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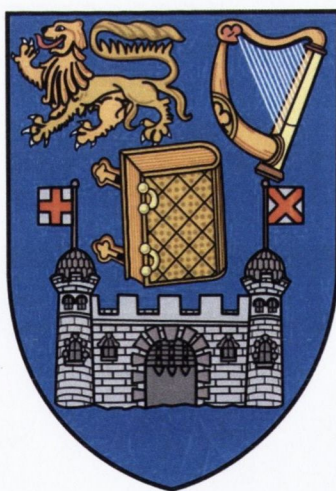
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DHX9: novel roles in cytokine induction and its antagonism by vaccinia virus protein E3

**Thesis submitted to the University of Dublin for the
degree of Doctor of Philosophy**

April 2015

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Declaration

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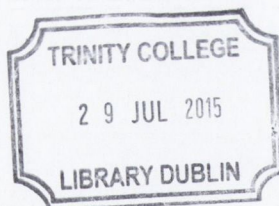
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Abstract

Vaccinia virus (VACV) is a dsDNA virus, which replicates in the cytoplasm of infected host cells. VACV, similar to other viruses, is detected by host innate immunity mainly by sensing of viral nucleic acids such as dsRNA, leading to type I interferon (IFN) and cytokine induction. A major contributor to the ability of VACV to target IFN is the dsRNA-binding protein E3. Mass spectrometry analysis previously identified DEAH-box polypeptide 9 (DHX9) as a novel interacting partner of E3. DHX9, an RNA helicase vital in various aspects of RNA metabolism, had previously been shown to bind to the p65 subunit of NF- κ B to enhance NF- κ B-dependent gene expression. Recently, DHX9 was proposed as a cytoplasmic nucleic acid sensor in dendritic cells of CpG-B DNA and dsRNA, signalling via MyD88 and MAVS, respectively. The aims of this project were to investigate immune functions of DHX9 and then to determine whether E3 could antagonise these.

Using DHX9 shRNA-expressing THP-1 cell lines, DHX9 was demonstrated to have no role in DNA- or RNA-stimulated IFN β mRNA induction or chemokine secretion in monocytes. DHX9 was found to be required for maximal IFN-stimulated ISG56 mRNA induction. However, this did not translate to an effect on ISG56 protein expression, possibly because the mRNA is not limiting for translation. DHX9 was discovered to be required for TLR- and RNA virus-induced cytokine production in THP-1 cells. Endosomal TLR7/8 signalling was found to be particularly dependent on DHX9, as all CL075-stimulated cytokines were reduced upon silencing of DHX9 expression. A role for DHX9 in endosomal TLR signalling is consistent with the fact that it has previously been reported to interact with MyD88. DHX9 was also found to be critical in IL-6 production by any stimuli tested in both THP-1 and BEAS-2B cells. Reporter gene assays showed DHX9 to potentiate activation of the IL-6 promoter.

DHX9 was demonstrated to enhance NF- κ B activation in response to any stimuli tested in HeLa cells, most likely due to its ability to bind p65. Interestingly, DHX9 was also shown to enhance transactivation of the transcription factors, IRF3 and IRF7. The ability of DHX9 shown here to transactivate p65 and IRF family members may provide an explanation as to why DHX9 loss-of-function affected NF- κ B- and IRF-dependent cytokines. It also raises the question of whether DHX9 is a bona fide nucleic acid sensor as its ability to activate transcription factors may provide an alternative explanation to previously published data.

Co-immunoprecipitation studies confirmed that E3 interacted with DHX9, and showed that the interaction was via its RNA-binding domain, and nucleic acid-independent. Interestingly, THP-1 cells stably expressing VACV E3 were found to have a very similar cytokine induction profile as DHX9 shRNA-expressing THP-1 cells following stimulation. In particular, E3-expressing cells displayed reduced expression of IL-6 in response to all stimuli tested. Notably, E3 was previously reported to inhibit IL-6 production by an unidentified mechanism, and IL-6 has a role in the clearance of VACV, consistent with the notion that E3 targets DHX9 as an immune evasion strategy. Further, E3 was capable of antagonising the ability of DHX9 to potentiate NF- κ B and IRF7 transactivation. Overall the results reveal novel roles for DHX9 in cytokine induction and new mechanisms whereby VACV E3 contributes to viral immune evasion.

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List of abbreviations

ADAM	A disintegrin and metalloprotease
AP-1	Activator protein 1
AIM2	Absent in melanoma 2
AKT	Thymoma viral proto-oncogene 1
ALR	AIM2-like receptor
APS	Ammonium persulphate
ASC	Apoptosis-associated speck-like protein containing a CARD
ATP	Adenosine triphosphate
ATPase	Adenosine triphosphatase
BAFF	B-cell activating factor
Bcl-10	B cell lymphoma/leukemia 10
BCR	B cell receptor
BD	Binding domain
BIR	Baculovirus inhibitor of apoptosis protein repeat
BMDC	Bone marrow-derived dendritic cell
BSA	Bovine serum albumin
BSF-3	B cell-stimulating factor 3
CARD	Caspase activation and recruitment domain
CBP	CREB-binding protein
CD	Cluster of differentiation
c-di-GMP	Cyclic diguanylate monophosphate
CDN	Cyclic dinucleotide
cDNA	Complementary DNA
C/EBP β	CCAAT-enhancer-binding protein β
CEV	Cell-associated enveloped virus
cGAMP	Cyclic-GMP-AMP
cGAS	cGAMP synthase
CIITA	MHC class II transactivator
CNTF	Ciliary neurotrophic factor
CpG	Cytidine-phosphate-guanosine

CRD	Carbohydrate recognition domain
CREB	cAMP-response element binding protein
CTD	C-terminal domain
CT-1	Cardiotropin-1
CTP	Cytidine triphosphate
DAI	DNA-dependent activator of IRF
DBD	DNA-binding domain
DC	Dendritic cell
DDX	DEAD-box
DECTIN-1	Dendritic-cell-associated C-type lectin 1
DExD/H	Aspartate-glutamate-any amino acid-aspartate/histidine
DHX	DEAH-box
DMEM	Dulbeccos modified eagles medium
DNA	Deoxyribonucleic acid
DNA-PK	DNA-dependent protein kinase
DNase	Deoxyribonuclease
ds	Double-stranded
ECACC	European collection of animal cell cultures
EEV	Extracellular enveloped virus
eIF2 α	Eukaryotic initiation factor 2 α subunit
EMCV	Encephalomyocarditis virus
ER	Endoplasmic reticulum
ERK	Extracellular signal-regulated kinase
FADD	Fas-associated death domain protein
FCS	Fetal calf serum
FLI1	Friend leukemia integration 1
GAS	IFN γ -activated sites
GRB2	Growth-factor-receptor-bound protein 2
GTP	Guanosine triphosphate
HBV	Hepatitis B virus
HCV	Hepatitis C virus
HEK	Human embryonic kidney
HIV	Human immunodeficiency virus

HSV	Herpes simplex virus
IEV	Intracellular enveloped virus
IFI16	IFN γ -inducible protein 16
IFIT	IFN-induced protein with tetratricopeptide repeats
IFN	Interferon
IFNAR	IFN α/β receptor
IFNGR	IFN γ receptor
IFNLR	IFN λ receptor
I κ B	Inhibitor of kappa B
IKK	I κ B kinase
IL	Interleukin
IL-1R	IL-1 receptor
IMV	Intracellular mature virus
IRAK	IL-1R-associated kinase
IRF	Interferon regulatory factor
IRS	Insulin receptor substrate
ISG	Interferon-stimulated gene
ISGF3	ISG factor 3
ISRE	IFN-sensitive response element
JAK	Janus kinase
JNK	c-Jun N-terminal kinase
KLF4	Kruppel-like factor 4
KSHV	Kaposi's sarcoma-associated herpesvirus
LBP	LPS-binding protein
LGP2	Laboratory of genetics and physiology 2
LIF	Leukemia inhibitory factor
LPS	Lipopolysaccharide
LRR	Leucine rich repeat
LRRFIP1	Leucine-rich repeat flightless-interacting protein 1
Mal	MyD88 adaptor-like protein
MAPK	Mitogen activated protein kinase
MAVS	Mitochondrial antiviral signalling protein
mbIL-6R α	Membrane-bound IL-6 receptor α

MCMV	Mouse cytomegalovirus
MCS	Multiple cloning site
MDA5	Melanoma differentiation-associated gene 5
mDC	Myeloid dendritic cell
MDP	Muramyl dipeptide
MD-2	Myeloid differentiation factor 2
MEF	Mouse embryonic fibroblast
MHC	Major histocompatibility complex
miRNA	micro RNA
MKK	MAPK kinase
MLK3	Mixed lineage kinase 3
MMTV	Mouse mammary tumor virus
mRNA	Messenger RNA
mTOR	Mammalian target of rapamycin
MyD88	Myeloid differentiation primary response gene 88
NAP1	NF- κ B-activating kinase-associated protein 1
NEMO	NF- κ B essential modulator
NFAT	Nuclear factor of activated T cells
NF- κ B	Nuclear factor- κ B
NIK	NF- κ B-inducing kinase
NK	Natural killer
NLR	NOD-like receptor
NLS	Nuclear localisation sequence
NMD	Nonsense mediated decay
NNT-1	Novel neurotrophin-1
NOD	Nucleotide-binding oligomerisation domain containing
NPN	Neuropoietin
NSC	Non-silencing control
NTD	N-terminal domain
OAS	2'5'-oligoadenylate synthetase
OSM	Oncostatin M
PAMP	Pathogen associated molecular pattern
PAM3	Pam3CSK4

PBMC	Primary peripheral blood mononuclear cell
PBS	Phosphate-buffered saline
PBS-T	PBS-Tween
pDC	Plasmacytoid dendritic cell
PI3K	Phosphoinositide 3 kinase
PKC	Protein kinase C
PKR	Protein kinase R
PMA	Phorbol-12-myristate-13-acetate
PML	Promyelocytic leukemia
Pol II/III	RNA Polymerase II/III
Poly(dA:dT)	Polydeoxyadenylic:polydeoxythymidylic
Poly(I:C)	Polyinosine-polycytidylic acid
PRR	Pattern recognition receptor
PTP	Protein tyrosine phosphatase
PVDF	Polyvinylidene difluoride
PYD	Pyrin domain
PYHIN	Pyrin and HIN domain-containing protein
qPCR	Quantitative real-time PCR
RD	Repressor domain
RHD	Rel homology domain
RIG-I	Retinoic-acid-inducible gene I
RIPK2	Receptor-interacting serine/threonine-protein kinase 2
RIP1	Receptor interacting protein 1
RLR	RIG-I-like receptor
RNA	Ribonucleic acid
RNA-BD	RNA-binding domain
RNase	Ribonuclease
RSV	Respiratory syncytial virus
rRNA	Ribosomal RNA
RT	Reverse transcription
SARM	Sterile α and armadillo motif-containing protein
SDS	Sodium dodecyl sulphate
SDS-PAGE	SDS polyacrylamide gel electrophoresis

SeV	Sendai virus
SF	Superfamily
sIL-6R α	Soluble IL-6 receptor α
siRNA	Short interfering RNA
SINTBAD	Similar to NAP1 TBK1 adaptor
SHP	SH2-domain containing protein tyrosine phosphatase
shRNA	Short hairpin RNA
SLE	Systemic lupus erythematosus
SOCS	Suppressor of cytokine signalling
Sp-1	Specificity protein-1
ss	Single-stranded
STAT	Signal transducers and activators of transcription
STING	Stimulator of interferon genes
TAB	TAK1-binding protein
TAK1	TGF β -activated kinase 1
TAB1	TAK1 binding protein 1
TAD	Transactivation domain
TANK	TRAF family member-associated NF- κ B activator
TBK1	TANK-binding kinase 1
TBS	Tris-buffered saline
TCR	T cell receptor
TEMED	N,N,N',N'-tetramethyldiamine
TGF	Transforming growth factor
T _h	Helper T cell
TIR	Toll/Interleukin-1 receptor
TLR	Toll-like receptor
TNF	Tumor necrosis factor
TNFR	Tumor necrosis factor receptor
Tpl2	Tumor progression locus 2
TRADD	TNFR1-associated DEATH domain protein
TRAF	TNFR-associated factor
TRAM	TRIF-related adaptor molecule
TRIF	TIR domain-containing adapter protein inducing IFN β

TYK2	Tyrosine kinase 2
UTP	Uridine triphosphate
VACV	Vaccinia virus
VSV	Vesicular stomatitis virus
WNV	West Nile virus
WB	Western blot
WR	Western Reserve

Chapter 1: Introduction

1.1 The first line of defence

In order to fight pathogens, all organisms require an immune response to mount a defence against infection. An organism's immune system can be divided into two; the innate and the adaptive immune systems. The innate immune system consists of the primary line of defence against pathogens and is said to be non-specific, in that, it will attack any invading pathogens [1]. The second line of defence, the adaptive immune system, requires the innate immune system for its activation and is said to be specific, in that it has the ability to recognise and remember specific pathogens [2]. Years of research have provided a much better understanding of the mechanisms involved in the innate immune response and its integral role in triggering a secondary immune response, which is vital to the clearance of microbial infection. The innate immune system is made up of several distinct components which provide the first barriers against infection. Amongst these are physical barriers such as the bodies' epithelia, and the cells (e.g. macrophages and neutrophils) and molecules (e.g. complement system) available which are capable of controlling or destroying invading pathogens [1]. These cells express pattern recognition receptors (PRRs) which are key to the initial recognition of invading pathogens, and the first constituent to the triggering of an intracellular innate immune response to infection, needed for the activation of the adaptive immune system [3]. PRRs recognise pathogen associated molecular patterns (PAMPs) which are molecules that are associated with groups of pathogens but discernible from host molecules, and are normally essential for pathogen viability [4]. The first described PRR family were the membrane-bound Toll-like receptors (TLRs), which are key in the recognition of a range of PAMPs and the triggering of an effective innate immune response to infection [5]. Other PRR families described include the nucleotide-binding oligomerisation domain (NOD)-like receptors (NLRs) which sense microbial products in the cytoplasm of cells and have a variety of functions in the regulation of inflammatory and apoptotic responses [6], and the retinoic-acid-inducible gene I (RIG-I)-like receptors (RLRs) which are vital in the detection of various forms of foreign ribonucleic acid (RNA) [7]. There are also the C-type lectin (CLR) receptors which are key in anti-fungal immunity, recognising the major carbohydrate structures found in fungal cell walls, and triggering the induction of anti-fungal effector mechanisms [8]. Upon activation, the majority of these PRR families share an ability to trigger the upregulation of a vast array of pro-inflammatory cytokines and type I

interferon (IFN), i.e. IFN α and IFN β . Pro-inflammatory cytokines (see Section 1.3) are generally expressed following activation of the transcription factor, nuclear factor-kappa B (NF- κ B), while IFNs are generally upregulated via activation of interferon regulatory factor (IRF) transcription factor family members (Section 1.4). There are also the recently defined family of PRRs involved in deoxyribonucleic acid (DNA) sensing, termed the absent in melanoma 2 (AIM2)-like receptors (ALRs), which include the pyrin and HIN domain-containing proteins (PYHINs) IFN γ -inducible protein 16 (IFI16), AIM2 and p204 [9]. This family have been shown to respond to double-stranded (ds)DNA of any form or species in the cytosol and trigger an upregulation in type I IFN [10]. The enzyme cGMP-AMP synthase (cGAS) has also been implicated in DNA sensing [11].

A number of infectious organisms can be detected and successfully destroyed by the innate immune system. However, adaptive immunity, which is restricted to vertebrates, comes into play when the innate defences have been evaded or overwhelmed by invading pathogens [2].

1.2 Adaptive immunity

While the innate immune response is restricted in its diversity of recognition, the adaptive immune system is mediated by a highly diverse set of receptors which are capable of specific antigen recognition. Unlike the innate immune response, which can be mounted within hours of infection, the adaptive immune response is characterised by a period of up to a few days clonal expansion before proliferating lymphocytes can mature into effector cells capable of eliminating the infection. The innate immune system generally repels a pathogen at the early stage of infection until an effective adaptive immune response can be mounted [12].

The adaptive immune system is mediated by highly diverse antigen receptors represented by antibodies and T cell receptors (TCRs), which are clonally expressed on B and T lymphocytes, respectively [2]. These receptors consist of immunoglobulin domains which are generated by somatic recombination of variable, diversity and joining gene segments. This process of somatic recombination provides a vast repertoire which allows the adaptive immune system to recognise virtually any antigen. Mature T

cells are activated upon interaction of their TCRs with antigenic peptides complexed with major histocompatibility complex (MHC) molecules [13]. Cluster of differentiation (CD) 8^+ T cells engage peptides which are in a complex with MHC class I molecules while CD 4^+ T cells engage peptides bearing MHC class II molecules. MHC class I molecules are constitutively expressed on all nucleated cells and MHC class I-restricted peptides are generally endogenous antigens or antigens from viruses or other pathogens which replicate intracellularly. Unlike MHC class I, MHC class II molecules are present only on antigen presenting cells, such as dendritic cells (DCs), and are inducible by innate immune stimuli [13]. Antigen presenting cells sample the environment for antigens and danger molecules and upon binding of antigen and activation, these cells undergo an upregulation of MHC class II molecules and migrate from skin and mucosal sites to regional lymph nodes where they interact with T lymphocytes to initiate an immune response. T lymphocytes get further stimulated by DC-secreted cytokines which direct the transition of the naïve T cell into the appropriate effector cell depending on the pathogen encountered. Upon activation, CD 4^+ T lymphocytes mature into helper T cells (T_h cells). There are six main CD 4^+ T_h cell subsets defined: T_h1 , T_h2 , T_h17 , T_h9 , follicular and regulatory T cells [14]. Each of these subsets regulate other immune cells through the secretion of selected cytokines. CD 8^+ T cells on the other hand, mature into what are referred to as CD 8^+ cytotoxic T cells upon activation [15]. Cytotoxic T cells are capable of inducing apoptotic cell death of an infected cell through the release of cytotoxins which include perforin, granzymes and granulysin. Perforin inserts itself into a target infected cell membrane, forming a pore, which allows entry of granzymes which can induce caspase-dependent apoptosis of the target cell. In contrast to T lymphocytes which are involved in a cell-mediated response to infection, B lymphocytes function “at a distance” by the secretion of highly specific antibodies into the blood upon activation, which bind and initiate elimination of extracellular pathogens and toxins [16]. This response is often referred to as humoral immunity. After antigen binding of the B cell receptor (BCR), naïve B cells develop into plasma cells which are capable of secreting large amounts of antigen-specific antibodies. Upon recognition of an antigen, both T and B lymphocytes undergo a process known as clonal selection in which antigen-specific effector cells proliferate in order to eliminate the pathogen during infection. Upon clearance of infection, some of

these cells are long-lived and establish an immune memory that facilitates a faster and more vigorous response upon a second encounter with the same pathogen [17].

Although defined as being two separate arms of the immune response, there is an important functional link between the innate and adaptive immune responses, with cross-talk between the systems being bidirectional [18]. For instance, TLR stimulation can upregulate cytokines capable of inducing dendritic cell maturation, expression of co-stimulatory molecules and T cell differentiation. Also, IFN γ secreted by T_h1 cells activates macrophages and interleukin (IL)-4 and IL-6 produced by T_h2 cells can stimulate some macrophage and dendritic cell subsets [18].

1.3 NF- κ B – a key transcription factor in pro-inflammatory cytokine induction

1.3.1 NF- κ B activation

There are five NF- κ B family members in mammals; p65 (RelA), RelB, c-Rel, p50/p105 (NF- κ B1), and p52/p100 (NF- κ B2) [19]. They are characterised by an N-terminal Rel homology domain (RHD). Binding to 9–10 base pair DNA sites (κ B sites) as either homodimers or heterodimers, they are capable of exerting both positive and negative effects on target gene transcription. p65, RelB and c-Rel initiate transcription through their C-terminal transactivation domains (TADs). Lacking TADs, p50 and p52 are generally not activators of transcription. They require dimerisation with other NF- κ B subunits or non-Rel proteins that have transactivation ability, in order to positively regulate transcription. p50 and p52 homodimers can negatively regulate transcription by competing with other TAD-containing dimers for κ B binding [19]. In non-stimulated cells, the NF- κ B proteins are localised to the cytoplasm in an inactive form through the interaction with inhibitor of kappa B (I κ B) family members. The I κ B family consists of eight bona fide members; I κ B α , I κ B β , I κ B ϵ , I κ B ζ , BCL-3, I κ BNS, p100 (the precursor of p52), and p105 (the precursor of p50), which are typified by the presence of multiple ankyrin repeat domains [20]. The prototypical and most extensively studied member is I κ B α , whose primary target is a p50/p65 heterodimer. I κ B members mask the nuclear localisation sequence (NLS) of NF- κ B family members, thereby inhibiting their translocation to the nucleus. NF- κ B is released following phosphorylation of conserved

residues on I κ B, which leads to its K48-linked polyubiquitination and targeting to the proteasome for degradation. NF- κ B is then free to translocate to the nucleus and initiate transcription of κ B-dependent genes.

The NF- κ B signalling pathways have been broadly classified into two types; canonical (or classical) and non-canonical (Figure 1.1). A common upstream regulatory step of both pathways is the activation of the I κ B kinase (IKK) complex [21]. Classical NF- κ B signalling occurs upon ligand binding to cytokine receptors such as tumor necrosis factor (TNF) receptor (TNFR) and IL-1 receptor (IL-1R), PRRs and antigen receptors, and involves the triggering of signalling events which culminates in the activation of an IKK complex consisting of the catalytic subunits IKK α , IKK β and the scaffold protein, NF- κ B essential modulator (NEMO) [19]. IKK β then phosphorylates I κ B, allowing release of NF- κ B. Classical NF- κ B signalling is mostly mediated by the p50 and p65 subunits of NF- κ B. On the other hand, non-canonical NF- κ B signalling is induced by specific members of the TNF family, which include CD40 ligand, B-cell activating factor (BAFF) and lymphotoxin- β [22]. The non-canonical pathway, which does not require NEMO, mainly depends on IKK α and an upstream kinase, NF- κ B-inducing kinase (NIK). Receptor activation leads to NIK activation, which phosphorylates an IKK complex consisting of two IKK α subunits. The IKK α complex then phosphorylates p100, leading to its partial proteolysis, and resulting formation of RelB/p52 complexes which translocate to the nucleus. Although NF- κ B has a vast array of target genes, it is best known for its involvement in pro-inflammatory cytokine induction.

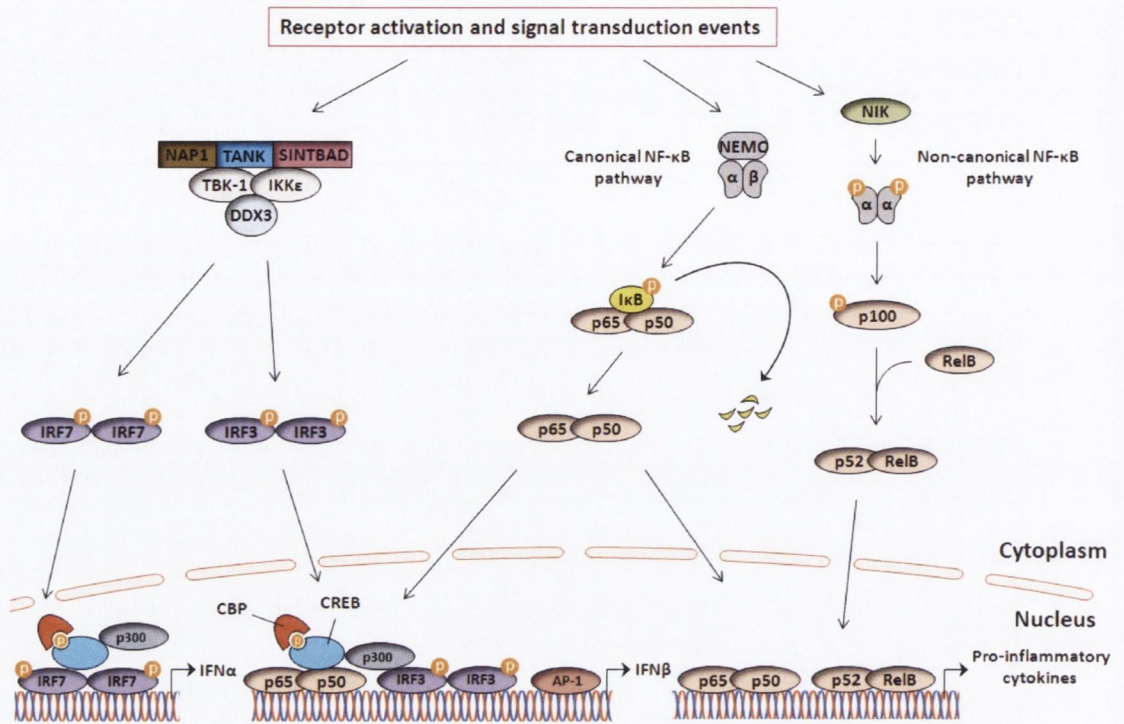


Figure 1.1 IRF3/7 and NF-κB activation

IRF3 and IRF7 are phosphorylated by the TBK1/IKKε complex, consisting of the TBK1 and IKKε kinases, alongside the adaptor proteins; NAP1, TANK, SINTBAD and DDX3. Upon phosphorylation, IRF homodimers translocate to the nucleus to upregulate IRF-dependent genes. IRF7 is mainly involved in IFNα production. IRF3, together with NF-κB, AP-1, and the coactivators CBP and p300, bind to the IFNβ promoter, resulting in its transcription. The canonical NF-κB pathway signals via an IKK complex consisting of the IKKα and IKKβ kinases and the scaffold protein NEMO. This pathway is mediated by the phosphorylation of the inhibitory IκB protein by IKKβ, resulting in its degradation and the release of p50/p65 heterodimer to translocate to the nucleus. In the non-canonical pathway, receptor-activated NIK phosphorylates an IKK complex consisting of two IKKα subunits. This IKKα complex then phosphorylates p100, resulting in its partial proteolysis and production of p52. p52 then forms a heterodimer with RelB and enters the nucleus. As well as being required for effective activation of the IFNβ promoter, NF-κB is also important in the expression of numerous pro-inflammatory cytokines, such as TNFα, IL-1 and IL-6.

1.3.2 Pro-inflammatory cytokines

Cytokines and chemokines are a heterogeneous group of secreted proteins with growth, differentiation, and activation functions that regulate and determine the nature of immune responses [23]. They mediate their actions through both autocrine and paracrine cell signalling. Although a simplistic means of classification, cytokines are often divided into pro- and anti-inflammatory cytokines [24]. A pro-inflammatory cytokine is a cytokine which promotes systemic inflammation [25]. Examples include IL-1, TNF α and IL-8. When administered to humans, IL-1 and TNF α can produce fever, inflammation, tissue destruction, and, in some cases, shock and death [26]. Blocking of IL-1 or TNF α have proved successful in the treatment of numerous inflammatory diseases such as rheumatoid arthritis and inflammatory bowel disease [27]. IL-8 is a chemoattractant cytokine, produced by a range of cell types, which attracts and activates neutrophils in inflammatory regions [28]. Anti-inflammatory cytokines are proteins which are capable of suppressing the action of pro-inflammatory cytokines [24]. Examples include IL-10, IL-4 and transforming growth factor (TGF) β , which are capable of suppressing the production of IL-1, TNF α and vascular adhesion molecules. There is a fine balance between the expression of pro- and anti-inflammatory cytokines. A dysregulation in pro- and/or anti-inflammatory cytokines can be seen at the heart of numerous autoimmune diseases, indicating the importance in their balance [29]. There are a number of cytokines which display both anti- and pro-inflammatory function and hence the classification system of anti- and pro-inflammatory cytokines is ambiguous. For example, although IL-4, IL-10 and IL-13 are potent activators of B lymphocytes, they are still referred to as anti-inflammatory cytokines due to their ability to suppress genes for pro-inflammatory cytokines such as IL-1 and TNF [25]. Likewise, although IL-6 is generally referred to as a pro-inflammatory cytokine, it also exhibits anti-inflammatory function [30]. As only pro-inflammatory cytokine induction was investigated in this project, the role of anti-inflammatory cytokines will not be further discussed. The cytokines TNF α , IL-1 and IL-6 are elevated in most, if not all, inflammatory states, and hence will be discussed further.

1.3.2.1 IL-1 signalling

IL-1 signalling occurs via the cell surface, membrane-bound, IL-1R (Figure 1.2). The IL-1R complex includes the type I IL-1R (IL-1RI) and the IL-1R accessory protein

(IL-1RAcP) [31]. IL-1RI contains a C-terminal cytoplasmic Toll/IL-1R (TIR) domain which facilitates recruitment of the TIR-domain containing adaptor, myeloid differentiation primary response gene 88 (MyD88). Upon the binding of IL-1 to IL-1RI, there is a rapid recruitment of IL-1RAcP, MyD88 and IL-1R-associated kinase (IRAK) family members [32]. IRAK proteins are kinases capable of the phosphorylation of downstream signalling components and the assembly of a platform for further signalling events [33]. Following phosphorylation, IRAK members associate with TNFR-associated factor (TRAF)6 and promote its polyubiquitination. Recruitment of TGF β -activated kinase 1 (TAK1)-binding protein (TAB)2/3 and TAK1 results in the phosphorylation of I κ B by the IKK complex, freeing NF- κ B, and allowing its translocation to the nucleus, where it upregulates a vast array of NF- κ B-dependent genes. IL-1 signalling also results in the activation of the mitogen activated protein kinases (MAPKs) p38 and c-Jun N-terminal kinase (JNK), via coupling of TAK1 to their upstream kinases [34]. Ultimately, IL-1-induced NF- κ B and MAPK activation results in the upregulation of a vast array of pro-inflammatory cytokines [32]. These signalling events are further described in Section 1.5, where TLR signalling via MyD88 occurs via a similar signalling cascade.

1.3.2.2 TNF signalling

TNF can be recognised by two types of receptors: TNFR1 or TNFR2 [35]. While TNFR1 is expressed on almost all cell types and is the major signalling receptor for TNF α (Figure 1.2), TNFR2, which binds both TNF α and TNF β , is mostly expressed on immune cells and mediates limited biological responses. TNFR1 contains a cytoplasmic death domain, while TNFR2 has a cytoplasmic TRAF-interacting motif. Both receptors can activate similar IKK and MAPK pathways which result in the activation of transcription factors such as NF- κ B and c-Jun and the expression of numerous cytokines and chemokines. However, the pro-inflammatory effects of TNF are thought to be predominantly TNFR1-mediated [36]. Upon TNF binding, TNFR1 receptor trimerization occurs and the receptor interacts with TNFR1-associated death domain (TRADD) via its death domain [37]. Subsequent recruitment of receptor interacting protein 1 (RIP1) and TRAF2 leads to the phosphorylation of the IKK complex and translocation of NF- κ B to the nucleus. TRAF2 and RIP1 can interact with upstream kinases of JNK and p38, respectively, leading to activator protein 1 (AP-1),

cAMP-response element binding protein (CREB) and CCAAT-enhancer-binding protein β (C/EBP β) transcription factor activation. Also, TNFR1 can transmit apoptotic signals through the recruitment of fas-associated death domain protein (FADD) and subsequent caspase-8 cleavage [36]. Similar to IL-1 signalling, the resulting activation in NF- κ B and MAPK family members results in a potent induction of pro-inflammatory cytokines. IL-6 is one such cytokine, being strongly NF- κ B-dependent [38], that is potently upregulated following both TNF α and IL-1 signalling.

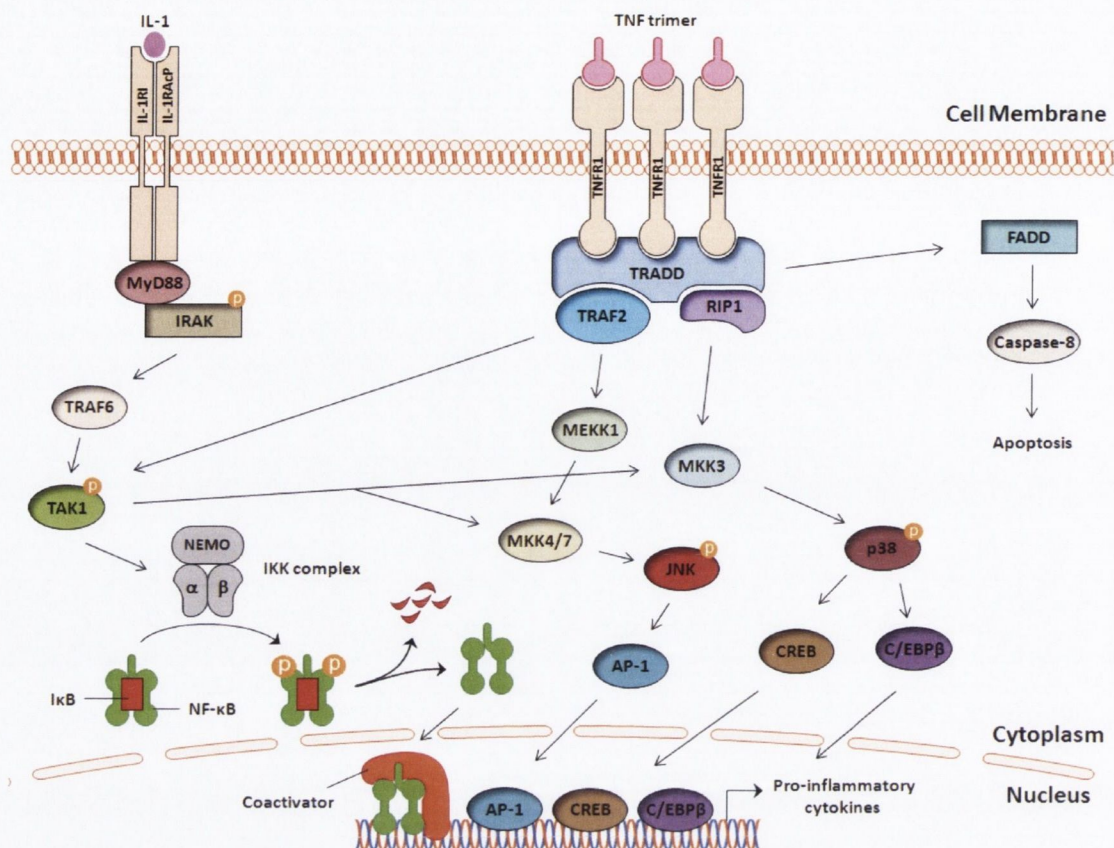


Figure 1.2 IL-1 and TNF signalling

Upon binding of IL-1 to the IL-1 receptor (IL-1R), there is a rapid recruitment of IL-1R accessory protein (IL-1RAcP), which associates with MyD88 via its TIR domain. IRAK family members are next recruited and assemble with TRAF6, which triggers a cascade of events that results in the autophosphorylation of TAK1. TAK1 activates the IKK complex which phosphorylates I κ B, freeing NF- κ B and allowing its translocation to the nucleus where it upregulates numerous pro-inflammatory cytokines. As well as activating the IKK complex, TAK1 is also capable of directing MKK4/7-dependent phosphorylation of JNK, ultimately resulting in activation of the AP-1 transcription factor. TAK1 can also signal via MKK3 to cause p38 phosphorylation, subsequently activating the CREB and C/EBP β transcription factors. All of these transcription factors culminate in the upregulation of numerous pro-inflammatory cytokines. Upon binding of TNF, TNFR1 receptor trimerisation occurs and the IKK complex is activated via a TRADD-TRAF2-TAK1 pathway. The TNF receptor can also recruit RIP1. TRAF2 and RIP1 can interact with upstream kinases of JNK and p38, respectively, leading to AP-1, CREB and C/EBP β transcription factor activation. The TNF receptor can also trigger apoptosis via the recruitment of TRADD and cleavage of caspase-8.

1.3.2.3 IL-6 function and signalling

IL-6 belongs to the IL-6 family of cytokines which includes, IL-11, leukemia inhibitory factor (LIF), oncostatin M (OSM), ciliary neurotrophic factor (CNTF), cardiotropin-1 (CT-1), novel neurotrophin-1 (NNT-1)/B cell-stimulating factor 3 (BSF-3), neuropoietin (NPN), IL-27, and IL-31 [30, 39]. Following infection or trauma, the production of PAMPs or danger associated molecular patterns (DAMPs), respectively, stimulates TLR signalling, which causes the production of IL-6 by monocytes and macrophages [40]. Signalling via cytokines such as TNF α and IL-1 also leads to the production of IL-6. As well as playing a role in immunity, IL-6 is also involved in bone metabolism and the endocrine, nervous and hematopoietic systems [41-43]. IL-6 is involved in the regulation of T_h1 and T_h2 cells, and acts on B lymphocytes to promote immunoglobulin production [44]. Amongst other functions, IL-6 also has the ability to stimulate B and T cell differentiation, activate macrophages and natural killer (NK) cells, and stimulate hepatocytes to produce acute phase proteins [45-48]. IL-6 is critical in the transition from innate to acquired immunity, being required for both the initial infiltration of neutrophils to the site of infection, and the subsequent recruitment of monocytes [49]. As well as exhibiting pro-inflammatory function, IL-6 also has a range of regenerative or anti-inflammatory functions [30]. For instance, IL-6 has been found to play a role in liver and peripheral nerve regeneration [50, 51].

IL-6 signals via the IL-6 receptor and the associated membrane glycoprotein, gp130 (Figure 1.3) [52]. The majority of the IL-6 family require the signal transducer protein gp130 for signalling transduction [39]. Upon IL-6 binding to the membrane-bound α IL-6 receptor (mbIL-6R α), gp130 associates with the complex, creating a gp130-homodimer formation, and initiating signalling. The activated IL-6 receptor complex is formed as a hexameric structure comprising of two molecules each of IL-6, IL-6R α and gp130 [53]. Gp130 is essential for signal transduction, binding the IL-6/IL-6R α complex at numerous sites [52]. Although it lacks kinase activity, gp130 is non-covalently associated with Janus kinases (JAKs - JAK1, JAK2 and tyrosine kinase 2 (TYK2)), which become autophosphorylated upon receptor engagement and phosphorylate gp130. Upon its phosphorylation, gp130 mediates the internalisation of the receptor complex and further signalling events [54]. Phosphorylated tyrosine residues on the JAKs and receptor serve as docking sites for SH2-domain containing

proteins. These include signal transducers and activators of transcription (STAT)3 and SH2-domain containing protein tyrosine phosphatase (SHP)2 [52]. Following its phosphorylation, STAT3 translocates to the nucleus and upregulates IL-6 responsive genes. SH2-domain-containing proteins link the receptor to MAPK, phosphoinositide 3 kinase (PI3K)/thymoma viral proto-oncogene 1 (AKT) and other cellular pathways [52]. These include, the SHP2 and Shc proteins, which can recruit the growth-factor-receptor-bound protein 2 (GRB2) adaptor and stimulate the Ras cascade [55], giving rise to extracellular signal-regulated kinase (ERK) phosphorylation [56]. STAT3-dependent upregulation of suppressor of cytokine signalling (SOCS)3 also indirectly promotes Ras signalling by binding to, and reducing the activity, of RasGAP, a negative regulator of Ras signalling [57]. Also, the recruitment and JAK phosphorylation of insulin receptor substrate (IRS) and PI3K, results in the activation of the PI3K/AKT pathway [58]. AKT can phosphorylate the IKK complex, resulting in NF- κ B translocation to the nucleus [59, 60]. Mammalian target of rapamycin (mTOR) has also been shown to be required for maximal STAT3 activation [61]. Phosphorylated ERK family members can proceed to activate numerous transcription factors (e.g. C/EBP β , AP-1) which, alongside NF- κ B and STAT3, come together to regulate a variety of complex promoters and enhancers that respond to IL-6, resulting in upregulation of numerous transcription factors, acute phase proteins and SOCS proteins (Figure 1.3) [52].

In addition to the mbIL-6R α , a soluble form of the receptor has also been described (sIL-6R α) [62]. In contrast to soluble receptors of IL-1 α and TNF α , which act as antagonists, inhibiting the biological activity of their ligands [63, 64], sIL-6R α amplifies IL-6-mediated signalling [65]. Although gp130 is ubiquitously expressed, mbIL-6R α is only expressed on a few cell types, including macrophages, neutrophils, and some types of T lymphocytes and hepatocytes [62]. Expression of sIL-6R α allows for a much broader range of signalling by IL-6. The sIL-6R α binds to IL-6 with a similar affinity as mbIL-6R α . Activation of cells that only express gp130 via the IL-6/sIL-6R α complex is referred to as trans-signalling, while activation via the IL-6/mbIL-6R α complex is called classic-signalling [65]. Activation of gp130 by trans-signalling has been demonstrated to be key in the trafficking of lymphocytes to sites of inflammation by controlling chemokine expression [66]. Amongst numerous other functions, trans-signalling has also been shown to promote T cell proliferation during

colon cancer development and is involved in regulating adhesion molecule expression on endothelial cells [67, 68]. There are two major mechanisms for the production of sIL-6R α . sIL-6R α can be produced from an alternative spliced IL-6R α messenger RNA (mRNA) which lacks the transmembrane and cytosolic domains, or mbIL-6R α can be proteolytically cleaved by metalloproteases of the a disintegrin and metalloprotease (ADAM) family [65]. ADAM proteins are type I transmembrane proteins which are involved in the shedding of numerous membrane-bound cytokine receptors, including IL-1R α and TNFR1/2 [69]. mbIL-6R α has been described as a substrate for the ADAM family members ADAM10 and ADAM17 [70]. ADAM10 is thought to cause slow constitutive shedding of the receptor, while ADAM17 can cause rapid IL-6R α proteolysis upon appropriate activation. Amongst others, ADAM17 can be activated by pro-inflammatory cytokines such as IL-1 and TNF α , bacterial toxins, or phorbol ester phorbol-12-myristate-13-acetate (PMA), a known activator of protein kinase C (PKC) [71]. IL-6 trans-signalling has been shown to mediate pro-inflammatory responses while classic signalling mediates regenerative anti-inflammatory responses [30]. Using models of inflammation, autoimmune disease, and inflammation-associated cancer *in vivo*, blocking of IL-6 trans-signalling was found to cease their inflammatory progress [65]. A protective effect of IL-6 was reported in IL-6 knockout mice where the regenerative abilities of IL-6 were needed for intestinal wound healing to *Citrobacter rodentium* infection [72]. This activity was concluded to be most likely mbIL-1R α -dependent.

As IL-6 is a highly pleiotropic cytokine, its signalling is highly regulated to prevent overstimulation. Phosphorylation is a key event in IL-6 signal transduction and there are numerous protein tyrosine phosphatases (PTPs) that are capable of dephosphorylating IL-6 signalling components, thus negatively regulating IL-6 transduction [73]. SHP2 was the first phosphatase identified as playing a role in the regulation of IL-6 signaling [74]. Containing two N-terminal SH2 domains and a C-terminal catalytic phosphatase domain, all tyrosine-phosphorylated signalling components are potential substrates of SHP2. Its enzyme activity is inhibited in the absence of tyrosine-phosphorylated binding partners by the covering of its active site by its SH2 domain. Binding of its SH2 domains to phosphotyrosine motifs of receptors or adaptors unfolds the protein, enabling enzyme activity. Several other phosphatases,

including PTP ϵ C, PTP1B, CD45 and SHP1 have also been reported to regulate JAK-STAT signalling [75]. The SOCS proteins are another family of proteins that regulate JAK-STAT signalling [76]. Upregulated following STAT3 translocation to the nucleus, SOCS1 and SOCS3 can bind to tyrosine phosphorylated-JAK and -gp130, respectively, to attenuate the IL-6 signalling cascade in a negative feedback loop [77, 78]. In addition to its signalling being regulated, a tight control of IL-6 mRNA synthesis is also evident based on the number of transcription factors which have been found to bind the IL-6 promoter and initiate IL-6 mRNA production [52]. Furthermore, generally not constitutively expressed in resting nontransformed cells, IL-6 upregulation is dependent on transcription factor activation following stimulation. Several groups have demonstrated that transcription of the IL-6 gene promoter requires, amongst others, binding of NF- κ B [38], C/EBP β (NFIL-6) [79], AP-1, CREB [80], IRF1, specificity protein-1 (Sp-1) [81], friend leukemia integration 1 (FLI1) and Kruppel-like factor 4 (KLF4) transcription factors in response to various stimuli [82, 83].

Similar to IL-6 signalling, type I IFNs signal via JAK-STAT proteins. Critical in innate immunity, type I IFNs are upregulated following phosphorylation and subsequent nuclear translocation of members of the IRF family of transcription factors.

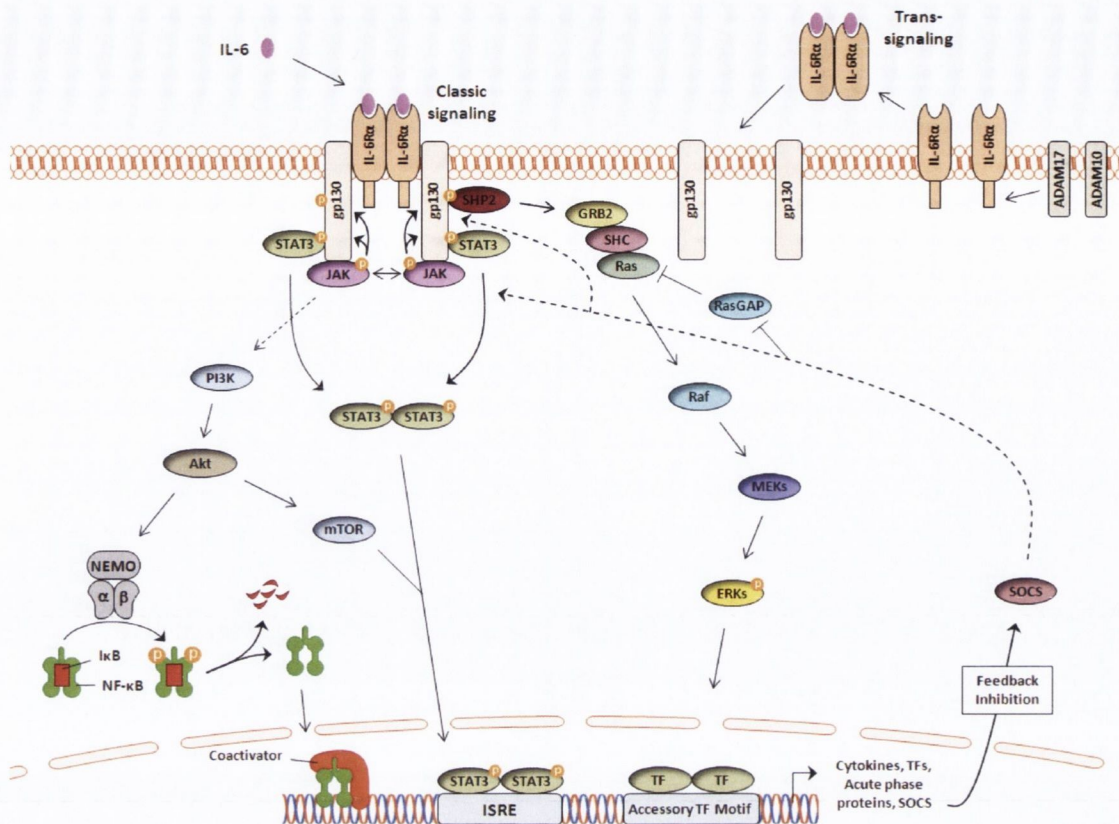


Figure 1.3 IL-6 signalling

After binding of IL-6 to the IL-6 receptor (IL-6R), the IL-6/IL-6R complex associates with gp130, inducing its homodimerisation, and triggering activation of JAKs. JAKs subsequently phosphorylate gp130. Phosphorylated gp130 then recruits STAT3, triggering its phosphorylation and translocation to the nucleus. Phosphorylated gp130 can also associate with SHP2 which can trigger a Ras-Raf-MEK signalling cascade, resulting in ERK MAPK phosphorylation. STAT3-dependent upregulation of SOCS3 also indirectly promotes Ras signalling by binding to, and reducing the activity, of RasGAP, a negative regulator of Ras signalling. JAKs are capable of phosphorylating PI3K, which recruits AKT. AKT is capable of phosphorylating the IKK complex, which then triggers IκB degradation and NF-κB translocation to the nucleus. mTOR has been shown to be required for maximal activation of STAT3. NF-κB, STAT3 and ERK-activated transcription factors, culminate in the nucleus to activate a range of complex promoters, giving rise to the production of numerous cytokines, acute phase proteins and SOCS. SOCS proteins negatively regulate upstream IL-6-induced signalling events. Classic IL-6 signalling refers to the binding of IL-6 to membrane-bound IL-6 receptor, its association with gp130 and initiation of signalling events. IL-6 trans-signalling refers to the binding of soluble IL-6 receptor to IL-6, its movement to membrane-bound gp130 and subsequent signalling events. Soluble IL-6 can be created by the proteolytic cleavage of membrane-bound IL-6R by the metalloproteases ADAM10 and ADAM17.

1.4 IRFs and the IFN signalling arm of innate immunity

1.4.1 IRF activation and signalling

The IRF family consists of nine members, IRF1-9. Each IRF member contains a highly conserved N-terminal DNA binding domain which forms a helix-turn-helix that recognises a DNA consensus sequence known as IFN-sensitive response element (ISRE) within the promoters of genes [84]. With the exception of IRF1 and IRF2, each of the IRFs have a C-terminal IRF association domain that facilitates homo- and heteromeric interactions with other family members or other transcription factors such as PU.1 and STATs. IRFs have diverse roles in the body. As well as playing a critical role in immunity, IRFs have also been found to play a role in the development of numerous immune cells and to take part in control of cell growth, cell survival and oncogenesis [85]. Following PRR stimulation, IRFs can cause upregulation in IFN, pro-inflammatory cytokines and other genes.

The IRF family are best known for their involvement in type I IFN production. Although IRF3 and IRF7 are primarily involved in the production of type I IFN following PRR activation, IRF1, IRF5 and IRF8 have also been found to contribute [86]. IRF3 is constitutively expressed in most cell types. In contrast, IRF7 is expressed at very low levels in most cells, with the exception of plasmacytoid DCs (pDCs), but is strongly induced following type I IFN stimulation. Following upstream signalling events, the TRAF family member-associated NF- κ B activator (TANK)-binding kinase 1 (TBK1)/IKK ϵ complex is known to phosphorylate the C-terminal region of IRFs (Figure 1.1) [85]. This results in their homodimerisation and translocation to the nucleus. Alongside other transcription factors including CREB-binding protein (CBP), p300, NF- κ B and AP-1, they form an enhanceosome that induces the transcription of type I IFNs. The TBK1/IKK ϵ complex consists of the serine threonine kinases TBK1 and IKK ϵ and a number of adaptor molecules; TANK, NF- κ B-activating kinase-associated protein 1 (NAP1), similar to NAP1 TBK1 adaptor (SINTBAD) and DEAD-box protein 3 (DDX3) [87]. *In vivo* studies using TBK1 and IKK ϵ knockout mice suggest that TBK1 has the most predominant role in type I IFN induction with IKK ϵ being redundant in many cases [88, 89]. The functional relationship of the adaptor proteins to TBK1 and IKK ϵ remains poorly defined.

IRF3 is known to be required for effective TLR3, TLR4 and many of the known cytosolic PRR responses, while TLR7 and TLR9 signalling in pDCs are more IRF7- and IRF5-dependent [85]. Both IRF7 and IRF5 have been reported to directly interact with MyD88 and TRAF6 and to have a role in effective TLR9 signalling [90, 91]. While IRF7 was found to be required for type I IFN production, IRF5 was needed for pro-inflammatory cytokine induction. Additionally, IRF4 has also been reported to interact with MyD88. It acts as a negative regulator of TLR signalling by competing with and inhibiting the sustained binding of IRF5 to MyD88 [92]. IRF8 has also been attributed a role in the TLR9-MyD88 signalling cascade. The use of IRF8^{-/-} cells showed an impairment in NF- κ B and MAPK activation in response to TLR9 stimulation [93]. Although not capable of binding MyD88, IRF8 was found to directly interact with TRAF6. Finally, forming part of the ISG factor 3 (ISGF3) complex required for IFN signalling, IRF9 has been shown to be required for induction of IRF7 expression following IFN signalling [86].

1.4.2 The Interferon system

The induction of IFN upon PRR activation, and its subsequent signalling via IFN receptors leading to the expression of a vast array of IFN-stimulated genes (ISGs), is crucial in an effective immune response. IFNs were first described in the 1950's as secreted soluble factors which were capable of interfering with viral infection [94]. Host defence against viral invasion involves the initiation of antiviral innate immune responses which ultimately culminate in the induction of expression of the type I IFNs and numerous pro-inflammatory cytokines [95]. The central importance of type I IFNs in antiviral responses has been well characterised and validated *in vivo*, with mice deficient in type I IFN receptor [96], or humans with genetic defects in IFN signalling components [97, 98], having increased susceptibility to viral infection. In addition to influencing innate immune responses, type I IFNs are capable of directing the adaptive immune response by priming cytotoxic T cells and T_h cells, thereby resulting in the induction of antigen-specific responses. Roles in modulating apoptosis, the cell cycle, and numerous other physiological processes have also been reported [99]. Based on their receptor usage, IFNs have been classified into three classes, type I, II and III [100]. The type I IFN family consists of 16 members; 12 IFN α subtypes, IFN β , IFN ϵ , IFN κ and IFN ω , the type II family has only one member; IFN γ , and the type III

family is the IFN λ family which includes IFN λ 1, IFN λ 2, IFN λ 3 and IFN λ 4. Each class of IFNs signal via different receptors, each of which is composed of two subunits. Type I IFNs signal via the IFN α/β receptor (IFNAR – composed of IFNAR1 and IFNAR2 subunits), type II IFNs signal via the IFN γ receptor (IFNGR – composed of IFNGR1 and IFNGR2 subunits) and type III IFNs signal via the IFN λ receptor (IFNLR – composed of IFNLR1 and IL-10R β subunits).

With IFNAR being broadly expressed, almost all cell types can respond to type I IFN. Although the signalling pathways to type I IFN induction vary between cell type and stimulus, they all ultimately lead to the activation of IRF3 or IRF7, their translocation to the nucleus and transcription of IFN α and IFN β [101]. The expression of IFN γ is more restricted, with IFN γ being primarily expressed by activated T cells and NK cells. IFN γ signalling is essential for the activation of macrophages to effectively respond to intracellular microorganisms, and it also promotes the development of CD4⁺ T cells and cytotoxic CD8⁺ T cells [102]. Although structurally very different from type I and type II IFNs, the most recently classified type III IFNs are also antiviral and capable of inducing the production of ISGs [103]. The IFNLR receptor is primarily expressed on epithelial cells and hence only type I and type II IFN signalling will be discussed further here.

Following secretion, IFNs bind to their cognate receptor and trigger a signalling cascade which ultimately results in the induction of hundreds of ISGs, a number of which enhance the resistance of cells towards potential viral infection. IFN signalling mainly occurs via JAK-STAT signalling (Figure 1.4). Each of the receptor subunits interacts with a member of the JAK family. For type I IFN receptor signalling, IFNAR1 associates with TYK2 and IFNAR2 with JAK1. For type II IFN (IFN γ) receptor signalling, IFNGR1 and IFNGR2 interact with JAK1 and JAK2, respectively [104]. Upon IFN binding to the corresponding receptor, the receptor dimerises, triggering autophosphorylation and activation of the JAKs. These JAKs subsequently phosphorylate STAT proteins which proceed to form hetero- or homodimers that translocate to the nucleus and bind to either ISRE or IFN γ -activated sites (GAS) within the promoters of ISGs, initiating their transcription [104]. The main transcription factor complex formed upon type I IFN receptor activation, ISGF3, consists of STAT1,

STAT2 and IRF9, and is known to specifically bind to ISRE elements. All other STAT homo- and heterodimers interact with GAS elements. The IFNGR mainly activates STAT1 homodimers which target GAS elements [104]. Although initially thought to only signal via STAT1 homodimers, further studies have reported that IFN γ may also recruit the ISGF3 complex [105].

As well as JAK-STAT signalling, other signalling cascades have been shown to be important in an effective IFN response, including MAPK and PI3K cascades [104]. Independent of STAT activation or the assembly of the ISGF3 complex, the p38 MAPK cascade was found to regulate IFNAR-mediated ISG expression. The IFNAR activates p38 via the MAPK kinases (MKK)3/6 [106]. The role of p38 in IFNGR signalling is more controversial but the receptor was reported to interact with MyD88 to mediate *TNF α* and *IP10* mRNA stability via a MyD88-mixed lineage kinase 3 (MLK3)-p38 'signalosome' [107]. There is also evidence to support a role for ERK MAPKs in effective signalling following both type I and type II IFN receptor activation. ERK2 has been demonstrated to be required for effective ISG expression following type I IFN stimulation [108], while ERK1 and ERK2 have been demonstrated to play a role in the activation of both C/EBP β - and AP-1-dependent gene transcription in response to IFN γ [109]. Although less extensively studied, there also appears to be a role for JNK MAPKs in effective IFN signalling, as both type I and type II IFN stimulation of cells were found to cause an increase in the phosphorylation of JNK [110, 111]. Type I IFNs have been shown to activate JNK for the induction of apoptosis in lymphoma cells [112], while the activation of JNK after type II IFN stimulation was reported to upregulate numerous genes required for antigen presentation such as MHC class II transactivator (CIITA), and genes encoding MHC class II molecules [111]. PI3K has also been shown to have an important role in STAT-mediated responses. Upon IFN treatment, PI3K phosphorylates PKC- δ , which in turn phosphorylates STAT1, enhancing its transcriptional activity [113]. PI3K activation is also known to activate AKT, which regulates apoptosis [114]. PI3K-AKT activation following IFN stimulation also regulates the activation of mTOR, which mediates the initiation of translation of numerous ISGs [104]. Furthermore, NF- κ B activation has also been reported in response to type I IFNs via both a PI3K-AKT-dependent pathway leading to classical

p50/p65 activation, and a TRAF2/NIK-dependent pathway leading to alternative p52/p65 activation [115].

Effective IFN signalling and ISG induction is vital in the effective clearance of viral infection. Numerous ISGs can directly counteract viral infection [116]. For example, translation of viral mRNA and viral replication can be antagonised by the upregulation of dsRNA-activated serine/threonine protein kinase R (PKR) following IFN signalling [117], and viral RNA can be degraded by the increase in expression of 2'5'-oligoadenylate synthetase (OAS) and ribonuclease (RNase) L [118]. Additionally, IFNs induce the production of hundreds of other ISGs that have roles in combating viral infection and limiting its spread [119]. The IFN-induced protein with tetratricopeptide repeats (IFIT) family are another example of ISGs which are involved in numerous processes in the response to viral infection. Humans have four IFIT family members; IFIT1 (also known as ISG56), IFIT2 (ISG54), IFIT3 (ISG60) and IFIT5 (ISG58) [120]. All members of the IFIT family of ISGs have been shown to be capable of antiviral activity. For example, ISG56 and ISG54 are capable of suppressing viral translation during protein synthesis through their direct association with the translational factor eIF3 [121]. Interestingly, further evidence of the importance of the IFIT family members in antiviral immunity came with the discovery that ISG56 can directly recognise RNA that carries a triphosphate group on its 5' terminus (5'ppp RNA), a pattern that discriminates pathogenic from self RNA [122]. ISG56 was found to recognise 5'ppp RNA, form a complex with ISG54 and ISG60, and exert antiviral activity most likely by sequestering viral RNA. Although it was not found to interact with the other IFIT family members, ISG58 was also found to associate with 5'ppp RNA [122]. As well as pro-inflammatory cytokine induction, activation of numerous PRRs can lead to IFN induction, for example, TLRs.

