunit of NF- κ B, but not the p50 subunit, also contains a transactivation domain (TAD) within its C-terminal domain which regulates the transcriptional activity of the prototypical p65/p50 complex (Diamant and Dikstein, 2013). Besides its phosphorylation at Ser276, p65 is also phosphorylated at Ser 536 within the TAD domain. Both TNF α and IL-1 induce p65 Ser536 phosphorylation by IKK β , and possibly a range of other kinases including IKK α , to regulate the transcriptional activity of p65 (Sakurai et al., 2003). Other sites for p65 phosphorylation, including Ser311 and Ser539, and other PTMs, including acetylation and ubiquitination, have been identified that serve as a mechanism of regulation in NF- κ B signalling downstream of the IKK complex (Schmitz et al., 2004). These multiple sites are thought to be involved in the recruitment of proteins and promoters required for the transcriptional activity of NF- κ B and the expression of NF- κ B target genes or are involved in the stability of p65.

1.3.5.6 The role of DUBs in NF- κ B signalling

The NF- κ B signalling pathway is under tight regulation to maintain homeostasis and prevent dysregulation of the pathway which can lead to the pathogenesis of many diseases including chronic inflammation and cancers (Pal et al., 2014). For this purpose inhibitors are required to balance out ubiquitination events in the pathway and many DUBs have been identified which act to provide this balance (Harhaj and Dixit, 2011).

A20

A20 is a well established OTU domain-containing DUB which acts as a negative regulator of NF- κ B activation. Overexpression of A20 inhibits TNF α - and IL-1-induced NF- κ B activation and A20-deficient mice show severe inflammation and hypersensitivity to treatment with LPS and TNF α resulting in premature death. Additionally, TNF α -stimulated A20-deficient cells exhibit persistent NF- κ B activation, clearly indicating that A20 is critical in the negative regulation of NF- κ B (Jaattela et al., 1996, Lee et al., 2000). A20 also has 7 C-terminal ZnF domains, one of which, ZnF4, displays intrinsic ubiquitin ligase activity meaning that, unusually, A20 is a novel ubiquitin-editing enzyme able to exhibit both deubiquitinase and E3 ligase properties. A likely model for the function of A20 in ubiquitin editing in TNF α -induced NF- κ B signalling is the initial suppression of NF- κ B through cleavage of K63-linked polyubiquitin chains on RIP1 by the OTU domain of A20 which is a prerequisite for the polyubiquitination of RIP1 with K48-linked polyubiquitin chains by the A20 ZnF4 domain for the targeting of RIP1 for proteasomal degradation to further downregulate NF- κ B activation (Wertz et al., 2004). A20 is also able to inhibit IL-1-induced NF- κ B activation through interactions with TRAF6, resulting in the deubiquitination of K63-linked TRAF6 polyubiquitin chains, however it does not mediate its degradation through the catlysis of K48-linked polyubiquitin chains as with RIP1 (Heyninck and Beyart, 1999, Boone et al., 2004).

Cylindromatosis tumour supressor

The cylindromatosis (*CYLD*) gene encodes a tumour suppressor, mutations of which have been linked to familial cylindromatosis, a genetic predisposition to multiple neoplasms of the skin appendages known as cylindromas (Bignell et al., 2000). *CYLD*-deficient mice have uncovered functions of *CYLD* in various biological processes including T-cell development and, in addition, *CYLD*-deficient mice are more susceptible to induced inflammation and tumourigenesis in a colitis-associated cancer model and exhibit enhanced NF- κ B and JNK activity (Reiley et al., 2006, Zhang et al., 2006). *CYLD* is a

NEMO-interacting enzyme able to deubiquitinate K63 polyubiquitinated substrates including TRAF2, RIP1 and TRAF6 and in this way downregulate NF- κ B activity (Brummelkamp et al., 2003, Kovalenko et al., 2003, Wright et al., 2007, Trompouki et al., 2003).

USP4

Whereas most reports to date have identified roles for DUBs in TNF α -induced NF- κ B signalling in particular, reports for a role of USP4 in TNF α -, IL-1- and LPS-induced NF- κ B signalling have also emerged. Initially, Fan et al. (2011b) reported that USP4 downregulates TAK1-induced NF- κ B activity. It was reported that TNF α induces association of USP4 with TAK1 via its C-terminal USP domain and this consequently leads to reduced ubiquitination of TAK1. Furthermore, USP4 was also reported to inhibit IL-1 β - and LPS-induced NF- κ B activity and IKK phosphorylation. In addition to identifying TAK1 as a substrate for USP4 in the downregulation of NF- κ B, it was also suggested that TRAF2 and TRAF6 may be substrates of USP4 due to the reduction in NF- κ B activity with overexpression of USP4 in TRAF2- and TRAF6-induced cells (Fan et al., 2011b). This observation was supported in another study which reported interactions of USP4 with both TRAF2 and TRAF6 targeting them for deubiquitination (Xiao et al., 2012). The interaction with TRAF6 occurs between the C-terminal USP domain of USP4 and the TRAF domain of TRAF6. USP4 was reported to inhibit TRAF2-, TRAF6-, TNF α - and IL-1 β -induced NF- κ B activity by deubiquitinating the non-degradative K63-linked polyubiquitination of TRAF2 and TRAF6. Similarly, Zhou et al. (2012) supported the role of USP4 as a negative regulator of NF- κ B signalling in line with these studies, with more focus on IL-1- and LPS-induced effects. As with the other studies, it was reported that USP4 suppresses IL-1-induced NF- κ B activity and affects the expression levels of NF- κ B target genes, including *TNF α* , *IL-1 β* and *IL-6* in response to IL-1 or LPS stimulation. Together these studies implicate USP4 as a negative regulator of NF- κ B activation through deubiquitination of mediators TRAF2, TRAF6 and TAK1 with TNF α - and IL-1-induced pathways (Figure 1.8) (Fan et al., 2011b, Xiao et al., 2012, Zhou et al., 2012).

Other USPs involved in the regulation of NF- κ B

USP21 downregulates TNF α -induced NF- κ B activity by interacting with and directly deubiquitinating RIP1 (Xu et al., 2010). In addition, USP2a inhibits TNF α -induced NF- κ B activity through association and deubiquitination of K63-linked polyubiquitination of

RIP1 and TRAF2 and also K48-linked polyubiquitination of RIP1. By deubiquitinating the K63 polyubiquitin chains of RIP1, USP2a represses NF- κ B activation and potentially causes conversion to the apoptotic pathway mediated by the TNFR1 pro-apoptotic complex II to mediate cell death (Mahul-Mellier et al., 2012).

Most negative regulators in the NF- κ B signalling pathway act upstream of the IKK complex but others act downstream of this. The key integral step in canonical NF- κ B activation is the ubiquitination and subsequent proteasomal degradation of I κ B. USP11 is able to reverse this by specific association and direct deubiquitination of I κ B α , thereby negatively regulating TNF α -induced NF- κ B activation by acting at an essential step in the signalling pathway and controlling the stability of I κ B α (Sun et al., 2010).

All the DUBs discussed so far act as negative regulators in the NF- κ B pathway but, additionally, DUBs can upregulate NF- κ B signalling. Ye et al. (2010) identified USP6 (also known as TRE17) as the first DUB to activate NF- κ B via an unusual mechanism. USP6 does not affect the phosphorylation or degradation of I κ B α but instead activates NF- κ B through stimulation of the nuclear accumulation of I κ B α -free p65 and the phosphorylation of p65 at Ser536 through associations with and activation of the IKK complex. IKK α , IKK β and NEMO are all required for this NF- κ B activation, unusual as IKK α is considered essential in non-canonical rather than canonical NF- κ B signalling (Pringle et al., 2012). Moreover, USP6 overexpression contributes to tumour pathogenesis in aneurysmal bone cysts, likely by the USP6-induced transcription of *MMP9* and *MMP13* via activation of NF- κ B (Ye et al., 2010).

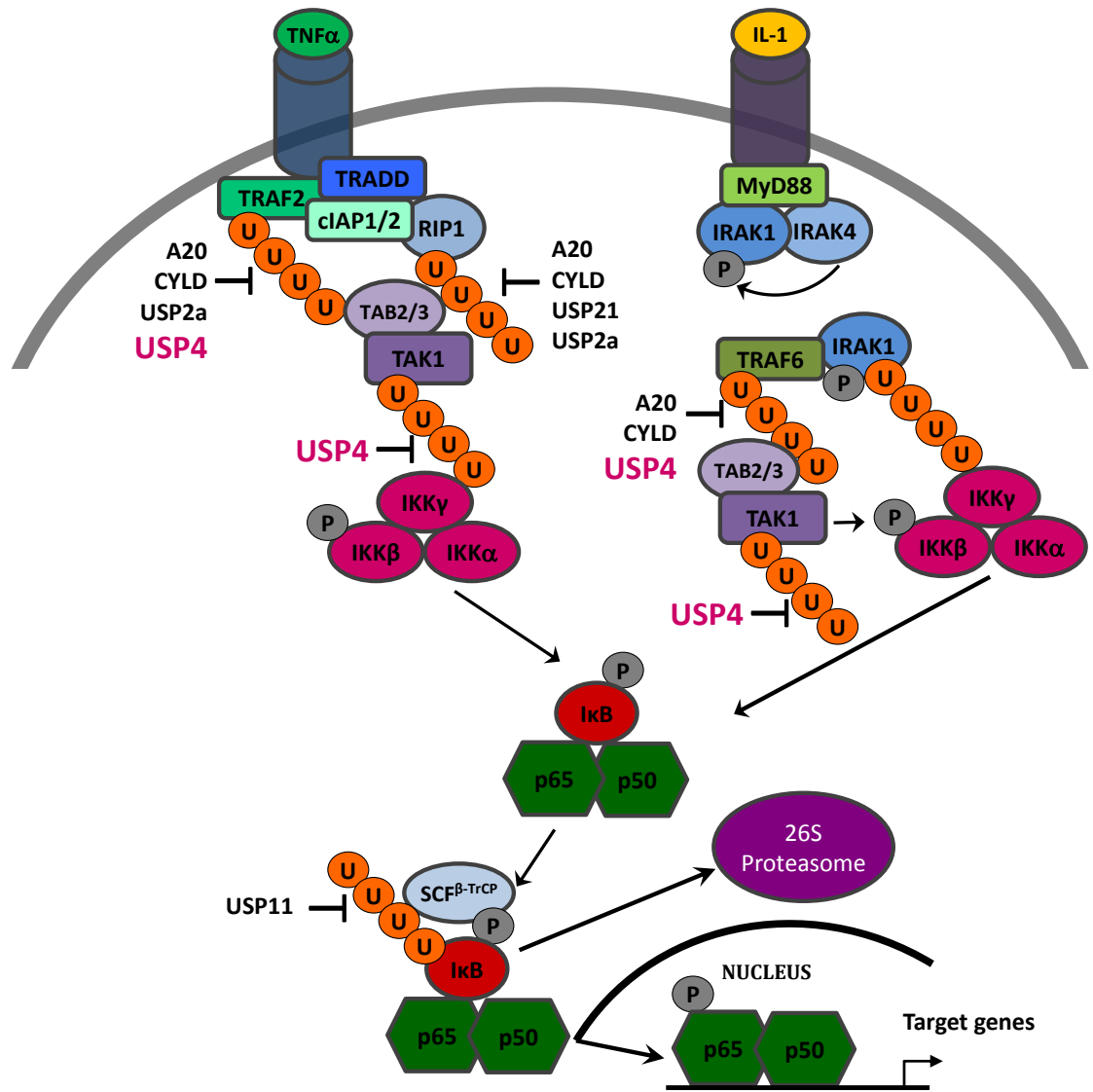


Figure 1.8. The NF- κ B signalling pathway. Diagram of the cascade of events in TNF α - and IL-1-induced NF- κ B activation. Ubiquitin-mediated recruitment of proteins, via K63-, K11- and linear polyubiquitin chains, results in the activation and K63-linked polyubiquitination of TAK1 which phosphorylates the IKK complex for subsequent I κ B phosphorylation, K48-linked polyubiquitination and degradation. Free from its inhibitory proteins, this allows the translocation of the NF- κ B dimer, p65/p50, to the nucleus for the transcriptional activation of a wide array of NF- κ B target genes. Ubiquitination of proteins is represented by orange circles. The phosphorylation of proteins is represented by grey circles. Many known DUBs are listed at their target substrates. USP4 is highlighted in pink.

1.3.5.7 Non-canonical NF- κ B signalling

The canonical (also called classical) NF- κ B signalling pathway is initiated by signals through receptors including TNFRs, IL-1Rs and TLRs and mainly leads to the IKK β -dependent phosphorylation of I κ B α for the nuclear translocation and activation of NF- κ B, usually p65/p50 heterodimers. The NEMO subunit of IKK is also required for this response, whereas the IKK α subunit may be redundant. Alternatively, NF- κ B can be activated through the less well studied non-canonical (also called alternative) pathway (Figure 1.9). The non-canonical pathway can be induced by ligands through members of the TNF family receptors; CD40 ligand (CD40L), B-cell activating factor belonging to the TNF family (BAFF) and lymphotoxin- β (LT- β) receptor (Coope et al., 2002, Claudio et al., 2002, Dejardin et al., 2002). This pathway is dependent on IKK α which phosphorylates p100 of a p100/RelB dimer to mediate its partial processing by ubiquitination and proteasomal degradation to yield NF- κ B subunit p52, resulting in the nuclear translocation of p52/RelB dimers for transcriptional activation (Bonizzi and Karin, 2004). Upstream of the IKK complex, the non-canonical pathway also differs to the canonical pathway in terms of the signalling cascade leading to the activation of IKK. A key component essential in the non-canonical pathway is the NF- κ B-inducing kinase (NIK), a Ser/Thr kinase in the MAPKKK family which, when activated, phosphorylates IKK α for downstream activation of NF- κ B (Ling et al., 1998). NIK is kept under strict regulation by ubiquitination to prevent uncontrolled activation of the pathway which can lead to disorders including inflammation and autoimmune disorders. This regulation requires the proteasomal degradation of NIK by a complex of TRAF2, TRAF3 and cIAP1/2. In unstimulated cells, TRAF2 recruits E3 ligases cIAP1/2 to TRAF3-bound NIK which binds to an IAP binding motif (IBM) on NIK and mediates its ubiquitination for degradation (Zarnegar et al., 2008, Varfolomeev et al., 2007). This results in suppression of p100 processing and reduced expression of NF- κ B target genes. When the non-canonical pathway is induced, this complex is dissociated and TRAF2 and TRAF3 are degraded, resulting in the stabilisation and activation of NIK, and consequently the downstream NIK-mediated phosphorylation of IKK α and processing of p100 required for NF- κ B activation (Lee et al., 2014).

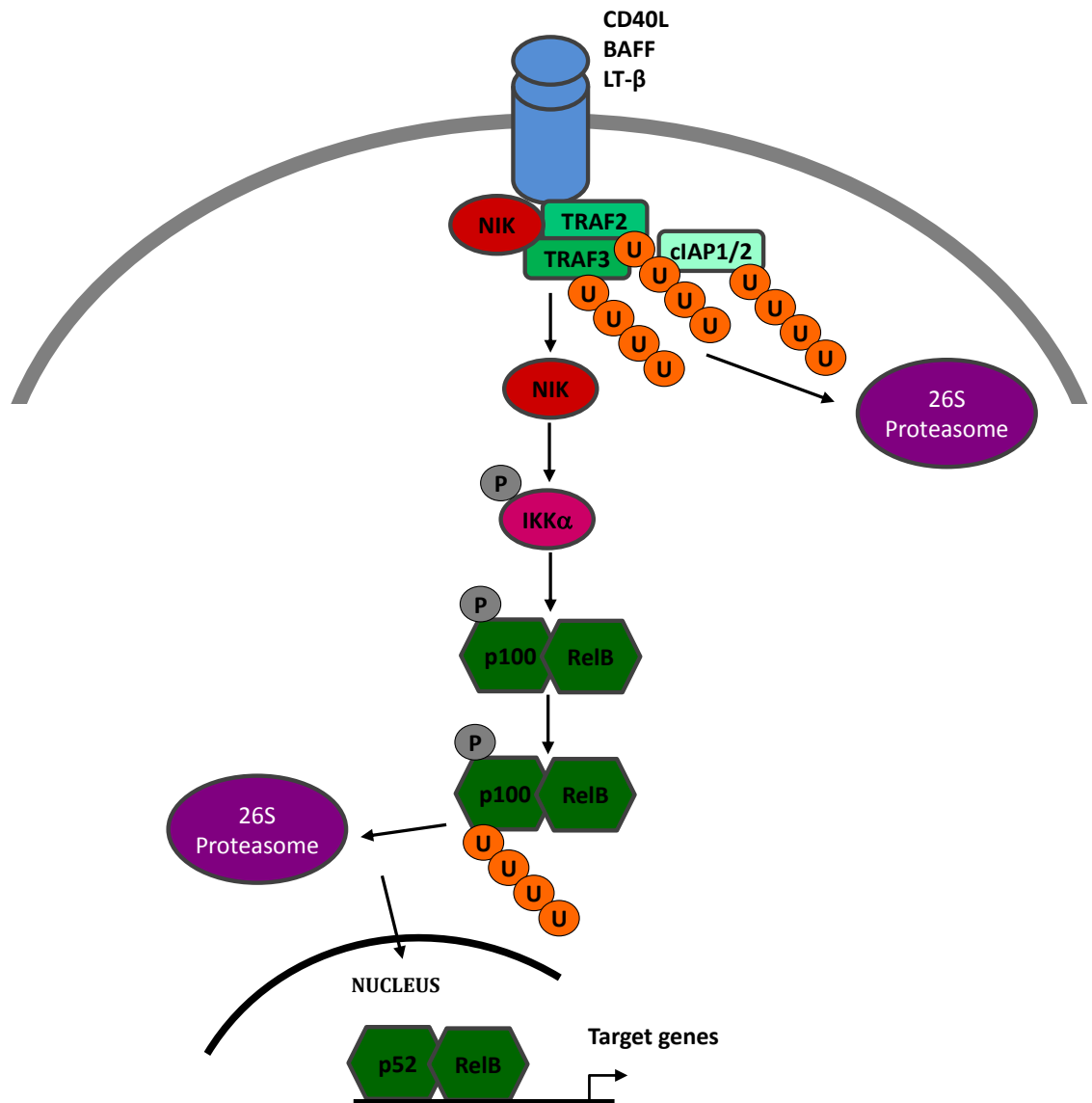


Figure 1.9. The non-canonical NF- κ B signalling pathway. Diagram of the cascade of events in non-canonical NF- κ B activation. Ubiquitin-mediated proteasomal degradation of the TRAF2-TRAF3-clAP1/2 complex allows the phosphorylation of IKK α by NIK which in turn phosphorylates p100 for its subsequent proteasome-mediated processing to p52. The NF- κ B dimer p52/RelB can then translocate to the nucleus for the transcriptional activation of NF- κ B target genes. Ubiquitination of proteins is represented by orange circles. The phosphorylation of proteins is represented by grey circles.

The many NF- κ B-inducing stimuli activating the canonical or non-canonical pathway, the array of NF- κ B target genes involved in different biological processes including immune and inflammatory responses, and the disorders and diseases linked to NF- κ B activity dysregulation all highlight the diversity of NF- κ B responses and the need for tight regulation through multiple levels of positive and negative regulators. Furthermore, there is crosstalk between NF- κ B and parallel signalling pathways to regulate its response (Oeckinghaus et al., 2011). As discussed, RLRs can activate NF- κ B transcription initiation as well as IRF3 activation for IFN- β production (Yoneyama et al., 2015). The gatekeeper for NF- κ B signalling is the IKK complex as this mediates the phosphorylation of I κ B for its ubiquitination, degradation and release of NF- κ B. The IKK complex is activated by RIG-I signalling for downstream activation of NF- κ B but many proteins upstream of the IKK complex, such as TAK1, are also critical in other signalling pathways including MAPK pathways (Ajibade et al., 2013).

1.3.6 The MAPK pathways

MAPKs are Ser/Thr kinases that mediate signalling pathways involved in diverse biological processes including inflammation, proliferation, differentiation, survival and apoptosis. The MAPK pathways are activated by stimuli such as cytokines, peptide growth factors and cellular stresses to induce a cascade of phosphorylation events leading to the activation of transcription factors, for instance AP-1 and NF- κ B, for the expression of a wide array of genes (Kim and Choi, 2010). The MAPK family comprises extracellular signal-related kinase 1/2 (ERK1/2), p38 $\alpha/\beta/\gamma/\delta$ and c-Jun NH₂-terminal kinases (JNKs) which form three distinct MAPK signalling cascades. All of these involve a MAPKKK which phosphorylates and activates a MAPK kinase (MAPKK) which can then phosphorylate and activate MAPK for the downstream signalling and phosphorylation of further components of the MAPK pathway (Figure 1.10). These signalling cascades rely on a series of scaffolding proteins for the formation of complexes required for transduction of the signal. Dysregulation in the MAPK signalling pathways has been implicated in the pathogenesis of diseases including cancers and neurodegenerative diseases (Kim and Choi, 2010).

1.3.6.1 The ERK pathway

Stimulation of the ERK MAPK pathway activates Ras GTPase which activates the MAPKKK Raf (A-Raf, B-Raf or Raf-1). This in turn phosphorylates and activates the MAPKK MEK1/2 which phosphorylates and activates ERK1/2.

1.3.6.2 The JNK pathway

Stimulation of the JNK MAPK pathway activates MAPKKKs, including apoptosis signal-regulating kinase 1 (ASK1), MAP and ERK kinase kinase (MEKK1) or TAK1; which in turn phosphorylate MAPKKs, including MKK4 or MKK7; which phosphorylate and activate JNK.

1.3.6.3 The p38 pathway

Stimulation of the p38 pathway activates the same MAPKKKs as in the JNK pathway; ASK1, MEKK1 or TAK1, which phosphorylate and activate MAPKKs, MKK3 or MKK6, to phosphorylate and activate p38.

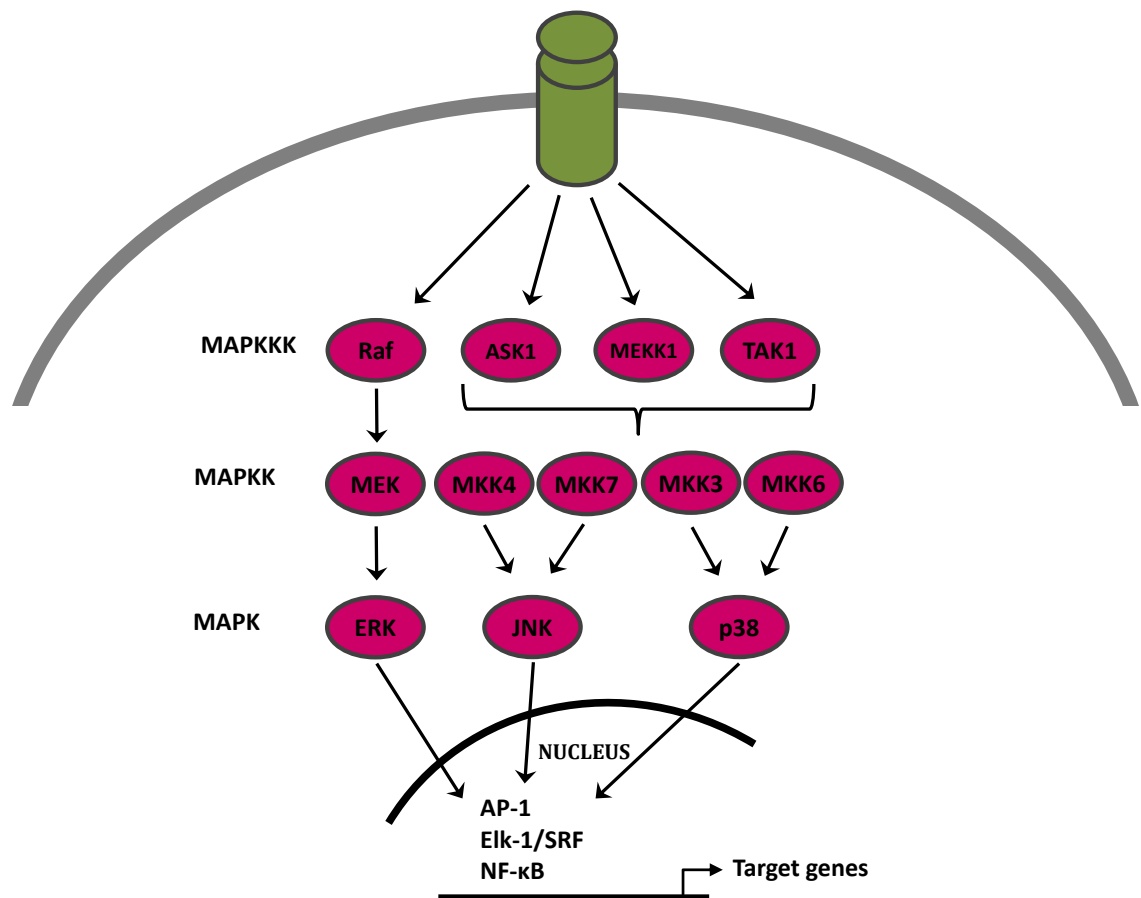


Figure 1.10. The MAPK pathways. Upon stimulation of the pathway, a MAPKKK phosphorylates and activates a MAPKK which in turn phosphorylates and activates a MAPK leading to the activation of transcription factors, such as AP-1, Elk-1/SRF and NF-κB.

1.3.7 Implications of USP4 functions in cell signalling pathways on disease pathogenesis

Due to the wide functioning of ubiquitin signalling in a variety of cellular processes including protein degradation, cell signalling, DNA damage repair and gene expression, dysregulation may have damaging consequences to the cell. It may lead to accumulation of proteins, improper activation or deactivation of cell signalling pathways or inefficient DNA repair which can all contribute to the pathogenesis of diseases such as autoimmune and neurodegenerative disorders and cancers (Popovic et al., 2014).

1.3.7.1 USP4 in cancer pathogenesis

As previously discussed, early studies documented elevated expression of USP4 in human lung cancers and primary cell lines, although in contrast, others reported reduced expression in small cell lung carcinoma cell lines (Gray et al., 1995, Frederick et al., 1998). More recently, evidence suggests that USP4 acts to regulate ubiquitination in multiple cellular pathways including both tumour-suppressing and tumour-promoting pathways which have been discussed in this chapter. In the p53 pathway USP4 stabilises ARF-BP1, a p53 E3 ligase which ubiquitinates p53 for proteasomal degradation thereby reducing levels of the p53 tumour suppressor (Zhang et al., 2011). Consistent with the potential oncogenic properties of USP4, the same study also reported overexpression of USP4 in human cancer samples, particularly in urinary bladder and prostate cancer samples and elevated levels of p53 in *Usp4* null MEFs with premature senescence and increased apoptosis of these cells. Together these data suggest that through regulation of p53 USP4 displays oncogenic properties. Moreover, USP4 enhances TGF- β signalling by deubiquitinating and maintaining the stability of T β R-I at the plasma membrane (Zhang et al., 2012). TGF- β is a known inducer of epithelial-to-mesenchymal transition (EMT), a highly regulated process in normal embryo development and also implicated in cancer progression and metastasis (Xu et al., 2009a). TGF- β production is increased in cancer cells which induces EMT through the function of T β R-I and causes invasion of cells. The mechanism of TGF- β -induced EMT can involve many of the Smad proteins or can occur through non-Smad activation of MAPKs, Rho GTPases and the PI3 kinase/Akt pathway, which all can contribute to the transcriptional activation of genes which suppress epithelial marker gene expression and activate mesenchymal gene expression that defines EMT (Deckers et al., 2006, Xu et al., 2009a). USP4 increases expression of EMT marker genes with TGF- β induction and increases T β R-I-dependent invasion in a zebrafish

embryo xenograft invasion-metastasis model, indicating that TGF- β -induced cancer invasion and metastasis is enhanced by USP4 (Zhang et al., 2012). This again supports an oncogenic function for USP4.

Besides p53 and TGF- β signalling, USP4 is also functions in TNF α - and IL-1-mediated NF- κ B signalling. Although predominantly recognised for its role in inflammation and immunity, NF- κ B signalling is also linked to the progression of cancers (Popovic et al., 2014, Ben-Neriah and Karin, 2011). Activation of the NF- κ B pathway activates proinflammatory cytokines and chemokines as well as many other genes including those involved in cell survival, proliferation, migration and invasion. Dysregulation of the NF- κ B pathway or persistent activation can lead to chronic inflammation and eventually promotion of cancer development and progression through NF- κ B-mediated gene expression. In fact, many cancers have constitutively active NF- κ B (Pal et al., 2014). TNF α signalling is linked to many cancers, including increasing migration of breast cancer cells (Wu et al., 2009). USP4 is reportedly a negative regulator of NF- κ B signalling with substrates including TAK1, TRAF2 and TRAF6. Further, depletion of USP4 promotes TNF α -induced migration in a human lung adenocarcinoma cell line, indicating USP4 may display tumour suppressor properties in the context of TNF α signalling, potentially through regulation of NF- κ B (Xiao et al., 2012). Additionally, USP4 acts as a negative regulator of the Wnt signalling pathway. Dysregulation of the Wnt pathway can lead to tumourigenesis and so USP4 may act as a tumour suppressor through its actions in suppressing this pathway (Zhao et al., 2009). These contrasting reports on the function of USP4 in the pathogenesis of cancers suggest USP4 may possess oncogenic and tumour suppressor properties depending on the substrate and in a context-dependent manner.

1.3.7.2 USP4 in inflammation and the immune response

The NF- κ B pathway is critical in inflammation and the immune response, the activation of which induces the expression of a variety of proinflammatory cytokines and chemokines. As such, aberrations in this signalling pathway have been linked to chronic inflammation and multiple immunological disorders (Makarov, 2000). USP4 is reported to act as a negative regulator by deubiquitinating components TAK1, TRAF2 and TRAF6 thus attenuating the TNF α - and IL-1-induced NF- κ B signal and proinflammatory response. Furthermore, *usp4*-depleted zebrafish embryos are more susceptible to LPS-mediated endotoxin challenge and express higher levels of proinflammatory cytokines

indicating that USP4 is essential in negatively regulating the IL-1-mediated immune response and in preventing chronic inflammation (Fan et al., 2011b, Zhou et al., 2012, Xiao et al., 2012).

USP4 is also implicated in the RIG-I immune signalling pathway which activates immune cells to produce type I IFNs and proinflammatory cytokines in response to viral infection (Yoneyama et al., 2015). In this context, USP4 is reported to deubiquitinate and stabilise RIG-I to enhance RIG-I signalling and IFN- β , a critical factor in innate immune responses. Moreover, overexpression of USP4 decreased viral replication in cells infected with VSV, a virus recognised by RIG-I, and siRNA-mediated depletion of USP4 increased viral replication in VSV infected cells (Wang et al., 2013). As well as controlling immune homeostasis through the negative regulation of NF- κ B, USP4 potentially plays an important role in the antiviral immune response through positive regulation of RIG-I-mediated IFN- β signalling.

Reports to date have identified roles of USP4 in cell signalling pathways which may potentially be important in the pathogenesis of cancers and inflammation. However, the physiological substrates, how USP4 itself is regulated and how dysregulation of USP4 may lead to pathologies in these pathways are incompletely defined. Understanding the mechanistic actions of USP4 in tightly regulating inflammatory, cell proliferation and apoptotic pathways may provide targets for therapies of inflammatory diseases and cancers.

1.4 Post-translational modifications (PTMs)

The function of a wide number of proteins can be regulated by PTMs: ubiquitination, sumoylation, phosphorylation, methylation and acetylation can all control the activity of proteins in a dynamic and reversible way. PTMs can influence the activity of enzymes, protein-protein interactions, protein stability and the subcellular localisation of proteins (Kessler and Edelman, 2011). The function of ubiquitination within cell signalling pathways is apparent and, similarly, other PTMs are crucial in the regulation of cell signalling pathways and other biological processes underlining the significance of this mechanism in regulating the activity of proteins. Proteins can undergo more than one modification and there is much crosstalk between the different types of PTMs. Recently it has been reported that ubiquitin itself can be subjected to phosphorylation to alter its structure, ubiquitin chain assembly and the hydrolysis of ubiquitin chains; thus adding

further complexity to ubiquitin signalling and the regulation of proteins by PTMs (Wauer et al., 2014).

1.4.1 Importance of PTMs in cell signalling pathways

As well as ubiquitination of proteins in cell signalling pathways, phosphorylation also plays a critical role in their co-ordination. In fact there is much crosstalk between ubiquitination and phosphorylation to mediate the tight regulation of these pathways (Karin and Ben-Neriah, 2000). This can be highlighted by the integral step in NF- κ B signalling in which the initial phosphorylation of I κ B is required for the ubiquitination and subsequent degradation of I κ B, catalysed by the E3 ligase SCF ^{β -TrCP}. SCF ^{β -TrCP} only recognises the phosphorylated form of I κ B and so phosphorylation mediates interactions between I κ B and this E3 ligase. This ensures it is only degraded when the pathway is activated and upstream events lead to the IKK-mediated phosphorylation of I κ B, thereby adding an extra level of control in the activation of NF- κ B (Spencer et al., 1999). As well as creating a phosphodegron for the recognition of E3 ligases, phosphorylation can crosstalk with ubiquitination by altering the subcellular localisation of ubiquitin signalling components to allow them to function at the correct location within the cell (Yuan et al., 2010). Conversely, ubiquitination can crosstalk with phosphorylation through the degradation of protein kinases or the activation of protein kinases, as occurs when TAK1 becomes polyubiquitinated within the NF- κ B pathway (Fan et al., 2010).

The most studied PTMs are phosphorylation and ubiquitination, however, other PTMs exist which also provide another level of regulation within signalling pathways. As mentioned, the phosphorylation of p65 at Ser276 by PKA promotes its interaction with CBP/p300 for p65 activation. This in turn leads to the acetylation of p65 at Lys310 to facilitate the recruitment of factors required for transcriptional activity (Diamant and Dikstein, 2013).

1.4.2 Regulation of DUBs by PTMs

DUBs are vital regulators in cell signalling pathways and many other diverse cellular processes and are themselves modulated by ubiquitin binding, binding of scaffold and adaptor proteins, and also PTMs to regulate their activity and function (Kessler and Edelmann, 2011). PTMs can regulate DUB protein interactions as already discussed using the example of USP1 for which its phosphorylation at Ser313 mediates its interaction with UAF1 (Villamil et al., 2012). PTMs can also enhance or reduce the activity of DUBs.

Under normal conditions, CYLD deubiquitinates TRAF2 to downregulate NF- κ B activation but its TNF α -mediated phosphorylation suppresses the ability of CYLD to deubiquitinate TRAF2 thus leading to downstream activation of NF- κ B (Reiley et al., 2005). Conversely, the phosphorylation of A20 by IKK β enhances the ability of A20 to suppress NF- κ B activation (Hutti et al., 2007). Additionally, PTMs can regulate the subcellular localisation of DUBs. In unstressed cells, USP10 stabilises p53 in the cytoplasm, but after induced DNA damage ATM-mediated phosphorylation of USP10 translocates it to the nucleus for the stabilisation of nuclear p53 (Yuan et al., 2010).

1.4.3 USP4 PTMs

Given the mechanism of regulation of other DUBs by their PTMs, it is likely that USP4 is subjected to PTMs for its regulation. One mechanism of regulation for USP4 in the TGF- β signalling pathway is through its phosphorylation at Ser445 by AKT. TGF- β -induced AKT activation leads to the translocation of USP4 from the nucleus to the cytoplasm and plasma membrane where USP4 stabilises T β R-I and enhances TGF- β signalling (Zhang et al., 2012). In Wnt signalling, USP4 interacts with the kinase Nlk which promotes its nuclear translocation. Although not examined in this study, Nlk may regulate the subcellular localisation of USP4 by phosphorylation, similarly to Akt-mediated phosphorylation in TGF- β signalling (Zhao et al., 2009).

In addition to phosphorylation, USP4 is also subjected to ubiquitination by the E3 ligase Ro52 (Wada and Kamitani, 2006b). Although this ubiquitination does not appear to affect the stability of USP4, other ways in which it may regulate USP4 have not been explored.

Further to these, and many other studies, which have identified examples of PTMs in the regulation of DUBs, several proteomic and high-throughput studies have mapped multiple other residues which are modified by PTMs. The mechanism of how these PTMs regulate DUBs is only just beginning to be revealed but the potential for the regulation of DUBs by these is extensive and may provide insight into the biological significance of these proteins. In addition to its phosphorylation at Ser445, studies have identified a multitude of phosphorylation and ubiquitination sites which may play a part in the function of USP4 (PhosphoSitePlusTM: www.phosphosite.org). Since many DUBs, including USP4, have been implicated in many diseases, knowledge on the PTM-mediated regulation of these DUBs may provide further therapeutic targets.

1.5 Aims

When this study first began the role of USP4 in any single cell signalling pathway had not been well studied. Ubiquitin signalling is key to the tight regulation of many cell signalling pathways in maintaining homeostasis and preventing pathological diseases and so the initial aim of this thesis will be to examine whether USP4 functions in any of these key pathways and what its role may be. As reports have emerged implicating USP4 in the regulation of NF- κ B signalling, more focus will be given to the mechanism by which USP4 acts in this pathway and immune responses. An RNAi approach to deplete USP4 and overexpression of Usp4 will be utilised to examine effects on NF- κ B signalling. Also, *Usp4* null mouse embryonic fibroblasts, established from *Usp4* null mice, which are phenotypically normal, will also be utilised for examining effects on the loss of Usp4 in NF- κ B signalling. In addition, establishing the effect of the phosphorylation of Usp4 through generation of a series of Usp4 phosphorylation mutants, particularly effects on NF- κ B signalling, will also be examined. Therefore the main aims of this thesis will be:

- Determine the cell signalling pathways that Usp4 may regulate
- Examine the function of USP4 in the IL-1- and TNF α -induced NF- κ B cell signalling pathway
- Identify potential target substrates of USP4 in the NF- κ B signalling pathway
- Examine the regulation of Usp4 by the PTM at serines 675 and 680, with particular focus on effects on the NF- κ B signalling pathway

Chapter 2 : Materials and Methods

2.1 Materials

2.1.1 Antibodies

All primary antibodies used are listed in Table 2.1. Polyclonal goat anti-mouse and anti-rabbit immunoglobulins/HRP were purchased from Dako (Cambridgeshire, UK).

Table 2.1. Antibodies used for immunoblotting and immunoprecipitation experiments. All antibodies were purchased from either Cell Signaling Technology (Beverly, MA, USA), Santa Cruz Biotechnology (CA, USA), Sigma-Aldrich (Poole, UK), Millipore (Watford, UK), Abcam (Cambridge, UK) or Caltag Medsystems Ltd. (Buckingham, UK) as stated.

Antibody	Species	Monoclonal /Polyclonal	Purchased from	Catalogue number	Dilution (Immunoblotting)
I κ B α	Mouse	Monoclonal	Cell Signaling Technology	#4814	1:1000
Phospho-I κ B α (Ser32)	Rabbit	Monoclonal	Cell Signaling Technology	#2859	1:1000
NF- κ B p65	Rabbit	Monoclonal	Cell Signaling Technology	#8242	1:1000
NF- κ B phosho-p65 (Ser536)	Rabbit	Monoclonal	Cell Signaling Technology	#3033	1:1000
IKK α	Rabbit	Polyclonal	Cell Signaling Technology	#2682	1:1000
IKK β	Rabbit	Monoclonal	Cell Signaling Technology	#2370	1:1000
Phospho-IKK α/β (Ser176/180)	Rabbit	Monoclonal	Cell Signaling Technology	#2697	1:1000
Phospho-p38 MAPK (Thr180/Tyr182)	Rabbit	Polyclonal	Cell Signaling Technology	#9211	1:1000
p44/42 MAPK (ERK1/2)	Rabbit	Polyclonal	Cell Signaling Technology	#9102	1:1000
Phospho-p44/42 MAPK (ERK1/2) (Thr202/Tyr204)	Rabbit	Polyclonal	Cell Signaling Technology	#9101	1:1000
Phospho-SAPK/JNK (Thr183/Tyr185)	Rabbit	Polyclonal	Cell Signaling Technology	#9251	1:1000

Chapter 2: Materials and Methods

TRAF6	Rabbit	Monoclonal	Cell Signaling Technology	#8028	1:1000
TRAF2	Rabbit	Polyclonal	Cell Signaling Technology	#4724	1:1000
TAK1	Rabbit	Polyclonal	Caltag Medsystems	PSI-3385	1:1000
NIK	Rabbit	Polyclonal	Caltag Medsystems	PSI-1129	1:1000
IRAK	Rabbit	Polyclonal	Caltag Medsystems	PSI-1007	1:1000
USP4	Rabbit	Polyclonal	Cell Signaling Technology	#2651	1:1000
GFP	Rabbit	Polyclonal	Santa Cruz Biotechnology	sc-8334	1:1000
Anti-FLAG M2	Mouse	Monoclonal	Sigma-Aldrich	F1804	1:1000
Anti-phosphoserine	Rabbit	Polyclonal	Millipore	AB1603	1:500
Anti-glyceraldehyde- 3-phosphate dehydrogenase (GAPDH)	Mouse	Monoclonal	Millipore	MAB374	1:40 000
Anti-HA tag (HA C5)	Mouse	Monoclonal	Abcam	ab18181	1:1000

2.1.2 Immunoblotting reagents

Ammonium persulphate (APS), β -mercaptoethanol, bovine serum albumin (BSA), polyoxyethylene sorbitan (Tween-20), N,N,N',N'-Tetramethylethylenediamine (TEMED), Triton X-100 and Kodak high-speed X-ray film were all purchased from Sigma-Aldrich (Poole, UK). A standard stock solution of BSA was purchased from Pierce & Warriner (Chester, UK). BradfordUltra reagent was purchased from Expedeon (Cambridge, UK). A 37.5:1 mix of acrylamide/bis-acrylamide was purchased from Severn Biotech Ltd. (Worcestershire, UK). PageRuler™ pre-stained protein ladder was purchased from Thermo Scientific (Leicestershire, UK). Immobilon-P polyvinylidene difluoride (PVDF) 0.45 μ M membrane and Immobilon western chemiluminescent HRP substrate were purchased from Millipore (Watford, UK). Enhanced chemiluminescence (ECL) and ECL-advanced western blotting detection reagents were purchased from GE Healthcare Life Sciences (Buckinghamshire, UK). Marvel non-fat dry milk powder was purchased from Premier Foods (St. Albans, UK).

2.1.3 Cell lines

2.1.3.1 SW1353

SW1353 cells were purchased from the American Type Culture Collection (ATCC) (HTB-94) and cultured in Dulbecco's Modified Eagle's Medium: Nutrient Mixture F-12 (DMEM/F-12) culture medium as described in section 2.2.1. This cell line initiated from a primary grade II chondrosarcoma in the right humerus of a 72 year old Caucasian female in 1977, full details of which can be found on the ATCC website (www.atcc.org).

2.1.3.2 HeLa

The HeLa cell line initiated from the cervical adenocarcinoma of a 31 year old female and were cultured in DMEM as described in section 2.2.1. Full details can be found on the ATCC website (www.atcc.org).

2.1.3.3 HEK 293T

HEK 293T cells were purchased from the ATCC and cultured in DMEM as described in section 2.2.1. This cell line initiated in the human embryonic kidney cells of a foetus, full details of which can be found on the ATCC website (www.atcc.org).

2.1.3.4 *Usp4*^{+/+} and *Usp4*^{-/-} mouse embryonic fibroblasts (MEFs)

MEFs were obtained from wild-type (*Usp4*^{+/+}) and *Usp4* null (*Usp4*^{-/-}) mouse embryos and prepared and cultured as described in section 2.2.1.1.

2.1.3.5 U2OS

U2OS cells were obtained from the ATCC (HTB-96) and cultured in DMEM as described in section 2.2.1. This cell line initiated from osteosarcoma in the bone of a 15 year old Caucasian female, full details of which can be found on the ATCC website (www.atcc.org).

2.1.3.6 NIH3T3

NIH3T3 cells were purchased from the ATCC and cultured in DMEM as described in section 2.2.1. This cell line initiated from mouse fibroblasts, full details of which can be found on the ATCC website (www.atcc.org).

2.1.4 Cell culture reagents

DMEM/F-12 and DMEM culture media were both purchased from Gibco, Life Technologies Ltd. (Paisley, UK). Foetal bovine serum (FBS), Dulbecco's phosphate buffered saline (PBS), trypsin-EDTA (derived from porcine pancreas), L-glutamine, penicillin-streptomycin, and dimethyl sulphoxide (DMSO), were purchased from Sigma-Aldrich (Poole, UK).

2.1.5 Transfection reagents

DharmaFECT Transfection Reagent 1 was used for transfection of cells with siGENOME SMARTpool small interfering RNAs (siRNAs) or siGENOME Non-targeting Pool #2 (siControl), all purchased from Dharmacon (Leicestershire, UK). For transient transfections with plasmid DNA, Attractene transfection reagent purchased from QIAGEN (West Sussex, UK), GeneJuice transfection reagent purchased from Merck Millipore (Watford, UK) or FuGENE HD transfection reagent purchased from Promega (Southampton, UK) were used.

2.1.6 Plasmids

pCMV6-AN-mGFP was purchased from OriGene Technologies (MD, USA) and used as the backbone for all *Usp4* mutant plasmids. The following plasmids were obtained from Addgene: FLAG-TRAF6-wt (21624) and p-EBG-TRAF2 (GST) (21586) both deposited

by John Kyriasis, pRK5 Myc-RIP1 (44159) and pcDNA3 TAK1/F (44161) both deposited by Xin Lin, psPAX2 (12260) deposited by Didier Trono, pCMV-VSV-G (8454) deposited by Bob Weinberg, pRK5-HA-Ubiquitin-WT (17608), pRK-HA-Ubiquitin-K48 (17605) and pRK5-HA-Ubiquitin-K63 (17606) all deposited by Ted Danson and pHAGE NF- κ B-TA-LUC-UBC-GFP-W (49343) deposited by Darrell Kotton. NF- κ B firefly luciferase reporter and pRL-TK *Renilla* expression vectors were a gift from Dr Matt Barter (Newcastle University, Newcastle, UK). pCDH-EF1-MCS-IRES-GFP lentiviral expression vector was purchased from System Biosciences (Cambridge, UK). pLVX-Tet-ON Advanced, pLVX-Tight-Puro and Lenti-X HTX Packaging Mix were all purchased from Clontech (Paris, France).

2.1.7 Cytokines and other stimuli

Tumour necrosis factor α (TNF α) was purchased from Sigma-Aldrich (Poole, UK) and stored at -20°C in sterile filtered PBS. Doxycycline hyclate was also purchased from Sigma-Aldrich (Poole, UK). Recombinant human interleukin-1 α (IL-1 α) was a gift from GlaxoSmithKline (Stevenage, UK).

2.1.8 Inhibitors

MG132 (carbobenzoxy-Leu-Leu-leucinal) proteasome inhibitor and N-ethylmaleimide (NEM) deubiquitinase inhibitor were purchased from Sigma-Aldrich (Poole, UK). NEM was reconstituted in ethanol as per the manufacturer's instructions.

2.1.9 Immunohistochemistry reagents and antibodies

The Vectastain® Elite® ABC kit used for immunohistochemical staining was purchased from Vector Laboratories (Peterborough, UK). VSV primary antibody was a gift from the laboratory of Dr John Bell (Ottawa Hospital Research Institute, Ottawa University, Ottawa, Canada). D.P.X. mounting medium was purchased from Sigma-Aldrich (Poole, UK).

2.1.10 General molecular biology reagents

Ampicillin and Luria-Broth (LB) EZMix™ powder were purchased from Sigma-Aldrich (Poole, UK). Bacto Agar was purchased from Scientific Laboratory Supplies (Nottingham, UK). Molecular biology grade agarose was purchased from Severn Biotech Ltd. (Worcestershire, UK). S.O.C medium and Subcloning Efficiency™ DH5 α ™ Competent Cells were purchased from Invitrogen, Life Technologies Ltd. (Paisley, UK).

GeneRuler™ 100bp and 1kb DNA ladders, Rapid DNA Ligation kit and FastDigest® restriction enzymes and FastDigest® Green buffer were purchased from Fermentas, Thermo Fisher Scientific (Leicestershire, UK). Cells-to-cDNA II lysis buffer was purchased from Ambion, Ambion (Europe) Ltd. (Huntingdon, UK). Random hexamers p(dN)₆ were synthesised by Integrated DNA Technologies (IDT) (Leuven, Belgium). Deoxyribonucleotide triphosphate (dNTP) mix was purchased from Bioline (London, UK). RNase-, DNase-free water and all real-time reverse transcriptase quantitative polymerase chain reaction (real-time qRT-PCR) primers and probes and regular desalted oligonucleotides were purchased from Sigma-Aldrich (Poole, UK). Probe library probes were purchased from Roche, Roche Diagnostics Ltd. (West Sussex, UK). Moloney Murine Leukaemia Virus (M-MLV) reverse transcriptase, 5X First Strand Buffer and 100mM DTT were all purchased from Invitrogen, Life Technologies Ltd. (Paisley, UK) and TaqMan® gene expression master mix was purchased from Applied Biosystems, Life Technologies Ltd. (Paisley, UK). Protein G PLUS-Agarose was purchased from Santa Cruz Biotechnology (CA, USA). Phire Reaction Buffer and Phire Hot Start II DNA Polymerase were purchased from Thermo Scientific (Leicestershire, UK). Stellar Competent Cells were purchased from Takara Bio Europe/Clontech (Paris, France). Vectorshield® Mounting Medium for Fluorescence was purchased from Vector Laboratories (Peterborough, UK).

All standard laboratory chemicals and reagents were commercially available from Sigma-Aldrich, Thermo Scientific or Invitrogen, Life Technologies unless otherwise indicated.

2.1.11 Commercially available kits

Rapid DNA ligation kit was purchased from Fermentas, Thermo Fisher Scientific (Leicestershire, UK). Signal Finder Reporter Array and QIAquick Gel Extraction kits were purchased from QIAGEN (West Sussex, UK). QuikChange II XL Site-directed Mutagenesis Kit was purchased from Agilent Technologies (Santa Clara, CA, USA). PureYield™ Plasmid Mini/Maxiprep, Dual-Luciferase® Reporter Assay and Dual-Glo® Luciferase Reporter Assay Systems were purchased from Promega (Southampton, UK). Mouse ProInflammatory 7-Plex Ultra-Sensitive Kit was purchased from Meso Scale Discovery®, Meso Scale Diagnostics (Gaithersburg, MD, USA). Mouse IL-6 Quantikine ELISA and Mouse CXCL1 Quantikine ELISA kits were purchased from R&D Systems® (Abingdon, UK). Extract-N-Amp™ Tissue PCR Kit was purchased from Sigma-Aldrich (Poole, UK). In-Fusion® HD Cloning kit and Lenti-X™ qRT-PCR Titration kit were

purchased from Takara Bio Europe/Clontech (Paris, France). NucleoSpin® Gel & PCR Clean-up kit was purchased from Machery-Nagel (Düren, Germany).

2.1.12 Culture vessels

Tissue culture plates (96-, 48-, 24-, 12- and 6-well), 75cm² and 162cm² vented cell culture flasks were purchased from Corning/Costar (Sunderland, UK). Flat-bottomed non-sterile 96-well plates and 30ml universals were purchased from Greiner Bio-One (Stonehouse, UK). Falcon tubes (15ml and 50ml), scrapers and 10cm cell culture dishes were purchased from Cellstar (Leicestershire, UK). Eppendorf-equivalent tubes (0.6ml and 1.5ml) were purchased from Starlab (Milton Keynes, UK).

2.2 Methods

2.2.1 Cell culture

Reagents

- HEK 293T, HeLa, U2OS, NIH3T3 and MEF culture medium: DMEM containing 2mM L-glutamine, 100U/ml penicillin, 100µg/ml streptomycin and 10% (v/v) FBS
- SW1353 culture medium: DMEM/F12 containing 2mM L-glutamine, 100U/ml penicillin, 100µg/ml streptomycin and 10% (v/v) FBS
- Freezing medium: 90% (v/v) FBS and 10% (v/v) DMSO

Method

Cells were plated into vented T75cm² flasks and cultured at 37°C with 5% CO₂ (v/v) in a humidified incubator until they reached approximately 80% confluence. Cells were washed with PBS and detached by incubation with trypsin-EDTA to be passaged into further T75cm² flasks for continuation of the line every 2-3 days, or into the appropriate culture vessel for experimentation. For long-term storage in liquid nitrogen, cells were resuspended in freezing medium, transferred to cryovials, and frozen slowly at -80°C overnight before being transferred for storage in liquid nitrogen.

2.2.1.1 *Usp4*^{+/+} and *Usp4*^{-/-} MEF preparation

Mice containing an inactivating retroviral provirus in the *Usp4* gene (strain TF 2497) (Figure 2.1) were purchased from Taconic Laboratories (Hudson, New York, USA). Crosses were performed between mice previously determined by PCR-based genotyping to be heterozygous (details of the genotyping are provided in section 2.2.9).

Pregnant female mice were sacrificed between day E13.5 and E15.5. Under sterile conditions the embryos were removed from the uterus, individual embryo sacs were cut and rinsed in PBS. After removing each embryo from its yolk sac, the head and internal organs of the embryos were removed and the remaining tissue was incubated in trypsin-EDTA at 37°C for 10 minutes before pipetting up and down to homogenise. To allow genotyping of the embryos, as described in section 2.2.9, a small piece of tail was cut and stored at -20°C until required. Next, 5ml of MEF culture medium (DMEM) was added and the cell suspension was centrifuged at 300 x g for 5 minutes. Medium was aspirated and cells resuspended in fresh DMEM before being plated into 10cm dishes at 37°C with 5% CO₂ (v/v) in a low oxygen (3%) (v/v) incubator until approximately 80% confluent. The cell culture was then continued, transferred to the relevant culture vessel for experimentation, or stored in liquid nitrogen as described in section 2.2.1.

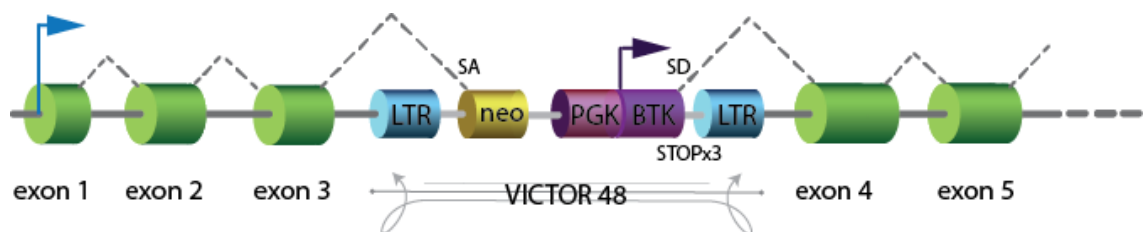


Figure 2.1. Schematic of the retroviral insertion in the *Usp4* gene.

2.2.2 Generation and use of expression vectors

Usp4 and mutant versions of Usp4 were transiently transfected into cells to study effects of Usp4 overexpression in NF- κ B signalling. First, a range of Usp4 mutant constructs were designed to incorporate Usp4 within a pCMV-AN-mGFP vector (OriGene Technologies, MD, USA). Wild-type Usp4 and a catalytically inactive version of Usp4 (C311A), a gift from Dr Doug Gray (Ottawa Hospital Research Institute, Ottawa, Canada), were cloned into the pCMV6-AN-mGFP vector (section 2.2.2.1). Other versions of Usp4 were then created by mutagenesis of the phosphorylation sites: serines 675 and 680 of the wild-type pCMV-AN-Usp4-mGFP vector (section 2.2.2.2).

2.2.2.1 Cloning

For cloning, all restriction enzymes used were FastDigest® enzymes in combination with 10X FastDigest® Green buffer and ligation was performed using the Rapid DNA ligation kit (Fermentas, Thermo Scientific, Leicestershire, UK).

Method

To subclone the catalytically inactive version of Usp4 (for which a cysteine at the catalytic site has been substituted for an alanine: C311A) into the correct vector, *Xba* I and *Cla* I restriction enzymes were used in a double digest reaction following the manufacturer's protocol. Briefly, reactions were prepared of 2 μ l 10X buffer, 1 μ l restriction enzymes and either donor or recipient vector made up to 20 μ l total in nuclease-free water. After mixing, reactions were incubated at 37°C for 10 minutes before heat killing at 80°C for 20 minutes to stop restriction endonuclease activity. Reactions were loaded onto a 0.8% (w/v) agarose gel as described in section 2.2.2.5.

Next, bands of the expected size on the gel were extracted using a QIAquick Gel Extraction kit according to the manufacturer's protocol. DNA was eluted in 50 μ l and the insert cut from the donor vector was ligated into the recipient vector by a reaction with insert and vector at a 3:1 ratio using the Rapid DNA Ligation kit, following the manufacturer's instructions. 1 μ l of the ligation was then used in bacterial transformation as described in section 2.2.2.3.

2.2.2.2 Site-directed mutagenesis

To examine the function of Usp4 phosphorylation, two phosphorylation sites at serines 675 and 680 were mutated by site-directed mutagenesis. Mutagenesis of the pCMV6-AN-Usp4-mGFP vector was performed using the QuikChange II XL kit (Agilent

Technologies, Santa Clara, CA, USA) following the manufacturer's protocol. Primers were designed approximately 16 base pairs (bp) either side of the region to be mutated (Table 2.2).

Method

For the mutagenesis, a reaction mix was prepared of 5µl 10X reaction buffer, 50ng double-stranded DNA (dsDNA) template, 1.25ng forward and reverse mutant primers, 0.01M dNTP mix, 3µl QuikSolution reagent and made up to 50µl with distilled H₂O (dH₂O) followed by the addition of 2.5U *PfuUltra* HF DNA polymerase. The reaction was cycled following the parameters: 95°C for 2 minutes followed by 18 cycles of 95°C for 1 minute, 60°C for 1 minute, 68°C for 1 minute per kb of plasmid length, and a final step at 68°C for 7 minutes. Next, 10U *Dpn* I restriction endonuclease was added to the reaction and incubated at 37°C for 1 hour to digest the parental methylated, supercoiled dsDNA.

After mutagenesis, the new mutated plasmid was then transformed into XL10-Gold ultracompetent cells following the manufacturer's protocol. The plasmid sample was added to ice-cold XL10-Gold cells with XL10-Gold β-mercaptoethanol mix (β-ME) and incubated on ice for 30 minutes. Following a 30 second heat-shock at 42°C, pre-heated (42°C) S.O.C. medium was added to the sample and incubated at 37°C with shaking at 225rpm. After 1 hour's incubation, the sample was spread onto LB-agar plates supplemented with ampicillin, and the mutated plasmids expanded as described in sections 2.2.2.3 and 2.2.2.4.

Table 2.2. Sequences of primers used for the generation of Usp4 mutants by site-directed mutagenesis and the sequencing primer used to verify the presence of the correct mutation in plasmids generated by site-directed mutagenesis and In-Fusion cloning.

Mutant	Template	Primer	Primer sequence	Amino acid sequence
S675A/S680S	pCMV-AN-Usp4-GFP	Forward	5' -AGGAAAAGAGCAGCTT GCG GAAAGTGGAAAGGCAGT-3'	GKEQL A EVEGS
		Reverse	5' -ACTGCCTTCCACTTC CGC AAGCTGCTCTTTTCCT-3'	
S675S/S680A	pCMV-AN-Usp4-GFP	Forward	5' -TTCCGAAGTGGAAAGGC GCG GGTGAGGACGATCGG-3'	S EVEGA A GEDDQ
		Reverse	5' -CTGATCGTCCTCACC CGC GCCTTCCACTTCGGAA-3'	
S675D/S680S	pCMV-AN-Usp4-GFP	Forward	5' -AGGAAAAGAGCAGCTT GAC GAAAGTGGAAAGGCAGT-3'	GKEQL D EVEGS
		Reverse	5' -ACTGCCTTCCACTTCG TC AAGCTGCTCTTTTCCT-3'	
S675S/S680D	pCMV-AN-Usp4-GFP	Forward	5' -TTCCGAAGTGGAAAGGC GAT GGTGAGGACGATCAG-3'	S EVEG D GEDDQ
		Reverse	5' -CTGATCGTCCTCACC AT CGCCTTCCACTTCGGAA-3'	
S675D/S680D	S675S/S680D	Forward	5' -AGGAAAAGAGCAGCTT GAC GAAAGTGGAAAGGCAGT-3'	GKEQL D EVEGD
		Reverse	5' -ACTGCCTTCCACTTCG TC AAGCTGCTCTTTTCCT-3'	
S675A/S680D	S675S/S680D	Forward	5' -AGGAAAAGAGCAAGCTT GCG GAAAGTGGAAAGGCAGT-3'	GKEQL A EVEGD
		Reverse	5' -ATCGCCTTCCACTTC CGC AAGCTGCTCTTTTCCT-3'	
S675D/S680A	S675D/S680S	Forward	5' -TGACGAAGTGGAAAGGC GCG GGTGAGGACGATCAG-3'	GKEQL D EVEGA
		Reverse	5' -CTGATCGTCCTCACC CGC GCCTTCCACTTCGTCA-3'	
Method			Sequencing primers	
Site-directed mutagenesis			5' -GCTGTTTGTGATCGTATCAG-3'	
In-Fusion Cloning EF1:			5' -CTCCACGCTTTGCCTGACC-3'	
IRES:			5' -CCTCACATTGCCAAAAGACG-3'	

2.2.2.3 Transformation

Reagents

- LB (EZMix (Sigma-Aldrich, Poole, UK) in dH₂O, autoclaved)
- LB-agar with ampicillin-resistance (LB, Bacto Agar and ampicillin (100µg/ml) in dH₂O, autoclaved)

Method

Plasmid DNA was added to Subcloning Efficiency DH5α Competent Cells and incubated on ice for 20 minutes before a 30 second heat-shock at 42°C. S.O.C medium was added to the cells and incubated at 37°C with shaking for 1 hour. Next, the transformation mixture was spread onto LB-agar plates and incubated at 37°C overnight. The following day individual colonies were selected and incubated in 5ml of LB supplemented with ampicillin (100µg/ml) at 37°C with shaking at 225rpm. For minipreps, bacteria were cultured overnight. For maxipreps, the 5ml bacteria was cultured for approximately 4 hours before transferring to 250ml of LB supplemented with ampicillin and cultured overnight.

2.2.2.4 Minipreps/maxipreps

Minipreps/maxipreps were performed using the PureYield™ plasmid mini/maxiprep system (Promega, Southampton, UK) following the manufacturer's protocol. Plasmid DNA was eluted in 30µl or 1ml nuclease-free water for minipreps and maxipreps, respectively. DNA concentration was determined using the NanoDrop ND-1000 spectrophotometer. Plasmids were electrophoresed on a 1% Tris-acetate-ethylenediamine tetraacetic acid (TAE) agarose gel as outlined in section 2.2.2.5 and sequenced by Sanger sequencing (Source BioScience Life Sciences, Nottingham, UK) to verify correct identity and confirm the presence of the correct mutation in the case of the Usp4 mutant constructs. The sequencing primer used for this verification is listed in Table 2.2.

