INNATE RESPONSE TO ENVELOPED VIRUS ENTRY

REGULATION OF INTERFERON STIMULATED GENES FOLLOWING ENVELOPED VIRUS ENTRY AND DELIVERY OF VIRAL NUCLEIC ACID

By

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Lay Abstract

Cells rely on pattern recognition receptors for innate antiviral defence. While the study of pattern recognition has focused on virus-associated nucleic acid motifs, disruptions of the cellular environment during virus infection could similarly warn the cell. Membrane perturbation during enveloped virus entry is associated with upregulation of antiviral interferon-stimulated genes. This thesis examines the mechanism of membrane perturbation sensing and different antiviral signalling pathways activated by non-replicating enveloped virus particles.

We found evidence that membrane perturbation triggers cytosolic Ca^{2+} signalling which may act as a co-stimulatory signal for recognition of incoming viral nucleic acid. We initially thought enveloped virus particles were recognized through a common pathway, but have since learned that recognition is more complex. Further work is necessary to understand how membrane perturbation and nucleic acid sensing interface during enveloped virus infection and what role this plays in antiviral defence.

Abstract

Innate antiviral defence depends on virus recognition and cytokines like interferon (IFN) that upregulate many interferon-stimulated genes (ISGs). Virus recognition normally relies on pattern recognition receptors binding to virus-associated nucleic acid motifs, but virus activity may provide an additional means for the cell to recognize infection. Enveloped viruses fuse with a cell membrane during entry and membrane fusion by virus-like particles or the purified protein p14 are sufficient to upregulate ISGs in the absence of viral nucleic acid. This thesis examines the mechanism by which cells recognize membrane fusion and how this affects downstream signalling and upregulation of ISGs.

We found that membrane perturbation by enveloped virus particles or p14 triggered cytosolic Ca^{2+} oscillations important for antiviral defence. Surprisingly, Ca^{2+} signalling seemed to act upstream of nucleic acid sensing pathways during enveloped virus infection. In the absence of viral nucleic acid, p14 triggered a Ca^{2+}-dependent antiviral response to dsRNA. It is still unclear how p14 might trigger recognition of endogenous dsRNA.

We found that enveloped virus particles trigger IRF3-mediated upregulation of interferon as well as direct IFN-independent upregulation of ISGs. Furthermore, while some viruses like HCMV trigger widespread IRF3 activation, other viruses like SeV upregulate IRF3 and IFN in a minority of

infected cells. This disparate response to infection can lead to different biological outcomes when measured at the population level.

Our work highlights the complexity of the response to enveloped virus particles, despite the absence of replication. Further work is necessary to understand how membrane perturbation is recognized and how this interfaces with nucleic acid sensing. While nucleic acid sensing is sufficient to upregulate antiviral ISGs, other signals like membrane perturbation may provide important contextual cues during infection. This will be important to understand moving forward as virus-like particles are used more and more for research and clinical applications.

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List of Abbreviations and Symbols

2-APB: 2- aminoethyl diphenylborinate
4-IPBA: 4-iodophenylboronic acid
αMEM: alpha modified Eagle's medium
ADAR: adenosine deaminase acting on RNA
AdV: adenovirus
ANOVA: analysis of variance
AP1: activator protein 1
ATF2: cAMP transcription factor 2
ATP: adenosine triphosphate
BAPTA-AM: 1,2-bis(2-aminophenoxy)ethane-N,N,N',N'-teteraacetic acetic acid tetrakis(acetoxymethyl ester)
cAMP: cyclic adenosine monophosphate
CARD: caspase activation and recruitment domain
CBP: CREB-binding protein
cDNA: complimentary DNA
cGAMP: 2'3'-cyclic GMP-AMP
cGAS: cyclic GMP-AMP synthase
CHX: cycloheximide
CREB: cAMP response element-binding protein
CRISPR: clustered regularly interspersed palindromic repeats
CVB: coxsackie virus B
CXCL10: C-X-C motif chemokine 10
Cas9: CRISPR-associated protein 9
DAI: DNA-dependent activator of IFN-regulatory factors
DDX41: DEAD box helicase 41
DMEM: Dulbecco's modified Eagle's medium
DMSO: dimethyl sulphoxide
DNA: deoxyribonucleic acid
DOPE: 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine
DOTAP: 1,2-dioleoyl-3-trimethylammoniumpropane
dsDNA: double-stranded DNA
dsRNA: double-stranded RNA
DVG: defective virus genome
EGTA: ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid
ELISA: enzyme-linked immunosorbent assay
ER: endoplasmic reticulum
FAK: focal adhesion kinase
FAST: fusion-associated small transmembrane protein
FBS: fetal bovine serum

fRIP-Seq: formaldehyde RNA co-immunoprecipitation sequencing
GAPDH: glyceraldehyde 3-phosphate dehydrogenase
GFP: green fluorescent protein
gRNA: guide RNA
gSeV: genomic SeV
GTP: guanosine triphosphate
HBSS: Hank's balanced salt solution
HBV: hepatitis B virus
HCMV: human cytomegalovirus
HCV: hepatitis C virus
HEK: human embryonic kidney
HEL: human embryonic lung
HEPES: 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HIV: human immunodeficiency virus
HSV: herpes simplex virus
hTERT: human telomerase reverse transcriptase
HTLV: human T cell leukemia virus
IE1: immediate early 1
IFI16: IFN-inducible protein 16
IFIT1: IFN-inducible transmembrane protein 1 (also known as ISG56 or p56)
IFN: interferon
IFNAR: interferon α/β receptor
IFNGR: IFN-γ receptor
IFNLR: IFN-λ receptor
IKK: IκB kinase
IRE: IRF-response element
IRF: interferon regulatory factor
ISG: interferon-stimulated gene
ISGF3: IFN-stimulated gene factor 3
ISRE: IFN-stimulated response element
JAK: janus kinase (just another kinase)
JEV: Japanese encephalitis virus
JNK: c-Jun N-terminal kinase
KO: knock-out
L-particles: light particles
LGP2: laboratory of genetics and physiology 2
LPS: lipopolysaccharide
MAM: mitochondria-associated ER membrane
MAPK: mitogen-activated protein kinase
MAVS: mitochondrial antiviral signalling
MDA5: melanoma-differentiation-associated protein 5

MEF: murine embryonic fibroblast
MeOH: methanol
miRNA: microRNA
MyD88: myeloid differentiation primary response 88
NADPH: nicotinamide adenine dinucleotide phosphate
NF-κB: nuclear factor κ-light-chain-enhancer of activated B cells
OAS1: 2'-5'-oligoadenylate synthetase 1
PAK1: p21 activated kinase
PAMP: pathogen-associated molecular pattern
PBS: phosphate buffered saline
PCR: polymerase chain reaction
pDC: plasmacytoid dendritic cell
PFU: plaque forming units
PI3K: phosphatidylinositol-3-OH kinase
PLC- γ: Phospholipase C-γ
PKR: protein kinase R
PML: promyelocytic leukemia protein
PRD: positive regulatory domain
PREP: pre-viral replication enveloped particles
PRR: pattern-recognition receptor
RIG-I: retinoic acid inducible gene I
RIPA: radioimmunoprecipitation assay buffer
RLR: RIG-I like receptor
RNA: ribonucleic acid
ROS: reactive oxygen species
rRNA: ribosomal RNA
RT: reverse transcriptase
RSV: respiratory syncytial virus
SDS: sodium dodecyl sulphate
SDS-PAGE: SDS poly-acrylamide gel electrophoresis
SeV: Sendai virus
siRNA: small interfering RNA
SKIV2L: SKI2 homolog, superkiller viralicidic activity 2-like
ssRNA: single-stranded RNA
STAT: signal transducer and activator of transcription
STING: stimulator of interferon genes
TANK: TRAF family member-associated NF-κB activator
TBK1: TANK-binding kinase
TBS: Tris-buffered saline
THF: telomerised human fibroblasts
TLR: toll-like receptor

TPCK: L-1-tosylamido-2-phenylethyl chloromethyl ketone
TREX1: three-prime repair exonuclease 1
TRIF: Toll/IL-1 receptor domain-containing adaptor inducing IFN-β
tRNA: transfer RNA
TRPS: tunable resistive pulse sensing
TYK2: tyrosine kinase 2
Ub: ubiquitin
UPR: unfolded protein response
UV: ultraviolet
VLP: virus-like particle
VRE: carriable response elements
VSV: vesicular stomatitis virus
WNV: West Nile virus
XBP1: X-box binding protein 1

Declaration of Academic Achievement

The manuscript contained in chapter 2 titled “Membrane perturbation-associated Ca^{2+} signalling and incoming genome sensing are required for the host response to low-level enveloped virus particle entry” was initially submitted October 14, 2015 and published February 24, 2016. The work builds on initial observations made by Susan Collins in 2011 that intracellular Ca^{2+} signalling is important for the antiviral response to enveloped virus particles. Susan carried out experiments in figure 4 and part of figure 7 and assisted with design and optimization of experiments throughout the paper. Time-lapse Ca^{2+} microscopy experiments were a collaborative effort between Subhendu Mukherjee in Luke Janssen’s lab, Susan and myself. Yueh-Ming Loo in Michael Gale’s lab created $MAVS^{-/-}TLR3^{-/-}TRIF^{-/-}$ MEFs that were used in figure 5. The remaining experiments were done by myself. The paper was written with assistance from Susan Collins and Karen Mossman.

The manuscript contained in chapter 3 titled “Virus-intrinsic differences and heterogeneous IRF3 activation influence IFN-independent antiviral protection” was submitted Feb 27, 2020 to Cell Reports and is currently under revision at iScience. Necessary revisions involve repeating key experiments in primary cells and different cell types. The necessary revisions have been delayed due to COVID-19 related restrictions, of which the editor is aware of and accommodating, but will be completed shortly. The idea for this work came from

my experiments in IFNAR KO fibroblasts in 2016 and discussions with Karen Mossman. Transcriptome sequencing was done by the Farncombe metagenomics facility and analyzed by Anna Dvorkin-Gheva (figure 2). Flow cytometry and sorting were done by Minomi Subapanditha (figure 5 and 6). Plaque reduction assays and RT-PCR involving RelA$^{-/-}$ THFs (figure 4) were done by Kaushal Baid who is assisting with a revision experiment. The remaining experiments were done by myself. The paper was written with assistance from Karen Mossman.

The manuscript contained in chapter 4 titled "Membrane perturbation by a viral fusogen triggers a RLR-dependent antiviral response" is awaiting a few last experiments before publishing. In particular, we aim to co-purify FLAG-RIG-I and associated RNA following p14 treatment and use high-throughput sequencing to identify the source of stimulatory RNA. The idea for this paper came from experiments I carried out with p14 in MAVS$^{-/-}$ and STING$^{-/-}$ THFs in 2016 and discussions with Karen Mossman. p14 was produced and purified by Roberto De Antueno in Roy Duncan's lab. Generation of THF cells with doxycycline inducible expression of p14 and initial experiments were carried out by Tetyana Murdza. Fusogenic liposomes were produced with assistance from Raquel Epand in Richard Epand's lab. Ca^{2+} microscopy and analysis were again carried out in collaboration with Subhendu Mukherjee. The remaining experiments were done by myself. The paper was written with assistance from Karen Mossman.

The remainder of the thesis was written by myself, unless noted otherwise, with editing and suggestions from Karen Mossman.

Chapter 1 - Introduction

Portions of this introduction have been adapted from published review articles written by myself with assistance from my supervisor Karen Mossman. One review concerns innate antiviral responses to physiological changes within cells during infection and is used to introduce the main research questions we pursued (Hare and Mossman 2013). The other review concerns defects in innate immunity incurred during cellular immortalization and is used to justify the models we have chosen to study (Hare, et al. 2016). Both reviews have been edited for brevity and clarity.

1.1 - Innate antiviral immunity

As an obligate intracellular parasite, a virus's success depends on strategies evolved to exploit host cells and the suitability of these strategies to overcome cellular antiviral defenses. Intrinsic defenses include passive features that protect the host from infection. Innate defenses are activated by environmental factors and pattern recognition to restrict virus replication. Viruses must overcome both intrinsic and innate barriers to infection by avoiding recognition or antagonizing cellular defences.

A major component of innate antiviral defense is the type 1 interferon (IFN-I) system that upregulates hundreds of IFN-stimulated genes (ISGs) that cumulatively restrict virus spread. The ability of a virus to prevent IFN-I production is critical for replication and is an important determinant of a virus's species tropism (reviewed by (McFadden, et al. 2009)). IFN-I signalling plays an important role in viral disease and IFN-I deficient mice display severe pathology following infection with otherwise innocuous viruses (Muller, et al. 1994). Conversely, mis-regulated IFN signalling is responsible for a number of autoimmune disorders (reviewed by (Lee-Kirsch 2017)).

1.2 - IFN-I signalling

Different IFN types

There are several types of IFN in humans, grouped by the receptor they bind to, which play different roles in innate immunity. IFN-II includes IFN-γ, is produced mainly be leukocytes and is recognized by the IFN-γ receptor (IFNGR). IFN-III includes 4 IFN-λ subtypes that are recognized by the IFN-λ receptor (IFNLR) and triggers similar signalling to IFN-I. IFNLR expression is mainly restricted to epithelial cells giving it an important role in barrier function (reviewed by (Lazear, Nice, and Diamond 2015)). IFN-I includes 13 subtypes of IFN-α, IFN-β, IFN-ε, IFN-κ and IFN-ω all recognized by the IFN-α/β receptor

(IFNAR). IFN-α and IFN-β are both highly upregulated in virus infected cells and play important roles in antiviral defence. Their regulation is largely dependent on IFN-regulatory factor 3 (IRF3), IRF7 and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB). IFN-ε is constitutively expressed in the female genital epithelium and is sensitive to estrogen levels (Fung, et al. 2013). IFN-κ and IFN-ω are expressed in keratinocytes and leukocytes and are also upregulated in response to virus infection and dsRNA (LaFleur, et al. 2001; Hauptmann and Swetly 1985). While IFN-ε, IFN-κ and IFN-ω likely play important roles in certain tissues, the host antiviral response to infection is largely dependent on IFN-α and IFN-β, in large part because they are highly upregulated in a wide variety of infected cells (reviewed by (Capobianchi, et al. 2015)).

Pattern recognition receptors

IFNs are usually released following recognition of pathogen-associated molecular patterns (PAMPs) by pattern recognition receptors (PRRs)(Figure 1). The best characterized PRRs include toll-like receptors (TLRs), retinoic acid inducible gene I (RIG-I)-like receptors (RLRs), and DNA receptors (reviewed by (Wu and Chen 2014)). TLRs are responsible for sensing a variety of extracellular PAMPs either at the cell surface or within endosomes. TLRs are activated by coordinated binding of ligands, leading to clustering of their intracellular

signalling domain (reviewed by (Moresco, LaVine, and Beutler 2011; Kawai and Akira 2006)). TLR2 and TLR4 are usually present on the cell surface and usually recognize bacterial peptidoglycan and lipopolysaccharide (LPS) respectively, (Yang, et al. 2010; Poltorak, et al. 1998). It has since been found that TLR2 and TLR4 can recognize a variety of viral glycoproteins from viruses including respiratory syncytial virus (RSV), measles virus, human cytomegalovirus (HCMV), herpes simplex virus type 1 (HSV-1), coxsackie virus B (CVB), ebolavirus and human immunodeficiency virus type 1 (HIV-1) (Kurt-Jones, et al. 2000; Bieback, et al. 2002; Compton, et al. 2003; Triantafilou and Triantafilou 2004; Kurt-Jones, et al. 2004; Yan, et al. 2005; Okumura, et al. 2010; Nazli, et al. 2013). The broad specificity of these TLRs may in part rely on interactions with co-receptors. TLR3 is expressed by a broad range of cells and recognizes dsRNA (Alexopoulou, et al. 2001). While it is found at the cell surface, only endosomal TLR3 is able to upregulate IFN (Funami, et al. 2004; Matsumoto, et al. 2003). TLR7, TLR8, and TLR9 are mainly present in the endosome compartment of professional IFN-producing cells like plasmacytoid dendritic cells (pDCs) (reviewed by (Colonna, Trinchieri, and Liu 2004). TLR7 and TLR8 are thought to recognize single-stranded RNA (ssRNA) degradation products (Tanji, et al. 2015; Zhang, et al. 2016). TLR9 recognizes unmethylated cytosine-gaunosine (CpG) motifs in bacterial or viral double-stranded DNA (dsDNA) (Lund, et al. 2003; Hemmi, et al. 2000).

The cytosolic domain of TLRs recruits different adaptor proteins including myeloid differentiation primary response 88 (MyD88) and/or Toll/IL-1 receptor domain-containing adaptor inducing IFN-β (TRIF). MyD88 adaptor complexes activate IRF7, TRIF complexes activate IRF3 and both MyD88 and TRIF complexes activate NF-κB. TLR3 associates with TRIF and is important for upregulating IFN-β in fibroblasts exposed to dsRNA. TLR7, TLR8 and TLR9 all associate with MyD88 and are important for upregulating IFN-α in pDCs. TLR2 also associates with MyD88 but may only upregulate IFN in monocytes exposed to viral PAMPs (Barbalat, et al. 2009). Other cell types exposed to viral TLR2 ligands produce inflammatory cytokines but not IFN (Juckem, et al. 2008). Unlike other TLRs, TLR4 associates with both TRIF and MyD88 but mainly upregulates IFN through a TRIF-IRF3-dependent pathway (Sakaguchi, et al. 2003). The current paradigm is that MyD88 associated with TLR7, TLR8 and TLR9 are mostly important for IRF7-dependent upregulation of IFN-α in pDCs, while TRIF associated with TLR3 and TLR4 are mostly important for IRF3-dependent upregulation of IFN-β in fibroblasts and other non-professional immune cells.

Endosomal localization plays an important role in the sensitivity and specificity of TLRs upstream of IFN. Indeed, all nucleic acid sensing TLRs signal from the endosome (reviewed by (Kawai and Akira 2006). Endosomal localization allows TLRs to recognize virus particles that have been internalized and degraded or nucleic acid that is taken up by scavenger receptors (DeWitte-Orr, et al. 2010).

Endosomal localization also prevents TLRs from recognizing endogenous nucleic acid and triggering inflammation. TLR9 engineered for surface expression was found to recognize self and non-self dsDNA, suggesting the importance of TLR localization for ligand specificity (Barton, Kagan, and Medzhitov 2006).

Cytosolic dsRNA is recognized by RIG-I and melanoma-differentiation-associated protein 5 (MDA5). RIG-I recognizes 5' tri-phosphorylated or di-phosphorylated uncapped ends of dsRNA and has a preference for short dsRNA molecules (Hornung, et al. 2006), while MDA5 has a preference for long dsRNA molecules (Kato, et al. 2008). dsRNA regions near the 5' terminus allow the assembly of RIG-I filaments in a stepwise manner (Peisley, et al. 2013; Myong, et al. 2009). In contrast, MDA5 binds internal portions of dsRNA molecules and forms filaments able to move up and down dsRNA (Peisley, et al. 2011; Peisley, et al. 2012). The different mechanisms of filament assembly and their rates of assembly/disassembly are believed to explain the preferences of RIG-I and MDA5 for short and long dsRNA respectively (reviewed by (Sohn and Hur 2016). dsRNA binding and assembly of RIG-I or MDA5 filaments exposes and clusters their caspase activation and recruitment domains (CARDs) which then interact with mitochondrial antiviral signalling (MAVS) via CARD-CARD interactions (Kowalinski, et al. 2011). MAVS is a transmembrane protein localized in the mitochondria and acts as a scaffold for activation of NF-κB and IRFs (Seth, et al. 2005). While other cytosolic dsRNA sensors like laboratory of genetics and

physiology 2 (LGP2), 2’-5’-oligoadenylate synthetase 1 (OAS1), and protein kinase R (PKR) play a role in immunity, they do not directly signal upregulation of antiviral ISGs (reviewed by (Wu and Chen 2014)).

Cytosolic dsDNA is primarily recognized by cyclic GMP-AMP synthase (cGAS) through a sequence-independent mechanism. cGAS binds to B-form dsDNA in the cytoplasm and generates 2’3’-cyclic GMP-AMP (cGAMP) through conjugation of guanosine triphosphate (GTP) and adenosine triphosphate (ATP) substrates (Sun, et al. 2013). The secondary messenger cGAMP is sensed by the endoplasmic reticulum (ER) / mitochondria-associated ER membrane (MAM) resident stimulator of IFN genes (STING) (Wu, et al. 2013). STING is also able to recognize other cyclic dinucleotides produced by bacteria (Burdette, et al. 2011). Cyclic dinucleotide binding stabilizes STING dimers which translocate to cytoplasmic signalling vesicles (Ishikawa, Ma, and Barber 2009; Saitoh, et al. 2009; Shu, et al. 2012; Ouyang, et al. 2012). Once there, STING acts as a scaffold for activation of NF-κB and IRFs. Other DNA sensors including DNA-dependent activator of IFN-regulatory factors (DAI), IFN-inducible protein 16 (IFI16) and DEAD box helicase 41 (DDX41) have all been implicated in the IFN response to DNA, but there are other reports that their depletion from cells does impact the response to cytosolic DNA. Thus, these sensors may play a role in recognition of particular forms of DNA or their function may be cell type or context dependent (reviewed by (Wu and Chen 2014)).

Regulation of IFN-β

The *ifnb1* promoter contains 4 critical regulatory elements, positive regulatory domain (PRD) IV, III, I and II in that order, known to play a role in its upregulation in response to infection (reviewed by (Negishi, Taniguchi, and Yanai 2018; Honda, Takaoka, and Taniguchi 2006). PRDIV is bound by a cyclic AMP transcription factor 2 (ATF2) / c-Jun heterodimer (AP1), PRDIII and PRDI are each bound by an IRF dimer and PRDII is bound by an NF-κB dimer. PRDIII and PRDI are mainly bound by IRF3 and IRF7. This transcriptional complex bound to PRDI-IV is called the IFN-β enhanceosome and was originally thought to be necessary to recruit transcriptional machinery to upregulate IFN-β transcription (Thanos and Maniatis 1995).

IRF3/7 and NF-κB are critical transcription factors for upregulation of IFN-β and bind regions in the *ifnb1* promoter to upregulate gene expression (Thanos and Maniatis 1995; Yoneyama, et al. 1998). TRIF, MyD88, MAVS and STING act as scaffolding proteins for phosphorylation of IRF3/7 and NF-κB (Liu, et al. 2015). These scaffold proteins are associated with the IκB kinase (IKK)-related kinases TANK-binding kinase (TBK1) and IKK-ε which are responsible for phosphorylation of IRF3/7 (Sharma, et al. 2003; Hemmi, et al. 2004), and IKK-β and IKK-α which phosphorylate NF-κB (Zandi, et al. 1997). Clustering of scaffold proteins leads to

autophosphorylation of TBK1 and phosphorylation of sites on the scaffold that recruit IRF3/7 (Liu, et al. 2015). Phosphorylation of IRF3/7 in C-terminal residues by TBK1 causes a conformational change that allows dimerization, nuclear accumulation and association with CREB-binding protein (CBP) (Mori, et al. 2004; Lin, et al. 1998). Under resting conditions active subunits of NF-κB are kept in the cytosol by the inhibitory subunit IκBα. IKK-β and IKK-α phosphorylate IκBα, signalling its ubiquitin (Ub)-mediated degradation, and the liberated active subunits of NF-κB accumulate in the nucleus (reviewed by (Pham and Tenoever 2010)).

ATF2/c-Jun also binds the *ifnb1* promoter and plays a role in IFN-β upregulation. ATF2 and c-Jun are phosphorylated by p38 mitogen-activated protein kinase (MAPK) and c-Jun N-terminal kinase (JNK) respectively (Iordanov, et al. 2000). Both MAPK and JNK stress-associated pathways can be activated by dsRNA through a pathway involving translation shutdown but the mechanism is unclear (Iordanov, et al. 2000; Taghavi and Samuel 2012). While ATF2/c-Jun likely enhances IFN-β upregulation during infection, additional work is necessary to understand its role.

Unlike IFN-β, which can be upregulated by IRF3, IFN-α upregulation requires activated IRF7 (Sato, et al. 1998; Sato, et al. 2000; Marié, Durbin, and Levy 1998). The IFN-α family includes 13 members in humans and these genes promoters contain PRDIII- and PRDI-like elements (PRD-LEs) also called variable

response elements (VREs) (Ryals, et al. 1985). The PRD-LEs in IFN-α promoters differ from IFN-β in that they have a strong binding preference for IRF7 (Andrilenas, et al. 2018). Because IRF7 has a very short half-life and its expression is nearly undetectable in most resting cells, IRF3-dependent upregulation of IFN-β is usually necessary before IRF7-mediated upregulation of IFN-α subtypes (reviewed by (Honda, Takaoka, and Taniguchi 2006)). pDCs are the exception to the rule because they constitutively express high levels of IRF7 and respond to viral PAMPs by immediately upregulating IFN-α (reviewed by (Colonna, Trinchieri, and Liu 2004)).

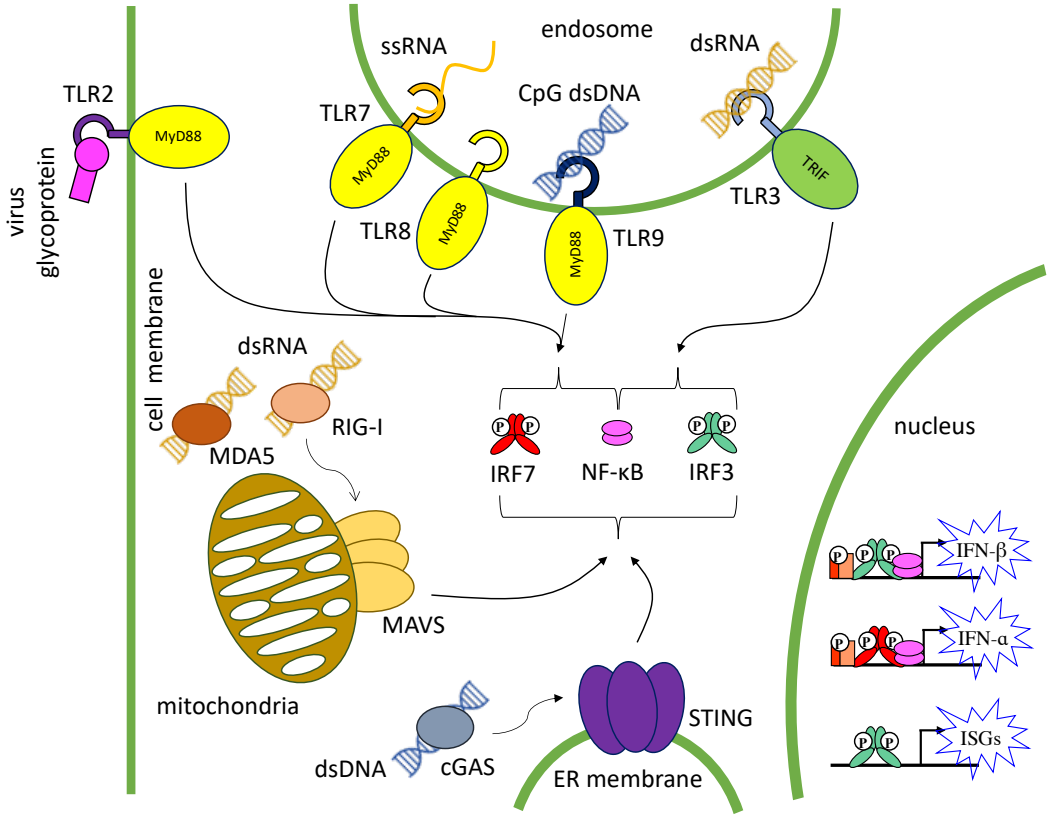

Figure 1 – Pattern recognition receptors and IFN upregulation. TLR2 recognizes viral glycoproteins at the cell surface, TLR7 and TLR8 recognize ssRNA and its degradation products in the endosome and TLR9 recognizes unmethylated CpG motifs in dsDNA in the endosome. All these TLRs associate with the adaptor MyD88 and can activate IRF7 and NF-κB in certain cell types. TLR3 recognizes dsRNA in the endosome, associates with the adaptor TRIF and activates IRF3 and NF-κB in most cells. MDA5 and RIG-I recognize dsRNA and un-capped RNA in the cytosol, activate the scaffold protein MAVS on the mitochondria which activates IRF3, IRF7 and NF-κB. cGAS recognizes dsDNA in the cytosol, activates the scaffold protein STING on the ER membrane which activates IRF3, IRF7 and NF-κB. ATF2/c-Jun, IRF3/IRF7 and NF-κB bind the promoters of IFN-β/IFN-α and upregulate their expression. IRF3/7 and NF-κB individually bind the promoters of a subset of ISGs and directly upregulate their transcription.

IFNAR signalling

IFNAR is composed of two chains, IFNAR1 and IFNAR2, expressed on the surface of most cell types. Binding of IFN-α/β to IFNAR activates the associated janus kinases (JAKs) tyrosine kinase 2 (TYK2) and JAK1 which phosphorylate both signal transducer and activator of transcription 1 (STAT1) and STAT2. STAT1, STAT2 and IRF9 form the IFN-stimulated gene factor 3 (ISGF3) complex (Darnell, Kerr, and Stark 1994; Haque and Williams 1994; Bluyssen, Durbin, and Levy 1996). This complex enters the nucleus and binds IFN-stimulated response elements (ISREs) in the promoters of hundreds of ISGs to up-regulate their expression. ISGs have a variety of functions including antiviral defence and initiation of adaptive immunity, and collectively create a non-permissive antiviral cellular state (Schoggins 2019). A number of IRFs are also upregulated by IFN, providing both negative and positive feedback. IRF1 is upregulated in response to IFN-α and directly upregulates a subset of ISGs (Panda, et al. 2019; Forero, et al. 2019). IRF2 negatively regulates upregulation of ISGs by binding to ISREs and competing with other IRFs (Harada, et al. 1989; Hida, et al. 2000). IRF7 is present in very low levels in resting cells but plays a role in positive feedback through upregulation of IFN-α subtypes (Sato, et al. 1998). Upregulation of IRF9 in response to IFN increases sensitivity to further IFN signalling (Bluyssen, Durbin, and Levy 1996). Together IRFs regulate upregulation of IFN and ISGs through a

complex network of positive and negative feedback loops (reviewed by (Michalska, et al. 2018)).

Tonic IFN-β signalling

IFN-β activity is nearly undetectable in unstimulated cells and aberrant ISG expression can be harmful and is associated with autoimmune disease. However, low-level basal IFN-β signalling plays an important role in priming cells to rapidly and robustly respond to infection and maintain homeostasis (reviewed by (Gough, et al. 2012; Taniguchi and Takaoka 2001)). For example, while IRF3 is constitutively expressed and quite stable, the more labile IRF7 requires a basal IFN-β signalling loop for its expression and is maintained at low to undetectable levels (Sato, et al. 1998; Sato, et al. 2000). Basal IFN-β signalling regulates a number of ISGs involved in antiviral defense, virus recognition and induction of IFN-α. This basal activity allows signalling components upregulated by IFN-β to amplify the response to virus infection. However, cells lacking such signalling also lack basal expression of important genes that regulate the induction of IFN-β.

1.3 - IFN-independent signalling

IRF3-mediated ISG upregulation

In addition to IFN-mediated upregulation of ISGs, IRF-binding to a subset of ISREs is sufficient to directly upregulate ISGs independent of IFN. Cell lines unable to respond to IFN-α nonetheless upregulated ISGs when treated with the dsRNA mimetic poly I:C (Tiwari, Kusari, and Sen 1987). Furthermore, the protein translation inhibitor cycloheximide (CHX) was used to demonstrate that ISG transcripts were upregulated in the absence of *de novo* protein translation (Bandyopadhyay, et al. 1995). Direct ISG upregulation in response to dsRNA involves phosphorylation, dimerization and nuclear translocation of IRF3 followed by the binding of an IRF3-CBP complex to the ISRE of a subset of ISGs (Yoneyama, et al. 1998; Weaver, Kumar, and Reich 1998).

IRF3-dependent upregulation of a subset of ISGs was also observed in HSV and HCMV infected fibroblasts in the presence of CHX (Mossman, et al. 2001; Browne, et al. 2001). To mimic IRF3 activation alone, in the absence of virus or dsRNA, a constitutively active IRF3 mutant (IRF3-5D) was created by replacing 5 C-terminal serine and threonine residues with aspartate, a negatively charged phosphomimetic (Lin, et al. 1998). Induced expression of IRF3-5D upregulated a similar subset of IFN-independent ISGs (Grandvaux, et al. 2002).

Interestingly, IRF3-5D binds to promoter sequences containing the PRDI-PRDIII element found in the IFN promoter as well as the core ISRE element (Lin,

et al. 1998). It is not totally understood what prevents activated IRF3 from binding any ISRE, including the IFN-β promoter, and upregulating transcription. The core ISRE sequence (GAAAXXGAAA) is conserved among all ISREs and the two GAAA motifs make contact with a single IRF (Williams 1991). Surrounding bases seem to control which IRFs bind and their relative affinity (Andrilenas, et al. 2018).

IRF3-dependent IFN-independent antiviral response

HSV expresses a variety of proteins to impair innate antiviral signalling, and effectively suppresses upregulation of ISGs during infection of human fibroblasts. However, if protein translation is inhibited with the drug CHX, HSV infected fibroblasts are able to upregulate mRNA from a subset of ISGs through an IRF3-dependent pathway (Mossman, et al. 2001). This ISG subset resembles the subset of IRF3-responsive ISGs identified previously (Grandvaux, et al. 2002; Elco, et al. 2005). Because both cellular and viral protein translation are impaired by CHX, ISG upregulation occurs independent of both IFN production and HSV gene expression. This suggests that cells recognize virus particles in the absence of virus replication, or production of stimulatory dsRNA, and upregulate ISGs through an IFN-independent pathway. Furthermore, upregulation of ISGs

requires HSV entry and is independent of TLRs (Mossman, et al. 2001; Paladino, et al. 2006).

Another way to examine the antiviral response to non-replicating virus particles is to genetically inactivate virus with ultraviolet (UV)-radiation. These viruses are capable of entry but not gene expression. Infection with a variety of non-replicating UV-inactivated enveloped viruses triggers an IRF3-dependent antiviral response (Mossman, et al. 2001; Collins, Noyce, and Mossman 2004). Interestingly, the antiviral response to enveloped virus particles occurs in the absence of detectable IFN and only with higher amounts of enveloped virus particles is NF-κB activation and IFN production detected (Paladino, et al. 2006)(Figure 2 from Paladino, et al., 2006). IRF3 is essential for the antiviral response to UV-inactivated enveloped virus particles. However, IRF3 activation markers, including phosphorylation, dimerization, nuclear translocation or 2D gel shift, are inconsistent across different viruses (Noyce, Collins, and Mossman 2009).

C-terminal phosphorylation of IRF3 and dimerization following virus infection is the prototypic mechanism of IRF3 activation (Lin, et al. 1998), but is not necessarily detectable, despite being required for subsequent ISG induction (Noyce, Collins, and Mossman 2009). N-terminal phosphorylation has also been observed in response to various stress-related stimuli (Servant, et al. 2001). S-Glutathionylation is present on IRF3 in uninfected cells and deglutathionylation is

necessary for IRF3 transcriptional activity via its interaction with CBP (Prinarakis, et al. 2008). IRF3 can also be positively regulated by conjugation of ISG15 or negatively regulated by ubiquitination or sumoylation (Bibeau-Poirier, et al. 2006; Kubota, et al. 2008; Shi, et al. 2010). The wide variety of IRF3 modifications suggests it serves a broader function than previously thought. Perhaps cell stress and a variety of danger signs converge on IRF3 to positively or negatively modulate its activity or determine the specific nature of the antiviral response, whether that is IFN-β production, apoptosis, or induction of a subset of ISGs.

