Vaccinia protein C16 blocks innate immune sensing of DNA by binding the Ku complex

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Declaration

The work detailed herein is the work of the candidate except where clearly indicated. The structural prediction of C16 was performed by Dr. Brian J Ferguson. In addition, the nuclear translocation of IRF-3 experiment and the biotinylated DNA experiment were performed in conjunction with Dr. Brian J Ferguson.

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Abstract

VACV gene C16L encodes a 37-kDa protein that is highly conserved in orthopoxviruses and functions as an immunomodulator. Intranasal infection of mice with a virus lacking C16L (vΔC16) induced less weight loss, fewer signs of illness and increased infiltration of leukocytes to the lungs compared with wild-type virus.

To understand C16's mechanism of action, tandem affinity purification and mass spectrometry were used to identify C16 binding partners. This revealed that Ku70, Ku80 and PHD2 interact with C16 in cells.

Ku70 and Ku80 constitute the Ku heterodimer, a well characterised DNA repair complex. MEFs lacking Ku, or the other component of the DNA-dependent protein kinase (DNA-PK) complex, the catalytic subunit of DNA-PK (DNA-PKcs), were shown to be deficient in the upregulation of IRF-3-dependent genes such as *Cxcl10*, *Il6* and *Ifnb* in response to transfection of DNA, but not poly (I:C). Furthermore, following infection of MEFs with VACV strain MVA the activation of *Cxcl10* or *Il6* transcription was dependent on DNA-PK. Therefore, DNA-PK is a DNA sensor capable of detecting poxvirus DNA and activating IRF-3-dependent innate immunity.

C16 inhibited the binding of Ku to DNA, and therefore inhibited DNA-mediated induction of Cxcl10 and Il-6 in MEFs. The role of C16 *in vivo* was also examined: infection with vΔC16 led to increased production of Cxcl10 and Il-6 following intranasal infection of mice compared with wild-type virus. C16 is therefore an inhibitor of DNA-PK-mediated DNA sensing and innate immune activation.

C16 was also shown to bind to PHD2, an enzyme involved in regulation of hypoxic signalling. VACV was found to activate the transcription of hypoxia-related genes, and C16 expression in cells was also capable of doing this. The role of hypoxic signalling in VACV infection remains poorly understood.

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List of Abbreviations

AGS	Aicardi-Goutières Syndrome
AIM2	Absent in melanoma 2
AP-1	Activated protein - 1
ASC	Apoptosis-associated speck-like protein containing a CARD
ATP	Adenosine tri-phosphate
BAL	Bronchoalveolar lavage
BMDDC	Bone-marrow derived dendritic cell
BMDM	Bone-marrow derived macrophage
bp	Base pair
CaMK2	Calmodulin kinase 2
CARD	Caspase activation and recruitment domain
CCI	CC Chemokine inhibitor
CD	Cluster of differentiation
cDC	Conventional dendritic cell
cDNA	Complementary deoxyribonucleic acid
CpG	Hypomethylated CpG motif
CPXV	Cowpox virus
CVA	Chorioallantois virus
DAI	DNA-activator of IFN
DAPI	6-diamidino-2-phenylindole
DBD	DNA-binding domain
DC	Dendritic cell
DDX3	Dead box RNA helicase 3
DEAD	Asp-Glu-Ala-Asp motif
DHX	DEAH (Asp-Glu-Ala-His) box helicase
DMEM	Dulbecco's modified eagle medium
DMOG	Dimethyloxalylglycine
DNA	Deoxyribonucleic acid
DNA-PK	DNA-dependent protein kinase
DNA-PKcs	DNA-dependent protein kinase catalytic subunit
dNTP	Deoxynucleotide tri-phosphate
ds	Double stranded
ECTV	Ectromelia virus
EDTA	Ethylene diamine tetraacetic acid
EEV	Extracellular enveloped virus
EGLN1	Egg-laying nine homolog 1
eIF	Eukaryotic initiation factor
ELISA	Enzyme-linked immunosorbant assay
EPO	Erythropoietin
ERK	Extracellular-regulated kinase
EYA4	Eyes-absent 4
FADD	Fas-associated death domain
FBS	Foetal bovine serum
FIH	Factor inhibiting HIF
FLAG	Peptide sequence (N' – C') DYKDDDDK
GAF	IFN-γ activated factor
GAG	Glycosaminoglycan
GAGA	DNA sequence (5' – 3') GAGAAAGAGAA
GAPDH	Glyceraldehyde phosphate dehydrogenase
GAS	IFN-γ–activated sequence
GLUT-1	Glucose transporter 1
HBV	Hepatitis B virus
HCV	Hepatitis C virus

HEK	Human embryonic kidney
HIF	Hypoxia-inducible factor
HIV	Human immunodeficiency virus
HPRT	Hypoxanthine phosphoribosyl transferase
HPV	Human papilloma virus
HRE	HIF response element
HSV-1	Herpes simplex virus 1
HTLV-1	Human T cell-leukaemia virus
icIL-1Ra	Intracellular IL-1 receptor antagonist
IFI16	Interferon-induced 16
IFN	Interferon
IFNAR	IFN-α receptor
IFNGR	IFN-γ receptor
IFNLR1	IFN-λ receptor
Ig	Immunoglobulin
I.I.	Integrated intensity
IKK	Inhibitor of NF-κB kinase
IL-	Interleukin-
IL10R	IL-10 receptor
IL-1Ra	IL-1 receptor antagonist
IMV	Intracellular mature virus
IP	Immunoprecipitation
IRAK	IL-1 receptor associated kinase
IRF	Interferon regulatory factor
ISD	Immunostimulatory DNA
ISG	Interferon stimulated gene
ISGF3	Interferon stimulated gene factor 3
ISRE	Interferon stimulated response element
ITR	Inverted terminal repeats
IκB	Inhibitors of κB
JAK	Janus activated kinase
JNK	Jun kinase
kDa	Kilodalton
KSHV	Kaposi's sarcoma associated herpesvirus
LB	Luria broth
LCMV	Lymphocytic choriomeningitis virus
LD50	Lethal dose, 50%
LGP2	Laboratory of genetics and physiology 2
LPS	Lipopolysaccharide
LRRFIP-1	Leucine rich repeat flightless interacting protein 1
Luc	Luciferase
MAPK	Mitogen activated protein kinase
MAVS	Mitochondrial antiviral signalling protein
MCMV	Murine cytomegalovirus
MDA-5	Melanoma differentiation associated 5
mDC	Myeloid dendritic cell
MEF	Murine embryonic fibroblast
MMTV	Murine mammary tumour virus
MOCV	Molluscum contagiosum
MPXV	Monkeypox virus
mRNA	Messenger RNA
MSF	Murine skin fibroblast
MVA	Modified VACV Ankara
MyD88	Myeloid-differentiation factor 88

MΦ	Macrophage
NAP	NAK-associated protein
NDV	Newcastle disease virus
NF-κB	Nuclear factor -κB
NHEJ	Non-homologous end joining
NK	Natural killer
NLR	NOD-like receptor
NLS	Nuclear localisation sequence
NOD	Nucleotide-oligomerisation domain
OAS	Oligoadenylate synthetase
OD	Optical density
ORF	Open reading frame
p.f.u	Plaque forming units
PAMP	Pathogen-associated molecular pattern
PBS	Phosphate-buffered saline
PBS-T	Phosphate-buffered saline, 0.05% Tween
PCR	Polymerase chain reaction
pDC	Plasmacytoid dendritic cell
PEI	Polyethylenimine
PFA	Paraformaldehyde
PHD	Prolyl-hydroxylase domain
PKR	Protein kinase R
PMN	Polymorphonuclear cells
Poly (dA:dT)	Poly A:T DNA
Poly (I:C)	Polyinosinic : Polycytidylic acid
pppRNA	5’ triphosphate RNA
PRR	Pattern recognition receptor
qRT-PCR	Quantitative real-time PCR
RIG-I	Retinoic-acid inducible gene I
RIP-1	Receptor interacting protein-1
RLR	RIG-I like receptor
RNA	Ribonucleic acid
RSV	Respiratory syncitial virus
RT	Room temperature
SCID	Severe combined immunodeficiency
SCR	Short consensus repeats
SDS-PAGE	Sodium dodecyl sulphate- polyacrylamide gel electrophoresis
SEM	Standard error of the mean
shRNA	Short hairpin RNA
SINTBAD	Similar to NAP-1 TBK-1 adaptor
siRNA	Small interfering RNA
SLE	Systemic lupus erythematosus
ss	Single stranded
STAT	Signal transducer and activator of transcription
STING	Stimulator of IFN genes
STREP	Peptide sequence (N’ – C’) WSHPQFEK
TAB	TAK-1 binding protein
TAE	Tris-acetate ethylene diamine tetraacetic acid
TAK	TGFβ activated kinase
TANK	TRAF family member-associated NF-κB activator
TAP	Tandem affinity protein purification
TBK	TANK-binding kinase
TEMED	Tetramethylethylenediamene
TGFβ	Transforming growth factor-β

Th1/2	T helper 1 / 2
TIR	Toll/Interleukin 1 receptor
TIRAP	TIR-containing adaptor protein
TLR	Toll-like receptor
TNF	Tumour necrosis factor
TRADD	TNF receptor associated death domain protein
TRAF	TNF receptor associated factor
TRAM	TRIF related adaptor molecule
TRIF	TIR-domain-containing adapter-inducing interferon-β
TRIM	Tri-partite motif
TYK2	Tyrosine kinase 2
V(D)J	Variable (Diversity) Joining
VACV	Vaccinia virus
VARV	Variola virus
VCP	Viral complement control protein
VEGF	Vascular endothelium derived growth factor
VHL	Von-Hippel Lindau
VIPER	Viral inhibitory peptide of TLR4
VSV	Vesicular stomatitis virus
WHO	World Health Organisation
WR	Western Reserve
XRCC5/6	X-ray repair complementing defective repair in Chinese hamster cells 5/6

Chapter 1: Introduction

1.1. Vaccinia virus (VACV)

1.1.1. Study of poxviruses

Poxviruses have had a profound influence on human history. Smallpox, caused by variola virus (VARV), was a highly contagious disease with a mortality rate approaching 40% and accounted for millions of deaths worldwide (Mack, 1972; Fenner, 1993). The eradication of smallpox was achieved by a dedicated effort from a World Health Organisation (WHO) team led by D.A. Henderson, using VACV as the live vaccine (Henderson, 1976; Henderson, 2009). The last natural case of smallpox was in Somalia in 1977, and the eradication was announced in 1980 (Henderson, 2009).

Although the initiation of vaccination against smallpox is widely attributed to the 1796 report of Edward Jenner inoculating a child with cowpox virus (CPXV) and demonstrating subsequent protection against smallpox, important advances predate this. Firstly, the direct inoculation of material from smallpox scabs into a recipients skin, termed variolation, was widely practiced in China and India in the 16th century, and this practice was introduced to the west from Turkey by Lady Montagu (Dinc and Ulman, 2007; Henderson, 2009). Secondly, others such as surgeon John Fewster and Benjamin Jesty, a Dorset farmer, practiced the inoculation of CPXV to prevent the development of smallpox many years prior to Jenner's report (Hopkins, 2002; Pead, 2003). The unique contributions of Jenner to the field, however, were the direct demonstrations of protection against smallpox, of the effectiveness of person to person inoculation of CPXV (circumventing the need for a cow for vaccination) and eventual publication of his studies (Hopkins, 2002).

Infection with CPXV conferred protection against smallpox owing to the antigenic similarity between the viruses (Macdonald and Downie, 1950). In 1939, Alan Downie showed that smallpox vaccines being used in the 20th century were distinct to CPXV, and thereafter the term VACV was used to describe this emerged virus. The exact origin of VACV is an enigma of virology.

The eradication of smallpox has been heralded as the greatest humanitarian achievement to date. Whilst many years have passed since the last naturally occurring case of smallpox, there are compelling reasons to continue studying VACV.

First, it is commonly used as a recombinant expression vector for vaccine development, because of the relative ease of constructing recombinant VACVs (Panicali and Paoletti, 1982), the wide range of cells that can be infected and the high expression levels of desired protein obtained with VACV (Moss et al., 1983; Smith et al., 1983; Mackett and Smith, 1986).

Secondly, VACV expresses multiple proteins which have increased the understanding of host-pathogen interactions involved in immunity and cell biology, and further study of these and additional host-pathogen interactions may have important implications for fields beyond virology (Smith et al., 1999; Bowie and Unterholzner, 2008).

This study involves the characterisation of the role of the VACV protein, C16, during infection by identifying interacting ligands and analysis of their potential roles in VACV infection.

1.1.2. Classification of VACV

The family *Poxviridae,* of which VACV is a member, is a family of large, double stranded (ds) DNA viruses which replicate exclusively in the cytoplasm of infected cells. Poxviruses are divided into *Chordopoxvirinae* and *Entomopoxvirinae* based upon, respectively, their vertebrate and insect host ranges. VACV belongs to the *Orthopoxvirus* genus of the *Chordopoxvirinae*. As well as the *Orthopoxvirus* genus, there are seven other genera belonging to the *Chordopoxvirinae*, these are *Parapoxvirus*, *Avipoxvirus*, *Capripoxvirus*, *Molluscipoxvirus*, *Suipoxvirus*, *Yatapoxvirus* and *Leporipoxvirus*. VACV is the prototypical *Orthopoxvirus* because it is the most extensively studied member of this genus and it has been used as the smallpox vaccine for over 150 years. VACV is no longer thought to exist in a natural host, although there are reports of VACV strains circulating amongst cattle in Brazil (Leite et al, 2005) and buffalo in India (Dumbell and Richardson, 1993).

The Western Reserve (WR) strain of VACV is the most frequently used strain in the laboratory and is the strain used principally in this study. Another strain used is modified vaccinia Ankara (MVA), a highly attenuated strain of VACV, derived from chorioallantois virus Ankara (CVA) and passaged more than 570 times in chicken embryo fibroblasts. As a result of the high number of passages, MVA has six major deletions of the parental virus genome (Antoine et al., 1998; Meisinger-Henschel et al., 2007) and has lost multiple virulence factors (Blanchard et al., 1998), as well as acquiring mutations in many more (Mayr et al., 1978; Meyer et al., 1991; Antoine et al., 1998). MVA cannot replicate fully in most mammalian cell lines yet is able to synthesise antigen within infected cells and is a popular vaccine vector (Moss et al., 1996; Carroll et al., 1997; Drexler et al., 1998). The fact that

MVA cannot replicate fully in mammalian cells makes vaccination with MVA safe even in the context of immunosuppression (Mayr and Danner, 1978).

1.1.3. VACV morphogenesis, entry and spread

There are two infectious forms of VACV called intracellular mature virus (IMV) and extracellular enveloped virus (EEV). EEV particles are IMV particles surrounded by an additional lipid envelope and associated viral antigens. During entry, enveloped viruses usually fuse their membranes with the plasma membrane or fuse with endocytic vesicles after endocytosis, and there is evidence to suggest that both methods are utilised by both the IMV and EEV forms of VACV (Moss, 2007).

IMV entry remains an intensively studied aspect of VACV biology. An IMV particle is surrounded by a single lipid envelope and can enter cells by fusion of its membrane with either the cell plasma membrane or the membranes of endoyctic vesicles. Electron microscopy studies have demonstrated direct fusion of IMV with the plasma membrane, allowing for the transfer of virus core into the cytoplasm of infected cells (Carter et al., 2005). In addition, there is evidence for a dependence on low pH during virus entry, which implicates an endosomal entry method (Townsley and Moss, 2007). Furthermore, a study has also provided evidence for an entry pathway based on apoptotic mimicry and macropinocytosis (Mercer and Helenius, 2008).

EEV, which contains an extra lipid envelope surrounding IMV, is thought to use separate entry mechanisms from IMV because the existence of an extra membrane raises topological difficulties. Two EEV entry models have been proposed. The first involves the endocytosis of the infecting virion, and the subsequent disruption of outer membrane by acidification which allows the second membrane to fuse directly with the endosomal

membrane (Ichihashi, 1996; Vanderplasschen and Smith, 1999; Townsley et al., 2006). The second model, termed ligand-dependent non-fusogenic dissolution of viral membrane, is based upon observations of rupture of the outer membrane of EEV at the point of contact with the infecting virion. This rupture allows direct contact and fusion of the IMV of the plasma membrane (Law et al., 2006)

1.1.4. VACV uncoating

After binding and entry, proteolysis of some virion proteins as well as hydrolysis of the virion phospholipid occurs (Zaslavsky, 1985). Subsequently, the disruption of the viral core commences. In the absence of early viral mRNA and/or proteins the complete disruption of the virus core does not commence, suggesting that release of the VACV genome from the virus core requires nascent viral proteins (Pedley and Cooper, 1987; Pedersen et al., 2000)

1.1.5. VACV genome structure

The VACV genome consists of a continuous, linear dsDNA molecule approximately 190 kilobase-pairs (kbp) in length, although this varies between strains (Table 3.2). At the two termini of the genome, all known poxviruses have inverted terminal repeats (ITRs) which vary in length between strains and contain identical yet oppositely orientated sequences. At the genome termini there are hairpin loops connecting the two DNA strands to complete the continuous genome (Baroudy et al, 1982). In addition to the hairpin loop, the ITRs also contain a highly conserved region less than 100- base pairs (bp) in length which is required for the resolution of concatameric replication intermediates termed the concatamer resolution

site. The ITRs contain a series of tandemly repeated sequences, and the number of repetitions of these sequences differs between strains of VACV and other poxviruses (Table 3.2).

Based upon published sequences of *Chordopoxvirinae* members, the overall genome structure (in terms of gene arrangement) can be generalised into two parts, (i) a central and highly conserved region of genes encoding proteins involved in essential replication functions such as transcription, replication and morphogenesis, and (ii) the regions located toward the ends of the genome, which tend to be involved in host interactions such as those concerned with modulation of the immune system (Gubser et al., 2004).

1.1.6. VACV DNA replication

As mentioned previously, poxvirus DNA replication takes place exclusively in the cytoplasm of infected cells. Discrete viral factories form during the course of infection, and each infectious particle has the potential to initiate the formation of a viral factory, thus the number of viral factories observed is a function of the multiplicity of infection. VACV DNA replication begins between 1 – 2 hours after infection, resulting in approximately 1×10^5 genome copies per infected cell (Salzman, 1960).

VACV encodes multiple enzymes involved in DNA replication, including those involved in DNA precursor metabolism such as thymidine kinase (Bajszar et al., 1983; Hruby et al., 1983), thymidylate kinase (Smith et al., 1989), ribonucleotide reductase (Slabaugh et al., 1988; Tengelsen et al., 1988) and a dUTPase (Broyles, 1993). Also, enzymes involved in DNA replication are produced by VACV such as a DNA polymerase, DNA topoisomerase, DNA ligase, a single stranded (ss) DNA binding factor and a processivity factor (Moss, 2007).

Whilst there is incomplete understanding of how poxvirus DNA replicates, several lines of evidence point toward a rolling hairpin strand displacement mechanism such as that proposed for parvovirus DNA (Tattersall and Ward, 1976). Briefly, this model involves the introduction of a nick near the genome terminus, with the terminal 200-bp being particularly important for the introduction of the nick (Du and Traktman, 1996). The nick provides a 3' end for priming of the replication complex (consisting of the VACV DNA polymerase, uracil DNA glycosylase, nucleotide triphosphate (NTP)-ase and the VACV A20 protein). The newly synthesised DNA strand then, by virtue of replicating the ITRs (including the hairpin loop), folds back on itself and the remainder of the genome is replicated (Moss, 2007).

1.2. The innate immune system

The interaction between a virus and the host immune system is of considerable interest, and much understanding of the function of the immune system has been derived from studies of viruses. Furthermore, future design of vaccines might benefit enormously from a better understanding of how a virus interacts with its host and, importantly, how a host detects infection.

The rapid detection of invading pathogens and the recruitment and activation of cells of both the innate and the adaptive immune system outlines the remit of the innate immune system. This system is comprised of both germ-line encoded molecules, such as Toll-like receptors (TLRs), and cells such as macrophages and neutrophils.

The recent advances in the study of innate immunity have been driven by the concept, developed over many years, including more recently by Charles A. Janeway, regarding the capability of the innate immune system to detect and respond to certain unchangeable

features of pathogens (Janeway, 1989; Medzhitov, 2009). Pathogens often have a series of molecules, termed pathogen-associated molecular patterns (PAMPs) which are essential for their existence, and the innate immune system has evolved to recognise these PAMPs with the use of pattern recognition receptors (PRRs). Examples of PAMPs include viral nucleic acid and lipopolysaccharide (LPS) of gram-negative bacteria.

Pattern recognition receptors (PRRs) are germline encoded molecules which detect PAMPs. Activation of PRRs leads to the activation of a number of transcription factors such as nuclear factor-κB (NF-κB) and the interferon regulatory factors (IRFs) which activate pro-inflammatory gene transcription. In addition, intracellular signalling molecular complexes such as inflammasomes, involved in the post-translational modification of pro-inflammatory molecules, are also activated during the course of infection. The activation of these factors results in a coordinated response against the invading pathogen such as the initiation of inflammation (Medzhitov, 2008) and the recruitment and activation of the adaptive immune system (Iwasaki and Medzhitov, 2010). The coordination of the resulting immune response is largely due to the production of cytokines and chemokines. Chemokines are involved in the recruitment of leukocytes to the site of infection, whereas cytokines play a more diverse role, such as initiating cell death and inducing an antiviral state within cells, and among the cytokines are the interleukins (IL), tumour necrosis factor (TNF) and the interferons (IFNs).

The role of the innate immune system during VACV infection has been studied by many groups and various facets have been examined, from cellular to molecular mechanisms. Cellular contributions are of paramount importance and these are described here, however much work is dedicated to the understanding of the initial detection events and the role of cytokines, such as IFNs, produced during virus infections.

1.2.1. Cells of the innate immune system

The cells of the innate immune system are ultimately responsible for dismantling of pathogens and also the activation of the adaptive immune system. Various cell types are employed during the course of infection to fulfil different roles and are briefly described here.

Neutrophils

Neutrophils (also termed polymorphonuclear cells, PMNs) are granular leukocytes of myeloid origin and are a key cell type in various inflammatory processes. PMNs are an important element in bronchial defence following infection, and are recruited rapidly to the airway epithelium (Malech, 2007), therefore suggesting importance in some poxvirus infections since smallpox was transmitted principally via airborne droplets. PMNs may play a role in phagocytosing VACV since a study using radiolabelled VACV showed uptake of VACV particles by PMNs *in vivo* (West et al., 1987).

One of the key effector mechanisms utilised by PMNs is the production and secretion of an enzyme termed neutrophil elastase upon stimulation of the neutrophil PRRs. Neutrophil elastase induces the production of pro-inflammatory mediators by bronchial epithelial cells (Korkmaz et al., 2008). VACV counters this role of neutrophils by inhibiting neutrophil elastase signalling pathways via two proteins, A46 and A52, which were found to inhibit both IL-1 and neutrophil elastase mediated NF-κB activation (Carroll et al., 2005).

Macrophages

Macrophages (MΦs) are another leukocyte population of myeloid origin which play a key role in inflammatory processes. MΦs represent 95% of resident phagocytic cells in the respiratory tract and lungs, the major route of VARV infection, and are derived from infiltrating monocytes (Curtis and Kaltreider, 1989). MΦs act as phagocytes but also activate T cells via cross presentation of antigens following phagocytosis of infected cells and in addition can secrete large amounts of IFNs and pro-inflammatory cytokines.

Cross presentation by MΦs to T cells occurs within 1 h of VACV infection (Ramirez and Sigal, 2002), and depletion of MΦs from mice during intranasal infection of VACV using liposomal clodronate led to increased weight loss of the mice and decreased viral clearance (Rivera et al., 2007), suggesting that MΦs play a protective role during VACV infection.

Natural killer (NK) cells

NK cells are granular cells of lymphoid origin that contribute to immediate antiviral immunity. NK cells act in two main ways to clear viral infection. Firstly, by secretion of IFN-γ, which then induces an antiviral state and activates other cell types, such as macrophages to undergo phagocytosis; and secondly, by direct lysis of infected cells (Vivier et al., 2008).

NK cells have been found to participate in the immune response to VACV. Firstly, VACV infection induces the accumulation and activation of NK cells at the site of infection following intradermal injection of virus (Jacobs et al., 2006), suggesting that chemokines and cytokines produced during VACV infection result in the chemoattraction of NK cells. Secondly, depletion of NK cells renders mice more susceptible to VACV infection (Bukowski et al., 1983), suggesting a protective role of NK cells.

Dendritic cells (DCs)

DCs are a fundamentally important cell type in the immune system and are needed for the full activation of naive cluster of differentiation- (CD) 4+ and CD8+ T cells, via antigen presentation, which results in the initiation of adaptive immune responses against viruses, including VACV. DCs can be broadly divided into two categories, conventional DCs (cDCs) and plasmacytoid DCs (pDCs). Using a quantitative proteomics approach, the responses of different subsets of cDCs to certain viruses have been studied, and distinct PRR expression profiles have been found amongst different DC populations, indicating complexity in the methods of virus detection among different cDCs (Luber et al., 2010).

DCs are important for defence against virtually all pathogens, and there are several lines of evidence suggesting a critical role for DCs in VACV infection. Depletion of DCs led to a decreased production of IFN-γ during VACV infection (Liu et al., 2008), and also DCs produce large quantities of type I IFN in response to VACV infection (Yao et al., 2007; Liu et al., 2008).

1.2.2 Extracellular molecules of the innate immune system

As well as powerful cellular components, the innate immune system also employs a complex series of molecules. These molecules are important for a diverse range of functions, for example enhancing phagocytosis in the case of complement, or chemoattraction of leukocytes to the sites of infection in the case of chemokines. These molecules are also therefore a prime target for viral countermeasures and there are multiple examples of interference by VACV in the following sections.

Complement

The complement system comprises a series of zymogens that are involved in the opsonisation of invading pathogens as well as serving as anaphylatoxins and chemoattractant proteins (Ricklin et al., 2010). The complement system is activated via three main pathways termed the classical, alternative, and lectin pathways.

The classical pathway of complement activation involves the binding of C1q to antibody-antigen complexes and activation of a sequence of complement components C4, C2 and C3. Activation of the lectin pathway involves recognition of microbial associated lectins by mannose-binding-lectin and ficolins. Activation of the alternative pathway is initiated through a proteolytic activation of C3 on microbial surfaces (a process which is inhibited on host cells by cell membrane bound factor H).

Complement & VACV

During VACV infection, complement is activated via the alternative pathway (Leddy et al., 1977). IMV is sensitive to complement in the absence of antibody. EEV, on the other hand, has an additional cell-surface membrane containing proteins, and is insensitive to complement in the absence of antibody due to the incorporation of host-cell complement regulatory proteins, such as CD55 and CD59 (Vanderplasschen et al., 1998).

One complement countermeasure is the VACV complement control protein (VCP), which is secreted from infected cells and consists of four copies of a repeated amino acid sequence, termed short consensus repeats (SCRs) which are found in host complement

regulatory proteins (Kotwal and Moss, 1988; Kotwal et al., 1990). VCP contributes to virulence by binding C4b and C3b and accelerating the decay of complement convertase enzymes critical to both the classical and alternative pathways (Isaacs et al., 1992). A second VACV protein, B5, contains short consensus repeats (SCRs) which are also found in complement regulatory proteins (Takahashi-Nishimaki et al., 1991), however no role in complement regulation has been reported.

Thus, as well as being of fundamental importance to the host, the complement system is also the target of pathogen countermeasures (Lambris et al., 2008).

Chemokines

Chemokines are a family of small (8-13 kDa) molecules involved in the recruitment of leukocytes to areas of infection and inflammation. After reaching the site of infection, chemokines on the luminal surface of the endothelium induce leukocytes to undergo diapadesis. Alternatively, chemokines can guide leukocytes through tissue matrix by binding to glycosaminoglycans (GAGs) and establishing a concentration gradient from the site of infection (Murphy, 2008).

Chemokines are grouped into C, CC, CXC or CX_3C subfamilies, where C represents highly conserved cysteine residues (Bacon et al., 2002). The biological actions of chemokines are mediated through their interactions with G-protein coupled receptors on leukocytes, and therefore a degree of specificity as to which leukocytes are recruited in a given inflammatory processes can be observed since the distribution of chemokine receptors can be differentially expressed depending on the cell type. Whilst a degree of specificity is observed, there is a high level of redundancy in the chemokine system, however, with multiple chemokine

receptors on single cells, multiple chemokines able to bind individual receptors and multiple receptors able to bind individual chemokines (Murphy, 2008).

Chemokines and VACV

VACV encodes proteins which modulate the function of chemokines because chemokines mediate the recruitment of leukocytes to sites of infection and as such they play a critical role in immunity and in VACV infection.

The CC chemokine inhibitor (CCI, also termed 'viral CC chemokine binding protein' – vCKBP) is expressed by VACV and is secreted from infected cells (Graham et al., 1997; Smith et al., 1997; Alcami et al., 1998). CCI binds to multiple CC- chemokines including CCL2 (Beck et al., 2001), CCL3, CCL5 and CCL11 (Seet et al., 2001) and secretion of this molecule inhibits recruitment of inflammatory cells to sites of VACV infection leading to decreased morbidity and reduced weight loss in mice (Reading et al., 2003).

VACV A41 is a 30-kDa glycoprotein secreted by infected cells that blocks the binding of chemokines to GAGs (Ng et al., 2001; Bahar et al., 2008). Interestingly, while this molecule does not inhibit the binding of chemokines to their receptors, the binding of chemokines to GAGs is inhibited. Consequently, the establishment of a chemokine gradient is obscured by A41. The obscuring of the chemokine concentration gradient results in decreased leukocyte chemotaxis in mice infected intranasally with VACV. Expression of A41 by VACV, compared to a deletion mutant, is associated with slightly increased virulence and increased immunogenicity *in vivo* (Clark et al., 2006).

1.2.2.1. Interferon (IFN)

IFNs are a powerful cytokine family described originally by Isaacs and Lindenmann (Isaacs and Lindenmann, 1957; Isaacs et al., 1957). There are three types of IFN, termed type I IFN, type II IFN and type III IFN and can be distinguished based upon their activating receptors. IFN is thought to be important for the control of most animal viruses, and is also often important for the clearance of other pathogens such as mycobacteria.

Type I IFN

Type I IFNs consist of a group of molecules termed IFN -α, –β, –ω, –ε, –κ, –δ, and –τ, however IFN-δ and IFN-τ have only been described in pigs and cattle and are not thought to have human counterparts (Pestka et al., 2004). The best characterised amongst the type I IFNs are IFN-α (of which there are 13 subtypes in man) and IFN-β, both of which are induced during the course of viral infection. There are less well characterised roles for the other type I IFNs in viral infection.

Type I IFN activates a receptor complex termed IFNAR consisting of an IFNAR1 and IFNAR2 heterodimer. Each receptor subunit is physically associated with a member of the Janus activated kinase (JAK) family. IFNAR1 is associated with tyrosine kinase-2 (TYK2), whereas IFNAR2 is associated with JAK1 (Figure 1.1). After binding of the heterodimeric receptor by type I IFN, the JAK family kinases are activated by a conformational change leading to autophosphorylation and activation of signal transducer and activator of transcription (STAT) -1 and STAT-2, leading to STAT-1/STAT-2 heterodimerisation (the JAK-STAT signalling pathway) (Platanias, 2005; Randall and Goodbourn, 2008). IFN regulatory factor (IRF)-9 subsequently becomes associated with the STAT-1/STAT-2

heterodimer (Horvath et al., 1996; Martinez-Moczygemba et al., 1997) and the complex of STAT-1/STAT-2 and IRF-9 is referred to as interferon-stimulated genes (ISG) factor 3 (ISGF3) (Schindler and Darnell, 1995). ISGF3 is a transcription factor complex which translocates to the nucleus and binds to interferon stimulated response element (ISRE) sites within promoters of ISGs (Kessler et al., 1990), as illustrated in Figure 1.1.

Type II IFN

There is only one type II IFN, IFN-γ, and this is typically produced in mitotically active T cells and NK cells after activation. Dimeric IFN-γ activates the IFN-γ receptor (IFNGR) consisting of a heterodimer of IFNGR1 and IFNGR2. Ligation of the IFNGR leads to activation of JAK1 and JAK2, associated with IFNGR1 and IFNGR2, respectively. The activation of these JAKs leads to the phosphorylation and homodimerisation of STAT-1, resulting in a complex referred to as IFN-γ activated factor (GAF) (Decker et al., 1991). After translocation to the nucleus, GAF binds to a specific DNA sequence termed IFN-γ – activated sequence (GAS) within gene promoters (Lew et al., 1991). Type I and type II IFN signalling has been reviewed extensively (Platanias, 2005; Randall and Goodbourn, 2008) and is illustrated in Figure 1.1.

Type III IFN

Type III IFNs represent a more recently described group consisting of IFNλ1, IFNλ2 and IFNλ3 (also known as IL-29, IL-28A and IL-28B, respectively). The role of these molecules are not as well characterised in the context of virus infection as type I or type II IFNs. Type III IFN activates a receptor consisting of a heterodimer of IL-10 receptor (IL10R)-2 and IFN-λ receptor (IFNLR)-1, which displays a limited tissue distribution,

notably to epithelial cells, and activate similar signalling pathways to IFN-α/β (Meager et al., 2005; Zhou et al., 2007). Type III IFN can also signal through non JAK-STAT pathways such as via mitogen activated protein kinases (MAPKs) Jun-kinase (JNK) and extracellular regulated kinase (ERK)-1/2 (Ank and Paludan, 2009). Whilst the same cell often produces type I and type III IFN during virus infection, liver biopsies of patients infected with hepatitis C virus (HCV) demonstrated upregulation of type III IFN but not type I IFN (Mihm et al., 2004), perhaps suggesting differential regulation of these IFNs. A fuller understanding of the role of type III IFN in immunity is required.

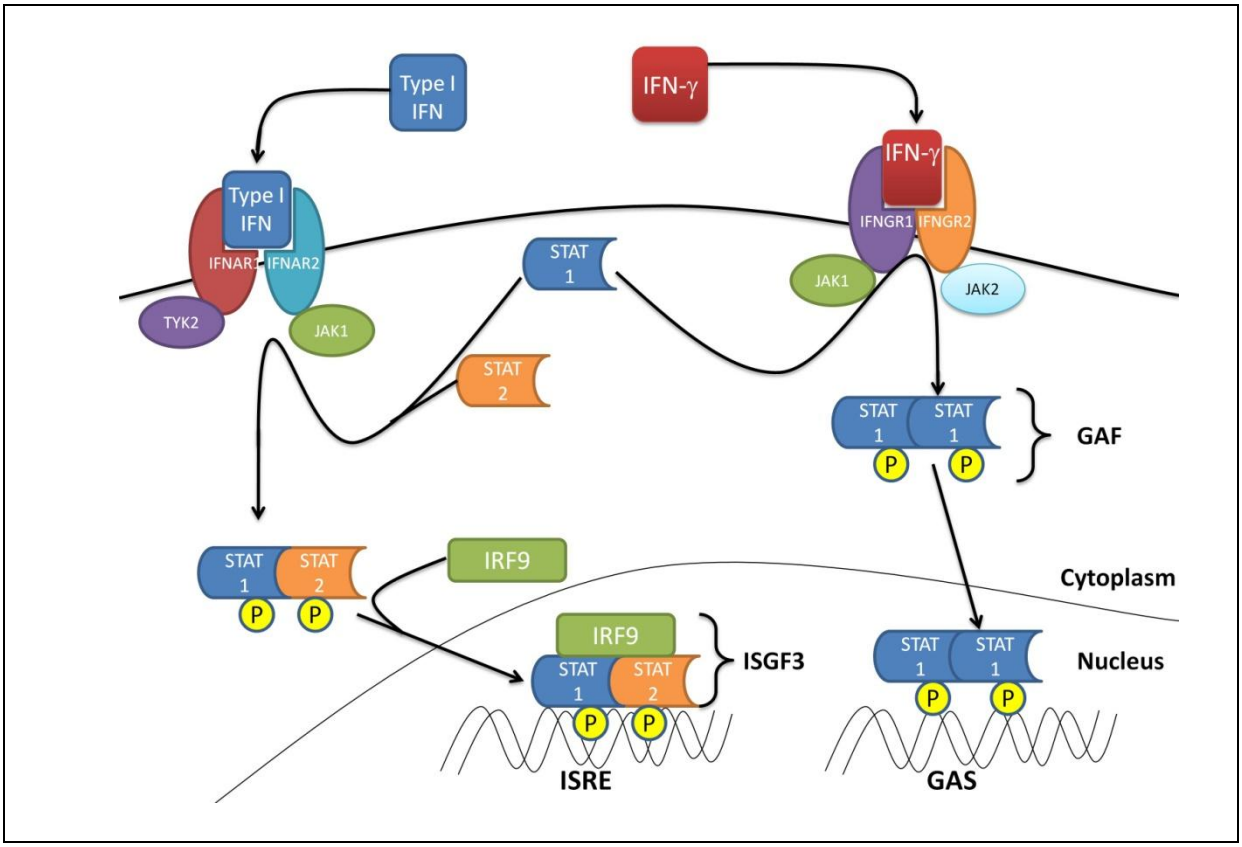

Figure 1.1. Overview of type I and type II IFN signalling. A STAT-1 and STAT-2 heterodimer is formed after binding of type I IFN to the type I IFN receptor (IFNAR) and the subsequent phosphorylation by the kinases JAK1 and TYK2. Subsequently, IRF-9 becomes associated with the complex which then translocates to the nucleus and is termed ISGF3, promoting the transcription of genes containing an ISRE site within the promoter. Type II IFN signalling is shown on the right hand side of the figure and shows that after binding of IFN-γ to the IFNGR, a STAT-1 homodimer is formed (termed GAF) and this translocates to the nucleus to activate transcription of genes containing a GAS in the promoter.

Role of IFN in VACV infection

Increased mortality occurs in mice infected with VACV when lacking the type I IFN receptor (van den Broek et al., 1995). Additionally, intraperitoneal administration of IFN-α/β severely inhibited virus replication after intraperitoneal infection of mice with a VACV expressing luciferase, and this effect was even observed with infection 4 days after pre-treatment with IFN (Rodriguez et al., 1991). Furthermore, studies using bioluminescence demonstrated a clear protective role for type I IFN during intranasal VACV infection, since infection of mice lacking IFNAR with a VACV expressing firefly luciferase led to increased bioluminescence compared with wild-type mice (Luker et al., 2005).

Both IFN-α/β and IFN-γ are found to have non-redundant roles as infection with VACV of mice lacking either IFNAR or IFNGR was much more severe compared with wild-type littermate controls. In contrast, other viruses such as lymphocytic choriomeningitis virus (LCMV) and Semliki Forest virus were more virulent in mice lacking IFNAR but not in mice lacking IFNGR (van den Broek et al., 1995).

Mice lacking IFNGR have also been demonstrated to be more susceptible to VACV infection (Huang et al., 1993). Importantly, this effect was independent of cytotoxic T cell generation since cells from IFNGR null mice showed unimpaired cytotoxicity in chromium release assays, suggesting that it is the action of IFN-γ on infected cells which has direct antiviral activity rather than IFN-γ being required for the generation of protective CD8+ T cells. Furthermore, treatment of mice with anti-IFN-γ antibodies resulted in increased mortality following infection with the mousepox virus, ectromelia virus (ECTV) (Karupiah et al., 1993).

In addition, whilst the roles of type III IFNs in viral infection remain to be well characterised, a protective effect of type III IFN *in vivo* in a poxvirus infection model has been noted, as intranasal infection of mice infected with VACV WR expressing either IFN-λ2 or IFN-λ3 were attenuated compared to wild-type viruses (Bartlett et al., 2005).

The importance of IFNs in the immune response to poxviruses is also illustrated by the many mechanisms these viruses have evolved to combat the production or function of IFNs, and the fact that deletion of genes responsible for these mechanisms reduces virulence.

Poxvirus IFN countermeasures

To combat the potent anti-viral host defence mechanisms conferred by IFNs, VACV employs countermeasures against different stages of the IFN response. For instance, poxviral IFN countermeasures include those which inhibit the upregulation of IFN (reviewed later), those which bind to IFN and those which inhibit IFN signalling. Examples of IFN countermeasures employed by VACV are illustrated in Figure 1.2.

Firstly, VACV protein B8 is a secreted glycoprotein with sequence similarity to IFNGRs that binds to, and inhibits the activity of, IFN-γ (Alcami and Smith, 1995). B8 has a broad species specificity and can inhibit the activity of rat, ovine, bovine and human, but not murine IFN-γ (Mossman et al., 1995; Symons et al., 2002b).

Secondly, VACV protein B18 is a decoy type I IFN receptor which is both secreted and present on the surface of infected cells. B18 binds soluble IFN-α/β thus preventing the transmembrane signalling of IFN-α/β (Colamonici et al., 1995; Symons et al., 1995). B18 and B8 differ due to the fact that whilst B8 has sequence similarity to the IFN-γ receptor

(Alcami and Smith, 1995), B18 does not have sequence similarity to the type I IFN receptor, but instead belongs to the immunoglobulin (Ig) protein superfamily (Symons et al., 1995).

Thirdly, VACV protein H1 is a phosphatase able to dephosphorylate STATs. Initially, H1 was found to dephosphorylate STAT-1 (Najarro et al., 2001) and later was reported to also dephosphorylate STAT-2 (Mann et al., 2008). Dephosphorylation of STATs in this way inhibits IFN-mediated signal transduction and thus dampens the upregulation of antiviral effectors. The H1 protein is packaged within virions, allowing for immediate STAT dephosphorylation without requiring nascent protein synthesis. VACV has also been shown to block STAT-independent IFN signalling (Mann et al., 2008), however the molecules involved in this have not yet been described.

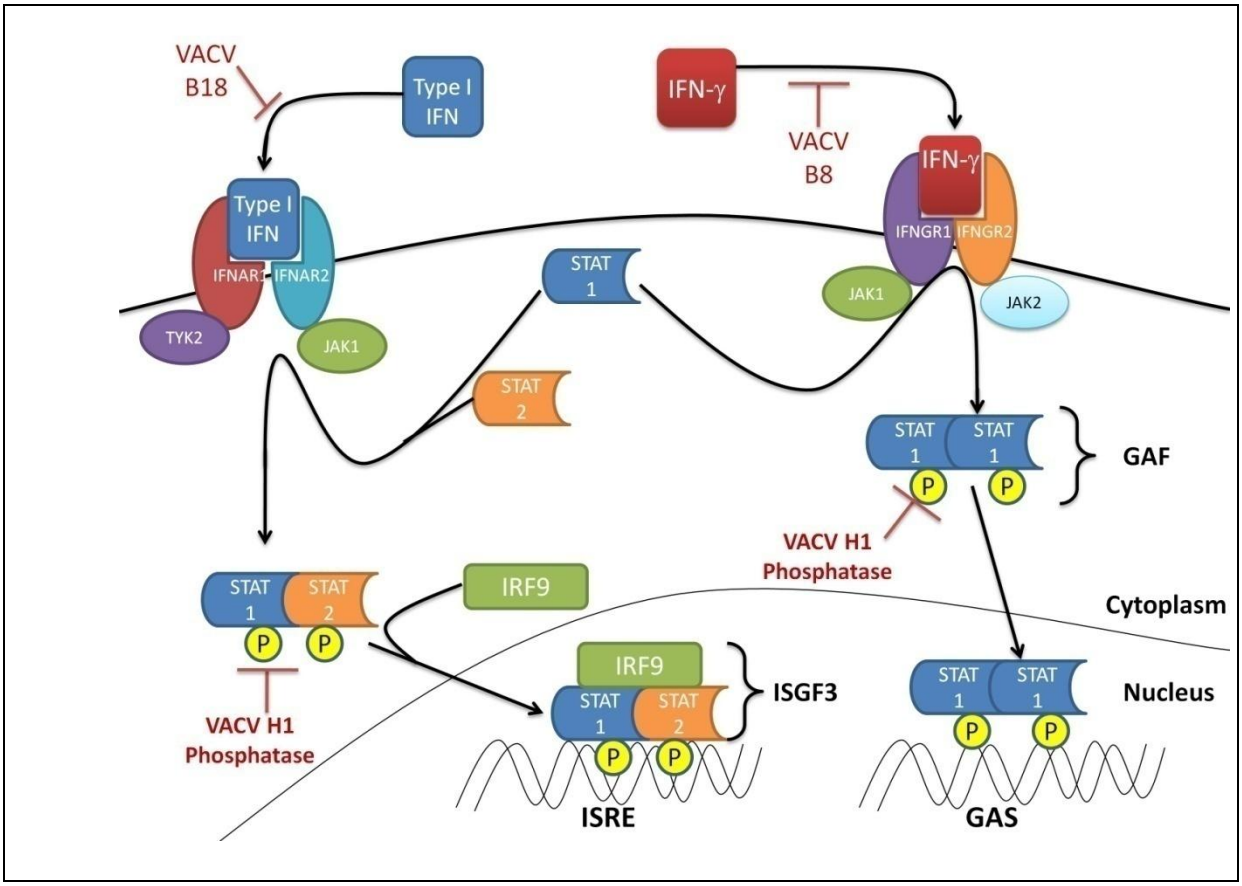

Figure 1.2. VACV inhibition of IFN signalling. This figure demonstrates how VACV interferes with IFN signalling (VACV mechanisms are shown in red). Two proteins, B18 and B8 interfere with type I and type II IFN, respectively, binding to the endogenous receptor. VACV H1 phosphatase reverses the phosphorylation of STATs thereby inhibiting downstream IFN signalling.

1.2.2.2. Interferon stimulated genes (ISGs)

After binding of IFN to IFN receptors and the subsequent activation of signalling pathways the cell enters what is termed the ‘antiviral state’, which involves the transcriptional upregulation of genes containing ISRE and GAS elements within their promoters (Sadler and Williams, 2008), and translation of the mRNAs produced. The products of this response are typically referred to as ISGs. Some of the ISGs with a documented role in VACV infection are described here.

Protein kinase R (PKR)

PKR recognises, and is activated by, double stranded RNA (dsRNA). Since dsRNA is not normally produced by the host cell, the activation of PKR usually only occurs during the course of infection. PKR contains a dsRNA binding domain and a serine/threonine kinase domain. Upon binding dsRNA, PKR becomes activated and phosphorylates eukaryotic initiation factor (eIF)-2α, an essential component of translational initiation. Phosphorylation of eIF-2α by PKR inhibits the recycling of this molecule, thus inhibiting the translation of viral, as well as cellular, mRNA.