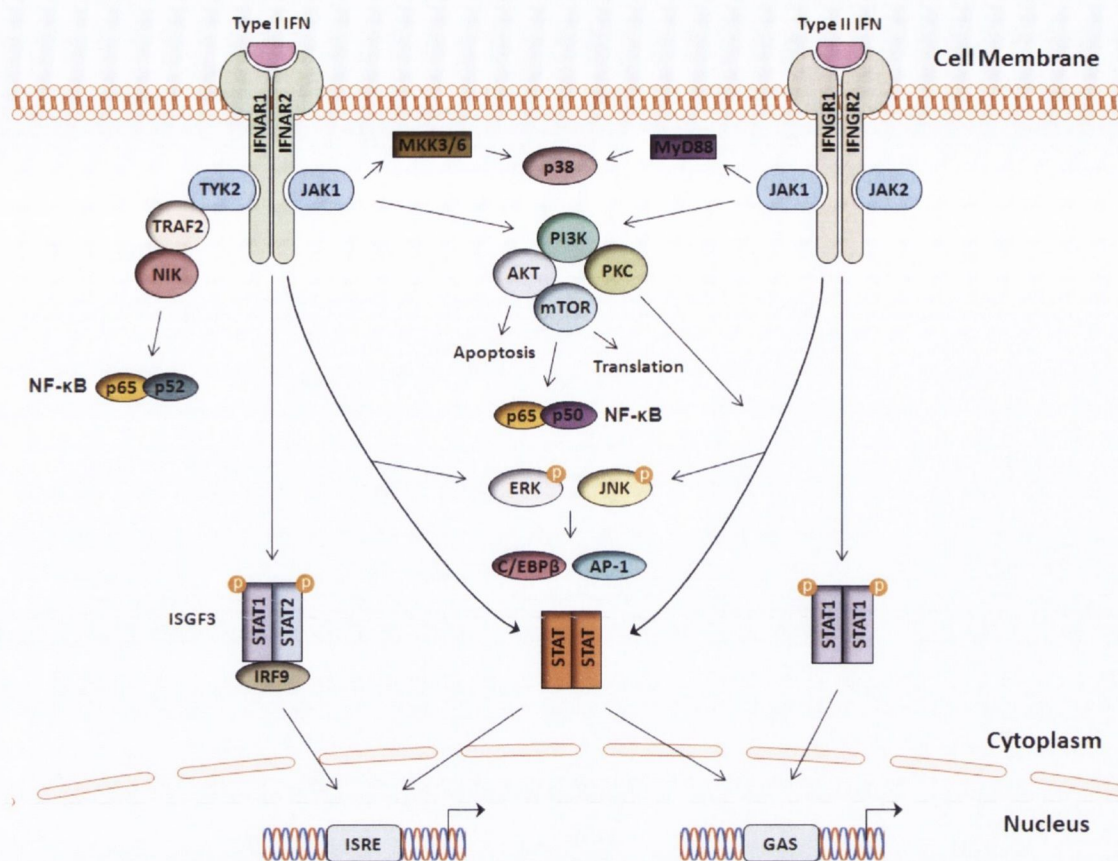


Figure 1.4 Type I and type II IFN signalling

Type I IFNs signal through the IFNAR receptor, consisting of IFNAR1 and IFNAR2 chains, which are associated with TYK and JAK1, respectively. Upon binding of type I IFN, IFNAR initiates receptor dimerisation and activation of its associated JAKs, leading to tyrosine phosphorylation of STAT1 and STAT2. STAT1 and STAT2 form a complex with IRF9 (ISGF3 complex), which then translocates to the nucleus and binds to ISRE promoter elements to initiate ISG transcription. Type II IFNs signal via the IFNGR receptor, consisting of IFNGR1 and IFNGR2 chains, which are associated with JAK1 and JAK2, respectively. Type II IFN signalling primarily results in the formation of STAT1 homodimers, and their induction of genes containing GAS elements. Both IFN pathways can also give rise to other STAT homo- and heterodimers that mostly bind GAS elements. Type I IFN signalling can also result in p38 activation via association of the receptor with MKK3/6. Following type II IFN binding, the IFNGR receptor can interact with MyD88 to mediate *IP10* and *TNFα* mRNA stabilization via a MyD88-MLK3-p38-dependent pathway. ERK and JNK MAPKs have been shown to play a role in effective ISG induction following both type I and type II IFN stimulation. Activation of both receptors can result in signalling via PI3K/AKT. This pathway results in PKC-δ-mediated enhancement of transcriptional activity of STATs, mTOR-mediated induction of translation, and AKT-mediated induction of apoptosis. Type I IFN signalling has also been reported to activate classical p50/p65 NF-κB activation via a PI3K-AKT-dependent pathway, or alternative p52/p65 NF-κB activation via a TRAF2-NIK-dependent pathway.

1.5 Toll-like receptors (TLRs)

1.5.1 TLRs and their ligands

TLRs are a major family of transmembrane PRRs, crucial in the innate response to infection. Humans encode 10 functional TLR members, each of them binding a distinct family of microbial molecules [123]. TLRs are composed of three main domains; an N-terminal leucine rich repeats (LRRs) ectodomain required for the recognition and binding of specific ligands, a single transmembrane helix and a C-terminal cytoplasmic TIR domain key to the recruitment of TIR domain-containing adaptor proteins via homotypic interaction. After ligand binding, these receptors dimerise, causing a change in the conformation of their intracellular TIR domains which results in the emergence of binding sites for TIR-domain-containing adaptor proteins, providing a scaffold for the recruitment of TIR adaptors and subsequent signalling events (Figure 1.5) [124]. TLRs can be divided into two groups based on their localisation in the cell. The TLRs found on the cell membrane (TLR 1, 2, 4, 5, and 6) recognise extracellular microbial components such as lipids and proteins, while the second group all reside intracellularly, expressed in endocytic compartments where they primarily recognise nucleic acids (TLR 3, 7, 8 and 9). The differential localisation of the TLRs is thought to be important for their accessibility to their specific ligands, with the cell membrane-bound TLRs recognising microbial surface components and the endocytic TLRs recognising nucleic acid derived from microorganisms following their endosomal degradation [125].

The founding member of the TLR family is TLR4 [126]. TLR4 recognises lipopolysaccharide (LPS), a crucial glycolipid component of the outer membrane of gram-negative bacteria. Upon activation, a homodimer of TLR4 forms a complex with myeloid differentiation factor 2 (MD-2), CD14 and LPS-binding protein (LBP) [127]. TLR4 has also been described to recognise the F protein of respiratory syncytial virus (RSV) and the envelope protein of mouse mammary tumor virus (MMTV) [128, 129]. TLR2 forms a heterodimer with either TLR1 or TLR6 to recognise a range of bacterial, viral and fungal PAMPs [130]. In a similar manner to TLR4 signalling, co-receptors participate in ligand recognition and effective signalling (CD36 and CD14). The heterodimer of TLR1 and TLR2 is known to recognise bacterial triacylated lipopeptides

produced by *Escherichia coli* and other gram negative bacteria. On the other hand, the TLR2/TLR6 heterodimer recognises diacylated lipopeptides from gram-positive bacteria, and a mixture of fungal cell membrane components [130]. Interestingly, TLR2 has been shown to be an important receptor in the recognition of numerous viruses including hepatitis C virus (HCV) and herpes simplex virus (HSV) [130]. TLR2 has also been implicated in the innate immune response to vaccinia virus (VACV). As well as having been reported to be involved in the activation of NK cells and control of VACV infection *in vivo* [131], TLR2 has also been proposed to cause non-nucleic acid-driven type I IFN induction in response to VACV in inflammatory monocytes [132]. TLR5, which is highly expressed in lamina propria DCs in the intestine, is capable of recognising bacterial flagellin, a major protein constituent of bacteria flagella which are employed by a range of microbial pathogens for their mobility [133].

The nucleic acid sensing TLRs (TLR 3, 7, 8 and 9) are restricted to endosomal membranes, where they mainly encounter ligands derived from phagocytosed pathogens or infected apoptotic cells [134]. TLR3 recognises viral genomic dsRNA [135]. Beyond detecting dsRNA from viruses which have a dsRNA genome (e.g. reoviruses), TLR3 can also detect dsRNA replication intermediates of single-stranded (ss)RNA viruses (e.g. RSV and West Nile virus (WNV)) and some DNA viruses (e.g. HSV and mouse cytomegalovirus (MCMV)) [136-139]. TLR3 is also capable of detecting synthetic dsRNA (polyinosine-polycytidylic acid (poly(I:C))) [140]. The co-receptor CD14 has also been shown to be required for effective TLR3 signalling. Interestingly, TLR3 is the only TLR which has been linked to a human disease to date. Patients with missense mutations in *TLR3*, or mutations in TLR3 signalling components [138, 141-143], all present with recurrent herpes encephalitis, an immunodeficiency disease which mainly occurs in early childhood during primary infection with HSV-1 [144]. The functional defect is most likely due to an abrogation in type I IFN production. However, why patients with this immunodeficiency are only susceptible to HSV remains to be elucidated [145]. Signalling from endosomes, the closely related TLR7 and TLR8 recognise ssRNA [146]. TLR7 has also been found to respond to synthetic short interfering RNA (siRNA), guanine analogues and imidazoquinoline derivatives, such as resiquimod and imiquimod. ssRNAs from vesicular stomatitis virus (VSV) and influenza viruses are the natural ligands for TLR7 [147]. Guanosine and uridine rich ssRNA from

human immunodeficiency virus (HIV)-1 is recognised by both TLR7 and TLR8 [148]. Synthetic TLR7/8 ligands include R848, a synthetic imidazoquinoline, which stimulates TLR8 signalling, and CL075, a thiazoloquinolone derivative which activates TLR8, and to a lesser extent, TLR7. There is data suggesting an involvement of TLR8 in the detection of VACV. TLR8 has been reported to be activated in murine pDCs by VACV DNA [149]. This finding was controversial as DNA has not been previously linked to TLR8 activation and murine pDCs are generally known to express TLR7 and TLR9, but little TLR8 [150]. TLR9 is a bona fide sensor of DNA [151], specifically binding unmethylated cytosine-phosphate-guanosine (CpG)-containing DNA motifs that are abundant in bacterial and viral DNA, but rare in the mammalian genome. Amongst others, TLR9 is capable of detecting MCMV, HSV-1/2 and adenovirus [139, 152, 153]. TLR9 has also been reported to sense hemozoin, a residual product of heme-detoxification by malaria infection [154]. However, more recent studies suggest no involvement of hemozoin in TLR9 activation, with malarial hemozoin-DNA complexes being the exclusive trigger of innate immunity [155]. These conflicting studies may be due to one having been carried out *in vivo* [154], and the other *in vitro* [155]. Therefore, further work will need to be carried out in order to clarify if hemozoin is involved in TLR9-mediated responses. Among the known 10 human TLRs, TLR10 is the only member whose agonist or function has yet to be fully characterised [156]. One study has suggested that TLR10 cooperates with TLR2 in the detection of triacylated lipopeptides, recruiting MyD88 to the activated complex [157]. The activated pathway remains unknown and may differ from the typical TLR2-induced signalling cascade. In another study, a role for TLR10 as an innate immune sensor of viral infection was suggested [158]. TLR10 expression was shown to increase upon influenza infection of peripheral blood monocyte-derived macrophages, with knockdown studies showing TLR10 to be required for effective pro-inflammatory cytokine and IFN production.

1.5.2 TLR signalling

Upon ligand binding and receptor dimerisation, the TIR domains of TLRs recruit TIR-domain containing adaptor proteins. There are four of these adaptors which initiate further signal transduction; MyD88, MyD88 adaptor-like protein (Mal), TIR domain-containing adapter protein inducing IFN β (TRIF) and TRIF-related adaptor molecule (TRAM) [159]. Upon activation, these adaptors initiate signalling cascades

which culminate in the activation of NF- κ B, MAPKs and IRFs (Figure 1.5). The fifth TIR-domain containing adaptor, sterile α and armadillo motif-containing protein (SARM), plays more of a negative regulatory role in TLR signalling [160].

TLR signalling can be roughly divided into two major pathways; the MyD88- and the TRIF-dependent (or MyD88-independent) pathways [161]. All of the TLRs, with the exception of TLR3, signal via MyD88. In the case of TLR2 and TLR4, the Mal protein acts as a bridging adapter to recruit MyD88 to the activated receptor. Upon association with the TLR complex, MyD88 is able to interact with IRAK family members via its death domain. IRAK4 is recruited, followed by IRAK1 and IRAK2. IRAK4 most likely activates IRAK1 and IRAK2 by phosphorylation [33]. Both IRAKs then dissociate from the TLR/MyD88 complex to interact with TRAF6, with IRAK2 promoting TRAF6 polyubiquitination. TRAF6, in conjunction with the E2 ubiquitin-conjugating enzymes Ubc13 and Uev1A, synthesises K63-polyubiquitin, which then recruits TAB2/3, leading to autophosphorylation and activation of TAK1 [162]. NEMO (also known as IKK γ) associates with TRAF6 ubiquitin chains via its ubiquitin-binding domain. NEMO forms a complex with IKK α and IKK β (known as the IKK complex), which are the catalytic kinases required for phosphorylating I κ B [163]. I κ B is a protein which binds to and sequesters NF- κ B in the cytoplasm. Upon phosphorylation, I κ B is ubiquitinated and targeted to the proteasome for degradation, freeing NF- κ B and allowing its translocation to the nucleus where it can upregulate a vast array of NF- κ B-dependent genes. In addition to being essential for the translocation of NF- κ B, TAK1 has also been reported to be capable of MAPK phosphorylation, activating the MAPK kinases MKK3/6 and MKK4/7, which subsequently phosphorylate p38 and JNK, respectively [164]. On the other hand, the IKK complex is involved in signalling via the ERK1/2 MAPKs, phosphorylating p105 which is a negative regulator of tumor progression locus 2 (Tpl2) [165]. Upon release from p105, Tpl2 can activate MKK1/2, the upstream kinases of ERK1/2. Activation of these MAPKs culminates in the phosphorylation of transcription factors such as CREB and AP-1. Activation of these transcription factors alongside NF- κ B, triggers the expression of numerous inflammatory cytokines and chemokines. The endosomally localised MyD88-dependent TLRs, TLR7, TLR8 and TLR9, differ slightly in that in addition to being capable of MyD88-dependent NF- κ B and MAPK activation as described, they can also trigger the

expression of type I IFNs, mainly IFN α [134]. In this case, MyD88 forms a complex with IRAK4, TRAF3, TRAF6, IRAK1 and IKK α , with the latter two kinases phosphorylating IRF7, leading to its translocation to the nucleus [90, 166, 167]. Similar to signalling from IRF7 [90], IRF5 also associates with MyD88 but is more specifically involved in the induction of IL-6 or IL-12 following any TLR activation [91]. IRF5 dimerisation, translocation to the nucleus and induction of inflammatory cytokines, has been recently shown to be dependent on the phosphorylation by IKK β [168, 169]. Other IRF family members also play a role in MyD88-dependent signalling. IRF4 has been found to compete with IRF5 for the binding to MyD88, thereby limiting the production of pro-inflammatory cytokines [92]. IRF1, also reported to interact with MyD88, mediates TLR9-dependent type I IFN production in myeloid dendritic cells (mDCs) [170]. As well as signalling from the plasma membrane, Mal has recently been shown to also take part in endosomal TLR signalling events. Although its mechanism of action has yet to be fully elucidated, recent studies indicate Mal is required for IFN responses induced by TLR7 or TLR9 [171].

TLR3 and TLR4 both utilise TRIF to signal via MyD88-independent pathways. The TRIF pathway differs from the MyD88 pathway in the signalling molecules used to activate NF- κ B and MAPKs [172]. Also, the TRIF pathway uses an additional pathway to upregulate IFN β through the activation of the transcription factor IRF3. TLR4 has the most complex signalling arrangement of the TLR family. Localised at both the plasma membrane and endosomes, TLR4 can signal via a Mal-MyD88 pathway to induce NF- κ B signalling, or it can signal via a TRAM-TRIF pathway to induce IRF3 signalling. Unlike TLR3, TLR4 requires TRAM as a bridging adaptor to TRIF. TRAM activation is regulated by myristoylation and phosphorylation by PKC ϵ [173, 174]. CD14-mediated internalisation of TLR4 and the bridging of TRAM to TRIF are required for effective signalling. Unlike TLR3 which is already localised at endosomes, TLR4 translocates to endosomes following ligand engagement [127]. After TLR3 or TLR4 activation, TRIF binds to TRAF3 and TRAF6 via its N-terminal TRAF-binding motif, with TRIF-TRAF3 activating the TBK1/IKK ϵ complex (non-canonical IKKs) [175], resulting in IRF3 activation and subsequent type I IFN induction, or TRIF-TRAF6 activating TAK1 and subsequently NF- κ B and MAPKs in a similar way as described for MyD88-dependent signalling [162]. Alternatively, TRIF recruits the adaptor RIP1

through a RIP homotypic interaction motif, forming a multi-protein signalling complex along with TRAF6, TRADD, RIP3 and the E3 ubiquitin ligase Pellino-1 [134]. This complex phosphorylates TAK1, which in turn activates the NF- κ B and MAPK pathways [176]. SARM has been described to negatively regulate TRIF signalling in humans [160]. However, ongoing work has shown that the role of SARM may be more complicated than previously envisaged. Although demonstrated as a negative regulator of TRIF-dependent signalling in human cell lines [160], a similar role for SARM was not found in mice. Data from SARM-deficient mice suggested a more positive role for SARM in antiviral immunity, with the knockout mice showing impaired TNF α production in the central nervous system following WNV infection [177]. Recently, SARM was reported to have a critical role in the production of RANTES in macrophages [178]. It was found to specifically promote the recruitment of transcription factors and RNA Polymerase II (Pol II) to the *RANTES* promoter. A general concept is that TLR-triggered production of type I IFN is initiated from endosomal compartments [134]. Although, a membrane-bound TLR which has been classically considered as only mediating pro-inflammatory responses, TLR2 has been recently reported to also traffick to endosomes and trigger type I IFN production [179]. This was demonstrated to involve a newly described, TLR2-TRAM-MyD88-IRF7 pathway. In a similar way to how the movement of TLR4 from the plasma membrane to interact with TRIF at endosomes is TRAM-dependent [127], TLR2-stimulated relocalisation of MyD88 to endosomes was TRAM-dependent [179]. However, unlike TLR4 signalling, relocalisation of TLR2 resulted in IRF7, rather than IRF3 signalling. Whether TRAF3 is involved in the TLR2-TRAM-IRF pathway, and what the downstream kinases for IRF7 phosphorylation are, have yet to be determined. Interestingly, this pathway may provide an explanation as to how TLR2 was previously shown to unexpectedly induce type I IFN in a subset of monocytes following VACV infection [132].

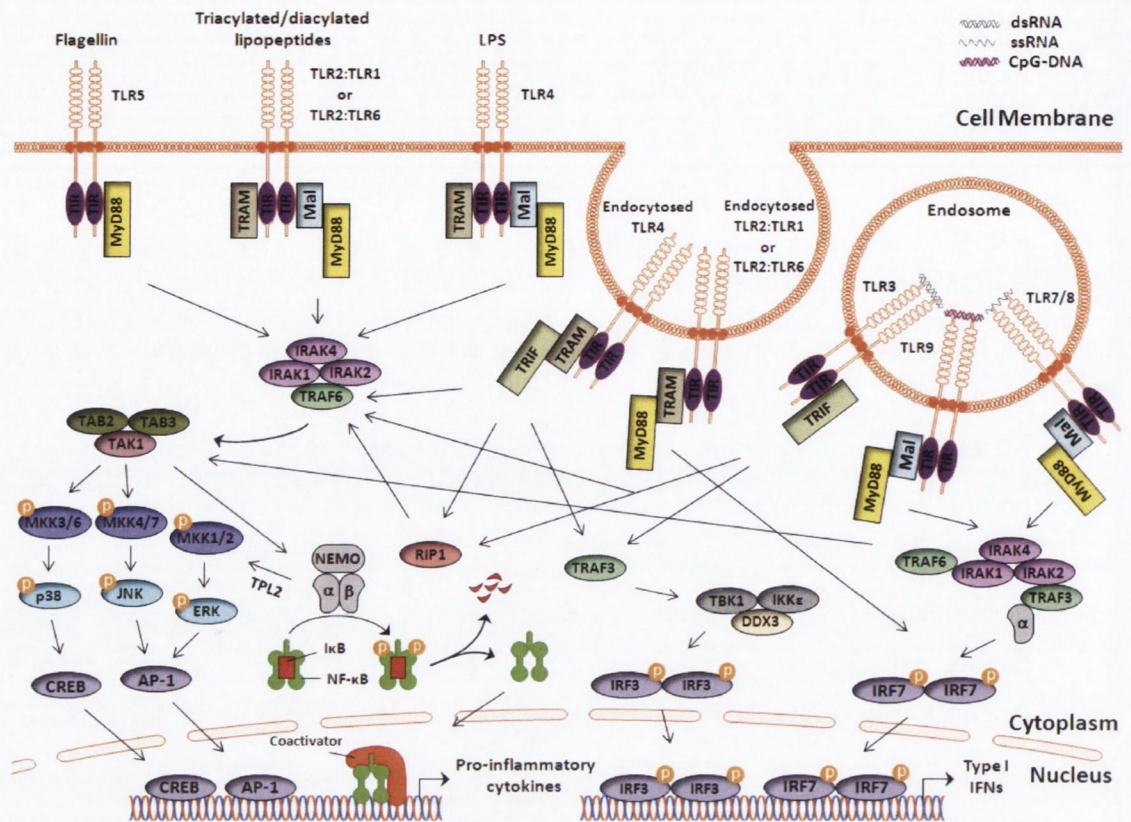


Figure 1.5 Overview of TLR signalling pathways

TLR1, 2, 4, 5 and 6 are all expressed on the cell surface while TLR3, 7, 8 and 9 all localise to endosomal compartments. All of the TLRs, except TLR3, activate MyD88-dependent signalling to induce NF- κ B and MAPK activation. TLR2 and TLR4 require Mal as a bridging adaptor for interaction with MyD88 to signal. Upon activation, TLR4 can traffic to endosomes where the bridging adaptor TRAM facilitates interaction with TRIF. TLR2 can also traffic to endosomes following ligand binding, signalling via TRAM and MyD88. TLR3 directly recruits TRIF upon ligand binding. After MyD88 recruitment, TLRs associate with IRAK family members which subsequently activate TRAF6. TAB2/3, in a complex with TAK1, then gets recruited most likely via free ubiquitin chains synthesised by TRAF6. This complex proceeds to phosphorylate upstream MAPK kinases and the IKK complex to give rise to MAPK and NF- κ B activation, respectively. TLR3- and endocytosed TLR4-bound TRIF can associate with TRAF6 to give rise to TAK1 activation and subsequent NF- κ B activation. Alternatively, they can activate TAK1 via ubiquitinated RIP1. Only endosomal TLRs are capable of type I IFN induction. Endosomal TLR3 and TLR4 can initiate IRF pathways via recruitment of TRAF3, which activates the non-canonical kinases TBK1 and IKK ϵ . These kinases phosphorylate IRF3 and IRF7, leading to their nuclear translocation and upregulation of type I IFN. For MyD88-dependent TLR7, 8 and 9, an association with IRAK family members and TRAF3 results in the phosphorylation of IRF7 by IKK α , and induction of IFN α . Endocytosed TLR2, whose signalling pathway has yet to be elucidated, can also cause IRF7 phosphorylation and subsequent IFN α production. As well as serving as a bridging adaptor for TLR2 and TLR4, Mal has also been found to be required for effective TLR7, 8 and 9 signalling.

1.6 Cytosolic RNA sensing

1.6.1 RIG-I-like receptors (RLRs) – Structure and functions

The RLRs form a family of cytosolic receptors which are critical in the induction of pro-inflammatory cytokines and type I IFN in response to viral and synthetic RNA in the cytosol (Figure 1.6) [180]. Unlike the nucleic acid sensing endocytic TLRs, which only recognise nucleic acid after it reaches endosomes following endocytosis [134], RLRs are cytosolic receptors which are capable of recognising RNA from actively replicating viruses [181]. Also, while TLR expression is often cell specific [182], RLR signalling is found to occur in most cell types due to its ubiquitous expression [183]. The RLR family consists of three highly related proteins; RIG-I, melanoma differentiation-associated gene 5 (MDA5) and laboratory of genetics and physiology 2 (LGP2) [184]. They each belong to the aspartate-glutamate-any amino acid-aspartate/histidine (DEXD/H) family, which are characterised by a central ATPase containing DEXD/H box helicase domain [180].

While all three members contain a C-terminal RNA-binding domain, only RIG-I and MDA5 contain N-terminal tandem caspase activation and recruitment domains (CARDs) [185]. Both RIG-I and LGP2 have been shown to have a repressor domain (RD) which is important in regulating their activation [186]. In non-infected cells, RIG-I is said to be in an inactive conformation, in which the RD masks the CARDs, thus preventing its interaction with other CARD-containing proteins. In contrast, MDA5, does not possess a RD and was not shown to have auto-inhibitory function [186]. Ligand binding to the C-terminal domain (CTD) causes the RLRs to undergo a helicase-dependent conformational change, exposing their CARDs and facilitating the homotypic CARD engagement with mitochondrial antiviral signalling protein (MAVS) [187]. MAVS is known to form aggregates upon interaction with RLRs [188], causing the formation of oligomeric platforms on mitochondrial and peroxisomal membranes, and the recruitment of both the canonical and non-canonical IKKs, resulting in NF- κ B and IRF3 activation, respectively (Figure 1.6).

1.6.2 RLR signalling

The IRF arm of RLR signalling is mediated by an interaction between MAVS and TRADD [189]. TRADD activates TRAF3, which in turn activates the TBK1/IKK ϵ complex which is associated with TANK, NAP1, SINTBAD and DDX3 [183]. This complex phosphorylates IRF3 and IRF7, triggering their translocation to the nucleus and expression of type I IFNs. TBK1 and IKK ϵ most likely form different forms of complexes with NAP1, TANK and SINTBAD [183]. The discovery of novel IKK ϵ splice variants showed that the splice variants vary in their ability to interact with TANK, NAP1, and SINTBAD, possibly allowing different signalling functions of IKK ϵ within the RLR signalling cascade [190]. TRADD is also involved in the activation of NF- κ B [183]. TRADD associates with TRAF family members, which recruits FADD and RIP1, leading to the activation of the canonical IKK complex and nuclear translocation of NF- κ B following the phosphorylation of its inhibitor I κ B. Activation of the canonical IKK complex has also been described following association of TRAF family members with a CARD9/B cell lymphoma/leukemia 10 (Bcl-10) complex [191], or association of FADD with caspase-8/10 [192]. Signalling through the MAVS-associated TRADD-TRAF-FADD-RIP1 complex results in the recruitment of TANK and NEMO to the MAVS signalosome [193, 194]. These facilitate IRF3 and IRF7 activation by TBK1 or IKK ϵ , and IKK α -IKK β -dependent activation of NF- κ B, respectively. A role for stimulator of interferon genes (STING), which is expressed mainly in the endoplasmic reticulum (ER) membrane [195], has also been suggested in RLR signalling. Although dispensable in MDA5-mediated responses, STING was shown to interact with both RIG-I and MAVS and to be important in effective RIG-I-mediated responses [195]. RIG-I, but not MDA5, has also been reported to be capable of inflammasome formation [196]. Capable of binding both apoptosis-associated speck-like protein containing a CARD (ASC) and caspase-1, RIG-I demonstrated the ability to trigger maturation of IL-1 β and IL-18 after RNA virus infection, independently of MAVS, CARD9 and NLRP3 [196]. This hence provides another means by which RIG-I can induce pro-inflammatory cytokine production.

Lacking CARDs associated with and recruiting and interacting with downstream signalling components, LGP2 has been suggested to have a regulatory role in RIG-I and MDA5 signalling [197]. LGP2 was initially thought to act as a negative regulator of

RLRs as overexpression of LGP2 was found to inhibit RIG-I signalling [198]. Coinciding with this, LGP2 knockout mice were reported to have enhanced IFN production following VSV infection [199]. However, subsequent studies have made the function of LGP2 quite controversial. Further work using LGP2-knockout mice, found LGP2 to enhance RIG-I- and MDA5-dependent IFN induction in response to picornaviridae infection [200]. Endogenous LGP2 was also shown to downregulate IFN production during infection by seasonal influenza A viruses that activate IRF3 and IFN transcription [201]. The controversial role of LGP2 in RLR signalling suggests that LGP2 may act as either an activator or inhibitor of RIG-I and MDA5, possibly depending on the concentration of LGP2 in the cell and the nature of the RNA ligand [202].

1.6.3 RLR ligands

Both MDA5 and RIG-I bind cytosolic dsRNA, but with a different dependency on length. While MDA5 recognises long dsRNA (>1000 bp) with no specificity, RIG-I preferentially recognises small “uncapped” dsRNA fragments (<300 bp) that have blunt ends and a 5'triphosphate (5'ppp) moiety [203, 204]. This size preference may explain the specificity in the viruses they both detect. While MDA5 senses picornaviruses such as encephalomyocarditis virus (EMCV) and Mengo virus [205, 206], RIG-I detects members of the paramyxoviridae family (e.g. Sendai virus (SeV) and RSV) [207, 208], rhabdoviridae (e.g. VSV) [209], orthomyxoviridae (influenza) and many others [207]. The 5'ppp moiety detected by RIG-I allows for non-self recognition as host transcripts are usually “capped” with a 7-methyl-guanosine group at the 5' end [204]. During infection, RIG-I ligands can also be generated by Pol III, which is capable of transcribing AT-rich dsDNA into 5'ppp dsRNA for RIG-I recognition [210]. Furthermore, the endoribonuclease, RNase L, is capable of cleaving microbial RNA to trigger MDA5 and RIG-I activation [211]. Crystal structure studies have demonstrated that although RIG-I and MDA5 have a similar domain structure, they bind dsRNA in a different manner [212, 213]. RIG-I is now known to undergo significant conformational change upon ligand binding. The CTD of RIG-I tightly binds to the 5'ppp and its helicase domain wraps around the dsRNA. The binding by RIG-I is sequence-independent as RIG-I primarily recognises the RNA through non-specific binding with the phosphate sugar backbone [213]. Although the helicase domain of

MDA5 similarly wraps around dsRNA, rather than preferentially binding 5'ppp like RIG-I, the MDA5 CTD is rotated by 20 degrees, bringing it closer to the dsRNA [212]. The CTD of MDA5 folds back onto its helicase domain, forming a closed ring around the dsRNA. Because of this CTD orientation, MDA5 can be observed to form filaments along dsRNA, as binding is initiated from internal sites in the dsRNA rather than from one of the ends, as is the case for RIG-I.

1.6.4 Further cytosolic RNA sensors

There have been a number of other cytosolic dsRNA-binding proteins implicated in the mounting of a type I IFN response. However, whether they serve as direct sensors of dsRNA or they promote RLR-mediated responses remains unclear. These proteins include PKR, NOD2, NLRP3, leucine-rich repeat flightless-interacting protein 1 (LRRFIP1) and numerous DExD/H family members. Although best known for its inhibition of translation by phosphorylation of the alpha subunit of eukaryotic initiation factor 2 (eIF2 α) [214], PKR is also capable of enhancing IFN β expression upon stimulation of cells with exogenous RNA [215]. However, whether this is mediated by RIG-I signalling is not clear. NOD2 has also been implicated in cytosolic RNA sensing. Belonging to the NLR family and primarily known for its ability to recognise bacterial peptidoglycan (discussed in Section 1.7) [216], NOD2 has also been reported to activate MAVS after stimulation with viral ssRNA or RSV infection [217]. Activation of the NLRP3 inflammasome has been demonstrated to be necessary for caspase-1 cleavage in response to SeV, influenza virus or bacterial mRNA [218, 219]. However, direct binding of microbial RNA by NOD2 or NLRP3 has yet to be demonstrated. LRRFIP1 was found to contribute to VSV- and *Listeria monocytogenes*-induced IFN β expression [220]. Mostly located in the cytosol, LRRFIP1 can bind both dsRNA and dsDNA and induce type I IFN production through β -catenin phosphorylation. Activated β -catenin translocates to the nucleus where it increases the expression of IFN β by binding to the CTD of IRF3 and promoting the recruitment of the transcriptional coactivator p300 to the *Ifnb1* promoter [220]. Finally, a number of the DExD/H helicase family members have been implicated in having a role in cytosolic RNA sensing (further discussed in Section 1.11). DDX3 has been shown to bind to VSV RNA, enhancing IFN production by an association with the RLR/MAVS complex [221]. DEAH-box polypeptide 9 (DHX9) has been shown in mDCs to bind dsRNA via

its dsRNA-binding motif, and signalling via MAVS, is required for type I IFN and pro-inflammatory cytokine expression in response to poly(I:C), influenza virus and reovirus [222]. DDX1, DDX21 and DHX36 have been proposed to form a complex in mDCs, recognising cytosolic RNA and signalling via TRIF, resulting in IFN and pro-inflammatory cytokine production [223]. DHX33 was found to be capable of binding both synthetic and viral RNA and activating the NLRP3 inflammasome [224]. Subsequently, another study showed DHX33 to be capable of recognising synthetic and reoviral RNA, and signalling via MAVS in mDCs, inducing the activation of NF- κ B, MAPK and IRF3 [225]. The DExD/H family member, DDX60, has also been implicated in cytosolic RNA sensing. DDX60 has been reported to enhance the type I IFN response to RNA stimulation by formation of complexes with RIG-I, MDA5 or LGP2, but not MAVS [226]. Further DExD/H protein family members proposed to sense cytosolic RNA, and trigger cytokine production following MAVS recruitment, include DHX15 and DHX29 [227].

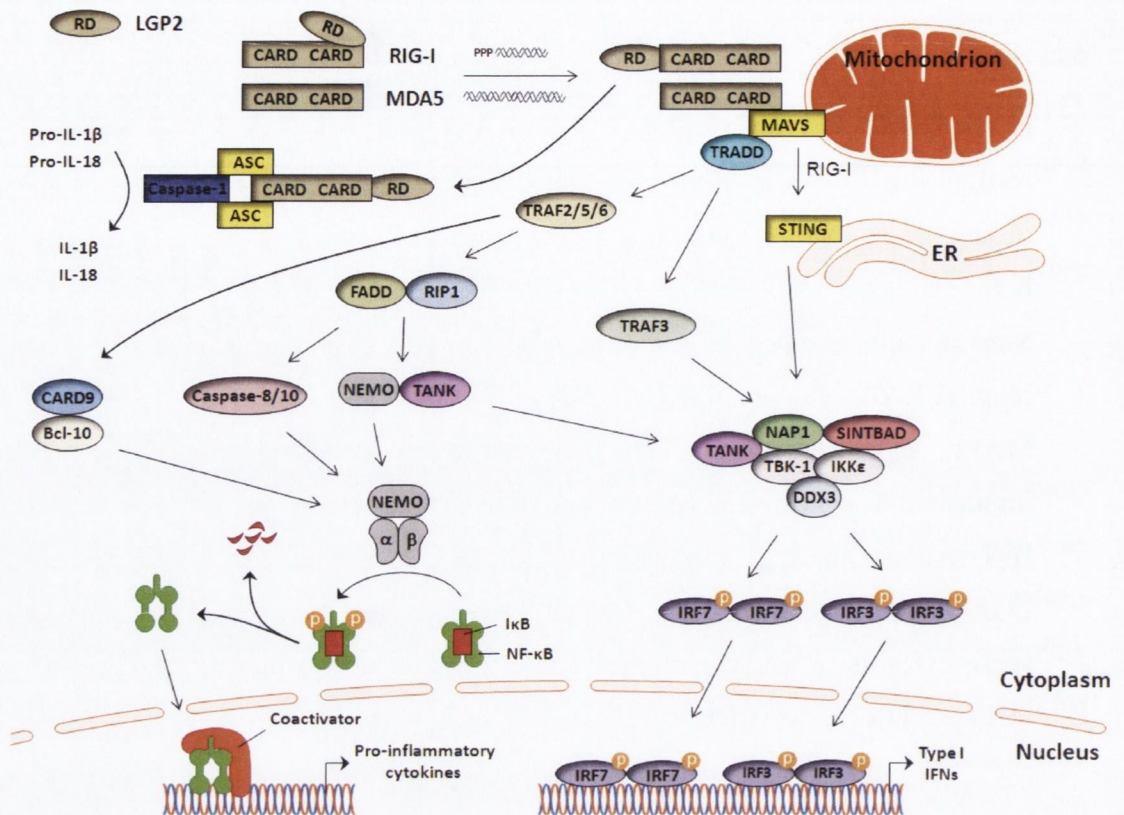


Figure 1.6 RLR signalling

The RIG-I-like receptor (RLR) family consists of three members; RIG-I, MDA5 and LGP2. RIG-I and MDA5 contain CARDs required for interaction with MAVS at the mitochondria. Both RIG-I and LGP2 contain a repressor domain (RD). In uninfected cells, RIG-I is said to be in an inactive conformation, with the RD masking its CARDs. MDA5 does not show this auto-inhibitory function as it lacks a RD. MDA5 and RIG-I bind long cytosolic dsRNA and short uncapped dsRNA that has a 5'triphosphate moiety, respectively. Upon RNA binding, they undergo a conformational change and interact with MAVS at the mitochondria. Recruitment of TRADD gives rise to signalling pathways resulting in NF-κB and IRF activation. The role of LGP2 is controversial as it has been shown to both negatively and positively regulate RLR signalling. TRADD recruitment of TRAF2/5/6 results in activation of the IKK complex via FADD and RIP1, and translocation of NF-κB to the nucleus. Activation of the canonical IKK complex has also been described following association of TRAF family members with a CARD9/Bcl-10 complex, or association of FADD with caspase-8/10. The transcription factors IRF3 and IRF7 are activated via TRADD recruitment of TRAF3. TRAF3 activates the TBK1/IKKε complex, which subsequently phosphorylates IRF3 and IRF7, resulting in their nuclear translocation and induction of type I IFNs. Although dispensable in MDA5-mediated responses, STING has been shown to interact with both RIG-I and MAVS and to be important in effective RIG-I-mediated responses. RIG-I, but not MDA5, has also been shown to be capable of inflammasome formation, capable of binding both ASC and caspase-1, and triggering maturation of IL-1β and IL-18 after RNA virus infection.

1.7 NOD-like receptors (NLRs)

1.7.1 NLR structure and function

The NLRs are a family of cytosolic proteins that drive inflammation in response to various microbial molecules (PAMPs) or endogenous danger signals following tissue damage (DAMPs). In humans, there are currently 22 family members while at least 34 have been identified in the mouse [228]. Not restricted to functions in innate immunity, the NLRs also have important roles in reproduction and embryonic development [229-231]. The NLRs have a tripartite structure, comprising of a variable N-terminal homotypic protein-protein interaction domain, a central NOD domain which is necessary for nucleotide binding and self-oligomerisation, and a C-terminal LRR which is believed to be involved in agonist sensing or ligand binding [232]. Although there have been numerous studies which have described the importance of individual NLRs against specific pathogens, a direct interaction between a putative ligand and its corresponding NLR has not been shown for most of the NLRs. Furthermore, a number of the NLR family can be activated by a broad range of stimuli, making it more likely that activation is mediated by an intermediate signalling molecule rather than a specific NLR-PAMP/DAMP interaction. Upon ligand binding, the auto-inhibitory LRR is thought to undergo a conformational change, exposing the N-terminal domain (NTD) and allowing it to form homotypic interactions with downstream signalling adaptors or effectors, forming an oligomeric complex [233]. The NLR family can be subdivided into subfamilies based on variation in their NTDs; NLRAs contain an acid TAD, NLRBs possess a baculovirus inhibitor of apoptosis protein repeat (BIR) domain, NLRCs have a CARD, and NLRPs a pyrin domain (PYD). There are also NLRs with an undefined NTD which are grouped into the NLRX subfamily.

The NLRs can be functionally divided based on their capability of inflammasome formation. The NLRs NOD1, NOD2, NLRP10, NLRX1, NLRC5 and NLRA do not require inflammasome formation for their main functions. Instead of engaging caspase-1, a number of these NLRs can activate NF- κ B, MAPKs and IRFs to trigger an innate immune response [233]. NOD1 and NOD2 were the first described NLRs [234, 235]. Belonging to the NLRC subfamily, they have been shown to recognise different structures of bacterial peptidoglycan and are implicated in the

response to numerous intracellular bacteria (e.g. *Shigella flexneri*, *Streptococcus pneumonia* and *Mycobacterium tuberculosis*) [236]. Upon stimulation, these NLRs both recruit receptor-interacting serine/threonine-protein kinase 2 (RIPK2) by a CARD-CARD homotypic interaction. Recruitment of RIPK2 results in generation of protein scaffolds necessary for transduction of downstream signalling and ultimately NF- κ B and MAPK activation [233]. NOD2 has also been proposed to have a role in non-bacterial infection. Through an interaction with MAVS, NOD2 has been shown to trigger a non-canonical signalling pathway that induces IRF3 activation and type I IFN production in response to viral ssRNA from RSV, VSV, and influenza virus [217].

1.7.2 NLR signalling

Upon ligand binding and self-oligomerisation, a number of the NLR family are capable of forming a platform for the recruitment and activation of the inflammatory protease caspase-1, referred to as inflammasomes [237]. Inactive pro-caspase-1 is recruited via homotypic CARD-CARD interactions and is activated by proximity-induced autoproteolytic cleavage. Caspase-1 is required for the processing of the precursor cytokines pro-IL-1 β and pro-IL-18 into their active forms, and for the induction of an inflammatory form of cell death known as pyroptosis [238]. The NLRs, NLRP1, NLRP3, NLRP6, NLRP7, NLRP12, NLRC4, and NAIP have been reported to form such inflammasomes [233]. Some NLR members, such as NLRP1 which contains both a CARD and PYD, are capable of directly activating caspase-1. Other members, such as NLRP3, do not have a CARD and hence recruit the bipartite adaptor protein ASC, which contains both a CARD and PYD, in order to form a complex with caspase-1 [232]. Formation of NLR inflammasomes has been observed in response to various pathogens, including viruses (e.g. SeV, influenza virus), bacteria (e.g. *Staphylococcus aureus* and *Listeria monocytogenes*), fungi (e.g. *Candida albicans*, *Saccharomyces cerevisias*), microbial products such as muramyl dipeptide (MDP), bacterial pore-forming toxins, DAMPs such as extracellular adenosine triphosphate (ATP) and various insoluble molecules or environmental insults such as monosodium urate crystals, β -amyloid, silica and asbestos [237].

1.8 C-type lectin receptors (CLRs)

CLRs are a group of membrane-bound PRRs which are critical in anti-fungal immunity [8]. They were initially designated C-type because of their calcium binding requirement. Characterised by their C-type lectin domain, these receptors bind to major carbohydrate structures in fungal cell walls, including β -glucan and mannose, and are capable of recognising most fungal species that cause disease in humans [239]. CLRs are a family of proteins which bind carbohydrates through one or more carbohydrate recognition domains (CRDs). They contain calcium-binding sites and CRDs in their extracellular domains. CLRs can be divided into 17 groups based on their functional and structural characteristics. Upon response to specific ligands, these CLRs are capable of activating signalling pathways which lead to the activation of transcription factors such as NF- κ B and the induction of an inflammatory response [240]. Expressed predominantly in myeloid cells [241], CLRs were originally thought to be mainly involved in anti-fungal immunity. However further studies have shown CLRs to also play a role in immune responses to other pathogens, including bacteria, viruses and nematodes [239].

One of the best characterised CLRs is dendritic-cell-associated C-type lectin 1 (Dectin-1). Dectin-1 recognises β -glucan and is involved in the detection of numerous fungal species including *Candida albicans*, *Aspergillus fumigatus* and *Pneumocystis carinii* [242]. Dectin-1 signalling can result in the induction of the respiratory burst, phagocytosis and endocytosis that mediates the killing of pathogens. Dectin-1 activation also results in NF- κ B and MAPK activation, and the production of inflammatory mediators, such as eicosanoids, TNF α , IL-1 β , IL-6, IL-23 and numerous chemokines [242]. Dectin-1 signalling can induce or modulate T_h1 and T_h17 responses [243], both of which are critical in the mounting of an effective adaptive immune response [2]. Interestingly, CLRs have also been found to have ‘crosstalk’ with the TLR family of PRRs [244]. CLR signalling has been found to be capable of synergising with the likes of TLR2 and TLR4 signalling, as a means of augmenting pro-inflammatory responses to fungi [245].

1.9 Cytosolic DNA receptors

1.9.1 Early identification of cytosolic DNA sensors

The immunostimulatory activity of exogenously added DNA has been known for over 50 years [246]. DNA is normally present in the nucleus of eukaryotic cells and when it is found in abnormal locations, such as the cytoplasm and endosomes, it is believed to trigger immune activation. Therefore, accumulation of unchromatinised DNA, whether pathogenic or self DNA, is thought to lead to immune activation [247]. The existence of cytosolic DNA receptors responsible for the detection of viral or bacterial DNA has long been proposed based on experiments *in vitro* [248, 249]. It had long been known that the cytosolic delivery of dsDNA could trigger immune responses independent of TLRs [250]. Before the identification of the first DNA sensor, it was shown that TBK1 and IRF3 were critical in mediating the type I IFN response to cytosolic DNA [249, 251].

In 2007, the first reported cytoplasmic DNA receptor was identified as DNA-dependent activator of IRFs (DAI) [252]. DAI, an IFN-inducible gene, was found to be capable of upregulating the expression of type I IFN through downstream induction of NF- κ B and IRF3 transcriptional activities [252]. DAI was shown to associate with TBK1 and IRF3 and recruit RIP1/3 to activate NF- κ B. However, while DAI appeared to be responsible for DNA-dependent IFN production in certain cell types, in other cell types, and DAI-knockout mice, normal responses to DNA were exhibited, indicating the presence of other cytosolic DNA receptors responsible for the detection of microbial DNA which were, as-of-yet, unidentified [251]. Subsequently, a new adaptor known as STING was identified as playing a crucial role in the response of cytosolic DNA, functioning upstream of TBK1/IRF3 activation [195]. STING is a central adaptor protein to most if not all cytosolic DNA sensors. Although not formally demonstrated, DAI is likely to signal via STING [253]. In addition to its adaptor function, STING was shown to directly recognise and induce a type I IFN response to the bacterial second messenger cyclic diguanylate monophosphate (c-di-GMP) [254].

1.9.2 PYHIN family members as sensors of microbial DNA

In 2009, further elucidation into the mechanism of DNA sensing was brought about by the discovery of the PYHIN family member AIM2, which was identified as being capable of detecting viral dsDNA in the cytoplasm [255, 256]. AIM2 was shown to form a distinct type of inflammasome with ASC and caspase-1, leading to the expression of the active forms of IL-1 β and IL-18, as in the case of the NLRs [232]. Later that year, two groups identified a further DNA sensing pathway which, surprisingly for a DNA response, involved RIG-I and MAVS [210, 257]. Pol III was found to be capable of transcribing AT-rich dsDNA, such as polydeoxyadenylic:polydeoxythymidylic (poly(dA:dT)), into an RNA-containing 5'-triphosphate moiety, which can then be sensed by RIG-I leading to IFN β induction [210, 257]. This provided an explanation for the involvement of MAVS in some DNA responses, as in the case of poly(dA:dT)-stimulated human embryonic kidney (HEK) 293 cells [248]. Many forms of pathogen DNA, not just AT-rich DNA, have been shown to induce IFN β through cytoplasmic PRR sensing [258]. Thus, given that the role of DAI is very cell type-specific, and that the Pol III-RIG-I pathway only detects AT-rich DNA, additional cytoplasmic DNA PRRs remained to be discovered [95].

In late 2010, the identification of a further cytoplasmic DNA sensing PRR, the PYHIN protein family member, IFI16, provided at least a partial answer to non-AT-rich DNA PRR recognition [259]. IFI16 was identified to be capable of directly associating with IFN β -inducing viral DNA motifs. Upon DNA stimulation, IFI16 was capable of recruiting STING, a critical mediator of IFN β responses, to DNA [259]. siRNA 'knockdown' of IFI16 or its murine counterpart, p204, was seen to inhibit gene induction induced by DNA and HSV-1, indicating their importance in immune responses to viral infections [259]. Importantly, IFI16 could account, at least partially, for the mechanism by which intracellular non-AT-rich dsDNA is recognised in mouse embryonic fibroblast (MEF) cells and some monocyte-derived cells, which was known to require IRF3, TBK1 and STING [259]. Furthermore, IFI16 has since been reported to be capable of detecting kaposi's sarcoma-associated herpesvirus (KSHV) viral DNA in the nucleus also [260]. IFI16 was shown to be capable of inflammasome formation, interacting with ASC and caspase-1, after detection of KSHV DNA in the nucleus of endothelial cells [260]. IFI16-dependent inflammasome formation has also been

demonstrated following HSV-1 infection of human fibroblasts [261]. A predominantly nuclear-expressed protein, IFI16 has been shown to shuttle from the nucleus to the cytoplasm through acetylation of its nuclear localisation signal [262].

The structure of the PYHIN family members is consistent with their proposed role as cytosolic DNA sensors, as they possess a defined signalling (pyrin) and DNA binding (HIN200) domain. IFI16, p204 and AIM2 form a new family of PRRs, termed the ALRs [9].

1.9.3 Identification of further cytoplasmic DNA sensors

Other proposed cytosolic DNA sensors include cGAS, Ku70, LRRFIP1, DNA-dependent protein kinase (DNA-PK), STING itself, Rad50, MRE11, and the DExD/H family members (further discussed in Section 1.11); DHX9, DHX36 and DDX41. LRRFIP1 can contribute to IFN β gene induction in response to dsRNA or dsDNA by promoting phosphorylation of the coactivator, β -catenin [220]. LRRFIP1 was not found to be required for NF- κ B or IRF3 activation. However, phosphorylated β -catenin is capable of recruiting p300 to the IFN β enhanceosome via IRF3 [263]. Therefore, by promoting β -catenin phosphorylation following nucleic acid detection, LRRFIP1 can indirectly upregulate IFN β [220]. Ku70 is primarily known for its involvement in the DNA repair process [264]. However, it has since been described as having a role in the cytoplasmic detection of dsDNA greater than 500 bp in length, triggering the production of type III IFN (IFN λ 1), rather than type I IFN, via IRF1 and IRF7 activation [265]. DNA-PK is localised to the nucleus where it has been shown to be involved in non-homologous end joining, playing a key role in the repairing of dsDNA breaks [266]. DNA-PK is a heterotrimeric protein complex consisting of Ku70, Ku80 and the catalytic subunit DNA-PKcs. A year following the identification of Ku70 as a DNA sensor [265], the DNA-PK complex was identified as a DNA sensor in mouse fibroblasts, capable of binding DNA in the cytoplasm and, signalling via STING, triggering a type I IFN response through the activation of TBK1/IRF3 [267]. Interestingly, although crucial for IRF3 activation in response to immunostimulatory DNA or VACV virus infection both *in vivo* and *in vitro*, DNA-PK was not required for DNA-dependent NF- κ B activation [267]. The exact mechanism of DNA-PK downstream signalling events is unclear. Having no role in NF- κ B activation,

investigating why this is so may provide a means of better understanding the mechanism of NF- κ B activation downstream of STING, which is currently unclear. Another DNA damage sensor protein, MRE11, has also been shown to mediate STING-dependent cytosolic responses to dsDNA (but not to DNA virus) [268]. In addition to its key role as a signalling adaptor in cytosolic DNA sensing, it has recently been reported that STING binds directly to DNA both *in vitro* and *in vivo*, possibly directly activating the STING signalling pathway by itself [269]. However, further work will need to be carried out in order to establish whether STING can directly sense foreign DNA without the aid of other DNA sensors. DHX9 and DHX36 have been reported to be involved in the recognition of CpG-B and CpG-A DNA in the cytosol of pDCs, respectively [270]. Although both CpG-A and CpG-B DNA contain a central palindromic CpG motif-containing sequence with a phosphodiester backbone, CpG-A differs in its presence of flanking poly G tails with phosphorothioate linkages not present in CpG-B. These two forms of CpG oligonucleotide have been identified based on distinct immunologic activities on human plasmacytoid dendritic cells and B cells [271, 272]. Signalling via MyD88, DHX9 and DHX36 mediate NF- κ B and IRF7 activation, respectively. Following cytosolic DNA binding, DDX41 has been demonstrated to induce NF- κ B and IRF3 activation via STING in mDCs and human monocytes [273]. As DDX41 is constitutively expressed, it was proposed as being an early sensor of cytosolic DNA, with subsequent IFN production inducing the expression of IFI16, amplifying the response [273]. Prior to this, DDX41 had also been shown to recognise bacterial secondary messengers to activate a type I IFN response [274].

At the beginning of 2013, a novel signalling pathway was described upon identification of a factor synthesised by mammalian cells, following stimulation with DNA, that was capable of activating STING. This factor was cyclic-GMP-AMP (cGAMP) and it was shown to directly bind STING and lead to IRF3 activation [275]. The binding of bacterial cyclic dinucleotides (CDNs) to STING is now thought to be a mechanism whereby bacteria mimic host cGAMP as a means of stimulating STING-dependent type I IFN induction to benefit the bacteria [254, 276]. At the same time, a further publication identified cGAS as a cyclase that generated cGAMP from ATP and guanosine triphosphate (GTP) following DNA stimulation [277]. Since this study, the binding of DNA by cGAS, its activation, and its production of cGAMP have been well

characterised, and it's key role in innate immunity has become evident [278]. Whether the cGAS-cGAMP pathway is broadly relevant in different cell types, or whether in certain cell types the likes of IFI16, DDX41 or other DNA sensors play more of a role in cytosolic DNA sensing, has yet to be established.

Rad50 was the most recently described cytosolic DNA sensor [279]. The DNA-damage sensor was demonstrated to directly interact with CARD9, and in a Rad50-CARD9-Bcl-10-dependent pathway, trigger activation of the IKK complex and subsequent NF- κ B translocation, following DNA stimulation. Rad50 was demonstrated to generate pro-IL-1 β and pro-inflammatory cytokines following DNA stimulation [279]. This publication hence provided insight into the previously unidentified mechanism by which cytosolic DNA can cause NF- κ B activation.

1.9.4 Self versus non-self DNA recognition

Unlike most PRRs which are activated by select microbial ligands, cytosolic DNA sensors are potentially capable of recognising both self and non-self DNA [247]. Autoimmune reactions are likely avoided by compartmentalisation, in that the cytoplasm should generally be free of DNA unless tissue damage or infection results in the appearance of cytosolic DNA, triggering an immune response. Therefore, cytosolic DNA receptors are likely to act as sensors of mislocated naked DNA rather than specific sensors of microbial DNA (recognising DAMPs, not PAMPs). This coincides with a number of the cytosolic DNA sensor family binding DNA in a non-sequence dependent manner. Structural studies have confirmed that for cGAS and a number of the PYHIN family, DNA binding occurs via electrostatic interactions with the sugar-phosphate backbone of dsDNA [280, 281]. The mechanism by which the likes of IFI16, which has been shown to respond to foreign DNA in the nucleus [260], does not respond to genomic DNA is not fully clear. There may be control mechanisms by which genomic DNA is not recognised, possibly because of its tight binding to proteins in chromatin. Another mechanism is provided by deoxyribonucleases (DNases), which are capable of degrading DNA and preventing the accumulation of DNA from apoptotic or necrotic cells for example [282]. Supporting the idea that cytosolic DNA sensors recognise mislocated rather than foreign DNA, functional loss of some DNases can lead to the development of autoimmune disorders such as systemic lupus erythematosus

(SLE) and Aicardi-Goutieres syndrome [283, 284], which are driven by self-DNA recognition and aberrant type I IFN production [285].

1.9.5 Large number of proposed DNA sensors

Since the initial description of DAI as a novel cytosolic DNA sensor in 2007 [252], there has been a vast array of proteins proposed as DNA sensors (Figure 1.7). However, a number of these molecules may not be bona fide DNA sensors. Whether some of these sensors work alongside one another, whether different cell types have different sensors, and if there is redundancy in their activity, has yet to be fully elucidated. For example, the DExD/H family members implicated in DNA sensing have been reported to predominantly function in DCs [270, 273]. Although DDX41 was also demonstrated to play a role in monocytes [273], its role, or that of DHX9 and DHX36 in other cell types, has yet to be fully characterised. Furthermore, whether these helicases act as direct sensors of cytoplasmic DNA is uncertain. As helicases, their association with DNA/RNA nucleic acid is to be expected. Also, rather than acting as a direct DNA sensor itself, the binding of DDX41 to bacterial secondary messengers may allow it to feed into the cGAS-cGAMP pathway [274, 278]. Finally, responses to CpG DNA in pDCs, as well as being DHX9- or DHX36-dependent, were also found to be TLR9-dependent [270]. Therefore, these helicase family members may be necessary for effective TLR9 signalling rather than acting as CpG DNA sensors themselves.

Further studies are necessary to determine whether these sensors function alongside one another or whether some are redundant in certain cell types. For instance, IFI16 and cGAS have both been shown to play a role in the sensing of herpesviral and lentiviral DNA [286]. IFI16 has been demonstrated to induce IFN β upon binding of HSV-1 DNA in the nucleus of human fibroblasts [261], while cGAS was shown to be required for IRF3 dimerisation in human monocytes and effective IFN β production in murine fibroblasts and bone marrow-derived DCs (BMDCs) [277]. Therefore, both nuclear IFI16 and cytosolic cGAS have been reported to be necessary for IRF3 signalling in different cell types in response to HSV-1. What remains unclear, is whether both are required in the same cell types. Preliminary work from Knipe et al. [286], indicates that both IFI16 and cGAS are necessary for IRF3 signalling in human foreskin fibroblasts. This may mean that IFI16 and cGAS cooperate for effective

signalling or that their signalling cascades may interact in some way. Interestingly, although there has been two herpesviral proteins demonstrated to target IFI16 (ICP0 and pUL83) [287, 288], there has been none shown to target cGAS to date. Similarly, both IFI16 and cGAS have been described to play a role in the sensing of HIV-1 DNA in different cell types. While cGAS was demonstrated to be required for IFN β induction in HIV-1-infected THP-1 cells [289], IFI16 was shown to colocalise with HIV DNA forms in the cytoplasm of human macrophages and be required for TBK1/IRF3/7 activation [290]. Interestingly, short hairpin RNA (shRNA) knockdown of IFI16, cGAS and STING indicated that both IFI16 and cGAS may be necessary for effective type I IFN induction following HIV infection [290]. Therefore, similar to the detection of HSV-1 [286], both IFI16 and cGAS have been demonstrated to be involved in HIV-1 sensing. This may indicate that they cooperate in DNA sensing or function in a common signalling cascade or two interconnecting pathways in the cell.

A vast array of cytosolic DNA sensors have been described in the past decade (Figure 1.7) [247]. This large number of potential DNA-sensing PRRs has raised the question as to why there is such a multitude of sensors. The large number of DNA sensors may have evolved as a means of coping with the coevolution of evasion strategies by DNA viruses. Alternatively, these sensors may have alternative sites of expression within the cell and therefore may be differentially required depending on the localisation of foreign DNA within the cell. The cooperation of these DNA sensors in the same cell and whether a number of them are bona fide DNA sensors requires further investigation.