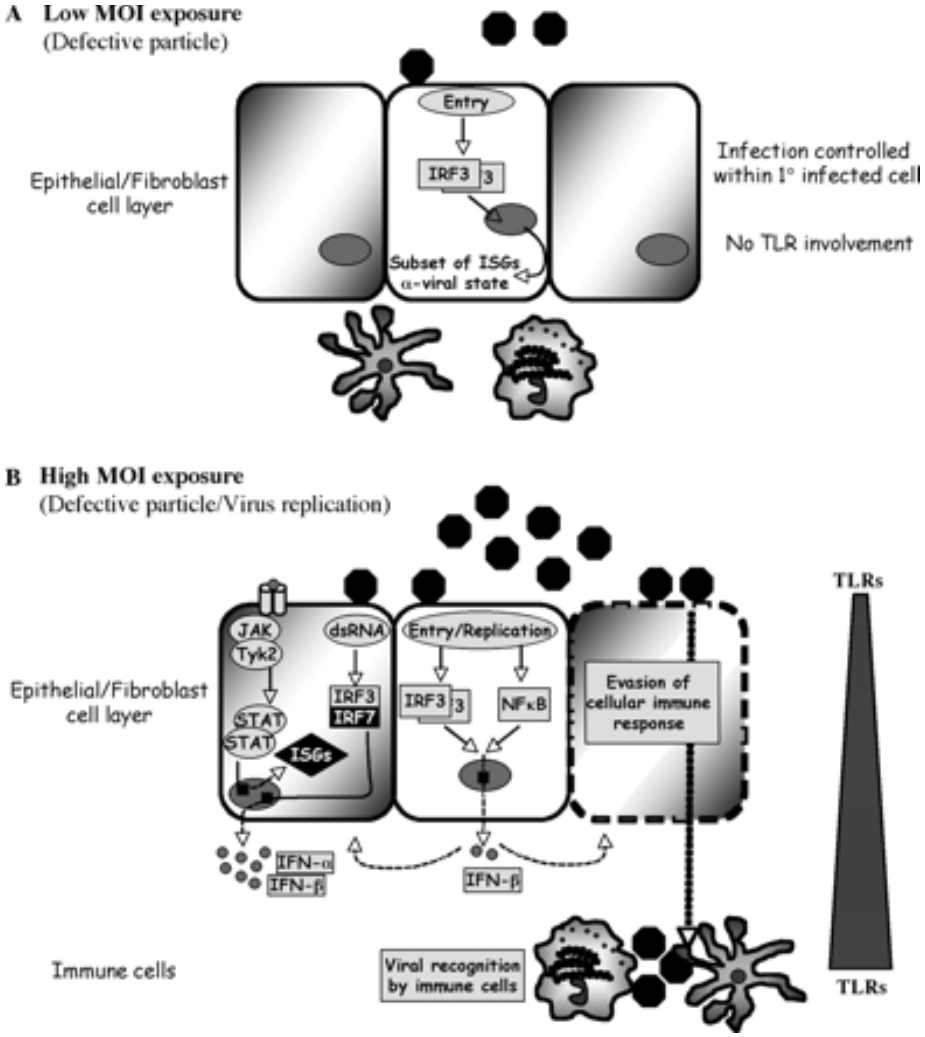

Figure 2 – The IFN-independent antiviral response to enveloped virus particles. *A*, During low level infection, the initial entry of enveloped virus into epithelial/fibroblast cells triggers an IFN-independent antiviral response that contains the viral spread to the primary infected cell. This response does not require the involvement of TLRs, RIG-I, or NF-κB but the participation of IRF3 is essential. *B*, A breach of the primary line of defense initiates a secondary line of cellular defense involving the activation of IRF3 and NF-κB, resulting in production of IFN and protection of neighboring cells. In the event that a virus infection evades the cellular immune response at the epithelial/fibroblast layer, immune cells such as monocytes/macrophages and dendritic cells (DCs) participate in another level of cellular defense, which involves IFN, cytokines, and initiation of an adaptive immune response. The involvement of TLRs is cell-type dependent and increases as the complexity of the immune response increases. This figure was reproduced from Paladino, et al., 2006.

1.4 - Recognition of virus-associated physiological perturbation

Although the established pathways leading to IRF3 activation involve different sensing and signaling components, they are functionally similar in that they detect viral components via receptor-ligand interactions. However, it seems unlikely that this is the only form of viral detection. It is well known that viruses alter regular cellular processes such as endocytosis and cytoskeletal remodeling during entry. Viruses must also dramatically alter cellular conditions to mediate their replication and, consequently, cause cell stress. It is intriguing to speculate that perturbation of physical or homeostatic conditions within the cell could act as a danger signal for viral infection (Figure 3). Indeed, recent findings support this hypothesis.

Moreover, given the evolution of multiple viral sensing pathways, viruses have evolved strategies to avoid exposing their viral components to PRRs. Viruses are capable of sequestering their genomes and/or degrading dsRNA by-products of replication to prevent recognition (Espada-Murao and Morita 2011; Hastie, et al. 2011). On the other hand, physical changes to the cell are harder to conceal because of their more global nature. For example, cytoskeletal rearrangements and various signs of cell stress cannot be sequestered or hidden. Another issue with reliance on detection of viral dsRNA for host defense is that

for many viruses, dsRNA does not accumulate until later in the viral replication cycle when the virus has had opportunity to subvert the antiviral response.

Membrane Perturbation

To enter a cell, all viruses must cross a cell membrane either at the surface or within endosomal compartments. For enveloped viruses, this entails membrane fusion. Membrane fusion is energetically unfavourable because of the need to disrupt hydrophobic interactions within the phospholipid bilayer. Enveloped viruses apply force with membrane fusion proteins to bring the membranes together and induce curvature and eventual fusion leading to incorporation of the envelope into the cellular membrane (reviewed by (Chernomordik and Kozlov 2003)). These alterations at the cellular membrane are characteristic of viral entry and could alert the cell to the presence of the virus.

Certain virus-like particles (VLPs) mimic enveloped viruses and are capable of membrane fusion but do not contain packaged virus genome or capsid. Light particles (L-particles) are produced during natural infection by alphaherpesviruses and are composed of an envelope without capsid or genome (Szilagyi and Cunningham 1991). They can be separated from replication competent virus by density gradient centrifugation or with mutant viruses incapable of capsid assembly (Roberts, et al. 2009; Szilagyi and Cunningham

1991). Pre-viral replication enveloped particles (PREPs) are produced during viral replication when the viral polymerase is blocked (Dargan, Patel, and Subak-Sharpe 1995). PREPs contain viral capsid and tegument within a fusion competent envelope but do not contain genome. Both types of VLPs induce ISGs (Holm, et al. 2012). However, because these particles are produced in the context of viral replication, there is still the possibility of protein or nucleic acid contaminants inadvertently packaged within the envelope. Fusion-associated small transmembrane (FAST) proteins are non-structural, syncytia forming proteins expressed by non-enveloped reoviruses (Corcoran and Duncan 2004). Purified p14 FAST protein in complex with lipofectamine (p14 lipoplexes) can upregulate ISGs in the absence of viral or cellular contaminants (Noyce, et al. 2011). Finally, fusogenic liposomes capable of spontaneous fusion with cells recapitulate a similar response (Holm, et al. 2012).

The mechanism of antiviral signaling following membrane fusion is largely unknown. Phospholipase C-γ (PLC-γ) and phosphatidylinositol-3-OH kinase (PI3K) pathways are associated with membrane signaling and inhibitors of PLC-γ and PI3K pathways interfere with the antiviral response to membrane perturbation (Holm, et al. 2012; Noyce, Collins, and Mossman 2006). However, the specific PI3K family members involved have yet to be identified as the response to UV-inactivated enveloped virus was shown to be independent of prototypic PI3K family members (Noyce, Collins, and Mossman 2006).

Several studies in primary fibroblasts suggest that IRF3 mediates the direct activation of a subset of ISGs independent of IFN production (Collins, Noyce, and Mossman 2004; Noyce, Collins, and Mossman 2006; Preston, Harman, and Nicholl 2001; Noyce, et al. 2011). Other findings, predominantly with immune cells, suggest that ISG upregulation in response to membrane perturbation is STING- and IFN-dependent (Holm, et al. 2012). The apparent discrepancy could be a cell specific phenomenon, or it could relate to the extent of membrane perturbation.

Cytoskeletal Perturbation

Cytoskeletal perturbation has also been implicated in antiviral signaling. The cytoskeleton is involved in multiple aspects of virus infection being both a physical barrier and necessary for transport of viral components within the cell. The cytoskeleton networks the cytoplasm and mediates transport of vesicles and organelles. For example, a mesh of actin called the cell cortex gives the membrane support and allows formation of membrane structures such as membrane ruffles, focal adhesions, and other specialized cell structures (Salbreux, Charras, and Paluch 2012). In epithelial cells, the cell cortex is highly developed at the apical membrane, and movement of pathogens across the membrane requires cytoskeletal disassembly and reorganization (Delorme-Axford and Coyne 2011). The cytoskeleton is also important for cell adhesion and

maintaining tight junction barriers that restrict access to underlying tissues. While disruption of microtubules has long been known to activate NF-κB (Rosette and Karin 1995), there is an emerging role of the actin cytoskeleton in innate immunity.

Perturbation of the cytoskeleton by either actin depolymerizing or polymerizing agents can also activate NF-κB, suggesting that unscheduled alteration of the actin cytoskeleton equilibrium activates innate pathways (Kustermans, et al. 2005). Interestingly, in intestinal epithelial cells, cytoskeleton disruption causes IRF3 activation and IFN-β upregulation (Mukherjee, et al. 2009). After actin depolymerization, the dsRNA sensor RIG-I translocates from cytoskeletal structures at the membrane of these cells to actin-rich cytoplasmic punctate structures (Mukherjee, et al. 2009). RIG-I normally interacts with MAVS during antiviral signaling and there are some data to suggest that these punctate structures might exist at the mitochondrial membrane (Bozym, et al. 2012; Ohman, et al. 2009). This response could alert the cell to virus disassembly of cortical actin at the membrane or disruption of tight junctions; however the sensing and signaling mechanisms and whether or not RIG-I is involved is currently uncertain (Mukherjee, et al. 2009).

There are other instances of cytoskeletal-associated proteins involved in innate immune pathways. Focal adhesion kinase (FAK) normally associates with focal adhesions, which link the actin cytoskeleton with the cell membrane, but

may also play a role in RIG-I signaling through MAVS (Bozym, et al. 2012). Interestingly, point mutation of Y397, a key autophosphorylation residue for FAK activation, enhances FAK mediated antiviral signaling, which suggests FAK's role is not as a kinase (Bozym, et al. 2012). Additionally, Rho GTPase Rac1 and p21-activated kinase 1 (PAK1) were shown to be involved in IRF3 activation and the IFN-I response to influenza virus and dsRNA (Ehrhardt, et al. 2004). Rac1 has many functions involving actin remodelling at the membrane but notably it acts through PAK1 to regulate formation of cellular protrusions (Citi, et al. 2011; Parrini, Matsuda, and de Gunzburg 2005). Although cytoskeleton-associated proteins FAK, Rac1 and PAK1 have been implicated in innate signaling pathways, it is not known whether they are involved in sensing cytoskeletal perturbation or if they play a structural role in either sensing or signaling following conventional PAMP recognition.

Mitochondria, ROS, and ER Stress

Reactive oxygen species (ROS) are toxic metabolic by-products and can be used by phagocytes for killing phagocytosed microbes. There have been emerging roles for ROS in innate signaling pathways as well as their role in cell oxidative stress (Kohchi, et al. 2009). Metabolic processes involving mitochondria produce superoxide anions (O_2^-) as a toxic by-product that is quickly converted to hydrogen peroxide (H_2O_2) or hydroxyl radicals (OH^-) in reactions catalyzed by

superoxide dismutases and other enzymes. The term ROS includes O_2^- and all the reactive downstream products. ROS are capable of chemically altering molecules they come in contact with and thereby modulate signaling cascades. For example, glutathione catalyzes oxidation of H_2O_2, and can donate an electron to form a disulphide bridge between two cysteine residues. This modification can alter protein function and activate or inhibit downstream signaling. While global increases in cellular ROS can occur in cases of cell stress, ROS scavengers neutralize ROS to prevent cell damage. Because scavengers confine ROS production and create a gradient surrounding its source, ROS signaling is compartmentalized allowing signal specificity (Chen, Craige, and Keaney 2009). The primary source of ROS production is the mitochondria, but the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase complex is the common source of intentional ROS production for microbe killing or signaling (Chen, Craige, and Keaney 2009).

ROS are induced by a number of different viruses including Respiratory syncytial virus (RSV), human T cell leukemia virus 1 (HTLV-1), hepatitis B virus (HBV), hepatitis C virus (HCV), herpes simplex virus (HSV), Sendai virus (SeV), Japanese encephalitis virus (JEV), and influenza virus. In many cases, the ROS induced by viral infection have been linked with innate antiviral signaling pathways (Gonzalez-Dosal, et al. 2011; Indukuri, et al. 2006; Soucy-Faulkner, et al. 2010). In addition to their role in signaling, it is possible that ROS originating

from mitochondrial stress could modulate the antiviral response. The inhibitor rotenone uncouples the mitochondrial electron transport chain causing massive ROS production and will amplify the antiviral response of cells in the context of infection (McGuire, et al. 2011; Tal, et al. 2009). This suggests oxidative stress could affect the cellular sensitivity to antiviral signaling.

ER stress has also been associated with antiviral signaling and is often associated with altered protein translation during viral infection. ER stress-inducing agents have been found to synergistically activate type I IFN signaling in response to either LPS or dsRNA (Liu, et al. 2012; Hu, et al. 2011). ER stress comprises multiple stress response pathways including oxygen and nutrient deprivation, calcium dysregulation, misfolded protein recognition, and N-linked glycosylation inhibition, but they all converge on the unfolded protein response (UPR) (Schroder and Kaufman 2005b; Schroder and Kaufman 2005a). X-box binding protein 1 (XBP1) is activated downstream of the UPR and has been reported to bind an enhancer site upstream of the *ifnb1* gene to enhance IFN-β induction (Zeng, et al. 2010). IRF3 has also been reported to translocate to the nucleus after ER stress, in the absence of additional stimuli, and bind to the *ifnb1* enhancer sequence (Liu, et al. 2012). Little is known about how IRF3 might be activated by ER stress, but the signaling pathway seems dependent on the type of ER stress. Ca^{2+} associated ER stress seems to depend on the innate signaling

molecules STING and TBK1 while other ER stressors appear to signal independently (Liu, et al. 2012).

Recognition of endogenous nucleic acid

Virus-associated physiological perturbation can also trigger recognition of endogenous nucleic acid. Cellular sensors are tasked with discriminating minute amounts of viral nucleic acid from an abundance of endogenous nucleic acid. RNA secondary structures contained within ribosomal rRNA, tRNA, miRNA and transposon transcripts contain stretches of dsRNA. While dsDNA is normally compartmentalized, mitochondrial turnover, DNA damage, transposable elements and cell division could expose endogenous dsDNA to cytosolic sensors. Thus, nucleic acid sensing PRRs must balance sensitivity with aberrant activation (reviewed by (Roers, Hiller, and Hornung 2016; Schlee and Hartmann 2016)).

One example of endogenous nucleic acid recognition in the context of virus infection comes from HSV-1. Somewhat paradoxically, RIG-I plays a role in antiviral defence against HSV-1, a DNA virus. It was assumed that dsRNA was produced as a by-product of HSV-1 replication, but evidence for this was somewhat lacking. It has since been found that late during HSV-1 infection RIG-I binds to a 5S rRNA pseudogene (RNA5SP141) transcript that is normally restricted to the nucleus and shielded from recognition by associated protein (Chiang, et al. 2018). The authors suggested that the combination of nuclear

envelope breakdown and depletion of cellular protein were responsible for recognition of RNA5SP141 during HSV-1 infection.

A number of DNA damaging agents trigger IFN through dsRNA or dsDNA recognition pathways (Ranoa, et al. 2016; Mackenzie, et al. 2017; Gehrke, et al. 2013). During normal conditions cells rely on DNA and RNA nucleases to digest potentially stimulatory nucleic acid, and the absence of these nucleases often results in aberrant IFN production (Ablasser, et al. 2014; Eckard, et al. 2014; Lan, et al. 2014; Hartlova, et al. 2015). Similarly, RNA modifications are critical for cells to discriminate between viral and endogenous RNA (reviewed by (Schoggins 2015)). A number of RNA modifications like 5'-caps prevent recognition of endogenous RNA by RIG-I (Schuberth-Wagner, et al. 2015). Other modifications, like adenosine deaminase acting on RNA (ADAR) editing of RNA hairpins and inverted repeats, prevent formation of long stretches of dsRNA and recognition by MDA5 (Liddicoat, et al. 2015; Ahmad, et al. 2018). Thus, the correct functioning of a number of cellular pathways is necessary to avoid nucleic acid recognition under resting conditions and failure of these pathways triggers nucleic acid recognition and IFN production.

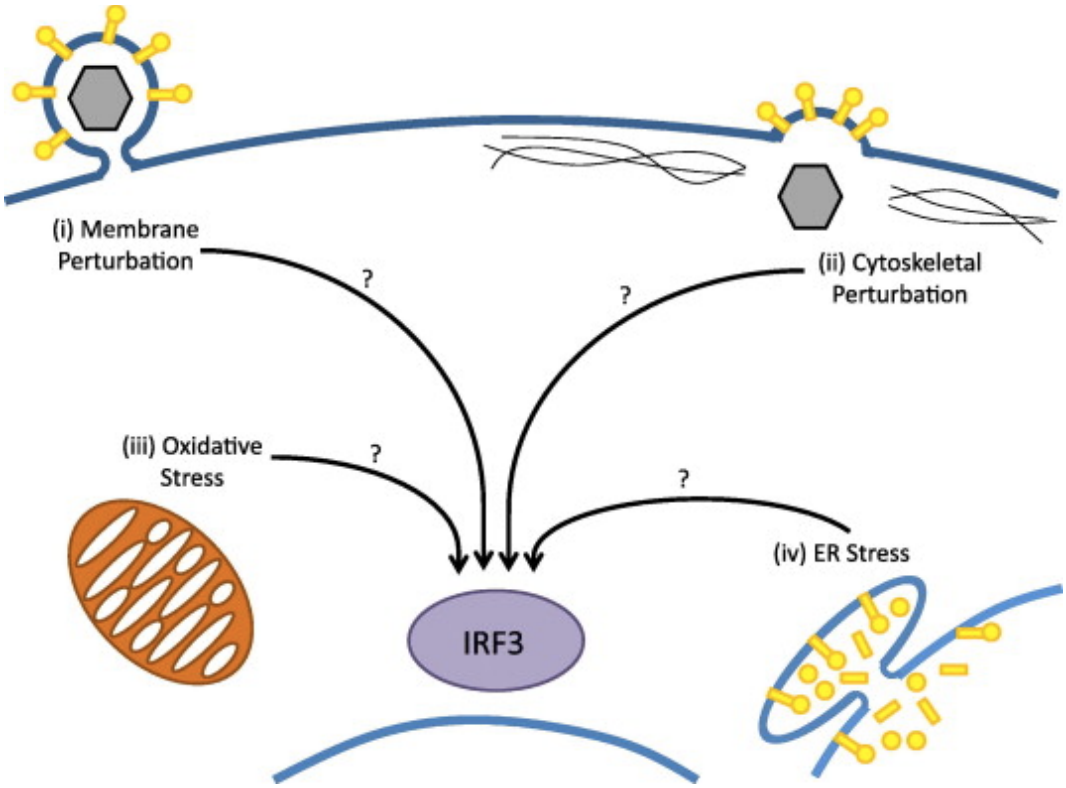

Figure 3 – Stress signalling pathways converge on IRF3 activation. Hallmarks of virus infection include the physical act of entry followed by cellular changes associated with viral replication. These physical changes modulate IRF3 through largely unknown mechanisms. (i) Viruses must cross the plasma membrane, directly of via endocytosis. Depending on the virus, cytoplasmic access may require membrane fusion. (ii) The actin cytoskeleton networks the cell and resides in a mesh beneath the cell membrane. Viruses must somehow modulate the cytoskeleton to move through the cytoplasm to their site of replication. (iii) Reactive oxygen species are released by mitochondria during situations of stress, and several viruses are associated with increases in the redox status of cells. (iv) Many ER stress pathways converge on the unfolded protein response. Broad changes to the cellular environment, including host translational inhibition and over-expression of viral proteins, could cause ER stress.

1.5 - Cellular immortalization and innate signaling

When studying viruses, the way they interact with their host is critical, and differences in the cellular model may lead researchers to false conclusions. While primary cells are more representative of the tissue they were isolated from, they are not a particularly tractable system. Stable expression of exogenous genes in primary cells is virtually impossible because they are limited to a certain number of population doublings and are often approaching senescence by the time selection is complete. Moreover, primary cells are often resistant to transfection, making transient manipulations using plasmids or small interfering RNA (siRNA) difficult. The low transfection efficiency of primary cells is often attributed to intrinsic and innate pathways activated by foreign DNA. IFI16, promyelocytic leukemia protein (PML) bodies, and other intrinsic factors recognize foreign DNA in the nucleus and epigenetically repress gene expression (reviewed by (Knipe 2015; Gu and Zheng 2016)). In addition to intrinsic defenses against exogenous gene expression, transfection of DNA is able to induce innate responses through the IFN-I pathway (Ishikawa, Ma, and Barber 2009; Reynolds, et al. 2006; Jensen, Anderson, and Glass 2014). Thus, even if transient transfection is successful, the ensuing innate response interferes with experiments.

The difficulties associated with manipulating primary cells make the use of other models a necessity. Transformed cells are generally much easier to

manipulate in culture because they have higher transfection efficiency, faster growth rate, and do not senesce. These qualities allow for the creation of cell lines suited to answering difficult research questions and are critical for a deeper understanding of virus-host interactions. However, transformed cells often do not resemble the original cell type. Cells accumulate mutations during tumorigenesis and continual culture that over time affect the phenotype and how cells respond to virus infection. Furthermore, innate signalling pathways may overlap with senescence pathways, making dysregulation of these pathways a natural consequence of spontaneous immortalization.

To immortalize cells in a way that preserves innate signalling pathways, it is important to understand how innate signalling pathways are dysregulated during immortalization and transformation. There is a great deal of overlap between cellular pathways involved in antiviral defense and tumour suppression. Both pathways can trigger anti-proliferative, pro-apoptotic and pro-inflammatory responses. It has been hypothesized that tumor suppressors and antiviral defense share a common evolutionary history and that many tumor suppressors first evolved as a defense against DNA viruses (Reddel 2010; Fridman and Tainsky 2008; Miciak and Bunz 2016). In support of this hypothesis, cells lacking antiviral genes are more easily immortalized in culture (Chen, et al. 2009). Additionally, cells lacking tumor suppressors are more susceptible to virus infection (Munoz-Fontela, et al. 2005; Ma-Lauer, et al.

2016). Antiviral genes may be silenced by the loss of certain tumor suppressors or selected against during tumorigenesis due to their anti-proliferative nature.

Senescence is controlled by molecular clocks within the cell which measure age, and perhaps the most important of these clocks are the telomeres (Hayflick and Moorhead 1961; Dell'Orco, Mertens, and Kruse 1973; Harley, Futcher, and Greider 1990). These regions of heterochromatin capping each chromosome become shorter each time the genome is replicated. Telomeres can be maintained in stem cells and other dividing cells by expression of the enzyme telomerase, which catalyzes template-dependent addition of telomeric repeats (Kim, et al. 1994). While replicative senescence in human cells appears to depend on telomere shortening, there may be additional aging clocks (reviewed by (Shay and Wright 2001)).

Others found that human telomerase reverse transcriptase (hTERT) immortalization does not impair the IFN-I response in human fibroblasts (Smith, et al. 2013). We tested a similarly hTERT-immortalized human foreskin fibroblast (THF) cell line (Bresnahan, Hultman, and Shenk 2000), and found they mount an antiviral response to UV-inactivated virus or the dsRNA mimetic poly I:C with similar efficiency as primary human fibroblasts. As with any cell type, continually cultured cells will gradually accumulate mutations, and higher passage hTERT immortalized cells will resemble the original primary cells less. However, directed

immortalization by expression of hTERT seems to avoid selective pressures during the bottleneck that occurs during spontaneous immortalization.

1.6 - Preliminary Experiments

At the outset of my thesis we knew that certain types of membrane fusion were sufficient to upregulate antiviral ISGs with little understanding of how membrane fusion was sensed or signalled. HSV-1 entry triggers an immediate spike in cytosolic Ca^{2+} which we suspected was involved in the antiviral response to membrane perturbation (Cheshenko, et al. 2003; Cheshenko, et al. 2007). We found that p14 lipoplexes and a variety of enveloped virus particles trigger cytosolic Ca^{2+} oscillations which are important for the subsequent antiviral response.

One early lead we investigated was the integrin-associated protein FAK. FAK has been shown to play an important role upstream of Ca^{2+} signalling during HSV entry, suggesting it may play a role in signalling membrane perturbation (Cheshenko, et al. 2005). FAK is also involved in the antiviral response of intestinal epithelial cells by controlling localization of the dsRNA sensor RIG-I (Bozym, et al. 2012). We investigated the role of FAK using chemical inhibitors and siRNA knockdown, but found that it played a minimal role in the antiviral response to different enveloped virus particles (Figure S1).

We also investigated the role of mechanosensitive channels in sensing membrane perturbation. Membrane fusion requires tight membrane curvatures that might be recognized by mechanosensitive Ca^{2+} channels. We found that Gd^{3+} ions, which broadly impair mechanosensitive channels, impaired the antiviral response to enveloped viruses (Figure S2). Gd^{3+} ions have a number of documented off-target effects and there are a large number of different mechanosensitive channels (Hamill and McBride 1996). To more specifically inhibit mechanosensitive channels we used the peptide inhibitor GsMTx4 (Gnanasambandam, et al. 2017). We found that GsMTx4 had no effect on the antiviral response to enveloped virus (data not shown).

ROS are produced during conditions of cell stress and can act as secondary messengers for cell signalling. We had preliminary data from another student suggesting that ROS are produced in response to enveloped virus entry and contribute to antiviral defence. However, we could not replicate experiments demonstrating a role for ROS in the response to enveloped virus (Figure S3).

Shortly after our lab published that p14-mediated fusion triggered an IRF3-dependent antiviral response, others found that HSV VLPs and fusogenic DOPE:DOTAP liposomes triggered STING- and IFN-dependent upregulation of ISGs (Holm, et al. 2012). The study by *Holm, et al.* prompted us to examine the role of STING in the antiviral response to enveloped virus particles and p14

lipoplexes. We also wanted to determine whether fusogenic liposomes triggered an IFN-independent IRF3-dependent antiviral response in human fibroblasts.

1.7 - Hypotheses and Thesis Objectives

Before transferring into the Ph.D. program, I considered how cells recognize membrane perturbation and how membrane perturbation contributes to IFN-independent IRF3-dependent upregulation of antiviral ISGs. These questions led to two central hypotheses that I have included below. The objective of my thesis would be to test these hypotheses and expand our understanding of how cells recognize enveloped virus particles.

We knew that treatment of cells with a variety of enveloped virus particles triggered Ca^{2+} oscillations which were important for subsequent upregulation of ISGs. Others had found that STING was important for upregulation of ISGs in response to membrane fusion. While IRF3 played an essential role in this response, we often failed to observe IRF3 activation in infected cells. We hypothesized that membrane perturbation during virus entry triggered Ca^{2+} oscillations critical for antiviral signalling. This signalling pathway culminated in activation of STING and IRF3 in a way that led to direct upregulation of a subset of antiviral ISGs. I planned to uncover the mechanistic details of this signalling pathway.

While enveloped virus-cell membrane fusion appeared to be sufficient for upregulation of ISGs and antiviral protection, we knew cells did not respond to all membrane fusion. Fusion between cellular membranes is necessary for uptake of extracellular vesicles, exocytosis, autophagy and membrane reorganization. We knew that p14-mediated fusion, and perhaps liposome fusion, were sufficiently different from routine membrane fusion events to trigger antiviral signalling. We hypothesized that contextual cues such as cellular localization and perhaps additional co-signals were required for the cell to discern routine membrane fusion from virus entry. To understand these cues, I planned to determine which types of membrane fusion triggered antiviral signalling.

Chapter 2

Membrane perturbation-associated Ca^{2+} signalling and incoming genome sensing are required for the host response to low-level enveloped virus particle entry

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Chapter Introduction

Fibroblasts respond to a wide variety of non-replicating enveloped virus particles by upregulating antiviral ISGs (Collins, Noyce, and Mossman 2004; Mossman, et al. 2001). We previously found that low level infection with UV-inactivated enveloped virus particles triggered an IFN-independent antiviral response and detectable IFN was only produced following infection with increasing levels of enveloped virus particles (Collins, Noyce, and Mossman 2004; Paladino, et al. 2006). In contrast, human fibroblasts failed to respond to non-replicating adenovirus (Collins, Noyce, and Mossman 2004). We previously speculated this difference in the response to enveloped and non-enveloped virus particles was because membrane perturbation during enveloped virus entry was sensed as a danger signal during infection and contributed to activation of IRF3 and upregulation of ISGs (Hare and Mossman 2013). We found that FAST p14-mediated membrane fusion was sufficient to trigger IRF3-dependent upregulation of ISGs and antiviral protection (Noyce, et al. 2011). Others found that HSV VLPs and fusogenic liposomes triggered a STING-dependent upregulation of ISGs in murine cells (Holm, et al. 2012). However, neither group found how cells recognize membrane fusion and how this leads to activation of IRF3 and/or STING.

To define the number of particles sufficient to trigger IFN-independent antiviral protection we determined that 13 and 84 particles/cell of SeV-UV and

HCMV-UV, respectively, were sufficient to trigger an antiviral response in HEL fibroblasts in the absence of detectable IFN. This low level of UV-virus infection was used in subsequent experiments to model the IFN-independent antiviral response to enveloped virus particles.

We next investigated pathways that were activated upon enveloped virus infection with links to innate immunity. Ca^{2+} has been previously implicated in IRF3 activation, suggesting a possible role in signalling membrane perturbation (Servant, et al. 2001). HSV infection triggers cytosolic Ca^{2+} influx from intracellular stores within minutes of addition of virus to cells (Cheshenko, et al. 2003; Cheshenko, et al. 2007). This Ca^{2+} response to HSV involved PI3K and FAK, which have both been implicated in IFN regulation and signalling (Noyce, Collins, and Mossman 2006; Bozym, et al. 2012). We found that cytosolic Ca^{2+} oscillations were triggered by a variety of diverse enveloped virus particles and played an important role in the antiviral response. The involvement of Ca^{2+} signalling in the antiviral response to diverse enveloped viruses suggests that Ca^{2+} signalling is triggered by membrane perturbation during enveloped virus entry and contributes to upregulation of antiviral ISGs.

Given the involvement of STING in the antiviral response to membrane fusion (Holm, et al. 2012), we tested the importance of STING for the antiviral response to SeV-UV and HCMV-UV. Surprisingly, we found that STING was necessary for the antiviral response to UV-inactivated enveloped DNA viruses

like HCMV, while MAVS was necessary for the antiviral response to RNA viruses like SeV. The involvement of well characterized nucleic acid sensing pathways suggests that packaged viral nucleic acid is recognized by cellular PRRs. Interestingly, we found that Ca^{2+} signalling lay upstream of STING activation during HCMV-UV infection, suggesting that both membrane perturbation and nucleic acid sensing culminate in activation of STING during infection with HCMV-UV.

Our work suggests that only a few virus particles are necessary to trigger an antiviral response, suggesting an exquisite sensitivity to non-replicating enveloped virus. The involvement of Ca^{2+} oscillations in the antiviral response to all enveloped virus particles tested suggests that Ca^{2+} signalling triggered in response to membrane perturbation is involved in signalling an antiviral response. It remains unclear how membrane perturbation is sensed or exactly how IRF3 is activated, but the involvement of Ca^{2+} signalling suggests pathways linking membrane perturbation to Ca^{2+} release and Ca^{2+}-dependent pathways upstream of IRF3. Additionally, we found that nucleic acid sensing is essential for the antiviral response to enveloped virus particles, even in the absence of replication. We previously thought that membrane perturbation was the primary driver of antiviral gene upregulation. These results suggest instead that membrane perturbation may compliment recognition of minute amounts of viral nucleic acid delivered within enveloped virus particles.

Membrane perturbation-associated Ca^{2+} signalling and incoming genome sensing are required for the host response to low-level enveloped virus particle entry

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Running title: Host response to low-level enveloped virus entry

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Abstract

The type-I interferon (IFN) response is an important aspect of innate antiviral defense and the transcription factor IRF3 plays an important role in its induction. Membrane perturbation during fusion, a necessary step for enveloped virus particle entry, appears sufficient to induce transcription of a subset of IFN-stimulated genes (ISGs) in an IRF3-dependent, IFN-independent fashion. IRF3 is emerging as a central node in host cell stress responses, although it remains unclear how different forms of stress activate IRF3. Here we investigated the minimum number of Sendai virus (SeV) and human cytomegalovirus (HCMV) particles required to activate IRF3 and trigger an antiviral response. We found that Ca^{2+} signalling associated with membrane perturbation and recognition of incoming viral genomes by cytosolic nucleic acid receptors are required to activate IRF3 in response to fewer than 13 particles of SeV and 84 particles of HCMV per cell. Moreover, it appears that Ca^{2+} signalling is important for activation of STING and IRF3 following HCMV particle entry, suggesting that Ca^{2+} signalling sensitizes cells to recognize genomes within incoming virus particles. To our knowledge, this is the first evidence that cytosolic nucleic acid sensors recognize genomes within incoming virus particles prior to virus replication. These studies highlight the exquisite sensitivity of the cellular response to low level stimuli and suggest that virus particle entry is sensed as a stress signal.

Importance

The mechanism by which replicating viruses trigger IRF3 activation and type I IFN induction through the generation and accumulation of viral pathogen associated molecular patterns has been well characterized. However, the mechanism by which enveloped virus particle entry mediates a stress response, leading to IRF3 activation and the IFN-independent response, remained elusive. Here, we find that Ca^{2+} signalling associated with membrane perturbation appears to sensitize cells to recognize genomes within incoming virus particles. To our knowledge, this is the first study to show that cytosolic receptors recognize genomes within incoming virus particles prior to virus replication. These findings not only highlight the sensitivity of cellular responses to low level virus particle stimulation, but provide important insights as to how non-replicating virus vectors or synthetic lipid-based carriers used as clinical delivery vehicles activate innate immune responses.

Introduction

Cells defend themselves from viral infection by producing antiviral proteins, which cumulatively make them non-permissive to virus replication (Fensterl and Sen 2009). Large sets of antiviral proteins are induced by type-I interferons (IFNs), and this response is critical for defense against viral infection (Muller, et al. 1994; Sancho-Shimizu, et al. 2011). IFN-β has no direct antiviral activity but signals induction of a set of IFN-stimulated genes (ISGs) encoding proteins with antiviral activity (Schoggins and Rice 2011; Fensterl and Sen 2015). IFN-β is produced by a wide array of cells, and as the first IFN subtype produced in response to virus infection, many subsequent immune responses hinge on this initial signal (Honda, Takaoka, and Taniguchi 2006; Crouse, Kalinke, and Oxenius 2015; Swiecki and Colonna 2011). Following viral recognition, the transcription factors NF-κB, ATF2/c-Jun, and IFN-regulatory factor 3 (IRF3) are activated and form an enhanceosome on the IFN-β promoter, critical for its induction (Fensterl and Sen 2009; Thanos and Maniatis 1995).

While certain stimuli activate the IFN pathway to induce ISGs, low-level infection with enveloped virus particles is sufficient to directly induce a subset of ISGs in the absence of IFN (Preston, Harman, and Nicholl 2001; Mossman, et al. 2001). Unlike IFN-β production, which can occur in an IRF3-independent fashion (DeWitte-Orr, et al. 2009; Sato, et al. 2000; Takaki, et al. 2014), IFN-independent induction of ISGs by virus particles occurs in an IRF3-dependent, NF-κB-

independent manner (Paladino, et al. 2006; Collins, Noyce, and Mossman 2004). Based on our observations that the threshold for activation of IRF3 is lower than that of NF-κB (Paladino, et al. 2006), we previously proposed a model in which the IFN-independent antiviral response serves to efficiently and quietly induce a localized and primarily intracellular protective response to low-level virus stimulation, without inducing unwanted or unnecessary immune activity (Paladino, et al. 2006). The ability of IRF3 to function independent of the IFN-β enhanceosome and in the absence of traditional markers of activation (Noyce, Collins, and Mossman 2009; Collins, Noyce, and Mossman 2004) suggests a means of IRF3 activation distinct from the canonical virus-activated signalling pathway.