Whilst VACV is a dsDNA virus, dsRNA is produced during the course of infection owing to transcription of the genome in opposite and complementary directions. However, the arrangement of genes transcribed early during the course of infection is such that these genes are orientated and are transcribed in the direction of the nearest genome terminus thereby reducing dsRNA synthesis. In addition, multiple sequences preventing transcription elongation are found in-between genes, thus preventing generation of complementary RNA strands.

VACV encodes two molecules which inhibit the activity of PKR: E3 and K3. Whilst E3 binds prevents activation of PKR by sequestration of dsRNA (Rice and Kerr, 1984; Chang et al., 1992; Chang and Jacobs, 1993; Beattie et al., 1995; Chang et al., 1995; Shors et al., 1998), K3 is a direct inhibitor of PKR-mediated antiviral activity by acting as a pseudosubstrate for PKR owing to an amino acid similarity to eIF-2α. Acting as a pseudosubstrate of PKR leads to prevention of phosphorylation of eIF-2α, and therefore dampens the PKR-mediated translational shutdown, as demonstrated by mutational analysis of residues conserved between K3 and eIF-2α (Kawagishi-Kobayashi et al., 1997).

2’ 5’ oligoadenylate synthetase (OAS)

OASs are a family of four molecules identified initially as enzymes able to inhibit cell-free protein synthesis (Kerr et al., 1977). OAS is activated by detection of dsRNA which induces the formation of an active OAS1 tetramer from inactive OAS1 monomers. Activated OAS inhibits protein synthesis by converting ATP into adenosine oligomers by forming 2’-5’ phosphodiester bonds (pppA(2’p5’A)n). These activate RNase L, which subsequently mediates the degradation of all RNAs, including mRNA, transfer RNA and ribosomal RNA (Clemens and Williams, 1978; Hovanessian and Justesen, 2007). RNase L contains two kinase-like domains and is constitutively inactive, however binding of pppA(2’p5’A)n leads to its homodimerisation and activation (Silverman, 2007). An additional feature of RNA degradation by RNase L is the formation of RNA molecules which may subsequently activate the retinoic acid inducible gene (RIG)-I like receptors (RLRs) RIG-I and MDA5 (Malathi et al., 2007).

There are thought to be at least 11 OAS genes in mice, in contrast to the 4 human genes. Whilst the products of these genes each have dsRNA binding activity, only the *OAS2* and *OAS3* murine homologs Oas1a and Oas1g have been shown to have 2’ 5’ OAS activity (Kakuta et al., 2002). A further study has shown that murine Oas1b negatively regulates 2’ 5’ OAS activity in cells, suggesting fine tuning of the OAS-RNase L system in mice (Elbahesh et al., 2011). However, Oas1b has been shown to be protective against flavivirus infection (Mashimo et al., 2002; Perelygin et al., 2002), suggesting alternative downstream pathways of murine OAS-like molecules.

The role of the OAS/RNase L pathway in VACV infection is not well defined, and this is likely due to the effect of VACV protein E3 on preventing detection of dsRNA by

OAS, however the fact that E3 performs this function might suggest an importance of this system in VACV infection (Rivas et al., 1998; Xiang et al., 2002).

ISG15

ISG15 is a prominent protein induced by virus infection and the ensuing IFN response. The function of this protein has only recently begun to be established. ISG15 was discovered soon after the discovery of ubiquitin, and was recognised as a ubiquitin-like protein (Loeb and Haas, 1992). Ubiquitylation occupies an increasingly important role within the immune system (Bhoj and Chen, 2009). Like ubiquitin, ISG15 is attached to protein molecules by a series of members of the E1, E2 and E3 ubiquitin ligase enzyme families, and these molecules include many involved in the antiviral response, such as JAK1, STAT1, RIG-I, PKR and RNase L (Zhao et al., 2005). The ISG15-like modification of proteins by ISG15 is hereafter referred to as ISGylation. ISGylation does not lead to protein degradation, unlike K48-linked ubiquitylation, but instead resembles the activating effects of K63-linked ubiquitylation. Accordingly, ISGylation has been shown to prevent virus-mediated degradation of IRF-3 and therefore enhance the IFN response (Lu et al., 2006). In addition, Sindbis virus expressing ISG15 did not induce mortality in *Ifnar*$^{-/-}$ mice, whilst wild-type virus induced mortality in these mice, suggesting the importance of ISG15 for control of virus infection (Lenschow et al., 2005).

The role of the antiviral response mediated by ISG15 in VACV infection is only partially understood, however the VACV E3 protein is reported to affect ISG15 antiviral functions. Firstly, VACV WR infection was enhanced in *Isg15*$^{-/-}$ murine embryonic fibroblasts (MEFs) compared to wild-type cells, and secondly an E3 knockout virus, which

was unable to grow in wild-type MEFs, was able to grow in *Isg15*$^{-/-}$ MEFs, suggesting modulation of ISG15 by E3 (Guerra et al., 2008).

1.3. Intracellular innate immunity

The production of chemokines and cytokines is usually controlled at the transcriptional level by transcription factors activated by intracellular signal transduction pathways originating at PRRs. However, in some cases, for example the cytokines IL-1β and IL-18, processing of these molecules is required before their release and biological activity, and this is regulated by post-translational activation by inflammasomes. Transcription factors and inflammasomes play key roles in the coordination of immune responses to VACV and are described in the following sections.

1.3.1. Nuclear factor-κB (NF-κB) and VACV

NF-κB was discovered as a transcription factor that binds to the intronic enhancer element of the kappa light chain gene in B cells (Sen and Baltimore, 1986a; Sen and Baltimore, 1986b) and was subsequently demonstrated to be a major regulatory factor in innate and adaptive immunity and has been reviewed recently (Vallabhapurapu and Karin, 2009). Activation of NF-κB results in the upregulation of pro-inflammatory molecules such as tumour necrosis factor (TNF)-α.

The NF-κB family of transcription factors is composed of p65 (also called RelA), c-Rel, RelB, p50 and p52- all of which are characterised by the presence of a C-terminal Rel-

homology domain (RHD) that mediates homo- and heterodimerisation. Whereas p65 and c-Rel typically heterodimerise with p50, RelB preferentially heterodimerises with p52, and its precursor p100 (Vallabhapurapu and Karin, 2009).

The subunits of NF-κB contain nuclear localisation signals (NLSs) but are usually held within the cytoplasm by a family of inhibitors, the inhibitors of κB (IκBs) such as IκBα. Activation of NF-κB requires degradation of these inhibitors for nuclear translocation of NF-κB to occur.

Engagement of various receptors, such as TLR4, IL-1R or TNFR with their specific ligands each lead to the activation of the inhibitors of the κB (IκB) kinase (IKK) complex, consisting of IKKα, IKKβ and IKKγ (also called NEMO). The IKK complex subsequently phosphorylates IκBα on serines 32 and 36 leading to its polyubiquitination and proteasomal degradation. This liberates the bound p50:p65, which then translocates to the nucleus to activate transcription of pro-inflammatory genes such as cytokines and chemokines. This process is illustrated in Figure 1.3.

The activation of NF-κB is inhibited at many stages by VACV (Figure 1.3), which highlights its importance in the control of VACV infection. VACV strain WR protein B14, is a Bcl-2-like protein which binds to IKKβ and prevents the activation of NF-κB signalling (Chen et al., 2008). N1 is another protein found to act intracellularly to contribute to virulence (Bartlett et al., 2002) by inhibiting NF-κB activation (DiPerna et al., 2004). In addition, the M2 protein acts on an alternative activation pathway of NF-κB by inhibiting the phosphorylation of ERK2, and importantly an endoplasmic reticulum localisation is essential for this function (Gedey et al., 2006; Hinthong et al., 2008). K1 is another VACV protein

thought to inhibit NF-κB signalling by inhibiting IκBα degradation, though its exact mechanism is yet to be established (Shisler and Jin, 2004).

There are a plethora of molecules found to inhibit NF-κB activation, however these are mostly thought to act at different stages of the pathway. Although multiple VACV proteins have been shown to inhibit NF-κB activation, they are not redundant as viruses lacking individual genes display an attenuated phenotype during infection (Bowie and Unterholzner, 2008). One explanation for this might be that by acting on different components of the divergent signalling pathways multiple phenotypes can exist. For instance, VACV proteins A52 and A46 inhibit TLR and IL-1R signalling, thus affecting NF-κB, IRF-3 and mitogen activated protein kinase (MAPK) signalling via TLRs (Bowie et al., 2000), but not TNF-mediated NF-κB activation. B14 binds to IKKβ and thus inhibits NF-κB activation via TLRs, IL-1R and tumour necrosis factor (TNF) receptor (TNFR) signalling, but does not affect IRF-3 or MAPK activation by these receptors (Chen et al., 2008).

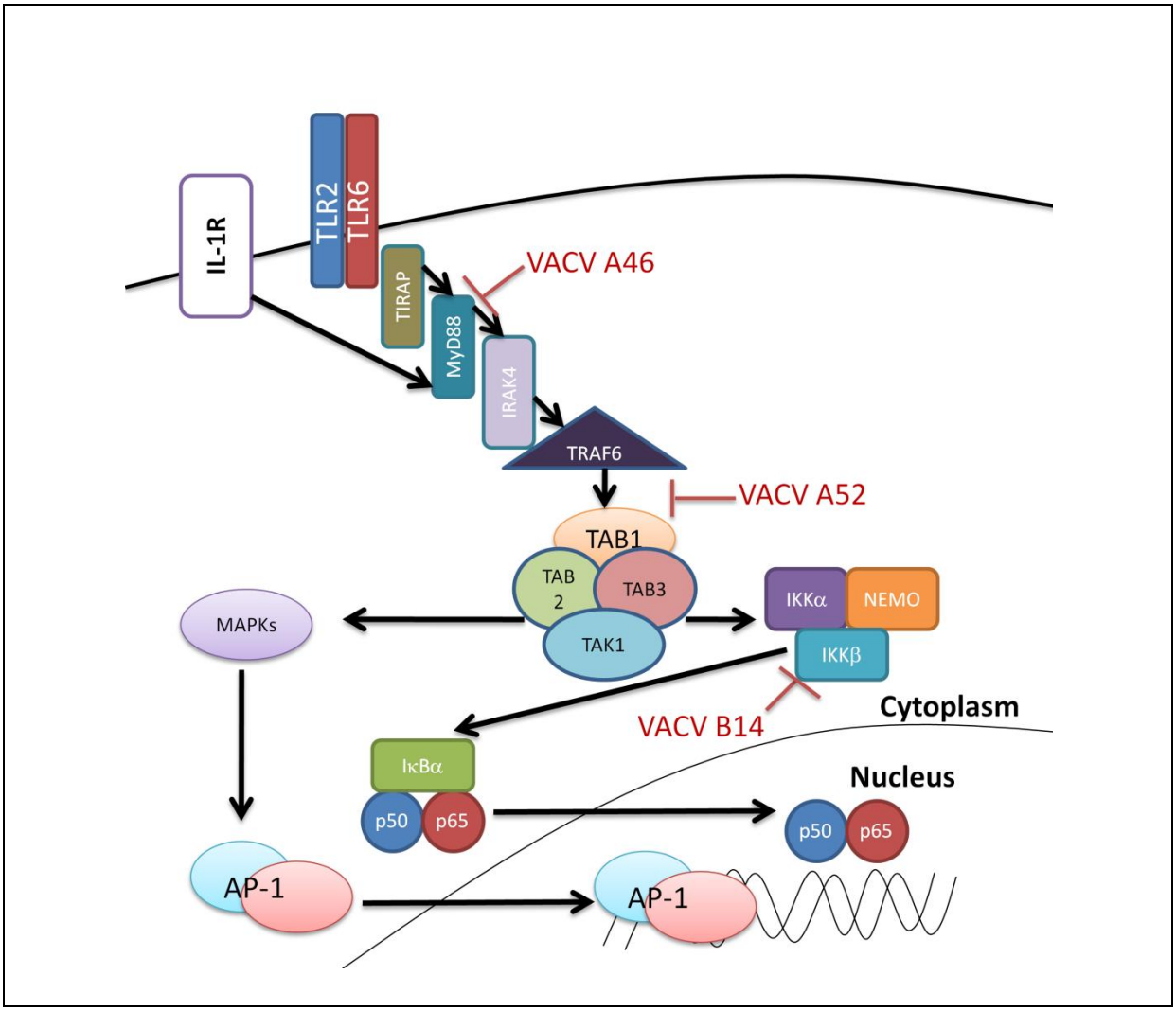

Figure 1.3. NF-κB signalling and interference by VACV. Myeloid differentiation factor 88 (MyD88) is activated by TLRs and IL-1R which in turn recruits TNF receptor associated factor (TRAF)-6. TRAF6 is responsible for binding and activating the TAK1 complex, which phosphorylates IKKβ. Phosphorylated IKKβ then phosphorylates IκBα, leading to its proteasomal degradation and the liberation of the p50:p65 heterodimer, which subsequently translocates to the nucleus to induce the transcription of pro-inflammatory genes. VACV-mediated inhibition of this pathway is indicated in red.

1.3.2. Interferon regulatory factors (IRFs) and VACV

The IRFs are major transcription factors which function in a variety of biological contexts and are particularly important in innate immune signalling. There are 9 members in humans and mice (IRFs 1-9) and these molecules each have an N-terminal DNA-binding domain (DBD) containing a helix-turn-helix motif which recognises a distinct DNA sequence (Honda and Taniguchi, 2006; Tamura et al., 2008).

IRF3 and IRF7 are responsible primarily for the induction of type I IFNs. Activation of the IRFs occurs following stimulation of multiple PRRs, such as RIG-I, melanoma differentiation associated (MDA)-5, TLR3 and TLR4 (Section 1.3.4). IRF activation is achieved following phosphorylation by two kinases, TRAF family member-associated NF-κB activator (TANK)-binding kinase-1 (TBK-1) and IKKε (Fitzgerald et al., 2003; Sharma et al., 2003; Hemmi et al., 2004; Perry et al., 2004). Whilst both TBK-1 and IKKε can both phosphorylate serines on both IRF3 and IRF7 at the C-terminal (regulatory) end, mutagenesis studies have demonstrated that TBK-1 is the major contributor to this process (McWhirter et al., 2004; Matsui et al., 2006).

Five IRFs (IRF1, IRF3, IRF5, IRF7 and IRF8) have been implicated in type I IFN gene expression in multiple cell types.

IRF1

Originally, IRF1 was considered the main IRF activating type I IFN expression (Fujita et al., 1989). However *Irf1*$^{-/-}$ MEFs produced comparable levels of type I IFN to WT MEFs in response to Newcastle disease virus (NDV), although a reduction in poly (I:C)-induced IFN was observed (Matsuyama et al., 1993). IRF1 is important for VACV infection

in mice, since IFN-γ – mediated antiviral activity was abolished in *Irf1*$^{-/-}$ MEFs, suggesting that a subset of IRF-1 stimulated genes are important for VACV control (Trilling et al., 2009).

IRF3 and IRF7

IRF3 and IRF7 are now thought to be the major contributors to the RIG-I/MDA-5-mediated IFN activation pathway. Upon activation, TBK-1 phosphorylates IRF3 at multiple C-terminal residues. This causes removal of autoinhibition allowing subsequent additional phosphorylation at Ser385 or Ser386, resulting in dimerisation and translocation to the nucleus (Mori et al., 2004; Panne et al., 2007). The current model suggests that IRF3 responds primarily to virus infection because this molecule is constitutively expressed in most cell types. IRF7 is only detected after induction by IRF3/IFN in most cell types (Sato et al., 2000; Taniguchi and Takaoka, 2002), with plasmacytoid dendritic cells (pDCs) being a notable exception (Gilliet et al., 2008).

IRF3 is thought to be important to VACV infection because of its importance in type I IFN production (Sato et al., 1998) and also since VACV K7 targets IRF3 activation by binding to Asp-Glue-Ala-Asp (DEAD) box RNA helicase 3 (DDX3), a component of the IRF3 activation machinery whose role is incompletely understood (Schroder et al., 2008). In addition, another VACV protein currently being studied within our laboratory, C6 inhibits IRF3 activation by binding to TANK, NF-κB activating kinase (NAK)-associated protein (NAP)-1 & Similar to NAP-1 TBK-1 adaptor (SINTBAD) (Unterholzner et al, submitted) which are adaptors for the activation of TBK-1 (Sasai et al., 2006; Guo and Cheng, 2007; Ryzhakov and Randow, 2007) .

1.3.3. Inflammasomes and VACV

Inflammasomes are multi-protein complexes found within various cell types, such as macrophages, and are involved in the processing of pro-IL-1β, pro-IL-18 and pro-IL-33 into their biologically active forms, IL-1β, IL-18 and IL-33 by caspase-1. Inflammasomes are activated following detection of a variety of ligands by distinct PRRs including Nod-like receptors (NLRs) (Lamkanfi and Dixit, 2009). Inflammasome formation begins when a PRR binds a PAMP, but instead of activating transcription factors, the PRR recruits caspase-1 via a caspase recruitment domain (CARD), either directly or via an adaptor such as apoptosis-associated speck-like protein containing a CARD (ASC).

VACV can activate inflammasomes via distinct mechanisms. Firstly, the absent in melanoma -2 (AIM2) inflammasome has been shown to be activated during VACV WR infection, *in vivo* (Rathinam et al., 2010). AIM2 binds to cytoplasmic DNA and activates caspase-1 through a homotypic interaction between a CARD present on both caspase 1 and the adaptor ASC (Burckstummer et al., 2009; Fernandes-Alnemri et al., 2009; Hornung et al., 2009; Roberts et al., 2009) resulting in the activation of caspase 1 and subsequent secretion of IL-1β and IL-18.

In addition, *in vitro* studies demonstrate that the NLRP3 inflammasome is activated during MVA infection (Delaloye et al., 2009) by an unknown ligand. Infection of *Nalp3*$^{-/-}$ bone-marrow derived macrophages (BMDMs) with MVA resulted in significantly reduced production of IL-1β compared to wild type BMDMs, and this was confirmed with small hairpin RNA (shRNA) knockdown of *NALP3*, *ASC* and *Caspase1* in human THP-1 cells.

Inhibition of inflammasomes by VACV

Inflammasomes are inhibited by VACV at multiple stages. VACV WR B13 is an ortholog of CPXV CrmA, which was found to inhibit IL-1β processing in baculovirus Sf9 cells (Howard et al., 1995). B13 inhibits caspase 1 in THP-1 cells, although this protein does not affect virulence in a murine infection model *in vivo* (Kettle et al., 1997).

VACV also inhibits the cytokines produced by inflammasome activation by producing decoy receptors to both IL-1β and IL-18. A molluscum contagiosum virus (MOCV), another member of the *Poxviridae*, protein was found to have similarity to the mammalian IL-18 binding protein and was shown to bind IL-18 and prevent IL-18-dependent activation of IFN-γ (Xiang and Moss, 1999). Subsequently, other poxvirus orthologs were investigated and were also found to bind IL-18 (Born et al., 2000; Smith et al., 2000). The VACV IL-18 binding protein, C12, was shown to bind IL-18 and intranasal infection of mice with a C12 deletion mutant demonstrated that the virus was attenuated compared with wild-type viruses (Symons et al., 2002a; Reading and Smith, 2003).

VACV WR also encodes an IL-1β binding protein, B15, which binds to and sequesters IL-1β (Alcami and Smith, 1992). Intracranial infection of mice with a B15 deletion virus demonstrated decreased virulence, as demonstrated by an increased dose of virus required to kill 50% of infected mice (LD50) (Spriggs et al., 1992). Intranasal infection of mice with a B15 deletion was not shown to significantly increase the LD50, although infection with the B15 deletion virus lead to an enhanced febrile response (Alcami and Smith, 1996) and an acceleration of the appearance of symptoms (Alcami and Smith, 1992), suggesting a role for IL-1β in generating fever in response to VACV and enhancing the acute phase response to VACV.

1.3.4. Pattern recognition receptor (PRRs)

The signalling molecules described above are activated ultimately after engagement of PRRs by PAMPs. PRRs are therefore of fundamental importance in the activation of innate immune responses and serve the host by acting as the initial sensors of infection (Takeuchi and Akira, 2010). There are multiple types of PRRs, including TLRs and RLRs, which detect a multitude of different ligands, and these are of considerable interest in the innate immune response to VACV as the activation of innate immunity by VACV remains incompletely defined.

1.3.4.1. Toll-like receptors (TLRs) and VACV

Toll-like receptors (TLRs) are type 1 transmembrane proteins with ectodomains containing leucine rich repeats (LRRs) that bind directly to PAMPs and a Toll/Interleukin-1 receptor (TIR) intracellular domain required for downstream signalling (Kawai and Akira, 2010). TLRs can either homodimerise or heterodimerise to form active receptors. PAMPs recognised by TLRs include lipopolysaccharide (LPS) from gram-negative bacteria, peptidogylcans and nucleic acids from fungi, bacteria, parasites and viruses. While most TLRs are found on the cell surface, a subset of TLRs are located on endosomes (TLR3, TLR7, TLR8 and TLR9).

1.3.4.1.1. Signalling pathways activated by TLRs: the MyD88-dependent pathway

Although there is a broad overlap in downstream signalling events after stimulation of different TLRs, there are certain levels of specificity. These specific signalling events are

conferred by the Toll-interleukin-1 like (TIR) domain containing adapters myeloid differentiation factor 88 (MyD88), TIR containing adaptor protein (TIRAP), TIR-domain-containing adapter-inducing interferon-β (TRIF) and TRIF related adaptor molecule (TRAM). TIRAP is involved in the recruitment of MyD88 to TLR2 and TLR4 (Fitzgerald et al., 2001; Kagan and Medzhitov, 2006). TRAM is involved in the recruitment of TRIF to TLR4 (Yamamoto et al., 2003b).

After binding to ligand, TLRs can activate the NF-κB or IRF transcription factors, or both, via two main pathways, the MyD88–dependent pathway and the TRIF-dependent pathway.

The MyD88-dependent pathway (Figure 1.3) is utilised by all TLRs except TLR3, and activates NF-κB and mitogen-activated protein kinases (MAPKs) to induce pro-inflammatory cytokines. Briefly, this pathway involves the recruitment of the IL-1-receptor associated kinases (IRAK) IRAK4, IRAK1, IRAK2 and IRAKM (Kawai and Akira, 2010). IRAK4 is activated first, followed by sequential activation of IRAK1 and IRAK2 (Kawagoe et al., 2008). IRAK activation leads to the recruitment of TRAF6 that induces K63-linked polyubiquitination of itself and IRAK1 along with Ubc13 and Uev1A. Subsequently, NEMO (part of the IKK complex) and transforming growth factor (TGF)-β activated kinase (TAK)-1 binding protein (TAB)-2/TAB3 (regulatory subunits of the TAK1 kinase) are recruited to the polyubiquitin chains and this brings the IKK complex into close proximity with an activating kinase TAK1 (Bhoj and Chen, 2009; Xia et al., 2009). TAK1 can also simultaneously activate MAPKs ERK1, ERK2, p38 and JNK which leads to the activation of the transcription factor activated protein-1 (AP-1) (Yamaguchi et al., 1995; Holtmann et al., 2001).

1.3.4.1.2. VACV and the MyD88-dependent pathway

During *ex vivo* VACV infection of pDCs, MyD88 is crucial for production of type I IFN (Martinez et al., 2010) and for development of CD8+ T cell responses, implicating TLR signalling in the development of immunity to VACV. However, *MyD88*$^{-/-}$ DCs developed and primed CD8+ T cells normally in response to VACV WR, suggesting both a MyD88 dependency of a full immune response and a MyD88-independent mechanism of DC activation (Zhao et al., 2009).

MyD88 is the target of a VACV immunomodulator, A46. A46 is expressed early during the course of infection (Stack et al., 2005; Assarsson et al., 2008) and was found to bind to MyD88. Over-expression of A46 prevented MyD88-mediated activation of NF-κB. Since MyD88 is a common adaptor molecule for TLRs, the role of A46 in modulating activation of NF-kB by multiple TLRs was examined and A46 was found to have a pleiotropic effect by inhibiting TLRs 1, 2, 4, 5, 6, 7 and 9 as well as IL-1R activation of NF-κB (Stack et al., 2005).

In addition, TRAF6 and IRAK2 have been proposed as binding partners of another VACV protein, A52. A52 is a member of the poxvirus Bcl-2 like family (Graham et al., 2008) and is thought to be expressed early during the course of infection (Assarsson et al., 2008). A52 has evolved to inhibit NF-κB activation by interfering with the TLR pathway by disrupting the interactions between TAB1 and TRAF6 and between IRAK2 and TIRAP, leading to inhibition of signalling mediated by TLRs 1, 2, 3, 4, 5 and 6 (Harte et al., 2003).

1.3.4.1.3. Signalling pathways activated by TLRs: the TRIF-dependent pathway

The TRIF-dependent pathway is utilised by TLR3 and TLR4 and is the only pathway utilised by TLR3, illustrated in Figure 1.4. The activation of the TRIF-dependent pathway culminates in the activation of both NF-κB and IRF3. TRIF activates NF-κB by recruiting TRAF6 (Sato et al., 2003), however the activation of NF-κB by TRIF also involves the recruitment of the adaptor receptor interacting protein (RIP)-1, which itself interacts with TNFR-associated death domain protein (TRADD), leading to K63-linked polyubiquitination of RIP1 (Ermolaeva et al., 2008; Pobezinskaya et al., 2008). In addition, Pellino-1 is also recruited to the complex containing TRIF, TRAF6, RIP1 and TRADD, and loss of Pellino-1 leads to loss of TRIF-dependent activation of NF-κB (Chang et al., 2009). The result of this multiprotein complex is the activation of TAK1 which phosphorylates the IKK complex and p38 MAPKs leading to the nuclear translocation of NF-κB and AP-1.

The TRIF-dependent pathway leads to IRF3 nuclear translocation via the recruitment of TRAF3, which is required for the activation of the non-canonical IKKs IKKε and TBK-1 (Hacker et al., 2006; Oganesyan et al., 2006), which are required for the phosphorylation of IRF3 (Fitzgerald et al., 2003; Sharma et al., 2003; Hemmi et al., 2004; Perry et al., 2004).

1.3.4.1.4. TRIF-dependent pathway and VACV

Whilst TRIF deficiency did not appear to result in impaired CD8+ T cell responses to VACV (Zhao et al., 2009), a thorough study evaluating the role of TRIF in VACV infection has not yet been conducted. However, VACV protein A46 has been demonstrated to bind to TIRAP and TRAM which inhibits TRIF recruitment to TLR4 (Stack et al., 2005), perhaps suggesting a role for TRIF in VACV infection.

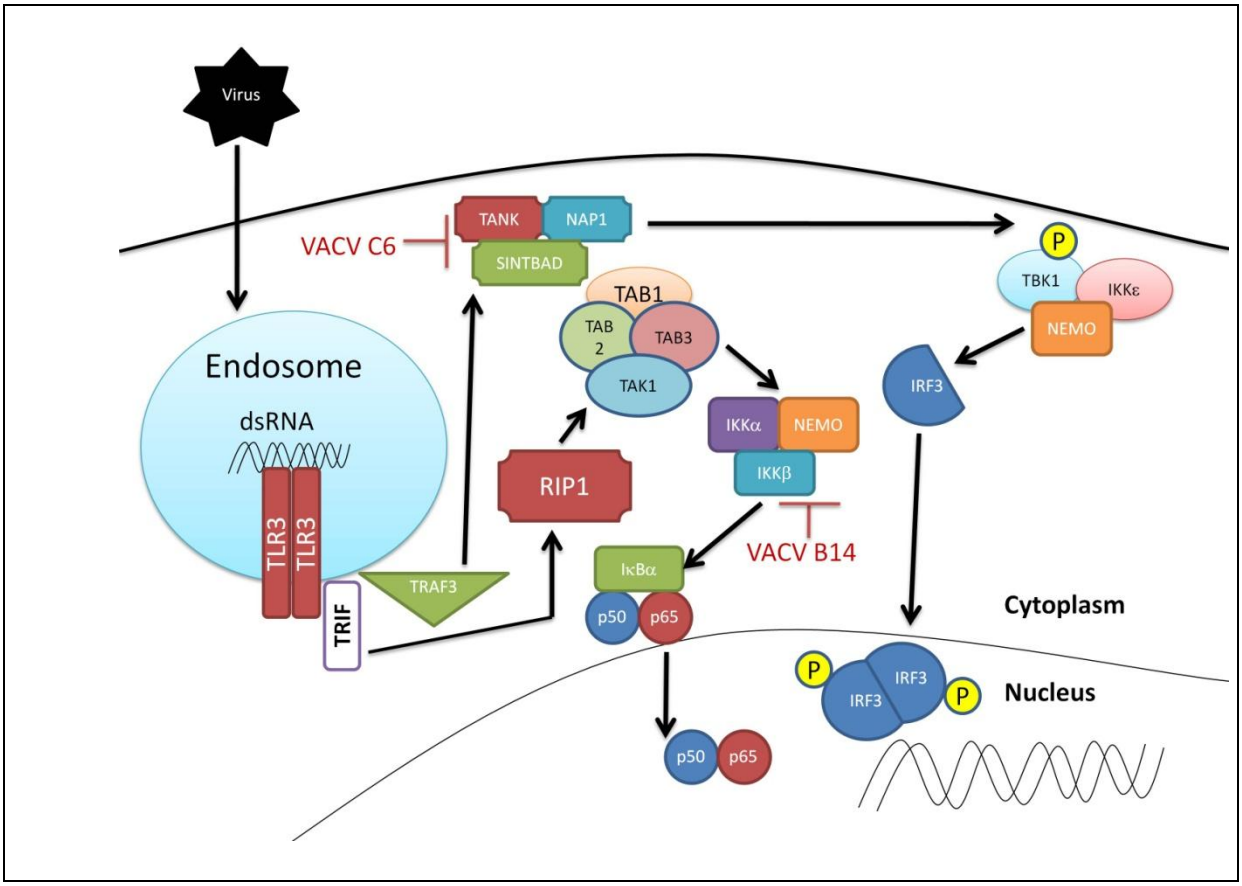

Figure 1.4. TRIF-dependent TLR pathway and its inhibition by VACV. An overview of TLR3 signalling leading to the activation of p50:p65 (NF-κB) and IRF3. After binding of dsRNA, or potentially another ligand, VACV induces TLR3 signalling via recruitment of TRIF leading to the activation of TRAF3 which activates the TANK/NAP1/SINTBAD complex which in turn activates the non-canonical IKK complex leading to the activation of IRF3. The TRIF-dependent pathway also leads to the activation of NF-κB via RIP1 which leads to the activation of the TAK1 complex resulting in nuclear translocation of NF-κB. VACV inhibition of this pathway is indicated in red.

1.3.4.1.5. Individual TLRs

TLR2

TLR2 has been shown to be important for activation of innate immunity in the context of VACV infection. TLR2 and MyD88 are required for the production of pro-inflammatory cytokines and control of VACV infection both *in vitro* (Zhu et al., 2007; Barbalat et al., 2009)

and *in vivo* (O'Gorman et al., 2011), although TLR-independent mechanisms are primarily responsible for the production of IFNβ (Zhu et al., 2007; O'Gorman et al., 2011).

Dendritic cells (DCs) deficient in TLR2 were unable to induce cytokines produced by WT DCs in response to VACV infection (Zhu et al., 2007). In a more recent study, intravenous infection with VACV induced production of IL-6 in a TLR2-dependent manner, which subsequently activated STAT3 signalling in DCs and T cells. However, ECTV infection did not elicit the production of this IL-6 (O'Gorman et al., 2011). The VACV ligand responsible for activating TLR2 is not known.

TLR3

TLR3 recognises dsRNA (Alexopoulou et al., 2001) and plays a key role in defence against many virus infections. Whilst TLR3 demonstrates quite a wide tissue distribution, particularly high levels are found on myeloid DCs (mDCs). The importance of TLR3 in protection against many viruses has been shown, including MCMV (Tabeta et al., 2004). However TLR3 is not required for effective protection against many other viruses such as LCMV, vesicular stomatitis virus (VSV) and reovirus (Edelmann et al., 2004). In the case of VACV, a *Tlr3*$^{-/-}$ genotype confers greater resistance to infection, and this is explained by TLR3 involvement in recruitment of inflammatory cells to sites of infection and induction of immunopathology. Although it is clear that TLR3 is activated in the course of VACV infection, the role of this activation remains to be studied in detail (Hutchens et al., 2008).

TLR2/6

TLR2/6 is reported to be important for innate immune sensing of MVA by BMDMs. BMDMs from TLR2, TLR6 and MyD88 null mice were unable to produce CXCL2 in response to MVA infection, but CXCL2 production was normal in cells lacking TRIF, TLR4

or TLR1. Type I IFN production by BMDMs infected with MVA, however, was not affected by the loss of TLR1, TLR2, TLR4, TLR6, MyD88 or TRIF but was dependent upon MDA-5 and mitochondrial antiviral signalling protein (MAVS) (Delaloye et al., 2009).

TLR 4

TLR4 inhibition by an 11 amino acid (aa) motif, termed viral inhibitory peptide of TLR4 (VIPER) found within the VACV protein A46 has suggested a role for TLR4 during the course of viral infection (Lysakova-Devine et al., 2010). VIPER inhibited TLR4 but did not interfere with other TLRs. The ligand responsible for the activation of TLR4 during VACV infection has not been identified.

TLR8

TLR8 has been implicated in the host response to VACV WR. The role of TLR8 in VACV infection was identified initially via an NF-κB luciferase reporter assay in human embryonic kidney (HEK) cells transfected with TLR7 or TLR8 and VACV DNA added to the supernatant. Small interfering (si) RNA-mediated knockdown of TLR8 in pDCs completely ablated type I IFN production in response to VACV and AT-rich DNA (Martinez et al., 2010).

TLR9

TLR9 recognises hypomethylated CpG motifs (Bauer et al., 2001) within endosomes and activates a MyD88-dependent signalling pathway (Barton et al., 2006). Data regarding the role of TLR9 in VACV infection are equivocal. One study has suggested a role for TLR9-

independent activation of pDCs in response to MVA infection, which also suggested a role for TLR independent activation of pDCs (Waibler et al., 2007) following 'mildly UV-inactivated MVA infection'. On the other hand, another study demonstrated that UV-inactivated MVA-mediated IFNα production by pDCs is entirely dependent on TLR9 (Samuelsson et al., 2008). However, both studies concluded that MVA encoded TLR9 antagonists, suggesting that TLR9 may be important for innate immune activation following VACV infection. A third study has shown a MyD88-dependent but TLR9-independent activation of pDCs in response to VACV WR (Martinez et al., 2010).

1.3.4.2. Soluble PRRs leading to detection of RNA and their downstream signalling pathways

Six receptors that detect intracellular RNA have been identified and these are of critical importance in the activation of the immune response to many viruses. The six receptors are PKR, OAS, RIG-I, MDA-5, laboratory of genetics and physiology 2 (LGP2) and nucleotide oligomerisation domain (NOD)-2 (Barbalat et al., 2011).

RIG-I was discovered using a cDNA screen to identify genes that enhanced the activation of the IFN-β promoter in response to poly (I:C) (Yoneyama et al., 2004) and was thus identified as a PRR responsible for the detection of viral RNA. RIG-I contains two CARDs and a central DEAD box helicase/ATPase domain essential for binding to RNA and IRF3 activation, as well as a C-terminal regulatory domain that prevents constitutive activation. RIG-I discriminates between self and non-self RNA by binding to uncapped 5'-triphosphate ssRNA (pppRNA), a motif commonly found during the course of infection with certain viruses, but not on host RNA (Hornung et al., 2006; Pichlmair et al., 2006). However, more recent studies have reported that small regions of base pairing may also be required for

activation of RIG-I dependent innate immune responses (Schlee et al., 2009; Schmidt et al., 2009). To complicate matters further, other studies have demonstrated that some species of dsRNA which lack pppRNA can also activate RIG-I (Kato et al., 2008; Takahasi et al., 2008).

After initial detection of viral RNA, a signal transduction pathway is activated resulting in the dimerisation and nuclear translocation of IRF-3 (Figure 1.5). The CARD domains of RIG-I and MDA-5 are the domains responsible for the interaction with an adaptor molecule termed MAVS/Cardif/VISA/IPS-1 (Kawai et al., 2005; Meylan et al., 2005; Seth et al., 2005; Xu et al., 2005), hereafter termed MAVS, required for the nuclear translocation of IRF-3. MAVS was found to interact with an E3 ubiquitin ligase, TRAF3, responsible for K63 polyubiquitin chain formation (Hacker et al., 2006; Oganesyan et al., 2006; Saha et al., 2006). TRAF3 null cells show severely impaired production of type I IFN induction after RNA virus infection. Subsequently, TRAF3 recruits and activates the non-canonical IKKs, TBK-1 and IKKε, which phosphorylate IRF-3 and IRF-7 leading to type I IFN induction.

In addition to IRF3, NF-κB is also activated in response to dsRNA (Visvanathan and Goodbourn, 1989) and this activation is mediated by TRAF6 recruitment to MAVS (Xu et al., 2005). In addition, RIP1 and Fas-associated death domain (FADD) are thought to be recruited to MAVS via TRADD, leading to the activation of NF-κB (Balachandran et al., 2004; Kumar et al., 2006; Michallet et al., 2008).

RIG-I binds to unanchored K63-linked ubiquitin chains via the CARD domain, and an inability to bind K63 linked ubiquitin abolishes the ability of RIG-I to respond to 5' triphosphate RNA (Zeng et al., 2010). Originally RIG-I was thought to require ubiquitination by TRIM25 on the K172 residue (Gack et al., 2007; Gack et al., 2008) and influenza virus NS1 inhibited this process (Gack et al., 2009). However, a more recent study suggests that the ubiquitination of the K172 residue is not required, and it is binding to unanchored

ubiquitin chains that is the more important factor for activation of RIG-I (Zeng et al., 2010). The role of ubiquitin binding versus ubiquitination in RIG-I signalling remains to be completely resolved.

MDA-5 was discovered originally as a ligand of paramyxovirus V proteins (Andrejeva et al., 2004) and has a domain structure similar to RIG-I: N-terminal CARD domains, a central DEAD box helicase/ATPase domain and a C-terminal regulatory region. MDA-5 functions in a similar way to RIG-I by binding viral RNA and activating downstream signalling pathways leading to the induction of IFN (Andrejeva et al., 2004; Yoneyama et al., 2005).

In contrast, LGP2 lacks the N-terminal CARD domains required for IRF3 activation and consists only of the central DEAD box helicase/ATPase domain and C-terminal regulatory domain, and was thought initially to be a negative regulator of the RIG-I/MDA5 pathways (Yoneyama et al., 2005). However, more recently it has been suggested to be a co-receptor for the other RLRs because generation of *LGP2*$^{-/-}$ mice showed that the absence of LGP2 leads to decreased type I IFN production in response to picornaviruses (Satoh et al., 2010). NOD2 also contains an N-terminal CARD domain, a central nucleotide binding domain and a C-terminal LRR domain and was identified as a cytosolic RNA sensor responsible for type I IFN production in response to ssRNA (Sabbah et al., 2009).

The different roles of MDA-5 and RIG-I have been studied extensively (Kato et al., 2006; Loo et al., 2008) and their different ligand specificities can lead to each receptor detecting distinct virus families. *Rig-I*$^{-/-}$ cells do not produce type I IFN in response to certain RNA viruses, including paramyxoviruses, VSV and influenza virus (Kato et al., 2005; Kato et al., 2006). Interestingly, MDA-5 was identified initially as an RNA sensor when it was characterised as a binding partner for the paramyxovirus PIV5 protein, and this viral protein

was shown to inhibit MDA-5-mediated IFN induction (Andrejeva et al., 2004). This perhaps explains why absence of MDA-5 does not significantly impair the response to paramyxovirus infection. In contrast, cells lacking MDA-5 are unable to respond to picornaviruses such as poliovirus and encephalomyocarditis virus (Gitlin et al., 2006; Kato et al., 2006). Several other viruses, including Newcastle disease virus and Sendai virus are thought to be detected by both RIG-I and MDA-5 (Yoneyama et al., 2005; Kato et al., 2006; Fredericksen et al., 2008).

MDA-5 and MAVS are reported to play an important role in the detection of poxviruses in macrophages since knockdown of either of these molecules by shRNA led to decreased type I IFN production after MVA infection of THP-1 cells and, importantly, RIG-I was not required for production of type I IFN in response to MVA infection of these cells (Delaloye et al., 2009).

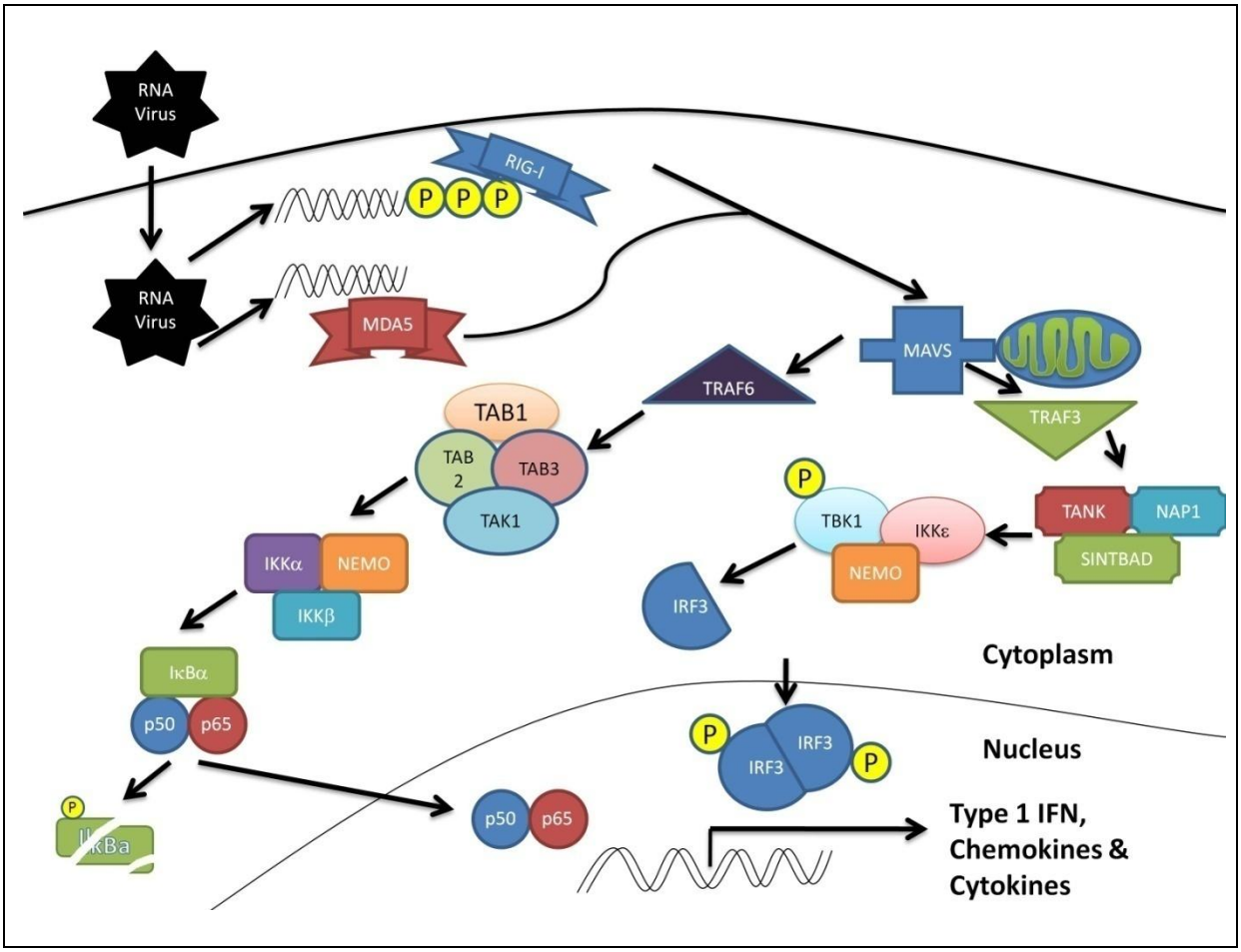

Figure 1.5. RNA sensing within host cells. This figure demonstrates the activation of NF-κB and IRF-3 after RNA sensing by the RLRs RIG-I and MDA-5. After binding either dsRNA (MDA-5) or pppRNA (RIG-I), MAVS becomes activated leading to the phosphorylation and dimerisation of IRF-3 via the non-canonical IKKs TBK-1 and IKKε. NF-κB is activated via TRAF6 which activates the TAK1 complex to phosphorylate IKKβ, which results in the phosphorylation and subsequent proteasomal degradation of IκBα, allowing for nuclear translocation of p50:p65 (NF-κB).

1.3.4.3. Soluble PRRs leading to the detection of DNA and downstream signalling pathways

Whilst the mechanisms governing RNA sensing in virus infection are relatively well known, the sensors and downstream signalling molecules involved in DNA sensing are less well characterised and have recently become the subject of intense study. DNA sensing in poxvirus infection is likely to be of fundamental importance in the activation of the innate

immune response since these viruses produce large quantities of cytoplasmic DNA and this presents a prime target for innate immune sensing.

In 2006, two independent papers described type I IFN induction by transfection of DNA (Ishii et al., 2006; Stetson and Medzhitov, 2006). Subsequently, a putative DNA sensor termed DNA-dependent activator of IFN (DAI) was proposed as the DNA sensor responsible for DNA mediated IRF3 activation (Takaoka et al., 2007). However, in further studies, the *in vivo* relevance of DAI was questioned and DAI was found to be dispensable for DNA-mediated activation of the innate immune system both *in vitro* and *in vivo* (Ishii et al., 2008; Wang et al., 2008) suggesting the existence of alternative DNA sensors.

