INVESTIGATING ANTIVIRAL RESPONSES: FROM INDUCTION TO EVASION

INVESTIGATION OF THE ACTIVATION OF INNATE ANTIVIRAL SIGNALING AND ITS COUNTERACTION BY THE HERPES SIMPLEX VIRUS PROTEIN ICP0

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TITLE:	Investigation of the activation of innate antiviral signaling and its counteraction by the herpes simplex virus protein ICP0
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ABSTRACT

The classical description of the innate antiviral response involves the production of type I interferon (IFN) and the subsequent expression of hundreds of interferon stimulated genes (ISGs), which cooperatively repress viral replication and spread. More recently, an IFN-independent antiviral response has also been described, in which the entry of an enveloped virus induces a subset of ISGs without requiring the production of IFN, although the details of this response remain unclear. In this work, multiple approaches were used to further characterize antiviral signaling pathways. Initially, the potential involvement in the IFN-independent response of the small GTPase Rac1, which has been implicated in both viral entry and antiviral signaling, was investigated. Here, Rac1 was shown to have a possible function in the negative regulation of ISG expression, although technical complications prevented definitive conclusions. As an alternative strategy to identify novel aspects of antiviral signaling, the mechanism of action of ICP0, a herpes simplex virus (HSV) protein involved in innate immune evasion, was investigated. Although ICP0 is generally thought to perform its actions in the nucleus, by tagging proteins for proteasome-mediated degradation via the E3 ubiquitin ligase activity of its RING finger domain, here it was shown that not only does cytoplasmic ICP0 have a RING-dependent but proteasome-independent ability to block antiviral signaling, but also that ICP0 has a previously unknown RING-independent function in the promotion of viral replication in the cytoplasm. To further investigate the cytoplasmic activities of ICP0, proteins interacting with ICP0 in the cytoplasm were identified using quantitative mass spectrometry. This revealed several intriguing binding partners for ICP0, including WDR11, a poorly-characterized cellular protein which was shown to undergo a dramatic relocation during HSV infection, although it was not required for viral replication in cultured cells. Therefore, this study has uncovered several new and unexpected insights into ICP0 behavior.

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LIST OF ABBREVIATIONS

2-5A	2’-5’-linked oligoadenylates
AIP1	Actin interacting protein 1
αMEM	Alpha minimal essential medium
AREB	Animal research ethics board
ATCC	American type culture collection
Atg	Autophagy related gene
ATP	Adenosine triphosphate
ATRX	α-thalassemia mental retardation, X-linked
B6	C57BL/6
BAC	Bacterial artificial chromosome
BSA	Bovine serum albumin
bZIP	Basic-region leucine zipper
C14orf166	Chromosome 14 open reading frame 166
CA	Constitutively active
cAMP	Cyclic adenosine monophosphate
CARD	Caspase activation and recruitment domain
CARDIF	CARD adaptor inducing IFN-β
CAST	CAZ-associated structural protein
CAZ	Cytomatrix at the active zone
CBP	CREB-binding protein
CBP	Calmodulin-binding peptide
CD83	Cluster of differentiation 83
Cdc	Cell division cycle
CENP	Centromere protein
cGAS	Cyclic GMP-AMP synthase
CLOCK	Circadian locomotor output cycles kaput
co-IP	Co-immunoprecipitation
CREB	cAMP-response element-binding protein
CYLD	Cyclindromatosis tumor suppressor protein
CypA	Cyclophilin A
DAI	DNA-dependent activator of IFN-regulatory factors
DAK	Dihydroxyacetone kinase
DDX3	DEAD-box protein 3
DDX41	DEAD (Asp-Glu-Ala-Asp) box polypeptide 41
DDX60	DExD/H-box helicase 60
DExD/H	Aspartate-glutamate-any amino acid-aspartate/histidine
DHX36	DExD/H-box helicase 36
DMEM	Dulbecco’s modified Eagle’s medium
DN	Dominant negative
DNA	Deoxyribonucleic acid
DNA-PK	DNA-dependent protein kinase
DNA-PKcs	Catalytic subunit of DNA-PK
DOCK2	Dedicator of cytokinesis 2

dsRNA	Double-stranded RNA
DUBA	Deubiquitinating enzyme A
E gene	Early gene
E2FBP1	E2F-binding protein 1
EBV	Epstein-Barr virus
EDTA	Ethylenediaminetetraacetic acid
EF-1δ	Elongation factor 1δ
eIF	Eukaryotic initiation factor
EMX1	Empty spiracles homeobox 1
ER	Endoplasmic reticulum
ERC1	ELKS/RAB6-interacting/CAST family member 1
ERIS	Endoplasmic reticulum IFN stimulator
FAST	Fusion-associated small transmembrane
FBS	Fetal bovine serum
FRET	Fluorescence resonance energy transfer
FXR1	Fragile X mental retardation protein 1
G3BP2	Ras GTPase-activating protein-binding protein 2
GAP	GTPase-activating protein
GDI	GDP dissociation inhibitor
GDP	Guanosine diphosphate
GEF	Guanine nucleotide exchange factor
GFP	Green fluorescent protein
GST	Glutathione-S-transferase
GTP	Guanosine triphosphate
HA	Hemagglutinin
HCF1	Human factor C1
HCMV	Human cytomegalovirus
HCV	Hepatitis C virus
HDAC	Histone deacetylase
HEL	Human embryonic lung fibroblast
Hey1	Hairy/enhancer-of-split related with YRPW motif 1
$(His)_6$	Six histidines
HIV	Human immunodeficiency virus
HMBA	Hexamethylene bisacetamide
HMGB	High mobility group box
HNRNPK	Heterogeneous nuclear ribonucleoprotein K
hpi	Hours post-infection
HPLC	High-performance liquid chromatography
HRP	Horseradish peroxidase
HSE	Herpes simplex encephalitis
HSP	Heat shock protein
HSV	Herpes simplex virus
HUL-1	Herpesvirus ubiquitin ligase 1
HVEM	Herpesvirus entry mediator

ICP	Infected cell protein
IE gene	Immediate-early gene
IFI16	IFNγ-inducible protein 16
IFN	Interferon
IFNAR	Type I interferon receptor
IFNα/β	Type I interferon
IGF2BP1	Insulin-like growth factor 2 mRNA binding protein 1
IKK	IκB kinase
IPS-1	Interferon-beta promoter stimulator
IRF	Interferon regulatory factor
ISG	Interferon-stimulated gene
ISGF3	Interferon-stimulated gene factor-3
ISRE	Interferon stimulated response element
JAK	Janus family protein kinase
JNK	c-Jun amino terminal kinase
KSHV	Kaposi sarcoma herpesvirus
L gene	Late gene
LAT	Latency-associated transcript
LB	Luria Bertani
LGP2	Laboratory of genetics and physiology 2
LoxP	Locus of Crossover in P1
LRRFIP1	Leucine-rich repeat in flightless I-interacting protein 1
LSD1	Lysine-specific demethylase 1
Lys	Lysine
Mal	MyD88 adapter-like
MAPK	Mitogen-activated protein kinase
MAVS	Mitochondrial antiviral signaling
MDA5	Melanoma differentiation-associated gene 5
MEM-F11	minimum essential medium F11
miRNA	MicroRNA
MITA	Mediator of IRF3 Activation
MOI	Multiplicity of infection
MPYS	Methionine-proline-tyrosine-serine
MRE11	Meiotic recombination 11
mRNA	Messenger RNA
MyD88	Myeloid differentiation factor 88
ND10	Nuclear domain 10
Ndrg4	N-Myc downstream-regulated gene 4
NEMO	NFκB essential modulator
NFκB	Nuclear factor κB
NK	Natural killer
NLRX1	NOD-like receptor family member X1
NLS	Nuclear localization signal
NOD2	Nucleotide-binding oligomerization domain 2

NPC	Nuclear pore complex
Nrdp1	Neuregulin receptor degradation protein-1
Nrxn3	Neurexin 3
OAS	2′–5′-oligoadenylate synthetase
Oct1	Octamer transcription factor-1
PAGE	Polyacrylamide gel electrophoresis
Pak1	p21-activated kinase
PAMP	Pathogen-associated molecular pattern
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
pDC	Plasmacytoid dendritic cell
PFL	Protein frequency library
pI:C	Polyinosinic/polycytidylic acid
Pin1	Peptidyl-prolyl cis-trans isomerase 1
PKC1	Protein kinase C1
PKR	Protein kinase R
PML	Promyelocytic leukemia protein
PRD	Positive regulatory domains
PRR	Pattern recognition receptor
PSMA7	Proteasome subunit alpha type 7
QUICK	Quantitative immunoprecipitation combined with knockdown
Rac1	Ras-related C3 botulinum toxin substrate 1
RAG	Recombination activating gene
rag2	Recombinase-activating gene
RAUL	RTA-associated ubiquitin ligase
RBCC	RING-B-box-coiled-coil
RBCK1	RBCC protein interacting with PKC1
REST	Repressor element 1 silencing transcription factor
Rho	Ras homolog
RIG-I	Retinoic acid inducible gene I
RING	Really interesting new gene
RIPA	Radioimmunoprecipitation assay
RISC	RNA-induced silencing complex
RLR	RIG-I-like receptor
RNA	Ribonucleic acid
RNAi	RNA interference
RNF	RING finger protein
Ro52	52 kD Ro protein
rRNA	Ribosomal RNA
RT	Reverse transcription
RTA	Replication and transcription activator
scid	Severe combined immunodeficiency
SDS	Sodium dodecyl sulfate
Serpin E1	Serpin peptidase inhibitor clade E member 1

SeV	Sendai virus
SF	Serum-free
SFK	Src Family Kinases
SG	Stress granule
SIAH-1	Seven in absentia homologue 1
SIKE	Suppressor of IKKε
SILAC	Stable isotope labeling with amino acids in cell culture
SIM	SUMO interaction motif
siRNA	Small interfering RNA
SLC25A5	Solute carrier family 25 member 5
SLS	SIM-like sequence
SSC	Saline-sodium citrate
STAT	Signal transducers and activation of transcription
STING	Stimulator of interferon genes
SUMO	Small ubiquitin-like modifier
Tagln2	Transgelin 2
TAK1	TGFβ-activated kinase 1
TANK	Traf family member-associated NFκB activator
TAP	Tandem affinity purification
TBK1	TANK-binding kinase 1
TBS	Tris buffered saline
TGFβ	Transforming growth factor
TGN	*Trans*-Golgi network
TIA-1	T cell internal antigen 1
TIR	Toll/interleukin-1 receptor/resistance
TLR	Toll-like receptor
TNFR	Tumor necrosis factor receptor
TRAF	TNFR-associated factor
TRAM	TRIF-related adaptor molecule
Trex1	Three prime repair exonucleases
TRIF	TIR domain-containing adaptor-inducing IFN-β
TRIM	Tripartite motif protein
TUBE	Tandem ubiquitin binding entities
Tyk2	Tyrosine kinase 2
UACA	Uveal autoantigen with coiled-coil domains and ankyrin repeats
UBA	Ubiquitin-associated
UBAP2L	Ubiquitin-associated protein 2-like
UBD	Ubiquitin-binding domain
UBX	Ubiquitin-regulatory X
UBXD7	UBX domain protein 7
UL	Unique long
US	Unique short
USP7	Ubiquitin-specific protease 7
UV	Ultraviolet

vhs	Virion host shutoff
VISA	Virus-induced signaling adaptor
VP	Viral protein
VSV	Vesicular stomatitis virus
VZV	Varicella-zoster virus
WDR11	WD repeat domain 11
WT	Wildtype

DECLARATION OF ACADEMIC ACHIEVEMENT

All experiments in this thesis were conceived, designed, executed, analyzed and written by the author of this thesis, under the guidance of Dr. K. Mossman, with the following exceptions: in Chapter 4, Dr. Mossman did the initial recombination and plaque screening for the generation of the D8/FXE virus, while I executed the plaque-purification, preparation, confirmation and characterization of the virus. As well, Dr. A. Ashkar and M. Chew performed the infection, monitoring, vaginal washes and ELISA for the *in vivo* murine experiments, while I prepared the viral inocula and titered the vaginal washes. In Chapter 5, mass spectrometry was performed by P. Ezzati, while I did the immunoprecipitations, trypsinization and sample preparation.

CHAPTER 1
GENERAL INTRODUCTION TO THE INNATE ANTIVIRAL RESPONSE AND THE HERPES SIMPLEX VIRUS

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1.1 The innate antiviral response

Despite almost constant exposure to pathogens, mammals are only rarely infected to the point where disease becomes evident. The first line of defense against this continuous onslaught is the innate immune system. Highly conserved, this response is activated almost immediately upon infection, providing non-specific mechanisms to detect and control infectious organisms ranging from fungi and protozoa to bacteria and viruses. This system also functions to activate the more specific adaptive immune system.

Innate immunity encompasses physical barriers to infection, such as the skin; intrinsic responses performed by constitutively expressed cellular proteins; and inducible responses, including interferon (IFN) signaling. The IFN family of soluble cytokines is a crucial component of the innate response to viral infection. IFNs mediate a wide variety of activities, including anticancer, antiproliferative, antiviral and immunomodulatory functions (1) by inducing the expression of more than 300 interferon-stimulated genes (ISGs) (2). There are three classes of IFNs which have distinct structures, bind separate receptors and have unique biological actions (reviewed in (3-5)). Type II IFN (IFN-γ) is made primarily by activated T-lymphocytes and natural killer cells and has important roles in mediating the functions of immune cells in both the innate and adaptive responses. Type III IFNs (IFN-λ1, - λ2, and - λ3) are a more recent addition to the IFN family, and seem to function primarily in the protection of epithelial cells, due to the restricted distribution of the type III IFN receptor.

Here, I focus on the type I IFNs (IFNα/β), which in humans, consists of five subtypes: IFNα, β, ε, κ, ω, with IFNα and IFNβ the best studied of these. Each subtype is produced from a single gene – with the exception of IFNα, which has 13 genes that encode 12 distinct IFNα proteins (6). All type I IFNs bind to the same receptor, can be produced by most cell types, and have potent antiviral activity. The type I IFN response is bimodal: first, recognition of an invading virus leads to IFNα/β production and secretion. Secondly, this newly-made IFNα/β is detected in an autocrine and paracrine manner, activating signaling pathways to induce ISGs, the products of which work collectively to

disrupt viral replication in the infected cell and to stop the spread of the virus to the surrounding cells.

1.1.1 The production of IFNα/β: Pattern recognition receptors

In the first step of the biphasic type I IFN response, a cell detects the presence of an infecting virus through the recognition of pathogen-associated molecular patterns (PAMPs), highly conserved structural features found in broad classes of pathogens. PAMPs are sensed by pattern recognition receptors (PRRs), which include the Toll-like receptors (TLRs), retinoic acid inducible gene I (RIG-I)-like receptors (RLRs) and several DNA sensors. Upon binding to viral PAMPs, PRRs trigger various signal transduction pathways that culminate in the activation of transcription factors involved in the production of IFNα/β, such as nuclear factor κB (NFκB) or interferon regulatory factor 3 (IRF3) (figure 1.1).

The TLR family includes 10 members in humans (reviewed in (7)). Of these, six recognize ligands likely to be present during viral infection: TLR2 and TLR4, traditionally thought of as cell surface TLRs (though endocytosis is required for IFNα/β production by TLR2) (8) detect viral glycoproteins while TLR3, TLR7, TLR8 and TLR9, as intracellular receptors within endosomes, bind viral nucleic acids. Although immune cells such as macrophages, neutrophils, and dendritic cells express the largest number and highest levels of TLRs, most of the cells in the body express at least some of the TLR classes (9, 10). Upon ligand binding, TLRs undergo a conformational change, with their cytoplasmic Toll/interleukin-1 receptor/resistance (TIR) domains forming a platform for the subsequent recruitment of TIR-containing adaptor proteins. These include myeloid differentiation factor 88 (MyD88); MyD88 adapter-like (Mal); TIR domain-containing adaptor-inducing IFN-β (TRIF); and TRIF-related adaptor molecule (TRAM). Different adaptors associate with particular TLRs, and are involved in the activation of distinct downstream signaling pathways (reviewed in (11)). For example, recruitment of MyD88, which can interact with every TLR except TLR3, generally results in the activation of NFκB, while engaging TRIF, which binds only TLR3 and TLR4, leads to the

phosphorylation of IRF3. MAL and TRAM act as bridging adaptors, with MAL required for the recruitment of MyD88 to TLR2 and TLR4, and TRAM needed for the association of TRIF with TLR4. MyD88-mediated signaling is also involved in the activation of mitogen-activated protein (MAP) kinases, which stimulate the activating transcription factor 2 (ATF2)/c-Jun transcription factors (12).

The RLRs are a second class of PRR, and include RIG-I itself as well as melanoma differentiation-associated gene 5 (MDA5) (reviewed in (13)). These proteins are important for the detection of double-stranded RNA (dsRNA), a common PAMP formed during viral replication. RIG-I and MDA5 have specificities for different types of dsRNA, and are thus involved in the detection of different viruses (14). The RLRs can be found in the cytoplasm of most cell types, though as they are both IFN-inducible proteins (15, 16), they may not be detectable in unstimulated cells (9), and the relative importance of the TLRs and RLRs in antiviral signaling varies with the cell type (17). RLRs have an RNA helicase domain, which is important for dsRNA interaction, and a caspase activation and recruitment domain (CARD), which is involved in mediating signal transduction, through the interaction with a matching domain in the adaptor protein mitochondrial antiviral signaling (MAVS) (18), also known as IPS-1, VISA, or CARDIF (19-21). MAVS, which is embedded in the outer leaflet of the mitochondrial membrane, acts as a platform for the recruitment of downstream signaling molecules (22) that ultimately permit the RLRs to activate both NFκB and IRF3 (16, 23).

Another common PAMP generated during viral infection is foreign DNA. However, although many have been proposed, the exact PRR(s) involved in the recognition of viral DNA and the activation of innate signaling remain unclear (reviewed in (24)). Though TLR9 may have a role in the detection of DNA, its endoplasmic location likely precludes it from recognizing viral genomes in the cytoplasm or nucleus, and accordingly, TLR-independent DNA sensors have been proposed to exist (25). For example, DAI (DNA-dependent activator of IFN-regulatory factors) has been suggested to detect cytosolic DNA, leading to the activation of IRF3 (26). However, IFNα/β production in response to various forms of cytoplasmic DNA has been reported in DAI-

deficient cells, suggesting that alternative pathways must exist (27). Indeed, a large number of additional sensors have been proposed, including HMGB proteins (28), DHX36 (29), LRRFIP1 (30), DDX41 (31), DDX60 (32), DNA-PK (33), LSm14A (34), cGAS (35) and MRE11 (36), although at this time, these are incompletely characterized. Intriguingly, IFNγ-inducible protein 16 (IFI16) has recently been proposed to detect foreign DNA in the nucleus as opposed to the cytoplasm, leading to the activation of IRF3 (37-40). This is consistent with the fact that some DNA viruses replicate completely within the nucleus and so cytoplasmic DNA may not be available for detection. However, IFI16 can be found in the cytoplasm under some circumstances, and it appears it can also recognize foreign DNA in this location (37, 39, 41). Additionally, it has been suggested that IFI16 may be more important in epigenetic regulation of DNA expression as opposed to IFNα/β production (42, 43). Therefore, while it is possible that multiple DNA sensors are involved in the detection of DNA viruses, depending on the specific localization and form of DNA and the cell type of interest (44), it is clear that further work is required to fully understand the contributions of these various proteins to the innate antiviral response.

An important adaptor protein involved in PRR signaling is stimulator of interferon genes (STING, also called MITA, MPYS or ERIS). Found on the endoplasmic reticulum (ER) membrane, STING has been identified as being important in the production of IFNα/β in response to cytosolic nucleic acids and viral infection (45-47). It has been suggested that STING is involved in signaling after the detection of cytoplasmic RNA (46, 47), and may interact with RIG-I and MAVS (45, 46, 48-50). However, the best characterized function for STING is its role as a central signaling hub upon sensing of cytoplasmic DNA, as several proteins implicated in DNA detection signal via STING, including DAI (26), IFI16 (39), DDX41 (31), DNA-PK (33), LSm14A (34), cGAS (35) and MRE11 (36). PRR activation may result in the movement of STING from the ER membrane to the cytoplasm (reviewed in (51)), where it acts as a scaffold, stabilizing the interaction between the kinase TBK1 (TRAF family member-associated NFκB activator

(TANK)-binding kinase 1) and its substrate IRF3 (52). However, the exact mechanism through which STING functions in innate immunity remains unclear.

1.1.2 The production of IFNα/β: Transcription factors

Once a virus has been sensed by a PRR, several complex signaling pathways are activated, ultimately leading to the activation of three main transcription factor complexes: NFκB, IRF3/IRF7, and ATF2/c-Jun (53) (figure 1.2). NFκB in fact refers to a family of transcription factors, consisting of 5 distinct proteins that form hetero- and homodimers in different combinations and recognize a common sequence motif in the promoters of responsive genes (reviewed in (54)). In resting cells, NFκB is held as an inactive complex in the cytoplasm by its inhibitor, IκBα (55), likely via masking of the NFκB nuclear localization signal (NLS) (56). PRR activation stimulates IκBα phosphorylation by IκB kinase (IKK), which consists of catalytic subunits IKKα and IKKβ (57-59) and regulatory subunit IKKγ/NEMO (60), leading to its polyubiquitination (61, 62) and degradation (63) via the proteasome – which releases NFκB to translocate to the nucleus and induce target genes.

The ATF2/c-Jun complex has received less attention than the other transcription factors involved in IFNα/β production. These proteins are members of the large family of transcription factors known as basic-region leucine zippers (bZIPs), and form a complex that is constitutively nuclear, even in its inactive form (reviewed in (64)). However, phosphorylation of the activation domain is required for its ability to stimulate IFNα/β transcription. This is performed by members of the stress-activated MAPK superfamily, specifically the c-Jun amino terminal kinases (JNKs) (65). These are activated in response to TLR agonists in a MyD88-dependent pathway, likely through the action of TGFβ-activated kinase 1 (TAK1) (66).

The IRF family of transcription factors has 9 members, which are expressed in different tissues and have a variety of roles. All IRFs have a homologous N-terminal, DNA-binding region, while their C-terminal domains are distinct, allowing them to participate in diverse protein-protein interactions, and providing their specificity of

action. In particular, IRF3, IRF7 and IRF9 are important in the IFNα/β-mediated antiviral response, with the first two crucially involved in the initial production of IFNα/β and the third required for signaling in response to IFNα/β detection, described further below (reviewed in (67)). IRF3, which is constitutively expressed in resting cells, is activated by the phosphorylation of serine and threonine residues in its carboxy terminus, although the specific residues required for activation remain controversial (68-70). This phosphorylation is performed upon initiation of PRR signaling by the IKK-related kinases IKKε and TBK1 (71), causing IRF3 to homodimerize and translocate to the nucleus.

These three groups of transcription factors – NFκB, IRF3/IRF7, and ATF2/c-Jun – come together to activate the virus-inducible enhancer of the IFNβ gene, which is differentially regulated from the other type I IFN subtypes. The IFNβ promoter has four positive regulatory domains, named PRDI though PRDIV. PRDII is the binding site for NF-κB, PRDIV is recognized by a heterodimer of ATF2 and c-Jun, and PRDI and PRDIII are bound by IRF3 and IRF7 (53). Unlike IRF3, IRF7 is constitutively expressed at very low to undetectable levels in most cells. Activation of IRF3, NFκB and ATF2/c-Jun leads to the low-level production of IFNβ and one subspecies of IFNα (IFNα4) (72). This is sufficient to induce the expression of some ISGs, including IRF7 (73). Like IRF3, IRF7 is phosphorylated by TBK1 and IKKε, causing it to interact with IRF3. This IRF3/IRF7 heterodimer can then cooperate with NFκB and ATF2/c-Jun to form a highly stable complex known as the enhanceosome (74). This creates a platform for the recruitment of proteins involved in transcription, including co-activators p300/CBP (cAMP-response element-binding protein (CREB)-binding protein) (64), which recruit the RNA polymerase holoenzyme to allow the expression of all type I IFN subtypes and the full induction of ISGs. The specific identities and amounts of each IFN subtype produced depends on both the cell type and the identity of the infecting virus (75).

1.1.3 IFNα/β signaling: Receptor binding and signal transduction

The second half of the biphasic response occurs after IFNα/β has been produced and secreted from the cell (figure 1.3). All type 1 IFN subtypes bind to a single cell-

surface type I interferon receptor (IFNAR), composed of two transmembrane subcomponents referred to as IFNAR1 and IFNAR2 (76, 77). Ligand binding brings the two subunits together into a ternary complex (78, 79), allowing the Janus family protein kinases (JAKs) Tyk2 (80) and Jak1 (81), which associate with IFNAR1 and IFNAR2, respectively, to reciprocally phosphorylate one another (82-84). This leads to the phosphorylation of the receptor molecules themselves, producing a docking site for the transcription factors signal transducers and activators of transcription (STAT)-1 and -2 (reviewed in (85)). The site-specific phosphorylation of tyrosine residues in STAT-1 and STAT-2 allows them to associate with IRF9 to form the heterotrimeric complex known as IFN-stimulated gene factor-3 (ISGF3) (86-88).

1.1.4 IFNα/β signaling: Interferon stimulated genes

Once activated, ISGF3 binds the IFN stimulated response element (ISRE) in the promoters of ISGs (reviewed in (89)), with IRF9 providing the core ISRE recognition component and STAT2 acting as a potent transactivator, via its ability to recruit various transcriptional co-activators (90, 91). This leads to the transcription of the ISGs, the products of which then work cooperatively to limit the replication and spread of the infecting virus.

Although IFNα/β causes the expression of hundreds of ISGs, the functions of many of these proteins, and how they limit viral replication, are not well understood (92). Several of the better characterized ISGs have roles in inhibiting translation, thus blocking the production of viral proteins. For example, protein kinase R (PKR) binds dsRNA, causing the activation of its kinase domain to phosphorylate the eukaryotic initiation factor 2α (eIF-2α), disrupting translation initiation (93). Another well-known example is 2'-5'-oligoadenylate synthetase (OAS), which also responds to dsRNA by producing 2'-5'-linked oligoadenylates (2-5A) from ATP, which subsequently activate the latent enzyme RNase L to degrade host and viral mRNAs (94). More recently, ISG-56 has been found to bind to and disrupt the action of another translation initiation factor, eIF3e (95). Interestingly, ISG-56 has additionally been found to have a role in negative feedback,

binding STING at late times post-induction, to help to terminate antiviral signaling after viral clearance (96). ISGs with antiviral activities distinct from effects on translation have also been characterized. For example, cholesterol-25-hydroxylase is an IFN-inducible enzyme producing 25-hydroxycholesterol, which modulates the lipid composition of the plasma membrane and disrupts viral entry (97). At the opposite end of the viral lifecycle, the ISG viperin alters the integrity of specialized plasma membrane domains known as lipid rafts, impairing the release of newly-made virions from the cell (98). ISG-15 acts as a ubiquitin-like modifier, which can be covalently linked to proteins in a process known as ISGylation (reviewed in (99)). ISGylation appears to preferentially target newly-made proteins, with the modification of viral proteins disrupting their function, while the targeting of cellular proteins, particularly those involved in innate signaling, may increase their stability or activity. Thus, ISGs target every stage of viral replication, from entry to egress.

1.1.5 The IFN-independent response

Intriguingly, in the absence of IFNα/β production, some ISGs can still be produced (100-116). Indeed, the expression of a small subset of ISGs is observed even when neither biologically active IFNα/β (9, 101, 103, 109) nor IFNα/β mRNA production (110, 113) is detected, as well as after treatment with neutralizing antibodies or soluble receptors against IFNα/β (107, 114, 116), and in cell lines deficient in Tyk2, Jak1, STAT1, IFNAR or the type I IFN genes themselves (100, 102, 106, 112, 113, 115). Additionally, blocking protein synthesis with cyclohexamide does not prevent the expression of ISG mRNA, meaning that IFNα/β production cannot be required for their induction (101, 103-109). The IFN-independent activation of ISGs is thought to occur through the direct binding of IRF3 to the ISRE found in the promoter of some ISGs (53, 100, 102-104, 108, 110, 112-116). NFκB activation, however, appears to result in IFNα/β production, leading to the expression of the full complement of ISGs (9). This has led to the following model (9): the IFN-independent response is activated as a result of low levels of viral entry, and attempts to limit viral replication in the initial infected cell via

the activation of IRF3, without requiring the potentially damaging release of cytokines and infiltration of immune cells. However, if this response is unable to contain the infection, a threshold of viral replication will be surpassed, leading to the additional activation of NFκB, the production of IFNα/β, and the full innate antiviral response.

IFN-independent signaling has been observed with a wide variety of viruses, including herpes simplex virus (HSV) (101, 103, 106, 109), human cytomegalovirus (HCMV) (102, 104, 105, 107), vesicular stomatitis virus (100, 103), Sendai virus (100, 103), vaccinia virus (103), Newcastle disease virus (103), influenza A virus (112), hepatitis C virus (114), Chikungunya virus (115) and dengue virus (115). These are diverse viruses from several unrelated families, ranging from having small single-stranded RNA to large double-stranded DNA genomes, with some replicating in the nucleus and others in the cytoplasm. In fact, viral replication is not necessary to induce IFN-independent signaling (101, 103, 107, 112). The only feature required appears to be the presence of a viral envelope, as the non-enveloped adenovirus cannot induce this response (103). It has also been observed that the binding of the virus to the cell is insufficient to stimulate this effect – the virus must actually penetrate into the host cell in order to be recognized (101, 108, 117). This suggests that an early step in the entry process, unique to enveloped viruses, is sufficient to activate IFN-independent signaling – such as the fusion of the viral envelope with a cellular membrane, or the resulting changes in cytoskeleton. In support of this, a viral glycoprotein involved in virus-cell fusion, the gB protein from HCMV, has been found to induce a small subset of ISGs in the absence of other viral components, with the specific region important in inducing membrane fusion required for this process (102, 118). Additionally, perturbing the cellular membrane with a viral protein that induces cell-cell fusion also activates this response (110). However, the exact mechanism through which the entry of enveloped viruses is detected remains unclear. This response appears to be independent of TLRs and RLRs (9), but it has recently been reported that STING may be important in the detection of viral fusion (119) as well as in IFN-independent signaling (113), and that MAVS specifically localized to the peroxisome, instead of the mitochondria, may be important

for the IFN-independent expression of ISGs (116). Interestingly, dysregulation of lysosome biogenesis in the absence of the exonuclease Trex1 has also been found to induce ISG expression in the absence of IFNα/β (113), although the significance of this remains unclear.

1.2 The herpes simplex virus

1.2.1 General introduction to the herpesviruses

The *Herpesvirales* order is a tremendously successful group of viruses, containing 3 families, 3 subfamilies, 17 genera and 90 species, and infecting a broad range of eukaryotic organisms, from the common, such as human, feline, canine, bovine, and equine viruses, to the more unusual, including viruses infecting the wildebeest, reindeer, hedgehog, badger, koi, eel or oyster (120). All members of this order are enveloped, double-stranded DNA viruses that cause lifelong, latent infections of their respective host (121, 122). *Herpesvirales* virions consist of four distinct structures: the core, containing the DNA genome; the capsid, an ordered icosahedral protein shell enclosing the genome; the tegument, a proteinaceous layer surrounding the capsid; and the envelope, a bilayer composed of host-derived lipids, embedded with viral glycoproteins (reviewed in (123)). These viruses are divided into Alpha-, Beta- and Gammaherpesvirinae subfamilies, originally based on biological factors, such as tropism, replicative cycles, and site of the establishment of latency (121, 122) and more recently using sequence-based phylogeny (120). There are 8 known human herpesviruses – three alphaherpesviruses (the closely related HSV subtypes 1 and 2 (HSV-1 and HSV-2) and varicella-zoster virus (VZV)); three betaherpesviruses (HCMV and human herpesviruses 6 and 7 (HHV-6 and HHV-7)); and two gammaherpesviruses (Epstein-Barr virus (EBV) and Kaposi sarcoma herpesvirus (KSHV)) (120).

Of all the human herpesviruses, HSV-1 is one of the most successful. Globally, HSV-1 prevalence increases steadily with age, tending to plateau beyond age 30 at the very high seroprevalence of approximately 60% - 90% (124). Regional differences account for this large range – for example, in Canadian women, the seroprevalence is 57.4% (125), while 97% of those greater than 5 years of age are seropositive in Eritrea (126). HSV-1 is most commonly associated with orofacial lesions, referred to as herpes labialis or cold sores. However, depending on the site of inoculation, HSV-1 can cause a wide variety of conditions (reviewed in (127)). For example, herpetic whitlow is an infection of the fingers that frequently affects health care professionals, while herpes

gladiatorum is a cutaneous infection, which may affect the neck, trunk or extremities, found in athletes practicing close contact sports. Herpes keratitis, caused by recurrent HSV-1 reactivation in the eye, is a major cause of blindness worldwide (128), and genital herpes infections, once thought to be almost exclusively caused by HSV-2, are now caused by HSV-1 in at least 50% of cases in developed countries (129-133). In neonatal or immunocompromised individuals, herpes simplex encephalitis (HSE) can be a fatal complication of HSV infection, with the mortality rate reaching 70% for untreated individuals (134) and 5-19% even after treatment with antiviral drugs, and survivors typically left with persistent neurological symptoms (135-139).

1.2.2 The lytic replication cycle of HSV

The HSV lytic replication cycle (figure 1.4) begins when viral particles are inoculated on the mucosa or skin, and pass through microabrasions to reach the basal layer of the epithelium (140). In the initial attachment step, the virus interacts with ubiquitous cell surface receptors on the basolateral membrane, with glycoproteins gC or gB binding to the proteoglycans heparan sulfate or chondroitin sulfate (141-144). This step is not essential for viral entry, but increases the contact between virus and cell to improve the probability of encountering a specific but less common entry receptor in the next stage of the entry process, thus increasing the efficiency of infection (145).

In the subsequent step, essential entry component gD (146) interacts with one of several unrelated receptors, including nectin-1 (147, 148), herpesvirus entry mediator (HVEM) (149), and 3-O sulfated heparan sulfate (150). This specific interaction is thought not only to direct the virus to infect only suitable cell types (151), but also triggers a conformational change in gD, allowing it to act as a catalyst to initiate the fusion of the viral envelope with a cellular membrane (152-156). This is achieved by activating the remaining members of the core fusion machinery – gB, gH and gL (146, 157-162). The exact mechanism through which these proteins drive fusion remains elusive, as this multipartite fusion apparatus is quite unusual (reviewed in (163)). Since gB shares several characteristics of known fusion proteins (164), it has been suggested

that this glycoprotein performs the fusion itself, while gH/gL is thought to act as a regulator of gB that may promote, as opposed to initiate, fusion (151, 163). This may be achieved by destabilizing the host membrane, as gH/gL has been proposed to interact with lipids (165-167). However, others have suggested it may simply activate gB, possibly by linking it to the changes in gD (168, 169). Interestingly, this fusion can occur either directly at the cell membrane, or within either acidic (170) or neutral (171) vesicles after being endocytosed. The specific route of entry depends on the cell type (171-173), with the same fusion machinery required regardless of the site of entry (171, 173).

Fusion of the viral envelope with a cellular membrane results in the release of the capsid and tegument proteins into the cytoplasm (reviewed in (151)). Many tegument components dissociate from the capsid during this process, but some inner tegument proteins remain capsid-associated (174). HSV capsids are then transported to the nucleus along microtubules via molecular motor proteins (175-179). Capsid-associated tegument proteins may be involved in capsid transport (180-183). In particular, pUL36 may direct capsids to the nucleus, potentially by interacting with the nuclear pore complex (NPC) (182, 184). Although the capsids dock at the NPC (182, 185), their diameter of 125nm makes them too large to fit through intact nuclear pores, which can accommodate macromolecules of a maximum size of approximately 36nm (186-188). Instead, the genomic DNA is ejected into the nucleus, in a process that may require the proteolytic cleavage of pUL36 (189, 190).

Once inside the nucleus, the genomic DNA circularizes (191-195), and transcription begins using the host RNA polymerase II (196). Gene expression in HSV proceeds in an sequential, cascade manner, and HSV genes can be divided into immediate-early (IE), early (E) and late (L) classes, based on their temporal order of promoter activation, as well as whether prior viral gene expression or DNA replication is required for their transcription (197). The synthesis of IE gene products is initiated when tegument protein VP16, released during entry, associates with host transcription factor Oct1 and chromatin modifying protein HCF1 (198-204). This complex transactivates the IE genes, of which there are 5 - ICP0, ICP4, ICP22, ICP27, and ICP47. These proteins are

important in the regulation of viral replication – in particular, ICP4 (205) and ICP27 (206) are essential for the downstream expression of E and L genes – while the others, though nonessential, optimize the cellular environment for maximal viral replication (reviewed in (207)).

Activation of the E genes is important for the replication of the DNA genome (208), encoding, for example, the DNA polymerase (209), a helicase-primase complex (210) and a ribonucleotide reductase (211), among others. The exact mechanism for HSV genomic replication remains unclear – though initially proposed to occur via rolling circle replication, recent evidence suggests recombination dependent replication may occur instead (212). Regardless of the exact mechanism, it is clear that DNA synthesis produces concatemeric DNA, comprising tandem repeats of the viral genome (213). DNA synthesis is required for the expression of L genes (214), which have important roles as structural proteins and in assembly and egress (reviewed in (207)). Concatemeric DNA is cleaved into individual genomes by an enzyme complex known as the terminase (215-217). This enzyme additionally inserts the DNA into preformed capsids composed of L gene products, with the inserted DNA replacing a temporary interior scaffold in a process known as encapsidation (218, 219).

The newly-made HSV capsids face the same problem as they did during entry – they are too large to pass through the nuclear pore. Although it has been proposed that the nuclear pore can become dilated during infection, allowing for egress via nuclear export (220), changes in nuclear pore architecture do not seem to correlate with efficient egress (221). Therefore, in order to escape the nucleus, it is generally agreed that capsids bud from the inner nuclear membrane, acquiring a primary envelope in the process (reviewed in (222)). This produces immature enveloped capsids in the space between the inner and outer nuclear membranes, known as the perinuclear space (223).

The subsequent steps of egress have been highly controversial. Originally, it was suggested that because the perinuclear space is continuous with the lumen of the ER, primary capsids could move directly through the ER cisternae to the Golgi for packaging into vesicles, which are then transported to the cell membrane for release (220, 224-228).

However, a second possibility has proposed, known as the de-envelopment/re-envelopment model (221, 229-234), which is supported by most of the recent evidence in the field (218, 222). In this case, the envelope of the primary virion will fuse with the outer nuclear membrane, releasing naked capsids into the cytoplasm. The specific mechanisms and components involved in de-envelopment are currently unclear. None of the proteins required for fusion during HSV entry are necessary for nuclear egress (222), and so these two fusion processes must be fundamentally different.

After escaping the nucleus, unenveloped capsids travel through the cytoplasm on route to the site of secondary envelopment, where they acquire their final, mature envelope (reviewed in (222)). As they travel, capsids sequentially recruit tegument proteins through a series of protein-protein interactions (235-237). Several sites of secondary envelopment have been proposed for HSV, including the Golgi apparatus (238), early and late endosomes (236, 239), multivesicular bodies (240, 241) and aggresomes (242). At this time, however, overwhelming evidence appears to support the *trans*-Golgi network (TGN) as the major site of secondary envelopment (236, 237, 239, 243-246). The TGN is a network of tubules connected with the *trans*-face of the Golgi apparatus, which functions in the secretory pathway as a sorting station (reviewed in (247, 248)). During HSV infection, a second subset of tegument proteins, along with the glycoproteins, assemble at the TGN (233, 236, 237, 249-258), where they interact with the capsid-associated tegument subassembly, helping to drive the secondary envelopment process to generate the mature particles (218, 223). This process results in an enveloped viral particle within a transport vesicle, which will then travel to and fuse with the plasma membrane, releasing the virus, in a manner similar to the release of secretory molecules (reviewed in (218)). Such secretory vesicles may preferentially travel to specific HSV egress sites at the plasma membrane on adherent surfaces, helping to direct newly-formed virus to nearby target cells (259). In addition to release into the environment, virions can also spread directly cell-to-cell, in a mechanism that appears to require gE/gI (260-262), although the details are unclear. This is thought to allow for rapid dissemination *in vivo* (263).

1.2.3 Latency of HSV

An important aspect of the success of HSV is its ability to establish a latent reservoir in neurons, lasting for the lifetime of the host (264-266). Latency can be defined as the stable maintenance of the viral genome in a state that does not produce progeny virions, but does allow for periodic reactivations. This strategy allows the virus to persist while escaping immune surveillance, and continuing to infect new individuals throughout the lifetime of its original host, helping the virus to disseminate widely (reviewed in (267)). In humans, a major site of HSV-1 latency is in the trigeminal ganglion (268-271), however latency has also been identified in several other sites, including the geniculate, facial, spinal, and vestibular ganglia (272-275).