Canonical activation of IRF3 by virus infection requires recognition of pathogen associated molecular patterns (PAMPs) by pattern recognition receptors (PRRs) (Kumar, Kawai, and Akira 2011; Hiscott 2007). The IFN-independent response is associated with the entry of enveloped virus particles (Collins, Noyce, and Mossman 2004; Mossman, et al. 2001; Preston, Harman, and Nicholl 2001; Tsitoura, et al. 2009). All enveloped viruses must fuse with cell membranes during entry, and reports suggest membrane fusion itself is sufficient to induce ISGs. Enveloped virus particles and lipoprotein complexes containing purified reovirus fusion-associated small transmembrane (p14-FAST) protein directly induce ISGs in primary fibroblasts (Collins, Noyce, and Mossman

2004; Noyce, et al. 2011) while in immune cells, virus-like particles and fusogenic liposomes induce type-I IFN (Holm, et al. 2012). Interestingly, membrane fusion by p14 lipoplexes induces the same non-canonical IRF3 activation and ISG subset as virus particles. A number of stress pathways have been associated with non-canonical IRF3 activation, leading us to speculate that like cell stress, membrane perturbation is sensed as a danger signal of infection (Collins and Mossman 2014; Hare and Mossman 2013).

One cellular pathway associated with both stress and innate signalling is the Ca^{2+} signalling pathway (Collins and Mossman 2014; Smith 2014). Ca^{2+} influx to the cytoplasm acts as a second messenger and can originate from outside the cell or from ER-associated stores (Berridge, Bootman, and Roderick 2003). Signalling often takes the form of spikes or oscillations in cytoplasmic Ca^{2+} and utilizes localization and/or oscillation frequency to confer signalling specificity (Dupont, et al. 2011; Berridge, Bootman, and Roderick 2003). Entry of herpes simplex virus (HSV) or virus-like particles (VLPs) causes rapid Ca^{2+} influx from intracellular stores (Cheshenko, et al. 2007; Holm, et al. 2012; Cheshenko, et al. 2003), providing evidence of a link between Ca^{2+} signalling and membrane perturbation. Furthermore, Ca^{2+} mobilized ER-stress is sufficient to activate IRF3 and enhance induction of certain ISGs, while other pathways require Ca^{2+} signalling for IRF3 activation and full ISG induction (Liu, et al. 2012; Liu, et al.

2008). These results suggest Ca^{2+} signalling acts as a danger signal priming the response to viral infection.

To explore the hypothesis that entry of low levels of enveloped virus particles is detected as a danger signal, prior to virus replication and a prototypic PAMP response, we set to investigate the cellular pathways induced by membrane perturbation and entry of low levels of enveloped virus particles. We found that recognition of low-level enveloped virus particle entry involves sensing both membrane perturbation and incoming viral genomes, and that Ca^{2+} signalling plays a central role.

Materials and Methods

Cells and reagents

Human embryonic lung (HEL) fibroblasts (American Type Culture Collection, ATCC) were maintained in DMEM supplemented with 10% FBS. Murine embryonic fibroblasts (MEF) were obtained from wildtype, STING$^{-/-}$ golden ticket (Jackson), and MyD88$^{-/-}$ MAVS$^{-/-}$ TLR3$^{-/-}$ triple knockout (TKO) mice and maintained in α-MEM supplemented with 12% FBS. All media was supplemented with 1% L-glutamine. The Ca^{2+} inhibitors 2-aminoethyl diphenylborinate (2-APB) and BAPTA-AM (Life Tech) were reconstituted in methanol or DMSO respectively and diluted in serum-free media to a working concentration of 200 µM or 10 µM. Cells were pretreated with 2-APB for 60 minutes or BAPTA-AM for 30 minutes prior to treatment and the inhibitor was present for the duration of the experiment. The synthetic dsRNA mimetic poly IC was resuspended in PBS and diluted in serum-free media to a working concentration of 20 µM. Antibodies against SeV (kind gift of Dr. Yoshiyuki Nagai) and HCMV IE1 (Rumbaugh-Goodwin Institute) were used for western immunoblotting.

Viruses and p14 lipoplexes

Herpes simplex virus 1 (HSV-1; KOS strain) and vesicular stomatitis virus expressing GFP (VSV-GFP; Indiana strain) were grown and titred on Vero cells, human cytomegalovirus (HCMV; Ad169 strain) was grown and titred on HEL fibroblasts, Sendai virus (SeV; Cantell strain) was purchased from Charles River Laboratories and titred on CV-1 cells with 1 µg/ml TPCK-treated trypsin overlay, and replication-deficient adenovirus (AdV E1/E3) was grown and titred on HEK 293 cells. HSV-1 was ultraviolet (UV)-inactivated with 575 mJ/cm^2 and used at a multiplicity-of-infection (MOI) of 10 pfu/ml while HCMV and SeV were inactivated with 800 mJ/cm^2 and used at MOI of 0.02 pfu/cell and 0.14 pfu/cell, respectively, unless otherwise stated. UV-inactivation was performed using a CL-1000 ultraviolet crosslinker (UVP). All infections were performed for 1 hour in minimal media at 37^{o}C, except VSV-GFP infections which were done in 40 minutes. p14 lipoplexes were created by diluting 4 µg of purified p14 (kind gift of Roy Duncan) and 3 µl of lipofectamine 2000 in PBS and nuclease-free water respectively and incubating separately for 5 minutes before mixing.

VSV-GFP plaque reduction assay

Cells were conditioned with virus particles or p14 lipoplexes, later challenged with VSV-GFP, and F11 overlay media containing 1% FBS and 1% methyl-cellulose added to restrict plaques. Green fluorescence from VSV-GFP

was measured using a Typhoon laser scanner (GE Healthcare) and quantified using ImageQuant software. Fluorescence was then expressed as a percentage of unconditioned cells challenged with VSV-GFP.

Particle counting

Particles of SeV or HCMV were counted by tunable resistive pulse sensing (TRPS), based on the coulter principle, using a qViro-X particle counter (Izon). Virus stocks were diluted in filtered pH 7.4 formulation buffer containing 10mM, 150mM NaCl and 4% sucrose, and sonicated briefly before measurement. Diluted samples were run through a ~400 nm diameter pore at constant stretch, pressure, and voltage. The amplitude and frequency of disruptions in the current signal trace correspond to size and concentration of particles, while beads of known size and concentration were run to calibrate the measurements and give quantitative data. All measurement and analysis were done using Izon protocols and software.

Calcium microscopy

HEL cells were grown on glass-bottomed Petri plates for fluorimetry, and changes in Ca^{2+} concentration were monitored using Oregon green (Invitrogen, USA), a Ca^{2+}-indicator dye. A stock solution of Oregon Green was prepared in

DMSO and 20% pluronic acid. Cells were incubated with Oregon Green (5 µM) and sulfobromophthalein (100µM) for 30 min at 37°C and then treated with different particles for another 30 min. Cells were infected with HSV-UV, HCMV-UV, or SeV-UV at 10 pfu/cell, 0.04 pfu/cell, or 8 x 10^{-5} HAU/cell respectively to visualize Ca^{2+} signalling under conditions where IFN is not induced. Cells were then placed in a Plexiglass recording chamber and perfused with HBSS solution for a period of 15-30 min prior to experimentation to allow for complete dye hydrolysis. Confocal microscopy was then performed at room temperature (21–23^0C) using a custom-built apparatus based on an inverted Nikon Eclipse TE2000-4 microscope (Mukherjee, et al. 2012); recording rate was generally 1 frame/s. Picture frames were stored in TIF stacks of several hundred frames on a local hard drive using image acquisition software (Video Savant 4.0; IO Industries, London, ON). Image files were then analyzed using ImageJ software.

RT-PCR

RNA was extracted using TRIzol reagent (Invitrogen) and treated with DNAase (Ambion) as per the manufacturers' instructions. 500 ng of RNA was reversed transcribed using SuperScript II Reverse Transcriptase (Invitrogen) and a random hexamer primer as per the manufacturer's instructions. The abundance of cDNAs were measured using specific Taqman probes and reagents on a StepOnePlus Q-PCR instrument (Applied Biosystems) as per the manufacturer's

instructions. Ct values were calculated and GAPDH was used as an endogenous control to calculate individual ΔΔCt values. ΔΔCt values of samples were compared with mock treated samples to calculate fold change. Specific probes for human GAPDH (Hs02758991_g1), ISG56 (Hs03027069_s1), STING (Hs00736958_m1), and cGAS (Hs00403553_m1) or murine GAPDH (Mm99999915_g1) and ISG56 (Mm00515153_m1) from ThermoFisher were used.

siRNA knockdown

Cells at 50% confluence were transfected with pooled Stealth siRNA sequences directed against STING or cGAS (HSS139156-58 and HSS132955-57 purchased from Life Technologies). Specific or non-targeting control siRNAs were diluted in OptiMEM media (Life Tech), combined with RNAiMAX lipofectamine (Life Tech), and added to cells, as per the manufacturer's instructions. Media were changed 6 hours after addition of siRNA to reduced serum DMEM and experiments carried out at 72 hours post-transfection, corresponding with optimal knockdown.

Immunofluorescence

Fibroblasts were seeded on acid-washed glass coverslips to reach approximately 50% confluency. Five hours post-infection, cells were formalin-fixed, permeabilized in 0.1% Triton-X PBS, and blocked in 3% FBS, 3% goat serum in PBS. IRF3 (Santa Cruz FL-425) diluted 1:250 or anti-STING (Abcam EPR13130) diluted 1:200 in block followed by anti-rabbit AlexaFluor488 (Life Tech) diluted 1:400 in block and Hoechst dye diluted in PBS. A Leica DM IRE2 microscope was used and IRF3 positive nuclei were counted using OpenLab software and calculated as a percentage of total nuclei.

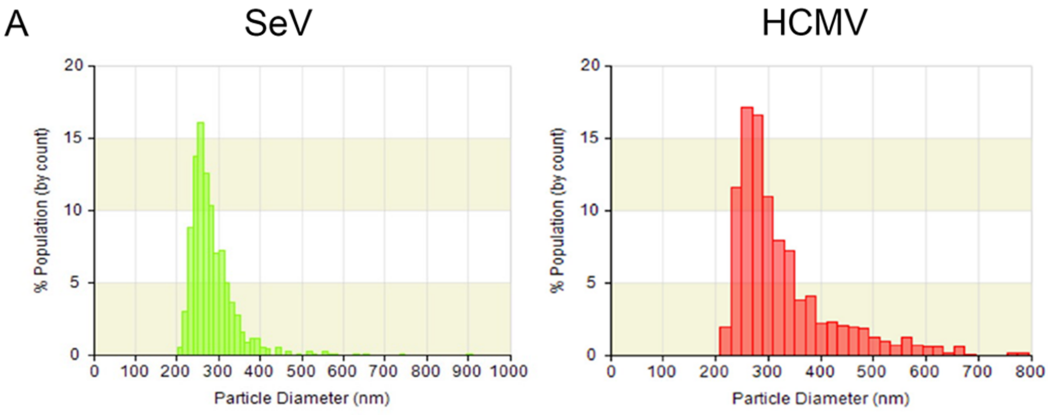
Results

Low numbers of enveloped virus particles are sufficient for IFN-independent ISG induction

We previously proposed that sensing membrane perturbation plays an important role in the first line of defense against enveloped viruses (Noyce, et al. 2011; Collins, Noyce, and Mossman 2004). Both membrane perturbation and low-level infection with enveloped virus particles induce a similar IFN-independent response (Mossman, et al. 2001; Noyce, et al. 2011; Paladino, et al. 2006). However, it remains unclear what signals are required to overcome the activation threshold of IRF3. To define low-level infection, we previously described the number of plaque forming units (pfu) sufficient to induce an IFN-independent antiviral response (Paladino, et al. 2006). Titres based on replication competency, however, rarely correspond to the number of virus particles, and our studies indicate that replication is not required for IRF3 activation. To understand how many physical virus particles cells are exposed to, we used tunable resistive pulse sensing (TRPS) to measure particle number, with SeV and HCMV used as representative RNA and DNA viruses respectively. We measured size distribution (Figure 1A), as well as particle concentration (Figure 1B), of our stocks. We determined that as few as 13 particles from the SeV preparation or 84 particles from the HCMV preparation per cell are sufficient for full antiviral protection in HEL cells. We further measured the number of particles in extracts

from uninfected cells, purified similarly to HCMV, and found particles (< 1 log in abundance) of similar size to HCMV that on their own do not contribute to antiviral protection (data not shown). Therefore, while we cannot determine the exact number of virus particles required to illicit a response, it is likely lower than we have estimated. Unlike virus particles, p14 lipoplexes adopt ill-defined size and shape, and thus cannot be counted using this technique.

A



B

	Titre (stock)	Concentration (stock)	MOI	Particles/cell
SeV-UV	2.7 x 10^7 pfu/ml	2.6 ± 0.2 x 10^9 particles/ml	0.14 pfu/cell	13
HCMV-UV	1.8 x 10^6 pfu/ml	7.6 ± 1.45 x 10^9 particles/ml	0.02 pfu/cell	84

Figure 1 – Low particle multiplicity sufficient to induce an antiviral response. Virus stocks of SeV or HCMV were diluted and their properties measured using tunable resistive pulse sensing. The size distribution (A) and particle concentration, with standard error (B), of virus stocks were calculated by comparing the amplitude and frequency of pore obstructions to calibration beads of known size and concentration. Plaque forming units were converted to total virus particles when determining how many particles are sufficient to induce an IFN-independent response.

Ca^{2+} oscillations are associated with lipid-based particle stimulation

To investigate whether Ca^{2+} signalling is an inherent feature of envelope fusion with the cell, we measured cytosolic Ca^{2+} following addition of virus particles. The minimum numbers of UV-inactivated SeV, HCMV, or HSV-1 particles necessary for an antiviral response were added, while 500 particles/cell of non-replicating AdV were added as a control. We found that all UV-inactivated enveloped virus particles, as well as p14 lipoplexes, induced Ca^{2+} oscillations (Figure 2). Although the amplitude and frequency of these oscillations varied between experiments and between cells, we reproducibly detected similar oscillation patterns with all of the lipid-based particles relative to mock-treated cells. In subsequent discussion of our results we use the term Ca^{2+} signalling to refer to the oscillation patterns observed. Non-replicating E1/E3-deleted adenovirus was used as a control and did not induce calcium oscillations. The variety of lipid-based particles which induce Ca^{2+} oscillations suggest that Ca^{2+} signalling is associated with entry of enveloped particles.

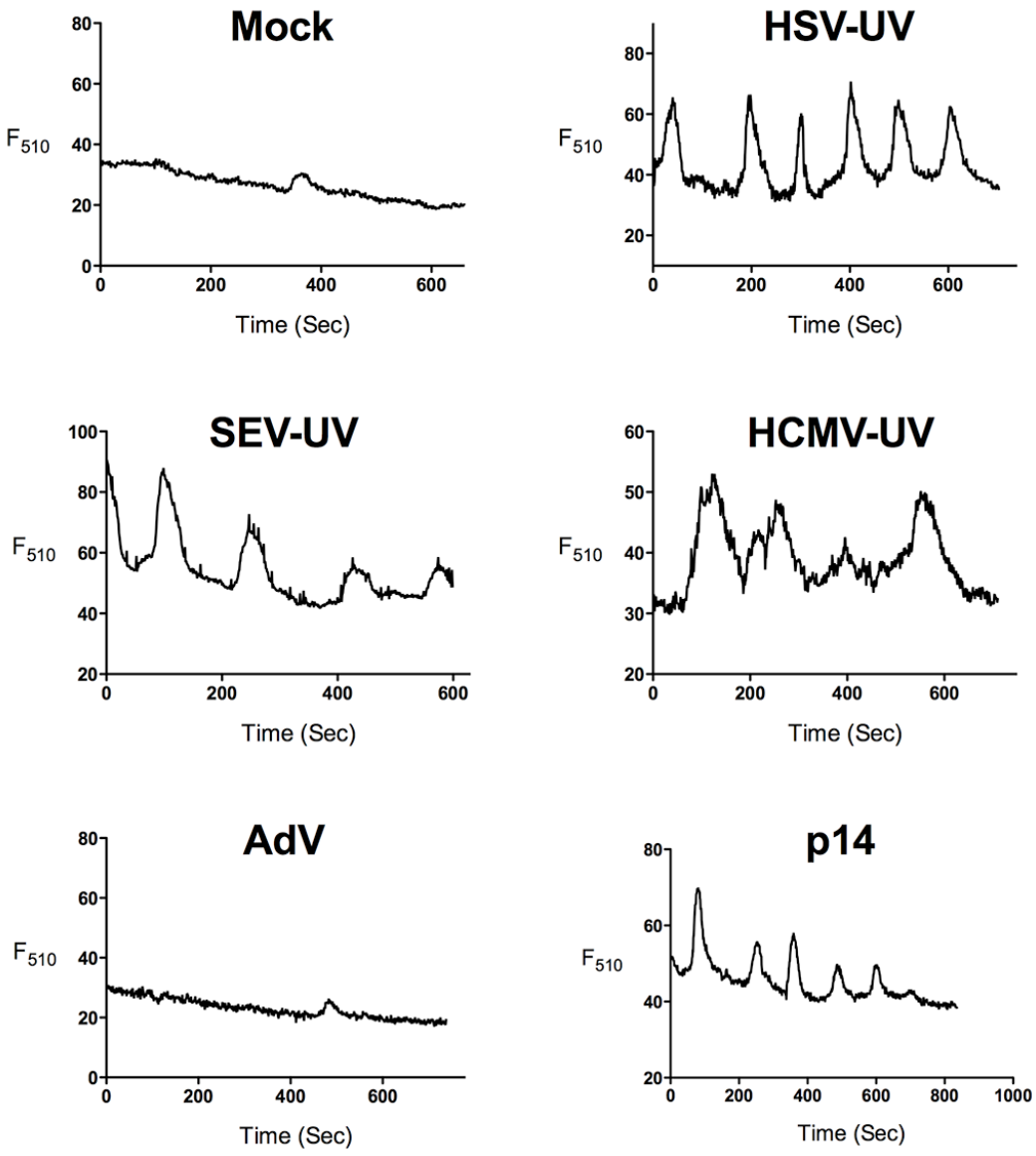


Figure 2 – Ca2+ oscillations are associated with lipid-based particles.
HEL fibroblasts were loaded with Ca^{2+} sensitive dye and mock infected, infected with UV-inactivated HSV (178 particles/cell), SeV (13 particles/cell), HCMV (84 particles/cell), live AdV (500 particles/cell), or treated with p14 lipoplexes. Fluorescence of representative cells, beginning 45 minutes after the addition of treatment, is plotted above.

Ca^{2+} oscillations are required for the response to membrane perturbation and enveloped virus particle entry

To determine the role of Ca^{2+} signaling in the antiviral response to membrane perturbation we used the inhibitor 2-APB, which broadly disrupts Ca^{2+} signalling. Treatment with 2-APB was sufficient to completely abolish Ca^{2+} signalling following HSV-UV infection (Figure 3A).

Membrane perturbation by p14 lipoplexes induces ISGs and antiviral protection in the absence of nucleic acid (Noyce, et al. 2011), making it the simplest lipid-based particle to examine pathways leading to activation of the key node protein IRF3. We treated HEL fibroblasts with p14 lipoplexes in the presence or absence of 2-APB, which broadly disrupts Ca^{2+} signalling (Bootman, et al. 2002; Peppiatt, et al. 2003), and measured antiviral protection. Disruption of Ca^{2+} signalling completely prevented the antiviral response to p14 lipoplexes, suggesting that Ca^{2+} is necessary for the antiviral response to membrane perturbation mediated by this stimulus (Figure 3B).

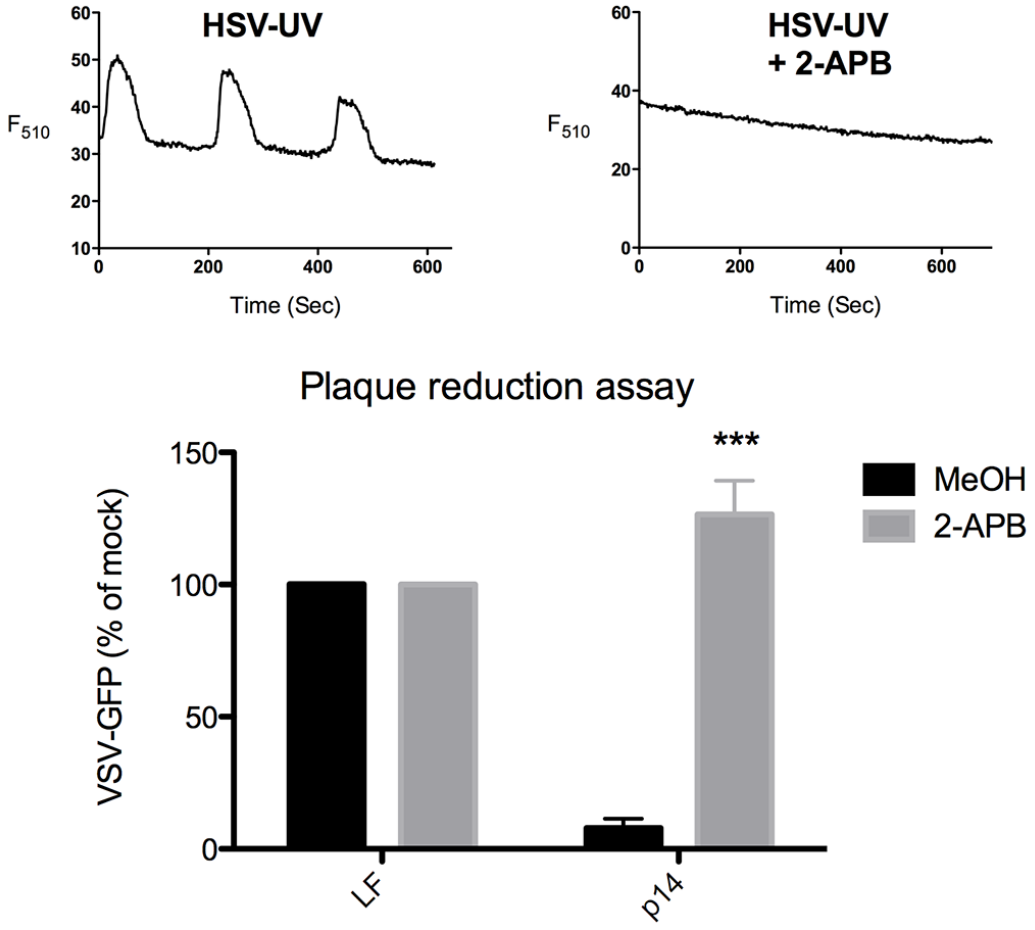


Figure 3 – Ca^{2+} signalling is required for the antiviral response to membrane perturbation.

HEL fibroblasts were infected with UV-inactivated HSV (178 particles/cell) in the presence of the inhibitor 2-APB or MeOH alone and fluorescence of representative cells, beginning 45 minutes after infection, is plotted above (A). HEL fibroblasts were treated with p14 lipoplexes or lipofectamine 2000 alone in the presence of 2-APB or MeOH alone and challenged with VSV-GFP in a plaque reduction assay 12 hours later (B). GFP fluorescence was quantified from plate scans and plotted as a percentage of unconditioned VSV-GFP infected cells. The average and standard error of 3 biological replicates was determined, and significance calculated by unpaired t-test.

Next, we examined the role of membrane perturbation and Ca^{2+} signalling in the response to enveloped virus particles. Disrupting Ca^{2+} with 2-APB during SeV-UV or HCMV-UV infection significantly reduced antiviral protection (Figure 4A). 2-APB also limited ISG induction under these conditions (Figure 4B). In addition, we observed that specifically chelating intracellular Ca^{2+} with BAPTA-AM significantly reduced the antiviral response to SeV-UV and HCMV-UV (Figure 4C). Similar experiments chelating extracellular Ca^{2+} with EGTA showed no effect (data not shown). Additionally, inhibition of Ca^{2+} signalling had no effect on the antiviral protection of poly IC, which robustly produces IFN under the experimental conditions used. Finally, we probed protein extracts with virus-specific antibodies, and saw that Ca^{2+} chelation had little to no effect on entry and early gene expression during live virus infection (Figure 4D).

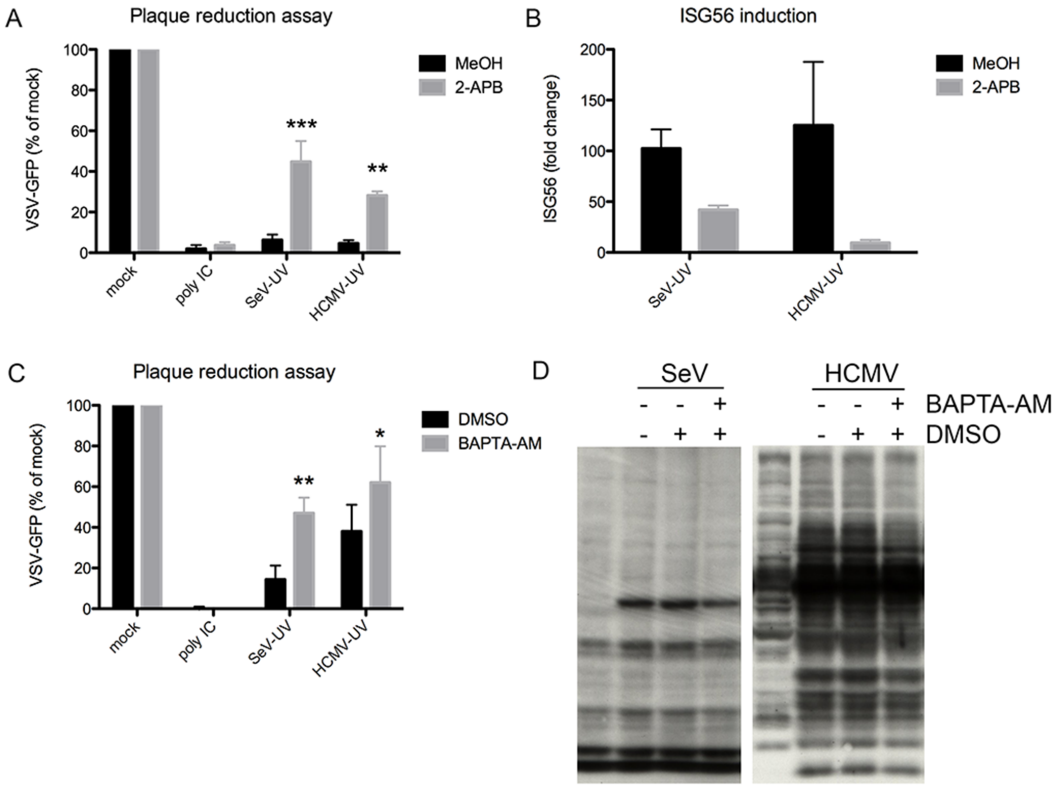


Figure 4 – Ca^{2+} signalling is involved in the antiviral response to enveloped virus particles.

HEL cells were infected with SeV-UV (13 particles/cell) or HCMV-UV (84 particles/cell) in the presence of 2-APB or MeOH alone and challenged with VSV-GFP in a plaque reduction assay 12 hours later (A) or ISG56 induction measured by quantitative RT-PCR, as described in the methods, after 6 hours (B). Cells similarly infected in the presence of BAPTA-AM or DMSO alone were challenged with VSV-GFP in a plaque reduction assay 7 hours after initial infection (C). GFP fluorescence is plotted as a percentage of mock treated VSV-GFP infected cells. Protein extracts from cells infected with live SeV (13 particles/cell) or HCMV (84 particles/cell), in the presence of BAPTA-AM or DMSO, were probed with virus-specific antibodies to measure viral entry and gene expression (D). All graphs report the average and standard error of 3 biological replicates. Significance was calculated by two-way ANOVA and Bonferroni post-tests.

Sensing of packaged genomic nucleic acid during low-level enveloped virus particle infection is required for the antiviral response

While disruption of Ca^{2+} signalling completely blocked the antiviral activity of p14 lipoplexes, only a partial, but statistically significant, block was observed in response to enveloped virus particles, suggesting that additional pathways are involved in particle recognition. Although cytoplasmic PRRs recognize viral nucleic acids that accumulate during viral replication, it is not known how sensitive these PRRs are to incoming packaged genomes. To determine whether nucleic acid sensing plays a role in response to low-level enveloped particle treatment, we asked whether known PRR signalling pathways contribute to IRF3 activation and subsequent ISG induction. We used primary MEFs lacking essential components of either RNA or DNA sensing pathways. Triple knock out (TKO) MEFs deficient in TLR3, MyD88 and MAVS were used to investigate the role of RNA sensing, while MEFs derived from Golden-ticket mice encoding a null mutation in STING were used to investigate the role of DNA sensing (Sauer, et al. 2011). Unlike wildtype MEFs, treatment of TKO MEFs with SeV-UV did not induce antiviral protection (Figure 5A) or upregulation of ISG56 (Figure 5B). Similarly, STING KO MEFs had an impaired response to HCMV-UV (Figure 5A & C). As expected, the absence of STING did not impair the response to SeV-UV, and TKO MEFs responded fully to HCMV-UV (Figure 5A).

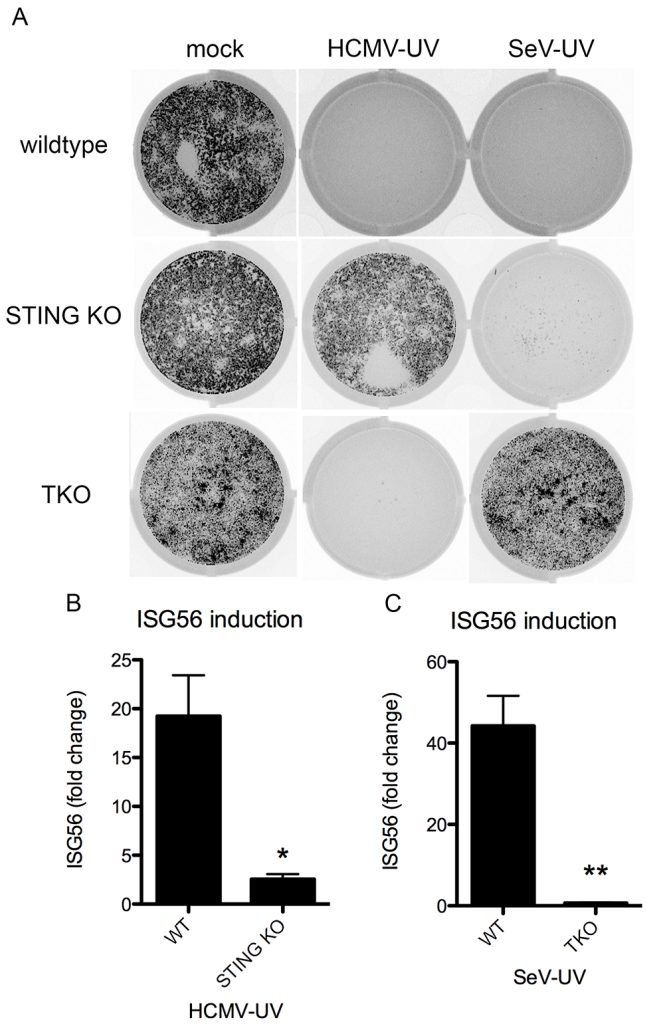


Figure 5 – Nucleic acid sensing pathways are necessary for the antiviral response to low-level incoming virus particles.
Wildtype or triple knockout (TKO) MEFs lacking MAVS, TLR3, and MyD88 were mock infected or infected with SeV-UV (7 particles/cell) or HCMV-UV (84 particles/cell). MEFs were then challenged with VSV-GFP in a plaque reduction assay and a representative plate scan included (A) or ISG56 induction measured by quantitative RT-PCR after 12 hours and plotted relative to mock infected cells (B). Wildtype or STING KO MEFs were infected and antiviral protection (C) and ISG56 induction measured similarly (D). Fold change from quantitative RT-PCR was calculated as described in the methods and graphed with the average and standard deviation of 3 biological replicates. Significance was calculated by unpaired t-test.

While STING has been characterized as a critical adaptor in DNA sensing (Tanaka and Chen 2012), additional roles for STING have been suggested, including recognition of envelope-membrane fusion (Liu, et al. 2012; Maringer and Fernandez-Sesma 2014; Holm, et al. 2012). Although the ability of SeV-UV particles to induce full antiviral protection in STING KO MEFs suggests that STING participates in sensing DNA genomes and not envelope-membrane fusion *per se*, we asked whether the associated cytoplasmic DNA sensor cGAS is required for the antiviral response to HCMV-UV. We first depleted STING or cGAS in HEL fibroblasts using pooled siRNA sequences and then infected cells with HCMV-UV. STING and cGAS mRNA were reduced by more than 90% for up to 72 hours post-transfection prior to experiments (Figure 6A). Both STING and cGAS knockdown similarly reduced antiviral protection and ISG56 induction in response to incoming HCMV-UV particles (Figure 6B-C), but had no effect on the response to SeV-UV particles (data not shown). Taken together, these data suggest that sensing of incoming packaged genomes is an essential component of the host response to enveloped virus particle entry, even with stimulation with as low as 7 (SeV) or 84 (HCMV) non-replicating virus particles per cell.

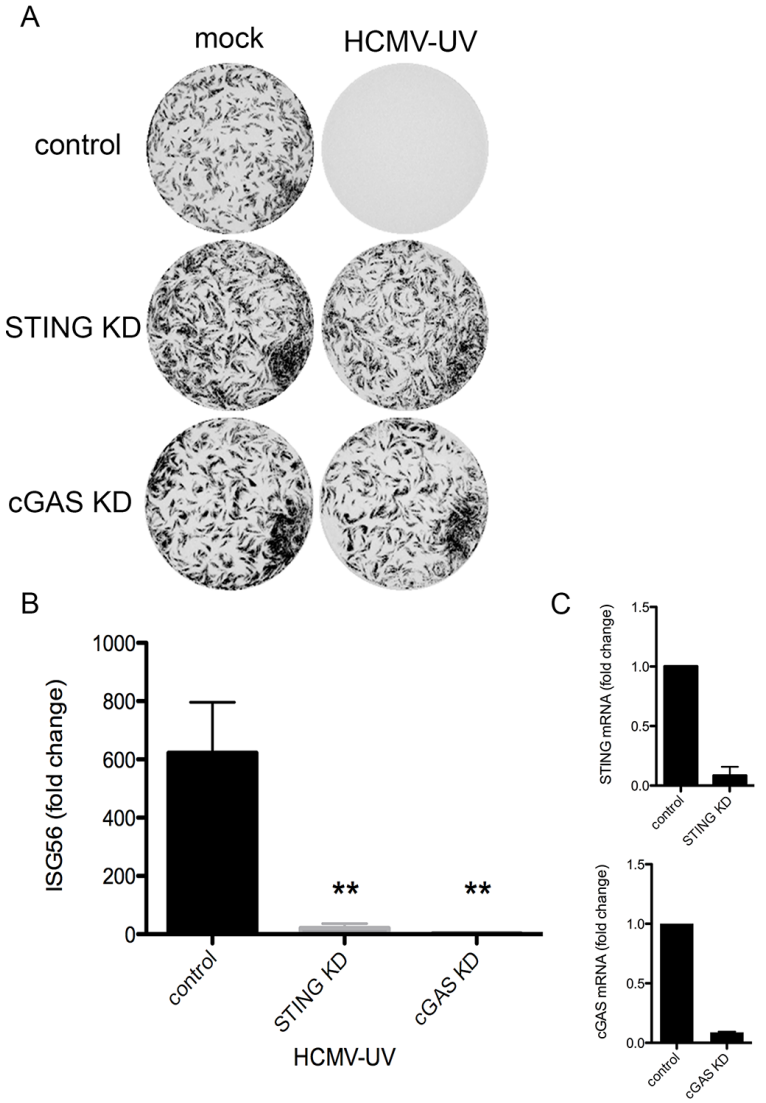


Figure 6 – The cytoplasmic DNA sensor cGAS is necessary for the antiviral response to incoming HCMV particles.

STING and cGAS were knocked down in HEL fibroblasts and transcript levels of STING and cGAS measured by quantitative RT-PCR after 72 hours and plotted relative to control siRNA transfection (A). After knockdown, cells were mock infected or infected with HCMV-UV (84 particles/cell) then challenged with VSV-GFP in a plaque reduction assay and a representative plate scan included (B) or ISG56 induction measured by quantitative RT-PCR and plotted relative to mock infected cells (C). Fold change from quantitative RT-PCR was calculated as described in the methods and graphed with the average and standard error of 3 biological replicates. Significance was calculated by two-way ANOVA and Bonferroni post-tests.

