

THE ROLE OF IRF-3 IN INNATE ANTIVIRAL SIGNALLING

THE ROLE OF INTERFERON REGULATORY FACTOR 3 IN THE INNATE
ANTIVIRAL RESPONSE

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ABSTRACT.

The transcription factor interferon (IFN) regulatory factor 3 (IRF-3) plays a central role in the innate immune response to viral stimulation. IRF-3 participates in both the type I IFN-dependent and -independent signalling pathways that result in the induction of an antiviral state. The work presented in this thesis characterizes the central role of IRF-3 in the IFN-independent response to virus particle entry. In addition, novel splice variants of human IRF-3 are identified and characterized, implying a role for splice-mediated regulation of IRF-3-mediated antiviral signalling. Finally, a role for reactive oxygen species in the activation of IRF-3 following virus particle entry is described, with virus particle entry inducing danger associated molecular patterns associated with IRF-3 activation and IFN-independent antiviral gene expression. Taken together, this thesis characterizes the role of IRF-3 in the innate antiviral signalling pathways activated following viral stimulation, and highlight the importance of danger-associated molecular patterns as important mediators of antiviral signalling.

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LIST OF ABBREVIATIONS AND SYMBOLS.

AAD	Apoptosis activation domain
AIM2	Absent in melanoma 2
ALR	Absent in melanoma 2-like receptor
AP-1	Activator protein 1
ASC	Apoptosis speck-like protein containing a caspase activation and recruitment domain
ATF-2	Activated transcription factor 2
ATP	Adenosine triphosphate
ASK1	Apoptosis signal regulating kinase 1
Bak	Bcl-2 homologous antagonist killer
Bax	Bcl-2-associated X protein
Bcl-2	B cell lymphoma 2
B-DNA	B-form DNA
BHA	Butylated hydroxyanisole
BH3	Bcl-2 homology domain 3
BIR	Baculovirus inhibitor of apoptosis protein repeat
CaMK	Ca ⁺⁺ /calmodulin dependent protein kinase
Carboxy-H ₂ DCFDA	6-carboxy-2',7'-dichlorodihydrofluorescein diacetate
CARD	Caspase activation and recruitment domain
Cardif	Caspase activation and recruitment domain adaptor inducing interferon β
CBP	Cyclic AMP response element binding protein
CD	Cluster of differentiation
cDNA	Complementary DNA
CEB	Cyclin E binding protein
CFDA-AM	5-Carboxyfluorescein Diacetate, Acetoxymethyl Ester
ciAP	Cellular inhibitor of apoptosis
CIITA	Class II major histocompatibility complex transactivator
CMV	Cytomegalovirus
CREB	Cyclic AMP response element binding
CRM1	Chromosome region maintenance protein 1
CXCR4	C-X-C chemokine receptor type 4
CYLD	Cylindromatosis protein
DAI	DNA-dependent activator of interferon regulatory factors
DAMP	Danger associated molecular pattern
DAP	Diaminopimelic acid
DBD	DNA binding domain
DC	Dendritic cell
DDX	DEAD-box helicase
DEAE	Diethylaminoethanol

DHX	DEHD-box helicase
DMEM	Dulbecco's modified Eagle's medium
DPI	Diphenyleneiodonium
DTT	Dithiothreitol
EBV	Epstein-Barr virus
ECM	Extracellular matrix
ϵ -DAP	ϵ -Diaminopimelic acid
EEA1	Early endosomal antigen 1
eEF	Eukaryotic elongation factor
eIF	Eukaryotic initiation factor
EMCV	Encephalomyocarditis virus
ER	Endoplasmic reticulum
ERK	Extracellular signal-regulated kinase
ESE	Exon splicing enhancer
ESS	Exon splicing silencer
EWS	Ewing sarcoma breakpoint region 1
FADD	Fas-associated protein with death domain
Fas	Apoptosis stimulating fragment
FBS	Fetal bovine serum
FIP	Feline infectious peritonitis
FRAS1	Fraser syndrome 1
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GAS	Gamma interferon activation site
GAS41	Glioma amplified sequence 41
GFP	Green fluorescent protein
GST	Glutathione S-transferase
GTP	Guanosine triphosphate
HAU	Hemagglutination units
HBSS	Hank's balanced salt solution
HBV	Hepatitis B virus
HCMV	Human cytomegalovirus
HCV	Hepatitis C virus
HECT	Homologous to the E6-AP Carboxyl Terminus
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HERC5	Homologous to the E6-AP Carboxyl Terminus domain and Regulator of chromosome condensation 1-like domain-containing protein 5
HET-E	Heterokaryon incompatibility protein
HHV	Human herpesvirus
HIF1 α	Hypoxia-inducible factor 1 α
HIV	Human immunodeficiency virus
HMGB1	High-mobility group protein B1
HPV	Human papillomavirus

Hsp90	Heat shock protein 90
HSV	Herpes simplex virus
HTLV-1	Human T cell leukemia virus 1
IAD	Interferon regulatory factor association domain
ICP0	Infected cell protein 0
IFI16	Interferon γ -inducible protein 16
IFIT	Interferon -induced protein with tetratricopeptide repeats
IFN	Interferon
IFNAR	Interferon α/β receptor
IFNGR	Interferon gamma receptor
Ig	Immunoglobulin
I κ B	Inhibitor of nuclear factor kappa light chain enhancer of activated B cells
IKK	Inhibitor of nuclear factor kappa light chain enhancer of activated B cells kinase
IL	Interleukin
iNOS	Inducible nitric oxide synthase
IP3R	Inositol triphosphate receptor
IPAF	Interleukin 1 β converting enzyme protease activating factor
IPS-1	Interferon β promoter stimulator 1
IRAK-4	Interleukin 1 receptor-associated kinase 4
IRF	Interferon regulatory factor
IRF-E	Interferon regulatory factor binding element
ISG	Interferon stimulated gene
ISGF3	Interferon stimulated gene factor 3
ISRE	Interferon stimulated regulatory element
JAK	Janus kinase
JEV	Japanese encephalitis virus
JNK	c-Jun N-terminal kinase
LB	Luria broth
LCMV	Lymphocytic choriomeningitis virus
LDL	Low-density lipoprotein
Lgp2	Laboratory of genetics and physiology 2
LPS	Lipopolysaccharide
LRR	Leucine rich region
LRRFIP1	Leucine-rich repeat flightless-interacting protein 1
MAPK	Mitogen activated protein kinase
MARCO	Macrophage receptor with collagenous structure
MAVS	Mitochondrial antiviral signalling protein
Mcl1	Myeloid leukemia cell differentiation protein 1
MCMV	Murine cytomegalovirus
MDA5	Melanoma differentiation associated gene 5
MDP	Muramyl dipeptide

MEF	Mouse embryonic fibroblast
MEK	Mitogen activated protein kinase-extracellular signal-regulated kinase
MHC	Major histocompatibility complex
MIP	Macrophage Inflammatory Protein
MITA	Mediator of IRF-3 activation
MMLV	Moloney murine leukemia virus
MOI	Multiplicity of infection
Msk1	Mitogen and stress activated kinase 1
MxA	Interferon-induced cellular resistance mediator protein
MyD88	Myeloid differentiation primary response gene
NAIP	Nucleotide-binding oligomerization domain-like receptor family, apoptosis inhibitory protein
Nalp	NAIP, CIITA, HET-E and TP1 NACHT, leucine rich region, and pyrin domain-containing protein
NACHT	<u>N</u> ucleotide-binding oligomerization domain-like receptor family, <u>a</u> poptosis inhibitory protein, <u>C</u> lass II major histocompatibility complex transactivator, <u>H</u> eterokaryon incompatibility protein, and <u>T</u> elomerase-associated protein 1
NADPH	Nicotinamide adenine dinucleotide phosphate, reduced
NDV	Newcastle disease virus
NEDD4	Neural precursor cell expressed developmentally down-regulated 4
NEIL1	Nei endonuclease VIII-like 1
NEMO	Nuclear factor kappa light chain enhancer of activated B cells essential modulator
NES	Nuclear export signal
NFκB	Nuclear factor kappa light chain enhancer of activated B cells
NK	Natural killer
NLR	Nucleotide-binding oligomerization domain-like receptor
NLRX1	Nucleotide-binding oligomerization domain, leucine rich repeat containing X1
NLRC5	Nucleotide-binding oligomerization domain-like receptor family, caspase activation and recruitment domain containing 5
NLS	Nuclear localization signal
NO	Nitric oxide
NOD	Nucleotide-binding oligomerization domain
NOS	Nitric oxide synthase
NOX	Nicotinamide adenine dinucleotide phosphate oxidase
NSP4	Nonstructural protein 4
OLFML2A	Olfactomedin-like 2A
OPHN1	Oligophrenin 1
Ori	Origin of replication

OTUB	Ovarian tumor domain, ubiquitin aldehyde binding
PAGE	Polyacrylamide gel electrophoresis
PAK1	p21 protein (cell division control protein 42/Ras-related C3 botulinum toxin substrate)-activated kinase 1
PAMP	Pathogen associated molecular pattern
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
pdAdT	Polydeoxyadeninic:polydeoxythyminic acid
pDC	Plasmacytoid dendritic cell
Pfu	Plaque forming units
PI3K	Phosphatidylinositol 3-kinase
pIC	Polyinosinic:polycytidylic acid
Pin1	Peptidylprolyl cis/trans isomerase, never in mitosis gene a-interacting 1
PKR	Protein kinase R
PML	Promyelocytic leukemia protein
PMN	Polymorphonuclear cells
PMSF	Phenylmethylsulfonyl fluoride
PRD	Positive regulatory domain
PRR	Pattern recognition receptors
PVDF	Polyvinylidene fluoride
PVM	Pneumonia virus of mice
PYD	Pyrin domain
Rac1	Ras-related C3 botulinum toxin substrate 1 (Rho family, small GTP binding protein)
RACE	Rapid amplification of cDNA ends
RANTES	Regulated upon activation, normal T cell expressed, and secreted
RAUL	Replication and transcription activator-associated ubiquitin ligase
Rb	Retinoblastoma protein
RBCK	Ran binding protein-type and C3HC4-type zinc finger containing 1
RCC1	Regulator of chromosome condensation
RIG-I	Retinoic acid-inducible gene I
RING	Really interesting new gene
RIP	Receptor-interacting protein
RLR	Retinoic acid-inducible gene I-like receptor
RNAP	RNA polymerase
RNF125	Really interesting new gene finger protein
RNS	Reactive nitrogen species
ROR γ t	Retinoic acid receptor-related orphan receptor γ t
ROS	Reactive oxygen species

Rpm	Revolutions per minute
RPS6KA5	Ribosomal protein S6 kinase α 5
RSV	Respiratory syncytial virus
RT	Reverse transcription
SAPK	Stress-activated protein kinase
SDS	Sodium dodecyl sulfate
SeV	Sendai virus
SF	Serum-free
SGT1	Suppressor of G2 allele of S-phase kinase-associated protein 1
SH	Small hydrophobic protein
SH2/3	Src homology 2/3
SH3GL2	Src homology 3 domain growth factor receptor bound 2-like 2
SHD	Src homology 2 domain containing transforming protein D
SLO	Slowpoke
snRNP	Small nuclear ribonucleoproteins
SNV	Sin Nombre Hantavirus
SR	Splice regulatory
SR-A	Class A scavenger receptor
SRR	Serine rich region
STAT	Signal transducer and activator of transcription
STING	Stimulator of interferon genes
TANK	Tumor necrosis factor receptor associated factor family member-associated NF κ B activator
TBK-1	Tumor necrosis factor receptor associated factor family member-associated NF κ B activator binding kinase 1
TFR	Transferrin receptor
T _h	T helper
TIR	Toll/Interleukin 1 receptor
Tks4	Tyrosine kinase substrate with four Src homology 3 domains
TLR	Toll-like receptor
TMX2	Thioredoxin-related transmembrane protein 2
TNF	Tumor necrosis factor
TP-1	Telomerase-associated protein 1
TRADD	Tumor necrosis factor receptor type 1-associated death domain protein
TRAF	Tumor necrosis factor receptor associated factor
TRAP β	Translocon-associated protein β
TRIF	Toll/Interleukin 1 receptor domain-containing adaptor inducing interferon β
TRIM25	Tripartite motif containing 25
TRIP	Tumor necrosis factor receptor associated factor-interacting protein
TRX	Thioredoxin

TXNDC14	Thioredoxin domain-containing protein 14
TXNIP	Thioredoxin-interacting protein
Tyk2	Tyrosine kinase 2
Ubc13	Ubiquitin conjugating enzyme 13
USP31	Ubiquitin specific peptidase 31
UV	Ultraviolet
VAF	Virus activated factor
VCP	Vaccinia complement control protein
VISA	Virus-induced signaling adaptor
VSV	Vesicular stomatitis virus
VV	Vaccinia virus
WNV	West Nile virus
X-gal	5-bromo-4-chloro-indolyl- β -D-galactopyranoside
Zyg-11B	Zyg-11 homolog of <i>Caenorhabditis elegans</i> B

Chapter I. Introduction.

I.I. Innate Antiviral Immunity

The immune response to viral infection encompasses multiple distinct and overlapping pathways that collectively serve to protect the host from disease. Antiviral immunity is comprised of both innate and adaptive immune mechanisms, with each arm playing important roles in the protection of a host from viral infection and pathogenesis. The innate immune system is responsible for the first line of defence against incoming pathogens and recognizes common pathogen associated molecular patterns (PAMPs), as well as generic danger associated molecular patterns (DAMPs), associated with infection. The adaptive immune system involves a long-term, antigen-driven, pathogen-specific response and leads to immunological memory and long-term immunity. Importantly, these distinct arms of the immune system play well-characterized and distinct roles against viral infection, with each arm playing a role in the regulation of the other.

The innate antiviral response encompasses a wide variety of defence strategies that remain highly conserved among multicellular organisms, and comprises the earliest physical, cellular, and molecular responses to incoming pathogens. While being characterized as largely pathogen non-specific, the innate arm of the immune system recognizes a wide variety of conserved and specific PAMPs and DAMPs, leading to signalling pathways involved in inflammation and clearance, control of viral replication, apoptosis of infected cells, and promotion of an adaptive immune response. Between transmission and antigen presentation, multiple physical, cellular, and molecular barriers contribute to antiviral protection and are described below.

I.I.i. Intrinsic Immunity

Intrinsic immunity comprises the anatomical and mucosal barriers that protect the host from microbial infection, and plays an important role protecting the host against viral transmission¹. Anatomical barriers in the body provide an efficient physical barrier against infection. Skin has a low pH and a lower temperature relative to core body temperature, making it a less hospitable environment for microbes. Constant sloughing of the outer layers of skin, as well as sweat production, provide a mechanism for mechanical removal of potential pathogens. In addition, antimicrobial peptides such as defensins and lysozyme are resident molecules on the skin's surface that antagonize microbial invasion.

Mucous membranes also provide a strong physical barrier against viral infection, particularly in the respiratory, gastrointestinal, and genital tracts. Mucus contains many molecules involved in innate defence against viral infection, including antimicrobial peptides, lactoferrin, high molecular weight glycoproteins, and immunoglobulins. In addition, ciliary action and peristalsis at mucosal sites promotes the physical clearing of viral pathogens. Finally, the flushing action of tears and saliva also promote viral clearance.

I.I.ii. Cell Type-Specific Antiviral Immunity

In the innate antiviral response, various cell types play distinct roles in the recognition of viral infection leading to the clearance of infected cells, inflammation and chemotaxis, and suppression of viral replication. The following section will review some of the major cell types involved in the innate immune response to viral infection, including natural killer (NK) cells, macrophages, plasmacytoid dendritic cells (pDCs), and polymorphonuclear leukocytes (PMNs).

I.I.ii.i. Natural killer cells

NK cells are well characterized in terms of their role in antiviral immunity *in vivo*. NK cells survey major histocompatibility complex (MHC) expression via activating and inhibitory receptor engagement, leading to the destruction of virally infected cells in which surface MHC expression is decreased². This surveillance mechanism provides a compensatory antiviral role against viruses that have evolved to downregulate surface MHC expression to avoid antigen presentation. Importantly, the many NK cell evasion strategies employed by a wide variety of viruses such as Human immunodeficiency virus (HIV), Cytomegalovirus (CMV), and Dengue virus highlights the importance of this cell type in antiviral immunity. In addition, NK cell responses to viral infection lead to the production of cytokines such as IFN γ and TNF α , which can ultimately lead to protection and viral clearance³. Finally, while principally characterized as an innate immune effector, a recent body of literature has characterized a recall response in innate immune cells such as NK cells⁴. The secondary NK cell response to viral infection has been shown to have distinct kinetics and amplitude than during primary infection, implying a distinct role for “experienced” NK cells in antiviral immunity.

I.I.ii.ii. Macrophages

Macrophages play an important role in the phagocytosis of virally infected cells, and are considered a critical aspect of both innate and adaptive responses to infection. In the innate response to infection, macrophages recognize and phagocytose foreign pathogens, typically via complement-dependent opsonization of pathogens. Macrophages are also important producers of antiviral cytokines and chemokines, which play important roles in antiviral defence in response to a wide variety of viruses including Lymphocytic choriomeningitis virus (LCMV), Vesicular stomatitis virus (VSV), Murine cytomegalovirus (MCMV), and Ectromelia virus⁵⁻⁹. Conversely, macrophage activation contributes to antibody-dependent enhancement of infection in macrophage-tropic viruses such as Dengue virus and Feline infectious peritonitis (FIP) virus, and therefore can play a role in the immune pathology associated with viral infection¹⁰.

I.I.ii.iii. Plasmacytoid dendritic cells

pDCs were discovered as a lymphocyte subpopulation with a morphology similar to plasma cells, but lacking classical B and plasma cell markers¹¹. These cells were later identified as a dendritic cell (DC) subset with low MHCII expression and poor T cell stimulatory capacity¹². Further, this cell population was shown to bear a unique set of surface markers and was demonstrated to potently induce type I IFN in response to viral infection^{13,14}. Further, pDCs have been shown to constitutively express interferon (IFN) regulatory factor 7 (IRF-7), which is required for the transcription of the full complement of IFN α subspecies as well as IFN stimulated genes (ISGs)^{15,16}. Not surprisingly, pDCs play an important role in antiviral immunity *in vivo* via IFN production in response to a wide variety of viruses including Herpes simplex virus (HSV) type 1 (HSV-1), MCMV, Influenza A and murine coronavirus¹⁷⁻¹⁹.

I.I.ii.iv. Other cell types

Other major cell types involved in the innate response to viral infection include the PMNs, the granulocyte population of which neutrophils comprise the bulk. Neutrophils are an important hallmark of the inflammatory response induced by viral infection. They play an important role in antiviral immunity via phagocytosis, production of antimicrobial peptides and innate immune cytokines, having demonstrated a protective role against viruses such as HIV, HSV, and Influenza A²⁰⁻²⁴. Conversely, the role of neutrophils in immune pathology in the context of various viral infections has been well-characterized, leading to lower survival rates in animal models of viral infection such as HSV-1 and influenza A as well as leading to inflammatory complications such as chronic obstructive pulmonary disease²⁵⁻²⁷.

Similarly, the contradictory role of eosinophils in viral clearance versus protection is represented in animal models of Respiratory syncytial virus (RSV) infection. On one hand, eosinophils have been shown to contribute to antiviral cytokine levels such as IL-4 and macrophage inflammatory protein 1 α (MIP1 α) in response to pneumonia virus of mice (PVM), a murine model of human RSV infection²⁸. Additionally, eosinophils have been shown to contribute to RSV clearance in mice by a mechanism involving nitric oxide (NO)²⁹. On the other hand, eosinophilic inflammation has been associated with RSV bronchitis in children³⁰. Similarly, in Influenza virus-infected children, virus-induced acute pneumonia was associated with increased recruitment of eosinophils to the respiratory tract as well as increased levels of IL-5, a cytokine typically associated with eosinophil responses³¹.

While classically characterized as mediators of allergic inflammation, basophils and mast cells have some demonstrated involvement in the immune response against various viral infections. The downregulation of basophil activation by a Human herpesvirus 8 (HHV-8)-encoded gene speaks to the involvement of this

cell type in host defence against HHV-8³². Additionally, basophils have been shown to be the predominant IL-4 producers in response to RSV infection *in vivo*, contributing to RSV-induced immunopathology³³. Mast cells have been shown to play a role in the chemotaxis of NK cells and/or cluster of differentiation (CD) 8+ T cells in response to polyinosinic:polycytidylic acid (pIC) treatment and Reovirus infection^{34,35}.

I.I.iii. Molecular Pathways Leading to Innate Antiviral Immunity

Viral infection is associated with cellular recognition of viral PAMPs by pattern recognition receptors (PRRs), leading to an innate antiviral response. An outline of the PAMPs associated with viral recognition, as well as their signalling consequences, is described in detail in section I.II.i. Similarly, viral infection leads to the cellular production of DAMPs, whereby signals of cellular stress activate signalling cascades that lead to antiviral immunity. An overview of some DAMPs associated with viral infection is discussed in section I.III.i. A comprehensive overview of PRR-mediated antiviral immunity is represented in Figure 1, and is described in further detail in the following sections.

Upon viral infection of a host cell, a number of diverse and converging antiviral responses are induced, leading to a variety of antiviral consequences. These include inflammation, antiviral gene expression, phagocytosis, and induction of adaptive immunity, among others. Not surprisingly, the specific antiviral effectors activated by infection are virus- and cell type-dependent. The following section will review the major molecular pathways associated with innate antiviral protection. First, the major inflammatory processes associated with viral infection, such as nuclear factor of kappa light polypeptide gene enhancer in B cells (NFκB)-dependent gene expression and inflammasome activation, will be discussed. Second, an overview of the three types of IFN, as well as their signalling consequences, will be given. Finally, other mediators of innate antiviral immunity, such as complement and antimicrobial peptides, will be discussed.

I.I.iii.i. Inflammation

Viral infection is often associated with inflammatory cytokine production. In response to viral infection, the major cytokines associated with inflammation and chemotaxis largely depend on the transcription factor NFκB, and include, but are not limited to, IL-6 and tumor necrosis factor (TNF). More recently, assembly of the inflammasome complex leading to caspase-1 activation has been shown to follow viral infection and leads to the proteolytic processing of IL-1β and IL-18, two proinflammatory cytokines.

I.I.iii.i.i. NFκB-dependent inflammatory cytokines

The cellular recognition of viruses by a wide variety of PRRs leads to the activation of NFκB and the expression of proinflammatory cytokines. There are a wide variety of NFκB-dependent target genes, and additional transcription factors play a determining role in the expression of specific NFκB-responsive genes³⁶. In the context of viral infection, multiple virus-induced signalling cascades lead to the activation of inflammatory cytokines, including components of the inflammasome cascade and the IFN signalling pathways (discussed below). In addition, IL-6 and the TNF family of cytokines play important roles in the inflammatory response to viral infection. IL-6 is a pleiotropic cytokine whose immune functions include the promotion of T and B cell activation and proliferation as well as NK cell function³⁷. TNF production following viral infection leads to the potentiation of NFκB-dependent gene expression, as well as being involved in apoptotic signalling cascades³⁸⁻⁴⁰. The wide variety of evasion mechanisms employed by viruses to subvert these signalling pathways highlights their importance in antiviral defence³⁹.

I.I.iii.i.ii. The inflammasome

Among the more recently characterized antiviral pathways, the inflammasome has been shown to play a crucial role in the defence against viral infection⁴¹. The inflammasome is comprised of multiple cellular components including a member of the nucleotide-binding oligomerization domain (NOD)-like receptor (NLR) family and activated caspase-1, often along with an adaptor molecule to facilitate molecular interactions. The major consequence of inflammasome assembly is the activation of caspase-1, which mediates the cleavage of pro-IL1β and pro-IL18 into their active, soluble forms, leading to an inflammatory signalling cascade that limits microbial replication and induces apoptosis. The virus-dependent expression of pro-IL1β and pro-IL18 is thought to require NFκB, which is activated following PRR engagement by viral PAMPs. The inflammasome pathway, therefore, is not directly involved in transcriptional activation, but synergizes with other signalling pathways to induce an inflammatory and apoptotic response.

Inflammasome assembly is activated by a wide variety of PRRs and senses a variety of PAMPs and pathogen-associated danger signals⁴². For example, multiple NLRs such as NOD1, NOD2, IL1β converting enzyme protease activating factor (IPAF), and NLR family, apoptosis inhibitory protein 5 (NAIP5) have been shown to recognize bacterial motifs such as *meso*-diaminopimelic acid (*meso*DAP), muramyl dipeptide (MDP), and bacterial flagellin⁴²⁻⁴⁴. Importantly, a variety of viral nucleic acids and proteins have been shown to activate the inflammasome via the NLR NAIP, class II major histocompatibility complex transactivator (CIITA), heterokaryon incompatibility protein (HET-E), and telomerase-associated protein 1 (TP1) (NACHT); leucine rich region (LRR); and pyrin domain (PYD) domain-containing protein 3 (Nalp3)⁴⁵⁻⁵¹. The antiviral actions of the inflammasome are an important current area of research in innate antiviral defence. Inflammasome activation is thought to require a secondary signal such as K⁺ efflux, P2X₇ receptor binding by adenosine triphosphate (ATP), ultraviolet (UV) irradiation, and reactive

oxygen species (ROS) production⁵²⁻⁵⁸. Therefore, the current model of inflammasome-mediated immune responses is a two-step process, in which PAMP recognition mediates the NF κ B-dependent transcription of pro-IL1 β and pro-IL18, while a secondary signal such as a DAMP mediates NLR-dependent activation of the inflammasome and caspase-1. Activated caspase-1 cleaves pro-IL1 β and pro-IL18 into their mature, soluble forms IL1 β and IL18. Importantly, the inflammasome is activated by a broad range of PAMPs and DAMPs, implying that a generic stress response lies at the core of this process.

I.I.iii.ii. Interferon Signalling

The IFNs constitute a family of pleiotropic and multifunctional cytokines, whose collective action leads to antiviral immunity at a variety of stages. The IFNs are classified into three groups, with distinct cell type specificity and mechanisms of action. They include the type I IFNs, produced by most cell types and exerting signalling effects to most cell types; type II IFN, which is produced primarily by T cells and NK cells and exerts its effects on a number of cell types including NK cells and phagocytes; and the type III IFNs, which are produced primarily in epithelial cells and DCs, and exert their effects on a variety of cell types.

I.I.iii.ii.i. Type I interferon

The type I IFNs are the best characterized group of cytokines involved in innate antiviral immunity. The type I IFNs (hereby referred to as IFN), comprised mostly of IFN α and β , are a family of pleiotropic cytokines whose expression is largely dependent on the transcription factors IRF-3 and IRF-7⁵⁹⁻⁶⁴. In humans, the type I IFNs are also comprised of IFN ϵ , IFN κ , and IFN ω ⁶⁵. Signalling of IFN through the IFN α/β receptor (IFNAR) complex, comprised of IFNAR1 and IFNAR2, induces the expression of a large number of antiviral genes whose promoters contain IFN-stimulated response elements (ISREs). The signalling pathways leading to both IFN production and IFN-dependent antiviral gene expression are described in detail in section I.II.ii. Type I IFN signalling ultimately leads to the expression of a wide variety of ISGs, which plays a variety of roles in antiviral immunity^{61,66,67}. These roles include, but are not limited to, inhibition of host and viral protein translation^{68,69}, recruitment of NK cells to sites of infection⁷⁰, regulation of cellular proliferation and apoptosis⁷¹, and regulation of adaptive immunity^{72,73}. Together, these antiviral effectors are sufficient to inhibit further viral gene expression and virus replication. IFN signalling also plays a role in promoting adaptive immune mechanisms by upregulating MHC I surface expression, promoting cross-presentation of antigen, and promoting the activation of NK cells, DCs and T cell subsets⁶⁵. A detailed description of some important antiviral actions of IFN appears in section I.II.iii.

I.I.iii.ii.ii. Type II interferon

Type II IFN, of which IFN γ is the only member, is a major cytokine involved in the adaptive immune response to viral infection. NK cells, macrophages, CD8⁺ T cells, and T helper 1 (T_h1) CD4⁺ T cells are the classical cell types responsible for IFN γ production in response to viral infection, although a wider variety of adaptive immune cell types are also known to produce this cytokine⁷⁴. Signalling of IFN γ through its receptor complex, comprised of IFN γ receptors 1 (IFNGR1) and 2 (IFNGR2), leads to the phosphorylation of Janus kinases 1 (JAK1) and 2 (JAK2) followed by phosphorylation and dimerization of signal transducer and activator of transcription 1 (STAT1) and transcription of genes with IFN γ -activated site (GAS) element-containing promoters. IFN γ production is a hallmark of activated T_h1 CD4⁺ cells and promotes cell-mediated immunity. CD8⁺ T cells also rely on IFN γ to mediate cytotoxic effects. In macrophages, IFN γ mediates nitric oxide (NO) and ROS production by promoting inducible nitric oxide synthase (iNOS) synthesis and the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase system, leading to the activation of a wide range of antiviral activities including inflammation and apoptosis (discussed in section I.V).

I.I.iii.ii.iii. Type III interferon

The type III IFNs (also known as IFN λ) are comprised of IL-28 and IL-29, and have been more recently characterized in terms of their antiviral activity. Type III IFN is primarily produced in epithelial cell types in response to viral infection, although production at other sites such as macrophages and DCs has also been reported⁷⁵⁻⁸⁴. In general, type III IFN signalling leads to the transcription of ISGs, and therefore these cytokines are thought to play a redundant role to the type I IFNs in terms of antiviral immunity⁷⁸⁻⁸⁰, where cell type specificity is determined by expression of the IL28 receptor complex (IL28R α /IL10R β), which is more restricted than the type I IFN receptor^{76,79,85}. However, recent studies have identified novel roles for type III IFNs in innate and adaptive immune mechanisms. For example, Koltsida *et al.*⁷⁵ reported a role for IL28A in DCs and their ability to promote T_h1 immunity. Liu *et al.*⁷⁶ also reported opposing roles for IL-29 and IFN α in terms of their ability to stimulate IL-12p40 in macrophages. Taken together, type III IFNs and their role in antiviral immunity represent a novel and interesting area of research, with these cytokines playing increasingly distinct roles in response to viral infection.

I.I.iii.iii. Other Innate Antiviral Mechanisms

In addition to the mechanisms described above, there are a number of other mechanisms governing innate antiviral defence. These include the complement pathway and antimicrobial peptides, as discussed below. In addition, the role of innate effectors in the regulation of adaptive antiviral immunity will be discussed.

I.I.iii.iii.i. Complement

The complement pathway functions primarily in the blood and mediates a variety of innate antiviral functions such as opsonization, chemotaxis, and direct lysis of pathogens. Comprised of several converging pathways, the complement system recognizes various signals of infection such as antigen-bound immunoglobulin M (IgM) or G (IgG) in the classical pathway, PAMPs in the alternative pathway, and pathogen-associated carbohydrate or glycoprotein moieties in the lectin pathway⁸⁶. Collectively, activation of the complement cascade by any of these pathways leads to convergence at the C3 molecule, where downstream activation leads to pathogen clearing, chemotaxis of leukocytes to sites of infection, mast cell activation, and inflammation. C3 is an acute phase protein that is a major regulator of complement-mediated immunity, whereby cleavage of C3 into its active fragments C3a and C3b leads to a variety of antiviral effects. C3a is a chemotactic factor that mediates inflammation and subsequent inflammatory cytokine production through its cognate receptor, C3aR⁸⁷. C3b can directly bind pathogens and serves as an opsonin to mediate phagocytosis⁸⁸. Additionally, C3b mediates the assembly of the membrane attack complex that mediates the direct lysis of pathogens by osmotic lysis⁸⁸. The importance of this system in the innate antiviral response is highlighted by the multitude of evasion strategies employed by various viruses to combat this system. For example, Vaccinia virus (VV) encodes a complement control protein (VCP) whose function leads to inhibition of complement-enhanced antibody neutralization^{89,90}. Hepatitis C virus (HCV) infection is also associated with inhibition of C4 in infected patients⁹¹. Further, activation of the complement cascade following viral infection has been shown to enhance immunopathology for viruses such as RSV⁹².

I.I.iii.iii.ii. Antimicrobial peptides

Antimicrobial peptides represent a structurally and functionally diverse and highly conserved mechanism of defence against pathogens. These molecules are generally 12-50 amino acids in length and are typically membrane-associated, and exert a broad range of antiviral functions at various locations including mucosal sites⁹³. Some examples of common antimicrobial peptides in humans include the defensins, a group of cysteine-rich cationic peptides that are expressed in a variety of cells types including neutrophils and epithelial cells⁹⁴. Among their antiviral functions, the α and β defensins have been shown to block virus:host binding and entry^{95,96}. In particular, human β -defensins 2 and 3 have been shown to be upregulated by HIV infection and perform two important antiviral roles. First, the production of these molecules leads to downregulation of C-X-C chemokine receptor type 4 (CXCR4) surface expression and prevention of viral binding and entry. Second, these molecules were shown to directly bind HIV particles, preventing infection and replication⁹⁶. Lactoferrin, an antimicrobial peptide found in breast milk, has been shown to exert multiple antiviral functions including blocking of receptor-mediated viral entry and inhibition of virus assembly^{97,98}.

I.I.iii.iii.iii. Regulation of adaptive immunity by innate effectors

While the innate and adaptive arms of antiviral immunity have unique hallmarks and functions, the complex interplay between these two classically distinct pathways is becoming increasingly appreciated. In this section, I will provide some examples of the intricate crosstalk between innate and adaptive immune effectors.

Adaptive immunity is characterized by long-term, antigen-specific responses to foreign pathogens. This arm of antiviral immunity relies on virus-specific antigen presentation on MHC molecules and leads to antibody production, cytolysis of infected cells, and long-term antigen memory. Cytokines such as the NF κ B-dependent inflammatory cytokines and IFN are associated with innate immunity but play significant roles in the regulation of adaptive immune functions. For example, IFN has been shown to increase MHCI surface expression leading to antigen presentation, as well to directly stimulate CD8+ T cell responses following viral infection^{99,100}. In addition, type I IFN signalling has been shown to activate gamma interferon activation site (GAS) elements in a cell type-specific manner, implying some redundancy and shared functions between the type I and type II IFN signalling pathways¹⁰¹. Likewise, IFN γ has been shown to induce ISRE-dependent genes via STAT1 and STAT2 phosphorylation and interferon stimulated gene factor 3 (ISGF3) complex formation¹⁰¹⁻¹⁰³. IL-6 upregulates IFN γ production and has been shown to stimulate IL-17 production in T_h17 cells in response to viral stimulus *in vivo*^{104,105}. IL1 β , a major product of inflammasome activation, signals through the p38, extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK), and NF κ B signalling pathways and mediates a wide variety of adaptive immune functions, including proliferation and survival of naïve and memory T cells through the IL-2 pathway, and differentiation and maintenance of T_h17 cells through IRF-4¹⁰⁶.

I.II. Type I Interferon Signalling

As previously described, type I IFN signalling is one of the best characterized innate antiviral responses, and plays a critical role in the ability of a host to combat infection. At the molecular level, IFN production is mediated by a wide variety of diverse and converging recognition events. In this section, I will give an overview of the mechanisms of viral recognition leading to IFN production, as well as outline some specific antiviral effector functions mediated by IFN.

I.II.i. Virus Recognition

Virus recognition leads to the activation of a number of distinct and overlapping immune signalling pathways and is mediated by PRRs recognizing a wide variety of conserved viral patterns or motifs as well as various signals of cell stress. While the innate immune response is classified as a non-specific response, the breadth and specificity of these PRRs to various foreign structures is becoming increasingly appreciated. In terms of virus recognition, the majority of viral ligands that are recognized by PRRs are nucleic acid in nature, as shown in Table 1. A representation of the PRRs involved in viral nucleic acid recognition leading to IFN production is depicted in Figure 1.1. The structure, length, and location of the nucleic acid are key determinants in the cellular receptors engaged and their downstream signalling consequences. In addition, the expression of individual components of the signalling pathway is a determinant of the cell type-specific responses to infection. The following section will cover the major classes of PRRs involved in antiviral signalling leading to type I IFN production. In particular, this section will focus on PRRs that are implicated in viral nucleic acid recognition and their downstream antiviral consequences.

I.II.i.i. Nod-Like Receptors

The NLRs are a group of structurally related, cytosolic PRRs identified by the presence of a NOD, and represent a major mechanism of innate defence against bacterial pathogens⁴². All the NLRs contain a central NACHT domain responsible for self-oligomerization and activation. The C-terminal LRR is, as in the TLRs, thought to be the site of ligand binding. The N-terminal domain carries out effector functions and is the basis for structural classification of the NLRs into Nods, Naips, and Nalps. In general, the Nod class of NLRs contains an N-terminal caspase activation and recruitment domain (CARD) and mediates the activation of NF κ B in response to various bacteria-associated PAMPs. The Naips and IPAF are evolutionarily grouped and, with the exception of IPAF, contain N-terminal baculovirus inhibitor of apoptosis protein repeat (BIR) domains that are associated with the induction of apoptosis. The Naip proteins exhibit ligand specificity, and have been shown to mediate inflammasome activation through IPAF¹⁰⁷⁻¹⁰⁹. Finally, the Nalps form the largest class of NLRs and contain an N-terminal pyrin domain

(PYD), which mediates protein:protein interactions leading to inflammasome assembly in response to a wide variety of PAMPs and DAMPs.

With respect to antiviral immunity, Nalp3 is the best characterized antiviral NLR, recognizing viral nucleic acid and leading to inflammasome assembly^{46-48,110-112} (Figure 1.1A). The Nalp3 inflammasome complex is comprised of Nalp3, the adaptor protein apoptosis speck-like protein containing a CARD (ASC), which interacts with Nalp3 via PYD:PYD interactions, and caspase-1, which interacts with ASC via CARD:CARD interactions. The ultimate consequence of inflammasome formation is the activation of caspase-1, which leads to the proteolytic processing of IL1 β and IL18. The heat shock protein Hsp90, the ubiquitin ligase-associated protein suppressor of G2 allele of S-phase kinase-associated protein 1 (SGT1), and the CARD-containing adaptor protein Cardinal have also been implicated in the Nalp3 inflammasome complex^{113,114}.

Activation of Nalp3 is also achieved in response to a wide variety of non-viral stimuli, including bacterial products, DNA transfection, uric acid, asbestos, and silica, as well as physiological changes such as K⁺ efflux, ATP binding, and ROS production^{48,52,54,57,112,115-122}. These generic cellular changes imply that a DAMP-mediated stress response underlies inflammasome assembly. Conversely, the requirement of a pathogen-associated moiety such as viral nucleic acid or bacterial cell wall components suggests that inflammasome assembly involves specific PAMP recognition by cognate cellular PRRs. Given the two-step model of caspase-1 activation, and given that Nalp3 has not been shown to directly bind viral nucleic acid, it is likely that in the context of viral infection, PAMP recognition is involved in signalling pathways leading to NF κ B-dependent gene expression, while DAMP recognition is involved in mediating inflammasome assembly leading to cytokine processing and their downstream consequences.

More recently, the newly identified NLR family, CARD domain containing 5 (NLRC5) has been demonstrated to serve an antiviral role in macrophages and primary fibroblasts, playing a role in promoting pIC and Sendai virus (SeV)-induced IFN β production¹²³. Kuenzel *et al.*¹²⁴ also report activation of both ISRE- and GAS-responsive promoters in response to NLRC5 overexpression *in vitro*. However, the precise involvement of this NLRC5 in viral recognition and signal transduction is not clear, as macrophages from *NLRC5*^{-/-} mice do not show differences in the transcription of IFN β or NF κ B-dependent inflammatory genes following viral infection or pIC treatment¹²⁵. Rather, overexpression of NLRC5 appears to activate IL1 β via caspase-1 in an inflammasome-independent manner. Additionally, NLRC5 has been implicated in the upregulation of MHCI, providing an additional mechanism of antiviral immunity¹²⁶. It is possible that this molecule plays dual but redundant roles in IFN β production and inflammasome activation following viral stimulation.

NLR-mediated regulation of antiviral gene expression has also been demonstrated in the absence of viral nucleic acid recognition. In particular, NOD, LRR containing X1 (NLRX1) has been implicated as a negative regulator of NF κ B and IFN β signalling pathways following pIC treatment as well as SeV and Influenza virus infection at multiple levels¹²⁷⁻¹²⁹. NLRX1 interacts with TNF associated factors 3

(TRAF3) and 6 (TRAF6) and has been shown to negatively regulate the interaction between NF κ B essential modulator (NEMO, also known as inhibitor of NF κ B γ (IKK γ)) and IKK α/β , inhibiting NF κ B activation. Additionally, NLRX1 expression negatively regulates the interaction between RIG-I and IPS-1, thereby inhibiting IFN β production.

I.II.i.ii. DNA Sensors

A number of cytosolic sensors of viral DNA have also been identified as antiviral immune activators. These include DNA-dependent activator of IRFs (DAI), absent in melanoma 2 (AIM2), IFN γ -inducible protein 16 (IFI16), RNA polymerase III (RNAPIII), the DEAD- and DEHD-box helicases DDX41 and the DDX1/DDX21/DHX36 complex, and LRR flightless-interacting protein 1 (LRRFIP1) (Figure 1.1B).

DAI was the first identified of the viral DNA sensors^{130,131}. Dimerization of DAI leads to its phosphorylation, recruitment of TRAF-associated NF κ B activator (TANK) binding kinase 1 (TBK-1), and activation of IRF-3, leading to IFN production. In addition, DAI activation leads to NF κ B activation through interactions with receptor-interacting proteins 1 (RIP1) and 3 (RIP3). This PRR was shown to recognize B-form DNA (B-DNA), a conformation shown to be mimicked by polydeoxyadeninic:polydeoxythymine acid (pdAdT), as well as HSV infection. Collectively, the activation of DAI by viral DNA leads to IFN- and NF κ B-mediated antiviral responses.

AIM2 is an inflammasome-activating molecule that responds to dsDNA and is essential for the VV and MCMV-induced inflammasome responses¹³²⁻¹³⁴. Like Nalp3, AIM2 contains a PYD that associates with ASC and leads to caspase-1 activation and IL1 β /IL18 processing¹³³. Surprisingly, the AIM2-like molecule IFI16 was also shown to be involved in viral DNA recognition, but instead of activating the inflammasome, IFI16 activates the IRF-3 and NF κ B pathways via the adaptor molecule stimulator of interferon genes (STING, also known as MITA)^{135,136}. Interestingly, AIM2 and IFI16 play a reciprocal negative regulatory role to one another, indicating a bifurcation of viral dsDNA-mediated signalling at the level of the AIM2-like receptors (ALRs)^{137,138}.

RNAPIII was shown to recognize B-DNA and use it as a template to synthesize 5'-triphosphate RNA, leading to retinoic acid inducible gene I (RIG-I) recognition and activation^{139,140}. As such, IFN β production in response to Adenovirus, HSV, and Epstein-Barr virus (EBV) was shown to be RNAPIII-dependent¹⁴⁰.

The DExD/H box helicase superfamily is a large family of nucleic acid binding proteins that have a growing role in antiviral immunity through viral nucleic acid recognition. The best-characterized member of this family is RIG-I, whose diverse signalling role in antiviral immunity is discussed in section 2.1.3. With respect to DNA recognition, DDX41 has been recently shown to recognize viral DNA and mediate IFN β production through interaction with STING and activation of TBK-1¹⁴¹.

The LRR domain containing protein LRRFIP1 has also recently been shown to mediate viral dsDNA- as well as pIC-dependent IFN β production by a mechanism involving β -catenin, which interacts with IRF-3 at the promoter site and recruits p300 to promote transcriptional activation¹⁴². Interestingly, the identification of TRAF-interacting protein (TRIP) as a LRRFIP1-binding protein as well as a sensor for dsRNA (and a weak sensor for dsDNA) implies an indirect role for this protein in viral nucleic acid recognition¹⁴³. It is also possible that TRIP is the PRR that is directly responsible for viral nucleic acid recognition, mediating its antiviral effects through interactions with LRRFIP1, leading to β -catenin-dependent recruitment of p300 to IRF-3.

I.II.i.iii. RIG-I-Like Receptors

The RIG-I like receptors (RLRs) represent a class of cytosolic RNA helicases within the DExD/H box helicase superfamily and include RIG-I, melanoma differentiation associated gene 5 (MDA5), and laboratory of genetics and physiology 2 (Lgp2), among others (Figure 1.1C). Members of this group of molecules contain a C-terminal regulatory domain that mediates nucleic acid binding, and an N-terminal CARD that mediates signalling through adaptor protein interactions. RIG-I and MDA5 are positive regulators of IFN production following viral infection and recognize viral RNA of distinct structures and lengths, while Lgp2 is a thought to serve as a negative regulator of this pathway, as described below.

