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Role of the Elongator complex in innate immune responses in macrophages

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Jamie Murphy

2023

Abstract

The innate immune system functions as the first line of defence against pathogen infection. Innate immune systems are functionally present across the animal kingdom and mediate host defence against infection via a myriad of complex pathways. One such pathway involvesrapid induction of gene expression of innate immune signalling molecules such as cytokines and chemokines, to mount and co-ordinate appropriate responses. In particular, detection of pathogens, for example by Toll-like receptor 4 (TLR4) sensing LPS, leads to gene induction of type I interferons (IFN-I), and IFN-Is subsequently stimulated a transcriptional programme of IFN-stimulated genes (ISGs). Intense investigation has been performed into the underlying mechanisms of transcriptional regulation of innate immune gene expression. Contrastingly, the mechanisms by which such gene expression is controlled at the level of translation is less well studied and understood.

The Elongator complex is an evolutionarily conserved multiprotein complex that is crucial in modifying Uridines at the wobble position of tRNA molecules, to ensure efficient and proper decoding of mRNA codons during translation. Elongator is known to regulate the translation of proteins involved in processes ranging from cell cycle and DNA damage response, to nutrient starvation and CNS development. However, the contribution of the Elongator complex to innate immunity is poorly understood. Therefore, the aim of this project was to assess the role of the Elongator complex in innate immune responses in macrophages.

To do this, we used macrophages lacking ELP3, the catalytic subunit of the Elongator complex. Quantitative proteomics analysis of *Elp3*$^{-/-}$ macrophages following LPS treatment, showed a strong impairment in the expression of proteins involved in IFN-I-mediated signalling. We demonstrated that LPS-mediated gene induction of IFN-I and ISGs was impaired in *Elp3*$^{-/-}$ cells. Furthermore, ELP3 was necessary for ISG induction mediated directly by IFN-I stimulation. TYK2 is essential for IFN-I signalling and ELP3 was likely required for TYK2 activation, as gene induction downstream of other cytokine cascades which utilise TYK2, were also impaired in the absence of ELP3. As regards the requirement of ELP3 for LPS-stimulated IFN-I induction, we found that ELP3 was

necessary for TLR4-mediated IRF3 activation. Thus we demonstrated a two-fold requirement for ELP3 in TLR4-mediated IFN-I induction and signalling.

Interestingly, although many pathogen sensing pathways utilise IRF3, ELP3 was only necessary for gene induction downstream of TLRs and the RNA sensor RIG-I, and not DNA sensing via STING-mediated signalling pathways. Supporting this, innate immune gene induction in response to RNA virus infection was abrogated in *Elp3*$^{-/-}$ cells. Interestingly however, ELP3 was also required for Influenza A virus replication, suggesting a bi-directional role for ELP3 in IFN-I induction and RNA virus replication. We also established that the PYHIN family of proteins were enriched in Elongator-dependent codons, and that ELP3 was required for p205 protein expression, but not mRNA induction following IFNγ treatment.

In summary, this work clarifies and reveals a pivotal role for Elongator in IFN-I gene induction and signalling in macrophages, as well as demonstrating a dual role for Elongator in innate responses to viruses and viral replication.

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Lastly, I would like to thank Orla Sherwood, without whom, I'm not sure I could have gotten through the last few years. Her support, humour and affection throughout the PhD have been invaluable.

Out in the copse after rain

(too late after dark to be here).

Warm soil, woodlice dripping

From the underside of leaves.

I root down to the tender stalks

And twist them free – soaked petals

Dip and touch my arm, kernels

Of bud, itch of foliage, of wildness

On my skin. The plants are carrying

The smell, earth-rich, too heavy

To lift above head-height, and my boots

And jeans are bleached with it.

I turn home, and all across the floor

The spiked white flowers

Light the way. The world is dark

But the wood is full of stars

- **Sean Hewitt**

The East wind blows gently,

The rising rays float

On the thick perfumed mist.

The moon appears, right there,

At the corner of the balcony.

I only fear in the depth of night

The flowers will fall asleep.

I hold up a gilded candle

To shine on their scarlet beauty

- **Su Dongpo**

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Abbreviations

5’dA	5’ deoxyadenosine
5’ppp	5’ triphosphate
AA	Amino acid
AIM2	Absent in melanoma 2
ALS	Amyotrophic lateral sclerosis
AP-1	Activator protein 1
APS	Ammonium persulfate
ASC	Apoptosis-associated speck-like protein containing a CARD
BMDM	Bone marrow-derived macrophage
BSA	Bovine albumin fraction
CARD	Caspase activation and recruitment domain
cGAMP	Cyclic guanosine monophosphate-adenosine monophosphate
cGAS	Cyclic GMP-AMP synthase
CHIKV	Chikungunya virus
cm	carboxymethyl
CNS	Central nervous system
CTU1/CTU2	Cytosolic thiouridylase 1 and 2
DAMP	Damage associated molecular pattern
DAMP	Damage-associated molecular pattern
DDX41	DEAD-Box Helicase 41
DENV	Dengue virus
DMEM	Dulbecco's Modified Eagle's Medium
dNTPs	deoxynucleoside triphosphates
dsDNA	Double-stranded DNA
dsRNA	Double-stranded RNA

DTT	Dithiothreitol
Elongator complex subunit 1-6	ELP1-6
ER	endoplasmic reticulum
FCS	Fetal calf serum
HAT	acetyltrasferase domain
HCV	hepatitis C virus
HMW	High molecular weight
hpRNA	5’-triphosphate hairpin RNA
IAV	Influenza A virus
iBMDM	immortalised BMDM
IFI16	IFN inducible protein 16
IFN	Interferon
IFNAR	Interferon-α/β receptor
IFN-I	Type I Interferon
IKAP	I-κB kinase complex associated protein
IKK	Inhibitor of nuclear factor-κB (IκB) kinase
IL	Interleukin
IRF	Interferon regulatory factor
ISG	Interferon stimulated genes
ISGF3	IFN-stimulated factor 3
JAK1	Janus kinase 1
LGP2	Laboratory of Genetics and Physiology 2
LPS	Lipopolysaccharide
MAL	MyD88-adaptor-like
MAVS	Mitochondrial antiviral-signaling protein
mcm^5	methoxycarbonylmethyl
MDA5	Melanoma differentiation associated gene 5
M-MLV	Moloney Murine Leukemia Virus
MOI	Multiplicity of infection

mtDNA	mitochondrial DNA
mTOR	mammalian target of rapamycin
MyD88	Myeloid differentiation primary response protein 88
NF-κB	Nuclear factor kappa light chain enhancer of B-cells
NLRP3	NLR family pyrin domain containing protein 3
OAS	oligoadenylate synthase
P204	IFN inducible protein 204
P205	IFN inducible protein 205
PKR	Protein kinase R
Poly(I:C)	Polyinosine-polycytidylic acid
PRR	Pattern recognition receptor
qPCR	Real-time quantitative PCR
qRT-PCR	Quantitative real-time polymerase chain reaction
RIG-I	Retinoic acid-inducible gene I
RLR	RIG-I-Like Receptor
RSV	Respiratory syncytial virus
SAM	S-adenosylmethionine
SARS-CoV-2	Severe acute respiratory syndrome coronavirus 2
SAVI	STING-associated vasculopathy with onset in infancy
SDS	Sodium dodecylsulfate
SDS-PAGE	SDS-polyacrilamide gel electrophoresis
SEM	Standard error of the mean
SLE	systemic lupus erythematosus
ssRNA	single-stranded RNA
STAT	Signal transducer and activator of transcription
STING	Stimulator of interferon genes
TBK1	TANK-binding Kinase 1
TBS	Tris-buffered saline
TEMED	Tetramethylethylenediamine

Th	T helper
TLR	Toll-like receptor
TRAM	TRIF-related adaptor molecule
Trex1	Three prime repair exonclease 1
TRIF	TIR-domain containing adaptor inducing interferon (IFN)-β
tRNA	transfer RNA
TYK2	Tyrosine kinase 2
VACV	Vaccinia virus

Chapter 1 Introduction

1.1 Innate immunity

Throughout the entirety of life, from birth until death, the human body is under constant microbial assault and stimulation. Humans possess a large surface area of mucosal surfaces, which are in constant state of sensing, interacting with and potentially being infected by pathogenic microbes in the environment. Infection and invasion by these pathogenic microbes is an ever-present and ubiquitous threat and hazard to the health and homeostasis of humans. By necessity, humans have innovated elaborate and sophisticated mechanisms to detect and respond effectively to the vast array of microbial agents they encounter. This enables us to maintain and preserve organismal integrity and homeostasis in a world of constant microbial stimulation and flux. Coordination of these mechanisms of antimicrobial defence are performed by our complex and multifaceted immune system. Two distinct arms constitute the mammalian immune system; innate and adaptive immunity. Innate immunity functions as the crucial first point of contact and front line defence against pathogenic infection in what was considered a non-specific manner. Its evolutionary conservation across the animal kingdom is abundantly clear (1). Adaptive immunity contrastingly, is considered the specialized modality of the immune system, deriving from its characteristics of antigen-specific responses, clonal expansion and the generation of immune memory to microbes (2).

Innate immunity is comprised of several mechanisms of defence. Firstly, physical and chemical barriers such as the skin, blood brain barrier, antimicrobial peptides, pH and complement, defend against infection. The second arm of innate immunity is cellular based, mediated by immune cells like macrophages, monocytes, dendritic cells, NK cells and granulocytes (neutrophils, basophils and eosinophils) (3).

For a long time, innate immunity was considered to lack specificity and sophistication and to primarily function as a basal mechanism by which to engage the more complex and specific adaptive immune system to fight infection. This narrow and uncomplicated paradigm into which innate immunity found itself contextualised began to shift and evolve based off an hypotheses proposed by Janeway (4). In the late 80s, Janeway

proposed the existence of innate immune receptors capable of recognising and binding conserved molecular patterns present in different classes of microorganisms. It has since been established, that innate immune cells possess the ability to recognise conserved motifs specific to microbes known as pathogen associated molecular patterns (PAMPs), against which they engage an immune response either dependent or independent of adaptive immunity (5). Thus, innate immune cells possess the capacity to recognize common and conserved molecular patterns present in microbes, without mounting responses to specific antigens in a clonal manner. Innate immune cells sense and respond to PAMPs via a number of germline-encoded pattern recognition receptors (PRRs) (6). PRRs are highly conserved, and function spatially and temporally across many aspects of innate immunity. A vital and fundamental function of PRRs is the ability to discern and distinguish PAMPs from 'self', which engages an immune response against a microbial agent rather than against the host itself (5). However, PRRs can also sense molecular signals from the host, related to damaging or deleterious actions such as tissue damage or cell death, which are called damage-associated molecular patterns (DAMPs) (7).

Innate immune cells utilise PRRs spatially to sense infection or damage. Cell surface PRRs can recognise PAMPs on extracellular pathogens, or intracellular PRRs become activated following phagocytosis and degradation of a pathogen. PRR activation initiates a network of complex and intricate signalling cascades, which engage an array of transcription factors to mediate expression of a diverse range of innate immune genes. PRR-mediated signalling in response to infection leads to a rapid and strong enhancement in expression of genes encoding pro-inflammatory cytokines, chemokines & interferons (7). The rapid inflammatory response representative of innate immunity is executed by pro-inflammatory cytokines. Recruitment of distal immune cells to the given site of infection is enabled by chemokine release by innate immune cells present. Type I interferons (IFN-I) secreted by immune cells signal in an autocrine and paracrine manner, to induce an antiviral immune state in neighbouring immune cells via the upregulation of hundreds of interferon stimulated genes (ISGs). These ISGs can either directly restrict viruses, or further amplify and enhance IFN-I signalling in a feedback loop.

Recent research into the functioning of innate immune cells has revealed that what was considered a rough and inelegant arm of immunity, actually possesses specificity, competence and complexity. Although innate immunity has been shown to not solely function to activate adaptive immunity, the discovery of PRRs and the greater complexity they engender, strongly illustrates how tight and complex the link between innate and adaptive immunity is.

1.2 Pattern recognition receptors

Innate immune cells possess several groups of PRRs which are delineated based on their spatial localization, structure and the molecular motif they recognise.

1.2.1 Toll-like receptors

Toll-like receptors (TLRs) are key PRRs utilised by innate immune cells for PAMP recognition and antimicrobial defence. TLRs are type I transmembrane glycoproteins made up of an extracellular domain for ligand binding, a transmembrane domain, and an intracellular domain for adaptor protein recruitment and signal transduction (3). Ten functional TLRs have been identified in humans (TLR1-10), and 12 in mice, with a reverse transcriptase insertion meaning murine TLR10 is non-functional (8, 9). TLRs are expressed either extracellularly at the plasma membrane (TLR1, 2, 4, 5, 6), or intracellularly at endosomal membranes (TLR3, 7-9). TLR1/TLR2, and TLR2/TLR6 heterodimers sense lipopeptides from gram-positive bacteria, whilst TLR4 senses lipopolysaccharide (LPS) from gram negative bacteria and TLR5 senses flagellin (10). Intracellular TLRs sense microbial nucleic acid, with TLR3 sensing double stranded RNA (dsRNA), TLR7/8 sense single-stranded RNA (ssRNA) whilst TLR9 senses hypomethylated CpG DNA (11).

The intracellular domain of TLRs contains a Toll/interleukin-1 receptor domain (TIR), which they utilize for interaction with TIR domains of signalling adaptor proteins. This family of TIR containing signalling adaptors are myeloid differentiation primary-response gene 88 (MyD88), MyD88-adaptor-like (MAL, also known as TIRAP), TIR-domain-containing adaptor 3 protein inducing IFNβ (TRIF), TRIF-related adaptor molecule (TRAM), and sterile α- and armadillo motif-containing protein (SARM1).

All TLRs apart from TLR3 utilise MyD88 for downstream signal transduction. MyD88 recruits MAL, and induces activation of transcription factors such as activator protein 1 (AP-1), nuclear factor kappa-light-chain-enhancer of activated B-cells (NF-κB), and interferon regulatory factor 3 (IRF3) and interferon regulatory factor 7 (IRF7). TLR3 signals exclusively through TRIF and TRAM to activate NF-κB and IRF3 (12). Uniquely, TLR4 possess the ability to signal through both MyD88/MAL and TRIF/TRAM, to activate and NF-κB and IRF3 respectively and induce innate immune gene expression (13). NF-κB and AP-1 mediate gene induction of pro-inflammatory cytokines downstream of TLR signalling. Chemokine induction is mediated by NF-κB and IRF3 . IRF3 and/or IRF7 control gene expression of IFN-Is in response to TLR activation (14). A schematic overview of TLR signalling is shown in Fig 1.1.

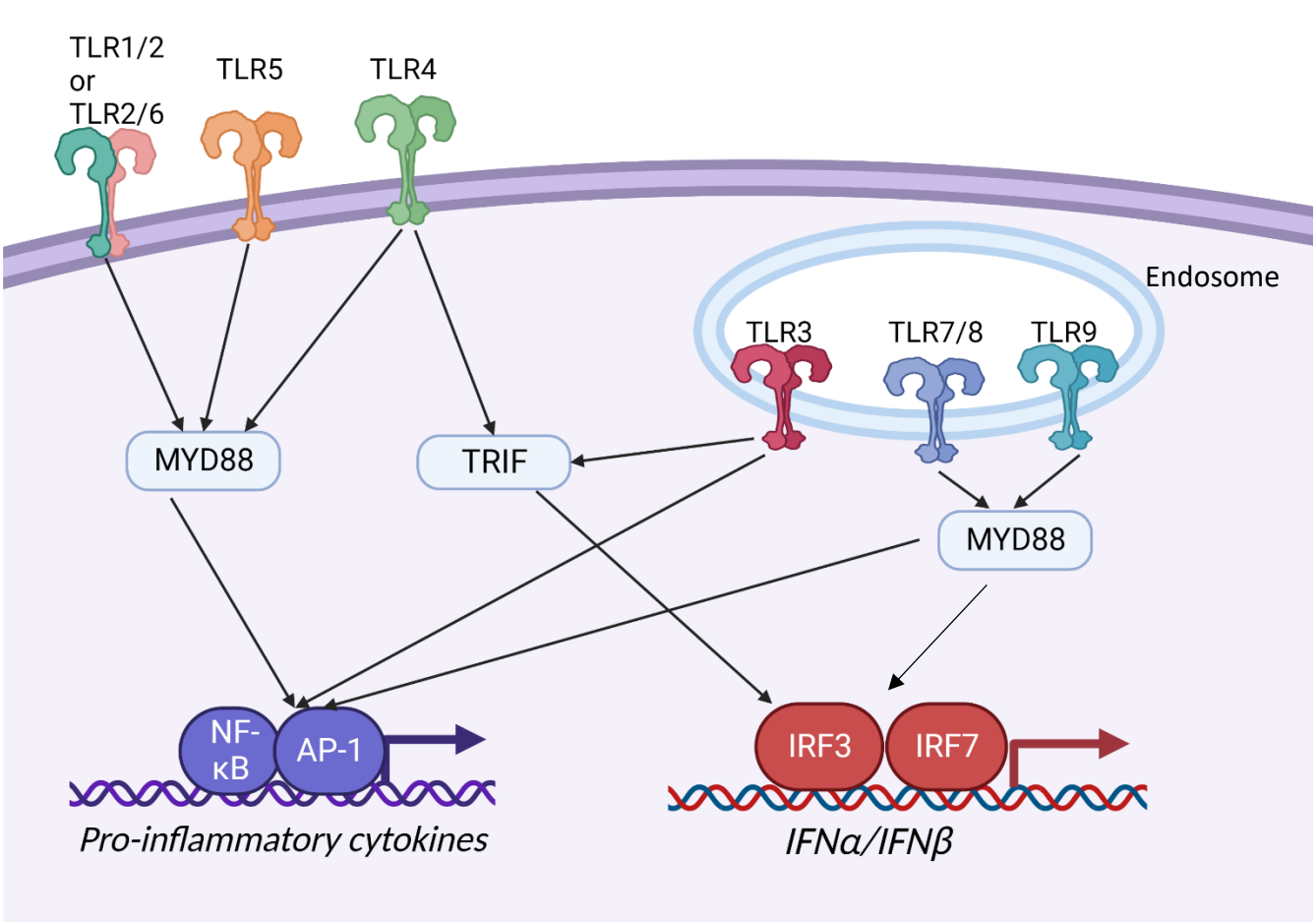


Fig 1.1 TLR signalling overview

TLRs function either at the cell surface or intracellularly at endosomal membranes. TLRs signal through the adaptors MyD88 and TRIF, with TLR4 uniquely able to utilise both, whilst TLR3 is the only TLR to solely signal through TRIF. MyD88 signalling downstream of cell surface TLRs activates the transcription factors AP-1 and NF-κB, leading to transcription of proinflammatory cytokines such as *Il-6* and *Tnf*. TRIF signalling enables activation of IRF3, which mediates transcription of IFN-I. MyD88 signalling downstream of TLR7/8 and TLR9 leads to IRF7 activation and IFN-I gene induction.

1.2.2 RIG-I-like receptors in RNA sensing

A key PAMP which PRRs sense and subsequently induce a strong innate immune response to, is microbial nucleic acid present in the cytosol during an infection. The RIG-I-like receptors (RLRs) are key mediators of intracellular RNA sensing and antiviral innate immunity. The RLR family is comprised of three members: RIG-I, melanoma differentiation-associated protein 5 (MDA5) and laboratory of genetics and physiology 2 (LGP2). RIG-I and MDA5 mediate viral RNA sensing, whilst LGP2 can enhance MDA5 signalling but inhibit RIG-I signalling (15,16). All three members possess a carboxy-terminal domain (CTD) and a central helicase domain. RIG-I and MDA5 each have two additional caspase activation and recruitment domains (CARD), to initiate downstream signalling (17). RIG-I recognizes specific motifs and structures related to viral RNA, to ensure its activation does not occur in the presence of the vast quantities of self-RNA that are present in the cytosol at a given time. RIG-I senses RNA with a 5' triphosphate group (5'ppp), which is a molecular feature of RNA derived from viruses (18,19). Viral RNAs with uncapped 5' diphosphate groups are also immunostiulatory for RIG-I (20). Cellular mRNA possess a methylated cap at the 2'O position of phosphates. Viral RNA has no such cap, and so RNA where the terminal phosphate is unmethylated at the 2'O position activates RIG-I (21). MDA5 does not discriminate RNA based on the presence or lack of a 5'ppp moiety, but rather, its activation is determined by the length of dsRNA it binds (22). Upon binding of viral dsRNA, RIG-I and MDA5 undergo conformation changes in an ATPase dependent manner. RIG-I subsequently oligomerizes via its CARD domain. Following this RIG-I interacts with mitochondrial antiviral signalling protein (MAVS) at the mitochondria via its CARD domain. MAVS subsequently activates TANK-binding kinase 1 (TBK1), which activates IRF3 and IRF7, which mediate IFN-I gene induction (23). RIG-I and MDA5 also mediate NF-κB activation via NEMO, IKKα and IKKβ (24). An overview of RLR signalling is illustrated schematically in Fig 1.2.

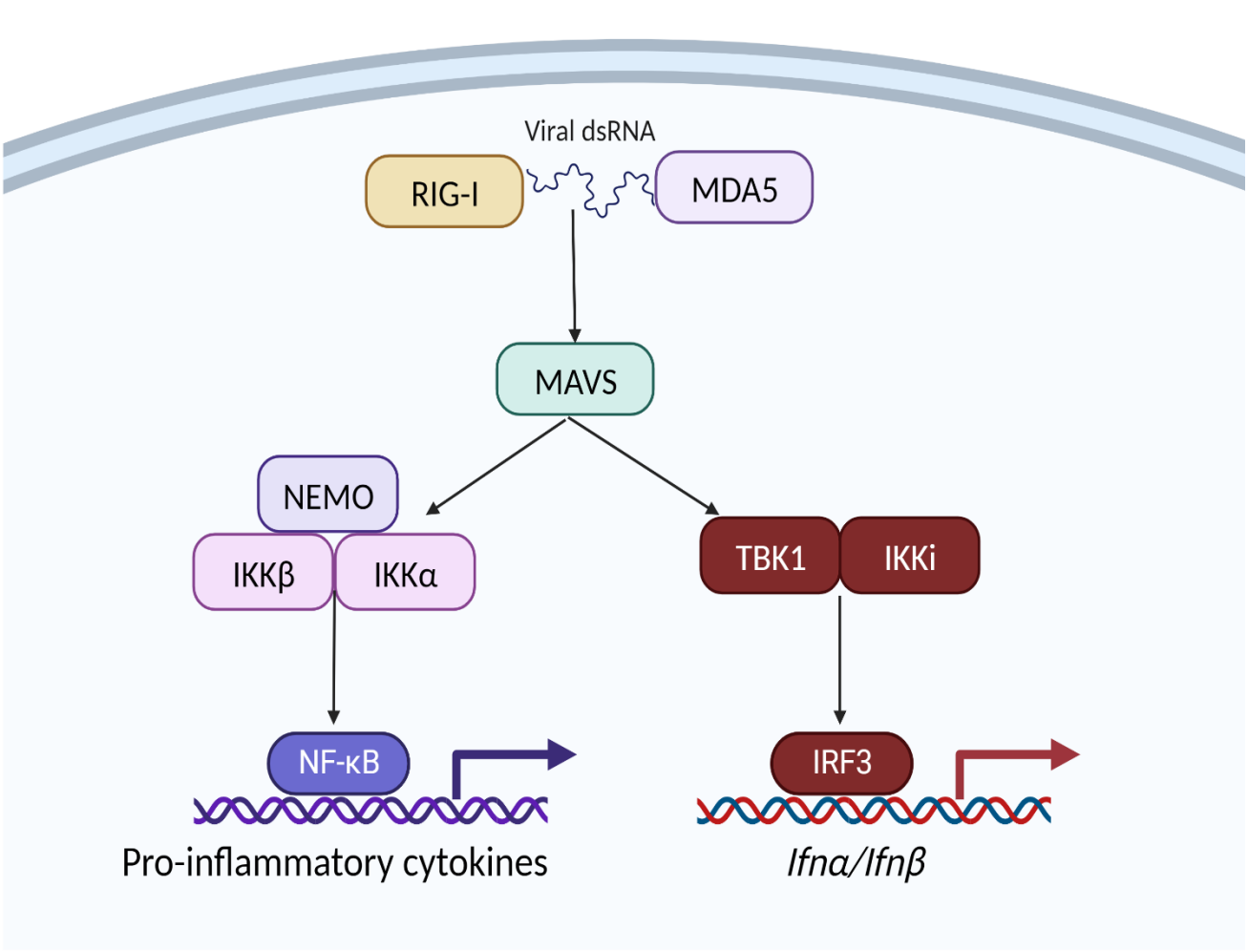


Fig 1.2 Overview of RLR signalling

RIG-I and MDA5 sense cytosolic dsRNA derived from viruses. Following activation of MAVS, activation of the IKK complex occurs, leading to NF-κB activation and proinflammatory cytokine induction. Alternatively, IRF3 activation occurs downstream of TBK1, enabling gene induction of IFN-I in response to viral RNA sensing. NEMO; NF-κB essential modulator, IKKα/β; IkappaB kinase α/β.

1.2.3 cGAS-STING pathway in DNA sensing

The introduction of DNA into cells has long been known to be strongly immunostimulatory (25). Since the discovery of PRRs and their role of innate immunity, many sensors of cytosolic DNA have been identified and studied. The presence of such sensors enables recognition of many different modalities of either host or microbial DNA, leading to activation of a multitude of complex and sophisticated innate immune signalling pathways.

Understanding of the molecular mechanisms of, and responses induced by innate DNA sensing has progressed significantly since the discovery of cyclic GMP-AMP synthase (cGAS) a decade ago (26). Signalling via cGAS and its downstream signalling effector, stimulator of interferon genes (STING), underpin DNA sensing and innate immune responses in a variety of contexts, aswell as being implicated in disease states such as autoimmunity and cancer (27). cGAS binds dsDNA, with longer dsDNA enhancing the enzymatic activity of cGAS by promoting its liquid-liquid phase separation, aswell as by inducing its oligomerization (Refs). Other DNA sensors engage adaptor proteins upon sensing of DNA, whereas cGAS catalyzes the production of a di-nucleotide second messenger for signal transduction. cGAS catalyzes the formation of the second messenger cyclic GMP-AMP (cGAMP) from GTP and ATP (26). cGAMP subsequently binds and induces conformational changes in STING, which resides at the endoplasmic reticulum (ER), leading to STING activation (28). Following activation, STING traffics to the Golgi via the ER-Golgi intermediate compartment, where it recruits and activates TBK1. TBK1 phosphorylates STING, which provides a docking site for IRF3, where it is subsequently activated by TBK1. IRF3 translocates to the nucleus following dimerization and mediates transcription of IFN-I (29). IFN-Is initiate and induce an antiviral state for host protection. cGAS-STING signalling also activates NF-κB and induction of proinflammatory cytokines (30,31).

The cGAS-STING pathway induces IFN-I in response to a number of DNA motifs. cGAS senses microbial DNA derived from viruses and bacteria upon infection (32). Microbial infection also activates STING via an alternative mechanism, whereby mitochondrial perturbation following infection leads to the release of mitochondrial DNA (mtDNA), which potently activates cGAS (33). Host-derived genomic DNA is also a strong activator

of cGAS-STING signalling. Genomic DNA from dead cells, debris from aberrant DNA damage, or genetic material of chromosomes in micronuclei exposed to the cytosol all activate cGAS (34,35). DNA is compartmentalised spatially within the cell, to the mitochondria and nucleus. Leakage of DNA into the cytosol has the ability to activate cytosolic DNA sensors and cause autoimmunity and pathology. Misregulation of the cGAS-STING pathway is detrimental to the host. Mutations in the DNase, three prime repair exonclease 1(*Trex1*), leading to the accumulation of nucleic acid, are associated with cGAS-STING-dependent IFN-I production in Aicardi-Goutieres syndrome (36). Gain of function mutations in STING lead to the development of the interfeonopathy termed, STING-associated vasculopathy with onset in infancy (SAVI) (37). cGAS has also been implicated in macular degeneration, whereby it senses mtDNA, leading to IFN-I production and noncanonical inflammasome activation (38). A range of other diseases and conditions are related to cGAS-STING dysregulation, highlighting its fundamental role in DNA sensing.

1.2.4 PYHIN family proteins in DNA sensing

In addition to cGAS, a plethora of proteins have been implicated in DNA sensing. One such group of proteins that play a key role in DNA sensing are members of the AIM2-like receptor family (ALRs). The ALRs are made up of members from the pyrin and HIN200-domain containing protein family (PYHINs), namely absent in melanoma 2 (AIM2), interferon-γ inducible protein 16 and murine p204. The PYHIN family is composed of 5 genes in humans and 13 in mice . PYHIN proteins contain a PYRIN domain for protein:protein interactions and a C-terminal HIN domain for DNA binding. POP3 in humans is the only PYHIN that lacks a HIN domain (39).

AIM2 and IFI16 have been established as innate immune DNA sensors. AIM2 functions in the cytosol, and senses DNA via its HIN domain. Following DNA ligation, AIM2 interacts with apoptosis-associated speck-like protein containing a CARD (ASC), to form a multiprotein inflammasome complex. This leads to caspase-1 activation, leading to IL-1β processing and secretion, and pyroptosis (40,41). AIM2 is important in the context of infection by bacteria such as *Francisella tularensis* and *Listeria monocytogenes* amongst others (42,43). In a viral context, AIM2 is protective against viruses such as vaccinia, mouse cytomegalovirus and human papillomaviruses (44). AIM2 has been implicated in

multiple disease states. Reduced AIM2 expression correlates with poor prognosis in colorectal cancer (45). DNA-mediated AIM2 activation can lead to IL-1β release and triggering of inflammation in psoriasis (46). Changes in AIM2 expression are implicated in the interferonopathy, systemic lupus erythematosus (SLE) (47).

IFI16 is another PYHIN family member implicated in mediating sensing of cellular DNA in a variety of innate immune settings. IFI16 was initially found to be a PRR for DNA when it was shown to bind a 70 base pair (bp) motif derived from vaccinia virus, and to induce IFN-β expression in a STING-dependent manner (48). IFI16 has been shown to sense DNA from HIV-1 and multiple other DNA viruses (49,50). Activation of IFI16 by DNA is dependent on the length of the dsDNA bound, with dsDNA of ~150 bp being optimal for activation (51). IFI16 is has the ability to shuttle between the nucleus and cytosol, and sense dsDNA in both subcellular locations. In the nucleus IFI16 has been shown to associate with ASC and caspase-1, in response to herpesvirus DNA, to form a multimeric inflammasome structure and shuttle to the cytosol (52). The murine ortholog of IFI16, p204, has also been shown to be required for HSV-1 mediated IFNβ induction in mouse macrophages (48). Intriguingly, IFI16 also possess a role in RNA virus infection, where it can bind viral RNA, and both enhance transcription and activation of RIG-I in response to influenza virus infection (53). Thus, IFI16 mediates innate immune responses to both DNA and RNA viruses. IFI16 has been implicated in anti-tumour immunity, where by it prevents DNA damage repair, leading to cytosolic accumulation of DNA, STING activation and IFNβ-mediated anti-tumour functioning (54). IFI16 is also targeted in autoimmune interferonopathy conditions, with SLE, Rheumatoid arthritis (RA) and Sjogren's syndrome patients possessing autoantibodies against IFI16 (55–57). A schematic overview of cGAS and ALR-mediated DNA sensing is shown in Fig 1.3.

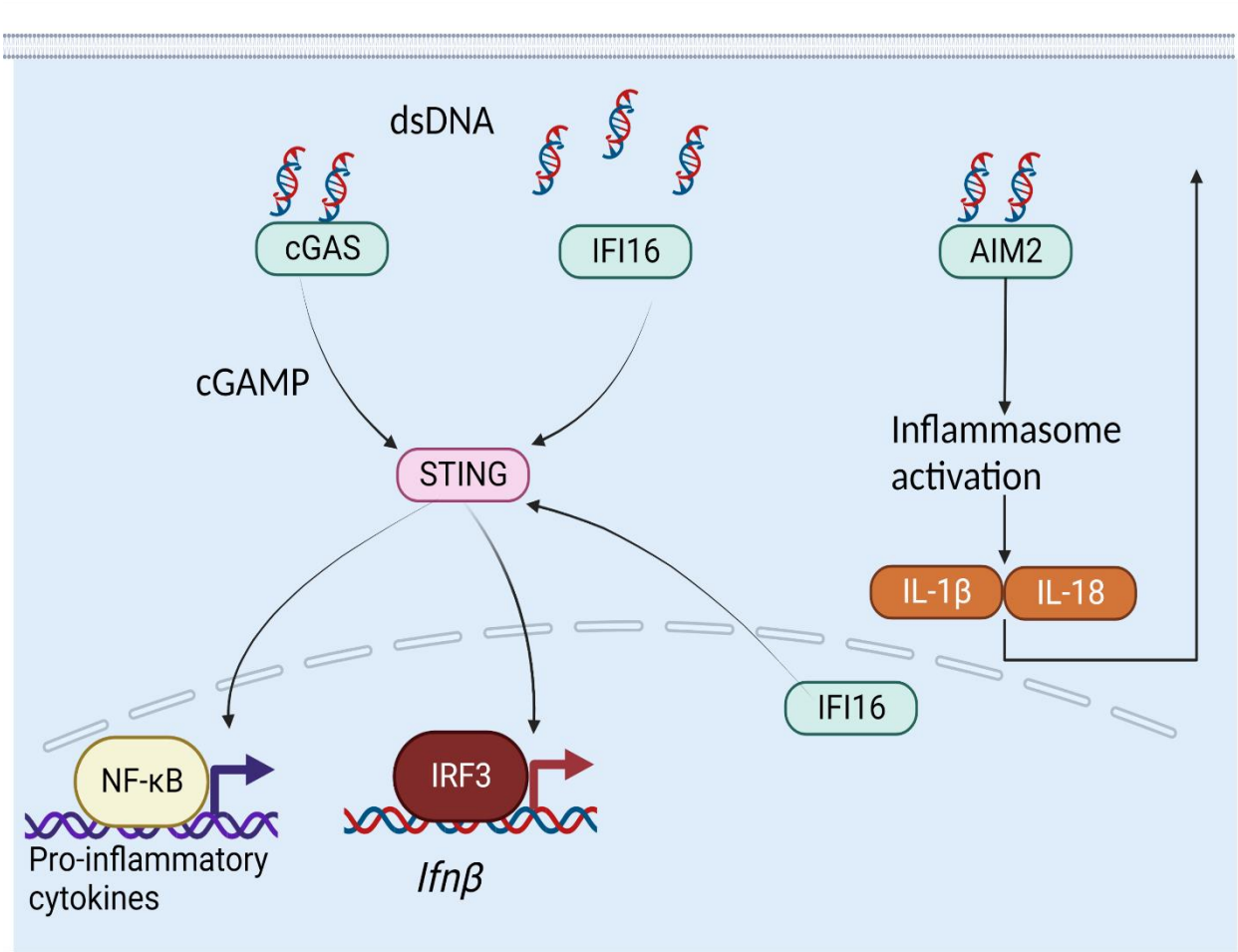


Fig 1.3 cGAS-STING/ALR-mediated DNA sensing

dsDNA in the cytosol leads to activation of cGAS and IFI16. IFI16 interacts with STING, whilst cGAS generates the cyclic dinucleotide second messenger cGAMP to activate STING. STING subsequently mediates activation of NF-κBa nd IRF3, leading to proinflammatory cytokine and *ifnb* gene induction respectively.

1.3 IFN-I induction and responses

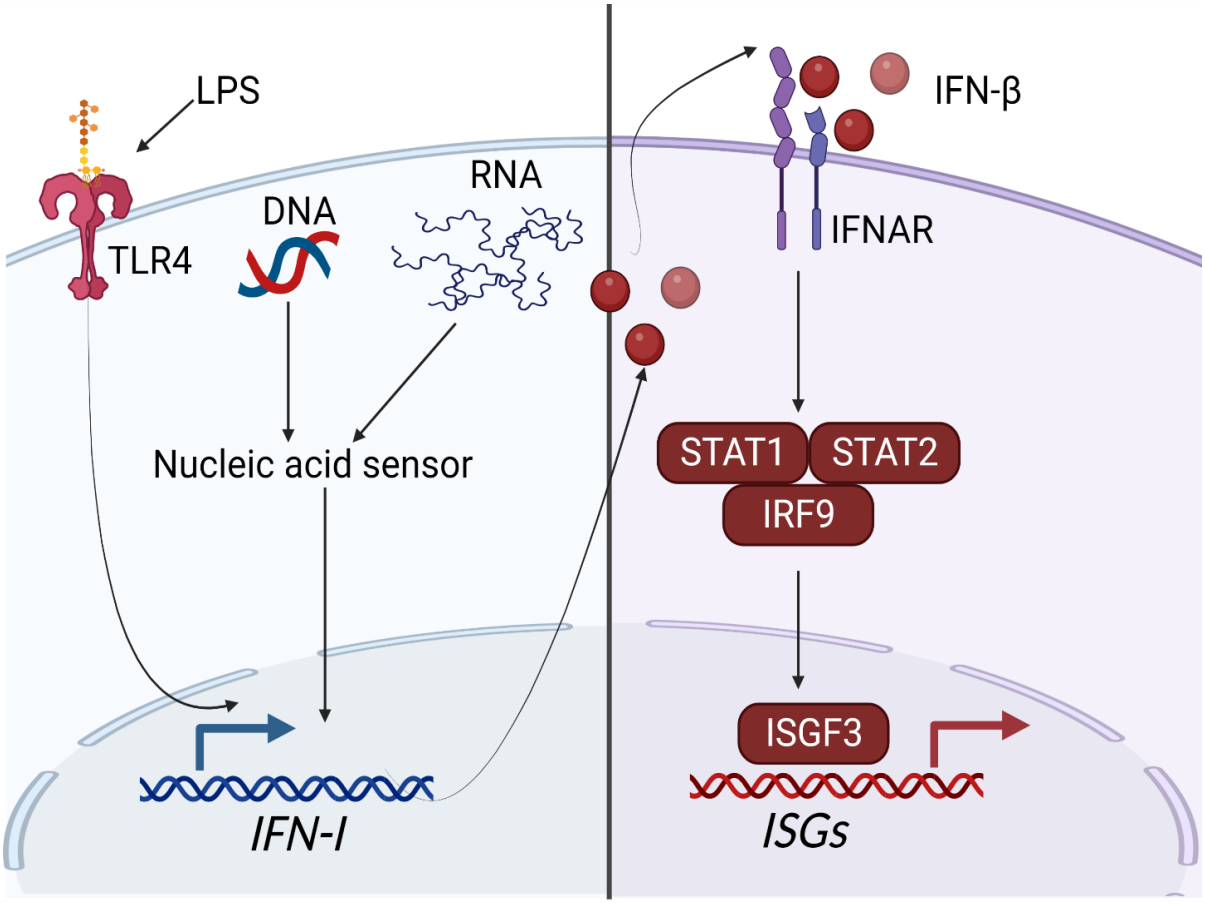
A cardinal output of activation of many of the aforementioned innate immune PRRs is the induction of IFN-I expression. The Interferon family of cytokines are divided into three separate groups. The IFN-I group are composed of IFNβ and 13 different genetic subtypes of IFNα (58). IFN-II has a sole member, IFNγ predominantly derived from T and NK cells (59). The IFN-III family is composed if IFNλ1-4 which mediate antiviral defence at epithelial surfaces (60).

IFN-I are crucial in orchestrating, coordinating and shaping innate immune responses. IFN-I execute and facilitate myriad functions across diverse facets of innate immunity

1.3.1 IFN-I signalling

IFN-I induction occurs in response to activation of multiple TLRs, RLRs, ALRs and cGAS-STING. IFNβ signals in an autocrine or paracrine feedback loop to activate IRF7 transcription, which further enhances transcription of IFN-I genes (predominantly IFNα subtypes) & ISGs (61). IFN-I signalling is transduced via a JAK-STAT pathway (Janus activated kinase and signal transducer and activator of transcription). IFN-I is bound by the interferon α receptor (IFNAR), a heterodimeric receptor composed of IFNAR1 and IFNAR2. IFNAR signalling leads to activation of the receptor associated kinases, Janus kinase 1 (JAK1) and tyrosine kinase 2 (Tyk2). JAK1 and Tyk2 subsequently enable the recruitment and phosphorylation of STAT1 and STA2, which heterodimerize before binding IRF9 to form the ISG factor 3 complex (ISGF3). ISGF3 translocates to the nucleus where it binds IFN-stimulated response elements in the promoters of ISGs, initiating their transcription (61). Basal tonic levels of IFN-I production in immune cells is facilitated by commensal bacteria, to enable strong basal levels of STAT1 to ensure rapid IFN-I induction on infection or PRR activation (62,63) signals through STAT1 homodimers to activate gene expression (64). As chronic or aberrant IFN-I production can be pathological and deleterious to the host, IFN-I signalling contains a number of built-in regulatory mechanisms. IFN-I can also activate STAT3 homodimers downstream of IFNAR, which possess the ability to restrain STAT1 responses (65,66). IFN-I signalling also induces transcription of SOCS1 and SOCS3, which block IFNAR-mediated JAK-STAT

activation to regulate the strength and temporal nature of IFN-I signalling (67,68). A schematic of IFN-I induction and signalling is shown in Fig 1.4



1.4 Overview of IFN-I induction and signalling

TLR4 sensing of LPS and nucleic acid sensor ligation of DNA or RNA leads to gene induction of IFN-I. IFN-I signal in an autocrine or paracrine manner to activate the heterodimeric IFNAR complex. With the aid of the receptor associated kinases JAK1 and TYK2, IFNAR activates STAT1 and STAT2 heterodimerization. STAT1-STAT2 bind IRF9 to form the ISGF3 complex, which translocates to the nucleus and induces expression of hundreds of ISGs.

1.3.2 Interferon stimulated genes

Foremost among its plethora of crucial functions, IFN-I enable and execute antiviral innate immunity. Secreted IFN-I signals in an autocrine and manner to induce a transcriptional programme in infected cells and uninfected neighbouring cells that restrains viral infection and replication. The cardinal output of IFN-I signalling that enforces antiviral innate immunity is the induction of hundreds of ISGs. Antiviral immunity enabled and shaped by ISGs is complex and multifactorial. ISGs implement innate responses that mediate viral sensing, inhibit viral replication as well as directly enhancing and amplifying IFN-I signalling. Over 300 ISGs are induced by IFN-I signalling, as such a number of key ISGs will be briefly discussed. A number of ISGs amplify IFN-I signalling in a positive feedback loop. IRF7 is strongly induced following IFN-I signalling. IRF7 enables amplification of IFN-I induction by mediating IFNα transcription and thus further induction of ISGs and propagation of antiviral immunity (69). STAT1 and IRF9, key mediators of IFN-I signalling, are ISGs themselves, facilitating positive feedback activation of IFN-I signalling (70). A number of ISGs function as PRRs for microbial sensing, leading to further IFN-I induction, ISG expression and amplification of IFN-I responses temporally and spatially within the host. RIG-I and MDA5, as previously described (section 1.2.2) are cytosolic PRRs that bind viral dsRNA. They themselves are IFN-inducible ISGs that sense viral infection initiating downstream signalling that also induce IFN-I and ISG expression. The PYHIN family of proteins are upregulated following IFN-I signalling. As well as the key role in nucleic acid sensing that AIM2, IFI16 and its murine ortholog p204 play, other members of the PYHINs can mediate a plethora of processes, such as proinflammatiory cytokine production and inflammasome activation (28, 29).