2.2.2.5 Agarose gel electrophoresis

Reagents

- TAE buffer (0.04M Tris (pH 8), 5.7% (v/v) glacial acetic acid and 0.001M ethylenediaminetetraacetic acid (EDTA))
- Loading buffer (Tris 0.125M (pH 6.8), 2% (w/v) SDS, 10% (v/v) glycerol and 0.001% (w/v) bromophenol blue)

Method

Agarose gels were prepared at 1% (w/v) in TAE with the addition of 0.2µg/ml ethidium bromide solution (EtBr). After restriction digest, the required amount of DNA was electrophoresed on the gel in loading buffer and bands visualised on a ChemiGenius II BioImager (Syngene, Cambridge, UK) using a GeneRuler™ 100bp or 1kb DNA ladder to determine DNA band size.

2.2.2.6 Transient transfection

To investigate effects of overexpression on NF-κB signalling cell lines were transfected with Usp4 wild-type and mutant constructs. For luciferase reporter assays, cells were transfected with NF-κB reporter gene and *Renilla* luciferase vectors. For immunoprecipitation (IP) experiments, cells were transfected with vectors to express the relevant tagged proteins.

Method

SW1353 or HeLa cells were seeded into the appropriate culture vessel, depending on experiment, at a density of 1.5×10^4 cells/cm². The following day, cells were transfected with DNA and FuGENE HD transfection reagent at an optimised 3:1 FuGENE:DNA ratio, according to manufacturer's instructions. Briefly, DNA was added to serum-free DMEM and incubated at room temperature for 5 minutes. FuGENE HD was then added to the DNA/DMEM mix and incubated at room temperature for 15 minutes to allow formation of transfection reagent/DNA complexes. After complex formation the transfection mix was added to the cell culture medium and incubated at 37°C with 5% (v/v) CO₂ for 24 hours. For stimulation of cells, overnight serum starvation took place as is described in section 2.2.2.7.

HEK 293T cells were seeded at a density of 4.5×10^4 cells/cm² in 10cm dishes and transfected with 5µg plasmid DNA at a 4:1 FuGENE:DNA ratio, optimised for transfection efficiency, as outlined above.

NIH3T3 cells were seeded at a density of 1.5×10^4 cell/cm² in 4-well glass chamber slides. The following day, cells were transfected with DNA and GeneJuice transfection reagent at an optimised 3:1 GeneJuice:DNA ratio, according to manufacturer's instructions. After transfection, cells were incubated at 37°C with 5% (v/v) CO₂ for 24 hours.

2.2.2.7 Cell stimulations

At 24 hours post-transfection, cells were washed in PBS and serum-containing culture media was replaced with serum-free culture media for 16-20 hours. Cells were then stimulated for the appropriate time point(s). For time course stimulations cells were stimulated ensuring cells for all time points were lysed at the same time.

2.2.3 Immunofluorescence

For subcellular localisation experiments, NIH3T3 cells were transfected as described in section 2.2.2.6. After the transfection period, cells were fixed in paraformaldehyde (PFA) for 2 minutes, washed with PBS and stained using Vectashield® Mounting Medium for Fluorescence with 4'-6 diamidino-2-phenylindole (DAPI). Mounted slides were incubated for a minimum of one hour at 4°C. A Zeiss Cell Observer Spinning Disk Microscope with a 63x objective lens was used to observe cells by confocal fluorescent microscopy. Images were scanned on two channels, blue for DAPI fluorescence and green for GFP expression, and analysed using Leica Application Suite Advanced Fluorescence imaging software. Results from the analysis were scored as predominantly nuclear, nuclear and cytoplasmic or predominantly cytoplasmic.

2.2.4 RNA interference (RNAi)

2.2.4.1 Small interfering RNAs (siRNAs)

Standard siGENOME SMARTpool siRNAs were purchased from Dharmacon (Leicestershire, UK). SMARTpool siRNAs contain 4 siRNAs that target different regions of the target gene to maximise potency and specificity; the sequences of which can be found in Table 2.3.

Table 2.3. The 4 sequences which make up the siGENOME SMARTpool siRNAs for USP4 and the non-targeting siRNA pool #2.

siRNA SMARTpool Target Sequences	
siGENOME USP4 siRNA	siGENOME Non-Targeting siRNA Pool #2
GAUCUGAACCGGGUAAAGA	UAAGGCUAUGAAGAGAUAC
GAAUUGGACUAUGUAUUGG	AUGUAUUGGCCUGUAUUAG
GGAAUAAACUACUAAACUG	AUGAACGUGAAUUGCUCAA
CCAAUUGGAUGAAGGUUUA	UGGUUUACAUGUCGACUAA

2.2.4.2 siRNA Transfection

Method

SW1353 or HeLa cells were seeded in a 96- or 12-well plate at a density of 1.5×10^4 cells/cm². The following day, at around 50% cell confluency, cells were transfected with 50nM siRNA targeting USP4 (siUSP4) or non-targeting control siRNA pool #2 (siCon) using DharmaFECT transfection reagent 1 (DF1). In two separate tubes, the siRNA and DF1 were diluted in the appropriate volume of serum-free, antibiotic-free DMEM and incubated at room temperature for 5 minutes. Both tubes were then combined and incubated at room temperature for a further 20 minutes. After incubation, the transfection mix was added to an appropriate volume of serum-containing DMEM, culture medium was aspirated off the cells in each well and replaced with the transfection mix. Cells were then incubated at 37°C in 5% (v/v) CO₂ for the required time period as determined in section 2.2.4.3.

The optimal concentration of siGENOME siRNAs has generally been found to be 50nM and as such was the concentration all siRNAs were used at in this thesis (thesis of Dr. Matthew Barter, Newcastle University, Newcastle upon Tyne, UK).

2.2.4.3 Optimisation of siRNA transfection time

To determine the optimal transfection time with siUSP4 for USP4 depletion, siRNA transfection was performed as described in section 2.2.4.2 but for a range of transfection time periods: SW1353 cells were transfected with siUSP4 or siCon for 16, 24, 48 or 72 hours before cell lysis and immunoblotting were performed as described in section 2.2.6. Blots were probed with an anti-USP4 antibody (Table 2.1) and the optimal transfection time was visually determined. Figure 2.2 indicates that 24 hours of siRNA transfection is the best minimum duration for depletion of USP4 protein levels and as such was the chosen siRNA transfection time for experiments in this thesis.

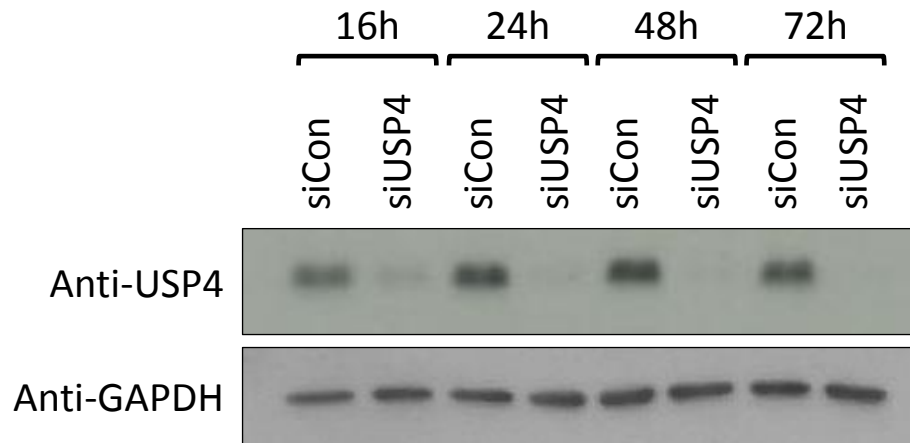


Figure 2.2. Optimisation of siRNA transfection time. SW1353 cells were transfected with non-targeting control siRNA (siCon) or siRNA targeting USP4 (siUSP4) at 50nM concentration for 16, 24, 48 or 72 hours (h). Cells were lysed and total protein extracted, resolved by SDS-PAGE and immunoblotted with an anti-USP4 antibody. GAPDH was used as a loading control. Figure is of one experiment but USP4 depletion was subsequently, routinely verified.

2.2.5 Luciferase reporter assays

2.2.5.1 NF- κ B reporter gene assay

Method

An NF- κ B reporter gene assay was conducted using the Dual-Luciferase Reporter Assay System (Promega, Southampton, UK) according to the manufacturer's protocol. Briefly, cells were seeded into 48-well plates and the next day transfected with 50ng NF- κ B firefly luciferase reporter and 3ng *Renilla* expression vectors as outlined in section 2.2.2.6. Following transient transfection of the expression vectors for appropriate times, cells were washed in PBS and lysed (if stimulation was required cells were serum-starved and stimulated as described in section 2.2.2.7 before lysis). Next, cell lysate was added to a cross-talk-free, white walled 96-well plate (PerkinElmer, Wellesley, MA, USA), luciferase assay reagent was injected to initiate the firefly luciferase reaction and luminescence was measured, both using a Glomax-Multi+ Detection System (Promega, Southampton, UK). After reading, a second reagent was injected to stop the reaction and luminescence for *Renilla* luciferase was also measured.

For experiments in which USP4 was depleted, cells were first transfected with NF- κ B reporter and *Renilla* expression vectors for a period of 6-8 hours before washing with PBS and transfecting with the relevant siRNAs for a further 24 hours, as described in section 2.2.4.2, before continuation of the reporter assay as described in this section.

For experiments in which Usp4 was overexpressed, cells were co-transfected with Usp4, NF- κ B reporter and *Renilla* expression vectors at a total DNA concentration of 250ng/well in a 48 well plate for 24 hours before continuation of reporter assay as described in this section.

2.2.5.2 Signal Finder multi-pathway reporter array

The multi-pathway reporter array consists of 10 dual-luciferase reporter assays for studying the gene activity of 10 signalling pathways in one experiment, listed in Table 2.4. The system employs a reverse transcription format in which cells are seeded onto transfection complexes present on the plate.

Table 2.4. Pathways included in the Cancer 10-pathway Reporter Array.

Pathway	Transcription Factor
Wnt	TCF/LEF
Notch	RBP-J κ
p53/DNA damage	p53
TGF β	SMAD2/3/4
Cell cycle/pRb-E2F	E2F/DP1
NF- κ B	NF- κ B
Myc/Max	Myc/Max
Hypoxia	HIF1A
MAPK/ERK	Elk-1/SRF
MAPK/JNK	AP-1

Method

The reporter array was performed using a Cignal Finder reporter array kit (QIAGEN, West Sussex, UK) following the manufacturer's protocol. Briefly, reporter assay constructs on the plate were resuspended in DMEM and incubated at room temperature for 5 minutes. After incubation, Attractene transfection reagent diluted at a 3:1 Attractene:DNA ratio in DMEM was added to the wells, mixed and incubated at room temperature for a further 20 minutes to allow transfection reagent/DNA complex formation. Next, the appropriate density of U2OS cells was added to the plate and mixed by gentle rocking. Cells were then incubated at 37°C with 5% CO₂ (v/v) in a humidified incubator for 24 hours. A luciferase assay was performed using the Dual-Glo Luciferase Assay System following manufacturer's instructions and luminescence measured on a luminometer as in section 2.2.5.1.

2.2.5.3 Optimisation of stimulation concentration and time*Method*

SW1353 cells were seeded into a 48-well plate at a density of 1.5×10^4 cells/cm². The following day cells were transfected with both the NF- κ B firefly reporter gene and *Renilla* expression vectors as described in section 2.2.2.6. After the experimental period, cells were stimulated with varying concentrations of either IL-1 α or TNF α for 6 hours before lysis. Figure 2.3A and Figure 2.3B show luminescence measured, indicating that 0.5ng/ml IL-1 α and 10ng/ml TNF α were the optimum concentrations for inducing NF- κ B activity.

Next the experiment was repeated but altering stimulation times before lysis. Figure 2.3C and Figure 2.3D show the optimum stimulation time for inducing NF- κ B activity was 6 hours for both IL-1 α and TNF α . Accordingly, stimulations in this thesis were performed using 0.5ng/ml and 10ng/ml for IL-1 α and TNF α , both for a period of 6 hours.

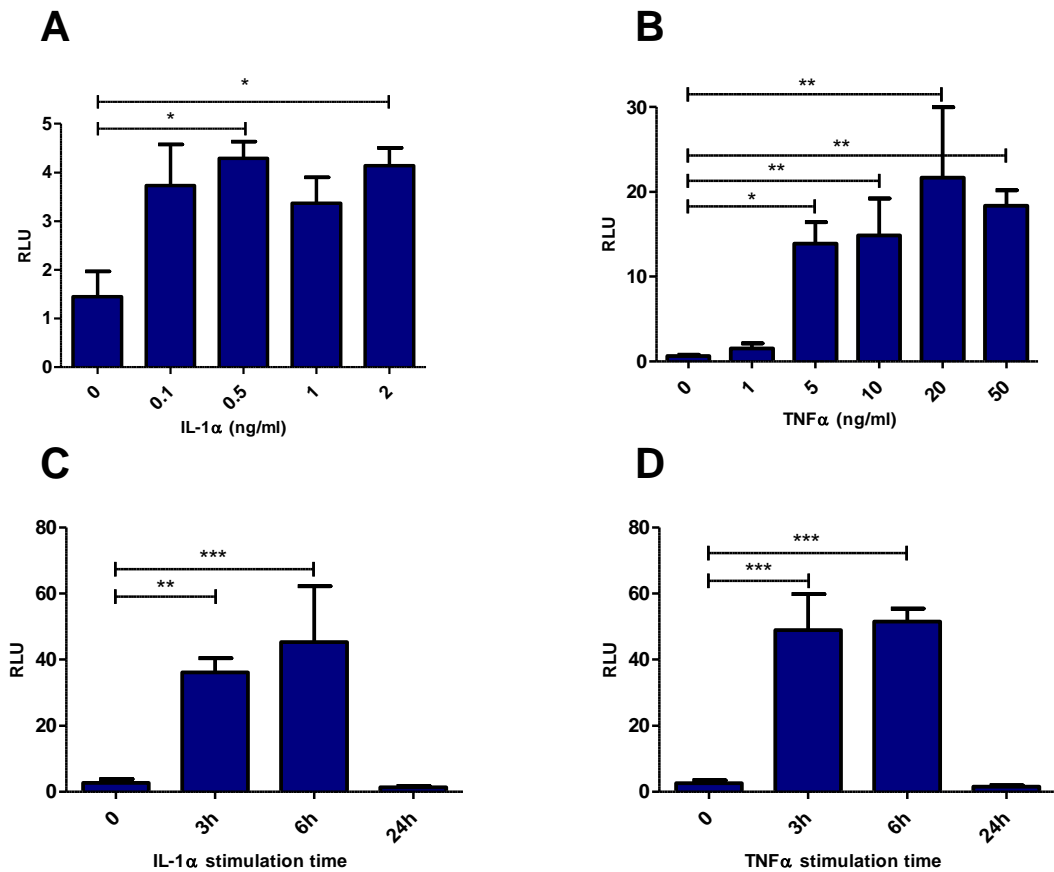


Figure 2.3. Optimisation of stimulation concentration and time on luciferase activity. SW1353 cells were transfected with NF- κ B firefly reporter gene and *Renilla* expression vectors. At 24h post-transfection cells were stimulated at varying concentrations or time points before cell lysis and measurement of luminescence. **A)** Cells were stimulated with 0.1, 0.5, 1 and 2ng/ml IL-1 α or left unstimulated (0). **B)** Cells were stimulated with 1, 5, 10, 20 and 50 ng/ml TNF α or left unstimulated (0). **C)** Cells were stimulated with IL-1 α (0.5ng/ml) for 3, 6 and 24h or left unstimulated (0). **D)** Cells were stimulated with TNF α (10ng/ml) for 3, 6 and 24h or left unstimulated (0). Values were normalised to *Renilla* and displayed as relative light units (RLU) as mean \pm SD (n=3/experiment).

2.2.6 Protein analysis

2.2.6.1 Whole cell lysis

Reagents

- Lysis buffer (50mM Tris, pH 7.4, 10% glycerol (v/v), 1mM EDTA, 1mM EGTA, 1mM Na₃VO₄, 5mM NaF, 10mM β-glycerol phosphate, 5mM Na₄P₂O₇, 1% Triton (v/v) X-100, 1μM microcystin-LF and 1 Complete protease inhibitor Mini tablet (Roche Diagnostics, West Sussex, UK) for 50ml of buffer)

Method

Cells were seeded in 12-well or 6-well plates at a density of 1.5×10^5 cells/cm². After the appropriate experimental period, culture medium was removed and cells were washed with ice-cold PBS. Ice-cold lysis buffer (containing 0.01% (v/v) β-mercaptoethanol) was added at 75μl/well or 100μl/well for 12- or 6-well plates, respectively. Cells were scraped and the cell suspension transferred to a cooled Eppendorf for a 20 minute incubation on ice. Following incubation the lysates were centrifuged at 4°C for 3 minutes at 10 000 x g. Supernatants were removed and immediately either stored at -80°C or a Bradford protein assay was performed.

2.2.6.2 Bradford protein assay

Method

Bradford protein assays were used to determine protein concentration in the lysates. A standard stock solution of BSA (2mg/ml) was diluted to 0.4mg/ml in dH₂O. A series of standards between 0-4mg/ml (at increments of 0.4mg/ml) and equal quantities of cell lysate samples and cell lysis buffer (as a control) were added to a flat-bottomed 96-well plate. BradfordUltra reagent was then added to the wells according to the manufacturer's protocol, followed by 5 minutes incubation at room temperature. Absorbance was read at 595nm using a Tecan Sunrise microplate absorbance reader and protein concentrations in the samples were calculated from this and equalised before sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis.

2.2.6.3 Immunoblotting

Reagents

- Separating gel solution: 4X lower gel buffer (LGB) (1.5M Tris Base pH8.8, 0.4% SDS (w/v)), 37.5:1 acrylamide/bis-acrylamide 40%, APS and TEMED

- Stacking gel solution: 4X upper gel buffer (UGB) (0.5M Tris-HCl pH6.8, 0.4% (w/v) SDS), APS, TEMED
- 5X Laemmli sample buffer (0.1M Tris-HCl, pH6.8, 0.35M SDS, 20% (v/v) glycerol, 0.01% bromophenol blue and 10% (v/v) β -mercaptoethanol)
- 10X Running buffer (250mM Tris Base, 2M Glycine and 10% (w/v) SDS)
- Transfer buffer (20mM Tris Base, 0.6M glycine and 20% (v/v) methanol)
- Tris buffered saline (TBS)-Tween (TBS-T) (150mM NaCl, 10mM Tris Base pH7.4, 0.1% (v/v) Tween-20)
- 5X Stripping buffer (500mM glycine, pH2)

Methods

Cell lysate samples were thawed on ice and Laemmli sample buffer added to the samples at a 5:1 ratio of total volume. Samples were then heated to 100°C for 5 minutes, cooled on ice and electrophoresed on 10% SDS-polyacrylamide gels. For the separating gel, acrylamide/bis-acrylamide 40% solution was diluted with 4X LGB and water at the correct volumes to give a 10% gel. TEMED and APS 0.2% (w/v) were added to the gel solution for polymerisation and the gel poured into 1.0mm Bio-Rad separator plates (Bio-Rad Laboratories, Hemel Hempstead, UK). Stacking solution made up of 4.5% acrylamide/bis-acrylamide and polymerised with TEMED and APS was added on top of the separating solution once set, with combs inserted. The SDS-PAGE gels were electrophoresed at 100-180V using the Bio-Rad mini-PROTEAN gel vertical electrophoresis system (Bio-Rad Laboratories, Hemel Hempstead, UK) in which the gel plates are inserted into a sealed casting frame and placed into a tank filled with 1X Running buffer. PageRuler™ pre-stained protein ladder was loaded onto the gel alongside the protein samples.

After electrophoresis, proteins were transferred from the gel to a PVDF membrane by electroblotting in transfer buffer using a Scie-Plas V20-SDB 20 x 20cm semi-dry blotter at 1mA/cm² for 1.5 hours. Membranes were blocked for 1 hour in TBS-T, 5% (w/v) non-fat dry milk powder or 1% BSA (according to antibody manufacturer's instructions). After thorough washing in TBS-T (3 times for 5 minutes each with gentle agitation) membranes were incubated overnight with gentle agitation at 4°C with primary antibody,

diluted according to manufacturer's instructions in primary antibody dilution buffer (TBS-T, 5% BSA/milk).

Following overnight incubation membranes were washed (3 x 5 minutes) before incubation with horseradish peroxidase (HRP)-conjugated secondary antibody (diluted 1:3000 in TBS-T, 5% milk) for one hour at room temperature with gentle agitation. Membranes were again washed for 3 x 5 minutes and then visualised on high-speed X-ray film using ECL detection reagents.

To allow probing with a different antibody, membranes were stripped. 1X Stripping buffer was heated to boiling and cooled to around 60°C before incubation with membranes for 2 x 45 minutes with gentle agitation. After washing with TBS-T for 3 x 5 minutes, membranes were then incubated with the desired primary antibody overnight as described above. An anti-GAPDH antibody was used to ensure equal loading.

2.2.7 Immunoprecipitation (IP)

Method

HEK 293T cells were seeded into 10cm dishes at a density of 4.5×10^4 cells/cm². The following day cells were transfected with the appropriate tagged protein for 24 hours as outlined in section 2.2.2.6. For experiments investigating effects of USP4 depletion, cells were transfected with siRNA 24 hours preceding plasmid transfection as outlined in section 2.2.4.2. Whole cell lysis was performed as in section 2.2.6.1 and cell lysate protein was quantified by Bradford assay as described in section 2.2.6.2. For IP experiments to investigate the polyubiquitination of proteins, 20mM NEM and 10µM MG132 were added to the extracts during lysis to inhibit deubiquitinase and proteasome activity, respectively. For IP experiments to investigate the phosphorylation of proteins 1mM Na₃VO₄ was added to all buffers to inhibit phosphatases.

First, the lysate was pre-cleared by adding 3µg of irrelevant antibody (IgG mouse/rabbit according to IP primary antibody species) to 300µg of protein sample (1µg/µl). Samples were incubated on ice for one hour followed by a 30 minute incubation at 4°C with end-to-end rotation with added Protein G PLUS-agarose conjugate suspension. After a 10 minute centrifugation at 14000 x g at 4°C, supernatants were removed for IP.

On ice, 3µg of the IP antibody was added to the pre-cleared lysate and incubated overnight with end-to-end rotation at 4°C. The following day Protein G PLUS-agarose conjugate

suspension was added to the samples and incubated for 3 hours with end-to-end rotation at 4°C. The bead pellet was collected by centrifugation at 10 000 x g for 5 minutes at 4°C and the supernatant discarded. The bead pellet was then washed 3 times with PBS, each time centrifuged at 10 000 x g for 1 minute at 4°C. After the last PBS wash was discarded, the bead pellet was resuspended in 2X Laemmli sample buffer and heated to 100°C for 5 minutes. Supernatant was collected and electrophoresis was performed as described in section 2.2.6.3.

2.2.7.1 Optimisation of the transfection of FLAG-tagged proteins

Method

SW1353 and HEK 293T cells were seeded into 10cm dishes at a density of 1.5×10^4 cells/cm² and 4.5×10^4 cells/cm², respectively. The following day cells were transfected with 1µg/ml of FLAG-TRAF6-wt plasmid DNA at FuGENE:DNA ratios of 1:1, 2:1, 3:1 and 4:1 or with 0.5µg/ml of plasmid DNA at a FuGENE:DNA ratio of 3:1 as previously described in section 2.2.2.6. After 24 hours, whole cell lysis and immunoblotting were performed as described in section 2.2.6.3. Blots were probed with anti-FLAG antibody. It can be seen in Figure 2.4 that at long exposure FLAG was detected in both SW1353 and HEK 293T cells. FLAG was detected at a higher level in HEK 293Ts indicating that these cells have higher transfection efficiency than the SW1353s. A ratio of 4:1 FuGENE:DNA (1µg/ml plasmid DNA) in HEK 293Ts was determined to be optimal for the transfection of plasmids, as such HEK 293Ts were chosen for use in IP experiments (Figure 2.4).

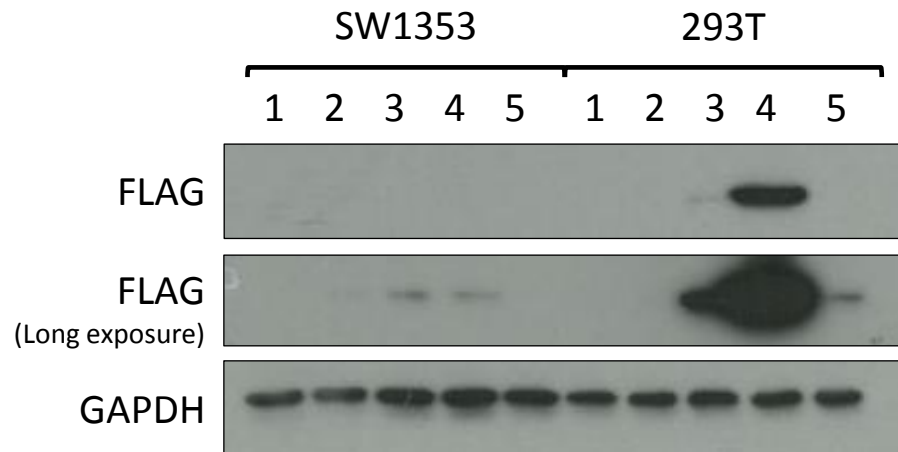


Figure 2.4. Optimisation of the transfection of FLAG-tagged proteins. SW1353 and HEK 293Ts were transfected with varying concentrations of plasmid DNA and varying transfection reagent:DNA ratios. At 24h post-transfection cells were lysed, immunoblotted and probed with anti-FLAG antibody. 1) 1:1, 2) 2:1, 3) 3:1, 4) 4:1, 5) 3:1 (0.5 μ g/ml). Ratios are shown as FuGENE HD transfection reagent:plasmid DNA. Plasmid DNA was transfected at a concentration of 1 μ g/ml unless otherwise stated. Figure is from one experiment. GAPDH was used as a loading control. Long exposure shows FLAG is present in SW1353s but at lower levels than in HEK 293Ts.

2.2.7.2 Optimisation of IP conditions with FLAG-tagged proteins

Method

Immunoprecipitation was performed as described previously in this section. To optimise IP conditions, lysates were incubated with varying concentrations of anti-FLAG antibody: 3µg of antibody was added to 300µg protein for a 1:100 antibody:protein ratio. Other concentrations of antibody were tested at ratios of 1:50 and 1:200. Conditions also included pre-cleared compared to not pre-cleared and washing with lysis buffer compared to washing with PBS. Pre-clearing the lysate and incubating with a 1:100 ratio of antibody:protein was optimal (Figure 2.5A). Washing in PBS rather than lysis buffer appeared to have no effect and so PBS was used to wash in all future immunoprecipitations (Figure 2.5B and E).

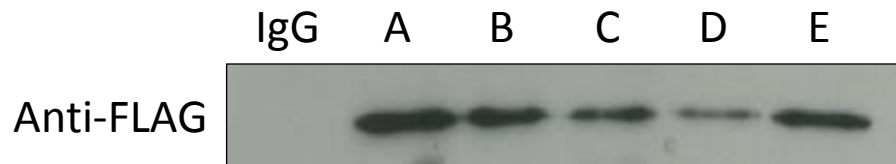


Figure 2.5. Optimisation of IP conditions. SW1353 cells were transfected for 24 hours with FLAG-TRAF6 plasmid before cell lysis and immunoprecipitation with anti-FLAG antibody under varying conditions. Immunoprecipitates were immunoblotted with anti-FLAG antibody. A) Pre-cleared, antibody ratio 1:100. B) Antibody ratio 1:100. C) Antibody ratio 1:50. D) Antibody ratio 1:200. E) Antibody ratio 1:100, washed with lysis buffer. No lysates were pre-cleared and all were washed in PBS unless otherwise stated. Figure is from one experiment.

2.2.8 RNA analysis

2.2.8.1 Cell lysis

Cells-to-cDNA II lysis buffer yields cell lysates for reverse transcription in a single step to lyse cells and inactivate RNases, removing the process of isolating RNA.

Method

Cell lysis was performed using Cells-to-cDNA II lysis buffer following the manufacturer's protocol. After siRNA transfection and the appropriate experimental time period as described in section 2.2.4.2, cell culture medium was removed and cells were washed in ice-cold PBS. Next, cells were lysed in 30µl ice-cold Cells-to-cDNA II lysis buffer per well, transferred to a 96-well PCR plate and heated to 75°C for 15 minutes to ensure RNase inactivation. Lysates were then stored at -80°C or reverse transcription was performed immediately.

2.2.8.2 Reverse transcription

Method

For reverse transcription, 8µl of the lysate was transferred to a new PCR plate to which final concentrations of 0.375mM dNTPs and 0.2µg random hexamers p(dN)₆ were added to each well and heated to 70°C for 5 minutes. The samples were then placed on ice and 5X First Strand Buffer, final concentration of 10mM DTT and M-MLV reverse transcriptase were added. The reaction was made up to 20µl total per well with H₂O, incubated at 37°C for 50 minutes followed by incubation at 75°C for 15 minutes. The stable cDNA was then diluted by addition of 30µl dH₂O for target gene quantification and diluted a further 1:50 for housekeeping gene (18S) quantification. The cDNA was stored at -20°C prior to analysis or quantified immediately by real-time qRT-PCR.

2.2.8.3 Primers for real-time qRT-PCR

Both forward and reverse primers for TaqMan® probe-based real-time qRT-PCR were designed using the Universal ProbeLibrary Assay Design Center (Roche Diagnostics, West Sussex, UK) and are shown in Table 2.5. Mouse 18S was an ABI assay on demand (Applied Biosystems, Life Technologies Ltd., Paisley, UK). The 18S ribosomal RNA gene was used as the housekeeping gene as an endogenous control for normalisation and to allow analysis of the relative expression of the gene of interest. To prevent amplification of any contaminating genomic DNA, all primers were designed to span an intron-exon boundary.

2.2.8.4 TaqMan® Probe-based real-time qRT-PCR

Method

For quantification of target genes, a subtotal 6µl reaction of 2X TaqMan gene expression master mix, 200nM of each forward and reverse primers, 150nM probe and made up with H₂O was prepared and added to each well of a 96-well qRT-PCR plate. Similarly, for quantification of the housekeeping gene a subtotal 5µl reaction of 2X TaqMan gene expression master mix, 300nM of each forward and reverse primers and 75nM probe was prepared. Next, 4µl of diluted cDNA or 5µl diluted cDNA, for target or housekeeping gene quantification respectively, was added to each well to give a total 10µl reaction. Real-time qRT-PCR was performed using the ABI Prism 7900HT PCR System (Applied Biosystems, Life Technologies Ltd., Paisley, UK). Thermocycling parameters were: 95°C for 10 minutes followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 minute.

Table 2.5. Sequences of primers used for real-time qRT-PCR.

Gene	Primer	Primer Sequence	Probe
Human IL6	Forward	5' -CAGGAGCCCAGCTATGAACT-3'	Probe library #45
	Reverse	5' -GAAGGCAGCAGGCAACAC-3'	
Human IL8	Forward	5' -AGACAGCAGAGCACACAAGC-3'	Probe library #72
	Reverse	5' -AGGAAGGCTGCCAAGAGAG-3'	
Human MMP13	Forward	5' -AAATTATGGAGGAGATGCCATT-3'	5' -CTACAACCTGTTTCTTGTGCTGCGCATGA-3'
	Reverse	5' -TCCTTGGAGTGGTCAAGACCTAA-3'	
Human USP4	Forward	5' -GATCGAGTTATGGAGGTTTTCT-3'	Probe library #18
	Reverse	5' -CGGCACAGTCACACGGTA-3'	
Human IκBα	Forward	5' -GTCAAGGAGCTGCAGGAGAT-3'	Probe library #38
	Reverse	5' -ATGGCCAAGTGCAGGAAC-3'	
Human 18S	Forward	5' -CGAATGGCTCATTAATCAGTTATGG-3'	5' -TCCTTTGGTCGCTCGCTCCTCTCC-3'
	Reverse	5' -TATTAGCTCTAGAATTACCACAGTTATCC-3'	
Mouse IL6	Forward	5' -TGATGGATGCTACCAAAGTGG-3'	Probe library #6
	Reverse	5' -TTCATGTACTCCAGGTAGCTATGG-3'	
Mouse CXCL1	Forward	5' -GACTCCAGCCACACTCCAAC-3'	Probe library #83
	Reverse	5' -TGACAGCGCAGCTCATTG-3'	
Mouse MMP13	Forward	5' -CAGTCTCCGAGGAGAACTATGA-3'	Probe library #62
	Reverse	5' -GGACTTTGTCAAAAAGAGCTCAG-3'	
Mouse USP4	Forward	5' -GCTGAATTGGTATGGCTGTG-3'	Probe library #66
	Reverse	5' -AGGCCATGCTCCACAACCTT-3'	

2.2.9 Genotyping mice

Genotypes of the mice/MEFs used in experiments were determined using the Extract-N-Amp™ Tissue PCR Kit (Sigma-Aldrich, Poole, UK).

Method

Extraction was performed following the manufacturer's protocol. Briefly, a small piece of ear or tail tissue was obtained from the mice and incubated in a 100µl mix of Extraction Solution and Tissue Preparation Solution for 10 minutes at room temperature. Next tissue extracts were incubated at 95°C for 3 minutes before the addition of 100µl of Neutralization Solution B. Samples were stored at 4°C or PCR was performed immediately.

All PCR reactions were prepared at room temperature as the JumpStart *Taq* antibody in the Extract-N-Amp PCR reaction mix is specific for hot start amplification. Added to a thin-walled PCR tube was 10µl Extract-N-Amp PCR reaction mix, 0.5µM of each of 3 primers (see Table 2.6) and 4µl tissue extract, made up with dH₂O to give a 20µl total reaction. Thermocycling parameters were: 94°C for 3 minutes followed by 32 cycles of 94°C for 30 seconds, 50°C for 30 seconds, 72°C for 1 minute and a final step of 72°C for 10 minutes. The amplified DNA was then loaded onto a 1% agarose gel, as described in section 2.2.2.5, to allow determination of genotype as shown in Figure 2.6. Mice were born at expected Mendelian ratios.

Table 2.6. Sequences of primers used for genotyping mice.

Primer	Primer Sequence
Ex3-F	5' -CCAGCAGCCTATTGTCAGAA -3'
In3-R	5' -TCAGTACTTAGGGATCTCTGA -3'
Neo-R	5' -AACCTGCGTGCAATCCATCT -3'

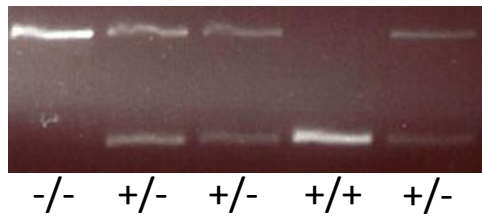


Figure 2.6. *Usp4* mouse genotypes. Extractions were performed using the ear or tail of mice before performing PCR using genotyping primers listed in Table 2.6 and loading onto a 1% agarose gel. Figure shows *Usp4*^{-/-} (-/-), *Usp4*^{+/-} (+/-) and *Usp4*^{+/+} (+/+) mice.

2.2.10 Cytokine array

A Meso Scale Discovery (MSD) cytokine array (Meso Scale Diagnostics, Gaithersburg, MD, USA) was performed using a Mouse ProInflammatory 7-Plex Ultra-Sensitive Kit. The principle of the assay is to allow measurement of levels of a number of cytokines and chemokines from one sample by use of antibodies pre-coated on a single well. The proinflammatory kit provides antibodies for IL-1 β , IL-12p70, IFN- γ , IL-6, KC/GRO, IL-10 and TNF- α in an immunoassay format. Electrochemiluminescent compounds are used to allow quantitative analysis of these cytokines and chemokines present in the sample by measuring light emitted.

Method

The MSD cytokine array was performed according to the manufacturer's protocol. First, blood samples were collected from wild-type (Usp4 $^{+/+}$), heterozygous (Usp4 $^{+/-}$) or Usp4 null ($+/-$) mice intravenously. Samples were left to clot for a minimum of 10 minutes at room temperature before centrifugation for 20 minutes at 4°C to remove clotted materials and obtain the mouse serum. Next, antibodies on the plate were reconstituted in buffer by vigorous shaking on a plate shaker (300-1000rpm) for 30 minutes. A range of calibrator standards or sample (25 μ l volume) were added to each well and subjected to vigorous shaking on a plate shaker (300-1000rpm) for 2 hours at room temperature. The plate was then washed in PBS (x3) and samples were incubated on a plate shaker (300-1000rpm) for 2 hours at room temperature with a detection antibody solution. After thorough washing in PBS (x3) read buffer was added to all wells and the plate was read on an MSD SECTOR Imager.

2.2.11 Enzyme-linked immunosorbent assay (ELISA)

To measure levels of IL-6 and CXCL1 levels in mouse serum, Mouse IL-6 and CXCL1 Quantikine kits (R&D Systems®, Abingdon, UK) were used. These immunoassays use antibodies raised against IL-6 and CXCL1 to quantitate levels of these cytokines.

Method

The serum of mice was obtained as described in section 2.2.10 and were sampled according to the manufacturer's protocol. Serum was diluted to a 1:2 concentration and 50 μ l of each sample was used. Briefly, standards, sample or control were added to wells containing a pre-coated antibody and incubated at room temperature for 2 hours. Wells were then washed 5 times to remove unbound substances and incubated with a HRP-

conjugated antibody at room temperature for a further 2 hours. After repeating the washing steps, a substrate solution to initiate an enzyme reaction followed by a stop solution were added to the wells and absorbance measured at 450nm on a Tecan Sunrise™ microplate absorbance reader (Tecan, Theale, UK). Standard curves for each ELISA were generated using a range of standards and are shown in Figure 2.7. Two standard curves are shown for CXCL1 as two separate experiments with two cohorts of mice were performed.

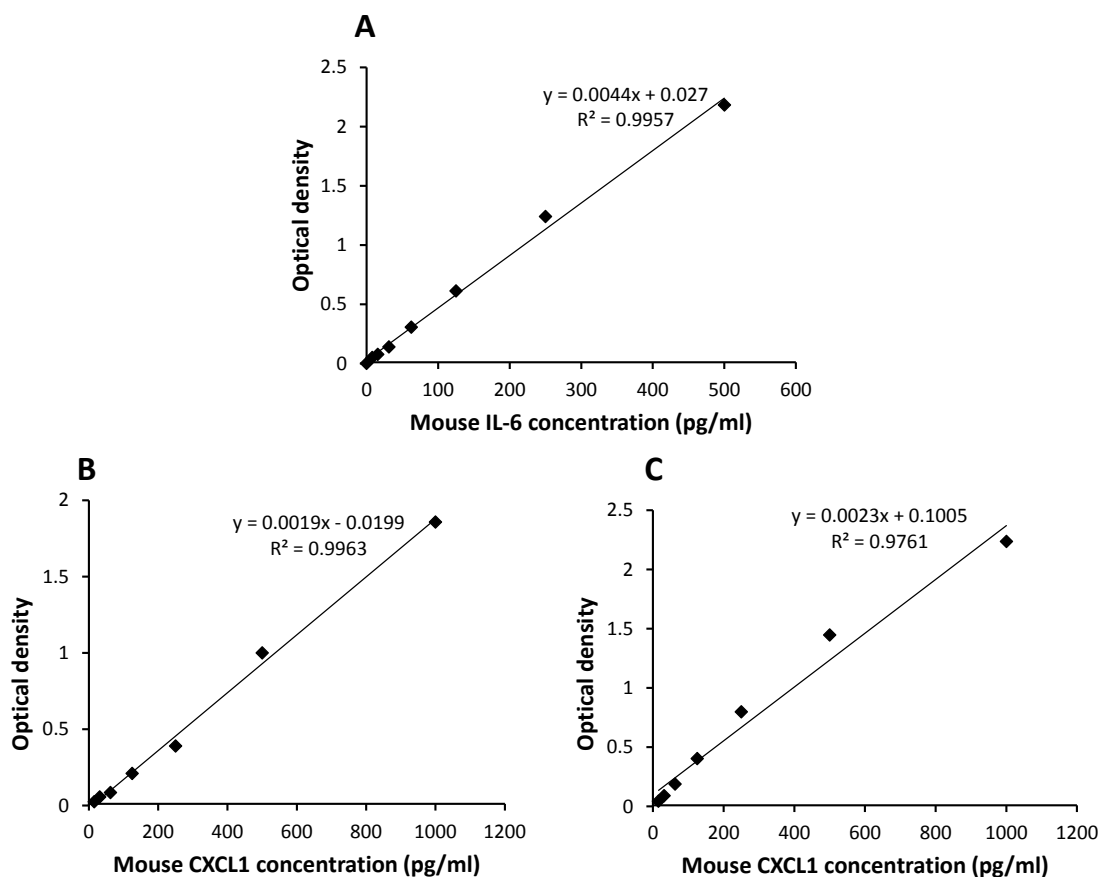


Figure 2.7. Standard curves for ELISAs to measure concentration of A) IL-6, B) CXCL1 and C) CXCL1 (second mouse cohort). A range of standards were prepared between 0-500pg/ml of either a mouse IL-6 standard or a mouse CXCL1 standard. Standards were added to wells containing a pre-coated antibody and the ELISA performed alongside samples. Absorbance was measured at 450nm.

2.2.12 Immunohistochemistry

2.2.12.1 Mouse infection

To compare viral replication in *Usp4*^{-/-} and *Usp4*^{+/+} mice to determine any effect on the immune response, immunohistochemical analysis on mouse brain sections infected with vesicular stomatitis virus (VSV) was performed. Mice were infected intranasally with 4×10^6 pfu VSV per mouse. The brains of these mice were harvested 48 hours later and fixed in formalin. In total 7 brains from *Usp4*^{+/+} and 8 mouse brains from *Usp4*^{-/-} mice were harvested.

2.2.12.2 Histology

Method

Mouse brains were embedded in paraffin blocks and cut into 5 μ m sections using a Leica RM2235 rotary microtome (Leica, Milton Keynes, UK) and placed on Superfrost Plus slides (Cellpath Ltd, Powys, UK).

2.2.12.3 Immunohistochemistry

Method

Sections were deparaffinised in 2 changes of xylene for 5 minutes each and rehydrated through graded concentrations of ethanol for 5 minutes each: 100% (x2), 95%, 70% and 50%, with a final wash in dH₂O. For heat-induced epitope retrieval the sections were placed in sodium citrate buffer pH 6.0 and microwaved for 2 x 5 minutes. The endogenous peroxidase activity was quenched by incubating the slides in 0.3% (v/v) H₂O₂ in dH₂O for 30 minutes. Sections were then blocked in 2% (v/v) normal horse blocking serum for 30 minutes before 30 minutes incubation with a 1:3000 dilution of primary antibody (diluted in PBS with 2% (v/v) normal horse blocking serum). Slides were then incubated with Biotin-conjugated secondary antibody for 30 minutes followed by a further 30 minutes incubation with Avidin/Biotin Complex (ABC). Peroxidase substrate 3,3'-diaminobenzidine (DAB) demonstrated peroxidase activity. Sections were washed in running tap water for 5 minutes and were counterstained with haematoxylin followed by dehydration through graded concentrations of ethanol for 5 minutes each: 50%, 70%, 95% (x2) and 100% (x2) with a final 5 minutes in xylene (x2). All incubation steps were carried out at room temperature and sections were washed thoroughly in between each step; all washing steps used PBS for 2 x 5 minutes unless otherwise stated. Slides were mounted

with D.P.X. mounting medium and covered with glass coverslips for visualisation under a Leica DM4000B microscope (Leica, Milton Keynes, UK).

Experimental work described in sections 2.2.12.1 and 2.2.12.2 was carried out by staff in the laboratory of Dr Doug Gray (Ottawa Hospital Research Institute, Ottawa, Canada). Experimental work in section 2.2.12.3 was carried out by Sharon Watson, Musculoskeletal Research Group (Institute of Cellular Medicine, Newcastle University, Newcastle, UK) with my assistance throughout.

2.2.12.4 Image analysis

All images were taken using a Leica SCN 400 slide scanner and viewed with the Slide Path software (Leica, Milton Keynes, UK).

Method

The percentage of area of mouse brain stained positive for VSV was calculated using FFramework for Image Dataset Analysis (FrIDA) software (Cornish et al., 2008). This software facilitated the use of image masks, enabling inclusion and exclusion of areas of the image or pixels of specific colours. For each image, only the brain area was selected and a pixel threshold for hue, saturation and brightness was set to identify only the brown stained pixels (VSV positive). The pixel threshold was the same for all images analysed, allowing a percentage of positive staining to be quantified in each brain area selected. All images were analysed blind to eliminate bias.

2.2.13 Lentivirus: inducible expression system

Lentiviral expression vectors are a powerful and effective tool for the delivery of a gene of interest into cells which can be difficult to transfect. Primary cell lines, such as MEFs, are notoriously difficult to transfect and so a lentiviral system was used to introduce an NF- κ B reporter vector for the study of NF- κ B activity and mutant Usp4 overexpression vectors for the examination of Usp4 phosphorylation in SW1353 cells and/or *Usp4*^{+/+} and *Usp4*^{-/-} MEFs.

Two methods were used for producing lentiviral supernatants, the first utilised the Lenti-X™ Tet-On® Advanced Inducible Expression System (Clontech, Paris, France).

2.2.13.1 Cloning

For lentiviral introduction of mutant Usp4 into cells, the GFP-tagged Usp4 constructs were cloned into a response vector, pLVX-Tight-Puro, which contains an inducible

promoter, P_{Tight} , that controls expression of the Usp4 gene. These response vectors were generated for wild-type, catalytically inactive Usp4 and all phosphorylation mutants of Usp4 generated in section 2.2.2.2. For cloning, all restriction enzymes used were FastDigest® enzymes in combination with 10X FastDigest® Green buffer and ligation was performed using the Rapid DNA ligation kit (Fermentas, Thermo Scientific, Leicestershire, UK).

Method

To clone the Usp4 vectors into the multiple cloning site (MCS) of the response vector, all vectors were double digested with *MluI* and *BamHI* restriction enzymes. Briefly, reactions were prepared of 2µl 10X buffer, 1µl of each restriction enzyme, 1µg DNA and made up to total 20µl with nuclease-free water. After mixing, reactions were incubated for 10 minutes at 37°C before loading onto a 0.8% (w/v) agarose gel as described in section 2.2.2.5.

Next, bands of the expected size on the gel were extracted using a QIAquick Gel Extraction kit, according to the manufacturer's protocol. DNA was eluted in 50µl and the insert cut from the Usp4 vector was ligated into the response vector by a reaction with insert and vector at a 5:1 ratio using the Rapid DNA Ligation kit, following the manufacturer's instructions. 1µl of the ligation was then used in bacterial transformation as described in section 2.2.2.3.

2.2.13.2 Lentivirus generation

To produce lentivirus, the lentiviral vectors constructed in the previous section were packaged into viral particles along with Lenti-X HTX Packaging mix which contains lentiviral vectors and envelope vectors. A second lentiviral supernatant was produced with a regulator vector, pLVX-Tet-On-Advanced, which contains a tetracycline-controlled transactivator, rtTA-Advanced, along with the Lenti-X HTX Packaging mix. Transduction of both lentiviral supernatants and treatment with doxycycline should induce expression of the Usp4 gene.

Method

HEK 293T cells were seeded into 10cm dishes at a density of 8×10^4 cells/cm². The next day media was replaced with DMEM (with 10% (v/v) FBS) and cells were transfected with the lentiviral response or regulator vector and packaging mix as in section 2.2.2.6. At 24 hours post-transfection, medium was replaced with DMEM (with 10% (v/v) FBS)

and incubated for a further 48 hours to allow viral production. Lentiviral supernatants were then harvested by centrifugation of the medium at 500 x I for 10 minutes to remove cellular debris and stored in 0.5ml aliquots at 4°C for short-term or -80°C for long-term storage.

2.2.13.3 Transduction

Transduction into target cells first needed to be optimised. This was tried in SW1353 cells using the lentiviral supernatant for wild-type Usp4.

Method

Target cells were seeded into 24-well plates at a density of 1.5×10^4 cells/cm². The following day, media was replaced with a transduction mix containing the appropriate amount of virus, DMEM (with 10% FBS), varying amounts of doxycycline and 8µg/ml polybrene. Different ratios of response and regulator lentiviral supernatants were used (1:1, 1:4 or 4:1) and different concentrations of doxycycline were added to different wells (0.125ng/ml, 250ng/ml, 500ng/ml, 1µg/ml or 2µg/ml). For control, some wells contained polybrene only. Cells were incubated at 37°C with 5% (v/v) CO₂ for 24 hours. After 24 hours, cell culture media was replaced with DMEM (with 10% (v/v) FBS) and doxycycline and cells were incubated for a further 24 hours. After the experimental period, cells were visualised on a fluorescent microscope for examination of GFP expression. No fluorescence was observed with any of the conditions so it was decided that a different lentiviral expression system would be approached.

2.2.14 Lentivirus: second generation expression system

The second lentiviral system utilised was a second generation lentiviral expression system.

2.2.14.1 Cloning

For lentiviral introduction of mutant Usp4 into cells, In-Fusion cloning (Takara Bio Europe/Clontech, Paris, France) enabled cloning of all mutant Usp4 overexpression vectors into the lentiviral expression vector pCDH-EF1-MCS-IRES-GFP, which contains the essential machinery for packaging, transduction and stable integration of the gene into the cells genomic DNA. Lentiviral expression vectors for wild-type, catalytically inactive Usp4 and all phosphorylation mutants of Usp4 generated in section 2.2.2.2 were produced by In-Fusion cloning using the In-Fusion® HD Cloning kit (Takara Bio Europe/Clontech, Paris, France).

Method

Before Usp4 could be fused into the lentiviral vector, primers were designed to isolate and linearise Usp4 from the pCMV6-AN-mGFP vector by PCR. Primers were designed to incorporate a KOZAK consensus sequence immediately prior to the start codon for efficient initiation of translation as well as 15 bases at the 5' end of the primers homologous to the vector (Kozak, 1987) (Table 2.7).

Table 2.7. Sequences of primers for infusion cloning.

Primer	Primer Sequence
mUSP4 Inf 1 F	5'-tagagctagcgaattGCCACCATGGCGGAAGGCCGGGGCAGCC-3'
mUSP4 Inf 1 R	5'-atttaaattcgaatttCAGTTGGTGTCCATGTTGTAAGCCT-3'

A PCR reaction was prepared of 4µl 5X Phire Reaction Buffer, 2.5mM dNTPs, 0.5µM forward and reverse infusion primers, 0.4µl Phire Hot Start II DNA Polymerase, 5ng plasmid DNA (pCMV-AN-Usp4-mGFP) and made up to 20µl with dH₂O. The reaction was cycled following the parameters: initial step of 98°C for 30 seconds followed by 35 cycles of 98°C for 5 seconds, 68°C for 5 seconds, 72°C for 1 minute 15 seconds (10-15 seconds per kb of plasmid length), and a final step at 72°C for 2 minutes. After cycling, the PCR reaction was purified using the NucleoSpin® Gel and PCR Clean-up kit (Machery-Nagel, Düren, Germany) following manufacturer's protocol and was eluted in 25µl Buffer NE. This PCR reaction was performed for wild-type and all phosphorylation mutants of Usp4.

To linearise the lentiviral expression vector, pCDH-EF1-MCS-IRES-GFP, 5µg of plasmid DNA was digested with *EcoRI* in a total 25µl reaction, at 37°C for one hour followed by a 15 minute heat-inactivation of *EcoRI* at 65°C.

Next, the In-Fusion Cloning reaction was prepared of 2µl In-Fusion HD enzyme premix, 50ng linearised lentiviral vector, 50ng PCR reaction insert and made up to 10µl total with dH₂O. The cloning reaction was then incubated for 15 minutes at 50°C before being placed on ice, ready for transformation.

Transformation of the cloning reaction mix was done using Stellar Competent Cells according to the manufacturer's protocol. On ice, cloning reaction mix was added to Stellar Competent Cells and incubated for 30 minutes. Following a 45 second heat shock

at 42°C, cells were returned to ice for 1-2 minutes before the addition of pre-warmed S.O.C. medium and a one hour incubation at 37°C with shaking at 200rpm. After incubation, the transformation mixes were spread onto LB-agar plates with ampicillin resistance and cultured as described in sections 2.2.2.3 and 2.2.2.4. Plasmids were sequenced by Sanger sequencing for verification: primer sequences can be found in Table 2.2.

2.2.14.2 Lentivirus generation

To produce lentivirus, the lentiviral vectors constructed in the previous section were packaged into viral particles using a second generation lentiviral system in which HEK 293T cells were transfected with two other plasmids along with each lentiviral vector: psPAX2 packaging vector and pCMV-VSV-G envelope vector. Lentivirus using the empty pCDH-EF1-MCS-IRES-GFP vector, as a control, was also produced.

Method

HEK 293T cells were seeded into 10cm dishes at a density of 8×10^4 cells/cm². The next day media was replaced with DMEM (with 10% (v/v) heat-inactivated FBS) and cells were transfected with the lentiviral vector, packaging vector and envelope vector as in section 2.2.2.6. At 24 hours post-transfection, medium was replaced with DMEM (with 10% (v/v) heat-inactivated FBS) and incubated for a further 48 hours to allow viral production. Lentiviral supernatants were then harvested by centrifugation of the medium at 500 x g for 10 minutes to remove cellular debris and stored in 0.5ml aliquots at 4°C for short-term or -80°C for long-term storage.

2.2.14.3 Virus titre

To determine the viral RNA genome content in the lentivirus supernatants produced, titration was performed using the Lenti-X™ qRT-PCR Titration kit (Takara Bio Europe/Clontech, Paris, France).

Method

For each of the lentiviral supernatant stocks produced, 150µl viral RNA was purified using the NucleoSpin RNA virus kit (part of the Lenti-X™ qRT-PCR Titration kit) following the manufacturer's protocol. RNA was eluted in 50µl RNase-free water. Residual plasmid DNA was removed in a DNase reaction with DNase I Buffer and DNase I enzyme, incubated at 37°C for 30 minutes, followed by incubation at 70°C for 5 minutes. Lentiviral genomic RNA was then amplified by qRT-PCR. Both Lenti-X RNA control

template and sample viral RNA were diluted in a series of 10-fold dilutions and reactions of 12.5µl 2X Quant-X Buffer, 0.4µM of each Lenti-X forward and reverse primers, ROX Reference Dye LMP, 0.5µl Quant-X enzyme, 0.5µl RT enzyme mix were made up to 25µl with RNase-free water. The enzyme reaction mix was added to the qRT-PCR plate and 2µl of either Lenti-X control, sample or a no-template control were added to the mix in duplicate. Thermocycling parameters were: 42°C for 5 minutes, 95°C for 10 seconds followed by 40 cycles of 95°C for 5 seconds, 60°C for 30 seconds and a final dissociation curve cycle at 95°C for 15 seconds, 60°C for 30 seconds and 60-95°C.

A standard curve was generated using average C_t values from the control dilution duplicates plotted against copy number (log scale) for the calculation of the quantity of viral RNA. Once an average C_t value for each sample was determined and a corresponding value from the standard curve read, the copy number of sample viral RNA per ml was then calculated from the following equation:

$$\text{Copies/ml} = \frac{(\text{copy number from } C_t\text{s})(1000\mu\text{l/ml})(2x \text{ DNase})(50\mu\text{l RNA elution})}{(150\mu\text{l sample})(2\mu\text{l added to well})}$$

The lentiviral supernatants produced were for a number of Usp4 mutants, wild-type Usp4 and for the control empty vector. For experimental procedures using the lentiviral supernatants, the values obtained from the above equation were used to transduce target cells with equal amounts of viral RNA/particles.

2.2.14.4 Transduction of Usp4 wild-type and phosphorylation mutants

Method

Target cells were seeded into 48- or 6-well plates at a density of 1.5×10^4 cells/cm². The following day, media was replaced with a transduction mix containing the appropriate amount of virus, DMEM (with 10% heat-inactivated FBS) and 8µg/ml polybrene. Cells were incubated at 37°C with 5% (v/v) CO₂ for 24 hours. After 24 hours, cell culture media was replaced with DMEM (with 10% (v/v) heat-inactivated FBS) or serum-free DMEM (if cells were to be stimulated) and cells were incubated for a further 24 hours. After the experimental period, cells were either lysed immediately (for western blots to examine overexpression) or stimulated before lysis (for NF-κB luciferase reporter assays).

2.2.15 Computational modelling

A computational model was constructed to incorporate USP4 in NF- κ B signalling. An initial model was constructed in collaboration with Dr Carole Proctor (Newcastle University, Newcastle, UK) and included key molecular mechanisms of NF- κ B signalling and assumptions based on results from work in this thesis. The model was encoded in the Systems Biology Markup Language (SBML) (Hucka et al., 2003). The model was constructed and stochastic simulations performed using a Complex Pathway Simulator (COPASI) (Hoops et al., 2006).

2.2.16 Statistical analysis

Luminescence values were normalised against *Renilla* values and combined experiment values were given as mean \pm standard error of the mean (SEM).

The relative levels of gene expression were calculated using appropriate standard curves, from C_t values which are inversely proportional to the expression of the gene. Data were normalised against the basal levels (or a set time point for time course stimulations) of the housekeeping gene 18S. Data were then plotted as gene of interest relative to 18S. Data from at least 2 experimental repeats were combined and values given as mean \pm standard error of the mean (SEM).

Statistical significance of differences between protein levels was determined using densitometry for which the relative density of each band was plotted at each stimulation time point. Data from at least 3 experimental repeats were combined and values given as mean \pm SEM.

An unpaired Student's t-test was performed to compare the means and determine any statistical significance between two independent sample groups. One-way analysis of variance (one-way ANOVA) was performed to compare the means of more than two samples, with a post-hoc Tukey comparison test, or two-way ANOVA was performed, with a post-hoc Bonferroni comparison test. Statistical significance is shown as * $p \leq 0.05$, ** $p \leq 0.01$ and *** $p \leq 0.001$.

Chapter 3 : USP4 in Inflammatory and Immune Pathways

3.1 Introduction

Multiple cell signalling pathways regulate the expression of a wide number of immunoregulatory genes to mediate the immune response. Ligand induction of pathways, including those mediated by IL-1Rs, can lead to the activation of transcription factors NF- κ B and AP-1 which control the expression of many proinflammatory cytokines such as TNF α , IL-6, IL-1 β and IL-8, which in turn amplify inflammatory signalling cascades and facilitate an immune response. Besides these proinflammatory cytokines, viral recognition by immune cell pattern-recognition receptors (PRRs), including RLRs, activates signal transduction to activate the transcription factor IRF3 and produce type I IFNs, such as IFN- β , to mediate an antiviral response (Yoneyama et al., 2015).

Sustaining tight regulation of these pathways is crucial in mediating the correct inflammatory and immune responses and maintaining immune homeostasis. When beginning this thesis, there were few reports on USP4 in cell signalling pathways. Since then, new findings have been published which have reported USP4 to play a regulatory role in pathways including the NF- κ B and RIG-I signalling pathways, and as such may be important in inflammatory and immune responses (Zhou et al., 2012, Wang et al., 2013). It has been suggested that USP4 negatively regulates TNF α , IL-1 and LPS-induced NF- κ B activation in HEK 293T and HeLa cell lines, through interaction and deubiquitination of substrates including TAK1, TRAF2 and TRAF6 (Fan et al., 2011b, Xiao et al., 2012, Zhou et al., 2012). In support of this, expression of many proinflammatory cytokines regulated by NF- κ B were increased with siRNA-mediated depletion of USP4 and in *Usp4* null mouse embryonic fibroblasts (MEFs) in these published studies.

USP4 has also been reported to positively regulate RIG-I signalling: it stabilises RIG-I to enhance RIG-I activation, and consequently downstream production of IFN- β in response to viral infection. Additionally, USP4 influences viral replication in HEK 293T cells infected with the VSV virus. RLRs recognise viral RNA, including VSV, to trigger an antiviral innate immune response indicating that USP4 is potentially involved in the RIG-I-mediated response to viral infection (Wang et al., 2013). The study examined the effect of USP4 on viral replication in a human embryonic kidney cell line, no *in vivo* examination has been performed.

There is some crosstalk between the RIG-I and NF- κ B pathways: detection of viral RNA by RLRs initiates a downstream signal to activate the transcription factor IRF3 through TBK1, and also to activate the NF- κ B transcription factor through the IKK complex. This leads to induction of IFN- β and proinflammatory cytokines, respectively, to mediate antiviral and inflammatory responses (Yoneyama et al., 2015). Given that USP4 has been reported to regulate RIG-I and NF- κ B pathways, USP4 may play important roles in facilitating antiviral and inflammatory immune responses.

When this project was initiated, the role of USP4 in cell signalling pathways was only just beginning to emerge and was still poorly defined in any single pathway. Therefore the first step was to identify whether USP4 may play a role in cell signalling pathways. To determine whether USP4 affects cell signalling pathways, the effects of overexpression of Usp4 on the activity of an array of pathways was first examined, as listed in Table 2.4. As the activation of these pathways leads to the production of a number of genes, including inflammatory cytokines and genes involved in the immune response, the wider effect USP4 may have on inflammatory and immune responses was also examined. Experiments were performed using wild-type (*Usp4*^{+/+}), heterozygous (*Usp4*^{+/-}) and Usp4 null mice (*Usp4*^{-/-}), and MEFs derived from these mice, to explore the potential impact Usp4 may have on cytokine production and viral replication *in vivo* and *in vitro*.