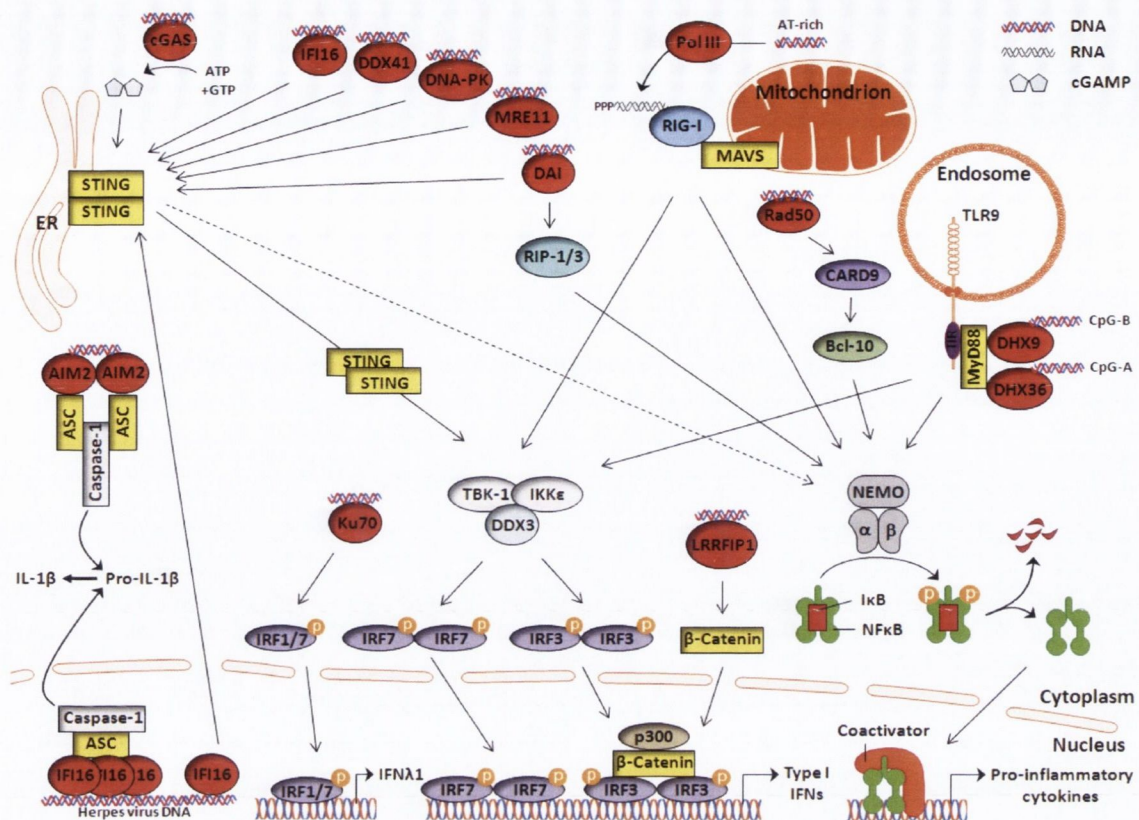


Figure 1.7 Cytosolic DNA sensors and their signalling pathways

A large number of cytosolic DNA sensors have been described. Upon recognition of dsDNA, IFI16, DDX41 and DAI signal via STING to trigger TBK1/IRF-dependent type I IFN production. IFI16 and DDX41 trigger a pro-inflammatory response through the nuclear translocation of NF-κB following its phosphorylation by the canonical IKK complex, DAI via RIP1/3 activation of the IKK complex. DNA-PK signals through STING following dsDNA binding to activate IRF3, but not NF-κB. MRE11 has been shown to mediate STING-dependent cytosolic responses to dsDNA. STING itself is capable of directly binding dsDNA and triggering type I IFN and pro-inflammatory cytokine production. Upon dsDNA recognition, the enzyme cGAS catalyses the production of cGAMP from ATP and GTP. Direct binding of cGAMP by STING, results in its activation and downstream signalling to IRFs and NF-κB. Pol III does not directly induce signalling, but transcribes AT-rich dsDNA into 5'-triphosphate dsRNA, which signals through RIG-I to activate IRF family members and NF-κB. Rad50 recognises dsDNA and triggers a Rad50-CARD9-Bcl-10-dependent pathway, resulting in NF-κB activation. In pDCs, DHX9 and DHX36 recognise CpG-B and CpG-A DNA, respectively. Signalling via MyD88, DHX9 and DHX36 give rise to NF-κB and IRF7 nuclear translocation. These responses are also TLR9-dependent. Via a coactivator pathway, LRRFIP1-dsDNA binding enhances IFNβ production by activating β-catenin and facilitating p300 recruitment to IRF3. After dsDNA binding, Ku70 signalling results in IFNλ1 expression via activation of IRF1 and IRF7. DNA-sensing AIM2, is capable of inflammasome formation with ASC and caspase-1, converting pro-IL-1β and pro-IL-18 into their active forms. IFI16 has also been implicated in inflammasome formation, having been shown to recognise both KSHV and HSV-1 DNA in the nucleus.

1.10 The DExD/H proteins: A family of multifunctional proteins critical in numerous cellular processes

The focus of this thesis is the RNA helicase DHX9. Nucleic acid helicases are NTP-dependent enzymes, capable of separating duplex oligonucleotides, which are critical to all aspects of DNA and RNA metabolism [291]. Amino acid analysis has identified several conserved sequence motifs which allows RNA and DNA helicases to be classified into six distinct groups (superfamilies SF1-SF6). SFs 3 to 6 comprise the ring-forming helicases (so called because they contain a core that consists of six individual RecA-like domains arranged in a ring), while SF1 and SF2 are non-ring-forming helicases (containing a monomeric core formed from the tandem repeat of a RecA-like fold) [291]. All eukaryotic RNA helicases belong to SFs 1 and 2 [292]. Nucleic acid helicases can be characterised by the presence of a helicase domain, which consists of eight conserved motifs (I, Ia, Ib, and II-VI). This helicase domain contains the amino acids necessary for ATP binding, hydrolysis, and nucleic acid binding and unwinding [293]. There are vast arrays of eukaryotic RNA helicases which are critical to the function of numerous cellular processes (Figure 1.8). The majority of putative RNA helicases fall into two families, DDX and DHX, which are named after the single letter designation of the amino acid sequence of motif II. The helicase domains however, also show differences in their motifs [293]. DDX and DHX proteins are often referred to as DExD/H box proteins (where x signifies any amino acid). DExD/H proteins are of major importance in the cell as they play critical roles in virtually every aspect of RNA synthesis and function, including pre-mRNA editing and splicing, ribosome biogenesis, RNA turnover, RNA export to the cytoplasm, translation initiation and termination, processes that involve multi-step association/dissociation of large RNP complexes, as well as the modulation of complex RNA structures (Figure 1.8) [294]. Although once deemed as only important in RNA metabolism, further research into these families of proteins has demonstrated that numerous members are critical to various aspects of the immune system; indicating their importance in immunity is much greater than previously predicted (Figure 1.9).

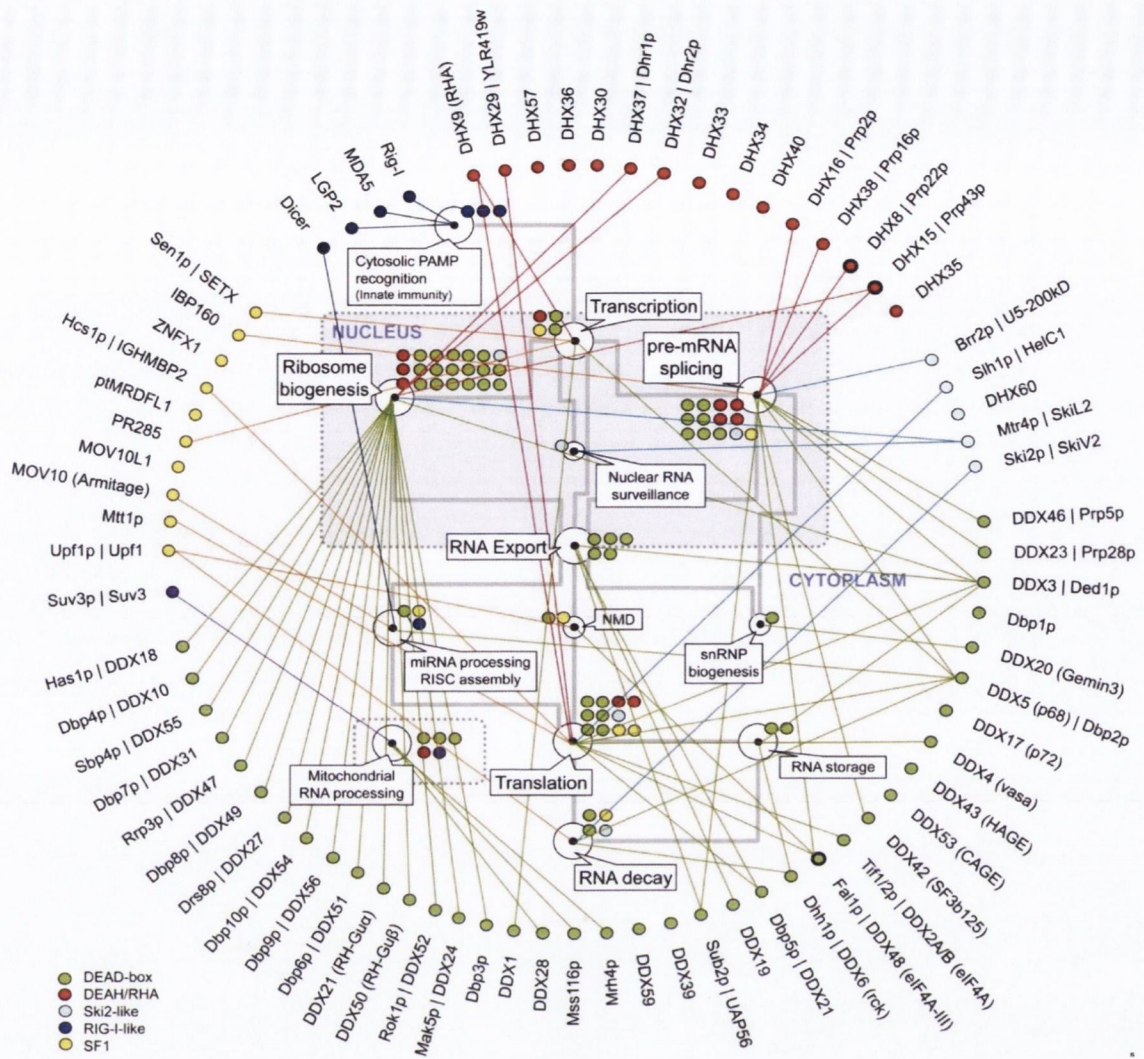


Figure 1.8 Cellular roles of eukaryotic RNA helicases

Selected, basic processes of eukaryotic RNA metabolism are represented by the white circles, as indicated by the callouts (NMD, nonsense mediated decay). The grey lines mark connections between processes. The coloured circles represent the number of individual RNA helicases involved in a given process. RNA helicases (yeast and human orthologs) are grouped and color-coded according to their families (see legend at left lower corner). Connectors indicate involvement in one or more processes of RNA metabolism (Figure taken from [292]).

1.11 A critical role for DExD/H proteins in innate immunity

1.11.1 The RLR family and NF- κ B-interacting helicases

Apart from playing a critical role in various aspects of RNA metabolism, transcription, translation, and numerous other cellular processes (Figure 1.8), a number of DExD/H helicase proteins have been implicated as integral in the immune response to infection (Figure 1.9). One such example of a DExD/H protein family member being important to the mounting of an effective immune response came with the identification of the DDX protein, RIG-I (DDX58), as being crucial in triggering an immune response to viral dsRNA [295]. Previously, it was unknown how IFN β induction occurred upon stimulation with a synthetic analog of dsRNA (poly(I:C)) or RNA virus infection in the absence of TLR3 or its intracellular adaptor molecule, TRIF [89, 296]. RIG-I, acting as an innate PRR of the immune system, was found to be capable of transmitting a signal leading to the activation of NF- κ B, IRF3 and IRF7, through recognition of dsRNA by its RNA helicase domain [295]. Research into the mammalian database led to the discovery of a further two closely related DExD/H proteins; MDA5 and LGP2 (DHX58). RIG-I, MDA5 and LGP2 comprise the RLR family (Section 1.6) [297].

As well as directly sensing RNA, a number of DExD/H protein family members have been found to be capable of interacting with the p65 (RelA) subunit of NF- κ B. Both DDX1 and DHX9 have been demonstrated to do so and consequently enhance NF- κ B-dependent transcription [298, 299].

1.11.2 DDX3 – a protein highly targeted by viruses

A further major role for DExD/H proteins in the innate immune response to infection was identified upon the discovery that VACV encodes a protein, K7, which is capable of inhibiting IRF activation [300]. K7 was found to inhibit PRR-induced IFN β induction by preventing TBK1/IKK ϵ -mediated IRF activation. Further investigation into the mechanism by which K7 antagonises IRF activation led to the discovery of DDX3. K7 was found to directly bind DDX3 and further research showed DDX3 to associate with the TBK1/IKK ϵ complex, being critical to TBK1/IKK ϵ -mediated IRF activation and IFN β promoter induction [300]. A further study published in this period [301], demonstrated DDX3 to be directly recruited to the IFN β promoter, suggesting

DDX3 acts as a transcriptional regulator after activation by TBK1. Further highlighting its importance in antiviral immunity, hepatitis B virus (HBV) pol has also been shown to target DDX3 to antagonise the production of type I IFNs [302]. The multifunctionality of numerous DExD/H family members is astounding. For instance, DDX3, in addition to its role in innate signalling pathways contributing to the induction of antiviral mediators such as type I IFNs [300, 301], it is involved in the nuclear export of proteins and various classes of RNAs through the nuclear pore complex [303], the suggested initiation of protein translation [304], and the regulation of transcription [305], cell cycle control, apoptosis and tumourigenesis [306]. More recent studies of DDX3 have shown further roles for its involvement in innate immunity. Binding to MAVS, DDX3 has been proposed as a sensor for viral RNA, alongside RIG-I and MDA5 [221]. At early stages of infection when levels of IFN-inducible RIG-I are low, the authors proposed that DDX3 is required to sensitise the RLR system to dsRNA ligands. The same authors later identified the HCV core protein, which had already been reported to target DDX3 [307], as having the potential to disrupt the DDX3-MAVS interaction and hence prevent IFN induction [308]. Contrary to this, a separate report showed HCV core protein actually triggers IFN induction through DDX3 [309]. Further research is necessary in order to clarify whether DDX3 is capable of acting as a direct viral RNA sensor and/or as a transcriptional activator. Although DDX3 has been shown to contribute to antiviral immunity [300, 301], there are also viruses which exploit DDX3 for their viral life cycle [310]. These include HIV and HCV [311, 312]. In HIV infection, DDX3 was shown to be required for the export of HIV RNA from the nucleus, making it an essential host factor for HIV replication [311]. It is also required for HCV replication [312], although the exact contribution of DDX3 is yet unclear. Whether the interaction of HCV core protein with DDX3 mediates the effects on HCV replication also remains to be elucidated [307].

1.11.3 A role for DDX41 in STING-dependent signalling

In order to identify the potential involvement of other DExD/H family members in innate immunity, Yong-Jun Liu's group carried out a siRNA screen with 59 members of the family. They identified DDX41 as a novel intracellular DNA sensor in mDCs [273]. DDX41 shRNA-expressing mDCs were shown to have impaired NF- κ B and IRF3 transcription factor activation, and IFN and pro-inflammatory cytokine

production, in response to transfected DNA and DNA virus infection. DDX41 was shown to bind DNA via its Walker A and B motifs and associate with both STING and TBK1, in keeping with its proposed role as a separate DNA receptor [273]. More recently, DDX41 was reported to directly bind the bacterial secondary messengers, cyclic-di-GMP and cyclic di-AMP [313]. These CDNs act as PAMPs which signal the presence of a bacterial infection and induce a type I IFN response. However, they may also be utilised as an immune escape mechanism where an induction of type I IFN may benefit the pathogen. Upon binding to CDNs, DDX41 was reported to be capable of inducing a type I IFN response via STING, TBK1 and IRF3 [313]. As DDX41 was reported to bind bacterial CDNs, it may have a role in cGAMP sensing which could account for its requirement in the mounting of an effective response to DNA viral infection. Thus the exact roles of DDX41 and STING in CDN sensing have yet to be fully clarified.

1.11.4 Emerging roles of the DExD/H protein family in nucleic acid sensing

Since the initial description of DDX41 as a DNA sensor in mDCs [273], there has been a myriad of publications implicating numerous DExD/H family members in innate immune signalling (Figure 1.9). DDX1, in a complex with DDX21 and DHX36, was shown to recognise dsRNA in the cytosol of mDCs [223]. DDX1 was demonstrated to bind poly(I:C), while DDX21 bound DDX1 and DHX36, suggesting that it bridges the interaction of these two helicases. Both DDX1 and DHX36 were reported to bind TRIF. Knockdown of DDX1, DDX21 or DHX36 inhibited IFN induction in response to poly(I:C), influenza virus and reovirus. It was therefore suggested that DDX1 binds dsRNA and signals via DDX21 and DHX36 to TRIF, activating type I IFN responses in the cytosol of mDCs [223]. However, depending on the nature of the RNA ligand, knockdown of RIG-I, MDA5 or TLR3 also strongly inhibited IFN induction [223]. Hence, this helicase complex may facilitate effective dsRNA sensing pathways, rather than acting as a receptor itself. DDX60 has also been identified as a component of innate immunity. DDX60 was demonstrated to have a role in RLR-mediated signalling [226]. It was shown to work alongside RIG-I and MDA5 to trigger responses to viral dsRNA. Knockdown of DDX60 reduced expression of type I IFN and IFN-inducible genes after transfected poly(I:C), VSV, poliovirus, HSV-1 or SeV infection [226].

A recent study has also implicated the DEAH proteins, DHX9 and DHX36, as potentially acting as PRRs and triggering an innate immune response to infection in pDCs [270]. DHX36 and DHX9 were demonstrated to selectively bind CpG-A and CpG-B DNA, respectively, and signal via a MyD88-dependent pathway. DHX36 was associated with IFN α production and IRF7 nuclear translocation in response to CpG-A DNA, while DHX9 was found to be important for TNF α and IL-6 production and NF- κ B activation in response to CpG-B DNA [270]. Additionally, knockdown of TLR9 almost completely abrogated CpG DNA-dependent responses [270], possibly indicating that DHX9 and DHX36 may feed into the TLR9 pathway, rather than acting as DNA sensors themselves. DHX9 has also been proposed as a dsRNA sensor in mDCs [222]. Knockdown of DHX9 by shRNA showed DHX9 to be required for the production of type I IFNs and pro-inflammatory cytokines in response to poly(I:C), influenza A and reovirus. DHX9 was reported to bind poly(I:C) via its dsRNA-binding domains and signal via MAVS to trigger activation of NF- κ B and IRF3 [222]. In a further study since then, DHX36 was shown to be capable of enhancing RIG-I signalling by binding PKR and facilitating PKR-mediated antiviral stress granule formation which has been shown to provide a critical platform for RIG-I signalling [314, 315]. DHX36 was found to form a complex with PKR, facilitating its binding to dsRNA and phosphorylation. Knockdown of DHX36 in MEF cells resulted in a reduction in type I IFN production and a higher susceptibility to RNA virus infection [314]. Two further publications demonstrated a role for DHX33 in innate immune signalling. DHX33 was proposed as a sensor of cytosolic RNA, necessary for the activation of the NLRP3 inflammasome [224]. This study suggested DHX33 was the previously unknown upstream sensor of dsRNA and bacterial RNA required for the activation of the NLRP3 inflammasome. Knockdown of DHX33 blocked the activation of caspase-1 and secretion of IL-1 β and IL-18 in human macrophages that were activated by cytosolic poly(I:C), reoviral RNA, or bacterial RNA [224]. Following stimulation with RNA, DHX33 was found to interact with NLRP3 and form the inflammasome complex required for signalling. Two months after this publication [224], a report claimed DHX33 to be a dsRNA sensor in mDCs, signalling via MAVS [225]. Using knockdown studies, they found DHX33 was necessary for effective type I IFN production in response to poly(I:C) and reovirus infection. A publication a few months prior to these studies, showed that MAVS mediates the recruitment of NLRP3 to mitochondria, promoting inflammasome

activation [316]. Thus, the interaction of DHX33 with NLRP3 may have been indirect and its ability to affect NLRP3-dependent responses may have been via its interaction with MAVS.

Numerous other DExD/H protein family members have also been shown to have roles in the innate immune response. In late 2013, a role for DDX24 as a negative regulator of cytosolic RNA-mediated signalling was described [317]. Overexpression of DDX24 was shown to inhibit dsRNA-induced RLR signalling, while its silencing by siRNA was found to enhance it. DDX24 was shown to influence signalling by associating with both FADD and RIP1 and preventing the activation of IRF7 [317]. In 2014, there was a further myriad of publications describing the involvement of other members of the DExD/H family in immunity. In April of 2014 [318], DHX15 became another helicase to be described as having a role in RLR-mediated antiviral signalling. DHX15 was shown to interact with MAVS and specifically stimulate activation of NF- κ B and MAPKs following poly(I:C) stimulation or RNA virus infection. Rather than claiming DHX15 to be a novel RNA sensor, they described DHX15 as contributing to RLR signalling [318]. Interestingly, it was found to not be involved in the activation of IRF3. They also demonstrated DHX15 was required for poly(I:C)- or RNA virus-dependent, MAVS-mediated apoptosis [318]. Within a few months, a further publication on the involvement of DHX15 in MAVS-dependent signalling was described [227]. However, unlike the previous study [318], DHX15 was described as an RNA sensor, signalling via MAVS, to upregulate type I IFN and pro-inflammatory cytokines in response to poly(I:C) or RNA virus in mDCs [227]. They also showed that as well as being required for NF- κ B and MAPK activation in response to RNA viral infection, DHX15 was also necessary for effective IRF3 activation. However, in the previous study DHX15 was not found to have an effect on IRF3 activation [318]. Therefore, although a role for DHX15 in MAVS-dependent antiviral signalling appears evident, whether it serves as a bona fide RNA sensor or as an adaptor for MAVS-dependent signalling is not fully clear. Finally, another study in 2014 showed DHX29 to act as a cosensor of dsRNA, alongside RIG-I, in human airway epithelial cells and fibroblasts [319]. Knockdown of DHX29 by siRNA, showed it was required for effective type I IFN and pro-inflammatory cytokine induction following stimulation with poly(I:C), poly(dA:dT) or RNA virus infection. DHX29 was found to directly bind

to nucleic acids, to interact with RIG-I and MAVS, and subsequently to activate RIG-I-MAVS-dependent signalling pathways [319].

With the vast array of DExD/H family members having been recently discovered to have roles in innate immunity (Figure 1.9), a much broader role for DExD/H proteins in viral sensing, beyond the RLRs, is now becoming apparent. Having been discovered to trigger an immune response upon sensing of both dsRNA and dsDNA [222, 270], DHX9 was of particular interest when it was identified, via mass spectrometry, as a target of the VACV protein E3 (S. Keating, personal communication). E3 is a critical virulence factor for VACV [320].

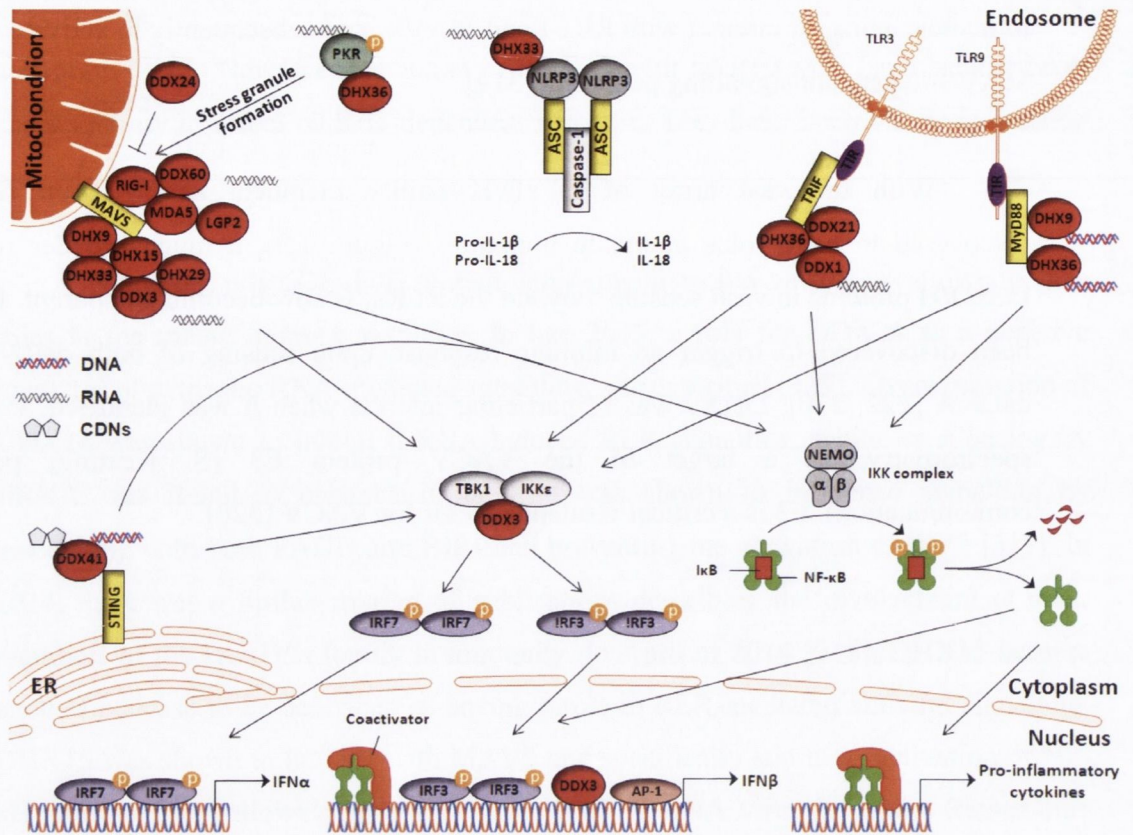


Figure 1.9 Proposed roles of the DExD/H protein family in nucleic acid sensing

While the RIG-like-receptors, RIG-I, MDA5 and LGP2, have defined roles in antiviral innate immunity, the exact function of other members of the DExD/H family is not as clear. DDX3 has been proposed as a dsRNA sensor in conjunction with the RLRs, as a component of the TBK1/IKK ϵ complex, and as a transcriptional regulator of the *ifn β* promoter. DHX33 has been proposed to directly sense dsRNA and signal via MAVS, as well as sense cytoplasmic RNA to activate the NLRP3 inflammasome. DDX60 can sensitise the RLRs to viral dsRNA ligands. DHX9, DHX15 and DHX29 have been described as MAVS-dependent dsRNA sensors. By association with FADD and RIP1, DDX24 negatively regulates RLR-mediated signalling. DDX41 can sense cytoplasmic DNA and signal in a STING-dependent manner. DDX1, DDX21 and DHX36 form a complex to recognise dsRNA and signal through TRIF. DHX9 and DHX36 sense CpG-A and CpG-B DNA, respectively, and signal via a MyD88-dependent pathway. Their binding of CpG DNA gives rise to IRF7 and NF- κ B activation, respectively. All of the proposed sensors are capable of NF- κ B activation via the canonical IKKs (IKK α/β), and IRF activation via the non-canonical TBK1/IKK ϵ complex, giving rise to the production of pro-inflammatory cytokines and type I IFN, respectively.

1.12 Vaccinia virus (VACV) evasion of the host immune response

Viruses are the most abundant, diverse, rapidly evolving pathogens against which the host immune system must protect itself on a regular basis. Having coevolved alongside the host immune system, viruses have mastered numerous strategies to evade and subvert the host antiviral response. With the ultimate goal of a virus being to inhabit its host and replicate for as long as possible, most viruses have refined an arsenal of strategies which enable them to interfere with the host defences and inhibit the mounting of an effective immune response. VACV is one such virus and encodes an array of proteins, many of which are multifunctional, that allow it to successfully avoid detection by the innate immune system and hence coexist with its host [321].

1.12.1 VACV structure and replication

VACV is a member of the genus *Orthopoxvirus* of the family Poxviridae, a family of large, dsDNA viruses that replicate in the cytoplasm of host cells [322]. VACV is best known for its use in the creation of live vaccines against smallpox, successfully allowing global eradication of the disease [323]. Poxviruses are characterised by having a large oval-shaped virion, containing enzymes and factors for mRNA synthesis and DNA replication, and a dsDNA genome of 130–300 kilobase pairs [322]. As with all poxviruses, VACV replicates within the cytoplasm of infected cells [324]. VACV encodes around 200 genes [325]. Depending on the time of their transcription during the infection process, the encoded genes can be divided into three groups; early, intermediate and late genes. Early class gene mRNAs appear within minutes of infection, and encode proteins required for evasion of the host antiviral responses (*E3L* being one – see Section 1.13), DNA replication and nucleotide biosynthesis [324]. Approximately half of the VACV genes belong to the early class [326]. Intermediate and late genes are transcribed after viral replication in the cell has initiated and are necessary for virion morphogenesis and assembly [324].

VACV replicates its DNA exclusively in the cytoplasm, in specifically designated regions called viroosomes [327]. VACV produces four different types of virion from each infected cell; intracellular mature virus (IMV), intracellular enveloped virus (IEV), cell-associated enveloped virus (CEV) and extracellular enveloped virus

(EEV) [328]. IMV particles are formed from non-infectious precursors called crescents and immature virus, within cytoplasmic viral factories in the cell. IMVs represent the majority of infectious progeny before cell lysis. Some IMV exit these cytoplasmic factories on microtubules [329]. During movement to the cell surface, they become wrapped in a double layer of intracellular membrane derived from early endosomes or the trans-Golgi network, forming IEV [328]. Physically indistinguishable, particles retained on the cell surface are referred to as CEV and those released from the cell, EEV. CEV induce the formation of actin tails that drives the virion away from the cell, facilitating cell to cell viral spread [330]. While IMV are poorly suited to viral spread due to their sensitivity to complement and antibodies, EEV are more effective in doing so as they have an additional lipid membrane. VACV can spread to other cells via CEV cell-to-cell mediated transmission or by the release of EEV and re-infection of distant or adjacent cells [331].

1.12.2 VACV evasion mechanisms

VACV can activate the innate immune response via both TLR-dependent and independent pathways, resulting in pro-inflammatory cytokine and type I IFN induction. Both TLR2 and TLR4 have been described in detecting VACV, most likely via surface proteins on IMV or EEV [332]. As well as having been reported to be involved in the activation of NK cells and control of VACV infection *in vivo* [131], TLR2 has been shown to cause non-nucleic acid-driven type I IFN induction in response to VACV in inflammatory monocytes [132]. TLR2 detection of VACV may signal via the recently described endocytic TLR2-TRAM-MyD88-IRF7 pathway [179]. Although controversial, TLR8 has also been implicated as having a role in VACV detection [149]. TLR8 activation following binding of VACV DNA in murine pDCs was described. However, as TLR8 has never been linked to DNA recognition, and murine pDCs have low levels of TLR8 protein expression, other authors have questioned the validity of their data [333]. Although other sensors of VACV have yet to be fully established, VACV dsRNA produced from bidirectional transcription may potentially be sensed by TLR3 or members of the RLR family. ssRNA replication intermediates may also be potentially recognised via TLR7/8. A role for TLR9, or possibly unidentified cytoplasmic DNA sensors, in direct recognition of the VACV dsDNA genome may also

be likely [334]. VACV has evolved an impressive array of proteins capable of specifically targeting various aspects of innate immune signalling (Figure 1.10).

VACV encodes for proteins capable of targeting numerous aspects of antiviral cell signalling and host defence. Analysis of VACV protein function resulted in the discovery of one of the first characterised viral inhibitors of a TLR signalling pathway, A46 [335]. A46 was found to directly target specific TIR-domain-containing adaptor proteins and contribute to virulence. Interestingly, although reported to interact with MyD88, TRAM, Mal and TRIF to block their signalling from TLRs, it was not found to interact with SARM [335]. Having been described to have a negative regulatory role in TLR signalling [160], the binding of A46 to all of the TIR-domain containing adaptors except for SARM [335], shows an impressive ability of VACV to target antiviral mediators of the immune system while not affecting host proteins that have functions beneficial to the virus. A52 is another example by which VACV can target multiple aspects of signalling to benefit its life cycle. A52 has been demonstrated to specifically target IL-1R and TLR signalling and contribute to virulence [336]. A52 interacts with TRAF6 and IRAK2, two signalling molecules that are downstream of the TIR-domain-containing adaptors. A52 inhibits NF- κ B activation by targeting IRAK2 but increases p38 MAPK activation by interacting with TRAF6 [336, 337]. This results in the inhibition of pro-inflammatory cytokine induction (e.g. IL-8) but enhancement in the production of the anti-inflammatory cytokine IL-10, generating an environment which is predominantly beneficial to viral life cycle. There have also been numerous VACV genes shown to encode viral protein inhibitors which are capable of antagonising the canonical and non-canonical IKK complexes. The targeting of the IKKs by VACV makes sense from a viral perspective, as it allows the virus to antagonise a vast array of antiviral signalling pathways which are dependent on these downstream signalling complexes. Targeting of the IKK α/β complex allows VACV to inhibit NF- κ B activation and subsequent pro-inflammatory cytokine induction, while targeting of the TBK1/IKK ϵ complex facilitates antagonism of type I IFN induction [338]. Both B14 and C4 are examples of VACV inhibitors of the canonical IKK complex. B14 has been demonstrated to preferentially bind IKK β , preventing its activation and thereby inhibiting the phosphorylation of I κ B in response to a range of stimuli [339]. C4 has similarly been demonstrated to inhibit NF- κ B activation [340]. Its target was mapped to

at, or downstream of, the IKK complex. VACV inhibitors of the non-canonical TBK1/IKK ϵ complex include N1, C6 and K7. N1 directly binds TBK1 and antagonises IRF3 transcription factor activation [341]. Interestingly, in the same study, they also demonstrated N1 to target the IKK complex to antagonise NF- κ B activation. C6 was reported to inhibit IRF activation via interaction with the TBK1/IKK ϵ complex scaffold proteins, TANK, SINTBAD and NAP1 [342]. The identification of the target of VACV K7 proved to be instrumental in the discovery of a novel component of the non-canonical IKK complex, DDX3 [300]. Determining that K7 inhibited PRR-induced IFN β induction by preventing TBK1/IKK ϵ -mediated IRF activation encouraged an investigation into the mechanism by which K7 antagonises IRF activation and led to the discovery of DDX3. K7 was found to directly bind DDX3 and further research showed DDX3 to associate with the TBK1/IKK ϵ complex, being critical to TBK1/IKK ϵ -mediated IRF induction and *IFN β* promoter induction [300].

It is evident that much can be learned about the host immune response by investigating the interacting partners and mechanism of action of viral protein inhibitors of the immune system. Another highly multifunctional VACV protein that has aided in our understanding of numerous aspects of antiviral immunity, is the *E3L* gene product, E3.

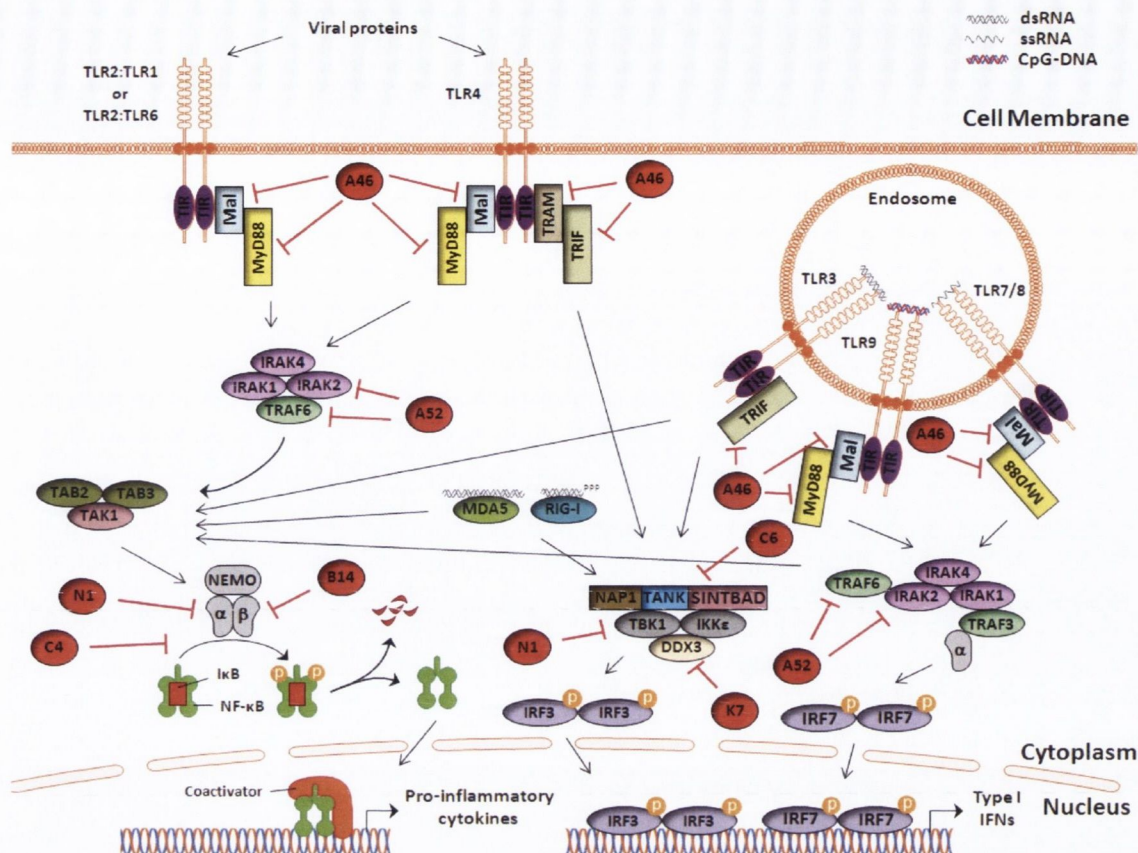


Figure 1.10 Evasion of the innate immune response by VACV

VACV expresses a vast array of proteins which are capable of inhibiting or subverting numerous aspects of innate immune signalling events. VACV B14, N1 and C4 have been described as inhibitors of the canonical IKK complex or it's downstream signalling to NF-κB activation. K7, N1 and C6 are capable of binding components of the TBK1/IKKε complex, thereby blocking effective IRF activation. A46 has been shown to directly bind all of the TIR-domain containing TLR adaptors except SARM, antagonising their downstream signalling events. A52 is capable of binding IRAK2 and TRAF6 to inhibit pro-inflammatory cytokine induction while also enhancing anti-inflammatory cytokine production, respectively.

1.13 VACV E3 – a multifunctional evasion protein

The VACV *E3L* gene is expressed early during infection, and is present in both the nucleus and cytoplasm of infected and transfected cells [343, 344]. The creation of VACV lacking the *E3L* gene (VACV Δ E3L) showed E3 to be necessary for efficient VACV replication in several cell lines and to be required for VACV pathogenesis [320, 345]. E3 is vital in conferring resistance to type I and type II IFNs. IFN sensitivity seems to be mediated primarily through the functions of PKR, as RNA interference against PKR in Huh7 cells reversed the IFN-sensitive phenotype of VACV Δ E3L [346]. The E3 protein is composed of a C-terminal dsRNA-binding domain (dsRNA-BD) and an N-terminal Z-DNA-BD, with both regions being required for virulence in mice [320]. While both domains are required for pathogenesis *in vivo* [320], only the dsRNA-BD appears essential for virus replication and IFN resistance in cell culture [347, 348].

The C-terminal dsRNA-BD serves to sequester dsRNA produced during viral replication, hence preventing the activation of PKR and RNase L, as well as, the activation of IRF3 [343, 347, 349-351]. Use of an E3 mutant with low dsRNA binding affinity demonstrated that the binding of dsRNA by E3 was necessary for PKR inhibition and this effect could be reversed using excess dsRNA [348]. The dsRNA-BD also inhibits poly(dA:dT)-induced IFN β expression, where Pol III-mediated RIG-I signalling appears to be required for NF- κ B activation and IFN β induction [352]. In addition to being crucial for IFN resistance, the broad-range phenotype of VACV has also been mapped to the dsRNA-BD of E3 [347, 348]. Knockdown of PKR demonstrated that the host range phenotype of E3 works primarily through its ability to antagonise PKR via its dsRNA-BD [353]. In another study, ISG15 was shown to act as a host defence molecule against VACV infection, its expression reducing VACV replication [354]. E3 was found to directly bind ISG15 via its dsRNA-BD and antagonise its antiviral actions [354].

Much less is known about the function of the N-terminal Z-DNA-BD. The Z-DNA-BD is known to bind Z-DNA and PKR [355-359]. There have been three forms of duplex DNA described; A-, B-, and Z-DNA [360]. The most common, B-DNA, has a canonical right-handed double helical structure. A-DNA also has a right-handed double helix but with a shorter, more compact, helical structure whose base pairs are not

perpendicular to the helix-axis as in B-DNA. Finally, Z-DNA differs from A- and B-DNA in that it takes on a left-handed double helical structure in which the double helix winds to the left in a zig-zag pattern [360]. While no definitive biological significance of Z-DNA has been found, it has been proposed to possibly provide torsional strain relief while DNA transcription occurs. The binding of Z-DNA by E3 has been demonstrated to be necessary for full pathogenesis as point mutations that decreased the affinity of Z-DNA binding were found to correlate with a decrease in neurovirulence in mice [357]. Although not capable of sequestering dsRNA like the dsRNA-BD [349], the Z-DNA-BD can antagonise PKR function by a direct protein-protein interaction [359, 361]. Although previously described as being dispensable for the IFN resistant phenotype of VACV [347, 348], more recent work has shown the Z-DNA-BD of E3 is required for inhibition of the IFN response in both mice and MEFs [361]. The molecular mechanism of inhibition in primary MEFs was shown to be through the inhibition of PKR [361]. This correlates with the Z-DNA-BD having been demonstrated to be necessary for full pathogenesis *in vivo* [320]. VACV has also been found to be capable of inhibiting apoptosis through transactivation of the human IL-6, nuclear factor of activated T cells (NFAT) and p53 genes, via its Z-DNA-BD [362]. Carrying out reporter gene assays, this study demonstrated that overexpression of E3 in HeLa cells enhanced the transactivation of these apoptotic genes.

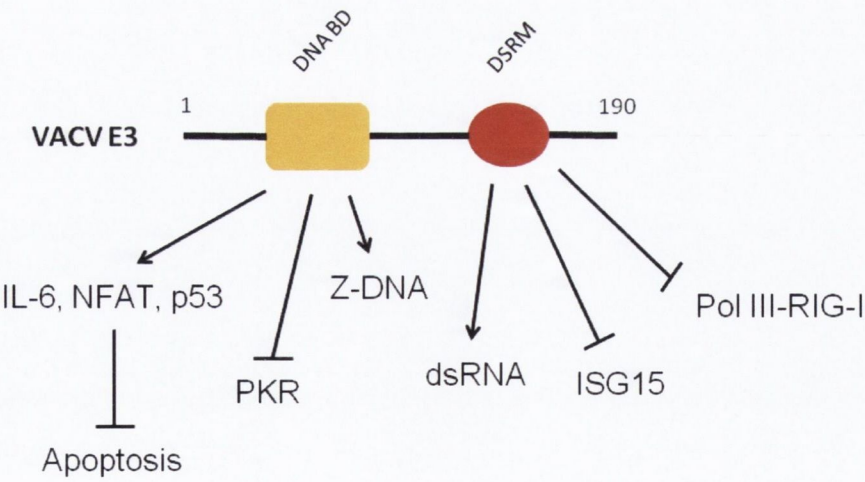


Figure 1.11 VACV E3 evasion mechanisms

VACV E3, a protein of 190 amino acids in length, encodes an N-terminal Z-DNA binding domain (BD) and a C-terminal dsRNA binding motif (DSRM). It is capable of immune evasion by an array of mechanisms. The Z-DNA BD can directly bind PKR to antagonise its function, and also inhibit apoptosis

by transactivation of the genes for IL-6, NFAT and p53. The binding of Z-DNA via the E3 DNA-BD has also been shown to be necessary for full pathogenesis. The DSRM is capable of sequestering dsRNA that would otherwise be recognised by PKR. The DSRM can also sequester dsRNA produced from Pol III-transcribed dsDNA. The binding of the DSRM to ISG15 inhibits its antiviral activity.

1.14 Further investigation into possible interacting proteins of E3

Due to the major importance of E3 in the VACV life cycle [320, 347], mass spectrometry was carried out in the lab to identify other possible proteins within the host cell which E3 interacts with (S. Keating, personal communication). Cells were infected with either wild-type Western Reserve (WR) VACV or VACV lacking the E3 gene (VACV Δ E3L), lysed, and then the lysates were subjected to immunoprecipitation with an E3 antibody. Immunoprecipitated protein was run on a gel, eluted from gel slices, and then sent for mass spectrometry analysis, with VACV Δ E3L-infected samples serving as a control. Host DHX9 was identified as a target in both HEK 293 cells and in MEF cells (S. Keating, personal communication). VACV 70mer, a dsDNA 70-base pair motif that is conserved and repeated multiple times in many poxviral genomes and capable of inducing IFN β in numerous cell types [259], had been previously identified via mass spectrometry, as also interacting with DHX9 (S. Keating, personal communication). 70mer is strongly conserved in different poxviral genomes such as VACV in the inverted terminal repeat region, often repeated multiple times, and is not known to encode for any gene. Because both VACV 70mer and E3 were found to interact with host DHX9 via mass spectrometry, it was thought that DHX9 may play a role as an innate DNA receptor which E3 might antagonise. Therefore, further investigation of this possibility was carried out.

1.15 DHX9 – a protein with newly emerging immunological importance

As previously discussed (Section 1.10), the DExD/H-box family of proteins include a large number of proteins that are involved in RNA metabolism. DHX9 is capable of binding both ssRNA and ssDNA, and unwinds double-stranded nucleic acids driven by hydrolysis of ATP, GTP, cytidine triphosphate (CTP), or uridine triphosphate (UTP) [363, 364]. In mammals, DHX9-knockout mice are embryonic lethal for homozygous DHX9 mutants [365]. DHX9 is therefore necessary for early embryonic development in mice. It was also suggested that DHX9 is required for the survival and differentiation of embryonic ectoderm [365]. DHX9 maps to chromosome 1q25 near a

major susceptibility locus for prostate cancer [366]. Despite its apparent steady-state nuclear expression, DHX9 is known to shuttle between the cytoplasm and nucleus [367]. DHX9 has been shown to have a C-terminal 19 amino acid NLS, which is highly conserved through species, and required for its shuttling to the nucleus [368]. DHX9 links the transcription and translation of both cellular and retroviral mRNAs [369, 370], as well as being capable of interacting with several transcriptional regulators [294]. It mediates the association of CBP with Pol II [371]. There are numerous studies showing DHX9 to have a more important role in innate immunity than previously envisaged (Figure 1.12). DHX9 has been identified as an interacting molecule of the p65 subunit of NF- κ B, capable of enhancing NF- κ B dependent reporter gene expression induced by p65, TNF α , or NIK [298]. DHX9 has also been identified as a PKR substrate, undergoing phosphorylation by PKR which disrupts its association with dsRNA [372]. This allows PKR to modulate transcription and inhibit replication of viruses such as bovine viral diarrhea virus which DHX9 transactivates by binding to the terminal non-translated regions of the viral RNA genome [373], and HIV-1, which is also transactivated by DHX9 [372, 374]. There are numerous other viruses which have been demonstrated to hijack DHX9 for their replication. Amongst others, DHX9 has been shown to enhance classical swine fever virus [375], foot-and-mouth disease virus [376], and HCV replication [377], by direct binding to their 5'- and/or 3'-untranslated regions, possibly modulating their translation. Numerous other publications have demonstrated a role for DHX9 in viral replication via siRNA studies (e.g. influenza A virus [378]), and speculated the requirement of DHX9 may be due to its capability to bind viral untranslated regions as previously demonstrated for other viruses. Interestingly, a recent publication identified DHX9 as a target of the myxoma virus protein M029 [379]. M029 is a truncated ortholog of VACV E3 which lacks a significant portion of the N-terminal Z-DNA-BD. Capable of binding both PKR and DHX9, M029 was shown to inhibit the PKR pathway, while hijacking DHX9 to promote viral replication [379].

As discussed in Section 1.11.4, DHX9 has been recently reported to play a role in nucleic acid sensing. In August of 2010 [270], DHX9 was proposed as sensor of CpG-B DNA in the cytosol of pDCs, being necessary for NF- κ B-dependent cytokine production in response to CpG-B DNA. Gene induction by HSV was seen to be impaired by DHX9 siRNA silencing in pDCs while no impairment to influenza A gene

induction occurred. Therefore, this publication concluded that DHX9 was a specific DNA sensor [270]. More recently, DHX9 was also proposed as a dsRNA receptor in mDCs. In this study, DHX9 was found to signal via MAVS, and was shown to be important in the production of type I IFN and pro-inflammatory cytokines in response to poly(I:C), influenza A and reovirus [222]. The fact that mass spectrometry identified DHX9 as being targeted by VACV E3 (S. Keating, personal communication), implied DHX9 to be potentially important in sensing VACV and other poxviruses.

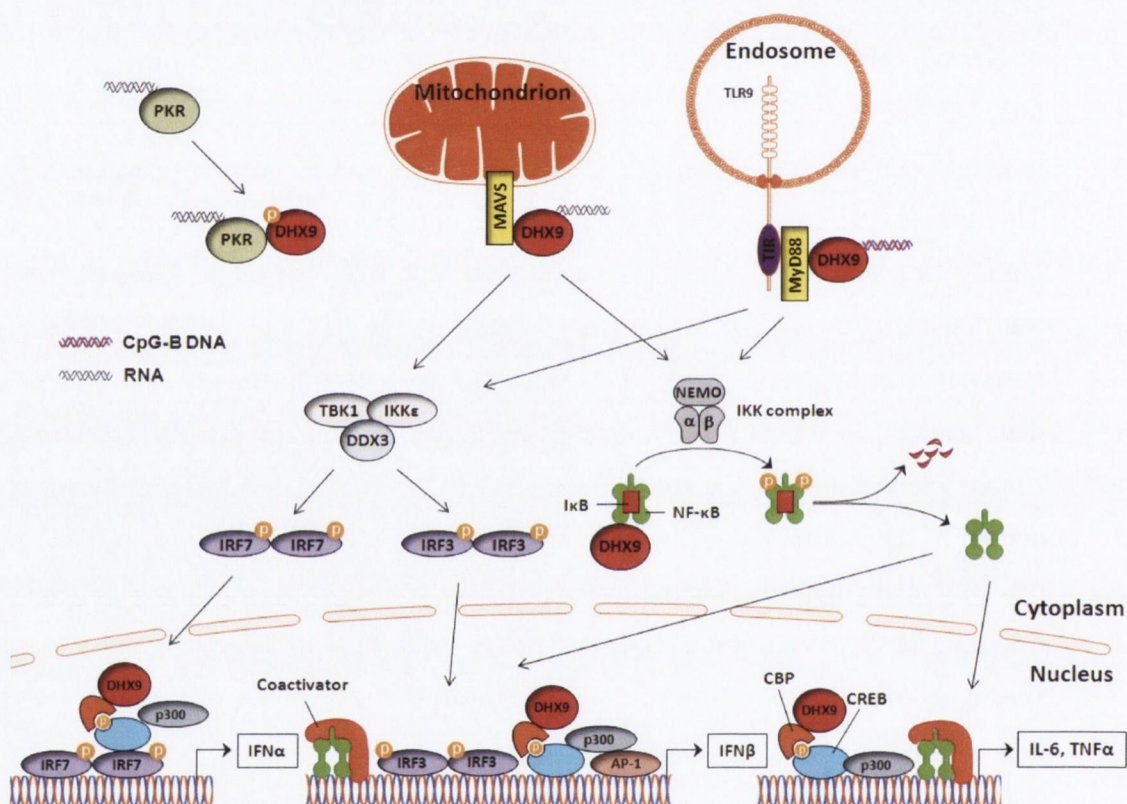


Figure 1.12 Proposed roles of DHX9 in innate immune signalling

PKR is capable of phosphorylating DHX9, disrupting its binding of dsRNA, to control transcription and viral replication. DHX9 binds p53 and is required for effective induction of NF-κB-dependent genes. DHX9 is also capable of binding CBP, mediating its association with Pol II and subsequent induction of CREB-dependent genes. DHX9 has been proposed as a sensor of CpG-B DNA in pDCs, signalling via MyD88 to activate NF-κB and upregulate IL-6 and TNFα. DHX9 was also required for effective IFNα production in response to CpG-B DNA. DHX9 was proposed as a dsRNA sensor in mDCs, signalling via MAVS to induce pro-inflammatory cytokines and type I IFN.

1.16 Aims

VACV E3 has evolved an astounding array of strategies by which it antagonises various aspects of host innate immune signalling events. Hence, when DHX9 was identified as a potential novel interacting partner of E3 by mass spectrometry (S. Keating, personal communication), the significance of this interaction was pursued. A previous mass spectrometry analysis carried out in the lab using VACV 70mer DNA as “bait”, showed DHX9 to potentially associate with VACV DNA (S. Keating, personal communication). Therefore, the aims of this project were to investigate innate immune functions of DHX9 and then explore the reason for why it is targeted by VACV E3. The following objectives were pursued:

1. Establish role(s) for DHX9 in the innate immune response
2. Map the interaction of VACV E3 and host DHX9 and test the ability of E3 to antagonise DHX9 function(s) in the innate immune response

Chapter 2: Materials and Methods

2.1 Materials

All cell culture plastics were from Corning Life Sciences and Greiner Bio-One, supplied by Fisher. All general laboratory chemicals were purchased from Sigma-Aldrich unless otherwise stated.