PKR also functions as a cytosolic receptor for viral dsRNA. PKR is expressed at a basal level constitutively, before being induced by IFN-I (73). Upon ligation of viral RNA, PKR dimerizes to an activated form, which leads to antiviral immunity via inhibition of eIF2α and subsequent translation inhibition (74). PKR-deficient mice are susceptible to infection with many RNA viruses such as Influenza virus, HIV-1 hepatitis D virus amongst others (74,75).

ISGs enable antiviral immunity via multiple mechanisms such as blocking viral entry, replication and egress. The myxoma resistance (Mx) family are GTPases that are potently induced following IFN-I signalling. MxA oligomerizes to bind and block activity of viral polymerase at an early stage post entry (74). MxA and MX1 also binds newly entered viral nuclocapsids, leading to their subsequent degradation. The interferon-inducible transmembrane family (IFITM) are ISGs that also play a key role in blocking viral entry. There are four IFITM members, which localize to endosomal surfaces within the cell and appear to possess selectivity with the viruses they restrict. IFITM1 restricts Ebola and Marburg as well as SARS coronavirus (76). Contrastingly, IFITM3 restricts influenza and HIV-1, which IFITM1 does not (77).

A number of ISGs function by inhibiting viral translation and replication. The oligoadenylate synthase (OAS) family of proteins are highly induced upon IFN-I signalling. They bind viral dsRNA, and catalyse the production of 2'-'5' oligoadenylates which mediate RNaseL activation, an endoribonclease which degrades viral RNA (78). This OAS-RNAseL axis prevents viral replication. As previously mentioned PKR activation leads to translational inhibition, thus blocking translation of viral RNA as well as cellular RNA. The interferon-induced protein with tetratricopeptide repeats (IFIT) family inhibit viral translation and replication. IFITs inhibit viral translation via eukaryotic initiation factor 3, or form a multiprotein complex to bind and sequester 5'ppp-containing viral RNA (79–81).

Thus, ISGs coordinate antiviral immunity in a complex & multifactorial manner following induction by IFN-I.

1.3.3 Role of IFN-I in bacterial infection

IFN-I and ISGs play an indispensable role in antiviral immunity. The function of IFN-I in bacterial infection is less understood. They appear to function as a double-edged sword, both giving protection and contributing to pathology in a context-dependent manner.

IFN-I are protective in *Chlamydia trachomatis* infection, inducing indoleamine 2,3-diocygenase to reduce the availability of tryptophan for bacterial survival (82). In the context of *Legionella pneumophilia* infection, IFN-I protect macrophages from infection by polarising them towards an M1-phenotype (83). IFN-I are critical for immunity to

group B streptococcus like *Streptococcus pneumonia*, where they enhance macrophage activation and cytokine induction in response to infection (84).

Contrastingly, IFN-Is are detrimental in the context of infection by *Listeria monocytogenes* and *Mycobacterium tuberculosis*. In the context of TB infection, IFN-I induce IL-10 and Il-1ra expression, which block inflammasome activation and IL-1 signalling, which are critical for antibacterial immunity (85). Patients with active infection by *M. tuberculosis* possess a IFN-I signature that correlates poorly with treatment (86). IFN-Is also dampen macrophage responsiveness to IFNγ in *M. tuberculosis* (87). In *L. monocytogenes* infection, IFN-Is mediate apoptosis of immune cells, which leads to strong expression of anti-inflammatory cytokines like IL-10 (88). Similar to TB, IFN-I suppress macrophage responses to IFNγ in Listeria infection (89).

1.3.4 IFN-I regulation of innate immunity

In addition to their functioning in infectious disease, IFN-I execute a plethora of crucial roles to coordinate innate immune responses. IFN-I regulate innate immune-mediated antigen presentation. DC's function as cardinal APCs to facilitate antigen-specific immune responses. IFN-I mediate and shape DC development, differentiation, whilst upregulating proteins involved in antigen presentation, to facilitate innate-mediated initiation of adaptive immune responses (18, 19). IFN-I induce expression of costimulatory molecules CD80/CD86 in DCs, increase expression of antigen presentation proteins such as TAP1/2, tapasin and MHC-I (92). IFN-Is also induce Il-12 expression by DCs, which is critical for activation of Th1 responses (93).

IFN-I induces natural killer (NK) cell mediated IFNγ production and effector functions in the context of viral infections. IFN-Is enhance cytolytic functions and IFNγ secretion by NK cells in influenza infection. IFN-Is also induce effector functions in NK cells in the context of murine cytomegalovirus infection (94,95).

1.3.5 IFN-I in adaptive immunity

Coupled with their facility to link innate and adaptive responses via APC regulation, IFN-I also directly regulate adaptive immunity. IFN-I shaping of adaptive responses is impacted at multiple levels. IFN-I can either directly inhibit or augment T-cell

differentiation and proliferation depending on IFN-I levels and signal strength, STAT expression or type of microbial infection (96–98).

1.3.6 IFN-I in disease

IFN-I engage multifaceted and counterbalancing responses and signals. Due to their fundamental importance in immunity, IFN-I are finely modulated and regulated. As such, alterations to or breakdowns in modulation of IFN-I responses can be deleterious to a host. Acute IFN-I generally facilitate host protection and antimicrobial immunity in the realm of infection. Chronic IFN-I induction can often have pathogenic consequences. In the context of autoimmunity, IFN-I are chronically and aberrantly induced, with autoimmune conditions such as SLE, rheumatoid arthritis and Sjogren's syndrome possessing a strong IFN-I signature (61). IFN-I can also be immunosuppressive during chronic microbial infection. In lymphocytic choriomeningitis virus infection (LCMV), chronic IFN-I induces IL-10 and PD-L1 expression, suppressing immune responses and blocking viral clearance (99).

IFN-I are integral mediators of innate immunity in a variety of contexts. They orchestrate and regulate a multitude of facets of innate immune responses.

1.4 Translational control of gene expression

A key mechanism by which IFN-I and ISGs counteract viral infection is via the regulation of translation. Regulation of translation has begun to emerge as an influential method for exerting an additional layer of control over expression of genes such as IFNs and ISGs. A number of intrinsic mechanisms are utilised to coordinate mRNA translation for proteostasis and general cellular homeostatic functioning.

1.4.1 Process of translation

Translation is the process that facilitates protein synthesis, via the decoding of mRNA into amino acids, which subsequently form the polypeptide chains that fold into functional proteins. The decoding of mRNA into protein via translation is a fundamental process across all domains of the animal kingdom. As such, it is highly controlled and regulated, and when dysfunctional, is highly detrimental to the host.

Translation in eukaryotes is composed of four stages: initiation, elongation, termination and ribosome recycling. Initiation involves translocation of a newly synthesized mRNA transcript to the ribosome, where it is bound by the 40S ribosomal subunit. Transfer RNA (tRNA) aminoacylated with a Methionine locates to the P-site of the ribosome. The ribosome scans along the transcript until the anticodon of the tRNA binds its cognate codon on the mRNA, the start codon AUG. The 40s subunit subsequently binds a 60s ribosomal subunit to give an 80s initiation complex with methionyl-tRNA in the P-site, that can proceed to the elongation phase of translation (100).

Aminoacylated-tRNA is delivered to the A-site by eukaryotic elongation factors. A peptide bond is formed between the amino acid (AA) of this tRNA, and the amino acid of the tRNA in the A-site. A and P-site tRNAs now translocate to the P and E-sites respectively, where deacylated tRNA is released from the E-site and recycled. Thus, the ribosome translocates along the mRNA transcript one codon at a time. Peptide bond formation between AAs bound to tRNAs in the A and P-site facilitate polypeptide formation and decoding of an mRNA transcript (101).

1.4.2 Importance of translational kinetics to proteostasis

Cells employ a variety of mechanisms to ensure the translational process gives rise to a stable and functional proteome. Cell-intrinsic non-uniform rates of translation elongation drive proteostasis. Ribosomes proceed at variable speeds along mRNA transcripts, balancing elongation, co-translational folding and fidelity of translation. Enabling disparate rates of elongation along an mRNA transcript balances differing requirements for proper translational fidelity. Slower elongation rates enable proper and efficient co-translational folding, whilst increased rates of ribosomal movement along mRNA transcripts can enhance efficiency and fidelity of translation (102). However, the kinetics of ribosomal movement and translational elongation necessitate potential compromises, with slower elongation rates potentially causing mRNA decay and protein degradation, and faster kinetics increasing the chances of mis-folding and aggregation (103).

These interlinked and counterbalancing measures ensure maintenance of proteostasis in a cellular environment in constant flux from alterations in environmental and stress-inducing conditions.

1.4.3 Codon bias, optimality and tRNA abundance as determinants of translational kinetics

The genetic code is composed of 64 different combinations of nucleotides, which compose 64 triplet codons that are transcribed to mRNA. 3 of these codons encode stop signals for translation, with the other 61 encoding the 20 AAs which comprise eukaryotic proteins. As there exists a discrepancy in the number of codons encoding a smaller number of AAs, multiple different codons can thus encode the same AA. This redundancy in the genetic code is known as codon degeneracy. Codons which encode the same AA are known as synonymous (Fig 1.5A). The interaction between the ribosome and each mRNA codon is discrete and distinctive, synonymous or not. Thus, there exists a phenomenon known as codon optimality, whereby there is a non-uniform rate of decoding of mRNA codons by the ribosome (104). Translational efficiency is crucially dependent on codon optimality. As such, there are synonymous codons represented disproportionately in mRNA transcripts, based on how optimally they are translated. This non-random distribution of synonymous codons is known as codon bias (105).

Codon bias and optimality are key determinants of translation elongation, efficiency, co-translational folding and mRNA stability. The presence of optimal synonymous codons affects translation elongation, with ribosomes spending less time decoding optimal codons, leading to reduced ribosomal occupancy and increased rates of elongation (106,107). An additional layer of influence pertaining to codon optimality and bias is the presence of cognate tRNA. Optimality can be determined by how well a cognate tRNA can be selected from the tRNA pool, and codons with bias for enriched and highly expressed tRNAs being abundant in highly expressed genes (108–110). Thus, the kinetics of elongation are regulated by codon optimality, where ribosomes stall when forced to wait for selection of a rare cognate tRNA for a non-optimal codon. The selection of this tRNA from a large pool of tRNA species is energetically and kinetically costly to elongation (111). Codon bias is strongly correlated with the efficiency of translation.

Highly expressed genes are biased towards an abundance of optimal codons to ensure high levels of expression . Interestingly however, there exists a group of non-optimal codons just downstream of the start codon, which slows down the start of the elongation process. This is important for spacing ribosomes along an mRNA transcript and preventing collisions along the transcript (112).

Co-translational folding and mRNA stability are affected by codon usage. Folding of nascent polypeptide chains occurs co-translationally, and is determined by the rate of mRNA decoding. Highly conserved protein domains are enriched in optimal codons, as optimal codons are not as prone to misfolding and thus these key structural domains are translated efficiently (113). Non-optimal codons are also associated with folding, whereby their slower rate of decoding can provide sufficient time for polypeptide folding before ribosomal dissociation and translational termination (114). mRNA stability appears to correlate with optimal codon usage. Abundance of optimal codons appears to stabilise mRNA transcripts and lead to enhanced levels of protein expression, by increasing the half-life of the mRNA (104,115). This codon usage seems to be related to protein function. For example in yeast, proteins whose expression are stimulus-dependent are enriched in non-optimal codons. The non-optimal codons engender mRNA instability, ensuring that mRNA decay occurs to quickly restrain protein expression once the activating stimulus dissipates (115).

1.4.4 tRNA wobble

Another critical and ubiquitous method employed to enhance translational fidelity is that of 'wobble' base pairing. There are 61 mRNA codons encoding AAs. However, there are far fewer tRNA molecules for decoding of these codons, and therefore each mRNA codon does not have a specific and unique cognate tRNA partner. As such, tRNAs possess the ability to interact with and decode multiple different mRNA codons. Standard interactions between an mRNA codon and the second and third nucleotide position of its cognate tRNA anticodon occur via Watson-Crick base pairing, with adenine-uridine (A-U), and guanine-cytosine interacting (G-C). 'Wobble' occurs at the first base (5') of the tRNA anticodon (position 34), and third base (3') of the mRNA codon. At this position, the anticodon base can interact with either its cognate pair, or

form a non-Watson Crick base pair with other nucleotides, such as Uridine at position 34 (U_{34}, wobble position) binding both A and G (Fig 1.5B).

However, wobble base pairs at U_{34} are unfavourable compared to canonical Watson-Crick interactions, due to poor base stacking and inefficient hydrogen bonding between unpaired nucleotides (116). To overcome the steric hindrance generated by unfavourable non-Watson-Crick wobble interactions, the U_{34} wobble position is chemically modified. Wobble modifications are extensive and complex, involving multiple different enzymatic cascades each adding their own chemical modification to facilitate wobble. These modifications enable wobble by stabilising the anticodon stem loop of the tRNA and enhancing base stacking, thereby reducing steric hindrance of the non-Watson-Crick base pair (117,118).

One key pathway that modifies tRNAs at U_{34} to facilitate wobble interactions is mediated by the evolutionarily conserved Elongator complex. Functioning of the Elongator complex in innate immunity is the focus of this thesis, and thus Elongator will now be introduced and described.

A.

UUU, UUC: Phe UUA, UUG: Leu	UCU, UCC, UCA, UCG: Ser	UAU, UAC: Tyr UAA, UAG: Stop	UGU, UGC: Cys UGA: Stop UGG: Trp
CUU, CUC, CUA, CUG: Leu	CCU, CCC, CCA, CCG: Pro	CAU, CAC: His CAA, CAG: Gln	CGU, CGC, CGA, CGG: Arg
AUU, AUC, AUA: Ile AUG: Met	ACU, ACC, ACA, ACG: Thr	AAU, AAC: Asn AAA, AAG: Lys	AGU, AGC: Ser AGA, AGG: Arg
GUU, GUC, GUA, GUG: Val	GCU, GCC, GCA, GCG: Ala	GAU, GAC: Asp GAA, GAG: Glu	GGU, GGC, GGA, GGG: Gly

B.

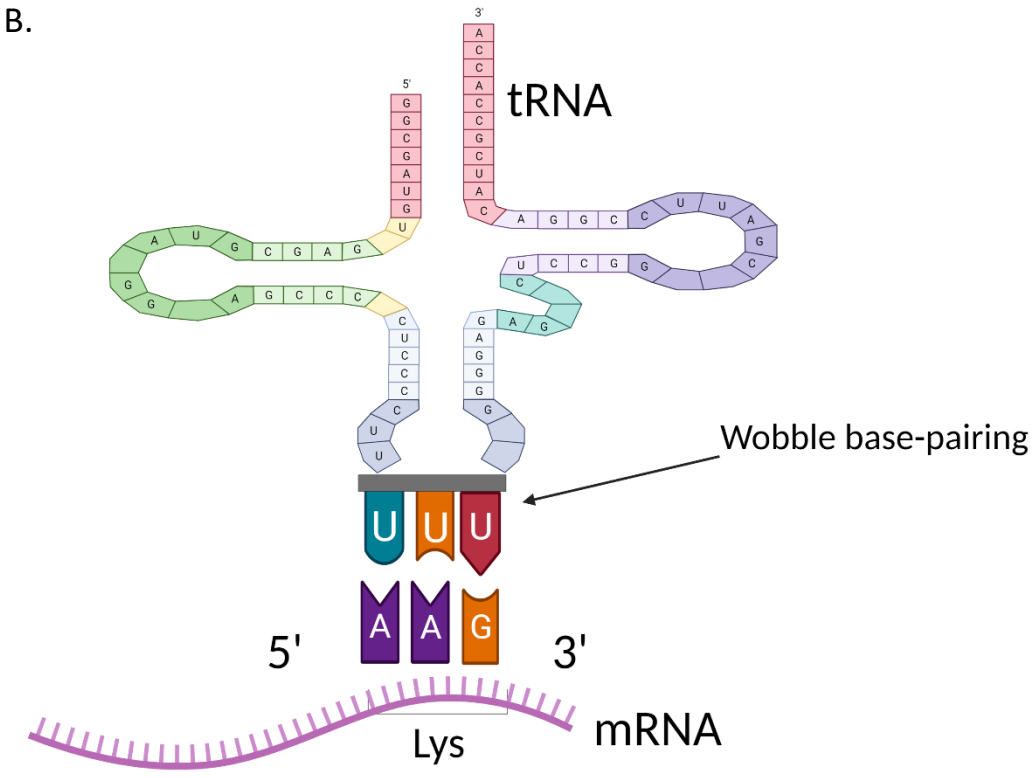


Fig 1.5 Codon degeneracy and wobble decoding

Illustration of the degeneracy of the genetic code (A). There are 64 codon combinations, with 3 encoding termination signals and 61 encoding amino acids. Amino acids can be encoded by multiple different codons. Wobble decoding of the AAG codon of Lys by its cognate $tRNA^{Lys}UUU$ at position 34 of the tRNA anticodon (B).

1.5 The Elongator complex

The Elongator complex is an ancient evolutionarily conserved hetero-dodecameric protein complex. When first discovered in 1999, it was implicated in binding hyper phosphorylated RNA polymerase II to facilitate transcriptional elongation (119). Since its initial discovery the Elongator complex has been shown to be highly evolutionarily conserved across species, as well as being associated with a number of critical functions ranging from histone acetylation (120) and exocytosis (121), to tRNA modification (122) and central nervous system development (123), on top of many others.

Elongator is composed of two copies each of six different subunits (ELP1-6), which form two separate subcomplexes of ELP123 and ELP456 (Fig 1.6). The structure of these individual subunits, and overall complex structure will be discussed in the following sections.

1.5.1 ELP1

ELP1 is the largest Elongator subunit, with a mass approximating ~150 kDa. Upon first discovery, ELP1 was characterised as inhibitor of the I-κB kinase complex associated protein (IKAP) (124), although this was soon disproven (125). Structurally, the N-terminus of ELP1 is composed of two WD40 domains, whilst the C-terminus of ELP1 contains a tetratricopeptide repeat domain (126). As will be discussed, ELP1 acts as complex scaffold, and its C-terminal dimerization is critical to assembly and functioning of the Elongator complex as a whole (127). ELP1 binds to and interacts with several of the complex subunits, and it has also been implicated in binding other cellular structures such as cytoskeletal components (128) as well as cellular proteins like Hrr25 kinase (129) and JNK-associated protein (130). It contains several phosphorylation sites critical to functioning of Elongator (131).

1.5.2 ELP2

ELP2 was first described as an interaction partner of signal transducer and activator of transcription 3 (STAT3), and was shown to modulate STAT localization (132). ELP2 is composed of two seven-bladed WD40 motifs (133). WD40 domains are key to maintaining stable protein-protein interactions within multi-protein complexes (134). In

keeping with this ELP2 is critical to both the structural integrity of the full Elongator complex, as well as to its functional role in regulating cellular processes (135–137).

1.5.3 ELP3

ELP3 is the enzymatic core of the complex. It harbours an acetyltrasferase domain (HAT) in its C-terminus and an S-adenosylmethionine (SAM) binding domain in its N-terminus (120). An iron-sulfur cluster (Fe4S4) also enables ELP3 to bind SAM with fidelity (138). The HAT domain of ELP3 has been shown to be highly related structurally to the Gcn5-related N-acetyltransferase (GNAT) domain family (120). ELP3 is the most evolutionarily conserved subunit of the complex, as bacteria and archaea possess homologs of ELP3 and not other Elongator subunits (139,140). ELP123 form a subcomplex within the structure of Elongator. As will be further discussed, the HAT, SAM and iron-sulfur cluster domains of ELP3 all work in concert to facilitate the tRNA modification carried out by Elongator across species.

1.5.4 ELP456

The second subassembly of the complex is made up of ELP456. ELP456 forms a heterohexameric ring, facilitated by RecA-like folds shared between the three subunits (126). Similar to other subunits, mutations in ELP4 has been shown to cause neurological disorders (141,142), whilst ELP5 and ELP6 have been associated with tumourigenesis (143,144). In terms of homeostatic function though, the Elp456 subcomplex has been shown to act like a Rec-like ATPase, regulating ATP hydrolysis which enables the Elongator to dissociate from modified tRNA (145).

1.5.5 Structure of the Elongator complex

Elongator assembles into two subcomplexes, ELP123 and ELP456, which combine to give a heterododecameric complex structure. Structurally, these subcomplexes assemble to generate an asymmetric bi-lobal structure. ELP123 form two wings of the complex, with dimerization of ELP1 bridging the two subcomplexes, functioning as an overall scaffold. Both copies of ELP456, which assemble independently of ELP123, are subsequently loaded onto ELP123 lobe, giving the complex a structural asymmetry (136,137). The N-terminus of ELP1 binds ELP3 and ELP4, and its C-terminus binds ELP2. ELP4 and ELP5

interact with each other, ELP3 and ELP6, whilst ELP6 only binds ELP4. The HAT and Sam domains of ELP3 form the active site, with the 4Fe-4S cluster localised at the dimerization interface of ELP3 (139). The central ring cavity of ELP456 sits over the active site and contacts the HAT and SAM domains of ELP3 (136).

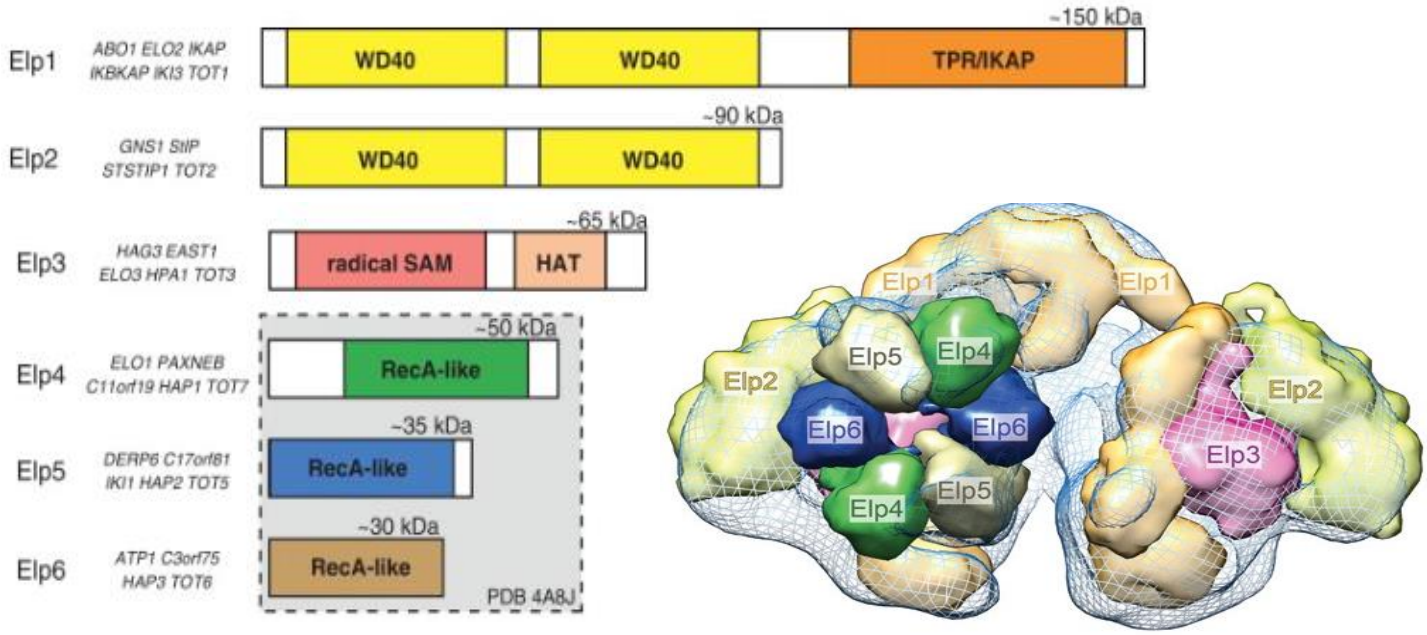


Fig 1.6 Schematic overview of both the structure of the individual Elongator subunits, and the configuration of the Elongator complex as a whole.

The Elongator complex forms a bi-lobal structure, bridged by the C-terminal of dimerized ELP1. ELP456 form a heterohexameric ring-like structure, which is loaded onto only a single lobe in an asymmetric manner. Adapted from Glatt & Muller, 2013,curr. opinions structural biology & Dauden et al, 2016, EMBO rep.

1.5.6 Evolutionary conservation of Elongator

As previously mentioned, bacteria and archaea have been shown to possess homologues of ELP3, the enzymatic core of the Elongator complex. Elongator has been shown to be conserved amongst eukaryotes, with all six subunits being detected across eukaryotic species. This evolutionary conservation is illustrated by rescue studies, whereby replacing corresponding subunits between species rescues Elongator complex function. Mouse neurons deficient in ELP1 can be rescued by human ELP1, and yeast deficient in ELP4 and ELP5 can have their phenotypes recovered with their corresponding human counterparts (146,147). An additional layer of evidence for the high levels of conservation of Elongator is that, across model organisms , *S. cerevisiae* (148), *Drosophila melanogaster* (149,150), *Arabidopsis thaliana* (151) and *Caenorhabditis elegans* (152), similar phenotypes arise upon deletion of each of the 6 subunits of the complex. This suggests that each subunit is both, conserved, as well as being necessary for full complex function, across eukaryotes. ELP3 is also present in lower level organisms of the animal kingdoms such as bacteria and archaea (138, 152).

1.5.7 Purported roles of Elongator

The purported functions of the Elongator complex have been a continual source of contention within the field. Its initial discovery and subsequent analysis of the complex strongly implicated Elongator as mainly functioning as a transcriptional regulator. However, the majority of data in recent years substantiate claims that the complex does not in fact function as a transcriptional regulator, but rather as a modifier of tRNA and thus translational fidelity.

As previously mentioned, the Elongator complex was first discovered in complex with hyperphosphorylated RNAPII and was suggested to enable transcriptional elongation. In several instances it has been shown to acetylate histone H3 and H4 (154), bind nascent mRNA (155) and to localize to the open reading frames of genes by chromatin immunoprecipitation (156). Although this all suggests a transcriptional function, the evidence is not conclusive. In contrast to the study showing Elongator localizing to genes (156), Elongator was not shown to ChIP to genes in yeast (157). Also in contrast to its initial discovery in yeast, other studies have not shown it to bind RNAPII (158). Although

Elongator was shown to acetylate histones *in vitro*, it wasn't found to acetylate nucleosomal histones (159). Furthermore, many studies assessing the localization of Elongator have found it to be predominantly cytosolic (160). Elongator has also been implicated in mediating α-tubulin acetylation (161,162). This has also been implicated as an indirect effect of Elongator, due to its tRNA modification of other cellular substrates (163).

Possibly the strongest evidence precluding a nuclear or transcriptional role for Elongator, occurs from studies showing that in mammals, observed phenotypes and transcriptional defects, in the absence of the complex can be traced back to its function in regulating translation (140). Further, the requirement for Elongator in transcriptional processes is bypassed by enhancing tRNA abundance to overcome defects in tRNA modification that occur in the absence of ELP3 (122).

1.5.8 Mechanism of Elongator-mediated tRNA modifications

As previously mentioned, uridine molecules present at the wobble base position (U34) of tRNA anticodons are chemically modified to enhance translational efficiency of non-optimal mRNA codons. This is especially important for AA-ending codons, whose poor steric interactions with anticodons leads to inefficient translation. Lysine, glutamine and glutamate are each encoded by two codons, one of which ends in AA, and these can all be decoded by a uridine at position 34 which has been chemically modified. These chemical modifications are critical to the ability to efficiently translate non-optimal codons (159, 160). Elongator plays a critical role in modifying tRNAs at the wobble base position to facilitate optimal mRNA decoding. Modifications facilitated by Elongator occur early in the process of tRNA chemical modification, and are rate-limiting U_{34} (166). The carbon 5 position of the uridine molecules are modified with methoxycarbonylmethyl (mcm^5) and 5-carbamoylmethyl (ncm^5), and Elongator enzymatic activity was shown to generate the carboxymethyl (cm), which is subsequently modified by other enzymatic pathways, before being attached to U34 nucleotides (Fig 1.7). Thiol groups (s^2) can also be used as part of this modification (xcm^5s^2), although the presence of a xcm^5 is a pre-requisite to thiolation (166) (Fig 1.7). Strong evidence for Elongator-mediated tRNA modification arose from the finding that yeast mutants lacking any of the complex subunits were resistant to killing by the

Kluyveromyces lactis toxin zymocin. Zymocin specifically targets tRNA modified at U34 positions, and Elongator subunit mutants were shown to be deficient in mcm^5 and ncm^5 (148).

Advances have been made in recent years towards elucidating the exact mechanism by which the Elongator complex modifies tRNA at the U34 position. The tRNA modifying ability of Elongator is present in all animal kingdoms, with archaea and bacteria only possessing homologues for ELP3. ELP3 is the catalytic subunit required for the enzymatic reaction leading to tRNA modification, although all the other subunits are critical for complex integrity and translational modification (123). The HAT and SAM domains of ELP3 form the active site of the complex, which binds tRNA with specificity and not histone peptides or nucleic acids, due to the spatial restriction imposed by the SAM domain effectively blocking access to the HAT domain (138,144). The anticodon stem loop is guided into the orientation for tRNA binding. tRNA ligation by ELP3 displaces an acetyl CoA blocking loop to facilitate acetyl CoA binding. Acetyl CoA is subsequently hydrolysed, and the acetyl group transferred to the SAM domain where the Fe-S cluster generates a 5' deoxyadenosine (5'dA) radical. This 5' dA radical chemical modifies the C5 of uridine at the wobble base position to give a cm^5 modification (140).

Elongator-mediated cm^5 modifications facilitate other U_{34}-modifying enzymes to chemically modify wobble bases. Cytosolic thiouridylase 1 and 2 (CTU1/CTU2), mediate thiolation of the C2 position of U_{34} following Elongator-mediated cm_5 modification (167). Trm9 in yeast and ALKBH8 in mammals methylate tRNAs following Elongator modifications, to generate ncm^5 or mcm^5 modifictaions, in addition to C2 thiolation, for proper decoding of AA-ending codons (160,168) (Fig 1.7).

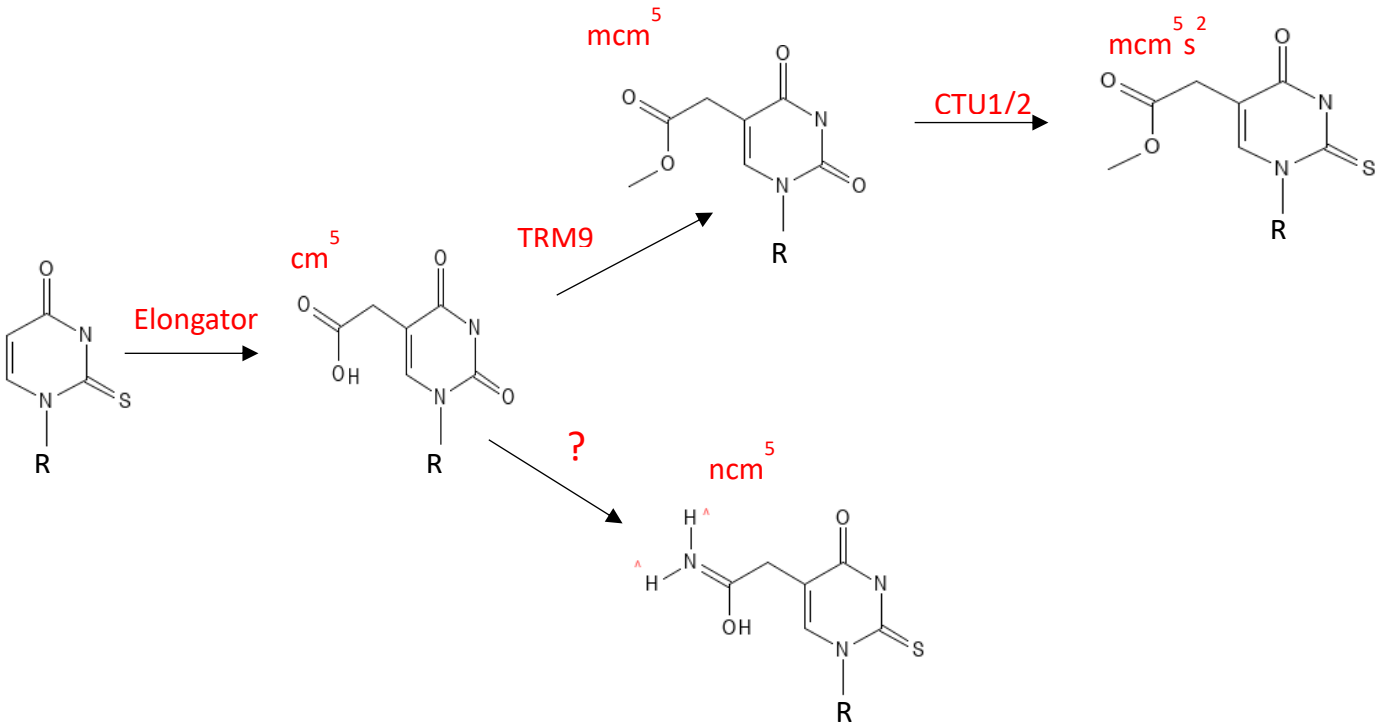


Fig 1.7 Elongator-dependent U_{34} modifications

Elongator mediates chemical modifications of Uridines at the C5 position with cm^5. This is subsequently methylated by TRM9 to give mcm^5. CTU1/CTU2 mediate thiolation of U_{34} at C2, contingent on Elongator modifications being already present at C5, to generate mcm^5s^2-modified U_{34} nucleotides. cm^5 can be further modified to give an ncm^5 modification. R; ribose sugar

1.5.9 Functional roles of Elongator

As previously mentioned, the initial implication of Elongator being involved in transcription has given away to the understanding that it functions in the cytosol in tRNA modification. Its purported functioning in yeast in the processes of transcription, exocytosis, telomeric gene silencing and DNA damage can all be linked to tRNA modification (121, 168). Elongator was shown to be essential for cell-cycle progression in fission yeast. Elongator is required for translation and protein expression of the kinase Cdr2, which is critical in coordinating mitosis and cytokinesis. Elongator mediates a translational control over cell division (169, 170). Thus, all functional roles of Elongator in yeast are likely mediated by tRNA modification.

In mammals and yeast, all evidence points to tRNA modification being the sole function of the Elongator complex. However, in the plant kingdom, Elongator appears to regulate disparate processes via transcriptional, epigenetic and translational mechanisms. Elongator appears to function both in the nucleus and cytoplasm. Nuclear Elongator regulates a variety of plant responses such as immune defence gene expression, auxin signalling, mitosis, root development and photomorphogenesis (172–175). Plant Elongator also is involved in micro RNA biogenesis (176). Elongator-mediated tRNA modifications are conserved in plants, with Elongator being required for tRNA modification in *Arabidopsis thaliana* (151). There is a body of evidence implicating Elongator in having a nuclear role potentially in transcription, suggesting that functioning of Elongator differs in plants to other eukaryotes.

Elongator is also highly conserved and functional in *Drosophila melanogaster* and *Caenorhabditis elegans*. Similar to other species, Elongator was implicated in α-tubulin acetylation in *C. elegans* (162), however this function and subsequent neurodevelopment observed in the absence of Elongator, were shown to be due to impairments in tRNA modification (152). In *D. melanogaster*, Elongator has been implicated in having a role in immunity, larval and neurodevelopment and neurotransmitter release (149,150,177,178).

1.5.10 Elongator in neurological disorders

A complex array of pathologies and diseases arise from defects in tRNA modifications (179). Defects in tRNA modifications and subsequent mRNA decoding and translation, oftentimes result in protein aggregation and accumulation. The brain is particularly susceptible thus, to dysregulated tRNA modification and protein aggregation. Elongator has been implicated in playing a vital role in neurodevelopment and neurodegeneration. Mutations in various Elongator subunits are associated with a profound spectrum of neurological conditions.

Elp1 mutations lead to development of the rare but fatal neuropathy, Familial dysautonomia (FD). A non-coding mutation in *Elp1* in the splice donor site of exon 20, leading to exon skipping and reduction in ELP1 expression (179, 180). Reduced Elongator-dependent modifications in peripheral neurons leads to serious reduction in expression proteins enriched in AA-ending codons (182). Fibroblasts and cerebrum from FD patients also have reduced Elongator-dependent modifications (183). Patients with FD suffer from sensitivity to pain and temperature, GI dysfunction, cardiovascular issues and reduced life expectancy (184). Inherited missense mutations in *Elp2* have been implicated in intellectual disability in several cases (184, 185). Amyotrophic lateral sclerosis (ALS), is commonly known as motor neuron disease, is a neurodegenerative disease characterised by motor neuron degeneration and muscle atrophy. Allelic variants of *Elp3* are associated with development of ALS (187). Reduced *Elp3* levels leads to impaired tRNA modifications, and accumulation of superoxide dismutase 1 and subsequent neuropathology (188). Mutations in *Elp4* are implicated in the development of rolandic epilepsy (141), as well as in autism and intellectual disability (142). Mutations in *Elp6* have defined as causative for the phenotype observed in the '*wobbly*' mouse. Mutations in *Elp6* lead to dysregulated complex integrity and reduced translational modification. This induces substantial death of purkinje neurons and neuroinflammation mediated by the NLRP3 inflammasome (189). The ensuing neurodegeneration leads to ataxic symptoms.

In summary, Elongator is critical to the development and homeostasis of the central nervous system.

1.5.11 Elongator in cancer

Aberrant activity of tRNA modifying-enzymes has the ability to induce the development of cancer, or translationally reprogram cells to a malignant phenotype (190). Just as impaired levels of different Elongator subunits can lead to the development of neurological conditions, enhanced levels and activity of Elongator can lead to cancer and tumourigenicity.

Germline loss-of-function in *Elp1* leads to a high level of genetic predisposition (40%) for paediatric medulloblastoma subgroup sonic hedgehog. Loss of ELP1 expression and function in these patients leads to destabilisation and disbanding of Elongator. In line with this, these paediatric patients had a drastic reduction in Elongator-dependent U_{34} modifications, with downregulated proteins enriched in AA-ending codons, and upregulated proteins abundant in the synonymous AG-ending lysine codon. Loss of ELP1 lead to a pronounced enhancement of the unfolded protein response, and strong downregulation of neurogenesis and synaptic signalling (191).

Elp5 deficiency has been implicated in regulating responses to treatment in gallbladder cancer. Gemcitabine is the front line treatment for patients with cancer of the gallbladder. However, patients show poor responsiveness to gemcitabine and mortality rates are high (191, 192). Mechanistically, *Elp5* deficiency leads to dysregulated integrity and functioning of Elongator. As a result, Elongator-mediated codon-dependent translation of heterologous nuclear ribonucleoprotein Q (hnRNPQ) is impaired. hnRNPQ enables translation of P53, thus loss of *Elp3* leads to abrogation of p53 translation and gemcitabine resistance. ELP5 and ELP6 have also both been found to be important for the motility and invasive capabilities of melanoma cells (143).

Altered expression of ELP3, the enzymatic core of the complex, has been implicated in several different malignant phenotypes. ELP3 was displayed to be upregulated in hepatocellular carcinoma, correlating with increased AKT phosphorylation and expression of the matrix metalloproteinases 2 and 9 and cancer metastasis (194). Elp3 plays a role in Wnt-dependent tumour development in the intestine. Wnt signalling enhances ELP3 expression in differentiated intestinal tuft cells expressing Lgr5, denoting their malignancy. ELP3 regulates translation of SOX9, which maintains the pool of these

cancerous stem cells (195). Elp3 is furthermore upregulated in breast cancer, where it enables invasiveness by mediating translation of DEK. DEK mediates IRES-dependent (internal ribosome entry site) translation of LEF1, which is a transcription cancer with proinvasive functioning in breast cancer (196). ELP3 also is important for therapy resistance in melanoma. Melanoma patients with the $BRAF^{V600E}$ mutation display enhanced resistance to therapeutic treatment (197). Enhanced PI3K signalling leads to enhanced ELP3 expression in an mTORC2-dependent manner. ELP3 subsequently mediates translation of hypoxia inducible factor 1 alpha (HIF-1α) in a codon-dependent manner, which leads to a metabolic reprogramming towards glycolysis and enhanced resistance to treatment (198).

One aspect of Elongator biology that is insufficiently studied and understood, is the role and functioning of the Elongator complex in the context of innate immunity. Concerted study and basic research is required to establish an understanding of how Elongator functions in innate immunity.

1.6 Aims

Elongator mediates modifications of tRNA to enable efficient decoding and translation of mRNA codons. The regulation of immune gene expression in response to pathogen infection has been extensively analysed at the level of transcription, translational regulation of immune responses is less well understood. Since Elongator plays a critical role in translational regulation the aim of this thesis was to appraise the function of Elongator to innate immune responses in macrophages.

Specifically, the aims of this thesis are:

1. Examine the contribution of Elongator to innate immune protein expression via quantitative proteomics.
2. Determine the mechanism by which Elongator regulates IFN-I induction and signalling in macrophages
3. Assess the contribution of Elongator to different PRR-mediated signalling pathways and to anti-viral immunity.

Chapter 2 - Materials & Methods

2.1 Materials

2.1.1 Buffers and solutions

Buffer/solution	Composition
10 X Tris-buffered saline (TBS)	1.5 M NaCl 200 mM Tris Adjust to pH 7.5
10 X Running Buffer – Western blot	250 mM Tris 1.9 M Glycine 35 mM SDS
10 X transfer buffer - Western blot	250 mM Tris 1.9 M Glycine
Washing buffer - Western blot	1 X TBS/0.1 % (v/v) Tween
3X sample buffer - Western blot	30 % (v/v) Glycerol 6 % (w/v) SDS 0.3 % (w/v) Bromophenol blue 187.5 mM Tris pH 6.8 150 mM dithiothreitol (DTT) added fresh

Table 2.1 Buffers and solutions

2.1.2 Cell Culture

Adherent cells were cultured in vented, cell culture treated flasks (T25, T75, or T175 depending on the cell number) from Corning. Cells were cultured at 37 ^{o}C and 5 % CO_2. Sterile cell scrapers were from Fisher Scientific.

2.1.2.1 Cells

In order to evaluate the contribution of Elongator to innate immune responses, we previously utilised CRISPR/Cas9 to knockout *Elp3*, the catalytic and enzymatic core of the complex. iBMDMs stably expressing Cas9 endonuclease were transfected with a gRNA targeting *Elp3*. Control WT iBMDMs utilised were transfected with a gRNA targeting GFP.