Absent in melanoma 2 (AIM2) was identified as a DNA sensor capable of activating an inflammasome (Burckstummer et al., 2009; Fernandes-Alnemri et al., 2009; Hornung et al., 2009; Roberts et al., 2009) in response to DNA, and the *in vivo* relevance of this molecule was later demonstrated as mice deficient in AIM2 had impaired inflammasome activation in response to various pathogens such as *Francisella tularensis*, murine cytomegalovirus (MCMV) and VACV WR (Fernandes-Alnemri et al., 2010; Rathinam et al., 2010). However, the initial observations that DNA activated IRF3 was not been explained by AIM2 since AIM2 was dispensable for type I IFN production, indicating that additional DNA sensors were also present.

RNA polymerase III was later identified as a DNA sensor (Figure 1.6). RNA polymerase III senses AT-rich DNA and transcribes this into AU-rich RNA which, importantly, has a 5’ triphosphate moiety and is therefore a ligand for activating RIG-I (Ablasser et al., 2009; Chiu et al., 2009). This pathway was only shown to be important in HEK293 cells and its *in vivo* importance is yet to be established. Importantly, the activation of this pathway is highly dependent on the DNA species used, and interruption of the AT rich

sequence leads to an abrogation of type I IFN induction (Chiu et al., 2009). Whilst poly (dA:dT), a synthetic ds-B-DNA molecule was used extensively in one paper demonstrating an IRF3-dependent activation of innate immunity by DNA (Ishii et al., 2006). Another report demonstrated the same effect with ISD DNA (a synthetic dsDNA molecule 45-bp in length engineered to contain no CpG motifs), which did not contain significant tracts of AT- rich DNA and therefore was incapable of activating RNA polymerase III (Stetson and Medzhitov, 2006).

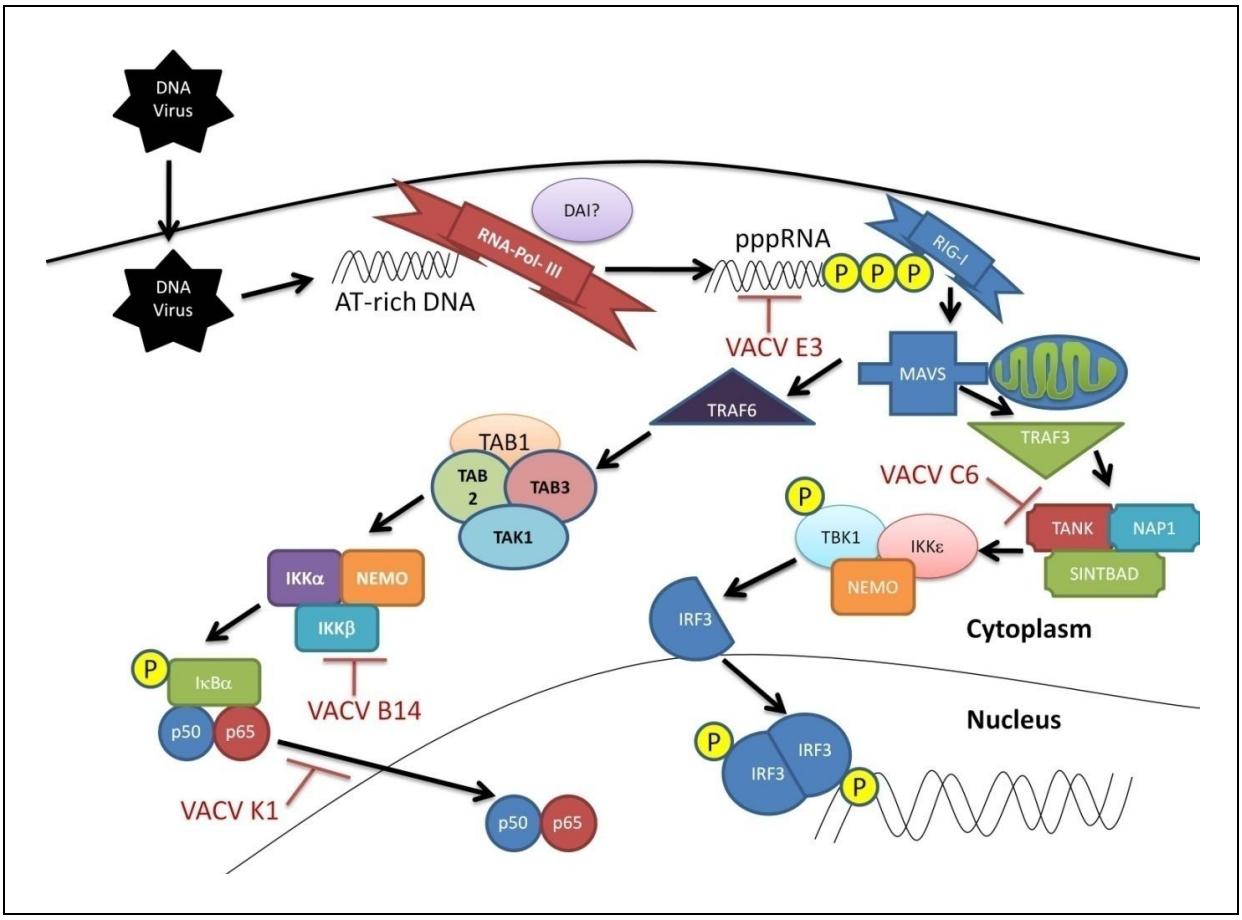

Figure 1.6. DNA sensing within host cells via the RNA Polymerase III pathway. This figure demonstrates the activation of NF-κB and IRF-3 after AT-rich DNA sensing by the RLRs RIG-I and MDA-5 after transcription by RNA-polymerase III. After binding either pppRNA (RIG-I), MAVS becomes activated leading to the phosphorylation and dimerisation of IRF-3 via the non-canonical IKKs TBK-1 and IKKε. NF-κB is activated via TRAF6 which activates the TAK1 complex to phosphorylate IKKβ, which results in the phosphorylation and subsequent proteasomal degradation of IκBα, allowing for nuclear translocation of p50:p65 (NF-κB).

Later, leucine rich repeat flightless interacting protein 1 (LRRFIP1) was characterised as a nucleic acid sensor active within macrophages capable of activating the β-catenin accessory pathway involved in the upregulation of type I IFN (Yang et al., 2010). DNA from *Listeria monocytogenes* and RNA from VSV both appeared to stimulate the LRRFIP1 mediated β-catenin accessory pathway.

Recently, a novel DNA sensor, IFI16, with similarity to AIM2 was shown to bind DNA and activate IRF-3 in response to DNA. This study involved principally the THP-1 macrophage cell line, however knockdown of IFI16 in MEFs also showed a partial dependency on this molecule for DNA sensing (Unterholzner et al., 2010). A subsequent study has implicated IFI16 in the activation of the inflammasome in response to Kaposi's sarcoma herpesvirus (KSHV) (Kerur et al., 2011). Interestingly, a nuclear-cytoplasmic divide between AIM2 and IFI16 appears to exist, with IFI16 responsible for detection of herpes simplex virus (HSV)-1 and KSHV in the nucleus, whereas AIM2 mediated innate immunity responds principally to pathogens in the cytoplasm (Rathinam et al., 2010). DNA sensing by AIM2 and IFI16 is summarised in Figure 1.7.

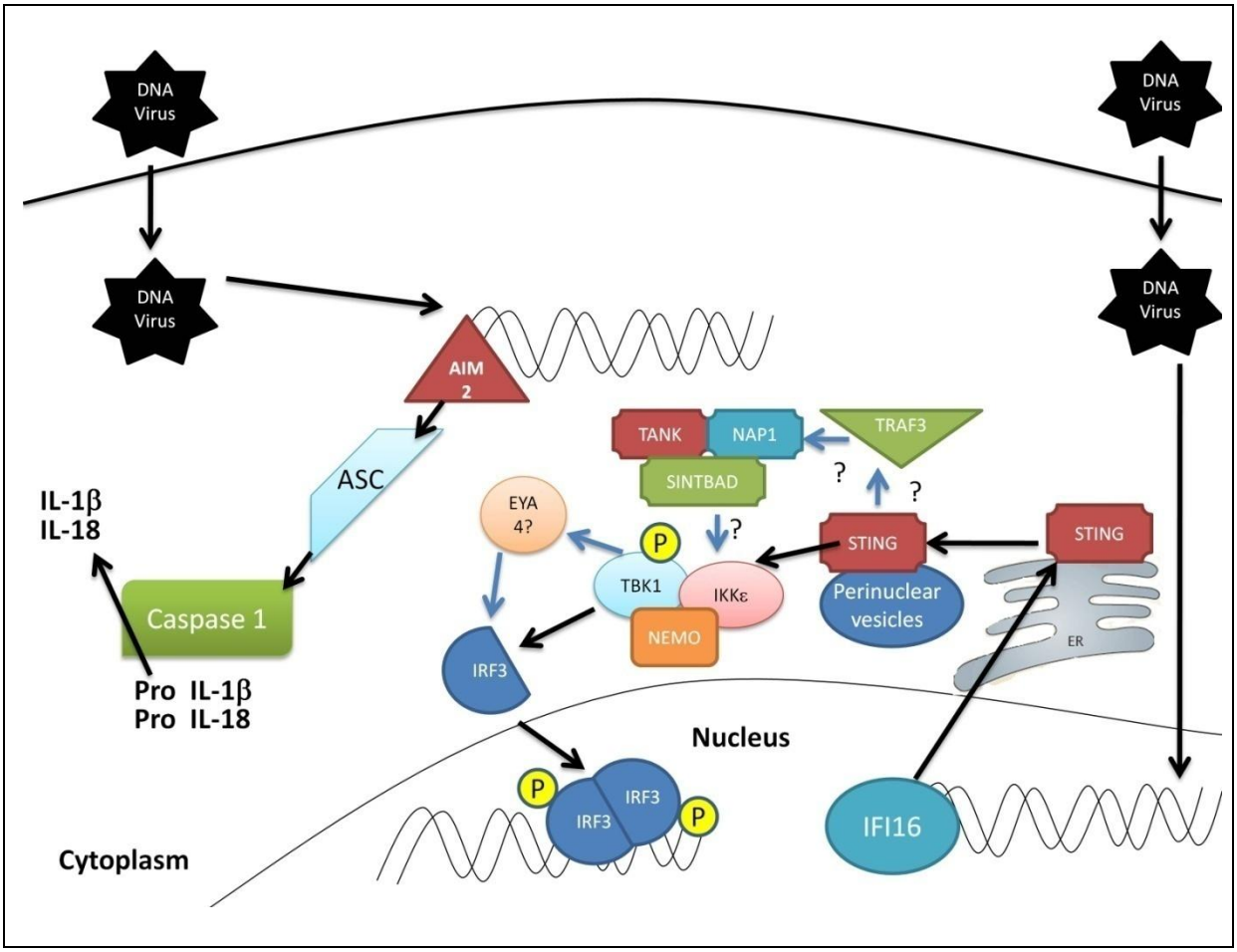

Figure 1.7. DNA sensing within macrophages. This figure demonstrates the activation of IRF-3 and the AIM2 inflammasome via DNA sensing by IFI16 and AIM2, respectively. It is not known with certainty where IFI16 binds DNA, but this is represented in the cytoplasm for the purposes of this diagram. IFI16 binds to DNA and activates a signalling pathway consisting of stimulator of IFN genes (STING), TBK-1 and IRF-3. Additional steps potentially involved in the DNA sensing pathway are indicated via red arrows and include TRAF3, the TANK/NAP1/SINTBAD complex and EYA4 which have not been shown definitively to be involved in DNA sensing as yet. AIM2 activates caspase-1 to convert pro-IL-1β and pro-IL-18 by binding to DNA and recruiting the inflammasome adaptor molecule ASC.

Asp-Glu-Ala-His (DEAH) box helicase (DHX)-9 and DHX36 appear to cooperate in pDCs to mediate upregulation of type I IFNs in response to CpG and HSV-1 DNA (Kim et al., 2010). These helicases act by recruitment of MyD88 and possibly IKKα–mediated

phosphorylation of IRF-7 and they represent an example of cell-type specific mechanisms beginning to typify this field of research (Table 1.1).

After detection of DNA by the various DNA sensors, a signalling pathway involving tri-partite motif (TRIM)-56 (Tsuchida et al., 2010), Atg9a (Saitoh et al., 2009), Eyes absent (EYA)-4 (Okabe et al., 2009), STING (Ishikawa et al., 2009), TBK-1 (Ishii et al., 2008) and IRF-3 (Ishii et al., 2006; Stetson and Medzhitov, 2006) is activated.

STING (also known as ERIS/MITA) is essential for induction of cytokines and chemokines after stimulation of cells with intracellular DNA. Independently, two groups have reported that following stimulation with DNA, STING undergoes a translocation to distinct sub-cellular punctuate perinuclear structures (Ishikawa et al., 2009; Saitoh et al., 2009). Furthermore, addition of an ER retention signal to STING decreases type I IFN production, suggesting the translocation is of functional significance for induction of DNA-mediated innate immune signalling (Saitoh et al., 2009). However, why the translocation of these molecules to distinct cellular compartments is of importance is not understood.

STING is also subject to regulation by TRIM56 and Atg9a. TRIM56 is an E3 ubiquitin ligase responsible for K63-linked polyubiquitination of STING, causing STING to dimerise, a prerequisite for recruitment of TBK-1 (Tsuchida et al., 2010), adding another example of the role of ubiquitin in innate immune signalling. Atg9a is a component of the autophagy machinery and negatively regulates DNA-mediated activation of IRF-3 by inhibiting the translocation of STING from the ER to the punctuate perinuclear p62/sequestosome 1 structures (Saitoh et al., 2009).

EYA4 is an enigmatic protein involved in innate immune responses to both DNA and RNA. EYA4 is a phosphatase with unknown substrates in the DNA/RNA-mediated

pathways, however overexpression of this molecule leads to upregulation of various genes including type I IFN and other cytokines and chemokines (Okabe et al., 2009).

DNA sensing has become the focus of intense study in recent years, yet there is much not yet known. An emerging theme is the segregation of different DNA sensing mechanisms occurring in different cell types. The information regarding the current state of knowledge is summarised in Table 1.1.

Component	**Responds to**	**Cell types role found in**	***In vivo* role**	**References**
DAI	Poly (dA:dT), not ISD dsDNA	L929 cells (not detectable prior to IFN stimulation)	Dispensable for IFN production in response to vaccinia and HSV-1.	(Takaoka et al., 2007; Ishii et al., 2008; Wang et al., 2008)
AIM2	Cytoplasmic, not nuclear, pathogens and dsDNA	Macrophages	Important for inflammasome activation to cytoplasmic pathogens (Francisella tularensis and VACV)	(Burckstummer et al., 2009; Fernandes-Alnemri et al., 2009; Hornung et al., 2009; Roberts et al., 2009; Fernandes-Alnemri et al., 2010; Rathinam et al., 2010)
IFI16	Nuclear pathogens (HSV, KSHV) and dsDNA	THP-1 cells, MEFs	Not determined	(Unterholzner et al., 2010; Kerur et al., 2011)
LRRFIP1	dsDNA and dsRNA	Macrophages	Not determined	(Yang et al., 2010)
STING	All DNA tested, HSV-1, VACV, Listeria	Multiple cell types	Important for cytokine and chemokine production in response to various DNA pathogens (VACV, HSV-1, Listeria)	(Ishikawa and Barber, 2008; Ishikawa et al., 2009; Saitoh et al., 2009; Tsuchida et al., 2010)
RNA-Polymerase III	Poly (dA:dT), not ISD DNA	HEK293T	Not determined	(Ablasser et al., 2009; Chiu et al., 2009)
DHX9	CpG and HSV-1 DNA	pDC	Not determined	(Kim et al., 2010)
TBK-1	dsRNA, dsDNA, multiple pathogens	Multiple cell types	Important for cytokine and chemokine production in response to various pathogens	(Ishii et al., 2008)

Table 1.1. DNA sensors of the innate immune system.

Various DNA sensors have been reported as being active in different cell types, and also are responsible for the detection of different DNA species. The *in vivo* role of many components of DNA sensing pathways described so far is not determined and therefore the relative contributions of these sensors to antiviral immunity is of interest.

DNA sensing is clearly of utmost importance in the context of VACV infection because VACV is a dsDNA virus. Not much is understood about the various interactions of VACV with the DNA sensors as these molecules have only been described recently, however AIM2 has been shown to be important for VACV-mediated inflammasome activation (Rathinam et al., 2010).

1.4. VACV C16

VACV C16 is a 37-kDa molecule encoded by the *C16L* gene which, in the VACV WR strain, is located within the ITRs and is therefore diploid. Analysis of *C16L* mRNA (Assarsson et al., 2008) and C16 protein expression (Fahy et al., 2008) each showed that C16 is produced early in the course of infection. VACV C16 was initially studied as a candidate immunoregulatory protein after the identification of a C-terminal six amino acid motif highly conserved with the IL-1 receptor antagonist, IL-1Ra (Kluczyk et al., 2002). This short amino acid sequence was critical for the activity of IL-1Ra to block IL-1R signalling and a mechanism of action of C16 was proposed based on this similarity. However, subsequently C16 was shown to be an intracellular protein located in the nucleus during transfection and in both the nucleus and cytoplasm during infection (Fahy et al., 2008), thus the primary mechanism of action of C16 was presumably not mediated by the IL-1Ra-like motif. Intranasal infection of mice with virus lacking both copies of the *C16L* gene lead to less

weight loss, decreased signs of illness, enhanced recruitment of leukocytes to lungs and increased virus clearance compared with wild-type and revertant viruses (Fahy et al, 2008).

Thus, C16 was known to be an intracellular protein made early during infection that contributes to virulence by an unknown mechanism. This project was to understand the mechanism of action of the C16 protein and how it contributes to virus virulence.

Chapter 2: Methods & Materials

2.1. Manipulation of nucleic acids

2.1.1. Polymerase chain reaction (PCR)

Amplification of DNA fragments was achieved using PCR. HiFi Taq (Invitrogen) and GoTaq (Promega) polymerases were used for molecular cloning and colony screening, respectively, according to the manufacturer's instructions. Briefly, reaction mixes consisted of 100 ng template DNA, 200 pM forward primer, 200 pM reverse primer, 0.25 μl polymerase enzyme, 800 μM deoxynucleotide triphosphates (dNTPs- Invitrogen), 2 mM $MgCl_2$, and either 10 x HiFi Taq (Invitrogen)- or 10 μl GoTaq Flexi (Promega) - buffers with deionised distilled H_2O (ddH_2O) making the volume up to 50 μl.

Reactions were performed in a programmable thermocycler. Template DNA was melted at 95 °C for 5 min and then a cycle consisting of melting at 95 °C for 30 s, annealing at 50 °C for 30 s and extension at 72 °C (HiFi Taq) or 68 °C (GoTaq) for 2 min was repeated a further 30 times.

2.1.2. Resolution of DNA fragments by agarose gel electrophoresis

DNA was resolved on 1-2 % agarose gels in Tris-acetate ethylene diamine tetraacetic acid (EDTA) (TAE) [40 mM Trisma, 40 mM sodium acetate, 1 M EDTA pH 8.0] buffer. Gels contained SYBR® Safe (Invitrogen) at a concentration of approximately 5 μl per 50 ml gel and were run at 80-120 V for between 30 and 100 min in TAE. Samples were mixed with 20 % volume per volume (v/v) DNA loading buffer (Bioline), which contained glycerol and

bromophenol blue dye. DNA was visualised using a transilluminator and was photographed using the Chemi-Doc gel documentation systems (Bio-Rad) with Quantity One software (Bio-Rad). DNA length was assessed using dsDNA hyperladder markers (Bioline).

2.1.3. Recovery of DNA from agarose gels

DNA bands were visualised using a SYBR® Safe imager (Invitrogen) imager and excised using a clean scalpel. The QIAquick gel extraction kit (Qiagen) was used according to the manufacturer's protocol. Briefly, the agarose gel was dissolved in a high-salt concentration buffer, allowing binding of the DNA to a silica membrane in a microcentrifuge column. DNA was eluted using between 30 and 100 µl deionised distilled H_2O.

2.1.4. Quantification of DNA

DNA concentration and purity was measured using a NanoDrop ND-1000 spectrophotometer according to the manufacturer's instructions using the OD_{260} reading. DNA purity was assessed using the OD_{260}/OD_{280} ratio, with a ratio between 1.8 and 2.0 being ideal.

2.1.5. Restriction endonuclease digestion of DNA

DNA was digested with restriction endonuclease enzymes in volumes of 20-50 µl containing at least 1 unit (U) of enzyme per µg of DNA. The enzymes and buffers were supplied by Roche and New England Biolabs. DNA was digested for at least 1 h at 37 °C and purified from agarose gels after electrophoresis.

2.1.6. Ligation of DNA fragments

Sticky-end ligations were carried out in a volume of 20 µl containing vector DNA and insert DNA in a 1:3 molar ratio combined with 1 U of T4 DNA-ligase (Roche) in 1 x T4 DNA ligase buffer (Roche) and incubated for 1 h at 18 °C followed by cooling to 4 °C until transformation.

2.1.7. Transformation of competent *E. coli* with plasmid DNA

Chemically competent XL-10 cells (30 µl) were thawed on ice and between 1 and 20 µl of a ligation reaction mixture containing DNA was added. Cells and DNA were incubated together on ice for 30 min and subsequently were heat shocked at 42 °C for 45 sec and then incubated on ice for a further 2 min. Three hundred microlitres of Super Optimal Catabolite (SOC) medium were added per reaction and the mix was then incubated at 37 °C for 1 h and subsequently spread onto agar plates containing the appropriate antibiotic. Plates were then dried, inverted and incubated at 37 °C for 16 h.

2.1.8. Screening of colonies by colony PCR

Colony PCR was performed by picking a colony with a pipette tip and re-suspending the bacteria in 20 µl H_2O. Then 15 µl was added to Luria broth (LB) containing the appropriate antibiotic, and the remaining 5 µl was used as template DNA for PCR using sequencing primers specific to the vector sequence. The presence of the correct length insert was determined by agarose gel electrophoresis with appropriate size markers.

2.1.9. Preparation of plasmid DNA (miniprep & maxiprep)

Plasmid DNA was purified by the QIAGEN miniprep or maxiprep method according to the manufacturer's instructions. Minipreps were prepared from 5 ml chemically competent XL-10 *E. coli* cultures and were used for screening plasmids. Maxipreps were prepared from 500 ml cultures and were used in assays described in the results chapters.

Below is a table detailing the plasmids used in this study.

Name of plasmid	Description
pcDNA4/TO-C16TAP	pcDNA4/TO (Invitrogen) based with C16 inserted using *BamHI* and *NotI* restriction sites. The TAP-tag sequence is as follows: AAAWSHPQFEKGGGSDYKDDDDKDYKDDDDK. Ampicillin resistant.
pcDNA4/TO-coC16	pcDNA4/TO (Invitrogen) based with codon optimised C16 inserted using *BamHI* and *NotI* restriction sites. Codon optimisation was performed by GenScript. Ampicillin resistant.
pcDNA4/TO-icIL-1RaTAP	pcDNA4/TO (Invitrogen) based with icIL-1Ra inserted using *BamHI* and *NotI* restriction sites. The TAP-tag sequence is as follows: AAAWSHPQFEKGGGSDYKDDDDKDYKDDDDK. Ampicillin resistant.
HRE-Luciferase	pGL2 (Promega) based with three tandem repeats of HIF-response element (HRE) sequence (5' TGTCACGTCCTGCACGACTCTAGT) upstream of firefly luciferase. Ampicillin resistant. Obtained from Dr. Eoin Cummins, University College Dublin
TK-Renilla	pRL-TK (Promega) based. Renilla luciferase is constitutively expressed, used for transfection control. Ampicillin resistant. Obtained from Dr. Andrew Bowie, Trinity College Dublin.

Table 2.1. Brief descriptions of plasmids used in this study.

2.1.10. Concatamerisation of oligonucleotides

Complementary oligonucleotides were mixed in a 1:1 molar ratio, heated to 80 °C for 30 sec and annealed at 60 °C for 15 min followed by cooling at room temperature (RT). A 1 mg/ml solution of annealed oligonucleotides was added to an appropriate volume of 10 x T4

DNA ligase buffer (Roche) with 3 U of polynucleotide kinase (New England Biolabs) per 100 μg DNA and incubated at 37 °C for 2 h and subsequently left to cool at room RT. Three units of T4 DNA ligase were then added per 100 μg of DNA and left to incubate at RT overnight. Successful concatamerisation was assessed using agarose gel electrophoresis. Concatamerised oligonucleotides were then purified using phenol-chloroform extraction and ethanol precipitation (see sections 2.1.9 and 2.1.10, respectively). The forward (sense) sequence used for stimulations in this study was as follows: 5'TACAGATCTACTAGTGATCTATGACTGATCTGTACATGATCTACA 3'. This sequence was annealed to a complementary DNA molecule.

2.1.11. Phenol-chloroform extraction of DNA

An equal volume of phenol:chloroform:isoamyl alcohol at a ratio of 25:24:1 (Fluka) was added to a solution of DNA and mixed thoroughly to form an emulsion. The mixture was then centrifuged at 13,000 rpm in a microcentrifuge for 1 min and the aqueous (upper) phase was transferred to a fresh tube. This was repeated until no remaining protein was visible at the interface of the organic and aqueous phase. Once the protein had been removed, an equal volume of chloroform: isoamyl alcohol at a ratio of 24:1 was added, and then mixed to form the emulsion. The mixture was then centrifuged at 13,000 rpm once more and the aqueous phase was transferred to a fresh tube. Ammonium acetate solution was then added to a final concentration of 2.5 M.

2.1.12. Ethanol precipitation of DNA

Two volumes of ice-cold ethanol were added to the solution of DNA and the ethanolic solution was stored at -20 °C for 30 min. The DNA precipitate was then collected by centrifugation at 13,000 rpm in a microcentrifuge for 10 min. The supernatant was then removed and the pellet was washed with 70% ethanol and re-centrifuged for a further 2 min at 4 °C. The supernatant was removed once again and the ethanol wash step was repeated. Lastly, the supernatant was removed and the tube was placed in a well ventilated area for 30 min in so that the remaining ethanol would evaporate. The DNA was then dissolved in ddH_2O.

2.1.13. Extraction of cellular RNA

The RNeasy kit (Qiagen) was used for extraction of cellular RNA according to the manufacturer's instructions. RNA was extracted from a minimum of 3×10^5 cells in tissue culture and also from homogenised frozen tissue. Precautions such as the use of commercial purified water (Sigma-Aldrich) and RNase free filtered pipette tips were taken to avoid RNase contamination.

2.1.14. Generation of cDNA from RNA

RNA (500 ng) was diluted into 11 µl of RNase free H_2O and mixed with 1 µl of 50 µM oligo dT (Qiagen) and 1 µl 40 mM dNTP mix and heated to 65 °C for 5 min. Next, a mix consisting of 4 µl 5 x First Strand cDNA synthesis buffer (Invitrogen), 1 µl 0.1 M DTT, 200 units RNaseOUT (Invitrogen) and 200 units Superscript Reverse Transcriptase (Invitrogen) was added to each tube, giving a total volume of 20 µl. The reaction mix was then incubated

at 50 °C for 1 h and denatured at 70 °C for 15 min. cDNA was subsequently diluted 1:5 with RNase free H_2O and analysed by Real Time-PCR.

2.1.15. Real Time PCR analysis of cDNA

Quantitative Real Time PCR (qRT-PCR) was performed on a 7900HT series qPCR machine (Applied Biosystems). Briefly, reactions were carried out on a 96-well format with a 20 µl reaction volume. Each reaction consisted of 10 µl Fast SYBR® Green Master Mix (Applied Biosystems), 3 µl H_2O, 5 µl template cDNA and 1 µl each of specific forward and reverse primers (10 µM). The reaction conditions were a 95 °C activation step for 20 s, followed by cycling of denaturing at 95 °C for 1 s and annealing/extension at 60 °C for 20 s. A total of 40 cycles in total were performed. Primers used are listed in Table 2.2.

Gene name	**Species**	**Sequence (5'-3')**
Cxcl10	*Mus musculus*	Forward: ACTGCATCCATATCGATGAC Reverse: TTCATCGTGGCAATGATCTC
Ccl5	*Mus musculus*	Forward: ACGTCAAGGAGTATTTCTACAC Reverse: GATGTATTCTTGAACCCACT
Ccl4	*Mus musculus*	Forward: GCCCTCTCTCTCCTCTTGCT Reverse: CTGGTCTCATAGTAATCCATC
Cxcl2	*Mus musculus*	Forward: GAGCTTGAGTGTGACGCCCCC Reverse: GTTAGCCTTGCCTTTGTTCAG
Ifnb	*Mus musculus*	Forward: CATCAACTATAAGCAGCTCCA Reverse: TTCAAGTGGAGAGCAGTTGAG
Isg54	*Mus musculus*	Forward: ATGAAGACGGTGCTGAATACTAGTGA Reverse: TGGTGAGGGCTTTCTTTTTCC
Nfkbia (IκBα)	*Mus musculus*	Forward: CTGCAGGCCACCAACTACAA Reverse: CAGCACCCAAAGTCACCAAGT
Xrcc6 (Ku70)	*Mus musculus*	Forward: GACAACAGGGGAAGAAGCAC Reverse: GGACAGAACTCGCTTTTTGG
Xrcc5 (Ku80)	*Mus musculus*	Forward: CAGCTGTTGTGCTGTGTGTG Reverse: GCTGCTGAGGTCAGTGAACA
Prkdc (DNA-PKcs)	*Mus musculus*	Forward: TCGGAGGAGGTTCATACAGG Reverse: CATCATGGCCTCGAATAACA

Il6	*Mus musculus*	Forward: GTAGCTATGGTACTCCAGAAGAC Reverse: GTAGCTATGGTACTCCAGAAGAC
Hprt	*Mus musculus*	Forward: GTTGGATACAGGCCAGACTTTGTTG Reverse: GATTCAACTTGCGCTCATCTTAGGC
MVA quantification	N/A (MVA)	Forward: TCGCCCTAATATGGTTACCG Reverse: CCTAAGCGGTGTTCTTCTGC
VEGF	*Homo sapiens*	Forward: CTTGCCTTGCTGCTCTACCT Reverse: CTGCATGGTGATGTTGGACT
GLUT1	*Homo sapiens*	Forward: TGGCATGGCGGGTTGT Reverse: CCAGGGTAGCTGCTCCAGC
HIF1A	*Homo sapiens*	Forward: CACCTCTGGACTTGCCTTTC Reverse: GGCTGCATCTCGAGACTTTT
18SRNA	*Homo sapiens*	Forward: GTAACCCGTTGAACCCCA Reverse: CCATCCAATCGGTAGTAGCG

Table 2.2. Oligonucleotides used for qRT-PCR

Expression levels were quantified by calculating ΔΔCt values relative to the housekeeping gene hypoxanthine-guanine phosphoribosyltransferase (Hprt), where

$$\Delta\Delta Ct = \Delta Ct_{\text{sample}} - \Delta Ct_{\text{calibrator}}$$

Data were analysed using the software RQ Manager (Applied Biosystems).

2.1.16. Quantification of MVA DNA by qRT-PCR

Murine embryonic fibroblast (MEF) cells were infected with MVA at 2 plaque forming units (p.f.u.) /cell and incubated for different periods of time. The cells were washed three times with phosphate-buffered saline (PBS) and frozen in HiFi Taq-buffer and 50 μg/ml proteinase K. Lysates were then homogenized by pippetting and incubated at 50 °C for 20 min and then heated to 80 °C for a further 20 min. qRT-PCR was performed using the primers specific for MVA that are listed in table 2.2. MVA DNA extracted from dilutions of sucrose-purified and titrated MVA were used as standards for absolute quantification using SDS 2.3 software (Applied Biosystems).

2.2. Protein analysis

2.2.1. Quantification of protein by BCA assay

Prior to SDS-PAGE, protein concentration was often assessed to ensure equal loading of gel lanes. The BCA assay kit (Pierce) was used according to the manufacturer's instructions. Briefly, the assay involves incubation of 5 µl of protein sample with a buffer containing Cu^{2+}, which is reduced to Cu^{+} in an alkaline buffer in the presence of protein (The Biuret reaction). Calorimetric detection of Cu^{+} with bicinchoninic acid is performed by measuring 562 nm absorbance.

2.2.2. Resolution of proteins by SDS-PAGE

Protein gels were assembled in vertical Mini-PROTEAN II apparatus (Bio-Rad) and consisted of approximately 1 ml of stacking gel atop 4 ml of 15 % polyacrylamide resolving gel. Stacking gels were composed of 5 % acrylamide solution (ProtoGel™, National Diagnostics), 125 mM Tris-HCl (pH 6.8) and 0.1 % SDS polymerised with 0.15 % ammonium persulphate (APS) and 0.001% tetramethylethylenediamene (TEMED) (both Sigma-Aldrich). Resolving gels consisted of 10-15 % polyacrylamide solution, 375 mM Tris-HCl (pH.8.8) and 0.1 % SDS polymerised with 0.15 % APS and 0.001 % TEMED.

Protein samples were mixed with 20 % (v/v) of 5 x Laemmli sample loading buffer (300 mM Tris-HCl (pH 6.8), 12 % SDS, 60 % glycerol and 0.6 % bromophenol blue with 10 % beta-mercaptoethanol (βME). Subsequently, the samples were heated at 90 °C for 5 min and loaded into the wells of the gel after 3 min cooling at RT. The samples were then electrophoresed alongside pre-stained molecular mass markers (Bio-Rad) in a Tris/Glycine

buffer (25 mM Tris HCl, 250 mM glycine, 0.1 % SDS). Samples were electrophoresed at 180 V for 1 h and processed subsequently for Coomassie staining or immunoblotting.

2.2.3. Detection of proteins by Coomassie staining

Acrylamide gels were stained for 30 min in Coomassie blue stain [0.25 % (w/v) Coomassie brilliant blue R-250 [BDH], 10 % (v/v) acetic acid, 45 % (v/v) methanol) and de-stained in de-stain buffer (10 % acetic acid (v/v), 20 % methanol (v/v)]. Images were obtained using the Chemi Doc gel documentation systems (Bio-Rad) with Quantity One software (Bio-Rad).

2.2.4.1. Detection of proteins by immunoblotting

Proteins that had been resolved by SDS-PAGE were transferred to a nitrocellulose membrane (Hybond ECL, Amersham) by electro-transfer in transfer buffer (25 mM Tris-HCl, 190 mM glycine, 0.037 % SDS (w/v) and 20 % methanol) at 300 mA for 30 min using a mini-electrophoretic trans-blot apparatus (BioRad) according to the manufacturer's instructions. Membranes were then blocked in 5 % non-fat dried milk in PBS with 0.05 % Tween-20 (PBS-T) for one h at RT.

Primary antibodies were also diluted in 5 % non-fat dried milk in PBS-T and incubated with the membranes for either 1 h at RT or alternatively at 4 °C overnight with gentle rocking. After incubation with primary antibody, the membrane was rinsed 3 times with PBS-T, 0.05 %, each time for 5 min before placing the membrane into secondary antibody diluted in 5 % milk in PBS-T 0.05 %. Secondary antibodies were either anti-goat (Dako), anti-rabbit or anti-mouse (both Sigma-Aldrich) horseradish peroxidise (HRP) –

conjugates. The membrane was incubated with secondary antibody for 1 h at RT and was rinsed 3 times with PBS-T, 0.05 %. Blots were developed using the Western Pico-Chemiluminescence kit (Thermo-Fisher) according to manufacturer's instructions. Finally, the membrane was wrapped in transparent plastic and exposed to film (X-OMAT/LS, Kodak) for an appropriate amount of time between 5 sec and 30 min.

Antigen	Supplier	Species	Dilution used
C16*	Harlan Sera-Lab	Rabbit	1 : 1000
Ku70	AbCam	Mouse	1 : 1000
Ku80	Santa-Cruz	Mouse	1 : 1000
DNA-PKcs	NeoMarkers	Mouse	1 : 1000
Tubulin	Millipore	Mouse	1 : 10000
PHD2	Millipore	Rabbit	1 : 1000
HIF-1α	BD Biosciences	Mouse	1 : 250
FLAG	Sigma-Aldrich	Mouse	1 : 1000
Mouse IgG (HRP)	Sigma-Aldrich	Goat	1 : 5000
Rabbit IgG (HRP)	Sigma-Aldrich	Mouse	1 : 10000
Mouse IgG (Licor)	Licor Biotechnology	Goat	1 : 10000
Rabbit IgG (Licor)	Licor Biotechnology	Goat	1 : 10000

Table 2.3. Details of antibodies used for immunoblotting. * Polyclonal C16 antibody was generated by Harlan Sera-Lab after production of recombinant C16 within our laboratory and described previously (Fahy et al, 2008).

2.2.4.2. Detection of proteins by immunoblotting & Licor development

Detection of proteins by immunoblotting was, where mentioned, carried out with the Licor development system. The protocol used was identical to section 2.2.4.1 up to the point

of application of secondary antibody, where instead a fluorophore-conjugated secondary antibody was diluted in milk and incubated with the membranes for 1 h in darkness. The membrane was washed 3 times in PBS-T, followed by one wash in PBS and allowed to dry at RT for 1 h. Images were obtained using a Licor Odyssey scanner and band intensity was analysed using Odyssey software (Licor Biotechnology). Licor western blotting was used when accurate quantification was required, such as when the protein in question was of a high abundance (e.g. Ku70 or DNA-PKcs)

2.2.5. Detection of proteins by immunofluorescent staining

Cells were grown on 13-mm sterilized glass coverslips (borosilicate glass, BDH) in 24-well plates. After infection or mock infection, the medium was removed and replaced with medium containing 4% paraformaldehyde (PFA) for 5 min. Cells were then washed 3 times in PBS and permeabilised in 0.1 % (v/v) Triton X-100 (Sigma-Aldrich) in PBS for 5 min. Cells were then blocked for 1 h at RT in 5 % milk in PBS-T and incubated with primary antibody in 1 % milk for 1 h at RT. The sample was then washed 3 times in PBS and stained with secondary antibody for 30 min at RT in darkness. Cells were then washed 3 times in PBS and once in ddH_2O and mounted in Mowiol-4',6-diamidino-2-phenylindole (DAPI) mounting medium. Samples were examined with a Zeiss Metaconfocal microscope using Zeiss LSM 5 software. Antibodies used for immunofluorescence are summarised in Table 2.4.

Antigen	Supplier	Species	Dilution used
Ku70	AbCam	Mouse	1 : 500
Ku80	Santa Cruz	Mouse	1 : 500
DNA-PKcs	NeoMarkers	Mouse	1 : 500
TBK-1	Millipore	Rabbit	1 : 500
Mouse IgG	Invitrogen	Goat	1 : 1000
Rabbit IgG	Invitrogen	Goat	1 : 1000

Table 2.4. Details of antibodies used for immunofluorescence.

2.2.6. Immunoprecipitation analysis

Protein G Sepharose™ 4 Fast Flow (GE Healthcare) beads (50 μl per sample) were washed four times by re-suspension in tandem-affinity protein purification (TAP)-wash buffer (0.1 % NP40 in PBS) and brief centrifugation in a bench top microcentrifuge to collect the beads. The beads were then conjugated to approximately 10 μl of antigen-specific antibody (Santa Cruz) or isotype control by incubating on a rotating platform for 1 h at 4 °C. Cell lysates were generated by the addition of 0.5 % NP40 in PBS (TAP-lysis buffer) to 10-cm dishes of cells after removal of culture medium and washing with PBS. Cell lysates were spun at 13,000 rpm in a microcentrifuge for 2 min to collect the DNA and organelles, and the supernatant was incubated with the conjugated beads for one h at 4 °C. The beads were then collected by centrifugation and re-suspended in TAP-wash buffer. The wash step was repeated a total of five times. On the final step, NuPAGE loading buffer was added to the beads and the protein constituents were separated by SDS-PAGE.

2.2.7. Tandem affinity protein purification pull-down assay.

A clone of stable inducible TAP-tagged cells, obtained by the process outlined in Section 2.3.4, was grown into twenty T175 tissue culture flasks. When the cells reached the level of 80 % confluency, tetracycline was added to the culture medium of ten T175 flasks to a final concentration of 2 μg/ml. The remaining flasks were left untreated.

The entire pull-down procedure has been described previously (Gloeckner et al., 2007). The procedure is carried out at 4 °C. TAP-lysis buffer supplemented with protease inhibitors (one complete EDTA-free protease inhibitor cocktail tablet (Roche) and one PhoSTOP (Roche) tablet per 50 ml of lysis buffer) and TAP–wash buffer supplemented with the same protease inhibitors were used during the procedure. The culture medium was removed from the flask and the cells were washed with sterile PBS, lysed with 1 ml of TAP-lysis buffer and incubated for 20 min. Cell lysates were then scraped and collected in a 15 ml falcon tube and centrifuged at 500 *g* for 10 min at 4 °C. The supernatant was collected and filtered through a 0.2 μm pore filter and 100 μl of Strep-Tactin superflow beads (IBA) were added and left to rotate slowly for 1 h. The beads were then spun at 500 *g* for 3 min, the supernatant was removed and the beads were re-suspended in 1 ml of TAP-wash buffer. The beads were washed a total of three times using sequential spinning, removal of supernatant and re-suspension in TAP-wash buffer. Protein complexes were then eluted using 10 x STREP-tag elution buffer (Desthiobiotin - IBA) containing desthiobiotin diluted in PBS and incubated on a rotating platform for 30 min. The eluate was added to 100 μl of anti-FLAG M2 beads (Sigma) and left incubating on a rotating platform for 1 h. The wash step for the STREP beads was repeated for the FLAG beads and the protein complexes were eluted using FLAG-peptide (Sigma) at 250 μg/ml for 30 min. The final eluate was then concentrated using Microcon® centrifugal filter devices (Millipore) with a 5 kilodalton (kDa) molecular mass

cut-off to a final volume of approximately 80 µl. To this, 30 µl of NuPAGE loading buffer was added and loaded onto a NuPAGE 4-12 % Bis-Tris gel (Invitrogen) to separate the individual proteins.

2.2.8. Identification of proteins by mass spectrometry

Coomassie stained bands from tandem affinity purification procedures were excised using a clean scalpel and placed in 100 µl H_2O (Sigma-Aldrich). Samples were analysed by liquid chromatography mass spectrometry (LCMS/MS) at the Centre for Systems Biology at Imperial College London.

2.2.9. Enzyme-linked immunosorbant assay (ELISA)

Cytokines & chemokines in supernatants from treated cells in tissue culture or bronchoalveolar lavage (BAL) samples were measured using Cxcl10, Il-6 and vascular endothelial growth factor (VEGF) DuoSET ELISA kits (R&D Diagnostics) according to the manufacturer's instructions.

2.2.10. Biotinylated DNA pulldown

HeLa or HEK293T cells were transfected using with 5 µg/ml of biotinylated DNA of the same sequence used for concatamerisation (Integrated DNA technology) and left to incubate at RT for at least 30 min. Cells were then lysed with VOPBA lysis buffer (see section 2.3.5) and the lysates were incubated with 20 µl Streptactin beads, that had been prewashed twice with H_2O and once with VOPBA lysis buffer, on a rotating wheel at 4 °C. The beads were then harvested by centrifugation at 3,000 rpm in a microcentrifuge and

washed three times with 1 ml PBS. After the final wash, NuPAGE loading buffer was added and the proteins were resolved by Lithium dodecyl-sulphate (LDS) - PAGE using precast NuPAGE gels (Invitrogen).

2.3. Tissue culture

2.3.1. Maintenance of cell stocks

Mammalian cell lines used included a human cervical cancer cell line (HeLa), HEK 293T, HEK 293 TRex and MEFs that were either wild type, or genetically matched *Xrcc5*$^{-/-}$ *p53*$^{-/-}$ (provided by E. Paul Hasty, University of Texas, USA), *Xrcc6*$^{-/-}$ (provided by Shigemi Matsuyama, Case Western Reserve University, USA), *Prkdc*$^{-/-}$ (provided by Penelope Jeggo, University of Sussex, UK), *Tmp173*$^{-/-}$ (STING knockout, provided by Glen Barber, University of Miami Miller Medical School, USA), *Tbk1*$^{-/-}$ (provided by Felix Randow, Cambridge University, UK), *Mavs*$^{-/-}$, *Irf3*$^{-/-}$, *Irf5*$^{-/-}$, *Irf7*$^{-/-}$ (all provided by Kate Fitzgerald, University of Massachusetts Medical School, USA), *MyD88/Ticam*$^{-/-}$, *Zbp1*$^{-/-}$ (both provided by Shizuo Akira, Osaka University, Japan) or *PHD1/2/3*$^{-/-}$ (provided by Peter Ratcliffe, University of Oxford).

HeLa cells were cultured in RPMI buffer supplemented with 10 % foetal bovine serum (FBS), HEK293T and HEK293TRex were cultured in Dulbecco's modified eagle medium (DMEM) supplemented with 10 % heat-inactivated (1 h at 56 FBS °C) (with the addition of 5 μg/ml blastocidin with HEK293TRex cells), MEF cell lines were cultured in DMEM with 15 % FBS. All cells were grown at 37 °C in a incubator (Heraeus) in the presence of humidified air containing 5 % (v/v) CO_2.

2.3.2. Passage of cell lines

Growth medium was discarded from tissue culture flasks and the cells were washed with sterile PBS. Cells were detached by adding 1 ml 0.125 % trypsin 500 μM EDTA (Gibco BRL) per 35 cm^2 flask area and incubating at 37 °C until cells were loosened and could be detached by tapping. Detached cells were collected by centrifugation at 2000 *g* for 5 min and re-suspended in an appropriate volume of culture medium with FBS, which was then diluted and inoculated into a fresh tissue culture flask.

2.3.3. Transfection of mammalian cells

FuGene 6.0 transfection reagent (Roche) was used for transfection according to the manufacturer's instructions. Briefly, 2 μl of FuGene were added per μg of DNA or poly (I:C) (Sigma-Aldrich) in OPTI-MEM (Gibco BRL). The mixture was then mixed gently, incubated at room temperature for 30 min and added to cells (approximately 80 % confluency) after dilution in the appropriate cell culture medium. Transfection media were removed after 4 h and replaced with appropriate culture media.

Polyethylenimine (PEI) and (Sigma-Aldrich) and Lipofectamine 2000 (Invitrogen) were also used for transfection using the same protocol as for FuGene 6.0.