3.2 Aims

- Determine whether Usp4 is involved in regulating cell signalling pathways associated with cancer
- Examine the levels of key cytokines in the serum of *Usp4*^{+/+}, *Usp4*^{+/-} and *Usp4*^{-/-} mice
- Examine viral replication in MEFs derived from *Usp4*^{+/+} and *Usp4*^{-/-} mice
- Identify whether viral replication is affected in the brains of *Usp4*^{+/+} and *Usp4*^{-/-} mice

3.3 Results

3.3.1 Usp4 overexpression affects cells signalling pathways

To explore the potential role of USP4 in cell signalling pathways, an initial experiment to examine the effect of USP4 using a multi-pathway reporter array, which examines the gene activity of 10 signalling pathways in one experiment, was performed. Human osteosarcoma U2OS cells were used as this cell line has wild-type p53, which is a key player in one of the pathways examined in this array. Cells were seeded onto the reporter array containing constructs for 10 pathways (listed in Table 2.4) with either wild-type Usp4, a catalytically inactive version of Usp4 (C311A) or a control vector (p-CMV6-AN-mGFP) and a luciferase assay was performed as described in section 2.2.5.2. The expression plasmid used was for mouse Usp4 which shares 94% homology with human USP4. The reporter array determined that overexpression of Usp4 particularly affected the basal activity of p53, NF- κ B, MAPK/ERK and MAPK/JNK pathways (Figure 3.1). Overexpression of wild-type Usp4 significantly reduced p53 activity compared to the control, but not overexpression of catalytically inactive Usp4. MAPK/ERK activity was significantly increased with overexpression of wild-type Usp4 but not with overexpression of catalytically inactive Usp4. Most notably, overexpression of wild-type Usp4 significantly increased both NF- κ B activity and MAPK/JNK activity, whereas overexpression of catalytically inactive Usp4 significantly reduced NF- κ B activity and MAPK/JNK activity compared to wild-type Usp4 (Figure 3.1).

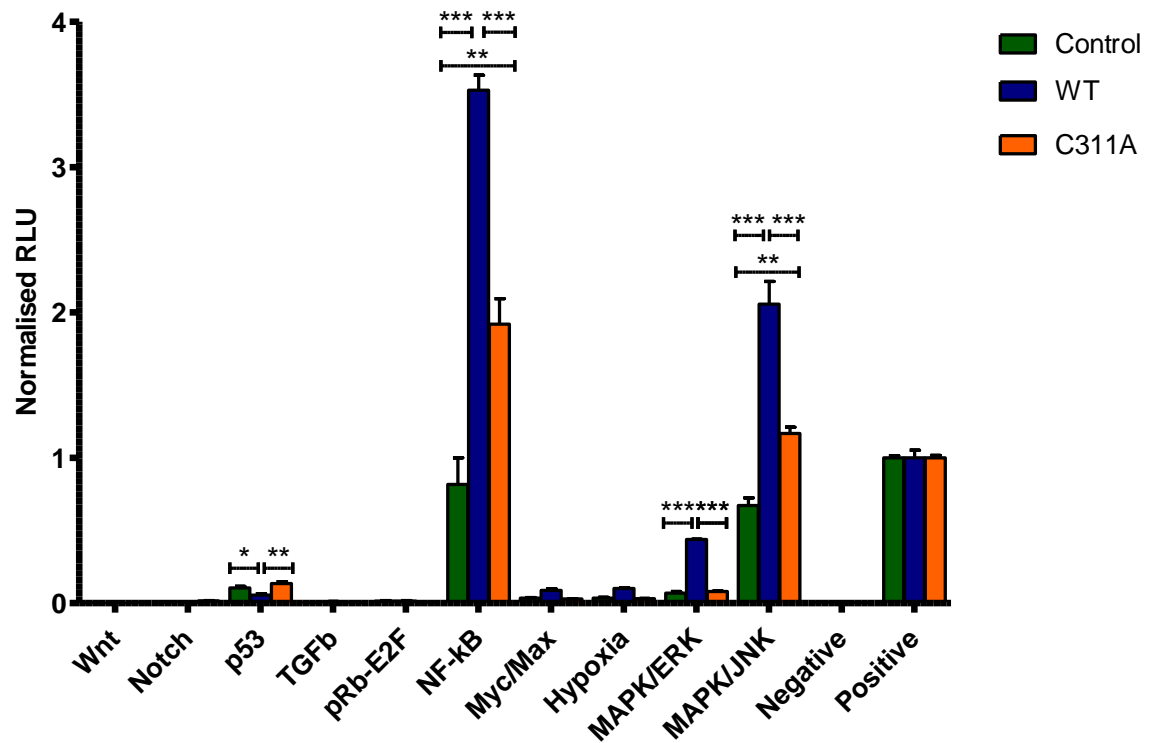


Figure 3.1. Overexpression of *Usp4* affected the activity of cell signalling pathways. A *Renilla* reporter vector and either p-CMV6-AN-mGFP control vector (Control), wild-type *Usp4* expression plasmid (WT) or catalytically inactive *Usp4* expression plasmid (C311A) were added to wells of a multi-pathway reporter array plate containing reporter constructs for 10 different pathways. After transfection reagent/DNA complex formation, U2OS cells were seeded onto the plates. At 24 hours post-transfection a luciferase assay was performed. Data were normalised to *Renilla* and to positive control values and are displayed as normalised relative light units (RLU). Figure is from one experiment and plotted as mean \pm SEM (n=4/experiment). For statistical analysis, one way ANOVA with a post-hoc Tukey comparison test was performed on data from each pathway separately. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$.

3.3.2 Cytokine levels in *Usp4*^{+/+}, *Usp4*^{+/-} and *Usp4*^{-/-} mice

3.3.2.1 The effect of *Usp4* on cytokine levels in mice

Overexpressing *Usp4*, but not catalytically inactive *Usp4*, affected the activity of some cell signalling pathways under basal conditions: NF- κ B activity and MAPK/ERK or JNK activity were significantly increased with *Usp4* overexpression (Figure 3.1). As these pathways regulate the expression of a number of target genes, including many inflammatory cytokines, the effect of USP4 on the levels of multiple cytokines was next examined.

A *Usp4* deficient mouse line was produced from gene-trap embryonic stem cells and a colony established. *Usp4* null (*Usp4*^{-/-}) mice were viable and were used to detect any differences in some key cytokines with loss of *Usp4*. Serum was obtained from *Usp4* wild-type (*Usp4*^{+/+}), heterozygous (*Usp4*^{+/-}) and *Usp4*^{-/-} mice and an MSD cytokine array was performed to measure the levels of a number of cytokines and chemokines in one experiment, as described in section 2.2.10. These preliminary data indicated there may be alterations in the levels of some cytokines when *Usp4* was absent, particularly Il-6 and C-X-C motif ligand 1(Cxcl1) which were reduced in the serum of *Usp4*^{-/-} mice compared to *Usp4*^{+/+} mice (Figure 3.2). The highest levels of Cxcl1 were observed in *Usp4*^{+/+} mouse serum, with lower levels in *Usp4*^{+/-} mouse serum and the lowest levels in *Usp4*^{-/-} mouse serum. Il-6 levels were also observed to be at the highest in *Usp4*^{+/+} mouse serum, with lower levels observed in *Usp4*^{-/-} mouse serum but levels of Il-6 in the serum of *Usp4*^{+/-} mice were the most reduced (Figure 3.2).

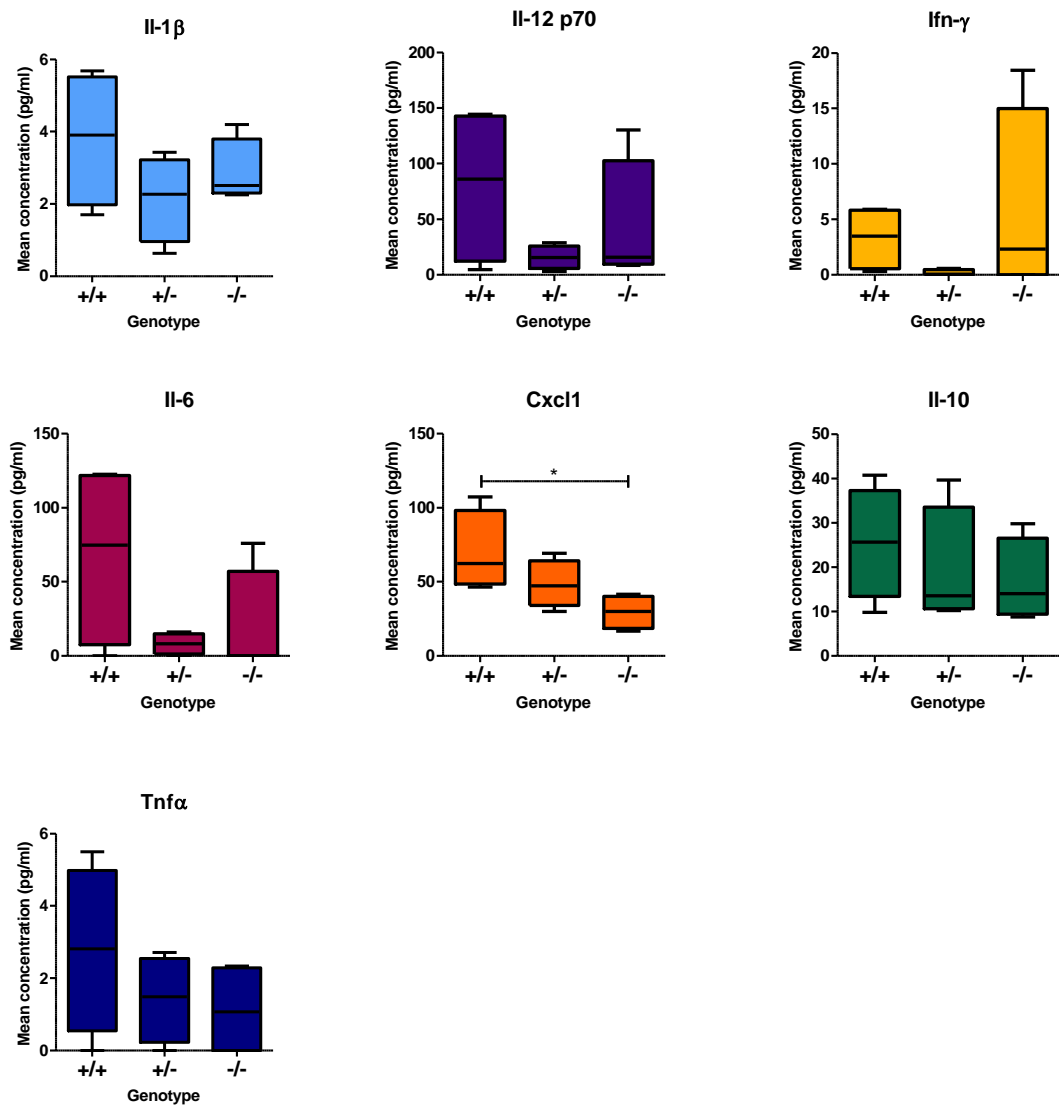


Figure 3.2. Cytokine levels in *Usp4*^{+/+} (+/+), *Usp4*^{+/-} (+/-) and *Usp4*^{-/-} (-/-) mouse serum. A MSD cytokine array was performed to measure levels of proinflammatory cytokines in the serum of *Usp4*^{+/+}, *Usp4*^{+/-} and *Usp4*^{-/-} mice. Serum samples were added to wells pre-coated with antibodies for IL-1 β , IL-12p70, Ifn- γ , IL-6, Cxcl1, IL-10 and Tnf α , and levels were detected. All mice were male (n=4/genotype). For statistical analysis, one way ANOVA with post-hoc Tukey comparison test was performed for each cytokine. * p \leq 0.05.

3.3.2.2 Levels of cytokines Il-6 and Cxcl1 in *Usp4*^{-/-} mice

To further examine the potential effect of *Usp4* on the levels of cytokines Il-6 and Cxcl1 in the serum of mice, ELISAs were performed which use antibodies raised against Il-6 and Cxcl1 to specifically detect levels in the serum of *Usp4*^{+/+}, *Usp4*^{+/-} and *Usp4*^{-/-} mice as described in section 2.2.11. In contrast to Figure 3.2 where Il-6 and Cxcl1 were detected at lower levels in *Usp4*^{-/-} compared to the wild-type, no definitive differences were observed between the different genotypes of either Il-6 or Cxcl1 and stratifying for male/female mice demonstrated no differences between gender (Figure 3.3).

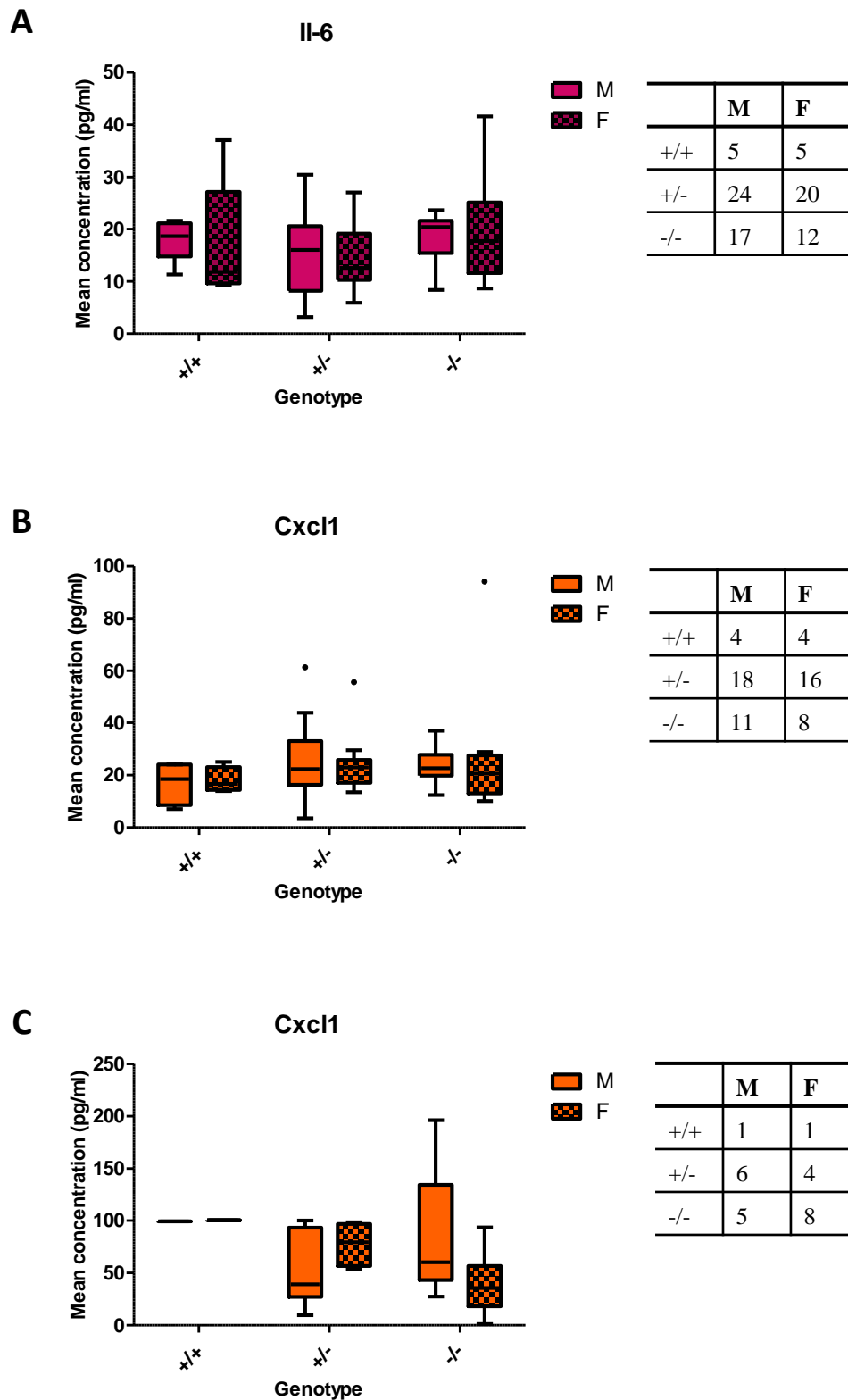


Figure 3.3. Levels of Il-6 and Cxcl1 in the serum of mice. ELISAs were performed using serum from *Usp4*^{+/+} (+/+), *Usp4*^{+/-} (+/-) and *Usp4*^{-/-} (-/-) mice (-/-). A) Il-6. Figure is of one experiment where total n=83. B) Cxcl1. Figure is of one experiment where total n=61, respectively. C) Cxcl1. Figure is of one experiment where total n=25. Male and female numbers for each genotype are displayed in tables (M and F, respectively). Dots represent outliers.

3.3.3 Viral replication in *Usp4*^{-/-} mouse embryonic fibroblasts

Usp4 has a role in the NF- κ B signalling pathway, as demonstrated in this chapter by the increase observed in NF- κ B activity with Usp4 overexpression (Figure 3.1), along with potential alterations in cytokine levels with loss of Usp4 (Figure 3.2). USP4 also has a reported role in the antiviral RIG-I signalling pathway, which can activate IRF3 and also NF- κ B transcription factors (Wang et al., 2013). These indicated that USP4 may affect immune and inflammatory responses, which if compromised in any way can affect the ability for clearing viral infection and lead to changes in viral replication. As a preliminary experiment prior to examining VSV replication in rodent brains, the laboratories of Dr John Bell and Dr Doug Gray, Ottawa Hospital Research Institute, Ottawa, Canada, used a model to determine whether Usp4 affects viral replication in *Usp4*^{-/-} MEFs. *Usp4*^{-/-} and *Usp4*^{+/+} MEFs were infected at various multiplicities of infection (MOIs) with a GFP-tagged VSV construct (WT VSV) or a GFP-tagged recombinant Δ 51 VSV virus (δ 51 VSV), for which a deletion of methionine 51 affects the interferon response (Stojdl et al., 2003). MEFs were visualised under the microscope and supernatants were collected for titrating at various time points. All work in this section (3.3.3) was performed by Dominic Roy in the Dr John Bell laboratory, Ottawa. Viral replication was increased in *Usp4*^{-/-} MEFs compared the *Usp4*^{+/+} MEFs when infected with WT VSV, as observed by fluorescent imaging (Figure 3.4). This was apparent at all MOIs and timepoints and was also confirmed by viral titre. Additionally, viral replication was increased in *Usp4*^{-/-} MEFs compared to *Usp4*^{+/+} MEFs infected with Δ 51 VSV (data not shown).

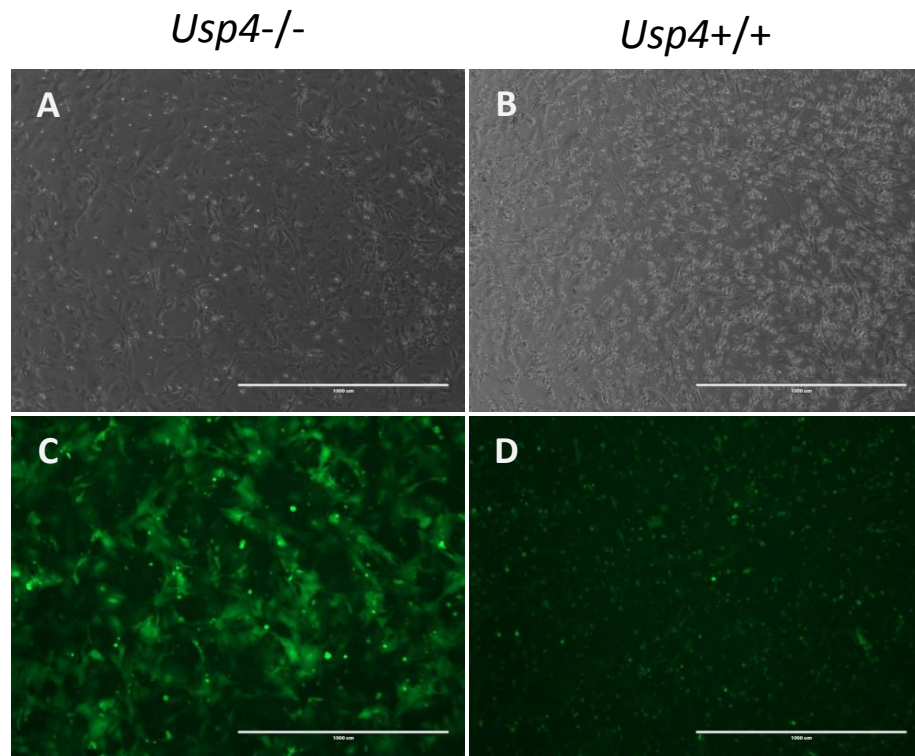


Figure 3.4. VSV viral replication in *Usp4*^{-/-} MEFs. *Usp4*^{-/-} and *Usp4*^{+/+} MEFs were infected with WT VSV at MOI 10 for 16 hours before visualisation for GFP under the microscope. Supernatants were also collected for titrating. A) *Usp4*^{-/-} MEFs B) *Usp4*^{+/+} MEFs C) GFP fluorescence of *Usp4*^{-/-} MEFs D) GFP fluorescence of *Usp4*^{+/+} MEFs. Bars represent 1000μm.

3.3.4 Viral replication in the brains of *Usp4*^{-/-} mice

Given that VSV viral replication was increased in *Usp4*^{-/-} MEFs, the effect of *Usp4* loss on viral replication *in vivo* was next explored. Mice were infected intranasally, through the left side, with the virus VSV and immunohistochemical analysis using an antibody raised against VSV was performed to determine viral replication in both hemispheres of the brains of *Usp4*^{-/-} and *Usp4*^{+/+} mice. Image analysis revealed that the percentage of VSV staining was higher in the left hemisphere than in the right hemisphere for both groups of mice (Figure 3.5A). Additionally, percentage of VSV staining was higher in the brain sections from *Usp4*^{-/-} mice compared to the brain sections from *Usp4*^{+/+} mice. This difference was statistically significant in the right hemisphere of the brain sections, indicating that viral replication in the right side of the mouse brain was increased in *Usp4*^{-/-} mice (Figure 3.5A, Figure 3.5B and Figure 3.5C).

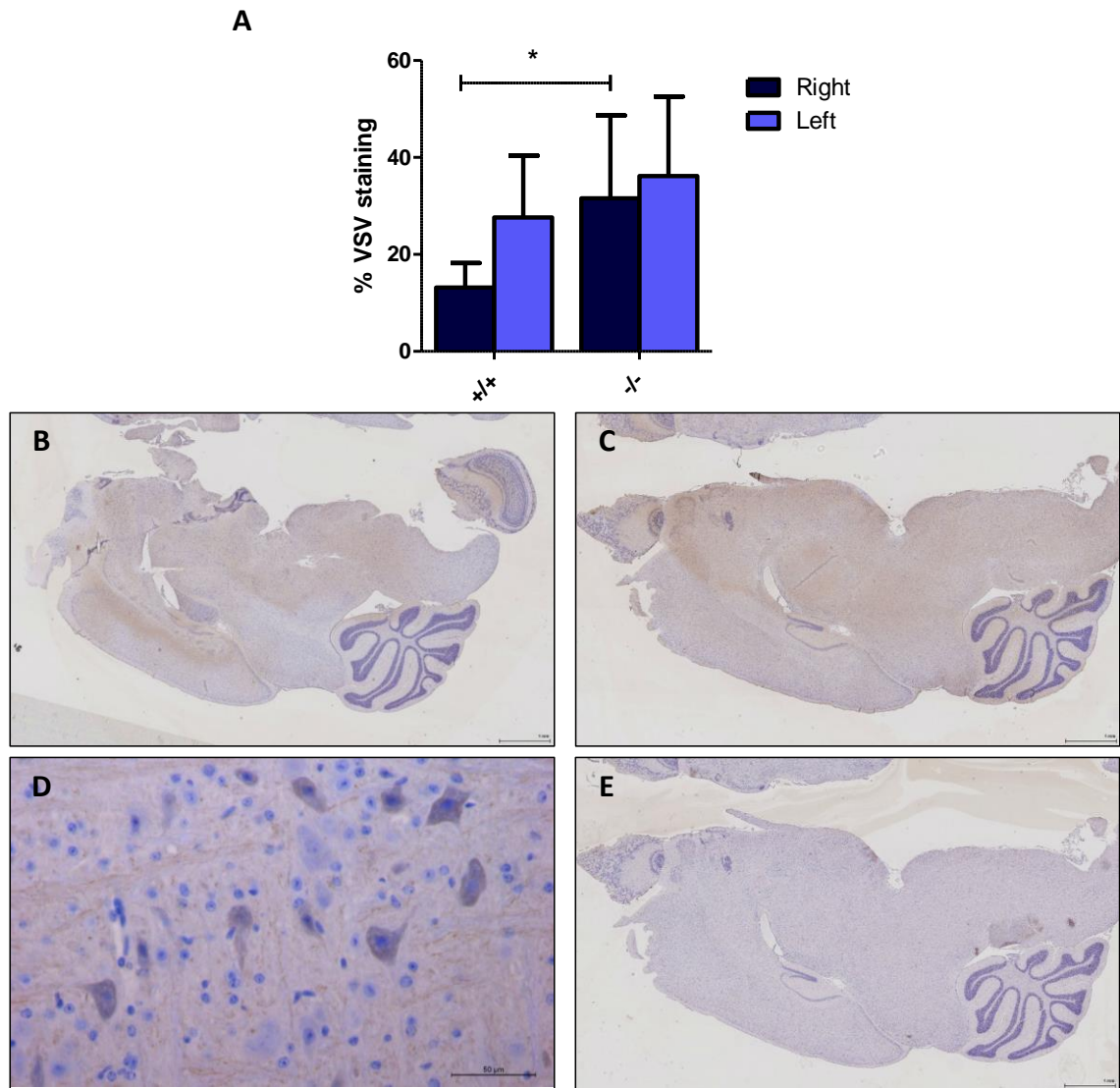


Figure 3.5. VSV viral replication in the brains of mice. *Usp4*^{+/+} and *Usp4*^{-/-} mice were infected with 4×10^6 pfu VSV intranasally (left side) and their brains were harvested and fixed in formalin 48 hours post-infection before immunohistochemistry using a VSV antibody and image analysis were performed. **A**) Percentage of VSV staining in *Usp4*^{+/+} (+/+) and *Usp4*^{-/-} (-/-) brain sections in the right hemisphere (right) and left hemisphere (left). * $p \leq 0.05$. **B**) *Usp4*^{+/+} brain section stained for VSV. Bar represents 1mm. **C**) *Usp4*^{-/-} brain section stained for VSV. Bar represents 1mm. **D**) Brain section magnified to 40X. Bar represents 50µm. **E**) *Usp4*^{-/-} brain section with no primary antibody.

3.4 Discussion

When beginning this thesis, the role of USP4 in any single pathway was not well studied. Therefore this chapter aimed to determine the effect of overexpression of Usp4 on key cell signalling pathways. Multiple cell signalling pathways regulate the expression of an array of genes which are crucial for cellular processes and responses. Many of these genes include immunoregulatory cytokines which mediate the immune response. The effect of USP4 on many of these cytokines and responses was also examined to further investigate the role of USP4 in some cell signalling pathways.

3.4.1 Usp4 overexpression affected the activity of cell signalling pathways

An initial experiment to determine whether USP4 affects some key cell signalling pathways identified significant differences with Usp4 overexpression in p53, NF- κ B, MAPK/ERK and MAPK/JNK pathways.

Zhang et al. (2011) identified USP4 as an inhibitor of the tumour suppressor p53 through stabilisation of the p53 E3 ligase, ARF-BP1. Overexpression of USP4, but not catalytically inactive USP4 for which the critical Cys 311 residue was mutated to an alanine (Ala) residue, reduced reporter activities controlled by the p53-targeting promoter p21 in DNA damage-induced human osteosarcoma U2OS cells. This suggests that USP4 acts as a negative regulator of p53 through its deubiquitinase activity. Using the same cell line, in this chapter it was demonstrated that overexpression of Usp4, but again not a catalytically inactive Usp4 (C311A), significantly reduced p53 activity in a reporter array, in line with the study by Zhang et al. (2011). The catalytically inactive Usp4 used here also had a mutation of the Cys of the catalytic site to an Ala. This mutation has been shown previously to reduce the DUB activity of human USP4 (Zhang et al., 2011).

The reporter array also identified USP4 as a potential regulator of the NF- κ B pathway, a signalling pathway critical in inflammation. Overexpression of wild-type Usp4 significantly increased NF- κ B activity compared to the control. Additionally, overexpression of catalytically inactive Usp4 significantly reduced NF- κ B activity compared to wild-type Usp4, indicating that the catalytic activity of USP4 may positively regulate NF- κ B. Recently, reports have emerged identifying USP4 as a regulator in the NF- κ B signalling pathway, although contrary to these data it has been suggested that USP4 acts as a negative regulator in downregulating NF- κ B signalling (Fan et al., 2011b,

Xiao et al., 2012). These studies in human embryonic kidney 293T cells and using NF- κ B-dependent luciferase reporter gene assays proposed that wild-type USP4, but not catalytically inactive USP4 C311A or C311S, suppressed TAK1-, TRAF2-, TRAF6-, TNF α -, IL-1 β - and LPS-induced NF- κ B activity. TAK1, TRAF2 and TRAF6 are adaptor proteins involved in TNF α - and/or IL-1 β -mediated NF- κ B signalling which are subject to K63-linked polyubiquitination for the recruitment and activation of downstream adaptors to mediate the signal and activation of NF- κ B. Fan et al. (2011b) reported that USP4 negatively regulates NF- κ B activation through TNF α -induced TAK1 deubiquitination and Xiao et al. (2012) further identified TRAF2 and TRAF6 as targets of USP4 deubiquitination for TNF α - and IL-1 β -induced negative regulation of NF- κ B. The data herein this chapter indicated that, conversely to these studies, USP4 positively regulated NF- κ B activity in U2OS cells.

The contrast in data could be due to a number of reasons. Both published studies were conducted in HEK 293T cells whereas the multi-pathway reporter array here was performed in U2OS cells meaning there could be differences in how USP4 functions between different cell lines. Additionally, the reporter array employed in this chapter was performed as a preliminary experiment to determine any signalling pathways Usp4 may affect, and as such was performed only at basal levels. The published studies reporting USP4 as a negative regulator either overexpressed components of the NF- κ B signalling pathway, such as TAK1, TRAF2 or TRAF6, or used known stimuli of the NF- κ B pathway, such as TNF α or IL-1 β , to induce NF- κ B activation. The mechanism of function of USP4 may differ upon activation of the pathway. NF- κ B is under tight regulation to ensure control of the pathway is maintained and prevent chronic inflammation. It is possible that upon stimulation of the NF- κ B signalling pathway, USP4 interaction with TAK1, TRAF2 and TRAF6 is induced to deubiquitinate their polyubiquitin chains and downregulate the signal to prevent uncontrolled activation of the pathway. Under basal conditions, USP4 may interact with other proteins to upregulate rather than downregulate the pathway. Fan et al. (2011b) did not examine differences between NF- κ B activity at basal levels with USP4 overexpression and although data from Xiao et al. (2012) did show NF- κ B basal levels with overexpression of wild-type and catalytically inactive USP4, differences were not clear and were not discussed in that study.

As the reporter array performed in this chapter used 10 different constructs for 10 different pathways on one plate, it would have been difficult to induce all the pathways,

however, it would have been interesting to determine effects of Usp4 overexpression when the pathways were induced. The activity of some of these pathways was clearly detectable without stimulation although this could be due to low levels of cytokines present in the serum added to the media in which the cells were cultured. Further examination of the function of USP4 in the NF- κ B signalling pathway was needed to determine how it may positively regulate NF- κ B activation. The next chapter will focus on this and further examine TNF α - and IL-1-mediated NF- κ B signalling.

The MAPK pathways are also involved in inflammation as well as proliferation, differentiation and apoptosis and signal through a three-tiered cascade of phosphorylation events involving a series of Ser/Thr kinases to activate transcription factors, including AP-1 and NF- κ B (Kim and Choi, 2010). There are three distinct MAPK signalling cascades for ERK, JNK and p38, all of which are phosphorylated by a MAPKK which itself has been phosphorylated by a MAPKKK upon activation of the pathway. Data from the multi-reporter array used transcription factors Elk-1/SRF and AP-1 to examine the activity of MAPKs ERK and JNK, respectively. The activity of the ERK pathway was significantly increased with overexpression of Usp4, but not with overexpression of catalytically inactive Usp4. In addition to affecting the activity of ERK, JNK activity was significantly increased with overexpression of Usp4. JNK activity was also significantly reduced with the catalytically inactive Usp4 compared to wild-type Usp4, suggesting that the deubiquitinase activity of Usp4 may play a role in these MAPK pathways.

To date there are no known reports to indicate USP4 functions directly within the MAPK pathways. Like other cell signalling pathways, MAPK signalling is under tight regulation to maintain control and mediate the correct transcriptional response. Many parallel cell signalling pathways crosstalk to co-ordinate and shape this transcriptional response leading to complex systems that interact at various steps within the signalling pathways. As critical in a variety of biological processes and cellular responses, NF- κ B and MAPK signalling pathways can crosstalk through the activation of a number of proteins including TAK1 and members of the TRAF family (Oeckinghaus et al., 2011). Further examination of the effects of USP4 on these key pathway adaptor proteins was needed to understand the function of USP4 in these pathways and will be the focus of the following chapters in this thesis.

3.4.2 *Usp4* may affect cytokine levels

Cell signalling pathways regulate the expression of many genes; NF- κ B and MAPK pathways are involved in inflammation and as such regulate the expression of many cytokines. Given that *Usp4* overexpression increased activity of these pathways, the effect of *Usp4* on cytokine levels was examined. This chapter demonstrated potential differences in the levels of cytokines with loss of *Usp4* by examination of serum cytokine levels in *Usp4*^{+/+}, *Usp4*^{+/-}, and *Usp4*^{-/-} mice. A preliminary cytokine array identified lower levels of some cytokines including Il-6 and Cxcl1 in *Usp4*^{-/-} mouse serum compared to *Usp4*^{+/+} mouse serum. The chemokine Cxcl1 is a murine homologue of IL-8 and a key mediator of inflammation. CXCL1 is overexpressed in breast, colon and ovarian cancers and is induced through NF- κ B and MAPK signalling pathways (Son et al., 2007, Lo et al., 2014). Another major inflammatory cytokine, IL-6, can also be induced through NF- κ B and MAPK pathways (Shimizu et al., 1990, Yang et al., 2008). Therefore, the reduction in Il-6 and Cxcl1 levels with loss of *Usp4* was supportive of the increase in NF- κ B and MAPK activity with *Usp4* overexpression also observed in this chapter.

Recently, a study challenged *usp4*-depleted zebrafish larvae with LPS, to stimulate TLR/IL-1R signalling, and reported higher levels of expression of proinflammatory cytokines, including TNF α and IL-1 β , with loss of *usp4* (Zhou et al., 2012). This is not in line with data from the multiplex cytokine array which indicated that cytokine levels, including TNF α and IL-1 β were lower with loss of *Usp4*. However, further examination of the levels of Cxcl1 and Il-6 cytokines, by performing ELISAs, identified no obvious differences between any of the mouse genotypes, suggesting no effect on these with loss of *Usp4*. Although a larger cohort of mice was used in the ELISAs than in the multiplex cytokine array, a limitation of the ELISA was that there were far fewer wild-type mice available for examination than *Usp4*^{+/-} or *Usp4*^{-/-} mice which may have reduced the reliability of these data.

Another difference between these two types of experiments was that only the serum taken from male mice was used in the multiplex cytokine array, whereas both male and female serum was examined in the ELISAs. To determine whether this was responsible for the differing results, data from the ELISAs were stratified by gender, however no differences in cytokine levels between genotypes was observed in either male or female mouse serum.

Differences between these data may also be due to different sensitivities of the two experiments. Concentrations detected in the MSD cytokine array were generally higher than for the ELISAs suggesting that the MSD cytokine array may be more sensitive than the ELISAs. All assays were within the standard curves.

These cytokine levels were measured at basal levels in the serum of these mice. As they are inflammatory mediators, it would have been interesting to use a model in which the mice were first challenged to induce an inflammatory response. As it has been demonstrated herein this chapter that *Usp4* has potential involvement in NF- κ B and MAPK pathways, challenging the mice with LPS could induce an inflammatory response through these pathways, perhaps making any differences in cytokine levels with loss of *Usp4* more evident. It would have been interesting to utilise an inflammatory model such as this, however, this was not an available possibility for this thesis. The potential role of USP4 in the NF- κ B signalling pathway will be addressed further in the next chapter and the expression levels of NF- κ B regulated genes, including cytokines IL-6 and IL-8 will be examined in cell lines stimulated to induce the NF- κ B pathway. As no model for examining an LPS-induced inflammatory response in *Usp4*^{-/-} mice was available, a model for which mice were induced with VSV to examine effects on viral replication was instead adopted.

3.4.3 Increased viral replication in *Usp4*^{-/-} mice

USP4 has reported roles in inflammatory and immune pathways RIG-I and NF- κ B (Wang et al., 2013, Fan et al., 2011b, Xiao et al., 2012). In this chapter, overexpression of *Usp4* affected the activity of pathways associated with inflammation and immune responses and loss of *Usp4* had possible effects on the levels of inflammatory cytokines, further indicating that *Usp4* may be involved in the inflammatory and immune responses. These responses are crucial in detecting viral infection and initiating signalling events to induce inflammatory mediators for the clearance of viruses. Given this, the laboratory of Dr John Bell used a model for examining the effect of *Usp4* loss on immune responses in MEFs. Imaging revealed there was more of the VSV virus in the *Usp4*^{-/-} MEFs than in the *Usp4*^{+/-} MEFs, also confirmed by viral titre of the cell supernatants. This demonstrated more viral replication with loss of *Usp4* indicating that the inflammatory or immune responses within these MEFs were compromised, potentially due the involvement of *Usp4* in cell signalling pathways.

A recent study identified USP4 as a positive regulator of RIG-I signalling, an important pathway in the antiviral immune response (Wang et al., 2013). It was reported that USP4 stabilises RIG-I to enhance RIG-I activation and consequently downstream production of IFN- β , a key mediator of antiviral activity, in response to viral infection. *In vitro* studies revealed that viral replication in HEK 293 cells infected with VSV increased viral replication with siRNA-mediated loss of USP4. This corresponds with data in this chapter for which viral replication was increased in *Usp4*^{-/-} MEFs.

Additionally, *in vivo* examination here (aided by myself) demonstrated an increase in viral replication in the brains of *Usp4*^{-/-} mice infected with VSV virus. Overall, there was increased VSV staining in the left hemisphere compared to the right hemisphere of the mouse brain, possibly due to the mice being intranasally infected through the left side. The difference in VSV between the *Usp4*^{-/-} mice and *Usp4*^{+/+} mice was only statistically significant when comparing staining in the right hemispheres, maybe due to a larger amount of virus infecting the left side of the brain and maximising antiviral pathways and meaning any differences with loss of *Usp4* are not as evident. The increased VSV in *Usp4*^{-/-} mouse brains is again supportive of the *in vitro* data and indicates that *Usp4* is potentially involved in the innate immune response against viral infection.

Further to activating the transcription factor IRF3 for the production of type I IFNs, RIG-I activation is also known to activate the NF- κ B transcription factor for the production of many inflammatory cytokines through recruitment of the IKK complex. Viral infection triggers the innate immune response, where receptors including Toll-like receptors (TLRs), IL-1Rs, and RLRs mediate signal transduction to activate transcription factors such as AP-1, NF- κ B and IRF3 to induce the production of inflammatory cytokines and type I IFNs. These inflammatory mediators induce inflammatory responses subsequently leading to the immune response to eradicate the viral infection (Negrate, 2012). Taken together, data herein this chapter suggest a role for USP4 in inflammatory and immune responses, potentially mediated through cell signalling pathways such as NF- κ B. As *Usp4* increased NF- κ B activity and potentially altered cytokine levels associated with NF- κ B signalling, the role of USP4 within the NF- κ B signalling pathway will be explored in more detail in the next chapter. The effect of USP4 on levels of adaptor proteins in the pathway and expression of NF- κ B-regulated genes will be examined with induction of the pathway to examine where and how USP4 may function as a positive regulator for the NF- κ B signalling pathway.

Chapter 4 : The Effect of USP4 Depletion on NF- κ B Signalling

4.1 Introduction

USP4 regulates aspects of many different cell signalling pathways including p53, Wnt/ β -catenin, TGF- β and NF- κ B pathways (Zhang et al., 2011, Zhao et al., 2009, Zhang et al., 2012, Zhou et al., 2012, Fan et al., 2011b, Xiao et al., 2012). The previous chapter discussed potential involvement of USP4 in NF- κ B signalling: the multi-pathway reporter assay performed demonstrated an increase in NF- κ B activity when overexpressing wild-type USP4, but not when overexpressing catalytically inactive USP4 (C311A mutant), signifying USP4 was upregulating NF- κ B signalling.

Ubiquitination of proteins involved in NF- κ B signalling play vital roles in the regulation of the pathway and many DUBs have been identified which act to facilitate this. Amongst these are A20 and CYLD which downregulate NF- κ B signalling through deubiquitination of K63-linked RIP1 polyubiquitin chains and TRAF2, TRAF6, NEMO, TAK1 and RIP1 polyubiquitin chains, respectively (Wertz et al., 2004, Kovalenko et al., 2003, Brummelkamp et al., 2003, Reiley et al., 2007). Additionally, DUBs can upregulate NF- κ B signalling: USP6 induces NF- κ B activation through association and activation of the IKK complex. Unusually, USP6 did not affect the phosphorylation or degradation of I κ B α but instead acted in an atypical manner by activating IKK for the phosphorylation of nuclear p65 at Ser536 (Pringle et al., 2012).

There are also reports of a role for USP4 in immune activation and NF- κ B signalling: Fan et al. (2011b) identified USP4 as a deubiquitinase for TAK1, acting as a downregulator of TAK1-mediated NF- κ B activation. In agreement with USP4 downregulating NF- κ B, Xiao et al. (2012) identified USP4 as a deubiquitinase for two members of the TRAF family; TRAF2 and TRAF6. Reports on the downregulation of this pathway through USP4 indicated that overexpression of USP4 inhibits TNF α -, IL-1 β - and LPS-induced NF- κ B activity, but not overexpression of catalytically inactive versions of USP4 (Fan et al., 2011b, Xiao et al., 2012, Zhou et al., 2012). Corresponding with this, these studies also observed an increase in TNF α - IL-1 β - and LPS-induced NF- κ B activity with USP4 depletion. A role for USP4 in regulating NF- κ B activation through upregulation of the pathway has not yet been elucidated. A more detailed understanding of the function of

USP4 was required in order to examine the mechanism by which it may upregulate NF- κ B activation.

One role of the NF- κ B family is in the immune system: NF- κ B can act as a transcriptional regulator for many genes including proinflammatory cytokines (Hayden et al., 2006). The previous chapter also discussed involvement of USP4 in the immune system; the preliminary data suggested levels of some cytokines were reduced in the serum of *Usp4* null mice compared to wild-type, suggesting a dampening down of the immune system. In conflict with this, Zhou et al. (2012) reported higher expression levels of proinflammatory cytokines in *Usp4* null MEFs and *usp4*-depleted zebrafish larvae.

To corroborate the involvement of USP4 in immune pathways, a more comprehensive examination of the effects of USP4 may enable identification of a mechanism for which USP4 upregulates stimulus-induced NF- κ B activation and provide a link between effects of USP4 in the immune system and NF- κ B signalling. This chapter used RNAi to knockdown USP4 and examine the functional consequences on various aspects of the NF- κ B pathway.

4.1.1 Aims

- Determine the effect of siRNA-mediated USP4 depletion on IL-1- and TNF α -induced NF- κ B activation in SW1353 cells
- Examine IL-1- and TNF α -induced NF- κ B activity in *Usp4*^{-/-} MEFs
- Identify whether IL-1- and TNF α -induced NF- κ B-regulated gene expression is affected in siRNA-mediated USP4 depleted SW1353 cells
- Examine IL-1- and TNF α -induced NF- κ B regulated gene expression in *Usp4*^{-/-} MEFs
- Establish the role of USP4 within the NF- κ B signalling pathway through examination of siRNA-mediated USP4 depletion on the stability, phosphorylation and ubiquitination of key NF- κ B adaptor proteins
- Determine whether the role of USP4 in the activation of NF- κ B may involve other signalling pathways

4.2 Results

4.2.1 Depletion of *USP4* gene expression and USP4 protein levels by targeted siRNA

To determine whether siRNA targeting USP4 (siUSP4) depletes *USP4* expression levels and protein levels, two parallel experiments were carried out in different cell lines for which USP4 depletion experiments were to be performed. Human SW1353 chondrosarcoma cells, human HeLa cervical adenocarcinoma cells and human embryonic kidney 293T cells were cultured, plated and transfected with siUSP4 or non-targeting control siRNA (siControl) as previously described in section 2.2.4.2. To ensure that transfection with siCon does not affect *USP4* expression, an initial experiment in SW1353 cells was performed including an additional control where cells were treated with only DharmaFECT transfection reagent (DF1) and no siRNA. At 24 hour post-siRNA transfection (or DharmaFECT treatment only) cells were lysed and *USP4* gene expression or USP4 protein levels in whole cell lysates were determined either by real-time qRT-PCR or by immunoblotting with anti-USP4 antibody, respectively. There was no significant difference in *USP4* expression between SW1353 cells treated with only DF1 compared to those also transfected with the control siRNA (Figure 4.1A). Transfection of siUSP4 resulted in significant depletion of *USP4* mRNA levels in SW1353 cells: *USP4* was more than 90% depleted compared to the control (Figure 4.1A). This reduction in *USP4* was also reflected in USP4 protein levels in SW1353, HeLa and HEK 293T cells (Figure 4.1A, Figure 4.1B and Figure 4.1C).

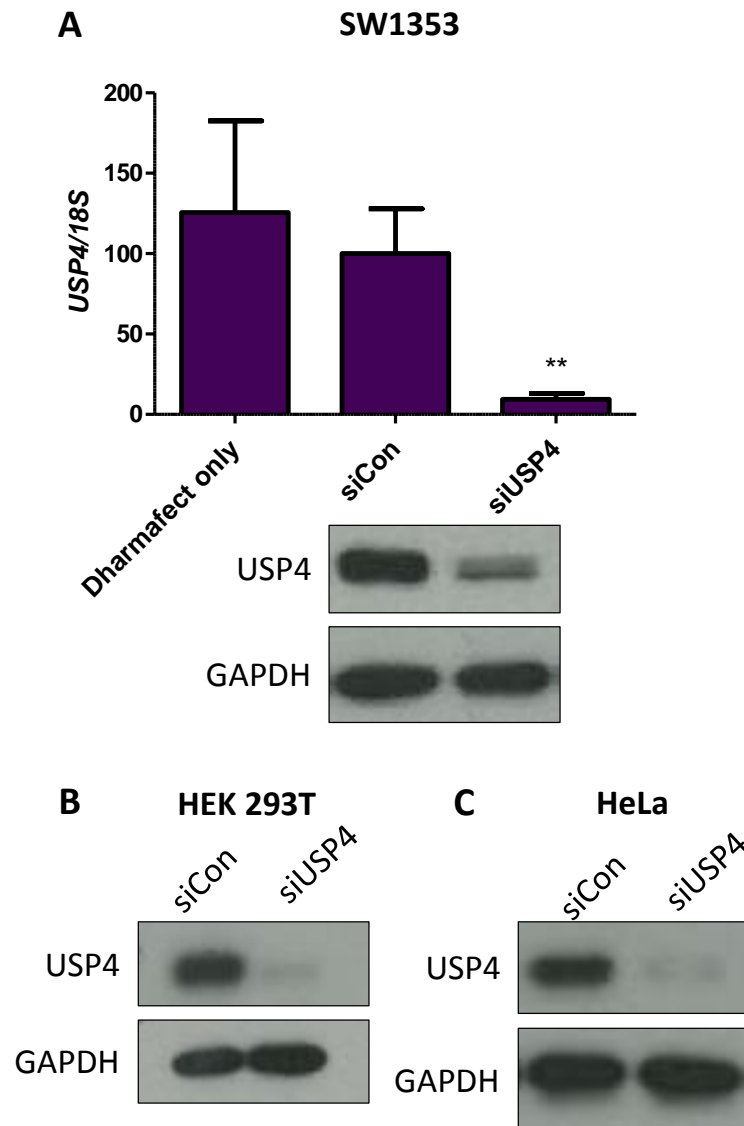


Figure 4.1. USP4 protein levels and *USP4* gene expression were reduced following transfection with a USP4-targeting siRNA. **A)** Relative gene expression analysis and protein expression analysis of USP4 in SW1353 cells transfected with either a non-targeting siRNA (siCon) or a USP4-targeting siRNA (siUSP4) or treated with DF1 but no siRNA (DharmaFECT only) for 24 hours. Gene expression data were normalised to 18S and are relative to siCon=100%. Data are plotted as mean \pm SEM (n=6). Student's t-test (unpaired) was performed as statistical analysis. ** $p \leq 0.01$. **B)** Protein expression analysis of USP4 in HEK 293T cells transfected with either siCon or siUSP4 for 24 hours. **C)** Protein expression analysis of USP4 in HeLa cells transfected with siCon or siUSP4 for 24 hours. Protein analysis for all cell lines was performed on whole cell lysates, GAPDH was used as a loading control.

4.2.2 The effect of USP4 depletion on TNF α - and IL-1-induced NF- κ B activity

To clarify the impact of USP4 depletion on the activation of the NF- κ B pathway, a luciferase reporter assay was performed. SW1353 cells were transfected with responsive NF- κ B firefly luciferase reporter and *Renilla* expression vectors before depletion of USP4 by RNAi. After stimulation with either tumour necrosis factor α (TNF α) or interleukin-1 (IL-1), luciferase activity was measured and normalised to *Renilla*. Optimal stimulation conditions were previously determined in section 2.2.5.3. The reporter assay determined that depleting USP4 significantly reduced both IL-1- and TNF α -induced NF- κ B activity compared to the control (Figure 4.2A and Figure 4.2B).

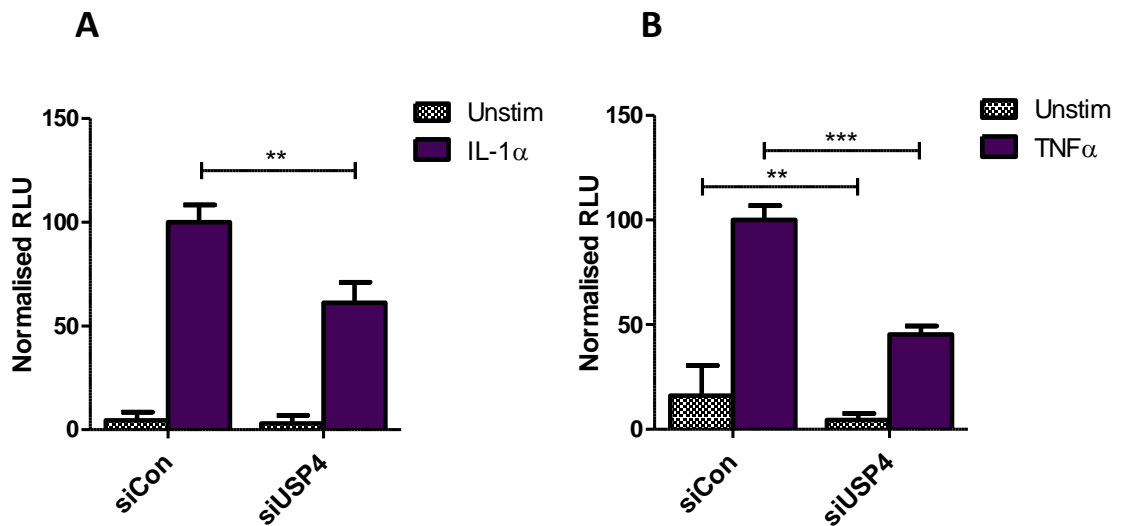


Figure 4.2. Depletion of USP4 significantly reduced NF- κ B activity. NF- κ B-dependent firefly luciferase and *Renilla* reporter vectors were transfected into SW1353 cells followed by USP4 depletion by transfection with siCon or siUSP4 after 6-8 hours. At 24 hours post-siRNA transfection cells were serum-starved overnight before a 6 hour stimulation, cell lysis and measurement of luciferase activity. Data were normalised to *Renilla* and are relative to siCon=100%. Data are displayed in normalised relative light units (RLU). A) SW1353 cells were stimulated with IL-1 α (0.5ng/ml) or remained unstimulated (Unstim). Figure is of 4 combined independent experiments \pm SEM (n=4/experiment). ** p \leq 0.01. B) SW1353 cells were stimulated with TNF α (10ng/ml) or remained unstimulated (Unstim). Figure is of 3 combined independent experiments and plotted as mean \pm SEM (n=4/experiment). **p \leq 0.01, *** p \leq 0.001. Student's t-test (unpaired) was performed for statistical analysis.

4.2.3 The effect of USP4 depletion on TNF α - and IL-1-induced expression of NF- κ B-regulated genes

Activation of the NF- κ B transcription factor through pathway activation by a variety of stimuli, such as IL-1 and TNF α , induces the expression of numerous genes including many proinflammatory cytokines. As depleting USP4 significantly reduced NF- κ B activity, the next stage was to examine whether there was any effect upon NF- κ B-regulated genes. Therefore, interleukins *IL-6*, *IL-8* and matrix metalloproteinase 13 (*MMP13*) were analysed. SW1353 cells were transfected and stimulated, as described in section 2.2.4.2, and *IL-6*, *IL-8* and *MMP-13* gene expression measured by real-time qRT-PCR. This quantitative analysis revealed that both IL-1 and TNF α -induced *IL-6* and *MMP13* relative gene expression were significantly reduced in USP4 depleted cells compared to the control (Figure 4.3A, Figure 4.3C, Figure 4.4A and Figure 4.4C). Although, IL-1- and TNF α -induced relative *IL-8* gene expression appeared reduced in USP4 depleted cells compared to the control this did not reach statistical significance (Figure 4.3B and Figure 4.4B). *USP4* expression was determined in each experiment and was significantly depleted in all experiments, with no change in expression with either IL-1 or TNF α stimulation (Figure 4.3D and Figure 4.4D).

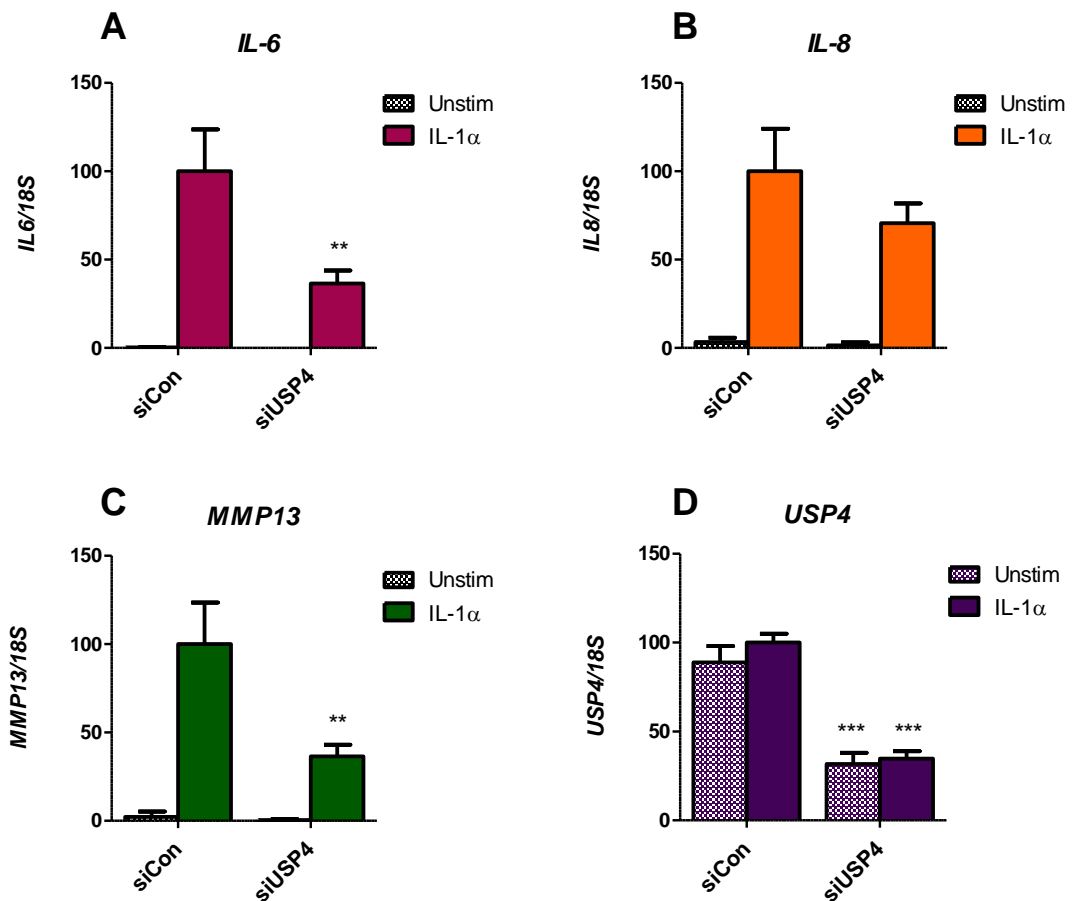


Figure 4.3. USP4 regulated IL-1-induced NF- κ B-regulated gene expression. SW1353 cells were transfected with siCon or siUSP4 for 24 hours and stimulated with IL-1 α (0.5ng/ml) for 6 hours or left unstimulated (Unstim) after overnight serum-starvation. Total RNA was extracted, reverse transcribed to cDNA and analysed by real-time qRT-PCR. Relative gene expression of IL-1 α -induced A) *IL-6*, B) *IL-8*, C) *MMP13* and D) *USP4* were measured. Data were normalised to 18S and are shown as relative to siCon=100%. Data are combined from at least 2 independent experiments (n=6/experiment) and plotted as mean \pm SEM. ** p<0.01, *** p<0.001. Student's t-test (unpaired) was performed for statistical analysis.

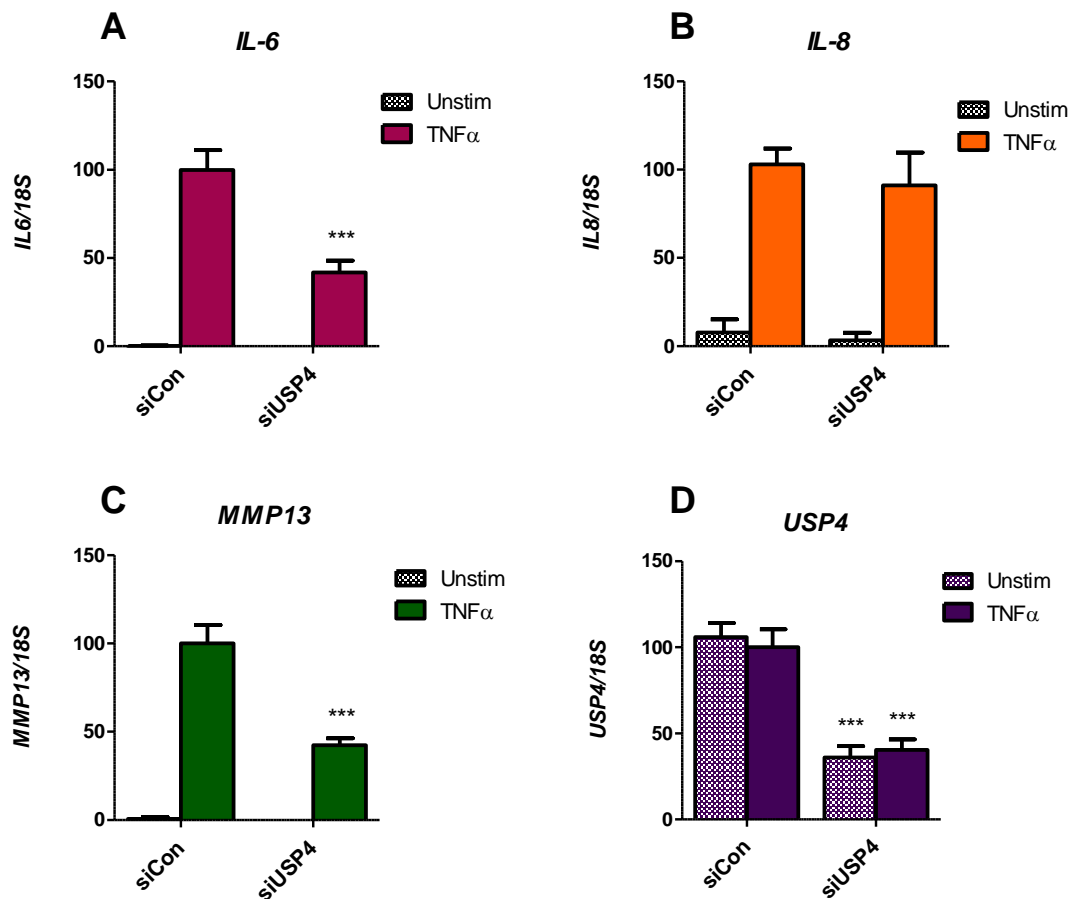


Figure 4.4. USP4 regulated TNF α -induced NF- κ B-regulated gene expression. SW1353 cells were transfected with siCon or siUSP4 for 24 hours and stimulated with TNF α (10ng/ml) for 6 hours or left unstimulated (Unstim) after overnight serum-starvation. Total RNA was extracted, reverse transcribed to cDNA and analysed by real-time qRT-PCR. Relative gene expression of TNF α -induced A) *IL-6*, B) *IL-8*, C) *MMP13* and D) *USP4* were measured. Data were normalised to 18S and are shown as relative to siCon=100%. Data are combined from at least 2 independent experiments (n=6/experiment) and are plotted as mean \pm SEM. ** p<0.01, *** p<0.001. Student's t-test (unpaired) was performed for statistical analysis.

4.2.3.1 USP4 and the regulation of IL-1-induced NF- κ B-regulated gene expression over a stimulation time course

To further confirm the effect of USP4 depletion on NF- κ B-regulated genes, cells were treated with IL-1 over a time course. Total RNAs were extracted from USP4 depleted and control SW1353 cells (treated with IL-1 for the time points indicated) and real-time qRT-PCR was performed. Both *IL-6* and *IL-8* gene expression increased after stimulation before declining after 6 hours IL-1 stimulation (Figure 4.5A and Figure 4.5B). *MMP13* gene expression increased after IL-1 stimulation and continued to increase at each time point over the 24 hour stimulation period (Figure 4.5C). As in Figure 4.3A, relative *IL-6* gene expression was reduced in USP4 depleted cells in comparison to the control, significantly so at 6 hours after stimulation (Figure 4.5A). Similarly, relative *IL-8* expression was reduced in USP4 depleted cells, significantly so at 3 hours after stimulation (Figure 4.5B). Relative *MMP13* expression was significantly reduced in USP4 depleted cells at 1, 3, 6 and 24 hours IL-1 stimulation (Figure 4.5C). From these data, depletion of USP4 reduced NF- κ B-regulated expression of *IL-6*, *IL-8* and *MMP13* genes after stimulation with IL-1 at different time points within a 24 hour period.

To confirm *USP4* depletion in these experiments, the relative gene expression levels of *USP4* were analysed by the same method. Figure 4.5D shows that levels of *USP4* were depleted in the cDNA of cells transfected with siUSP4 and that the levels did not change significantly with IL-1 stimulation in control cells.