RIG-I is the best characterized IFN-inducing RNA helicase, and has been implicated in the recognition of a wide variety of viruses and viral ligands¹⁴⁴. At the molecular level, RIG-I has been shown to bind RNA containing 5' triphosphate modifications¹⁴⁵⁻¹⁴⁹. This RNA modification is characteristic of viral RNAs, since cellular RNAs are typically capped or otherwise modified. Nucleic acid structures recognized by RIG-I are also void of 3' nucleotide overhangs, a feature of 'self' RNAs such as siRNA¹⁵⁰. RIG-I activation leads to the activation of three so-called 'signalosomes'¹⁵¹. First, RIG-I association with TRAF3, IFN β promoter stimulator 1 (IPS-1, also known as MAVS, VISA, and Cardif), STING, and TBK-1 and/or I κ B kinase ϵ (IKK ϵ , also known as IKK ι) mediate the activation of IRFs leading to IFN production¹⁵²⁻¹⁵⁹. Second, RIG-I association with TRAF6, IPS-1, and NEMO (also known as IKK γ) leads to the activation of NF κ B leading to proinflammatory cytokine production^{53,153-156,159-162}. Finally, the association of RIG-I with ASC leads to the assembly of a Nalp3-independent inflammasome leading to IL1 β processing¹⁵¹.

MDA5 shares close structural homology to RIG-I and serves as a positive regulator of virus-induced IFN production. The distinct nature of viral RNA recognition between these two molecules appears to occur at the level of RNA length. For example, Kato *et al.*¹⁶³ demonstrated a virus-specific distinction between RNA recognition, showing that *in vitro* transcribed RNAs of lengths no more than 1000 base pairs were capable of activating RIG-I, whereas pIC, which is typically in the range of thousands of base pairs of average length, was shown to engage MDA5. Later, it was demonstrated that long pIC strands were capable of exclusively activating MDA5, whereas digestion of pIC by RNaseIII into <1000 base pair lengths

led to activation of the RIG-I, but not MDA5, pathway¹⁶⁴. In addition, while the adaptor molecule STING has been shown to interact with RIG-I to promote TBK-1 activation leading to IFN production, interactions between MDA5 and STING were not detected, highlighting some mechanistic differences between these two helicases over and above ligand specificity¹⁵².

Lgp2 contains a regulatory domain but lacks a CARD. Not surprisingly, this molecule has been shown to serve as a negative regulator of virus-induced IFN signalling by binding to viral RNA, but not interacting with CARD-containing adaptors required to mediate signalling¹⁶⁵⁻¹⁶⁷. However, reports of positive regulation of IFN responses by Lgp2 in response to viral infection complicate the precise role of this helicase in antiviral signalling¹⁶⁸. It is possible that Lgp2 serves context-dependent positive and negative regulatory roles in IFN production, and/or that Lgp2-mediated IFN production is cell type-dependent.

In addition to the three classical RLRs described above, a growing list of DExD/H box family members have been implicated in virus-mediated antiviral signalling. Some have been shown to recognize viral DNA (described in section I.II.i.ii), while others have been shown to recognize viral RNA and signal through RIG-I and/or MDA5. These include DDX3 and DDX60, which have both been shown to bind dsRNA as well as RIG-I and MDA5, leading to IFN production¹⁶⁹⁻¹⁷¹. Additionally, DDX3 has been shown to be involved in the STING-dependent upregulation of IFN β following human cytomegalovirus (HCMV) infection, implying that this helicase acts to recognize both RNA and DNA associated with infection¹⁷². In addition, DDX1, DDX21 and DHX36 have been shown to form heterotrimeric complexes in response to pIC and lead to the activation of both NF κ B and IRF-3^{173,174}. Finally, DHX9 has been shown to interact with IPS-1 following virus and pIC treatment, leading to transcription of IFN β and NF κ B-dependent genes¹⁷⁵. Taken together, it appears that the DExD/H box superfamily contains multiple DNA- and RNA-binding proteins that mediate STING- and IPS-1-dependent activation of TBK-1, respectively, and ultimately lead to IRF-3 activation and IFN β production.

I.II.i.iv. Toll-Like Receptors

The Toll-like receptors (TLRs) are thought to function in the extracellular and endosomal compartments and recognize a wide variety of PAMPs. In terms of nucleic acid recognition, TLRs 3, 7, and 8 have been shown to mediate antiviral signalling pathways in the endosomal compartment (Figure 1.1D).

TLR3 has been shown to serve as an endosomal receptor for dsRNA through early studies involving pIC treatment¹⁷⁶. In contrast to all other known human TLRs, TLR3 does not signal through the adaptor molecule myeloid differentiation primary response gene (MyD88)^{177,178}. Instead, TLR3 associates with Toll/IL1 receptor domain-containing adaptor inducing interferon β (TRIF) via TIR:TIR domain interactions, where the signalling pathway bifurcates into two distinct downstream pathways¹⁷⁹⁻¹⁸¹. In complex with TRAF6, activation of TLR3:TRIF by pIC leads to activation of the NF κ B pathway and to the expression of inflammatory

cytokines such as IL-6 and TNF α ^{179,180}. In complex with TRAF3, activation of TLR3:TRIF by pIC leads to IRF-3-mediated signalling and IFN production^{180,181}.

Additionally, TLRs 7 and 8 have been shown to play an antiviral role in dendritic cell subsets in response to ssRNA^{182,183}. Activation of these TLRs through MyD88- and IL1 receptor-associated kinase 4 (IRAK-4)-dependent mechanism leads to both NF κ B activation and IFN production, and plays demonstrated roles in the regulation of adaptive immune processes¹⁸⁴.

TLRs not associated with nucleic acid recognition have also been implicated in the antiviral response. For example, HSV-1 and VV recognition by TLR2 has been shown to lead to inflammation and oxidative damage¹⁸⁵⁻¹⁸⁸. Human rhinovirus capsid has also been shown to be recognized by TLR2¹⁸⁹. HIV positive patients show a relative increase in the expression of TLRs 2, 3, 4, 6, 7, and 8 compared to uninfected controls, implying a role for these molecules in the response against infection¹⁹⁰. Recognition of DNA viruses by TLR9 has also been demonstrated for viruses such as HSV-1, HSV-2, and MCMV¹⁹¹⁻¹⁹⁴. However, the specific recognition of viral DNA by TLR9 is speculative, since this has not been directly demonstrated. Recently, a novel TLR was identified in humans, TLR13, which was shown to be activated in response to VSV infection in a MyD88-dependent manner¹⁹⁵.

I.II.i.v. Scavenger Receptors

Finally, the scavenger receptors are a group of structurally diverse, largely membrane bound receptors whose common characteristic is the ability to bind modified low-density lipoprotein (LDL). While classically thought to be expressed primarily in macrophages, with functions involving phagocytosis and clearing of pathogens and byproducts of cellular damage, it is now known that this class of molecules is widely expressed and serves important antiviral functions. *In vivo*, the class A scavenger receptors (SR-As) have been demonstrated to be critical for the antiviral responses to viruses such as HSV-1 and HCMV^{196,197}. Macrophage receptor with collagenous structure (MARCO) has also been shown to recognize Influenza A virus *in vivo*, leading to immune pathogenesis¹⁹⁸. At the molecular level, recognition of pIC, viral dsRNA, or virus-mediated inosine-containing RNA by SR-As leads to activation of signalling pathways such as p38, protein kinase R (PKR), and IFN¹⁹⁹⁻²⁰¹. The current model suggests that SR-As are responsible for the uptake of viral PAMPs and their delivery to other PRRs such as the TLRs or other nucleic acid receptors, leading to inflammation and antiviral protection (Figure 1.1E).

I.II.ii. Signalling Pathways Leading to Interferon Production

The IFN-mediated antiviral response consists of a series of distinct recognition events by PRRs, whose activation ultimately leads to the expression of a large number of ISGs^{202,203}. The collective function of ISGs is to suppress viral replication and spread, a characteristic known as the antiviral state. While there are many cellular receptors that recognize viral infection, in the context of IFN

signalling, these pathways appear to converge at the activation of TBK-1 or IKK ϵ . The relative contribution of TBK-1 and IKK ϵ to the activation of IRF-3 following viral stimulation is not entirely clear, and is likely to be both cell type- and stimulus-dependent. The pathways leading to IFN and ISG production following viral infection are depicted in Figures 1.1 and 1.2, and are described below.

I.II.ii.i. Converging pathways lead to the activation of TBK-1 and/or IKK ϵ

As described in section I.II.i, the recognition of viral nucleic acid occurs through many distinct cellular receptors. The structure, source, length, and location of viral nucleic acid appear to play important roles in their recognition by a particular PRR. In general, the signalling pathways activated by PRRs leading to IFN production via TBK-1 and/or IKK ϵ utilizes three adaptor molecules, IPS-1, STING, and NEMO (depicted in Figure 1.1). The differential contributions of TBK-1 and IKK ϵ to IRF-3 activation are not clear, and it is probable that these kinases play both redundant and cell-type specific roles in the antiviral signalling pathway leading to IFN production.

IPS-1 has been characterized as an essential adaptor to the RIG-I and MDA5-mediated production of IFN following viral infection^{153,160}, as has been shown to associate with RNA-binding RLRs^{153,155,156,160,170}. This molecule is localized to the mitochondria and has been shown to associate with IKK ϵ ¹⁵⁵. In addition, IPS-1 has been shown to interact with a number of proteins implicated in the assembly of a mitochondrial activation complex leading to TBK-1/IKK ϵ activation. These proteins include TRIF, TNF receptor type 1-associated death domain protein (TRADD), TRAF3, TANK, and NEMO^{156,158,160}. The interaction of IPS-1 with TRADD, TRAF3 and TANK via TRIF leads to the recruitment and activation of TBK-1/IKK ϵ . IPS-1 has also been shown to interact with Fas-associated protein with death domain (FADD) and RIP1 via TRADD association, leading to NF κ B activation^{153,156}. Furthermore, the association of IPS-1 with TRAF6 has also been shown to promote NF κ B activation^{160,161}. Taken together, it is clear that IPS-1 plays an important role at multiple stages of antiviral signalling pathways following viral RNA recognition, and bifurcates into the IRF-3-mediated and NF κ B-mediated transcription of antiviral genes.

STING has been characterized as an essential adaptor molecule in the viral DNA-mediated production of IFN^{152,204}. The localization of this molecule is unclear, as STING has been shown to localize to both the endoplasmic reticulum and to the mitochondria in unstimulated cells^{152,205}. In addition, STING has been shown to localize to perinuclear vesicles and associate with proteins associated with the endosome, translocon, and exocyst compartments, including early endosome antigen 1 (EEA1), transferrin receptor (TFR), translocon-associated protein β (TRAP β), and the exocyst component SEC5^{152,204,205}. It is probable that the mitochondria and endoplasmic reticulum interact closely during viral infection and signalling, or that perinuclear vesicles mediate the interaction of STING with other signalling components located in either the endoplasmic reticulum or mitochondria

in unstimulated cells. STING associates with DNA-binding PRRs and with TBK-1/IKK ϵ , IRF-3, and IRF-7 leading to IFN production^{136,141,152,204,205}. While STING does not appear to be essential for signalling in response to viral RNA recognition²⁰⁴, association with RIG-I, but not MDA5, has been demonstrated, implying a role for this molecule in viral RNA recognition^{152,205}. Therefore, STING does not merely serve an analogous role to IPS-1 in the DNA-dependent antiviral signalling pathway, as has been classically described.

Finally, NEMO has been shown to be essential for the activation of IFN following viral infection^{162,206}. While classically characterized for its essential role in NF κ B activation, studies in NEMO-deficient cells demonstrate that antiviral signalling bifurcates at NEMO into both the IRF-3- and NF κ B-dependent pathways, and that NEMO is essential for both¹⁶². NEMO forms a complex with TANK and TBK-1, leading to activation of IFN, and has been shown to interact with IPS-1 in a TRAF3- and TRAF5-dependent manner²⁰⁷.

I.II.ii.ii. IFN β Production

The production of IFN and establishment of an antiviral state occurs in a series of signal transduction events. First, the recognition of viral nucleic acid by PRRs leads to the activation of TBK-1/IKK ϵ and IFN β expression. Second, autocrine and paracrine signalling by IFN β result in the expression of ISGs, including IRF-7. Finally, the activation of IRF-3 and IRF-7 leads to a positive feedback mechanism via the production of the full complement of IFN α subspecies. A representation of these signalling events is depicted in Figure 1.2.

The activation of TBK-1/IKK ϵ by the aforementioned pathways leads to the phosphorylation of IRF-3²⁰⁸⁻²¹⁰ (Figure 1.2A). Phosphorylation of this transcription factor leads to a conformational change allowing homotypic interactions and dimerization, followed by nuclear translocation and binding to the IFN β promoter at the positive regulatory domain (PRD)III/I sites of the IRF binding element (IRF-E)²¹¹⁻²¹⁸. In cooperation with additional transcription factors NF κ B, activated transcription factor 2 (ATF2), and c-Jun and cofactors cyclic AMP response element binding (CREB) binding protein (CBP) and p300, IRF-3 mediates the expression of IFN β as well as IFN α ⁴^{213,214,218-223}.

Additionally, activated IRF-3 is capable of binding to the promoter region of a subset of ISGs, bypassing the requirement for IFN production in the establishment of an antiviral state. The production of this subset of IFN-independent ISGs is discussed in section I.3.

I.II.ii.iii. Interferon Stimulated Genes and Interferon Regulatory Factor 7 Production

IFN β is a soluble cytokine, and acts in an autocrine and paracrine fashion via a JAK/STAT pathway to promote ISG expression (Figure 1.2B). Extracellular IFN β binds the IFN receptor comprised of IFNAR1 and IFNAR2, the engagement of which leads to the activation of Jak1 and tyrosine kinase 2 (Tyk2). Activation of these

kinases, in turn, leads to phosphorylation of STAT1 and STAT2 and recruitment of IRF-9²²⁴⁻²²⁹. Activated STAT1, STAT2 and IRF-9 form the transcription factor complex known as ISGF3, which translocates to the nucleus and mediates the transcription of ISGs via binding to the ISRE on the promoters of these genes^{224,227,228,230}. In particular, the expression of IRF-7 is mediated by the ISGF3 complex²³¹, and activation of this transcription factor leads to a positive feedback loop, as described below.

III.ii.iv. IFN α Production and Positive Feedback

Activated IRF-7 is capable of binding to the *IFN α* and *IFN β* gene promoters, creating a positive feedback loop that potentiates the production of ISGs and the induction of an antiviral state²²⁰ (Figure 1.2C). At this stage, activated TBK-1/IKK ϵ mediates the phosphorylation of both IRF-3 and IRF-7 following viral infection. Activation of these transcription factors leads to heterotypic interactions that result in IRF-3:IRF-7 dimerization, nuclear translocation, and transcription of the full spectrum of IFN α s. Interestingly, the IRF-7 promoter bears an IRF-E in addition to an ISRE; therefore, the expression of IRF-7 may be achieved through both ISGF3 and the virus-activated factor (VAF) consisting of IRF-3, IRF-7, CBP, and p300²³². Autocrine and paracrine signalling by IFN α leads to a positive feedback loop that amplifies ISG expression and ultimately leads to the establishment of an antiviral state.

Taken together, it is clear that both IRF-3 and IRF-7 contribute to the antiviral state via their interactions with IFN promoters. However, homodimers of activated IRF-3 are capable of binding to the IFN β promoter, but not to most IFN α promoters, making IRF-3 a critical transcription factor at early times post infection in many cell types. Conversely, IRF-7 is itself an ISG, is capable of binding the promoters of all IFN genes, and has been shown *in vitro* and *in vivo* to be a critical component to the innate response to viral infection¹⁵. As such, IRF-7 is an important component to later stages of IFN production in many cell types. For this reason, IRF-7 is considered to be a “master regulator” of IFN responses¹⁵. Indeed, IRF-7 is constitutively expressed in pDCs, the major producers of IFN α *in vivo*^{15,16,233}. Collectively, the production of IFN and ISGs leads to an antiviral state, characterized by the inability of cells to support viral replication.

Interestingly, a number of studies have demonstrated antiviral activity in the absence of IRF-3 and IRF-7. Work from our own lab in *IRF-3*^{-/-} MEFs has demonstrated that both IRF-3 and IPS-1 are dispensable for the production of ISGs and the induction of an antiviral state in response to long viral dsRNA molecules derived from West Nile virus (WNV)²³⁴. Additionally, Andersen *et al.* identify a number of antiviral genes, including ISGs, induced in *IRF-3*^{-/-} MEFs following Newcastle disease virus (NDV) infection²³⁵. Studies in *IRF-3*^{-/-} and *IRF-7*^{-/-} mice have shown that in both MEFs and pDCs, IRF-7 appears to be essential for the production of both IFN α and IFN β , while IRF-3 is dispensable only for the production of IFN β , following HSV-1, VSV, and/or encephalomyocarditis virus (EMCV) infection¹⁵. Finally, both IRF-3 and IRF-7 have been shown to be dispensable for the production

of IFN β and ISGs following WNV infection²³⁶. In this study, IPS-1 was required for both IFN α and β production following WNV infection. Moreover, total ablation of WNV-induced IFN β production in *IRF-3*^{-/-}/*IRF-7*^{-/-} MEFs was achieved after pharmacological inhibition of NF κ B and ATF-2/c-Jun. The authors propose a model in which some, but not all, of the canonical transcription factors comprising the IFN β enhanceosome are required to activate transcription of this gene following viral infection.

Despite these findings, the importance of IRF-3 and IRF-7 in the antiviral response has been demonstrated in both *in vitro* and *in vivo* studies. In *IRF7*^{-/-} MEFs, the production of both IFN α and IFN β is abolished in response to HSV-1, VSV, and EMCV infection¹⁵. *IRF-7*^{-/-} mice are highly susceptible to HSV-1 and EMCV infection *in vivo*, showing lower levels of IFN α and decreased survival following infection¹⁵. Additionally, *IRF-7*^{-/-} mice show increased susceptibility to WNV infection, demonstrating decreased survival, increased viral titres, and susceptibility to viral invasion of the central nervous system²³⁷. Studies in *IRF3*^{-/-} MEFs showed highly diminished levels of IFN β following HSV-1, VSV, and EMCV infection, as well as a strong decrease in IFN α production following EMCV infection¹⁵. *In vivo*, IRF-3 deficiency does not lead to significant changes in serum IFN α production, but does decrease survival following EMCV infection¹⁵. IRF-3 has also been shown to play an important role *in vivo* to reovirus infection, mediating viral clearance as well as IFN β production at early times post-infection²³⁸. Taken together, IRF-3 and IRF-7 make an important contribution to the production of IFN and antiviral immunity both *in vitro* and *in vivo*, and are considered to be central mediators of the IFN signalling pathway.

I.II.ii.v. Other Interferon Regulatory Factors as Regulators of Interferon

IRF-3 and IRF-7 are among the best-characterized IRFs in terms of their role in IFN signalling in response to viral infection. However, other transcription factors participate in the expression of the type I IFN genes, as described below.

There are currently 10 known IRFs in humans, classified by their homologous N-terminal DNA binding domains. The IRFs have a wide variety of cellular expression profiles, and regulate innate and adaptive antiviral immunity in a wide variety of contexts.

IRF-1 and IRF-2 are structurally similar transcription factors with antagonistic functions. IRF-1 is a positive regulator of IFN expression and is involved in antiviral responses against rhinovirus and Human papillomavirus (HPV)^{239,240}. IRF-1 has also been shown to be type I and type II IFN-inducible, and is involved in promoting adaptive immune responses by upregulating MHC I expression²⁴¹. Finally, many of the initial studies regarding IRF-1 function have characterized its ability to act as a tumor suppressor²⁴²⁻²⁴⁴. In contrast, IRF-2 has been shown to serve as a negative regulator of IRF-1 and its activity is, not surprisingly, oncogenic in nature^{243,244}.

IRF-4 and IRF-8 both appear to participate in immune cell differentiation, promoting myeloid DC differentiation from the common DC precursor^{245,246}. In addition, they both appear to participate in the inhibition of inflammatory processes stimulated by various TLR ligands and physiological stresses²⁴⁷⁻²⁵⁰. IRF-4 has also been shown to be critical to the differentiation of the T_h17 subset through the expression of retinoic acid receptor-related orphan receptor γ t (ROR γ t), a crucial transcription factor for T_h17 differentiation. In terms of antiviral immunity, these transcription factors regulate the expression of a subset of T_h2 cytokines such as IL10 and IL4^{251,252}. HTLV-1 infection has also been shown to induce IL15 receptor expression in an IRF-4-dependent manner²⁵³. Studies in *IRF-4*^{-/-} cells demonstrate a negative regulatory role for IRF-4 in the production of proinflammatory cytokines following TLR stimulation^{247,248}.

IRF-5 has been well characterized as a positive regulator of IFN in myeloid cells, interacting with IRF-7 to amplify IFN α expression²⁵⁴⁻²⁵⁶. Interestingly, the profile of IFN α subspecies produced by activated IRF-5/IRF-7 heterodimers following viral infection is distinct from the profile associated with activated IRF-3/IRF-7, implying a unique role for this transcription factor in IFN responses to viral infection. Further, the profile of IFN α subspecies expressed following virus-induced activation of IRF-5 demonstrates a splice variant-dependent specificity²⁵⁶. *In vivo*, *IRF-5*^{-/-} mice demonstrate lower serum IFN and TNF α levels compared to wild-type mice in response to NDV infection, although IRF-5 is not essential for the production of these cytokines²⁵⁷. In addition, IRF-5 has been shown to mediate cell cycle arrest, presumably through the upregulation of proapoptotic molecules including B cell lymphoma 2 (Bcl-2), Bcl-2 homologous antagonist killer 1 (Bak1), and Bcl-2-associated X protein (Bax)^{256,258,259}.

IRF-6 appears to play a very limited role in antiviral immunity, but has been shown to act as a transcriptional activator in ovine uterine cells via ISRE binding²⁶⁰. IRF-6 is also a positive regulator of the NO pathway in response to smoke exposure, binding the NOS2 promoter²⁶¹. Finally, IRF-6 appears to be involved in embryonic development²⁶².

IRF-10 is a virus-inducible transcription factor²⁶³, having delayed kinetics relative to other ISGs and implying a role in late phase antiviral immunity^{263,264}. Upregulation of MHC I and guanylate binding protein, an IFN γ target gene, are among the functions of this transcription factor²⁶⁴.

I.i.iii. Consequences of Interferon Signalling

The antiviral state is established by the collective functions of ISGs. These effectors have multiple distinct and overlapping functions, including inhibition of viral protein expression, apoptosis, and recruitment of immune cells to sites of infection. The individual antiviral functions of the most highly upregulated and well-characterized ISGs are described below.

I.II.iii.i. ISG 56

Among the most abundant of the ISGs is ISG 56 (also known as IFIT1), which is induced downstream of IFN signalling, as well as being directly inducible by activated IRF-3^{202,235,265}. ISG 56 elicits its antiviral function by binding to and blocking the activity of the eukaryotic translation initiation factor eIF3, leading to a reduction in both cellular and viral gene expression²⁶⁶⁻²⁷². Specifically, ISG 56 has been shown to bind the c-subunit of eIF3, leading to inhibition of this translation initiation factor's ability to stabilize the ternary initiation complex comprised of eIF2, guanosine triphosphate (GTP), and Met-tRNA(i)^{267,270-272}.

In addition to inhibiting translation initiation, ISG 56 has been shown to interact with the eukaryotic translation elongation factor eEF1A, leading to inhibition of viral gene expression at multiple levels²⁷³. In fact, the specific inhibition of HPV gene expression has also been demonstrated, as ISG 56 has been shown to inhibit the HPV Ori-specific DNA binding as well as the activity of the viral transcriptional transactivator protein E1^{274,275}. Very recently, ISG 56 was also shown to be a receptor for 5' triphosphate RNA and contribute to the inhibition of viral replication and pathogenesis²⁷⁶. However, the downstream consequences of this interaction are unclear, and it is likely that viral RNA binding leads to antiviral protection by sequestering viral RNA from translational machinery.

Finally, ISG 56 is also thought to be a negative regulator of the innate antiviral response. Overexpression and RNAi experiments demonstrate that NFκB-dependent promoter activity and IFNβ promoter activity are both negatively regulated by ISG 56. In addition, coimmunoprecipitation experiments demonstrated that ISG 56 blocked the interaction between IPS-1 and TBK-1, leading to a reduction in SeV- and pIC-induced antiviral activity²⁷⁷. In this way, ISG 56 provides both positive and negative regulation of virus-induced IFN responses.

I.II.iii.ii. ISG 54

ISG 54 (also known as IFIT2) is structurally similar to ISG 56, belonging to the family of interferon-stimulated genes containing tetratricopeptide repeats (IFITs). ISG 54 has been shown to bind both the e- and c-subunits of eIF3, leading to inhibition of translation initiation²⁷¹. In addition, this molecule induces apoptosis in a Bax/Bak-dependent manner, leading to control of viral spread²⁷⁸. Finally, ISG 54 has been shown to interact with the microtubule network, by specifically co-localizing with β-tubulin²⁷⁹, and it is thought that this interaction affects viral assembly and transport.

I.II.iii.iii. ISG 15

ISG 15 is a ubiquitin-like modifier of proteins involved in a wide variety of processes including antiviral gene activity. It is thought that ISGylation, the modification of proteins by ISG 15 conjugation, regulates the activity, stability,

and/or degradation of both viral and cellular proteins involved in either antiviral protection or viral pathogenesis.

ISG 15 has more than 100 cellular targets with a broad range of functions, including protein synthesis and modification, carbohydrate metabolism, stress responses, and microtubule components²⁸⁰⁻²⁸². Among the genes known to be involved in antiviral processes, ISG 56, IFN-induced cellular resistance mediator protein (MxA), and RIG-I have been shown to be targets for ISG 15 conjugation²⁸². It is possible that ISG 15 competes with ubiquitin for the conjugation and regulation of various proteins involved in antiviral defence. In terms of ISGylation of viral genes, Influenza virus NS1B has been demonstrated to be a target for ISGylation, acting as a viral evasion mechanism by sequestering ISG 56 and preventing the ISGylation of other target proteins^{283,284}.

ISG 15 is also thought to be a negative regulator of the ubiquitination system at the level of enzyme activity, inhibiting the activity of many ubiquitin-conjugating enzymes such as ubiquitin conjugating enzyme 13 (Ubc13) and neural precursor cell expressed developmentally downregulated 4 (Nedd4)²⁸⁵⁻²⁸⁷. In this regard, ISG 15 is thought to affect the stability and hence activity of many proteins and processes.

I.II.iii.iv. Other ISGs

The antiviral activities of ISGs are diverse and affect both viral and cellular processes including adaptive immune regulation and chemotaxis. For example, promyelocytic leukemia (PML) protein has been implicated in a number of cellular antiviral processes, including the induction of apoptosis and MHC I antigen presentation^{288,289}. The chemokine IP10 is thought to be a T_h1-attracting chemokine²⁹⁰⁻²⁹², as well as being involved in the migration of a broader range of peripheral blood lymphocytes²⁹³. Regulated upon activation, normal T cell expressed, and secreted (RANTES) protein has been shown to act as a chemoattractant for a wide variety of cell types including eosinophils, B cells, mast cells, neutrophils, monocytes, and activated T cells in response to viral stimuli²⁹⁴. The reader is referred to a detailed review of the individual functions of many ISGs by de Veer *et al.*, who provide a functional classification for ISGs in terms of their role in antiviral immunity²⁰².

I.III. Interferon-Independent Antiviral Signalling

The production and subsequent signalling of IFN in response to viral infection is largely thought to be dependent upon the recognition of viral nucleic acid. In many cases, viral nucleic acid is a hallmark of viral replication. However, it is increasingly appreciated that the physiological changes associated with virus particle entry mediate signalling cascades that are antiviral in nature, even in the absence of viral gene expression. These changes may be mediated by cellular proteins during entry, or may be mediated by viral proteins, as described below.

I.III.i. Viral Entry Acts as a Danger Associated Molecular Pattern

The detection of changes to various hallmarks of cellular homeostasis is a complex and precise process. Changes in parameters such as membrane permeability, ion concentration, and cytoskeletal integrity lead to the activation of a variety of signalling pathways, whose collective function is thought to protect the cell from stress, damage, and infection. Analogous to the cellular recognition of PAMPs as an activator of molecular signalling pathways, these physiological changes represent so-called DAMPs, and their expression or activation induces molecular signalling pathways to combat cognate danger.

In terms of antiviral immunity, the entry of a virus particle leads to a series of physiological changes which are largely pathogen non-specific. These changes lead to antiviral signal transduction, and therefore serve as DAMPs, with signalling consequences analogous to PRRs in terms of antiviral immunity. These changes include cytoskeletal rearrangement as well as changes to membrane permeability and subsequent ion mobilization, and are described below.

I.III.i.i. Cytoskeletal Rearrangement

While viruses use a variety of mechanisms to enter a host cell, viral entry ultimately involves actin or tubulin rearrangement, which has been shown to serve as a DAMP. For example, multiple enveloped viruses such as parvovirus, HCV, and Bornavirus enter the host cell via clathrin-mediated endocytosis, leading to microtubule reorganization²⁹⁵⁻²⁹⁷. Other viruses use clathrin-independent mechanisms of endocytosis²⁹⁸⁻³⁰⁰, which also utilize molecules involved in cytoskeletal rearrangement. Still others such as Ebola virus utilize a cholesterol-dependent, clathrin-independent mechanism of entry³⁰¹. The entry of viruses such as Dengue virus and HIV has been shown to require the Rho GTPase Ras-related C3 botulinum toxin substrate 1 (Rac1), which is involved in actin rearrangement^{302,303}. Furthermore, many viruses encode their own fusion proteins to facilitate entry³⁰⁴⁻³⁰⁸; however, all viruses hijack some aspect of the cellular machinery to facilitate their entry.

During virus replication, many viruses require cytoskeletal rearrangements to facilitate virus transport to the nucleus for replication, utilize cytoskeletal components during assembly, or require cytoskeletal rearrangements for release of

infectious virions. Interestingly, HSV-1, SeV, and Influenza virus infection, as well as pIC treatment, have been shown to activate Rac1^{309,310}. Furthermore, Rac1 has been shown to be essential to the activation of IRF-3 following viral infection³⁰⁹, indicating that cytoskeletal rearrangement is an important DAMP that mediates antiviral signalling. Precisely how Rac1 regulates signalling components upstream of IRF-3 is not clear, but the involvement of this protein in the NADPH oxidase complex provides a role for Rac-1-mediated ROS production in the regulation of antiviral signalling components. The role of ROS in antiviral signalling is discussed in detail in section I.5.

I.III.i.ii. Membrane Permeability and Ion Mobilization

The viral entry event can lead to physiological changes which are largely pathogen non-specific. For example, changes in the concentration of ions such as intracellular calcium and potassium are associated with viral entry during changes in membrane permeability. In many cases, virus encoded proteins known as viroporins are responsible for facilitating changes in membrane permeability that lead to changes in ion concentration and facilitate virus particle entry. Some examples of viroporins include the small hydrophobic (SH) protein of RSV, gp41 of HIV, non-structural protein 4 (NSP4) of rotavirus, 2B of poliovirus, and p13 of HTLV-1³¹¹⁻³¹⁵. The entry and uncoating of viruses such as HSV and rotavirus, for example, have been shown to depend on calcium mobilization³¹⁶⁻³¹⁸. The consequences of virus-induced ion mobilization are diverse, and lead to cellular responses such as apoptosis and inflammasome activation^{57,319,320}. It is also known that changes in ion mobilization from various cellular compartments such as the endoplasmic reticulum, mitochondria, and cytosol contribute to DAMP-mediated signalling and are tightly linked with ROS production, and that ROS production has a wide variety of antiviral and inflammatory processes^{315,320-325}.

I.III.ii. Viral Entry Induces an Interferon-Independent Antiviral State

Virus particle entry leads to a cellular response that is characterized by ISG production and an antiviral state. Our lab and others have shown that the induction of an antiviral state does not require virus replication and occurs in the absence of IFN signalling^{67,326-330}. The establishment of an IFN-independent antiviral state involves the production of a subset of ISGs whose promoters interact directly with IRF-3 via the ISRE, bypassing a requirement for IFN production and signalling. Importantly, the IRF-3-dependent, IFN-independent response to virus particle entry occurs independently of any detectable soluble factor, since supernatants from treated cells are unable to confer protection to naïve cells^{331,332}. In addition, virus particle-induced transcription of ISGs has been shown to occur in the presence of cycloheximide, a translational inhibitor, demonstrating that production of IFN and subsequent signalling are not required for this response^{333,334}. Therefore, this process is likely to be an intracellular immune response, occurring independently of

secreted cytokines such as IFN, whose signalling components are constitutively expressed in uninfected cells.

I.III.ii.i. Components of the Signalling Pathway

A schematic representation of the IFN-independent signalling pathway is depicted in Figure 1.3. We have previously demonstrated that the cellular response to virus particle entry involves TBK-1 and IRF-3, and induces ISGs in the absence of IFN production and viral gene expression in primary fibroblasts^{331,332}. Although the TLR and RIG-I pathways are not essential for this response to occur³³², additional viral and/or cellular components that are required for this response remain largely unknown. Importantly, our lab has recently shown that the fusion event itself, in the absence of any viral component, is capable of stimulating this signalling pathway, leading to an antiviral state³³⁵. A member of the phosphatidylinositol 3-kinase (PI3K) family has been implicated in IRF-3 activation in response to virus particle entry³³⁶; however, the precise family member involved and the signalling pathway used by this protein have not been elucidated. Given that the inhibition of this molecule does not affect IRF-3 nuclear translocation, it is possible that this molecule acts on nuclear IRF-3 to prevent promoter binding, or that it acts upstream of the activation of another transcription factor required for IFN-independent ISG production.

Upon virus particle entry, an unknown component of the signalling pathway is thought to activate TBK-1. Studies from our lab suggest that TLRs and RIG-I do not appear to participate in the recognition of virus particle entry³³⁷. Since non-replicating virus particles contain very little nucleic acid to serve as a ligand, it is unlikely that the nucleic acid sensors are involved in the recognition of incoming particles. However, the ability of PRRs such as RIG-I to detect viral genomic nucleic acid does not rule out nucleic acid recognition in the response to virus particle entry³³⁸. The signalling components lying upstream of TBK-1 activation are currently not known.

TBK-1 is responsible for the activation of IRF-3 in response to viral entry. Activated IRF-3 translocates to the nucleus and directly binds the promoters of a subset of ISGs containing IRF-3 recognition sites. In the IFN-independent response, NF κ B does not appear to be activated, and does not participate in the transcription of antiviral genes³³⁷. It is thought that both NF κ B and IRF-3 activation are required for the transcription of the full set of IFNs and ISGs; hence, it is likely that in the absence of a nucleic acid ligand to induce activation of NF κ B, the virus particle is capable only of inducing the IRF-3-dependent, IFN-independent subset of ISGs, which are sufficient to establish an antiviral state and lead to protection against challenge virus³³⁹. This phenomenon is dependent on MOI, however, as NF κ B and IFN production were observed at higher MOIs of virus particle treatment. These observations imply that MOI is a determinant in whether cells will elicit a full-blown antiviral response characterized by NF κ B activation, IFN production, and inflammatory cytokine production in response to virus particle entry. This also implies that there is a threshold whereby the amount of a viral PAMP, or cellular

DAMP, determines the nature of the antiviral response, leading to inflammation and immunopathology.

Interestingly, Prescott *et al.* described the induction of ISGs independently of IRF-3, IRF-7, TLR3, RIG-I, and MDA5 in response to UV inactivated Sin Nombre Hantavirus (SNV) in the Huh7 human hepatoma cell line³⁴⁰. In this study, virus particle entry was not required, as siRNA directed against the cellular receptor for HNV did not affect ISG production following UV-inactivated SNV (SNV UV) treatment. While it is possible that HNV utilizes another cellular receptor in Huh7 cells for entry, these data suggest that a membrane-bound PAMP or DAMP facilitates the antiviral response to non-replicating virus particles. Additionally, siRNA-mediated knockdown of IPS-1 appeared to have a modest effect of SNV UV-mediated ISG production; however, statistical analysis was not performed on this data set, making it difficult to assign a definitive role for this molecule in the antiviral response to UV virus particles. Whether IPS-1 plays a role in the IFN-independent response to virus particle entry in primary human fibroblasts has not been established.

Importantly, the non-essential role of IRF-3 in the response to SNV UV appears to be in contrast to our current understanding of the IFN-independent antiviral signalling response to virus particle entry. In our hands, siRNA-mediated knockdown of IRF-3 significantly decreases, but does not abolish, ISG 56 production following NDV UV or HCMV UV treatment in primary human fibroblasts³⁴¹. However, the IFN-independent antiviral state stimulated by membrane fusion as well as UV virus treatment has been shown to be IRF-3 dependent in MEFs^{335,337}. Importantly, studies by Prescott *et al.* did not measure IFN production in SNV UV-treated Huh7 cells, and we have observed IFN production at high multiplicities of infection (MOIs) following UV virus treatment³³⁷. Therefore, the IRF-3-independent production of IFN by SNV UV may account for differences observed between these experimental approaches. Taken together, the current model of IFN-independent antiviral signalling involves the recognition of virus particles by yet unidentified PAMPs and/or DAMPs, and leads to the induction of ISGs in a manner that is highly dependent on IRF-3.

I.III.ii.ii. Comparison to Interferon-Dependent Signalling

The antiviral state induced by either replicating virus or non-replicating virus particles has both shared and distinct components. While the IFN-dependent response to replicating viruses is both an autocrine and paracrine response, the IFN-independent response to virus particle entry appears to be an intracellular response, where any soluble factors produced during this pathway do not induce antiviral protection in naïve cells. The IFN-dependent response has been shown to involve the nucleic acid sensors such as the TLRs, RLRs, and NLRs as well as the DNA sensors. In contrast, the IFN-independent response does not rely on TLRs or RIG-I. Whether this response is dependent on the other known nucleic acid sensors has not been tested. In the IFN-dependent response, many distinct and overlapping signalling pathways have been characterized to lead to antiviral protection, including mitogen activated protein kinase-extracellular signal-regulated kinase

(MEK)/ERK, c-Jun N-terminal kinase (JNK), and stress-activated protein kinase (SAPK) pathways in addition to the IRF-3 pathway. In the IFN-independent response, the aforementioned pathways besides IRF-3 have been ruled out as being involved in entry-induced signalling³³⁶. Activation of the IFN-dependent response involves the transcription factor IRF-3 as well as activated NF κ B, ATF2, c-Jun, and CBP/p300. Activation of the IFN-independent response relies on IRF-3 and does not require NF κ B. Other transcriptional factors involved in the IFN-independent induction of ISGs have not yet been elucidated. However, the requirement of CBP/p300 interaction with activated IRF-3 to allow DNA binding suggests that this cofactor is involved in IRF-3-mediated transcriptional activation, either in an IFN-dependent or -independent manner²²³. Finally, the IFN-dependent response involves a widespread induction of ISGs leading to an antiviral state. Conversely, the IFN-independent response involves a restricted ISG profile; however, expression of these ISGs is sufficient to lead to an intracellular antiviral state.

Taken together, it is clear that there are many differences between IFN-dependent and -independent signalling, with IRF-3 playing a central role in both. Many mechanistic questions regarding the initiation of antiviral signalling regarding virus particle entry remain unanswered. In addition, the mechanisms of IRF-3 activation following virus particle entry have not been elucidated, and provide strong rationale for the study of cellular components involved in the IRF-3-mediated, IFN-independent response to virus particle entry.

I.IV. IRF-3 Regulation in the Antiviral Signalling Cascade

I.IV.i. Structure and Functional Domains of IRF-3

IRF-3 is a constitutively expressed transcription factor in a wide variety of tissues⁶⁰. In the absence of stimulus, IRF-3 has been shown to shuttle between the cytoplasmic and nuclear compartments³⁴². A schematic representation of the functional domains of wild-type IRF-3 is depicted in Figure 1.4. The N-terminal DNA binding domain (DBD) is responsible for mediating DNA binding at ISRE and IRF-E sites, leading to activation of transcription of responsive genes^{213,218}. The nuclear export signal (NES) and nuclear localization signal (NLS) sequences are responsible for the subcellular localization of IRF-3, and exposure of these recognition signals is conformation dependent^{218,342}. The IRF association domain (IAD) is responsible for mediating interactions with other IRFs, as well as with IRF-3 itself^{212,215,216}. IRF-3 has been shown to form homodimers via homotypic interactions of the IAD, as well as heterodimers with IRF-7 via heterotypic interactions of the IADs of these transcription factors. Finally, the C-terminal serine rich region (SRR) contains a number of serine residues thought to be essential for the phosphorylation and activation of IRF-3 in response to stimulus^{211,212,214-216,343}.

The activation of IRF-3 involves a series of phosphorylation events, a conformational change leading to dimerization and nuclear translocation of the transcription factor, and finally DNA binding and activation of transcription. The precise kinetics of these hallmarks of activation are a matter of debate, and likely differ between stimuli. Given the limits of detection of assays used to detect these hallmarks, it is possible that in some cases, either not all features are required for activation, or that not all features occur at a detectable level.

I.IV.ii. Hallmarks of IRF-3 Activation

I.IV.ii.i. Phosphorylation

Upon stimulation, IRF-3 is phosphorylated at a number of serine/threonine residues. However, the roles of these sites in response to various viral stimuli as well as the minimum requirements for phosphorylation-mediated activation of IRF-3 are subject to interpretation^{211,216,343-345}. For example, while paramyxovirus infection and pIC treatment result in the phosphorylation of both S386 and S396 residues, a S396D mutation is sufficient to constitutively induce IFN and ISG promoter activity. However, S→D mutation of five C-terminal serine residues is necessary to detect constitutive hyperphosphorylation of IRF-3, suggesting that S396 is necessary but insufficient for full IRF-3 activation. Phosphorylation of T135 has been demonstrated in response to SeV infection; however, the involvement of T135 in response to other viruses or pIC has not been observed. Bergstroem *et al.*²¹¹ recently identified T390 as a target for phosphorylation, where

phosphorylated T390 is responsible for the positive regulation of S396 phosphorylation.

Meanwhile, SP339-40 have also been implicated in phosphorylation in response to pIC treatment, resulting in both positive and negative regulation^{212,345}. Phosphorylation of S339 appears to be involved in both dimerization and CBP association; however, this also leads to the recruitment of the prolyl isomerase enzyme peptidylprolyl cis/trans isomerase, never in mitosis gene a-interacting 1 (Pin1) to P140, leading to ubiquitination and degradation of activated IRF-3. Finally, the constitutive phosphorylation of S173 and S175 has also been demonstrated²¹¹. Taken together, it is apparent that exactly how and where IRF-3 is phosphorylated is unclear and may depend on the stimulus. Given that IRF-3 signals through both IFN-dependent and -independent pathways, it is also possible that the differential phosphorylation of IRF-3 determines its differential downstream effects.

In response to virus particle entry, our group failed to observe phosphorylation by either one-dimensional or two-dimensional polyacrylamide gel electrophoresis (PAGE) analysis³³⁶. Whether phosphorylation is dispensable for the antiviral response to virus particle entry is unclear, as phosphorylation may occur below detectable levels. It is also possible that a distinct post-translational modification such as ubiquitination, S-nitrosylation, or redox regulation is involved in the activation of this transcription factor, leading to the distinct transcriptional profile observed in this system. Indeed, the ubiquitination of proteins involved in innate antiviral immunity have been shown crucial to their activation. RIG-I ubiquitination at its second CARD domain by the E3 ubiquitin ligase enzyme tripartite motif containing 25 (TRIM25) has been shown essential for the recruitment of the adaptor protein IPS-1, leading to antiviral signalling^{116,346}. Likewise, the ROS-mediated regulation of thioredoxin (TRX)-interacting protein (TXNIP), as essential component of the Nalp3 inflammasome, by the ROS-sensitive molecule TRX, provides a mechanism of redox-dependent regulation of antiviral processes and signal transduction³⁴⁷.

I.IV.ii.ii. Dimerization

Upon phosphorylation, IRF-3 undergoes a conformational change that exposes its IRF association domain (IAD) and leads to dimer formation via homotypic and heterotypic IAD interactions. The crystal structure of IRF-3 demonstrates a three-dimensional autoinhibitory domain that hides the IAD until phosphorylation^{215,217}.

In response to virus particle entry, homodimerization of IRF-3 is observed in the absence of detectable phosphorylation³³⁶. This may be attributed to distinct kinetics of each hallmark of IRF-3 activation in this pathway, or may be a detection issue as aforementioned. It is likely that IRF-3 must undergo a post-translational modification prior to dimerization, as the exposure of the IAD is required for this interaction to occur. Whether this modification is a phosphorylation event that occurs below the limit of detection of current assays, or whether it is, in fact, another type of post-translational modification, is not currently known.