2.1.2.2 Cell culture reagents

REAGENT	COMPANY	IDENTIFYING CODE
Dulbecco's modified eagle medium (DMEM) plus GlutaMAX™	Gibco	61965059
Foetal calf serum (FCS)	Gibco	10500-064
Penicillin/Streptomycin (pen/strep)	Sigma-Aldrich	P4333-100ML
Dulbecco's phosphate buffered saline (PBS)	Sigma-Aldrich	D8537-500ML
Dimethyl sulfoxide (DMSO)	Sigma-Aldrich	D8418-250ML
Trypan blue	Fisher Scientific	10593524
Lipofectamine™	Invivogen	11668019
Opti-MEM™	Gibco	31985070

Table 2.2 Cell culture reagents

2.1.2.3 Cell culture stimulants & viruses

STIMULANTS	DESCRIPTION	CONCENTRATION	COMPANY	IDENTIFYING CODE
LPS	Lipopolysaccharide from *E. coli* serotype EH100(ra)	100 ng/ml	Enzo	ALX-581-010
CLO75	Thiazoloquinoline compound	5 µg/ml	InvivoGen	tlrl-c75-5
3p-hpRNA	5'triphosphate hairpin RNA	100 ng/ml	Invivogen	Tlrl-hprna
VACV-70mer	dsDNA motif derived from Vaccinia virus genome	2.5 µg/ml	N/A	N/A
High molecular weight Poly (I:C)	Synthetic dsRNA analog	10 µg/ml	Invivogen	Tlrl pic
2'-3' cGAMP	Cyclic di-nucleotide second messenger	5 µg/ml	Invivogen	Tlrl-nacga23
IFNγ	Recombinant murine IFNγ	50 ng/ml	Peprotech	315-05
Influenza virus	A/Puerto Rico/8/34 (PR8): mouse adapted H1N1 IAV strain	MOI of 5	Gifted by Dr. Bernadette Van Den Hoogen (Erasmus MC)	N/A
Respiratory syncytial virus	RSV A2 strain	MOI of 5	Gifted by Dr. Bernadette Van Den Hoogen (Erasmus MC)	N/A

Table 2.3 Cell culture stimulants & viruses

Gene	Forward (5'- 3')	Reverse (5'- 3')
Actin	TCCAGCCTTCCTTCTTGGGT	GCACTGTGTTGGCATAGAGGT
Ifnb	ATGGTGGTCCGAGCAGAGAT	CCACCACTCATTCTGAGGCA
Ifna	ACCCTCCTAGACTCATTCTG	GTTTCTTCTCTCTCAGGTACAC
Irf7	TTGGATCTACTGTGGGCCCA	CTTGCCAGAAATGATCCTGGG
Irf3	TGAGTTTGTGACTCCAGGGG	GTAGGTTTTCCTGGGAGTGAG
Irf2	CTGGAGGAGCAGATAAATTCC	GTATGGATCGCCCAGTTTC
Irf5	GGCTTCAGTGGGTCAAC	GTGTACTTCCCTGTCTCTTTAG
Il-6	AAGAGTTGTGCAATGGCAATTCTG	ATAGGCAAATTTCCTGATTATATCCAGT
Il-1b	GTGAAATGCCACCTTTTGACAGTGATGAG	CTGCTGCGAGATTTGAAGCTGGATG
Cxcl10	TCTGAGTGGGACTCAAGGGAT	TCGTGGCAATGATCTCAACACG
Ccl5	CTCACCATATGGCTCGGACA	ACAAACACGACTGCAAGATTGG
Il12p40	GTGTAACCAGAAAGGTGCGTTC	TCGGACCCTGCAGGGAAC
Eif2ak2	CGTTTCTTGCCTCCTGCTTTG	TCGGACCCTGCAGGGAAC
Stat1	TCACAGTGGTTCGAGCTTCAG	GCAAACGAGACATCATAGGCA
Stat3	GGGCATTTTTATGGCTTTCAAT	GTTAACCCAGGCACACAGACTTC
Arg1	CTCCAAGCCAAAGTCCTTAGA	AGGAGCTGTCATTAGGGACATC
Socs3	GCAGGAGAGCGGATTCTACT	ACGCTCAACGTGAAGAAGTG
Irf1	CCATTCACACAGGCCGATAC	GCCCTTGTTCCTACTCTGATC
Isg15	CTAGAGCCTGCAGCAATG	CACCAATCTTCTGGGCAATC
p205	GTATGAGTGAAGAAAAGACTGAC	GGATATTGGTGACTGGCATG
Matrix protein	ATGAGYCTTYTAACCGAGGTCGAAACG	TGGACAAANCGTCTACGCTGCAG

2.1.3 qRT-PCR primers

Table 2.4 List of qRT-PCR primers used

2.1.4 Antibodies

Antibody	Raised in	Company	Cat. #	Dilution
β-Actin	Mouse	Sigma-Aldrich	A5316	1:2000
ELP3	Rabbit	Abcam	ab190907	1:2000
IRF3	Rabbit	Santa cruz	sc-9082	1:1000
P-IRF3	Rabbit	Cell signalling technology (CST)	4947	1:500
STAT1	Rabbit	CST	9172	1:1000
P-STAT1	Rabbit	CST	9167	1:1000
STAT2	Mouse	CST	sc-514193	1:1000
P-STAT2 (Y689)	Rabbit	Millipore	07-224	1:1000
STAT3	Rabbit	CST	4904T	1:1000
P-STAT3	Rabbit	CST	9145T	1:1000
NF-κB: P65	Mouse	Santa cruz	sc-8008	1:1000
NF-κB: P-P65	Rabbit	CST	3033S	1:1000
ASC	Rabbit	CST	67824S	1:500
TYK2	Rabbit	Proteintech	16412-1-AP	1:1000
JAK1	Rabbit	CST	3332S	1:1000
IFNAR1	Rabbit	Thermo Fisher	PA5-79442	1:1000
PKR	Rabbit	Santa cruz	sc-708	1:500
P205	N/A	Generated by K. Fitzgerald lab, described in (72)	N/A	1:500
Mouse IRDye 680LT secondary	Goat	LiCOR	926-68070	1:10,000
Rabbit IRDye 800CW secondary	Goat	LiCOR	926-32211	1:10,000

Table 2.5 List of Antibodies used for immunoblotting

2.1.5 Method-specific materials

2.1.5.1 SDS-PAGE & western blot

SDS-PAGE & western blot	Company	Identifying code
Protogel (30% Acrylamide (w/v), 0.8% Bisacrylamide (w/v))	National Diagnostics	NAT1260
Tetramethylethylenediamine (TEMED)	Sigma Aldrich	T7024-50ML
PageRuler plus protein ladder, 10 to 250 kDa	ThermoFisher scientific	26620
Amersham Protran 0.45 µm Nitrocellulose membrane	Cytiva	GE10600007

Table 2.6 SDS-PAGE specific materials

2.1.5.2 qRT-PCR & RNA isolation

RNA isolation, reverse transcription and qPCR	Company	Idenifying code
High Pure total RNA Isolation Kit	Roche	11828665001
Ultrapure DNase/RNase-free water	Invitrogen	10977035

dATP, dCTP, dGTP, dTTP, each [100 mM]	Brennan & Co	N0446S
Random Hexamer	IDT	N/A
Moloney Murine Leukemia Virus Reverse Transcriptase (M-MLV RT)	Promega	M1701
M-MLV RT 5X Buffer	Promega	M531A
RNase OUT recombinant ribonuclease inhibitor	ThermoFisher Scientific	10777019
PowerUp SYBR Green Master Mix	ThermoFisher Scientific	A25778
MicroAmp fast optical 96-well plates	Applied Biosystems	N8010560

Table 2.7 qRT-PCR-specific materials

2.2 Methods

2.2.1 Cell culture

Immortalised Bone marrow derived macrophages (iBMDMs) were grown in DMEM with GlutaMAX medium supplemented with 10% (v/v) FCS & penicillin/streptomycin (50 µg/ml). Cells were cultured at 37 ^{o}C and 5 % CO_2. Cells were passaged/subcultured upon reaching 80-85 % confluency. Cells were scraped using a sterile cell scraper, and centrifuged at 180 g for 5 minutes. The supernatant was subsequently discarded and the cell pellets were resuspended with pre-warmed media. Cells were either passaged into 75 cm^3 vented cell culture flasks, or counted for experiments. Cells were counted using a TC20 automated cell counter (BioRad). Prior to counting, cells were diluted 1:1 with trypan blue, to stain and account for dead cells. Cells were subsequently seeded at a density of 5 x 10^5 cells/ml in appropriate cell culture plates, before being placed in an incubator to allow adherence prior to stimulation or infection.

2.2.2 Stimulation of cells

The TLR ligands, LPS and CLO75, were used to stimulate cells at the concentrations indicated in Table 2.3.

3p-hpRNA, poly(I:C) and cGAMP were transfected into cells using lipofectamine 2000 for stimulation. For transfection, lipofectamine was used at a concentration of 1 µg/ml. Both lipofectamine and the nucleic acid were diluted to the appropriate concentration in Opti-MEM medium and allowed to stand for 5 mins in microcentrifuge tubes. The two tubes were then mixed to give a homogenous solution, and incubated for 20 mins at room temperature before addition to cells seeded in cell culture plates.

VACV-70mer strands of DNA were annealed at 95 ^{o}C for 5 minutes, before being left at room temperature to cool down. 70mer was transfected into cells utilising the same method as with cGAMP and RNA ligands.

2.2.3 RSV & IAV PR8 infection

The volume of virus needed for an experiment was calculated using:

Vol. of virus = # cells per well*MOI/viral titre

The Multiplicity of infection utilised in experiments is indicated in Table 2.3. The medium was removed from adhered cells in cell culture plates, and cells were washed using 1 X PBS. The inoculum, composed of virus and media, was subsequently added to cells. Plates were placed in incubator at 37 ^{0}C and 5 % CO_2 with the inoculum for 1 h with IAV and 2 h for RSV. The inoculum was then removed and cells washed with 1 X PBS, and DMEM added to the wells. Cells were incubated for the times indicated in figure legends at 37 ^{o}C and 5 % CO_2.

2.2.4 RNA isolation

Cells were stimulated for times indicated in figure legends, and RNA was extracted from cells in culture using the High Pure RNA isolation kit (Roche). Medium was removed from stimulated cells, and Lysis/binding buffer added to wells at a ratio of 2:1 with PBS. Total RNA was isolated according to manufacturer's instructions.

2.2.5 RNA analysis via qRT-PCR

2.2.5.1 Reverse transcription

Complementary DNA was synthesized from RNA samples using reverse transcription. A reaction master mix, shown in Table 2.8, was made up on ice. 5 µl of RNA was mixed with 5 µl of reaction master mix to give a homogenous solution. The solutions were briefly centrifuged, and underwent the reverse transcription reaction in a Nexus gradient thermocycler (Eppendorf), using the conditions shown in table 2.9. cDNA was subsequently diluted 1:2 with RNA and DNA free ultrapure water, before being analysed by qPCR immediately, or stored at -20 ^{o}C for future analysis.

RT reaction components	Volume/reaction (µl)
M-MLV RT	0.25
M-MLV 5 X RT buffer	2
dNTPS	2
RNAse OUT	0.25
Random hexamer	0.5
Final volume	5

Table 2.8 Reverse transcription master mix

Time (mins)	Temperature (^{o}C)
10	25
50	42
3	95

Table 2.9 Reverse transcription thermocycling conditions

2.2.5.2 qRT-PCR

2 µl of cDNA was combined with 8 µl of a master mix containing gene-specific primers (table 2.4) and PowerUp SYBR green, shown in Table 2.10 . the cDNA being analysed was initially added to MicroAmp fast plates, before 8 µl of master mix was added. Plates were ealed and centrifuged for 5 mins to ensure homogenous mixing of cDNA and master mix. The qRT-PCR reaction was performed using a QuantStudio 3 Real-Time PCR machine (Apllied Biosystems). The conditions utilised are described in Table 2.11. Results were analysed using the comparative C_T method. Samples were normalised to the housekeeping gene *β-actin*, and calculated as relative expression to the WT unstimulated control, set to 1.

qRT-PCR reaction	Volume (µl)
Forward primer (5 µM)	0.5
Reverse primer (5 µM)	0.5
Ultrapure water	2
SYBR green	5

Table 2.10 qRT-PCR master mix

Time	Temperature (°C)	Cycles
2 mins	95	1
10 secs	95	40
30 secs	60	" "
hold	4	N/A

Table 2.11 qRT-PCR thermocycling conditions

2.2.6 SDS PAGE & Western blotting

2.2.6.1 Cell lysate preparation

Cells were harvested post stimulation on ice. Supernatants were removed and cells were washed with 1 X ice-cold PBS. Sample buffer containing DTT was added into the wells and cells were scraped and transferred to microcentrifuge tubes. Lysates were boiled for 5 mins at 95 °C. Lysates were subjected SDS-PAGE or stored at -20 °C for future analysis.

2.2.6.2 SDS-Poly acrylamide gel electrophoresis (PAGE)

SDS-PAGE gels were composed a day or two prior to running given samples. Different resolving gel percentages were utilised depending on the molecular weight of the protein of interest, with gel compositions being shown in Table 2.12. 5 µl of prestained protein marker was loaded to provide a molecular weight ladder against which the size of protein of interest could be identified. 15 – 20 µl of sample were loaded into the subsequent wells. Using a Minj-protean tetra cell system (BioRad), gels were run in 1 X running buffer at 80 volts until they entered the resolving gel from the stacking gel. The current was then raised to 120 volts until the dye reached the end of the resolving gel.

2x gels	8 % resolving	10 % resolving	15 % resolving	Stacking gel
H_2O	9.3 ml	8.2 ml	4.6 ml	5.5 ml
30 % acrylamide	5.3 ml	6.6 ml	10 ml	1.3 ml
SDS (10 %)	200 µl	200 µl	200 µl	80 µl
1.5 M Tris pH 8.8	5 ml	5 ml	5 ml	-
1 M Tris pH 6.8	-	-	-	1
APS (10 %)	200 µl	200 µl	200 µl	80 µl
TEMED	12 µl	10 µl	8 µl	8 µl

Table 2.12 SDS-PAGE gel composition

2.2.6.3 Semi-dry transfer

Proteins were transferred from gels to 0.45 µm nitrocellulose membrane. Gel, nitrocellulose membrane and 6 X filter paper were soaked in cold 1 X transfer buffer. Air bubbles were removed from the gel, membrane filter paper sandwich by rolling with a 25 ml pipette. Proteins were transferred using a semi-dry transfer method (Biometra), ran at 75 mA per gel.

2.2.6.4 Immunoblotting

Following semi-dry transfer, membranes were blocked in 10 ml of 5 % BSA/TBS-Tween 0.1 % (v/v) for 1 h at room temperature, to prevent non-specific binding of antibodies.

Membranes were then incubated with primary antibodies at the indicated concentrations (Table 2.5) overnight at 4 ^{o}C. Following primary antibody incubation, membranes were washed 4 times for 5 mins, in TBS-Tween 0.1 % (v/v). Washed membranes were incubated with secondary for 1 h at room temperature. Membranes were again washed 4 times for 5 mins and images using the Odyssey Imaging System (LI-COR Biosciences).

2.2.7 Quantitative Proteomics

2.2.7.1 Sample preparation

WT and *Elp3*$^{-/-}$ iBMDMs were seeded in quadruplicate in 6-well plates at 5 x 10^5 cells/ml. Cells were stimulated with LPS (100 ng/ml) for 6 or 12 h. Cells were harvested on ice, before being spun down at 180 g for 5 mins, and washed with ice-cold 1 X PBS. Cell suspensions were centrifuged again before being snap-frozen in liquid nitrogen. Samples were stored at -80 ^{o}C briefly before being sent for unbiased quantitative proteomics analysis to collaborators in TU Munich (Darya Haas & Andreas Pichlmair). Workflow for sample preparation is shown schematically in Fig 3.2.

2.2.7.2 Cell viability

The exact experimental setup for proteomics analysis was repeated. Harvested cells were diluted 1:1 with trypan blue to identify and exclude dead cells, and counted using a TC_{20} automated cell counter (BioRad).

2.2.7.3 Profile Plots

Profile plots for proteins of interest were generated based off their Label-free quantification intensity as detected by mass spectrometry

2.2.7.4 Ingenuity pathway (IPA) & upstream regulator analysis (URA)

Prior to proteomic analysis by mass spec, protein concentrations were equalised across all WT and *Elp3*$^{-/-}$ samples. Initial differential expression of proteins in *Elp3*$^{-/-}$ relative to WT cells, were based of a Log2 fold difference of 2 or greater. Differential protein expression analysis was performed by Darya Haas (TU Munich). Proteins were estimated as differential expressed in *Elp3*$^{-/-}$ cells relative to their expression in their WT stimulated

counterpart. Ingenuity Pathway analysis (IPA) was performed to group differentially expressed proteins in *Elp3*$^{-/-}$ into biological processes, based on pairwise comparison for each timepoint. The enrichment cut-off was relaxed to –Log10 p-val<1.3, so as to broaden the scope of analysis of over-represented pathways. IPA analysis was also analysed by z-score, or highest degree of change. Upstream regulator analysis was performed utilising IPA package with enrichment displayed as –Log10 p-val or z-score.

Initial IPA analysis was performed by Darya Haas, I selected the enrichment cutoffs following discussion with D. Haas, and subsequently generated graphs shown in chapter 3 from the data files of differentially enriched pathways provided.

2.2.8 Cycloheximide chase assay

Cells were seeded at 5 X 10^5 cells/ml and left to adhere at 37 ^oC and 5 % CO_2. Cells were treated with cycloheximide (50 µg/ml) for 8 h. Samples were washed and scraped in ice-cold 1 X PBS. Cells were spun down at 180 g for 5 mins. Cell pellets were washed in 1 X PBS and spun down again. Samples were lysed in sample buffer and prepped for SDS-PAGE as described in 2.2.6.1.

2.2.9 Codon usage

Coding sequences of mRNA were taken from the NCBI Consensus coding sequence (ccds) database. Codon content analysis was performed using Sequence Manipulation Suite V2.

2.2.10 Statistical analysis

Graphpad prism 9 was used to analyse all data. Data are presented as mean $\pm$ SEM of three independent experiments. Significance compared to WT counterpart is represented as *p<0.05. Statistical tests used are described in the appropriate figure legends.

Results

Chapter 3 – Quantitative proteomics analysis of mock and LPS-treated macrophages deficient in *Elp3*

3.1 Introduction

The roles and functions regulated by the Elongator complex are multifaceted and variable. Elongator is highly conserved across the animal kingdom, with homologs of ELP3, its catalytic core, being functionally present in higher organisms like humans, all the way back to bacterial Archaea that inhabit hydrothermal chimneys in the earth's crust 3000 metres below the sea (140). In mammals, Elongator chemically modifies tRNA molecules at the wobble base position of the anticodon loop, to enable the fidelity and proper efficiency of mRNA transcript translation. This impacts and regulates processes ranging from CNS functioning and neuronal migration, to cell division and cancer development (123,170,182). In lower organisms, Elongator appears to function in translational regulation, and also in epigenetic regulation of gene expression. An intriguing function of Elongator in lower organisms such as *D. melanogaster* and *A.thaliana,* is its vital and necessary role in immune and defence responses. Genetic deletion of *Elp3* in *Drosophila* has potent consequences for immunity. Pupal stage larval lethality occurs upon deletion of *Elp3*. Furthermore, melanotic nodules develop in mutant larvae, which is a physiological consequence of impaired innate immunity (149). The drosophila protein poly, which is a functional homolog of ELP6, was shown to interact with the insulin receptor and regulate Insulin-TOR signalling (177). In the model plant system of *A. thaliana*, Elongator has been uncovered as a critical and indispensable regulator of immunity and defence responses. Elongator is a crucial mediator of both basal and pathogen-triggered immunity. ELP2 interacts with and regulates the activation of NPR1, a pivotal coactivator of Arabidopsis transcription and induction of immunity. Elongator also directly activates immune defence genes, thus exerting control over transcriptional reprogamming in response to infection (172,199). Elongator exerts further influence over *A. thaliana* immunity by mediating processes like oxidative stress resistance and NAD^+-induced defence to pathogen infection (200,201).

Though not as abundant as the literature pertaining to plants, there are data both directly and indirectly implicating Elongator in the regulation of mammalian immune systems and responses. The ELP2 subunit of the Elongator protein was initially termed, STAT3 Interacting Protein 1, due to the fact that as its name suggests, it was found to bind STAT3 and potentially regulate its stimulant-dependent activation (132). This ELP2-STAT3 interaction has been implicated in the regulation of renal fibrosis and inflammation (202). Elp1 has been found to potentially interact with IRF4, in an analysis of networks regulating IFN-I production (203). Cellular oxidative stress responses appear to be modulated by Elongator. ELP3 appears to inactivate G6PD, which carries out the rate-limiting step of the pentose phosphate pathway (204). Dysfunction and dysregulation of the Elongator complex in mammals can lead to aberrant activation of immune responses. A single point mutation in ELP6 is sufficient to cause NLRP3 inflammasome-mediated neuroinflammation, leading to neurodegeneration and ataxia(189). In familial dysautonomia, where mutations in ELP1 destabilize the Elongator complex and cause neurodegeneration, there is potently enhanced mitochondrial depolarization, oxidative stress and caspase-mediated neuronal apoptosis (205,206). Intriguingly in a cancer setting, Elongator is required for the translation of HIF1α which enables resistance to targeted therapy. HIF1α is a key regulator of metabolism and immune gene expression during hypoxia and inflammation (198). In recent years data has begun to emerge implicating Elongator in the regulation and orchestration of adaptive and innate immunity. The loss of Elp3 from HSCs in mice led to a complete abrogation and blockade of haematopoiesis and subsequent bone marrow failure (207). In an adaptive context, ELP3 appears to be upregulated following T-cell activation. Loss of ELP3 leads to impairment of Tfh cell functioning (208). The potential functioning and contribution of the Elongator complex in mammalian innate immunity is poorly understood and investigated. Macrophages are key orchestrators of innate immunity. They sense, integrate and respond to a diverse and multifaceted array of signals and stimuli. Macrophages perform critical functions in the shaping and coordinating of innate responses to exogenous microbial stimulus and infection, to host intrinsic processes like sterile inflammation, wound healing and resolution of inflammation (209). We became interested by the juxtaposition of a highly conserved protein complex like Elongator, which is necessary for the fundamental process of translation, and an under-

assessed but potentially burgeoning function in mammalian innate immunity. Accordingly, we sought to examine and assess the functioning of Elongator in mouse macrophages to evaluate its contribution to innate immunity.

3.2 Results

3.2.1 Analysis of the proteome in Elp3$^{-/-}$ mouse macrophages

In order to evaluate the contribution of Elongator to innate immune responses, we previously utilised CRISPR/Cas9 to knockout *Elp3*, the catalytic and enzymatic core of the complex. iBMDMs stably expressing Cas9 endonuclease were transfected with a gRNA targeting Elp3. Control WT iBMDMs utilised were transfected with a gRNA targeting GFP. Figure 3.1 shows a western blot confirming the knockout of ELP3.

As a model of a canonical inflammatory stimulus, LPS-mediated TLR4 stimulation was used to assess the role of ELP3 in the context of macrophage-mediated innate immune response. In lower level organisms such as plants, Elongator has been shown on occasion, to regulate protein expression at the level of gene expression by localizing to the nucleus (172). In a mammalian setting however, all processes or roles mediated by ELP3 appear to derive from its role in translation, via the modification of tRNA molecules at the wobble base position. Due to this well understood role in translation, we utilised an unbiased quantitative proteomics approach to analyse the proteome of *Elp3*$^{-/-}$ iBMDMs both basally and following LPS stimulation. This experimental approach was facilitated by a collaboration with Prof. Andreas Pichlmair's group at the Technical University of Munich, where mass spectrometry was performed.

WT and *Elp3*$^{-/-}$ cells were seeded in 6-well plates and were untreated, or stimulated with LPS for 6 or 12 hours in quadruplicate (Fig 3.2). Cells were subsequently harvested and samples generated for proteomic analysis by Andreas Pichlmair's group. Prior to samples being sent for proteomic analysis by mass spectrometry, the experimental setup was replicated identically. This was performed to assess the viability of the cells lacking ELP3 basally and following LPS stimulation. Trypan blue staining was used to assess cell viability. Viability of *Elp3*$^{-/-}$ cells was only slightly diminished relative to their mock and LPS-stimulated WT counterparts (Fig 3.3). This suggests that any potential effects on

protein expression levels in the absence of ELP3 does not result from elevated levels of cell death.

Label free quantification (LFQ) intensity of peptides detected by mass spectrometry was used to generate protein expression plots for Elongator complex subunits and key transcription factors. This confirmed the western blot analysis (Fig 3.1), since ELP3 peptides were not detected in the *Elp3* CRISPR/Cas9 KO iBMDMs (Fig 3.4). Furthermore, the absence of ELP3 affected the expression of other Elongator complex subunits, ELP2 and ELP4, which are key to the structural integrity of the complex (Fig 3.4).

3.2.2 Role for ELP3 in IRF7 and STAT1 transcription factor expression

IRF3 and IRF7 transcription factors are critical regulators of the innate immune response, functioning as pivotal nodes for integrating intracellular and extracellular pathways. IRF3 protein expression was not impaired by the absence of ELP3. LPS-mediated IRF7 expression however, was ablated in *Elp3*$^{-/-}$ cells (Fig 3.5). Furthermore, expression of antiviral effectors IFIT1 and IFIT3 were completely impaired both basally and following LPS treatment in *Elp3*$^{-/-}$ cells (Fig 3.5). STAT proteins function as key and integral transducers of innate immune signalling, as well as functioning as transcription factors to mediate the resulting immune gene expression and induction. Intriguingly STAT1 protein expression was diminished in cells lacking ELP3 (Fig 3.6). In contrast, STAT3 and STAT6 expression appears to be unaffected in *Elp3*$^{-/-}$ cells, suggesting the effect is more specific to STAT1, rather than a general effect on STAT family protein expression (Fig 3.6). The NF-κB family of transcription factors are fundamental to the co-ordination and execution of innate immune signalling and responses. Expression of NFκB1 (p50) and NFκB2 (p52) appears to be unaffected in cells lacking ELP3 (Fig 3.6).

3.2.3 Altered proteome in Elp3$^{-/-}$ *macrophages compared to WT cells*

Unbiased Quantitative proteomic analysis showed a number of differentially expressed proteins in *Elp3*$^{-/-}$ cells compared to WT cells. Proteins were considered significantly up or downregulated if there was a log2 fold difference of 2 or greater in KO cells relative to their WT mock or LPS-stimulated counterpart. 330 proteins were found to be upregulated in *Elp3*$^{-/-}$ cells compared to WT. A significant proportion of these upregulated proteins were seen basally in mock stimulated samples (50 %, 190 proteins)

rather than after LPS stimulation. In *Elp3*$^{-/-}$ cells stimulated with LPS for 6 & 12 h there were 50 and 93 upregulated proteins respectively, relative to WT cells. 47 proteins were found to be upregulated in both LPS-stimulated samples and not mock samples (Fig 3.7a). 380 proteins were found to be downregulated in *Elp3*$^{-/-}$ iBMDMs. 167 downregulated proteins were basal differences occurring in mock stimulated samples in the absence of ELP3. The remaining 163 proteins were downregulated in the absence of ELP3 not basally but following LPS stimulation. 20 of these proteins were downregulated in both 6 & 12 h stimulated samples, while 58 and 85 proteins were downregulated solely after 6 and 12 h LPS respectively (Fig 3.7b).

Next we decided to analyse the distribution of differential protein expression in mock and LPS-stimulated *Elp3*$^{-/-}$ cells relative to WT samples. The distribution of differentially expressed proteins in mock, 6 & 12 h LPS samples are represented in volcano plots (Fig 3.8, 3.9 & 3.10 respectively). The right and left arms of the plots correspond to down and upregulated proteins respectively.

3.2.4 The ELP3-dependent proteome is enriched with proteins involved in antigen presentation and Interferon signalling

Differentially expressed proteins in Elp3$^{-/-}$ relative to WT cells were grouped using Ingenuity pathway analysis (IPA). This enabled the assessment and analysis of biological processes and functions most impacted by the absence of ELP3. In order to capture a broader range of biological processes potentially influenced by loss of ELP3 an enrichment cut-off of –log10 (p-value) < 1.3 was utilised. Basally and following 6 h LPS treatment, there was strongest enrichment of downregulated proteins involved in the process of antigen presentation in *Elp3*$^{-/-}$ cells (Fig 3.11a, b). Although not the most highly impaired biological process after 12 h LPS stimulation, proteins involved in antigen presentation were clearly strongly affected by the absence of ELP3. Intriguingly, IPA analysis showed a distinct and potent impairment of IFN signalling in *Elp3*$^{-/-}$ cells. IFN signalling was significantly diminished basally in *Elp3*$^{-/-}$ cells. Moreover, this effect becomes even clearer following stimulation, with IFN signalling second only to antigen presentation as the most downregulated process after 6 h LPS, and it being the most impaired biological process following 12 h LPs stimulation (Fig 3.11a, b). Expression of crucial regulators of IFN signalling such as STAT1, IRF7 & IRF9 were downregulated in

Elp3$^{-/-}$ cells. Expression of IRF7 was suppressed following LPS treatment, while STAT1 protein levels appear to be diminished basally in mock samples as well as following LPS stimulation. This was previously observed in Fig 3.6.

It also appears that in cells lacking ELP3, there was a strong suppression of proteins involved in the recognition and sensing of bacteria and viruses. A number of these proteins are involved in dsRNA binding and the inhibition of viral replication. Several members of the 2'-5' oligoadenylate synthetase family (OAS1-3), EIF2AK2 (PKR) and DDX58 (RIG-I) were downregulated (Fig 3.11). The majority of these proteins, OAS2 being an exception, are downregulated basally as well as following LPS stimulation in the absence of ELP3. Similar to OAS2, anti-viral effectors IFIT2, IFIT3 and ISG20 expression is not impaired basally, whilst their LPS-induced expression significantly diminished. IRFs play an imperative and fundamental role in co-ordinating signals from PRRs following the detection of bacterial and viral pathogens. Conjointly with the sensing of microbes, a clear and potent downregulation of proteins that mediate cytosolic PRR activation of IRFs was observed in *Elp3*$^{-/-}$ cells. Expression of cytosolic PRRs ADAR, RIG-I & DHX58 appear to be diminished basally and following stimulation, with other downregulated proteins such as ISG15 and STAT1, also being involved in this process. Moreover, many of these proteins also appear to be involved in systemic lupus erythematosus (SLE) signalling, which appears to be impaired following 12 h LPS treatment. Furthermore, SLE signalling appears to be strongly inhibited when the grouping of differentially expressed proteins is represented based on activation z-score, or the processes with the highest degree of change relative to WT samples (Fig 3.12). There appears to be a strong interlink between the impairment of IFN signalling and processes related to pathogen sensing and IRF activation. A strong majority of downregulated proteins mediating pathogen sensing and IRF activation are ISGs (PKR, RIG-I, OAS1-3, ADAR, ISG15, ISG20, IFIT2, IFIT3, STAT1, IRF7 & IRF9) (Fig 3.11 & 3.12). Simultaneously, a number of the downregulated proteins involved in both antigen presentation and SLE signalling are also involved processes related to signalling of and responses to IFN. Thus, ELP3 appears to be required for the regulation and proper functioning of IFN production, subsequent cellular responses to IFN and induction of anti-viral immunity via the expression of a number of key ISGs.

In addition to impaired type I interferon, a number of proteins involved in Inflammasome and LPS signalling were impaired in *Elp3*$^{-/-}$ cells. LPS-induced expression of both IL-1α and IL-1β is impaired, whilst their basal expression was unaltered. In accordance with this, CCL4 expression, which is induced in response to IL-1, was downregulated following 6 h LPS stimulation in cells lacking Elp3. The inflammasome protein NLRP3, was similarly downregulated in the absence of ELP3, although at a basal level rather than as an LPS-induced effect. A number of proteins involved in death receptor signalling such as caspase-6 and multiple PARP protein family members were also downregulated in *Elp3*$^{-/-}$ cells.

3.2.5 Cholesterol biosynthesis is upregulated in Elp3$^{-/-}$ *macrophages*

In contrast to the plethora of biological processes impaired in cells lacking ELP3, cholesterol biosynthesis was upregulated in *Elp3*$^{-/-}$. Basally, no enhancement is observed, however, a strong LPS-mediated upregulation of proteins involved in several pathways of cholesterol biosynthesis was observed in the *Elp3*$^{-/-}$ cells. This data suggests that the absence of ELP3 may release some sort of repression on cellular metabolic activity, or possibly that the cells are ramping up their metabolism in some sort of compensatory manner to account for the loss of ELP3 and the dysregulated cellular processes it controls following LPS stimulation.

3.2.6 Upstream regulator analysis of the proteome of Elp3$^{-/-}$ *cells*

Altered expression of functionally-related proteins in the absence of ELP3 could be due to hierarchical regulation of sets of proteins by ELP3-depedent upstream regulators . To explore this, differentially expressed proteins were further subjected to upstream regulator analysis (URA) to try to determine which immune signalling molecules are responsible for the observed changes in protein expression. Potential upstream regulators are presented by –log (p-value) (Fig 3.13a) or by activation z-score (Fig 3.13b). Corresponding to the IPA analysis where proteins involved in PRR-mediated activation of IRFs were downregulated, IRF3 and IRF7 were the top hits for potential upstream proteins regulating the expression of impaired proteins in *Elp3*$^{-/-}$, based on both p-value and z-score(Fig 3.13a & 3.13b respectively). Moreover, in accordance with the observation that IFN-signaling & IFN-mediated processes are impaired in the absence of

Elp3 (Fig 3.10-3.12), STAT1, MAVS, IFNAR1, DDX58 and IFNB1 arose as potential key upstream regulators responsible for the observed impairment of protein expression in *Elp3*$^{-/-}$ cells (Fig 3.13a). With regards to z-score, degree of change relative to WT, STAT1, MAVS & IFNAR1 once again arose as top hits as upstream regulators. TLR4 also appears, unsurprisingly considering it functions as a receptor for LPS (Fig 3.13b). This URA data further strengthens the observation that in *Elp3*$^{-/-}$ cells, there is a severe impairment in IFN signalling and processes.

Thus, unbiased quantitative proteomics analysis of *Elp3*$^{-/-}$ cells has revealed potential functions for the Elongator complex regulation of protein expression for proteins involved in innate immunity. ELP3 appears to be necessary for the expression of proteins required for IFN signalling. Furthermore, ELP3 regulates sensing of bacteria and viruses by PRRs. Interestingly, in response to LPS, an extracellular TLR4 stimulus, ELP3 appears to be responsible activation of IRFs via cytosolic PRRs. Overall, the data suggest ELP3 is potentially important for IFN induction and signalling, as well as processes downstream of IFN signalling pertaining to ISG expression and potentially subsequent antiviral immunity.

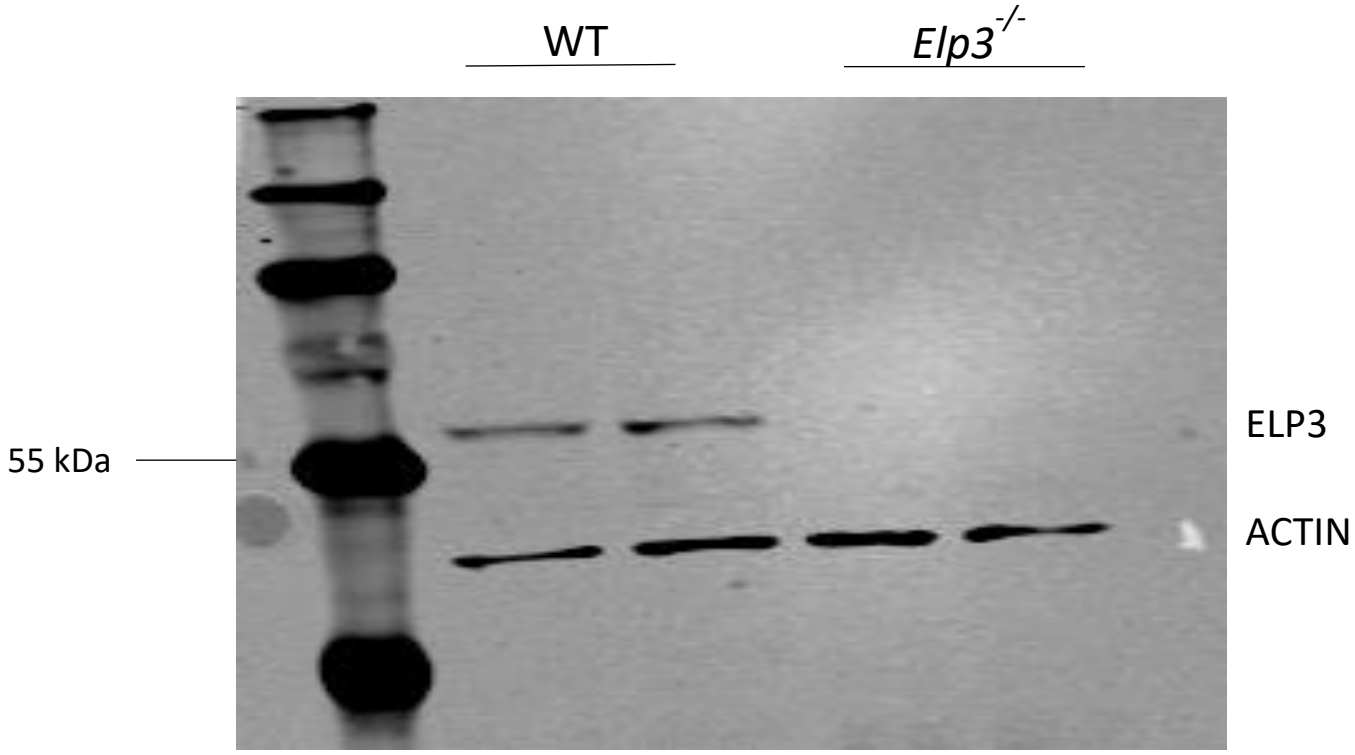
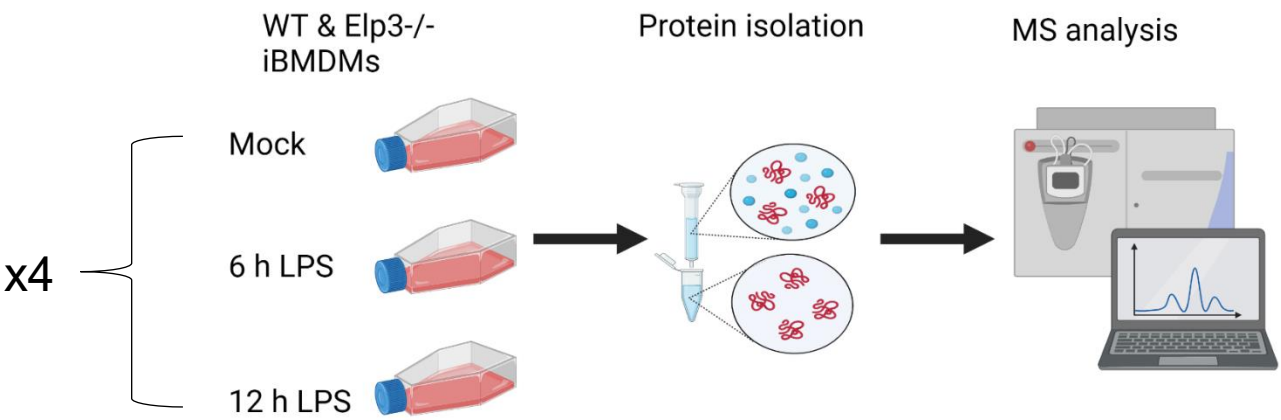


Fig 3.1 CRISPR/Cas9-mediated knockout of Elongator complex catalytic subunit Elp3 in immortalized bone marrow derived macrophages

WT and *Elp3* CRISPR/Cas9 knockout (KO) iBMDMs were seeded at 5 x 10^5 cells/ml before being harvested and assessed for Elp3 expression by immunoblot. Representative of three independent experiments



3.2 Workflow for unbiased quantitative proteomic analysis of iBMDMs lacking Elp3

WT & *Elp3*$^{-/-}$ iBMDMs were seeded at 5 x 10^5 cells/ml at a volume of 2 ml in 6-well plates in quadruplicate and then stimulated mock or with LPS (100 ng/ml) for 6 & 12 h. Cell proteins were subsequently isolated and concentrations equalised before being processed by mass spectrometry for quantitative proteomic analysis.

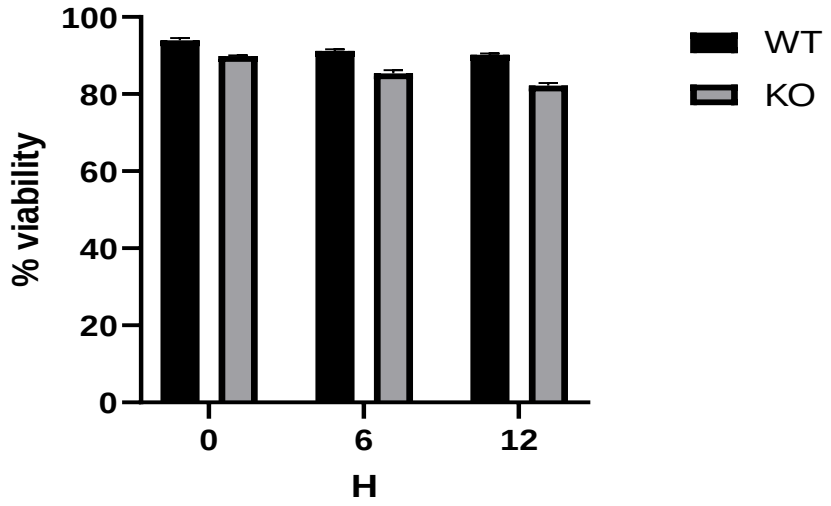


Fig 3.3 Cell Viability of WT & *Elp3*$^{-/-}$ iBMDMs following treatment with LPS

WT & *Elp3*$^{-/-}$ iBMDMs were seeded at 5 x 10^5 cells/ml in 6-well plates in quadruplicate and then stimulated mock or with LPS (100 ng/ml) for 6 & 12 h. Cells were analysed for viability via trypan blue staining prior to the experiment being repeated for quantitative proteomic analysis by Mass Spectrometry. Data are mean ± SEM of three experiments.