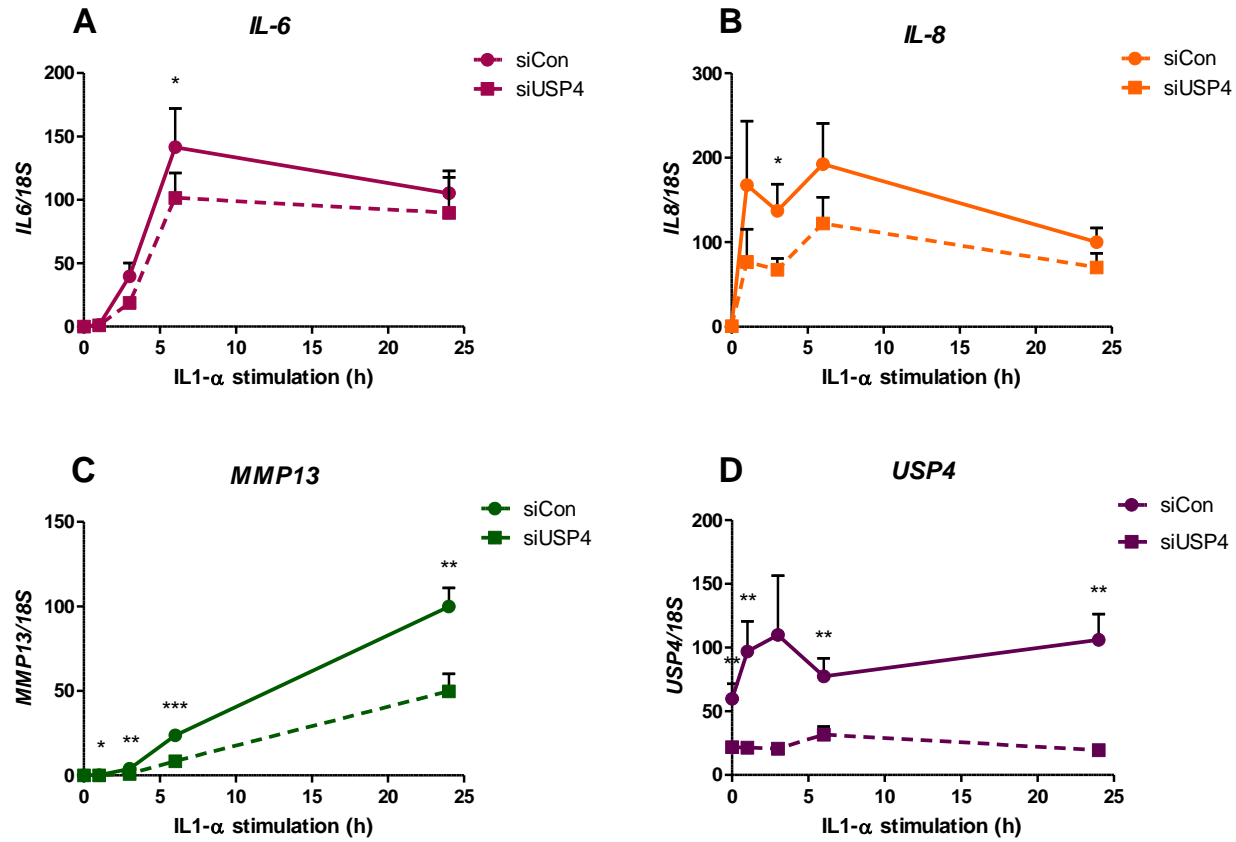


Figure 4.5. USP4 regulated IL-1 α -induced NF- κ B-regulated gene expression. SW1353 cells were transfected with siCon or siUSP4 for 24 hours and, after a period of serum-starvation, were stimulated with IL-1 α (0.5ng/ml) for 0, 1, 3, 6 and 24 hours (h). Total RNA was extracted, reverse transcribed to cDNA, and analysed by real-time qRT-PCR. Relative gene expression of A) *IL-6*, B) *IL-8*, C) *MMP13* and D) *USP4* were measured. Data are normalised to 18S and show as relative to siCon 24h = 100%. Data are combined from at least 2 independent experiments (n=6/experiment). Student's t-test (unpaired) were performed as statistical analysis. Data are plotted as mean \pm SEM. * p<0.05, ** p<0.01, *** p<0.001.

4.2.3.2 USP4 and the regulation of TNF α -induced NF- κ B-regulated gene expression over a stimulation time course

In addition to IL-1, TNF α stimulation after 6 hours resulted in less NF- κ B-regulated gene expression in USP4 depleted cells in comparison to the control (Figure 4.4), therefore relative gene expression after TNF α stimulation over a time course was also examined. Unlike previously where the relative *IL-6* expression was reduced in USP4 depleted cells after 6 hours stimulation (Figure 4.4A), USP4 depletion resulted in significantly increased relative *IL-6* expression after 3, 6, and 24 hours TNF α stimulation (Figure 4.6A). Similarly, relative *IL-8* expression appeared increased in USP4 depleted cells compared to the control, although this was only significant after 3 hours stimulation (Figure 4.6B). As with IL-1 stimulation, TNF α -induced relative *MMP13* expression was significantly reduced in USP4 depleted cells compared to the control, specifically after 6 and 24 hours stimulation (Figure 4.6C).

To confirm the depletion of *USP4* in these experiments, *USP4* gene expression was also measured. Transfection with siRNA against USP4 resulted in significantly lower levels of relative *USP4* expression compared to the control. This expression did not change significantly with TNF α stimulation (Figure 4.6D).

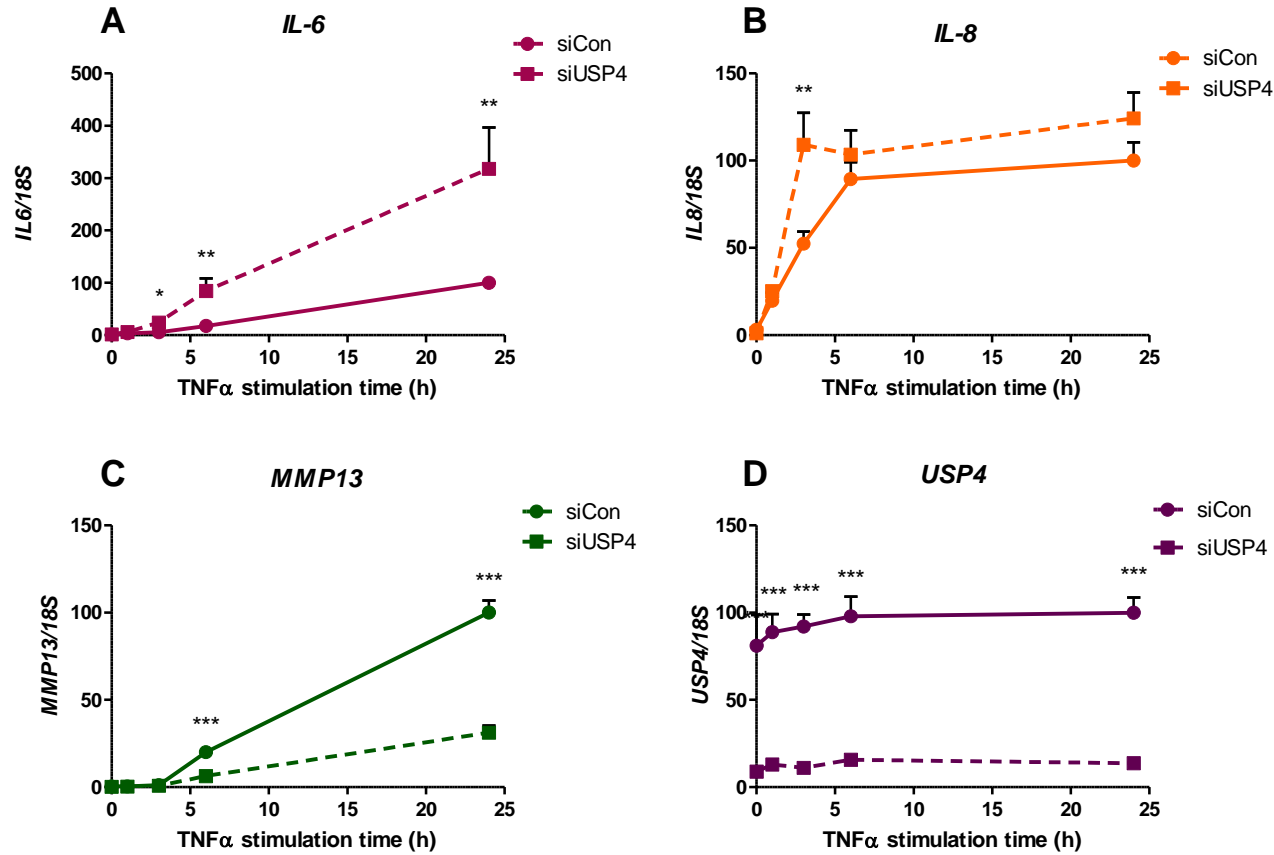


Figure 4.6. USP4 regulated TNF α -induced NF- κ B-regulated gene expression. SW1353 cells were transfected with siCon or siUSP4 for 24 hours and, after a period of serum-starvation, were stimulated with TNF α (10ng/ml) for 0, 1, 3, 6 and 24 hours (h). Total RNA was extracted, reverse transcribed to cDNA and analysed by real-time qRT-PCR. Relative gene expression of A) *IL-6*, B) *IL-8*, C) *MMP13* and D) *USP4* were measured. Data are normalised to 18S and relative to siCon 24h = 100%. Data are combined from at least 2 independent experiments (n=6/experiment). Student's t-test (unpaired) were performed as statistical analysis. Data are plotted as mean \pm SEM. * p<0.05, ** p<0.01, *** p<0.001.

4.2.4 The effect of USP4 depletion on levels of key NF- κ B signalling pathway adaptor proteins

Evidence from the data so far indicates that NF- κ B activation was reduced by the depletion of USP4 and, in addition, the IL-1-induced expression of genes which are regulated by NF- κ B were also reduced. This suggests USP4 plays a role in the regulation of NF- κ B signalling so further analysis was performed to try and uncover the function of USP4 in NF- κ B signalling. There are many key adaptor proteins important for the activation of NF- κ B and so the effect of USP4 depletion on the levels of some of these key proteins were next explored to identify whether their stability or phosphorylation, generally a marker of their level of activation, are regulated by USP4. USP4 was depleted in SW1353 cells before treatment with IL-1 for the indicated time points. Cells were then lysed, total protein extracted, resolved by electrophoresis and immunoblotted with the relevant antibody. In addition to visually examining protein levels, differences were verified by quantification of the protein bands through densitometry.

4.2.4.1 The effect of USP4 depletion on the IL-1-induced degradation of I κ B α

The liberation of the NF- κ B dimer from its inhibitory proteins is an integral step in the NF- κ B signalling pathway. This occurs after the phosphorylation of I κ B α by IKK which allows recognition by the E3 ligase, SCF $^{\beta}$ -TrCP, thus triggering the K48-linked polyubiquitination and subsequent degradation of I κ B α (Spencer et al., 1999). As such a crucial event in the activation of NF- κ B, the levels of I κ B α with USP4 depletion were first examined.

Upon depletion of USP4, there were higher levels of I κ B α compared to the control (Figure 4.7A). This was particularly evident after 5 and 15 minutes stimulation with IL-1 which densitometric analyses confirmed were statistically significant differences (Figure 4.7B). Higher I κ B α levels indicate either increased stability of I κ B α or increased expression when USP4 is depleted, consistent with downregulation of the pathway. As phosphorylation of I κ B α occurs prior to I κ B α ubiquitination, levels of phosphorylated I κ B α were also examined. The phosphorylation of I κ B α appeared to be induced in a biphasic manner with levels increasing after 5 and then 15 minutes of IL-1 stimulation, then decreasing at 30 minutes before another increase at 60 minutes (Figure 4.7A). This was the same for both control and USP4 depleted cells. There was less phosphorylation of I κ B α in USP4 depleted cells but densitometry analysis determined that this difference was not statistically significant (Figure 4.7B).

4.2.4.2 The effect of USP4 depletion on IL-1-induced I κ B α gene expression

As the loss of USP4 resulted in a significant change in I κ B α protein levels, the effect on its gene expression was then examined. As when examining the effect on the expression of other NF- κ B-regulated genes, USP4 depleted SW1353 cells were stimulated with IL-1 over a time course before the extraction of total RNA and analysis by real-time qRT-PCR.

Figure 4.7C illustrates a reduction in *I κ B α* gene expression in USP4 depleted cells compared to the control, further suggesting downregulation of NF- κ B signalling with USP4 depletion, and suggesting the mechanism that leads to altered I κ B α following USP4 depletion is not due to an increase in I κ B α expression.

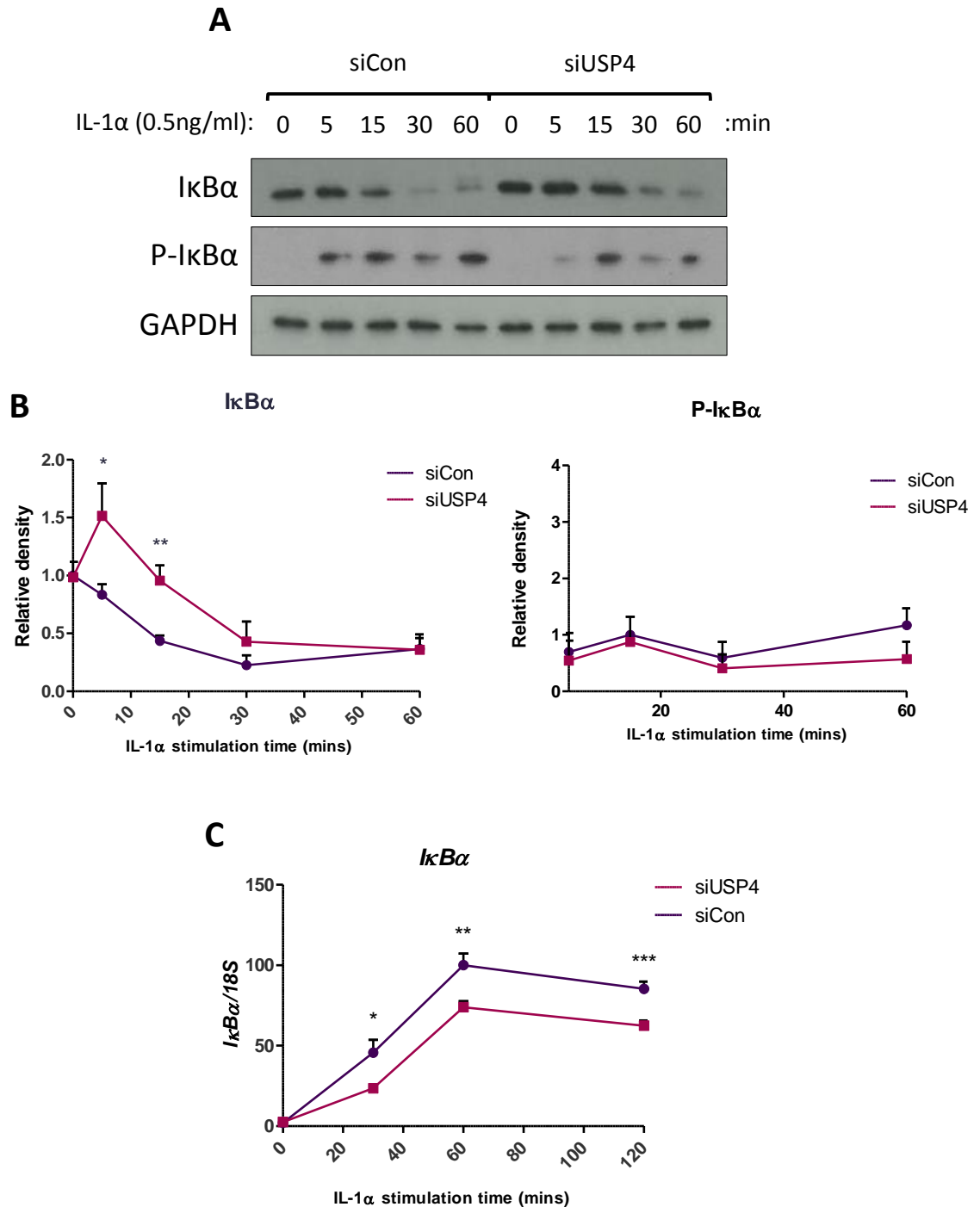


Figure 4.7. USP4 depletion affected I κ B α protein levels and I κ B α gene expression. SW1353 cells were transfected with siCon or siUSP4 for 24 hours, serum-starved and treated with IL-1 α (0.5ng/ml) for the indicated times (minutes). **A**) Total protein was extracted, resolved by SDS-PAGE and immunoblotted with anti-I κ B α and anti-phospho-I κ B α (P-I κ B α) antibodies. GAPDH was used as a loading control. Figure is representative of 5 independent experiments. **B**) Quantification of I κ B α and P-I κ B α protein levels. I κ B α : Data are plotted as mean \pm SEM * p <0.05, ** p <0.01. Data are combined from 5 independent experiments for I κ B α and standardised to siCon 0=1. P-I κ B α : data are plotted as mean \pm SEM. Data are combined from 4 independent experiments for P-I κ B α and standardised to siCon 15=1. **C**) Total RNA was extracted, reverse transcribed to cDNA and analysed by real-time qRT-PCR. Relative I κ B α gene expression was measured and normalised to 18S. Data are combined from 3 experiments (n=6/experiment) and plotted as mean \pm SEM. * p <0.05, ** p <0.01, *** p <0.001. Student's t -test (unpaired) were performed as statistical analysis.

4.2.4.3 The effect of USP4 depletion on the IL-1-induced polyubiquitination of I κ B α

For degradation of I κ B α to occur, it first becomes ubiquitinated with K48-linked polyubiquitin chains by the E3 ligase SCF ^{β -TrCP} (Spencer et al., 1999). Figure 4.7 indicates that USP4 depletion resulted in increased stability of I κ B α . This would suggest there may be a difference in the ubiquitination of I κ B α which leads to this change in its degradation kinetics; therefore the ubiquitination of I κ B α was next examined.

An initial experiment determined whether inhibiting USPs in general resulted in a change in the ubiquitination levels of I κ B α . SW1353 cells were stimulated with IL-1 for the indicated time points and treated with either ethanol as a control/vehicle or the DUB inhibitor, N-ethylmaleimide (NEM). Additionally, either DMSO (again as a control/vehicle) or proteasome inhibitor MG132 (carbobenzoxy-Leu-Leu-leucinal), to prevent degradation of I κ B α , were also added to additional cells. Cells treated with MG132 showed smeared bands at high exposure (indicative of polyubiquitination), particularly after 30 and 60 minutes treatment. However, treatment with NEM had no effect. I κ B α appeared to become increasingly ubiquitinated after stimulation with IL-1 as would be expected but treatment with NEM did not alter the amount of I κ B α ubiquitination (Figure 4.8A).

Next, rather than inhibiting DUBs in general, the effect of specifically targeting USP4 depletion on the ubiquitination of I κ B α was examined. USP4 was depleted in SW1353 cells as previously, and again stimulated with IL-1 for the indicated time points. Additionally, cells were treated with MG132 at the time of stimulation to prevent degradation of I κ B α and allow visualisation of ubiquitinated I κ B α . Analysis by immunoblotting with an anti-I κ B α antibody and a longer exposure time when developing revealed a stronger smeared band at 60 minutes IL-1 stimulation in the control than in the USP4 depleted cells, indicating that I κ B α does become more ubiquitinated and is therefore degraded more rapidly in the control cells than in the USP4 depleted cells (Figure 4.8B). MG132 severely inhibited the degradation of I κ B α , but even with this, the effect of USP4 loss on the degradation that did occur was evident.

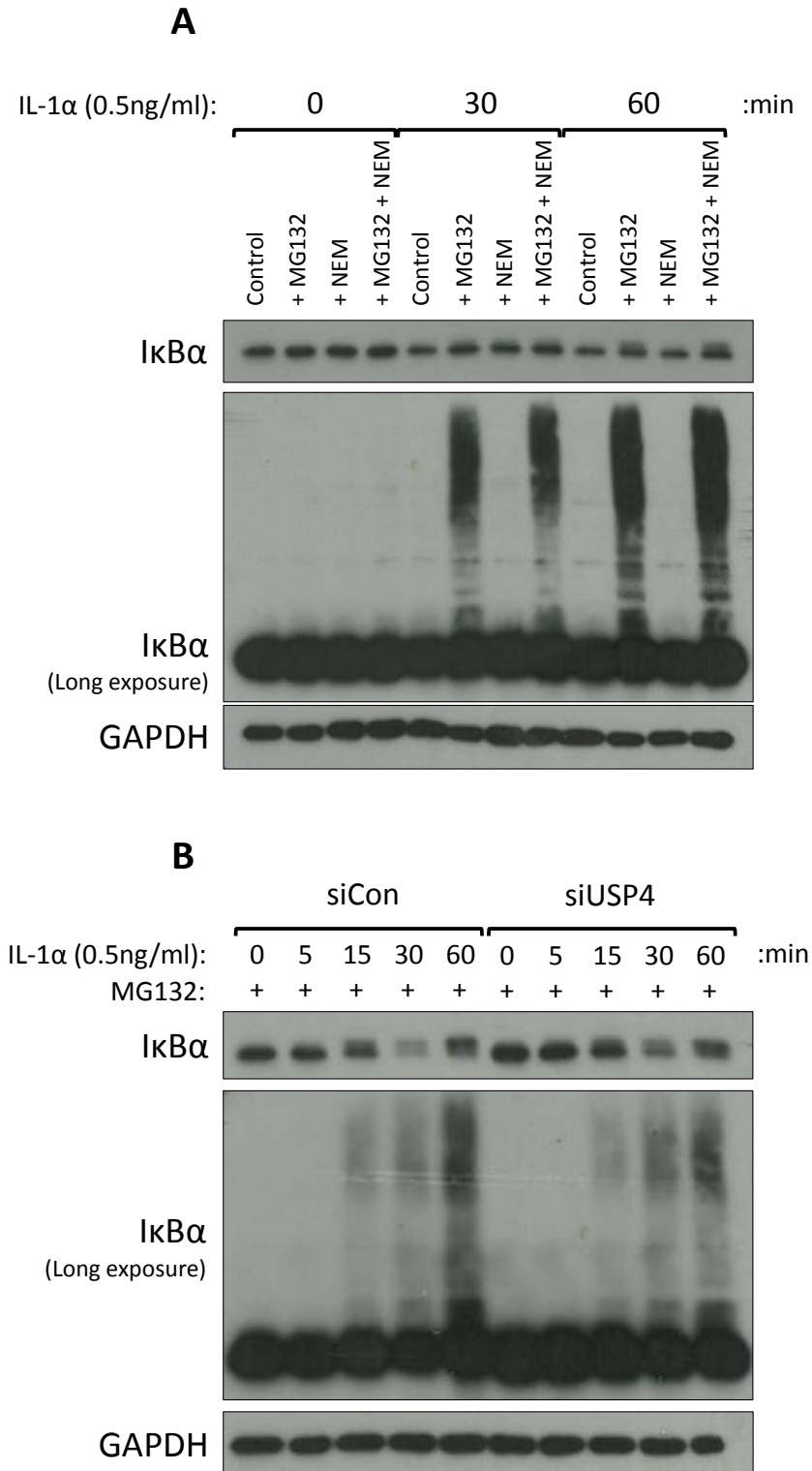


Figure 4.8. I κ B α ubiquitination. A) MG132 inhibits proteasome. SW1353 cells were stimulated with IL-1 α (0.5ng/ml) for 0, 30 and 60 minutes and treated with MG132 (5 μ M) and/or NEM (2mM) or DMSO and/or ethanol controls as indicated. Total protein was extracted, resolved by SDS-PAGE and immunoblotted with an anti-I κ B α antibody. B) Reduced I κ B α ubiquitination with USP4 depletion. SW1353 cells were transfected with siCon or siUSP4, serum-starved and treated with IL-1 α (0.5ng/ml) and MG132 (5 μ M) for 0, 5, 15, 30 and 60 minutes before lysis and immunoblotting with an anti-I κ B α antibody. GAPDH was used as a loading control. Figure is representative of 2 independent experiments.

4.2.4.4 The effect of USP4 depletion on IL-1-induced phosphorylation of p65

Consistent with downregulation of the NF- κ B pathway, USP4 depletion increased the ubiquitination of I κ B α and decreased the degradation of I κ B α with IL-1 stimulation (Figure 4.7A and Figure 4.8B). The phosphorylation, ubiquitination and subsequent degradation of I κ B α to release bound dimers of NF- κ B exposes the NF- κ B nuclear localisation site (NLS), allowing its translocation to the nucleus where it activates the transcription of many genes. In canonical NF- κ B signalling, NF- κ B exists as mostly p65 heterodimers with p50, which becomes phosphorylated as part of the regulation in the activation of NF- κ B; the phosphorylation of p65 occurs in accordance with the phosphorylation of I κ B α (Sakurai et al., 2003). As USP4 depletion affected levels of I κ B α , the levels of p65 and P-p65 further downstream in the pathway were examined.

Figure 4.9A indicates the levels of p65 were equal; there was no change when stimulated with IL-1 or between USP4 depleted and control cells implying that USP4 does not affect the stability of p65. The phosphorylation of p65 increased with IL-1 stimulation in both USP4 depleted and control. P-p65 was reduced following USP4 depletion, however, this reduction was not fully reproducible and therefore failed to reach statistical significance when quantified (Figure 4.9B).

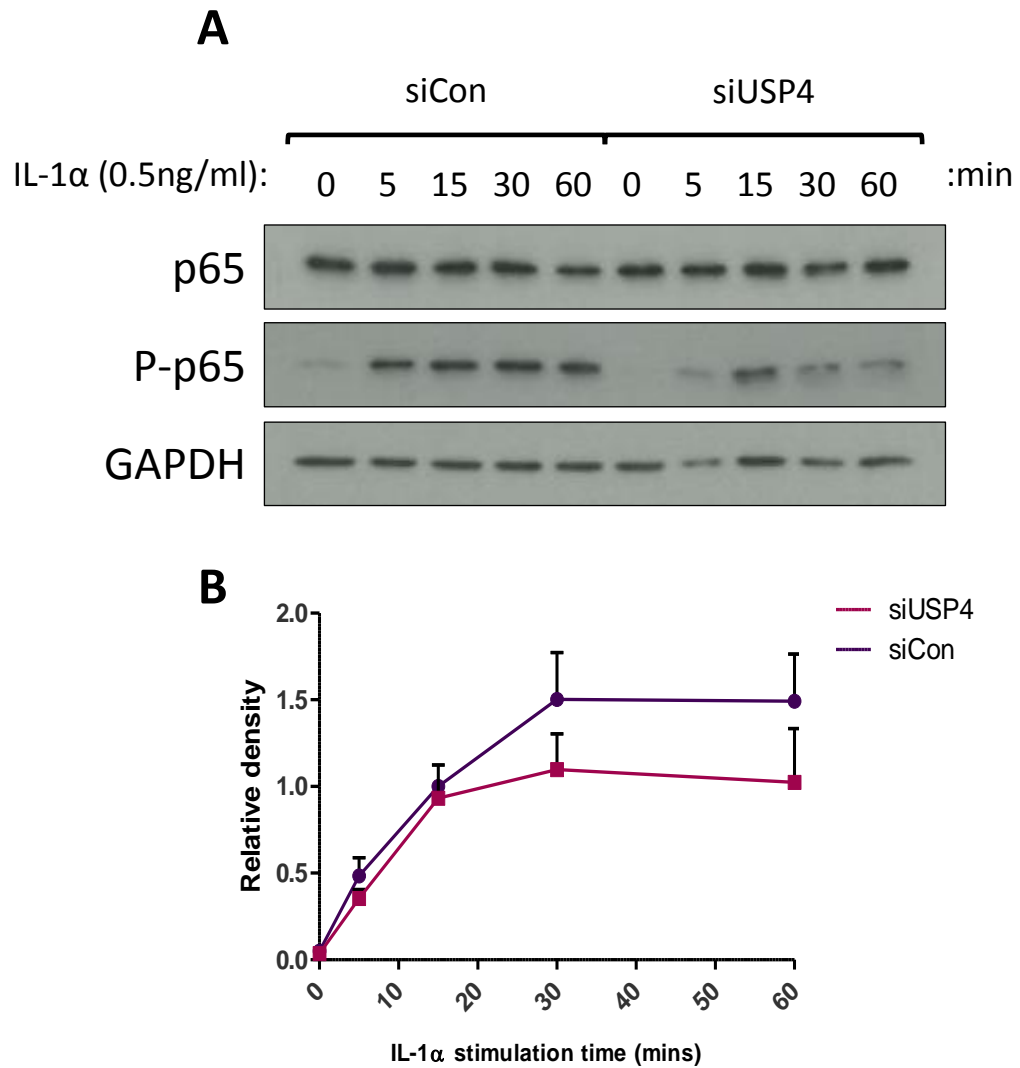


Figure 4.9. USP4 depletion and the effect on the levels of phosphorylation of NF- κ B subunit, p65. SW1353 cells were transfected with siCon or siUSP4, serum-starved and treated with IL-1 α (0.5ng/ml) for the indicated times (minutes). **A)** Total protein was extracted, resolved by SDS-PAGE and immunoblotted with anti-p65 and anti-P-p65 antibodies. GAPDH was used as a loading control. Figure is representative of 5 independent experiments. **B)** Quantification of protein levels. Data are plotted as mean \pm SEM. Student's *t*-tests (unpaired) were performed as statistical analysis. Data are combined from 5 independent experiments.

4.2.4.5 The effect of USP4 depletion on IL-1-induced phosphorylation levels of IKK α / β

Further upstream of p65 and I κ B α , the IKK complex is involved in the phosphorylation of I κ B α . As such, the protein levels of IKK α , IKK β and the phosphorylated form, P-IKK α / β , were examined following USP4 depletion and IL-1 stimulation.

The levels of total IKK α and IKK β remained unchanged with IL-1 stimulation in both control and USP4 depleted cells indicating their stability was not affected by the loss of USP4 (Figure 4.10A). Upon activation of the NF- κ B pathway, IKK subunits become phosphorylated to allow I κ B α phosphorylation. Measurement of levels of phosphorylated IKK α / β revealed a reproducible reduction in IL-1-stimulated phosphorylation of IKK α / β in USP4 depleted cells compared to the control (Figure 4.10A). Quantification confirmed the significance of this reduction after 15, 30 and 60 minutes (phospho-protein levels at time points earlier than 15 minutes were unquantifiable) (Figure 4.10B).

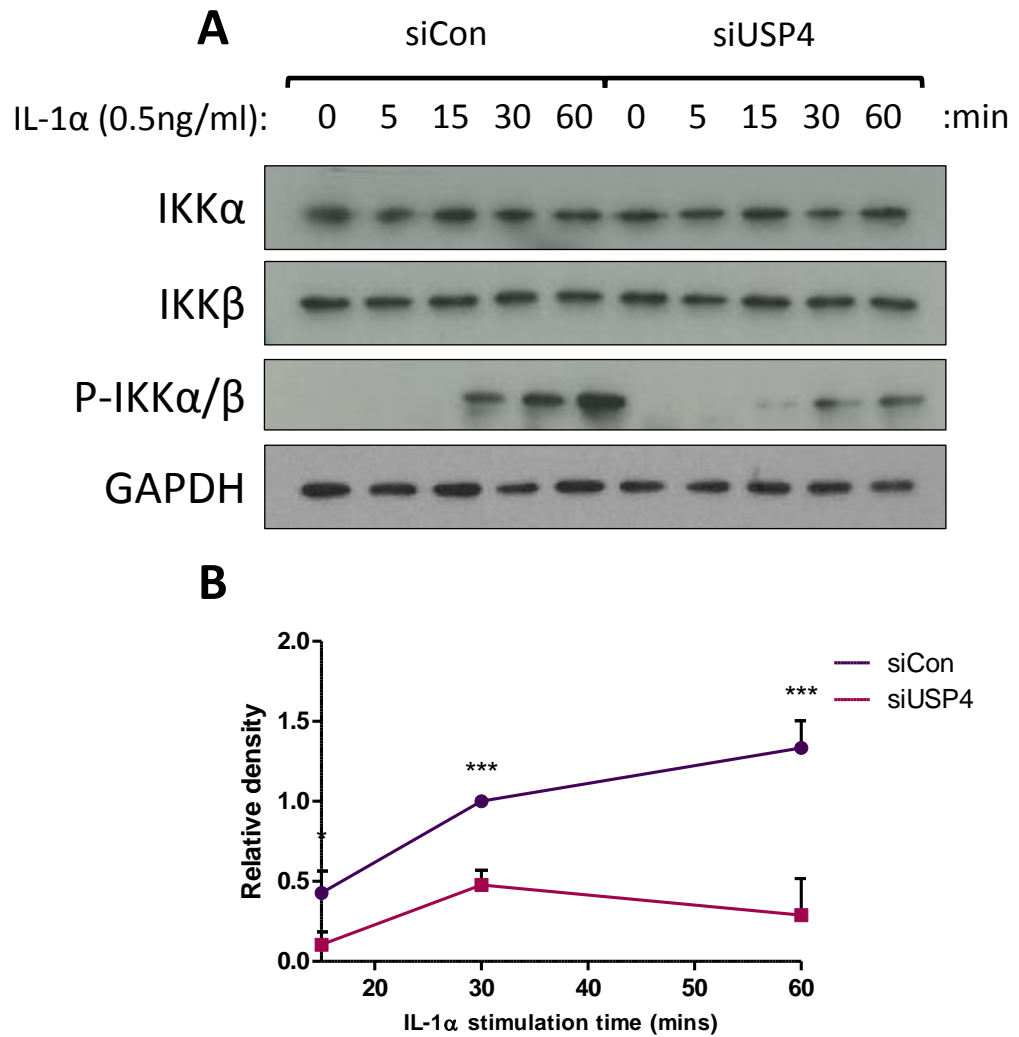


Figure 4.10. USP4 depletion and the effect on IKK α , IKK β and phosphorylated IKK α/β protein levels. SW1353 cells were transfected with siCon or siUSP4, serum-starved and treated with IL-1 α for the indicated times (minutes). **A)** Total protein was extracted, resolved by SDS-PAGE and immunoblotted with anti-IKK α , anti-IKK β and anti-P-IKK α/β antibodies. GAPDH was used as a loading control. Figure is representative of 4 independent experiments. **B)** Quantification of protein levels. Data are plotted as mean \pm SEM. *** \leq 0.001. Student's t-tests (unpaired) were performed as statistical analysis. Data are combined from 4 independent experiments and standardised to siCon15=1.

4.2.4.6 The effect of USP4 depletion on the phosphorylation levels of JNK

The IKK β subunit of the IKK complex is phosphorylated by TAK1, an essential step in the activation of NF- κ B. TAK1 is a MAPKKK, also essential in the activation of JNK and p38. As depleting USP4 clearly had an effect on the levels of phosphorylated IKK α/β , levels of phosphorylated p38, ERK and JNK were also examined through immunoblotting.

Depleting USP4 had no effect on IL-1-induced phosphorylation of p38 (Figure 4.11A). Basal levels of P-p38 appeared higher following USP4 depletion, although this was not reproducible. Additionally, USP4 depletion had no effect on the phosphorylation of ERK, but as ERK phosphorylation was not increased with IL-1 stimulation in either control or USP4 depleted cells, the ERK pathway may not have been activated by IL-1 (Figure 4.11A). However, there was a striking reduction in the phosphorylation of JNK in USP4 depleted cells compared to the control (Figure 4.11A). Quantification of the phosphorylation at Thr183 and Tyr183 of two isoforms of JNK, JNK1 and JNK2, which both have two different splicing forms, 54kDa (the prominent splicing form of JNK2) and 46 kDa (the prominent splicing form of JNK1) identified a significant difference in the phosphorylation of the larger JNK (54kDa) after 15, 30 and 60 minutes stimulation with IL-1 (Figure 4.11B). There also appeared to be a reduction in the phosphorylation of the smaller JNK (46kDa) after 5, 15, 30 and 60 minutes stimulation with IL-1 although this was not statistically significant with the densitometry method used (Figure 4.11). Protein levels at stimulation time points lower than those mentioned were unquantifiable by this technique.

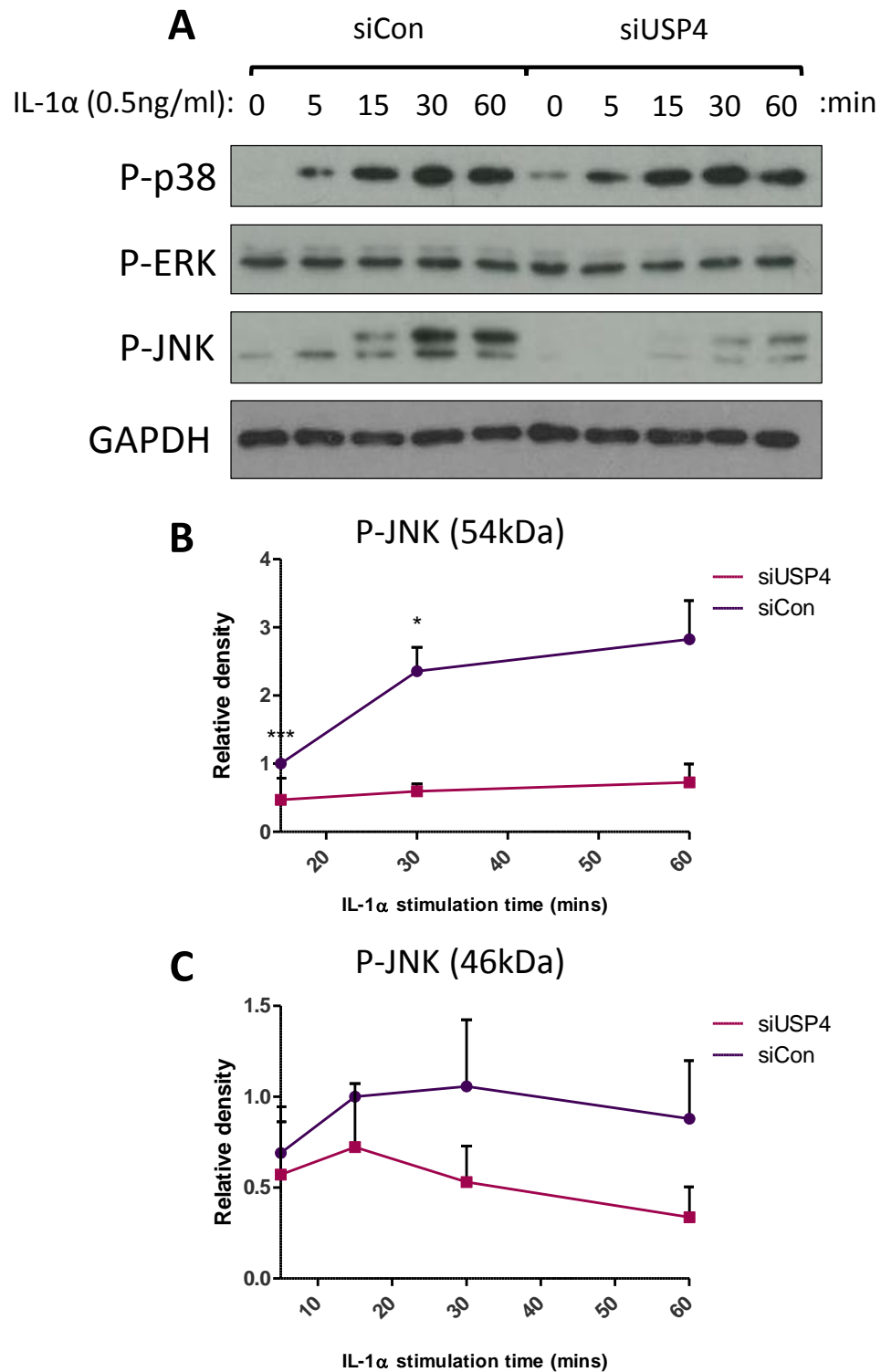


Figure 4.11. USP4 depletion and the effect on MAPK protein levels. SW153 cells were transfected with siCon or siUSP4, serum-starved and treated with IL-1 α for the indicated times (minutes). **A)** Total protein was extracted, resolved by SDS-PAGE and immunoblotted with anti-P-p38, anti-P-ERK and anti-P-JNK antibodies. GAPDH was used as a loading control. Figure is representative of 3 independent experiments. **B)** Quantification of P-JNK protein levels (54 kDa band). **C)** Quantification of P-JNK protein levels (46 kDa band). Data are plotted as mean \pm SEM. * $p \leq 0.05$, *** $p \leq 0.001$. Student's *t*-tests (unpaired) were performed as statistical analysis. Data are combined from 3 experiments and standardised to siCon₁₅=1.

4.2.4.7 The effect of USP4 depletion on TNF α -induced levels of key NF- κ B adaptor proteins

NF- κ B activation occurs via a number of stimuli, in addition to IL-1 the pathway can be induced by TNF α via TNFRs. To further investigate the effect of USP4 on NF- κ B signalling, SW1353 cells were stimulated with TNF α after USP4 depletion before lysis and western blot analysis. Immunoblotting was performed using antibodies for those key proteins found to be of interest with USP4 depletion in IL-1-induced cells (sections 4.2.4.1, 4.2.4.4, 4.2.4.5 and 4.2.4.6).

IL-1-induced I κ B α levels were higher in USP4 depleted cells (Figure 4.7) and with TNF α induction there was an increase in I κ B α levels at basal levels and after 5 and 15 minutes induction in USP4 depleted cells compared to the control (Figure 4.12). Additionally, phosphorylated p65 was moderately reduced in USP4 depleted cells and phosphorylated IKK α / β after 30 minutes induction with TNF α appeared to be lower in USP4 depleted cells than the control. Overall, any differences between the levels of these key NF- κ B adaptor proteins with and without USP4 depletion were not as definitive in TNF α -induced SW1353 cells as in IL-1-induced SW1353 cells.

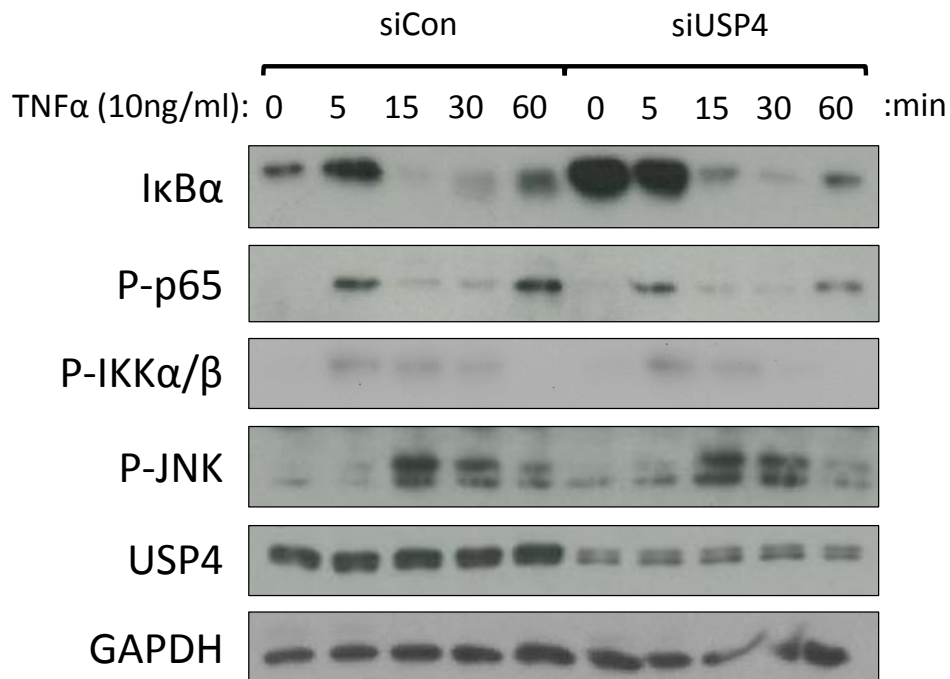


Figure 4.12. USP4 depletion and the effect on TNF α -induced NF- κ B adaptor protein levels. SW1353 cells were transfected with siCon or siUSP4, serum-starved and treated with TNF α (10ng/ml) for the indicated times (minutes). A) Total protein was extracted, resolved by SDS-PAGE and immunoblotted with anti-I κ B α , anti-P-p65, anti-P-IKK α / β , anti-P-JNK and anti-USP4 antibodies. GAPDH was used as a loading control. Figure is representative of 3 independent experiments.

4.2.4.8 The effect of USP4 depletion on IL-1- and TNF α -induced levels of key NF- κ B adaptor proteins in HeLa cells

To verify the findings in this chapter on the effect of USP4 depletion on levels of proteins involved in NF- κ B signalling and to establish if this was due to cell-specific effects, levels of key NF- κ B adaptor proteins were examined in cervical adenocarcinoma HeLa cells. Depletion of USP4 protein levels in HeLa cells with transfection of siUSP4 was confirmed in section 4.2.1. As with SW1353 cells, USP4 was depleted before treatment with IL-1 or TNF α for the indicated time points. Cells were then lysed, total protein extracted, resolved by electrophoresis and immunoblotted with the relevant antibody. As with TNF α induction in SW1353 cells, only the key NF- κ B adaptor proteins of interest, for which differences in levels with USP4 depletion were previously identified, were examined.

Similarly to IL-1-induction in SW1353 cells, levels of phosphorylated p65 were reduced when USP4 was depleted, particularly after 15, 30 and 60 minutes of stimulation (Figure 4.13A). Again there was a dramatic reduction in the phosphorylation of both IKK α/β and JNK in SW1353 cells, as observed in Figure 4.10 and Figure 4.11. Surprisingly, there did not appear to be any I κ B α degradation in either the control or USP4 depleted cells, known to be a key step in NF- κ B activation. Considering there were changes in the levels of both phosphorylated IKK α/β and p65 which are upstream and downstream of I κ B α , respectively, IL-1 was evidently inducing the pathway. Rather than loss of I κ B α degradation, it is possible that resynthesis of I κ B α was balancing its degradation: following stimulation, as much I κ B α was being synthesised as degraded which would explain why levels of I κ B α were unchanged.

Contrary to IL-1 stimulation, TNF α stimulation induced clear degradation of I κ B α (Figure 4.13B). Furthermore, levels of I κ B α were higher in USP4 depleted cells compared to the control, particularly evident at basal levels and after 5 and 60 minutes TNF α stimulation. This suggests less or slower degradation of I κ B α in USP4 depleted cells, consistent with the effects of USP4 depletion on I κ B α degradation in SW1353 cells, or increased expression of USP4 as I κ B α was induced after 5 minutes stimulation with TNF α . Again, dramatic reductions in the phosphorylation of both IKK α/β and JNK were observed in USP4 depleted cells. Overall, effects of USP4 depletion with IL-1 and TNF α induction on NF- κ B adaptor proteins in HeLa cells were consistent with effects observed in SW1353 cells, although the effect in HeLas appeared to be more pronounced with

TNF α induction than in SW1353s and more representative of observations with IL-1 α induction. There was also no apparent degradation of I κ B α in IL-1-induced HeLa cells.

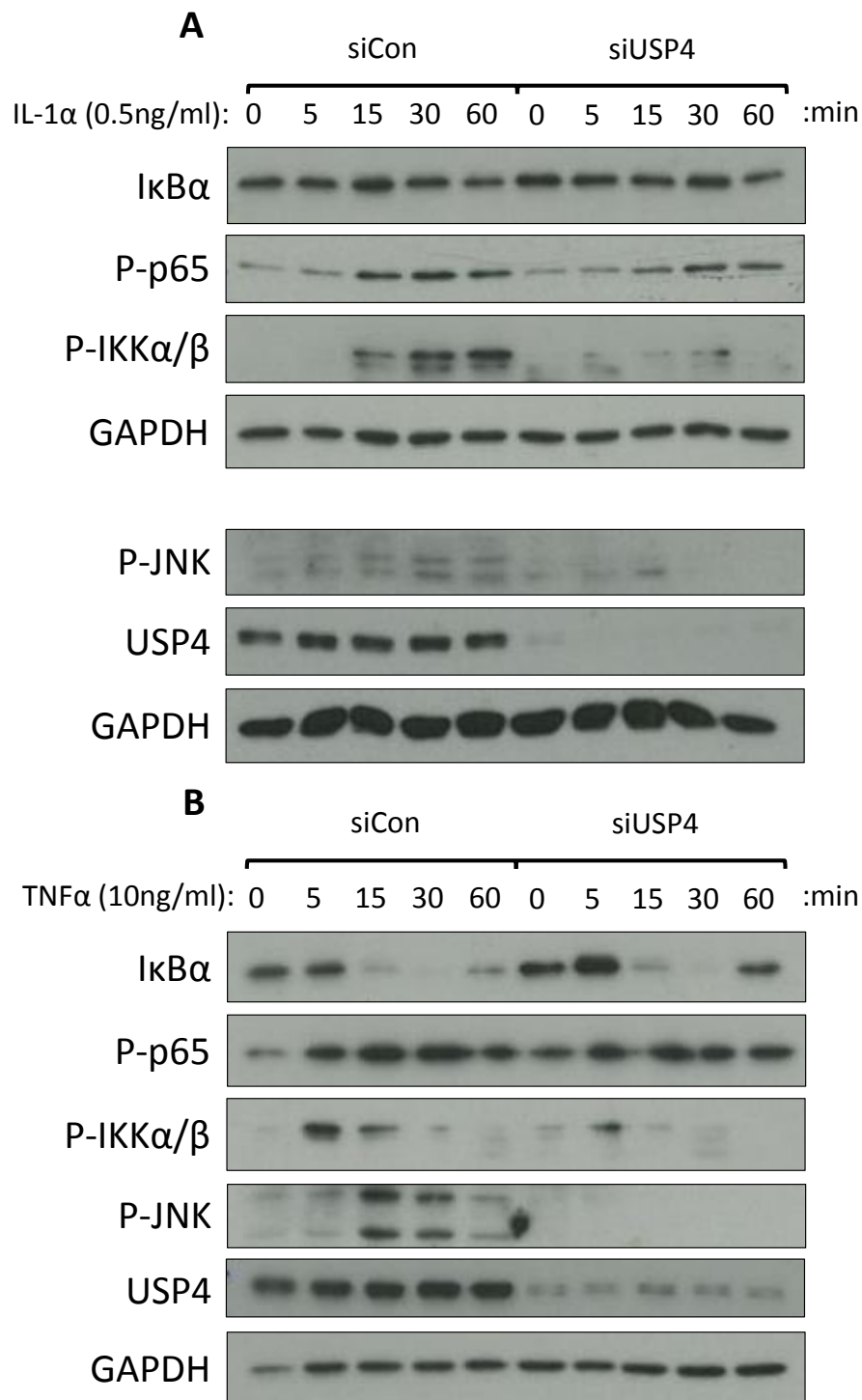


Figure 4.13. USP4 depletion and the effect on NF- κ B adaptor protein levels in stimulated HeLa cells. HeLa cells were transfected with siCon or siUSP4, serum-starved and stimulated for the indicated times (minutes). Total protein was extracted, resolved by SDS-PAGE and immunoblotted with anti-I κ B α , anti-P-p65, anti-P-IKK α / β , anti-P-JNK and anti-USP4 antibodies. GAPDH was used as a loading control. A) Cells were stimulated with IL-1 α (0.5ng/ml). Figure is taken from 2 experiments. B) Cells were stimulated with TNF α (10ng/ml). Figure is representative of one experiment.

4.2.5 NF- κ B signalling in *Usp4*^{-/-} mouse embryonic fibroblasts

Mouse embryonic fibroblast (MEF) cell lines were established from wild-type and *Usp4*^{-/-} mouse embryos (described in section 2.2.1.1). MEFs can be used as a powerful model to address protein function and cell signalling (Lovas et al., 2012, Zhang et al., 2011). As data has shown in this chapter, depleting USP4 in SW1353 and HeLa cells significantly affected NF- κ B signalling therefore MEFs were next used for validating these results.

4.2.5.1 IL-1-induced NF- κ B activity in *Usp4*^{+/+} and *Usp4*^{-/-} MEFs

USP4 depletion reduced NF- κ B activity in SW1353 cells in section 4.2.2 and so the first step was to examine IL-1-induced NF- κ B activity in MEFs from *Usp4*^{-/-} mouse embryos (*Usp4*^{-/-}) and MEFs from wild-type mouse embryos (*Usp4*^{+/+}). Initially, MEFs were transfected with a NF- κ B responsive firefly luciferase reporter and a constitutive *Renilla* expression vector. After a period of serum-starvation cells were stimulated with IL-1 before relative luciferase activity was measured. Data showed light units only detectable at background levels indicating no transfection of NF- κ B firefly luciferase reporter or *Renilla* expression vectors (data not shown). Primary cell lines, such as MEFs, are notoriously difficult to transfect and so the next approach was to transduce a lentiviral NF- κ B reporter vector, pHAGE-NF κ B-TA-LUC-UBC-GFP-W, into the MEFs.

Lentiviral supernatant was produced by transfection of lentiviral expression vector, pHAGE-NF κ B-TA-LUC-UBC-GFP-W, and viral packaging plasmids into HEK 293T cells (section 2.2.14.2). Viral supernatant was collected and the MEFs were transduced with the supernatant before overnight serum starvation and stimulation with IL-1. MEFs were then lysed and luminescence measured. To verify successful introduction of the lentiviral expression vector into cells, a preliminary experiment in SW1353 cells was performed. Cells were either infected with lentiviral preparations with/without IL-1 stimulation or treated with polybrene only. Infection with lentiviral preparations increased light units compared to the control, which was increased more than 500 fold with IL-1 stimulation, indicating that transduction was successful (Figure 4.14).

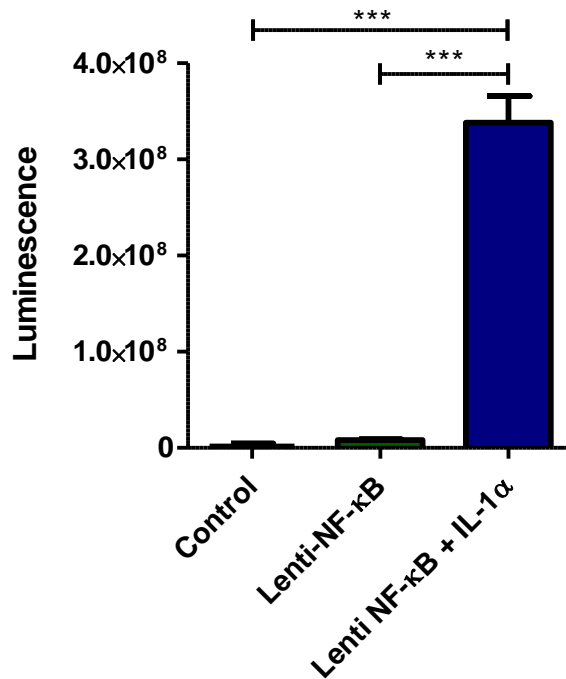


Figure 4.14. IL-1-induced NF- κ B activity in SW1353 cells. Lentiviral supernatant for a NF- κ B-dependent firefly luciferase expression vector (pHAGE-NF κ B-TA-LUC-UBC-GFP-W) was transduced into cells. SW1353 cells were infected with lentiviral preparations with IL-1 α stimulation (0.5ng/ml) for 6 hours (Lenti-NF- κ B + IL-1 α), without IL-1 α (Lenti-NF- κ B) or with polybrene only (Control). Luminescence was measured after cell lysis. One way ANOVA with a post-hoc Tukey comparison test was performed as statistical analysis. Data are plotted as mean \pm SEM (n=4/experiment). *** p \leq 0.001.

Next, both *Usp4*^{-/-} and *Usp4*^{+/+} MEFs were infected with lentiviral preparations for the NF- κ B luciferase reporter vector and the reporter assay performed as described above. IL-1-induction increased NF- κ B activity by over 300 fold and 200 fold in *Usp4*^{+/+} and *Usp4*^{-/-} MEFs, respectively. When induced with IL-1, NF- κ B activity was significantly reduced in *Usp4*^{-/-} MEFs compared with *Usp4*^{+/+} MEFs by over 35% (Figure 4.15).

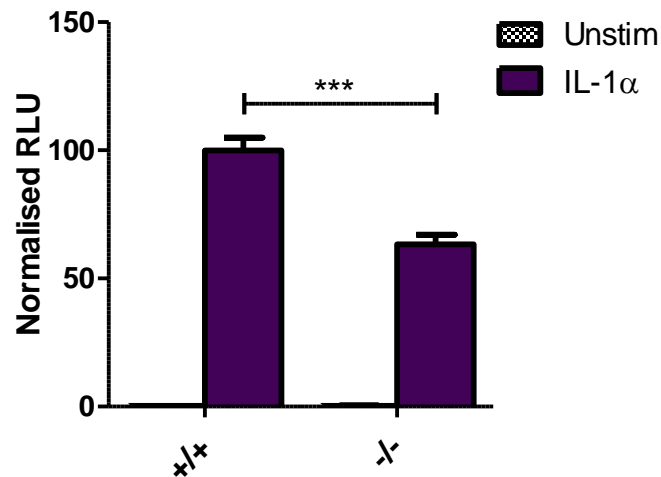


Figure 4.15. IL-1-induced NF- κ B activity in MEFs. Lentiviral preparations were transduced into wild-type (+/+) and *Usp4* null (-/-) MEFs for 24 hours before overnight serum starvation. Cells were stimulated with IL-1 α (0.5ng/ml) for 6 hours, or left unstimulated (Unstim), before lysis and measurement of luminescence. Data are shown relative to IL-1 α -induced +/+ = 100%. Student's t-tests (unpaired) were performed as statistical analysis. Data are combined from 2 independent experiments and plotted as mean \pm SEM (n=4/experiment). *** p \leq 0.001.

4.2.5.2 IL-1-induced expression of NF- κ B-regulated genes in *Usp4*^{-/-} MEFs

As with SW1353 cells, expression of NF- κ B-regulated genes was measured in the MEFs. The day after cells were seeded, the MEFs were subjected to overnight serum-starvation. After stimulation with IL-1 (0.5ng/ml) total RNAs were extracted from *Usp4*^{-/-} and *Usp4*^{+/+} MEFs and real-time qRT-PCR was performed.

Relative *Usp4* gene expression was essentially undetectable in *Usp4*^{-/-} compared to *Usp4*^{+/+} MEFs with and without IL-1 stimulation (Figure 4.16D). In *Usp4*^{+/+} MEFs there was a significant increase in relative *Usp4* gene expression after stimulation with IL-1 α , not previously observed in SW1353 cells (section 4.2.3). As expected, IL-1 α stimulation significantly increased expression of *Il-6*, *Cxcl1* (the mouse homologue of *IL-8*) and *Mmp13* (Figure 4.16A to Figure 4.16C). IL-1-induced relative *Il-6* and *Mmp13* gene expression were significantly reduced in the *Usp4*^{-/-} MEFs (Figure 4.16A and Figure 4.16C). IL-1-induced relative *Cxcl1* expression was not significantly reduced in *Usp4*^{-/-} compared to *Usp4*^{+/+} (Figure 4.16B). These data concur with IL-1-induced expression of NF- κ B-regulated genes in SW1353 cells, supporting the previous evidence that absence of USP4 downregulates inflammatory gene expression, presumably by regulation of the NF- κ B pathway.

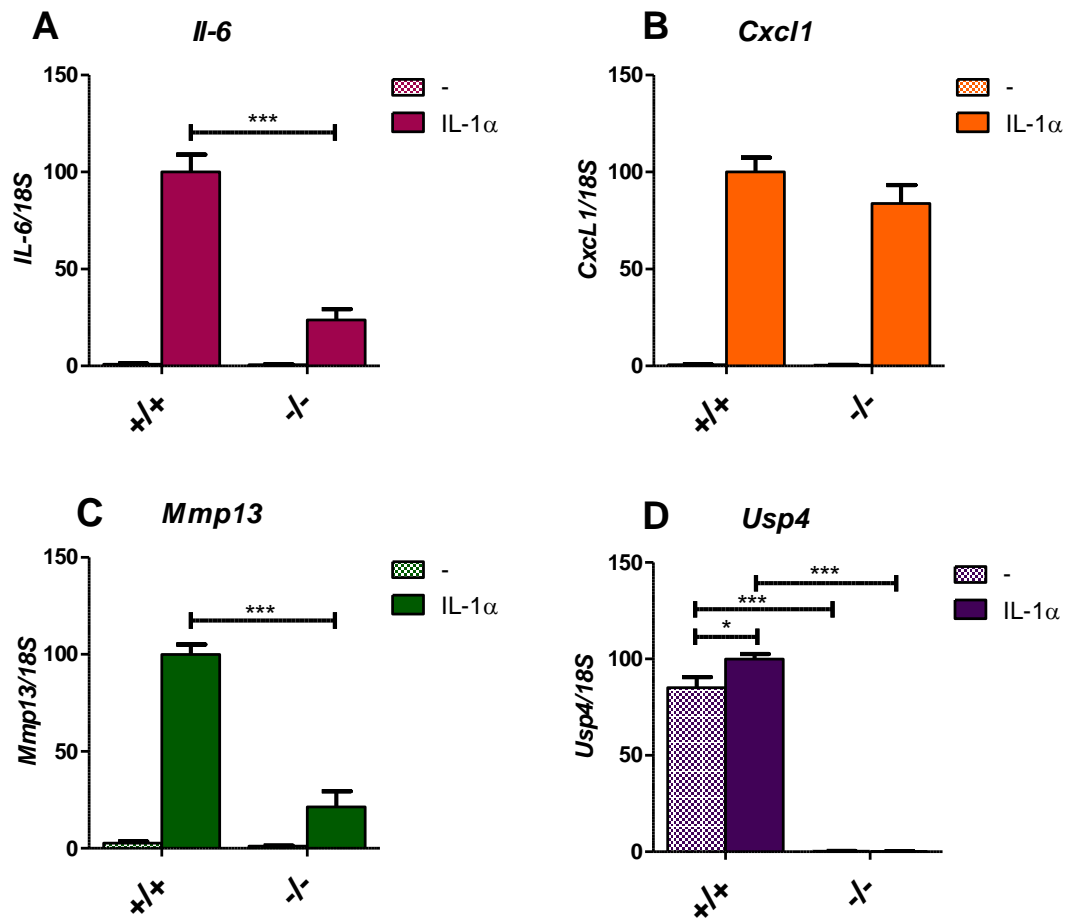


Figure 4.16. USP4 regulated IL-1-induced NF- κ B-regulated gene expression in MEFs. Wild-type (+/+) and *Usp4* null (-/-) MEFs were stimulated with IL-1 α (0.5ng/ml) for 6 hours after overnight serum-starvation. Total RNA was extracted, reverse transcribed to cDNA and analysed by real-time qRT-PCR. Relative gene expression of IL-1 α -induced A) *Il-6*, B) *Cxcl1*, C) *Mmp13* and D) *Usp4* were measured. Data were normalised to 18S and shown as relative to IL-1 α -induced +/+ =100%. Data are combined from at least 2 experiments (n=6/experiment). Student's *t*-test (unpaired) were performed as statistical analysis. Data are plotted as mean \pm SEM. *p<0.05, ** p<0.01, *** p<0.001.