2.1.1 Buffers

Buffer	Composition
Luciferase assay mix	• 20 mM Tricine • 1.07 mM $(\text{MgCO}_3)_4\text{Mg}(\text{OH}_2)\cdot 5\text{H}_2\text{O}$ • 2.67 mM MgSO_4 • 0.1 M EDTA • 33.3 mM DTT • 270 M Coenzyme A • 470 mM Luciferin (Biosynth) • 530 mM ATP
NP-40 IP buffer	• 50 mM HEPES • 200 mM NaCl • 1 mM EDTA • 10 % (v/v) Glycerol • 1 % (v/v) NP-40 → pH 7.5 → Store at 4 °C, add fresh: • 10 µl/ml Aprotinin • 1 mM Sodium Orthovanadate • 1 mM PMSF
10x PBS	• 1.45 M NaCl • 39 mM NaH_2PO_4 • 227 mM Na_2HPO_4
Stacking Gel buffer	• 0.5 M Tris → pH 6.8
5x RT buffer	• 250 mM Tris-HCl (pH 8.3) • 375 mM KCl • 15 mM MgCl_2 • 50 mM DTT
50x TAE buffer	• 2 M Tris acetate • 0.05 M EDTA (pH 8.0)

10x TBS	• 250 mM Tris • 1.37 M NaCl • 27 mM KCl → pH 7.4
10x WB Running buffer	• 0.25 M Trizma base • 1.9 M Glycine • 35 mM SDS
3x WB Sample buffer	• 10 % (v/v) Glycerol • 10 % (w/v) SDS • 0.1 % (w/v) Bromophenol blue • 62.5 mM Trizma base (Fisher) → pH 6.8
1x WB Transfer buffer	• 10 % (v/v) 10x WB Transfer buffer • 20 % (v/v) Methanol
10x WB Transfer buffer	• 0.25 M Trizma Base • 1.9 M Glycine
WB Washing buffer	• 0.1 % (v/v) Tween-20/PBS

Table 2.1 Buffers

2.1.2 Cells

Cell type	Description	Medium
BEAS-2B	• Human bronchial epithelial cell line • A gift from S. Miggin (NUI Maynooth, Ireland)	• DMEM (Gibco) • 10 % (v/v) FCS (Biosera) • 10 µg/ml Ciprofloxacin (Sigma-Aldrich)
shRNA BEAS-2B	• Human bronchial epithelial cell line • Stably transfected with pTRIPZ lentiviral vector encoding DHX9-targeting or non-silencing control shRNA • Puromycin resistant	• DMEM • 10 % (v/v) FCS • 10 µg/ml Ciprofloxacin • 1 µg/ml Puromycin (Sigma-Aldrich) → 1 µg/ml doxycycline (Sigma-Aldrich) added for 72 hr to induce shRNA expression
HEK 293T	• Human Embryonic Kidney 293 cells • Expressing SV40 Large T-antigen	• DMEM • 10 % (v/v) FCS • 10 µg/ml Ciprofloxacin

HEK 293-TLR2	• Human Embryonic Kidney 293 cells • Expressing TLR2 • Neomycin resistant	• DMEM • 10 % (v/v) FCS • 10 µg/ml Ciprofloxacin • 1 mg/ml G418 (Sigma-Aldrich)
HEK 293-TLR3	• Human Embryonic Kidney 293 cells • Expressing TLR3 • Blasticidin resistant	• DMEM • 10 % (v/v) FCS • 10 µg/ml Ciprofloxacin • 10 µg/ml Blasticidin (Sigma-Aldrich)
HEK 293-TLR8	• Human Embryonic Kidney 293 cells • Expressing TLR8 • Blasticidin resistant	• DMEM • 10 % (v/v) FCS • 10 µg/ml Ciprofloxacin • 10 µg/ml Blasticidin
HeLa	• Human cervical epithelial cell line • A gift from J. O'Sullivan (TBSI, Ireland)	• DMEM • 10 % (v/v) FCS • 10 µg/ml Ciprofloxacin
Primary monocytes	• Monocytes isolated from blood buffy coat PBMCs through the use of CD14 microbeads	• RPMI (Sigma-Aldrich) • 10 % (v/v) FCS • 2 mM L-Glutamine (Sigma-Aldrich) • 50 µg/ml Gentamicin (Sigma-Aldrich)
THP-1	• Human monocyte cell line • Purchased from ECACC	• RPMI • 10 % (v/v) FCS • 2 mM L-Glutamine • 50 µg/ml Gentamicin
pMSCV THP-1	• Human monocyte cell line • Stably transfected with pMSCV plasmid alone or pMSCV plasmid encoding the VACV E3 sequence • Neomycin resistant	• RPMI • 10 % (v/v) FCS • 2 mM L-Glutamine • 50 µg/ml Gentamicin • 1 mg/ml G418

pTRIPZ THP-1	• Human monocyte cell line • Stably transfected with pTRIPZ lentiviral vector encoding DHX9-targeting or non-silencing control (NSC) shRNA • Puromycin resistant	• RPMI • 10 % (v/v) FCS • 2 mM L-Glutamine • 50 µg/ml Gentamicin • 1 µg/ml puromycin → 1 µg/ml doxycycline added for 72 hr to induce shRNA expression
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Table 2.2 Cells

2.1.3 Cell receptor ligands and virus

Receptor ligand/virus	Description	Company
cGAMP	• 3'3'-cGAMP • Cyclic dinucleotide • Binds STING and causes subsequent TBK1/IRF3 activation	Invivogen
CL075	• TLR7/8 agonist	Invivogen
IFN α	• Recombinant human IFNα	PBL Interferon Source
IL-1 α	• Recombinant human IL-1α	NCI BRB
LPS	• Lipopolysaccharide • TLR4 agonist	Alexis Biochemicals
PAM3	• Pam3CSK4 • TLR1/2 agonist	Invivogen
Poly(dA:dT)	• Double-stranded AT-rich DNA	Sigma-Aldrich
Sendai virus	• ssRNA virus • RIG-I agonist	Gift from S. Paludan (Aarhus University, Denmark)
TNF α	• Recombinant human TNF α	Peptrotech
VACV Δ E3L	• VACV lacking the <i>E3L</i> gene	Gift from B. Jacobs (ASU Biodesign Institute, USA)

VACV 70mer	F:5'-CCATCAGAAAGAGGTTTAATATTTTGTGAGACCATCGAAGAGAGAAAGAGATAAACTTTTTTACGACT-3' R:5'-AGTCGTAAAAAAGTTTTATCTCTTCTCTCTTCGATGGTCTCACAAAAATATTAAACC TCTTTCTGATGG-3' Complementary strands were annealed by boiling for 5 min at 95°C and then cooling to room temperature before use	MWG Biotech
WR VACV	- Western Reserve Vaccinia virus - dsDNA Poxvirus family member	Gift from G. Smith (Imperial College London, UK)

Table 2.3 Cell receptor ligands and virus

2.1.4 DHX9 shRNA target sequences

Name	Sequence	Company
A1	5'-ATAGTTAACAAAGTCTCTG-3'	Thermo Scientific
A7	5'-ATGACATAAACAAACATCGT-3'	Thermo Scientific
A9	5'-TTCTTGAAGTGGGTAAGTC-3'	Thermo Scientific
B8	5'-TTGGTTACTTCAACAACCA-3'	Thermo Scientific
D11	5'-TTCGTTCAATTGAGACATG-3'	Thermo Scientific
E8	5'-ATTTGATCCATGTTCTCGT-3'	Thermo Scientific
G2	5'-TGCACTTCTTGTTCTTCCT-3'	Thermo Scientific
H6	5'-TGAATGATGTTTTGCAGCT-3'	Thermo Scientific

Table 2.4 DHX9 shRNA target sequences

2.1.5 Antibodies

Antibody (anti-)	Conc.	Dilution	Company
DHX9, rabbit	0.2 mg/ml	1/1000	Novus Biologicals
HA, mouse	1 mg/ml	1/1000	Covance
IgG, rabbit	12.5 mg/ml	1/62.5 then 4 µl/IP	Sigma-Aldrich
ISG56, rabbit	Not provided	1/1000	Gift from A. Pichlmair (Max Planck Institute, Germany)
IκBα, mouse	Not provided	1/1000	Gift from R. Hay (University of Dundee, Scotland)
Mouse-IRDye® 680LT	1 mg/ml	1/10000	LI-COR
Myc, mouse	1 mg/ml	1/1000	Sigma-Aldrich
Phospho-p38, rabbit	Not provided	1/1000	Cell Signalling
Phospho-p65, rabbit	Not provided	1/1000	Cell Signalling
Phospho-STAT1, rabbit	Not provided	1/1000	Cell Signalling
Rabbit-IRDye® 800CW	1 mg/ml	1/5000	LI-COR
Total p38, rabbit	Not provided	1/1000	Cell Signalling
Total p65, mouse	0.2 mg/ml	1/1000	Santa Cruz
Total STAT1, rabbit	Not provided	1/1000	Cell Signalling
Tubulin, mouse	1 mg/ml	1/50000	Millipore
VACV E3	Not provided	1/3000	Gift from B. Jacobs (ASU Biodesign Institute, USA)
β-actin, mouse	2 mg/ml	1/50000	Sigma-Aldrich

Table 2.5 Antibodies

2.1.6 Primers

2.1.6.1 Real-time

Name	Sequence	Company
DHX9	• F: 5'-CCAGCTTTTGGGGTAGCATC-3' • R: 5'-TGAGAGCCAGATGTGGAGGA-3'	MWG Biotech
IFN β	• F: 5'-CTGCATTACCTGAAGGCCAAG-3' • R: 5'-TTGAAGCAATTGTCCAGTCCC-3'	MWG Biotech
IL-6	• F: 5'-GGTACATCCTCGACGGCATCT-3' • R: 5'-GTGCCTCTTTGCTGCTTTCAC-3'	MWG Biotech
ISG56	• F: 5'-CACCATTGGCTGCTGTTTAGCTCC-3' • R: 5'-GGCAGCCGTTCTGCAGGGTTTT-3'	MWG Biotech
β -actin	• F: 5'-CGCGAGAAGATGACCCAGATC-3' • R: 5'-GCCAGAGGCGTACAGGGATA-3'	MWG Biotech

Table 2.6 Real-time primers

2.1.6.1 Cloning

Name	Sequence	Company
DHX9 1-1270	• F: 5'-GATGAATTCTGATGGGTGACGTTAAA-3' • R: 5'-GATGGTACCTTAATAGCCGCCACC-3'	MWG Biotech
DHX9 1-300	• F: 5'-GATGAATTCTGATGGGTGACGTTAAA-3' • R: 5'-GATGGTACCTTACGGGGGCAAAATCTC-3'	MWG Biotech
DHX9 1-600	• F: 5'-GATGAATTCTGATGGGTGACGTTAAA-3' • R: 5'-GATGGTACCTTAACCATCATCATCCTT-3'	MWG Biotech
DHX9 1-779	• F: 5'-GATGAATTCTGATGGGTGACGTAAA-3' • R: 5'-GATGGTACCTTATCGGCTGCACAGGTG-3'	MWG Biotech
DHX9 301-1270	• F: 5'-GATGAATTCTGCCTGAAGATCCTTCT-3' • R: 5'-GATGGTACCTTAATAGCCGCCACC-3'	MWG Biotech

DHX9 780-1270	• F: 5'-GATGAATTCTGGCTCGTTTTG AGAGA-3' • R: 5'-GATGGTACCTTAATAGCCGCC ACC-3'	MWG Biotech
pMSCV E3	• F: 5'-GATGAATTCATGTACCCATAC GATGTTCCAGATTACGCTATGTCT AAAATCTAT-3' • R: 5'-GATATGTACTTAGACGATGTT GTAGATTACGCTCTCGAGTTAGAA TCTAATGAT-3'	MWG Biotech

Table 2.7 Cloning primers

2.1.7 Plasmids

All plasmids were ampicillin resistant unless otherwise stated.

Plasmid	Description	Source
pCMV-HA	• CMV immediate early promoter • Expresses protein containing an N-terminal hemagglutinin (HA) epitope tag • For inserts VACV E3, DHX9, IFI16, STING, MyD88 and p65	Clontech
pCMV-Myc	• CMV immediate early promoter • Used to create DHX9 fragments • Expresses protein containing an N-terminal Myc epitope tag	Clontech
IFN β P-luc	• Firefly luciferase reporter under IFNβ promoter control	Gift from T. Taniguchi (University of Tokyo, Japan)
NF- κ B-luc	• Firefly luciferase reporter under control by promoter containing decameric κB motif	Gift from R. Hofmeister (University of Regensburg, Germany)
IL-6P-luc	• Firefly luciferase reporter under IL-6 promoter control	Gift from M. Wewers (The Ohio State University, USA)
IL-6P Δ NF- κ B-luc	• Firefly luciferase reporter under IL-6 promoter control (lacking the NF-κB-binding site)	Gift from M. Wewers (The Ohio State University, USA)

IL-6PΔAP1-luc	• Firefly luciferase reporter under IL-6 promoter control (lacking the AP-1-binding site)	Gift from M. Wewers (The Ohio State University, USA)
pFR-luc	• Encodes a promoter with five tandem repeats of the yeast Gal4 binding sites that controls expression of the luciferase gene	Stratagene
Gal4-IRF3	• Plasmid encoding the IRF3 transcription factor fused to a Gal4 DNA-binding domain	Gift from K. Fitzgerald (University of Massachusetts Medical School, USA)
Gal4-IRF7	• Plasmid encoding the IRF7 transcription factor fused to a Gal4 DNA-binding domain	Gift from K. Fitzgerald (University of Massachusetts Medical School, USA)
Gal4-p65	• Plasmid encoding the full-length p65 subunit of NF-κB fused to a Gal4 DNA-binding domain	Gift from C. Jefferies (RCSI, Ireland)
pGL3 <i>Renilla</i> -luc	• <i>Renilla</i> luciferase reporter under pGL3 promoter	Promega
pTRIPZpuro	• A lentiviral shRNA inducible vector • Induction of shRNA expression in the presence of doxycycline • Eight DHX9-targeting shRNA expressing pTRIPZ plasmids purchased and NSC shRNA • Puromycin resistant	Thermo Scientific
psPAX2	• Lentiviral packaging vector	Addgene
pMD2.G	• VSV-G envelope expressing plasmid • Used in generation of lentiviruses	Addgene
pMSCVneo	• Murine stem cell virus vector derived from retroviral vectors • With packaging, integrates and stably expresses a gene of interest (used to stably express VACV E3 in THP-1 cells) • Neomycin resistant	Gift from K. Fitzgerald (University of Massachusetts Medical School, USA)

VSVg	Envelope protein-expressing plasmid used for generation of retroviruses	Gift from K. Fitzgerald (University of Massachusetts Medical School, USA)
Gag-pol	- Expresses the retroviral structural proteins - The gag region encodes genes which comprise the capsid proteins and the pol region encodes the reverse transcriptase and integrase proteins	Gift from K. Fitzgerald (University of Massachusetts Medical School, USA)

Table 2.8 Plasmids

2.2 Methods

2.2.1 Cell handling

Cells were handled under aseptic conditions in a laminar flow hood and all reagents and materials were sterile.

2.2.1.1 Cell resuscitation from liquid nitrogen stocks

Cells taken from liquid nitrogen were thawed at room temperature until only small ice crystals remained. They were then immediately poured into 20 ml pre-warmed complete medium in a 75 cm² flask (see Table 2.2 for details on medium used). All cells were maintained in a 37 °C incubator in a humidified atmosphere of 5 % CO₂.

2.2.1.2 Cell culture

All cells were grown in complete medium (Table 2.2) in either 75 cm² flasks containing 15-20 ml medium or 175 cm² flasks containing 25-30 ml medium. Cells were grown at 37 °C in a humidified atmosphere of 5 % CO₂. Once roughly 80 % confluent, cells were split and excess cells were used for experiments.

Adherent cells (HEK 293, HeLa and BEAS-2B cells) were split by trypsinization. The cell supernatants were decanted or pipetted off. The cells were washed in 5-10 ml PBS. Trypsin solution in PBS was then added to the cells (1 ml/75

cm² flask, 2 ml/175 cm² flask) and the cells were incubated at room temperature until the cells detached. The cells were then resuspended in 10 ml pre-warmed complete medium and centrifuged in 50 ml centrifuge tubes at 1000 g for 5 minutes. The supernatant was discarded and the cells resuspended in pre-warmed complete DMEM before being transferred into flasks.

Non-adherent cells (THP-1 cells and primary monocytes) were cultured in complete RPMI (see Table 2.2 for medium details) in flasks as above. The cells were split by removing some of the supernatant and adding pre-warmed complete medium directly to the flask. THP-1 cells and primary monocytes were undifferentiated for all experiments.

Cell number and viability was determined through the use of 0.4 % (w/v) trypan blue solution and a haemocytometer. Cells resuspended in supplemented medium were counted by taking an aliquot and diluting it in 0.4 % (w/v) trypan blue solution. 10 µl of this solution was taken and applied to a haemocytometer. The trypan blue is excluded by viable cells, giving them a translucent appearance under the microscope, while membrane damaged cells are seen to incorporate trypan blue and stain blue. Cells were then seeded at the density required for the experiment.

2.2.1.3 Freezing cells

A cell pellet was reconstituted in 10 % (v/v) DMSO (Fisher) in FCS to give a cell suspension of roughly 1×10^6 cells/ml. 1 ml aliquots of this suspension were immediately transferred to cryovials and they were placed in a Nalgene® “Mr. Frosty” freezing container and placed in a -80 °C freezer overnight before long term storage in liquid nitrogen.

2.2.2 Cell transfection

2.2.2.1 Transfection of cells using GeneJuice®

For 6 well plates, cells were seeded at the appropriate density, 2 ml per well, 24 hours prior to GeneJuice® (Millipore) transfection. 2 µg of DNA was transfected per well of a 6 well plate. 8 µl GeneJuice® was mixed with 92 µl serum free DMEM per

transfection and then incubated for 5 minutes at room temperature. Following incubation, the mixture was added directly to the DNA to be transfected, mixed, and left to incubate for a further 20 minutes at room temperature. The DNA-GeneJuice® mixture was then added dropwise to the seeded cells. Plates were tapped or swirled to ensure mixing, and then left to incubate for 20-24 hours at 37 °C, 5 % CO₂. For 96 well plate transfections (200 µl medium per well), the above was carried out except 0.8 µl of GeneJuice® was added to 9.2 µl serum free DMEM per transfection, with a total of 230 ng DNA transfected per well. For transfection of 10 cm plates (15 ml medium per plate), 15 µl of GeneJuice® was added to 235 µl of serum free DMEM, with a total of 8 µg DNA transfected per plate. Transfection of 15 cm plates (30 ml medium per plate) was carried out using twice the reagents used for transfection of a 10 cm plate.

2.2.2.2 Transfection of cells using Lipofectamine®2000

Cells were seeded at the appropriate density in 96 (200 µl/well) or 12 well (1 ml/well) plates, 24 hours prior to transfection. A lipofectamine®2000 (Invitrogen)-serum free medium mix was first made. Per 96 plate well to be transfected, this consisted of 0.2 µl lipofectamine®2000 added to the required amount of serum free medium required to give a total of 10 µl after addition to DNA/RNA/cGAMP. For transfection of 12 well plates, 1 µl of lipofectamine®2000 to a total volume of 50 µl serum free medium after addition of DNA/RNA was added per well. The lipofectamine®2000 and serum free medium were mixed and incubated at room temperature for 5 minutes. This mix was then added directly to the DNA, RNA or cGAMP to be transfected, mixed, and incubated for a further 20 minutes at room temperature. The DNA/RNA/cGAMP-lipofectamine®2000 mixture was then added to the seeded cells. Plates were tapped or swirled to ensure mixing and then left to incubate for the appropriate time at 37 °C, 5 % CO₂.

2.2.3 Stimulating and infecting cells

Cells were seeded at the required density 24 hours prior to stimulation/infection. Cells were either transfected with RNA, DNA or cGAMP as described in Section 2.2.2.2, stimulated with TLR ligands, cytokine receptor ligands, or infected with SeV, WR VACV or VACVΔE3L. All ligands were appropriately diluted in serum free medium so that addition of 10 µl per well gave the desired end concentration. VACV

70mer, poly(dA:dT) and cGAMP were transfected as outlined in Section 2.2.2.2. Cells transfected with lipofectamine®2000 alone served as a control. Cells were stimulated with LPS, CL075, TNF α , PAM3, IL-1 α or IFN α by adding them directly to the cell medium (after appropriately diluting). Medium alone was added to cells as a control. Poly(I:C) was either transfected with lipofectamine®2000 to activate intracellular RNA sensors (Section 2.2.2.2) or added directly to the cell medium to activate TLR3. WR VACV and VACV Δ E3L were used at a multiplicity of infection (M.O.I.) of 1 and SeV was used at a dilution of 1/200. A mock infection control was included with all infections. Cells were then incubated for the required time at 37 °C, 5 % CO₂.

SeV was a gift from S. Paludan (Aarhus University, Denmark). No virus titer was obtained and hence serial dilutions of the viral stock were carried out in order to investigate what dilution gave the most potent IFN β induction without dramatically affecting cell viability. A dilution of 1/200 was found to be optimal.

2.2.4 Quantitative real-time PCR

2.2.4.1 RNA isolation

RNA was isolated using a Roche High Pure RNA isolation Kit, following the protocol supplied. The RNA was stored at -80 °C until reverse transcription was carried out.

2.2.4.2 Reverse transcription PCR

Complementary DNA (cDNA) was synthesised from isolated RNA using M-MLV reverse transcriptase enzyme (Promega) and a self-made reverse transcription (RT) buffer. Two negative control samples were prepared for each RT in order to control for any remaining genomic DNA amplified in the real-time PCR reaction. One was prepared by replacing the enzyme with water (no RT control), the other by replacing RNA with water (no RNA control). Per reaction, 5 μ l RNA (approximately 0.5-1 μ g RNA) was mixed with 5 μ l RT mix (Table 2.9) in 8-tube PCR strips. The reaction was performed in a Thermo Hybrid PxE 0.2 PCR machine using the program described in Table 2.9. The cDNA was then diluted 1:3 by adding 20 μ l molecular grade water, and stored at -20 °C until real-time PCR analysis.

RT reaction mix	Volume (μl)	Company
Random hexamer primer (1 μg/μl)	0.5	MWG Biotech
5x RT buffer	2	Self-made (see Table 2.1)
dNTPs (2.5 mM each)	2	Promega
RNaseOUT	2	Invitrogen
Reverse Transcriptase	0.25	Promega

Temperature (°C)	Time (min)
25	10
42	60
95	3
4	hold

Table 2.9 Reverse transcription protocol

2.2.4.3 Real-time PCR

Real-time PCR was carried out using GoTaq® Master Mix and a MX3000P real-time machine (Stratagene). 7 μl of the PCR reaction mix (see Table 2.10), containing primers of the specific gene of interest, was added per well of a Thermo-Fast 96 well PCR plate alongside 3 μl of cDNA (diluted 1:3 from RT). The plate was sealed with adhesive film (Thermo Scientific), briefly centrifuged to remove air bubbles (10 seconds at 1000 g), and then applied to a MX3000P real-time PCR machine using the PCR cycle conditions in Table 2.10. All real-time data was normalised to the expression of the housekeeping gene, *β-actin*.

Real-time PCR reaction mix	Volume (μl)	Company
Foward primer (5 pmol/μl)	0.5	MWG Biotech
Reverse primer (5 pmol/μl)	0.5	MWG Biotech
GoTaq qPCR Master Mix	2.5	Promega
Molecular biology water	3.4	Promega
ROX reference dye	0.1	Promega

Temperature (°C)	Time (sec)	Number of cycles
95	120	1
95	3	40
60	30	

Table 2.10 Real-time PCR protocol

Data was analysed using the MxPro quantitative real-time PCR (qPCR) software (Agilent Technologies) and the comparative C_t method. The calculations are based on comparing the cycle numbers where fluorescence crossed a certain threshold (C_t). The mRNA expression levels were normalised to the β -actin mRNA and the RQ values were calculated from the following equations:

$$\Delta C_t = C_{t\text{target}} - C_{t\beta\text{-actin}}$$

$$\Delta C_{t\text{mean control}} = (\Delta C_{t\text{control1}} + \Delta C_{t\text{control2}} + \Delta C_{t\text{control3}})/3$$

where $\Delta C_{t\text{control1-3}}$ are ΔC_t values of biological replicates of the control sample

$$\Delta \Delta C_t = \Delta C_t - \Delta C_{t\text{mean control}}$$

$$RQ = 2^{-\Delta \Delta C_t}$$

The mean RQ values calculated from three corresponding replicates was presented as fold induction relative to the control sample set to 1. Error bars represent the standard deviation calculated using GraphPad Prism 5 software.

2.2.5 Enzyme-linked immunosorbent assay (ELISA)

Capture antibodies were made up to the manufacturer's (R&D) specified dilution in PBS and 50 μ l per well was added to high binding 96 well ELISA plates (Greiner Bio-One). Plates were then incubated overnight at 4 °C. After incubation, plates were washed three times in PBS-T (0.05 % (v/v) Tween) and tapped dry. Plates were then blocked with 200 μ l appropriate blocking solution and incubated for 1 hour at room temperature. After incubation, plates were washed in PBS-T three times and tapped dry. Cell supernatants were applied to the plates at the required dilution (diluted in blocking buffer). A blank triplicate was left on each plate containing the diluent alone. Standards were prepared at the starting concentration as specified by the manufacturer, transferred to a 96 well plate and serially diluted (1:2). All standards and samples were applied to plates at 50 μ l per well total volume for incubation overnight at 4 °C. After incubation, plates were washed three times with PBS-T and tapped dry. Detection antibody was then diluted in assay diluent according to the manufacturer's instructions and added to plates at 50 μ l per well. The plates were left at room temperature for the indicated time. They were then washed in PBS-T three times and

tapped dry. Streptavidin-HRP was diluted according to the manufacturer's instructions in assay diluent and 50 µl was added per well. This was incubated at room temperature for 20 minutes in the dark. Plates were once again washed in PBS-T three times and tapped dry before 50 µl per well of TMB substrate solution (BD Biosciences) was added. ELISA assays were then stopped by the addition of 25 µl per well of 1 M H₂SO₄. The absorbance at 450 and 540 nm was measured using a VERSAmax tunable microplate reader (Molecular Devices) and the data analysed using SoftMax Pro v4.8 software.

2.2.6 Luciferase reporter gene assay

Direct activation of the IFN β and IL-6 promoter was assayed by using a luciferase reporter gene under the control of the IFN β or IL-6 promoter, respectively. NF- κ B transcription factor activation was assayed by reporter gene assay through the use of a plasmid in which the decameric κ B motif, which binds to and responds to NF- κ B, was incorporated into a promoter driving the firefly *luciferin* gene. These reporter genes were transfected into cells and the ability of other expression vectors, or stimulation, to activate the transfected promoter was assayed by measuring the appearance of firefly luciferase protein in the lysates. The presence of the enzyme was assayed by adding a luciferase substrate to the cell lysates. The subsequent luminescence produced was measured in a luminometer and was directly proportional to the amount of promoter activation which had occurred (Figure 2.1).

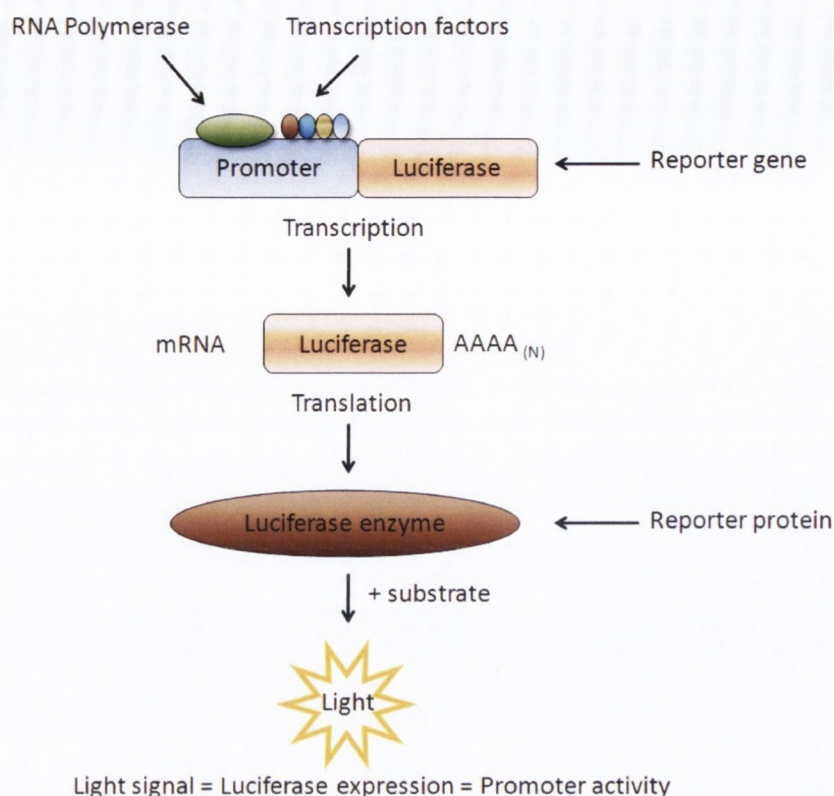


Figure 2.1 Direct activation luciferase reporter assay

A related assay was used to assay the transactivation of p65, IRF3 and IRF7. Rather than a direct measurement of transcription factor activation, this assay was based on the ability of upstream kinases to phosphorylate the transcription factor of interest in its TAD. Expression vectors encoding full-length p65, IRF3 or IRF7 fused to a Gal4 DNA-binding domain (DBD) were utilised. Their activation was assayed by the ability of the Gal4 fusion protein to transactivate the pFR luciferase reporter, which contains Gal4 binding sites in its promoter. Once bound, the pFR luciferase reporter induces the expression of firefly luciferase enzyme. Addition of luciferase substrate gives luminescence that is directly proportional to the amount of transcription factor transactivation (Figure 2.2).

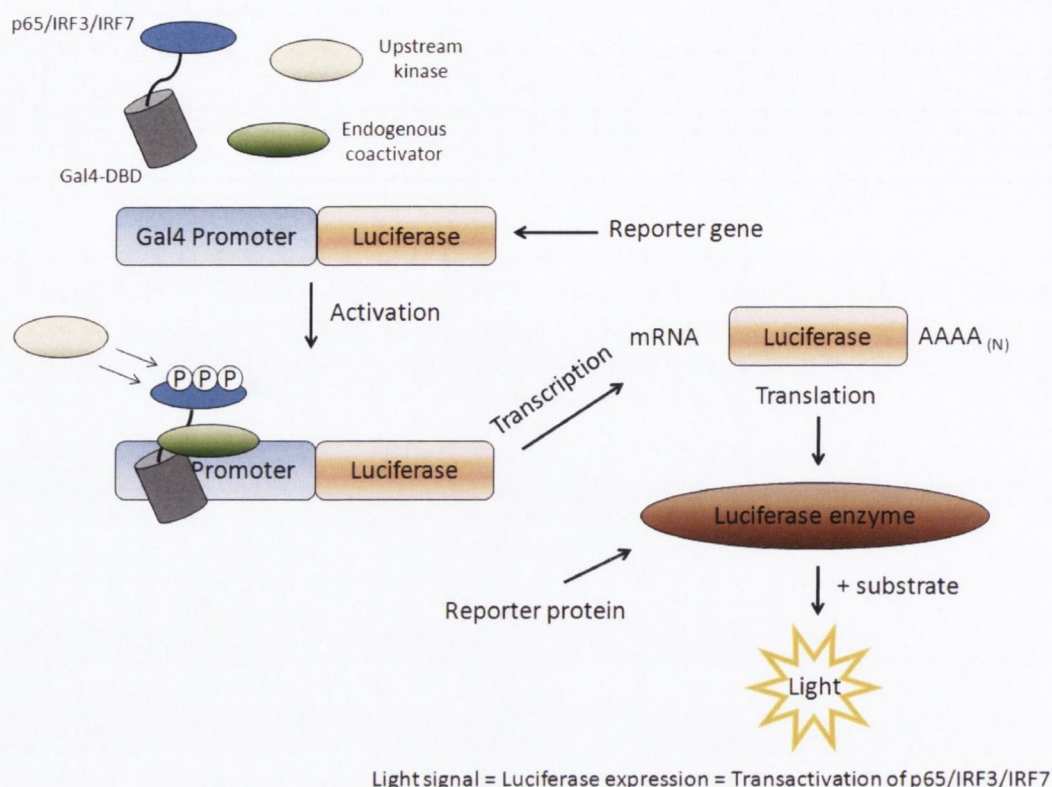


Figure 2.2 Transactivation luciferase reporter assay

For all of the luciferase reporter assays, it was important to control for transfection efficiency and any toxic effects that overexpressed proteins may have had on the cell. To do this, a *Renilla* luciferase reporter gene was cotransfected with the firefly luciferase reporter. In a similar reaction to firefly luciferase, *Renilla* luciferase uses coelenterazine as its substrate to produce light. Unlike the firefly luciferase gene, the *Renilla* luciferase gene is constitutively active and therefore should give off a similar light signal in all transfected cells regardless of stimulation. Thus the final luciferase activity was always expressed as a function of the firefly activity relative to the *Renilla* activity, in order to control for variable transfection efficiency and toxicity.

All reporter gene assays were performed in HeLa cells seeded at 0.5×10^5 cells/ml, 200 μ l per well, in 96 well plates. For NF- κ B, IFN β promoter and IL-6 promoter reporter gene assays, cells were transfected 24 hours later using GeneJuice® (see Section 2.2.2.1) with 60 ng/well of luciferase reporter expression vector, 20 ng/well of pGL3 *Renilla* as the transfection efficiency control, and the combination of expression vectors of choice. For the Gal4 assays, cells were transfected with 60 ng/well

of pFR luciferase, 20 ng/well of pGL3 *Renilla* as the transfection efficiency control, 3 ng of the Gal4 construct (Gal4-IRF3, Gal4-IRF7 or Gal4-p65), and the combination of expression vectors of choice.

The total amount of DNA transfected per well was kept at a constant 230 ng by addition of pCMV empty vector. 24 hours after transfection, cells to be stimulated were treated with ligand. After a further 24 hours, the cells were lysed with 50 µl per well of 1x passive lysis buffer (Promega). 20 µl of the lysates were transferred into two different white 96 well plates. 40 µl of luciferase assay mix (see Table 2.1 for constituents) or 40 µl of coelenterazine (2 µg/ml in PBS), as a substrate, was added to the 20 µl lysates, assaying the firefly and *Renilla* luciferase activities, respectively. Reporter gene activity was measured spectrophotometrically using a Luminoskan machine (Thermo Scientific) and Ascent Software v2.6. The final reporter activities were normalised to the activities of the *Renilla* luciferase to account for transfection efficiency within the samples. Data were presented as fold induction compared to reporter gene only-transfected cells.

2.2.7 Western Blot analysis

2.2.7.1 Preparation of cell lysates

Plates were kept on ice at all times. The supernatant was removed and the cells gently washed in PBS. For 6 well plates, the PBS was removed and the cells were scraped into 100 µl 1x gel sample buffer (see Table 2.1 for constituents). For THP-1 cells, the cells were centrifuged at 1000 g for 5 minutes in order to remove the supernatant and subsequent PBS. Samples were then sonicated, boiled at 99 °C for 5 minutes and then centrifuged briefly. For 15 cm plates used for co-immunoprecipitation studies, cells were scraped into 1 ml PBS, centrifuged at 13,400 g for 5 minutes, the PBS removed, and the cells resuspended in 450 µl 1 % (v/v) NP-40 lysis buffer containing freshly added inhibitors (see Table 2.1). They were incubated on ice for 1 hour, pipetting vigorously every 20 minutes, then centrifuged at 15,700 g (to remove cell debris) and the supernatants removed. 25 µl of 3x gel sample buffer was added per 50 µl of lysate. They were boiled for 5 minutes, briefly centrifuged and then electrophoresed as described in Section 2.2.7.2.

2.2.7.2 SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

Proteins from collected western blot samples (described in Section 2.2.7.1) were separated according to their molecular weight by SDS-PAGE. SDS gels consisted of a stacking gel casted over a resolving gel of the appropriate polyacrylamide (Protogel from National diagnostics) percentage to gain best separation (Table 2.11). 20 μ l of sample was loaded per well and the gels were electrophoresised at 100 V using a Mini-Protean Tetra cell system (Bio-Rad) and 1x running buffer (Table 2.1). Additionally, 7 μ l of prestained protein markers (NEB) were loaded per gel in order to determine the molecular weight of proteins of interest. A set of two 0.75 mm polyacrylamide gels were prepared as follows:

Resolving Gel						
Gel type (%)	H ₂ O (ml)	Protogel (ml)	1 M Tris-HCl pH 8.8 (ml)	10 % SDS (ml)	10 % APS (μ l)	TEMED (μ l)
8	4.7	2.7	2.5	0.1	50	5
12	3.4	4	2.5	0.1	50	5
15	2.4	5	2.5	0.1	50	5

Stacking gel					
H ₂ O (ml)	Protogel (ml)	0.5 M Tris-HCl pH 6.8 (ml)	10 % SDS (μ l)	10 % APS (μ l)	TEMED (μ l)
1.7	0.5	0.75	30	30	3

Table 2.11 SDS gel compositions

2.2.7.3 Transfer

The resolved proteins were transferred to 0.2 μ m PVDF membrane (Millipore) using a Biometra semi-dry transfer apparatus (constant 75 mA/membrane) for the appropriate time depending on the size of protein to be detected. The membrane was first activated by submersion in methanol and then immersed in 1x transfer buffer (see Table 2.1 for constituents). A “sandwich” was set up per transfer, consisting of three sheets of whatman paper, the membrane, the gel, and a further three sheets of whatman

paper, all soaked in transfer buffer. Air bubbles were removed by rolling a 10 ml pipette over the transfer sandwich.

2.2.7.4 Immunoblotting

Following transfer of protein, non-specific binding sites on the membrane were blocked by incubation in 3 % (w/v) BSA (Sigma-Aldrich) in TBS for 1 hour. The membrane was then incubated in primary antibody, appropriately diluted (Table 2.5) in 4 ml blocking buffer, rolling in a 50 ml Falcon® centrifuge tube overnight at 4 °C. The membrane was then washed three times, 10 minutes per wash, in 1x TBS/0.1 % (v/v) Tween (TBS-T), before incubating it in the appropriate fluorescent-labelled secondary antibody diluted in 4 ml blocking buffer (Table 2.5), rolling for 1 hour at room temperature. The membrane was then washed six times, 5 minutes per wash, in TBS-T (the final wash in TBS without Tween) and then the proteins were detected using the Odyssey Imaging system (LI-COR).

2.2.8 Co-immunoprecipitation

HEK 293T cells were seeded at a density of 2×10^5 cells/ml, 30 ml/plate, in 15 cm plates, 24 hours prior to transfection. Cells were then transfected with the required plasmids using genejuice (Section 2.2.2.1). 48 hours following transfection, the cells were scraped into 1 ml PBS, transferred to 1.5 ml microcentrifuge tubes, and lysed with 1 % (v/v) NP-40 lysis buffer as described in Section 2.2.7.1. For testing if an interaction was nucleic acid-dependent, lysates were rolled at 4 °C with or without 200 U benzonase for 1 hour before carrying out the immunoprecipitation. A fraction of each sample was removed and kept as a whole cell lysate sample. The remaining lysates were pre-cleared by adding 40 µl of a 50 % (v/v) slurry of protein A sepharose beads (Sigma-Aldrich) to each lysate and rolling them at 4 °C for 1 hour. Samples were then centrifuged for 3 minutes, 1500 g at 4°C, and the lysates transferred to fresh microcentrifuge tubes. Per immunoprecipitation, each lysate was next added to 30 µl of a 50 % (v/v) slurry of protein A beads alongside 4 µl of anti-DHX9 antibody, and rolled at 4 °C for 2 hours. An IgG control immunoprecipitation, using an equal concentration of antibody, was also carried out to ensure immunoprecipitation was not non-specific. After the 2 hours, the beads were centrifuged for 3 minutes, 1500 g at 4 °C, the supernatant removed and the beads washed five times with 1 ml lysis buffer containing

freshly added inhibitors (Table 2.1). Following the last wash, the beads were carefully pipette dry using gel loading tips and then 30 µl 3x gel sample buffer (Table 2.1) was added per sample. Samples were boiled for 5 minutes at 99 °C, centrifuged at 15700 g for 1 minute, and then electrophoresised as in Section 2.2.7.2. 10 µl of sample was resolved per control immunoprecipitation, 18 µl per experimental immunoprecipitation.

2.2.9 Amplification and purification of plasmid DNA

2.2.9.1 Transformation

NovaBlue SinglesTM Competent *E. Coli* cells (Clontech) were taken from the -80 °C freezer and thawed on ice. 5 µl was transferred to a 1.5 ml microcentrifuge tube under a bunsen flame and 1 µl of the appropriate plasmid DNA to be transformed was added. The cells were then placed on ice for 5 minutes before heat shocking for 40 seconds at 42 °C, and then placing them on ice for a further 2 minutes. 100 µl of sterile SOCS media was added to the competent cells. They were mixed and the cell suspension was spread on a LB agar (Fisher) plate containing 100 µg/ml of the selective antibiotic, ampicillin. The plate was left at room temperature for 10 minutes to allow the inoculum to soak into the agar, and was then inverted and incubated overnight at 37 °C, before picking of single colonies the following day for Miniprep or Maxiprep purification. The agar plates were stored at 4 °C.

2.2.9.2 Miniprep

A single colony was picked from an agar plate, using a yellow tip, and inoculated into 5 ml LB broth (Fisher) containing 100 µg/ml ampicillin. The culture was grown overnight, shaking at 200 rpm, 37 °C. The following day, 2 ml of the bacterial culture was centrifuged at 5000 g for 5 minutes and plasmid DNA was isolated from the bacterial pellet using a QIAprep Miniprep kit (Qiagen) following the manufacturer's instructions. DNA was eluted in 50 µl elution buffer and was stored at -20 °C until further use. Plasmid DNA obtained from Minipreps was used for cloning, restriction enzyme digestions and sequencing.

2.2.9.3 Maxiprep

As a starter culture, 3 ml of LB broth containing 100 µg/ml ampicillin was inoculated with a single bacterial colony in a 15 ml centrifuge tube and incubated for 6-8 hours shaking at 200 rpm, 37 °C. 1 ml of this culture was then taken and added to 100 ml of LB broth containing 100 µg/ml ampicillin and incubated overnight, shaking at 200 rpm, 37 °C. The following day, the culture was centrifuged in a Sorval centrifuge at 6000 g, 4 °C, for 15 minutes. Plasmid DNA was isolated from the cell pellet using a Endofree Plasmid Purification Kit (Qiagen). The DNA obtained was resuspended in 500 µl buffer TE and its concentration was determined spectrophotometrically at 260 nm using a NanoDrop (Thermo Scientific). Plasmid DNA was stored at 4 °C (or -20 °C for long term storage).

2.2.10 Cloning

2.2.10.1 Cloning of DHX9 fragments into pCMV-Myc

Full-length DHX9 and fragments based on its domains (Figure 4.3 A), were subcloned from HA-tagged DHX9 plasmid (DHX9 in a pCMV-HA vector) into a pCMV-Myc vector (Figure 2.3). In order to subclone DHX9 fragments, primers were designed (Table 2.7) with *EcoRI* and *KpnI* restriction enzyme sites that bound to the 5'- and 3'-ends of the DHX9 fragment to be cloned into pCMV-Myc vector. The DHX9 inserts were amplified using PfuTurbo® polymerase (Stratagene) according to the optimised protocol presented in Table 2.12.

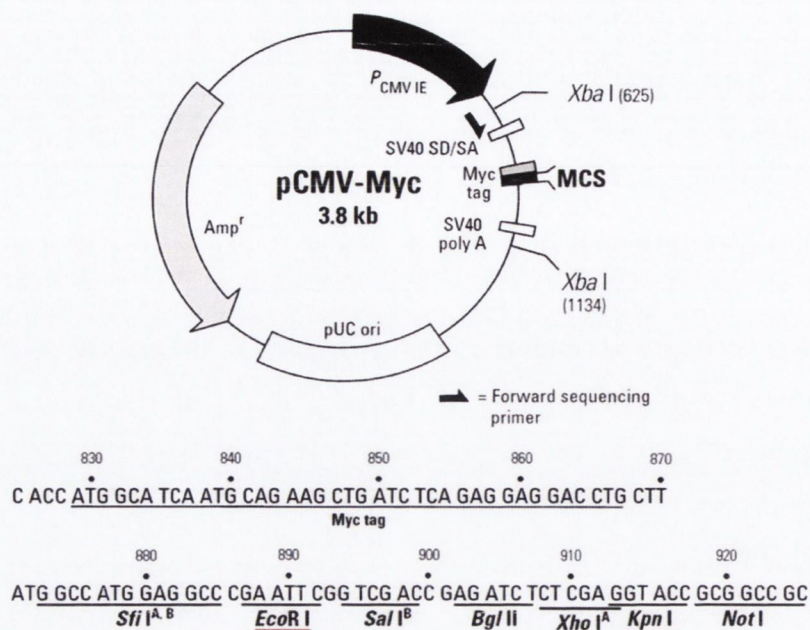


Figure 2.3 pCMV-Myc vector map and multiple cloning site (MCS)

PCR reaction mix (50 µl total)	Volume (µl)	Temperature (°C)	Time (min)	Number of cycles
10x Pfu buffer	5	95	10	1
dNTPs (25 mM each)	0.4	95	0.5	35
100 ng DNA template (100 ng/µl stock)	1	58	1	
20 pmol Forward primer (20 pmol/µl stock)	1	68	4.5	
20 pmol Reverse primer (20 pmol/µl stock)	1	68	10	1
PfuTurbo® polymerase	1	4	hold	
Molecular biology water	40.6			

Table 2.12 PCR protocol to amplify DHX9 fragments

Following PCR amplification, the PCR products were resolved on a 1 % (w/v) agarose gel (see Section 2.2.11), visualised under UV, excised from the gel, and purified using an Agarose GelExtract Mini Kit (5 Prime). Purified PCR products and

the target pCMV-Myc expression vector were digested overnight with *EcoRI* and *KpnI* (NEB) (Table 2.13).

PCR product digestion mix	Volume (μl)	pCMV-Myc digestion mix	Volume (μl)
PCR DNA product	50	1 μg pCMV-Myc	x
<i>EcoRI</i>	1	<i>EcoRI</i>	1
<i>KpnI</i>	1	<i>KpnI</i>	1
10x NEB Buffer 1	6	10x NEB Buffer 1	2
100x BSA	0.6	100x BSA	0.2
Molecular biology water	1.4	Molecular biology water (to final 20 μl)	x

Table 2.13 Digestion protocols for DHX9 inserts and pCMV-Myc plasmid

Digested DNA products were purified from a 1 % (w/v) agarose gel using a gel extraction kit. Digested DHX9 inserts and pCMV-Myc vector were then ligated using a Clonables™ Ligation/Transformation Kit. The ligation mix was incubated for 15 minutes at 16 °C in a PCR machine (Table 2.14).

Ligation reaction mix	Volume (μl)
2x Ligation mix	2.5
Digested vector	0.5
Digested insert	1.5
Molecular biology water	0.5

Table 2.14 Ligation reaction mix

1 μl of the ligated plasmid DNA was transformed into 5 μl competent bacteria as described in Section 2.2.9.1. The plasmid DNA was then isolated by minipreps as described in Section 2.2.9.2 and insert-containing clones were selected by double digesting 20 μl of the minipreps (similar to Table 2.13 protocol) with *EcoRI* and *KpnI* and resolving the DNA on a 1 % (w/v) agarose gel. Miniprep clones found to have an

insert of the correct estimated size were then sent for DNA sequencing. Upon sequence verification, maxipreps of the clones were carried out as described in Section 2.2.9.3 and their expression was tested by overexpression in HEK 293T cells by genejuice transfection (Section 2.2.2.1) and Western Blot analysis (Section 2.2.7).

2.2.10.2 Cloning of VACV E3 into pMSCVneo

VACV E3 was subcloned from HA-tagged E3 plasmid (E3 in a pCMV-HA vector) into a pMSCV vector (Figure 2.4). Primers were designed (Table 2.7) with *EcoRI* and *XhoI* restriction enzyme sites that bound to the 5'- and 3'-ends of the E3 to be cloned into pMSCV vector. The forward primer encoded a HA tag for detection of the construct by immunoblot with a HA antibody. E3 was amplified using PfuTurbo® polymerase according to the optimised protocol presented in Table 2.15.

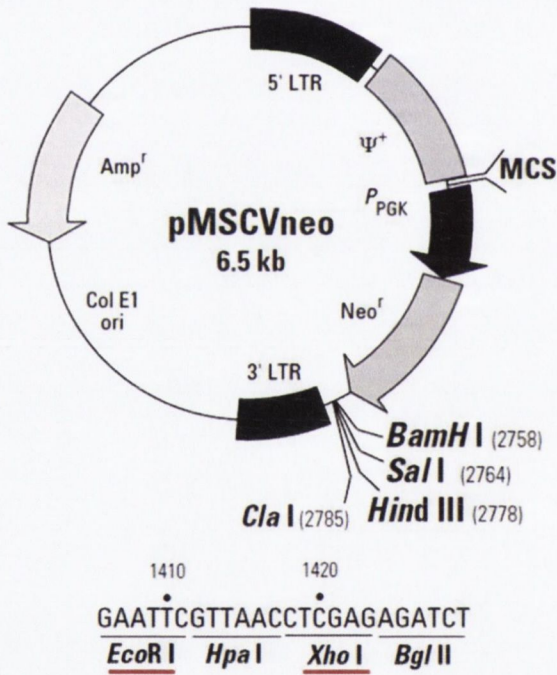


Figure 2.4 pMSCVneo vector map and multiple cloning site (MCS)

PCR reaction mix (50 µl total)	Volume (µl)
10x Pfu buffer	5
dNTPs (25 mM each)	0.4
100 ng DNA template (100 ng/µl stock)	1
20 pmol Forward primer (20 pmol/µl stock)	1
20 pmol Reverse primer (20 pmol/µl stock)	1
PfuTurbo® polymerase	1
Molecular biology water	40.6

Temperature (°C)	Time (min)	Number of cycles
95	1	1
95	0.5	35
60	0.5	
72	1	
72	10	1
4	hold	

Table 2.15 PCR protocol to amplify E3

Following PCR amplification, the PCR product was resolved on a 1 % (w/v) agarose gel (see Section 2.2.11), visualised under UV, excised from the gel and purified using an agarose gel extraction kit. Purified PCR product and the target pMSCV expression vector were digested overnight with *EcoRI* and *XhoI* (Table 2.16).

PCR product digestion mix	Volume (µl)
PCR DNA product	50
<i>EcoRI</i>	1
<i>XhoI</i>	1
10x NEB Buffer 4	6
100x BSA	0.6
Molecular biology water	1.4

pCMV-Myc digestion mix	Volume (µl)
1 µg pMSCV	x
<i>EcoRI</i>	1
<i>XhoI</i>	1
10x NEB Buffer 4	2
100x BSA	0.2
Molecular biology water (to final 20 µl)	x

Table 2.16 Digestion protocols for E3 insert and pMSCV plasmid

Digested E3 insert and pMSCV were purified, ligated, and transformed into competent bacteria (as described in Section 2.2.10.1). As in Section 2.2.10.1, colonies were tested for inserts, sent for sequencing and then tested for expression in HEK 293T cells.

2.2.11 Agarose gel electrophoresis

DNA was separated based on size using a 1 % (w/v) agarose gel. 1 g of agarose powder added to 100 ml of TAE buffer (see Table 2.1), was microwaved until completely dissolved. Once cooled to below 60 °C, 10 µl of ethidium bromide (5 mg/ml stock) was added, and the solution was mixed through swirling. The solution was then poured into an agarose gel loading rig containing a comb. The gel was let set for 30-40 minutes and then submerged in TAE buffer in an agarose gel running apparatus. Samples were appropriately diluted in 6x DNA loading dye (NEB), and loaded in the gel. 1 µg of 2 log DNA ladder (NEB) was loaded on each gel in order to quantify the size of DNA fragments present in the samples. The samples were then electrophoresed at 100 V until appropriate separation of DNA bands had been acquired. The gel was then taken out and placed in a UVP BioImaging system darkroom cabinet where DNA bands could be viewed and excised from the gel where necessary.

2.2.12 Generating DHX9 and NSC shRNA-expressing cells

Generating THP-1 and BEAS-2B cells stably expressing doxycycline-inducible shRNA involved making shRNA encoding-lentiviruses in HEK 293T cells and then infecting THP-1 and BEAS-2B cells with the lentivirus-containing supernatants. Infected target cells were then selected for shRNA-expressing cells. The process is summarised in Figure 2.5.

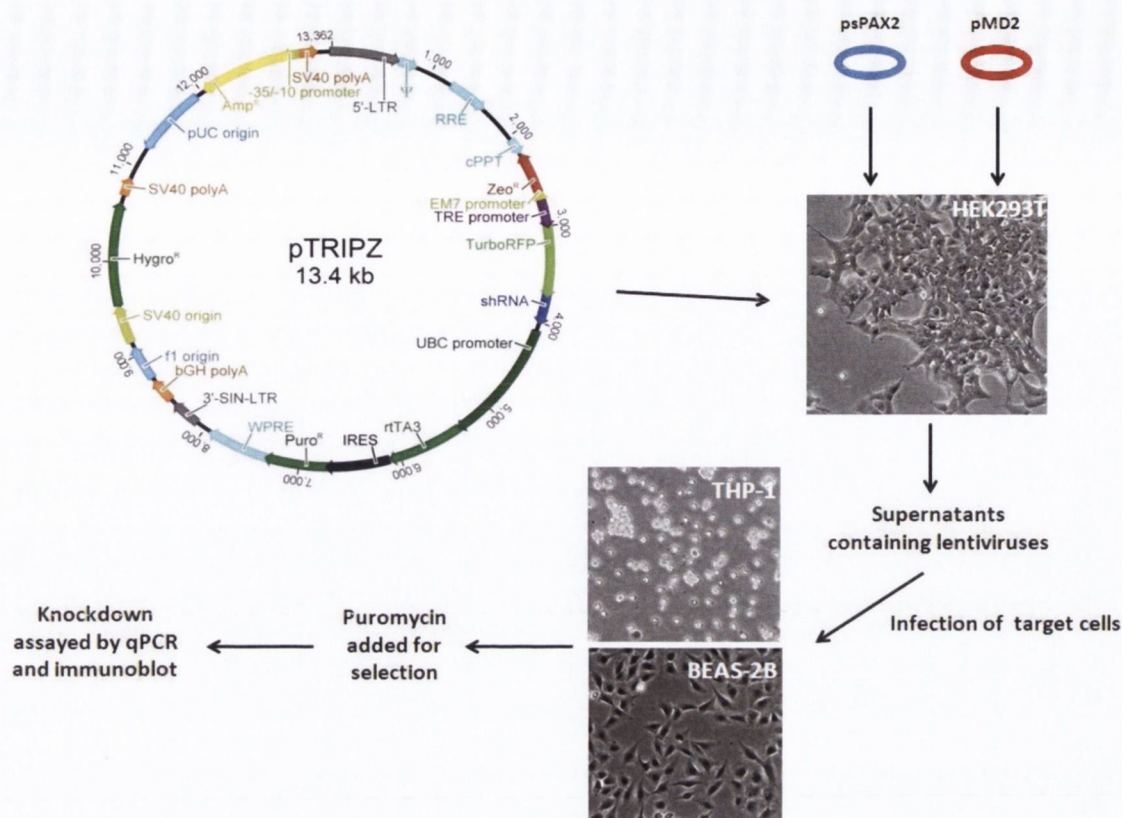


Figure 2.5 Generating doxycycline-inducible shRNA-expressing cells

2.2.12.1 Generating lentiviruses

Eight DHX9-targeting shRNA pTRIPZ vectors, purchased from Thermo Scientific Open Biosystems, were initially employed (target sequences in Table 2.4). A NSC shRNA designed with minimal homology to known mammalian genes was also used. HEK 293T cells, seeded at 1×10^5 cells/ml in 10 cm plates 24 prior, were genejuice-transfected (see Section 2.2.2.1) with 4 μ g shRNA-expressing pTRIPZ vector, 3 μ g psPAX2 vector (lenticorn packaging) and 1 μ g pMD2 vector (lenticorn envelope). 48 hours later, the supernatants were harvested and replaced with fresh media. The lentivirus-containing supernatants were centrifuged at 1000 g for 5 minutes to remove any contaminating cells, and then filtered through 0.45 μ m filters. They were stored at 4 $^{\circ}$ C until use. The supernatants were harvested again at 72 hours, centrifuged, and filtered.

2.2.12.2 Infection and selection of target cells

THP-1 or BEAS-2B cells were seeded at 2×10^6 cells per 10 cm plate (10 ml medium per plate). 24 hours later, THP-1 cells were centrifuged at 1000 g for 5 minutes and resuspended in 50 % (v/v) complete RPMI (5 ml) and 50 % (v/v) lentivirus supernatant medium (5 ml) with 4 μ g polybrene (hexadimethrine bromide - made up in molecular biology water and filter sterilised). Adherent BEAS-2B cells had their supernatant pipetted off and then 50 % (v/v) complete DMEM and 50 % (v/v) lentivirus supernatant (with polybrene) was gently added to the cells. The cells were incubated at 37 °C for 48 hours. The transduction step was then repeated in the absence of polybrene i.e. the cells were resuspended in 50 % (v/v) RPMI/DMEM and 50 % (v/v) lentiviral supernatant. After a further 48 hours the cells were selected by the addition of 1 μ g/ml puromycin. The cells were then left for 5-7 days at 37 °C and then tested for shRNA expression. shRNA expression was assayed by incubating the cells in 1 μ g/ml doxycycline for 24, 48 and 72 hours and looking for expression of GFP by fluorescent microscopy. After confirmation of GFP expression upon the addition of doxycycline, DHX9 protein and mRNA knockdown were assayed. A total of eight clones were tested (see Table 2.4) and the three clones (A7, G2 and D11) found to give the most effective knockdown of DHX9 mRNA and protein were later employed, alongside the NSC cell line.

2.2.13 Generation of THP-1 cells stably expressing VACV E3

THP-1 cells stably expressing VACV E3 were generated using a retroviral system. E3 was firstly subcloned from pCMV-HA vector into the retroviral vector, pMSCVneo (see Section 2.2.10.2). The protocol carried out for stable expression of E3 in THP-1 cells was very similar to that for generating cells stably expressing shRNA through the use of a lentiviral system (Figure 2.5). The protocol only differed in the plasmids, amount of polybrene, and the antibiotic for selection, used (refer to Section 2.2.12.1). HEK 293T were transfected with 4 μ g of pMSCV vector encoding E3, 1 μ g VSVg (expresses envelope proteins) and 1 μ g of Gag-pol (expresses packaging proteins). HEK 293T cells were also transfected with pMSCV empty vector (pMSCV-EV), VSVg and Gag-pol to serve as control cells not expressing E3. The supernatants were collected as in Section 2.2.12.1 and then THP-1 cells were transduced with the retroviral supernatants as was done for lentiviral infection, except 0.5 μ g (rather than 4

µg) of polybrene was used (Section 2.2.12.2). Selection of the cells was carried out using 1 mg/ml G418 (instead of puromycin) and the same protocol as for selection of shRNA-expressing THP-1 cells was carried out (Section 2.2.12.2). After selection, lysates of pMSCV-EV- and pMSCV-E3-infected THP-1 cells were generated and the expression of E3 was tested by immunoblotting for HA (Section 2.2.7).

2.2.14 Isolation of human PBMCs and primary monocytes

Human primary peripheral blood mononuclear cells (PBMCs) were isolated from buffy coats of heparinised blood provided by the Irish blood transfusion clinic at St. James's Hospital, Dublin. The buffy coat was diluted 1:7 in PBS and then 35 ml aliquots of it were carefully layered onto 15 ml lymphoprep (Axis-Shield) in 50 ml centrifuge tubes. The tubes were then centrifuged at 350 g for 30 minutes at room temperature in a swing-out rotor with the brake off. After centrifugation, the PBMCs comprised a white layer of cells between the lymphoprep/serum interface. Careful not to disturb the interface, the cells were transferred to 20 ml complete RPMI in a 50 ml centrifuge tube using a transfer pipette. The cells were then centrifuged at 350 g for 5 minutes, washed again in complete RPMI, and then resuspended in 10 ml 1x red blood cell lysis solution (Miltenyi Biotec), and incubated at room temperature for 10 minutes. After the incubation, the cells were washed in complete RPMI and then resuspended in an appropriate volume of complete RPMI for cell counting.

Primary monocytes were purified from PBMCs isolated from blood buffy coats, through the use of CD14 microbeads and a magnetic LS column (Miltenyi Biotec), according to the manufacturer's instructions.

2.2.15 Statistical analysis

Graphs were generated following transfer of data into GraphPad Prism v.5.02 software. Statistical analysis was performed using the two-tailed unpaired student *t* test.

**Chapter 3: Investigating innate
immune functions of the host helicase
DHX9**

3.1 Introduction

Having coevolved alongside the host immune system, viruses have mastered numerous strategies to evade and subvert the host antiviral response. VACV encodes a number of proteins which interact with and manipulate numerous aspects of innate immune signalling [380]. One gene encoding such a protein is VACV *E3L*, which encodes the protein E3. E3 is a multifunctional protein which has been found to be vital in VACV replication and pathogenesis [362]. Because of the major importance of E3 in VACV viral evasion, mass spectrometry was previously carried out in the lab by Dr. Sinead Keating in order to identify other possible target proteins within the host with which E3 interacts (S. Keating, personal communication). This experiment was carried out using an E3 antibody to immunoprecipitate possible E3-interacting proteins from cells infected with either wild type WR VACV or virus lacking the *E3L* gene (VACV Δ E3). Immunoprecipitated proteins were then resolved on a SDS gel and the entire gel was dissected and analysed in slices and compared to the control immunoprecipitation protein bands. Proteins eluted from gel slices were sent for analysis by Liquid Chromatography-Mass Spectrometry. Based on these analyses, DHX9 was identified as a potential E3-interacting protein in both HEK 293 and MEF cells (S. Keating, personal communication).

VACV 70mer, a dsDNA 70-base pair motif that is conserved and tandemly repeated in the termini of many poxviral genomes, is capable of inducing IFN β in numerous cell types [259]. This motif has also previously been shown, via mass spectrometry, to interact with DHX9 (S. Keating, personal communication). Given that DHX9 was identified as an interacting protein of both E3 and VACV dsDNA 70mer, we hypothesised that DHX9 was likely to be important in the VACV viral life cycle or as an antiviral protein limiting viral spread. We further hypothesised that VACV, in turn, may be either associating with DHX9 in order to inhibit the immune response or it may be sequestering DHX9 for its viral replication. In order to investigate the former possibility, innate immune functions of DHX9 were first studied before investigating the ability of VACV E3 to antagonise its activity.

DHX9 has been shown to play a role in almost all aspects of RNA metabolism and is capable of unwinding both dsRNA and dsDNA [294]. DHX9 couples the

transcription and translation of both cellular and retroviral mRNAs [369, 370], as well as interacting with several transcription regulators [294]. There is now evidence that DHX9 plays a significant role in innate immune signalling. DHX9 has previously been shown to interact with the p65 subunit of NF- κ B and be involved in mediating the transcriptional activity of NF- κ B [298]. DHX9 has also been found to be a component of the STAT6-dependent enhanceosome, where it is required for IL-4 induced STAT6-mediated transcription [381]. A further publication showed DHX9 is required for Pol II recruitment to CBP and subsequent CREB-dependent transcription [371].

Furthermore, recent work has identified DHX9 as playing an integral role in nucleic acid sensing. In 2010, DHX9 was proposed as a novel CpG-B DNA sensor in pDCs [270]. DHX9 was demonstrated to be important in TNF α and IL-6 production and NF- κ B activation in response to CpG-B DNA. DHX9 was reported to do so in a MyD88-dependent fashion, associating with the TIR domain of MyD88 via its HelicC-HA2-DUF domains. DHX9 was proposed to directly bind CpG-B DNA via its DUF domain [270]. Interestingly, although primarily characterised as a regulator of NF- κ B-dependent genes by these authors, DHX9 was also shown to be partially required for CpG-B-DNA-stimulated IFN α , where siRNA knockdown of DHX9 significantly dampened IFN α secretion [270]. These authors later discovered that DHX9 paired MAVS to sense dsRNA in mDCs [222]. DHX9 was demonstrated to associate with the CARD domain of MAVS via its HelicC-HA2-DUF domains and with dsRNA via its dsRNA-binding motifs. Interestingly, the cytokine readouts affected were similar to those published in their earlier work, with IFN α , TNF α and IL-6 secretion being significantly affected in response to poly(I:C) and reovirus infection [222]. Fascinatingly, while in their previous study they claimed that DHX9 is necessary for CpG-B and not CpG-A DNA-stimulated IFN α secretion in pDCs [270], their later work demonstrated that DHX9 was crucial for CpG-A DNA stimulated IFN α in mDCs [222], indicating that the role of DHX9 in nucleic acid sensing may be cell type-specific.