To initiate latency, virions produced during the initial lytic infection of epithelial cells enter the axon termini innervating the infected tissue, in the same mechanism as in non-neuronal cells, with the released capsid then moving along the axon to the neuronal cell body via retrograde axonal transport (276-278). It has long been known that the neuronal nucleus is the site where the latent genome is maintained (266, 268, 279). However, the mechanism through which this arises is less clear. It has been suggested that instead of being actively promoted, latency simply results when the virus fails to successfully initiate the lytic replication cycle. For example, it has been found that HSV infection of a neuron via the axon favors the development of latency, whereas infection through the cell body results in a lytic infection (280). This supports the concept that the transport of tegument proteins – particularly VP16 – is inefficient over the long distance from the axon termini to the cell body, resulting in the failure of the virus to initiate the productive replication cycle (281). Indeed, provision of exogenous VP16 to the cell body inhibits the development of latency (280).

The latent genome is thought to adopt a heterochromatic structure, with repressive epigenetic marks (282-284), which may inhibit viral gene expression (reviewed in (285)). In fact, only one viral transcript is abundantly expressed during latency – known as the LAT, or latency-associated transcript (286, 287). The LAT is thought to function in the repression of viral protein expression (288), though it is not absolutely required for either

latency or reactivation (282, 289-291), and the importance of the LAT to the latency-reactivation process varies considerably with the experimental conditions used (290, 292-295). Interestingly, the LAT has been proposed to be a precursor for a number of microRNAs (miRNAs) with functions in the regulation of latency (reviewed in (296)). For example, one of these miRNAs, mir-H2, has been found to downregulate the expression of ICP0, (296, 297), a viral protein thought to be important in the reactivation process (see below). The LAT may additionally have roles in the inhibition of apoptosis, ensuring the persistence of the latent reservoir (298, 299).

How HSV reactivates from latency is also uncertain, with several proteins proposed to be involved but none found to be essential for this process, and little consensus on the mechanism. In animal models, a large number of different stresses have been found to promote HSV reactivation, including ultraviolet (UV) light (300), hyperthermia (301), skin trauma (302) and immunosuppression (303), and a similar pattern is observed in humans (304-309). The mechanism through which stress leads to the reactivation of HSV is unclear. Several viral proteins have been suggested to stimulate reactivation, including VP16 (310, 311), and ICP0 (312-315), though others have found that ICP0 is not required for reactivation (316-318), and still others have found that it is the cooperative action of multiple proteins that activates this process, including ICP4 in addition to VP16 and ICP0 (319).

It is currently unknown whether gene expression upon initiation of reactivation follows the IE-E-L progression observed during lytic infection, with current indications suggesting that the temporal order may be disorganized or that all gene classes may be activated simultaneously (320-325). The pathway to egress in neurons for virions produced as a result of reactivation is thought to be different from that observed in non-neuronal cell types, given that movement from the nucleus in the cell body to the axon termini in sensory neurons may require HSV virions to be transported as far as 10-100cm, requiring specialized adaptations for egress (reviewed in (326)). There are two competing models for this process – the first, referred to as the subassembly model, suggests that naked capsids move along microtubules, while the tegument and envelope proteins are

transported separately in vesicles, with the final assembly process occurring at the axon terminus when the two meet (234, 327). The second, called the "married" model, suggests that mature virions are assembled in the cell body and then transported within vesicles (328). One possible explanation for this disparity is that in neurons, virus egress can occur both from the cell body, via the same mechanism as in cultured cells, as well as via the axon terminus, which requires specialized transport mechanisms (326). The reactivation of HSV results in the recurrent symptomatic diseases associated with this virus (reviewed in (329-332)). Interestingly, viral shedding can also occur in the absence of clinical symptoms, and asymptomatic shedding may be more common that symptomatic reactivations, potentially accounting for a significant amount of viral transmission (333-336).

1.2.4 The ability of the Type I IFN response to control HSV replication

As described above, the type I IFN response is a powerful weapon, capable of inhibiting the replication and spread of diverse viruses, and HSV is no exception. It has long been known that IFNα/β has a key role in controlling the replication of HSV in mice. Initial observations suggested that differences in the sensitivity of various strains of mice to HSV infection could be explained by their ability to mount a rapid type I IFN response (337-339), with survival correlating with the production of IFNα/β (340-342). Additionally, it has been found that antibody-mediated depletion of IFNα/β increased viral replication and decreased survival after HSV-1 infection *in vivo* (337, 343-346), while provision of exogenous IFNα/β had the opposite effect (347-352) on both HSV-1 and the closely-related HSV-2. Likewise, the ability of different TLR ligands to induce IFNβ correlated exactly with their ability to cause the survival of mice infected with HSV-2 (353), and a protective effect for type I IFN was observed in IFNαβ receptor knockout mice after HSV-2 challenge (353-355). HSV-1 replication and pathology were similarly increased in Stat1-deficient mice (356). The loss of the type I IFN receptor caused both elevated viral titers and augmented mortality after intracranial infection of HSV-1 (357), increased HSV-1 dissemination to various organs after localized footpad or

ocular inoculation (358) and strongly promoted the replication of even highly attenuated HSV mutants (359). Remarkably, mice deficient in the recombination activating gene (RAG), which have intact IFNα/β responses but no mature T or B cells, showed resistance to intraperitoneal HSV-1 infection, while mice that lacked type I IFN signaling, yet with intact adaptive responses, were found to have strongly increased susceptibility to HSV-1 (360). Ultimately, work by Halford *et al.* using *scid* mice, which lack lymphocytes but have normal innate immune responses (361) confirmed that it is the ability of mice to raise a rapid IFNα/β response, as opposed to physical differences in the capacity of their cells to support viral replication, that determines the susceptibility of various mouse strains to HSV infection (362).

Although the majority of *in vivo* studies on the impact of the type I IFNs on HSV replication have been done in mice, in actuality, this is an exclusively a human virus that does not naturally infect rodents. Therefore, although useful, it is difficult to be sure that the results obtained in mouse models are truly representative of the situation in humans. Recently, the study of human patients suffering from unusually severe infections with HSV has demonstrated that IFNα/β also plays an important role in combating HSV in its natural host. For example, herpes simplex encephalitis (HSE) is a rare complication of infection with HSV, resulting in a life-threatening infection of the central nervous system (reviewed in (363)). Recently, it has been reported that HSE is associated with certain genetic deficiencies involved in the type I IFN pathway. For example, two unrelated patients presenting with HSE were found to have an autosomal recessive deficiency in a protein known as UNC-93B (364) which plays a role in signaling through TLRs to produce IFNα/β in both mice and humans (364-366). Another group has reported that two patients showing increased susceptibility to HSE, but not to other pathogens, had the same dominant negative mutation in TLR3, which was not found in 1581 healthy controls (367). Patients deficient in STAT-1 and thus IFNα/β signaling also show increased vulnerability to both mycobacteria and viruses, including, but not specific to, HSE (368). Finally, a patient with increased susceptibility to HSE has been identified to have a specific defect in NEMO (369). Although the small number of patients involved in such

studies makes it difficult to draw definitive conclusions, it appears that as in mice, IFNα/β is important for the control of HSV replication in humans.

1.2.5 The ability of HSV to inhibit IFNα/β production and signaling

Given the powerful effect of the IFNα/β response in reducing the replication of HSV, it is not surprising that this virus has several mechanisms to counteract the effects of the innate antiviral response. A major player involved in this is the IE protein ICP0, which is capable of both blocking the activation of IRF3 (38, 101, 370-375) as well as preventing the cell from fully responding to exogenous IFNα/β (370, 376-379). ICP0 and its various important roles in enhancing viral replication are discussed at length below.

However, HSV has an abundance of proteins that are also thought to have functions in blocking the antiviral response. Another IE protein, ICP27, has been suggested to play a role in both the inhibition of IFNα/β signaling (380, 381), as well as the disruption of IFNα/β production (382). Overexpressed ICP27 was found to block the nuclear translocation of STAT1 (383), which may explain how the former is achieved, but the mechanism behind the latter is less clear. ICP27 has roles in transcription, mRNA splicing, export and translation, and may nonspecifically block antiviral signaling by causing a general decrease the expression of host genes (reviewed in (384)). On the other hand, others have found the lack of ICP27 did not affect ISG induction (371), and an ICP27-null virus was able to block IRF3 activation by Sendai virus (SeV) (374).

It is not only IE proteins involved in evasion of type I IFN signaling. For example, an E protein, pUS3, has been found to play a role in this function. It has been demonstrated that pUS3-deficient viruses show increased sensitivity to IFNα/β (385) and increased IRF3 activation (386). More recently, it has been suggested that IRF3 is a substrate of pUS3, resulting in atypical IRF3 phosphorylation that blocks its dimerization and nuclear translocation (387). Another component of innate evasion is ICP34.5, a leaky late protein, which unlike standard L proteins, is expressed at early times post-infection (197). ICP34.5 is well known to counteract one of the downstream effects of IFNα/β production by blocking the action of the ISG PKR (388, 389), by recruiting protein

phosphatase 1α to dephosphorylate eIF2α (388-391). More recently, ICP34.5 has been found to block the initial production of IFNα/β by interfering with the activity of TBK1, possibly by competing with IRF3 for TBK1 binding (392, 393). Another leaky late protein, VP16, has been found to block the production of IFNα/β by SeV, by disrupting the action of IRF3 through competition for the coactivator CBP (394).

In addition, late protein pUS11 has been suggested to have roles in preventing both the initial induction and signaling of IFNα/β. pUS11 is thought to bind and sequester dsRNA, which may be important in preventing this PAMP from activating a number of sensors – including the ISG OAS (395). pUS11 has additionally been found to block IRF3 activation by disrupting RLR signaling, binding directly to RIG-I and MDA5 (396), and may also bind and inhibit PKR (397-400). Similarly, other late proteins, including pUL36 and vhs, have roles in impairing antiviral signaling. pUL36 was recently found to disrupt the action of TRAF3, a signaling molecule upstream of TBK1 (401), while the RNase vhs nonspecifically degrades both host and virus mRNAs (402, 403) and may block ISG production via the loss of their transcripts (404). Interestingly, it has been reported that vhs can block the activation of IRF3 in HSV-2 (405), though in HSV-1, vhs appears to be unable to directly interact with IRF3 (406) and only has a noticeable effect on inhibiting ISG induction in the absence of ICP0 (371). This selective IRF3 binding is consistent with the fact that HSV-2 vhs mutants are more attenuated than HSV-1 viruses with the same mutations (407-409). For HSV-1, vhs-null viruses are less resistant to IFNα/β (407), and vhs is thought to function at later times post-infection to block ISG induction (371), possibly by decreasing the accumulation of dsRNA and thus the activation of innate sensors (407).

The large number of proteins involved in IFNα/β evasion by HSV may be a consequence of the ordered cascade of viral gene expression, with different proteins expressed at different times ensuring that ISG expression is prevented at each stage of the replication cycle. However, since it is generally thought that it is the prevention of innate responses at early times that determines the ultimate success of a viral infection (109), it seems surprising that so many late proteins have been implicated in blocking type I IFN

signaling. However, although many ISGs would be powerless to block viral replication in the initial infected cell by the time viral assembly has been initiated, preventing the production of IFNα/β and the resulting protection of the surrounding cells remains important. Another possible explanation is that almost all of the IFN-antagonizing proteins are components of the tegument – including pUS11, VP16, pUL36, pUS3, and vhs, and thus might be expected to block antiviral signaling prior to viral gene expression (reviewed in (326)). However, both UV-inactivated HSV particles (103) and an HSV strain with mutations in all five IE genes (38), which are impaired in terms of gene expression but still capable of entering the host cell and releasing the tegument proteins, strongly induce ISG expression, suggesting that tegument proteins are incapable of blocking the antiviral response. It is also possible that the different proteins may be important in different cell types. For example, ICP34.5 is a major neurovirulence determinant (410), and so it may be required to block antiviral responses in neurons, while ICP27 and vhs have been shown to be involved in the inhibition of IFN production or signaling in dendritic cells (382, 411).

1.2.6 The infected cell protein 0 (ICP0)

The IE protein ICP0 is a particularly interesting viral protein, involved in many diverse aspects of HSV replication, from augmenting viral gene expression during the lytic cycle as well as promoting reactivation from latency, to blocking both intrinsic and interferon-mediated antiviral responses (reviewed in (412)). Several protein-protein interaction sites and functional domains involved in these assorted roles have been identified in ICP0 (figure 1.5). A particularly important feature of ICP0, that has been implicated in most of its functions, is its really interesting new gene (RING) finger domain, which acts as an E3 ubiquitin ligase (413). This makes ICP0 a component of the ubiquitination pathway, which involves linking the 76 amino acid ubiquitin protein into chains that can be conjugated to proteins as a post-translational modification (reviewed in (414)). The ubiquitination process begins when an E1 ubiquitin activating enzyme first forms a thioester linkage between a cysteine in its active site and the carboxy terminus of

ubiquitin in an ATP-dependent process. Next, the activated ubiquitin is passed to an E2 ubiquitin conjugating enzyme. This generates an isopeptide bond between the lysine of the target protein and ubiquitin, with the help of an E3 ubiquitin ligase, which binds to both the E2 and the substrate protein to catalyze the transfer of ubiquitin. There are seven lysines within the ubiquitin protein itself, any of which can act as acceptors for activated ubiquitin moieties, and so repeated rounds of ubiquitination can produce polyubiquitin chains. When such chains are made using the lysine residues at position 48 (Lys 48) within ubiquitin, they serve as the most common signal targeting a protein for proteasomal degradation. However, any of the other lysine residues, such as Lys 63, can also be used to link the ubiquitin moieties into what are known as atypical chains, which are associated with non-degradative, signaling roles (reviewed in (415)).

ICP0 has been implicated in the proteasome-dependent degradation of a tremendous variety of cellular targets including PML (416), Sp100 (417), CENP-A (418), CENP-C (419), DNA-PKcs (420), USP7 (421), p53(422), IκBα (423) CD83 (424), Mal and MyD88(425), E2FBP1 (426), RNF8 and RNF168 (427), and IFI16 (38), as well as viral protein pUL46 (428). The mechanism through which ICP0 identifies its substrates is still unclear (429). Specific binding sites have been identified in ICP0 for several proteins, but only some (430, 431) but not all (432, 433) of these proteins are targeted for degradation. Small ubiquitin-like modifier (SUMO) interaction motifs (SIMs) have been identified in ICP0 (434), with SIM-like sequence 4 (SLS-4) reported to have a role in substrate recognition (429). Indeed, the sumoylated forms of some target proteins have been found to be preferentially degraded; however, ICP0 can direct proteins to the proteasome in a SUMO-independent manner (416, 434, 435). ICP0 has also been suggested to interact with at least three cellular E3 ubiquitin ligases – SIAH-1, RNF8, and RNF168 – which may increase its repertoire of targets (427, 431, 436). Additionally, ICP0 has been reported to have a second domain with E3 ubiquitin ligase activity, known as HUL-1, but at this time only one target of this region has been proposed – the E2 enzyme cdc34 (437).

It is well-known that ICP0, though non-essential, has an important ability to stimulate virus replication. It has been suggested that ICP0 accomplishes this by increasing the probability that an incoming genome will launch a productive infection (reviewed in (3)). This concept is derived from the observation that at low multiplicity in cultured cells, ICP0-null viruses enter the cell but have a tendency to remain in a quiescent, transcriptionally silent state (438, 439) comparable to latency, instead of initiating lytic replication. Interestingly, the provision of exogenous ICP0 can reactivate such quiescent genomes (440-443). However, the exact mechanism through which it produces this effect is still not clear.

A potential explanation for this involves one of the first functions identified for ICP0, which is that it acts as a promiscuous transactivator, able to increase the expression of genes driven by both viral and cellular promoters, independently of any specific *cis*-acting sequence (444-447). Though neither ICP0 nor its RING finger have been found to directly bind DNA (448-450), it may produce its effects by interacting with or degrading proteins that can. Indeed, it has been proposed that ICP0 induces gene expression by acting as a DNA template remodeler (451), inhibiting the formation of repressive, higher-order structures of chromatin, and thus allowing enhanced transcription. Such repressive marks are found on the latent genome (282-284), and may explain the lack of viral gene expression observed in this phase of the HSV lifecycle. Correspondingly, ICP0 has been found to block heterochromatin formation (452), as well as to decrease repressive and increase activating epigenetic marks (43, 452-454). This may be achieved via the stabilization of the histone acetyltransferase CLOCK (455, 456), by direct interaction with class II histone deacetylases (HDACs) (457), through the disruption of the CoREST/REST/HDAC1/2/LSD1 repressor complex (458, 459), or via the degradation of IFI16, which is thought to promote repressive epigenetic modifications of foreign DNA (42, 43). However, the initiation of transcription by ICP0 during reactivation has been found to occur before changes in epigenetic markers are observed (460), suggesting that ICP0 may enhance transcription via a different method.

Another proposed mechanism for the ICP0-mediated enhancement of viral replication is that the cell may have a defense mechanism to repress the incoming genome, unless ICP0 is present to counteract it. This is termed an intrinsic antiviral response, which does not require *de novo* protein production, but instead involves the recruitment of constitutively expressed cellular proteins which work to block viral activities (reviewed in (461)). Key players in this are the components of nuclear bodies known as nuclear domain 10 (ND10), including promeylocytic leukemia protein (PML), Sp100, hDaxx and ATRX (462-464). These proteins are recruited to the viral genome in one of the earliest detectable cellular events during HSV infection (465) – except in the presence of ICP0, which efficiently blocks their association with the genome (466, 467). This is achieved through the proteasome-dependent degradation of PML (416) and Sp100 (417), though a direct interaction of ICP0 with either ATRX or hDaxx has not been detected (464). These proteins are thought to function cooperatively to repress viral growth – while the individual depletion of PML (462), Sp100 (463), hDaxx or ATRX (464) increases the replication of ICP0-null viruses by only 5-10 fold, the simultaneous depletion of both PML and Sp100 has a greater effect, with the additional depletion of hDaxx increasing viral titers still more (463, 468). The exact mechanism through which ND10 components repress viral growth is unclear, but it has been proposed that these structures limit viral transcription by sequestering viral genomes, preventing their interaction with host transcriptional components (434, 468). It is also worth noting that ATRX and hDaxx form a chromatin modifying complex, interacting with HDACs and histones (469-471).

In addition to inhibiting the intrinsic response, it is well established that ICP0 is involved in blocking the cellular response to exogenous IFNα/β. The replication of HSV mutants lacking ICP0 is strongly impaired by IFNα/β pretreatment of cells, while the wildtype virus is not (370, 376, 377). The pre-expression of nuclear ICP0 in the absence of other viral proteins can reduce the sensitivity of HSV to exogenous IFNα/β (378). This may be accomplished via the degradation of ND10 component PML (416), which is itself an ISG (472). Interestingly, yields of ICP0-null HSV were not different in PML-positive

or PML-deficient cells, except after IFNα/β pretreatment, which dramatically reduced viral yields only in the PML-expressing cells (379). This suggests that PML is important in mediating the cellular antiviral response to exogenous IFNα/β.

The function of ICP0 in inhibiting the initial production of IFNα/β is more controversial. It has been reported that ICP0 can block the activation of NFκB after TLR stimulation via two different mechanisms, either directly, via the degradation of adaptor proteins MyD88 and Mal (425), or indirectly, by recruiting deubiquitinating enzyme USP7 to the cytoplasm to remove non-degradative, activating Lys-63 chains from adaptor proteins TRAF6 and IKKγ (473). ICP0 has also been found to decrease both IRF3 activation and ISG expression (38, 101, 370-375). Initial work suggested this function required both the RING finger domain, as well as the activity of the proteasome, but despite considerable effort, no component of IRF3 signaling that is subject to ICP0-mediated degradation has been found (371, 372). For example, no change in the protein levels of TBK1, IKK-ε, CBP, DDX3 or HSP90 was observed during a wild-type HSV-1 infection (371, 373). An enhancement in IRF3 degradation by ICP0, as well as the sequestration and inactivation of nuclear IRF3, has been proposed, but the SeV virus co-infection model used in these studies complicates interpretation of these data (374, 375), and others have found no such effect on IRF3 levels in the context of a single HSV infection (371, 373). Another potential target is the catalytic subunit of DNA-dependent protein kinase (DNA-PKcs), which is degraded by ICP0 (420, 474) and is also thought to be involved in the activation of IRF3 (33, 475) – but cells deficient in DNA-PKcs still produced ISGs after HSV infection (109). Additionally, ICP0 has been reported to block IRF3 activation by targeting the nuclear DNA sensor IFI16 to the proteasome (38), but under conditions allowing for efficient viral replication, IFI16 has been found to be degraded during infection with an ICP0-null virus (476). Finally, it has been proposed that αvβ3-integrin is involved in both TLR-mediated NFκB activation and TLR-independent IRF3 phosphorylation, and that this is counteracted by ICP0, but the mechanism behind this is currently unclear (477).

It has recently been found that the localization of ICP0 is crucial to its ability to block IRF3 activation (373). While nuclear ICP0 may be important for inhibiting cellular responsiveness to IFNα/β (378) via its disruption of PML (379), ICP0 is not an exclusively nuclear protein, but instead moves from the nucleus to the cytoplasm during the course of viral infection (478-482). Interestingly, it has been found that when ICP0 is restricted to the nucleus during infection, by mutation or treatment with proteasome inhibitors, it loses its ability to block IRF3 signaling, while cytoplasmic ICP0 efficiently disrupts the antiviral response, even in the absence of a functional proteasome (373). Similarly, ectopic expression of wild-type ICP0, which causes its retention in the nucleus, fails to prevent IRF3-mediated ISG induction (373, 378). Intriguingly, others have demonstrated that cytoplasmic bICP0 from the related bovine herpesvirus 1 also opposes IRF3 activation (483). However, the potential mechanism through which ICP0 inhibits IRF3 in the cytoplasm remains unknown.

1.3 Objectives

The IFN-independent response is an intriguing but incompletely characterized aspect of innate antiviral signaling. In particular, it is unclear how the physical entry of an enveloped viral particle into the cell is recognized, leading to the activation of this response. Good candidates for proteins potentially involved in this process are those that are both implicated in viral entry, potentially by associating with the plasma membrane or the underlying cytoskeleton, and also have functions in antiviral signaling. The small GTPase Rac1 is one such protein. Rac1 has been found to be involved in a variety of processes involving the actin cytoskeleton, ranging from morphogenesis to migration and from phagocytosis to cytokinesis (reviewed in (484)). Rac1 has also been implicated in TLR signaling (485, 486), as well as the activation of NF-κB (486-488), as well as in the activation of IRF3 in response to both dsRNA and replicating RNA viruses (489), although this has not been fully characterized or confirmed. Since Rac1 has been found to be activated during the entry of multiple enveloped viruses (490-496), Rac1 could represent a link between the cytoskeletal rearrangements required during the entry process and the activation of innate signaling. **Therefore, I tested the hypothesis that Rac1 is involved in the IFN-independent response to enveloped viruses (Chapter 3).**

The HSV IE protein ICP0 is involved in several aspects of viral replication, almost all of which are thought to require the E3 ubiquitin ligase activity of its RING finger domain (413, 497-499). Thus, the ability of ICP0 to target various cellular proteins to the proteasome for degradation has been proposed as the means through which it produces its various effects (38, 378, 416-427). The ability of ICP0 to block the activation of IRF3 in response to HSV infection is particularly controversial (38, 101, 370-375). Initial work suggested that both the RING finger and the proteasome were required for this process (371, 372), but although multiple proteins involved in antiviral signaling have been proposed to be degraded by ICP0 (38, 374, 420, 474), it has not been confirmed that the loss of any of these proteins is required for the ability of ICP0 to prevent IRF3 activation. Recently, it was shown that ICP0 blocks antiviral signaling only when localized to the cytoplasm, and not to the nucleus, and surprisingly, that cytoplasmic ICP0

remains capable of inhibiting IRF3 phosphorylation even in the absence of a functional proteasome (373). This had been previously overlooked because the inhibition of the proteasome causes ICP0 to localize exclusively to the nucleus, where it is incapable of blocking IRF3. This suggests that ICP0 is preventing innate signaling via a new and previously unknown mechanism. Given that the disruption of the RING finger similarly restricts ICP0 to the nucleus (500), **I tested the hypothesis that the RING finger domain of ICP0 is not required for its ability to impair the antiviral response (Chapter 4).**

In general, the functions of ICP0 in the cytoplasm are poorly understood, with only a few activities proposed for ICP0 in this location, none of which are fully characterized (423, 479, 501). During the studies in Chapter 4, I unexpectedly found that cytoplasmic ICP0 is able to promote viral replication (502) – and that it does so in a RING finger-independent manner. To further investigate the mechanism through which ICP0 is producing this effect, I identified proteins interacting with ICP0 in the cytoplasm using co-immunoprecipitation and quantitative mass spectrometry. In doing so, I found a novel cytoplasmic interaction partner for ICP0, WD repeat domain 11 (WDR11), and **tested the hypothesis that the WDR11-ICP0 interaction is involved in the ability of ICP0 to promote HSV-1 replication (Chapter 5).**

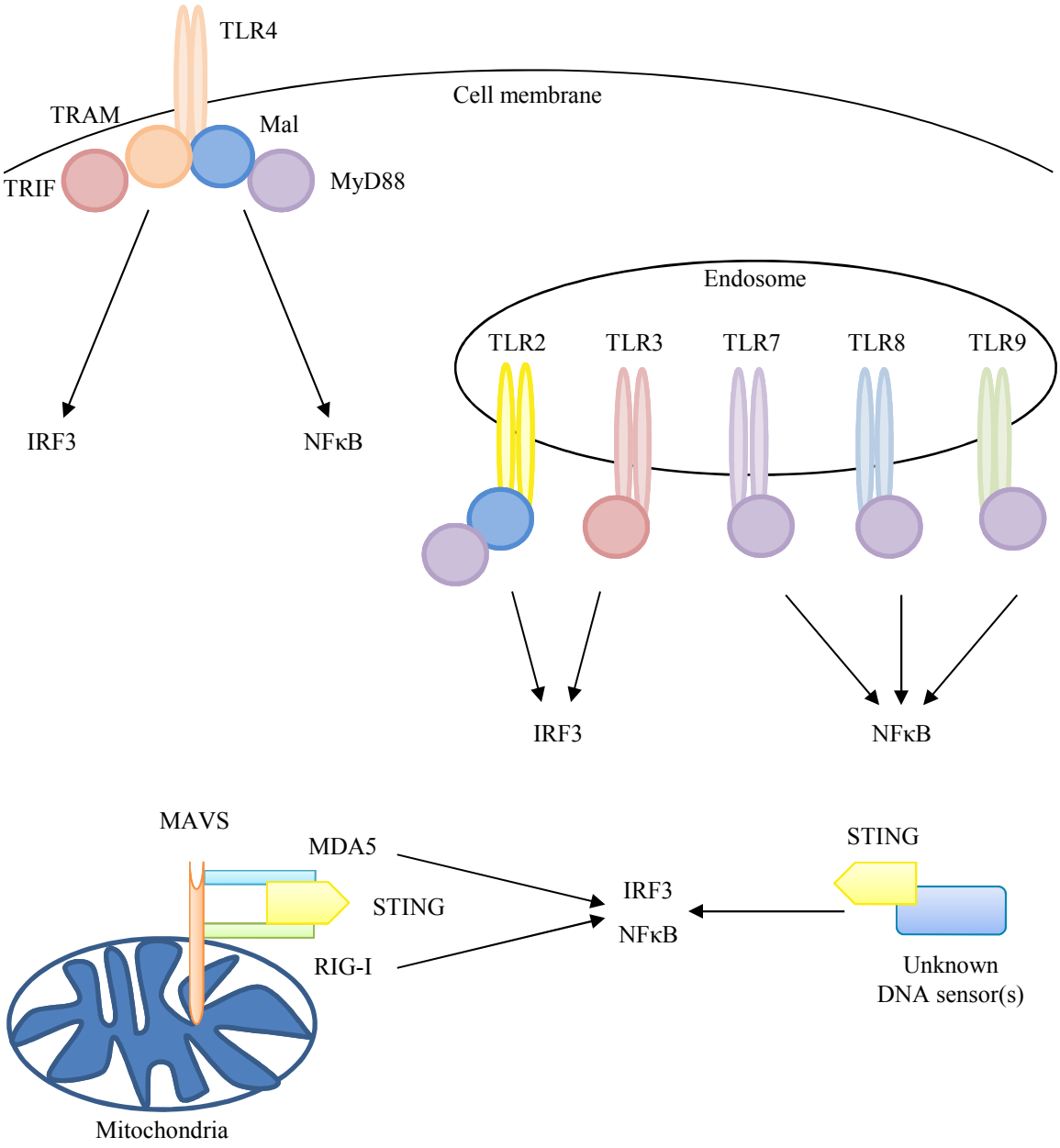

Figure 1.1: The innate antiviral response: pathways to virus detection. Viral components are recognized by various pattern recognition receptors (PRRs), including the TLRs, which are located both at the plasma membrane and in endosomes and signal through adaptor proteins TRIF, TRAM, Mal and MyD88 to lead to the activation of transcription factors such as IRF3 and NFκB. As well, RLRs RIG-I and MDA5 detect viral dsRNA and signal through the adaptor MAVS to cause IRF3 and NFκB activation. Additionally, the adaptor protein STING is recruited from the endoplasmic reticulum to the cytoplasm, where it participates in both RLR signaling as well as in the detection of DNA through sensor(s) that remain incompletely characterized.

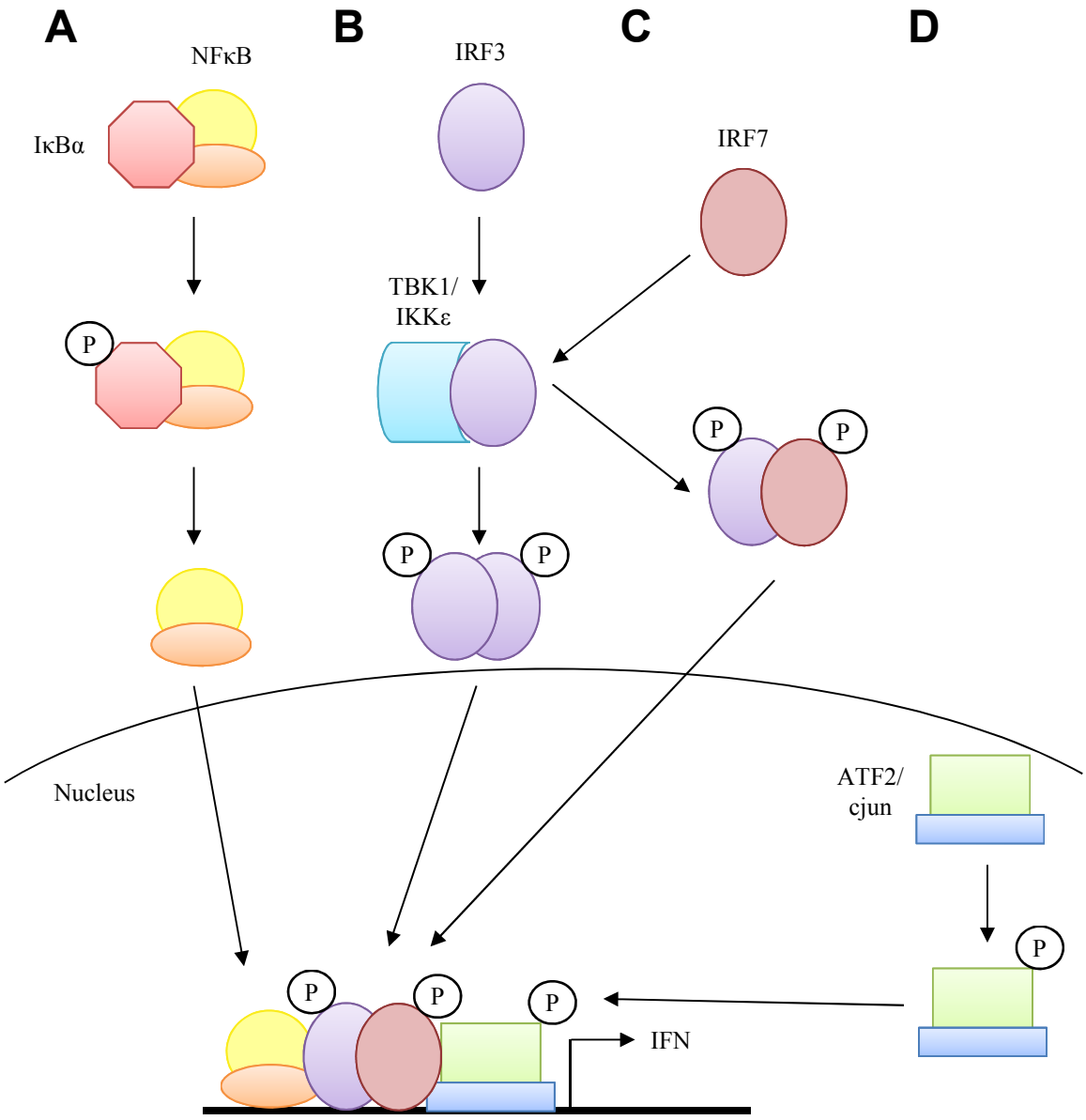

Figure 1.2: The innate antiviral response: from transcription factor activation to IFNα/β production. Detection of viral components by PRRs leads to the activation of a variety of transcription factors. (A) NFκB is held in an inactive cytoplasmic complex via interaction with its inhibitor, IκBα. Upon virus recognition, IκBα is phosphorylated and subsequently degraded, freeing NFκB to translocate to the nucleus and bind the IFNα/β promoter. (B) Upon virus detection, constitutively-expressed IRF3 is phosphorylated by the kinases TBK1 and IKKε, leading to its dimerization and nuclear translocation. (C) Although there is little basal IRF7 expression in most resting cell types, low level IFNβ production induces the expression of IRF7, leading to its phosphorylation by TBK1/IKKε, heterodimerization with IRF3, and nuclear translocation. (D) The constitutively nuclear ATF2/c-Jun is phosphorylated upon virus detection by stress-activated members of the mitogen-activated protein (MAP) kinase superfamily, leading to the activation of the complex. Cooperative binding of NFκB, IRF3/IRF7 and ATF2/c-Jun to the IFNα/β promoter leads to full expression of type I IFN genes.

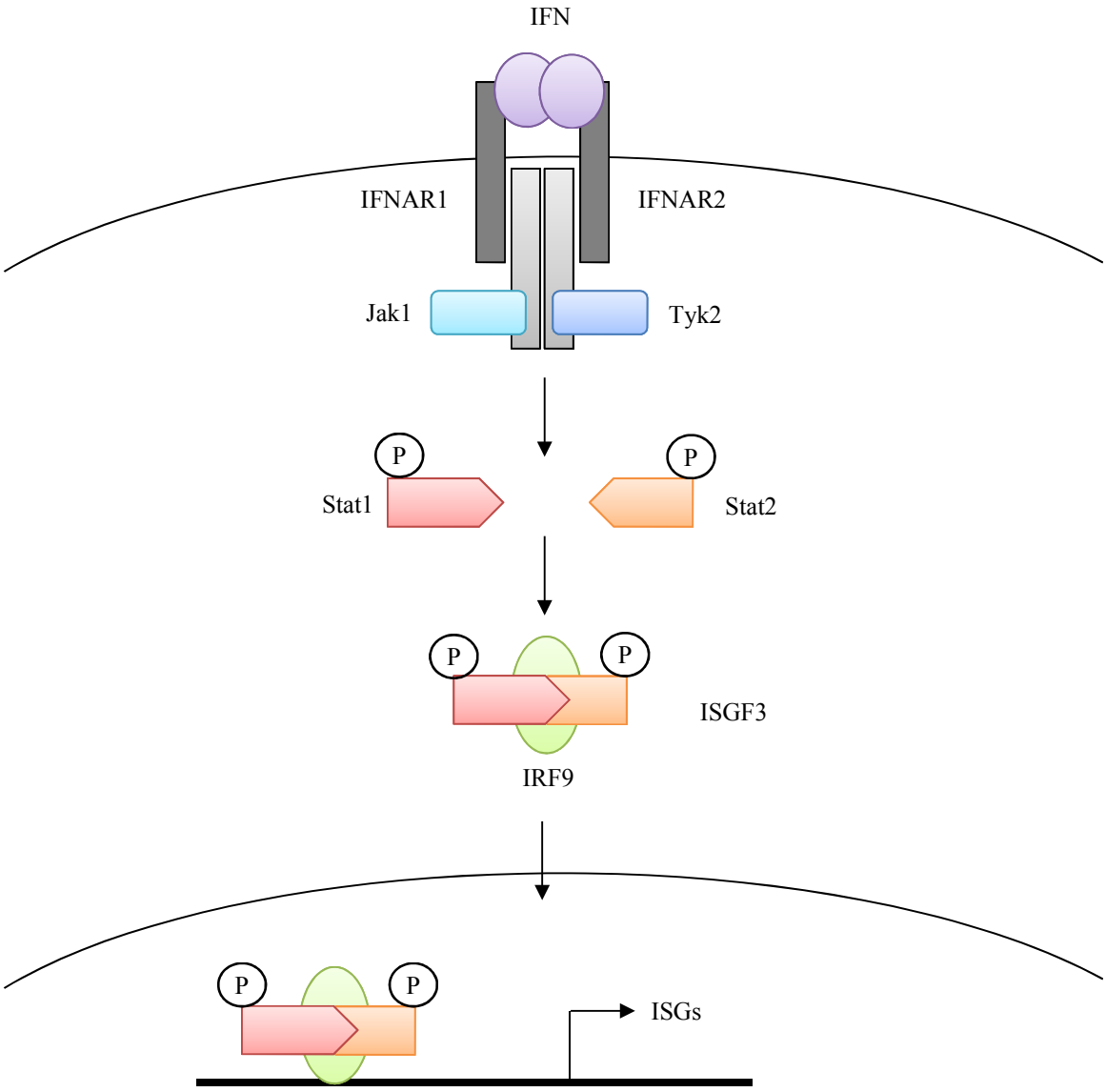

Figure 1.3: The innate antiviral response: from IFNα/β detection to ISG expression. Secreted IFNα/β binds to the cell-surface receptor IFNAR, leading to the activation of kinases Tyk2 and Jak1, which phosphorylate and activate proteins STAT1 and STAT2, resulting in the formation of a heterotrimeric complex containing IRF9, known as interferon-stimulated gene factor-3 (ISGF3). Binding of ISGF3 to the promoters of ISGs leads to their transcriptional activation, and the collective actions of the hundreds of ISGs induced by IFNα/β inhibit both virus replication and spread.