2.3.4. Generation of stable inducible C16 TAP-tagged cell lines

Eight micrograms of the pcDNA-4 T/O plasmid vector containing a C16-TAP-tagged gene under control of a tetracycline-inducible promoter (C16-TAP) was transfected into a 10-cm dish of HEK293T-Rex cells using FuGene 6.0 as a transfection reagent. Transfection into HEK-293 Trex cells places the promoter of C16-TAP under the control of a Tet-repressor

protein, which in turn is inactivated by the addition of tetracycline or analogs such as doxycycline. After initial transfection, individual clones were obtained by diluting the cells at a range of concentrations from 1 in 10 to 1 in 1,000 by volume and adding 5 μg/ml zeocin (Invitrogen) to select for clones expressing the zeocin resistance protein provided by the pcDNA-4 T/O plasmid. Individual clones were then picked and grown into larger volumes. Test expression of the clones of the C16-TAP tagged protein was carried out by the addition of tetracycline (or the analog doxycycline) and is demonstrated in Figure 3.1.

2.3.5. Cell fractionation

Crude cell fractionation was performed by washing cells with PBS, lysing the cells in VOPBA lysis buffer (100 mM Tris-HCl, ph 8.0, 2 mM $MgCl_2$, 1 mM EDTA, 0.2% Triton X-100) and centrifuging the lysate at 600 *g* for 3 min to remove nuclei and cell debris. The supernatant was collected and was centrifuged at 20,000 *g* to sediment membranes and debris. The remaining supernatant represents the cytoplasmic fraction.

2.3.6. Infection of cells with VACV

VACV stocks that had been purified by sedimentation in sucrose density gradients were thawed and virus was diluted as required into a minimal volume of either DMEM containing 15 % FBS (for MEF infections) or MEM containing 2.5 % FBS (for all other cell types). The appropriate dilution was then added to the cells and incubated at 37 °C for 1 h with gentle rocking every 15 min to distribute the virus evenly. After this time, the inoculum was aspirated and the cells were incubated in pre-warmed culture medium. Mock infection

refers to cells treated in an identical fashion, but with no virus being included in the initial inoculums.

2.4. Functional protein analysis

2.4.1. Luciferase reporter assay

Cells were seeded at 1×10^5 cells per well of a 96-well plate the day prior to transfection. Each well of cells was then transfected with 70 ng of expression plasmid, 70 ng and a plasmid encoding a firefly luciferase reporter driven by a specified promoter and 10 ng TK-renilla-luciferase. Cells were lysed in passive lysis buffer (Promega) and freeze-thawed to lyse the cells. Firefly luciferase activity was measured by adding luciferase reagent (8 mg/ml luciferin (Lux biotechnology) in a buffer consisting of 270 μM acetyl coenzyme-A (Sigma-Aldrich), 530 μM ATP, 33 mM DTT, 0.1 mM EDTA, 2.67 mM $MgSO_4$, 20 mM Tricine) to the cell lysates. Renilla luciferase activity was measured by adding renilla luciferase reagent (2 μg/ml coelenerazine (Lux Biotechnology) in PBS) to the cell lysates.

The relative stimulation of luciferase activity was measured as relative light units (RLU's) in a FLUOstar Omega luminometer (BMG Labtech). Reporter activity was calculated by normalising firefly luciferase with renilla luciferase activity.

For details of plasmids used in luciferase reporter assays see Table 2.1.

2.5. In vivo assays

2.5.1. Intranasal infection of mice

Groups of five female BALB/c mice between six and eight weeks old were anaesthetised and inoculated intranasally with 5×10^4 p.f.u. of purified VACV that was diluted in 20 µl PBS (Alcami and Smith, 1992). Mice were sacrificed at the specified time points.

2.5.2 Harvesting bronchoalveolar lavage (BAL) fluid

In order to harvest BAL fluid, mice were anaesthetized with isofluorane and sacrificed by exsanguination from the subclavian artery and vein. After removal of the lung, a pipette tip was inserted into the top of the trachea and the 200 µl PBS containing heparin (10 units/ml) was inoculated into the lungs and removed using the same pipette tip This process was repeated a further four times. BAL was collected into an eppendorf tube and centrifuged at 800 x g for 5 min at 4 °C to remove BAL cells and the supernatant was immediately used to perform cytokine analysis using ELISA (see section 2.2.9).

2.5.3. Harvesting RNA from infected tissue

Freshly harvested tissue was flash frozen in liquid nitrogen and stored at -80 °C until extraction of RNA. Tissue was crushed using a pestle and mortar with freezing in liquid nitrogen to ensure thorough homogenization of the sample. Homogenised tissue was then lysed with RLT buffer (Qiagen) and subjected to the RNeasy RNA extraction process described in section 2.1.13.

Chapter 3: Identification of C16 ligands leading to the characterisation of DNA-PK as a cytoplasmic DNA sensor

3.1. Identification of C16 binding partners

3.1.1. Introduction

Previous work in our laboratory identified VACV strain Western Reserve (WR) protein C16 as an intracellular virulence factor (Fahy et al., 2008). It was shown that the deletion of the *C16L* gene from VACV WR attenuated the virus, such that intranasal infection with a C16 knockout virus (vΔC16) induced fewer signs of illness, less weight loss and greater recruitment of leukocytes into the respiratory tract compared to wild type and revertant control viruses. However, the mechanism by which C16 promoted virulence remained unknown.

To understand how C16 was modulating the immune response to infection and promoting virulence, molecules with which it interacts (binding partners) were sought since intermolecular interactions usually mediate the function of viral immunomodulators. Previous attempts at direct immunoprecipitations with known components of innate immune signalling cascades had not revealed any potential interactions with C16, and furthermore it was not entirely clear which pathway C16 interfered with. Tandem affinity protein purification (TAP) and identification by mass spectrometry provided an unbiased proteomics-based approach to identification of C16 binding partners.

3.1.2. Identification of C16 binding partners

To identify C16 ligands, the C16 open reading frame (ORF) was cloned from VACV WR genomic DNA into the pcDNA4/TO vector (Invitrogen) with an engineered TAP-tag consisting of a C-terminal STREP and FLAG tag (pcDNA4/TO-C16TAP) for tandem affinity protein purification as described previously (Gloeckner et al., 2007). This process involves sequential purification of protein complexes by using two separate affinity tags on a target molecule and subsequent large scale purification to identify binding partners via mass spectrometry (Section 2.2.7).

Transfection of pcDNA4/TO-C16TAP into HEK293T-Rex cells placed the promoter under the control of a Tet-repressor protein, as described originally by Gossen (Gossen and Bujard, 1992). Stable cell lines were generated in which expression of the protein of interest can be induced by the addition of tetracycline. Individual clones of cells were selected in the presence of increasing doses of zeocin (Section 2.3.4) to generate stable cell lines that would express TAP-tagged C16 upon addition of tetracycline (or doxycycline) (Figure 3.1).

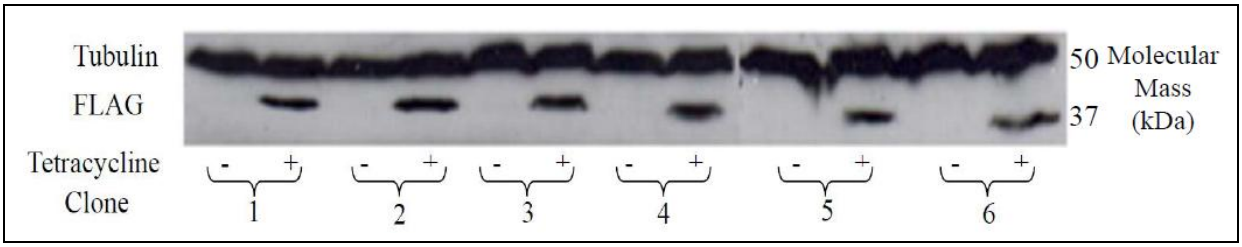

Figure 3.1 Inducible expression of C16 from clones of transfected HEK293T-Rex cells. Clones of zeocin-resistant cells were grown in 6-well plates and either left untreated or treated with tetracycline at a concentration of 2 μg/ml for 24 h. The protein concentration of the cell lysate was determined by a BCA assay and the lysates were resolved by SDS-PAGE and analysed by immunoblotting with anti-tubulin or anti-FLAG monoclonal antibodies. The positions of molecular mass markers are indicated in kDa.

In each of the clonal cell lines generated, there was a marked increase in the production of a 37 kDa band (corresponding to the size of C16) which stained positively with an anti-FLAG antibody following the addition of tetracycline. Furthermore, the cells showed a high stringency of repression when untreated with tetracycline and each clone appeared to express similar levels of C16. Clone 1 was selected for amplification and a large scale tandem affinity protein purification was performed (Section 2.2.7). As a control, a cell line inducibly expressing the intracellular interleukin-1 receptor antagonist (icIL-1Ra) using the icIL-1Ra ORF cloned into pcDNA4/TO vector with an identical TAP-tag (pcDNA4/TO-icIL-1RaTAP) was also generated and was used in a parallel TAP experiment. The ic-IL-1Ra ORF was selected for study because C16 was thought originally to act in a similar fashion to icIL-1Ra due to its intracellular localisation and the conservation of a C-terminal amino acid sequence highly similar to IL-1Ra (VTKFYF in icIL-1Ra and VTRFYF in C16) (Kluczyk et al., 2002). As a further control, another cell line inducibly expressing the TAP-tag alone was included in the purification procedure.

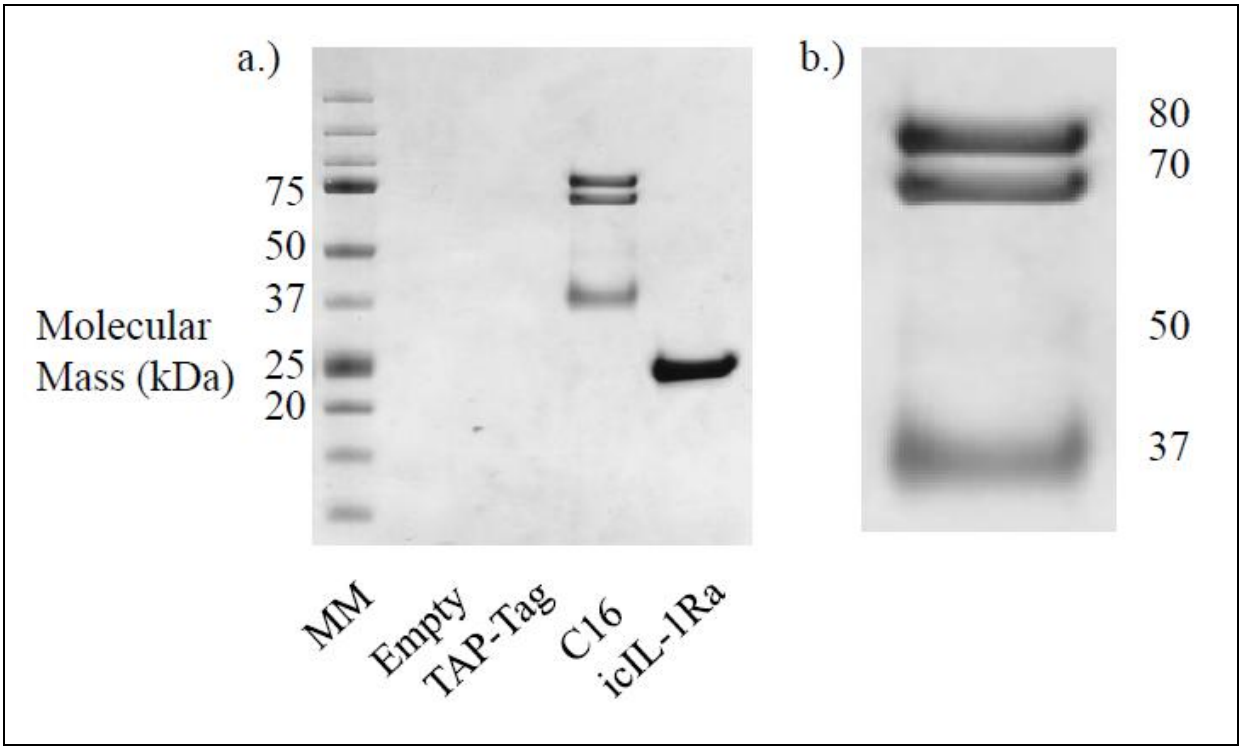

Figure 3.2 Tandem affinity protein purification of C16. Ten T175 flasks of each of the indicated cell lines were grown and after induction with tetracycline at 2 μg/ml for 24 h, protein complexes were purified by tandem affinity protein purification. Final eluates were resolved by NuPAGE (Invitrogen) and stained with Coomassie brilliant blue. Panel (a.) shows the total gel and panel (b.) an enlarged view of the C16 lane with bands identified with their approximate molecular masses, which were used as identifiers for mass spectrometric analysis. Empty, no protein loaded. TAP-Tag, Cells expressing the TAP-Tag alone.

Tandem affinity protein purification of C16 yielded four Coomassie stained bands (Figure 3.2). These had the approximate molecular masses of 37 (the mass of C16), 50, 70 and 80 kDa. The cell line expressing the TAP-tag alone pulled down no bands visible by this method. The purification procedure using the icIL-1Ra cell line pulled down a band of approximately 25 kDa, corresponding to the predicted molecular mass of this molecule. This band was detected with immunoblotting with an anti-FLAG antibody (Figure 4.3), suggesting it was indeed icIL-1Ra with a C-terminal TAP-tag. This result indicated that the 50, 70 and 80 kDa bands correspond to proteins which bind specifically to C16 and not to the affinity

beads used in the procedure or to the TAP-tag. Collectively, these data provide solid evidence that the proteins pulled down when C16 is expressed are specific to the induction of C16 and hence correspond to host-cell binding targets of this VACV protein. Furthermore, C16 bound to proteins which were not bound by icIL-1Ra (Figure 3.2 and 4.1), suggesting that C16 has distinct intracellular functions to icIL-1Ra. However, this does not rule out a role for ic-IL-1Ra-like properties of C16 because there could be common binding partners not detected by this method.

The four bands derived from the C16-TAP cell line were excised from the gel and sent for mass spectrometry analysis (Section 2.2.8), which provided unequivocal identification of proteins within the bands as Egg-laying 9 homolog 1 (Egln1)/Prolyl-hydroxylase domain containing protein (PHD)-2 (50 kDa band), Ku70 (70 kDa band) and Ku80 (80 kDa band) as molecules that interact with C16 (Table 3.1). Ku70 and Ku80 (also termed Ku86) form a heterodimer, which is known to be involved in DNA repair (Downs and Jackson, 2004; Collis et al., 2005), whereas Egln1/PHD2 is a prolyl 4-hydroxylase involved in hypoxic signalling (Kaelin, 2005). Mass spectrometric analysis of the excised bands did reveal a significant level of keratin contamination; however this was not sufficient to obscure the positive identification of cellular proteins in these bands.

Band	Rank	Total Ion Score	Identification	Molecular Mass (kDa)	Peptide Count
37	1	833.34	VACV C16	37.5	10
37	2	226.19	Keratin	66.0	5
50	1	367.28	EGLN1/PHD2	55.0	5
50	2	216.97	Keratin	66.0	5
70	1	1473.11	Ku70/XRCC6	69.8	26
70	2	259.03	Keratin	66.0	4
80	1	1369.82	Ku80/XRCC5	82.7	32
80	2	450.39	Keratin	66.0	8

Table 3.1. Mass spectrometric analysis of excised bands from tandem affinity purification assay. The two highest ranked hits for each protein are listed here. Band names relate to the approximate molecular masses as indicated in Figure 3.2. Samples were analysed by Dr. Paul Hitchen, Biochemistry Department, Imperial College London.

3.1.3. Confirmation of C16-Ku70/Ku80 interaction

After initial identification of putative C16 binding partners, it was essential to confirm these interactions by alternative methods. This confirmation was achieved firstly by immunoblotting of affinity purified proteins with antibodies specific to the proteins identified by mass spectrometric analysis. And, secondly, by reciprocal co-immunoprecipitation of these binding partners with C16 in the context of virus infection. The remainder of this chapter will focus on the interaction between C16 and the Ku heterodimer, see Chapter 4 for work relating to Egln1/ PHD2.

Ku70 and Ku80 constitute the constitutively formed Ku heterodimer (Downs and Jackson, 2004), an abundant protein complex that is present in many organisms ranging from humans to *Saccharomyces cerevisiae* to humans (Dynan and Yoo, 1998). DNA-dependent protein kinase (DNA-PK) is a large heterotrimeric complex consisting of the 70 kDa Ku70, 80 kDa Ku80 (also termed Ku86) and 460 kDa catalytic subunit of DNA-PK (DNA-PKcs) (Gottlieb and Jackson, 1993; Collis et al., 2005).

DNA-PK is involved in dsDNA-repair (Collis et al., 2005; Meek et al., 2008). The most highly characterised function of DNA-PK to date is its ability to bind to disruptions of the DNA double helix such as dsDNA breaks (Mimori and Hardin, 1986; Paillard and Strauss, 1991). Upon binding to dsDNA breaks, Ku translocates along the DNA molecule, often resulting in multiple Ku subunits being bound to DNA, akin to beads on a string (Paillard and Strauss, 1991). Subsequently, the Ku heterodimer recruits DNA-PKcs via a C-terminal domain of Ku80 (Gell and Jackson, 1999) and once this heterotrimeric complex is loaded onto both ends of a DNA break, the process of non-homologous end joining occurs (Collis et al., 2005; Weterings and Chen, 2007).

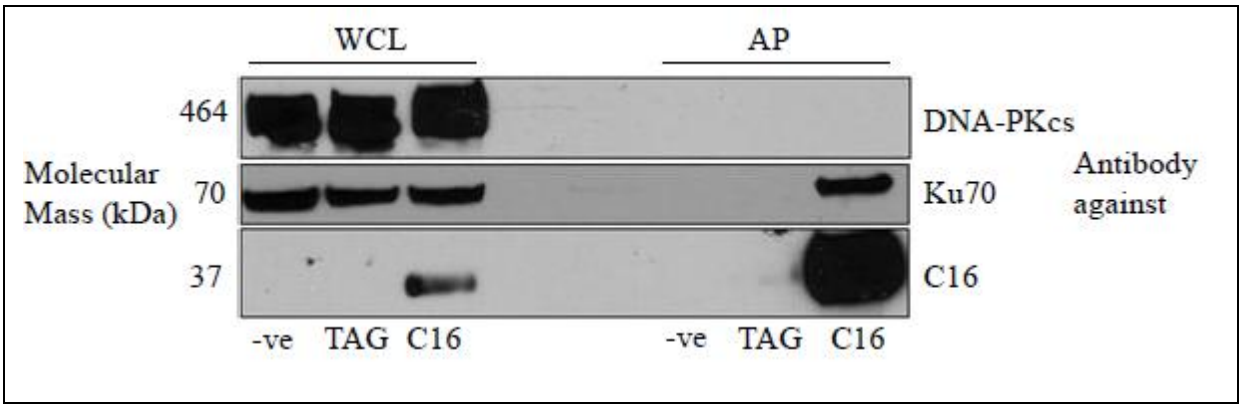

Figure 3.3 Ku70 co-precipitates with C16. One T175 flask of each of the indicated cell lines was induced with 2 μg/ml tetracycline for 24 h. Protein lysates were prepared and C16 protein complexes were purified with streptavidin beads. Whole cell lysate (WCL) and affinity purified proteins (AP) were resolved by SDS-PAGE and immunoblotted with antibodies against Ku70, DNA-PKcs or C16. –ve, C16 cell line without addition of tetracycline; TAG, HEK293TRex cell line expressing the TAP-tag only; C16, HEK293TRex cells expressing C16 induced with tetracycline.

Immunoblotting was performed using eluates from a small scale affinity purification of C16 using streptavidin beads (Figure 3.3). Ku70 was found to co-precipitate with C16 but not with the TAP-tag alone. Interestingly, DNA-PKcs did not co-precipitate with C16. Similar data were obtained using an anti-Ku80 antibody (data not shown), and importantly

the co-precipitation of C16 with Ku80 was confirmed in the context of virus infection (Figure 3.4). The Ku heterodimer is an extremely stable heterodimer. There are extensive protein-protein interaction regions between the two subunits, demonstrated both biochemically (Wu and Lieber, 1996) and crystallographically (Walker et al., 2001), hence interaction with Ku70 implies interaction Ku80 and vice versa. Additionally, absence of one subunit leads to the structural instability and degradation of the other subunit (Errami et al., 1996; Wu and Lieber, 1996; Singleton et al., 1997).

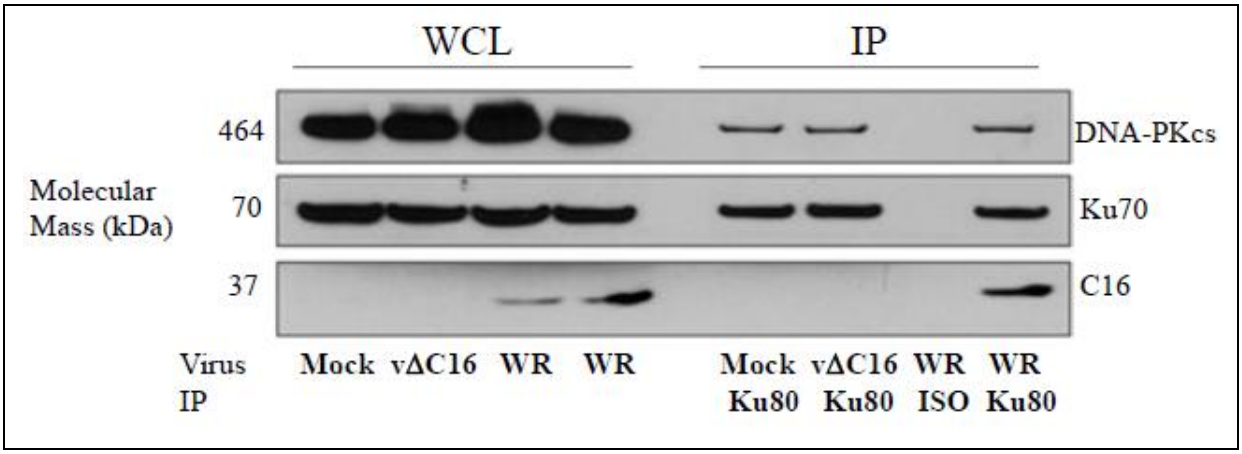

Figure 3.4 C16 immunoprecipitates with Ku80 from VACV-infected cells. HeLa cells were mock-infected or infected with either a C16 knockout virus (vΔC16) or VACV WR at 2 p.f.u. per cell for 4 h. Cells were then lysed and anti-Ku80 or isotype control (ISO) antibodies conjugated to protein G sepharose beads were incubated with lysates for 1 h. Subsequently whole cell lysates (WCL) and immunoprecipitates (IP) were resolved by SDS-PAGE and immunoblotted with antibodies against the indicated proteins. IP lanes represent complexes purified with the indicated antibody.

Confirmation of the interaction between the Ku heterodimer and C16 was achieved by co-immunoprecipitation of the complex from cells infected with VACV (Figure 3.4). Protein complexes were precipitated from infected cell lysates with an anti-Ku80 antibody, or an isotype control antibody, and the precipitates were analysed by immunoblotting with antibodies against C16, Ku70 and DNA-PKcs. This experiment showed that C16, Ku70 and

DNA-PKcs were co-immunoprecipitated with anti-Ku80, but not isotype control, antibody. As expected, C16 was absent from mock-infected cells and from cells infected with the VACV strain lacking the *C16L* gene (vΔC16). These data confirm a specific interaction between the Ku heterodimer and C16 at endogenous protein levels found during the course of a virus infection.

Interestingly, immunoprecipitation of Ku80 pulled down C16 and DNA-PKcs, yet purification of C16 shows binding to the Ku heterodimer, but not DNA-PKcs. This suggests the existence of a mixed group of complexes containing either Ku80 with DNA-PKcs or Ku80 with C16, but a complex containing Ku80, C16 and DNA-PKcs has not been demonstrated. This might suggest an inhibition of DNA-PKcs binding to Ku by C16 and this is explored further below.

3.2. Characterisation of DNA-PK as a cytoplasmic DNA sensor of innate immunity

DNA-PK is an abundant protein complex found in most cell types (Collis et al., 2005). DNA-PK components are essential for V(D)J recombination (Blunt et al., 1995; Errami et al., 1996; Jeggo et al., 1996), and loss of these components results in a severe-combined immunodeficiency (SCID) phenotype, indicating a critical role for DNA-PK in the development of the adaptive immune system. The identification of Ku as an interacting partner of C16 was interesting, but the purpose of this interaction was not evident initially. Whilst V(D)J recombination is a critical aspect of immunity for generating T and B cell receptors, and defects in this process lead to a SCID phenotype, it was unlikely that C16 was binding to Ku for the purpose of interfering in this process specifically. Firstly, V(D)J recombination occurs early in the development of lymphocytes in the bone marrow or thymus for B and T cells, respectively. C16 was shown to be a virulence factor during an intranasal infection (Fahy et al., 2008) and cytokine production was shown to be affected by 24 h (Fahy, unpublished data)- which would not represent a long enough period of time for a derangement of B or T cell development to manifest. Secondly, VACV is a highly lytic virus and is not known to persist in B or T cells. Therefore, in the instance that C16 was produced by infection of a developing B or T cell, it is likely to have little significance since the cell would soon by lysed, independently of C16, before V(D)J recombination and subsequent lymphocyte maturation occurs.

Consequently, it was unclear why Ku was a binding target for a VACV protein, or how the binding of C16 to Ku was linked to the characterisation of C16 as a virulence factor. Ku and the larger DNA-PK complex have been described principally as a nuclear protein,

however a cytoplasmic fraction has been noted (Fewell and Kuff, 1996; Collis et al., 2005) and the function of this cytoplasmic DNA-PK is unclear.

DNA-PK has been shown previously to interact with IRF-3 (Karpova et al., 2002), a component of innate immunity critical for DNA sensing (Ishii et al., 2006; Stetson and Medzhitov, 2006).

DNA-PK, therefore, is a protein complex with a well characterised DNA-binding activity, and in addition it is also present in the cytoplasm and interacts with IRF-3. Since C16 was an immunomodulator, and the effects of this immunomodulation can be observed early in the course of infection, it was hypothesised that DNA-PK was acting as a cytoplasmic DNA sensor and that C16 might be modulating a DNA-mediated innate immune response.

3.2.1. DNA-PK binds to DNA in the cytoplasm

Since C16 is a VACV protein, and poxviruses replicate their DNA exclusively in the cytoplasm, if Ku were to play a role in DNA sensing it would have to bind cytoplasmic DNA. To assess the ability of DNA-PK to bind cytoplasmic DNA, biotinylated DNA pull downs were performed on cytoplasmic extracts.

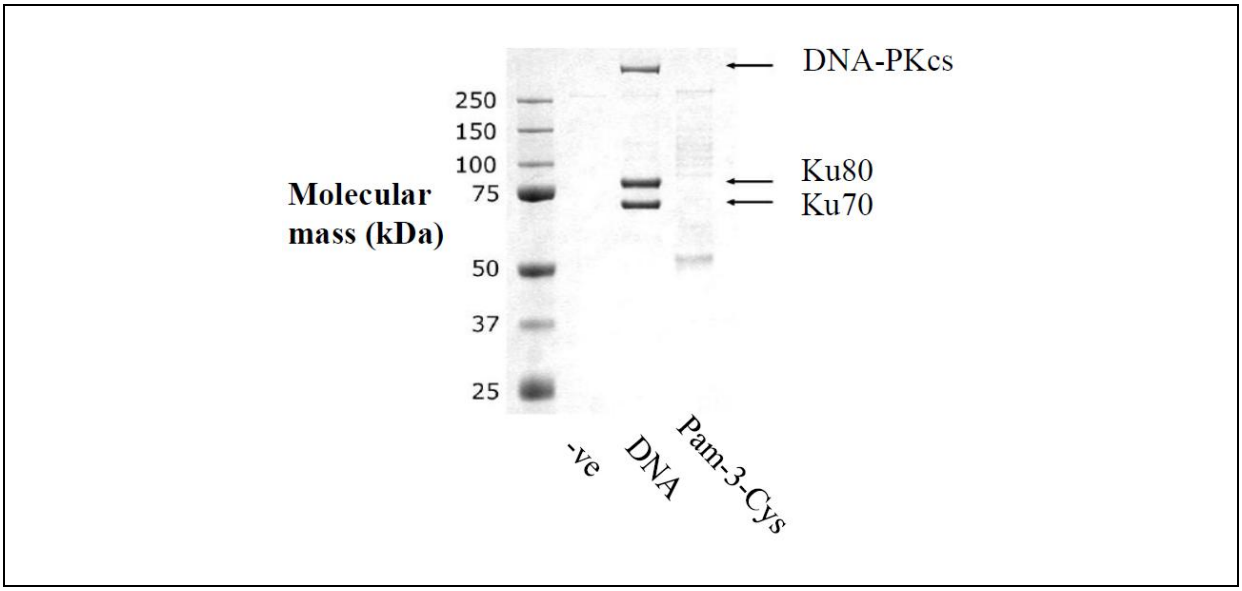

Figure 3.5 DNA-PK binds to DNA purified from the cytoplasm. HEK cells were mock transfected (-ve), transfected with biotinylated DNA (DNA) or transfected with biotinylated Pam-3-Cys (Pam3cys) for 1 h. Cytoplasmic extracts were generated, incubated with streptavidin beads and protein complexes purified in this manner were analysed by SDS-PAGE. The gel was stained with Coomassie brilliant blue and the stained bands were excised and the proteins present were identified using mass spectrometry. Molecular mass markers are labelled in kDa. Samples were analysed by Dr. Paul Hitchen, Biochemistry Department, Imperial College London. This work was carried out with Dr. Brian Ferguson.

Biotinylated DNA purifications from cytoplasmic extracts of HEK cells demonstrated that DNA-PK bound to DNA in the cytoplasm, consistent with a previous report (Frasca et al., 2001). Notably, all three components of DNA-PK co-purified with biotinylated DNA but not with biotinylated Pam-3-Cys, indicating that DNA-PK specifically interacts with DNA and is not co-purified with biotin or streptavidin beads.

Further data generated within our laboratory have shown a refined nuclear-cytoplasmic separation with actin and tubulin immunoblotting to control for adequate nuclear and cytoplasmic exclusion, respectively. With this more stringent nuclear-cytoplasmic

fractionation, DNA-PK components are still found to be both cytoplasmic and nuclear in unstimulated cells (Ferguson et al, submitted).

The ability of DNA-PK to bind to DNA in the cytoplasm is consistent with a role for DNA-PK in DNA sensing in the context of a poxvirus infection. However, DNA-PK had previously been ruled out as a candidate DNA sensor, because bone marrow derived monocytes from mice lacking Ku70 or DNA-PKcs demonstrated no deficit in innate immune sensing of DNA (Stetson and Medzhitov, 2006). This clearly directly conflicted with the hypothesis that DNA-PK acts as a DNA sensor.

3.2.2. DNA-PK is not expressed by macrophages

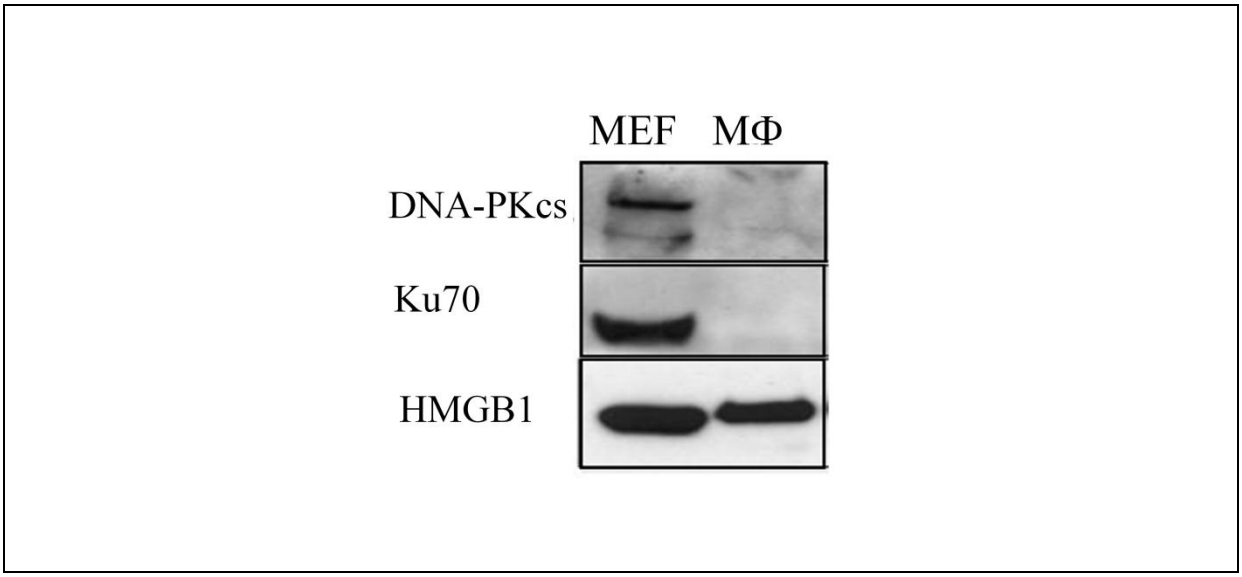

Figure 3.6. DNA-PK is expressed in MEFs, but not macrophages. Cell lysates from BALB/c primary peritoneal macrophages and MEFs were analysed by SDS-PAGE and immunoblotting with the indicated antibodies. This work was performed with Drs. Brian Ferguson and Daniel Mansur.

Given the findings of Stetson & Medzhitov that the Ku heterodimer and DNA-PKcs are dispensable for DNA sensing in BMDCs (Stetson and Medzhitov, 2006) the presence of

these molecules in this cell type was investigated. Whilst Ku70 and DNA-PKcs can be easily detected in MEFs by immunoblotting, these molecules were not detectable within macrophages, despite the quantities of HMGB1, a control protein, being similar between both cell types. This result demonstrates an uneven distribution of DNA-PK across cell types, and suggests that the elimination of DNA-PK as a candidate DNA sensor is premature, at least across all cell types.

DNA sensing in macrophages has been studied intensively, and a recent DNA sensor, interferon-induced 16 (IFI16) is likely to be the major DNA sensor responsible for activating the innate immune system in this cell type (Unterholzner et al., 2010). However, MAVS and MDA-5 have also been reported to be critical for the induction of IRF-3-mediated innate immune signalling during poxvirus infection in macrophages (Delaloye et al., 2009), which suggests a complex induction of innate immune signalling and leaves open the question as to whether poxvirus DNA is acting as a PAMP during the course of infection.

Poxviruses have a broad tropic range and are capable of infecting many cell types. Therefore, the likely sites of initial VACV replication following intranasal infection are the first cells which VACV encounters, and these are typically the epithelial cells and fibroblasts of the airways and lungs. The presence of DNA-PK in these cell types is consistent with a role for DNA-PK as a primary DNA-sensor during VACV infection. Additionally, the absence of DNA-PK in macrophages is consistent with the dispensability of the genes encoding these molecules for the induction of *Ifnb* mRNA induction (Stetson and Medzhitov, 2006).

3.2.3. DNA-PK components are upregulated upon DNA stimulation

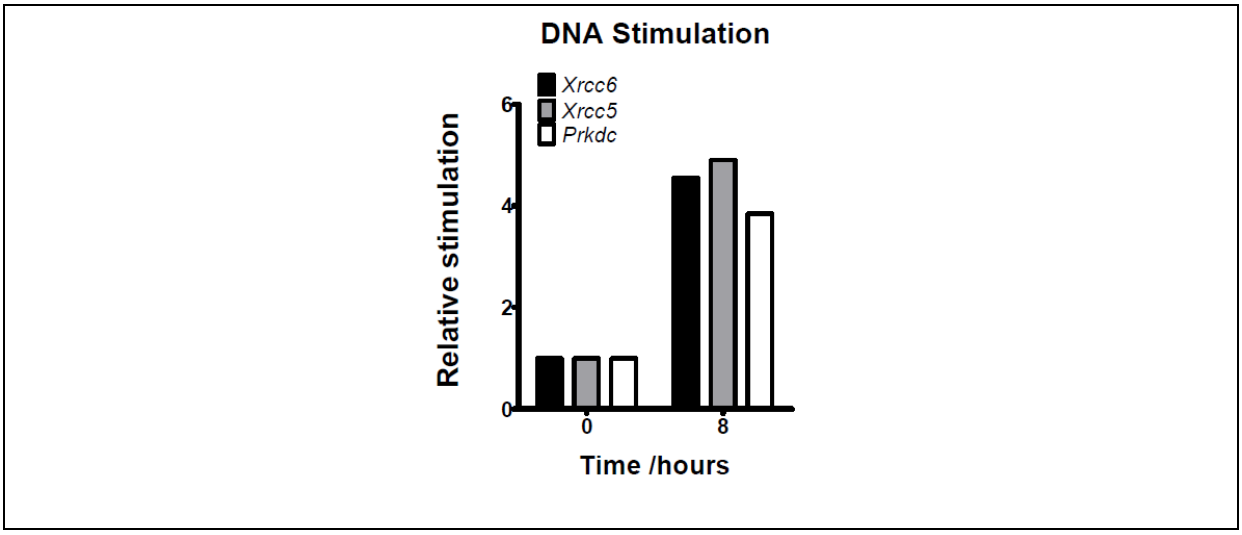

Figure 3.7. DNA-PK component mRNAs are upregulated following DNA stimulation. Primary MEFs from BALB/c mice were stimulated with 10 μg/ml of 200-bp ISD dsDNA for the indicated times. qRT-PCR analysis was performed on RNA extracted from these cells with primers specific for *Ku80* (*Xrcc6)*, *Ku70* (*Xrcc5)* & *DNA-PKcs* (*Prkdc)*. Stimulation was calculated relative to *Hprt*.

A feature of several proteins that function in innate immune signalling is that they are upregulated in the presence of IFN or pro-inflammatory stimuli. To assess if DNA-PK was induced upon stimulation with DNA, the mRNA levels for Ku70, Ku80 and DNA-PKcs were measured by qRT-PCR before and after DNA transfection (Figure 3.7). This showed that by 8 h following DNA transfection each mRNA was upregulated approximately 4 fold, relative to the housekeeping gene *Hprt*. This relatively modest increase in mRNA is significant given the fact that all three proteins are already expressed at high levels in resting MEFs and are easily detectable by immunoblotting (Figure 3.6). Interestingly, upregulation of DNA-PK components upon DNA transfection was only noted in primary MEFs, and not transformed MEFs (data not shown).

Data showing that DNA-PK is able to bind DNA in the cytoplasm, is constitutively expressed at high levels at the sites of poxvirus infection and is also inducible upon DNA transfection are consistent with basic characteristics of a DNA sensor acting in the detection of poxvirus infection. The role of Ku and DNA-PKcs in the activation of innate immunity following DNA transfection was then investigated.

3.2.4. Cells lacking the Ku heterodimer are deficient in DNA, but not RNA sensing

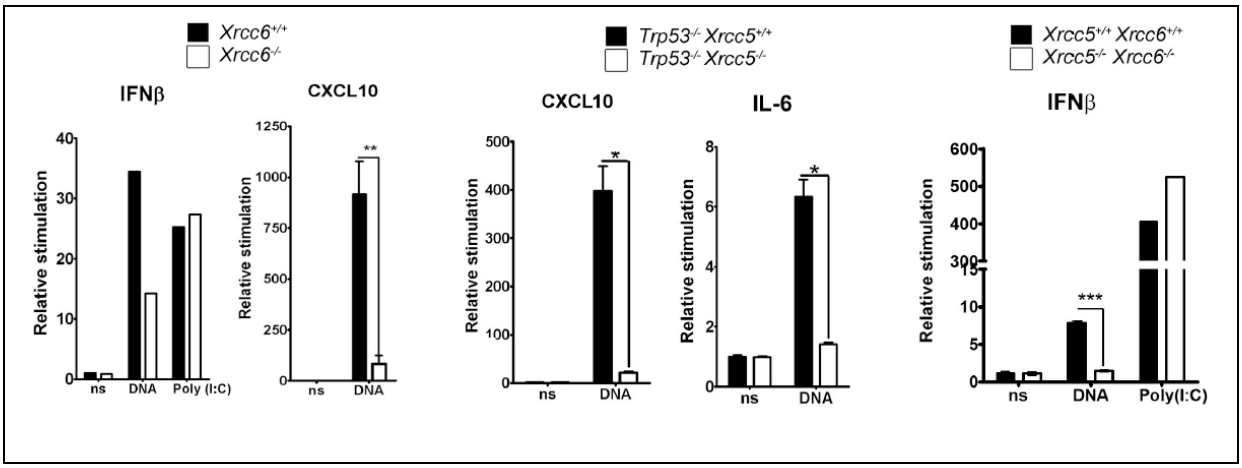

Figure 3.8. Ku is required for innate immune signalling following DNA stimulation, but not RNA stimulation. MEFs lacking Ku70 (*Xrcc6*$^{-/-}$), Ku80 (*Trp53*$^{-/-}$*Xrcc5*$^{-/-}$) or adult murine skin fibroblast (MSFs) lacking both Ku70 and Ku80 (far right panel) were stimulated with 10 μg/ml 200-bp ISD dsDNA or 5 μg/ml poly(I:C) for 8 h. qRT-PCR analysis was performed on RNA extracted from these cells. ns, Non-stimulated. Stimulation was calculated relative to *Hprt*. Error bars +/- Standard error of the mean (SEM) (N≥3), * p<0.05 ** p<0.01 *** p<0.001.

Previous reports have shown that MEFs transfected with DNA induced various IRF-3 dependent cytokines and chemokines such as Il-6, Cxcl10 and Ifnβ. Wild-type MEFs and those lacking either Ku70 (*Xrcc6*$^{-/-}$) or Ku80 (*Trp53*$^{-/-}$*Xrcc5*$^{-/-}$) components were stimulated by transfection with DNA or the dsRNA mimentic poly (I:C) (Figure 3.8). This showed that

the induction of *Ifnb* mRNA in response to DNA was impaired in cells lacking Ku70, however in response to poly (I:C), transcription was equivalent between wild-type and knockout cells (Figure 3.8). Similarly, upregulation of the transcription of *Cxcl10* mRNA in response to DNA was also found to be dependent on Ku70 as the transcriptional up-regulation was severely inhibited in *Xrcc6*$^{-/-}$ MEFs. *Xrcc5*$^{-/-}$ MEFs were also found to have abrogated *Cxcl10* and *Il6* responses to DNA, suggesting that both Ku components are required for complete DNA sensing. This is consistent with the fact that loss of the Ku70 gene, *Xrcc6*, leads to destabilisation and consequent absence of Ku80 protein and, and vice versa (Errami et al., 1996; Singleton et al., 1997). Adult murine skin fibroblasts (MSFs) lacking both *Xrcc5* and *Xrcc6* were also found to be deficient in *Ifnb* mRNA upregulation in response to DNA, but not dsRNA.

Together, these data confirm that cells lacking the Ku heterodimer are deficient in innate immune activation by DNA, but not poly (I:C) and furthermore that this response is found in both MEFs and adult MSFs, suggesting Ku-mediated innate immune activation by DNA is not specific to embryonic cells, and that the phenoytype observed is consistent amongst several knockout fibroblast cell lines generated independently.

3.2.5. Cells lacking DNA-PKcs are deficient in DNA sensing

C16 was found to bind to the Ku heterodimer, but not DNA-PKcs. Having established a requirement for the Ku70 and Ku80 proteins in DNA sensing, the requirement for DNA-PKcs in DNA-mediated innate immune signalling was investigated. To assess the contribution of DNA-PKcs to DNA-mediated innate immune activation, wild-type MEFs and

MEFs lacking the *Prkdc* gene (encoding DNA-PKcs) were stimulated with DNA or control ligands.

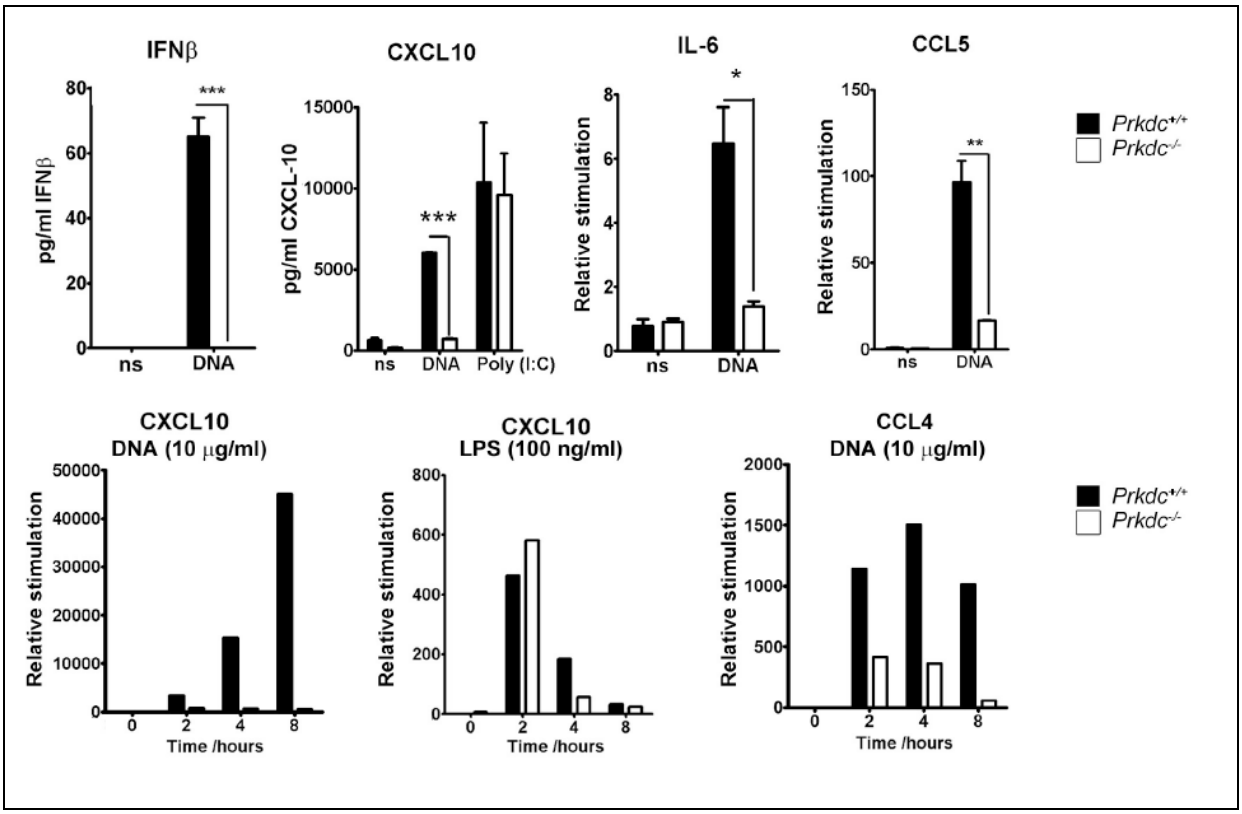

Figure 3.9. DNA-PKcs is required for innate immune sensing of DNA, but not RNA or LPS. MEFs lacking DNA-PKcs (*Prkdc*$^{-/-}$) and MEFs from WT littermate controls were stimulated with 5 μg/ml poly (I:C), 5 μg/ml 200-bp ISD dsDNA or LPS at an indicated concentration for 8 h or indicated times. RNA was extracted and mRNAs were quantified by qRT-PCR. Stimulation was calculated relative to *Hprt*. ns, Non-stimulated; Error bars +/- SEM (N≥3), * $p<0.05$ ** $p<0.01$ *** $p<0.001$.