I.IV.ii.iii. Nuclear Translocation

The localization of IRF-3 is mediated by both a NES and a NLS^{218,342}, which leads to shuttling of monomeric IRF-3 between the nuclear and cytoplasmic compartments. The dominant cytoplasmic localization of inactive IRF-3 has been shown to depend on recognition of the NES by chromosome region maintenance protein 1 (CRM1), a nuclear export protein³⁴². Activation of IRF-3 is thought to lead to a conformational change that no longer exposes the NES involved in cytoplasmic localization of the transcription factor^{218,342}, leading to nuclear retention. Here, IRF-3 binds to the promoters of both the *IFN β* gene and to a subset of IFN-independent ISGs.^{214,215,217,342,348,349}

In response to virus particle entry, nuclear translocation of IRF-3 is observed in the absence of detectable phosphorylation. However, it is possible that post-translational modifications leading to DNA binding and transcription occur in the nucleus. Indeed, Noyce *et al.*³³⁶ demonstrated that a PI3K-related family member is involved in the activation of IRF-3 in the nuclear compartment following virus particle entry, since inhibiting this protein did not inhibit IRF-3 translocation to the nucleus but did block transcription of IRF-3-dependent genes. It is also possible that this family member activates other transcription factors that comprise the enhanceosome that mediates IFN-independent, IRF-3-dependent genes. Additionally, the recent identification of the role of β -catenin in IRF-3:CBP/p300 association and histone acetylation at the *IFN β* promoter suggests that regulation of nuclear IRF-3 is an important factor in the ability of this transcription factor to activate gene expression¹⁴².

I.IV.ii.iv. Pro-Apoptotic Function of IRF-3

In addition to acting as a transcription factor for IFN and ISGs, IRF-3 has also been characterized as a pro-apoptotic protein, mediating the expression of multiple genes involved in both apoptosis and survival²³⁵. A Bcl-2 homology domain 3 (BH3) has been identified in the IRF-3 protein that has been shown to mediate binding to Bax following activation, leading to both antiviral gene expression and apoptosis via the mitochondrial apoptotic pathway^{255,350,351}. Dogusan *et al.* also demonstrated that pIC-mediated apoptosis in pancreatic beta cells is IRF-3-dependent³⁵².

I.IV.iii. Post-Translational Regulation of IRF-3 Activity

Little is known about the regulation of IRF-3 expression itself. The gene is constitutively expressed in a wide variety of tissues, and changes in the expression of this gene are largely a consequence of degradation following activation. At the level of activity, multiple genes have been identified to play a role in regulating the IRF-3 pathway, either by directly or indirectly interacting with the transcription factor.

The IRF-3-dependent antiviral signalling pathway is a target for both virus-mediated regulation and cell-mediated positive and negative regulation, both at the level of IRF-3 and at components both up- and downstream of IRF-3. The viral countermeasures to IRF-3 signalling are numerous and occur at multiple levels of the pathway, including, but not limited to, TBK-1 activation, IPS-1 expression, competitive antagonism of IRF-3, and competitive binding to IRF-3-dependent promoters³⁵³⁻³⁵⁷. The numerous viral evasion strategies for IRF-3-mediated antiviral signalling highlight the importance of this pathway in antiviral immunity.

At the cell-mediated level, IRF-3 is regulated at multiple stages by both positive and negative regulators of activity. For the most part, the cellular regulation of IRF-3 and other components of the signalling pathway appear to occur at the level of ubiquitination. This occurs at the level of components upstream of IRF-3, as well as at the level of IRF-3 itself. The consequences of ubiquitination and ubiquitin-like modification are both positive and negative feedback loops.

Upstream targets of ubiquitination include TRAF3, RIG-I, and IPS-1. For example, TRAF3, a component of the mitochondrial complex containing TBK-1, IPS-1, STING, and IRF-3, is subject to ubiquitin-mediated regulation by multiple E3 ubiquitin ligase enzymes including ovarian tumor domain, ubiquitin aldehyde binding 1 and 2 (OTUB1/2), cellular inhibitor of apoptosis 1 and 2 (cIAP1/2), and A20³⁵⁸⁻³⁶¹, the consequence of which leads to both positive and negative regulation of the pathway. RIG-I has been shown to be a target for ubiquitin-conjugation by TRIM25 and Riplet (also known as RING finger protein 135 (RNF135)), leading to activation of the pathway^{346,362}, and by RNF125 and Cyldromatosis protein (CYLD) leading to degradation and inhibition of the pathway^{363,364}. RNF125 has also been shown to ubiquitinate MDA5 and IPS-1, leading to degradation³⁶³.

IRF-3 itself has also been shown to be a target for ubiquitination and ubiquitin-like modification. Recently, Ran binding protein-type and C3HC4-type zinc finger containing 1 (RBCK1) has been identified as an enzyme responsible for the ubiquitin-mediated degradation of IRF-3, acting as a negative regulator of the pathway. The ubiquitin E3 ligase replication and transcription activator-associated ubiquitin ligase (RAUL) has been shown to lead to polyubiquitination and subsequent degradation of IRF-3³⁶⁵. Saitoh *et al.*³⁴⁵ identified a novel serine phosphorylation site at residue 339, and the recognition motif phosphoserine 339-proline 340 serves as a binding site for the prolyl isomerase Pin 1. Modification of IRF-3 by Pin 1 was shown to promote polyubiquitination of activated IRF-3, leading to proteasome-mediated degradation. Whether RBCK1 or RAUL enzymes are involved in the Pin1-mediation regulation of IRF-3 is not known.

In addition, IRF-3 has been shown to be regulated by ISGylation in a positive feedback loop via the enzyme homologous to the E6-AP carboxy terminus (HECT) domain and regulator of chromosome condensation 1 (RCC1)-like domain-containing protein 5 (HERC5)^{366,367}. HERC5 is responsible for the conjugation of ISG 15 to activated IRF-3, leading to inhibition of Pin1 binding and ubiquitin-mediated degradation. Taken together, it appears that the post-translational regulation of IRF-3 occurs at the level of protein stability of the activated transcription factor by ubiquitin and ubiquitin-like modifiers.

Finally, cellular components of the IRF-3 pathway are subject to regulation at the level of splicing. An alternate splice variant of IKK ϵ has been identified, and functions by forming a dominant negative dimer with wild-type IKK ϵ and leads to inhibition of function³⁶⁸. Splicing-mediated regulation of IRF-3 itself is discussed below.

I.IV.iv. Splice-Mediated Regulation of IRF-3

Many genes are subject to regulation by splicing. Alternative splice variants play a role regulating both the expression and activity of genes. In this section, I will cover mechanisms of splicing and alternative splicing, as well as known mechanisms of splice-mediated regulation of IRF-3.

I.IV.iv.i. Mechanisms of Splicing and Alternative Splicing

RNA splicing was discovered simultaneously by Phillip Sharp and Richard Roberts in 1977, when they showed that the mRNA transcribed from the adenoviral genome was non-contiguous with the corresponding DNA genome³⁶⁹⁻³⁷¹. The 'R loop' structures that formed as a result of the hybridization of genomic negative-strand DNA to its antisense mRNA were shown to consist of ssDNA, corresponding to an intron sequence which had been removed during RNA processing. This was later shown to be a feature of eukaryotic genes as well, with IgM being the first characterized eukaryotic gene to feature introns and undergo RNA splicing^{372,373}. It is estimated that 15-60% of genetic diseases in humans can be attributed to defects in some aspect of RNA splicing, making this cellular process a very important mechanism of regulating both the expression and function of cellular genes.

All protein-coding genes are transcribed into by RNAPII in the nucleus, and quickly undergoes 5' capping and polyadenylation³⁷⁴. These modifications are involved in the stability of mRNA as well as mediating nuclear export, among other functions. As previously discussed, many cellular PRRs recognize 5' triphosphate RNA, making the 5' methylguanosine cap an important feature distinguishing self from non-self RNA by immune activators. Splicing precludes, and appears to be required for, the export of pre-mRNA into mature mRNA that can be recognized by translational machinery in the cytoplasm.

The molecular mechanisms of splicing have been well characterized, and are loosely conserved^{375,376}. A schematic representation of the splicing mechanism is depicted in figure 1.5³⁷⁵. In general, the consensus sites for splicing involved a GU sequence at the 5' end of the intron, and an AG sequence at the 3' end, with a loosely conserved branch point sequence close to the 3' end of the intron. Many branch point consensus sequences have been identified, the most important feature being an adenine residue that is critical to the splicing mechanism. RNA splicing involves a two-step reaction and is mediated by a number of small nuclear riboproteins (snRNPs) that facilitate positioning and perform catalytic functions. First, a nucleophilic attack by the branch point adenine residue to the 5' intron consensus site releases the first exon and forms a lariat intermediate structure. Second, a

nucleophilic attack by the 3' end of the first exon to the 3' intron consensus site leads to exon ligation and release of the intron and snRNPs. Two homologous systems have been identified, the classical being the U2-type spliceosome because it utilizes the U2 snRNP, and the U12-type spliceosome using the U12 snRNP as well as structurally and functionally homologous snRNPs to the classical model. The U12-type spliceosome is utilized by a minority of genes, *IRF-3* being among them³⁷⁷. Given the loose conservation of sequence and recognition of the splicing system, it is possible that there are other uncharacterized splicing factors that mediate a redundant or compensatory splicing process.

I.IV.iv.ii. RNA Splicing Regulates Gene Expression and Function

The consequences of alternative splicing lie in the regulation of both gene expression and function. In general, the splice variants of a gene function to antagonize or otherwise modify the functional activities of the wild-type gene. Often, this is accomplished by competitive binding, since the variants typically share a large proportion of their nucleotide sequence. In *IRF-3*, many splice variants have been identified in the literature and have been shown to have competitive binding activity, as discussed in the next section.

Many gene products are involved in the regulation of alternative splicing, the majority of which are serine-arginine rich proteins termed SR proteins³⁷⁶. SRs can bind exon sequences to serve as either exon splicing enhancers (ESEs) or exon splicing silencers (ESSs), which positively or negatively regulated the inclusion of a given exon. The concentration of SRs has been shown to have an effect on exon recognition, implying that the induction of SR expression can mediate alternative splicing.

The Slowpoke (*SLO*) gene product is a prototypic example of signalling-mediated regulation of alternative splicing. The *SLO* gene encodes a potassium channel, the splicing variants of which have varying levels of sensitivity to intracellular calcium. It has been shown that calcium concentration affects the splicing of the *SLO* gene to express potassium channels with increased calcium sensitivity, and that this can be attributed to a calcium-dependent signalling via calcium/calmodulin-dependent protein kinase (CaMK)³⁷⁸. The exon included in calcium-sensitive variants of the *SLO* gene was shown to contain a CaMK response element, implying that CaMK signalling directly leads to exon inclusion by ESE formation. Taken together, it is clear that signal transduction is an important mediator of regulating the alternative splicing of cellular genes.

I.IV.iv.iii. Splice Variation of IRF-3

Wild-type *IRF-3* is a 427aa protein in humans, with a variety of conserved functional domains, depicted in Figure 1.4. The inclusion or exclusion of various functional domains in a splice variant of *IRF-3*, therefore, have profound effects on the ability of a given splice variant to undergo the classical hallmarks of activation, and has effects on wild-type function through competitive interactions.

Splicing has been shown to be a target for regulation of IRF-3. At the genesis of my PhD project, only one example, IRF-3a, was characterized by Peter Howley's lab, in which the N-terminal DBD was largely truncated and replaced by non-wild-type sequence due to alternate start codon usage^{379,380}. This variant of IRF-3 has been shown to be a negative regulator of wild-type IRF-3 function, by forming a non-functional heterodimer with activated wild-type IRF-3. The identification of multiple splice variants of IRF-3 following this initial variant further confirms the importance of IRF-3 splicing in the regulation of antiviral signalling, and is discussed in Chapter IV.

Taken together, the importance of splice-mediated regulation of gene expression and function, as well as the susceptibility of *IRF-3*-encoded RNA to undergo RNA splicing, provides strong rationale for delineating a role for splicing in IRF-3 signalling over and above IRF-3a.

I.V. Reactive Oxygen Species as Antiviral Signalling Molecules

ROS comprise a family of constitutively generated products of various cellular processes. Physiologic concentrations of ROS are tightly regulated by a number of antioxidant enzymes that control the overproduction of ROS that could otherwise lead to oxidative stress and cellular damage. However, high concentrations of ROS have been shown to serve as DAMPs for stress responses such as apoptosis and inflammation. ROS have also been elucidated as sensors for viral infection, leading to a number of cellular antiviral processes including IFN and ISGs, as discussed below.

I.V.i. Cellular Sources of Reactive Oxygen Species

Reactive oxygen species are generated in a cell as a by-product of a number of constitutive cellular processes as well as in response to various pathogen-dependent and -independent stresses. In the mitochondria, ROS are largely a by-product of the citric acid cycle and electron transport chain, and physiological concentrations of ROS are tightly regulated by a number of cellular enzymes including superoxide dismutase, catalase, TRX, glutathione peroxidase, or related enzymes. Elsewhere, ROS are produced via the NADPH oxidase complex, consisting of a number of subunits including p47phox, p67phox, p40phox, and Rac1/2 cytoplasmic subunits and gp91phox and p22phox membrane-bound subunits, along with a member of the NADPH oxidase (NOX) enzyme family. A schematic representation of the mitochondrial and plasma membrane-bound complexes involved in ROS generation is depicted in Figure 1.6.

The family of reactive oxygen species includes hydrogen peroxide (H_2O_2), superoxide anion (O_2^-), and hydroxyl radical ($HO\bullet$), each of which has varying substrate specificities, stability, and downstream consequences³⁸¹. Hydroxyl radical is thought to be highly indiscriminate in its substrate recognition, while superoxide anion has a more restricted specificity, targeting Fe-S cluster-containing moieties. However, being relatively unstable, O_2^- makes a poor signalling molecule. Hydrogen peroxide is relatively stable and has a restricted specificity, modifying cysteine residue-containing proteins and often forming disulfide bridges on target proteins, modifying their binding ability and function. For this reason, hydrogen peroxide is thought to be a predominant ROS-related signalling molecule.

I.V.ii. Consequences of ROS-Mediated Regulation of Gene Expression and Function

As a signalling molecule, ROS production in response to various stimuli leads to the activation of a number of apoptotic, necrotic, inflammatory, and antiviral signalling pathways. The precise mechanisms of ROS-dependent signalling have been characterized, and are described below.

I.V.ii.i. Apoptosis and Necrosis

As a positive regulator of cell death, ROS release from the outer mitochondrial membrane is a well-characterized mechanism of caspase-3 activation and apoptosis via the intrinsic apoptotic pathway. Oxidative stress is also thought to have consequences that lead to telomere shortening and senescence in tissue culture, by a mechanism thought to involve p53 and retinoblastoma protein (Rb)^{382,383}. High-mobility group protein B1 (HMGB-1) has been characterized as a DAMP and is a target for oxidation by ROS in response to injury. HMGB-1 activation by ROS following glucose deprivation has been shown to lead to gene expression and activation of necrotic responses³⁸⁴. Conversely, ROS have been shown to activate cell survival signals, and ROS-mediated tissue repair mechanisms are associated with tumor progression and oncogene expression. For example, ROS-mediated activation of Akt leads to β 1-integrin expression, which is involved in cell growth and survival³⁸⁵. Hypoxia-induced ROS production has also been shown to favour the expression of anti-apoptotic genes such as myeloid leukemia cell differentiation protein 1 (Mcl1) and apoptosis stimulating fragment (Fas) in a hypoxia-inducible factor 1 α (HIF1 α)-dependent manner^{386,387}.

I.V.ii.ii. Inflammation

ROS mediate inflammatory responses through a variety of molecular pathways. ROS-mediated oxidation of HMGB-1 allows it to interact with TLR2, mediating a variety of inflammatory signalling pathways³⁸⁸⁻³⁹⁰. ROS have also been shown to mediate cysteine oxidation of activator protein 1 (AP-1) transcription factor components, leading to transcription of a wide variety of genes including those involved in inflammation^{389,391}. ROS components have also been implicated in NF κ B activation following lipopolysaccharide (LPS) stimulation³⁹².

ROS are also thought to be required for activation of the inflammasome. In particular, the antioxidant molecule TRX has been implicated in the ROS-mediated activation of the inflammasome³⁴⁷. Oxidation of TRX leads to its disassociation from TXNIP, allowing it to bind to Nalp3 and leading to activation of the inflammasome. This represents an important mechanism of ROS regulation of cellular pathways, as the TRX superfamily are thought to bind to and regulate a large number of cellular proteins, and also because in this process, ROS-mediated responses do not involve *de novo* protein synthesis or modification of transcription factors.

I.V.ii.iii. Antiviral Signalling

As mentioned, TRX is a negative regulator of a number of signalling proteins via binding and is a target for oxidation by ROS. TRX is also known to negatively regulate apoptosis signal regulating kinase 1 (ASK1), an adaptor protein for the JNK and p38 antiviral signalling pathways^{393,394}. ROS also have roles in regulating TLR-mediated inflammatory pathways, which may serve to combat viral infection³⁸⁹.

ROS are also involved in the activation of the viral nucleic acid sensor Nalp3, leading to inflammasome activation. A recent body of literature has implicated a wide variety of viruses in the production of ROS upon infection, including RSV, human T cell leukemia virus 1 (HTLV-1), hepatitis B virus (HBV), HCV, HSV, SeV, Japanese encephalitis virus (JEV), and Influenza virus^{186,315,324,395-401}. In addition to the aforementioned signalling pathways, virus-induced ROS have also been shown to activate the IFN pathways via induction of IPS-1 and activation of IKK ϵ and IRF-3^{395,399,402}.

I.V.iii. Reactive Nitrogen Species

ROS and reactive nitrogen species (RNS) are highly related on a functional level in terms of their ability to regulate gene expression and function in response to DAMPs. RNS are a family of highly reactive nitrogen-containing molecules whose overproduction leads to nitrosative stress, analogous to ROS-induced oxidative stress⁴⁰³. Members of the RNS family include nitric oxide (NO), peroxynitrite (ONOO-), and nitrogen trioxide (N₂O₃), among others. RNS are also produced downstream of ROS, as superoxide ion can react with nitric oxide to produce other family members of the RNS family, implying a cooperative effect of these two groups of molecules in regulating gene expression and function. Similar to ROS, RNS function to modify target proteins, leading to regulation of their activity and function, and can function as signalling molecules; however, the targets and specific modifications mediated by RNS are distinct from ROS-mediated regulation of gene function. An important post-translational modification carried out by RNS is the S-nitrosylation of cysteine-containing target proteins by NO. Many cellular proteins are targets for S-nitrosylation⁴⁰⁴, and include surfactant proteins, heat shock associated proteins, and even glyceraldehyde 3-phosphate dehydrogenase (GAPDH)⁴⁰⁵⁻⁴⁰⁷. In particular, GAPDH modification leads to the transnitrosylation of a number of other target proteins, creating a broader variety of targets for nitrogen-mediated protein modification. NO is an important molecule in innate immune signalling pathways, particularly in macrophages and other phagocytes, whereby phagocytosis triggers the production of ROS and RNS, the production of which activates various immune signalling pathways leading to immune protection.

Interestingly, NADPH oxidase itself was recently identified as a target for S-nitrosylation, leading to negative regulation of ROS production and signalling⁴⁰⁸. Taken together, it appears that the ROS and RNS pathways are tightly linked and provide both positive and negative regulatory functions for each other.

I.V.iv. Infection by a Variety of Viruses Induces ROS Production

The downstream consequences of virus-induced ROS production are varied based on the virus and cell type; therefore, the exact mechanism of ROS-induced antiviral signalling is not clear and presumably overlaps with other virus-specific responses. Recently, a number of examples of virus-mediated ROS production have

been elucidated, with a variety of signalling and phenotypic consequences such as DNA damage, inflammation, and antiviral gene expression.

HTLV-1 and HCV have both been shown to induce ROS production, leading to DNA damage^{397,398}. In particular, HCV-induced ROS leads to the regulation of Nei endonuclease VIII-like 1 (NEIL1), a DNA repair enzyme³⁹⁸. RSV and HBV infections have been shown to correlate with ROS-mediated inflammatory processes^{324,396}. RSV infection has been shown to upregulate a subset of NFκB-dependent genes in a ROS-dependent manner, via activation of mitogen- and stress-activated kinase 1 (Msk1) and subsequent NFκB p65 phosphorylation³⁹⁶. Finally, ROS production in response to viral infection has been shown to activate genes involved in IFN signalling. In response to SeV infection, the ROS synthase NOX2 is required for IFNβ and ISG induction³⁹⁹. In response to RSV, ROS mediates the activation of IKKε and IRF-3, as well as being involved in STAT phosphorylation and IRF-1 and IRF-7 expression^{395,409}.

The widespread consequences of virus-mediated ROS signalling are not surprising, considering the variety of cellular targets of oxidative modification. Indeed, JEV infection has been shown to lead to the downregulation of TRX, an antioxidant enzyme involved in both regulating ROS homeostasis and in the binding and regulation of a wide variety of cellular proteins⁴⁰¹.

Interestingly, virus particle entry is associated with Rac1 activation, a component of the NADPH oxidase complex^{303,310,410}. The importance of Rac1 in the activation of IRF-3 following HSV-1 infection provides a strong link between virus particle entry and IRF-3 activation through ROS³⁰⁹.

I.VI. Rationale and Hypothesis

I have outlined our current understanding of IRF-3-mediated antiviral immunity, and provided a detailed description of both the IFN-dependent and -independent arms of innate antiviral signalling. The precise role of IRF-3 in the response to virus particle entry is an important area of research, and has implications in areas such as antiviral drug design and viral vaccine development. Given the critical role of IRF-3 in innate antiviral immunity, my PhD thesis has focused on delineating the role of this transcription factor in the antiviral signalling pathways governing ISG production and the antiviral state *in vitro*.

First, given that differences between IFN-dependent and -independent signalling cascades have been identified, and that a cell line deficient for IFN production has been identified and characterized, I chose to use this cell line as a model for investigating IFN-independent responses to virus particle entry. Vero cells bear a genetic lesion in the *IFN β* gene⁴¹¹, providing a useful tool for the characterization of IFN-independent responses. Given the demonstrated role for IRF-3 in the IFN-independent signalling pathway in HEL cells and in MEFs, and given the IFN-independent nature of the antiviral response in Vero cells, I hypothesized that **IRF-3 activation is essential to the IFN-independent signalling pathway in response to viral stimuli in Vero cells.**

Second, the identification of splice-mediated regulation of IRF-3 provided a strong rationale for the role of splicing in the regulation of IRF-3 signalling. Given that preliminary evidence from our lab identified multiple IRF-3 species at the mRNA level, I hypothesized that **the antiviral functions of IRF-3 are regulated by splicing.**

Finally, the role of ROS in antiviral signalling, and in IFN signalling in particular, has been recently characterized. Given that ROS components such as Rac1 are associated with virus particle entry, and that ROS have been implicated in the activation of IRF-3 in response to viral infection, I hypothesized that **virus particle entry induces a cellular antiviral response involving ROS production.**

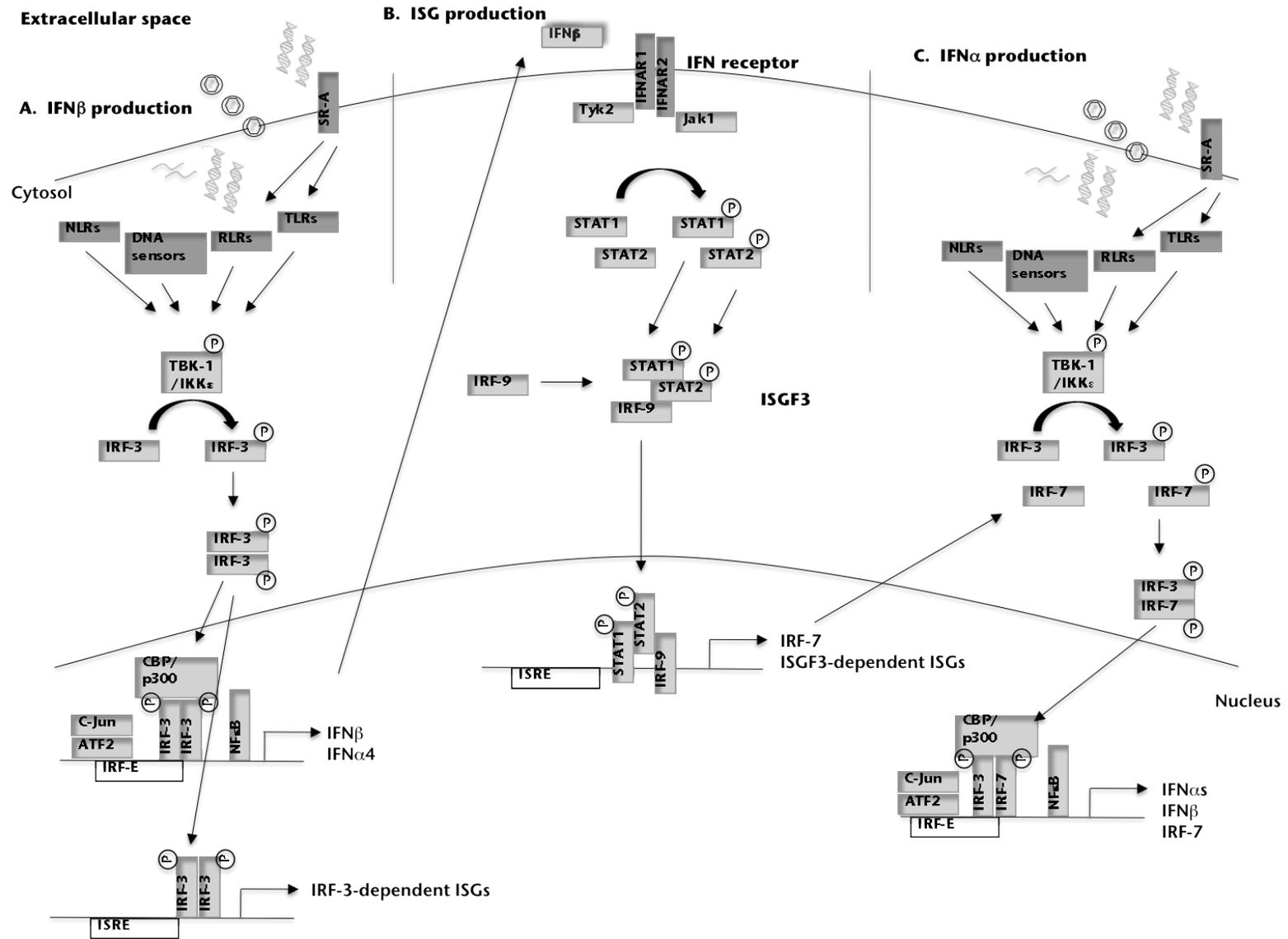
Table 1. Recognition of viral nucleic acid by PRRs.

Class	Subtype	Viral Ligand	References
NLRs	Nalp3 (NLRP3, Cryopyrin)	Adenovirus HCV HSV dsDNA pIC	Barlan <i>et al.</i> 2011 ¹¹⁰ Burdette <i>et al.</i> 2011 ¹⁴⁶ Ichinohe <i>et al.</i> 2009 ¹¹¹ Kanneganti <i>et al.</i> 2006 ⁴⁷ Muruve <i>et al.</i> 2008 ⁴⁸
	NLRC5 (NOD27)	HCMV SeV pIC	Kuenzel <i>et al.</i> 2010 ¹²⁴ Kumar <i>et al.</i> 2011 ¹²⁵ Neerincx <i>et al.</i> 2010 ¹²³
	NLRX1 (NOD9)	Influenza A SeV pIC	Allen <i>et al.</i> 2011 ¹²⁷ Moore <i>et al.</i> 2008 ¹²⁸ Xia <i>et al.</i> 2011 ¹²⁹
DNA sensors	DAI	HCMV HIV HSV B-DNA Z-DNA	DeFilippis <i>et al.</i> 2010 ¹⁷² Hayashi <i>et al.</i> 2010 ⁴¹² Takaoka <i>et al.</i> 2007 ¹³¹ Wang <i>et al.</i> 2008 ⁴¹³
	AIM2	MCMV VV B-DNA	Fernandes-Alnemri <i>et al.</i> 2009 ¹³² Hornug <i>et al.</i> 2009 ¹³³ Rathinam <i>et al.</i> 2010 ¹³⁴
	IFI-16	KHSV VV B-DNA	Kerur <i>et al.</i> 2011 ¹³⁵ Unterholzer <i>et al.</i> 2010 ¹³⁶
	RNAPIII	Adenovirus EBV HSV B-DNA dsDNA	Ablasser <i>et al.</i> 2009 ¹³⁹ Chiu <i>et al.</i> 2009 ¹⁴⁰
RLRs	RIG-I (DDX58)	Classical swine fever virus Dengue virus Influenza A Influenza B JEV NDV Rabies virus Reovirus RSV SeV VSV WNV 5'-triphosphate RNA 5'-OH dsRNA pIC <1000bp dsRNA <1000bp viral genomic RNA	Ablasser <i>et al.</i> 2009 ¹³⁹ Allam <i>et al.</i> 2009 ⁴¹⁴ Chiu <i>et al.</i> 2009 ¹⁴⁰ Fredericksen <i>et al.</i> 2006 ⁴¹⁵ Hausmann <i>et al.</i> 2008 ⁴¹⁶ Hornung <i>et al.</i> 2006 ⁴⁴⁵ Husser <i>et al.</i> 2011 ⁴¹⁷ Jiang <i>et al.</i> 2011 ¹⁴⁶ Kato <i>et al.</i> 2006 ⁴¹⁸ Le Goffic <i>et al.</i> 2006 ⁴¹⁹ Loo <i>et al.</i> 2008 ⁴²⁰ Nasirudeen <i>et al.</i> 2011 ⁴²¹ Rehwinkel <i>et al.</i> 2010 ³³⁸
	MDA5 (Ifih1, Helicard)	Classical swine fever virus Coxsackie virus Dengue virus EMCV Foot and mouth disease virus Influenza A NDV Reovirus Rhinovirus RSV Semliki forest virus SeV Theiler's murine encephalitis virus VSV VV WNV pIC	Childs <i>et al.</i> 2008 ⁴²² Fredericksen <i>et al.</i> 2006 ⁴²³ Gitlin <i>et al.</i> 2010 ⁴²⁴ Huhn <i>et al.</i> 2010 ⁴²⁵ Husser <i>et al.</i> 2011 ⁴¹⁷ Kato <i>et al.</i> 2006 ⁴¹⁸ Loo <i>et al.</i> 2008 ⁴²⁰ Nasirudeen <i>et al.</i> 2011 ⁴²¹ Pilchmair <i>et al.</i> 2009 ⁴²⁶ Triantafilou <i>et al.</i> 2011 ¹⁸⁹

	Lgp2 (DHX58)	EMCV JEV Mengovirus NDV Reovirus SeV VSV 5' -triphosphate RNA ssRNA dsRNA pIC	Bamming <i>et al.</i> 2009 ⁴²⁷ Murali <i>et al.</i> 2008 ¹⁶⁵ Rothenfusser <i>et al.</i> 2005 ¹⁶⁶ Satoh <i>et al.</i> 2010 ¹⁶⁸ Yoneyama <i>et al.</i> 2005 ¹⁶⁷
	DDX1:DDX21:DHX36	Influenza A Reovirus pIC	Zhang <i>et al.</i> 2011 ¹⁷⁴
	DDX3	HCMV dsRNA pIC	DeFilippis <i>et al.</i> 2010 ¹⁷² Oshiumi <i>et al.</i> 2010 ¹⁷⁰
	DHX9	Reovirus pIC	Zhang <i>et al.</i> 2011 ¹⁷⁵
	DDX41	Adenovirus HSV dsDNA	Zhang <i>et al.</i> 2011 ¹⁴¹
	DDX60	HSV Poliovirus SeV VSV dsRNA pIC	Miyashita <i>et al.</i> 2011 ¹⁶⁹
	LRRFIP1	VSV pIC B-DNA Z-DNA	Yang <i>et al.</i> 2011 ¹⁴²
TLRs	TLR3	Baculovirus Classical swine flu virus Coxsackievirus Dengue virus HCV Influenza A NDV Rhinovirus dsRNA pIC polyI	Aloxopoulou <i>et al.</i> 2001 ¹⁷⁶ Chen <i>et al.</i> 2009 ⁴²⁸ Husser <i>et al.</i> 2011 ⁴¹⁷ Jelinek <i>et al.</i> 2011 ⁴²⁹ Kuo <i>et al.</i> 2011 ⁴³⁰ Le Goffic <i>et al.</i> 2007 ⁴¹⁹ Marshall-Clarke <i>et al.</i> 2007 ⁴³¹ Nasirudeen <i>et al.</i> 2011 ⁴²¹ Orinska <i>et al.</i> 2005 ³⁵ Richer <i>et al.</i> 2009 ⁴³² Slater <i>et al.</i> 2010 ⁴³³ Wang <i>et al.</i> 2009 ⁴³⁴ Wang <i>et al.</i> 2011 ⁴³⁵
	TLR 7/8	Friend virus HIV Measles virus Rhinovirus VV ssRNA	Browne 2011 ⁴³⁶ Clifford <i>et al.</i> 2011 ⁴³⁷ Mandl <i>et al.</i> 2008 ⁴³⁸ Martinez <i>et al.</i> 2010 ⁴³⁹ Triantafilou <i>et al.</i> 2011 ¹⁸⁹
Scavenger receptors	Class A scavenger receptors (SR-As)	HCMV HSV Influenza A Inosine-containing ssRNA dsRNA	DeWitte-Orr <i>et al.</i> 2010 ¹⁹⁹ Ghosh <i>et al.</i> 2011 ¹⁹⁸ Liao <i>et al.</i> 2011 ²⁰⁰ Limmon <i>et al.</i> 2008 ²⁰¹ Suzuki <i>et al.</i> 1997 ¹⁹⁶ Yew <i>et al.</i> 2010 ¹⁹⁷

Figure 1.1. *Multiple PRRs are involved in the recognition of viral nucleic acid.* A. The recognition of viral nucleic acid by NLR family members Nalp3 and NLRC5 leads to the activation of caspase-1, a hallmark of the inflammasome. B. The recognition of viral dsDNA by various DNA sensors leads to diverging signalling pathways, the most predominant of which is the STING-mediated activation of TBK-1 leading to IFN production. Exceptions to this are AIM2, whose activation leads to inflammasome assembly, and RNAPIII, whose recognition of dsDNA leads to the production of 5'-triphosphate RNA, a ligand for RIG-I. DAI has also been shown to mediate NF κ B activation, leading to inflammatory cytokine production. C. The recognition of viral RNA by a wide variety of RLRs leads to diverging signalling pathways, the most predominant of which is the IPS-1-mediated activation of TBK-1/IKK ϵ , leading to IFN production. A number of RLRs have been shown to function upstream of RIG-I, whose activation leads to TRAF3-mediated activation of the mitochondrial complex containing the adaptor molecule IPS-1, NEMO, and a number of other signalling molecules. The overall consequence of this complex is the recruitment of TBK-1, whose activation leads to IFN production. MDA5 and the recently described DDX1:DDX21:DHX26 trimer are involved in IPS-1 activation, but unlike other RLRs, their activity does not rely on RIG-I association. Lgp2 is classically characterized as a negative regulator of RIG-I- and MDA5-mediated antiviral signalling. D. The TLRs recognizing viral dsRNA or ssRNA function in the endosomal compartment. TLR3 activation bifurcates into both IFN production via TRAF3 activation, and inflammatory cytokine production via TRAF6 activation. Additionally, the activation of NEMO by TRAF6 itself bifurcates into both IFN and inflammatory cytokine production, as NEMO is a requirement in the activation of TBK-1. E. The membrane bound class A scavenger receptors are involved in the recognition of extracellular viral dsRNA. Scavenger receptor engagement has been shown to lie upstream of other PRRs such as TLR3.

Figure 1.2. *The induction of the antiviral state involved multiple waves of IFN and ISG production.* A. The initial recognition of viral infection leads to PRR engagement and activation of TBK-1, which phosphorylates IRF-3. Phosphorylated IRF-3 forms a homodimer and translocates to the nucleus, and binds to the promoters of IFN β , along with IFN α 4 and a subset of IFN-independent ISGs via interactions with the IRF-E and ISRE sites on the promoters of these genes. Activated IRF-3 interacts with the transcriptional coactivator CBP/p300 and binds to the IFN β promoter along with NF κ B, ATF2 and c-Jun transcription factors. B. IFN signalling occurs through engagement of the cell surface receptor comprised of IFNAR1 and IFNAR2. Receptor engagement leads to the Tyk2- and Jak1-mediated phosphorylation of STAT1 and STAT2, which subsequently recruit IRF-9 to form the ISGF3 transcription factor complex. ISGF3 mediates the transcription of a wide variety of IFN-dependent ISGs, including IRF-7, via interaction with the ISRE site on the promoters of these genes. C. Virus recognition by PRRs in cells expressing IRF-7 leads to the TBK-1-mediated phosphorylation of both IRF-3 and IRF-7. Heterodimer formation and promoter binding leads to the activation of the spectrum of IFN α subspecies as well as IFN β via interactions with the IRF-E site on the promoters of these genes. Activation of both IRF-3 and IRF-7 in stimulated cells leads to the positive regulation of IFN production and signalling via this feedback system.



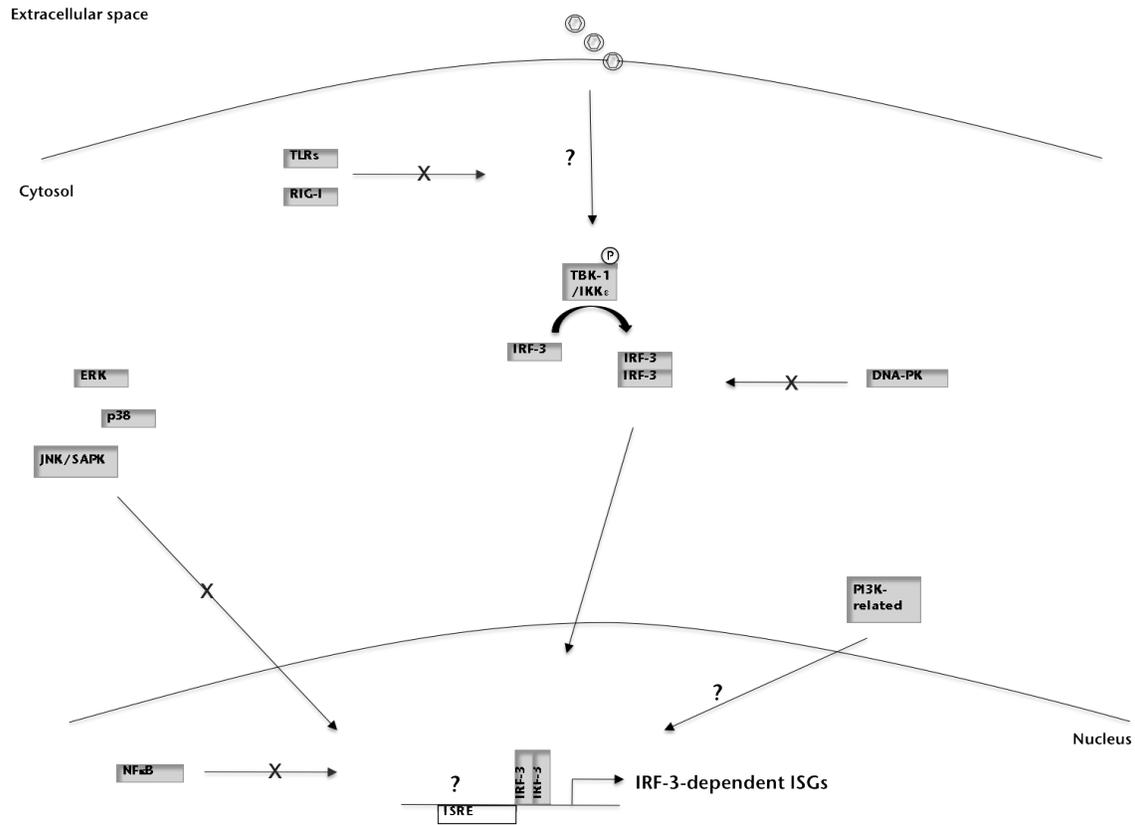


Figure 1.3. *The IFN-independent antiviral signalling pathway requires TBK-1 and IRF-3, and leads to an antiviral state mediated by a subset of IFN-independent ISGs. Viral entry induces TBK-1 activation, the mechanism of which is currently unknown. TBK-1-mediated activation of IRF-3 leads to homodimerization, nuclear translocation and promoter binding. A PI3K family member is involved in mediating transcription of IFN-independent ISGs, but the precise mechanism by which this occurs is not known³³⁶. The TLRs and RIG-I are not essential to entry-induced activation of this pathway³³⁷. Chemical inhibitors show that the ERK, p38, and JNK/SAPK signalling pathways are not essential to IRF-3-mediated ISG transcription following entry³³⁶. The activity of DNA-PK, which has been shown to be involved in N-terminal IRF-3 phosphorylation, is likewise not involved in this pathway³³⁶. Finally, NFκB activation is not required for this pathway to occur³³⁷.*

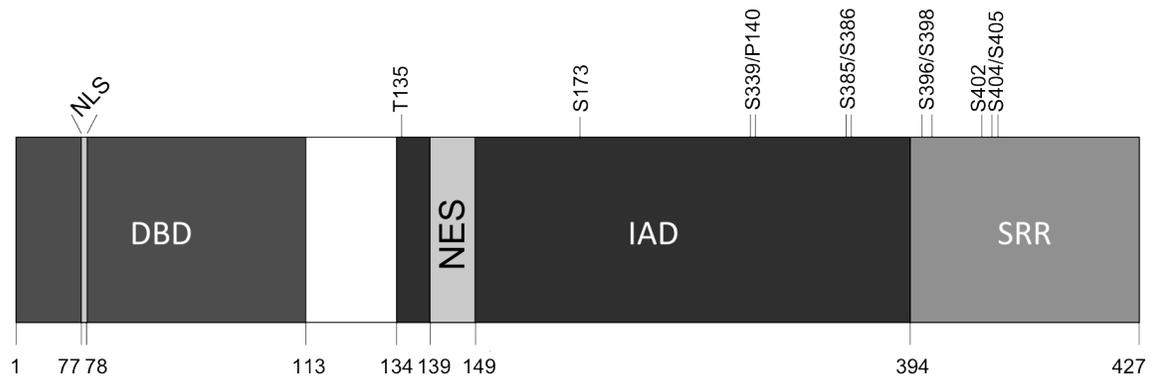


Figure 1.4. *Functional domains of IRF-3.* The N-terminal DNA binding domain (DBD) is conserved between IRFs, and mediates promoter binding. The IRF association domain (IAD) mediates homotypic and heterotypic interactions which lead to the transcription of IFN α/β and ISGs. The C-terminal serine rich region (SRR) contains a number of phosphorylation targets that have been deemed crucial to the activation of IRF-3. The NLS and NES sequences have been identified to mediate the cytoplasmic/nuclear shuttling of inactive IRF-3³⁴². Residues indicated above the domain structure represent known targets of phosphorylation^{212,214-216,344,440}. Not shown, C-terminal apoptosis activation domain (AAD)³⁵⁰, and autoinhibitory domain, comprised of the H3 and H4 helices of the IAD²¹⁷

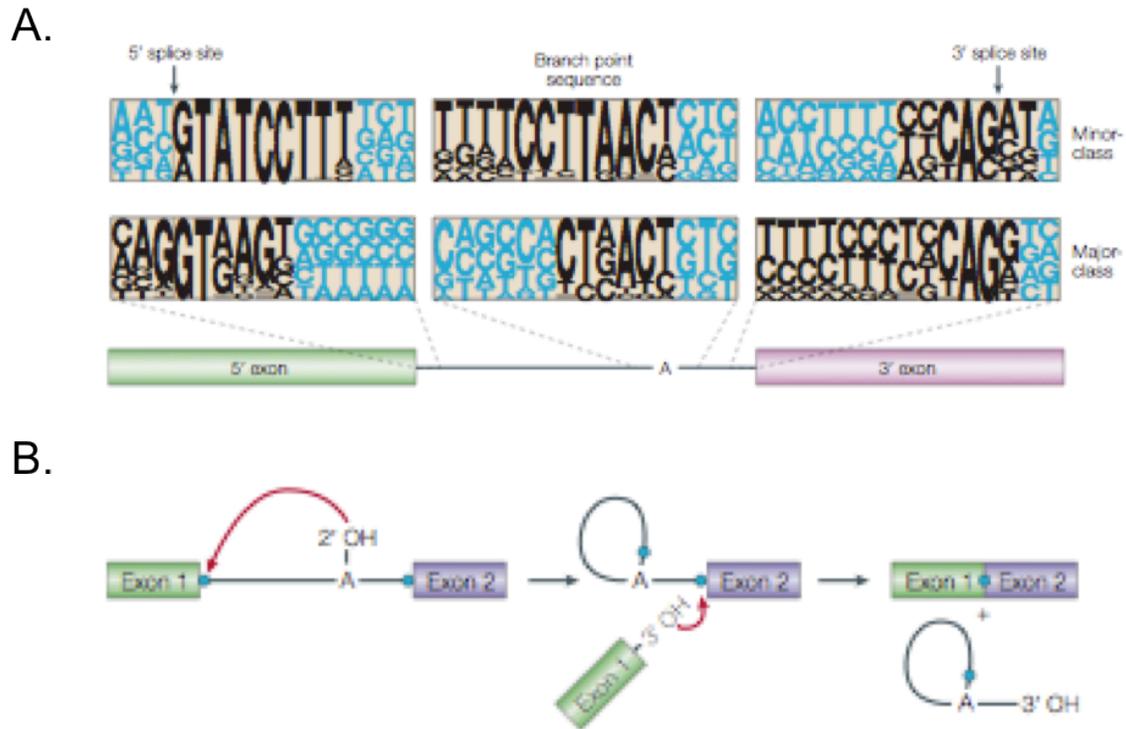
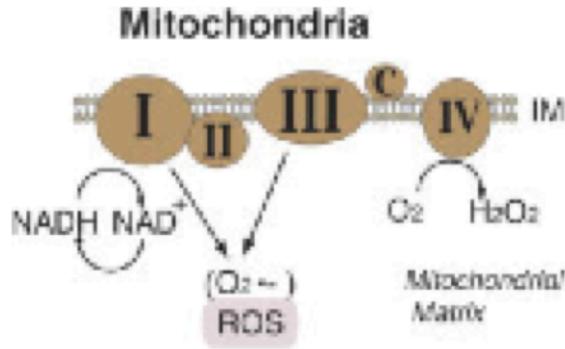


Figure 1.5. *RNA splicing is mediated by conserved intron sequences and occurs by two trans-esterification reactions.* Taken from Patel and Steitz 2003³⁷⁵. A. Consensus sequences of major and minor class introns. The majority of major (U2-dependent) and minor (U12-dependent) class intron recognition sites bear a 5' GU/GT sequence and a 3' AG sequence. The branch point sequence is loosely conserved, but generally contains a critical adenine residue that mediates the two-step splicing reaction. B. Splicing occurs in two steps. In the first step, a nucleophilic attack of the phosphodiester bonds at the 5' splice site leads to the formation of a lariat structure and the release of exon 1. In the second step, a nucleophilic attack of the 3' splice site leads to ligation of exons 1 and 2 and the release of the lariat structured intron, along with the bound splicing factors.