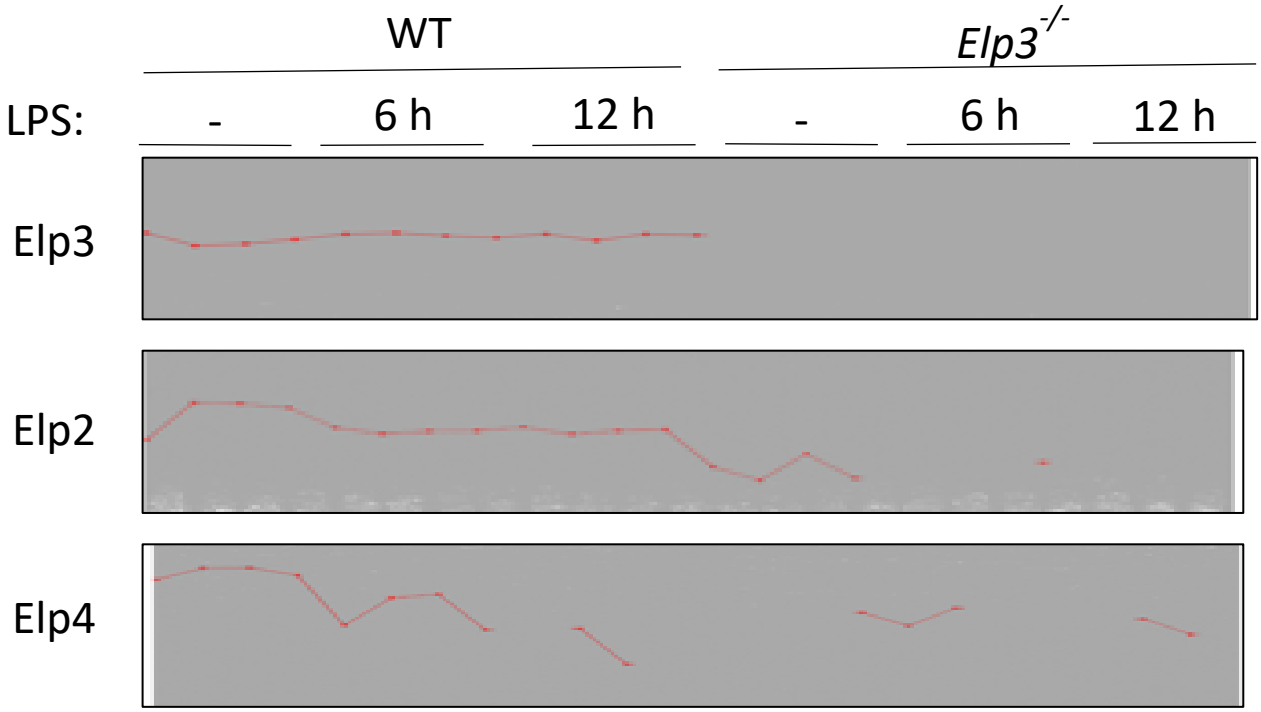


Fig 3.4 Expression plots for Elongator complex subunits Elp2, Elp3 & Elp4 in WT & *Elp3*$^{-/-}$ cells

Profile Plots showing the protein expression of ELP2, ELP3 & ELP4 based on their relative Label free quantification (LFQ) intensity detected by mass spectrometry in WT & *Elp3*$^{-/-}$ cells basally and following 6 or 12 h LPS treatment.

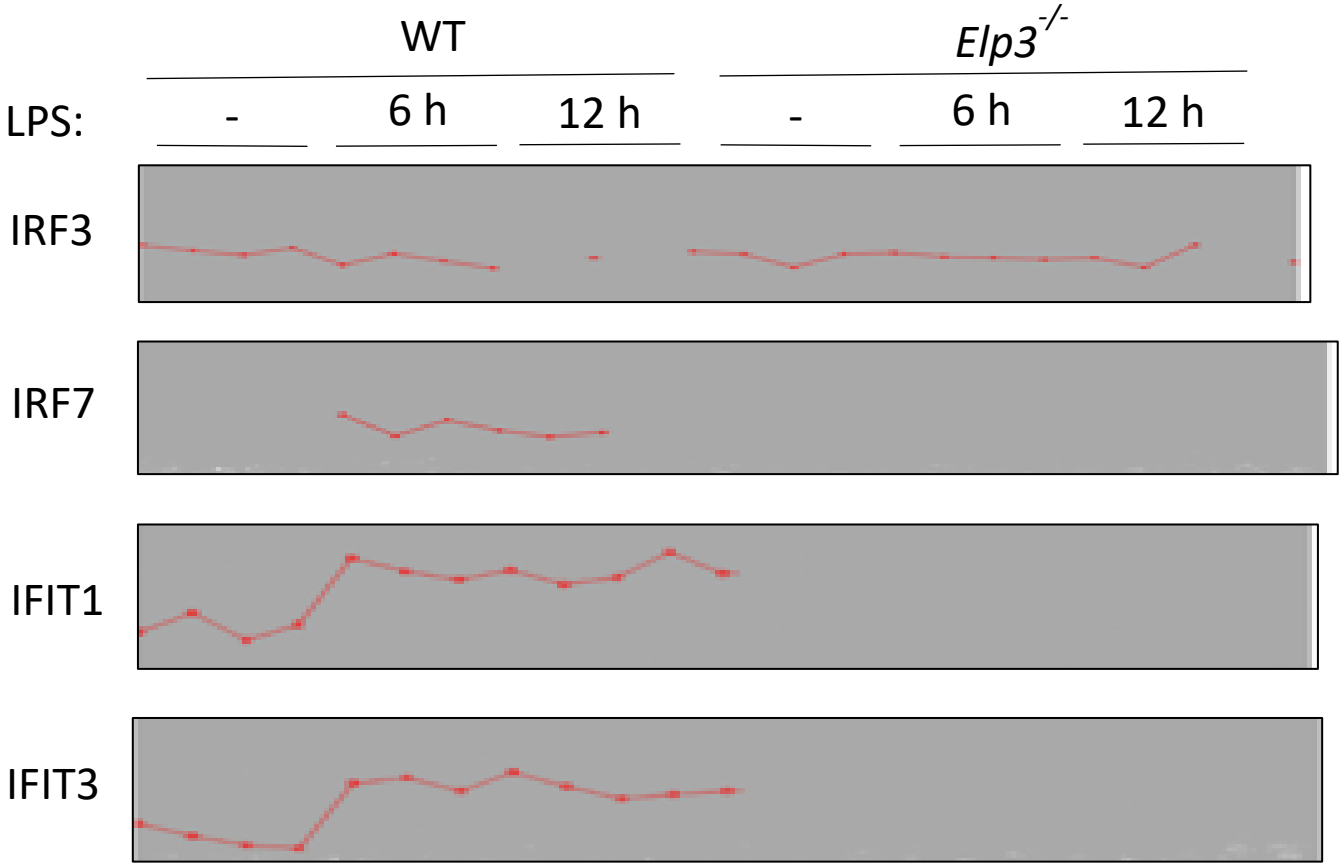


Fig 3.5 Profile plots of transcription factors & IFIT proteins differentially expressed in WT & *Elp3*$^{-/-}$ cells

Profile Plots showing the protein expression of IRF3, IRF7, IFIT1 & IFIT3 based on relative LFQ intensity by mass spectrometry in WT & *Elp3*$^{-/-}$ iBMDMs basally & following 6 or 12 h LPS treatment.

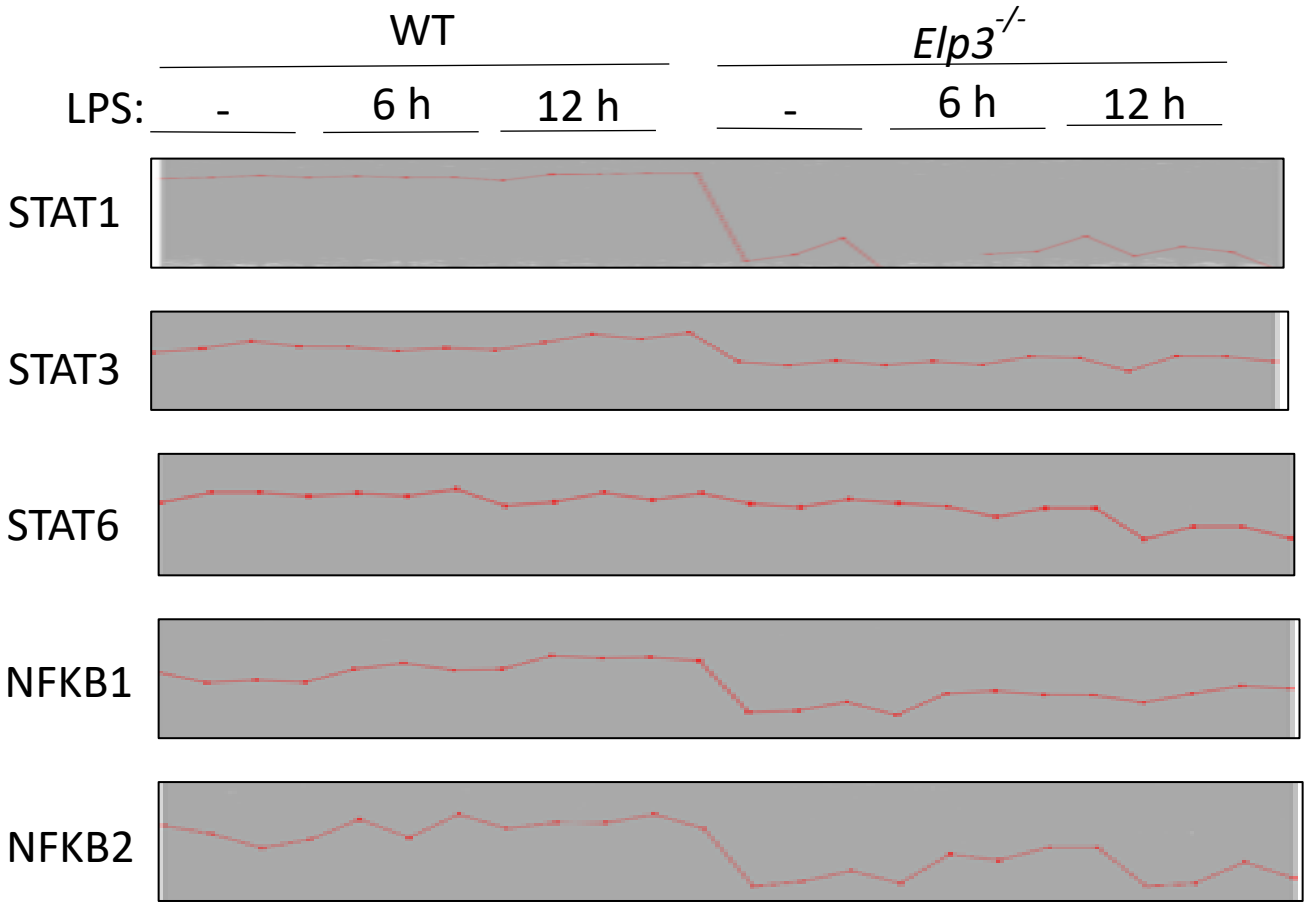


Fig 3.6 Profile plots of STAT & Nf-κB family proteins in WT & *Elp3*$^{-/-}$ cells

Profile Plots showing the protein expression of STAT1, STAT3 & STAT6 based on relative LFQ intensity by mass spectrometry in WT & *Elp3*$^{-/-}$ iBMDMs basally & following 6 or 12 h LPS treatment.

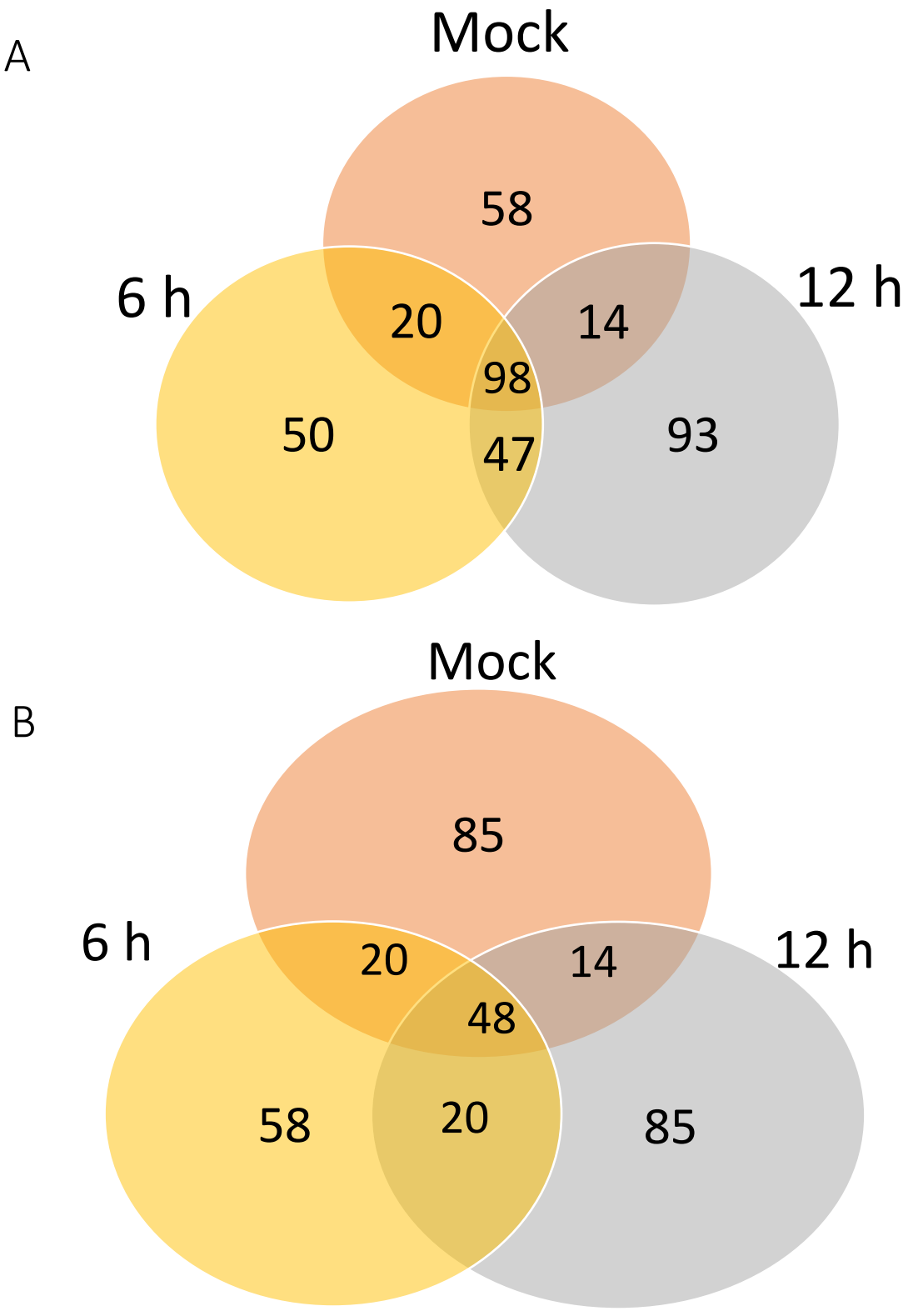


Fig 3.7 Distribution of differentially expressed proteins in *Elp3*$^{-/-}$ iBMDMs

Venn diagrams presenting distribution of both down (A) and upregulated (B) proteins in *Elp3*$^{-/-}$ cells compared to WT counterpart, based on a log2 fold difference of 2 or greater.

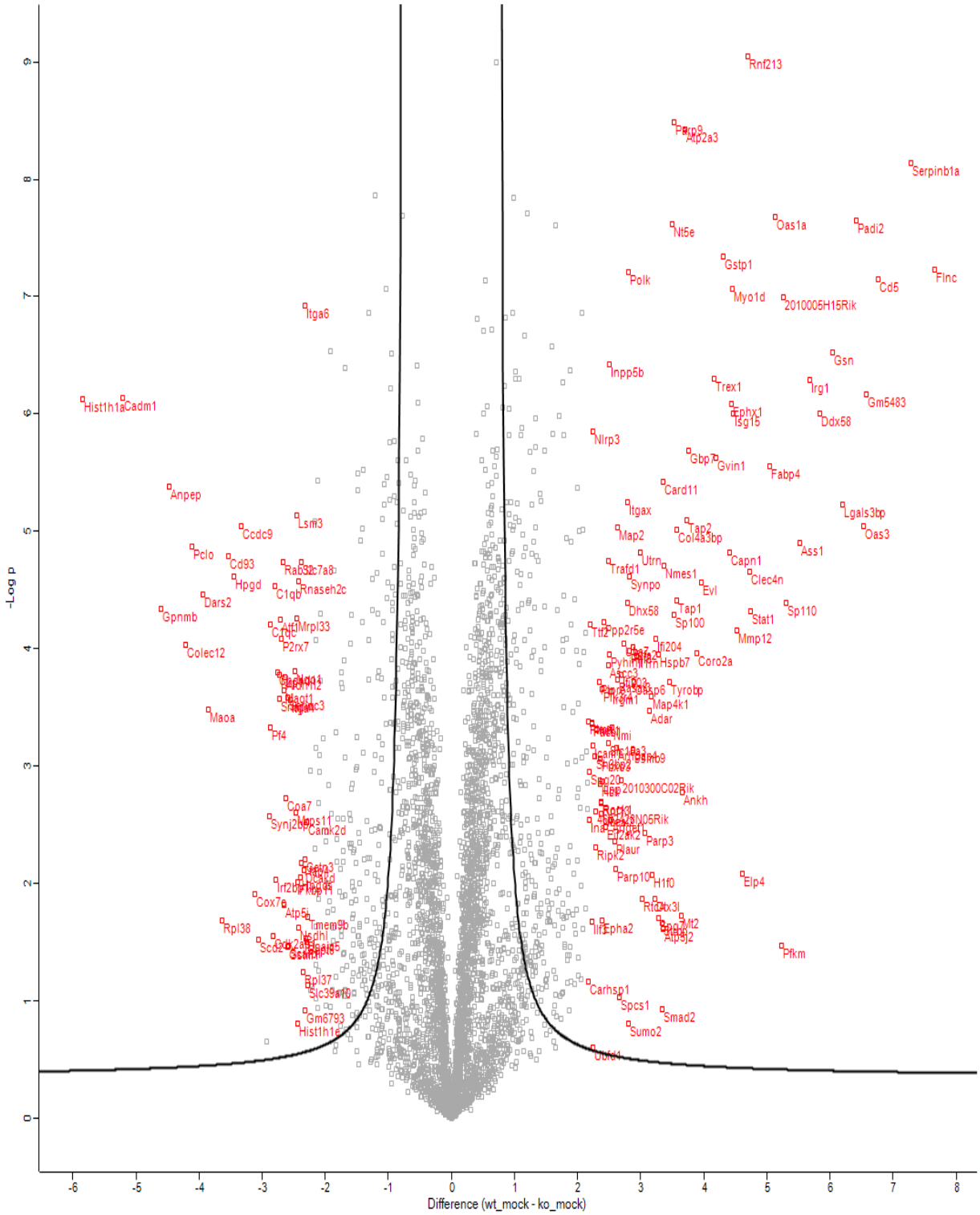


Fig 3.8 Differential protein expression in mock treated *Elp3*$^{-/-}$ iBMDMs relative to WT cells

Volcano plots showing differentially expressed proteins in mock *Elp3*$^{-/-}$ samples vs wild-type cells. Data is presented as –Log P value vs difference in *Elp3*$^{-/-}$ relative to WT. Proteins were considered significantly up- or down-regulated if there was a log2 fold difference of 2 or greater in KO cells relative to WT cells (shown in red). Positive differences correspond to protein downregulation & negative differences to upregulation in *Elp3*$^{-/-}$ cells compared to WT cells

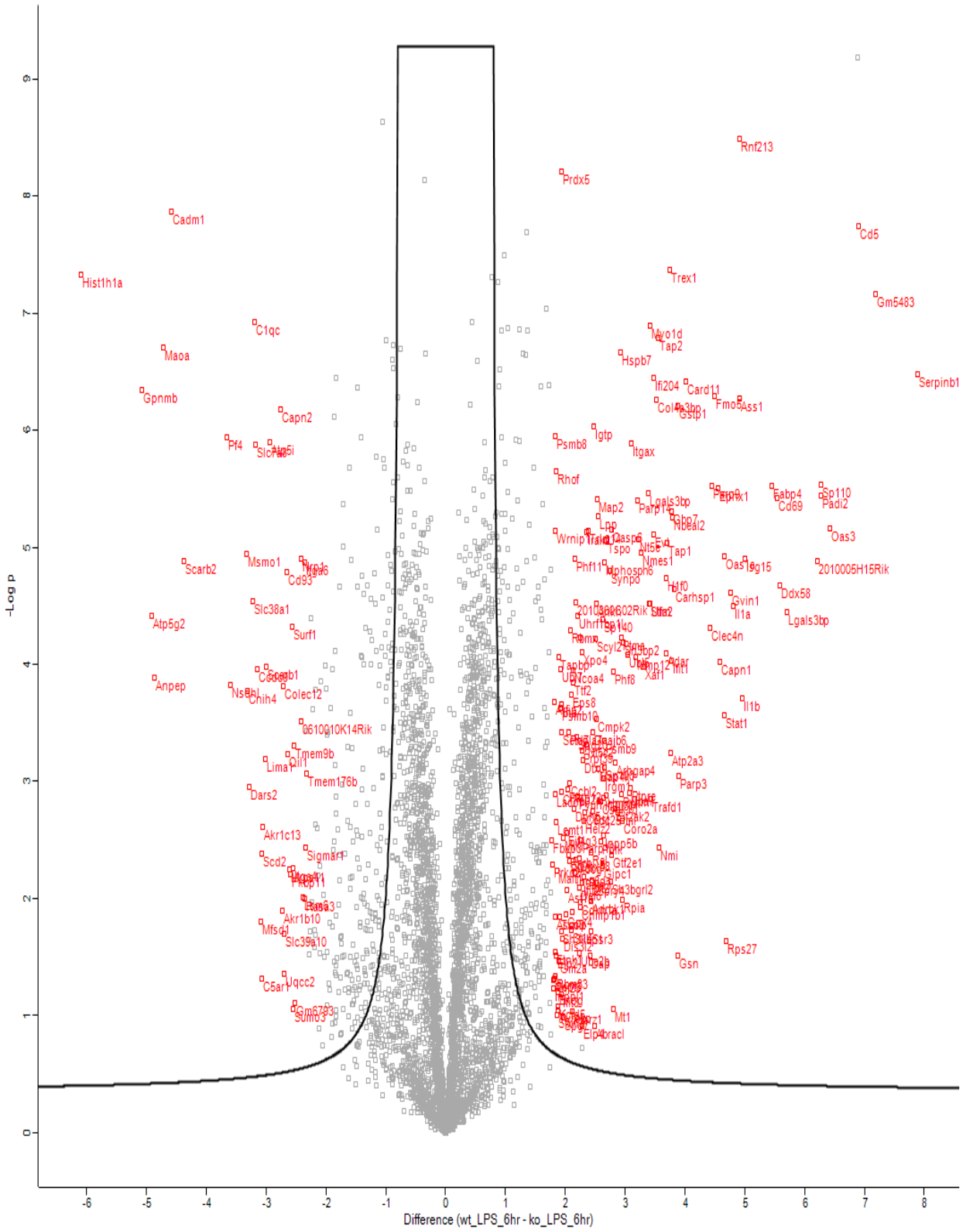

-Log p
Difference (wt_LPS_6hr - ko_LPS_6hr)
Rnf213
Prdx5
Cadm1
Cd5
Hist1h1a
Trex1
Gm5483
C1qc
Maoa
Hspb7
Serpinb1
Gpnmb
Capn2
Pf4
Igtp
Psmb8
Itgax
Rhof
Map2
Oas3
Scarb2
Msmo1
Cd93
Phf11
Oas1g
2010005H15Rik
Slc38a1
Ddx58
Atp5g2
Surf1
Gvin1
Il1a
Lgals3bp
Clec4n
Anpep
Colec12
Capn1
Il1b
Stat1
0610010K14Rik
Tmem9b
Atp2a3
Lima1
Tmem176b
Parp3
Dars2
Akr1c13
Scd2
Sigmar1
Nmi
Akr1b10
Slc39a10
Rps27
Gsn
C5ar1
Uqcc2
Mt1

Fig 3.9 Differential protein expression in *Elp3*$^{-/-}$ iBMDMs stimulated with LPS for 6 h relative to WT cells

Volcano plots showing differentially expressed proteins in 6 h LPS-treated *Elp3*$^{-/-}$ samples vs wild-type cells. Data is presented as –Log P value vs difference in *Elp3*$^{-/-}$ relative to wild-type. Proteins were considered significantly up- or down-regulated if there was a log2 fold difference of 2 or greater in KO cells relative to WT cells (shown in red). Positive differences correspond to protein downregulation & negative differences to upregulation in *Elp3*$^{-/-}$ cells compared to WT cells

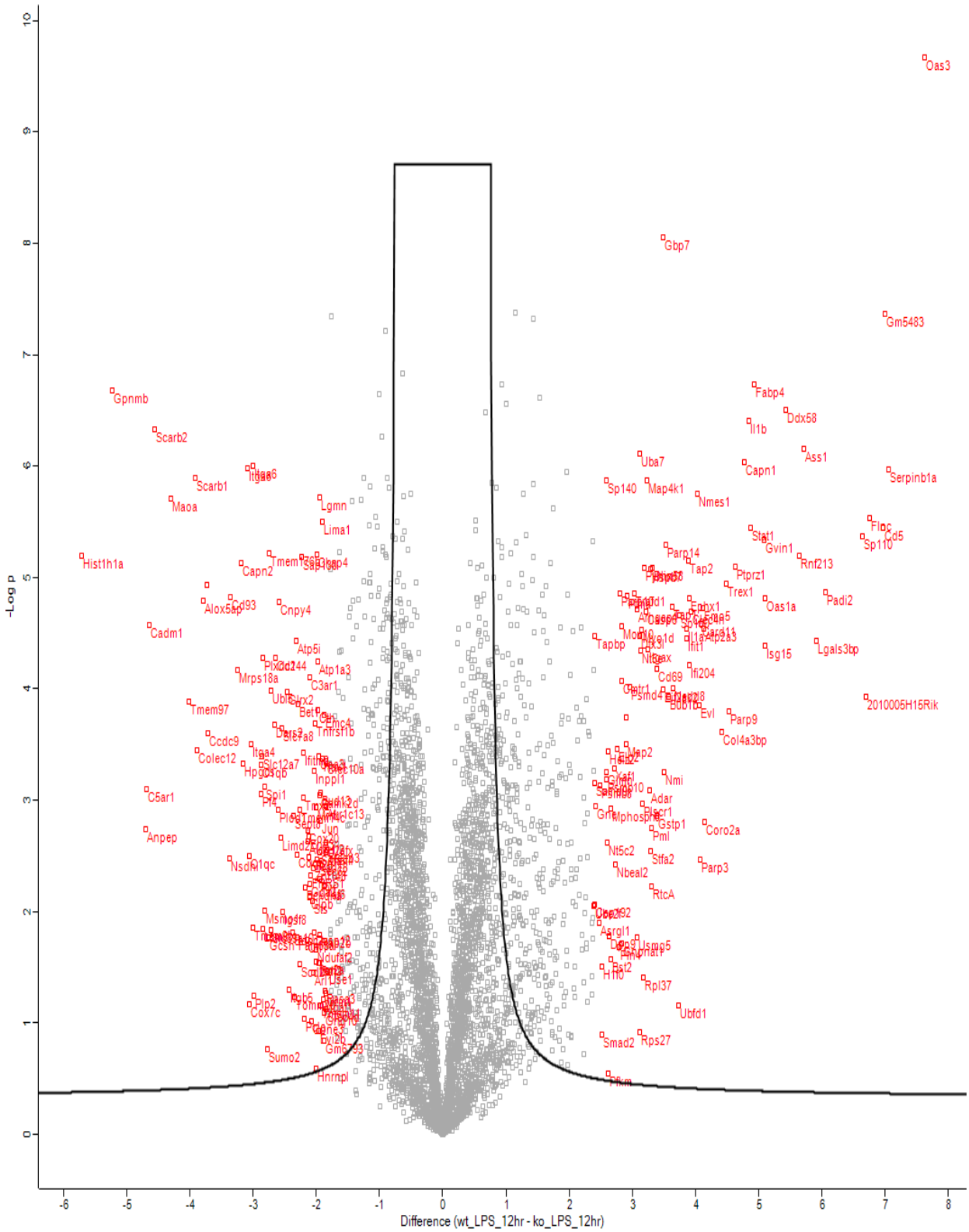
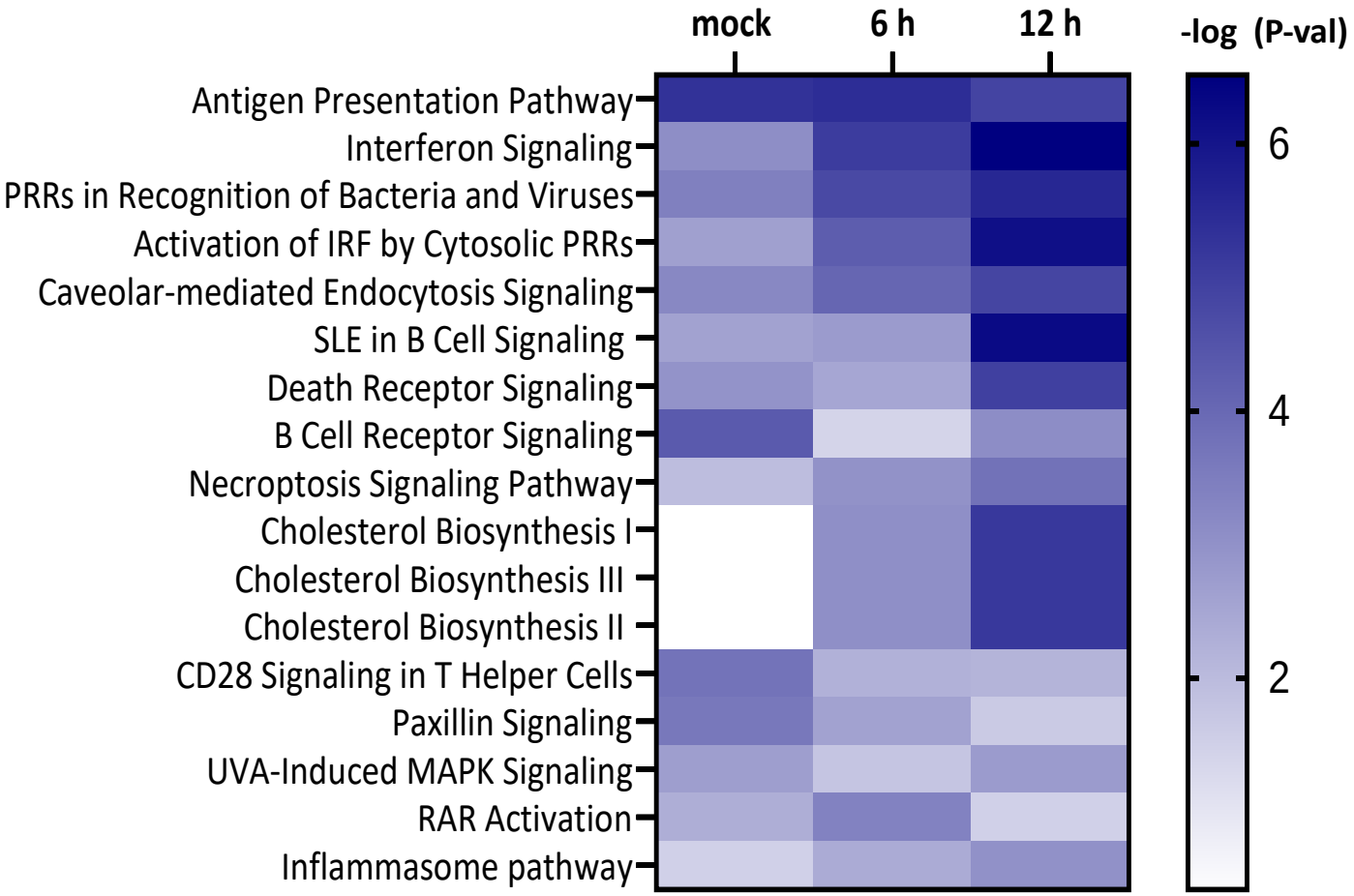

-Log p
Difference (wt_LPS_12hr - ko_LPS_12hr)
Oas3
Gbp7
Gm5483
Gpnmb
Fabp4
Ddx58
Il1b
Scarb2
Ass1
Uba7
Capn1
Serpinb1a
Scarb1
Sp140
Map4k1
Nmes1
Maoa
Lgmn
Lima1
Stat1
Gvin1
Sp110
Cd5
Hist1h1a
Parp14
Tap2
Rnf213
Capn2
Ptprz1
Trex1
Padi2
Alox5ap
Cd93
Cnpy4
Oas1a
Cadm1
Tapbp
Isg15
Lgals3bp
Atp5i
Atp1a3
Mrps18a
C3ar1
Cd69
Ifi204
Tmem97
2010005H15Rik
Evl
Parp9
Ccdc9
Col4a3bp
Colec12
Nmi
C5ar1
Adar
Gstp1
Coro2a
Anpep
Pml
Nt5c2
Stfa2
Parp3
Nbeal2
RtcA
Rpl37
Ubfd1
Smad2
Rps27
Sumo2
Hnrnpl

Fig 3.10 Differential protein expression in 12 h LPS-treated *Elp3*$^{-/-}$ iBMDMs relative to WT cells

Volcano plots showing differentially expressed proteins in mock *Elp3*$^{-/-}$ samples vs WT cells. Data is presented as –Log P value vs difference in *Elp3*$^{-/-}$ relative to WT. Proteins were considered significantly up- or down-regulated if there was a log2 fold difference of 2 or greater in KO cells relative to WT cells (shown in red). Positive differences correspond to protein downregulation & negative differences to upregulation

A


mock
6 h
12 h
-log (P-val)
6
4
2
Antigen Presentation Pathway
Interferon Signaling
PRRs in Recognition of Bacteria and Viruses
Activation of IRF by Cytosolic PRRs
Caveolar-mediated Endocytosis Signaling
SLE in B Cell Signaling
Death Receptor Signaling
B Cell Receptor Signaling
Necroptosis Signaling Pathway
Cholesterol Biosynthesis I
Cholesterol Biosynthesis III
Cholesterol Biosynthesis II
CD28 Signaling in T Helper Cells
Paxillin Signaling
UVA-Induced MAPK Signaling
RAR Activation
Inflammasome pathway

B

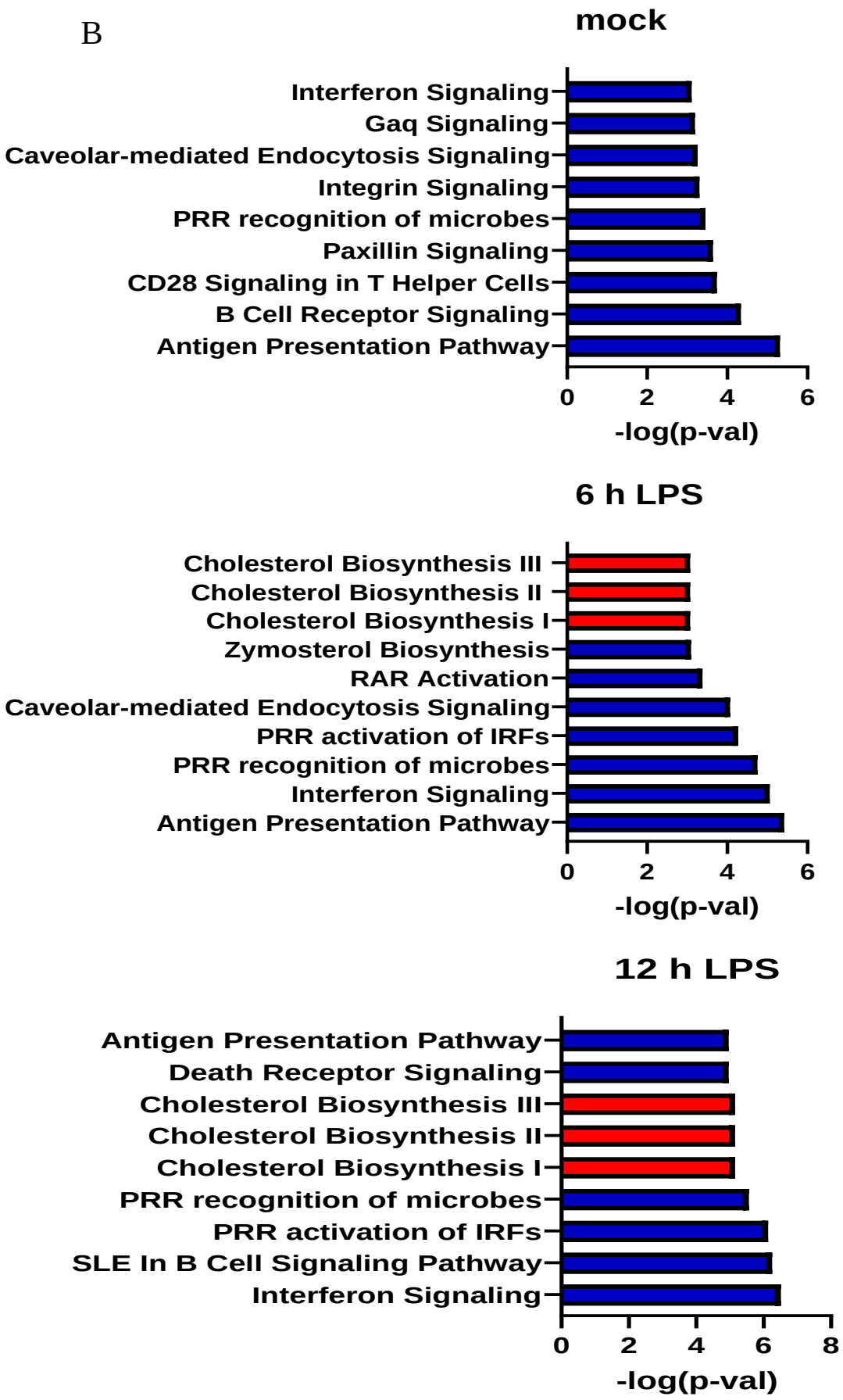

mock
Interferon Signaling
Gaq Signaling
Caveolar-mediated Endocytosis Signaling
Integrin Signaling
PRR recognition of microbes
Paxillin Signaling
CD28 Signaling in T Helper Cells
B Cell Receptor Signaling
Antigen Presentation Pathway
0
2
4
6
-log(p-val)
6 h LPS
Cholesterol Biosynthesis III
Cholesterol Biosynthesis II
Cholesterol Biosynthesis I
Zymosterol Biosynthesis
RAR Activation
Caveolar-mediated Endocytosis Signaling
PRR activation of IRFs
PRR recognition of microbes
Interferon Signaling
Antigen Presentation Pathway
0
2
4
6
-log(p-val)
12 h LPS
Antigen Presentation Pathway
Death Receptor Signaling
Cholesterol Biosynthesis III
Cholesterol Biosynthesis II
Cholesterol Biosynthesis I
PRR recognition of microbes
PRR activation of IRFs
SLE In B Cell Signaling Pathway
Interferon Signaling
0
2
4
6
8
-log(p-val)

Fig 3.11 Ingenuity pathway analysis of differentially expressed proteins in unstimulated & LPS-treated *Elp3*$^{-/-}$ iBMDMs relative to WT cells

Differentially expressed proteins in mock and LPS-stimulated *Elp3*$^{-/-}$ iBMDMs relative to WT cells were grouped into differing biological processes using Ingenuity Pathway Analysis (IPA) based on –log10 (p-value). (A) Heatmap of different biological processes mediated by differentially expressed proteins in *Elp3*$^{-/-}$ cells. (B) breakdown of IPA analysis of mock and LPS-stimulated differential protein expression, with red corresponding to biological processes mediated by upregulated proteins and blue to downregulated proteins in *Elp3*$^{-/-}$ cells relative to their WT stimulated counterparts. P-value was calculated using a right-tailed fisher's exact test with an enrichment cut-off of –log10 p-value<1.3.

