Investigating NF-κB ubiquitination: An *in vitro* study

Jennifer P. Mitchell
LL.B, B.Sc (Hons)

Submitted in fulfilment of the requirements for the degree of
Doctor of Philosophy

College of Medical, Veterinary and Life Sciences
Institute of Infection, Immunity and Inflammation
University of Glasgow

May 2019

Abstract

NF-κB is a transcription factor family that controls the expression of hundreds of biologically important genes, many of which have essential roles in the regulation of inflammation. When its activity is dysregulated, this can lead to the development of chronic inflammatory diseases such as rheumatoid arthritis. Therefore, it is critical that NF-κB is tightly controlled. The p50 subunit of NF-κB lacks a transactivation domain so when present as a homodimer, it acts as a transcriptional repressor in macrophages to limit the expression of pro-inflammatory cytokines and promote the resolution of inflammation. The stability of p50 homodimers is an important determinant of this repressor function and is controlled by ubiquitin-triggered degradation. Despite this, relatively little is known about the molecular mechanisms that target p50 for degradation, or the cellular components that mediate p50 ubiquitination. By identifying the components of the ubiquitin-proteasome system (UPS) that target p50 for degradation, and in particular, the identity of an E3 ligase for p50, we can intervene therapeutically to prevent ubiquitination and degradation from occurring, and regulate NF-κB activity in a gene-specific manner. In this thesis, the relationship between the known E3 ligases for the other NF-κB subunits and p50 was explored through a series of *in vitro* assays. SOCS1 was observed to promote the ubiquitination and degradation of p50, although this was in some capacity that is independent of both its E3 ligase activity and the proteasome. The role of a known site of ubiquitination of p50 was investigated using a mutant monocytic cell line and was found to have influence over the levels of the other subunits of NF-κB. Furthermore, transcriptomic analysis of two E3 ligase knock-out macrophage cell lines revealed that these selectively control the expression of NF-κB target genes in response to TLR activation. Collectively, the data presented in this thesis advances our understanding of the ubiquitination-controlled regulation of NF-κB transcriptional activity.

Table of Contents

List of figures

List of tables

Abbreviations

ANK	Ankyrin
AP-1	Activator protein 1
ATP	Adenosine triphosphate
βTrCP	Beta-transducin repeat containing
BAFF	B-cell activating factor
BARD1	BRCA1 associated RING domain 1
BCL-3	B-cell lymphoma 3
BCR	B cell receptor
BDP2	BCL-3-derived peptide 2
BMDM	Bone marrow derived macrophage
BRCA1	Breast cancer type 1
BSA	Bovine serum albumin
bp	Base pair
CBL	Casitas B-lineage lymphoma
CCL	C chemokine ligand
CCR	C chemokine receptor
CD40	Cluster of differentiation 40
ChIP	Chromatin immunoprecipitation
cIAP2	Cellular inhibitor of protein 2
COMMD1	Copper metabolism mouse U2af1-rs1 region 1-domain-containing protein 1

COX2	Cyclo-oxygenase
CRISPR	Clusters of regularly interspaced short palindromic repeats
CXCL	Chemokine CXC motif ligand
DAMP	Damage associate molecular patterns
DMEM	Dulbecco's modified eagle medium
DSB	Double stranded break
DTT	Dithiothreitol
DUB	Deubiquitinase
EDTA	Ethylenediaminetetraacetic acid
ERK	Extracellular signal-regulated kinases
FBS	Foetal bovine serum
FIMO	Find individual motif occurances
FPKM	Fragments per kilobase million
GM-CSF	Granulocyte macrophage-colony stimulating factor
gRNA	Guide RNA
GRR	Glycine rich region
GSEA	Gene set enrichment analysis
GSH	Glutathione
GST	Glutathione S-transferases
HDAC-1	Histone deacetylase 1
HECT	Homologous to the E6-AP carboxyl terminus
HEK293T	Human embryonic kidney 293T

HRP	Horseradish peroxidase
ICAM1	Intercellular adhesion molecule 1
IFN	Interferon
IgG	Immunoglobulin G
IκB	Inhibitor of κB
IKK	IκB kinase
IL	Interleukin
IL-1R	Interleukin-1 receptor
Indel	Insertion or deletion mutation
ING4	Inhibitor of growth 4
IP	Immunoprecipitation
IRAK	Interleukin-1 receptor-associated kinase
IRF	Interferon regulatory transcription factor
JNK	c-Jun N-terminal kinase
kDa	Kilodalton
KEGG	Kyoto encyclopaedia of genes and genomes
KPC	Kip1 ubiquitination-promoting complex
LB	Lysogeny broth
LPS	Lipopolysaccharide
LTβR	Lymphotoxin β receptor
LZ	Leucine zipper
MAP3K	MAP kinase kinase kinase

MAPK	Mitogen activated protein kinase
M-CSF	Macrophage colony stimulating factor
MEK	Mitogen/extracellular signal regulated kinase
MHC	Major histocompatibility complex
MKRN2	Makorin ring finger protein 2
MS	Mass spectrometry
MyD88	Myeloid differentiation primary response protein 88
NEMO	NF-κB essential modulator
NES	Nuclear export signal
NF-κB	Nuclear factor-kappa B
NHEJ	Non-homologous end joining
NIK	NF-κB-inducing kinase
NLS	Nuclear localisation signal
N-terminal	Amino terminal
PAGE	Polyacrylamide gel electrophoresis
PAM	Protospacer-associated motif
PAMP	Pathogen-associated molecular patterns
PBS	Phosphate buffered saline
PBS-T	Phosphate buffered saline tween
PCA	Principal component analysis
PCR	Polymerase chain reaction
PDLIM2	PDZ and LIM domain 2

PELI1	Pellino 1
PKA	Protein kinase A
PMA	Phorbol 12-myristate 12-acetate
PPARγ	Peroxisome proliferator activated receptor gamma
PRR	Pattern recognition receptor
PTM	Post-translational modification
RA	Rheumatoid arthritis
RHD	Rel homology domain
RING	Really interesting new gene
RIPA	Radioimmunoprecipitation assay buffer
RNA-seq	RNA sequencing
RPMI	Rosewell park memorial institute
SCF	Skp cullin F-box-containing complex
SCID	Severe combined immunodeficiency
SD	Standard deviation
SDS	Sodium dodecyl sulphate
SEM	Standard error of the mean
SOCS1	Suppressor of cytokine signalling 1
ssODN	Single-stranded oligonucleotide
TAD	Transactivation domain
TBE	Tris borate EDTA
TBP	TATA-binding protein

TCR	T cell receptor
TFBS	Transcription factor binding site
Th	T helper cell
TIR	Toll/interleukin-1 receptor
TIRAP	TIR-domain-containing adapter protein
TLR	Toll-like receptor
TNFα	Tumour necrosis factor alpha
TNFR	Tumour necrosis factor receptor
TPL-2	Tumour progression locus 2
TRAF	Tumour necrosis factor receptor-associated factor
TRAM	TRIF-related adaptor molecule
TRIF	TIR-domain-containing adaptor protein inducing IFNß
Ub	Ubiquitin
UBC	Ubiquitin-conjugating enzyme
USP7	Ubiquitin specific peptidase 7
UV	Ultraviolet
VCAM1	Vascular cell adhesion protein 1
w/v	Weight per volume
w/w	Weight per weight
WB	Western blot
WT	Wild-type
ZFN	Zinc finger nuclease

Amino acid codes

Glycine	Gly	G
Alanine	Ala	A
Methionine	Met	M
Cysteine	Cys	C
Tyrosine	Tyr	Y
Lysine	Lys	K
Arginine	Arg	R
Serine	Ser	S
Threonine	Thr	T

Acknowledgements

I would like to begin by thanking my supervisor, Dr. Ruaidhrí Carmody. You have provided invaluable guidance and mentoring to me throughout my PhD and I have greatly appreciated the time you have spent on my development as a scientist. To my co-supervisors Prof. Carl Goodyear, Prof. Paul Garside and Prof. Andy Clarke, and my advisors Prof. Maggie Harnett and Dr. Pasquale Maffia, thank you so much for your help and advice over the last 3+ years. Furthermore, thanks to everyone involved in the RACE programme and to Versus Arthritis who funded my project.

To every one of my professors at law school – thank you for making me realise that I couldn't care less about selling houses or writing wills (or making money) and that I actually wanted a career in science. In addition, thank you to H&M for frequently updating their range of checked shirts and stripey tops so that I always had something fresh and unique to wear in the lab.

I feel incredibly lucky to have met some of my favourite people during my PhD and without the support and friendship I have encountered I undoubtedly would not have reached this point. Sam and Robin, thank Jebus the universe brought us together that very first day when we all sat in the exact same row at induction. I think we have learned a little bit about science in the last 3.5 years, and a whole lot about what we should be eating based on our star signs. Paul, as one arm of Thursday Club, your shared love of horror movies and boring couple vlogs has provided many welcome distractions from the stress of lab life. You definitely made this experience feel like RuPaul's Best Friend Race. Catherine, though it didn't feel like it at the time, your enthusiasm for 6.30am gym sessions honestly kept what little was left of my sanity together in the last few months, and I miss my gym buddy no end. To level three pals past and present, your ever-willingness to take a break to do the Guardian crossword and for evening therapy sessions watching Bake Off, Drag Race or Game of Thrones has been greatly appreciated.

To my NF-κBaes: Dave, Dom, Emma, Felix, Izaskun and Trish, you kept me smiling even when everything around me in the lab was falling apart, literally and metaphorically. I'm sorry you had to endure me presenting one failed

Western blot after another every week in lab meeting, and I miss us all working together. I must make special mention to Dave who toiled endlessly to make some of the cell lines I used and for generally babysitting me for much of my first year, and to Dom for being the bioinformatician I didn't have the time to become. Before, and after she departed for the Windy City, Trish Collins provided immeasurable lab and life support, and I am eternally grateful. You taught me so much, particularly how quickly I should move when an eppendorf is being fired in my general direction. Thanks to you, when I grow up, I'm going to Bovine University. Emma, towards the end we only had each other (and lysine and the big fat pen) and if I had to go through thesis hell with someone, I'm really glad it was with you.

However, I have also been delighted to be reminded that there is actually a world outside the lab. So, to Aileen, Caroline, Catriona, and my Law Buddies: thank you so much for enduring my rants, for understanding when I couldn't come visit due to work or because I wasn't paid enough to buy a train ticket, and for having such cute daughters/nieces/puppies/bunnies/parrots who never failed to make me smile even when I was feeling blue.

To Dad, thanks for always pretending to understand what I am doing and for spell checking, if not fact checking my manuscripts. I hope that this will at last lead to me being a fully functioning adult with a real grown-up job and you will no longer have to lend me a tenner every time we see each other (however, I will never refuse).

Finally, to Mum: You have been with me every step of the way throughout this experience, and this is for you.

I hope I would have made you proud.

Author's declaration

I declare that, except where reference is made to the contribution of others, that this thesis is the result of my own work and has not been submitted for any other degree at the University of Glasgow, or any other institution.

Signature:

Printed name:

Chapter 1

Introduction

1.1 General

The cellular microenvironment undergoes constant challenge from injury, infection and other stresses causing it to be highly dynamic in nature as it adapts to maintain the physiological integrity of the host. The response of the immune system must be readily inducible, specific and tightly regulated to restore the microenvironment to its homeostatic state. One mechanism by which the immune system achieves this is through the evolutionarily conserved transcription factor, NF-κB. Nuclear Factor-κB (NF-κB) controls the expression of hundreds of biologically important genes, many of which have essential roles in the immune system, which warrants its description as the 'master regulator' of the inflammatory response. In particular, it regulates the expression of genes encoding pro-inflammatory cytokines, chemokines and others that are important for the development of the immune system. NF-κB is activated by a wide array of inducers, including microbial components known as pathogen associated molecular patterns (PAMPs) which are recognised by pattern recognition receptors (PRRs) such as Toll-like receptors (Carmody & Chen, 2007) (figure 1.1). For example, the bacterial component lipopolysaccharide (LPS) is a ligand of TLR4. In addition, endogenous ligands and damage associated molecular patterns (DAMPs) that occur in response to cell death rather than microbial assault have also been implicated in activating TLR responses (Feldman *et al.*, 2015). Once such an activating stimulus has been identified by the cell, the activation of NF-κB is closely regulated by a multitude of elements at various stages throughout the signalling pathway. Considering its extensive influence in a diverse range of biological systems, failure to regulate the activation of NF-κB can result in devastating consequences for the host, such as cancer, neurodegenerative disorder, cardiovascular disease and autoimmune and other chronic inflammatory diseases.

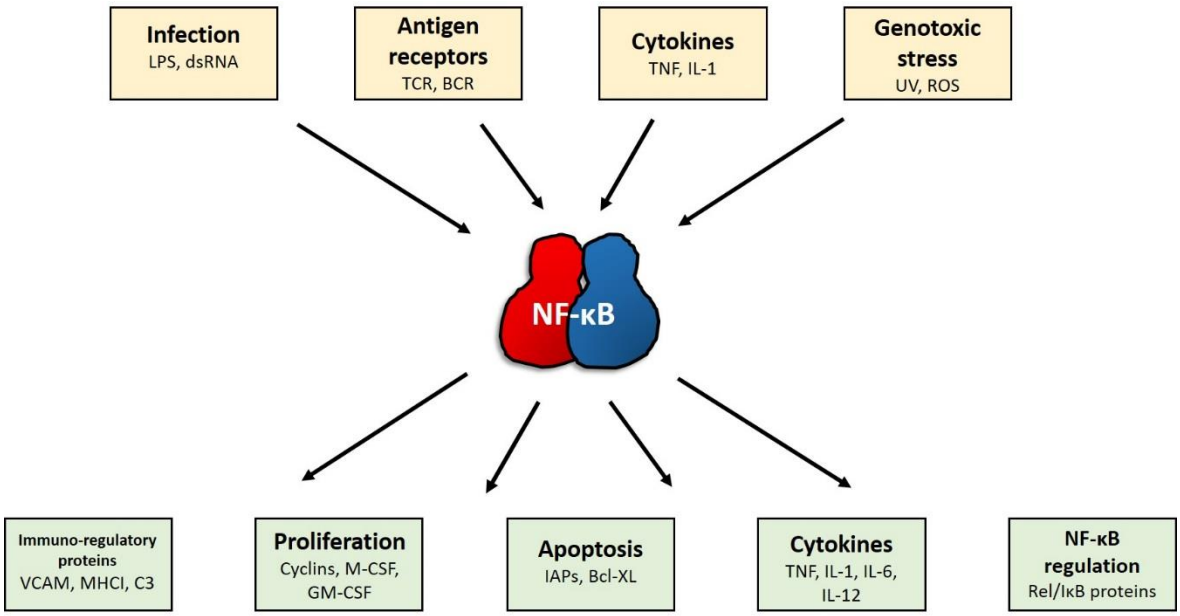


Figure 1.1: Input and output of NF-κB activity.
NF-κB is activated by a diverse array of stimuli such as pathogens, cytokines, environmental and chemical stresses, mitogens and hormones, among others. Once activated, NF-κB controls a great number of transcriptional outputs including the expression of immuno-regulatory proteins, cell cycle factors, cytokines, and regulators of NF-κB creating a negative feedback loop.

1.2 NF-κB

1.2.1 History of NF-κB

Since its discovery over 30 years ago by David Baltimore and Ranjan Sen, NF-κB has firmly rooted itself as the main regulator of inducible gene expression within the immune system (Sen & Baltimore, 1986). NF-κB was initially reported to be a specific binding activity in B cells that recognised an enhancer element in the gene encoding the immunoglobulin-κ light chain, and was named accordingly. However, it was later found to be expressed in almost all cell types but the name NF-κB persisted (Baeuerle & Baltimore, 1988). It was soon understood that NF-κB was an inducible transcription factor fulfilling essential roles in the regulation of the development of and homeostasis within the immune system, as well as orchestrating the inflammatory response. Although it is essential in inflammation, the role of NF-κB in the transcription of the κ light chain for which it is named remains unknown.

1.2.2 Subunits of NF-κB

Despite often being misunderstood as just a single entity, NF-κB in mammals is in fact a family of five related proteins that can bind together to form dimers that are capable of either positively or negatively regulating gene transcription. These five subunits are p65, also known as RelA (and encoded by the gene *rela*), RelB (*relb*), c-Rel (*c-rel*) and p105 (*nfkb1*) and p100 (*nfkb2*) which are further processed into the active subunits p50 and p52 respectively (figure 1.2). All of the subunits share a common, evolutionarily conserved 300 amino acid long Rel homology domain (RHD) at the N-terminal, which allows these transcription factors to bind to the κB sites of gene promotors and to form either homo- or heterodimers with each other, of which there is a possible 15 combinations (figure 1.3). Additionally, p65, RelB and c-Rel also have a transactivation domain (TAD) at their C-terminal that enables these subunits to positively regulate gene transcription. On the other hand, p50 and p52 lack TADs, so when they are present as homodimers they are thought to act as repressors of transcription as they compete with the transcriptionally active dimers to bind to their DNA targets, preventing them from doing so. These subunits can also drive transcription when they are bound to a TAD-containing subunit in the form of a heterodimer, highlighting their dual role in the regulation of transcription.

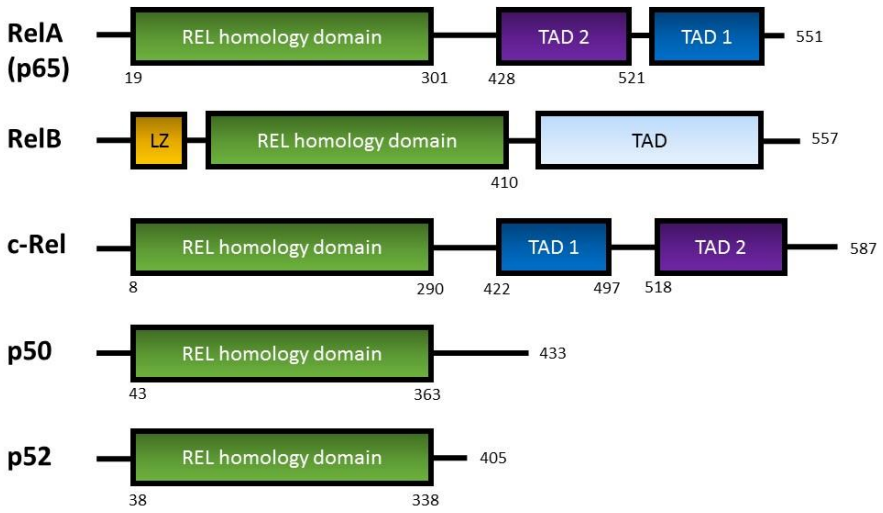


Figure 1.2: Members of the NF-κB Rel protein family.
The mammalian Rel protein family consists of five members: RelA (p65), RelB, c-Rel, p50 and p52 that are derived from the limited proteasomal processing of precursors p105 and p100 respectively. Numbers refer to amino acid sequence. LZ: leucine zipper; TAD: transactivation domain.

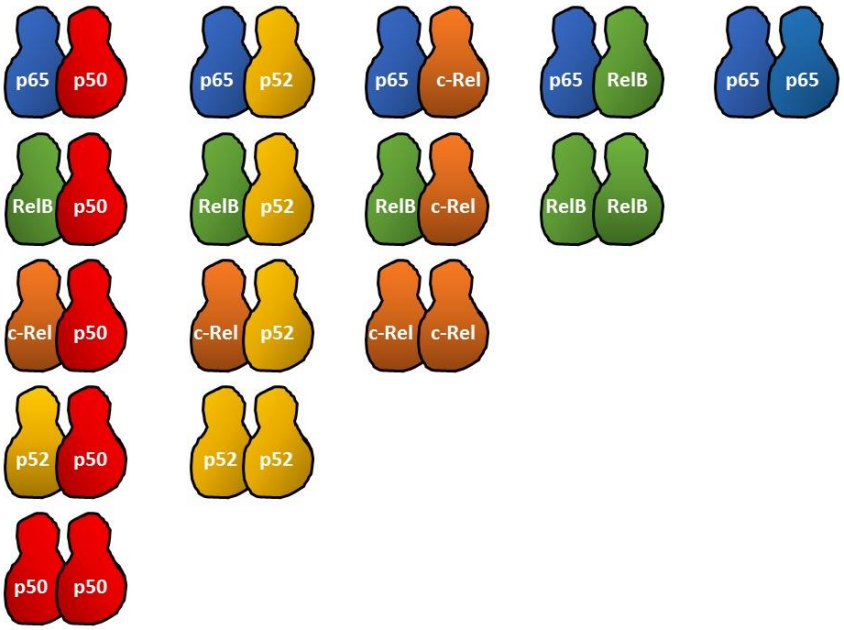


Figure 1.3: Dimer combination of NF-κB subunits.
The presence of the RHD allows NF-κB subunits to form either homo- or heterodimers. p65, RelB and c-Rel contain a TAD domain that confer transcriptional activity upon dimers containing these subunits, whereas p50 and p52 lack this domain and correspondingly lack transcriptional activity. *In vivo*, RelB has only been observed to form dimers with p50 and p52 so there are only 12 dimer configurations that exist biologically.

1.2.3 Regulation by IκB proteins

NF-κB signalling functions through both positive and negative regulation following its induction by a stimulus. In resting cells, NF-κB dimers are sequestered in the cytoplasm in an inactive state by members of a family of inhibitor of κB (IκB) proteins. The typical members of this family are IκBα (*ikba*), IκBβ (*ikbb*), IκBε (*ikbe*) and the precursors of p50 and p52, p105 and p100 (Hayden & Ghosh, 2014). As well as these, there are two atypical IκB proteins: BCL-3 (B-cell lymphoma-3) and IκBζ (figure 1.4). Common among the IκB proteins are multiple ankyrin repeat domains (ANK). Of the IκB proteins, the most extensively studied is IκBα. In the absence of an activating signal, IκBα binds to NF-κB dimers in the cytoplasm, masking their nuclear localisation signals (NLS) and thus preventing them from translocating into the nucleus and maintaining them in an inactive state. Upon receiving an activating signal, the IκB proteins are rapidly phosphorylated at two serine residues (Hayden & Ghosh, 2014) by the IκB kinase (IKK) complex (Baker & Ghosh, 2010) (C Scheidereit, 2006). This phosphorylation event ultimately results in lysine48- (K48) linked polyubiquitination by the Skp cullin F-box-containing complex (SCF)/UbcH5

ubiquitin ligase complex (Wertz & Dixit, 2010) which causes proteasomal degradation of the IκB proteins. In this event, NF-κB dimers are now free to move into the nucleus where they can bind to their DNA targets and regulate gene expression. Although complexes of IκBα:p65:p50 are able to dynamically shuttle between the nucleus and the cytoplasm (Tam *et al.*, 2000), the masking of the NLS on p65 results in NF-κB dimers being maintained in the cytoplasm. IKK contains the catalytic kinases IKKα or IKKß plus a regulatory element, IKKγ (also known as NF-κB essential modifier, or NEMO). It is upon the IKK complex that both activation pathways of NF-κB converge.

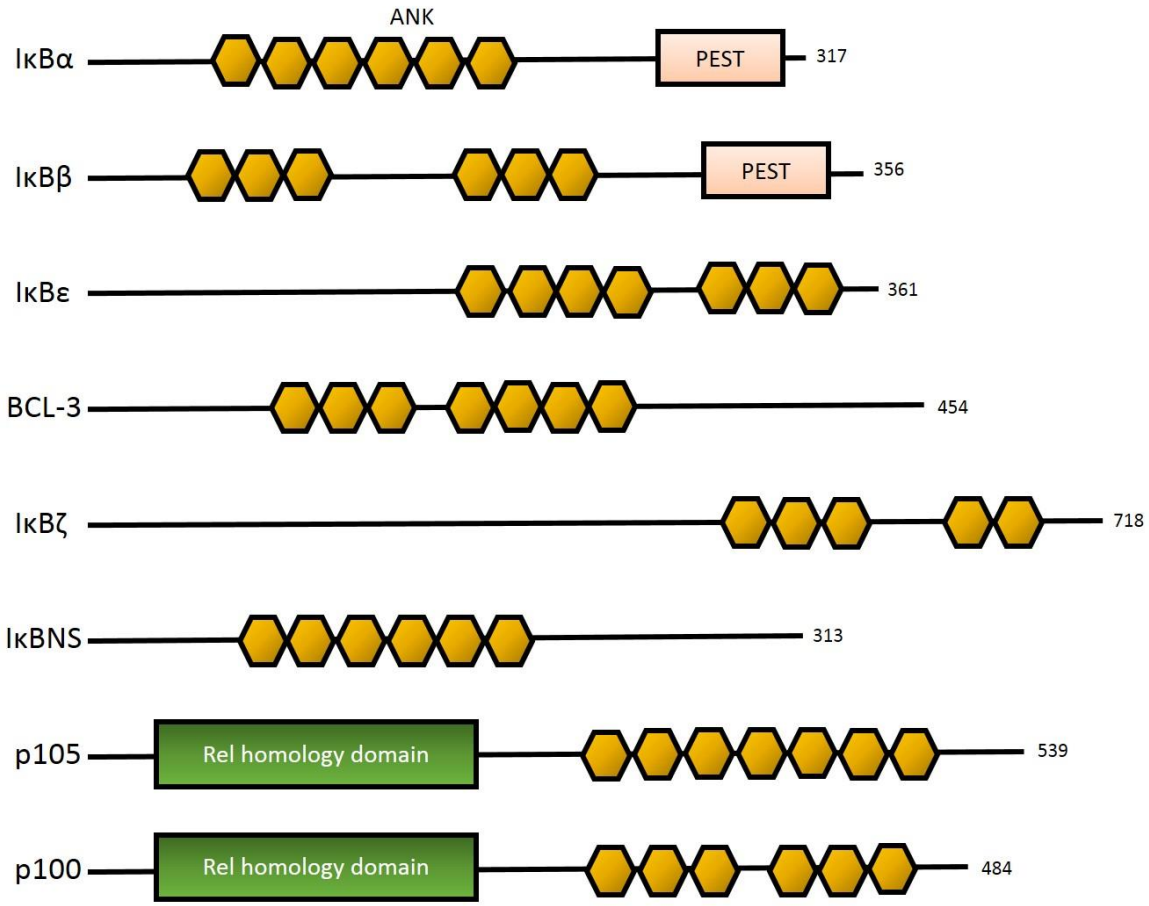


Figure 1.4: Members of the IκB protein family.
There are 8 members of this family, which are IκBα, IκBβ, IκBε, BCL-3, IκBζ, IκBNS, p105 and p100 and are typified by the presence of multiple ankyrin repeats. Numbers refer to amino acid sequence. ANK: ankyrin repeat domains; PEST: PEST domain.

1.2.4 Activation pathways

The two pathways through which NF-κB signalling can progress are the canonical and the non-canonical (Bonizzi & Karin, 2004). The first step of both is the post-

translational modification of the IκB proteins, and as stated above, both converge on the activation of one of two IKK complexes.

1.2.4.1 Canonical pathway

The canonical, or classical, pathway is the general model of NF-κB regulation. The activation stimulus in this activation pathway is the binding of a ligand displaying a pathogen associated molecular pattern (PAMP), or a pro-inflammatory cytokine to a cell surface receptor, such as the members of the Toll-like receptor (TLR), interleukin-1 (IL-1) receptor or tumor necrosis factor (TNF) receptor families. This results in the activation of the IKK complex, which in the canonical pathway, is predominantly the IKKß catalytic subunit that exists in a complex along with IKKα and regulatory NEMO. This phosphorylates IκBα which is bound to the NF-κB subunits at positions serine 32 (S32) (Senftleben *et al.*, 2001a) and S36 (or S19 and S23 on IκBß). This phosphorylation tags IκBα for polyubiquitination by the SCF beta-transducin repeats-containing ($SCF^{\beta TrCP}$) ubiquitin ligase (at positions K21 and K22 on IκBα) and subsequently IκBα is degraded by the 26S proteasome, therefore allowing NF-κB dimers (in this case predominantly p65:p50) to move into the nucleus, bind to DNA and activate the transcription of NF-κB target genes (figure 1.5).

1.2.4.2 Non-canonical pathway

The end result of the non-canonical, or alternative pathway, is the activation of p100:RelB complexes and is reliant upon on IKKα alone, without IKKß or NEMO (figure 1.5). Stimuli that activate the non-canonical pathway lead to the stabilisation of NF-κB-inducing kinase (NIK). NIK phosphorylates and thus activates IKKα, which itself then phosphorylates p100 at S176 and S180 in its C-terminal. Again, this leads to polyubiquitination by the $SCF^{\beta TrCP}$ ubiquitin ligase and degradation by the proteasome, however in this case degradation is only partial. Only the inhibitory C-terminal of p100 is degraded, leaving the N-terminal – the NF-κB subunit p52. This pathway is triggered by a limited number of stimuli including B cell activating factor (BAFF) and lymphotoxinß (LTß), and occurs during the generation of lymphoid organs (Senftleben *et al.*, 2001b). There has been the observation that the non-canonical pathway is almost entirely independent of other pathways except for the MyD88 and mitogen-

activated protein kinases (MAPK) activation pathways. However, the canonical pathway is able to mediate cross-communication with different signalling pathways such as p53, MAPK and interferon regulatory factors (IRF), so this must be taken into consideration when investigating the regulation of the NF-κB activation pathways (Ghosh & Dass, 2016). Whilst the canonical pathway is a rapid responder to infection, the non-canonical pathway is slow and its kinetics depend on the synthesis of new proteins, which is consistent with a role in organogenesis (Baltimore, 2011).

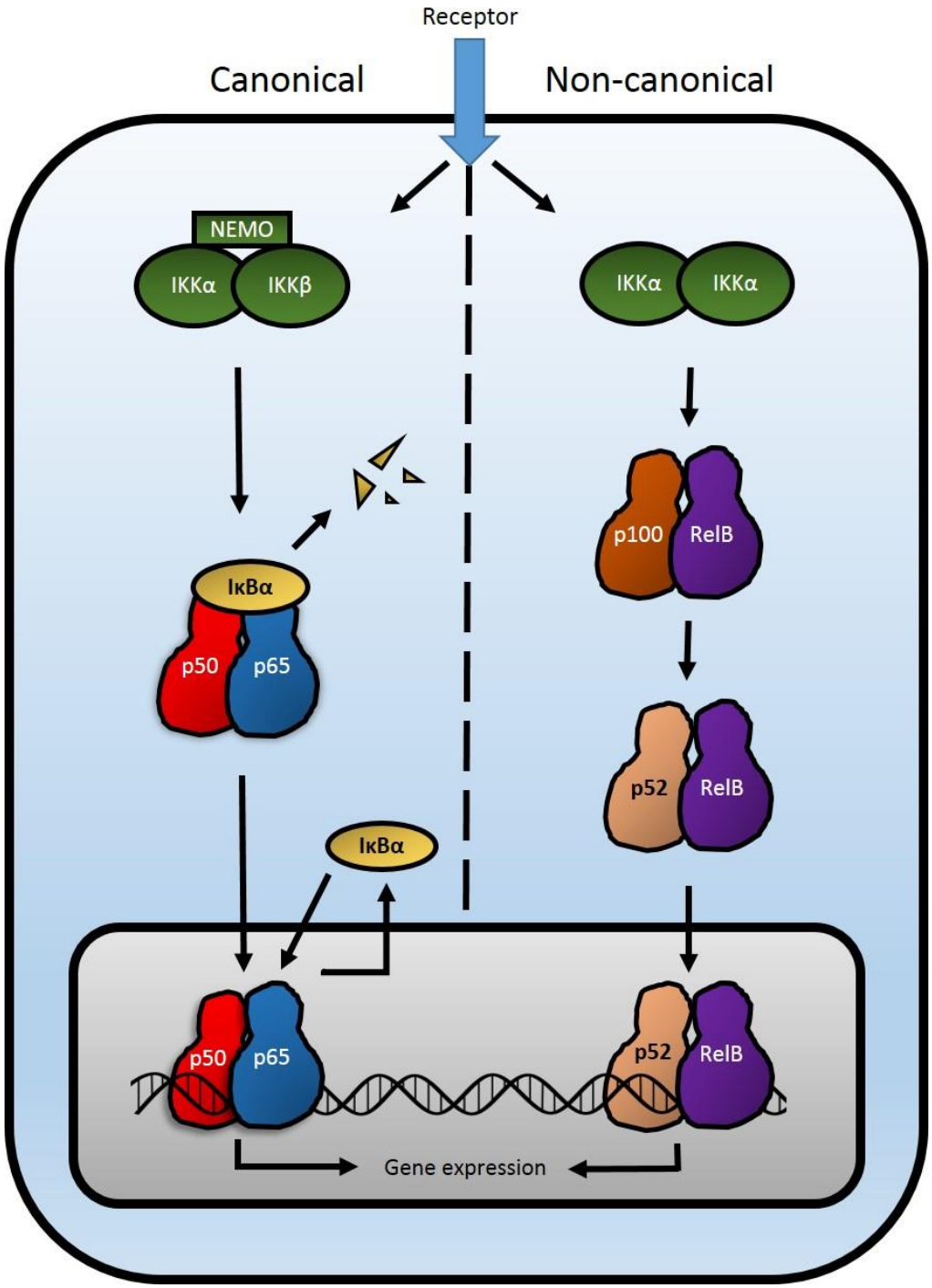


Figure 1.5: Canonical and non-canonical activation pathways of NF-κB.
Upon receiving an activating signal, IκB proteins, which sequester NF-κB dimers in the cytoplasm, are phosphorylated by the IKK complex, marking them for ubiquitination and proteasomal degradation. This frees the NF-κB dimers that can now move into the nucleus and activate gene transcription. In the canonical pathway, the triggering stimuli may be the binding of a TLR ligand resulting in the phosphorylation and degradation of IκBα, following which, p65:p50 dimers move into the nucleus and bind to their DNA targets. The non-canonical pathway is induced by certain TNF family cytokines such as CD40L, BAFF and LTβ and leads to the IKKα-mediated phosphorylation of p100 into p52 that is bound to RelB and is transcriptionally active.

1.2.4.3 Oscillations of NF-κB signalling

It has been observed that NF-κB activity can occur in oscillations between the cytoplasm and nucleus. As detailed above in section 1.2.3, IκB proteins sequester NF-κB dimers in the cytoplasm of resting cells and upon receiving an activating signal, NF-κB is released and translocates to the nucleus. However, NF-κB also controls the transcription of IκBα (Sun *et al.*, 1993) resulting in a negative feedback loop that causes oscillations in NF-κB activity leading to dynamic changes in gene expression that may depend on the number, duration and intensity of such oscillations (Nelson *et al.*, 2004). For example, treatment of cells with TNFα at time intervals that represented pulsative inflammatory responses resulted in synchronised oscillations of translocation of NF-κB from the cytoplasm into the nucleus, with different stimulation intervals resulting in different patterns of gene expression (Ashall *et al.*, 2009). Nuclear:cytoplasmic oscillations were recorded over a period of 100 minutes for a range of TNFα concentrations, although as the concentration was lowered, a smaller fraction of the cells responded which supports the concept of a threshold for activation (Turner *et al.*, 2010).

1.3 The role of NF-κB in inflammation

NF-κB controls the expression of hundreds of biologically important genes (an ever-increasing list can be found at https://www.bu.edu/nf-kb/gene-resources/target-genes/), thus living up to its reputation as the 'master regulator', however, one of its most critical roles is in the regulation of genes that control inflammation. Considering there are 15 possible combinations of NF-κB subunit dimers, this provides some diversity in approaching this complex task. However, there are nuances surrounding the specificity of NF-κB targets genes that influence how this transcription factor controls genes expression, including which other transcription factors are also involved in the signalling pathway, and what the specific binding site sequence is for a particular gene. The hundreds of genes that are activated by NF-κB do so via κB sites in their enhancer or promotor regions. The consensus κB site is 5'-GGGRNWYCC-3' (where N is any base, R is a purine, W is adenine or thymine and Y is a pyramidine) and it is this

sequence that binds to the RHD of NF-κB dimers (Smale, 2012). The different dimer combinations can recognise variations of the κB sequence with different affinities and this leads to the vast array of gene regulatory patterns that emerge from just five protein subunits (Zhang *et al.,* 2017). In order to further discern how such a modest family of transcription factors can respond to a large number of activators and in turn activate the required patterns of gene expression in multiple tissue types we can look to knock-out studies conducted in mice, and more recently to human genetic deficiencies. Mice that have the individual NF-κB subunits knocked out or otherwise modified have provided huge insight into the roles of these subunits themselves as well as their activators and regulators and have highlighted the complexity of this signalling pathway. Almost every technique available for manipulating the mouse genome has been employed in the analysis of NF-κB signalling and the challenging nature of evaluating the individual roles *in vivo* has been revealed due to this complexity and the many redundancies that exist between the various factors (Pasparakis *et al.*, 2006). Additionally, continual progress in the study of human genetic diseases that affect the components of the NF-κB signalling pathway will significantly contribute to our future understanding of the regulation of NF-κB in human health and disease.

1.3.1 NF-κB subunits

1.3.1.1 p105/p50 (*Nfkb1*)

The loss of p50 is not fatal and indeed results in viable adult development when *Nfkb1* is knocked out (Sha *et al.,* 1995). However, there is evidence of impaired innate and adaptive immune function (Cariappa *et al.*, 2000; Artis *et al.*, 2005). As pointed out by Gerondakis *et al.* (2006), it is problematic to blame all of the defects seen in these mice solely on the loss of p50 because a high proportion of p105 subunits act as scaffolds for Tumour progression locus 2 (Tpl2), the apical kinase of the MAPK cascade (Beinke & Ley, 2004). In resting cells, all cellular Tpl2 is bound by p105, while Tpl2-bound p105 represents only a small pool of the total p105 in the cell. This interaction is required to keep Tpl2 in an inactive but stable state. Activation of the MAPK pathway requires the IKKß-induced phosphorylation of p105 and Tpl2, which triggers the proteasomal degradation of

p105 and the activation of Tpl2 (Roget *et al.*, 2012). In the absence of p105, there is little to no Tpl2 found in cells, as it is rapidly degraded. Therefore, *Nfkb1*$^{-/-}$cells essentially have defects in two signalling pathways – NF-κB and MAPK (Yang *et al.*, 2012). In *Nfkb1*$^{-/-}$macrophages, following TLR stimulations there is reduced expression of interleukin-6 (IL-6), IL-10 and cyclooxygenase-2 (COX-2), which are all immune effectors. IL-6 and COX-2 are regulated by both NF-κB and extracellular-signal-regulated kinase (ERK) so this effect was found to result from a combined defect in both pathways (Banerjee *et al.*, 2006).

Natural killer (NK) cells that lack *Nfkb1* however, exhibit enhanced proliferation and increased expression of IFNγ *in vitro* when stimulated with *Toxoplasma gondii* which suggests p105/p50 is a negative regulator of these cells (Tato *et al.*, 2006). B cells from *Nfkb1*$^{-/-}$ mice show some variation: marginal zone and CD5^{+} peritoneal B cells are reduced, however, follicular B cell numbers are normal but turn over more quickly (Grumont *et al.*, 1998; Pohl *et al.*, 2002). B cell activating factor of the TNF family (BAFF) promotes follicular B cell survival by inducing the expression of p52 (Claudio *et al.*, 2002), however, it was discovered that BAFF also activates p50 (Hatada *et al.*, 2003), which may suggest that p50 has a role in BAFF-dependent B cell survival as well. B cells lacking *Nfkb1* exhibit a poor response to LPS, a ligand of Toll-like receptor 4 (TLR4), although B cell receptor proliferation is normal (Sha *et al.*, 1995). In the case of T cells, *Nfkb1* is not essential for maturation but it is indispensable for the development of normal Th2 responses involving the effector cytokines IL-4, IL-5 and IL-13 (Das *et al.*, 2001; Artis *et al.*, 2005). On the other hand, when the C-terminal of p105 is deleted as in *Nfkb1*$^{\Delta CT/\Delta CT}$ mice, this results in overexpression of p50 homodimers in the nucleus and thus highlights the C terminal as an important regulatory domain (Gerondakis *et al.*, 2006). Mice with this mutation exhibit lymphoid infiltration into various organs and enlarged lymph nodes and spleen (Ishikawa *et al.*, 1998). Additionally, B cell numbers are increased and are hyper-responsive, however T cells are less able to proliferate and their capacity to produce cytokines is diminished.

In human studies, heterozygous mutations in *NFKB1* result in common variable immunodeficiency (CVID) which presents as poor antibody responses and ineffective isotype class switching. Furthermore, these mutations result in a

reduction of p105 and p50 in resting cells and greatly diminished p105 phosphorylation following stimulation (Fliegauf *et al.*, 2015; Boztug *et al.*, 2016).

1.3.1.2 p100/p52 (*Nfkb2*)

As with *Nfkb1*$^{-/-}$ mice, *Nfkb2*$^{-/-}$ mice are viable but develop a multitude of immune deficiencies such as the inability to develop normal secondary lymphoid organs, an impairment in B cell maturation and abnormal T cell functionality (Beinke & Ley, 2004). They have disrupted structures of their spleen and lymph nodes in that the perifollicular marginal zone of the spleen is absent and B cell follicular areas are greatly diminished (Caamano *et al.*, 1998).

The link between NF-κB and other signalling pathways was highlighted by *Nfkb2*$^{-/-}$ mice because similar defects were seen in mice that lack lymphotoxinß (LTß), LTß-receptor (LTßR), NIK or RelB (Beinke & Ley, 2004). A signalling pathway that operates downstream of LTßR and which in stromal cells is essential for the p52/RelB-dependent expression of chemokines that are crucial for lymphoid organogenesis was identified (Bonizzi & Karin, 2004). Other TNF-receptor superfamily ligands in addition to LTß, such as BAFF and CD40, also induce nuclear expression of p52/RelB dimers. This is done via the NIK-IKKα-dependent processing of p100, so defects (such as the poor survival rates of B cells) that are seen in mice that lack these ligands will overlap with mice that lack *Nfkb2* (Beinke & Ley, 2004) (Gerondakis & Strasser, 2003).

Activated T cells *in vitro* that lack *Nfkb2* proliferate at a normal rate and exhibit no defects in cytokine production (Franzoso *et al.*, 1998). However, the Th1 responses of *Nfkb2*$^{-/-}$ mice appear to vary depending on the challenging pathogen. These mice are particularly susceptible to assault by *Leishmania major* due to their inability to mount an effective IFNγ response (Speirs *et al.*, 2002) but this is due to diminished CD40-dependent IL-12 production by *Nfkb2*$^{-/-}$ macrophages rather than an inability of *Nfkb2*$^{-/-}$ T cells to mount at Th1 response. Furthermore, challenge by *Toxoplasma gondii* also results in increased susceptibility due to an inefficient IFNγ response, however in this case it is due to a Fas-dependent reduction in the number of T cells (Caamano *et al.*, 2000). Studies conducted in *Nfkb2*$^{\Delta CT/\Delta CT}$ mice, which have deletions in the ankyrin

repeats of the C-terminal, and which cause overexpression of p52 again highlights the importance of the C-terminal as an inhibitory domain. These mice exhibit many abnormalities such as lymphocytic infiltrates into organs, enlarged lymph nodes and granulocytosis (Ishikawa *et al.*, 1997) all of which are hyper-proliferative defects and are consistent with the induction of p52-containing dimers being regulated with many TNF-R superfamily ligands (Beinke & Ley, 2004). The C-terminal of p100 is highlighted as an important inhibitory domain because, when mutated, higher than usual levels of p52 are detected in tissues and there is increased expression of NF-κB target genes. The hyper-proliferative defects observed in mice that overexpress p52 indicate the importance of controlling cell proliferation by regulating the nuclear levels of p52 dimers (Gerondakis *et al.*, 2006). This is further entrenched by observations made in some human lymphomas that have similar alterations in *NFKB2* to the *Nfkb2*$^{\Delta CT/\Delta CT}$ mice (Courtois & Gilmore, 2006).

In human genetic diseases, heterozygous mutations in *NFKB2* are present in an autosomal-dominant common variable immune deficiency (CVID) where patients present with chronic respiratory infections and autoimmune infiltration of the scalp causing baldness, among other symptoms (Shi *et al.*, 2016).

1.3.1.3 c-Rel

Like, p105/p50, c-Rel is essential for the normal function of B and T cells, macrophages and dendritic cells (DCs). c-Rel is responsible for co-ordinating G1- to S-phase of the cell cycle (Grumont *et al.*, 1998) as it is able to induce the expression of E2F3a which is required for cycle progression (Cheng *et al.*, 2003). It also promotes the survival of B cells by upregulating the B-cell lymphoma 2 (BCL-2)-like survival genes, *A1* and *Bcl-xl*. Additionally, B cells with c-Rel knocked-out are defective in isotype switching (Pohl *et al.*, 2002).

In T cells, c-Rel is not required for positive or negative selection (Strasser *et al.*, 1999) but is essential for the control of $CD4^+$ and $CD8^+$ immunity. In $CD4^+$ responses, c-Rel regulates the development of Th1 cells and cytokine expression (Hilliard *et al.*, 2002; Mason *et al.*, 2004). Although c-Rel-dependent production of IL-12 by professional antigen presenting cells (APCs) is important in influencing the development of Th1 cells in a mouse model of multiple sclerosis

(MS) (experimental autoimmune encephalomyelitis, or EAE) (Hilliard *et al.*, 2002), c-Rel is not required for the generation of Th1 cells and IL-12 production by APCs when challenged by *T. gondii* (Mason *et al.* , 2004). In this case, the defective Th1 response in *Rel*$^{-/-}$ mice appears to stem from defective clonal expansion of Th1 effector cells. Furthermore, c-Rel has a role in the production of cytokines by T cells. In CD4^{+} T cells, c-Rel is essential for pro-inflammatory cytokine priming of immune responses leading to greater expression of IFNγ and IL-2 by naïve T cells (Banerjee *et al.,* 2005). When CD4^{+} T cells are activated, c-Rel assists in the production of GM-CSF and IL-2 (Gerondakis *et al.*, 1996) by directly regulating IL-2 transcription via chromatin remodelling on the *Il2* promotor (Chen *et al.*, 2005).

In terms of APCs, *Rel*$^{-/-}$ mice exhibit reduced numbers of plasmacytoid dendritic cells (pDCs) which produce type I IFNs (O'Keeffe *et al.*, 2005). The lack of c-Rel does not negatively affect the development of conventional DCs (cDCs) nor their co-stimulatory protein expression, but it is essential for the promotion of antigen-specific cytotoxic T cell responses (Mintern *et al.*, 2002) and for the expression of the IL-12 gene, *p53* (Grumont *et al.*, 2001). This is in contrast with the requirement for c-Rel in the expression of the IL-12 gene, *p40*, by macrophages (Sanjabi *et al.*, 2000). The reasons for the different requirements for regulation of IL-12 subunit production by c-Rel across different APCs are not understood, however it is speculated that it may be that cDC and macrophages are the essential producers of IL-12 in primary and sustained immune responses (Gerondakis *et al.*, 2006).

Of interest, a role for c-Rel has been implicated in the formation of long-term memories (LTM). Although known to promote the survival of neurons (Pizzi *et al.*, 2002), it was later found that c-Rel binding sites were enriched in the upstream regions of genes that are regulated in the consolidation of LTM and later confirmed by studies using *Rel*$^{-/-}$ mice (Levenson *et al.*, 2004).

1.3.1.4 p65 (*Rela*)

Also known as RelA, the p65 subunit of NF-κB has been difficult to study *in vivo* because p65 KO (*Rela*$^{-/-}$) mice die on embryonic day 15 due to TNFα-mediated foetal hepatocyte apoptosis (Sha *et al.*, 1995), however this is overcome by

either removing TNFα (Doi Marino *et al.*, 1999) or TNF-receptor 1 (Alcamo *et al.*, 2001). Therefore, *Rela*$^{-/-}$*Tnfr*$^{-/-}$ mice are born without major overt abnormalities, including a protective role for p65 against TNFα-induced toxicity. Shortly after birth however, these mice die at increased rates possibly due to greater susceptibility to infection (Alcamo *et al.*, 2001). The protective effect of p65 against TNFα toxicity has also been observed in other cell types such as macrophages (Beg & Baltimore, 1996), B cells (Prendes *et al.*, 2003) and T cells (Senftleben *et al.*, 2001b). However, its anti-apoptotic effect extends beyond TNFα as p65 also protects against apoptosis induced by double-stranded RNA (dsRNA) which is a TLR9 ligand (Li *et al.*, 2001). This feature of p65 derives from its regulation of many genes that encode proteins like cellular FLICE (FADD-like IL-1ß-converting enzyme)-inhibitory protein (c-FLIP), the transcription factor A20 and growth arrest and DNA damage inducible beta (GADD45ß), among others (Dutta *et al.*, 2006).

In *Rela*$^{-/-}$*Tnfr*$^{-/-}$ mice, the structure of the spleen is disrupted and there is an absence of lymph nodes (Alcamo *et al.*, 2002). It was revealed that the role of p65 in the development of secondary lymphoid organs is dependent on radio-resistant stromal cell development rather than haemopoietic cells. Along with p50, p65 reduces sensitivity to LPS-induced toxic shock that can be a consequence of an unregulated innate immune system (Gadjeva *et al.*, 2004). Furthermore, during the initiation of the innate immune response, p65 is required for the recruitment of leukocytes, and in adaptive immunity it is essential for T cell responses (Alcamo *et al.*, 2002) and for B cell isotype switching to IgG3 (Horwitz *et al.*, 1997).

1.3.1.5 RelB

RelB has not been observed to form homodimers unlike the other subunits of NF-κB. Instead, it forms heterodimers with p50 and p52, the highest concentrations of which are found in the thymus, lymph nodes and Peyer's patches suggesting its main role is in the development of secondary lymphoid organs and the regulation of immune cell responses. Mice that lack *Relb* exhibit an abnormal inflammatory phenotype (Burkly *et al.*, 1995) with T cell infiltrates in multiple organs, inflammatory dermatitis, T-cell dependent myeloid hyperplasia and splenomegaly all being observed. As with *Nfkb1*$^{-/-}$ and *Nfkb2*$^{-/-}$mice, *Relb*$^{-/-}$ mice

exhibit disrupted development of secondary lymphoid organs (Yilmaz *et al.*, 2003). These mice do not develop Peyer's patches nor splenic germinal centres and follicular DCs (fDCs) in response to exposure to antigen. In addition, RelB is indispensable for the proper development of the marginal zone and its infiltration by macrophages and B cells (Weih & Caamano, 2003). As with *Nfkb2*$^{-/-}$ mice, the defects in secondary lymphoid organogenesis observed in *Relb*$^{-/-}$ mice are due to the p52/RelB-dependent expression of chemokines regulated by LTβ signalling (Bonizzi & Karin, 2004).

Relb$^{-/-}$ mice have a complete deficiency of certain thymic and splenic DCs which is thought to be a consequence of defective stromal cells and haemopoietic stem cells (Wu *et al.*, 1998). The function of *Relb*$^{-/-}$ B cells *in vitro* appears to be normal. However, *Relb*$^{-/-}$ mice exhibit defects in humoral immunity, perhaps because of an impairment in CD4^{+} T cell responses from deficient APC or T cell function (Weih *et al.*,1997). The expression of RelB by T cells is essential for the proliferation of single positive T cells throughout the latter stages of development (Guerin *et al.*, 2002). It is also required in mature CD4^{+} T cells for normal Th1 function and IFNγ production because *Relb*$^{-/-}$ mice exhibit impaired differentiation to Th1 cells, possibly due to decreased expression of Stat4 (Corn *et al.*, 2005).

In human studies, an autosomal recessive RelB deficiency has been observed in three patients from the same family, who all presented with combined immunodeficiency (CID) from a young age, experiencing frequent infections and severe autoimmune skin diseases (Sharfe *et al.*, 2015).

1.3.1.6 Double knock-out mice

Individual NF-κB knock-out mutant mice provide much information about the unique roles of the subunits within the control of inflammation, however they do not fully reveal their functions because overlapping pattern expressions and redundant roles that are performed by more than one subunit will prevent the full story from unravelling. Therefore, mice with more than one subunit knocked out reveal a wider picture.

Lo et al. (2006) performed analysis of *Nfkb1*$^{-/-}$*Nfkb2*$^{-/-}$ mice, which indicated a role for these subunits in co-ordinating the LTβ-dependent formation of secondary lymphoid organs. The phenotype of mice that lack LTβ is similar to these mice and is more severe than mice with single gene knock-outs. Accordingly, LTβ is an inducer of both p50- and p52-containing dimers in the nucleus. Double knock-outs of *Nfkb1* and *Relb* in mice exhibit more severe organ inflammation than that seen in single *Relb*$^{-/-}$ mice (Weih *et al.*, 1997). The double knock-out mice experience inflammatory infiltrates that are entirely devoid of B cells, indicative of a critical impairment in B cell development. Mice with both *Nfkb1* and *Rel* knocked out display similar defects as the single knock-outs but more severe, for example in the reduction in the CD5^{+} peritoneal B cell population in *Nfkb1*$^{-/-}$ mice is even further diminished in the double knock-outs (Pohl *et al.*, 2002). The embryonic death observed in *Rela*$^{-/-}$ mice due to hepatocyte apoptosis in response to TNFα toxicity occurs at an even earlier stage of development in *Nfkb1*$^{-/-}$*Rela*$^{-/-}$ mutants (Horwitz *et al.*, 1997). Furthermore, this double knock-out mutation reveals a joint role for p50 and p65 in the control of mature B cell activation because although normal haemopoietic cells can rescue the development of these double knock-out mutants, they do not divide in response to mitogenic signals (Horwitz *et al.*, 1999).

1.3.2 IKK complex

1.3.2.1 IKKα

In the canonical activation pathway of NF-κB, IKKβ appears to be the most important kinase while IKKα alone is required for activation of the non-canonical pathway (Scheidereit, 2006). Initial studies demonstrated that mutant mice lacking IKKα (*Ikka*$^{-/-}$) exhibit many skin and skeletal defects that are not seen in mice lacking one or more NF-κB subunits. These developmental defects are not seen in mice with a catalytically inactive allele of IKKα (*Ikka*$^{AA/AA}$), thus revealing a kinase-independent role for IKKα (Cao *et al.*, 2001). The importance of IKKα in the activation of the non-canonical pathway is obvious in these mice, which are phenotypically similar to *Nfkb2*$^{-/-}$, *Ltb*$^{-/-}$ and *Nik*$^{-/-}$ mice, in that they exhibit defects in the development of secondary lymphoid organs and do not produce mature fDCs (Bonizzi *et al.*, 2004; Gerondakis *et al.*, 2006). Somewhat counter-

intuitively, *Ikkα*$^{AA/AA}$ mice also display a heightened inflammatory response to LPS due to increased pro-inflammatory cytokine expression by macrophages (Lawrence *et al.*, 2005). The mechanism behind this response is unclear and has been proposed that IKKα phosphorylation of c-Rel and p65 promotes their degradation. However, it has also been suggested that in the absence of IKKα kinase activity there is a decreased post-stimulus induction of IκBα due to increased IKKß activity, indicating a role for IKKα in the regulation of the IKK complex (Li *et al.*, 2005). This is further supported by studies from IKKα-null bone marrow chimeras that demonstrate immune system abnormalities including altered NF-κB activation in macrophages and defective B-cell function (Senftleben *et al.*, 2001a).

In humans, autosomal recessive loss-of-function mutations in IKKα result in foetal lethality, whereas IKKß deficiency causes severe combined immunodeficiency (SCID) (Pannicke *et al.*, 2013).

1.3.2.2 IKKβ

The importance of IKKß in the classical pathway of NF-κB activation is apparent from the phenotype of mice that lack it which broadly encompasses the phenotypes of *Nfkb1*$^{-/-}$, *Rel*$^{-/-}$ and *Rela*$^{-/-}$ mice. As with *Rela*$^{-/-}$ mice, *Ikkb*$^{-/-}$ mice die as embryos due to TNFα-induced hepatocyte apoptosis (Li *et al.*, 1999). This has meant that much of our understanding of IKKß function *in vivo* has come from studies performed using tissue or cell-specific deletion of IKKß, identifying important cell-specific roles for IKKß in regulating inflammation.

IKKß expression in macrophages is required for the production of pro-inflammatory cytokines (Lawrence *et al.*, 2005) and there has been a link established between IKKß and the inflammation observed during obesity-induced insulin resistence (Arkan *et al.*, 2005). Hepatocyte-specific inactivation of IKKß led to the liver still being responsive to insulin, whereas muscle and fat tissue became resistant. However, mice with IKKß deleted in myeloid cells retained systemic sensitivity to insulin suggesting that the production of pro-inflammatory mediators by myeloid cells as regulated by NF-κB is key to the development of systemic insulin resistance that is the hallmark of type II diabetes. In keratinocytes, the opposite finding was observed, wherein inactivating IKKß

resulted in inflammation (Pasparakis *et al.*, 2002). Mice with epidermis-specific deletion of IKKß developed severe, TNFα-dependent inflammation in the skin shortly after birth. These studies suggest that IKKß in epidermal keratinocytes has a critical function in maintaining immune homeostasis of the skin. This highlights the complex role IKKß plays in the regulation of inflammation, whereby for monocytes IKKß is essential for inflammatory responses, whereas in skin cells, its inactivation leads to an inflammatory response.