A.



B.

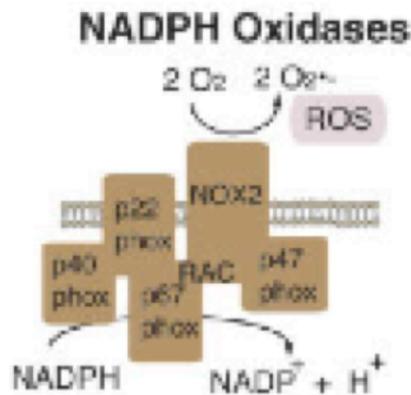


Figure 1.6. *Mitochondrial and cytoplasmic sources of reactive oxygen species.* Taken from Martinon 2010⁵⁵. A. Mitochondrial ROS are produced during the electron transport chain during cellular respiration. B. Cytoplasmic ROS are produced in response to a variety of stimuli and require the NADPH oxidase complex comprised of cytoplasmic p40phox, p68phox, p47phox, and Rac, along the membrane bound p22phox and NADPH oxidase enzyme (NOX), the best characterized of which is NOX2.

Chapter II. Materials and Methods

II.I. Cells and Cell Lines

II.I.i. Cell Lines

The African green monkey kidney cell lines Vero (epithelial), Cos7 (fibroblast) and CV-1 (fibroblast) were maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 5% fetal bovine serum (FBS), 0.03% l-glutamine, and antibiotics (5% DMEM). Human embryonic lung (HEL) fibroblasts and osteosarcoma (U2OS) cells were maintained in 10% DMEM. A549 human lung epithelial cells were maintained in 10% α MEM. ARPE human retinal epithelial cells were maintained in 10% DMEM-F12 supplemented with l-glutamine and antibiotics as well as 0.011% sodium pyruvate. All cell lines were obtained from American Type Culture Collection.

II.I.ii. Stable Cell Lines

Vero-huIRF-3 cells were derived from Vero cells and stably express the human IRF-3 protein under the control of a cytomegalovirus (CMV) promoter (pCMVTnT-huIRF3, backbone from Promega). Stable expression was achieved by cotransfecting pCMVTnT-huIRF3 and pBABE-Puro, which expresses a puromycin resistance cassette. Cells were selected in 10 μ g/ml puromycin and validated for human IRF-3 expression by immunofluorescence microscopy.

ARPE-retHEL cells were derived from ARPE cells. ARPE-retHEL stably express a heterogeneous cDNA library reverse transcribed from mRNA extracted from unstimulated HEL cells. The HEL cDNA library is expressed as a heterogeneous population of cDNAs in the pFB retroviral backbone (pFB-retHEL, backbone from Agilent).

For retroviral cDNA library preparation, the pFB/pVPack plasmid system was employed (Stratagene). Transgenes are expressed under the control of the Moloney Murine Leukemia Virus (MMLV) 5' long terminal repeat. To prepare a retrovirus stock, pFB-retHEL or pFB-LacZ was cotransfected along with pVPack-GagPol and pSG5-VSV-G, which express the retroviral packaging genes, into 293T cells for 48h and viral supernatants harvested and stored at -80°C. ARPE-retHEL cells were obtained by infecting ARPE19 cells with 300 μ l retroviral supernatant per well of a 6-well plate supplemented with 0.1% diethylaminoethyl dextran (DEAE-Dextran) for 4h, then maintenance media added. Stably transduced cells were selected and maintained in 500 μ g/ml G418 (neomycin). ARPE-retLacZ cells were prepared as a control cell line using a retroviral vector expressing β -galactosidase. Transgene expression was verified in control cells by histochemical staining containing 5mM K₃Fe[CN]₆, 5mM K₄Fe[CN]₆, 2mM MgCl₂, and 1mg/ml 5-bromo-4-chloro-indolyl- β -D-galactopyranoside (X-gal) in PBS.

HEL cells stably expressing shRNAs against TMX-2, Msk1, and Zyg-11B were established by infecting HEL cells with a heterogeneous pool of five shRNAs expressed in a the lentiviral backbone.

For lentiviral shRNA preparation, the pLKO vector and MISSION™ shRNA system was employed (Sigma-Aldrich). For each target gene, five shRNA sequences expressed in pLKO.1 were pooled and used to cotransfect 293 cells along with pCMV.DR.8.91 and pCMV-VSV-G, which encode the lentiviral packaging genes. Supernatants from transfected cells were harvested 24 and 48h post transfection, pooled and filtered through a 0.22 µm filter, and stored at -80°C. HEL-shRNA cell lines were obtained by serially infecting HEL cells twice with 300ul lentiviral supernatant for 1h at 37°C. 24h post infection, infected cells were selected with 5µg/ml puromycin in 30% DMEM until surviving cells grew to confluency. Stable cell lines were maintained in 10% DMEM containing 5µg/ml puromycin. As a control for lentiviral infection, stable selection, and shRNA expression, HEL cells stably expressing shRNA against firefly luciferase were also derived, as well as HEL cells stably expressing green fluorescent protein (GFP) under the CMV promoter, using a plasmid derived from the pLKO backbone. Transgene expression was verified in control cells by monitoring GFP expression by immunofluorescence microscopy.

293G cells were derived from 293 human embryonic kidney cells, and stably express the envelope glycoprotein from VSV, and were a kind gift from Dr. B.D. Lichty. 293G cells were maintained in 5% DMEM containing 2µg/ml puromycin and 2µg/ml tetracycline.

II.I.iii. Mouse Embryonic Fibroblasts and Adult Lung Fibroblasts

Mouse embryonic fibroblasts (MEFs) from wild-type (C57Bl/6), *IRF-3*^{-/-}, *IRF-3*^{-/-}*IRF-9*^{-/-}, and *ASC*^{-/-} mice were derived by harvesting embryos at 13.5 days post coitus and mincing into 15% αMEM. Media was changed every 2 days until cells grew to confluency, then fibroblasts maintained in 10% αMEM.

Adult lung fibroblasts from wild-type (Balb/c) or *ASC*^{-/-} mice were derived by harvesting whole lung from animals and mincing into 30% αMEM in a 10cm² plate. Media was changed every 2 days until cells grew to confluency, then fibroblasts maintained in 10% αMEM.

II.II. Viruses and Polyinosinic:Polycytidylic Acid

II.II.i. Viruses

The enveloped DNA viruses HSV-1 (KOS strain) and VV (Copenhagen strain) were prepared by infecting Vero cells at a multiplicity of infection (MOI) of 0.01 plaque forming units (pfu)/cell in serum-free (SF) media for 1h at 37°C, then

maintained in 5% DMEM. Virus was harvested approximately 48h later or when cells showed signs of cytopathic effect. Infected cells were spun at 1500rpm in a Beckman Coulter Allegra™ 6R centrifuge for 15 minutes, supernatants collected and freeze-thawed at -80°C, and cell pellet homogenized using a glass homogenizer. Homogenates were spun at 1500rpm for 10 minutes, then supernatant collected and sonicated three times for 30 seconds each at 65% amplitude on a Fisher Scientific Sonic Dismembrator Model 500. Sonicated supernatants were spun at 1500rpm for 10 minutes and supernatants collected. All supernatants were pooled and virus pelleted at 25 000 rpm on a Beckman Coulter Optima™ L-90K ultracentrifuge for 2h on a cushion of 36% sucrose in 10 mM Tris pH 8.0. Virus was resuspended in SF media and stored at -80°C.

HCMV (strain AD169) was prepared as described above, with viral supernatants harvested every 3 days until cells showed signs of CPE, approximately 14 days. Virus titration was performed by infecting Vero cells with serial dilutions of stock virus in SF DMEM for 1h at 37°C, then maintaining cells in F-11 media containing 1% methylcellulose until plaque formation. Cells were then fixed in 100% methanol for one minute and Giemsa stained with a 1:10 dilution of stock staining solution to visualize viral plaques.

SeV (Cantell strain) was obtained from Charles River Laboratories International, Inc. Vesicular stomatitis virus (VSV) and VSV-GFP were prepared by infecting Vero cells at a MOI of 0.01pfu/cell in SF DMEM for 1h at 37°C, then maintaining cells in 5% DMEM until cell show signs of cytopathic effect. Viral supernatants were collected and freeze thawed, and cell debris spun out for 10 minutes at 1500rpm and cleared supernatant stored at -80°C. VSV-ΔG-GFP lacks the gene encoding the envelope glycoprotein. VSV-ΔG-GFP was prepared by infecting 293G cells as described for VSV.

AdhIRF3, a replication-deficient adenovirus encoding full-length human IRF-3, and AdΔE1E3, its backbone control virus, (serotype 5) were kind gifts from Drs. J.L. Bramson and F.L. Graham, respectively.

Viral infections were performed in HEL, Vero, or ARPE19 cells in SF media for 1h at 37°C, then inoculum replaced with 5% medium until harvest. Adenoviral infections were performed in phosphate buffered saline (PBS) containing 0.9mM CaCl₂ and 0.5mM MgCl₂ for 30 minutes at room temperature, then inoculum supplemented with 5% DMEM.

II.II.ii. Polyinosinic:Polycytidylic Acid

pIC (Amersham Biosciences) treatment was performed by heating to 65°C for 10 minutes, then vortexing before diluting to a final concentration of 100ug/ml in complete media.

II.III. Antiviral Assays

II.III.i. VSV-GFP Antiviral Assay

To quantify antiviral state induction, monolayers of treated cells were challenged with VSV-GFP at a dilution of 10^{-4} of a stock solution (to yield a final concentration of approximately 10^{-3} pfu/cell) in SF media for 30 minutes at 37°C. Viral inoculum was replaced with F-11 media containing 1% methylcellulose for 24h. Challenged cells were scanned 24h post infection on a Typhoon Trio™ variable mode imager (GE Life Sciences) and quantified for GFP fluorescence as a measure of viral gene expression.

II.III.ii. G-less Antiviral Assay

To quantify antiviral state induction in transfected cells, a modification of the G-less antiviral assay was employed⁴⁴¹. Vero cells were cotransfected with a plasmid of interest along with pSG5-VSV-G for 24h. Cells were then treated with viral stimulus for 24h and challenged with VSV-ΔG-GFP, which replicates only in VSV-G-expressing cells, for 24h. Supernatants from challenged cells were titered on naïve Vero cells to assess replication of challenge virus in cotransfected, treated cells. 24h following supernatant transfer, cells were scanned on a Typhoon imager for GFP fluorescence as a measure of viral gene expression.

II.IV. Protein Isolation and Western Blot Analysis

II.IV.i. Protein Isolation and Quantification

Whole cell lysates from mock-infected or treated cells were harvested 24h post treatment. Cells were scraped into 1ml PBS and pelleted at 1500rpm. Cell pellets were lysed for 10 minutes on ice in 1 ml whole cell lysis buffer containing 20mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) pH 7.4, 100mM NaCl, 10mM β-glycerophosphate, 0.2% Triton X-100, 50mM NaF, 1mM Na₃VO₄, 1mM phenylmethylsulfonyl fluoride (PMSF), 2mM dithiothreitol (DTT) and 1x protease inhibitor cocktail (Sigma). Samples were centrifuged at 14000rpm for 10 minutes and supernatants collected. Aliquots of whole cell lysate were used for quantification by Bradford assay (BioRad) as directed, and the remainder of the sample was boiled in 1x sample buffer containing sodium dodecyl sulfate (SDS) and β-mercaptoethanol. Protein samples were stored at -20°C.

To purify GST-TC1 protein for splice-specific antibody production, the TC1 splice-specific sequence was cloned into pGEX4T1 bacterial expression vector to yield a glutathione S-transferase (GST) fusion construct (pGEX4T1-TC1). BL21 *E.*

coli cells were transformed with pGEX4T1-TC1 by electroporation. 50µl BL21 cells were incubated with 500ng plasmid on ice for 30 minutes in an electroporation cuvette, then pulsed at 2.25V and recovered in 1ml cold Luria Broth (LB) for 2 minutes on ice. Transformed cells were shaken for 30 minutes at room temperature before plating. Protein purification was performed using GSTrap™ 4B GST affinity purification column (GE Healthcare) according to the manufacturer's instructions. Affinity purified protein was run on SDS PAGE, Coomassie stained, and protein excised for antibody production. Polyclonal antibody production was performed by Cedarlane (Burlington, ON) using this gel fragment.

II.IV.ii. Western Blot Analysis

Protein samples were run on a 5-12% polyacrylamide gel containing SDS. Protein was transferred to a polyvinylidene fluoride (PVDF) membrane, blocked in 5% skim milk and probed with primary antibody diluted in PBS, and HRP-conjugated secondary antibody diluted in 5% skim milk. Blots were incubated with 10% H₂O₂, 1.25 mM luminol (3-aminophtalhydrazide), and 0.198 mM paracoumaric acid for 1 minute and exposed to film.

Quantitative Western blots were performed as above, but using ECL Advance™ Western blotting detection kit (GE Healthcare) as directed. Chemifluorescent signal was scanned and quantified on a Typhoon imager as above. Relative fluorescent intensity was expressed as a percentage of HEL or Vero IRF-3 protein and normalized to actin loading control.

II.V. Immunofluorescence Microscopy

Cells were seeded at half confluency on coverslips, and treated 24h later with viral stimulus. Treated cells were harvested by fixing in 4% paraformaldehyde or 10% formalin in PBS for 10 minutes, permeabilized in 0.1% (v/v) Triton-X 100 in PBS, and incubated overnight at 4°C in blocking solution containing 3% bovine serum albumin, 3% FBS, and 0.02% Tween-20 in PBS. To monitor IRF-3 localization, cells were stained with rabbit anti-IRF-3 (15-02, a kind gift from Dr. M. David) diluted 1:1000 for 30 minutes at room temperature, then stained with secondary antibody conjugated to Alexafluor fluorophores (Invitrogen) at a dilution of 1:500 for 30 minutes at room temperature. To monitor ISG 15 expression in Vero cells, cells were stained with mouse anti-ISG 15 diluted 1:250 for 30 minutes at room temperature, then stained with biotinylated anti-mouse antibody (Jackson ImmunoResearch) diluted 1:500 for 30 minutes at room temperature, followed by streptavidin conjugated to Texas Red (Jackson ImmunoResearch) diluted 1:500 for 30 minutes at room temperature. All antibodies were diluted in PBS. Cells were incubated with Hoescht nuclear stain (Invitrogen) at a dilution of 1:10 000 in PBS for 5 minutes at room temperature, then coverslips washed and mounted on slides using mounting media containing 1 mg/ml p-phenylenediamine in glycerol. Cells

were visualized and/or counted on a Leica DM IRE2 deconvolution microscope.

For quantification of nuclear IRF-3, a minimum of 100 Hoescht positive cells were counted from five fields of view in each of three independent experiments. IRF-3 nuclear translocation was reported as a % nuclear IRF-3 out of total Hoescht-positive cells.

II.VI. RNA Isolation and RT-PCR Analysis

To determine relative changes in mRNA expression, RNA was harvested from treated cells at the timepoints indicated using Trizol reagent (Invitrogen) according to the manufacturer's instructions. All primer sequences are outlined in Table 2.

For reverse transcription polymerase chain reaction (RT-PCR) analysis, purified RNA was reverse transcribed using SuperScript II reverse transcriptase enzyme (Invitrogen) according to the manufacturer's protocol, and cDNA was subjected to PCR analysis using Taq polymerase (Invitrogen) as directed using gene-specific primer sets (Sigma). For IRF-3 cloning, cDNA was amplified using Platinum Taq HIFI (Invitrogen) as directed using primers flanking the coding sequence of wild-type IRF-3 (Sigma), then incubated for 10 minutes at 72°C to add adenine overhangs. PCR products were purified by gel extraction or from PCR solution using QIAquick gel extraction kit or PCR purification kit as directed (Qiagen), then cloned into pMOS-Blue (GE Healthcare) or pCST (a kind gift from Dr. FL Graham) for sequence analysis. IRF-3 was subsequently subcloned into the mammalian expression vector pCMV-TnT (Promega) for *in vitro* studies.

For quantitative PCR (qPCR) analysis, RNA was DNase-treated using DNase I (Ambion) as instructed prior to reverse transcription. cDNA was diluted 1:5 in water and utilized for TaqMan analysis according to the manufacturer's protocol and using pre-designed gene-specific primer sets (ABI). Alternatively, DNase-treated RNA was reverse transcribed and subjected to SYBR green qPCR analysis using Brilliant SYBR® Master Mix (Agilent Technologies) according to the manufacturer's protocol and using gene-specific primer sets. Primer sets were validated by standard curve analysis of each primer set as described by the manufacturer.

II.VII. ROS Detection

To detect ROS, treated cells were washed 2x with Hanks balanced salt solution (HBSS) and stained with 20µM 5-(and-6)-carboxy-2',7'-dichlorodihydrofluorescein diacetate (carboxy-H₂DCFDA, Invitrogen) diluted in HBSS. Stained cells were scanned on a Safire²™ microplate reader (Tecan) at various times post-infection for fluorescent metabolite detection as a measure of ROS production (excitation wavelength = 495nm, emission wavelength = 520nm). For timecourse studies, cells were infected for 15 minutes before adding carboxy-H₂DCFDA to allow binding and entry of virus. Cells were placed on ice for the

duration of the experimental setup so as to allow for a synchronous infection³¹⁷. For inhibitor studies, cells were pretreated for 1h with the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase inhibitor diphenyleneiodonium chloride (DPI, Calbiochem) or the free radical scavenger butylated hydroxyanisole (BHA, Sigma) at the concentrations indicated, then infected as described with inhibitor present for the duration of the experiment.

II.VIII. Viability Assays

In order to measure viability, treated cells were washed twice in PBS and stained with 5%(v/v) Alamar Blue (Invitrogen), diluted in PBS. Cells were stained for 30 minutes at 37°C, and then scanned on a Safire reader (excitation wavelength = 530nm, emission wavelength = 590nm) for the presence of fluorescent metabolite as an indicator of cell metabolism. In addition, treated wells were stained with 4µM 5-carboxyfluorescein diacetate acetoxyethyl ester (CFDA-AM, Invitrogen) as above and scanned on a Safire microplate reader (excitation wavelength = 485nm, emission wavelength = 530nm) for the presence of fluorescent metabolite as an indicator of both cell metabolism and membrane integrity.

Table 2. Primers used for PCR analysis.

a. PCR primers (non-quantitative)

Gene	5' primer sequence	3' primer sequence
Human ISG 56 (human and Vero)	AGCGCTGCCTAATTTACAGC	AGTGGCTGATATCTGGGTGC
Human GAPDH (human and Vero)	CGGAGTCAACGGATTTGGTCGTA	AGCCTTCTCCATGGTGGTGAAGAC
IRF-3 for cloning (human and Vero)	IRF3A: CCCGACCTTGGATCGTAGGC IRF3Nco: CCGGATGGGAACCCCAAAGCCACGG IRF3-5': CAACTGGAAGAGGAATTTCCGC VeIRF3A1: CCTGGAAGAGGAATTTCCG	IRF3B: ACACCATGCCGAGCGAGGGC IRF3Xba: CCCGGACCCCTCTGGACGAGATCTGG IRF3-3': CAGAACCAGAGGCATAGCGTG VeIRF3B1: TACACACCGTCACGGGATTGGC VeIRF3-B2: CACCAACAGCCGCTTCA

b. TaqMan qPCR primer sets (Life Technologies)

Gene	Product Number
Human ISG 56	Hs01911452_s1
Human GAPDH	Hs99999905_m1
Murine ISG 56	Mm00515153_m1
Murine IFN β	Mm00439552_s1
Murine GAPDH	Mm99999915_g1

c. SYBR green qPCR

Gene	5' primer sequence	3' primer sequence
Human TMX2	AGAACCCGAGATCCATCACTG	GGTTTGCACGTCATCAGGAAC
Human Msk1	CTCATGACCGAAGTTTGACGTG	TGGATCTACTGTGAGAAGTCCTTGG
Human Zyg11B	TGCCTCATTGCCAAGATTAGAGA	TAAACATTTCAAGTGGTGCATGGT
Human GAPDH	GCACCGTCAAGGCTGAGAAC	GCCTTCTCCATGGTGGTGAA

Chapter III. Results: Part One

Portions of this chapter are published in:
Chew, T., Noyce, R., Collins, S. E., Hancock, M. H. & Mossman, K. L. Characterization of the interferon regulatory factor 3-mediated antiviral response in a cell line deficient for IFN production. *Molecular immunology* **46**, 393-399, doi:10.1016/j.molimm.2008.10.010 (2009)

III.I. Preamble

In primary fibroblasts, virus particle entry has been shown to elicit an IFN-independent antiviral state leading to protection against virus challenge. The biological significance of this response is likely realized under conditions of low multiplicity infection or in response to defective virus particles, and is predicted to be the predominant response in the first cells to be infected within a given host³³⁷. Aside from TBK-1 and IRF-3, few components of the IFN-independent antiviral signaling pathway have been identified. While many cellular antiviral components have been tested and deemed non-essential for this response to occur, very little information is available regarding what cellular components are involved in the response. Indeed, the TLRs and RIG-I have been shown to be non-essential for the establishment of the IFN-independent antiviral state, as have the p38, JNK, and classical PI3K pathways^{336,337}. Similarly, NF- κ B does not appear to be activated following treatment with UV-inactivated virus particles³³⁷. Components such as CBP/p300 and AP-1 have not been identified in the IFN-independent antiviral response, and components besides IRF-3 and TBK-1 have not been identified.

Vero cells are a renal epithelial cell line derived from the African green monkey (*Cercopithecus aethiops*), and are routinely used for virus propagation and for *in vitro* studies of viral pathogenesis and antiviral immunity. Vero cells are incapable of producing IFN β due to a genetic lesion within the interferon locus^{411,442,443}. However, they bear an intact IFN signalling pathway, and are used to titer IFN concentration using a standard VSV plaque reduction assay. They also produce ISGs and undergo an antiviral state in response to various viral stimuli. Therefore, this cell line provides a useful tool for the study of IFN-independent antiviral responses.

III.II. Hypothesis and Approach

Given that Vero cells are deficient in IFN production but have been historically considered to be phenotypically normal in terms of antiviral immunity, it is reasonable to suggest that these cells provide a good model for the study of IFN-independent antiviral responses, and that their phenotype mimics our observations with respect to the cellular response to virus particle entry. I therefore hypothesized that **IRF-3 activation is essential to the IFN-independent signalling pathway in response to viral stimuli in Vero cells.**

In order to test this hypothesis, the cellular response to virus particle entry was monitored using the classical hallmarks of antiviral signalling and the antiviral state in Vero cells. Viruses used in the experiment were rendered replication-deficient by UV irradiation, which leads to crosslinking of genomic nucleic acid and prevents virus replication, and virus inactivation was confirmed by immunofluorescence to determine viral gene expression in cells treated with UV virus. The hallmarks of antiviral signalling were assessed and compared to the response previously characterized in HEL primary fibroblasts⁴⁴⁴. Sequence analysis

of IRF-3 from Vero cells was performed and compared to human IRF-3 sequence. Finally, the contribution of IRF-3 to the IFN-independent antiviral response in Vero cells was assessed using overexpression studies.

III.III. Results

III.III.i. Vero Cells Fail to Mount an IFN-Independent Antiviral Response to pIC or Virus Particle Treatment

The ability of Vero cells to establish an antiviral state in response to various viral stimuli was assessed by pretreating cells with IFN, pIC or UV-inactivated HSV-1 (HSV-1 UV) or SeV (SeV UV) and subsequent challenge with VSV-GFP. Exogenous IFN treatment resulted in >75% reduction in GFP fluorescence, however neither pIC nor virus particle treatment significantly affected GFP fluorescence (Figure 3.1). To determine whether Vero cells elicit a dose-dependent response to pIC, cells were treated with increasing doses of pIC and assessed for antiviral state. As shown in Figure 3.2, Vero cells do not respond to high doses of pIC treatment. These results demonstrate that, in contrast to primary human fibroblast cells⁴⁴⁴, Vero cells fail to mount an antiviral response against pIC or virus particle treatment.

III.III.ii. Expression of Human IRF-3 Restores Antiviral Immunity Against pIC But Not UV-Inactivated Virus

The IFN-independent induction of antiviral genes has been shown to rely on IRF-3 activity^{265,444,445}. To understand the contribution of IRF-3 to antiviral signalling in Vero cells, human IRF-3 was expressed using an adenoviral vector (AdhuIRF-3). In control adenovirus treated cells, reproducible accumulation of ISG 56 mRNA was only observed following treatment with IFN or infection with replication competent SeV (Figure 3.3A). Pretreatment with AdhuIRF-3 stimulated ISG 56 mRNA accumulation in response to pIC and enhanced the response to SeV. Likewise, ISG 56 protein expression was observed in response to pIC treatment in cells pretreated with AdhuIRF-3 (Figure 3.3B). Similar results in ISG enhancement were observed with ISG 15 protein expression as monitored by immunofluorescence microscopy (Figure 3.4). Consistent with this observation, AdhuIRF-3 pretreatment led to restoration of a pIC-induced antiviral state in a standard antiviral assay (Figure 3.5A-B). Supernatants transferred from treated cells were unable to confer antiviral protection to naïve Vero cells (Figure 3.5C), indicating that production of soluble factors do not play a role in the generation of an innate antiviral response. Collectively, these results suggest that expression of human IRF-3 restores the pIC-mediated antiviral response in Vero cells.

III.III.iii. Sequence Analysis of IRF-3 Cloned From Vero Cells

Since exogenous huIRF-3 expression restored selective antiviral functions in Vero cells, we cloned Vero IRF-3 to investigate sequence differences. Vero IRF-3 is 96% identical to human IRF-3 at the amino acid level and contains two deletion sites corresponding to aa164–168 and aa301 of human IRF-3 (Figure 3.6). These deletion sites are situated within the IAD, which is important for dimerization and nuclear translocation of IRF-3 following stimulation. Therefore, it is possible that these differences play a role in the ability of endogenous IRF-3 to undergo dimerization and induce antiviral gene expression in Vero cells. Additionally, sequence differences in the IAD and DBD may underlie an inability of Vero IRF-3 to either dimerize or bind to IRF-3-dependent promoters following stimulation.

III.III.iv. Endogenous Vero IRF-3 Demonstrates Altered Mobility and Abundance Relative to Human Cells

To further characterize differences between the two species of IRF-3, immunoblot analysis for IRF-3 between these cell lines was performed. While the ~55 kDa IRF-3 doublet was observed in varying abundance between human cell lines, IRF-3 from Vero cells displayed a doublet with an apparent faster migration and at a significantly lower level of expression (Figure 3.7). The altered migration of Vero IRF-3 could be explained by the five amino acid difference in protein length, differences in post-translational modification of individual IRF-3 residues that differ between the two species, or to differences in the post-translational modification machinery in human and monkey cells. Regardless of the mechanism, this observation suggests that IRF-3 in Vero cells has altered mobility and is expressed at significantly lower levels than in human cells that have an intact IRF-3-dependent signalling pathway.

A similar phenotype was observed in CV-1 African green monkey fibroblast cells with respect to IRF-3 migration and pIC-mediated responses (Figure 3.8). However, in contrast to Vero, supernatants from pIC-treated CV-1 cells confer protection against virus challenge in naïve Vero cells, implying that the production of a soluble antiviral cytokine mediated antiviral activity in CV-1. Indeed, CV-1 cells do not bear a genetic lesion in the *IFN β* gene as Vero do, accounting for this difference.

III.III.v. Abundance of IRF-3 in Vero Cells is a Determinant of pIC-Induced Antiviral Immunity

Given that endogenous IRF-3 in Vero cells is expressed at lower levels relative to IRF-3 in human cells, and demonstrates differences in virus-mediated activation relative to human IRF-3, the next question was whether Vero IRF-3 is functional in response to viral stimulation when expressed at levels comparable to those observed in responsive cell lines such as HEL. Cells were co-transfected with a

parental vector, human IRF-3 or Vero IRF-3, along with a plasmid encoding the envelope glycoprotein from VSV. Transfected cells were treated as indicated and then challenged with VSV-GFP(Δ G), which can only replicate in cells transfected with the glycoprotein expression vector, thus permitting the quantification of antiviral activity of various IRF-3 constructs independent of transfection efficiency⁴⁴⁶. Virus contained within supernatants from challenged cells was subsequently titered on naive Vero cells, and relative GFP expression was quantified as a measure of virus production. Relative expression of IRF-3 from mock and transfected cells is depicted in Figure 3.9A-B. As observed with AdhuIRF-3 treatment, transfected human IRF-3 restored pIC-induced antiviral immunity that approached the level of IFN-induced protection (Figure 3.9C). Furthermore, transfection of Vero IRF-3 also restored responsiveness to pIC. Interestingly, no difference in antiviral activity against UV virus was detected in cells transfected with either control plasmid or plasmid expressing human or Vero IRF-3. These data imply that the endogenous IRF-3 expression level in Vero cells plays a dominant role in determining antiviral activity against pIC. Furthermore, since the difference in mobility between human IRF-3 and Vero IRF-3 is maintained following IRF-3 expression in Vero cells, differences in the post-translational modification machinery in human cells versus monkey cells likely does not account for the altered mobility. More importantly, these data show that Vero IRF-3 protein is indeed functional despite differences and deletions at the amino acid level.

III.III.vi. IFN β Pretreatment Does Not Affect IRF-3 Expression or pIC-Mediated Antiviral State Induction in Vero Cells

Given that Vero cells are deficient in both IFN β production and IRF-3-mediated gene expression in the IFN-independent pathway, it is possible that the two defects are related, and that IFN β deficiency affects the ability of pIC to mediate IRF-3 activation in Vero. Indeed, a few groups have demonstrated tissue and cell-type specific constitutive expression of both IFN α and β ⁴⁴⁷⁻⁴⁴⁹. It is thought that this constitutive activation primes cells to efficiently produce IFN in response to viral infection. In order to determine whether constitutive IFN β plays a role upstream of IRF-3 in the IFN-independent antiviral response, cells were pretreated with various concentrations of IFN β and the response of these cells to pIC treatment was assessed in an antiviral assay. As shown in Figure 3.10, IFN β pretreatment does not appear to affect the inability of Vero cells to mount an antiviral response against pIC treatment. Increasing doses of IFN β led to antiviral state induction in both mock and pIC treated cells. Therefore, the inability of Vero cells to constitutively produce IFN β does not appear to underlie the additional defects in antiviral signalling observed in Vero cells.

III.IV. Summary and Conclusions

Vero cells are widely used in the study of virus:host interactions and are commonly used for growing virus and vaccine stocks. Furthermore, viruses that normally demonstrate a very narrow host range, due to their evolutionary history within a single species, are often propagated and/or titered in Vero cells or related African green monkey kidney cells^{450,451}. Here we provide evidence that Vero cells have a dysfunctional intracellular antiviral signalling pathway over and above their inability to produce IFN β , which likely explains the permissive nature of Vero cells to viral infection. We also demonstrate that the endogenous abundance of IRF-3 protein in Vero cells is a key determinant in their ability to respond to pIC stimulation.

It was observed that AdhuIRF-3 pretreatment restored pIC-induced ISG 56 mRNA accumulation and antiviral activity in a VSV challenge model. Interestingly, Ad Δ E1E3 infection reduced VSV-GFP fluorescence in response to pIC to ~70% of mock, suggesting that adenovirus infection may sensitize cells to pIC. However, full sensitization to pIC required AdhuIRF-3 pretreatment, demonstrating a significant involvement of IRF-3 in the antiviral response. This protection is intracellular, as supernatants failed to confer antiviral protection on naïve cells. Thus, in addition to the failure to produce IFN β , Vero cells fail to induce soluble antiviral cytokines in response to virus particle treatment. This may indicate that IFN β production is a requirement for IFN α production, as suggested in fibroblast cells⁴⁵², thus demonstrating a further impairment in the antiviral response in this cell line. Importantly, the inability of supernatants to confer antiviral protection to naïve cells implies a lack of production of any effective antiviral cytokine, which may include not only the type I IFNs, but also other species of IFN such as IFN λ , which has been shown to be an important antiviral cytokine in epithelial cells^{79,453}. It is possible that naïve Vero cells do not bear the receptors to engage some soluble antiviral cytokines, and that the antiviral state in Vero cells leads to both an upregulation of soluble antiviral cytokines as well as their cognate surface receptors. However, this is unlikely given the ability of soluble cytokines produced by pIC-treated CV-1 cells to elicit an antiviral state in naïve Vero.

The ability of SeV but not pIC, a dsRNA mimetic, to induce ISGs in Vero cells was initially unexpected. One explanation for this observation may be the level of stimulation between the two treatments. SeV replication involves multiple rounds of amplification of its genome and hence of viral dsRNA production. However, treatment of Vero cells with up to 200 μ g/ml pIC failed to elicit an antiviral response. Since these studies, the intricate differences between the recognition of pIC and nucleic acid of various viral sources have been elucidated, and may provide an explanation for the differential response to SeV and pIC. Interestingly, evidence of SeV recognition by both RIG-I and MDA5 has been demonstrated^{163,424}, and the length-dependent recognition of pIC by either RIG-I or MDA5 has been defined¹⁶⁴. Taken together, it is clear that there are molecular differences between the signalling pathways that govern antiviral immunity against SeV and pIC. Given that overexpression of IRF-3 can restore the antiviral response against pIC but does not significantly affect SeV-mediated IRF-3-dependent gene expression, it is possible that SeV infection activates IRF-3-independent pathways leading to ISG production

and an antiviral state. Alternatively, it is possible that at this level of SeV stimulation, downstream components of the IRF-3 signalling pathway are saturated, explaining the similar phenotype between low and high expression of IRF-3.

As expected, we failed to observe induction of ISGs in response to replication competent HSV-1, as mechanisms of blocking antiviral gene expression have been described. For example, infected cell protein 0 (ICP0) of HSV-1 has been shown to block IFN and ISG production via IRF-3 and IRF-7^{454,455}. In addition, the ability of HSV-1 to affect global gene expression was reflected in changes in relative GAPDH signal, and has been previously observed late times post-infection⁴⁴⁴.

Sequence analysis of human and Vero IRF-3 revealed differences in the sequence, mobility, and abundance of endogenous IRF-3 between Vero cells and human cells. The mobility and abundance differences are likely attributed to species-specific differences, since IRF-3 from CV-1 cells demonstrated similar mobility and abundance to IRF-3 from Vero cells on SDS-PAGE, and expression of exogenous human IRF-3 in Vero cells retained its slower migration pattern relative to expression of exogenous Vero IRF-3, despite similar expression levels. While we have demonstrated that IRF-3 abundance is a major determinant to pIC-induced antiviral signalling in the absence of IFN, we cannot rule out the possibility that sequence differences or deletions contribute, in part, to the function and activation of IRF-3 and subsequent signalling.

The exogenous expression of either human or Vero IRF-3 restored pIC-induced antiviral protection, suggesting that the endogenous abundance of IRF-3 is a major determinant in the ability of Vero cells to respond to pIC. However, these cells remained unresponsive to virus particle entry, highlighting the distinct nature of IRF-3 activation in response to different viral stimuli. The selective restoration of pIC-induced antiviral immunity suggests that in response to virus particle entry, one or multiple cellular components that underlie entry-mediated IRF-3 activation are dysfunctional, but is/are not required for pIC-mediated signalling. Alternatively, Vero cells may express a cellular component that selectively inhibits IRF-3 activity in response to virus entry.

The nature of pIC-mediated IRF-3 activation differs from that of virus particle-mediated IRF-3 activation. Indeed, our lab has observed differential modification of IRF-3 following virus particle entry³⁴¹, implying differential requirements for activation of gene transcription by IRF-3. It is possible that the signalling pathways governing pIC stimulation lead to specific modifications of IRF-3 that resulting in activation, and that in Vero cells, the differential modification of IRF-3 required for virus particle-mediated activation is mediated by one or more cellular components that are not constitutively expressed.

Taken together, it was demonstrated in this chapter that IRF-3 is an important cellular component in the IFN-independent response to viral stimuli, and that its endogenous abundance underlies the ability of Vero cells to respond to viral and synthetic nucleic acid. I have also demonstrated that while IRF-3 is essential for the response to virus particle entry, it is insufficient to induce antiviral protection and requires additional, currently uncharacterized cellular components.

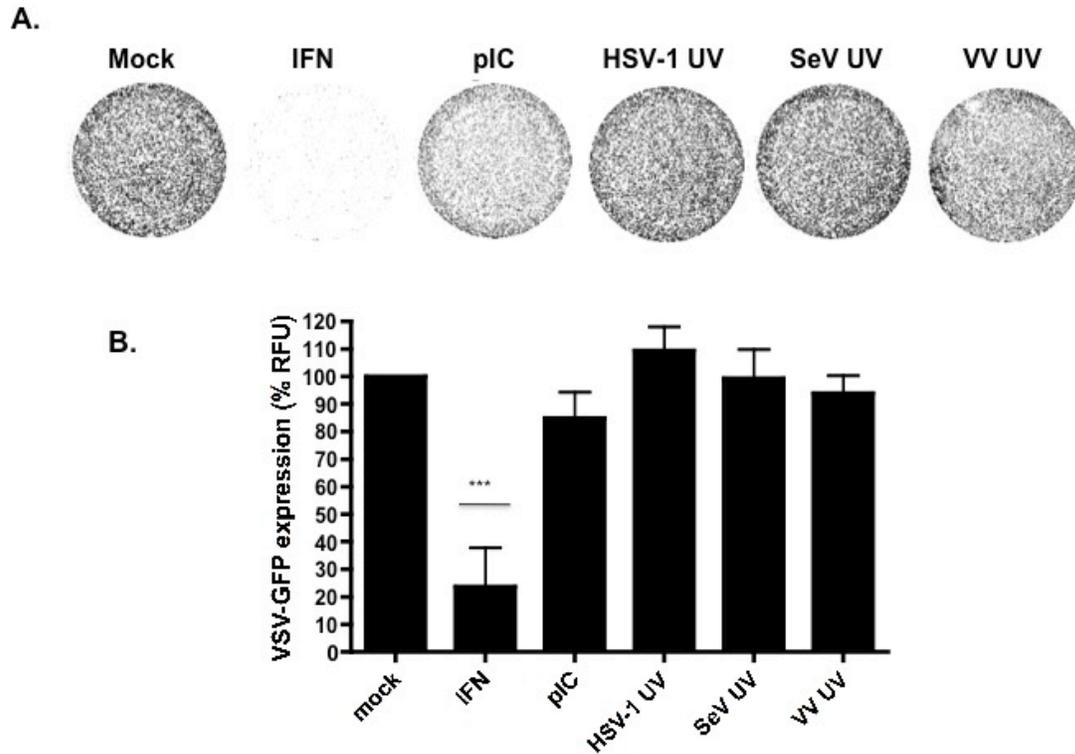


Figure 3.1. *Vero cells fail to mount an IFN-independent antiviral response.* A. Cells were treated for 24h with IFN, pIC, HSV-1 UV, or SeV UV then challenged with VSV-GFP. Monolayers were imaged 24h post challenge for GFP fluorescence, indicative of viral replication. B. Quantification of GFP fluorescence from (A). Error bars represent SEM from three independent experiments. *** represents statistical significance by one-way ANOVA using Tukey's post-hoc test ($p < 0.001$).

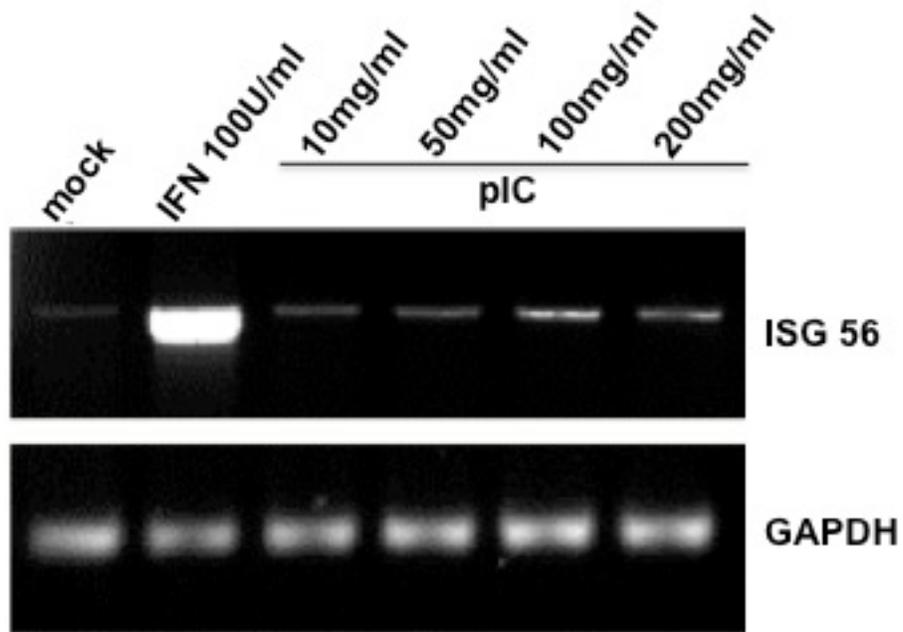


Figure 3.2. *Vero cells do not mount an antiviral response to pIC even at high concentrations.* Vero cells were treated for 8h as indicated, then RNA harvested and subject to RT-PCR analysis to assess the expression of ISG 56, a hallmark of antiviral state induction. GAPDH expression was assessed as a loading control.