4.3 Discussion

Data from a multi-pathway reporter assay in the previous chapter alluded to a potential role for USP4 as a positive regulator of NF- κ B signalling, something not previously seen. Therefore this chapter further examined USP4 by using RNAi as a molecular biological tool to deplete USP4 in cells. Following this, work was replicated in MEFs isolated from *Usp4*^{-/-} mice and their wild-type littermates. Effects were examined using a combination of approaches to study proteins and genes involved in NF- κ B signalling in the hope of identifying a previously unfound role for USP4 in upregulating NF- κ B activation.

USP4 has actually been reported as a negative regulator of the NF- κ B signalling pathway (Fan et al., 2011b, Zhou et al., 2012, Xiao et al., 2012). Results in this thesis chapter ultimately indicated that depleting USP4 downregulates IL-1-, and possibly less pronounced, TNF α -induced NF- κ B signalling, suggesting USP4 upregulates NF- κ B signalling, in stark contrast with the literature.

It was first established that NF- κ B activity was significantly reduced when cells with USP4 depletion were induced with both IL-1 and TNF α ; consistent with data in the previous chapter for which overexpression of wild-type, but not catalytically inactive, *Usp4* increased NF- κ B activity. This is conflicting with published data which reported an increase in TNF α - and IL-1 β -induced NF- κ B activity with USP4 depletion and a reduction in TNF α - and IL-1-induced NF- κ B activity with overexpression of wild-type, but not catalytically inactive, USP4 (Fan et al., 2011b, Zhou et al., 2012, Xiao et al., 2012). To begin to elucidate the mechanism for USP4 in enhancing NF- κ B activity, and why data might be conflicting with published data, more studies were performed.

4.3.1 IL-1- and TNF α -induced NF- κ B-regulated gene expression

NF- κ B is an important transcription factor responsible for the regulation of many genes involved in the immune and inflammatory response, and any changes in the activation of NF- κ B may be reflected in the expression levels of these target genes. IL-6 and IL-8 are cytokines which function as major mediators in inflammation. The activation of *IL-6* and *IL-8* gene expression can be mediated through NF- κ B by inducers including IL-1 and TNF α (Shimizu et al., 1990, Kunsch and Rosen, 1993). It was demonstrated in this chapter that USP4 enhanced IL-1-induced expression of proinflammatory cytokines: after stimulation with IL-1 at certain time points over a 24 hour period there was a significant

decrease in *IL-6* and *IL-8* gene expression with USP4 depletion. This is again in contrast to literature which reported enhanced expression of IL-1-induced NF- κ B-regulated gene expression, including *IL-6* (Zhou et al., 2012). Whereas in this chapter it was demonstrated that IL-1-induced expression of *IL-6* was reduced with siRNA-mediated depletion of USP4 in SW1353 cells after stimulation for 6 hours or after stimulation over a 24 hour time course, Zhou et al. (2012) reported that IL-1-induced expression of *IL-6* was enhanced with lentivirus shRNA-mediated USP4 knockdown in MEFs after stimulation for 4 hours. Differences could therefore be due to variations in cell lines or species utilised or method of USP4 depletion. Even though the IL-1 stimulation times varies slightly between published data and data in this chapter, at no time point was *IL-6* expression enhanced here. All IL-1-induced gene expression experiments herein were performed at least twice and the depletion of USP4 was consistently confirmed for each experiment. No known study has reported effects of USP4 depletion on IL-1-induced expression of *IL-8* although other IL-1-induced NF- κ B-regulated genes were measured which were also reported to be enhanced with USP4 depletion.

With TNF α -induced expression of proinflammatory cytokines, the data were less clear. Initially, experiments demonstrated a reduction in *IL-6* with USP4 depletion after 6 hours stimulation with TNF α , but over a time course of stimulations *IL-6* appeared to increase with USP4 depletion. The latter is more in line with other studies in which TNF α induction enhanced expression of NF- κ B-regulated genes including *IL-6* and *IL-8* with the knockdown of USP4 (Fan et al., 2011b, Xiao et al., 2012). Fan et al. (2011b) reported that TNF α -induced expression of *IL-6* was enhanced in stable USP4 knockdown HeLa cells, and Xiao et al. (2012) reported that TNF α -induced expression of *IL-6* and *IL-8* were enhanced in siRNA-mediated USP4 depleted lung adenocarcinoma epithelial A549 cells, whereas the expression of *IL-6* and *IL-8* herein was measured in SW1353 cells with siRNA-mediated USP4 depletion, therefore differences could be cell-specific. The period of TNF α stimulation in published data (30-120 minutes) was also much shorter than here (1-24 hours) and it was reported that the expression of *IL-6* peaked after 60 minutes of stimulation before decreasing whereas it was observed here that *IL-6* was consistently increased over the 24 hour period. The reduction in TNF α -induced *IL-6* expression with USP4 depletion after 6 hours stimulation could therefore be due to longer stimulation times.

The cascade of proteins and their activation as part of NF- κ B activation does differ between IL-1 induction through the IL-1R and TNF α stimulation through the TNFR which may account for the differences in the alterations in gene expression levels depending on where USP4 functions in the pathway. However, this would not account for differences between the data observed with a 6 hour TNF α stimulation and the data collected at various TNF α stimulation times (including 6 hours) and so the reason for these observations remain unclear. Experimental procedure was the same when cells were stimulated for 6 hours and cells were stimulated over a time course: all experiments were performed in 96-well plates with the same density of cells, the same protocol was followed and cells were stimulated with the same concentration of TNF α (10ng/ml). The only difference was that cells were serum-starved for a longer period when stimulations were performed over a time course to allow cells stimulated for different lengths of time to be lysed at the same time. All gene expression experiments were replicated at least twice to validate results.

In addition to proinflammatory cytokines, NF- κ B is involved in the regulation of many other important genes. MMP13 has been majorly implicated in osteoarthritis and rheumatoid arthritis as a mediator of cartilage degradation when overexpressed. The regulation of *MMP13* is complex and can be expressed by induction with TNF α , IL-1 and through TLRs, largely induced via AP-1, composed of c-Jun and c-Fos proteins, and NF- κ B transcription factors, both of which can often be induced by the same stimulus e.g. IL-1 (Mengshol et al., 2000, Otero et al., 2012, Schmucker et al., 2012). In USP4 depleted cells, *MMP13* expression was reduced with both IL-1 and TNF α induction across a 24 hour period, indicating that USP4 is involved in IL-1- and TNF α -induced pathway(s) which regulate the expression of *MMP13*. No other studies examined the effect of USP4 depletion on *MMP13* but this is in line with data for the reduced IL-1-induced *IL-6* and *IL-8* expression also observed in this chapter.

4.3.2 Depletion of USP4 altered IL-1 α -induced NF- κ B adaptor protein levels

Although some gene expression data appeared to be contrasting, it did suggest USP4 affected NF- κ B-regulated genes. As IL-1 stimulation proved to give the most consistent and profound differences on NF- κ B-regulated gene expression with USP4 depletion, focus was given to IL-1-induced effects on NF- κ B adaptor proteins.

For NF- κ B to become activated, it relies on a cascade of events involving the recruitment and phosphorylation of many adaptor proteins. With both TNF α and IL-1 stimulus, the transactivation of NF- κ B can occur through phosphorylation of Ser-536 on the p65 subunit of NF- κ B after its translocation to the nucleus to induce expression of its target genes by binding to consensus sequence, GGGATTTCC (Sakurai et al., 1999, Sakurai et al., 2003, Buss et al., 2004). Data in this chapter demonstrated a potential reduction in IL-1-induced p65 phosphorylation when USP4 was depleted, suggesting decreased NF- κ B activation in accordance with decreased target gene expression. Studies have shown dynamic translocation of NF- κ B. Oscillations in NF- κ B translocation arise from a negative feedback loop involving I κ B α : degradation of I κ B α allows NF- κ B translocation to the nucleus for the transcriptional activation of NF- κ B target genes, including I κ B α which then binds to NF- κ B to aid the nuclear export of NF- κ B (Nelson et al., 2004, Hoffmann et al., 2002a). It would therefore have been interesting to study the effects of USP4 depletion on p65 translocation to and from the nucleus.

Prior to p65 phosphorylation, NF- κ B translocation to the nucleus occurs after the K48-linked polyubiquitination and degradation of I κ B α so it is no longer sequestered in the cytoplasm. Consistent with reduced phosphorylation of p65, signifying reduced NF- κ B activation, IL-1-induced I κ B α degradation was also reduced with USP4 depletion. The I κ B family includes other members I κ B β , I κ B γ , I κ B ϵ which can regulate NF- κ B, possibly through more complex regulation (Napetschnig and Wu, 2013). Although the effect of USP4 depletion on these other isoforms could have been studied, the best characterised is I κ B α with knowledge on how its degradation is key in IL-1-induced NF- κ B activation and as such was studied here.

As I κ B α is degraded by the proteasome after K48-linked polyubiquitination, changes in its degradation suggest changes in its ubiquitination; therefore I κ B α ubiquitination was examined. As many DUBs, including USPs, regulate NF- κ B activation, the effect of inhibiting USPs using NEM, which acts by blocking DUB active sites, and the proteasome inhibitor MG132, was first examined. MG132 is a peptide aldehyde which inhibits the proteolytic activity of the 26S proteasome, leading to accumulation of K48-linked polyubiquitinated proteins. At longer exposures, higher molecular weight I κ B α 'smears', which were attributed to K48-linked polyubiquitination of I κ B α , were visible with MG132 treatment. There was no obvious difference in I κ B α ubiquitination with DUB inhibition. This could be due to the low dose of NEM used: higher doses were toxic

to cells but the dose used may not have been high enough for successful full inhibition of DUBs. Previous reports utilising NEM for the inhibition of DUBs used it at a concentration of 10mM, however this was only added to the lysis buffer and this concentration was found to be toxic when directly added to cells here (Shenoy and Lefkowitz, 2002, Hjerpe et al., 2009). Therefore, a lower dose of 2mM NEM was used.

Alternatively, inhibiting DUBs may result in their counteraction which may mask any specific effects on I κ B α . The tight network of regulation of NF- κ B signalling involves multiple DUBs which deubiquitinate different proteins within the NF- κ B pathway to both upregulate and downregulate NF- κ B activation. The effect of inhibiting DUBs which reduce I κ B α ubiquitination may therefore be masked by inhibiting other DUBs which have other effects on other proteins in the NF- κ B pathway. Confirmation of DUB inhibition with NEM at the concentration used would be needed to determine whether this is the case.

As MG132 allowed visualisation of I κ B α , the effect of specifically targeting USP4 depletion was then studied: the IL-1-induced bands of ubiquitinated I κ B α were less abundant when USP4 was depleted. This corresponded with higher levels of the protein observed with USP4 depletion; the two experiments overall representing reduced or slower degradation of I κ B α .

In addition to genes including *MMP13* and cytokines *IL-6* and *IL-8*, the translocation of NF- κ B to the nucleus upon I κ B degradation activates the transcription of other genes, including I κ B α itself. This allows a negative feedback mechanism for the temporal control of NF- κ B activation (Hoffmann et al., 2002a). Reduced *I κ B α* gene expression observed with USP4 depletion also implied downregulation of the pathway, differing to Fan et al. (2011b) who reported enhanced *I κ B α* expression in USP4 knockdown cells. However, this was with TNF α stimulation and *I κ B α* expression was not measured with IL-1 stimulation in this published study.

There is no evidence to suggest that USP4, as a deubiquitinase, directly acts on I κ B α as ubiquitination appears decreased in absence of USP4, rather more it hypothesises that USP4 functions upstream of I κ B α . For I κ B α to be recognised by the specific E3 ligase SCF ^{β -TrCP} and K48-linked polyubiquitinated for degradation, it is first phosphorylated by the IKK complex (Spencer et al., 1999). IKK is itself activated through TAK1-mediated

phosphorylation of the IKK β subunit at two serine residues within the activation loop. As NF- κ B activation and I κ B α degradation were affected by USP4 depletion, the effect on the phosphorylation of the IKK complex was studied to determine whether USP4 may act upstream of I κ B α . Phosphorylation of IKK α/β was dramatically reduced with USP4 depletion, indicating there was less phosphorylation of IKK by TAK1 upon IL-1 stimulation in these cells, and again tying in with downregulation of NF- κ B activation. Zhou et al. (2012) reported a decrease in IL-1 β -induced I κ B α levels, again in opposition to data in this chapter, however, no study reported on IL-1-induced levels of either P-p65 or P-IKK α/β . Published data on the effects of IL-1 induction on the level of I κ B α also utilised a lentivirus-mediated shRNA knockdown of USP4 in HeLa cells, rather than the siRNA-mediated depletion of USP4 in SW1353 cells here. Differences in experimental methods and cell lines used could therefore account for these conflicting data. Protein level experiments were all performed at least three times for reproducibility and densitometry combined from these experiments were performed to confirm the consistency of any observations and to allow analysis of statistically significant differences. As a good antibody against USP4 has been lacking, it is possible the antibodies may recognise USP15, which shares significant sequence homology with USP4 and so in some initial experiments, the depletion of USP4 was not definitively confirmed. The antibody chosen did demonstrate depletion of USP4 with an siRNA targeting USP4 and was used to confirm USP4 depletion in most protein analysis experiments. Therefore, use of a more accurate USP4 antibody would be helpful.

Results presented in this chapter consistently pointed towards involvement of USP4 in the upregulation of NF- κ B activation, in direct contrast to reports in the literature. The differences in levels of key adaptor proteins involved in IL-1-induced NF- κ B signalling were remarkable, particularly the change in the amount of phosphorylated IKK α/β . However, previous reports mostly focussed on TNF α -induced NF- κ B signalling and here the focus was on IL-1 induction. To determine if the differences are due to this, levels of key adaptor proteins altered with IL-1 stimulation in USP4 depleted SW1353 cells were also examined with TNF α stimulation. Although there may be small differences, the effects were not as clear as with IL-1 stimulation. As well as conflicts in data potentially arising from differences in cell lines used and methodology, USP4 may function differently with different stimuli. Further experimentation is needed to determine where USP4 may function to positively regulate IL-1-induced NF- κ B signalling. As multiple

proteins are regulated by their ubiquitination in NF- κ B signalling, as will be discussed further, potential USP4 deubiquitination targets will be examined further in the next chapter.

4.3.3 IL-1-induced phosphorylation of JNK was reduced with USP4 depletion

The particularly significant effect of USP4 depletion on IL-1-induced IKK α/β phosphorylation alludes to a role for USP4 acting upstream of the IKK complex. Although reports have been inconsistent with the findings in this chapter they have found a function for USP4 as a deubiquitinase for TAK1, a kinase which acts upstream of the IKK complex and activates IKK β by phosphorylation (Wang et al., 2001, Fan et al., 2011b). The K63-linked polyubiquitination of TAK1 is essential for this kinase activation and downstream signalling effects in transduction pathways (Fan et al., 2010). In addition to acting as an IKK kinase in NF- κ B signalling, TAK1 can act as a MAPKKK in activating MAPK pathways including p38 and JNK (Moriguchi et al., 1996, Ninomiya-Tsuji et al., 1999, Shirakabe et al., 1997). Other NF- κ B signalling proteins that are crucial for the activation of the IKK complex also feature in MAPK activation to mediate crosstalk between parallel signalling pathways. As an E3 ligase, TRAF6 ubiquitinates IRAK1, TAK1 and self-ubiquitinates with K63-linked polyubiquitination, critical for the proximal recruitment of TAK1 and IKK for downstream NF- κ B signal transduction in IL-1-mediated signalling (Conze et al., 2008, Fan et al., 2010, Lamothe et al., 2007). Another member of the TRAF family, TRAF2 is involved in the recruitment of cIAP1/2 to the TNFR1 complex for TNF α -mediated NF- κ B activation (Vince et al., 2009). TRAFs can also function as a divergent platform in signal transduction pathways: the activation of TRAF6 in IL-1-mediated signalling mediates the activation of TAK1 that can lead to activation of not only the NF- κ B but also the MAPK pathway. Alternatively, TRAF2, as well as activating NF- κ B, can associate with several MAPKs for activation of MAPK pathways in response to TNF α (Chung et al., 2002). TRAF6 and TRAF2, as well as TAK1, are reported substrates for USP4 deubiquitination (Xiao et al., 2012, Zhou et al., 2012). That data in this thesis alluded to a potential function for USP4 upstream of the IKK complex, and that these proteins can also regulate MAPK pathways, led to examination of the effects of USP4 depletion on the phosphorylation of MAPKs JNK, p38 and ERK.

USP4 depletion with IL-1 stimulation in SW1353 cells, and with both IL-1 and TNF α stimulation in HeLa cells, abrogated the phosphorylation of JNK, indicative of a reduction in activation of the JNK MAPK pathway. However, it should be noted that JNK total protein levels were not measured. In line with reduced JNK phosphorylation, in Chapter 3 a multi-pathway reporter assay demonstrated an increase in MAPK/JNK activity with USP4 wild-type overexpression, but not catalytically inactive USP4 overexpression. It has not been reported previously as to whether USP4 is involved in the IL-1-induced regulation of JNK phosphorylation and activation. However, conflicting with these data, Zhou et al. (2012) reported reductions in the TRAF6- and bacterial lipopolysaccharide (LPS)-induced activity of AP-1, a transcription factor which is activated upon activation of MAPK pathways, with overexpression of USP4. It would be of interest to determine whether depletion of USP4 affects the IL-1-induced activity of AP-1 by performing an AP-1-dependent luciferase reporter assay.

Previously in this chapter, the effect of USP4 on the expression of NF- κ B-regulated genes were examined with reductions in *IL-6* and *MMP13* with the depletion of USP4. As well as NF- κ B, IL-1 induces other signalling pathways through its IL-1R including MAPK pathways p38, ERK and JNK. Along with requiring NF- κ B translocation for its induction *MMP13* also requires JNK activation for IL-1-induction of *MMP13* in some systems (Mengshol et al., 2000). The reduction in the phosphorylation of JNK and the reduction in this NF- κ B- and JNK-regulated IL-1-induced gene with USP4 depletion, further indicates possible crossover between NF- κ B and MAPK pathways or USP4 functioning upstream of the IKK complex, possibly acting on TAK1 or TRAF6 and thereby having more widespread effects than on NF- κ B signalling.

Many of the proteins involved in NF- κ B and MAPK signalling pathways are ubiquitinated for their regulation, stability and recruitment of proteins for downstream signal transduction, leading to the possibility that USP4 functions upstream of the IKK complex to positively regulate NF- κ B signalling through the deubiquitination of these proteins. In IL-1-induced NF- κ B signalling, K63-linked polyubiquitin chains act as a scaffold for the recruitment of proteins and are crucial for NF- κ B activation (Xu et al., 2009b). TRAF6 both self-ubiquitinates and ubiquitinates TAK1 and IRAK1 with K63-linked polyubiquitin chains to mediate recruitment and activation of the IKK complex in IL-1 signalling (Lamothe et al., 2007, Fan et al., 2010, Conze et al., 2008). Further, TRAF6, TAK1 and IRAK1 can also be ubiquitinated with K48-linked polyubiquitin

chains to target them for degradation in the regulation of NF- κ B signalling (Zhou et al., 2010, Fan et al., 2012, Cui et al., 2012). Many DUBs which have been identified to function in the NF- κ B signalling pathway do so by the removal of polyubiquitin chains on key adaptor proteins such as these to maintain tight functioning of NF- κ B activation (Harhaj and Dixit, 2012). As USP4 has been demonstrated to function in the NF- κ B pathway herein this chapter, potentially by acting upstream of the IKK complex, this gives rise to the possibility that USP4 may function by deubiquitinating substrates such as TRAF6, TAK1 or IRAK1. Literature reports TRAF6 and TAK1 as targets of USP4 deubiquitination of K63-linked polyubiquitination (Xiao et al., 2012, Fan et al., 2011b). However, these reports were largely conflicting with data in this thesis. Further, Zhou et al. (2012) reported that USP4 mediated the removal of TRAF6 K48-linked polyubiquitin chains indicating that the mechanism of function of USP4 in NF- κ B signalling may be complex, with multiple substrates of USP4. To gain further insight into the role of USP4 within NF- κ B signalling, identifying potential substrates and determining whether USP4 affects the polyubiquitination of these will therefore be further explored in the next chapter.

4.3.4 Altered levels of IL-1- and TNF α -induced NF- κ B adaptor proteins were not cell-specific

This study utilised the chondrosarcoma SW1353 cell line whereas other studies were carried out using other cell lines including HEK 293T and cervical adenocarcinoma HeLas. To determine whether the effects observed were specific to SW1353 cells, the levels of these key adaptor proteins were also examined in HeLa cells. Similarly to in SW1353 cells, there appeared to be slightly lower levels of IL-1-induced phosphorylated p65, indicating less NF- κ B activation in USP4 depleted HeLa cells, and again a markedly reduced amount of phosphorylated IKK α / β thereby verifying effects seen in this study.

Additionally, there was less TNF α -induced I κ B α degradation and less phosphorylation of IKK α / β . These effects were much clearer in the HeLa cells than in SW1353 cells suggesting that USP4 depletion may have stronger, or different, effects in some cell lines than others.

4.3.5 IL-1-induced NF- κ B activity and NF- κ B-regulated gene expression were reduced in *Usp4*^{-/-} MEFs

Further to this, NF- κ B activity and NF- κ B gene regulation were measured in *Usp4*^{+/+} and *Usp4*^{-/-} MEFs. Null MEFs are a powerful biological tool for exploring the effects of USP4, the loss of which was confirmed by measuring *Usp4* gene expression alongside all gene expression experiments by real-time qRT-PCR. Exactly the same as in USP4 depleted SW1353 cells, IL-1-induced *Il-6* and *Mmp13* were significantly reduced in *Usp4*^{-/-} MEFs whereas there may have been a small but insignificant reduction in *Cxcl1* (*IL-8* mouse homologue) after 6 hours stimulation. These data also tie in with data in the previous chapter which identified reductions in *Il-6* and *Cxcl1* in the serum of *Usp4*^{-/-} mice using a cytokine array. Differing to this, Zhou et al. (2012) reported an increase *IL-6* expression in *Usp4*^{-/-} MEFs, however this was in response to stimulation with LPS which signals through toll-like receptors (TLRs) to activate NF- κ B. Whereas TLRs recognise microbial patterns, such as LPS, IL-1Rs bind IL-1-related cytokines. Both TLR and IL-1 signalling share many of the same mechanisms, and both the TLRs and the IL-1R are characterised by an intracellular toll/IL-1 receptor domain (TIR). Similarly to IL-1-mediated NF- κ B activation through the IL-1R, LPS-mediated NF- κ B activation through TLR-4 involves the recruitment of MyD88, IRAK4 and IRAK1 for IRAK4-mediated phosphorylation, recruitment of TRAF6 and similar downstream signal transduction. However, whereas binding of IL-1 to the IL-1R leads to the formation of a receptor complex with IL-1RAcP and recruitment of MyD88 through TIR domains, LPS-induced recruitment of MyD88 to TLR-4 requires a TIR domain-containing adaptor protein (TIRAP). Moreover, LPS-induced NF- κ B signalling through TLR-4 can occur independently of MyD88, involving TRIF-related adaptor molecule (TRAM), which associates with TRAF6 and RIP1 to induce NF- κ B activation (Verstrepen et al., 2008). The differences in *IL-6* expression in *Usp4*^{-/-} MEFs as reported with LPS induction, and as observed here with IL-1 induction could therefore be due to differences in the signal transduction between TLR- and IL-1-mediated signalling.

In addition to reductions in NF- κ B-regulated gene expression, IL-1-induced NF- κ B activation was reduced in *Usp4*^{-/-} MEFs in a lentiviral NF- κ B reporter assay, confirming data observed in SW1353 cells with siRNA-mediated depletion of USP4.

4.3.6 Summary

The aims of this chapter were to further elucidate a role for USP4 in NF- κ B signalling through examination of the effects of siRNA-mediated USP4 depletion on NF- κ B activation, NF- κ B-regulated gene expression and on the stability, phosphorylation or ubiquitination of key NF- κ B adaptor proteins. Studies in this chapter have demonstrated a role for USP4 in positively regulating NF- κ B activation: IL-1-induced NF- κ B activity and the expression of NF- κ B-regulated genes were reduced in siRNA-mediated USP4 depleted SW1353 cells and in *Usp4*^{-/-} MEFs. In addition, there was diminished phosphorylation of proteins within the pathway, and reduced I κ B α ubiquitination and degradation with USP4 depletion. Taken together, the data in this thesis chapter indicated that loss of USP4 downregulated IL-1-induced NF- κ B signalling.

Conclusions on the role of USP4 in TNF α -induced NF- κ B signalling were not as straightforward. Where TNF α -induced NF- κ B activity was reduced, opposing data on the effect of TNF α -induced NF- κ B-regulated gene expression was observed.

Data in this chapter is opposing to previous reports on the function of USP4 in NF- κ B signalling. Although the reason for this remains unclear, differences in cell lines used and the type and duration of stimulations could possibly account for this. SW1353 cells were predominantly used in these experiments but to determine if effects were cell-specific, the levels of some proteins altered by the loss of USP4 were also examined in HeLa cells. Although, similarly to in SW1353 cells, there were reductions in the IL-1-induced phosphorylation of p65 and IKK α/β , I κ B α was not degraded. Additionally, effects on TNF α -induced protein levels were more dramatic in HeLa cells than in SW1353 cells, indicating that there may be differences in the mechanism of NF- κ B signalling between cell lines and therefore differences with USP4 depletion. The increased NF- κ B activity observed with overexpression of *Usp4* in the previous chapter conflicted with published data, possibly due to mouse *Usp4* rather than human USP4 being overexpressed. However, experiments in this chapter used an RNAi approach to deplete endogenous USP4 in human SW1353 cells and a reduction in NF- κ B activity was observed, in line with the increase in NF- κ B activity with mouse *Usp4*. Additionally, reductions in IL-1-induced NF- κ B activity and NF- κ B-regulated gene expression in USP4 depleted cells were mimicked in *Usp4*^{-/-} MEFs.

Due to more significant and consistent results, focus was given to the role of USP4 in IL-1-induced NF- κ B signalling, whereas the literature mainly focuses on TNF α -induced or LPS-induced NF- κ B signalling. The mechanism of NF- κ B activation differs downstream of IL-1Rs, TNFRs and TLRs as discussed, meaning that USP4 could have different functions in each of these pathways. Thorough examination of the effects of USP4 depletion on aspects of NF- κ B signalling was performed with validation of results by replication of experiments and regular confirmation of the depletion of USP4 which consistently alluded to a role for USP4 as a positive regulator of IL-1-induced NF- κ B signalling.

As IL-1 induced USP4 effects appeared to be the most significant in identifying a possible mechanism for the function of USP4 in NF- κ B signalling, further elucidating how USP4 may function to positively regulate IL-1-induced NF- κ B activation would be interesting and as yet remained undefined. Data in this chapter has indicated USP4 could act upstream of the IKK complex. There are many NF- κ B adaptor proteins upstream of IKK, including IRAK1, TAK1 and TRAF6, which rely on ubiquitination, mainly K63-linked polyubiquitination, for the activation and recruitment of proteins crucial for the activation of the pathway. Additionally, the K48-linked polyubiquitination and degradation of proteins is important in regulating NF- κ B, often by preventing persistent activation of the pathway. The next chapter will try to identify potential substrates of USP4 and elucidate whether USP4, as a DUB, affects the polyubiquitination of these potential targets upstream of IKK in the pathway.

4.3.7 Conclusions

USP4 depletion in SW1353 cells:

- Reduced IL-1- and TNF α -induced NF- κ B activity
- Reduced IL-1-induced NF- κ B-regulated gene expression and in some cases TNF α -induced NF- κ B-regulated gene expression
- Reduced the IL-1 ubiquitination of I κ B α and correspondingly reduced the degradation of I κ B α in SW1353 cells and HeLa cells

- Non-significantly reduced the IL-1-induced phosphorylation of p65 and significantly reduced the phosphorylation of IKK α/β and JNK in SW1353 cells and HeLa cells
- Reduced the TNF α -induced degradation of I κ B α and the phosphorylation of IKK α/β and JNK in HeLa cells

Chapter 5 : Target(s) of USP4 within the NF- κ B Pathway

5.1 Introduction

The previous chapter demonstrated IL-1-induced effects on the levels and phosphorylation of specific NF- κ B adaptor proteins and effects on the expression of NF- κ B-regulated genes with loss of USP4. Together, data alluded to a potential role for USP4 upstream of the IKK complex which will be explored further in this chapter.

Upon binding of IL-1 to the IL-1R, a series of adaptor proteins are recruited for the cascade of signalling events required for the activation of the IKK complex and downstream NF- κ B activation. Many of these events are ubiquitin-dependent and rely on different types of polyubiquitin chains for the recognition and activation of key adaptor proteins (Iwai, 2014). Amongst others, K63-linked polyubiquitin chains feature in the NF- κ B signalling pathway, functioning as scaffolds for the recruitment of a multitude of proteins upstream of the IKK complex. IL-1 induction leads to the phosphorylation of IRAK1 and subsequent recruitment of TRAF6 followed by the TRAF6-mediated K63-linked polyubiquitination of IRAK1, at Lys134/Lys180, and K63-linked self-polyubiquitination of TRAF6, at Lys124, in conjunction with E2 enzyme Ubc13/Uev1A (Lamothe et al., 2007, Conze et al., 2008). These K63 polyubiquitin chains serve to recruit the IKK complex via the NEMO subunit to ubiquitinated IRAK1, and to recruit TAK1 via TAB2 and TAB3 UBDs to ubiquitinated TRAF6 for its activation of TAK1 and the downstream activation of IKK. Furthermore, NEMO also binds to K63-linked polyubiquitin chains on TAK1, the ubiquitination of which is mediated again by TRAF6 upon IL-1 induction for the activation of TAK1 and occurs at Lys158 on TAK1 (Fan et al., 2010). These K63-linked polyubiquitin chains together serve as a platform for recruiting the IKK complex proximally to the activated TAK1, required for the activation of NF- κ B. Equally, these polyubiquitin chains can be specifically removed by a number of DUBs for the negative regulation of NF- κ B and prevention of uncontrolled NF- κ B activity (Harhaj and Dixit, 2011). Two well-studied DUBs with functions in the NF- κ B pathway, A20 and CYLD, deubiquitinate substrates including the K63-linked polyubiquitin chains on TRAF6 for the downregulation of NF- κ B signalling (Heyninck and Beyart, 1999, Trompouki et al., 2003). CYLD has also been shown to remove TAK1 K63-linked polyubiquitin chains for the downregulation of NF- κ B (Reiley et al., 2007). Both A20- and CYLD-deficient mice show enhanced susceptibility to induced

inflammation, highlighting the importance of DUBs in the regulation of NF- κ B signalling (Lee et al., 2000, Zhang et al., 2006). The full spectrum of DUBs involved in the regulation of NF- κ B signalling is not yet fully understood, for example there are no known reports to identify the DUB(s) responsible for the removal of K63-linked polyubiquitin chains on IRAK1.

In addition to the integral step in NF- κ B signalling; the K48-linked polyubiquitination and degradation of I κ B α , degradative K48-linked chains are crucial in the regulation of the pathway at other signalling nodes. The key adaptor protein IRAK1 undergoes IL-1 induced phosphorylation, followed by SCF ^{β -TrCP}-mediated K48-linked polyubiquitination, at Lys134, and is subsequently degraded (Cui et al., 2012). Equally, TRAF6 is subjected to K48-linked polyubiquitination and subsequent degradation, reportedly through interactions with NUMBL (Zhou et al., 2010). Similarly, TAK1 is subject to K48-linked polyubiquitination, at Lys72, for its degradation (Fan et al., 2012). The degradation of TRAF6 or TAK1 leads to inhibition of NF- κ B activation to suppress the pathway, indicating that the negative regulation of TRAF6 and TAK1 can either occur through the removal of K63-linked polyubiquitin chains by DUBs or through the K48-linked polyubiquitination and degradation of TRAF6 and TAK1. There are few studies on the importance of the K48-linked polyubiquitination of some of these key adaptor proteins in the regulation of NF- κ B signalling and, again, limited knowledge on potential DUBs that may remove these K48-linked polyubiquitin chains, furthering the regulation of NF- κ B.

Although K63- and K48-linked polyubiquitin chains are the most well studied chain topologies, others have also been identified in the NF- κ B pathway (Iwai, 2014). The IL-1-induced K63-linked polyubiquitination of IRAK1 induces the formation and covalent attachment of linear chains to the K63 chains by the LUBAC complex. This again serves as a platform for recruitment of the NEMO subunit of the IKK complex which binds to the linear chains (Emmerich et al., 2013).

As a DUB involved in NF- κ B activity, USP4 has been reported to suppress IL-1-induced NF- κ B activity through the deubiquitination of K63-linked polyubiquitin chains of both TAK1 and TRAF6 (Fan et al., 2011b, Xiao et al., 2012). However, data in the previous chapter have alluded to a role for USP4 in positively regulating NF- κ B rather than suppressing its activation and there are currently no known reports to identify substrates

for which USP4 may deubiquitinate for this positive regulation. As data in the previous chapter indicated USP4 may function upstream of the IKK complex, further examination of the effect of USP4 on the ubiquitination of some of these key proteins in the IL-1-induced NF- κ B pathway needs to be explored. Computational modelling was utilised as a predictor of USP4 substrates in IL-1 NF- κ B signalling. Additionally, an RNAi approach to deplete USP4 and examine the functional consequences on the K48- and K63-linked polyubiquitination of TAK1 and TRAF6, through immunoprecipitation and immunoblotting procedures, were also performed.

5.1.1 Aims

- Use computer modelling to identify potential deubiquitination substrates of USP4 in the positive regulation of the NF- κ B pathway
- Confirm the ubiquitination of TAK1 and TRAF6
- Determine the effect of depleting USP4 on the IL-1-induced K48- and K63-linked polyubiquitination of TAK1 and TRAF6

5.2 Results

5.2.1 Computational modelling of USP4 in the IL-1-induced NF- κ B signalling pathway

Data in the previous chapter indicated that USP4 acts as a positive regulator of NF- κ B signalling, potentially upstream of the IKK complex. Therefore, a computational modelling approach was utilised to investigate potential substrates of USP4 in NF- κ B signalling. The majority of DUBs with identified roles in NF- κ B signalling act as negative regulators through the removal of K63-linked polyubiquitin chains (Harhaj and Dixit, 2012). However, the stability of proteins can be mediated through their K48-linked polyubiquitination for proteasomal degradation. Upstream of the IKK complex, key mediators, TAK1, TRAF6 and IRAK1 have all been reported to be subjected to K48-linked polyubiquitination (Fan et al., 2012, Zhou et al., 2010, Cui et al., 2012). Therefore, the removal of these K48-linked polyubiquitin chains on TAK1, TRAF6 or IRAK1 by USP4 were factored into the NF- κ B pathway computer model, as three separate models, and the output of stochastic simulations for each observed. Additionally, as USP4 is reported in the literature to remove TAK1 and TRAF6 K63-linked polyubiquitin chains,

the USP4-mediated removal of these chains was factored into a fourth model. All components and reactions in the model are displayed in Appendix Table 1 and Appendix Table 2.

5.2.1.1 Model assumptions

Under normal conditions, NF- κ B is in complex with I κ B which prevents activation of NF- κ B. IL-1 signalling by the binding of IL-1 to the IL-1R, activates MyD88 and IRAK4 (Martin and Wesche, 2002). Activated IRAK4 enables phosphorylation of IRAK1 and subsequent activation of TRAF6 for TRAF6 K63-linked polyubiquitination and TRAF6-mediated TAK1 K63-linked polyubiquitination (Lamothe et al., 2007). The K63-linked polyubiquitination of TAK1 enables the phosphorylation of IKK, leading to the phosphorylation and degradation of I κ B to release NF- κ B (Fan et al., 2010). NF- κ B is then phosphorylated to activate its transcriptional activity (Sakurai et al., 2003). Included are the transcription and translation of NF- κ B target genes IL-6, IL-8, MMP13 and I κ B. Also included are the K48-linked polyubiquitination of IRAK1, TAK1 or TRAF6, in three separate models, to mediate their degradation (Fan et al., 2012, Zhou et al., 2010). These polyubiquitin chains can be removed by a DUB. For each of the 3 models, the specified DUB was USP4 for IRAK1, TAK1 or TRAF6. In a fourth model, USP4 was the specified DUB for removal of K63-linked polyubiquitin chains on TAK1 and TRAF6 (Fan et al., 2011b, Xiao et al., 2012). A simplified diagram of the model components and reactions is displayed in Figure 5.1. For all four models, simulations were performed with USP4 at control and depleted levels (USP4=100 or USP4=5, respectively).

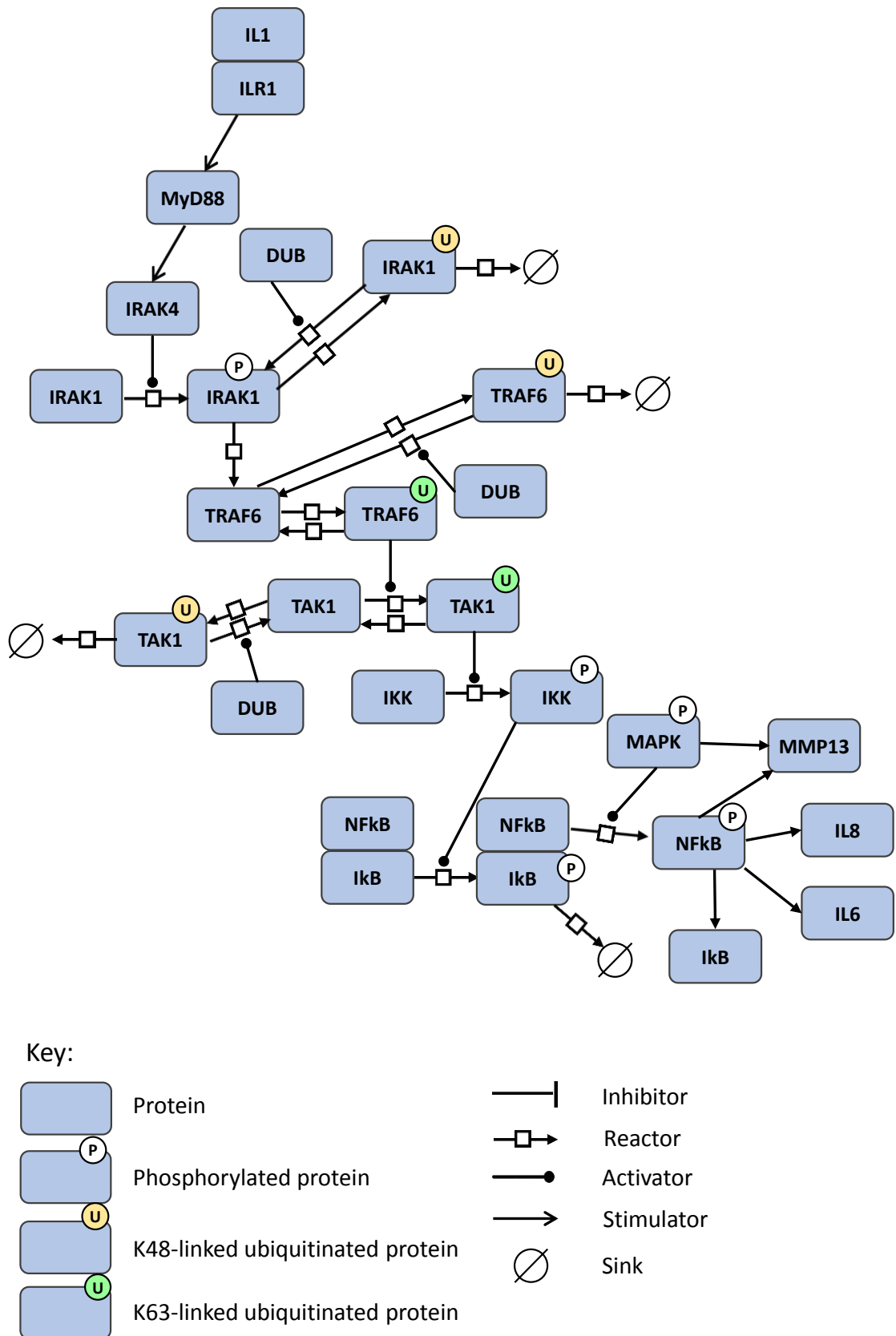


Figure 5.1. IL-1-induced NF-κB signalling pathway model diagram. For each of the three models, DUB was replaced with USP4 for removal of K48-linked polyubiquitin chains on IRAK1, TAK1 or TRAF6. In a fourth model, USP4 was added for the removal of TAK1 and TRAF6 K63-linked polyubiquitin chains. Sink refers to the degradation of a protein. Full details of all reactions are displayed in Appendix Table 2.

5.2.1.2 USP4 in the computational model

In the first model, where USP4 was hypothesised to deubiquitinate K48-linked polyubiquitin chains on IRAK1, the phosphorylation of IKK was reduced when the level of USP4 was reduced, and there were small reductions in the phosphorylation of NF- κ B (Figure 5.2A and Figure 5.2B). Additionally, there were reduced levels of IL-6, IL-8 and MMP13 (Figure 5.2C, Figure 5.2D and Figure 5.2E). This closely matches the levels of phosphorylated IKK and p65 proteins observed in Figure 4.9 and Figure 4.10, and the reductions in NF- κ B regulated gene expression with USP4 depletion as observed in Figure 4.5.

Similarly, in the second model, where USP4 was hypothesised to deubiquitinate K48-linked polyubiquitination on TAK1, the phosphorylation of IKK was reduced when the level of USP4 was reduced, particularly at early time points (Figure 5.3A). There were also small reductions in the level of phosphorylated NF- κ B and reductions in IL-6, IL-8 and MMP13 (Figure 5.3B, Figure 5.3C, Figure 5.3D and Figure 5.3E). Again, this closely matches observations on protein levels and NF- κ B-regulated gene expression observed with USP4 depletion in Chapter 4.

In the third model, where USP4 was hypothesised to deubiquitinate K48-linked polyubiquitination on TRAF6, IL-6, IL-8 and MMP13 were also reduced when reducing the level of USP4 (Figure 5.4C, Figure 5.4D and Figure 5.4E). However, there were no discernible differences in the levels of phosphorylated IKK or NF- κ B (Figure 5.4A and Figure 5.4B).

In the fourth model, where USP4 was hypothesised to deubiquitinate K63-linked polyubiquitination on TAK1 and TRAF6, there were no differences in the levels of phosphorylated IKK or NF- κ B with loss of USP4 (Figure 5.5A and Figure 5.5B). There were moderate increases in IL-6, IL-8 and MMP13 when the level of USP4 was reduced (Figure 5.5C, Figure 5.5D and Figure 5.5E). Although USP4 has been reported to deubiquitinate TAK1 and TRAF6 K63-linked polyubiquitin chains, there was very little impact of USP4 on NF- κ B signalling in this model, and outputs did not match data in this thesis, contrasting with published data (Fan et al., 2011b, Xiao et al., 2012).

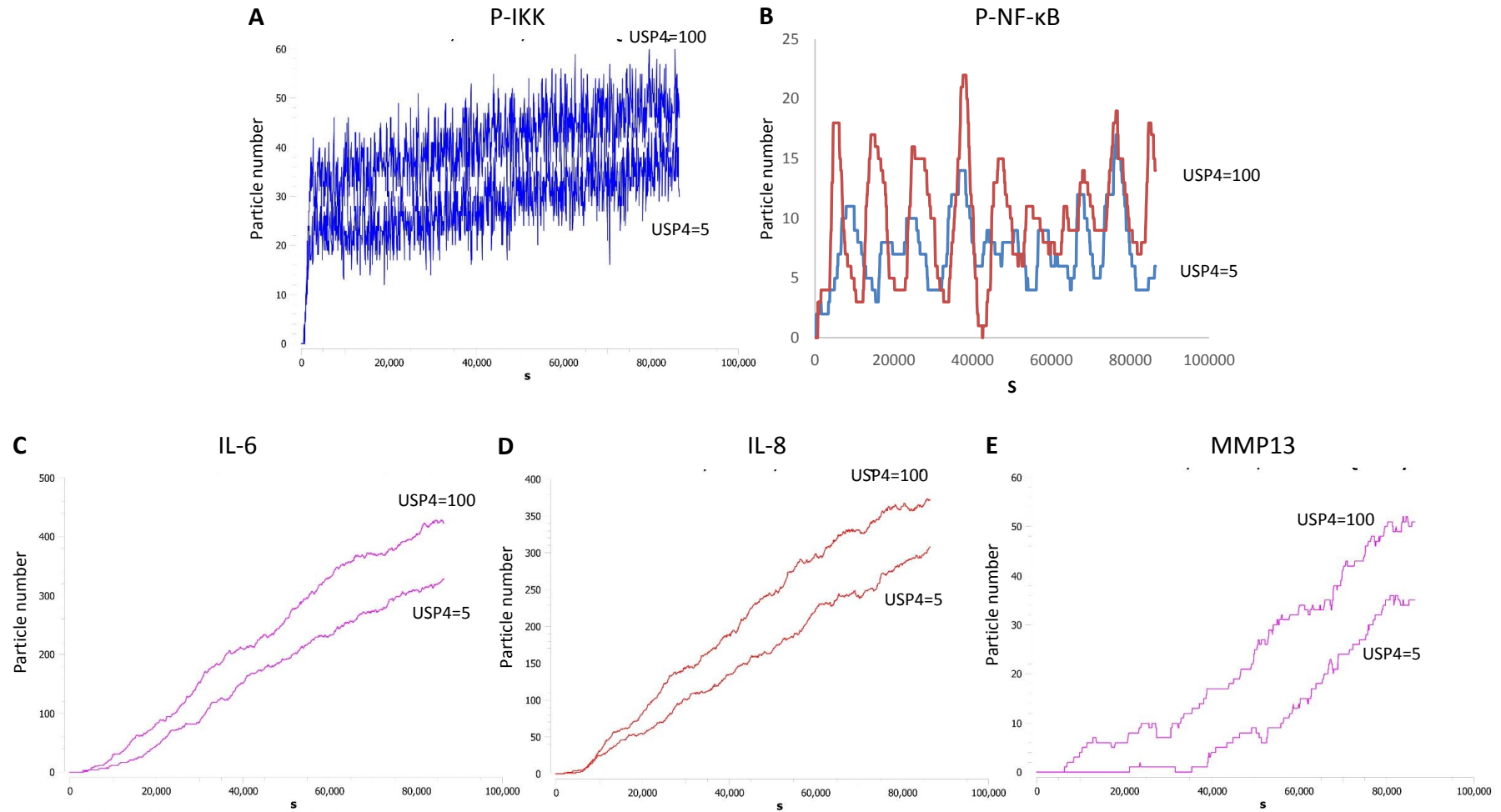


Figure 5.2. Simulation when USP4 deubiquitinates K48-linked polyubiquitinated IRAK1. Simulation output from one stochastic simulation showing changes in A) phosphorylated IKK (P-IKK), B) phosphorylated NF- κ B (P-NF- κ B), C) IL-6, D) IL-8 and E) MMP13 particle numbers. Particle number was plotted over time (seconds, s).

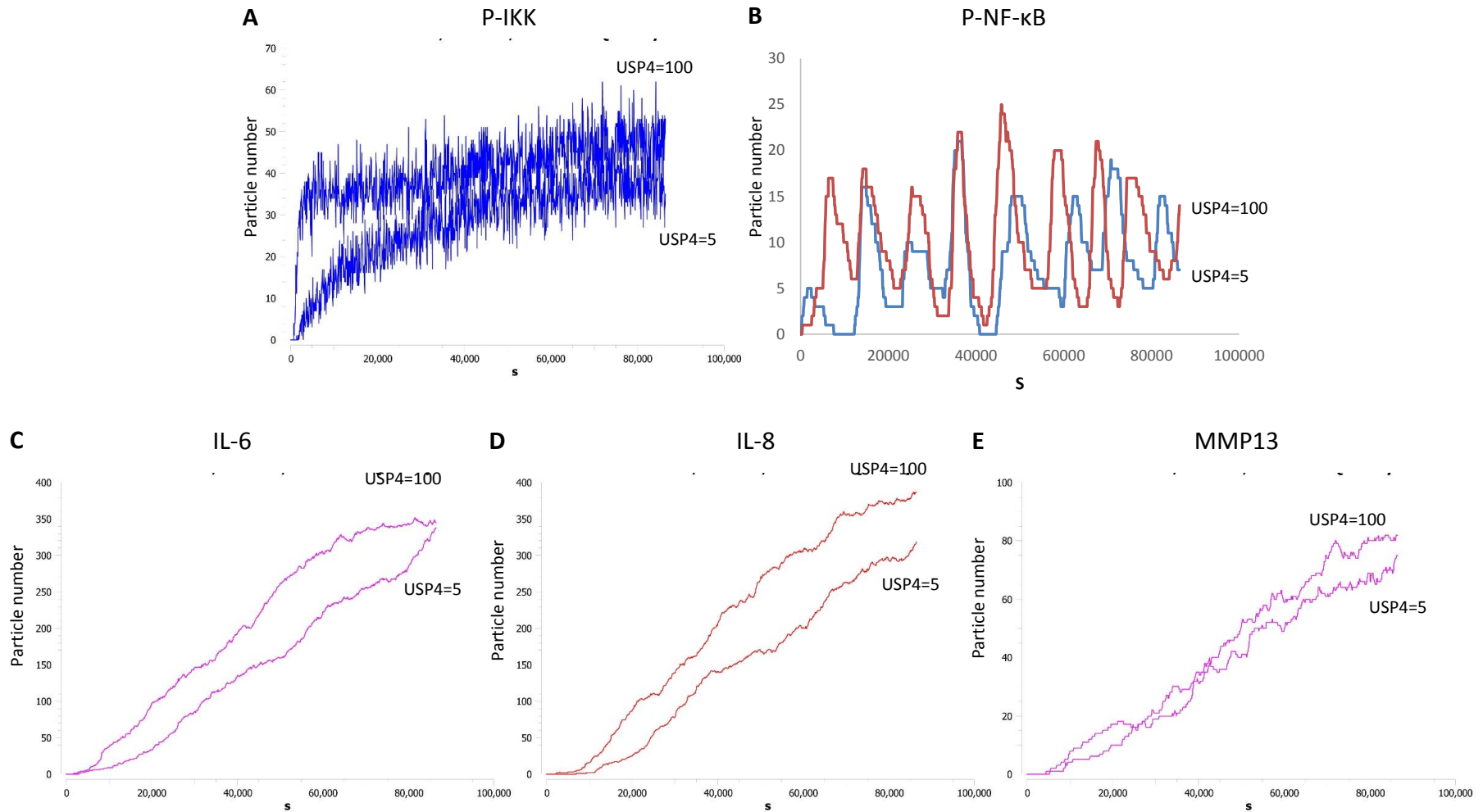


Figure 5.3. Simulation when USP4 deubiquitinates K48-linked polyubiquitinated TAK1. Simulation output from one stochastic simulation showing changes in A) phosphorylated IKK (P-IKK), B) phosphorylated NF- κ B (P-NF- κ B), C) IL-6, D) IL-8 and E) MMP13 particle numbers. Particle number was plotted over time (seconds, s).

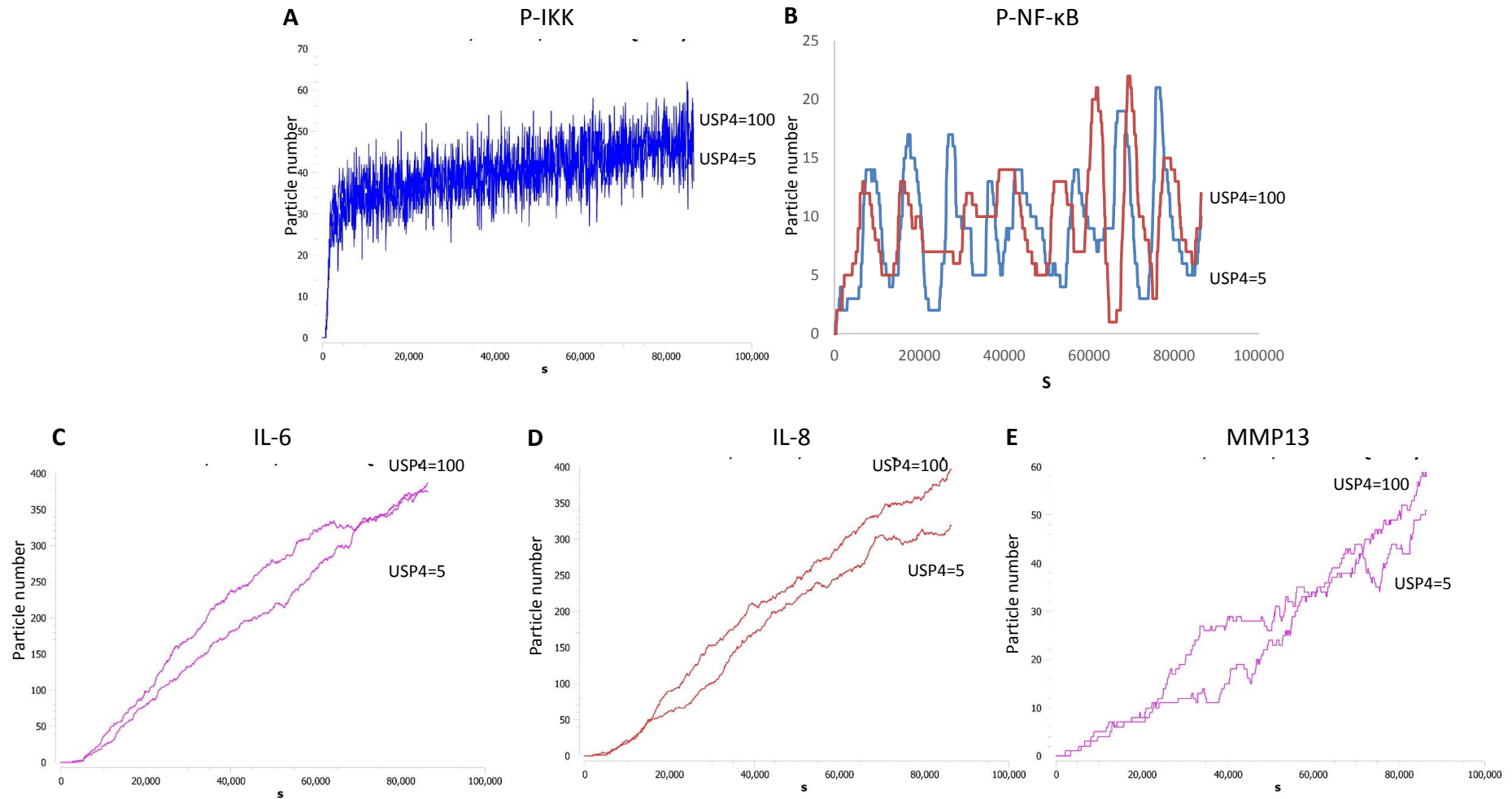


Figure 5.4. Simulation when USP4 deubiquitinates K48-linked polyubiquitinated TRAF6. Simulation output from one stochastic simulation showing changes in A) phosphorylated IKK (P-IKK), B) phosphorylated NF- κ B (P-NF- κ B), C) IL-6, D) IL-8 and E) MMP13 particle numbers. Particle number was plotted over time (seconds, s).

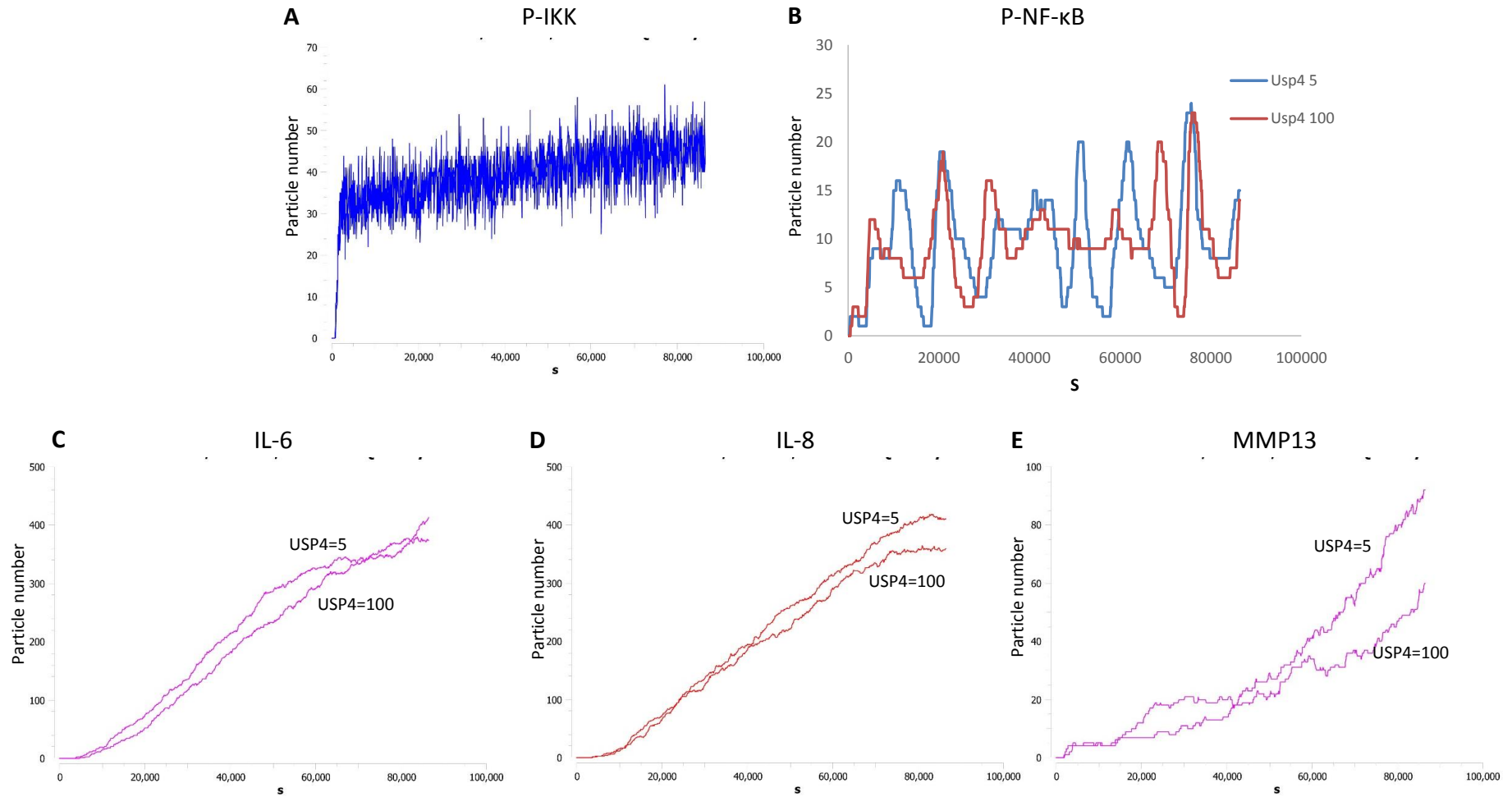


Figure 5.5. Simulation when USP4 deubiquitinates K63-linked polyubiquitinated TAK1 and TRAF6. Simulation output from one stochastic simulation showing changes in A) phosphorylated IKK (P-IKK), B) phosphorylated NF- κ B (P-NF- κ B), C) IL-6, D) IL-8 and E) MMP13 particle numbers. Particle number was plotted over time (seconds, s).

5.2.2 IL-1-induced TAK1 and TRAF6 ubiquitination, K48- and K63-linked polyubiquitination

Computational modelling indicated that if TAK1 was a target of USP4, this could result in reduced NF- κ B-regulated genes, and reduced phosphorylation of IKK and NF- κ B with USP4 depletion, as observed in the previous chapter. Additionally, if TRAF6 was a target of USP4, this could result in reduced levels of NF- κ B-regulated genes. Therefore, as potential USP4 substrates, the IL-1-induced polyubiquitination of TAK1 and TRAF6 with USP4 depletion was examined.

5.2.2.1 TAK1 and TRAF6 are polyubiquitinated

There are many reports on the polyubiquitination of TAK1 and TRAF6 in NF- κ B signalling (Iwai, 2014, Fan et al., 2010, Liang et al., 2013, Conze et al., 2008, Zhou et al., 2010). To confirm that TAK1 and TRAF6 are polyubiquitinated with both K48- and K63-linked polyubiquitin chains and that this could be visualised by the chosen method in this thesis, HEK 293T cells were transfected with either FLAG-TAK1 or FLAG-TRAF6 and either HA-tagged wild-type ubiquitin (HA-Ub WT), a ubiquitin mutant with only K48 (HA-Ub K48) or a ubiquitin mutant with only K63 (HA-Ub K63). All other Lys residues in the ubiquitin mutants had been mutated to arginine. Immunoprecipitation using an anti-FLAG antibody was performed (as optimised and described in section 2.2.7), followed by immunoblotting with the relevant antibodies. FLAG immunoprecipitates, when transfected with either FLAG-TAK1 or FLAG-TRAF6, showed smeared bands with all HA-tagged wild-type and mutant ubiquitin, indicative that FLAG immunoprecipitates, presumably TAK1 and TRAF6, were polyubiquitinated with wild-type ubiquitin and K48 and K63 polyubiquitin chains following IL-1 stimulation (Figure 5.6).