In human studies, patients that lack IKKß are born without any obvious defects but develop SCID after birth that is in contrast to *Ikkb*$^{-/-}$ mice, which die as embryos, and further highlights a major physiological difference in the role of IKKß in mice and humans. Cells from patients with a homozygous mutation in *IKKB* had impaired responses to stimulation through T-cell receptors, B-cell receptors, TLRs, inflammatory cytokine receptors and mitogens (Zhang *et al.*, 2017). In the absence of IKKß, the IKK complex consists of IKKα homodimers thus altering the response to some stimuli; for example, phosphorylation and degradation of IκBα and induction of IL-6 is eliminated in response to TLR5 stimulation, but only marginally affected following IL-1ß stimulation (Pannicke *et al.*, 2013). These studies demonstrate that IKKα homodimers can mediate canonical pathway signalling for some stimuli, suggesting IKKα and IKKß may serve different receptors (Solt *et al.*, 2007; Zhang *et al.*, 2017).

1.3.2.3 NEMO

NEMO is a regulatory component of the IKK complex that is essential for the activation of the canonical NF-κB pathway. Mice that lack NEMO (*Ikbkg*$^{-/-}$), similar to *Rela*$^{-/-}$ and *Ikkb*$^{-/-}$ mice, die during embryogenesis due to hepatocyte apoptosis induced by TNFα (Cho *et al.*, 2003). The *Ikbkg* gene is located on the X chromosome so heterozygous null females are viable but exhibit an inflammatory skin disease soon after birth that is similar to incontinentia pigmenti, a human genetic condition caused by NEMO mutations (Makris *et al.*, 2000). Indeed, in humans complete loss of function or amorphic mutations cause pre-natal lethality in males (Zhang *et al.*, 2017)). Hypomorphic mutation in *Ikbkg* cause anhidrotic ectodermal dysplasia with immunodeficiency (EDA-ID) (Döffinger *et al.*, 2001). Cells without NEMO also lack activation of the canonical pathway in response to a number of immune mediators such as IL-1ß, TNFα and LPS (Hubeau

et al., 2011). TNFα activates both apoptotic and NF-κB pathways and so the immune activation of cells requires NF-κB to induce genes that inhibit apoptosis (Karin & Lin, 2002). When NEMO is disrupted, NF-κB does not induce the expression of these anti-apoptotic genes leading to TNFα-induced apoptosis and dysregulated immunity.

Mice with a conditional deletion of NEMO in intestinal epithelial cells ($NEMO^{\Delta IEC}$) revealed a key role for NF-κB in maintaining epithelial barrier function and immune homeostasis in the gut (Nenci *et al.*, 2007). As a result, $NEMO^{\Delta IEC}$ mice develop spontaneous colitis due to defective epithelial barrier function. Of note, this phenotype is observed in mice with the conditional deletion of both *Ikka* and *Ikkb* in intestinal epithelial cells, but not in mice with a conditional deletion of either alone, providing further proof that IKKα and IKKß share some functional redundancy.

T cell-specific deletion of NEMO or the replacement of IKKß with a dominant-negative mutant prevents the development of mature peripheral T cells, demonstrating that mature T cells require NF-κB activity (Schmidt-Supprian *et al.*, 2003). Hepatocyte-restricted NEMO deletion leads to spontaneous chronic hepatitis in adult mice, which ultimately progresses to hepatocellular carcinoma through compensatory hepatocyte proliferation (Luedde *et al.*, 2007). Indeed, this finding that was the first indication that the pharmaceutical inhibition of the IKK complex may not be a viable strategy (Herrington *et al.*, 2016).

1.3.3 IκB proteins

1.3.3.1 Prototypical

The main control of NF-κB transcriptional activity is by the IκB proteins. In resting cells, IκB proteins sequester NF-κB dimers in the cytoplasm where they are unable to bind to their DNA targets in the nucleus and regulate gene expression. However, the nuances of the regulatory role of these proteins are further clarified by studying mice with their encoding genes knocked out. The prototypical IκB proteins are IκBα, IκBß and IκBε, however the regulatory roles of IκBα and IκBß were found to be almost identical (Cheng *et al.*, 1998). When IκB proteins are absent, there is no inhibition of NF-κB activity and so the

consequences on the regulation of inflammation will be detrimental. *Ikba*$^{-/-}$ mice die very soon after birth because of severe inflammatory dermatitis (Sha *et al.*, 1995). IκBα is essential in regulating the expression of pro-inflammatory genes as controlled by NF-κB, therefore in the inflammatory dermatitis observed in *Ikba*$^{-/-}$ mice there is increased levels of TNFα, granulocyte-colony stimulating factor (G-CSF), macrophage inflammatory protein 2 (MIP-2, also known as CXCL2) and eotaxin (Klement *et al.*, 1996). Conversely, mice that lack IκBε are viable and present with only minor immune defects (Memet *et al.*, 1999), such as B cell hyper-proliferation (Alves *et al.*, 2014). Also, mice that lack IκBß have been observed to have hypo-inflammatory responses, with a reduction in TNFα expression and resistance to endotoxin shock and collagen-induced arthritis (CIA) (Rao *et al.*, 2010; Scheibel *et al.*, 2010). More recently, research using mathematical modelling and experimental approaches revealed that fibroblasts and macrophages that lack IκBß also lack p65 homodimers, which are a strong activator of inflammatory gene expression (Tsui *et al.*, 2015). Previous models were unable to account for this phenotype as they assumed that the amount of NF-κB dimers was constant and calculated the control of their subcellular localisation and DNA-binding activity based on IκB degradation and synthesis. This investigation based their model on biophysical measurements of recombinant proteins and *in vivo* experimentation and thus revealed that IκBß acts as an essential positive regulator of the formation of p65 homodimers, whereas IκBα is the main regulator of the dynamics of NF-κB activity, not only of p65:p50 heterodimers but also p65 homodimers (Tsui *et al.*, 2015). They concluded that the prototypical IκB proteins could be categorised into two classes: IκBα and –ε primarily function within the IκB-NF-κB signalling module that is responsive to inflammatory stimuli, whereas IκBß's main function is as a positive regulator of NF-κB dimer formation, which explains the different phenotypes of mice that lack each of these IκB proteins.

1.3.3.2 Atypical IκB proteins

BCL-3 is an atypical IκB protein that, unlike the prototypical proteins, is mainly nuclear in its localisation and is not degraded upon activation of the IKK complex. It binds only to homodimers of p50 or p52 (Nolan *et al.*, 1993), the two transcriptionally inactive subunits of NF-κB. It has the effect of stabilising p50 homodimers by preventing their ubiquitination and subsequent degradation by

the proteasome. BCL-3, therefore, is a negative regulator of TLR-activated immune responses. When BCL-3 is knocked out, *Bcl3*$^{-/-}$ macrophages are hyper-responsive to TLR stimulation and do not develop TLR tolerance (Carmody *et al.*, 2007). This was attributed to p50 homodimers undergoing greater ubiquitination and proteasomal degradation, thus shifting the balance between repressive and activating NF-κB dimers in the nucleus toward the activating end of the spectrum. The result of this is dysregulated cytokine production due to improper NF-κB dimer loading and exchange (Palmer & Chen, 2008) as indicated by the increased expression of pro-inflammatory genes such as *Tnfa* and *Il6*. Furthermore, the formation of secondary lymphoid organs is disrupted in *Bcl3*$^{-/-}$ mice as the structure of the germinal centre of the spleen is diminished which is accompanied by a reduction in follicular B cells and marginal zone macrophages (Franzoso *et al.*, 1997; Schwarz *et al.*, 1997; Gerondakis *et al.*, 2006). Similar defects were found in mice that lacked the *Nfkb1* and *Nfkb2* genes, which reinforces the link between BCL-3 and these subunits. BCL-3 was reported to have a role independent of being a negative regulator of inflammation by O'Carroll et al. (2013) when *Bcl3*$^{-/-}$ mice were observed to be less sensitive to an induced model of colitis than wild-type (WT) controls and retained more integrity in the structure of colonic tissue. There was no difference between the mutants and WT counterparts in the expression of pro-inflammatory cytokines, but *Bcl3*$^{-/-}$ mice exhibited greater proliferation of intestinal epithelial cells.

1.4 The ubiquitin-proteasome system and NF-κB

Considering the ability of NF-κB to control the expression of many genes, it is essential that its activity is regulated, and that this regulation occurs at various levels to prevent the negative consequences of dysregulation such as autoimmune disease, cancer and sepsis. The primary point of control of NF-κB transcriptional activity is through the IκB proteins, which, as outlined in section 1.2.3, sequester NF-κB dimers in the cytoplasm in resting cells. When an activating signal is received, the IKK complex phosphorylates the IκB protein resulting in its subsequent ubiquitination and degradation by the proteasome. NF-κB dimers are now free to translocate into the nucleus, bind κB sites on promoter and regulate gene expression (Hayden & Ghosh, 2012). However, the

degradation of the IκB proteins by themselves in not sufficient to result in an optimal NF-κB response (Oeckinghaus & Ghosh, 2009) and the human and mouse studies detailed in section 1.3.1 do not explain the full story of how NF-κB regulates transcriptional activity. In recent years, a number of investigations have shed light on further regulatory mechanisms that are essential for the control of NF-κB transcription (Christian *et al.*, 2016). In addition to the phosphorylation of the IκB proteins and the IKK complex, they also undergo other post-translational modifications (PTMs), and the NF-κB subunits themselves are subject to a number of modifications that contribute to the regulation of the signalling system including phosphorylation, ubiquitination, acetylation, glycosylation and nitrosylation (Huang *et al.*, 2010). A clear emerging theme from studies on NF-κB modification is that many regulate NF-κB transcriptional activity in a gene-specific manner, and demonstrate the further complexity of NF-κB regulatory networks that incorporate the local environment of promoter and enhancer regions.

1.4.1 Background

Ubiquitin was discovered some 40 years ago when a small (8.5 kDa), highly conserved polypeptide of 76 amino acids was isolated from bovine thymus (Schlesinger *et al.,* 1975) (figure 1.6). It was so named because ubiquitin is found in all eukaryotes, and is so highly conserved that human and yeast share 96% sequence identity. This highlights its essential role within biological systems. The initial studies that elucidated ubiquitin's role in ATP-dependent proteolysis following the covalent attachment of ubiquitin to a substrate protein outlined the beginning of what is now known as the ubiquitin-proteasome system (UPS).

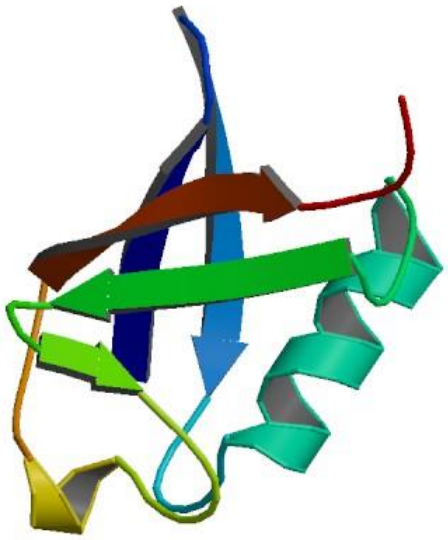


Figure 1.6: Crystal structure of ubiquitin monomer.
Prominent features include three and a half alpha-helix turns, a short 3(10)-helix, a mixed beta-sheet that contains five strands and seven reverse turns. There is a hydrophobic core between the alpha-helix and beta-sheet (Vijay-Kumar *et al.*, 1987).

Ubiquitination is the process of these ubiquitin molecules being covalently attached to the lysine (K) residues of a substrate protein, and it controls a diverse array of cellular processes (table 1.1). These include cell cycle and division, DNA repair, transcriptional regulation and signal transduction (Geng *et al.*, 2012). However, its most prominent role is in the regulation of protein stability as ubiquitination acts as a molecular signal, targeting proteins for degradation by the proteasome, most commonly in the form of K48-linked poly-ubiquitin chains. Thus, the turnover of proteins and the elimination of damaged or misfolded proteins is tightly controlled.

Table 1.1: List of poly-ubiquitin linkages and their function

Residue	Function	Reference
M1	NF-κB activation	Tokunaga *et al.*, 2009 Walczak *et al.*, 2012 Gerlach *et al.*, 2011
K6	DNA damage response Parkin-mediated mitophagy	Morris & Solomon, 2004 Ordureau *et al.*, 2014
K11	Cell cycle regulation Proteasomal degradation Endoplasmic reticulum-associated degradation (ERAD) Wnt/β-catenin signalling	Matsumoto *et al.*, 2010 Meyer and Rape, 2014 Bremm & Komander, 2011 Hay-Koren *et al.*, 2011
K27	PKC signalling pathway DNA damage response Parkin-mediated mitophagy Differentiation of T cells	Okumura *et al.*, 2004 Gatti *et al.*, 2015 Glauser *et al.*, 2011 Peng *et al.*, 2011
K29	Differentiation of T cells AMPK-related kinases regulation Wnt/β-catenin signalling	Chastagner *et al.*, 2006 Al-Hakim *et al.*, 2008 Hay-Koren *et al.*, 2011
K33	Differentiation of T cells AMPK-related kinases regulation Post-Golgi trafficking	Huang *et al.*, 2010 Al-Hakim *et al.*, 2008 Yuan *et al.*, 2014
K48	Proteasomal degradation Lysosomal degradation	Chau *et al.*, 1989 Zhang *et al.*, 2013
K63	DNA damage response NF-κB signalling	Liu *et al.*, 2018 Ohtake *et al.*, 2016

1.4.2 Ubiquitin cascade

The covalent attachment of an ubiquitin molecule to a substrate protein is a process involving three enzymes: an ubiquitin-activating enzyme (E1), an ubiquitin-conjugating enzyme (E2) and an ubiquitin ligase (E3) (figure 1.7). Firstly, ubiquitin is activated in a process that requires ATP, whereby the C-terminal carboxyl group on ubiquitin is linked to the sulfhydryl group of an E1 enzyme by a thioester bond. Following this, the activated ubiquitin is transferred to the active site cysteine on an E2 enzyme and finally the E2 works with the E3 ligase to conjugate ubiquitin to a substrate protein by an isopeptide bond between the C-terminal glycine (G) of the ubiquitin molecule and a lysine residue of the substrate. This attachment may also occur non-conventionally at residues other than lysine (Kravtsova-Ivantsiv *et al.*, 2015). Ubiquitination may cease after the addition of a single ubiquitin molecule (monoubiquitination), or many ubiquitin molecules may attach via the preceding ubiquitin forming a polyubiquitin chain. Multiple ubiquitin molecules may attach at different lysine residues to create a multi-monoubiquitinated substrate protein, or multiple chains of ubiquitin may attach at multiple residues resulting in multi-polyubiquitination (figure 1.8). Polyubiquitin chains may form via one of the seven lysine residues on ubiquitin itself (K6, K11, K27, K29, K33, K48 and K63), or the N-terminal methionine residue (Met or M1) (Komander and Rape, 2012).

Only two E1 enzymes have been identified in humans (UBA1 and UBA5), but there are around 50 E2s and upwards of 600 E3s (Berndsen and Wolberger, 2014) which allows for great specificity within this process (Metzger *et al.*, 2014). A single E3 enzyme can interact with several other E3 ligases which themselves selectively bind multiple substrate proteins. It is via this mechanism that the many thousands of proteins within the proteome can be tightly regulated by a relatively small number of enzymes. Additionally, E4 ligases have been shown to enhance the activity of E3 ligases in some cases. These factors can bind to a single conjugated ubiquitin or polyubiquitin chain, extend, and regulate the length of the chain (Baranes-Bachar *et al.*, 2018). For example, ubiquitin fusion degradation 2 (UFD2) was the first identified family of E4 enzymes in yeast, and is characterised by a C-terminal U-box that is structurally similar to the RING domain of RING E3 ligases, which are described in more detail below. It binds to substrates that have been conjugated with between one and three ubiquitin

molecules and facilitates the addition of further molecules in the presence of E1, E2 and E3 enzymes, resulting in multi-ubiquitinated substrates that are targeted by the proteasome (Hoppe, 2005).

Furthermore, once a protein has been ubiquitinated, this can be recognised by other proteins that contain a ubiquitin-binding domain (UBD) that can bind non-covalently to ubiquitin signals, most commonly around isoleucine at position 44 (I44) of the ubiquitin molecule (Husnjak & Dikic, 2012). Over 20 families of UBDs have been identified and have different specificities for the different ubiquitin chain linkages. This allows for ubiquitination to act as a signal that triggers other molecular events within cells such as the regulation of protein stability, receptor trafficking in the endosome, DNA damage responses and inflammatory pathways (Dikic *et al.*, 2009).

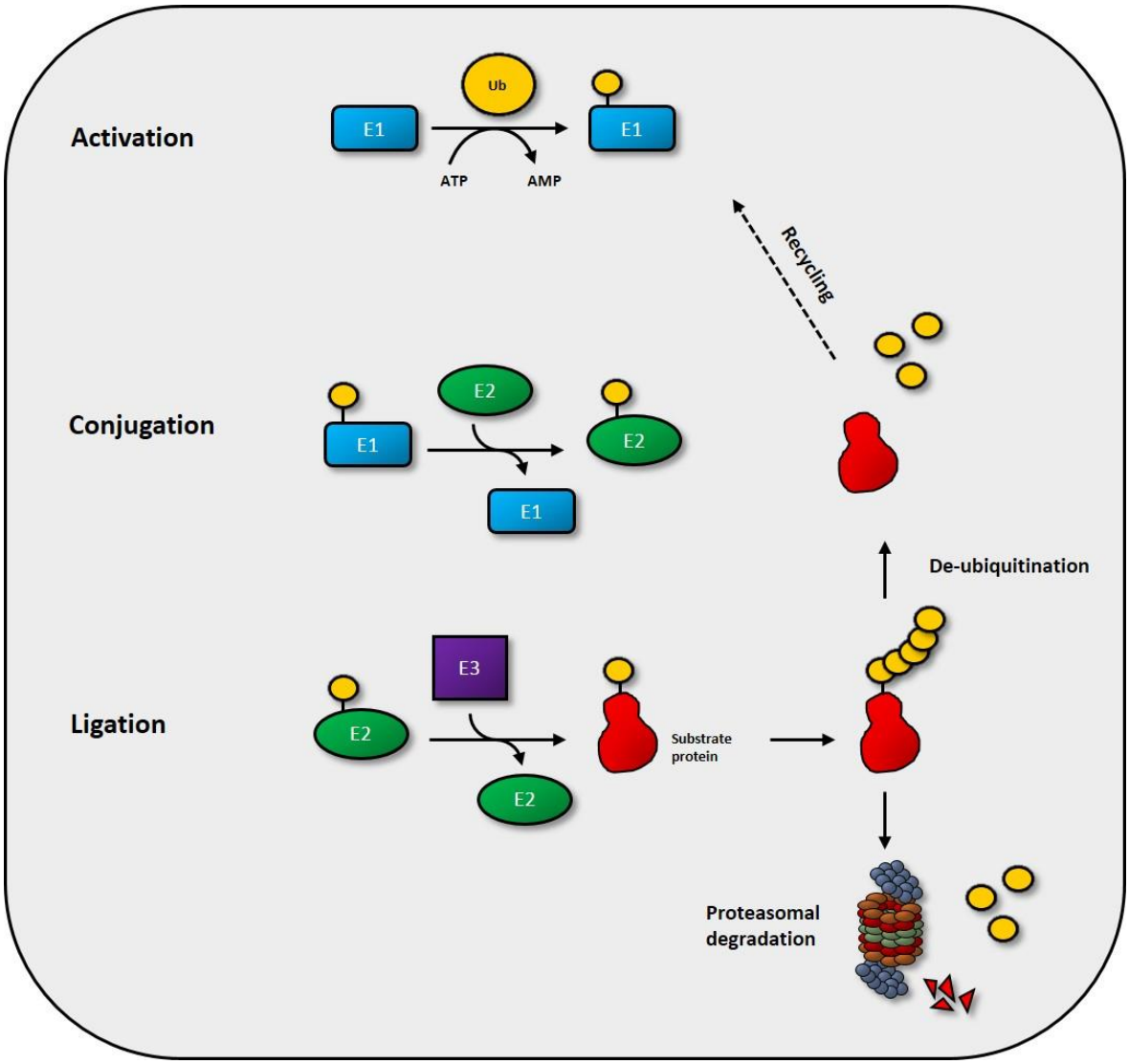


Figure 1.7: The ubiquitin cascade.
Ubiquitination of a substrate protein occurs via a three-step enzymatic reaction. Firstly, ubiquitin is activated in an ATP-dependent reaction by the E1 ubiquitin-activating enzyme by way of adenylation of the C-terminal carboxyl group. Then ubiquitin is transferred via a thioester linkage to the active site cysteine on the E1, releasing AMP and pyrophosphates. Next, ubiquitin is transferred to a sulfhydryl group on the E2 ubiquitin-conjugating enzyme by way of a trans-thioesterification reaction. Finally, an E3 ubiquitin-ligase catalyses the transfer of ubiquitin to the substrate protein. Ubiquitination can be reversed by de-ubiquitinating enzymes (DUBs) which hydrolyse the isopeptide bond between ubiquitin and the substrate, or the peptide bond between ubiquitin molecules to remove the modification partially or completely. Ubiquitin is then recycled back into the UPS maintaining a constant available pool.

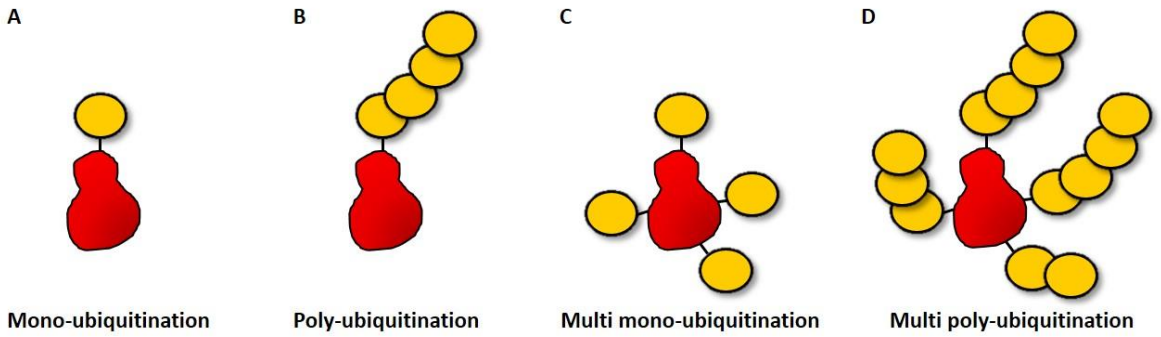


Figure 1.8: Different types of ubiquitin attachments.
(A) A single ubiquitin molecule is attached via an isopeptide bond to the substrate protein, usually at a lysine residue. Either this can occur at a specific residue, or it can be confined to a domain. (B) When the N-terminus of one of the seven lysine residues present on ubiquitin itself is modified this leads to poly-ubiquitination. Ubiquitin chains may be short, consisting of just two molecules, or can incorporate upwards of ten. The chains may be homogenous if the same K residue is modified, or mixed if the residues are different. (C) Multiple K residues on the substrate protein may be modified by single ubiquitin molecules, leading to multi mono-ubiquitination. (D) Multiple K residues on the substrate protein may be modified by multiple ubiquitin chains, leading to multi-polyubiquitination. These may be homogenous or mixed causing branched chains.

1.4.3 Ubiquitination and the control of transcription by NF-κB

Current research has begun to explore the relationship between the UPS and transcriptional regulation. It is surprising that, in addition to controlling the steady state levels of transcription factors, their proteolysis has been implicated in both the repression and promotion of gene activity. Although as important in transcriptional regulation are the non-proteolytic functions of ubiquitination, such as determining the subcellular localisation of transcription factors (Hoppe *et al.*, 2000), their interaction with other co-activators (Kaiser *et al.*, 2000) and the duration of their occupancy of promoters (Saccani *et al.*, 2004).

Even so, the proteolysis of transcription factors as induced by ubiquitination remains the prominent way in which the UPS regulates transcription. It controls the abundance of protein that is available for activation and destabilises the interaction between transcription factors and DNA thus preventing uncontrolled gene expression (Molinari *et al.*, 1999).

With regards to NF-κB activity, and as mentioned above in section 1.2.3, ubiquitination of the IκB proteins by the E3 ligase complex $SCF^{\beta\text{-}TrCP}$ follows their initial phosphorylation at S32 and S36 by the IKK complex as triggered by a stimulating event (Brown *et al.*, 1995). This induces the proteasomal degradation of IκBα that has, until now, sequestered NF-κB dimers in the

cytoplasm and so these dimers are now free. The inducible degradation of IκBα provides a highly sensitive and fast system allowing NF-κB dimers to translocate into the nucleus, bind their κB target sites on DNA and activate gene expression. This is also a negative feedback loop, as NF-κB also induces the expression of the *Nfkbia* gene that encodes IκBα, thus limiting its own response. When IκBα is re-synthesised it enters the nucleus, dissociates NF-κB that is bound to DNA and shuttles it back into the cytoplasm (Collins *et al.*, 2016). In fact, it was in studying *Ikba*$^{-/-}$ cells that the role of ubiquitination in regulating NF-κB activity was initially identified. Despite lacking the critical negative feedback loop, NF-κB activity was still terminated following stimulation, which turned out to be dependent on the ubiquitination and degradation of p65, which in turn is dependent on the binding of p65 to DNA (Saccani *et al.*, 2004).

Although the ability to respond rapidly to changes in the cellular environment is essential in mounting an immune response to pathogenic invasion, it is also important that NF-κB activity be tightly controlled to avoid the damaging effects of prolonged expression of pro-inflammatory genes. In contrast to the previously held belief, this regulation is not only by way of the blanket repression of NF-κB by the IκB proteins, but its activity is selectively controlled in a gene-specific manner, and this is achieved by the post-translational modification of not only the upstream elements but of the NF-κB subunits themselves (Perkins, 2006).

1.4.3.1 Ubiquitination of p65

Most of the available information concerning the regulation of NF-κB by ubiquitination comes from work done on the p65 subunit. Ubiquitination results in degradation of p65, as occurs with the factors contributing to its activation. However, unlike IκBα, the degradation of p65 is partial rather than complete (Collins *et al.*, 2016). It occurs mostly in the nucleus with DNA binding being an essential stimulus as demonstrated by the observations that DNA-binding defective p65 mutants are ubiquitination-resistant (Saccani *et al.*, 2004). When p65 degradation is prevented by the expression of an ubiquitination-resistant mutant, p65 remains bound to the promoter for longer, thus extending the duration of NF-κB target gene expression. A number of important forms of poly-ubiquitination have been identified for p65. For degradation, K48 poly-ubiquitination is the predominant mechanism, however there are a number of

others including K29, K33 and K63, the function of which remains unknown (Li *et al.*, 2012). There is also evidence of non-degradative mono-ubiquitination that may promote the retention of p65 in the nucleus (Hochrainer *et al.*, 2012). To prevent p65 ubiquitination it is essential that almost all lysine residues be abolished, therefore it is thought that its ubiquitination is highly promiscuous (Li *et al.*, 2012), but it is unclear whether one p65 molecule is ubiquitinated at many sites at the same time, or if an individual lysine residue is modified in isolation.

1.4.3.2 Deubiquitination and other PTMs

Also pertinent in the regulation of NF-κB is the role of deubiquitinases (DUBs). As the name suggests, these remove poly-ubiquitin chains from residues, which causes increased transcription as the activating NF-κB subunits are no longer degraded by the proteasome. Ubiquitin specific protease 7 (USP7) is known to play an essential role in regulating transcription with it being reported that p65 is a unique substrate and that deubiquitination by USP7 increases p65 stability (Colleran *et al.*, 2013). Mass spectrometry analysis revealed that a number of ubiquitination sites also overlap with acetylation sites, which is another PTM that happens to NF-κB subunits. It appears that when ubiquitination of p65 is increased, acetylation decreases and vice versa. The mechanism of this effect is unknown (Li *et al.*, 2012). Furthermore, methylation occurs on p65 lysine residues that have been modified by acetylation and ubiquitination so there may be some interplay between a number of PTMs, contributing to the regulation of p65 and the control of transcription on a gene-specific level (Ea and Baltimore, 2009).

1.4.4 E3 ligases

As outlined in section 1.4.2, E3 ligases are the final catalysts in the ubiquitin cascade that function to bind an E2-Ub complex and a substrate protein to assist in the formation of an isopeptide bond between the C-terminal carboxyl of the ubiquitin molecule and the amino group of the substrate.

1.4.4.1 Classification

Common among all E3s is the E2-Ub-binding domain and what distinguishes them is the structure of this particular domain and the mechanism by which they transfer ubiquitin from the E2 to the substrate (figure 1.9).

RING (really interesting new gene) E3s act directly to catalyse the transfer of ubiquitin from the E2 to the substrate. They make up the largest sub-group of E3s in humans (Li *et al.*, 2008) and are characterised by the presence of a RING (or U-box) catalytic domain, which is required to recruit an E2 and stimulate the transfer of ubiquitin (Buetow & Huang, 2016). RING E3s may act as monomers such as casitas B-lineage lymphoma (CBL) (Zheng *et al.*, 2000), or as oligomers in the case of cellular inhibitor of apoptosis protein 2 (cIAP2) (Mace *et al.*, 2008) or TNF-receptor-associated factor 6 (TRAF6) (Yin *et al.*, 2009). Other RING E3s only gain ligase activity upon forming a heterodimer with a RING domain-containing partner, such as BRCA1-associated RING domain 1 (BARD1) with breast cancer type 1 associated protein (BRCA1) (Brzovic *et al.*, 2001), and others are actually large multi-subunit complexes such as the cullin-RING E3 ligases (CRLs) (Lydeard *et al.*, 2013).

HECT (homologous to E6AP carboxyl terminus) E3s, by contrast, facilitate ubiquitination in a two-step process, firstly via a catalytic cysteine residue that receives ubiquitin from an E2 to form an E3-Ub thioester intermediate. This is followed by the transfer of ubiquitin to the substrate protein. At 28, as opposed to 600, there are much fewer HECT than RING E3 ligases in humans (Rotin & Kumar, 2009). They contain an N-terminal substrate-binding domain and a C-terminal HECT domain that has the catalytic components for conjugating and transferring ubiquitin. The HECT domain itself comprises an N-terminal lobe that binds E2-Ub complexes and this is connected by a flexible hinge to the C-terminal lobe that contains the catalytic cysteine.

Finally, RBR (RING-between-RING) E3 ligases also facilitate ubiquitination in an indirect, two-step process. They have in common a RING1-IBR-RING2 motif. Thus far, 14 have been identified in humans, and they are RING-HECT hybrids that use the RING domain to recruit an E2, and contain a catalytic cysteine to form a

thioester intermediate with the C-terminus of ubiquitin, like HECT E3s (Dove & Klevit, 2017).

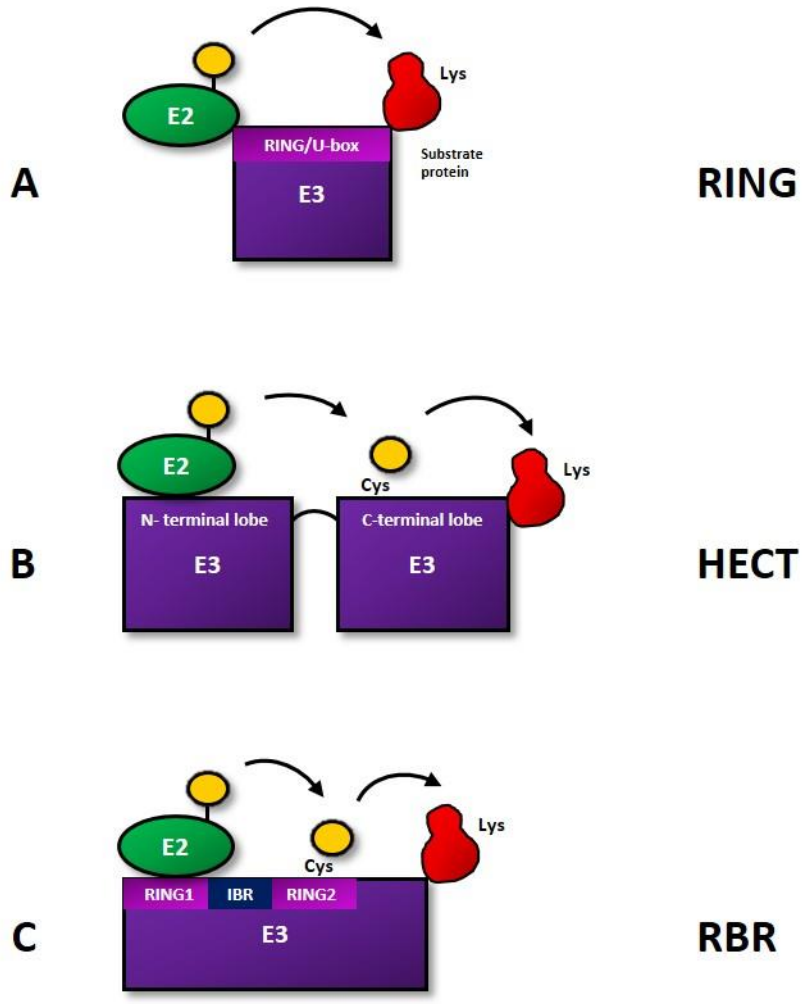


Figure 1.9: RING, HECT and RBR E3 ligases.
(A) RING and RING-like E3s serve as scaffolds that bring together the E2 and substrate in a direct transfer of ubiquitin. They can function as monomers, dimers or as part of multi-subunit complexes. (B) HECT E3s are bi-lobed, consisting of an N-terminal lobe that interacts with E2 enzymes, and a C-terminal lobe that contains the active-site cysteine that forms the thioester bond with ubiquitin. The two lobes are connected via a flexible hinge that allows them to come nearer together during ubiquitin transfer. HECT E3s transfer ubiquitin in an indirect manner, with the initial formation of a thioester intermediate between the active-site cysteine and ubiquitin, followed by transfer to the substrate protein. (C) RBR E3s consist of a RING, an in-between RING and a RING-like domain, and are considered hybrids of RING and HECT E3s. The RING domain recruits the E2 and then ubiquitin is transferred to an active-site cysteine in the RING-like domain, again forming a thioester intermediate before finally ubiquitin is transferred to the substrate. IBR: in-between RING domain. Cys: active-site cysteine. Lys: lysine residue.

1.4.4.2 Known E3 ligases of NF-κB

A number of E3 ligases for p65 and c-Rel have been identified, although to date none has been found for p50 or RelB (see table 1.2). RelB has been demonstrated to be ubiquitinated however, and unlike p65, its ubiquitination does not require DNA binding and appears to promote rather than inhibit

transcriptional activity (Leidner *et al.*, 2008), perhaps due to non-degradative poly-ubiquitination. It remains unclear whether there is redundancy in the activity of E3 ligases on the NF-κB subunits but studies suggest that the E3 ligases for p65 may control specific subsets of NF-κB target genes (gene-specific effects).

Table 1.2: Table of E3 ligases of NF-κB subunits and their target residues where known

Subunit	E3 ligase	Target residue
p65	SOCS1 COMM1 ING4 PDLIM2 PPARγ MKRN2 ?	 K62 K28 K195
RelB	?	
c-Rel	PELI1 cIAP	
p105	$SCF^{β\text{-}TrCP}$ KPC1	
p100	$SCF^{β\text{-}TrCP}$	
p50	?	K128
p52	?	

1.4.4.2.1 SOCS1

Suppressor of cytokine signalling 1 (SOCS1) is a member of a family of proteins that regulate cytokine responses in many cells (Alexander, 2002). They each have a central SH2 domain, an amino terminal of variable length and divergent sequence, and a C-terminal SOCS box (Hilton *et al.*, 1998) (figure 1.10). SOCS1 has a number of roles within the immune system. By way of its SH2 domain, it can interact with and inhibit members of the Janus kinase (JAK) family. JAKs function to phosphorylate tyrosine residues on intracellular receptors leading to the recruitment and activation of signal transduction proteins such as the signal transducer and activator of transcription (STAT) family of transcription factors, which results in the production of cytokines. Beyond this function in the case of NF-κB, SOCS1 was found to bind to and induce the ubiquitination and degradation of p65 when it was part of a multi-subunit complex alongside elongin c, cullin2 and Rbx1 known collectively as $ECS^{(SOCS1)}$ (Ryo *et al.*, 2003). This complex interacts with copper metabolism Murr1 domain-containing 1

(COMMD1) which stabilises the interaction between SOCS1 and p65, further enhancing the ability of SOCS1 to ubiquitinate p65 (Maine *et al.*, 2007). This interaction has been demonstrated in both the cytoplasm and nucleus (Strebovsky *et al.*, 2011).

Figure 1.10: Schematic of SOCS1 structure.
Like all members of the SOCS family, SOCS1 contains a Src homology 2 (SH2) domain and a C-terminal SOCS box immediately preceded by a nuclear localisation signal (NLS). SOCS1 also contains a kinase inhibitory region (KIR) upstream of the SH2.

1.4.4.2.2 ING4

ING4, as a tumour suppressor and member of the inhibitor of growth family, has an important role in a number of cellular processes including cell cycle progression, proliferation and tumour angiogenesis (Zhang *et al.*, 2004; Russell *et al.*, 2006). When dysregulated, it has been implicated in a number of cancers including gastric, breast, brain and melanomas among others. It had been observed to inhibit NF-κB target gene expression; however, the mechanism by which it did this was unknown. It became clear that ING4 was functioning as an E3 ligase via its plant homeodomain (PHD) motif inducing K48-linked poly-ubiquitination and subsequent degradation of p65 at its K62 residue (Hou *et al.*, 2014). Since p65 is an activating subunit of NF-κB, its degradation results in the termination of NF-κB activity.

1.4.4.2.3 PPARγ

Peroxisome proliferator activated receptor-γ (PPARγ) is a transcription factor in its own right that, alongside NF-κB, performs distinct but also overlapping functions in cell regulation. It has an important role in glucose metabolism and in negatively regulating the immune response and the expression of inflammatory cytokines in macrophages. Many studies had observed that PPARγ bound to p65 and inhibited NF-κB transcriptional activity *in vitro* however again the mechanism remained unknown until relatively recently. It was shown that PPARγ, via its RING domain, acts as an E3 to induce the K48-linked poly-ubiquitination and degradation of p65 at its K28 residue (Hou *et al.*, 2012).

1.4.4.2.4 PDLIM2

PDLIM2 is a protein that contains both PDZ and LIM domains and can be either nuclear or cytoplasmic in its location depending on the cell type. PDLIM2 was first identified as an E3 ligase for STAT4, binding to and promoting its ubiquitination and degradation, thus inhibiting STAT-induced gene expression (Tanaka *et al.,* 2005). Similarly, it was also found to bind to and promote the ubiquitination of p65 via its LIM domain which is similar to the RING domain (Tanaka *et al.*, 2007), thus negatively regulating NF-κB activity. The lack of PDLIM2 was observed to result in an accumulation of p65 in the nucleus, its ubiquitination was defective and the production of pro-inflammatory cytokines in response to stimuli was increased. Additionally, PDLIM2, via its PDM domain, sequestered p65 in discrete intra-nuclear compartments called promyelocytic leukaemia protein (PML) bodies where it was degraded by the proteasome (Shin *et al.*, 2017).

1.4.4.2.5 PELI1

PELI1 is a member of the Pelino family of RING ligases. For many years, the ubiquitination of c-Rel had been reported but the mechanism remained elusive. Recently, it was reported that PELI1 is responsible for promoting the K48-linked poly-ubiquitination and subsequent degradation of c-Rel in T cells (Chang *et al.*, 2011). In this manner, PELI1 acts as a negative regulator of T cell activation and prevents the development of autoimmunity. This was further established by observing PELI1-deficient activated T cells that accumulated c-Rel in the nucleus, were hyper-responsive to TCR-CD28 stimulation, and *Peli1*-deficient mice that developed autoimmunity. Since, PELI1 does not affect upstream TCR signalling, it may be a potential therapeutic target for T cell therapy.

1.4.4.2.6 MKRN2

Although much more has become known about how NF-κB activity is negatively controlled, the full picture is still emerging. Recently it emerged that MKRN2, a RING finger domain-containing protein that belongs to the makorin ring finger gene family, is a novel E3 ligase of p65 (Shin *et al.*, 2017). The researchers in this study employed yeast two-hybrid screening to identify and isolate proteins that interacted with PDLIM2 and were critical for supressing NF-κB signalling.

They observed that MKRN2 and PDLIM2 worked synergistically to promote the ubiquitination and degradation of p65. It was also recorded that the knock-down of MKRN2 resulted in a larger amount of p65 in the nucleus of dendritic cells and increased pro-inflammatory cytokine production in response to innate stimuli.

1.4.4.2.7 KPC1

Thus far has been discussed the E3 ligases responsible for ubiquitinating and degrading the active subunits of NF-κB. However, recently characterised was the mechanism that leads to the limited proteasomal processing of the NF-κB1 precursor, p105, to p50. KPC1 (KIP1 ubiquitination-promoting complex) was identified as the E3 ligase that binds to the ankyrin repeats domain of p105 and ubiquitinates it. This leads to it being processed, both under basal and stimulated conditions (Kravtsova-Ivantsiv *et al.*, 2015). This study observed that overexpression of KPC1 inhibits the growth of tumours, probably via the increased generation of repressive p50. Furthermore, this overabundance of p50 leads to the downregulation of p65, which suggests that p50 homodimers may regulate transcription in place of the prototypical p50:p65 and this suppresses tumour growth.

1.4.5 BCL-3 and p50

Thus far, the published data on the role of ubiquitination in the regulation of NF-κB activity has come mainly from work done on p65 with relatively little known about the mechanisms involved in p50 ubiquitination. Therefore, much of what is known about the UPS and p50 has been derived from studies on the atypical IκB protein, BCL-3.

BCL-3 was initially detected by cloning chromosomal breakpoints containing a t (14;19) translocation found in some patients with B cell chronic lymphocytic leukaemia (Ohno *et al.*, 1990). It is considered an IκB protein because it contains several central ANK domains that facilitate its interaction with NF-κB subunits (Palmer & Chen, 2008), but is atypical because unlike its typical counterparts it is predominantly nuclear in localisation as opposed to sequestering the subunits in the cytoplasm, and it is not degraded upon activation of the IKK complex.

Furthermore, BCL-3 has a well-defined transactivation domain that is absent from the typical IκB proteins (Ghosh & Hayden, 2008).

There has been much investigation into the nature and function of the interaction between BCL-3 and NF-κB, with early studies showing that BCL-3 interacts preferentially with p50 and p52 (Nolan *et al.*, 1993b). BCL-3 associates with p50 homodimers and has the effect of prolonging their half-life and thus occupancy of target gene promoters by inhibiting K48-linked poly-ubiquitination and subsequent proteasomal degradation, as opposed to increasing their binding affinity (Carmody & Chen, 2007). Because p50 lacks a transactivation domain it is considered to be a repressor of NF-κB target gene transcription when present as a homodimer, so when it is stabilised by BCL-3 it can inhibit the expression of pro-inflammatory genes for longer as it competes with the transcriptionally active subunits to bind to DNA κB target sites (figure 1.11). When BCL-3 is not present in macrophages, p50 becomes hyper-ubiquitinated and its half-life drastically reduced. These *Bcl3*$^{-/-}$ macrophages are hypersensitive to TLR stimulation as demonstrated by the increase in TNFα and IL-6 (Carmody *et al.*, 2007).

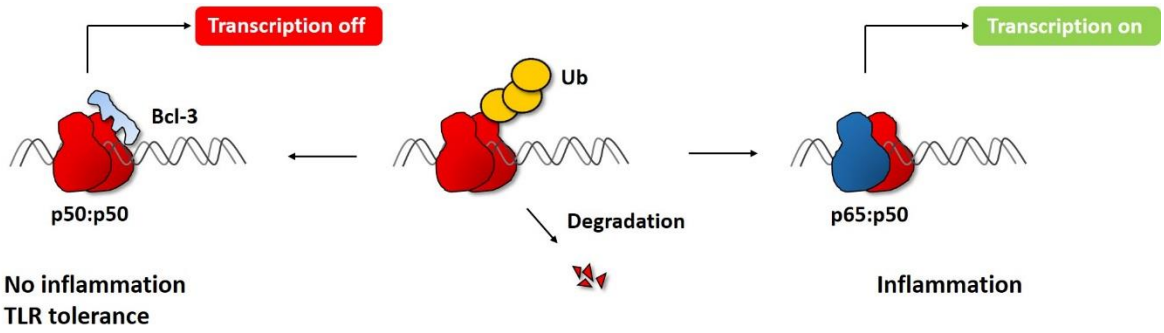


Figure 1.11: BCL-3 stabilises p50 homodimers.
When p50 homodimers are bound to DNA in the nucleus, this triggers their ubiquitination and degradation by the proteasome. This allows transcriptionally active NF-κB dimers such as p65:p50 to bind to κB targets on DNA and promote the transcription of pro-inflammatory target genes. BCL-3 binds preferentially to p50 and has the effect of inhibiting its ubiquitination and degradation, therefore stabilising the complex allowing it to remain bound to DNA for longer. This reinforces p50 homodimers' repressive effects on the transcription of pro-inflammatory genes, therefore inflammation is reduced, and the cellular state known as TLR tolerance is mimicked.

It is not currently known how BCL-3 is able to inhibit p50 ubiquitination but it has been established that a direct interaction with p50 is essential for this stabilisation and anti-inflammatory effect (Collins *et al.*, 2014). Further investigations indicated that there are many points of interaction between p50 homodimers and BCL-3, in particular with the ANK 1, 6 and 7 domain and the N-terminal region of BCL-3 (Collins *et al.*, 2015). This study used this data to generate a short mimetic peptide consisting of the essential amino acid sequence of BCL-3 that is required for interaction with p50 homodimers, which emulates the inhibitory effects that BCL-3 has on NF-κB transcription *in vitro*. Furthermore, the peptide, known as BDP2, had the effect of repressing the expression of pro-inflammatory cytokines *in vitro* and reducing paw swelling in carrageenan-induced mouse models of arthritis. This highlights a potential role for BCL-3 and BDP2 to be used as part of a therapeutic strategy to reduce chronic inflammation by inducing the cellular state known as TLR tolerance. This is in addition to a role for BCL-3 as a regulator of central immunologic tolerance to self, whereby it functions within stroma to create medullary thymic epithelial cells, which are required for the negative selection of auto-reactive T cells (Zhang *et al.*, 2007).

1.5 NF-κB as a therapeutic target

Considering the ubiquity of NF-κB in the control of hundreds of biologically important genes and its critical role in the regulation of inflammation and immune development, it is a highly attractive pharmaceutical target. If its activity can be attenuated this may provide potential treatments for chronic inflammatory diseases and cancer. Current research is underway that hopes to take advantage of the increasing understanding of the molecular mechanisms involved in all aspects of the NF-κB signalling pathway, particularly in the context of inflammation, and the problems this can cause when it becomes dysregulated. Indeed, already in some cancers there has been some success in the use of inhibitors of NF-κB (Herndon *et al.*, 2013).

Dysregulated NF-κB activation has been implicated in the development of pathologies such as autoimmune disease. Patients with rheumatoid arthritis have presented with NF-κB activation in their joint synovium (Handel *et al.*,1995) which contributes to the expression of pro-inflammatory genes that promote lymphocyte recruitment, cartilage destruction and pannus formation (Han *et al.*, 1998). On the other hand, it is not only increased activity that is the consequence of dysregulated NF-κB activity, as patients with systemic lupus erythematosus (SLE) have exhibited reduced levels of NF-κB binding to DNA in peripheral blood lymphocytes (Oikonomidou *et al.*, 2006).

Currently, the anti-inflammatory therapies used in the treatment of autoimmune diseases such as non-steroidal anti-inflammatory drugs (NSAIDs) and disease-modifying anti-rheumatic drugs (DMARDs) have non-specific effects on various stages of the NF-κB signalling pathway, which incidentally result in the dampening down of pro-inflammatory transcription (Herrington *et al.*, 2016). The development of specific inhibitors of NF-κB is an active area of research following on from the discoveries that the individual NF-κB subunits can be targeted as each plays a unique role in the signalling pathway (Ghosh *et al.*, 1998). This is in addition to the various stages and co-factors of the NF-κB pathway that can be attenuated such as the IKK complex, IκB proteins, ubiquitination and other PTMs, and the DNA-binding ability of NF-κB. There are already drugs on the market that have the effect of inhibiting NF-κB activity, such as raloxifene, which removes p65 from its binding site through estrogen

receptor α interaction with p65 (Olivier *et al.*, 2006). Given the vast number of components of NF-κB signalling, there are many potential therapeutic targets that could be efficacious against autoimmune and lymphoproliferative diseases. However, these may also result in unwanted side effects. Thalidomide is an IKK inhibitor and has been effective in treating myeloma. However, its side effects include nephrotoxicity and neuropathy (Mina *et al.*, 2016). The development of NF-κB-focussed drugs that are efficacious in treating disease but do not cause undesirable side effects therefore remains a difficult task for researchers.

1.6 Thesis hypothesis and aims

The transcription factor NF-κB is regulated post-translationally by components of the ubiquitin proteasome system and this affects both the stability of its subunits and the transcription of its target genes.

To confirm or dismiss this hypothesis, this thesis aims to:

- Identify potential E3 ligases of p50.
- Investigate the mechanisms that cause SOCS1 to promote the ubiquitination and degradation of p50.
- Map the transcriptional landscapes of macrophages that are deficient in either ING4 or SOCS1, both of which are known E3 ligases of NF-κB p65.

Chapter 2

Materials and methods

2.1 Materials

2.1.1 Antibodies

Table 2.1: Primary antibodies

Antibody and clone	Cat #	Supplier
c-Myc (9E10)	sc-40	Santa Cruz
c-Rel (C)	sc-71	Santa Cruz
FLAG M2	F1804	Sigma Aldrich
GFP	ab6556	Abcam
HA	sc-805	Santa Cruz
HDAC	QC8205	Sigma Adrich
ING4	ab108621	Abcam
ING4 (BTIM-4)	MABE1156	Millipore
ING4	10617-1-AP	ProteinTech
IκBα (44D4)	4812S	Cell Signalling
NF-κB p65	A301-824A	Bethyl
NF-κB1 p50/p105 (D7H5M)	12540	Cell Signalling
p44/42 MAPK (ERK1/2)	9102	Cell Signalling
Phospho-p44/42 MAPK (P-ERK1/2)	9101	Cell Signalling
RelB (C-19)	sc-226	Santa Cruz
SOCS1 (4H1)	04-002-C	Millipore
SOCS1	25852-1-AP	ProteinTech
Ubiquitin (VU-1)	VU101	LifeSensors
Xpress	PN 46-0528	Thermo Scientific
β-actin	SAB1305567	Sigma Aldrich
α-tubulin	T6074	Sigma Aldrich
β-tubulin (9F3)	5346S	Cell Signalling

Table 2.2: Secondary antibodies

Antibody and clone	Cat #	Supplier
Anti-mouse IgG HRP	LNXA931V/AF	GE Healthcare
Anti-rabbit IgG HRP	LNA934V/AH	GE Healthcare
Mouse anti-goat IgG HRP	Sc-2768	Santa Cruz

2.1.2 BCL-3 mimetic peptides

Table 2.3: BDP2 mimetic peptide sequences

BDP2	mBDP2
YGRKKRRQRRAAVYRILSLFKLGSR	YGRKKRRQRRWAWGYILSLDCLGSY
Both supplied by GenScript (Hong Kong)	

2.1.3 Plasmids

Table 2.4: Plasmids used
All plasmids were from Carmody lab stocks and were cloned using murine cDNA with the exception of the SOCS1 plasmids, which used human cDNA.

Protein	Supplier (if external)	Backbone	Tag	Antibiotic-resistance
BCL-3		pRK5	Flag	Ampicillin
Empty		pcDNA3.1	Flag	Ampicillin
ING4		pcDNA3.1	Flag	Ampicillin
p50		peF4a	Xpress	Ampicillin
$p50^{K49R}$		peF4a	Xpress	Ampicillin
$p50^{K74,76,77R}$		peF4a	Xpress	Ampicillin
$p50^{K83R}$		peF4a	Xpress	Ampicillin
$p50^{K114R}$		peF4a	Xpress	Ampicillin
$p50^{K128R}$		peF4a	Xpress	Ampicillin
$p50^{K191R}$		peF4a	Xpress	Ampicillin
$p50^{K249R}$		peF4a	Xpress	Ampicillin
$p50^{K272,275R}$		peF4a	Xpress	Ampicillin
$p50^{K312,315R}$		peF4a	Xpress	Ampicillin
$p50^{K334}$		peF4a	Xpress	Ampicillin
$p50^{K352,354R}$		peF4a	Xpress	Ampicillin
$p50^{K360,363R}$		peF4a	Xpress	Ampicillin
$p50^{Y57A}$		peF4a	Xpress	Ampicillin
p65		pcDNA3.1	-	Ampicillin
PDLIM2		pcDNA3.1	Ha	Ampicillin
PDLIM2		pcDNA3.1	Myc	Ampicillin
PELI1		pcDNA3.1	HA	Ampicillin
PPARγ		pcDNA3.1	Flag	Ampicillin
SOCS1		peGFP	GFP	Kanamycin
SOCS1	GenScript	pcDNA3.1	Flag	Ampicillin
$SOCS1^{6R/A}$	A gift from the Dalpke lab	peGFP	GFP	Kanamycin
$SOCS1^{R172X}$		peGFP	GFP	Kanamycin
Ubiquitin			Flag	Ampicillin
Ubiquitin			Ha	Ampicillin
UBR5	A gift from Darren Saunders (Addgene #52050)	pcDNA6.2	GFP	Ampicillin
$UBR5^{\Delta HECT}$	A gift from Darren Saunders (Addgene #52051)	pcDNA6.2	GFP	Ampicillin

2.1.4 Primers

2.1.4.1 RT-qPCR

Table 2.5: qPCR primers used

Primer	Sequence	Supplier	Catalogue #
CCL2	F: AGCCAACTCTCACTGAAGCC R: GCGTTAACTGCATCTGGCTG	A gift from Chemokine Research Group	-
CCL7	F: TGAAAACCCCAACTCCAAAG R: TTAGGCGTGACCATTTCACA	A gift from Chemokine Research Group	-
CXCL1	Quantitect Primer Assay	Qiagen	QT00115647
CXCL2	Quantitect Primer Assay	Qiagen	QT00113253
FAM63A	Quantitect Primer Assay	Qiagen	QT00159075
hc-Rel	Quantitect Primer Assay	Qiagen	QT00052472
hCXCL2	Quantitect Primer Assay	Qiagen	QT00013104
hIL-6	Quantitect Primer Assay	Qiagen	QT00083720
hRelB	Quantitect Primer Assay	Qiagen	QT00038640
hTBP	Quantitect Primer Assay	Qiagen	QT00000721
hTNFα	Quantitect Primer Assay	Qiagen	QT00029162
IFNß	Quantitect Primer Assay	Qiagen	QT00249662
IL-10	Quantitect Primer Assay	Qiagen	QT00106169
IL-1ß	Quantitect Primer Assay	Qiagen	QT01048355
ORM1	Quantitect Primer Assay	Qiagen	QT00101409
PALM3	Quantitect Primer Assay	Qiagen	QT02330804
SHISA3	Quantitect Primer Assay	Qiagen	QT01049811
TBP1	TGTTGGTGATTGTTGGT	Eurofins	
TBP2	AACTGGCTTGTGTGGGAAAG	Eurofins	
TNFα	Quantitect Primer Assay	Qiagen	QT00104006
Mouse unless specified otherwise			

2.1.5 Reagents

All general salts and chemicals were stored and prepared according to the manufacturer's instructions.