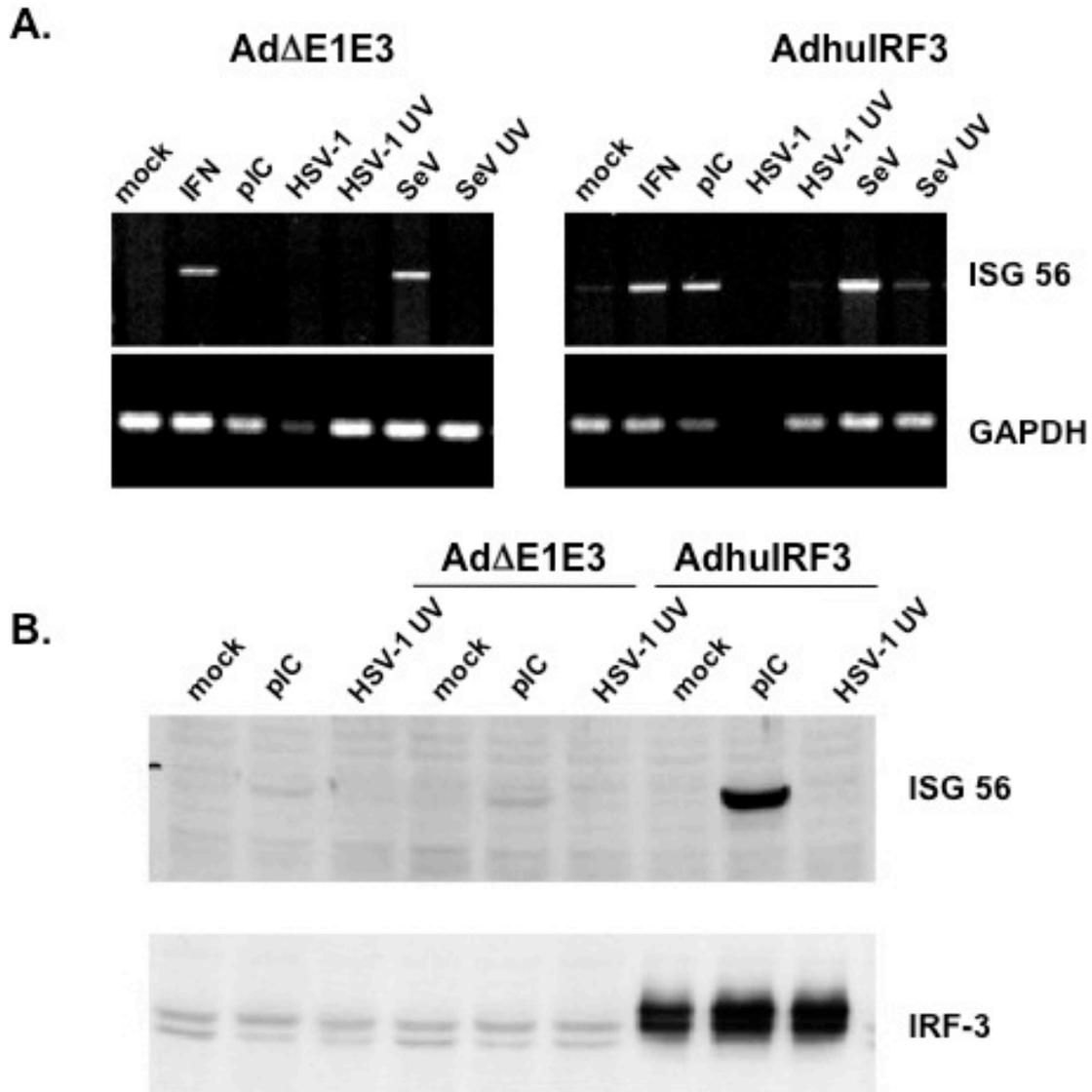
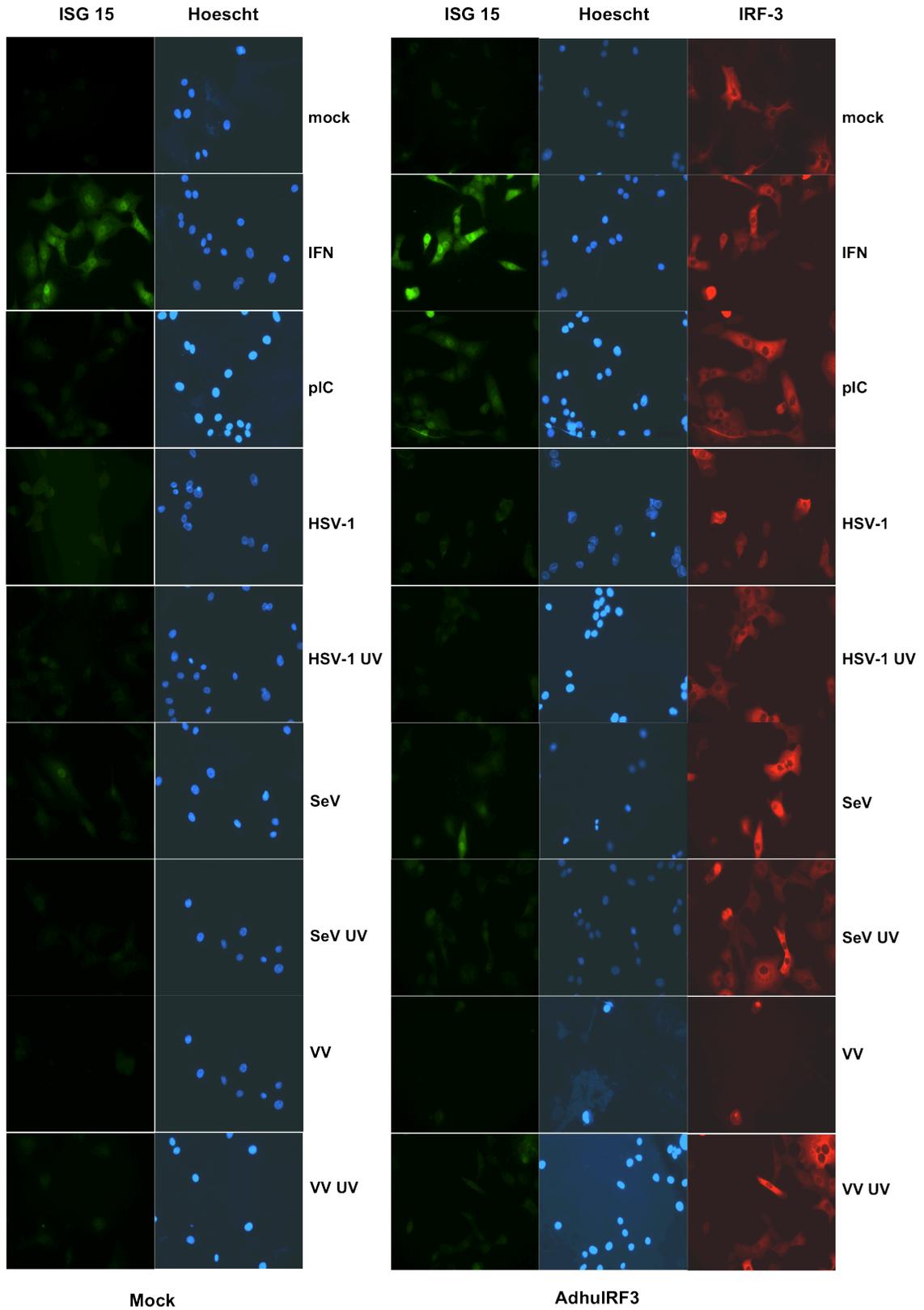


Figure 3.3. *Human IRF-3 expression restores ISG 56 expression in response to pIC but not virus particle treatment.* Cells were preloaded for 24h with AdhuIRF-3 or its control vector, then treated as indicated. A. RNA was harvested 8h post-treatment and subject to RT-PCR analysis for ISG 56 or GAPDH as indicated. B. Protein samples from mock- or Ad-pretreated cells were subjected to Western blot analysis for IRF-3. For visualization purposes, 40 μ g of whole cell extract from mock and Ad Δ E1E3 treated cells were loaded along with 10 μ g of whole cell extract from AdhuIRF-3 treated cells.

Figure 3.4. *Human IRF-3 expression restores ISG 15 expression in response to pIC but not virus particle treatment.* Cells were preloaded for 24h with AdhuIRF-3 or its control vector, then treated as indicated. Cells were harvested 6h post-treatment and subject to immunofluorescence analysis for ISG 15 expression as a hallmark of antiviral state induction. Hoescht nuclear stain was added to visualize viable cells and protein localization. AdhuIRF3-treated cells were stained with an IRF-3 antibody (right) to confirm overexpression.



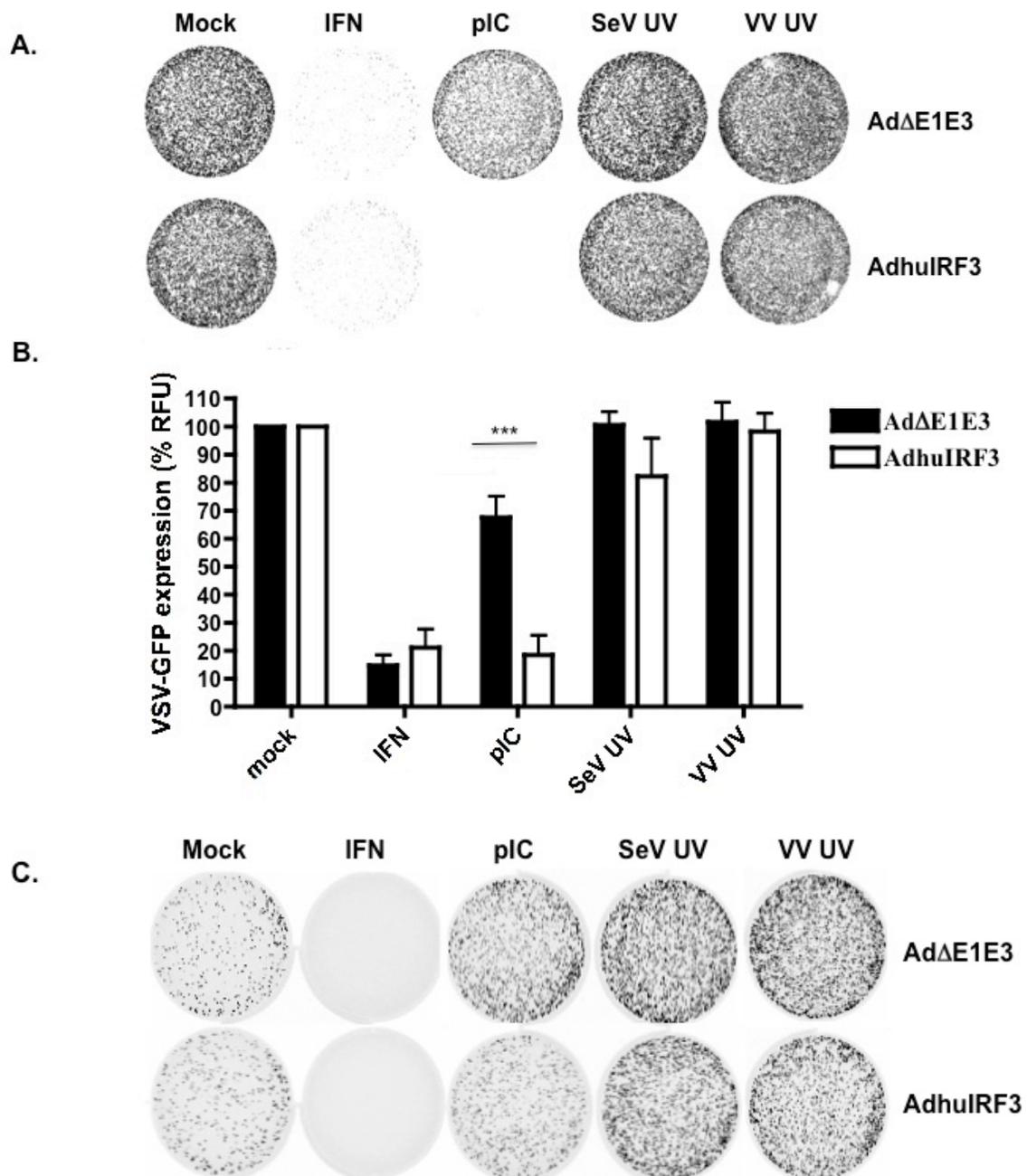


Figure 3.5. Human IRF-3 expression restores antiviral state induction in response to pIC but not virus particle treatment. A. Cells were pretreated for 24h with AdhuIRF-3 or its control vector, then treated as indicated. Cells were challenged 24h post-treatment with VSV-GFP and monolayers scanned 24h post challenge for GFP fluorescence as a measure of viral gene expression. B. Quantification of relative GFP fluorescence from (A). Error bars represent SEM from three independent experiments. *** represents statistical significance by two-way ANOVA and Bonferroni's post-hoc test compared to control treatment group ($p < 0.001$). C.

Supernatants from cells treated in (A, B) were transferred to naïve Vero cells for 24h, then challenged with VSV-GFP and monolayers scanned for GFP fluorescence.

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                                DBD                                NLS
HEL  MGTFKPRILPWLVSQDLGQLEGVAWVNSRTRFRIPWKHGLRQDAQCEDFPIQAWAEATGAYVPGRDAPDLPTWKRNFRSALNRKGLGLAEDRSKDPHPHKIYE 108
Vero  MGTFKPRILPWLVSQDLGQLEGVAWVNSRTRFRIPWKHGLRQDAQCEDFPIQAWAEATGAYVPGRDAPDLPTWKRNFRSALNRKGLGLAEDRSKDPHPHKIYE 108

                                NES                                IAD
HEL  FVNSGVGDFSQPDTSPDTNGGGSTSDTQENILDELLGNMVLAPLPDPGPPSLAVAPEPCQPLRSPSLDNPTFPFNLSPSENPLKRLLVFGEEWEPEVTAFYRGRQVF 216
Vero  FVNSGVGDFSQPDTSPDTNGGGSTSDTQENILDELLGNMVLAPLPDPGPPSLAVA---PEPCQPLRSPSLDNPTFPFNLSPSENPLKRLLVFGEEWEPEVTAFYRGRQVF 212

                                IAD
HEL  QQTISCPEGLRLVGSEVGDRTLPGWFTLPDPGMSLDRGVMSYVRHVLSSLGGGLALRAGQWLMAQRLGHCHTYNAVSELLNSGHGPDGEVPKDEGGVFDLGP 324
Vero  QQTISCPEGLRLVGSEVGDRTLPGWFTLPDPGMSLDRGVMSYVRHVLSSLGGGLALRAGQWLMAQRLGHCHTYNAVSELLNSGHGPDGEVPKDEGGVFDLGP 319

                                IAD                                SRR
HEL  FIVDLITFTEGSGRSPRYALWFCVGESWPQQPWTKRLVMVKVVPTCLRALVEARVGGASLENTVDLHISNSHPLSLTSQYKAYLQDLVEGMDFQGGPGET 427
Vero  FIVDLITFTEGSGRSPRYALWFCVGESWPQQPWTKRLVMVKVVPTCLRALVEARVGGASLENTVDLHISNSHPLSLTSQYKAYLQDLVEGMDFQGGPGET 422

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Figure 3.6. Amino acid sequence of IRF-3 in Vero cells compared to HEL. Differences and deletions are represented in bold and highlight. Functional domains are indicated. DBD, DNA binding domain; NLS, nuclear localization signal; NES, nuclear export signal; IAD, IRF association domain; SRR, serine rich region.

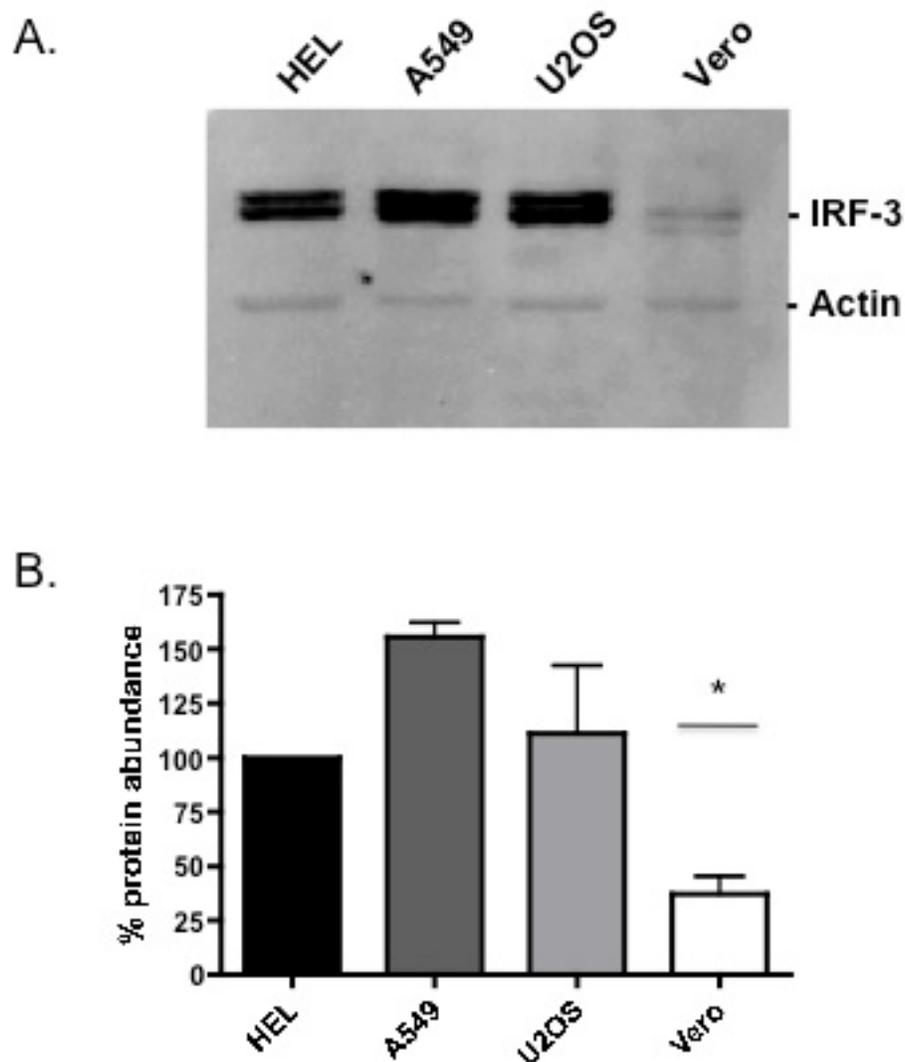
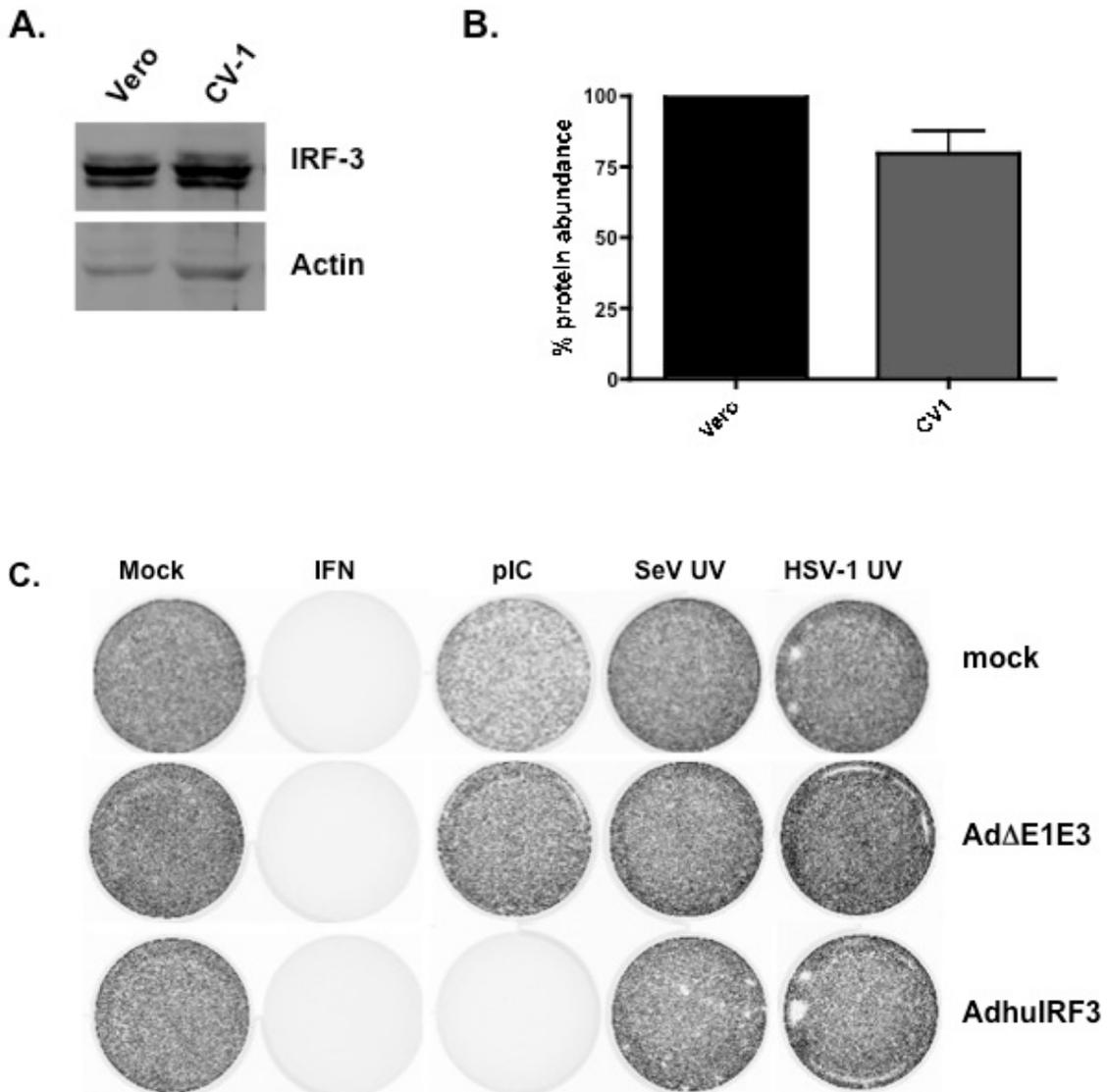
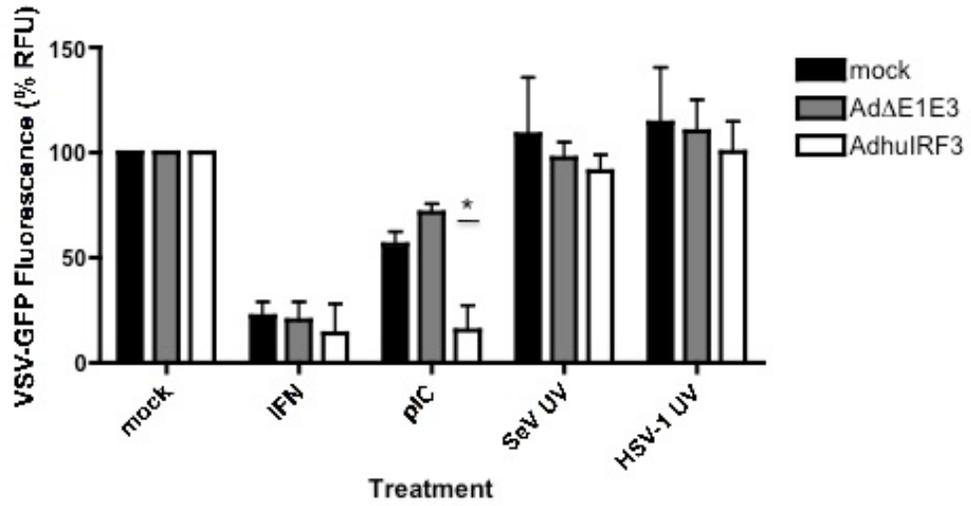


Figure 3.7. *IRF-3 in Vero cells has lower abundance and faster migratory pattern on SDS-PAGE.* A. 15 μ g of whole cell lysate was subjected to Western blot analysis from four different cell lines: HEL primary lung fibroblasts, A549 lung epithelia, U2OS osteosarcoma, and Vero monkey kidney epithelia. Blots were first probed for IRF-3 and visualized by enhanced chemifluorescence, and subsequently reprobed for actin. B. Relative chemifluorescence was quantified and normalized to actin loading control. * represents statistical significance by one-way ANOVA and Newman-Keul's post-hoc test ($p < 0.05$).

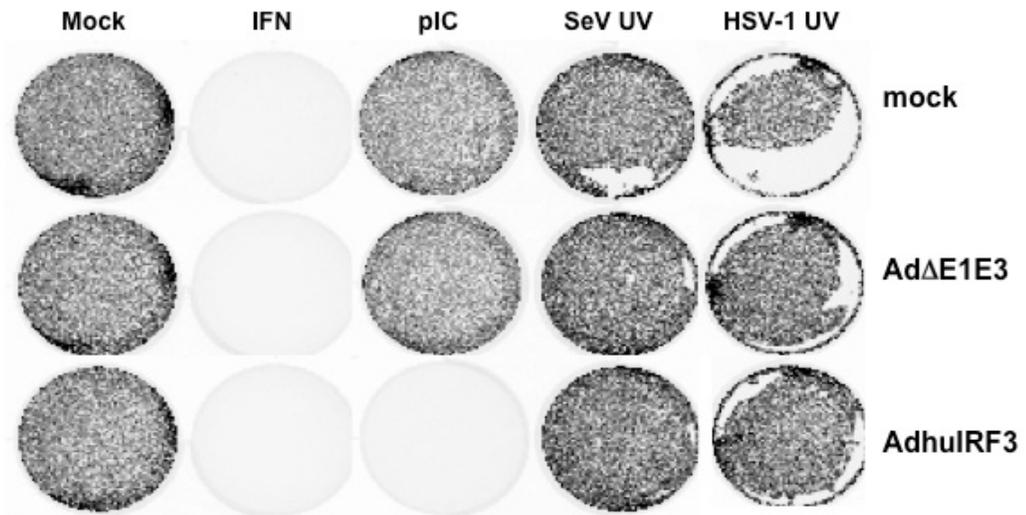
Figure 3.8. *CV-1 IRF-3 follows a similar pattern to Vero with respect to protein migration and pIC-mediated responses.* A. 30µg whole cell lysate was subjected to Western blot analysis as in Figure 3.7 and chemifluorescent intensity quantified and normalized to actin loading control. B. Statistical analysis showed no significance by student's t test ($p < 0.05$). C. Cells were treated for 24h as indicated, then challenged with VSV-GFP. Monolayers were imaged 24h post challenge for GFP fluorescence as a measure of viral replication. D. Quantification of GFP fluorescence from (C). Error bars represent SEM from three independent experiments. * represents statistical significance by one-way ANOVA using Tukey's post-hoc test ($p < 0.05$). E. Supernatants from treated cells were transferred to naïve Vero cells for 24h, then challenged with VSV-GFP and monolayers scanned for GFP fluorescence.



D.



E.



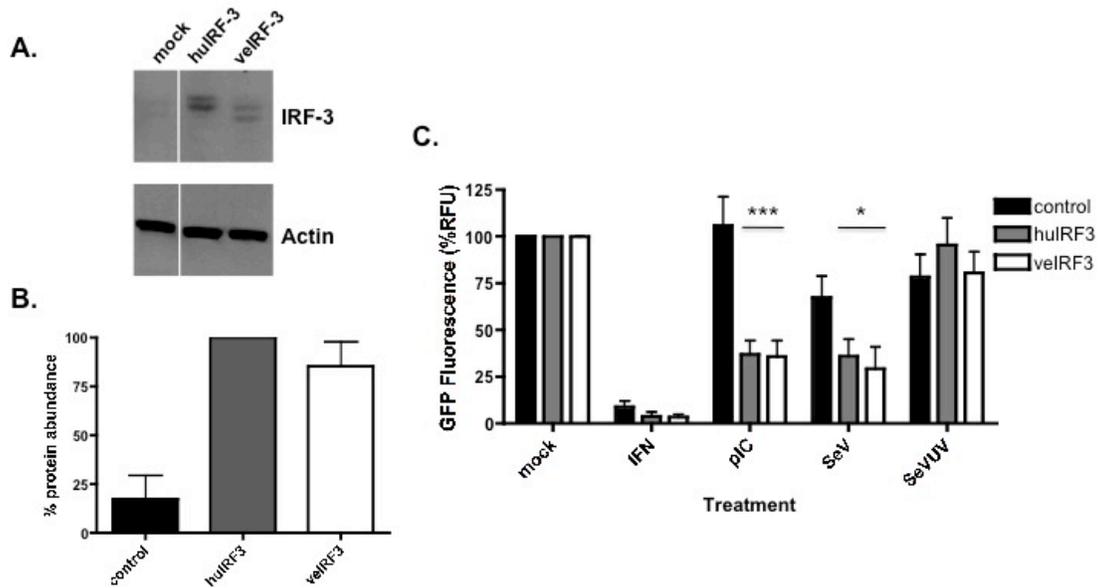


Figure 3.9. Human and Vero IRF-3 overexpression restore the ability of Vero cells to inhibit viral replication in response to pIC in a transfection system. A. Whole cell lysates from Vero cells transfected with either human or Vero IRF-3 were prepared and 5 μ g lysate were subjected to Western blot analysis, and chemifluorescent intensity quantified and normalized to actin loading control (B). C. Cells were co-transfected with a control vector, human IRF-3, or Vero IRF-3 along with pSG5-VSV-G. Transfected cells were treated as indicated and challenged 24h later with VSV-GFP- Δ G. 24h post challenge, supernatants were collected and titered on naive Vero cells, monitoring GFP fluorescence as a measure of relative viral titres. Error bars represent SEM from a minimum of three independent experiments. Statistical significance by two-way ANOVA and Bonferroni's post-hoc test is represented by * ($p < 0.05$) and *** ($p < 0.001$), compared to control treatment group.

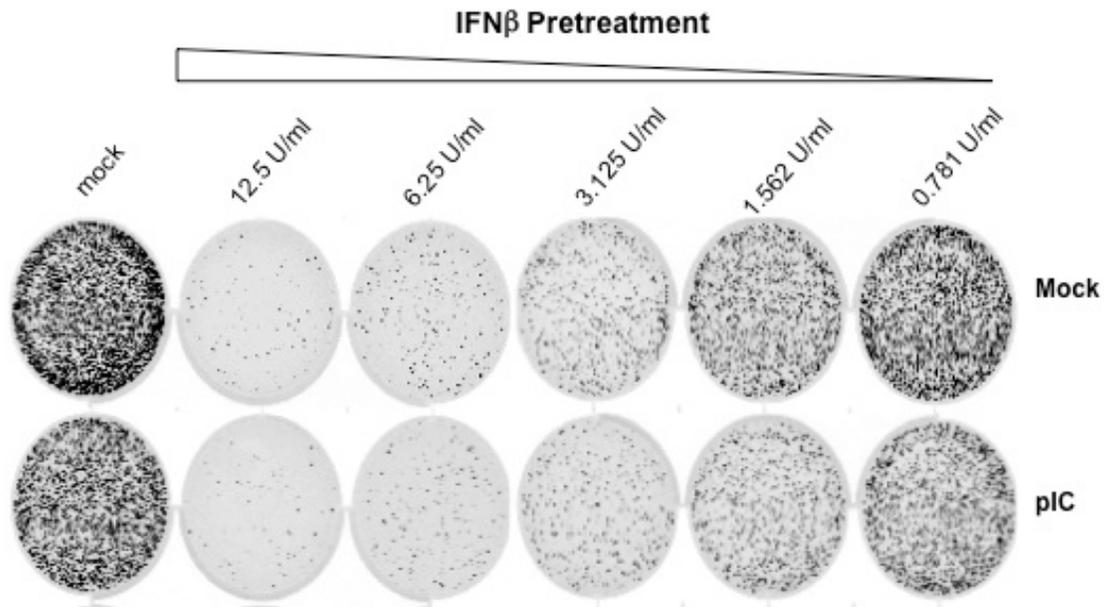


Figure 3.10. *IFN β pretreatment does not sensitize Vero cells to the effects of pIC treatment.* Vero cells were pretreated for 24h with IFN β at the concentrations indicated. Cells were then left untreated or treated with pIC for 24h, then challenged with VSV-GFP and imaged 24h post challenge for GFP fluorescence as a measure of viral gene expression.

Chapter IV. Results: Part Two

IV.I. Preamble

While the downstream effects of IRF-3 activation are relatively well-characterized, regulation of this pathway has not been explored extensively in the literature. A number of mechanisms of IRF-3 regulation have been characterized, and are largely attributed to ubiquitin and ubiquitin-like modification, as described in section I.IV.iii. At the level of RNA splicing, many of the IRFs, including IRF-1, -5, and -7 have been shown to be subject to regulation by multiple splice variants^{256,456,457}. IRF-3 itself has been shown to be regulated by a splice variant of IRF-3 lacking a wild-type DBD^{379,380}. Therefore, the tissue-specific and stimulus-specific regulation of IRF-3 by splice variation is of interest, as it would provide a mechanism for the differential activation of IRF-3 observed between viral stimuli^{214,216,255,336,344,350,380,458}.

Previous evidence from our lab has identified multiple forms of IRF-3 at both the protein and mRNA levels in human, mouse, and African Green monkey cell lines. In the case of IRF-5, it has been shown that splice variants of the transcription factor vary in their responsiveness to viral stimuli, and that the repertoire of IFN α subsets is distinct between variants^{255,256}. Therefore, it appears that splice variants of an IRF can vary in their IFN induction profile. Given the tissue-specific distribution of splice variants of IRF-5, is it probable that the cell type specific IRF-5 profile determines the cell type specific IFN response to viral stimuli. It is reasonable to suggest, therefore, that IRF-3 activation involves the same fine-tuning at the level of splicing. Indeed, at the genesis of this project, a splice variant of IRF-3, denoted IRF-3a, was characterized as a negative regulator of wtIRF-3 following viral infection^{379,380}. IRF-3a was demonstrated to be ubiquitously expressed; interestingly, the relative levels of IRF-3a to wtIRF-3 showed tissue-specific variation, implying a tissue-specific role for this splicing variant in the regulation of antiviral responses mediated by wtIRF-3³⁷⁹.

In the case of IRF-1, a splice variant of the protein was found to be upregulated in patients with myelodysplasia/myeloid leukemia, while the wild-type protein was downregulated. Given that IRF-3 is involved in anti-tumour immunity, it is likely that the profile of IRF-3 splice isoforms varies between primary and immortalized cells as well. Indeed, preliminary evidence from our lab suggests that the immortalization of primary MEFs is associated with changes in IRF-3 signalling and the antiviral response.

IV.II. Hypothesis and Approach

Based on preliminary identification of multiple splice variants of IRF-3 in our lab, and given that splicing serves an important regulatory function for many antiviral genes including the IRFs, I hypothesized that **the antiviral functions of IRF-3 are regulated by splicing**.

In order to address this hypothesis, novel splice variants were identified by RT-PCR analysis from various human cell lines, and were isolated and sequenced.

These sequences were then compared to wild-type IRF-3 to determine differences in critical functional domains and amino acid residues that may affect function. Lastly, transient and stable overexpression experiments were employed to determine the function of a potential negative regulator of wild-type IRF-3.

IV.III. Results

IV.III.i. Western Blot Analysis of IRF-3 Reveals Multiple Isoforms at the Protein Level

To identify various splicing products of IRF-3 at the protein level, whole cell extracts were isolated from various human cell lines as well as Vero cells, which have been shown in Chapter III to be dysfunctional in some IRF-3-mediated antiviral responses. Western blot analysis of HEL, A549, and U2OS cell lines using a polyclonal antibody against full length wtIRF-3 revealed multiple products in addition to wtIRF-3, which runs as a doublet at 55kDa (Figure 4.1). Whether this doublet represents two distinct sequences of IRF-3 has not been determined, but phosphatase treatment does not affect the appearance of this doublet, implying that phosphorylation does not account for this migration pattern (data not shown). In addition, bands corresponding to ~45kDa and ~30kDa were also identified. These bands could correspond to an alternatively spliced product of IRF-3, or could be the result of IRF-3 degradation. However, given that protein extracts were taken from unstimulated cells, and that IRF-3 degradation has only been shown to occur following viral stimulation^{214,459}, it is likely that these bands correspond to a splicing product of IRF-3.

IV.III.ii. RT-PCR and Sequence Analysis of IRF-3 Reveals Multiple Isoforms at the RNA Level

Next, RNA was harvested from untreated cell lines and RT-PCR analysis was performed for IRF-3 using primers flanking the wild-type start and stop codons. As shown in Figure 4.2, multiple products of IRF-3 were observed in all cell lines tested. I decided to focus on the identification of human isoforms of IRF-3, since much of the characterization of this gene has been done in human cell lines, and since our laboratory's characterization of the IRF-3-mediated response to virus particle entry has been performed in human cell lines.

RT-PCR products were either isolated from agarose gel or cloned into a sequencing vector for sequence analysis. A schematic representation of the IRF-3 splicing products is shown in Figure 4.3. In addition to wild-type IRF-3 (wtIRF-3), three novel splicing products were identified. The first splicing variant was denoted IRF-3(Δ E6), due to its lack of exon 6. This exon corresponds to a large portion of the IAD, which is responsible for protein:protein interactions with other IRFs, leading to

transcriptional activation. Presumably, a gene product missing the IAD might be capable of competitively binding to promoter regions of IRF-3-dependent genes and inhibiting the transcriptional activity of wtIRF-3.

The remaining two splice forms appear to utilize an alternate splice acceptor site at the 5' end of exon 7. Two consensus AG sequences lie 16 nucleotides apart, and use of the first sequence results in a frame shift mutation relative to wtIRF-3. These two splicing products were denoted IRF-3(+16) and IRF-3(Δ E6+16). This frame shift results in a novel amino acid sequence at the C terminus which does not contain the serine residues previously identified as critical phosphorylation sites for activation of IRF-3. In addition, this frame shift results in the use of a stop codon downstream from the wild-type stop codon, leading to a protein that is 25 amino acids in length longer than each non-frameshift counterpart. Presumably, these isoforms cannot be phosphorylated, an event thought to be required for the conformational changes that allow homotypic and heterotypic protein interactions through the exposed IAD, rendering these isoforms non-functional. Alternatively, these isoforms may have different three-dimensional structures altogether that lead to constitutive exposure or hiding of the IAD, which would render these isoforms constitutively active or inactive.

IV.III.iii. Overexpression of IRF-3 Isoforms in Vero Cells

In order to assess the function of these isoforms in response to various viral stimuli, it was necessary to clone each form into a mammalian expression vector. While attempts to clone and express the +16 forms of IRF-3 into an expression vector were unsuccessful or yielded inconclusive results, IRF-3(Δ E6) was successfully cloned into the mammalian vector pCMV-TnT for functional analysis. The remainder of the chapter will focus on functional studies using this isoform of IRF-3.

Figure 4.4A shows the overexpression of wtIRF-3 and IRF-3(Δ E6) in Vero cells by Western blot analysis. The constitutively active form of IRF-3 contains phosphomimetic residues at critical phosphorylation sites and was denoted IRF-3(5D)²¹⁴. The dominant negative form of IRF-3 is deleted in the N-terminal DBD and was denoted IRF-3(Δ N)²¹⁴. Both are represented in Figure 4.4A and serve as positive and negative controls for subsequent functional assays. In addition, the localization of these forms of IRF-3 during transient transfection was assessed by immunofluorescence microscopy (Figure 4.4B). As shown, wtIRF-3, IRF-3(Δ E6) and IRF-3(Δ N) appear to localize to the cytoplasm in the absence of stimulus in the majority of cells overexpressing these proteins. Conversely, the constitutively active IRF-3(5D) appears to localize to the nucleus in many cells overexpressing this protein, as expected.

IV.III.iv. Development of a Splice-Specific Polyclonal Antibody

In order to determine whether these isoforms are endogenously expressed at the protein level, a fragment of IRF-3 corresponding to the +16 frameshift sequence was cloned into an N-terminal GST fusion vector. The corresponding protein, denoted GST-TC1, was expressed and purified in *E. coli* cells (strain BL21), concentrated by membrane filtration and centrifugation, and separated by size exclusion chromatography to minimize contaminants (Figure 4.5A). This peptide was then used to inoculate two rabbits for polyclonal antibody production, a service performed by Cedarlane Laboratories. Presumably, this polyclonal antibody has the ability to cross-react with the IRF-3(+16) and IRF-3(Δ E6+16) isoforms, allowing detection of these splicing products at the protein level.

Two stocks of polyclonal antibody were produced from this procedure. Both were used at various concentrations to detect protein in either untreated HEL whole cell extract or crude GST-TC1 protein preparation (Figure 4.5B). One stock of antibody cross-reacted with a band between 25-30kDa, which corresponds to the size of IRF-3(Δ E6) or IRF-3(Δ E6+16). Alternatively, this band corresponds to the approximate size of the mammalian GST enzyme. I attempted to immunoprecipitate IRF-3 using a goat polyclonal antibody followed by Western blot analysis with the splice-specific rabbit antibody; however, the results of this experiment were ultimately inconclusive due to the lack of detection of positive control. Taken together, while the nature of the band detected by splice specific antibody Lot 2 is ultimately inconclusive, it is possible that this splice-specific polyclonal antibody was able to identify IRF-3(Δ E6+16) expression in HEL cells at the protein level.

IV.III.v. Transient Overexpression of IRF-3(Δ E6) Does Not Significantly Affect Antiviral Responses

The ability of IRF-3(Δ E6) to elicit an antiviral effect against various viral stimuli was then assessed using the G-less antiviral assay as previously described. Vero cells, as shown in Chapter III, do not endogenously respond to pIC or UV virus, but do respond to IFN and replicating viruses at the transcriptional level and in an antiviral assay. As opposed to many primary cells and cell lines, they also do not produce ISGs in response to plasmid transfection, making them an ideal cell line for transfection studies in antiviral signalling. To address the effect of IRF-3(Δ E6) on antiviral signalling, the G-less cotransfection antiviral assay was utilized to determine whether expression of IRF-3(Δ E6) affects antiviral responses to various viral stimuli. As shown in Figure 4.6, expression of IRF-3(Δ E6) does not appear to change the phenotype of untransfected Vero cells with regard to their response against IFN, pIC, or SeV. While differences in titres of challenge virus in response to SeV UV were consistently observed, this did not reach statistical significance. However, it is possible that the kinetics or breadth of response to SeV UV in IRF-3(Δ E6)-expressing cells is different from what has been observed in primary

fibroblasts, and that either the dose or timepoint are a critical factor in the antiviral response in this system.

IV.III.vi. Vero Cells Stably Overexpressing IRF-3(Δ E6) Do Not Demonstrate Obvious Differences in Antiviral Responses

Given the transient nature of the transfection experiments performed above, and given that a low transfection efficiency may affect the overall assessment of antiviral activity against a viral stimulus, Vero cells stably expressing IRF-3(Δ E6) were derived. Briefly, Vero cells were cotransfected with IRF-3(Δ E6) along with a puromycin resistance cassette, then selected and maintained in puromycin for IRF-3(Δ E6) expression, and denoted this cell line Vero-E6. IRF-3(Δ E6) expression and localization was confirmed by immunofluorescence microscopy (Figure 4.7A). Interestingly, this cell line showed a largely nuclear localization of IRF-3. These cells were then assessed for their ability to respond to viral stimuli by qualitative and quantitative RT-PCR analysis. As shown in Figure 4.7B-C, no significant difference in phenotype in Vero-E6 cells was observed compared to Vero cells. Like Vero and VePuro, Vero-E6 cells do not significantly upregulate ISG 56 mRNA expression upon pIC or SeV UV treatment, implying that IRF-3(Δ E6) does not play an antiviral role in this context. Likewise, Vero-E6 stimulation with SeV leads to similar levels of ISG 56 expression compared to control cells, implying neither a positive nor negative regulatory role of IRF-3(Δ E6) in IRF-3-mediated antiviral signalling. Taken together, these experiments do not reveal an effect of IRF-3(Δ E6) in mediating antiviral signalling.

IV.III.vii. IRF-3(Δ E6) Does Not Regulate IRF-3-Mediated Responses to Viral Stimuli

Finally, it was important to determine whether IRF-3(Δ E6) affects wtIRF-3 function in response to viral stimulation. To this end, Vero3 cells were transfected with various amounts of IRF-3(Δ N) or IRF-3(Δ E6), then treated with viral stimuli and harvested cells 8hpi for RT-PCR analysis to measure ISG 56 mRNA expression (Figure 4.8). While IRF-3(Δ N) appears to affect pIC-induced IRF-3 expression, significant differences in ISG 56 mRNA accumulation in IRF-3(Δ E6)-expressing cells were not observed with respect to IFN, pIC, SeV, or SeV UV treatment. While this observation is not conclusive due to the qualitative nature of the RT-PCR analysis, there is no obvious difference in antiviral gene expression between control and IRF-3(Δ E6)-treated cells. Quantitative analysis would be useful to confirm this result. Taken together, these data do not reveal a regulatory role for IRF-3(Δ E6) in the wtIRF-3-mediated antiviral response.