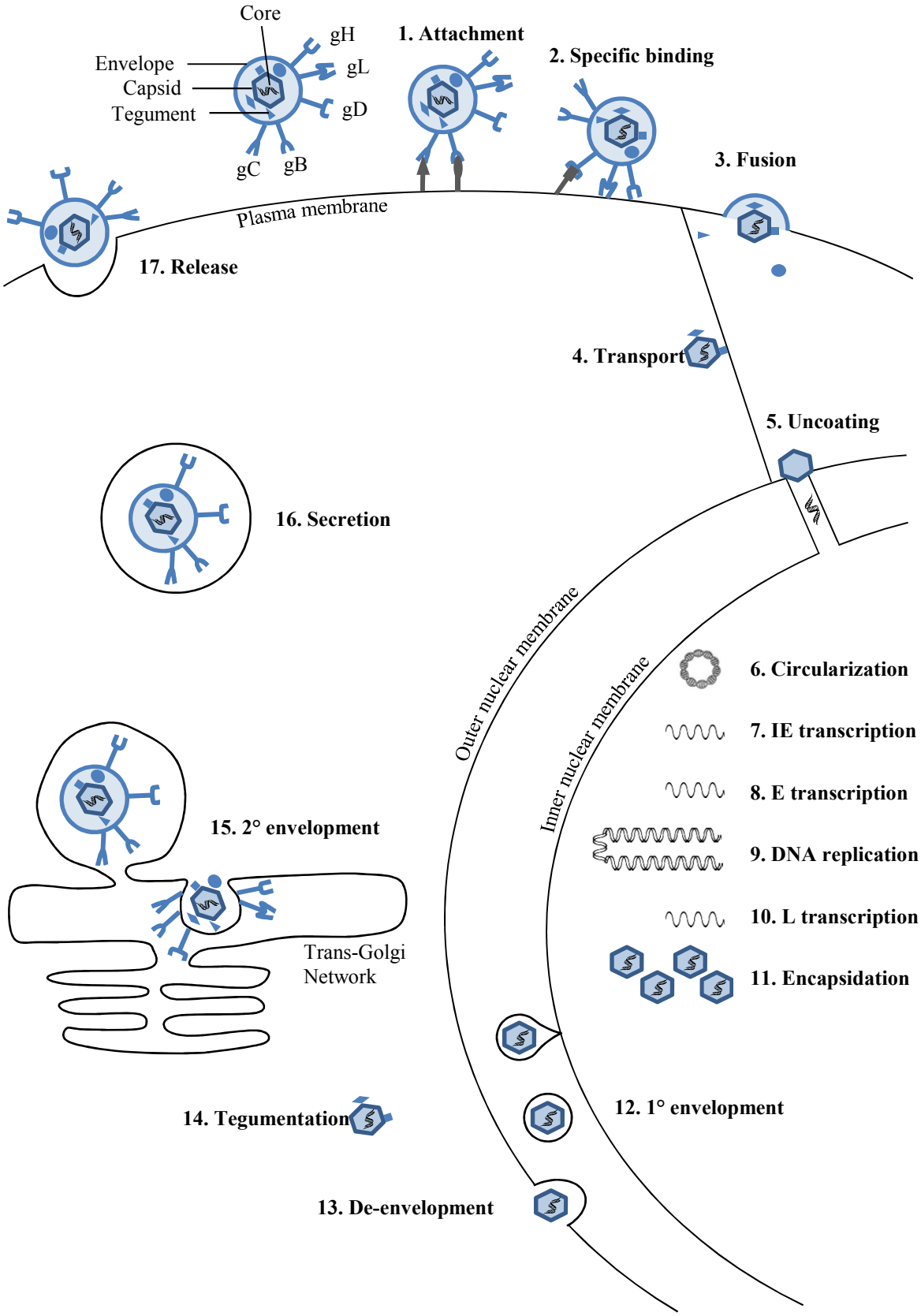
Core
gH
gL
gD
gB
gC
Envelope
Capsid
Tegument
1. Attachment
2. Specific binding
3. Fusion
Plasma membrane
17. Release
4. Transport
5. Uncoating
16. Secretion
Outer nuclear membrane
Inner nuclear membrane
6. Circularization
7. IE transcription
8. E transcription
9. DNA replication
10. L transcription
11. Encapsidation
15. 2° envelopment
Trans-Golgi
Network
12. 1° envelopment
14. Tegumentation
13. De-envelopment

Figure 1.4: The HSV replication cycle. The HSV particle consists of a double-stranded DNA genome in the core, enclosed within an icosahedral capsid, surrounded by a proteinaceous tegument layer and enveloped in a lipid membrane with embedded glycoproteins, such as gB, gC, gD, gH and gL. Initial attachment to the cell surface is mediated by gB and gC (1), increasing the likelihood of the interaction of essential entry component gD with its receptor (2), triggering the fusion machinery consisting of gB, gH and gL. Fusion of the viral envelope with the plasma membrane allows the release of the viral capsid and tegument proteins into the cytoplasm (3). Some tegument proteins dissipate, while others remain associated with the capsid while it is transported along microtubules to the nucleus (4). The capsid then docks at the nuclear pore complex, and releases the viral genome into the nucleus (5). There, the genome circularizes (6), and the initial transcription of the immediate-early (IE) genes begins (7), allowing for the subsequent transcription of early (E) genes (8). This results in the initiation of viral DNA replication (9) and the subsequent transcription of late (L) genes (10). The concatemeric viral DNA is cleaved into individual genomes and packaged into capsids (11), which bud from the inner nuclear membrane, acquiring a primary envelope in the process (12). Primary virions then fuse with the outer nuclear membrane, releasing capsids, with some associated tegument proteins, into the cytoplasm (13). Here, the capsids acquire more tegument proteins (14) while traveling to the trans-Golgi network, where the capsids associate with additional tegument proteins as well as the glycoproteins and acquire the final envelope (15). Mature particles are transported to the cell membrane in secretory vesicles (16), where they are released into the extracellular space (17).

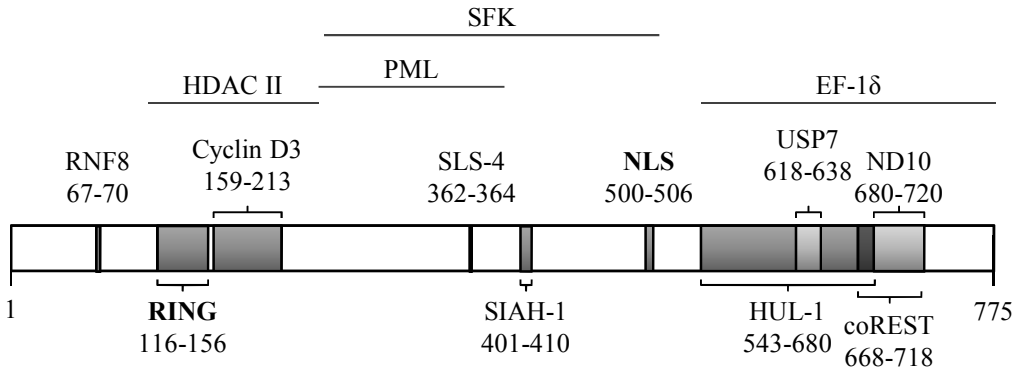

Figure 1.5: Schematic of ICP0 domains. The RING finger domain and NLS are indicated in bold. Binding sites for RNF8, Cyclin D3, SIAH-1, USP7, and coREST are indicated, as is the location of the SIM-like sequence SLS-4. ND10 indicates the region required for the localization of ICP0 to the ND10 domains, and the secondary domain with E3 ubiquitin ligase activity, HUL-1, is shown. Bars indicate the general regions thought to be important for ICP0 binding to HDAC II, PML, the SFKs and EF-1δ.

CHAPTER 2
MATERIALS AND METHODS

Sections of this chapter written by Kathryne Taylor have been published:

Taylor KE, Chew MV, Ashkar AA, Mossman KL. 2014. Novel roles of cytoplasmic ICP0: proteasome-independent functions of the RING finger are required to block interferon-stimulated gene production but not to promote viral replication. J Virol. 88:8091-8101.

2.1 Cell lines

Human embryonic lung (HEL) fibroblasts, U2OS, HEK293, and Vero cells were purchased from the American Type Culture Collection (ATCC). The generation of Vero cells stably expressing human IRF3 (Vero-IRF3) has been previously described (503). Primary wildtype dermal fibroblasts derived from the back skin of an adult C57BL/6 mouse were obtained from Dr. A. Ashkar.

All cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (HEL and U2OS) or 5% (Vero) fetal bovine serum (FBS), MEM-F11 +10% FBS (HEK293), or αMEM +15% FBS (dermal fibroblasts). In all cases, 2mM L-glutamine, 100U/mL penicillin, and 100mg/mL streptomycin was added, except for the dermal fibroblasts, which were grown in 200U/mL penicillin and 200mg/mL streptomycin. All cells used TrypLE Express (Life Technologies) for trypsinization, except for HEK293 cells, which were passaged using citric saline.

2.2 Viruses

2.2.1 Viral strains and virus preparation

The HSV-1 strains used in this study are described in Table 2.1. To grow up viral stocks, Vero cells were infected with 17 syn and D8/FXE-R at a multiplicity of infection (MOI) of 0.01, while all ICP0 mutant strains were grown on U2OS in the presence of 3mM hexamethylene bisacetamide (HMBA) at an MOI of 0.1. Infections were allowed to progress for 2-3 days, until all cells showed evidence of cytopathic effect but had not yet detached. Cells were then resuspended in the media via pipetting, and spun at 1500rpm in a GH-3.8 rotor (Beckman Coulter) for ten minutes, and supernatant fractions were collected, frozen at -80°C, then thawed and spun at 25000rpm in a SW32Ti rotor (Beckman Coulter) for two hours. Meanwhile, the cell-associated fraction was resuspended in serum-free (SF) DMEM, freeze-thawed three times, treated with 30 strokes with a glass homogenizer and spun at 1000rpm in a GH-3.8 rotor for ten minutes. The supernatant media was conserved, while the cell pellet was resuspended, sonicated at 50% amplitude for 1 minute in Sonic Desmembrator Model 550 (Fisher), and spun again

at 1000rpm for ten minutes. The cell pellet was discarded, and the supernatants pooled, then purified on a 36% sucrose cushion by spinning at 30,000rpm in a SW41 rotor (Beckman Coulter). The final viral pellet was then resuspended in SF DMEM. All viruses were titered on U2OS cells in the presence of HMBA, with the exception of 17 syn and D8/FXE-R, which were titered on Vero cells.

Sendai virus (SeV, Cantell strain) was commercially obtained (Charles River Laboratories International, Inc.), while VSV and VSV-GFP were obtained from B. Lichty, McMaster University. UV inactivation of SeV was performed by placing 1mL of diluted virus in a well of a 6 well dish and exposing it to 8000µjoules in a Stratalinker 2400 (Stratagene), which was sufficient to block viral gene expression as determined via immunofluorescence with an anti-SeV antibody (provided by Y. Nagai, University of Tokyo). Viral infections were performed for one hour in SF media at 37°C, and at an MOI of 10 pfu/mL for HSV and VSV and 80 HAU/10^6 cells for SeV, and unless otherwise stated.

2.2.2 Preparation of infectious HSV DNA

Vero cells or U2OS cells were infected with 17 syn at MOI 0.01 or D8/FXE in the presence of HMBA at MOI 0.1, respectively, and grown until all cells showed evidence of cytopathic effect. Cells were pelleted at 2500rpm in a GH-3.8 rotor for ten minutes, washed once with PBS and re-pelleted, then resuspended in 1mL of 200mM EDTA pH 8.0, to which 25µL of 20% SDS and 10µL of 10mg/mL proteinase K (Invitrogen) were then added, and incubated overnight at 37°C. DNA was extracted 4 times with phenol:chloroform:isoamyl alcohol and one time with chloroform only. DNA was then twice dialyzed in a 10,000 molecular weight cut-off Slide-A-Lyzer dialysis cassette (Thermo Scientific) for 12 hours against 0.1X SSC buffer (15mM NaCl and 1.5mM sodium citrate dehydrate, pH 7.0).

2.2.3 Construction of recombinant viruses

To create ICP0 containing the D8 and FXE lesions, a XhoI-KpnI fragment from p110-FXE (481), which contains the FXE lesion, was inserted into XhoI-KpnI cut p110-D8 (481). The resulting plasmid, p110-D8/FXE, was sequenced to verify the presence of the D8 and FXE lesions. The D8/FXE virus was constructed using homologous recombination following co-transfection of U2OS cells with infectious DNA from 17 syn and the linearized p110-D8/FXE plasmid. Plaques were screened on Vero cells, which are only semi-permissive for ICP0-deficient viruses, and small plaques were isolated for further characterization. Viruses containing D8/FXE ICP0 were plaque purified three times and the presence of the D8 and FXE lesions were confirmed by Western blot analysis and sequencing. The D8/FXE-R revertant was generated by homologous recombination in U2OS cells of infectious DNA from D8/FXE with the p110 plasmid, and screened on Vero cells for large plaques. Plaque purification and confirmation were done as with D8/FXE.

2.2.4 Sequencing of viral genomic DNA

$1x10^9$ pfu of purified viral stock was diluted in pronase working buffer (0.5% SDS, 10mM Tris pH 7.4, 10mM EDTA), and 0.5mg/mL pronase (Roche) was added and incubated at 37°C overnight. An equal volume of buffer stabilized phenol was then added, incubated for 3 minutes at room temperature, then spun at 12000g for 2 minutes. The top layer was taken and an equal volume of isoamyl alcohol-stabilized chloroform was added, incubated for 3 minutes, and spun at 12000g for 2 minutes. The top layer was taken, and NaCl was added to a final concentration of 0.2M. Sodium acetate was then added to a final concentration of 0.3M, and the sample was combined with 2 volumes of ice-cold 95% ethanol and incubated on ice for 15-30 minutes. The sample was then spun at 18,000g for ten minutes, the supernatant was discarded, and 750µL 70% ethanol was added, and spun at 18,000g for 2 minutes. The 70% ethanol wash was repeated, and the tube was stored open at room temperature until all liquid had evaporated. The pellet was then dissolved in 50µL 10mM Tris and adjusted to a concentration of 0.25µg/µL.

Sequencing of the prepared viral genomic DNA was performed by MOBIX (McMaster University) with the following primers: 5'-AGGAAGACCCCGGCAGTTGC-3' and 5'-GCGGGGGCCGTCGGGTACTC-3' using their hairpin protocol.

2.2.5 Virus particle quantification via tunable resistive pulse sensing

Purified viral stocks were diluted 1:100 for 17 syn, D8 and D8/FXE and 1:200 for dl1403 in standard electrolyte buffer (Izon) and quantified using a qViro-X (Izon) with an NP400 nanopore (Izon). Particle size and concentration were determined by calibration with polystyrene beads (Izon) of known diameter (335nm) and concentration ($7.0x10^{10}$ particles/mL).

2.2.6 Plaque assays

HEL cells were infected at the indicated MOIs, grown in 1mL media for 24h, and then cells were scraped into the media and freeze-thawed three times. Serial dilutions of the resulting samples were used to infect U2OS cells in the presence of HMBA and 2% human serum, and after three days, cells were fixed with methanol, stained with Giemsa (Sigma) and plaques were counted.

2.2.7 VSV-GFP protection assays

24 hours after siRNA transfection of HEL cells, or control treatment with IFNα, 1mL of the supernatant media was transferred onto Vero cells. After 24 hours of incubation with the supernatant, cells were challenged with VSV-GFP using a 10^{-5} dilution in SF media for one hour at 37°C, and then the media was replaced with 1.5% methylcellulose. Levels of GFP fluorescence were visualized using a Typhoon scanner (GE Healthcare) 24 hours after infection.

2.3 Drug treatments

Polyriboinosinic polyribocytidylic acid (poly I:C) (GE Healthcare) was reconstituted in phosphate buffered saline (PBS) (137mM NaCl, 2.7mM KCl, 10mM

Na_2HPO_4, and 1.8mM KH_2PO_4) at a concentration of 5mg/mL. When used, it was thawed at room temperature, heated at 56° for 10 minutes, cooled at room temperature for 5 minutes, and then added directly to the culture media at a concentration of 100µg/mL. Human IFNα (Sigma) was reconstituted in PBS at a concentration of 100U/µL, and was used at a concentration of 200U/mL.

NSC23766 (Calbiochem) was resuspended in sterile water at a concentration of 100mM, and used at a concentration of 200µM. 4-Hydroxytamoxifen (Sigma) was resuspended at 10mM in ethanol by placing the vial in a boiling water bath for a few seconds, and was used at a concentration of 100nM. MG132 (Sigma) was resuspended in dimethyl sulfoxide at a concentration of 5µg/uL and used at a concentration of 5µM.

MG132 and NSC23766 were added to the culture medium 30 minutes prior to infection, and were maintained in the medium throughout the experiment. Tamoxifen was added to the regular culture medium of dermal fibroblasts and replaced daily for 3 days. Cells were then cultured in the absence of tamoxifen for the indicated times.

2.4 DNA techniques

2.4.1 Plasmids and transformations

The GFP-Rac1 expression vector set containing T17N DN-Rac1 and Q61L CA-Rac1 was commercially available (Cell Biolabs), with pcDNA3 used as the empty vector control. Recombination vectors p110, p110-D8, and p110-FXE, and expression vectors pCI110, pCI-D8 and pCI-FXE (504) were obtained from R. Everett, University of Glasgow, and p110-D8/FXE and pCI-D8/FXE were generated as described above for the recombinant virus. The TAP tag was added to these constructs by cutting the appropriate p110 or pCI110-derived plasmid with Sal1, which cuts 27 nucleotides upstream of the stop codon in ICP0. To add these nucleotides to the TAP tag, PCR was performed on the pSHZTAP plasmid (provide by M. Weitzman, Salk Institute) with the following primers: 5'-TAGTCGACCCGGGACGAGGGAAAACAAAAGCGACGATGGAAAAAGAAT TTC-3' and 5'-GCGTCGACTTAGCCCAGCTTGCAGCCGCC-3', with the PCR product then ligated into the TOPO vector using the TOPO TA cloning kit (Life

Technologies), according to manufacturer's instructions. This plasmid was then treated with Sal1, and the resulting fragment was then inserted into the cut ICP0 vectors.

Plasmids were used to transform competent *Escherichia coli* strain DH5α via electroporation with an E. Coli Pulser (BioRad), and positive clones were selected using Luria Bertani (LB) media containing ampicillin (50μg/mL). These clones were grown in 1L of LB overnight at 37°C with shaking, and the plasmid DNA was isolated using the HiSpeed Plasmid Midi Kit (Qiagen) according to manufacturer's instructions.

2.4.2 Plasmid transfection

Vero-IRF3 cells were transfected with 5μg of the indicated Rac1 plasmid and 5μL Lipofectamine 2000 (Invitrogen), while HEK293 cells received 3μg plasmid and 5μL Lipofectamine, per well of a 6 well dish. Lipofectamine-DNA complexes were made up in Optimem (Invitrogen) according to the manufacturer's instructions and added to cells grown in drug-free 5% DMEM.

For nucleofection of TAP-tagged ICP0 constructs, HEL cells were trypsinized, counted, and $1x10^6$ cells per reaction were pelleted at 90g and resuspended in Nucleofector Solution (VP1-1002, Amaxa). 2μg of DNA was added per sample, and the cell/DNA suspension was transferred to cuvettes and treated via program U-023 in a Nucleofector II (Amaxa). Cells were then immediately placed into pre-equilibrated culture media and seeded into 6-well plates.

2.5 RNA techniques

2.5.1 RT-PCR

6 hours after infection with the indicated viruses, total cellular RNA was obtained using Trizol reagent (Invitrogen), then 2μg of RNA was reverse transcribed using 200ng of random hexamer primer and SuperScript II Reverse Transcriptase (Invitrogen), according to manufacturer's instructions. Samples were then analyzed via PCR with primers for human ISG-56 and GAPDH, as previously described (103). 10μL of each

product was then separated on a 0.8% agarose gel containing 1.25mM ethidium bromide (Invitrogen) and visualized using an AlphaImager (Alpha Innotech).

2.5.2 Quantitative RT-PCR

RNA was harvested as above. 2.5µg of RNA was DNase-treated (DNA-free kit, Ambion), then 150ng of each sample was reverse transcribed as above. Samples were then analyzed in triplicate with Universal PCR Master Mix and gene-specific TaqMan primers (Life Technologies) - Hs01911452_s1 for IFIT1 and Hs99999905_m1 for GAPDH. Gene expression was normalized to GAPDH via the $\Delta\Delta C_T$ method, and expressed as a fold change relative to the internal control of poly I:C.

2.5.3 siRNA transfection

Rac1 experiments were performed using the Rac1 Validated Stealth RNAi DuoPak (Invitrogen) (sequences: 5'-UGGAGAAUAUAUCCCUACUGUCUUU-3' and 5'-AGGGUCUAGCCAUGGCUAAGGAGAU-3'), and the Stealth RNAi Negative Control Lo (36%) and Med (52%) GC, which were obtained from Invitrogen as 20µM stocks. The Rac1 ON-TARGETplus SMARTpool (Dharmacon) (sequences: 5'-GAACUGCUAUUUCCUCUAA-3', 5'-AUGAAAGUGUCACGGGUAA-3', 5'-GUAGUUCUCAGAUGCGUAA-3', and 5'-GUGAUUUCAUAGCGAGUUU-3') and the ON-TARGETplus Non-Targeting Pool (Dharmacon) were also used, and were both resuspended in sterile nuclease-free water to a concentration of 20µM. For WDR11 experiments, the WDR11 ON-TARGETplus SMARTpool (Dharmacon) (sequences: 5'-GCCAAGAAAGCUCUAAAUA-3', 5'-GGAUGUAGCAGCAGGAGUA-3', 5'-GGUAUUGAAUGGACAAGUU-3', and 5'-GCAGUCGUAUUCAGAGAUA-3') was resuspended in sterile nuclease-free water to make a 5µM stock. The same negative control as above was used, resuspended to a 5µM stock.

For the Invitrogen Rac1 siRNA experiments, the transfection was performed by quarter-seeding HEL cells in drug-free 5% DMEM. Cells were then placed in pure Optimem and treated with 100nM siRNA and 1.5µL Lipofectamine RNAiMAX

(Invitrogen) per well of a 12-well dish, made up in Optimem according to the manufacturer's instructions. After 7 hours, the transfection was repeated a second time. Cells were left in Optimem overnight, then media was replaced with 2% drug-free DMEM and cells were left to incubate for four days.

For the Dharmacon Rac1 siRNA experiments, 150nM siRNA was combined with 2μL Dharmafect 1 (Dharmacon) in serum-free DMEM according to the manufacturer's instructions, and added to HEL cells that had been quarter-seeded in drug-free 5% DMEM. Cells were transfected only once, and media was replaced after an overnight incubation. Cells were left to grow for 4 days.

For the WDR11 siRNA experiments, transfections were performed as above, except that 25nM siRNA and 3μL Dharmafect 1 were used, and cells were incubated only 3 days.

2.6 Protein techniques

2.6.1 Cell extracts

For RIPA extracts, cells were washed twice in ice-cold PBS, then scraped into RIPA buffer (50mM Tris-HCl, pH 7.4, 100mM NaCl, 5mM EDTA, 50mM sodium fluoride, 40mM β-glycerophosphate, 1% Triton X-100, 0.1% SDS, 1% sodium deoxycholate, 1mM sodium orthovanadate, 1mM phenylmethylsulfonyl fluoride, 2mM dithiothreitol, and 1x protease inhibitor cocktail (Sigma)), passed through a 25-gauge needle 5 times, then centrifuged at 14000rpm in a Microfuge 18 F241.5P (Beckman Coulter) for 20 minutes at 4°C. For cytoplasmic extracts, cells were washed once with ice-cold 1x PBS, once with ice-cold 0.2x PBS, then scraped into cytoplasmic buffer (10mM HEPES, pH 7.3, 10mM potassium chloride, 1.5mM magnesium chloride, 50mM sodium fluoride, 1mM sodium orthovanadate, 1mM phenylmethylsulfonyl fluoride, 2mM dithiothreitol, and 1x protease inhibitor cocktail (Sigma)). Lysates were incubated on ice for ten minutes, then Triton X-100 was added to the final concentration of 1%, and samples were spun at 12,000g for 3 min. Extracts were quantified via Bradford assay (Bio-Rad Laboratories).

2.6.2 Immunoprecipitation

5µg anti-ICP0 antibody (Virusys Corporation) or 2µg anti-WDR11 antibody (Abcam ab93871) was incubated with 30µl of Protein G Plus agarose (Thermo Scientific) for 1 hour at 4°C. Beads were washed three times with immunoprecipitation (IP) buffer (50mM Tris pH 8.0, 150mM NaCl, and 1% NP-40) and incubated with 500µg of cytoplasmic extract for an additional 4 hours. Beads were washed 5 times in IP buffer, then eluted with two consecutive 2 minute incubations with 20µL of elution buffer (100mM Glycine, pH 2.0), then neutralized with 4µL 2M Tris, pH 8.0.

2.6.3 TUBE agarose pulldown assays

Infections were performed in the presence of MG132 to stabilize ubiquitinated proteins. 20µL of TUBE-1 agarose (LifeSensors) was then incubated with 500µg cell extract in the presence of 50µM PR619 (LifeSensors), a ubiquitin protease inhibitor, and 5µM 1,10-phenanthroline (Sigma), a chelator that inhibits metalloproteases and stabilizes K63 chains, overnight at 4°C. Samples were washed 3 times in TBS-T (20mM Tris pH8.0, 0.15M NaCl, 0.1% Tween-20) and boiled in sample buffer containing SDS and β-mercaptoethanol.

2.6.4 Western blotting

25ug of the indicated extracts were separated via electrophoresis on 10% (or 12% for Rac1) denaturing polyacrylamide gels, transferred onto polyvinylidene difluoride (Millipore) membranes, and blocked in 5% skim milk. Blots were probed with 1:1000 of the following primary antibodies, as indicated: anti-ICP0, anti-actin (Santa Cruz SC-1616), anti-ISG-56 (provided by G. Sen, Cleveland Clinic), anti-USP7 (R2B2, provided by L. Frappier, University of Toronto), anti-Rac1 (Pierce) or anti-WDR11. Anti-rabbit and anti-goat (Sigma) and anti-mouse (Chemicon) secondary antibodies conjugated to horseradish peroxidase (HRP) were used at 1:5000, and signal was visualized via chemiluminescence.

2.6.5 Quantitative Western blotting

2-fold serial dilutions of purified viral stocks were made in 10mM Tris pH 8.0, then boiled in SDS loading buffer for 10 minutes. The SDS-PAGE, transfer, and blocking steps were performed as above, except that nitrocellulose membranes were used instead of polyvinylidene difluoride. The blots were treated with 1:60,000 anti-VP5 (provided by J. Brown, University of Virginia) overnight at 4°C, then incubated with 1:10,000 anti-rabbit HRP for 1h at room temperature. Blots were then visualized using the ECL Prime reagent (Amersham) according to the manufacturer's instructions, and imaged using the Typhoon scanner.

2.6.6 SYPRO Ruby staining

After electrophoresis, the gel was placed in 100mL of fix solution (50% methanol and 7% acetic acid) for 30 minutes at room temperature, which was then discarded and replaced with a fresh 100mL of fix solution for an additional 30 minutes, then discarded. 60mL of SYPRO Ruby protein gel stain (Molecular Probes) was then added and shaken overnight at room temperature, covered in foil. The gel was then washed once in wash solution (10% methanol and 7% acetic acid) for 30 minutes at room temperature, twice in pure water, and then immediately imaged using the AlphaImager.

2.6.7 Immunofluorescence and direct fluorescence

HEL cells were seeded onto coverslips and infected at 50% confluency. At the indicated time, cells were fixed with 10% formalin (Sigma), permeabilized with 0.1% Triton X-100, and blocked with 3% goat serum, 3% bovine serum albumin (BSA), and 0.02% Tween 20. Cells were incubated with 1:250 anti-ISG-15 (provided by E. Borden , Cleveland Clinic), 1:250 anti-ICP0 or 1:250 anti-WDR11, then 1:250 anti-mouse or anti-rabbit Alexa fluor 488 or 594-conjugated secondary (Invitrogen). For the Rac1 plasmids, Vero-IRF3 cells or HEK293 cells were seeded onto coverslips at 50% confluency, transfected as above, and the GFP tag on the constructs was visualized via direct fluorescence. In all cases, nuclei were stained with 1:10000 Hoechst 33258 dye. All

images were captured with a Leica DM-IRE2 inverted microscope and analyzed using Openlab software (Improvision).

2.7 SILAC

2.7.1 Culturing

HEL cells were passaged three times (6 doublings) in DMEM with no glutamine, arginine or lysine (Invitrogen) supplemented with 10% dialyzed FBS (Invitrogen), 2mM L-glutamine, 100U/mL penicillin, 100mg/mL streptomycin, and 100mg/mL each of $^{12}C_6$, $^{12}N_4$-Arginine and $^{12}C_6$-Lysine (light), $^{13}C_6$-Arginine and 4,4,5,5-D4-Lysine (medium) or $^{13}C_6$, $^{15}N_4$-Arginine and $^{13}C_6$, $^{15}N_2$-Lysine (heavy) (Cambridge Isotope Laboratories).

2.7.2 Large-scale immunoprecipitations

Cells were seeded into dishes and infected with D8/FXE (light), D8 (medium) or dl1403 (heavy) at MOI 10 for 8 hours. Cytoplasmic extracts were then performed, and pre-cleared by incubation with 100µL Protein G-coupled Dynabeads (Invitrogen) for one hour at 4 degrees. Separate immunoprecipitations were then performed for each population. For each sample, 10µL of anti-ICP0 antibody was allowed to bind to 100µL Protein G-coupled Dynabeads for one hour at 4 degrees, and then beads were washed three times in IP buffer, and incubated with 2.5mg of protein in 1.5mL of cytoplasmic extract buffer at 4°C for 12 hours. Beads were then washed twice in 1mL 100mM Tris pH 8.0; twice in 20mM Tris pH 8.0, 150mM NaCl, 1% NP-40 and 0.1% SDS; three times in sterile water; then twice in freshly-made 100mM ammonium bicarbonate.

2.7.3 Trypsinization and sample preparation

Washed beads were transferred to a new tube, and resuspended in 100µL of 100mM ammonium bicarbonate. 1µg of sequencing grade Trypsin (Promega) was added and then incubated with shaking at 37°C for 24h. 100µL 3% trifluoroacetic acid was then added, and rotated for 5 minutes, and the supernatant was conserved. 100µL of 80% acetonitrile was then added to the beads, rotated for 5 minutes, then the supernatant was

added to the conserved fraction and the beads were discarded. The samples were then frozen at -80°C, and then dried by Vacufuge (Eppendorf). Dried peptides were then frozen at -80°C degrees. Mass spectrometry analysis was performed as previously described (505).

2.8 Mouse techniques

2.8.1 Mice and HSV-1 infection

Female C57BL/6 mice, 6-8 weeks of age, were purchased from Charles River Laboratory. Mice were housed in Biosafety Level 2 conditions in the Central Animal Facility at McMaster University. All mice were housed in level B rooms that followed a 12-hour day and 12-hour night schedule, and were maintained under standard temperature controlled conditions. All animal experiments were approved by the Animal Research Ethics Board (AREB) of McMaster University. To prepare mice for intravaginal infection, female mice were injected with 50µL of 50mg/mL Depo-Provera hormone (Pfizer Canada Inc.) subcutaneously four days prior to HSV-1 infection. To infect mice, they were anaesthetized by injectable anesthetic (150µL of ketamine-xylazine (0.75mL: 0.25mL)) given intraperitoneally, and infected intravaginally with 10^5 PFU of the indicated viruses in 10µL of PBS. Mice were kept on their backs under the influence of anesthesia for one hour to allow for infection. Infected mice were followed daily for genital pathology and survival. Genital pathology was scored daily as follow: 0, no apparent infection changes; 1, redness of external vagina; 2, severe redness and swelling of external vagina; 3, severe redness and swelling of external vagina and surrounding tissues and hair loss; 4, genital ulceration with severe redness, swelling and hair loss of genital and surrounding tissues; 5, severe genital ulceration extending to surrounding tissue or any sign of hind limb paralyses. To examine viral shedding and cytokines in the vaginal mucosa, vaginal lavages were collected on days 1-5 post infection by pipetting 2x 30µL of PBS into and out of the vagina six to eight times. Virus in the vaginal washes was quantified via plaque assay as described above.

2.8.2 ELISA

To measure IFN-γ concentrations in the vaginal lumen of infected mice, vaginal lavages were pooled from several mice and were assayed for IFNγ production by the DuoSet ELISA kit, according to manufacturing protocol (R&D Systems). The plates were read using the Safire plate reader (Tecan) at 450nm wavelength.

2.9 Statistical analysis

Analysis was performed using GraphPad Prism. Where necessary, values were first transformed, via logarithmic transformation to equalize variance, or arcsine-square-root transformation for proportions.

Table 2.1: ICP0 mutants used in this study.

Virus	Mutation	Phenotype	Reference
17 syn	None	Wildtype	[506]
D8	Deletion of amino acids 475–548 (NLS)	ICP0 restricted to cytoplasm	[438]
FXE	Deletion of amino acids 106-150 (RING)	ICP0 lacks a functional RING finger and is mainly nuclear	[438]
D8/FXE	Deletion of amino acids 106-150 (RING) and 475–548 (NLS)	ICP0 restricted to cytoplasm; lack of a functional RING finger	This work
D8/FXE-R	D8 and FXE mutations are repaired to wildtype sequence	Wildtype	This work
dl1403	2 Kb deletion in ICP0	ICP0 null	[507]

CHAPTER 3
INVESTIGATION OF THE ROLE OF THE SMALL GTPASE RAC1 IN THE INNATE ANTIVIRAL RESPONSE

KE Taylor and KL Mossman conceived and designed the experiments. KE Taylor performed the experiments.

3.1 Abstract

The innate immune response to viral infection is thought to be activated by the detection of viral components and byproducts of replication, and to require IFN-mediated signaling. However, there is recent evidence to suggest that the entry process of an enveloped virus itself may be sufficient to induce an IFN-independent antiviral response. How viral entry might be detected is unknown. The small Rho GTPase Rac1 plays a role in regulating the rearrangements of the cytoskeleton that occur during the entry process of multiple viruses, and has also been implicated in IRF3 signaling, making it an intriguing candidate for potential involvement in the IFN-independent response. Here, I examined this possibility, but found instead some evidence to suggest that Rac1 may have a function in the negative regulation of the innate antiviral response. However, this investigation was beset with technical difficulties and despite the use of several approaches, including chemical inhibition, siRNA-mediated knockdown, overexpressed plasmid constructs and a conditional knockout cell line, the potential negative regulatory effect of Rac1 was found to be intermittent and unpredictable. Because it is involved in a tremendous variety of cellular processes, disrupting Rac1 with the relatively blunt tools currently available disturbs countless downstream pathways, resulting in unmanageable variability. Therefore, a full characterization of the function of Rac1 in the innate antiviral response awaits the development of more specific tools.

3.2 Introduction

As discussed in Chapter 1, the innate antiviral response is classically described as involving the production of type I IFNs, in response to the detection of highly conserved viral components. However, more recently it has become apparent that while this model is valid for high multiplicity infections with replicating virus, it does not accurately represent the events which occur following stimulation with low multiplicities of infection or with replication-deficient enveloped virus particles. Under these circumstances, an IFN-independent response is induced, which involves the direct expression of a small subset of ISGs, mediated by the transcription factor IRF3, without requiring the production of IFNα/β (101, 103, 107, 112). It is currently hypothesized that the physical entry process of an enveloped virus into the host cell is sufficient to activate IFN-independent signaling (101, 108, 117) – but the mechanism through which this could be detected remains unknown.

A common feature of enveloped viral entry into the host cell may be perturbations of the actin cytoskeleton. The cortical shell is a dense layer of actin filaments associated with the cytoplasmic face of the plasma membrane, with roles in determining cell shape, providing mechanical strength, and producing membrane protrusions (reviewed in (508)). Long considered to be simply a barrier to traffic into and out of the cell, the cortical shell has been more recently found to have a positive function in the projections and invaginations of the plasma membrane involved in vesicular trafficking (509, 510). The polymerization of cortical actin has also been suggested to produce the mechanical force required to drive membrane fusion (511-513). Accordingly, the entry of several enveloped viruses is prevented by disrupting the actin cytoskeleton, including HSV (514, 515), murine ecotropic leukemia virus (516), human immunodeficiency virus (HIV) (517), vaccinia virus (492), influenza virus (518), and dengue virus (490, 493). Interestingly, the cytoskeleton has been suggested to have a potential role in transmitting signals if rearranged (519). For example, both actin depolymerizing and stabilizing agents were found to induce the activation of NFκB, hinting that cells can detect the perturbation of actin dynamics (520), although the exact mechanism behind this is currently unclear.

An important protein involved in the regulation of cytoskeletal dynamics is Rac1 (Ras-related C3 botulinum toxin substrate 1), which is one of the 22 members of the Rho GTPase subfamily of low molecular weight GTPases found in mammalian cells. These proteins act as molecular switches, alternating between an active GTP-bound state and an inactive GDP-bound form. Early work on Rac1 demonstrated that it played a role in reorganizing the actin cytoskeleton to produce membrane protrusions known as lamellipodia or membrane ruffles (521). This observation led to a flurry of studies linking Rac1 to a vast number of cellular processes involving the actin cytoskeleton, including pinocytosis (521), phagocytosis (522, 523), morphogenesis (524, 525), migration (526-528), cell-cell adhesion (529), cytokinesis (530) and smooth muscle contraction (531). However, it soon became clear that Rac1 is not limited to the control of cellular activities directly involving the actin cytoskeleton – for example, it has also been implicated in the regulation of gene expression (532, 533), cell cycle progression (534), ion channel activity (535), endothelial permeability (536), cell proliferation (537), reactive oxygen species production (538), and cellular transformation (539-542).

The staggering diversity of functions regulated by Rac1 can be explained by the large number of effector proteins whose activation it controls in response to various signals. A general model for the Rac1 cycle is shown in figure 3.1. In a resting cell, Rac1 is bound by a GDP dissociation inhibitor (GDI), which works nonenzymatically to sequester the GDP-bound GTPase in the cytosol (reviewed in (543, 544)). This is accomplished by masking the hydrophobic, isoprenylated C-terminus of Rac1, as well as by preventing the spontaneous dissociation of GDP. A variety of extracellular stimuli can lead to Rac1 activation, including the binding of soluble molecules such as cytokines, growth factors and hormones to cell surface receptors; mechanical stresses, including fluid shear stress, tension and compression; and adhesive interactions between cells or to the extracellular matrix (reviewed in (545, 546)). This results in the induction of signaling pathways leading to the activation of guanine nucleotide exchange factors (GEFs). GEFs interact with Rac1, and catalyze the exchange GDP for GTP by destabilizing GDP binding, allowing it to dissociate and GTP to bind in its place, producing a

conformational change in Rac1 (reviewed in (547, 548)). This releases the GDI from Rac1, exposing its isoprenyl group and thus allowing it to insert into the plasma membrane. There, effector proteins, which specifically recognize the GTP-bound form of Rac1, interact with it and become activated. The exact mechanism varies with the protein of interest, but in some cases, Rac1 binding may produce a conformational change in the effector, disrupting an autoinhibitory interaction and thus unmasking its active site – which can result, for example, in effector autophosphorylation, decreasing its affinity for the GTPase as well as maintaining it in an active conformation even after dissociation (549, 550). Termination of signaling is achieved by GTPase-activating proteins (GAPs), which inactivate Rac1 by increasing its low intrinsic rate of GTP hydrolysis, returning it to its inactive conformation and thus resulting in its reassociation with the GDI, ending the response (reviewed in (551)).

Rac1 has also been implicated in innate immune signaling. For example, Rac1 is activated in response to IFNα signaling (552, 553), which may be mediated by the Rac1 GEF Vav, which has been found to interact with JAK-STAT component Tyk2 (554). This activation of Rac1 is thought to be important in mediating alternative signaling pathways that produce the antiproliferative, as opposed to antiviral, functions of IFNα (555). In addition, the stimulation of either TLR2 or TLR4 leads to Rac1 activation (485, 486). The Rac1 GEF DOCK2 (556, 557) has been implicated in the production of IFNα in plasmacytoid dendritic cells (pDCs) after TLR7 and TLR9 stimulation during viral infection (558). It is thought that triggering Rac1 through DOCK2 may result in the activation of IKKα – which in pDCs, unlike non-immune cells, is required for IRF7 phosphorylation, instead of TBK1/IKKε (559). Consistent with this, Rac1 inhibition has been found to block IFNα production by pDCs (560). Rac1 has also been reported to be involved in the activation of NFκB through a variety of mechanisms (485-488, 561-564). However, NFκB has roles in a broad range of cellular functions in addition to its activities in IFNα/β signaling – from the production of pro-inflammatory cytokines to functions in proliferation, differentiation, and survival (reviewed in (565)) – and so this does not necessarily implicate Rac1 in the initial production of IFNα/β.

More pertinent to antiviral signaling is a report demonstrating that Rac1 is involved in the activation of IRF3 in response to both dsRNA as well as a replicating RNA virus (489). The expression of an IRF3 reporter construct, and IRF3 phosphorylation and dimerization, was found to be increased by exogenous wildtype Rac1 and inhibited by a dominant negative (DN) Rac1 mutant. A modest increase in influenza replication was also observed in Rac1-inhibited cells, potentially due to an impairment of the antiviral response. Although thought-provoking, this study relied mainly on overexpressed IRF3 and reporter constructs instead of endogenous proteins, and therefore their conclusions remain controversial.

The potential role of Rac1 in the innate antiviral response is particularly interesting when considered in combination with its functions in the regulation of the actin cytoskeleton, as this makes it an appealing candidate for potential involvement in the detection of viral entry in the IFN-independent response. Indeed, Rac1 is activated during the entry of multiple enveloped viruses, including vaccinia virus (492, 566), influenza virus (489), Kaposi's sarcoma-associated herpesvirus (567), murine leukemia virus (568), HSV (496, 569), Ebola virus (495) and dengue virus (490). For HIV, it has been shown that it is specifically the fusion step of viral entry that is mediated though Rac1-dependent signaling (491, 494, 570-572). Similarly, cell-cell fusion induced by a fusion-associated small transmembrane (FAST) protein has also been shown to require Rac1 activation (573) – which is particularly intriguing, because membrane perturbation by a FAST protein has been previously shown to induce IFN-independent signaling (110). Rac1 likely mediates these effects on viral entry through its regulation of the cortical shell (reviewed in (508, 574)). Consistent with a potential link between Rac1 activation during viral entry and the induction of IRF3 signaling is the observation that although Rac1 is rapidly activated during HSV infection, this GTPase is not essential for virus entry (496, 569). In contrast, overexpression of a constitutively active mutant of Rac1 decreased viral gene expression without affecting the internalization or cytoplasmic transport of the virus (496, 569), which could be explained by the possible induction of ISGs by Rac1, which then act to prevent viral replication.

In light of its potential to bridge the detection of viral entry and the activation of antiviral signaling, the possible function of Rac1 in the IFN-independent antiviral response was investigated. Unexpectedly, I found instead that Rac1 has a negative regulatory role, decreasing ISG expression in response to both IFN-dependent and –independent stimuli, as well as in unstimulated cells. However, despite the use of a variety of different techniques to perturb Rac1 activity, including chemical inhibition, siRNA-mediated knockdown, overexpressed plasmid constructs and a conditional knockout cell line, a number of technical challenges were encountered, and the inhibitory effect of Rac1 was found to be inconsistent, with low reproducibility. Despite considerable effort, I was unable to resolve this discrepancy using the currently available techniques. Consequently, although the data hints of a role for Rac1 in the innate antiviral response, its precise contribution remains unclear.