As with the Ku heterodimer, *Prkdc*$^{-/-}$ MEFs were also found to be deficient in DNA-mediated upregulation of various genes, including *Cxcl10, Il6, Ccl5,* and *Ccl4* but responded normally to both transfected poly (I:C) and LPS. ELISA analysis on supernatants from wild-type and knockout MEFs demonstrated that *Prkdc*$^{-/-}$ MEFs had a profound defect in Ifn-β and

Cxcl10 production following stimulation with DNA for 8 h, but production of Cxcl10 was unimpaired following stimulation with poly (I:C).

The dependence on DNA-PKcs-mediated innate activation by DNA was also observed over a range of different time points. The upregulation of *Cxcl10* and *Ccl4* mRNA was impaired in MEFs lacking DNA-PKcs at all time points tested compared with wild-type MEFs, yet *Cxcl10* mRNA upregulation in response to LPS was found to be equivalent between the two cell types. Whilst upregulation of *Cxcl10* mRNA peaked at 2 h after LPS stimulation, consistent with existing literature (Hao and Baltimore, 2009), upregulation of this gene continued to increase after this time following transfection with DNA. Whether this temporal difference was due to kinetics of transfection or whether the pathway is subject to less stringent regulation than the pathway activated by LPS remains to be determined.

These data suggest that the entire DNA-PK complex is required for DNA sensing, not just the Ku heterodimer. Interestingly, the defect in DNA-mediated innate immune activation in *Prkdc*$^{-/-}$ MEFs appears more severe than in MEFs lacking either *Xrcc5* or *Xrcc6*. This is consistent with the reported ability of DNA-PKcs to bind to DNA in the absence of Ku, albeit with a lower affinity (Yaneva et al., 1997; Hammarsten and Chu, 1998).

Cxcl10 has been shown previously to be consistently upregulated in response to multiple species of DNA (Ishii et al., 2006). In addition, it has been demonstrated in Figures 3.8 and 3.9 that there is a similar dependence on Ku and DNA-PKcs for multiple cytokines and chemokines, including *Cxcl10*, *Il6* and *Ifnb* being upregulated in response to DNA stimulation. *Cxcl10* was therefore used as the default molecule measured in remaining experiments, with additional molecules assessed if necessary or informative.

3.2.6. DNA activates an innate immune signalling pathway involving STING, TBK-1 and IRF-3

In addition to DNA-mediated innate immune stimulation of MEFs being dependent on DNA-PK, these responses were also found to be dependent on other components of the IRF-3 pathway. Previous reports have implicated components of this pathway, including IRF-3 itself, (Ishii et al., 2006; Stetson and Medzhitov, 2006), the IRF-3 kinase TBK-1 (Ishii et al., 2008) , and STING (Ishikawa and Barber, 2008; Ishikawa et al., 2009) in DNA sensing and the requirement for these molecules was also assessed in our laboratory to confirm a requirement for these components.

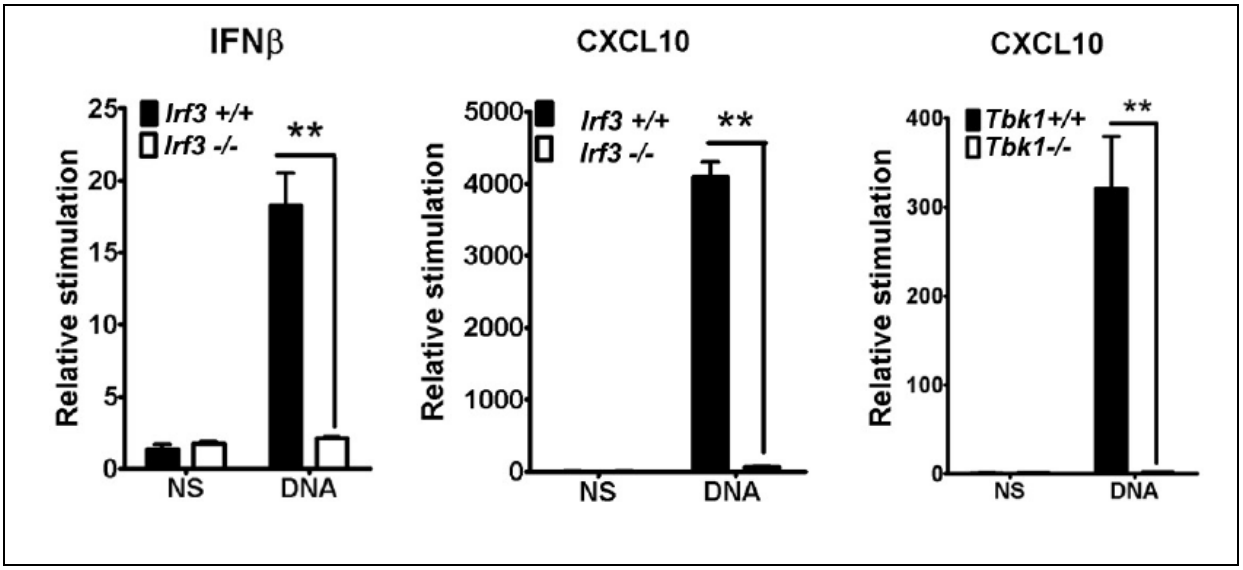

Figure 3.10. *Irf*3 and *Tbk*1 are both required for innate immune sensing of DNA. MEFs lacking *Irf*-3 or *Tbk*1 and those from wild-type littermate control cells were stimulated with 5 μg/ml 200-bp ISD dsDNA for 8 h and qRT-PCR was performed on RNA extracted from these cells. NS, non stimulated. Stimulation was calculated relative to *Hprt.* Error bars +/- SEM (N≥3) ** $p<0.01$

Irf3 knockout MEFs had a profound defect in innate immune responses to DNA with a near total abrogation of both *Ifnb* and *Cxcl10* mRNA upregulation following DNA

stimulation (Figure 3.10, middle and left panels), consistent with previous reports (Ishii et al., 2006; Stetson and Medzhitov, 2006). In addition, the upstream kinase of IRF-3, TBK-1 was also found to have a dramatic effect on *Cxcl10* mRNA upregulation following DNA stimulation, since absence of the gene *Tbk1* from MEFs lead to a profound deficit in innate immune activation (Figure 3.10, right panel), confirming the importance of TBK-1 in DNA-mediated innate immune activation as reported previously (Ishii et al., 2008).

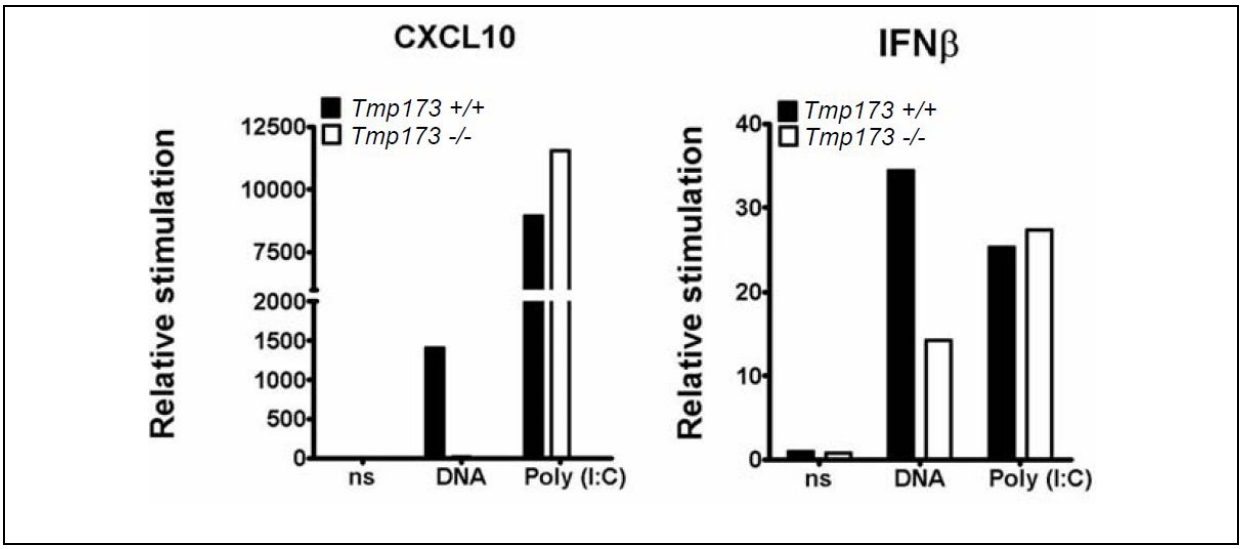

Figure 3.11. STING is required for innate immune sensing of DNA, but not RNA. Primary MEFs from mice lacking STING (*Tmp173*$^{-/-}$) or wild-type littermate control MEFs were stimulated with 5 μg/ml 200-bp ISD dsDNA or 5 μg/ml poly (I:C) for 8 h. qRT-PCR analysis was performed on RNA harvested from these cells. Stimulation was calculated relative to *Hprt.*

MEFs lacking STING, encoded by the gene *Tmp173*, were also shown to be deficient in innate immune sensing of DNA (Figure 3.11). *Tmp173*$^{-/-}$ MEFs had highly reduced *Cxcl10* and *Ifnb* mRNA upregulation compared with wild-type littermate control MEFs following DNA stimulation but not poly (I:C) stimulation, confirming a DNA specific defect as reported previously (Ishikawa et al., 2009).

3.2.7. DNA in primary, but not transformed, MEFs also activates NF-κB

During the studies of induction of various cytokines and chemokines in response to DNA, it was noted that, while *Cxcl10* and *Il-6* were upregulated substantially following transfection of DNA, the upregulation of *Ifnb* was not robust in some instances and in several experiments was completely absent. Our observations correlated with a recent study which reported that repeated passage of MEFs lead to a loss of the inducibility of *Ifnb* mRNA in response to DNA (Chiu et al., 2009). Our study, by necessity, used MEFs of various passage numbers and so a cause for this loss of *Ifnb* mRNA inducibility by DNA was investigated.

It has been known for some time that the *Ifnb* promoter contains multiple binding sites for transcription factors involved in innate immune signalling including IRF-3, NF-κB and AP-1 (Visvanathan and Goodbourn, 1989; Thanos and Maniatis, 1995; Merika and Thanos, 2001). However, whilst IRF-3 signalling in transformed MEFs was established to be intact, the state of NF-κB signalling in response to DNA in primary and transformed MEFs had not been investigated thoroughly. To examine the role of NF-κB signalling in DNA-mediated innate immune activation *Nfkbia* (encoding IκBα) transcription was measured, because this is specifically upregulated by NF-κB activation (Sun et al., 1993), and is therefore an indicator of NF-κB transcriptional activity.

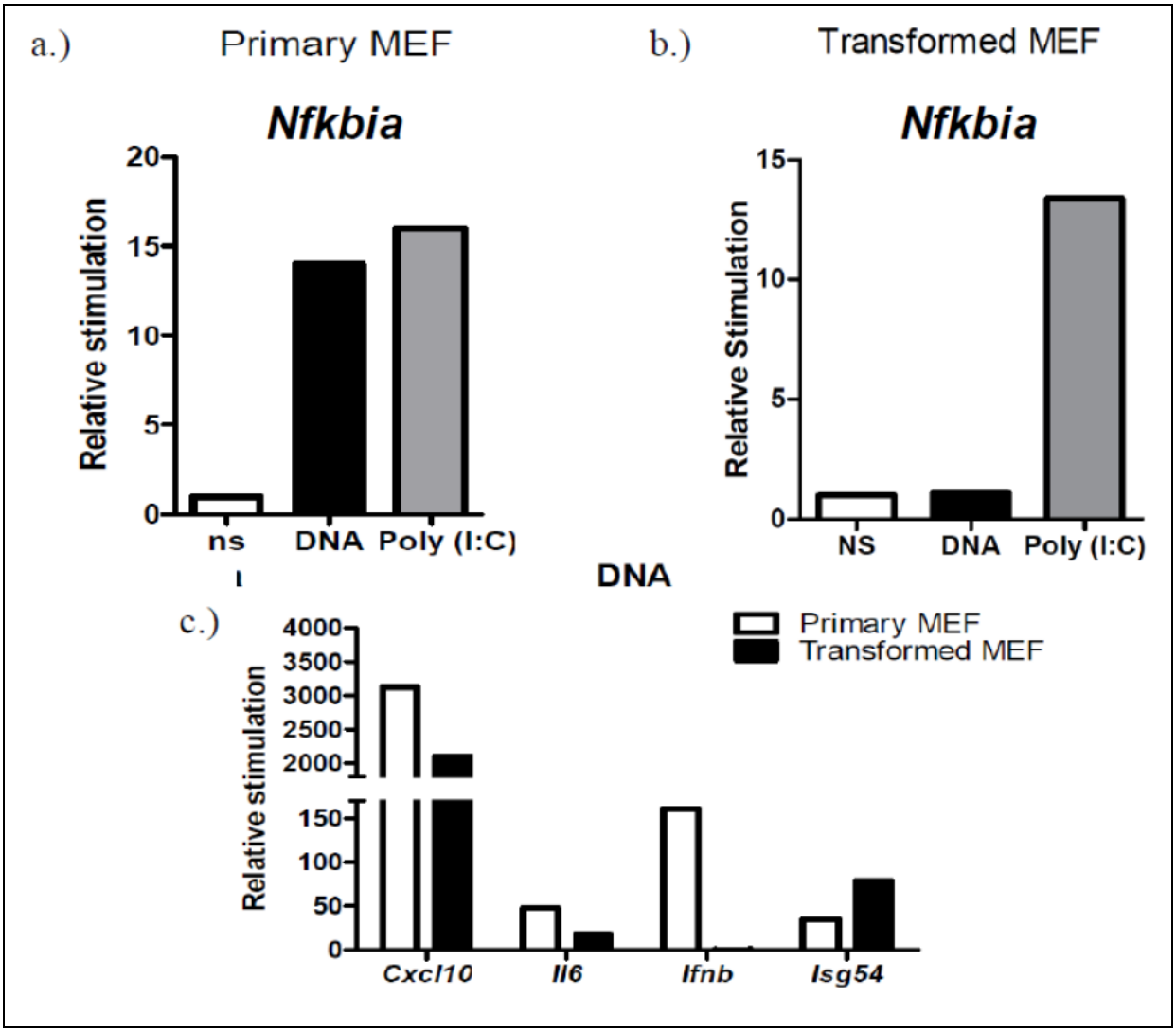

Figure 3.12. Differential NF-kB responses in primary and transformed MEFs. Primary BALB/c MEFs or spontaneously transformed BALB/c MEFs were stimulated with 5 μg/ml 200-bp ISD DNA or 5 μg/ml poly (I:C). qRT-PCR was performed on RNA harvested from these cells 8 h post stimulation. Stimulation was calculated relative to *Hprt*. qRT-PCR of *Nfkbia* following stimulation of (a) primary MEFs, and (b) transformed MEFs, with DNA or poly (I:C). (c) Parallel comparison of *Cxcl10*, *Il6*, *Ifnb* and *Isg54* upregulation in primary and transformed MEFs

In primary MEFs it was noted that NF-κB was activated in response to DNA transfection and poly (I:C) transfection (Figure 3.12a) as indicated by upregulation of *Nfkbia* mRNA transcription. In contrast, whilst *Nfkbia* mRNA was upregulated in transformed MEFs

in response to poly (I:C), *Nfkbia* was not upregulated in response to DNA transfection of transformed MEFs, indicating a lack of NF-κB activation (Figure 3.12b). *Cxcl10* and *Il-6* mRNA upregulation was still observed in transformed MEFs, however *Ifnb* mRNA upregulation in response to DNA was notably absent (Figure 3.12c). From these data, it appears that with transformation, MEFs lose the capability to activate NF-κB in response to DNA, but not in response to other stimuli, and that activation of NF-κB is necessary for an *Ifnb* induction in response to DNA stimulation.

3.2.8. NF-κB stimulation in response to DNA is enhanced by STING and independent of IRF-3

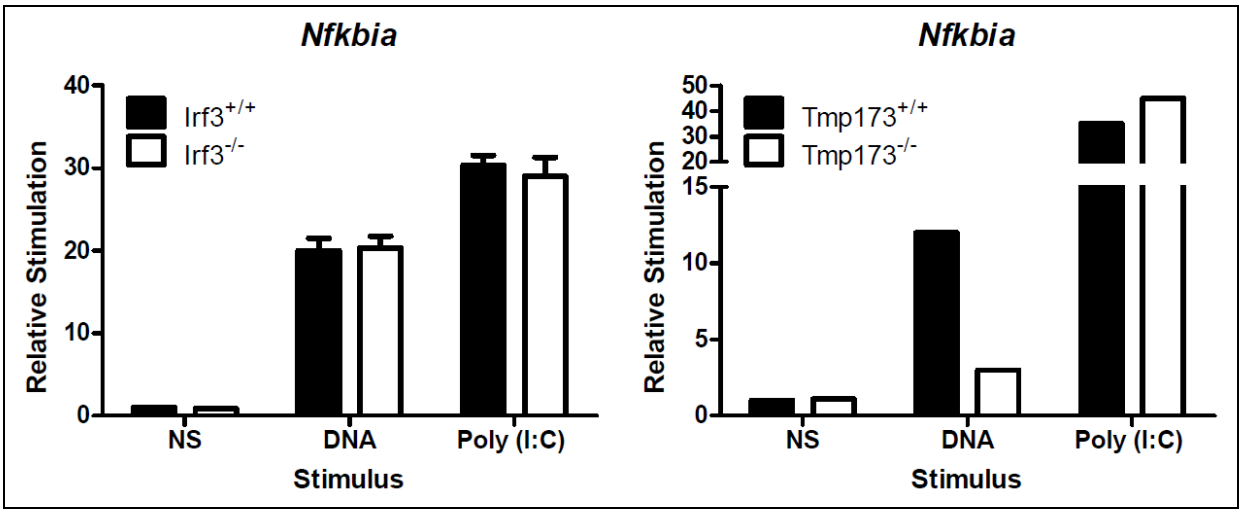

Figure 3.13. *Nfkbia* transcription in *Irf3*$^{-/-}$ and *Tmp173*$^{-/-}$ MEFs. *Irf3*$^{-/-}$,*Tmp173*$^{-/-}$ and WT littermate controls were stimulated with 5 μg/ml 200-bp ISD DNA or 5 μg/ml poly (I:C). qRT-PCR was performed on RNA harvested from these cells 8 h post stimulation. Relative stimulation was calculated relative to *Hprt* transcription.

To ascertain the relative contributions of other signalling molecules to NF-κB stimulation, qRT-PCR was used to test the upregulation of *Nfkbia* mRNA following DNA

stimulation of several primary MEFs. Consistent with work published previously (Ishikawa et al., 2009), NF-κB activation following DNA transfection was found to be enhanced by STING, since the absence of the *Tmp173* gene led to a decrease in *Nfkbia* mRNA upregulation in MEFs (Figure 3.13, right panel). In addition, the absence of *Irf3* in MEFs had no effect on the upregulation of NF-κB by DNA (Figure 3.13, left panel). These data indicate that activation of NF-κB is likely to be mediated intracellularly by a distinct adaptor or receptor, and not secondary to IRF-3 dependent gene transcription/translation and subsequent activation of cytokine receptors. Together, these data might indicate the existence of an additional DNA sensor or adaptor which activates NF-κB in response to DNA stimulation.

The roles of other molecules identified in the DNA-sensing pathway in the DNA-mediated NF-κB activation are yet to be assessed, and study of the relative roles of other molecules will lead to further understanding of the innate immune response to DNA.

Given that transcription factors such as IRF-3 are found to be activated following stimulation with DNA, and that DNA-PK components are also implicated, the order of the components in the proposed signalling pathway following DNA stimulation was investigated.

3.2.9. DNA-PK is upstream of IRF-3 in the DNA sensing pathway in MEFs

To assess whether DNA-PK acts in the same pathway as IRF-3, the transcriptional control of *Isg54* and *Cxcl10* was assessed in response to DNA stimulation in MEFs lacking either *Irf3* or *Prkdc*.

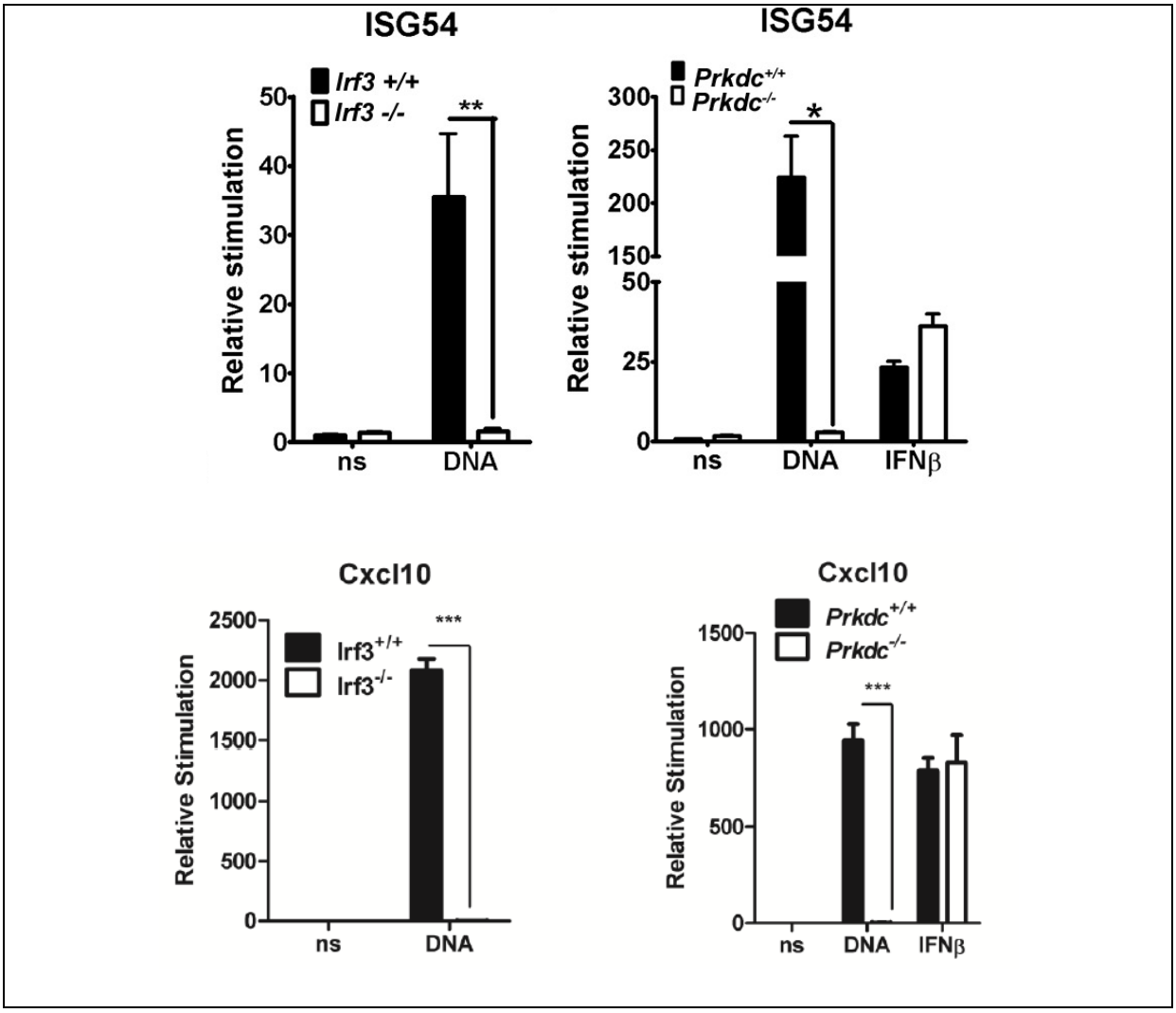

Figure 3.14. *Isg54* and *Cxcl10* upregulation after DNA stimulation is dependent upon both IRF-3 and DNA-PKcs. MEFs lacking *Irf3* or *Prkdc* were stimulated with 5 μg/ml 200-bp ISD dsDNA or 2000 U/ml murine IFNβ for 8 h and qRT-PCR analysis was performed on RNA extracted from these cells. ns, Non-stimulated. Stimulation was calculated relative to *Hprt*. Error bars +/- SEM (N≥3), * $p<0.05$ ** $p<0.01$

Transcription of the genes *Isg54* and *Cxcl10* was shown to be IRF-3-dependent in response to DNA (Figure 3.14, left panels). Stimulation of MEFs with DNA leads to a marked upregulation of both *Isg54* and *Cxcl10* mRNA levels, however in *Irf3*$^{-/-}$ MEFs this response is completely abrogated. In addition, the transcriptional upregulation of *Isg54* and *Cxcl10* following stimulation with DNA is also dependent upon DNA-PKcs as the response

is also ablated in *Prkdc*$^{-/-}$ MEFs, but the upregulation following stimulation with Ifn-β is still intact (Figure 3.14, right panels). Together, these data suggest that DNA-PK and IRF-3 fall within the same pathway activated by DNA because the absence of either molecule ablates the upregulation of both *Cxcl10* and *Isg54* mRNA following DNA stimulation.

Next, the ability of IRF-3 to translocate from the cytoplasm to the nucleus in the absence of DNA-PK was assessed by immunofluorescence (Figure 3.15).

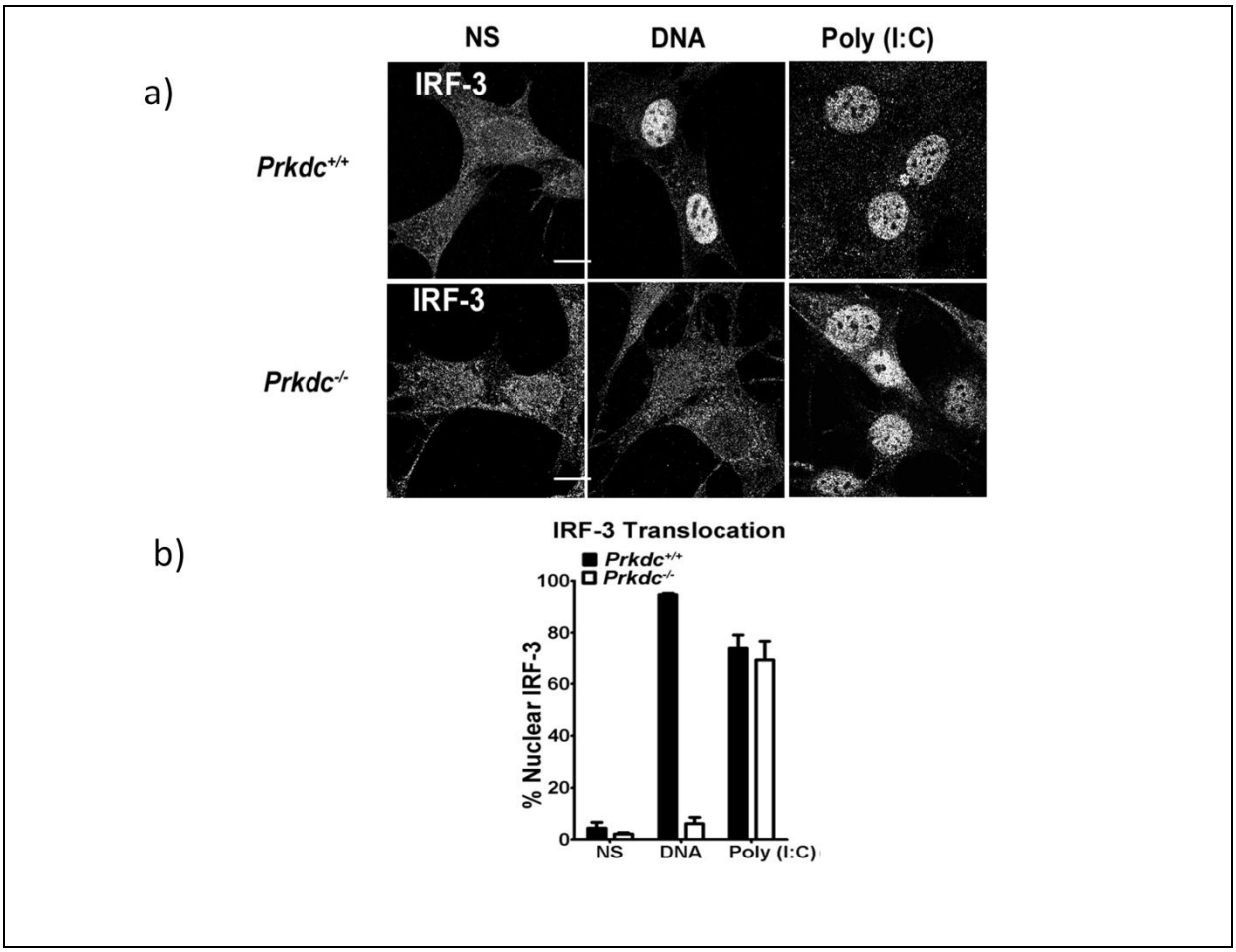

Figure 3.15. IRF-3 translocation to the nucleus in response to DNA, but not RNA, is dependent upon DNA-PK, but not in response to RNA. Wild-type MEFs or *Prkdc*$^{-/-}$ MEFs were stimulated with 5 μg/ml 200-bp ISD dsDNA or 5 μg/ml poly (I:C) for 1 h and stained for IRF-3. Top panel (a) shows representative images of cells with or without stimulation. The bottom panel (b) represents the percentage of cells in which IRF-3 is present within the nucleus, n=100. NS, non-stimulated. Error bars +/- SEM (n≥3). Scale bar, 5 μm. This work was carried out with Dr. Brian Ferguson.

Unstimulated MEFs showed a dispersed cytoplasmic staining of IRF-3, consistent with its cytoplasmic location in resting cells (Lin et al., 1998). Both wild-type and *Prkdc*$^{-/-}$ MEFs showed a strong translocation of IRF-3 to the nucleus of approximately 70% of cells following stimulation with poly (I:C), as expected. However, *Prkdc*$^{-/-}$ MEFs displayed no translocation of IRF-3 to the nucleus following stimulation with dsDNA, whereas *Prkdc*$^{+/+}$ MEFs showed IRF-3 translocation in more than 90% of cells (Figure 3.15a). The translocation of IRF-3 was quantified (Figure 3.15b) and a statistically significant effect of DNA-PKcs on DNA-mediated, but not poly (I:C)-mediated, IRF-3 translocation was demonstrated. These data are consistent with DNA-PKcs having a role in DNA, but not RNA, sensing. This suggests that DNA-PKcs is upstream of IRF-3 in the DNA sensing pathway operating within MEFs, and is consistent with DNA-PK binding to DNA in the initial event of DNA sensing within infected cells.

3.2.10. DNA sensing in MEFs is independent of DAI/ZBP1, MyD88/TRIF, MAVS, IRF-5 and IRF-7

Whilst DNA sensing in MEFs was found to involve a pathway involving, at a minimum, DNA-PK, STING, TBK-1 and IRF-3, a large cohort of innate immune signalling components had not been assessed for a role in DNA-mediated innate immune signalling. To address the input, if any, of these molecules to intracellular DNA sensing, knockout MEFs and genetically matched controls were transfected with DNA and the upregulation of *Cxcl10* mRNA was used as a marker for DNA stimulation (Figure 3.16).

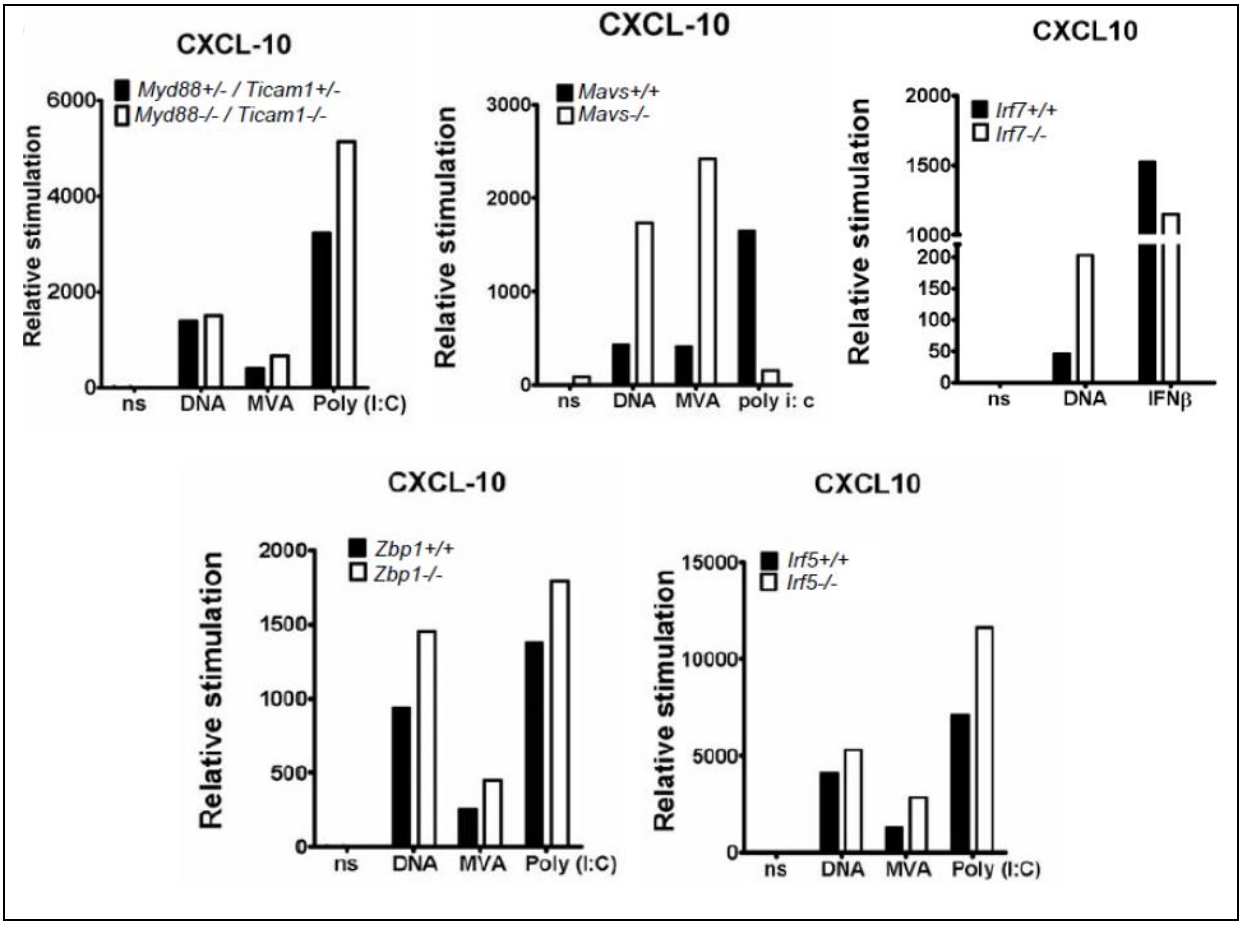

Figure 3.16. DNA sensing is not impaired in the absence of *MyD88, Trif, Mavs, Irf7, Irf5* or *Dai/Dlm1/Zbp1*. *MyD88/Ticam1*$^{-/-}$, *Mavs*$^{-/-}$, *Irf7*$^{-/-}$, *Zbp1*$^{-/-}$, *Irf5*$^{-/-}$ & WT littermate controls were transfected with 5 μg/ml DNA or 5 μg/ml poly (I:C), infected with MVA (5 p.f.u. per cell) or treated with murine Ifn-β (2000 U/ml) for 8 h. Quantitative RT-PCR analysis was performed on RNA extracted from these cells. Fold-stimulation was calculated relative to *Hprt.*

MEFs from a mouse with both *Myd88* and *Ticam1* (encoding TRIF) genes knocked out demonstrated no deficiency in upregulation of *Cxcl10* mRNA compared to wild-type controls in response to MVA infection, DNA or poly (I:C) transfection (Figure 3.16, upper left panel), consistent with literature published previously (Ishii et al., 2006). Since removal of these molecules ablates all known TLR signalling (Yamamoto et al., 2003a), a role for TLRs in intracellular DNA sensing in MEFs can be ruled out.

The role of RNA-polymerase III, RIG-I and MDA-5 in these assays was assessed using MEFs deficient in MAVS (*Mavs*$^{-/-}$) since this molecule is a common signalling adaptor to each of the aforementioned nucleic acid sensors. MEFs deficient in *Mavs* showed no reduction in *Cxcl10* mRNA upregulation following DNA transfection (Figure 3.16, upper centre panel), suggesting that the RLRs were not involved in DNA-mediated innate immune activation as described in this study. Interestingly a trend toward upregulation of *Cxcl10* in response to both DNA and MVA the absence of *Mavs* was observed (n=2). Although this observation requires reproduction to confirm the trend, it could suggest a regulatory role of Mavs in DNA sensing. There was, however, a marked reduction in *Cxcl10* mRNA following poly (I:C) transfection in *Mavs* knockout MEFs compared to wild-type cells in accordance with its documented role RNA sensing (Kawai et al., 2005; Meylan et al., 2005; Seth et al., 2005; Xu et al., 2005). Interestingly, despite VACV having an A:T rich genome (67% A+T), *Mavs*$^{-/-}$ MEFs demonstrated no impaired *Cxcl10* mRNA upregulation following MVA infection, suggesting that RNA-polymerase III is not involved in the detection of MVA in MEFs, at least at the time point tested, consistent with previous reports that MAVS is dispensable for poly (dA:dT)-mediated upregulation of *Ifnb* mRNA in MEFs (Kumar et al., 2006; Sun et al., 2006). A report published previously suggested that MDA-5 and MAVS were required for the MVA-mediated induction of IFNβ in macrophages (Delaloye et al., 2009). This provides further evidence for differential mechanisms of DNA sensing in alternate cell types, the reason for this is unknown.

Similarly, *Irf5* (Figure 3.16, lower right panel), *Irf7* (Figure 3.16, upper right panel) and *Dai/Dlm1/Zbp1* (Figure 3.16, lower left panel) null MEFs did not display a reduction in *Cxcl10* mRNA upregulation following DNA stimulation compared with WT littermate controls, which suggests IRF-5 and IRF-7 are not involved in DNA sensing in MEFs. As with *Mavs*, there appears to be an increase in *Cxcl10* upregulation in the absence of *Irf7*. This data

has not yet been reproduced and may not withstand further scrutiny, however in the context of this study both IRF-7 and Mavs can likely be excluded from DNA sensing since there is not a profound impairment of *Cxcl10* upregulation following DNA stimulation as is the case for MEFs lacking *Irf3, Tmp173, Tbk1* or *Prkdc*. In the case of *Dai/Dlm1/Zbp1* this is in agreement with data published previously (Ishii et al., 2008; Wang et al., 2008). DAI is only present at very low quantities in resting cells, but is induced upon activation of cells by IFN (Takaoka et al., 2007; DeFilippis et al., 2010), suggesting that DAI might play a secondary role in DNA sensing and is dispensable for initial DNA-mediated innate immune activation.

Collectively, these data provide evidence that DNA-mediated upregulation of *Cxcl10* mRNA following DNA stimulation of MEFs is dependent upon DNA-PK, STING, TBK-1 and IRF-3 and independent of TLRs, RLR's, IRF-5, IRF-7 and DAI.

3.3. DNA-PK is essential for innate immune responses to VACV

3.3.1 DNA-PK components co-localise with VACV virus factories

Since DNA-PK was found to bind DNA in the cytoplasm, it seemed possible that it might also bind to VACV factories in the cytoplasm that contain many copies of VACV genomic DNA. To address this, the location of DNA-PK components was compared in infected and mock-infected cells by immunofluorescence. In uninfected cells, Ku70 is found predominantly within the nucleus (Figure 3.17a.). However, Ku70 was shown to have an additional location within an infected cell. In keeping with a role in the nucleus there was a strong signal from the nucleus of HeLa cells stained with Ku70 antibody, but there was also a clear co-localisation with DAPI stained viral DNA factories in the cytoplasm. Likewise,

DNA-PKcs also had a predominantly nuclear localisation in uninfected cells, but co-localised with viral DNA factories in the cytoplasm (Figure 3.17b).

These data represents the first demonstration of a candidate DNA sensor associated with sites of VACV replication.

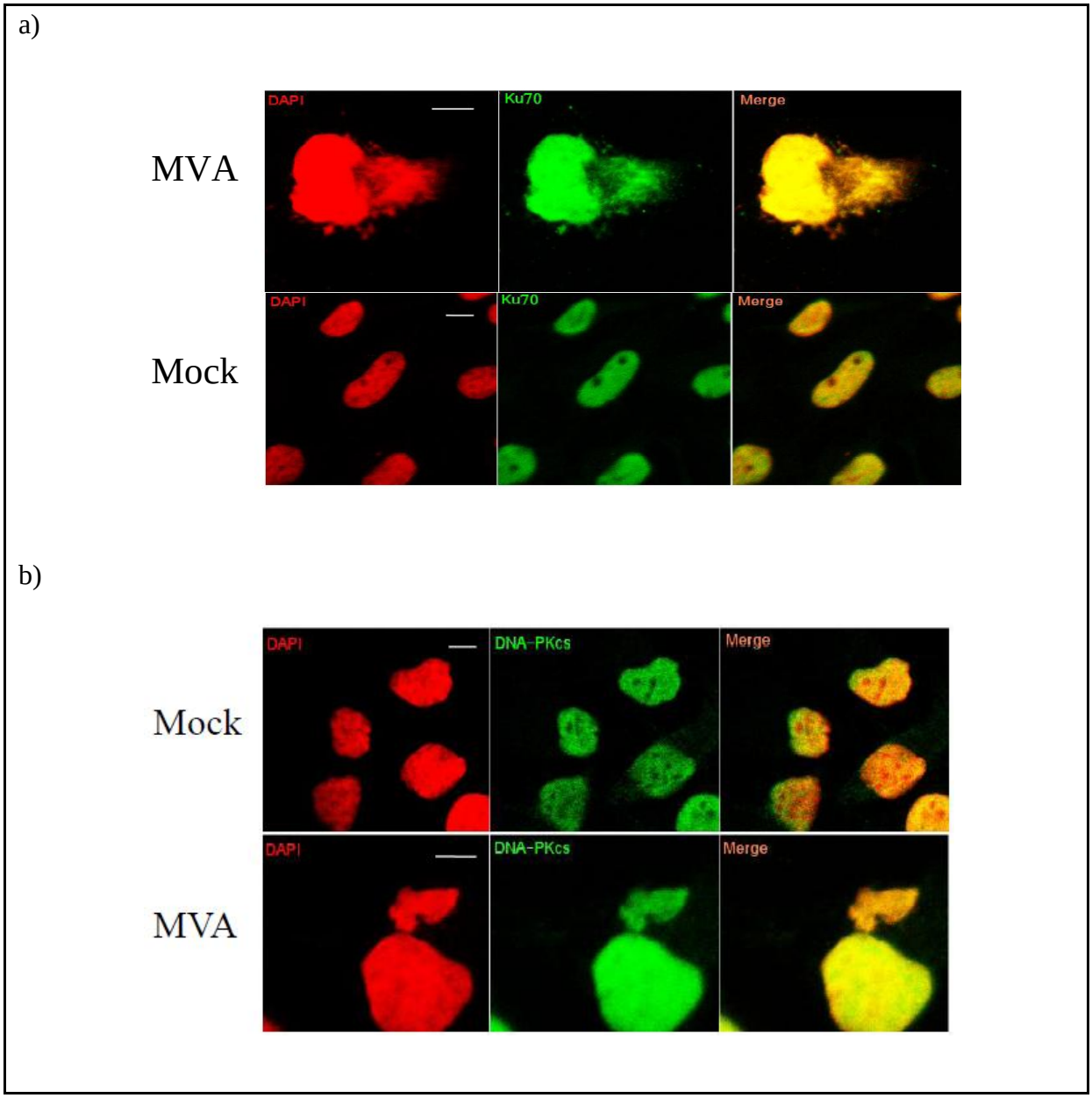

Figure 3.17. DNA-PK components co-localise to viral DNA factories. HeLa cells were infected with MVA for 16 h at 2 p.f.u. / cell or mock-infected. Cells were then fixed with paraformaldehyde and stained with DAPI and antibodies raised against either (a) Ku70 or (b) DNA-PKcs followed by an anti-mouse-FITC-conjugated secondary antibody. Scale bars, 5 μm.

These data are consistent with data presented in Figure 3.5 showing that DNA-PK components are capable of binding to DNA in the cytoplasm and suggests that DNA-PK is recruited to sites of viral DNA replication, presumably to viral DNA itself.

3.3.2. DNA-PK components are essential for MVA-mediated innate immune signalling in MEFs.

To investigate if DNA-PK was needed for innate immune sensing in response to poxvirus infection, wild type MEFs or MEFs lacking components of the DNA-PK complex were infected with differing amounts of MVA, and the amounts of mRNA for specific markers of innate immune activation (*Cxcl10* and *Il6*) were measured by qRT-PCR (Figure 3.18).