Table 2.6: List of reagents

Reagent	Supplier	Catalogue #
30 % acrylamide	Sigma Aldrich	A3574
Agarose ultrapure	Invitrogen	16500
Ammonium persulfate (APS)	Sigma Aldrich	A3678
Ampicillin sodium salt	Sigma Aldrich	A9518
Aprotinin	Sigma Aldrich	A1153

ß-mercaptoethanol	Sigma Aldrich	M6250
Bafilomycin		
Bovine serum albumin (BSA)	Sigma Aldrich	A7906
Bromophenol blue	Sigma Aldrich	B8026
Dimethyl sulfoxide (DMSO)	Fisher Chemical	D/4120/PB08
DPBS 1X	Gibco	14190-094
Dithiothreitol (DTT)	Melford Biolaboratories	MB1015
Dulbecco's Modified Eagle's Medium-High Glucose (DMEM)	Sigma Aldrich	D6429
Ethylenediaminetetraacetic acid (EDTA)	Fisher Chemical	D/0700/60
Ethanol	VWR	20821-330
GSH beads	Sigma Aldrich	G4510
L-glutamine	Sigma Alrich	G7513
Glycerol	Fisher Scientific	G/0650/17
Glycine	Fisher Scientific	G/0800/60
Heat-inactivated foetal bovine serum (FBS)	Gibco	10500-064
Hydrochloric acid	Fisher Chemical	H/1200/PB17
Isopropanol	Sigma	24137
Kanamycin	Melford Laboratories	K0126
NP-40	Sigma Aldrich	18896
Leupeptin	Sigma Life Science	L2884
Lipopolysaccharides from *E.coli* 055:B5 (LPS)	Sigma Aldrich	L2880
Methanol	Fisher Chemical	M/4000/PC17
MG132 (Z-leu-leu-leu-al)	Sigma	C2211-5MG
Nuclease-free water	Qiagen	1039480
Penicillin/streptomycin	Sigma Aldrich	P0781
Pepstatin A	Sigma Life Science	P5318
Potassium chloride	Analar Normapur	26764
Potassium dihydrogen phosphate	Analar Normapur	26936
Protein Assay Dye Reagent Concentrate	Bio-Rad	500-0006
Protein G agarose beads	EMD Millipore	16-266
Ponceau S Solution	Sigma Aldrich	P7170
Quinacrine	Sigma Life Science	Q3251-100G
Restore Plus Westen Blot Stripping Buffer	Thermo Scientific	46430
RPMI	Sigma Life Science	R8758-500ML
Sodium chloride	VWR	27810.295
Sodium deoxycholate	Sigma Aldrich	D6750
Sodium dihydrogen phosphate dihydrate	Riedel de Haen	04269
Sodium dodecyl sulfate (SDS)	VWR	UN1325
Sodium fluoride	Sigma Aldrich	S7920
Sodium hydrogen phosphate	Analar Normapur	102494C
Sodium hydroxide pellets	Fisher Chemicals	S/4920/53
Sodium orthovanadate	Sigma Aldrich	S6508
Sulfuric acid	Sigma Aldrich	258105
Tetramethylethylenediamine	Sigma Aldrich	T9281

Tris HCl	Fisher Scientific	BP 152-1
0.05% Trypsin-EDTA 1X	Gibco	25300-054
Tween-20	Sigma Life Science	P2287
Western Bright ECL	Advansa	K12045
Western Bright Sirius	Advansa	K12043

2.1.6 Buffers

2.1.6.1 Lysis buffers

Table 2.7: Cell lysis buffer recipes

Buffer	Ingredients
Radioimmunoprecipitation assay (RIPA)	50 mM Tris-HCl pH7.4 0.1 – 1% NP-40 0.25% sodium deoxycholate 150 mM NaCl 1 mM EDTA pH8 1 mM PMSF* 1 mM NaF* 1 mM Na_3VO_4* 2 µg/mL aprotinin* 2 µg/mL leupeptin* 1 µg/mL pepstatin*

* Added to buffer immediately before use.

2.1.6.2 Electrophoresis buffers for Western blotting

Table 2.8: Western blot buffer recipes

Buffer	Ingredients
1X Tris-glycine running buffer	25 mM Tris-HCl 250 mM glycine 0.1% SDS H_2O
1X Tris-glycine transfer buffer	28 mM Tris-HCl 39 mM glycine 0.038% SDS 20 % methanol H_2O

2.1.6.3 SDS-PAGE loading buffer

Table 2.9: SDS-PAGE loading buffer recipe

2X SDS loading buffer	4X SDS loading buffer
20% glycerol	40 % glycerol
0.2% bromophenol blue	0.04% bromophenol blue
4% SDS	8% SDS
200 mM ß-mercaptoethanol	5% ß-mercaptoethanol
100 mM Tris-HCl pH6.8	240 mM Tris-HCl pH6.8
H_2O	H_2O

2.1.6.4 Tris-borate-EDTA (TBE) buffer

Table 2.10: Agarose gel electrophoresis buffer

Ingredient
5.4 g Tris 2.75 g boric acid 2 mL 0.5 M EDTA

2.1.7 Tris-glycine SDS-polyacrylamide gels

Table 2.11: SDS-PAGE gel recipes

Ingredient	Gel		
	5% stacking (1 mL)	*10% resolving (5 mL)*	*8% resolving (5 mL)*
H_2O	0.68 mL	1.9 mL	
30% acrylamide	0.17 mL	1.7 mL	
1 M Tris-HCl (pH 6.8)	0.13 mL	-	-
1.5 M Tris-HCl (pH 8.8)	-	1.3 mL	
10% SDS	0.01 mL	0.05 mL	
10% APS	0.01 mL	0.05 mL	
TEMED	0.001 mL	0.002 mL	

2.1.8 Electrophoresis gels

Table 2.12: Agarose gel recipe

Ingredient
1.5 % agarose 0.5X TBE buffer 1X GelRed

2.1.9 Mice

WT C57BL6/J mice (Harlan, UK) aged between 8 and 12 weeks were sacrificed in order to generate bone marrow derived macrophages (BMDM). The University of Glasgow Ethical Review Committee under a United Kingdom Home Office Licence approved Mouse handling and experimental procedures. Licence and ethical approval number: 60/4314.

2.2 Methods

2.2.1 Cell biology

2.2.1.1 Cell culture maintenance

RAW 264.7, 3T3, L929 and human embryonic kidney 293T (HEK293T) cells were obtained from in-lab stocks and maintained in high glucose Dulbecco's Modified Eagle Medium (DMEM) (Gibco Life Technologies) containing 10% foetal bovine serum (FBS) (Gibco), L-glutamine (2 mM) (Sigma), and penicillin/streptomycin (100 units/mL) (Sigma) (complete DMEM). THP-1 cells were obtained from in-lab stocks and cultured in Roswell Park Memorial Institute (RPMI) 1640 media, supplemented with 10% heat-inactivated FBS, L-glutamine and penicillin/streptomycin. All cells were maintained at 37°C in a humidified environment with 5% CO_2. Cells were sub-cultured three times per week either by mechanical (RAW 264.7, L929) or chemical detachment (3T3 and HEK 293T) with 0.05% Trypsin-EDTA solution (Gibco).

2.2.1.2 THP-1 differentiation

THP-1 cells were re-suspended and plated at a density of 1.5 x 10^6/mL as described in table 2.13. Following a protocol optimised in the lab, cells were treated with 25 ng/mL PMA for 72 hours. Following differentiation, PMA-containing media was removed, cells were washed once in THP-1 culture media and fresh media was replaced. Cells either were treated immediately or were rested in culture for 4 days before treatment.

2.2.1.3 Bone Marrow Derived Macrophage

2.2.1.3.1 Isolation

WT C57BL6/J mice (Harlan, UK) aged between 8 and 12 weeks were sacrificed in order to generate primary bone marrow derived macrophages (BMDM) *in vitro*. Mice were euthanized by exposure to a rising concentration of CO_2 and cervical dislocation. Hind legs were then removed at the hip joint and surrounding tissue removed so that the femur and tibia bones were easily accessed. Under sterile conditions, the bones were cleaned in sterile phosphate buffered saline (PBS) (-$CaCl_2$ -$MgCl_2$) (Gibco) and then cut at each end and flushed with cold PBS using a

21-gauge needle and syringe to extract the bone marrow. Bone marrow was then collected in sterile PBS and re-suspended to generate a single cell suspension. Debris was removed by passing the bone marrow through a 40 μM cell strainer. The bone marrow suspension was washed twice in complete DMEM and centrifuged at 4°C at 300 g for 5 minutes and re-suspended in complete DMEM for culture or in freezing media (complete DMEM and 10% DMSO) for cryopreservation. Generally, bone marrow was collected and pooled from three mice. The University of Glasgow Ethical Review Committee under a United Kingdom Home Office Licence approved Mouse handling and experimental procedures. Licence number 60/4314.

2.2.1.3.2 Differentiation

Once bone marrow was isolated from mice or recovered from cryopreservation it was cultured in complete DMEM supplemented with 30% L929 conditioned media (BMDM differentiation media) in sterile petri dishes for 7 days. Differentiation media was replaced on day 3 and non-adherent cells removed. By day 7 adherent monocyte progenitors had differentiated into BMDM. BMDM were removed from petri dishes by incubating with 5 mM EDTA in sterile PBS at 37°C for 2 minutes. Cells were then washed twice in complete DMEM at 4°C at 300 g for 5 minutes. Cells were then re-suspended in complete DMEM without L929 supplement and re-plated overnight at the appropriate density in tissue culture-treated dishes for experimentation purposes (Yan *et al.*, 2012).

2.2.1.4 Cryopreservation

Cells were centrifuged for 5 minutes at 300 g to form a pellet, which was then re-suspended in a solution of FBS containing 10% DMSO at a density of 2-4 x 10^6 per mL. Re-suspended cells were frozen at -80°C for one day and then moved to liquid nitrogen for long-term storage.

2.2.1.5 Plating conditions

Table 2.13: Plating conditions of different cell types

Plate/dish	Media volume	No. HEK293T	No. RAW 264.7	No. BMDM	No. THP-1
24 well	500 µL	1×10^5	2×10^5	1×10^5	
12 well	1 mL	2.5×10^5	5×10^5	2.5×10^5	
6 well	2 mL	5×10^5	1×10^6	5×10^5	1.5×10^6
6 cm	5 mL	2×10^6	4×10^6	2×10^6	
10 cm	10 mL	5×10^6	1×10^7	5×10^6	

2.2.2 Molecular biology methods

2.2.2.1 Agarose gel electrophoresis

DNA samples were resolved by agarose gel electrophoresis, using the Mupid-One electrophoresis system (Eurogentect). Samples were diluted in 6X loading dye (New England Biolabs) and were resolved on GelRed-stained 1.5% agarose gels (table 2.12) at 100-135 V for 30 minutes in 0.5X TBE buffer (table 2.10). Gels were visualised using a UV transilluminator (Alpha Innotech). DNA size was determined using a 100 bp ladder (Promega). DNA that was required for further cloning steps was excised and purified using QIAquick gel extraction kit (Qiagen), according to the manufacturer's instructions.

2.2.2.2 Polymerase chain reaction (PCR)

Primers for PCR were designed using Primer3 software. Genomic DNA was amplified using HotStarTaq Master Mix Kit (Qiagen) according to manufacturer's instructions. If amplified DNA was required for restriction digest, PCR products were purified using QIAquick PCR purification kit (Qiagen) according to manufacturer's instructions. Concentrations of purified PCR products were measures using a NanoDrop spectrophotometer (ThermoFisher Scientific).

2.2.2.3 Restriction digest

Restriction digests were prepared by mixing either 1 µg plasmid DNA or 500 ng purified PCR product with the required enzymes (NEB), according to manufacturer's instructions, in a total volume of 20 µL.

2.2.2.4 Ligation

Ligation reactions were prepared by mixing insert DNA and plasmid backbone at a molar ratio of 5:1 with T4 DNA ligase (NEB) according to manufacturer's instructions, in a total volume of 20 µL. Ligation reactions were incubated for 1 hour at room temperature.

2.2.2.5 DNA transformation for routine plasmid preparation

10 – 20 µL of competent bacteria (XL1-Blue, Agilent Technologies) was thawed on ice and 10 ng plasmid DNA (or 1 µL ligation reaction) was added. This mixture was incubated on ice for 2 minutes. Bacteria was then heat shocked at 37°C in a water bath for 1 minute and immediately returned to ice. Bacteria was recovered in 100 µL of super optimal broth with catabolite repression (SOC) media (Invitrogen) and transferred to luria broth (LB) agar plates containing the appropriate antibiotic (100 µg/mL ampicillin from Sigma Aldrich or 50 µg/mL kanamycin from Melford). Plates were then incubated overnight at 37°C inverted.

2.2.2.6 Plasmid extraction

2.2.2.6.1 Midiprep

A single bacterial colony was inoculated in 2 mL L-Broth supplemented with the appropriate antibiotic and this starter culture incubated at 37°C shaking at 150 rpm for 6 – 8 hours. 100 mL L-Broth supplemented with the appropriate antibiotic was spiked with 100 µL of the starter culture and incubated at 37°C shaking at 150 rpm for 16 – 20 hours. Plasmid DNA was extracted using the PureYield Plasmid Midiprep System (Promega) according to the manufacturer's instructions and plasmid purity and concentration determined using a NanoDrop or Denovix DS-11+ Spectrophotometer.

2.2.2.6.2 Miniprep

2 mL of L-Broth supplemented with the appropriate antibiotic was inoculated with a single bacterial colony and incubated overnight at 37°C shaking at 150 rpm. DNA was extracted using Qiaprep Spin Miniprep Kit (Qiagen) according to the manufacturer's instructions.

2.2.2.7 DNA sequencing

Plasmid DNA sequencing was performed by GATC Biotech Ltd. (Germany) using either common universal primers provided by the company or custom primers. Sequencing results were analysed using Geneious Molecular Biology and NGS Analysis Tools (Biomatters Ltd., New Zealand).

2.2.2.8 Transfection

HEK293T cells were transiently transfected using Turbofect transfection reagent (Thermo Scientific). Plasmids were incubated with serum-free media (SFM) and Turbofect for 15 minutes at room temperature and added drop-wise to the cells. The ratio of SFM (µL):DNA (µg):Turbofect (µL) was 100:1:2 (table 2.14). Within each experiment, total plasmid concentrations were kept constant between samples by the addition of an empty expression vector.

Table 2.14: Transfection conditions.

Plate/dish	Cell no.	SFM	DNA	Turbofect
12 well	2.5×10^5	50 µL	0.5 µg	1 µL
6 well	5×10^5	100 µL	1 µg	2 µL
6 cm	2×10^6	300 µL	3 µg	6 µL
10 cm	5×10^6	500 µL	5 µg	10 µL

2.2.2.9 Gene expression analysis by RT-qPCR

For real-time quantitative PCR (qPCR), total RNA was extracted from cells using a RNeasy Kit (Qiagen), Qiashredders (Qiagen) and RNase Free DNase Set (Qiagen) according to the manufacturer's instructions. RNA was primed with random hexamer oligonucleotides and reverse-transcribed into complimentary DNA (cDNA) using a nanoScript RT Kit (Primerdesign) following the manufacturer's instructions. RT-qPCR was performed using PerfeCta SYBR Green FastMix with ROX (Quanta Bioscience) using QuantiTect Primers (Qiagen) (table 2.15) in a 384-well plate using a QuantStudio 7 Flex (Applied Biosystems by Life Technologies). Data were normalised to TATA-binding protein (TBP) and gene expression changes using the $2^{-\Delta\Delta Ct}$ method (Livak & Schmittgen, 2001).

Table 3.15: RT-qPCR cycle settings

Stage	Cycle	Rate	Temperature	Time
Hold	1	(1.6˚C/s) (1.6˚C/s)	50˚C 95˚C	2 min 10 min
PCR	40	(1.6˚C/s) (1.6˚C/s)	95˚C 60˚C	15 sec 1 min
Melt curve	2 x 10^6	(1.6˚C/s) (1.6˚C/s) (1.6˚C/s)	95˚C 60˚C 95˚C	15 sec 1 min 15 sec

2.2.3 Protein methods

2.2.3.1 Protein extraction

2.2.3.1.1 Non-denatured whole cell extraction

Culture media was aspirated and tissue culture plates were washed gently with 4˚C PBS. Cells (HEK293T, RAW264.7, THP-1) were detached with 4˚C PBS or with mechanical agitation and centrifuged at 11,000 g for 45 seconds at 4˚C to create a pellet. Pellets were re-suspended in 20 – 200 µL radio-immunoprecipitation assay (RIPA) buffer (table 2.7) that was freshly supplemented with protease and phosphatase inhibitor cocktail (table 2.16). This was incubated on ice for 30 minutes and vortexed every 5 minutes. Lysate was cleared by centrifugation at 16,000 g for 10 minutes at 4˚C. Supernatant was collected and analysed straight away or stored at -20˚C or -80˚C for long-term storage.

Table 2.16: Non-denaturing lysis conditions

Plate/dish	RIPA volume
12 well	40 – 50 µL
6 well	80 – 100 µL
6 cm	150 – 180 µL
10 cm	300 – 350 µL

2.2.3.1.2 Denatured whole cell extracts

Cells were incubated with 10 mM n-ethylmaleimide (NEM) for 30 seconds before cells were harvested. Culture media was aspirated and tissue culture plates were washed gently with 4˚C PBS with 10 mM NEM. Cells were detached with 4˚C PBS and centrifuged at 11,000 g for 45 seconds at 4˚C to form a pellet. Pellets were re-suspended in 100 µL 1% SDS and heated at 95˚C for 5 minutes. Cell pellet was disrupted by sonication for 10 seconds (30% amplitude, 50% duty cycle) (Bandolin SONOPULS Ultrasonic Homogeniser HD 2070 with MS 73 Microtip). Lysate was cleared by centrifugation at 16,000 g for 10 minutes at 4˚C

and supernatant collected and analysed straight away or stored at -20°C or -80°C for long-term storage.

2.2.3.2 Quantification

Whole cell protein extracts were quantified by Bradford assay. 1 µL of cell lysate was diluted in 1 mL of 1X Bradford assay reagent (Bio-Rad) and assayed in triplicate on a spectrophotometric 96-well plate reader with absorbance measured at 595 nM. To determine unknown protein concentrations a standard curve was generated using bovine serum albumin (BSA) at concentrations of 0-9 µg/mL.

2.2.3.3 Western blotting

Protein samples were separated by performing denaturing sodium dodecyl sulphate (SDS) polyacrylamide gel electrophoresis (PAGE) on the Mini-PROTEAN Tetra Cell System (Bio-Rad). Cell lysate was diluted in either 2X or 4X SDS loading buffer then boiled at 95°C for 5 minutes and resolved on 8% or 10% acrylamide gels (table 2.11). Gels were run at 120 V for 80-120 minutes in 1X tris-glycine running buffer (table 2.8).

Once resolved, protein was transferred to Amersham Protran 0.45 µM nitrocellulose membrane (GE Healthcare) using the Mini Trans-Blot Electrophoretic Transfer System (Bio-Rad) and 1X Tris-glycine transfer buffer (table 2.8). Membranes were incubated in Ponceau solution (Sigma Aldrich) and washed with PBS-Tween 20 0.05% (PBS-T) to confirm proper transfer of protein. To block non-specific binding, membranes were incubated in 5% non-fat milk (Marvel)/PBS-T solution for 1 hour at room temperature. Membranes were probed with primary antibody in 5% milk/PBS-T or 5% BSA/PBS-T overnight at 4°C or for 1-2 hours at room temperature with gentle agitation, then incubated with secondary antibody in 5% milk/PBS-T for 1 hour at room temperature. Three 5-minute washes were performed using PBS-T after incubation with antibody.

Bound protein was detected using WesternBright ECL Chemiluminescent Substrate (Advansta), as all secondary antibodies used were horseradish peroxidase (HRP)-conjugated. For low abundance proteins, WesternBright Sirius

Chemiluminescent HRP substrate was used. Membranes were scanned using the LI-COR c-Digit Model 3600 (USA) on either standard or high sensitivity settings. For membranes requiring multiple antibody re-probes for proteins of similar size, they were stripped using Restore Plus Western Blot Stripping Buffer (Thermo Scientific) according to the manufacturer's instructions. Protein sizes were determined by the addition of a protein ladder (Thermo Scientific) or a biotinylated ladder followed by the addition of an anti-biotin antibody (Cell Signalling Technologies).

2.2.4 Functional assays

2.2.4.1 Immunoprecipitation

Non-denatured whole cell lysates were prepared as described above in section 2.2.3.1.1. Equal amounts of protein (30 µg) were diluted in RIPA buffer (0.1% NP-40) up to 20 µL and 20 µL 2X SDS loading buffer, boiled at 95°C for 5 minutes and kept frozen for subsequent analysis. To immunoprecipiate (IP) the desired protein, equal amounts of protein (1 mg) were diluted in 1 mL RIPA buffer (0.1% NP-40). Samples were pre-cleared for 30 minutes on a rotator at 4°C by the addition of 20 µL protein G agarose beads (EMD Millipore). Beads were discarded by centrifugation at 14,000 g at 4°C for 2 minutes. Samples were incubated with 20 µL protein G agarose beads (Millipore) and 1 µL of the appropriate antibody overnight at 4°C on a rotator. Immunoprecipitated proteins were washed 3 times in 1 mL RIPA buffer and centrifuged at 11,000 g for 1 minute at 4°C. Proteins were eluted from the beads by the addition of 30 µL 2X SDS loading buffer and then boiled at 95°C for 5 minutes, incubated on ice for 2 minutes and centrifuged at 14,000 g for 2 minutes at 4°C. Samples were stored at -20°C or analysed immediately by Western blot.

2.2.4.2 Ubiquitination assay

Denatured whole cell lysates were prepared as described above in section 2.2.2.1.2. Equivalent volumes of lysate (5 µL) were diluted in 5 µL 2X SDS loading buffer, boiled at 95°C for 5 minutes and used as input samples. The remaining volumes of lysate (95 µL) were diluted up to 1 mL with RIPA buffer (1% NP-40) supplemented with 20 mM NEM. Samples were pre-cleared by adding 20 µL protein G agarose beads (Millipore) for 30 minutes on rotation at 4°C. Pre-

cleared lysate was centrifuged for 2 minutes at 14,000 g and at 4°C and transferred to a new Eppendorf tube. 20 µL of fresh protein G agarose beads were added to pre-cleared lysate and immunoprecipitated with 1 µL antibody overnight rotating at 4°C. Immunoprecipitated protein was washed 3 times in 1 mL RIPA buffer. To elute immunoprecipitates, beads were re-suspended in 30 µL of 2X SDS loading buffer and heated to 95°C for 5 minutes. Samples were vortexed for 10 seconds and centrifuged for 2 minutes at 16,000g at 4°C to isolate eluate. Eluate was stored at -20°C or -80°C, or analysed immediately by Western blot (see section 2.2.2.3).

2.2.5 Bioinformatic methods

Bioinformatic analysis was performed with the kind assistance of Dr. Domenico Somma and Mr John Cole (GLAZgo Discovery Centre).

2.2.5.1 RNA sequencing (RNA-seq)

WT and *Ing4*$^{-/-}$ and *Socs1*$^{-/-}$ RAW 264.7 cells were left untreated or were treated for 3 hours with 100 ng/mL LPS. Total RNA was extracted from the cells using the RNeasy kit (QIAGEN) according to the manufacturer's instructions. Duplicate samples of each condition were sent to the University of Glasgow Polyomics facility for sample QC, polyA library preparation (Truseq stranded mRNA kit). Illumina NextSeq™ 500 platform was used to sequence single-end 75bp reads to a depth of 20 million.

2.2.5.2 RNA-seq analysis

2.2.5.2.1 CuffDiff pipeline

Quality control of raw sequence reads was determined using the Fast QC tool. Reads were aligned to mouse reference sequence GRCm38.p6 using HISAT (v. 2.0.3.2) (Pertea *et al.*, 2016). Aligned reads were assembled into transcripts using Stringtie (v. 1.2.3). Significant differential fragments per million reads (FPKM) values were calculated using CuffDiff (v. 2.2.1.3) (Trapnell *et al.*, 2010). The above analyses were performed using Glasgow University Galaxy server (http://heighliner.cvr.gla.ac.uk/). Clustering analysis was performed with default options (K-means clustering with Jensen-Shannon distance) using the

Bioconductor package CummeRbund. Visualisation of RNA-seq data was performed using Papillon (v. 0.1.1) developed by Dr. Domenico Somma (University of Glasgow) (http://github.com/domenico-somma/Papillon).

2.2.5.2.2 DESeq2 pipeline

Quality control and read alignment was performed as described in the section above. Aligned reads were counted using HTseq-count (v. 0.6.1) (Anders *et al.*, 2015) using Glasgow University Galaxy server. Read counts were used as input to SearchLite (Beta), an automated pipeline for analysis and visualisation of RNA-seq data, developed by Mr John Cole and colleagues (GLAZgo Discovery Centre, University of Glasgow). Differential gene expression was calculated using DESeq2 (Love *et al.*, 2014).

2.2.5.3 GEO submission details

RNA-seq data is available in the NCBI Gene Expression Omnibus database with the following accession number: GSE 134456

2.2.5.4 Transcription factor binding site (TFBS) analysis

2.2.5.4.1 Motif analysis

Motif analysis was based on the GREAT approach (McLean *et al.*, 2010) which incorporates both proximal and distal regulatory regions for enrichment analyses. Regulatory regions for genes were obtained from the Ensembl Regulatory Build (Zerbino *et al.*, 2015). Genomic locations were obtained using the 'fetch closest non-overlapping feature' tool. These analyses were performed using the Glasgow University Galaxy Server.

De novo motif searches were performed using the motif search program HOMER, developed by C. Benner (Heinz *et al.*, 2010), using the findMotifs.pl command with default parameters (promoter region 200bp upstream 50bp downstream of transcription start site). Custom motifs were generated using the seq2profile.pl command.

Chapter 3

The effects of NF-κB E3 ligases on p50

3.1 Abstract

NF-κB is a transcription factor family that controls the expression of hundreds of biologically important genes, many of which have essential roles in the immune response. Its dysregulation results in a number of pathologies and so it must be tightly controlled at all stages of its activation. The ubiquitin proteasome system (UPS) targets proteins for degradation and has long been established as important in the upstream activation of NF-κB. However, it has become clear in the last few years that ubiquitination of the NF-κB subunits themselves is also an important mechanism for the regulation of gene expression. Most studies have focussed on p65, with a number of E3 ligases identified that target this, and other subunits for ubiquitination. In comparison, little is known about p50. A particularly insightful piece of the puzzle would be the identity of the E3 ligase that is responsible for ubiquitinating p50, resulting in its proteasomal degradation. This would open up novel therapeutic targets that seek to stabilise p50 homodimers and reinforce their repressive effects on the transcription of pro-inflammatory genes. Within this chapter, we looked to the published studies and used a panel of putative E3 ligases that included SOCS1, ING4, PPARγ, PDLIM2 and UBR5 in a series of *in vitro* assays to determine whether any of these also promoted the ubiquitination of p50. Furthermore, the consequences of mutating a site of p50 ubiquitination are explored via the use of a mutant monocytic cell line. It was observed that SOCS1 and UBR5 are able to promote the ubiquitination of p50, whereas ING4 has no effect on p50 and so was ruled out as an E3 ligase for this subunit. Furthermore, the K130 site of human p50 is found to have influence over the abundance of the other NF-κB subunits, but did not affect the TLR-induced expression of certain cytokines.

3.2 Introduction

NF-κB is an essential regulator of immune gene expression, in particular those critical for the inflammatory response. When dysregulated, this leads to the development of a number of diseases including cancer (Rayet and Gélinas, 1999), neurodegenerative disease, viral infections and autoimmune diseases such as rheumatoid arthritis and multiple sclerosis (Baldwin, 2001; Kumar *et al.*, 2004). It is therefore crucial that NF-κB activity is tightly controlled to avoid these undesirable outcomes.

The role of the ubiquitin proteasome system (UPS) in the upstream activation of both the canonical and non-canonical NF-κB pathways is well established (Wertz & Dixit, 2010; Kanarek & Ben-Neriah, 2012). However, it is emerging that ubiquitination of the NF-κB subunits themselves is a critical post-translational modification contributing to the degradation of NF-κB and the termination of its activity. Thus, it is an area that could lead to greater understanding of how to exert control over aberrant pro-inflammatory gene transcription in a gene-specific, rather than signal-specific manner (Saccani *et al.*, 2004; Bosisio *et al.*, 2006; Carmody *et al.*, 2007).

The majority of published work regarding the relationship between the UPS and NF-κB has focussed on the p65 subunit (Ryo *et al.*, 2003; Maine *et al.*, 2007; Tanaka *et al.*, 2007; Strebovsky *et al.*, 2011; Hou *et al.*, 2012; Hou *et al.*, 2014; Shin *et al.*, 2017). There have also been some studies published on c-Rel (Chang *et al.*, 2011) and the precursor p105 (Kravtsova-Ivantsiv *et al.*, 2015). Processing of p105 to p50 may occur constitutively or after induction, and whilst both are dependent on ubiquitination, they seem to be regulated by different mechanisms (Palombella *et al.*, 1994; Orian *et al.*, 1995). Phosphorylation of p105 at serine (Ser, S) 927 and 932 by IKKβ following stimulation creates a destruction motif that is recognised by the $SCF^{\beta TrCP}$ ubiquitin ligase and this leads to the complete proteolysis of p105 (Orian *et al.*, 2000). KPC1 is the E3 ligase that monoubiquitinates p105 at multiple lysine residues, mediating its partial processing to p50 (Kravtsova-Ivantsiv *et al.*, 2015). Ubiquitin-mediated degradation of p65 is induced by many TLR and TNFR ligands (Saccani *et al.*, 2004) and is a major limiting factor of pro-inflammatory gene expression (Carmody & Chen, 2007). In addition to the growing number of E3 ligases

identified for p65 so far, a number of target lysine (Lys, K) residues have been identified including K28, K62 and K310. Furthermore, non-degradative ubiquitination of p65 there has been demonstrated although its function remains unknown (Hochrainer *et al.*, 2012; Li *et al.*, 2012). Despite this increasing understanding of how the UPS regulates NF-κB activity through the targeting of p65 for degradation, much remains unknown. For example, it is not clear if a single p65 molecule is ubiquitinated at multiple K residues at the same time, or if modification occurs in isolation (Collins *et al.*, 2016). There have been many K residues identified as acceptor sites on p65 from mass spectrometry analysis and perhaps this accounts for the binding specificities of the different E3 ligases that act upon the subunit. Thus far, there has been no overlap of ubiquitination sites for the E3 ligases identified for p65. It is also not known why there are so many E3 ligases that target p65 and why some NF-κB target genes such as IL-6 are regulated by multiple ligases, whereas others are regulated more selectively. It has been suggested that some E3 ligases might only target p65 when part of a specific dimer combination, or depending on the cell type or activating stimulus (Collins *et al*, 2016).

Considering the growing body of work into the relationship between the UPS and NF-κB, relatively little is understood about the mechanisms that lead to the ubiquitination and degradation of p50. Due to its lack of transactivation domain, p50 is considered a repressor of the transcription of pro-inflammatory genes when present as a homodimer, competitively binding to κB sites of DNA and preventing transcriptionally active NF-κB dimers from doing so. In unstimulated macrophages, p50 homodimers occupy the promoters of *Tnfa* and *Cxcl2,* both pro-inflammatory genes. However, in *Bcl3*$^{-/-}$ cells that undergo hyper-ubiquitination of p50, these promoters are occupied by the transcriptionally active p65 and c-Rel (Carmody *et al.*, 2007), therefore resulting in increased NF-κB transcriptional activity and pro-inflammatory gene expression. DNA binding is known to trigger p50 ubiquitination and degradation, so stabilisation of these p50:p50:DNA complexes would be a desirable objective to dampen the unwanted chronic inflammation that is characteristic of many human diseases. Indeed, in human head and neck tumours and glioblastoma, there is a significant decrease in p50 levels in the nucleus compared to healthy tissue (Kravtsova-Ivantsiv *et al.*, 2015). One approach to increasing p50 homodimer stability would be to

prevent their ubiquitination and degradation. This will require a detailed understanding of the underlying mechanisms. Previously, the K128 residue of murine p50 (K130 in human p50) was identified as a site of its ubiquitination, although the function of ubiquitination at this site is unknown (thesis of Patricia Collins, 2014). Furthermore, the E3 ligases that mediate p50 ubiquitination have yet to be identified.

In this chapter, the known E3 ligases for the other NF-κB subunits were used as a starting point to investigate the relationship between the UPS and p50. Since so little is known about how the UPS regulates p50, this was considered a reasonable starting point. Using a panel of putative E3 ligase constructs, a series of cell-based assays were conducted to explore the effects, if any, these had on p50. In addition, the function of the K130 site of ubiquitination of p50 is investigated using mutant cell lines that have this residue abolished. It is discovered in this chapter that an interesting relationship exists between p50 and SOCS1, previously found to increase the ubiquitination and degradation of p65 (Ryo *et al.*, 2003; J. Strebovsky *et al.*, 2011). SOCS1 increases p50 ubiquitination and promotes its degradation. Furthermore, ING4 is ruled out as having specificity for p50. Finally, it is clarified that the K130 residue does not regulate the pro-inflammatory gene expression of a number of genes analysed, as induced by LPS. However, it does affect the stability of other NF-κB subunits, both basally and following stimulation. The findings outlined here provide a basis on which additional avenues of work can be established, and indeed are explored further in later chapters.

3.3 Results

3.3.1 Interaction between p50 and some putative E3 ligases

To determine whether SOCS1, ING4, PPARγ or PDLIM2 could interact with, and therefore possibly have an effect on p50 stability, a co-immunoprecipitation (co-IP) assay was performed. HEK293T (293T) cells were transiently transfected with plasmids previously generated within the lab to co-express wild-type p50-Xpress (p50-XP) and either GFP-tagged SOCS1 (eGFP-hSOCS1, herein referred to as GFP-SOCS1), Flag-tagged ING4 (ING4-Flag) or PPARγ (PPARγ-Flag), or Myc-tagged PDLIM2 (PDLIM2-Myc), or not. Whole-cell lysates were used and XP-tagged p50 was immunoprecipitated out using anti-XP antibody, and the E3 ligases detected via Western blot (WB) using antibodies against their respective protein tags. A clear interaction was detected between p50 and SOCS1, and p50 and PDLIM2. A weaker interaction was seen between p50 and PPARγ, and no interaction was observed between p50 and ING4 (figure 3.1). This may not rule out ING4 as a potential binding partner of p50, it might be the case that the interaction is short-lived and so not captured within the time course adopted in this assay. This data provides a foundation on which to proceed to investigate further the nature of the interactions these candidate E3 ligases have with p50.

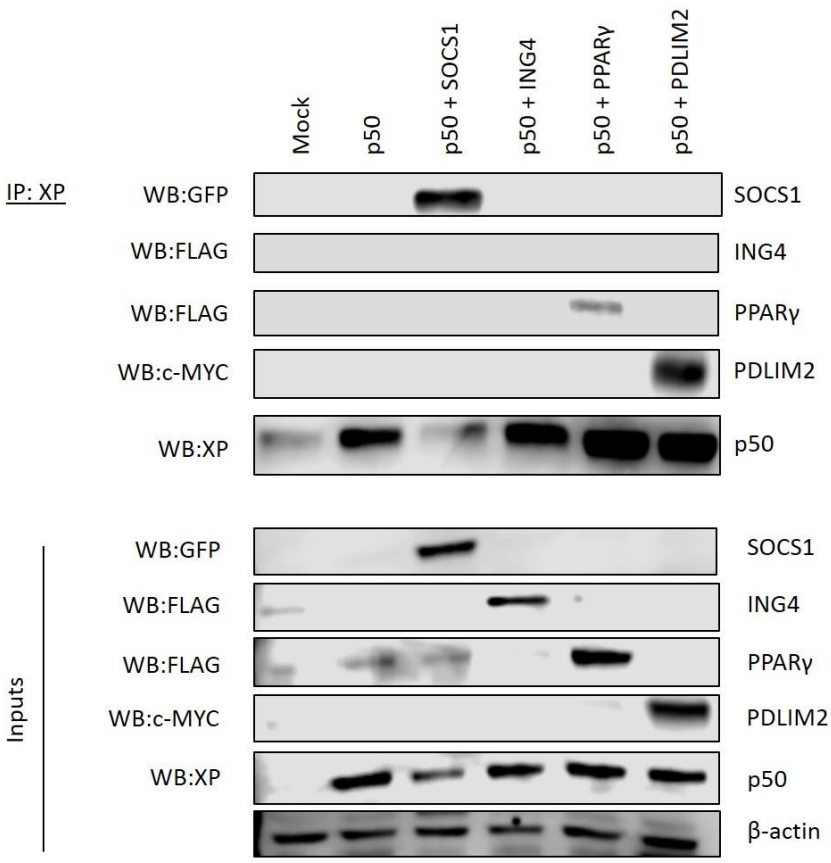


Figure 3.1: Interaction between p50 and SOCS1, ING4, PPARγ and PDLIM2.
HEK293T (293T) cells were co-transfected to express p50-XP and either GFP-SOCS1, ING4-FLAG, PPARγ-FLAG or PDLIM2-MYC, or with empty plasmid (mock). Total amount of DNA transfected was 3 µg, using 1 µg of p50 and 0.5 µg E3 ligase, making up the rest with empty vector. Cells were treated with 20 µM of proteasome inhibitor MG132 for 1 hour prior to harvest. Protein interaction was analysed via co-immunoprecipitation (co-IP) of XP and then Western blot (WB) against the respective tags of the putative E3 ligases. Figure is representative of three individual experiments.

3.3.2 SOCS1 causes most prominent degradation of p50

Previous studies have demonstrated that SOCS1, ING4, PPARγ and PDLIM2 are able to affect the stability of their NF-κB subunit targets, by ubiquitinating them and directing them towards degradation by the proteasome. To determine whether the candidate E3 ligases had any such degradative effect on p50, 293T cells were co-transfected to express p50-XP, and either GFP-SOCS1, ING4-Flag, PPARγ-Flag or PDLIM2-Myc, or not. Following harvest, expression levels of p50 were analysed via immunoblot using anti-XP antibody. There is a stark decrease in p50 stability when it is present with SOCS1 when compared to p50 alone. This is also the most marked decrease compared to the other candidate E3 ligases (figure 3.2). There is a slight loss of p50 stability in the presence of PDLIM2 as well. This interesting result indicates that SOCS1's repertoire of roles within the regulation of the immune system extends to affecting the stability of the p50 subunit of NF-κB, in addition to ubiquitinating p65 (Strebovsky *et al.*, 2011) and as a JAK/STAT inhibitor (Liau *et al.*, 2018).

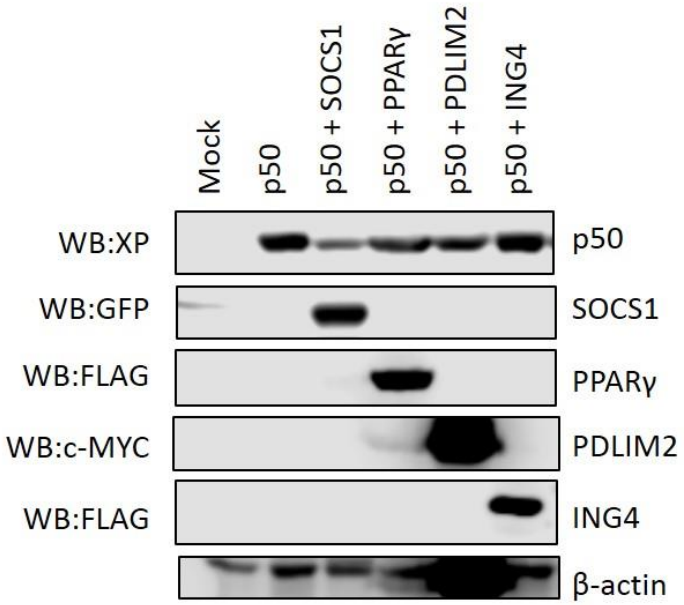


Figure 3.2: Most prominent degradation of p50 observed in the presence of SOCS1. 293T cells were co-transfected to express p50-XP and either GFP-SOCS, PPARγ-FLAG, PDLIM2-MYC, or ING4-FLAG, or not. Total amount of DNA transfected was 3 µg, using 1 µg of p50 and 0.5 µg E3 ligase, making up the rest with empty vector Cells were lysed and expression levels of p50 analysed via WB against XP. The figure is representative of three individual experiments.

3.3.3 SOCS1 most strongly promotes p50 ubiquitination

It was important to discover more about the nature of the p50 degradation observed in the previous section. It was hypothesised that the putative E3 ligases might be promoting the ubiquitination of p50, and this was causing the observed degradation. To investigate if the E3 ligases increased p50 ubiquitination, 293T cells were co-transfected with plasmids expressing p50-XP, ubiquitin-HA (Ub-HA), and the putative E3 ligases as described earlier, or not. 20 μM MG132 was added to inhibit proteasomal degradation because any ubiquitination activity might mediate such degradation. One hour after this, cell lysates were harvested and denatured prior to being immunoprecipitated with anti-XP antibody and immunoblotted with anti-HA to detect ubiquitin. Compared to controls, SOCS1 clearly promotes the ubiquitination of p50 (figure 3.3A), and this is further clarified by densitometry analysis (figure 3.3B). PDLIM2 appears to promote p50 ubiquitination although to a lesser extent. No promotion of ubiquitination is observed with PPARγ nor ING4, and indeed, they may reduce p50 ubiquitination. This is further indication that SOCS1 might be acting as an E3 ligase for p50, promoting its ubiquitination and causing it to be degraded.

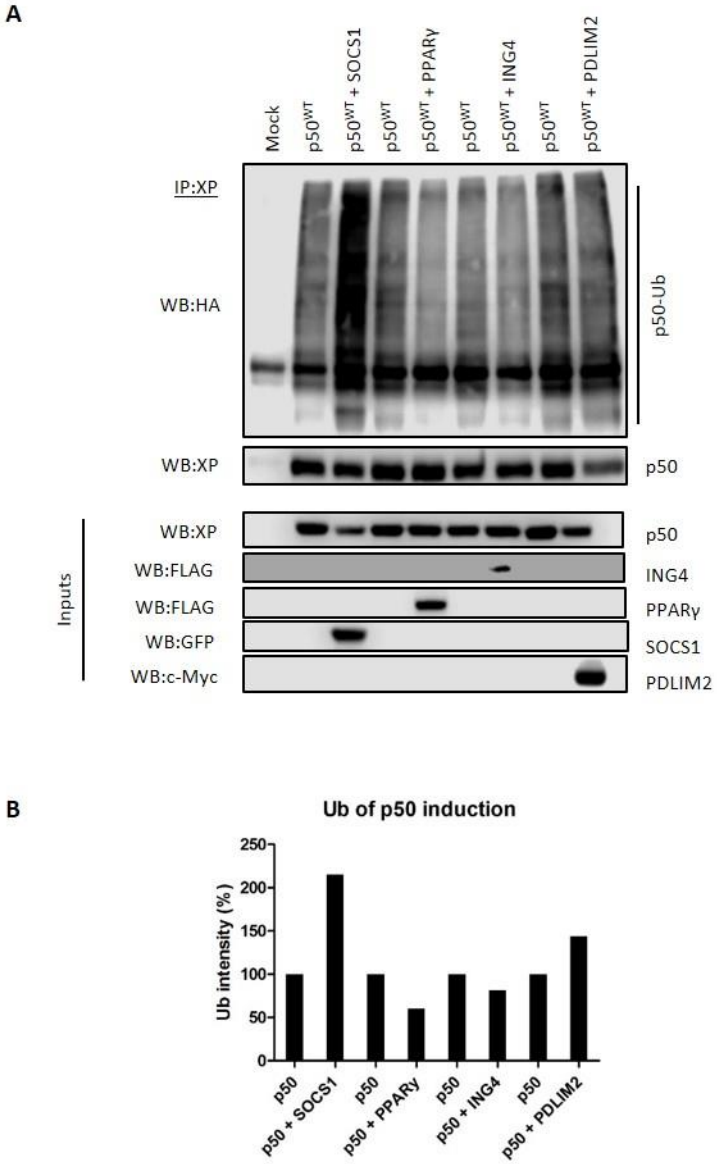


Figure 3.3: Effect of E3 ligases on p50 ubiquitination.
(A) 293T cells were co-transfected to express p50-XP, Ub-HA and either GFP-SOCS1, PPARγ-FLAG, ING4-FLAG or PDLIM2-MYC, or not. Total amount of DNA transfected was 3 µg, using 1 µg of p50, 1 µg of ubiquitin and 0.5 µg E3 ligase, making up the rest with empty vector. Cells were treated with 20 µM MG132 for 1 hour prior to harvest. Ubiquitination of p50 was analysed via immunoprecipitation (IP) of XP and WB against the HA tag of ubiquitin. Data shown is representative of three individual experiments. (B) Densitometry analysis was performed using Image Studio Lite software and the level of ubiquitination (Ub) was normalised to the amount of p50 immunoprecipitated. Data shown indicates induction of ubiquitination relative to p50 alone.

3.3.4 UBR5 promotes ubiquitination of p50

A previous mass spectrometry analysis performed in the lab identified UBR5 to be a binding partner of p50. UBR5 is an E3 ligase that has been identified as a key regulator of the UPS in cancer and development. Many substrates have been identified for this HECT domain-containing ligase, although the context of these substrates is elusive and it remains unknown if its activity is dependent upon particular circumstances within different cell types and in different health states (Shearer *et al.*, 2015). For instance, it negatively regulates the stability of TIP60, a tumour suppressor, by targeting it for ubiquitination and degradation by the proteasome (Subbaiah *et al.*, 2016). It has also been identified as a substrate of ERK2 in the MAPK signalling cascade (Eblen *et al.*, 2003). Constructs that express WT UBR5 ($UBR5^{WT}$) and a mutant that is missing the HECT domain ($UBR5^{\Delta HECT}$) were employed in a series of ubiquitination assays with p50 to explore the relationship between these two proteins. 293T cells were co-transfected with plasmids expressing p50-XP, Ub-HA, and either GFP-$UBR5^{WT}$ or GFP-$UBR5^{\Delta HECT}$, or not. 20 μM MG132 was added to inhibit proteasomal degradation and one hour after this, cell lysates were harvested and denatured prior to being immunoprecipitated with anti-XP antibody and immunoblotted with anti-HA to detect ubiquitin. Due to the large molecular mass of UBR5 (309 kDa) it was difficult to resolve this protein via SDS-PAGE alongside the other proteins of interest, so instead to determine its expression its GFP tag was visualised by fluorescence microscopy (figure 3.4C). Ubiquitination assays indicated that UBR5 is able to promote the ubiquitination of p50 compared to controls (figure 3.4A). As expected, a UBR5 mutant that lacked ubiquitination activity (ΔHECT) was unable to promote the ubiquitination of p50. Quantification by densitometry analysis confirmed that ubiquitination of p50 is increased by about 50% in the presence of UBR5, and reduced by about 75% with the mutant (figure 3.4B). Collectively, with previous UBR5-p50 binding data, these data suggest that UBR5 interacts with p50 and might act as an E3 ligase via its HECT domain.

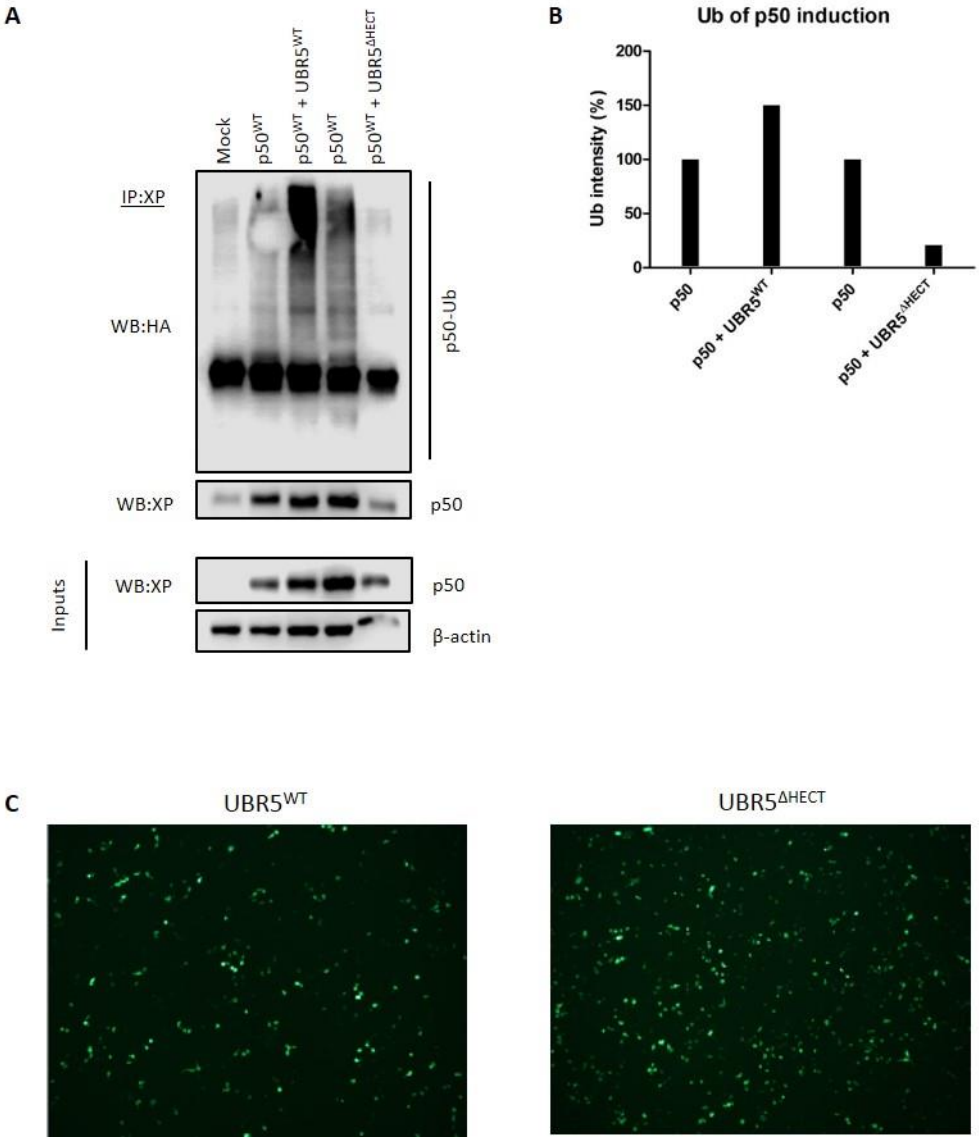


Figure 3.4: UBR5 promotes ubiquitination of p50.
(A) 293T cells were co-transfected to express p50-XP, Ub-HA and either GFP-UBR5^{WT} or GFP-UBR5$^{\Delta HECT}$, or not. Total amount of DNA transfected was 3 µg, using 1 µg of p50, 1 µg of ubiquitin and 0.5 µg E3 ligase, making up the rest with empty vector. Cells were treated with 20 µM MG132 for 1 hour prior to harvest. Ubiquitination of p50 was analysed via immunoprecipitation (IP) of XP and WB against the HA tag of ubiquitin. Figure is representative of two individual experiments. (B) Densitometry analysis was performed using Image Studio Lite software and the level of ubiquitination was normalised to the amount of p50 immunoprecipitated. Data shown indicates induction of ubiquitination relative to p50 alone. (C) Due to the large size of UBR5, it was difficult to resolve via SDS-PAGE. Instead, the GFP tag was observed under a fluorescent microscope to determine UBR5 expression.

3.3.5 ING4 does not promote ubiquitination of p50

Although RING/U-box and HECT domains are the main types of E3s in eukaryotes, LIM and PHD domains have also been seen to have the ability to ubiquitinate substrates. It has been established that ING4, which contains a PHD domain, acts as an E3 ligase that induces the ubiquitination and degradation of p65 via its K62 residue, and this is essential for the termination of NF-κB activity (Hou *et al.*, 2014). Although ING4 was not seen to interact with p50 (figure 3.1), this did not rule out the possibility that ING4 could have an effect on p50 ubiquitination via an indirect mechanism. To investigate further, 293T cells were co-transfected with plasmids expressing p50-XP, Ub-HA, and ING4-Flag, or not. A concentration gradient of MG132 was added to inhibit proteasomal degradation and one hour after this, cell lysates were harvested and denatured prior to being immunoprecipitated with anti-XP antibody and immunoblotted with anti-HA to detect ubiquitin, and the other antibodies as indicated. Across the three different MG132 concentrations, there is no observable increase of p50 ubiquitination (figure 3.5A). When densitometry analysis is performed and the levels of ubiquitination normalised to the amount of p50 immunoprecipitated out, there is actually a decrease in the level of ubiquitination compared to p50 alone at the higher MG132 concentrations (figure 3.5B). This data, coupled with the finding that ING4 does not appear to interact with p50 (figure 3.1) appears to rule out ING4 as an E3 ligase for p50 and instead suggests that ING4 may cause p50 to be deubiquitinated to some extent.

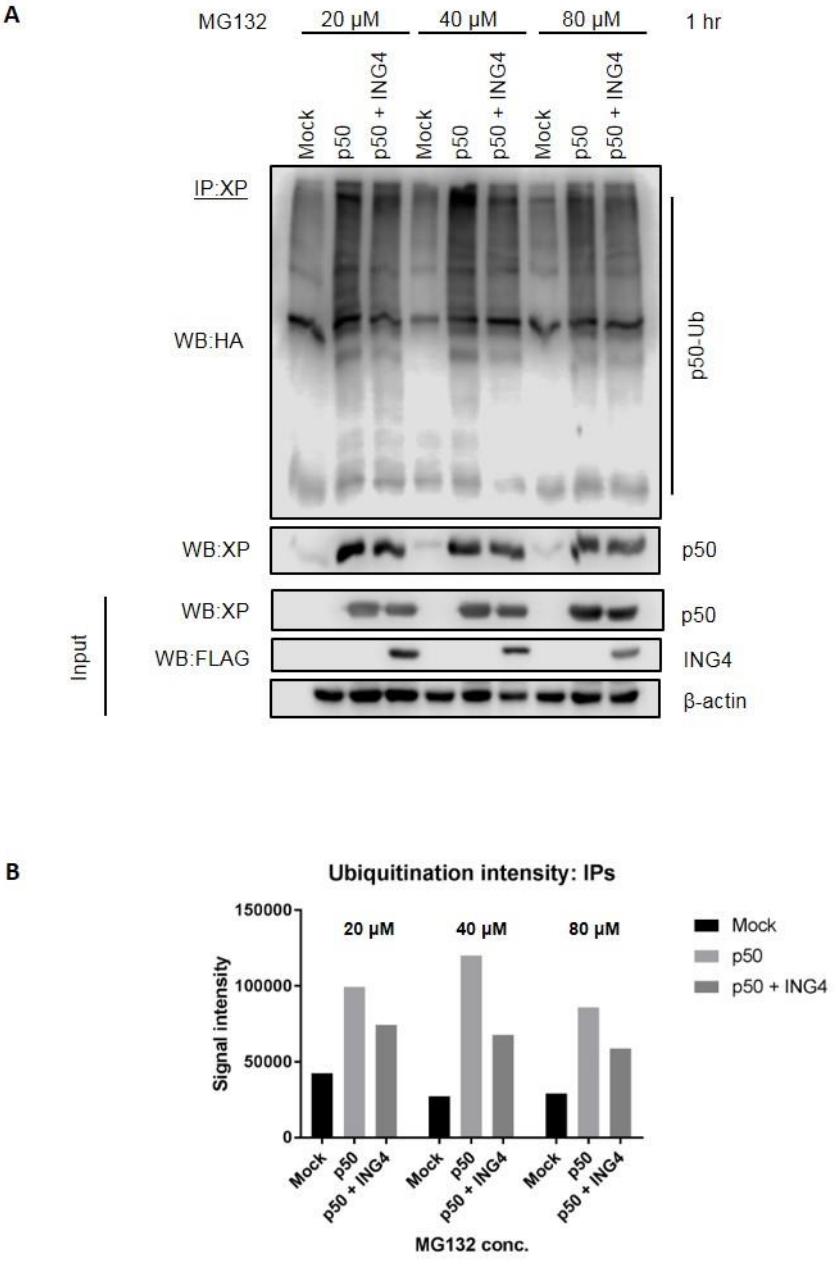


Figure 3.5: ING4 does not promote ubiquitination of p50.
(A) 293T cells were co-transfected to express p50-XP, Ub-HA and ING4-FLAG, or not. Total amount of DNA transfected was 3 μg, using 1 μg of p50, 1 μg of ubiquitin and 0.5 μg E3 ligase, making up the rest with empty vector. Cells were treated with increasing concentrations of MG132 for 1 hour prior to harvest, as indicated. Ubiquitination of p50 was analysed via immunoprecipitation (IP) of XP and WB against the HA tag of ubiquitin. (B) Densitometry analysis was performed using Image Studio Lite software and the level of ubiquitination was normalised to the amount of p50 immunoprecipitated.