3.3 Results

3.3.1 Chemical inhibition of Rac1 inconsistently disrupts antiviral signaling

To begin to investigate the potential role of Rac1 in the innate antiviral response, the ability of a chemical Rac1 inhibitor known as NSC23766 (575) to disrupt the production of ISG-15, one of the subset of ISGs produced during the IFN-independent response (101), in response to SeV was investigated. HEL cells were treated with either a high dose of replicating SeV, which would be expected to induce IFN-dependent signaling, or a low dose of non-replicating virus, SeV-UV (that had had its genome inactivated with UV light), which would active the IFN-independent response (9, 103). ISG-15 production was then determined via immunofluorescence. Interestingly, initial results suggested that Rac1 inhibition may, in fact, impair only the response to SeV-UV but not SeV (figure 3.2A). This appears to implicate Rac1 in IFN-independent signaling, required for the detection of SeV-UV, which enters the cell but does not replicate, but not SeV, which produces several PAMPs during replication and thus activates various PRRs required for IFNα/β production. However, further study demonstrated that these results were very difficult to reproduce. For example, in some cases, the ISG-15 response was decreased in drug-treated cells after challenge with replicating but not non-replicating SeV (figure 3.2B), a direct contradiction of the previous results.

To determine whether this variability was an artifact of the immunofluorescence technique, I examined the effect of NSC23766 on the expression of ISG-56, another member of the IFN-independent subset (101), using RT-PCR. Initial observations suggested that Rac1 inhibition impaired both the response to replicating viruses (two different strains of ICP0-null HSV-1) and non-replicating viruses (SeV-UV as well as UV-inactivated vesicular stomatitis virus (VSV-UV)) (figure 3.3A). However, once again this effect was found to be inconsistent, with other replicates showing no effect of NSC23766 treatment on either SeV or SeV-UV (figure 3.3B). This poor reproducibility precludes interpretation of this data, although the results may suggest some involvement for Rac1 in the antiviral response.

3.3.2 siRNA-mediated Rac1 knockdown intermittently suggests Rac1 has a role in the negative regulation of IFNα/β signaling

To attempt to resolve this variability, I used siRNA-mediated knockdown of Rac1 as an alternative strategy to investigate the potential function of Rac1 in antiviral immunity. Rac1 protein levels were monitored via Western blotting in HEL cells transfected with Rac1 or control siRNA, which were reproducibly found to be lowest by day 4 after Rac1 siRNA transfection (figure 3.4A). To determine whether Rac1 deficiency had any effect on the antiviral response, HEL cells were mock-treated, or challenged with replicating or non-replicating SeV, pI:C or IFNα, four days after siRNA transfection. Unexpectedly, decreased Rac1 protein levels caused increased production of ISG-15 in both unstimulated (figure 3.4B) and stimulated (figure 3.4C and D) cells. This increase in ISG-15 was very striking, and reached levels not normally observed during routine stimulation. This does not fit with the hypothesized role for Rac1 in the detection of viral entry and the IFN-independent response. Instead, this suggests that Rac1 may decrease the basal expression of ISGs, preventing the activation of antiviral signaling in the absence of an appropriate stimulus, and may also function to limit ISG expression induced by replicating or non-replicating virus, dsRNA, or IFNα/β itself.

In order to interpret these results, it was important to rule out a confounding effect of the siRNA transfection itself. It has previously been shown that siRNA can be detected as foreign dsRNA, leading to the production of IFNα/β (576). In an attempt to avoid this caveat, in these experiments I used Stealth siRNA (Invitrogen), which has been altered with proprietary chemical modifications to prevent its recognition by dsRNA sensors. However, it is possible that particular characteristics of the Rac1 siRNA had rendered this modification ineffective, and that it is the response to the siRNA molecules themselves, as opposed to the depletion of Rac1, that is causing the observed increase in the antiviral response. To test this, I took advantage of the relatively long half-life of the Rac1 protein (577) by examining the antiviral response 24 hours after siRNA transfection. At this time, no change in Rac1 protein levels was observed (figure 3.5A), and so any effects on the antiviral response seen at this time cannot be explained by Rac1 deficiency. To do this, I

used a VSV-GFP protection assay (103), which tests whether supernatant media from siRNA-treated HEL cells can produce a protective antiviral state in Vero cells, which would indicate the presence of biologically active IFNα/β. Vero cells are used because they cannot produce IFNα/β (578-580) or IFN-independent ISGs (503) themselves, but can respond normally to exogenous IFNα/β (581). Therefore, HEL cells were treated with siRNA, and 24 hours post-transfection, the supernatant media was transferred to Vero cells. These cells were subsequently challenged with VSV-GFP (582), an IFN-sensitive virus expressing GFP under the control of a viral promoter. Decreased GFP expression would indicate that an antiviral response has been induced in the Vero cells as a result of IFNα/β produced by the HEL cells. However, no such protection was observed for any of the supernatants tested, suggesting that siRNA transfection does not induce IFNα/β production (figure 3.5B). To test for possible IFN-independent ISG induction by the siRNA, HEL cells were challenged with SeV-UV one day after siRNA transfection, and analyzed for ISG-15 production via immunofluorescence. There was no increase in ISG-15 in the cells that had received the Rac1 siRNA at this time (figure 3.5C), suggesting that the cells are not responding to the transfection event itself. Instead, this argues that the observed phenotype correlates with Rac1 levels.

However, although preliminary results using the Rac1 Stealth siRNA seemed to consistently show an increase in antiviral responses in Rac1-deficient cells, further analysis showed this effect to be inconsistent – with a strongly increased antiviral response in the Rac1-deficient cells observed via immunofluorescence in one replicate and no difference seen in the next. To quantify these results, OpenLab software was used to measure the total fluorescence in each image, relative to the number of cells in that image. The fluorescence per cell for the Rac1-deficient cells relative to that of the control cells for each treatment was then determined, and then averaged for 9 independent replicates (figure 3.6A). This clearly shows the tremendous variability of the results, meaning that on average, there was no significant difference between the control cells and the Rac1-deficient cells. Again, this variability was not found to be specific to the immunofluorescence technique, as RT-PCR data confirmed that HEL cells treated with

Rac1 siRNA intermittently show elevated ISG expression after viral challenge (figure 3.6B), while in other cases, no such difference was observed (figure 3.6C).

In an attempt to circumvent this inconsistency in the results, siRNA against Rac1 was purchased from a different manufacturer, Dharmacon. Transfection and challenge were performed in the same manner as the Invitrogen siRNA. Unfortunately, excessive variation was again observed (figure 3.7), with Rac1-deficient cells showing an intermittently increased response when unstimulated or treated with IFNα. In addition, this reagent resulted in pronounced toxicity, in both control and Rac1 siRNA-treated cells, making the results difficult to interpret.

3.3.3 Overexpression of Rac1 mutant constructs supports a role for Rac1 in the suppression of innate signaling but is also limited by high variability

The siRNA experiments showing an enhancement in antiviral signaling in the absence of Rac1 were very interesting, and had occurred sufficiently often to be conceivable, yet the poor reproducibility of these results precluded drawing any clear conclusions from these data. Therefore, I sought another tactic to investigate this phenomenon. To do so, I overexpressed Rac1 plasmid constructs in Vero cells that had been engineered to stably express human IRF3 (Vero-IRF3) (503). Like the parental Vero cells, these cells cannot produce IFNα/β (578-580), but they do express ISGs in response to some stimuli, including replicating virus and dsRNA (503). These cells are useful because unlike HEL cells, they do not produce a confounding antiviral response when transfected with plasmid DNA, but they are capable of expressing ISGs when treated with an appropriate stimulus. The Vero-IRF3 cells were transfected with one of three plasmids encoding GFP-tagged Rac1 constructs – wildtype (WT) Rac1, a constitutively active (CA) mutant, and a DN mutant. The transfection efficiency of the Vero-IRF3 cells with the Rac1 plasmids was found to be low – despite extensive optimization, less than 10% of the cells were positive for GFP (figure 3.8A). Despite this, Vero-IRF3 cells expressing CA-Rac1 had lower expression of ISG-56 after challenge with SeV (figure 3.8B). This is the reciprocal of the siRNA results – the knockdown of Rac1 increased ISG expression,

whereas elevated Rac1 activity decreased ISG expression. No difference in ISG-56 expression was observed in cells transfected with the WT or DN Rac1 constructs, but the low transfection efficiency of these cells makes it is difficult to draw definitive conclusions, particularly because DN mutants, which act by sequestering GEFs, must be expressed at high levels to produce their effects (583). However, repeated experiments revealed that once again, this effect was inconsistent between replicates (figure 3.8C).

HEK293 cells are widely used for overexpression studies due to their ease of transfection, which has been suggested to result from the absence of DNA sensor IFI16 in this cell type (43). As a transformed cell line (584), these cells are not ideal for the study of innate immunology, as a common feature in the immortalization or transformation process seems to be the disruption of antiviral signaling pathways and their associated antiproliferative effects (585-587). Indeed, HEK293 cells have been previously shown to have an impaired antiviral response (588). However, these cells did respond to IFNα/β with the production of ISG-56 (see below), and so the effect of the Rac1 plasmid constructs on antiviral signaling was tested in this cell type. As expected, the transfection efficiency was greatly enhanced in HEK293 cells (figure 3.9A). However, although initial experiments showed an effect that matched previous results, with a decrease in ISG production in cells expressing WT or CA-Rac1 and an increase in cells with DN-Rac1 (figure 3.9B), these effects were small, and did not correlate with the increased transfection efficiency observed in this cell type. Additionally, as with previous techniques, such trends were found to be inconsistent between replicates (figure 3.9C).

3.3.4 Conditional Rac1-deficient dermal fibroblasts are unsuitable for studies of the antiviral response due to the confounding effects of the inducing agent, tamoxifen

Because the various experimental manipulations of Rac1 used thus far had produced such inconsistent results, an alternative approach was clearly needed. It has been suggested that Rac1 knockout cells represent an improvement over traditional techniques for the study of Rac1 (589). Although Rac1 deficiency is embryonic-lethal (590), Rac1 conditional knockout mice, with a fibroblast-specific deletion of Rac1, have

been described (591). Therefore, I used Rac1-null fibroblasts as a final strategy to investigate the potential role of Rac1 in antiviral immunity. To do this, dermal fibroblasts derived from explants of the Rac1 conditional knockout mice were obtained. These cells have the Rac1 gene flanked by LoxP sites, and also express a tamoxifen-dependent Cre recombinase under the control of a fibroblast-specific promoter, meaning that the Rac1 gene can be deleted by treatment with tamoxifen (592).

Prior to using the Rac1 conditional knockout cells, I initially characterized the antiviral response in wildtype dermal fibroblasts, which were found to respond normally to challenge with a variety of stimuli by expressing ISG-56 (figure 3.10A). I next examined the effect of tamoxifen treatment itself on antiviral signaling, in wildtype cells lacking the Cre recombinase transgene. Tamoxifen is an antagonist of the estrogen receptor, and there is some evidence to suggest that this drug may have an effect on the IFNα/β response (593-596). Therefore, I treated wildtype dermal fibroblasts with tamoxifen, and then challenged with various stimuli, and found that tamoxifen treatment itself impaired ISG-56 expression in response to both replicating and non-replicating SeV as well as the synthetic dsRNA analogue polyinosinic/polycytidylic acid (pI:C) (figure 3.10B). This decreased response persisted at least 12 days after tamoxifen had been removed, although responsiveness partially recovered over time (figure 3.10C). Since dermal fibroblasts are primary cells, they do not grow in culture beyond 3-4 passages, and so a further delay between tamoxifen treatment and challenge is not possible. Given the effect of tamoxifen itself on the antiviral response, the use of the conditional Rac1 knockout cells was not pursued further, as it would be difficult to differentiate between the effect of Rac1 knockout and the contribution of tamoxifen treatment itself.

3.4 Discussion

As a protein implicated in both the entry process of multiple enveloped viruses, as well as in the activation of innate immune signaling, Rac1 appeared to be an excellent candidate for a possible role in the activation of the IFN-independent response. Indeed, initial work with the Rac1 chemical inhibitor suggested that Rac1 may be involved in producing the antiviral response to low levels of non-replicating virus, but not to IFN-inducing levels of replicating virus, suggesting a potential function for Rac1 in IFN-independent signaling. However, further study using siRNA against Rac1 as well as overexpressed Rac1 mutant constructs implied an opposing possibility – that Rac1 may, in fact, have a role in the negative regulation of antiviral responses, decreasing both basal signaling in unstimulated cells as well limiting the response to both replicating and non-replicating virus, dsRNA and IFNα itself. However, all of this data was beset with excessive variability, and these effects could not be reproducibly demonstrated, which precludes me from drawing any definite conclusions from these results. An attempt to overcome the drawbacks of classic techniques for Rac1 investigation, which are increasingly being found to have serious caveats, using conditional Rac1-defecient fibroblasts was hindered by a technical problem, with the reagent required for the deletion of Rac1 also disrupting antiviral signaling. Therefore, although it remains entirely possible that it may play a role in innate antiviral immunity, at this time I can neither conclusively exclude nor confirm the participation of Rac1 in this process.

3.4.1 Pitfalls in classic techniques for the investigation of Rac1

The excessive variability observed during the course of this investigation may be due, at least in part, to the use of flawed techniques. For example, NSC23766 has received criticism (597) because of its mechanism of action, which involves blocking the GEF-recognition groove of Rac1 (575). Importantly, this drug does not prevent the interaction of Rac1 with some GEFs that bind to Rac1 outside of this region, such as Vav1, and thus it cannot prevent the activation of Rac1 under all circumstances (575). Additionally, a caveat of all small molecule inhibitors is that they are prone to off-target

effects (598), meaning that the outcome observed may not be directly caused by the inhibition of Rac1, and could instead result from the unintended targeting of other proteins with roles in antiviral signaling. Therefore, not only could this drug be failing to block the activation of Rac1 by the specific stimulus involved in its negative regulatory mechanism, explaining why the increase in ISG expression observed with the siRNA and DN-Rac1 construct was not seen during drug treatment, but the observed inhibition of antiviral responses that did occur during NSC23766 treatment could be caused by the unintended interaction of the drug with an alternative target. For example, though NSC23766 was found not to interact with the two of the best characterized members of the Rho GTPase family, RhoA and Cdc42 (575), there are 22 members of this family, some of which, like RhoG, have similar structures to Rac1, and have not been shown to be resistant to NSC23766 binding (reviewed in (599)). A completely unrelated protein may also happen to have a region that unexpectedly interacts with the drug. The fact that NSC23766 is routinely used at a relatively high concentration of 100μM (575) may enhance the risk of this, as it has been suggested that drug concentrations greater than 10μM should be avoided, to minimize such off-target binding (600). Finally, the mechanism of action of NSC23766 means that it cannot inactivate CA-Rac1 (575). This is relevant because an alternative splice variant of Rac1, known as Rac1b, does not require GEFs for its activation and is thus predominantly found in cells in an activated form (601). Rac1b cannot activate all Rac1 effectors (602), but it is capable, for example, of inducing NFκB (603). Since Rac1 was found to inhibit antiviral signaling even in unstimulated cells, it is possible that such effects could be mediated by Rac1b, which would not require a signal to activate it to repress ISG production, and which would not be inhibited by NSC23766.

It is important to note that like chemical inhibitors, siRNA reagents are also susceptible to off-target effects. RNA interference occurs when the double-stranded siRNA molecule interacts with Argonaute proteins to form the RNA-induced silencing complex (RISC) (reviewed in (604)). One siRNA strand is then thermodynamically chosen as the “guide” strand, which is antisense to the targeted mRNA and thus drives its

sequence-specific recognition and subsequent cleavage. The other strand is thought to be quickly lost or degraded. However, if this "sense" strand is used as the guide strand instead, it can lead to the targeting of an undesired mRNA to which it has homology, resulting in the knockdown of an unexpected protein. Additionally, although it was initially thought that siRNA targeting was highly sequence-specific (605), others have reported siRNA-mediated decreases of mRNAs with limited sequence similarity to the antisense siRNA (606), in a mechanism that is not fully understood. However, in my study, such effects were reduced through the use of siRNA that had been chemically modified in such a way as to ensure that only the desired antisense strand enters the siRNA pathway (607). Off-target effects of the antisense strand were also reduced, by additional chemical modification in the case of Dharmacon, or the use of proprietary algorithms that perform mismatch alignment searches for the design of the siRNA sequence for Invitrogen. To further decrease non-specific interactions, the reagents obtained from both manufacturers consisted of pools of more than one siRNA sequence targeting Rac1. Since each individual siRNA duplex in these pools targets the same mRNA, their effects on Rac1 levels will be additive, and thus each can be used at a lower concentration, meaning their off-target effects, which are presumably different for each sequence, will therefore be correspondingly smaller (reviewed in (608)).

Although the potential for off-target effects of siRNA may have been minimized, this reagent did present a problem that is more specific to the investigation of Rac1. Because of the relatively long half-life of Rac1 (577), I found that Rac1 protein levels did not reach their lowest point until 4 days post-infection. In order to avoid excessive confluency, which can impair viral infection (data not shown), cells were seeded at a low density and allowed to grow to confluence over the course of the experiment. However, Rac1 has been shown to be required for cell cycle progression (537). Therefore, as Rac1 protein levels decrease, so does cellular growth, and thus the Rac1-deficient cells reached lower densities than the control-treated cells. Although these differences in cell numbers were accounted for in MOI calculations, variations in cell-cell contacts can influence many aspects of cellular behaviour – including the protein levels and activation state of

Rac1 itself (see below). It is possible that this is involved in the observed differences in the innate response between the control and Rac1-deficient cells. Slight deviations in cellular growth rate and transfection efficiency between experiments meant that the exact confluency achieved by the cells that received the Rac1 siRNA fluctuated somewhat between experiments, adding a further level of variation.

Another technique more specific to the analysis of Rho GTPases that has recently been called into question is the use of overexpressed Rac1 mutants. One issue is that there are only three RhoGDIs in humans, with different expression patterns in different tissue types, which regulate all of the Rho GTPase family members (reviewed in (543)). A RhoGDI forms a 1:1 complex with a Rho GTPase, and the total number of RhoGDIs in a cell is roughly equal to the combined amounts of all Rho GTPases (609). Therefore, RhoGDIs are limiting, and the overexpression of any particular Rho GTPase will result in increased competition for the RhoGDIs, resulting in the release of some of the endogenous proteins – something that will affect all members of the Rho GTPase family. This has been found to result in both the degradation of a large proportion of the released endogenous proteins as well as the inappropriate activation of the remainder, meaning that signaling pathways affected by all Rho GTPases can be disrupted by the overexpression of only one (610). Similarly, the overexpression of DN mutants of Rac1 in particular presents an additional problem. Such mutants have an increased affinity for GEFs but decreased binding to GTP, and so they sequester GEFs needed for the activation of the endogenous protein (611). However, many GEFs interact with more than one Rho GTPase, and so this strategy not only blocks Rac1 activation but also the function of additional Rho GTPases (612, 613). This problem is clearly demonstrated by the following example: initial studies using DN-Rac1 suggested that Rac1 is required for chemotaxis (614), while a more recent study using Rac1-null cells found no impairment in their chemotaxis (589). However, the expression of DN-Rac1 in the Rac1-null cells strongly disrupted chemotaxis (589) – meaning that however this construct produced its effect, it was not via Rac1 itself, demonstrating how the use of DN GTPases can generate misleading results. Therefore, chemical inhibition, siRNA treatment, and protein

overexpression can all produce effects on proteins unrelated to Rac1, meaning not only can a function attributed to Rac1 be instead the result of the disruption of unconnected pathways, but also that all of these techniques can influence a large number of uncontrolled variables.

3.4.2 Local versus global regulation of Rho GTPases: a key problem in the investigation of Rac1

The imprecise effect of DN mutants highlights a larger, more fundamental issue encountered when studying Rac1, which may also partially account for the low reproducibility of my experiments. Though a single GEF can indeed activate multiple Rho GTPases, under biological conditions, tight spatial and temporal regulation allow such a promiscuous GEF to specifically induce only one member of the Rho GTPase family (reviewed in (615)). Similarly, a large number of extracellular signals, and therefore multiple GEFs, can activate the same Rho GTPase, which in turn has the ability to activate numerous effectors (reviewed in (547, 548)). However, Rac1 stimulation by a particular GEF does not result in the activation of every one of its effectors – instead, Rac1 can simultaneously be induced in very precise and distinct sites in the cell, where it induces completely independent signaling cascades (616). Indeed, it is thought that at most, only 2-5% of the total available Rac1 is activated by a particular stimulus (617). This specificity is likely achieved through compartmentalization and scaffolding proteins, which allow Rac1 to associate with different GEFs, GAPs and effectors in separate complexes at precise subcellular locations (reviewed in (484, 618)). In this way, the cellular context – meaning not only the cell type involved and the particular signal being transduced, but also the specific intracellular localization and the precise timing of the signal – is crucial in controlling which Rac1-dependent pathway is activated (519, 550, 619). This tight regulation gives Rac1 its ability to modulate such a wide variety of functions, under highly specific conditions (618).

The difficulty that arises from the careful spatio-temporal regulation of Rac1 is that many of the classic techniques used to investigate Rho GTPase activities, including

chemical inhibition, overexpression of Rac1 constructs, siRNA-mediated knockdown, and even conditional Rac1 knockout cells, are relatively "blunt tools" – meaning that they affect the entire population of Rac1 molecules in the cell at once. This broad disruption is, however, not biologically relevant, and completely disrupts the precise regulation of Rac1 that exists in an intact cell. For example, the overexpression of a CA mutant, which is unable to hydrolyze GTP and thus cannot be inactivated, means that activated Rac1 is no longer restricted to a specific intracellular zone, and potentially allows it to induce all downstream effectors simultaneously (reviewed in (618)). This shifts Rac1 regulation from local to global, potentially resulting in artifacts.

Although it has often been proposed that knockdown or knockout cell lines provide an improved strategy for the study of Rac1, such techniques are also prone to producing misleading results. For instance, conclusions about the effect of Rac1 on cell motility differ when Rac1 knockout (589) is used instead of Rac1 knockdown (620). This likely results because the global inhibition or loss of Rac1 disrupts all of the carefully regulated, Rac1-mediated pathways at once. With over 60 GEFs, 70 GAPs and 60 effectors identified for Rho GTPases, it is difficult to identify a cellular process that is not, either directly or indirectly, influenced by the loss of Rac1, particularly given how highly interconnected these proteins are, and their abilities to activate, cooperate with, antagonize or inhibit one another (reviewed in (484, 615)). This is potentially the source of the excessive variability seen in my experiments – techniques that disrupt Rac1 influence a tremendous number of interrelated variables, meaning that even a slight change in conditions may produce an entirely different outcome.

The reciprocal of the above statement is also true – a large number of variables influence Rac1 activation. One practical example of this is that increased cell confluency leads to a strong elevation in Rac1 protein levels and a resulting rise in its activity (621, 622), and so differences in cell density would have significant effects on Rac1 accumulation and function (623). Additionally, Rac1 activation has been previously shown to be highly variable in subconfluent cells (569). Therefore, experiments involving Rac1 are exceedingly sensitive to differences in cell density. Moreover, it has also been

shown that even small changes in the level of Rac1 protein in a cell can have large effects on cellular behavior, including cell shape and motility (620). In this way, a slight alteration in cell density could shift the level of Rac1 protein, in turn modifying various cellular characteristics. Although my experiments were performed, as much as possible, in cells of similar confluency, a primary cell line such as HEL fibroblasts can be prone to slight variations in cellular growth rate with changing conditions, making it difficult to precisely control for cell density. Because Rac1 is so sensitive to minor differences, this could result in increased Rac1 protein levels in one experiment compared to another, meaning not only would the same dose of inhibitor or siRNA not always produce the same relative effect, but also that the cells themselves will behave differently, again potentially explaining some of the variation seen between my replicates.

3.4.3 Improved strategies for the investigation of Rac1

Clearly, the commonly used techniques for the investigation of Rac1, including the strategies employed here, are limited by a number of shortcomings. In some cases, there are relatively simple changes that could potentially improve the results reported in this study. For example, an alternative chemical inhibitor for Rac1 known as EHT 1864 has been designed, although it is not yet commonly used. EHT 1864 disrupts GTP association with Rac1, and thus unlike NSC23766, it presumably prevents the interaction of Rac1 with all effectors (597). This could make the effect of Rac1 inhibition more clear, though it does not resolve the problem of off-target effects of small molecules. Similarly, the problem of the confounding effects induced by tamoxifen could likely be overcome through the use of alternative strategies for inducing the expression of Cre recombinase. A variety of Cre-inducible systems exist (reviewed in (624)), including an IFN-inducible construct, which would clearly not avoid the issue of off-target effects on antiviral signaling, as well as a doxycycline-inducible system, though this drug has also been reported to have antiviral effects (625) as well as to reduce cytokine expression, even at low doses (626). Interestingly, a cell-permeant version of the Cre protein has been described (627), which could solve the problem of unanticipated side effects of small

molecule treatment. However, it is important to note that neither Rac1 inhibition nor Rac1 knockout prevents the issue of the global disruption of Rac1 causing broad effects on a large number of pathways, and thus high variability.

The most successful strategy would likely be one that allows for more precise observations of Rac1 signaling. One such technique employs probes that detect the activation of a particular Rho GTPase *in situ* (reviewed in (618)). For example, an effector domain that binds only to a specific GTP-bound Rho GTPase can be flanked with a different fluorescent label on either side (628). The binding of this construct to the appropriate endogenous Rho GTPase, such as Rac1, can be monitored via fluorescence resonance energy transfer (FRET), which involves the nonradiative transfer of energy from a donor fluorophore to an acceptor fluorophore when the two are in close proximity, due to an overlap of the emission spectrum of the donor and the excitation spectrum of the acceptor (reviewed in (629)). Therefore, when the probe is free in the cytoplasm, the two fluorophores are in close proximity and a decrease in donor and increase in acceptor fluorescence is seen. However, upon Rac1 activation, the effector domain will bind the GTPase and undergo a conformational change, separating the two fluorophores and therefore decreasing the FRET effect. This technique is useful for monitoring the precise location and timing of Rac1 activation in response to a particular stimulus, thus addressing the spatio-temporal aspect of Rac1 activation. However, it does not clarify the exact role for the GTPase in a particular cellular function.

An alternative mechanism that has been widely employed to narrow the effect of Rac1 manipulation is the use of a set of effector domain mutants (541). This involves expressing Rac1 constructs with specific mutations in the effector-binding region, which are incapable of interacting with certain effectors. These mutants are frequently used to identify which effectors have a role in a particular activity of Rac1 – however, in theory, such mutants could also be used to limit the number of downstream pathways affected by the overexpression of CA or DN Rac1 mutants. However, such a strategy still requires transfection and expression of foreign protein, the specific binding site of all effectors are

not characterized and may overlap, and mutations disrupting the binding of all but one effector are not currently known.

Recently, siRNA screens have been described that target all identified Rho GTPase GEFs and GAPs (630-634). This may represent a more biologically relevant strategy, allowing the precise disruption of specific downstream pathways instead of the global inhibition of Rac1 signaling. With over 130 proteins currently known to be directly involved in Rho GTPase signaling, some form of high throughput screen is necessary for such studies. For example, FRET probes can be a useful assay readout, allowing screening with fluorescence microscopy to determine if a particular siRNA treatment prevents the activation of a specific Rho GTPase in response to a stimulus (634). By identifying a GEF or GAP with a role in a particular process, much more exact manipulation of a particular pathway becomes possible, without influencing countless additional processes, making this a much improved mechanism for the study of Rac1. However, such assays are expensive, and require specialized expertise and equipment (reviewed in (635)). Also, new GEFs are still being discovered (636), and so the use of current screens do not guarantee the identification of the proteins required for every Rac1-mediated activity. Therefore, although representing a significant advance over conventional "blunt tool" approaches, even the best of the current techniques do not provide a rapid or straightforward mechanism to investigate the possible function of Rac1 in antiviral immunity.

3.4.4 Could Rac1 be negatively regulating antiviral responses?

Although the excessive variability of the results of the current study makes them far from convincing, the possibility that Rac1 may have activities in the negative regulation antiviral signaling remains intriguing. Interestingly, such an inhibitory role for Rac1 is not without precedent. For example, although Rac1 has been found to positively regulate NFκB activation via TLR2 (485), it has also been reported to negatively regulate NFκB induction through NOD2 (nucleotide-binding oligomerization domain 2), a PRR involved in the detection of intracellular bacteria (637, 638). Because the Rho GTPases

are involved in several aspects of immune defense against bacteria, including the phagocytosis of invading organisms, migration of immune cells, generation of toxic oxygen radicals, and TLR signaling, a large number of bacterial pathogens have evolved mechanisms to inhibit Rho GTPase activity (reviewed in (639, 640)). Therefore, an increase in NFκB activation by a sensor of bacterial PAMPs during the inhibition of Rac1 could provide a cellular counter defense to overcome this method of bacterial immune evasion. Interestingly, dengue virus has been found to inhibit Rac1 early during infection (493), though the significance of this is not yet clear.

Rac1 may also play a role in the routine negative regulation of antiviral signaling. Cells have a variety of strategies to prevent IFNα/β signaling from being activated to harmfully high levels and to terminate the response after viral clearance (reviewed in (641)). For example, several cellular proteins are involved in targeting components of IFN-mediated signaling for degradation via the proteasome, including Pin1(642), RNF125 (643), Ro52 (644), RBCK1 (645) and RNF5 (646). Others, such as DUBA (647), CYLD (648) and A20 (649), have functions in preventing or removing the post-translational modification of Lys 63 ubiquitin chains, which play a role in activating several signaling proteins in the antiviral response. Still other negative regulators physically associate with various constituents of the antiviral response and prevent their activity, including LGP2 (650), DAK (651), Atg5–Atg12 (652), NLRX1 (653), PSMA7 (654), ISG-56 (96) and SIKE (655). It is entirely possible that Rac1 has a function in the activation of any of these proteins, or in as-yet-uncharacterized aspects of the negative regulation of antiviral immunity.

An activity of Rac1 in decreasing ISG production contradicts the original study demonstrating the involvement of Rac1 in the activation of IRF3 (489). However, as mentioned earlier, that study relied on somewhat outdated techniques and their conclusions remain controversial. Indeed, the results of this initial report have been challenged by others – for example, CA-Rac1 has more recently been found to be unable to activate IRF3 (569). Additionally, while the original study suggested that the Rac1 effector Pak1 may have a role in inducing IRF3-mediated signaling (489), further study

suggested that Pak1 was not essential for the production of ISGs after viral infection (109). Therefore, the conclusions of the initial paper may not be entirely reliable.

A final explanation for my results comes from a quirk of IFNα/β signal transduction. It has been found that clathrin-dependent endocytosis of IFNAR occurs upon IFNα/β binding, and blocking this effect decreases JAK-STAT signaling (656). Interestingly, Rac1 negatively regulates clathrin-dependent endocytosis (657), and the reduction of endogenous Rac1 enhances clathrin-mediated endocytosis (658). In this way, the resulting increase in IFNAR internalization in the absence of Rac1 could explain the enhanced antiviral response observed here.

3.4.5 What can be learned from the Rac1 project?

Bill Gates once said that "It is fine to celebrate success, but it is more important to heed the lessons of failure." What, therefore, can be learned from the Rac1 project? My consistently inconsistent results prevent drawing any definitive conclusions about the potential involvement of Rac1 in antiviral responses. However, this does not mean that this undertaking was entirely fruitless, so long as it can be used to improve future work. Accordingly, the first lesson to be taken away from this study is that a target for a molecular investigation must be chosen carefully. Although there was compelling evidence to suggest Rac1 could play a role in IFN-independent signaling, its direct and indirect participation in countless basic cellular processes made it a poor candidate for analysis. This is particularly true when studying the endogenous antiviral responses in primary cultured cells – a system that is already inherently sensitive to subtle variations (an unpublished observation of many in our group). A better strategy would be to analyze the effector proteins that truly produce the functions of Rac1, which provides a more targeted and therefore likely a more rewarding mechanism to understand Rac1-mediated processes. This concept does not apply only to Rho GTPases, but instead is an important consideration when beginning an investigation of any cellular process. As opposed to attempting to monitor the phenotype observed after disrupting a crucial signaling hub that impacts large numbers of different processes, which results in a variety of confounding

effects, it is better to concentrate on downstream proteins that are more intimately associated with the pathway of interest.

Another key lesson to be learned from the Rac1 project is that the specific techniques used in an investigation must also be chosen carefully. The drawbacks of overexpressed mutants and chemical inhibitors are broadly applicable and bear careful consideration during experimental design. Additionally, during this investigation it became clear that the conventional RT-PCR technique was not suitable for examining subtle differences in ISG induction. Although useful for revealing whether a particular treatment induces ISGs, compared to one that does not, conventional RT-PCR is, at best, a semi-quantitative technique, and cannot be used to accurately compare the degree of ISG induction between samples. This is because the readout of this method is the visualization of band intensity of PCR products after ethidium bromide staining of agarose gels, which cannot resolve differences of less than ten-fold between samples (reviewed in (659)). Also, because this method measures the total amount of PCR products generated at the end of the PCR cycle, after the reaction has stopped due to limiting reagents, it shows elevated variability, which could, once more, explain some of the poor reproducibility seen in my investigation. Therefore, for the remainder of the thesis, I have instead used quantitative RT-PCR via the Taqman system. Although not without its own caveats, this technique can detect as little as a 2-fold difference between samples, and because quantification is performed during the exponential amplification phase of the PCR cycle, it shows greatly decreased variability, making it more suitable for my investigations (reviewed in (660)).

The final lesson to be learned from the Rac1 project is less tangible – and that is knowing when to stop. The intermittent positive results obtained during this investigation made it difficult to determine whether further optimization or different approaches could resolve the low reproducibility observed. Although perseverance is often rewarded, it is also possible to drift into nonproductive persistence on an insoluble problem (661). There is clearly no definitive answer to the question of when to continue to struggle, and when to move on (662). However, the analysis performed here, using four different techniques

and countless attempts to standardize experiments and reduce variability, is likely sufficient to conclude that this line of research will not produce publishable results. Although it is possible that with sufficient further work, this investigation may have eventually yielded reproducible data, and though it is costly and difficult to abandon a project in which I have invested significant effort, both time and resources are finite, and the need to focus my attention on a more attainable goal was apparent (663). However, the lesson learned here is that it is far better to quickly determine whether a project is likely to be productive, and if not, to move on, before a significant amount of time has been devoted to it.

In conclusion, I present some data to suggest that Rac1 activity dampens the innate antiviral response to infection, both in the absence and the presence of stimulation. However, although this response was investigated using multiple distinct techniques, high levels of variability means that no absolute conclusions can be drawn from this data. This likely resulted from a combination of problems, including off-target effects of the reagents used in this investigation, the involvement of Rac1 in a tremendous variety of interconnected cellular processes, and the limitations of the techniques used to monitor the activation of the antiviral response. Although this investigation did not produce publishable results, several important lessons were learned, which will be implemented in the design and execution of my future studies.

3.5 Acknowledgements

I thank Dr. Andrew Leask for providing the conditional Rac1 deficient dermal fibroblasts, and Dr. Ali Ashkar for providing the wildtype dermal fibroblasts.

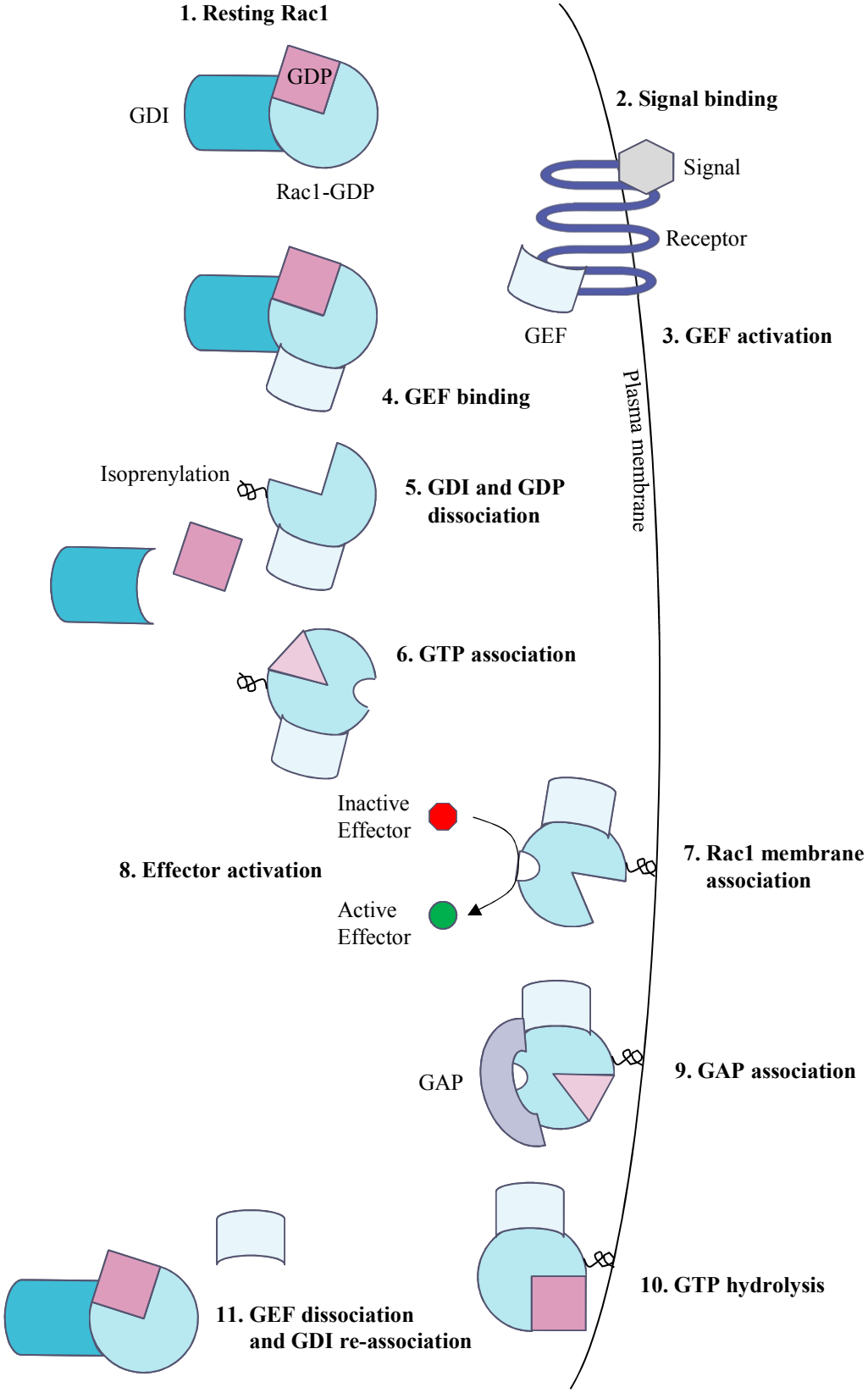
1. Resting Rac1
GDP
GDI
Rac1-GDP
2. Signal binding
Signal
Receptor
GEF
3. GEF activation
Plasma membrane
4. GEF binding
Isoprenylation
5. GDI and GDP dissociation
6. GTP association
Inactive Effector
8. Effector activation
Active Effector
7. Rac1 membrane association
GAP
9. GAP association
10. GTP hydrolysis
11. GEF dissociation and GDI re-association

Figure 3.1: The Rac1 GTPase activation cycle. In resting cells, Rac1 is found in the cytoplasm, associated with GDP and the GDP dissociation inhibitor (GDI) (1). The induction of the appropriate signaling pathway (2) leads to the activation of a guanine nucleotide exchange factor (GEF) (3), which then associates with Rac1 (4), releasing it from the GDI (5) and catalysing the exchange of GDP for GTP (6). This reveals the isoprenylated C-terminus of Rac1, allowing it to associate with the plasma membrane (7). Rac1 then interacts with its downstream targets, activating various effector pathways (8). Termination of signaling is achieved by the association of a GTPase-activating protein (GAP) (9), which increases the intrinsically low rate of GTP hydrolysis of Rac1 (10), leading to its reassociation with the GDI and its relocation to the cytoplasm (11).