As DHX9 is a helicase which has been demonstrated to bind and unwind both RNA and DNA nucleic acid [294], it is likely to interact with most forms of nucleic acid, including poly(I:C) and CpG DNA. Therefore, it is difficult to conclude with certainty that DHX9 is a bona fide DNA or RNA sensor. With previous studies on

DHX9 having reported it to be capable of binding p65 and several transcriptional coactivators, it is difficult to rule out the possibility that DHX9 may be functioning further downstream in signalling events, possibly more at a promoter or transcriptional level [294, 298]. Although it is evident that DHX9 has a role in PRR signalling, further work needs to be carried out in order to elucidate the specific functions of DHX9 and where it precisely functions in signalling.

We therefore investigated the role of DHX9 in both nucleic acid sensing and non-nucleic acid sensing PRR signalling, focusing on the human monocytic cell type THP-1, both a useful model system for PRR signalling and amenable to shRNA loss-of-function studies.

3.2 Results

3.2.1 No role for DHX9 in DNA- or RNA-induced responses in THP-1 cells

As noted earlier (Section 1.15), DHX9 has been proposed as both a DNA- and RNA-sensor, capable of inducing type I IFN [222, 270]. DHX9 was also found to interact with VACV 70mer (S. Keating, personal communication), a dsDNA 70-base pair motif that is conserved and repeated multiple times in many poxviral genomes and capable of inducing IFN β in numerous cell types [keating259]. As VACV E3 was found to interact with host DHX9 via mass spectrometry (S. Keating, personal communication), it was hypothesised that the interaction may be a means by which E3 antagonises the sensing of VACV nucleic acid. Type I IFNs are vital in antiviral innate immunity. Hence, the ability of DHX9 to induce the IFN β promoter in HeLa cells was firstly examined using a luciferase-based assay system. As a number of the currently identified DNA receptors require STING for downstream signalling [382], DHX9 was overexpressed with and without STING to investigate its ability to stimulate the IFN β promoter. IFI16, a known cytosolic DNA sensor which is dependent on STING [259], was used as a positive control for promoter activation. Interestingly, while both IFI16 and DHX9 were unable to enhance the IFN β promoter by themselves, they were capable of strongly inducing the promoter in a dose-dependent manner upon the addition of STING (Figure 3.1). Although STING was capable of activating the IFN β promoter to some degree by itself, the highest dose of DHX9 was able to potentiate this activity approximately five-fold at all concentrations of STING used. IFI16 was also capable of dose-dependently activating the IFN β promoter upon the addition of STING, but to a lesser extent than that of DHX9 (Figure 3.1).

As DHX9 was found to activate the IFN β promoter in HeLa cells, we next investigated its ability to modulate this promoter in a more physiologically relevant cell line. Stable cell lines were generated using a pTRIPZ lentiviral system in which a doxycycline-inducible shRNA construct targeting DHX9 mRNA, was introduced into THP-1 cells. Three stable cell lines knocking down DHX9 mRNA were selected, A7, G2 and D11, alongside a NSC shRNA cell line designed with minimal sequence homology to any known mammalian genes. Before carrying out any experiments, each of the cell lines were incubated with or without 1 μ g/ml doxycycline for 72 hours, and

the level of DHX9 protein and mRNA was assessed by SDS-PAGE and qPCR, respectively. DHX9 mRNA was found to be reduced by up to 70-80 % for all three stable cell lines (G2, A7 and D11) compared to the NSC cell line (Figure 3.2 B). This correlated with DHX9 protein, with effective knockdown observed for each of the DHX9 shRNA-expressing cell lines when compared to the uninduced, or NSC cell line upon addition of doxycycline (Figure 3.2 A). Also, the addition of doxycycline to the NSC stable cell line did not appear to affect the basal levels of DHX9 protein. The NSC cell line, alongside the A7 and G2 cell lines, were stimulated with VACV 70mer, cGAMP or poly(dA:dT). cGAMP is a second messenger produced in mammalian cells by cGAS in response of dsDNA in the cytoplasm, to activate innate immune responses by binding to STING and subsequently inducing the TBK1/IRF3-dependent production of IFN β [383]. Poly(dA:dT) is a synthetic DNA ligand which is recognised by several sensors including AIM2, DAI and indirectly by RIG-I [210]. Like IFN β , RANTES and IP10 are IRF3-dependent and are more convenient to measure than IFN β production in human cells. 70mer-, cGAMP- or poly(dA:dT)-stimulated RANTES (Figure 3.3 A) or IP10 (Figure 3.3 B) were unaffected upon silencing of DHX9. IFN β mRNA upregulation upon RNA and DNA stimulation was also assayed upon silencing of DHX9 (Figure 3.4). There was an initial difficulty in the selection of the NSC shRNA stable cell line. Thus, for these experiments, gene induction was compared to cells treated or not treated with doxycycline. Effective DHX9 mRNA knockdown was seen for each of the cell lines when compared to doxycycline negative control cells (Figure 3.4 A, B). DHX9 was not found to be required for poly(I:C)- or 70mer-induced IFN β mRNA (Figure 3.4 C, D). Thus, it was concluded that although DHX9 was capable of activating the IFN β promoter alongside STING by reporter gene assay in HeLa cells, it was found to have no role in RNA- or DNA-stimulated chemokines or IFN β mRNA in THP-1 cells.

3.2.2 Requirement for DHX9 in IFN α -stimulated ISG56 mRNA induction but not for ISG56 protein production

IFN signalling is critical in the upregulation of a vast array of ISGs which are necessary for mounting an effective antiviral response [384]. Therefore, whether DHX9 may have a role in the IFN signalling pathway was next investigated. Numerous ISGs were measured by qPCR after 6 hours stimulation with 70mer, poly(I:C) or IFN α .

Effective DHX9 mRNA knockdown was seen for each of the cell lines when compared to doxycycline negative control cells (Figure 3.5 A, B). ISG56 upregulation upon IFN α stimulation was found to be roughly 50 % less than that of the non-doxycycline control for all three stable cell lines (Figure 3.5 C, D, G2 not shown). Other ISGs tested, namely ISG54, OAS2 and PKR, were not found to be as reproducibly affected as ISG56 mRNA (data not shown). Thus, a more detailed analysis was carried out using an IFN α -stimulation time course (Figure 3.6). Each cell line, with and without the addition of doxycycline for 72 hours, was stimulated for a range of different times over a 24 hour period. With both DHX9 mRNA (Figure 3.6 A, B) and protein (data not shown) being greatly reduced upon addition of doxycycline, ISG56 mRNA levels were found to be decreased at all time points tested (Figure 3.6 C, D). Upon generating a NSC cell line, each of the DHX9-silencing cell lines, alongside the NSC cell line, were incubated in doxycycline for 72 hours prior to each experiment. Each experiment was then compared to the NSC cell line with the addition of doxycycline. Controlling with the NSC stable line, DHX9 was still found to be required for ISG56 mRNA induction, with ISG56 mRNA being reduced by roughly 50 % for each of the A7, G2 and D11 cell lines (data not shown).

As DHX9 is necessary for effective IFN α upregulation of ISG56 mRNA, whether DHX9 is somehow required in IFN signalling was next assayed. It was thought that DHX9 may be functioning at some point in the JAK-STAT signalling pathway. This was investigated by SDS-PAGE in which activation of the JAK-STAT signalling cascade was assayed through the use of a phospho-STAT1 antibody. The phosphorylation of STAT1 was found to be unaffected in all three DHX9-silencing stable cell lines (only A7 is shown) compared to NSC stable cell line lysates (Figure 3.7). Phosphorylation of STAT1 was found to occur rapidly, at 15 minutes, and then to dissipate at later time points, with very little phospho-STAT1 being detected at 24 hours IFN α stimulation. ISG56 protein was also assayed and although DHX9 appeared to be necessary for ISG56 mRNA induction, DHX9 was not required for ISG56 protein expression. In the immunoblot in Figure 3.7, DHX9 appears to be subtly required for both total STAT1 and ISG56 protein expression. However in repeating the experiment numerous times, this was not reproducible and may be explained here due to variation

in the quantity of protein loaded. DHX9 may therefore be necessary for the transcription of ISG56 mRNA but this did not translate to an effect on ISG56 protein expression.

3.2.3 Role for DHX9 in TLR- and RNA virus-stimulated cytokines in THP-1 cells

Having found no role for DHX9 in nucleic acid sensing in THP-1 cells, further roles for DHX9 in innate immunity were investigated in these cells by stimulating the shRNA stable cell lines with a range of stimuli and then measuring cytokine secretion by ELISA. The NSC, A7 and G2 cell lines were incubated in doxycycline for 72 hours in order to induce production of the required shRNA, and were then stimulated for 24 hours with a number of ligands. The cells were firstly infected with SeV and DHX9 was found to have a key involvement in SeV-stimulated cytokine responses (Figure 3.8). Although no effect was found on SeV-stimulated RANTES (Figure 3.8 A), TNF α (Figure 3.8 B) or IP10 (Figure 3.8 E) production upon knockdown of DHX9, both SeV-stimulated IL-6 (Figure 3.8 C) and IFN α (Figure 3.8 D) secretion were significantly reduced in two separate DHX9 shRNA-expressing cell lines compared to the NSC cell line.

Numerous TLR agonists were employed to test if DHX9 was required for optimal TLR-dependent cytokine production (Figure 3.9). The NSC cell line, alongside two DHX9-targeting shRNA cell lines, were stimulated for 24 hours with a range of TLR agonists and then the supernatants were removed and analysed by ELISA. PAM3 was used to activate TLR1/2, CL075 to activate TLR8, and to a lesser extent, TLR7, and LPS to activate TLR4. Surprisingly, DHX9 was found to play a critical role in the regulation of numerous TLR-stimulated cytokines. Cytokine readouts in which significant differences occurred upon shRNA knockdown of DHX9 included RANTES, TNF α , IL-6 and IP10 production, depending on the exact TLR stimulus. Although PAM3- and LPS-stimulated RANTES production was unaffected upon DHX9 silencing, CL075-stimulated RANTES production was consistently observed to be reduced by up to 50 % (Figure 3.9 A). A requirement of DHX9 for effective CL075-stimulated TNF α production was also identified, LPS-stimulated TNF α was unaffected, and PAM3-stimulated TNF α production appeared to be significantly increased upon DHX9 knockdown (Figure 3.9 B). Reduction in IL-6 protein production upon silencing

of DHX9 was also seen, with IL-6 secretion being reduced by over 50 % for all of the stimuli tested (Figure 3.9 C). DHX9 was also necessary for CL075-dependent IP10, while it was not required for effective PAM3- or LPS-stimulated IP10 (Figure 3.9 E). Thus DHX9 was required for all CL075-stimulated cytokines tested, and for all the stimulants of IL-6 tested.

3.2.4 DHX9 is critical to effective IL-6 production

IL-6 secretion was found to be significantly lower in response to all the stimuli tested upon silencing of DHX9 in THP-1 cells. As DHX9 has been implicated in both transcription and translation [298, 370], qPCR was next carried out to examine whether the lower levels of IL-6 protein upon silencing of DHX9 were due to DHX9 having a role pre- or post-transcription. As IL-6 protein levels were dramatically reduced following both PAM3 and CL075 stimulation, a stimulation time course for both of these ligands was carried out and DHX9 and IL-6 mRNA were measured by qPCR (Figure 3.10, Figure 3.11). DHX9 mRNA was found to be dramatically reduced at all of the time points tested for both time courses (Figure 3.10 A, Figure 3.11 A). IL-6 mRNA was not found to be upregulated until 3 hours post PAM3 (Figure 3.10 B) and CL075 (Figure 3.11 B) stimulation, with IL-6 mRNA continuously rising up to 24 hours stimulation. IL-6 mRNA was found to be over 50% lower in the DHX9-shRNA targeting cell lines compared to the NSC cell line at 24 hours PAM3 stimulation. IL-6 mRNA at 24 hours CL075 stimulation was similarly found to be significantly reduced, but to a greater extent than that observed for PAM3. This correlates with the extent to which IL-6 protein was found to be reduced by ELISA, with CL075-stimulated IL-6 protein being reduced more than that for PAM3 stimulation.

As DHX9 was found to be crucial in IL-6 production, gain-of-function studies were next carried out to investigate whether DHX9 could enhance IL-6 production after overexpression. IL-8 secretion was also measured to examine if DHX9 could also upregulate other NF- κ B-dependent cytokines. As all of the TLR ligands employed activate pathways which are MyD88-dependent, MyD88 was transfected into HeLa cells alongside DHX9 expression vector and then, 48 hours later, the supernatants were measured for cytokine production by ELISA. It was found that while DHX9 was unable to induce IL-6 or IL-8 production alone, it was able to potentiate both MyD88-driven

IL-6 (Figure 3.12 A) and IL-8 production (Figure 3.12 B) over twofold, in a dose-dependent manner.

Whether DHX9 is also critical in the upregulation of IL-6 in other human cells was next investigated. Unfortunately, DHX9 siRNA studies in primary human cells were not possible as primary monocytes isolated from blood buffy coats were not found to express a detectable level of DHX9 protein by SDS-PAGE (Figure 3.13 A). Hence, the protein level of DHX9 in numerous cell lines was assayed by SDS-PAGE (Figure 3.13 B). Although DHX9 protein could not be detected in primary monocytes or primary PBMCs (Figure 3.13 A and data not shown), DHX9 protein was detectable in all of the cell lines tested. Thus, DHX9 may be expressed to a higher degree in immortalized cells. BEAS-2B, an epithelial cell line transformed from bronchial tissue, were chosen to test whether DHX9 plays a role in IL-6 production in cell types other than THP-1 cells. The lentiviral pTRIPZ system was again employed to generate cell lines stably expressing doxycycline-inducible DHX9 shRNA or a NSC shRNA. The same shRNAs were used as for the generation of the THP-1 stable cell lines. Each of the cell lines were incubated with doxycycline for 72 hours and then lysates were collected, resolved by SDS-PAGE, and immunoblotted for DHX9 to assay DHX9 knockdown (Figure 3.14 A). Each of the three DHX9 shRNA stable cell lines were found to have dramatically reduced DHX9 protein levels compared to the NSC cell line. The NSC cell line and the two cell lines which showed the most effective DHX9 protein knockdown (A7 and D11) were incubated with doxycycline for 72 hours and then stimulated with a range of ligands for 24 hours. The supernatants were removed and IL-6 secretion was measured by ELISA (Figure 3.14 B). IL-6 production was found to be greater than 50% reduced for all the ligands tested. Upon knockdown of DHX9, IL-6 protein was consistently found to be the most significantly reduced following transfected poly(I:C) stimulation. DHX9 has therefore a critical role in IL-6 protein which is pre-translational and not cell type-specific.

3.2.5 DHX9 is not required for TLR2 or TLR8 upstream signalling

In both loss- and gain-of-function studies, DHX9 was found to have a role in the upregulation of numerous cytokines in response to a range of stimuli in a number of different cell types (HeLa, BEAS-2B and THP-1 cells). As PAM3- and CL075-induced

IL-6 mRNA was found to be reduced upon silencing of DHX9 in THP-1 cells, one can conclude that DHX9 is not functioning at a post-transcriptional level. Therefore, the next step was to determine whether DHX9 is functioning at a transcriptional level, for example, by regulating upstream signalling events. MAPK and NF- κ B activation are major signalling events stimulated by TLRs [161]. Thus, whether DHX9 is necessary for effective I κ B degradation, p65 phosphorylation and MAPK activation after PAM3- and CL075-stimulation was next assayed (Figure 3.15). As a number of the cytokine readouts affected upon DHX9 knockdown in THP-1 cells are NF- κ B-dependent, I κ B degradation and p65 phosphorylation were assayed upon DHX9 knockdown. DHX9 shRNA-expressing THP-1 stable cell lines, which had been incubated in doxycycline for 72 hours to allow production of shRNA and target silencing, were stimulated for the indicated times with PAM3 (Figure 3.15 A) or CL075 (Figure 3.15 B). Cells were lysed, and the lysates resolved by SDS-PAGE. NSC cell lysates were resolved alongside G2 cell lysates in order to identify by immunoblot analysis whether silencing of DHX9 had an effect on I κ B degradation, p65 phosphorylation (both hallmarks of NF- κ B activation), or p38 MAPK activation. For all of the time points tested, DHX9 protein expression was found to be strongly reduced in the G2 cell line compared to the NSC cell line. For PAM3 stimulation (Figure 3.15 A), I κ B degradation was not seen to be affected upon knockdown of DHX9. I κ B disappearance was seen as early as 5 minutes PAM3 stimulation, and to be almost complete by 15 minutes. I κ B was observed to begin reappearing again at 1 hour and was almost fully replenished by 2 hours stimulation for the NSC and G2 cell lines. DHX9 was also not found to be required for phosphorylation of p65 at serine 536 after PAM3 stimulation. The phosphorylation of p65 was found to coincide with the disappearance of I κ B. p65 phosphorylation was seen to begin at 5 minutes PAM3 stimulation, peak at 30 minutes stimulation and begin disappearing again by 1 hour stimulation for the NSC and G2 cell lines. p38 MAPK activation was also found to be unaffected as measured by the appearance of phospho-p38 (Figure 3.15 A). DHX9 silencing also had no effect on CL075-induced I κ B degradation or p38 phosphorylation (Figure 3.15 B). The kinetics of I κ B degradation upon CL075 stimulation was more delayed than that of PAM3 stimulation, with degradation not being observed to occur until 90 minutes CL075 stimulation, reappearing again at 150 minutes. Similar data was found when comparing the A7 cell line to the NSC cell line for both time courses (data not shown). Also, CL075- and

PAM3-induced phosphorylation of JNK was also found to be unaffected (data not shown). It was thus concluded that, upon stimulation with CL075 or PAM3, TLR signalling events upstream of transcription were unaffected in THP-1 cells.

3.2.6 DHX9 is capable of potentiating activation of the IL-6 promoter

DHX9 was shown to be critical for the upregulation of a number of cytokines upon TLR stimulation, particularly IL-6, which was reduced for all the stimuli tested upon knockdown of DHX9 in both THP-1 and BEAS-2B cells. Although TLR signalling events were unaffected, IL-6 mRNA was reduced following PAM3 and CL075 stimulation in THP-1 cells, which would implicate DHX9 as playing a role in regulation of transcription. Therefore, it was likely that DHX9 might be acting at the promoter level. The next step was to investigate whether DHX9 could cause the direct activation of the IL-6 promoter by reporter gene assay. For this, the promoter sequence of interest (i.e. the IL-6 promoter) controls expression of the luciferase gene, which encodes the firefly luciferase enzyme. The reporter gene was transfected into cells, alongside expression vectors of interest, and activation of the transfected promoter was assayed by measuring the appearance of the firefly luciferase protein in the lysates. A wild type IL-6 promoter luciferase construct, as well as IL-6 promoter mutants lacking either its NF- κ B-binding site or its AP-1 binding site, were employed. These luciferase constructs were transfected into HeLa cells with DHX9 and/or MyD88 expression vector, to assay whether DHX9 could directly activate the IL-6 promoter or potentiate its activation by MyD88, an upstream TIR adaptor known to be capable of activating NF- κ B-dependent genes upon TLR stimulation (Figure 3.16). DHX9 was found to be capable of activating the IL-6 promoter roughly twofold. However, it was able to dramatically potentiate MyD88-driven IL-6 promoter activation in a dose-dependent manner (Figure 3.16 A). Whether this potentiation by DHX9 was dependent on the IL-6 NF- κ B-binding site or its AP-1-binding site was also investigated through the use of IL-6 promoter luciferase constructs that were lacking these sites. Interestingly, although the activation of the IL-6 promoter by MyD88 or its potentiation by DHX9 was unaffected in a mutant that lacked the AP-1-binding site (Figure 3.16 C), the activation by MyD88 or its potentiation by DHX9 of a IL-6 promoter construct lacking the NF- κ B-binding site was abrogated (Figure 3.16 B). These results indicate that DHX9 has a role in the transcriptional regulation of IL-6, which is likely NF- κ B-dependent.

3.2.7 DHX9 enhances NF- κ B activation by multiple stimuli in a helicase-independent manner

Since DHX9 enhanced activation of the IL-6 promoter, and this seemed to be dependent on the κ B motif, the effect of DHX9 on NF- κ B-dependent reporter gene assays was next examined. As DHX9 was found to play a critical role in TLR8 and TLR2 responses in THP-1 cells, whether DHX9 is similarly involved in HEK 293 cells stably expressing TLR8 or TLR2, was first examined by reporter gene assay (Figure 3.17). The activation of NF- κ B was assayed through the use of the decameric κ B motif, which binds and responds to NF- κ B, contained within a promoter controlling expression of the luciferase gene, which encodes the firefly luciferase enzyme. Overexpression of DHX9 was found to dose-dependently, significantly enhance both TLR8- (Figure 3.17 A) and TLR2- (Figure 3.17 B) dependent NF- κ B activation by luciferase assay, while having no effect alone. Also, overexpression of DHX9 was found to enhance CL075-dependent IL-8 production almost twofold upon transfection with the highest concentration of DHX9 expression vector (Figure 3.17 C).

DHX9 has been previously described to directly bind p65, acting as a transcriptional coactivator of NF- κ B-dependent genes [298]. This function of DHX9 may explain some of the effects on cytokine readouts upon silencing of DHX9, especially in the case of IL-6, since the ability of DHX9 to potentiate MyD88-driven activation of the IL-6 promoter was antagonised when a mutant promoter lacking the NF- κ B-binding site was employed. This may explain why DHX9 was found to be required for effective IL-6 cytokine induction for any stimuli tested in both THP-1 and BEAS-2B cells. To further characterise this, additional luciferase assays were carried out, in which the activation of NF- κ B was assessed in more detail (Figure 3.18). Similar to the case with ligand stimulation (Figure 3.17), DHX9 was found to be capable of significantly enhancing MyD88-dependent NF- κ B activation (roughly fivefold compared to MyD88 alone transfected cells – Figure 3.18 A). Whether this enhancement by DHX9 was a direct effect on NF- κ B was next tested by assessing whether DHX9 could stimulate p65 transactivation of the NF- κ B-dependent reporter gene. HeLa cells were transfected with p65 expression vector, with or without, DHX9 (Figure 3.18 B). Interestingly, DHX9 dose-dependently enhanced p65 transactivation of the NF- κ B-dependent reporter gene. Next, in a related assay, the phosphorylation and

activation of p65 by upstream kinases was examined by luciferase assay (Figure 3.18 C). This system employs an expression plasmid encoding the TAD of the p65 subunit of NF- κ B fused to the DNA-binding domain of Gal4 and a Gal4-responsive reporter plasmid, pFR luciferase. The transactivation of p65 is assayed by an increase in the ability of the Gal4-p65 fusion protein to transactivate the pFR luciferase reporter, which contains Gal4-binding sites in its promoter. DHX9 was found to cause p65 transactivation by itself and could also dose-dependently enhance MyD88-driven p65 transactivation. Thus, DHX9 can directly increase the ability of p65 to transactivate genes.

Having shown that DHX9 can directly stimulate p65 function, whether DHX9 could enhance other NF- κ B-dependent stimuli, apart from TLR8 and TLR2 (Figure 3.17), was next assayed (Figure 3.19). HeLa cells or HEK 293 stably expressing TLR3, with or without transfected DHX9 expression vector, were stimulated with numerous ligands, and their ability to activate NF- κ B was assayed. DHX9 was found to significantly enhance poly(I:C)- (Figure 3.19 A), IL-1 α - (Figure 3.19 B), TNF α - (Figure 3.19 C) and transfected poly(I:C)- (Figure 3.19 C) induced NF- κ B activation.

Having found that DHX9 can positively regulate NF- κ B transactivation, the requirement for its helicase activity was next tested. Helicases unwind RNA/DNA via ATP hydrolysis. An ATP-binding mutant, which has been previously described and shown to lack its ATPase activity [298, 371, 381, 385], was created by mutating lysine 417 of DHX9 to an arginine, via a single nucleotide mutation (Figure 3.20 A). The wild type DHX9 construct and the helicase mutant were both cloned into a pCMV-Myc expression vector and were found to be expressed to a similar extent by immunoblot analysis (data not shown). The helicase mutant of DHX9 was found to enhance both TLR8- (Figure 3.20 B) and MyD88- (Figure 3.20 C) mediated NF- κ B activation, to a similar extent as wild type DHX9 expression vector.

3.2.8 DHX9 expression enhances IRF transactivation

Having shown that DHX9 enhances p65 transactivation, we hypothesised that DHX9 may have a similar function in the biology of other transcription factors, thereby explaining some of its other NF- κ B-independent effects. For example, DHX9 was found

to be required for an effective IFN α response to SeV infection in THP-1 cells (Figure 3.8). IFN α is known to be a very IRF7-dependent cytokine. Therefore, whether DHX9 may be involved in the transcriptional activation of IRF7-dependent genes was assessed by carrying out Gal4-IRF7 luciferase assays. The Gal4-IRF7 assay is based on the ability of host kinases (e.g. TBK1) to phosphorylate and activate the IRF7 transcription factor. This is assayed by an increase in the ability of a Gal4-IRF7 fusion protein to transactivate the pFR luciferase reporter, which contains Gal4-binding sites in its promoter. Interestingly, DHX9 was observed to dose-dependently enhance TLR8- (Figure 3.21 A), TLR3- (Figure 3.21 B), and MyD88- (Figure 3.21 C) driven IRF7 transactivation. DHX9 was also found to enhance MyD88-driven IRF3 transactivation (Figure 3.21 D). Thus DHX9 may have a broad role in PRR and type I IFN stimulated gene induction by enhancing the transcriptional activity of multiple transcription factors.

3.3 Discussion

3.3.1 DHX9 has no role in nucleic acid sensing in THP-1 cells

Upon recognition of CpG-B DNA, DHX9 has been reported to induce a MyD88-dependent signalling pathway leading to an upregulation of NF- κ B-dependent cytokines such as TNF α and IL-6 [270]. Although primarily characterised as a regulator of NF- κ B-dependent genes in this study, there is also data showing that DHX9 is necessary for CpG-B-DNA-stimulated IFN α , with knockdown of DHX9 by siRNA significantly dampening IFN α secretion [270]. Less than one year later, the same authors also identified DHX9 as a sensor of dsRNA, signalling via MAVS, again being required for effective IFN α , IL-6 and TNF α production [222]. In their first publication, they identified DHX9 as being necessary for CpG-B but not CpG-A DNA stimulated IFN α production in pDCs [270]. Interestingly, their later work demonstrated a requirement for DHX9 in effective CpG-A DNA-stimulated IFN α production in mDCs [222]. Thus, it appears that the functions of DHX9 may be strongly cell type-specific. DHX9 is an RNA helicase which has already been demonstrated to unwind both dsRNA and dsDNA [294]. Hence, it is likely that DHX9 will directly bind to most forms of nucleic acid. It is therefore still uncertain whether DHX9 is a bona fide innate immune receptor for nucleic acids. Although DHX9 appears to be required in innate immune signalling events, the question of exactly what point within signalling cascades DHX9 has a role, has yet to be fully elucidated.

Upon identification of an interaction between VACV E3 and host DHX9 (S. Keating, personal communication), research was carried out in order to firstly investigate functions of DHX9 in innate immune signalling (this chapter), before then examining if VACV E3 is capable of inhibiting these (Chapter 4). As DHX9 was shown to be required for effective IFN α production in response to both CpG-B DNA and dsRNA [222, 270], overexpression studies in HeLa cells were firstly carried out to see if DHX9 was capable of inducing the IFN β promoter by reporter gene assay. IFI16, a previously identified DNA receptor in the lab, which is capable of signalling through STING to give an upregulation in IFN β [259], was used as a positive control. Both DHX9 and IFI16 were unable to activate the IFN β luciferase promoter on their own, but once STING was cotransfected, both DHX9 and IFI16 could strongly induce the IFN β

promoter in a dose-dependent manner. DHX9 was found to be more effective at activating the IFN β promoter than IFI16. However, the IFN β promoter requires both IRFs and NF- κ B for efficient activation. DHX9 has been demonstrated to interact with the p65 subunit of NF- κ B, functioning as a transcriptional coactivator of NF- κ B [298]. Hence, its ability to enhance the IFN β promoter demonstrated here, may be a consequence of this. Whether DHX9 is actually capable of signalling via STING is therefore uncertain. Therefore, it was next decided to generate DHX9 shRNA-expressing stable THP-1 cell lines so that loss-of-function studies in a more physiologically relevant cell type could be carried out.

Whether DHX9 has a role in nucleic acid sensing was further investigated through loss-of-function studies in THP-1 cells. Stable cell lines were generated using a pTRIPZ lentiviral system in which shRNA, which is doxycycline-inducible, was introduced into THP-1 cells. Three stable cell lines knocking down DHX9 mRNA were generated; A7, G2 and D11. Initially the NSC cell line proved difficult to generate. Hence, experiments were carried out using each of the cell lines with and without the addition of doxycycline, where the absence of doxycycline served as the control for each cell line. The first experiment carried out in these stable cell lines was to investigate if DHX9 was necessary for DNA- or RNA-induced IFN β . The cells were stimulated with VACV 70mer and synthetic dsRNA (poly(I:C)). VACV 70mer, a dsDNA 70-base pair motif that is conserved and repeated multiple times in many poxviral genomes and capable of inducing IFN β in numerous cell types [259], had been previously identified, via mass spectrometry, as also interacting with DHX9 (S. Keating, personal communication). For each of the three cell lines, it was found that DHX9 was not required for 70mer- or poly(I:C)-induced IFN β mRNA induction (G2 data not shown). These results were repeated upon generation of the NSC stable cell line and similar data was obtained (data not shown). To further characterise whether DHX9 has a role in nucleic acid sensing in THP-1 cells, IP10 and RANTES production were assayed by ELISA after silencing of DHX9 and stimulation with 70mer, cGAMP and poly(dA:dT). IP10 and RANTES are critical antiviral chemokines, which are both IRF3-dependent, and often used as a readout for IRF3-dependent PRR signalling [387, 388]. cGAMP is a second messenger produced in mammalian cells by cGAS in response to dsDNA in the cytoplasm, which binds STING to activate innate immune

responses and a type I IFN response [383]. As it was not possible to obtain synthetic dsRNA which could effectively elicit an effective cytokine response from THP-1 cells, poly(dA:dT) was employed instead. Poly(dA:dT) is a synthetic DNA ligand which is recognised by several sensors including AIM2 and DAI. However, its presence can also be recognised indirectly by RIG-I in a similar pathway to that activated by poly(I:C), where Pol III can transcribe poly(dA:dT) into an RNA-containing 5'-triphosphate moiety, which can then be sensed by RIG-I, leading to IFN β induction [210, 257]. DHX9 silencing was also not found to have an effect on 70mer-, cGAMP-, or poly(dA:dT)-stimulated IP10 or RANTES production. Therefore, DHX9 silencing had no effect on antiviral chemokine production or IFN β mRNA upregulation in response to dsDNA or RNA in THP-1 cells. It was thus concluded that DHX9 does not play a role in nucleic acid sensing in THP-1 cells. As DHX9 was identified as both a DNA and RNA sensor in human pDCs and mDCs, respectively [222, 270], the role of DHX9 in nucleic acid sensing may be cell specific.

3.3.2 DHX9 is required for IFN α -stimulated ISG56 mRNA induction but not for ISG56 protein production

As the upregulation of ISGs is crucial in the immune response to viruses, whether DHX9 had a role in IFN signalling was next tested. DHX9 shRNA-expressing THP-1 stable cells lines were incubated with and without doxycycline for 72 hours and then stimulated with 70mer, poly(I:C) and IFN α . Numerous ISGs tested were not found to be reproducibly affected by silencing of DHX9 (ISG54, OAS2, PKR – data not shown). However, for all three cell lines, it appeared that DHX9 was required for IFN α -induced ISG56 mRNA induction. ISG56 upregulation, upon IFN α stimulation, was found to be roughly 50 % less than that of the non-doxycycline control for all three stable cell lines (G2 not shown). A more detailed IFN α stimulation time course confirmed that DHX9 was critical for effective ISG56 upregulation after IFN α stimulation, while other ISGs were unaffected. Upon generating a NSC line, the experiment was repeated and similar data were obtained. Immunoblotting for the phosphorylation of STAT1 after IFN α stimulation in NSC and DHX9 shRNA-expressing cells, showed that JAK-STAT signalling was unaffected. ISG56 protein was also assayed, and although DHX9 appeared to be necessary for ISG56 mRNA induction, DHX9 was not required for ISG56 protein expression. DHX9 appears to be

subtly required for both total STAT1 and ISG56 protein expression in the experiment shown. However, in repeating the experiment numerous times, this was not reproducible and it may be explained here due to variation in the quantity of protein loaded. DHX9 may therefore be necessary for the transcription of ISG56 mRNA but this does not translate to an effect on ISG56 protein expression. This may be because ISG56 mRNA is very effectively translated to ISG56 protein or that the mRNA is not limiting for translation. Although DHX9 may be required for effective ISG56 upregulation, there may be redundancy in ISG56 mRNA translation, with other helicase family members being sufficient for effective translation. Interestingly, DHX9 has been demonstrated to be recruited to promyelocytic leukemia (PML) nuclear bodies after IFN α stimulation, where it associated with nascent mRNA transcripts [389]. This may suggest a role for DHX9 in regulating transcription of ISGs or subsequent processing of ISG mRNAs [389]. Hence, ISG56 may belong to a specific group of ISGs which DHX9 regulates.

3.3.3 DHX9 may have both gene-specific and TLR-specific roles in THP-1 cells

Next, cytokine responses upon stimulation with numerous ligands, were measured upon knockdown of DHX9 via shRNA. Firstly, cytokine responses to the RNA virus, SeV, were investigated upon the knockdown of DHX9 in the pTRIPZ stable cell lines. Interestingly, upon knockdown of DHX9 in the A7 and G2 stable cell lines, SeV-stimulated RANTES, IP10 and TNF α cytokine production were unaffected while both IL-6 and IFN α cytokine production were significantly reduced in comparison to that of the NSC stable cell line. The signalling pathway described in which DHX9 signals via MAVS upon recognition of dsRNA, might be involved in this case [222]. DHX9 could possibly be required for the recognition of SeV dsRNA and effective upregulation of type I IFNs and pro-inflammatory cytokines, in response to SeV infection.

Given that DHX9 was not found to have a role in nucleic acid sensing in THP-1 cells, the cells were next stimulated with a range of TLR ligands in order to investigate whether DHX9 has a role in TLR signalling. PAM3 was used in order to activate TLR1/2, CLO75 was used to activate TLR8, and to a lesser extent, TLR7, and LPS was

used to activate TLR4. It was found that there was a strong requirement of DHX9 for all the CL075- stimulated cytokines tested (i.e. RANTES, TNF α , IL-6 and IP10). Interestingly, while DHX9 was required for PAM3- and LPS-stimulated IL-6, it was not essential for PAM3- or LPS-stimulated RANTES, TNF α or IP10 protein production.

As IL-6 protein was reduced for any stimuli tested, the role of DHX9 may be gene-specific rather than stimulus-dependent for effective IL-6 production. From collectively analysing all of the ELISA data upon silencing of DHX9, DHX9 may play a role in endosomal TLR signalling in THP-1 cells. DHX9 was found to be critical in the upregulation of all TLR8-dependent cytokines, an endosomal-localised TLR. TLR2 and TLR4 are expressed on the cell surface [134]. Only TLR2- and TLR4-stimulated IL-6 production was found to be reduced upon silencing of DHX9, with all other cytokine readouts being unaffected. This may be due to DHX9 having a role in the regulation of IL-6 at a transcriptional level, which was indeed found to be the case. A role for DHX9 in endosomal TLR signalling may explain why DHX9 was proposed as a sensor of CpG-B DNA in pDCs [270]. In this study DHX9 was found to be necessary for CpG-B-stimulated cytokine production. However, knockdown of TLR9 also almost completely abolished transcription factor activation and cytokine secretion, following CpG-B DNA stimulation, suggesting both TLR9 and DHX9 are required for effective CpG-B DNA sensing in pDCs [270]. As DHX9 is a helicase which binds and unwinds nucleic acid, its binding to CpG-B DNA may have been misinterpreted, with DHX9 not directly sensing CpG-B DNA, but rather serving a role further downstream in signalling events. DHX9 may thus be required for the effective signalling via endosomal TLR9 upon sensing of CpG-B DNA. The interaction studies of DHX9 with MyD88 in this study may have been physiologically relevant, with DHX9 possibly being required for MyD88-dependent endosomal signalling [270]. The ability of DHX9 to enhance MyD88-dependent IL-6 and IL-8 secretion in HeLa cells, as well as transcription factor activation by reporter gene assay, would correlate with this. As CpG DNA was not found to stimulate cytokine production in THP-1 cells, a role for DHX9 in its sensing was not pursued.

Alternatively, some of the differences in TLR-stimulated cytokine readouts upon knockdown of DHX9 in THP-1 cells, may be due to functions of DHX9 which have

been previously described. DHX9 has been previously reported to interact with p65 and function as a transcriptional coactivator [298]. As p65 is required for the transcription of any NF- κ B-dependent genes, this may explain some of the differences seen in cytokine readouts. As the IFN β promoter is both NF- κ B- and IRF-dependent, this may also explain how DHX9 is capable of augmenting STING-dependent IFN β promoter activation by reporter gene assay. The strongest effects of DHX9 knockdown appeared to be on TNF α and IL-6 cytokine production. These effects may be partially due to the fact that both of the genes for these cytokines contain κ B-binding sites, and hence p65 is required for their transcription. RANTES production is also somewhat affected, but to a lesser extent than that of TNF α or IL-6. RANTES is also NF- κ B-dependent but, known to also be regulated by IRFs [390], the RANTES promoter may be less NF- κ B-dependent than that of TNF α and IL-6. However, differences in κ B-binding sites would not explain why DHX9 appears to be differentially required for cytokine production depending on the stimulus. For example, while DHX9 appears to be required for CL075-stimulated RANTES production, it is not required for PAM3-stimulated RANTES. It may be possible that although both TLR ligands induce MyD88-dependent pathways, the pathways taken by each ligand may vary in their dependency and activation of NF- κ B. Alternatively, there may be several functions of DHX9 at play. As DHX9 was found to be necessary for the upregulation of all TLR8-dependent cytokines, DHX9 may have a specific role in TLR8 signalling, as well as binding to p65 and being required for effective activation of NF- κ B-dependent genes (Figure 3.22). Another function of DHX9, which may be involved in the differences in cytokine production, is its requirement for the complex formation between CBP and Pol II [371]. CBP is a transcriptional coactivator which is vital in the activation of the transcription factor CREB. As the RANTES, TNF α , IL-6, IFN α and IP10 genes all have CREB-binding sites, knockdown of DHX9 may affect the regulation of these genes. The importance of CREB in the activation of these genes may vary and hence lead to differences in their dependence on DHX9.

Interestingly, in the publications which describe DHX9 as being a sensor of both dsRNA and CpG-B in mDCs and pDCs, respectively, the cytokine readouts affected upon silencing of DHX9 are the same, namely IL-6, TNF α and type I IFNs [222, 270]. In the presented work, we have also found these cytokines to also require DHX9,

depending on the stimulus, in THP-1 cells. This may correlate with DHX9 possibly having a role in gene regulation, rather than nucleic acid sensing or upstream signalling cascades. A requirement of DHX9 for the transactivation of p65 may be why IL-6 and TNF α secretion is affected upon silencing of DHX9 [298], while in this work DHX9 was found to similarly enhance the transactivation of IRF3 and IRF7, possibly the mechanism by which DHX9 is required for effective type I IFN production.

Similar to its binding of CpG-B DNA [270], the binding of DHX9 to dsRNA may be solely due to its capability of unwinding both RNA and DNA complexes, rather than it acting as a nucleic acid sensor. The association of DHX9 with MyD88 and MAVS for the sensing of CpG-B DNA and dsRNA, respectively [222, 270], may not be direct. With both the RIG-I-MAVS and TLR9-MyD88 complexes being vital in the detection of nucleic acid, DHX9 may be non-specifically immunoprecipitated alongside these complexes due to its binding of nucleic acid possibly upstream or downstream of nucleic acid sensing. In order to verify whether DHX9 directly binds MyD88 or MAVS, GST pull-downs would need to be carried out using recombinant protein.

3.3.4 DHX9 is critical to effective IL-6 upregulation

Silencing of DHX9 was found to have the most robust effect on IL-6 cytokine production, with DHX9 being necessary for all the stimuli tested, i.e. PAM3-, CL075-, LPS- and SeV-stimulated IL-6 protein. Therefore, the mechanism of IL-6 regulation by DHX9 was further investigated. IL-6 mRNA analysis after PAM3 and CL075 stimulation of the DHX9 shRNA-expressing THP-1 cells, showed DHX9 to be critical in effective IL-6 mRNA induction. The mRNA data was found to correspond with IL-6 protein levels upon knockdown of DHX9. By both qPCR and ELISA, IL-6 was found to be more dramatically reduced after CL075 stimulation than PAM3 stimulation. This may again indicate a separate role of DHX9 in effective TLR8 signalling. The general reduction in IL-6 mRNA may be due to the ability of DHX9 to transactivate p65, while the greater reduction in IL-6 mRNA following CL075 stimulation may be a consequence of a separate role for DHX9 in TLR8 signalling [298]. DHX9 has been previously reported to be involved in the translation of selected mRNAs [370]. Based on the mRNA data, it is possible to place DHX9 upstream of the transcription of IL-6, ruling out a translational role for DHX9.

Next, whether DHX9 is also critical to IL-6 production in other cell types was investigated. Gain-of-function studies in HeLa cells showed that transfected DHX9 expression vector could significantly enhance MyD88-driven IL-6 and IL-8 secretion. IL-8 secretion was measured alongside IL-6, as DHX9 is known to bind to p65 [298], and thus whether DHX9 could enhance the induction of other NF- κ B-dependent cytokines was assayed. Overexpression of MyD88 was found to drive both IL-8 and IL-6 production by itself. Although DHX9 was unable to drive cytokine production by itself, it was capable of significantly enhancing MyD88-driven IL-6 and IL-8 production, in a dose-dependent manner. This fits with a role for DHX9 in the transactivation of p65 rather than it functioning further upstream in signalling events, as an upstream signal and activator of p65 (MyD88) was required before DHX9 was capable of enhancing cytokine secretion. One would expect DHX9 to drive cytokine secretion by itself if it functioned as a sensor or upstream signalling molecule.

Whether DHX9 also has a role in IL-6 production in other cell types was also examined. Although DHX9 protein was undetectable in any primary cell lysates examined by immunoblot analysis (primary monocytes and PBMCs), DHX9 was detectable in any immortalized cell line lysate tested. This may indicate it is upregulated in immortalized cells for some reason. The pTRIPZ lentiviral shRNA system was employed in BEAS-2B cells. After confirming effective DHX9 silencing upon addition of doxycycline, the cells were stimulated with a range of ligands, and IL-6 secretion was measured by ELISA. DHX9 was found to be critical for effective IL-6 production in response to any stimuli tested. Interestingly, the most dramatic reduction of IL-6 production in BEAS-2B cells was consistently seen to be for transfected poly(I:C). DHX9 has been proposed as a dsRNA sensor in mDCs [222]. The stronger reduction in IL-6 in response to poly(I:C) may be accumulative effect of DHX9 playing a role in RNA sensing, as well as functioning further downstream in the signalling events culminating in IL-6 secretion. DHX9 may therefore have an RNA sensing role in epithelial cells. As DHX9 appears to be required for IL-6 production in response to any ligand tested in both THP-1 and BEAS-2B cells, it is likely that DHX9 is functioning at a transcriptional level.

3.3.5 DHX9 is not required for upstream TLR2 or TLR8 signalling events

DHX9 was found to play a role in the upregulation of numerous TLR-dependent cytokines in THP-1 cells. Having found that DHX9 is most likely involved in the transcriptional regulation of IL-6, as it is required for effective IL-6 production in response to any stimuli tested, whether DHX9 plays any role upstream in TLR signalling events was next examined. The ligands PAM3 and CL075 were employed in order to assay whether DHX9 is necessary for I κ B degradation, p65 phosphorylation and MAPK activation in THP-1 cells. As RANTES, TNF α , IP10 and IL-6 are all NF- κ B-dependent, whether DHX9 is required for effective NF- κ B activation in response to PAM3 and CL075 stimulation was tested. This was assayed by measuring I κ B degradation and p65 phosphorylation in response to PAM3 and CL075 stimulation, both hallmarks of NF- κ B activation. MAPK activation is also critical to the upregulation of these cytokines, so the phosphorylation of p38 and JNK were also assayed. DHX9 was not found to be required for I κ B degradation in response to PAM3 or CL075 stimulation, as a similar pattern of degradation was observed for the NSC, G2 and A7 cell lines (A7 not shown). As DHX9 is known to directly bind p65 [298], the phosphorylation of p65 was also assayed upon PAM3 stimulation. No difference in phosphorylation of p65 was seen upon knockdown of DHX9 compared to the NSC cell line. However, as the antibody used recognises the phosphorylation of p65 at only one site (serine 536), a requirement of DHX9 for the phosphorylation of p65 at other phosphorylation sites may yet be a possibility. DHX9 was also not found to be required for p38 or JNK phosphorylation (JNK data not shown) in response to PAM3 or CL075 stimulation. It can hence be concluded that DHX9 does not play a role in TLR2 or TLR8 signalling events upstream of transcription in THP-1 cells. As immediate signalling events are unaffected, but IL-6 mRNA was found to be dramatically reduced following stimulation of DHX9 shRNA-expressing THP-1 cells, DHX9 is most likely functioning at a transcriptional level.

3.3.6 DHX9 is capable of enhancing the transactivation ability of p65

Having found that DHX9 is crucial in the regulation of IL-6 in both THP-1 and BEAS-2B cells, the next step was to investigate whether DHX9 could cause the direct activation of the IL-6 promoter by reporter gene assay. This was done using a construct which had the firefly luciferase gene under the control of the IL-6 promoter.

Transfection of this luciferase construct alongside DHX9 and/or MyD88 expression vector, showed that DHX9 was able to significantly enhance MyD88-dependent activation of the IL-6 promoter. Two mutant IL-6 promoter constructs were also used to further map the site on the promoter by which DHX9 enhances its activation. Interestingly, although a mutant IL-6 promoter lacking the AP-1 site had no effect on its activation by MyD88 or DHX9, a construct lacking its NF- κ B-binding site showed less activation in response to MyD88 overexpression or its enhancement by DHX9. One can conclude from these observations that DHX9 has an important role in the regulation of IL-6, which is partially NF- κ B-dependent. This partial requirement of the NF- κ B-binding site for the enhancement in activation of the promoter by DHX9 may be a consequence of its ability to enhance p65 transactivation [298].

Having found that the ability of DHX9 to enhance p65 transactivation may explain its requirement in effective IL-6 upregulation, whether this function of DHX9 could explain more of the TLR-stimulated cytokine data obtained following silencing of DHX9 in THP-1 cells, was further examined. As DHX9 was necessary for the effective secretion of numerous cytokines in response to TLR2 and TLR8 stimulation in THP-1 cells, reporter gene assays were employed in HEK 293 cells stably expressing TLR2 or TLR8, to further map the mechanism by which DHX9 is required in these signalling cascades. Although DHX9 overexpression was found to do little without stimulation, it was found to significantly enhance both TLR8- and TLR2-dependent NF- κ B activation in HEK 293 cells after stimulation. This correlates with DHX9 having more of a transcriptional role, as was the case in THP-1 cells, where signalling events prior to NF- κ B or MAPK activation were unaffected. It was also found that overexpression of DHX9 enhanced CL075-dependent IL-8 production almost twofold upon transfection of the highest amount of DHX9 expression vector. DHX9 has been shown to be capable of directly binding to p65 and acting as a transcriptional coactivator of NF- κ B-dependent genes [298]. NF- κ B reporter gene assays demonstrated that, while DHX9 was found to have little effect on NF- κ B activation when overexpressed by itself, it was capable of dose-dependently enhancing MyD88-dependent NF- κ B activation. Although these data correlate with the ability of DHX9 to bind p65, it is also consistent with the idea of DHX9 being required in a MyD88-dependent signalling cascade in the sensing of CpG-B DNA [270]. To take a more detailed look at whether it is the ability of DHX9 to

affect p65 transactivation that is involved in its ability to activate NF- κ B by reporter gene assay, a p65 construct and a Gal4-p65 luciferase construct were next employed. DHX9 was found to potentiate p65-dependent transactivation of NF- κ B in a similar manner to that of MyD88 overexpression. More convincingly, DHX9 was also capable of enhancing MyD88-dependent Gal4-p65 transactivation. Therefore, it appears that the enhancement of NF- κ B activation upon DHX9 overexpression is dependent on its ability to directly affect the transactivation ability of p65. This data is consistent with the fact that DHX9 was found to be less efficient in its ability to enhance the activation of the IL-6 promoter construct which was lacking its NF- κ B-binding site. Thus, knowing that DHX9 is potentiating the activation of NF- κ B by enhancing p65 transactivation ability, one would expect DHX9 to be capable of enhancing NF- κ B activation in response to any stimuli. This was found to be the case, with DHX9 being found to be capable of enhancing poly(I:C)-, IL-1 α -, TNF α - or transfected poly(I:C)-driven NF- κ B activation. It was hence concluded that DHX9 could enhance any NF- κ B-dependent stimulus and this was most likely due to its ability to act as a transcriptional coactivator through its binding of p65. This fits with DHX9 not being necessary for the phosphorylation of p65 after TLR2- or TLR8-stimulation, as DHX9 is most likely not required for p65 phosphorylation. The precise mechanism by which DHX9 mediates the transcriptional activity of NF- κ B has yet to be fully unravelled. DHX9 was found to specifically interact with p65 and was demonstrated to enhance NF- κ B-dependent reporter gene expression induced by p65, TNF α or NIK [298]. The transactivation ability of p65 is mediated by the interactions of its TAD with co-transcriptional regulators and the basal transcriptional machinery [391]. DHX9 was found to interact with the N-terminal TAD of p65 [298]. As this domain of p65 recruits CBP/p300 coactivators, DHX9 appears to further facilitate coactivator recruitment or the assembly of the transactivation complex by interaction with Pol II.

A helicase mutant of DHX9 was created to assess whether its helicase activity is required for its ability to enhance the transactivation of p65. The ability of DHX9 to enhance NF- κ B activation through its interaction with p65 was previously shown to be dependent on its helicase activity, with the mutant acting as a dominant negative, antagonising the activation of NF- κ B by endogenous DHX9 after numerous stimuli [298]. Here, whether the helicase mutant could potentiate TLR8- or MyD88-dependent

NF- κ B activation was assayed. Surprisingly, the ATP-binding mutant was capable of enhancing both CL075-dependent activation of NF- κ B in HEK 293-TLR8 cells and MyD88-dependent NF- κ B activation in HeLa cells, to a similar extent as wild type DHX9. In published data on the role of DHX9 in the transcriptional coactivation of NF- κ B, whether DHX9 could potentiate TLR8- or overexpressed MyD88-dependent activation of NF- κ B was not assessed. It may be that separate functions of DHX9 are at play in its ability to enhance TLR8- or MyD88-enhanced NF- κ B activation, and hence whether this is the case will need to be further investigated.

3.3.7 A broad role for DHX9 in transcription factor transactivation

Upon recognition of CpG-B DNA, DHX9 has been proposed to induce a MyD88-dependent signalling pathway, leading to an upregulation in NF- κ B-dependent cytokines such as TNF α and IL-6 [270]. Although primarily characterised as a regulator of NF- κ B-dependent genes in this study, there is also data showing that DHX9 is required for CpG-B-DNA-stimulated IFN α , with knockdown of DHX9 by siRNA significantly dampening IFN α secretion [270]. Less than one year later, the same authors also identified DHX9 as a sensor of dsRNA, signalling through MAVS, again being required for effective IFN α , IL-6 and TNF α production [222]. In their first publication, they identified DHX9 as being necessary for CpG-B, but not CpG-A, DNA stimulated IFN α production in pDCs [270]. Interestingly, in their second publication they show DHX9 being required for effective CpG-A DNA-stimulated IFN α production in mDCs [222]. Thus, the function of DHX9 may be very cell type-specific. Here, DHX9 has been found to be important in the upregulation of IFN α after SeV infection, in THP-1 cells. The pathway proposed in which DHX9 signals via MAVS upon recognition of dsRNA might also be involved in this case [222]. DHX9 could possibly be required for the recognition of SeV dsRNA and effective upregulation of type I IFNs and pro-inflammatory cytokines in response to SeV infection. As DHX9 has been found to have an important role in the transactivation of p65 and upregulation of NF- κ B-dependent genes, whether DHX9 may similarly enhance the activation of other transcription factors was examined. IFN α is known to be strongly dependent on IRF7 [392]. Therefore, whether DHX9 is somehow involved in the transcriptional activation of IRF7-dependent genes was assessed by carrying out Gal4-IRF7 luciferase assays. Interestingly, in a similar manner to its activation of NF- κ B, DHX9 was found to dose-

dependently enhance TLR8-, TLR3- and MyD88-dependent transactivation of IRF7. DHX9 was also found to be capable of enhancing MyD88-dependent transactivation of IRF3. Therefore, DHX9 may have a much greater role in transcription factor activation than previously envisaged. NF- κ B appears to form a functional transactivation complex containing DHX9, CBP, Pol II and general transcription factors. Here, preliminary data may implicate a role for DHX9 in the transcriptional regulation of members of the IRF transcription factor family also. NF- κ B, IRF3 and IRF7 all require CBP for effective activation. Therefore, there may be a correlation between the genes affected upon silencing of DHX9 and their dependency on CBP. For example, although all of the genes tested are CBP-dependent, IL-6 may be more reliant on CBP than some of the other genes examined. This would explain why IL-6 was dramatically reduced in response to any stimuli tested.

Previous work in which DHX9 has been proposed as playing a role in nucleic acid sensing, will hence need to be reassessed on whether the data can be explained based on its numerous activities downstream in transcriptional regulation. DHX9 has been shown here to have a strong involvement in PRR signalling. This may provide a rationale for the binding of VACV E3 to DHX9, with E3 possibly antagonising DHX9 function. Whether this was the case was explored next.

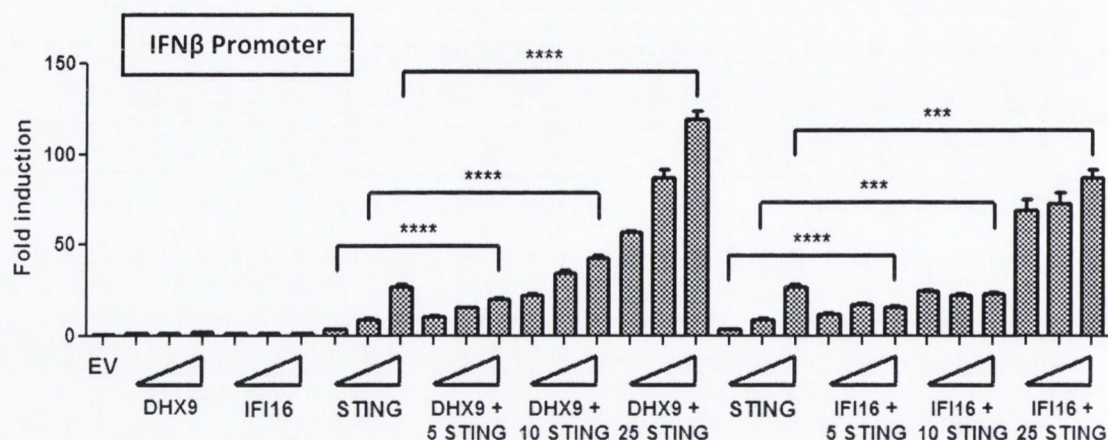


Figure 3.1 DHX9 enhances STING-dependent IFN β promoter luciferase activity

HeLa cells were seeded at 0.5×10^5 cells/ml, 24 hours prior to GeneJuice® transfection. Cells were transfected with IFN β promoter reporter gene, the pGL3-Renilla constitutive reporter, empty vector (EV), or the indicated amounts (ng) of STING, DHX9 or IFI16 expression vectors. Wedges indicate increasing amounts of DHX9 (25, 50, 100 ng), IFI16 (25, 50, 100 ng) or STING (5, 10, 25 ng). The cells were lysed 48 hours after transfection, and the lysates assayed for luciferase activity using a luminometer. Data is shown as relative fold induction normalised to EV alone-transfected cells. The data is mean $\pm$ SD of triplicate samples and is representative of three experiments. ***= $p < 0.001$, ****= $p < 0.0001$ compared to STING alone-transfected cells.

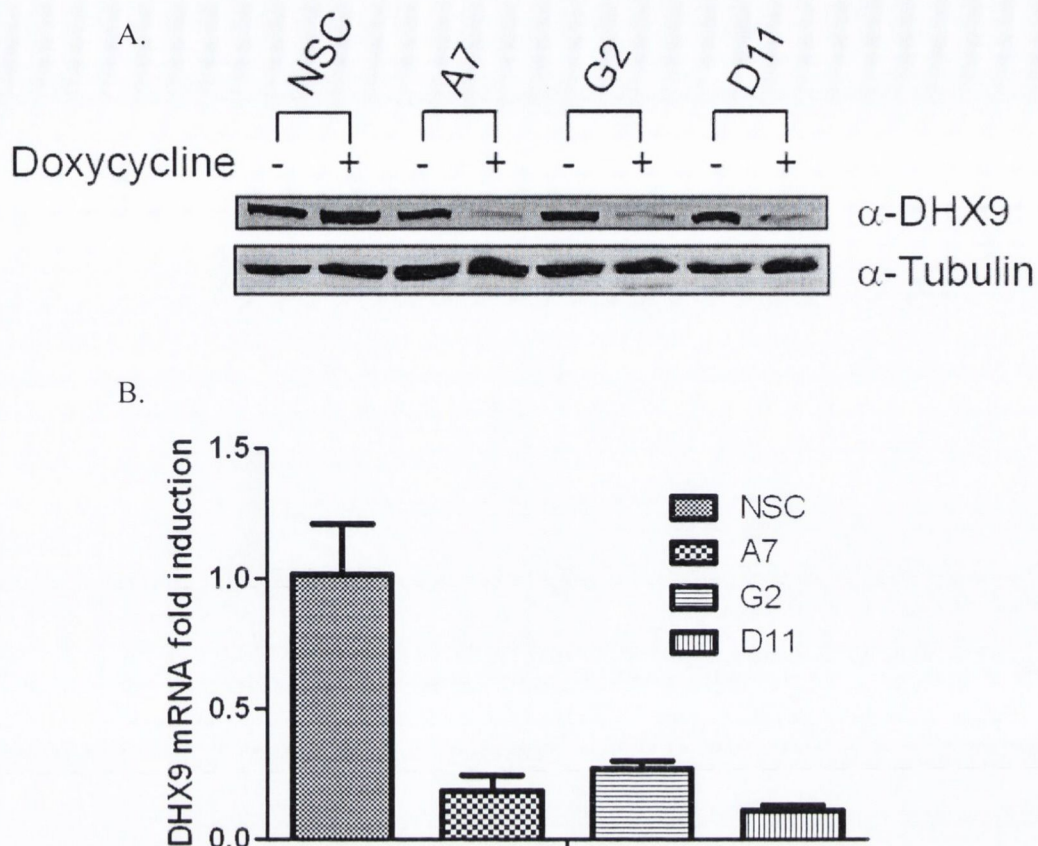


Figure 3.2 Generation of THP-1 cells stably expressing doxycycline-inducible DHX9 shRNA

THP-1 cells were transduced with lentiviruses encoding DHX9-targeting shRNA (A7, G2 or D11) or a non-silencing control (NSC) shRNA designed with minimal sequence homology to known mammalian genes. After transduction, the THP-1 cells were incubated in 1 μ g/ml puromycin for 1 week. Each THP-1 cell line was either left untreated (-) or incubated with 1 μ g/ml doxycycline (+) for 72 hours to induce the required shRNA. Lysates were generated, resolved by SDS-PAGE, and immunoblotted for DHX9 and tubulin (A). Cells treated with doxycycline were harvested and DHX9 mRNA levels, relative to β -actin, were assayed by qPCR (B). Data is expressed as mRNA values relative to β -actin and then fold induction is relative to the NSC THP-1 cell line. The data is mean $\pm$ SD of triplicate samples and is representative of three experiments.