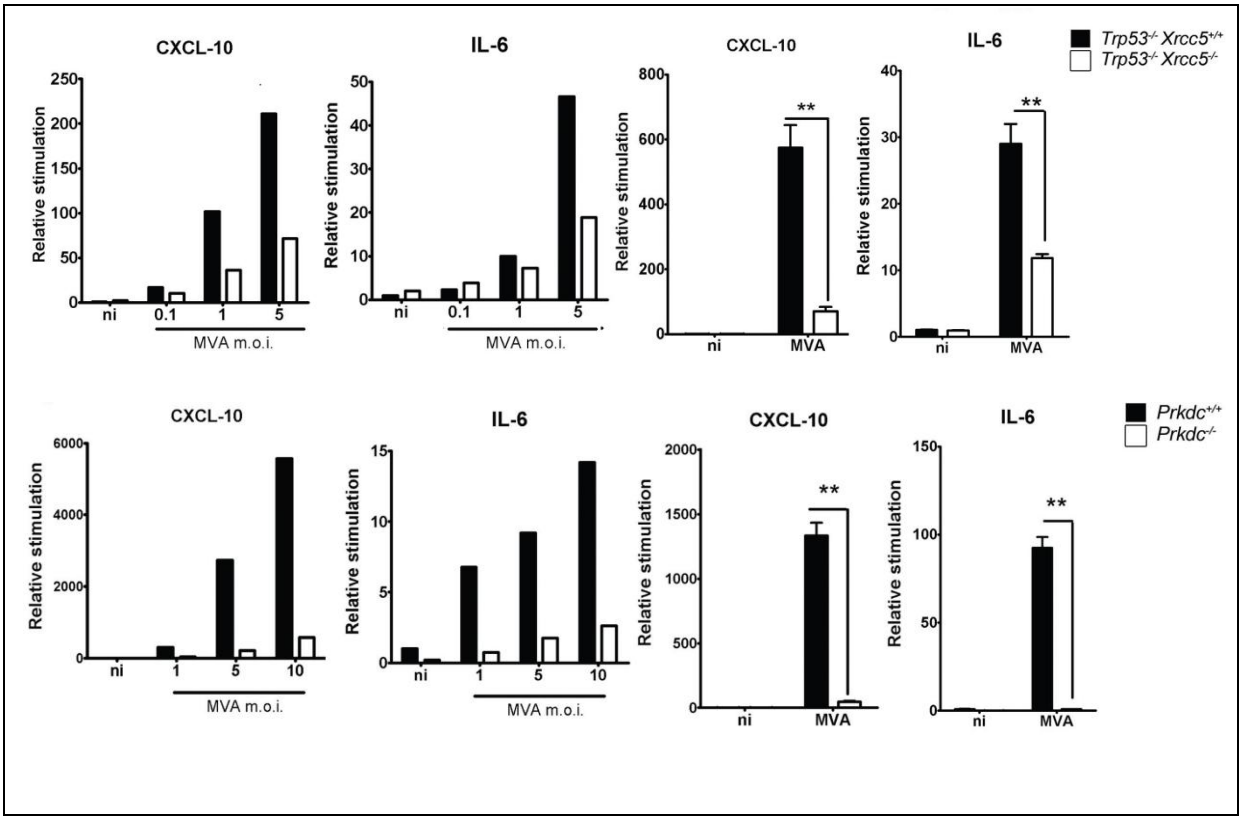

Figure 3.18. Upregulation of *Cxcl10* and *Il-6* following infection by VACV MVA is dependent upon DNA-PK components. MEFs lacking either Ku80 (*Xrcc5*$^{-/-}$, top panel) or DNA-PKcs (*Prkdc*$^{-/-}$, bottom panel) and MEFs from wild-type littermate controls were infected with MVA at the indicated p.f.u. per cell. RNA was extracted after 8 h and analysed by qRT-PCR with primers specific for *Il6* or *Cxcl10*. ni, non-infected. Stimulation was calculated relative to *Hprt*. Error bars +/- SEM (n ≥3), ** p<0.01 .

An increased production of *Cxcl10* and *Il6* mRNA was observed with increasing quantities of MVA used to infect cells (Figure 3.18, left-hand panels). Whilst MVA infection induced cytokine and chemokine production in wild-type MEFs, this response was markedly reduced in MEFs lacking Ku80 (Figure 3.18, upper panels). A defect in chemokine and cytokine up-regulation was also observed in MEFs lacking DNA-PKcs (Figure 3.18, lower panels), indicating that loss of these components of DNA-PK caused impaired innate immune responses to VACV. The deficiency in chemokine and cytokine upregulation following infection appeared to be more severe in MEFs lacking DNA-PKcs than Ku80, though both were statistically significant. Quantification of these changes showed that MEFs lacking Ku80 showed at least a 60% decrease in production of *Il6* mRNA compared to wild-type cells, whereas this figure approached 95% in MEFs lacking DNA-PKcs (Figure 3.18).

These data suggest that upon MVA infection of fibroblasts, viral genomic DNA is itself the PAMP responsible for the activation of transcriptional upregulation of pro-inflammatory cytokines and chemokines.

3.3.3. DNA-PK is not required for VACV replication

Whilst DNA-PK components were shown to be important for the upregulation of pro-inflammatory cytokines and chemokines, it was important to assess whether this complex was important for VACV replication since one interpretation of Figure 3.18 could be that VACV DNA simply does not accumulate at similar levels in MEFs with or without DNA-PK. However, it was known already that viruses with or without C16 replicated well (Fahy et al., 2008), suggesting that C16 and therefore Ku are not principally involved in a replicative process within infected cells. In order to determine the replicative capacity of VACV DNA during infection with MVA in the presence or absence of the Ku heterodimer, wild-type and

Xrcc5$^{-/-}$ MEFs were infected with MVA and levels of viral DNA were measured at different time points (Figure 3.19). It should be noted that if either Ku70 or Ku80 is absent, the other component of the Ku heterodimer is unstable and is essentially also absent. As such, *Xrcc5*$^{-/-}$ cells can be considered Ku null cells (Errami et al., 1996; Singleton et al., 1997).

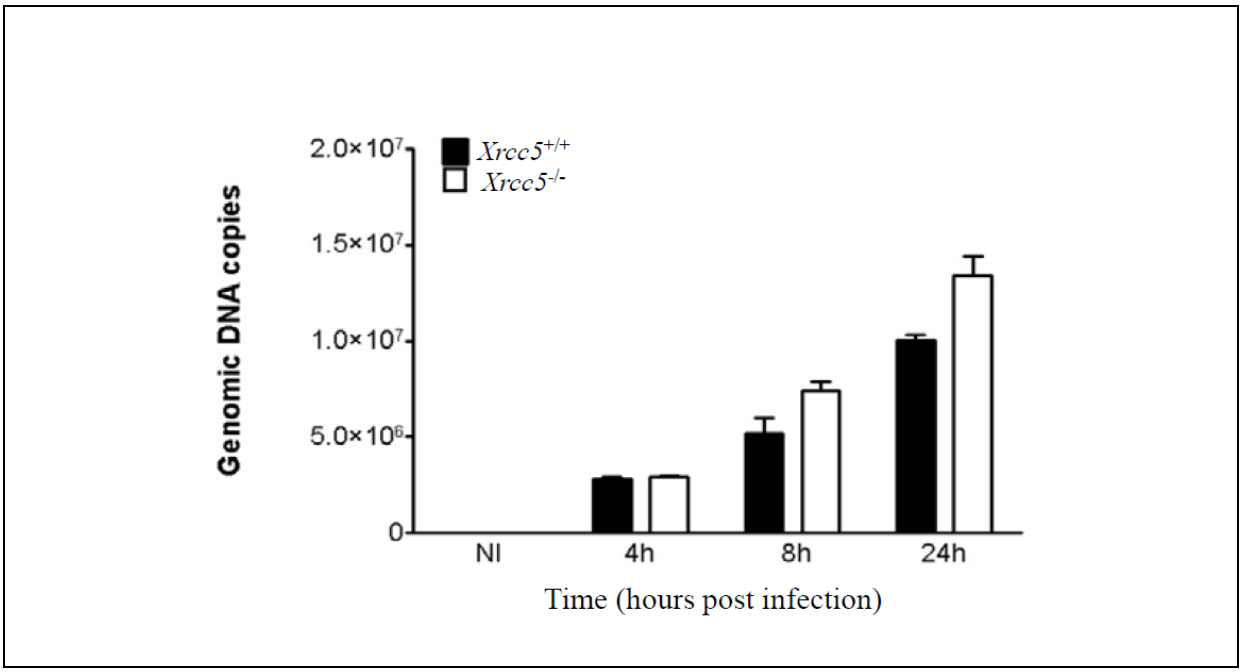

Figure 3.19. MVA DNA replication is not affected by the absence of the Ku heterodimer. Wild-type and *Xrcc5*$^{-/-}$ MEFs were infected with MVA with 2 p.f.u./cell for the indicated times. Cells were lysed, treated with proteinase K and the number of VACV genomic DNA copies was assessed with qRT-PCR using primers specific to the MVA genome. The absolute quantification based upon dilutions of proteinase K-treated purified virus of a known infectious titre (for a detailed description of the experimental procedure refer to section 2.1.16). Error bars +/- SEM (n≥3).

Whilst the copy number of MVA genomes increased over time, there was no statistically significant difference in MVA DNA replication in the presence or absence of Ku80, although there was a slight tend towards increased viral DNA loads in *Xrcc5*$^{-/-}$ MEFs

(Figure 3.19). This shows that the Ku heterodimer is not required for VACV DNA replication and that the defect in *Cxcl10* mRNA upregulation noted in Figure 3.18 is not likely to be due to a lack of viral DNA accumulation.

The fact that Ku is targeted by the VACV C16, an immunomodulatory VACV protein, further supports the hypothesis that Ku has an alternative role during the course of VACV infection, such as DNA sensing.

3.3.4. *Cxcl10* mRNA upregulation in MEFs upon MVA infection is dependent on IRF-3 and TBK-1.

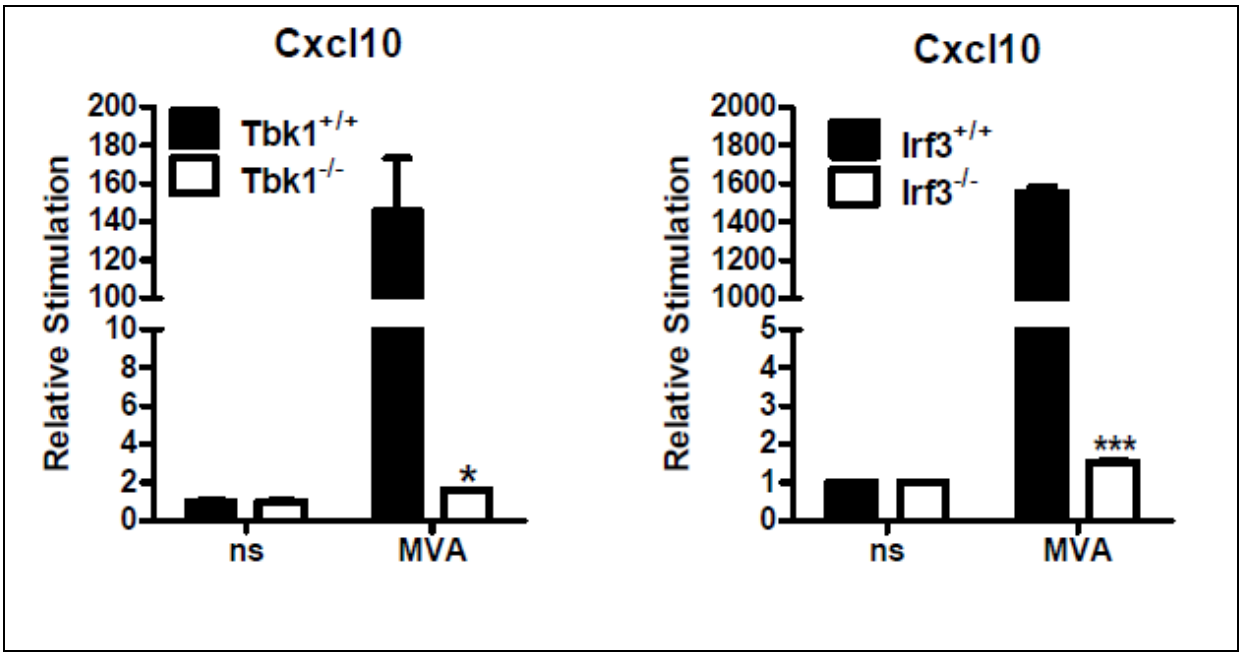

Figure 3.20. Response to MVA is abrogated in cells lacking *Irf3* and *Tbk1*. *Irf3*$^{-/-}$, *Tbk1*$^{-/-}$ and wild-type littermate control MEFs were infected with MVA at 5 p.f.u. per cell for 8 h. qRT-PCR analysis was performed on RNA extracted from these cells with primers specific for *Cxcl10*. ns, non-stimulated. Stimulation was calculated relative to *Hprt*. Error bars +/- SEM (n ≥3), *** p<0.001, * p<0.05.

Next, the pathway through which DNA-PK sensing of MVA signalled was investigated using MEFs lacking specific signalling molecules or transcription factors. Whereas, infection of MEFs with MVA induced the upregulation of various chemokine and cytokine RNA species, such as *Cxcl10* & *Il-6*, in MEFs lacking *Irf3* there was no detectable *Cxcl10* upregulation (Figure 3.20).

Since IRF-3 is phosphorylated at its C terminus by TBK-1 (Fitzgerald et al., 2003; Sharma et al., 2003), the role of TBK-1 in MVA induced *Cxcl10* mRNA upregulation was also investigated. $Tbk1^{+/+}$ MEFs induced *Cxcl10* robustly after infection with MVA for 8 h, however this induction was completely absent in MEFs lacking *Tbk1* (Figure 3.20). This indicates that TBK-1, as well as its kinase substrate IRF-3, are both required for the upregulation of *Cxcl10* mRNA following infection with MVA. These data indicate that DNA-PK, TBK-1 and IRF-3 are all required for innate immune activation following MVA infection, as they are for DNA transfection, and this implies that during the course of infection MVA DNA stimulates IRF-3 dependent genes in MEFs.

Since DNA-PK localised to viral factories and was found to be required for full activation of innate immune signalling in response to MVA, and that IRF-3 and TBK-1 were also found to be required for innate immune signalling activation in response to MVA, the localisation of both TBK-1 & IRF-3 during MVA infection was subsequently investigated. To assess the localisation of these molecules during MVA infection, HeLa cells were infected with MVA and immunofluorescent staining was performed (Figure 3.21).

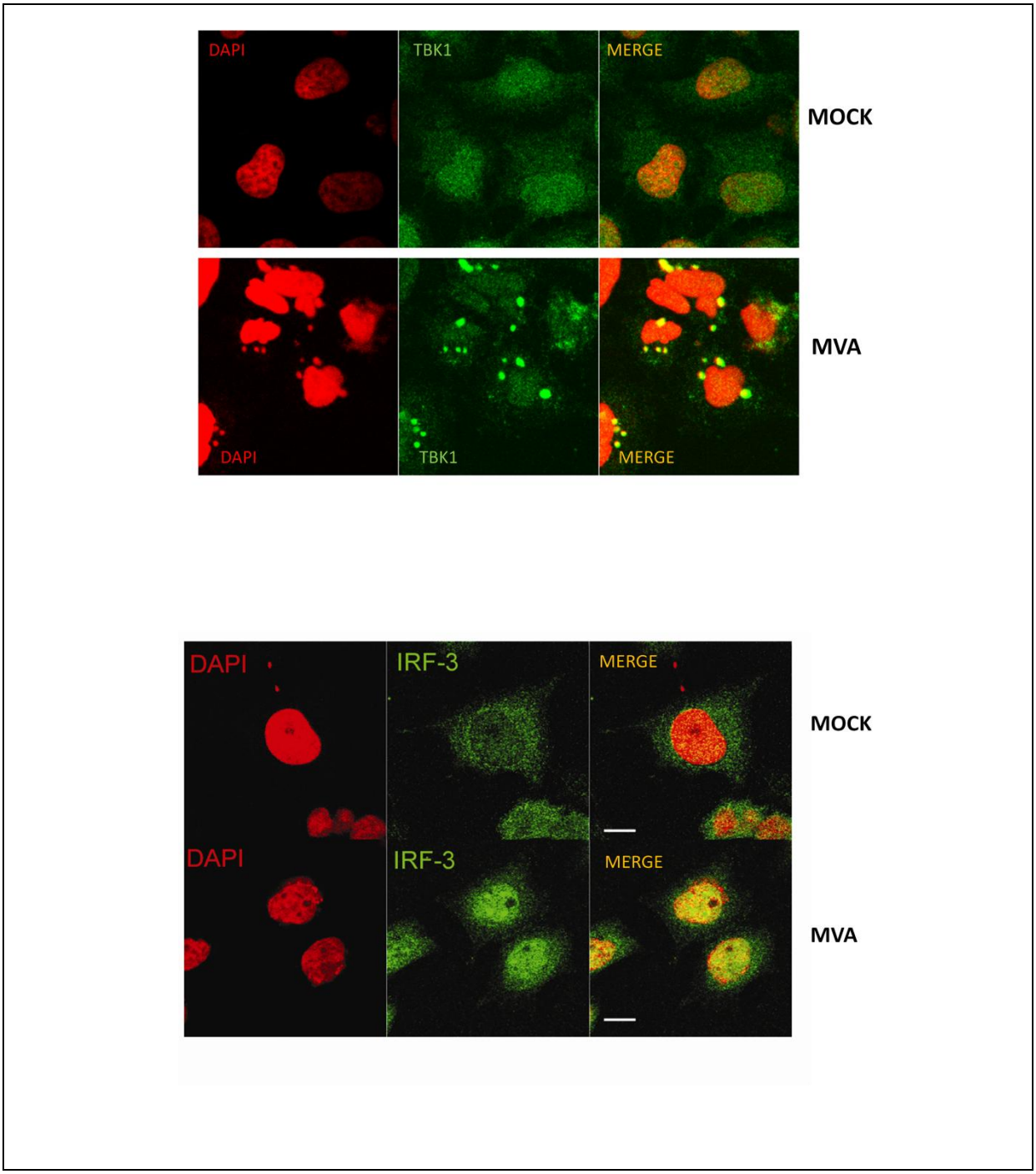

Figure 3.21. Localisation of TBK-1 and IRF-3 during MVA infection. HeLa cells were infected for 16 h at 2 p.f.u. / cell, fixed with paraformaldehyde and then stained with DAPI and either antibodies raised against TBK-1 or IRF-3 followed by an anti-mouse-FITC-conjugated secondary antibody. Scale bars, 5 μm.

Infection of HeLa cells with MVA lead to the formation of characteristic cytoplasmic virus factories. TBK-1 in uninfected cells had a diffuse localisation, however upon infection with MVA, TBK-1 was found to co-localise with the discrete cytoplasmic MVA virus factories (Figure 3.21), indicating that TBK-1 is recruited to sites of viral replication, as is the case with DNA-PK (Figure 3.17).

IRF-3, was found to have a broadly cytoplasmic localisation in uninfected cells, consistent with previous literature, reviewed in previously (Honda and Taniguchi, 2006). However, in contrast to TBK-1, upon infection with MVA IRF-3 was found to translocate to the nucleus (Figure 3.21), consistent with its role as a transcription factor and the activation of IRF-3 in response to MVA infection (Figure 3.21 and Ishii *et al*., 2006).

Together, these data suggest that TBK-1 is found at sites of DNA replication during VACV infection, along with Ku70 and DNA-PKcs and that IRF-3 is activated during VACV infection.

DNA-PK has been shown to phosphorylate IRF-3 at its N terminus (Karpova et al., 2002). Phosphorylation of IRF-3 at its C terminus, however, is the phosphorylation event which leads to the dimerisation and nuclear translocation involved in its role in innate immune signalling (Lin et al., 1999; Kumar et al., 2000). The role of the N-terminal phosphorylation by DNA-PK, if any, is unlikely to play a part in DNA-mediated innate immune activation since DNA sensing operates normally in cells lacking the kinase domain of DNA-PK (Shirota et al., 2006). In addition, if DNA-PK-mediated phosphorylation of IRF-3 was sufficient for activation of innate immune signalling, TBK-1 and other intermediate molecules would likely be dispensable- and the evidence presented demonstrate that this is not the case. The role of N-terminal phosphorylation of IRF-3 by DNA-PK, if any, in innate immune signalling remains to be elucidated.

The observation that TBK-1, is recruited to sites of MVA replication, along with DNA-PK suggests that phosphorylation of IRF-3 occurs close to the viral factories. However, STING has been shown to translocate to perinuclear vesicles after stimulation by DNA (Ishikawa et al., 2009; Saitoh et al., 2009). STING is likely to be upstream of TBK-1 in the DNA-mediated innate immune activation pathway since STING shows DNA-specific activation whereas TBK-1 is activated by DNA and RNA, so the location of STING during DNA virus infection, such as MVA, merits attention. The role of the documented perinuclear vesicles during infection, and indeed whether or not these vesicles are formed upon infection, or whether these are recruited to viral factories is also of interest. However, the localisation of STING during MVA infection has not yet been assessed due to reagent availability.

Collectively, these data strongly suggest that viral DNA acts as a PAMP during MVA infection. Understanding that it is this DNA which leads to innate immune activation upon infection with MVA might lead to a better basis for MVA-based vaccine design since it is commonly used as a vaccine vector.

3.4. GAGA is an immunostimulatory DNA sequence

Previously, it had been noted that Ku binds to a DNA sequence, termed NRE1, in the mouse mammary tumour virus (MMTV) genome, specifically 5'GAGAAAGAGAA3' (which we have termed 'GAGA') on both ssDNA (Torrance et al., 1998) and dsDNA (Giffin et al., 1996). Intriguingly, by inspection of the genomic sequences of several orthopoxviruses, it was found that this sequence also occurs at a high frequency in the genomes of some of these viruses. For example, despite the number of base pairs required to have expected the sequence to occur once being approximately 4×10^6, the MVA genome, with a genome length of approximately 1.78×10^5 contains 134 copies of GAGA (Table 3.2). Notably, the

GAGA sequences were found exclusively at the termini of the genome. In highly passaged VACV strains (e.g. MVA), multiple copies of GAGA were observed, however in all variola virus strains examined (such as India 1953 New Delhi, Tanzania 1965 & UK 1946 Harvey), only one copy of GAGA was noted.

Virus	Genome Length (kbp)	Occurrences of GAGAAAGAGA (range of distance from genome terminus in kbp)	Occurrences of GAGAAAGAGAA (range of distance from genome terminus in kbp)
VACV-WR	194.7	**88** (0.2-3.3)	**26** (1.1-3.3)
VACV-COP	191.7	**104** (0.2-4.0)	**28** (2.8-4.0)
VACV-MVA	177.9	**170** (0.3-6.0)	**134** (0.8-6.0)
VARV- UK1946 Harvey	185.7	**4** (0.1-0.6)	**1** (0.6)
VARV- India 1953 New Delhi	186.7	**17** (0.1-1.4)	**1** (1.4)
VARV- Tanzania 1965	185.8	**10** (0.1-0.5)	**1** (0.5)
ECTV-Moscow	209.8	**20** (0.4-1.3)	**2** (1.3)
MOCV – Subtype 1	190.3	**2** (28.7)	**1** (28.7)
Camelpox-CMS	202.2	**8** (0.1-0.3)	**2** (0.3)
Cowpox- Brighton Red	224.5	**68** (0.2-2.4)	**68** (0.2-2.4)
Monkeypox – Sierra Leone	198.8	**2** (0.1)	**0** (0)
Fowlpox- Iowa	288.5	**1** (133.3)	**1** (133.3)
Fowlpox- FP9	266.0	**1** (117.0)	**0** (0)

Table 3.2 Table showing the prevalence of GAGA in several orthopoxviruses and molluscum contagiosum virus. The above genome sequences were interrogated for 5' GAGAAAGAGA 3' and 5' GAGAAAGAGAA 3' occurrences. The distances of the GAGA sequences from the genome termini are given in kbp to represent nearest and furthest occurrences of GAGA from the genome terminus. VARV; variola virus, ECTV; ectromelia virus, MOCV, molluscum contagiosum virus.

Table 3.2 demonstrates several interesting features of GAGA and poxviruses. Firstly, GAGA is highly prevalent across multiple members of the *Orthopoxvirus* genus, including VACV, variola virus, molluscum contagiosum virus, camelpox virus, cowpox virus, monkeypox virus and ectromelia virus. An abbreviated version of GAGA, lacking the final 3' adenine was also searched for and in many instances there were many more copies of the shortened sequence of GAGA. The minimum sequence required for Ku binding to GAGA has not been determined and it is possible that the final adenine is not required for specific Ku binding.

Secondly, with the exception of MOCV, GAGA is found exclusively at the genome termini. For example; MVA has 134 copies of GAGA, yet these are not evenly distributed across the genome but are packaged tightly between 849-bp and 5,963-bp from each end of the genome. In addition, when only one copy of GAGA is found, such as the three strains of VARV examined- GAGA is located between 544-bp (Tanzania) and 1426-bp (India, New Delhi, 1953) from the genome terminus.

Thirdly, there is a loose correlation between the number of passages of virus in tissue culture and GAGA copy number. Primary virus isolates, such as the variola virus strains, tend to have fewer copies of GAGA, whereas extensively passaged viruses, such as MVA and CPXV strain Brighton Red, appear to have accumulated GAGA copies. The reason for this difference in GAGA copy number requires further investigation. It is possible that the low copy number of GAGA in primary virus isolates might imply negative selection in the presence of a functional immune system.

Furthermore, GAGA was only shown to be present once in the Fowlpox virus strains Iowa and FP9, indicating that GAGA is not common to all poxviruses. In the MOCV and Fowlpox virus strains containing only one copy of GAGA, it was not found in the ITRs.

Poxvirus ITRs vary enormously in length and it is thought that ITR sequences can be duplicated and transposed (Moyer et al., 1980), and the amount of duplication and transposition will likely depend on passage number. Therefore, the variability of GAGA occurrence across different strains is likely to be function of chance and passage number of the virus strain.

However, since Ku has been found previously to bind to GAGA, that Ku has been characterised as a potential cytoplasmic DNA sensor, and that poxviruses, especially highly attenuated strains, had large numbers of GAGA in their genomes, the relative immunogenicity of DNA molecules with or without GAGA was investigated.

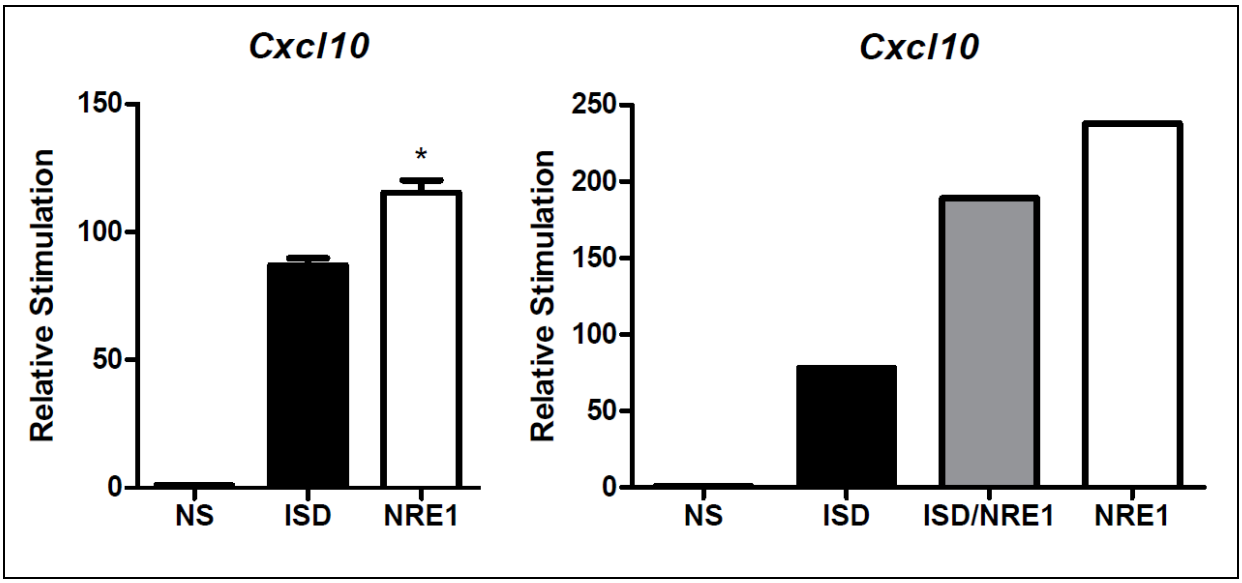

Figure 3.22 NRE1 is more immunostimulatory than a control DNA sequence. WT MEFs were stimulated with 5 μg/ml 90-bp oligonucleotides of the indicated DNA species. qRT-PCR analysis was performed on RNA extracted 8 h post stimulation. NS, Non-stimulated. Stimulation was calculated relative to *Hprt*. Error bars +/- SEM (N≥3), * $p<0.05$

Quantitative RT-PCR analysis was performed in MEFs transfected with different DNA species (Figure 3.22). ISD DNA, a DNA sequence used extensively in the literature and does not contain a GAGA sequence was compared with the NRE1 sequence, which is a 45-bp sequence with one copy of GAGA, originally identified as the target for Ku binding (Giffin et al., 1996). Relative to the control ISD sequence, NRE1 was more immunostimulatory than ISD as measured by qRT-PCR analysis of *Cxcl10* mRNA induction. In addition, this immunostimulatory potential was observed to be dose-dependent because 100-bp oligonucleotides containing 2 copies of the NRE1 sequence was more immunostimulatory than an oligonucleotide containing 1 copy of NRE1 and 1 copy of ISD, which in turn stimulated higher levels of *Cxcl10* mRNA induction than an oligonucleotide containing 2 copies of the ISD control sequence (Figure 3.22, right panel).

Together, these observations provide evidence that GAGA is an immunostimulatory DNA sequence presumably mediated by its specific interaction with Ku. Further work will be required to confirm these initial findings and explore the potential contributions GAGA has to poxviral replication and any potential role for inclusion of GAGA in DNA vaccines for enhanced immunostimlatory effects.

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3.5. The role of C16 in VACV infection

3.5.1. C16 blocks DNA sensing, but not RNA sensing

As C16 interacts with Ku, and Ku binds to DNA and activates an IRF-3-dependent innate immune response, it was hypothesised that C16 might be acting to inhibit this pathway to avoid activation of the immune response mediated by VACV DNA during infection. To assess this, MEFs were transfected with a plasmid containing a C16 open reading frame that had been codon optimised for expression in mammalian cells (pcDNA4/TO-coC16) or an empty vector (pcDNA4/TO) along with greater masses of linear DNA or poly (I:C) (Figure 3.23). Transfection of MEFs with plasmid DNA induces activation of the innate immune system in the manner described previously. To assess the contribution of C16 to subversion of DNA-mediated innate immune activation, simultaneous transfection of plasmid and linear DNA or poly (I:C) was required.

Codon optimisation of the C16 ORF was required since the virally-encoded C16 mRNA, along with many other VACV mRNAs, was found to have cryptic splice sites. During the course of infection, the splice sites are not significant as the transcription of these ORFs occurs in the cytoplasm. However, during expression of C16 via plasmid transfection the mRNA is exposed to nuclear splicing machinery and therefore the relative protein production is dramatically reduced compared with virus infection (data not shown).

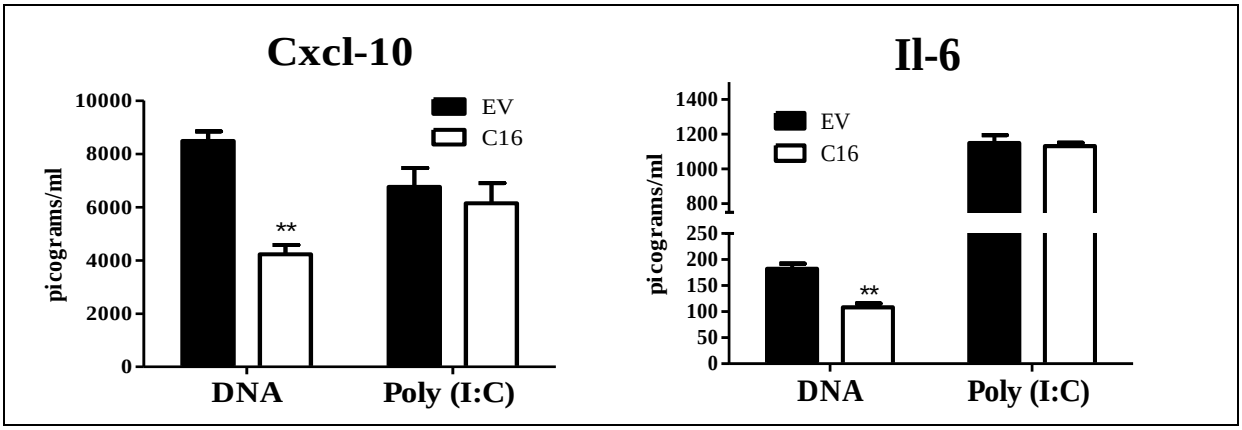

Figure 3.23. Cxcl10 and Il-6 ELISA following DNA and RNA stimulation of MEFs. Wild type (*Trp53*$^{-/-}$ *Xrcc5*$^{+/+}$) MEFs were transfected with 200 ng/ml pcDNA4/TO (EV) or pcDNA4/TO encoding codon optimised C16 (pcDNA4/TO-coC16) and 5 μg/ml concatamerised DNA or 2 μg/ml poly (I:C). The presence of Cxcl10 or Il-6 were measured in the supernatants at 24 h post transfection. Error bars +/- SEM (N≥3), ** $p<0.01$.

Transfection of cells with plasmid and either DNA or poly (I:C) produced robust responses to these molecules, including the production of Cxcl10 and Il-6. In the presence of C16, there was significantly lower production of both Cxcl10 and Il-6 in the supernatants of transfected cells when DNA was co-transfected, however no such difference was observed in either Cxcl10 or Il-6 secretion when poly (I:C) was co-transfected (Figure 3.23), indicating that the inhibitory effect of C16 is specific to DNA and not RNA sensing pathways. These observations strongly indicate that the C16 protein can act to inhibit the innate immune response to DNA.

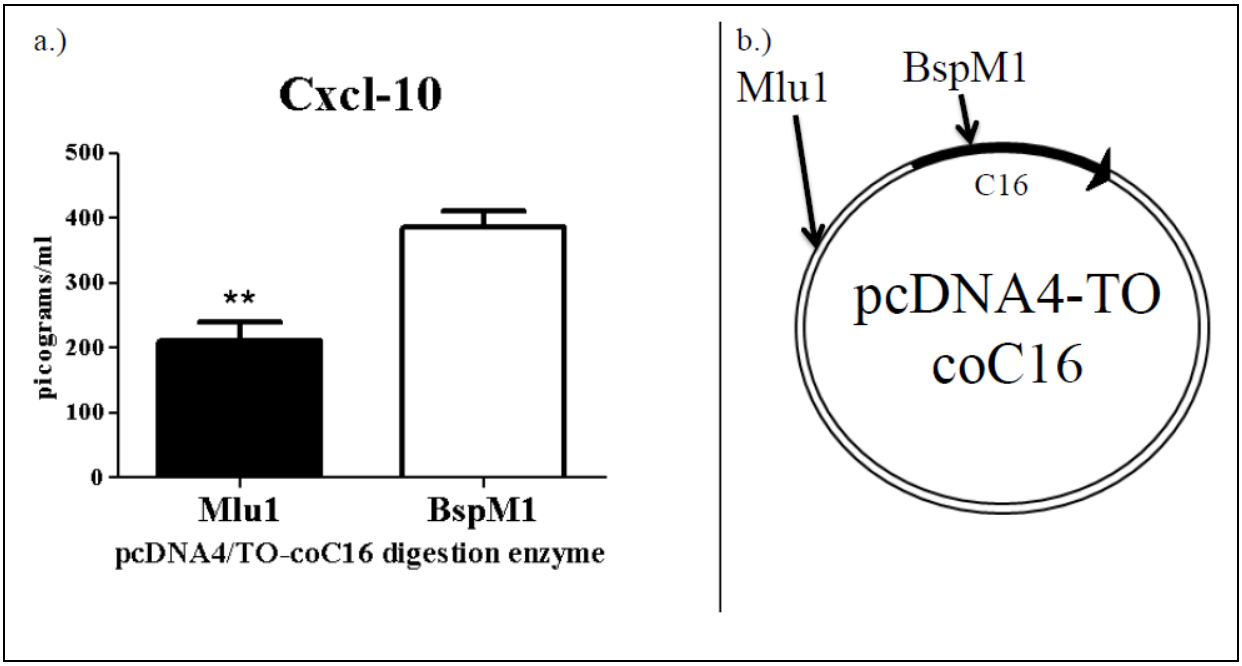

Figure 3.24. Disruption of the C16 ORF ablates inhibition of DNA sensing. (a.) WT MEFs were transfected with 5 μg/ml of pcDNA4/TO-coC16 that had been linearised by digestion with either *Bsp*MI or *Mlu*I. Agarose gel electrophoresis and subsequent purification was used to isolate digested plasmid. After 16 h the amount of Cxcl10 in the culture medium was measured by ELISA. Error bars +/- SEM (N≥3), ** $p<0.01$. (b.) Schematic demonstrating locations of *Bsp*MI and *Mlu*I restriction digestion sites on the pcDNA4/TO-coC16 plasmid.

To control for any irregularities presented by co-transfection of circular plasmid and linear dsDNA oligonucleotides, the pcDNA4/TO-coC16 plasmid was linearised by digestion with either the enzyme *Mlu*I, which leaves the C16 ORF and promoter intact, or *Bsp*MI which cleaves the plasmid within the C16 ORF rendering the plasmid unable to express C16 protein after transfection. In this way, the stimulus is also the delivery vehicle for C16 expression, thus no cell stimulated with DNA would be without the pcDNA4/TO-coC16 plasmid.

Transfection of MEFs with pcDNA4/TO-coC16 digested with *Mlu*I (i.e. with the C16 ORF intact) lead to a significantly lower production of Cxcl10 compared with pcDNA4/TO-

coC16 digested with *Bsp*MI (which destroys the C16 ORF). These data are consistent with, and support, the hypothesis that C16 inhibits innate immune activation following DNA stimulation, and it was hypothesised that this inhibitory function was mediated by its interaction with Ku.

3.5.2. The inhibition of cytokine/chemokine production mediated by C16 is dependent on Ku

To investigate if this immunomodulatory effect of C16 was mediated by targeting Ku, the same transfection assay with empty vector control or plasmid expressing C16 in combination with immunostimulatory linear DNA was performed in *Xrcc5*$^{-/-}$ MEFs (Figure 3.25) and Cxcl10 and Il-6 protein production were measured by ELISA.

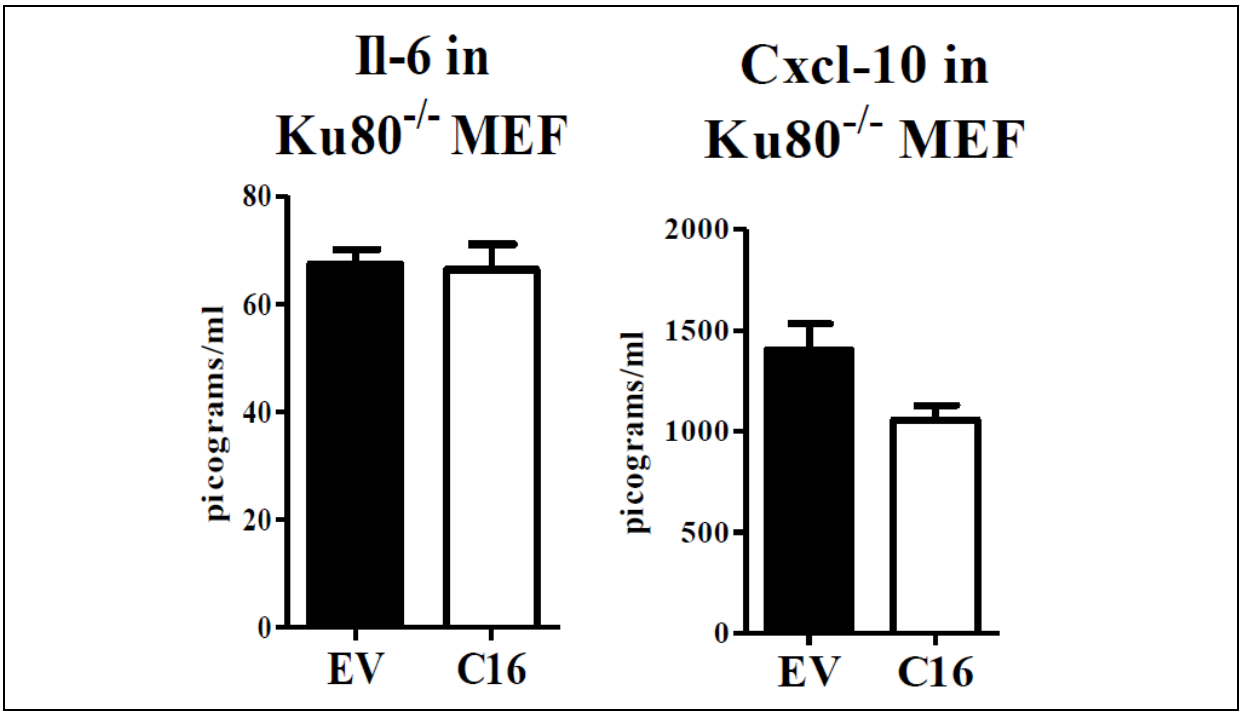

Figure 3.25. Cxcl10 and Il-6 ELISA following DNA and RNA stimulation of Ku80$^{-/-}$ MEFs. *Trp53*$^{-/-}$*Ku80*$^{-/-}$ MEF were transfected with 200 ng/ml pcDNA4/TO (EV) or pcDNA4/TO encoding codon optimised C16 (C16) and 5 μg/ml concatamerised DNA or 2 μg/ml poly (I:C). The presence of Cxcl10 and Il-6 were measured by ELISA in the supernatant at 24 h post transfection. Error bars +/- SEM (N≥3).

The transfection of plasmid and immunostimulatory DNA induced Cxcl10 and Il-6 production in *Xrcc5*$^{-/-}$ MEFs (Figure 3.25), however the concentrations of these proteins were considerably lower compared to those observed in wild-type MEFs on the same plate on the same day (Figure 3.23), which supports the hypothesis that Ku is a DNA sensor. The fact that some activation of *Xrcc5*$^{-/-}$ cells was observed can be explained by either the activation of an alternative DNA sensor such as IFI16 (Unterholzner et al., 2010) occurring in *Xrcc5*$^{-/-}$ MEFs, or the Ku heterodimer aiding detection of DNA by DNA-PK but not being an absolute requirement. This latter explanation would be consistent with the ability of DNA-PKcs to bind to DNA in the absence of Ku, albeit less efficiently (Yaneva et al., 1997; Hammarsten and Chu, 1998).

Whilst C16 displayed a strong negative immunomodulatory effect to DNA sensing in wild-type cells, no such effect was seen in *Xrcc5*$^{-/-}$ MEFs, since transfection with either C16 or empty vector induced similar levels of Cxcl10 and Il-6 production (Figure 3.25), suggesting that the function of C16 is dependent on its interaction with the Ku heterodimer. A slight reduction was observed in Cxcl10 production following transfection with C16; however this was not statistically significant.

3.5.3. Absence of C16 leads to increased production of cytokines and chemokines *in vivo* following infection by VACV

Whilst C16 blocked DNA sensing in cell culture, and this effect was mediated by Ku, it was not clear what effect this host-virus protein interaction might have *in vivo* due to the redundancy reported previously in DNA sensing (Wang et al., 2008) and also because VACV produces many other inhibitors of innate immune signalling. To address the role of C16, and indirectly the role of Ku, *in vivo,* BALB/c mice were infected intranasally with wild-type

(WR), C16-knockout (vΔC16) and revertant (rev) viruses and the innate immune response was measured over the first 72 h (Figure 3.26).

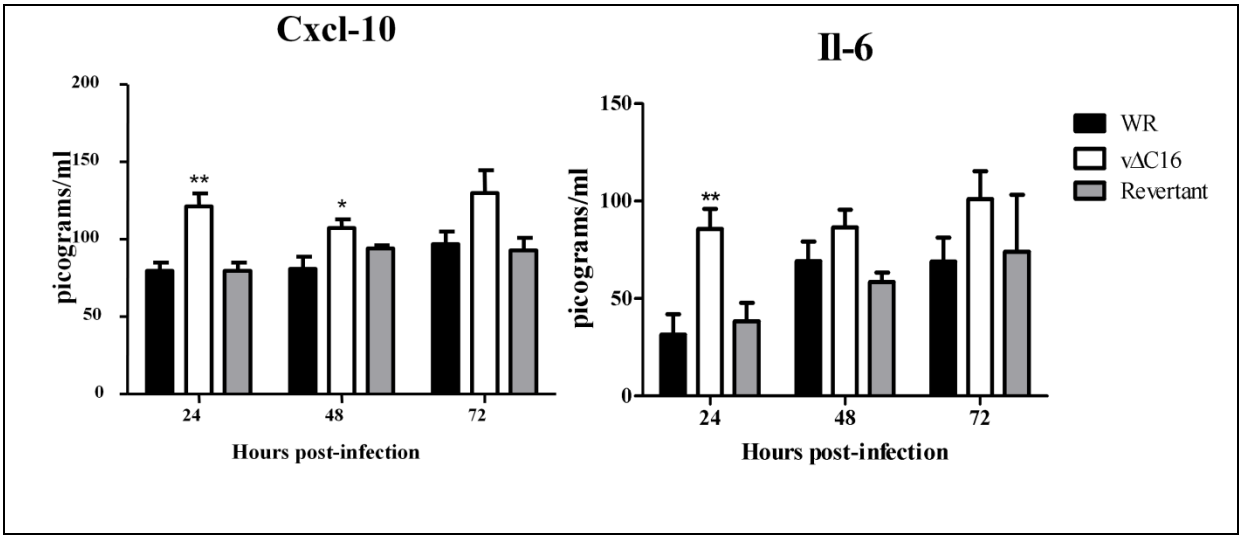

Figure 3.26. C16 affects Cxcl10 and Il-6 production in vivo. Groups of five BALB/c mice were infected intranasally with 5×10^4 p.f.u. per mouse of wild-type (WR) C16 knockout (vΔC16) or revertant viruses. Mice were sacrificed at the indicated time points and the amount of Cxcl-10 and Il-6 in BAL fluid were measured by ELISA. Error bars +/- SEM ($N \geq 3$). * $p<0.05$, ** $p<0.01$.