3.3.6 Investigating the functional impact of the p105^{K130} mutation in THP-1 cells on TLR-induced gene expression

Previous data generated in the lab identified lysine 130 (K128 mouse) of p50 was a site of ubiquitination. In this unpublished study, 293T cells were transfected with plasmids expressing murine p50-XP or p50-XP in which K128 of p50 was mutated to arginine (R), as well as Ub-HA. This mutation dramatically reduced p50 ubiquitination when measured by immunoprecipitation and immunoblot. It also had a repressive effect on transcription, as observed in an IL-23p19 reporter luciferase assay. Transfection of p105 and p105 in which K128 was mutated to R into mouse embryonic fibroblast (MEF) cells that lacked *Nfkb1* resulted in reduced TNFα-stimulated reporter activity. THP-1 is a human monocytic cell line that is an *in vitro* model of LPS-activated gene expression and so is an excellent tool for investigating the immune response. Using a retroviral vector approach, two THP-1 mutants were created that had the K130 residue mutated to an alanine (K130A) and an arginine (K130R). K130 in human is analogous to K128 in mouse. Although it eliminates the site of ubiquitination, a K to A mutation changes a positively charged amino acid to a hydrophobic one and this could have other effects on the protein such as a structural change. K and R on the other hand are both positively charged so it is highly unlikely that such a mutation will have any biochemical effect other than the elimination of the site of ubiquitination, and is thus the more biologically relevant mutant. However, both give insight into the role of this residue. In these experiments, WT THP-1s were not transduced with a virus and instead expressed endogenous p105/p50. It was hypothesised that the loss of this site of p50 ubiquitination may result in changes in NF-κB target gene expression. More specifically, if p50 is no longer ubiquitinated and degraded by the proteasome, then its stability is increased which might result in a greater abundance of repressive p50 homodimers within the nucleus causing a reduction in pro-inflammatory gene expression. To explore this, WT, p105^{K130A}- and p105^{K130R}-expressing THP-1 cells were left either undifferentiated or differentiated into macrophages by treatment with 25 ng/mL PMA for 72 hours as described in section 2.2.1.2 of Materials and Methods. They were then stimulated with LPS over a time course or left unstimulated and NF-κB target gene expression measured by performing RT-qPCR and fold changes analysed using the $2^{-\Delta\Delta Ct}$ method. RT-qPCR revealed no differences that could be reproduced in the expression of *TNFa*, *IL6*, or *CXCL2* (figure 3.6), which are all

pro-inflammatory genes controlled by NF-κB following TLR activation, either in the undifferentiated or differentiated state.

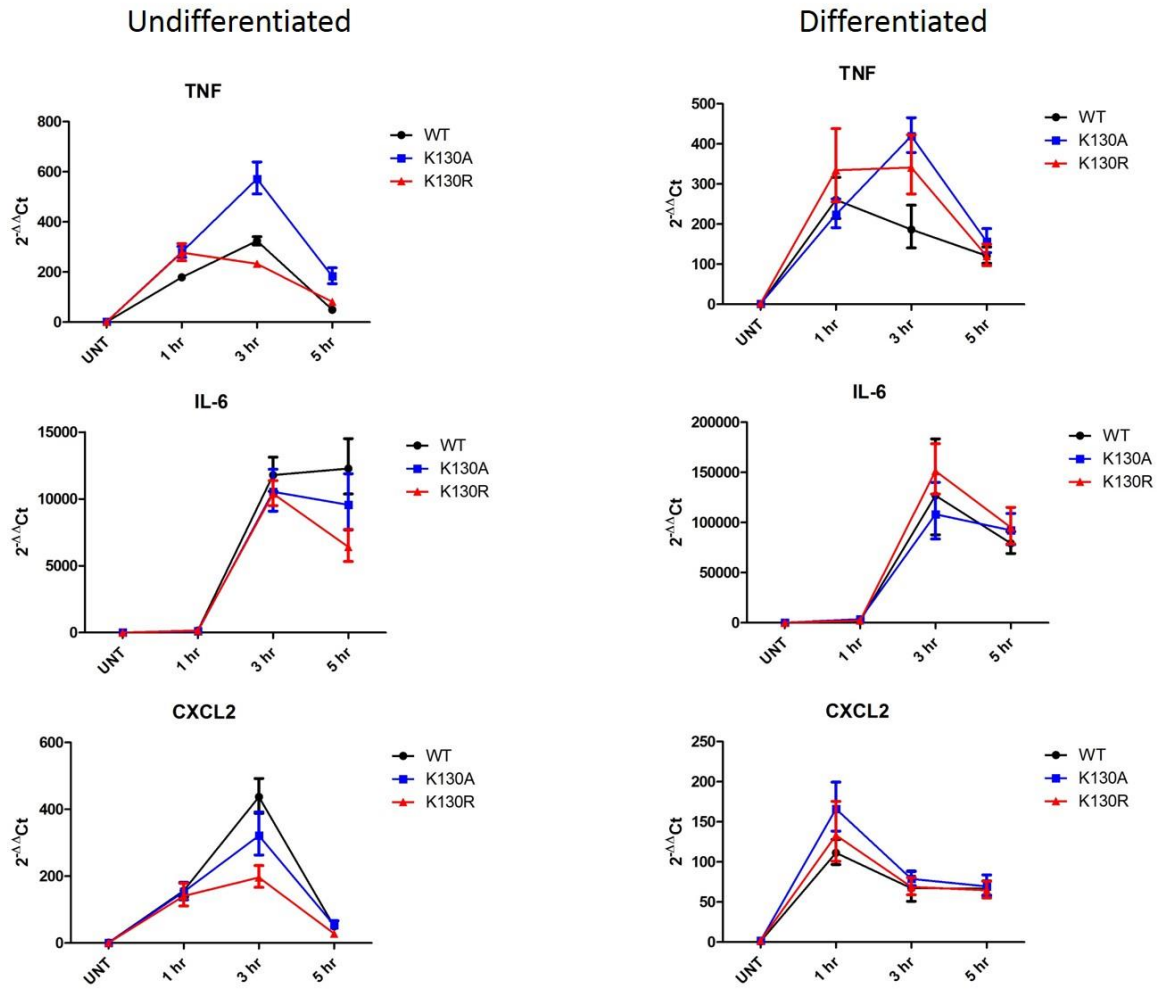


Figure 3.6: p105$^{K130A/R}$ THP1 mutation does not affect cytokine expression.
WT, p105^{K130A} and p105^{K130R} THP-1 cells were either left undifferentiated or differentiated by treatment with 25 ng/mL PMA for 72 hr as described above and then stimulated with 100 ng/mL LPS for the indicated time. Gene expression levels were determined by real-time quantitative PCR (RT-qPCR). Data shown are representative of three independent experiments showing fold change with corresponding standard deviation. TNF: Tumour necrosis factor alpha.

3.3.7 K130 mutation in p50 may influence other NF-κB subunit abundance and ratio

Considering the lack of any noticeable effects on NF-κB target gene transcription as outlined above, the consequence, if any, of the K130 mutation of p50 remained unknown. We next assessed the impact on p50 protein levels and those of the other NF-κB subunits. To determine this, undifferentiated WT, $p105^{K130A}$ and $p105^{K130R}$ THP-1 cells were left either untreated, or treated with 100 ng/mL LPS for 4 hours. Cells were harvested and protein levels measured via immunoblot using antibodies specific for the proteins of interest. There was little change in the levels of p50 across WT and either mutant, either basally or after induction with LPS (figure 3.7A). The levels of p105 were increased in both unstimulated mutants, and p105 is still processed to p50. There is a stark decrease in the levels of RelB and c-Rel in the unstimulated mutants compared to WT. There is a decrease in IκBα levels in the $p105^{K130R}$ mutant in the resting state. The more biologically relevant mutant, $p105^{K130R}$ has lower levels of p65 both with and without treatment with LPS (figure 3.7B). To determine whether the decrease in the protein levels of both RelB and c-Rel was also seen at the transcriptional level for the biologically relevant $p105^{K130R}$ mutant, relative gene expression was measured by performing RT-qPCR. The results revealed that the decrease in expression occurs at the mRNA level, with a smaller fold change seen in the expression of both genes in the mutant compared to WT over the indicated time course (figure 3.7C), and in untreated cells (figure 3.7D). This indicates that it is not the effect of a post-translational modification of p50 causing the marked decrease in RelB and c-Rel abundance since it occurs before transcription both basally and after induction. K130 of p50 may therefore have a regulatory role in NF-κB subunit abundance and dynamics, repressing the transcription of some subunits.

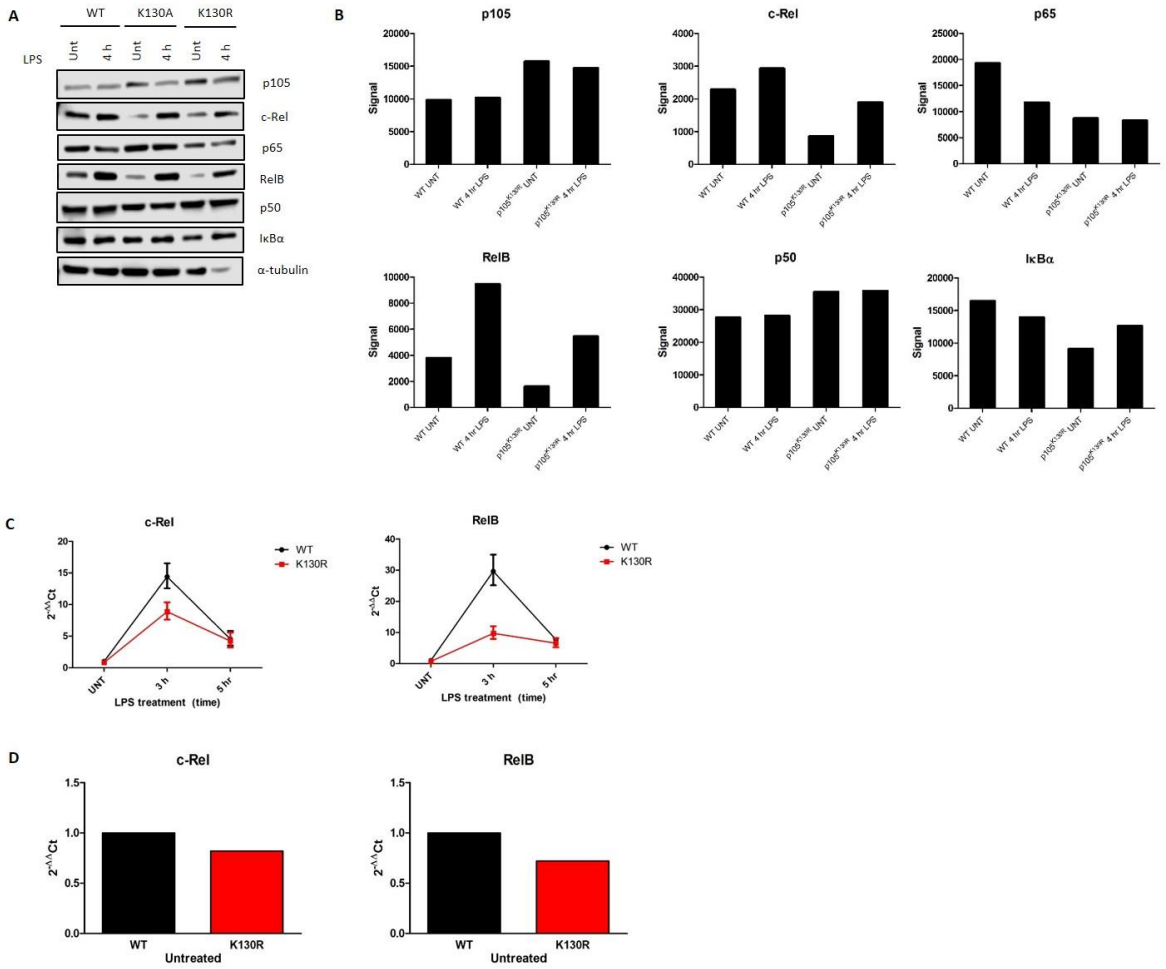


Figure 3.7: K130R mutation of p105/p50 may influence NF-κB subunit dynamics at the transcriptional as well as protein level.
(A) Undifferentiated WT, p105^{K130A} and p105^{K130R} THP-1 cells were treated with 100 ng/mL LPS for 4 hours or left untreated and then whole cell extracts were analysed for endogenous levels of components of the NF-κB pathway via WB with the antibodies indicated. Data shown is representative of two individual experiments. (B) Densitometry analysis was performed using Image Studio Lite software. Data was not normalised due to the poor detection of α-tubulin in the last lane that was likely due to uneven incubation of the blot in antibody. (C) Undifferentiated WT THP-1 cells (black) or p105^{K130R} THP-1 cells (red) were stimulated with 100 ng/mL LPS for the indicated time. Gene expression levels were determined by RT-qPCR. Data shown are representative of two independent experiments showing fold change with corresponding standard deviation. (D) Bar graphs indicating fold change in gene expression in untreated cells as before.

3.4 Discussion

The results in this chapter indicated that SOCS1 is the most promising candidate to be an E3 ligase for the p50 subunit of NF-κB. This is the focus of the following chapter where an in-depth discussion is conducted, so will not be addressed in detail here. There were, however, two other interesting results to emerge from the initial investigations on p50 ubiquitination, and that was concerning PDLIM2 and UBR5. PDLIM2 was shown to interact with p50 (figure 3.1), it decreased its stability (figure 3.2) and promoted its ubiquitination (figure 3.3), although to a lesser extent than SOCS1. PDLIM2 contains a PDZ and a LIM domain, and it is via the latter that PDLIM2 has been shown to function as an E3 ligase as it is structurally similar to the RING domain (Capili *et al.*, 2001). The ability of p50 to interact with PDLIM2 is in direct contrast to a previously published study that established PDLIM2 as an E3 ligase that targets p65 for ubiquitination and degradation (Tanaka *et al.*, 2007). This study was unable to detect overexpressed PDLIM2 binding to endogenous p50 in 293T cells. This discrepancy might be explained by PDLIM2 only being able to bind p50 when it is present in abundance in cells, such as in an overexpression assay. Endogenously, perhaps PDLIM2 has greater affinity for p65 than p50 so will preferentially bind it, whereas the interaction can only be detected when there is no competition for p50.

UBR5 has emerged as an E3 ligase that is a key regulator in cancer and development. Localised in both the cytoplasm and nucleus, overexpressed UBR5 is reported in many cancer types however the mechanisms by which it is thought to control DNA damage responses, metabolism, transcription and apoptosis remain poorly understood (Shearer *et al.*, 2015). Although NF-κB is most commonly considered to promote inflammatory cancers (DiDonato *et al.*, 2012), repressive p50 homodimers can act as tumour suppressors, for example in neutrophil-driven hepatocellular carcinoma (Wilson *et al.*, 2015). Additionally, lower levels of KPC1, the E3 ligase responsible for the partial processing of p105 into p50 have been reported in human tumour tissues from head and neck and glioblastoma cancers (Kravtsova-Ivantsiv *et al.*, 2015). It was observed in the current study that UBR5 increased p50 ubiquitination, whereas a mutant that lacked the HECT domain did not (figure 3.4). With high levels of UBR5 and low levels of p50 homodimers reported in some cancers, it is possible that UBR5,

acting as an E3 ligase for p50, promotes its ubiquitination and degradation and this leads to the development of tumours. There are some challenges in exploring this relationship further given the large size of the protein, however future experiments to determine the stability of p50 in the presence of UBR5 could be performed to shed more light on the situation.

ING4 is an established E3 ligase for p65. It has been demonstrated to induce p65 degradation, to interact with the RHD of p65 via its PHD domain and to increase the K48-linked polyubiquitination of p65 at residue K62 (Hou *et al.*, 2014). Our data shows that ING4 does not appear to interact with p50, nor promote its ubiquitination and degradation. In fact, over three different concentrations of MG132, ubiquitination p50 was observed to decrease with the addition of ING4. Although it is anticipated that p50 is being ubiquitinated by more than one E3 ligase, there appears to be no overlap in the function of ING4 as an E3 ligase for both p65 and p50.

As mentioned in the introduction to this chapter, residue K130 of p50 has been identified as a site of ubiquitination. A mutant that had the residue mutated to arginine was not ubiquitinated when overexpressed in HEK293T cells however, the functional consequences of ubiquitination at this site were not known. It was hoped that the consequences of eliminating this site in a monocytic cell line would shed light on the role of this residue in the context of the inflammatory response. It was therefore unexpected that this mutation appeared to elicit no distinct phenotype in the context of NF-κB target inflammatory gene expression. A previous luciferase reporter assay conducted in the lab had indicated that this mutation resulted in reduced activity of the BCL-3-regulated and NF-κB-dependent IL-23p19 gene as stimulated by TNFα, although this was a reconstitution assay in MEF cells that lacked Nfkb1 (thesis of Patricia Collins, 2014), and so represent a different species and different activating stimulus. Expression of the pro-inflammatory genes *TNFa, IL6* and *CXCL2* was not greatly affected by this mutation in THP-1 cells. It is possible that this mutation does affect the inflammatory response to TLR activation in a gene-specific manner, but a wider transcriptional analysis such as RNAseq is required to detect the profile, or the response is dependent on cell type, time-point or activating stimulus.

On the other hand, the effects observed on the stability of the other NF-κB subunits were intriguing. For both mutants, an increase in the basal level of p105 was detected. This suggests that K130 might be important for the processing of p105 to p50 in resting cells, with its loss resulting in less degradation. The E3 ligase $SCF^{\beta TrCP}$ is responsible for the ubiquitination and complete degradation of not only IκB proteins but also p105 (Heissmeyer *et al.*, 2001), and KPC1 was recently identified as the E3 ligase that promotes the ubiquitination and partial processing of p105 to p50 (Kravtsova-Ivantsiv *et al.*, 2015). These studies also highlighted that all 30 lysine residues in the IκBγ domain of p105 are involved in its ubiquitination by $SCF^{\beta TrCP}$ (Cohen *et al.*, 2004). However, they focussed only on the residues that reside downstream of the glycine rich domain (GRR) which are degraded when p105 is processed into p50 and did not examine the lysine residues within the p50 domain of p105. Perhaps K130 is also important for the functioning of these E3 ligases. The more biologically relevant mutant, $p105^{K130R}$, had lower levels of p65 than WT, particularly compared to baseline levels. However, the most prominent effects were observed for RelB and c-Rel, particularly in unstimulated cells. This is curious because levels of p50 in the mutants did not vary greatly compared to WT, both unstimulated and following LPS stimulation, with densitometry analysis only revealing a slight increase in expression levels. These data may indicate a role for K130 in regulating the relative abundance of NF-κB subunits that are available in both resting and stimulated cells. RelB and c-Rel are little studied although both are transcriptionally active subunits, with RelB being activated by the non-canonical NF-κB pathway and c-Rel the canonical. Both RelB and c-Rel are implicated in lymphoid organogenesis so this is worth bearing in mind should an *in* vivo mouse model of $p105^{K130R}$ be considered (Weih *et al.*, 1995). The relative mRNA levels of both RelB and c-Rel are reduced in the mutant compared to WT, which demonstrates that K130 affects the regulation of these subunits on the transcriptional level. There have been few studies into the regulation of RelB transcription, although it has been shown to be dependent on p65 and possibly other transcription factors such as CREB as well (Bren *et al.*, 2001). Evidence exists that the genes of the NF-κB family, and its inhibitors, are transcriptionally regulated by the NF-κB members themselves, including c-Rel by p65, and c-Rel by c-Rel itself (Hannink & Temin, 1990; Ten *et al.*, 1992; Liptay *et al.*, 1994). The degradative effect of the K130R mutation of p105 on the other

subunits of NF-κB indicates that the loss of this site of ubiquitination has implications for the other members of the NF-κB family. It is possible that RelB and c-Rel are now the focus for increased ubiquitination and degradation in lieu of p105/p50, however the inhibition seems to occur at the transcriptional level. p50 is able to promote transcription as a dimer partner for p65, c-Rel and RelB or repress it as a homodimer, therefore the composition of NF-κB dimers is critical in the progression of the inflammatory response. The choice of p50 dimer partner may occur co-translationally (Lin & Kobayashi, 2003) but there is also evidence that it is a dynamic process that is signal-dependent (Cartwright *et al.*, 2016). Many mutations and PTMs have been identified that regulate the functionality or stability of NF-κB1 proteins, such as K274A:V275S, F320A and H326A:R327S which disrupt heterodimerisation with RelB (Bressler *et al.*, 1993). A future study might examine the composition of NF-κB dimers following mutation of K130 in p105, as it is possible this mutation affects the dynamics of dimer partner choice, perhaps favouring p50 homodimerisation, or altering the duration and affinity p50 has for the other subunits.

The results from this chapter provide a foundation on which the next experimental steps can be based. ING4 can be eliminated from consideration whereas other putative E3 ligases have generated interesting results, in particular SOCS1, which most noticeably promoted the ubiquitination and degradation of p50. Furthermore, the K130 residue of p105/p50 demonstrated unexpected influence over the stability of not only these subunits, but of other members of the NF-κB transcription factor family. This might have a consequence for NF-κB dimer composition, stability and possibly gene expression, and opens up avenues for future research.

Chapter 4

An investigation into the role of SOCS1 as a regulator of p50

4.1 Abstract

When present as a homodimer, the p50 subunit of NF-κB acts to repress the transcription of pro-inflammatory genes. Stabilising p50:p50:DNA immunosuppressor complexes therefore, would be a desirable outcome of any therapeutic intervention that seeks to treat chronic inflammatory diseases. The ubiquitin proteasome system (UPS) targets proteins for degradation. Most of the studies on the relationship between NF-κB and the UPS thus far have focussed on the p65 subunit whilst the molecular mechanisms that lead to p50 ubiquitination and degradation are yet to be determined. Preventing p50 from being targeted for degradation would be advantageous in stabilising p50 homodimers and thus reinforcing their repressive effects on pro-inflammatory gene expression. Based on the results of the previous chapter and studies identifying SOCS1 as an E3 ligase for p65, we hypothesised that SOCS1 was a putative E3 ligase responsible for ubiquitinating and degrading p50 via the proteasome and so a series of *in vitro* assays were performed to investigate this relationship further. Instead, a novel function for SOCS1 was discovered, whereby it causes the ubiquitination and degradation of p50 by some mechanism outside of its capacity as an E3 ligase. This study extends the list of functions that SOCS1 has in the regulation of the immune response, and further highlights its importance as a potential target for therapeutics that mean to repress dysregulated inflammatory gene expression.

4.2 Introduction

In the previous chapter, the growing body of work surrounding the relationship between the ubiquitin proteasome system (UPS) and NF-κB was outlined. Most of what is known so far is based on studies on the p65 subunit, with p50 receiving relatively little attention. However, identifying the components of the UPS that target p50 for ubiquitination and degradation is critical for stabilising p50 homodimers and thus reinforcing their repressive effects on the transcription of pro-inflammatory genes during periods of immune system dysregulation.

Suppressor of cytokine signalling 1 (SOCS1) is one member of a family of eight related proteins. All members share a central Src homology 2 (SH2) domain, a C-terminal SOCS box that is ~40 amino acids long and a variable length N-terminal. SOCS1 also has a kinase inhibitory region (KIR) domain. SOCS1 has a number of roles within the immune system. Initially, it was found to act as a negative regulator in the JAK/STAT cytokine signalling pathway (Davey *et al.*, 2006) by binding to JAK via the SH2 domain and inhibiting its kinase activity and signalling via the KIR domain (Narazaki *et al.*, 1998). This is a classical negative feedback loop whereby cytokine signalling induces SOCS1 that can inhibit the pathways that stimulated its production. Additionally, SOCS1 is able to modulate cytokine production via its SOCS box. Mice expressing a mutant form of SOCS1 that lacked the SOCS box exhibited prolonged JAK/STAT signalling in response to cytokines (Zhang *et al.*, 2001). This was reinforced by the finding that SOCS1 acts to ubiquitinate and degrade JAK2 by way of its SOCS box (Frantsve *et al.*, 2001).

More recently, SOCS1 was identified as an E3 ligase for the p65 subunit of NF-κB (Ryo *et al.*, 2003; Strebovsky *et al.*, 2011). Via its SOCS box domain, it functions to polyubiquitinate p65 leading to its proteasomal degradation within the nucleus. This has the effect of terminating the prolonged expression of NF-κB inducible genes (Saccani *et al.*, 2004). It has also been shown to function as part of a multi-subunit complex containing elongin c, cullin2 and rbx1, known as $ECS^{(SOCS1)}$, and this cooperates with COMMD1 to ubiquitinate p65. In this case, the SOCS box domain of SOCS1 functions as the substrate recognition component, whilst COMMD1 stabilises the interaction between SOCS1 and p65 promoting its ubiquitination.

The data presented in the previous chapter strongly suggested that SOCS1 promotes the ubiquitination and degradation of p50 as well, therefore in this chapter this relationship was explored in greater detail. A series of *in vitro* assays were performed in cells that had the proteins of interest overexpressed so that the fundamental mechanisms of p50 ubiquitination and degradation in the presence of the putative E3 ligase SOCS1 could be better understood. These data demonstrated that SOCS1 promotes the ubiquitination and degradation of p50, but this is independent of its E3 ligase activity because a SOCS1 mutant that lacks a functional SOCS box is still able to promote degradation and a proteasome inhibitor did not prevent it. Furthermore, SOCS1 and p50 are demonstrated to interact even when SOCS1 has a defective nuclear localisation signal and SOCS box. Ultimately, the promotion of ubiquitination and degradation of p50 might be due to SOCS1 being one component of a larger, unidentified complex. Regardless, SOCS1 is established as an important factor in the maintenance of p50 stability. SOCS1 could prove to be a desirable target for future therapies that aim to reinforce the repressive effects p50 homodimers have on the expression of pro-inflammatory genes.

4.3 Results

4.3.1 SOCS1 promotes the degradation of p50

To determine what effect, if any, SOCS1 has on the stability of p50, HEK293T (293T) cells were transiently transfected with plasmids previously generated within the lab to co-express wild-type p50-Xpress (p50-XP) and wild-type human GFP-tagged SOCS1 (eGFP-hSOCS1, herein referred to as GFP-SOCS1). Cells were harvested and expression levels of p50 analysed by Western blotting with anti-XP antibody. This revealed a marked decrease in the level of p50 when co-expressed with SOCS1 in comparison to p50 expressed on its own (figure 4.1). This result indicates that the presence of SOCS1 causes p50 to be degraded although the mechanism is yet unknown.

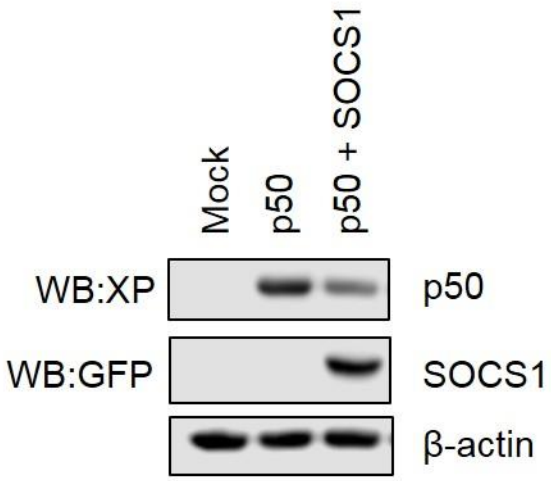


Figure 4.1: SOCS1 promotes degradation of p50.
HEK293T (293T) cells were co-transfected to express WT p50-XP and WT GFP-SOCS1 or not. Total amount of DNA transfected was 3 µg, using 1 µg of p50 and 0.5 µg E3 ligase, making up the rest with empty vector. Cells were lysed and expression levels of p50 were analysed via Western blotting (WB) against XP. The figure is representative of three individual experiments.

4.3.2 SOCS1 promotes ubiquitination of p50

Given its ability to induce p50 degradation, it was important to determine whether SOCS1 is able to poly-ubiquitinate p50 *in vitro*, and whether this might be the mechanism causing p50 to be degraded. To achieve this, a cellular ubiquitination assay was performed. 293T cells were co-transfected with plasmids expressing p50-XP, GFP-SOCS1 and ubiquitin-HA (Ub-HA). 20 μM MG132 was added to inhibit proteasomal degradation because any ubiquitination activity might mediate such degradation. One hour after this, cell lysates were harvested and denatured prior to being immunoprecipitated with anti-XP antibody and immunoblotted with anti-HA to detect ubiquitin, and the other antibodies as indicated. Compared to the controls, there is a noticeable increase in the level of ubiquitination of p50 in the presence of SOCS1 (figure 4.2), which suggests that SOCS1 is promoting p50 ubiquitination.

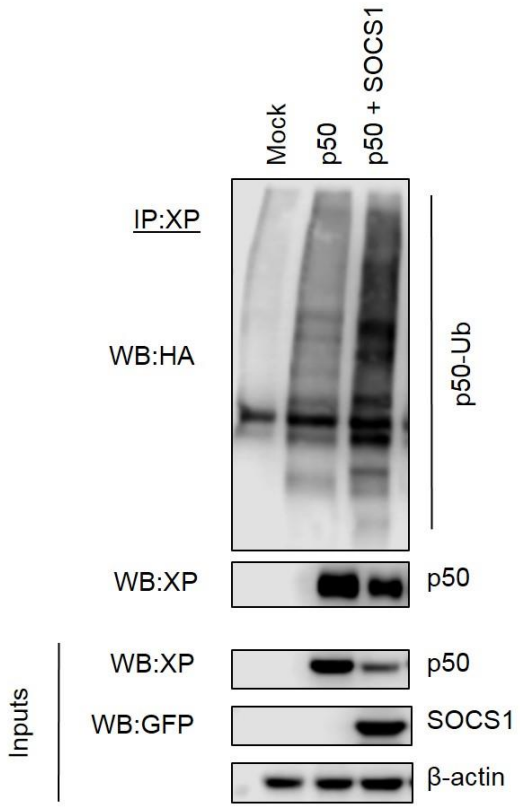


Figure 4.2: SOCS1 promotes ubiquitination of p50.
293T cells were co-transfected to express p50-XP, Ub-HA and GFP-SOCS1 or not. Total amount of DNA transfected was 3 µg, using 1 µg of p50, 1 µg of ubiquitin and 0.5 µg E3 ligase, making up the rest with empty vector. Cells were then treated with 20 µM of the proteasome inhibitor MG132 for 1 hour before harvest. Ubiquitination of p50 was analysed via immunoprecipitation (IP) of XP and WB against the HA tag of ubiquitin. Figure is representative of three individual experiments.

4.3.3 SOCS1 interacts with p50

To determine whether the effect SOCS1 has on p50 stability is due to an interaction between the two, a co-immunoprecipitation (Co-IP) experiment was performed. 293T cells were transiently transfected to co-express p50-XP and GFP-SOCS1. Next, whole cell lysates were used and XP-tagged p50 was immunoprecipitated and immunoblotted using anti-GFP to detect SOCS1. This analysis revealed the interaction of p50 and SOCS1 (figure 4.3). At this stage, it is not known what domain of SOCS1 or p50 is important for this interaction, or in what sub-cellular location it occurs.

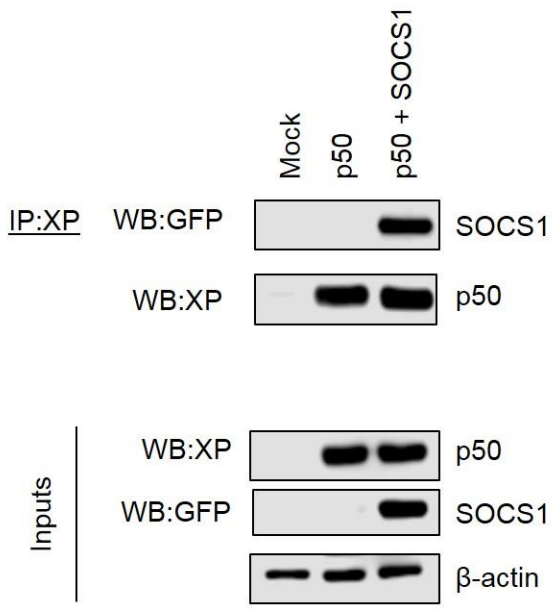


Figure 4.3: SOCS1 interacts with p50.
293T cells were co-transfected to express p50-XP and GFP-SOCS1 or not. Total amount of DNA transfected was 3 µg, using 1 µg of p50 and 0.5 µg E3 ligase, making up the rest with empty vector.Cells were treated with 20 µM MG132 for 1 hour before harvest. Protein interaction was analysed via co-immunoprecipitation (co-IP) of XP and then WB against the GFP tag of SOCS1. Figure is representative of three individual experiments.

4.3.4 SOCS1 promotes ubiquitination of p50 more strongly than p65

293T cells were co-transfected with plasmids expressing p50-XP, p65, GFP-SOCS1 and Ub-HA. It was decided to increase the concentration of MG132 that was used to treat the cells due to the level of p50 degradation that was still observed with the addition of 20 μM. Therefore, cells were treated with 80 μM MG132 and after 1 hour lysates were harvested and an ubiquitination assay performed as described above. SOCS1 is able to induce more strongly the ubiquitination of p50 than it can p65, and the degradative effect on p50 appears more pronounced than p65 (figure 4.4A). The baseline level of ubiquitination of p65 is already much greater than p50 (figure 4.4B), however when normalised to the amount of p50 and p65 that is immunoprecipitated, SOCS1 induces a greater relative increase in the ubiquitination of p50 compared to p65, although both are increased by SOCS1 (figure 4.4C). Densitometry analysis was employed because the relative levels of ubiquitination of p50 and p65 are so different that it is difficult to discern by eye the increase that is induced by SOCS1. This finding indicates that not only is SOCS1 important for repressing the NF-κB response by causing the degradation of p65, but it may also have a role to play in promoting its activity by de-stabilising p50.

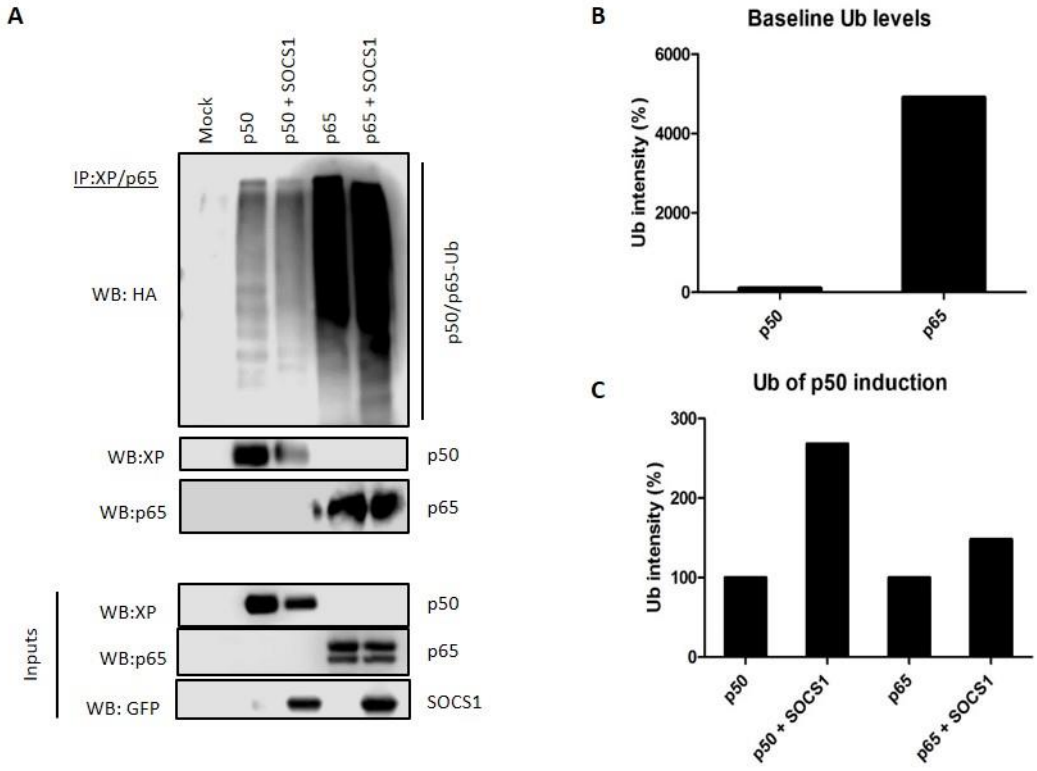


Figure 4.4: SOCS1 promotes ubiquitination of p50 more strongly than p65.
(A) 293T cells were co-transfected to express p50-XP, p65, Ub-HA, and either GFP-SOCS1 or not. Total amount of DNA transfected was 3 µg, using 1 µg of NF-κB subunit, 1 µg of ubiquitin and 0.5 µg E3 ligase, making up the rest with empty vector. Cells were then treated with 80 µM MG132 for 1 hour before harvest. Ubiquitination of p50 and p65 was analysed via IP of XP and p65 and WB against the HA tag of ubiquitin. (B) Densitometry analysis was performed using Image Studio Lite software and the baseline level of p65 measured as compared to p50. (C) Densitometry analysis was performed as above. Levels of p50 and p65 ubiquitination were normalised to the amount of p50 and p65 that was immunoprecipitated and compared to p50 or p65 without SOCS1. Figure is representative of two individual experiments.

4.3.5 SOCS1 does not ubiquitinate p50 at a single lysine residue

Lysine (Lys, K) residues are the target sites for ubiquitination. Ubiquitination can consist of one single moiety of ubiquitin (mono-) or multiple moieties linked via K residues within ubiquitin (poly-), and these attachments can occur at single or multiple K residues on the substrate protein. Previous data generated in the lab demonstrated that p50 is K48-linked poly-ubiquitinated (Carmody *et al.*, 2007), and that this occurs at a single K residue because ubiquitination assays using a mutant of ubiquitin incapable of forming K48-linked chains produced a single ubiquitination band at 58 kDa, which is the size of p50 modified with one ubiquitin molecule. However, this refers to constitutive p50 ubiquitination, which may be different from SOCS1-induced ubiquitination. If SOCS1 is responsible for ubiquitinating p50 then it would be doing it at one or more of the 29 K residues present on p50. It has already been demonstrated in our lab that K128 is an important site of murine p50 ubiquitination but the E3 ligase responsible remains unknown. To determine if SOCS1 ubiquitinates p50 at a single specific lysine, we next used a series of K to arginine (Arg, R) mutants of p50 that had been generated previously in the lab and employed them in ubiquitination and expression assays in the presence and absence of SOCS1 as described previously (figure 4.5A). Arginine is also a positively charged, polar amino acid, and so most of the biochemical properties are retained but R cannot be ubiquitinated. Therefore, it was hypothesised if a target K residue was identified, there would be a dramatic decrease in p50 ubiquitination in the presence of SOCS1, or the degradation would not be observed. The results of this analysis allowed for no conclusion to be drawn with regards to the levels of ubiquitination of the p50 lysine mutants compared to wild-type (figure 4.5B). However, there is the obvious degradation of wild-type and all p50 mutants in the presence of SOCS1 compared to without it (figure 4.5C). This might be indicative of a mechanism separate from the UPS that is causing SOCS1 to degrade p50. However, our library covers 18 of the 29 total K residues of p50 so it could also be that the relevant residue has not yet been tested.

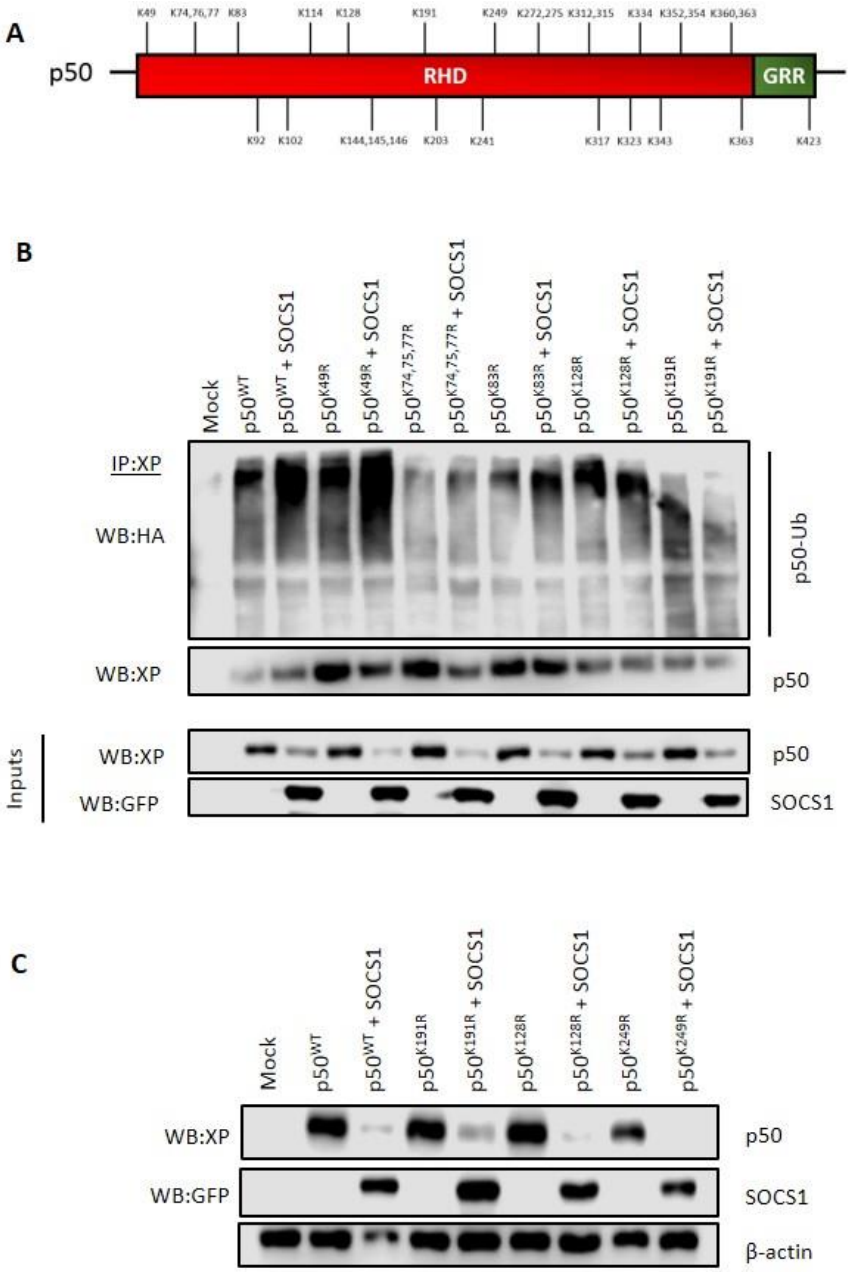


Figure 4.5: SOCS1 does not ubiquitinate p50 at a single lysine residue.
(A) Schematic of p50 with all lysine residues highlighted. Residues above are those tested using in-lab library of p50 mutants and those below are yet to be tested. RHD: Rel homology domain. GRR: Glycine rich region. (B) 293T cells were co-transfected to express WT or mutant p50-XP, Ub-HA and GFP-SOCS1 or not. Total amount of DNA transfected was 3 µg, using 1 µg of p50, 1 µg of ubiquitin and 0.5 µg E3 ligase, making up the rest with empty vector. Cells were then treated with 80 µM MG132 for 1 hour before harvest. Ubiquitination of p50 was analysed via IP of XP and WB against the HA tag of ubiquitin. Figure is representative of more than three individual experiments. (C) 293T cells were co-transfected to express WT or mutant p50-XP and GFP-SOCS1 or not. Total amount of DNA transfected was 3 µg, using 1 µg of p50 and 0.5 µg E3 ligase, making up the rest with empty vector. Cells were lysed and expression levels of p50 were analysed via WB against XP. Figure representative of more than three experiments.

4.3.6 GFP tag of SOCS1 is not responsible for degradation of p50

It has previously been reported that the enhanced GFP tag, which is what SOCS1 in this study is tagged with, can inhibit NF-κB activation perhaps due to an ability to interfere with both K48- and K63-linked poly-ubiquitination (Baens *et al.*, 2006). There has also been a link reported between expression of GFP and cell apoptosis (Liu *et al.*, 1999), impaired actin-myosin interaction in heart muscle cells (Zhang *et al.*, 2003), and the induction of HSP70 causing up-regulation of COX2 (Agbulut *et al.*, 2006). To rule out any adverse effects the tag might have had on the experimental outcomes, a FLAG-tagged SOCS1 plasmid was purchased and employed in a series of cellular ubiquitination and expression assays, performed as described previously. No discernible difference was seen between the ubiquitination levels of p50 when co-expressed with GFP-SOCS1 or SOCS1-FLAG (figure 4.6A), however more revealing was the expression level of p50 in the absence of a proteasome inhibitor. When either GFP-SOCS1 or SOCS1-FLAG is present, the expression level of p50 is decreased compared to controls (figure 4.6B) indicating that it is SOCS1 itself, and not any associated protein tag, that promotes the degradation of p50.

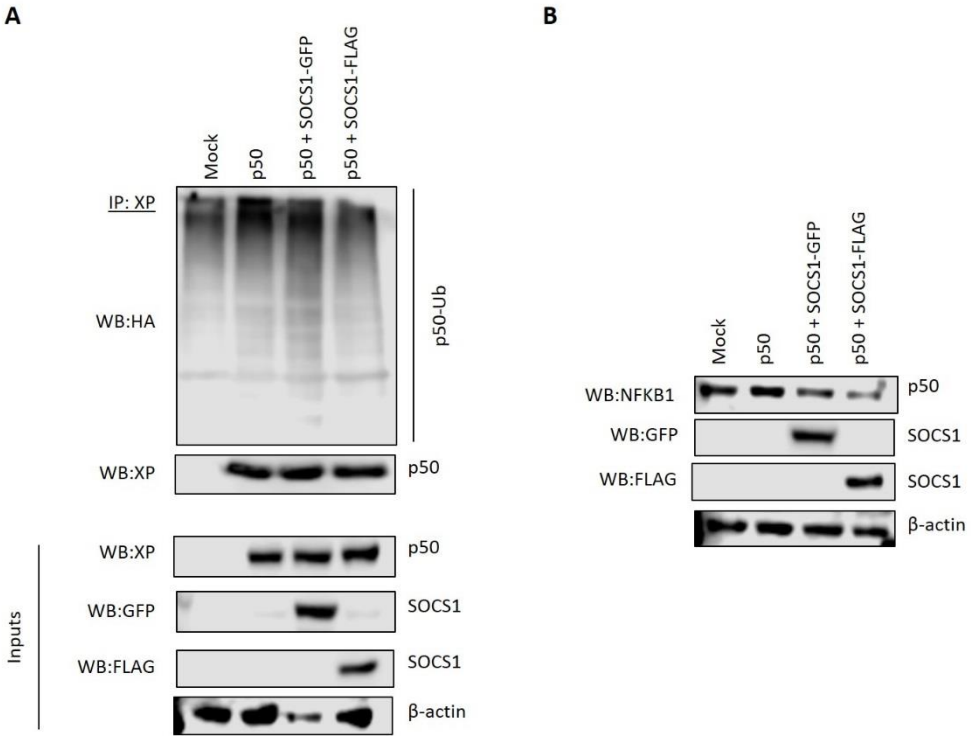


Figure 4.6: GFP tag not responsible for degradation of p50.
(A) 293T cells were co-transfected to express p50-XP, Ub-HA and GFP-SOCS1 or SOCS1-FLAG or not. Total amount of DNA transfected was 3 µg, using 1 µg of p50, 1 µg of ubiquitin and 0.5 µg E3 ligase, making up the rest with empty vector. Cells were then treated with 80 µM MG132 for 1 hour before harvest. Ubiquitination of p50 was analysed via IP of XP and WB against the HA tag of ubiquitin. Figure is representative of two individual experiments. (B) 293T cells were co-transfected to express p50-XP and GFP-SOCS1 or SOCS1-FLAG or not. Total amount of DNA transfected was 3 µg, using 1 µg of p50 and 0.5 µg E3 ligase, making up the rest with empty vector. Cells were lysed and expression levels of p50 were analysed via WB using anti-p50.

4.3.7 SOCS1 interacts with and promotes degradation of DNA-binding mutant of p50

The binding of p50 homodimers to DNA is an essential step that triggers their ubiquitination and subsequent proteasomal degradation (Carmody *et al.*, 2007). The p50:p50:DNA complex is inherently unstable for this reason. A mutant of p50 wherein the tyrosine at position 57 (Y57A) was substituted for alanine and glycine at position 60 substituted for aspartate (G60D), herein referred to as $p50^{Y57A,G60D}$, had previously been generated in the lab. These mutations in the DNA-binding domain of p50 render it unable to interact with DNA, but still able to bind to other protein partners. It was previously demonstrated that ubiquitination of this mutant is diminished compared to wild-type, and this translated to a longer half-life and greater stability. Therefore, it was relevant to investigate if SOCS1 had any noticeable effect on the ubiquitination status or stability of this DNA-binding mutant compared to wild-type. 293T cells were co-transfected with plasmids expressing $p50^{WT}$-XP, $p50^{Y57A,G60D}$-XP, Ub-HA and GFP-SOCS1 or not and a cellular ubiquitination assay performed as described previously. The results indicate that SOCS1 expression degrades this mutant. p50 is noticeably degraded compared to the mutant alone, and even compared to wild-type p50 in the presence of SOCS1 (figure 4.7A). Furthermore, although baseline levels of ubiquitination of the DNA-binding mutant are less than WT as expected, SOCS1 is able to ubiquitinate p50 even when it is unable to bind to DNA (figure 4.7B). Additionally, a co-immunoprecipitation assay revealed that there is a weak interaction between SOCS1 and the DNA-binding mutant of p50 (figure 4.7C). This is intriguing because it suggests SOCS1 interacts with and has an effect on the stability of non-DNA-bound p50, thus having an impact on the dynamic pool of p50 that is available to form dimers within the cell.

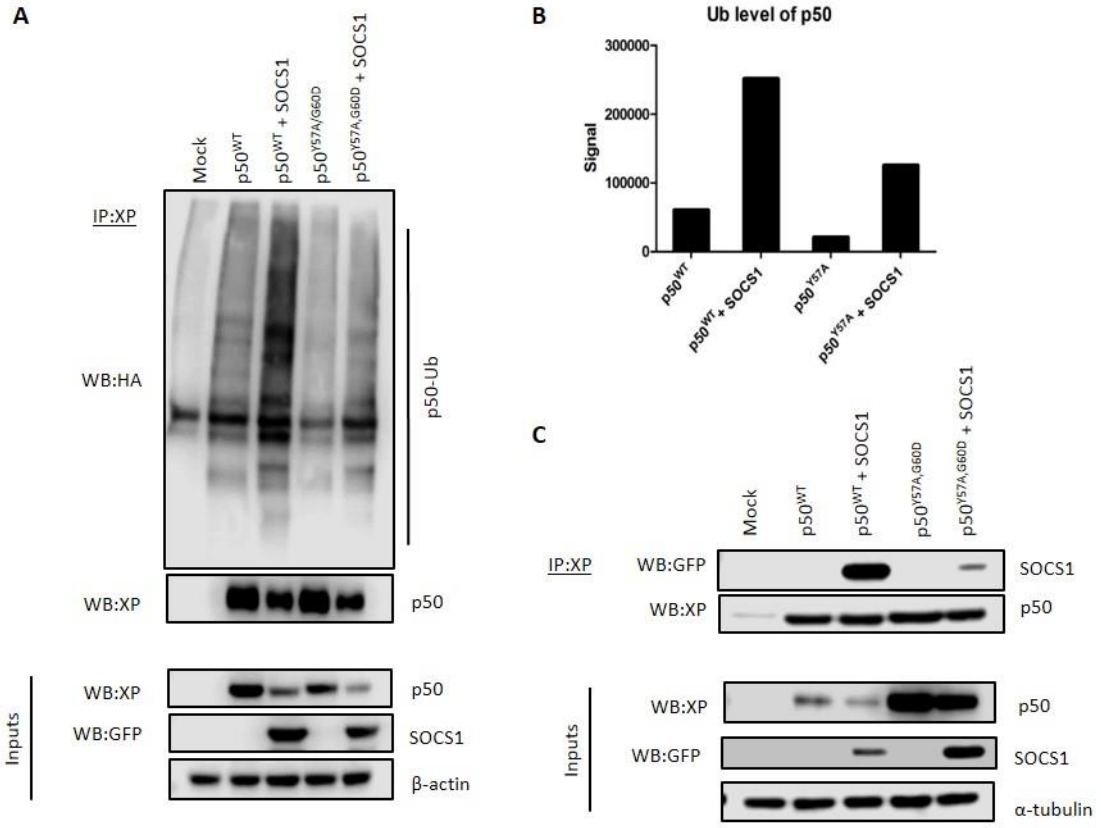


Figure 4.7: SOCS1 interacts with, ubiquitinates and promotes degradation of DNA-binding mutant of p50.
(A) 293T cells were co-transfected to express $p50^{WT}$-XP or $p50^{Y57A,G60D}$-XP, Ub-HA and GFP-SOCS1 or not. Total amount of DNA transfected was 3 µg, using 1 µg of p50, 1 µg of ubiquitin and 0.5 µg E3 ligase, making up the rest with empty vector. Cells were then treated with 80 µM MG132 for 1 hour before harvest. Ubiquitination of p50 was analysed via IP of XP and WB against the HA tag of ubiquitin. (B) Densitometry analysis was performed using Image Studio Lite software and the levels of ubiquitination were normalised to the amount of p50 that was immunoprecipitated. Figure is representative of two individual experiments. (C) 293T cells were co-transfected to express $p50^{WT}$-XP or $p50^{Y57A,G60D}$-XP and GFP-$SOCS1^{WT}$ or not. Total amount of DNA transfected was 3 µg, using 1 µg of p50 and 0.5 µg E3 ligase, making up the rest with empty vector. Cells were treated with 80 µM MG132 for 1 hour before harvest. Protein interaction was analysed via co-IP of XP and then WB against the GFP tag of SOCS1.

4.3.8 Nuclear localisation signal-defective SOCS1 mutant promotes the degradation of p50

SOCS1 localises to the nucleus because of its nuclear localisation sequence (NLS) situated between the SH2 domain and the SOCS box (Baetz *et al.*, 2008). This was a hitherto unexpected property of SOCS1 because of its prominent role as an inhibitor of JAK activity in the cytoplasm. However, the finding opened up the potential repertoire of activity of SOCS1. Previous studies had demonstrated that SOCS1 was able to limit NF-κB activity by mediating nuclear degradation of p65 (Strebovsky *et al.*, 2011). However, this effect was not seen when a NLS mutant of SOCS1 was employed. This mutant was also shown not to bind to p65 in the cytoplasm. Leading on from the findings in the previous section, it was of interest to determine whether SOCS1 was able to exert any effect on p50 outside of the nucleus. For this purpose, a NLS mutant of SOCS1 was generated and kindly donated by the Dalpke lab, referenced previously (GFP-SOCS1$^{6R/A}$). This mutant had 6 basic arginine-to-alanine residues substituted within the NLS and exhibited predominant if not exclusive cytoplasmic localisation. To determine what effect, if any, this mutant had on the ubiquitination status of p50, a cellular ubiquitination assay was conducted. 293T cells were co-transfected with plasmids expressing p50-XP, Ub-HA and GFP-SOCS1^{WT} or GFP-SOCS1$^{6R/A}$ or not and a cellular ubiquitination assay performed as previously described. No discernible effects on p50 ubiquitination by the non-nuclear SOCS1 mutant were observed (figure 4.8A). However, it proved challenging to quantify any changes in the level of p50 ubiquitination due to its degradation in the presence of SOCS1, even with the addition of high concentrations of MG132. Therefore, expression of p50 was investigated instead by performing an expression assay. As previously observed, there is a decrease in the expression of p50 in the presence wild-type SOCS1, although an even more noticeable decrease in the presence of the NLS mutant can be seen (figure 4.8B). Furthermore, to determine if this mutant interacts with p50, a co-immunoprecipitation assay was conducted and performed as previously outlined. As seen in figure 4.8C, a clear binding was detected between p50 and both wild-type and the SOCS1 mutant that cannot access the nucleus. This is surprising considering the previous finding that wild-type SOCS1 binds to p65 within the nucleus and this leads to p65 degradation, but the NLS mutant did not.