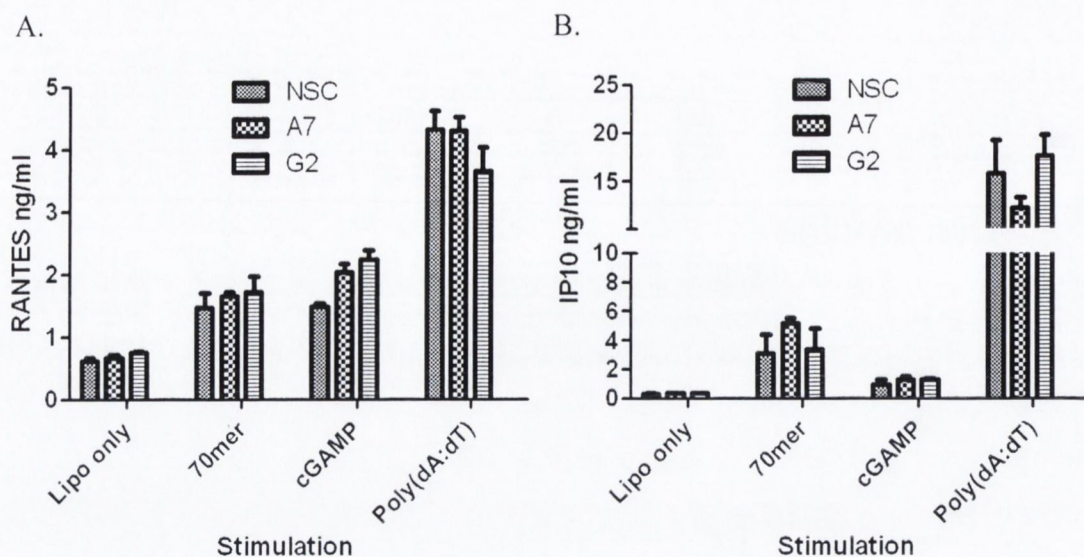


Figure 3.3 No role for DHX9 in DNA- or RNA-induced chemokine production in THP-1 cells

Two THP-1 cell lines (A7 and G2) expressing doxycycline-inducible DHX9-specific shRNA, and one which expresses a non-silencing control (NSC) shRNA, were incubated with 1 $\mu\text{g/ml}$ doxycycline for 72 hours. They were then seeded at 0.8×10^6 cells/ml and transfected 24 hours later with 1 $\mu\text{g/ml}$ 70mer, 5 $\mu\text{g/ml}$ cGAMP, 1 $\mu\text{g/ml}$ poly(dA:dT) or lipofectamine only (Lipo only). The supernatants were removed 24 hours later and RANTES (A) and IP10 (B) secretion was measured by ELISA. The data is mean $\pm$ SD of triplicate samples and is representative of three experiments.

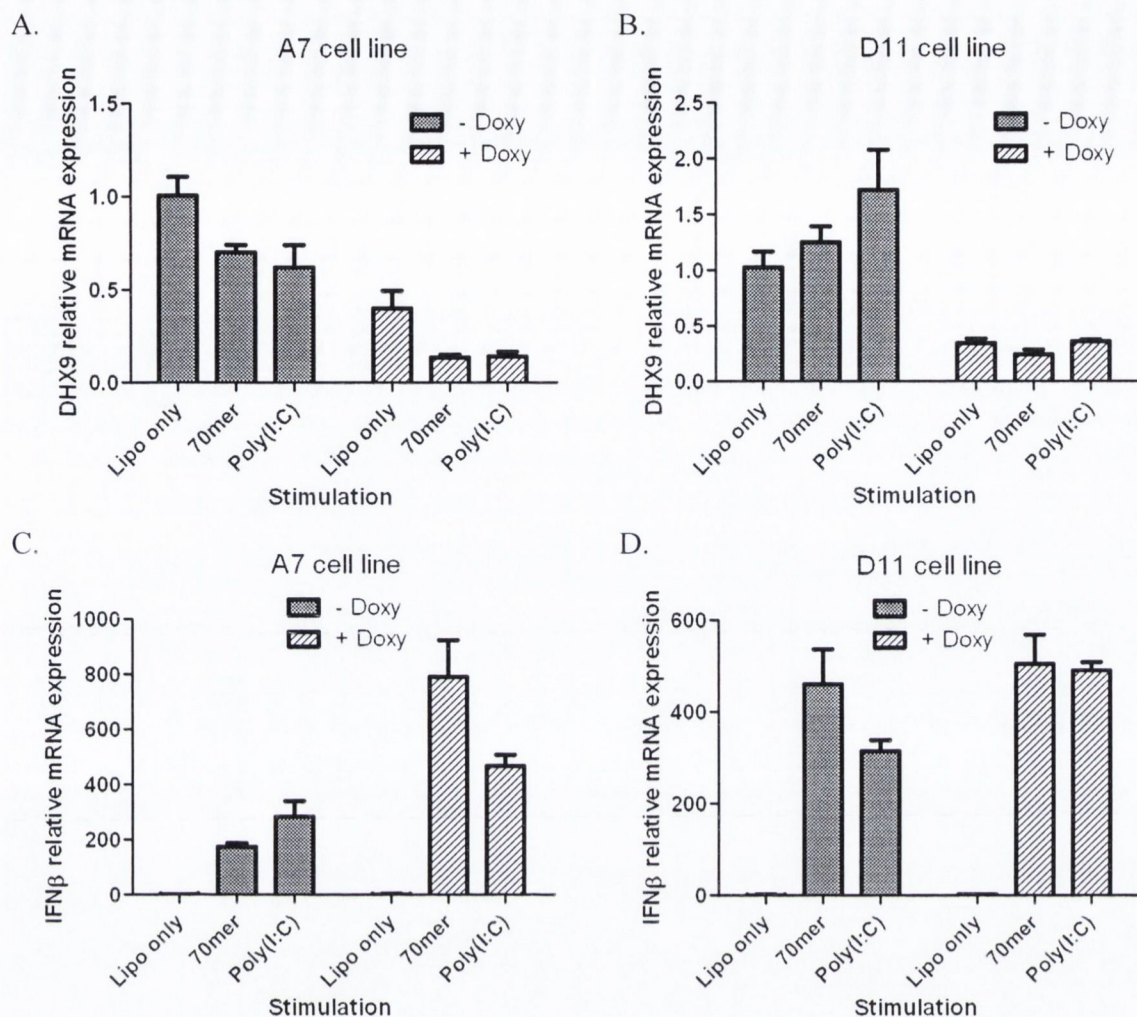


Figure 3.4 No role for DHX9 in DNA- or RNA-stimulated IFN β induction in THP-1 cells

Two THP-1 cell lines (A7 and D11) expressing doxycycline-inducible DHX9-specific shRNA were incubated with (+ Doxy) or without (- Doxy) 1 μ g/ml doxycycline for 72 hours. Each cell line was then transfected with lipofectamine alone (Lipo only), 1 μ g/ml 70mer or 1 μ g/ml poly(I:C) for 6 hours. DHX9 (A, B) and IFN β (C, D) mRNA expression was assayed by qPCR. Data are expressed as mRNA values relative to β -actin and the fold induction is relative to the lipofectamine only without doxycycline for each cell line. The data is mean $\pm$ SD of triplicate samples and is representative of three experiments.

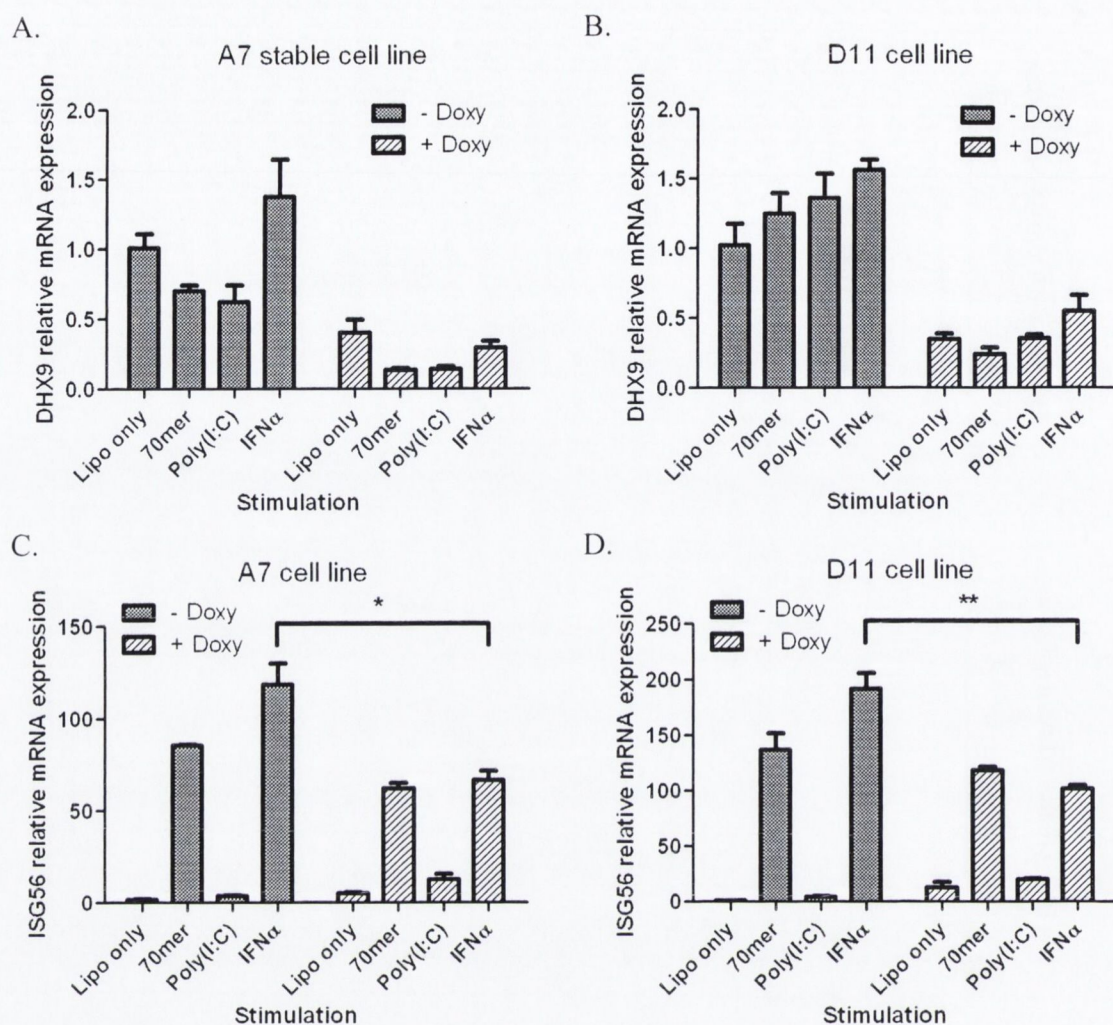


Figure 3.5 Role for DHX9 in IFN α -stimulated ISG56 mRNA induction in THP-1 cells

Two THP-1 cell lines (A7 and D11) expressing doxycycline-inducible DHX9-specific shRNA were incubated with (+ Doxy) or without (- Doxy) 1 μ g/ml doxycycline for 72 hours. Each cell line was then stimulated with 1000 U/ml IFN α or transfected with lipofectamine alone (Lipo only), 1 μ g/ml 70mer or 1 μ g/ml poly(I:C) for 6 hours. DHX9 (A, B) and ISG56 (C, D) mRNA expression was assayed by qPCR. Data are expressed as mRNA values relative to β -actin and the fold induction is relative to the lipofectamine only without doxycycline for each cell line. The data is mean $\pm$ SD of triplicate samples and is representative of three experiments. *= p <0.05, **= p <0.01 compared to without doxycycline.

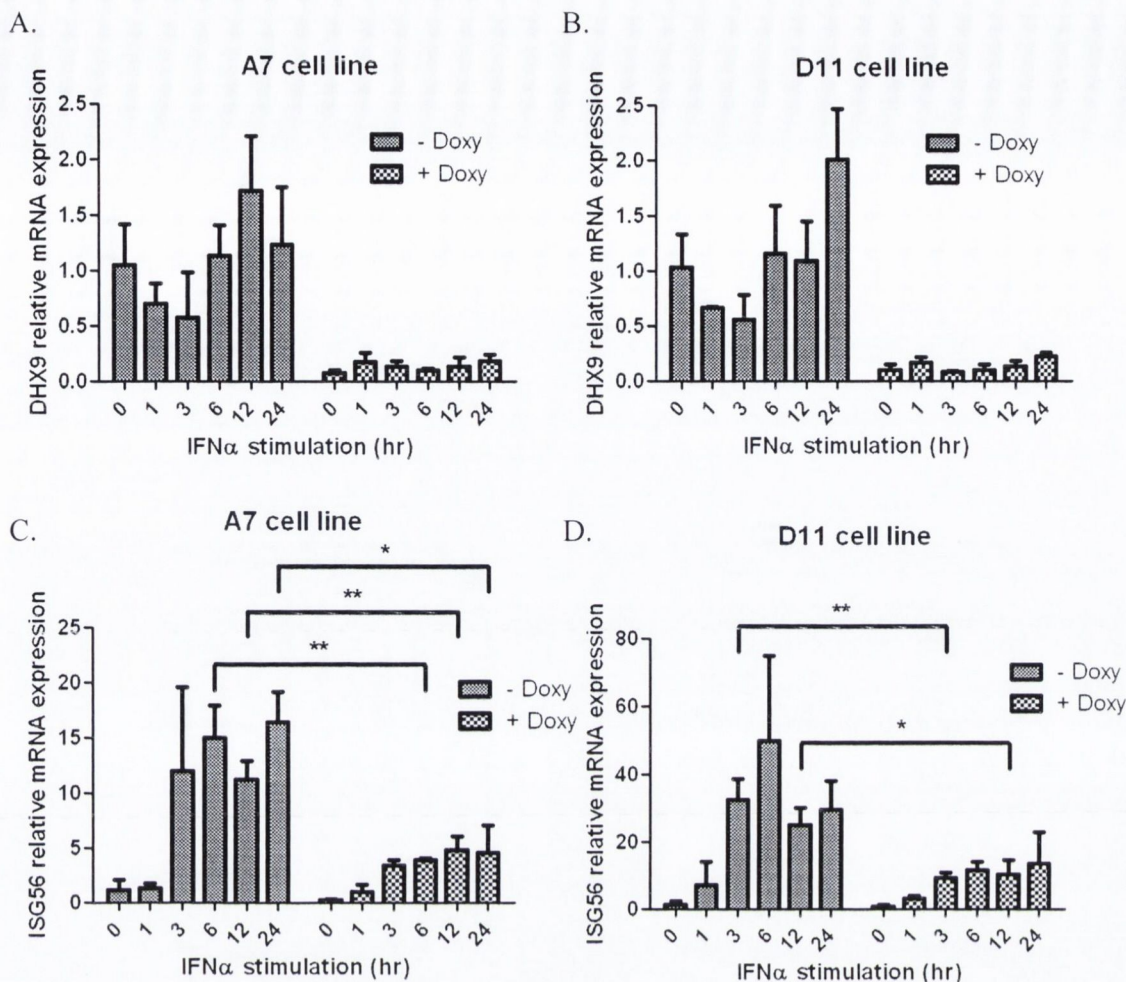


Figure 3.6 Requirement for DHX9 in IFN α -stimulated ISG56 induction

Two THP-1 cell lines (A7 and D11) expressing doxycycline-inducible DHX9-specific shRNA were incubated with (+ Doxy) or without (- Doxy) 1 μ g/ml doxycycline for 72 hours. Each cell line was then stimulated with 1000 U/ml IFN α for the indicated times. DHX9 (A, B) and ISG56 (C, D) mRNA expression was assayed by qPCR. Data is expressed as mRNA values relative to β -actin and the fold induction is relative to the non-stimulated cells without doxycycline. The data is mean $\pm$ SD of triplicate samples and is representative of three experiments. *= p <0.05, **= p <0.01 compared to without doxycycline.

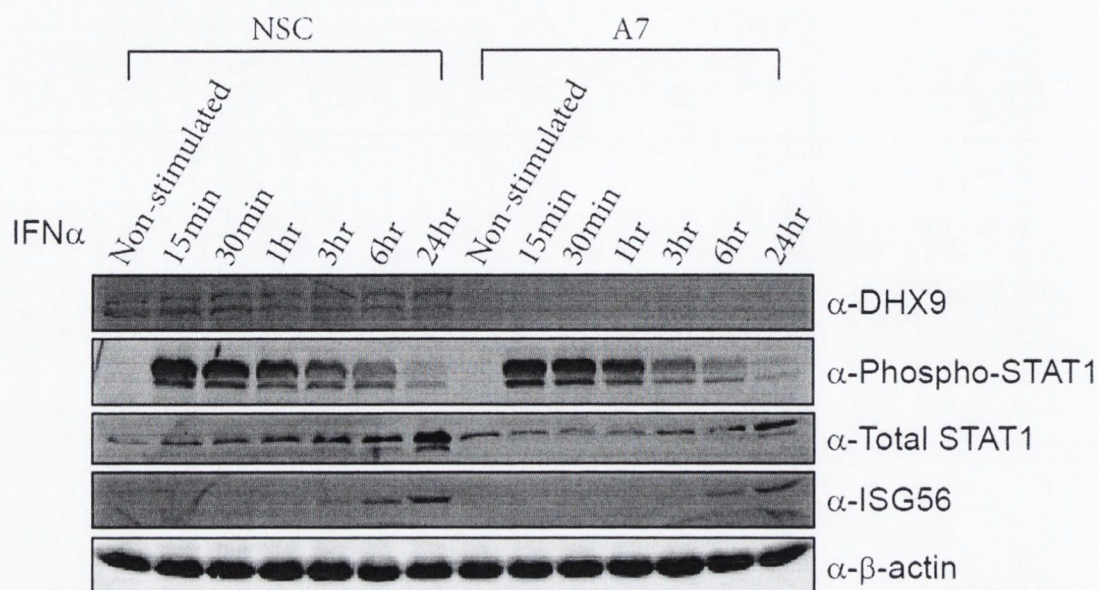


Figure 3.7 DHX9 is not required for IFN α -stimulated ISG56 protein

A THP-1 cell line (A7) expressing doxycycline-inducible DHX9-specific shRNA and one which expresses a non-silencing control (NSC) shRNA were incubated with 1 μ g/ml doxycycline for 72 hours. The cells were then seeded at 0.8×10^6 cells/ml in 6 well plates. 24 hours later, each cell line was stimulated with 1000 U/ml IFN α for the indicated times. Lysates were generated, resolved by SDS-PAGE, and immunoblotted for DHX9, total STAT1, phospho-STAT1, ISG56 and β -actin. This data is representative of three experiments.

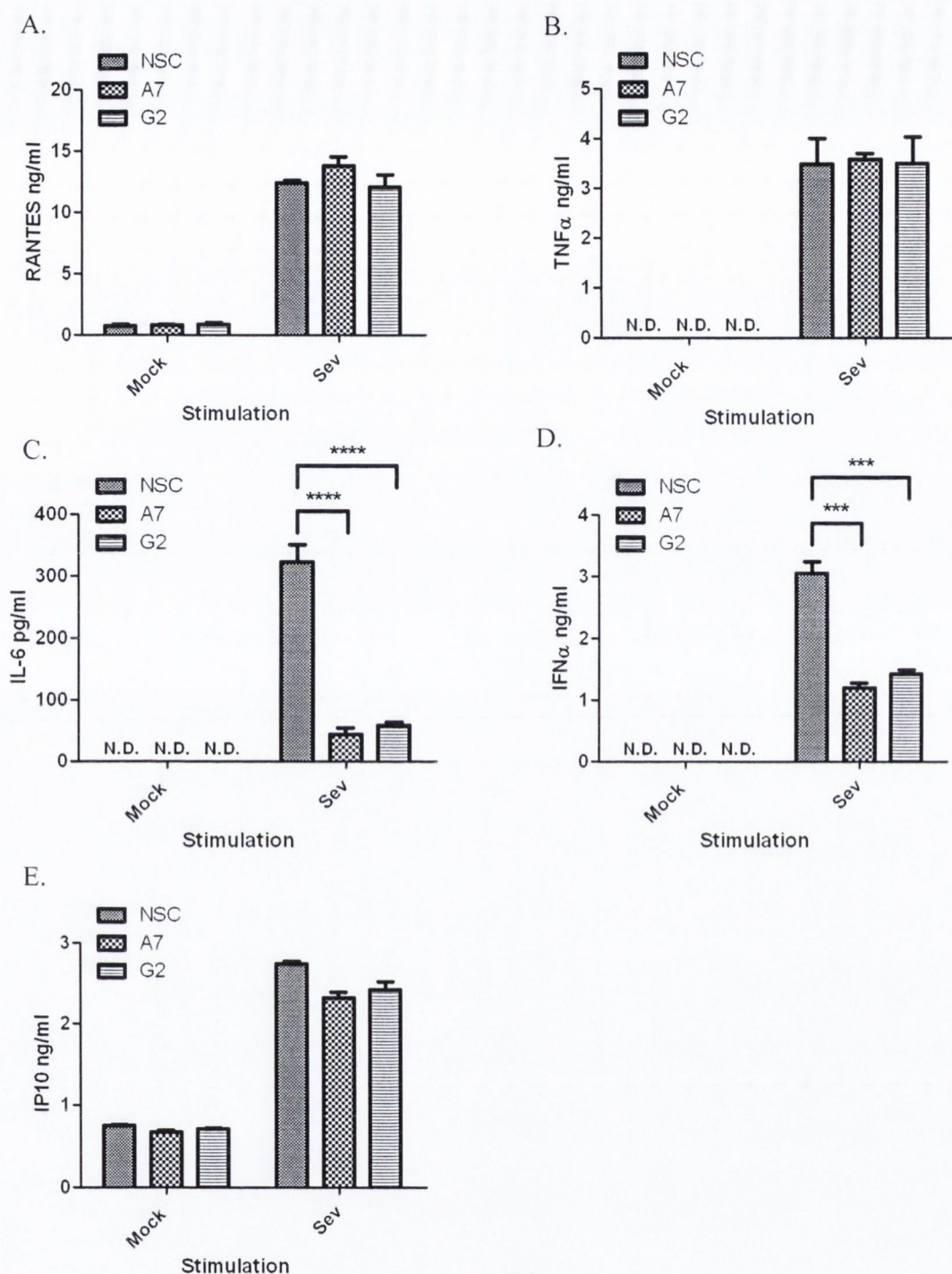


Figure 3.8 Role for DHX9 in RNA virus-stimulated IL-6 and IFNα production in THP-1 cells

Two THP-1 cell lines (A7 and G2) expressing doxycycline-inducible DHX9-specific shRNA and one which expresses a non-silencing control (NSC) shRNA were incubated with 1 µg/ml doxycycline for 72 hours. They were then seeded at 0.8×10^6 cells/ml and mock-infected (Mock) or infected with Sendai virus (SeV) 24 hours later. The supernatants were removed 24 hours later and RANTES (A), TNFα (B), IL-6 (C), IFNα (D) and IP10 (E) secretion were measured by ELISA. The data is mean ± SD of triplicate samples and is representative of three experiments. ***= $p < 0.001$, ****= $p < 0.0001$ compared to NSC. N.D., non-detectable.

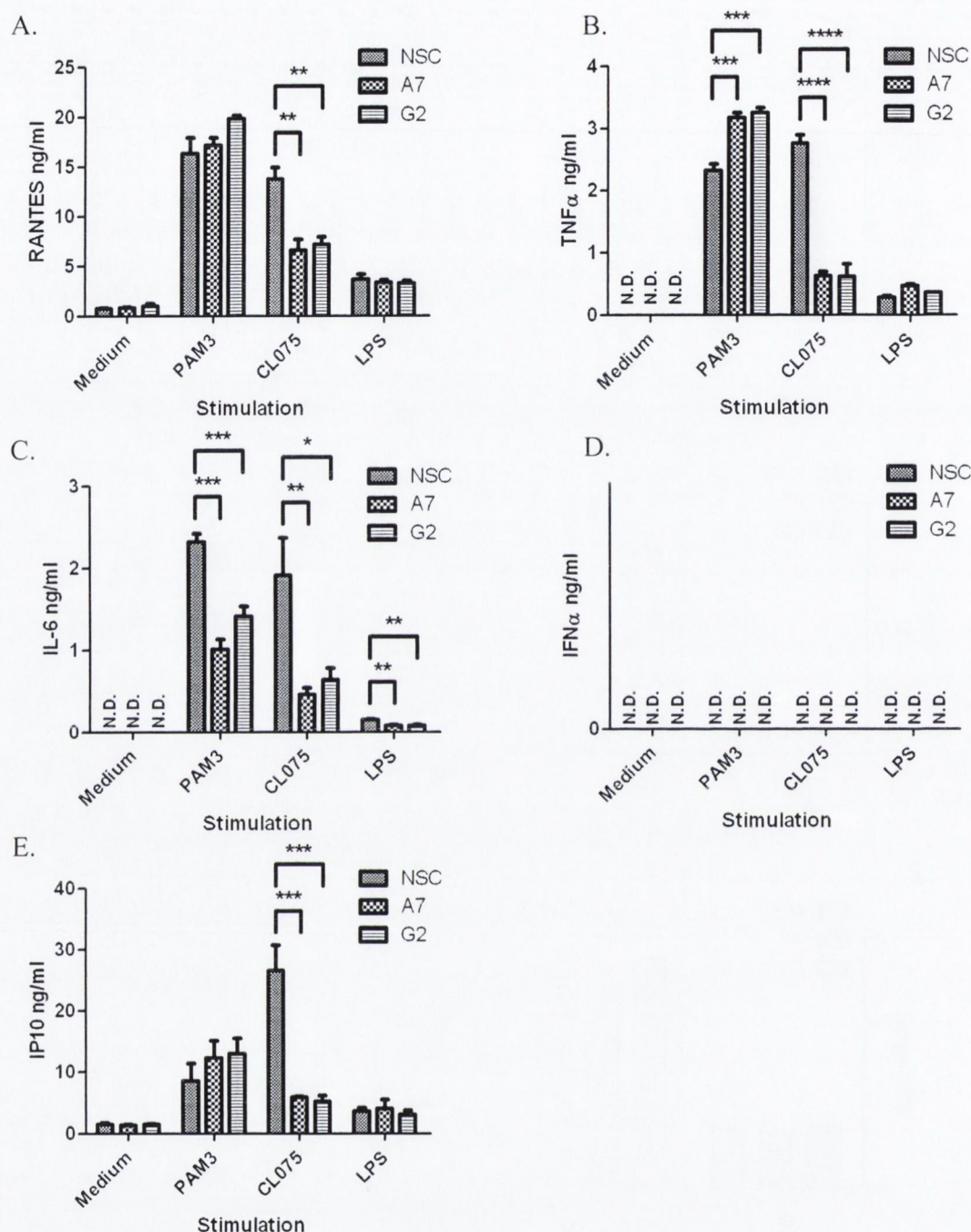


Figure 3.9 Role for DHX9 in TLR-induced cytokines in THP-1 cells

Two THP-1 cell lines (A7 and G2) expressing doxycycline-inducible DHX9-specific shRNA and one which expresses a non-silencing control (NSC) shRNA were incubated with 1 μ g/ml doxycycline for 72 hours. They were then seeded at 0.8×10^6 cells/ml and stimulated 24 hours later with 2 μ g/ml Pam3CSK4 (PAM3), 2.5 μ g/ml CLO75, 100 ng/ml lipopolysaccharide (LPS) or medium alone. The supernatants were removed 24 hours later and RANTES (A), TNF α (B), IL-6 (C), IFN α (D) and IP10 (E) secretion were measured by ELISA. The data is mean $\pm$ SD of triplicate samples and is representative of three experiments. *= $p < 0.05$, **= $p < 0.01$, ***= $p < 0.001$, ****= $p < 0.0001$ compared to NSC. N.D., non-detectable.

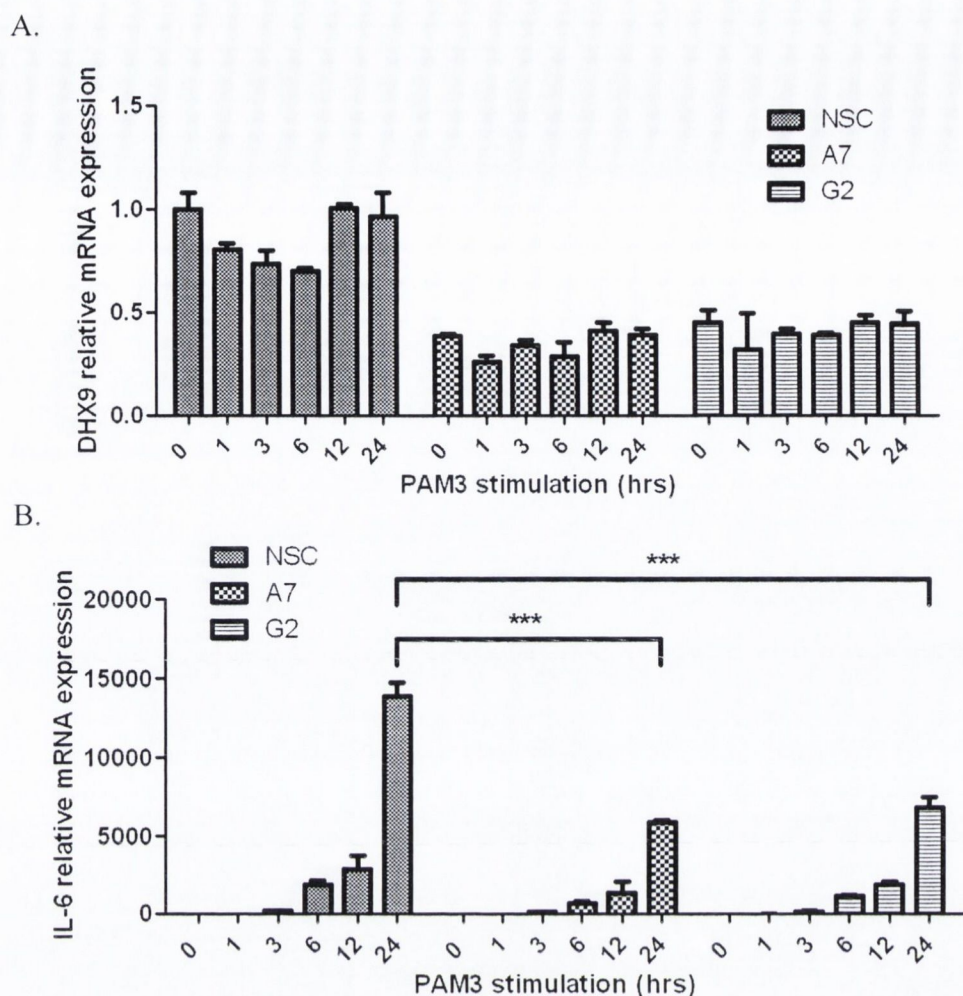


Figure 3.10 DHX9 is required for TLR2-induced IL-6 mRNA

Two THP-1 cell lines (A7 and G2) expressing doxycycline-inducible DHX9-specific shRNA and one which expresses a non-silencing control (NSC) shRNA were incubated with 1 μ g/ml doxycycline for 72 hours. They were then stimulated with 2 μ g/ml Pam3CSK4 (PAM3) for the indicated times. DHX9 (A) and IL-6 (B) mRNA were assayed by qPCR. Data is presented as relative expression of measured gene normalised to β -actin compared to non-stimulated NSC shRNA-expressing cells. The data is mean $\pm$ SD of triplicate samples and is representative of three experiments. ***= $p < 0.001$ compared to NSC shRNA-expressing cells.

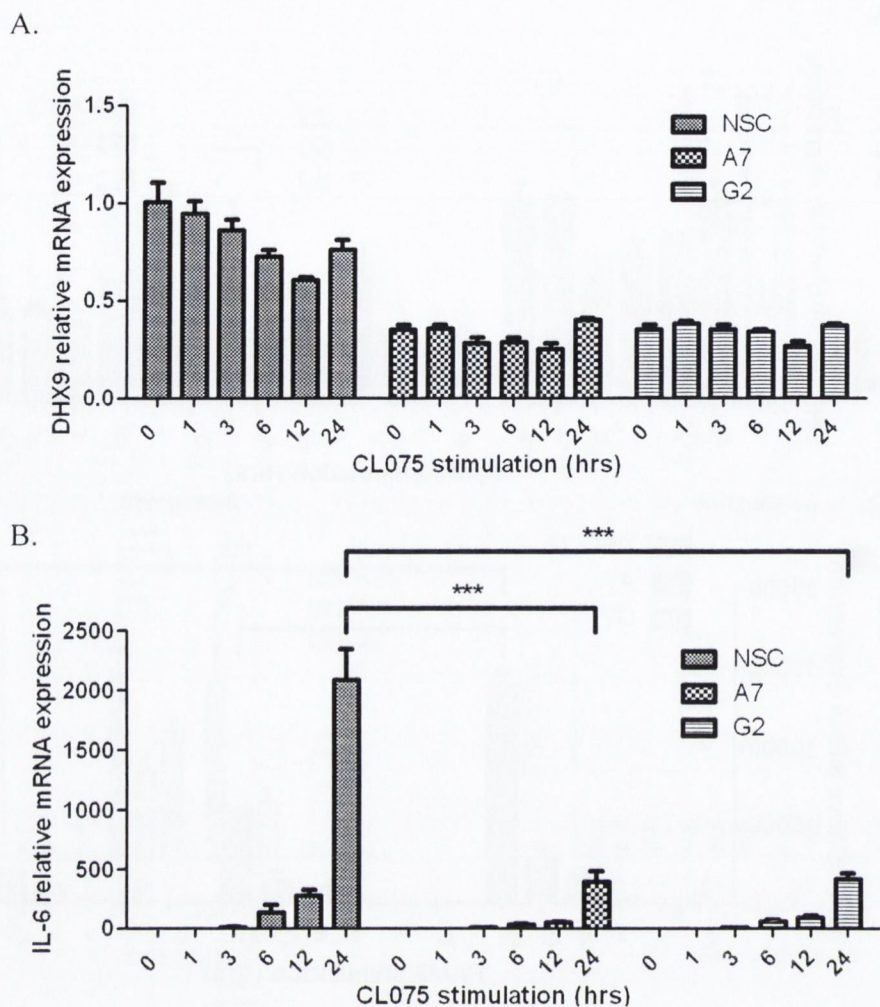


Figure 3.11 DHX9 is required for TLR8-induced IL-6 mRNA

Two THP-1 cell lines (A7 and G2) expressing doxycycline-inducible DHX9-specific shRNA and one which expresses a non-silencing control (NSC) shRNA were incubated with 1 μ g/ml doxycycline for 72 hours. They were then stimulated with 2.5 μ g/ml CL075 for the indicated times. DHX9 (A) and IL-6 (B) mRNA were assayed by qPCR. Data is presented as relative expression of measured gene normalised to β -actin compared to non-stimulated NSC shRNA-expressing cells. The data is mean $\pm$ SD of triplicate samples and is representative of three experiments. ***= $p < 0.001$ compared to NSC shRNA-expressing cells.

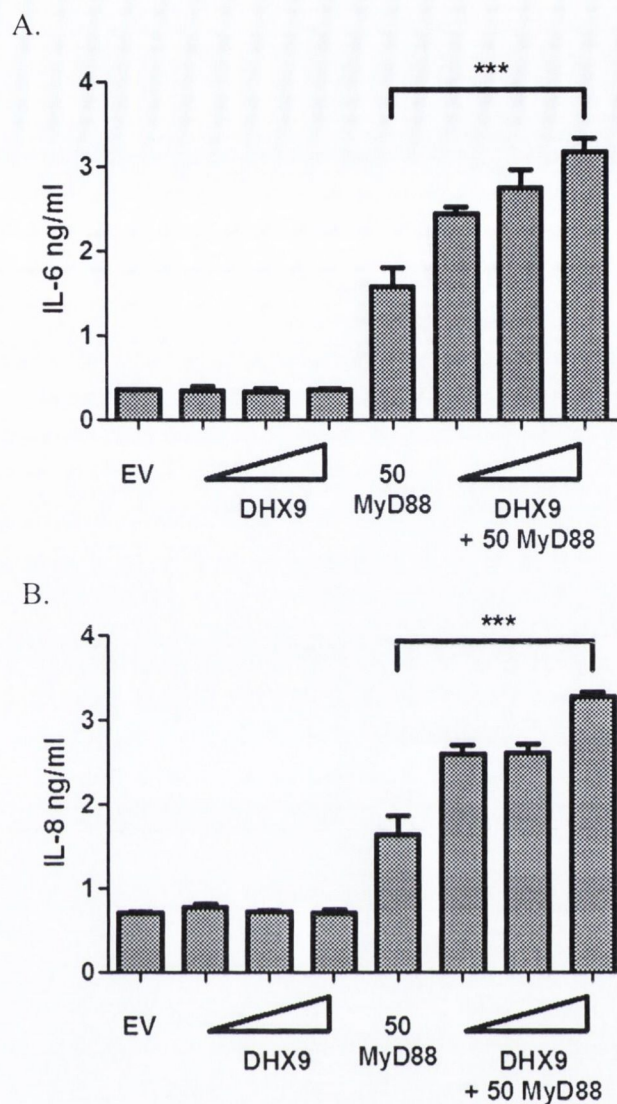


Figure 3.12 DHX9 enhances MyD88-dependent IL-6 and IL-8 production

HeLa cells were seeded at 0.5×10^5 cells/ml 24 hours prior to GeneJuice® transfection. Cells were transfected with empty vector (EV), 50 ng MyD88 or 25, 50 or 100 ng of DHX9 expression vector (wedge) with or without MyD88. Supernatants were removed 48 hours after transfection and analysed for IL-6 (A) and IL-8 (B) by ELISA. The data is mean $\pm$ SD of triplicate samples and is representative of two experiments. ***= $p < 0.001$ compared to MyD88-transfected cells.

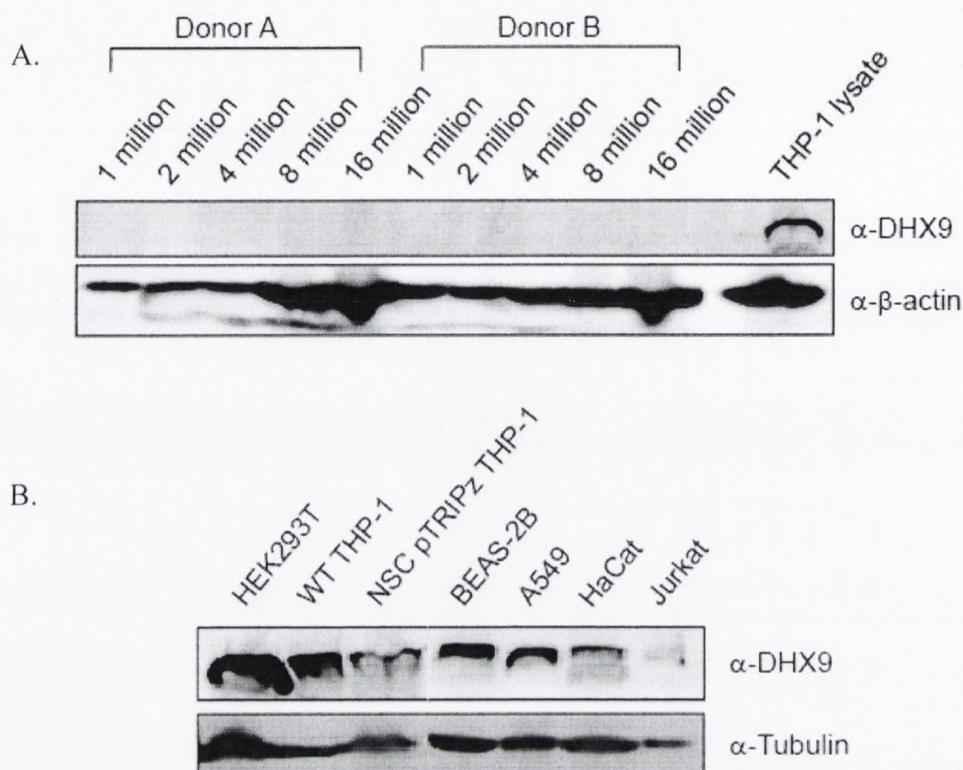


Figure 3.13 DHX9 protein is undetectable in primary monocytes but detectable in a wide range of immortalized cell lines

Primary monocytes were isolated from two blood buffy coats (Donor A and B) using CD14 microbeads. The cells were counted and the indicated amount of cells were lysed (1-16 million). The lysates were resolved by SDS-PAGE alongside a THP-1 cell lysate and immunoblotted for DHX9 and β -actin (A). Numerous cell lines were lysed and resolved by SDS-PAGE to compare levels of DHX9 expression, with tubulin serving as a loading control (B). (A) is representative of three experiments, (B) is from one experiment.

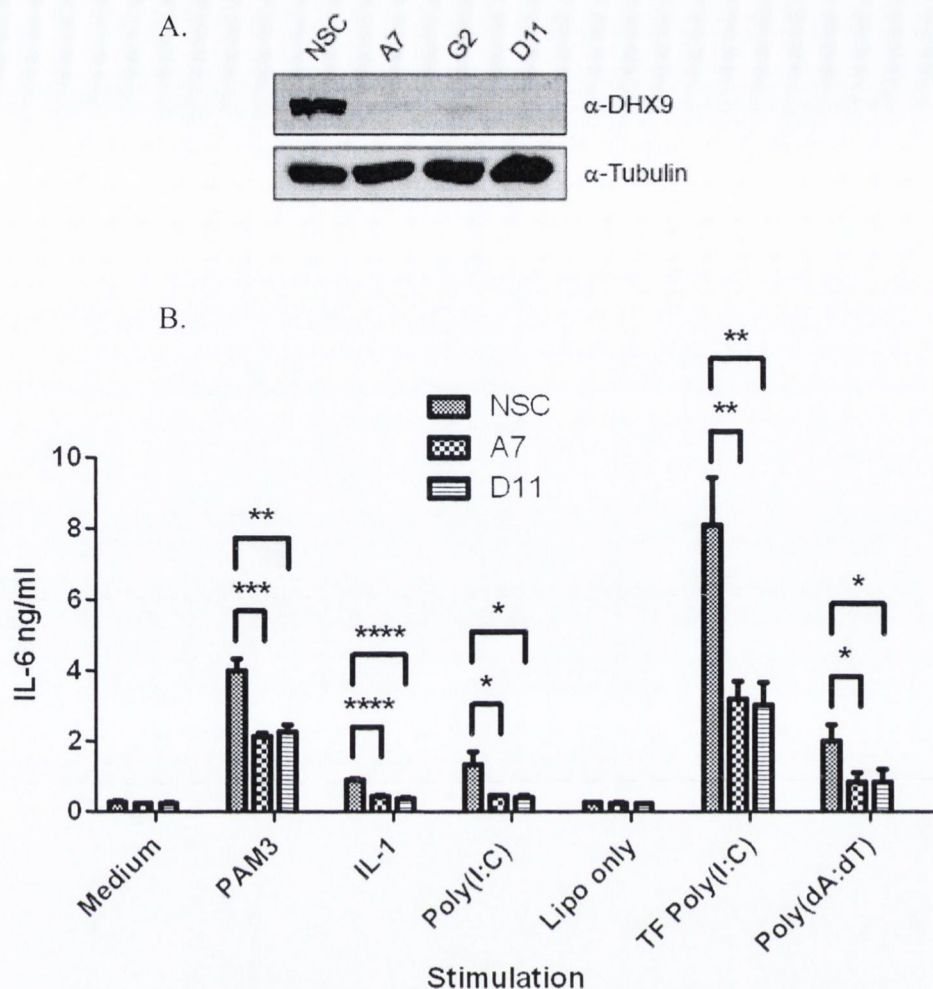


Figure 3.14 DDX9 is required for effective IL-6 protein production in BEAS-2B cells

BEAS-2B cells were transduced with lentiviruses encoding DDX9-targeting shRNA (A7, G2 or D11) or a non-silencing control (NSC) shRNA designed with minimal homology to known mammalian genes. After transduction and puromycin selection, the cells were incubated with 1 μ g/ml doxycycline for 72 hours to induce the required shRNA. Lysates were generated, resolved by SDS-PAGE, and immunoblotted DDX9 and tubulin (A). The shRNA cell lines found to give best DDX9 protein silencing (A7 and D11) and the NSC shRNA-expressing BEAS-2B cells were incubated with 1 μ g/ml doxycycline for 72 hours. They were then non-stimulated, stimulated with 10 μ g/ml Pam3CSK4 (PAM3), 100 ng/ml IL-1, 125 μ g/ml poly(I:C), or transfected (TF) with lipofectamine only (Lipo only), 25 μ g/ml poly(I:C) or 5 μ g/ml poly(dA:dT) for 24 hours. The supernatants were removed and IL-6 secretion was measured by ELISA (B). The data is mean $\pm$ SD of triplicate samples and is representative of three experiments. *= p <0.05, **= p <0.01, ***= p <0.001, ****= p <0.0001 compared to NSC stimulated/transfected cells.

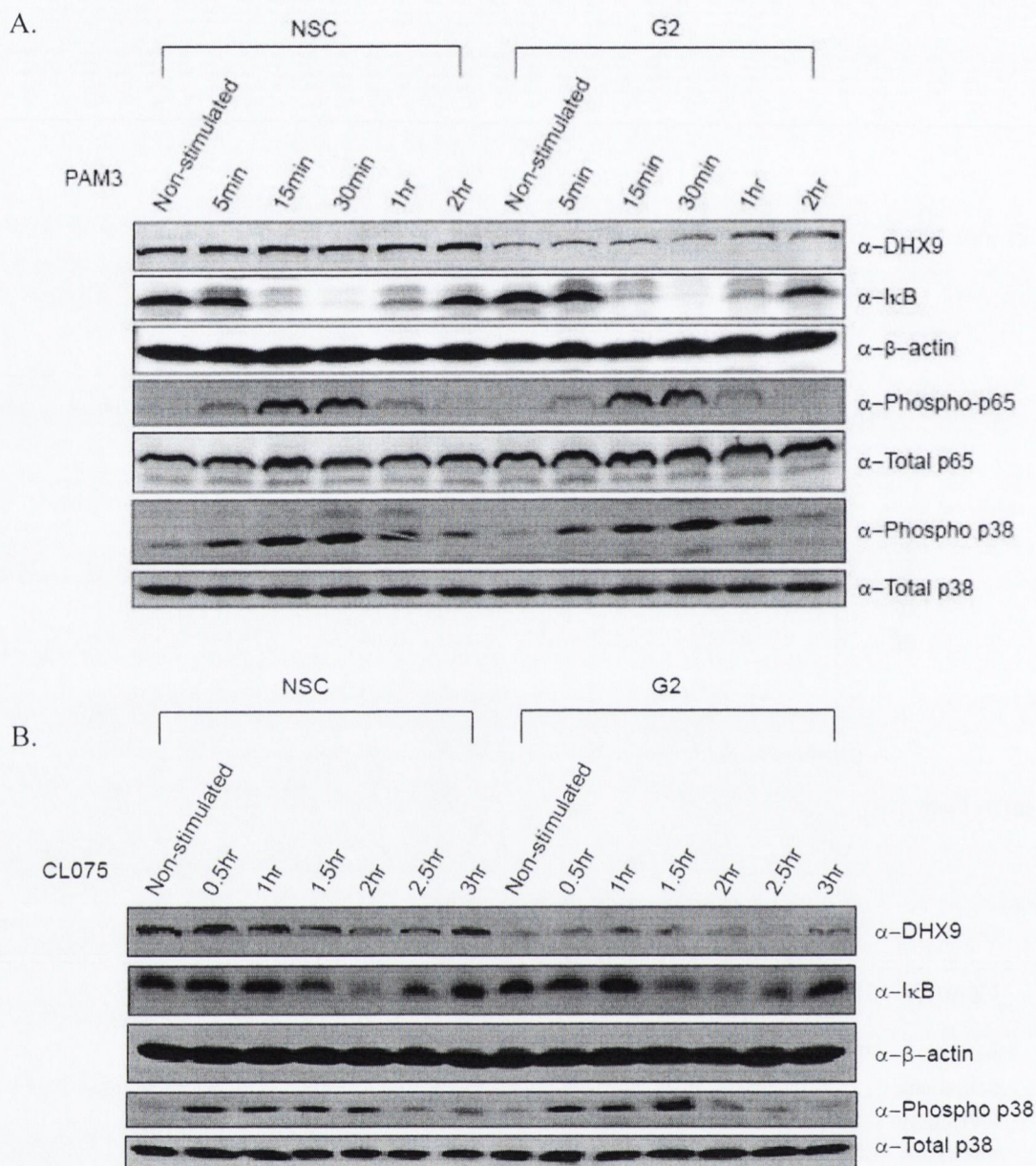


Figure 3.15 DHX9 is not required for TLR2-induced IκB degradation, p65 activation and p38 activation or TLR8-induced IκB degradation or p38 activation

A THP-1 cell line expressing doxycycline-inducible DHX9-specific shRNA (G2) and one which expresses a non-silencing control (NSC) shRNA, were incubated with 1 μg/ml doxycycline for 72 hours and then stimulated with either 2 μg/ml Pam3CSK4 (PAM3) (A) or 2.5 μg/ml CL075 (B) for the indicated times. Lysates were created and resolved by SDS-PAGE. Gels were immunoblotted for DHX9, IκB, β-actin, phospho-p65, total p65, phospho-p38 and total p38 as indicated. This data is representative of three experiments.

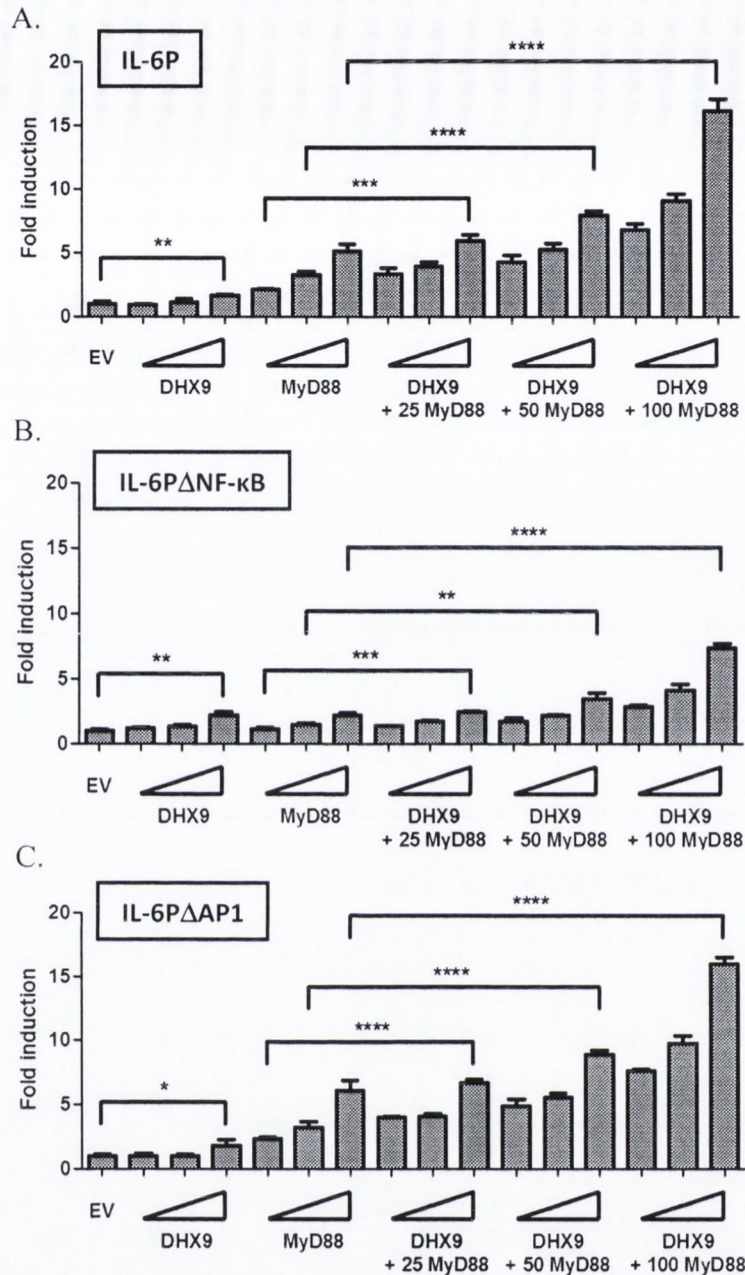


Figure 3.16 DHX9 enhances MyD88-dependent transactivation of the IL-6 promoter and this is partially dependent on the κ B site of the promoter

HeLa cells were seeded at 0.5×10^5 cells/ml, 24 hours prior to GeneJuice® transfection. Cells were transfected with wild type (IL-6P) IL-6 promoter reporter gene (A), an IL-6 promoter reporter lacking (IL-6PΔNF- κ B) its κ B-binding site (B) or an IL-6 promoter reporter (IL-6PΔAP1) lacking its AP1-binding site (C), alongside the pGL3-Renilla constitutive reporter and empty vector (EV) or increasing amounts of DHX9 and/or MyD88 expression vector. Wedges indicate increasing amounts of DHX9 or MyD88 (25, 50, 100 ng). 48 hours after transfection, the cells were lysed and luciferase activity in the lysates was assayed using a luminometer. Data is shown as relative fold induction normalised to EV only-transfected cells. The data is mean $\pm$ SD of triplicate samples and is representative of two experiments. *= $p < 0.05$, **= $p < 0.01$, ***= $p < 0.001$, ****= $p < 0.0001$ compared to EV alone-transfected cells or cells transfected with only MyD88 expression vector.

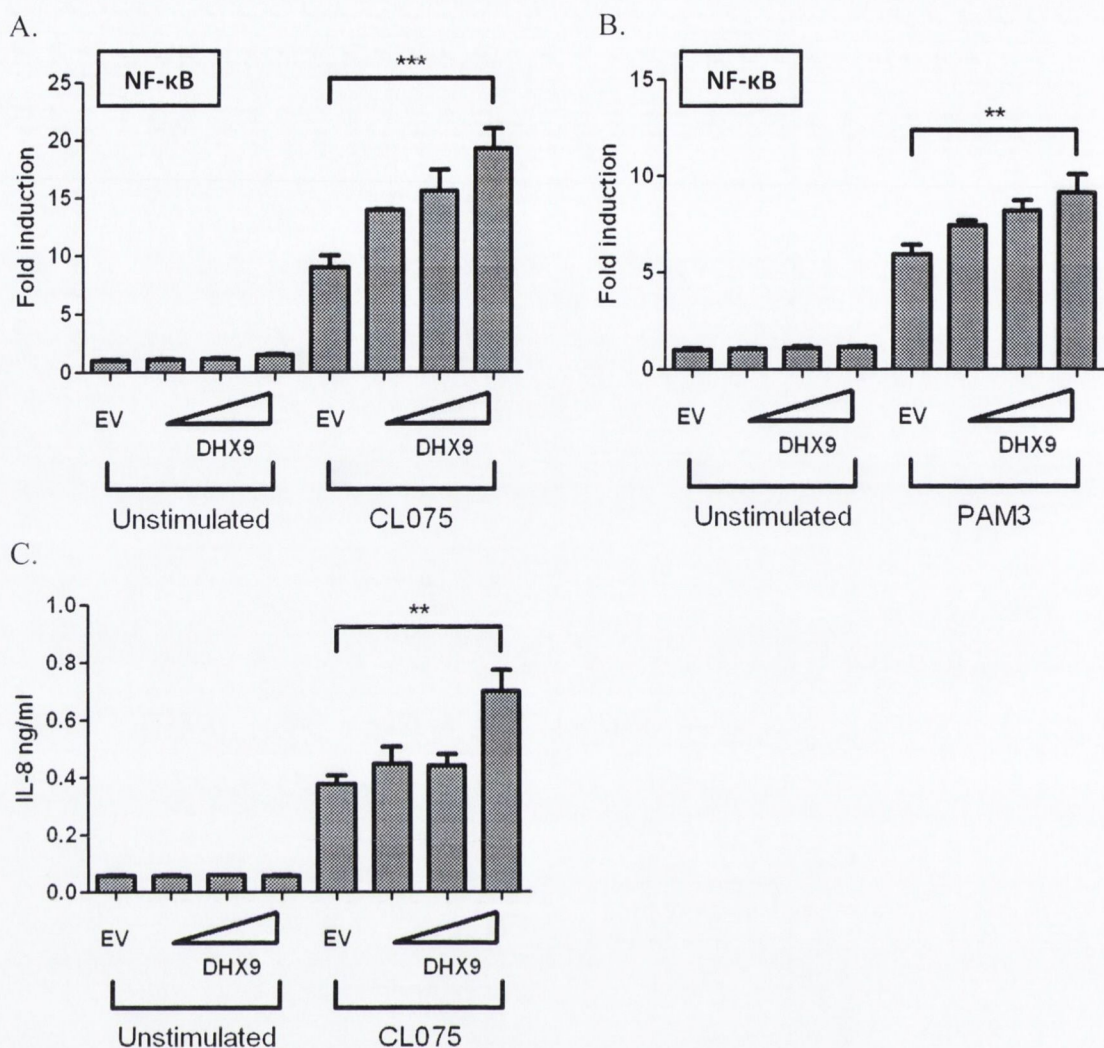


Figure 3.17 DHX9 enhances TLR8- and TLR2-dependent NF-κB activation and TLR8-dependent IL-8 production

HEK-TLR8 (A, C) and HEK-TLR2 (B) cells were seeded at 1×10^5 cells/ml, 24 hours prior to GeneJuice® transfection. (A, B) Cells were transfected with NF-κB promoter reporter gene, the pGL3-Renilla constitutive reporter and empty vector (EV) or increasing amounts of DHX9 expression vector. Wedges indicate increasing amounts of DHX9 (25, 50, 100 ng). 24 hours later, the cells were either unstimulated or stimulated with 2.5 μg/ml CL075 (A) or 2 μg/ml Pam3CSK4 (PAM3) (B). After a further 24 hours the cells were lysed and the lysates assayed for luciferase activity using a luminometer. Data is shown as relative fold induction normalised to unstimulated, EV only-transfected cells. (C) Cells were transfected with empty vector (EV) or 50, 100 or 200 ng of DHX9 expression vector (wedge). Cells were either unstimulated or stimulated with 2.5 μg/ml CL075 24 hours later. Supernatants were removed 24 hours after stimulation and analysed for IL-8 by ELISA. The data is mean $\pm$ SD of triplicate samples and is representative of three experiments. **= $p < 0.01$, ***= $p < 0.001$ compared to CL075-stimulated, EV only-transfected cells (A, C) or PAM3-stimulated, EV only-transfected cells (B).