ELISA analysis of bronchoalveolar lavage (BAL) fluid from mice infected intranasally with 5×10^4 p.f.u. showed that infection with VACV induced the production of Cxcl10 and Il-6, however infection with vΔC16 lead to an enhanced production of both of these molecules (Figure 3.26). This effect was statistically significant at 24 and 48 h post infection for Cxcl10, and at 24 h for Il-6, but by 72 h post infection the presence of C16 made no statistically significant difference to the concentration of these molecules in BAL fluid (Figure 3.26).

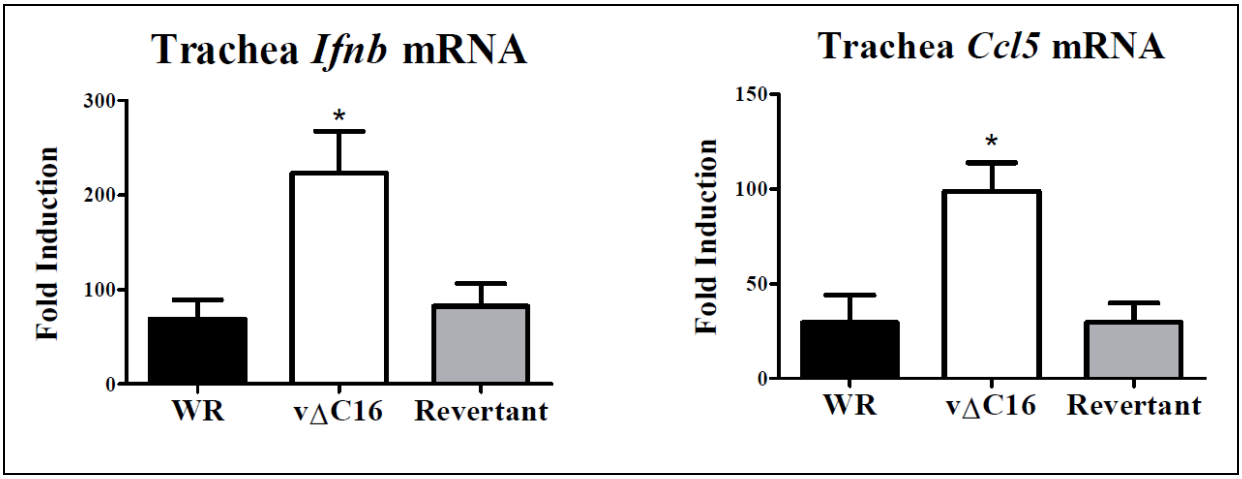

Figure 3.27. C16 affects *Ccl5* and *Ifnb* mRNA transcription in vivo. Groups of five BALB/c mice were infected intranasally with 5 x 10^4 p.f.u. of wild-type (WR) C16 knockout (vΔC16) or revertant viruses. Mice were sacrificed 24 h post infection and qRT-PCR analysis was performed on RNA extracted from tracheas. Data were normalised to *Hprt.* Fold induction refers to induction relative to mock-infected mice. Error bars +/- SEM (N≥3). * $p<0.05$.

Ifnβ was undetectable in BAL fluid from mice infected with this dose of virus in this model at these time points tested. Explanations for this might be that the levels of Ifnβ produced in this system are below the detection limits of current ELISA kits, or that the VACV of B18 protein, which binds type-1 IFNs (Symons et al., 1995) prevents detection of Ifnβ by ELISA. To circumvent these potential difficulties, *Ifnb* mRNA levels in tracheal cells were analysed by qRT-PCR (Figure 3.27). This demonstrated that *Ifnb* mRNA transcription was at a higher level in vΔC16 infected mice than either vC16 or revC16 infected mice at 24 h post infection. A similar result was observed for *Ccl5,* indicating that C16 inhibits the transcription of type I IFN as well as Cxcl10, Il-6 and *Ccl5 in vivo.*

Previous work within our laboratory had demonstrated that after infection of mice intranasally the production of IL-1β was not affected by the absence of C16 (Fahy, unpublished data). This was consistent with the previous observation that the production of

this molecule in response to VACV infection is largely due to DNA sensing leading to the activation of the AIM2 inflammasome (Rathinam et al., 2010) and that Ku is not likely to be involved in the formation of this inflammasome (Burckstummer et al., 2009).

These data add to data published previously by our laboratory showing that more leukocytes were recruited to the lungs of mice infected with vΔC16 compared with wild-type and revertant viruses early in the course of infection (Fahy et al., 2008). This data adds more detail to the report published previously regarding the mechanism of action of C16 in virus infection and strongly supports the hypothesis that DNA-PK-mediated activation of the innate immune response to VACV is of biological significance. Although indirect, this data provides the first *in vivo* example of an IRF-3-activating DNA sensor having significant *in vivo* effects in the context of virus infection.

3.5.4. C16 inhibits the interaction of Ku with DNA

Data presented thus far indicate that C16 bound to Ku, blocked Ku-dependent DNA-mediated innate immune signalling *in vitro,* and reduced cytokine production in VACV-infected mice. However, the mechanism of action of C16 was not yet clear.

To address how C16 might be exerting its immunomodulatory effects on Ku, the interaction of the DNA-PK components with DNA in the presence or absence of C16 was investigated using biotinylated DNA to pull down protein complexes from cells .

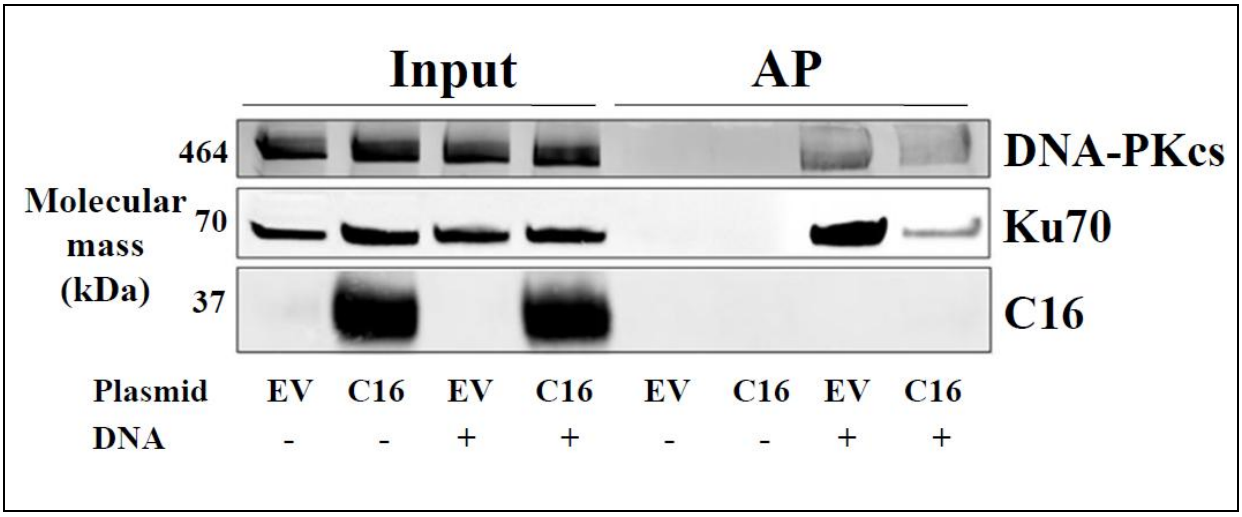

Figure 3.28. C16 affects binding of DNA-PK to DNA. HeLa cells were transfected with 2 μg/ml pcDNA4/TO (EV) or pcDNA4/TO-coC16 (C16). After 24 h, cells were transfected with 5 μg/ml biotinylated DNA for 30 min. Cells were then lysed and biotinylated DNA was affinity purified using streptavidin beads. Inputs and Affinity Purified (AP) complexes were resolved by SDS-PAGE and immunoblotted with antibodies against proteins indicated on the right. Approximate molecular masses of proteins are indicated in kDa. Images were obtained using a Licor Odyssey scanner (Section 2.2.4.2).

In the whole cell lysates it was noted that the level of DNA-PKcs and Ku70 were similar in the presence or absence of DNA, and whether or not C16 was expressed. Quantification of the expression levels using LICOR was performed, and showed a slight but statistically insignificant different increase in DNA-PK components in the presence of C16 (Figure 3.29). The equivalent levels of DNA-PK component molecules rule out the possibility that C16 might degrade, or mark for degradation, the Ku heterodimer.

In the affinity purified samples, C16 did not co-precipitate with DNA (Figure 3.28). This finding ruled out the possibility that C16 bound to DNA directly and thereby would prevent either Ku or DNA-PKcs from binding to DNA. This finding also suggests that it is also unlikely that C16 inhibits Ku after Ku has bound to DNA, since no C16 was detected to co-precipitate with DNA, although Ku was bound to DNA. In addition it was seen that in

cells expressing C16, the amounts of both Ku and DNA-PKcs that co-purified with DNA were reduced compared with cells transfected with empty vector. This inhibition was much more profound for Ku70 than for DNA-PKcs.

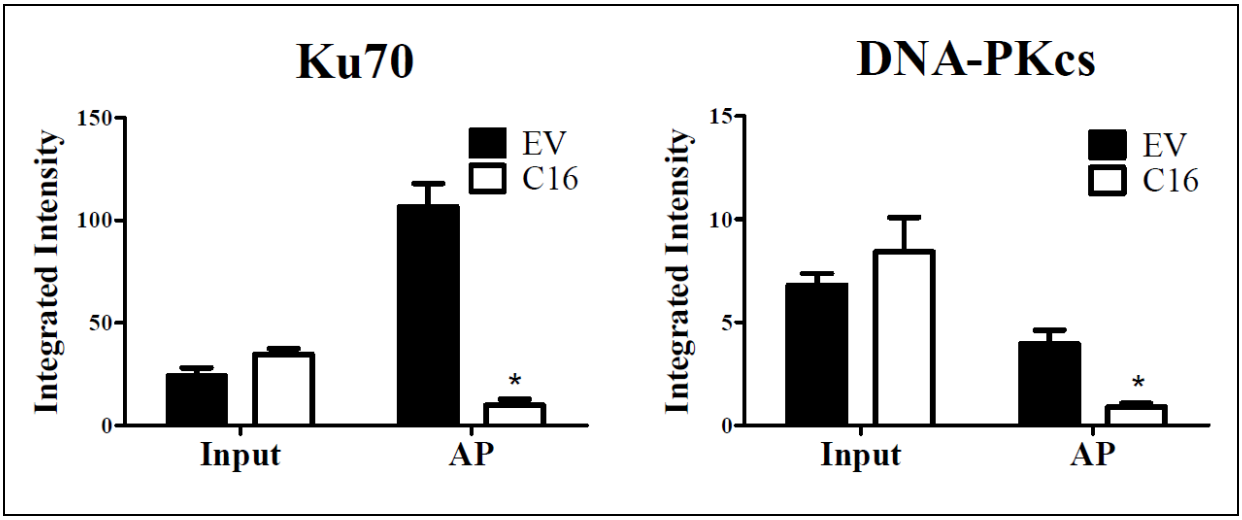

Figure 3.29. C16 affects binding of DNA-PK to DNA. Three wells each of a 6-well plate of HeLa cells were transfected with 2 μg/ml pcDNA4/TO (EV) or pcDNA4/TO-C16 (C16). After 24 h, cells were then transfected with 5 μg/ml biotinylated DNA for 30 min. Affinity purification (AP) was performed on the biotinylated DNA using streptavidin beads. Inputs and affinity purified complexes were resolved by SDS-PAGE and immunoblotted with Ku70 and DNA-PKcs antibodies. Integrated intensity was calculated using infrared imaging by a Licor Odyssey scanner.

The experiment documented in Figure 3.28 was repeated in triplicate and the integrated intensity of the bands was assessed using infrared imaging (Figure 3.29). A statistically significant reduction in Ku70 and DNA-PKcs binding to DNA in the presence of C16 compared with empty vector was demonstrated. The effect of C16 inhibiting the recruitment of Ku to DNA is both quantifiable and reproducible, providing strong evidence that C16 inhibits the recruitment of Ku to DNA and that this is the mechanism by which C16 blocks innate immune signalling in response to DNA.

These data suggest that C16 prevents Ku from binding to DNA, and that DNA-PKcs binding to DNA was reduced in consequence. It is known that DNA-PKcs binding to DNA is increased in the presence of Ku via recruitment to DNA by the C-terminal domain of Ku80 (Gell and Jackson, 1999), but can also occur less efficiently without it (Hammarsten and Chu, 1998). This could explain why C16 does not bind to DNA-PKcs, but nonetheless affects the binding of this molecule to DNA.

Upon binding of DNA by Ku, DNA-dependent conformational changes occur in the Ku heterodimer, and these conformational changes contribute to the recruitment of DNA-PKcs (Lehman et al., 2008). The indication that C16 inhibits the binding of DNA by Ku is entirely consistent with the finding described in Figure 3.3 that C16 interacts with Ku, but not DNA-PKcs, since C16 is inhibiting DNA binding by Ku and therefore inhibits the formation of DNA-PK. Put another way, C16-bound Ku has inhibited DNA-binding activity, and therefore the DNA-PK complex is not assembled, thus C16 would not be expected to also interact indirectly with DNA-PKcs.

Inhibition of Ku binding to DNA by C16 therefore inhibits the assembly of the DNA-PK complex on DNA and it is likely that through this mechanism C16 modulates the host innate immune response to VACV.

3.6. Summary – C16 and Ku

Using an unbiased proteomics-based approach, binding partners for VACV C16 were identified. C16 was found to bind to the Ku heterodimer, a well characterised DNA-repair complex involved in the non-homologous end joining (NHEJ) DNA repair pathway (Downs and Jackson, 2004). Whilst binding partners were found using this method, it is possible that other C16 ligands may exist but were not detected, due to binding being obscured by the TAP-tag.

C16 was not found to have any common binding partners with icIL-1Ra with this method. However, it is possible that common binding partners do exist. For instance, the region of homology between C16 and icIL-1Ra is a short 6 amino acid peptide at the C terminus of both molecules, and the use of a C-terminal TAP-tag might have obscured the positive identification of common binding partners. N-terminal TAP-tagging of C16 might avoid this problem. Alternatively, C16 and icIL-1Ra might share common binding partners pulled down during the TAP experiment but were not identified with Coomassie or silver-staining.

Additionally, another technique now used in our laboratory is the generation of VACV expressing TAP-tagged proteins of interest. Generation of VACV expressing TAP-tagged C16 might highlight binding partners which only interact upon virus infection. For instance, C16 has been shown previously using yeast two-hybrid assays to interact with VACV protein K1 (McCraith et al., 2000), which might suggest that C16 could interact with additional proteins when other virus proteins are produced in the same cell.

However, the finding that C16 interacts with Ku presented an intriguing issue as to the role of C16, a VACV virulence factor, interacting with a protein principally characterised

as a nuclear DNA repair protein. Subsequently, DNA-PK was shown to interact with cytoplasmic DNA, and importantly it also co-localised with sites of viral DNA replication in VACV-infected cells. Binding of DNA-PK to cytoplasmic DNA, and, perhaps more importantly, to VACV factories suggested a role in DNA sensing.

DNA-PK components, Ku70, Ku80 and DNA-PKcs were shown to be important for the transcription of multiple genes including *Cxcl10* and *Il-6* upon stimulation of cells with DNA, this provided the first evidence that DNA-PK was acting as a DNA sensing PRR. Importantly, DNA-PK was found to be responsible for DNA-mediated activation of innate immunity but was not required for LPS- or poly (I:C)-mediated activation. This suggests a DNA specific effect, and is consistent with DNA-PK binding to DNA therefore acting upstream in the DNA-mediated IRF-3 activation pathway.

Previously, DNA-PK had been ruled out as a candidate DNA sensor because BMDMs from mice lacking Ku70 or DNA-PKcs were shown to be as responsive as wild-type cells to DNA-mediated IFNβ upregulation (Stetson and Medzhitov, 2006). Our study, however, has demonstrated that DNA-PK is not expressed in these cells, and therefore this supports the findings of the aforementioned report. In contrast, our study shows that DNA-PK is expressed in MEFs and, furthermore, is crucial for DNA sensing in these cells. Since VACV is capable of infecting many cell types, the epithelial cells and underlying fibroblasts are likely to be the first cells infected during a poxvirus infection and therefore the activation of innate immunity in these cells is of fundamental importance. The presence of DNA-PK at high levels in these cells is also consistent with a role as a primary sensor of infection, unlike other putative DNA sensors, such as DAI which is not detectable at a steady-state but is induced subsequently by IFN (Takaoka et al., 2007; DeFilippis et al., 2010).

IRF-3 is well characterised for its role in innate immune signalling (Tamura et al., 2008), and is also critical for the induction of various cytokines and chemokines in response to transfected DNA (Ishii et al., 2006; Stetson and Medzhitov, 2006). DNA-PK was shown to be required for the translocation of IRF-3 in response to DNA, but not RNA, suggesting that DNA-PK is upstream of IRF-3 in a DNA-specific innate immune signalling pathway. A role has also been demonstrated for the signalling components TBK-1 (Ishii et al., 2008) and STING (Ishikawa et al., 2009) in DNA-mediated activation of innate immunity, and these findings were confirmed in this study. Along with Ku70 and DNA-PKcs, TBK-1 was also found to be recruited to viral factories during MVA infection. TBK-1 is likely to be downstream of STING in the DNA-sensing pathway, because it is the IRF-3 kinase (Fitzgerald et al., 2003; McWhirter et al., 2004). However, the location of STING during MVA infection has not been determined, due to a lack of reagent availability. STING has been reported to translocate to perinuclear vesicles after DNA transfection (Ishikawa et al., 2009; Saitoh et al., 2009), and is also ubiquitylated by TRIM56, resulting in its interaction with TBK-1 (Tsuchida et al., 2010). Therefore, it will be interesting in the future to determine the location of STING during MVA infection and also the role, if any, of the STING-docked perinuclear vesicles during virus infection.

DNA-PK was also shown to be important for the transcriptional induction of *Cxcl10* and *Il6* in response to infection with VACV. MEFs lacking either Ku80 or DNA-PKcs had impaired induction of pro-inflammatory molecules during MVA infection, suggesting that DNA-PK is required for the full activation of the innate immune response to VACV. Also, it was demonstrated that the activation of innate immune signalling pathways during MVA infection of fibroblasts is likely to be principally due to viral DNA activating a DNA-PK-dependent DNA-sensing pathway. This is in contrast with a previous study that reported that

MDA-5 is required for activation of the innate immune response to MVA in macrophages (Delaloye et al., 2009).

GAGA is a DNA sequence reported previously to be bound by Ku with a particularly high affinity (Giffin et al., 1996). GAGA was also noted to be present at a particularly high frequency in the termini of various poxvirus strains. Stimulation of cells with a DNA sequence including GAGA was found to stimulate cells more strongly than a control DNA sequence lacking GAGA, suggesting that this sequence might represent a particularly immunogenic sequence detected by DNA-PK. However, why poxviruses would maintain this sequence in the genome at all merits attention. Notably, virus strains heavily passaged in tissue culture such as MVA and WR had high copy numbers of GAGA whereas clinical isolates of VARV and MOCV contained very few copies. It is possible that GAGA is under constant negative selective pressure *in vivo*, but in tissue culture the absence of a functioning immune system might permit the expansion of GAGA copy numbers. Interestingly, MVA, a highly immunogenic VACV strain was found to have the highest number of GAGA copies in its genome, suggesting that GAGA might play a role in its immunogenicity. Further work on GAGA insertions or deletions into VACV strains will help to establish the role, if any, of GAGA in the innate immune response to VACV and GAGA in poxvirus biology.

The fact that DNA-PK is required V(D)J recombination raises issues with the interpretation of *in vivo* experiments using Ku or DNA-PKcs deficient mice. The lack of V(D)J recombination leads to a SCID phenotype, and SCID mice are highly susceptible to a variety of pathogens, independently of any role DNA-PK plays in DNA sensing. Accordingly, infection of DNA-PK deficient mice with VACV will result in a much more severe phenotype than a wild-type control mouse, but attributing any difference in disease severity to either the adaptive or innate immune systems would be highly problematic. One argument is that assessment of production of chemokines and cytokines very early on in the

course of infection would only allow for the innate immune response to be assessed, however this temporal separation will not account for differences in local leukocyte populations secondary to impaired B and T cell development. For example, SCID mice lack Thy-1^{+} dendritic epidermal cells (Nixon-Fulton et al., 1987), which may play a role in intradermal infections of mice with VACV.

One way around this difficult *in vivo* problem is the use of viral antagonists of the sensor. After identification of DNA-PK as a DNA sensor, the role of C16 in DNA sensing was investigated since Ku was initially studied and proposed as a DNA sensor due to its interaction with C16. Ku was found to bind to C16 via a cell line-based over-expression and proteomics-based system and was subsequently confirmed in the context of virus infection.

It was found that C16 was able to inhibit DNA, but not RNA, sensing and that this inhibition was likely due to its interaction with Ku since the absence of Ku lead to the loss of the inhibitory function of C16. C16 appeared to inhibit the binding of Ku to DNA. The use of biotinylated DNA pull-downs demonstrated that the presence of C16 reduced the amount of Ku binding to DNA, and it is likely that through this mechanism C16 exerts its effects on DNA sensing. This can also explain the observation that C16 does not bind to DNA-PKcs, since the recruitment of DNA-PKcs by Ku is dependent on its interaction with DNA (Lehman et al., 2008).

It is likely that C16 has evolved as a viral countermeasure to DNA detection by DNA-PK, and as such the loss of C16 contributes to the attenuation of the virus *in vivo*. The role of C16 *in vivo* has been described previously (Fahy et al., 2008), and data in the present study add to the findings of this report. During intranasal VACV infection with wild-type, vΔC16, or revertant control viruses, it was found that infection with vΔC16 caused enhanced production of the chemokines Cxcl10, Ccl5 and the cytokine Il-6. Given the chemoattractive

properties of Cxcl10 and Ccl5, this is consistent with earlier findings that infection with vΔC16 lead to enhanced recruitment of leukocytes to lungs during infection. This effect was particularly noticeable early in the course of infection and by day 3 the absence of C16 had no statistically significant effect on the production of Cxcl10 or Il-6 in BAL fluid, although the trend remained, suggesting that C16 modulation of the innate immune response affects principally the initial stages of infection, in agreement with the hypothesis that DNA-PK acts as a frontline DNA sensor.

Whilst there have been multiple DNA sensors proposed recently, only AIM2 so far has been shown to biologically relevant *in vivo* (Fernandes-Alnemri et al., 2010; Rathinam et al., 2010). Therefore, the finding that C16 affects the production cytokines and chemokines *in vivo* provides the first, albeit indirect, example of a DNA sensor being important for the induction of IRF-3-mediated innate immune signalling *in vivo*, and the fact that VACV has evolved an inhibitor of this DNA sensor also adds significant support to the hypothesis that DNA-PK acts as a DNA sensor *in vivo*. This represents a major advance in the understanding of induction of immune responses against VACV and possibly other pathogens.

Together, this work has identified a novel DNA sensor responsible for the detection of cytoplasmic DNA in fibroblasts and has also characterised a viral inhibitor of this PRR. These findings contribute towards to an enhanced understanding of the immune response to ectopic DNA.

Chapter 4: VACV and Hypoxia-Inducible Factor (HIF)

4.1. The N-terminal half of C16 is predicted to have a PHD2-like fold

Analysis of the primary amino-acid sequence indicated that this protein has no identifiable homology to known cellular proteins other than in the final ten residues which show homology to icIL1-ra (Fahy 2008, Kluczyk 2002), and there is no structure of C16 available. Therefore, in an attempt to learn more about the potential three-dimensional structure of C16, sequence-structure homology analysis was carried out using the FUGUE software (Shi et al., 2001).

The first 205 amino acid residues of C16 were subjected to a sequence-structure homology recognition analysis using the FUGUE software (Shi et al., 2001). Notably, when the entire C16 sequence was used, FUGUE returned no positive matches. FUGUE takes a given query sequence and scans the HOMSTRAD database of structure profiles for potential matches. The summation of environment-specific substitution tables and structure-dependent gap penalties for each amino acid alignment can be used to generate a compatibility score, indicating the accuracy of each prediction. The homology modelling software MODELLER (Sali et al., 1995) was used to create 30 potential models of this domain. The model with the lowest energy and fewest spatial violations was selected as the most accurate representation of this domain. Three-dimensional structure visualization and image generation was carried out using Pymol software (DeLano, 2002) (Figure 4.2).

Rank	Hit	Species	z-score
1	Prolyl-4-Hydroxylase	*Shewanella Baltica*	9.26
2	Prolyl-4-Hydroxylase	*Chlamydia Reinhardtii*	8.84
3	Egln-1/PHD2	*Homo Sapiens*	5.14

Table 4.1 Structural predictions of the N-terminal half of C16. The N-terminal 205 amino acids of C16 were uploaded to the FUGUE structural prediction program (http://tardis.nibio.go.jp/fugue/) and the HOMSTRAD database was used to find structural predictions. The Z-score represents a confidence rating, with >4 meaning 'likely' (90% confidence) and >6 meaning 'very likely' (95% confidence) >8 meaning 'certain' (99% confidence).

Uploading of the N-terminal half of C16 to the FUGUE structural prediction program yielded several strong hits from a family of prolyl-4-hydroxylases, with the prolyl-4-hydroxylases from *Shewanella baltica* and *Chlamydia reinhardtii* having the highest z-scores and *Homo sapiens* Egln-1/PHD2 having the third highest z-score. Similar analysis of the C-terminal region of C16 yielded no predicted hits and, as such, structural information of this region of the protein remains unavailable. Alignment of the primary sequences of C16 residues 1 - 205 and the sequence of human PHD2 corresponding to the C-terminal region, consisting of amino acids 181-426, of this protein whose structure has been solved (McDonough et al., 2006) (Fig 4.1) indicated that the overall secondary structure is conserved although the first two N-terminal α-helices of the known PHD2 structure are absent in C16. Overall this analysis provided a strong indication that the N-terminal region of C16 may have a three-dimensional structure similar to that of this large family of enzymes.

Figure 4.1. JOY alignment of C16 and PHD2. Primary amino acid sequence alignment between C16 and PHD2 (PDB code: 2g19) as generated by FUGUE. Letters in red correspond to predicted alpha-helices and are denoted with 'a' underneath the corresponding amino acids. Blue letters correspond to beta-sheets and are denoted with 'b' beneath the corresponding amino acids. Maroon letters correspond to a predicted 3_{10} helix and are denoted with a '3' beneath the corresponding amino acids.

Using this sequence alignment it was possible to generate a structure prediction for corresponding the region of C16 using the Modeller software (Sali et al., 1995). This analysis led to a predicted structure of C16 based upon the known structure of PHD2 (Fig 4.2). The structures show the typical double stranded β helix fold of this enzyme family, although the C16 structure is missing the first two alpha-helices the remainder of structural features of the PHD2 fold predicted to be conserved in the C16 model.

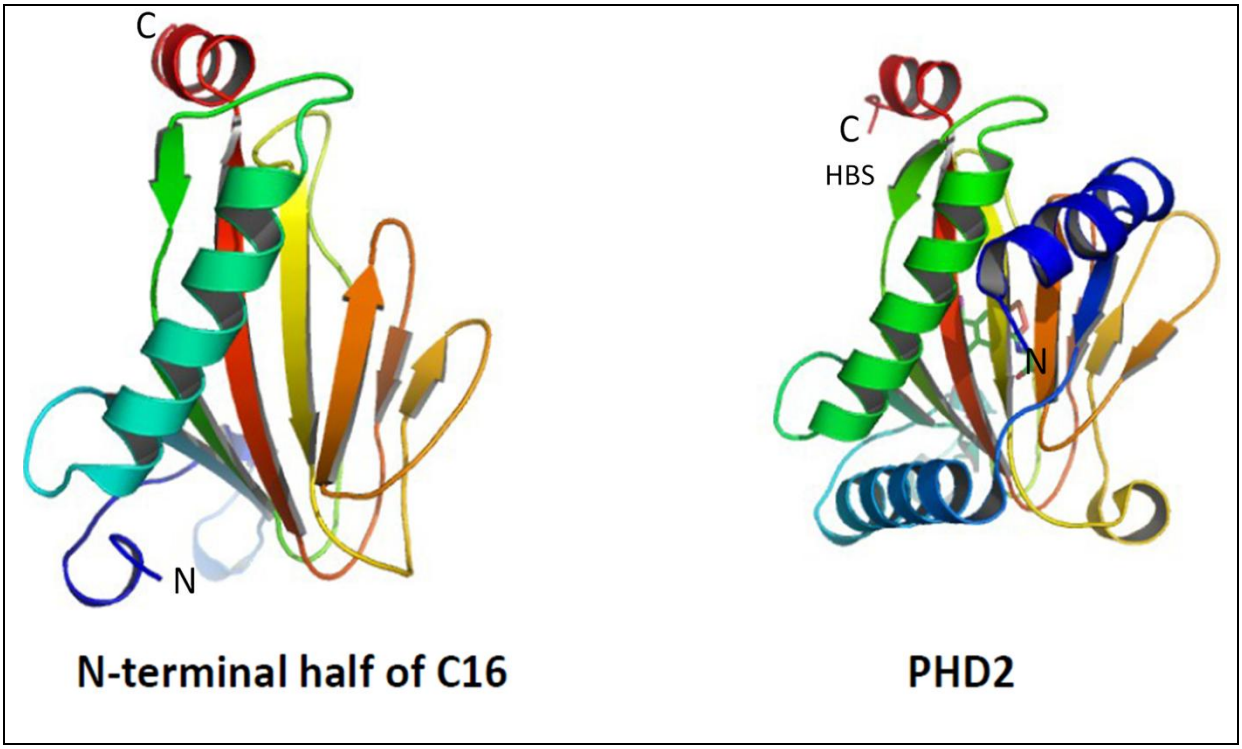

Figure 4.2. Structural prediction of C16 based on PHD2 using modeller. The left panel shows the predicted structure of residues 1 - 205 of C16 based on the solved structure of amino acids 181-426 of PHD2 (right panel).

The C-terminal structural domain has been shown to be the site responsible for the interaction with HIF-1α (Chowdhury et al., 2009) and is indicated with 'HBS' in Figure 4.2. This structural domain is conserved between the modelled C16 structure and PHD2, however key catalytic residues of PHD2 (His 313, Asp 315 and His 372) required for binding Fe^{2+} (McDonough et al., 2006) are not conserved in C16. Together, these data suggest that C16 might adopt a general PHD2-like conformation, but it is not likely to be catalytically active.

4.2. C16 binds PHD2

Using an unbiased proteomics approach, C16 was found to bind PHD2 as well as the Ku complex (see section 3.1.2). The interaction with PHD2 was particularly interesting given

that the N-terminal part of C16 is predicted to have a PHD2-like fold. However, independent evidence was needed to confirm the mass spectrometry data, and this was obtained by immunoblotting with PHD2 specific antibodies (Figure 4.3).

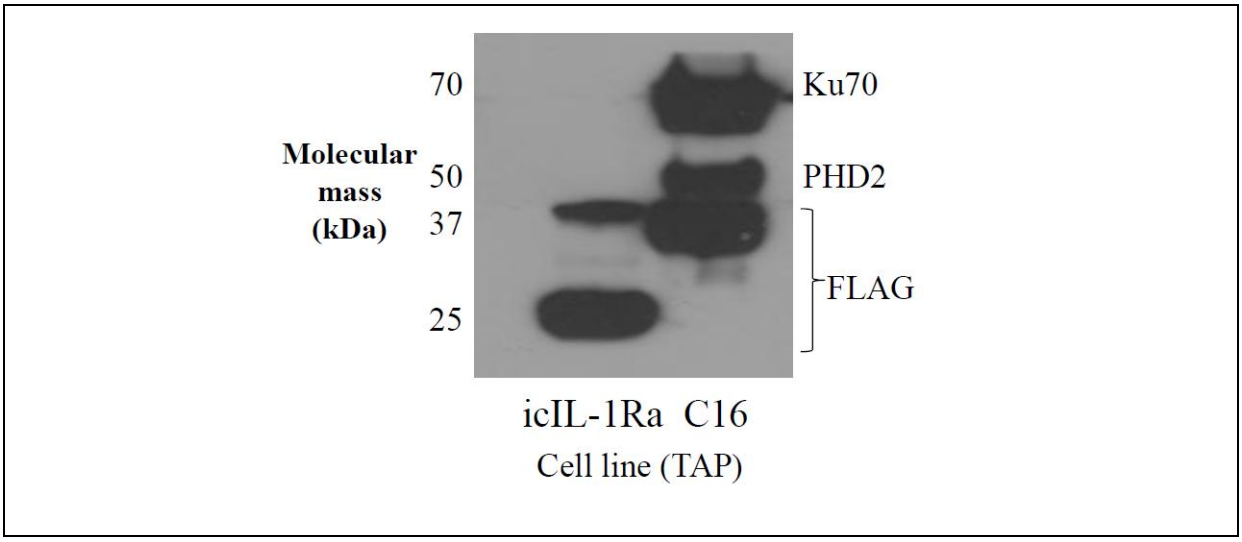

Figure 4.3. Confirmation of C16 binding partners using immunoblotting. Ten flasks of each of the indicated cell lines were grown and expression of either C16 or icIL-1Ra was induced by the addition of tetracycline for 24 h. Protein complexes were then purified by tandem affinity protein purification using STREP and FLAG beads and the final eluates were resolved by SDS-PAGE and immunoblotted with the antibodies against the indicated proteins. Molecular mass markers are indicated in kDa.

Immunoblotting of the final elutions from the TAP procedure with the indicated antibodies showed that, as well as a band of approximately 70 kDa, which was recognised with the anti-Ku70 antibody, there was a band of approximately 50 kDa which was recognised by an anti-PHD2 antibody (Figure 4.3). This confirmed the mass spectrometric analysis that identified PHD2 as a binding partner of C16. Importantly, neither Ku70 nor PHD2 co-precipitated with a TAP-tagged icIL-1Ra, indicating that the co-precipitation of these molecules with C16 was neither due to non-specific binding to beads nor due to nonspecific binding to the TAP-tag.

As was the case with the C16-Ku interaction, the biological relevance of C16 binding PHD2 was not immediately obvious.

4.3. PHD2 and hypoxia

PHD2 is an approximately 50-kDa prolyl-hydroxylase enzyme best characterised for its role in the hydroxylation of HIF-1α (Bruick and McKnight, 2001).

The ability of cells to respond to hypoxia (low oxygen concentration) is conferred by a system which culminates in the activation of HIFs 1 and 2, though HIF-1 is the better studied to date. HIF-1 is composed of a regulatory subunit (HIF-1α) and a constitutive beta subunit (HIF-1β) (Wang et al., 1995). Under normoxic conditions, HIF-1α is constitutively degraded, meaning that the hypoxic transcriptional program consisting of genes such as vascular endothelium derived growth factor (VEGF), glucose transporter-1 (GLUT1) and erythropoietin (EPO) is not active. This hydroxylation is achieved by a system consisting of prolyl- and asparaginyl-hydroxylases and an E3 ubiquitin ligase, Von Hippel-Lindau (VHL) and has been extensively reviewed (Fong and Takeda, 2008; Kaelin and Ratcliffe, 2008; Walmsley et al., 2008).

Briefly, prolyl-hydroxylases (PHDs) (Bruick and McKnight, 2001; Masson et al., 2001; Yu et al., 2001b; Yu et al., 2001a), mainly PHD2 (Berra et al., 2003) and an asparaginyl-hydroxylase (Lando et al., 2002), factor inhibiting HIF (FIH), use molecular oxygen as a substrate to hydroxylate HIF-1α on specific proline and asparagine residues. This hydroxylation leads to the binding of VHL which polyubiquitinates HIF-1α resulting in its proteasomal degradation (Ivan et al., 2001; Jaakkola et al., 2001; Yu et al., 2001a). When the oxygen concentration falls, this process can no longer occur and HIF-1α translocates to

the nucleus, where it associates with HIF-1β to form the transcription factor HIF-1 (Wang et al., 1995). HIF-1 activates a hypoxic transcriptional programme whereby genes containing a HIF-response element (HRE) in their promoter are transcribed (Wenger, 2002). The HIF-pathway is illustrated in Figure 4.4.

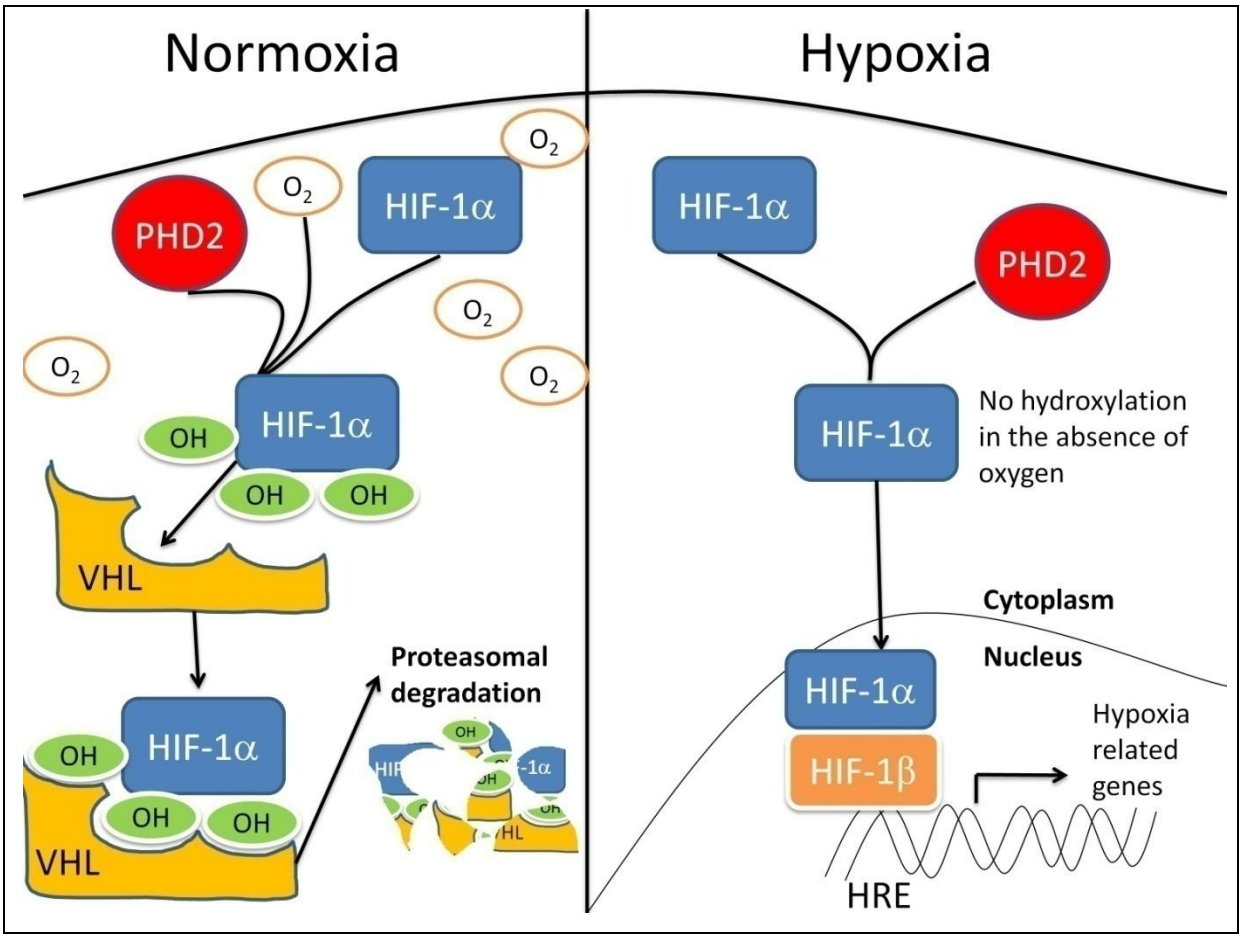

Figure 4.4. Illustration of hypoxic signalling. In the presence of molecular oxygen, PHD2 hydroxylates HIF-1α, which allows for interaction with the E3 ubiquitin ligase Von-Hippel Lindau (VHL), resulting in proteasomal degradation. In the absence of molecular oxygen (hypoxia), PHD2 can no longer hydroxylate HIF-1α, which results in the nuclear translocation of HIF-1α, upon which the transcription factor HIF-1 is formed by the interaction of HIF-1α and HIF-1β. HIF-1 binds to the HIF-response element (HRE) in the promoters of hypoxia-related genes.

The finding that C16 bound to PHD2 was surprising given the work showing a prolyl-hydroxylase domain-like fold prediction preceding the discovery. PHDs have not been shown previously to dimerise or oligomerise, although crystallographically PHD2 has been shown to homotrimerise (McDonough et al., 2006). Since PHD2 has been described principally hitherto as an oxygen sensor and in the degradation of HIF, the state of HIF-1α in cells infected with VACV was investigated.

4.4. VACV infection stabilises HIF-1α

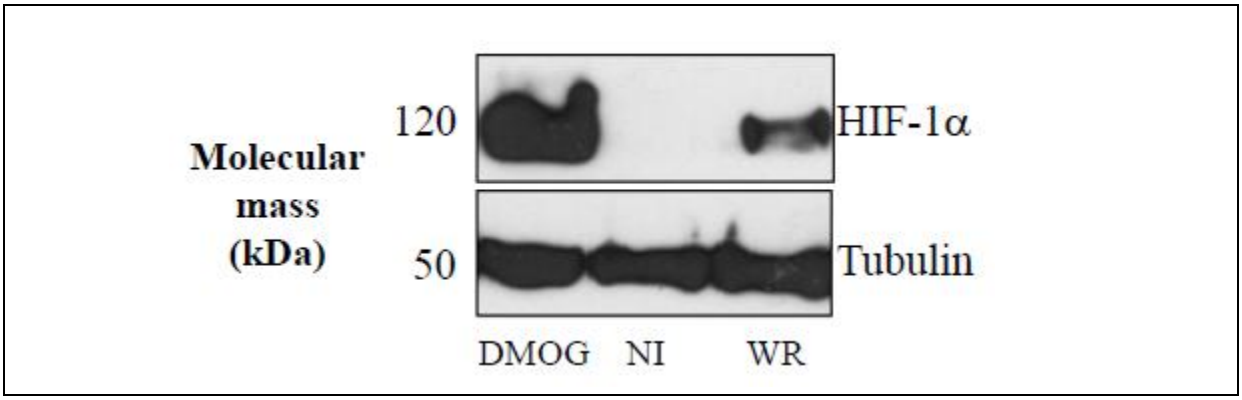

Figure 4.3 VACV infection leads to HIF-1α stabilisation. HeLa cells were either mock-infected (NI), treated with 1 mM dimethyloxalylglycine (DMOG) or infected with VACV strain WR at 1 p.f.u. / cell. Cell lysates were prepared 16 h later and proteins were resolved by SDS-PAGE and analysed by immunoblotting with the indicated antibodies. The positions of molecular mass markers are shown on the left hand side.

To assess the state of hypoxic signalling within VACV-infected cells, HeLa cells were infected with VACV for 16 h at 1 p.f.u per cell and cell lysates were immunoblotted with anti-HIF-1α antibody (Figure 4.3). In the non-infected cells, no band was detected with the anti-HIF-1α antibody, consistent with its well documented degradation under normoxic conditions. The addition of DMOG, a pan-hydroxylase inhibitor, induced the stabilisation of

the 120-kDa HIF-1α as expected. Interestingly, infection with VACV strain WR also induced the stabilisation of HIF-1α suggesting that hypoxic signalling within VACV-infected cells might be activated. This is the first demonstration of the stabilisation of HIF-1α during a poxvirus infection.

HIF-1α stabilisation has been demonstrated during infection with other pathogens, such as bacteria and parasites (Nizet and Johnson, 2009) and other viruses including respiratory syncitial virus (RSV) (Kilani et al., 2004), vesicular stomatitis virus (VSV) (Hwang et al., 2006), hepatitis B virus (HBV) (Yoo et al., 2003), hepatitis C virus (HCV) (Ripoli et al., 2009), human papilloma virus (HPV) type 16 (Lu et al., 2007; Tang et al., 2007; Nakamura et al., 2009; Bodily et al., 2010), and human herpesvirus 8 (Carroll et al., 2006; Cai et al., 2007). Importantly, the stabilisation of HIF-1α during viral infection is without hypoxia, i.e. the hypoxic signalling is modulated rather than there being a drop in O_2 concentration. This suggests that HIF-1α stabilisation is a common feature of infection with viruses.

To assess whether HIF-1α stabilisation was accompanied by transcriptional activation of HIF-1α-responsive genes, RNA was extracted from cells treated as before and analysed by qRT-PCR (Figure 4.4).