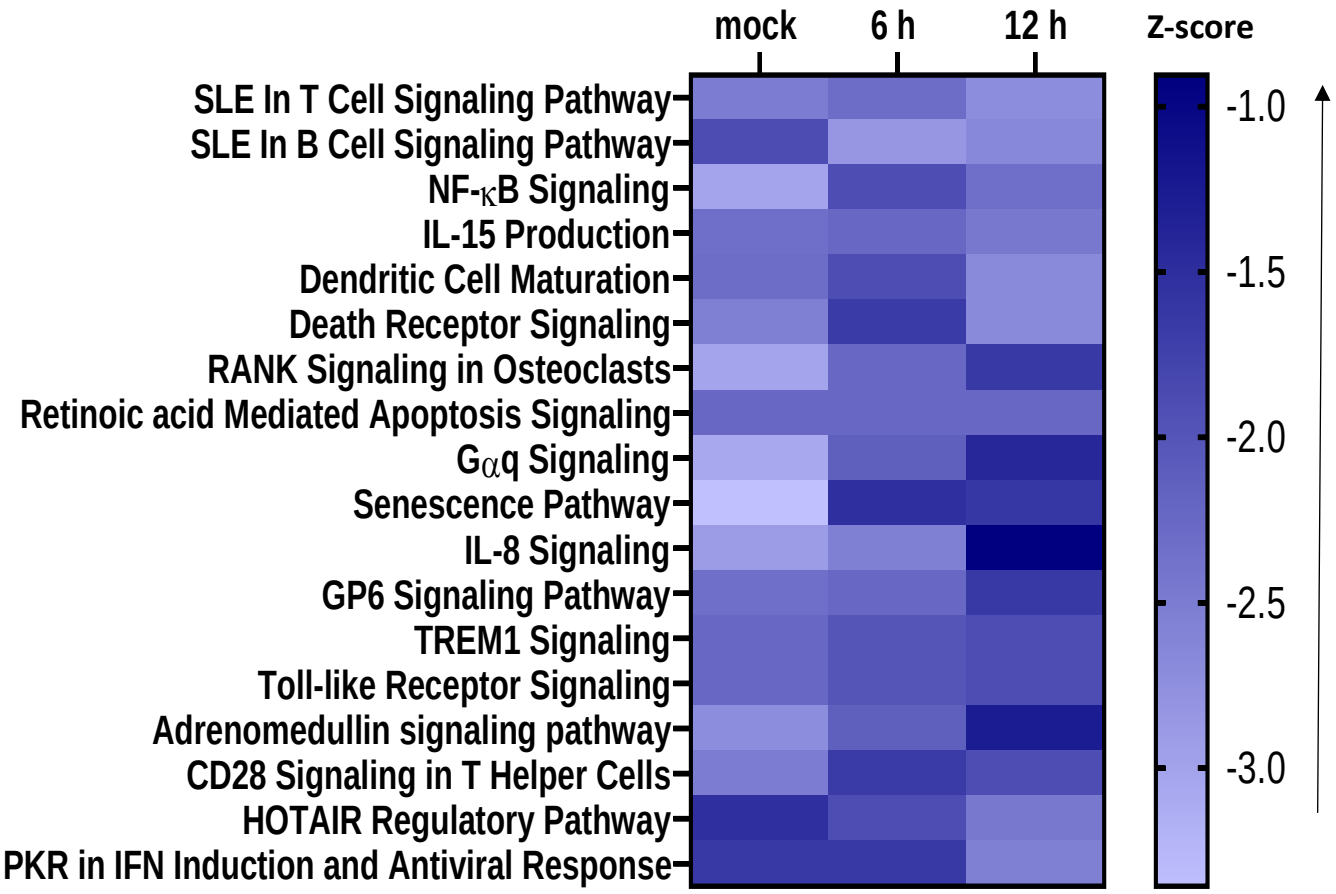


Fig 3.12 Differentially regulated biological processes in *Elp3*$^{-/-}$ iBMDMs relative to WT cells based on activation z-score

IPA analysis-mediated grouping of differentially expressed proteins into biological processes in mock and LPS-treated *Elp3*$^{-/-}$ iBMDMs relative to WT cells based on activation z-score (highest degree of change)

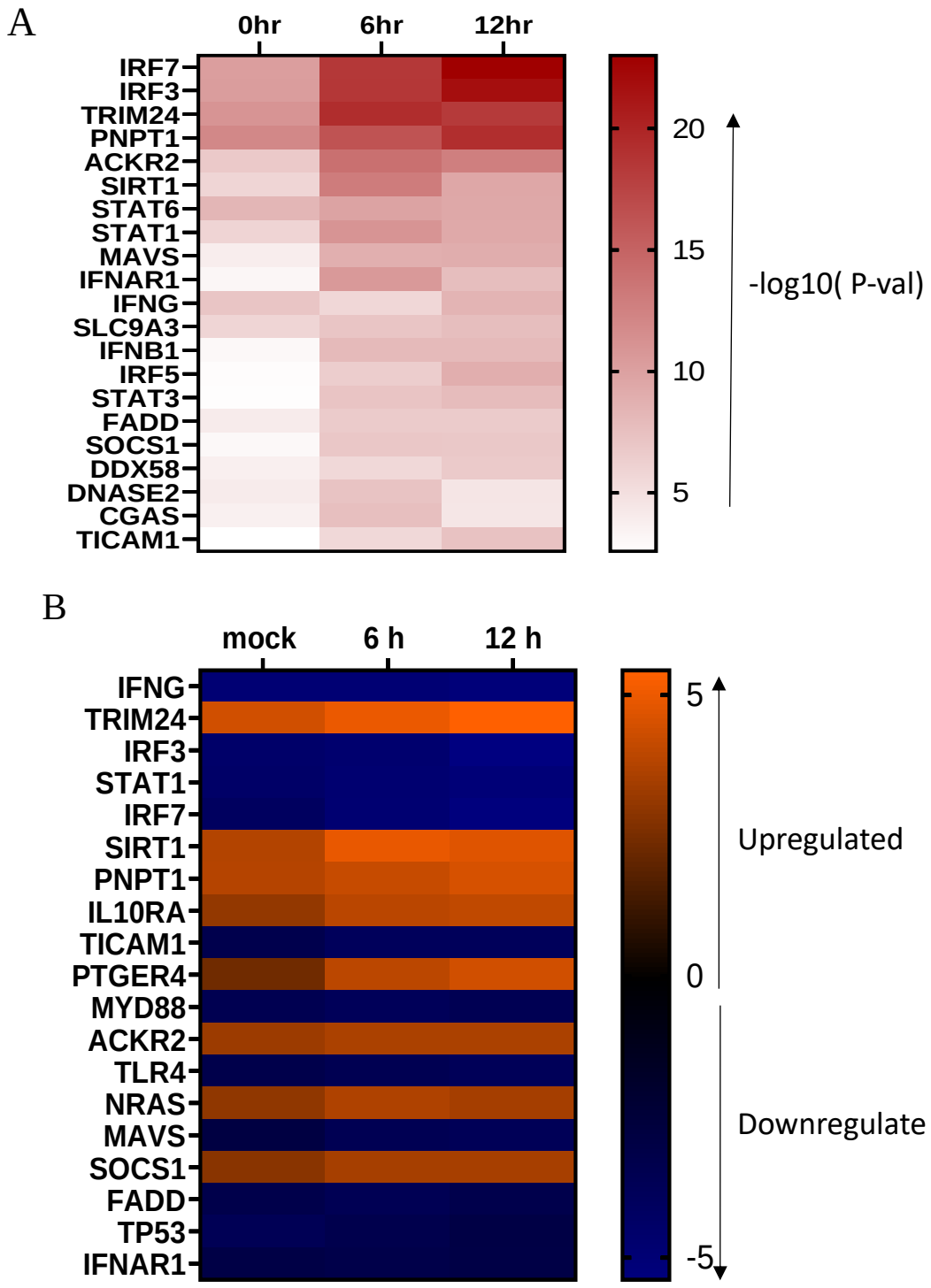


Fig 3.13 Identification of upstream regulator networks associated with changes in protein expression in the absence of ELP3

IPA was used to subject differentially expressed proteins in *Elp3*$^{-/-}$ iBMDMs to upstream regulator analysis. Differentially expressed proteins were grouped into upstream regulator networks by either (A) –log p-value or (B) activation z-score where orange corresponds to activated pathways and blue to suppressed

3.3 Discussion

We resolved to appraise the role of Elongator in innate immunity via its functioning in macrophages. As an initial step towards this appraisal, we stimulated both WT and iBMDMs with a CRISPR/Cas9 KO of *Elp3*, the enzymatic core of the complex, with the TLR4 activator LPS and subjected them to unbiased quantitative proteomic analysis. This proteomic interrogation demonstrated that macrophages lacking ELP3 show a potent abrogation of proteins involved IFN signalling and processes such as microbial sensing and IRF activation following LPS treatment. Our data indicate that Elongator is a key regulator of proteins involved in LPS-induced IFN signalling and processes in macrophages.

Elongator plays a key role in regulating the proper and efficient translation of mRNA transcripts. We demonstrate that loss of ELP3 expression alters the proteomic landscape of macrophages. Expression of numerous proteins were altered, and trend toward upregulation or downregulation in cells lacking ELP3. A significant proportion of DEPs occur basally in unstimulated cells. ELP3 appears to be required for basal protein expression in and functioning of macrophages. Proteomic analysis did reveal a small proportion of proteins were only differentially expressed in *Elp3*$^{-/-}$ cells following LPS treatment (IL-1α and IL-1β), suggesting that in addition to regulating basal protein expression, ELP3 can regulate stimulus-dependent protein expression in macrophages. Proper stoichiometry and presence of each of the six Elongator subunits is required for proper integrity and functioning of the complex since genetic deletion of any given subunit of Elongator leads to defective complex stability and activity across species (150–152). CRISPR/Cas9-mediated deletion of *Elp3* leads to impaired expression of the Elongator subunits, ELP2 and ELP4. This data suggests that in murine macrophages, sufficient expression of a complex subunit regulates and influences protein levels of related subunits. Accordingly, faithful complex stoichiometry is necessary for sufficient stability and cohesiveness of Elongator in macrophages.

LPS-mediated TLR4 activation catalyses downstream activation of IRF and NFκB family memebers, enabling the induction of IFNs and proinflammatory cytokines. IFN-I subsequently signals in a feedback loop to induce expression of ISGs which mediate sensing or direct restriction of viruses. Organisation of differentially expressed proteins

in *Elp3*$^{-/-}$ cells into biological processes demonstrates that ELP3 was necessary for expression of proteins involved in IFN signalling and processes. Consistent with the observation that loss of ELP3 affects basal protein expression, IFN signalling was predicted to be impaired in unstimulated macrophages and to become highly pronounced following stimulation, with IFN signalling arising as the most compromised biological process following LPS treatment. This prediction will be tested in the next chapter. Processes such as antigen presentation and signalling in SLE are also predicted to be defective in *Elp3*$^{-/-}$ cells. A significant proportion of downregulated proteins involved in these pathways (TAP1, IFIT2, IFIT3, ISG15 etc), are interferon-inducible. SLE is known to be an interferonopathy, and antigen presentation to be upregulated by IFN (92,210). Therefore it is likely that these processes would be strongly downregulated in *Elp3*$^{-/-}$ cells not as a result of a direct regulation by ELP3, but as a collateral effect of impaired IFN signalling. This further strengthens the notion that restrained and diminished IFN processes arise in the absence of ELP3.

This data strongly suggests IFN signalling pathways will be affected by loss of ELP3 and thus puts forward the question as to which point in the IFN pathway is ELP3 exerting an influence. Proteins and processes related to IFN induction, IFN signalling and IFN-mediated gene induction were affected by the absence of ELP3. Therefore, regulation of ELP3 on IFN –related processes may potentially be varied and multifaceted. STAT1 is a key mediator of IFN signalling and subsequent induction of ISG expression (61). STAT1 protein expression was strongly abrogated in *Elp3*$^{-/-}$ cells, suggesting that ELP3 is required for IFN-mediated signalling. Many ISGs that mediate viral restriction and sensing were downregulated in the absence of Elp3. This data proposes several possibilities. Absence of ELP3 may affect ISG expression via defective STAT1 expression, and subsequent impairment of IFN signalling and ISG induction. URA analysis of *Elp3*$^{-/-}$ iBMDMs suggests that proteins involved in IFN signalling like IFNAR1 and STAT1 may be the key regulators of proteins whose expression are suppressed in cells lacking ELP3. Alternatively, it is possible that ELP3 may directly regulate ISG expression directly following LPS treatment, independently of regulating upstream IFN signalling.

A further intriguing possibility to assess is whether ELP3 regulates TLR4-mediated IFN-I expression, at the level of gene induction or direct translation of IFN-I mRNA. IRFs are

key regulators of PRR signalling and pivotal mediators of subsequent gene induction and expression. Proteomics analysis showed that ELP3 is required for PRR-mediated IRF activation. URA analysis of the proteomic landscape of *Elp3*$^{-/-}$ macrophages suggested that IRF3 and IRF7 are potentially the crucial effectors that mediate induction of proteins who are dysregulated in the absence of ELP3. LPS-mediated IRF7 expression was ablated in *Elp3*$^{-/-}$ cells. IRF3 expression however, was unaffected in cells lacking Elp3, raising the possibility that ELP3 is required not for IRF3 protein expression, but for TLR4-mediated IRF3 phosphorylation and activation.

As mentioned TLR4 activation leads to downstream NF-κB activation and proinflammatory cytokine induction. Expression of NFκB subunits was unaffected in *Elp3*$^{-/-}$ cells. Although IPA analysis suggests that processes related to NFκB signalling are not impaired, whether ELP3 regulates NFκB activation remains to be analysed.

The most strongly predicted upregulated process that arose in cells lacking ELP3 is related to cholesterol biosynthesis. Expression of several enzymes involved in the cholesterol synthesis pathway were enhanced in *Elp3*$^{-/-}$. This may represent a compensation mechanism engaged by the cells to respond to defective translation that occurs in the absence of ELP3. Potentially ELP3 could regulate the expression of a protein which restricts cholesterol synthesis in macrophages, leading to a de-repression of the enzymatic flux through the pathway. Alternatively, enhanced cholesterol synthesis could derive from reduced IFN induction following LPS treatment. IFN has been shown to induce reduced cholesterol synthesis in macrophages to enable antimicrobial resistance (211), and therefore reduced IFN in the absence of ELP3 may enable enhanced flux throught the cholesterol biosynthesis pathway.

In summary unbiased quantitative proteomics analysis of *Elp3*$^{-/-}$ cells revealed intriguing roles for the Elongator complex in regulation of proteins involved in innate immunity. ELP3 was necessary for the expression of proteins required for IFN signalling. ELP3 was necessary for activation of IRFs by cytosolic PRRs. Therefore ELP3 possess' a varied and multifaceted role in the orchestration of IFN induction and signalling, in addition to shaping processes downstream of IFN signalling in the form of ISG expression and potentially subsequent antiviral immunity. In the next chapter will we assess the mechanism by which ELP3 contributes to IFN-I signalling in macrophages.

Chapter 4 – Regulation of IFN-I signalling by the Elongator complex

4.1 Introduction

The Interferon family of cytokines are divided into three separate groups. The IFN-I group are composed of IFNβ and 13 different genetic subtypes of IFNα (58). IFN-II has a sole member, IFNγ predominantly derived from T and NK cells (59). The IFN-III family is composed if IFNλ1-4 which mediate antiviral defence at epithelial surfaces (60).

IFN-I are pivotal coordinators and shapers of innate immune responses. IFN-I execute and facilitate a diverse array of functions across the spectrum of innate immunity. IFN-I induction occurs in response to an array of microbial stimuli. IFN-I signalling has been covered in the main introduction (section 1.3.1 & Fig 1.4). Briefly, IFNβ signals in an autocrine manner or to neighbouring cells, to activate IFNAR, which leads to ISGF3 complex formation and ISG induction (Fig 4.1). A number of ISGs either directly restrict viruses, or enhance and amplify the IFN-I signalling pathway itself. As chronic or aberrant IFN-I production can be pathological and deleterious to the host, IFN-I signalling contains a number of built-in regulatory mechanisms. IFN-I can also activate STAT3 homodimers downstream of IFNAR, which possess the ability to restrain STAT1 responses (65,66). IFN-I signalling also induces transcription of SOCS1 and SOCS3, which block IFNAR-mediated JAK-STAT activation to regulate the strength and temporal nature of IFN-I signalling (67,68).

IFN-I execute a plethora of crucial roles to coordinate innate immune responses in the realms of microbial infection, antigen presentation and NK cell function. IFN-I regulate innate immune-mediated antigen presentation. DC's function as cardinal APCs to facilitate antigen-specific immune responses. IFN-I mediate and shape DC development, differentiation, whilst upregulating proteins involved in antigen presentation, to facilitate innate-mediated initiation of adaptive immune responses (90,91). IFN-I induces NK cell mediated IFNγ production and effector functions in the context of viral infections (94,95). Coupled with their facility to link innate and adaptive responses via APC regulation, IFN-I also directly regulate adaptive immunity. IFN-I shaping of adaptive responses is impacted at multiple levels. IFN-I can either directly inhibit or augment T-

cell differentiation and proliferation depending on IFN-I levels and signal strength, STAT expression or type of microbial infection (96–98).

Foremost among its plethora of crucial functions, IFN-I enable and execute antiviral innate immunity. Secreted IFN-I signals in an autocrine and manner to induce a transcriptional programme in infected and uninfected neighbouring cells that restrains viral infection and replication. The cardinal output of IFN-I signalling that enforces antiviral innate immunity is the induction of hundreds of ISGs. Antiviral immunity enabled and shaped by ISGs is complex and multivectoral. ISGs implement immune responses that mediate viral sensing, inhibit viral replication and enhance IFN signalling. IRF7 is strongly induced following IFN-I signalling, and as previously mentioned enables amplification of IFN-I induction by mediating IFNα transcription (69). STAT1 and IRF9, key mediators of IFN-I signalling, are ISGs themselves, facilitating positive feedback activation of IFN-I signalling (70). RIG-I and MDA5 are IFN-inducible cytosolic PRRs that bind viral dsRNA. They initiate downstream signalling that also induce IFN-I and ISG expression in an amplification feedback loop. PKR is also a key cytosolic receptor for viral dsRNA. PKR activates eIF2α causing translation inhibition and antiviral immunity (74). MxA is a GTPase that is potently induced following IFN-I signalling. MxA binds and blocks activity of viral polymerase following its oligomerization (74). Oligoadenylate synthase (OAS) family of proteins are also highly induced upon IFN-I signalling. They bind viral dsRNA, and catalyse the production of 2’-‘5’ oligoadenylates which mediate RNaseL activation, an endoribonclease which degrades viral RNA (78). The PYHIN family of proteins are upregulated following IFN-I signalling. The PYHINS play key role in nucleic acid sensing in the context of infection, and can mediate a plethora of processes, from proinflammatiory cytokine production, inflammasome activation and IFN-I induction (71). Thus, ISGs coordinate antiviral immunity in a complex & multifactorial manner following induction by IFN-I.

IFN-I engage multifaceted and counterbalancing responses and signals. Due to their fundamental importance in immunity, IFN-I are finely modulated and regulated. As such, alterations to or breakdowns in regulation of IFN-I responses can be deleterious to a host. Acute IFN-I induction generally facilitates host protection and antimicrobial immunity in the realm of infection. Chronic IFN-I induction can often have pathogenic

consequences. In the context of autoimmunity, IFN-I are chronically and aberrantly induced, with autoimmune conditions such as SLE, rheumatoid arthritis and Sjogren's syndrome possessing a strong IFN-I signature (61). IFN-I can also be immunosuppressive during chronic microbial infection (99). In the context of TB infection, IFN-I induce IL-10 and Il-1ra expression, which block inflammasome and IL-1 function, which are critical for antibacterial immunity (85).

IFN-I are integral mediators of innate immunity in a variety of contexts. They orchestrate and regulate a multitude of facets of innate immune responses and as such are highly regulated, with aberrant IFN-I responses being detrimental to the host in a myriad of settings.

In the previous chapter I showed that ELP3 was required for protein expression of components of the signalling pathways eliciting IFN-I production and controlling IFN-I signalling. Therefore here I examined the impact of loss of ELP3 on these signal transduction pathways.

4.2 Results

4.2.1 LPS-mediated IFN-I and Irf7 gene induction is impaired in the absence of Elp3

Proteomic analysis of *Elp3*$^{-/-}$ cells in the last chapter revealed a strongly impaired IFN signature in the absence of ELP3. As previously mentioned, in mammalian systems, cellular processes regulated by the Elongator complex are dependent on its function in modifying tRNA molecules to enable efficient translation of mRNA transcripts. Based on unbiased quantitative proteomics data, we wanted to assess if ELP3 regulates IFN production at a translational level, or whether ELP3 is exerting influence further upstream by regulating another protein required for IFN gene induction. LPS induces IFNβ and IFNα through a well-defined signalling cascade and in a sequential manner (Fig. 4.1A). LPS-induced IFN-I gene expression is impaired in *Elp3*$^{-/-}$ cells. TLR4-mediated *Ifna and Ifnb* mRNA induction were strongly diminished in cells lacking ELP3 (Fig 4.1B & C).

IRF7 expression gets upregulated via autocrine IFN-I signalling following LPS stimulation, and consistent with the impaired IFN-I gene induction, mRNA expression of *Irf7* was abolished following treatment with LPS (Fig 4.1C). As well as the role of IRF7 in *Ifna*

induction, IRFs play a fundamental role in regulating PRR-mediated *Ifnb* gene induction, and so a general effect of ELP3 on IRF mRNA induction could contribute to the impaired LPS-stimulated IFNβ mRNA in *Elp3*$^{-/-}$cells (Fig. 4.1A). Therefore, we decided to assess whether this effect on *Irf7* mRNA induction was a specific effect, or a general effect on IRF gene expression. However, gene expression levels of *Irf3*, *Irf2* and *Irf5* were unaffected in *Elp3*$^{-/-}$ cells (Fig 4.2A-C). Thus, ELP3 absence affects *Irf7* gene expression, but does not have a global effect on IRF mRNA expression.

Hence, although the proteomics data suggested that ELP3 is required for IFN signalling and responses, the data suggests ELP3 is not directly regulating translation of IFN-I at the protein level, but rather is functioning further upstream by exerting control of gene induction of IFN-I following LPS stimulation.

4.2.2 ELP3 is required for IFN-I-mediated ISG induction and STAT activation

Our cellular based gene induction experiments and unbiased proteomics approach suggests a role for the Elongator complex in the regulation of the IFN-I signalling pathway. Therefore, we sought to determine at which point in the IFN-I signalling pathway ELP3 is exerting its effect. IFN-I signalling cascade is shown in Fig 4.1A. STAT1 expression was downregulated in *Elp3*$^{-/-}$ cells in our quantitative proteomics. Thus, we assessed whether this autocrine feedback loop arm of IFN-I is dysregulated in cells lacking ELP3, which could explain the observed defect in IFN processes (Proteomics data, Fig 3.11-3.13) and *IFN-I* mRNA induction (Fig 4.1). To eliminate TLR4-mediated IFN-I gene induction, and activate IFNAR signalling directly, WT and *Elp3*$^{-/-}$ cells were stimulated directly with IFNβ and mRNA induction of several ISGs was assessed. Mirroring LPS-induced gene expression, IFNβ-mediated *Irf7* mRNA induction was abolished in *Elp3*$^{-/-}$ cells relative to WT cells (Fig 4.3B). Similarly, IFNβ-mediated gene induction of *Isg15*, *Stat1* & *Stat3* were severely diminished in *Elp3*$^{-/-}$ cells (Fig 4.3C-E). This data intriguingly raises the possibility that, dysregulation of the IFN feedback arm of the pathway may be responsible for impaired *Irf7*, *ISG* and *IFN-I* gene induction in the absence of ELP3 as opposed to upstream regulators.

Based on this impaired gene induction, and to confirm the data from our quantitative proteomics, we assessed STAT1 protein expression by western blot. Total STAT1 protein

expression was strongly diminished in cells lacking ELP3 relative to WT cells. This is in line with our proteomics data. As a result, total abolishment of STAT1 phosphorylation and hence activation was observed in *Elp3*$^{-/-}$ cells following IFN-I stimulation. STAT3 activation and phosphorylation was similarly impaired, though in accordance with our proteomics data, total STAT3 expression is unaffected in *Elp3*$^{-/-}$ cells, suggesting ELP3 absence does not affect expression of all STATs (Fig 4.4).

4.2.3 LPS and IFN-I, but not IFNγ-mediated STAT activation and gene induction are impaired in Elp3$^{-/-}$ *cells*

Next we sought to ascertain the mechanism by which ELP3 affects STAT activation and subsequent gene induction following IFN-I stimulation. As we observed, total STAT1 levels were diminished in *Elp3*$^{-/-}$ cells. This raises the possibility that there is insufficient total STAT1 protein to enable proper phosphorylation, activation and signal transduction in cells lacking ELP3. We hypothesized that if total STAT1 protein levels were the restrictive and rate-limiting factor for dysregulated LPS and IFN-I responses, then other signalling pathways mediated by STAT1 should be similarly impaired in *Elp3*$^{-/-}$ cells. IFN-I signals through the IFNAR complex, which via its receptor associated kinases, JAK1 and TYK2, activates STAT1 and STAT2 phosphorylaton and heterodimerisation. Comparably to IFN-I, IFNγ, also induces STAT1 activation (Fig 4.5A). A number of differences exist however, between IFN-I and IFNγ signalling pathways. Analogous to IFNAR signalling, the IFNγR utilizes JAK1 as a receptor associated kinase, but whereas IFNAR binds TYK2, the IFNγR binds JAK2. In contrast to the STAT1-STAT2 heterodimer formed during IFN-I signalling, IFNγ stimulation leads to the formation and activation of a STAT1 homodimer, which translocates to the nucleus and enables transcription of ISGs such as *Irf1* (Fig 4.5a). To evaluate the possibility that total STAT1 protein levels are the restrictive factor for signalling in the absence of ELP3, STAT1 activation and subsequent gene induction was examined following stimulation with IFNγ. Interestingly, contrary to stimulation with IFN-I, IFNγ-mediated STAT1 activation and phosphorylation was unaffected in *Elp3*$^{-/-}$ cells (Fig 4.5B). Correspondingly, IFNγ-mediated IRF1 mRNA induction is unimpaired in cells lacking Elp3. Thus, as IFNγ-mediated STAT1 activation and downstream gene induction is not dysregulated, this indicates that not all STAT1 regulated processes are

impaired in *Elp3*$^{-/-}$ cells and that a requirement for ELP3 for normal STAT1 total protein expression is not the main mechanism by which the absence of ELP3 is affecting IFN-I expression and signalling. This was further illustrated in Figure 4.6, where STAT activation and IRF1 induction was assessed following LPS, IFNβ & IFNγ stimulation performed in parallel. LPS and IFNβ-mediated STAT1 phosphorylation was ablated in *Elp3*$^{-/-}$ cells relative to WT cells. Also, LPS and IFNβ-mediated STAT2 phosphorylation was similarly defective in Elp3$^{-/-}$ cells, even though total STAT2 protein expression levels were similar in WT and KO cells, further suggesting impaired formation of a functional ISGF3 complex in the absence of ELP3. In contrast, IFNγ-mediated STAT1 phosphorylation was unaffected in *Elp3*$^{-/-}$ cells, with no STAT2 activation occurring (Fig 4.6A). LPS, IFNβ & IFNγ-mediated IRF1 gene induction was also assessed in parallel, with *Irf1* mRNA levels in WT cells set to 100 %, and *Elp3*$^{-/-}$ relative mRNA levels of IRF1 expressed as a percentage of their WT stimulated counterpart (Fig 4.6b). LPS and IFNβ *Irf1* gene induction was severely diminished in Elp3$^{-/-}$ cells, with IFNγ-stimulated *Irf1* mRNA induction unaffected. Absence of ELP3 dysregulates LPS and IFNβ signalling and exerts no effect on IFNγ-mediated processes. This suggests that the diminished STAT1 total protein expression in *Elp3*$^{-/-}$ cells, is not the reason for impaired IFN expression and signalling.

4.2.4 TYK2-mediated signalling pathways are abrogated in cells lacking Elp3

As STAT1 activation but not expression is impaired following LPS & IFNβ stimulation, we decided to evaluate the expression of the IFNAR complex and its associated proteins. The expression of IFNAR1, JAK1 and TYK2 were unaffected by the absence of ELP3 (Fig 4.7). This data raises the possibility that LPS and IFN-I-mediated activation of JAK1 or TYK2 is defective in cells lacking ELP3. As mentioned, both IFNAR and IFNγR both bind JAK1 for intracellular signal transduction. However, due to the observation that IFNγ signalling and responses are unaffected in *Elp3*$^{-/-}$ cells, precluding JAK1 activation as the dysregulated factor restricting LPS & IFN-I signalling in the absence of Elp3. Consequently, we decided to examine whether signalling pathways regulated by the other tyrosine kinase used by IFNAR, TYK2, were dysregulated in *Elp3*$^{-/-}$ cells. IL-4 signalling induces alternative 'M2' macrophage activation, leading to upregulation of proteins such as arginase-1, Ym1 and Fizz (212). IL-4 signals through a receptor complex

that utilizes TYK2, and interestingly, it has been shown that conversion of murine macrophages to an M2-like phenotype and expression of M2 markers following IL-4 stimulation is defective in the absence of ELP3 (213). Supporting this observation, we found that IL-4-mediated upregulation of M2 marker expression, *Arg1*, in *Elp3*$^{-/-}$ iBMDMs was completely abolished (Fig 4.8a). IL-10 similarly signals through a receptor complex which utilizes TYK2 for intracellular signal transduction. The IL-10-mediated gene induction responses, as measured by *Socs3* mRNA expression, was also severely impaired in *Elp3*$^{-/-}$ cells (Fig 4.8B). Thus, multiple signalling pathways, namely IFN-I, IL-4 and IL-10, all utilizing TYK2 to enable signal transduction, are dysregulated in cells lacking ELP3. Tyk2 total protein expression was unaffected in *Elp3*$^{-/-}$ cells (Fig 4.7), indicating that aberrant TYK2 activation may be culpable in the observed restriction of IFN-I signalling, STAT1 activation and ISG and IFN-I mRNA induction in the absence of ELP3.

4.2.5 PKR protein expression is unaffected in Elp3$^{-/-}$ *cells*

Having established a mechanism for the requirement for ELP3 for IFN-I signalling, namely a requirement for LEP3 for TYK2-depednent responses, we turned to examine other aspects of the IFN response that might be regulated by ELP3. PKR plays a pivotal role in RNA sensing and antiviral innate immunity. Quantitative proteomics data suggest that PKR expression was downregulated in *Elp3*$^{-/-}$ *cells*. PKR is also enriched in codons requiring Elongator for their proper translation (13.2 %). Thus, we decided to evaluate PKR expression as an ISG whose expression is potentially ElP3-dependent. As previously observed with IRF1, mRNA induction of PKR was severely impaired following LPS and IFN-I, but not IFNγ stimulation (Fig 4.9A). As PKR gene induction was unaffected for IFNγ stimulation, and is enriched for ELP3-dependent mRNA codons, we examined PKR protein expression to assess whether ELP3 is required for IFNγ-mediated PKR translation. However, Fig 4.9B shows that PKR protein expression was unaffected in *Elp3*$^{-/-}$ cells, in the presence of absence of stimulation. This data suggest that although PKR is enriched with Elongator-dependent mRNA codons, that potentially PKR protein expression is sufficiently stable to overcome the translational restriction, or that in the absence of ELP3 cells engage a compensatory mechanism to enable sufficient PKR expression.

4.2.6 ELP3 is required for protein expression, but not gene induction of PHYIN protein p205 following IFNγ stimulation

The PYHIN family of proteins are IFN-inducible genes that play a myriad of roles in innate immunity, ranging from nucleic acid sensing and viral restriction, to inflammasome activation and regulation of gene expression (39,71). Previous work from the lab has focused on a range of PYHIN proteins and their functions in innate immunity. Intriguingly, murine PYHIN family members are highly enriched with mRNA codons that are Elongator-dependent (Fig 4.10). Expression of murine PYHINs at the protein level are often difficult to detect. Consequently, we decided to evaluate expression of the PYHIN protein p205 in *Elp3*$^{-/-}$, due to its potential high level of Elongator-dependency and the availability of an antibody capable of detecting p205 expression by immunoblot (Antibody generated and gifted by Kate Fitzgerald lab in UMASS). Mirroring trends previously observed, LPS and IFN-I but not IFNγ-mediated *p205* mRNA induction was dysregulated in *Elp3*$^{-/-}$ cells (Fig 4.11A). In accordance with this impairment of gene expression, LPS and IFN-I induction of p205 protein expression was similarly abolished in cells lacking ELP3. However for IFNγ stimulation, even though *p205* mRNA expression, IFNγ-induced p205 protein expression was strongly impaired in *Elp3*$^{-/-}$ cells (Fig 4.11B). As evident from the gene induction analysis, IFNγ signalling is fully functional in *Elp3*$^{-/-}$ cells. Consequently, the impaired p205 protein expression following IFNγ stimulation suggests ELP3 is necessary for regulating protein expression, but not gene induction, of p205. This is the first instance whereby Elongator has been shown to be required for the protein, but not mRNA expression of an innate immune protein, and strongly suggest a direct role for ELP3 in translation of p205. P205 has been implicated in regulating transcription and subsequent expression of the inflammasome adaptor protein ASC in BMDMs (72). Therefore, we decided to assess ASC expression to the impaired induction of p205 in the absence of ELP3. However, ASC expression was not reduced in *Elp3*$^{-/-}$ cells, but was actually enhanced in the absence of ELP3 (Fig 4.12). Thus, reduced expression of p205 protein had no restrictive effect on ASC. It may be possible that ELP3 regulates expression of a protein that restricts ASC expression, and *Elp3*$^{-/-}$ leads to a de-repression on ASC which overcomes p205 deficiency.

4.2.7 ELP3 does not regulate ACLY expression and stability in macrophages

ATP-citrate lyase (ACLY) catalyses the production of acetyl CoA, which is critical for epigenetic regulation of gene promoters. It has been shown to be required for LPS-induced acetylation and subsequent gene induction of IL-6 and IL-12 (214). Across multiple animal models, Elongator was observed to interact with ACLY and regulate microtubule-dependent transport in neurons (215). It appears to do this not be mediating ACLY translation, but by regulating ACLY protein stability. Due to the ready availability of a an antibody for ACLY, and its purported role in innate immunity, we decided to mirror the technique used to assess this in neurons, by performing a cycloheximide chase assay, which determines protein stability and degradation kinetics. WT and *Elp3*$^{-/-}$ iBMDMs were treated with cycloheximide for 8 hours before being harvested and assessed for ACLY expression and stability by immunoblot. In murine macrophages, ELP3 deficiency had no discernible effect on ACLY stability following cycloheximide treatment (Fig 4.13). This data suggest that possibly Elongator-mediated stabilization of ACLY may be limited to a neuronal setting, and may not effect ACLYs purported role in innate immunity.

Overall, the results show that ELP3 is required for IFN-I signalling. IFN-I signalling was impaired in *Elp3*$^{-/-}$ cells due to abrogated TYK2 activation. Other TYK2-mediated pathways were impaired in the absence of ELP3, suggesting TYK2 activation is restrictive for IFN-I signalling in cells lacking ELP3. We found that the PYHIN family of proteins were enriched in Elongator-dependent codons, and that ELP3 was required for IFNγ-mediated p205 protein expression, but not *p205* mRNA induction. ELP3 was shown to not be required for PKR and ACLY protein expression.

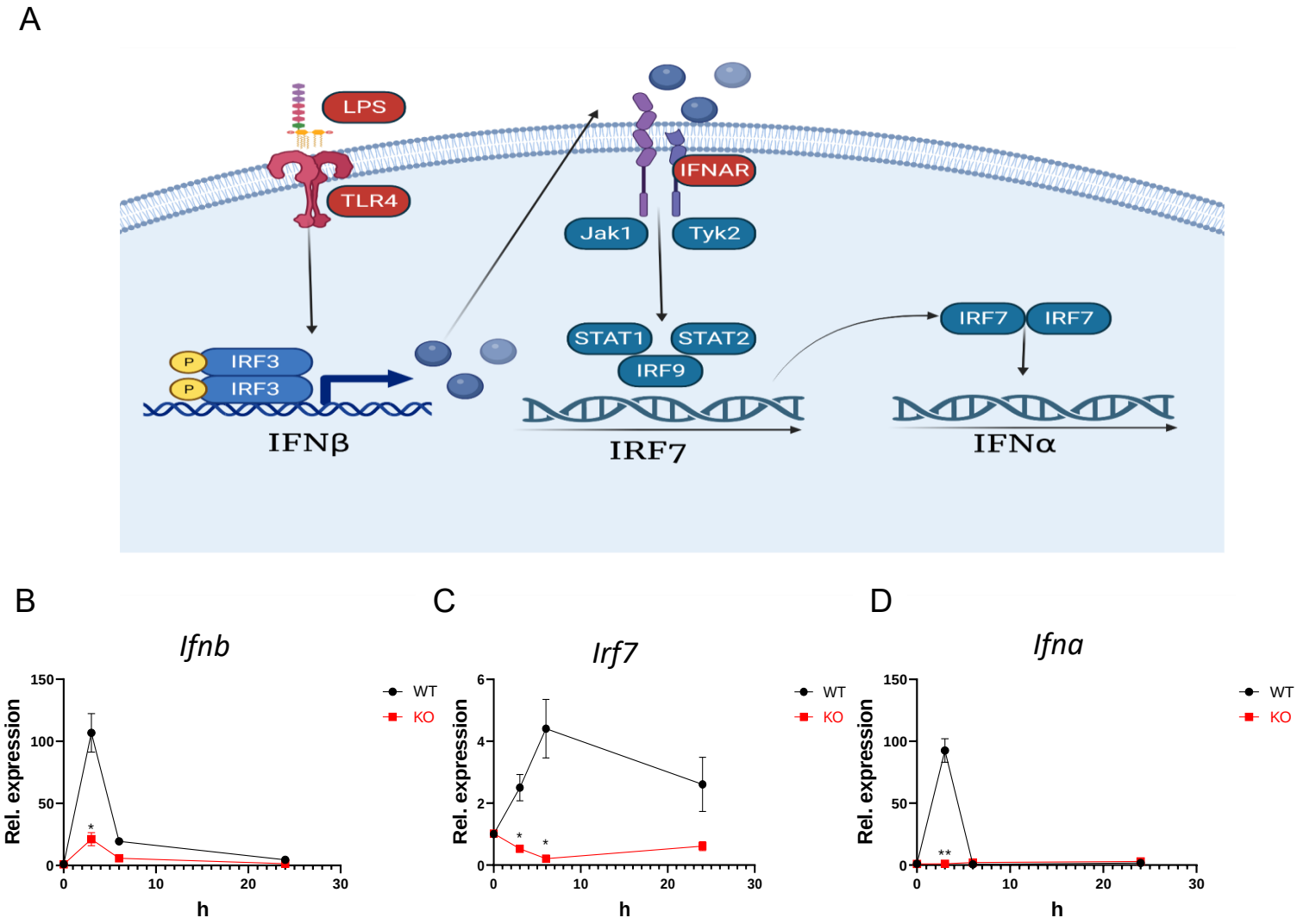


Fig 4.1 TLR4-mediated IFN-I induction is ELP3-dependent

Schematic of LPS-mediated IFN-I induction and subsequent signalling (A). Wild-type (WT) and *Elp3*$^{-/-}$ iBMDMs were seeded at 5 x 10^5 cells/ml and stimulated with 100 ng/ml LPS. RNA was isolated following 3, 6 and 24 h LPS stimulation and gene expression of *Ifnb* (B), *Irf7* (C) & *Ifna* (D) was measured by qRT-PCR. mRNA levels are presented relative to mRNA levels of β-actin. Data are mean ± SEM and is average of three independent experiments. *P < 0.05, ***P < 0.001 compared to WT, based on students t-test

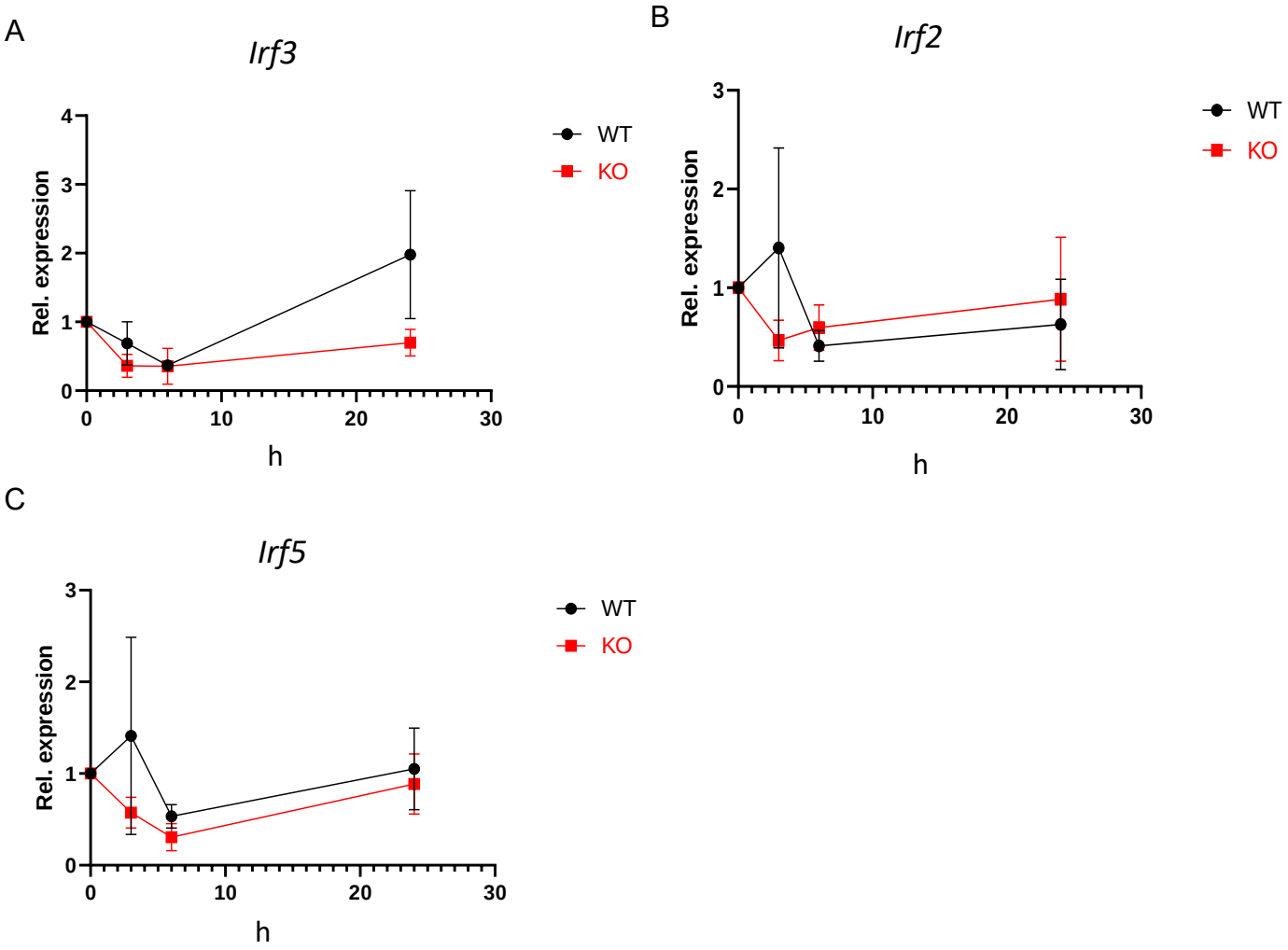


Fig 4.2 Gene expression of IRF2, IRF3 & IRF5 is unimpaired in *Elp3*$^{-/-}$ iBMDMs following LPS stimulation

Wild-type (WT) and *Elp3*$^{-/-}$ iBMDMs were seeded at 5 x 10^5 cells/ml and stimulated with 100 ng/ml LPS. RNA was isolated following 3, 6 and 24 h LPS stimulation and gene expression of (A) *Irf3*, (B) *Irf2* and (C) *Irf5* was measured by qRT-PCR. mRNA levels are presented relative to mRNA levels of β-actin. Data are mean ± SEM of 3 experiments.