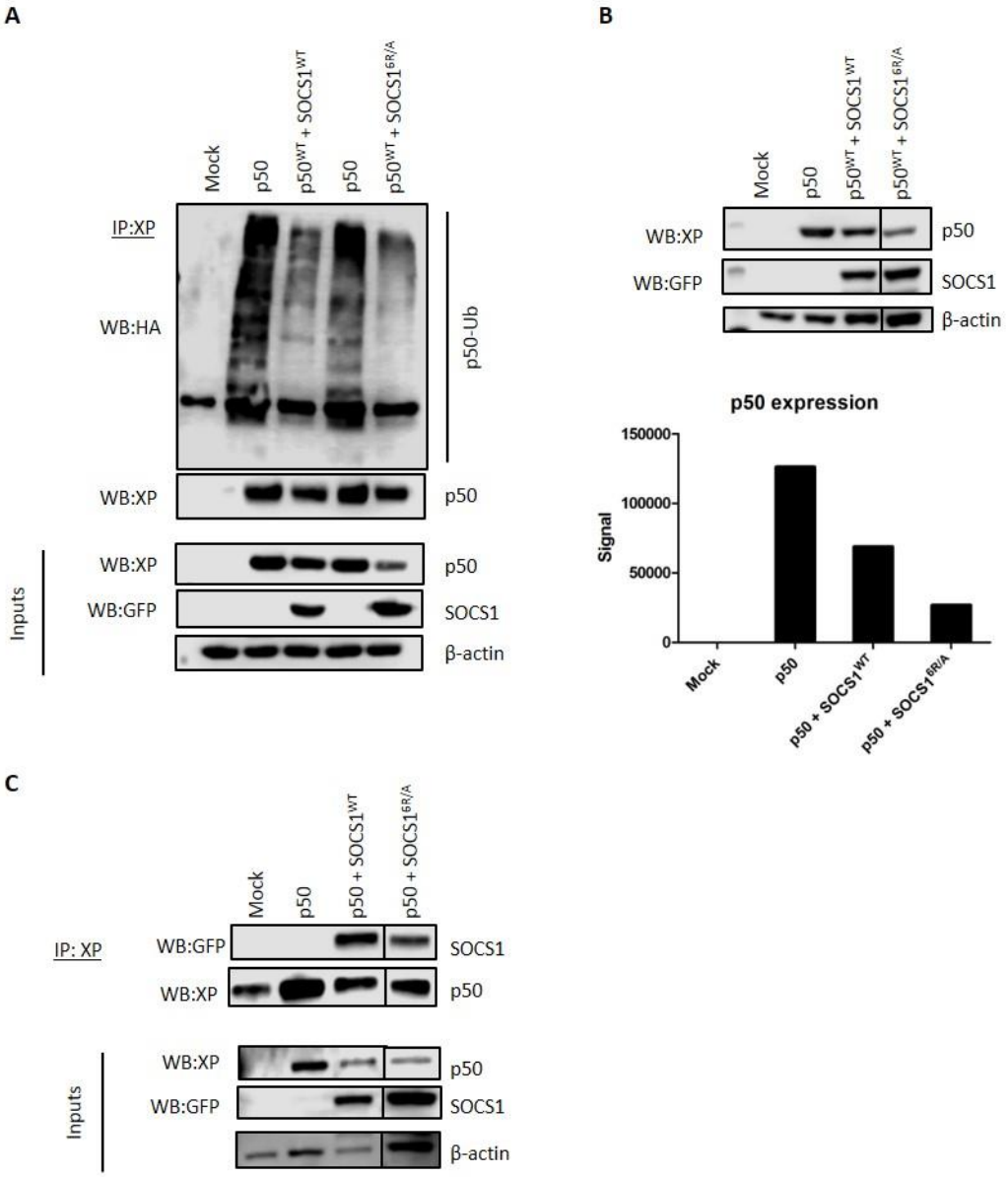


Figure 4.8: NLS mutant of SOCS1 promotes the degradation of p50 but may not promote ubiquitination.

(A) 293T cells were co-transfected to express p50-XP, Ub-HA and GFP-SOCS1^{WT} or GFP-SOCS1$^{6R/A}$ or not. Total amount of DNA transfected was 3 µg, using 1 µg of p50, 1 µg of ubiquitin and 0.5 µg E3 ligase, making up the rest with empty vector. Cells were then treated with 80 µM MG132 for 1 hour before harvest. Ubiquitination of p50 was analysed via IP of XP and WB against the HA tag of ubiquitin. Figure is representative of three individual experiments. (B) 293T cells were co-transfected to express p50-XP and GFP-SOCS1^{WT} or GFP-SOCS1$^{6R/A}$, or not. Total amount of DNA transfected was 3 µg, using 1 µg of p50 and 0.5 µg E3 ligase, making up the rest with empty vector. Cells were lysed and expression levels of p50 were analysed via WB against XP. Figure edited to remove unrelated sample but image is taken from one scan using one exposure. Densitometry analysis was performed using Image Studio Lite software and the levels of p50 were normalised to β-actin. (C) 293T cells were co-transfected to express p50-XP and GFP-SOCS1^{WT} or GFP-SOCS1$^{6R/A}$ not. Total amount of DNA transfected was 3 µg, using 1 µg of p50 and 0.5 µg E3 ligase, making up the rest with empty vector. Cells were treated with 80 µM MG132 for 1 hour before harvest. Protein interaction was analysed via co-IP of XP and then WB against the GFP tag of SOCS1. Figure edited to remove unrelated sample but image is taken from one scan using one exposure. Figure is representative of three individual experiments.

4.3.9 SOCS1 promotes the ubiquitination of p50 in the presence of BCL-3

It has been established that the atypical IκB protein, BCL-3, is an essential regulator of NF-κB during TLR and TNFR signalling. It stabilises DNA-bound p50 homodimers by preventing their ubiquitination and subsequent degradation (Carmody *et al.*, 2007). Thus, the repressive effect of p50 homodimers on pro-inflammatory gene expression is reinforced. Furthermore, a direct interaction between p50 and BCL-3 is necessary and sufficient for this stabilising effect and for the anti-inflammatory role of BCL-3 (Collins *et al.*, 2014). p50 that is unable to interact with BCL-3 is hyper-ubiquitinated and therefore has a reduced half-life compared to wild-type. Thus far, it has been observed that SOCS1 has a de-stabilising effect on p50. To determine whether SOCS1 promotes p50 ubiquitination in the presence of BCL-3, a ubiquitination assay was performed as previously described. Ubiquitination of p50 is increased in the presence of SOCS1, and as expected is inhibited in the presence of BCL-3. In the presence of both SOCS1 and BCL-3, some ubiquitination of p50 appears to be restored (figure 4.9A). Interestingly, when p50, SOCS1 and BCL-3 are co-expressed, the degradation of BCL-3 was observed. The mechanism causing this remains unknown.

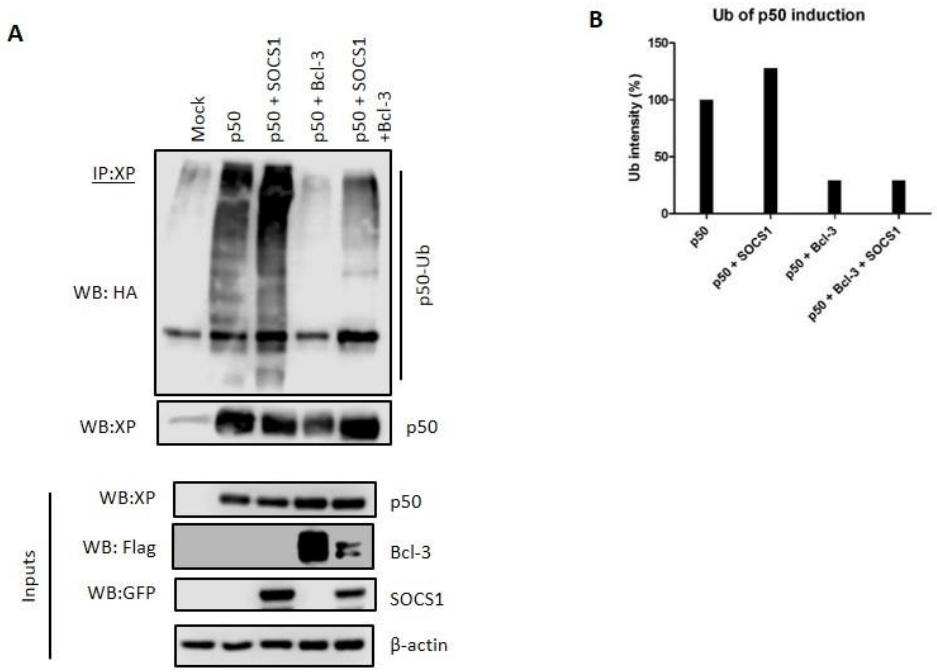


Figure 4.9: The interplay between SOCS1 and BCL-3 when present with p50.
(A) 293T cells were co-transfected to express p50-XP, Ub-HA and GFP-SOCS1 or not, and BCL-3-FLAG or not. Total amount of DNA transfected was 3 µg, using 1 µg of p50, 1 µg of ubiquitin and 0.5 µg E3 ligase and 0.5 g BCL-3. Cells were then treated with 80 µM MG132 for 1 hour before harvest. Ubiquitination of p50 was analysed via IP of XP and Western blotting against the HA tag of ubiquitin. Figure is representative of three individual experiments. (B) Densitometry analysis was performed using Image Studio Lite software and the levels of ubiquitination were normalised to the amount of p50 that was immunoprecipitated.

4.3.10 Degradation of p50 occurs in the presence of a SOCS1 mutant that lacks SOCS box domain

Based on the data gathered thus far in this investigation, it was hypothesised that SOCS1-bound p50 was ubiquitinated and degraded due to the SOCS box of SOCS1, which is known to mediate E3 ligase activity. To test this hypothesis, a truncated mutant that had been generated previously in the lab, and which lacked a functional SOCS box, was used (GFP-SOCS1^{R172X}) (figure 4.10A). A mutant with this same mutation had been employed in a separate study and had been unable to mediate the ubiquitination of p65 (Strebovsky *et al.*, 2011). In the first instance, a cellular ubiquitination assay was performed as previously described. Unexpectedly, the presence of the R172X mutant was still able to induce some ubiquitination of p50 (figure 4.10B). When this was quantified by densitometry and normalised to the amount of p50 immunoprecipitated, it appeared that p50 was more ubiquitinated in the presence of the mutant SOCS1 than the wild-type (figure 4.10C). This surprising result led us to determine whether the mutant was able to cause the degradation of p50 and so an expression assay was performed as described before. Surprisingly again, there was a detectable level of degradation of p50 in the presence of the SOCS box mutant of SOCS1 compared the controls, and even in comparison to p50 with wild-type SOCS1 (figure 4.10D and E). Considering the other mutants of SOCS1 had demonstrated clear binding to p50 this property was also investigated for this mutant. A co-immunoprecipitation assay was conducted as described earlier. A clear binding of SOCS1^{R172X} to p50 was detected, which demonstrates that this interaction is not dependent upon the SOCS box (figure 4.10F). However, taken together, these data indicate that the interaction between SOCS1 and p50 promotes the ubiquitination and degradation of p50 independently of the E3 ligase activity of SOCS1.

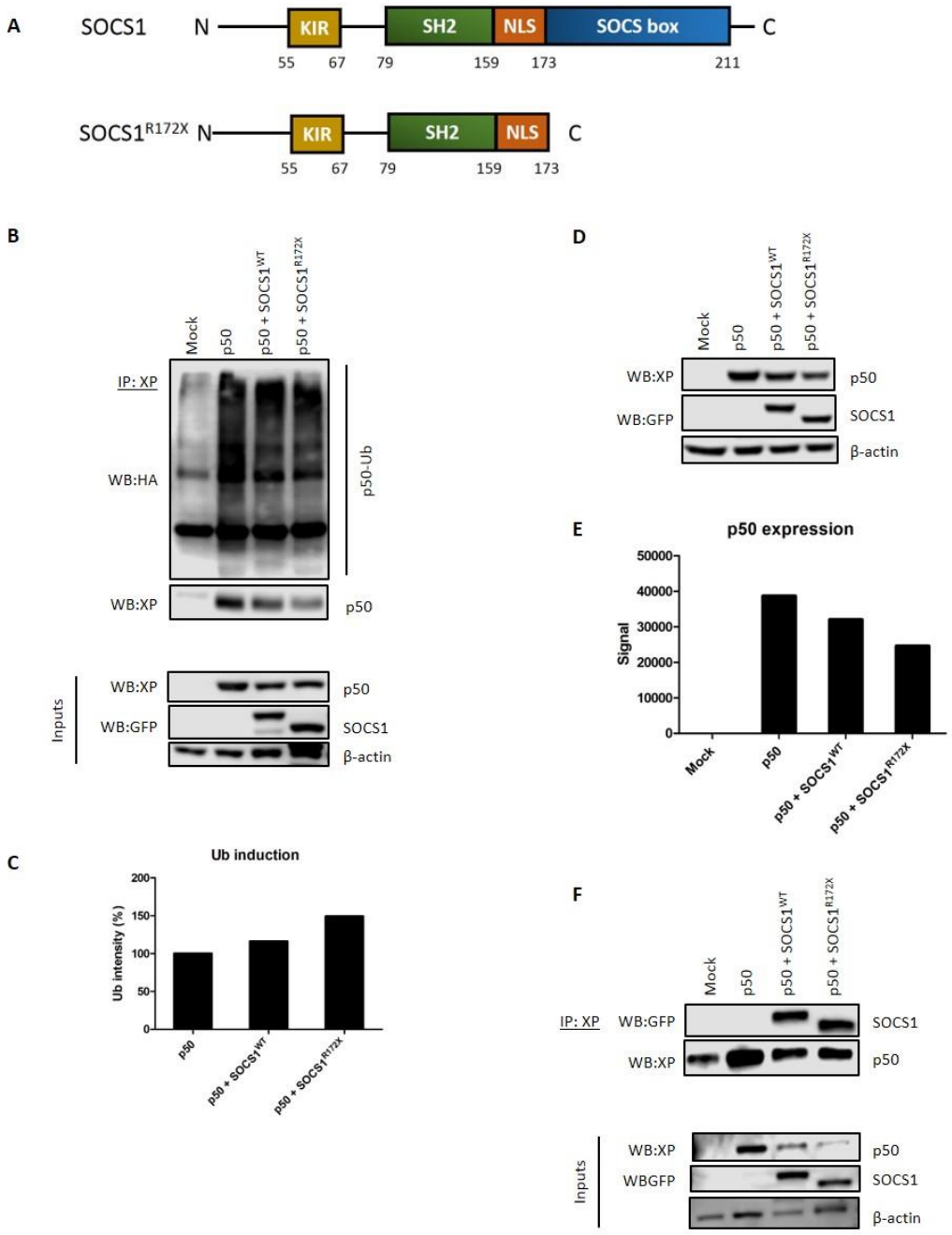


Figure 4.10: Degradation of p50 occurs in the presence of a SOCS1 mutant that lacks a SOCS box domain.

(A) Schematic drawing of SOCS1 and SOCS-box mutant. Numbers represent amino acid location. (B) 293T cells were co-transfected to express p50-XP, Ub-HA and GFP-SOCS1^{WT} or GFP-SOCS1^{R172X} or not. Total amount of DNA transfected was 3 µg, using 1 µg of p50, 1 µg of ubiquitin and 0.5 µg E3 ligase, making up the rest with empty vector. Cells were then treated with 80 µM MG132 for 1 hour before harvest. Ubiquitination of p50 was analysed via IP of XP and Western blotting against the HA tag of ubiquitin. Figure is representative of three individual experiments. (C) Densitometry analysis was performed using Image Studio Lite software and the levels of ubiquitination were normalised to the amount of p50 that was immunoprecipitated. (D) 293T cells were co-transfected to express p50-XP and GFP-SOCS1^{WT} or GFP-SOCS1^{R172X} or not. Total amount of DNA transfected was 3 µg, using 1 µg of p50 and 0.5 µg E3 ligase, making up the rest with empty vector. Cells were lysed and expression levels of p50 were analysed via Western blotting against XP. (E) Densitometry analysis was performed using Image Studio Lite software and the levels of p50 were normalised to β-actin. (F) 293T cells were co-transfected to express p50-XP and GFP-SOCS1^{WT} or GFP-SOCS1^{R172X} not. Total amount of DNA transfected was 3 µg, using 1 µg of p50 and 0.5 µg E3 ligase, making up the rest with empty vector. Cells were treated with 80 µM MG132 for 1 hour before harvest. Protein interaction was analysed via co-IP of XP and then Western blotting against the GFP tag of SOCS1. Figure is representative of three individual experiments.

4.4 Discussion

The results in this chapter indicate that SOCS1 interacts with p50 (figure 4.3), and that SOCS1 causes p50 to de-stabilise (figure 4.1). It is unclear how this interaction causes p50 degradation, because although SOCS1 increases p50 ubiquitination and degradation (figure 4.2), this also occurred in the presence of a SOCS1 mutant that lacked a functional SOCS box meaning it was independent of its E3 ligase activity (figure 4.10). Furthermore, this degradation did not seem to be proteasomal because treatment with a proteasome inhibitor did not prevent it. These data therefore suggest that the effect SOCS1 has on p50 stability is not due to its capacity as an E3 ligase, ubiquitinating it and directing it towards proteasomal degradation.

SOCS1 might be acting in concert with an unidentified mediator(s) that induces the increased ubiquitination of p50. The presence of the SOCS box confers E3 ligase capacity on SOCS1, allowing it to promote the ubiquitination and degradation of p65 to which it binds within the nucleus (Strebovsky *et al.*, 2011). However, SOCS1 also functions as part of larger complexes that have ubiquitin ligase ability. For example, SOCS1 forms a heterodimeric E3 ligase with the protein von Hippel-Lindau (VHL) that targets phosphorylated JAK2 for ubiquitin-mediated degradation (Russell *et al.*, 2011). It interacts with elongins B and C, cullin2 and Rbx1 to form a multimeric ubiquitin ligase (ECS^{SOCS1}). COMMD1 was shown to accelerate the ubiquitination and degradation of p65 and this was due to its association with the ECS^{SOCS1} (Maine *et al.*, 2007). In the complex SOCS1 is the probable substrate recognition component. In the current investigation, a SOCS box mutant of SOCS1 was still able to bind to p50 so it is possible that SOCS1 recognises and binds to p50 via a different domain, and another component that interacts with the ubiquitin ligase complex is responsible for increasing its ubiquitination. Alternatively, SOCS1 may have a degradative effect on p50 due to it interfering with a different PTM of p50. Phosphorylation of the NF-κB subunits has a number of consequences, one of which is stabilisation. For instance phosphorylation of p65 on serine (S) 276 by Pim-1 kinase was shown to protect it from degradation by preventing SOCS1-mediated ubiquitination (Nihira *et al.*, 2010). Phosphorylation of p50 is less understood and to date, no residue has been identified that leads to increased p50 stability. However, this does not preclude the possibility that one exists and that SOCS1 is preventing such a

stabilising step from happening, perhaps by binding to p50 in such a way that the pertinent phosphorylation (or other PTM) site is inaccessible by the kinase. On the other hand, instead of ubiquitinating p50, SOCS1 could be preventing p50 from being deubiquitinated by inhibiting the action of a deubiquitinase (DUB). Ubiquitination is a reversible modification, and to counteract the action of E3 ligases, DUBs mediate the removal of ubiquitin from substrate proteins (Komada, 2008; Clague *et al.*, 2009). USP7 has been shown to interact with p50 but its deubiquitinase activity has only been demonstrated for p65 (Colleran *et al.*, 2013). Perhaps USP7, or another yet unidentified DUB, is being blocked by SOCS1 from hydrolysing ubiquitin moieties, which has the effect of increased p50 ubiquitination and degradation when SOCS1 is present.

Had time not been a limiting factor in the current investigation, an appropriate experiment would have been to separate the nuclear and cytoplasmic fractions of the cells and determine where the interaction between SOCS1 and p50 was occurring, and where the degradative effect was predominantly observed. This question was somewhat addressed by the finding that the NLS SOCS1 mutant is able to interact with SOCS1 and that p50 is degraded in its presence (figure 4.8). This is in contrast with Strebovsky and colleagues' finding (2011) that SOCS1 was able to limit NF-κB activity by binding to and mediating p65 degradation only within the nucleus. They speculated this was because SOCS1 has no access to p65 in the cytoplasm due to it being bound by IκB proteins there. Although their study also indicated that IκBα and SOCS1 might bind at different sites on p65 because SOCS1 was still able to negatively influence p65 when nuclear export of both SOCS1 and IκBα were blocked. The ability of SOCS1 to bind p50 outside the nucleus might indicate that the binding site of SOCS1 and p50 is inherently different from that of SOCS1 and p65. Whilst p50 is also bound by IκBα in the cytoplasm in such a way that blocks the NLS of p50 (Beg *et al.*, 1992), SOCS1 might interact at a different domain that is still accessible. To identify the specific amino acid regions of p50 essential for interaction with SOCS1, a computational structural analysis followed by an immobilized peptide array could be employed. This would facilitate the generation of a p50 mutant that was unable to interact with SOCS1 but would retain its DNA binding, nuclear localisation and dimerization ability, allowing insight into whether a direct

interaction with SOCS1 is required for the degradative effect on p50 that is observed.

The effect SOCS1 has on p50 may occur both constitutively and upon activation by an external stimulus. Without information on the endogenous proteins involved, this is difficult to deduce. When p50 is overexpressed, it moves into the nucleus without the need for TLR activation because there are not enough IκB proteins in the cytoplasm to retain it there. Furthermore, there is a significant number of p50 homodimers in the nuclei of unstimulated cells indicating that p50 is important for both basal and inducible transcription of target genes (Cheng *et al.*, 2011). In the current study, it was observed that SOCS1 promotes the degradation of a DNA-binding mutant of p50, and some ubiquitination is seen. Furthermore, a weak interaction between $p50^{Y57A}$ and SOCS1 was detected which is in contrast to previous published studies on p65 whereby a DNA binding mutant was unable to interact with SOCS1 (Strebovsky *et al.*, 2011). This suggests SOCS1 may mediate p50 degradation under basal conditions. To confirm or refute this, a possible approach would be to silence the expression of SOCS1 using small interfering RNA (siRNA) and observe the effects on p50 in both unstimulated and TLR stimulated cells. If SOCS1 is able to cause p50 to be ubiquitinated and degraded even under basal conditions, then SOCS1 may have role in directing p50 degradation via basal autophagy. Although it has been demonstrated that degradation of ubiquitinated proteins is not dependent on basal autophagy (Takayama *et al.,* 2017), in this case ubiquitination of p50 by SOCS1 does not appear to be the mechanism directing it for degradation. This may implicate a novel role of ubiquitination in p50 regulation that is not dependent upon DNA-binding, nor directs it towards the proteasome.

An interesting feature is the relationship between SOCS1 and the atypical IκB protein, BCL-3, when both are present with p50. BCL-3 is predominantly a nuclear protein and is not degraded upon activation of the IKK complex. It has been demonstrated to interact with and stabilise p50 homodimers, preventing their ubiquitination and subsequent degradation by the proteasome and thus inhibiting NF-κB transcriptional activity. For this reason, BCL-3 is essential for establishing TLR tolerance – a state characterised by diminished expression of

pro-inflammatory genes and is critical for limiting the inflammatory response (Carmody *et al.*, 2007). In this study, BCL-3 was degraded when present with both p50 and SOCS1, and this restored some of the ubiquitination of p50 that was blocked by BCL-3 (figure 4.9). Although BCL-3 has the effect of stabilising p50 homodimers, p50 is also required for the stabilisation of BCL-3 (Wessells *et al.*, 2004). Therefore, it cannot be assumed that SOCS1 causes BCL-3 to be degraded as it does p50. Rather, it might be that SOCS1 prevents p50 from stabilising BCL-3 in some manner. This may have an implication for TLR tolerance since both p50 homodimers and BCL-3 are necessary for establishing this cellular state.

Treatment with MG132, a proteasome inhibitor, did not prevent SOCS1-induced degradation of p50 despite it being able to promote p50 ubiquitination, suggesting a different mechanism is responsible. A recent study identified that the DUB Cezanne is essential for regulating the degradation of the transcription factor HIF-1α, but this action is independent of the proteasome (Bremm *et al.*, 2014). Furthermore, a study identified that the zinc metalloprotease NleC targets IκBα, p65 and p50 for degradation by a proteasome-independent mechanism (Mühlen *et al.*, 2011). The researchers demonstrated that NleC inhibits NF-κB activity both basally and after TNFα stimulation and this is due to it causing a decrease in both p65 and p50 levels. In this case, although SOCS1 is an established E3 ligase for p65, perhaps it regulates the degradation of p50 in a capacity that is independent of its E3 ligase activity.

The ability of SOCS1 to interact with and destabilise p50 is a novel role for this multifaceted protein, which is already an established mediator of the inflammatory response. The finding that ubiquitination of p50 is increased in its presence, but this does not appear to be reliant on SOCS1's capacity as an E3 ubiquitin ligase, has not been described previously and generates questions that future lines of enquiry might seek to answer. It is established that p50 stability is controlled by the UPS. However, it remains to be seen if the degradation of p50 in the presence of SOCS1 is due to an indirect effect that subsequently facilitates p50 ubiquitination and guides it toward a degradative pathway that is distinct from the proteasome. The observation that SOCS1 is able to interact with and cause the degradation of p50 when SOCS1 is not able to access the

nucleus and when p50 is unable to bind to DNA is in direct contrast to previous published data on p65. SOCS1 is therefore an essential regulator of NF-κB subunit stability and may influence subunit turnover and abundance in a subunit specific manner.

Chapter 5

Mapping the transcriptional landscapes of *Ing4*$^{-/-}$ and *Socs1*$^{-/-}$ macrophages

5.1 Abstract

ING4 and SOCS1 are both known to act as E3 ubiquitin ligases for the p65 subunit of NF-κB, causing it to be degraded by the proteasome and this terminates NF-κB activity. Furthermore, our data suggests that SOCS1 also promotes the degradation of the p50 subunit. ING4 and SOCS1 are therefore essential regulators of NF-κB activity. However, their function extends well beyond this and so to explore the changes that occur in the transcriptional landscape of cells that lack each of these components, knock-out RAW 264.7 macrophages were generated using CRISPR/Cas9 technology and RNAseq analysis was performed on both unstimulated and TLR-activated conditions. This revealed distinct clusters of genes that are differentially expressed between wild-type and mutant cells, and highlighted that NF-κB target genes are regulated in a gene-specific manner by each of these E3 ligases. Anti-inflammatory genes such as *Il10* are significantly upregulated in *Ing4*-deficient cells, and pro-inflammatory genes including *Ccl2* and *Ccl7* are significantly downregulated in cells that lack SOCS1. Gene ontology analysis of biological processes also provides insight into the functions of the differentially expressed genes. Therefore, these mutant cell lines are useful tools to identify the wider roles that ING4 and SOCS1 have in macrophages, both in an immune context and within other biological systems.

5.2 Introduction

ING4 is a member of the inhibitor of growth (ING) family, of which there are five evolutionarily conserved members, all characterized by a C-terminal plant-homeodomain-like zinc finger (Campos *et al.*, 2004; Coles & Jones, 2009). Like SOCS1, ING4 has a number of roles within many biological processes. It interacts with acetylation and de-acetylation complexes therefore having a role in chromatin remodelling and gene expression (Doyon *et al.*, 2006; Soliman & Riabowol, 2007). It is also a proposed tumour suppressor as its expression is reduced in human cancers and the gene mutated in cancer cell-lines (Coles & Jones, 2009). ING4 interacts with the p65 subunit of NF-κB and its inhibition in U87MG glioblastoma cells led to increased tumour vascularization in immunocompromised mice and the downregulation of many NF-κB-target genes involved in angiogenesis (Garkavtsev *et al.*, 2004). It was later revealed that ING4 functions as an E3 ligase for p65, inducing its ubiquitination and proteasomal degradation, and this inhibits NF-κB transcriptional activity (Hou *et al.*, 2014). In the previous chapter, SOCS1 was shown to promote the ubiquitination and degradation of p50 in some capacity that is independent of its E3 ligase activity, and its wider role was covered in depth there so will not be discussed again here to avoid repetition. Therefore, they are both important regulators of NF-κB activity and it is of interest to determine what effect their loss has in macrophages.

The use of knock-out macrophages is a powerful tool to explore the function of certain genes in an immune context. Previous studies have used knock-out mice to examine the roles that ING4 and SOCS1 play in various biological processes, however the generation of knock-out RAW macrophages provides a novel and straight-forward way to perform a number of *in vitro* assays to give researchers a fundamental understanding of what these genes do in different contexts. A previous study that used *Ing4*$^{-/-}$ mice found that they were fully viable, although they were highly sensitive to LPS (Coles *et al.*, 2010). *Socs1*$^{-/-}$ mice die within 3 weeks of birth due to severe inflammation (Naka *et al.*, 1998; Starr *et al.*, 1998) however heterozygous knock-outs and *Ifng*$^{-/-}$*Socs1*$^{-/-}$ double knock-outs are also highly sensitive to LPS (Kinjyo *et al.*, 2002).

Exploratory gene expression analyses that compare different populations under different conditions allows researchers to gain a deeper understanding of gene functions in both health and disease states. High-throughput assays such as RNAseq can identify candidate target genes that are significantly differentially expressed between groups that can be further explored in molecular biology experiments. It can detect genes that are expressed at very low or high levels, and is not limited by prior knowledge of the genome of the organism, which are drawbacks of other transcriptome analyses such as microarrays.

Two RAW 264.7 (RAW) macrophage mutants were generated via CRISPR/Cas9 by lab technician, Mr David Kerrigan: one that lacks ING4 (*Ing4*$^{-/-}$) and one that lacks SOCS1 (*Socs1*$^{-/-}$), so that the physiological roles of these two E3 ligases in an inflammatory context could be explored. Although their function in relation to the expression of NF-κB target genes was of particular interest, it was kept in mind that these two components play multiple roles within the immune and other biological systems in addition to their E3 ligase activity, so the broader landscape of transcriptional changes was considered as analysis was performed. To this end, RNAseq analysis was carried out on unstimulated and LPS stimulated samples, so that the changes in the genetic landscapes of these populations could be mapped. Comparing the knock-outs not only to wild-type RAW cells, but to each other, revealed whether any redundancies exist between which gene sets are controlled by either or both of these components under both basal and inducible conditions. The results of this chapter demonstrate that cells that lack ING4 or SOCS1 express some pro-inflammatory genes to a lesser extent than wild-type, and some anti-inflammatory genes are indeed upregulated in these knock-outs, which is a desirable phenotype in the quest for chronic inflammatory disease gene therapies. The loss of these components also highlights that some, but not all NF-κB target genes are differentially expressed following LPS challenge, indicating that these E3 ligases influence NF-κB activity in a gene-specific manner. The RNAseq analysis also reveals that, although some inflammatory genes are differentially expressed in the knock-out populations, the wider effects of these mutations on biological processes such as development and viability mean that these would not be target mutations in any therapy that sought to treat chronic inflammatory disease in a precise manner with few detrimental side effects.

5.3 Results

The results of this chapter are separated into three distinct groups. Firstly, the *Ing4*$^{-/-}$ cells are compared to wild-type. Secondly, the *Socs1*$^{-/-}$ cells are compared to wild-type, and lastly, the two mutant cell lines are compared with each other to discern what genes are uniquely differentially expressed by each knock-out, and which are common to both. This should highlight genes that are under the direct influence of each E3 ligase, and where redundancy lies between the two. The bioinformatic analyses in this chapter were performed with the great assistance of Mr John Cole of the GLAZgo Discovery Centre, and Dr. Domenico Somma. WT, *Ing4*$^{-/-}$ and *Socs1*$^{-/-}$RAW cells were left either untreated or treated with 100 ng/mL LPS for 3 hours. Cells were harvested and RNA extracted as described previously. Duplicate samples of each condition were sent to the University of Glasgow Polyomics facility for sample QC and polyA library preparation. Single-end 75bp reads were sequenced to a depth of 20 million.

5.3.1 WT vs *Ing4*$^{-/-}$ RAW 264.7 macrophages

5.3.1.1 Principal component analysis

Principal component analysis (PCA) was performed on the RNAseq data to determine what factors caused the greatest variability between the samples. The principal components that cause the greatest difference between samples is the treatment with LPS (57%) and the loss of ING4 (21%) (figure 5.1). This indicates there is indeed a difference between WT and mutant cells, and the mutation is the cause.

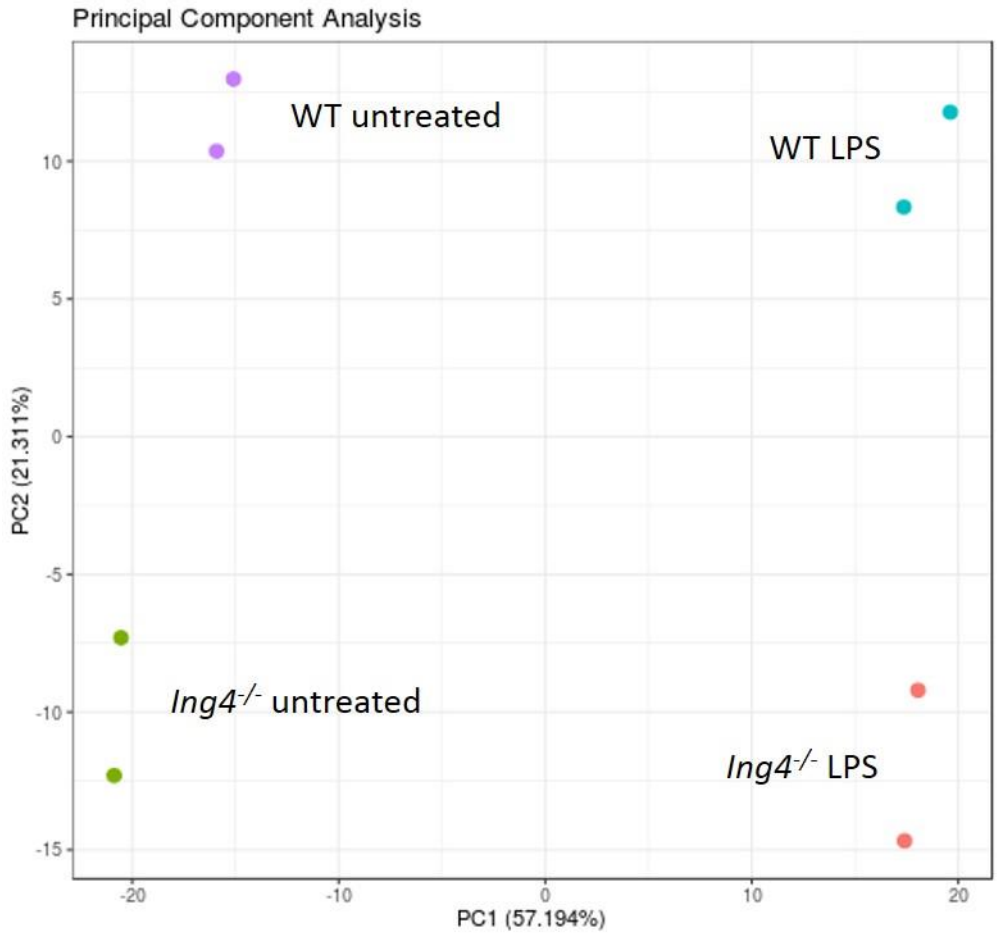
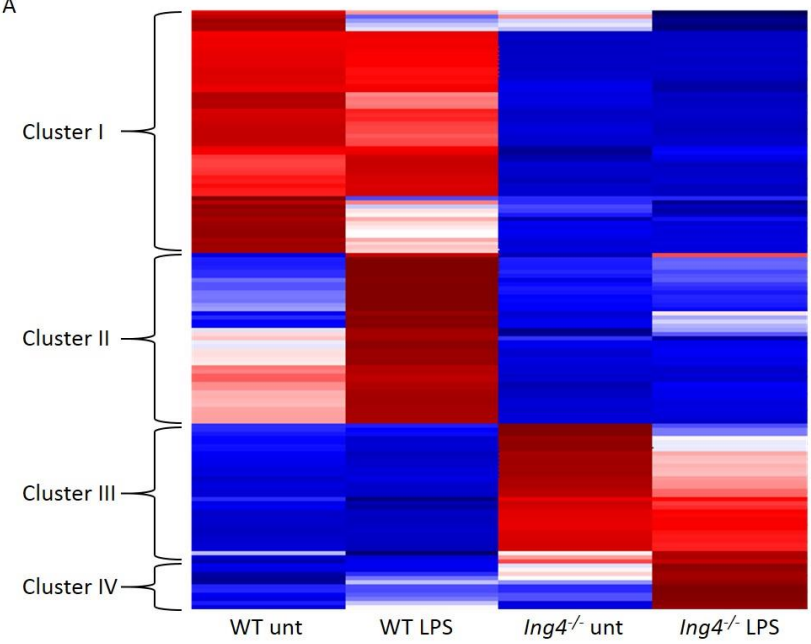


Figure 5.1: Principal component analysis (PCA) plot and significantly differentially expressed genes between wild-type and *Ing4*$^{-/-}$ RAW 264.7 macrophages.
Gene expression variances between wild-type (WT) and *Ing4*$^{-/-}$ RAW macrophages both unstimulated and stimulated by 100 ng/mL LPS for 3 hours are displayed as PCA plots. Each dot represents a sample. The percentage of total variation explained by each component is given. The expression values were pre-transformed under the formula log10(n+1).

5.3.1.2 Heatmap of differentially expressed genes

The heatmap combines the two replicates from each condition to create a mean. This makes it clear where the significantly differentially expressed (DE) genes are and identifies clusters of genes (figure 5.2A). For instance, in cluster I are genes that are constitutively expressed in WT, but are decreased in both the unstimulated mutants and mutants stimulated with LPS. Cluster II are genes that are off in both WT and mutant when unstimulated and turned on in the WT following stimulation with LPS, but are lowly expressed in the mutant when stimulated. Cluster III are genes that are constitutively expressed at low levels in WT, but are changed to being constitutively expressed at higher levels in the mutant. Finally, cluster IV are genes that change from being constitutively expressed at low levels in the WT, but that are induced by LPS in the mutant cells. This indicates that ING4 is instrumental for the expression and control of many genes. Some insight is given by identifying the ten most highly up and down regulated genes following TLR activation by LPS. Of particular interest is *Il10* at position ten in the list (figure 5.2B). IL-10 is an anti-inflammatory cytokine, which in macrophages represses MHC class II expression and strongly inhibits cytokine expression following LPS stimulation. This is a desirable outcome of any therapeutic strategy seeking to reduce chronic inflammation. Similarly, *Pf4* is among the top most downregulated genes, which is a cytokine thought to have a role in inflammation.

A



B

10 most upregulated genes (LPS)

Gene name	Log2 fold	P adj. value
Agap2	4.0972149	3.2446644E-13
Mkx	3.6185253	5.9432502E-09
Peg12	3.3767080	2.2889469E-07
Sema5a	3.3453597	2.6770863E-32
Shisa3	3.0825987	1.0045687E-05
Cpq	2.8849317	9.7455693E-06
Tmem26	2.8844017	0.0001247720
Six1	2.8020864	4.4264485E-06
Palm3	2.7365837	0.0004489763
Il10	2.6827016	0.0007159537

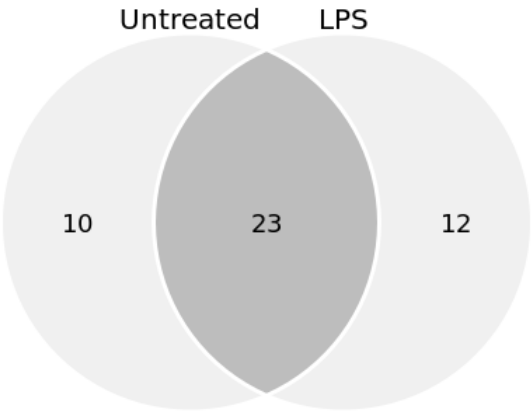
10 most downregulated genes (LPS)

Gene name	Log2 fold	P adj. value
Cers4	-4.847908971	8.025182E-22
Enpp4	-4.836878724	6.347333E-18
Tanc1	-4.235809374	2.361714E-42
Chchd10	-4.024815834	5.718145E-20
Emp2	-3.708749336	4.265854E-08
Lamb2	-3.495613998	2.471944E-13
Gypc	-3.394761084	5.727756E-10
Acap1	-3.355663072	4.030665E-08
Pf4	-3.302547938	6.882912E-08
Prkg2	-3.268760918	4.186639E-06

Figure 5.2: Heatmap of significantly differentially expressed genes between WT and *Ing4*$^{-/-}$ RAW macrophages.
(A) Heatmap of significantly differentially expressed (p.adj < 0.05, absolute log2 fold > 1.0) genes between WT control and *Ing4*$^{-/-}$ macrophage, both unstimulated and stimulated for 3 hours with LPS. Colour intensity represents expression level, with red high expression and blue low expression. (B) Table indicating the ten most highly upregulated and downregulated genes by log2 fold change in the *Ing4*$^{-/-}$ cells compared to WT.

5.3.1.3 Significantly upregulated genes in *Ing4*$^{-/-}$ RAW macrophages

A number of genes are significantly upregulated in the mutant cells compared to WT, at both the basal level and following TLR activation by LPS. Thirty-three genes are upregulated in the *Ing4*$^{-/-}$ cells under resting conditions. Ten genes are uniquely upregulated only in untreated cells such as *Cxcl16* and *Trip6,* meaning the absence of ING4 turns these genes on in resting cells but they are not expressed following activation by LPS (figure 5.3). Due to the low number of significantly differentially expressed genes, the method of Gene Set Enrichment Analysis (GSEA) deemed appropriate on this gene set was the Enrichr tool (Chen *et al.*, 2013; Kuleshov *et al.*, 2016). Gene ontology (GO) of the biological processes of the 10 unique genes indicate they are involved in metabolic pathways (figure 5.4A). The number of upregulated genes in the *Ing4*$^{-/-}$ mutant following stimulation by LPS is 35, with 12 of these being uniquely upregulated only in stimulated cells, including *Il10, Six1* and *Bcl6*. The most common role for these 12 genes is the regulation of B cell apoptosis (figure 5.4B), therefore loss of *Ing4*$^{-/-}$ may lead to aberrant B cell function following TLR activation. Furthermore, cytoplasmic sequestering of NF-κB is a biological process of this gene set, although to a lesser extent. Of the 45 genes that are significantly upregulated overall, 23 are common to both untreated and LPS-treated cells, meaning they are constitutively turned on regardless of receiving an activating stimulus. GO analysis of their biological processes suggest roles in cardiac function (figure 5.4C).



Untreated (10)	LPS (12)	All conditions (23)
Arhgef40	*Il10*	*Aldh1l1*
Cbfa2t3	*Col15a1*	*Zic2*
Cacna1s	*Trim36*	*Cpq*
Zdhhc2	*Six1*	*Sp8*
Xkr5	*Dst*	*Slc22a17*
Ppm1e	*Zs3h12c*	*Cds1*
Cxcl16	*Fam126b*	*Sort1*
Trip6	*Rpl3l*	*Slc8a1*
Chka	*Bcl6*	*Agap2*
Zmynd15	*Adgb*	*Tmem26*
	Ocstamp	*Ank2*
	Shisa3	*2610528A11RIK*
		Pltp
		Rnls
		Plcb2
		Sema5a
		Peg12
		AC166344.1
		Hoxc4
		Adgrl1
		Palm3
		Mkx
		Tmem246

Figure 5.3: Significantly upregulated genes in *Ing4*$^{-/-}$ RAW macrophages.
Venn diagram indicating the number of genes that are significantly upregulated in the *Ing4*$^{-/-}$ cells compared to WT at the basal level, after stimulation with LPS for 3 hours, and under both conditions. These genes are listed in the table below.

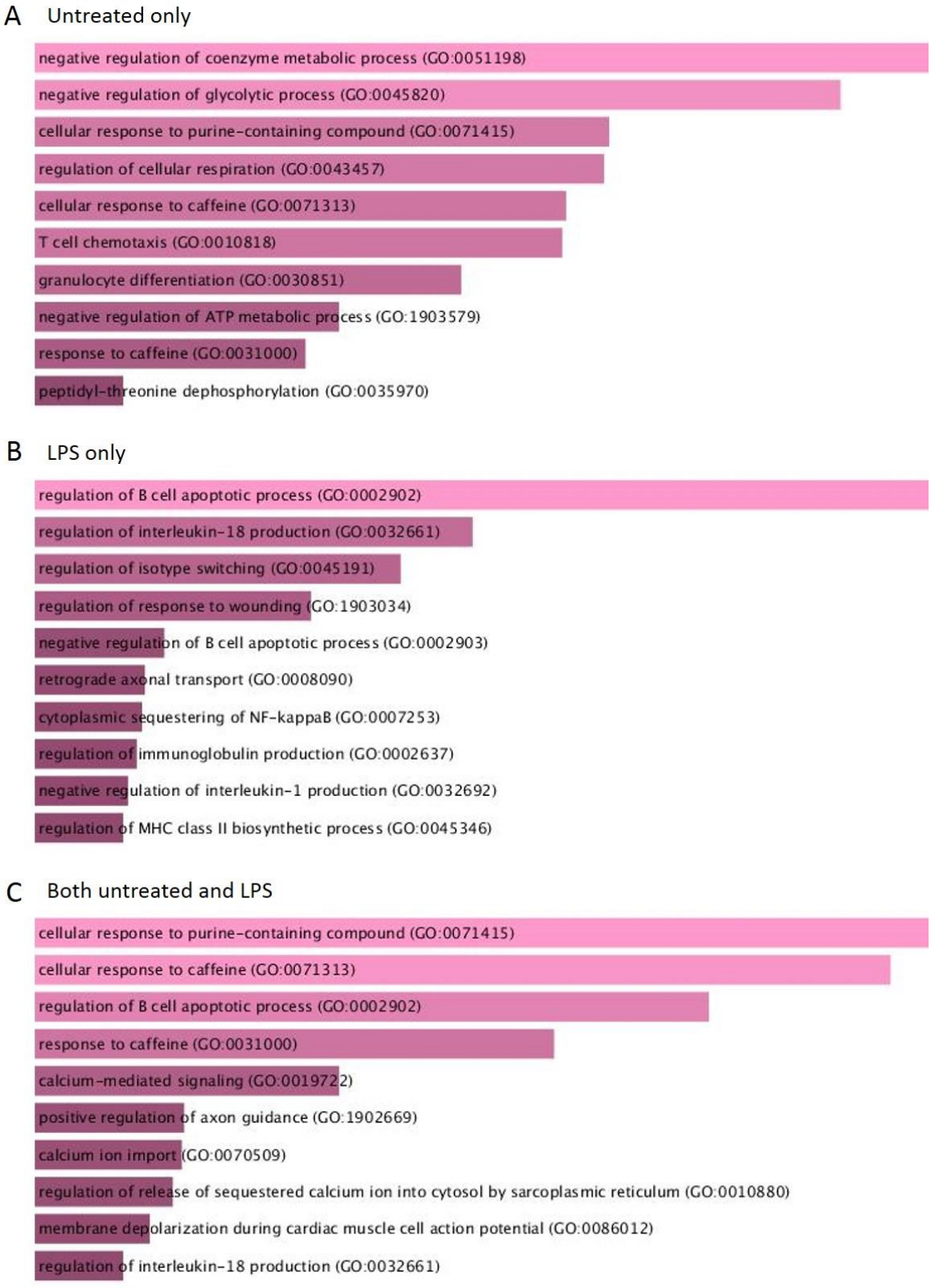
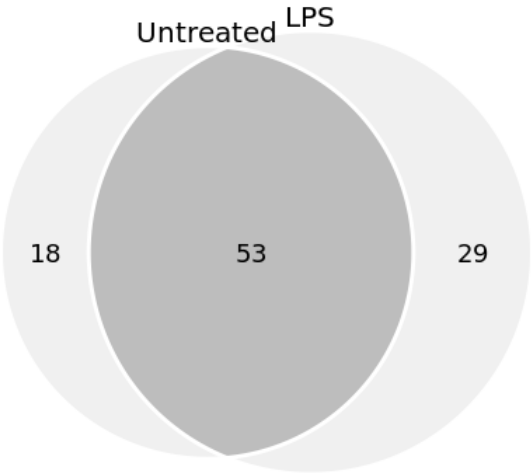


Figure 5.4: Enrichment analysis of Gene Ontology (GO) biological processes of the significantly upregulated genes in *Ing4*$^{-/-}$ RAW macrophages using Enrichr.
The classification terms and their serial numbers are displayed within the bars. Bars are ordered and coloured in descending *p*-value cutoffs.

5.3.1.4 Significantly downregulated genes in *Ing4*$^{-/-}$ RAW macrophages

More genes are downregulated in the mutant cell line than upregulated. Overall, 100 genes are significantly downregulated in the mutants, either unstimulated, LPS stimulated or under both conditions. Eighteen genes are downregulated only in unstimulated cells including *Dusp9* and *Dcstamp* (figure 5.5) and GO analysis of their biological processes indicate roles in positive regulation of monocyte differentiation and the response to IL-4 (figure 5.6A). In response to LPS, 29 genes are uniquely downregulated in the mutant cells including *Ccl2* and *Ccl7,* and these have roles in a number of immune-related biological processes (figure 5.6B). Fifty-three genes are downregulated in both untreated and LPS activated cells. Of all the 100 genes that are significantly downregulated in *Ing4*$^{-/-}$ cells regardless of activation status, the most common biological processes are related to natural killer cell chemotaxis and chemotaxis generally, leukocyte differentiation, and positive regulation of the ERK1/2 cascade. These suggest the loss of *Ing4*$^{-/-}$ results in the repression of the immune response.



Untreated (18)	LPS (29)		All conditions (53)	
Tnfrsf10b	*Adgrl2*	*Ccl2*	*Ppic*	*Nes*
Acy1	*Klf9*	*Trim47*	*Map6*	*Epb41l1*
Abhd14a	*Dner*		*Gypc*	*Maf*
Zfp2	*Chst11*		*Qpct*	*Odc1*
Antrx1	*Trpv4*		*Kif21a*	*E330009J07RIK*
Layn	*Jund*		*Pcbp4*	*Tmsb10*
Hpgd	*Ehd2*		*Coro2a*	*Ptgs1*
Fbxo15	*Anxa6*		*Lamb2*	*Mgl2*
Shc2	*Sash3*		*Cep170b*	*Flt1*
Afp	*Tnc*		*Arhgap25*	*Eid2*
9130008F23RIK	*Mamld1*		*Fat3*	*Prkg2*
Dusp9	*Cd276*		*Mmp12*	*Igsf11*
Zbtb46	*Prkd3*		*Nsd1*	*Nkain1*
Dcstamp	*Evl*		*Il11ra1*	*Gpr179*
Ccdc127	*Ccl12*		*Vopp1*	*Ctse*
Car2	*Slc6a9*		*Abtb2*	*Ccr1*
Acot1	*Ccl7*		*Pf4*	*Cd24a*
Dmwd	*Stard10*		*Ier3*	*Btbd3*
	Fam149a		*Glipr2*	*Ccdc69*
	Zfp984		*Icosl*	*Col18a1*
	Ggta1		*Sdr39u1*	*Itpr2*
	Mapk11		*Enpp4*	*H2-m3*
	Cebpe		*Tanc1*	*Emp2*
	Abcg2		*Notch1*	*Mindy1*
	Osm		*Tmem98*	*Chchd10*
	Ptger2		*Cers4*	*Acap1*
	Serpinb2		*Pkd1l2*	

Figure 5.5: Significantly downregulated genes in *Ing4*$^{-/-}$ RAW macrophages.
Venn diagram indicating the number of genes that are significantly downregulated in the *Ing4*$^{-/-}$ cells compared to WT at the basal level, after stimulation with LPS for 3 hours, and under both conditions. These genes are listed in the table below.

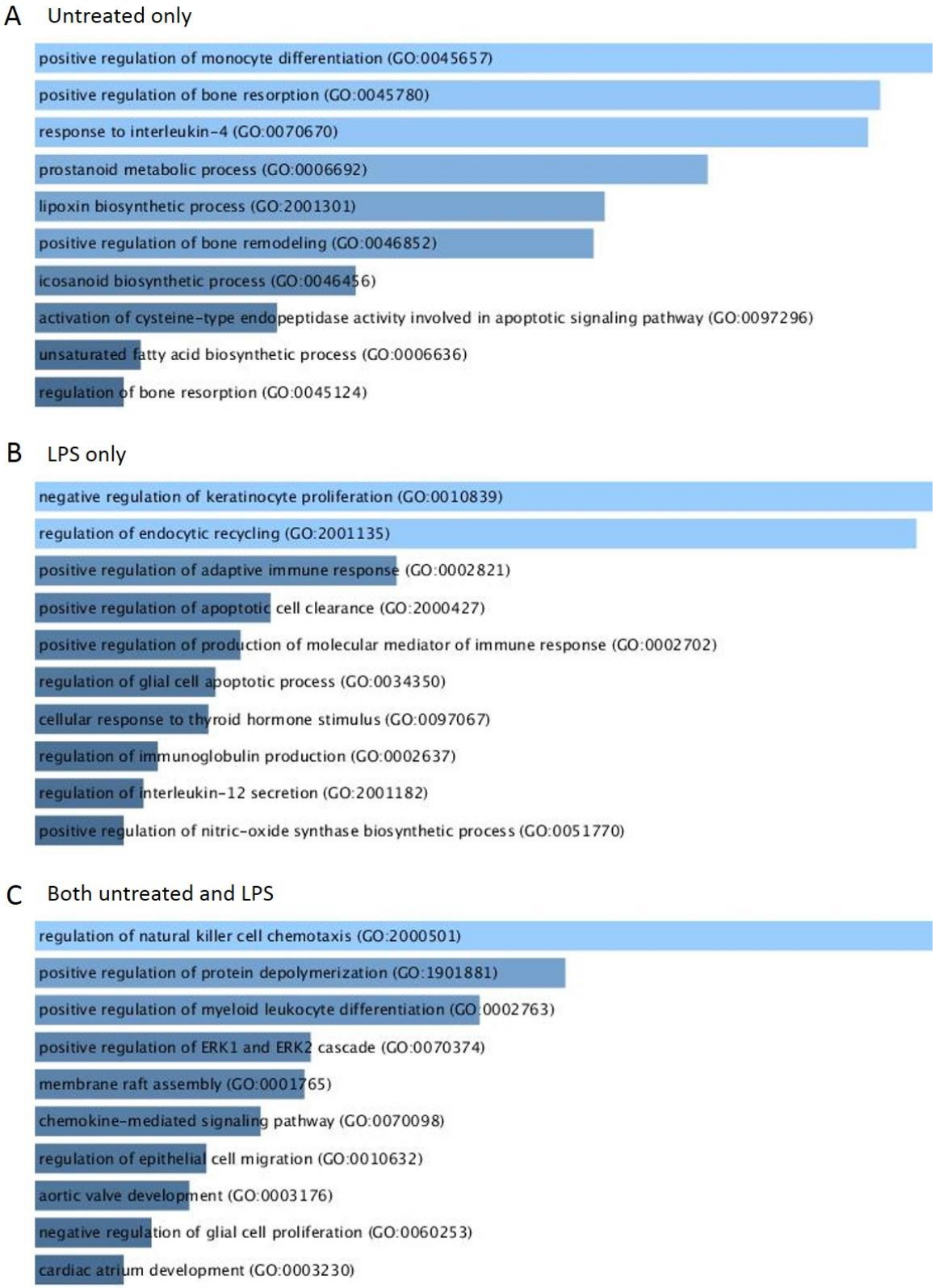


Figure 5.6: Enrichment analysis of GO biological processes of the significantly downregulated genes in *Ing4*$^{-/-}$ RAW macrophages using Enrichr.
The classification terms and their serial numbers are displayed within the bars. Bars are ordered and coloured in descending *p*-value cutoffs.

5.3.1.5 RNAseq analysis of NF-κB target genes in *Ing4*$^{-/-}$ RAW macrophages

Due to the function of ING4 as an E3 ligase for the p65 subunit of NF-κB, the effect its absence has on the expression of NF-κB targets was of interest. A number of genes that are under the direct control of NF-κB were chosen for further analysis, and which have various roles within the immune system. Their normalised read counts following TLR activation by LPS were extracted from the RNAseq data and plotted. It is clear that the loss of ING4 affects the expression of NF-κB target genes selectively (figure 5.7). There is no blanket promotion or suppression of transcription. Instead, some genes are expressed to a greater level in the *Ing4*$^{-/-}$ cells, such as *Baff*, *Bcl3*¸ and *Fas*, whereas others are repressed, for instance *Cd80*, *Cd86* and *Il1b*. Others are not affected by the loss of ING4, such as *Il1a* and *Irf1*. Transcript levels of the NF-κB subunits themselves vary to some extent, with slight increases in the expression of *Nfkb1* and *Nfkb2*, and greater increases in *Rela* and *Relb*. The expression of *Nfkbia* (IκBα) is decreased in the knock-out.