Ca^{2+} signalling is upstream of STING and IRF3 in HCMV particle recognition

The host response to enveloped virus particles appears to require multiple signals, including both Ca^{2+} signalling and viral genome recognition, prompting the question of where these signals converge upstream of antiviral gene induction. In this experiment, we used the fewest number of HCMV-UV particles that elicit clear activation of both STING and IRF3. Upon activation, STING translocates from the ER to cytoplasmic vesicles (Ishikawa, Ma, and Barber 2009; Dobbs, et al. 2015), where it acts as a scaffold for the activation of IRF3, which subsequently translocates to the nucleus. Both translocation events can be visualized using immunofluorescence microscopy. Preventing Ca^{2+} signalling with 2-APB reduced the relocation of STING to cytoplasmic foci (Figure 7A) as well as nuclear translocation of IRF3 following infection with HCMV-UV (Figure 7B), suggesting Ca^{2+} signalling lies upstream of STING activation in the response to HCMV-UV.

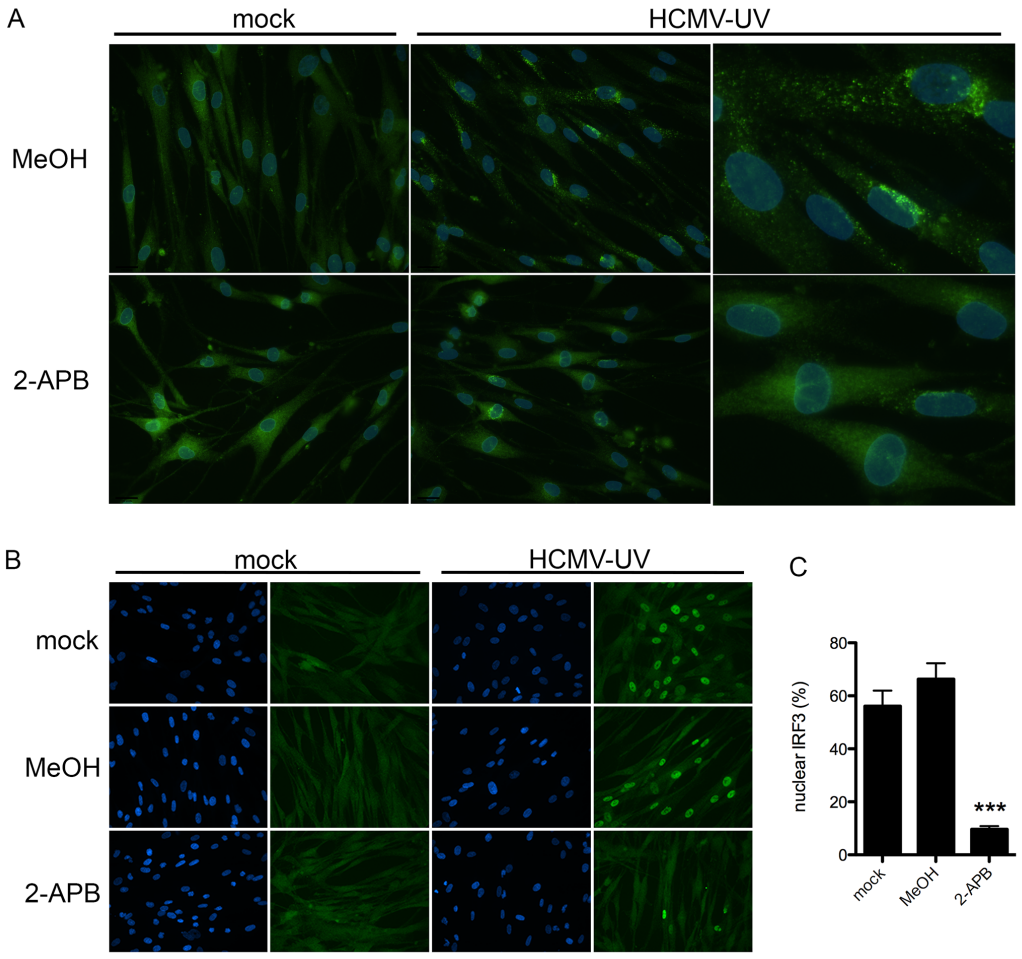


Figure 7 – Ca^{2+} signalling is important for activation of STING and IRF3 following entry of HCMV particles. HEL fibroblasts were infected with HCMV-UV (840 particles/cell) in the presence of 2-APB or MeOH alone and STING relocalization visualized by immunofluorescence 4 hours post-infection (A), or IRF3 translocation visualized 5 hours post-infection (168 particles/cell) (B). IRF3 translocation was graphed as the percentage of IRF3 positive nuclei and standard error from 4 biological replicates (C). Significance was calculated by unpaired t-test.

Discussion

We previously reported that enveloped virus particle entry or membrane perturbation by p14 lipoplexes induces an ISG subset through an IFN-independent, IRF3-dependent pathway (Collins, Noyce, and Mossman 2004; Noyce, et al. 2011). However, since the known pathways upstream of IRF3 involve nucleic acid sensing or TLR signalling through TRIF, neither of which are necessary for the IFN-independent induction of ISGs (Noyce, et al. 2011; Paladino, et al. 2006; Tsitoura, et al. 2009), the mechanisms by which low level enveloped virus particle exposure activates IRF3 remained elusive. Of interest, IRF3 is also activated in response to different forms of cellular stress, such as redox and ER stress, and cytoskeleton disruption (Mukherjee, et al. 2009; Tal, et al. 2009; Liu, et al. 2012; Servant, Grandvaux, and Hiscott 2002). Consistent with IRF3 being increasingly recognized as a key mediator of diverse host stress responses, it is likely that the activation profile of IRF3 is equally diverse. While activation of IRF3 following virus replication and accumulation of viral PAMPs can lead to common modifications, such as Ser385 and Ser396 hyperphosphorylation, the host response to more subtle stimuli, such as enveloped virus particle entry, often lacks canonical, or indeed any, IRF3 activation markers (Noyce, Collins, and Mossman 2009). Accordingly, we proposed that membrane perturbation serves as a stress or danger signal prior

to, or in the absence of, a prototypic pathogen-sensing response (Collins and Mossman 2014; Hare and Mossman 2013).

An intriguing pathway to investigate was the Ca^{2+} signalling pathway, as HSV-1 infection or VLP treatment induces rapid Ca^{2+} fluxes from intracellular stores (Cheshenko, et al. 2007; Holm, et al. 2012; Cheshenko, et al. 2003), and Ca^{2+} signalling is associated with stress responses, homeostatic regulation and innate signalling (Collins and Mossman 2014; Smith 2014). The Ca^{2+} activity we observed in primary fibroblasts appears to be a common feature following treatment with diverse lipid-based particles, but not non-enveloped viruses, despite the assumption that non-enveloped virus particles would need to perturb a cellular membrane in some capacity to access the interior of a susceptible cell. Enveloped viruses enter at either the cell membrane or from within endosomes, and thus any signal triggered during entry could originate from either membrane.

Our investigation has not uncovered how membrane perturbation is sensed and signals the release of Ca^{2+} from intracellular stores. The PI3K pathway commonly lies upstream of Ca^{2+} store-mediated signalling, and we and others have shown that the PI3K inhibitor LY294002 (LY) blocks the antiviral response to enveloped virus particles (Holm, et al. 2012; Noyce, Collins, and Mossman 2006). However, the exact means by which LY blocks antiviral gene induction is unknown. LY does not inhibit IRF3 nuclear translocation following entry of

enveloped virus particles but instead appears to modulate a pathway downstream of IRF3 activation (Noyce, Collins, and Mossman 2006). Moreover, the prototypic p85/p110 PI3K complex is not involved in the IFN-independent antiviral response (Noyce, Collins, and Mossman 2006). Thus, the relationship between Ca^{2+}-mediated and PI3K-mediated signalling during this response is unclear, and requires further investigation.

Although a common Ca^{2+}-dependent pathway exists for enveloped particle recognition, we found that recognition of incoming genomes, by cytoplasmic DNA and RNA sensing pathways, significantly contributed to the IFN-independent response. While previous findings suggested that neither virus replication nor TLR signalling are required (Collins, Noyce, and Mossman 2004; Paladino, et al. 2006; Tsitoura, et al. 2009), the contributions of incoming viral genomes or cytoplasmic nucleic acid sensing pathways were not fully evaluated. While diverse enveloped virus particles induce the IFN-independent antiviral response (Collins, Noyce, and Mossman 2004), we used SeV and HCMV particles as representative RNA and DNA virus particles, respectively. SeV belongs to the paramyxovirus family of non-segmented negative stranded RNA viruses, and releases its genome into the cytoplasm following entry and uncoating. RIG-I recognizes genomes of SeV and other negative stranded RNA viruses when transfected into the cell and activates the signalling adaptor MAVS to induce IFN (Rehwinkel, et al. 2010; Kato, et al. 2006). However, the ability of RIG-I and

MAVS to recognize incoming virus particles has not been described, especially in the range of 7-13 particles/cell used here. HCMV belongs to the herpesvirus family of dsDNA viruses, and following entry, capsids translocate to the nucleus and inject their genomes through the nuclear pore (Kobiler, et al. 2012). HCMV DNA can be recognized by DAI (ZBP2) in fibroblasts (DeFilippis, et al. 2010) and additionally IFI16 in macrophages (Horan, et al. 2013), but to our knowledge, no role for the cytoplasmic sensor cGAS has been shown. Conceptually, while incoming HCMV genomes should not be exposed within the cytoplasm, virus entry is an imperfect process and HCMV capsid degradation by the proteasome, and thus viral DNA release, has been demonstrated in macrophages (Horan, et al. 2013).

Unlike herpesviruses, AdV capsids uncoat in the cytoplasm at the nuclear pore prior to import, which leaves AdV genomes exposed at this bottleneck (Wang, et al. 2013). The cGAS/STING/TBK-1 pathway is capable of sensing cytoplasmic AdV DNA, leading to IRF3 activation (Lam, Stein, and Falck-Pedersen 2014; Stein and Falck-Pedersen 2012). In our studies, however, infection with 1000 pfu/cell of AdV particles failed to elicit a response (data not shown), while infection with 0.02 pfu/cell of HCMV particles efficiently induced a response. This represents greater than 25,000 particles/cell of AdV versus fewer than 100 particles/cell of HCMV. Thus, the simplest interpretation is that in human fibroblasts, DNA sensing of incoming viral genomes alone is insufficient to

activate IRF3, and requires additional signals such as Ca^{2+} oscillations. However, the nature, timing or amplitude of the Ca^{2+} signal is likely important (Berridge, Bootman, and Roderick 2003; Dupont, et al. 2011); indeed Ca^{2+} mobilization induced by ionomycin is insufficient to activate IRF3 (31). Consistent with these findings, preliminary experiments combining ionomycin treatment with AdV infection failed to elicit IRF3 activation and ISG induction (data not shown).

Here we show that Ca^{2+} signalling plays a role upstream of both STING and IRF3 activation following HCMV-UV infection. Rather than converging on the activation of IRF3 as we had hypothesized, Ca^{2+} signalling associated with membrane perturbation appears necessary for activation of STING. While no role for Ca^{2+} in STING activation had been observed previously, crystal structures of STING show an important Ca^{2+}-binding pocket at the interface of 2 STING dimers (Shu, et al. 2012; Burdette, et al. 2011). While these results suggest that Ca^{2+} signalling is similarly upstream of MAVS activation following stimulation with enveloped RNA virus particles, we were unable to reproducibly detect MAVS activation in primary fibroblasts to test this hypothesis.

p14 lipoplexes do not contain nucleic acid and the sensor upstream of Ca^{2+} is currently unknown. While one explanation is that the p14 protein is sensed by an unknown receptor, it is unlikely that p14 serves as a PAMP due to its size and structural dissimilarity with enveloped virus fusion proteins (Boutilier and Duncan 2011). It is intriguing that while we routinely detect ISG and antiviral

state induction to envelope virus particle entry within 6-8 hours in fibroblasts, the response to p14-lipoprotein complexes requires ~12 hours, despite induction of the same subset of ISGs (Mossman, et al. 2001; Noyce, et al. 2011). Studies are currently ongoing to elucidate the mechanism(s) by which p14-lipoprotein complexes and Ca^{2+} signalling lead to IRF3 activation.

Although we found that viral nucleic acid sensing was required for the antiviral response to incoming virus particles, we do not observe typical markers of IRF3 activation such as phosphorylation of S386 or S396. While this could be explained by simple limitations of detection when using so few particles, there are clear differences in IRF3 activation following infection with different live or inactivated virus preparations, despite equal induction of ISGs (Noyce, Collins, and Mossman 2009), suggesting that a linear relationship between canonical activation of IRF3 and ISG induction does not exist. Accumulating data instead suggest that non-canonical and undefined modifications additionally play a role in the activation of IRF3. Together with previous reports, our study underscores the complexity of the host intrinsic/innate response and how different cell types and species uniquely respond to incoming stimuli. For example, VLPs and fusogenic liposomes efficiently induce type I IFN and ISGs in human immune cells (Holm, et al. 2012). Similarly, AdV activates the cGAS/STING pathway and elicits IRF3 activation in murine macrophages (Lam, Stein, and Falck-Pedersen 2014; Stein and Falck-Pedersen 2012) while AdV infection of primary mouse lung

fibroblasts leads to type I IFN induction (Nociari, et al. 2007). Our studies also highlight the exquisite sensitivity of cells to incoming low level virus particle entry.

Little is known about how membrane perturbation leads to IRF3 activation and ISG induction, but our data suggest Ca^{2+} plays an important role in this process. Both Ca^{2+} signalling and recognition of packaged viral genomes contribute to IRF3 activation following low-level enveloped virus particle entry. However, we know very little about the interaction between pathways or how membrane perturbation alone leads to IRF3 activation. There is an emerging cross talk between cell stress responses and intrinsic/innate immunity (Collins and Mossman 2014; Hare and Mossman 2013), and further work examining the role of Ca^{2+} and related pathways in innate signalling will help uncover these pathways.

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Chapter 3

Virus-intrinsic differences and heterogeneous IRF3 activation influence IFN-independent antiviral protection

This manuscript was originally submitted to Cell Reports on Feb. 27, 2020 and was later transferred to iScience. It is currently under revision while an additional experiment is being completed.

Chapter Introduction

We previously observed upregulation of an IRF3-dependent subset of ISGs in the absence of detectable IFN when fibroblasts were infected with enveloped virus particles (Collins, Noyce, and Mossman 2004; Mossman, et al. 2001). As fibroblasts were exposed to increasing amounts of enveloped virus, activation of the transcription factor NF-κB and production of IFN were observed (Paladino, et al. 2006). Based on these results, we proposed that NF-κB-dependent upregulation of IFN has a higher activation threshold than IRF3-dependent upregulation of ISGs. Consequently, low levels of virus particles trigger IRF3-dependent upregulation of ISGs in the absence of IFN production.

The IFN-β promoter contains one of the earliest and best characterized enhancer elements. When cells detect virus infection, a transcriptional complex called the IFN-β enhanceosome containing ATF2/c-Jun, two IRF3/7 dimers and NF-κB forms on the IFN-β promoter. While assembly of the entire IFN-β enhanceosome was initially assumed to be necessary for IFN-β upregulation, subsequent studies have suggested that ATF2/c-Jun and NF-κB play accessory roles (Basagoudanavar, et al. 2011; Wang, et al. 2007; Peters, et al. 2002). Canonical IRF3 activation depends on phosphorylation of a cluster of C-terminal serine residues and Ser386 and Ser396 are both essential for IRF3 activation (Lin, et al. 1998; Mori, et al. 2004).

Because IRF3 is necessary for the IFN-independent antiviral response to enveloped virus particles we expected that markers of IRF3 activation would be detected in the majority of infected cells, consistent with antiviral protection throughout the monolayer. While some enveloped virus particles triggered clearly detectable IRF3 activation markers, other enveloped virus particles triggered an IRF3-dependent IFN-independent antiviral response in the absence of detectable IRF3 activation markers (Noyce, Collins, and Mossman 2009). While IRF3 activation is well characterized, there are many different post-translational modifications (Robitaille, et al. 2016). Thus, detection of IRF3 activation is complicated by the diversity of activated forms and our ability to detect these forms with conventional assays. Given the absence of detectable IRF3 activation markers in response to certain enveloped virus particles, we speculated that IRF3 activation occurs through a non-canonical pathway that confounds conventional assays for IRF3 activation (Noyce, Collins, and Mossman 2009).

This work updated what we knew about the IFN-independent antiviral response to enveloped virus particles and takes into account more recent advances in the field. We found that low level infection with enveloped virus particles triggered production of minute amounts of IFN below the detection threshold of traditional methods. We found that HCMV particles activated IRF3 and upregulated IFN and ISGs in the majority of cells while SeV particles activated IRF3 and upregulated IFN and ISGs in only a small fraction of cells.

Heterogeneous IRF3 activation and IFN-β production has been described for replicating SeV and there is some debate about whether this is due to heterogeneity in virus particles or stochastic signalling events. We detected little difference between IFN-β producing and non-producing cells in the number of SeV genomes or stimulatory defective viral genomes.

These findings advance our understanding of how cells respond to different enveloped viruses and modify our existing model. Instead of a multi-tiered response to infection, cells respond to infection by activating IRF3 and upregulating both IFN-β and a subset of ISGs. Once cell-to-cell variations in signalling and the involvement of IFN are taken into account, canonical IRF3 activation is sufficient to explain our observations. While heterogeneous IFN-β production has been described by others in the context of replicating virus, we found it interesting that heterogeneous IFN-β production occurred in the absence of virus replication.

Virus-intrinsic differences and heterogeneous IRF3 activation influence IFN-independent antiviral protection

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Summary

Type 1 interferon (IFN) plays a critical role in early antiviral defense and priming of adaptive immunity by signalling upregulation of host antiviral interferon-stimulated genes (ISGs). Certain stimuli trigger strong activation of IFN regulatory factor 3 (IRF3) and direct upregulation of ISGs in addition to IFN. It remains unclear why some stimuli are stronger activators of IRF3 and how this leads to IFN-independent antiviral protection. We found that UV-inactivated human cytomegalovirus (HCMV) particles triggered an IFN-independent ISG signature that was absent in cells infected with UV-inactivated Sendai virus (SeV) particles. HCMV particles triggered mostly uniform activation of IRF3 and low-level IFN-β production within the population while SeV particles triggered a small fraction of cells producing abundant IFN-β. These findings suggest that population level activation of IRF3 and antiviral protection emerges from a diversity of responses occurring simultaneously in single cells. Moreover, this occurs in the absence of virus replication.

Keywords: Interferon, innate, immunity, sensing, antiviral, enveloped, virus, particles, vector, dsRNA

Introduction

Virus infection is sensed by pattern recognition receptors (PRRs) that recognize viral particle components or by-products of replication, such as cytosolic double-stranded RNA (dsRNA) and DNA (dsDNA), and upregulate pro-inflammatory and antiviral interferon-stimulated genes (ISGs) through production of type 1 interferon (IFN). Antiviral ISGs collectively create an antiviral state in infected and surrounding cells to restrict virus spread while pro-inflammatory ISGs recruit immune effector cells. IFN signalling is critical for early control of infection, and productive virus replication is usually dependent on the virus's ability to antagonize IFN signalling (reviewed in (Fensterl and Sen 2009)).

Both replicating and non-replicating viruses are recognized and contribute to IFN upregulation but most replicating viruses simultaneously suppress IFN upregulation and signalling. In fact, many viruses trigger stronger IFN responses in the absence of virus gene expression and replication (Mossman, et al. 2001; Preston, Harman, and Nicholl 2001; Weber, et al. 2013; Collins, Noyce, and Mossman 2004). Consequently, the immune response to many important viral pathogens and vaccines is dependent on recognition of defective virus particles or abortive infection (O'Neal, et al. 2019; Drayman, et al. 2019; Yount, et al. 2006; Ho, et al. 2016). Recognition of virus particles can involve a combination of stimulatory components, such as viral glycoproteins or packaged nucleic acid, along with cellular perturbations, such as virus entry (Hare, et al.

2015a; Holm, et al. 2012; Juckem, et al. 2008). How cells recognize and respond to virus particles are important because these processes play a critical role in the immune response to virus infection as well as the immune response to non-replicating viral vectors.

IFN-β is the first IFN subtype produced during infection and can be produced by most cell types. The IFN-β promoter contains binding sites for IFN regulatory factors (IRF), NF-κB and AP1, which together modulate IFN-β expression (reviewed in (Balachandran and Beg 2011; Honda, Takaoka, and Taniguchi 2006)). IRF3 is a critical transcription factor for the upregulation of IFN-β, and is constitutively expressed in a majority of cells (Au, et al. 1995; Hiscott 2007). When IRF3 is phosphorylated by the kinase tank-binding kinase 1 (TBK1) it undergoes a conformational change that promotes its dimerization, nuclear accumulation and binding to the IFN-β promoter (Yoneyama, et al. 1998; Lin, et al. 1998; Sharma, et al. 2003; Fitzgerald, et al. 2003). Since NF-κB was first observed binding to the IFN-β enhanceosome, it has been shown to be important for early IFN-β upregulation, but it is not essential (Apostolou and Thanos 2008; Basagoudanavar, et al. 2011; Peters, et al. 2002). After IFN-β is produced and secreted, it binds to the IFN-α/β receptor (IFNAR) on the surface of nearby cells. IFNAR initiates Jak-STAT signalling and formation of a complex containing STAT1, STAT2 and IRF9 named IFN-stimulated gene factor 3 (ISGF3). The ISGF3 complex binds to IFN-stimulated response elements (ISREs) in the

promoters of ISGs, thus upregulating hundreds of genes involved in antiviral defence (reviewed in (Schoggins 2018)).

In addition to upregulating IFN-β, activated IRF3 can directly upregulate a subset of ISGs by binding to ISREs and forming a transcriptional complex with CBP/p300 (Bandyopadhyay, et al. 1995; Grandvaux, et al. 2002; Weaver, Kumar, and Reich 1998). IRF3-mediated upregulation of ISGs is thought to act as a first line of defense to limit virus replication in the infected cell, while IFN-β upregulates ISGs in nearby cells to halt spread of the virus. Low-level infection with non-replicating enveloped virus particles causes IRF3-mediated upregulation of ISGs in the absence of detectable IFN (Collins, Noyce, and Mossman 2004; Mossman, et al. 2001; Paladino, et al. 2006). While this response is IRF3-dependent, the extent of observable IRF3 posttranslational modifications fail to correlate with ISG induction (Noyce, Collins, and Mossman 2009). Activation of NF-κB and subsequent production of IFN were found to require a higher level of virus particle stimulation (Paladino, et al. 2006).

We and others have suggested that non-replicating enveloped virus particles are recognized upon membrane fusion that occurs during virus entry (Collins, Noyce, and Mossman 2004; Holm, et al. 2012; Noyce, et al. 2011; Tsitoura, et al. 2009). However, we found that sensing of genomic nucleic acid is critical for the antiviral response to low levels of enveloped virus particles (Hare,

et al. 2015a). Given that nucleic acid recognition is known to activate transcription factors necessary for IFN-β production, we revisited the role of IFN-β signalling in the antiviral response to two different enveloped virus particles. Sendai virus (SeV) belongs to the paramyxovirus family and is an enveloped, negative stranded, non-segmented RNA virus. Human cytomegalovirus (HCMV) belongs to the herpesvirus family and is an enveloped dsDNA virus. While SeV and HCMV are known to cause disease in mice and humans respectively, they are also commonly used models to study innate antiviral pathways.

Results

Fibroblasts mount an IFN-independent antiviral response to HCMV particles but not SeV particles

While we and others previously described ISG induction in the absence of detectable IFN production, we investigated the role of IFN signalling in the antiviral response to representative enveloped virus particles. We infected wildtype or IFNAR1 KO hTERT-immortalized human fibroblasts (THFs) with either UV-inactivated HCMV (HCMV-UV) or SeV (SeV-UV) particles and measured the antiviral response. IFNAR1 KO THFs are not responsive to IFN-I but remain capable of mounting IFN-independent antiviral responses to appropriate stimuli. Consistent with previous findings, wildtype THFs infected with low levels of

HCMV or SeV particles were protected from VSV-GFP in a plaque reduction assay and IFN was only detected in supernatants after THFs were infected with higher amounts of HCMV or SeV particles. Surprisingly, IFNAR1 KO THFs had a lower antiviral response to HCMV particles, requiring a higher number of particles to achieve full protection (figure 1A) and IFNAR1 KO THFs did not mount a protective antiviral response to SeV particles at all, regardless of input (figure 1B). These results suggest that the antiviral response to low levels of HCMV or SeV particles relies on low levels of IFN that are not detected by conventional supernatant transfers. To validate our hypothesis, we concentrated protein in supernatants from wildtype THFs infected with low levels of SeV particles and found that cells treated with these concentrated supernatants were protected (supplementary figure 1)

To further test whether IFN was necessary for the antiviral response to infection with virus particles in wildtype THFs, we blocked IFN signalling with B18R, a recombinant vaccinia virus IFN antagonist(Alcami, Symons, and Smith 2000; Symons, Alcami, and Smith 1995), prior to infection with HCMV or SeV particles, and measured the antiviral response using a plaque reduction assay. Similar to IFNAR1 KO THFs, B18R-treated wild type THFs mounted a protective antiviral response to infection with HCMV particles at high MOI but were not protected following infection with SeV particles (figure 1C-D)

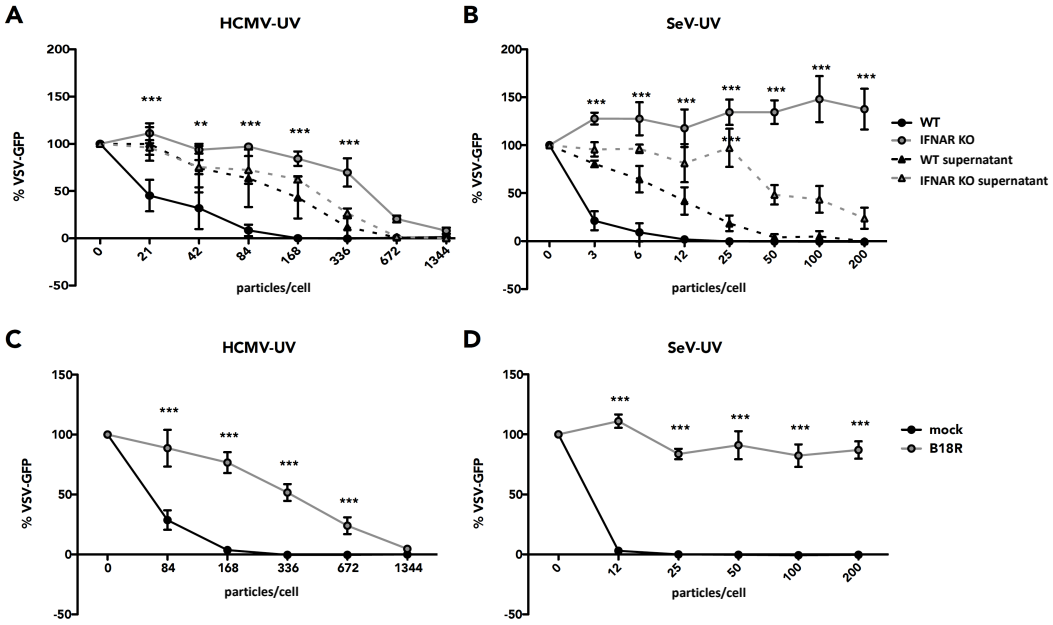

Figure 1 - Antiviral response to HCMV-UV and SeV-UV in the presence/absence of IFN signalling. (A-B) Wildtype or IFNAR1 KO THFs were infected with increasing levels of UV-inactivated HCMV before challenging with VSV-GFP 16 hours later. The presence of IFN in the supernatants was assayed by transferring supernatants from infected THFs to naïve wildtype THFs and challenging them with VSV-GFP 6 hours later (WT supernatant / IFNAR KO supernatant). (C-D) Wildtype THFs pretreated with B18R or media alone were infected with increasing levels of HCMV-UV or SeV-UV and challenged with VSV-GFP. Mean fluorescence intensity and standard error from 3 biological replicates was plotted as a percentage of mock infected cells challenged with VSV-GFP. Wildtype and IFNAR KO, wildtype and IFNAR KO supernatant, or mock and B18R treated THFs were compared by two-way ANOVA with Bonferroni post-tests (* for $p < 0.05$, ** for $p < 0.01$ and *** for $p < 0.001$).

IFN-III has been shown to upregulate a similar set of ISGs in cells expressing the IFN-λ receptor (IFNLR)(Sheppard, et al. 2003; Kotenko, et al. 2003), thus creating a protective antiviral state independent of IFNAR. IFNLR is expressed in cells of epithelial origin but is believed to be absent in fibroblasts. To determine whether THFs respond to IFN-λ we treated THFs or A549 cells, derived from lung epithelial carcinoma, with increasing amounts of recombinant IFN-λ1. We found that A549 cells responded to IFN-λ1 in a dose-dependent manner while THFs did not, consistent with THFs lacking IFNLR (supplementary figure 2). Therefore, the antiviral responses we observed in IFNAR1 KO THFs are independent of both IFNAR and IFNLR.

A subset of IFN-independent ISGs are upregulated in response to HCMV but not SeV particles

Our antiviral data suggested that HCMV particles were capable of stimulating both IFN-independent and IFN-mediated responses while SeV particles only stimulated IFN-mediated responses. To better understand the gene products involved, we employed whole transcriptome sequencing of wildtype or IFNAR1 KO THFs infected with either UV-inactivated HCMV or SeV particles. Among genes significantly upregulated >2 fold, IFNAR1 KO THFs infected with HCMV particles upregulated 51% (49/96) of genes upregulated in infected wildtype THFs. In contrast, IFNAR1 KO THFs infected with SeV particles

upregulated 6% (5/79) of genes upregulated in infected wildtype THFs. The 30 genes most highly upregulated by either virus had varying degrees of IFN-independence in THFs infected with HCMV particles, while there was very little IFN-independent ISG upregulation in THFs infected with SeV particles (figure 2A). There was a high degree of overlap between all genes significantly upregulated by THFs infected with either virus (figure 2A and supplementary table 1).

To validate our transcriptome data, we chose two representative ISGs that were mostly IFN-independent in THFs infected with HCMV particles but not SeV particles (IFIT1 and ISG15) and one representative ISG whose upregulation was similarly IFN-dependent in THFs infected with either virus (CXCL10). Quantitative RT-PCR findings validated the transcriptomic data for all three genes, following both high and low level infection (figure 2B-D). A similar trend in IFN-dependent/independent ISG upregulation is seen in non-transformed, non-immortalized human fibroblasts (data not shown). The combined transcriptomic and quantitative RT-PCR results indicate that infection with HCMV particles upregulates a subset of ISGs in an IFN-independent fashion while infection with SeV particles upregulates ISGs almost entirely through IFN-dependent signalling.

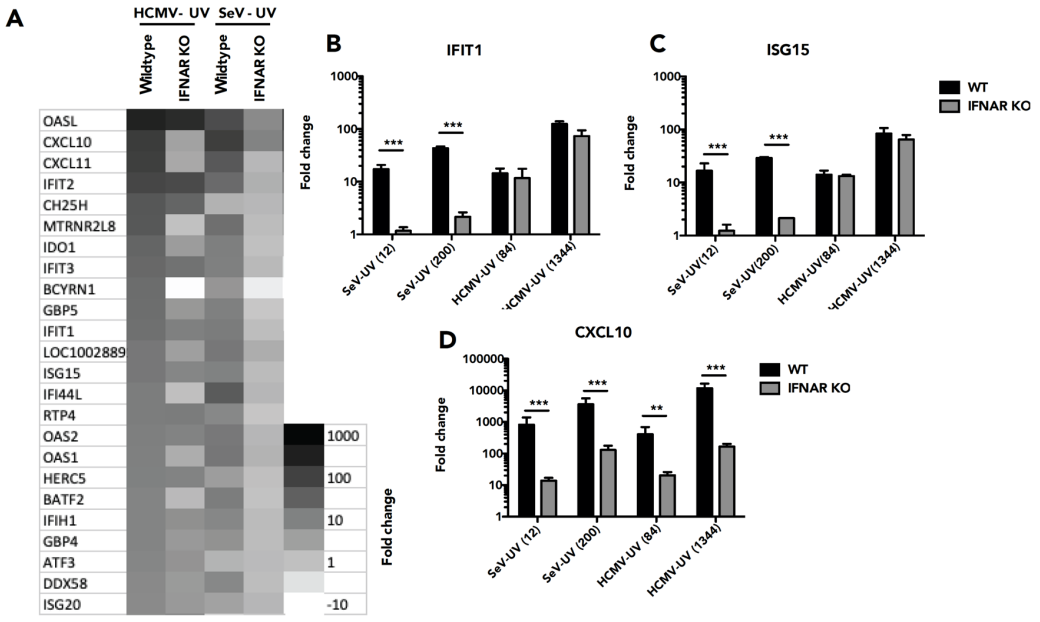

Figure 2 - ISG upregulation in response to HCMV or SeV particles. (A) Wildtype or IFNAR1 KO THFs were treated with 84 particles/cell HCMV-UV or 12 particles/cell SeV-UV and RNA harvested for high-throughput RNA sequencing analysis. The 30 most highly upregulated genes across all samples are shown. (B-D) Wildtype or IFNAR1 KO THFs were treated with 84 or 1344 particles/cell of HCMV-UV or 12 or 200 particles/cell of SeV-UV and RNA harvested 6 hours post-treatment. Transcript levels of IFIT1 (B), ISG15 (C) and CXCL10 (D) were measured by quantitative RT-PCR. Mean fold change over mock and standard error from 3-6 biological replicates was graphed and groups compared by two-way ANOVA with Bonferroni post-tests (* for $p < 0.05$, ** for $p < 0.01$ and *** for $p < 0.001$).

IFN-independent ISG induction depends on virus-intrinsic differences

Given that infection with HCMV particles triggers a STING-dependent antiviral response while SeV particles trigger a MAVS-dependent antiviral response (DeFilippis, et al. 2010; Seth, et al. 2005; Hare, et al. 2015a), we hypothesized that dsDNA recognition through STING directly upregulates ISGs more effectively than dsRNA recognition through MAVS. To test this hypothesis, we transfected wildtype and IFNAR1 KO THFs with either dsDNA or dsRNA of a matched 1000 base pair sequence and harvested cellular RNA 6 hours later. Transfection with either dsDNA or dsRNA was sufficient to upregulate IFIT1, ISG15 & CXCL10 in IFNAR1 KO THFs (figure 3A-C). Therefore, it appears the differences in IFN-independent response to infection with either HCMV and SeV particles cannot be explained by differences in STING and MAVS signalling and appear to be due to virus-intrinsic differences.

Next we chose to test whether the differences in IFN-independent responses between HCMV and SeV particles also held true between replicating HCMV and SeV. Under similar experimental conditions, ISG induction to replicating HCMV (figure 3D-F) mirrored that of non-replicating HCMV (figure 2B-D), while ISG induction by replicating SeV (figure 3D-F) more closely mirrored that of transfected dsRNA (figure 3A-C) as opposed to SeV-UV (figure 2C-E). Thus, IFIT1 and ISG15 upregulation by SeV is less IFN-dependent than what we

observed with SeV-UV. Moreover, similar to UV-inactivated particles, there are clear differences in the antiviral response to replicating HCMV or SeV.