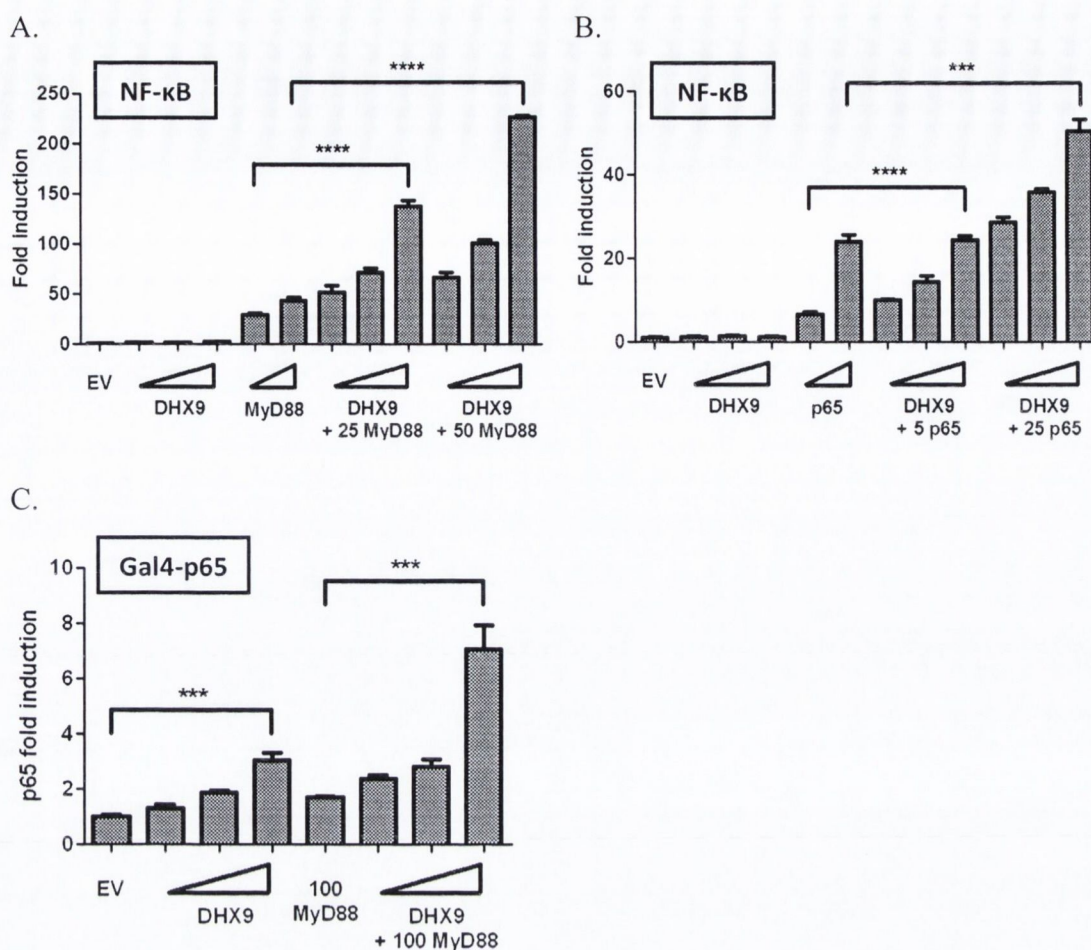


Figure 3.18 DHX9 enhances MyD88- and p65-dependent NF-κB activation and p65 transactivation

HeLa cells were seeded at 0.5×10^5 cells/ml, 24 hours prior to GeneJuice® transfection. Cells were transfected with NF-κB promoter reporter gene (A, B) or Gal4-p65 with pFR luciferase (C), with the pGL3-Renilla constitutive reporter and empty vector (EV) or the indicated amounts (ng) of MyD88, p65 or DHX9 expression vectors. Wedges indicate increasing amounts of DHX9 (25, 50, 100 ng), MyD88 (25, 50 ng) or p65 (5, 25 ng). The cells were lysed 48 hours after transfection and the lysates assayed for luciferase activity using a luminometer. Data is shown as relative fold induction normalised to EV only-transfected cells. The data is mean $\pm$ SD of triplicate samples and is representative of three experiments. ***= $p < 0.001$, ****= $p < 0.0001$ compared to MyD88 (A) or p65 only (B) -transfected cells, or EV only-transfected cells or MyD88 only-transfected cells (C).

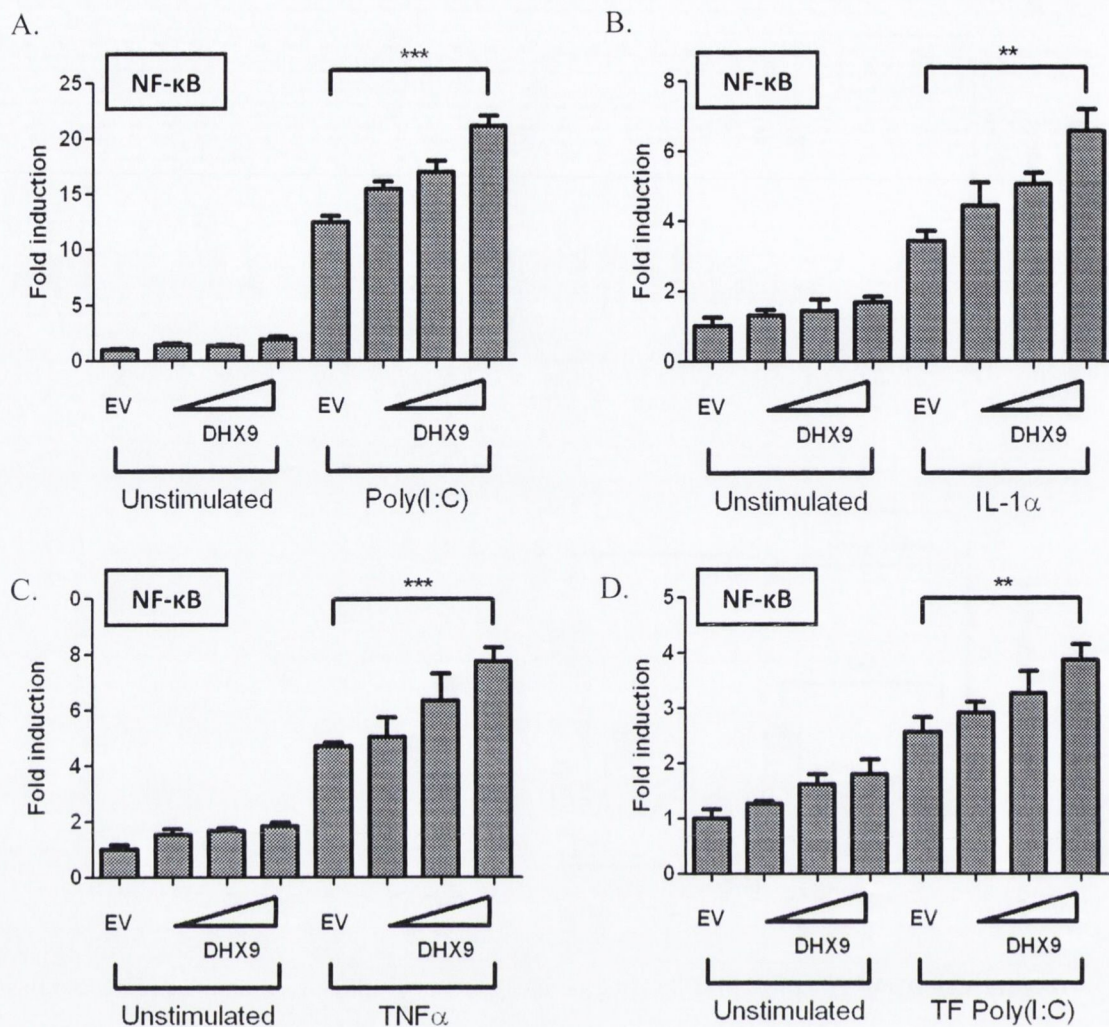


Figure 3.19 DHX9 enhances NF- κ B activation by multiple stimuli

HEK-TLR3 cells (A) and HeLa cells (B-D) were seeded at 1×10^5 cells/ml and 0.5×10^5 cells/ml, respectively, 24 hours prior to GeneJuice® transfection. Cells were transfected with NF- κ B promoter reporter gene, the pGL3-Renilla constitutive reporter and empty vector (EV) or increasing amounts of DHX9 expression vector. Wedges indicate increasing amounts of DHX9 (25, 50, 100 ng). 24 hours after transfection HEK-TLR3 cells were unstimulated or stimulated with 25 μ g/ml poly(I:C) (A). HeLa cells were unstimulated or stimulated with 20 ng/ml IL-1 α (B), 20 ng/ml TNF α (C) or transfected (TF) with 25 μ g/ml poly(I:C) (D). The cells were lysed 24 hours after stimulation and luciferase activity in the lysates was assayed using a luminometer. Data is shown as relative fold induction normalised to unstimulated, EV only-transfected cells. The data is mean $\pm$ SD of triplicate samples and is representative of two experiments. **= $p < 0.01$, ***= $p < 0.001$ compared to stimulated, EV only-transfected cells.

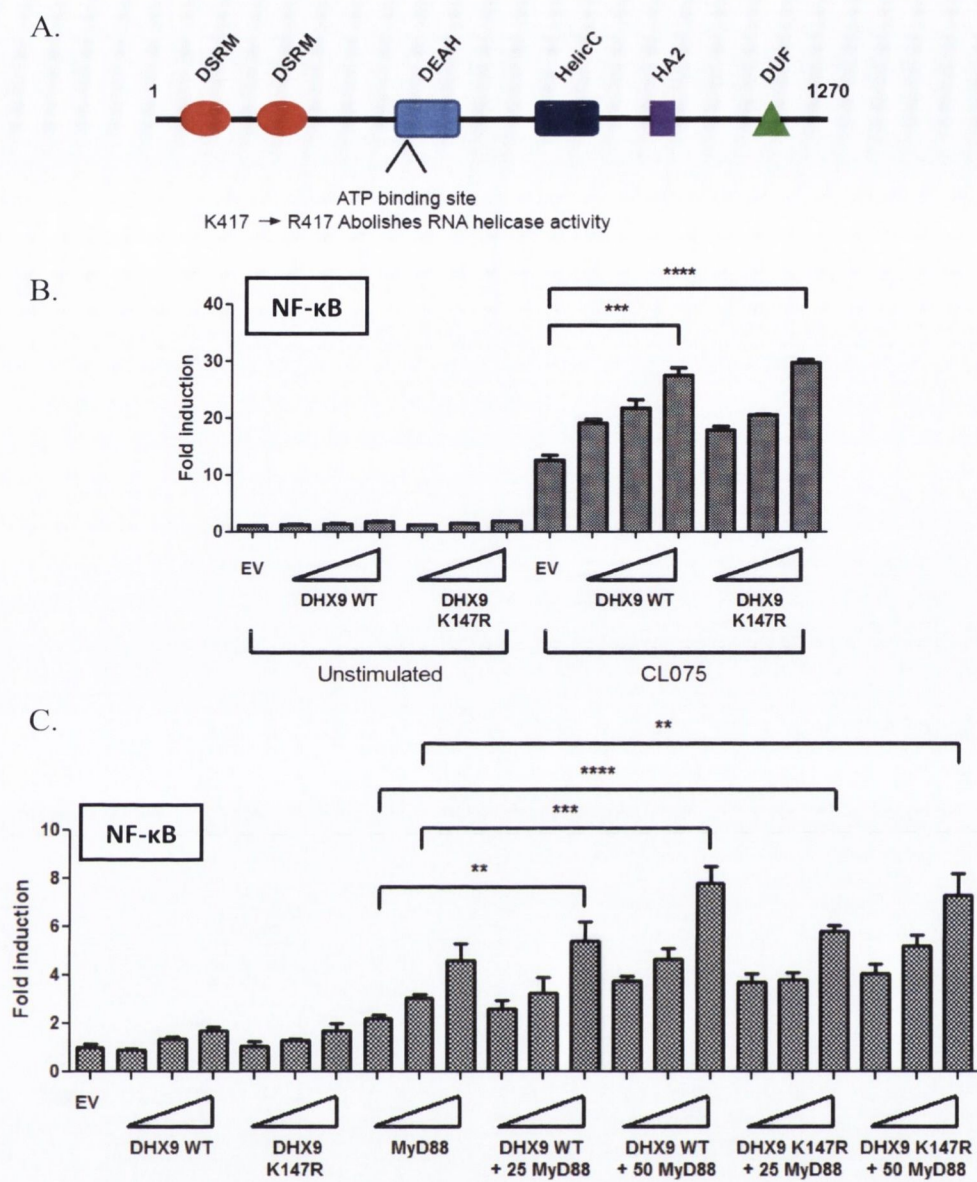


Figure 3.20 DHX9 enhancement of NF-κB activation does not require its helicase activity

(A) Schematic of DHX9 structure. A single nucleotide substitution to change lysine 417 to an arginine (K417R) in the DHX9 ATP binding site was carried out to create a DHX9 mutant which lacks its helicase activity. DSRM, Double-stranded RNA binding motif; DEAH, Aspartate-glutamate-alanine-histidine box motif; HelicC, Helicase C-terminal domain; HA2, Helicase-associated domain 2; DUF, Domain of unknown function. HEK-TLR8 cells (B) and HeLa cells (C) were seeded at 1×10^5 cells/ml and 0.5×10^5 cells/ml, respectively, 24 hours prior to GeneJuice® transfection. Cells were transfected with NF-κB promoter reporter gene, the pGL3-Renilla constitutive reporter and empty vector (EV) or the indicated amounts (ng) of DHX9 expression vector and MyD88 expression vector. Wedges indicate increasing amounts of DHX9 (25, 50, 100 ng) or MyD88 (25, 50, 100 ng). 24 hours after transfection HEK-TLR8 cells were unstimulated or stimulated with 2.5 μg/ml CL075 (B). HeLa cells were lysed 48 hours after transfection (C) and HEK-TLR8 cells 24 hours after stimulation (B), and luciferase activity in the lysates was assayed using a luminometer. Data is shown as relative fold induction normalised to unstimulated, EV only-transfected cells. The data is mean ± SD of triplicate samples and is representative of three experiments. **= $p < 0.01$, ***= $p < 0.001$, ****= $p < 0.0001$ compared to stimulated, EV only-transfected cells or MyD88 only-transfected cells.

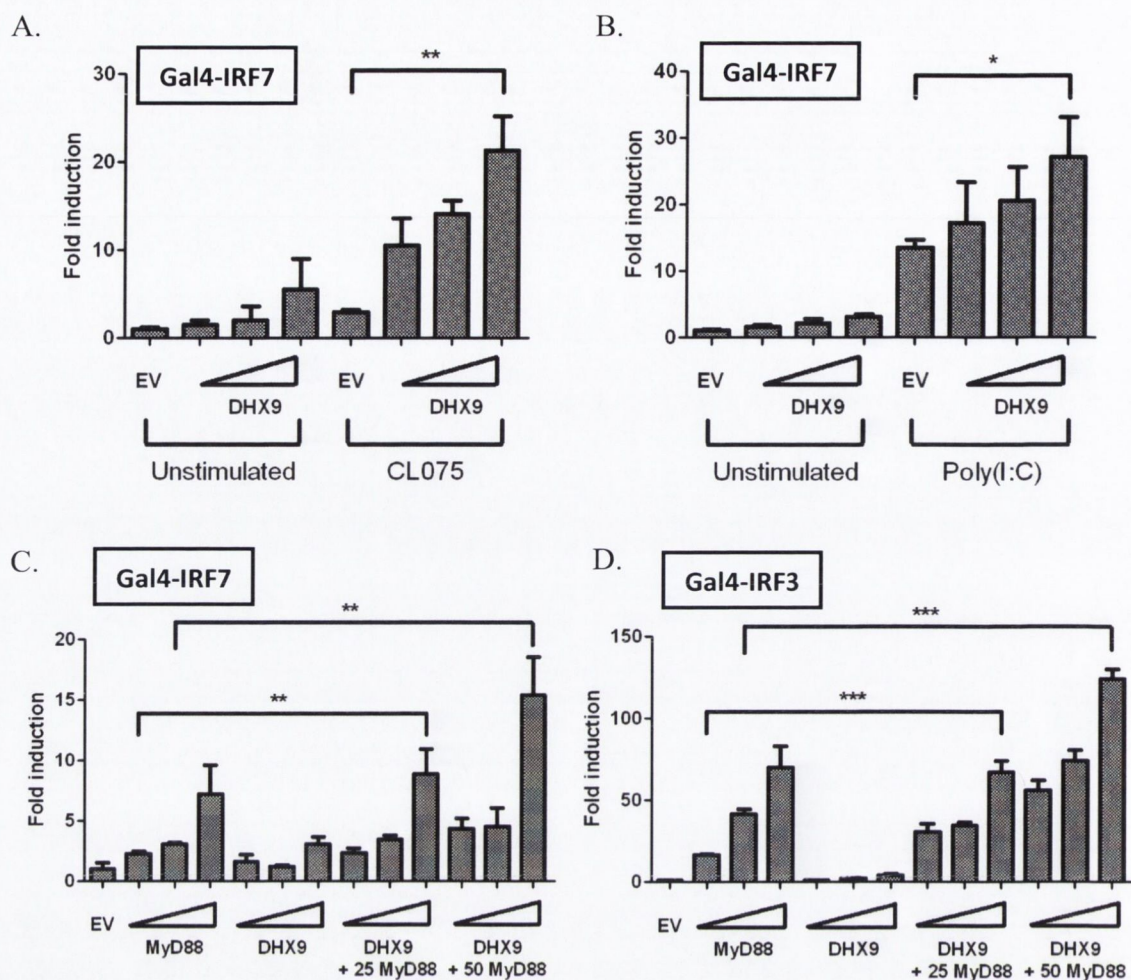


Figure 3.21 DHX9 expression enhances IRF7 and IRF3 transactivation

HEK-TLR8 cells (A), HEK-TLR3 cells (B) and HeLa cells (C, D) were seeded at 1×10^5 cells/ml, 1×10^5 cells/ml, and 0.5×10^5 cells/ml, respectively, 24 hours prior to GeneJuice® transfection. Cells were transfected with Gal4-IRF7, pFR luciferase, the pGL3-Renilla constitutive reporter and empty vector (EV), or the indicated amounts of DHX9 or MyD88 expression vector. Wedges indicate increasing amounts of DHX9 (25, 50, 100 ng) or MyD88 (25, 50, 100 ng). 24 hours after transfection, HEK-TLR8 cells were unstimulated or stimulated with 2.5 μ g/ml CL075 (A), and HEK-TLR3 cells were unstimulated or stimulated with 25 μ g/ml poly(I:C) (B). HeLa cells were lysed 48 hours after transfection (C, D) and HEK-TLR8 and TLR3 cells 24 hours after stimulation, and luciferase activity in the lysates was assayed using a luminometer. Data is shown as relative fold induction normalised to unstimulated, EV only-transfected cells. The data is mean $\pm$ SD of triplicate samples and is representative of three experiments. *= $p < 0.05$, **= $p < 0.01$, ***= $p < 0.001$ compared to stimulated, EV only-transfected cells (A, B) or MyD88 only transfected cells (C, D).

A.

Ligand	IFN α	IL-6	TNF α	RANTES	IP10
PAM3	-	↓	1/2↑	n.a	n.a
CL075	-	↓↓	↓↓	↓	↓↓
SeV	↓	↓↓	n.a	n.a	n.a
LPS	-	↓	n.a	n.a	n.a
Poly(dA:dT)	-	-	n.a	n.a	n.a
70mer	-	-	-	n.a	n.a
cGAMP	-	-	-	n.a	n.a

B.

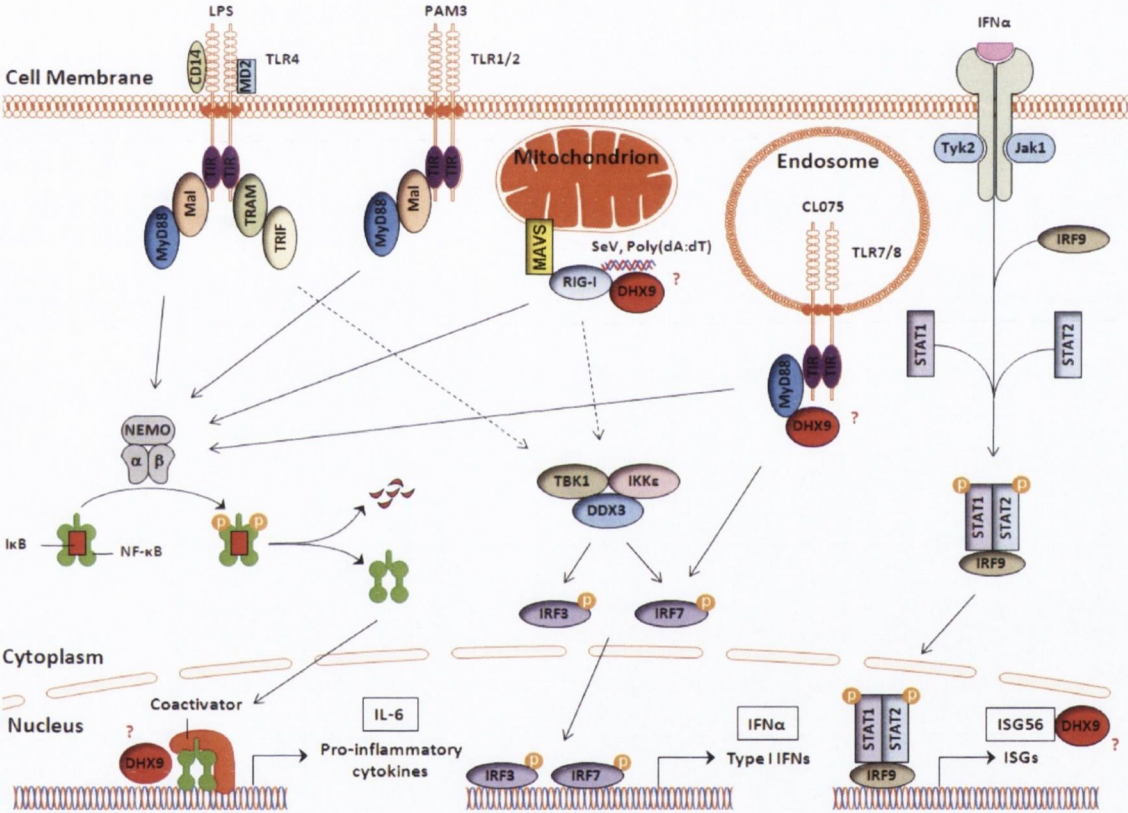


Figure 3.22 A role for DHX9 in innate immune signalling in THP-1 cells

(A) Schematic of cytokine responses affected upon DHX9 knockdown. PAM3, Pam3CSK4; SeV, Sendai virus; LPS, Lipopolysaccharide; n.a, non-affected; -, non-induced; ↓, ≥50% down; ↓↓, ≥70% down; 1/2↑, ≥25% up. (B) DHX9 was found to be required for effective NF-κB activation in response to any stimuli tested. DHX9 was found to be critical for effective IL-6 production, most likely functioning at the promoter as upstream signalling cascades were found to be unaffected. SeV-induced IFN α and IL-6 cytokine production was also found to be DHX9-dependent. DHX9 has been found to signal via MAVS upon recognition of dsRNA and hence may be recognising SeV dsRNA. DHX9 may be required for MyD88-dependent endosomal signalling. TLR7/8-dependent cytokine upregulation was particularly dependent on DHX9. DHX9 was also found to be required for efficient IFN α -induced ISG56 mRNA induction.

**Chapter 4: Investigating innate
immune functions of the host helicase
DHX9 which E3 targets**

4.1 Introduction

VACV encodes a range of immunomodulatory proteins which are vital in establishing early infection. One gene encoding such a protein is VACV *E3L*, which encodes the protein E3. E3 is a multifunctional protein which has been found to be vital in VACV replication and pathogenesis [362]. E3 is 190 amino acids in length and encodes a C-terminal dsRNA-BD and an N-terminal Z-DNA-BD, both of which can antagonise various aspects of innate immune signalling (Section 1.13). VACV deleted of *E3L* has been reported to be more IFN sensitive, which is mediated primarily by PKR [346, 393]. The inhibition of PKR by E3 is primarily through its ability to sequester dsRNA via its C-terminal RNA-BD [348]. However, there is also evidence that E3 can directly bind PKR to antagonise its function [394]. Other functions of the E3 RNA-BD include its ability to inhibit the Pol III dsDNA-sensing pathway, and its direct interaction, and inhibition, of the antiviral protein, ISG15 [352, 395]. The N-terminal DNA-BD is less understood. Although dispensable for replication and IFN resistance in cell culture [347, 348], it has been shown to be necessary for full pathogenesis in mice [320, 396]. The Z-DNA-binding capability of the DNA-BD has been shown to be important in neurovirulence in mice [320]. Other functions of the Z-DNA-BD of E3 include its ability to inhibit the PKR pathway, and its ability to inhibit apoptosis through transactivation of IL-6, NFAT and p53 [362].

Due to the major importance of E3 in VACV viral evasion, mass spectrometry was carried out in the lab by Dr. Sinead Keating to identify other possible proteins within the host which E3 interacts with (S. Keating, personal communication). This experiment was carried out using an E3 antibody to immunoprecipitate possible interacting partners from both wild-type WR VACV-infected and, as a control, VACV Δ E3-infected cell lysates. Immunoprecipitated proteins were then electrophoresed on a SDS gel and the entire gel was analysed in slices and visually compared to the control immunoprecipitation protein bands. Proteins eluted from gel slices were sent for Liquid Chromatography-Mass Spectrometry. DHX9 was identified as a potential E3-interacting protein, with DHX9 interacting with E3 in both HEK 293 and MEF cells (S. Keating, personal communication).

The E3 protein is composed of a C-terminal dsRNA-BD and an N-terminal Z-DNA-BD, with both regions being required for virulence in mice [320]. Hence, one of the first goals of this chapter was to investigate what region of E3 is necessary for the DHX9 interaction. Also, to identify what region of DHX9 was required for the interaction, truncations of DHX9 needed to be created, so that immunoprecipitation studies could be carried out.

VACV may be targeting DHX9 to antagonise the host immune response or it may be sequestering DHX9 to aid in its replication and life cycle. There is currently no definitive study in which a virus has been shown to directly target host DHX9 as a means of antagonising the host immune response. However, knockdown of DHX9 has shown that DHX9 is necessary for an effective cytokine response to HSV in pDCs [270], and to influenza A and reovirus in mDCs [222]. On the other hand, there are a number of published studies in which viruses have been shown to hijack host DHX9 and use it to enhance viral replication, transcription and translation. Amongst them are; influenza A virus, HIV-1, human T cell leukemia virus type 1, foot-and-mouth disease virus and hepatitis C virus [376-378, 397, 398]. In 2013, a publication identified DHX9 as being a target of the myxoma virus protein M029 [379]. M029 is a truncated ortholog of VACV E3 which lacks a significant portion of the N-terminal Z-DNA-BD. In this publication, M029 was found to play a vital role in the myxoma viral life cycle. Capable of binding both PKR and DHX9, M029 was demonstrated to inhibit the PKR pathway, while recruiting DHX9 to promote viral replication [379].

DHX9 has been shown to be required for the production of type I IFNs and pro-inflammatory cytokines in response to both RNA and DNA in DCs [222, 270]. That E3 would bind DHX9 to antagonise the potential role of DHX9 in the sensing of VACV genomic DNA or RNA replication intermediates, signalling via MyD88 or MAVS, respectively, was therefore a possibility. This is however unlikely, as DHX9 was found to not be required for effective chemokine or IFN β production in response to VACV 70mer DNA or synthetic dsRNA (poly(I:C)) in THP-1 cells (Chapter 3). Since DHX9 was demonstrated here to be required for the effective transactivation of NF- κ B, IRF7 and IRF3, as a means of evading the innate immune response, the antagonism of DHX9 by VACV E3 would hence be logical from the virus's perspective.

Of particular interest is the role of DHX9 in IL-6 production. DHX9 was found to be vital in the production of IL-6 in response to any stimuli tested in either THP-1 or BEAS-2B cells. Overexpression of DHX9 was demonstrated to enhance both MyD88-dependent activation of the IL-6 promoter, and IL-6 protein secretion, in HeLa cells. All experiments carried out to date, indicate DHX9 to play a transcriptional role in the production of IL-6, with upstream transcription factor and MAPK activation not being affected, but IL-6 mRNA being reduced. IL-6 has been reported to play a pivotal role in the VACV life cycle and VACV E3 has been demonstrated to inhibit its production by an unidentified mechanism [399]. IL-6 deficient mice have been reported to support up to a 1000-fold-greater viral replication in the lungs, due to a defective cytotoxic T cell response against VACV [400]. As the mechanism by which E3 inhibits IL-6 production has yet to be elucidated, the next goal was to investigate whether it may be via its association with DHX9.

To explore whether E3 antagonised innate immune functions of DHX9 discovered in the previous chapter, THP-1 cells stably expressing E3 were generated, and overexpression studies of DHX9 co-expressed with E3 were carried out.

4.2 Results

4.2.1 E3 interacts with DHX9 via its RNA-binding domain

Upon discovery of a possible interaction of host DHX9 with VACV E3 via mass spectrometry, the interaction needed to be confirmed *in vitro*. An endogenous co-immunoprecipitation was carried out in VACV-infected HEK 293T cells and an interaction of DHX9 with VACV E3 was confirmed (data not shown, S. Keating, personal communication). The first goal of this chapter was to map the region of VACV E3 required for the interaction with host DHX9. This was done by carrying out semi-endogenous immunoprecipitations, in which HA-tagged E3 expression vector constructs were transfected into HEK 293T cells (Figure 4.1 A). Cells were transfected with full-length E3 (1-90), a construct encoding the E3 DNA-BD (1-83), or a construct encoding the RNA-BD (84-190). The cells were lysed 48 hours later, and the lysates immunoprecipitated with DHX9 antibody. As a control, the lysates were also immunoprecipitated with an equal concentration of IgG antibody, in order to ensure that the DHX9 endogenous antibody was specifically pulling down DHX9 and that a non-specific antibody would not do the same. With similar input levels of DHX9 protein, an interaction between endogenous DHX9 and overexpressed full-length E3 was seen only upon immunoprecipitation with DHX9 antibody and not for the IgG control (Figure 4.1 B). Although expressed at comparable levels, only the RNA-BD protein construct of E3 (E3 84-190), and not the DNA-BD protein construct (E3 1-83), was consistently seen to interact with endogenous DHX9.

4.2.2 The E3-DHX9 interaction is nucleic acid-independent

Because both DHX9 and E3 are known to bind both RNA and DNA, whether the interaction was nucleic acid-dependent or not was next investigated. This was examined through the use of benzonase, a nuclease that catalyses the degradation of all forms of DNA and RNA. Now knowing that the interaction required the RNA-BD of VACV E3, only full-length E3 (E3 1-190) and the RNA-BD (E3 84-190) expression vectors were used. The immunoprecipitations were carried out both in the presence and absence of benzonase. To ensure that the benzonase was functioning as expected, a small amount of each lysate, with or without benzonase treatment, was resolved on an agarose gel. The total nucleic acid was found to be dramatically degraded upon

benzonase treatment (Figure 4.2 lower panel). The interaction of endogenous DHX9 with both full-length E3 protein and its RNA-BD truncation were still present even in the presence of benzonase (Figure 4.2). Interestingly, DHX9 appeared to be more easily immunoprecipitated upon the addition of benzonase. The interaction of DHX9 with E3 appears stronger with benzonase treatment. However, more non-specific immunoprecipitation of E3 was also seen for the IgG control upon benzonase treatment.

4.2.3 Cloning of DHX9 into pCMV-Myc expression vector

Knowing the RNA-BD of E3 was necessary for its interaction with DHX9, the next goal was to examine what region of host DHX9 is required for the interaction. In order to do so, DHX9 was subcloned from a pCMV-HA into a pCMV-Myc expression vector, as the E3 constructs were HA-tagged. With the aim of investigating what region of DHX9 is required for the interaction, numerous truncations of DHX9 were created and cloned into a pCMV-Myc expression vector. The truncations created were based on the defined domains of DHX9, so that it would be possible to identify the exact region of DHX9 which is required for its interaction with E3 (Figure 4.3 A). After cloning each of the constructs into a pCMV-Myc expression vector, each vector was double digested with the required restriction enzymes to ensure an insert was present of the appropriate size, and then each plasmid was sent for sequencing (data not shown). Upon sequence verification, each of the constructs were transfected into HEK 293T cells to confirm expression. Cell lysates were resolved by SDS-PAGE, and immunoblotted with Myc antibody. All of the constructs were found to have a comparable level of protein expression (Figure 4.3 B).

4.2.4 Viral expression of E3 reduces DHX9 expression

In investigating the endogenous interaction of VACV E3 with host DHX9 in HEK 293T cells, it was noted that the endogenous expression levels of DHX9 were diminished in WR VACV-infected cells compared to VACV Δ E3-infected cells (Figure 4.4). This was consistently observed in a number of experiments and immunoblotting for β -actin confirmed equal protein loading.

4.2.5 THP-1 cells stably expressing VACV E3 have an impaired response to 70mer or cGAMP

To examine why VACV E3 binds to the endogenous helicase DHX9, THP-1 cells stably expressing E3 were generated using a pMSCVneo plasmid retroviral system. Whether THP-1 cells stably expressing E3 would phenocopy DHX9 shRNA-expressing THP-1 cells was investigated. THP-1 cells expressing the pMSCVneo plasmid with no insert were used as control cells. E3 was cloned with a HA tag so its expression could be assayed by immunoblot. After retroviral transduction and selection of the cells, cell lysates were generated, resolved by SDS-PAGE, and tested for HA-E3 expression (Figure 4.5 A). A HEK 293T lysate, in which the cells had been transfected with the HA-E3 retroviral construct, was resolved as a positive control. Although E3 expression was found to be much greater in the HEK 293T lysate, HA-E3 expression was also detectable in the THP-1 cells which had been infected with the HA-E3-expressing retroviruses. The protein band for E3 was detectable at the correct size (roughly 25 kDa) and was not present in the retroviral empty vector-infected cells.

Having generated THP-1 cells stably expressing VACV E3, the next step was to determine whether expression of E3 in THP-1 cells would give a similar cytokine profile to cells in which DHX9 had been silenced, following stimulation. As E3 has been implicated in the targeting of numerous aspects of innate immune signalling, THP-1 cells stably expressing E3 would be expected to have numerous defects in innate immune signalling, a number of which may be unrelated to its association with DHX9. Therefore, although DHX9 was not found to have a role in nucleic acid sensing, the E3-expressing THP-1 cells were firstly transfected with VACV 70mer or cGAMP to determine if E3 can antagonise innate immune signalling in response to VACV DNA or direct activation of STING by cGAMP, a second messenger produced in mammalian cells by cGAS in response to dsDNA [401]. Interestingly, the IP10 response of THP-1 cells stably expressing E3, was found to be almost completely abolished in response to 70mer, compared to control cells. Expression of E3 also reduced the IP10 response to cGAMP, but to a lesser extent to that of 70mer (Figure 4.5 B).

4.2.6 E3-expressing THP-1 cells have a similar cytokine profile as DHX9 shRNA-expressing THP-1 cells

DHX9 knockdown in THP-1 cells was found to effect the regulation of numerous TLR- and RNA virus-induced cytokines. Whether expression of E3 in THP-1 cells similarly affected some of these cytokines was next tested. THP-1 cells stably expressing E3, alongside control cells, were stimulated with the TLR ligands; PAM3, CL075 and LPS, and infected with SeV. RANTES, TNF α , IL-6, IFN α , and IP10 secretion were measured by ELISA. RANTES production was only reduced for TLR8-stimulation in the E3-expressing cells compared to the empty vector control cells (Figure 4.6). This correlated with the cytokine profile observed in the DHX9 shRNA-expressing cells (Figure 3.8 A, Figure 3.9 A). TNF α production was reduced following CL075 stimulation in E3-expressing cells, PAM3-induced TNF α was enhanced, and LPS- and SeV-induced TNF α were unaffected (Figure 4.7), again correlating to the cytokine profile of DHX9 shRNA-expressing cells (Figure 3.8 B, Figure 3.9 B). IL-6 protein was reduced in E3-expressing cells (Figure 4.8) for all the stimuli which gave effective IL-6 secretion (PAM3 and CL075). Silencing of DHX9 by shRNA also gave a similar cytokine profile (Figure 3.8 C, Figure 3.9 C). As seen for DHX9 shRNA-expressing THP-1 cells (Figure 3.8 D, Figure 3.9 D), IFN α production upon SeV infection was also found to be significantly reduced in E3-expressing THP-1 cells (Figure 4.9). Finally, in a similar pattern to RANTES production, IP10 was only observed to be reduced after TLR8 stimulation in THP-1 cells stably expressing E3 (Figure 4.10), again exhibiting a similar IP10 profile as DHX9 shRNA-expressing THP-1 cells (Figure 3.8 E, Figure 3.9 E). Overall, when stimulated with the same ligands, E3-expressing THP-1 cells were found to have a similar cytokine profile to DHX9 shRNA-expressing THP-1 cells (Figure 4.11).

4.2.7 E3 can antagonise the ability of DHX9 to enhance MyD88-driven IL-6 and IL-8 protein production

The cytokine profile of E3-expressing THP-1 cells was found to be similar to DHX9 shRNA-expressing THP-1 cells. Similar to silencing of DHX9, IL-6 production was found to be dramatically reduced for any stimuli tested upon stable expression of VACV E3. Levels of IL-6 have been previously demonstrated to be significantly enhanced upon infection of HeLa cells with a mutant form of VACV lacking the *E3L*

gene, compared to infection with a revertant virus, where the *E3L* gene has been reinserted into the viral genome of a VACV strain lacking the *E3L* gene [399]. This production in IL-6 was shown to be heavily dependent on NF- κ B. As IL-6 has been implicated in the effective clearance of VACV [400], the inhibition of its production by VACV E3 is crucial to the viral life cycle. Here, overexpression of DHX9 in HeLa cells was capable of enhancing MyD88-dependent IL-6 and IL-8 production. This enhancement in cytokine production may be due to its binding to p65 and role in transcriptional coactivation [298]. As the upregulation in IL-6 upon VACV infection was found to be strongly NF- κ B-dependent, this upregulation may be DHX9-dependent. Before testing whether this is the case in VACV infection, the ability of E3 to inhibit the enhancement of MyD88-driven IL-6 (Figure 4.12 A) and IL-8 (Figure 4.12 B) production in HeLa cells, was assayed. DHX9 or E3 overexpression by themselves had no effect on IL-6 or IL-8 production in HeLa cells. MyD88 overexpression was capable of causing significant IL-6 and IL-8 secretion. Cotransfection with DHX9 expression vector was found to enhance this roughly twofold. Interestingly, when E3 was transfected at increasing doses alongside MyD88 and DHX9 expression vector, it was capable of antagonising the ability of DHX9 to potentiate IL-6 and IL-8 production. At the highest dose of E3, both IL-6 and IL-8 production are reduced to a similar level to that of transfection with MyD88 alone.

4.2.8 E3 can antagonise the ability of DHX9 to enhance NF- κ B and IRF7 transactivation

E3-expressing THP-1 cells were found to have a similar cytokine profile to DHX9 shRNA-expressing THP-1 cells and E3 was able to inhibit the ability of DHX9 to potentiate MyD88-dependent cytokine production in HeLa cells. It was found by reporter gene assays, that DHX9 was capable of enhancing NF- κ B activation for any stimuli tested, and that this was most likely due to its ability to directly bind to the p65 subunit of NF- κ B, and play a role in the transcriptional coactivation of NF- κ B-dependent genes [298]. Therefore, whether the inhibition by E3 of NF- κ B-dependent cytokine production could be explained by an inhibition of downstream transcriptional activity of DHX9 was next examined by reporter gene assay (Figure 4.13). Cells were transfected with or without DHX9 and/or E3 expression vector and then stimulated with a number of NF- κ B-dependent stimuli, and the ability of E3 to antagonise DHX9-

enhanced NF- κ B activation was assessed by reporter gene assay. VACV E3 was found to dose-dependently antagonise the potentiation by DHX9 of both TLR8- (Figure 4.13 A) and TLR3- (Figure 4.13 B) dependent NF- κ B activation. E3 overexpression by itself was found to have little effect on TLR8- or TLR3-dependent NF- κ B activation. Whether this inhibition by E3 was a result of its ability to impede the positive role of DHX9 in NF- κ B activation through its association with p65 [298], was next investigated (Figure 4.13 C). DHX9 was co-expressed with, and without, p65 expression vector, and the ability of E3 to antagonise the potentiation by DHX9 of p65-dependent NF- κ B luciferase reporter was assessed. DHX9 was able to significantly enhance p65 transactivation of the NF- κ B-dependent reporter gene and E3 was found to dose-dependently inhibit this.

As well as being necessary for the effective activation of NF- κ B, DHX9 was also found to play a role in the transactivation of IRF family members, particularly IRF7. Whether E3 could also antagonise this function of DHX9 was next assayed. E3 was found to be capable of dose-dependently reducing the ability of DHX9 to enhance both TLR8- and TLR3-dependent transactivation of IRF7. Therefore, suppression of cytokine induction by E3 is likely, in at least some cases, to be due to E3 antagonising DHX9-dependent transcription factor transactivation.

4.3 Discussion

4.3.1 E3 interacts with DHX9 via its RNA-binding domain in a nucleic acid-independent manner

VACV E3 is a multifunctional protein, vital to the VACV life cycle, which has a range of mechanisms of evading the host immune response (Section 1.13) [399]. Due to its critical role in VACV pathogenesis [362], novel host-interacting partners of E3 were explored by carrying out an immunoprecipitation of E3 from HEK 293 and MEF cells, and then identifying possible interacting partners by mass spectrometry analysis (S. Keating, personal communication). The RNA helicase, DHX9, was identified as a potential E3-interacting protein. Dr. Sinead Keating confirmed this interaction after WR VACV infection. An endogenous interaction of VACV E3 and host DHX9 was confirmed in HEK 293T cells after immunoprecipitation with an E3 antibody showed an interaction of E3 with DHX9 (S. Keating, personal communication). Cells infected with VACV lacking the E3L gene (VACV Δ E3L) served as an excellent control, in that no DHX9 protein was co-immunoprecipitated from these lysates with an E3 antibody. In a separate mass spectrometry analysis carried out in the lab at the time, DHX9 had also been identified to associate with VACV 70mer DNA (S. Keating, personal communication). Thus, upon identification of an interaction of VACV E3 with host DHX9, it was initially hypothesised that DHX9 may possibly be a novel DNA sensor of VACV DNA and E3 may be binding DHX9 in order to antagonise its role in nucleic acid sensing. The E3 protein is composed of a C-terminal dsRNA-BD and an N-terminal Z-DNA-BD, with both domains having been shown to antagonise innate immunity through various mechanisms (Figure 1.11) [320]. Truncated forms of E3 were cloned into a pCMV-HA vector. Full-length VACV E3 (E3 1-190), an E3 1-83 construct (encoding the Z-DNA-BD) and an E3 84-190 construct (encoding the dsRNA-BD), were cloned to determine (Figure 4.1 A), via overexpression studies, the mechanism by which E3 binds host DHX9 (S. Keating, personal communication).

The aims of this project were firstly to investigate innate immune functions of DHX9, and secondly to map the interaction of VACV E3 and DHX9 and to investigate whether E3 is capable of antagonising DHX9 function. Having investigated the role of DHX9 in innate immunity, the next step of the project was to identify what region of

VACV E3 was required for the interaction with host DHX9 and vice versa. Due to technical issues with the antibody (where the antibody light chain in immunoprecipitations was found to run at the same size as E3), a semi-endogenous immunoprecipitation was opted for, in which endogenous DHX9 was immunoprecipitated and overexpressed VACV E3 immunoblotted for. A strong interaction of full-length E3 (E3 1-190) was observed with DHX9, and the RNA-BD of E3 (E3 84-190) was found to be necessary for this interaction. No interaction with the N-terminal Z-DNA-BD (E3 1-83) was consistently observed. As both DHX9 and VACV E3 contain dsRNA-binding domains, the interaction may occur between these domains in a homotypic domain interaction. This has yet to be elucidated using the DHX9 truncations generated.

As both DHX9 and E3 can bind both RNA and DNA, whether the interaction was direct or via nucleic acid needed to be addressed. This was examined by the use of benzonase. Benzonase is a nuclease which is capable of degrading all forms of DNA and RNA. Benzonase is often used as a means of testing whether a protein-protein interaction is nucleic acid dependent or not [402, 403]. Knowing the C-terminal dsRNA-binding domain of E3 is necessary for the interaction with endogenous DHX9, only the full-length- and RNA-BD-expressing constructs were employed. Upon treatment of the lysates with benzonase, a strong interaction with VACV E3 and the RNA-BD truncation was still observed. Running the lysates on an agarose gel confirmed effective nucleic acid degradation. Interestingly, upon treatment with benzonase, DHX9 appeared to be more easily immunoprecipitated than in the absence of benzonase. This cannot be explained by difference in lysate protein concentration as the same number of HEK 293T cells were used for generating both untreated and benzonase-treated lysates, and the input lysates before immunoprecipitation had a similar level of DHX9 protein regardless of benzonase treatment. The increase in DHX9 pull-down upon treatment with benzonase, has numerous possible causes. As DHX9 is an RNA helicase required for the unwinding of DNA and RNA in various aspects of cell metabolism [404], it may be in a complex with nucleic acid in the cell. This nucleic acid may be masking the epitope of which the DHX9 antibody is recognising and hence, when the lysates are treated with benzonase, this nucleic acid is being degraded and DHX9 may be more freely available and immunoprecipitated by the DHX9 antibody.

The antibody specifications indicate that the epitope it recognises maps to a region between residue 1220 and the C-terminus (residue 1270). This region is not required for nucleic acid binding (see Figure 4.3 A for domain structure). However, DHX9 protein may aggregate on nucleic acid or have a different conformation upon nucleic acid binding, which may be altered upon degradation of nucleic acid, possibly making DHX9 more accessible to the antibody. More non-specific pull-down of E3 protein in the IgG control immunoprecipitation was found upon treatment with benzonase. However, a much greater concentration of E3 was found to be pulled down in the DHX9 immunoprecipitation compared to the IgG control. This enhancement in non-specific pull-down may be related to an increase in the detection of DHX9 upon benzonase treatment as discussed above.

DHX9 truncation constructs were then generated and cloned into a pCMV-Myc expression vector in order to map the region of host DHX9 needed for the interaction with VACV E3. Antibody issues proved to make this quite difficult. As both the HA- and Myc-tag antibodies were raised in mice, anti-mouse secondary antibody needed to be used in immunoblotting for both tags. As E3 protein is a similar size to the secondary antibody light chain, immunoprecipitation of E3 was difficult to detect. Different primary antibodies raised in rabbit were also employed, but these antibodies were not as effective at detecting overexpressed E3 or the DHX9-Myc constructs. As a result, full-length E3 has now been cloned into a GST vector to map the interaction via GST pull-downs.

4.3.2 A possible reduction in DHX9 expression by VACV E3

In carrying out endogenous co-immunoprecipitations of DHX9 with VACV E3, a dramatic decrease in DHX9 protein expression was observed in the WR VACV-infected cells, compared to the VACV Δ E3-infected cells. This was consistently observed in a number of experiments. Immunoblotting for β -actin confirmed equal loading and also indicated that the reduction in DHX9 observed in wild-type WR VACV-infected cells was not simply as a result of cell death induced by viral infection or a global host protein shut off mechanism triggered by VACV infection. A preliminary experiment to test whether VACV E3 was capable of inducing the proteasomal degradation of DHX9 was next carried out through the use of the

proteasomal inhibitor, MG132. Initial data showed that E3 may somehow trigger the proteasomal degradation of DHX9 (data not shown). This experiment requires repetition and the ability of E3 to induce the degradation of host DHX9 further tested via ubiquitination immunoprecipitations before any conclusions can be made. If E3 is confirmed to promote the degradation of host DHX9, this may rule out the possibility of VACV E3 sequestering DHX9 for its viral life cycle.

4.3.3 VACV E3 is capable of antagonising the innate immune response to 70mer and cGAMP

DHX9 has been previously shown to play a role in various aspects of innate immunity (Section 1.15). Initial reports on DHX9 in innate immune signalling placed DHX9 at a transcriptional level. Amongst other innate immune functions, DHX9 was shown to interact with the p65 subunit of NF- κ B, functioning as a transcriptional coactivator [298], to be a component of the STAT6-dependent enhanceosome [381], and to mediate the association of CBP with Pol II and subsequent CREB-dependent transcription [371]. However, in the last five years, DHX9 has been proposed to play more of a role further upstream in innate immune signalling events, acting as a nucleic acid PRR. In particular, two publications from Yong-Jun Liu's group within a year of each other, proposed DHX9 as both a sensor of CpG-B DNA in pDCs and dsRNA in mDCs [222, 270]. In the work carried out here, DHX9 was not found to have a role in RNA or DNA sensing in THP-1 cells. This may be because the function of DHX9 is very cell type-specific. However, DHX9 was found to play a crucial role in the effective upregulation of TLR- and RNA-virus stimulated cytokines in THP-1 cells. The cytokine readouts affected were similar to those in which DHX9 was proposed as being a RNA or DNA sensor in mDCs and pDCs, respectively [222, 270]. A closer look at upstream signalling events and downstream transcription factor activations, showed DHX9 to play more of a transcriptional role in innate immune signalling. This fits with work published previous to the studies from Yong-Jun Liu's group. The work from this group, in which DHX9 has been proposed as a RNA/DNA sensor [222, 270], may have been misinterpreted, as a large quantity of their data can be explained from previously published functions of DHX9.

In order to assay whether VACV E3 is directly binding DHX9 to antagonise its roles in innate immunity, THP-1 cells stably expressing VACV E3 were generated using a retroviral vector. VACV E3 was cloned into a pMSCVneo vector with a HA tag so that it could be detected by immunoblot analysis. The pMSCVneo vector with no insert was used as a control (empty vector). Although the expression of E3 was much weaker than that seen for overexpression of E3 in HEK 293T cells, E3 expression was detectable in THP-1 cells by immunoblot analysis. E3 was found to run at the correct size (roughly 25 kDa) and a corresponding band was not observed in the empty vector-transduced THP-1 cells. Silencing of DHX9 in THP-1 cells, showed DHX9 to not be necessary for an effective immune response to DNA or RNA in THP-1 cells. However, E3 is a multifunctional protein which has been shown to antagonise the innate immune response through various mechanisms (Section 1.13). Hence, although the aim was to identify whether the E3-expressing THP-1 cells showed a similar phenotype to DHX9 shRNA-expressing THP-1 cells, it was expected that the E3-expressing cells may show numerous other defects in innate immune signalling, which are independent of DHX9 function. The E3-expressing and control cells were both transfected with VACV 70mer and cGAMP to test whether E3 can antagonise the innate immune response to VACV DNA and whether the same would be the case for direct activation of STING by cGAMP, a second messenger produced in mammalian cells by cGAS in response to dsDNA [401]. Intriguingly, E3 expression was found to antagonise both 70mer- and cGAMP-stimulated IP10. 70mer-stimulated IP10 was observed to be almost completely abolished in THP-1 cells stably expressing E3, while cGAMP-induced IP10 was also affected, but to a lesser extent to that of 70mer. There may be numerous mechanisms by which E3 expression is effecting IP10 induction by 70mer and cGAMP. VACV E3 has been reported to be capable of binding to Z-DNA *in vivo*, with its binding of DNA effecting viral pathogenicity [356, 357]. Therefore, it is possible that stably expressed E3 is capable of sequestering transfected 70mer, thus preventing its detection by DNA sensors in the cell, and an effective upregulation in IP10. E3 expression also appears to antagonise the direct activation of STING by cGAMP or subsequent signalling downstream of STING activation, by an unidentified mechanism. 70mer- or cGAMP-stimulated IP10 was found to be unaffected upon silencing of DHX9. Therefore, the interaction of VACV E3 with host DHX9 is unlikely to be a means of antagonising the ability of DHX9 to sense VACV genomic DNA as initially hypothesised. E3 is however

capable of antagonising the innate immune response to VACV 70mer and cGAMP by a mechanism(s) which is unclear at present.

4.3.4 VACV E3 is capable of antagonising DHX9 cytokine induction, possibly by inhibition of transcription factor transactivation by DHX9

Although DHX9 was not found to have a role in nucleic acid sensing in THP-1 cells, it was found to have a role in non-nucleic acid PRR signalling. THP-1 cells stably expressing E3 were next stimulated with numerous ligands which DHX9 was required for an effective cytokine response to. Interestingly, the cytokine profile in response to all the ligands tested was found to correlate to that observed upon silencing of DHX9 protein by shRNA (Figure 4.11). Similar to knockdown of DHX9 in THP-1 cells, THP-1 cells stably expressing E3 showed a reduction in all CL075-dependent cytokines tested, and a reduction in IL-6 for all the stimuli examined. Whether E3-expressing THP-1 cells showed a similar cytokine profile to THP-1 cells expressing DHX9 shRNA is because of a direct antagonism of endogenous DHX9 function by E3 had to be further investigated. Of particular interest was the reduction of IL-6 secretion for all the stimuli tested in E3-expressing THP-1 cells. IL-6 has been previously reported to play an important role in the clearance of VACV, and VACV E3 has been demonstrated to inhibit its production by an unidentified mechanism [399, 400]. In addition to being necessary for IL-6 production to any stimuli tested in THP-1 and BEAS-2B cells, overexpression of DHX9 alongside MyD88 expression vector, was found to significantly enhance MyD88-driven IL-6 and IL-8 protein production in HeLa cells. Whether E3 could antagonise the ability of DHX9 to enhance MyD88-driven IL-6 or IL-8 production was next assayed. Interestingly, E3 expression was found to dose-dependently reduce the ability of DHX9 to enhance both MyD88-driven IL-6 and IL-8 production. Expression of E3 was seen to reduce IL-6 and IL-8 production to a similar level to cells transfected with MyD88 alone. As E3 expression was never found to reduce IL-6 or IL-8 secretion below that of MyD88 overexpression alone, it is likely that the reduction in cytokine secretion is a result of a direct antagonism of DHX9 function by E3. Whether the upregulation in IL-6 protein upon infection with VACV lacking the *E3L* gene is DHX9-dependent, and whether E3 can antagonise this, will next need to be examined. The ability of E3 to antagonise IL-6 production in HeLa cells conflicts with a previous publication which demonstrated E3 to transactivate IL-6 for

inhibition of apoptosis [362]. However, IL-6 protein was not analysed in this study and hence the luciferase reporter assay data obtained in this study, may not correlate with IL-6 protein levels.

DHX9 was found to be capable of enhancing the transcriptional activity of multiple transcription factors. Its ability to transactivate NF- κ B may explain a requirement for DHX9 in NF- κ B-dependent cytokine secretion in THP-1 cells, while its ability to enhance IRF transactivation could explain its role in type I IFN gene induction. Whether E3 could suppress the ability of DHX9 to enhance transcription factor transactivation was next examined by reporter gene assay. Overexpression of E3 was found to significantly inhibit the enhancement of both TLR8- and TLR3-dependent NF- κ B activation by DHX9, while having no effect alone. E3 was also found to be capable of dose-dependently inhibiting the ability of DHX9 to enhance p65 transactivation of the NF- κ B-dependent reporter gene. In a similar manner, E3 was also found to be capable of antagonising the enhancement of TLR8- and TLR3-dependent IRF7 transactivation by DHX9. Regardless of the stimulus, E3 overexpression alone had little effect on NF- κ B or IRF7 transactivation. The inhibition by E3 was only observed for the enhancement in transactivation by DHX9, rather than transactivation after stimulation alone. Therefore, it appears that the inhibition by VACV E3 is a direct antagonism of DHX9 function, rather than an inhibition of the general signalling network. DHX9 is predominantly nuclear in localisation, capable of regulating gene expression at transcriptional and post-transcriptional levels [405]. This fits with its ability to enhance the transactivation of transcription factors shown in this project. VACV belongs to the poxvirus family, which are known to replicate in the cytoplasm of the cell. However, E3 has been shown to localise to both the nucleus and the cytoplasm of VACV-infected cells, and hence, would have access to DHX9 during viral infection [344]. Therefore, the inhibition of DHX9 function by VACV E3 may be a consequence of its direct association with DHX9 in the nucleus.

DHX9 has been shown here to play a crucial role in the transcriptional regulation of a vast array of genes integral to mounting an effective response to infection. All of the data collated places DHX9 downstream in PRR signalling, important in gene regulation and enhancement of the transcriptional activity of

transcription factors. Therefore, the targeting of DHX9 by VACV E3 may be an effective means of inhibiting the innate immune response. Consistent with this, E3 was found to be capable of successfully inhibiting any innate immune functions of DHX9 discovered in THP-1, HEK 293 or HeLa cells.