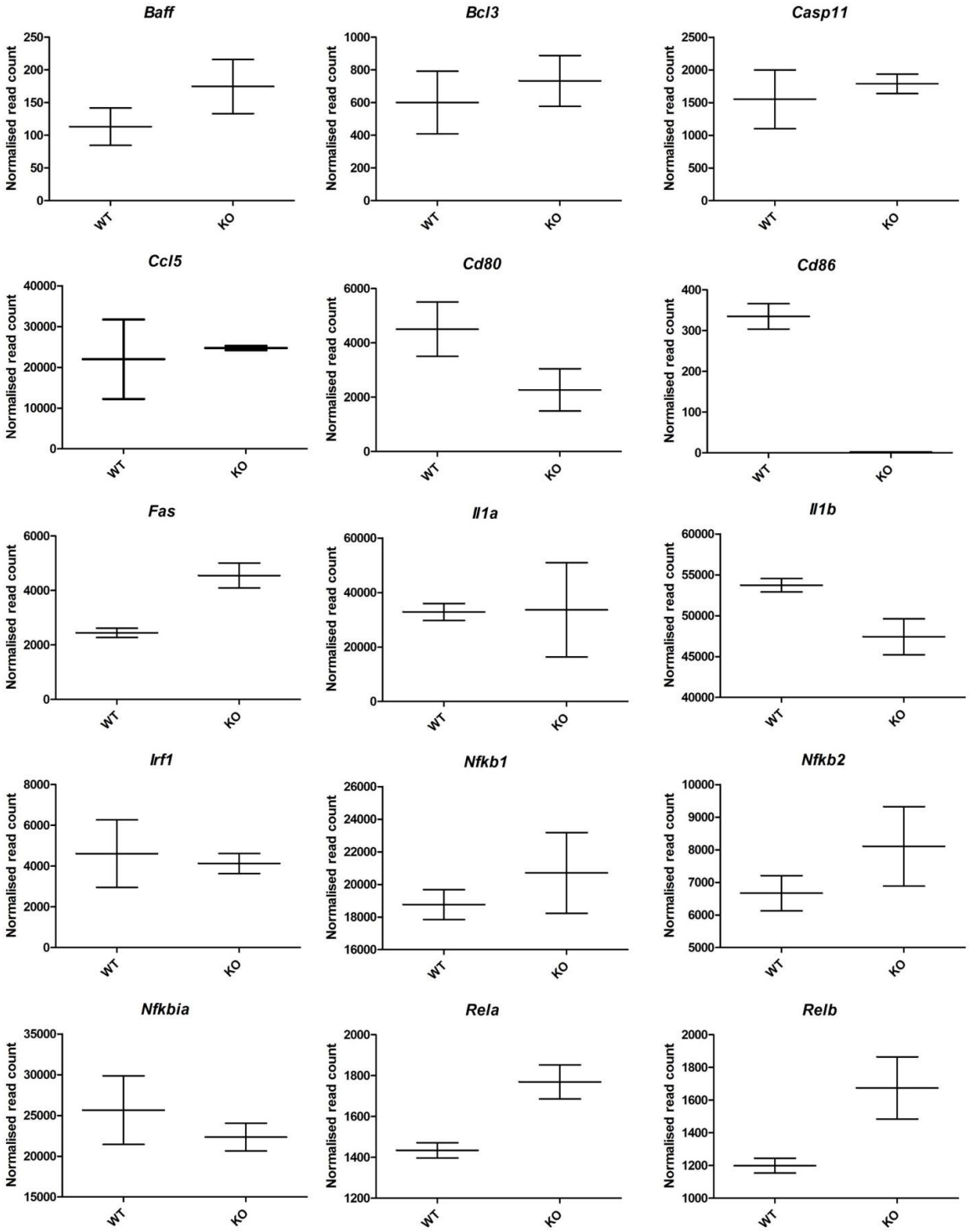


Figure 5.7: Expression of a selection of NF-κB target genes in WT and *Ing4*$^{-/-}$ macrophages after 3-hour LPS stimulation from RNAseq data.
WT and *Ing4*$^{-/-}$ cells were prepared for RNAseq analysis as described above. A number of NF-κB target genes with various roles within the immune system were chosen and their expression levels analysed following stimulation with LPS for 3 hours by plotting their copy number. Means represented with whiskers denoting minimum and maximum values.

5.3.1.6 Validation of RNAseq by RT-qPCR

Validation of the RNAseq data was performed by RT-qPCR of a number of NF-κB target genes involved in the inflammatory response, as well as genes that were highlighted as being significantly differentially expressed. WT and *Ing4*$^{-/-}$ cells were left either untreated or treated with 100 ng/mL LPS for 3 hours. Relative fold change was calculated using the $2^{-\Delta\Delta Ct}$ method. This not only confirmed the RNAseq data but again demonstrated that the loss of ING4 causes selective changes in the way NF-κB controls the transcription of its target genes (figure 5.8). Some pro-inflammatory cytokines are repressed in the mutant, such as *TNFa* and *Cxcl2*, whereas *Il6* expression is increased. *Ccl2* and *Ccl7* are chemokines that attract monocytes and other immune cells to sites of inflammation and both are significantly downregulated in *Ing4*$^{-/-}$ RAW cells following stimulation with LPS for 3 hours. The anti-inflammatory cytokine, *Il10,* is expressed much more in the mutant following LPS stimulation than WT, which was highlighted in section 5.3.1.2 as a significantly upregulated gene.

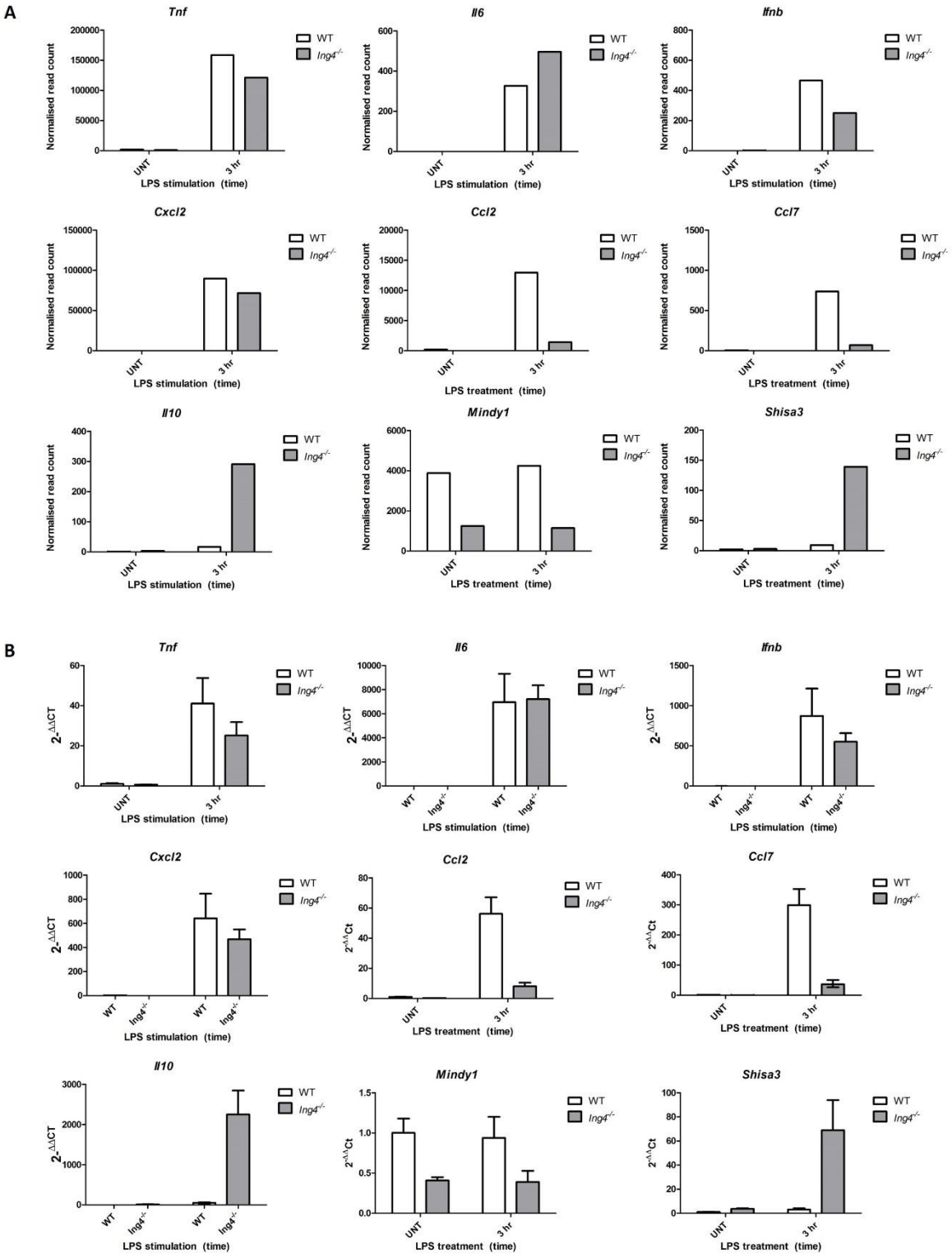


Figure 5.8: Validation of RNAseq by RT-qPCR.
(A) WT and *Ing4*$^{-/-}$ cells were prepared for RNAseq analysis as described above. RNAseq data graphed using the mean of normalised read counts from the two replicates (n=2). (B) WT and *Ing4*$^{-/-}$ cells were left either untreated or treated with 100 ng/mL LPS for 3 hours. RT-qPCR data shows combined results from three independent experiments indicating fold change calculated using the $2^{-\Delta\Delta Ct}$ method with corresponding standard deviation (n=3).

5.3.1.7 Expression of NF-κB subunits in WT and *Ing4*$^{-/-}$ RAW macrophages

It was essential to determine whether the loss of ING4 resulted in any changes in the protein levels of the NF-κB subunits, or of their upstream regulator IκBα. Some variation was observed in their mRNA levels in section 5.3.1.5, so to determine whether this was translated at the protein level, WT and *Ing4*$^{-/-}$ RAW macrophages were left either untreated, or treated with 100 ng/mL LPS for the time course indicated. Cells were harvested and protein levels measured via Western blot (WB) using antibodies specific for the indicated proteins. No great differences in the expression levels of the NF-κB components were detected (figure 5.9). Importantly, no changes in the expression pattern of IκBα were observed, which confirms that the changes in NF-κB target gene expression observed in section 5.3.1.5 are not due to any upstream effects.

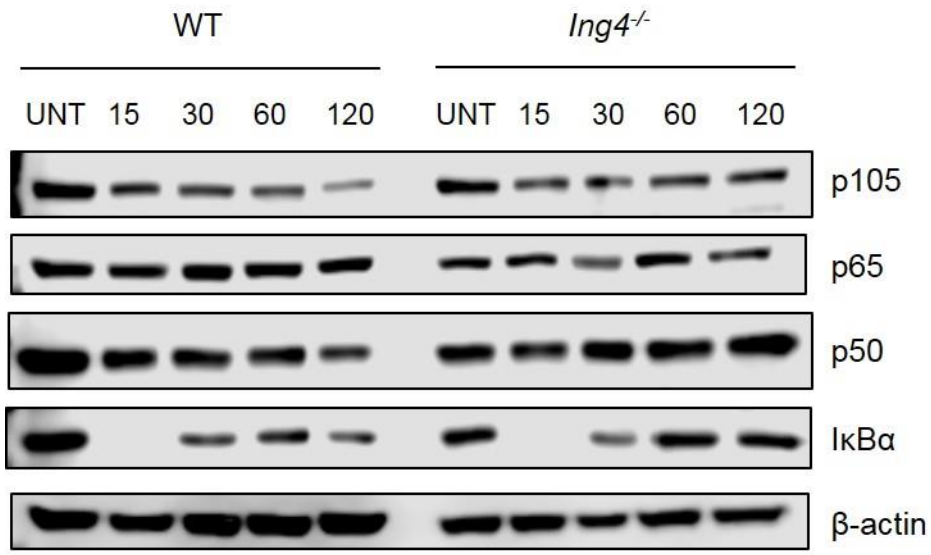


Figure 5.9: Expression levels of NF-κB proteins in WT and *Ing4*$^{-/-}$ RAW macrophages.
WT and *Ing4*$^{-/-}$ RAW cells were treated with 100 ng/mL LPS for the indicated time (minutes) or left untreated (unt). Whole cell extracts were analysed for levels of the indicated proteins via Western blot (WB). Figure representative of three individual experiments.

5.3.2 WT vs *Socs1*$^{-/-}$ RAW 264.7 macrophages

5.3.2.1 Principal component analysis

Principal component analysis (PCA) was performed on the RNAseq data to determine what factors caused the greatest variability between the samples. The principal components that cause the greatest difference between samples is the treatment with LPS (52%) and the loss of SOCS1 (19%) (figure 5.10). This indicates there is indeed a difference between WT and mutant cells, and the mutation is the cause. Unfortunately, this analysis revealed a discrepancy between the two *Socs1*$^{-/-}$ mutant replicates, which do not cluster together in the same quadrant. Validation was performed by RT-qPCR and outlined in section 5.3.2.6 below and based on these results, replicate 1 was discarded from further analysis because it varied greatly from the RT-qPCR measurements whereas replicate 2 corresponded with the RT-qPCR results more accurately.

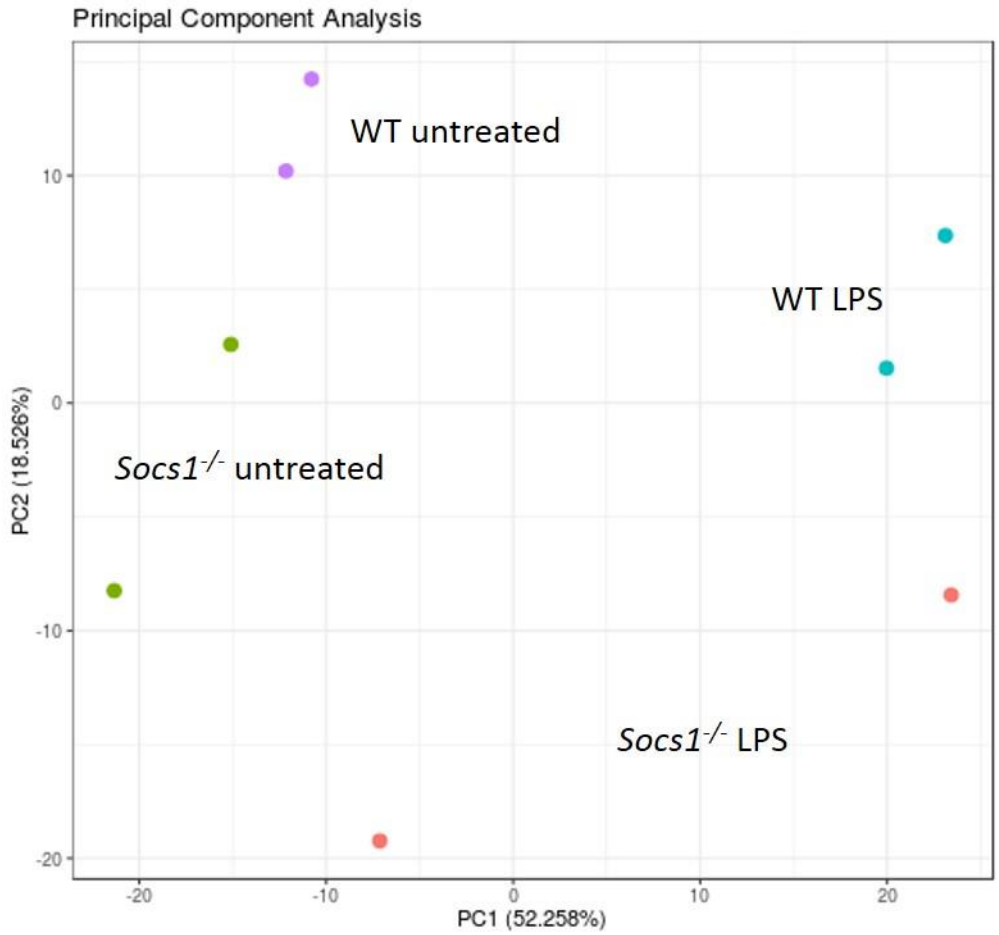
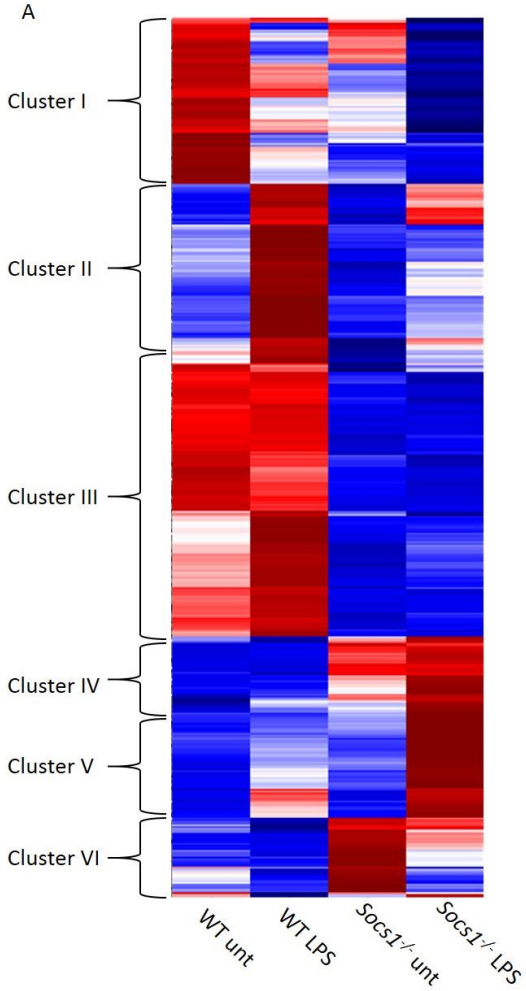


Figure 5.10: PCA plot and significantly differentially expressed genes between wild-type and *Socs1*$^{-/-}$ RAW macrophages.
Gene expression variances between wild-type (WT) (blue) and *Socs1*$^{-/-}$ (pink) RAW macrophages both unstimulated and stimulated by 100 ng/mL LPS for 3 hours are displayed as PCA plots. Each dot represents a sample. The percentage of total variation explained by each component is given. The expression values were pre-transformed under the formula log10(n+1).

5.3.2.2 Heatmap of differentially expressed genes

Due to the discrepancy between the two mutant replicates, only replicate 2 from the *Socs1*$^{-/-}$ mutants is represented in the heatmap below. Again, distinct clusters of genes can be identified (figure 5.11A). Cluster I are genes that change from being expressed highly in untreated WT to being expressed at low levels after LPS stimulation and under all conditions in the *Socs1*$^{-/-}$ mutant. Cluster II includes genes that are induced by LPS stimulation in the WT, but are lowly expressed after stimulation in the mutant. Cluster III are genes that are expressed under all conditions in WT only but are downregulated under all conditions in the mutant. Cluster IV represents genes that are expressed at low levels under all conditions in the WT, but are expressed more highly under all conditions in the mutant. In cluster V are genes that either are off or constitutively expressed at low levels in WT and in untreated mutants but whose expression is induced following LPS stimulation. Finally, cluster VI represents genes that expressed at low levels in all groups except untreated mutants where there is high expression. Identifying the ten most highly up and down regulated genes following TLR activation by LPS also provides some insight into the consequence of losing SOCS1. Amongst the ten most highly upregulated genes following TLR activation is *H2-aa* and *H2-Eb1,* which encode histocompatibility antigens important in adaptive immunity (figure 5.11B). In the list of most downregulated genes is *Prkg2*, which is a protein kinase, and *Ccr1,* a chemokine receptor.

A



B

10 most upregulated genes (LPS)

Gene name	Log2 fold	P adj. value
Dio2	4.925563259	1.79074E-12
P2ry13	4.497563793	5.05008E-11
Fam49a	4.395851975	7.56164E-22
H2-Ea-ps	4.154640114	1.6328E-11
H2-Aa	3.982895048	1.02805E-12
H2-Eb1	3.947934474	5.91876E-09
Prdm1	3.405095725	2.69393E-07
Ppfibp2	3.278603	3.02975E-05
Gm973	3.172880303	5.98398E-05
Mreg	3.11512375	3.11672E-05

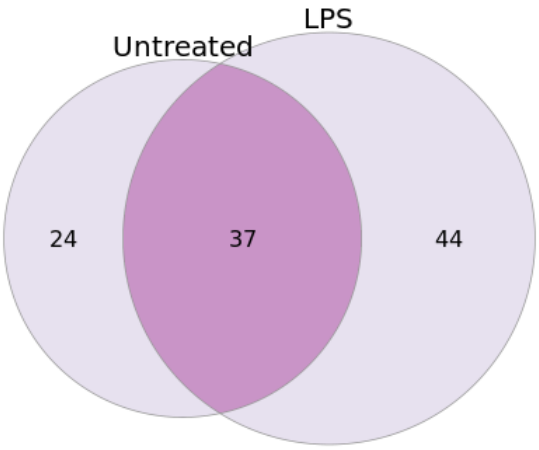
10 most downregulated genes (LPS)

Gene name	Log2 fold	P adj. value
Hbegf	-5.527874	1.31E-23
Scarf1	-4.64188	7.19E-15
Cers4	-4.451627	1.16E-14
Layn	-3.987423	6.6E-19
Prkg2	-3.977215	1.33E-08
Car12	-3.722097	2.94E-22
B3galnt1	-3.714091	6.57E-11
Cd93	-3.645569	1.59E-15
Dner	-3.530352	2.22E-11
Ccr1	-3.432891	2.66E-08

Figure 5.11: Heatmap of significantly differentially expressed genes between WT and *Socs1*$^{-/-}$ RAW macrophages.
(A) Heatmap of significantly differentially expressed (p.adj < 0.05, absolute log2 fold > 1.0) genes between WT control and *Socs1*$^{-/-}$ macrophage, both unstimulated and stimulated for 3 hours with LPS. Colour intensity represents expression level, with red high expression and blue low expression. (B) Table indicating the ten most highly upregulated and downregulated genes by log2 fold change in the *Socs1*$^{-/-}$ cells compared to WT.

5.3.2.3 Significantly upregulated genes in *Socs1*$^{-/-}$ RAW macrophages

In untreated cells, there are 61 genes that are significantly upregulated in the *Socs1*$^{-/-}$ cells compared to WT. Of these, 24 are uniquely upregulated in untreated cells including *Tlr8* and *Il1a*, whereas the remaining 37 are also significantly upregulated after LPS stimulation for 3 hours as well. 81 genes are significantly upregulared following LPS stimulation , and of these, 44 are more greatly expressed only in LPS stimulated cells including *Mreg* and *Cpq*. Overall, 105 genes are significantly upregulated in the mutant RAW cells, whether under resting conditions or following activation by LPS (figure 5.12). Again, due to the low number of significantly differentially expressed genes, the Enrichr tool was deemed the most appropriate for gene ontology analysis. GO analysis of the biological processes revealed that the genes that are significantly upregulated only in untreated cells appear to have roles in the positive regulation of IL-6 secretion (figure 5.13). It also indicates roles in the negative regulation of NF-κB signalling, and other immune functions. The upregulation of genes involved in these processes in the *Socs1*$^{-/-}$ mutant is feasible given SOCS1's prominent role as a suppressor of cytokine signalling. The genes that are significantly upregulated only after TLR activation by LPS have roles in T helper cell function. However, SOCS1 has been previously demonstrated as being important for differentiation of T cells into Th17, so for genes relating to the regulation of the Th17 response to be upregulated in the *Socs1*$^{-/-}$ mutant is intriguing. Overall, the genes that are significantly upregulated in the knock-out cells regardless of stimulation with LPS or not are mainly associated with cytoplasmic translation and the general inflammatory response.



Untreated (24)	LPS (44)		All conditions (37)	
Cpeb1	*Dio2*	*Cd48*	*Mreg*	*Csf3r*
Card14	*Prdm1*	*Siglece*	*Fam49a*	*Slamf7*
Greb1	*Gfi1*	*Peli1*	*H2-Aa*	*Ms4a7*
Fabp4	*Edn1*	*Ms4a6d*	*H2-Ea-ps*	*Ccdc85c*
Stc2	*Adra1a*	*Fam126b*	*H2-Eb1*	*Trem3*
Ctdspl	*Rap1gap2*	*Ms4a6b*	*P2ry13*	*Adgb*
Tlr8	*Zfp334*	*Plxna2*	*Arhgap27os3*	*Mfsd2a*
Ubxn11	*Bambi-ps1*	*Ly9*	*Gm973*	*Traf3ip3*
Cytip	*Pdcd1*	*Flcn*	*Treml2*	*Sort1*
Arhgef40	*Adora2a*	*Ppp1r15a*	*Hal*	*BE692007*
Il1a	*Il6*	*Slc43a3*	*Ccbe1*	*Cyfip2*
Cd33	*Zfp579*	*Bcl6*	*Pilrb1*	*Clec4a3*
Clec4n	*F630028O10Rik*	*Cpeb4*	*Treml4*	*Lsp1*
Plaat3	*Fgd4*	*Tmem273*	*H2-Ab1*	*Csf3r*
Cd84	*Ctsc*	*Tnfrsf1b*	*Tspan10*	
Nlrc3	*Tanc2*	*Syk*	*Clec4a1*	
Slc5a3	*Gm6377*	*Irak3*	*Ocstamp*	
Nlrp10	*S1pr1*	*Sowahc*	*Ppfibp2*	
Rab32	*Ms4a6c*	*D030028A08Rik*	*Slc22a17*	
Trip6	*Clec4a2*	*Rapgef5*	*Cpq*	
Rhoq	*Slpi*		*Plpp3*	
Neurl3	*Ldlrad3*		*Lair1*	
Pou2f2	*Itgal*		*Nxf7*	
Smpdl3a	*Zfp703*		*Pilrb2*	

Figure 5.12: Significantly upregulated genes in *Socs1*$^{-/-}$ RAW macrophages.
Venn diagram indicating the number of genes that are significantly upregulated in the *Socs1*$^{-/-}$ cells compared to WT at the basal level, after stimulation with LPS for 3 hours, and under both conditions. These genes are listed in the table below.

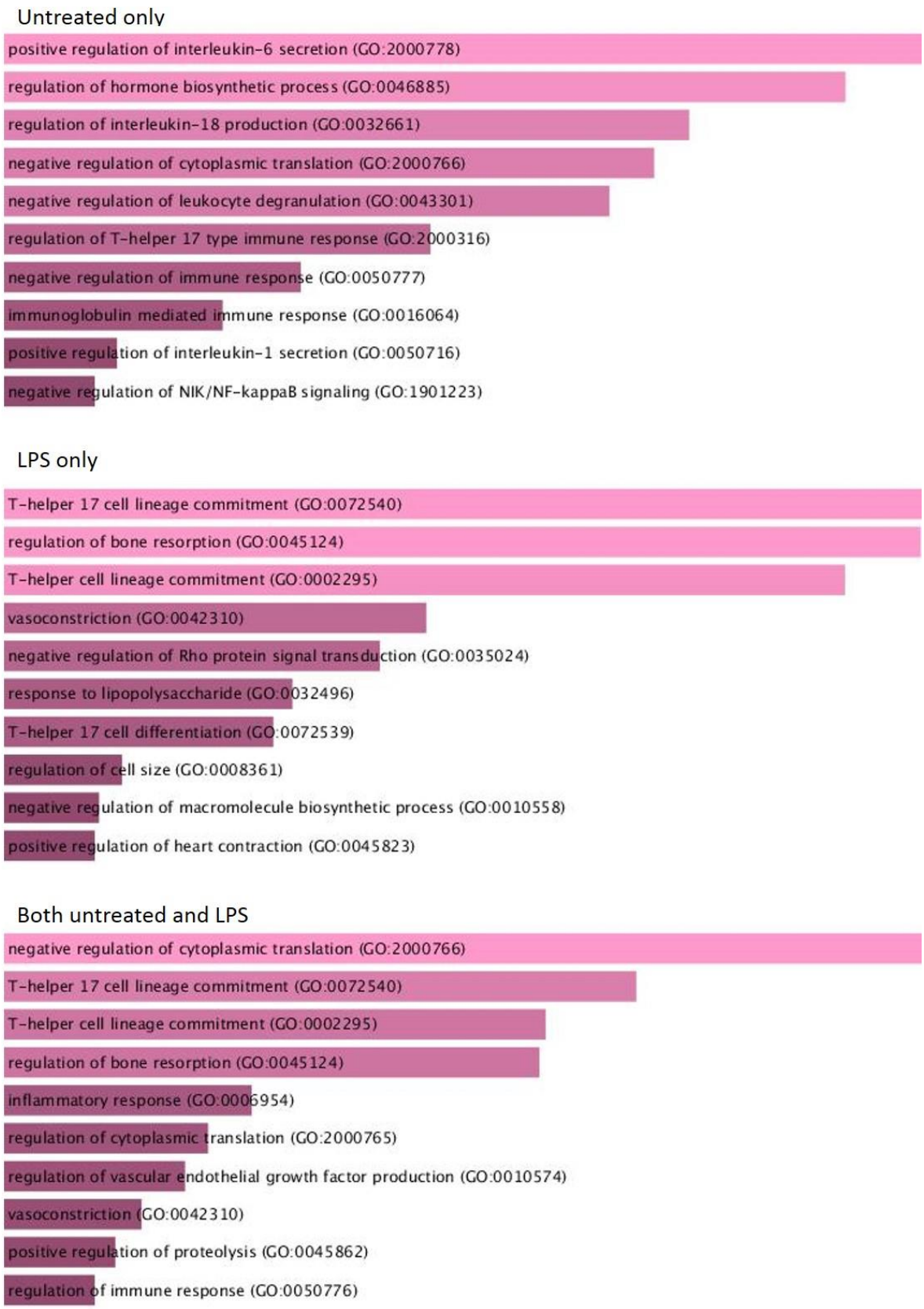
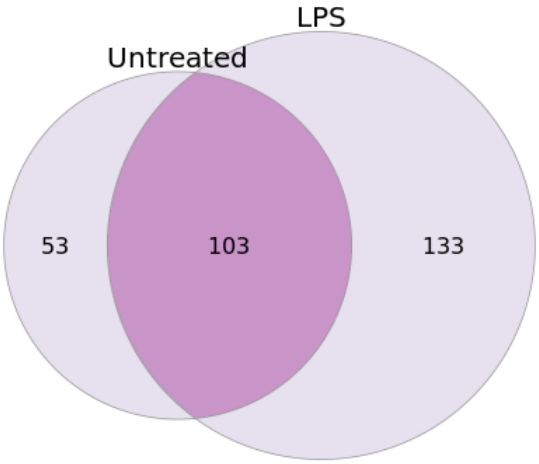


Figure 5.13: Enrichment analysis of GO biological processes of the significantly upregulated genes in *Socs1*$^{-/-}$ RAW macrophages using Enrichr.
The classification terms and their serial numbers are displayed within the bars. Bars are ordered and coloured in descending *p*-value cutoffs.

5.3.2.4 Significantly downregulated genes in *Socs1*$^{-/-}$ RAW macrophages

Overall, 289 genes are significantly downregulated in the *Socs1*$^{-/-}$ cells compared to WT, under both basal and LPS stimulated conditions. 156 genes are significantly downregulated in unstimulated cells, and of these 53 are uniquely downregulated only in unstimulated cells and include *Ccl4, Irak2* and *Il1rn* (figure 5.14). After performing GO analysis using the Enrichr tool, the biological processes of these 53 genes are associated with calcium ion transport. Following LPS treatment, 236 genes are significantly downregulated in the knock-out cells, with 133 genes being uniquely downregulated after TLR activation, such as *Dusp9, Mindy1* and *Pf4*. GO analysis suggests that these genes have roles in cell migration and wound healing. As a whole, the genes that are significantly downregulated in the *Socs1*$^{-/-}$ cells compared to WT regardless of LPS stimulation or not, are associated with biological processes that include cell migration, NK cell chemotaxis and the positive regulation of the ERK1/2 cascade (figure 5.15).



Untreated (53)	LPS (133)		All conditions (103)	
Rab15	*Scarf1*	*Gm28875*	*Nes*	*Csf2rb2*
Egr2	*Cers4*	*Eps8*	*Abtb2*	*Slc30a4*
Spp1	*Layn*	*Cd200r1*	*Sulf2*	*H2-Q5*
Egr1	*Prkg2*	*Vegfa*	*Dner*	*Adssl1*
Egr3	*B3galnt1*	*Tmem171*	*Nkain1*	*Atxn1*
Dpys	*Ccr1*	*Procr*	*Gm17928*	*Ninj1*
Src	*Cd200r2*	*Ptchd1*	*Pf4*	*Rab7b*
Ranbp3l	*Ccl2*	*Bhlhe40*	*Morc4*	*Clic1*
Ccl4	*Pkd1l2*	*Hilpda*	*Rassf8*	*Dok2*
Slc22a4	*Emp2*	*Nubpl*	*Runx2*	*Gm23935*
Il1rn	*Tpbg*	*Mical2*	*H2-M3*	*Ifitm6*
C5ar1	*Lpar1*	*Dusp7*	*Gypc*	*Hmga1*
Eid2	*Gm9260*	*Gm2a*	*Btbd3*	*CT010467.1*
Flt1	*Serpinb2*	*Irf4*	*Car12*	*Snx9*
Oasl1	*Nptxr*	*Cyth4*	*Map3k15*	*Actn1*
Pgm5	*Chst11*	*Serpinb1c*	*Slc17a6*	*Cd9*
Ccl9	*Axl*	*Pip5k1c*	*Tnc*	*Hmga2*
Plk2	*Mamld1*	*Lasp1*	*Sdc1*	*Lyz1*
Rhoc	*Plau*	*Car5b*	*Gm29340*	*Kif3c*
Rgs1	*Carmil1*	*Zdhhc14*	*Lims2*	*Abcb4*
Gm10419	*Mmp12*	*Rasgrp3*	*Atp6v0d2*	*Ncs1*
Dgkh	*Gm22748*	*Smim3*	*Efr3b*	*Fxyd2*
Micall2	*Vopp1*	*Lyl1*	*Olfr99*	*Psmb8*
Bcl2l1	*H2-M2*	*Lmna*	*Coro2a*	*Hmga1b*
Zfhx4	*Plek2*	*Susd3*	*Cyp2s1*	*Glrx*
Rasal1	*Acsbg1*	*Gmpr*	*Dcstamp*	*Gm26917*
Siglec1	*F3*	*Inf2*	*Fosl1*	*Dab2*
Epop	*Gm7993*	*Slc48a1*	*Qpct*	*Nrp1*
Etv1	*Ptgs1*	*Smox*	*Acap1*	*Pdgfa*
A230028O05Rik	*Vegfc*	*Nrm*	*Svil*	*Dhrs9*
Ndrg1	*Ccl7*	*Heatr5a*	*Gm17749*	*H2-DMa*

Tnfsf9	*Mcam*	*Cebpa*	*Dusp9*	*Igf2bp2*
Ccl3	*Gm7278*	*S100a6*	*Cd36*	*Bcar3*
Zfp651	*Mmp14*	*Slc36a1*	*Rhoj*	*Clec7a*
Agrn	*Rnf183*	*Ica1*	*Cd93*	*Fat1*
Slc30a1	*Angptl2*	*Cd300lb*	*Pcdh7*	*Kcnn4*
Ercc1	*Gm9115*	*Orai1*	*Spink5*	*Pxdn*
Pdgfb	*Gm42635*	*Nek6*	*Map6*	*Ppic*
Klf6	*E330009J07Rik*	*Arpin*	*Gfod1*	*Plxdc1*
Cass4	*Mt2*	*Tnfrsf12a*	*Zfp462*	*4932441J04Rik*
H2-Q6	*Xirp1*	*Ahi1*	*Tfcp2l1*	*Cst7*
Pip5k1b	*Irf2bp2*	*St8sia4*	*Chst3*	*Bcl6b*
Mpzl1	*Ptpn5*	*St14*	*Slamf8*	*Prkar2b*
Fosl2	*Arhgap31*	*Sema4b*	*Col18a1*	*Epb41l1*
Lpl	*Cadps*	*Cdc42se2*	*Mindy1*	*Dusp5*
Ube2l6	*Ptpn22*	*Rnf19b*	*Slc24a5*	*Edil3*
Pmm1	*Dync2li1*	*Mt1*	*Padi2*	*Odc1*
Sdc4	*Nr1d1*	*Irx2*	*Slc7a4*	*Ctsl*
Thap7	*Ier3*	*Phlda1*	*Ndrg4*	*Xylt1*
Irak2	*Ccnd1*	*Tnni2*	*Hbegf*	*Adam8*
Gnl1	*Gm49774*	*Dyrk3*	*Adamts1*	*Sema4g*
Mir6236	*Eef2k*	*Jarid2*	*S100a11*	
Rbpj	*Tiam1*	*Slc43a2*		
	Eepd1	*Hpcal1*		
	Gm38158	*Eif4ebp1*		
	Elk3	*Kbtbd11*		
	Hhex	*Rab11fip5*		
	AA414768	*Fut4*		
	Abcd2	*Rnasel*		
	Notch1	*Tec*		
	Epm2a	*Klf10*		
	Gm16201	*Mgst2*		
	Tlr4	*Cuedc1*		
	Zfp36l2	*Syne3*		
	Endod1	*Emp1*		
	3300005D01Rik	*Gm18445*		
		Apbb2		

Figure 5.14: Significantly downregulated genes in *Socs1*$^{-/-}$ RAW macrophages.
Venn diagram indicating the number of genes that are significantly downregulated in the *Socs1*$^{-/-}$ cells compared to WT at the basal level, after stimulation with LPS for 3 hours, and under both conditions. These genes are listed in the table below.

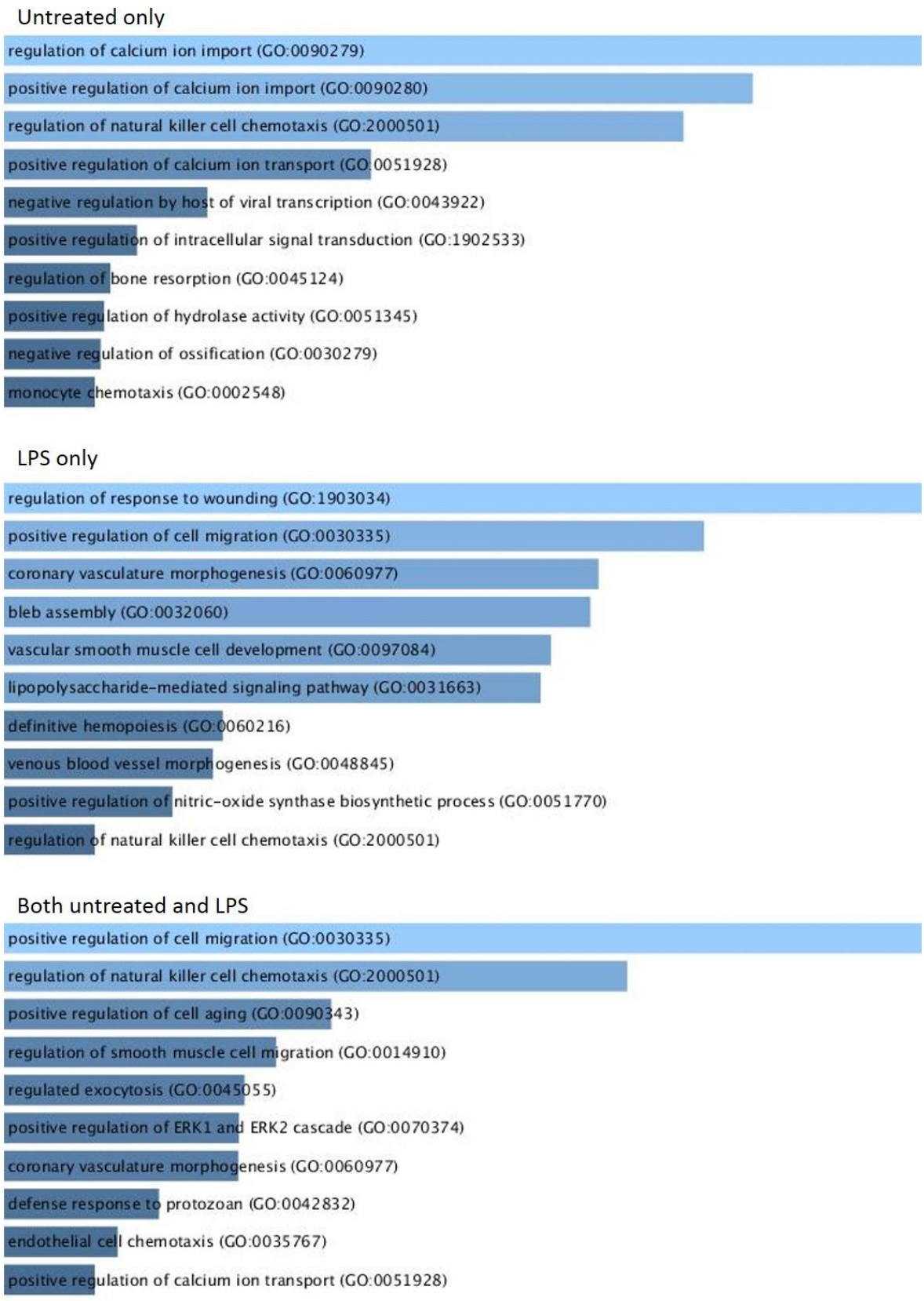


Figure 5.15: Enrichment analysis of GO biological processes of the significantly downregulated genes in *Socs1*$^{-/-}$ RAW macrophages using Enrichr.
The classification terms and their serial numbers are displayed within the bars. Bars are ordered and coloured in descending *p*-value cutoffs.

5.3.2.5 RNAseq analysis of NF-κB target genes in *Socs1*$^{-/-}$ RAW macrophages

Again, considering the role of SOCS1 as an E3 ligase for the p65 subunit of NF-κB, and its destabilising effects on p50 as outlined in the previous chapter, the effect its absence has on the expression of NF-κB targets was of interest. A number of genes that are under the direct control of NF-κB were chosen for further analysis, and which have various roles within the immune system. Their normalised read counts following TLR activation by LPS were extracted from the RNAseq data and plotted, using only sample 2 from the mutants due to the discrepancy between samples as mentioned above. As with the loss of ING4, the loss of SOCS1 affects the expression of NF-κB target genes selectively (figure 5.16). Some genes are expressed to a greater level in the *Socs1*$^{-/-}$ cells, such as *Fas*, *Il1a*¸and *Il1b*, whereas others are repressed, for instance *Cd80*, *Cd86* and *Baff*. Others are not affected by the deletion of SOCS1, such as *Bcl3* and *Caspase11*. Transcript levels of the NF-κB subunits themselves vary to some extent, with increases in the expression of *Nfkb1* and *Rela*. The expression of *Nfkb2* and *Nfkbia* (IκBα) remains relatively unchanged.

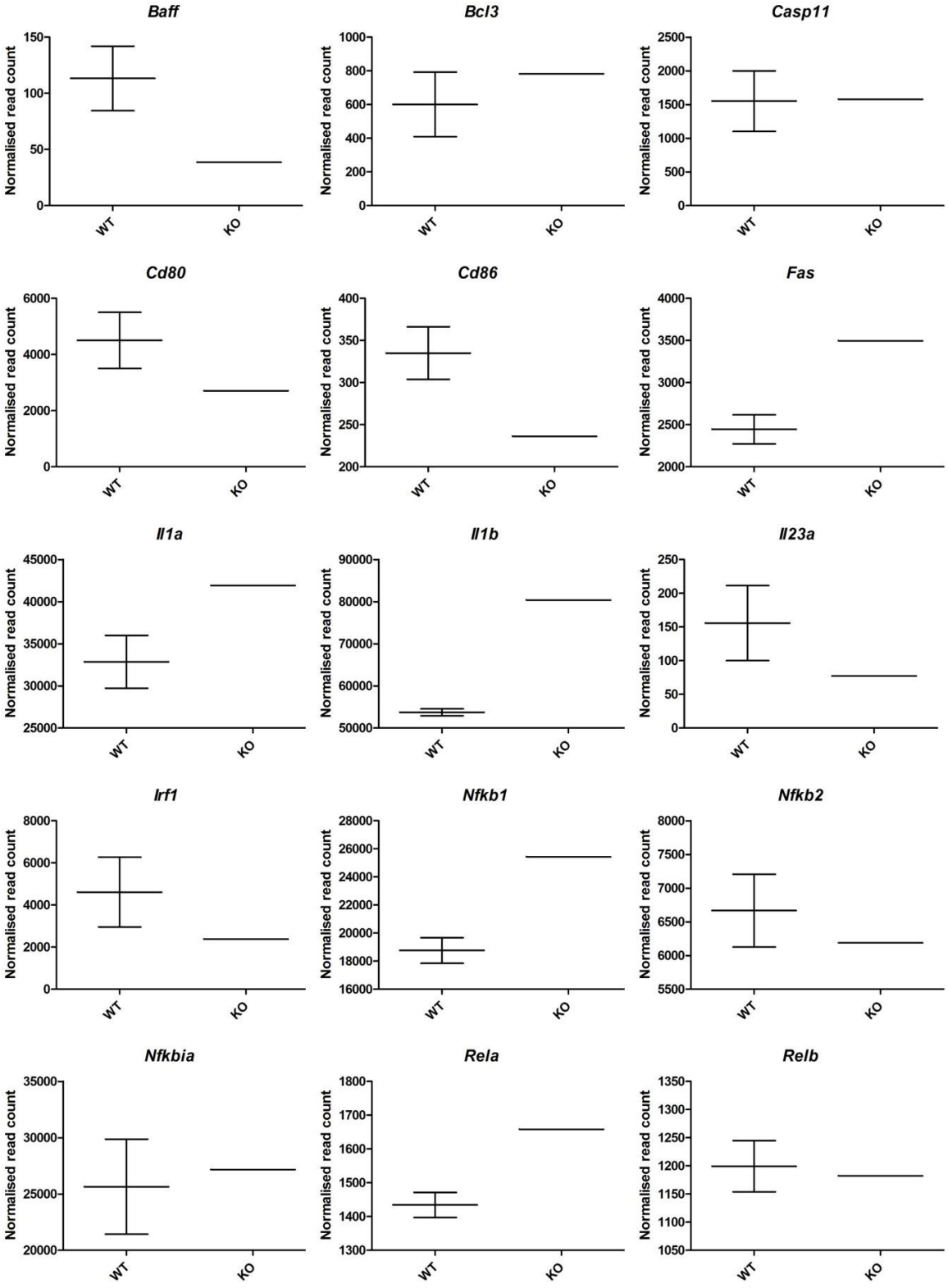


Figure 5.16: Expression of selection of NF-κB target genes in WT and *Socs1*$^{-/-}$ cells after 3-hour LPS stimulation from RNAseq data.
A number of NF-κB target genes with various roles within the immune system were chosen and their expression levels analysed following stimulation with LPS for 3 hours by plotting their copy number. Means represented with whiskers denoting minimum and maximum values.

5.3.2.6 Validation of RNAseq by RT-qPCR

Validation of the RNAseq data and the confirmation that replicate 2 was the reliable sample was performed by RT-qPCR of a number of NF-κB target genes involved in the inflammatory response, as well as genes that were highlighted as being significantly differentially expressed. WT and *Socs1*$^{-/-}$ cells were left either untreated or treated with 100 ng/mL LPS for 3 hours. Relative fold change was calculated using the $2^{-\Delta\Delta Ct}$ method. This not only confirmed the RNAseq replicate 2 data but also again demonstrated that the loss of SOCS1 causes selective changes in the way NF-κB controls the transcription of its target genes (figure 5.17). Some pro-inflammatory cytokines are repressed in the mutant, such as *Ccl2* and *Ccl7*, whereas *Tnfa* expression is increased. The anti-inflammatory cytokine, *Il10,* is expressed much more in the mutant following LPS stimulation than WT. The results from RT-qPCR largely mimic those from the RNAseq, which confirms their validity. Furthermore, these results are not the same as those seen in the *Ing4*$^{-/-}$ RAW cells, and indicates that each of these components has control over the expression of different NF-κB genes, with some overlap of control between genes.

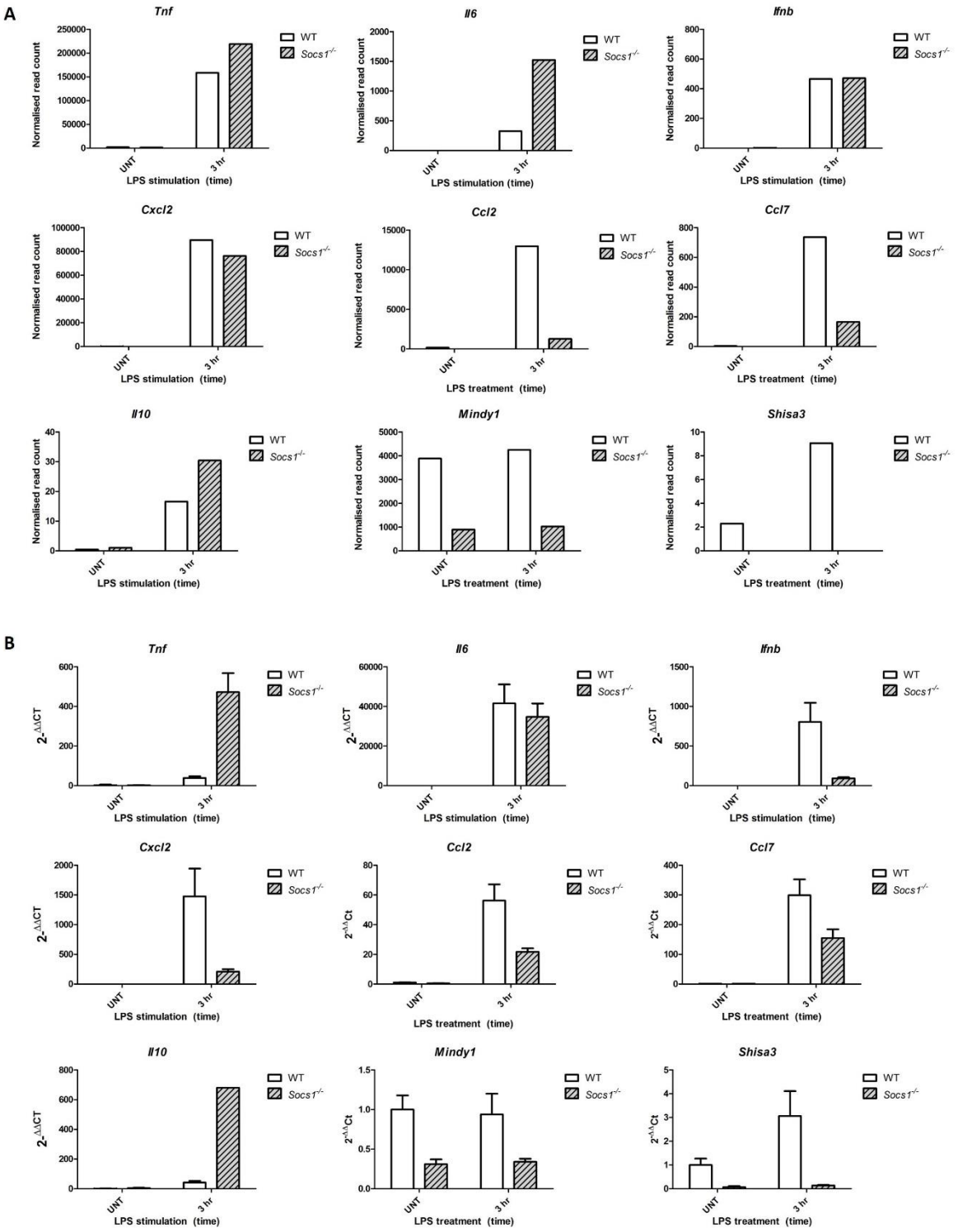


Figure 5.17: Validation of RNAseq by RT-qPCR.
(A) WT and *Socs1*$^{-/-}$ cells were prepared for RNAseq analysis as described above. RNAseq data graphed using the normalised read count from replicate 2 (n=1). (B) WT and *Socs1*$^{-/-}$ cells were left either untreated or treated with 100 ng/mL LPS for 3 hours. RT-qPCR data shows combined results from three independent experiments indicating fold change calculated using the $2^{-\Delta\Delta Ct}$ method with corresponding standard deviation (n=3).

5.3.2.7 Expression of NF-κB subunits in WT and *Socs1*$^{-/-}$ RAW macrophages

As with the *Ing4*$^{-/-}$ cells, it was essential to determine whether the loss of SOCS1 resulted in any changes in the protein levels of the NF-κB subunits, or of their upstream regulator IκBα. Some variation was observed in their mRNA levels in section 5.3.2.5, so to determine whether this was translated at the protein level, WT and *Socs1*$^{-/-}$ RAW macrophages were left either untreated, or treated with 100 ng/mL LPS for the time course indicated. Cells were harvested and protein levels measured via Western blot (WB) using antibodies specific for the indicated proteins. As with the *Ing4*$^{-/-}$ cells, no great differences in the expression levels of the NF-κB components were detected (figure 5.18). Importantly, no changes in the expression pattern of IκBα were observed, which confirms that the changes in NF-κB target gene expression observed in section 5.3.2.5 are not due to any upstream effects.

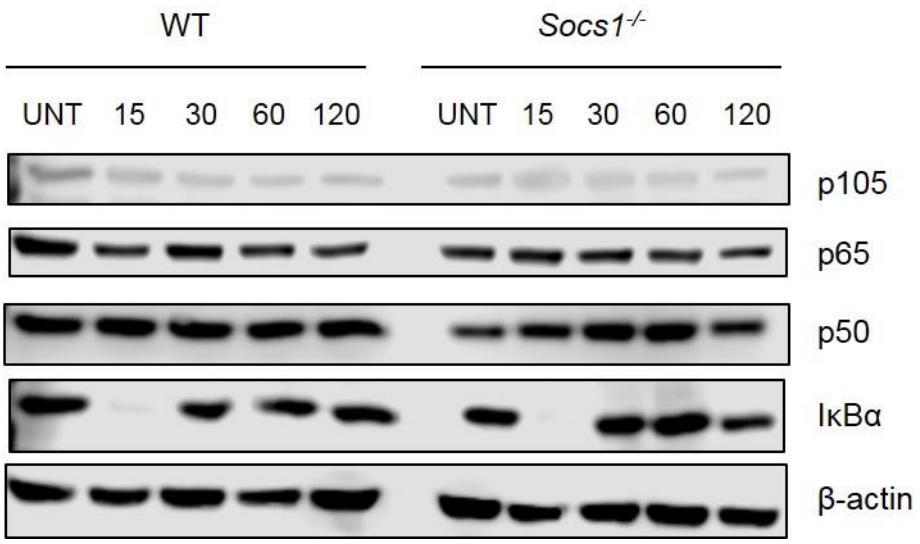


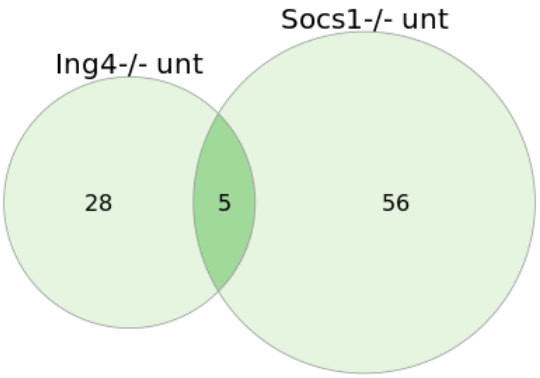
Figure 5.18: Expression levels of NF-κB proteins in WT and *Socs1*$^{-/-}$ RAW macrophages. WT and *Socs1*$^{-/-}$ RAW cells were treated with 100 ng/mL LPS for the indicated time (minutes) or left unstimulated. Whole cell extracts were analysed for levels of the indicated proteins via WB. Figure representative of three individual experiments.

5.3.3 *Ing4*$^{-/-}$ vs *Socs1*$^{-/-}$ RAW 264.7 macrophages

Both ING4 and SOCS1 are known E3 ligases for the p65 subunit of NF-κB so it was of interest to compare directly the genes whose expression is uniquely affected by the deletion of each element, and which are common to both knock-out cell lines. This will reveal the overlap in transcriptional control between the two E3 ligases

5.3.3.1 Genes significantly upregulated in untreated mutant RAW 264.7 macrophages

By creating a Venn diagram of the 89 genes that are significantly upregulated in the mutant cell lines compared to WT, it becomes apparent where overlaps in transcriptional control fall. Overall, 33 genes are significantly upregulated in untreated *Ing4*$^{-/-}$ cells compared to WT, and of these, 28 are unique to *Ing4*$^{-/-}$ cells, such as *Agap2* and *Tmem26*. There are 5 genes that are significantly upregulated in both untreated *Ing4*$^{-/-}$ and *Socs1*$^{-/-}$ cells (figure 5.19). In total, 61 genes are significantly upregulated in untreated *Socs1*$^{-/-}$ cells and of these, 56 are unique to this mutant including *Ocstamp* and *Cd33*. Therefore, in untreated cells, there is little overlap between the genes that are upregulated in the absence of ING4 and SOCS1.