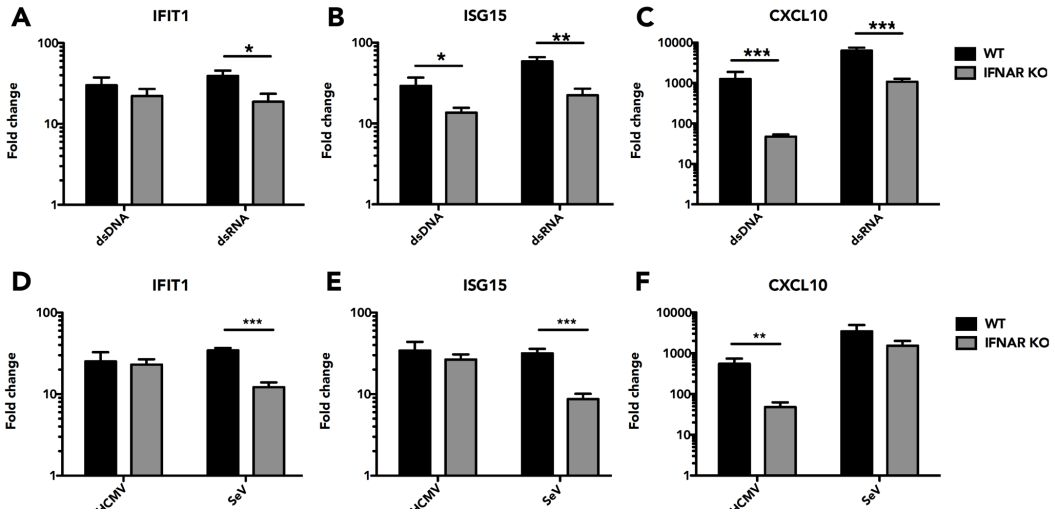

Figure 3 - ISG upregulation in response to transfected nucleic acid or replicating virus. Wildtype or IFNAR1 KO THFs were transfected with matched sequence dsRNA or dsDNA (A-C) or infected with replication competent HCMV or SeV (D-F). Transcript levels of IFIT1 (A and D), ISG15 (B and E) and CXCL10 (C and F) were measured by quantitative RT-PCR. Mean fold change over mock and standard error from 3-7 biological replicates was graphed and groups compared by two-way ANOVA with Bonferroni post-tests (* for $p < 0.05$, ** for $p < 0.01$ and *** for $p < 0.001$).

HCMV and SeV particles differentially activate IRF3

We previously demonstrated that IRF3 is essential for the antiviral response to a variety of enveloped virus particles and that activation of RelA, a subunit of NF-κB, is associated with a higher level of particle stimulation and detectable IFN-β production (Collins, Noyce, and Mossman 2004; Paladino, et al. 2006). The involvement of IFN signalling prompted us to re-examine the relative contribution of these two transcription factors in THFs treated with either HCMV or SeV particles.

To test the relative importance of IRF3 and NF-κB, we infected wildtype, IRF3 KO or RelA KO THFs with increasing levels of HCMV or SeV particles and measured antiviral responses, IFN production and upregulation of representative ISGs. Consistent with previous findings, IRF3 was essential for antiviral protection (figure 4A-B), IFN production (figure 4C-D) and upregulation of IFIT1, ISG15 and CXCL10 (figure 4E-G) in response to infection with either HCMV or SeV particles. RelA was dispensable for antiviral protection, IFN production and upregulation of ISGs after treatment with HCMV particles, but did play a role in response to SeV particles (figure 4A-G).

Given the importance of IRF3 for the antiviral response to infection with either HCMV or SeV particles, we measured IRF3 activation in wildtype THFs infected with either HCMV or SeV particles. We observed IRF3 nuclear translocation in the majority of cells following infection with HCMV particles, but

only in a small proportion of THFs infected with SeV particles (figure 4H-I). Collectively, these data suggest that SeV particles may trigger an IRF3-dependent IFN response in a minority of cells that protects the monolayer through paracrine signalling. By virtue of HCMV particles stimulating IRF3 more evenly across the monolayer, cells are less reliant on paracrine IFN signalling for antiviral protection.

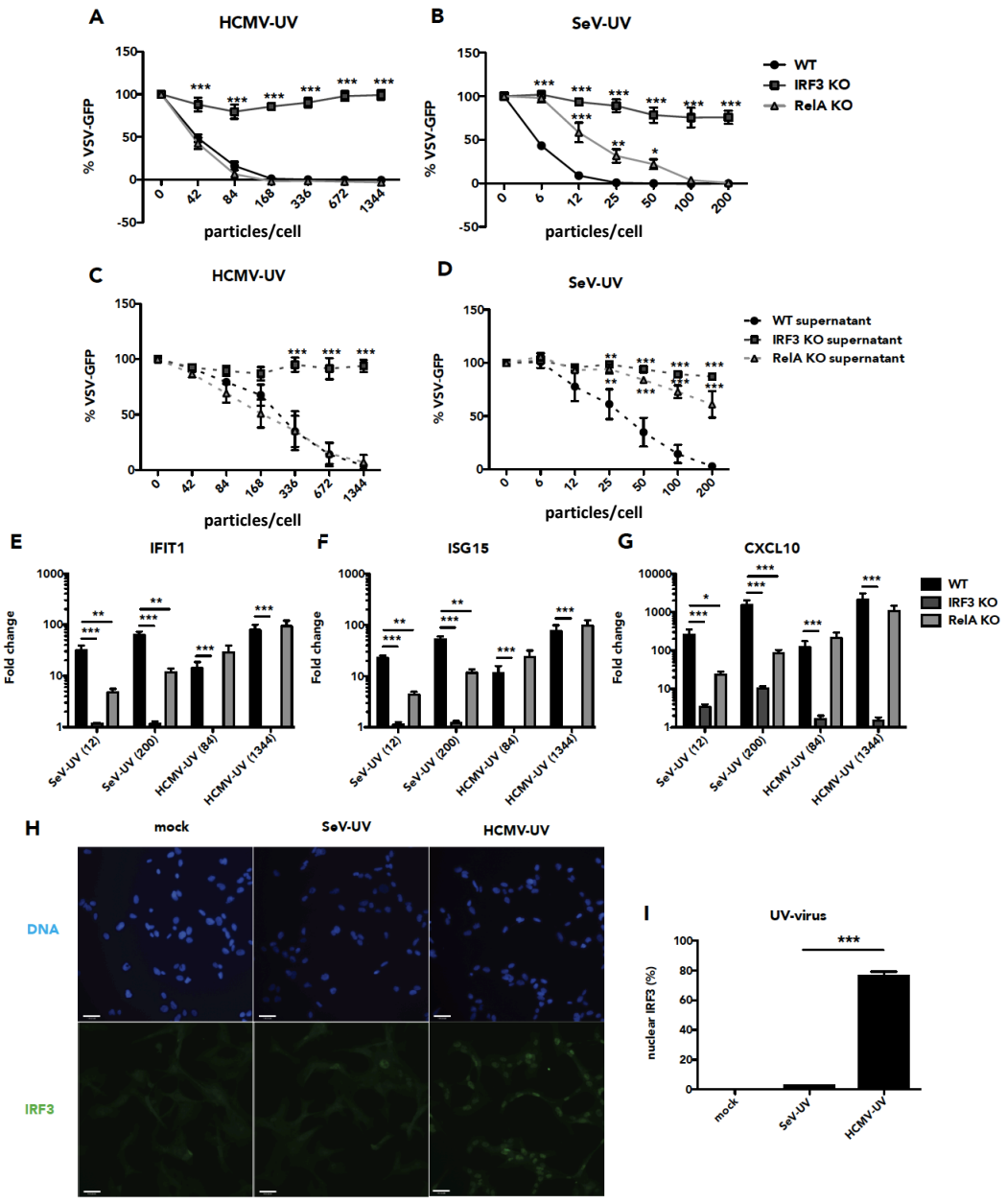
A
HCMV-UV
% VSV-GFP
particles/cell
B
SeV-UV
WT
IRF3 KO
RelA KO
C
HCMV-UV
D
SeV-UV
WT supernatant
IRF3 KO supernatant
RelA KO supernatant
E
IFIT1
F
ISG15
G
CXCL10
Fold change
SeV-UV (12)
SeV-UV (200)
HCMV-UV (84)
HCMV-UV (1344)
H
mock
SeV-UV
HCMV-UV
DNA
IRF3
I
UV-virus
nuclear IRF3 (%)

Figure 4 - Role of IRF3 and RelA in the antiviral response to HCMV and SeV particles. (A-D) Wildtype, IRF3 KO or RelA KO THFs were treated with increasing levels of UV-inactivated HCMV or SeV (A-B) and 16 hours post-treatment supernatants were transferred to naïve wildtype THFs (C-D). All THFs were subsequently challenged with VSV-GFP. Mean GFP fluorescence and standard error from 3 biological replicates were plotted as a percentage of mock treated, VSV-GFP infected cells. (E-G) Wildtype, IRF3 or RelA KO THFs were treated with 84 or 1344 particles/cell of HCMV-UV or 12 or 200 particles/cell of SeV-UV and transcript levels of IFIT1, ISG15 and CXCL10 measured by quantitative RT-PCR. Mean fold change relative to mock and standard error from 7 biological replicates were graphed. (H) Wildtype THFs were treated with 1344 particles/cell of HCMV-UV or 200 particles/cell of SeV-UV, fixed 4 hours post-treatment and IRF3 and cell nuclei visualized by immunofluorescence microscopy. IRF3 +ve nuclei in SeV-UV infected THFs are marked. (I) IRF3 positive nuclei as a percentage of total nuclei were calculated from 3 independent fields of view and mean % IRF3 positive nuclei and standard error from 3 biological replicates were graphed. All groups were compared by two-way ANOVA with Bonferroni post-tests (A-G) or by student's t-test (I) (* for $p < 0.05$, ** for $p < 0.01$ and *** for $p < 0.001$).

HCMV and SeV particles induce heterogeneous IFN-β production

To measure IFN-β production in individual cells, we generated THFs with a stably integrated GFP reporter controlled by the IFN-β promoter sequence (IFN-β-GFP THFs). We used flow cytometry to measure upregulation of GFP (IFN-β) and ISG15 in our IFN-β-GFP THF reporter line. IFN-β-GFP THFs treated with 1000 U/ml recombinant IFNα had increased ISG15 expression but no change in GFP (figure 5A). Infection with HCMV particles increased GFP expression in the majority of cells and increased ISG15 expression in nearly all cells. Infection with SeV particles increased GFP expression in < 5% of cells but increased ISG15 expression in nearly all cells.

To determine the contribution of IFN signalling to ISG15 expression in individual cells, we pretreated IFN-β-GFP THFs with B18R prior to infection with HCMV or SeV particles to block IFN signalling (but not production). As expected, ISG15 upregulation by recombinant IFNα was nearly absent when IFN-β-GFP THFs were pretreated with B18R (figure 5A). When IFN-β-GFP THFs were infected with either HCMV or SeV particles, pre-treatment with B18R primarily prevented ISG15 upregulation in cells lacking GFP upregulation. This observation is consistent with $ISG15^{high}$ GFP^{high} cells upregulating ISG15 directly, while $ISG15^{high}$ GFP^{low} cells upregulate ISG15 through IFN-dependent signalling.

To confirm our flow cytometry data and better visualize cell populations, we observed GFP (IFN-β) and ISG15 in adherent IFN-β-GFP THFs using immunofluorescence microscopy. As with flow cytometry, GFP was upregulated in the majority of cells treated with HCMV particles and highly upregulated in < 5% of cells treated with SeV particles, with ISG15 observed throughout both treated populations (figure 5B). Similar to our flow cytometry data, treatment with B18R predominantly affected ISG15 expression in SeV particle treated cells, and predominantly in cells lacking IFN-β production.

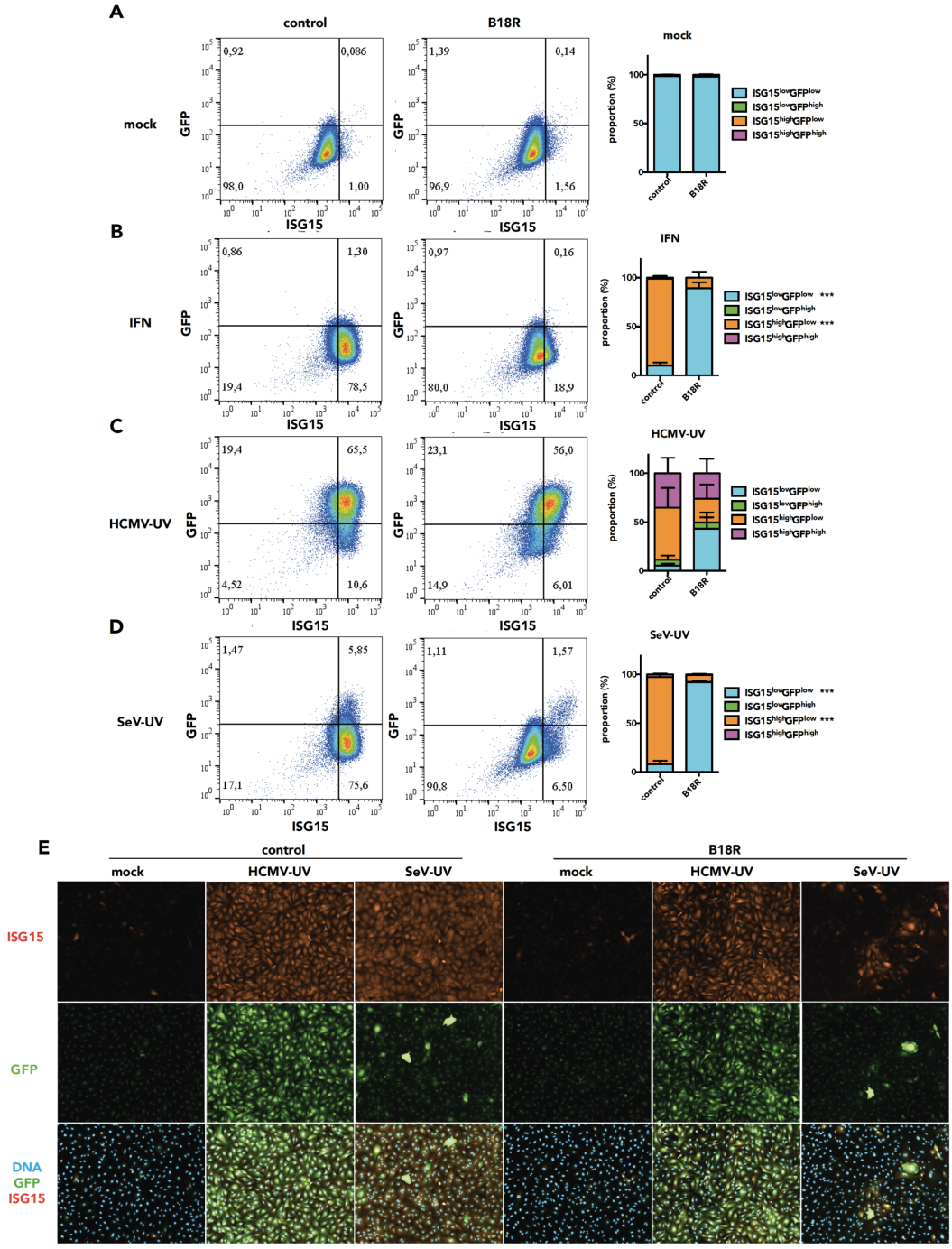
A
control
B18R
mock
mock
GFP
ISG15
0,92
0,086
98,0
1,00
1,39
0,14
96,9
1,56
proportion (%)
ISG15lowGFPlow
ISG15lowGFPhigh
ISG15highGFPlow
ISG15highGFPhigh
B
IFN
IFN
0,86
1,30
19,4
78,5
0,97
0,16
80,0
18,9
***
C
HCMV-UV
HCMV-UV
19,4
65,5
4,52
10,6
23,1
56,0
14,9
6,01
D
SeV-UV
SeV-UV
1,47
5,85
17,1
75,6
1,11
1,57
90,8
6,50
E
control
B18R
mock
HCMV-UV
SeV-UV
ISG15
GFP
DNA
GFP
ISG15

Figure 5 - Single cell dynamics of IFN-β and ISG15 upregulation in response to HCMV or SeV particles. (A-E) IFN-β-GFP THFs were pretreated with B18R or media alone and mock treated, treated with IFNα (1000 U/ml), infected with HCMV-UV (1344 particles/cell) or infected with SeV-UV (200 particles/cell). Cells were fixed 16 hours later and levels of GFP and ISG15 expression measured by flow cytometry (A-D). Cells were categorized as $GFP^{low/high}$ and $ISG15^{low/high}$ based on measurements of mock treated cells, and mean proportion and standard error from 3 biological replicates graphed. Mean proportions from control and B18R pretreated cells were compared by two-way ANOVA with Bonferroni post-tests (*** for $p<0.001$). Similarly treated IFN-β-GFP THFs were visualized by fluorescence microscopy and representative images from 3 biological replicates shown (E).

The simplest explanation for heterogeneous IFN-β production in THFs infected with SeV-UV is that IFN-β producing THFs contain more SeV particles. We infected IFN-β-GFP THFs with replicating SeV or SeV-UV, measured SeV proteins using a pan-specific SeV antibody and were able to detect SeV protein in both SeV and SeV-UV infected IFN-β-GFP THFs. However, we found no clear correlation between GFP expression and SeV protein in IFN-β-GFP THFs infected with SeV particles (Figure 6A). SeV is known to produce and package defective viral genomes (DVGs) recognized by RIG-I during SeV infection, and these may be responsible for IFN-β production in cells infected with SeV particles (Baum, Sachidanandam, and Garcia-Sastre 2010; Sanchez-Aparicio, et al. 2017). We infected IFN-β-GFP THFs with SeV particles and sorted GFP^{high} and GFP^{low} cells before harvesting RNA. We measured both genomic SeV (gSeV) and SeV DVG-546, the predominant DVG found in the SeV stocks used in this study, using quantitative RT-PCR with primers designed by *Genoyer, et al.* (Genoyer and

Lopez 2019). We detected gSeV and DVG-546 in GFP^{high} and GFP^{low} cells but found no difference in gSeV and a noticeable but not significant increase in DVG-546 in GFP^{high} cells (Figure 6B-C).

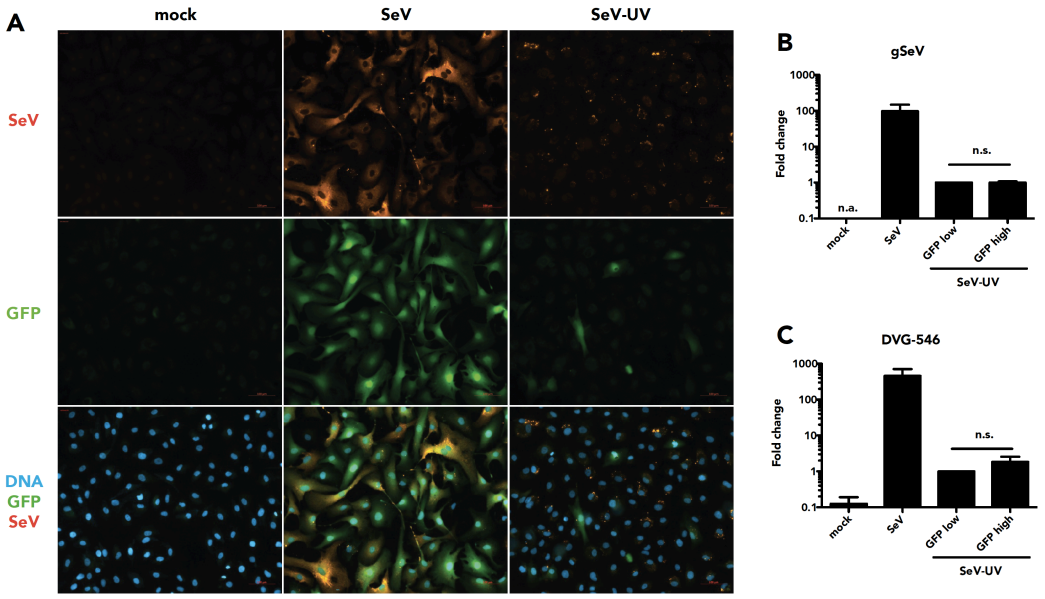

Figure 6 - Relative SeV abundance in IFN-β producing cells. (A-C) IFN-β-GFP THFs were mock infected, infected with replicating SeV (200 particles/cell) or infected with SeV-UV (200 particles/cell). (A) Cells were fixed 16 hours later and GFP and SeV proteins visualized by fluorescence microscopy. (B-C) SeV-UV infected cells were sorted for GFP fluorescence 16 hours post-infection and gSeV (B) or DVG-546 (C) measured by quantitative RT-PCR. Mean fold change relative to SeV-UV-GFP^{low} and standard error from 3 biological replicates were graphed. SeV-UV-GFP^{low} and SeV-UV-GFP^{high} populations were compared by student's t-test (n.a.: no amplification, n.s.: not significant).

Discussion

Our previous reporting of an IRF3-dependent IFN-independent response to incoming virus particles stemmed from examination of individual ISGs by RT-PCR and an inability in multiple assays to detect IFN (Mossman, et al. 2001; Collins, Noyce, and Mossman 2004). Similar to other studies, our early interpretation was that membrane perturbation during virus entry stimulates a similar response to all enveloped virus particles (Collins, Noyce, and Mossman 2004; Noyce, et al. 2011; Holm, et al. 2012). Along with previous findings (Hare, et al. 2015a), this study clearly demonstrates a difference in the cellular response to HCMV and SeV particles, despite both being enveloped virus particles. Although HCMV and SeV contain DNA and RNA genomes respectively, differential nucleic acid sensing alone does not explain the differences in the antiviral response to each, as THFs responded similarly to transfected dsRNA and dsDNA.

Consistent with previous studies, HCMV particles induced IRF3-mediated upregulation of ISGs. Unexpectedly, low levels of HCMV particles also induced IRF3-mediated upregulation of IFN. It is difficult to determine the relative contribution of IFN-dependent versus IFN-independent ISG upregulation to antiviral defence in wildtype cells, as IRF3 is essential for both IFN-β production and direct ISG upregulation in this system. While IFNAR1 KO cells upregulate an IRF3-dependent subset of ISGs that are protective against subsequent challenge,

a higher level of particle stimulation is required for full protection. Therefore, it appears that IFN-β and IRF3 upregulate complimentary and overlapping sets of ISGs that act in concert to protect cells from infection.

In contrast to HCMV, we found that SeV particles strongly stimulate a minority of cells that protect the surrounding monolayer through production of IFN-β. Heterogeneous production of IFN-β has been described in response to several different replicating viruses, even following high multiplicity or *in vivo* infection (O'Neal, et al. 2019; Drayman, et al. 2019; Killip, et al. 2017; Rand, et al. 2012; Chen, et al. 2010; Zawatzky, De Maeyer, and De Maeyer-Guignard 1985; Kallfass, et al. 2013). In many of these cases, IFN-β production occurs in abortively infected cells where the virus fails to suppress antiviral signalling (Drayman, et al. 2019; O'Neal, et al. 2019). The percentage of IFN-β producing cells varies widely amongst viruses and infected cell types and results from a combination of virus-and cell-intrinsic factors (Killip, et al. 2017; Rand, et al. 2012; Zhao, et al. 2012). While previous studies have found heterogeneous IFN-β production in cells infected with replicating virus or treated with dsRNA, we are the first to report heterogeneous IFN-β production in cells infected with inactivated virus particles.

We found SeV genomes and defective viral genomes in cells regardless of whether they were producing IFN-β. While SeV defective viral genomes contain

complimentary ends that form stimulatory dsRNA recognized by RIG-I, packaged SeV defective genomes are normally tightly bound by nucleocapsid protein which prevent formation of the stimulatory hairpin structure (Kolakofsky, 1976). However, not all SeV genomes may be properly packaged in nucleoprotein owing to errors in replication exacerbated by the presence of defective genomes (Genoyer and Lopez, 2019). This additional requirement may explain why only a small fraction of cells infected with SeV particles produced IFN-β. Unfortunately, we were not able to measure whether SeV genomes and SeV DVG-546 were exposed in all SeV-UV infected cells or only in a subset. However, it appears that delivery of packaged SeV RNA is insufficient for recognition by RIG-I and additional factors are required for immune activation.

While in this study we used HCMV and SeV as representative DNA and RNA viruses, respectively, we do not propose that all enveloped DNA or RNA viruses will respond in a similar fashion. Indeed, the diversity of responses is likely to mirror the diverse composition of different virus particles. As the innate antiviral response to virus infection is heavily dependent on recognition of non-replicating virus particles, understanding intrinsic and innate responses to diverse infections helps explain why some infections are associated with greater disease. Moreover, understanding the innate response to non-replicating virus particles is becoming more important as virus-based therapies engineered in the lab make their way to the clinic. Introducing genes by *in vivo* or *ex vivo*

transduction of cells relies on minimizing the innate response to virus vectors to maximize engraftment and stable expression of therapeutic genes (Brown, et al. 2007). In contrast, viral vaccines or oncolytic virus require a robust innate response in order to recruit and activate antigen presenting cells (Proietti, et al. 2002; Davola and Mossman 2019; Walsh, et al. 2019). Notably, SeV is being developed as a vector for gene therapy as well as vaccination, and the ability to modulate immunogenicity of SeV particles will be important for either application (reviewed in (Nakanishi and Otsu 2012; Baltusnikas, Satkauskas, and Lundstrom 2019; Saga and Kaneda 2015)). Lipid-based delivery of nucleic acid is used in addition to virus-based vectors and also triggers innate immune activation. Our results suggest that formulation and delivery route for nucleic acid may influence the innate response as much as the nucleic acid itself and must be taken into account.

Limitations of the study

The experiments described in this study were done in life-extended human fibroblasts with HCMV and SeV used as representative enveloped viruses. Fibroblasts are often used as a model system in which to study regulation of IFN-β, sometimes referred to as fibroblast IFN, because they play an important role in the first wave of IFN-β production during infection. Although nearly all cells

are capable of producing and responding to IFN-β there is some variation in signalling pathways present across different cell types. It remains to be seen how heterogenous IFN-β production affects antiviral protection in tissue made up of many different cell types. HCMV and SeV are both well studied enveloped viruses in the context of innate immune activation and seem to activate a dramatically different proportion of cells. These results show that differences may exist in the antiviral response to non-replicating virus particles, but further experiments with other virus particles are necessary to understand what type of innate response they induce.

Resource availability

Lead Contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Karen L Mossman (mossk@mcmaster.ca)

Materials Availability

All unique/stable reagents generated in this study are available from the Lead Contact with a completed Materials Transfer Agreement.

Data and Code Availability

The RNA-seq data generated during this study are available at GEO (GEO: GSE149705).

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Author contributions

D.H. and K.M. conceived and designed the study. D.H. and K.B. performed experiments. A.D.-G. analyzed transcriptome data. D.H. and K.M. wrote the paper.

Declaration of Interests

The authors declare no competing interests.

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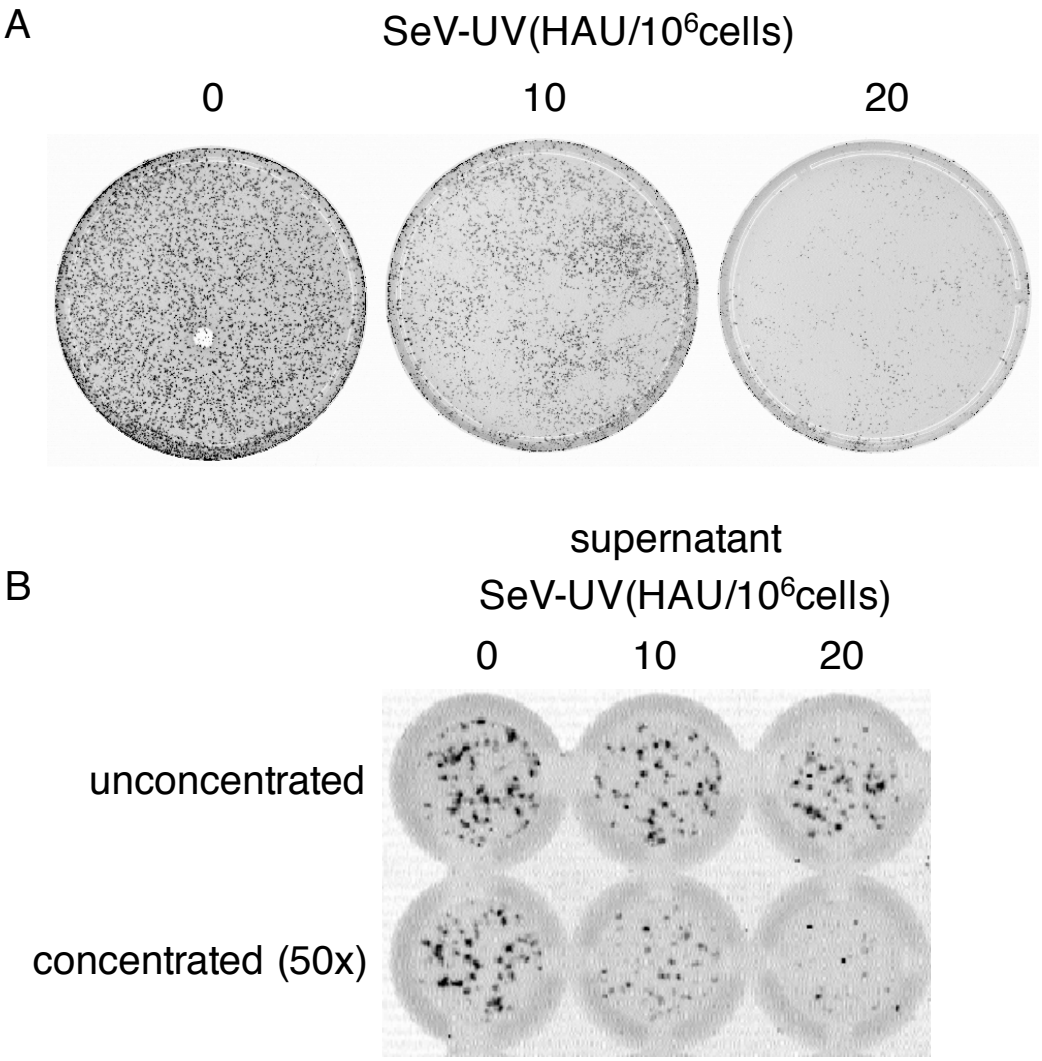
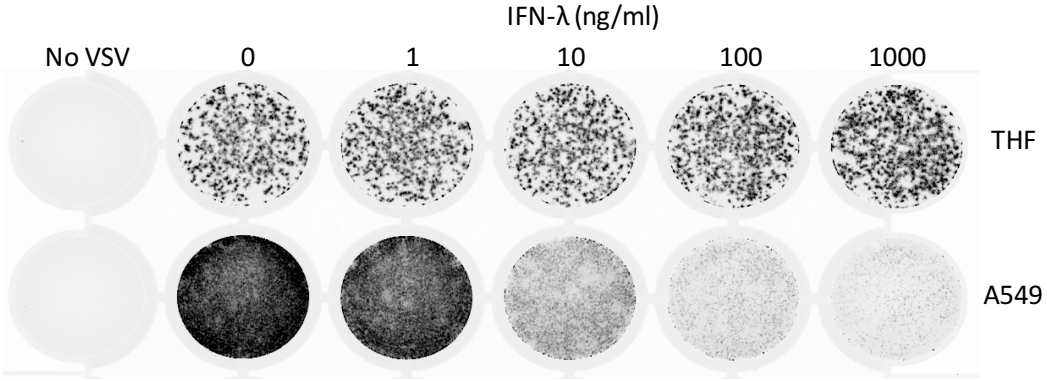
Supplementary Data



Supplementary figure 1 - IFN detected in concentrated supernatants from SeV-UV infected THFs (Related to Figure 1). 1 x 10^6 THFs were treated with the indicated amount of SeV-UV and incubated for 16 hours before concentrating the supernatant 50-fold and transferring concentrated or non-concentrated supernatants in 100 µl to 1 x 10^4 THFs and performing a plaque reduction assay. THFs were challenged with VSV-GFP either 16 hours after SeV-UV treatment (A) or 6 hours after supernatant transfer (B).



Supplementary figure 2 - A549 but not THFs protected following treatment with recombinant IFN-λ (Related to Figure 1). THFs or A549 were treated with the indicated concentration of IFN-λ and challenged with VSV-GFP 6 hours later in a plaque reduction assay.