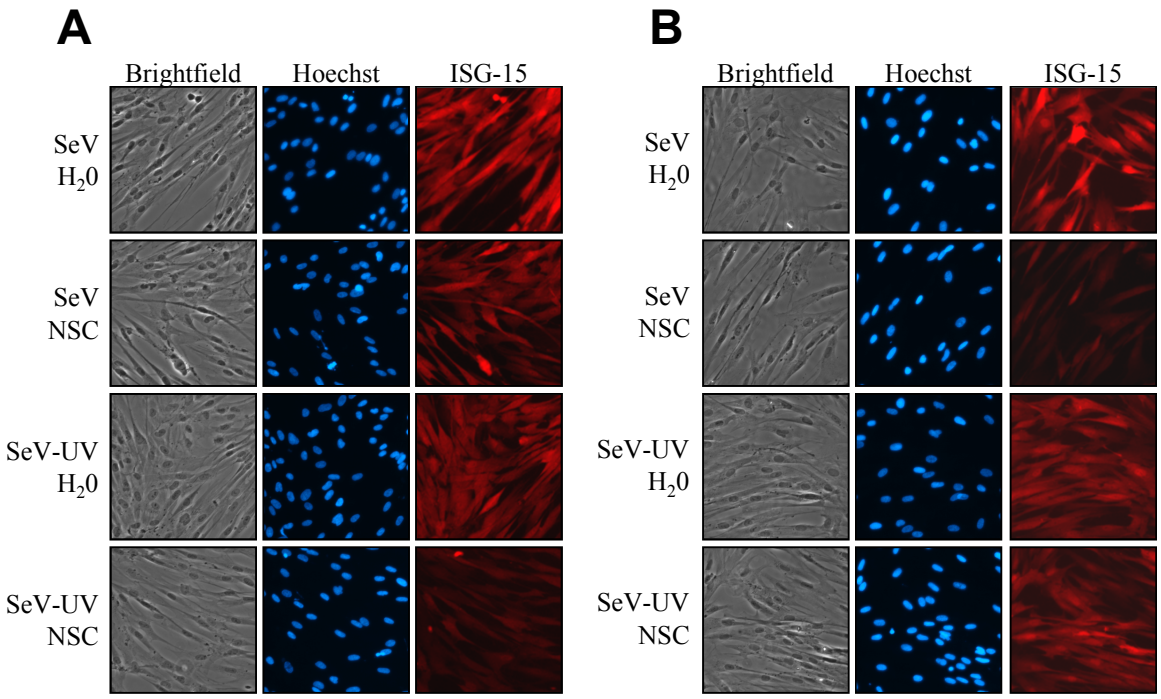

Figure 3.2: The effect of Rac1 chemical inhibition on ISG-15 protein expression is poorly reproducible. HEL cells were pretreated with 200µM NSC23766/water for 30 minutes prior to infection. Cells were mock-treated or infected as indicated with SeV (80 HAU/10^6 cells) or SeV-UV (20 HAU/10^6 cells) in serum-free media with 200µM NSC23766/water for 1 hour. Fresh media was then added for 24 hours before cells were fixed, permeabilized and analyzed for ISG-15 via immunofluorescence. Nuclei were stained with Hoechst dye. (A) and (B) are independent replicates of the same experiment.

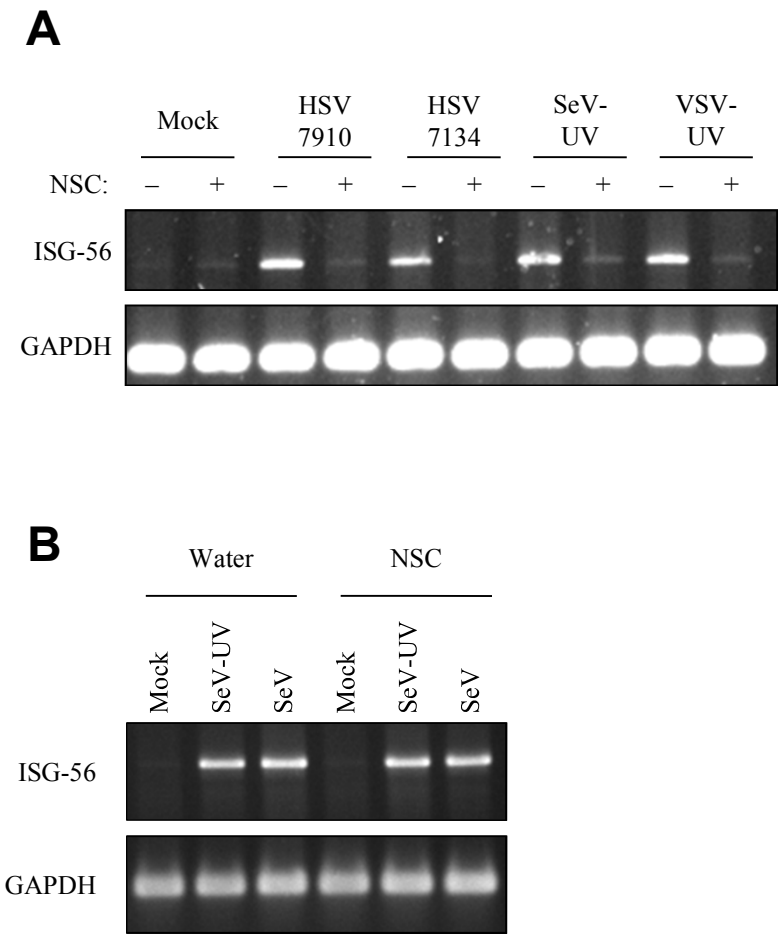

Figure 3.3: The effect of Rac1 inhibition on ISG-56 mRNA expression is also poorly reproducible. HEL cells were pretreated with 200μM NSC23766/water for 30 minutes prior to infection. Cells were mock-treated or infected as indicated with HSV ICP0 mutants, 7910 and 7134 (10 pfu/mL), SeV (80 HAU/10^6 cells), SeV-UV (20 HAU/10^6 cells) or VSV-UV (10 pfu/mL) in serum-free media with 200μM NSC23766/water for 1 hour. Fresh media was then added for 6 hours before RNA was harvested and analyzed for the expression of ISG-56 and GAPDH via RT-PCR. (A) and (B) represent independent experiments.

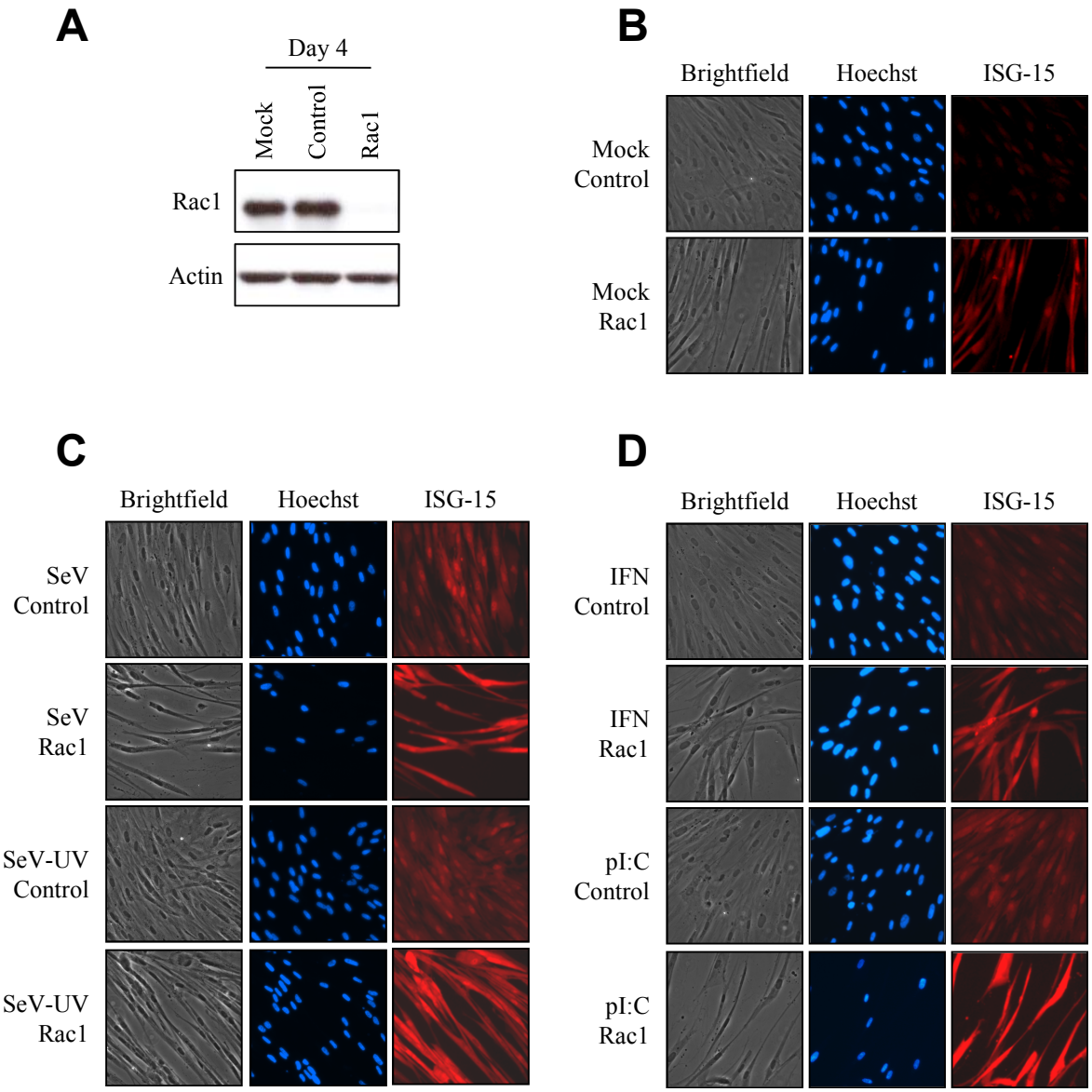

Figure 3.4: Rac1 siRNA treatment increases the antiviral response. HEL cells were transfected twice with 100nM Rac1 or control Invitrogen siRNA, cultured for 4 days, then either harvested for protein and analyzed for Rac1 and actin levels via Western blotting in (A), or analyzed via immunofluorescence 24 hours after mock treatment in (B), infection with replicating or non-replicating SeV in (C), or treatment with IFNα or pI:C in (D). Nuclei were stained with Hoechst dye.

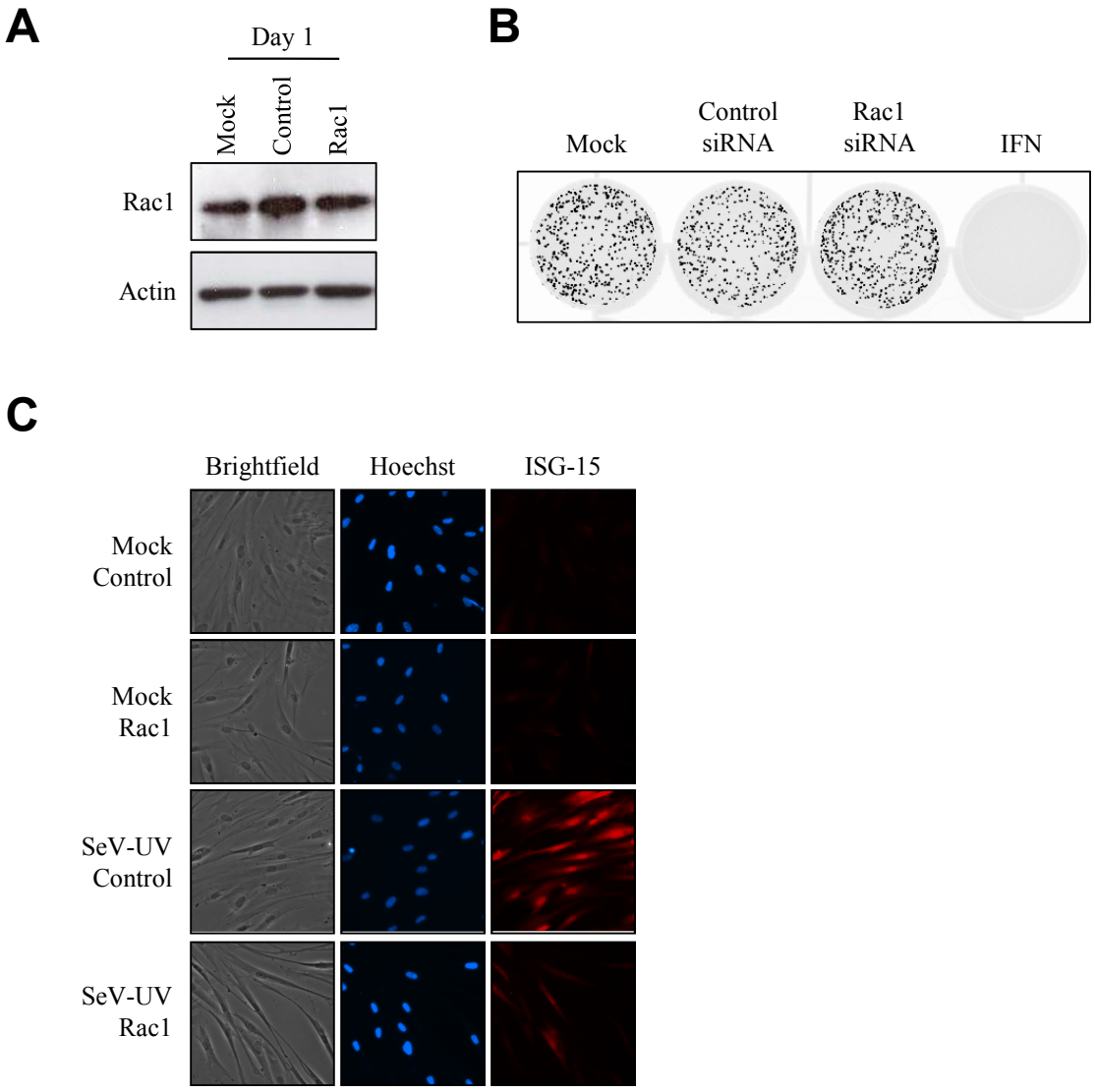

Figure 3.5: Stealth siRNA transfection itself does not induce the antiviral response. HEL cells were transfected twice with 100nM Rac1 or control Invitrogen siRNA and cultured for 24 hours. In (A), protein was then harvested and analyzed for Rac1 and actin levels via Western blotting. In (B), the supernatant media was removed and transferred to Vero cells for 24 hours, which were then challenged with VSV-GFP for 24 hours and then visualized for GFP expression via the Typhoon scanner. In (C), cells were analyzed via immunofluorescence for ISG-15. Nuclei were stained with Hoechst dye.

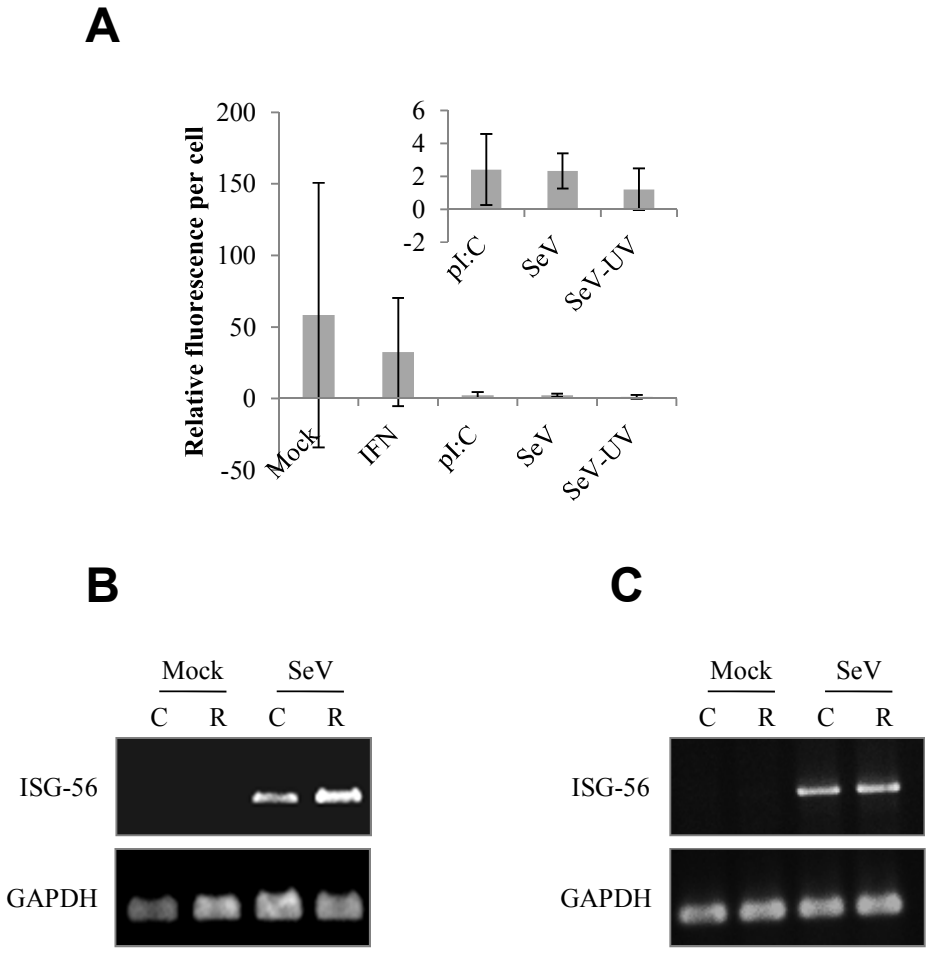

Figure 3.6: The effect of Rac1 deficiency on ISG expression is poorly reproducible. HEL cells were transfected twice with 100nM Rac1 (R) or control (C) siRNA from Invitrogen. 4 days after transfection, cells were mock-treated or challenged with SeV, SeV-UV, IFNα or pI:C. In (A), immunofluorescence for ISG-15 was performed 24 hours after challenge. Quantification of ISG-15 was then performed from the immunofluorescence images. Total fluorescence due to ISG-15, and total cell number (as determined by Hoechst staining of nuclei), were quantified for each field of view with OpenLab software, and used to determine the fluorescence per cell (F) value for each treatment. 3 fields of view were quantified to create an average for each treatment. Results are shown as the ratio between the Rac1-deficient and control cells ($F_{Rac1}/F_{control}$). Results are shown graphically as the average of 9 independent replicates. In (B) and (C), RNA was harvested 6hpi and analyzed for ISG-56 and GAPDH expression via RT-PCR. (B) and (C) represent two independent replicates of the same experiment.

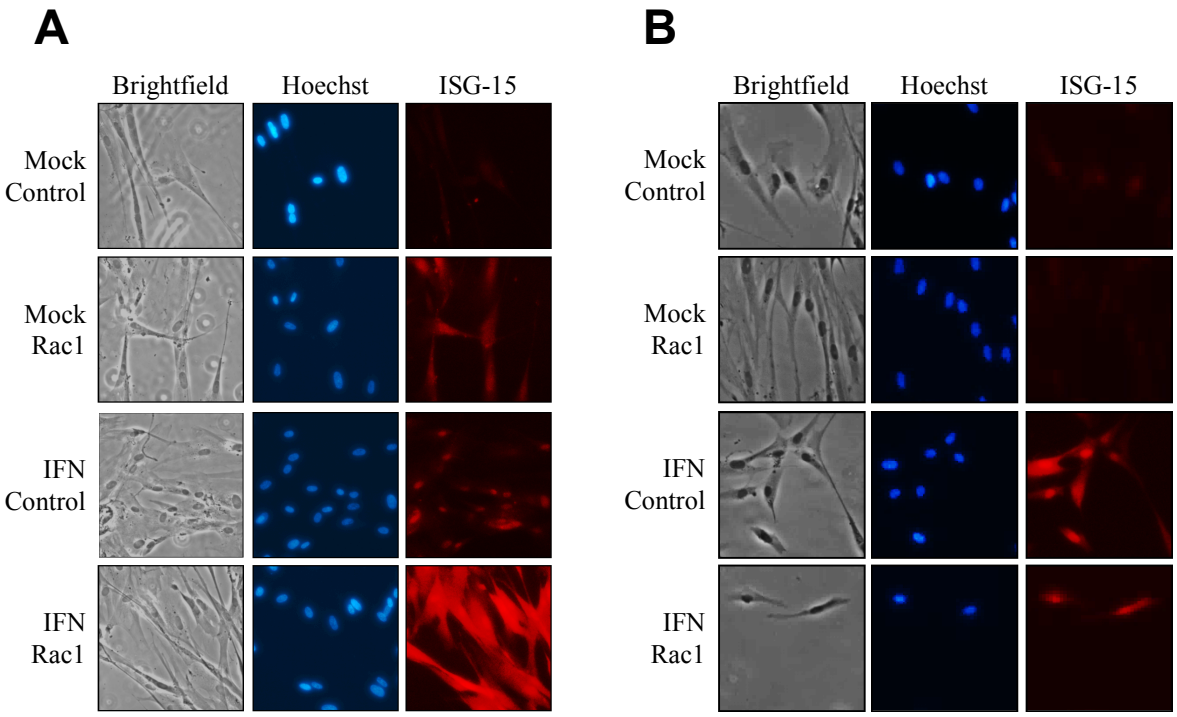

Figure 3.7: Use of Dharmacon siRNA to decrease Rac1 protein levels also results in an intermittent effect on ISG-15 expression. HEL cells were transfected with 150nM Rac1 or control siRNA from Dharmacon. 4 days after transfection, cells were mock-treated, or challenged with IFNα. Immunofluorescence for ISG-15 was performed 24 hours after challenge. Nuclei were stained with Hoechst dye. (A) and (B) are independent replicates of the same experiment.

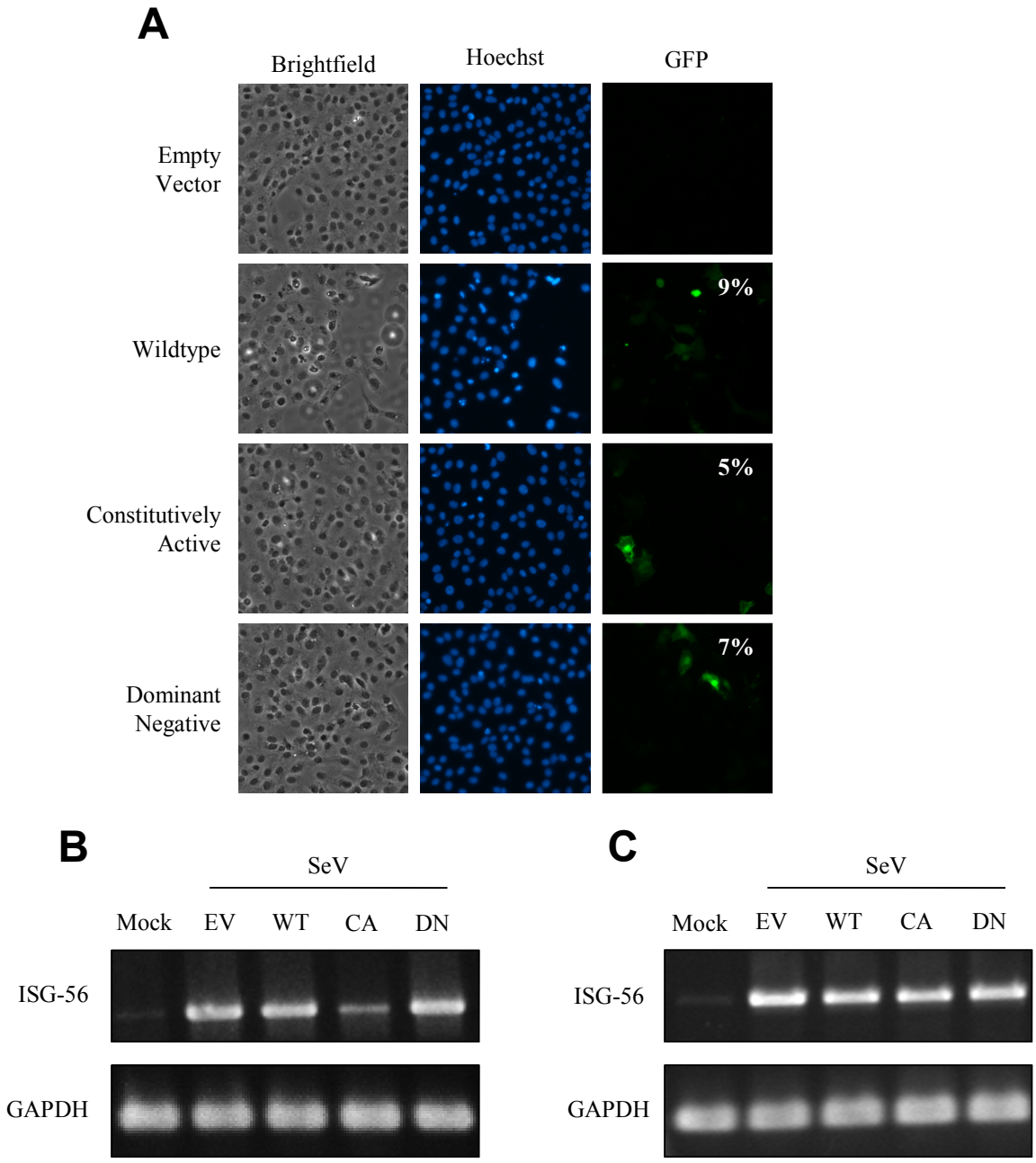

Figure 3.8: Overexpression of Rac1 mutant constructs in Vero-IRF3 cells produces an intermittent effect on ISG-56 expression. Vero-IRF3 cells were transfected with 2.5µg per well of a 12 well dish in (A) or 5µg per well of a 6 well dish in (B) with plasmids expressing various GFP-tagged Rac1 constructs, as indicated. In (A), GFP fluorescence was determined 48 hours later, and the number of positive cells relative to the total number of cells, as counted via Hoechst staining of nuclei, was determined. In (B) and (C), cells were infected with SeV 48 hours after transfection, and then RNA was harvested 8hpi. RT-PCR was then performed for ISG-56 and GAPDH. (B) and (C) are independent replicates of the same experiment.

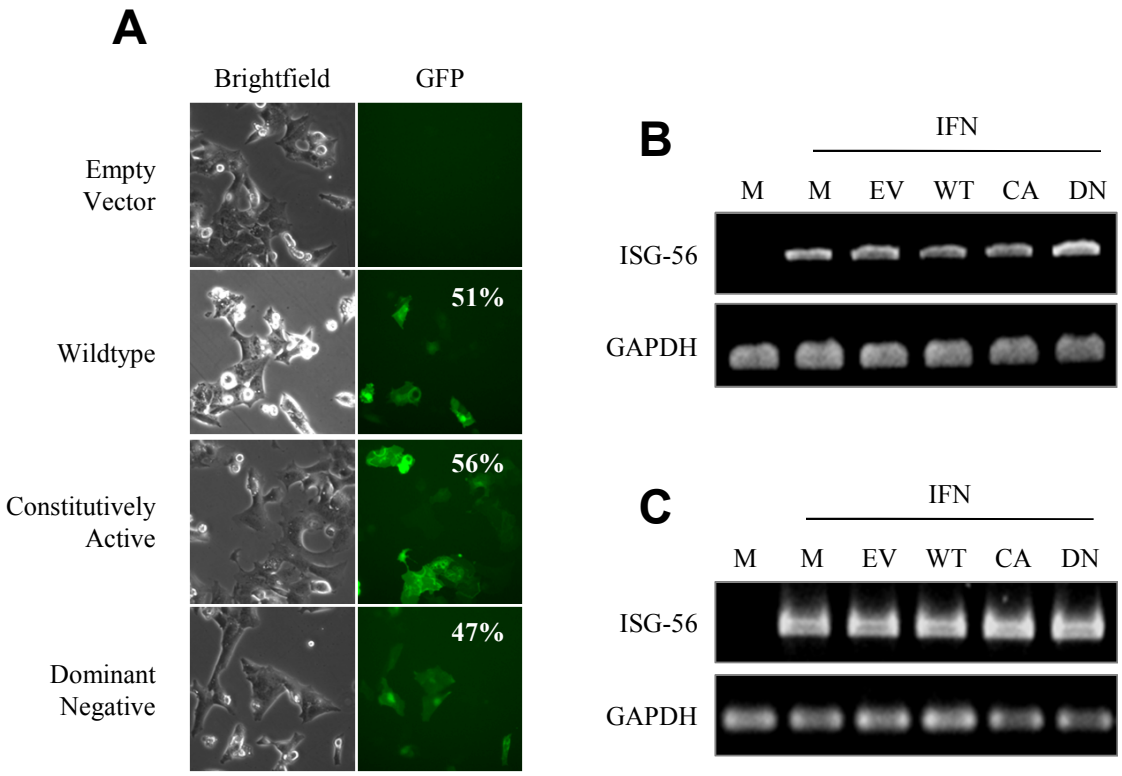

Figure 3.9: Increased transfection efficiency of Rac1 constructs in HEK293 cells does not clarify the effect of Rac1 on the antiviral response. HEK293 cells were mock-treated (M), or transfected with 3µg of the empty vector (EV) or the indicated Rac1 constructs, per well of a 6-well dish. In (A), GFP expression was determined 24 hours post-transfection. (B) and (C) are independent replicates of the same experiment, where cells were challenged with IFNα 24 hours after transfection, and RNA was harvested 8 hours later, with ISG-56 or GAPDH expression determined via RT-PCR.

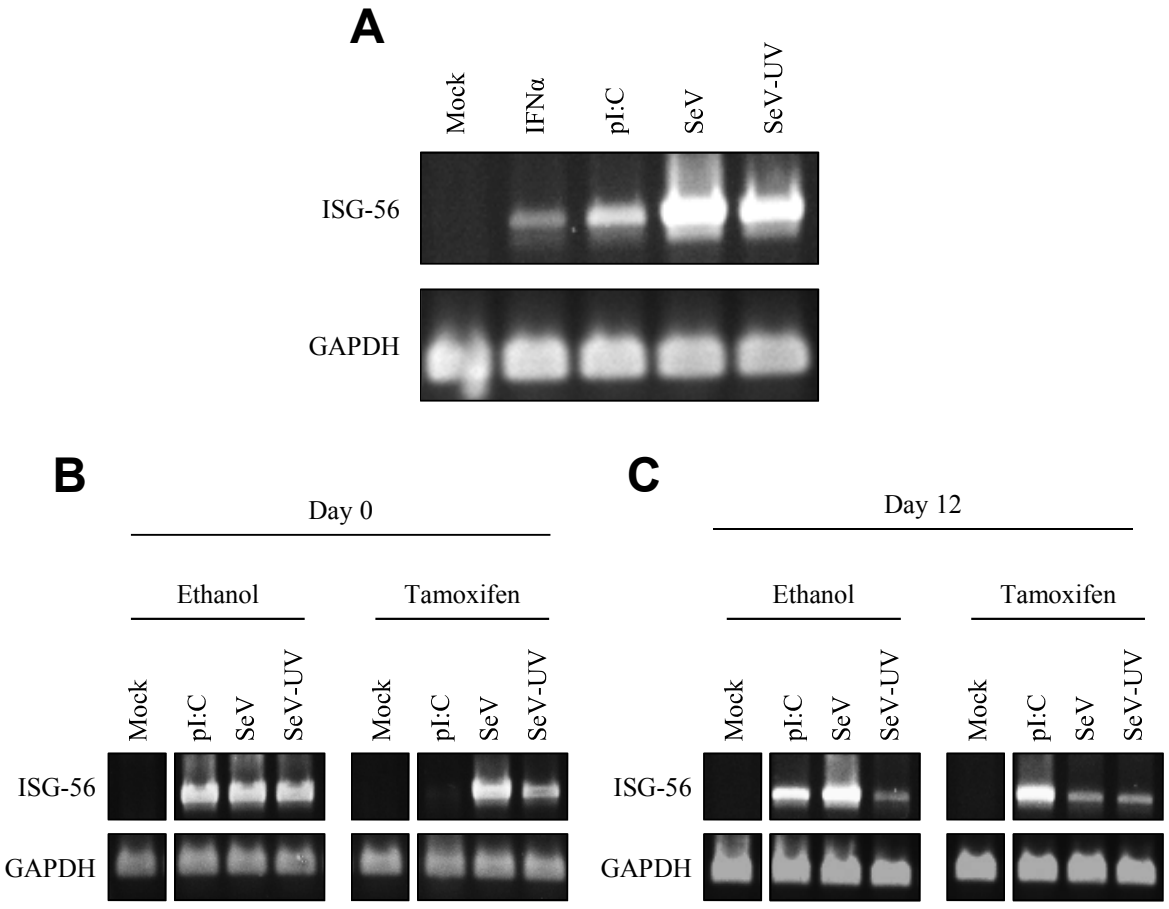

Figure 3.10: Tamoxifen treatment impairs the antiviral response in wildtype dermal fibroblasts. In (A), wildtype dermal fibroblasts were challenged with various stimuli, as indicated. 8 hours after treatment, RNA was harvested and RT-PCR was performed for ISG-56 and GAPDH. In (B), wildtype dermal fibroblasts were treated with 100nM of tamoxifen or ethanol in αMEM, replaced daily for 3 days. Cells were then placed in fresh αMEM, and at the indicated times after removal of tamoxifen, cells were challenged with pI:C, SeV or SeV-UV, or mock-treated. 8 hours after challenge, RNA was harvested and RT-PCR was performed as above.

CHAPTER 4
NOVEL ROLES OF CYTOPLASMIC ICP0: PROTEASOME-INDEPENDENT FUNCTIONS OF THE RING FINGER ARE REQUIRED TO BLOCK ISG PRODUCTION BUT NOT TO PROMOTE VIRAL REPLICATION

Sections of this chapter written by Kathryne Taylor have been published:

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Taylor KE, Chew MV, Ashkar AA, Mossman KL. 2014. Novel roles of cytoplasmic ICP0: proteasome-independent functions of the RING finger are required to block interferon-stimulated gene production but not to promote viral replication. J Virol. 88:8091-8101.

KE Taylor and KL Mossman conceived and designed the experiments. KE Taylor performed the experiments, with KL Mossman contributing to the generation of the D8/FXE virus, and MV Chew and AA Ashkar assisting with the *in vivo* murine experiments.

4.1 Abstract

The immediate-early protein ICP0 from HSV-1 plays pleiotropic roles in promoting viral lytic replication and reactivation from latency. Most of the known actions of ICP0 occur in the nucleus and are thought to involve the E3 ubiquitin ligase activity of its RING finger domain, which targets proteins for degradation via the proteasome. Although ICP0 translocates to the cytoplasm as the infection progresses, little is known about its activities in this location. Recently it was found that cytoplasmic ICP0 blocks the activation of IRF3, and surprisingly, it did so in a mechanism that does not require a functional proteasome, although the exact means through which it produces this effect remains unclear. Here, I investigated this further, and found that the RING finger of ICP0 is required for the ability of ICP0 to block antiviral signaling, implicating the RING domain in a proteasome-independent function for the first time. Additionally, I found that in both primary cell cultures and in an intravaginal mouse model, cytoplasmic ICP0 promotes viral replication – and interestingly, it does so in the absence of an intact RING finger domain. Collectively, these results underscore the importance of cytoplasmic-localized ICP0 and the diverse nature of its activities.

4.2 Introduction

The innate antiviral response is astonishingly complex, with a vast number of sensors, adaptors, scaffolding proteins, ubiquitinases, kinases, transcription factors, chromatin remodelers, cytokines, receptors, effectors and negative regulators all working together to inhibit viral replication. It is, therefore, a challenging task to unravel the details of these pathways. Attempting to identify novel components involved in antiviral signaling based on literature searches for likely candidates is not always successful, as shown in Chapter 3. However, an alternative strategy can be derived from the old saying "know thy enemy, know thyself." In order to successfully replicate, viruses must overcome innate antiviral signaling. To do this, they have evolved many diverse strategies, targeting every aspect of innate signaling, from the initial detection of viral components to the ultimate action of specific ISGs (reviewed in (664)). By characterizing how viruses manipulate these pathways, we in turn further our understanding of how antiviral signaling operates in the first place. A recent example comes from the investigation of how the human adenovirus protein E1A evades type I IFN signaling (665). It was shown that E1A blocks the expression of ISGs by preventing the monoubiquitination of histone 2B at lysine 120, a posttranslational modification associated with transcriptionally active chromatin, in both the transcribed regions and the promoters of ISGs – a process not previously known to be required for antiviral signaling (665). This demonstrates how studying the virus can lead to a better understanding of the host.

As discussed in Chapter 1, HSV is a virus that is particularly adept at evading IFNα/β signaling, with several proteins and strategies involved in this process. A key player in this is ICP0. This multifunctional protein also has roles in regulating viral gene expression, promoting reactivation from latency, and optimizing the cellular environment to maximize replication (reviewed in (666)). To date, almost all of the assorted actions of ICP0 are thought to require the E3 ubiquitin ligase activity of its RING finger domain (413, 497, 498). Additionally, although much of the work on ICP0 has focused on its roles in the nucleus, where it initially localizes due to its NLS (667), this protein later

moves to the cytoplasm as the infection progresses (478-482), and there is accumulating data suggesting that it also has important functions in this compartment (373, 501). Recently, our group reported that ICP0 can block the activation of IRF3 – but only when localized to the cytoplasm, while ICP0 that has been restricted to the nucleus loses its ability to impede antiviral signaling (373). Interestingly, cytoplasmic ICP0 was able to impair the action of IRF3 even in the absence of a functional proteasome (373). Although it was traditionally thought that E3 ubiquitin ligases such as ICP0 only produce chains of ubiquitin moieties linked via their lysine residues at position 48 (Lys 48), which target a protein for proteasomal degradation, it has since been found that ubiquitin can be linked using alternative lysine residues, resulting in atypical chains that modify protein activities without causing their degradation (reviewed in (415, 668-673)). The best characterized of these are Lys 63 chains, which have extensive non-degradative signaling roles in antiviral responses (647, 674-677). Therefore, it is possible that ICP0 produces its proteasome-independent effects via atypical ubiquitination. However, ICP0 may instead block antiviral signaling in a mechanism that is completely distinct from its function as an E3 ubiquitin ligase. Further study is needed to distinguish between these two possibilities.

Because the process through which ICP0 blocks IRF3 activation is unclear, and appears to be unusual, I hoped to gain new insight into the manner through which cells detect and respond to viral infection by further investigating the mechanism of action of ICP0. Given that the proteasome is not required for cytoplasmic ICP0 to impede antiviral signaling, I was first interested in determining whether the RING finger itself is involved in this process. Since RING finger mutants have been found to be largely restricted to the nucleus (500), to investigate the role of the RING domain in the context of cytoplasmic ICP0, I generated a virus in which ICP0 lacks both the NLS and the RING finger. Intriguingly, I found that despite the ability of cytoplasmic ICP0 to block ISG production in the absence of a functional proteasome, the RING finger is essential for ICP0 to inhibit the antiviral response. Unexpectedly, I also observed that cytoplasmic ICP0 has an important activity in promoting viral replication both in cell culture and in mice, even in the absence of the RING finger domain. These observations highlight two unknown

aspects of ICP0: the RING finger can act in a proteasome-independent manner, and ICP0 has RING finger-independent functions in the cytoplasm.

4.3 Results

4.3.1 Generation of an HSV-1 mutant expressing cytoplasmic ICP0 lacking the RING finger

To investigate the role of the RING finger in the cytoplasmic activity of ICP0, I generated an ICP0 construct containing both the FXE deletion (Δ106-150), which removes the RING finger domain, and the D8 deletion (Δ475-548), which disrupts the NLS (438) (figure 4.1A). This construct was introduced into the wildtype HSV-1 strain 17 syn genome via homologous recombination, and the presence of the expected mutations was verified via DNA sequencing. Western blot analysis confirmed the size reduction of the ICP0 protein expressed by the double mutant virus, designated D8/FXE (figure 4.1B). To ensure that the presence of two deletions did not result in gross protein misfolding, I confirmed that the D8/FXE ICP0 was capable of binding to USP7, a well-characterized interaction partner whose binding site in the C-terminus of ICP0 would not be directly impacted by FXE and D8 deletions, via co-immunoprecipitation (co-IP) (430, 678, 679) (figure 4.1C). As expected, the amount of ICP0 recovered in the cytoplasmic extracts differed among the viruses. Due to its exclusive cytoplasmic localization, a greater quantity of D8 ICP0 was present compared to WT ICP0, a portion of which is still nuclear at this time. D8/FXE ICP0 was found at the highest level in accordance with its increased stability as a result of the loss of the RING finger (see below). However, the amount of USP7 recovered in each co-IP was proportional to the amount of ICP0 in the particular sample, demonstrating that all forms of ICP0 interact with USP7 to a comparable degree.

I next investigated the localization of D8/FXE ICP0 via immunofluorescence microscopy (figure 4.2). Unlike the FXE mutant, which was found predominantly in the nucleus, D8/FXE ICP0 was found exclusively in the cytoplasm. This localization pattern was confirmed in primary mouse fibroblasts and primary human genital epithelial cells (data not shown).

4.3.2 Cytoplasmic ICP0 cannot block antiviral signaling in the absence of the RING finger

Next, I determined whether ICP0 is capable of blocking antiviral signaling in the absence of the RING finger domain. HEL cells were infected with D8/FXE, the single mutant D8, the ICP0-null dl1403, and the wild-type parent strain 17 syn in the presence or absence of the proteasome inhibitor MG132, and antiviral state induction was monitored by determining the accumulation of ISG-56 message at 8 hours post-infection (hpi), via quantitative RT-PCR (figure 4.3A). 17 syn efficiently blocked the accumulation of ISG-56 message when the proteasome was active, but lost this ability after treatment with MG132, while D8 blocked ISG-56 accumulation, even when the proteasome was inhibited, in accordance with previous results (373). Intriguingly, D8/FXE was unable to prevent ISG-56 expression, regardless of the status of the proteasome, suggesting that cytoplasmic ICP0 requires the RING finger, but not the proteasome, for its ability to block antiviral signaling. To confirm that the inability of D8/FXE to impede ISG-56 induction was due to the absence of the ICP0 RING finger domain, as opposed to secondary site mutations, I generated the revertant virus D8/FXE-R, in which mutant ICP0 is replaced with WT ICP0. As expected, D8/FXE-R efficiently prevented the activation of innate signaling (figure 4.3B). Similarly, examining ISG-56 protein levels via Western blot analysis confirmed that D8 efficiently blocked the activation of the antiviral response, while D8/FXE could not (figure 4.3C).