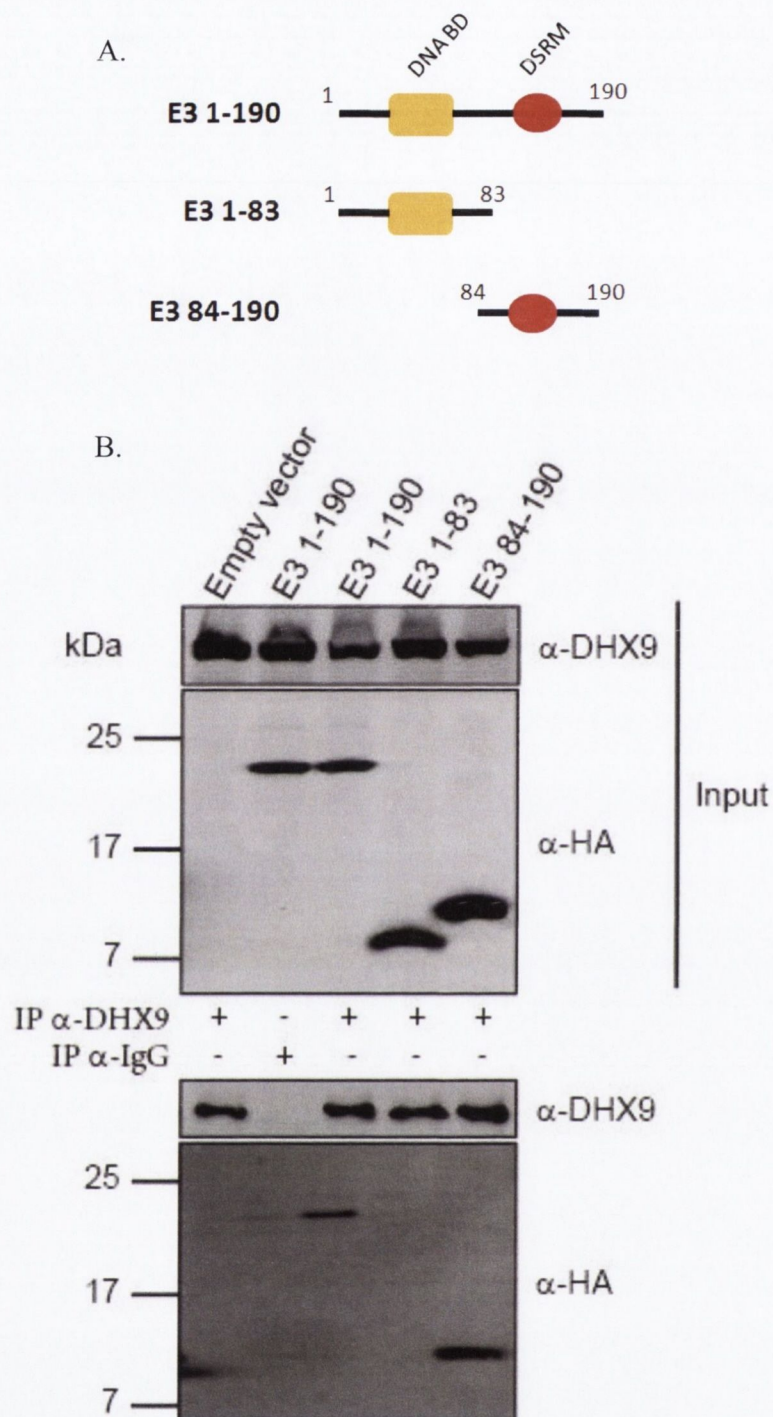


Figure 4.1 E3 interacts with DHX9 via its RNA-binding domain

(A) Schematic of E3 domain structure and truncations. DNA BD, DNA binding domain; DSRM, Double-stranded RNA binding motif. (B) HEK 293T cells were seeded at 2×10^5 cells/ml in 15 cm dishes. 24 hours later, they were transfected using GeneJuice® with empty vector or plasmid encoding HA-E3 (1-190), HA-E3 (1-83) or HA-E3 (84-190), and incubated for 48 hours. Cell lysates were then subjected to immunoprecipitation (IP) using DHX9 or IgG antibody and then resolved by SDS-PAGE. E3 and DHX9 interactions were analysed by immunoblotting for HA and DHX9, respectively. Representative of three experiments.

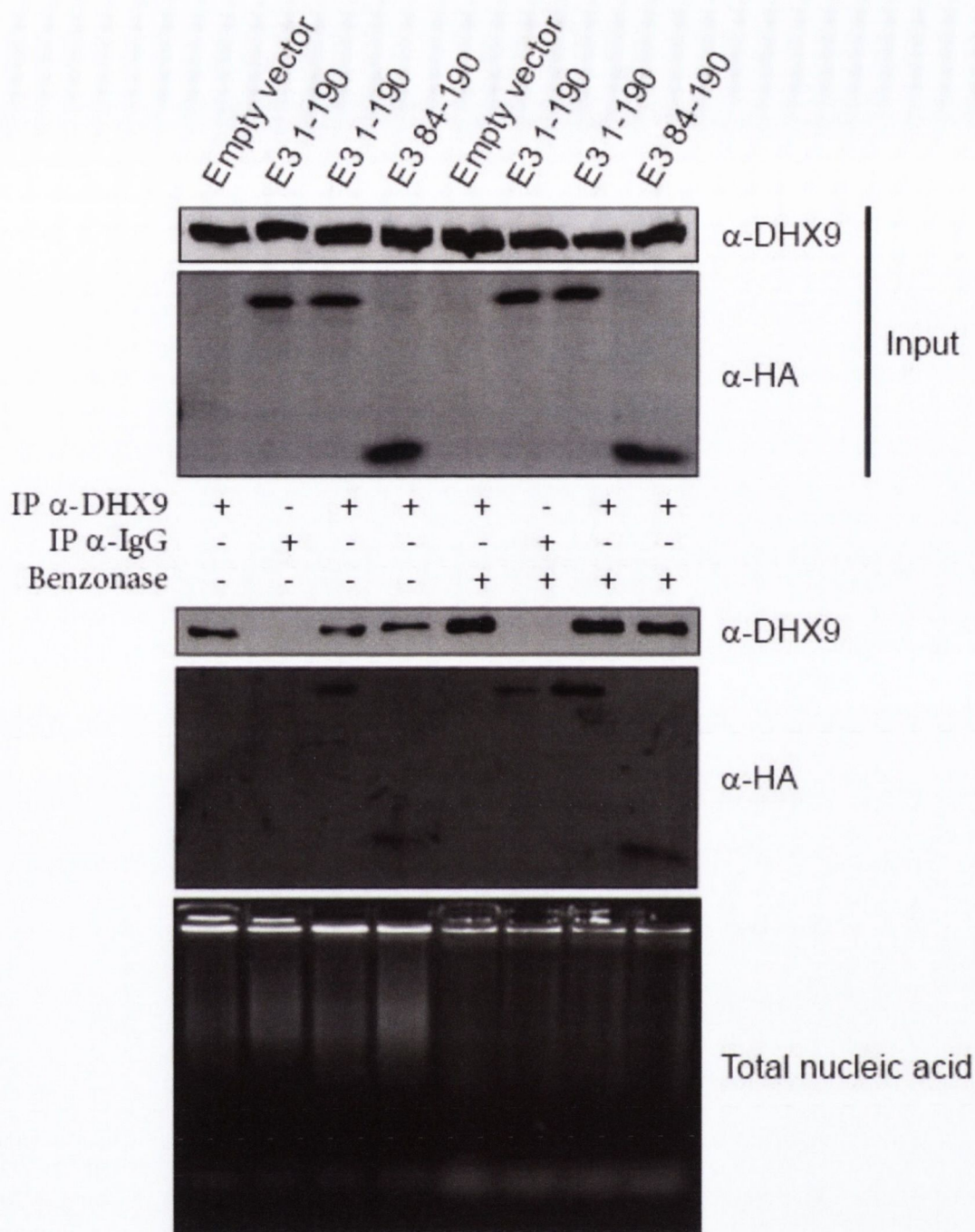


Figure 4.2 The E3-DHX9 interaction is nucleic acid-independent

HEK 293T cells were seeded at 2×10^5 cells/ml in 15 cm dishes. 24 hours later, they were transfected using GeneJuice® with empty vector or plasmid encoding HA-E3 (1-190) or HA-E3 (84-190), and incubated for 48 hours. Cell lysates were treated with (+) or without (-) 200 U of benzonase and were then subjected to immunoprecipitation (IP) using DHX9 or IgG antibody. E3 and DHX9 interactions were analysed by immunoblotting for HA and DHX9, respectively. After benzonase treatment, lysates were resolved on a 1 % (w/v) agarose gel and total nucleic acid was viewed under UV. Representative of two experiments.

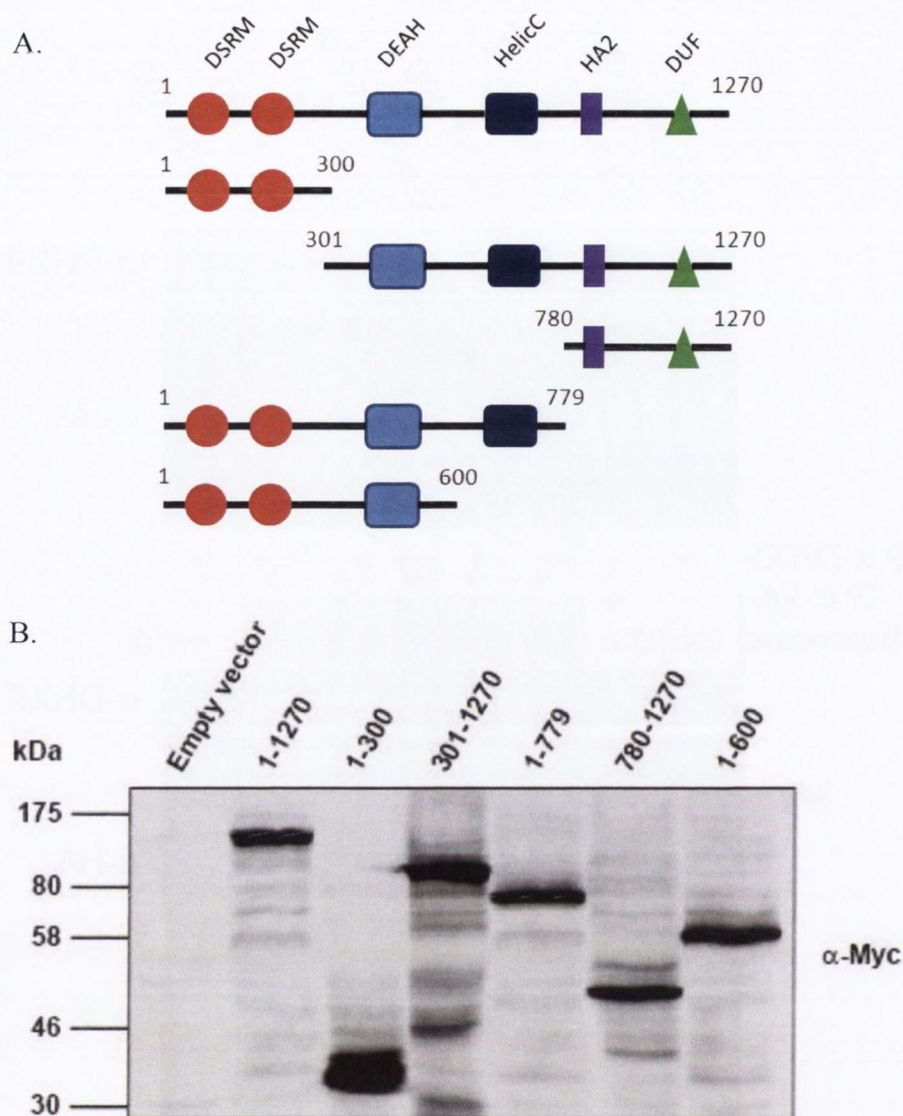


Figure 4.3 Cloning of DHX9 fragments into pCMV-Myc expression vector

(A) DHX9 constructs were cloned into a pCMV-Myc vector. Schematics show the domain structure of DHX9 and the constructs cloned. DSRM, Double-stranded RNA binding motif; DEAH, Aspartate-glutamate-alanine-histidine box motif; HelicC, Helicase C-terminal domain; HA2, Helicase-associated domain 2; DUF, Domain of unknown function. (B) HEK 293T cells seeded in 6 well plates at 1×10^5 cells/ml, were GeneJuice®-transfected 24 hours later, with with 2 μ g empty vector or the cloned Myc-DHX9 constructs. Cells were harvested 24 hours later, lysates resolved by SDS-PAGE, and protein expression assayed by immunoblotting for Myc. Representative of three experiments.

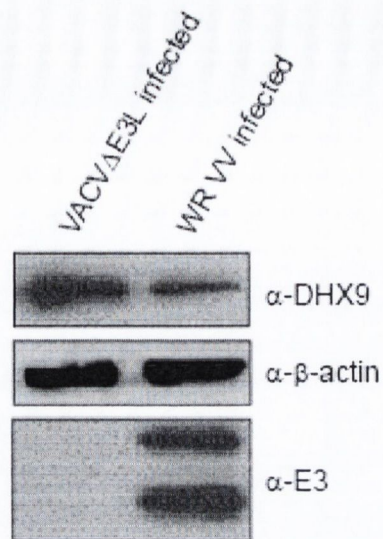


Figure 4.4 Viral expression of E3 reduces DHX9 expression

HEK 293T cells seeded at 2×10^5 cells/ml in 10 cm dishes, were infected 24 hours later with either Western Reserve vaccinia virus (WR VACV) or VACV lacking the *E3L* gene (VACVΔE3L) at an M.O.I. of 1, for 24 hours. The lysates were harvested, resolved by SDS-PAGE, and immunoblotted for DHX9, β-actin and E3. Representative of three experiments.

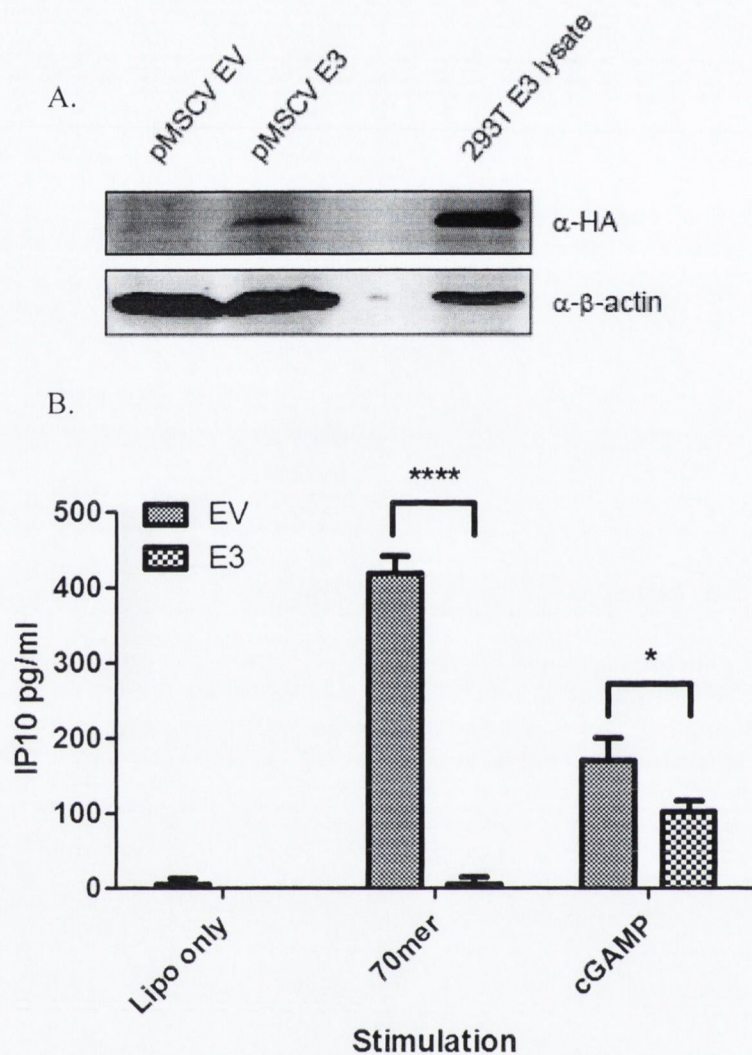


Figure 4.5 THP-1 cells stably expressing VACV E3 have an impaired response to 70mer or cGAMP

THP-1 cells were transduced with retroviruses encoding HA-tagged E3 expression vector (E3) or empty vector (EV). After transduction and neomycin selection, lysates were generated, resolved by SDS-PAGE, and immunoblotted for DHX9 and β -actin (A). THP-1 cells stably expressing E3 and THP-1 cells transduced with EV, were seeded at 0.8×10^6 cells/ml, and transfected 24 hours later with lipofectamine alone (Lipo only), 1 μ g/ml 70mer or 5 μ g/ml cGAMP. The supernatants were removed 24 hours later and IP10 secretion was measured by ELISA (B). The data is mean $\pm$ SD of triplicate samples and is representative of two experiments. *= $p < 0.05$, ****= $p < 0.0001$ compared to EV-containing THP-1 cells.

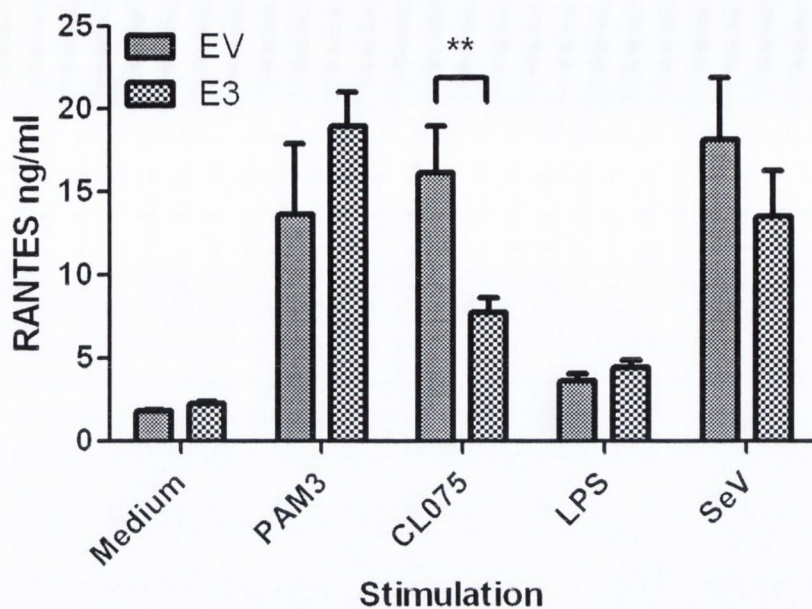


Figure 4.6 E3-expressing THP-1 cells have a similar TLR- and SeV-stimulated RANTES cytokine profile as DHX9 shRNA-expressing THP-1 cells

THP-1 cells stably expressing E3 (E3) and THP-1 cells containing an empty vector (EV) were seeded at 0.8×10^6 cells/ml, and stimulated 24 hours later with 2 μ g/ml Pam3CSK4 (PAM3), 2.5 μ g/ml CL075, 100 ng/ml lipopolysaccharide (LPS), Sendai virus (SeV) or medium alone. The supernatants were removed 24 hours later and RANTES secretion was measured by ELISA. The data is mean $\pm$ SD of triplicate samples and is representative of three experiments. **= $p < 0.01$ compared to EV-containing THP-1 cells.

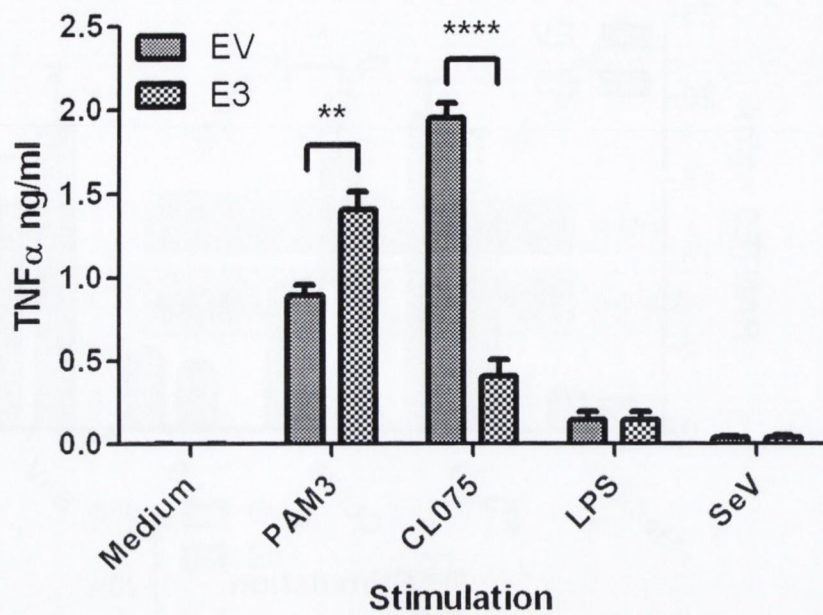


Figure 4.7 E3-expressing THP-1 cells have a similar TLR- and SeV-stimulated TNF α cytokine profile as DHX9 shRNA-expressing THP-1 cells

THP-1 cells stably expressing E3 (E3) and THP-1 cells containing an empty vector (EV) were seeded at 0.8×10^6 cells/ml, and stimulated 24 hours later with 2 μ g/ml Pam3CSK4 (PAM3), 2.5 μ g/ml CLO75, 100 ng/ml lipopolysaccharide (LPS), Sendai virus (SeV) or medium alone. The supernatants were removed 24 hours later and TNF α secretion was measured by ELISA. The data is mean $\pm$ SD of triplicate samples and is representative of three experiments. **= $p < 0.01$, ****= $p < 0.0001$ compared to EV-containing THP-1 cells.

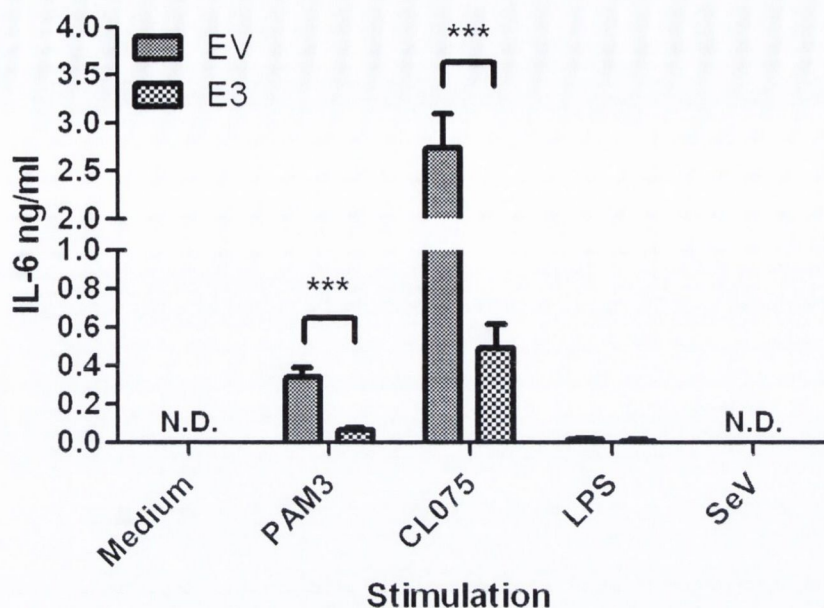


Figure 4.8 E3-expressing THP-1 cells have a similar TLR-stimulated IL-6 cytokine profile as DHX9 shRNA-expressing THP-1 cells

THP-1 cells stably expressing E3 (E3) and THP-1 cells containing an empty vector (EV) were seeded at 0.8×10^6 cells/ml, and stimulated 24 hours later with 2 μ g/ml Pam3CSK4 (PAM3), 2.5 μ g/ml CL075, 100 ng/ml lipopolysaccharide (LPS), Sendai virus (SeV) or medium alone. The supernatants were removed 24 hours later and IL-6 secretion was measured by ELISA. The data is mean $\pm$ SD of triplicate samples and is representative of three experiments. ***= $p < 0.001$ compared to EV-containing THP-1 cells. N.D., non-detectable.

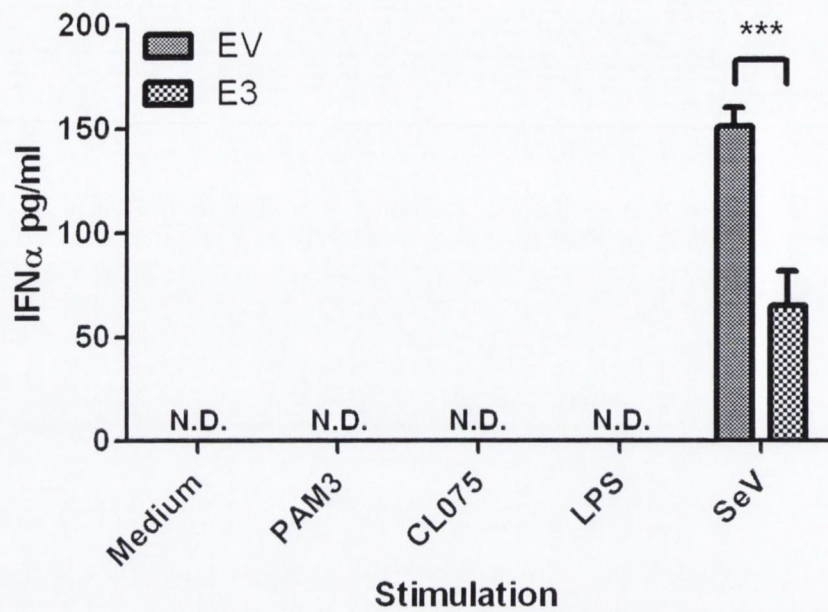


Figure 4.9 E3-expressing THP-1 cells have a similar SeV-stimulated IFN α cytokine profile as DHX9 shRNA-expressing THP-1 cells

THP-1 cells stably expressing E3 (E3) and THP-1 cells containing an empty vector (EV) were seeded at 0.8×10^6 cells/ml, and stimulated 24 hours later with 2 μ g/ml Pam3CSK4 (PAM3), 2.5 μ g/ml CLO75, 100 ng/ml lipopolysaccharide (LPS), Sendai virus (SeV) or medium alone. The supernatants were removed 24 hours later and IFN α secretion was measured by ELISA. The data is mean $\pm$ SD of triplicate samples and is representative of three experiments. ***= $p < 0.001$ compared to EV-containing THP-1 cells. N.D., non-detectable.

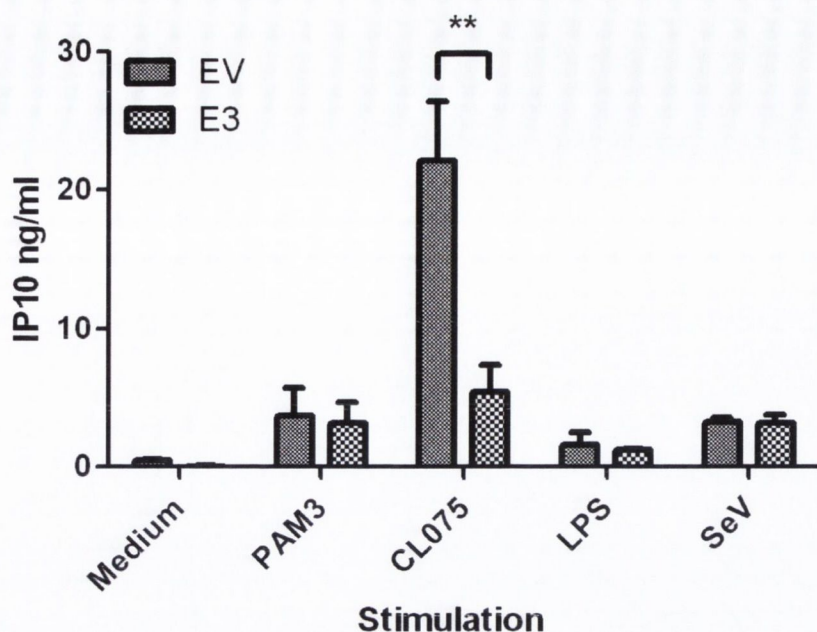


Figure 4.10 E3-expressing THP-1 cells have a similar IP10 cytokine profile as DHX9 shRNA-expressing THP-1 cells

THP-1 cells stably expressing E3 (E3) and THP-1 cells containing an empty vector (EV) were seeded at 0.8×10^6 cells/ml, and stimulated 24 hours later with 2 μ g/ml Pam3CSK4 (PAM3), 2.5 μ g/ml CLO75, 100 ng/ml lipopolysaccharide (LPS), Sendai virus (SeV) or medium alone. The supernatants were removed 24 hours later and IP10 secretion was measured by ELISA. The data is mean $\pm$ SD of triplicate samples and is representative of three experiments. **= $p < 0.01$ compared to EV-containing THP-1 cells.

	IFN α		IL-6		TNF α		RANTES		IP10	
Ligand	shRNA	E3	shRNA	E3	shRNA	E3	shRNA	E3	shRNA	E3
PAM3	-	-	↓	↓↓	1/2↑	1/2↑	n.a	n.a	n.a	n.a
CL075	-	-	↓↓	↓↓	↓↓	↓↓	↓	↓	↓↓	↓↓
SeV	↓	↓	↓↓	-	n.a	n.a	n.a	n.a	n.a	n.a
LPS	-	-	↓	-	n.a	n.a	n.a	n.a	n.a	n.a

Figure 4.11 E3-expressing THP-1 cells have a similar cytokine profile to DHX9 shRNA-expressing THP-1 cells in response to TLR agonists and SeV infection

Schematic of cytokine responses affected upon DHX9 shRNA knockdown (shRNA) or stable expression of E3 (E3) in THP-1 cells. PAM3, Pam3CSK4; SeV, Sendai virus; LPS, Lipopolysaccharide; n.a, non-affected; - , non-induced; ↓, ≥50% down; ↓↓, ≥70% down; 1/2↑, ≥25% up.

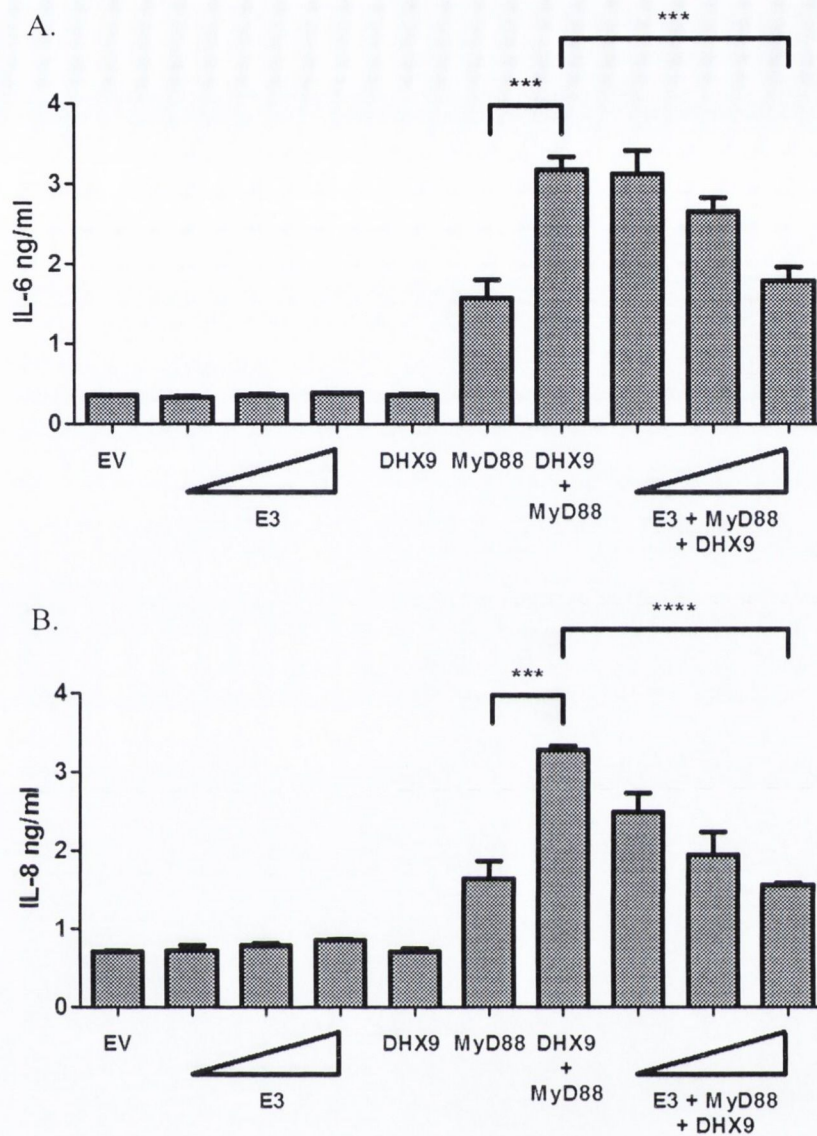


Figure 4.12 Effect of E3 on the ability of DHX9 to enhance MyD88-dependent IL-6 and IL-8 protein production

HeLa cells were seeded at 0.5×10^5 cells/ml, 24 hours prior to GeneJuice® transfection. Wedges indicate increasing amounts of E3 (25, 50, 100 ng). E3 was transfected in increasing doses with and without 100 ng DHX9 and 50 ng MyD88. The supernatants were removed 48 hours after transfection and IL-6 (A) and IL-8 (B) protein secretion was measured by ELISA. The data is mean $\pm$ SD of triplicate samples and is representative of two experiments. ***= $p < 0.001$, ****= $p < 0.0001$ compared to cells transfected with MyD88 or both DHX9 and MyD88.

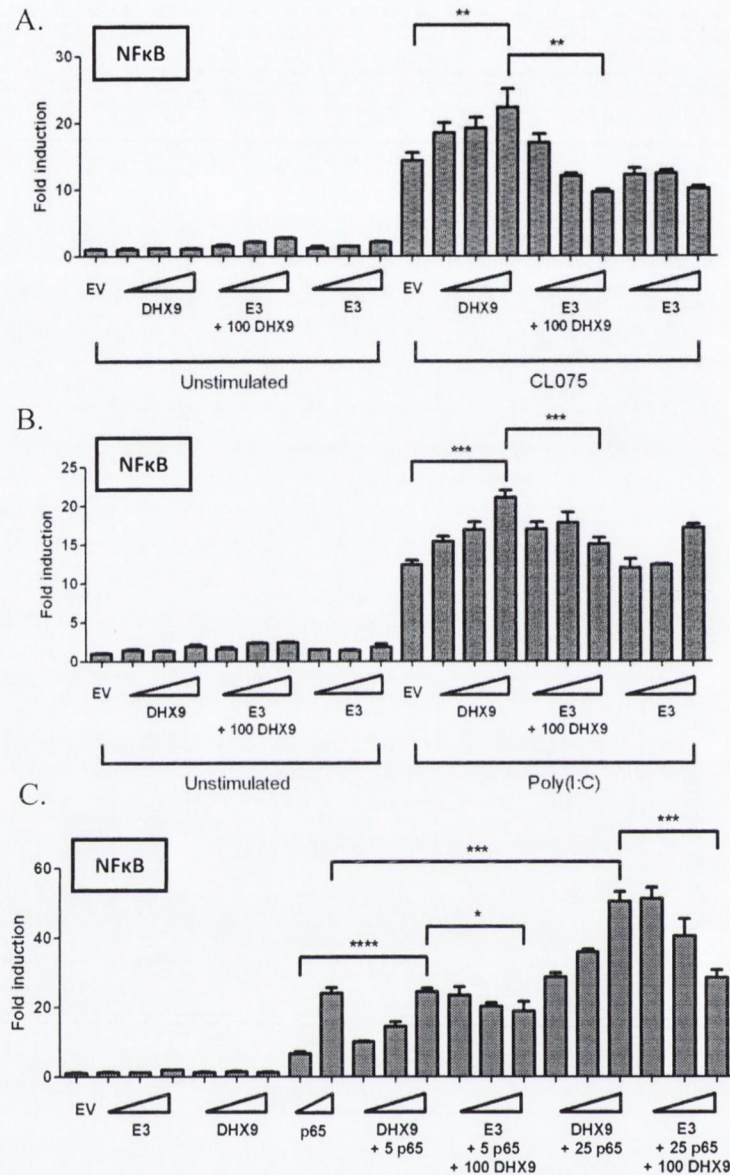


Figure 4.13 E3 antagonises the ability of DHX9 to enhance TLR8-, TLR3- and p65-dependent NF-κB activation

HEK-TLR8 (A), HEK-TLR3 (B) and HeLa (C) cells were seeded at 1×10^5 cells/ml, 1×10^5 cells/ml and 0.5×10^5 cells/ml, respectively, 24 hours prior to GeneJuice® transfection. After 24 hours, the cells were transfected with NF-κB promoter reporter gene, the pGL3-Renilla constitutive reporter and empty vector (EV) or the indicated amounts (ng) of DHX9, p65 or E3 expression vector. Wedges indicate increasing amounts of DHX9 (25, 50, 100 ng), p65 (5, 25 ng) or E3 (25, 50, 100 ng). 24 hours later, the HEK-TLR8 cells were either unstimulated or stimulated with 2.5 μg/ml CL075 (A) and HEK-TLR3 cells were either unstimulated or stimulated with 25 μg/ml poly(I:C) (B). After a further 24 hours, the cells were lysed and the lysates assayed for luciferase activity using a luminometer. Data is shown as relative fold induction normalised to unstimulated, EV only-transfected cells. The data is mean ± SD of triplicate samples and is representative of three experiments. *= $p < 0.05$, **= $p < 0.01$, ***= $p < 0.001$, ****= $p < 0.0001$ compared to stimulated, EV only-transfected cells or cells transfected with the highest dose of DHX9 expression vector (A-B) or p65-transfected cells or cells transfected with p65 and the highest dose of DHX9 expression vector (C).

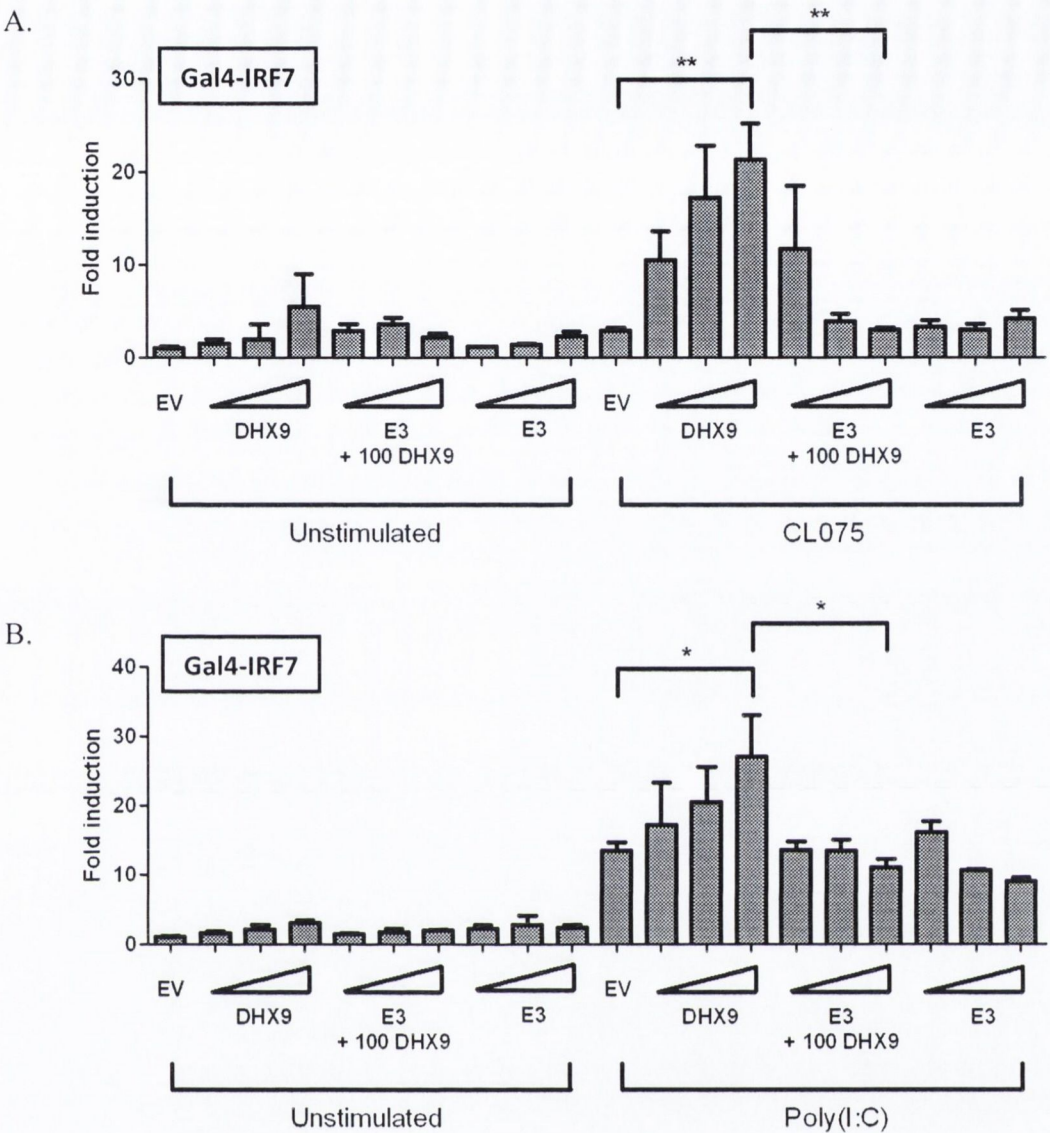


Figure 4.14 E3 antagonises the ability of DHX9 to enhance TLR8- and TLR3-dependent IRF7 transactivation

HEK 293-TLR8 (A) and -TLR3 (B) cells were seeded at 1×10^5 cells/ml, 24 hours prior to GeneJuice® transfection. After 24 hours the cells were transfected with Gal4-IRF7, pFR luciferase, the pGL3-Renilla constitutive reporter and empty vector (EV) or the indicated amounts (ng) of DHX9 or E3 expression vector. Wedges indicate increasing amounts of DHX9 (25, 50, 100 ng) or E3 (25, 50, 100 ng). 24 hours later, the HEK 293-TLR8 cells were either unstimulated or stimulated with 2.5 µg/ml CL075 (A) and HEK 293-TLR3 cells were either unstimulated or stimulated with 25 µg/ml poly(I:C) (B). After a further 24 hours, the cells were lysed and the lysates assayed for luciferase activity using a luminometer. Data is shown as relative fold induction normalised to unstimulated, EV only-transfected cells. The data is mean $\pm$ SD of triplicate samples and is representative of three experiments. *= $p < 0.05$, **= $p < 0.01$ compared to stimulated, EV only-transfected cells or cells transfected with the highest dose of DHX9 expression vector.

Chapter 5: Final conclusions

The innate immune response is crucial in the early defence against infection and for induction of an appropriate response. Having evolved alongside the host, viruses have developed numerous strategies of avoiding detection and antagonising various aspects of innate immunity. VACV is an example of a virus which encodes a vast array of proteins, sculpted through evolution, to inhibit a range of innate immune signalling components (Section 1.12.2). One such protein, VACV E3, is capable of inhibiting numerous aspects of innate immunity (Section 1.13). Amongst others, these include its sequestering of dsRNA and its antagonism of the antiviral proteins PKR and ISG15 [354, 394]. Mass spectrometry analysis identified host DHX9 as a potential E3 interacting partner (S. Keating, personal communication). The ability of DHX9 to associate with VACV 70mer DNA in a separate mass spectrometry analysis (S. Keating, personal communication), made it attractive as a potential novel poxviral nucleic acid sensor. Hence, a lentiviral shRNA inducible system was employed here to generate THP-1 cells in which DHX9 had been silenced. THP-1 cells were chosen as a suitable cell model as they are optimal for shRNA knockdown studies and are routinely employed for investigating nucleic acid sensing pathways due to their high level in expression of innate immune sensors and the signalling components involved. The presented work demonstrates no role for DHX9 in nucleic acid sensing in THP-1 cells. DHX9 does however appear to have an important role in cytokine induction, most likely through its regulation of transcription factor activation. Given that THP-1 cells are an immortalised monocytic cell line, a role for DHX9 in primary monocytes from blood will next need to be investigated to confirm a more physiological role for DHX9 in cytokine induction.

During this project, there have been two publications, from the same authors, which described DHX9 as both a DNA and RNA sensor in pDCs and mDCs, respectively [222, 270]. However, in THP-1 cells studied here, there was no requirement of DHX9 for effective IFN β mRNA induction, or RANTES and IP10 secretion in response to either RNA or DNA. This may be due to cell type differences, with DHX9 having more of a nucleic acid sensing role in DCs rather than monocytes. DHX9 was however, found to have an important role in TLR- and SeV-stimulated cytokine production. Interestingly, it was noted that a number of the cytokine readouts affected were the same as those shown to be affected in the studies in which DHX9 was

described as playing a role in nucleic acid sensing [222, 270], namely, IL-6, TNF α and IFN α . Although described as being required for pro-inflammatory cytokine induction rather than type I IFN in the publication proposing DHX9 as a sensor of CpG-B DNA [270], closer analysis of the data shows that IFN α secretion is also significantly reduced upon knockdown of DHX9. Therefore, both publications show knockdown of DHX9 to give an abrogation in a common set of NF- κ B- and IRF-dependent genes.

A requirement of DHX9 for NF- κ B-dependent cytokine induction could be explained by its ability to bind p65 and act as a transcriptional coactivator [298]. Here, it has been demonstrated that, as well as enhancing p65 transactivation, DHX9 is also capable of enhancing the transactivation of IRF family members. Also, DHX9 may have a role in effective endosomal TLR signalling, with TLR8- and TLR3-dependent signalling being abrogated upon DHX9 silencing. Collectively, these data lead one to question the validity of whether DHX9 is a bona fide nucleic acid PRR or whether the data may have been misinterpreted, with DHX9 playing a transcriptional role rather than functioning further upstream in signalling events. DHX9 is a helicase which is capable of unwinding both RNA and DNA. Hence, binding to biotinylated CpG-B DNA or poly(I:C), as shown in the aforementioned studies as evidence of a nucleic acid sensing role [222, 270], would not be unexpected. DHX9 was proposed to recognise CpG-B DNA in pDCs and signal via MyD88 to cause an upregulation in NF- κ B- and IRF-dependent cytokines [270]. As silencing of TLR9 abolished responses to a similar degree to DHX9 silencing, it is possible that DHX9 may serve as an adaptor in the signalling cascade rather than a sensor of the CpG-B DNA. As TLR9 is an endosomal localised TLR, this would fit with a role for DHX9 in endosomal TLR signalling events as demonstrated in this thesis for TLR8. The interaction of DHX9 with MyD88 reported by Liu et al. may not have been direct, in that pull-down of MyD88 may have been a consequence of DHX9 immunoprecipitating the whole signalling complex [270]. However, given that it has been found here that DHX9 may be required for TLR8 signalling, the MyD88-DHX9 interaction may be physiologically relevant. DHX9 may be required for MyD88-dependent endosomal signalling. Similarly, for the proposed role of DHX9 in RNA sensing [222], its interaction with RNA may have led to its non-direct immunoprecipitation of MAVS rather than it directly recruiting MAVS for downstream signalling events. Whether these interactions would still be observed using

recombinant protein and GST pull-down assays remains uncertain. The ability of DHX9 shown here to transactivate p65 and IRF family members may provide an alternative explanation as to why its knockdown affected NF- κ B- and IRF-dependent cytokines [222, 270].

Coinciding with more of a role in transcriptional regulation, DHX9 was also found to have a role in effective ISG56 mRNA induction after stimulation with IFN α in THP-1 cells. Other ISG members were not found to be affected upon DHX9 silencing. Interestingly, this did not translate to an effect on ISG56 protein expression, which may indicate that ISG56 mRNA is very effectively translated or that the mRNA is not limiting for translation. Alternatively, there may be a redundancy in the translation of ISG56 mRNA, where other DExD/H family members are sufficient for effective ISG56 translation. DHX9 has been reported to be recruited to PML bodies after IFN α stimulation, where it associates with nascent mRNA transcripts [406]. Therefore, ISG56 may belong to a specific subset of ISGs which DHX9 regulates.

DHX9 was found to be necessary for effective IL-6 production in both THP-1 and BEAS-2B cells. Unlike other NF- κ B-dependent cytokines assayed, IL-6 was found to be reduced for any stimuli tested. Thus, it is likely that DHX9 may be required for the assembly or effective activation of the IL-6 promoter complex. This correlated with data obtained from overexpression studies. Although DHX9 was not able to activate an IL-6 promoter luciferase construct by itself, dose-dependent enhancement of MyD88-dependent transactivation of the promoter was observed. Similarly, overexpression of DHX9 in HeLa cells demonstrated that, although DHX9 could not stimulate IL-8 or IL-6 production by itself, it was capable of enhancing both MyD88-dependent IL-8 and IL-6 production. The ability of DHX9 to transactivate the IL-6 promoter was only partially dependent on the promoter's NF- κ B-binding site. Thus, in addition to its ability to interact with p65 and act as a transcriptional coactivator [298], DHX9 appears to be capable of regulating IL-6 promoter activation by a further mechanism. IL-6 levels are elevated in most, if not all, inflammatory states, and hence it has been recognised as a therapeutic target. IL-6 is involved in the onset and maintenance of multiple diseases, including rheumatoid arthritis, Castleman's disease and osteoporosis [407]. Tocilizumab, a humanised monoclonal antibody against the IL-6 receptor, is currently

used as an immunosuppressive drug to treat rheumatoid arthritis, Castleman's disease, SLE and several other conditions [408]. IL-6 plays a pivotal role in the transition from innate to acquired immunity. During acute inflammation, there is initially a massive recruitment of neutrophils to the site of infection. After 24-48 hours, these are replaced by monocytes and T cells in order to prevent increased tissue damage by neutrophil-secreted proteases and reactive oxygen species. IL-6 plays a crucial role in both of these events. As well as being key in the initial recruitment of neutrophils, IL-6 also signals via the IL-6 receptor in invading neutrophils and leads to a switch from neutrophil to monocyte recruitment by suppressing the expression of neutrophil-attracting chemokines and enhancing the production of mainly monocyte-attracting chemokines [49]. Amongst numerous other important roles, IL-6 is also known to play an important role in B and T cell differentiation [30]. As IL-6 has a pivotal role in immunity, the mechanism by which it is regulated is of vast importance to immune signalling research. The discovery of DHX9 as a potential regulator of IL-6 expression may provide a novel means of therapeutically targeting IL-6 expression. Chromatin immunoprecipitation assays may help provide more evidence as to whether DHX9 is required for effective IL-6 promoter activation. Employing the DHX9 shRNA-expressing THP-1 cell lines, immunoprecipitation with a Pol II antibody followed by qPCR amplification of the IL-6 promoter, would show whether DHX9 is required for effective Pol II recruitment. A further immunoprecipitation with DHX9 antibody could determine whether DHX9 itself is recruited to the IL-6 promoter following stimulation. Alternatively, rather than acting at the IL-6 promoter itself to regulate IL-6 induction, DHX9 may be involved in IL-6 mRNA stability. This could be tested via the use of actinomycin D in mRNA stability assays in the DHX9-shRNA expressing THP-1 cell lines.

The importance of DHX9 in the host immune response is shown by its targeting by the VACV protein E3. Although numerous viruses are reported to hijack DHX9 to promote their replication [377, 378], it is unlikely the case for VACV, as preliminary observations suggest that E3 may promote the degradation of DHX9. Therefore, although myxoma virus M029, a truncated ortholog of E3, was demonstrated to hijack DHX9 to promote viral replication [379], this may not be the case for E3. Retroviral expression of E3 in THP-1 cells gave a similar altered cytokine profile as knockdown of DHX9 by shRNA. Overexpression studies showed that E3 could inhibit the ability of

DHX9 to transactivate NF- κ B and IRF family members. It is likely that E3 is directly binding DHX9 to antagonise its involvement in the secretion of pro-inflammatory cytokines and type I IFN. Of particular interest is the ability of E3 to inhibit the effective production of IL-6. IL-6 has been reported to be crucial in the effective clearance of VACV, and E3 has been implicated in its inhibition by an unidentified mechanism [399, 409]. IL-6 production was reduced for all stimuli tested in THP-1 cells stably expressing VACV E3. Interestingly, ectopic expression of E3 in HeLa cells was able to antagonise the ability of DHX9 to enhance both MyD88-driven IL-6 and IL-8 production. It is thus likely that E3 is inhibiting the production of IL-6 by directly antagonising DHX9 function. This may provide the mechanism by which VACV is capable of antagonising IL-6 production *in vivo* [409]. DHX9 shRNA-expressing THP-1 cells will next need to be infected with either WR VACV or VACV Δ E3L to determine whether VACV lacking the E3 gene induces a greater IL-6 response upon live infection in this system.

The presented data demonstrates DHX9 to have a more transcriptional role than previously envisaged, playing an important role in transcription factor transactivation. Although previously reported to bind p65 and act as a transcriptional coactivator [298], its ability to transactivate IRF family members was previously uncharacterised. As discussed, this would lead one to question whether DHX9 is a bona fide nucleic acid sensor or whether its ability to transactivate numerous transcription factor members could explain previous published data on the helicase. Since the description of DDX41 as a DNA sensor [273], a huge interest in the roles of DExD/H family members in innate immunity, has led to a myriad of publications describing their roles in nucleic acid sensing (Section 1.11). The majority of these publications demonstrate nucleic acid binding through the use of biotinylated nucleic acid, and recruitment of downstream adaptor proteins via co-immunoprecipitation studies. Neither of these techniques are sufficient to demonstrate with certainty that these DExD/H family members are bona fide receptors, as all helicases are capable of nucleic acid binding, and co-immunoprecipitation studies cannot definitively demonstrate direct protein-protein interactions. In addition, the mapping of the interaction of these family members with downstream adaptors has been extremely erratic with some family members found to bind several adaptors via the same domains, or using the same domains to interact with

both nucleic acid and 'downstream' signalling components. There has been little consistency between the family members on what region interacts with nucleic acid or downstream signalling adaptors. For instance, DHX9 is reported to interact with both MyD88 and MAVS via its C-terminal HA2-DUF domain to sense either CpG-B DNA or dsRNA, respectively [222, 270]. As well as recruiting MyD88 via its C-terminus, DHX9 was also proposed to bind CpG-B DNA via its C-terminus [270]. Another example is DHX36, which has been shown to form a dsRNA-sensing complex with DDX1 and DDX21 [223]. Here, its HA2-DUF domain is said to interact with TRIF. In a separate publication, DHX36 is also shown to interact with CpG-A DNA and MyD88 via its HA2-DUF domain [270]. The mapping of the regions required for nucleic acid binding and recruitment of signalling components have been carried out through the use of truncation mutants in co-immunoprecipitation studies. However, although a truncated protein may be detectable following overexpression, there is no guarantee that the protein is still folded correctly. The huge variance in what regions of these helicases interact with nucleic acid or other 'interacting' partner's, may be a result of misfolding of these truncations. Some truncations may result in the misfolding of the protein so that its helicase activity or ability to bind nucleic acid are no longer intact. No longer capable of nucleic acid binding or helicase activity, its complex with nucleic acid and associated proteins may be disrupted and hence no 'interaction' would be seen with associated proteins. Therefore truncated regions of the protein could be misinterpreted as being necessary for interactions. In a similar mechanism by which Pol III transcribes AT-rich dsDNA for RIG-I recognition [210], DExD/H family members may be required for the unwinding and presentation of nucleic acid to PRRs, rather than serving as nucleic acid sensors themselves. A further possibility is that they may form a tighter association with the nucleic acid than innate immune sensors and thus provide a form of platform or reservoir of nucleic acid for other sensors to interact with. To conclude, although it is evident that numerous DExD/H family members have an important role in innate immune signalling events, a more detailed analysis will need to be carried out before concluding whether a number of these molecules are bona fide nucleic acid sensors.

Millions of years of evolution alongside the host have led to viruses developing precise and intricate mechanisms by which they subvert and evade the host defence

systems. Similarly, mammals have evolved a complex intracellular signalling system that allows them to counteract viral infection. Their coevolution alongside the host immune system, makes viruses an excellent model of interrogating the host for antiviral signalling molecules. Here, VACV E3 was used to probe for novel molecules involved in the host response to VACV. VACV E3 was found to directly associate with host DHX9. Further study showed that this is most likely a means of antagonising the ability of DHX9 to transactivate NF- κ B and IRF family members, as well as antagonising its ability to upregulate IL-6 protein to counteract VACV infection. Hence, a novel mechanism by which VACV E3 antagonises the innate immune response has been identified and further work will now need to be carried out to identify the exact mechanism whereby DHX9 contributes to promoter transactivation and how E3 inhibits DHX9 function.

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Appendix

List of suppliers

Company	Address
Alexis Biochemicals	Enzo Life Sciences LTD., Palatine House, Matford Court, Exeter EX2 8NL, United Kingdom
Axis-Shield	Axis-Shield Diagnostics Ltd., Luna Place, The Technology Park, Dundee DD2 1XA, Scotland
BD Biosciences	Edmund Halley Road – Oxford Science Park, OX4 4DQ Oxford, United Kingdom
Biometra	Biometra GmbH, Rudolf-Wissell-Str. 30, D-37079 Goettingen, Germany
Bio-Rad	Bio-Rad Laboratories Ltd., Bio-Rad House, Maxted Road, Hemel Hempstead, Hertfordshire HP2 7DX, United Kingdom
Biosera	Biosera Ltd – Head Office, 1 Acorn House, The Broyle, Ringmer, East Sussex, United Kingdom
Biosynth	Biosynth AG, Rietlistr. 4, 9422 Staad, Switzerland
Cell Signalling	Brennan and Company, 61 Birch Avenue, Stillorgan Industrial Park, Stillorgan, Co. Dublin, Ireland
Clontech	2 Avenue du President Kennedy, 78100 Saint-Germain-en- Laye, France
Corning Life Sciences	Corning B.V. Life Sciences, Fogostraat 12, 1060 LJ Amsterdam, The Netherlands
Covance	Covance Inc., 6 Roxborough Way, Maidenhead Berkshire, SL6 3UD, United Kingdom
Fisher	Fisher Scientific Ireland, Suite 3, Plaza 212, Blanchardstown Corporate Park 2, Ballycoolin, Dublin 15
Gibco	Bio-Sciences, 3 Charlemont Terrace, Crofton Road, Dun Laoghaire, Co. Dublin, Ireland
Greiner Bio-One	Greiner Bio-One GmbH, Maybachstrasse 2, 72636 Frickenhausen, Germany

Invitrogen	Bio-Sciences, 3 Charlemont Terrace, Crofton Road, Dun Laoghaire, Co. Dublin, Ireland
Invivogen	5 rue Jean Rodier, F-31400 Toulouse, France
LI-COR	St. John's Innovation Centre, Cowley Road, Cambridge, CB4 0WS, United Kingdom
Millipore	Bioscience Division, Millipore Ireland B.V., Tullagreen, Carfigtwchill, Co. Cork, Ireland
Miltenyi Biotec	Miltenyi Biotec Ltd., Almac House, Church Lane Bisley, GU24 9DR Surrey, United Kingdom
Molecular Devices	Molecular Devices, LLC, 1311 Orleans Drive, Sunnyvale, CA 94089-1136, USA
MWG Biotech	Eurofins MWG Operon, Anzingerstr. 7a, 85560 Ebersberg, Germany
National Diagnostics	Bio-Sciences, 3 Charlemont Terrace, Crofton Road, Dun Laoghaire, Co. Dublin, Ireland
NCI BRB	Biological Resources Branch, DCTD, DTP, NCI-Frederick, ATRF, Suite E3900, Fort Detrick, Frederick, Maryland, USA
NEB	New England Biolabs Ltd., 75/77 Knowl Piece, Wilbury Way, Hitchin, Herts. SG4 0TY, United Kingdom
PBL Interferon Source	Quansys Biosciences, 365 North 600 West, Logan, Utah, USA
Peptotech	Peptotech Corporate Headquarters, Princeton Business Park, 5 Crescent Avenue, Rocky Hill, NJ 08553, USA
Promega	Medical Supply Co. Ltd. Distributor, Damastown, Mulhuddart, Dublin 15, Ireland
Qiagen	Qiagen House, Fleming Way, Crawley, West Sussex, RH10 9NQ, United Kingdom
R&D	R&D Systems Europe Ltd., 19 Barton Lane, Abingdon Science Park, Abingdon, OX14 3NB, United Kingdom
Roche	Roche Products Ltd., 3004 Lake Drive, Citywest, Dublin 24, Ireland

Santa Cruz	Santa Cruz Biotechnology Inc., Bergheimer Str. 89-2, 69115 Heidelberg, Germany
Sigma-Aldrich	Sigma-Aldrich Ireland Ltd., Vale Road, Arklow, Co. Wicklow, Ireland
Stratagene	Agilent Technologies UK, Life Sciences & Chemical Analysis Group, Lakeside Cheadle Royal Business Park, Stockport, Cheshire, United Kingdom
Thermo Scientific	Thermo Fisher Scientific, 81 Wyman Street, Walkham, MA 02454, USA
5 Prime	5 Prime GmbH, D-22767 Hamburg, Germany

Table 4.1 List of suppliers