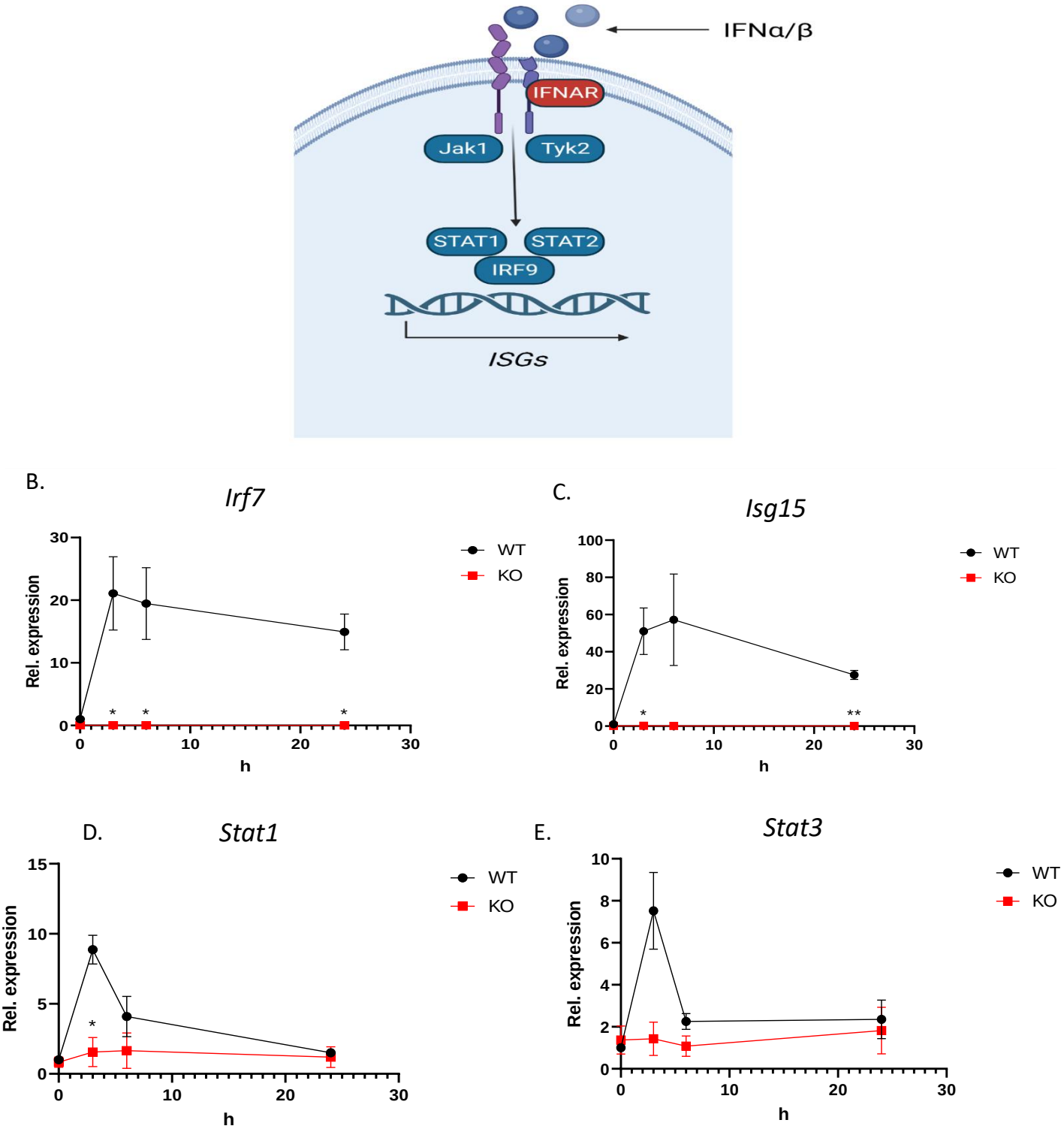


Fig 4.3 IFNβ-mediated ISG expression is impaired in the absence of ELP3

(A) IFNβ-mediated signalling. (B-E)WT and *Elp3*$^{-/-}$ iBMDMs were seeded at 5 x 10^5 cells/ml and stimulated with IFNβ (1000 U/ml). RNA was isolated following 3, 6 and 24 h and gene expression of *Irf7, Isg15, Stat1 and Stat3* was measured by qRT-PCR. mRNA levels are presented relative to β-actin. Data are mean ± SEM. *p<0.05 compared to WT, based on students t-test

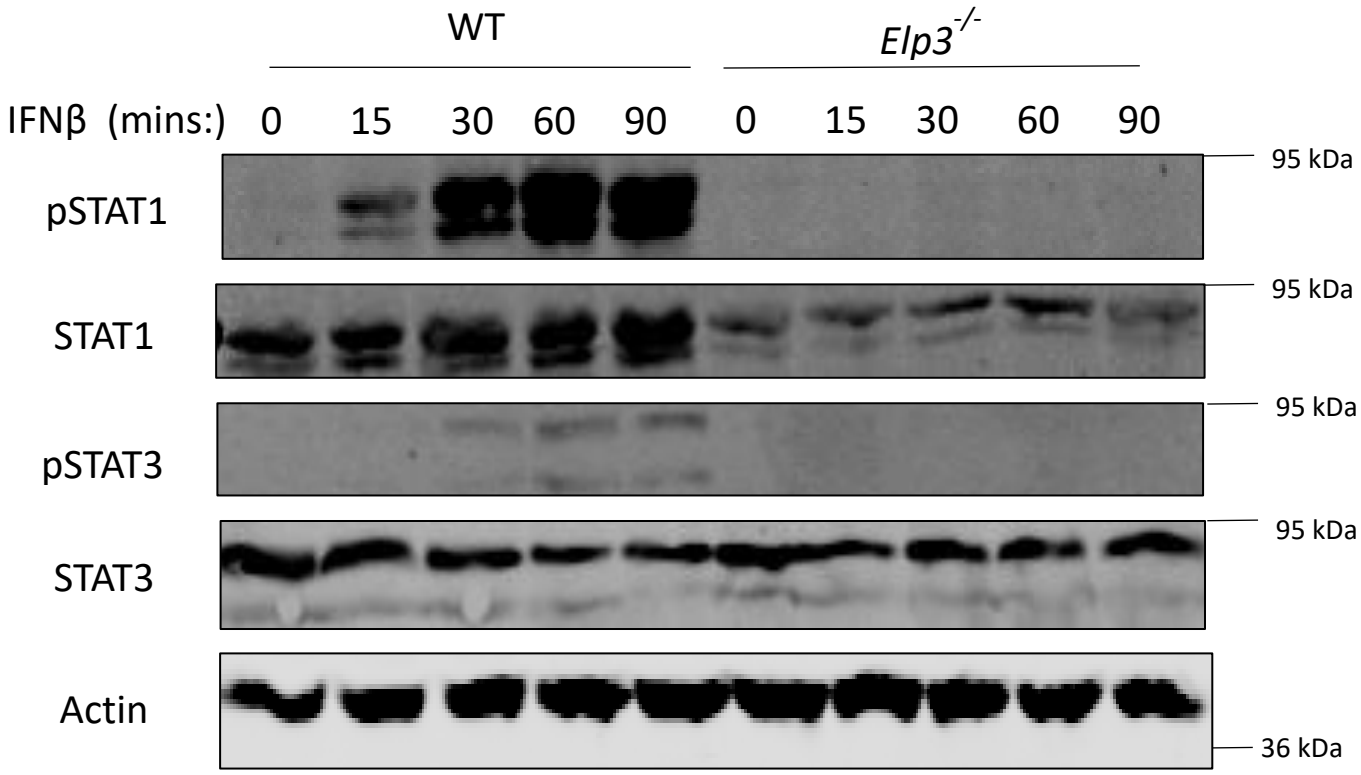
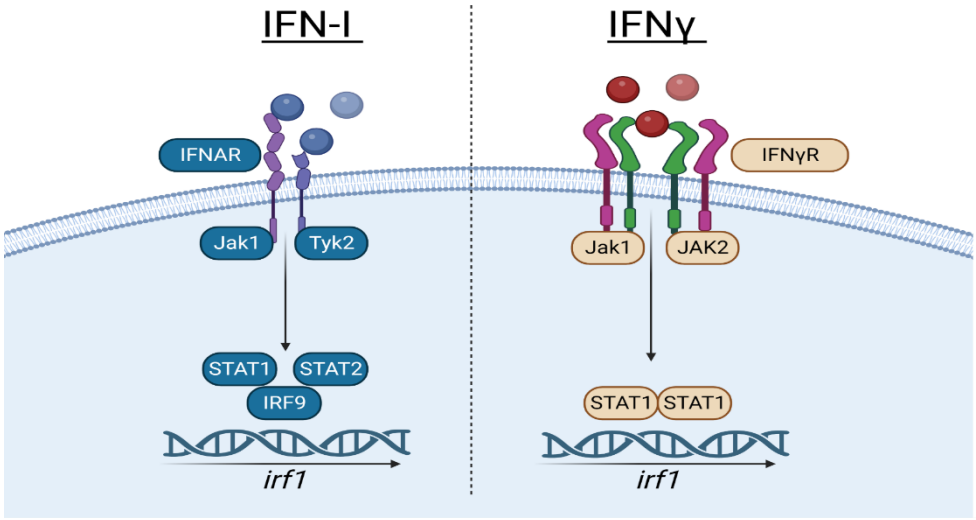


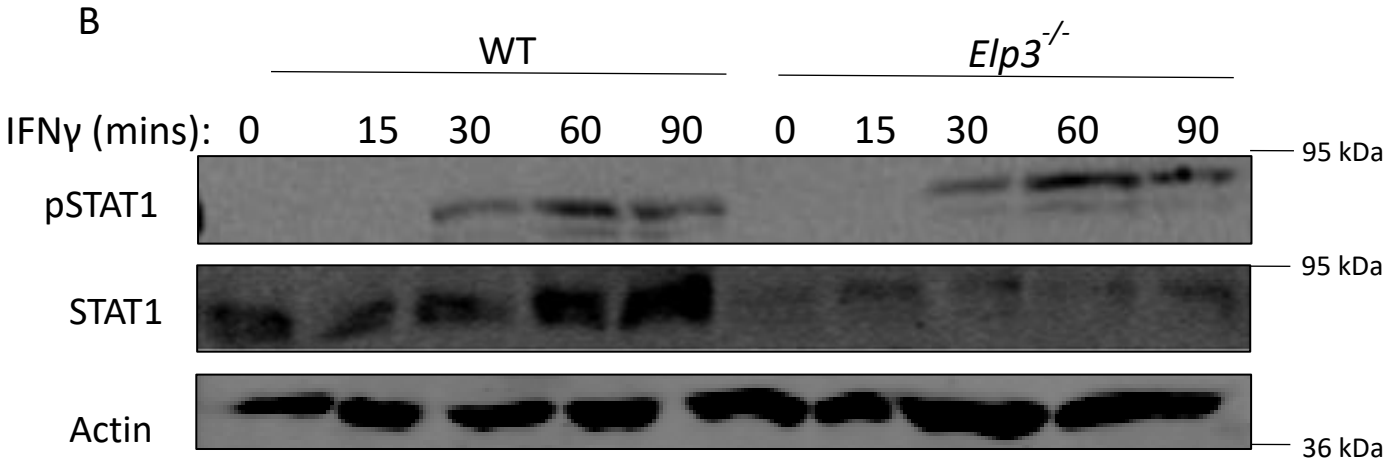
Fig 4.4 IFNβ-mediated STAT activation is impaired in the absence of ELP3

WT & *Elp3*$^{-/-}$ iBMDMs were seeded at 5 x 10^5 cells/ml and stimulated with IFNβ (1000 U/ml) for the indicated times. Cells were harvested and total & phosphorylated STAT1 & STAT3 was assessed by immunoblot. Representative of three independent experiments

A


IFN-I
IFNγ
IFNAR
Jak1
Tyk2
STAT1
STAT2
IRF9
irf1
IFNγR
Jak1
JAK2
STAT1
STAT1
irf1

B


WT
Elp3-/-
IFNγ (mins): 0 15 30 60 90 0 15 30 60 90
pSTAT1
STAT1
Actin
95 kDa
95 kDa
36 kDa

C

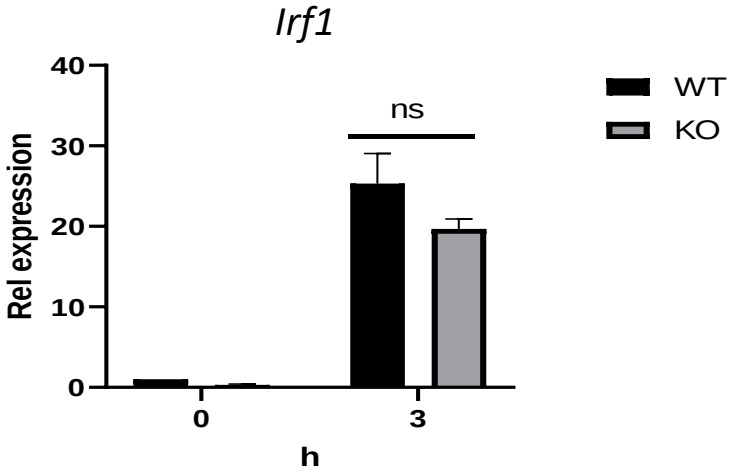

Irf1
Rel expression
40
30
20
10
0
ns
0
3
h
WT
KO

Fig 4.5 IFNγ-mediated STAT activation & ISG induction is unimpaired in iBMDMs lacking ELP3

(A)IFN-I and IFNγ-mediated signalling pathways. (B) WT & *Elp3*$^{-/-}$ iBMDMs were seeded at 5 x 10^5 cells/ml and stimulated with IFNγ (50 ng/ml) for the indicated times . Cells were harvested and total & phosphorylated STAT1 was assessed by western blot. Representative of N=3. (C) *Irf1* gene expression was assessed by qRT-PCR following 3 h stimulation with with IFNγ (50 ng/ml). mRNA levels are represented relative to β-actin. Data are mean ± SEM and is average of 3 experiments.

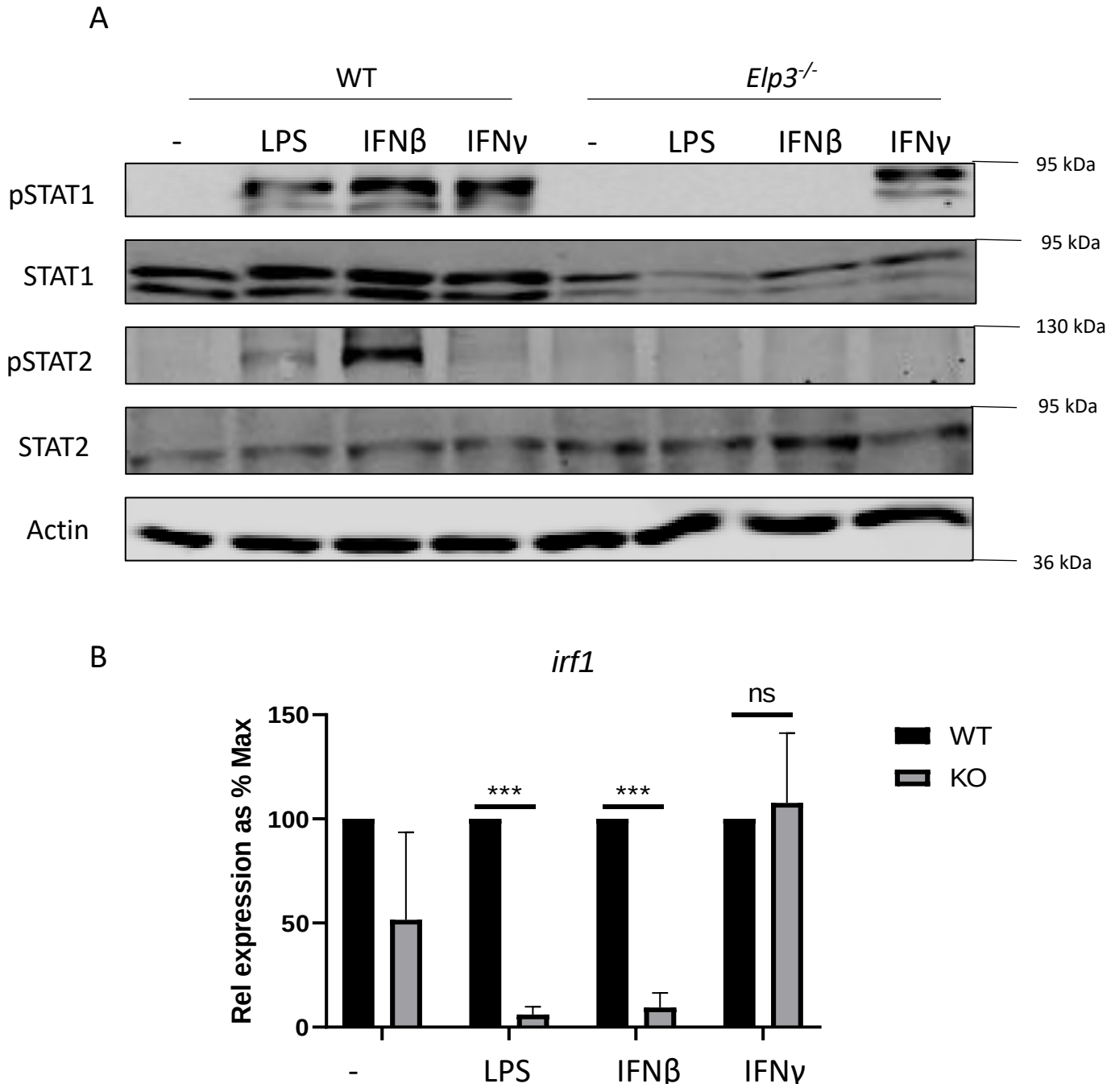


Fig 4.6 LPS and IFNβ, but not IFNγ-mediated STAT activation and downstream gene induction are suppressed in cells lacking ELP3

WT & *Elp3*$^{-/-}$ iBMDMs were seeded at 5 x 10^5 cells/ml and stimulated with either LPS (100 ng /ml), IFNβ (1000 U/ml) or IFNγ (50 ng/ml) for 90 mins. Cells were harvested and total & phosphorylated STAT1 & STAT2 was assessed by immunoblot. Representative of 3 independent experiments. (B) *Irf1* gene expression was assessed by qRT-PCR following 3 h stimulation with with LPS (100 ng /ml), IFNβ (1000 U/ml) or IFNγ (50 ng/ml). mRNA levels are represented relative to β-actin, with each WT sample set to 100% and KO levels expressed as a percentage of their WT stimulated counterpart. Data are mean ± SEM and is average of 3 experiments. *p<0.001 compared to WT, based on students t-test

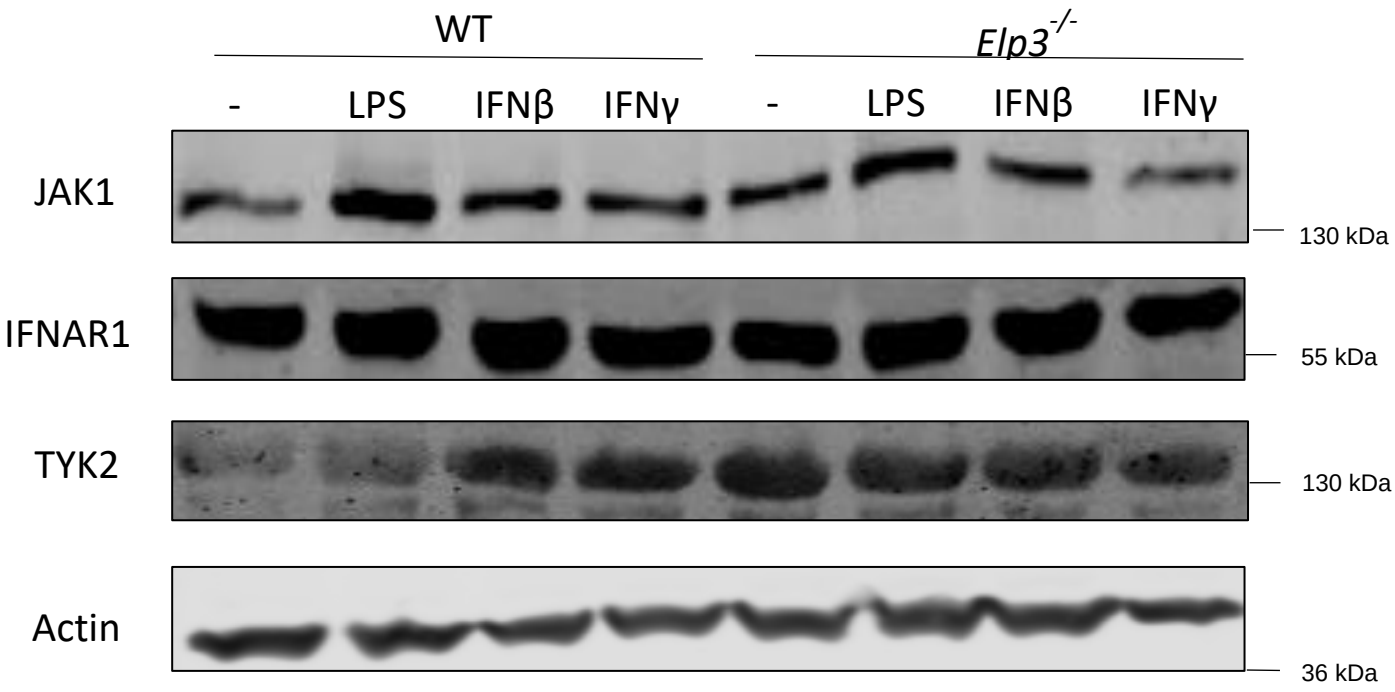


Fig 4.7 IFN-I receptor complex expression is unaffected by the absence of ELP3

WT & *Elp3*$^{-/-}$ iBMDMs were seeded at 5 x 10^5 cells/ml and stimulated with either LPS (100 ng /ml), IFNβ (1000 U/ml) or IFNγ (50 ng/ml) for 90 minutes. Cells were harvested and expression of JAK1, IFNAR1 & TYK2 was assessed by immunoblot. Representative of three independent experiments

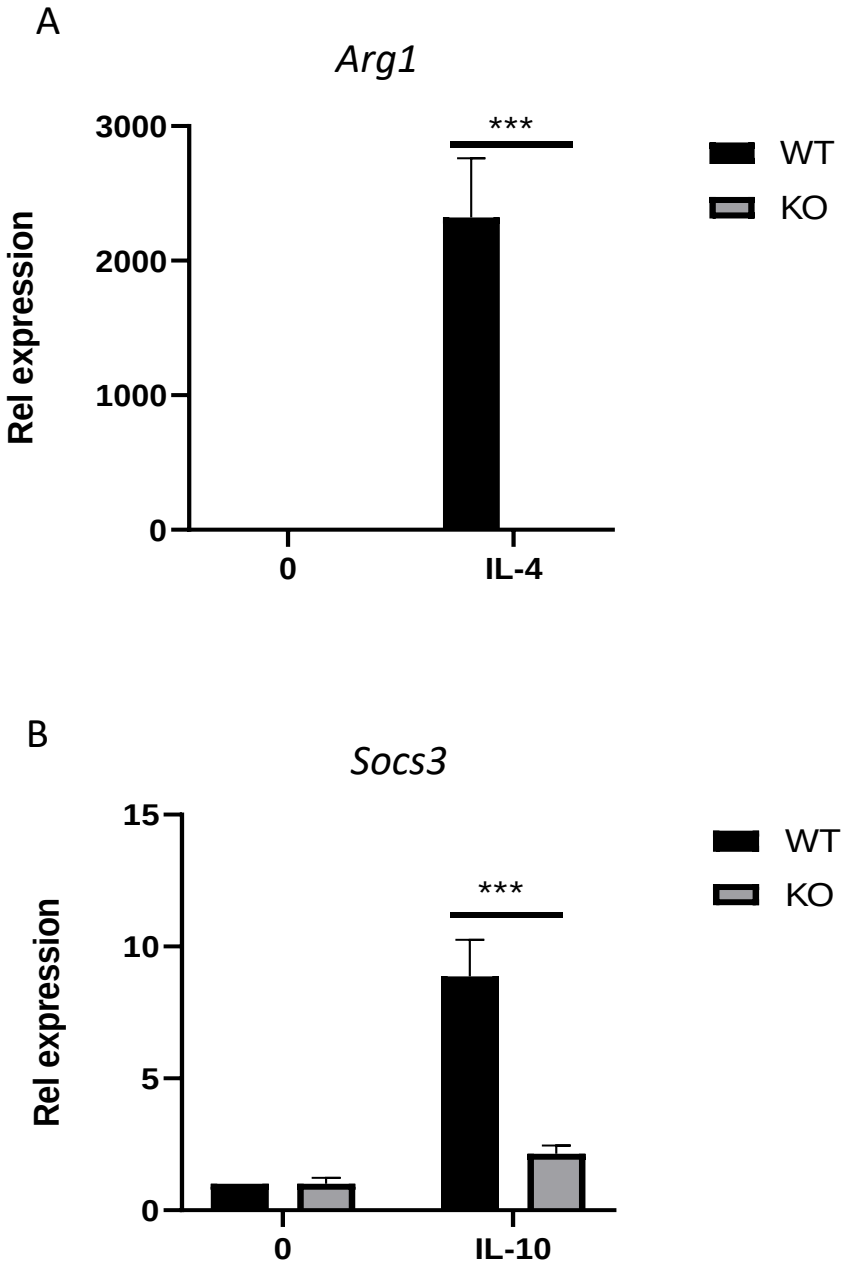


Fig 4.8 IL-4 & IL-10-mediated signalling pathways are dysregulated in *Elp3*$^{-/-}$ iBMDMs

WT and *Elp3*$^{-/-}$ iBMDMs were seeded at 5 x 10^5 cells/ml and stimulated with IL-4 (10 ng/ml) (A) or IL-10 (10 ng/ml) (B). RNA was isolated following 3 h and gene expression of *Arg1* and *Socs3* was measured by qRT-PCR. mRNA levels are presented relative to β-actin. ***p<0.001 compared to WT based on students t-test

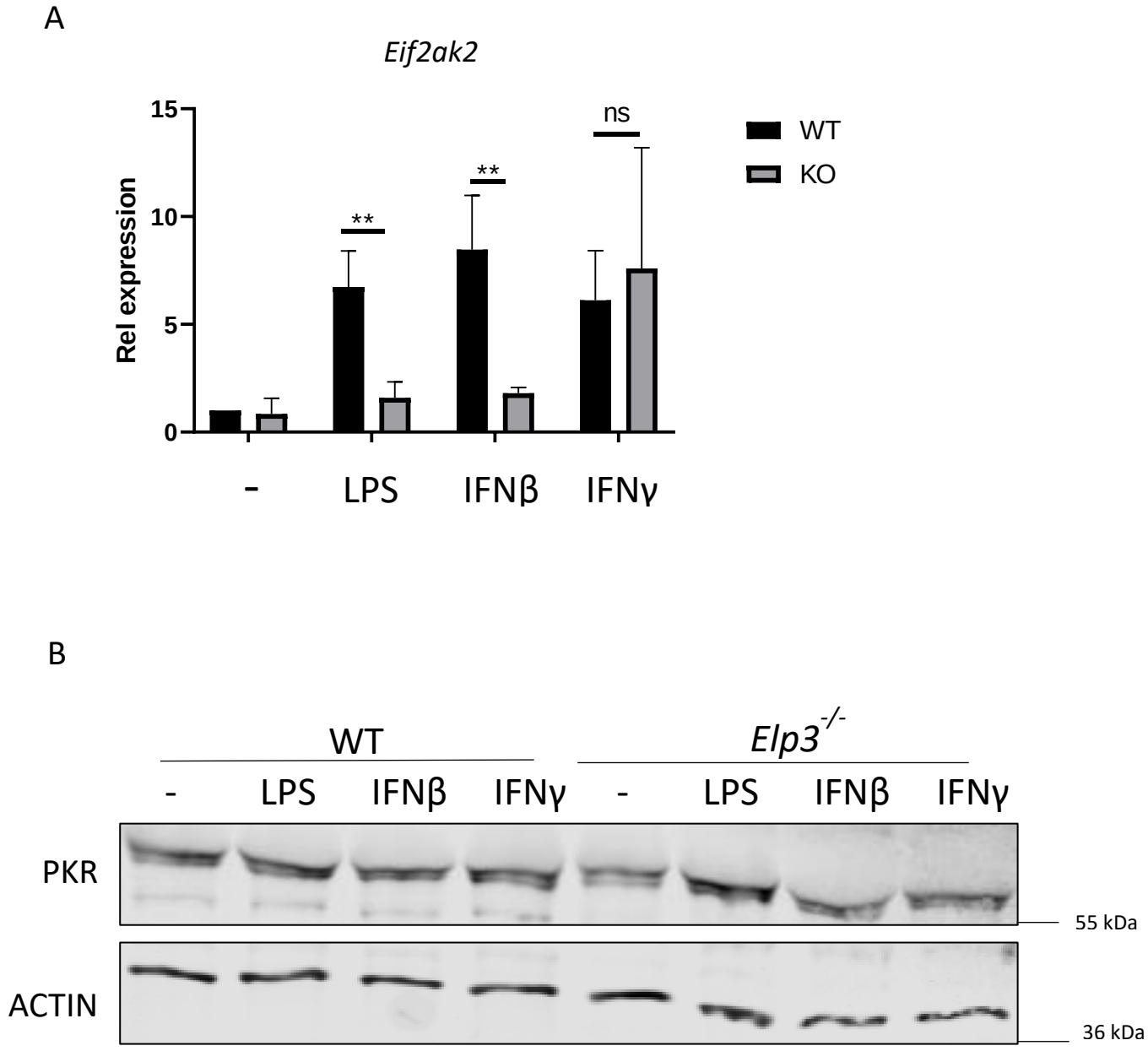


Fig 4.9 Effect of *Elp3*$^{-/-}$ on PKR gene induction & protein expression

WT & *Elp3*$^{-/-}$ iBMDMs were seeded at 5 x 10^5 cells/ml and stimulated with either LPS (100 ng /ml), IFNβ (1000 U/ml) or IFNγ (50 ng/ml). RNA was isolated after (A) 3 h and *Eif2ak2* gene expression was analysed by qRT-PCR. mRNA levels are represented relative to β-actin. Data are mean ± SEM and of 3 experiments. ** $p<0.01$(B) Cells were harvested 3 h post stimulation and PKR protein expression was assessed by immunoblot. Representative of three independent experiments

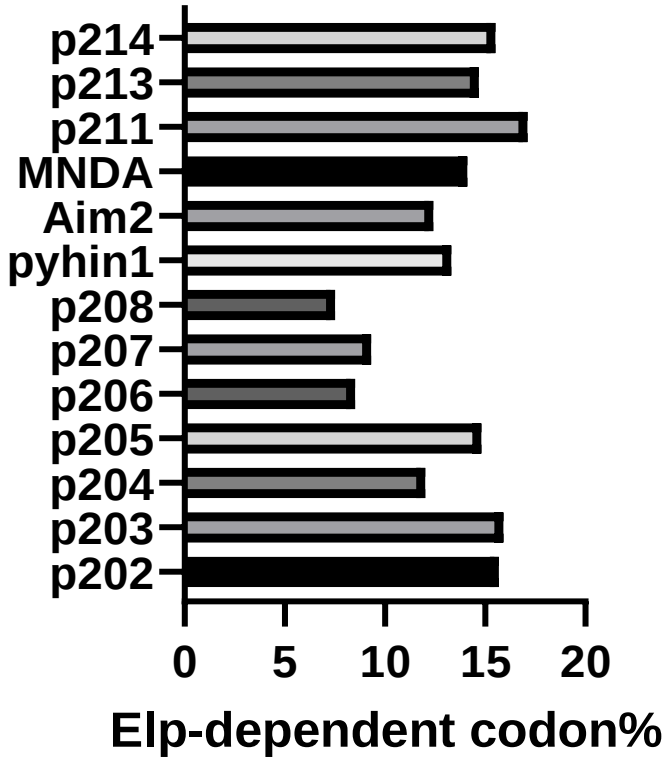


Fig 4.10 Mouse Pyhin mRNA transcripts are enriched with Elongator-dependent codons

Mouse pyhin mRNA coding sequences were obtained and analysed for the presence of CAA, GAA & AAA codons. The proportion of these codons are shown as a % of total codons in each mRNA transcript

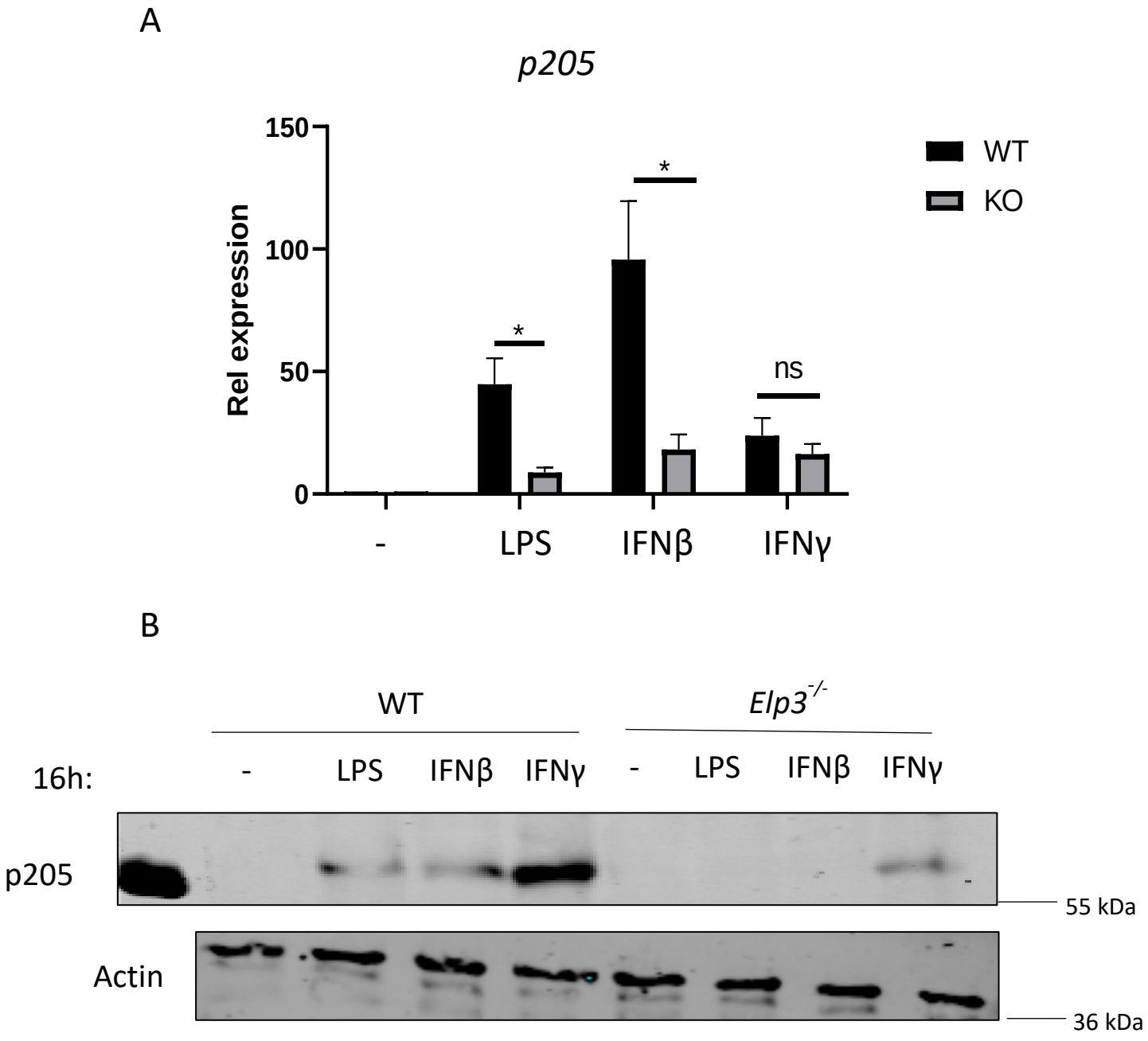


Fig 4.11 IFNγ-mediated p205 protein but not mRNA expression is affected in *Elp3*$^{-/-}$ iBMDMs

WT & *Elp3*$^{-/-}$ iBMDMs were seeded at 5 x 10^5 cells/ml and stimulated with either LPS (100 ng /ml), IFNβ (1000 U/ml) or IFNγ (50 ng/ml). RNA was isolated after (A) 3 h and *p205* gene expression was analysed by qRT-PCR. mRNA levels are represented relative to β-actin. Data are mean ± SEM and is average of 3 experiments. *p<0.0.5 compared to WT, based on students t-test (B) Cells were harvested 16 h post stimulation and p205 protein expression was assessed by immunoblot. Data are representative of 3 independent experiments.

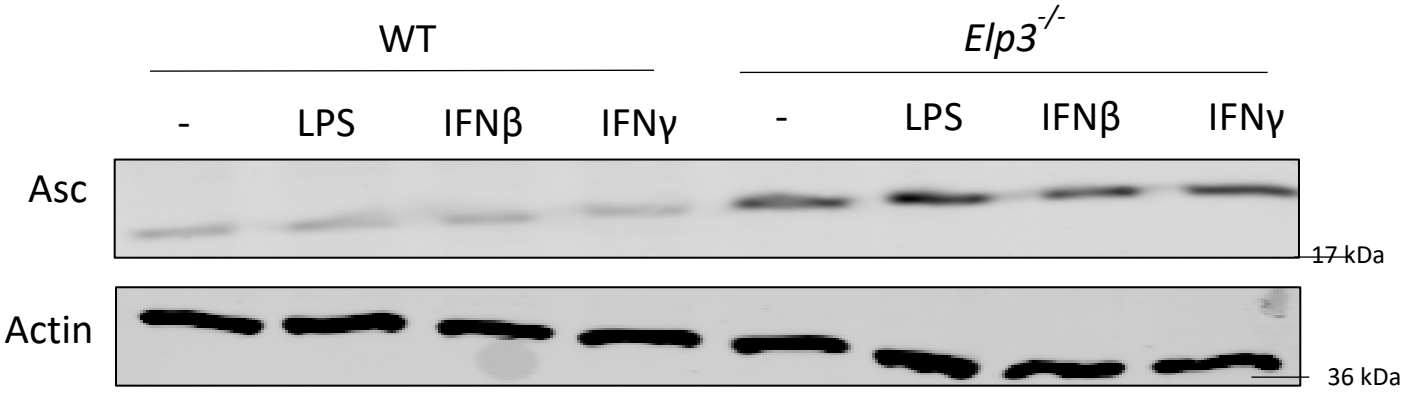


Fig 4.12 ASC expression is unimpaired in the absence of ELP3

WT & *Elp3*$^{-/-}$ iBMDMs were seeded at 5 x 10^5 cells/ml and stimulated with either LPS (100 ng /ml), IFNβ (1000 U/ml) or IFNγ (50 ng/ml). Cells were harvested 3 h post stimulation and ASC protein expression was assessed by immunoblot. Representative of three independent experiments.

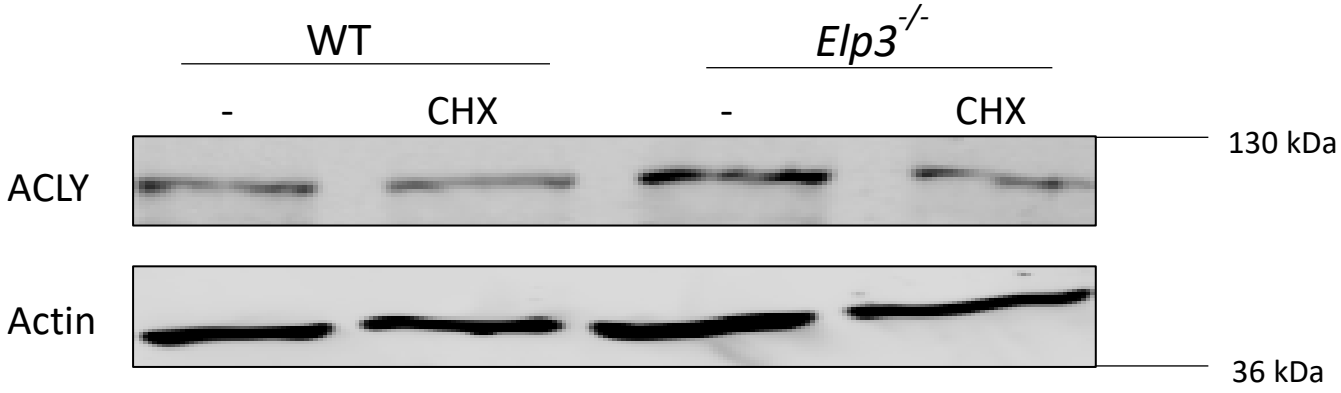


Fig 4.13 ACLY stability is unaffected by the absence of ELP3

WT & *Elp3*$^{-/-}$ iBMDMs were seeded at 5 x 10^5 cells/ml before being treated with cyclohexamide (50 µg/ml) for 8 h. Cells were subsequently harvested and ACLY expression assessed by immunoblot. Representative of three independent experiments

4.3 Discussion

Proteomic analysis of *Elp3*$^{-/-}$ macrophages revealed an impaired IFN-I signature in the absence of ELP3. We sought to appraise how ELP3 influences and regulates IFN-I. LPS-mediated IFN-I gene induction was impaired in *Elp3*$^{-/-}$. We demonstrate that IFN-I signalling is strongly abrogated, with STAT activation and ISG induction ablated in *Elp3*$^{-/-}$ cells. IFNγ signalling and responses were unaffected. IFNAR complex protein expression is unaffected. However, other Tyk2-mediated pathways like IL-4 and IL-10, are abrogated, suggesting ELP3 is necessary for Tyk2 activation in macrophages. Thus overall, the data indicate that ELP3 is crucial for IFN-I-mediated feedback signalling and gene induction, likely due to a requirement for ELP3 for TYK2 activation.

Proteomic analysis of *Elp3*$^{-/-}$ cells illustrated that the most abrogated process in cells lacking ELP3 was IFN-mediated signalling and processes. URA analysis also revealed IFNβ as a potential regulator that is required for expression of downregulated proteins in *Elp3*$^{-/-}$ cells. Intriguingly, we discovered that following LPS treatment IFN-I gene induction is ablated in the absence of ELP3. Following IFN-I gene induction, IFNβ signals in a feedback loop to activate *Irf7* expression, which mediates subsequent transcription of *Ifna*, and generally amplifies IFN-I expression (69). Impaired *Ifna* induction is likely due to abrogated expression of *Irf7*. *Irf7* expression likely derives from impaired *Ifnb* gene induction following LPS stimulation. This data suggests that the effect of ELP3 on LPS-induced IFN-I is not a translational regulation of IFN-I expression.

In plants and yeast, Elongator can directly regulate gene expression via epigenetic mechanisms (172). In mammalian systems however, initially purported roles for Elongator in transcription appear to result indirectly from its translational role in decoding of mRNA. As mentioned, IFN-I signals in an autocrine manner to induce both ISG and further IFN-I expression, to amplify IFN-I responses. Proteomics analysis of *Elp3*$^{-/-}$ cells showed that expression of a number of proteins involved in IFN signalling (eg STAT1) was impaired. Based on this data we decided to examine and analyse IFN-I signalling pathway in *Elp3*$^{-/-}$ cells. We showed that IFN-I mediated ISG induction and STAT1 activation was ablated in the absence of ELP3. Reduction of STAT1 expression occured at the level of both protein and mRNA. Tonic IFN-I expression, induced by commensal microbes, is required to maintain steady state levels of STAT1 and other IFN

signalling proteins (62). As STAT1 is down at the mRNA level, this suggests that rather than ELP3 regulating STAT1 translation, basal and tonic IFN-I signalling requires ELP3.

We resolved to investigate whether diminished total protein expression, is the rate-limiting factor for abrogated activation of STAT1 in *Elp3*$^{-/-}$ cells. Where IFN-I signals predominantly through STAT1-STAT2 heterodimers, IFNγ induces activation of STAT1 homodimers. We displayed that LPS and IFNβ-mediated STAT1 & STAT2 phosphorylation is abrogated, but in contrast, IFNγ-mediated STAT1 activation was unaffected. Conjointly, IFNγ-mediated induction of IRF1 gene expression was unaffected in *Elp3*$^{-/-}$. We concluded that as IFNγ retains the ability to activate STAT1 and activate ISG induction in *Elp3*$^{-/-}$ cells, total STAT1 protein expression is not rate-limiting for STAT1 phosphorylation and IFN-I signalling and responses in cells lacking ELP3. This data also exhibit that ELP3 does not globally affect all aspects of IFN in macrophages, but rather that the influence exerted by ELP3 is specific to the IFN-I family and its responses.

STAT1 expression does not appear to be prohibitive for IFN-I signalling. This proposes that the restrictive influence enforced by the absence of ELP3 occurs upstream at the level of receptor-mediated signalling. IFN-I stimulates IFNAR1-IFNAR2 heterodimerization, which bind and activate receptor-associated kinases TYK2 and JAK1 respectively, to facilitate STAT activation for signal transduction. Expression levels of IFNAR1, JAK1 and TYK2 are unaffected, suggesting JAK1 or TYK2 activation is impaired in *Elp3*$^{-/-}$ cells. IFNγ signals through JAK1 and JAK2 to activate STAT1. IFNγ signalling is unimpaired in *Elp3*$^{-/-}$ cells, indicating that JAK1 activation is unaffected and unlikely to explain abrogated IFN-I signalling. In addition to IFN-I signalling, other TYK2-mediated signalling pathways, IL-4 and IL-10, are abrogated in *Elp3*$^{-/-}$ cells. This data suggests that ELP3 is required for TYK2 activation in murine macrophages. The impairment in IL-4 induced gene expression in macrophages lacking Elp3 has previously been observed, further enforcing the hypothesis that ELP3 regulates TYK2 activation (213). The mechanism by which ELP3 regulates TYK2 activation is unclear. An inbuilt regulatory mechanism to restrain IFN signalling is the induction of SOCS proteins. SOCS1 and SOCS3 have been shown to block TYK2 activity via the binding of conserved tyrosines through their SH2 domains (216). IFNα can induce TYK2 degradation in a SOCS1-dependent manner, with a SOCS1 mimetic peptide also being shown to inhibit TYK2 (217). Whether

enhanced SOCS expression is the causative restrictive factor in *Elp3*$^{-/-}$ cells is hard to know, as SOSC1 and SOCS3 can inhibit other JAKs (JAK1-3), aswell as blocking IFNγ signalling, which is unaffected in *Elp3*$^{-/-}$ cells (218). Potentially, ELP3 may regulate the translation of an unknown protein which is required for IFN-I-mediated TYK2 activation.