The requirement for the RING finger in the absence of the proteasome to prevent ISG induction suggested that ICP0 may be blocking antiviral signaling via the production of atypical ubiquitin modifications. To test this possibility, I examined whether IRF3 is modified by ubiquitin chains in an ICP0-dependent manner, using tandem ubiquitin binding entities (TUBE) 1-linked agarose (680). TUBEs consist of multiple copies of the ubiquitin-associated (UBA) domain from the protein ubiquilin 1 connected by flexible linkers and coupled to agarose beads, and have a 100-1000-fold increased affinity for polyubiquitin chains compared to antibodies. TUBE 1 in particular has a 10 fold higher affinity for Lys 63 chains compared to Lys 48 chains, and so this reagent preferentially

associates with atypically modified proteins. Pull-down assays using cytoplasmic extracts from cells infected with the various HSV-1 mutants were performed, and eluents were analyzed for the presence of IRF3 (Figure 4.4). Although ubiquitin-modified IRF3 was detectable even in mock-treated samples (visualized as a smear at high molecular weights due to the variable lengths of polyubiquitin chains (681)), there were no clear differences in the level of modified IRF3 after infection with any of the HSV-1 mutants. Minor variations in the extent of IRF3 ubiquitination do not correlate with the ability of the particular mutants to inhibit antiviral signaling – for example, the results for the wildtype virus, which prevents IRF3 activation, look more similar to D8/FXE than D8, despite the fact that only the latter and not the former can block antiviral signaling. These minor differences were also not reproducible in repeated experiments and are most likely explained simply by experimental variation. Therefore, it does not appear that ICP0 targets IRF3 with atypical ubiquitin chains in order to disrupt its function.

4.3.3 Cytoplasmic ICP0 promotes virus replication in cell culture in the absence of the RING finger

Since most of the known roles of ICP0 are thought to occur in the nucleus and require an intact RING finger domain, I expected the D8/FXE virus to be highly attenuated. However, while investigating the ability of D8/FXE to block antiviral signaling, I observed that it replicated more strongly than anticipated. To investigate this further, HEL cells were infected with the various HSV-1 mutants, at both high (10) and low (0.1) multiplicities of infection (MOIs), for 24 hours. Virus was then titered on U2OS cells in the presence of HMBA. Remarkably, the D8/FXE virus, as well as the D8 virus, grew significantly better than either dl1403 or the single FXE mutant, at both high and low MOIs (figure 4.5A and C). Although neither D8/FXE nor D8 replicated as well as 17 syn, both viruses caused a productive infection at MOI 10, reaching titers approximately 8-fold higher than dl1403, which produced little more than the input virus. A similar pattern was observed even at the low MOI of 0.1, indicating that cytoplasmic ICP0 is still able to promote viral replication, even in the absence of a functional RING finger domain.

As expected, repaired strain D8/FXE-R replicated to similar titers as 17 syn at both high and low MOIs (figure 4.5B and D).

To further characterize the point in the replication cycle affected by cytoplasmic ICP0, the expression of the immediate-early protein ICP4 was determined via immunofluorescence microscopy after infection with 17syn, D8, D8/FXE or dl1403. The number of cells positive for ICP4 was counted, relative to the total number of cells in each field of view (figure 4.5E). The pattern of ICP4 expression mirrored the observations for viral replication, with both D8 and D8/FXE showing a significantly greater number of cells expressing ICP4, relative to dl1403.

In terms of both titer and ICP4 expression, D8/FXE showed a slight but reproducible improvement in replication over D8. This may be explained by ICP0 auto-ubiquitination and subsequent degradation (682), which would be expected to occur in D8 but not the RING-deficient D8/FXE. In support of this possibility, D8/FXE ICP0 was found to accumulate to higher levels than D8, but the addition of MG132 normalized the levels of the two cytoplasmic ICP0 mutants (figure 4.5F). I next determined whether differences in the particle to pfu ratios between the ICP0 mutants could account for the increased replication of the viruses expressing cytoplasmic ICP0. First, VP5 levels in serial dilutions of the purified viral stocks were determined via Western blotting (figure 4.6A). The HSV capsid consists of 150 hexons and 12 pentons (683), which contain 6 and 5 copies of VP5, respectively (684), with one vertice replaced with a portal structure (685) – meaning that each particle contains exactly 955 copies of VP5. Therefore, the amount of VP5 accurately reflects the number of particles in the sample. D8, D8/FXE and dl1403 had VP5 levels that were within approximately 2 fold of each other, while the particle to pfu ratio was much lower for 17 syn, as expected (686). Counting viral particles using tunable resistive pulse sensing (687) gave equivalent results (figure 4.6B). Therefore, differences in particle number cannot explain the disparities in the replication of the ICP0 mutant viruses. Together, these data suggest that cytoplasmic ICP0 stimulates the replication of HSV-1 in cell culture in a mechanism independent of the RING finger.

4.3.4 Cytoplasmic ICP0 does not require the RING finger to promote virus replication in vivo

To determine whether the differences in viral replication observed in cell culture were reproducible *in vivo*, an intravaginal mouse model was used. Despite increasing clinical data linking HSV-1 with genital infections, few murine studies utilize HSV-1 in intravaginal inoculation. Thus, in preliminary studies, 17 syn and dl1403 were tested in parental C57BL/6 (B6) and IRF3$^{-/-}$ mice (figure 4.7). One day post-infection (figure 4.7A), there was a trend towards increased viral titers in the vaginal washes for both 17 syn and dl1403 in IRF3$^{-/-}$ mice compared to B6 infected mice, suggesting that IRF3 contributes to the host response to vaginal HSV-1 challenge. While this general trend was maintained at day 2 post-infection (figure 4.7B), dl1403 was being cleared in both strains of mice at this time. Of note, the ICP0-null virus did not reach wild-type titers in IRF3-deficient mice, indicating that ICP0 contributes important activities in addition to blocking IRF3.

From the *in vitro* data, I was particularly intrigued to observe that cytoplasmic ICP0 promotes HSV-1 replication in the absence of a RING finger. Since D8/FXE cannot block the IRF3-mediated antiviral response, while D8 can (figure 4.3), I chose to assess its ability to support virus replication *in vivo* in mice deficient for IRF3. Thus, IRF3-deficient mice were challenged with 17 syn, D8, D8/FXE or dl1403, and I determined viral titers in the vaginal washes at two days post-infection (figure 4.8A). Similar to the *in vitro* findings, both D8 and D8/FXE replicated to significantly higher titers than dl1403, with the enhanced stability of ICP0 in D8/FXE likely responsible for the increase in titers over D8. Vaginal pathology results correlated closely with viral titers (figure 4.8B), and a similar pattern was also observed when levels of the cytokine IFNγ were quantified in the vaginal washes (figure 4.8C). Collectively, these data show that *in vivo*, as well as in cell culture, cytoplasmic ICP0 has a growth-promoting activity that is distinct from its ability to block antiviral signaling, and which does not require the RING finger.

4.4 Discussion

This study demonstrates that while ICP0 mediates significant functions in the nucleus, its cytoplasmic roles also have a largely unappreciated significance in viral replication. I show that ICP0 restricted to the cytoplasm can promote viral growth both in cell culture and in mice, and surprisingly, that it can achieve this equally well in the presence or absence of a functional RING finger domain. Additionally, I found that the RING finger contributes to blocking antiviral signaling in a proteasome-independent fashion.

4.4.1 The unappreciated importance of cytoplasmic ICP0

Partial or complete restriction of ICP0 to the nucleus results from a wide variety of experimental manipulations (478, 500, 688-692), including commonly used strategies such as DNA transfection prior to infection, the use of proteasome inhibitors, or ICP0 expression in the absence of other viral proteins. Thus unintentionally, many studies have exclusively considered the roles of nuclear ICP0, which can produce misleading results. This is clearly demonstrated by the previous report (373), confirmed here, showing the ability of cytoplasmic ICP0 to block IRF3 activation in the absence of a functional proteasome. Because proteasome inhibition leads to the nuclear restriction of ICP0, earlier investigations had concluded that the proteasome was required for the antiviral-inhibiting actions of ICP0 (372), while it was, in fact, the cytoplasmic localization that was essential, and not the action of the proteasome at all. Similarly, studies using an inducible ICP0-expressing cell line, where ICP0 is restricted to the nucleus, had suggested that ICP0 is incapable of blocking ISG expression (378), when again, it is ICP0 in the cytoplasm that is necessary. Therefore, care must be taken when using conditions that impair the translocation of ICP0, as this is akin to generating a “cytoplasmic-null” ICP0 mutant. For example, results showing that proteasome inhibition disrupts a particular activity of ICP0 could equally mean that cytoplasmic ICP0 is involved in that specific function.

Likewise, as shown here (figure 4.2) and elsewhere (500), RING finger mutations cause ICP0 to be largely restricted to the nucleus. This is consistent with the previous suggestion that ICP0 must complete its nuclear functions before it can translocate to the cytoplasm (688), and so preventing ICP0 from performing these activities by disrupting its RING finger results in its nuclear retention. My results are in agreement with the vast amount of literature demonstrating that impairing the RING finger of ICP0 results in a virus as attenuated as an ICP0-null strain (438, 443, 693-696), and suggest that ICP0 absolutely requires the RING finger to perform its functions specifically within the nucleus. However, most studies using RING finger mutants conclude that ICP0 requires a RING finger for all of its biological roles, without taking the effect of ICP0 localization into consideration. By including the additional D8 deletion to remove the NLS and create D8/FXE, I have overcome the nuclear restriction of FXE, allowing me to investigate the activity of RING-deficient ICP0 in the cytoplasm. It is important to note that immunofluorescence studies cannot conclusively demonstrate that a small amount of the NLS-deleted ICP0 is not reaching the nucleus. Indeed, it has been observed that under high multiplicity infections in certain circumstances, D8 ICP0 can overcome the loss of its NLS, and can be found in the nucleus late in infection in some cell types (438, 480). However, we have previously shown that in the cell type and at the MOI used here, the D8 virus does not degrade nuclear PML (373). Since it is well-established that even a small amount of nuclear ICP0 leads to the loss of PML (695), it can thus be concluded that this mutation prevents a biologically relevant amount of ICP0 from localizing to the nucleus in HEL cells. Additionally, since FXE is mainly nuclear and yet is inactive, a small amount of D8/FXE reaching the nucleus cannot explain these results.

4.4.2 The RING finger-dependent but proteasome-independent function of cytoplasmic ICP0 in blocking antiviral signaling

Cytoplasmic ICP0 failed to block antiviral signalling in the absence of the RING domain. At first glance, it is difficult to understand how the RING finger, with its ubiquitin ligase activity, could be required for the ability of ICP0 to prevent antiviral

signaling, while the proteasome itself is dispensable. This observation is consistent with our previous work demonstrating the involvement of the RING finger in antiviral inhibition (371), as well as with the fact that despite this requirement, no factor involved in IRF3 signaling has been unequivocally identified as being degraded by ICP0 during an HSV-1 infection (371, 373). As discussed in Chapter 1, a few reports have suggested that ICP0 can block antiviral signalling in a proteasome-dependent manner, but some of these studies relied on a confounding SeV co-infection model (374, 375), while another reported mechanism (38) has since been found to be ICP0-independent (476).

In recent years, there has been a tremendous increase in our understanding of the proteasome-independent activities of ubiquitin modifications (reviewed in (415, 668-673)). The traditional signal for proteasomal degradation consists of ubiquitin moieties conjugated into chains via Lys 48 (697). Yet ubiquitin contains seven lysine residues, any of which can be used to make chains, and so ubiquitin can also be linked in a variety of atypical manners via residues other than Lys 48 (698). These alternative chains are associated with non-degradative, signaling roles (699). Atypical linkages result in chains that adopt a variety of conformations (700), which can be recognized by different ubiquitin-binding domains (UBDs) found in a wide diversity of proteins, in a linkage-specific manner. Thus, ubiquitin modification can control protein-protein interactions, and are therefore involved in cellular processes ranging from receptor endocytosis to DNA repair (reviewed in (670)). Of particular interest is that the Lys 63 linkage, one of the best characterized of these alternative chains, plays an extensive role in antiviral signaling (647, 674-677). For example, Lys 63-linked chains, but not Lys 48-linked chains, are essential for the activation of IRF3 (674), although the exact target(s) of atypical ubiquitination remain unclear. IRF7 has been found to be directly modified by Lys 63 chains, initiating its activity (675). Similarly, Lys 63 chains are also important for inducing RLRs (701). Several deubiquitinating enzymes have been found to inhibit IFNα/β signaling – for example, DUBA blocks antiviral responses by specifically cleaving Lys 63 chains (647). Finally, NFκB activation is extensively regulated by atypical ubiquitin modification of signal transduction proteins (reviewed in (702)).

Though the activity of the RING finger of ICP0 as a ubiquitin ligase has been confirmed *in vitro* (422, 497) as well as *in vivo* (413), the linkage type found in the ubiquitin chains produced by ICP0 has never been determined. The loss of ICP0-targeted proteins from the cell certainly appears to implicate the RING finger in the generation of Lys 48 linkages and proteasomal degradation. However, this is not incompatible with the potential role of the RING finger in generating alternatively linked chains involved in proteasome-independent signaling roles, since it has been found that many E3 ubiquitin ligases can produce both Lys 48 and Lys 63-linked chains, depending on the particular E2 enzyme associated with it, as an E3 protein can interact with more than one E2 (reviewed in (672, 703)). For example, the cellular protein Nrdp1 is a RING-finger ubiquitin ligase that differentially mediates both Lys 48 and Lys 63 polyubiquitination of proteins involved in antiviral signaling (704). Therefore, ICP0 may be able to modify target proteins either with Lys 48 chains, to direct them to the proteasome, or with chains with atypical linkages, altering protein function without causing protein degradation. Accordingly, ICP0 could be preventing IRF3 activation by attaching a Lys 63 chain to a protein involved in the antiviral response, disrupting its function in a manner that requires its RING finger but not the proteasome. It is also possible that the RING finger acts in a completely ubiquitin-independent manner. Recently, it has been found that the cellular retrovirus restriction factor TRIM-CypA is able to inhibit virus infection in a mechanism that requires its RING domain but neither the proteasome nor ubiquitin conjugation (705).

The ubiquitination of IRF3 is not fully characterized at this time. It was originally reported that IRF3 is ubiquitinated in a manner that increased after viral infection (706), though the linkage type was not determined. It was later shown that IRF3 is targeted for degradation by the virus-inducible E3 ubiquitin ligase RBCK1 in a negative feedback mechanism (645), and it was likewise found that RO52 could cause the ubiquitination and subsequent degradation of IRF3 after PRR-mediated activation (644). The E3 ubiquitin ligase RAUL has also been observed to catalyze the addition of Lys 48 chains on both resting and activated IRF3 to maintain optimal levels of IRF3 protein (707). Interestingly, it has been demonstrated that exogenous IRF3 is modified by Lys 63-linked chains (708),

and endogenous IRF3 has been reported to be conjugated to Lys 63-linked chains of exogenous ubiquitin in response to SeV infection (709). However, the significance of this potential Lys 63 modification of IRF3 is not understood at this time. The fact that the TUBE 1 pulldown assay did not reveal any reproducible increase in IRF3 ubiquitination after infection with HSV-1 suggests that ICP0 does not produce its RING-dependent but proteasome-independent effect on antiviral responses by targeting IRF3. However, ICP0 may instead modify any of the large number of proteins involved in antiviral signaling upstream of IRF3 activation with atypical ubiquitin chains. Further study is needed to investigate this. The failure here to detect the previously observed increased ubiquitination of IRF3 during viral infection may represent differences in experimental technique, particularly because previous studies have relied heavily on overexpressed, tagged ubiquitin and IRF3 as opposed to endogenous protein. Also, because the TUBE 1 reagent recognizes Lys 48 as well as Lys 63 chains, albeit with a lower affinity, it is possible that the ubiquitination observed in the mock sample represents basal levels of Lys 48-modified IRF3 involved in routine protein turnover. However, in the virus infected samples, an increase in Lys 63 ubiquitination with a corresponding decrease in Lys 48 modification might cause the total level of ubiquitinated protein to remain unchanged, and may therefore not be detectable using this technique. Making use of the recently developed Lys 63-selective TUBEs (710) may help to resolve this possibility.

While unable to control the antiviral response, D8/FXE grows equally well as D8 in cell culture. This observation is consistent with previous studies demonstrating that neither the depletion of IRF3 nor STAT-1 in cultured cells could improve the replication of an ICP0-null virus (711). In contrast, as described in Chapter 1, the type I IFN response is crucial in controlling HSV replication in mouse models (355, 357, 359, 360). Conversely, IRF3-deficient mice survive intravenous infection with wild-type HSV-1 (712), and show no increased viral replication in peripheral tissues (712-714), though augmented replication was observed in the central nervous system (713, 714). While ICP0-null mutants are attenuated in wild-type mice (314, 715, 716), viruses lacking ICP0 have not been studied in IRF3-deficient mice. Here, I used $IRF3^{-/-}$ mice to investigate the

replication of the ICP0 mutants, using an intravaginal model of infection, as HSV-1 is now responsible for at least 50% of new genital herpes episodes in developed countries (reviewed in (717)). I found that while there was a trend towards higher titers in IRF3$^{-/-}$ mice for both 17 syn and dl1403, this was not statistically significant. It is probable that this results from the compensatory role of IRF7 in IRF3$^{-/-}$ mice, as these mice continue to make IFNα via constitutively expressed IRF7 in plasmacytoid dendritic cells, and thus survive infection with wild-type HSV-1, while IRF7$^{-/-}$ mice lack IFNα production and succumb (712). This is analogous to previous work demonstrating that ICP0-null viruses show augmented replication in STAT1$^{-/-}$ and IFNAR$^{-/-}$ mice (359, 716), but remain attenuated in PML$^{-/-}$ mice (716). Therefore, IRF3$^{-/-}$ mice are useful for these studies, allowing me to compare the two cytoplasmic ICP0 mutants without the confounding effects of IRF3 activation, which D8 controls and D8/FXE cannot. However, these mice are not so deficient as to eliminate the requirement for ICP0 to achieve maximal viral replication.

My observations in IRF3$^{-/-}$ mice confirm the ability of cytoplasmic ICP0 to support virus growth in a RING-independent manner. Virus growth is mirrored by the observed pathology in these mice, and further confirmed by measurement of IFNγ levels, a cytokine produced by natural killer (NK) cells that represents a characteristic feature of the innate immune response to replicating genital HSV (718-720). Consistent with my data, previous work in lymphocyte-deficient *rag2*$^{-/-}$ mice demonstrated that an HSV-2 virus expressing an NLS mutant of ICP0 was lethal in 80% of mice, compared to only 20% infected with a RING finger mutant (721), and while attenuated and asymptomatic in wildtype mice, NLS-mutant HSV-2 was capable of inducing protective immunity, while the RING finger mutant could not (722). This supports my observation that cytoplasmic ICP0 has a positive effect on viral replication *in vivo*. Surprisingly, D8/FXE reaches titers even higher than D8, and also induces higher levels of IFNγ. This may be explained by the increased stability of RING finger mutants (682, 693), as ICP0 undergoes RING-dependent autoubiquitination. Indeed, I observed that ICP0 accumulates to higher levels in D8/FXE compared to D8.

4.4.3 RING-independent replication-promoting effects of cytoplasmic ICP0

How cytoplasmic ICP0 induces virus replication, particularly in the absence of the RING finger domain, is currently unclear. Few RING-independent functions of ICP0 are known – FXE has been found to block rRNA degradation at late stages after infection (499), and a RING finger mutant has been found to disrupt coREST-REST interactions, albeit at a reduced level (500). Daubeuf *et al.* showed that a RING finger mutant of ICP0 was still capable of blocking NFκB signaling (473), though these results were in direct contrast to those of a second group, who found that the RING is necessary for ICP0-mediated inhibition of NFκB (425). Finally, it has recently been found that ICP0 can promote the degradation of the surface marker CD83 in mature dendritic cells in a mechanism that appears to require neither the RING finger nor ubiquitination (723). I also cannot rule out that HUL-1, the second region of ICP0 with E3 ubiquitin ligase activity (437), is playing a role in the replication-promoting activities of D8/FXE in the absence of the RING, though to date only one target of HUL-1 has been identified.

In terms of cytoplasmic functions, ICP0 has been found to bind to EF-1δ, a cytoplasmic elongation factor involved in translation (479), although little is known about the significance of this interaction. Other potential cytoplasmic activities identified for ICP0, such as the degradation of IκBα (423) or the disruption of the cellular microtubule network (501), appear to require RING-dependent ubiquitination. It has also been found that ICP0 must be cytoplasmic in order to be packaged into the viral tegument (724, 725), and accordingly, RING finger mutants are not found in the tegument (726, 727), though it is currently unknown whether the RING finger itself is required for packaging, or whether RING mutants simply fail to be packaged because of their restriction to the nucleus (727). Interestingly, capsids from viruses lacking tegument ICP0 have been found to have disrupted transport to the nucleus, which has been suggested to partially explain the decreased replication of ICP0-null viruses (727). Therefore, the cytoplasmic localization of D8 and D8/FXE may allow their packaging into the tegument, resulting in more direct capsid transport and greater efficiency of replication.

I also showed here that ICP0 continues to interact with its well-known partner USP7 (430, 678, 679), a cellular deubiquitinating enzyme, even when in the cytoplasm. It has previously been reported that ICP0 transports USP7 from the nucleus to the cytoplasm in a RING-independent but NLS-dependent manner (473). Conversely, USP7 translocation can occur after TLR stimulation in the absence of ICP0 (473), and I observed that ICP0 restricted to the cytoplasm can still interact with USP7. Loss of USP7 binding by ICP0 has been found by some groups to decrease viral growth (421, 504), while others have reported that such viruses show increased gene expression, though decreased cell-to-cell spread (728). It can be difficult to interpret studies about ICP0-USP7 interactions, because the loss of USP7 binding decreases ICP0 stability (682), and so effects observed as a result of the loss of USP7 binding may correspond instead to the overall decrease in ICP0 levels (421). Since only D8, and not D8/FXE, is capable of RING-mediated USP7 degradation, any involvement of USP7 in the promoting viral replication would have to be due to its deubiquitination function, as opposed to a benefit derived from its ICP0-mediated depletion. However, currently the function of USP7 in HSV infection is unknown, and the question remains that if USP7 is performing a function that is useful to viral replication, why would it be targeted for degradation by ICP0?

A role for cytoplasmic ICP0 in promoting viral growth via increased translation, capsid transport, or USP7 binding would be a departure from the current paradigm in ICP0 biology, which suggests that ICP0 stimulates virus replication by increasing the probability that an incoming genome will launch a productive infection (reviewed in (666)). As discussed in Chapter 1, ICP0 may achieve this by overcoming intrinsic resistance factors, such as ND10 components (416, 417, 462-464, 468), by acting as a DNA template remodeler (451), decreasing repressive and increasing active histone modifications and counteracting the formation of heterochromatin, or by disrupting various repressor proteins, such as the coREST-REST complex (43, 418, 453-460, 729). However, it is difficult to understand how cytoplasmic ICP0 could affect such nuclear components, though NLS mutants of ICP0 have been found to associate with PML in the

cytoplasm (482), PML has been found at specific cytoplasmic locations (480), and the coREST-REST complex has been found to translocate to the cytoplasm in a HSV-dependent but ICP0-independent manner (458).

My results are consistent with early observations that ICP0 mutants lacking normal cytoplasmic translocation were attenuated, suggesting that ICP0 may have functions in the cytoplasm (482). In fact, it has been previously shown that the D8 virus replicates better than FXE (438), but little has been made of such observations, though it has been more recently proposed that the defect in replication of a VP22 phosphorylation mutant may be attributed to the loss of cytoplasmic-localized ICP0 observed with this virus (692). In contrast, in an inducible cell line system, the expression of cytoplasmic ICP0 was unable to complement the growth of a superinfected ICP0-null virus (695). However, ICP0 expression in this model was low, reaching only approximately the level observed between 2 and 4hpi with a wildtype virus (695). ICP0 has been found to reach much higher levels in the cytoplasm of infected cells, in comparison to what is observed in the nucleus (501), and so the level of ICP0 in the inducible cell line may simply be too low to produce an effect. As opposed to functions involving the RING finger, non-enzymatic roles may require larger amounts of the ICP0 protein (460).

There have also been occasional reports of RING finger mutants having some activity. For example, Ferenczy *et al*. observed that a virus expressing ICP0-RF, which has point mutations disrupting RING finger activity, replicated better than an ICP0-null virus (729). This study did not investigate the potential cytoplasmic localization of their RING mutant, but it is possible that ICP0 was able to escape from the nucleus in this cell type, despite the disruption of the RING, allowing it to promote replication. The rate of translocation of wildtype ICP0 to the cytoplasm is well known to vary between cell types, tending to occur sooner in primary cells compared to continuous cell lines (451, 479). In fact, in some cell types, such as mature dendritic cells (424), foreskin fibroblasts (479) and corneal fibroblasts or keratocytes (730), ICP0 is located predominantly in the cytoplasm from the onset of infection. A recent study also supports the idea of location-specific functions of ICP0 in the absence of the RING domain (731). They found that a

virus expressing a RING finger mutant of ICP0 that was retained exclusively at ND10 domains was more highly attenuated than a virus encoding a RING domain mutant with additional deletions allowing it to disperse away from the ND10, and potentially into the cytoplasm, again suggesting the reason for the complete attenuation of RING mutants is their entrapment in the nucleus (731).

In conclusion, this work demonstrates that cytoplasmic ICP0 has two independent activities: blocking ISG production in a mechanism that involves the RING finger but not the proteasome, and promoting virus replication in a RING-independent manner. These observations underscore the importance of cytoplasmic ICP0, and suggest alternative functions for the RING finger domain, opening new avenues for the investigation of this multifaceted viral protein.

4.5 Acknowledgements

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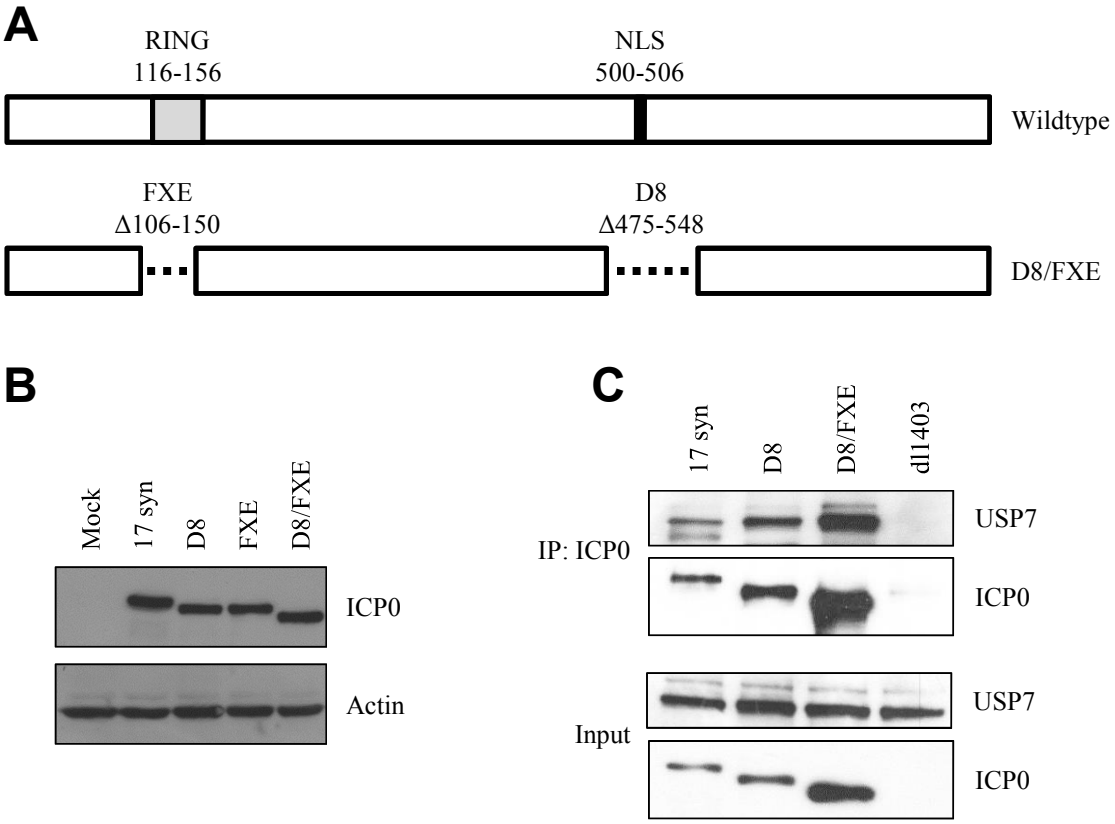


Figure 4.1: Generation of an HSV mutant expressing cytoplasmic ICP0 lacking the RING finger. (A) Schematic of the deletions in the *icp0* gene found in the wildtype and D8/FXE viruses. In (A) and (B), HEL cells were infected with the indicated viruses at an MOI 10 for 8 hours. In (B), cells were harvested via RIPA extract and analyzed for ICP0 size and expression relative to actin via Western blot. In (C) a cytoplasmic extract was performed and samples were then immunoprecipitated with an αICP0 antibody. The eluent and input was then analyzed via Western blotting for ICP0 and USP7.

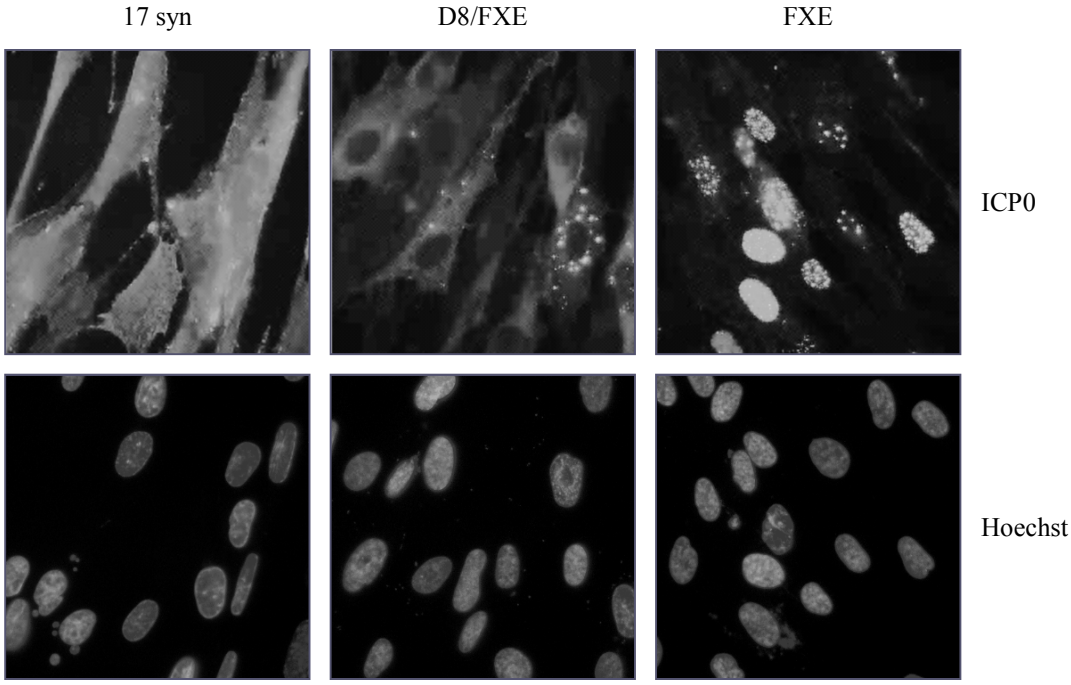


Figure 4.2: An ICP0 NLS mutant lacking the RING finger localizes to the cytoplasm. HEL cells were infected with the indicated viruses at MOI 10 for 8 hours, then immunofluorescence microscopy was performed to determine the subcellular location of ICP0, and nuclei were stained with Hoechst dye.

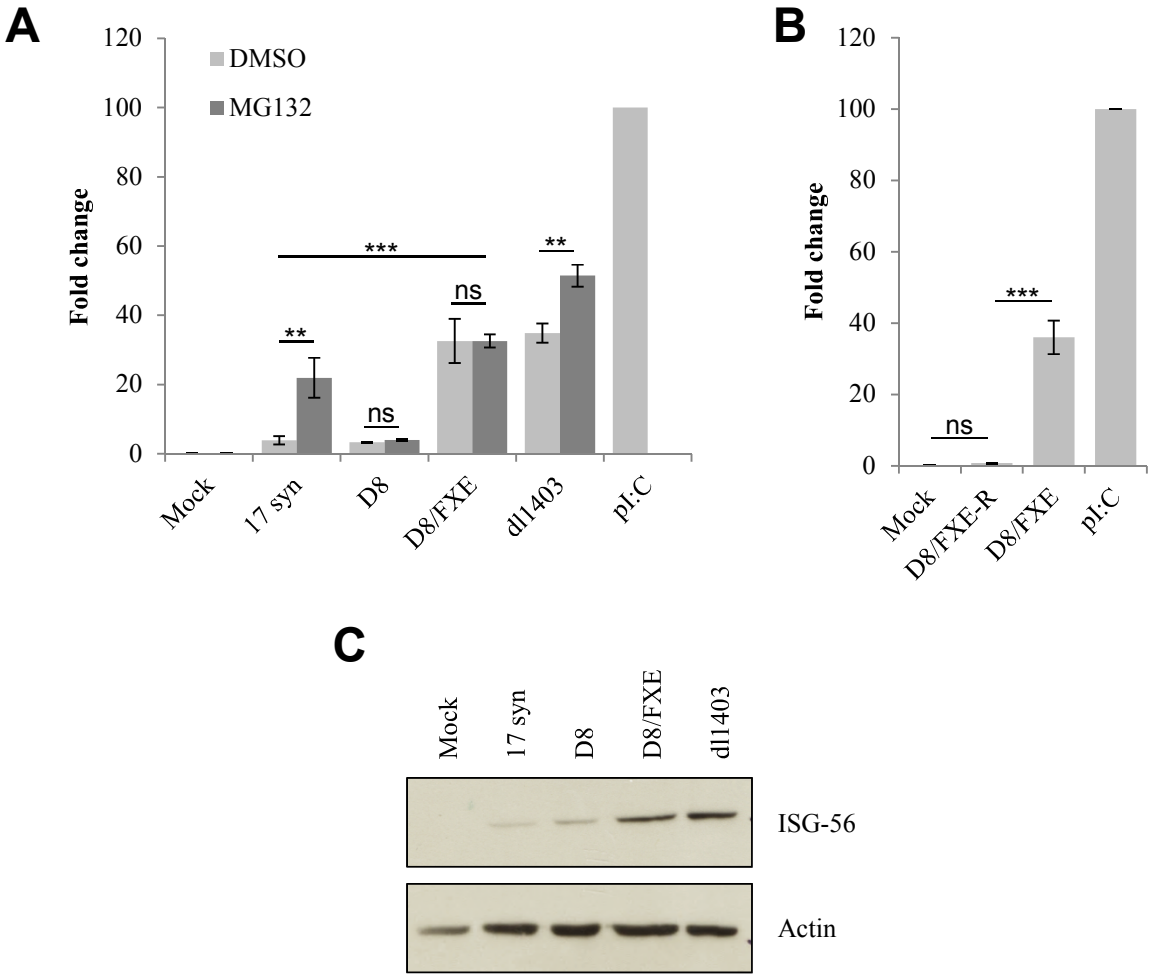


Figure 4.3: Cytoplasmic ICP0 requires the RING finger but not the proteasome to block ISG expression. HEL cells were infected with the indicated viruses at an MOI of 10. In (A), infections were performed in the presence or absence of the proteasome inhibitor MG132, as indicated. For (A) and (B), RNA was harvested after 6 hours and the expression of ISG-56 relative to GAPDH was determined using the Taqman system of quantitative RT-PCR. Values are reported relative to pI:C treated cells, whose fold change was set to 100. These data are the average of three independent experiments ± SEM. Statistical analysis was performed using one-way ANOVA and Tukey's post test, ** $p<0.01$, ***$p<0.001$. In (C), protein was harvested after 8 hours and analyzed for ISG-56 and actin expression via Western blot.

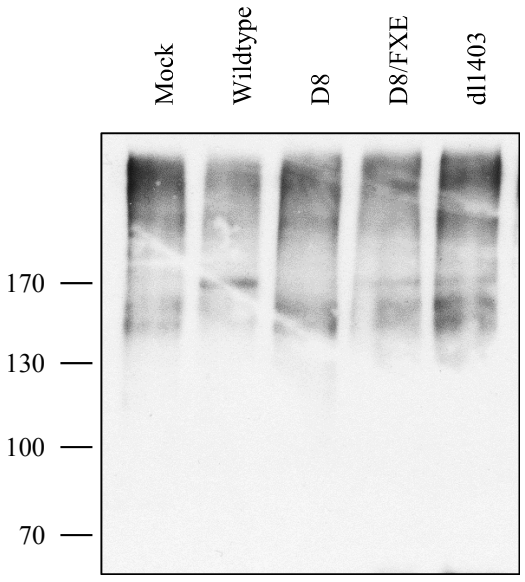


Figure 4.4: Differences in IRF3 Lys-63 ubiquitination do not correlate with the ability of ICP0 mutants to block antiviral signaling . HEL cells were infected with the indicated viruses at an MOI of 10 for 8 hours, then cytoplasmic extracts were harvested. 500µg of extract was then incubated with 20µL of TUBE-1 agarose for 16 hours at 4°C. Beads were then washed 3 times, boiled in SDS loading buffer and the eluent was then analyzed via Western blot for IRF3.

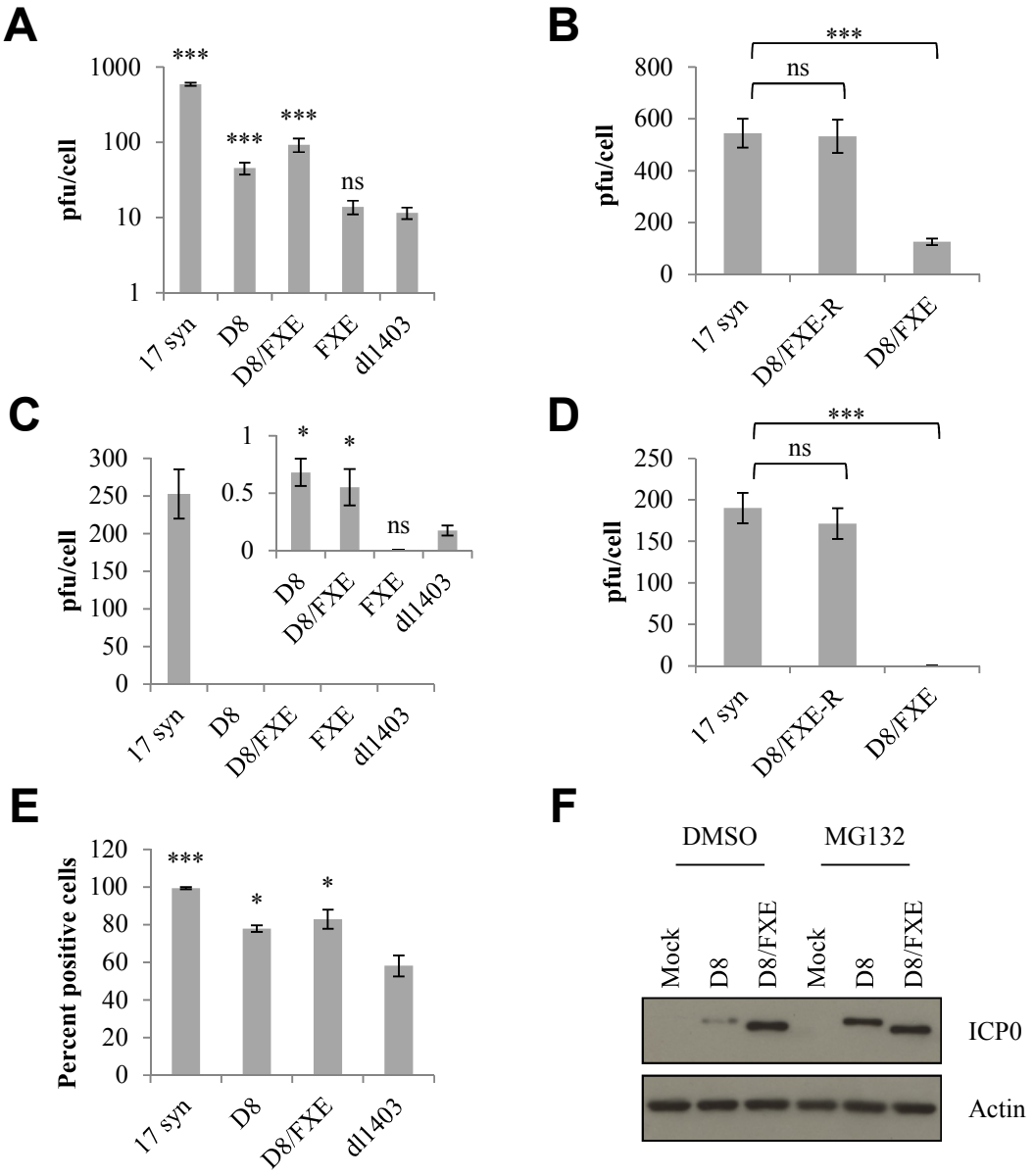
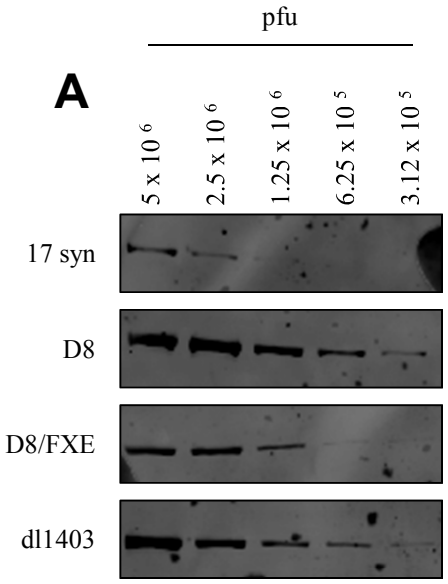


Figure 4.5: Cytoplasmic ICP0 promotes viral replication in the absence of the RING finger domain in primary fibroblasts. HEL cells were infected with the indicated viruses at a high MOI of 10 in (A) and (B) or the low MOI of 0.1 in (C) and (D) for 24 hours. Cells and supernatants were harvested, sent through 3 freeze-thaw cycles, and then titered on U20S cells in the presence of HMBA. In (E), HEL cells were infected with the indicated viruses at MOI 10 for 12 hours, then immunofluorescence microscopy was performed to determine the number of cells expressing ICP4 relative to the total number of cells, as determined by staining nuclei with Hoechst dye. These data are the average of three independent experiments ± SEM. Statistical analysis was performed using one-way ANOVA and Tukey's (B) and (D) or Dunnett's (A) and (C) post test relative to dl1403. *$p<0.05$, ** $p<0.01$, ***$p<0.001$. In (F), HEL cells were infected with the indicated viruses at MOI 10 for 8 hours in the presence of MG132 or the carrier DMSO, then harvested via cytoplasmic extract. Western blot analysis was then performed to determine levels of ICP0 and actin.