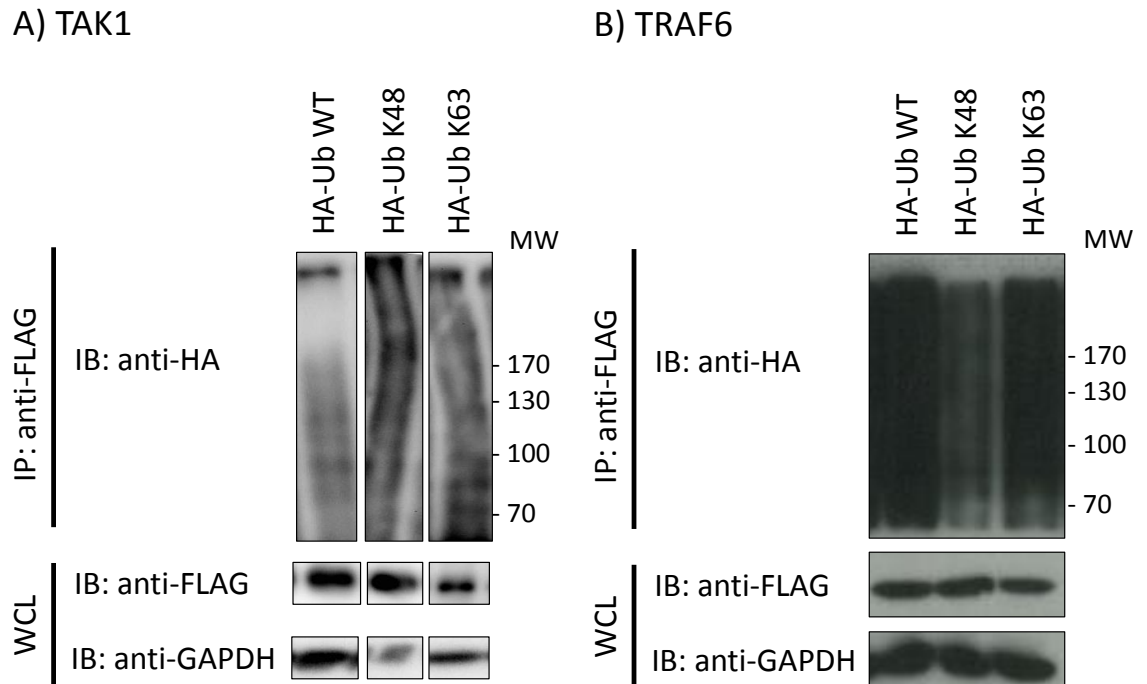


Figure 5.6. FLAG immunoprecipitates were polyubiquitinated following IL-1 induction. HEK 293T cells were transfected with HA-Ub WT, HA-Ub K48 or HA-Ub K63, and either A) FLAG-TAK1 or B) FLAG-TRAF6. At 24 hours post-transfection cells were stimulated with IL-1 α (0.5ng/ml) for 15 minutes before lysis. NEM (20mM) and MG132 (10 μ M) were added to the cell extracts. Total proteins were extracted, resolved by SDS-PAGE and immunoblotted with an anti-FLAG antibody to confirm transfection in whole cell lysates (WCL). GAPDH was used for matching transfection efficiency. Immunoprecipitation (IP) was performed with an anti-FLAG antibody and immunoblotting (IB) with an anti-HA antibody. Molecular weights (MW) (kDa) are indicated.

5.2.2.2 The effect of USP4 depletion on the IL-1-induced polyubiquitination of TAK1

To determine whether USP4 affects the wild-type ubiquitination and both K48- and K63-linked polyubiquitination of TAK1, USP4 was depleted in HEK 293T cells followed by transfection with FLAG-TAK1 and either wild-type or mutant versions of ubiquitin before stimulating with IL-1 and performing immunoprecipitation and immunoblotting experiments.

Initially, the wild-type ubiquitination of TAK1 with USP4 depletion was examined. The ubiquitination of FLAG immunoprecipitates, presumably TAK1, was reduced with USP4 depletion compared to the control. This was particularly evident before stimulation with IL-1 (Figure 5.7A). However, these data were not definitively confirmed upon replication of the experiment.

Next, the effect of USP4 depletion specifically on the K48-linked polyubiquitination of TAK1 was examined. There was no effect on the K48-linked polyubiquitination of FLAG immunoprecipitates with IL-1 induction, neither did the depletion of USP4 have any effect (Figure 5.7B).

Additionally, the effect of USP4 depletion specifically on the K63-linked polyubiquitination of TAK1 was examined. Although, as with K48-linked polyubiquitination, there was no change in the level of K63-linked polyubiquitination of FLAG immunoprecipitates with IL-1 induction, there was less IL-1-induced K63-linked polyubiquitination with depletion of USP4. This indicates that USP4 may be involved in the ubiquitination status of K63-linked polyubiquitination of TAK1 (Figure 5.7C).

Initial experiments included a negative control for which immunoprecipitation was performed using an IgG antibody. As expected, no FLAG or HA was detected when immunoblotting with an anti-FLAG or anti-HA antibodies, respectively (data not shown).

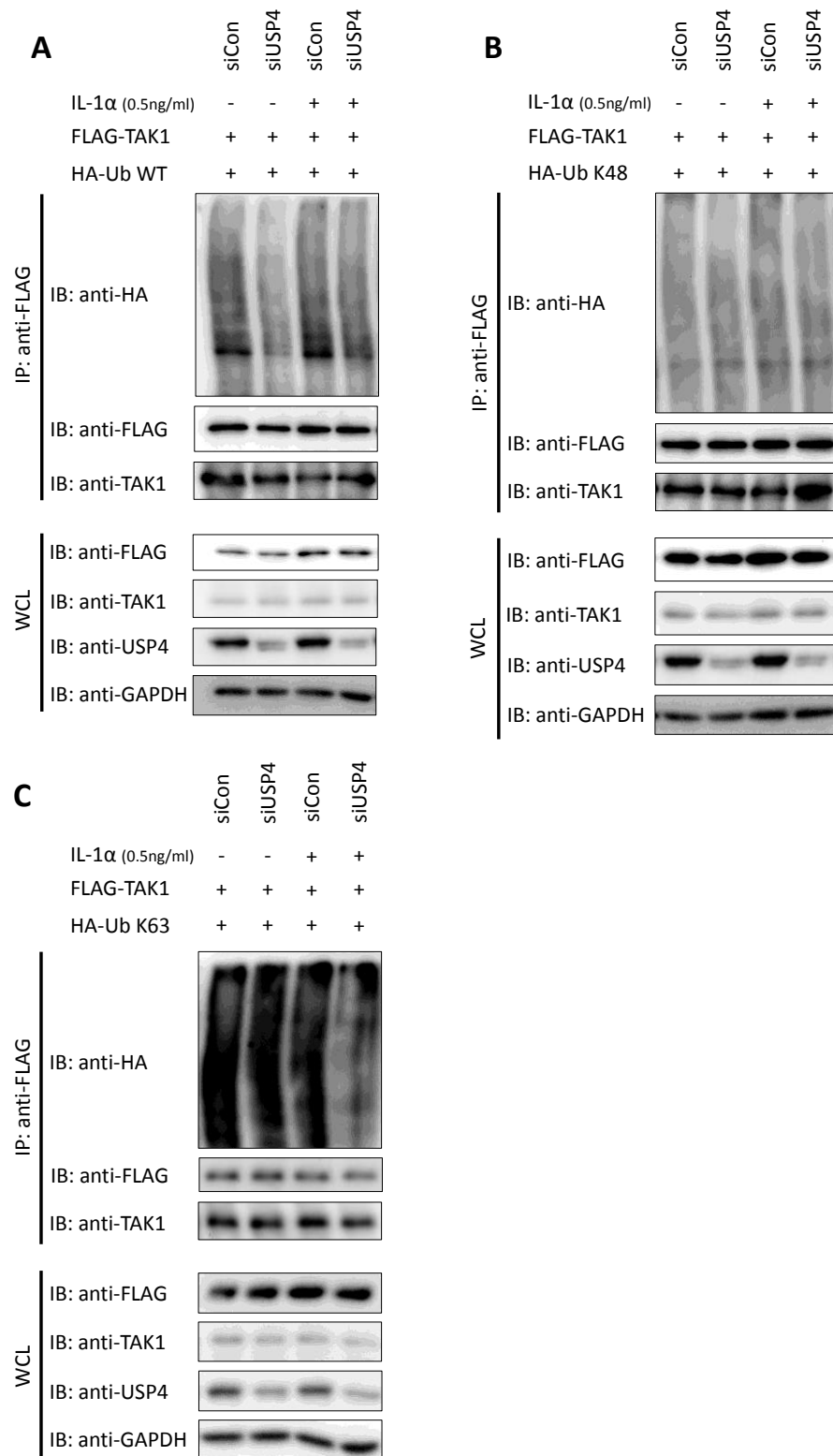


Figure 5.7. IL-1-induced TAK1 ubiquitination with USP4 depletion. HEK 293T cells were transfected with siCon or siUSP4 24 hours before transfection with FLAG-TAK1 and A) HA-Ub WT, B) HA-Ub K48 or C) HA-Ub K63. At 24 hours post-transfection, cells were left unstimulated or stimulated with IL-1 α (0.5ng/ml) for 15 minutes before lysis. Total protein was extracted, resolved by SDS-PAGE and immunoblotted (IB) with anti-FLAG, anti-TAK1, anti-USP4 antibodies to confirm transfection and USP4 depletion in whole cell lysates (WCL). Immunoprecipitation (IP) was then performed using an anti-FLAG antibody and immunoblotting with anti-HA, anti-FLAG and anti-TAK1 antibodies. Figure is representative of three independent experiments for each ubiquitin mutant.

5.2.2.3 The effect of USP4 depletion on the IL-1-induced polyubiquitination of TRAF6

As another key protein which is ubiquitinated in NF- κ B signalling, the polyubiquitination of TRAF6 with USP4 depletion was next examined. Similarly to the examination of TAK1, HEK 293T cells were transfected with FLAG-TRAF6 and HA-tagged wild-type or mutant versions of ubiquitin before stimulating with IL-1 and performing immunoprecipitation and immunoblotting experiments.

First, the wild-type ubiquitination of TRAF6 was examined. The ubiquitination of FLAG immunoprecipitates, presumably TRAF6, was not affected by stimulation with IL-1, nor did depleting USP4 have any effect (Figure 5.8A).

The K48- and K63-linked polyubiquitination of FLAG immunoprecipitates were not affected by stimulation with IL-1 for the control. There was, however, a reduction in the IL-1-induced K48-linked polyubiquitination of FLAG immunoprecipitates with USP4 depletion (Figure 5.8B). The IL-1-induced K63-linked polyubiquitination was also slightly reduced with USP4 depletion (Figure 5.8C).

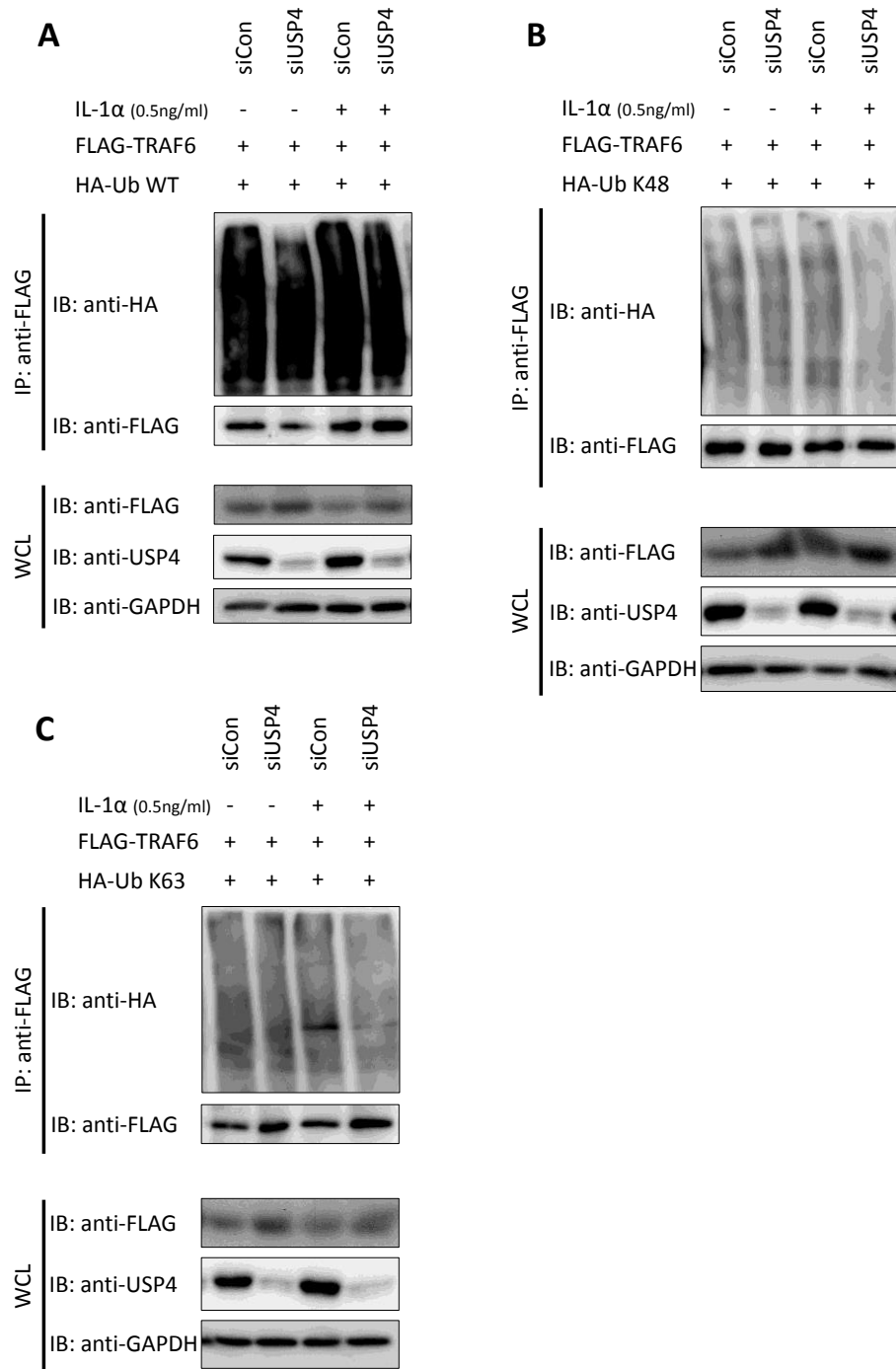


Figure 5.8. IL-1-induced TRAF6 ubiquitination with USP4 depletion. HEK 293T cells were transfected with siCon or siUSP4 24 hours before transfection with FLAG-TRAF6 and A) HA-Ub WT, B) HA-Ub K48 or C) HA-Ub K63. At 24 hours post-transfection, cells were left unstimulated or stimulated with IL-1 α (0.5ng/ml) for 15 minutes before lysis. Total protein was extracted, resolved by SDS-PAGE and immunoblotted (IB) with anti-FLAG or anti-USP4 antibodies to confirm transfection and USP4 depletion in whole cell lysates (WCL). GAPDH was used as a loading control. Immunoprecipitation (IP) was then performed using an anti-FLAG antibody and immunoblotting with anti-HA or anti-FLAG antibodies. Figure is representative of two independent experiments for each ubiquitin mutant.

5.2.2.4 The effect of USP4 depletion on IL-1-induced levels of key NF- κ B adaptor proteins in HEK 293T cells

In this chapter, HEK 293T cells were selected for all immunoprecipitation experiments due to the high transfection efficiency of this cell line. Optimisation experiments, in section 2.2.7.1, demonstrated higher transfection of FLAG-tagged expression plasmids in HEK 293T cells compared to SW1353 cells. However, the data from the previous chapter was from experiments in SW1353 cells. There were some dramatic changes in the levels of key NF- κ B adaptor proteins with USP4 depletion in SW1353 cells and so to determine whether the same effects could be observed in HEK 293T cells, the levels of these key adaptor proteins were also examined in this cell line. After USP4 depletion, HEK 293T cells were treated with IL-1 for the indicated time points, lysed, total protein extracted and resolved by electrophoresis before immunoblotting with the relevant antibodies.

The phosphorylation of p65 was not induced in USP4 depleted cells as strongly as in the control. However, the level of phosphorylation was less after 15, 30 and 60 minutes IL-1 induction with USP4 depletion, similar to observations in SW1353 cells (Figure 5.9). There were no changes in the levels of I κ B α with IL-1 treatment, an observation matching I κ B α levels in HeLa cells in the previous chapter. Again though, both the IL-1-induced phosphorylation of IKK α/β and JNK were reduced with USP4 depletion, however, these effects were not as clear as in SW1353 cells and HeLa cells.

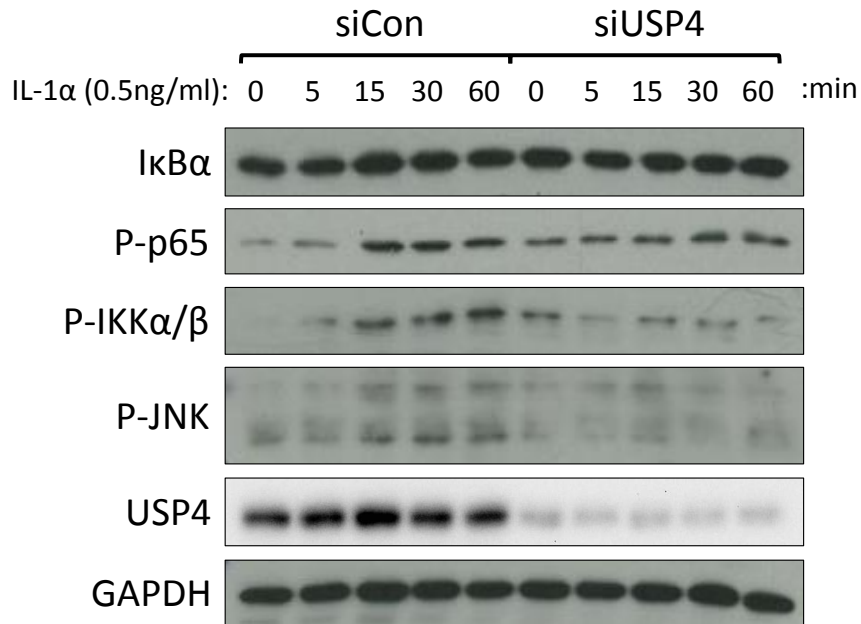


Figure 5.9. The effect of USP4 depletion on levels of NF- κ B adaptor proteins in HEK 293T cells. HEK 293T cells were transfected with siCon or siUSP4 for 24 hours, serum-starved overnight and stimulated with IL-1 α (0.5ng/ml) for the indicated times (minutes). Total protein was extracted, resolved by SDS-PAGE and immunoblotted with anti-I κ B α , anti-P-p65, anti-P-IKK α / β , anti-P-JNK and anti-USP4 antibodies. GAPDH was used as a loading control.

5.2.2.5 The effect on the IL-1-induced polyubiquitination of TAK1 with USP4 depletion in SW1353 cells

As the effects of USP4 depletion on IL-1-induced NF- κ B protein and phosphorylation levels in HEK 293T cells were not as clear as in SW1353 cells in the previous chapter, the effect of USP4 depletion on the IL-1-induced polyubiquitination of TAK1 was next examined in SW1353 cells. SW1353 cells were transfected with FLAG-TAK1 and either wild-type or mutant versions of ubiquitin before stimulating with IL-1 and performing immunoprecipitation and immunoblotting experiments.

Unlike in HEK 293T cells, the overall ubiquitination of FLAG immunoprecipitates, presumably TAK1, was slightly increased with depletion of USP4 but again IL-1 treatment did not affect the polyubiquitination of TAK1 (Figure 5.10A).

Whereas there were no effects of USP4 depletion on the K48-linked polyubiquitination of FLAG immunoprecipitates in HEK 293T cells, USP4 depletion reduced the K48-linked polyubiquitination in SW1353 cells both with and without IL-1 induction (Figure 5.10B).

Again, IL-1 treatment did not alter the K63-linked polyubiquitination of FLAG immunoprecipitates in SW1353 cells. There was a strong band of K63-linked polyubiquitination in control cells stimulated with IL-1 that was reduced with USP4 depletion (Figure 5.10C). Overall this was indicative that the IL-1-induced K63-linked polyubiquitination of TAK1 was reduced with depletion of USP4 in HEK 293T cells and SW1353 cells.

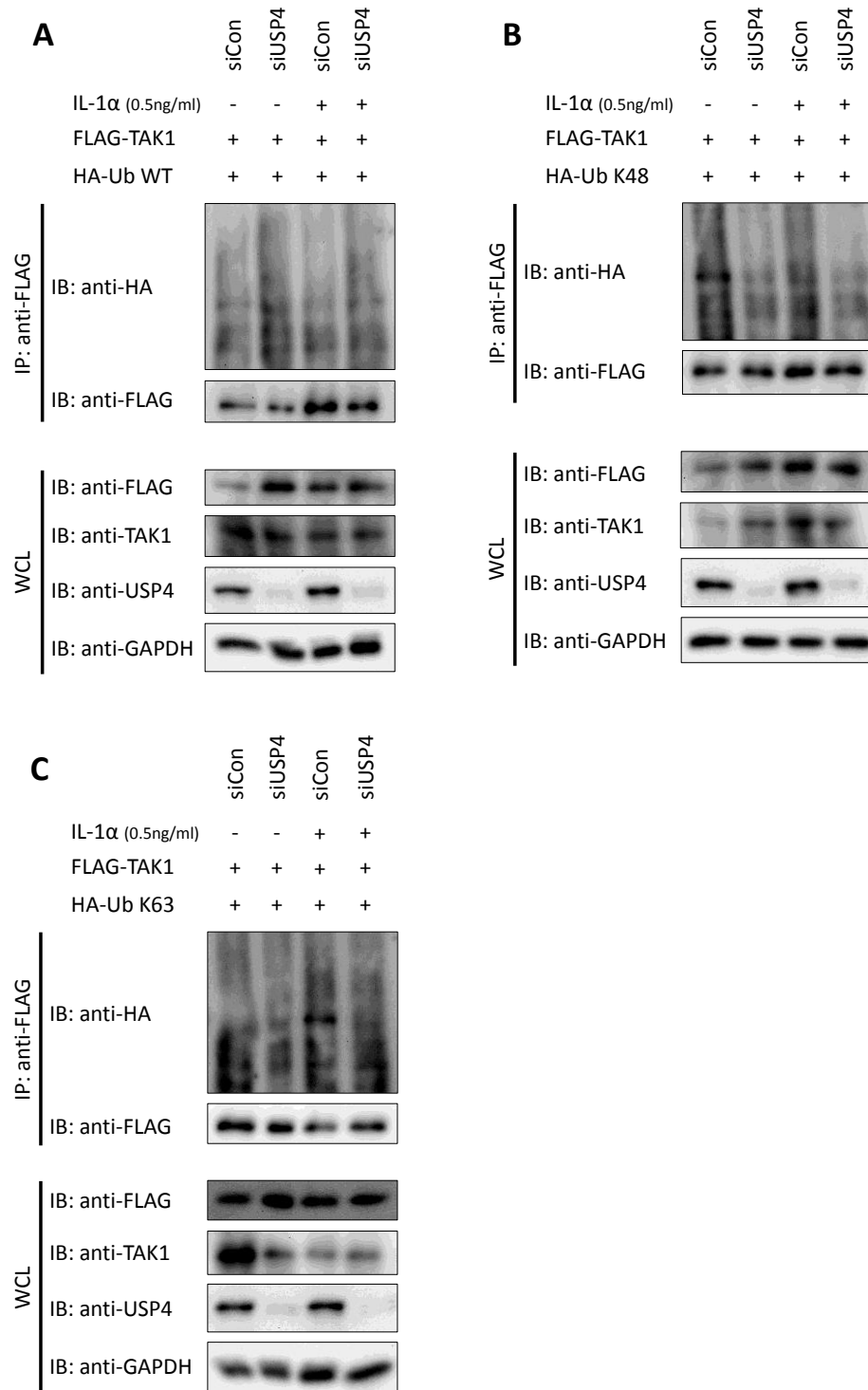


Figure 5.10. IL-1-induced TAK1 ubiquitination with USP4 depletion in SW1353 cells. SW1353 cells were transfected with siCon or siUSP4 24 hours before transfection with FLAG-TAK1 and A) HA-Ub WT, B) HA-Ub K48 or C) HA-Ub K63. At 24 hours post-transfection, cells were left unstimulated or stimulated with IL-1 α (0.5ng/ml) for 15 minutes before lysis. Total protein was extracted, resolved by SDS-PAGE and immunoblotted (IB) with anti-FLAG, anti-TAK1, anti-USP4 antibodies to confirm transfection and USP4 depletion in whole cell lysates (WCL). GAPDH was used as a loading control. Immunoprecipitation (IP) was then performed using an anti-FLAG antibody and immunoblotting with anti-HA and anti-FLAG antibodies.

5.2.3 The effect of USP4 depletion on the IL-1-induced levels of proteins upstream of the IKK complex

The depletion of USP4 potentially affected the K48-linked polyubiquitination of TAK1 and TRAF6. As this ubiquitin linkage type is associated with targeting proteins for proteasomal degradation, the effect of USP4 depletion on the levels of these (and other NF- κ B adaptor proteins upstream of the IKK complex not previously examined) were next observed. The depletion of USP4 did not affect protein levels of TAK1, TRAF2, TRAF6 or IRAK1, indicating that USP4 did not affect the stability of these proteins (Figure 5.11).

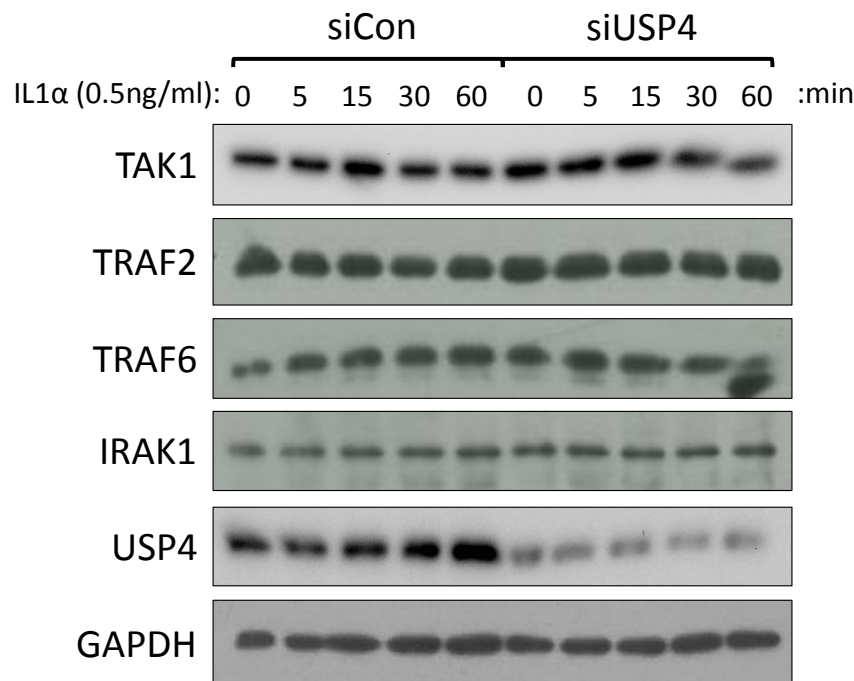


Figure 5.11. The effect of USP4 depletion on levels of NF- κ B adaptor proteins upstream of IKK. SW1353 cells were transfected with siCon or siUSP4 for 24 hours, serum-starved overnight and stimulated with IL-1 α (0.5ng/ml) for the indicated times (minutes). Total protein was extracted, resolved by SDS-PAGE and immunoblotted with anti-TAK1, anti-TRAF2, anti-TRAF6, anti-IRAK1 and anti-USP4 antibodies. GAPDH was used as a loading control.

5.3 Discussion

The aims of this chapter were to examine potential substrates of USP4 through use of computational modelling and to examine the ubiquitination of these potential substrates with loss of USP4.

5.3.1 Computational modelling to determine potential USP4 substrate(s)

Computational modelling is a useful tool for the prediction of interactions with cell signalling networks and was utilised to identify potential targets of USP4 within the NF- κ B signalling pathway. Data within this thesis indicated that USP4 functions upstream of the IKK complex and so the potential effects of USP4 in deubiquitinating proteins in this part of the pathway was examined by hypothesising that USP4 may deubiquitinate K48-linked polyubiquitin chains on TAK1, TRAF6 and IRAK1 to mediate proteasomal degradation. Outputs from stochastic simulations of the model demonstrated a reduction in phosphorylated IKK, phosphorylated NF- κ B and levels of IL-6, IL-8 and MMP13 with reduced USP4 levels when USP4 was hypothesised to deubiquitinate K48-linked polyubiquitination chains on TAK1 and IRAK1. This is consistent with experimental data where USP4 depletion reduced levels of phosphorylated IKK and p65, and expression of *IL-6*, *IL-8* and *MMP13* (Chapter 4). Simulations also demonstrated a reduction in levels of IL-6, IL-8 and MMP13 when USP4 was hypothesised to deubiquitinate TRAF6, but there were no obvious differences in phosphorylated IKK or NF- κ B. USP4 has been reported to interact with both TAK1 and TRAF6 in the NF- κ B signalling pathway and so perhaps further differences would have been observed if USP4 was set to deubiquitinate multiple adaptors in the same model (Xiao et al., 2012, Fan et al., 2011b).

USP4 has been reported to deubiquitinate K63-linked polyubiquitinated substrates TAK1 and TRAF6 to downregulate NF- κ B (Fan et al., 2011b, Xiao et al., 2012). Given this, a fourth model was designed which incorporated USP4-mediated removal of K63 polyubiquitin chains on TAK1 and TRAF6. Data in previous chapters were conflicting with reports that USP4 acts as a negative regulator of NF- κ B signalling, and outputs from stochastic simulations indicated moderate increases in IL-6, IL-8 and MMP13 with reduced USP4, again conflicting with data herein this thesis. Together, data in the previous chapter and model outputs in this chapter indicated that it is unlikely that USP4

targets TAK1 and TRAF6 for K63-linked deubiquitination for the positive regulation of NF- κ B.

Within the IL-1 NF- κ B signalling pathway, phosphorylated IRAK1 undergoes SCF ^{β -TrCP}-mediated K48-linked polyubiquitination and degradation to allow dissociation of TRAF6-TAK1 from the complex with IRAK1 for downstream activation (Cui et al., 2012). This indicates that the degradation of IRAK1 is important to transduce the signal down the pathway for NF- κ B activation. The TRAF6-TAK1 dissociation from IRAK1 was not incorporated into the model utilised in this thesis, and as the degradation of IRAK1 is reported to enhance rather than suppress NF- κ B activation, it seems unlikely that USP4 mediates the removal of these ubiquitin chains as USP4 was demonstrated to positively regulate NF- κ B signalling in the previous chapter. Both TAK1 and TRAF6 have been reported to be polyubiquitinated with K48-linked polyubiquitin chains for proteasomal degradation and the prevention of persistent NF- κ B activation (Fan et al., 2012, Zhou et al., 2010). Outputs from the models hypothesising that USP4 deubiquitinates K48-linked polyubiquitination of TAK1 or TRAF6 fitted with the reduced expression of NF- κ B-regulated genes observed with USP4 depletion. Therefore, it was decided that TAK1 and TRAF6 are more likely targets for deubiquitination by USP4 than IRAK1 and experiments to observe the effects of USP4 depletion on the polyubiquitination of these proteins were performed.

5.3.2 The effect of USP4 depletion on the IL-1-induced ubiquitination of TAK1 and TRAF6

5.3.2.1 TAK1 polyubiquitination

In signalling pathways, K63-linked polyubiquitin chains can serve as a platform for the assembly of protein complexes. It is well documented that TAK1 is polyubiquitinated with K63-linked polyubiquitin chains upon activation of the NF- κ B pathway as a key step for downstream NF- κ B activation (Fan et al., 2010, Liang et al., 2013, Li et al., 2011, Fan et al., 2011a). IL-1-induced K63-linked polyubiquitination of TAK1 by TRAF6 at Lys158 mediates activation of TAK1 and recruitment of the IKK complex for the TAK1-mediated phosphorylation of IKK β required for NF- κ B activation (Fan et al., 2010). Further, NF- κ B can later be downregulated at the level of TAK1 through the K48-linked polyubiquitination of TAK1 and subsequent degradation (Fan et al., 2012). TNF α -induced K48-linked polyubiquitination of TAK1 at Lys72 was reported to occur at a later

time point than the K63-linked polyubiquitination of TAK1 to mediate TAK1 degradation and prevent persistent activation of NF- κ B. There are no known reports to have specifically identified IL-1-induced K48-linked polyubiquitination of TAK1.

As the K63- and K48-linked polyubiquitination of TAK1 play important roles in the activation and downregulation of NF- κ B, respectively, and as USP4 was demonstrated to act as a potential positive regulator of NF- κ B in previous chapters in this thesis, TAK1 was examined as a potential USP4 substrate for deubiquitination. Mutant versions of ubiquitin, for which the only Lys present was K48 or K63, allowed visualisation of these specific ubiquitin topologies. It was first confirmed that, as according to reports, FLAG immunoprecipitates can be polyubiquitinated with K63-linked polyubiquitin chains upon IL-1 stimulation. Additionally, FLAG immunoprecipitates, presumably TAK1, were also polyubiquitinated with K48-linked polyubiquitin chains with IL-1 stimulation, as identified previously with TNF α stimulation (Fan et al., 2012). This is presumably TAK1 polyubiquitination, although other proteins could have been co-immunoprecipitated with TAK1 which are immunoblotted with HA. Therefore, although it can be assumed that the polyubiquitination in these experiments represents the polyubiquitination of TAK1 (or TRAF6 in later experiments) it has not been directly confirmed to be specifically polyubiquitination of TAK1.

Next, the effect of USP4 depletion on the polyubiquitination of TAK1 was examined, initially in HEK 293T cells. FLAG immunoprecipitates were polyubiquitinated with wild-type ubiquitin and K48- and K63-linked polyubiquitin with and without the presence of IL-1. This observation was surprising considering that TAK1 has been documented to be polyubiquitinated upon stimulation of the NF- κ B pathway (Fan et al., 2010, Fan et al., 2012). It would be expected that the ubiquitination should be increased with induction, something not observed here in control cells. The same concentration of IL-1 was used in these experiments as in SW1353 cells in previous experiments, for which elements of the NF- κ B signalling pathway were induced (Chapter 4). HEK 293T cells are smaller in size than SW1353 cells. As such, more cells were seeded onto plates meaning that perhaps a higher concentration of IL-1 needed to be used. However, there was also no effect on the polyubiquitination with IL-1 induction when the experiment was repeated in control SW1353 cells. The observed ubiquitination with no IL-1 induction suggests that overexpressing TAK1 was enough to induce the pathway and its ubiquitination.

Additionally, some differences were observed in USP4 depleted cells with IL-1 induction suggesting that IL-1 induction was successful.

When examining the effects of USP4 depletion on the levels and phosphorylation of NF- κ B adaptor proteins, experiments were performed over a time course. The most apparent differences with USP4 depletion on the levels of proteins, for example I κ B α and phosphorylated p65 and IKK α/β , were observed after 15 minutes of stimulation. This was therefore the time point chosen for IL-1 stimulation in these experiments. As the degradation of I κ B α and the phosphorylation of IKK α/β and p65 occur downstream of TAK1 K63-linked polyubiquitination, it is possible that an earlier time point may have demonstrated more differences. Furthermore, many DUBs counteract the action of E3 ligases by deubiquitinating K63-linked polyubiquitinated proteins to prevent persistent activation of NF- κ B (Harhaj and Dixit, 2012). It is possible that no differences were observed in the polyubiquitination of FLAG immunoprecipitates with IL-1 induction after 15 minutes as the action of DUBs may have resulted in a balanced level of ubiquitination at this time point in control cells. This would also explain why depleting USP4 demonstrated differences with IL-1 induction but not control; depleting USP4 may have disrupted the balance between ubiquitination/deubiquitination within the NF- κ B pathway. It would have been interesting to have been able to perform these experiments over a time course of IL-1 induction to observe whether TAK1 polyubiquitination is altered at different times after induction. The K48-linked polyubiquitination of TAK1 was reported with TNF α induction at a later time point than the K63-linked polyubiquitination of TAK1 (after one hour TNF α stimulation) (Fan et al., 2012). It would therefore be interesting to see if this is the same with IL-1 induction by performing a time course experiment with longer stimulation times.

Overall, loss of USP4 reduced the polyubiquitination of FLAG immunoprecipitates, presumably TAK1, particularly with IL-1 induction. Specifically, K63-linked polyubiquitination was reduced with USP4 depletion and IL-1 induction. This concurs with data in Chapter 4 of this thesis where IL-1-induced NF- κ B signalling was suppressed with depletion of USP4. Previously, it has been reported that USP4 deubiquitinates TAK1 for the downregulation of NF- κ B (Fan et al., 2011b). However, the reported data conflict with data in this thesis which have indicated USP4 positively regulates NF- κ B. Fan et al. (2011b) reported that USP4 interacted with TAK1 and that TNF α -induced polyubiquitination of TAK1 was enhanced with depletion of USP4 and reduced with

overexpression of wild-type, but not catalytically inactive, USP4. Whether these reported changes in TAK1 ubiquitination were due to USP4 deubiquitinating K63- or K48-linked polyubiquitin chains was not determined. The published data are again in contrast with observations in this chapter where USP4 depletion reduced the IL-1-induced overall, and specifically K63-linked, polyubiquitination of FLAG immunoprecipitates, presumably TAK1. TNF α -induced TAK1 polyubiquitination was not examined in this chapter, potentially the differences could be due to the difference between IL-1- and TNF α -induced NF- κ B signalling. However, the previous chapter demonstrated TNF α -induced NF- κ B activity, NF- κ B-regulated gene expression and levels of NF- κ B adaptor proteins, in some cases, were consistent with IL-1, upon USP4 depletion. Additionally, Fan et al. (2011b) reported inhibition of IL-1-induced NF- κ B activity with overexpression of USP4, again inconsistent with data in this thesis.

Given that optimisation of transfection revealed higher transfection efficiency in HEK 293T cells than SW1353 cells, it was the original chosen cell line for immunoprecipitation experiments. Considering previous experiments were predominantly performed in SW1353 cells rather than HEK 293T cells, the effect of USP4 depletion on the IL-1-induced levels of key proteins which were altered in SW1353 cells were examined in HEK 293T cells. Similar to SW1353 cells, extracts from HEK 293T cells demonstrated lower levels of p65 and IKK α/β phosphorylation with loss of USP4, although these effects were not as clear. Therefore, examination of the IL-1-induced polyubiquitination of TAK1 with USP4 depletion was also performed in SW1353 cells via immunoprecipitation. In contrast to HEK 293T cells, the wild-type ubiquitination of FLAG immunoprecipitates, presumably TAK1, was increased with depletion of USP4, suggesting that USP4 may deubiquitinate TAK1 polyubiquitin chains. To determine whether USP4 may be involved in the deubiquitination of specifically K48 or K63 polyubiquitin chains, the K48- and K63-linked polyubiquitination of TAK1 were also examined. Loss of USP4 resulted in a reduction of both IL-1-induced K48- and K63-linked polyubiquitination, indicating that USP4 does not deubiquitinate these TAK1 polyubiquitin chains. The reduction in K63-linked polyubiquitination is in line with the downregulation of IL-1-induced NF- κ B signalling observed in the previous chapter. As the wild-type ubiquitination was enhanced with USP4 depletion, this could point to USP4 deubiquitinating a different TAK1 ubiquitin chain topology. The importance of polyubiquitin chains other than K48- and K63-linked in NF- κ B signalling is only just

beginning to emerge (Iwai, 2014). Similarly to K63-linked chains, linear chains have been reported to serve as a platform for the recruitment of the IKK complex for the activation of NF- κ B. With IL-1 induction, the LUBAC complex attaches linear ubiquitin chains to K63-linked polyubiquitinated IRAK1 which NEMO of the IKK complex binds to, indicating that different ubiquitin topologies can interact for further regulation of NF- κ B signalling (Emmerich et al., 2013). In addition, in TNF α -mediated NF- κ B signalling, RIP1 can be polyubiquitinated with K11 chains by cIAP ligases (Dynek et al., 2010). Although different chain linkages have been reported to be involved in NF- κ B activation, the exact roles played by these and the substrate targets for these ubiquitin chains have not been fully identified. Therefore, USP4 may be involved in the removal of linear, or other ubiquitin chains on TAK1 that have not yet been reported. If TAK1 is ubiquitinated with an unidentified type of polyubiquitin chain, the removal of these may be required before TAK1 can become K63 polyubiquitinated for the downstream activation of NF- κ B. By depleting USP4, the removal of these unknown polyubiquitin chains may be prevented, thus preventing full K63-linked polyubiquitination of TAK1 and K48-linked polyubiquitination of TAK1. Hence reduced polyubiquitin levels would be observed and, further, the suppression of NF- κ B activation. Similar to the immunoprecipitation experiments performed here, it would be interesting to determine whether TAK1 is subject to ubiquitination via other linkage topologies and whether the depletion of USP4 affects this.

Moreover, through replacement of ubiquitin with a K63 mutant ubiquitin, it has been demonstrated that K63 polyubiquitination is required for IL-1-induced IKK activation, but not TNF α (Xu et al., 2009b). In the previous chapter, there were clearer effects on IL-1-induced NF- κ B signalling than TNF α -induced NF- κ B signalling with USP4 depletion. If USP4 is involved in enabling K63-linked polyubiquitination of substrates involved in NF- κ B, this may not be a requirement for TNF α -mediated signalling and could somewhat explain the differences in effects between TNF α - and IL-1-induced NF- κ B signalling.

Another possibility for the reduction of K63- and K48-linked polyubiquitination could be due to interactions of USP4 with another protein upstream of TAK1, rather than direct interaction with TAK1. Other reported TNF α -induced protein-protein interactions include IKK β and TRAF6 with USP4, and so it may be that changes in the polyubiquitination of TAK1 with USP4 depletion is a result of interaction with a different protein within the NF- κ B pathway (Fan et al., 2011b). Alternatively, many E3 ligases

exist in complexes with DUBs for the tight control of the ubiquitination/deubiquitination of proteins. For example, USP7 binds to p53 ligase Mdm2 for the tight control of p53 stability (Li et al., 2004). USP4 could therefore be interacting with a TAK1 E3 ligase for the regulation of its polyubiquitination. Upstream of TAK1 is TRAF6 which is itself polyubiquitinated and also acts as a TAK1 E3 ligase. As such, TRAF6 could be a potential substrate of USP4.

Co-immunoprecipitation experiments to determine whether IL-1 induces a specific interaction of USP4 with TAK1, as was reported with TNF α induction previously, or other known NF- κ B adaptor proteins upstream of the IKK complex, would be informative. Immunoprecipitating USP4 would allow examination of potential interactions with proteins upstream of the IKK complex and/or E3 ligases which USP4 may potential form a complex with to mediate effects on the IL-1-induced polyubiquitination of proteins.

5.3.2.2 TRAF6 polyubiquitination

TRAF6 is a key adaptor protein in the NF- κ B signalling pathway, for which its ubiquitination is essential for the regulation of this pathway. IL-1-induced self-ubiquitination of TRAF6 with K63-linked polyubiquitin chains serves as a platform for the recruitment of TAK1 via the UBDs of TAB2 and TAB3 (Xia et al., 2009, Lamothe et al., 2007). DUBs have been identified which can remove this K63 polyubiquitination of TRAF6 for downregulation of the NF- κ B pathway including CYLD (Trompouki et al., 2003). Additionally, the NF- κ B pathway can be downregulated by the K48-linked polyubiquitination and degradation of TRAF6 (Zhou et al., 2010). Moreover, TRAF6 acts as an E3 ligase for self-ubiquitination and the K63-linked polyubiquitination of TAK1 (Lamothe et al., 2007, Fan et al., 2010). Due to the importance of TRAF6 in ubiquitin signalling in the NF- κ B pathway, and given that TRAF6 has been shown to directly associate with USP4, the effect of IL-1-induced polyubiquitination of TRAF6 with USP4 depletion was also examined (Xiao et al., 2012).

It was first confirmed that FLAG immunoprecipitates, presumably TRAF6, can be polyubiquitinated with K48- and K63-linked polyubiquitin chains upon IL-1 stimulation. Next, examination of whether USP4 affects this polyubiquitination revealed that the IL-1-induced wild-type ubiquitination was not affected by the loss of USP4 but both IL-1-induced K48- and K63-linked polyubiquitination were reduced with loss of USP4. This

is in contrast with reports that USP4 directly deubiquitinates K48- and K63-linked polyubiquitinated TRAF6 (Xiao et al., 2012, Zhou et al., 2012). Xiao et al. (2012) reported that overexpression of USP4, but not catalytically inactive USP4, reduced the ubiquitination of TRAF6 in HEK 293T cells under basal conditions and, equally, the siRNA-mediated depletion of USP4 enhanced the ubiquitination of TRAF6. This is not in line with data in this chapter for which the ubiquitination of FLAG immunoprecipitates, presumably TRAF6, was unaffected with USP4 depletion at basal levels in HEK 293T cells. Zhao et al. (2012a) reported that overexpression of USP4, but not catalytically inactive USP4, reduced the wild-type, K48- and K63-linked polyubiquitination of TRAF6 in HEK 293T cells using K48 and K63 ubiquitin mutants. They also reported that USP4, but not catalytically inactive USP4, reduced the IL-1-induced TRAF6 K63-linked polyubiquitination in HeLa cells, and that shRNA-mediated depletion of USP4 enhanced the basal and IL-1-induced TRAF6 K63-linked polyubiquitination in HeLa cells and macrophages. Again, this is in contrast with data here, where IL-1-induced K63-linked polyubiquitination was reduced with USP4 depletion. It would have been interesting to examine here whether overexpression of wild-type and catalytically inactive Usp4 would affect polyubiquitination and whether this would also have been contradictory to the literature. Given that experiments were previously predominantly performed in SW1353 cells, if time allowed it would have been potentially beneficial to also examine the effects of IL-1-induced TRAF6 polyubiquitination with USP4 depletion in this cell line, in addition to HEK 293T cells.

As with reductions observed herein this chapter in the K63-linked polyubiquitination of FLAG immunoprecipitates, presumably TAK1, with USP4 depletion, the reduction in the K63-linked polyubiquitination of FLAG immunoprecipitates, presumably TRAF6, also concurs with the downregulation in NF- κ B signalling as observed in Chapter 4. Again, this could potentially be due to interactions with either a protein upstream of TRAF6 or due to interactions with an E3 ligase responsible for the ubiquitination of TRAF6.

5.3.2.3 Other potential substrates of USP4

Computational modelling indicated that if USP4 removes K48-linked polyubiquitin chains from IRAK1, this could result in outputs matching data to implicate USP4 is involved in positively regulating NF- κ B signalling. As literature have reported that the K48-linked polyubiquitination and degradation of IRAK1 allows TAK1-TRAF6 to dissociate from IRAK1 and further the signal for activating NF- κ B, it was decided that

this was an unlikely target for USP4 (Cui et al., 2012). As the ubiquitination of TRAF6 and TAK1 was potentially affected by USP4 depletion, but not indicative that USP4 directly deubiquitinated either of these proteins, it is possible that USP4 acts upstream of this part of the pathway. As IRAK1 is known to be not only K63- and K48-polyubiquitinated but also linearly ubiquitinated, it would be interesting to observe whether USP4 affects any of these types of ubiquitination (Conze et al., 2008, Cui et al., 2012, Emmerich et al., 2013).

Although TRAF6 is reported to self-ubiquitinate with K63 polyubiquitin chains, other ligases able to catalyse TRAF6 K63-linked polyubiquitination include; the U-box E3 ligase, Act1, within IL-17-mediated signalling pathways, including NF- κ B, and the RING E3 ligase, tripartite motif (TRIM) 38 (Liu et al., 2009, Zhao et al., 2012b). This gives rise to the possibility that, in addition to self-ubiquitination, other E3 ligases may be responsible for the K63-linked polyubiquitination of TRAF6. Equally, some E3 ligases which mediate the K48-linked polyubiquitination for the proteasomal degradation of TRAF6 have been identified including; WW domain containing E3 ubiquitin protein ligase 1 (WWP1) and TRIM38 in TLR signalling, and NUMBL (Lin et al., 2013, Zhao et al., 2012b, Zhou et al., 2010). As mentioned, E3 ligases can exist in complex with DUBs, presumably for controlled regulation of the ubiquitination/deubiquitination of proteins. Within the NF- κ B signalling pathway, the DUB CYLD has been documented to downregulate NF- κ B activation through the deubiquitination of K63-linked polyubiquitin chains (Trompouki et al., 2003). Additionally, CYLD can interact with the E3 ligase Itch and form a ubiquitin-editing complex for the removal of Tak1 K63-linked ubiquitination and catalysis of Tak1 K48-linked ubiquitination for proteasomal degradation (Ahmed et al., 2011). Similarly, USP4 may form a complex with an E3 ligase, perhaps TRAF6 itself, to tightly regulate the ubiquitination/deubiquitination of TRAF6.

Ro52 (also known as TRIM21) is a RING E3 ligase able to monoubiquitinate phosphorylated IKK β for the downregulation of NF- κ B signalling (Wada et al., 2009). Interestingly, USP4 and Ro52 interact to form a transregulatory protein complex in which USP4 is able to deubiquitinate self-ubiquitinated Ro52, and Ro52 is able to monoubiquitinate USP4 and USP4 potentially removes this Ro52-mediated ubiquitination (Wada et al., 2006, Wada and Kamitani, 2006b). Given that Ro52 is involved in the regulation of the NF- κ B pathway, it would be interesting to investigate further whether the function of USP4 in the positive regulation of NF- κ B is mediated by

interactions with Ro52 and whether this ubiquitinating/deubiquitinating complex is important in the regulation of the NF- κ B signalling pathway.

As discussed, although it was presumed that the polyubiquitination observed in these experiments was of TAK1 or TRAF6, other proteins may have been co-immunoprecipitated with TAK1 or TRAF6. The polyubiquitination observed may therefore also represent polyubiquitination of other proteins which were co-immunoprecipitated. This further indicates the importance of determining protein-protein interactions upstream of the IKK complex for USP4 and also for TAK1 and TRAF6 through co-immunoprecipitation experiments.

5.3.2.4 The effect of USP4 depletion on the levels of NF- κ B adaptor proteins upstream of the IKK complex

Although the substrate(s) of USP4 within the NF- κ B signalling pathway have not been fully determined from data here, there were potential effects on the IL-1-induced K48-linked polyubiquitination of TAK1 and TRAF6 with USP4 depletion. Therefore, the levels of proteins upstream of the IKK complex, including TAK1 and TRAF6, were examined to determine any effects of USP4 depletion on the stability of these proteins. Neither the levels of TAK1, TRAF2, TRAF6 nor IRAK1 were altered with loss of USP4 across a time-course of IL-1-induction. This indicates that USP4 does not affect the stability of these proteins. However, timepoints chosen were based on those from experiments performed in Chapter 4. Studies reporting the proteasomal degradation of TAK1 and TRAF6 have indicated that the K48-linked polyubiquitination and subsequent degradation of these proteins occurs after their K63-linked polyubiquitination as a measure of downregulating the pathway to prevent persistent activation. For example, TNF α -induced degradation of TAK1 occurred after one hour of treatment (Fan et al., 2012). Therefore, examination of protein levels at later time points may be more beneficial in determining potential effects of USP4 on the stability of these proteins. Additionally, the deubiquitination of these adaptor proteins could alter their cellular distribution. As such, it would be interesting to examine the levels of these proteins in nuclear and cytoplasmic extracts in addition to the whole cell extracts examined here.

5.3.3 Summary

Ubiquitin signalling is crucial in the regulation of the NF- κ B signalling pathway. As USP4 is a DUB with a role in the positive regulation of IL-1-induced NF- κ B signalling,

as demonstrated in this thesis, potential substrates within this pathway were explored in this chapter. As the majority of DUBs with known roles in NF- κ B signalling function to negatively regulate the pathway through the removal of K63-linked polyubiquitin chains, it was difficult to determine how USP4 may function to positively regulate NF- κ B signalling. Computational modelling predicted that if USP4 was deubiquitinating K48-linked chains, rather than K63-linked chains, on TAK1 and TRAF6, outputs consistent with suppression of NF- κ B signalling with USP4 depletion could be observed. Upon examination of the ubiquitination of TAK1 and TRAF6, it was demonstrated that loss of USP4 led to potentially reduced IL-1-induced K48- and K63-linked polyubiquitination of TAK1 and TRAF6. Although not indicative of direct deubiquitination of these proteins, but rather indicative of enhanced ubiquitination or reduced deubiquitination, it is possible that USP4 may interact with another protein upstream of TAK1 and TRAF6 or may regulate an E3 ligase responsible for ubiquitinating them. Alternatively, USP4 may deubiquitinate a different type of ubiquitin chain, as yet unidentified. USP4 has been shown previously to have a higher specificity for the removal of linear ubiquitin chains, than either K48- or K63-linked chains (McGouran et al., 2013). Linear chains have known involvement in the regulation of IL-1-induced NF- κ B signalling, and so it is possible that USP4 may target this type of chain. Further examination to determine the substrate(s) of USP4, and of the effect of USP4 depletion on chains other than K48- and K63-linked chains would provide further insight into where and how USP4 functions to positively regulate IL-1-induced NF- κ B signalling.

5.3.4 Conclusions

- Computational modelling predicted that removal of TAK1 or TRAF6 K48-linked polyubiquitin chains by USP4 could produce outputs consistent with USP4 as a positive regulator of IL-1-induced NF- κ B signalling
- FLAG immunoprecipitates, presumably TAK1 and TRAF6, were ubiquitinated with both K48- and K63-linked polyubiquitin chains
- Depleting USP4 potentially reduced the IL-1-induced K48- and K63-linked polyubiquitination of TAK1 and TRAF6

Chapter 6 : USP4 Phosphorylation and NF- κ B Signalling

6.1 Introduction

USP4 has many known substrates, including PDK1, A_{2A} adenosine receptor and spliceosome components (Uras et al., 2012, Milojević et al., 2006, Song et al., 2010). It is also involved in the regulation of many cell signalling pathways, including p53, TGF- β , NF- κ B pathways, and in cellular processes including neuronal morphogenesis (Zhang et al., 2011, Zhang et al., 2012, Fan et al., 2011b, Anckar and Bonni, 2015). However, the mechanisms by which USP4 is regulated itself are largely unknown.

PTMs regulate the function of a wide array of proteins. The phosphorylation of proteins is one of the most well-studied PTMs and plays a critical role in the regulation of many cell signalling pathways: MAPK pathways are based on signalling through a series of phosphorylation events: ERK, p38 and JNK MAPKs require activation by a series of other kinases leading to a cascade of phosphorylations and the transcriptional activation of target genes (Kim and Choi, 2010). Additionally, NF- κ B signalling is regulated by phosphorylation events: the key step in the activation of NF- κ B relies on the phosphorylation of I κ B to mediate its ubiquitination and degradation for NF- κ B release.

As well as regulating protein activity and stability, PTMs can regulate protein-protein interactions and the subcellular localisation of proteins and many DUBs are regulated in this way. The phosphorylation of USP1 facilitates the protein interaction with UAF1 to mediate its catalytic activity, and the phosphorylation of USP10 facilitates its translocation to the nucleus to allow for the deubiquitination of nuclear p53 (Villamil et al., 2012, Yuan et al., 2010).

When this project first began there were no reports on how PTMs may regulate the function of USP4. Recently, it was revealed that the AKT-mediated phosphorylation of USP4 at Ser445 results in its translocation from the nucleus to the cytoplasm and plasma membrane. This allows USP4 to interact with T β R-I at the plasma membrane resulting in the deubiquitination and stabilisation of the receptor for the downstream activation of the TGF- β pathway (Zhang et al., 2012). This mechanism regulates the subcellular localisation of USP4 in the TGF- β signalling pathway, but how USP4 might be regulated

by PTMs for other functions of USP4 and in other cell signalling pathways has still not been reported.

In addition to phosphorylation at Ser445, other residues which can be modified by PTMs have been mapped in high-throughput and proteomic studies which may also regulate the function of USP4 (PhosphoSitePlus™: www.phosphosite.org). These phosphorylation sites and other USP4 modifications are depicted in Figure 6.1. Thin-layer chromatography of isotope-labelled USP4 identified USP4 was serine phosphorylated. This was mapped to two serines located at Ser675 and Ser680 by proteomics by Dr Doug Gray in collaboration with the Ottawa Institute for Systems Biology (Figure 6.1). In parallel to experiments to examine the functional consequences of USP4 depletion on NF-κB signalling in the previous chapters, the function of the phosphorylation of USP4 at these two sites was examined, with particular focus on NF-κB signalling.

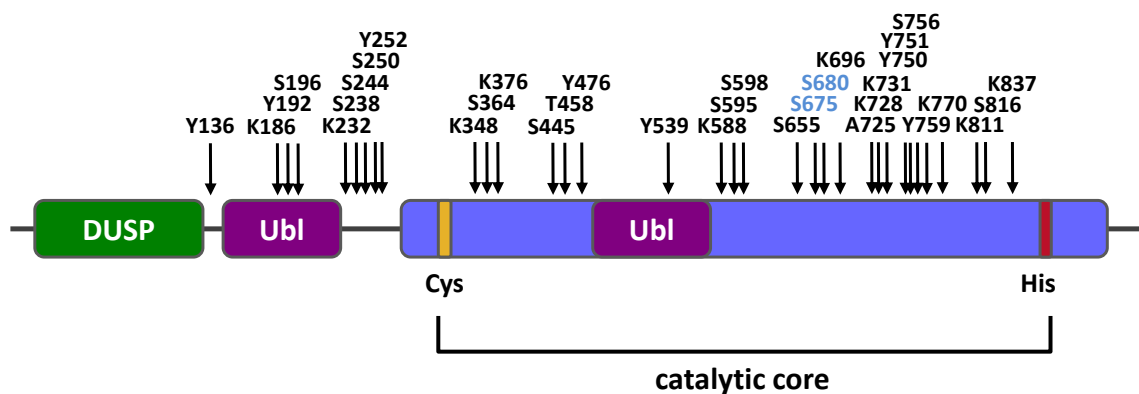


Figure 6.1. USP4 modification sites. Diagram indicating the PTMs of human USP4. Serines 675 and 680 are highlighted in blue. Adapted from PhosphoSitePlus™: www.phosphosite.org.

First, a readout of function of USP4 was needed to study these PTMs. Previous chapters in this thesis identified a function for USP4 in the NF- κ B signalling pathway allowing the effect of Usp4 phosphorylation in NF- κ B signalling to next be examined. To establish a useful method for addressing the effects of Usp4 PTMs at Ser675/Ser680, a range of phosphorylation and phosphomimetic mutants were generated to enable examination of Usp4 phosphorylation on its subcellular localisation and effects on the activity of cell signalling pathways, specifically the NF- κ B pathway. Additionally, RNAi knockdown of an identified kinase for USP4 allowed examination on the effect of this in NF- κ B signalling.

6.1.1 Aims

- Determine the effect of overexpressing a non-phosphorylatable version of Usp4 on its subcellular localisation
- Determine whether Usp4 phosphorylation is involved in regulating cell signalling pathways
- Confirm the phosphorylation status of Usp4 phosphorylation variants
- Determine the effect of overexpressing a non-phosphorylatable version of Usp4 on IL-1-induced NF- κ B activation
- Generate a range of phosphorylation variants and lentiviral phosphorylation variants
- Examine the effect of Usp4 PTM on the IL-1-induced NF- κ B activation in SW1353 cells and MEFs
- Examine the effect of siRNA-mediated depletion of the kinase GRK2 on NF- κ B signalling

6.2 Results

6.2.1 Usp4 phosphorylation at Ser675/Ser680 does not affect subcellular localisation

USP4 has been located in both the nucleus and the cytoplasm, displaying nucleocytoplasmic shuttling properties (Soboleva et al., 2005). Both NLS and NES motifs have also been identified. The localisation of USP4 differs between cell types and the regulation of its subcellular localisation may be crucial to its function. Therefore, this chapter aimed to explore effects of these PTMs and the effect on the subcellular localisation of USP4 was first examined. After experiments to examine the effects of modification at Ser675/Ser680 were performed in this thesis, Zhang et al. (2012) reported that the phosphorylation of USP4 at Ser445 is important for its translocation as part of its function within the TGF- β signalling pathway. When beginning this thesis, two Ser residues at 675 and 680 were novel phosphorylation sites.

Mouse NIH3T3 fibroblasts were transfected with GFP-tagged Usp4 wild-type (Usp4 WT), a mutant version of Usp4 for which Ser675 and Ser680 were mutated to alanine (Ala) rendering it non-phosphorylatable at these sites (Usp4 S675A/S680A), or a catalytically inactive version of Usp4 (C311A). Cells were fixed and mounted for visualisation by confocal fluorescent microscopy and analysed by scoring GFP fluorescence (and so Usp4) as being predominantly nuclear, both nuclear and cytoplasmic or predominantly cytoplasmic (Figure 6.2A).

In NIH3T3 cells, Usp4 WT was predominantly cytoplasmic in approximately 65% of cells, predominantly nuclear in approximately 15% of cells and was observed throughout the cell in approximately 20% cells (Figure 6.2B). There were no significant differences between the localisation of Usp4 WT, Usp4 C311A or Usp4 S675A/S680A, indicating that neither the catalytic activity of Usp4 nor the phosphorylation at Ser675/680 regulates the subcellular localisation of Usp4 under the conditions investigated.

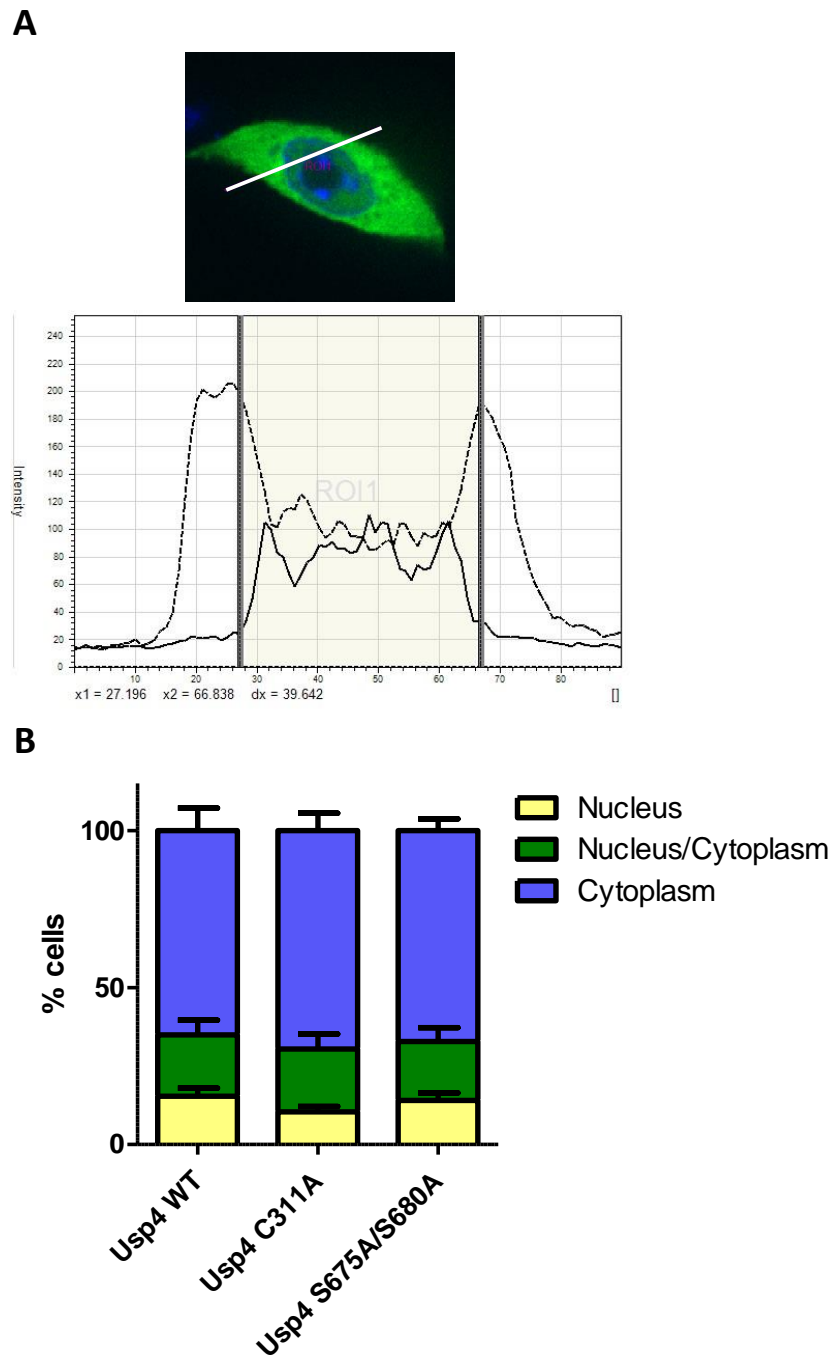


Figure 6.2. The subcellular localisation of Usp4 in NIH3T3 cells. NIH3T3 cells were transfected with GFP-tagged Usp4 wild-type (Usp4 WT), a non-phosphorylatable version of Usp4 (Usp4 S675A/S680A), or a catalytically inactive version of Usp4 (Usp4 C311A). At 24 hours post-transfection, cells were fixed in 4% PFA, mounted and stained with DAPI for nuclei staining before visualisation on a confocal fluorescent microscope. A) Example image of a cell in which Usp4 was scored as predominantly cytoplasmic. Region of interest (ROI) was drawn through the cell and represented as a graph of fluorescent intensity for determining the localisation. Usp4 was scored as being predominantly nuclear (nucleus) when fluorescent intensity increased at the nucleus, predominantly cytoplasmic (cytoplasm) when fluorescent intensity decreased at the nucleus, or throughout the cell (nucleus/cytoplasm) when there were no changes in fluorescent intensity. Channel 1= DAPI, Channel 2= GFP. B) Percentage of cells for which Usp4 was predominantly nuclear, cytoplasmic or throughout the cell. Data are presented as a percentage of total cells analysed and are combined from 3 independent experiments. Statistical analysis was performed using two-way ANOVA with posthoc Bonferroni analysis.