PKR was shown to be downregulated in our proteomic analysis of *Elp3*$^{-/-}$ cells. We found this intriguing as PKR is a sensor for viral dsRNA, regulates cellular translation like Elongator and appears to be enriched in Elongator-dependent codons (~13 %). LPS and IFN-I-mediated PKR gene induction was impaired, but no effect on PKR protein expression was observed in *Elp3*$^{-/-}$ cells. This was surprising due to the high level of Elongator-dependent codons it possess'. PKR is constitutively expressed in cells and is present as an inactive monomer, before ligation of viral dsRNA induces its phosphorylation, activation and oligomerization (74). Potentially, basal levels of inactive PKR protein expression can overcome and compensate for defects in PKR gene induction following LPS and IFNβ stimulation. Based on its high level of Elongator-dependency, it could be hypothesized that lack of ELP3 would lead to ribosomal stalling along PKR mRNA transcripts, causing defective PKR translation and protein expression. Potentially the stability of basal PKR expression overcomes slower translational kinetics, or perhaps a compensation mechanism is engaged upon absence of ELP3, which stabilizes translation of some mRNA transcripts which possess a high level of Elongator-dependency. PKR phosphorylation and activation was not assessed, and though ELP3 does not appear to regulate PKR translation, perhaps absence of ELP3 abrogates PKR activation following sensing of viral RNA.

The PYHIN family of proteins are enriched in codons requiring Elongator for translation. We resolved to ascertain whether ELP3 is required for gene induction and/or translation of the mouse PYHIN p205. As observed with other innate immune genes, LPS and IFN-I-mediated *p205* gene induction is impaired in cells lacking ELP3, perhaps unsurprising given the discovered abrogation of IFN-I signalling. Similar to IRF1, IFNγ-mediated p205 gene induction is unaffected in *Elp3*$^{-/-}$ cells. Compellingly however, IFNγ-mediated p205 protein expression is potently diminished in *Elp3*$^{-/-}$ macrophages. This data suggests that ELP3 is required for proper protein expression of p205. P205 has been shown to regulate ASC expression in macrophages, however lack of ELP3 appears to lead to strongly

enhanced ASC expression. The impairment of p205 expression in the absence of ELP3 may possibly induce a compensation mechanism to enable sufficient ASC expression. It is unclear as to why ELP3 is necessary for translation of p205 but not PKR when they share similar levels of Elongator-dependent codons. It has been proposed that Elongator-dependent codons are required but not sufficient to predict the translational fate of a protein. It was proposed that following Elongator-dependent codon-specific translational defects, that the presence of a specific hydrophilic motif mediates protein aggregation and aberrant expression (219). Neither PKR nor p205 possess the supposed hydrophilic motif, yet one is properly translated and the other not. Contrastingly, Elp3 has been shown to regulate the translation of a guanine nucleotide exchange factor, Ric8b, in a codon specific manner in macrophages following IL-4 treatment (213). Ric8b does not possess the proposed hydrophilic motif, and also possess' a much lower levels of Elp-dependent codons (9.45%) when compared to PKR or p205. This suggests that possibly mRNA codon content/hydrophilic motif presence is not sufficient to predict protein fate in the absence of ELP3. Possibly some other motif or mechanism as yet undiscovered is necessary to delineate with specificity as to whether a protein will or will not be properly translated in the absence of ELP3. Protein and situation-specific compensatory mechanisms may also be engaged by the cell to account for the absence of a functional Elongator Complex.

Elongator has been to interact with and be required for stability and expression of ACLY in *d. melanogaster* and in neurons in mammals (163). Loss of Elp3 does not appear to affect ACLY stability or regulation in murine macrophages following cycloheximide pulse-chase assay. This suggests that influence of Elongator on ACLY functioning may be restricted to a neuronal setting and not extend to immune cells.

In summary, ELP3 is required for IFN-I feedback signalling in macrophages. LPS and IFN-I-mediated STAT activation and gene induction is potently abrogated in the absence of ELP3. This specific to IFN-I, as IFNγ-mediated signalling and gene induction responses are fully functional in *Elp3*$^{-/-}$ cells. Expression of IFNAR complex and its associated kinases is stable in cells lacking ELP3. Interestingly, multiple TYK2-mediated signalling pathways are suppressed in *Elp3*$^{-/-}$ macrophages, suggesting that ELP3 is required for proper TYK2 activation. ELP3 appears to be necessary for translation, but not gene induction of the

mouse PYHIN protein p205 following IFNγ treatment. In the context of murine macrophages, ELP3 does not appear to regulate stability or expression of ACLY.

We have analysed the role ELP3 plays in multiple extracellular signalling pathwyas in innate immunity. In the next chapter, we will assess the role ELP3 plays in intracellular signalling and gene induction-mediated by cytosolic PRRs, in conjunction with whether ELP3 is necessary for antiviral immunity in the context of viral infection.

Chapter 5: Regulation of cytosolic PRR signalling by ELP3

5.1 Introduction

IFN-Is and inflammatory cytokines perform various roles across multiple facets of innate immunity. IFN-I and proinflammatory cytokine induction occurs in response to an array of microbial and host intrinsic stimuli. The varied nature of innate immune gene induction requires diverse PRRs, which function throughout the cell spatially and temporally to sense and respond to diverse stimuli.

Spatially distinct TLRs enable recognition of a variety of microbial stimuli, transducing signals to execute induction of IFN-Is and proinflammatory cytokines. TLR4 functions at the cell surface, and in response to ligation of bacterial LPS or the F protein of Respiratory syncytial virus, is internalised which enables transcription of IFN-Is and cytokines (220). Multiple TLRs function intracellularly at endosomes to facilitate sensing of microbial nucleic acids. TLR3, TLR7 and TLR8 sense viral RNA, and TLR9 senses hypomethylated CpG DNA (11). TLR3 signals through TRIF to activate IRF3 and enable IFNβ transcription, or activate NF-κB and AP-1 to facilitate proinflammatory cytokine induction (12). pDCs are professional IFN-I producing cells. pDCs utilise TLR7 and TLR9 which signal through MyD88 to activate IRF7 and induce IFN-I transcription (11). The compartmentalization of TLRs to the endosome enables discrimination of self from non-self nucleic acids, since host nucleic acid material is normally localised to nuclei and mitochondria. However, when this spatial dissociation of self-nucleic acid from endosomal TLRs breaks down, autoimmunity and disease can emerge. TLR7 and TLR9 sensing of self-nucleic acid leaked into the cytosol, contributes to autoantibody production inn SLE (221). Insufficient nucleic acid degradation leads to aberrant nucleic acid sensor activation and autoimmunity, such as in the case of mutations in TREX1 endonuclease, which is related to SLE, myocarditis and other autoimmune conditions (222,223).

Cytosolic PRRs derive from a variety of protein families, and are critical and cardinal orchestrators of innate immunity in response to nucleic acid and intracellular pathogen sensing. A multitude of cytosolic PRRs induce IFN-Is and innate immune genes upon sensing of nucleic acids. cGAS, ZBP1 and PYHINs mediate innate immune responses to a

plethora of diverse DNA motifs. In response to DNA binding, cGAS generates the nucleotide second messenger cGAMP, which activates STING, leading to IRF3-dependent IFN-I induction and NF-κB-dependent proinflammatory cytokine induction (23). cGAS functions as a central node for sensing of DNA from a diverse array of sources. cGAS enables sensing of microbial DNA, extranuclear chromatin and mitochondrial DNA which leaks into the cytosol following DNA/RNA virus infection (224). This multifaceted sensing by cGAS, enables IFN-I and cytokine induction in a variety of contexts, ranging from infection to cases of genotoxic stress (23). The PYHIN family proteins, IFI16 and AIM2, mediate intracellular DNA sensing, leading to a multitude of responses, ranging from IFN-I production to inflammasome activation (71). A wide array of other DNA sensors function in the cytosol, such as ZBP1 which can both form inflammasomes and induce IFN-Is and cytokines in response to virus infection (225).

The RLRs are the cardinal PRRs that facilitate sensing of cytosolic RNA upon RNA virus infection. The RLRs are made up of RIG-I, MDA5 and LGP2, with RIG-I and MDA5 mediating antiviral signalling in response to RNA, whereas LGP2 functions by enhancing MDA5 signalling (23). RIG-I and MDA5 recognize RNA derived from RNA virus infection. However, in contrast to DNA sensing, where self-DNA is spatially compartmentalised to prevent its recognition by DNA sensors, cellular RNA is abundant in the cytosol in many forms. To prevent aberrant sensing of cellular RNA, RIG-I and MDA5 sense duplex RNA structures, with RIG-I sensing viral RNAs which all contain a 5’ppp group (17). MDA5 does not recognize the 5’ppp motif, but rather dsRNA length is important for RNA recognition by MDA5 (226). Upon RNA binding, both RIG-I and MDA5 enagage MAVS at mitochondria, leading to TBK1 activation and subsequent stimulation of IRF3 and NF-κB and induction of IFN-Is and proinflammatory cytokines (17). RLR sensing of viral RNA in the cytosol, is critical for IFN-I induction, subsequent ISG expression and suppression of viral replication. Due to this integral function in innate immunity, RLR signalling is tightly regulated and fine-tuned at the level of the RNA ligand, its interacting partners and at the level of the sensor itself via a plethora of regulatory mechanisms.

In chapter 4 we demonstrated that ELP3 is indispensable for IFN-I signalling in macrophages. IFN-I feedback signalling was potently impaired in the absence of ELP3. We observed that gene induction of IFN-I ablated in response to activation of the

extracellular PRR TLR4. Unbiased quantitative proteomic analysis showed that expression of proteins involved in cytosolic PRR signalling was abrogated in *Elp3*$^{-/-}$ cells. Based on these observations, we decided to assess the relative contribution of ELP3 to TLR, DNA and RNA sensing pathways in macrophages and their gene induction outputs, as well as assessing whether ELP3 is required for innate immunity in the context of RNA virus infection.

5.2 Results

5.2.1 Loss of ELP3 abrogates TLR4-mediated IRF3 phosphorylation and activation

We previously demonstrated that ELP3 is necessary for functional IFN-I signalling and gene induction responses. We further determined that LPS-mediated IFN-I and IRF7 gene induction is ablated in cells lacking ELP3. Impaired induction of *Ifna* is likely due to defective *Irf7* expression. IFN-I feedback signalling induces ISG expression, but also amplifies and potentiates IFN-Is expression itself, so the requirement of ELP3 for LPS-stimulated IFN-I could be solely due to an effect on the IFN-I signalling pathway. However, aberrant initial *Ifnb* induction following LPS stimulation due to a direct role for ELP3 in TLR4 signalling could also contribute to impaired *Irf7* induction. Therefore, we decided to examine whether ELP3 is exerting influence solely on LPS-induced IFN-I signalling and potentiated induction, or whether ELP3 also regulates TLR4 signal transduction and initial induction of IFN-I expression. Supporting previous data, LPS-induced STAT1 and STAT3 activation were ablated in *Elp3*$^{-/-}$ cells (Fig 5.1, panels 5 & 7). p65, also known as RelA, is a key component of the NFκB complex, which gets phosphorylated following TLR4 stimulation, leading to nuclear translocation and induction of proinflammatory cytokines. p65 following LPS treatment was unaffected by the absence of ELP3 (Fig 5.1, panel 3). IRF3 phosphorylation and activation downstream of TLR4 stimulation mediates an initial wave of *Ifnb* gene induction. IRF3 protein expression was unimpaired in cells lacking Elp3 (Fig 5.1, panel 2). Intriguingly, LPS-mediated IRF3 phosphorylation was potently abrogated in *Elp3*$^{-/-}$ cells. This data suggests that ELP3 is required for proper activation of IRF3 downstream of TLR4 signaling.

This data indicates a two-step requirement for ELP3 in TLR4-mediated IFN-I processes. Firstly, ELP3 is necessary for proper TLR4-mediated IRF3 activation and initial IFNβ gene

induction. Secondly, ELP3 coordinates IFN-I feedback signalling, STAT activation and gene induction responses via regulation of TYK2 activation.

5.2.2 ELP3 is required for intracellular TLR-mediated gene induction

We established that ELP3 is necessary for TLR4-mediated gene induction and IRF3 activation. We next determined to analyse whether this influence is restricted to the cell surface TLR4, or whether ELP3 also regulates intracellular TLR responses. The thiazoloquinone derivative CLO75 is an agonist for murine TLR7, which functions intracellularly at endosomes, normally sensing ssRNA (11). We stimulated WT and *Elp3*$^{-/-}$ cells with CLO75 to investigate whether ELP3 regulates intracellular TLR responses. TLR7-mediated gene induction of *il1b* and *il12p40* was strongly impaired in ELP3$^{-/-}$ cells following CLO75 treatment (Fig 5.2A & B).

This data suggest that ELP3 is required not solely for TLR4 signalling, but also plays an essential role in intracellular TLR7-dependent gene induction responses.

5.2.3 ELP3 is not required for STING-mediated gene induction.

We have demonstrated that extracellular TLR4-mediated IFN-I induction is ablated in cells which lack ELP3, and that ELP3 regulates LPS-induced IFN-I processes in a twofold manner, by regulating both IFN-I signalling, and IRF3 activation leading to IFNβ gene induction. Interestingly, unbiased quantitative proteomics analysis of LPS-stimulated cells (Chapter 3) demonstrate that expression of proteins predicted to be involved in signalling downstream of cytosolic PRRs was impaired in *Elp3*$^{-/-}$. Deriving from this, we decided to evaluate whether ELP3 exerts influence over cytosolic PRR-mediated signalling processes. In response to sensing of cytosolic dsDNA, cGAS generates cGAMP, a nucleotide second messenger, which activates STING leading to IRF3/NF-κB phosphorylation and subsequent induction of IFNβ and proinflammatory cytokines (224). STING uses the same kinase as TLR4, TBK1, to phosphorylate IRF3 so it was of interest to assess whether STING responses were also ELP3-dependent. To evaluate STING-mediated responses, we transfected WT and Elp3$^{-/-}$ cells directly with 2'-3' cGAMP and examined gene induction responses. cGAMP-mediated induction of the key ISG

Cxcl10 was unaffected in Elp3$^{-/-}$ cells (Fig 5.3A). Accompanying this, gene induction of the chemokine *Ccl5*, and the proinflammatory cytokine *Il-6* were unimpaired in the absence of ELP3 (Fig 5.3B & C). This data indicates that ELP3 does not globally regulate innate immune signalling, and is not necessary for STING-mediated gene induction responses.

5.2.4 ELP3 is not necessary for dsDNA VACV 70mer-mediated gene induction

STING-mediated gene induction responses function independent of ELP3. We sought to appraise whether this finding translates to innate immune sensing of dsDNA in general, which can involve STING-dependent and –independent pathways. The 70-bp long double-stranded DNA motif, derived from the poxvirus vaccinia virus, is recognized multiple DNA sensors leading to IRF3 and NF-κ activation and IFNβ, ISG and inflammatory cytokine gene induction (2, 3). We transfected WT and *Elp3*$^{-/-}$ cells with VACV 70mer to mimic a DNA virus infection and assess whether ELP3 contributes to cytosolic DNA sensing. Following 70mer transfection, gene induction of *Ifnb* and the chemokine *Ccl5* were unaltered by the absence of ELP3 (Fig 5.4A & B). This data suggest that ELP3 is not required for dsDNA-mediated responses in macrophages.

The confluence of data derived from both cGAMP and VACV 70mer transfection indicate that ELP3 does not play a role in regulating STING and cytosolic DNA-sensing pathways.

5.2.5 ELP3 is required for RLR and cytosolic RNA-sensing pathway-mediated gene induction responses.

Cytosolic nucleic acid sensing is facilitated by an array of intracellular receptors that bind and recognise cytosolic DNA or RNA. Upon RNA virus infection or RNA transfection multiple receptors such as endosomal TLR3, and cytosolic PKR and the RIG-like receptors (RLRs) RIG-I/MDA5 are engaged. Cytosolic pathways that mediate DNA-sensing are unimpacted by the absence of ELP3. This data in conjunction with the fact our proteomic analysis of Elp3$^{-/-}$ cells suggests that proteins involved cytosolic PRR signalling are impaired, led us to appraise whether cytosolic sensing of RNA and subsequent responses are impacted by the absence of ELP3.

Poly(I:C) is a synthetic dsRNA analog that mimics an RNA virus infection. Poly(I:C) transfection leads to sensing via multiple cytosolic pathways, such as by TLR3, PKR and

RIG-I/MDA5 (15, 16). We transfected WT and *Elp3*$^{-/-}$ cells with poly(I:C) for 3, 6 and 24 hours to simulate a RNA viral infection. Gene induction of *Ifnb* and *Ccl5* was completely ablated in cells lacking ELP3 (Fig 5.5A & B). This data indicate that whilst cytosolic DNA sensing pathways do not require ELP3, RNA-sensing pathway stimulated IFN-I and *Ccl5* induction, was regulated by ELP3.

RLRs are pivotal mediators of RNA virus sensing and innate immunity. MDA5 induce IFN-I and proinflammatory cytokine induction in response to dsRNA ligation, but poly(I:C) may also activate RIG-I. We decided to evaluate whether ELP3 is required specifically for the RIG-I pathway of RNA sensing. To exclude other RNA sensing pathways, we transfected WT and Elp3$^{-/-}$ cells with the RIG-I specific ligand 3p-hpRNA, which is a double stranded hairpin RNA derived from influenza (H1N1) (230). hpRNA-mediated induction of *Ifnb, Cxcl10 and Il-6* was potently diminished in cells lacking ELP3 (Fig 5.6).

The data thus far suggests that ELP3 regulates nucleic acid sensing pathways of cytos olic RNA, but not of DNA. This appraisal was further supported when we compare TLR4, RIG-I and STING-mediated responses simultaneously. LPS and hpRNA-mediated induction of *Ifnb, Cxcl10 and Ccl5* was strongly impaired in Elp3$^{-/-}$ cells, while contrastingly cGAMP-mediated activation of STING and subsequent gene induction was unimpaired (Fig 5.7A-C).

Overall the data clearly shows that ELP3 regulates signalling and downstream gene induction of TLR and RLR pathways, whilst ELP3 was not necessary for DNA sensing STING-mediated responses.

5.2.6 ELP3 is necessary for innate immune gene induction following RNA virus infection of macrophages

Since we found that ELP3, whereas ELP3 is required for examined RNA sensing pathways when utilising PAMPs, we decided to analyse innate immune gene induction in *Elp3*$^{-/-}$ macrophages following RNA virus infection.

We infected WT and *Elp3*$^{-/-}$ macrophages with the mouse-adapted PR8 H1N1 strain of influenza A virus (IAV), which is a negative sense ssRNA virus. Upon IAV infection, innate immune cells upregulate IFN-Is which induce expression of ISGs, which enable restriction

of viral replication and proliferation. Upon PR8 infection, *Elp3*$^{-/-}$ cell-mediated IFN-I expression was completely ablated relative to WT cells (Fig 5.8.A & B). IRF7 is an ISG strongly upregulated following IAV infection, which subsequently enables a second wave of IFN-I expression in a feedback loop to combat infectious virus (231). *Irf7* gene induction was potently impaired in *Elp3*$^{-/-}$ cells following IAV infection, suggesting the impaired IFN-I induction leads to subsequent abrogation of ISG expression (Fig 5.8C). The chemokine CCL5 has been show to play a protective role in the context of IAV infection via the recruitment of leukocytes and subsequent antiviral immunity (232). Intriguingly, gene induction of *Ccl5* following IAV infection was not ELP3-dependent. *Elp3*$^{-/-}$ cells upregulated *Ccl5* to levels comparable with WT cells following viral infection (Fig 5.8D). This data suggests that ELP3 is required for macrophage-mediated induction of IFN-I and *Irf7*, but not the inflammatory chemokine *Ccl5* in response to IAV infection.

Due to the impaired levels of IFN-Is and IRF7, which are important for restricting viral infection, we decided to assess viral replication indirectly via assessing gene expression of IAV *Matrix protein* (a key structural protein) following infection of WT and *Elp3*$^{-/-}$ cells. Interestingly, expression of *Matrix protein* was abrogated in cells lacking ELP3, indicating reduced viral replication in the absence of ELP3 (Fig 5.9). This data was surprising as ELP3$^{-/-}$ cells have diminished IFN-I gene induction following IAV infection, which are important for restricting viral replication. This data indicates that in macrophages ELP3 is required for proper replication of IAV.

We decided to determine whether ELP3 was required for innate immune gene induction with other RNA viruses, or whether it is an effect specific to IAV. We infected WT and *Elp3*$^{-/-}$ cells with RSV, as it is sensed by both RLRs and extracellular TLR4 (220), and assessed gene induction responses. RSV-mediated induction of IFN-Is was undetectable following infection in both WT and *Elp3*$^{-/-}$ cells (data not shown), likely due to immune evasion strategies employed by the virus. However, other genes such as *Irf7* and *Cxcl10* were inducible by RSV infection. ELP3 was necessary for RSV-mediated *Irf7* gene induction following virus infection (Fig 5.10A). The chemokine CXCL10 has been shown to mediate antiviral immunity in the context of RSV infection (233). Gene induction of *Cxcl10* following RSV infection was strongly impaired in cells lacking ELP3 (Fig 5.10B). These data indicate that ELP3 is required for induction innate immune genes that are

important in coordinating antiviral immunity to RSV, aswell as to other RNA viruses like IAV.

In summary, we have shown that ELP3 is necessary for multiple PRR-mediated signalling pathways. We demonstrated that TLR4-mediated activation of IRF3 was abrogated in cells lacking ELP3. This delineates a twofold role for ELP3 in TLR4-mediated processes, in IRF3 activation and IFN-I feedback signalling. Intracellular TLR-mediated gene induction was abrogated in *Elp3*$^{-/-}$ cells, with TLR7-enabled cytokine induction impaired in cells lacking ELP3. We showed that ELP3 was not required for STING and cytosolic DNA sensing pathways in macrophages. However, we established that ELP3 was in fact necessary for intracellular RNA sensing pathways and subsequent gene induction in response to poly(I:C) and the RIG-I ligand 3p-hpRNA. Finally, ELP3 was necessary for innate immune gene induction in response to infection of macrophages with RNA viruses, and interestingly was also required for optimal expression of IAV matrix protein in infected cells.

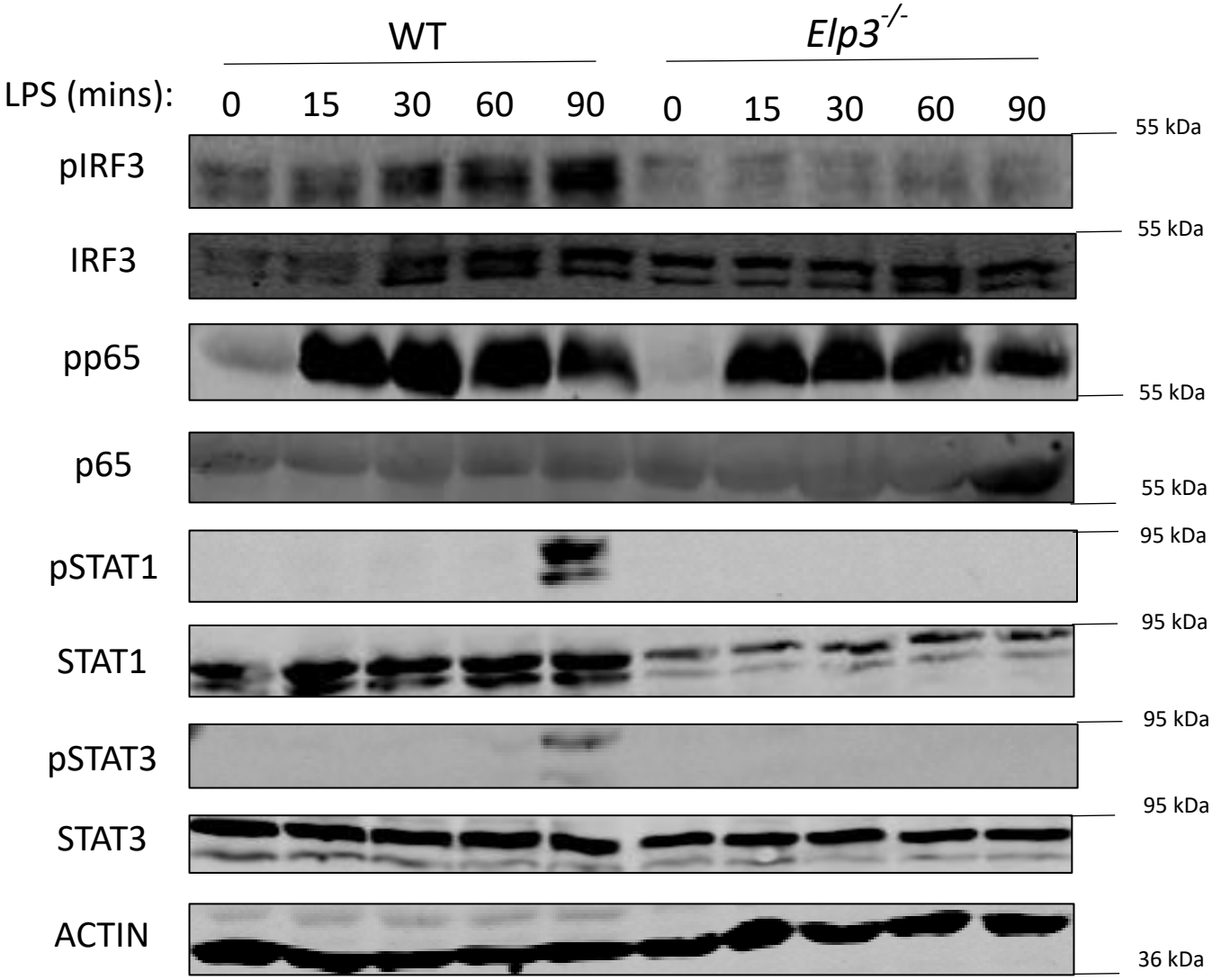


Fig 5.1 TLR4-mediated IRF3 & STAT activation is abrogated in the absence of ELP3

WT and *Elp3*$^{-/-}$ iBMDMs were seeded at 5 x 10^5 cells/ml and stimulated with LPS (100 ng/ml) for the indicated times. Cells were harvested and total and phosphorylated IRF3, STAT1, STAT3 & p65 was assessed by immunoblot. Representative of three independent experiments

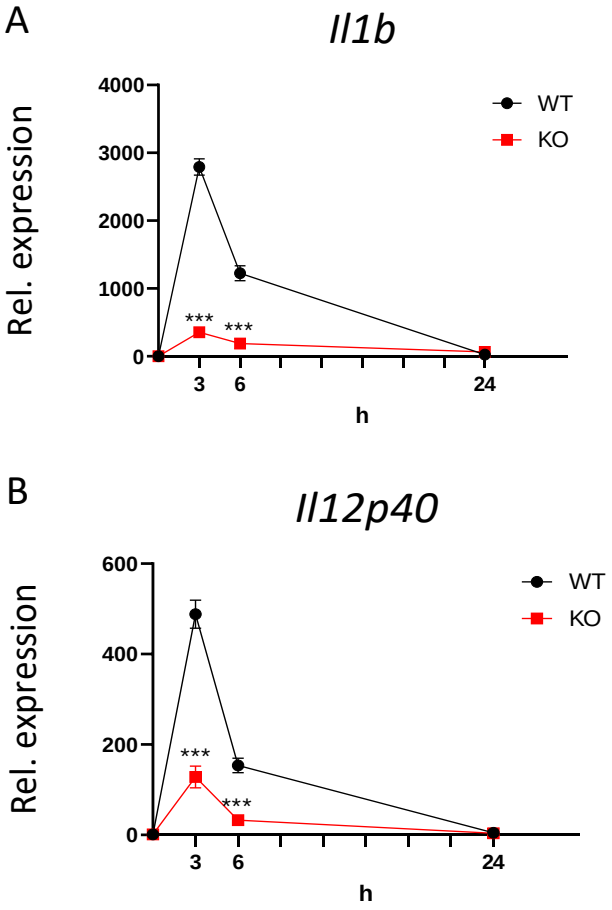


Fig 5.2 ELP3 is required for TLR7-mediated gene induction

WT and *Elp3*$^{-/-}$ iBMDMs were seeded at 5 x 10^5 cells/ml and stimulated withCLO75 (5 µg/ml). RNA was isolated following 3, 6 and 24 h stimulation and gene expression of (A) *Il1b* and (B) *Il12p40* was measured by qRT-PCR. mRNA levels are presented relative to mRNA levels of β-actin. Data are mean ± SEM and is average of 3 experiments. *P < 0.05, ***P < 0.001 compared to WT, based on student's t-test

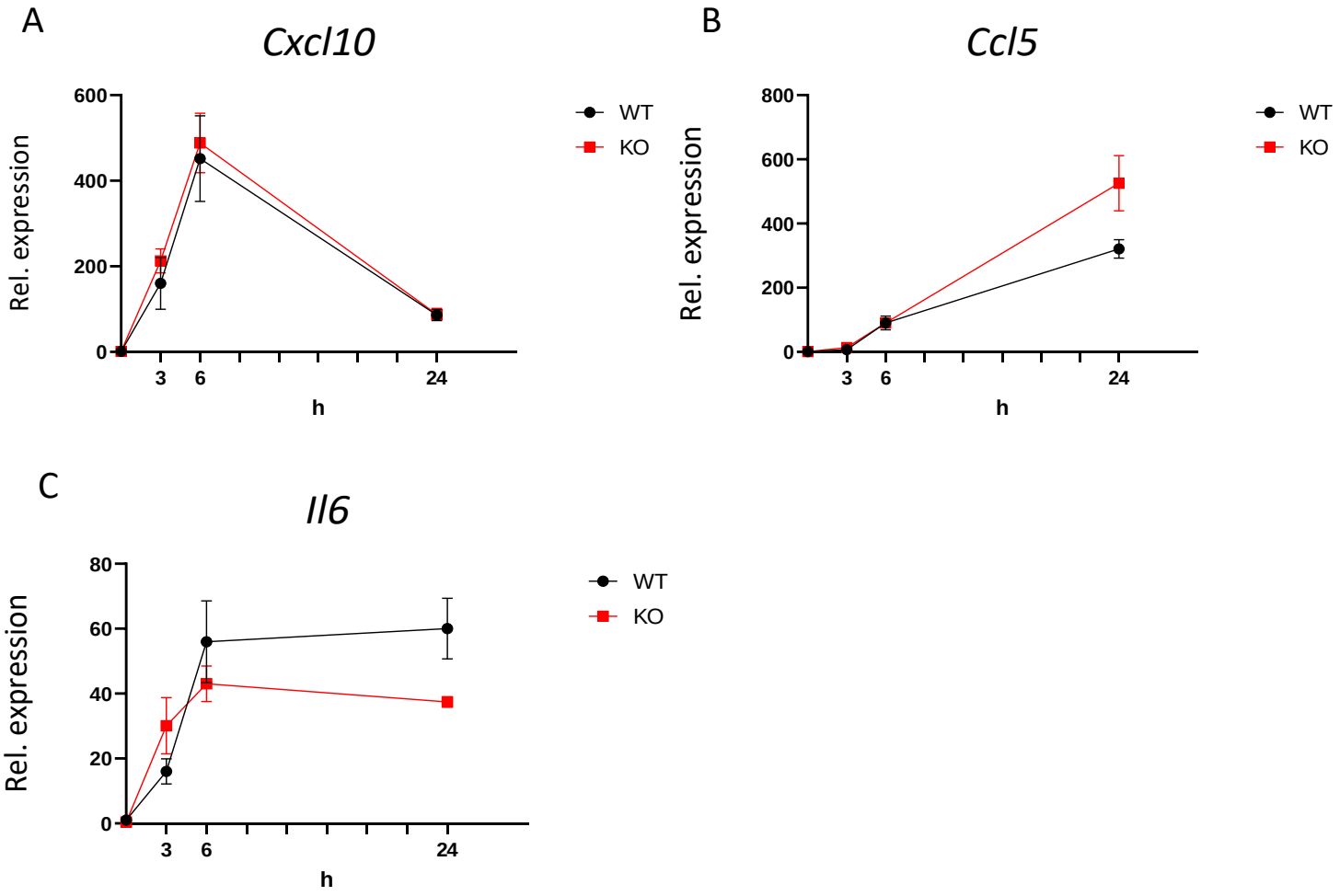


Fig 5.3 cGAMP-mediated gene induction is unimpaired in *Elp3*$^{-/-}$ iBMDMs

WT and *Elp3*$^{-/-}$ iBMDMs were seeded at 5 x 10^5 cells/ml and transfected with 2'-3' cGAMP (5 µg/ml). RNA was isolated following 3, 6 and 24 h and gene expression of (A) *Cxcl10*, (B) *Ccl5*, (C) *Il6* was measured by qRT-PCR. mRNA levels are presented relative to β-actin. Data are mean ± SEM of three independent experiments

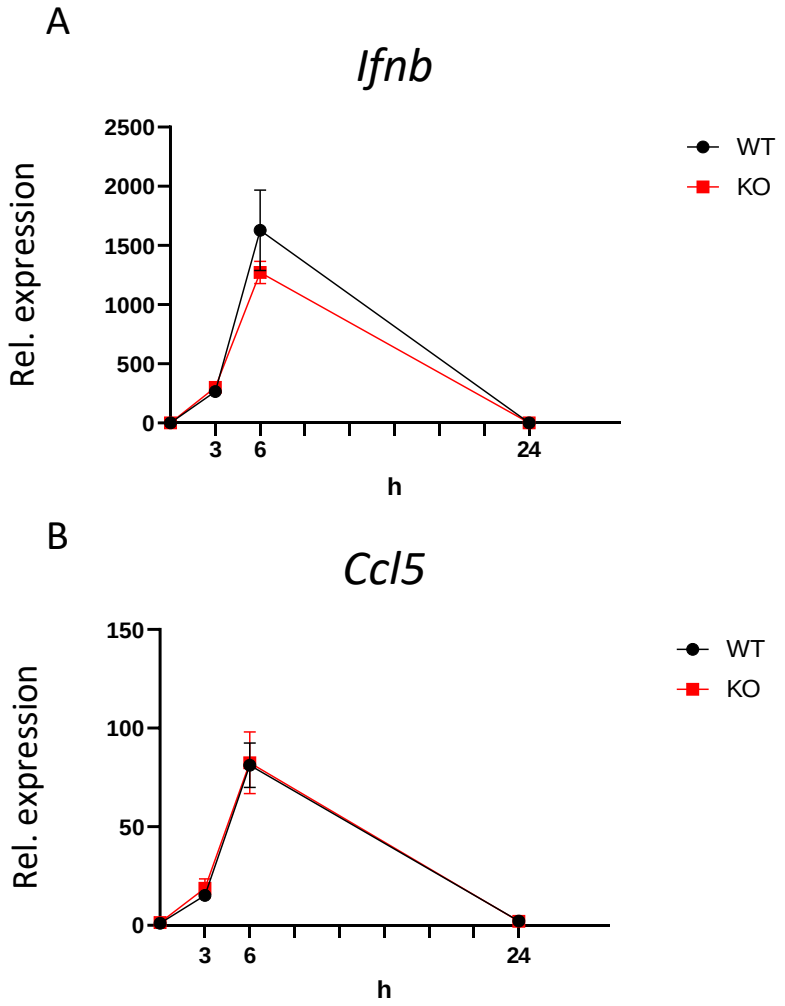


Fig 5.4 Double-stranded VACV-70mer DNA mediated gene induction does not require ELP3

WT and *Elp3*$^{-/-}$ iBMDMs were seeded at 5 x 10^5 cells/ml and transfected with double-stranded VACV-70mer DNA (2.5 µg/ml). RNA was isolated following 3, 6 and 24 h and gene expression of (A) *Ifnb* & (B) *Ccl5* was measured by qRT-PCR. mRNA levels are presented relative to β-actin. Data are mean ± SEM of three independent experiments

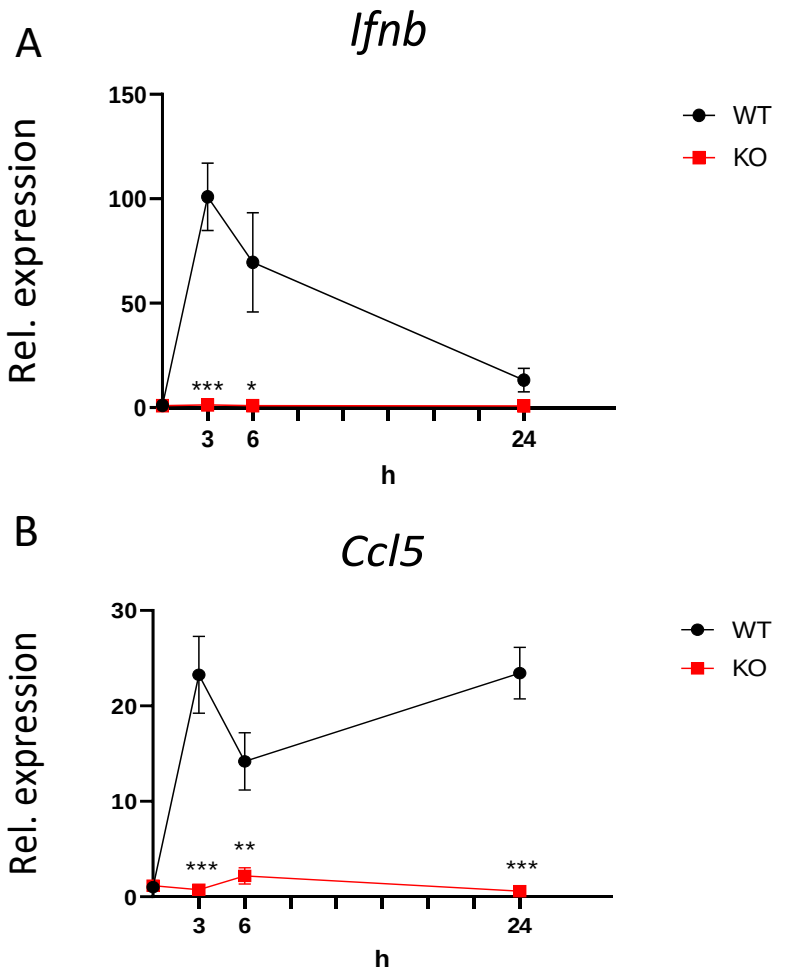


Fig 5.5 poly(I:C)-mediated gene induction is ELP3-dependent

WT and *Elp3*$^{-/-}$ iBMDMs were seeded at 5 x 10^5 cells/ml and transfected with HMW poly(I:C) (10 µg/ml). RNA was isolated following 3, 6 and 24 h and gene expression of (A) *Cxcl10* & (B) C*cl5* was measured by qRT-PCR. mRNA levels are presented relative to β-actin. Data are mean ± SEM and is average of 3 experiments. *p<0.05, **p<0.01, ***p<0.001 compared to WT, based on students t-test

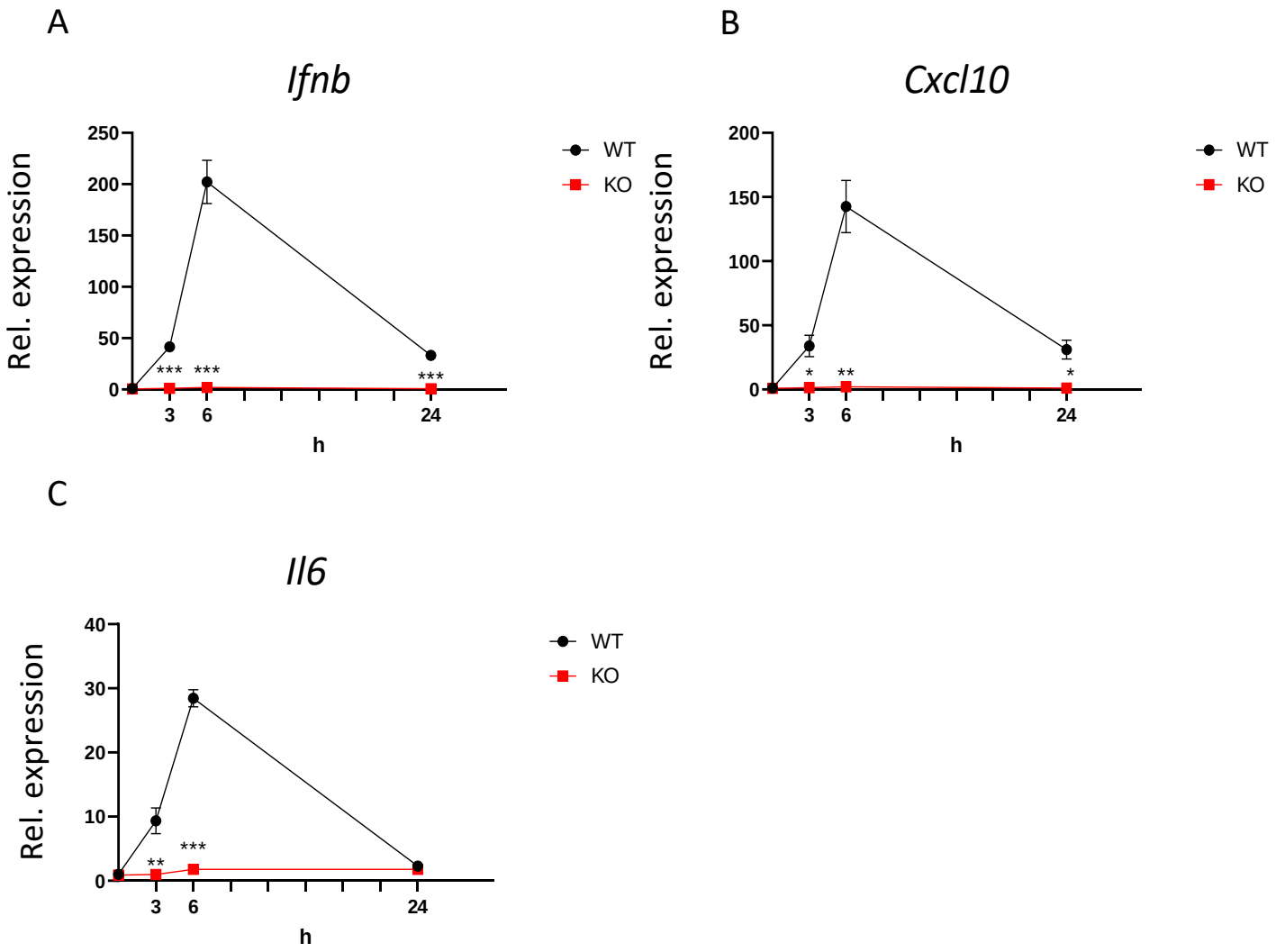


Fig 5.6 3p-hpRNA-mediated gene induction is ELP3-dependent

WT and *Elp3*$^{-/-}$ iBMDMs were seeded at 5 x 10^5 cells/ml and transfected with 3p-hpRNA (100 ng/ml). RNA was isolated following 3, 6 and 24 h and gene expression of (A) *Ifnb*, (B) *Cxcl10*, (C) *il6* was measured by qRT-PCR. mRNA levels are presented relative to β-actin. Data are ± SEM and is average of 3 experiments. *p<0.05, **p<0.01, ***p<0.001 compared to WT, based on students t-test

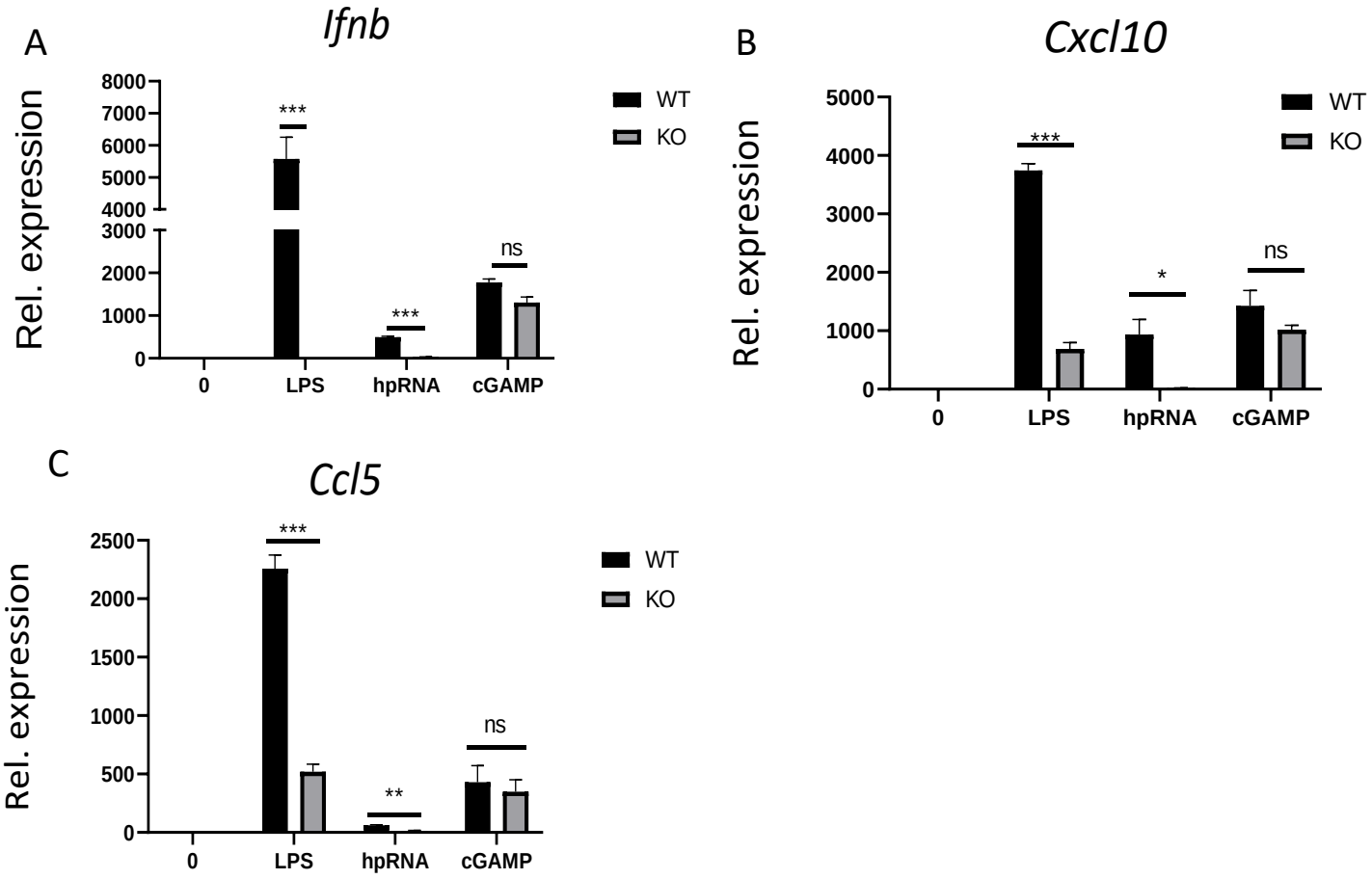


Fig 5.7 TLR4 and RIG-I but not STING-mediated gene induction is suppressed in *Elp3*$^{-/-}$ cells.