Supplementary Table 1 - Differential expression of genes significantly upregulated in HCMV-UV or SeV-UV treated wildtype or IFNAR1 KO THFs (Related to Figure 2)

Gene	Fold change				Gene	Fold change			
	wildtype		IFNAR KO			wildtype		IFNAR KO	
	HCMV-UV	SeV-UV	HCMV-UV	SeV-UV		HCMV-UV	SeV-UV	HCMV-UV	SeV-UV
OASL	**303.44**	**64.35**	**215.92**	7.28	RNF19B	**1.52**	**1.53**	1.13	1.05
CXCL10	**120.79**	**112.51**	2.89	**9.60**	SLFN5	**1.51**	**2.31**	1.16	1.14
CXCL11	**109.44**	**42.17**	2.33	1.40	LAP3	**1.51**	**1.68**	1.01	-1.05
IFIT2	**85.17**	**21.80**	**72.92**	1.83	RGAG1	1.50	-1.33	**2.06**	1.07
CH25H	**46.06**	1.73	**27.37**	1.37	SOX13	**1.50**	1.04	1.24	-1.05
IDO1	**27.33**	**11.02**	3.66	1.00	TMEM126A	**1.50**	1.09	-1.15	1.09
IFIT3	**24.11**	**10.90**	**16.89**	1.30	SERPINB2	**1.49**	**1.61**	1.20	1.35
GBP5	**19.69**	**10.79**	**4.39**	-1.23	SKIDA1	1.48	1.30	**2.09**	1.29
IFIT1	**18.69**	**12.57**	**10.90**	1.11	VSIG10L	1.48	**1.61**	-1.07	-1.05
LOC100288911	**14.45**	**14.57**	3.26	2.03	OGFR	**1.48**	**1.76**	1.10	1.02
ISG15	**14.33**	**9.91**	**8.28**	1.21	XKR8	**1.47**	1.07	1.29	1.07
IFI44L	**12.17**	**39.63**	1.05	1.43	RPS6KC1	**1.46**	1.08	1.31	1.08
RTP4	**12.01**	**7.75**	**12.47**	-1.20	FAM26E	**1.46**	1.10	1.34	1.03
OAS2	**10.95**	**13.91**	**9.80**	1.44	ELF1	**1.45**	**1.48**	1.09	1.02
OAS1	**10.66**	**14.83**	**2.00**	1.66	STAT2	**1.45**	**1.85**	1.27	1.08
HERC5	**10.25**	**3.48**	**9.66**	1.03	RASGRP3	1.44	**1.87**	-1.10	-1.09
BATF2	**9.38**	**12.16**	1.28	-1.09	DDX60L	**1.44**	**2.43**	1.07	-1.12
IFIH1	**9.26**	**8.05**	**6.11**	1.18	APOBEC3G	1.43	**1.69**	1.21	1.28
GBP4	**9.17**	**5.42**	**4.03**	1.13	FNDC3A	**1.43**	1.09	1.23	1.06
ATF3	**8.21**	1.57	**5.31**	1.22	LCP2	**1.43**	-1.00	1.24	1.11
DDX58	**7.57**	**7.70**	**3.94**	1.11	TMEM38A	**1.43**	1.12	-1.06	**-1.45**
ISG20	**7.15**	**2.93**	**4.01**	1.43	TMEM51	**1.42**	1.05	1.19	-1.03
CD7	**6.23**	**2.85**	1.43	-1.40	HMOX1	1.42	1.65	1.50	**1.78**
AIM2	**6.08**	2.00	**4.10**	-3.02	ZBTB42	**1.41**	**1.48**	-1.03	-1.02
XAF1	**5.99**	**11.98**	1.29	1.23	DNAJB5	**1.41**	-1.09	1.25	-1.08
IFI44	**5.93**	**4.98**	**6.65**	1.30	SP100	**1.41**	**1.83**	-1.00	-1.04
RARRES3	**5.93**	1.18	**3.04**	-1.17	LYSMD2	**1.40**	1.33	-1.08	-1.16
THEMIS2	**5.83**	**6.74**	1.60	-1.87	RP6-109B7.3	**1.39**	-1.08	1.05	1.15
CD48	**5.65**	2.35	1.38	1.35	TBXA2R	**1.39**	1.14	-1.02	1.02
USP18	**5.65**	**8.37**	1.87	-1.26	TMEM62	**1.39**	**1.39**	1.02	1.06
RHEBL1	**5.63**	1.39	**4.15**	-1.02	KLF9	**1.38**	1.27	**1.44**	**1.44**
NFKBIZ	**5.41**	1.37	**4.53**	1.03	MTERFD3	**1.38**	1.11	-1.23	-1.07
DDX60	**5.28**	**3.08**	**3.44**	-1.01	WARS	**1.37**	1.23	1.10	1.07
TNFSF10	**5.26**	**6.41**	1.75	1.39	IFITM3	**1.37**	**1.40**	1.03	1.05
NCF2	**5.07**	1.07	**2.33**	-1.38	KIAA0040	**1.37**	1.24	1.06	1.01
IRF1	**4.94**	**2.89**	**1.89**	1.08	PRKD2	**1.37**	**1.70**	1.07	1.06
HELZ2	**4.63**	**8.05**	1.11	1.17	PHF11	1.36	**1.73**	1.01	-1.00
KLF4	**4.58**	1.24	**2.91**	-1.07	B4GALT5	**1.36**	1.02	1.14	-1.01
MX2	**4.49**	**7.86**	**1.58**	1.02	GBP3	**1.36**	**1.48**	1.08	1.04
RANBP3L	**4.26**	-1.10	1.19	-1.03	PNPT1	1.36	**2.22**	1.00	1.00
IL6	**3.93**	1.15	**9.44**	1.95	GTPBP1	**1.36**	1.23	1.21	1.00
SNPH	**3.90**	1.06	**3.00**	-1.06	FENDRR	**1.36**	1.17	**1.39**	1.13
HERC6	**3.75**	**3.06**	**2.21**	-1.03	MASTL	**1.35**	**1.61**	1.01	-1.01
ZC3HAV1	**3.67**	**2.44**	**2.55**	1.06	GCA	**1.35**	1.20	-1.17	-1.10
SAMD9	**3.62**	**3.96**	**1.67**	1.00	BTN3A3	**1.34**	1.16	-1.04	-1.03
CEACAM1	**3.49**	1.70	**2.44**	1.07	IFNAR2	**1.34**	1.03	1.13	-1.08
ADAP1	**3.43**	-1.12	**2.50**	1.26	TRIM56	**1.33**	**1.57**	1.18	1.03
SLC15A3	**3.39**	**4.68**	1.47	1.22	RBM43	1.33	**1.52**	1.07	1.06
APOL6	**3.35**	**2.88**	**2.37**	1.10	FBXO30	**1.33**	1.05	**1.44**	1.17
DTX3L	**3.31**	**4.57**	1.22	-1.04	ZNF107	**1.33**	**1.41**	1.12	1.09
DHX58	**3.27**	**2.97**	**2.52**	1.10	ADAR	**1.33**	**1.71**	1.01	-1.02

PTGS2	**3.24**	1.14	1.21	1.42	CASP7	**1.33**	**1.52**	1.00	1.02
NEDD9	**3.15**	1.15	**2.16**	-1.05	MCL1	**1.32**	1.18	1.25	-1.00
IFI6	**3.13**	**3.84**	1.34	1.01	FAM76A	**1.32**	**1.36**	1.28	1.23
KRT17	**3.11**	1.78	1.45	1.06	GALM	**1.31**	1.09	-1.03	-1.01
PMAIP1	**3.09**	1.19	**2.44**	-1.05	STAMBPL1	**1.31**	-1.20	1.21	-1.06
APOL1	**3.09**	**1.87**	**2.31**	1.09	CNP	**1.30**	**1.37**	1.00	-1.01
TMEM229B	**3.02**	**1.83**	**2.26**	1.00	TMEM219	**1.30**	1.10	1.13	-1.05
PARP9	**2.95**	**4.90**	1.17	-1.09	BTN3A1	**1.30**	1.15	1.13	1.01
TMEM140	**2.93**	**2.97**	1.81	1.18	MOB3C	**1.30**	**1.43**	-1.01	1.06
IFITM1	**2.80**	**4.16**	1.24	-1.40	MAP4K4	**1.30**	-1.00	**1.22**	-1.01
MX1	**2.80**	**4.53**	1.38	1.02	IL1R1	**1.30**	1.07	**1.32**	1.09
FAM46A	**2.77**	**1.83**	**1.63**	-1.01	COX7B2	**1.30**	1.21	-1.00	1.03
DAPP1	2.68	**3.54**	1.21	1.21	CYTH1	**1.29**	**1.37**	1.05	-1.07
GRIP2	**2.63**	1.71	1.58	1.07	TRIM22	1.29	**1.97**	1.13	-1.02
ZNF43	**2.62**	1.47	2.25	1.10	RNF31	**1.29**	**1.49**	1.07	-1.01
IFIT5	**2.60**	**2.88**	1.21	-1.05	SHISA5	**1.29**	**1.28**	1.02	-1.00
ZNFX1	**2.57**	**2.47**	**1.88**	1.06	OBFC1	**1.28**	-1.06	1.08	1.05
APOL2	**2.56**	**1.75**	**1.89**	1.08	TAF8	**1.27**	1.09	**1.30**	1.10
CCRN4L	**2.53**	1.15	**2.22**	1.10	BCL2L13	**1.27**	**1.32**	-1.03	1.04
PLEKHA4	**2.52**	**2.38**	1.52	-1.03	SERTAD1	**1.27**	**1.29**	1.06	1.11
PLSCR1	**2.50**	**3.81**	1.01	-1.12	CCDC6	**1.27**	1.03	1.15	-1.06
PTGER4	**2.48**	1.08	1.81	-1.09	BLZF1	1.27	**1.50**	-1.08	-1.01
PARP10	**2.37**	**3.65**	1.19	1.08	TIPARP	**1.26**	1.20	**1.29**	1.22
IRF9	**2.37**	**3.02**	1.13	1.07	UBA7	1.26	**1.63**	-1.04	1.01
SECTM1	**2.37**	**1.86**	1.33	1.09	DCP1A	**1.26**	**1.40**	1.13	1.09
G0S2	**2.35**	1.29	1.44	1.19	PCGF5	**1.25**	1.11	1.08	1.00
TRIM21	**2.33**	**2.68**	1.10	1.01	PANX1	**1.25**	**1.21**	1.14	1.01
LOC102724224	**2.32**	1.89	1.37	1.03	PLEKHA3	**1.25**	1.05	1.07	-1.02
SAMD9L	**2.29**	**3.26**	**1.40**	1.08	PHACTR4	1.25	**1.38**	1.09	1.03
ASPHD2	**2.27**	1.37	**1.60**	1.09	LGALS3BP	**1.24**	**1.24**	1.02	-1.01
REC8	**2.24**	**2.71**	1.09	1.26	COA6	**1.24**	1.14	-1.04	1.06
USP32P1	2.23	**3.20**	1.88	1.34	GPBP1	**1.23**	1.19	1.14	1.06
APOL3	**2.22**	**1.89**	**2.05**	-1.10	ASNA1	**1.23**	1.05	-1.05	-1.01
NRG2	**2.22**	1.44	1.15	1.09	PELO	**1.23**	1.02	1.11	1.08
FGF2	**2.21**	1.01	**2.26**	1.05	PSME2	**1.23**	**1.22**	-1.01	1.04
ZFP36L2	**2.18**	1.03	**2.11**	1.03	SPATS2L	**1.23**	1.19	1.05	-1.02
PARP14	**2.18**	**3.64**	**1.48**	1.20	MLKL	1.22	**1.56**	1.00	1.10
PPM1K	**2.17**	**2.07**	**1.77**	1.06	CHMP5	**1.22**	**1.29**	-1.03	-1.02
PARP12	**2.16**	**2.80**	1.04	-1.10	SLC35C2	**1.22**	1.08	-1.00	-1.03
TRANK1	**2.15**	**2.08**	**1.98**	1.01	GCLM	1.21	1.20	1.17	**1.29**
BAMBI	**2.09**	-1.00	**2.41**	1.04	B2M	**1.21**	1.09	-1.01	-1.06
NEURL1	**2.08**	**1.83**	1.24	1.30	PSME1	**1.20**	**1.18**	1.03	-1.01
RNF149	**2.07**	1.12	**1.65**	1.06	SLC2A13	**1.20**	1.09	1.08	1.04
SOCS1	**2.04**	**1.69**	1.05	1.08	PRDX2	**1.20**	1.08	1.11	1.00
TNFAIP3	**2.04**	1.06	**1.75**	1.02	DNAJA1	**1.20**	**1.22**	1.02	-1.01
SLC25A28	**2.02**	**2.39**	-1.02	1.04	MRPL22	**1.20**	1.16	**-1.19**	-1.10
FAM65B	**2.02**	1.11	-1.00	1.02	SHOX2	1.20	**1.45**	-1.03	1.05
TRPC4	**2.00**	1.01	1.20	-1.01	TRIM5	1.20	**1.53**	1.08	1.12
SP110	**1.97**	**2.95**	1.06	-1.09	ATP5SL	**1.20**	1.09	-1.01	-1.03
OAS3	**1.97**	**2.71**	1.17	1.02	FMR1	1.19	**1.26**	1.09	-1.00
GBP1	**1.95**	**2.29**	1.09	1.01	GPR180	1.18	**1.32**	1.04	1.10
PIK3AP1	**1.93**	1.40	1.38	1.23	EHD4	1.18	**1.36**	1.07	1.01
GMPR	**1.92**	**2.93**	1.32	1.23	TRIQK	**1.18**	1.05	1.04	1.03
ZCCHC2	**1.92**	**2.37**	1.09	1.08	RBCK1	1.18	**1.30**	1.07	1.01
COL24A1	**1.92**	**2.00**	-1.12	1.13	STAMBP	**1.18**	1.05	-1.05	-1.01
LOC102724927	**1.91**	1.62	-1.08	-1.15	NUP54	**1.18**	1.08	-1.05	-1.03
SAMHD1	**1.91**	**2.40**	1.17	-1.03	SPCS1	**1.16**	1.06	-1.04	-1.02
UBE2L6	**1.91**	**2.16**	1.25	1.11	CMTR1	1.16	**1.39**	1.05	-1.01
ARID5A	**1.90**	1.47	1.14	-1.22	HOXD10	1.16	**1.28**	-1.04	-1.08

LOC728769	**1.88**	1.62	1.02	-1.09	ETS2	1.16	**1.39**	-1.06	1.01
HES4	**1.87**	1.78	1.50	-1.15	CCDC113	1.16	1.04	1.92	**2.60**
TRIM14	**1.85**	**2.55**	1.25	1.03	GTPBP2	1.16	**1.41**	-1.04	1.01
N4BP1	**1.84**	**1.90**	1.25	1.04	FHL3	**1.15**	1.10	-1.10	-1.05
CD274	**1.82**	**1.65**	1.12	-1.07	DUSP16	1.14	**1.29**	1.13	-1.00
TLR3	1.82	**2.44**	-1.19	1.04	TNFRSF21	1.14	1.05	**1.27**	1.07
BST2	**1.81**	**2.12**	1.11	1.04	SEMA3A	1.14	-1.07	**1.53**	-1.03
MYD88	**1.81**	**2.46**	-1.05	-1.08	IER2	1.13	**1.29**	-1.13	-1.05
TRIM25	**1.80**	**2.57**	-1.02	1.01	MED25	1.12	**1.20**	1.05	-1.07
SIX2	**1.79**	1.11	1.32	-1.35	ZNF844	1.12	**1.47**	1.10	1.01
C19orf66	**1.79**	**2.64**	1.00	1.04	KIAA0226	1.11	**1.24**	1.09	-1.03
TDRD7	**1.79**	**2.26**	1.22	1.07	APLP1	1.10	**1.28**	-1.08	1.02
SQRDL	**1.79**	1.16	**1.58**	1.12	TBX15	1.10	**1.26**	-1.05	1.01
TTC39B	**1.78**	**1.48**	**1.39**	1.17	NLRC5	1.09	**1.81**	-1.07	1.01
ZBED5-AS1	**1.78**	1.02	1.28	1.42	C6orf62	1.09	**1.18**	-1.03	-1.01
MSX1	**1.77**	**1.45**	1.36	1.13	ATP10A	1.08	**1.61**	1.06	1.08
CDK5R2	**1.76**	1.26	1.10	-1.04	ZGLP1	1.06	1.06	**1.82**	1.41
IFI35	**1.74**	**2.34**	-1.07	1.01	S1PR3	1.06	**1.25**	-1.06	1.08
EIF2AK2	**1.73**	**2.56**	1.03	-1.08	ETV6	1.06	**1.20**	1.01	1.05
HIP1R	**1.73**	1.00	1.37	1.04	RICTOR	1.05	**1.29**	1.10	1.06
PML	**1.71**	**2.05**	1.02	1.01	ANKFY1	1.05	**1.26**	1.01	-1.00
SIX1	**1.70**	1.24	1.14	-1.03	SOX9	1.05	**1.28**	**-1.29**	-1.13
TREX1	**1.69**	**1.77**	-1.04	-1.07	GNB4	1.05	**1.28**	-1.06	-1.05
FOXF1	**1.68**	1.07	**1.57**	1.09	BAZ1A	1.05	**1.24**	-1.00	1.00
CYP2J2	**1.67**	1.55	-1.15	-1.12	LOC100506714	1.04	1.28	1.25	**1.40**
ZFP42	**1.64**	**1.67**	1.10	1.17	ADAT2	1.04	-1.07	**1.55**	-1.00
NMI	**1.63**	**1.94**	1.10	-1.00	REEP2	1.04	1.15	**1.58**	1.27
SLAMF7	**1.63**	1.10	1.18	1.02	ZFP36	1.03	1.09	1.12	**1.22**
HIAT1	**1.62**	-1.03	1.17	-1.07	LETM2	1.02	-1.04	1.25	**1.41**
C18orf56	**1.62**	1.59	-1.43	-1.27	EBF4	-1.00	1.02	**3.36**	1.41
SPRY2	**1.62**	1.15	1.21	1.14	DCLRE1C	-1.00	**1.26**	-1.01	-1.06
IFI16	**1.60**	**1.96**	1.06	1.04	SLC30A4	-1.02	1.03	**1.25**	1.09
ZNF232	**1.60**	1.06	1.27	-1.25	KCNN3	-1.06	-1.05	**2.64**	**2.54**
SRGAP3	**1.59**	1.01	1.08	-1.00	INO80B	-1.06	-1.39	**1.92**	1.48
PIK3R3	**1.59**	1.07	**1.33**	-1.01	CDC14A	-1.09	-1.13	**1.30**	1.16
FZD4	**1.58**	-1.08	**1.55**	1.08	PHF10	-1.10	-1.10	**1.36**	1.09
VEGFC	**1.58**	1.18	1.32	1.05	ABCA3	-1.11	1.14	**5.12**	4.00
IRF2BPL	**1.58**	1.15	1.23	1.09	C4orf36	-1.13	-1.08	**1.65**	1.38
STAT1	**1.58**	**2.15**	-1.01	-1.01	AP1S3	-1.16	-1.02	1.23	**1.48**
TSKU	**1.57**	1.15	1.29	-1.04	LOC102724023	-1.17	1.84	**2.38**	1.24
GIMAP2	**1.57**	**1.57**	1.00	1.09	ADAMTS9	-1.19	-1.08	**1.92**	1.28
ZC3H12C	**1.56**	1.20	1.41	1.09	TMEM176A	-1.20	-1.22	1.43	**3.15**
PNP	**1.56**	1.06	1.33	1.03	OLFM4	-1.22	-1.10	**3.96**	-1.01
TRIM38	**1.54**	**1.80**	1.03	1.07	LOC100506258	-1.38	-1.08	2.22	**3.30**
IRF2	**1.52**	**1.55**	1.09	-1.00	NDP	-1.39	-1.67	**3.36**	1.43

Fold change calculated relative to mock infected wildtype or IFNAR KO cells
Values in bold are significantly different from mock
IFNAR: IFN-α/β receptor, HCMV-UV: UV-inactivated human cytomegalovirus, SeV-UV: UV-inactivated Sendai virus

Transparent Methods

Cells and viruses

Telomerized human fibroblasts (THFs) and THF IFNAR1 KOs from Victor DeFilippis were immortalized through expression of hTERT in BJ fibroblasts (Bresnahan et al., 2000). THF IRF3 KO and RelA KO were generated by transducing THF with lentivirus encoding Cas9 and gene specific gRNA, followed by selection of single cell clones lacking protein expression for the gene of interest. THF IFN-β-GFP cells were generated by transducing THF with lentivirus encoding green fluorescence protein (GFP) under the control of the IFN-β promoter region (Cellomics). SeV (Charles River) Cantell strain was produced in eggs and titred by plaque assay on CV-1 cells, HCMV strain AD169 (from Theresa Compton) was propagated and titred in human embryonic lung (HEL) fibroblasts using a standard plaque assay, and vesicular stomatitis virus expressing GFP (VSV-GFP) was propagated in Vero cells. Virus particles were counted by tunable resistive pulse sensing with a qViro-X particle counter (Izon). Virus inactivation was carried out in a stratolinker UV-crosslinker using working concentration of virus with the amount of energy optimized to yield a 5-log reduction in infectious titre. Treatments were done in minimal serum-free media for 1 hour at 37^{o}C with periodic rocking. E1000 dsDNA and dsRNA derived from the WNV genome was transcribed in vitro and purified as previously described (DeWitte-Orr, et al. 2009). Transfections were carried out with lipofectamine 3000 (ThermoFisher)

according to the manufacturer's instructions. B18R was used to block IFN at 50 ug/ml in serum-free media and incubated with cells for 30 minutes prior to treatment.

Plaque reduction assay

Cells were conditioned with virus or other treatment and incubated 16 hours at 37^{o}C before challenge with VSV-GFP infection and an overlay containing 1% methyl-cellulose to restrict plaques. Immediately before VSV-GFP challenge, supernatants from treated cells were transferred to naive cells and allowed to sit for 6 hours before the supernatant conditioned cells were also challenged with VSV-GFP. Plates were scanned with a Typhoon fluorescence scanner 24 hours post-infection with VSV-GFP to assay antiviral protection.

Transcriptome sequencing and analysis

RNA was extracted using an RNeasy RNA extraction kit (Quiagen) and treated with DNAase (Ambion) according to the manufacturer's instructions. cDNA libraries were created by polyA enrichment using NEBNext poly(A) magnetics isolation module (NEB) and reverse transcribed using NEBNext ultra II directional RNA library prep kit (NEB) according to the manufacturer's instructions. cDNA libraries were sequenced using an Illumina HiSeq rapid V2 (1 x 50 bp sequence reads) at the Farncombe Metagenomics Facility (McMaster University). Sequencing yielded ~1 x 10^7 reads/sample.

First, reads were filtered by quality (at least 90% of the bases must have a quality score of 20 and higher). Then the mapping of the remaining reads was performed using *HISAT2* (Kim, Langmead, and Salzberg 2015) with hg38 (UCSC) reference genome; reads were counted by using *HTSeq count* (Anders, Pyl, and Huber 2015). Genes showing less than 10 counts in more than 30% of the samples per group were removed using *filterByExpr* function in *EdgeR* package (Robinson, McCarthy, and Smyth 2010; McCarthy, Chen, and Smyth 2012) in R, resulting in 13,134 genes. These remaining count values were normalized with *TMM* normalization method (Robinson and Oshlack 2010) and then transformed with *voom* transformation (Law, et al. 2014). Next, batch effect was removed using ComBat (Johnson, Li, and Rabinovic 2007), with experiment date used as the batch information. *Limma* package (Ritchie, et al. 2015) in R was used to examine differential expression between the groups of interest; p-values obtained from the analysis were corrected with BH correction for multiple testing (Benjamini and Hochberg 1995), and corrected values <0.05 were considered to be significant.

Quantitative RT-PCR

RNA was extracted using TRIzol reagent (Invitrogen) and treated with DNAase (Ambion) according to the manufacturers' instructions. 500 ng of RNA was reversed transcribed using SuperScript II Reverse Transcriptase (Invitrogen) and random hexamer primers as per the manufacturer's instructions.

Quantitative PCR reactions contained Taqman probes and Universal PCR Master Mix (Applied Biosystems) or primers and SsoFast EvaGreen Supermix (BioRad) were used as indicated, along with PCR amplification on a StepOnePlus Q-PCR instrument (Applied Biosystems). Ct values were calculated and GAPDH was used as an endogenous control to calculate individual ΔΔCt values. ΔΔCt values of samples were compared with mock treated samples to calculate fold change. Taqman probes for human GAPDH (Hs02758991_g1), IFIT1 (Hs03027069_s1), ISG15 (Hs00192713_m1) and CXCL10 (Hs00171042_m1) and PCR primers for human GAPDH (F-5'-GGAGCGAGATCCCTCCAAAAT-3' and R-5'-GGCTGTTGTCATACTTCTCATGG-3'), genomic SeV (F-5'-GACCAGGAAATAAAGAGTGCA-3' and R-5'-CGATGTATTGGCATATAGCGT-3') and SeV DVG-546 (F-5'-TCCAAGACTATCTTTATCTATGTCC-3' and R-5'-GGTGAGGAATCTATACGTTATAC-3') were used.

Immunofluorescence

Cells were fixed on glass coverslips using 10% formalin, permeabilized in 0.2% Triton-X 100 in phosphate buffered saline (PBS) and blocked in 3% fetal bovine serum (FBS), 3% goat serum, 0.02% Tween-20 in PBS for 1 hour. The following antibodies were used for 1 hour at the indicated dilution in blocking buffer: anti-IRF3 (Millipore)(1:400), AlexaFluor488-conjugated anti-GFP (Invitrogen)(1:1000), anti-ISG15 (gift from Dr. EC Borden)(1:10), anti-SeV (1:2000), AlexaFluor488-conjugated anti-rabbit (Invitrogen)(1:400) and

AlexaFluor594-conjugated anti-mouse (1:50). Hoechst 33258 (Invitrogen) was diluted 1:5000 in PBS and added to cells for 15 minutes. A Leica DM IRE2 microscope was used and IRF3 positive nuclei were calculated as a percentage of total nuclei using OpenLab software (Leica).

Flow cytometry

Cells were fixed and permeabilized using a cytofix/cytoperm fixation/permeabilization kit (BD Biosciences) according to the manufacturer's instructions. Cells were stained with anti-ISG15 (diluted 1:10) and APC-conjugated anti-mouse (Biolegend) (diluted 1:400) for 30 minutes each in perm/wash buffer (BD Biosciences). Flow cytometry of fixed cells was carried out in 1% BSA, 5mM EDTA in PBS using a MoFlow XDP cell sorter (Beckman Coulter). Cell populations were analyzed using FlowJo.

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Chapter 4

Membrane perturbation by a viral fusogen triggers a RLR-dependent antiviral response

This work was compiled and written as a complete manuscript but is awaiting key experiments before publication. In particular, FLAG-RIG-I RNA co-purification is necessary to identify any dsRNA recognized by RIG-I following p14 lipoplex treatment.

Chapter Introduction

Previous work in our lab established lipid-based particles containing purified FAST protein p14 as a model for enveloped virus-cell membrane fusion (Noyce, et al. 2011). p14 in complex with lipofectamine 2000 (p14 lipoplexes) was sufficient to trigger antiviral ISG upregulation. The antiviral response to p14 was dependent on the fusion domain of p14, as a deletion mutant lacking this domain failed to trigger an antiviral response. Importantly, these data established that membrane fusion is recognized by cells and is sufficient to trigger an antiviral response. The bulk of my thesis work has focused on understanding how cells recognize membrane fusion and how this culminates in an antiviral response.

We discovered that cytosolic Ca^{2+} oscillations play an important role in the antiviral response to enveloped virus particles, but Ca^{2+} oscillations were insufficient to explain the antiviral response to enveloped virus (Hare, et al. 2015b). Instead, Ca^{2+} oscillations appeared to be involved in viral nucleic acid sensing pathways critical for virus recognition. Furthermore, enveloped virus infection triggered IRF3 activation and IFN-I production at low but protective levels (Hare, et al. 2020b). While enveloped viruses are complex particles containing nucleic acid, p14 lipoplexes are a simplified model of membrane perturbation. Given what we had observed with enveloped virus particles, we

chose to revaluate the importance of IFN-I signalling and nucleic acid sensing in the antiviral response to p14 using CRISPR KO THF cell lines.

We found that the antiviral response to p14 involved IFN-I signalling and required the mitochondrial scaffold protein MAVS. MAVS is activated by the cytosolic RNA sensors RIG-I and MDA5, which were redundantly necessary for the antiviral response to p14 lipoplexes. Further data suggested that dsRNA recognition by RIG-I was important for the antiviral response to p14 despite strong evidence that p14 lipoplexes do not deliver sufficient amounts of dsRNA to explain the antiviral response.

Compartmentalization, RNA modifications and RNA degradation are all important for cells to avoid recognition of endogenous RNA, and impairment of any of these systems can trigger an IFN response (reviewed by (Roers, Hiller, and Hornung 2016; Schlee and Hartmann 2016)). We are particularly interested in whether p14-mediated fusion exposes stimulatory RNA to cytosolic sensors or triggers recognition of otherwise innocuous RNA. We are in the process of identifying the nature of the RIG-I bound ligand.

We know that not all membrane fusion leads to upregulation of ISGs and antiviral protection because routine membrane fusion between cellular vesicles required for cellular homeostasis does not trigger an antiviral response. Furthermore, lipofectamine alone is insufficient to trigger an antiviral response. Thus, we were interested in what characteristics of membrane fusion were

recognized by the cell and led to an antiviral response. While we were studying the antiviral response to p14, another group found that fusogenic liposomes formulated to fuse at the cell membrane were sufficient to trigger a STING-dependent antiviral response in murine cells (Holm, et al. 2012; Holm, et al. 2016). When we treated primary human fibroblasts with fusogenic liposomes, we found that they triggered Ca^{2+} oscillations but no antiviral response. Recently, we found that induced expression of p14 was sufficient to upregulate antiviral ISGs. Further work will be necessary to define the characteristics of membrane fusion that are recognized by the cell and what role the various signals play in antiviral signalling.

Membrane perturbation by a viral fusogen triggers a RLR-dependent antiviral response

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Abstract

Interferon (IFN) is critical for early defence against virus infection through upregulation of antiviral IFN-stimulated genes (ISGs). ISG upregulation requires virus recognition that is mostly limited to recognition of viral nucleic acid. Many viruses are surrounded by a lipid envelope that must fuse with a cell membrane during virus entry. We previously described a Ca^{2+}- and IRF3-dependent IFN-independent antiviral response to membrane fusion involving direct upregulation of ISGs. Here we used lipid complexes (lipoplexes) containing purified fusion associated small transmembrane (FAST) p14 protein to mimic fusion in the absence of exogenous nucleic acid. We found that the antiviral response to p14 lipoplexes triggers IFN-dependent upregulation of ISGs through a pathway dependent on MAVS and the cytosolic RNA sensors RIG-I and MDA5. RNA sensing appears to be involved despite the absence of exogenous RNA. While investigating the antiviral response to other forms of membrane fusion we found that inducible expression of p14 was also sufficient to upregulate antiviral ISGs but treatment with fusogenic liposomes was not. These data suggest that certain types of membrane fusion may trigger upregulation of antiviral ISGs through nucleic acid sensing pathways. It remains to be seen what ligand is recognized by nucleic acid sensors in p14 lipoplex treated cells and how membrane fusion contributes to its recognition.

Introduction

IFN-I signalling is an important early form of innate defence against viral infection. Impaired IFN-I signalling allows unchecked virus growth and sensitivity to otherwise innocuous virus infections (Muller, et al. 1994). Virus recognition and IFN upregulation are primarily dependent on recognition of viral nucleic acid. Cells must effectively discriminate viral nucleic acid amongst a sea of self-nucleic acid to quickly respond to infection while avoiding aberrant inflammation (reviewed in (Schlee and Hartmann 2016)). To discriminate between self and viral nucleic acid, cellular pattern recognition receptors (PRRs) recognize and respond to virus-associated motifs or mislocalized nucleic acid. RIG-I and MDA5 recognize uncapped 5' triphosphorylated RNA, long stretches of dsRNA and perhaps elements of RNA secondary structure (Kato, et al. 2008; Hornung, et al. 2006; Pichlmair, et al. 2006; Pichlmair, et al. 2009). Additional PRRs are responsible for sensing DNA and endosomal nucleic acid (reviewed in (Wu and Chen 2014)). While cells can recognize other viral components, such as viral glycoproteins (Juckem, et al. 2008), viral nucleic acid is the predominant cause of IFN-I production in the context of infection.

Despite the importance of viral nucleic acid sensing, cells recognize virus-associated physiological stress through a wide variety of pathways (reviewed in (Hare and Mossman 2013; Colaco and Moita 2016)). Virus entry is an obligate step in the virus life cycle and an appealing target for cellular recognition. All

viruses must cross a cell membrane, at the cell surface or within endosomes, prior to replication. Viruses surrounded by a lipid envelope do this by fusion of their envelope with a cellular membrane. There is growing evidence to suggest that enveloped virus fusion with a cell membrane is recognized and contributes to IFN-I upregulation (Noyce, et al. 2011; Holm, et al. 2012; Holm, et al. 2016; Hare, et al. 2015a).

We previously found that membrane perturbation by the reovirus fusion-associated small transmembrane protein (FAST) p14 was sufficient to upregulate ISGs through an IRF3-dependent IFN-independent pathway (Noyce, et al. 2011). The antiviral response to p14 can be induced by expression of p14 in co-cultured cells or by delivery of purified p14 in lipid complexes (lipoplexes). Membrane fusion is required for the antiviral response as a mutant lacking the 30 N-terminal amino acids comprising the fusion peptide did not induce an antiviral response. Cytosolic Ca^{2+} oscillations were important for the antiviral response to different enveloped viruses and particularly important for the antiviral response to p14 lipoplexes (Hare, et al. 2015a). Furthermore, we found that cytosolic Ca^{2+} oscillations triggered by enveloped virus entry were essential for IRF3 activation (Hare, et al. 2015a).

Of interest, virus-like particles, which lack viral nucleic acid, or fusogenic liposomes were found to be sufficient to upregulate IFN through a STING-dependent cGAS-independent pathway in murine cells (Holm, et al. 2012; Holm,

et al. 2016). STING activation in response to membrane fusion occurred through a distinct mechanism that did not involve cyclic di-nucleotides (Holm, et al. 2016). This non-canonical STING activation played an important role in antiviral defence against enveloped but not non-enveloped viruses (Holm, et al. 2016).

Previous data suggested that low level enveloped virus entry was sufficient to trigger an IRF3-dependent IFN-independent antiviral response. Recent findings suggest that nucleic acid sensing pathways and IFN signalling are critical for the antiviral response to enveloped virus particles (Hare, et al. 2015a; Hare, et al. 2020a). Given the importance of IFN in the antiviral response to enveloped virus particles, which contain viral nucleic acid along with other potential PAMPs, we decided to test the extent to which IFN signalling was involved in the antiviral response to p14 lipoplexes, which trigger a fusion-dependent antiviral response independent of viral nucleic acid.

Results

The antiviral response to p14 lipoplexes involves IFN-I signalling and MAVS

We have previously shown that p14 lipoplexes trigger Ca^{2+} and IRF3-dependent IFN-independent upregulation of antiviral genes in the absence of exogenous nucleic acid or traditional viral PAMPs (Hare, et al. 2015a; Noyce, et al. 2011). IFN involvement in the antiviral response to enveloped virus particles

and virus-like particles prompted us to investigate the role of IFN signalling in the antiviral response to p14 lipoplexes. To test the importance of IFN we pretreated primary human embryonic lung (HEL) fibroblasts with recombinant B18R, a vaccinia virus derived IFN-I antagonist, before treatment with p14 lipoplexes, lipofectamine 2000 (LF2000) or media alone and challenged cells with VSV-GFP in a plaque reduction assay. We found that HEL fibroblasts mounted an antiviral response to p14 lipoplexes which was impaired by B18R pre-treatment (Figure 1A). We additionally tested the importance of IFN-I signalling by treating wildtype or IFNAR$^{-/-}$ telomerised human fibroblasts (THFs) with p14 lipoplexes and found that wildtype, but not IFNAR$^{-/-}$, THFs mounted an antiviral response to p14 (Figure 1B). This suggests that IFN-I signalling is critical for the antiviral response to p14.

Canonical IRF3 activation requires scaffold proteins, such as STING or MAVS, to bring together IRF3 and its kinase TBK1 (Liu, et al. 2015). To test the importance of these scaffold proteins in the antiviral response to p14 we treated wildtype, STING$^{-/-}$ or MAVS$^{-/-}$ THFs with p14 lipoplexes. We found that wildtype and STING$^{-/-}$ THFs responded normally to p14 lipoplexes while MAVS$^{-/-}$ THFs had an impaired antiviral response (Figure 1B). This suggests that p14 lipoplexes upregulate antiviral ISGs through a MAVS, IRF3 and IFNAR-dependent pathway.

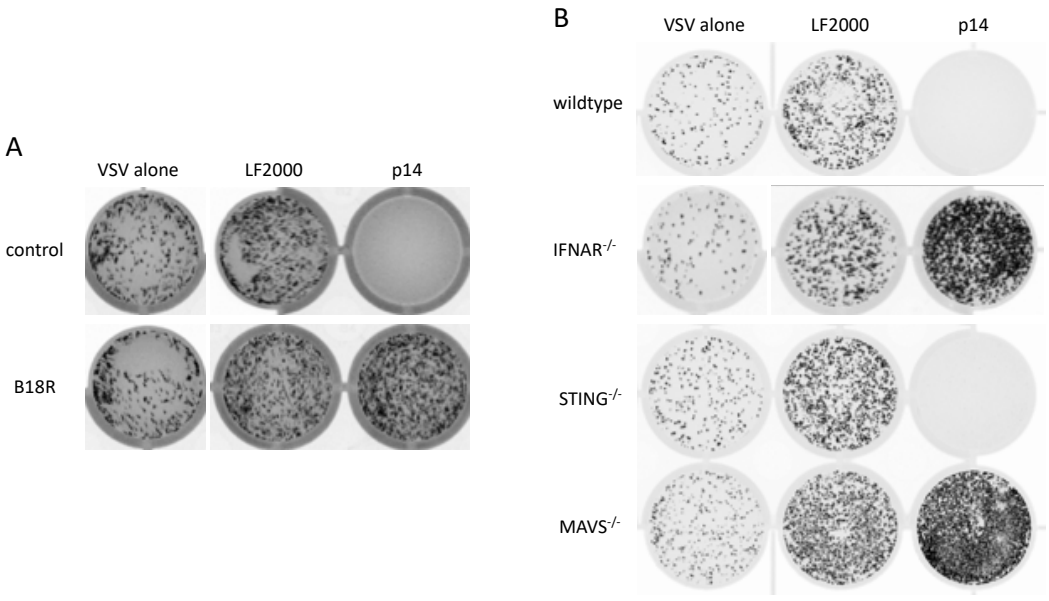

Figure 1 – The antiviral response to p14 lipoplexes involves MAVS and IFN-I signalling. HEL fibroblasts were pretreated with B18R or media alone before treatment with p14 lipoplexes, lipofectamine 2000 or media alone and challenged with VSV-GFP in a plaque reduction assay (A). Wildtype, IFNAR$^{-/-}$, STING$^{-/-}$ or MAVS$^{-/-}$ THFs were treated with p14 lipoplexes, lipofectamine 2000 or media alone and challenged with VSV-GFP in a plaque reduction assay (B). Representative GFP plate scans are shown from 2-3 biological replicates.

RIG-I and MDA5 are redundantly necessary for the antiviral response to p14 lipoplexes

MAVS is canonically activated by the cytosolic RNA sensors RIG-I and MDA5. To test the importance of these proteins we similarly treated wildtype, RIG-I$^{-/-}$, MDA5$^{-/-}$ or RIG-I$^{-/-}$MDA5$^{-/-}$ THFs with p14 lipoplexes. We found that both RIG$^{-/-}$ and MDA5$^{-/-}$ THFs upregulated representative ISGs and mounted an antiviral response to p14 lipoplex treatment but RIG-I$^{-/-}$MDA5$^{-/-}$ THFs had much lower ISG upregulation and did not mount an antiviral response (Figure 2A-C). The highly impaired antiviral response in RIG-I$^{-/-}$MDA5$^{-/-}$ THFs, but not individual knockouts, suggests that RIG-I and MDA5 act redundantly in the antiviral response to p14 lipoplexes.