IV.IV. Summary and Conclusions

The investigation into splicing-mediated regulation of IRF-3 has been interesting, because it provides one of few lines of evidence of IRF-3 regulation aside from ubiquitin and ubiquitin-like modification. At the time this project began, few examples of IRF regulation by splicing had been published. The first example of alternative splicing of IRF-3 was characterized by Peter Howley's lab, in which the N-terminal DBD was largely truncated and replaced by non-wild-type sequence due to alternate start codon usage^{379,380}. This variant of IRF-3, denoted IRF-3a, has been shown to be a negative regulator of wild-type IRF-3 function, by forming a non-functional heterodimer with activated wild-type IRF-3.

Since then, a number of splice variants of IRF-3 have been characterized, and many correspond to those that have been identified in this study. The cell type-specific expression of an alternative splice variant of IRF-3, termed IRF-3nirs, was shown in hepatocellular carcinoma cell types from various sources⁴⁶⁰. This splice variant is deleted in the 6th exon containing a large portion of the IAD, and has been shown to act as a negative regulator of wild-type IRF-3 function by competing for DNA binding sites. Indeed, IRF-3nirs is identical in sequence to IRF-3(Δ E6). The expression of this splice variant in cancer cell types appears to affect IFN β expression in response to both pIC and VSV-M51R, an IFN-inducing mutant strain of VSV. The expression of IRF-3nirs in cancer cell lines provides one mechanism of downregulation of IFN-sensitivity that is often observed in a variety of cancer cells.

Regulation of pIC, SeV, and SeV UV responses by IRF-3(Δ E6) was not observed in terms of ISG 56 mRNA accumulation or induction of an antiviral state. In terms of pIC treatment, Marozin *et al.*⁴⁶⁰ report negative regulation of wtIRF-3-mediated responses to transfection of 5 μ g/ml pIC. Importantly, the recognition of extracellular pIC by cell surface receptors is distinct from the intracellular recognition of pIC at an endosomal surface or in the cytoplasmic compartment, which may provide an explanation for the difference observed between the authors' system and the system utilized in this study. Indeed, endosomal and cytoplasmic pIC have been shown to be recognized by TLR3 and MDA5, respectively^{176,461}, and the recognition of extracellular pIC has been shown to be recognized by the class A scavenger receptors¹⁹⁹. Therefore, cell-type specific differences in these receptors may provide an explanation for the differences observed between these systems. In terms of viral infection, it is difficult to compare responses to VSV infection at a MOI of 1pfu/cell, used by Marozin *et al.* with responses to SeV infection at a MOI of 20HAU/10⁶ cells. Both the virus type and the MOI may play roles in the activation of IRF-3 in the cell types tested and in the ability of IRF-3(Δ E6) to negatively regulate these pathways.

While a demonstrated dominant negative regulatory function of IRF-3(Δ E6) has been described in the literature⁴⁶⁰, the experiments in this study did not reveal differences *in vitro* in response to various viral stimuli. Some preliminary evidence suggests that IRF-3(Δ E6) expression leads to an augmented response to virus particle entry; however, these results need to be confirmed. Vero-E6 cells do not mimic the phenotype observed in the transient system. This could be because IRF-

3(Δ E6) truly does not play a role in the antiviral response to UV virus. However, given the nuclear localization of IRF-3(Δ E6) in Vero-E6 cells, it is possible that this clone in particular harbours a unique localization and subsequent phenotype. Screening of IRF-3(Δ E6)-expressing cells during the stable selection procedure yielded clones with highly variable expression and localization of IRF-3 by immunofluorescence analysis. It is possible that this observation is a consequence of the stable selection protocol, and that another approach to overexpression of this protein would be required to confirm these observations.

We observed via qualitative RT-PCR analysis that IRF-3(Δ E6) expression does not significantly affect wtIRF-3-mediated antiviral gene expression in Vero3 cells. However, it is possible that IRF-3(Δ E6) overexpression leads to slight changes in wtIRF-3-mediated responses that are not obvious in the qualitative system employed here. qRT-PCR analysis may be useful to definitely ascribe a role for this splice variant in IRF-3(Δ E6)-mediated regulation of wtIRF-3 function. However, given the already published role for this splicing variant at the time these experiments took place, and given that preliminary evidence did not reveal an obvious role for IRF-3(Δ E6) either in overexpression experiments or in co-expression experiments with wtIRF-3, we decided not to pursue the *in vitro* regulatory role of this splicing product further in our system.

More recently, an additional splice variant of IRF-3, termed IRF-3CL, was shown to be ubiquitously expressed and to act as a negative regulator of virus- and IKK ϵ -dependent IRF-3 activation and IFN β expression⁴⁶². This variant has an alternative 3' splice acceptor site in the intron preceding exon 7 of wtIRF-3, leading to a frame shift that changes the sequence of the C-terminal portion of the protein. Indeed, this splice variant is identical in sequence to the isoform denoted IRF-3(+16) identified in this study. This variant has also been shown to dimerize with activated wild-type IRF-3 and inhibit its nuclear translocation and transcription of IFN β .

It was difficult to conclusively detect either endogenous IRF-3(+16) or IRF-3(Δ E6+16) at the protein level in HEL fibroblasts, despite having identified IRF-3(+16) at the mRNA level in untreated HEL cells. This could be due to the fact that the polyclonal antibodies produced against GST-TC1 peptide do not sufficiently cross-react with either +16 isoform. Alternatively, it is possible that neither isoform is expressed at the protein level in HEL cells, despite their expression at the mRNA level. Indeed, the ubiquitous expression of IRF-3CL at the protein level, as reported by Li *et al.*⁴⁶², is difficult to discern, since detection of this isoform at the protein level was not adequately distinguished from wtIRF-3. The authors raise a polyclonal antibody against the entire isoform, which shares significant sequence homology to wtIRF-3, and given the similarity in size between wtIRF-3 and IRF-3CL, it is difficult to distinguish whether this antibody truly detected ubiquitous IRF-3CL. However, it is also possible that this isoform is expressed in many cell types and cell lines but not in HEL cells at the protein level.

More recently, four novel splicing variants of IRF-3 were identified by RT-PCR and sequence analysis from various cell lines⁴⁶². These isoforms use a start site in the 5th exon of wtIRF-3, and contain combinations of exons 3, 4, and 6 with

respect to wild-type exon usage, and produce two distinct protein isoforms, one of which is deleted in exon 6. These splicing products are found more frequently in tumor tissues compared to their normal counterparts, and the exon 6-bearing variant has been shown to interact with wtIRF-3 upon pIC stimulation, implying that this variant acts in a negative regulatory manner. This study did not detect these splicing variants of IRF-3 at the RNA level. This is not surprising, since the approach utilized to identify splice variants of IRF-3 used primers designed based on the start and stop codons of wtIRF-3. In this study, these isoforms are not obviously present at the protein level by Western blot analysis. It is possible that the additional bands revealed by Western blot analysis in Figure 4.1 correspond to these splicing products. Indeed, these splicing products correspond to the ~30kDa band observed in this experiment. Further studies are needed to determine whether these isoforms are indeed present in either HEL cells or other cell lines tested.

Finally, two novel splicing variants of IRF-3 have been identified by rapid amplification of cDNA ends (RACE) experiments, and use a novel promoter binding site in intron 2⁴⁶³. However, no evidence of these variants has been demonstrated at the protein level. In addition, while the expression of these variants was identified in various tissues by RT-PCR using splice-specific primers, various explanations for this have not been ruled out, including DNA or heterologous nuclear RNA contamination, or the existence of a splice variant not identified by the authors which contains intron 2. Finally, no functional analysis of these variants has been performed. Again, these isoforms are not obviously present at the protein level by Western blot analysis in this study, and were also not detected at the RNA level due to the primer design procedure used for cloning.

Taken together, three splicing variants of human IRF-3 were identified at the RNA level in this study. Overexpression studies using IRF-3(Δ E6) failed to ascribe either a positive or negative regulatory function in the context of pIC treatment and viral infection. However, alternative approaches to address the function of IRF-3(Δ E6) in the response to viral infection or virus particle entry would be useful to verify this observation. Based on the high degree of splicing variation of IRF-3, it is reasonable to speculate that the regulation of IRF-3 by RNA splicing provides a useful mechanism for the tissue- and stimulus-specific regulation of the function of this protein.

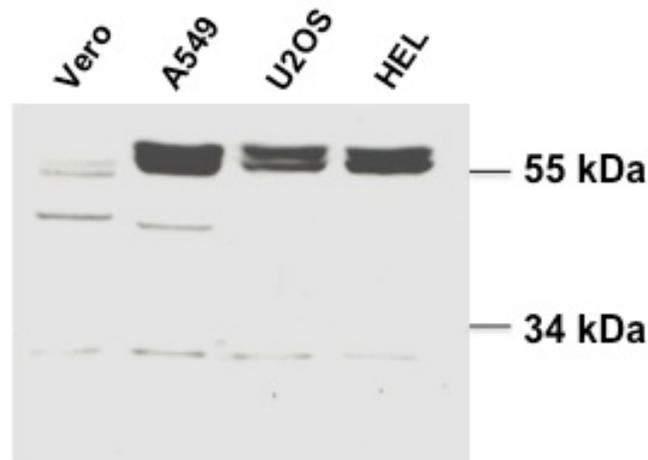


Figure 4.1. Multiple species of IRF-3 are observed at the protein level in Vero and human cell lines. Whole cell lysate from Vero, A549 human lung epithelia, U2OS osteosarcoma, and HEL primary lung fibroblasts were harvested, and 40 μ g protein subject to Western blot analysis for IRF-3.

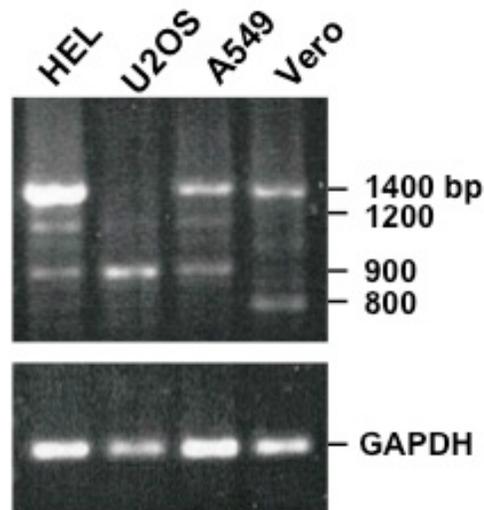
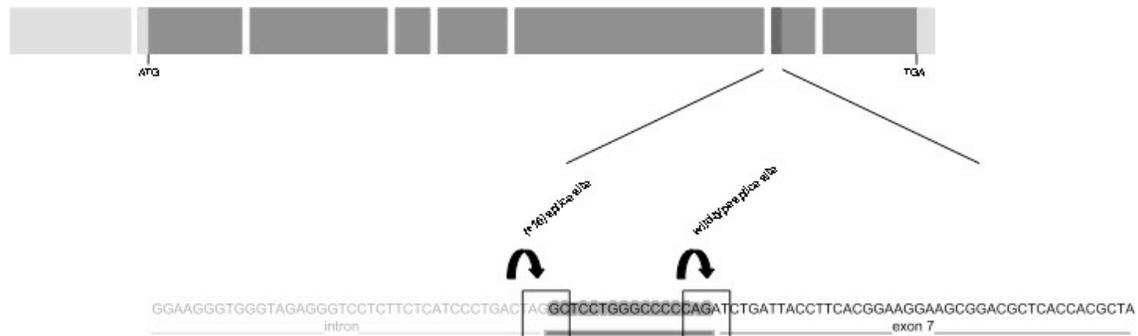


Figure 4.2. Multiple RNA species of IRF-3 are observed at the RNA level in Vero in human cell lines. RNA was harvested from HEL, U2OS, A549, and Vero, and used for RT-PCR analysis using primers flanking the start and stop codons of wild-type human IRF-3.

A.



B.

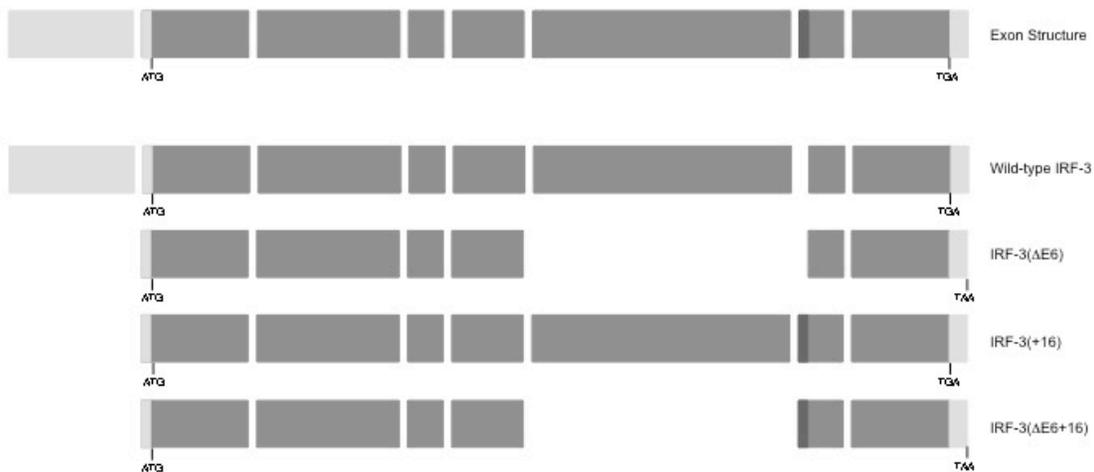


Figure 4.3. Identification of novel human *IRF-3* mRNA products by alternative splicing. A. Exon structure of the *IRF-3* gene. Wild-type *IRF-3* contains 8 exons. Coding sequence is represented in dark grey and non-coding regions are represented in light grey (above). Alternative splice site usage at exon 7 is highlighted (above) and magnified to show sequence (below). Highlighted sequence represents (+16) frame shift sequence. B. Cloning and sequence analysis of multiple RNA products following RT-PCR analysis as described in Figure 4.2. *IRF-3(+16)* utilizes an alternative splice acceptor site represented in (A), leading to a frame shift relative to wild-type *IRF-3* at the C-terminus. *IRF-3(ΔE6)* has its 6th exon spliced out relative to wild-type *IRF-3*. *IRF-3(ΔE6+16)* bears both of these splicing differences.

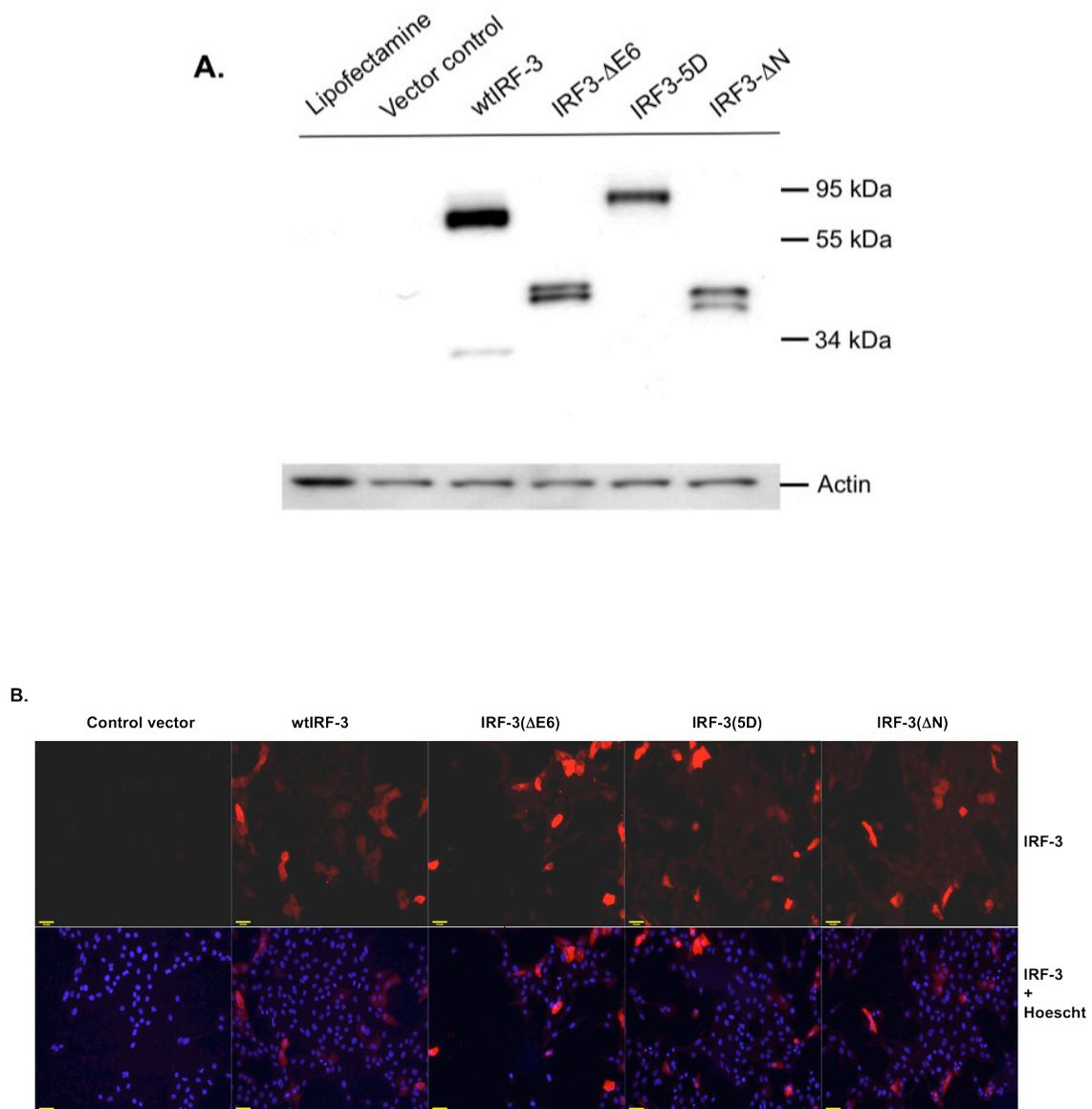
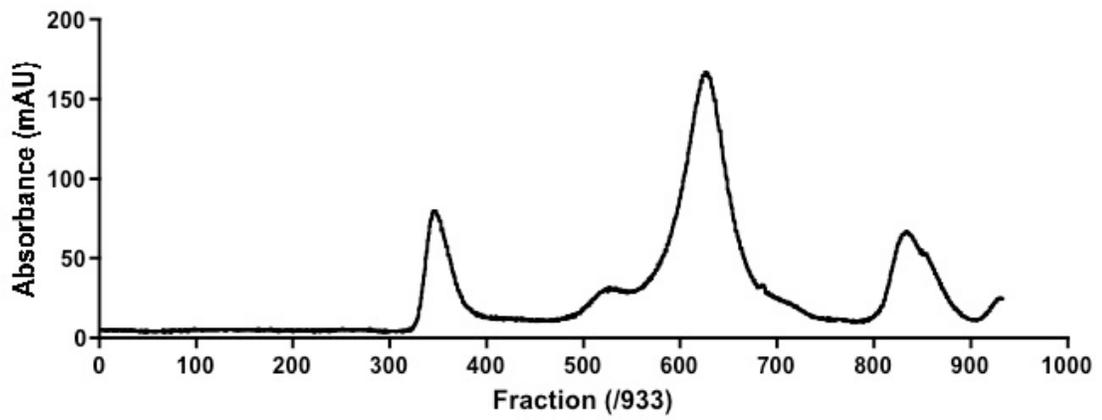


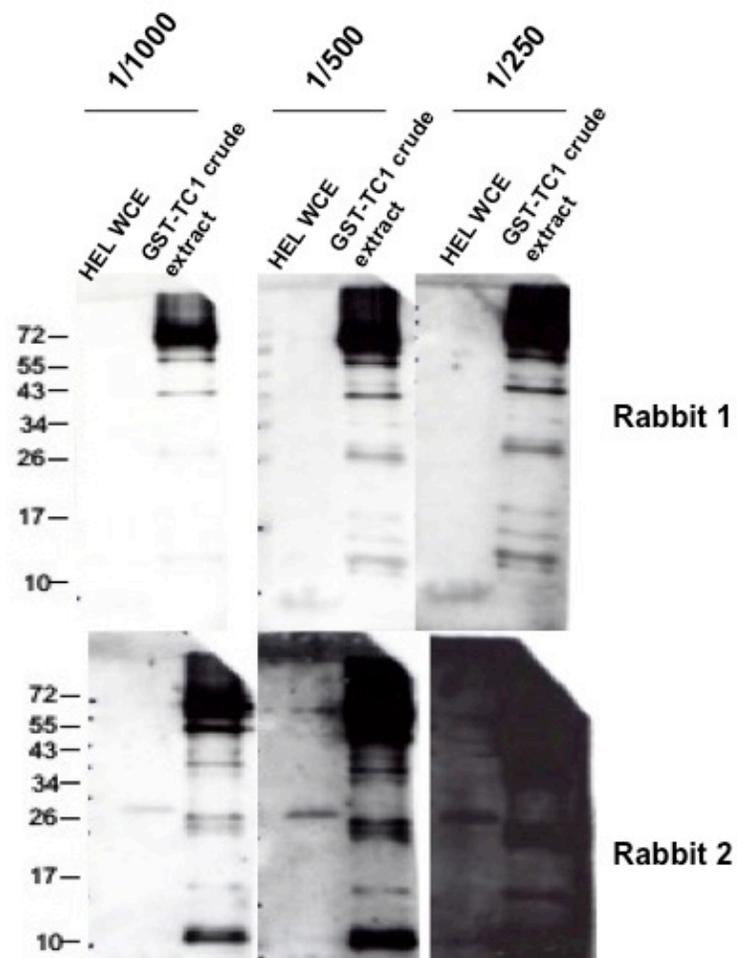
Figure 4.4. *Expression and localization of IRF-3(ΔE6) in transfected Vero cells.* A. Vero cells were transfected as indicated for 24h, then whole cell lysate harvested and subject to Western blot analysis for IRF-3 or actin loading control. B. Vero cells were transfected as indicated for 24h, then cells harvested for immunofluorescence analysis to monitor IRF-3 expression and localization. Above, IRF-3 expression in transfected cells. Below, IRF-3 expression (red) merged with Hoescht nuclear stain (blue) to visualize localization.

Figure 4.5. *Preparation and analysis of splice-specific IRF-3 antibody in rabbits.* A. GST-TC1 peptide was prepared and purified by size exclusion chromatography. The three major peaks correspond to three major species observed in crude extracts which likely include the common contaminants GST (~23kDa, right peak) and DNA-K (~70kDa, left peak). The peak corresponding to a 26kDa species (middle peak), likely representing GST-TC1, was collected, extracted from polyacrylamide gel, then used to inject two rabbits. B. Purified antibody preparations were used at 1:1000, 1:500, and 1:250 of stock to assess splice-specific IRF-3 protein in HEL cells. Crude GST-TC1 purified protein was used as a positive control for antibodies.

A.



B.



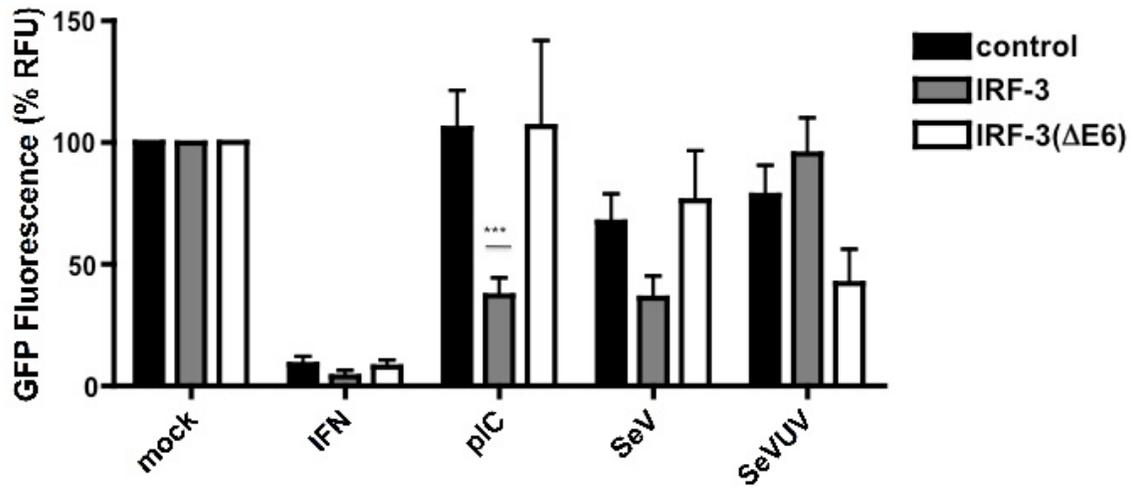
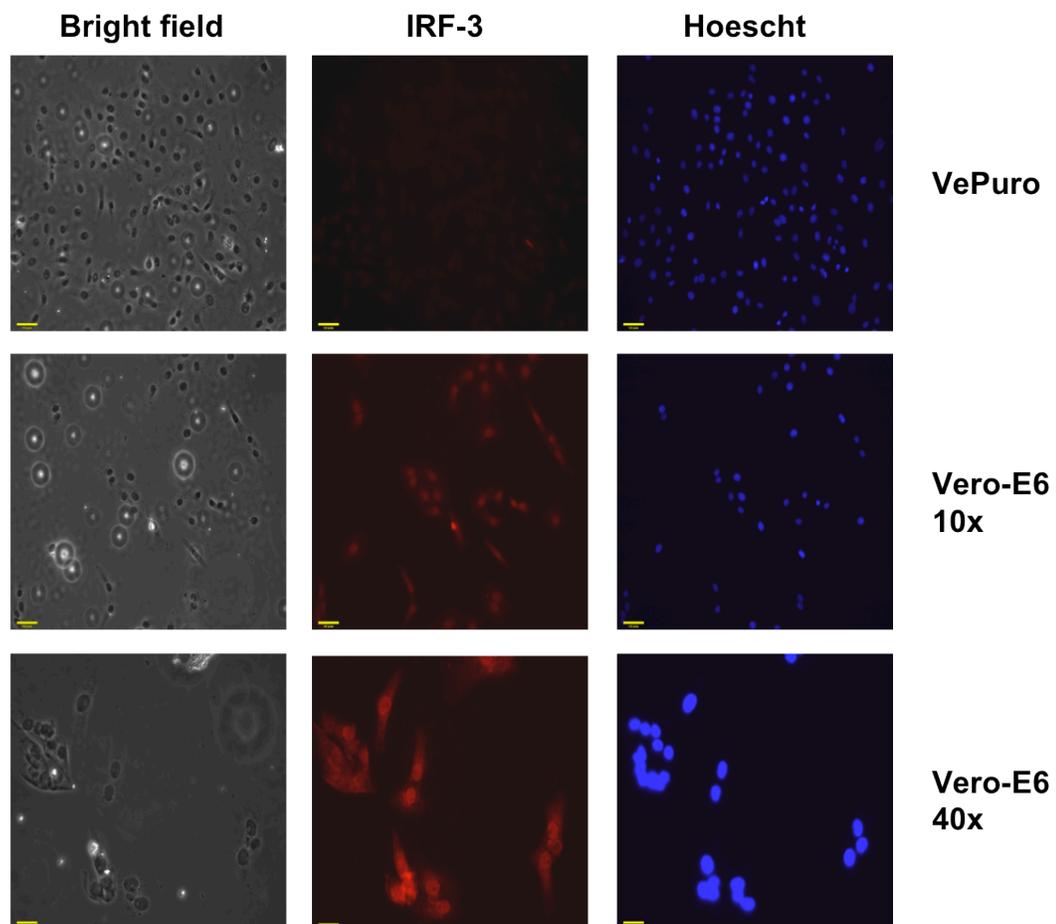
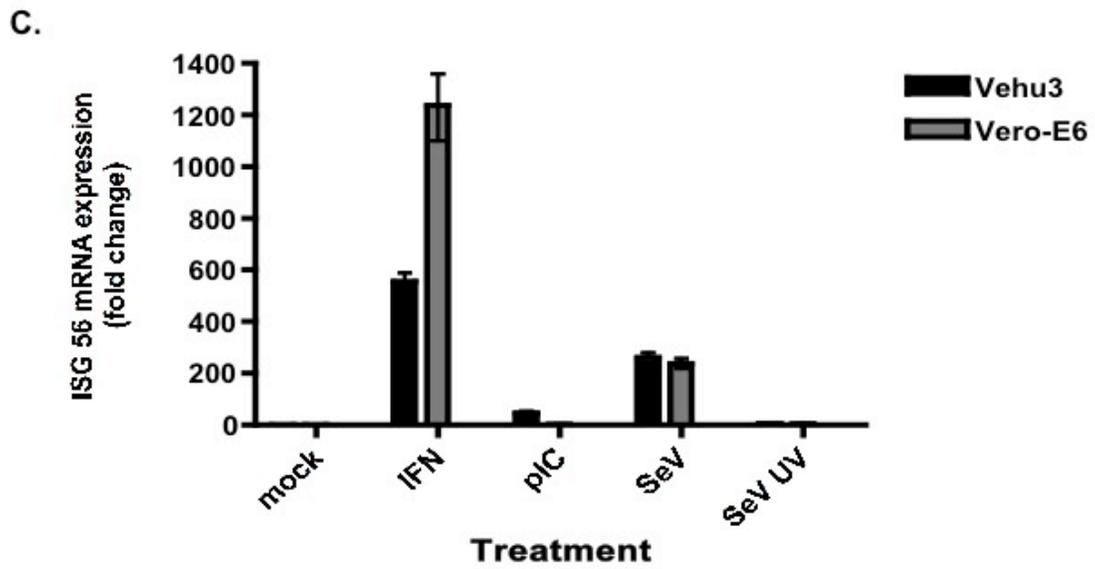
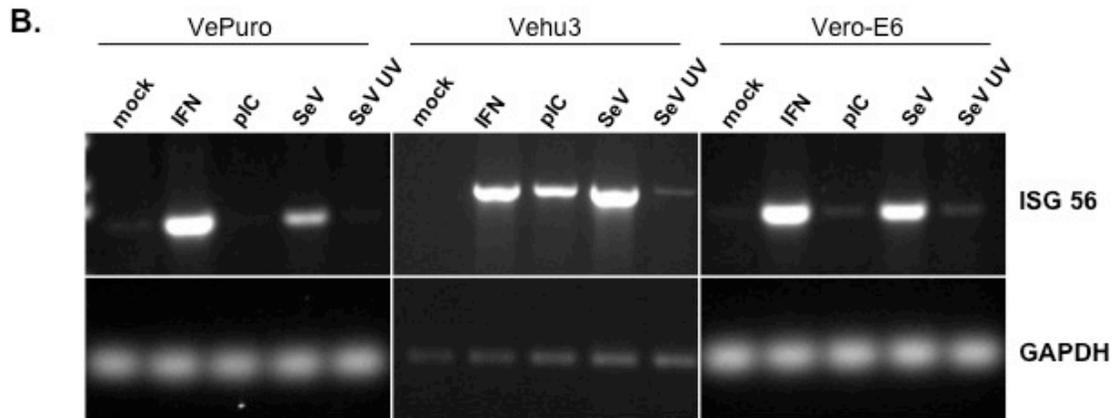


Figure 4.6. Overexpression of wild-type IRF-3, but not IRF-3 Δ E6, restores the ability of Vero cells to inhibit viral replication in response to pIC in a transfection system. A. Cells were cotransfected with a control vector, human IRF-3, or Vero IRF-3 along with pSG5-VSV-G. Transfected cells were treated as indicated and challenged 24h later with VSV-GFP- Δ G. 24h post challenge, supernatants were collected and titered on naive Vero cells, monitoring GFP fluorescence as a measure of relative viral titres. Error bars represent SEM from a minimum of three independent experiments. Statistical significance by two-way ANOVA and Bonferroni's post-hoc test is represented by *** ($p < 0.001$), compared to control treatment group.

Figure 4.7. *Vero cells stably expressing IRF-3($\Delta E6$) do not elicit differential responses to viral stimuli.* A. Vero cells were co-transfected with IRF-3($\Delta E6$) or control vector along with a puromycin resistance cassette, then selected and maintained in 10 μ g/ml puromycin and denoted Vero-E6 and VePuro, respectively. Stable cell lines were harvested for immunofluorescence analysis for IRF-3 expression and localization. B. VePuro, Vehu3, and Vero-E6 cells were treated as indicated and RNA was harvested 8h post treatment to assess ISG 56 expression. C. Vehu3 and Vero-E6 were treated as in (B), with RNA harvested for qRT-PCR analysis for ISG 56 expression.

A.





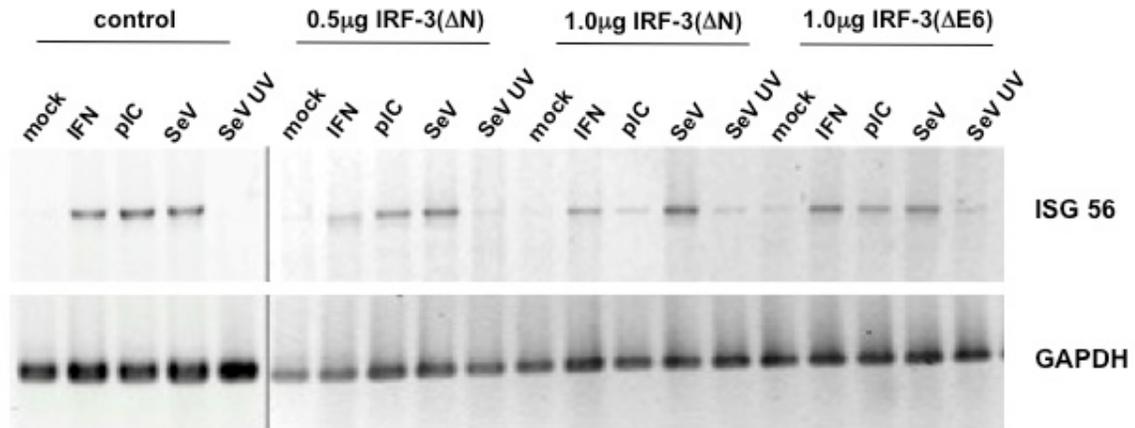


Figure 4.8. *IRF-3(ΔE6)* expression does not negatively regulate *IRF-3*-mediated responses to viral stimuli. Vehu3 cells were transfected for 24h with *IRF-3(ΔN)* or *IRF-3(ΔE6)*, then treated as described. RNA was harvested 8h post-treatment to assess ISG 56 expression as a measure of antiviral state induction, or GAPDH as a loading control.

Chapter V. Results: Part Three

V.I. Preamble

The cellular response to virus particle entry constitutes one of the earliest cellular responses to pathogen invasion. As described in Chapter I, this response does not require viral replication and leads to an IFN-independent signalling pathway resulting in the production of a subset of IRF-3-dependent ISGs and the induction of an antiviral state. The cellular components that lead to the activation of TBK-1 and IRF-3 in this pathway have not been elucidated.

In the literature, viral infection from a number of viruses has been shown to induce the cellular production of reactive oxygen species (ROS), in many cases leading to antiviral protection through IRF-3, NF κ B, and/or p38 MAPK activation^{186,315,324,395-401}. A wide range of cellular signalling pathways are subject to regulation by ROS; therefore, it is not surprising that the downstream consequences of virus-induced ROS production have been widely varied based on the virus and cell type. In this way, ROS are considered DAMPs that recognize generic cellular stresses induced by viral infection.

Among the cellular processes regulated by ROS are components of the IFN-dependent and -independent signalling pathways. ROS activate the IFN pathways via induction of IPS-1 and activation of IKK ϵ and IRF-3 in response to viral infection^{395,402}. In addition, components of many antiviral pathways including the Nalp3 inflammasome, p38, and JNK, and AP-1 have been shown to be regulated by a redox-sensitive inhibitory binding proteins such as TRX^{347,389,391,393,394}.

In order to identify cellular components involved in the cellular response to virus particle entry, we used a high-throughput reverse genomics approach. Here, we identified a number of putative genes involved in the establishment of the IFN-independent antiviral state. Among these putative genes were several involved in the production of ROS and ROS-mediated signalling.

V.II. Hypothesis and Approach

Given that the entry of enveloped virus particles induces a virus non-specific cellular signalling pathway leading to an antiviral state, and given that ROS have been shown to regulate various virus-induced signalling pathways, I hypothesized that **virus particle entry induces a cellular antiviral response involving ROS production**. To test this hypothesis, ROS production was measured in HEL cells treated with various enveloped virus particles. Then, both specific and non-specific inhibitors of ROS were used to assess the contribution of ROS to the IFN-independent antiviral signalling pathway. The ability of cells deficient in their cellular response to virus particle entry to produce ROS was also assessed. Finally, attempts to stably knock down ROS-related genes in HEL cells were performed to determine their involvement in the IFN-independent antiviral response.

V.III. Results

V.III.i. A High-Throughput Approach to Delineating Components Involved in the Cellular Response to Virus Particle Entry Identifies Genes Associated with the ROS Pathway

The characterization of the IRF-3-mediated antiviral response in Vero cells in Chapter III revealed some important information about virus particle-mediated antiviral signalling. First, IRF-3 has been identified as a central mediator of the innate response to virus particle entry using experiments in HEL cells⁴⁴⁴. I have shown through experiments in Vero cells that IRF-3, while central to this pathway, is insufficient for IFN-independent antiviral signalling. Vero cells, while expressing TLR3 and RIG-I (³³⁷ and data not shown), do not respond to pIC treatment unless pretreated with vectors expressing IRF-3, highlighting IRF-3 abundance as a determinant in antiviral signalling in Vero. Furthermore, the inability of Vero cells expressing IRF-3 to elicit an antiviral response to UV virus highlights the requirement of an additional cellular component responsible for activation of this signalling pathway. Whether this component lies upstream of IRF-3, or whether this component is required for the activation of additional transcription factors that comprise the enhanceosome of IFN-independent ISGs is not known. In either case, Vero cells overexpressing IRF-3 provide a useful tool for a high-throughput approach to delineating components of the IFN-independent antiviral signalling pathway.

First, human IRF-3 was stably expressed in Vero, and this cell line was denoted Vehu3. This cell line, as in the transient system, responds to pIC by inducing ISG 56 and an antiviral state (Figure 5.1). Additionally, Vehu3 cells do not elicit an antiviral response against UV virus, implying that an additional cellular component is not expressed, or is dysfunctional, in this cell line. Furthermore, these cells do not respond to plasmid transfection, which is thought to mimic the cellular changes that occur during virus particle entry. Conversely, HEL cells bear an intact antiviral response to virus particle entry, as previously characterized^{336,337,444}. Studies using cycloheximide to prevent viral protein translation demonstrate antiviral gene transcription in response to UV virus, implying that *de novo* protein synthesis is not required for this response to occur, and that cellular components are constitutively expressed in HEL cells^{336,337}. I therefore proposed that a cDNA library expressing the heterogeneous pool of mRNAs that are constitutively expressed in HEL cells could be employed to restore the antiviral response in Vehu3 cells to UV virus.

In order to perform a high-throughput screen of genes involved in the IFN-independent antiviral response, the following reverse genomics approach was performed. A HEL cDNA library was constructed by harvesting RNA from untreated HEL cells, reverse transcribing using a polyT primer specific to mRNA, and cloning into the retroviral construct pFB-neo. Presumably, this pool of cDNA constructs contains all constitutively expressed mRNAs from resting HEL cells, including one or more cellular components involved in the IFN-independent antiviral response. This heterogeneous plasmid pool was used for direct transfection of Vero cells or

retrovirus preparation. As shown in Figure 5.2A, Vero cells do not express transgenes efficiently from the pFB vector. Compared to U2OS cells, the effective virus titre of the same stock of retrovirus preparation is approximately 1000 times lower in Vero cells. While Vero cells are thought to be efficiently transducible, this particular vector utilizes an MMLV-based promoter, which is not commonly used in Vero cells. In order to determine the efficiency of this pFB-based promoter in Vero cells, Vero cells were transfected with either pCMV-Luc or pFB-Luc and luciferase activity was measured to indicate relative promoter strength. As shown in Figure 5.2B, the relative luciferase activity in pFB-Luc-transfected cells is approximately 200 times lower than in pCMV-Luc-transfected cells. Taken together, it appears that the pFB-based retrovirus does not yield efficient transgene expression in Vero cells, making the use of Vehu3 difficult in this system.

ARPE19 cells are a human retinal epithelial cell line that bears an intact antiviral response to IFN and pIC treatment (Figure 5.3A). However, these cells do not undergo an antiviral response to virus particle entry, a similar phenotype to Vehu3 cells. In addition, the use of a human cell line for the stable expression of the HEL cDNA library eliminates issues that may arise due to the expression of human genes in a non-human cell line such as Vehu3 cells. We therefore expressed the retroviral cDNA library in this cell line and monitored transduction efficiency using pFB-LacZ as a control. As shown in Figure 5.3B, ARPE19 cells are transduced with similar efficiency to U2OS cells, making them an ideal candidate for this reverse genomics screen. ARPE19 cells were therefore transduced with HEL cDNA-expressing retrovirus, and were selected and maintained in puromycin. The cell line stably expressing the HEL retroviral library was denoted ARPE-retHEL. The control cell line expressing β -galactosidase was denoted ARPE-retLacZ. RNA was harvested from stable cell lines and subject to RT-PCR and sequencing analysis using retrovirus specific primers to confirm transgene expression (data not shown).

In order to screen for candidate genes involved in the IFN-independent antiviral response, I performed the following screen: A minimum of 10^7 cells of either ARPE-retHEL or ARPE-retLacZ were plated to ensure that each HEL cDNA copy was adequately represented in the mixed population. Cells were treated for 24h with HSV-1 UV at a MOI of 5 pfu/cell. Treated cells and untreated controls were then challenged with VSV(Δ G)-GFP at a dilution of 1:5 of a stock solution, which infects approximately 100% of cells (data not shown). Challenged cells were left to die off with media changed every two days, and cells were monitored for surviving outgrowths of colonies, which represent cells having undergone an antiviral state. DNA from either surviving colonies or single cells was amplified by PCR using primers specific for the common retroviral backbone of each transgene. Amplified products were sequenced to identify the transgene of interest. A small number of surviving colonies were observed in negative control samples, and these transgenes were identified as described and eliminated from the list of candidate genes.

From this screen, 13 candidates were identified. These genes and their described functions are outlined in Table 3. Of interest, three candidate genes were chosen from this screen for further analysis: thioredoxin-related transmembrane protein 2 (TMX2), mitogen- and stress-activated protein kinase 1 (Msk1), and Zyg-

11 homolog of *Caenorhabditis elegans* B (Zyg-11B). The demonstrated functions of these genes are described below.

TMX2 (also known as TXNDC14) is a member of the TRX superfamily, whose role in general involves oxidation-reduction reactions at the endoplasmic reticulum. TMX2 has been implicated as a resistance factor against oncolytic HSV-1 in our lab, and has also been shown to bind to the HBV major antigen HBsAg⁴⁶⁴. While enzymatic activity has not been shown for this protein, it is highly homologous to TMX, whose oxidoreductase activity has been implicated in redox regulation at the endoplasmic reticulum^{465,466}. The TRX superfamily has been well characterized in the ROS-mediated regulation of gene expression and function. As described previously, proteins bound to TRX are subject to regulation when TRX is oxidized and subsequently removed from target gene products following ROS production^{347,394}. Additionally, TRX reductase has been implicated in the IFN and retinoic acid pathways leading to cell death⁴⁶⁷. Whether TMX2 binds to and regulates antiviral genes in a similar manner has not been tested.

Msk1 (also known as RPS6KA5) is a member of the ribosomal S6K superfamily, which is a broad family of mitogen-activated protein kinases (MAPKs) involved in a wide spectrum of signal transduction pathways. Msk1 has been shown to be a target of phosphorylation by p38 MAPK and ERK. Of interest, Msk1 activation following RSV infection leads to the phosphorylation of the p65 subunit of NFκB, leading to NFκB target gene expression³⁹⁶. Importantly, NFκB activation by Msk1 is thought to be mediated by ROS⁴⁶⁸. Rift Valley Fever Virus infection has also been shown to induce the phosphorylation of both ERK and Msk1⁴⁶⁹, which mirrors the pathway induced by oxidative stress⁴⁷⁰. Msk1 activation has also been shown to lead to activation of the transcription factors ATF-2 and c-Jun⁴⁷⁰, which are known components of the IFN-dependent antiviral signalling pathway^{221,222,471}. Msk1 activation has been implicated in Bad-dependent apoptosis following Ca⁺⁺ influx⁴⁷², which has important implications in the cellular response to virus particle entry presumably via Ca⁺⁺ mobilization. Finally, Msk1 activation has been shown to lie downstream of IFNα by a mechanism involving p38 signalling⁴⁷³.