B

Virus	Particles	Titer	Particle:pfu
17 syn	3×10^{10}	8.5×10^{9}	3.5
D8	4.3×10^{10}	9.55×10^{8}	45
D8/FXE	1.6×10^{11}	4.4×10^{9}	36
dl1403	2.7×10^{10}	8.31×10^{8}	32

Figure 4.6: Differences in particle:pfu ratio between the ICP0 mutants cannot account for differences in their replication. In (A), serial dilutions of purified virus stocks were disrupted via boiling in SDS loading buffer then analyzed via quantitative Western blot for VP5 levels. In (B), particle numbers were determined using tunable resistive pulse sensing, and used to determine the particle:pfu ratios.

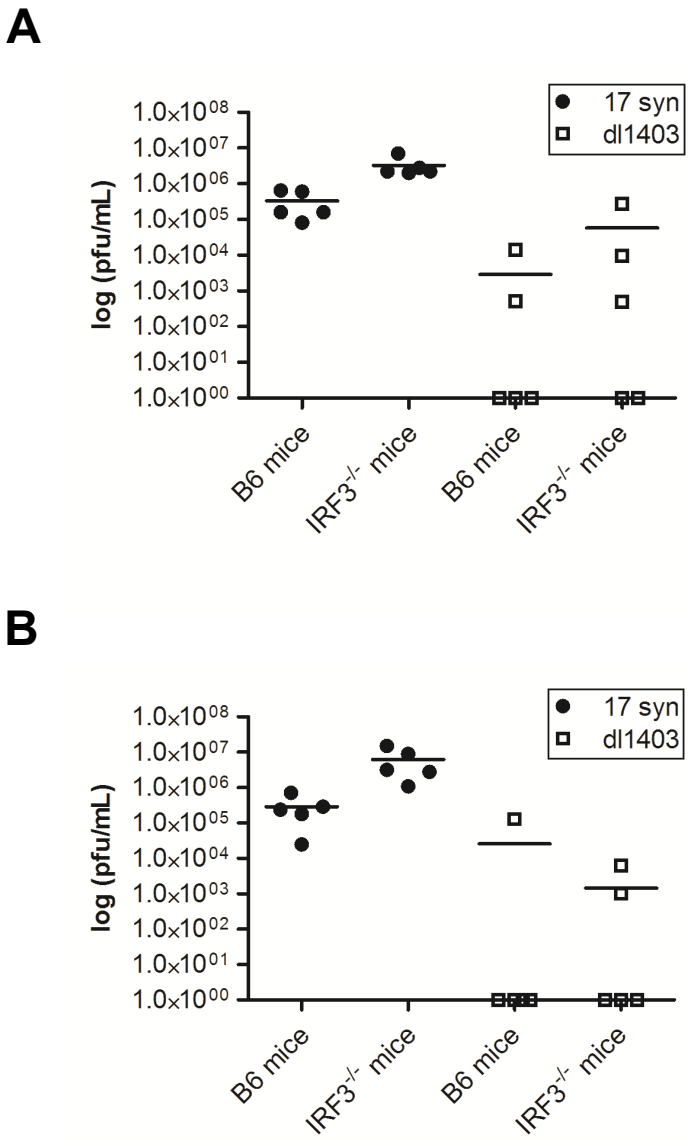


Figure 4.7: IRF3 deficiency cannot compensate for the loss of ICP0 in a mouse model of genital HSV infection. Wildtype B6 or *irf3*$^{-/-}$ mice were intravaginally infected with 1 x 10^5 pfu of the indicated viruses (n=5 for each treatment). Vaginal washes were collected after 1 day in (A) and two days in (B) and titered on U2OS cells in the presence of HMBA.

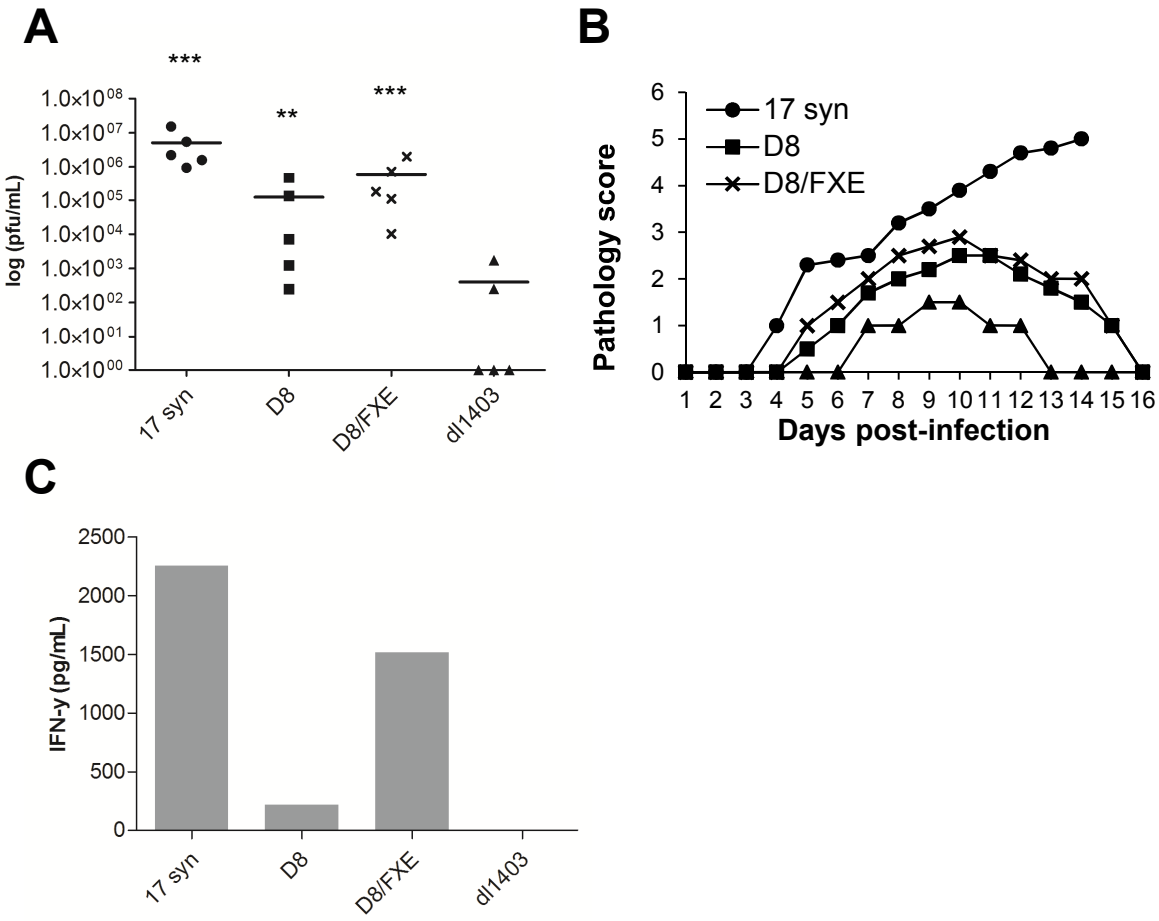


Figure 4.8: Cytoplasmic ICP0 promotes viral replication in the absence of the RING finger domain in a genital mouse model of HSV infection. 1 x 10^5 pfu of the indicated viruses were used to intravaginally infect *irf3*$^{-/-}$ mice (n=5 for each treatment). In (A) vaginal washes were collected after 2 days, and titered on U2OS cells in the presence of HMBA. Statistical analysis was performed using one-way ANOVA and Dunnett's post test relative to dl1403, ** $p<0.01$, ***$p<0.001$ In (B), vaginal pathology was monitored daily and scored on a 5 point scale. In (C), vaginal washes collected after 2 days were pooled, and ELISA was used to measure IFNγ levels.

CHAPTER 5
INVESTIGATION OF INTERACTION PARTNERS FOR CYTOPLASMIC ICP0

KE Taylor and KL Mossman conceived and designed the experiments. KE Taylor performed the experiments.

5.1 Abstract

The novel functions I identified for ICP0 in the cytoplasm – namely, its ability to both block antiviral responses and promote virus replication – are intriguing, but precisely how ICP0 performs these activities is unclear. To further characterize its mechanism of action, I investigated several strategies to identify novel cytoplasmic interaction partners for ICP0. Addition of a tandem affinity purification (TAP) tag to either terminus of ICP0 was found to negatively affect its localization and expression. However, I found several potential binding partners for ICP0 using untagged protein, co-immunoprecipitation, and quantitative mass spectrometry. I further investigated one of these prospects, WD repeat domain 11 (WDR11), confirming its interaction with ICP0. I also found that this protein undergoes a dramatic change in localization at late times after HSV-1 infection, moving from defined perinuclear structures to a dispersed cytoplasmic distribution. This reorganization is specific to HSV infection, as it does not occur during infection with alternative viruses, and appears to require the presence of ICP0. However, knockdown of WDR11 was not found to affect the ability of either wildtype or ICP0-null HSV-1 to replicate in cultured human fibroblasts, meaning that further study is required to understand the significance of this interaction.

5.2 Introduction

It has been said that "if you need to know the measure of a man, you simply count his friends" (732). Likewise, to truly understand how a protein functions, it is essential to characterize its binding partners (733). It is exceedingly rare for a protein to act in isolation; instead, cellular functions rely on the combined actions of associated proteins. Protein-protein interactions can be defined as the non-random physical association of two or more proteins occurring in response to a specific cellular condition (734). Such interactions may be stable – for example, the binding of structural components such as tubulin α and β – or more transient, which includes most interactions involved in signal transduction, that must respond to changes in the cellular environment (735). Many proteins are multifunctional, and a single protein can have entirely different functions depending on which specific partner it is interacting with – which can in turn be influenced by a variety of factors, including its location, its post-translational modifications, the environmental conditions, the cell type, the levels of co-factors and binding partners, and the phase of the cell cycle (733-735). Studying protein-protein interactions can be particularly useful in characterizing the role of an unknown protein, by linking it to partners of known function.

There are many strategies to identify proteins interacting with a specific target. One of the most common is co-immunoprecipitation (co-IP), where an antibody specific for the protein of interest is immobilized to a resin, and then incubated with the cellular lysate, resulting in the retention of both the target protein as well as its interaction partners. Co-purifying proteins are commonly identified using mass spectrometry. As the success of a co-IP can be limited by the availability of a high affinity, specific antibody for the desired protein, the related strategy of affinity purification can serve as an alternative. In this case, the protein of interest is tagged with one of a large number of affinity tags, which include small epitope flags such as six histidines $(His)_6$, FLAG, and hemagglutinin (HA), or larger tags such as calmodulin-binding peptide (CBP), GFP, and glutathione-S-transferase (GST). Although this strategy requires experimental manipulation and expression of exogenous protein, and tagging can disrupt protein

localization or function and may increase non-specific binding, it does have the advantage of commercially available, highly specific reagents that allow for straightforward recovery of the target protein.

A significant concern when using single step co-IP or affinity purification protocols is that they produce relatively high rates of false positive interaction partners (736). In general, there are four classes of proteins found in the eluent after co-IP or affinity purification – the target protein itself, specific binding partners of the target protein, proteins non-specifically binding to the beads, tubes, antibodies, or tags, and proteins that cross-react with the antibody (737). All of these proteins will be identified by analysis of the eluent by mass spectrometry, and it is difficult to know which belong to the first two, desirable, classes, and which to the second two, undesired, groups. It can be particularly challenging to differentiate specific yet low abundance interaction partners from highly abundant but low affinity background proteins (738). Since the validation of potential binding partners is a time consuming and expensive process, it is important to minimize the risk, as much as possible, of inadvertently characterizing a protein that is in fact nothing but a background contaminant.

Simply increasing the stringency of washing steps in purification protocols may decrease background binding, but will also result in the loss of low abundance or low affinity true interaction partners, which may have biological relevance. Therefore, alternative strategies have been devised. For example, the TAP tag combines gentle washing with two successive purification steps to decrease background proteins without losing true interaction partners (739). The original TAP tag consisted of a protein A tag linked to the CBP tag (740), but several variations and enhancements on the original TAP tag have since been created (739). The consecutive purification steps result in decreased co-purification of non-specific background proteins, as contaminants surviving the first purification step may be lost in the second round. However, though gentle, the extensive washing and long times involved in this multiple phase approach still tends to cause the loss of specific but low-affinity or transient binding partners. Additionally, the large size of TAP tags may have a more disruptive effect on protein folding and function (736).

An alternative strategy is to combine low-stringency washes along with a mechanism to identify and exclude contaminant proteins. For example, the protein frequency library (PFL) is a database that records the frequency with which every protein in the human proteome is detected in co-IP and pulldown experiments, helping to determine whether a particular protein is a frequent contaminant (741). Perhaps the most useful approach of all, however, is to have a mechanism for differentiating background proteins from specific interaction partners within a particular experiment. This can be accomplished with a technique known as quantitative immunoprecipitation combined with knockdown (QUICK) (737), which unites a form of quantitative mass spectrometry referred to as stable isotope labeling with amino acids in cell culture (SILAC) (742), with RNA interference (RNAi) and co-IP. The SILAC strategy relies on two facts – first, mammalian cells are unable to synthesize several amino acids, referred to as essential amino acids, which must be supplied in the growth media. Secondly, isotopically labeled amino acids are available that, though chemically identical to naturally occurring amino acids, have a greater mass. Therefore, if a heavier analog of an essential amino acid is provided in the growth media instead of the corresponding natural version, then the heavy amino acid will be incorporated into new proteins as they are produced. After sufficient cell doublings, every protein in the cell will contain the heavy amino acid, with no change in protein function. Two populations of cells can thus be maintained – those grown in light media, with light proteins, and those grown in heavy media, with correspondingly heavier proteins. Next, RNAi is used to knock down the protein of interest in only the heavy population, and co-IP experiments are then performed in parallel with extracts from the light and heavy cells. The eluents are then analyzed using mass spectrometry, which can differentiate whether a particular peptide is derived from the cells grown in heavy versus light media due to the corresponding differences in their mass. Because the target protein is not present in the heavy population, eluents from these cells will lack both the protein of interest and its associated interaction partners – but background proteins binding to alternative components will remain unchanged. Eluents from the light cells, however, will be enriched for the target and its associates – but levels of the

contaminants will be approximately equal to that found in the heavy eluents. Thus, the ratio of light to heavy peptides will be high for the target protein and specific binding partners of that protein, but 1:1 for background proteins (737). This allows non-specific proteins to be differentiated from true interaction partners.

Although the functions of cytoplasmic ICP0, both in blocking the antiviral response in a RING-dependent but proteasome-independent manner, and in promoting viral replication in a RING-independent manner, are highly intriguing, the mechanism through which it produces these effects is unknown. To further characterize these actions of ICP0, I investigated proteins interacting with ICP0 in the cytoplasm. Initial attempts to identify binding partners using the TAP tag strategy proved unsuccessful, as the TAG disrupted the localization and expression of ICP0. However, an adapted version of the QUICK technique was used to successfully identify novel binding partners for ICP0 in the cytoplasm. The significance of one of these potential associates for ICP0, the poorly characterized cellular protein WDR11, was investigated in detail. Although depletion of this protein did not affect viral replication in cultured cells, WDR11 was found to undergo a dramatic change in cellular localization at late time post-infection, in an HSV-specific manner that appears to be dependent on ICP0 expression, suggesting that this interaction may be important only in certain cellular contexts or purely *in vivo*.

5.3 Results

5.3.1 The TAP tag disrupts ICP0 expression, localization, and function

In the pursuit of proteins involved in the cytoplasmic actions of ICP0, I first investigated whether the TAP tag affected the localization and function of ICP0. A virus expressing an ICP0 construct tagged at the N-terminus with the Stratagene Interplay TAP tag had previously been generated in our lab, but had not been characterized. I infected HEL cells with wildtype HSV-1 or the virus expressing the TAP-tagged ICP0 (TAP), and ICP0 localization was determined via immunofluorescence at various times post-infection (figure 5.1A). As expected, wildtype ICP0 initially localized to the nucleus but had mostly moved to the cytoplasm by 8hpi. In contrast, the expression of the TAP-tagged ICP0 appeared to be reduced, as ICP0 was undetectable at 4hpi and was only very faintly visible at 8hpi. In addition, the TAP tag caused ICP0 to be retained in the nucleus, even as late as 24hpi. I then determined the ability of the TAP-tagged ICP0 to inhibit the antiviral response by examining the expression of ISG-56 after infection via RT-PCR (figure 5.1B). While wildtype HSV-1 efficiently blocked antiviral signaling, TAP was unable to prevent the expression of ISG-56, which is consistent with the nuclear restriction of the TAP-tagged ICP0. Since the goal of this work is to identify proteins interacting with ICP0 in the cytoplasm, especially those involved in its ability to inhibit the antiviral response, this virus is clearly unsuitable.

It has been reported that if the function of a protein is impaired by a TAP tag, simply switching the tag to the opposite terminus can restore the protein's activity (743). Therefore, I commenced the production of a virus with an ICP0 construct tagged with TAP at the C-terminus, in the hopes that this would be less disruptive to the behavior of ICP0. I generated plasmid constructs with a C-terminal TAP tag for wildtype ICP0 as well as for cytoplasmic mutants D8 and D8/FXE, to be used for homologous recombination to create the desired virus. Prior to producing the virus, I determined the expression and localization of the ICP0 constructs by transfecting the plasmids into HEL cells (figure 5.2). Although, as expected, D8/FXE-TAP localized to the cytoplasm, while wildtype ICP0-TAP was found in the nucleus when expressed exogenously, the presence

of the TAP tag decreased the expression of all ICP0 constructs. In particular, no expression of D8-TAP could be detected whatsoever, suggesting that the TAP tag may disrupt the structure or stability of ICP0, even when located at the C-terminus. Because of these concerns, I did not pursue the construction of these viruses.

5.3.2 Single-step purification of ICP0 does not reveal novel cytoplasmic interaction partners via total protein stain of SDS-PAGE gels

Given that the tandem affinity method appeared to be unsuitable for the purification of ICP0, I next investigated a single-step purification approach. Therefore, I obtained a virus expressing FLAG-tagged ICP0 from a collaborator (744). Although a single tag does not allow for as stringent a purification as the TAP tag system, the FLAG tag is considerably smaller and less disruptive to protein folding and function (736). Indeed, a FLAG-tagged ICP0 expressing virus has been found to replicate normally at low MOI, suggesting that the FLAG tag does not disrupt ICP0 function (744). Consistent with the low stringency of this approach, standard affinity purification with anti-FLAG reagents (figure 5.3A) revealed high levels of background contaminants, with many proteins present in both the mock and FLAG-ICP0 samples, compared to only a few unique bands in the presence of ICP0. Unexpectedly, performing equivalent co-IP experiments using wildtype virus with untagged ICP0, and an ICP0-specific antibody, produced similar results to the affinity purification for FLAG, suggesting that the ICP0 antibody has a sufficiently high affinity and specificity to be used to identify interaction partners, and thus the FLAG tagged construct provided no clear advantage (figure 5.3B).

Performing co-IP experiments using the anti-ICP0 antibody has the distinct benefit of allowing me to identify binding partners for untagged ICP0 mutants. Of particular interest is to compare proteins interacting with the D8 versus the D8/FXE ICP0 mutants – since D8 blocks antiviral signaling, and D8/FXE does not, any partner that associates with D8 and not D8/FXE may be involved in the mechanism through which ICP0 prevents the activation of IRF3. However, no novel bands were observed for D8 which were not present in D8/FXE (figure 5.3C). In fact, only two bands were

reproducibly observed after co-IP, which were not present in control samples. To identify these, eluents for co- IP experiments were split in two and used to run two duplicate gels. One gel was then stained for total protein via Sypro Ruby while the second gel was transferred and analyzed via Western blotting (figure 5.3D). Comparison of the total protein stained gel with the Western blot showed that one of the unique bands probably corresponded to ICP0 itself, while the other was likely USP7, a well-known interaction partner for ICP0 (430, 678, 679).

5.3.3 Novel cytoplasmic interaction partners for ICP0 are identified using SILAC

Since few unknown, unique bands were observed after co-IP in the presence of ICP0, compared to control reactions performed in the absence of ICP0, standard mass spectrometry analysis, where specific bands are cut from the gel to be analyzed and identified, was not appropriate. However, it has been previously shown that while two eluents may not have any remarkable differences visible after total protein staining of an SDS-PAGE gel, distinctions in the composition of these samples can be detected using the SILAC technique (745). Therefore, I used an adaptation of the QUICK strategy to further investigate potential interaction partners for cytoplasmic ICP0 (figure 5.4). Since my protein of interest is a viral rather than a cellular protein, I used an ICP0-null virus instead of RNAi. Also, to allow for the simultaneous comparison of three samples, intermediate or "medium" weight isotope-substituted amino acids were used in addition to the traditional heavy and light isotopes (746).

Therefore, I used the adapted QUICK strategy combined with triple SILAC to identify interaction partners for the D8 and D8/FXE ICP0 mutants. HEL cells grown in SILAC media containing $^{13}C_6$, $^{15}N_4$-Arginine and $^{13}C_6$, $^{15}N_2$-Lysine (heavy) or $^{13}C_6$-Arginine and 4,4,5,5-D4-Lysine (medium) or with natural amino acids $^{12}C_6$, ^{12}N4-Arginine and $^{12}C_6$-Lysine (light) were infected with dl1403, D8, or D8/FXE, respectively. Co-IPs were performed on cytoplasmic extracts with an anti-ICP0 antibody. Quantitative mass spectrometry was then used to compare the peptide abundance ratio for particular proteins between the light and heavy isotopes, or the medium and heavy isotopes, thus

comparing proteins found in the D8/FXE or D8 samples with those found in the dl1403 control sample. Since any protein appearing in the ΔICP0 (heavy) sample could not be a true interaction partner for ICP0, any protein with a 1:1 ratio between light/medium and heavy peptides can be ruled out as a non-specific contaminant. In contrast, higher ratios suggested a protein specifically interacting with the D8 or D8/FXE ICP0. Results are summarized in table 5.1 (cellular proteins) and table 5.2 (viral proteins). Several known or proposed interaction partners for ICP0 were identified, including the well-characterized USP7, confirming the validity of this technique. The detection of DNA-PKcs in the D8 and D8/FXE eluents is also intriguing, as an ICP0-DNA-PKcs interaction has been previously proposed but could not be confirmed (420).

Initially, I had intended to compare peptide ratios between the D8/FXE and D8 samples (light: medium ratio), to identify components potentially involved in the ability of ICP0 to inhibit IRF3. However, as discussed in Chapter 4, I found that ICP0 levels were much higher after infection with D8/FXE compared to D8 (figure 4.5). Therefore, the levels of the two ICP0 mutants were sufficiently different as to make a direct comparison of peptide ratios impractical. However, I did identify some proteins that appeared to interact with D8 ICP0 but not D8/FXE ICP0 (table 5.3), which may play a role in the ability of ICP0 to block antiviral signaling.

5.3.4 WDR11 is relocated to the cytoplasm during HSV-1 infection

Of the possible binding partners identified for ICP0 using SILAC, the most intriguing is WDR11, a poorly characterized member of the WD-repeat family of proteins. WDR11 has a very high abundance ratio for both D8/FXE and D8, yet it is very rarely identified in eluents after co-IP/pull-down assay, as determined by the PFL database (741), making it an excellent candidate for further investigation. I first validated the ICP0-WDR11 interaction by reciprocal co-IP (figure 5.5). Accordingly, WDR11 was confirmed to be present after immunoprecipitation with an anti-ICP0 antibody, and ICP0 was present after immunoprecipitation with an anti-WDR11 antibody. Next, I investigated the localization of WDR11 during an HSV-1 infection using

immunofluorescence (figure 5.6). In uninfected cells, WDR11 was found to have a characteristic perinuclear distribution. However, treatment with wildtype HSV-1 caused a dramatic relocalization of WDR11, which became distributed throughout the cytoplasm. To determine the timing of this rearrangement, HEL cells were fixed at 2, 4, 6, 8, 10, and 12hpi, and examined via immunofluorescence. Representative images are shown in figure 5.7. No change in WDR11 localization was observed until relatively late after infection, at approximately 10-12hpi.

To investigate whether this re-localization was dependent on ICP0, immunofluorescence was performed after infection with dl1403, as well as D8, FXE, D8/FXE and 17syn (figure 5.8). At 12hpi, only 17 syn was able to alter the location of WDR11. At 16hpi, however, both D8 and D8/FXE seemed to join 17 syn in the ability to disperse WDR11, while FXE and dl1403 did not. By 24hpi, cells infected by all viruses other than dl1403 showed WDR11 distributed throughout the cytoplasm, suggesting that ICP0 lacking a functional RING finger domain, restricted to the cytoplasm, or both, was capable of altering the location of WDR11. However, this effect is delayed when ICP0 is mutated, and seems to correlate with viral replication, as shown in figure 4.5 – the fastest kinetics occurred in 17 syn, which also replicates the best, followed by D8 and D8/FXE, which replicate to intermediate titers, and finally by FXE, which is a highly attenuated virus. However, though dl1403 reaches similar titers as FXE at 24hpi, redistribution of WDR11 was not observed in cells infected by this virus, suggesting that ICP0 itself is required for this effect.

To investigate the idea that WDR11 re-localization correlates better with efficient viral replication as opposed to a specific activity of ICP0, I determined the localization of WDR11 after infection with HCMV, SeV or VSV, or after treatment with pI:C or IFNα (figure 5.9). MOIs were adjusted so that an approximately equal cytopathic effect was observed after all viral infections. Interestingly, no change in the location of WDR11 was observed after any of these treatments. I also confirmed that my observations with HSV-1 are truly a relocation of WDR11 as opposed to a degradation by determining the level of WDR11 in whole-cell extracts at 16hpi (figure 5.10). No change in WDR11 protein levels

was observed after treatment with either wildtype HSV-1 or any of the mutant ICP0-expressing viruses.

5.3.5 WDR11 is not required for HSV-1 replication in cultured cells

In light of the dramatic reorganization of WDR11 observed during HSV-1 infection, I next investigated whether WDR11 plays a role in viral replication. HEL cells were treated with siRNA against WDR11, which resulted in a strong decrease in WDR11 protein levels (figure 5.11A). However, there was no significant difference in the replication of either 17 syn or dl1403 at high or low MOI in the WDR11-deficient cells, compared to control siRNA-treated cells (figure 5.11B and C). WDR11-defiency also did not affect viral growth after pre-treatment of cells with IFNα (figure 5.11D), nor did examining viral replication at earlier times reveal a difference between WDR11-deficient and control cells (figure 5.11E). Therefore, despite the profound relocation of WDR11 during HSV-1 infection, the protein does not appear to be important for viral replication in cultured human fibroblasts.

5.4 Discussion

Identifying and characterizing protein-protein interactions is a powerful strategy to reveal the inner workings of complex cellular phenomena. Using triple SILAC and an adapted version of the QUICK strategy, I identified several potential cytoplasmic cellular and viral interaction partners for ICP0. Since little is known about the activities of ICP0 in this compartment, these findings are a valuable starting place to further our understanding of this key viral protein. Importantly, I also validated a novel interaction of ICP0 with the poorly understood cellular protein WDR11. This protein was found to have a distinct perinuclear localization in uninfected cells, but underwent a profound re-localization at late times after HSV-1 infection. This redistribution of WDR11 was unique to HSV, and was not induced by infection with other viruses or by activation of antiviral signaling. WDR11 reorganization was not observed after infection with an ICP0-null virus, and was delayed but not absent for HSV-1 expressing ICP0 restricted to the cytoplasm, lacking a functional RING finger domain, or both. However, depletion of WDR11 had no effect on the ability of wildtype or ICP0-null HSV-1 to replicate in cultured human fibroblasts. Although additional study is needed to determine the significance and purpose of this interaction, this provides an additional clue to the mechanism of action of cytoplasmic ICP0.

5.4.1 Strategies for the identification of cytoplasmic interaction partners for ICP0

The disruption of the function of ICP0 by the addition of the TAP tag is perhaps not surprising. The movement of ICP0 from the nucleus to the cytoplasm is exquisitely sensitive to experimental manipulations, and partial or complete nuclear restriction of ICP0 can result from everything from the expression of exogenous ICP0 in the absence of other viral proteins (478, 695), the disruption in ICP0 of the binding site for coREST (500) or cyclin D3 (691), the use of viruses lacking ICP27 (690) or with constitutively phosphorylated VP22 (692), to the transfection of any DNA prior to infection (688), or the use of drugs inhibiting the proteasome (478), HDACs (688), viral DNA replication (478) or the activity of cdk4 (689). Therefore, the retention of ICP0 with an N-terminal

TAP tag in the nucleus is not unexpected. The decrease in the expression observed for ICP0 tagged with a C-terminal TAP tag, even when simply expressed from a plasmid, is less easily explained. In general, it has been found that tags at the N- versus the C-terminus are superior in increasing the expression of recombinant proteins (reviewed in (747)). The observed decrease in TAP-tagged ICP0 levels could represent anything from impaired protein production due to formation of deleterious secondary structures in the mRNA itself, to increased protein degradation as a result of misfolding (747). It is also possible that the structure of the tagged ICP0 had simply been altered sufficiently as to impair antibody binding, decreasing the detection of the protein. Regardless of the exact cause, however, the outcome is clear – TAP-tagged ICP0 is not suitable for the investigation of cytoplasmic binding partners.

The strategy that was ultimately successful was, in fact, the opposite of the TAP approach – using endogenous, untagged protein combined with a low-stringency purification that preserved both low-abundance and low-affinity true interaction partners as well as, inevitably, many background proteins. Instead of trying to eliminate these non-specific proteins with harsh washing conditions and risking the loss of potentially biologically relevant proteins, I instead used an adapted version of the QUICK technique to identify and exclude proteins associating with the tubes, beads, or having non-specific or cross-reactive interactions with the antibody. It is important to note that this strategy cannot identify as background proteins those that non-specifically interact with ICP0 or its binding partners. Such binding can occur, for example, because cell lysis disrupts compartmentalization, allowing proteins that do not normally have access to one another to come into contact (733). In some cases, these proteins can be ruled out through the use of the PFL, since a protein appearing frequently in this database is often found in eluents of co-IPs regardless of the specific target protein – and may therefore represent a “sticky” protein with promiscuous binding that is therefore unlikely to represent a true interaction partner. Indeed, USP7, which has a well-characterized binding site in ICP0 (430, 678, 679), has a PFL frequency of only 7%.

It is also worth mentioning that although cytoplasmic extracts were used for the immunoprecipitations, a number of nuclear proteins were identified in the mass spectrometry results, particularly several histone proteins, which had elevated SILAC ratios. This may be explained by the alterations of the nuclear envelope that occur as a result of the high level of traffic of HSV capsids from the nucleus to the cytoplasm during assembly (222), or via the dramatic expansion of the nuclear pore that has been reported to be induced by HSV (220), which may allow nuclear components to escape into the cytosol. Indeed, I consistently found that while cytoplasmic extracts from uninfected cells were free of nuclear proteins, samples prepared after HSV infection frequently contained nuclear contaminants (data not shown). The high PFL frequencies of these histone proteins suggest that they likely interact nonspecifically with ICP0 upon disruption of compartmentalization.

5.4.2 The significance of the WDR11-ICP0 interaction

At this time, it is difficult to determine the significance of the WDR11-ICP0 interaction identified in this work. WDR11 was found in only 0.5% of co-IP results in the PFL database, yet had a very high SILAC ratio of 15.49 for D8/FXE and 2.92 for D8, indicating that this protein likely had a strong and specific interaction with ICP0, making it an excellent candidate for further investigation. Accordingly, I was able to confirm the ICP0-WDR11 interaction in reciprocal co-IP reactions, demonstrating the validity of this methodology. However, during the course of this investigation, I made two puzzling observations – first, while WDR11 undergoes a dramatic re-localization in a manner unique to HSV infection, this protein is not necessary for the replication of HSV-1 in cultured cells. Secondly, though ICP0 specifically interacts with WDR11, and ICP0 is one of the first proteins produced during an HSV infection, the relocation of WDR11 does not occur until late times post-infection. This is true even with viruses expressing ICP0 mutants that are constitutively cytoplasmic, so it is not simply a matter of requiring sufficient accumulation of ICP0 in the cytoplasm. Nor is the RING finger of ICP0 required for this effect. Most surprising of all is that a virus expressing the FXE mutant of

ICP0, which is largely restricted to the nucleus, still causes the redistribution of WDR11. This is difficult to explain if cytoplasmic ICP0 is required for this effect, but may represent the leakage of FXE ICP0 to the cytoplasm at late times post infection (data not shown). In general, viruses expressing mutated ICP0 show a delay, but not an absence, of WDR11 dispersion, which could reflect the partially impaired replication of these viruses. Indeed, the kinetics of WDR11 displacement mirrored the final titers reached by the ICP0-mutant expressing viruses. Additionally, since ICP0 has been found to prevent the activation of IRF3 at early times post-infection (373), while the relocation of WDR11 occurs late, and since even ICP0 mutants that cannot block antiviral signaling can eventually disperse WDR11, it seems unlikely that the ICP0-WDR11 interaction is involved in preventing the activation of IRF3.

It is important to note that given the timing of WDR11 movement, it can be envisioned that a viral late protein is also involved with ICP0 in this relocation, which must accumulate to a sufficient level prior to causing the diffusion of WDR11, and that expression of this protein is delayed when ICP0 is mutated. Although further work is needed to examine this hypothesis, the situation with WDR11 could be analogous to that of cyclin D3 and IFI16. Both of these proteins have been found to interact with ICP0, and both undergo changes (stabilization for cyclin D3 and degradation for IFI16) during infection with wildtype but not ICP0-null viruses (38, 432). However, in both cases, when conditions were adjusted so that the ICP0-null virus replicated as well as the wildtype, the alleged "ICP0-mediated" effect was found to, in fact, occur in the absence of ICP0 (476, 748). This highlights the importance of considering the indirect effects of the presence of ICP0 on the promotion of viral replication when a phenotype is observed during wildtype but not ICP0-null infection. However, this also makes it unclear whether the strong WDR11-ICP0 interaction is truly involved in producing the relocation of WDR11.

5.4.3 What is the cellular function of WDR11?

The purpose of the WDR11-ICP0 interaction is unclear, particularly since although it is widely expressed in human tissues (749) and highly conserved in

vertebrates (750), the function of WDR11 is poorly understood. Disruption of the WDR11 gene has been found in both human glioma cells (749) and breast cancer cells (751) – suggesting that it may have a role as a tumor suppressor – as well as in in patients with idiopathic hypogonadotropic hypogonadism or the related Kallmann syndrome, conditions characterized by low sex steroids and delayed puberty (750, 752). The mechanism through which the absence of WDR11 produces these diverse phenotypes is not known. The WD-repeat family of proteins, of which WDR11 is a member, comprises over 250 human proteins, with roles in diverse cellular functions, including transcription, chromatin assembly, cell cycle progression, signal transduction, cytoskeletal organization, apoptosis, vesicular fusion and pre-mRNA splicing (reviewed in (753)). First identified in the G protein transducin (754), the WD-repeat domain is a loosely conserved motif that can be defined by a regular expression – an algebraic description of a peptide sequence, specifying the most probable residues at any given location and the range of possible spacings between amino acids, while permitting alternative residues in any particular position (753). Each WD-repeat domain folds into four antiparallel β strands, and repeated WD motifs in a protein form a propeller-like arrangement, with each WD-repeat resulting in a blade in a circular structure (755-757). A protein model was generated for WDR11 based on its homology to actin interacting protein 1 (AIP1), a WD-repeat protein for which the crystal structure has been determined (758). This suggested that WDR11 contains 12 WD-repeats, nine of which form two consecutive β-propellers (750).

Despite their shared sequence motif and predicted structure, WD-repeat proteins have a great deal of functional diversity, and the WD-repeats themselves do not seem to exhibit any catalytic activity (759). Instead, the common role of all members of the WD-repeat family seems to be the coordination of multi-protein complexes, with the propeller structure providing a stable scaffold for several simultaneous protein-protein interactions (753, 759). Indeed, WDR11 has been suggested to interact with multiple proteins. For example, screens have identified WDR11 as a potential interaction partner for STAT3 (760), as well as EMX1, Tagln2, Ndrg4, Nrxn3, and Hey1 (750), although the significance of these interactions is not yet known. Hey1 is of particular interest here, as it

has been found to be degraded via the ubiquitin ligase activity of the RTA protein from the gammaherpesvirus KSHV (761). Like ICP0, RTA is an activator of viral replication and transcription, is involved in the switch between the latent and lytic phases of the replication cycle (reviewed in (762)), and degrades both repressors of viral gene expression (763) and components involved in antiviral signaling (764). Hey1 is a cellular transcriptional repressor (765) that has been suggested to be involved in the maintenance of KSHV latency, which is combated by RTA (761). A similar role for Hey1 in HSV latency can be envisioned, which could be inhibited by the ICP0-WDR11 interaction. However, Hey1 was not identified in my SILAC results, and so a direct interaction between Hey1 and ICP0 may not occur.

WDR11 was also identified in a large screen for interaction partners for the multifunctional p97 protein (766), which has been implicated in shuttling ubiquitinated proteins to the proteasome for degradation, as well as membrane fusion, cell-cycle regulation, and transcriptional control (reviewed in (767)). Relevant to ICP0, almost all the varied tasks of p97 have been found to involve the recognition of ubiquitinated substrates (768). Further characterization revealed that WDR11 interacted with the UBX domain-containing protein UBXD7, which subsequently binds to p97 (766). UBXD7 recognizes ubiquitin chains, and acts as one of many adapter proteins that allow p97 to bind to its substrates (769). Interestingly, UBXD7 was also found to directly interact with several E3 ubiquitin ligases, and immunoprecipitates of UBXD7 were found to contain a higher proportion of K11-linked versus K48-linked ubiquitin, potentially implicating this protein in atypical ubiquitin signaling (766) – which makes the potential interaction of UBXD7 with ICP0, via WDR11, intriguing.

The distinctive perinuclear distribution I observed for WDR11 in uninfected human fibroblasts is worthy of further investigation. WDR11 was initially described as having a cytoplasmic distribution in neuroblasts and U2OS osteosarcoma epithelial cells (750). However, the characteristic pattern observed for WDR11 in HEL cells suggests that it may be associated with specific cytoplasmic organelles such as the endoplasmic reticulum, TGN, autophagosome, mitochondria, or early endosome. In keeping with this,

analysis of WDR11 with the algorithm PSORT II (770, 771), which predicts protein localization based on the amino acid sequence, suggested that WDR11 was most likely to be found in the cytoplasm and in vesicles of the secretory system. This second group includes both proteins targeted to the lysosome for degradation via autophagy as well as proteins passing through the *trans*-Golgi network.