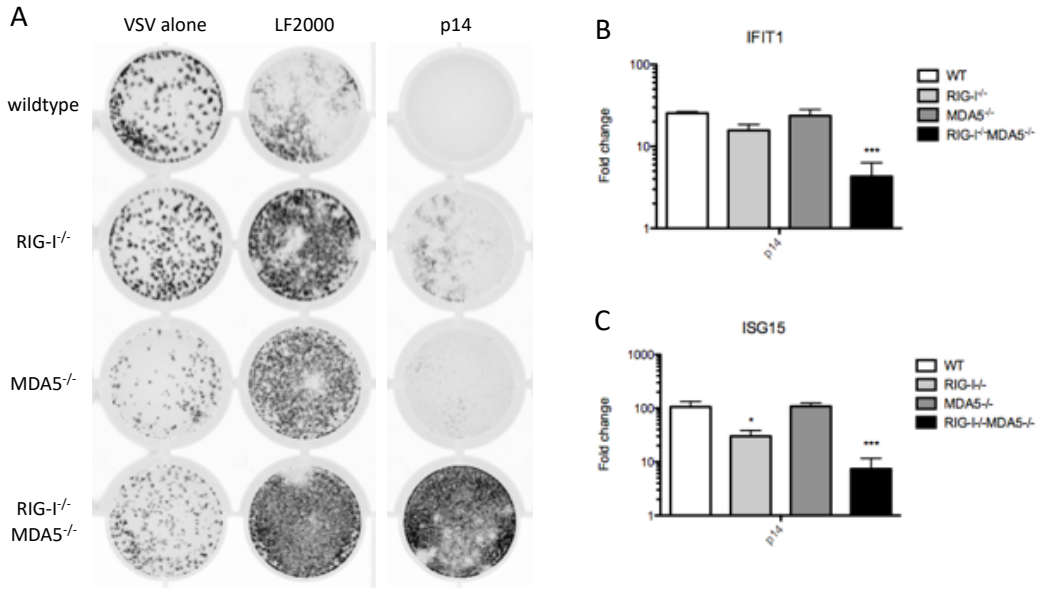

Figure 2 - Antiviral response to p14 lipoplexes depends on MAVS and RLRs. Wildtype, RIG-I$^{-/-}$, MDA5$^{-/-}$ or RIG-I$^{-/-}$MDA5$^{-/-}$ THFs were treated with p14 lipoplexes, lipofectamine 2000 or media alone and challenged with VSV-GFP 16 hours later in an antiviral assay (A). Wildtype, RIG-I$^{-/-}$, MDA5$^{-/-}$ or RIG-I$^{-/-}$MDA5$^{-/-}$ THF cells were treated with p14 lipoplexes and cellular RNA harvested 12 hours post-treatment. Levels of IFIT1 and ISG15 transcripts were measured using quantitative RT-PCR and shown relative to mock treated THF cells (C-D). Groups were compared by two-way ANOVA with Bonferroni post-tests after log-transformation (* for $p < 0.05$ and *** for $p < 0.001$).

RIG-I RNA recognition motifs are involved in the antiviral response to p14 lipoplexes

RIG-I and MDA5 are well studied cytosolic RNA sensors. RNA recognition by RIG-I or MDA5 involves RNA binding to a central helicase domain, and exposure of N-terminal CARD domains that interact with MAVS (reviewed by (Gack 2014)). We chose to focus our studies on the role of RIG-I given the available knowledge and tools surrounding its activation. To determine whether RIG-I ATPase activity and/or RNA binding are required for the antiviral response to p14 we employed the K270A mutant, which lacks ATPase activity necessary for formation of RIG-I filaments (Yoneyama, et al. 2004), and the K888/902A mutant, which lacks 2 lysine residues critical for RNA binding (Takahasi, et al. 2008). We found that reconstitution of RIG-I$^{-/-}$MDA5$^{-/-}$ THF cells with FLAG-RIG-I restored the antiviral response to p14 lipoplexes, but reconstitution with either FLAG-RIG-I K270A or FLAG-RIG-I K888/902A failed to do so (Figure 3A-B). This observation suggests that ATPase activity and RNA binding by RIG-I are important for the antiviral response to p14 lipoplexes.

We previously found that p14-mediated fusion was essential for the antiviral response to p14 lipoplexes (Noyce, et al. 2011). In addition, we detected no dsRNA in purified p14 using dot-blots probed with a dsRNA specific antibody (supplementary figure 1). This assay was sensitive to as little as 0.1 ng of dsRNA while >1 ng must be transfected with lipofectamine to induce an antiviral

response. Thus, while RNA recognition is required for the antiviral response to p14 lipoplexes, contaminating dsRNA is insufficient to explain our results.

To identify RIG-I bound RNA in the context of p14 lipoplex treatment we plan to use formaldehyde RNA co-immunoprecipitation and high throughput sequencing (fRIP-Seq). This technique involves using formaldehyde to cross-link and co-immunoprecipitate RNA-RIG-I complexes followed by RNA purification and sequencing. Using fRIP-Seq, we hope to identify RNA bound by RIG-I specifically following p14 lipoplex treatment. To optimize our fRIP protocol we used samples from SeV infected cells. RIG-I$^{-/-}$MDA5$^{-/-}$ THF cells stably reconstituted with FLAG-RIG-I were infected with SeV or media alone, formaldehyde fixed 4 hours later and FLAG-RIG-I-RNA complexes purified. When RNA was further purified and transfected into THFs we found that only RNA co-purified from SeV infected cells triggered antiviral protection (supplementary figure 2). Optimization experiments are ongoing to co-purify FLAG-RIG-I bound RNA from p14 lipoplex treated cells (data not shown).

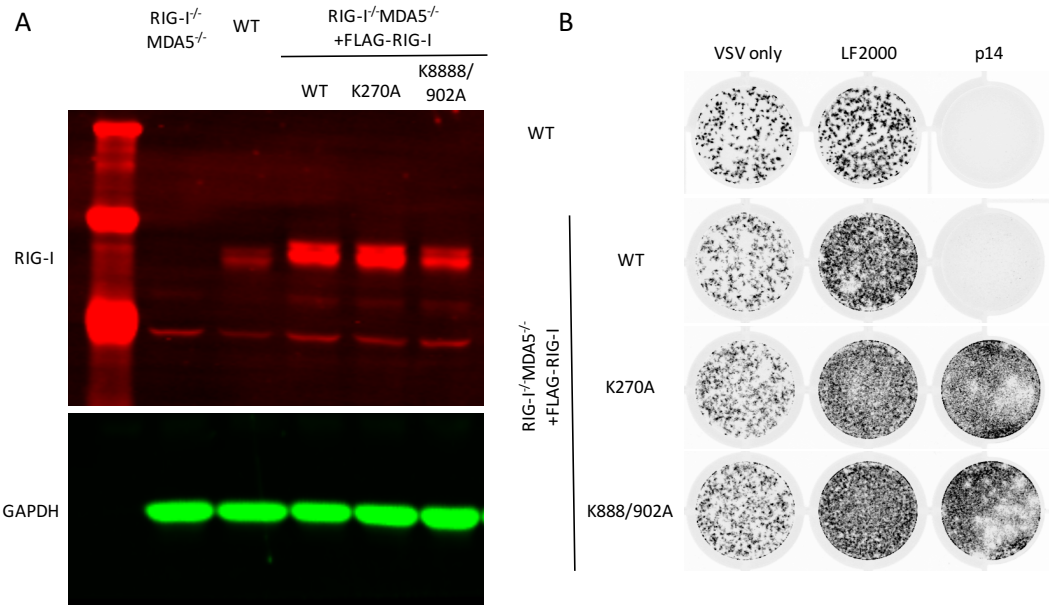

Figure 3 - RIG-I RNA binding mutants fail to reconstitute the antiviral response to p14 lipoplexes. Protein extracts from RIG-I$^{-/-}$MDA5$^{-/-}$, wildtype (WT) or RIG-I$^{-/-}$ MDA5$^{-/-}$ THF cells stably reconstituted with FLAG-RIG-I, K270A or K888/902A were separated by SDS-PAGE and probed with RIG-I- and GAPDH-specific antibodies (A). Wildtype (WT) or RIG-I$^{-/-}$MDA5$^{-/-}$ THF cells stably reconstituted with FLAG-RIG-I, K270A or K888/902A were treated with p14 lipoplexes, lipofectamine 2000 alone, or mock treated and challenged with VSV-GFP in an antiviral assay 16 hours post-treatment. A fluorescence plate scan is shown (B).

p14 expression and syncytia formation between cells is sufficient to upregulate ISGs

Our data so far suggests that exogenously delivered p14 triggers an antiviral IFN-I response to RNA. To answer whether this same response could be elicited by p14 expressed from within cells we generated THFs with doxycycline (dox) inducible p14 using a Tet-On system (THF-dox-p14). THF-dox-p14 began forming visible syncytia 24-48 hours after addition of dox and began upregulating ISGs 16 hours after addition of dox (Figure 4A-B). It appears that p14-mediated fusion is sufficient to trigger upregulation of antiviral ISGs whether p14 is delivered exogenously or expressed from within the cell.

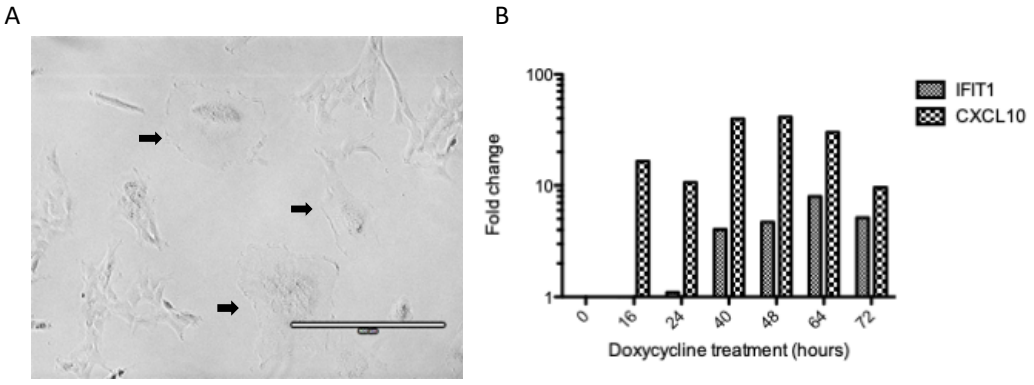

Figure 4 - Induced expression of p14 mediates cell-cell fusion and ISG upregulation. 1 ug/ml doxycycline (dox) was added to THF-dox-p14 cells for the indicated amount of time. Syncytia began forming 40 hours after addition of dox and a representative brightfield image from 48 hours post-dox is included with syncytia marked with black arrows (A). RNA was harvested at the indicated time post-dox treatment. Levels of IFIT1 and CXCL10 transcripts were measured using quantitative RT-PCR and shown relative to THF cells at time 0 (B).

Liposome fusion triggers Ca^{2+} oscillations but is insufficient to trigger antiviral defence

Our results so far suggest that the antiviral response to p14 lipoplexes differs from what is known about the antiviral response to enveloped virus, virus like particles (VLPs) and fusogenic liposomes (Hare, et al. 2015a; Holm, et al. 2012). To better understand how cells respond to membrane fusion we investigated how human fibroblasts respond to fusogenic liposomes, which others have found trigger a STING-dependent cGAS-independent antiviral response in murine cells (Holm, et al. 2012; Holm, et al. 2016).

We created small unilamellar liposomes containing a 1:1:0.1 mix of DOTAP, DOPE and DOPE-lissamine-rhodamine by sonicating resuspended lipid films as described previously (Csiszar, et al. 2010). These liposomes bound to and stained HEL fibroblasts within 1 hour of treatment suggestive of membrane fusion (Figure 5A). Previously, we observed cytosolic Ca^{2+} oscillations in fibroblasts treated with a variety of lipid-based particles that were important for downstream antiviral signalling (Hare, et al. 2015a). We found that liposome fusion triggered robust Ca^{2+} oscillations similar to what we previously described following treatment with enveloped virus particles or p14 lipoplexes (Figure 5B). We measured the antiviral response to fusogenic liposomes alone or poly I:C as a positive control using a plaque reduction assay. While HEL fibroblasts treated with poly I:C were protected from subsequent VSV-GFP replication, fusogenic

liposomes failed to trigger an antiviral response (Figure 5C). These data suggest that additional signals beyond membrane perturbation and Ca^{2+} oscillations must be necessary to trigger an antiviral response.

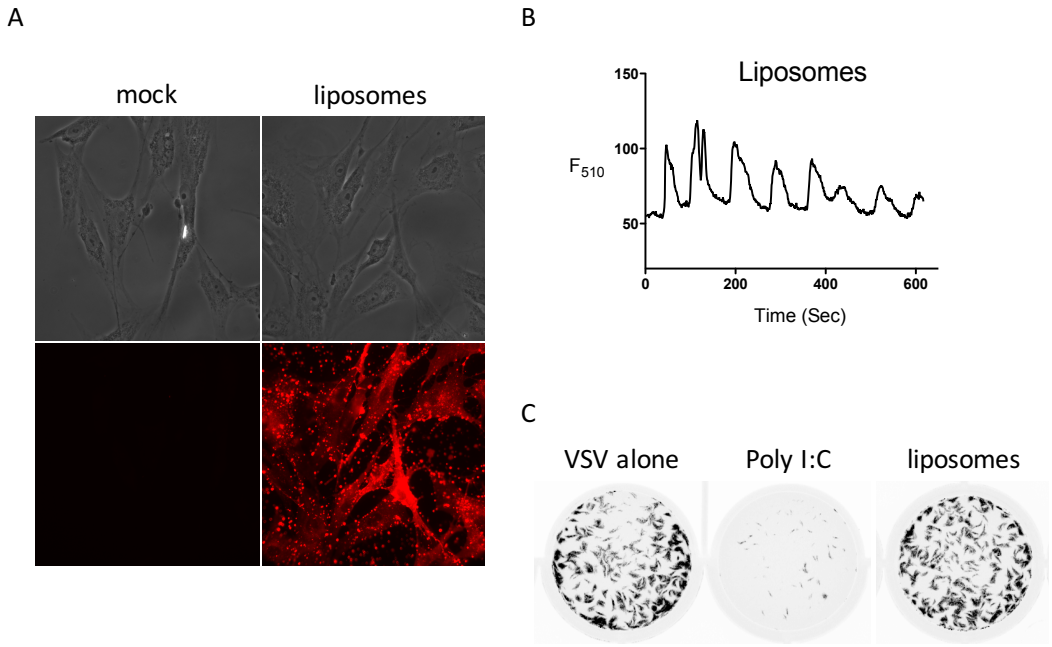

Figure 5 - Fusogenic liposomes trigger Ca^{2+} oscillations but no antiviral response. HEL fibroblasts were treated with fusogenic liposomes containing lissamine rhodamine-conjugated lipids or mock treated and fixed 1 hour later before imaging liposome incorporation by fluorescence microscopy (A). HEL fibroblasts pre-loaded with a Ca^{2+} sensitive dye were treated with fusogenic liposomes and subsequent Ca^{2+} oscillations imaged by live-cell fluorescence microscopy. Fluorescence from multiple cells was plotted over a time period beginning 45 mins after treatment and a representative plot shown (B). HEL fibroblasts were treated with poly I:C or fusogenic liposomes and challenged with VSV-GFP in a plaque reduction assay 16 hours post-treatment.

Discussion

We previously found that membrane fusion by p14 lipoplexes was sufficient to upregulate ISGs in the absence of viral nucleic acid (Noyce, et al. 2011). The inability of FLAG-RIG-I K270A and K888/902A to reconstitute the antiviral response to p14 in RIG-I$^{-/-}$MDA5$^{-/-}$ THFs strongly suggests that dsRNA recognition is involved in the antiviral response to p14. However, this raises the question of how dsRNA sensing is necessary for the antiviral response to a purified membrane fusion protein.

The simplest explanation is that contaminating dsRNA is transfected alongside p14. However, the fusion-defective p14Δ30 mutant did not induce an antiviral response, suggesting that p14-mediated fusion is necessary (Noyce, et al. 2011). Furthermore, dsRNA-specific dot-blots suggest any contaminating dsRNA is below levels sufficient to explain the antiviral response. Finally, the antiviral response to p14 lipoplexes is entirely blocked by the Ca^{2+} inhibitor 2-APB while the antiviral response to transfected dsRNA is unaffected (supplementary figure 3), suggesting p14 lipoplexes and transfected dsRNA are dependent on different signalling pathways. In totality, the data suggests that contaminating dsRNA alone is insufficient to explain the antiviral response to p14 lipoplexes.

p14-mediated fusion may enhance the sensitivity of cells to low levels of contaminating dsRNA that would otherwise go unnoticed. Alternatively, p14-

mediated fusion may enhance delivery of contaminating dsRNA. Both possibilities would be consistent with our p14 lipoplex data. However, doxycycline-induced expression of p14 was sufficient to trigger upregulation of ISGs in the absence of any exogenous dsRNA. Further work is necessary to determine if this response involves a similar signalling pathway as the response to p14 lipoplexes.

If the antiviral response to p14 is independent of exogenous dsRNA, then it must be coming from within the cell. There are a surprising number of ways that cells recognize endogenous dsRNA (reviewed by (Roers, Hiller, and Hornung 2016)). Given that p14 is capable of cell-cell membrane fusion, it is plausible that p14 fuses internal membranes and breaks down cellular compartmentalization, similar to radiation exposure or HSV infection (Chiang, et al. 2018; Ranoa, et al. 2016). Stimulatory dsRNA can also be generated in cells through the activation or inhibition of different RNA-associated machinery (Ahmad, et al. 2018; Malathi, et al. 2007; Eckard, et al. 2014). p14-mediated fusion might lead to activation or inhibition of parts of this machinery. Finally, it is possible endogenous dsRNA is unchanged, but p14 causes dsRNA sensing pathways become more sensitive. RIG-I and MDA5 point mutants with enhanced activity can spontaneously recognize endogenous dsRNA and are associated with autoimmune interferonopathies (reviewed by (Kato and Fujita 2015)). Thus, if antiviral

signalling increased the sensitivity of RLRs it may trigger a response to endogenous dsRNA.

The absence of an antiviral response to liposome fusion at the plasma membrane suggests that not all membrane fusion is sufficient to trigger upregulation of ISGs and an antiviral response. Our data with fusogenic liposomes differs significantly from what was reported by *Holm, et al*. (Holm, et al. 2012). While they observed a STING-dependent, Ca^{2+}-independent IFN response to fusogenic liposomes, we observed Ca^{2+} oscillations in the absence of antiviral signalling. There are a number of differences that may explain this discrepancy including cell-type or species specific differences since our work was done in human fibroblasts while their work was done in a variety of murine cells. Our results with p14 suggest that some element of the response may be related to cell stress and stimulatory nucleic acid, the levels of which may differ substantially between cells and experimental designs.

Materials and methods

Materials

Human embryonic lung (HEL) fibroblasts (American Type Culture Collection (ATCC)) and telomerized human fibroblasts (THF) (kind gift from Victor DeFilippis) were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum. Vesicular stomatitis virus expressing green fluorescent protein (VSV-GFP)(Indiana strain) was grown using Vero cells. The double stranded RNA (dsRNA) mimetic poly I:C was resuspended in phosphate-buffered saline (PBS) and diluted in serum-free medium to a working concentration of 20 uM. The Ca^{2+} inhibitor 2-aminoethyl diphenylborinate (2-APB) was reconstituted in MeOH and diluted in serum-free media to a working concentration of 200 uM. B18R (Millipore) was used to block IFN at 50 ug/ml in serum-free media and incubated with cells for 30 minutes prior to treatment.

Fusogenic liposomes and p14 lipoplexes

Fusogenic liposomes were made as previously described (Csiszar, et al. 2010). In brief, 1,2-Dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE), 1,2-Dioleoyl-3-triethylammonium-propane, chloride salt (DOTAP) and 1,2-Dioleoyl-*sn*-glycero-3-phosphoethanolamine-N-(lissamine rhodamine B sulfonyl) (ammonium salt) (LR-DOPE) were purchased from Avanti Polar Lipids, Inc. Lipids were reconstituted in a 1:1 methanol and chloroform mixture and mixed in a weight ratio of DOPE/DOTAP/LR-DOPE of 1:1:0.1. The solvent was evaporated

using nitrogen gas and stored at -20 ^{o}C under argon gas until lipid mixtures were resuspended in 20 uM HEPES buffer at a working concentration of 1 mg/ml. The solution was vortexed until resuspended and then sonicated in a bath sonicator for 20 minutes until clarity to form small unilamellar vesicles. Liposomes were diluted 1:100 in serum-free media before a 1 hour incubation with cells.

p14 was purified as described previously (Noyce, et al. 2011). To make p14 lipoplexes, 4 ug of purified p14 diluted in 100 ul PBS and 3 ul of lipofectamine 2000 diluted in 100 ul of nuclease-free water were combined and incubated 1 hour at room temperature before diluting to 1 ml in serum-free DMEM and addition to cells.

Quantitative RT-PCR

RNA was extracted using TRIzol reagent (Invitrogen) and treated with DNAase (Ambion) according to the manufacturers' instructions. 500 ng of RNA was reversed transcribed using SuperScript II Reverse Transcriptase (Invitrogen) with random hexamer primers as per the manufacturer's instructions. Quantitative PCR reactions containing Taqman probes and Universal PCR Master Mix (Applied Biosystems) with a StepOnePlus Q-PCR instrument (Applied Biosystems) were done according to the manufacturer's instructions. Ct values were calculated and GAPDH was used as an endogenous control to calculate individual ΔΔCt values. ΔΔCt values of samples were compared with mock treated samples to calculate fold change. Taqman probes for human GAPDH

(Hs02758991_g1), IFIT1 (Hs03027069_s1), ISG15 (Hs00192713_m1) and CXCL10 (Hs00171042_m1) were used.

Fluorescence microscopy

Cells were washed 3 times in PBS and incubated with 10% formalin for 10 minutes to fix. A Leica DM IRE2 microscope was used to visualize cells. Ca^{2+} imaging was carried out as previously described (Hare, et al. 2015a).

Plaque reduction assay

Cells were conditioned with virus particles or p14 lipoplexes, later challenged with VSV-GFP, and F11 overlay media containing 1% FBS and 1% methyl-cellulose added to restrict plaques. Green fluorescence from VSV-GFP was measured using a Typhoon laser scanner (GE Healthcare) and quantified using ImageQuant software. Fluorescence was then expressed as a percentage of mock infected cells challenged with VSV-GFP.

Western blotting

Protein extracts were harvested by scraping cells into modified RIPA buffer (50 mM Tris pH 7.5, 150mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.05% SDS, 1mM EDTA and protease inhibitor cocktail (Invitrogen)) followed by sonication and clarification by centrifugation. SDS sample loading buffer was added before boiling samples 5 minutes. Protein was separated by SDS-poly-acrylamide gel electrophoresis (SDS-PAGE) and transferred to low-fluorescence

PVDF membrane (LI-COR). Membranes were blocked with Odyssey Blocking Buffer in Tris-buffered saline (TBS) (LI-COR) for 1 hour, incubated with anti-RIG-I (Alme-1) (AdipoGen) diluted 1:1000 or anti-GAPDH (Santa Cruz) diluted 1:5000 overnight, followed by IRDye 800 conjugated anti-rabbit (LI-COR) diluted 1:5000 for 1 hour and scanned on an Odyssey CLx (LI-COR). All antibodies were diluted in 50% blocking buffer in TBS.

Formaldehyde RNA co-immunoprecipitation (fRIP)

Approximately $1x10^6$ Cells were resuspended with trypsin-EDTA, washed 3 times in PBS and incubated with 10% formalin for 10 minutes followed by 2 minutes of quenching in 1M glycine in PBS. Cells were resuspended in modified RIPA buffer and lysed (as above). Cell lysates were incubated with antibody conjugated magnetic beads (Invitrogen) overnight at 4^oC with gentle rocking, washed in modified RIPA buffer and eluted from beads in 50 ul of 2M glycine pH 2.0. Samples for protein analysis had SDS sample buffer added and were boiled for 5 minutes. Samples for RNA analysis had 300 ul of Trizol (Invitrogen) added and RNA purified according to the manufacturers protocol.

Dot blots

Diluted dsRNA (E200 synthesized as described previously (DeWitte-Orr, et al. 2009)) or purified p14 were applied to dry Hybond-N+ positively charged nylon membrane (Amersham) and crosslinked with 100 uJ/cm^2 UV-light using a

Stratalinker 1800 (Stratagene). The membrane was blocked in 5% skim milk in TBS for 1 hour, incubated with J2 antibody (SCICONS) diluted 1:2000 in 1% BSA in TBS for 2 hours, followed by HRP-conjugated anti-mouse diluted 1:5000 in 1% skim milk in TBS for 1 hour. An ECL solution of luminol, 4-IPBA and H_2O_2 in 100 mM Tris was added prior to X-ray film exposures.

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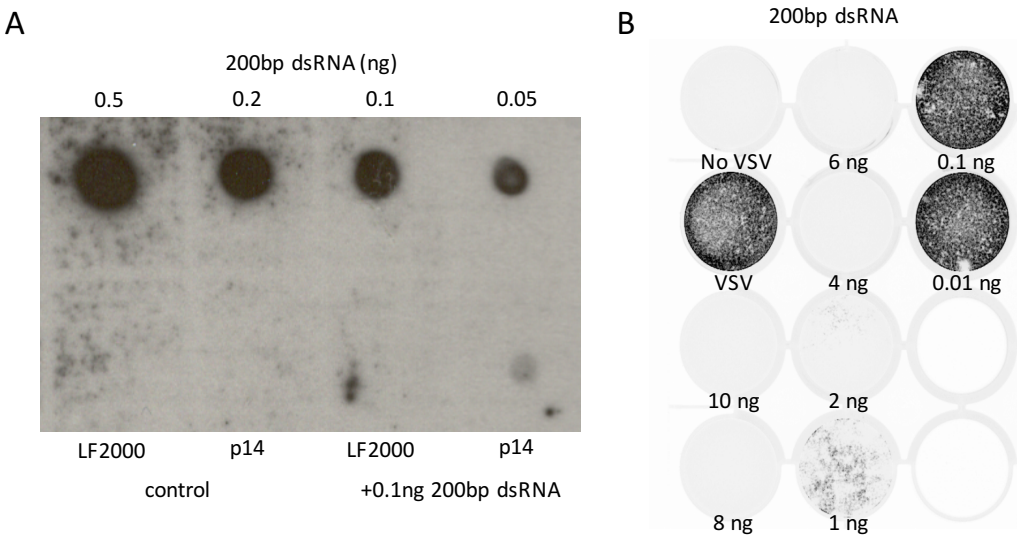
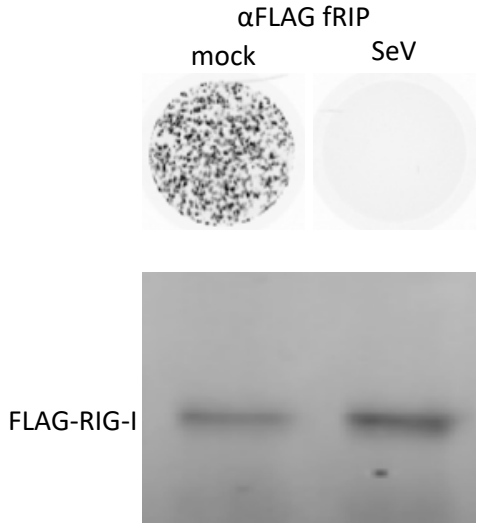
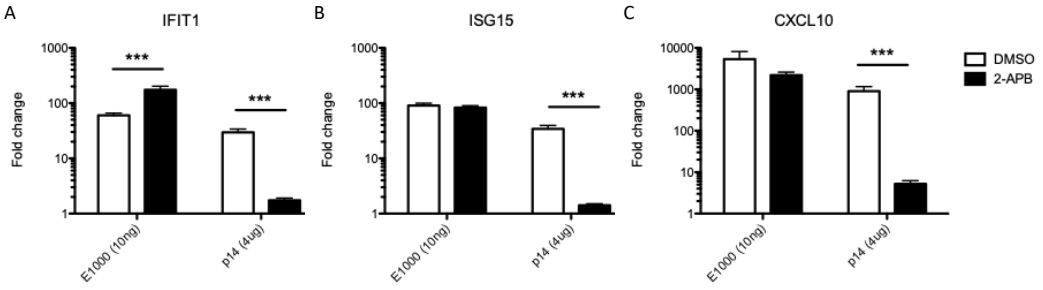
Supplementary Data



Supplementary figure 1 - Absence of detectable dsRNA in purified p14. lipofectamine 2000 (LF2000) or 4 ug of purified p14 were incubated 1 hour with or without addition of 1 ng of dsRNA in 5 ul total volume. LF2000, p14 and serially diluted dsRNA were cross-linked to nylon membrane and probed for the presence of dsRNA using specific antibodies and chemi-luminescence (A). THF fibroblasts were transfected with serial dilutions of dsRNA and challenged with VSV-GFP 16 hours later in a plaque reduction assay (B).



Supplementary figure 2 – Stimulatory RNA co-purified from RIG-I$^{-/-}$MDA5$^{-/-}$ THFs stably expressing FLAG-RIG-I and infected with SeV. FLAG-RIG-I was purified from RIG-I$^{-/-}$MDA5$^{-/-}$ FLAG-RIG-I THFs infected with SeV or mock infected, and co-purified RNA transfected into wildtype THFs. Purified FLAG-RIG-I was measured by western blot (bottom) while co-purified stimulatory RNA was measured by plaque-reduction assay of transfected THFs (top).



Supplementary figure 3 – Inhibition of Ca^{2+} signalling with 2-APB has a greater impact on the antiviral response to p14 than transfected dsRNA. THFs were pretreated for 30 minutes and maintained in 200 µM 2-APB during treatment with lipoplexes containing 1000 bp dsRNA (E1000) or p14. RNA was harvested 16 hours post-treatment and mean fold change in IFIT1 (A), ISG15 (B) or CXCL10 (C) over mock treated cells determined by qRT-PCR for 3 biological replicates and graphed. Groups were compared by 2-way ANOVA with Bonferonni post-tests after log transformation (*** for $p < 0.001$)

Chapter 5 – Conclusion

5.1 - Updated model of the antiviral response to enveloped virus particles

This thesis set out to uncover the mechanism underlying an IRF3-dependent IFN-independent antiviral response to membrane perturbation. Not only do we now know more about how cells sense membrane perturbation, but our interpretation of how cells recognize enveloped viruses has changed substantially.

We previously proposed a model for how fibroblasts respond to increasing levels of enveloped virus infection (Figure 2). Fibroblasts recognize low-level infection with enveloped virus entry and directly upregulate expression of antiviral ISGs in the absence of IFN. Infection with increasing amounts of enveloped virus particles, replicating virus or treatment with dsRNA activates NF-κB and upregulates IFN-β.

While it appears that membrane perturbation contributes to the antiviral response, nucleic acid sensing pathways are critical for the antiviral response to UV-inactivated enveloped viruses in our model. It was previously found that nucleic acid sensing PRRs capable of recognizing enveloped virus particles were

disposable or absent in human fibroblasts (Paladino, et al. 2006). The discovery of new PRRs and a better understanding of how viruses are sensed allowed us to explain how cells recognize incoming viral genomes from UV-inactivated virus.

While we still observe an antiviral response to enveloped virus particles in the absence of detectable IFN, we now know that IFN is produced in minute levels that are nonetheless critical for antiviral protection (Hare, et al. 2020b). The bioassay for IFN, which measures the ability of transferred supernatants to create an antiviral response in naïve cells, is the most sensitive and reliable assay we have used. Direct comparison in our lab found that IFN bioassays were an order of magnitude more sensitive than IFN-β ELISA kits (data not shown). Nonetheless, IFN produced following infection with low levels of enveloped virus is only detectable by bioassay after concentrating protein in supernatants (Hare, et al. 2020b).

IRF3 markers were inconsistent among cells infected with different enveloped viruses despite the antiviral response to each virus being similarly IRF3-dependent (Noyce, Collins, and Mossman 2009). Because of inconsistent IRF3 activation markers we speculated that additional non-canonical IRF3 activation markers were involved and possibly confounding IRF3 detection. We now know that some enveloped virus particles (i.e. SeV) activate IRF3 in a minority of cells and protect the monolayer through paracrine IFN signalling

(Hare, et al. 2020b). This explains the absence of detectable IRF3 activation in conventional assays, most of which rely on bulk population measurements.

The new data presented here prompted us to revaluate our model (Figure 4). Here we propose that recognition of membrane perturbation and nucleic acid during enveloped virus entry signals activation of IRF3. Activated IRF3 concurrently binds PRD-I/III and IREs to directly upregulate expression of IFN-β and a subset of ISGs. Autocrine and paracrine IFN-β signaling upregulates additional IFN-dependent ISGs. Both sets of antiviral ISGs act in concert to create an antiviral state and restrict virus spread through the cell population.

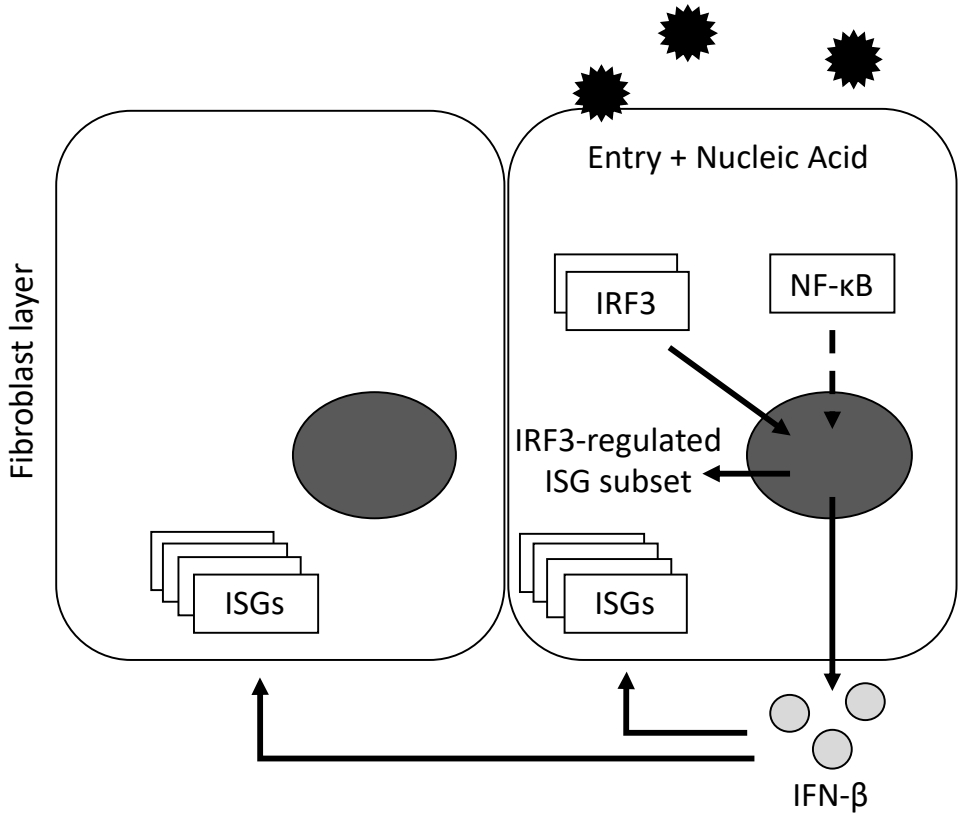


Figure 4 – Updated model depicting the antiviral response to enveloped virus particles. A combination of enveloped virus entry and nucleic acid sensing activate IRF3 which directly upregulates a subset of IRF3-regulated genes including IFN-β. NF-κB acts to enhance IFN-β production under certain conditions. IFN-β is secreted and upregulates ISGs in autocrine and in paracrine through binding to IFNAR and Jak-STAT signalling. IRF3- and IFN-regulated gene upregulation create an antiviral state that restricts virus spread through the cell population.

5.2 - Transcription factors involved in IFN-dependent / independent ISG upregulation

NF-κB binds to PRDII in the IFN-β promoter and was described as being part of the IFN-β enhanceosome. We previously detected activation of IRF3, and not NF-κB, following infection with low levels of UV-inactivated HCMV (Paladino, et al. 2006). At the time, we concluded that essential components of the IFN-β enhanceosome were not activated by low level enveloped virus infection, and this explained the absence of detectable IFN-β. We now know that NF-κB is involved but not essential for upregulation of IFN-β by dsRNA (Basagoudanavar, et al. 2011; Wang, et al. 2007; Peters, et al. 2002). Given the importance of the NF-κB subunit RelA in the antiviral response to SeV-UV, we suspect that NF-κB activation in a minority of cells infected with SeV-UV enhances the kinetics of IFN-β production. Given the unimportance of RelA in the antiviral response to HCMV-UV, NF-κB is likely not activated, consistent with previous data (Paladino, et al. 2006). The difference in NF-κB activation may be due to differences in the signalling complex assembled on MAVS or STING, or could be due to the level of stimulation each virus provides to responding cells.