6.2.2 The effect of Usp4 phosphorylation at Ser675/Ser680 on cell signalling pathways

As the prevention of the phosphorylation of Usp4 at Ser675/Ser680 did not affect the subcellular localisation of Usp4, the effect of overexpressing the non-phosphorylatable version of Usp4 on the activity of cell signalling pathways was next explored.

6.2.2.1 Overexpression of non-phosphorylatable Usp4 at Ser675/Ser680 affects cell signalling pathways

To examine the potential effect of the phosphorylation of Usp4 on cell signalling pathways, the effect of overexpressing a mutant phosphorylation version of Usp4 using a multi-reporter array was performed. Osteosarcoma U2OS cells were seeded onto the reporter array containing constructs for 10 pathways with either Usp4 WT, Usp4 S675A/S680A or a control vector (pCMV6-AN-mGFP) and a luciferase assay was performed as described in section 2.2.5.2.

As seen previously in section 3.3.1, the reporter array determined that overexpression of Usp4 significantly increased the activity of p53, NF- κ B, MAPK/ERK and MAPK/JNK pathways (Figure 6.2). Overexpression of the version of Usp4 which is non-phosphorylatable at Ser675/S680 ablated the ability of Usp4 to enhance these pathways, restoring levels to those containing the empty vector (Figure 6.3).

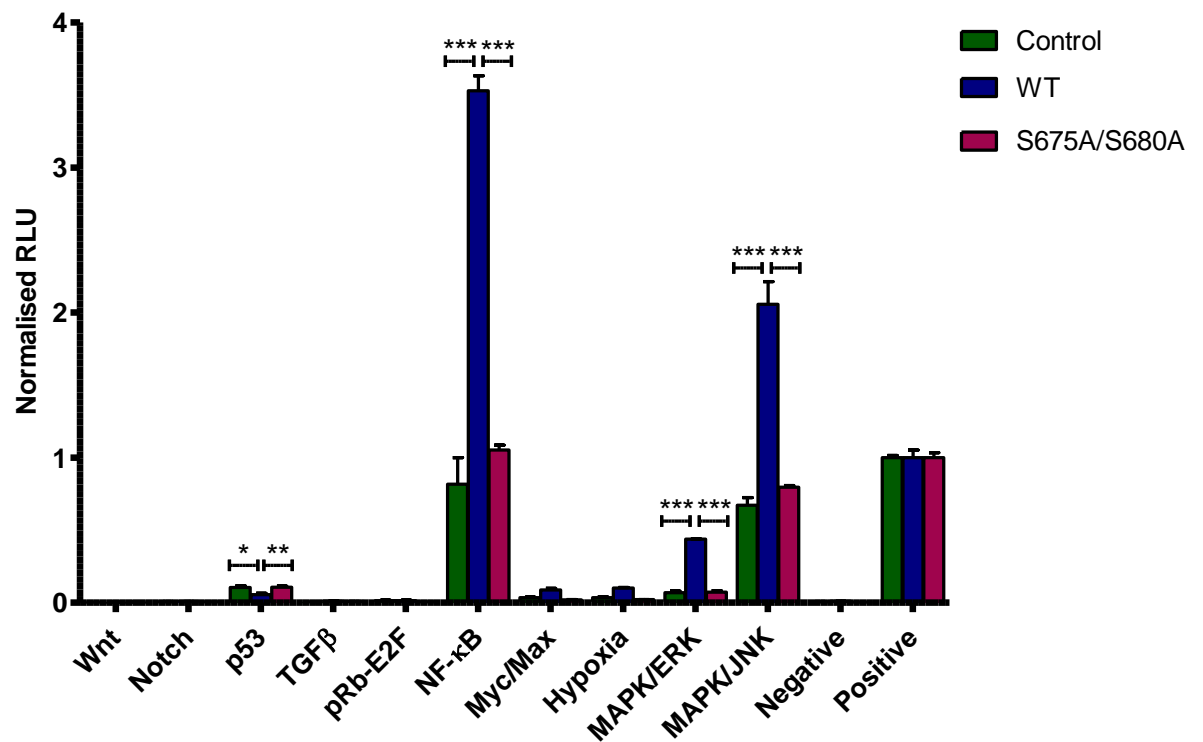


Figure 6.3. Overexpression of Usp4 affects the activity of cell signalling pathways, potentially regulated by the phosphorylation of Usp4. A *Renilla* reporter vector and either p-CMV6-AN-mGFP control vector (Control), wild-type Usp4 expression plasmid (WT) or non-phosphorylatable Usp4 expression plasmid (S675A/S680A) were added to wells of a multi-pathway reporter array plate containing reporter constructs for 10 different pathways. After transfection reagent/DNA complex formation, U2OS cells were seeded onto the plates. At 24 hours post-transfection a luciferase assay was performed. Data were normalised to *Renilla* and to positive control values and are displayed as normalised relative light units (RLU). Figure is from one experiment \pm SEM (n=4/experiment). For statistical analysis, one way ANOVA with a post-hoc Tukey comparison test was performed on data from each pathway separately. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$.

6.2.2.2 Overexpression of non-phosphorylatable Usp4 at Ser675/Ser680 affects IL-1- and TNF α -induced NF- κ B activity

As overexpression of Usp4, non-phosphorylatable at Ser675/S680, reduced the increase in NF- κ B activity that was observed with overexpression of wild-type Usp4, its effect on IL-1- and TNF α -induced NF- κ B activity was next examined by performing an NF- κ B pathway reporter assay. SW1353 cells were co-transfected with NF- κ B responsive firefly luciferase reporter and constitutive *Renilla* expression vectors alongside control, Usp4 WT, Usp4 C311A or Usp4 S675A/S680A expression vectors. After stimulation with either IL-1 or TNF α , luciferase activity was measured and normalised to *Renilla*. Additionally, the expression of Usp4-GFP was confirmed by transfection of the Usp4 expression vectors into SW1353. Cells were then lysed, total protein extracted, resolved by electrophoresis and immunoblotted with an anti-GFP antibody. Wild-type and mutant versions of Usp4 were expressed at similar levels (Figure 6.4A).

The reporter assay demonstrated a significant increase in both IL-1- and TNF α -induced NF- κ B activity with overexpression of wild-type Usp4 compared to the control. The increase was attenuated with overexpression of catalytically inactive Usp4, indicating that the increase in IL-1- and TNF α -induced NF- κ B activity is due to the deubiquitinase activity of Usp4 (Figure 6.4B and Figure 6.4C). Further, the overexpression of non-phosphorylatable Usp4 at Ser675/Ser680 significantly reduced the activity of NF- κ B compared to overexpression of wild-type Usp4. This significant reduction led to restored control NF- κ B activity levels with IL-1 stimulation and significantly reduced NF- κ B activity to levels below that of the control with TNF α stimulation, indicating that the phosphorylation of Usp4 may be involved in the regulation of its function within the NF- κ B signalling pathway (Figure 6.4B and Figure 6.4C).

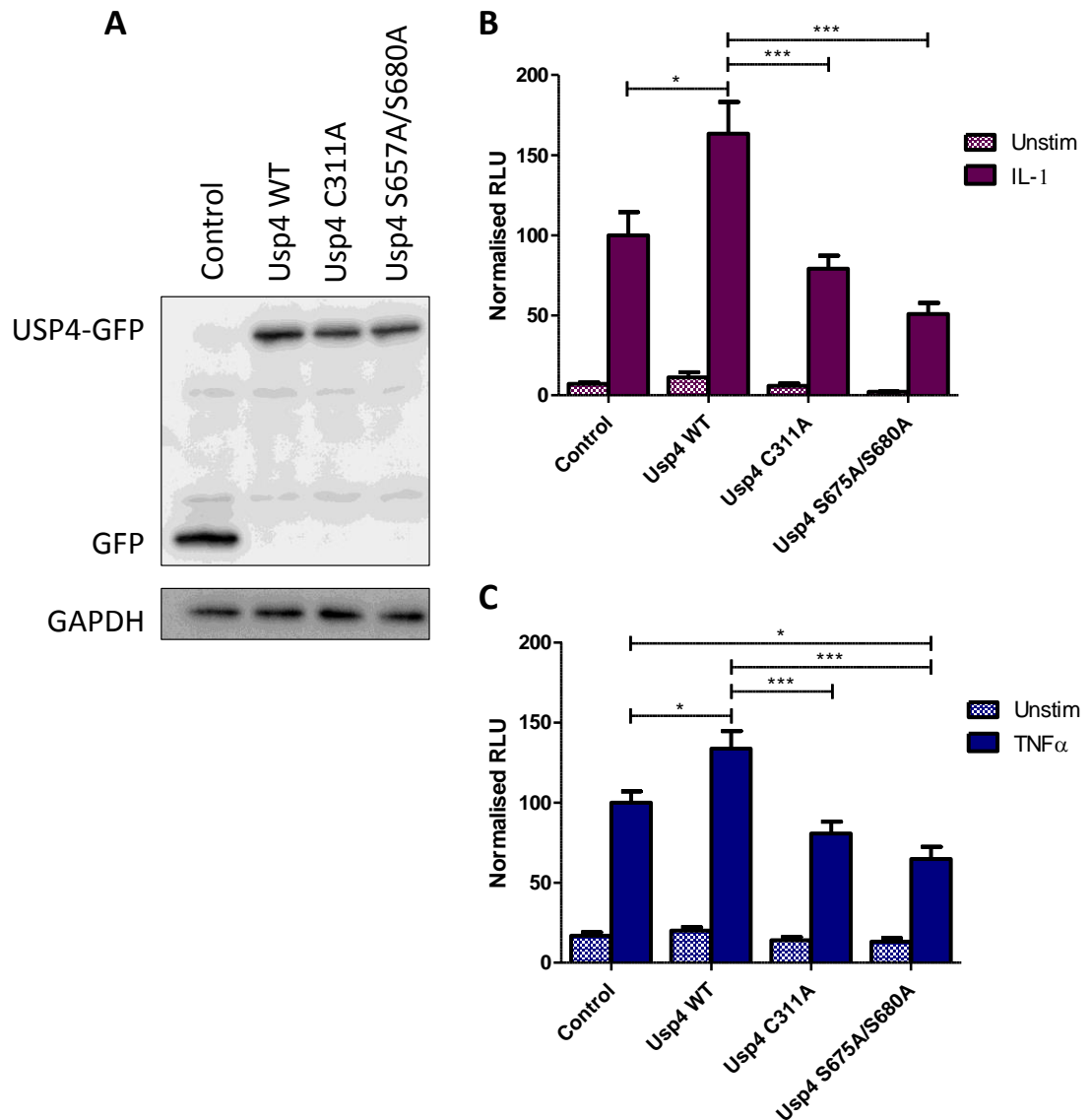


Figure 6.4. Overexpression of Usp4 and Usp4 variants affected NF- κ B activity. A) SW1353 cells were transfected with control, Usp4 WT, Usp4 C311A or Usp4 S675A/S680A vectors for 24 hours before being lysed. Total protein was extracted, resolved by SDS-PAGE and immunoblotted with an anti-GFP antibody. GAPDH was used as a loading control. B) and C) NF- κ B responsive firefly luciferase and *Renilla* reporter vectors were co-transfected into SW1353 cells with either control (pCMV6-AN-mGFP), wild-type Usp4 (Usp4 WT), catalytically inactive Usp4 (Usp4 C311A) or non-phosphorylatable Usp4 (Usp4 S675A/S680A) vectors. At 24 hours post-transfection, cells were serum-starved overnight before a 6 hour stimulation with the relevant stimulus. B) Cells were stimulated with IL-1 α (0.5ng/ml). Data are combined from 2 independent experiments \pm SEM (n=4/experiment). * $p \leq 0.05$, *** $p \leq 0.001$. C) Cells were stimulated with TNF α (10 μ g/ml). Data are combined from 3 independent experiments \pm SEM (n=4/experiment). * $p \leq 0.05$, *** $p \leq 0.001$.

6.2.3 The generation of Usp4 phosphorylation variants

6.2.3.1 Usp4 phosphorylation variant generation

The data presented in this chapter to date have utilised a phosphorylation variant of Usp4 for which both of two closely located phosphorylation sites, Ser675 and Ser680 were mutated to Ala residues to prevent phosphorylation of Usp4 at these sites. Alternatively, these sites could be mutated to aspartic acid (Asp) residues which has been shown to mimic phosphorylation (Léger et al., 1997, Zhang et al., 2012). To further explore the function of Usp4 phosphorylation, a range of different Usp4 phosphorylation variants were generated which incorporate mutations at one or both sites to prevent or mimic the phosphorylation of Usp4.

Site-directed mutagenesis of the pCMV6-AN-Usp4-mGFP vector was performed to generate a series of phosphorylation variants. For some variants, one or both of the Ser sites were mutated to Ala, referred to herein as non-phosphorylatable. For other variants, one or both of the Ser sites were also mutated to Asp, referred to herein as phosphomimetic. Presence of the correct mutations were confirmed by sequencing and are displayed in Table 6.1.

Table 6.1. Usp4 phosphorylation variants generated by mutagenesis. Mutations at sites 675 and 680 are highlighted in red, wild-type sites are highlighted in green.

Usp4 Variant	Sequence	Non-phosphorylatable/ phosphomimetic?
Usp4 WT	GGAAAAGAGCAGCTT TCC GAAGTGGAAAGGC AGT GGTGAGGACGATCAG	N/A
Usp4 S675A	GGAAAAGAGCAGCTT GCG GAAGTGGAAAGGC AGT GGTGAGGACGATCAG	Non-phosphorylatable
Usp4 S680A	GGAAAAGAGCAGCTT TCC GAAGTGGAAAGGC GCG GGTGAGGACGATCAG	Non-phosphorylatable
Usp4 S675A/S680A	GGAAAAGAGCAGCTT GCC GAAGTGGAAAGGC GCC GGTGAGGACGATCAG	Non-phosphorylatable
Usp4 S675D	GGAAAAGAGCAGCTT GAC GAAGTGGAAAGGC AGT GGTGAGGACGATCAG	Phosphomimetic
Usp4 S680D	GGAAAAGAGCAGCTT TCC GAAGTGGAAAGGC GAT GGTGAGGACGATCAG	Phosphomimetic
Usp4 S675D/S680D	GGAAAAGAGCAGCTT GAC GAAGTGGAAAGGC GAT GGTGAGGACGATCAG	Phosphomimetic
Usp4 S675A/S680D	GGAAAAGAGCAGCTT GCG GAAGTGGAAAGGC GAT GGTGAGGACGATCAG	Non-phosphorylatable/ Phosphomimetic
Usp4 S675D/S675A	GGAAAAGAGCAGCTT GAC GAAGTGGAAAGGC GCG GGTGAGGACGATCAG	Non-phosphorylatable/ Phosphomimetic

6.2.3.2 Identification of the phosphorylation status of Usp4

To determine the phosphorylation status of wild-type Usp4 (Usp4 WT), non-phosphorylatable Usp4 (Usp4 S675A/S680A) and phosphomimetic Usp4 (Usp4 S657D/S680D), the GFP-tagged expression vectors and a control vector (pCMV-AN-mGFP) were transfected into HEK 293T cells, immunoprecipitated with an anti-GFP antibody, resolved by electrophoresis and immunoblotted with an anti-phosphoserine antibody.

Usp4 WT, Usp4 S675A/S680A and Usp4 S657D/S680D were all immunoprecipitated successfully as demonstrated by the presence of GFP and Usp4, but not in the control (Figure 6.5). All variants of Usp4 were serine phosphorylated, with less phosphorylation of the Usp4 S675A/S680A variant than Usp4 S657D/S680D, indicating that the non-phosphorylatable Usp4 at sites Ser675 and Ser680 was less phosphorylated than the phosphomimetic Usp4 variant.

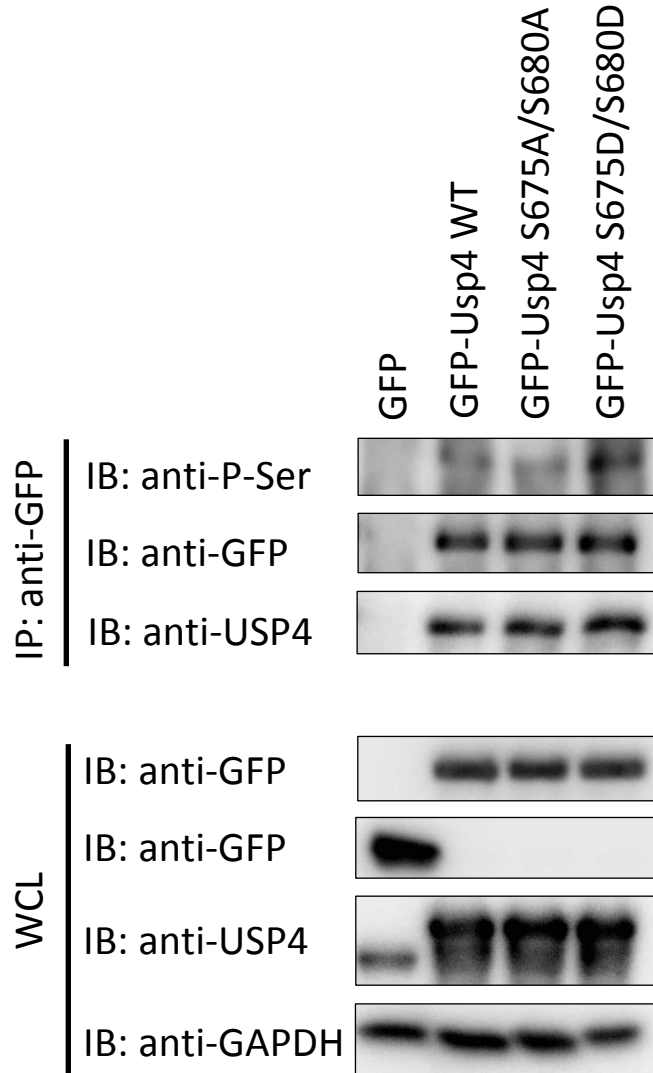


Figure 6.5. Usp4 is serine phosphorylated. HEK 293T cells were transfected with a control vector (pCMV-AN-mGFP) or Usp4 wild-type (Usp4 WT) or phosphorylation variants (Usp4 S675A/S680A or Usp4 S675D/S680D). At 24 hours post-transfection, cells were lysed, total protein extracted, resolved by SDS-PAGE and immunoblotted (IB) with anti-GFP and anti-Usp4 antibodies to confirm successful transfection in whole cell lysates (WCL). GAPDH was used as a loading control. Extracts were pre-cleared with rabbit IgG before immunoprecipitation (IP) with an anti-GFP antibody overnight, resolved by SDS-PAGE and immunoblotted (IB) with anti-phosphoserine (P-Ser), anti-GFP and anti-USP4 antibodies. Figure is representative of 3 independent experiments.

6.2.3.3 The effect of overexpressing Usp4 phosphorylation variants on NF- κ B activity

As overexpression of the non-phosphorylatable Usp4 variant, Usp4 S675A/S680A, restored the level of IL-1-induced NF- κ B activity to control levels in comparison to the overexpression of Usp4 WT, the effect of the overexpression of the other variants was next examined. SW1353 cells were co-transfected with NF- κ B responsive firefly luciferase reporter and constitutive *Renilla* expression vectors alongside control, Usp4 WT and Usp4 phosphorylation variant expression vectors. After stimulation with IL-1, luciferase activity was measured and normalised to *Renilla*.

An example of one experiment is shown in Figure 6.6. All phosphorylation mutants significantly reduced IL-1-induced NF- κ B activity, compared to wild-type Usp4, in this experiment. However, data from replicative experiments were very variable and not reproducible, therefore no conclusive effects could be observed. For this reason it was decided a new approach to introduce the Usp4 phosphorylation variants into cells would be utilised.

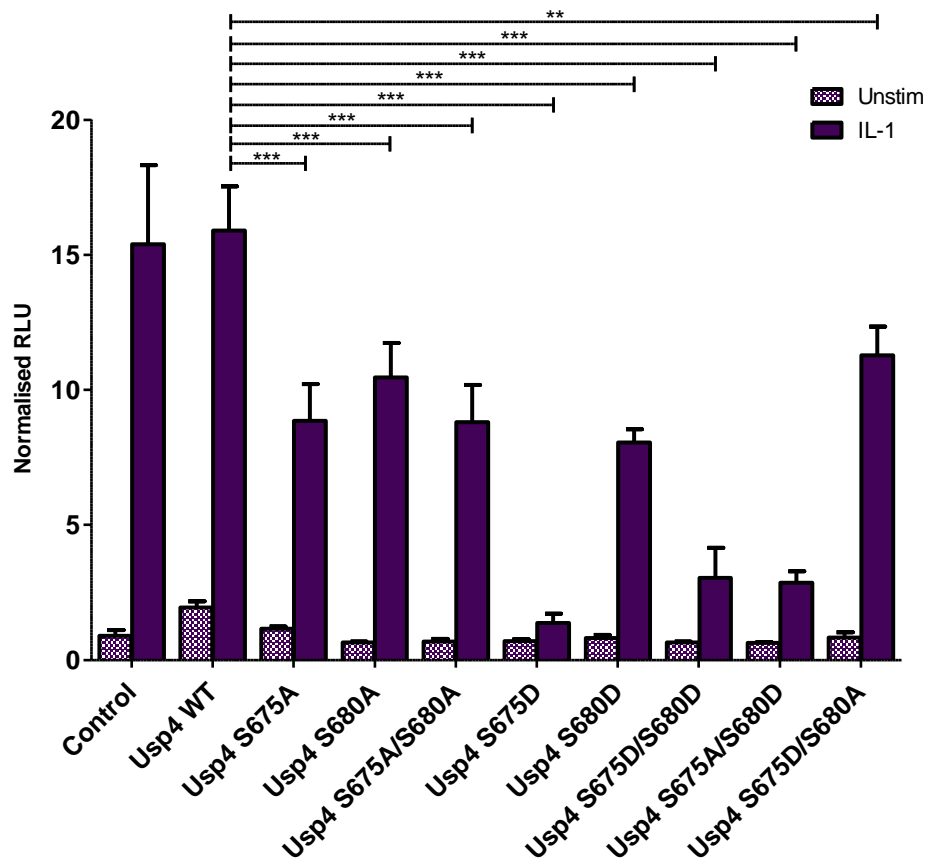


Figure 6.6. Overexpression of Usp4 and Usp4 phosphorylation variants on IL-1-induced NF- κ B activity. SW1353 cells were co-transfected with NF- κ B-dependent firefly luciferase and *Renilla* expression vectors along with either empty vector (Control), wild-type Usp4 (Usp4 WT) or Usp4 phosphorylation variants. At 24 hours post-transfection, cells were serum-starved overnight before being left unstimulated (Unstim) or stimulated with IL-1 α (0.5ng/ml) for 6 hours (IL-1). Data is an example of one experiment (n=4). Data are plotted as mean \pm SEM. Two way ANOVA with post-hoc Bonferroni comparison test was performed as statistical analysis. ** p \leq 0.01, *** p \leq 0.001.

6.2.3.4 Lentiviral generation

To overcome difficulties in the reproducibility of data from NF- κ B responsive luciferase assays with overexpression of Usp4 WT and phosphorylation variants, a lentiviral-mediated delivery approach was developed.

Initially, a doxycycline-inducible expression system was utilised. Usp4 WT and variants, with the GFP, were cloned into a response vector, pLVX-Tight-Puro, and transfected into cells with lentiviral packaging vectors to produce response lentiviral supernatants. Additionally, a regulator vector, pLVX-Tet-On Advanced, was transfected into HEK 293T cells with lentiviral packaging vectors to produce regulator lentiviral supernatants. Transduction of both regulator and response lentiviral supernatants into cells should activate transgene expression upon doxycycline induction. Various conditions to optimise the production of lentivirus in HEK 293T cells and transduction into SW1353 cells were tested as described in section 2.2.14. As the lentiviral particles should express a GFP protein if successfully transduced, cells were visualised on a fluorescent microscope, however no GFP was observed indicating unsuccessful lentiviral production or transduction.

The next approach was to utilise a different lentiviral expression system more commonly used in our laboratory, a second generation packaging and envelope plasmid system. Usp4 WT, Usp4 C311A and all Usp4 phosphorylation variants were first cloned into the lentiviral expression vector, pCDH-EF1-MCS-IRES-GFP. Lentiviral supernatants were produced by transfection of the lentiviral constructs, or control vector, pCDH-EF1-MCS-IRES-GFP, a packaging vector and an envelope vector into HEK 293T cells before harvesting. Successful transduction into SW1353 cells was confirmed by visualisation of GFP expression on a fluorescent microscope.

6.2.4 The effect of Usp4 phosphorylation on IL-1-induced NF- κ B activity

6.2.4.1 Lentiviral-mediated overexpression of Usp4 phosphorylation variants on IL-1-induced NF- κ B activity in SW1353 cells using viral titre normalisation

To ensure cells were transduced with equal viral particles across all lentiviral supernatants used, the viral RNA genome was measured by titration, as described in section 2.2.14.3, and normalised for all of the Usp4 variants and control to the lowest viral RNA copy

number. To determine the effect of Usp4 phosphorylation on NF- κ B activity, an NF- κ B responsive luciferase assay was performed using lentiviral preparations for control, wild-type Usp4, catalytically inactive Usp4 and non-phosphorylatable and phosphomimetic Usp4. SW1353 cells were co-transduced with the lentiviral preparations and NF- κ B luciferase lentiviral preparations, followed later by stimulation with IL-1 and lysis for use in a luciferase assay. Successful transduction of NF- κ B luciferase reporter, pHAGE-NF- κ B-TA-LUC-GFP-W, was previously verified (Figure 4.14).

There was no difference in IL-1-induced NF- κ B activity with overexpression of Usp4 WT compared to the control, in contrast with data in Figure 6.4. Overexpression of Usp4 C311A increased IL-1-induced NF- κ B activity compared to overexpression of Usp4 WT, again in contrast to data in Figure 6.4. In line with data in Figure 6.4, overexpression of the non-phosphorylatable Usp4, Usp4 S675A/S680A, reduced IL-1-induced NF- κ B activity compared to the overexpression of Usp4 WT. IL-1-induced NF- κ B activity was also reduced with overexpression of a variant with a single phosphorylation mutant, Usp4 S675A, and in both single phosphomimetic variants, Usp4 S675D and Usp4 S680D, in addition to a variant in which one site is non-phosphorylatable and one phosphomimetic, Usp4 S675D/S680D. The only Usp4 phosphorylation variant to increase the activity of NF- κ B compared to the Usp4 WT was a variant with the second phosphorylation site mutated to non-phosphorylatable, S680A (Figure 6.7).

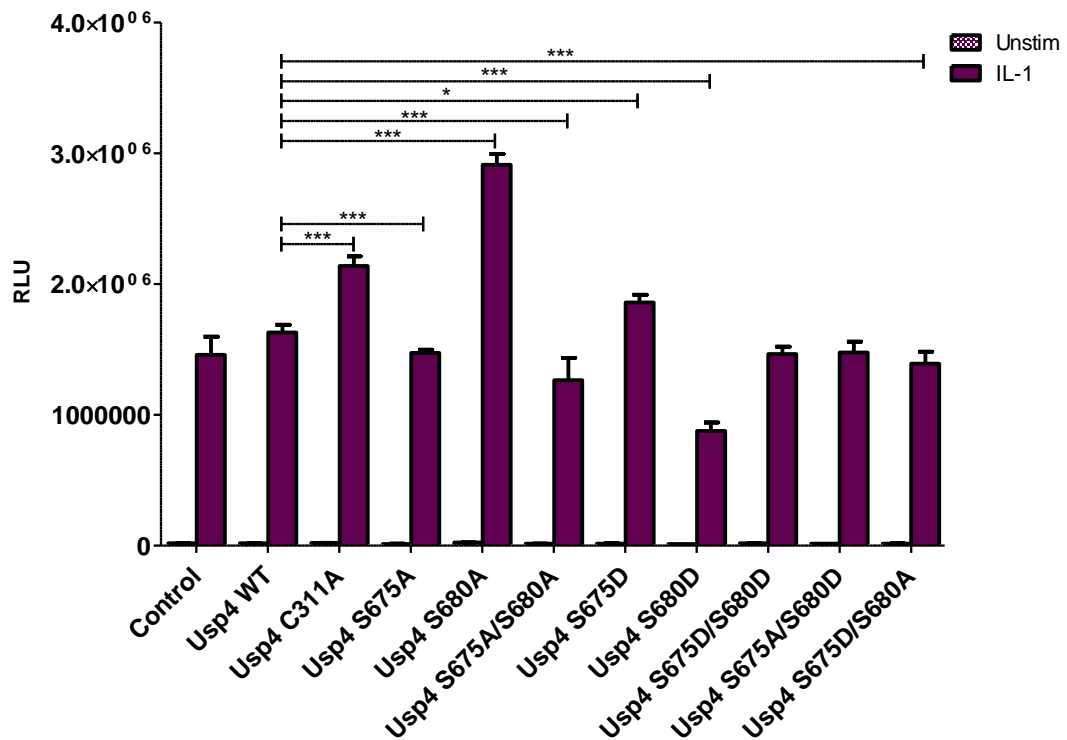


Figure 6.7. Lentiviral-mediated expression of Usp4 and Usp4 phosphorylation mutants on IL-1-induced NF-κB activity in SW1353 cells with normalised amounts of viral preparations. SW1353 cells were transduced with lentiviral preparations for an NF-κB responsive firefly luciferase expression vector (pHAGE-NF-κB-TA-LUC-UBC-GFP-W) and either lentiviral preparations for a control (pCDH-EF1-MCS-IRES-GFP), wild-type Usp4 (Usp4 WT), catalytically inactive Usp4 (Usp4 C311A) or Usp4 phosphorylation variants, and treated with polybrene (8μg/ml). After 48 hours, cells were stimulated with IL-1α (0.5ng/ml) for 6 hours, or left unstimulated, before cell lysis and luminescence was measured. Data are displayed in relative light units (RLU) ± SEM (n=4). Two way ANOVA with post-hoc Bonferroni comparison test was performed as statistical analysis. * p ≤ 0.05, *** p ≤ 0.001.

6.2.4.2 Expression of Usp4 with lentiviral-mediated overexpression of Usp4 phosphorylation variants

As the data in Figure 6.7 is conflicting with previous data in this thesis, the expression of Usp4 was examined for each of the Usp4 phosphorylation variants when transducing cells with equal viral particles, as determined by viral titre. SW1353 cells were transduced with lentiviral preparations for all Usp4 variants, cells were lysed, resolved by electrophoresis and immunoblotted with an anti-USP4 antibody.

Expression of Usp4 was observed for all Usp4 phosphorylation variants, however, the levels of Usp4 expression varied between the different lentiviral preparations, possibly indicating there was unequal transduction (Figure 6.8).

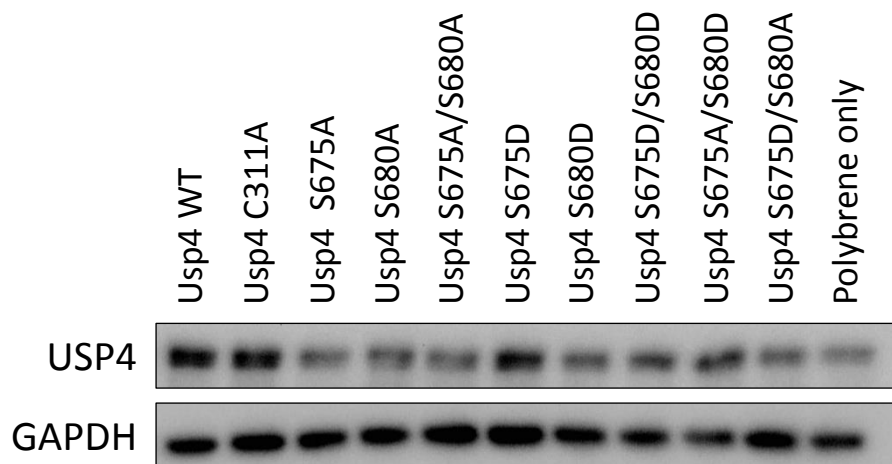


Figure 6.8. USP4 expression with lentiviral Usp4 and Usp4 phosphorylation variants. SW1353 cells were transduced with Usp4 WT, Usp4 C311A or Usp4 phosphorylation variants and treated with polybrene (8 μ g/ml), or treated with polybrene only (8 μ g/ml) (Polybrene only). After 48 hours, cells were lysed, resolved by SDS-PAGE and immunoblotted with an anti-USP4 antibody. GAPDH was used as a loading control.

6.2.4.3 Lentiviral-mediated overexpression of Usp4 phosphorylation variants on IL-1-induced NF- κ B activity in SW1353 cells with equal viral preparation volume

As the expression of Usp4 was unequal between the different Usp4 phosphorylation variants when normalising the volume of viral preparations used according to viral titre, and the NF- κ B responsive reporter assay produced data conflicting to other data in this thesis, the IL-1-induced NF- κ B activity was next examined using equal volumes of viral preparations with an assumption that viral preparations contain similar amounts of viral particles. The lentiviral particles generated were produced using the exact same procedure and the same lentiviral expression vector, therefore it would be expected that the amounts of viral particles produced would be similar across all preparations. Again, SW1353 cells were co-transduced with the lentiviral preparations and NF- κ B luciferase lentiviral preparations, followed later by stimulation with IL-1 and lysis for use in a luciferase assay.

Consistent with the effects on NF- κ B activity in Figure 6.4, lentiviral-mediated overexpression of wild-type Usp4 significantly increased IL-1-induced NF- κ B activity compared to the control. This was restored to control levels with lentiviral-mediated overexpression of catalytically inactive Usp4 and the non-phosphorylatable Usp4 variant, S675A/S680A. All other phosphorylation variants also significantly reduced the IL-1-induced NF- κ B activity compared to wild-type Usp4 (Figure 6.9).

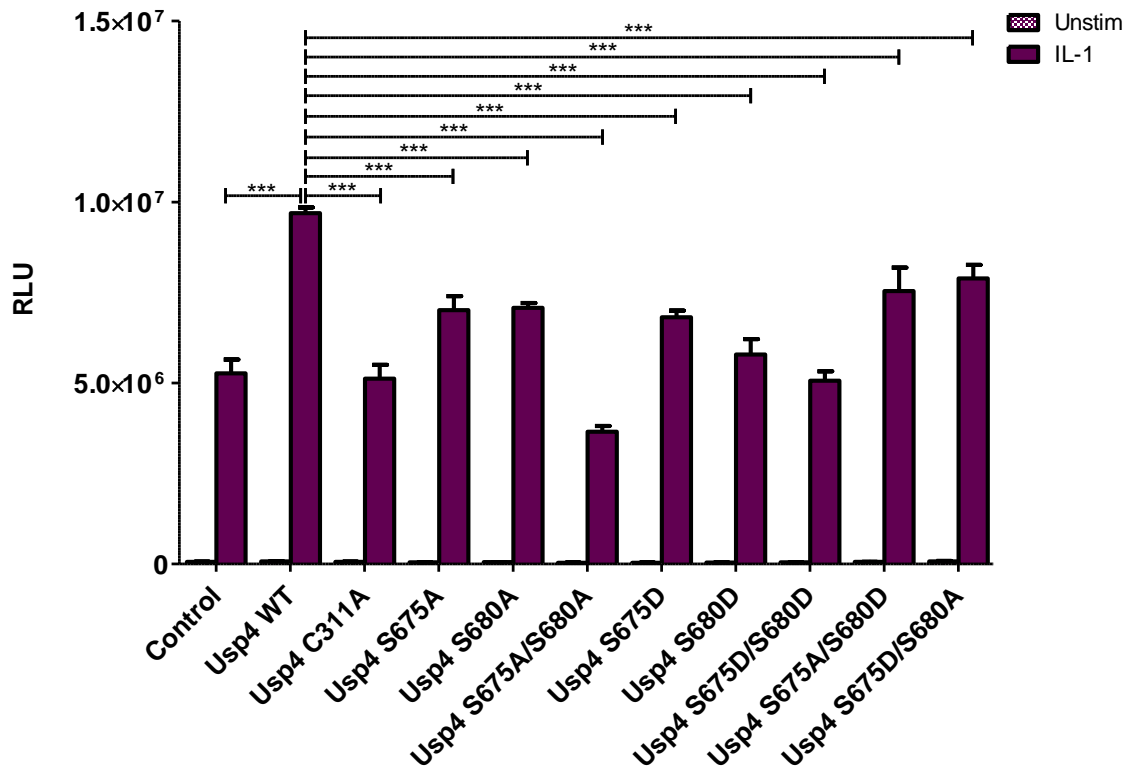


Figure 6.9. Lentiviral-mediated expression of Usp4 and Usp4 phosphorylation mutants on IL-1-induced NF- κ B activity in SW1353 cells with equal volumes of preparations. SW1353 cells were transduced with equal volumes of lentiviral preparations for an NF- κ B responsive firefly luciferase expression vector (pHAGE-NF- κ B-TA-LUC-UBC-GFP-W) and equal volumes of either lentiviral preparations for a control (pCDH-EF1-MCS-IRES-GFP), wild-type Usp4 (Usp4 WT), catalytically inactive Usp4 (Usp4 C311A) or Usp4 phosphorylation variants and treated with polybrene (8 μ g/ml). After 48 hours, cells were stimulated with IL-1 α (0.5ng/ml), or left unstimulated, for 6 hours before cell lysis and luminescence measured. Data are displayed in relative light units (RLU) \pm SEM (n=4). Two way ANOVA with post-hoc Bonferroni comparison test was performed as statistical analysis. *** p \leq 0.001.

6.2.4.4 Lentiviral-mediated overexpression of Usp4 phosphorylation variants on IL-1-induced NF- κ B activity in Usp4^{-/-} MEFs

Lentiviral expression vectors are also an effective way for gene delivery into cells which can be difficult to transfect ordinarily, such as MEFs. Therefore, this method was also utilised to examine the effect of Usp4 phosphorylation in Usp4^{-/-} MEFs. This allowed a model for which no endogenous Usp4 would be present, only the Usp4 variant introduced into the cell via lentiviral transduction would be present in the cells, eliminating any masking of effects by endogenous Usp4. The Usp4^{-/-} MEFs were transduced with equal volumes of control or Usp4 lentiviral preparations, and with NF- κ B luciferase lentiviral preparations, followed later by stimulation with IL-1 and lysis for use in a luciferase assay.

As in SW1353 cells (Figure 6.9), IL-1-induced NF- κ B activity was increased with overexpression of wild-type Usp4 compared to the control, and restored to control levels with overexpression of catalytically inactive Usp4. Overexpression of the single and double non-phosphorylatable variants, Usp4 S675A and Usp4 S675A/S680A, also significantly reduced IL-1-induced NF- κ B activity compared to wild-type, as did all of the phosphomimetic variants, Usp4 S675D, Usp4 S680D and Usp4 S675D/S680D. No differences in the IL-1-induced NF- κ B activity were observed with the single non-phosphorylatable variant, Usp4 S680A, or either of the combined variants, S675A/S680D and S675D/S680D (Figure 6.10).

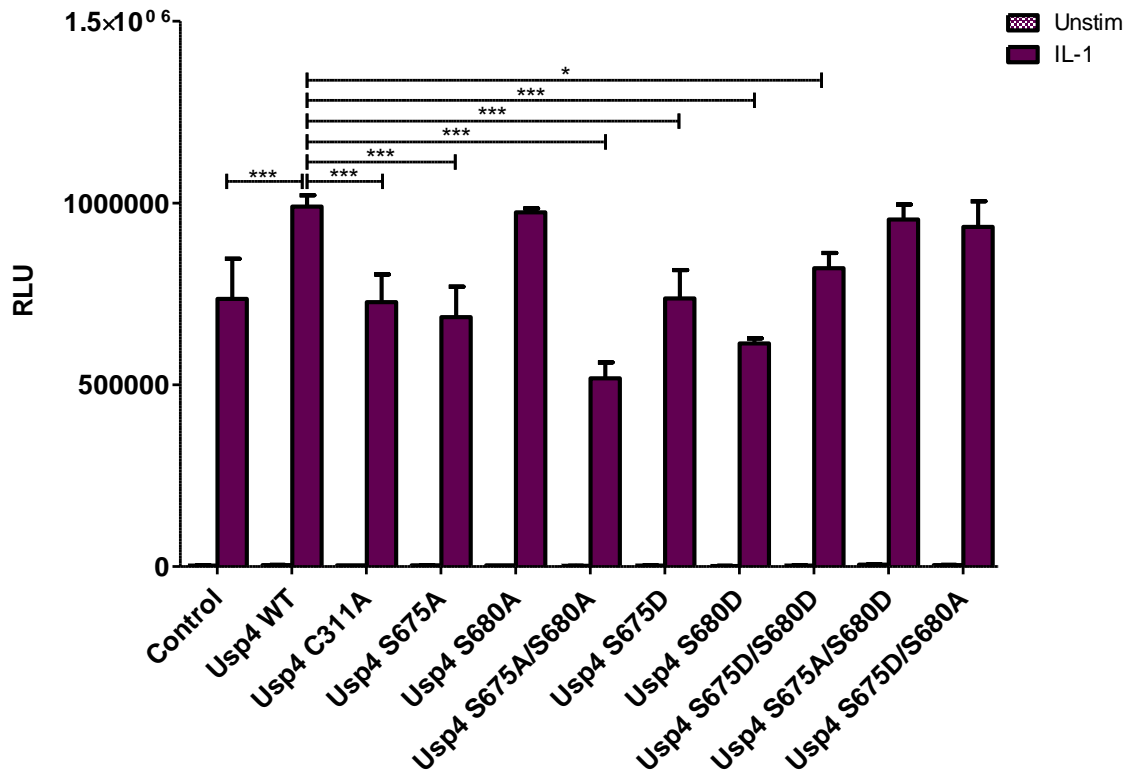


Figure 6.10. Lentiviral-mediated expression of Usp4 and Usp4 phosphorylation mutants on IL-1 α -induced NF- κ B activity in *Usp4*^{-/-} MEFs. *Usp4*^{-/-} MEFs were transduced with lentiviral preparations for an NF- κ B responsive firefly luciferase expression vector (pHAGE-NF- κ B-TA-LUC-UBC-GFP-W) and equal volumes of either lentiviral preparations for a control (pCDH-EF1-MCS-IRES-GFP), wild-type Usp4 (Usp4 WT), catalytically inactive Usp4 (Usp4 C311A) or Usp4 phosphorylation variants and treated with polybrene (8 μ g/ml). After 48 hours, cells were stimulated with IL-1 α (0.5ng/ml), or left unstimulated, for 6 hours before cell lysis and luminescence measured. Data are displayed in relative light units (RLU) \pm SEM (n=4). Two way ANOVA with post-hoc Bonferroni comparison test was performed as statistical analysis. * p \leq 0.05, *** p \leq 0.001.

6.2.5 The effect of depleting a USP4 kinase on NF- κ B signalling

6.2.5.1 Identification of USP4 protein kinases

To identify the protein kinases that phosphorylate the Ser675 and Ser680 residues, the laboratory of Dr. Robert Screaton in collaboration with Dr. Doug Gray, performed an *in vitro* kinase screen. A fusion of GST and a USP4 fragment, containing the two relevant serines, was exposed to a panel of recombinant kinases in the presence of γ - 32 P-labelled ATP. For methodology see report by Jansson et al. (2008).

Several kinases were identified that could phosphorylate USP4, including G-protein-coupled receptor kinases (GRKs) 1, 2 and 6 (Figure 6.11).

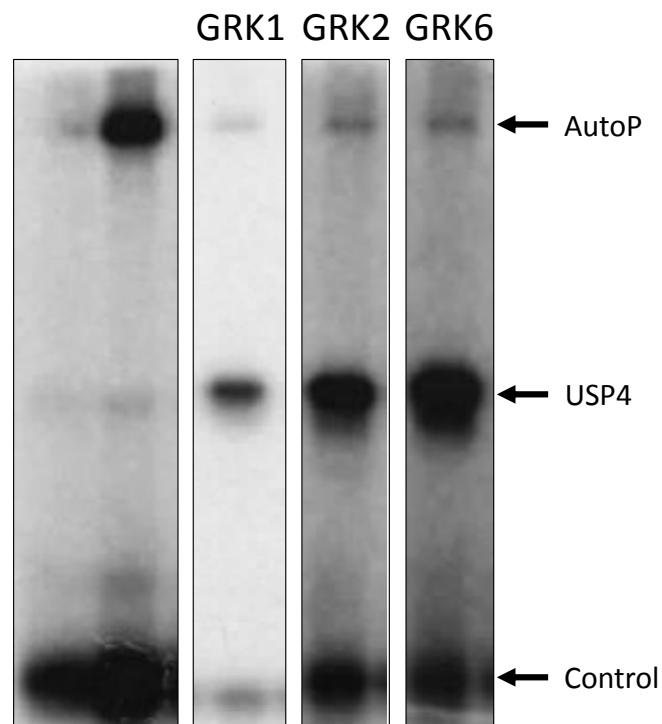


Figure 6.11. A kinase screen identified GRK1, 2 and 6 as USP4 kinases. A fragment of USP4 incorporating Ser675 and Ser680 was exposed to a panel of kinases in the presence of γ - 32 P-labelled ATP. A control fragment of myelin basic protein was also included in the screen as a control (Control). Phosphorylated USP4 fragment is indicated (USP4) as is kinase autophosphorylation (AutoP).

GRKs are a family of Ser/Thr protein kinases known for phosphorylating activated G-protein-coupled receptors (GPCRs) to mediate their desensitisation as a key mechanism of regulation of cell signalling pathways (Ribas et al., 2006). GRK2 is a β -adrenergic receptor kinase with substrates including Akt, R-Smads, p38 and A_{2A} adenosine receptor, and is involved in cell signalling pathways other than those mediated by GPCRs including TGF- β , MAPK and NF- κ B pathways (Ribas et al., 2006, Ho et al., 2005, Peregrin et al., 2006, Robinson and Pitcher, 2013, Patial et al., 2009). Given that USP4 affected the IL-1-induced activity of NF- κ B and that the phosphorylation of Usp4 potentially has a role in this, it would be interesting to explore the effect of USP4 kinases on NF- κ B signalling. As an identified USP4 kinase with some shared substrates of USP4, and involvement in the regulation of some of the same pathways as USP4, the effect of GRK2 on NF- κ B signalling was examined using an RNAi approach to deplete GRK2.

6.2.5.2 The effect of GRK2 depletion on IL-1-induced NF- κ B activity

To determine the effect of GRK2 depletion on the IL-1-induced activation of NF- κ B, an NF- κ B pathway luciferase reporter assay was performed. SW1353 cells transfected with an NF- κ B responsive firefly luciferase reporter and a constitutive *Renilla* expression vector were GRK2 depleted by RNAi before stimulation with IL-1 and lysis for the luciferase assay. Luciferase activity was normalised to *Renilla*.

The reporter assay demonstrated no significant difference in IL-1-induced NF- κ B activity with depletion of GRK2 compared to the control (Figure 6.12).

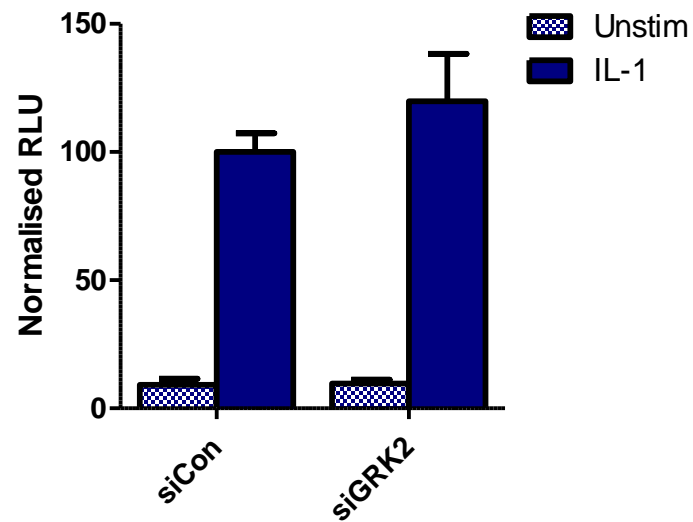


Figure 6.12. Depletion of GRK2 did not affect IL-1-induced NF- κ B activity. NF- κ B responsive firefly luciferase and *Renilla* reporter vectors were transfected into SW1353 cell followed by transfection with siCon or siGRK2 after 6-8 hours. At 24 hours post-transfection, cells were serum-starved overnight before a 6 hour stimulation with IL-1 α (0.5ng/ml), cell lysis and measurement of relative luciferase activity. Data were normalised to *Renilla* and to siCon IL-1=100% and are displayed as normalised relative light units (RLU). Figure is combined of three experiments \pm SEM (n=4/experiment). One-way ANOVA with a post-hoc Tukey test was performed as statistical analysis.

6.2.5.3 The effect of GRK2 depletion on IL-1-induced NF- κ B-regulated gene expression

As the depletion of USP4 reduced IL-1-induced expression of some NF- κ B-regulated genes, the effect of GRK2 depletion on the expression of *IL-6*, *IL-8* and *MMP13* were next examined. SW1353 cells were transfected with siCon, siUSP4 or siGRK2, total RNAs extracted and real-time RT-PCR performed.

GRK2 depletion with transfection of an siRNA targeting GRK2 (siGRK2) was confirmed (Figure 6.13E). Similarly to USP4 depletion, GRK2 depletion significantly reduced the IL-1-induced expression of *IL-6* and *MMP13* but there was no effect with GRK2 depletion on the IL-1-induced expression of *IL-8* (Figure 6.13A, Figure 6.13B and Figure 6.13C).

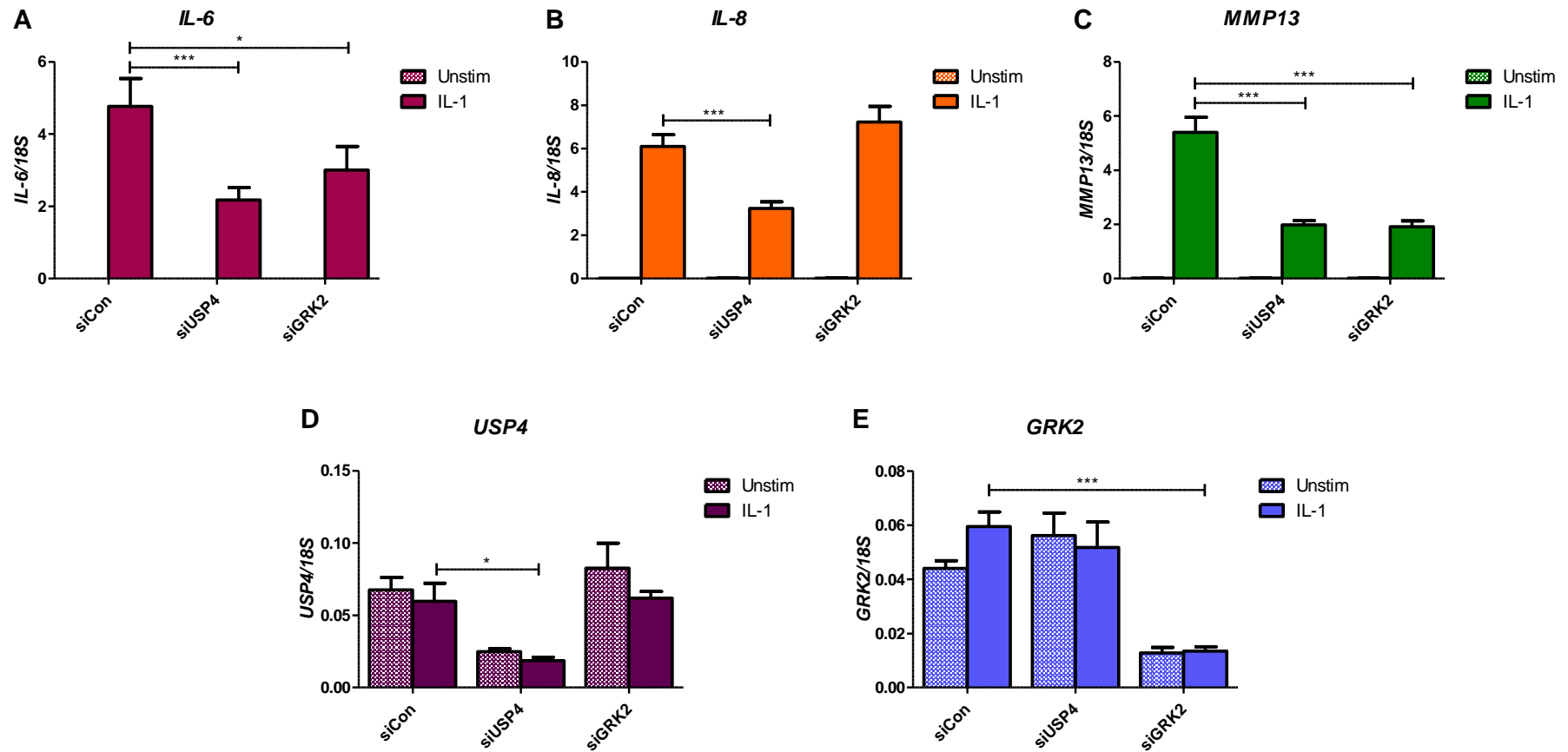


Figure 6.13. GRK2 depletion reduced IL-1-induced expression of NF- κ B-regulated genes. SW1353 cells were transfected with siCon, siUSP4 or siGRK2 for 24 hours. After overnight serum-starvation, cells were stimulated with IL-1 α (0.5ng/ml) for 6 hours before total RNA was extracted, reverse transcribed to cDNA and analysed by real-time RT-PCR. Relative gene expression of A) *IL-6*, B) *IL-8*, C) *MMP13*, D) *USP4* and E) *GRK2* were measured. Data are normalised to 18S and are plotted as mean \pm SEM (n=6). Two-way ANOVA with post hoc test Bonferroni was performed as statistical analysis. * $p \leq 0.05$, *** $p \leq 0.001$

6.2.5.4 The effect of GRK2 depletion on the levels of NF- κ B adaptor proteins

Next, the effect of depleting GRK2 on the levels of key proteins involved in NF- κ B signalling, for which depleting USP4 affected, were examined by immunoblotting total protein extracts from control, USP4 depleted and GRK2 depleted SW1353 cells with the relevant antibodies.

Whereas USP4 depletion reduced the levels of phosphorylated IKK α / β and phosphorylated JNK and reduced the degradation of I κ B α , the depletion of GRK2 had no effect on the levels of any of these proteins compared to the control (Figure 6.14).

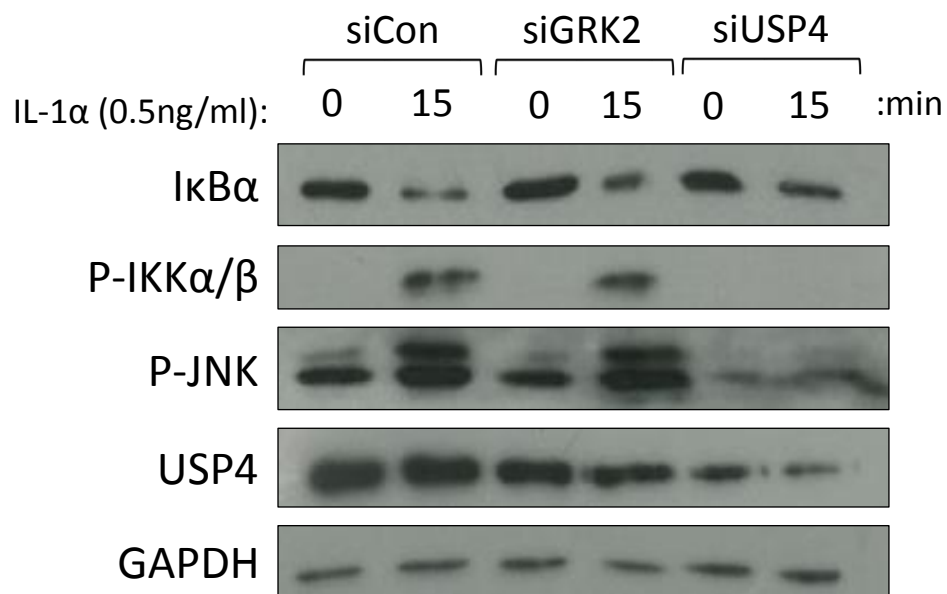


Figure 6.14. The effect of GRK2 depletion on levels of NF- κ B pathway proteins. SW1353 cells were transfected with siCon, siUSP4 or siGRK2 for 24 hours, serum-starved overnight and treated with IL-1 α (0.5ng/ml) for the indicated time (minutes). Total protein was extracted, resolved by SDS-PAGE and immunoblotted with anti-I κ B α , anti-P-IKK α / β , anti-P-JNK and anti-USP4 antibodies. GAPDH was used as a loading control.

6.3 Discussion

The aim of this chapter was to explore how the PTM of USP4 could affect its function. A series of phosphorylation mutants were generated to examine the functional consequences of their overexpression on the subcellular localisation of Usp4 and on cell signalling pathways. Experiments were performed in parallel with experiments to determine the role of USP4 on the NF- κ B signalling pathway, as discussed in previous chapters in this thesis. Given that data demonstrated that USP4 functions in the NF- κ B signalling pathway, the main focus was to examine effects of its phosphorylation on the activity of NF- κ B. Additionally, a potential USP4 kinase, GRK2, was also examined in relation to NF- κ B signalling.

It should be noted that although the Ser residues 675 and 680 were shown to be phosphorylated, other PTMs can modify Ser residues, including acetylation and *O*-glycosylation, thereby further diversifying regulation of the protein (Mukherjee et al., 2007, Huber and Hardin, 2004). A series of phosphorylation mutants were generated to examine the function of the phosphorylation at Ser675/Ser680, however, there is a possibility that this may affect other PTMs at these sites which should be kept in mind.

6.3.1 Overexpression of a non-phosphorylatable Usp4 variant, at Ser675 and Ser680, did not affect the subcellular localisation of Usp4

The phosphorylation of DUBs has been demonstrated to regulate their translocation between subcellular locations (Yuan et al., 2010, Zhang et al., 2012, Kessler and Edelmann, 2011). The effect of USP4 phosphorylation on its subcellular localisation was therefore examined by overexpression of a Usp4 phosphorylation variant which has two phosphorylation sites, Ser 675 and Ser680, mutated to Ala to prevent phosphorylation at these sites. Both overexpression of wild-type Usp4 and the Usp4 phosphorylation variant of Usp4 were localised mainly in the cytoplasm of NIH3T3 cells with no differences observed in the localisation between either version of Usp4, indicating that the phosphorylation status at these sites does not regulate its subcellular localisation. Although performed without stimulation of the cells, differences in the distribution of Usp4 between cells could be due to the presence of cytokines in the serum which mean the cells are in different phases.

USP4 is known to regulate many pathways, including p53, Wnt, RIG-I, TGF- β and NF- κ B pathways (Zhang et al., 2011, Zhao et al., 2009, Wang et al., 2013, Zhang et al., 2012, Fan et al., 2011b). The mechanism of the regulation of USP4 in TGF- β signalling is through the AKT-mediated phosphorylation of USP4 at Ser445 which facilitates its translocation from the nucleus to the cytoplasm and plasma membrane to spatially locate it to the T β R-I, required for its function in this pathway (Zhang et al., 2012). The reported data on the regulation of USP4 by its phosphorylation at Ser445 was published after experiments to evaluate Usp4 PTM at Ser675/Ser680. The mechanism of USP4 regulation in other pathways is largely unexplored but it is possible that regulation of its subcellular localisation is important for its function. Although, under conditions tested here, the PTM of Usp4 at Ser675/Ser680 did not regulate its subcellular localisation, it would be interesting to examine whether stimulating with IL-1 affects this, as this was shown to be regulated by phosphorylation of Usp4. Further, expressing or inhibiting known Usp4 kinases or expressing phosphomimetic Usp4 variants, as generated later in these studies, may give further insight into the effect of Usp4 PTM on its subcellular localisation.

6.3.2 Usp4 PTM at Ser675/Ser680: effects on cell signalling pathways

It was demonstrated in Chapter 3 that Usp4 affected cell signalling pathways in a multi-pathway reporter array, including p53, NF- κ B, MAPK/ERK and MAPK/JNK pathways. This was potentially through the deubiquitinase activity of Usp4 as a catalytically inactive Usp4 partially or fully attenuated the ability of Usp4 to enhance NF- κ B activity, restoring activity to control levels. In parallel, the effect of Usp4 phosphorylation on cell signalling pathways was examined, by overexpression of non-phosphorylatable Usp4 at Ser675/Ser680, and was demonstrated to attenuate the ability of Usp4 to enhance activity of pathways p53, NF- κ B, MAPK/ERK and MAPK/JNK, restoring activity to control levels. As wild-type Usp4 was demonstrated to increase the activity of these pathways, it suggests that PTM of Usp4 at one or both of these serines is required for the function of Usp4 within these pathways.