WT and *Elp3*$^{-/-}$ iBMDMs were seeded at 5 x 10^5 cells/ml and stimulated with 2'-3' cGAMP (5 µg/ml). RNA was isolated following 3, 6 and 24 h and gene expression of (A*) Ifnb* (B) *Cxcl10*, (C) *Ccl5* was measured by qRT-PCR. mRNA levels are presented relative to β-actin. Data are mean ± SEM and is average of 3 experiments. *p<0.05, **p0.01, ***p<0.001 compared to WT, based on students t-test

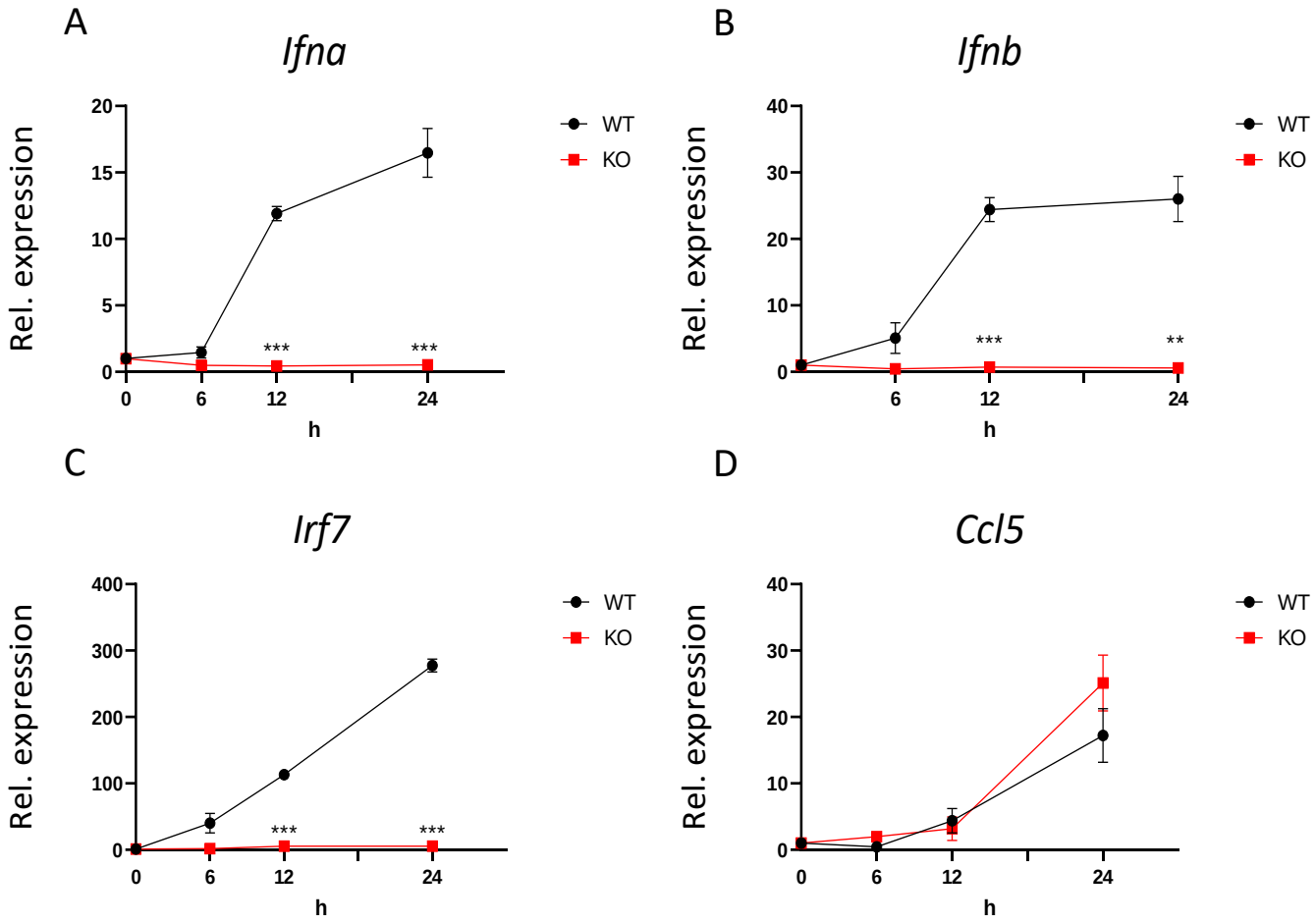


Fig 5.8 IAV-mediated gene induction is ELP3-dependent.

WT and *Elp3*$^{-/-}$ iBMDMs were seeded at 5 x 10^5 cells/ml and infected with the PR8 strain of IAV (MOI of 5). RNA was isolated following 6, 12 and 24 h PR8 infection and gene expression of (A) *Ifna*, (B) *Ifnb,* (C) *Irf7 &* (D) *Ccl5* was measured by qRT-PCR. mRNA levels are presented relative to mRNA levels of β-actin. Data are mean ± SEM and is average of 3 experiments. *P < 0.05, ***P < 0.001 compared to WT, based on students t-test

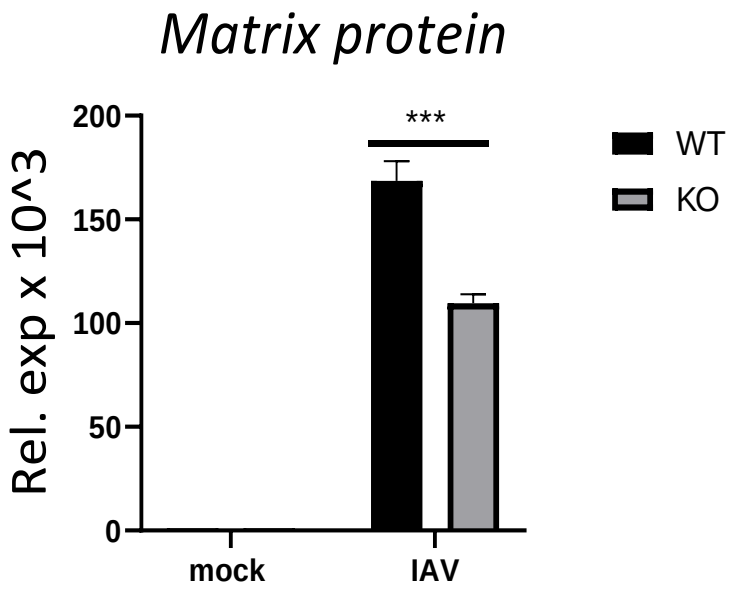


Fig 5.9 ELP3 is necessary for IAV replication in macrophages

WT and *Elp3*$^{-/-}$ iBMDMs were seeded at 5 x 10^5 cells/ml and infected with the PR8 strain of IAV (MOI of 5). RNA was isolated following 24 h PR8 infection. Gene expression of IAV *Matrix protein* was measured by qRT-PCR. mRNA levels are presented relative to mRNA levels of β-actin. Data are mean ± SEM and is average of 3 experiments. ***P < 0.001 compared to WT, based on students t-test

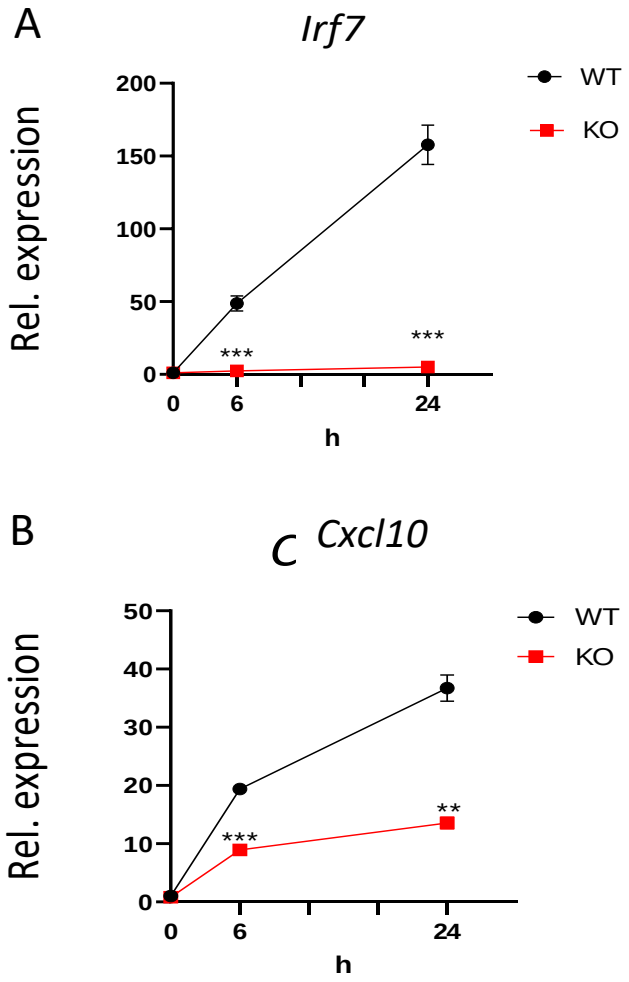


Fig 5.10 RSV-mediated gene induction is ELP3-dependent.

WT and *Elp3*$^{-/-}$ iBMDMs were seeded at 5 x 10^5 cells/ml and infected with the RSV (MOI of 5). RNA was isolated following 6, 12 and 24 h of infection and gene expression of (A) *Irf7* and (B) *Cxcl10* was measured by qRT-PCR. mRNA levels are presented relative to mRNA levels of β-actin. Data are mean ± SEM and is average of 3 experiments. *P < 0.05, ***P < 0.001 compared to WT, based on students t-test

5.3 Discussion

We previously demonstrated that ELP3 is crucial for IFN-I feedback signalling, likely due to regulation of TYK2 activation. In this chapter, we sought to appraise whether ELP3 was required for cytosolic PRR signalling in response to nucleic acids, and whether ELP3 is require for innate immunity to RNA virus infection. We demonstrated that there is a two-fold requirement for ELP3 in TLR4 signalling, via IRF3 activation and IFN-I gene induction as well as IFN-I feedback signalling. We illustrated that ELP3 is not necessary for gene induction in response to cytosolic sensing of DNA or activation of STING. However, ELP3 is required for innate immune gene induction in response to sensing of RNA in the cytosol. ELP3 is furthermore essential for immune gene activation in response to RNA virus infection. Overall, the data indicate that ELP3 is required for TLR and RIG-I, but not DNA and STING signalling and responses.

We previously showed that LPS-mediated induction of IFN-I gene expression was impaired in the absence of ELP3. We demonstrated that IFN-I signalling was abrogate by the absence of ELP3 due to aberrant TYK2 activation. As IFN-I feedback signalling can enhance and amplify IFN-I expression itself, it was possible that impaired LPS-mediated IFN-I induction was due to aberrant feedback signalling. However, we illustrated that in addition to impaired IFN-I signalling, LPS-mediated IRF3 activation and phosphorylation was abrogated in the absence of ELP3. This data proposes a two-step requirement for ELP3 in TLR4-mediated IFN-I responses: firstly in IFN-I gene induction via IRF3 activation, and secondly in IFN-I feedback signalling via TYK2 activation. IRF3 total protein expression was unaffected in cells lacking ELP3, suggesting that ELP3 possibly regulates the expression of an interacting partner of IRF3, which is necessary for its proper phosphorylation and activation. Numerous interacting proteins regulate IRF3 activation, via a variety of mechanisms (234). Thus, it also possible that ELP3 deficiency leads to a repression on the expression of a protein, which is able to interact with IRF3 and prevent its activation, via a multitude of possible mechanisms.

In addition to extracellular expression at the plasma membrane, TLRs are also expressed intracellularly at endosomal surfaces to facilitate antimicrobial immunity. We established that TLR7-mediated gene induction responses in macrophages lacking ELP3

were abrogated. This data implies that ELP3 is necessary for gene induction downstream of both extracellular and intracellular TLRs in macrophages.

Proteomic analysis of *Elp3*$^{-/-}$ cells suggested that proteins involved in cytosolic PRR-mediated signalling and responses were abrogated in cells lacking ELP3. This data was supported by the fact that gene induction downstream of the intracellular PRR, TLR7 were abrogated. We sought to appraise whether ELP3 was required for cytosolic PRR-mediated nucleic acid sensing pathways. Following transfection with a dsDNA motif derived from vaccinia virus, mRNA induction of *Ifnb* and the chemokine *Ccl5* was unaffected by the absence of ELP3. Conjointly, gene induction of *Ifnb* and proinflammatory cytokines in response to STING activation with cGAMP were unimpaired in cells lacking ELP3. VACV 70mer can by multiple DNA sensors (48,227). These data suggest that ELP3 is not required for induction of IFN-Is or cytokines mediated by STING or cytosolic DNA sensing pathways.

As ELP3 is not required for DNA sensing pathways in macrophages, we assessed whether ELP3 plays a functional role in gene induction downstream of RNA-sensing pathways. Upon its transfection, poly(I:C) activates multiple RNA sensing pathways such as TLR3, RIG-I and MDA5 (228,229). poly(I:C)-mediated gene induction of *Ifnb* and *Ccl5* were ablated in ELP3$^{-/-}$ cells. 3p-hpRNA specifically activates the RIG-I pathway. hpRNA-mediated *Ifnb* and proinflammatory cytokine mRNA induction were abolished in cells lacking ELP3. This data suggests that IFN-I and cytokine gene induction responses-mediated by RNA sensing pathways require ELP3. Poly(I:C) activates both intracellular TLRs and RLRs, so it is difficult to determine whether ELP3 regulates responses downstream of all PRRs poly(I:C) activates or whether its influence is restricted to a specific pathway. ELP3 does specifically regulate RIG-I-mediated gene induction in response to hpRNA stimulation. Overall these data establish that ELP3 regulates gene induction in response to activation of RNA-sensing, but not STING and DNA-sensing pathways. As to why ELP3 is not required for DNA and STING responses, but is for TLR and RLR-mediated gene induction, is intriguing. TLRs, RIG-I and STING mediate IRF3 activation via TBK1, suggesting TBK1 is not the restrictive factor. Potentially DNA and STING responses do not engage, and put less stress on the host translational machinery. Possibly RNA sensing pathways place a higher demand on translation and translational

efficiency for the proper functioning and signalling, compared to DNA and STING pathways. As such, if the energetic and kinetic demand of translation increases, possibly the requirement for ELP3 is enhanced, to facilitate efficient decoding of mRNA so a cell enhance protein expression in response to an RNA stimulus.

In accordance with this data, we demonstrated that ELP3 was necessary for innate immune gene induction in response to RNA virus infection. IAV-mediated induction of IFN-I and *Irf7* gene expression was impaired in *Elp3*$^{-/-}$ cells. *Ccl5* gene expression however was unaffected, demonstrating that not all innate immune gene induction is impaired in *Elp3*$^{-/-}$ following IAV infection. In response to RSV infection, gene induction of *Irf7* and *Cxcl10* was abrogated in *Elp3*$^{-/-}$ macrophages. This data showed that in the context of viral infection ELP3 is necessary for innate immune gene induction. Sensing and gene induction in response to IAV is mediated by RLRs, whereas RLRs and TLR4 mediate innate responses to RSV infection (233). This data corresponds to our observations that TLR4 and RLR-mediated IFN-I gene induction requires ELP3. However, replication of IAV was abrogated in *Elp3*$^{-/-}$ cells, which contrasts with the observed impairment of IFN-I, which mediates ISG induction to block viral replication. This indicates that ELP3 may function in a bidirectional manner in the context of infection, regulating both host innate immune responses and viral replication. Intriguingly, it has been observed that RNA viruses reprogram the translational machinery of the host to favour their translation in a codon-specific manner. A number of RNA viruses, chikungunya (CHIKV), SARS CoV-2, dengue, Zika, are enriched in –AA ending codons, which require Elongator-mediated tRNA modifications for their translation. CHIKV and dengue virus have been shown to upregulate pathways which enhance and enable translation of their own –AA ending codons (235). Thus, it is possible that the Elongator complex can be co-opted by RNA viruses, to enable translation of –AA ending codons in which they are enriched. There is a dynamic interplay ensuing, where ELP3 is require for innate immune responses to viral infection, yet is also required for proper viral replication.

Collectively the data signify an essential requirement for ELP3 in the regulation of TLR and RLR, but not DNA and STING-mediated responses in macrophages. Critically, we have shown that IFN-I induction in response to TLR4 and RLR activation with PAMPs,

aswell as with IAV infection is dependent on ELP3. These data indicate that ELP3 is crucial in orchestrating and regulating IFN-I induction, in response to an array of stimuli sensed by multifaceted signalling pathways. Intriguingly, ELP3 is not necessary for STING or DNA-mediated IFN-I and cytokine induction. Thus, ELP3's influence is specific and restricted to TLR and RNA-sensing pathways, as further supported by its role in IFN-I induction in response to RNA virus infection. Cumulatively the data implies a central and pivotal role for the Elongator complex in regulating IFN-I in innate immune responses. As such, as research into mechanisms regulating and coordinating innate immunity progress, there is strong evidence to suggest Elongator will be implicated as being an important regulator of IFN-I responses across a multitude of facets of innate immunity, possibly ranging from infectious disease to autoimmunity.

Chapter 6 – Discussion

Elongator influences and regulates a range of disparate processes, from the control of cancer and malignancy, to ensuring the proper functioning and development of the central nervous system. In recent years, the data has begun to hint at a potential immunological function for Elongator, regulating processes such as haematopoiesis and adaptive immunity via Tfh development (207,208). The structure and function of Elongator is highly conserved across eukaryotes, with bacteria and archaea even possessing functional similar homologs of ELP3, the catalytic subunit of the complex (139,140,145,151). Although mammals possess adaptive immune systems, innate immune systems are found across the animal kingdom, all the way from invertebrates to humans (236). However, very little is understood about how Elongator functions in the context of innate immunity. We became intrigued by the concept of a highly conserved protein complex like Elongator, which is necessary for the ubiquitous process of translation, and an under-assessed but potentially compelling role in innate immunity. Therefore to contribute to the understanding of how Elongator functions in innate immunity, I sought to elucidate how Elongator regulates innate immune responses in macrophages. The data in this thesis begin with unbiased quantitative proteomic analysis of *Elp3*$^{-/-}$ murine macrophages firstly demonstrating expression of proteins involved in IFN-I signalling and processes were impaired in cells deficient in ELP3. 2, LPS-mediated gene induction of IFN-I is impaired in the absence of ELP3. 3, IFN-I feedback signalling and gene induction responses are impaired in cells lacking ELP3. This occurs likely due to impaired TYK2 activation, as other TYK2-mediated signalling pathways are abrogated in *Elp3*$^{-/-}$ cells. 4, ELP3 is required for the translation of the murine PYHIN protein p205. 5, There is a two-step requirement for ELP3 in IFN-I responses, with ELP3 being necessary for LPS-mediated IRF3 phosphorylation and activation as well as IFN-I feedback signalling. 6, ELP3 is required for cellular responses to RNA but not DNA, with RNA virus-mediated gene induction also requiring ELP3.

6.1 Initial observations

Utilising *Elp3*$^{-/-}$ iBMDMs developed by CRISPR/Cas9, I sought to appraise the contribution of ELP3 to innate immune responses. I treated WT and *Elp3*$^{-/-}$ cells with the commonly utilised PAMP, LPS, to activate TLR4 as a common inducer of innate immunity. Due to

the well-established role for Elongator in regulating translation, we subjected mock and LPS-stimulated cells to unbiased quantitative proteomics analysis with help from collaborators at TU Munich (D. Haas & A. Pichlmair). Ingenuity pathway analysis was performed on differentially expressed proteins in *Elp3*$^{-/-}$ cells relative to WT cells, to illuminate innate immune processes that are ELP3-dependent. Compellingly, pathway analysis of processes downregulated in the absence of ELP3 showed a strong impairment in the expression of proteins involved in IFN-I signalling and responses. Following stimulation, IFN signalling arose as the most compromised biological process following LPS treatment. URA analysis suggested that key innate immune mediators such as IRF3, STAT1 and IFNβ were potentially responsible for the observed downregulation of gene expression observed in *Elp3*$^{-/-}$ cells.

6.2 Requirement for ELP3 in TYK2-mediated signalling

We demonstrated that the requirement for ELP3 in LPS-induced IFN-I processes was two-fold: firstly ELP3 was required for TLR4-mediated IRF3 activation and IFN-I gene induction, whilst secondly, ELP3 was necessary for TYK2 activation in response to IFN-I signalling, and subsequent ISG induction (Fig 6.1). Thus in murine macrophages, ELP3 exerts influence over LPS-mediated IFN-I processes in a bi-fold manner, at the level of gene induction and signalling. STAT1 protein levels were clearly diminished in the absence of ELP3, but were not the rate-limiting factor for signalling as IFNγ-mediated STAT1 activation was unimpaired in *Elp3*$^{-/-}$ cells. Expression levels of IFNAR, JAK1 and TYK2 were unaffected in cells lacking ELP3. As IFNγ also signals through a receptor associated with JAK1, this leaves TYK2 activation as the restrictive factor for IFN-I signalling in *Elp3*$^{-/-}$ cells. This hypothesis was supported by the fact that, two other distinct signalling pathways that utilise TYK2, IL-4 and IL-10, were abrogated in the absence of ELP3. IL-4 is important for polarization of macrophages to an M2-like phenotype (212). Intriguingly, ELP3 was shown in one recent study to be required for M2 polarisation of macrophages following IL-4/IL-13 treatment (213). TYK2 activation was similarly impaired in ELP3 deficient macrophages, although this was likely due to impaired IL-13Rα expression. Il-13Rα expression was not assessed in this thesis, but TYK2 activation is the likely causative factor in this setting, as both IFN-I and IL-10 pathways are also abrogated. LPS-induced IFN-I has previously shown to be impaired in

Tyk2$^{-/-}$ macrophages (237). These data suggest that ELP3 is necessary for receptor-mediated signalling via TYK2. IL-12 and IL-23 induce Th1 and Th17 responses respectively, and both cytokine receptors signal through TYK2 (238). It would be compelling to assess whether T-cells deficient in ELP3 would be able to differentiate to Th1 and Th17 phenotypes in response to IL-12 or IL-23 treatment. *Tyk2*$^{-/-}$ mice are resistant to EAE and colitis, with TYK2 being a susceptibility gene for psoriasis, due to reduced IL-23 and Th17 differentiation (239). The potential contribution of Elongator to these disease states is an interesting and an unanalysed concept. IL-4 plays a key role in Th2 responses in antiparasitic immunity and allergy. In response to parasitic larvae infection, basophils release IL-4 which induces Th2 differentiation and B-cell IgE class switching (240). Asthma is driven by eosinophilc airway infiltration-induced inflammation. IL-4 released by eosinophils is associated with macrophage-mediated pathology of allergic asthma (241,242). M2 polarisation which is regulated by ELP3, is involved in the pathology of allergic asthma (243). M2 macrophages are also associated with mucosal repair in colitis (244). In the context of the gut, ELP3 has been shown to regulate intestinal tumour initiation by mediating tuft cell differentiation (195). Parasites trigger tuft cells to activate ILC2s, which are key in immunity to *Nippostrongylus brasiliensis* (245). STAT6, which was shown to enhance ELP3 expression (213), promotes tuft cell expansion, intestinal epithelial remodelling and Th2 immunity to helminths (246). Influenza virus has also been shown to induce tuft cell expansion in the small intestine (247). Thus, ELP3 may function as a key node in different T-cell mediated pathologies and antimicrobial immunity, by regulating TYK2-mediated cell differentiation in response to cytokines. It remains to be seen whether ELP3 plays a definitive functional role in these responses, but the present study suggests that analysing the contribution of Elongator to these TYK2-mediated processes and pathologies would make for a compelling future line of enquiry.

6.3 Elongator and mTORC2

The axis of reciprocal regulation between Elongator and mTORC2 was not analysed in this thesis, but generates interesting questions regarding functioning of Elongator in immune responses. In the context of M2 macrophage polarisation, ELP3 expression was shown to be elevated in an mTORC2 and STAT6-dependent manner, with STAT6

localising to the promoter of the *Elp3* gene (213). Elongator was illustrated previously to be required for translation of the mTORC2 complex protein RICTOR in the setting of melanoma, and thus regulate mTORC2 activity, and mTORC2-dependent upregulation of Elongator complex subunits (198). STAT6 and mTORC2 are required for Th2 differentiation (248–250), giving an additional layer of support to the hypothesis that ELP3 may be upregulated during, and play a crucial role, in Th2 differentiation and functioning. *Rictor*$^{-/-}$ and subsequent mTORC2 deficiency have been related to impeded STAT1 and STAT2 activation (251). RICTOR knockdown in the U937 monocytic cell line also led to abrogated TYK2 activation, reduced ISG expression and IFN-I-mediated Akt phosphorylation (252). Thus there is an interplay between mTORC2 and the IFN-I pathway, with Elongator possibly functioning as a focal point between the two. mTORC2 also functions in a variety of other manners. mTORC2 mediates neuronal cell survival following primary HSV-1 infection, preventing systemic infection (253). Chemotaxis of mast cells (254) and neutrophils (255) has been demonstrated to be mTORC2-dependent. mTORC2 also regulates differentiation of different cell types such as cardiomyocytes (256), and pancreatic beta cells (257). mTORC2 also regulates insulin sensitivity in the liver (258). Thus, there appears to be an insulin-mTORC2 axis, suggesting a possible role for Elongator in insulin generation and sensitivity in diabetes.

6.4 Elongator-mediated regulation of PYHINs

The PYHIN family of proteins execute myriad functions in innate immunity. Here it was demonstrated that ELP3 was necessary for expression of the murine PYHIN p205 independent of transcriptional induction. mRNA induction of p205 was abrogated following LPS and IFN-I treatment due to impaired TYK2 activation and downstream signalling. IFNγ-mediated gene induction of *p205* was not ELP3-dependent, but its protein expression was, establishing that ELP3 was required for p205 protein expression. The PYHIN family carry out an array of roles in the immune system, from cell cycle regulation, to cytokine induction and DNA sensing (39,40,48). An intriguing aspect discovered in this project was that, as a family, the PYHINs are enriched in Elongator-dependent codons. Due to the well-established role of the PYHINs in innate immunity, this implicates Elongator as being required for functioning of innate immunity. As discussed in section 1.4.3, the human transcriptome is enriched in synonymous codons

that do not require Elongator-mediated wobble modifications, as this necessitates the diversion of cellular resource towards the decoding of non-optimal codons. Thus, it is fascinating that a whole family of innate immune proteins are enriched in codons which require Elongator for their efficient translation. p205 was previously shown to be required for ASC expression and NLRP3 inflammasome activation in BMDMs (72). Functionally p205 translational deficiency in the absence of ELP3 did not reduce ASC expression but significantly enhanced it. Elongator may mediate translation of a repressor on ASC expression to regulate inflammasome activation, which potentially was expressed at a higher level in *Elp3*$^{-/-}$ cells, overcoming the observed p205 deficiency. The '*wobbly*' mouse phenotype was shown to derive from a mutation in *Elp6*, which destabilised the complex and compromised its functioning (189). This lead to neuronal death induced by NLRP3 inflammasome-dependent microgliosis and neuroinflammation. Intriguingly, expression of the key inflammasome effectors, ASC and cleaved caspase-1, were significantly enhanced in the CNS of *wobbly* mice lacking a functional Elongator complex. ASC nucleation and speck formation was also enhanced in the *Elp6* mutant mice. In the CNS, NLRP3 inflammasome is activated in pathologies related to protein aggregation (259). Therefore it is possible that destabilisation of the Elongator complex can either lead to protein aggregation which enhances inflammasome activation, or Elongator is required for translation of a cellular repressor of inflammasome. It remains to be seen whether inflammasome activation and IL-1β & IL-1α release is enhanced in *Elp3*$^{-/-}$ iBMDMs. This would be an intriguing process to examine, as ASC expression is enhanced, but both IL-1β & IL-1α are enriched in Elongator-dependent codons (both ~9.6%). Proteins with similar Elongator-dependent codon content to this have been shown to have impaired expression in the absence of ELP3 (213), so it would be interesting to assess whether there is enhanced NLRP3 activity in *Elp3*$^{-/-}$ cells, or whether IL-1β & IL-1α secretion is abrogated due to a translational requirement for ELP3. Elongator has been clearly demonstrated to regulate CNS development and neurodegeneration (123,188). As neuroinflammation is a key causative factor for neurodegeneration, it would be intriguing to analyse whether Elongator plays a homeostatic role in microglia, the resident immune cells of the CNS.

6.5 Elongator-mediated regulation of IRF3 activation

We demonstrated that ELP3 was required for TLR and RIG-I, but not STING mediated signalling and gene induction. In the context of TLR4, this study illustrated that ELP3 deficiency leads to abrogated IRF3 phosphorylation and activation, impairing IFN-I mRNA induction (Fig 6.1). However, RIG-I and STING both signal through IRF3 for IFN-I induction in response to RNA and cGAMP respectively. IRF3 total protein expression was unaffected by ELP3 deficiency. This suggests that ELP3 is regulating the expression of a protein which is required for IRF3 phosphorylation and activation downstream of TLRs and RIG-I, but not STING. This is possibly via impaired translation of a factor which helps enable IRF3 phosphorylation, dimerization and nuclear translocation. Alternatively, the presence of a dysregulated Elongator complex may lead to enhanced expression of a negative regulator of IRF3 in response to TLR and RLR, but not STING activation. A variety of proteins mediate inhibition of IRF3 activation via a variety of mechanisms, such as ubiquitination (234), dephosphorylation (260–262), blockade of dimerization (263) and of nuclear translocation (264). Further work is needed to identify a factor, restricted to TLR and RLR but not STING pathway, that is required for IRF3 activation and is Elongator-dependent.

6.6 Consequence of Elongator-mediated regulation of IFN-I

The effect of ELP3 deficiency on innate immune gene induction were restricted to LPS and RNA, not DNA-mediated pathways. cGAMP and VACV 70mer-mediated gene induction was unaffected by the absence of ELP3, whilst poly (I:C) and hpRNA-enabled gene induction was abrogated in *Elp3*$^{-/-}$. These data pertaining to IFN-I raise questions relating to the functional consequence of Elongator-mediated regulation of IFN-I induction and signalling. As has been discussed in chapter 1 of this thesis, IFN-I, whilst being critical regulators of processes relating to innate immune function and antiviral immunity, can be deleterious in the context of autoimmunity. IFN-I are amongst the causative agents of pathology in diseases such as SLE, Sjogren's and rheumatoid arthritis. The understanding of the role of Elongator in IFN-I-dependent disease processes would be advanced by utilising samples derived from say SLE patients, and assessing the expression pattern of Elongator, and whether its elevated, as well as analysing whether RNA interference of Elongator expression abrogates the enhanced

IFN-I signature observed in these autoimmune conditions. IFN-I expression also correlates poorly with outcome in the context of bacterial infection with *M. tuberculosis* (29, 30). Thus it would be intriguing to assess whether the absence of Elongator in immune cells leads to reduced IFN-I and enhanced bacterial clearance. Hence, there are a myriad of avenues of discovery and inquiry to be made in the future, with the regard to Elongator functioning at the interface of IFN-I signalling and processes.

6.7 Elongator and Viral infection

This requirement for Elongator in RNA sensing pathways was further illustrated by the fact that gene induction of IFN-I and ISGs was impaired following RNA virus infection. The RLR-dependent virus IAV, and TLR and RLR-dependent RSV, failed to activate innate immune gene induction in infected *Elp3*$^{-/-}$ macrophages (Fig 6.1). An intriguing prospect not assessed in this study would be a comparative analysis between DNA and RNA virus infection of immune cells deficient in ELP3, as DNA sensing pathways were exhibited not to require ELP3. However, the RLRs have been shown to function in the context of DNA virus infection, via the recognition DNA virus encoded RNA, RNAPIII-mediated transcription of AT rich viral DNA to immunostimulatory RNA, and sensing of aberrant host RNA released by infection (265). Due to this crosstalk of RNA & DNA sensing pathways during infection, future work will need to be performed to assess whether Elongator regulates innate immune gene induction to solely RNA viruses, or to DNA viruses via a similar mechanism also. Compellingly, although induction of antiviral genes such as *Ifna and Ifnb* was abrogated in response to IAV infection, it was exhibited that IAV replication was abrogated in *Elp3*$^{-/-}$. This suggests a fascinating scenario, where Elongator functions as a node for both innate immune gene induction to viruses, but also as a host factor required for viral replication. RNA viruses hijack the host translational machinery to enable translation of their own viral RNA, such as by enriching the tRNA pool of the host cells for decoding of codons that the viruses are enriched in (266). Chikungunya virus was recently illustrated to reprogram host cell translational machinery to facilitate translation of its rare codons (235). CHIKV is enriched in rare codons ending in AA, which require tRNA modification for their efficient decoding. CHIKV and dengue virus (DENV) upregulated the expression of a protein, KIAA1456, which is required for the methylation of mcm^5 modified wobble Uridines at

position 34. CHIKV infection led to enhancement of these modifications and favoured translation of viral RNA, or host mRNAs enriched in AA-ending codons, such as those involved in DNA damage or cell cycle. Compellingly, Elongator mediates cm^5 modifications of wobble position Uridines, and this Elongator-dependent modification is rate-limiting and a prerequisite for additional methylation to give mcm^5 modifications. Hepatitis C virus, which is not enriched in these codons, did not induce enhancement of this tRNA modification pathway. DENV has also been illustrated to activate mTORC2 to prevent cell death and provide a reservoir for infection (267). This suggests that RNA viruses co-opt this evolutionarily conserved mechanism of tRNA modification, to favour viral replication. Additionally, other RNA viruses such as Zika virus and SARS CoV-2 are enriched in Elongator-dependent codons. Thus functional analysis of the role of Elongator in SARS CoV-2 infection, would be an intriguing new line of analysis into the immunology of the pandemic. A compelling pathway of inquiry is thus opened, into assessing the relative contribution of Elongator during viral infection, both to host innate immune responses, and co-opting by viruses by replication. These data thus place Elongator at the interface of innate immunity and viral infection.

How Elongator is regulated during immune responses remains an open question. Expression of Elongator subunits were shown to be enhanced in the context of cancer, and in M2 macrophage polarisation (198,213). In response to mTORC2 activation, Elongator activity has also been shown to be enhanced, as measured by ELP1 phosphorylation, which is known to enhance its tRNA modification capabilities (198). Whether Elongator enzymatic activity gets enhanced via post translational modification, or Elongator expression upregulated in the context of an immune response needs to parsed out and analysed in the future. It could be highly context and location specific, with enhanced expression or activation of Elongator occurring in specific immune cells, or in a specific immune setting or tissue. In the context of IFN-I-mediated pathological disease, Elongator expression may potentially be enhanced in an aberrant manner, supporting enhanced and deleterious IFN-I production. Elongator may be co-opted following RNA virus infection, to support decoding of viral codons and viral replication. Thus, Elongator expression or enzymatic activity may be modulated in a variety of immune contexts and settings.

The objective of this project was to assess the relative contribution of the Elongator complex to innate immune responses in macrophages. Unbiased quantitative proteomics analysis of macrophages deficient in *Elp3* illustrated a drastic impairment in the expression proteins involved in IFN-I signalling and responses following LPS treatment. I exhibited a two-step requirement for ELP3 in TLR4-mediated IFN-I, both at the level of IFN-I signalling via TYK2 activation, and at the level of IFN-I gene induction via a requirement for ELP3 in IRF3 activation. I illustrated that the PYHIN family of proteins are enriched in Elongator-dependent codons, and that ELP3 was required for translation of p205 following IFNγ treatment. Upon further examination of the role of Elongator in innate immunity, I discovered that ELP3 is necessary for RNA, but not DNA sensing pathways in macrophages. Furthermore, ELP3 is required for innate immune gene induction following RNA virus infection. However, there is bi-directional role for ELP3 in viral infection, with ELP3 also being necessary for IAV replication in macrophages.

In summary, this work illustrates that the Elongator complex plays a role in innate immune processes, being necessary for induction and signalling of IFN-I in macrophages, as well as regulating antiviral immune gene induction and IAV replication.

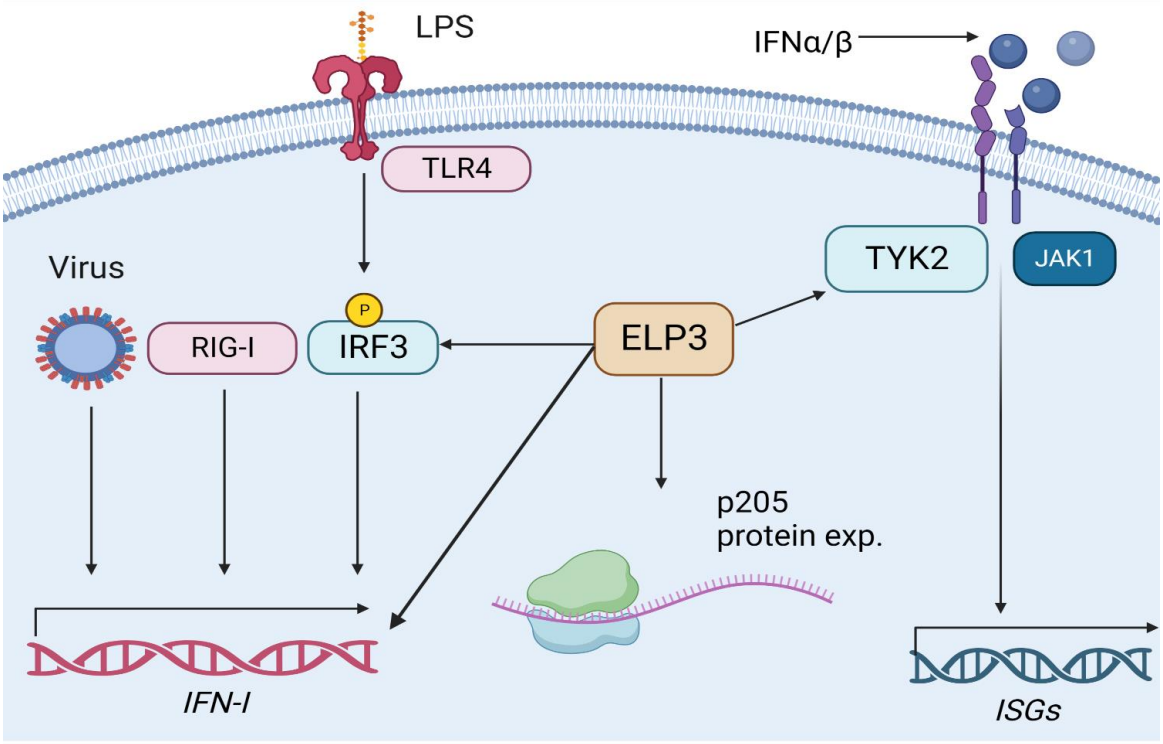


Fig 6.1 Overview of ELP3-mediated regulation of innate immune responses in macrophages

We demonstrated that ELP3 is required for IFN-I signalling, likely via regulation of TYK2 activation. ELP3 was required for IRF3 activation downstream of TLR4 activation, and thus regulated TLR4-mediated IFN-I gene induction. ELP3 was also necessary for RIG-I-mediated RNA sensing and subsequent IFN-I gene induction.

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