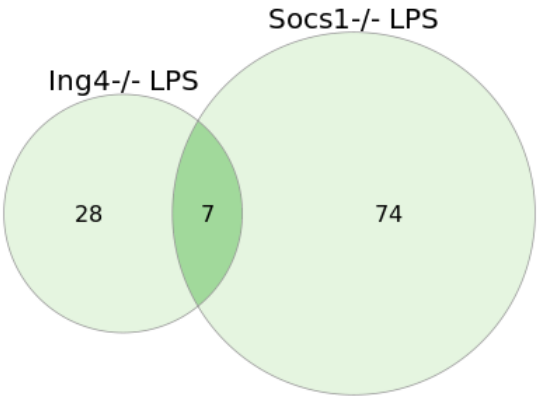


Ing4$^{-/-}$ only	Both	*Socs1*$^{-/-}$ only	
Aldh1l1	*Cpq*	*Mreg*	*Csf3r*
Zic2	*Slc22a17*	*Fam49a*	*Tlr8*
Sp8	*Arhgef40*	*H2-Aa*	*Ubxn11*
Cds1	*Sort1*	*H2-Ea-ps*	*Slamf7*
Slc8a1	*Trip6*	*H2-Eb1*	*Cytip*
Cbfa2t3		*P2ry13*	*Ms4a7*
Agap2		*Arhgap27os3*	*Ccdc85c*
Tmem26		*Gm973*	*Trem3*
Cacna1s		*Treml2*	*Il1a*
Zdhhc2		*Hal*	*Cd33*
Ank2		*Ccbe1*	*Clec4n*
2610528A11RIK		*Pilrb1*	*Plaat3*
Pltp		*Cpeb1*	*Adgb*
Xkr5		*Card14*	*Mfsd2a*
Rnls		*Treml4*	*Traf3ip3*
Ppm1e		*H2-Ab1*	*Cd84*
Cxcl16		*Tspan10*	*BE692007*
Plcb2		*Clec4a1*	*Cyfip2*
Sema5a		*Greb1*	*Nlrc3*
Peg12		*Ocstamp*	*Slc5a3*
Ac166344.1		*Ppfibp2*	*Nlrp10*
Hoxc4		*Plpp3*	*Rab32*
Adgrl1		*Lair1*	*Clec4a3*
Palm3		*Nxf7*	*Lsp1*
Mkx		*Fabp4*	*Rhoq*
Tmem246		*Stc2*	*Neurl3*
Chka		*Ctdspl*	*Pou2f2*
Zmynd15		*Pilrb2*	*Smpdl3a*

Figure 5.19: Significantly upregulated genes in unstimulated knock-out RAW macrophages. Venn diagram indicating the number of genes that are significantly upregulated in either the *Ing4*$^{-/-}$ cells alone, *Socs1*$^{-/-}$ cells alone, or in both knock-outs, at the basal level. These genes are listed in the table underneath. Unt = untreated/unstimulated.

5.3.3.2 Genes significantly upregulated in mutant RAW macrophages treated with LPS

The Venn diagram depicting the 109 genes that are significantly upregulated in the mutant cell lines compared to WT following 3 hours of stimulation with LPS indicates that overall, 35 genes are significantly upregulated in *Ing4*$^{-/-}$ cells compared to WT and of these, 28 are unique to this mutant such as *Il10* and *Ank2* (figure 5.20). In total, 81 genes are significantly upregulated in *Socs1*$^{-/-}$ cells stimulated with LPS, and of these, 74 are unique to this mutant including *Il6, Dio2* and *Bcl6*. Of the 109 genes in total that are significantly upregulated in the knock-out cells compared to WT, 7 are common to both *Ing4*$^{-/-}$ and *Socs1*$^{-/-}$ cells. Therefore, there again is very little overlap between genes that are upregulated in the absence of ING4 or SOCS1 following LPS stimulation.

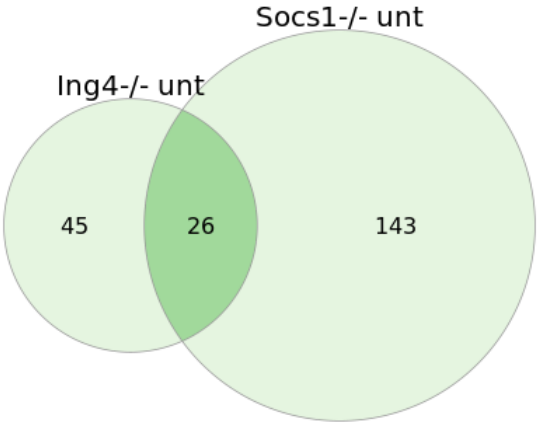


Ing4$^{-/-}$ only	Both	*Socs1*$^{-/-}$ only		
Aldh1l1	*Slc22a17*	*Dio2*	*Il6*	*Siglece*
Zic2	*Sort1*	*P2ry13*	*Plpp3*	*Peli1*
Sp8	*Cpq*	*Fam49a*	*Ms4a7*	*Ms4a6d*
Il10	*Fam126b*	*H2-Ea-ps*	*Cyfip2*	*Ms4a6b*
Col15a1	*Bcl6*	*H2-Aa*	*Zfp579*	*Plxna2*
Trim36	*Adgb*	*H2-Eb1*	*Nxf7*	*Ly9*
Cds1	*Ocstamp*	*Prdm1*	*Trem3*	*Flcn*
Six1		*Ppfibp2*	*Ccdc85c*	*Ppp1r15a*
Dst		*Gm973*	*F630028O10Rik*	*Slc43a3*
Slc8a1		*Mreg*	*BE692007*	*Cpeb4*
Agap2		*Gfi1*	*Fgd4*	*Tmem273*
Tmem26		*Treml2*	*Ctsc*	*Tnfrsf1b*
Zc3h12c		*Slamf7*	*Ccbe1*	*Syk*
Ank2		*Hal*	*Clec4a3*	*Irak3*
2610528A11RIK		*Clec4a1*	*Traf3ip3*	*Sowahc*
Pltp		*Edn1*	*Tanc2*	*D030028A08Rik*
Rnls		*Pilrb1*	*Gm6377*	*Lsp1*
Plcb2		*Pilrb2*	*S1pr1*	*Rapgef5*
Sema5a		*Tspan10*	*Ms4a6c*	
Peg12		*Arhgap27os3*	*Csf3r*	
Ac166344.1		*Adra1a*	*Clec4a2*	
Hoxc4		*Rap1gap2*	*Lair1*	
Adgrl1		*Zfp334*	*Slpi*	
Palm3		*H2-Ab1*	*Ldlrad3*	
Mkx		*Bambi-ps1*	*Itgal*	
Rpl3l		*Pdcd1*	*Mfsd2a*	
Tmem246		*Treml4*	*Zfp703*	
Shisa3		*Adora2a*	*Cd48*	

Figure 5.20: Significantly upregulated genes in knock-out macrophages stimulated for 3 hours with LPS.
Venn diagram indicating the number of genes that are significantly upregulated in either the *Ing4*$^{-/-}$ cells alone, *Socs1*$^{-/-}$ cells alone, or in both knock-outs, following stimulation with LPS for 3 hours. These genes are listed in the table below.

5.3.3.3 Genes significantly downregulated in untreated mutant RAW macrophages

By creating a Venn diagram of the 214 genes that are significantly downregulated in the mutant cell lines compared to WT, it is clear overall, 71 genes are significantly downregulated in untreated *Ing4*$^{-/-}$ cells compared to WT and of these, 45 genes are uniquely downregulated in the *Ing4*$^{-/-}$ macrophages including *Lamb2* and *Notch1* (figure 5.21). In total, 169 genes are significantly downregulated in untreated *Socs1*$^{-/-}$ cells and 143 are unique to the *Socs1*$^{-/-}$ cells, such as *Dusp5, Ccl2* and *Ccl9*. Of the 214 genes in total that are significantly downregulated in the knock-out cells compared to WT, 26 are common to both *Ing4*$^{-/-}$ and *Socs1*$^{-/-}$ macrophages. Therefore, in untreated cells, there is some overlap between the genes that are downregulated in the absence of ING4 and SOCS1.



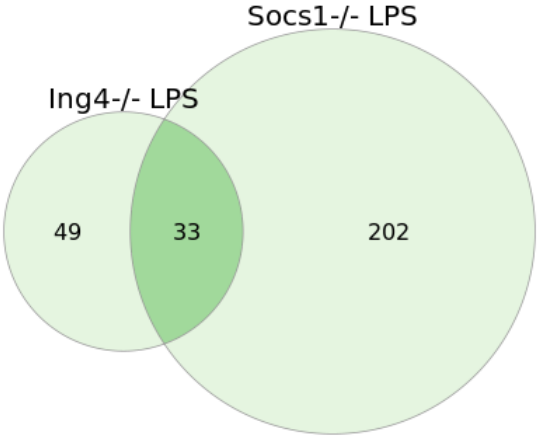
***Ing4*$^{-/-}$ only**	**Both**	***Socs1*$^{-/-}$ only**		
Tnfrsf10b	*Ppic*	*Axl*	*Adam8*	*Mpzl1*
Kif21a	*Map6*	*Scarf1*	*Sema4g*	*Fosl2*
Pcbp4	*Gypc*	*Chst11*	*Pxdn*	*Lpl*
Acy1	*Qpct*	*Nptxr*	*Ccl9*	*Gm23935*
Abhd14a	*Coro2a*	*Ccl2*	*Plxdc1*	*Ifitm6*
Lamb2	*Mmp12*	*B3galnt1*	*4932441J04Rik*	*Hmga1*
Zfp2	*Layn*	*Cd200r2*	*Plk2*	*Ube2l6*
Cep170b	*Abtb2*	*Sulf2*	*Cst7*	*Etv1*
Arhgap25	*Pf4*	*Dner*	*Rhoc*	*Snx9*
Fat3	*Btbd3*	*Gm17928*	*Bcl6b*	*Pmm1*
Nsd1	*Col18a1*	*Morc4*	*Prkar2b*	*Actn1*
Il11ra1	*Cers4*	*Rassf8*	*Rgs1*	*Cd9*
Vopp1	*Nes*	*Runx2*	*Dusp5*	*Hmga2*
Antxr1	*Epb41l1*	*Car12*	*Gm10419*	*Lyz1*
Hpgd	*Odc1*	*Map3k15*	*Dgkh*	*Sdc4*
Ier3	*Dusp9*	*Slc17a6*	*Edil3*	*Thap7*
Fbxo15	*Flt1*	*Tnc*	*Micall2*	*Kif3c*
Glipr2	*Eid2*	*Sdc1*	*Ctsl*	*Abcb4*
Icosl	*Dcstamp*	*Rab15*	*Xylt1*	*Irak2*
Cd24a	*Nkain1*	*Gm29340*	*Bcl2l1*	*Ncs1*
Ccdc127	*Ccr1*	*Lims2*	*Zfhx4*	*Gnl1*
Ccdc69	*Emp2*	*Egr2*	*Gm26917*	*Fxyd2*
Car2	*Mindy1*	*Atp6v0d2*	*Dab2*	*Psmb8*
Itpr2	*Acap1*	*Spp1*	*Rasal1*	*Hmga1b*
Sdr39u1	*Pkd1l2*	*Egr1*	*Nrp1*	*Mir6236*
Enpp4	*H2-m3*	*Efr3b*	*Pdgfa*	*Rbpj*
Tanc1		*Olfr99*	*Siglec1*	*Glrx*
Shc2		*Cyp2s1*	*Dhrs9*	*Slc7a4*
Notch1		*Fosl1*	*Epop*	*Ndrg4*
Tmem98		*Egr3*	*H2-DMa*	*Oasl1*
Maf		*Svil*	*CT010467.1*	*Pgm5*
E330009J07RIK		*Gm17749*	*A230028O05Rik*	*Hbegf*

Afp		*Cd36*	*Ndrg1*	*Adamts1*
Tmsb10		*Rhoj*	*Igf2bp2*	*S100a11*
9130008F23RIK		*Dpys*	*Bcar3*	*Rab7b*
Ptgs1		*Cd93*	*Tnfsf9*	*Klf6*
Mgl2		*Pcdh7*	*Ccl3*	*Clic1*
Zbtb46		*Spink5*	*Clec7a*	*Cass4*
Prkg2		*Src*	*Fat1*	*H2-Q6*
Igsf11		*Ranbp3l*	*Kcnn4*	*Dok2*
Gpr179		*Ccl4*	*Csf2rb2*	*Pip5k1b*
Ctse		*Gfod1*	*Zfp651*	
Acot1		*Zfp462*	*Agrn*	
Chchd10		*Slc22a4*	*Slc30a1*	
Dmwd		*Il1rn*	*Ercc1*	
		Tfcp2l1	*Pdgfb*	
		Chst3	*Slc30a4*	
		Slamf8	*H2-Q5*	
		C5ar1	*Adssl1*	
		Slc24a5	*Atxn1*	
		Padi2	*Ninj1*	

Figure 5.21: Significantly downregulated genes in unstimulated knock-out macrophages. Venn diagram indicating the number of genes that are significantly downregulated in either the *Ing4*$^{-/-}$ cells alone, *Socs1*$^{-/-}$ cells alone, or in both knock-outs, at the basal level. These genes are listed in the table below.

5.3.3.4 Genes significantly downregulated in mutant RAW macrophages treated with LPS

The Venn diagram depicting the 317 genes that are significantly downregulated in the mutant cell lines compared to WT following 3 hours of stimulation with LPS indicates that overall, 82 genes are significantly downregulated in *Ing4*$^{-/-}$ cells compared to WT and of these, 81 genes are uniquely downregulated in the *Ing4*$^{-/-}$ macrophages, including *Ccl2* and *Ccl7* (figure 5.22). In total, 236 genes are significantly downregulated in *Socs1*$^{-/-}$ cells stimulated with LPS and 235 are unique to the *Socs1*$^{-/-}$ cells, such as *Tlr4* and *Dusp9*. Of the 165 genes in total that are significantly upregulated in the knock-out cells compared to WT, only 33 are common to both *Ing4*$^{-/-}$ and *Socs1*$^{-/-}$ macrophages. Therefore, in cells treated with LPS, there again is some overlap between the genes that are upregulated in the absence of ING4 and SOCS1.



***Ing4*$^{-/-}$ only**	***Both***	***Socs1*$^{-/-}$ only**		
Adgrl2	*Dner*	*Hbegf*	*Eef2k*	*Lyl1*
Klf9	*Ppic*	*Scarf1*	*Kcnn4*	*Lmna*
Kif21a	*Map6*	*Layn*	*Tiam1*	*Susd3*
Pcbp4	*Gypc*	*Car12*	*Eepd1*	*Gmpr*
Lamb2	*Qpct*	*B3galnt1*	*Gm38158*	*Inf2*
Cep170b	*Chst11*	*Cd93*	*Elk3*	*Slc48a1*
Trpv4	*Coro2a*	*Cd200r2*	*Hhex*	*Ncs1*
Arhgap25	*Prkg2*	*Morc4*	*AA414768*	*Smox*
Fat3	*Pf4*	*Slc17a6*	*Dcstamp*	*Hmga2*
Nsd1	*Mmp12*	*Tpbg*	*Dhrs9*	*Nrm*
Jund	*Vopp1*	*Sulf2*	*Rab7b*	*Lyz1*
Il11ra1	*Tnc*	*Lpar1*	*Sema4g*	*Heatr5a*
Ehd2	*Mamld1*	*Rassf8*	*Abcd2*	*Cebpa*
Anxa6	*Abtb2*	*Lims2*	*Dusp5*	*S100a6*
Sash3	*Ier3*	*Padi2*	*Epm2a*	*Slc36a1*
Gpr179	*Notch1*	*Atp6v0d2*	*Pxdn*	*Ica1*
Cd276	*Ccl7*	*Gm9260*	*Gm16201*	*Cd300lb*
Prkd3	*Cers4*	*Nptxr*	*Tlr4*	*Orai1*
Glipr2	*Nes*	*Gm17928*	*Clic1*	*Fosl1*
Icsol	*Epb41l1*	*Svil*	*Adssl1*	*Nek6*
Evl	*Odc1*	*Sdc1*	*Gm17749*	*Arpin*
Sdr39u1	*Ptgs1*	*Zfp462*	*Cst7*	*Fxyd2*
Ccl12	*Nkain1*	*Cyp2s1*	*Zfp36l2*	*Fat1*
Slc6a9	*Ccr1*	*Map3k15*	*Endod1*	*Kif3c*
Enpp4	*Btbd3*	*Axl*	*Kbtbd11*	*Tnfrsf12a*
Tanc1	*Col18a1*	*Bcl6b*	*Rab11fip5*	*Ahi1*
Cd24a	*Serpinb2*	*Plau*	*Fut4*	*St8sia4*
Stard10	*Emp2*	*Adamts1*	*Rnasel*	*St14*
Tmem98	*Ccl2*	*Tfcp2l1*	*Tec*	*Sema4b*
Fam149a	*Mindy1*	*Carmil1*	*Slc7a4*	*Cdc42se2*
Maf	*Acap1*	*Gm22748*	*Klf10*	*Atxn1*
Zfp984	*Pkd1l2*	*H2-M2*	*Spink5*	*Rnf19b*

E330009J07RIK	*H2-m3*	*Plek2*	*Mgst2*	*Mt1*
Tmsb10		*Runx2*	*Cuedc1*	*Xylt1*
Mgl2		*Acsbg1*	*Syne3*	*Psmb8*
Flt1		*Rhoj*	*Emp1*	*Irx2*
Eid2		*Pdgfa*	*Dab2*	*Cd9*
Igsf11		*F3*	*Gm18445*	*Dok2*
Ggta1		*Gm7993*	*Apbb2*	*H2-Q5*
Mapk11		*Slc24a5*	*3300005D01Rik*	*Phlda1*
Cebpe		*Dusp9*	*Glrx*	*Tnni2*
Ctse		*Pcdh7*	*Gm28875*	*Dyrk3*
Abcg2		*Slamf8*	*Gm23935*	*Jarid2*
Ccdc69		*Plxdc1*	*Eps8*	*Slc43a2*
Osm		*Vegfc*	*H2-DMa*	*Hpcal1*
Ptger2		*Mcam*	*Clec7a*	*Eif4ebp1*
Itpr2		*Adam8*	*Cd200r1*	*Chst3*
Chchd10		*Gm7278*	*Vegfa*	*Ptpn22*
Trim47		*Mmp14*	*Hmga1*	*Dync2li1*
		Rnf183	*Tmem171*	*Nr1d1*
		Gfod1	*Procr*	*Edil3*
		Ndrg4	*Ptchd1*	*Bcar3*
		4932441J04Rik	*Gm26917*	*Ccnd1*
		Angptl2	*Bhlhe40*	*Ctsl*
		S100a11	*Hilpda*	*Gm49774*
		Gm29340	*Nrp1*	*Prkar2b*
		Gm9115	*Nubpl*	*Pip5k1c*
		Cd36	*Csf2rb2*	*Lasp1*
		Gm42635	*Mical2*	*Snx9*
		E330009J07Rik	*Abcb4*	*Car5b*
		Mt2	*Dusp7*	*Zdhhc14*
		Olfr99	*CT010467.1*	*Rasgrp3*
		Xirp1	*Gm2a*	*Actn1*
		Irf2bp2	*Irf4*	*Ninj1*
		Ptpn5	*Cyth4*	*Hmga1b*
		Efr3b	*Igf2bp2*	*Smim3*
		Arhgap31	*Serpinb1c*	
		Cadps	*Slc30a4*	

Figure 5.22: Significantly downregulated genes in knock-out macrophages stimulated for 3 hours with LPS.
Venn diagram indicating the number of genes that are significantly down regulated in either the *Ing4*$^{-/-}$ cells alone, *Socs1*$^{-/-}$ cells alone, or in both knock-outs, following stimulation with LPS for 3 hours. These genes are listed in the table below.

5.4 Discussion

The results of this chapter represent the first comparative analysis of the role of two known E3 ligases of NF-κB in the regulation of transcription. They provide insight into the functional consequences of ING4 and SOCS1 in a high-throughput manner, as well as the ability to focus on specific genes as required. Indeed looking at certain genes in detail reveals a number of interesting observations. The observation from both the RNAseq and RT-qPCR analysis that expression levels of *Il6*, *Il10* and *Ccl5* (RANTES) are increased in *Ing4*$^{-/-}$ cells following activation with LPS concurs with a previous study conducted in *Ing4*-null mice (Coles *et al.*, 2010). The researchers identified a role for ING4 in negatively regulating a subset of NF-κB responsive genes following macrophage exposure to LPS. They detected an increased amount of nuclear p65 in non-stimulated *Ing4*$^{-/-}$ macrophages and increased *Il6* promoter occupancy at 0 and 1 hour of LPS stimulation, which correlated with increased levels of promoter histone H4 acetylation. In contrast, they also found that occupancy of the *TNFa* promoter by p65 was increased in *Ing4*$^{-/-}$ macrophage but there was far less H4 acetylation following LPS stimulation than in WT cells, which tied in with the lower levels of *TNFa* expression they observed, and that were also detected in this investigation (figure 5.8). This indicated that a subset of NF-κB-responsive cytokine promoters require ING4 for promoter activation following p65 binding. This might explain the variances in the expression of NF-κB target genes observed in the current study, whereby the genes that are upregulated in the mutant cells do not require ING4 for H4 acetylation of their promoters, and those that are downregulated do require ING4 for this acetylation to occur. To confirm this, chromatin immunoprecipitation (ChIP) could be performed to determine how much promoter occupancy by p65 and H4 acetylation is detected in the *Ing4*$^{-/-}$ macrophages compared to WT. Analysis of the NF-κB subunit protein levels did not reveal a detectable increase in p65 expression in the *Ing4*$^{-/-}$ cells (figure 5.9), however transcript levels were increased (figure 5.7). The nuclear and cytoplasmic fractions were not analysed separately in the current study, however, so this may reveal differences in future experiments. The study mentioned above also detected a slower re-synthesis of IκBα (*Nfkbia)* in the *Ing4*$^{-/-}$ macrophages following LPS stimulation and both protein and gene levels remained lower overall. The reduced expression of *Nfkbia* in LPS stimulated *Ing4*$^{-}$

$^{-/-}$ cells was due again to decreased H4 acetylation of the *Nfkbia* promoter in the absence of ING4. In this study, transcript levels of *Nfkbia* were reduced in the *Ing4*$^{-/-}$ macrophages 3 hours after stimulation with LPS, although no great change in protein re-synthesis dynamics was observed over the shorter time course. Future investigations should incorporate a 3-hour time point and re-measure IκBα protein levels to determine if they are lower overall in the *Ing4*$^{-/-}$ RAW macrophages. The current data indicates that a reduction in IκBα levels and thus more NF-κB subunits translocating to the nucleus is not the cause of the increased expression of some NF-κB target genes. If it were then all NF-κB target genes would be upregulated and this is not what is seen. Upstream effects of ING4 on IκBα levels can therefore be ruled out as affecting NF-κB expression, and instead it appears that ING4 is affecting transcription when NF-κB has already moved into the nucleus.

It is not possible to perform *in vivo* experiments using *Socs1*$^{-/-}$ mice because they die within 3 weeks after birth due to severe inflammatory responses in almost all organs resulting from excessive action of IFNγ (Naka *et al.*, 1998; Starr *et al.*, 1998; Alexander *et al.*, 1999). Therefore, studies have used heterozygous or double knock-outs with IFNγ, which have demonstrated hypersensitivity of these mice to challenge with LPS (Hashimoto *et al.*, 2009). Indeed, in this investigation, levels of pro-inflammatory *Tnfa*, *Il1b* and *Cxcl1* are increased following LPS stimulation compared to WT, as is the expression of anti-inflammatory *Il10*, although not to as great an extent as in the *Ing4*$^{-/-}$ cells. However, as with the *Ing4*$^{-/-}$ macrophages, this attenuation is only detected in some and not all pro-inflammatory cytokines, suggesting that SOCS1 does not merely function as part of a negative feedback loop, but affects transcription on a gene-specific level. Of course, TLR activation by LPS does not only lead to the activation of the NF-κB pathway, but other signalling cascades as well. SOCS1 is a well-known suppressor of the JAK/STAT pathway (Sakamoto *et al.*, 1998; Yasukawa *et al.*, 1999), as well as of the p38 MAPK signalling cascade (Souma *et al.*, 2012) so the variations in gene expression observed in the *Socs1*$^{-/-}$ macrophages may be due to aberrations in these signalling pathways. Indeed, GO analysis of the biological processes of the genes significantly downregulated in the *Socs1*$^{-/-}$ cells includes those involved in the ERK1/2 pathway and LPS signalling generally (figure 5.16). For both knock-outs, had time not been a

limiting factor in the current investigation an important experiment would be to measure the levels of phosphorylated STAT1, ERK, p38, JNK, IκBα and p65 to determine the signal transduction in response to LPS in the knock-out. Additionally, the re-introduction of ING4 and SOCS1 into each of the cell lines to determine if this rescues the phenotype and mimics the WT transcriptional landscape would also be insightful.

Selective targeting of inflammatory genes as observed within this chapter is desirable in the treatment of chronic inflammatory disease as it retains some immune system functionality rather than current therapeutics that result in widespread immunosuppression and can result in greater susceptibility to infection and injury for the host. The selectivity in which genes are controlled by which E3 ligase might be achieved by a number of mechanisms. Perhaps a prior post-translational modification determines which E3 ligase ubiquitinates NF-κB, acting as a molecular switch. For example, phosphorylation of p65 at S468 as induced by TNFα allows the binding of COMMD1 and cullin 2 resulting in the inducible ubiquitination and degradation of p65, whereas this was not a pre-requisite for SOCS1 binding (Geng *et al.*, 2009). Alternatively, as mentioned above, perhaps certain E3 ligases cause increased occupancy of certain gene promoters by NF-κB, or some genes require the presence of an E3 ligase for H4 acetylation of their promoters and others do not. The data outlined in this chapter opens many avenues for future research.

Considering the multitude of roles ING4 and SOCS1 play within the immune system and other biological processes, in addition to their function as E3 ligases for p65 and effects on the other NF-κB subunits, knocking out either component will unavoidably have far-reaching effects on the phenotype of the host organism. In this case, whilst the expression of some pro-inflammatory genes are repressed and pro-resolution genes increased in the knock-outs, there are other, less desirable effects. For example, in the *Ing4*$^{-/-}$ cells, there is increased expression of *Agap2* and *Peg12* that are associated with tumour development, and *Sema5a*, which is linked to autism. Many of the downregulated genes in the *Socs1*$^{-/-}$ cells had roles in cardiac development and wound healing. There is a plethora of genes whose expression is altered in the knock-outs that, whilst not of interest from the point of view of the immune response, are still critical to

cellular development and viability. Therefore, in the case of chronic inflammatory diseases, whilst therapeutically it would be desirable to lose SOCS1 so that its degradative effect on repressive p50 homodimers is avoided, the consequences are not precise enough and could be considered the molecular equivalent of removing a patient's brain to treat Alzheimer's. Indeed, knocking out either of these components would not be the aim of any therapy that sought to terminate p65-driven transcription since both ubiquitinate and degrade p65. Rather, the use of these knock-out macrophages provides an in-depth picture of the genes that are directly influenced by each of these components, identifies where the redundancy between ING4 and SOCS1 lies by comparing the two knock-outs, and therefore gives researchers a deeper understanding of their function.

Chapter 6

General discussion

6.1 Overview of findings

The ubiquitination of NF-κB is an important mechanism that regulates its transcriptional activity. Although much is known about the relationship between the ubiquitin-proteasome system and the p65 subunit of NF-κB, relatively little is understood about the mechanisms that lead to the ubiquitination and degradation of p50. In this thesis, we sought to fill some of the gaps in our knowledge of how p50 is de-stabilised. By using a variety of biochemical and molecular approaches, it was revealed that SOCS1 is able to promote the ubiquitination and degradation of p50 in some capacity that is independent of its activity as an E3 ligase. By using a panel of putative E3 ligases, we were able to rule out ING4 as being an E3 ligase for p50, and others such as UBR5 provided interesting results that future lines of enquiry would seek to explore. A site of ubiquitination of p50 was examined in detail using mutant THP-1 cells that had the lysine residue at position 130 of p105 mutated to arginine. It was found that this site does not affect the expression of TNFα, IL-6 or CXCL2 in response to TLR activation, although a wider transcriptional analysis might reveal genes that are regulated by this site. However, mutation of this site causes a stark reduction in the levels of RelB and c-Rel. This is intriguing as it indicates that the post-translational modification of p50 directly affects the abundance and cellular dynamics of the other NF-κB subunits. Finally, transcriptomic analysis of mutant macrophages that had either ING4 or SOCS1 knocked-out, both of which are known E3 ligases for p65, revealed that different gene sets are directly influenced by each of these components, and that they affect the expression of NF-κB target genes in a gene-specific manner. Overall, the data presented within this thesis furthers our understanding of the mechanisms that regulate NF-κB activity, in particular the role of p50 and how it is affected by post-translational modifications.

6.2 Identifying further E3 ligases of NF-κB

Most of the studies into how ubiquitination controls the activity of NF-κB have focussed their attention on the p65 subunit. Understanding how p65 activity is terminated through ubiquitin-mediated proteasomal degradation is critical because this subunit is the main driver of transcription. However, p50 has a dual

role as both a promoter of transcription when dimerised with p65, and as a repressor when present as a homodimer due its lack of transactivation domain, and so discerning the mechanisms underlying its ubiquitination and degradation is equally as important. This investigation employed some of the known E3 ligases for the other NF-κB subunits as a starting point in determining whether any also had the ability to ubiquitinate p50. A combinatorial approach using this as well as high-throughput screening techniques such as yeast two hybrid (Y2H) or affinity purification coupled to mass spectrometry (MS) would be a robust next step to identify further candidates.

6.3 Exploring the NF-κB ubiquitin code

During this study, the exact sites of p50 ubiquitination were explored using constructs that express p50 with different lysine residues mutated to arginine. This approach is feasible in the case of p50, which is a relatively small protein that has few lysine residues. However, without the prior knowledge of the identity of the E3 ligase that ubiquitinates p50, the possible combinations of mutants with putative E3s in co-transfection assays is large. To gain a wider perspective of the ubiquitome of NF-κB as a whole, future lines of enquiry might employ the use of screens using antibodies that provide information on the exact sites of ubiquitination following MS. For example, the K-ε-G-G antibody recognises peptides with the diglycine remnant that is present on ubiquitinated lysines following tryptic digestion (Xu *et al.*, 2010). However, it has been reported that it has a bias toward certain amino acid contexts near to the ubiquitinated lysine (Wagner *et al.*, 2012), it does not recognise N-terminal ubiquitination, and it cannot distinguish between ubiquitin and other ubiquitin-like proteins (UBLs). A newly emerging technology is UbiSite, which is a monoclonal antibody that specifically recognises the unique ubiquitin that remains on protein targets after proteolytic digestion with LysC, an endopeptidase (Akimov *et al.*, 2018), which could be taken advantage of to identify sites of ubiquitination of p50. Wide scale screens such as MS can return false positive results when certain protease and deubiquitinase inhibitors are used as they can cause the formation of protein adducts that have the same mass as ubiquitin (Nielsen *et al.*, 2008), so this should be kept in mind.

Indeed, proteins display great promiscuity in respect of ubiquitination, receiving ubiquitin at multiple residues and from multiple E3 ligases. Already, many E3 ligases have been identified for p65 and their target sites do not overlap (Collins *et al.,* 2016). This could result in heterogeneous populations of differentially ubiquitinated NF-κB within cells, further highlighting the complexity of NF-κB regulation. It takes the mutation of almost all lysine residues of p65 to abolish its ubiquitination (Li *et al.*, 2012). Although thus far, K130 of human p50 is a known site of ubiquitination, it is likely that there are a number of others. In this investigation, mutation of K130 to R did not result in a reproducible pro- or anti-inflammatory phenotype in monocytes, at least not one that was detectible from the genes tested, but a wider transcriptomic screen may reveal otherwise. Instead, the loss of this site of ubiquitination led to changes in the abundance of other NF-κB subunits both basally and after LPS stimulation. The ubiquitin code describes the multitude of configurations of ubiquitin modifications that are possible on a protein. Historically, it was thought that K48-linked ubiquitin chains are proteolytic and K63-linked chains are non-proteolytic. However, it is now known that the situation is more complex than this, and K63-linkages can drive degradation and K48-linkages can function non-proteolytically, for example in transcription factor regulation (Flick *et al.*, 2006). Additionally, other ubiquitin linkages are possible through the other lysine residues or N-terminal methionine, and these can form number of different topologies. This, along with other factors such as deubiquitination, localisation and timing, has influence over the functional outcome of ubiquitination (Komander & Rape, 2012), and may explain the results seen in this investigation. Future experiments might explore the composition of NF-κB dimers in cells that have the K130R mutation in p105 and measure the ratios, as this might have some influence over which genes are ultimately being transcribed. Analogous to the proposed phosphorylation of NF-κB barcode hypothesis (Moreno *et al.*, 2010), it is possible that the individual ubiquitination events on p50 (and the other NF-κB subunits) lead to transcriptional outcomes that are highly gene specific. This hypothesis seeks to explain how different NF-κB activating stimuli causes the expression of distinct gene sets. Identifying the patterns of ubiquitination induced by particular E3 ligases and their target sites on p50 and their transcriptional consequences is an important task in cracking the ‘ubiquitination code’, and would allow targeting of NF-κB to specifically downregulate the expression of

pro-inflammatory genes during chronic inflammation. There is evidence emerging that phosphorylation at specific sites of p50 causes gene-specific transcriptional effects and this is mediated by the differential binding of phosphorylated p50 to κB sites of DNA, so it is possible that ubiquitination at different sites has a similar effect.

Indeed, this effect was observed during the RNAseq analysis of the specific E3 ligase-deficient RAW 264.7 macrophages in chapter 5. Some sets of NF-κB target genes were up or downregulated in *Ing4*$^{-/-}$ cells compared to WT, some were up or downregulated in *Socs1*$^{-/-}$ cells compared to WT, and these genes were not the same between the two mutants indicating that differential ubiquitination of NF-κB results in distinct transcriptional outcomes. However, also apparent was that these two components have wide-ranging roles in not only the ubiquitin pathway but many other biological processes as well. It must be kept in mind for both these mutants and in particular, the p105^{K130R} THP-1 mutant, that the possibility exists that genetic compensation is occurring. This mechanism leads to 'transcriptional adaptation' resulting in increased expression of related genes that are able to assume the function of the mutated gene and leads to observations whereby engineered mutants do not exhibit an obvious phenotype (El-Brolosy *et al.*, 2019).

6.4 Unravelling the relationship between SOCS1 and p50

The unexpected observation that SOCS1 is able to promote the ubiquitination and degradation of p50 in some capacity that is outside of its E3 ligase activity has provided a basis for further lines of enquiry. Due to the limitations of time, the exact nature of the mechanism of degradation was not deduced. The use of a proteasome inhibitor did not prevent p50 degradation which is at odds with previous studies carried out on p65 (Ryo *et al.*, 2003), but other degradative pathways are possible. The use of inhibitors such as quinacrine, Z-VAD-FMK or bafilomycin might shed light on whether SOCS1 promotes p50 degradation via the lysosome, caspases or by autophagy. In fact, ubiquitin-tagging has also been observed to route proteins towards these pathways as well as its prototypical role within the UPS (Clague & Urbé, 2010). Additionally, the expression of NF-κB target genes could be measured by RT-qPCR following the over-expression of

SOCS1 and TLR activation to determine what effect its presence or absence has on the transcriptional activity of NF-κB. This would perhaps be better performed in cells such as RAW 264.7 macrophages rather than HEK293Ts as these are more relevant from an inflammatory perspective, although they are more difficult to transfect. It must be borne in mind that overexpression assays may not accurately represent what is happening biologically. SOCS1 could be silenced using siRNA in future experiments to determine the effect it has endogenously on levels of p50. Similarly, the endogenous levels of p50 ubiquitination could be measured using an approach like tandem ubiquitin-binding entities (TUBEs), which is a 'molecular trap' that binds to and protects polyubiquitinated proteins (Hjerpe *et al.*, 2009). This would overcome issues that surround the study of ubiquitination such as the fact that the modification is highly reversible and that the act of ubiquitination itself marks proteins for degradation, and would reinforce the effects of deubiquitinase and proteasome inhibitors. It is apparent that SOCS1, in affecting the stability of both p65 and p50, highlights the layers of complexity in regulating not only the abundance and ratio of NF-κB subunits in cells, but its transcriptional activity as well.

6.5 Concluding remarks

In the context of the innate immune response, during which dysregulated gene expression can cause catastrophic damage to the host, the role of transcriptional repressors such as the p50 homodimer are of particular importance. Understanding the mechanisms that underlie the degradation of this and the other subunits of NF-κB will allow researchers to exert control over its activity and intervene when it becomes dysregulated. The ubiquitin-proteasome system is a major pathway that has implications on protein homeostasis and many other cellular signalling processes. Biotechnological approaches are being developed that harness this natural housekeeping ability of cells. Proteolysis-targeting chimeras (PROTACs) are synthetic small molecules that recruit proteins into proximity with E3 ligases to induce degradation of the target (Toure & Crews, 2016). Already, this has been demonstrated to utilize $CRL2^{VHL}$ and $CRL4^{CRBN}$ to induce the degradation of the epigenetic regulators BRD2, BRD3, BRD4 and ERRα both *in vitro* and *in vivo* (Winter *et al.*, 2015; Zengerle *et al.*, 2015). This provides great potential for future therapeutics and target validation as

PROTACs could be used to hijack the E3 ligases of NF-κB, or potential E3 ligases, and either degrade the subunits in a directed manner to control transcription, or to discover the identity of more NF-κB E3 ligases, in particular those which target p50. From an inflammatory disease perspective, stabilising repressive p50 homodimers through the manipulation of the ubiquitin proteasome system remains a promising avenue for the development of novel therapeutics.

Chapter 7

Appendix

7.1 *Socs1*$^{-/-}$ RAW 264.7 macrophage validation

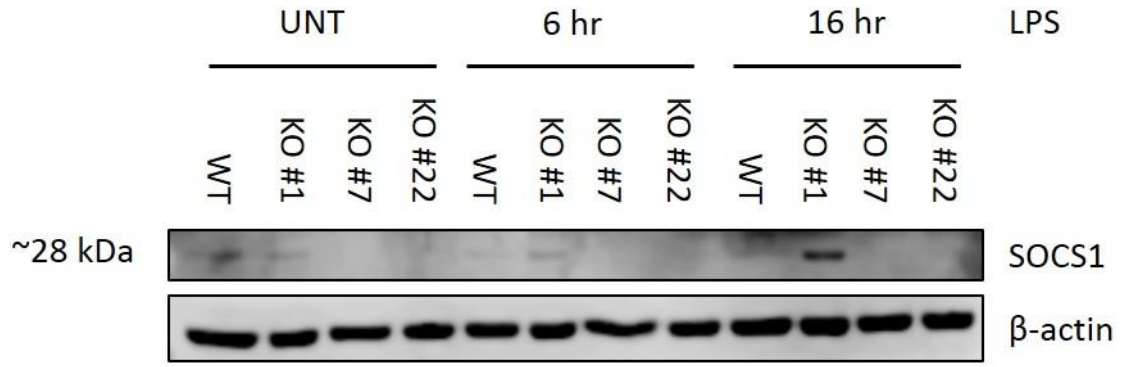


Figure 7.1: *Socs1*$^{-/-}$ RAW 264.7 macrophage validation
WT and *Socs1*$^{-/-}$ clones were left either untreated or treated with 100 ng/mL LPS for 6 or 16 hours. Whole cell lysate was harvested and expression levels of Socs1 was measured via Western blot using anti-SOCS1 antibody.

References

Agbulut, O. *et al.* (2006) 'GFP expression in muscle cells impairs actin-myosin interactions: implications for cell therapy', *Nature Methods*. Nature Publishing Group, 3(5), pp. 331–331. doi: 10.1038/nmeth0506-331.

Akimov, V. *et al.* (2018) 'UbiSite approach for comprehensive mapping of lysine and N-terminal ubiquitination sites', *Nature Structural & Molecular Biology*. Nature Publishing Group, p. 1. doi: 10.1038/s41594-018-0084-y.

Al-Hakim, A. K. *et al.* (2008) 'Control of AMPK-related kinases by USP9X and atypical Lys(29)/Lys(33)-linked polyubiquitin chains.', *The Biochemical journal*. Portland Press Limited, 411(2), pp. 249–60. doi: 10.1042/BJ20080067.

Alcamo Hacohen, N., Schulte, L.C., Rennert, P.D., Hynes, R.O., Baltimore, D., E. (2002) 'Requirement for the NF-κB family member RelA in the development of secondary lymphoid organs', *Journal of Experimental Medicine*, 195(2), pp. 233–244.

Alcamo, E. *et al.* (2001) 'Targeted Mutation of TNF Receptor I Rescues the RelA-Deficient Mouse and Reveals a Critical Role for NF- B in Leukocyte Recruitment', *The Journal of Immunology*, 167(3), pp. 1592–1600. doi: 10.4049/jimmunol.167.3.1592.

Alexander, W. S. *et al.* (1999) 'SOCS1 Is a Critical Inhibitor of Interferon γ Signaling and Prevents the Potentially Fatal Neonatal Actions of this Cytokine', *Cell*. Cell Press, 98(5), pp. 597–608. doi: 10.1016/S0092-8674(00)80047-1.

Alexander, W. S. (2002) 'Suppressors of cytokine signalling (SOCS) in the immune system', *Nature Reviews Immunology*, 2(6), pp. 410–416. doi: 10.1038/nri818.

Alves, B. N. *et al.* (2014) 'IκBε is a key regulator of B cell expansion by providing negative feedback on cRel and RelA in a stimulus-specific manner.', *Journal of immunology (Baltimore, Md. : 1950)*. NIH Public Access, 192(7), pp. 3121–32. doi: 10.4049/jimmunol.1302351.

Anders, S., Pyl, P. T. and Huber, W. (2015) 'HTSeq--a Python framework to work with high-throughput sequencing data', *Bioinformatics*. Narnia, 31(2), pp. 166–169. doi: 10.1093/bioinformatics/btu638.

Arkan Hevener, A.L., Greten, F.R, Maeda, S., Li, Z., Long, J.M., Wynshaw-Boris, A., Poli, G., Olefsky, J., Karin M., M. C. (2005) 'IKKβ links inflammation to obesity-induced insulin resistance', *Nature Medicine*, 11(2), pp. 191–198.

Artis, D. *et al.* (2005) 'Dendritic cell-intrinsic expression of NF-kappa B1 is required to promote optimal Th2 cell differentiation.', *Journal of immunology (Baltimore, Md. : 1950)*, 174(11), pp. 7154–7159. doi: 174/11/7154 [pii].

Ashall, L. *et al.* (2009) 'Pulsatile stimulation determines timing and specificity of NF-kappaB-dependent transcription', *Science*, 324(5924), pp. 242–246. doi: 10.1126/science.1164860.

Baens, M. *et al.* (2006) 'The dark side of EGFP: Defective polyubiquitination', *PLoS ONE*. Public Library of Science, 1(1), p. e54. doi: 10.1371/journal.pone.0000054.

Baetz, A. *et al.* (2008) 'Identification of a nuclear localization signal in suppressor of cytokine signaling 1', *The FASEB Journal*. Federation of American Societies for Experimental Biology, 22(12), pp. 4296–4305. doi: 10.1096/fj.08-116079.

Baeuerle Baltimore, D., P. A. (1988) 'Activation of DNA-binding activity in an apparently cytoplasmic precursor of the NF-κB transcription factor', *Cell*, 53, pp. 211–217.

Baker, R. and Ghosh, S. (2010) 'Direct activation of protein kinases by ubiquitin', *J Mol Cell Biol*, 2(1), pp. 20–22. doi: 10.1093/jmcb/mjp029.

Baldwin, A. S. and Jr. (2001) 'Series introduction: the transcription factor NF-kappaB and human disease.', *The Journal of clinical investigation*. American Society for Clinical Investigation, 107(1), pp. 3–6. doi: 10.1172/JCI11891.

Baltimore, D. (2011) ‘NF-kappaB is 25’, *Nat Immunol*, 12(8), pp. 683–685. doi: 10.1038/ni.2072.

Banerjee, A. *et al.* (2006) ‘Diverse Toll-like receptors utilize Tpl2 to activate extracellular signal-regulated kinase (ERK) in hemopoietic cells’, *Proc Natl Acad Sci U S A*, 103(9), pp. 3274–3279. doi: 10.1073/pnas.0511113103.

Banerjee, D., Liou, H. C. and Sen, R. (2005) ‘c-Rel-dependent priming of naive T cells by inflammatory cytokines’, *Immunity*, 23(4), pp. 445–458. doi: 10.1016/j.immuni.2005.09.012.

Baranes-Bachar, K. *et al.* (2018) ‘The Ubiquitin E3/E4 Ligase UBE4A Adjusts Protein Ubiquitylation and Accumulation at Sites of DNA Damage, Facilitating Double-Strand Break Repair’, *Molecular Cell*. Cell Press, 69(5), pp. 866-878.e7. doi: 10.1016/J.MOLCEL.2018.02.002.

Beg Baltimore, D., A. A. (1996) ‘An essential role for NF-κB in preventing TFNα-induced cell death’, *Science*. 1/11/1996, 274, pp. 782–784.

Beg Sha, W.C., Bronson, R.T., Baltimore, D., A. A. (1995) ‘Constitutive NF-κB activation, enhanced grunulopoiesis, and neonatal lethality in IκBα-deficient mice’, *Genes Dev*, 9, pp. 2736–2746.

Beg, A. A. *et al.* (1992) ‘I kappa B interacts with the nuclear localization sequences of the subunits of NF-kappa B: a mechanism for cytoplasmic retention.’, *Genes & development*, 6(10), pp. 1899–913.

Beinke Ley, S.C., S. (2004) ‘Functions of NF-κB1 and NF-κB2 in immune cell biology’, *Biochem J*, 382, pp. 393–409.

Berndsen, C. E. and Wolberger, C. (2014) ‘New insights into ubiquitin E3 ligase mechanism’, 21(4). doi: 10.1038/nsmb.2780.

Bonizzi Bebien, M., Otero, D.C., Johnson-Vroom, K.E., Cao, Y., Vu, D., Jegga, A.G., Aronow, B.J., Ghosh, G., Rickert, R.C., Karin, M., G. (2004) ‘Activation of IKKα target genes depends on recognition of specific κB binding sites by RelB:p52

dimers', *EMBO*, 23, pp. 4202–4210. doi: 10.1038/.

Bonizzi, G. and Karin, M. (2004) 'The two NF-kappaB activation pathways and their role in innate and adaptive immunity', *Trends Immunol*, 25(6), pp. 280–288. doi: 10.1016/j.it.2004.03.008.

Bosisio, D. *et al.* (2006) 'A hyper-dynamic equilibrium between promoter-bound and nucleoplasmic dimers controls NF-kappaB-dependent gene activity.', *The EMBO journal*. European Molecular Biology Organization, 25(4), pp. 798–810. doi: 10.1038/sj.emboj.7600977.

Boztug, H. *et al.* (2016) 'NF-kappaB1 Haploinsufficiency Causing Immunodeficiency and EBV-Driven Lymphoproliferation', *J Clin Immunol*, 36(6), pp. 533–540. doi: 10.1007/s10875-016-0306-1.

Bremm, A. *et al.* (2014) 'Cezanne (OTUD7B) regulates HIF-1α homeostasis in a proteasome-independent manner.', *EMBO reports*. European Molecular Biology Organization, 15(12), pp. 1268–77. doi: 10.15252/embr.201438850.

Bremm, A. and Komander, D. (2011) 'Emerging roles for Lys11-linked polyubiquitin in cellular regulation', 36(7).

Bren, G. D. *et al.* (2001) 'Transcription of the RelB gene is regulated by NF-κB', *Oncogene*. Nature Publishing Group, 20(53), pp. 7722–7733. doi: 10.1038/sj.onc.1204868.

Bressler, P. *et al.* (1993) 'Mutational analysis of the p50 subunit of NF-kappa B and inhibition of NF-kappa B activity by trans-dominant p50 mutants.', *Journal of virology*. American Society for Microbiology (ASM), 67(1), pp. 288–93.

Brown, K. *et al.* (1995) 'Control of I kappa B-alpha proteolysis by site-specific, signal-induced phosphorylation.', *Science (New York, N.Y.)*, 267(5203), pp. 1485–8.

Brzovic, P. S. *et al.* (2001) 'Structure of a BRCA1-BARD1 heterodimeric RING-RING complex.', *Nature Structural Biology*, 8(10), pp. 833–837. doi:

10.1038/nsb1001-833.

Buetow, L. and Huang, D. T. (2016) ‘Structural insights into the catalysis and regulation of E3 ubiquitin ligases’, *Nature Reviews Molecular Cell Biology*. Nature Publishing Group, 17(10), pp. 626–642. doi: 10.1038/nrm.2016.91.

Burkly Hession, C., Ogata, L., Reilly, C., Marconi, L.A., Olson, D., Tizard, R., Cate, R., Lo, D., L. (1995) ‘Expression of relB is required for the development of thymic medulla and dendritic cells’, *Nature*, 373, pp. 531–536.

Caamano Rizzo, C.A., Durham, S.K., Barton, D.S., Raventos-Suarez, C., Snapper, C.M., Bravo, R., J. (1998) ‘Nuclear factor (NF)-κB (p100/p52) is required for normal splenic microarcitechture and B cell-mediated immune responses’, *Journal of Experimental Medicine*, 187(2), pp. 185–196.

Caamano, J. *et al.* (2000) ‘Identification of a Role for NF- B2 in the Regulation of Apoptosis and in Maintenance of T Cell-Mediated Immunity to Toxoplasma gondii’, *The Journal of Immunology*, 165(10), pp. 5720–5728. doi: 10.4049/jimmunol.165.10.5720.

Campos, E. I. *et al.* (2004) ‘Biological functions of the ING family tumor suppressors’, *Cellular and Molecular Life Sciences*. Birkhäuser-Verlag, 61(19–20), pp. 2597–2613. doi: 10.1007/s00018-004-4199-4.

Cao Bonizzi, G., Seagroves, T.N., Greten, F.R., Johnson, R., Schmidt, E.V., Karin, M., Y. (2001) ‘IKKα provides an essential link between RANK signalling and cyclin D1 expression during mammary gland development’, *Cell*, 107, pp. 763–775.

Capili, A. D. *et al.* (2001) ‘Solution structure of the PHD domain from the KAP-1 corepressor: structural determinants for PHD, RING and LIM zinc-binding domains.’, *The EMBO journal*. European Molecular Biology Organization, 20(1–2), pp. 165–77. doi: 10.1093/emboj/20.1.165.

Cariappa Liou, H., Horwitz, B.H., Pillai, S., A. (2000) ‘Nuclear factor κB is required for the development of marginal zone B lymphocytes’, *Journal of*

Experimental Medicine, 192(8), pp. 1175–1182.

Carmody Chen, Y.H., R. J. (2007) 'Nuclear factor-κB: ACtivation and regulation during toll-like recptor signalling', *Cellular and Molecular Immunology*, 4(1), pp. 31–41.

Carmody Ruan, Q., Palmer, S., Hilliard, B., Chen, Y.H., R. J. (2007) 'Negative regulation of Toll-like receptor signalling by NF-κB p50 ubiquitination blockade', *Science*, 317, pp. 675–678.

Cartwright, T., Perkins, N. D. and L. Wilson, C. (2016) 'NFKB1: a suppressor of inflammation, ageing and cancer', *The FEBS Journal*. John Wiley & Sons, Ltd (10.1111), 283(10), pp. 1812–1822. doi: 10.1111/febs.13627.

Chang, M. *et al.* (2011) 'The ubiquitin ligase Peli1 negatively regulates T cell activation and prevents autoimmunity', *Nature Immunology*, 12(10), pp. 1002–1009. doi: 10.1038/ni.2090.

Chastagner, P., Israël, A. and Brou, C. (2006) 'Itch/AIP4 mediates Deltex degradation through the formation of K29-linked polyubiquitin chains', *EMBO reports*. EMBO Press, 7(11), pp. 1147–1153. doi: 10.1038/sj.embor.7400822.

Chau, V. *et al.* (1989) 'A multiubiquitin chain is confined to specific lysine in a targeted short-lived protein.', *Science (New York, N.Y.)*. American Association for the Advancement of Science, 243(4898), pp. 1576–83. doi: 10.1126/SCIENCE.2538923.

Chen, E. Y. *et al.* (2013) 'Enrichr: interactive and collaborative HTML5 gene list enrichment analysis tool.', *BMC bioinformatics*. BioMed Central, 14, p. 128. doi: 10.1186/1471-2105-14-128.

Chen, X. *et al.* (2005) 'Histone dynamics on the interleukin-2 gene in response to T-cell activation', *Mol Cell Biol*, 25(8), pp. 3209–3219. doi: 10.1128/MCB.25.8.3209-3219.2005.

Cheng Ryseck, R., Attar, R.M., Dambach, D., Bravo, R., J. D. (1998) 'Functional

redundancy of the nuclear factor κB inhibitors IκBα and IκBβ', *Journal of Experimental Medicine*, 188(6), pp. 1055–1062.

Cheng, C. S. *et al.* (2011) 'The specificity of innate immune responses is enforced by repression of interferon response elements by NF-κB p50', *Science Signaling*. American Association for the Advancement of Science, 4(161), pp. ra11–ra11. doi: 10.1126/scisignal.2001501.

Cheng, S. *et al.* (2003) 'Cyclin E and Bcl-xL cooperatively induce cell cycle progression in c-Rel-/- B cells', *Oncogene*, 22(52), pp. 8472–8486. doi: 10.1038/sj.onc.1206917.

Cho, S. K. *et al.* (2003) 'Functional characterization of B lymphocytes generated in vitro from embryonic stem cells', *PNAS*. National Academy of Sciences, 96(17), pp. 9797–9802. doi: 10.1073/pnas.96.17.9797.

Christian, F., Smith, E. L. and Carmody, R. J. (2016) 'The Regulation of NF-kappaB Subunits by Phosphorylation', *Cells*, 5(1). doi: 10.3390/cells5010012.

Clague, M. J. and Urbé, S. (2010) 'Ubiquitin: same molecule, different degradation pathways.', *Cell*. Elsevier, 143(5), pp. 682–5. doi: 10.1016/j.cell.2010.11.012.

Clague, M. J., Urbé, S. and Komander, D. (2019) 'Breaking the chains: deubiquitylating enzyme specificity begets function', *Nature Reviews Molecular Cell Biology*. Nature Publishing Group, p. 1. doi: 10.1038/s41580-019-0099-1.

Claudio, E. *et al.* (2002) 'BAFF-induced NEMO-independent processing of NF-kappa B2 in maturing B cells', *Nat Immunol*, 3(10), pp. 958–965. doi: 10.1038/ni842.

Cohen, S., Achbert-Weiner, H. and Ciechanover, A. (2004) 'Dual effects of IkappaB kinase beta-mediated phosphorylation on p105 Fate: SCF(beta-TrCP)-dependent degradation and SCF(beta-TrCP)-independent processing.', *Molecular and cellular biology*. American Society for Microbiology Journals, 24(1), pp. 475–86. doi: 10.1128/MCB.24.1.475-486.2004.

Coles, A. H. *et al.* (2010) 'Inhibitor of growth-4 promotes IkappaB promoter activation to suppress NF-kappaB signaling and innate immunity.', *Proceedings of the National Academy of Sciences of the United States of America*, 107(25), pp. 11423–8. doi: 10.1073/pnas.0912116107.

Coles, A. H. and Jones, S. N. (2009) 'The ING gene family in the regulation of cell growth and tumorigenesis.', *Journal of cellular physiology*. NIH Public Access, 218(1), pp. 45–57. doi: 10.1002/jcp.21583.

Colleran Collins, P.E., O'Carroll, C., Ahmed, A., Mao, X., McManus, B., Kiely, P.A., Burstein, E., Carmody, R.J., A. (2013) 'Deubiquitinase of NF-κB by ubiquitin-specific protease-7 promotes transcription', *Proc Natl Acad Sci U S A*, 110(2), pp. 618–623.

Collins, P. E. *et al.* (2015) 'Mapping the Interaction of B Cell Leukemia 3 (BCL-3) and Nuclear Factor kappaB (NF-kappaB) p50 Identifies a BCL-3-mimetic Anti-inflammatory Peptide', *J Biol Chem*, 290(25), pp. 15687–15696. doi: 10.1074/jbc.M115.643700.

Collins, P. E., Kiely, P. A. and Carmody, R. J. (2014) 'Inhibition of transcription by B cell Leukemia 3 (Bcl-3) protein requires interaction with nuclear factor kappaB (NF-kappaB) p50', *J Biol Chem*, 289(10), pp. 7059–7067. doi: 10.1074/jbc.M114.551986.

Collins, P. E., Mitxitorena, I. and Carmody, R. J. (2016) 'The Ubiquitination of NF-kappaB Subunits in the Control of Transcription', *Cells*, 5(2). doi: 10.3390/cells5020023.

Corn, R. A. *et al.* (2005) 'Opposing Roles for RelB and Bcl-3 in Regulation of T-Box Expressed in T Cells, GATA-3, and Th Effector Differentiation', *The Journal of Immunology*, 175(4), pp. 2102–2110. doi: 10.4049/jimmunol.175.4.2102.