The multi-pathway reporter array was performed without stimulation, and as no effect on the subcellular localisation was observed under the same conditions, it seems likely that the subcellular localisation of Usp4 is not regulated by phosphorylation at Ser675/Ser680 for its function in these pathways. Instead, its phosphorylation may facilitate protein-

protein interactions or alter the stability or activity of Usp4 to allow it to function in these pathways. However, as mentioned previously, the reporter array was performed with serum present in the media meaning that these pathways could have been induced by low levels of cytokines present in the serum. Again, it would be interesting to individually induce the pathways in the multi-pathway reporter array to further examine effects on pathway activity.

6.3.3 The effect of Usp4 PTM on IL-1-induced NF- κ B activity

The previous chapters in this thesis demonstrated a role for USP4 in TNF α - and particularly IL-1-induced NF- κ B signalling. The multi-pathway reporter array also demonstrated differences in NF- κ B activity with a phosphorylation variant of Usp4, compared to the wild-type, indicating that Usp4 phosphorylation may be involved in regulating Usp4 for its role in NF- κ B signalling. Initially, an NF- κ B responsive luciferase reporter assay was performed for wild-type, catalytically inactive Usp4 and the phosphorylation variant of Usp4. Consistent with data from RNAi experiments in which USP4 depletion reduced NF- κ B activity, overexpression of wild-type Usp4 significantly enhanced NF- κ B activity, but not overexpression of catalytically inactive Usp4. This suggests Usp4 enhanced NF- κ B activity due to its deubiquitinase activity. In line with the multi-reporter array, the increase in IL-1-induced NF- κ B activity was attenuated when phosphorylation at Ser675/Ser680 was prevented. TNF α -induced NF- κ B activity was actually reduced to below control levels of activity when phosphorylation was prevented, indicating that the PTM of Usp4 may alter the function of Usp4 in TNF α -mediated NF- κ B signalling. Overall, it suggested that the phosphorylation of Usp4 at one or both of these serines is important in the IL-1- and TNF α -induced activation of NF- κ B.

Protein levels of Usp4 were also examined for wild-type and both Usp4 variants. Phosphorylation and ubiquitination can crosstalk, adding to the complexity of regulation of proteins. The presence of a phosphodegron or phosphorylation of a protein can serve as a prerequisite for the ubiquitination and subsequent degradation of proteins, as occurs in NF- κ B signalling where I κ B α is phosphorylated by the IKK complex for recognition by the E3 ligase SCF- β -TrCP (Spencer et al., 1999). This is also potentially a mechanism by which DUBs may be regulated. Phosphorylation of USP25 by a tyrosine kinase led to a decrease in USP25 protein levels in the cell indicating that its phosphorylation may affect the stability of the protein (Cholay et al., 2010). Examination of protein levels revealed

there were similar levels of GFP-tagged Usp4 with expression of both Usp4 variants and wild-type Usp4, suggesting that neither the phosphorylation of Usp4 at these sites, nor its catalytic activity, regulate its stability.

Although preliminary experiments demonstrated that overexpression of the non-phosphorylatable Usp4 at Ser675/Ser680 attenuated NF- κ B activity compared to wild-type Usp4, it did not give indications as to which phosphorylation site(s) may be involved. To further explore Usp4 phosphorylation, a series of Usp4 phosphorylation variants were generated next.

6.3.3.1 Generation of Usp4 phosphorylation variants

Overexpression of proteins is an important molecular biological technique for observing the effects of a protein on a particular system. It also allows manipulated versions of the protein to be introduced into cells to allow observation of effects on these systems. It was therefore decided that a series of Usp4 phosphorylation variants would be generated to allow observations of effects on NF- κ B activity with modifications to the phosphorylation status of Usp4.

Initially, Usp4 phosphorylation variants generated by site-directed mutagenesis were used in NF- κ B responsive luciferase assays, as for the non-phosphorylatable Usp4 S675A/S680A variant. The lack of reproducibility of the data in these experiments led to a different, lentiviral-mediated approach as a more stable vehicle for the delivery of the Usp4 variants into cells. One limitation of overexpressing Usp4 in cells is that endogenous USP4 is still present which could affect observations made in these experiments. The overall aim of generating lentiviral preparations for the delivery of variants of Usp4 was for use in *Usp4*^{-/-} MEFs, where the only Usp4 present would be the mutant variant transduced in the cells. Lentiviral-mediated gene expression has many advantages over transient transfection of expression vectors. Primary cells are notoriously difficult to transfect, and transient transfection of MEFs with a lentiviral NF- κ B expression vector previously in this thesis proved unsuccessful. Lentiviral expression vectors are effective vehicles for the efficient delivery and stable expression of genes into primary cells (Stewart et al., 2003). As such, it was decided that lentiviral-mediated gene expression would be used.

The first method approached was a tetracycline-inducible lentiviral expression system. This system required production of lentiviral particles for a response vector, containing an inducible promoter, P_{Tight} , and Usp4 variant, and lentiviral particles for a regulator vector, containing a tetracycline-controlled transactivator, rtTA-Advanced. When transduced into cells, induction with doxycycline should enable rtTA-Advanced to bind to the P_{Tight} promoter to activate transcription of Usp4. After lentiviral production, different conditions including varying the ratio of regulator:response lentiviral preparations and varying the concentration of doxycycline added were tested to try and optimise transduction in SW1353 cells. However, no transduction occurred, as determined by the absence of any expression of GFP (which would be fused to Usp4) when visualised on a fluorescent microscope. Doxycycline concentrations and lentiviral volumes tested were within manufacturer recommended amounts. Viral RNA genome was measured which confirmed the presence of viral particles in both the regulator and response lentiviral preparations although this does not confirm the presence of Usp4. Whether the unsuccessful transductions were due to production of the lentiviral preparations or unsuccessful doxycycline induction were not determined. After several efforts, it was decided to use a different system for lentiviral delivery of Usp4.

The second method approached was a second generation lentiviral expression system. This method uses a constitutive promoter for the expression of the gene and required the production of only one lentiviral preparation for each of the Usp4 variants. As such it was deemed a more appropriate system for these experiments. All 10 Usp4 variants, previously generated by site-directed mutagenesis of the pCMV-AN-Usp4-mGFP vector, were cloned into the lentiviral expression vector, ensuring that Usp4 contained a KOZAK sequence for efficient translation initiation. The Usp4 endogenous stop codon was re-introduced to ensure the GFP fusion was not cloned into the vectors as the lentiviral expression vector contains an IRES GFP. Steps were taken to confirm successful lentiviral preparation and transduction: the presence of the Usp4 insert into the lentiviral expression vectors were confirmed by sequencing, transduction in SW1353 cells was confirmed by the presence of GFP, and to confirm the presence of viral particles in the lentiviral preparations (and to control for equal amounts of viral particles across the series of Usp4 variants) viral titration was performed. The time spent cloning all the Usp4 variants into the expression vectors and confirming successful cloning and the presence of viral particles in the lentiviral preparations allowed confidence in an effective system

for introducing these mutants into cells for examination of Usp4 phosphorylation at the two Ser residues.

6.3.3.2 The effect of Usp4 PTM at Ser675/Ser680 on IL-1-induced NF- κ B activity in SW1353 cells and Usp4^{-/-} MEFs

In conflict to overexpression of wild-type Usp4 by transient transfection, lentiviral-mediated expression of wild-type Usp4 did not significantly increase IL-1-induced NF- κ B activity and catalytically inactive Usp4 significantly increased IL-1-induced NF- κ B activity compared to wild-type Usp4. Similarly to previously, the non-phosphorylatable Usp4 at Ser675/Ser680 significantly decreased NF- κ B activity compared to the wild-type, as did the phosphomimetic Usp4 variants and the single non-phosphorylatable variant at Ser675. The surprising conflict in data was examined by determining the expression of Usp4 with all of the lentiviral Usp4 variants. Many of the Usp4 phosphorylation variants were expressed at much lower levels than wild-type Usp4, possibly indicating unequal or unsuccessful transduction. Another possibility could be that the phosphorylation status of Usp4 does affect the stability of this protein, however, levels of Usp4-GFP were previously examined for the original non-phosphorylatable Usp4 at Ser675/Ser680 and no differences were observed.

Viral titration allowed measurement of the viral RNA genome in the viral preparations. As the same lentiviral expression vector backbone was used for the production of all viral particles and the same protocol and conditions performed, it would be expected that viral titres would be similar for all preparations. However, viral titres revealed differences between viral particles of up to nearly 20 fold in some cases. As the viral titration was only performed once, it is possible that these titres are not reliable. Considering this, it was decided that the NF- κ B responsive luciferase assay should be repeated using equal volumes of viral preparations rather than volumes normalised to the determined titre values.

By doing this, data were in line with data from experiments where overexpression of Usp4 was achieved through transient transfection. Lentiviral-mediated expression of wild-type Usp4 significantly enhanced IL-1-induced NF- κ B activity compared to the control. Again, expression of catalytically inactive Usp4 and non-phosphorylatable Usp4 at both serines reduced the IL-1-induced NF- κ B activity compared to the wild-type, with the lowest levels of NF- κ B activity observed with non-phosphorylatable Usp4, at

Ser675/Ser680. Additionally, Usp4 variants with non-phosphorylatable sites at either Ser675 or Ser680 significantly reduced IL-1-induced activity compared to wild-type Usp4. This indicated that the phosphorylation of both of these sites is important for the function of Usp4 in NF- κ B signalling. Prevention of phosphorylation at one Ser partially restored NF- κ B activity to control levels, and prevention of phosphorylation at both sites reduced NF- κ B activity further. If the phosphorylation of Usp4 at both of these Ser residues does regulate Usp4 in NF- κ B signalling, the phosphorylation of Usp4 at only one Ser may partly compensate for the prevention of phosphorylation at the other Ser, explaining why levels were further reduced when both Ser residues were mutated.

Further, altering one or both of the Usp4 Ser sites to the phosphomimetic Asp significantly reduced the enhanced IL-1-induced NF- κ B activity observed with wild-type Usp4. This indicated that the mechanism of regulation of Usp4 by phosphorylation is more complex than simply requiring phosphorylation at Ser675 and/or Ser680 to function in the NF- κ B signalling pathway. Phosphorylation is a dynamic process and the regulation of Usp4 may require both the phosphorylation and dephosphorylation at one or both sites to function in NF- κ B signalling pathway. Indeed the reduction in NF- κ B activity compared to wild-type Usp4, with variants for which one Ser site was non-phosphorylatable and one Ser site was phosphomimetic, S675A/S680D or S675D/Ser680A, were not as big as reductions in NF- κ B activity where both Ser sites were either non-phosphorylatable or phosphomimetic, S675A/S680A or S675D/S680D.

Although data has shown that Usp4 is phosphorylated at these Ser675/Ser680, there is a possibility that other modifications, such as acetylation or *O*-glycosylation, may also occur at these sites. These PTMs may be required for the regulation of Usp4 and could have been prevented by mutating one or both sites. This could therefore be another explanation for the attenuated ability of Usp4 to enhance NF- κ B activity observed when both sites were mutated to Ala or Asp.

The endogenous USP4 present in these cells may also be altering or masking effects of changing the phosphorylation status of Usp4. Therefore, this experiment was also performed in *Usp4*^{-/-} MEFs where the only Usp4 present was the Usp4 variant lentivirally expressed.

Similarly to previous data, expression of wild-type Usp4 significantly increased NF- κ B activity compared to the control. This increase was attenuated with expression of catalytically inactive Usp4, or when Usp4 is non-phosphorylatable at Ser675 or both Ser sites, Ser675/Ser680, or when Usp4 is phosphomimetic at Ser675, Ser680 or Ser675/Ser680. However, when Usp4 was non-phosphorylatable at Ser675 or Ser sites were both either non-phosphorylatable or phosphomimetic, S675A/S680D or S675D/S680A, IL-1-induced NF- κ B activity was similar to wild-type Usp4 levels. This again indicates that the phosphorylation/dephosphorylation of Usp4 at these sites is important in the role of Usp4 in NF- κ B activation, with perhaps phosphorylation of one site being able to compensate for the prevention of phosphorylation at the other. How Usp4 may be regulated by its phosphorylation may be complex. Although many studies have identified PTMs which regulate DUBs, the full spectrum of PTM-mediated regulation of DUBs is still not well understood and crosstalk between different PTMs add further complexity to the field (Kessler and Edelman, 2011). Further knowledge of USP4 phosphorylation is needed to understand how it may regulate its function. In addition, whether these Ser residues are subject to other modifications needs to be explored.

Given that the phosphorylation status of Usp4 affected the IL-1-induced activity of NF- κ B, it would be interesting to explore effects of Usp4 phosphorylation on NF- κ B-regulated gene expression and on the levels and phosphorylation of NF- κ B adaptor proteins, as was examined with USP4 depletion, but using lentiviral-mediated expression of Usp4 and Usp4 phosphorylation variants in *Usp4*^{-/-} MEFs.

6.3.3.3 Limitations

It should be noted that although the lentiviral-mediated expression of Usp4 variants in these experiments demonstrated data consistent with previous experiments in this thesis, due to time constraints, these assays were only performed once. Reproducibility of these data would enable more definite conclusions to be made. Additionally, equal transduction needs to be confirmed and the effect of Usp4 phosphorylation on the stability of Usp4 further examined. Data herein have demonstrated that the PTM of Usp4 at one or both of these sites does regulate the ability of Usp4 to enhance IL-1-induced NF- κ B activity. It would be interesting to uncover the mechanism of regulation of Usp4 phosphorylation further. No known studies to date have examined the regulation of USP4 phosphorylation at Ser675 and Ser680 and consequently it is not currently known whether phosphorylation

at these sites affects the catalytic activity of Usp4 or affects protein-protein interactions which may enable Usp4 to function in NF- κ B signalling. It was interesting that catalytically inactive Usp4 and non-phosphorylatable Usp4 both attenuated the increase in NF- κ B activity that wild-type Usp4 demonstrated. The phosphorylation of some DUBs regulates their catalytic activity (Reiley et al., 2005, Hutti et al., 2007). The USP4 Ser sites 675 and 680 are located within the catalytic core of USP4 meaning that phosphorylation could regulate its catalytic activity. A DUB assay would allow examination of the catalytic activity of wild-type Usp4, catalytically inactive Usp4 and all Usp4 phosphorylation variants, with and without IL-1 stimulation, and therefore assess whether the phosphorylation of Usp4 regulates its activity with IL-1 induction. This would be needed to further assess the regulation of Usp4 by its PTM at these Ser residues.

6.3.3.4 The phosphorylation status of Usp4 phosphorylation variants

The serine phosphorylation of Usp4 was examined for Usp4 wild-type and phosphorylation variants. This confirmed the serine phosphorylation of wild-type Usp4 and indicated that this serine phosphorylation was less in the non-phosphorylatable variant of Usp4. However, the antibody used detects any serine phosphorylation, not specifically at Ser675 and Ser680. The phosphorylation of USP4 at Ser445 regulates its function in TGF- β signalling and other Ser sites in USP4 have also been identified, including Ser196, Ser 595 and Ser598 amongst others, which could have been detected by the phosphoserine antibody (Zhang et al., 2012)(www.phosphosite.org). Design of phosphospecific antibodies which specifically recognise phosphorylation at Ser675 and Ser680 would allow confirmation of the phosphorylation status of Usp4 and Usp4 phosphorylation variants.

6.3.4 The effect of depletion of a USP4 kinase on NF- κ B signalling

A kinase screen identified GRK family members, especially GRK2, as kinases able to phosphorylate USP4 at Ser675 and/or Ser680. As such, further examination of whether GRK2 may be the kinase regulating the phosphorylation of USP4, potentially for its role in NF- κ B signalling was performed. It has been reported that GRK2 enhances TNF α -induced NF- κ B activity in macrophages, possibly through interactions with I κ B α (Patil et al., 2009). Knockdown of GRK2 in that report resulted in reduced I κ B α phosphorylation and reduced I κ B α but only with TNF α stimulation and basal levels were not affected. This indicates that GRK2 acts in the NF- κ B signalling pathway only upon

stimulation. There are no known studies which have examined GRK2 in IL-1-induced NF- κ B signalling. In this chapter it was demonstrated, using an RNAi approach, that GRK2 depletion did not affect the IL-1-induced activity of NF- κ B. It would be interesting to examine the effect of GRK2 depletion on TNF α -induced NF- κ B signalling.

To further explore the effects of GRK2 on NF- κ B signalling, in comparison to USP4, the effect of GRK2 depletion on the expression of NF- κ B-regulated genes was also examined. Similar to the depletion of USP4, depletion of GRK2 significantly reduced the IL-1-induced expression of *IL-6* and *MMP13*. Although the level of IL-1-induced expression of *IL-8* was significantly reduced with depletion of USP4, no effect was observed with depletion of GRK2. GRK2 has many identified roles in cell signalling pathways, independent of its effects on GPCR signalling including roles in MAPK pathways. GRK2 has been reported to act as a RhoA-activated scaffold protein to mediate binding to Raf1, MEK1 and ERK2 for ERK MAPK activation, downstream of the epidermal growth factor (EGF) receptor (EGFR) (Robinson and Pitcher, 2013). It has also been shown to enhance EGF-induced ERK MAPK activation through complex formation with the EGFR (Gao et al., 2005). Alternatively, GRK2 is also reported to suppress p38 MAPK signalling by phosphorylating p38 to prevent phosphorylation and activation by MKK6 (Peregrin et al., 2006). Clearly, the function of GRK2 is complex. Although the reports mentioned demonstrate effects of GRK2 in EGF-mediated ERK MAPK signalling, it is possible it may have a function in regulating other IL-1-induced pathways. The expression of genes including *IL-6* and *MMP13* are regulated by multiple pathways, including MAPK (Mengshol et al., 2000, Liu et al., 2015). Given that no effect on NF- κ B activity was observed with GRK2 depletion, the differences in gene expression observed here could be due to changes in the GRK2-mediated regulation of pathways other than NF- κ B.

The effect of GRK2 depletion on the levels and the phosphorylation of some of the key NF- κ B proteins that were affected by USP4 depletion was also examined. As previously demonstrated in this thesis, IL-1-induced phosphorylation of IKK α/β and degradation of I κ B α were reduced with depletion of USP4. Depletion of GRK2 had no effect on either of these proteins.

The absence of any effects on IL-1-induced NF- κ B activity or levels of key NF- κ B adaptor proteins, indicated that GRK2 did not directly regulate IL-1 NF- κ B signalling.

Although Usp4 phosphorylation was demonstrated to potentially regulate the function of Usp4 in NF- κ B signalling, and GRK2 was identified as a kinase of USP4, it is possible that GRK2 is not the kinase responsible for this mechanism of regulation in NF- κ B signalling. Several other kinases were also identified in the kinase screen, including GRK1 and GRK6. Alternatively, the effects with GRK2 depletion on NF- κ B signalling may be masked by the presence of other kinases able to phosphorylate USP4. Further study is required to identify which kinase(s) may phosphorylate USP4 at Ser675 and Ser680 for its function within the NF- κ B signalling pathway. By identifying the kinases responsible for USP4 phosphorylation, experiments could be performed using kinase inhibitors or siRNA-mediated depletion of the kinase to monitor effects on NF- κ B signalling, and with Usp4 phosphorylation variants.

6.3.5 Summary

The main aim of this chapter was to further explore Usp4 modification at Ser675/Ser680 through the generation of a series of Usp4 phosphorylation variants. As previous chapters demonstrated a role for USP4 in the NF- κ B signalling pathway, the effect of Usp4 phosphorylation on NF- κ B activity was examined. Time was spent to develop a system for examining the PTM of Usp4 at these sites through generation of lentiviral expression vectors containing all 10 different Usp4 phosphorylation variants for lentiviral-mediated delivery into SW1353 cells and *Usp4*^{-/-} MEFs. Studies in this chapter have identified the PTM of Usp4 at Ser675 and Ser680 as a potential mechanism of regulation for Usp4 within the NF- κ B signalling pathway. By altering the phosphorylation status of Usp4 at these sites, the enhancement of IL-1-induced NF- κ B activity by wild-type Usp4 was attenuated. This indicates that the regulation of Usp4 by its phosphorylation is a complex process. As phosphorylation is a dynamic process, the interplay between the phosphorylation and dephosphorylation at each Ser residue may be crucial to understanding how Usp4 is regulated in this pathway. Alternatively, these Ser sites may also be modified by other PTMs. Use of phospho-specific antibodies for these Ser sites would allow observation of the phosphorylation status of USP4 upon induction with IL-1 to examine in more depth as to how the phosphorylation/dephosphorylation may regulate the function of USP4 in relation to NF- κ B.

Under conditions tested, the subcellular localisation of Usp4 was not affected by preventing phosphorylation at Ser675 and Ser680, but stimulation of the pathways

activated by Usp4 overexpression would be needed to fully determine if this is how Usp4 is regulated within these pathways. Additional examination of whether the phosphorylation of Usp4 regulates its stability, activity or protein-protein interactions is needed to further understanding of how USP4 is regulated in NF- κ B signalling and could be performed using the lentiviral Usp4 phosphorylation variants generated in this chapter.

Identifying the kinase responsible for phosphorylation at these sites would also enable further examination of Usp4 phosphorylation in NF- κ B signalling. GRK2 depletion studies did not suggest a specific role for this kinase in NF- κ B signalling, but multiple USP4 kinases were identified which could potentially phosphorylate USP4 in relation to NF- κ B signalling.

6.3.6 Conclusions

- Preventing Usp4 phosphorylation at Ser657/Ser680 did not affect the subcellular localisation of Usp4 under the conditions assessed
- Prevention of Usp4 phosphorylation at Ser675/S680 attenuated effects of wild-type Usp4 on the activity of cell signalling pathways
- A series of Usp4 phosphorylation variants and lentiviral variants were generated
- Altering the phosphorylation status of Usp4 altered effects of wild-type Usp4 on IL-1-induced NF- κ B activity
- Depletion of the USP4 kinase, GRK2, did not affect IL-1-induced NF- κ B activity or the levels or phosphorylation of proteins involved in the NF- κ B signalling pathway

Chapter 7 : General Discussion

Ubiquitin signalling is a tightly regulated process, critical in multiple cellular processes and cell signalling pathways for the correct functioning of cells. Detrimental effects from aberrations in ubiquitin signalling can lead to the development of diseases and pathologies including many cancers and inflammatory disorders, osteoarthritis, and even the normal ageing process (Kessler, 2013, Popovic et al., 2014, Löw, 2011). Further insight into the regulation of the components of ubiquitin signalling could provide knowledge leading to potential new therapeutic targets for these diseases and pathologies.

USP4 is a member of the ubiquitin-specific protease family of DUBs, responsible for reversing the ubiquitination process. USP4 expression is upregulated in human lung, urinary and prostate cancers and primary cell lines but has also been reported to be reduced in small cell lung carcinoma cell lines (Gray et al., 1995, Frederick et al., 1998, Zhang et al., 2011). USP4 increased migration of breast cancer cells, and in a zebrafish embryo xenograft invasion-metastasis model increased invasion and metastasis of breast cancer cells, indicating that USP4 may possess oncogenic properties and have involvement in the progression of cancers (Zhang et al., 2012). Consistent with this, USP4 stabilises ARF-BP1, an E3 ligase responsible for the ubiquitin and degradation of the p53 tumour suppressor (Zhang et al., 2011).

When this thesis was first started, although many substrates of USP4 had been identified, including the A_{2A} adenosine receptor, spliceosome U4 component Prp3, PDK1 and the S9/Rpn6 subunit of the proteasome, there were limited reports about its involvement in cell signalling pathways or about mechanisms of USP4 regulation (Milojević et al., 2006, Uras et al., 2012, Song et al., 2010, Zhao et al., 2012a). As ubiquitin signalling is key in the tight regulation of many cell signalling pathways, a more comprehensive understanding of the functioning of USP4 within cell signalling pathways and the mechanism of regulation of USP4 would contribute to research into the role of USP4 and in pathologies, including cancers. Further, this could provide understanding which could lead to novel therapeutic targets. In view of this, the aim of this thesis was to establish cell signalling pathways for which USP4 is involved and begin to determine how it functions in these pathways. Moreover, as the regulation of multiple DUBs is dependent on PTMs, including phosphorylation, another aim of this thesis was to explore the potential mechanism of regulation of USP4 by its PTM at Ser675/Ser680.

As this thesis was in progress, reports emerged to implicate functioning of USP4 in cell signalling pathways including Wnt/ β -catenin, NF- κ B, TGF- β and RIG-I (Zhao et al., 2009, Fan et al., 2011b, Xiao et al., 2012, Zhou et al., 2012, Zhang et al., 2012, Wang et al., 2013). Further, preliminary data alluded to a role for USP4 in the positive regulation of NF- κ B signalling and potential effects on inflammatory and immune responses, leading to further focus on the function of USP4 and its regulation in the NF- κ B signalling pathway.

7.1 USP4 in cell signalling pathways

The first aim of this thesis was to establish a role for USP4 in cell signalling pathways. Preliminary experiments indicated that Usp4 may function to suppress p53 activity. This was in line with Zhang et al. (2011) who reported inhibition of p53 due to the USP4-mediated deubiquitination and stabilisation of the p53 E3 ligase ARF-BP1. Additionally, experiments demonstrated effects of Usp4 on other pathways, including enhanced NF- κ B activity and enhanced MAPK/ERK and MAPK/JNK activity. Consistent with initial observations of enhanced MAPK activity with Usp4 overexpression, it was later demonstrated in this thesis that depletion of USP4 suppressed the IL-1-induced phosphorylation of JNK. Therefore overall, initial findings in this thesis elucidated potential functions for USP4 in p53, MAPK and NF- κ B pathways, the latter of which was further addressed.

7.2 Usp4 in immune and inflammatory pathways

In addition to initial observations suggesting a role for Usp4 in pathways associated with inflammation, effects on the circulating levels of specific inflammatory cytokines in *Usp4*^{-/-} mice further implicated a role for the DUB in the immune or inflammatory response. MEFs derived from *Usp4*^{-/-} mice and infected with VSV demonstrated increased VSV compared to wild-type MEFs. Consistent with this, there was increased VSV staining in the brains of *Usp4*^{-/-} mice compared to the brains of wild-type mice infected with VSV. Given that USP4 positively regulates RIG-I signalling, an antiviral immune response pathway, the enhanced levels of virus observed with loss of Usp4 could be indicative of dysregulation of antiviral pathways leading to reduced viral clearance and increased viral replication (Wang et al., 2013). Alternatively, this could be due to dysregulation of anti-inflammatory pathways and an altered inflammatory response.

Further examination would be needed to determine the mechanism of increased VSV with loss of Usp4.

7.3 USP4 in NF- κ B signalling

Preliminary experiments to examine Usp4 in cell signalling pathways, and the effects of Usp4 phosphorylation at Ser675/Ser680 were initially performed in parallel. This examination revealed that overexpression of Usp4 enhanced NF- κ B activity and that this could be attenuated by overexpression of catalytically inactive or non-phosphorylatable Usp4. Therefore, further examination of USP4 in the NF- κ B signalling pathway was carried out as a prerequisite to examining the mechanism of phosphorylation of USP4 in NF- κ B signalling later in this thesis.

During the progression of this thesis studies reported that USP4 negatively regulates NF- κ B signalling (Fan et al., 2011b, Xiao et al., 2012, Zhou et al., 2012). Conversely, preliminary results in this thesis indicated that USP4 positively regulated NF- κ B activity. Further results presented in this thesis confirmed USP4 as a positive regulator of NF- κ B activity. In addition to enhancing NF- κ B activity at basal levels in preliminary experiments, the overexpression of Usp4 was also shown to enhance IL-1- and TNF α -induced NF- κ B activity. In all cases, the ability of Usp4 to enhance NF- κ B activity was attenuated to control levels with overexpression of catalytically inactive Usp4, indicating that Usp4 catalytic activity was required to enhance NF- κ B. The regulation of NF- κ B signalling was further evaluated using an RNAi approach to deplete levels of USP4 in SW1353 chondrosarcoma cells. Consistent with enhanced NF- κ B activity with overexpression of Usp4, suppression of IL-1- and TNF α -induced NF- κ B activity was observed with USP4 depletion. A strength of this work was that the effect of USP4 on NF- κ B was examined by both depleting USP4 and overexpressing Usp4 and catalytically inactive Usp4, and effects were observed in multiple cell lines, with two different stimuli and with transient and lentiviral reporters.

NF- κ B activation ultimately leads to the transcriptional activation of a number of target genes including proinflammatory cytokines, *IL-6* and *IL-8*, and *MMP13* (Shimizu et al., 1990, Kunsch and Rosen, 1993, Mengshol et al., 2000). These genes were chosen due to their transcriptional activation via NF- κ B with both TNF α and IL-1 stimulation: *IL-8* is a classic NF- κ B-regulated gene whereas the regulation of *MMP13* is more complex, requiring NF- κ B and MAPK for its delayed induction. Differences observed between

these IL-1-induced and TNF α -induced NF- κ B-regulated genes with USP4 depletion indicated that USP4 may function differently between IL-1 and TNF α NF- κ B signalling. At varying IL-1 stimulation times over a 24 hour period, USP4 depletion reduced the expression of *IL-6*, *IL-8* and *MMP13*, consistent with suppression of NF- κ B. Although initially TNF α stimulation reduced *IL-6* and *MMP13*, further analysis revealed only reduced *MMP13* expression and enhanced *IL-6* and *IL-8* expression with USP4 depletion and TNF α stimulation. Discrepancies between stimulation with TNF α at a single time point and over a time course are difficult to explain, considering the experimental procedure was controlled and replications performed. Additionally, enhanced NF- κ B-regulated gene expression was not consistent with reduced NF- κ B activity observed, indicating TNF α -induced NF- κ B regulation by USP4 to be complex. The activation of many genes which are regulated by NF- κ B, can also be activated by co-ordination of other IL-1- and TNF α -induced pathways, including *IL-6*, *IL-8* and *MMP13* which are also activated by MAPK pathways (Hoffmann et al., 2002b, Mengshol et al., 2000, Schmucker et al., 2012). Experiments herein also indicated that Usp4 may also function in MAPK pathways, including JNK and ERK, therefore, effects observed on the expression of these genes could also be due to effects on pathways other than NF- κ B or due to crosstalk between NF- κ B and MAPK signalling pathways.

To further explore how USP4 regulates NF- κ B signalling, the effect of USP4 depletion on the IL-1-induced level and phosphorylation of a number of key proteins important for signal transduction were assessed. The aim was to elucidate where USP4 may act within the NF- κ B signalling pathway. As an integral step in the activation of NF- κ B, the degradation of I κ B α was first examined. USP4 depletion enhanced levels of I κ B α at certain timepoints, suggesting alterations in its degradation. Itself a transcriptional target of NF- κ B, expression levels of *I κ B α* were reduced with USP4 depletion, indicating that enhanced protein levels were due to less or slower degradation of I κ B α rather than increased expression. In line with less or slower degradation of I κ B α , USP4 depletion reduced polyubiquitination levels of I κ B α . The reduced expression, ubiquitination and degradation of I κ B α , as a result of reduction of USP4, are all in agreement with suppression of NF- κ B signalling. Together with effects on I κ B α degradation, the downstream effects of USP4 depletion on reducing IL-1-induced phosphorylation of p65 and the upstream effects of USP4 depletion on IL-1 induced phosphorylation of IKK α / β all confirm USP4 as a positive regulator of IL-1 induced NF- κ B signalling. The

phosphorylation of IKK is crucial for downstream I κ B phosphorylation and downstream signal transduction (Zhang et al., 2014). That USP4 depletion dramatically reduced the phosphorylation of IKK α/β indicates that USP4 positively regulates IL-1-induced NF- κ B signalling by functioning upstream of the IKK complex.

In contrast, reports have indicated that USP4 negatively regulates NF- κ B signalling. In opposition to this thesis, it has been reported that overexpression of USP4 inhibited IL-1- and TNF α -induced NF- κ B activity and phosphorylation of IKK α/β , whereas USP4 knockdown enhanced IL-1- and TNF α -induced NF- κ B activity, NF- κ B-regulated gene expression and phosphorylation and degradation of I κ B α (Fan et al., 2011b, Xiao et al., 2012, Zhou et al., 2012). These published studies used IL-1 β for stimulation of the NF- κ B pathway through the IL-1RI, whereas IL-1 α was used herein. Both IL-1 β and IL-1 α bind to the IL-1R, followed by recruitment of the IL-1RAcP to initiate the same signal and therefore exert their biological activity in a similar way (Dinarello, 2011). However, there are differences between IL-1 β and IL-1 α ; for instance both are synthesised as precursor forms, but only IL-1 β requires cleaving to its mature form before it is able to bind to the IL-1R (Contassot et al., 2012). Additionally, the IL-1 α precursor form is able to activate transcription in the nucleus, as well as binding to the IL-1RI at the cell surface (Werman et al., 2004). Whilst the reason for these contradictions are not clear it could be due to type of stimulus, stimulation times, method of USP4 knockdown/overexpression, or cell type or species.

The use of an RNAi approach to deplete USP4 in SW1353 cells was a good, useful technique for examining effects with loss of USP4. Alternatively, the use of null MEFs are a powerful tool for examining effects with loss of Usp4 and as such, were used for observing effects on NF- κ B signalling. *Usp4*^{-/-} MEFs exhibited reductions in IL-1-induced NF- κ B activity and NF- κ B-regulated gene expression, mimicking effects observed in SW1353 USP4 depleted cells and confirming that Usp4 positively regulates IL-1-induced NF- κ B signalling, thus we re-introduced Usp4 into MEFs in lentivirus.

7.4 Targets of USP4 in the NF- κ B signalling pathway

After identifying a role for USP4 upstream of the IKK complex in the IL-1 NF- κ B signalling pathway, this thesis next aimed to identify possible target substrates for deubiquitination by USP4. Multiple proteins within the NF- κ B signalling pathway are subject to various polyubiquitin chain topologies for their regulation and for the

recruitment of downstream signal adaptors (Iwai, 2014). Equally, these polyubiquitin chains are subject to removal by DUBs, adding another level of complexity to the regulation of NF- κ B signalling (Harhaj and Dixit, 2012, Napetschnig and Wu, 2013). Given that overexpression of catalytically inactive Usp4 attenuated the increase observed in NF- κ B with wild-type Usp4, it is likely that USP4 regulates NF- κ B signalling through its deubiquitinating activity. A number of potential targets upstream of the IKK include IRAK1, TAK1 and TRAF6 which have been demonstrated to be polyubiquitinated with chains including linear, and/or K48- and K63-linked polyubiquitin chains (Conze et al., 2008, Cui et al., 2012, Emmerich et al., 2013, Fan et al., 2010, Fan et al., 2012, Lamothe et al., 2007, Zhou et al., 2010). Indeed, USP4 has previously been reported to interact with and deubiquitinate TAK1 and TRAF6 (Fan et al., 2011b, Xiao et al., 2012). However, these reports identified a role for USP4 in downregulating NF- κ B, no reports exist to elucidate how USP4 may positively regulate NF- κ B signalling.

As likely substrates of USP4 deubiquitination, use of computational modelling to hypothesise that USP4 may deubiquitinate the K48-linked polyubiquitination of TAK1 and TRAF6 gave simulation outputs consistent with results demonstrating positive regulation of NF- κ B signalling by USP4. Further evaluation of the polyubiquitination of these potential substrates was performed by examining the effects with loss of USP4. Analysis of the polyubiquitination of FLAG-tagged TAK1 and TRAF6 with USP4 depletion demonstrated reductions in both the K48- and K63-linked polyubiquitination when FLAG was immunoprecipitated after IL-1 stimulation. This was in contrast to previously published data which reported enhanced TNF α -induced TAK1 polyubiquitination of TRAF6 with USP4 depletion and enhanced IL-1-induced TRAF6 polyubiquitination, and specifically K48- and K63-linked polyubiquitination with USP4 depletion (Fan et al., 2011b, Xiao et al., 2012, Zhou et al., 2012). As many DUBs are reported to negatively regulate NF- κ B signalling by the removal of K63-linked polyubiquitin chains, thereby preventing the activation or recruitment of proteins required for signal transduction, it is difficult to predict how USP4 may be positively regulating NF- κ B activity. However, the reduced polyubiquitination of TAK1 and TRAF6 were consistent with downregulation of IL-1-induced NF- κ B signalling, as observed when examining effects on the level and phosphorylation of NF- κ B adaptor proteins and when examining effects on IL-1-induced NF- κ B activity and NF- κ B-regulated gene expression. These alterations in K48- and K63-linked polyubiquitination of immunoprecipitates

therefore suggest, rather than targeting these proteins specifically for deubiquitination of K48- and K63-linked polyubiquitin chains, USP4 may alternatively be responsible for the removal of other, as yet unidentified, polyubiquitin chains. These could be linear chains which are known to be involved in the recruitment of the NEMO subunit of the IKK complex (Emmerich et al., 2013, Iwai, 2014). Although progress has been made in understanding the significance of ubiquitin signalling within cell signalling pathways, and many critical ubiquitination events have been identified, there is still limited information on how different ubiquitin chain topologies may be involved and the exact ubiquitin signalling components involved. Alternatively, the substrate(s) of USP4 may be further upstream of TAK1 and TRAF6 or USP4 may interact with another protein, possibly an E3 ligase, to mediate effects on the K48- and K63-linked polyubiquitination of these proteins. Identification of the substrate(s) which USP4 may specifically target for the positive regulation of NF- κ B therefore remains to be confirmed. Further examination of the polyubiquitination of proteins upstream of the IKK complex and protein-protein interactions of USP4 need to be addressed to fully elucidate the mechanism of function for USP4 in IL-1-mediated NF- κ B signalling.

7.5 The PTM of Usp4 at Ser675/Ser680

In addition to further elucidating a role for USP4 in the NF- κ B signalling pathway, another aim of this thesis was to examine the regulation of Usp4 by its phosphorylation at two serine residues in close proximity to each other, Ser675/Ser680. The mechanism of regulation of DUBs often involves PTMs which have been demonstrated to regulate their stability, activity, protein-protein interactions and subcellular localisation (Kessler and Edelmann, 2011). Given this, effects of the PTM of Usp4 at Ser675/Ser680, presumably phosphorylation, were assessed.

The phosphorylation of proteins has been demonstrated to regulate the translocation of proteins to and from the nucleus to spatially locate the protein to the correct subcellular site for its function. Indeed, whilst this thesis was in progress, the Akt-mediated phosphorylation of USP4 at Ser445 was demonstrated to translocate USP4 to the plasma membrane for interactions with the T β R-I as part of the activation of the TGF- β signalling pathway (Zhang et al., 2012). Unlike its phosphorylation at Ser445, the PTM of Usp4 at Ser675/Ser680 did not affect the subcellular localisation. Whilst the Akt-mediated phosphorylation of USP4 occurred with activated Akt or induction with TGF- β ,

subcellular localisation here was only determined at basal levels. Whether the localisation of Usp4 by its phosphorylation at Ser675/Ser680 is altered in response to induction, perhaps to specifically locate Usp4 only when required for the regulation of a pathway, remains to be determined. The Ser445 phosphorylation mutant could also have been generated to verify localisation changes with alteration of the phosphorylation status of Usp4 at this Ser residue.

In parallel to examining the effect of Usp4 on the activity of cell signalling pathways, the effect of the phosphorylation of Usp4 on the activity of cell signalling pathways was also examined. Preventing phosphorylation of Usp4 at Ser675/Ser680 attenuated effects seen with wild-type Usp4 on the activity of some pathways, including NF- κ B. Given this preliminary experiment identified potential regulation of Usp4 by its phosphorylation for mediating effects on NF- κ B signalling, and that a role for USP4 in NF- κ B signalling was further elucidated previously in his thesis, establishing a method to further assess this was essential. Overexpression of proteins with mutations is a useful way to study the effects on cellular processes, and lentiviral-mediated delivery of the mutated gene into cells is a stable way to introduce the gene into cells, particularly useful for delivery into cells which can be difficult to transiently transfect, such as MEFs. To fully assess the effects of phosphorylation at these sites, a series of phosphorylation mutants were generated, with one or both Ser sites mutated to Ala or Asp to represent non-phosphorylatable or phosphomimetic sites, respectively. Lentiviral preparations of these Usp4 mutant variants allowed evaluation of the effects of Usp4 PTM on NF- κ B signalling in both SW1353 cells and *Usp4*^{-/-} MEFs. The PTM of USP4 at Ser675/Ser680 had not previously been studied and was demonstrated here to be a potential mechanism of regulation of Usp4 within the NF- κ B signalling pathway. Altering the phosphorylation status of Usp4 attenuated IL-1-induced effects of wild-type Usp4 on NF- κ B activity. Phosphorylation is a dynamic process, potentially requiring interplay between the phosphorylation and dephosphorylation of Usp4 at Ser675/Ser680 to regulate its function in the IL-1-induced NF- κ B pathway. Further examination of whether these may regulate Usp4 subcellular localisation when induced with IL-1, or whether they regulate the catalytic activity or protein-protein interactions is needed to fully understand the mechanism of regulation of Usp4 at these Ser sites with relation to NF- κ B signalling.

7.6 Future work

The work presented in this thesis has furthered understanding of USP4 by establishing a role for USP4 in the positive regulation of NF- κ B signalling, potentially regulated by its phosphorylation at two serine residues. Discussion of this work highlighted a number of potential mechanisms by which USP4 could regulate NF- κ B signalling and itself be regulated through its PTMs. Therefore, future work would be required to address these and confirm some of the findings in this thesis, including:

- Overexpression of Usp4 affected the activity of some cell signalling pathways under basal conditions in a multi-pathway reporter array. To further assess the pathways that Usp4 may be regulating, these arrays could be repeated with induction of the pathways.
- *Usp4*^{-/-} mice demonstrated potentially reduced levels of some cytokines, indicating changes in the inflammatory response in these mice. Although the model used identified alterations in the replication of viral infection in the *Usp4*^{-/-} mice, indicative of compromised immune responses, use of an inflammatory model would be more suitable to evaluate the involvement of Usp4 in the inflammatory response. Mice could be injected with LPS to induce an inflammatory response as LPS mimics infection, and effects, such as effects on cytokine levels, between *Usp4*^{+/+} and *Usp4*^{-/-} mice examined.
- To confirm effects on NF- κ B signalling observed with USP4 depletion, overexpression of wild-type and catalytically inactive Usp4 would allow effects examination of the effects of Usp4 on NF- κ B-regulated gene expression and the levels and phosphorylation of NF- κ B adaptor proteins.
- Further examination of the involvement of USP4 in TNF α -mediated NF- κ B signalling could be performed to attempt to elucidate the contradictory observations made in this thesis.
- To further evaluate potential targets of USP4 deubiquitination in the IL-1-induced NF- κ B pathway, USP4 protein-protein interactions could be examined. USP4 could be immunoprecipitated and probed with antibodies for proteins which function upstream of the IKK complex in NF- κ B signalling, including TAK1,

TRAF6 and IRAK1. This would be important in determining where USP4 interacts with the NF- κ B signalling pathway. In addition, other types of polyubiquitination of these proteins, such as linear, could be examined, similarly to K48- and K63-linked polyubiquitination.

- A quantitative proteomics approach could be used to identify USP4 substrates. Tandem-repeated ubiquitin-binding entities (TUBEs) recognise tetra-ubiquitin and allow enrichment of polyubiquitinated substrates from cell extracts. TUBEs could be used to enrich polyubiquitinated substrates in the presence and absence of USP4 with IL-1 stimulation, and identified by mass spectrometric analysis. Alternatively, a stable isotope labelling by amino acids in cell culture (SILAC) strategy could be employed to compare proteomic expression profiles in the presence and absence of USP4 with IL-1 stimulation.
- Altering the phosphorylation status, through mutations, attenuated the enhanced NF- κ B activity observed with Usp4. The phosphorylation status of these mutants needs to be confirmed through use of phospho-specific antibodies.
- Effects of lentiviral-mediated expression of Usp4 and the phosphorylation variants of Usp4 need to be confirmed by confirming equal transduction of cells. Once this has been completed, use of these lentiviral preparations in *Usp4*^{-/-} MEFs would allow further validation of the effects on NF- κ B activity and could be used to examine effects of Usp4 phosphorylation on NF- κ B-regulated gene expression and the levels and phosphorylation of NF- κ B adaptor proteins.
- Importantly, a DUB assay could be performed, using the Usp4 phosphorylation variants, to examine whether the phosphorylation of Usp4 regulates its catalytic activity. To examine whether the PTM of Usp4 at Ser 675 and Ser680 regulates the catalytic activity of Usp4, and whether this is further regulated by IL-1 induction, wild-type Usp4, the C311A Usp4 mutant, and all Usp4 phosphorylation variants would need to be purified from transfected cells which have been stimulated with IL-1 (or left unstimulated). These could then be incubated with a fluorogenic substrate, e.g. ubiquitin-7-amido-4-methylcoumarin (AMC) (Boston Biochem), the cleavage of which can be monitored fluorometrically to determine the deubiquitinase activity of each of the Usp4 variants.

- As specifically depleting the identified USP4 kinase, GRK2, indicated no effect on NF- κ B signalling, use of kinase inhibitors may give more clues as to the regulation of USP4 by its phosphorylation. The phosphorylation of USP4 in the presence of a kinase inhibitor and upon stimulation with IL-1 could be examined by immunoprecipitating USP4 and using phospho-specific antibodies.

7.7 Summary

In summary of the findings in this thesis, it has been demonstrated that USP4 functions in cell signalling pathways, including NF- κ B signalling. Further evaluation revealed involvement of USP4 in the regulation of NF- κ B-regulated gene expression, the ubiquitination and degradation of I κ B α and the phosphorylation of p65 and IKK α/β . Overall, data have shown USP4 to positively regulate IL-1-induced NF- κ B signalling, not previously reported. Data alluded to a role for USP4 upstream of the IKK complex, and indeed further evaluation revealed differences in the K48- and K63-linked polyubiquitination of immunoprecipitated proteins, presumably TAK1 and TRAF6, consistent with USP4 as a positive regulator of IL-1-induced NF- κ B signalling.

Additionally, a series of Usp4 phosphorylation variants were generated, and a series of lentiviral phosphorylation variants, to examine effects of altering two Ser residues, Ser675/Ser680, on NF- κ B signalling. Altering the phosphorylation status of Usp4 attenuated effects of Usp4 on NF- κ B activity, indicating that the PTM at these Ser residues, presumably phosphorylation, is involved in regulating the mechanism of function of Usp4 within the NF- κ B signalling pathway.

These findings have furthered understanding of USP4 and how it may be regulated, contributing to the growing research of ubiquitin signalling, the importance of this in regulating cell signalling pathways, and how components of ubiquitin signalling are themselves regulated. Given the importance of tight regulation of NF- κ B signalling in preventing persistent activation, which can lead to chronic inflammation and cancers, this research will aid understanding in how USP4 may contribute to this regulation.

Appendix

Appendix Table 1. List of model species in the NF- κ B computational model.

Species name	Description	Initial value
DUB	Generic DUB	100
I κ B	Inhibitor of κ B	10
I κ B_mRNA	I κ B messenger RNA	0
I κ B_P	Phosphorylated I κ B	0
I κ B_NF κ B	Complex of I κ B and NF- κ B	100
I κ B_P_NF κ B	Complex of phosphorylated I κ B and NF- κ B	0
IKK	I κ B kinase	100
IKK_P	Phosphorylated IKK	0
IL1	Cytokine interleukin-1	100
IL1_IL1R	IL-1 bound to IL-1 receptor	0
IL6	Cytokine interleukin-6	0
IL6_mRNA	IL-6 messenger RNA	0
IL8	Cytokine IL-8	0
IL8_mRNA	IL-8 messenger RNA	0
ILR1	IL-1 receptor 1	100
IRAK1	IL-1R associated kinase 1	100
IRAK1_P	Phosphorylated IRAK1	0
IRAK1_P_K48Ub[x]	Phosphorylated IRAK1 ubiquitinated with x K48-linked ubiquitins	0
IRAK1_A	Active IRAK1	0
IRAK1_I	Inactive IRAK1	100
MMP13	Matrix metalloproteinase 13	0
MMP13_mRNA	MMP13 messenger RNA	0
MyD88_A	Active myeloid differentiation factor	0
MyD88_I	Inactive MyD88	100
NK κ B	Nuclear factor of κ B	0
NF κ B_P	Phosphorylated NF- κ B (active)	0
MAPK	Mitogen-activated protein kinase	100
MAPK_P	Phosphorylated MAPK	0

Appendix

PP2A	MAPK phosphatase	0
ROS	Reactive oxygen species	0
Sink	Protein degraded	0
Source	Protein synthesised	1
Tak1	Transforming growth factor- β -kinase 1	100
Tak1_K48Ub[x]	Tak1 ubiquitinated with x K48-linked ubiquitins	0
Tak1_K63Ub[x]	Tak1 ubiquitinated with x K63-linked ubiquitins	0
Tak1_Traf6_A_K63Ub6	Complex of Tak1 with active, K63 polyubiquitinated TNF α receptor-associated factor 6	0
Tak1_Ub[x]_Traf6_A_K63Ub6	Complex of Tak1 ubiquitinated with x K63-linked ubiquitins and active, K63 polyubiquitinated TRAF6	0
Traf6_A	Active TRAF6	0
Traf6_A_K48Ub[x]	Active TRAF6 ubiquitinated with x K48-linked ubiquitins	0
Traf6_A_K63Ub[x]	Active TRAF6 ubiquitinated with x K63-linked ubiquitins	0
Traf6_I	Inactive TRAF6	100
Ub	Ubiquitin	1500
Usp4	Ubiquitin-specific protease 4	5, 100

Appendix

Appendix Table 2. Reactions for the NF- κ B computational model.

Reaction name	Reaction	Parameter name	Value
I κ B_degradation	I κ B_P_NF κ B \rightarrow NF κ B	kdegI κ B	5e-3 s ⁻¹
I κ B_NF κ B_binding	I κ B + NF κ B \rightarrow I κ B_NF κ B	kbinI κ BNF κ B	0.1 mol ⁻¹ s ⁻¹
I κ B_NF κ B_P_binding	I κ B + NF κ B_P \rightarrow I κ B_NF κ B	kbinI κ BNF κ Bnuc	1e-5 mol ⁻¹ s ⁻¹
I κ B_NF κ B_release	I κ B_NF κ B \rightarrow I κ B + NF κ B	krelI κ BNF κ B	0.001 s ⁻¹
I κ B_phosphorylation	I κ B_NF κ B + IKK_P \rightarrow I κ B_P_NF κ B + IKK_P	kphosI κ B	1e-5 mol ⁻¹ s ⁻¹
I κ B_transcription	NF κ B_P \rightarrow I κ B_mRNA + NF κ B_P	ksynI κ BmRNA	0.004 s ⁻¹
I κ B_translation	I κ B_mRNA \rightarrow I κ B_mRNA + I κ B	ksynI κ B	0.001 s ⁻¹
I κ BmRNA_degradation	I κ B_mRNA \rightarrow Sink	kdegI κ BmRNA	0.002 s ⁻¹
IKK_dephosphorylation	IKK_P \rightarrow IKK	kdephosIKK	0.1 s ⁻¹
IKK_phosphorylation	Tak1_K63Ub6_Traf6_A _K63Ub6 + IKK \rightarrow Tak1_K63Ub6_Traf6_A _K63Ub6 + IKK_P	kphosIKK	5e-4 mol ⁻¹ s ⁻¹
IL1_degradation	IL1 \rightarrow Sink	kdegIL1	2e-5 s ⁻¹
IL1_ILR1_binding	IL1 + ILR1 \rightarrow IL1_ILR1	kbinIL1ILR1	1e-5 mol ⁻¹ s ⁻¹
IL1_ILR1_release	IL1_ILR1 \rightarrow IL1 + ILR1	krelIL1ILR1	0.001 s ⁻¹
IL1_synthesis	NF κ B_P \rightarrow IL1 + NF κ B_P	ksynIL1	1e-4 s ⁻¹
IL6_degradation	IL6 \rightarrow Sink	kdegIL6	2e-5 s ⁻¹
IL6_transcription	NF κ B_P \rightarrow IL6_mRNA + NF κ B_P	ksynIL6mRNA	0.001 s ⁻¹
IL6_translation	IL6_mRNA \rightarrow IL6_mRNA + IL6	ksynIL6	1e-4 s ⁻¹
IL6_mRNA_degradation	IL6_mRNA \rightarrow Sink	kdegIL6mRNA	1e-4 s ⁻¹
IL8_degradation	IL8 \rightarrow Sink	kdegIL8	2e-5 s ⁻¹
IL8_transcription	NF κ B_P \rightarrow IL8_mRNA + NF κ B_P	ksynIL8mRNA	0.001 s ⁻¹

Appendix

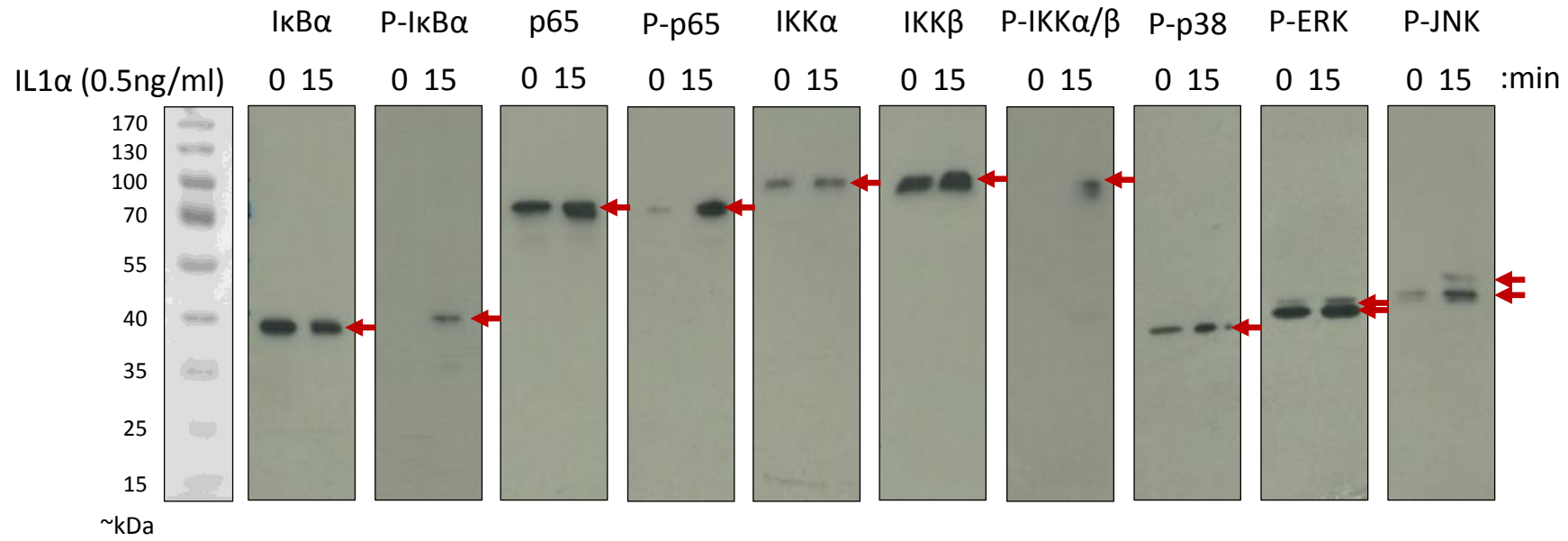
IL8_translation	IL8_mRNA \rightarrow IL8_mRNA + IL8	ksynIL8	1e-4 s ⁻¹
IL8_mRNA_degradation	IL8_mRNA \rightarrow Sink	kdegIL8mRNA	1e-4 s ⁻¹
IRAK1_degradation	IRAK1_P_K48Ub4 \rightarrow 4 * Ub	kdegIRAK1	0.01 s ⁻¹
IRAK1_dephosphorylation	IRAK1_P \rightarrow IRAK1	kdephosIRAK1	0.1 s ⁻¹
IRAK1_K48Deubiquitination[x]	IRAK1_P_K48Ub[x] + DUB \rightarrow IRAK1_P_K48Ub[x-1] + Ub + DUB	kdeubK48IRAK1	0.01 mol ⁻¹ s ⁻¹
IRAK1_K48Ubiquitination[x]	IRAK1_P + Ub \rightarrow IRAK1_P_K48Ub[x]	kubK48IRAK1	1e-4 mol ⁻¹ s ⁻¹
IRAK1_phosphorylation	IRAK1 + IRAK4_A \rightarrow IRAK1_P + IRAK4_A	kphosIRAK1	0.001 mol ⁻¹ s ⁻¹
IRAK1_synthesis	Source \rightarrow IRAK1	ksynIRAK1	0.001 mol s ⁻¹
IRAK4_activation	IRAK4_I + MyD88_A \rightarrow IRAK4_A + MyD88_A	kactIRAK4	1e-4 mol ⁻¹ s ⁻¹
IRAK4_inactivation	IRAK4_A \rightarrow IRAK4_I	kinactIRAK4	0.01 s ⁻¹
MMP13_degradation	MMP13 \rightarrow Sink	kdegMMP13	2e-5 s ⁻¹
MMP13_transcription	NFkB_P \rightarrow NFkB_P + MMP13_mRNA	ksynMMP13mRN A	3e-5 s ⁻¹
MMP13_transcription_by_MAPK	MAPK_P \rightarrow MAPK_P + MMP13_mRNA	KsynMMP13mR NAbyMAPK	3e-5 s ⁻¹
MMP13_translation	MMP13_mRNA \rightarrow MMP13_mRNA + MMP13	ksynMMP13	1e-4 s ⁻¹
MMP13mRNA_degradation	MMP13_mRNA \rightarrow Sink	kdegMMP13mR NA	6.4e6 s ⁻¹
MyD88_activation	MyD88_I + IL1_ILR1 \rightarrow MyD88_A + IL1_ILR1	kactMyD88	1e-4 mol ⁻¹ s ⁻¹
MyD88_inactivation	MyD88_A \rightarrow MyD88_I	kinactMyD88	0.01 s ⁻¹
NFkB_activation	NFkB + MAPK_P \rightarrow NFkB_P + MAPK_P	kactNfkb	0.001 mol ⁻¹ s ⁻¹
MAPK_dephosphorylation	MAPK_P \rightarrow MAPK	kdephosMAPK	0.001 s ⁻¹

Appendix

MAPK_dephosphorylation_PP2A	MAPK_P + PP2A → MAPK + PP2A	kdephosMAPKPP 2A	0.01 mol ⁻¹ s ⁻¹
MAPK_phosphorylation_by_ROS	MAPK + ROS → MAPK_P + ROS	kphospMAPK	1e-4 mol ⁻¹ s ⁻¹
MAPK_phosphorylation_by_IL1	MAPK + IL1_ILR1 → MAPK + IL1_ILR1	kphosMAPK	1e-4 mol ⁻¹ s ⁻¹
PP2A_degradation	PP2A → Sink	kdegPP2A	1e-4 s ⁻¹
PP2A_synthesis	NFkB_P → PP2A + NFkB_P	ksynPP2A	0.001 s ⁻¹
ROS_basal_generation	Source → ROS	kbasalROS	1e-5 mol s ⁻¹
ROS_generation_by_IL6	IL6 → IL6 + ROS	kgenROSIL6	1e-5 s ⁻¹
ROS_removal	ROS → Sink	kremROS	1e-4 s ⁻¹
Tak1_binding_to_Traf6	Tak1 + Traf6_A_K63Ub6 → Tak1_Traf6_A_K63Ub6	kbinTak1Traf6	1e-4 mol ⁻¹ s ⁻¹
Tak1_degradation	Tak1_K48Ub4 → 4 * Ub	kdegTak1	1e-5 s ⁻¹
Tak1_K63Deubiquitination[x]	Tak1_K63Ub[x]_Traf6_ A_K63Ub6 → Tak1_K63Ub[x- 1]_Traf6_A_K63Ub6 + Ub	kdebTak1	1e-7 s ⁻¹
Tak1_K48Deubiquitination[x]	Tak1_K48Ub[x] + DUB → Tak1_K48Ub[x-1] + Ub + DUB	kdeubK48Tak1	0.01 mol ⁻¹ s ⁻¹
Tak1_K48Ubiquitination[x]	Tak1 + Ub → Tak1_K48Ub[x]	kubK48Tak1	1e-4 mol ⁻¹ s ⁻¹
Tak1_K63Ubiquitination[x]	Tak1_Traf6_A_K63Ub6 + Ub → Tak1_K63Ub[x]_Traf6_ A_K63Ub6	kubTak1	5e-6 mol ⁻¹ s ⁻¹
Tak1_release_from_Traf6	Tak1_Traf6_A_K63Ub6 → Tak1 + Traf6_A_K63Ub6	krelTak1Traf6	0.001 s ⁻¹
Tak1_synthesis	Source → Tak1	ksynTak1	0.001 mol s ⁻¹
Traf6_activation	Traf6_I + IRAK1_P → Traf6_A + IRAK1_P	kactTraf6	0.001 mol ⁻¹ s ⁻¹
Traf6_degradation	Traf6_A_K48Ub4 → 4 * Ub	kdegTraf6	1e-5 s ⁻¹

Appendix

Traf6_inhibition	Traf6_A \rightarrow Traf6_I	kinactTraf6	0.01 s ⁻¹
Traf6_K48Deubiquitination[x]	Traf6_A_K48Ub[x] + DUB \rightarrow Traf6_A_K48Ub[x-1] + Ub + DUB	kdeubK48Traf6	0.01 mol ⁻¹ s ⁻¹
Traf6_K48Ubiquitination[x]	Traf6_A + Ub \rightarrow Traf6_A_K48Ub[x]	kubK48Traf6	1e-4 mol ⁻¹ s ⁻¹
Traf6_K63Deubiquitination[x]	Traf6_A_K63Ub[x] \rightarrow Traf6_A_K63Ub[x-1] + Ub	kdeubTraf6	0.01 s ⁻¹
Traf6_K63Ubiquitination[x]	Traf6_A + Ub \rightarrow Traf6_A_K63Ub[x]	kubK63Traf6	1e-4 mol ⁻¹ s ⁻¹
Traf6_synthesis	Source \rightarrow Traf6_I	ksynTraf6	0.001 mol s ⁻¹



Appendix Figure 1. Full length blots of antibodies used in Chapter 4. SW1353 cells were seeded, serum-starved overnight and stimulated with IL-1 (0.5ng/ml) for 15 minutes (min), or left unstimulated, before lysis. Total protein was extracted, resolved by SDS-PAGE and immunoblotted with the relevant antibodies. Red arrows indicate the protein of interest.

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