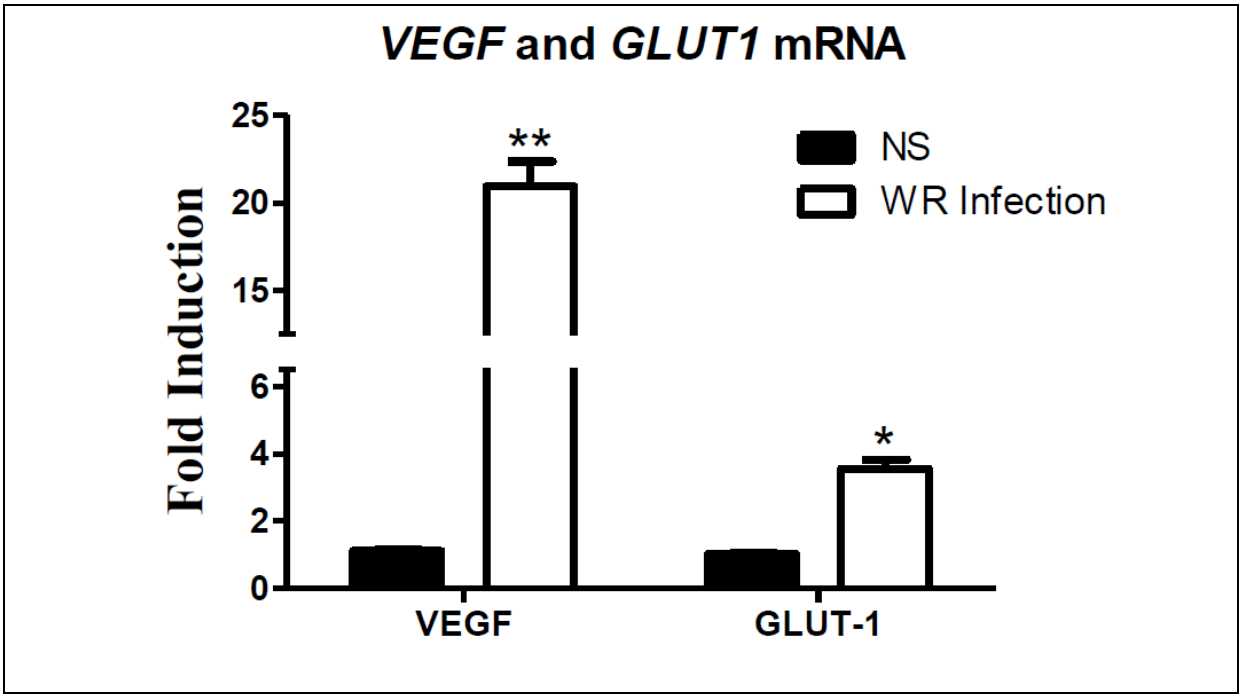

Figure 4.4. qRT-PCR analysis of-VEGF and GLUT1 in VACV infected cells. HeLa cells were infected with VACV strain WR at 2 p.f.u. / cell for 16 h. RNA was extracted from, these cells and the levels of mRNA for vascular endothelium derived growth factor (VEGF) and GLUT-1 were determined by qRT-PCR. mRNA induction was calculated relative to the glyceraldehyde phosphate dehydrogenase (*GAPDH)* gene. Error bars +/- SEM, ** $p<0.01$, * $p<0.05$

Intriguingly, infection with VACV lead to the statistically significant transcriptional upregulation of the hypoxia-related genes *VEGF* and *GLUT1* (Figure 4.4). Therefore, VACV infection is not only inducing stabilisation of HIF-1α but HIF-1α–dependent gene transcription. Additionally, HIF-1α nuclear translocation has now been shown in our laboratory by immunofluorescent staining of HIF-1α during VACV infection (Stuart Ember, unpublished data).

Whilst VACV infection induces HIF-1α stabilisation, the factors involved in the stabilisation needed to be determined. A plausible hypothesis was that the interaction of C16

with PHD2 inhibits PHD2 activity leading to HIF-1α stabilisation. This hypothesis was tested next.

4.5. C16 induces hypoxic gene up-regulation

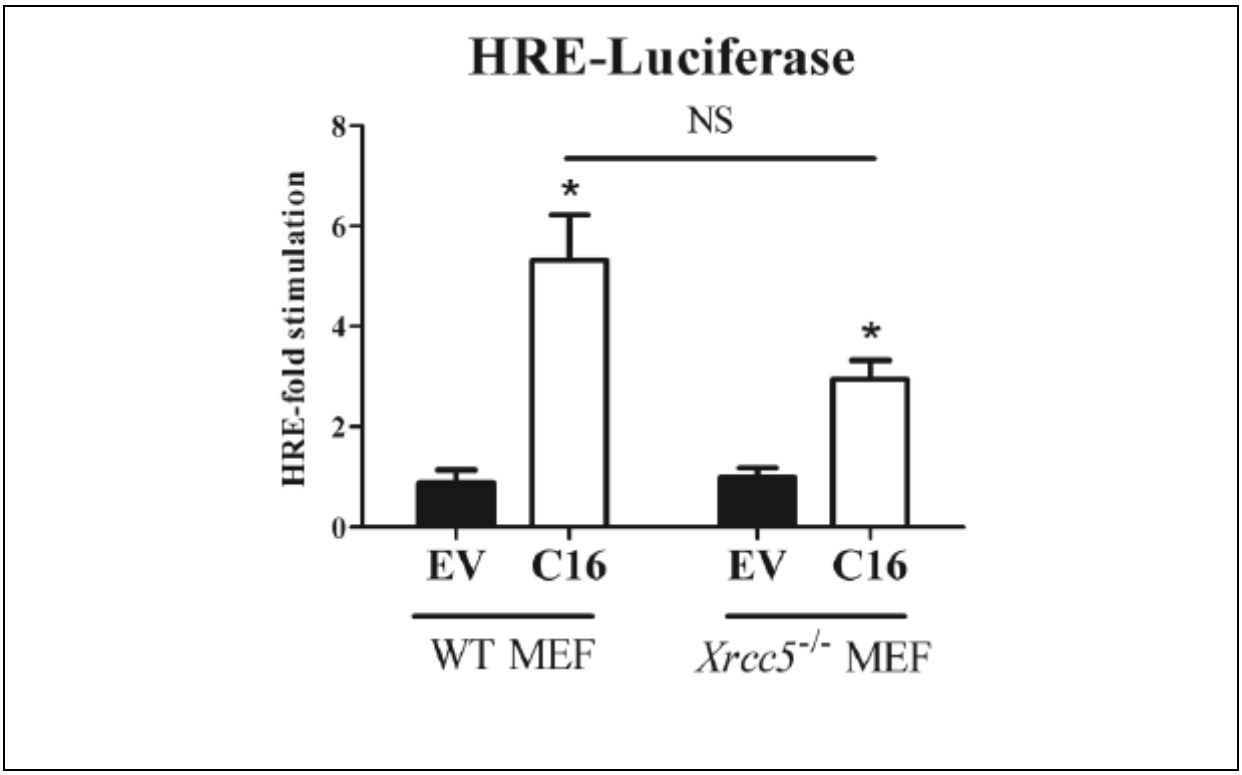

Figure 4.5. C16 can activate hypoxic signalling, and this is independent of Ku. WT and *Xrcc5*$^{-/-}$ MEFs were transfected with 100 ng of either pcDNA4/TO (Empty vector- EV) or pcDNA4/TO-C16 (C16) along with 100 ng HRE-luciferase and 10 ng TK-Renilla luciferase (Table 2.1) and left to incubate for 24 h. Cells were then lysed with passive lysis buffer and luminescence was measured. Fold-inductions are relative to empty vector. Error bars +/- SEM, * $p<0.05$.

To assess whether C16 altered HIF-1α stability, a plasmid encoding luciferase (Luc) driven from a Hypoxia response element (HRE-Luc) was co-transfected into cells with either empty plasmid vector control or the same plasmid expressing C16 (Figure 4.5). In WT MEFs C16 induced a roughly 6-fold stimulation of HRE-Luc, indicating that C16 promotes HIF-1α-

dependent transcription. Therefore C16 alone, is actively promoting hypoxic signalling, possibly via interacting with PHD2.

Interestingly, this effect was also noted in *Xrcc5*$^{-/-}$ MEFs, indicating that the effect of C16 on hypoxic signalling is mechanistically distinct from its effects on DNA-sensing (Figure 4.5). As C16 promotes HRE-Luc activity, it seemed likely that PHD2 is inhibited by C16 and so HIF1-α may be stabilised due to PHD2 inhibition.

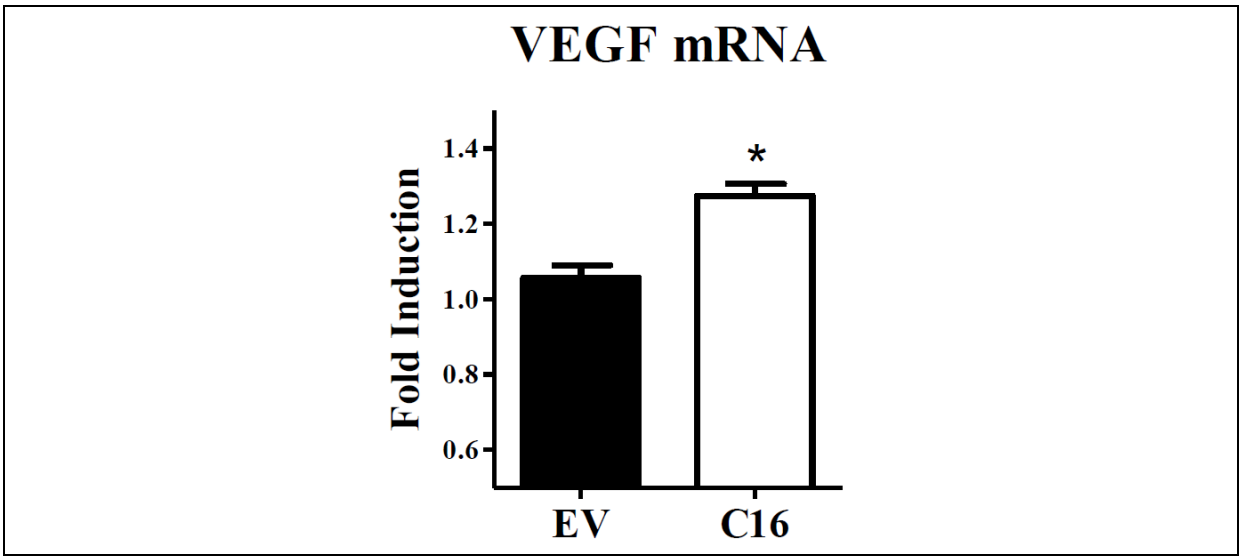

Figure 4.6. C16 can activate hypoxic signalling. HeLa cells were transfected with 1 μg/ml of either pcDNA4/TO (Empty vector- EV) or pcDNA4/TO-C16 (C16) for 24 h. RNA was extracted and the amount of VEGF mRNA was determined by qRT-PCR with the amount of 18S rRNA serving as a control. Error bars +/- SEM, * $p<0.05$

Concurrent with the observation that C16 activates HRE-Luc activity, it was observed that C16 activates expression of a HIF-1α responsive gene, *VEGF* (Figure 4.6). Cells transfected with C16 induced approximately 30% more VEGF mRNA compared with empty vector control-transfected cells. Combined, these data suggest that C16 can induce the transcription of hypoxia-related genes.

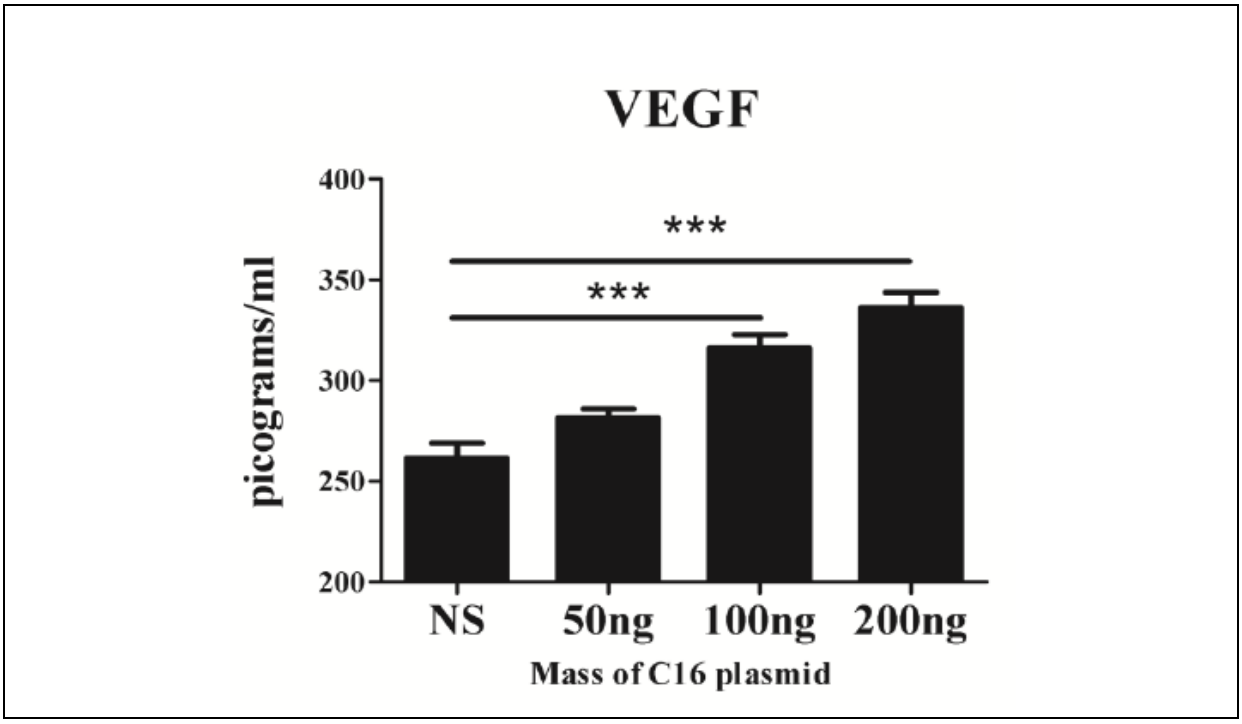

Figure 4.7. C16 can activate hypoxic signalling. HeLa cells in a 96- well plate were transfected with the indicated mass of pcDNA4/TO-C16 (C16) per well for 24 h. VEGF ELISA was performed on supernatants. NS, non-stimulated. Error bars +/- SEM, *** $p<0.001$

To assess whether the effect of C16 on hypoxic gene induction could also be seen at the protein level, the amount of VEGF in the culture medium of cells expressing C16 was measured by ELISA (Figure 4.7). Cells transfected with increasing amounts of a plasmid encoding C16 produced a dose-dependent, significantly-higher amount of VEGF. This indicates that C16 has an effect on hypoxic signalling at the transcriptional level that can also be observed at the protein level.

Having addressed the effects of C16 on hypoxic signalling, the role of PHD2 in this process was assessed next using *PHD1/2/3*$^{-/-}$ MEFs (Figure 4.8).

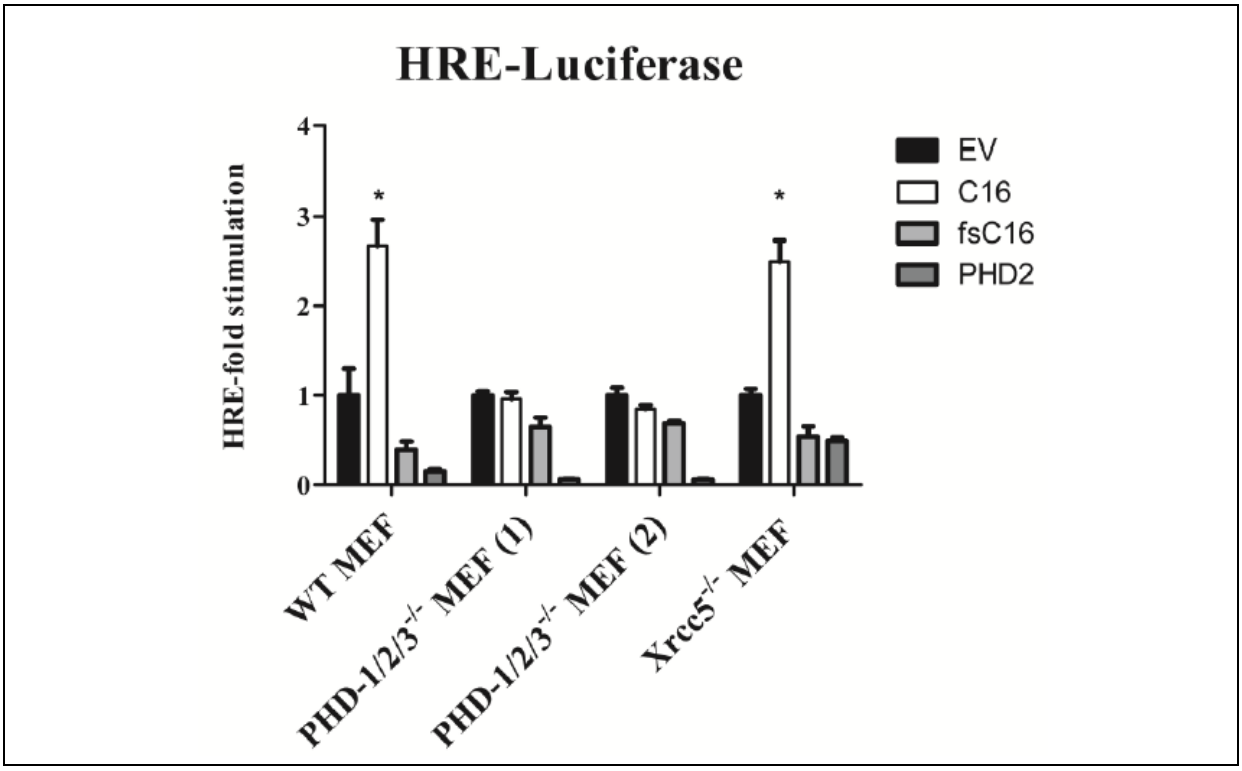

Figure 4.8. C16 can activate hypoxic signalling, and this is dependent on PHDs and independent of Ku. WT, two individual clones of *PHD1/2/3*$^{-/-}$ (all 3 hypoxia-related PHD molecules knocked out) and *Xrcc5*$^{-/-}$ MEFs were transfected with 100 ng of either pcDNA4/TO (Empty vector- EV), pcDNA4/TO-C16 (C16), pcDNA4/TO-frame shifted C16 (fsC16) or pcDNA3.1-PHD2 (PHD2) along with 100 ng HRE-Luciferase and 10 ng TK-Renilla luciferase and incubated for 24 h. Cells were then lysed with passive lysis buffer and luminescence was measured. Fold stimulations for each cell type are relative to empty vector. Error bars +/- SEM, * $p<0.05$, *** $p<0.001$

As noted previously, in wild-type MEFs C16 enhanced hypoxic signalling, as indicated by increased luminescence in an HRE-Luciferase assay. In contrast, over-expression of PHD2 has an inhibitory effect, compared to the empty vector control (Figure 4.8). This suggests an antagonistic relationship between the two molecules. In MEFs with all three hypoxia-related PHD genes deleted, C16 no longer has an effect on HRE-Luc, whereas over-expression of PHD2 maintains an inhibitory effect on hypoxic signalling. A plasmid encoding the C16 gene in which the translational initiation codon was mutated so that the

protein could not be expressed (frame-shifted C16) also has no effect on HRE driven luminescence, confirming that C16 protein, and not mRNA is responsible for this effect.

The fact that C16 does not have a hypoxia-enhancing effect in the absence of PHD molecules suggests that C16 requires the presence of at least PHD2 to enhance hypoxic signalling above the basal level. This result also confirms that C16 does not interact with the luciferase protein to enhance luminescence.

4.6. Potential additional role of PHDs in innate immunity

Whilst the effect of C16 on hypoxic signalling is reproducible, there has been increasing attention on non-hypoxia related functions of PHD molecules (Kaelin and Ratcliffe, 2008). A recent study provided evidence that PHD1 negatively-regulated IKKβ under normoxic conditions, and that this negative effect was alleviated under hypoxic conditions to potentiate NF-κB signalling (Cummins et al., 2006). Another study reported a different mechanism for activation of NF-κB by hypoxia involving calcium/calmodulin kinase (CaMK2)- dependent activation of TAK1 (Culver et al., 2010).

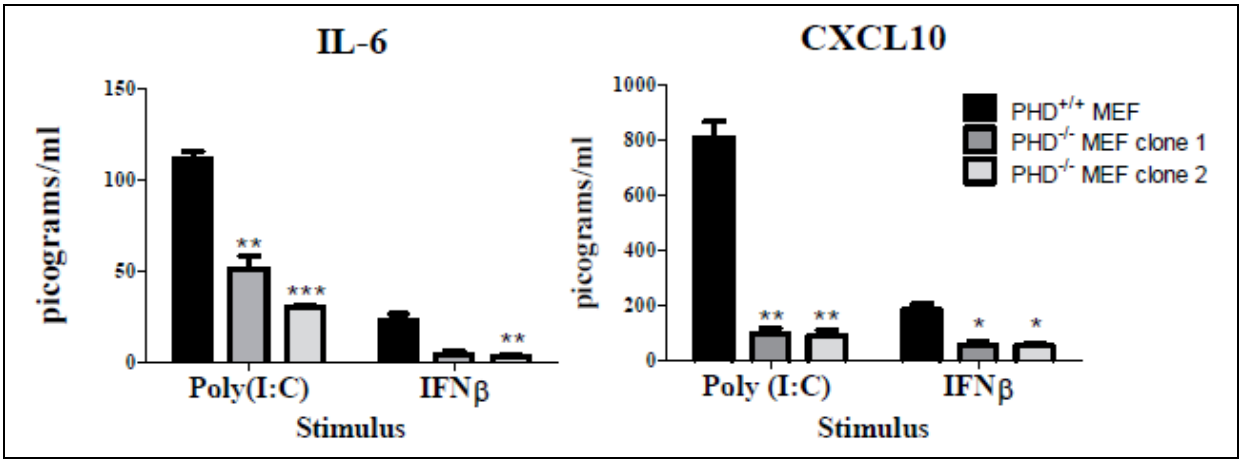

Figure 4.9. *PHD1/2/3*$^{-/-}$ MEFs are deficient in responses to poly (I:C) and Ifnβ. WT or *PHD1/2/3*$^{-/-}$ MEFs were transfected with 5 μg/ml poly (I:C) or treated with Ifn-β (2000 U/ml). After 24 h, supernatants were taken and levels of IL-6 and CXCL10 were assessed by ELISA. Error bars +/- SEM, *p<0.05 ** p<0.01 ***p<0.001

To assess any potential role for PHD molecules in innate immune signalling, a variety of different innate immune ligands were tested on WT and *PHD1/2/3*$^{-/-}$ MEFs. Intriguingly, *PHD1/2/3*$^{-/-}$ MEFs had decreased production of both Cxcl10 and Il-6 following stimulation with both Ifnβ and transfection with poly (I:C). These results suggest either a potential role of one PHD molecule in the ISRE pathway (since the ISRE pathway is a common transduction network activated with both poly (I:C) transfection and Ifn-β treatment) or alternatively that multiple PHDs might have a role in various innate immune signalling networks. Notably, C16 has previously been demonstrated to inhibit the ISRE pathway within our laboratory (Aodhnait Fahy, unpublished data).

Whilst these preliminary data are intriguing, it should be noted here that a stimulus evoking an equal activation of innate immune signalling across the wild-type and knockout MEFs has not been found, perhaps suggesting a general defect in innate immune signalling within these cells in our laboratory, which might be independent of a bona fide *in vivo* role.

4.7. Summary – C16 and Hypoxia

PHD2 was identified as a binding partner of C16 using an unbiased approach of TAP-tagging and identification by mass spectrometry. Interestingly, the N-terminal half of C16 was also predicted to adopt a PHD2-like conformation using unbiased structural prediction programs. C16 was also found to induce hypoxic signalling by using a hypoxia-luciferase reporter assay approach, and furthermore C16 was found to induce the production of VEGF after transfection into HeLa cells. For the first time, VACV was shown to induce the stabilisation of HIF-1α and this stabilisation also coincided with the transcriptional induction of HIF1α-dependent genes including *VEGF* and *GLUT1*.

HIF-1α stabilisation during the course of viral infection has had numerous explanations. In the case of RSV, HIF-1α stabilisation is reported to be due to RSV-mediated nitric oxide release as blockade of nitric oxide inhibited RSV-mediated HIF-1α stabilisation (Kilani et al, 2004) and as such is thought to represent a host-response to a noxious stimulus. A report regarding VSV-mediated HIF-1α stabilisation demonstrated that HIF-1 enhanced antiviral innate immune responses such as the transcription of IFNβ, and suggested that HIF-1α stabilisation was part of a host defence against viruses (Hwang et al, 2006). In the case of HBV, HCV, and HPV-type 16, HIF-1α stabilisation has been investigated in the context of oncogenesis, where HIF-1α stabilisation is promoted by virally encoded proteins leading to neovascularisation and tumour growth, perhaps explaining the propensity of these viruses to cause cancer, but few insights into the reasons why viruses might promote hypoxic signalling in the first place are offered by the literature.

Hypoxia and hypoxic signalling have been shown to have an increasingly important role in inflammation (Nizet and Johnson, 2009), and literature in this field is growing rapidly.

Hypoxia has largely been demonstrated to enhance inflammatory responses, such as TNF-mediated NF-κB activation (Cummins et al., 2006; Culver et al., 2010). In addition, an interdependent link between HIF-1α and NF-κB has been described (Rius et al., 2008), showing that HIF-1α mRNA transcription is dependent on NF-κB signalling. It has also been shown that hypoxia increases the expression and signalling potential of TLRs 2 and 6 (Kuhlicke et al., 2007). Furthermore, myeloid cells such as neutrophils have HIF-dependent ways of functioning in oxygen-depleted environments (Cramer et al., 2003), and HIF-1α has been shown to be important for phagocyte clearance of bacteria (Cramer et al., 2003; Peyssonnaux et al., 2005). HIF-1α activation in neutrophils has also been shown to enhance survival via NF-κB activation (Walmsley et al., 2005).

Together, the majority of the available literature suggests that hypoxia is a feature of inflammation, and the two systems cooperate to tackle infection: NF-κB upregulates *Hif1a* mRNA (Rius et al., 2008), and hypoxia potentiates NF-κB signalling (Cummins et al., 2006; Culver et al., 2010). The question, then, as to why VACV encodes a protein which seems to be enhancing hypoxic signalling by inhibiting PHD2 is pertinent and merits further attention.

One important point is the distinction between hypoxia and HIF-1 activity. Studies demonstrating enhancement of NF-κB signalling by hypoxia show that it is independent of HIF-1 activity (Cummins et al, 2006), and it is the low oxygen concentration which enhances the NF-κB signalling. There has not been a prominent example of a virus inducing hypoxia, rather virus infection often induces HIF-1 activity instead.

Two findings might support a potentially advantageous activation of hypoxia by VACV. Firstly, HIF-1α stabilisation leads to the transcription of EPO. The HRE-luciferase plasmid used in this study was in fact based on the EPO promoter, containing several HRE

sites, driving the expression of luciferase protein, so C16 is expected to enhance the transcription of EPO. EPO was recently reported to act on macrophages to suppress NF-κB pathways (Nairz et al., 2011), though the exact mechanism for this is not yet established.

A second hypoxia-upregulated gene, netrin-1, was also found to be upregulated in epithelial cells when HIF-activity was induced. Netrin-1 was also found to attenuate inflammation by inhibiting trans-epithelial migration of neutrophils by binding A2B adenosine receptors on neutrophils (Rosenberger et al., 2009). These studies combined illustrate the HIF-1 activity, and not hypoxia per se, upregulate anti-inflammatory molecules which could benefit VACV during the course of infection.

Secondly, HIF-1α stabilisation has a negative effect on T cell microbicidal activity. HIF-1α stabilisation was shown to negatively regulate T cell receptor signalling (Neumann et al., 2005), demonstrating that HIF-1 activity is not universally pro-inflammatory. Additionally, increased production of HIF-1α induces a CD4+ T cell polarisation shift from a T helper (Th)-1 to Th2 type, decreasing IFN-γ production by T cells (Ben-Shoshan et al., 2009), however the exact mechanism of this is unclear. Specifically, it is unclear whether or not it is T cell-intrinsic expression of HIF-1α (i.e. whether it is the T cell itself in which HIF-1 is active) that leads to this phenotype, or other cell types producing hypoxia-related cytokines results in a T cell shift. Furthermore, T cell intrinsic HIF-1α activation promotes the differentiation of regulatory T cells (Ben-Shoshan et al., 2008).

Together, the literature suggests that hypoxia plays a dual role in inflammation. Whilst myeloid cell-intrinsic hypoxia, i.e. HIF-1α stabilisation and HIF-1 transcriptional activity within myeloid cells appears to promote activation and phagocytosis, HIF-1 activation in other tissues conversely leads to the production of EPO and netrin-1, which results in dampening of myeloid cell NF-κB activation. On the other hand, hypoxia and HIF-

1 activation appears to attenuate lymphoid cell-mediated inflammation. Whether C16 is modulating any of these effects merits further attention.

C16-mediated inhibition of PHD2 might also be primarily independent of inflammatory effects of hypoxia. PHDs have been shown to have targets other than HIF-1α for prolyl-hydroxylation (Kaelin and Ratcliffe, 2008), and it might be one of these other targets that is the prime target for C16-mediated inhibition, and perhaps hypoxic signalling is a by-product of inhibiting PHD2 for these other functions.

A third possible reason for modulation of hypoxic signalling might be that activation of a hypoxic signalling programme, such as genes involved in glycolysis, might favour the metabolic demands of a cell for producing virus. Cell lines constitutively expressing HCV proteins were shown to induce the upregulation of hypoxia-related glycolytic genes, and this resulted in an 80% increase in cellular ATP levels (Ripoli et al., 2009). Preliminary data within our laboratory suggest that VACV growth is equivalent in WT and *PHD1/2/3-/-* MEFs (data not shown), suggesting that hypoxic signalling does not affect VACV production, though these data are preliminary and the role of hypoxia in virus production has not been fully assessed. However, another study regarding the usage of VACV in oncolytic therapy for hypoxic tumours has reported that VACV replicates equally well in hypoxic or normoxic conditions, and VACV protein expression was not affected either (Hiley et al., 2010). Furthermore, deletion of C16 did not appear to affect virus growth in tissue culture, though a slightly reduced plaque size was observed (Fahy et al., 2008), although *in vivo* models would be better suited to address this.

Whilst the role of the C16-PHD2 interaction in VACV infection is yet to be fully established, the demonstration that VACV induces hypoxic signalling and appears to actively promote this via encoding a PHD2-like molecule represents a novel finding. Modulation of

hypoxic signalling in various physiological or pathological conditions is attracting attention, and small molecules based on a C16-PHD2 interaction could potentially lead to novel therapeutic approaches to various conditions in which hypoxia may play a role in disease (Fraisl et al., 2009).

Chapter 5: Discussion and future work

5.1. DNA-sensing

5.1.1. Redundancy in DNA sensing molecules

Broadly speaking, there are two major mechanisms of intracellular ectopic DNA-mediated (hereafter termed DNA-mediated) activation of the innate immune system; the first is the activation of the inflammasome and the second is the activation of IRF-3 and NF-κB. Activation of the inflammasome by VACV is thought to occur via AIM2 (Rathinam et al., 2010) and NALP3 (Delaloye et al., 2009) in macrophages. In the case of AIM2, there is *in vivo* evidence that the absence of AIM2 leads to diminished production of IL-1β and IL-18 (Rathinam et al., 2010). However, despite a large number of molecules shown to activate IRF-3 and NF-κB in response to DNA, including DAI (Takaoka et al., 2007), RNA polymerase III (Ablasser et al., 2009; Chiu et al., 2009), LRRFIP1 (Yang et al., 2010), DHX9 (Kim et al., 2010) and IFI16 (Unterholzner et al., 2010), the roles of these molecules, if any, *in vivo* have not been established.

Data presented in this thesis support the conclusion that DNA-PK is important for the full activation of IRF-3-dependent cytokines and chemokines in response to DNA and VACV *in vitro*. Furthermore, the absence of an inhibitor of this complex, C16, leads to an enhanced production of the same cytokines and chemokines during VACV infection *in vivo*, suggesting that DNA-PK plays a role in the immune response to poxviruses. However, the role of the other DNA sensors is of interest. For example, many DNA sensors have so far only been reported in certain cell types such as macrophages in the case of LRRFIP1 (Yang et al., 2010), and plasmacytoid dendritic cells in the case of DHX9 (Kim et al., 2010). Furthermore,

RNA-polymerase III is not thought to act to upregulate IFNβ in fibroblastic cells because MAVS is not required for the upregulation of this molecules in response to poly (dA:dT) DNA (Kumar et al., 2006; Sun et al., 2006). It may be the case that different cell types employ different sensing mechanisms to elicit similar responses to DNA.

In addition, the putative DNA sensor DAI is not detectable in resting cells, but is upregulated substantially in cells treated with type I IFN (Takaoka et al., 2007; DeFilippis et al., 2010), suggesting temporal differences in DNA sensing mechanisms. Even further complexity might result from the nuclear/cytoplasmic divide of DNA sensing mechanisms, as AIM2 responds to cytoplasmic, but not nuclear pathogens to activate an inflammasome (Rathinam et al., 2010), whereas IFI16 is shown to activate an inflammasome in response to the nuclear pathogen KSHV (Kerur et al., 2011).

These reports suggest a great deal of complexity in the cell-type, temporal and cell-fraction specific mechanisms of DNA sensing (Table 1.1). In addition, data from this thesis show that DNA-PK is expressed in fibroblasts but not macrophages and that it is expressed at constitutively high levels. The cellular location in which DNA-PK acts as a DNA sensor has not been established, though investigation of which viruses activate IRF-3 via DNA-PK-dependent mechanisms may shed some light on this aspect of DNA-PK-mediated activation of innate immunity.

IFI16 & DNA-PK

Whilst data presented in this thesis are consistent with a key role for DNA-PK in DNA-mediated innate immune activation in MEFs, another study published recently has demonstrated a role for IFI16 in DNA sensing in MEFs (Unterholzner et al., 2010). There are, however, differences between these studies. Firstly, the DNA used to stimulate cells was different: in MEFs the DNA used to stimulate cells in the IFI16 study was 70-bp in length,

whereas DNA used in this study was either MVA genome via MVA infection or DNA at least 200-bp in length. Secondly, in primary MEFs, the absence of DNA-PKcs did not completely abrogate the induction of cytokines (Brian Ferguson, unpublished data), in contrast to transformed MEFs. Likewise, when IFI16 is knocked down in MEFs, some residual upregulation of *Ifnb* mRNA is detectable. This suggests that DNA-PK and IFI16 might somehow cooperate in MEFs to achieve full activation of the DNA-mediated innate immune response, for example DNA-PK might enhance the binding of IFI16 to DNA, or IFI16 might bring into STING into proximity with DNA-PK as IFI16 is reported to interact with STING (Unterholzner et al., 2010). The characterisation of the differential roles these molecules play *in vitro* and *in vivo* requires further work.

5.1.2. Characterisation of DNA-PK-mediated DNA sensing *in vivo*

Given the SCID phenotype of DNA-PK-deficient mice, the characterisation of DNA-PK-mediated innate immune activation by DNA is highly problematic. However, it will be necessary, given the redundancy in DNA sensing systems, to assess the role of DNA-PK *in vivo*.

Our laboratory is now in possession of *Prkdc*$^{-/-}$ mice and these are currently being bred with a view to performing *in vivo* experiments in the near future. Reconstitution of DNA-PKcs-deficient mice with wild-type bone marrow is one way of avoiding the problem of differing leukocyte populations presented by the SCID phenotype, but the role of DNA-PKcs in DNA repair means that knockout mice would be exquisitely sensitive to the radio-ablation of the bone marrow used during this procedure, perhaps resulting in anomalies such as enhanced pro-inflammatory cytokine production due to persistent DNA damage as reported previously (Rodier et al., 2009).

It is also possible that a conditional knockout mouse could be generated such that there is targeted deletion of DNA-PKcs or Ku in fibroblastic cells, this would allow for a more rigorous assessment of the contribution of DNA-PK in innate immune sensing of pathogens.

Further work on the *in vivo* contributions of DNA-PK to viral innate immunity will occur within our laboratory, however the significance and relevance of various other DNA sensors have not yet been characterised. The roles of these different sensors in different cell types are not known, therefore a serious effort using various pathogens with different cell tropisms will be required to begin to unravel the relevance of certain DNA sensors occupying biological niches. Understanding the contributions of these DNA sensors to different pathogens will enhance our understanding of the innate immune response and is likely to become a focus of research in future years.

5.1.3. N-terminal phosphorylation of IRF-3 by DNA-PK, the role of the kinase domain of DNA-PK

The N-terminal phosphorylation of IRF-3 by DNA-PK has been reported previously (Karpova et al., 2002). Activation of IRF-3 for transcriptional activity normally occurs at the C terminus (Fitzgerald et al., 2003; Sharma et al., 2003). However, preliminary data from our laboratory have shown that the kinase activity of DNA-PK is dispensable for the upregulation of Cxcl10 in response to DNA. This conclusion comes from two separate experiments: firstly, treatment with the DNA-PK kinase inhibitor, NU7026, created originally for enhancing radiotherapy by decreasing radiotherapy-induced DNA damage repair (Willmore et al., 2004), did not affect DNA sensing capacity (Brian Ferguson, unpublished data). Secondly, the use of MEFs from Bosma SCID mice, which have a point mutation ablating

DNA-PKcs kinase activity (Blunt et al., 1995; Blunt et al., 1996), also showed normal upregulation of *Cxcl10* mRNA in response to stimulation with DNA (Brian Ferguson, unpublished data). However, the role, if any, of the kinase activity of DNA-PK in DNA sensing is of interest given the reported phosphorylation of IRF-3 by DNA-PK.

The question as to the signalling mechanism initiated by DNA-PK remains open. Given the preliminary data that the kinase domain of DNA-PK is dispensable for DNA-PK-mediated innate immune activation by DNA, it is probable that DNA-PK provides a structural scaffold for the assembly of a complex which initiates the pathway leading to IRF-3 activation. Study of this mechanism is key to a full understanding DNA-PK-mediated DNA sensing.

5.1.4. Potential role for DNA-PK in and autoimmunity and detection of retroviruses

Autoimmune conditions are characterised by immune responses against host molecules and tissues. These conditions are frequently associated with inflammation, and the causative factors of many autoimmune conditions are not yet understood, yet deregulation of immune signalling may lead to autoimmunity in some instances (Diamond, 2008).

TRex1, a cytosolic exonuclease, has been shown to be linked to Aicardi-Goutières syndrome (AGS) (Crow et al., 2006). AGS is characterised by early onset severe encephalitis, lymphocytosis in the cerebrospinal fluid and elevated type I IFN production (Stephenson, 2008). TRex1 is now thought to be involved in the degradation of DNA, preventing cell-intrinsic responses to cytoplasmic DNA (Stetson et al., 2008). In addition, mutations of TRex1 inhibiting the catalytic domain are associated with systemic lupus erythematosus (SLE), which is the prototypic systemic autoimmune condition characterised by

autoantibodies to DNA and is thought to be caused by inappropriate activation of the immune system by DNA, often following apoptosis (Rahman and Isenberg, 2008). Chilblain lupus is characterised by lesions at the extremities such as fingers, toes and ears. Mutations in TRex1 impairing catalytic activity are linked to both regular and chilblain SLE (Lee-Kirsch et al., 2007a; Lee-Kirsch et al., 2007b).

The function of TRex1 is the cytoplasmic degradation of DNA, and failure of this process leads to intracellular accumulation of DNA and a subsequent innate immune response, including MEFs (Stetson et al., 2008). Therefore, it is possible that DNA-PK is also associated with the induction of this type I IFN response, and therefore might play a role in AGS.

Along similar lines, mice lacking DNAse ii, another enzyme involved in the degradation of DNA have been shown recently to exhibit a severe autoinflammatory polyarthritis. Intracellular accumulation of undigested DNA in macrophages is thought to be responsible (Kawane et al., 2006) and this DNA activates an innate immune response dependent on IRF-3 and IRF-7 (Okabe et al., 2008).

Collectively, there appears to be a role for DNA sensing in these conditions associated with the failure of DNA digestion. Furthermore, the role of DNA sensing in SLE is also coming under intense investigation (Kontaki and Boumpas, 2010). AIM2 was shown to be expressed at higher levels in a murine model of lupus, the BXSB mouse (Roberts et al., 2009), and subsequent investigation of renal biopsies from patients with SLE-mediated nephritis have also been shown to have upregulated AIM2 (Kimkong et al., 2009). The exact role of AIM2 in SLE, if any, is unclear. However, because SLE is characterised by inappropriate immune responses to DNA, the role of DNA sensing in this disease should be investigated. Interestingly, a large proportion of patients with SLE and systemic sclerosis

have autoantibodies to Ku (Cavazzana et al., 2008), and interestingly another DNA sensor, RNA polymerase III is also the target of autoantibodies in systemic sclerosis (Cavazzana et al., 2009).

There is increasing interest in the role of antiviral nucleic acid sensing and autoimmunity, since many autoimmune conditions are associated with an IFN signature. This subject has been reviewed recently (Stetson, 2009; Kontaki and Boumpas, 2010). The answer to the question as to whether DNA-PK has a role in nucleic acid driven autoimmunity could be very interesting and knowledge of this role, if any, could open important therapeutic avenues.

TRex1, in addition to the previously described role in Aicardi-Goutières syndrome, has also been shown to be important in innate immune activation following Human immunodeficiency virus (HIV) infection. It was reported that TRex1 bound to cytoplasmic HIV pro-viral DNA and digested it. However, in the absence of TRex1, the HIV DNA accumulated and this lead to an enhanced activation of IRF-3 and production of type I IFN (Yan et al., 2010). This suggests that HIV DNA is a PAMP involved in HIV infection and the study has lead to an enhanced knowledge regarding the innate immune detection of HIV. Whether DNA-PK is involved in the innate immune recognition of HIV remains to be determined, however DNA-PK is expressed in T cells as it has a critical role in V(D)J recombination, so therefore could be involved in the innate immune detection of HIV and other retroviruses such as human T cell leukaemia virus (HTLV)-1. HIV infects macrophages and CD4+ T cells, and data presented in this thesis suggest that DNA-PK is not expressed in macrophages.

Extensive study elsewhere has demonstrated that DNA-PK is critical for the development of T cells, so proving a role for DNA-PK in HIV-mediated innate immune

activation would be technically difficult, but would merit further study given the profound impact this virus has worldwide.

5.1.5. GAGA

The role of GAGA, if any, in the innate immune response to VACV is of great interest. Specifically, whether GAGA can enhance innate immune responses and increase potency of DNA vaccines *in vivo* should be given much further attention. Studies are underway within our laboratory to assess the role of GAGA during vaccination. It will also be interesting to study the role of GAGA in virus infection, and results from these studies may enhance understanding of poxvirus biology. Notably, GAGA is also present in strains of other DNA viruses, such as HSV-1, which might imply a broader role for GAGA in DNA viruses.

5.1.6. Possible role for alternative IRF molecules in DNA-PK-mediated DNA sensing

During the writing of this thesis, a study was published reporting that Ku70 acts as a DNA sensor in HEK cells (Zhang et al., 2011). HEK cells have been reported to be unresponsive to DNA types other than poly (dA:dT) as evidenced by lack of production of type I IFN (Ablasser et al., 2009; Chiu et al., 2009), however this study shows a potent upregulation of type III IFN in response to non-poly (dA:dT) DNA which was dependent on Ku70, and also on IRF1 and IRF7. Whilst this study did not show a role for Ku80 or DNA-PKcs in this system, it will be of interest to investigate the roles of IRF1 and IRF7 in the context of DNA-PK-mediated innate immune activation in other cell types, such as MEFs.

5.2. HIF and VACV

The role of hypoxic signalling in VACV infection remains uncharacterised. However, whilst data presented suggest that C16 is promoting HIF stabilisation, the timing of this response remains unclear. C16 is expressed early in infection (Assarsson et al., 2008; Fahy et al., 2008), yet the work regarding C16 and HIF stabilisation assessed the HIF activity after longer periods of infection. Assessment of the timing of HIF activity during VACV infection may help to guide future characterisation of the role of hypoxic signalling in virus infection. For instance, if HIF stabilisation occurs very late in infection, then it is perhaps unlikely that VACV is targeting this signalling pathway in order to induce the upregulation of EPO and netrin-1 as described in Section 4.6, because later on during the course of infection there is a severe impairment to the production of any new host proteins due to VACV infection. If HIF activity is only induced late, then VACV may instead be targeting PHD2 for some other as yet undefined function.

The use of the vΔC16 virus will help to further establish a role for C16 in hypoxic signalling. Preliminary data show that infection with vΔC16 induces less stabilisation of HIF-1α compared with wild type virus (Stuart Ember, unpublished data) although this has yet to be assessed thoroughly. However, qRT-PCR studies with WR and vΔC16 could also be used to define a role for C16 in hypoxic signalling.

C4, a closely related VACV protein to C16, is also predicted to encode a PHD2-like protein and the role of C4, if any, in hypoxic signalling merits further attention. Preliminary work suggest that C16 and C4 both contribute to HIF-1α stabilisation, and a double deletion virus (vΔC16ΔC4) has been made within the laboratory and *in vivo* studies with this virus are underway.

Recombinant C16 produced and purified from *E. coli* can also be used to assess PHD2 hydroxylase activity *in vitro*. To this end, collaboration has been established with a group based at Oxford University, and initial data are showing that C16 is inhibiting PHD2 hydroxylase activity, consistent with work presented in this thesis (Christoph Loenarz, unpublished data). This assay will prove useful in confirming other biochemical and functional data regarding C16 and secondly might be used to assess mutants of C16 for their ability to impair PHD2 enzymatic function and further characterise the molecular mechanism of this inhibition.

5.3. C16

C16, a highly conserved protein found in VACV and many other poxviruses, such as VARV, was shown previously to be a virulence factor *in vivo* (Fahy et al., 2008), however the mechanism of enhancing virulence was not understood. Two functions of C16 have been described in this thesis via the identification of binding partners and subsequent work. However, ascribing the *in vivo* phenotype (namely both the enhanced production of chemokines and cytokines and recruitment of leukocytes in vΔC16 infection relative to wild-type virus) to the DNA sensing inhibition or HIF-1 stabilising functions of C16 will require further work.

In order to distinguish the roles of C16, structural studies are ongoing with C16 in collaboration with two laboratories to examine the structure of C16 by crystallography, and studies of co-crystal structures of C16 with Ku or PHD2 are also being attempted. If a co-structure with either of these molecules is obtained, it might be possible to generate mutant C16 molecules capable of performing one function (i.e. inhibition of DNA sensing or enhancing hypoxic signalling) but not the other. Generation of viruses lacking one of these

functions would help to establish the relative contributions of these roles of C16 to the *in vivo* phenotype observed.

Many avenues of research have been opened up by the discovery of C16 binding partners, and this adds support to the continued research of host-pathogen interactions occurring within VACV infection. This study provides yet another example of how investigation of a virus, even a vaccine for an eliminated disease, can enhance the understanding of other fields of research.

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