An ATF2/c-Jun heterodimer (AP1) is part of the IFN-β enhanceosome and it may also be dispensable for upregulation of IFN-β. Similar to NF-κB, AP1 binding is important for recruitment of IRF3 to the IFN-β promoter (Falvo, et al. 2000). Early work on the IFN-β enhanceosome suggested that NF-κB and AP1 are

both dispensable for upregulation of ISGs and IFN-β (Peters, et al. 2002). *Peter, et al.* found that cells lacking NF-κB, p38 MAPK or JNK activation nonetheless upregulate IFN-β, and NF-κB and p38 MAPK inhibitors fail to fully block upregulation of IFN-β. The ability of cells to upregulate IRF3 in the absence of NF-κB and AP1 activation suggests that under certain conditions IRF3 alone is sufficient to upregulate both IFN-β and IRF3-responsive ISGs.

We previously described an IRF3-dependent IFN-independent antiviral response to enveloped virus particles. We have since shown that the antiviral response to enveloped virus particles involves combined IRF3-mediated upregulation of an ISG subset and IFN-β. While we previously failed to detect canonical IRF3 activation, this seems to be because IRF3 was activated in only a small minority of cells. We do not know whether additional IRF3 modifications are required for full upregulation of ISGs in response to enveloped virus particles. Inducible expression of the constitutively active IRF3-5D mutant was sufficient to directly upregulate a subset of ISGs (Grandvaux, et al. 2002), proving that sufficient amounts of C-terminally phosphorylated IRF3 are enough to upregulate ISGs. IRF3 may be additionally modified in response to enveloped virus entry, but such modifications are not required to explain our observations.

Interestingly, the similarities between regulation of IFN-β and IRF3-responsive ISGs suggest very similar enhancer elements and a possible common evolutionary origin. IFN signalling appeared in a common ancestor of jawed

vertebrates around the same time as adaptive immunity. On the other hand, IRFs are widespread in nature and would have appeared in early multicellular life (Nehyba, Hrdlicková, and Bose 2009). It is possible to imagine that ancestral antiviral genes were regulated by through cell autonomous signalling, similar to the direct IRF3-dependent ISG upregulation we observe today (Negishi, Taniguchi, and Yanai 2018). One such IRF-responsive enhancer element may have been duplicated upstream of an ancestral IFN gene. Once a feed forward loop was established, the ability to quickly upregulate antiviral protein in nearby uninfected cells would provide the selection necessary to hone this signalling pathway.

5.3 - Recognition of membrane perturbation during enveloped virus entry

Because the antiviral response to p14 cannot be fully explained by contaminating nucleic acid, it is unclear how p14-mediated fusion triggers a RLR-MAVS dependent response. Disruptions of cellular homeostasis can cause recognition of endogenous nucleic acid by altering the amounts, location or motifs of cellular nucleic acid (reviewed in (Schlee and Hartmann 2016; Roers, Hiller, and Hornung 2016)). Viral infection, DNA damage or metabolic stress can release stimulatory RNA or DNA from the nucleus or mitochondria (Chiang, et al.

2018; Ranoa, et al. 2016; Mackenzie, et al. 2017; Kim, et al. 2019). Oxidative stress or ER stress can also cause accumulation of stimulatory RNA or DNA (Gehrke, et al. 2013; Cho, et al. 2013), and bacterial toxins exist that can stimulate these pathways (Cho, et al. 2013; Hu, et al. 2019). Normally, endogenous nucleic acid sensing is tempered by RNA editing enzymes like ADAR1 that prevent duplex formation (Liddicoat, et al. 2015) and nucleases like TREX1 and SKIV2L that prevent accumulation of nucleic acid. Malfunction of these enzymes is associated with aberrant IFN production when stimulatory nucleic acid accumulates in the cytosol (Ablasser, et al. 2014; Eckard, et al. 2014). While there are many conditions that result in RLRs recognizing endogenous nucleic acid, identifying RIG-I-associated transcripts following p14-mediated fusion may suggest how p14 triggers an antiviral response. While our current techniques are sufficient to co-precipitate stimulatory RNA in the context of SeV infection, we must improve the sensitivity and stringency of our immunoprecipitation technique for comparatively weaker stimuli like p14 lipoplexes.

Our data with enveloped viruses and fusogenic liposomes suggest that not all membrane fusion is sufficient to trigger an antiviral response. The importance of cellular nucleic acid sensing pathways in the antiviral response to HCMV and SeV particles suggests that enveloped virus entry, and associated Ca^{2+} signalling, is not sufficient for an antiviral response (Hare, et al. 2015b). Rather, the data suggest that HCMV and SeV particles are recognized by nucleic acid

sensors and entry-associated Ca^{2+} signalling plays an accessory role (Figure 5). SeV-UV infects and causes Ca^{2+} oscillations in the majority of cells but only activates IRF3 in a minority of cells (Hare, et al. 2015b; Hare, et al. 2020b). Additionally, liposome fusion with the cell membrane triggers Ca^{2+} oscillations but fails to trigger detectable ISG upregulation. This raises the question of what role membrane perturbation and Ca^{2+} signalling are playing in the antiviral response to enveloped virus.

One possibility is that membrane perturbation is only recognized at certain locations. The fusogenic liposomes we used were characterized for their ability to fuse with the plasma membrane (Csiszar, et al. 2010). While examining enveloped virus entry pathways we found that dynasore, an inhibitor of clathrin-dependent endocytosis, impaired ISG upregulation in response to HCMV-UV and p14 lipoplexes but not SeV-UV (Figure S4). This is consistent with reports that SeV enters through fusion at the plasma membrane (Fan and Sefton 1978). It is possible that membrane perturbation is only sensed in endosomal compartments, similar to nucleic acid recognition by TLR, because this is the most common viral entry location and allows signalling machinery to be concentrated in a specific compartment. This theory would explain why fusogenic liposomes and SeV don't trigger antiviral signalling in the majority of cells but doesn't explain why they still cause Ca^{2+} oscillations.

It is also possible that virus-mediated fusion is recognized differently from regular membrane fusion events. This would explain why p14 lipoplexes trigger an antiviral response but fusogenic liposomes do not. Distinct mechanisms of fusion, forced membrane curvature for example, might be differentially recognized by the cell. However, this theory again does not explain why fusogenic liposomes and SeV trigger Ca^{2+} oscillations in the absence of IRF3 activation and upregulation of ISGs.

An appealing explanation for the involvement of both membrane perturbation and nucleic acid sensing is that membrane perturbation enhances sensing of nucleic acid or downstream signalling. This could be by activating pathways like autophagy or stress granule formation that expose nucleic acid to recognition by cellular sensors, or by acting as a co-stimulatory signal during STING or MAVS activation. Inhibition of Ca^{2+} signalling during HCMV-UV infection impaired STING activation, suggesting that Ca^{2+} signalling acts upstream of STING. We decided to use $STING^{-/-}$ and $MAVS^{-/-}$ MEFs test whether membrane perturbation by an enveloped RNA virus enhances the antiviral response to an enveloped DNA virus or *vice versa*. We tried to test whether fusogenic liposomes, which cause Ca^{2+} oscillations, could enhance the antiviral response to adenovirus (AdV), a non-enveloped virus. However, we found that fusogenic liposomes did not enhance ISG upregulation in response to AdV, except under conditions that enhanced AdV entry into cells (Figure S5 A-B). Additionally, when

we co-infecting MAVS$^{-/-}$TLR3$^{-/-}$TRIF$^{-/-}$ MEFs with AdV and SeV we did not see an enhancement in the antiviral response over AdV alone (Figure S5 C). It may be possible that the kinetics or localization of Ca^{2+} signalling are critical to enhance nucleic acid sensing and Ca^{2+} signalling cannot be provided in *trans* through separate particles. However, this seems at odds with the Ca^{2+} oscillations we observed which encompassed the whole cell and persisted up to an hour after infection.

While Ca^{2+} signalling is associated with membrane perturbation, it still isn't clear how this contributes to antiviral signalling. Ca^{2+} signalling plays an important role in the activation of STING and may also play a role in the activation of MAVS (Shu, et al. 2012; Srikanth, et al. 2019; Mathavarajah, Salsman, and Dellaire 2019). However, Ca^{2+} signalling also plays an important role in many intracellular trafficking and membrane fusion events (reviewed by (Hay 2007)). While we observed that cytosolic Ca^{2+} was important for antiviral signalling, this could be for a variety of reasons unrelated to the Ca^{2+} oscillations we observed. Therefore, it will be important to confirm that the cytosolic Ca^{2+} oscillations are a *bona fide* antiviral signal in the context of enveloped virus entry. To do this, future experiments may need to employ more specific forms of Ca^{2+} inhibition and more refined methods of Ca^{2+} detection.

It is possible that membrane perturbation affects other transcription factors like NF-κB or ATF2/c-Jun that bind to the IFN-β promoter region. On their

own these transcription factors are insufficient for IFN-β or ISGs upregulation, but they may stabilize IRF3 binding and enhance IFN-β production. This is consistent with our theory that membrane perturbation acts like a co-stimulatory signal for nucleic acid recognition to upregulate ISGs. While NF-κB is well studied and we previously failed to detect its activation following enveloped virus infection (Paladino, et al. 2006), very little is known about ATF2/c-Jun. ATF2 and c-Jun are activated by MAPK and JNK respectively but little is known about how they are activated during virus infection. It would be interesting to examine whether certain types of membrane perturbation or Ca^{2+} signalling are important for activation of these transcription factors that lie upstream of IFN-β but not IRF3-mediated ISG upregulation.

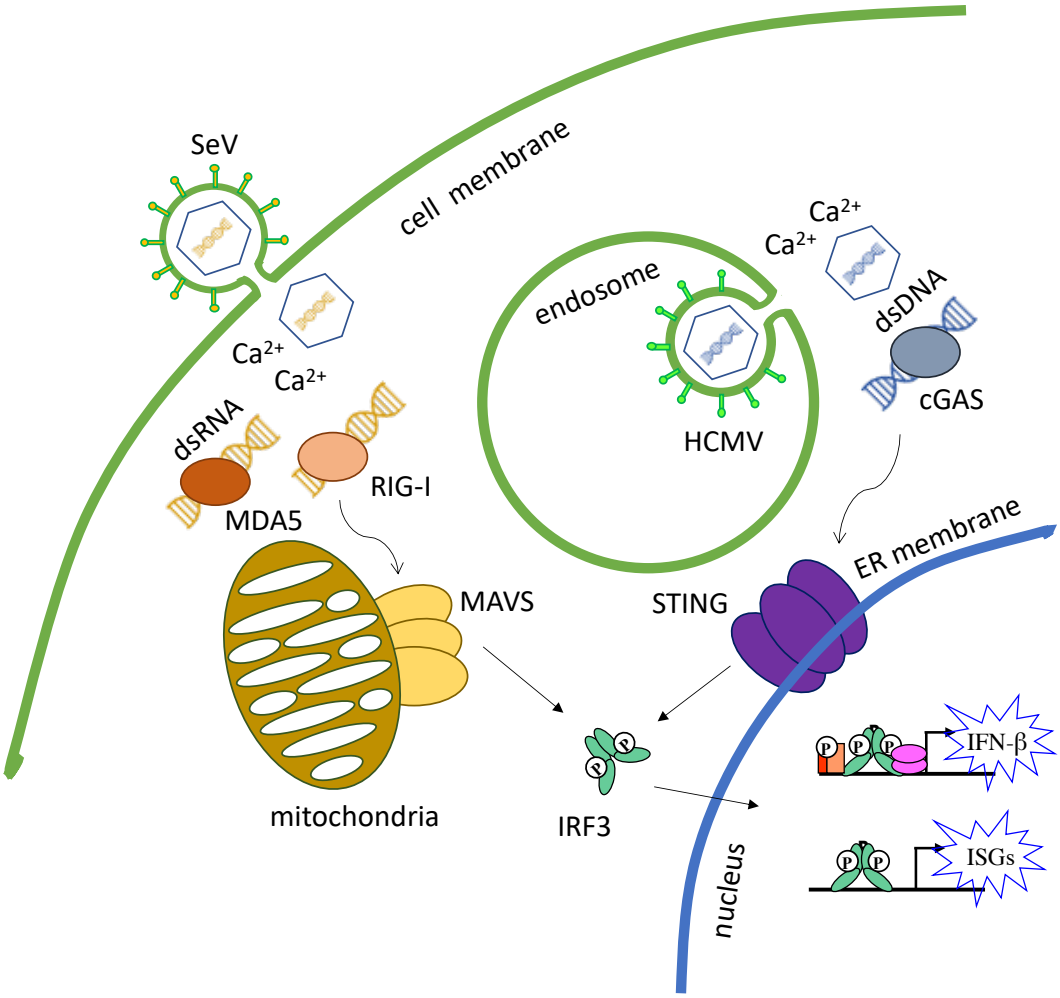


Figure 5 – Updated model depicting enveloped virus particle recognition by pattern recognition receptors. SeV particles enter by fusion at the cell membrane and viral RNA is exposed in the cytosol to recognition by RIG-I like receptors which IRF3 via MAVS. HCMV particles enter by fusion in endosomes and viral DNA is exposed in the cytosol to cGAS which activates IRF3 via STING. Activated IRF3 enters the nucleus and upregulates expression of IFN-β and a subset of antiviral ISGs. Cytosolic Ca^{2+} oscillations are associated with SeV and HCMV entry. Cytosolic Ca^{2+} contributes to activation of nucleic acid recognition pathways.

5.4 - Other potential membrane perturbation signalling pathways

There is still a lot unknown about how cells sense and signal membrane perturbation, but with a few promising leads to investigate. While investigating different classes of membrane channels we found that Gd^{3+}, an inhibitor of stretch-gated channels, and carbenoxelone (CBX), an inhibitor of gap-junctions, both impair the antiviral response to a variety of enveloped viruses (Figure S6). While we were unable to see effects with more specific inhibitors of stretch-gated channels and gap-junctions, it is interesting to note that both these inhibitors have documented effects on connexin hemichannels (Kang, et al. 2008).

We suspected kinases might be involved in the antiviral response to p14 lipoplexes, so we screened a library of 1600 putative kinase inhibitors for their effect on ISG upregulation in cells treated with p14 lipoplexes. This screen identified a collection of compounds with inhibitory effect and varying degrees of specificity (Figure S7 and Table S1). We used published data on the GSK2 library's ability to impair *in vitro* kinase activity to identify possible targets of our compounds (Drewry, Willson, and Zuercher 2014). Interestingly, many of the more specific compounds inhibited PI3K family members. We previously found that the PI3K inhibitor LY294002 blocked the antiviral response to enveloped virus particles (Noyce, Collins, and Mossman 2006). However, these data must be

interpreted cautiously given the PI3K/Akt pathway may regulate antiviral ISGs downstream of IFN (Kaur, et al. 2008; Ezell, et al. 2012). Furthermore, in our previous work PI3K inhibition failed to prevent IRF3 activation following HSV-UV and HCMV-UV infection (Noyce, Collins, and Mossman 2006). Thus, there is a strong possibility that LY294002 and the compounds identified in our screen act on kinases downstream of IFN rather than kinases involved in signalling membrane perturbation.

5.5 – Concluding remarks

Since I started my thesis, our model for how cells respond to enveloped virus particles has evolved considerably. We previously proposed that cells recognize membrane perturbation during enveloped virus entry and directly upregulate antiviral ISGs through a non-canonical pathway involving IRF3. We now know that cells directly upregulate antiviral ISGs and IFN-β through an IRF3-dependent pathway in response to low levels of enveloped virus particles. Furthermore, canonical IRF3 activation appears to be sufficient to explain antiviral protection when observed at the level of single cells. While we previously considered membrane perturbation by enveloped virus entry sufficient to upregulate antiviral ISGs, we now know that nucleic acid sensing

plays a critical role in enveloped virus recognition. While membrane perturbation may enhance the antiviral response to nucleic acid, this role remains unclear.

The findings of this thesis have furthered our understanding of how cells recognize and respond to virus particles. This is not only important for our understanding of viral disease, but also to understand our bodies' reaction to interventions like vaccines, viral vectors and oncolytic viruses. Further work will hopefully explain our unanswered questions about how cells recognize membrane perturbation during enveloped virus entry.

Appendix – Supplementary data

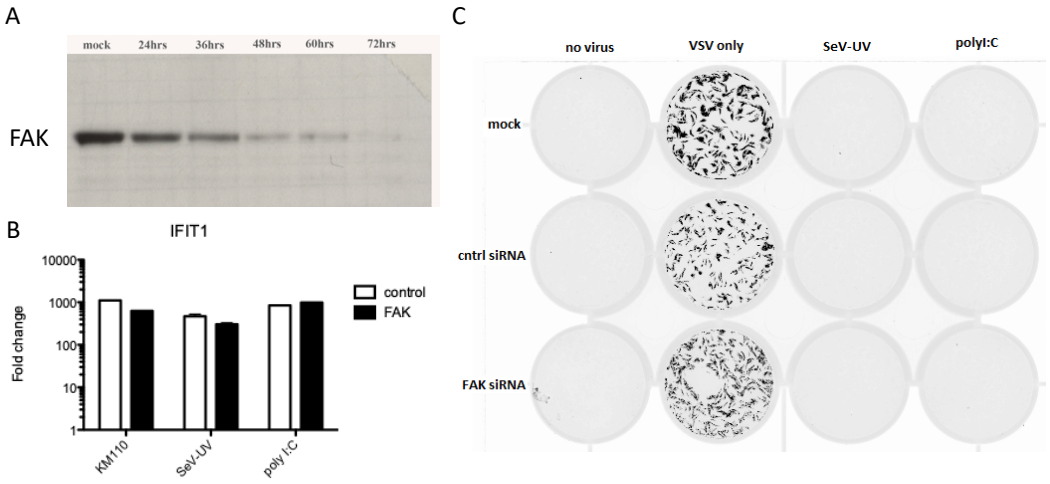


Figure S1 – FAK siRNA knockdown has little to no impact on antiviral response. HEL fibroblasts were transfected with pooled FAK-specific or control Stealth siRNA (Thermo). A decline in FAK protein was measured by immunoblot of extracts harvested from cells 24-72 hours post-transfection (A). Following 72 hour knockdown with FAK or control siRNA, HEL fibroblasts were infected with KM110 (VP16- and ICP0-null HSV mutant), SeV-UV or treated with poly I:C. RNA was harvested 6 hours post-treatment and fold-change in IFIT1 transcript measured by qRT-PCR and 1-2 replicates graphed (B). Similarly treated cells were challenged 16 hours later with VSV-GFP in a plaque reduction assay and representative fluorescence plate scan shown (C).

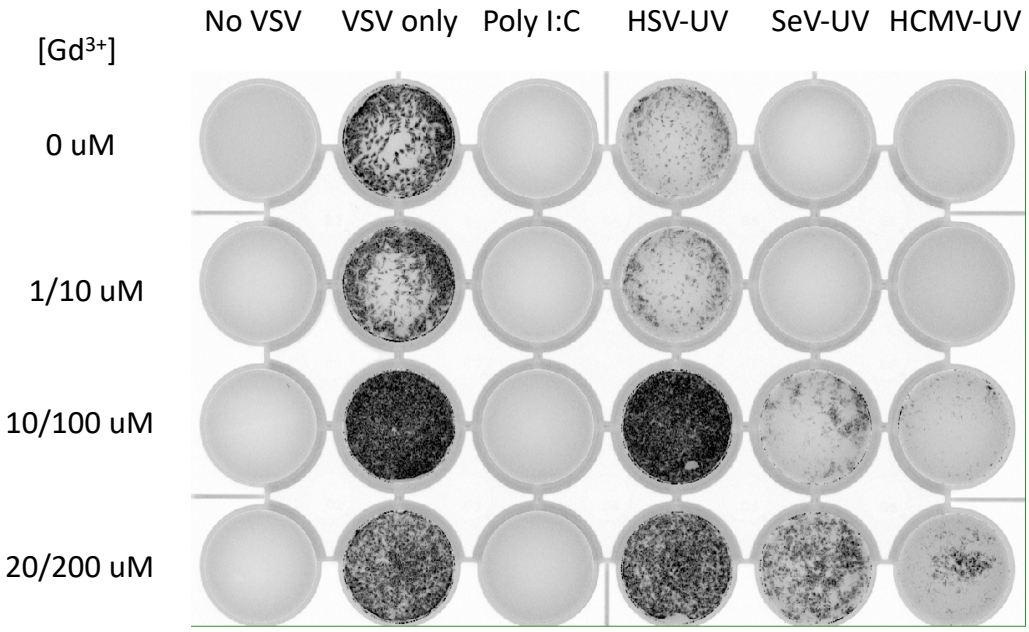


Figure S2 – Stretch channel inhibitor Gd^{3+} impairs the antiviral response to enveloped viruses and affects VSV-GFP replication. HEL fibroblasts were pretreated for 30 minutes and maintained with the indicated concentration of Gd^{3+} (10 times less during pretreatment) during treatment with 20 µg/ml poly I:C, infection with 10 pfu/cell HSV-UV, 80 HAU/10^6 cells SeV-UV or 0.02 pfu/cell HCMV-UV. Cells were challenged with VSV-GFP 16 hours post-treatment in a plaque reduction assay and a representative fluorescence plate scan shown.

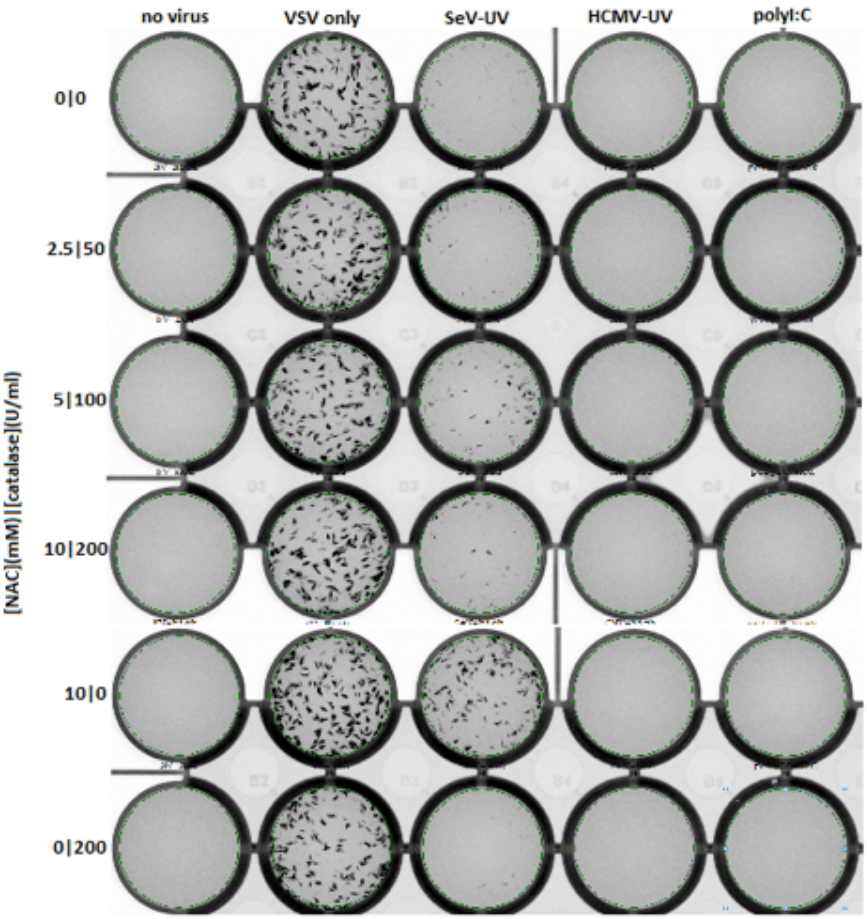


Figure S3 – ROS scavengers fail to consistently inhibit the antiviral response to SeV-UV and HCMV-UV. HEL fibroblasts were pretreated with the indicated concentration of NAC or catalase for 30 minutes and infected with SeV-UV, HCMV-UV or treated with poly I:C. 16 hours later HEL fibroblasts were challenged with VSV-GFP in a plaque reduction assay and representative fluorescence plate scan shown.

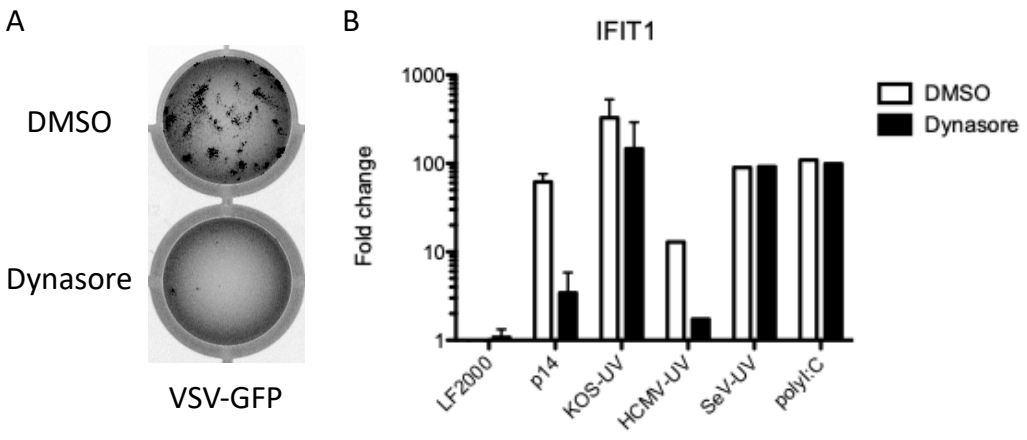


Figure S4 – The dynamin inhibitor dynasore has variable effects on the antiviral response to lipid-based particles. HEL fibroblasts were pretreated with 40 µM dynasore for 30 minutes before infection with VSV-GFP in the presence of dynasore. Impaired VSV-GFP entry was observed when cells were scanned 20 hours post-infection with a fluorescence scanner (A). Similarly pretreated HEL fibroblasts were infected with HSV(KOS)-UV, HCMV-UV, SeV-UV or treated with p14 lipoplexes or poly I:C in the presence of dynasore. RNA was harvested 6 hours post-treatment, change in IFIT1 transcripts determined by qRT-PCR and mean-fold change of 1-2 replicates graphed (B).

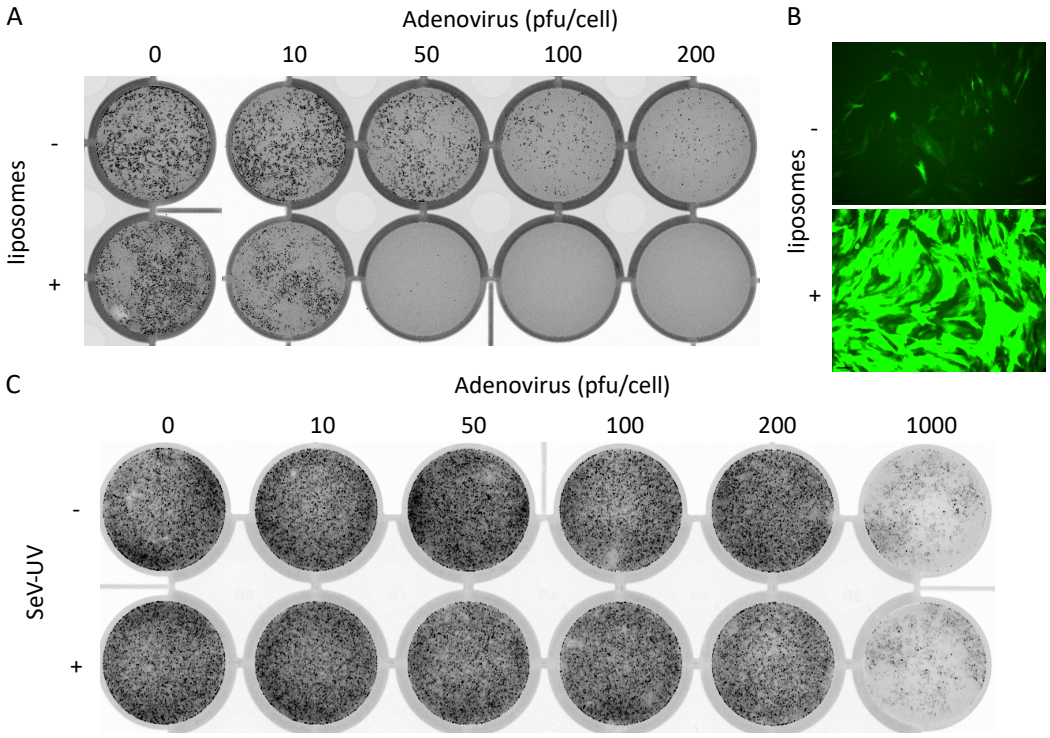


Figure S5 – Co-treatment with liposomes or SeV-UV fails to sensitize MEFs to adenovirus particles. MAVS$^{-/-}$TLR3$^{-/-}$TRIF$^{-/-}$ MEFs were infected with E1/E3-null adenovirus vector (AdV) at the indicated multiplicity in the presence of absence of liposomes (A) or 400 HAU/10^6 cells SeV-UV (C). MEFs were challenged with VSV-GFP 16 hours post-indection in a plaque reduction assay and representative fluorescence plate scans shown. MAVS$^{-/-}$TLR3$^{-/-}$TRIF$^{-/-}$ MEFs were infected with 50 pfu/cell E1/E3-null adenovirus vector expressing GFP (AdV-GFP) in the presence or absence of liposomes and GFP+ cells imaged with a fluorescence microscope 16 hours post-infection (B).

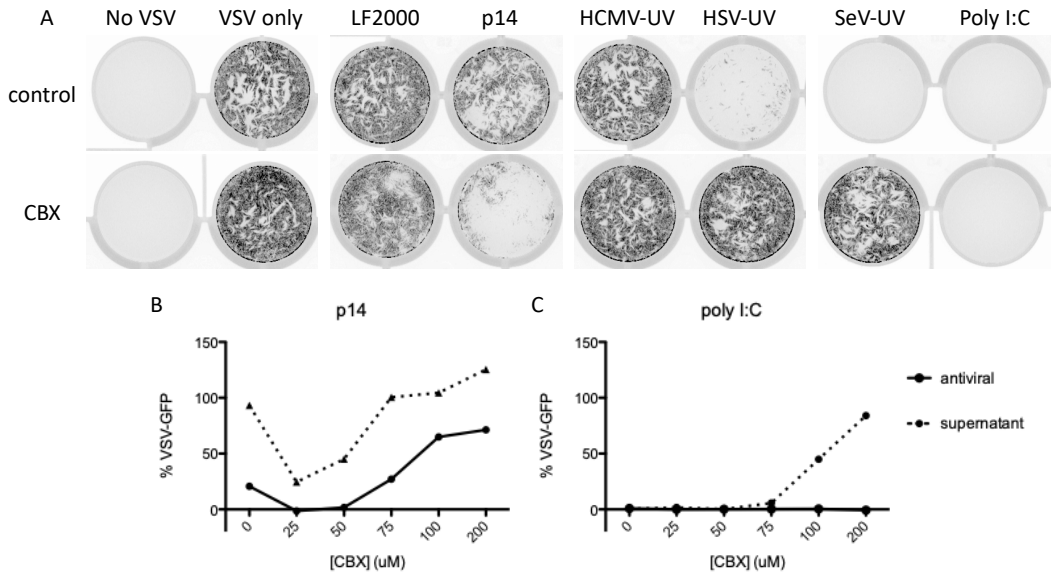


Figure S6 – Gap-junction inhibitor CBX impacts the antiviral response to p14 lipoplexes and enveloped virus in a dose-dependent manner. HEL fibroblasts were pretreated for 1 hour and maintained in 100 µM carbenoxelone (CBX) during treatment with LF2000, p14 lipoplexes, 20 µg/ml poly I:C, infection with 0.02 pfu/cell HCMV-UV, 10 pfu/cell HSV-UV, 80 HAU/10^6 cells SeV-UV or media alone. HEL fibroblasts were then challenged with VSV-GFP 12 hours post-treatment in a plaque reduction assay and a representative fluorescence plate scan shown (A). HEL fibroblasts similarly treated with media, LF2000, p14 lipoplexes or poly I:C with the indicated concentration of CBX were challenged with VSV-GFP in a plaque reduction assay. Total fluorescence of p14 treated wells was graphed as a percentage of LF2000 (B) while poly I:C was graphed as a percentage of media alone (C).

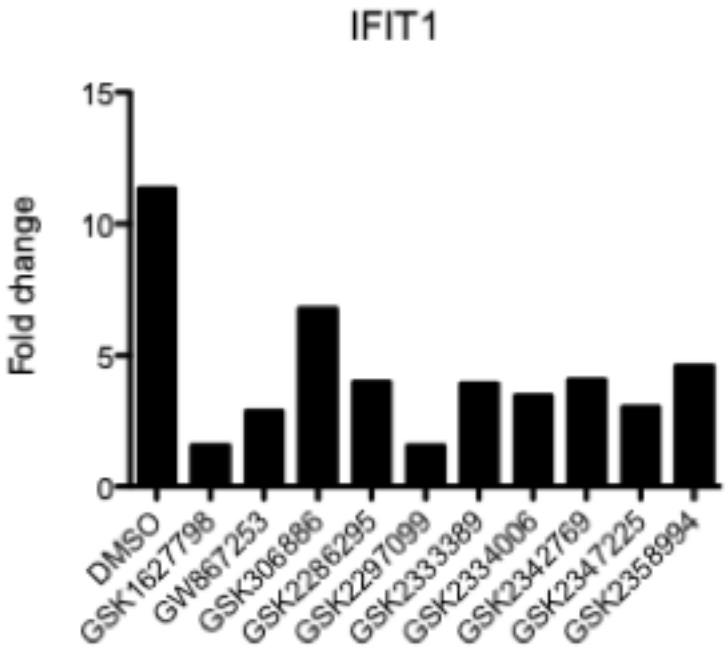


Figure S7 – Kinase inhibitors prevent IFIT1 upregulation by p14 lipoplexes. THF ISRE-luc cells were pretreated for 30 minutes and maintained in DMSO or inhibitor during treatment with p14 lipoplexes. RNA was harvested 12 hours post-treatment and mean fold change in IFIT1 over LF2000 and DMSO treated cells determined by qRT-PCR and graphed.

	4-6-di-Ar-quinoline		4-anilino-quinazoline and analogues	morpholino-imidazo-pyrimidinones				morpholino-triazolo-pyrimidinones		
	GSK1627798	GW867253	GSK306886	GSK2286295	GSK2297099	GSK2333389	GSK2334006	GSK2342769	GSK2347225	GSK2358994
AXL	36	0	0	100	0	0	0	4	45	3
BLK	100	0	10	35	13	21	37	18	52	0
CLK3	55	0	0	13	26	0	11	16	100	14
DLK	32	9	0	0	6	0	21	12	100	0
EGFR	18	0	99.2	20	18	0	45	25	39	0
ERBB2	0	11	98.7	0	0	0	20	0	0	0
FLT4	0	0	98.1	3	0	8	39	34	0	10
JAK2	0	8	2	0	100	17	2	6	18	6
LYN	0	0	0	97.8	2	0	4	0	10	0
MTOR	99.3	32	12	17	12	0	37	45	28	0
PFTK1	31	0	21	2	14	0	0	91.7	20	0
PIK3C2B	99.5	58	28	22	31	0	36	89	80	70
PIK3C2G	99.1	52	0	0	0	0	1	56	33	64
PIK3CA	100	38	0	20	11	0	3	5	15	8
PIK3CB	77	24	0	98.7	96.2	99.7	88	89	100	100
PIK3CD	94.1	39	21	89	75	89	100	99.6	85	82
PIK3CG	99.5	88	5	50	23	3	22	69	56	45
PIK4CB	96.1	99.1	6	0	20	6	0	0	0	0
RET	0	0	97.9	1	0	80	21	72	11	8
SRPK1	0	0	100	34	0	29	21	19	13	17
TRKA	90.2	0	0	0	0	0	17	0	1	1
TRKB	90.7	0	0	0	0	0	0	0	0	0
VPS34	93.3	78	0	97	95.2	98	96.7	95.4	97.1	100

Table S1 – In vitro screen identifies kinases inhibited by chosen compounds. GSK2 compounds used in our screen were screened for in vitro inhibitory activity by Drewry, et al., 2017. Compounds identified in our screen with <12 targets (>90% inhibition) are listed above and all kinases targeted by one or more compounds are shown along with their percent inhibition.

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