Courtois, G. and Gilmore, T. D. (2006) 'Mutations in the NF-kappaB signaling pathway: implications for human disease', *Oncogene*, 25(51), pp. 6831–6843. doi: 10.1038/sj.onc.1209939.

Das, J. *et al.* (2001) 'A critical role for NF-kappa B in GATA3 expression and TH2 differentiation in allergic airway inflammation.', *Nature immunology*, 2(1), pp. 45–50. doi: 10.1038/83158.

Davey, G. M., Heath, W. R. and Starr, R. (2006) 'SOCS1: a potent and multifaceted regulator of cytokines and cell-mediated inflammation', *Tissue Antigens*. John Wiley & Sons, Ltd (10.1111), 67(1), pp. 1–9. doi: 10.1111/j.1399-0039.2005.00532.x.

DiDonato, J. A., Mercurio, F. and Karin, M. (2012) 'NF-κB and the link between inflammation and cancer', *Immunological Reviews*. John Wiley & Sons, Ltd (10.1111), 246(1), pp. 379–400. doi: 10.1111/j.1600-065X.2012.01099.x.

Dikic, I., Wakatsuki, S. and Walters, K. J. (2009) 'Ubiquitin-binding domains — from structures to functions', *Nature Reviews Molecular Cell Biology*. Nature Publishing Group, 10(10), pp. 659–671. doi: 10.1038/nrm2767.

Döffinger, R. *et al.* (2001) 'X-linked anhidrotic ectodermal dysplasia with immunodeficiency is caused by impaired NF-kappaB signaling.', *Nature Genetics*, 27(3), pp. 277–285. doi: 10.1038/85837.

Doi Marino, M.W., Takahashi, T., Yoshida, T., Sakakura, T., Old, L.J., Obata, Y., T. S. (1999) 'Absence of tumor necrosis factor rescues RelA-deficient mice from embryonic lethality', *Proc Natl Acad Sci U S A*, 96, pp. 2994–2999.

Dove, K. K. and Klevit, R. E. (2017) 'RING-Between-RING E3 Ligases: Emerging Themes amid the Variations.', *Journal of molecular biology*. NIH Public Access, 429(22), pp. 3363–3375. doi: 10.1016/j.jmb.2017.08.008.

Doyon, Y. *et al.* (2006) 'ING Tumor Suppressor Proteins Are Critical Regulators of Chromatin Acetylation Required for Genome Expression and Perpetuation', *Molecular Cell*. Cell Press, 21(1), pp. 51–64. doi: 10.1016/J.MOLCEL.2005.12.007.

Dutta, J. *et al.* (2006) 'Current insights into the regulation of programmed cell death by NF-kappaB', *Oncogene*, 25(51), pp. 6800–6816. doi:

10.1038/sj.onc.1209938.

Ea, C. K. and Baltimore, D. (2009) ‘Regulation of NF-kappaB activity through lysine monomethylation of p65’, *Proc Natl Acad Sci U S A*, 106(45), pp. 18972–18977. doi: 10.1073/pnas.0910439106.

Eblen, S. T. *et al.* (2003) ‘Identification of novel ERK2 substrates through use of an engineered kinase and ATP analogs.’, *The Journal of biological chemistry*. American Society for Biochemistry and Molecular Biology, 278(17), pp. 14926–35. doi: 10.1074/jbc.M300485200.

El-Brolosy, M. A. *et al.* (2019) ‘Genetic compensation triggered by mutant mRNA degradation’, *Nature*. Nature Publishing Group, 568(7751). doi: 10.1038/s41586-019-1064-z.

Feldman, N., Rotter-Maskowitz, A. and Okun, E. (2015) ‘DAMPs as mediators of sterile inflammation in aging-related pathologies’, *Ageing Res Rev*, 24(Pt A), pp. 29–39. doi: 10.1016/j.arr.2015.01.003.

Flick, K. *et al.* (2006) ‘A ubiquitin-interacting motif protects polyubiquitinated Met4 from degradation by the 26S proteasome’, *Nature Cell Biology*. Nature Publishing Group, 8(5), pp. 509–515. doi: 10.1038/ncb1402.

Fliegauf, M. *et al.* (2015) ‘Haploinsufficiency of the NF-kappaB1 Subunit p50 in Common Variable Immunodeficiency’, *Am J Hum Genet*, 97(3), pp. 389–403. doi: 10.1016/j.ajhg.2015.07.008.

Frantsve, J. *et al.* (2001) ‘Socs-1 inhibits TEL-JAK2-mediated transformation of hematopoietic cells through inhibition of JAK2 kinase activity and induction of proteasome-mediated degradation.’, *Molecular and cellular biology*. American Society for Microbiology (ASM), 21(10), pp. 3547–57. doi: 10.1128/MCB.21.10.3547-3557.2001.

Franzoso Carlson, L., Xing, L., Poljak, L., Shore, E.W., Brown, K.D., Leonardi, A., Tran, T., Boyce, B.F., Siebenlist, U., G. (1997) ‘Requirement for NF-κB in osteoclast and B-cell development’, *Genes Dev*, 11, pp. 3482–3496.

Franzoso Carlson, L., Xing, L., Poljak, L., Shores, E.W., Epstein, S, Leonardi, A., Grinberg, A., Tran, T., Scharton-Kersten, T., Anver, M., Love, P., Brown, K., Siebenlist, U., G. (1998) 'Mice deficient in nuclear factor (NF)-κB/p52 present with defects in humoral responses, germinal centre reactions, and splenic microarcitechture', *Journal of Experimental Medicine*, 187(2), pp. 147–159.

Fujita Nolan, G.P., Liou, H., Scott, M.L., Baltimore, D., T. (1993) 'The candidate proto-oncogene bcl-3 encodes a transcriptional coactivator that activates through NF-κB p50 homodimers', *Genes Dev*, 7, pp. 1354–1363.

Gadjeva, M. *et al.* (2004) 'A Role for NF- B Subunits p50 and p65 in the Inhibition of Lipopolysaccharide-Induced Shock', *The Journal of Immunology*, 173(9), pp. 5786–5793. doi: 10.4049/jimmunol.173.9.5786.

Garkavtsev, I. *et al.* (2004) 'The candidate tumour suppressor protein ING4 regulates brain tumour growth and angiogenesis', *Nature*. Nature Publishing Group, 428(6980), pp. 328–332. doi: 10.1038/nature02329.

Gatti, M. *et al.* (2015) 'RNF168 Promotes Noncanonical K27 Ubiquitination to Signal DNA Damage', *Cell Reports*. Cell Press, 10(2), pp. 226–238. doi: 10.1016/J.CELREP.2014.12.021.

Geng, F., Wenzel, S. and Tansey, W. P. (2012) 'Ubiquitin and Proteasomes in Transcription', *Annual Review of Biochemistry*, 81(1), pp. 177–201. doi: 10.1146/annurev-biochem-052110-120012.

Geng, H. *et al.* (2009) 'Phosphorylation of NF-κB p65 at Ser468 controls its COMMD1-dependent ubiquitination and target gene-specific proteasomal elimination', *EMBO reports*. EMBO Press, 10(4), pp. 381–386. doi: 10.1038/EMBOR.2009.10.

Gerlach, B. *et al.* (2011) 'Linear ubiquitination prevents inflammation and regulates immune signalling', *Nature*. Nature Publishing Group, 471(7340), pp. 591–596. doi: 10.1038/nature09816.

Gerondakis Strasser, A., Metcalf, D., Grigoriadis, G., Scheerlinck, J.Y.,

Grumont, R.J., S. (1996) ‘Rel-deficient T cells exhibit defects in production of interleukin 3 and granulocyte-macrophage colony-stimulating factor’, *Proc Natl Acad Sci U S A*, 93, pp. 3405–3409.

Gerondakis, S. *et al*. (2006) ‘Unravelling the complexities of the NF-kappaB signalling pathway using mouse knockout and transgenic models’, *Oncogene*, 25(51), pp. 6781–6799. doi: 10.1038/sj.onc.1209944.

Gerondakis, S. and Strasser, A. (2003) ‘The role of Rel/NF-kappaB transcription factors in B lymphocyte survival.’, *Seminars in immunology*, 15(3), pp. 159–66.

Ghosh May, M.J., Kopp, E.B., S. (1998) ‘NF-κB and rel proteins: Evolutionarily conserved mediators of immune responses’, *Annu Rev Immunol*, 16, pp. 225–260.

Ghosh, S. and Dass, J. F. (2016) ‘Study of pathway cross-talk interactions with NF-kappaB leading to its activation via ubiquitination or phosphorylation: A brief review’, *Gene*, 584(1), pp. 97–109. doi: 10.1016/j.gene.2016.03.008.

Ghosh, S. and Hayden, M. S. (2008) ‘New regulators of NF-kappaB in inflammation’, *Nat Rev Immunol*, 8(11), pp. 837–848. doi: 10.1038/nri2423.

Glauser, L. *et al*. (2011) ‘Parkin promotes the ubiquitination and degradation of the mitochondrial fusion factor mitofusin 1’, *Journal of Neurochemistry*. John Wiley & Sons, Ltd (10.1111), 118(4), pp. 636–645. doi: 10.1111/j.1471-4159.2011.07318.x.

Grumont Hochrein, H., O’Keeffe, M., Gugasyan, R., White, C., Caminschi, I., Cook, W., Gerondakis, S., R. (2001) ‘c-Rel regulates interleukin 12 p70 expression in CD8+ dendritic cells by specifically inducing p35 gene transcription’, *Journal of Experimental Medicine*, 194(8), pp. 1021–1031.

Grumont Rourke, I.J., O’Reilly, L.A., Strasser, A., Miyake, K., Sha, W., Gerondakis, S., R. J. (1998) ‘B lymphocytes differentially use the Rel and nuclear factor κB1 (NF-κB1) transcription factors to regulate cell cycle progression and apoptosis in quiescent and mitogen-activated cells’, *Journal of Experimental Medicine*, 187(5), pp. 663–674.

Guerin Baron, M., Valero, R., Herrant, M., Auberger, P., Naquet, P., S. (2002) 'RelB reduces thymocyte apoptosis and regulates terminal thymocyte maturation', *European Journal of Immunology*, 32, pp. 1–9.

Han, Z. *et al.* (1998) 'AP-1 and NF-kappaB regulation in rheumatoid arthritis and murine collagen-induced arthritis', *Autoimmunity*, 28(4), pp. 197–208. doi: 10.3109/08916939808995367.

Handel McMorrow, L.B., Gravallese, E.M., M. L. (1995) 'Nuclear Factor-κB in rheumatoid synovium', *Arthritis & Rheumatism*, 38(12), pp. 1762–1770.

Hannink, M. and Temin, H. M. (1990) 'Structure and autoregulation of the c-rel promoter.', *Oncogene*, 5(12), pp. 1843–50.

Hashimoto, M. *et al.* (2009) 'Silencing of *SOCS1* in macrophages suppresses tumor development by enhancing antitumor inflammation', *Cancer Science*. John Wiley & Sons, Ltd (10.1111), 100(4), pp. 730–736. doi: 10.1111/j.1349-7006.2009.01098.x.

Hatada, E. N. *et al.* (2003) 'NF-κB1 p50 Is Required for BLyS Attenuation of Apoptosis but Dispensable for Processing of NF-κB2 p100 to p52 in Quiescent Mature B Cells', *The Journal of Immunology*. American Association of Immunologists, 171(2), pp. 761–768. doi: 10.4049/JIMMUNOL.171.2.761.

Hay-Koren, A. *et al.* (2011) 'The EDD E3 ubiquitin ligase ubiquitinates and up-regulates beta-catenin.', *Molecular biology of the cell*. American Society for Cell Biology, 22(3), pp. 399–411. doi: 10.1091/mbc.E10-05-0440.

Hayden, M. S. and Ghosh, S. (2012) 'NF-kappaB, the first quarter-century: remarkable progress and outstanding questions', *Genes Dev*, 26(3), pp. 203–234. doi: 10.1101/gad.183434.111.

Hayden, M. S. and Ghosh, S. (2014) 'Regulation of NF-kappaB by TNF family cytokines', *Semin Immunol*, 26(3), pp. 253–266. doi: 10.1016/j.smim.2014.05.004.

Heinz, S. *et al.* (2010) 'Simple Combinations of Lineage-Determining Transcription Factors Prime cis-Regulatory Elements Required for Macrophage and B Cell Identities', *Molecular Cell*. Cell Press, 38(4), pp. 576–589. doi: 10.1016/J.MOLCEL.2010.05.004.

Heissmeyer, V. *et al.* (2001) 'Shared pathways of IkappaB kinase-induced SCF(betaTrCP)-mediated ubiquitination and degradation for the NF-kappaB precursor p105 and IkappaBalpha.', *Molecular and cellular biology*. American Society for Microbiology (ASM), 21(4), pp. 1024–35. doi: 10.1128/MCB.21.4.1024-1035.2001.

Herndon, T. M. *et al.* (2013) 'U.s. Food and Drug Administration approval: carfilzomib for the treatment of multiple myeloma', *Clin Cancer Res*, 19(17), pp. 4559–4563. doi: 10.1158/1078-0432.CCR-13-0755.

Herrington, F. D., Carmody, R. J. and Goodyear, C. S. (2016) 'Modulation of NF-kappaB Signaling as a Therapeutic Target in Autoimmunity', *J Biomol Screen*, 21(3), pp. 223–242. doi: 10.1177/1087057115617456.

Hilliard, B. A. *et al.* (2002) 'Critical roles of c-Rel in autoimmune inflammation and helper T cell differentiation', *Journal of Clinical Investigation*, 110(6), pp. 843–850. doi: 10.1172/jci0215254.

Hilton, D. J. *et al.* (1998) 'Twenty proteins containing a C-terminal SOCS box form five structural classes.', *Proceedings of the National Academy of Sciences of the United States of America*, 95(1), pp. 114–9.

Hjerpe, R. *et al.* (2009) 'Efficient protection and isolation of ubiquitylated proteins using tandem ubiquitin-binding entities.', *EMBO reports*. European Molecular Biology Organization, 10(11), pp. 1250–8. doi: 10.1038/embor.2009.192.

Hochrainer, K. *et al.* (2012) 'Monoubiquitination of nuclear RelA negatively regulates NF-kappaB activity independent of proteasomal degradation', *Cell Mol Life Sci*, 69(12), pp. 2057–2073. doi: 10.1007/s00018-011-0912-2.

Hoppe, T. *et al*. (2000) 'Activation of a Membrane-Bound Transcription Factor by Regulated Ubiquitin/Proteasome-Dependent Processing', *Cell*. Cell Press, 102(5), pp. 577–586. doi: 10.1016/S0092-8674(00)00080-5.

Hoppe, T. (2005) 'Multiubiquitylation by E4 enzymes: "one size" doesn't fit all', *Trends in Biochemical Sciences*. Elsevier Current Trends, 30(4), pp. 183–187. doi: 10.1016/J.TIBS.2005.02.004.

Horwitz Scott, M. L., Cherry, S. R., Bronson, R. T., Baltimore, D., B. H. (1997) 'Failure of lymphopoiesis after adoptive transfer of NF-κB-deficient fetal liver cells', *Immunity*, 6, pp. 765–772.

Horwitz Zelazowski, P., Shen, Y., Wolcott, K. M., Scott, M. L., Baltimore, D., Snapper, C. M., B. H. (1999) 'The p65 subunit of NF-κB is redundant with p50 during B cell proliferative responses, and is required for germline Ch transcription and class switching to IgG3', *Journal of Immunology*, 162, pp. 1941–1946.

Hou, Y. *et al*. (2014) 'Inhibitor of growth 4 induces NFκB/p65 ubiquitin-dependent degradation', *Oncogene*. Nature Publishing Group, 33(15), pp. 1997–2003. doi: 10.1038/onc.2013.135.

Hou, Y., Moreau, F. and Chadee, K. (2012) 'PPARgamma is an E3 ligase that induces the degradation of NFkappaB/p65', *Nat Commun*, 3, p. 1300. doi: 10.1038/ncomms2270.

Huang, B. *et al*. (2010) 'Posttranslational modifications of NF-kappaB: another layer of regulation for NF-kappaB signaling pathway.', *Cellular signalling*. NIH Public Access, 22(9), pp. 1282–90. doi: 10.1016/j.cellsig.2010.03.017.

Huang, H. *et al*. (2010) 'K33-Linked Polyubiquitination of T Cell Receptor-ζ Regulates Proteolysis-Independent T Cell Signaling', *Immunity*. Cell Press, 33(1), pp. 60–70. doi: 10.1016/J.IMMUNI.2010.07.002.

Hubeau, M. *et al*. (2011) 'New mechanism of X-linked anhidrotic ectodermal dysplasia with immunodeficiency: impairment of ubiquitin binding despite

normal folding of NEMO protein', *Blood*, 118(4), pp. 926–935. doi: 10.1182/blood-2010-10-315234.

Husnjak, K. and Dikic, I. (2012) 'Ubiquitin-Binding Proteins: Decoders of Ubiquitin-Mediated Cellular Functions', *Annual Review of Biochemistry*. Annual Reviews , 81(1), pp. 291–322. doi: 10.1146/annurev-biochem-051810-094654.

Ishikawa Carrasco, D., Claudio, E., Ryseck, R., Bravo, R., H. (1997) 'Gastric hyperplasia and increased proliferative responses of lymphocytes in mice lacking the COOH-terminal ankyrin domain of NF-κB2', *Journal of Experimental Medicine*, 186(7), pp. 999–1014.

Ishikawa Claudio, E., Dambach, D., Raventos-Suarez, C., Ryan, C., Bravo, R., H. (1998) 'Chronic inflammation and susceptibility to bacterial infections in mice lacking the polypeptide (p)105 precursor (NF-κB1) but expressing p50', *Journal of Experimental Medicine*, 187(7), pp. 985–996.

Kaiser, P. *et al.* (2000) 'Regulation of Transcription by Ubiquitination without Proteolysis: Cdc34/SCFMet30-Mediated Inactivation of the Transcription Factor Met4', *Cell*. Cell Press, 102(3), pp. 303–314. doi: 10.1016/S0092-8674(00)00036-2.

Kanarek, N. and Ben-Neriah, Y. (2012) 'Regulation of NF-κB by ubiquitination and degradation of the IκBs', *Immunological Reviews*. John Wiley & Sons, Ltd (10.1111), 246(1), pp. 77–94. doi: 10.1111/j.1600-065X.2012.01098.x.

Karin Lin, M., M. (2002) 'NF-κB at the crossroads of life and death', *Nat Immunol*, 3(3), pp. 221–227.

Kinjyo, I. *et al.* (2002) 'SOCS1/JAB Is a Negative Regulator of LPS-Induced Macrophage Activation', *Immunity*. Cell Press, 17(5), pp. 583–591. doi: 10.1016/S1074-7613(02)00446-6.

Klement Rice, N. R., Car, B. D., Abbondanzo, S. J., Powers, G. D., Bhatt, H., Chen, C., Rosen, C. A., Stewart, C. L., J. F. (1996) 'IκBa deficiency results in a sustained NF-κB response and severe widespread dermatitis in mice', *Mol Cell*,

16(5), pp. 2341–2349.

Komada, M. (2008) ‘Controlling Receptor Downregulation by Ubiquitination and Deubiquitination’, *Current Drug Discovery Technologies*, 5(1), pp. 78–84. doi: 10.2174/157016308783769469.

Komander, D. and Rape, M. (2012) ‘The Ubiquitin Code’, *Annual Review of Biochemistry*. Annual Reviews, 81(1), pp. 203–229. doi: 10.1146/annurev-biochem-060310-170328.

Kravtsova-Ivantsiv, Y. *et al.* (2015) ‘KPC1-Mediated Ubiquitination and Proteasomal Processing of NF-κB1 p105 to p50 Restricts Tumor Growth’, *Cell*. Elsevier, 161(2), pp. 333–347. doi: 10.1016/j.cell.2015.03.001.

Kuleshov, M. V *et al.* (2016) ‘Enrichr: a comprehensive gene set enrichment analysis web server 2016 update.’, *Nucleic acids research*. Oxford University Press, 44(W1), pp. W90-7. doi: 10.1093/nar/gkw377.

Kumar, A. *et al.* (2004) ‘Nuclear factor-?B: its role in health and disease’, *Journal of Molecular Medicine*. Springer-Verlag, 82(7), pp. 434–448. doi: 10.1007/s00109-004-0555-y.

Lawrence, T. *et al.* (2005) ‘IKKα limits macrophage NF-κB activation and contributes to the resolution of inflammation’, *Nature*. Nature Publishing Group, 434(7037), pp. 1138–1143. doi: 10.1038/nature03491.

Leidner, J. *et al.* (2008) ‘Identification of lysine residues critical for the transcriptional activity and polyubiquitination of the NF-kappaB family member RelB.’, *The Biochemical journal*. Portland Press Limited, 416(1), pp. 117–27. doi: 10.1042/BJ20080432.

Levenson, J. M. *et al.* (2004) ‘A bioinformatics analysis of memory consolidation reveals involvement of the transcription factor c-rel’, *J Neurosci*, 24(16), pp. 3933–3943. doi: 10.1523/JNEUROSCI.5646-03.2004.

Li, H. *et al.* (2012) ‘Regulation of NF-kappaB activity by competition between

RelA acetylation and ubiquitination', *Oncogene*, 31(5), pp. 611–623. doi: 10.1038/onc.2011.253.

Li, M. *et al.* (2001) 'The Rela(p65) subunit of NF-kappaB is essential for inhibiting double-stranded RNA-induced cytotoxicity', *J Biol Chem*, 276(2), pp. 1185–1194. doi: 10.1074/jbc.M006647200.

Li, Q. *et al.* (2005) 'Enhanced NF-kappaB activation and cellular function in macrophages lacking IkappaB kinase 1 (IKK1)', *Proc Natl Acad Sci U S A*, 102(35), pp. 12425–12430. doi: 10.1073/pnas.0505997102.

Li, W. *et al.* (2008) 'Genome-Wide and Functional Annotation of Human E3 Ubiquitin Ligases Identifies MULAN, a Mitochondrial E3 that Regulates the Organelle's Dynamics and Signaling', *PLoS ONE*. Edited by H. Ploegh, 3(1), p. e1487. doi: 10.1371/journal.pone.0001487.

Liau, N. P. D. *et al.* (2018) 'The molecular basis of JAK/STAT inhibition by SOCS1', *Nature Communications*. Nature Publishing Group, 9(1), p. 1558. doi: 10.1038/s41467-018-04013-1.

Lin, L. and Kobayashi, M. (2003) 'Stability of the Rel homology domain is critical for generation of NF-kappa B p50 subunit.', *The Journal of biological chemistry*. American Society for Biochemistry and Molecular Biology, 278(34), pp. 31479–85. doi: 10.1074/jbc.M304140200.

Liptay, S. *et al.* (1994) 'Transcriptional regulation of NF-kappa B2: evidence for kappa B-mediated positive and negative autoregulation.', *Molecular and cellular biology*. American Society for Microbiology (ASM), 14(12), pp. 7695–703.

Liu, H.-S. *et al.* (1999) 'Is Green Fluorescent Protein Toxic to the Living Cells?', *Biochemical and Biophysical Research Communications*. Academic Press, 260(3), pp. 712–717. doi: 10.1006/BBRC.1999.0954.

Liu, P. *et al.* (2018) 'K63-linked polyubiquitin chains bind to DNA to facilitate DNA damage repair.', *Science signaling*. American Association for the Advancement of Science, 11(533), p. eaar8133. doi: 10.1126/scisignal.aar8133.

Livak, K. J. and Schmittgen, T. D. (2001) 'Analysis of Relative Gene Expression Data Using Real-Time Quantitative PCR and the 2−ΔΔCT Method', *Methods*. Academic Press, 25(4), pp. 402–408. doi: 10.1006/METH.2001.1262.

Lo, J. C. *et al.* (2006) 'Coordination between NF-κB family members p50 and p52 is essential for mediating LTβR signals in the development and organization of secondary lymphoid tissues', *Blood*. American Society of Hematology, 107(3), pp. 1048–1055. doi: 10.1182/BLOOD-2005-06-2452.

Love, M. I., Huber, W. and Anders, S. (2014) 'Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2', *Genome Biology*. BioMed Central, 15(12), p. 550. doi: 10.1186/s13059-014-0550-8.

Luedde, T. *et al.* (2007) 'Deletion of NEMO/IKKγ in Liver Parenchymal Cells Causes Steatohepatitis and Hepatocellular Carcinoma', *Cancer Cell*. Cell Press, 11(2), pp. 119–132. doi: 10.1016/J.CCR.2006.12.016.

Lydeard, J. R., Schulman, B. A. and Harper, J. W. (2013) 'Building and remodelling Cullin-RING E3 ubiquitin ligases.', *EMBO reports*. European Molecular Biology Organization, 14(12), pp. 1050–61. doi: 10.1038/embor.2013.173.

Mace, P. D. *et al.* (2008) 'Structures of the cIAP2 RING Domain Reveal Conformational Changes Associated with Ubiquitin-conjugating Enzyme (E2) Recruitment', *Journal of Biological Chemistry*, 283(46), pp. 31633–31640. doi: 10.1074/jbc.M804753200.

Maine, G. N. *et al.* (2007) 'COMMD1 promotes the ubiquitination of NF-κB subunits through a cullin-containing ubiquitin ligase', *EMBO Journal*. European Molecular Biology Organization, 26(2), pp. 436–447. doi: 10.1038/sj.emboj.7601489.

Makris Godfrey, V. L., Krahn-Senftleben, G., Takahashi, T., Roberts, J. L., Schwarz, T., Feng, L., Johnson, R. S., Karin, M., C. (2000) 'Female mice heterozygous for IKKγ/NEMO deficiencies develop a dermatopathy similar to the human x-linked disorder incontinentia pigmenti', *Mol Cell*, 5, pp. 969–979.

Mason, N. J., Liou, H. C. and Hunter, C. A. (2004) 'T Cell-Intrinsic Expression of c-Rel Regulates Th1 Cell Responses Essential for Resistance to Toxoplasma gondii', *The Journal of Immunology*, 172(6), pp. 3704–3711. doi: 10.4049/jimmunol.172.6.3704.

Matsumoto, M. L. *et al.* (2010) 'K11-linked polyubiquitination in cell cycle control revealed by a K11 linkage-specific antibody.', *Molecular cell*. Elsevier, 39(3), pp. 477–84. doi: 10.1016/j.molcel.2010.07.001.

McLean, C. Y. *et al.* (2010) 'GREAT improves functional interpretation of cis-regulatory regions.', *Nature biotechnology*. NIH Public Access, 28(5), pp. 495–501. doi: 10.1038/nbt.1630.

Memet Laouini, D., Epinat, J., Whiteside, S. T., Goudeau, B., Philpott, D., Kayal, S., Sansonetti, P. J., Berche, P., Kanellopoulos, J., Israel, A., S. (1999) 'IκBe-deficient mice: Reduction of one T cell precursor subspecies and enhanced Ig isotype switching and cytokine synthesis', *Journal of Immunology*, 163, pp. 5994–6005.

Metzger, M. B. *et al.* (2014) 'RING-type E3 ligases: Master manipulators of E2 ubiquitin-conjugating enzymes and ubiquitination', *Biochimica et Biophysica Acta (BBA) - Molecular Cell Research*, 1843(1), pp. 47–60. doi: 10.1016/j.bbamcr.2013.05.026.

Meyer, H.-J. and Rape, M. (2014) 'Enhanced protein degradation by branched ubiquitin chains.', *Cell*. Elsevier, 157(4), pp. 910–21. doi: 10.1016/j.cell.2014.03.037.

Mina, R. *et al.* (2016) 'New pharmacotherapy options for multiple myeloma', *Expert Opin Pharmacother*, 17(2), pp. 181–192. doi: 10.1517/14656566.2016.1115016.

Mintern, J. D. *et al.* (2002) 'The Cross-Priming APC Requires a Rel-Dependent Signal to Induce CTL', *The Journal of Immunology*, 168(7), pp. 3283–3287. doi: 10.4049/jimmunol.168.7.3283.

Molinari, E., Gilman, M. and Natesan, S. (1999) ‘Proteasome-mediated degradation of transcriptional activators correlates with activation domain potency in vivo.’, *The EMBO journal*. EMBO Press, 18(22), pp. 6439–47. doi: 10.1093/emboj/18.22.6439.

Moreno, R. *et al.* (2010) ‘Specification of the NF-κB transcriptional response by p65 phosphorylation and TNF-induced nuclear translocation of IKKε’, *Nucleic Acids Research*. Oxford University Press, 38(18), pp. 6029–6044. doi: 10.1093/nar/gkq439.

Morris, J. R. and Solomon, E. (2004) ‘BRCA1 : BARD1 induces the formation of conjugated ubiquitin structures, dependent on K6 of ubiquitin, in cells during DNA replication and repair’, *Human Molecular Genetics*, 13(8), pp. 807–817. doi: 10.1093/hmg/ddh095.

Mühlen, S., Ruchaud-Sparagano, M.-H. and Kenny, B. (2011) ‘Proteasome-independent degradation of canonical NFkappaB complex components by the NleC protein of pathogenic Escherichia coli.’, *The Journal of biological chemistry*. American Society for Biochemistry and Molecular Biology, 286(7), pp. 5100–7. doi: 10.1074/jbc.M110.172254.

Naka, T. *et al.* (1998) ‘Accelerated apoptosis of lymphocytes by augmented induction of Bax in SSI-1 (STAT-induced STAT inhibitor-1) deficient mice.’, *Proceedings of the National Academy of Sciences of the United States of America*. National Academy of Sciences, 95(26), pp. 15577–82.

Narazaki, M. *et al.* (1998) ‘Three distinct domains of SSI-1/SOCS-1/JAB protein are required for its suppression of interleukin 6 signaling.’, *Proceedings of the National Academy of Sciences of the United States of America*. National Academy of Sciences, 95(22), pp. 13130–4.

Nelson, D. E. *et al.* (2004) ‘Oscillations in NF-kappaB signaling control the dynamics of gene expression’, *Science*, 306(5696), pp. 704–708. doi: 10.1126/science.1099962.

Nenci, A. *et al.* (2007) ‘Epithelial NEMO links innate immunity to chronic

intestinal inflammation', *Nature*. Nature Publishing Group, 446(7135), pp. 557–561. doi: 10.1038/nature05698.

Nielsen, M. L. *et al.* (2008) 'Iodoacetamide-induced artifact mimics ubiquitination in mass spectrometry', *Nature Methods*. Nature Publishing Group, 5(6), pp. 459–460. doi: 10.1038/nmeth0608-459.

Nihira, K. *et al.* (2010) 'Pim-1 controls NF-κB signalling by stabilizing RelA/p65', *Cell Death & Differentiation*. Nature Publishing Group, 17(4), pp. 689–698. doi: 10.1038/cdd.2009.174.

Nolan Fujita, T., Bhatia, K., Huppi, C., Liou, H., Scott, M. L., Baltimore, D., G. P. (1993) 'The bcl-3 proto-oncogene encodes a nuclear IκB-like molecule that preferentially interacts with NF-κB p50 and p52 in a phosphorylation-dependent manner', *Molecular and Cellular Biology*, 13(6), pp. 3557–3566.

O'Carroll, C. *et al.* (2013) 'Bcl-3 deficiency protects against dextran-sodium sulphate-induced colitis in the mouse', *Clin Exp Immunol*, 173(2), pp. 332–342. doi: 10.1111/cei.12119.

O'Keeffe, M. *et al.* (2005) 'Distinct roles for the NF-kappaB1 and c-Rel transcription factors in the differentiation and survival of plasmacytoid and conventional dendritic cells activated by TLR-9 signals', *Blood*, 106(10), pp. 3457–3464. doi: 10.1182/blood-2004-12-4965.

Oeckinghaus, A. and Ghosh, S. (2009) 'The NF-kappaB family of transcription factors and its regulation', *Cold Spring Harb Perspect Biol*, 1(4), p. a000034. doi: 10.1101/cshperspect.a000034.

Ohno Takimoto, G., McKeithan, T. W., H. (1990) 'The candidate proto-oncogene bcl-3 is related to genes implicated in cell lineage determination and cell cycle control', *Cell*, 60, pp. 991–997.

Ohtake, F. *et al.* (2016) 'The K48-K63 Branched Ubiquitin Chain Regulates NF-κB Signaling', *Molecular Cell*. Cell Press, 64(2), pp. 251–266. doi: 10.1016/J.MOLCEL.2016.09.014.

Oikonomidou, O. *et al.* (2006) 'Glucocorticoid receptor, nuclear factor kappaB, activator protein-1 and C-jun N-terminal kinase in systemic lupus erythematosus patients', *Neuroimmunomodulation*, 13(4), pp. 194–204. doi: 10.1159/000100474.

Okumura, F. *et al.* (2004) 'Functional regulation of FEZ1 by the U-box-type ubiquitin ligase E4B contributes to neuritogenesis.', *The Journal of biological chemistry*. American Society for Biochemistry and Molecular Biology, 279(51), pp. 53533–43. doi: 10.1074/jbc.M402916200.

Olivier, S. *et al.* (2006) 'Raloxifene-induced myeloma cell apoptosis: a study of nuclear factor-kappaB inhibition and gene expression signature', *Mol Pharmacol*, 69(5), pp. 1615–1623. doi: 10.1124/mol.105.020479.

Ordureau, A. *et al.* (2014) 'Quantitative proteomics reveal a feedforward mechanism for mitochondrial PARKIN translocation and ubiquitin chain synthesis.', *Molecular cell*. Elsevier, 56(3), pp. 360–75. doi: 10.1016/j.molcel.2014.09.007.

Orian Gonen, H., Bercovich, B., Fajerman, I., Eytan, E., Isreal, A., Mercurio, F., Iwai, K., Schwartz, A. L., Ciechanover, A., A. (2000) 'SCFb-TrCP ubiquitin ligase-mediated processing of NF-κB p105 requires phosphorylation of its C-terminus by IκB kinase', *EMBO*, 19(11), pp. 2580–2591.

Orian, A. *et al.* (1995) 'Ubiquitin-mediated Processing of NF-κB Transcriptional Activator Precursor p105', *Journal of Biological Chemistry*. American Society for Biochemistry and Molecular Biology, 270(37), pp. 21707–21714. doi: 10.1074/JBC.270.37.21707.

Palmer, S. and Chen, Y. H. (2008) 'Bcl-3, a multifaceted modulator of NF-κB-mediated gene transcription', *Immunologic Research*. Humana Press Inc, 42(1–3), pp. 210–218. doi: 10.1007/s12026-008-8075-4.

Palombella, V. J. *et al.* (1994) 'The ubiquitinproteasome pathway is required for processing the NF-κB1 precursor protein and the activation of NF-κB', *Cell*. Cell Press, 78(5), pp. 773–785. doi: 10.1016/S0092-8674(94)90482-0.

Pannicke, U. *et al.* (2013) 'Deficiency of innate and acquired immunity caused by an IKBKB mutation', *N Engl J Med*, 369(26), pp. 2504–2514. doi: 10.1056/NEJMoa1309199.

Pasparakis Courtois, G., Hafner, M., Schmidt-Supprian, M., Nenci, A., Toksoy, A., Krampert, M., Goebeler, M., Gillitzer, R., Israel, A., Krieg, T., Rajewsky, K., Haase, I., M. (2002) 'TNF-mediated inflammatory skin disease in mice with epidermis-specific deletion of IKK2', *Nature Letters*, 417, pp. 861–866.

Pasparakis, M., Luedde, T. and Schmidt-Supprian, M. (2006) 'Dissection of the NF-kappaB signalling cascade in transgenic and knockout mice', *Cell Death Differ*, 13(5), pp. 861–872. doi: 10.1038/sj.cdd.4401870.

Peng, D.-J. *et al.* (2011) 'Noncanonical K27-linked polyubiquitination of TIEG1 regulates Foxp3 expression and tumor growth.', *Journal of immunology (Baltimore, Md. : 1950)*. American Association of Immunologists, 186(10), pp. 5638–47. doi: 10.4049/jimmunol.1003801.

Perkins, N. D. (2006) 'Post-translational modifications regulating the activity and function of the nuclear factor kappa B pathway', *Oncogene*. Nature Publishing Group, 25(51), pp. 6717–6730. doi: 10.1038/sj.onc.1209937.

Pertea, M. *et al.* (2016) 'Transcript-level expression analysis of RNA-seq experiments with HISAT, StringTie and Ballgown', *Nature Protocols*, 11(9), pp. 1650–1667. doi: 10.1038/nprot.2016.095.

Pizzi, M. *et al.* (2002) 'Opposing roles for NF-kappa B/Rel factors p65 and c-Rel in the modulation of neuron survival elicited by glutamate and interleukin-1beta', *J Biol Chem*, 277(23), pp. 20717–20723. doi: 10.1074/jbc.M201014200.

Pohl, T. *et al.* (2002) 'The combined absence of NF-kappa B1 and c-Rel reveals that overlapping roles for these transcription factors in the B cell lineage are restricted to the activation and function of mature cells', *Proc Natl Acad Sci U S A*, 99(7), pp. 4514–4519. doi: 10.1073/pnas.072071599.

Prendes, M., Zheng, Y. and Beg, A. A. (2003) 'Regulation of Developing B Cell

Survival by RelA-Containing NF- B Complexes', *The Journal of Immunology*, 171(8), pp. 3963–3969. doi: 10.4049/jimmunol.171.8.3963.

Rao, P. *et al.* (2010) 'IκBß acts to inhibit and activate gene expression during the inflammatory response', *Nature*, 466(7310), pp. 1115–1119. doi: 10.1038/nature09283.

Rayet, B. and Gélinas, C. (1999) 'Aberrant rel/nfkb genes and activity in human cancer', *Oncogene*. Nature Publishing Group, 18(49), pp. 6938–6947. doi: 10.1038/sj.onc.1203221.

Roget, K. *et al.* (2012) 'IκB kinase 2 regulates TPL-2 activation of extracellular signal-regulated kinases 1 and 2 by direct phosphorylation of TPL-2 serine 400.', *Molecular and cellular biology*. American Society for Microbiology (ASM), 32(22), pp. 4684–90. doi: 10.1128/MCB.01065-12.

Rotin, D. and Kumar, S. (2009) 'Physiological functions of the HECT family of ubiquitin ligases', *Nature Reviews Molecular Cell Biology*, 10(6), pp. 398–409. doi: 10.1038/nrm2690.

Russell, M. *et al.* (2006) 'Grow-ING, Age-ING and Die-ING: ING proteins link cancer, senescence and apoptosis', *Experimental Cell Research*, 312(7), pp. 951–961. doi: 10.1016/j.yexcr.2006.01.020.

Russell, R. C. *et al.* (2011) 'Loss of JAK2 regulation via a heterodimeric VHL-SOCS1 E3 ubiquitin ligase underlies Chuvash polycythemia', *Nature Medicine*, 17(7), pp. 845–853. doi: 10.1038/nm.2370.

Ryo, A. *et al.* (2003) 'Regulation of NF-κB Signaling by Pin1-Dependent Prolyl Isomerization and Ubiquitin-Mediated Proteolysis of p65/RelA', *Molecular Cell*. Cell Press, 12(6), pp. 1413–1426. doi: 10.1016/S1097-2765(03)00490-8.

Saccani, S. *et al.* (2004) 'Degradation of promoter-bound p65/RelA is essential for the prompt termination of the nuclear factor kappaB response', *The Journal of Experimental Medicine*. Rockefeller University Press, 200(1), pp. 107–113. doi: 10.1084/jem.20040196.

Sakamoto, H. *et al.* (1998) 'A Janus Kinase Inhibitor, JAB, Is an Interferon-γ–Inducible Gene and Confers Resistance to Interferons', *Blood*, 92(5).

Sanjabi, S. *et al.* (2000) 'Selective requirement for c-Rel during IL-12 P40 gene induction in macrophages', *Proc Natl Acad Sci U S A*, 97(23), pp. 12705–12710. doi: 10.1073/pnas.230436397.

Scheibel, M. *et al.* (2010) 'IkappaBbeta is an essential co-activator for LPS-induced IL-1beta transcription in vivo.', *The Journal of experimental medicine*. The Rockefeller University Press, 207(12), pp. 2621–30. doi: 10.1084/jem.20100864.

Scheidereit, C (2006) 'IkappaB kinase complexes: gateways to NF-kappaB activation and transcription', *Oncogene*, 25(51), pp. 6685–6705. doi: 10.1038/sj.onc.1209934.

Scheidereit, Claus (2006) 'IκB kinase complexes: gateways to NF-κB activation and transcription', *Oncogene*. Nature Publishing Group, 25(51), pp. 6685–6705. doi: 10.1038/sj.onc.1209934.

Schlesinger, D. H., Goldstein, G. and Niall, H. D. (1975) 'Complete amino acid sequence of ubiquitin, an adenylate cyclase stimulating polypeptide probably universal in living cells', *Biochemistry*. American Chemical Society, 14(10), pp. 2214–2218. doi: 10.1021/bi00681a026.

Schmidt-Supprian, M. *et al.* (2003) 'Mature T Cells Depend on Signaling through the IKK Complex', *Immunity*. Cell Press, 19(3), pp. 377–389. doi: 10.1016/S1074-7613(03)00237-1.

Schwarz Krimpenfort, P., Berns, A., Verma, I. M., E. M. (1997) 'Immunological defects in mice with a targeted disruption in Bcl-3', *Genes Dev*, 11, pp. 187–197.

Sen, R. & Baltimore, D., R. (1986) 'Inducibility of κ immunoglobulin enhancer-binding protein NF-κB by a posttranslational mechanism', *Cell*, 47, pp. 921–928.

Senftleben Cao, Y., Xiao, G., Greten, F. R., Krahn, G., Bonizzi, G., Chen, Y.,

Hu, Y., Fong, A., Sun, S., Karin, M., U. (2001) ‘Activation by IKKa of a second, evolutionary conserved, NF-κB signalling pathway’, *Science*, 293, pp. 1495–1499.

Senftleben Li, Z., Baud, V., Karin, M., U. (2001) ‘IKKb is essential for protecting T cells from TNFa-induced apoptosis’, *Immunity*, 14, pp. 217–230.

Sharfe, N. *et al.* (2015) ‘The effects of RelB deficiency on lymphocyte development and function’, *Journal of Autoimmunity*. Academic Press, 65, pp. 90–100. doi: 10.1016/J.JAUT.2015.09.001.

Shearer, R. F. *et al.* (2015) ‘Functional Roles of the E3 Ubiquitin Ligase UBR5 in Cancer’, *Molecular Cancer Research*. American Association for Cancer Research, 13(12), pp. 1523–1532. doi: 10.1158/1541-7786.MCR-15-0383.

Shi, C. *et al.* (2016) ‘NFKB2 mutation in common variable immunodeficiency and isolated adrenocorticotropic hormone deficiency: A case report and review of literature.’, *Medicine*. Wolters Kluwer Health, 95(40), p. e5081. doi: 10.1097/MD.0000000000005081.

Shin, C. *et al.* (2017) ‘MKRN2 is a novel ubiquitin E3 ligase for the p65 subunit of NF-κB and negatively regulates inflammatory responses’, *Scientific Reports*. Nature Publishing Group, 7(1), p. 46097. doi: 10.1038/srep46097.

Smale, S. T. (2012) ‘Dimer-specific regulatory mechanisms with the NF-κB family of transcription factors’, *Immunological Reviews*, 246, pp. 193–204.

Soliman, M. A. and Riabowol, K. (2007) ‘After a decade of study-ING, a PHD for a versatile family of proteins’, *Trends in Biochemical Sciences*. Elsevier Current Trends, 32(11), pp. 509–519. doi: 10.1016/J.TIBS.2007.08.006.

Solt, L. A. *et al.* (2007) ‘Interleukin-1-induced NF-kappaB activation is NEMO-dependent but does not require IKKbeta.’, *The Journal of biological chemistry*. American Society for Biochemistry and Molecular Biology, 282(12), pp. 8724–33. doi: 10.1074/jbc.M609613200.

Souma, Y. *et al.* (2012) ‘Antiproliferative effect of SOCS-1 through the

suppression of STAT3 and p38 MAPK activation in gastric cancer cells', *International Journal of Cancer*. John Wiley & Sons, Ltd, 131(6), pp. 1287–1296. doi: 10.1002/ijc.27350.

Speirs, K. *et al.* (2002) 'NF- B2 Is Required for Optimal CD40-Induced IL-12 Production but Dispensable for Th1 Cell Differentiation', *The Journal of Immunology*, 168(9), pp. 4406–4413. doi: 10.4049/jimmunol.168.9.4406.

Starr, R. *et al.* (1998) 'Liver degeneration and lymphoid deficiencies in mice lacking suppressor of cytokine signaling-1.', *Proceedings of the National Academy of Sciences of the United States of America*. National Academy of Sciences, 95(24), pp. 14395–9.

Strasser Grumont, R. J., Stanley, M. L., Gerondakis, S., A. (1999) 'The transcriptional regulator Rel is essential for antigen receptor-mediated stimulation of mature T cells but dispensable for positive and negative selection of thymocytes and T cell apoptosis', *European Journal of Immunology*, 29, pp. 928–935.

Strebovsky, J. *et al.* (2011) 'Suppressor of cytokine signaling 1 (SOCS1) limits NF B signaling by decreasing p65 stability within the cell nucleus', *The FASEB Journal*, 25(3), pp. 863–874. doi: 10.1096/fj.10-170597.

Strebovsky, Julia *et al.* (2011) 'Suppressor of cytokine signaling 1 (SOCS1) limits NFκB signaling by decreasing p65 stability within the cell nucleus', *The FASEB Journal*. Federation of American Societies for Experimental Biology Bethesda, MD, USA, 25(3), pp. 863–874. doi: 10.1096/fj.10-170597.

Subbaiah, V. K. *et al.* (2016) 'E3 ligase EDD1/UBR5 is utilized by the HPV E6 oncogene to destabilize tumor suppressor TIP60', *Oncogene*. Nature Publishing Group, 35(16), pp. 2062–2074. doi: 10.1038/onc.2015.268.

Sun, S. C. *et al.* (1993) 'NF-kappa B controls expression of inhibitor I kappa B alpha: evidence for an inducible autoregulatory pathway', *Science*, 259(5103), pp. 1912–1915.

Takayama, K., Matsuura, A. and Itakura, E. (2017) 'Dissection of ubiquitinated protein degradation by basal autophagy', *FEBS Letters*. John Wiley & Sons, Ltd, 591(9), pp. 1199–1211. doi: 10.1002/1873-3468.12641.

Tam Lee, L. H., Davis, L., Sen, R., W. F. (2000) 'Cytoplasmic Sequestration of Rel Proteins by IkBa Requires CRM1-Dependent Nuclear Export', *Molecular and Cellular Biology*, 20(6), pp. 2269–2284.

Tanaka, T, Grusby, M. J. and Kaisho, T. (2007) 'PDLIM2-mediated termination of transcription factor NF-kappaB activation by intranuclear sequestration and degradation of the p65 subunit', *Nat Immunol*, 8(6), pp. 584–591. doi: 10.1038/ni1464.

Tanaka, Takashi, Grusby, M. J. and Kaisho, T. (2007) 'PDLIM2-mediated termination of transcription factor NF-κB activation by intranuclear sequestration and degradation of the p65 subunit', *Nature Immunology*. Nature Publishing Group, 8(6), pp. 584–591. doi: 10.1038/ni1464.

Tanaka, T., Soriano, M. A. and Grusby, M. J. (2005) 'SLIM Is a Nuclear Ubiquitin E3 Ligase that Negatively Regulates STAT Signaling', *Immunity*. Cell Press, 22(6), pp. 729–736. doi: 10.1016/J.IMMUNI.2005.04.008.

Tato, C. M. *et al.* (2006) 'Opposing roles of NF-kappaB family members in the regulation of NK cell proliferation and production of IFN-gamma', *Int Immunol*, 18(4), pp. 505–513. doi: 10.1093/intimm/dxh391.

Ten, R. M. *et al.* (1992) 'The characterization of the promoter of the gene encoding the p50 subunit of NF-kappa B indicates that it participates in its own regulation.', *The EMBO journal*. European Molecular Biology Organization, 11(1), pp. 195–203.

Tokunaga, F. *et al.* (2009) 'Involvement of linear polyubiquitylation of NEMO in NF-κB activation', *Nature Cell Biology*. Nature Publishing Group, 11(2), pp. 123–132. doi: 10.1038/ncb1821.

Toure, M. and Crews, C. M. (2016) 'Small-Molecule PROTACS: New Approaches to

Protein Degradation', *Angewandte Chemie International Edition*. John Wiley & Sons, Ltd, 55(6), pp. 1966–1973. doi: 10.1002/anie.201507978.

Trapnell, C. *et al.* (2010) 'Transcript assembly and quantification by RNA-Seq reveals unannotated transcripts and isoform switching during cell differentiation', *Nature Biotechnology*. Nature Publishing Group, 28(5), pp. 511–515. doi: 10.1038/nbt.1621.

Tsui, R. *et al.* (2015) 'IκBβ enhances the generation of the low-affinity NFκB/RelA homodimer.', *Nature communications*. NIH Public Access, 6, p. 7068. doi: 10.1038/ncomms8068.

Turner, D. A. *et al.* (2010) 'Physiological levels of TNFalpha stimulation induce stochastic dynamics of NF-kappaB responses in single living cells', *J Cell Sci*, 123(Pt 16), pp. 2834–2843. doi: 10.1242/jcs.069641.

Vijay-Kumar, S., Bugg, C. E. and Cook, W. J. (1987) 'Structure of ubiquitin refined at 1.8Åresolution', *Journal of Molecular Biology*. Academic Press, 194(3), pp. 531–544. doi: 10.1016/0022-2836(87)90679-6.

Wagner, S. A. *et al.* (2012) 'Proteomic analyses reveal divergent ubiquitylation site patterns in murine tissues.', *Molecular & cellular proteomics : MCP*. American Society for Biochemistry and Molecular Biology, 11(12), pp. 1578–85. doi: 10.1074/mcp.M112.017905.

Walczak, H., Iwai, K. and Dikic, I. (2012) 'Generation and physiological roles of linear ubiquitin chains', *BMC Biology*. BioMed Central, 10(1), p. 23. doi: 10.1186/1741-7007-10-23.

Weih Caamano, J., F. (2003) 'Regulation of secondary lymphoid organ development by the nuclear factor-kB signal transduction pathway', *Immunological Reviews*, 195, pp. 91–105.

Weih Durham, S. K., Barton, D. S., Sha, W. C., Baltimore, D., Bravo, R., F. (1997) 'p50–NFkB Complexes Partially Compensate for the Absence of RelB: Severely Increased Pathology in p50-/-relB-/- Double-knockout Mice', *Journal of*

Experimental Medicine, 185(7), pp. 1359–1370.

Weih, F. *et al.* (1995) 'Multiorgan inflammation and hematopoietic abnormalities in mice with a targeted disruption of RelB, a member of the NF-κB/Rel family', *Cell*. Cell Press, 80(2), pp. 331–340. doi: 10.1016/0092-8674(95)90416-6.

Wertz, I. E. and Dixit, V. M. (2010) 'Signaling to NF-kappaB: regulation by ubiquitination', *Cold Spring Harb Perspect Biol*, 2(3), p. a003350. doi: 10.1101/cshperspect.a003350.

Wessells, J. *et al.* (2004) 'BCL-3 and NF-kappaB p50 attenuate lipopolysaccharide-induced inflammatory responses in macrophages.', *The Journal of biological chemistry*. American Society for Biochemistry and Molecular Biology, 279(48), pp. 49995–50003. doi: 10.1074/jbc.M404246200.

Wilson, C. L. *et al.* (2015) 'NFkappaB1 is a suppressor of neutrophil-driven hepatocellular carcinoma', *Nat Commun*. Nature Publishing Group, 6(1), p. 6818. doi: 10.1038/ncomms7818.

Winter, G. E. *et al.* (2015) 'DRUG DEVELOPMENT. Phthalimide conjugation as a strategy for in vivo target protein degradation.', *Science (New York, N.Y.)*. American Association for the Advancement of Science, 348(6241), pp. 1376–81. doi: 10.1126/science.aab1433.

Wu D'Amico, A., Winkel, K. D., Suter, M., Lo, D., Shortman, K., L. (1998) 'RelB Is Essential for the Development of Myeloid-Related CD8a2 Dendritic Cells but Not of Lymphoid-Related CD8a1 Dendritic Cells', *Immunity*, 9, pp. 839–847.

Xu, G., Paige, J. S. and Jaffrey, S. R. (2010) 'Global analysis of lysine ubiquitination by ubiquitin remnant immunoaffinity profiling', *Nature Biotechnology*. Nature Publishing Group, 28(8), pp. 868–873. doi: 10.1038/nbt.1654.

Yan Carmody, R. J., Qu, Z., Ruan, Q., Jager, J., Mullican, S. E., Lazar, M. A., Chen, Y. H., Q. (2012) 'Nuclear factor-κB binding motifs specify Toll-likereceptor–induced gene repression through aninducible repressosome', *Proc*

Natl Acad Sci U S A, 109(35), pp. 14140–14145.

Yang, H.-T. *et al.* (2012) 'Coordinate regulation of TPL-2 and NF-κB signaling in macrophages by NF-κB1 p105.', *Molecular and cellular biology*. American Society for Microbiology (ASM), 32(17), pp. 3438–51. doi: 10.1128/MCB.00564-12.

Yasukawa, H. *et al.* (1999) 'The JAK-binding protein JAB inhibits Janus tyrosine kinase activity through binding in the activation loop', *The EMBO Journal*. EMBO Press, 18(5), pp. 1309–1320. doi: 10.1093/EMBOJ/18.5.1309.

Yilmaz Weih, D. S., Sivakumar, V., Weih, F., Z. B. (2003) 'RelB is required for Peyer's patch development: differential regulation of p52-RelB by lymphotoxin and TNF', *EMBO*, 22(1), pp. 121–130.

Yin, Q. *et al.* (2009) 'E2 interaction and dimerization in the crystal structure of TRAF6', *Nature Structural & Molecular Biology*, 16(6), pp. 658–666. doi: 10.1038/nsmb.1605.

Yuan, W.-C. *et al.* (2014) 'K33-Linked Polyubiquitination of Coronin 7 by Cul3-KLHL20 Ubiquitin E3 Ligase Regulates Protein Trafficking', *Molecular Cell*. Cell Press, 54(4), pp. 586–600. doi: 10.1016/J.MOLCEL.2014.03.035.

Zengerle, M., Chan, K.-H. and Ciulli, A. (2015) 'Selective Small Molecule Induced Degradation of the BET Bromodomain Protein BRD4', *ACS Chemical Biology*. American Chemical Society, 10(8), pp. 1770–1777. doi: 10.1021/acschembio.5b00216.

Zerbino, D. R. *et al.* (2015) 'The Ensembl Regulatory Build', *Genome Biology*. BioMed Central, 16(1), p. 56. doi: 10.1186/s13059-015-0621-5.

Zhang, F. *et al.* (2003) 'Green fluorescent protein selectively induces HSP70-mediated up-regulation of COX-2 expression in endothelial cells.', *Blood*. American Society of Hematology, 102(6), pp. 2115–21. doi: 10.1182/blood-2003-01-0049.

Zhang, J. G. *et al.* (2001) 'The SOCS box of suppressor of cytokine signaling-1 is

important for inhibition of cytokine action in vivo.', *Proceedings of the National Academy of Sciences of the United States of America*. National Academy of Sciences, 98(23), pp. 13261–5. doi: 10.1073/pnas.231486498.

Zhang, L. *et al*. (2013) 'Both K63 and K48 ubiquitin linkages signal lysosomal degradation of the LDL receptor.', *Journal of lipid research*. American Society for Biochemistry and Molecular Biology, 54(5), pp. 1410–20. doi: 10.1194/jlr.M035774.

Zhang, Q, Lenardo, M. J. and Baltimore, D. (2017) '30 Years of NF-kappaB: A Blossoming of Relevance to Human Pathobiology', *Cell*, 168(1–2), pp. 37–57. doi: 10.1016/j.cell.2016.12.012.

Zhang, Qian, Lenardo, M. J. and Baltimore, D. (2017) '30 Years of NF-κB: A Blossoming of Relevance to Human Pathobiology', *Cell*. doi: 10.1016/j.cell.2016.12.012.

Zhang, X. *et al*. (2004) 'ING4 induces G2/M cell cycle arrest and enhances the chemosensitivity to DNA-damage agents in HepG2 cells', *FEBS Letters*. John Wiley & Sons, Ltd, 570(1–3), pp. 7–12. doi: 10.1016/j.febslet.2004.06.010.

Zhang, X. *et al*. (2007) 'A role for the IkappaB family member Bcl-3 in the control of central immunologic tolerance.', *Immunity*. Elsevier, 27(3), pp. 438–52. doi: 10.1016/j.immuni.2007.07.017.

Zheng, N. *et al*. (2000) 'Structure of a c-Cbl-UbcH7 complex: RING domain function in ubiquitin-protein ligases.', *Cell*, 102(4), pp. 533–9.