Zyg-11B is a human homolog of the cullin-binding protein Zyg11 from *C. elegans*. While Zyg-11B has not been characterized in humans, its role as a cullin-binding protein in *C. elegans* has been established⁴⁷⁴, and its best characterized function involves mediating meiotic events⁴⁷⁵⁻⁴⁷⁷. In the antiviral signalling cascade, the family of cullin-based ubiquitin ligases are involved in the ubiquitin conjugation IRF-3, STAT1 and STAT2 following viral infection^{459,478}. Together, this implies a role for Zyg-11B in the regulation of antiviral genes via ubiquitination and/or degradation.

Taken together, it is likely that the response to UV virus involves a redox/ROS pathway leading to antiviral responses, and that one or multiple of these genes are involved in the response. Therefore, the contribution of ROS to the IFN-independent antiviral response is of great interest.

V.III.ii. Virus Particle Entry Induces ROS Production in HEL Cells

In order to determine whether ROS are involved in the cellular response to virus particle entry, we treated HEL cells with various enveloped DNA and RNA virus particles. Cells were infected for 15 minutes to allow viral entry, then the fluorescent indicator dye carboxy-H₂DCFDA was added to measure ROS production over time. As shown in Figure 5.4, ROS production was observed in response to the HCMV and SeV particles. ROS production occurred at early times post-infection and reached a statistically significant level of production at approximately 2 hours post-infection. HSV-1 UV infection did not lead to a statistically significant increase in ROS production, but a trend towards a time-dependent increase in ROS was observed over the two-hour timecourse. It is possible that the kinetics of HSV-1 UV-induced ROS production are slower than in response to other virus particles, and that statistically significant levels of ROS production would be observed later than at 2h post treatment. Collectively, we determined that enveloped virus particles of varying size and nucleic acid composition were capable of inducing ROS at early times following infection.

V.III.iii. ROS Inhibitors Reduce Virus Particle-Driven ROS Production

The next step was to determine the role of virus particle-induced ROS in the antiviral response. HEL cells were treated with either diphenyleneiodonium chloride (DPI), an NADPH oxidase enzyme inhibitor, or with butylated hydroxyanisole (BHA), a free radical scavenger, and determined changes in ROS levels in response to virus particle entry at 2h post treatment. Only HCMV UV was used as a virus particle stimulus for this and subsequent experiments, as it showed the most potent ROS activity compared to the other viruses tested. As shown in Figure 5.5A, pretreatment with either inhibitor abrogated the HCMV UV-induced production of ROS following treatment. Cell metabolism was assessed as an indicator of viability using the fluorescent indicator dyes Alamar Blue and CFDA-AM at this time, and significant changes in viability due to inhibitor treatment were not detected (Figure 5.5B-C).

V.III.iv. ROS Are Critical to the Activation of IRF-3 Following Virus Particle Entry

In order to characterize the contribution of ROS to IRF-3 activation and signalling in response to virus particle entry, HEL cells were pretreated with DPI or BHA and the response to UV virus treatment was assessed by monitoring nuclear translocation of IRF-3 following virus particle treatment. As shown in Figure 5.6, cells pretreated with either ROS inhibitor showed a marked decrease in IRF-3 activation as assessed by nuclear localization following HCMV UV treatment. This demonstrates a crucial role for ROS in the activation of IRF-3 following HCMV UV treatment.

V.III.v. ROS are Critical to the Expression of ISG 56 Following Virus Particle Entry

Similarly, the role of ROS in the expression of IRF-3-dependent genes was also assessed. Cells were pretreated with ROS inhibitors as in Figure 5.6, and treated with UV virus to assess ISG 56 mRNA expression 6h following treatment. As shown in Figure 5.7, inhibitor pretreated cells demonstrated a dose-dependent decrease in ISG 56 expression following virus particle treatment. Interestingly, carrier pretreatment alone leads to a decrease in HCMV UV-mediated gene transcription. However, this abrogation is potentiated in the presence of ROS inhibitor, implying a strong role for ROS in the activation of IRF-3-dependent genes. Collectively, these data demonstrate that ROS production is responsible for the activation of IRF-3 and subsequent antiviral gene expression in response to virus particle treatment.

V.III.vi. Virus Particle Entry Does Not Induce ROS Upregulation in ARPE Cells

Since ROS appear to be responsible for the IRF-3-mediated response to virus particle entry, it was of interest to determine whether ARPE19 cells, which do not respond to UV virus, produce ROS in response to treatment. Importantly, this cell line does not produce measurable ROS at early times following virus particle treatment (Figure 5.8A), highlighting the importance of these molecules in the first line response to virus particle entry. Viability in this cell line following treatment was also assessed by Alamar Blue and CFDA-AM (Figure 5.8B-C), as changes in cellular viability could be reflected in ROS detection. A similar phenotype was also observed in Vero cells (data not shown), which have been previously characterized as unresponsive to UV virus treatment. These experiments, taken together with the observation that ROS inhibition reduces the IFN-independent response to virus particle entry, suggests a strong role for ROS in the activation of IRF-3, and subsequent antiviral gene expression, following virus particle entry.

V.III.vii. Stable Knockdown of Genes Involved in ROS Production

In order to validate putative genes identified from the retroviral screen, attempts to stably knock down each gene in HEL cells using shRNA constructs in a lentiviral backbone were performed. The methodology of generating HEL cells stably expressing each shRNA of interest, control shRNA, or GFP to monitor transduction efficiency, is described in Materials and Methods. Briefly, five pre-designed shRNA sequences were expressed in a lentiviral vector using The RNAi Consortium's algorithm for shRNA design. These vectors were pooled and transfected into 293 cells along with lentiviral packaging constructs, and lentivirus-containing supernatants were harvested 24h and 48h post infection. Viral

supernatants were filtered and used to infect HEL cells, then treated with puromycin to select for transduced cells. RNA from stable cells was harvested and subject to qRT-PCR using SYBR green primers specific for each target gene. The relative expression of TMX2, Msk1, and Zyg-11B in each stable cell line is represented in Figure 5.9. As the qPCR data shows significant variation between experiments, the knockdown of these genes has not been confirmed. However, preliminary data suggests that stable cell lines harbour non-specific changes in gene expression peripheral to their target gene of interest.

In the meantime, preliminary experiments to determine the effect of UV virus treatment in shRNA-expressing HEL cells were performed. Cells were treated with IFN, pIC, HCMV or HCMV UV for 6h, and RNA harvested to assess relative ISG 56 mRNA expression. As shown in Figure 5.10, it appears that there are no obvious differences between cell lines in terms of their response to various viral stimuli. Given the high degree of error between experiments, these results are ultimately inconclusive and need to be verified. However, preliminary analysis suggests that these genes do not play a significant role in the IRF-3-mediated response in HEL cells.

The expression of control shRNA alone appears to dampen the IRF-3-mediated antiviral response to stimulus relative to HEL control cells. In particular, the response against HCMV UV in HEL-Luc cells appears markedly lower than in HEL cells, implying that shRNA expression, or the stable selection protocol itself, has an effect on the cellular antiviral response. HEL cells are highly sensitive to changes in confluency and have been observed to undergo senescence under conditions of low confluency. Indeed, it was observed that HEL cells stably expressing transgenes appear to grow more slowly than untreated HEL cells, and global non-specific changes may account for phenotypic differences between HEL cells and their shRNA-expressing counterparts.

Taken together, it appears that there is no obvious effect of these genes on the IFN-independent antiviral response in HEL cells. Based on the preliminary nature of these experiments, there are multiple explanations for this observation. Most importantly, it is possible that the stably selected cells are not efficiently knocked down in target gene expression. In addition, it is possible that the stable selection protocol or that shRNA expression has a global effect on the ability of HEL cells to respond to viral stimuli. Further experiments are needed to confirm this observation.

V.IV. Summary and Conclusions.

Viral nucleic acid serves as an important virus-specific ligand for antiviral signalling. As such, a number of specific and functionally redundant cellular receptors have been shown to bind and respond to viral nucleic acid. Our lab has previously shown that neither TLR3 nor RIG-I is required for the cellular response to virus particle entry³³⁷. This evidence points to the generic nature of the entry event as a DAMP rather than a classical PAMP. The lack of requirement for viral gene expression in the IFN-independent antiviral response also highlights the non-

specific nature of this pathway, and implies that a generic damage or stress signal is likely to be involved in this response, rather than a pathogen-specific cellular receptor.

Previous work has implicated ROS in the activation of antiviral signalling pathways in response to infection. A wide range of viruses have been shown to induce ROS^{186,315,324,395-401}, and RSV infection in particular has been shown to activate IRF-3 in a ROS-dependent fashion³⁹⁹. The specific nature of virus-induced ROS induction, however, has not been elucidated. Here, we provide evidence that the viral entry event is the DAMP responsible for ROS production and subsequent IRF-3 activation. This study demonstrates that ROS are a crucial element in the signalling pathway that responds to incoming viruses and results in the induction of an antiviral state. While the specific mechanism by which viral entry induces ROS is not known, this data presented here implicate this class of molecules in the early antiviral response.

The source of cytoplasmic upregulation of ROS in response to viral stimulation is unclear. Both the cytoplasm and mitochondria are rich sources of ROS, either through the NADPH oxidase complex or through mitochondrial membrane leakage. The mitochondria are a rich source of ROS via the citric acid cycle and electron transport chain, and basal levels of mitochondrial ROS are rendered inert through steady-state metabolic processes. The mitochondrial release of excess ROS, or a substantial upregulation of cytoplasmic ROS, however, cross a homeostatic threshold and result in the activation of various signalling pathways that ultimately lead to inflammatory processes, antiviral signalling, and apoptosis. In the antiviral cascade, many nucleic acid receptors signal at the mitochondria where a common adaptor molecule IPS-1 couples nucleic acid receptor engagement with TBK-1 phosphorylation and subsequent IRF-3 activation^{154,479}. In the response against virus particle entry, it is unlikely that nucleic acid recognition is involved, given the low amount of genomic nucleic acid available for PRR binding. It is possible that the ROS serves a compensatory response at the mitochondria that ultimately leads to TBK-1 activation in the absence of PRR engagement.

Interestingly, DPI is a broad-spectrum inhibitor of the NADPH oxidase enzyme family, and inhibits ROS accumulation and IRF-3 activation following virus particle entry. This implies that the cytoplasmic and not mitochondrial pathway is a major contributor to cytoplasmic ROS upregulation and subsequent signalling. Indeed, Rac1 is a component of this complex and has also been shown to lie upstream of IRF-3 in the cellular response against viral infection or pIC treatment³⁰⁹. Rac1 is also involved in cytoskeletal rearrangement and has been implicated in viral entry^{302,303,410}. Together, this implies a role for Rac1 in the entry-induced generation of ROS and subsequent antiviral signalling. Preliminary experiments in our lab have failed to define a role for Rac1 in the activation of IRF-3 following virus particle entry. However, the involvement of this protein has not been definitively ruled out, and it is possible that Rac1, or a compensatory or redundant Rho GTPase, is involved in the entry-induced, ROS-mediated activation of IRF-3 in this system.

Furthermore, the mitochondrial involvement in IRF-3 activation through nucleic acid sensors and their adaptor proteins is interesting, due to the fact that this organelle is a potent generator of ROS. Indeed, TBK-1 and IRF-3 have been shown to localize to the mitochondria and interact with the RLR adaptor IPS-1 following viral infection^{160,205}. Therefore, the role of mitochondrial ROS in the activation of IRF-3 following virus particle entry is interesting, and requires further study. While neither IPS-1 nor any nucleic acid sensor has been implicated in the IFN-independent antiviral signalling pathway, their involvement cannot be ruled out. Furthermore, it is possible that the activation of ROS following virus particle entry results in activation of a compensatory mitochondrial activating complex that leads to TBK-1 and IRF-3 activation and ISG expression by a mechanism not involving IPS-1 or known PRRs.

ROS signalling has been shown to lead to inflammatory processes, largely through the activation of the transcription factor NF κ B. We have previously shown that NF κ B is not activated in the cellular response to virus particle entry³³⁷, therefore the signalling pathway induced by viral entry is likely to be limited in its inflammatory consequences. It is not known precisely how ROS activate NF κ B in response to viral infection, and it is possible that either the amount of ROS required to activate NF κ B has not been reached in this pathway, or that other viral components, or virus-induced cellular components, are required for ROS-induced activation of NF κ B during an infection.

Recent work has implicated Nalp3 in viral dsRNA recognition and activation of the inflammasome through the NLR adaptor protein ASC, ultimately leading to IL1 β and IL18 secretion. Inflammasome activation by any of the NLRs is thought to be dependent on ROS release from the mitochondria. Based on preliminary experiments, significant differences in antiviral gene induction in *ASC*^{-/-} mouse embryonic fibroblasts in response to virus particle entry were not observed (data not shown), implying that the inflammasome is not an upstream component of the response to virus particle entry. However, it is possible that the inflammasome is indeed activated in response to virus particle entry, and that an ASC-dependent, inflammasome-dependent process lies upstream of IRF-3 activation. Alternatively, ROS production following virus particle entry may bifurcate into both pathways, leading to multiple mechanisms of antiviral protection.

In conclusion, we have implicated ROS as a cellular DAMP in response to virus particle entry. Virus particle-induced upregulation of ROS leads to cellular signalling which results in the activation of IRF-3 and ISGs and the induction of an antiviral state. The results of this study highlight the importance of generic stress signals induced by pathogens that are capable of inducing a meaningful antiviral response. These data also add a novel cellular component to the IFN-independent antiviral signalling cascade.

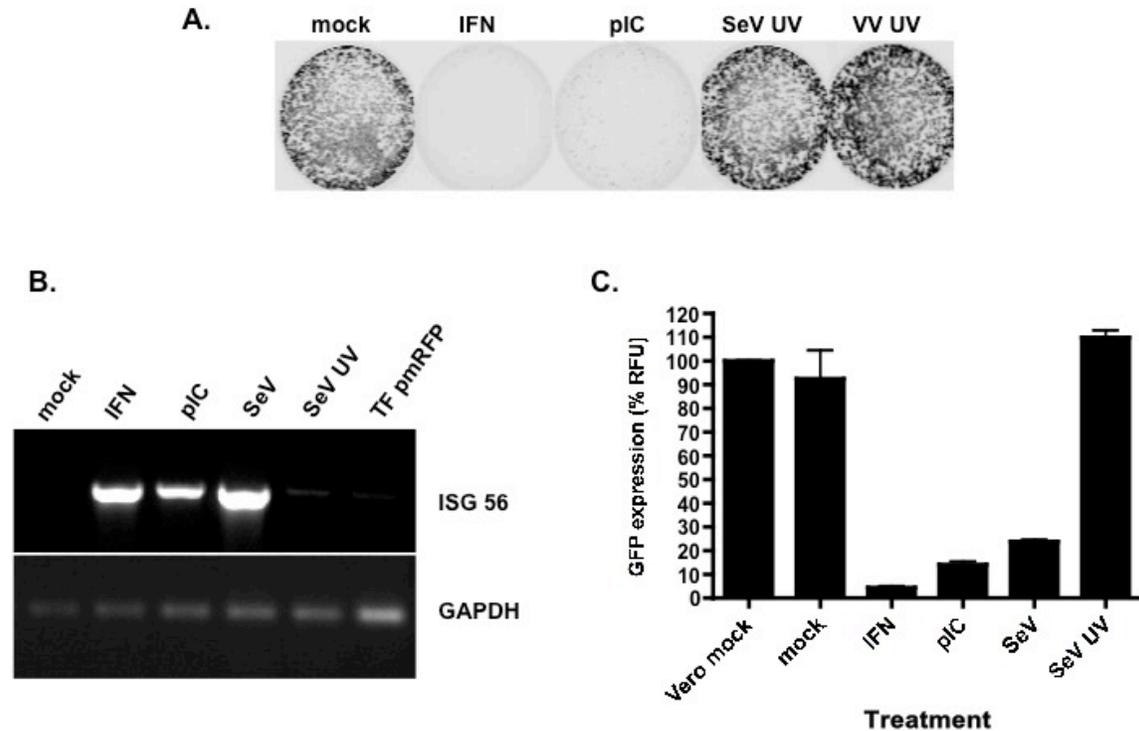


Figure 5.1. *Vehu3* cells overexpress human IRF-3 and respond to pIC, but not UV virus. A. *Vehu3* cells were treated as indicated and challenged with VSV-GFP 24h post treatment. Monolayers were scanned 24h later to measure GFP fluorescence as a measure of viral gene expression. B. *Vehu3* cells were treated as indicated and RNA was harvest 8h post treatment to assess ISG 56 expression by RT-PCR. GAPDH expression was also used as a loading control. TF pmRFP denotes transfection of *Vehu3* cells with pmRFP, a plasmid encoding red fluorescent protein. C. *Vehu3* cells were subject to the G-less antiviral assay as previously described, using only pSG5-VSV-G plasmid to express VSV envelope glycoprotein. Transfected cells were treated 24h later as indicated, and challenged with VSV(Δ G) 24h later. Virus containing supernatants were collected 24h post challenge and transferred to naïve Vero cells, and monolayers scanned 24h post transfer to measure GFP fluorescence as a measure of viral titres. Vero cells were also transfected with pSG5-VSV-G as a control.

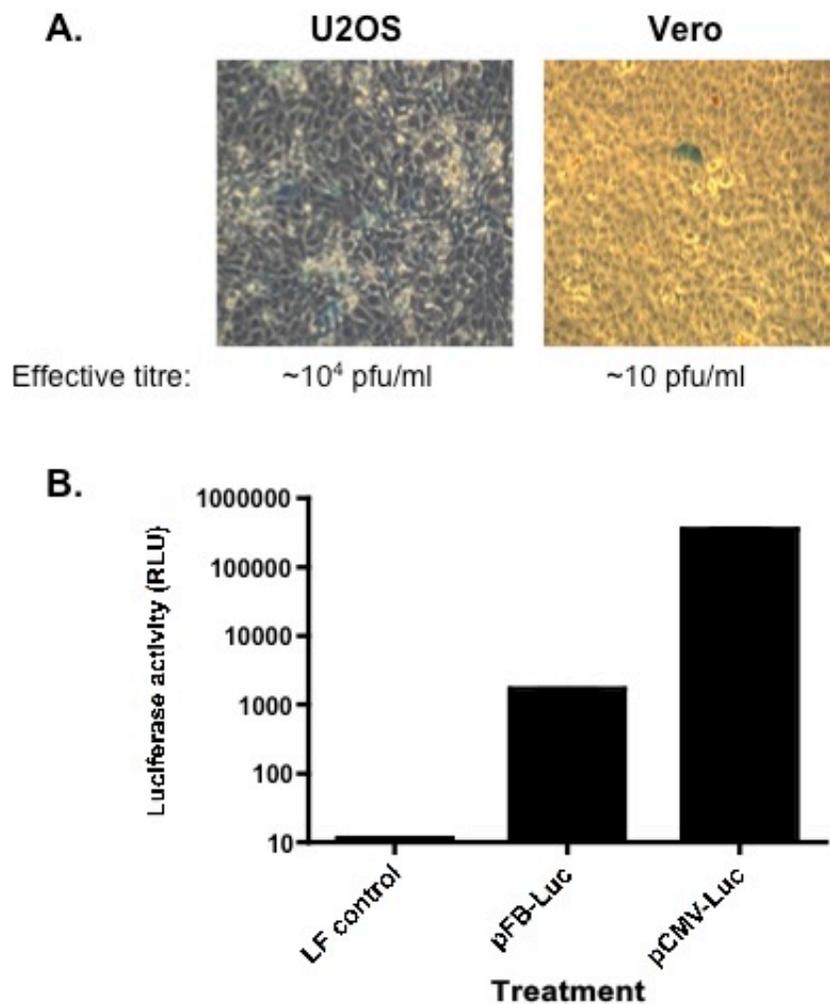


Figure 5.2. *Vero cells are poorly transduced by the pFB-based vector system.* A. A retrovirus stock of the HEL cDNA library was prepared in the pFB-neo backbone as previously described. Retroviral supernatant was used to infect U2OS or Vero cells for 48h, then stained for LacZ expression and positive cells counted by inspection under a light microscope. B. Vero cells were transfected with either pFB-Luc or pCMV-Luc, and luciferase activity assessed 24h later as a measure of promoter strength.

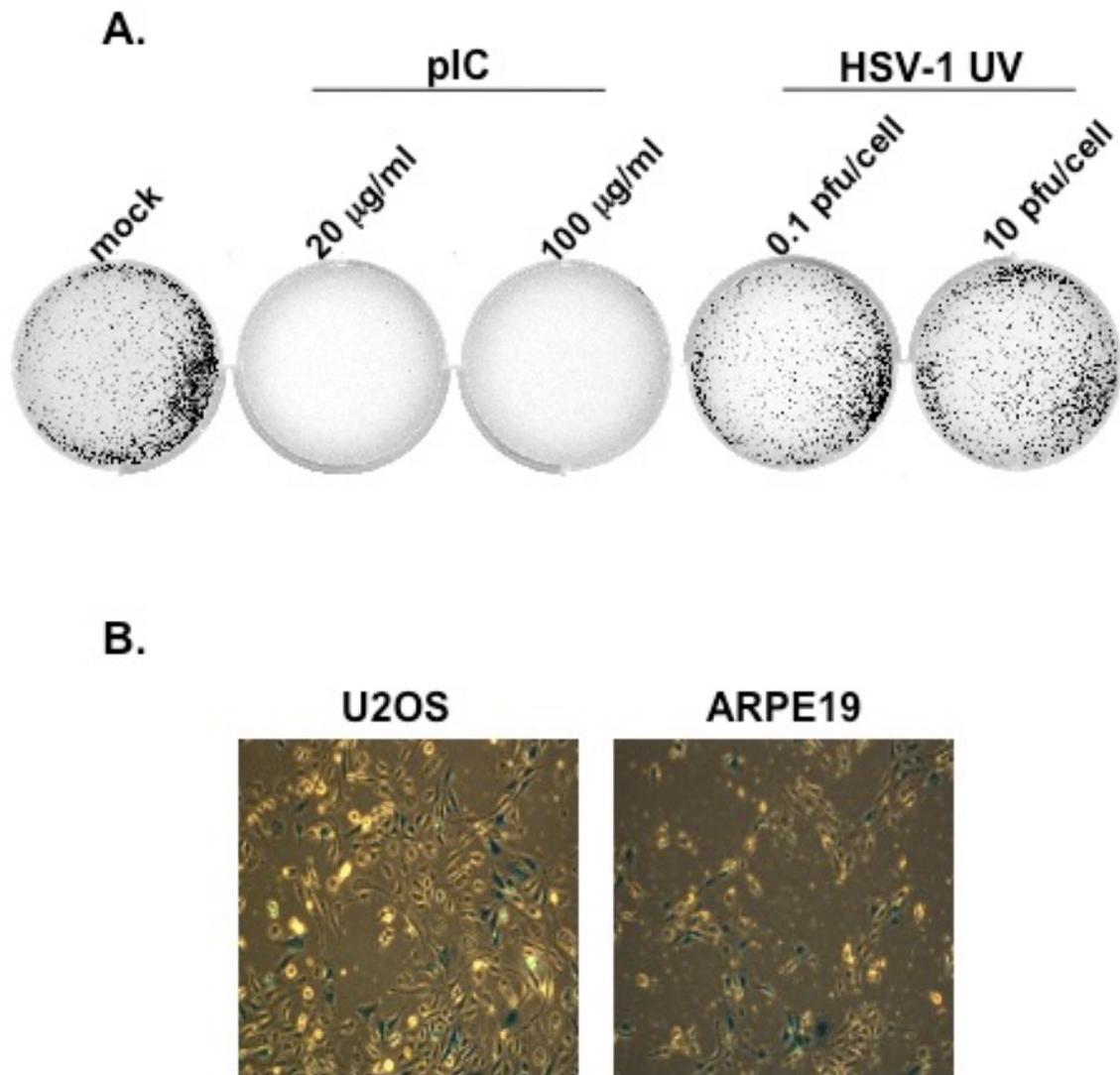


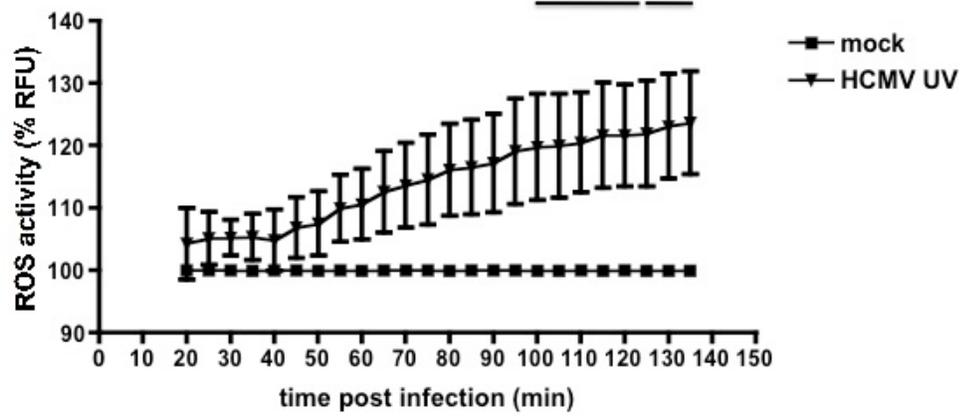
Figure 5.3. *ARPE19 cells have a similar phenotype to Vehu3 cells and are efficiently transduced by the pFB-based system.* A. ARPE19 cells were treated as indicated and challenged with VSV-GFP 24h post treatment. Monolayers were scanned 24h later to measure GFP fluorescence as a measure of viral gene expression. B. A retrovirus stock of the HEL cDNA library was prepared in the pFB-neo backbone as previously described. Retroviral supernatant was used to infect U2OS or ARPE19 cells for 48h, then stained for LacZ expression.

Table 3. Putative antiviral genes and functions in the IFN-independent antiviral signalling pathway.

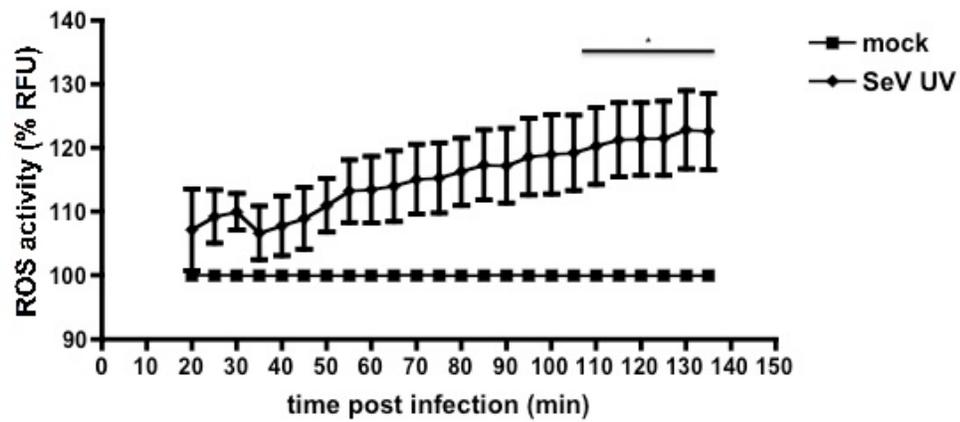
Gene	Gene Symbol	Class	Accession #	Putative or Demonstrated Function(s)	References
Ewing sarcoma breakpoint region	EWS	Transcription factor	NM_005243	Transcription factor Oncogene HCV IRES binding protein	Lu <i>et al.</i> 2004 ⁴⁸⁰ Ohno <i>et al.</i> 1994 ⁴⁸¹ Owen <i>et al.</i> 2008 ⁴⁸² Smith <i>et al.</i> 2006 ⁴⁸³
Fraser syndrome 1	FRAS1	ECM protein	NM_025074	Organogenesis Embryonic development Tumor survival factor STAT3 and NFκB target gene	Pavlikis <i>et al.</i> 2008 ⁴⁸⁴ Pitera <i>et al.</i> 2008 ⁴⁸⁵ Short <i>et al.</i> 2007 ⁴⁸⁶ (review) Yoon <i>et al.</i> 2011 ⁴⁸⁷
Glioma amplified sequence 41	GAS41	Transcription factor	NM_006530	Transcription factor Oncogene p53 regulation	Fischer <i>et al.</i> 1997 ⁴⁸⁸ Llanos <i>et al.</i> 2006 ⁴⁸⁹ Park <i>et al.</i> 2011 ⁴⁹⁰ Zimmermann <i>et al.</i> 2002 ⁴⁹¹
Ribosomal protein S6 kinase, 90kDa, polypeptide 5	RPS6KA5 (Msk1)	Signalling	NM_004755	ERK/p38 signalling pathway components Ca⁺⁺, ROS, IFN responsive Activation of NFκB, CREB, AP-1 Proinflammatory Anti-inflammatory	Aggeli <i>et al.</i> 2006⁴⁷⁰ Ananieva <i>et al.</i> 2008⁴⁹² Bertelsen <i>et al.</i> 2011⁴⁹³ Clark <i>et al.</i> 2007⁴⁷² Jamaluddin <i>et al.</i> 2009³⁹⁶ Kefaloyianni <i>et al.</i> 2006⁴⁶⁸ Li <i>et al.</i> 2004⁴⁷³ Popova <i>et al.</i> 2010⁴⁶⁹ Riis <i>et al.</i> 2011⁴⁹⁴ Vicent <i>et al.</i> 2006⁴⁹⁵
Myosin, light chain 6B, alkali, smooth muscle and non-muscle	MYL6B (MLC1SA)	Structural	NM_002475	Cytoskeleton component ATPase Rac/Pak signalling pathway components Ca ⁺⁺ responsive	Adelstein <i>et al.</i> 1981 ⁴⁹⁶ Hailstones <i>et al.</i> 1989 ⁴⁹⁷ Sanders <i>et al.</i> 1999 ⁴⁹⁸
Olfactomedin-like 2A	OLFML2A	ECM protein	NM_182487	Binds chondroitin-sulphate E and heparin	Furutani <i>et al.</i> 2005 ⁴⁹⁹
Oligophrenin 1	OPHN1	Signalling	NM_002547	RHO-GTPase activating protein F-actin binding protein Synaptic transmission and synaptic vesicle endocytosis Up-regulated in colorectal tumors	Fauchereau <i>et al.</i> 2003 ⁵⁰⁰ Kasri <i>et al.</i> 2009 ⁵⁰¹ Nakano-Kobayashi <i>et al.</i> 2009 ⁵⁰² Pinheiro <i>et al.</i> 2001 ⁵⁰³ Valnegri <i>et al.</i> 2011 ⁵⁰⁴ Xiao <i>et al.</i> 2004 ⁵⁰⁵
SH3-domain containing GRB2-like	SH3GL2 (Endophilin A1)	Signalling	NM_003026	ERK signalling pathway component Tumor suppressor EGFR and clathrin-mediated endocytosis Synaptic exocytosis	Ghosh <i>et al.</i> 2009 ⁵⁰⁶ Kaneko <i>et al.</i> 2005 ⁵⁰⁷ Mizuno <i>et al.</i> 2010 ⁵⁰⁸ Nonis <i>et al.</i> 2008 ⁵⁰⁹ Shang <i>et al.</i> 2010 ⁵¹⁰ Sinha <i>et al.</i> 2008 ⁵¹¹ Sundborger <i>et al.</i> 2011 ⁵¹²
SH3 and PX domain containing protein 2B	SH3PXD2B (Tks4)	Signalling	NM_001017995	ECM degradation Podosome/invadosome formation Actin reorganization P47 ^{phox} family member ROS production	Buschman <i>et al.</i> 2009 ⁵¹³ Gianni <i>et al.</i> 2009 ⁵¹⁴ Gianni <i>et al.</i> 2010 ⁵¹⁵ Gianni <i>et al.</i> 2010 ⁵¹⁶ Lanyi <i>et al.</i> 2011 ⁵¹⁷
SH2-domain containing adaptor protein D	SHD	Signalling	NM_020209	Abl tyrosine kinase substrate	Oda <i>et al.</i> 1997 ⁵¹⁸
Thioredoxin-like transmembrane protein 2	TMX2 (TXNDC14)	Redox Transmembrane	NM_015959	HBsAg interaction ER resident protein Member of redox-sensitive regulatory protein family	Ellgaard and Ruddock 2005⁵¹⁹ Meng <i>et al.</i> 2003⁵²⁰ Toh <i>et al.</i> 2005⁵²¹
Ubiquitin specific peptidase 31	USP31	Ubiquitin system component	NM_020718	Deubiquitinating enzyme NFκB regulation	Lockhart <i>et al.</i> 2004 ⁵²² Quesada <i>et al.</i> 2004 ⁵²³ Tzimas <i>et al.</i> 2006 ⁵²⁴
Zyg-11 homolog (<i>C. elegans</i>)-like	ZYG11B	Ubiquitin system component	NM_024646	Ubiquitin ligase complex adaptor Embryogenesis	Sato <i>et al.</i> 2009⁵²⁵ Vasudevan <i>et al.</i> 2007⁴⁷⁴

Figure 5.4. *Virus particle entry induces ROS production.* HEL cells were treated for 15 minutes with HCMV UV (A), SeV UV (B), or HSV-1 UV (C) to allow particle entry, then carboxy-H₂-DFCDA added and fluorescence measured every 5 minutes to detect ROS. Error bars represent SEM from a minimum of three independent experiments. Statistical significance by two-way ANOVA and Bonferroni's post-hoc test is represented by * (p<0.05) and ** (p<0.01), compared to control treatment group.

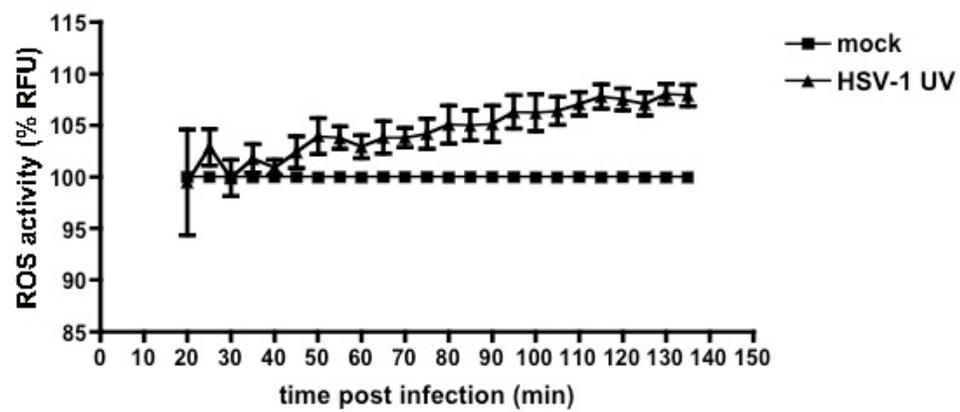
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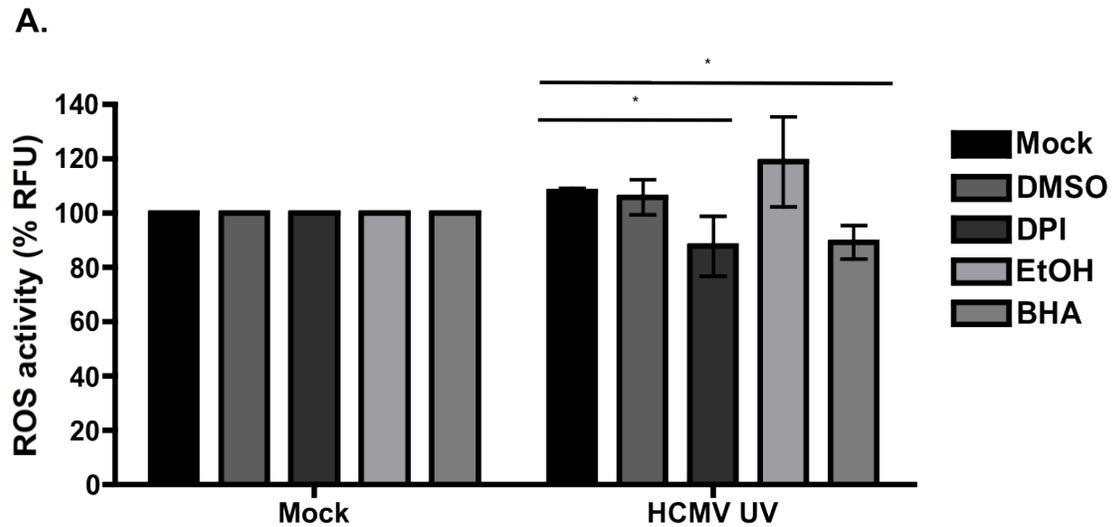
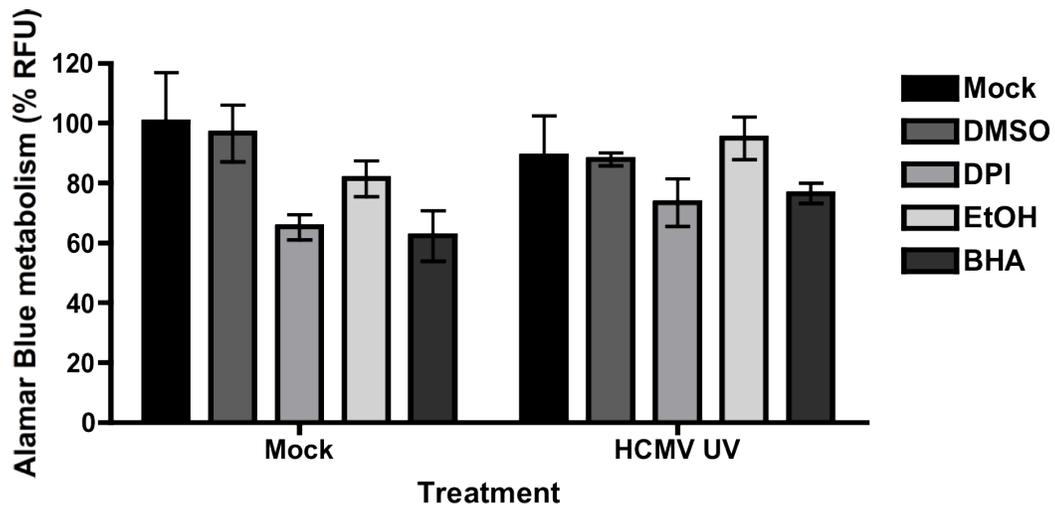
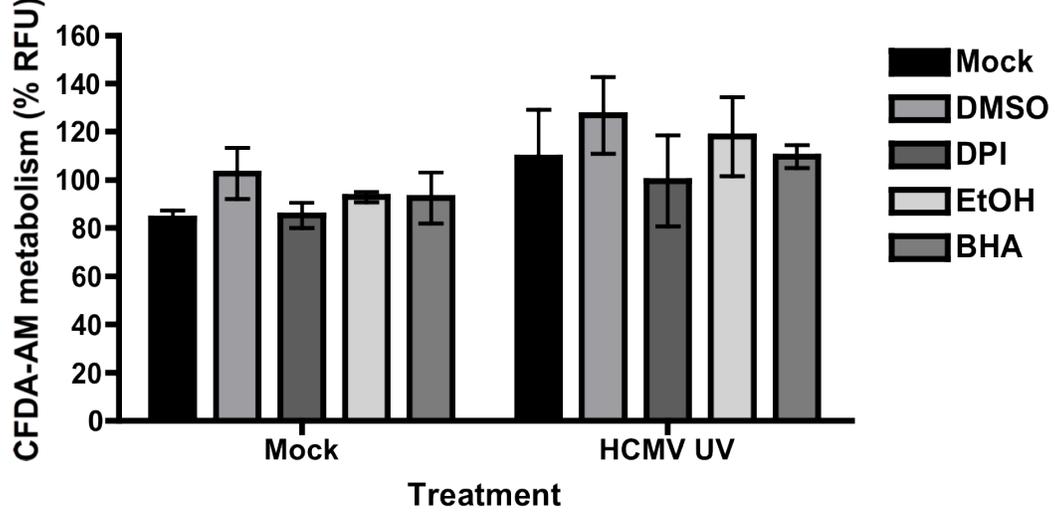


Figure 5.5. *ROS inhibitors reduce HCMV UV-induced ROS production.* (A) HEL cells were pretreated with ROS inhibitor as indicated, then infected with HCMV UV for 2h and carboxy-H₂-DCFDA added to detect ROS. Inhibitor-treated cells were also assessed for cell metabolism as an indicator of viability using AlamarBlue (B) or CFDA-AM (C). Statistical significance by two-way ANOVA and Bonferroni's post-hoc test is represented by * ($p < 0.05$) and ** ($p < 0.01$), compared to control treatment group.

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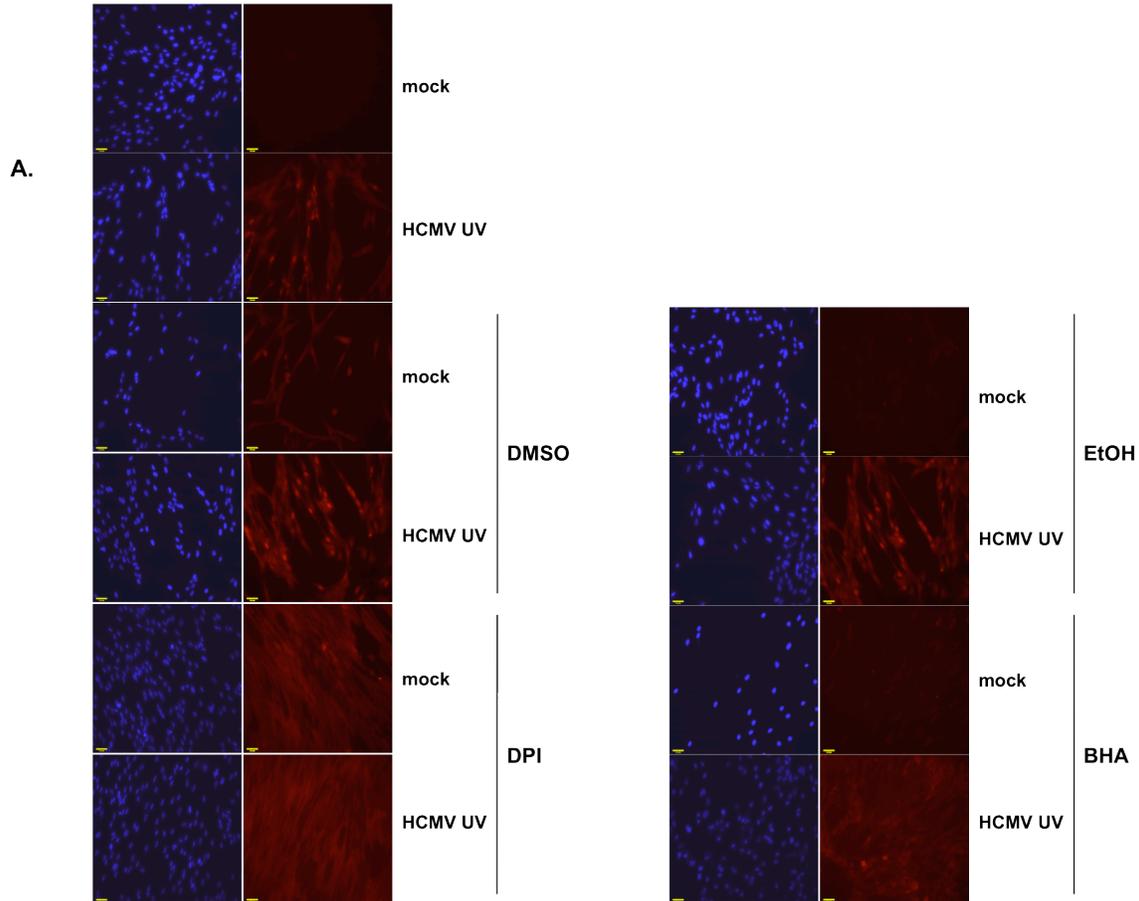


Figure 5.6. *Inhibitors of ROS reduce HCMV UV-induced activation of IRF-3.* HEL cells were pretreated for 1h with inhibitor then treated for 4h with HCMV UV and cells harvested for immunofluorescence analysis. A. Representative images of treated cells. Cells were stained for IRF-3 or Hoescht nuclear stain to monitor localization. B. Quantification of (A). IRF-3 activation is represented by number of cells showing nuclear translocation of IRF-3 in each sample as a percentage of the total number of Hoescht-positive cells in that sample. Statistical significance by two-way ANOVA and Bonferroni's post-hoc test is represented by * ($p < 0.05$), ** ($p < 0.01$), and *** ($p < 0.001$), compared to control treatment group.

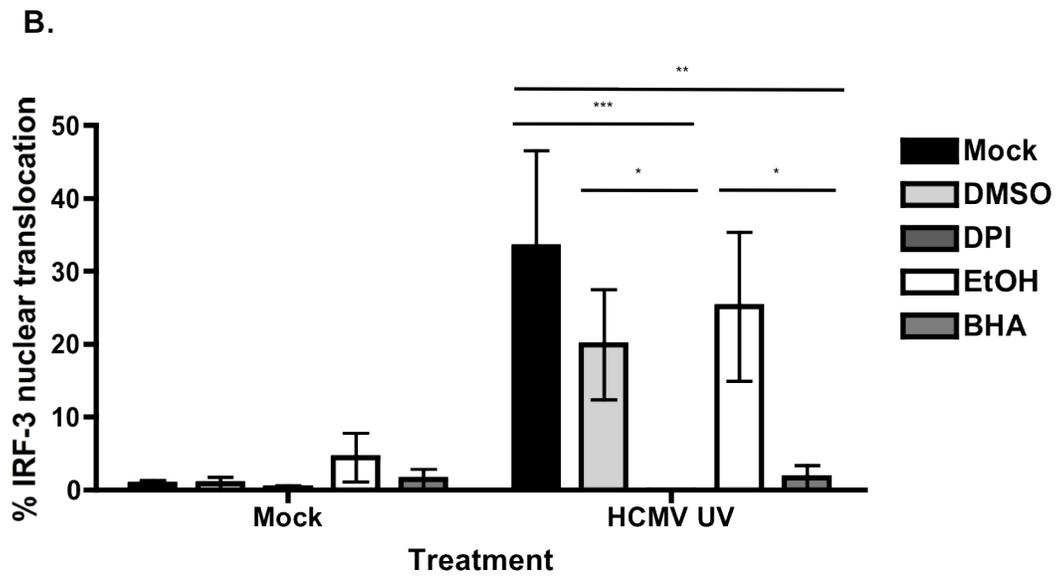


Figure 5.7. *Inhibitors of ROS reduce the antiviral response to virus particle entry.* HEL cells were pre-treated with inhibitor for 1h at the concentrations indicated, then treated with HCMV UV for 6h and RNA harvested to determine ISG 56 expression by qRT-PCR analysis. Statistical significance by two-way ANOVA and Bonferroni's post-hoc test is represented by * ($p < 0.05$), ** ($p < 0.01$), and *** ($p < 0.001$), compared to control treatment group.

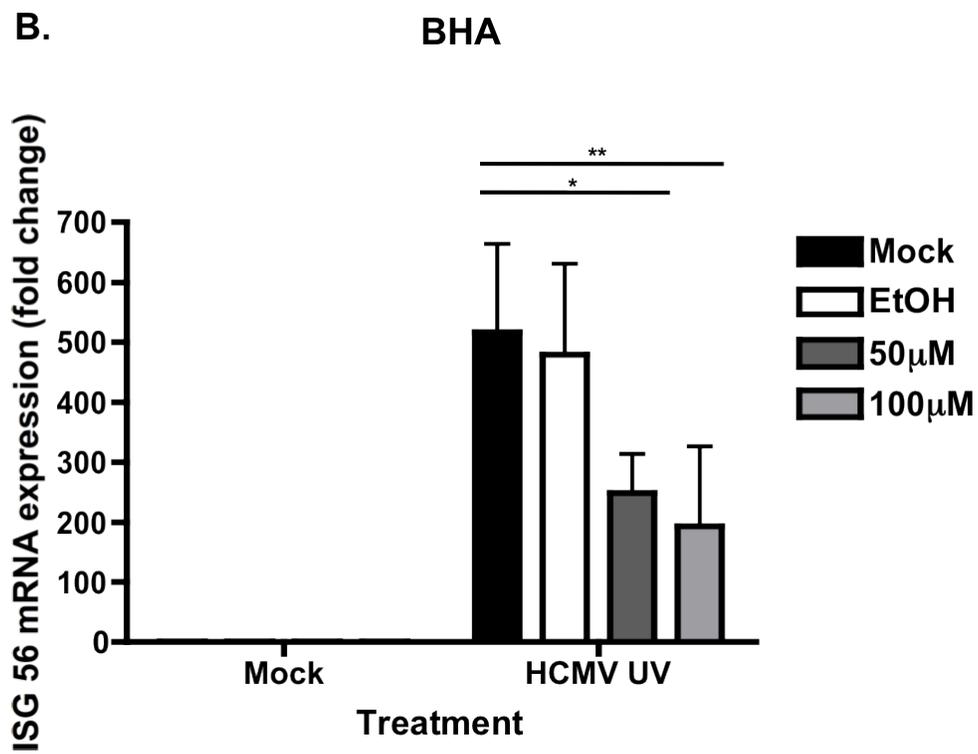
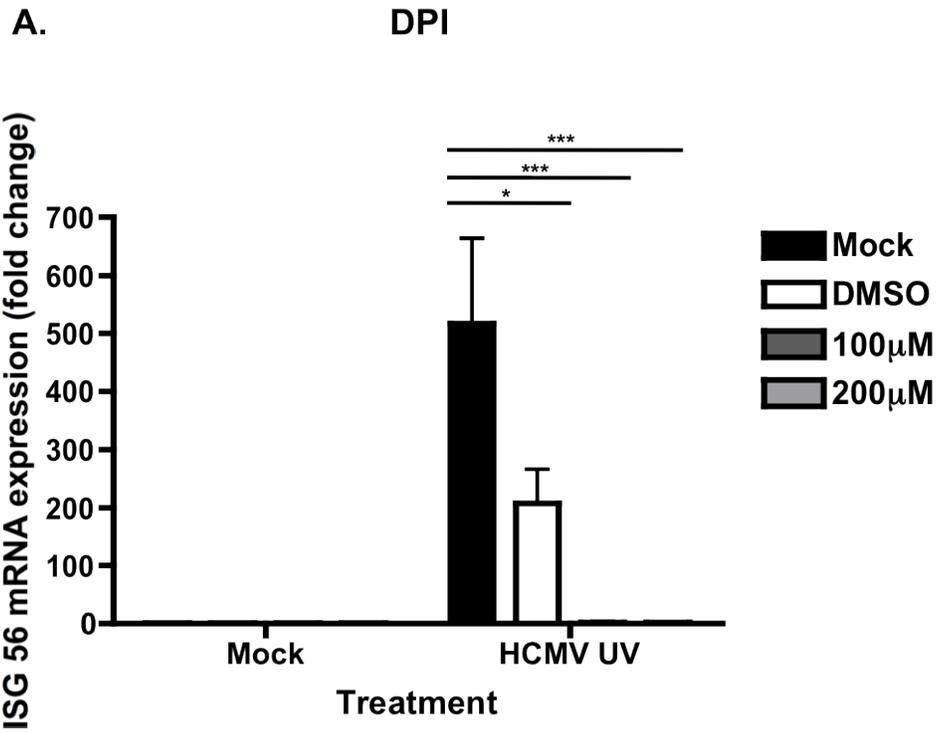
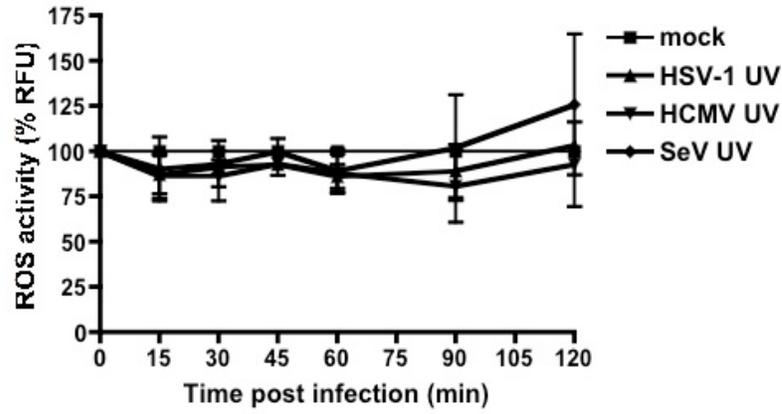
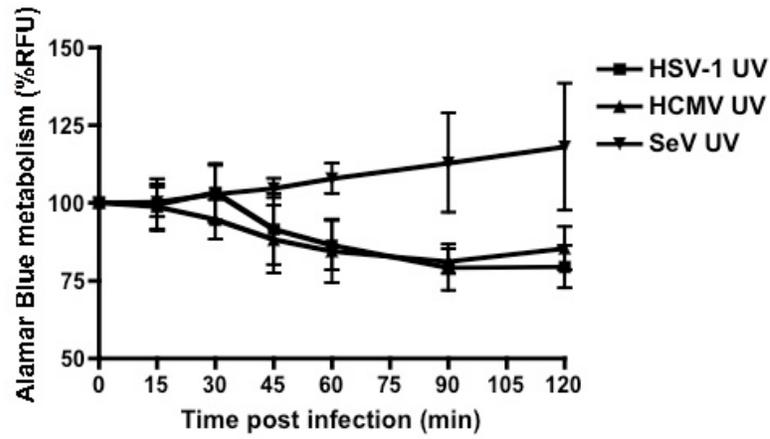


Figure 5.8. *ARPE cells are unresponsive to UV virus treatment and do not induce ROS upregulation following virus particle entry.* A, Cells were treated as indicated for 15 minutes, then carboxy-H₂-DCFDA added to measure ROS production. Cells were treated with as in (A) and Alamar blue and CFDA-AM were added to measure cell metabolism as an indicator of viability (B-C), as previously described. Error bars represent SEM from three independent experiments. No statistical significance was observed between treatment groups and untreated controls in any of the three assays by two-way ANOVA.

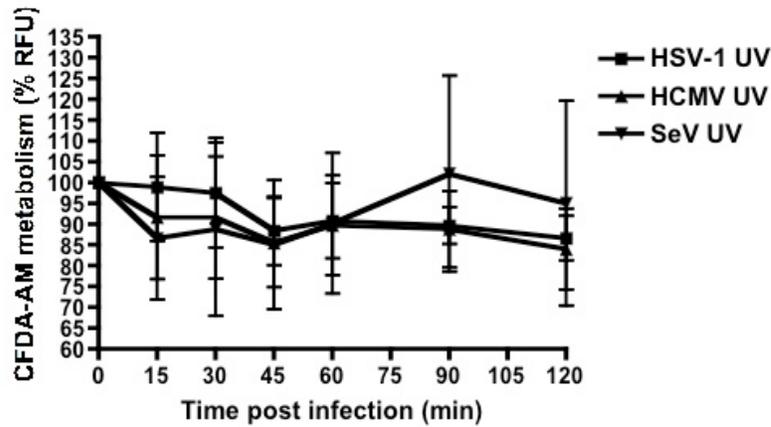
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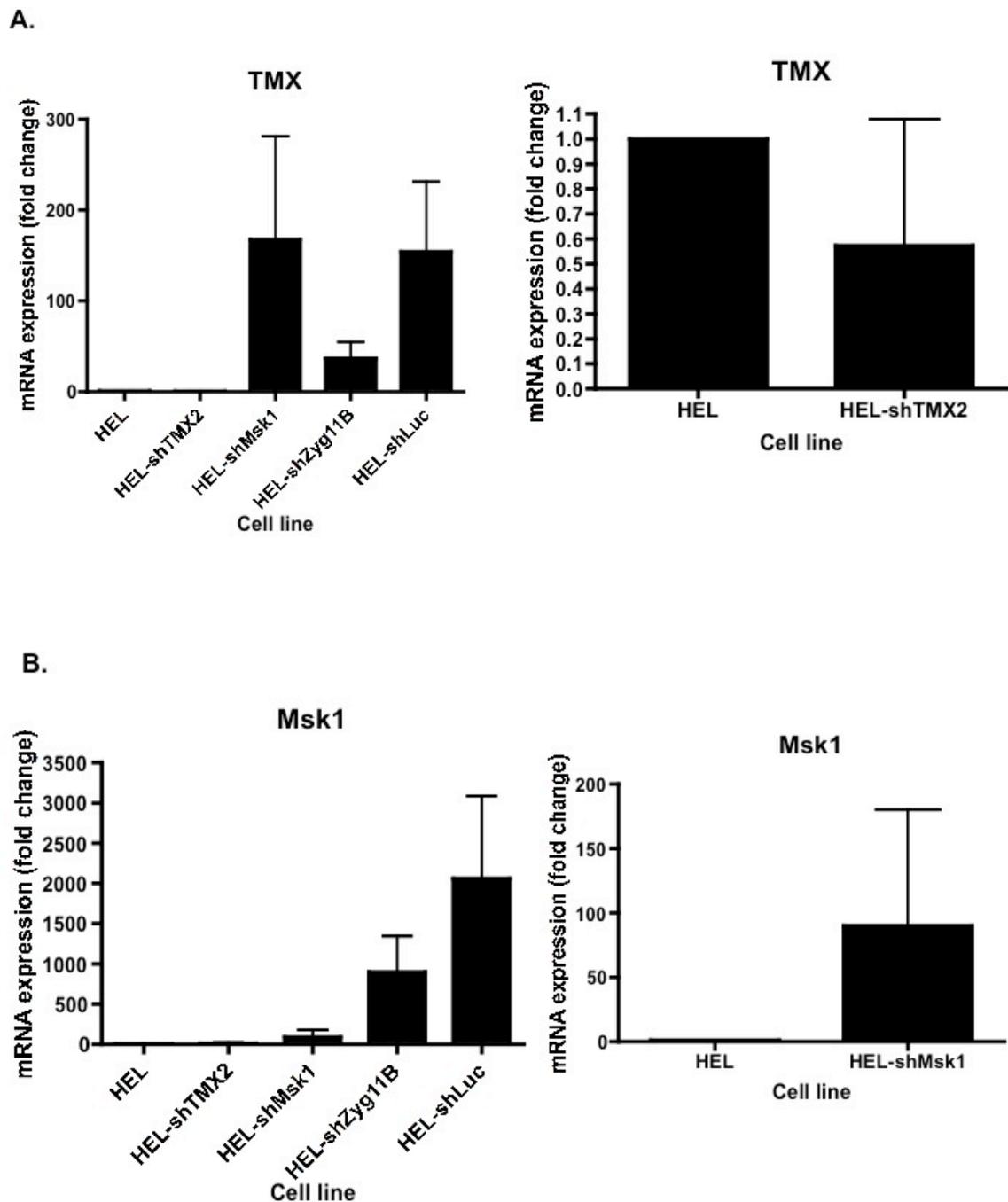
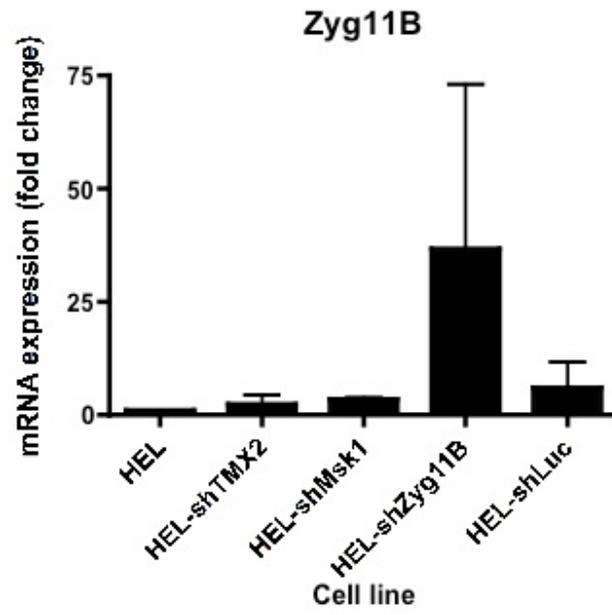


Figure 5.9. Target gene expression of HEL cells stably expressing shRNA targeting putative genes in the ROS-dependent, IFN-independent antiviral signalling pathway. RNA from shRNA-expressing HEL cells was harvested, reverse transcribed, and subjected to SYBR green qPCR analysis to measure relative expression of TMX2 (A), Msk1 (B), and Zyg-11B (C). HEL-shLuc denotes HEL cells stably expressing shRNA targeting luciferase, and is used as a control for shRNA expression and the stable selection protocol.

C.



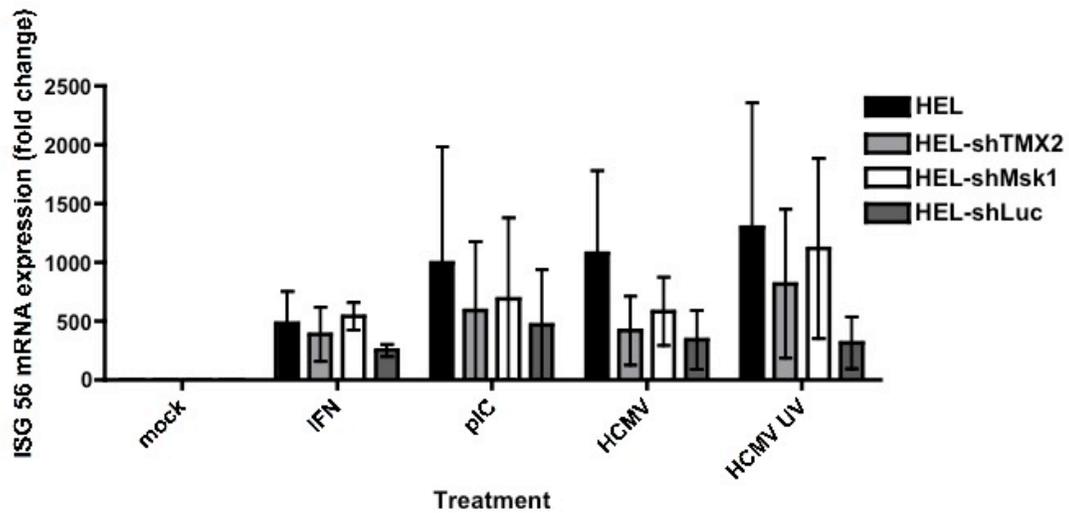


Figure 5.10. *shRNA-mediated knockdown of target ROS-related genes does not obviously affect the antiviral response in HEL cells.* HEL cells and shRNA-expressing HEL cell lines were treated as indicated, and RNA harvested 6h post treatment for qRT-PCR analysis for ISG 56 expression. Error bars represent SEM from two independent experiments. Statistical analysis was not performed on this data set.

Chapter VI. Discussion

VI.I. Summary of Work and Contributions to the Field

The innate immune response to viral infection represents an important area of infectious disease research, because it characterizes one of the first lines of antiviral defence. We are faced with constant immune stimulation in the form of exposure to defective, non-replicating, or non-pathogenic viruses, and the cells responsible for our constant defence against these stimuli face the challenge of establishing both an immediate protective response, and of minimizing the cellular damage caused by both the virus and the cellular response to the virus. Indeed, the IFN-independent response to virus particle entry represents an immediate early response against viral infection that leads to protection while minimizing the inflammatory responses associated with the IFN- and NF κ B-dependent signalling pathways.

The signalling pathways that govern the cellular response to various stages of viral infection are numerous and overlapping, and are subject to reciprocal regulation. During my PhD tenure, the scope of knowledge regarding antiviral signalling has changed dramatically, with many PRRs being identified and characterized, as well as new antiviral signalling pathways being characterized. In the context of virus particle entry, the requirement of IRF-3, but little else, was established at the start of my tenure. We now have a relatively detailed model for PAMP-mediated mechanisms of antiviral signalling, as well as some novel insight into components of the IFN-independent antiviral signalling pathway.

For my part, I have highlighted some differences between the cellular response to replicating and non-replicating virus, using Vero cells as an *in vitro* model. I have shown that while IRF-3 is an important component of the antiviral response to pIC, it is insufficient to elicit antiviral immunity in response to virus particle entry. Studies in Vero cells have further highlighted the distinct nature of stimulus-specific IRF-3 activation, as demonstrated through phenotypic differences between pIC treatment and viral infection. Furthermore, I have identified some splicing variants of IRF-3, highlighting the importance of RNA splicing in the regulation of antiviral signalling. Finally, I have implicated ROS as a DAMP responsible for the recognition of virus particle entry and the subsequent activation of IRF-3 and the expression of downstream antiviral genes.

VI.II. A Revised Model for IRF-3-Mediated Antiviral Signalling

VI.II.i. Sources of Entry-Induced ROS

I have summarized the known antiviral signalling pathways in Figure 1.1. Based on the work presented in this thesis, I have proposed a modified model that implicates ROS as an essential component feeding into both the IFN-dependent and independent antiviral signalling pathways (Figure 6.1). I believe that ROS represent an important DAMP whose production is a consequence of viral infection during

multiple stages of infection. In addition, I believe that ROS-mediated regulation of innate immune signalling occurs at multiple components of the antiviral pathway, and ultimately has both positive and negative consequences with regard to immune protection and pathology.

At the level of viral entry, ROS are produced following cytoskeletal rearrangement, which activate known members of the NADPH oxidase complex such as the Rho GTPases^{299,302,303,410}. In particular, Rac1 has been implicated in the activation of ROS following cytoskeletal rearrangement^{303,526-528}. In addition, Rac1 has been implicated in the activation of IRF-3 following SeV infection³⁰⁹. Together, these observations imply a role for ROS in the activation of IRF-3 following virus particle entry. Indeed, some preliminary experiments from our lab have identified a potential role for Rac1 in the IFN-independent antiviral response leading to ISG production.

In addition, there are many links between Ca⁺⁺ mobilization, endocytosis, and ROS production, all of which have been shown to be consequences of viral infection in various systems⁵²⁸⁻⁵³⁹. Indeed, preliminary evidence from our lab links virus particle entry with Ca⁺⁺ influx, leading to ROS production. Furthermore, several NADPH oxidase enzymes are thought to be directly regulated by calcium⁵⁴⁰. The Ca⁺⁺:calmodulin:calcineurin axis has been characterized in a number of *in vitro* models as a requisite for many types of endocytosis^{317,532,534,535,541,542}; indeed, in neural models of synaptic endocytosis, Ca⁺⁺ influx has been shown to initiate all forms of endocytosis. Whether Ca⁺⁺ is an absolute requirement for viral endocytosis has not been established; however, it is feasible that the Ca⁺⁺ is an important component involved in viral entry, as has been shown for viruses such as HSV-1 and WNV^{316,317,543}. Interestingly, the intracellular increase in Ca⁺⁺ concentration in response to viral infection is thought to follow viral entry and mediate downstream antiviral signalling pathways, despite the characterization of Ca⁺⁺ as being an important precursor to many forms of endocytosis⁵⁴³. Therefore, the exact role of Ca⁺⁺ during viral infection is not clear, and may lie both upstream and downstream of virus entry. Alternatively, viral entry may coincide with changes in intracellular Ca⁺⁺ concentrations, due to changes in ion permeability following membrane disruption.

Importantly, the contribution of Ca⁺⁺ mobilization to the regulation of ROS is complex, and has important implications in the IFN-independent antiviral signalling pathway. Indeed, the mitochondrial release of ROS following physiological stresses such as serum starvation and hypoxia has been shown to lead to the release of mitochondrial Ca⁺⁺ stores into the cytoplasm through engagement of inositol triphosphate receptor (IP3R), a Ca⁺⁺ ion channel⁵⁴⁴⁻⁵⁴⁷. Mitochondrial ROS production has been shown to activate Rac1 in addition to PI3K, phospholipase C, and members of the protein kinase C family, whose activation ultimately results in increases to intracellular Ca⁺⁺ concentration as well as activation of components of the NADPH oxidase complex. In this manner, mitochondrial ROS induces signalling pathways that result in a positive feedback loop, inducing cytoplasmic ROS production and subsequent signalling. Indeed, HSV entry has been shown to lead to increases in intracellular Ca⁺⁺ concentration due to IP3R-mediated Ca⁺⁺ release³¹⁷.

VI.II.ii. Mechanisms of ROS-Mediated Regulation of Antiviral Signalling

The mechanisms by which ROS may mediate antiviral signalling are numerous. ROS are promiscuous regulators of gene expression and function, and it is likely that ROS act at multiple levels along the pathway to positively regulate ISG production following virus particle entry.

In the IFN-dependent response, ROS have been shown to activate IFN production on multiple levels. First, ROS have been shown to stimulate the activation of ASK1 and Msk1, two members of the p38 MAPK signalling pathway^{393,394,470,548}. Msk1 is required for the activation of ATF-2 and c-Jun transcription factors following H₂O₂ stimulation⁴⁷⁰. Interestingly, ATF-2 and c-Jun are transcriptional activators of IFN, implying a role for Msk1 in the ROS-mediated production of IFN following stimulus⁴⁷¹. p38-mediated Msk1 activation has also been shown to activate CREB⁵⁴⁹, which is also implicated in IFN transcription^{348,550}. In addition, the ROS-mediated activation of ASK1 has been shown to activate IRF-3 in response to LPS stimulation⁵⁴⁸.

Additionally, ROS production has also been shown to stimulate NFκB activation. Interestingly, Rac1-mediated activation of NFκB has been shown to require ROS⁵⁵¹. In addition, ROS-mediated activation of Msk1 has been shown to lead to the activation of NFκB via the p38 and ERK signalling pathways following H₂O₂ stimulation⁴⁶⁸. Finally, IFNα treatment has also been shown to activate Msk1⁴⁷³, implying a role for Msk1 and ROS both up- and downstream of IFN production.

In the IFN-independent antiviral response, I have shown that ROS play a crucial role in the activation of IRF-3 following virus particle entry. Given that a PI3K-related family member has been previously implicated in this pathway³³⁶, it is possible that Ca⁺⁺ mobilization following virus particle entry stimulates ROS production in a pathway involving PI3K-dependent Ca⁺⁺ release into the cytoplasm and NADPH oxidase component activation, including Rac1.

I have also implicated Msk1 in the IFN-independent antiviral signalling pathway. Given the involvement of this molecule in the activation of transcription factors that comprise the IFN enhanceosome, it is possible that Msk1 plays a role in the ROS-mediated activation of transcription factors comprising the enhanceosome of IFN-independent ISGs. In particular, Msk1 is associated with the activation of CREB, and it is likely that CBP/p300 are involved in the binding of activated IRF-3 to the promoters of ISGs that are upregulated in the IFN-independent signalling pathway.

Finally, I have implicated TMX2 in the IFN-independent antiviral signalling pathway. Given the role of the TRX superfamily in the redox-sensitive regulation of gene expression and function, it is possible that TMX2 binds to and inhibits one or more components of the IFN-independent signalling pathway, and that ROS production releases TMX2 from this component to allow signalling. Indeed, ROS activation has been shown to lead to the oxidation and release of TRX from ASK, allowing activation and downstream signalling³⁹⁴. Given the role of ASK in LPS-

mediated activation of IRF-3, it is possible that TMX2 plays a role in the redox-sensitive regulation of molecules such as ASK that are involved in controlling the activation of IRF-3 or other signalling molecules leading to an antiviral state.

Taken together, I propose a model in which virus particle entry involves a change in intracellular Ca^{++} concentration via a PI3K-related family member, which leads to ROS production through the NADPH oxidase complex (Figure 6.1). ROS production leads to the redox-mediated regulation of proteins associated with the activation of IRF-3. This may occur via a direct mechanism through oxidation of a redox-sensitive inhibitory protein bound to IRF-3, or via an indirect mechanism through the regulation of upstream cellular proteins. In addition, ROS may directly regulate the activity of additional transcription factors comprising the enhanceosome of ISGs in this system. The exact mechanism of ROS-mediated regulation of this pathway is not known; however, it is likely that there are multiple stages of regulation and that redox-sensitive adaptor molecules control the activation of one or more molecules upstream of IRF-3. The ROS-mediated activation of IRF-3 then leads to the direct transcription of IFN and ISGs, and to an antiviral state. Indeed, ROS have been implicated in the activation of IFN-independent antiviral signalling at a point upstream of the nuclear translocation of IRF-3. Additionally, ROS production may lead to the activation of additional transcription factors comprising the enhanceosome of both IFN genes and ISGs in the IFN-dependent and -independent pathways.

VI.III. Other Potential Mechanisms of IFN-Independent Antiviral Signalling

Results from the reverse genomic approach to identifying components of the IFN-independent antiviral signalling pathway revealed putative genes with a variety of potential antiviral roles in response to virus particle entry (Table 3). Experimental validation of these putative genes is required; however, various roles in cytoskeletal rearrangement, signalling, RNA binding, and extra-cellular matrix stability provide a potential role for these genes at a variety of stages in the antiviral signalling cascade and warrant further investigation.

As previously discussed, ROS have been shown to play a role in the response to virus particle entry, presumably through cytoskeletal rearrangements leading to mitochondrial ROS production, Ca^{++} release to the cytoplasm, NADPH oxidase activation, and activation of redox-sensitive regulatory proteins. Similarly, oligophrenin 1 (OPHN1) and Src homology 3 (SH3) domain growth factor receptor bound 2-like 2 (SH3GL2, also known as Endophilin A1) have been shown to mediate synaptic vesicle endocytosis and are activated by Rho GTPase, a family to which Rac1 belongs^{502,505,507-509,552}. OPHN1 has also been shown to interact with F-actin⁵⁰⁰. Taken together, this implies a role for these proteins in the Rac1-mediated generation of ROS following viral endocytosis.

Similarly, the myosin light chain (MLC) family member MYL6B provides cytoskeletal integrity and is involved in cell motility and muscle contraction. Interestingly, this pathway is activated by increases in cytosolic Ca^{++} concentration

and has been shown to be regulated by p21 protein (cell division control protein 42/Rac)-activated kinase 1 (PAK1), a downstream target of Rac1⁴⁹⁸.

Additionally, SH3 and PX domain-containing protein 2B (SH3PXD2B, also known as Tks4) has been shown to mediate cytoskeleton reorganization and is a member of the p47phox family, a component of the NADPH oxidase complex leading to ROS production⁵¹⁵⁻⁵¹⁷. SH3PXD2B is thought to be required for Rac1-mediated ROS production, leading to extracellular matrix (ECM) degradation and cell motility^{513,514}. Indeed, the ECM plays a role in the inflammatory response to viral infection, with both proviral and antiviral consequences⁵⁵³⁻⁵⁵⁸. Current models suggests that ECM degradation produces bioactive fragments with various chemotactic properties, leading to inflammation^{559,560}. Similarly, Fraser syndrome 1 (FRAS1) and olfactomedin-like 2A (OLFML2A) are ECM proteins^{486,499}, implying a role for ECM integrity in antiviral immunity with respect to virus particle entry. Alternatively, it is possible that the ECM provides a barrier to viral challenge in our system which results in protection against VSV(Δ G)-GFP challenge, as opposed to having a direct role in the signalling pathway induced by virus particle entry.

Finally, some of the putative gene products identified in the reverse genomic screen have roles in signalling that may be related to antiviral immunity. Ewing sarcoma breakpoint region 1 (EWS) is a transcription factor that has been shown to bind the HCV internal ribosomal entry site^{480,482,483}. EWS has also been characterized as an RNA binding protein^{480,481}, making it a putative PRR. While the response to virus particle entry appears to be nucleic acid independent, the recognition of viral genomic RNA by PRRs in this system has not been ruled out. However, having used a DNA virus particle to induce an IFN-independent antiviral state, the role of EWS as a PRR is unlikely. Despite this, EWS may still play a role as a transcription factor in the expression of antiviral genes following virus particle entry. Similarly, glioma amplified sequence 41 (GAS41) is a transcription factor and has an inhibitory role in apoptosis via p53 regulation⁴⁸⁹⁻⁴⁹¹. While there is no direct link between GAS41 and antiviral signalling, the contribution of IRF-3 to both the antiviral signalling cascade as well as the apoptotic pathway may justify the validation of GAS41 as a putative transcription factor for antiviral genes. Likewise, SH2 domain containing transforming protein D (SHD) is a substrate for the tyrosine kinase c-Abl, and has been shown to mediate phosphotyrosine binding via its SH2 domains⁵¹⁸, implying a signalling role for this gene product. Lastly, ubiquitin specific peptidase 31 (USP31) is a deubiquitinating enzyme and has been shown to negatively regulate NF κ B signalling^{522,524}. While NF κ B is not involved in the IFN-independent signalling pathway following virus particle entry³³⁷, many components of this pathway are subject to regulation via ubiquitination and ubiquitin-like modification, as previously discussed. Therefore, the role of USP31 in the ubiquitin-mediated regulation of signalling components in this pathway is of interest.

VI.IV. Concluding Thoughts

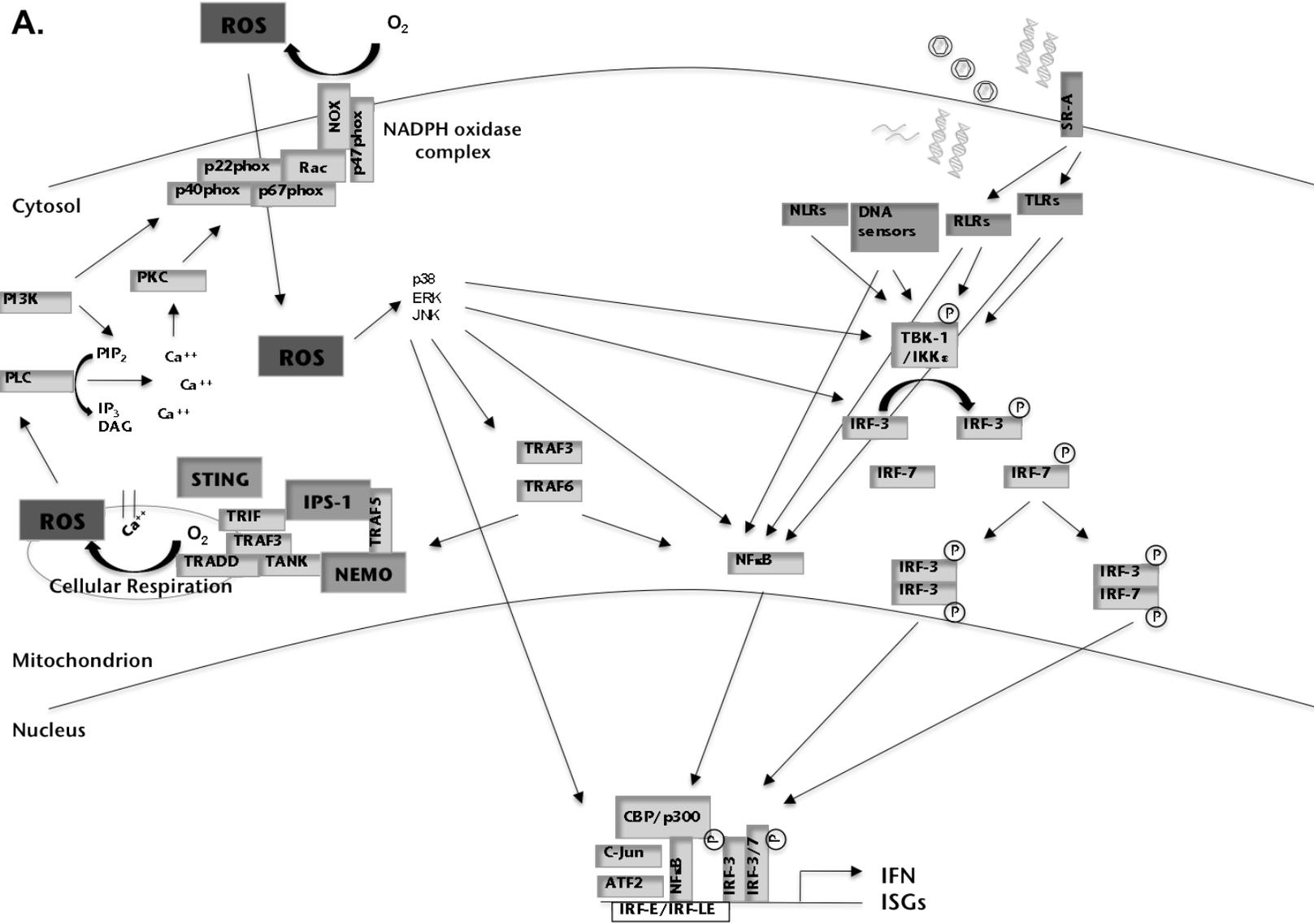
Given the burst of knowledge in the field of antiviral signalling in recent years, we are left with a greater wealth of knowledge in the field, accompanied by a growing list of unresolved questions. From my work, future directions in research should explore the specific mechanism of ROS-mediated activation of IRF-3, identifying the target of ROS-mediated oxidation and regulation of activity. I have implicated TMX2 in this process; however, whether TMX2 directly binds to and regulates IRF-3, TBK-1, or another upstream protein has not been confirmed. In addition, is it possible that other redox-sensitive binding partners of IRF-3 or upstream proteins exist and are targets of ROS induced by viral entry. If ROS-sensitive regulators of the IFN-independent signalling pathway are indeed found to exist, it begs the question of whether ROS are required for the global activation IRF-3 in response to all stimuli. ROS have indeed been implicated in the activation of components of the IFN pathway in response to various viruses^{395,396,399,409,561}, but whether ROS are absolutely required for IRF-3 activation has not been established in the literature.

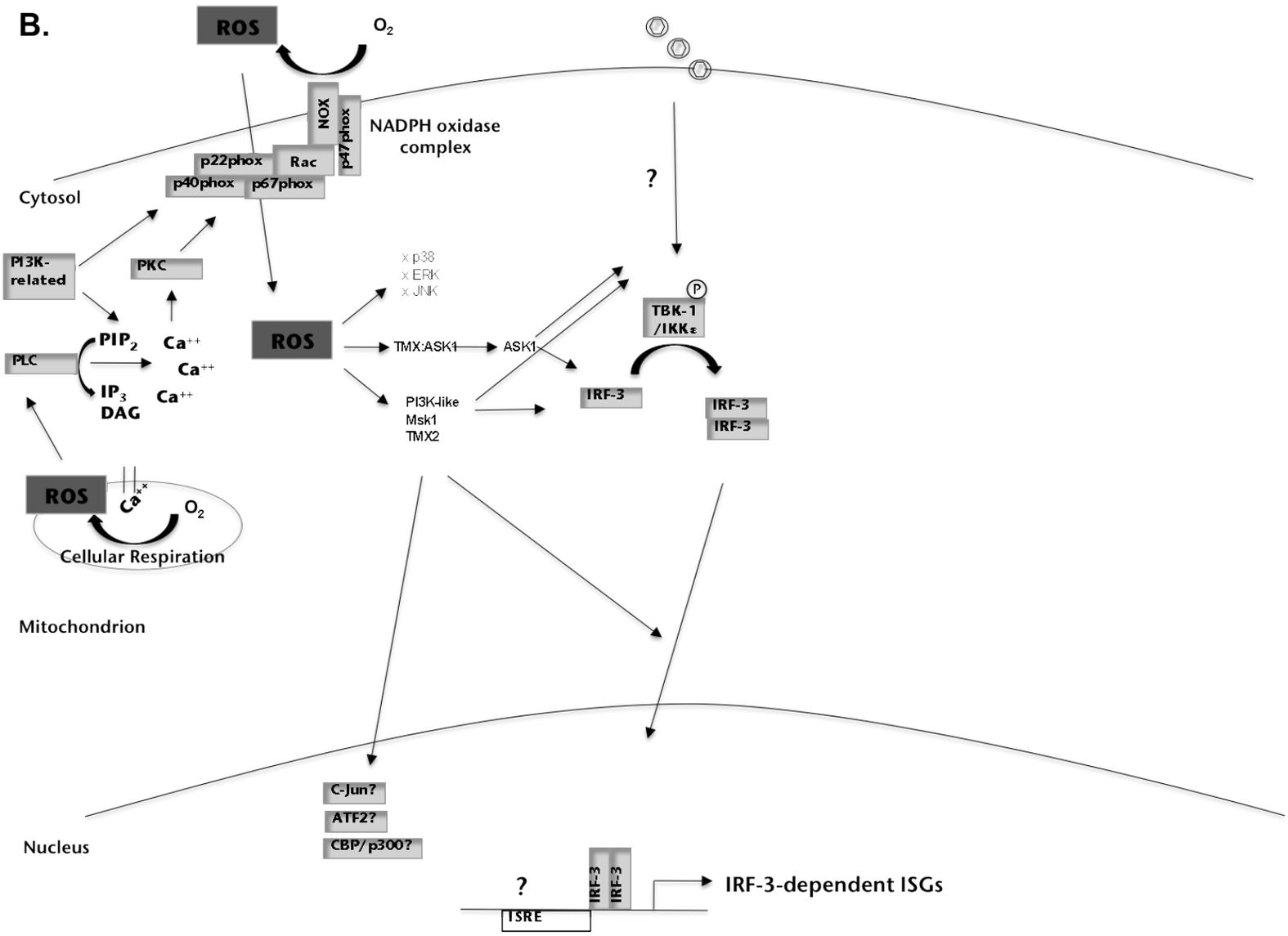
In addition, the reciprocal regulation of ROS and RNS is an important one, given that nitric oxide is an important component in antiviral immunity and innate pathogen sensing, particularly in macrophages. The crosstalk between ROS and RNS is extremely complex, as ROS and NADPH oxidase are subject to RNS-mediated modification such as S-nitrosylation⁴⁰⁸. In addition, RNS-mediated modifications to molecules such as cGMP are subject to regulation by ROS^{562,563}. In fact, peroxynitrite (ONOO⁻), a member of the reactive nitrogen species family, is produced by the reaction of superoxide ion (O₂⁻) with nitric oxide (NO), highlighting the intimate interactions between these groups of reactive molecules. In most cell types, NO production is thought to be dependent on *de novo* protein synthesis, as most cell types produce iNOS, an inducible enzyme. From work in our lab, we know that the cellular response to virus particle entry does not require *de novo* protein synthesis, since cells pretreated with the translational inhibitor cycloheximide do transcribe ISGs in response to UV virus. Therefore, there is not likely to be a contribution of NO to the IFN-independent signalling pathway despite its intimate role in the regulation of ROS, which are an essential component to the pathway. In response to replicating viruses, however, the system is much more complicated as many more PRRs and DAMPs are engaged following infection. It is therefore conceivable that RNS play a redundant or compensatory role in the response to viral infection, minimizing the importance of ROS in the activation of IRF-3 following infection.

Finally, the role of ROS in viral pathogenesis is still unclear, and may have implications in viral gene therapy and vaccine development, as well as in oncolytic virotherapy. While many cases of ROS-induced IFN signalling following viral infection have been reported^{395,399,409}, others have reported that infection-induced ROS production leads to the promotion of viral spread and/or clinical complications associated with infection^{537,564-568}. The precise role that ROS play in the pathogenesis of a viral infection has important clinical implications for antiviral drug design. Indeed, the use of antioxidant molecules in the treatment of influenza infection has proven successful in animal models of infection as well as in clinical

trial^{400,569}. Furthermore, understanding mechanisms of protection or viral spread of a viral gene therapy vector or vaccine has important implications for clinical efficacy, affecting the breadth and sustained expression of a transgene or antigen. Finally, the use of ROS-modifying compounds in the context of oncolytic therapy would have important effects on the ability of an oncolytic virus to disseminate into target tissues and/or affect apoptotic pathways. Indeed, preliminary experiments from our lab implicate TMX2 as a resistance factor in the response to oncolytic HSV-1 *in vitro*, implying a role for oxidative stress in the antiviral response in healthy cells, as well as a dysregulation of oxidative stress responses in cancer cells that are sensitive to viral oncolysis. Taken together, it is clear that the contribution of ROS to antiviral immunity represents a novel and important area of research with important therapeutic implications.

Figure 6.1. *A Revised Model for IRF-3-Mediated Antiviral Signalling.* A, ROS generated at both the mitochondrial membrane and at the cell surface play roles in the activation of signalling molecules leading to IFN and ISG production. Mitochondrial ROS production is associated with the activation of enzymes such as PI3K and PLC, whose enzymatic activity ultimately leads to increases in intracellular Ca^{++} , activation of PKC, and the activation of membrane-bound NADPH oxidase enzymes leading to cytoplasmic ROS production. Extracellular ROS production by plasma membrane-bound NADPH oxidase is thought to passively diffuse into the cytoplasm and initiate downstream signalling pathways. PI3K has also been shown to lead to the activation of Rac1 in the NADPH oxidase complex. ROS production results in the activation of a number of the p38, ERK, and JNK signalling pathways, which ultimately lead to the activation of components of the IFN-dependent antiviral signalling pathways such as the activation of IRF-3 via ASK1, activation of TRAF3/6 and NF κ B, and upregulation of TBK-1/IKK ϵ . B, ROS generated following virus particle entry acts to positively regulate IRF-3-mediated ISG transcription by a number of potential pathways. ROS may play a role in the activation of IRF-3 through the redox-sensitive regulation of signalling components responsible for IRF-3 activation, as has been shown with ASK1 in the ROS-mediated activation of IRF-3 following LPS stimulation. It is also possible that redox regulatory components such as those identified using reverse genomics in ARPE19 cells contributes to the activation of IRF-3 or upstream signalling molecules. Finally, it is possible that redox regulatory components contribute to the activation of additional transcription factors that comprise the enhancersome of IFN-independent ISGs.





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