5.4.4 A potential role for the WDR11-ICP0 interaction in the regulation of autophagy

Clearly, co-localization experiments using organelle markers are necessary to definitively characterize the subcellular location of WDR11 in primary human fibroblasts. However, the potential localization of this protein to the autophagic pathway is of particular interest. Autophagy is a process used for the turnover of long-lived proteins as well as the removal of larger entities, including redundant or damaged organelles and protein aggregates (reviewed in (772, 773)). Targets are engulfed into double lipid bilayer vesicles known as autophagosomes, which then fuse with the lysosome, leading to the degradation of their cargo (773). The mechanism through which cytoplasmic components are directed to the autophagic pathway is incompletely understood. Interestingly, like the proteasomal system, this is thought to involve the recognition of ubiquitin chains, via the action of adaptor proteins that bind both the polyubiquitinated cargo and components of the autophagy machinery (reviewed in (774)). Autophagy is involved in the maintenance of homeostasis, as well as the degradation of nonessential cellular components under starvation conditions, so that their constituents can be recycled to support vital cellular processes until growth conditions improve (775, 776). Moreover, autophagy has been found to contribute to the immune response in multiple ways, ranging from the simple degradation of intact pathogens (775, 777) to more complex roles in adaptive immunity (reviewed in (773)). In the innate antiviral response, autophagy can help deliver cytoplasmic PAMPs to endosomal TLRs (778), while reciprocally, several TLRs can activate autophagy upon ligand binding (779, 780). In contrast, autophagy can negatively regulate RLR signaling (652, 781, 782), and autophagy pathway component Atg9a has also been found to be involved in the inhibition of STING (783). The details of how these

apparently opposing actions of autophagy ultimately impact on IFNα/β production are currently unclear (775).

Consistent with its potential antiviral effects, autophagy has been found to have an inhibitory action against several viruses (784-788), though a variety of others have found ways to exploit autophagy, or components of autophagy, for their own uses (789-807). HSV has been reported to inhibit the induction of autophagy through the actions of ICP34.5, both through its ability to counteract PKR activity (808, 809) as well as via direct inhibition of Beclin 1, a cellular protein essential for promoting autophagy (787). Activation of autophagy in response to the detection of HSV-1 genomic DNA requires not IRF3 (810) but STING (811), in a pathway that is not fully understood. In pDCs (778) or bone marrow-derived dendritic cells and macrophages (811), the inhibition or absence of autophagy leads to decreased IFNα/β production after HSV infection.

Pertinent to this discussion, WDR11 has been identified in a large screen for proteins and interactions involved in autophagy (812). More recently, a second screen found that depletion of WDR11 sensitized cells to the AB-type toxin ricin (813). Interestingly, in many ways ricin behaves like a viral pathogen, binding cell-surface receptors, entering via endocytosis, appropriating intracellular trafficking pathways for its own uses, and causing translational arrest and cell death via interaction with the ribosome (reviewed in (814, 815)). Given the association of ICP0 with WDR11, and the well-known propensity for ICP0 to counteract intrinsic antiviral defenses (412), this could suggest that WDR11 is involved in cellular resistance to viruses, in addition to toxins. It has been suggested that WDR11 may achieve this effect by playing a role in toxin degradation, as depletion of WDR11 lead to an increase in intracellular ricin (813). Inhibition of autophagy similarly leads to increased ricin levels, and WDR11 was found to partially colocalize with the autophagosome marker LC3 (813), seemingly confirming the involvement of this protein in the autophagic system. Interestingly, this study also found that WDR11 interacts with C17orf75 (813). Although the function of this protein is unknown as well, like WDR11, its depletion increases cellular susceptibility to ricin, and these two proteins are thought to be components of the same pathway (813). C17orf75

was identified in my SILAC results, and found to have a high SILAC ration of 10.3 for D8/FXE and 3.9 for D8, although only one peptide was identified for C17orf75, meaning that these results were not considered to be significant. Additionally, C17orf75 was consistently identified in preliminary pre-SILAC optimization experiments as interacting with ICP0, but was never found in ICP0-null eluents. This supports the idea of a WDR11-C17orf75 interaction and suggests that this protein may also be involved in the activity of ICP0 mediated through WDR11.

Could ICP0 be targeting WDR11 to interfere with autophagic response? The fact that the knockdown of WDR11 via siRNA did not have any effect on the replication of either wildtype or ICP0-null HSV-1 in human fibroblasts is consistent with previous literature showing that in mouse embryonic fibroblasts, autophagy does not limit viral replication (652, 816), though autophagy does appear to inhibit HSV in L929 murine fibroblasts (817). Additionally, a virus expressing ICP34.5 deficient in Beclin-1 binding, and thus the inhibition of autophagy, showed no growth defect in cultured human SK-N-SH neuroblastoma cells (787). *In vivo*, however, the importance of ICP34.5-mediated inhibition became more significant, as a virus incapable of blocking Beclin-1 activation was neuroattenuated in mice, in terms of both survival and viral replication (787). This is a pattern that has been observed on multiple occasions – the effect on viral replication of the loss of a particular cellular or viral protein in cultured cells does not necessarily reflect the outcome in an animal. For example, although a virus lacking viral protein vhs replicates to wildtype levels in cultured cells, it is highly attenuated in mice (359). Therefore, the absence of an effect of WDR11 depletion on viral replication does not preclude a function for this protein in viral replication *in vivo*. A related point is that HSV does not exclusively replicate in fibroblasts in a living organism – a key portion of the viral lifecycle takes place in neurons. Therefore, although the function of WDR11 may be dispensable for viral replication in cells of the periphery, it may have a crucial function in viral activities in neurons. Along these lines, the role of autophagy in resistance to HSV-1 has been found to be cell-type specific even *in vivo*, as it is important for the protection of neurons, and not for keratinocytes of the vaginal mucosa, after intravaginal infection of

mice (818). This may occur because apoptosis of neurons, which are post-mitotic, is not a feasible antiviral strategy, and so autophagy may be more significant to the control of virus in this cell type (775).

The relocation of WDR11 cannot simply be explained by the activation of autophagy by HSV, since I did not observe a similar change in distribution after infection with VSV or HCMV, both of which have been found to induce autophagy with similar kinetics to HSV (788, 810). In particular, like HSV, the HCMV genome can induce autophagy at early times post-infection, but this is counteracted by viral inhibition of Beclin-1 (819). This suggests that the ICP0-WDR11 interaction itself results in the change in WDR11 localization, and could represent an additional mechanism of decreasing autophagy. Although ICP34.5 has already been implicated in blocking autophagy, the inhibition of antiviral signaling by both ICP0 and ICP34.5 provides an interesting precedent for this idea (392, 393). Alternatively, it is possible that the relocation of WDR11 by ICP0 is another example of a virus commandeering a component of autophagy for its own uses. Further investigation is required to differentiate between these two possibilities.

5.4.5 A potential role for the WDR11-ICP0 interaction in viral egress

It is also possible that HSV is, in fact, exploiting WDR11 for its own uses – but that WDR11 is not actually involved in autophagy. Instead of being localized to components of the autophagy pathway, it is possible that WDR11 is found associated with the TGN – an organelle that is thought to have a key role in HSV egress. As described in Chapter 1, the current model of HSV egress is the de-envelopment/re-envelopment model, which states that the final envelope is derived from a cytoplasmic organelle, currently thought to be the TGN (218). This network of tubules connected with the *trans*-face of the Golgi apparatus functions in the secretory pathway as a sorting station, directing cargo into distinct carriers which transport them to their final cellular destinations (reviewed in (247, 248)). HSV egress is thought to be mediated via the classical pathway of cargo transport from the TGN to the plasma membrane (246).

The dramatic redistribution of WDR11 that occurs at late time points after infection – approximately 10-12 hpi – is difficult to reconcile with the autophagy process, which has been found to be induced as early as 2hpi (810). During an HSV infection, capsids first appear in the nucleus at 4hpi, envelopment at the nuclear membrane occurs by 6hpi, followed by a striking increase in cytoplasmic capsids from 8-10hpi, and association of new capsids with glycoproteins from 10-12hpi (221, 820). Exponential viral release occurs between 8-15hpi (259, 821), with greater surface budding of mature particles at 12hpi relative to 8hpi (238), and cell lysis not occurring until 24-48hpi (820). Interestingly, it has been reported that both the Golgi and the TGN are disrupted during HSV infection (236, 237, 251, 822-824), with markers for both structures losing their tight perinuclear organization and becoming scattered throughout the cytoplasm at approximately 8-12hpi (237, 823). This correlates nicely with the dispersal I observed for WDR11. The reason and mechanism behind this movement is currently unknown. It has been suggested that the redistribution of TGN components is a specific effect of HSV late proteins, as opposed to a byproduct of envelopment (825), as blocking viral DNA synthesis prevented Golgi dispersal (823). However, neither inhibiting nor augmenting the fragmentation of the Golgi impacts viral replication in cell culture (822), casting into doubt whether this redistribution is required for efficient egress.

Therefore, the change in location of WDR11 late in infection is consistent with a potential localization of this protein to the TGN. In addition, the perinuclear localization of WDR11 at early times post-infection is similar to perinuclear accumulations of host and viral proteins that have been previously observed during HSV infection, which are thought to consist of sites of assembly (428, 826-833). Indeed, ICP0 itself has been previously identified within such perinuclear aggregates, where it was found to interact with pUL46 (428). Could WDR11 be important in HSV egress? Clearly further study is necessary to answer this question. I found that siRNA-mediated downregulation of WDR11 did not decrease viral titers. However, interfering with the transport of viral particles to the cell surface has been previously shown to disrupt viral yields in the supernatant media to a larger degree than yields of cell-associated virus (227). Therefore,

if WDR11 is involved in a step downstream of secondary envelopment, such as the transport of mature virions in vesicles to the plasma membrane, my strategy of harvesting both cells and supernatants may have concealed this effect. Titering the virus found only in the supernatant media would help to resolve this. Additionally, it is relevant to note that there are connections between the autophagy response and the TGN. For example, inhibiting TGN trafficking also blocks autophagy, and the small GTPase Rab1 has been implicated in both autophagy and in the secondary envelopment of HSV-1 (834, 835). Therefore, the potential role of WDR11 in autophagy does not preclude its localization in the TGN or a function in HSV envelopment or egress.

Consistent with a potential role of ICP0 (and WDR11) in viral assembly and egress, several of the viral proteins I identified in SILAC have been implicated in these processes, including pUL26 (836), pUL34 (222), US3 (232), pUL48 (837), gB (240, 838), gE (839), pUL36 (840) and pUL37 (841, 842) and VP22 (833). The potential interaction of ICP0 with proteins involved in egress, coupled with its binding to WDR11, a protein potentially located at a key site in HSV envelopment, could indicate a possible function for ICP0 in the assembly and egress pathway of the virus. However, since many of these interaction partners are also virion components, this may simply be a consequence of the intricate protein-protein interactions occurring between tegument proteins. In fact, of the 25 top proteins with the highest SILAC ratios, 16 are known components of the mature HSV virion, with an additional 3 that have been identified as potential components (pUL26 (843), TK (844) and ICP22 (845)), and a fourth that is a component of the primary virion, though lost during maturation (pUL34 (232)). Since ICP0 itself is a tegument component (846), this may represent the fact that tegument proteins bind to one another, as well as to the cytoplasmic tails of envelope glycoproteins on one side, and the capsid proteins on the other side – a feature that is important in linking all virion components together into a whole (reviewed in (222)). Therefore, my SILAC results for viral proteins may simply reflect how highly interconnected the constituents of the HSV particle are.

The activities of ICP0 in the cytoplasm remain mysterious, and yet it accomplishes vital functions in this location, both blocking the activation of innate antiviral signaling and stimulating viral replication. To truly understand the functions of ICP0 in the cytoplasm, the mechanisms through which it produces these effects need to be determined. Here, I have identified several potential binding partners for cytoplasmic ICP0, which may be implicated in its various roles. I also confirmed the novel interaction of WDR11 with ICP0. Although WDR11 undergoes a dramatic relocation in an HSV and possibly ICP0-specific manner, it is not yet clear whether this redistribution has a role in mediating the function of the ICP0-WDR11 interaction, or is simply a downstream consequence. Additionally, though depletion of WDR11 did not impair the replication of HSV-1 in cultured cells, this does not preclude a possible role for this complex in neurons or *in vivo*. Indeed, WDR11, and thus ICP0, may well be involved in the regulation of autophagy or in viral egress. Therefore, while further characterization is needed to understand the significance of this complex, this represents an intriguing development in our understanding of both the cytoplasmic actions of ICP0, as well as the role of the little-known cellular protein WDR11.

5.5 Acknowledgements

I thank M.D. Weitzman for the N-terminal TAP-tagged ICP0 construct, W.P. Halford for the FLAG-tagged ICP0 expressing virus, and A.L. Kroeker, P. Ezzati and K.M. Coombs for their assistance with the SILAC experiments.

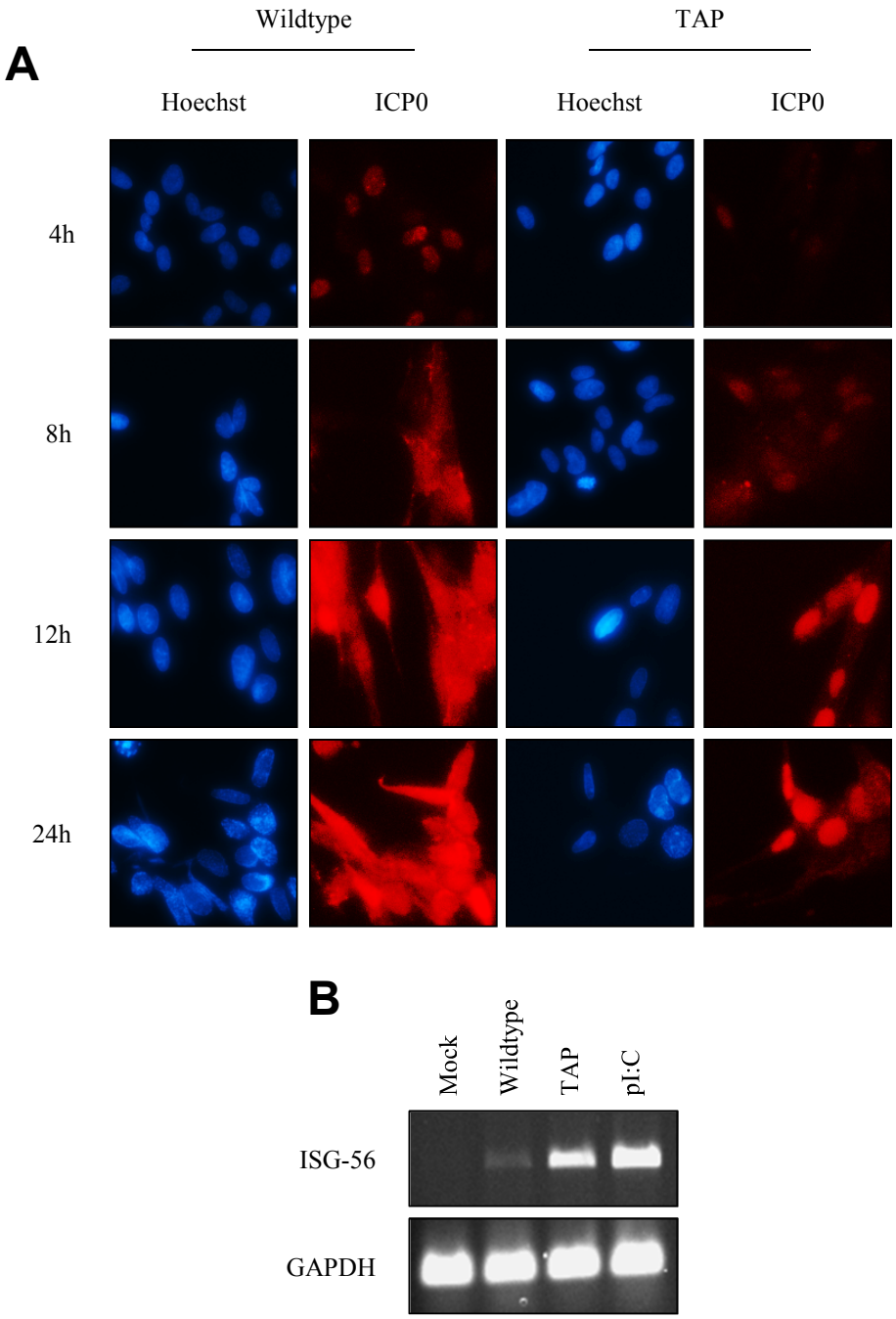


Figure 5.1: **An N-terminal TAP tag disrupts the localization and function of ICP0.** HEL cells were infected with wildtype HSV-1 or virus expressing TAP-tagged ICP0 (TAP) at an MOI of 10. In (A), cells were fixed at the indicated times (in hours) after infection, and analyzed for ICP0 localization via immunofluorescence. Nuclei were identified using Hoechst stain. In (B), RNA was harvested at 6hpi, and expression of ISG-56 and GAPDH was determined via RT-PCR.

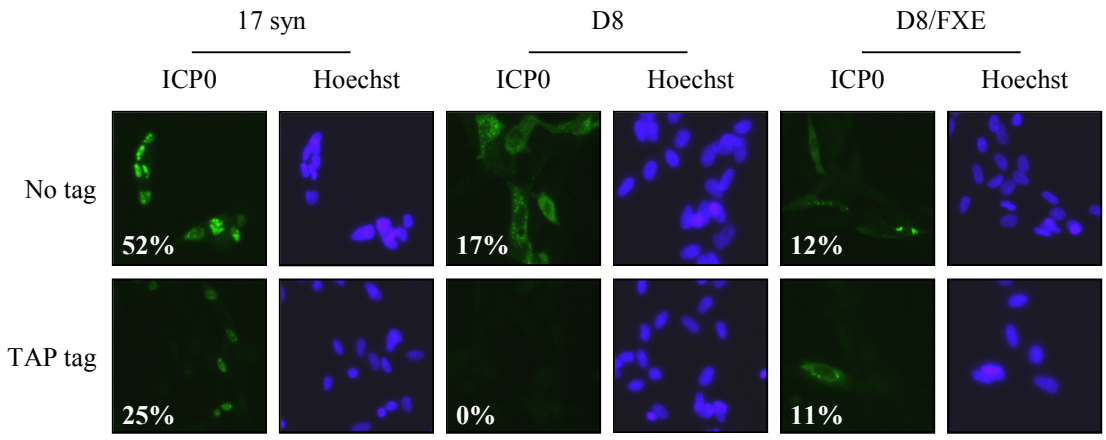


Figure 5.2: **ICP0 mutants expressed from a plasmid in HEL cells localize correctly but show decreased expression due to a C-terminal TAP tag.** HEL cells were nucleofected with 2µg of plasmids encoding wildtype ICP0 (WT), or ICP0 deletion mutants lacking either the nuclear localization signal (NLS, D8) or both the RING finger domain and the NLS (D8/FXE), in the presence or absence of a C-terminal TAP tag. 24 hours after nucleofection, cells were analyzed for the expression of ICP0 via immunofluorescence. Transfection efficiencies are indicated. Nuclei were identified using Hoechst stain.

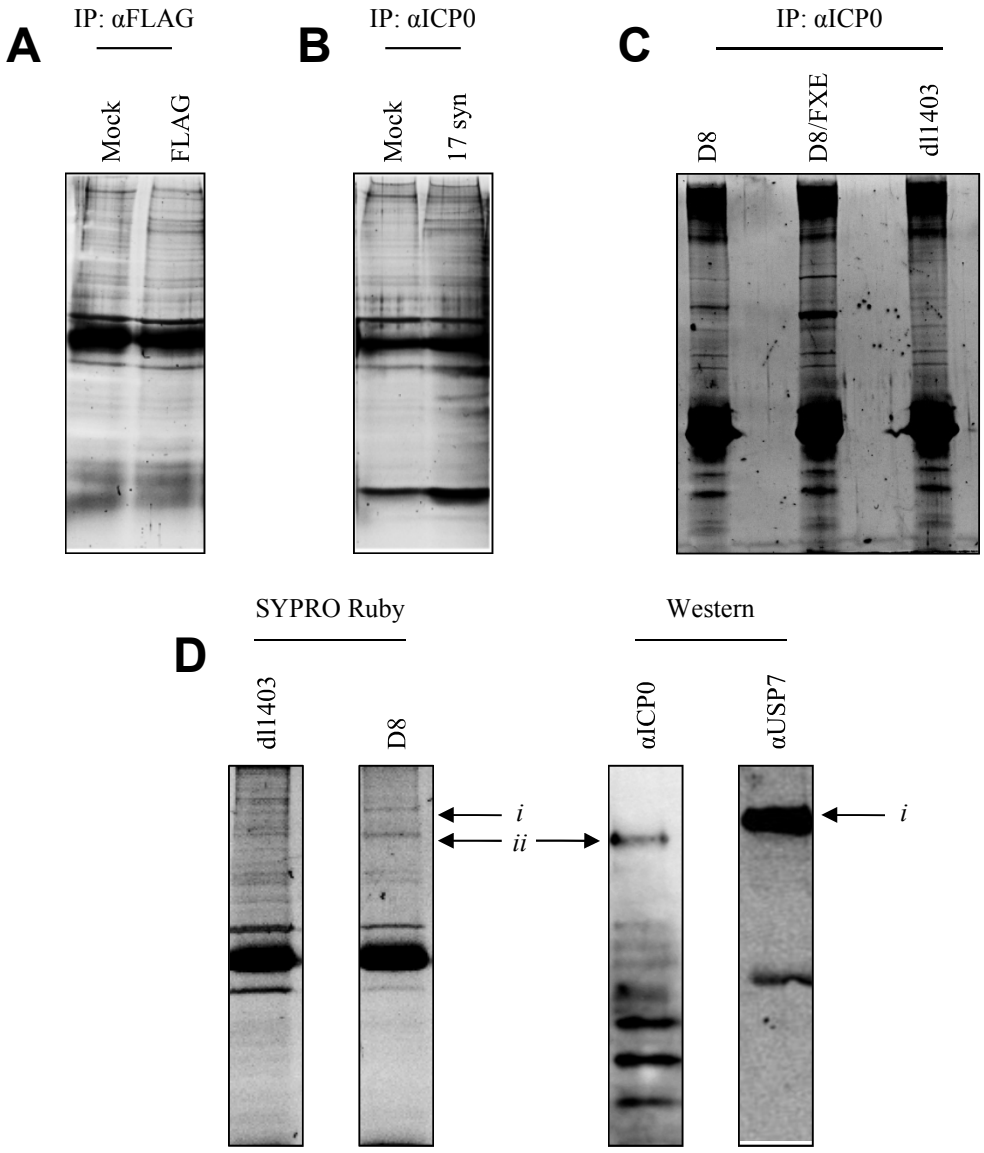


Figure 5.3: **Comparison of control and specific eluents after co-immunoprecipitation of FLAG-tagged and endogenous ICP0 revealed no evident novel cytoplasmic interaction partners.** HEL cells were infected with the indicated viruses at an MOI of 10. After 8 hours, cytoplasmic extracts were performed, and processed via immunoprecipitation, with the indicated antibody in (A) and (B), or with an αICP0 antibody in (C) or (D). Eluents were run on an SDS-PAGE gel. In (A), (B) and (C), gels were subsequently stained using SYPRO Ruby and visualized using UV light, while in (D), the eluent was split in two, and two duplicate gels were run. One gel was then stained with SYPRO Ruby and the second was analyzed via Western blot with the indicated antibodies, as shown. Arrows in (D) show the corresponding locations between the gels and the blots.

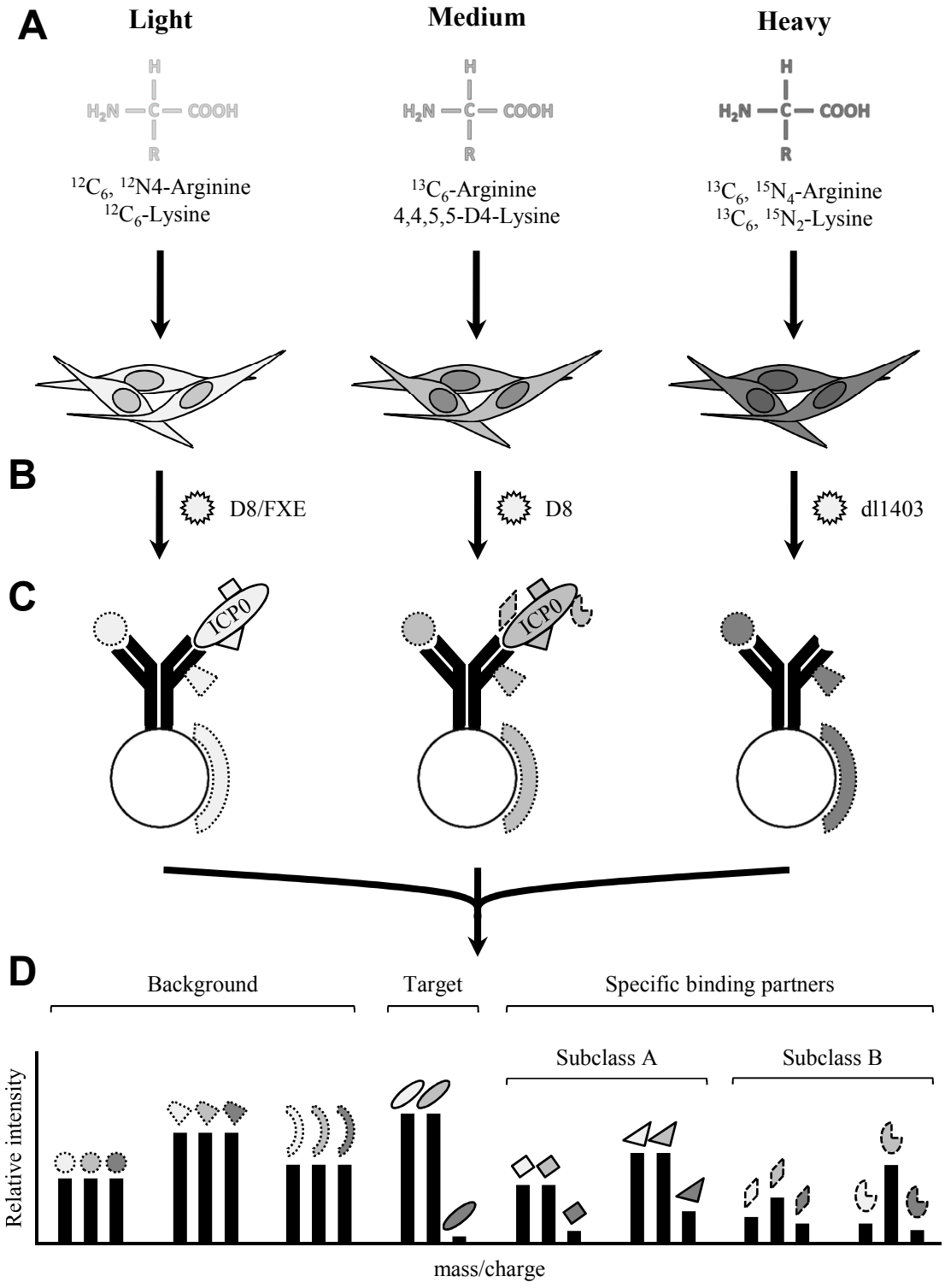

A
Light
Medium
Heavy
$^{12}C_6$, ^{12}N4-Arginine
$^{12}C_6$-Lysine
$^{13}C_6$-Arginine
4,4,5,5-D4-Lysine
$^{13}C_6$, $^{15}N_4$-Arginine
$^{13}C_6$, $^{15}N_2$-Lysine
B
D8/FXE
D8
dl1403
C
ICP0
ICP0
D
Background
Target
Specific binding partners
Subclass A
Subclass B
Relative intensity
mass/charge

Figure 5.4: **A general overview of an adapted QUICK strategy using triple SILAC and an ICP0-null virus.** In this technique, growth media is first supplemented with natural amino acids (light), or isotopically labelled amino acids of medium or heavy weight. HEL cells are then passaged in this media until all proteins have incorporated the labelled amino acids (A). Each population of cells is then infected with a different virus – the light cells with D8/FXE, the medium cells with D8, and the heavy cells with the ICP0-deficient dl1403 (B). At 8hpi, cytoplasmic extracts are generated and co-IPs performed for ICP0. The cells infected with dl1403 lack ICP0 and so the heavy proteins recovered in eluents from these cells must consist of non-specific background proteins, while light and medium proteins in the eluents from the cells infected with D8/FXE and D8 are expected to consist of both these same background proteins as well as specific interaction partners for ICP0. Since D8 blocks antiviral signaling and D8/FXE does not, the medium eluent may also contain proteins involved in preventing IRF3 activation, which would be lacking in light eluents (C). Samples are then analyzed via quantitative mass spectrometry, with a schematic illustration shown in (D). Peptides derived from light, medium and heavy proteins can be differentiated, and the relative abundance of a particular protein in each population determined. Proteins found in a 1:1 ratio for medium: heavy or light: heavy are considered to be non-specific contaminants, while proteins with a higher ratio have an increased probability of being true binding partners for ICP0. Of these specific associates, those with a high ratio in the medium but not the light samples (subclass B) may play a role in the ability of ICP0 to block IRF3 activation, while those found to have elevated ratios in both the light and medium cells (subclass A) may be involved in alternative functions of ICP0, such as promoting viral replication. Figure adapted from Selbach and Mann, 2006 (6).

Table 5.1: Potential cellular binding partners for D8 and D8/FXE ICP0 identified by SILAC

	D8/FXE: dl1403	D8: dl1403	PFL (%)
WD repeat-containing protein 11 (WDR11)	15.49	2.92	0.5
Histone H4 (HIST1H4A)	13.85	1.52	77.8
Ubiquitin carboxyl-terminal hydrolase 7 (USP7)	12.28	2.90	7.0
DNA-dependent protein kinase catalytic subunit (PRKDC)	5.65	2.48	53.0
Histone H2B type F-S (H2BFS)	5.40	1.60	81.6
Histone H2A type 1-D (HIST1H2AD)	4.46	1.57	63.2
Ras GTPase-activating protein-binding protein 2 (G3BP2)	2.66	2.16	35.7
ELKS/Rab6-interacting/CAST family member 1 (ERC1)	2.30	2.96	17.8
Ribosome-binding protein 1 (RRBP1)	2.18	1.74	31.9
ADP/ATP translocase 2 (SLC25A5)	2.15	2.28	65.4
Tubulin alpha-1A chain (TUBA1A)	2.14	2.56	53.0
Protein FAM164A (FAM164A)	2.14	1.66	0
Isoform 2 of Polyadenylate-binding protein 4 (PABPC4)	2.13	2.20	48.1
ADP/ATP translocase 3 (SLC25A6)	2.13	2.83	56.2
Matrin-3 (MATR3)	2.09	1.49	53.5
Pleckstrin homology-like domain family B member 1 (PHLDB1)	2.07	1.83	0.5
Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)	2.07	2.14	53.5
Elongation factor 2 (EEF2)	2.07	2.03	57.3
Heterogeneous nuclear ribonucleoprotein K (HNRNPK)	2.01	1.78	61.1

HEL cells were grown in DMEM supplemented with light, medium or heavy amino acids for 3 doublings, then infected with D8/FXE, D8 or dl1403, respectively, at an MOI of 10. After 8 hours, cytoplasmic extracts were performed, and processed via immunoprecipitation with an αICP0 antibody overnight at 4°C. Beads were washed and bound proteins were then subjected to on-bead digestion with trypsin overnight at 37°C. Samples were then pooled, and analyzed via high-performance liquid chromatography (HPLC)/mass spectrometry. Highlighted proteins have been previously identified as potential or confirmed interaction partners for ICP0. Ratios higher than 2.0 were considered to indicate a potential interaction. PFL indicates the frequency with which the protein is detected after co-immunoprecipitation/pull-down in all experiments in the PFL database.

Table 5.2: Potential viral binding partners for D8 and D8/FXE ICP0 identified by SILAC

	D8/FXE: dl1403	D8: dl1403	Location
Trans-acting transcriptional protein ICP0	68.77	7.89	Tegument
Capsid protein VP26 (UL35)	41.30	3.56	Capsid
Protease precursor (UL26)	17.52	1.44	Capsid?
Trans-acting transcriptional protein ICP4	16.76	2.56	Tegument
Virion-packaging protein UL17	13.03	1.90	Tegument
Virion-packaging protein UL25	10.88	3.33	Tegument
Tegument protein VP22	10.51	1.59	Tegument
Tegument protein UL47	8.63	0.99	Tegument
Envelope glycoprotein B	7.84	1.56	Envelope
Deoxyuridine 5'-triphosphate nucleotidohydrolase (UL50)	7.56	3.35	Tegument
Alpha trans-inducing factor 78 kDa protein (UL46)	7.45	2.02	Tegument
Serine/threonine-protein kinase (US3)	7.32	1.86	Tegument
Tegument protein VP16 (UL48)	6.68	1.42	Tegument
Ribonucleoside-diphosphate reductase large subunit (UL39)	6.57	1.78	Nucleus
Large tegument protein UL36	6.56	1.74	Tegument
Major capsid protein VP5 (UL19)	5.72	1.28	Capsid
DNA polymerase catalytic subunit (UL30)	5.53	0.56	Nucleus
Transcriptional regulator ICP27 (UL54)	5.34	1.51	Tegument
Virion egress protein UL34	5.13	2.04	1° virion
Thymidine kinase	3.76	1.35	Tegument?
Alkaline exonuclease (UL12)	3.71	1.79	Nucleus
Capsid assembly protein UL37	3.61	0.14	Tegument
Envelope glycoprotein E	3.03	0.30	Envelope
Major DNA-binding protein (DBP)	2.74	1.07	Nucleus
Transcriptional regulator ICP22	2.09	0.51	Tegument?

HEL cells were grown in DMEM supplemented with light, medium or heavy amino acids for 3 doublings, then infected with D8/FXE, D8 or dl1403, respectively, at an MOI of 10. After 8 hours, cytoplasmic extracts were performed, and processed via immunoprecipitation with an αICP0 antibody overnight at 4°C. Beads were washed and bound proteins were then subjected to on-bead digestion with trypsin overnight at 37°C. Samples were then pooled, and analyzed via high-performance liquid chromatography (HPLC)/mass spectrometry. Highlighted proteins have been previously identified as potential or confirmed interaction partners for ICP0. Ratios higher than 2.0 were considered to indicate a potential interaction. The locations of identified proteins in the viral particle (or in the cell, for proteins not forming part of the viral particle) are indicated.

Table 5.3: Potential cellular binding partners for D8 but not D8/FXE ICP0 identified by SILAC

	D8: dl1403	D8/FXE: dl1403	PFL (%)
Plasminogen activator inhibitor 1 (SERPINE1)	4.63	1.21	0
Dolichol-phosphate mannosyltransferase (DPM1)	3.59	0.87	27.6
EMILIN-1 (EMILIN1)	3.49	1.19	0
Lamin-A/C (LMNA)	3.45	1.60	53.0
Collagen alpha-1(VI) chain (COL6A1)	3.31	1.40	0
Tenascin (TNC)	3.11	1.17	1.1
UPF0568 protein C14orf166	2.85	1.30	38.4
Uveal autoantigen with coiled-coil domains and ankyrin repeats (UACA)	2.51	1.97	2.2
Tubulin alpha-1C chain (TUBA1C)	2.50	1.87	62.2
Collagen alpha-1(I) chain (COL1A1)	2.39	1.64	2.2
Protein-glutamine gamma-glutamyltransferase 2 (TGM2)	2.32	1.48	9.7
Triple functional domain protein (TRIO)	2.32	1.84	4.3
Eukaryotic initiation factor 4A-I (EIF4A1)	2.29	1.72	45.9
Fragile X mental retardation syndrome-related protein 1 (FXR1)	2.18	1.30	33.5
Myosin-9 (MYH9)	2.18	1.40	63.8
Insulin-like growth factor 2 mRNA-binding protein 1 (IGF2BP1)	2.17	1.55	31.4
Isoform 4 of Ubiquitin-associated protein 2-like (UBAP2L)	2.16	1.59	25.4
Tubulin beta-3 chain (TUBB3)	2.16	1.71	53.0
Collagen alpha-3(VI) chain (COL6A3)	2.16	1.28	15.1
Collagen alpha-2(I) chain (COL1A2)	2.07	0.73	0
Isoform 7 of Fragile X mental retardation 1 protein (FMR1)	2.03	1.52	26.5

HEL cells were grown in DMEM supplemented with light, medium or heavy amino acids for 3 doublings, then infected with D8/FXE, D8 or dl1403, respectively, at an MOI of 10. After 8 hours, cytoplasmic extracts were performed, and processed via immunoprecipitation with an αICP0 antibody overnight at 4°C. Beads were washed and bound proteins were then subjected to on-bead digestion with trypsin overnight at 37°C. Samples were then pooled, and analyzed via high-performance liquid chromatography (HPLC)/mass spectrometry. Highlighted proteins have been previously identified as potential or confirmed interaction partners for ICP0. Ratios higher than 2.0 were considered to indicate a potential interaction. PFL indicates the frequency with which the protein is detected after co-immunoprecipitation/pull-down in all experiments in the PFL database.

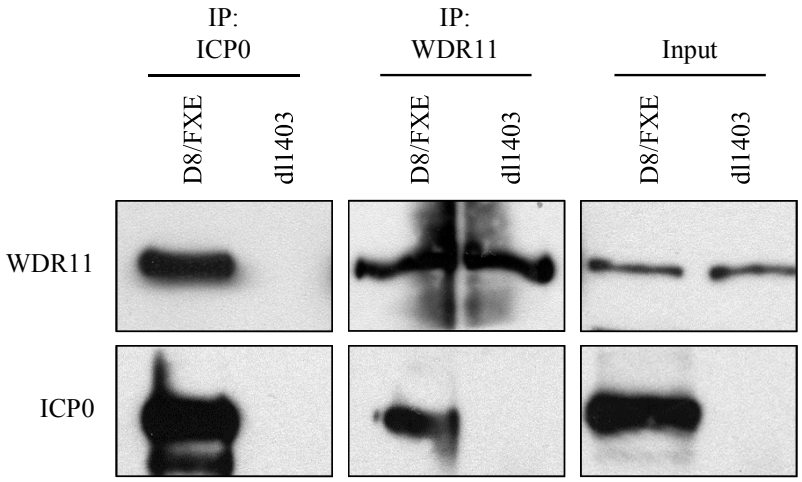


Figure 5.5: ICP0 interacts with WDR11. HEL cells were infected with the indicated viruses for 8 hours, then harvested via cytoplasmic extract and immunoprecipitated with the indicated antibodies. Eluents were then analyzed via Western blot for ICP0 and WDR11.

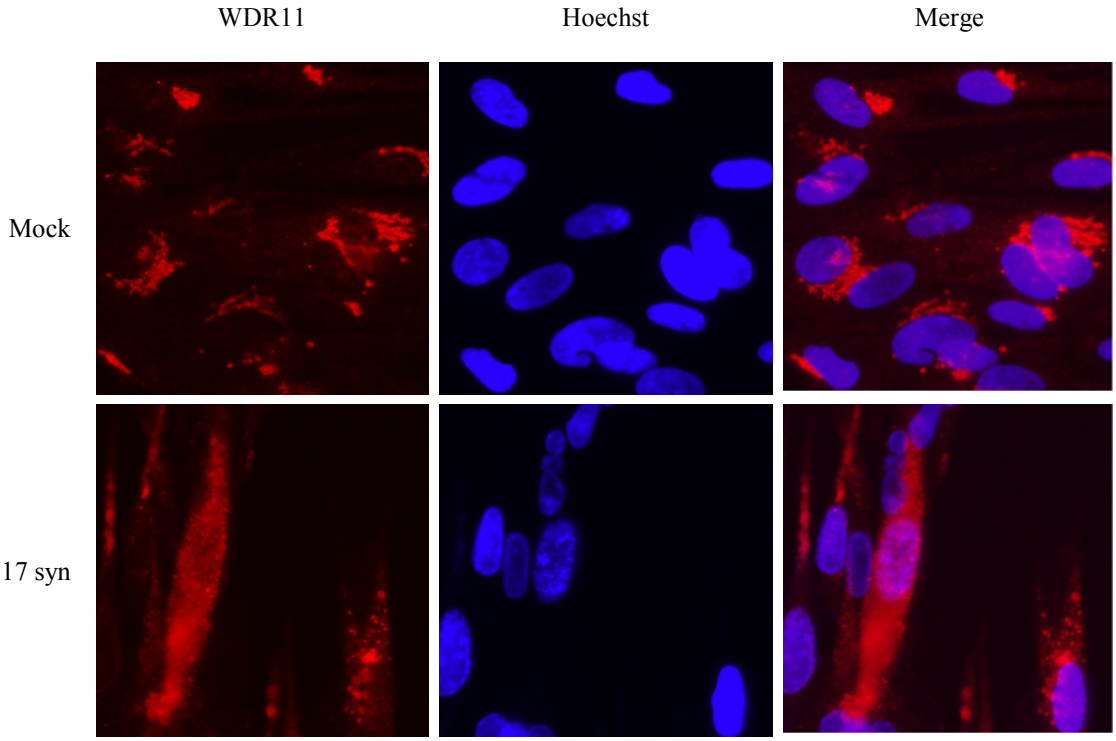


Figure 5.6: WDR11 is redistributed during infection with HSV-1. HEL cells were infected with the indicated viruses for 12 hours, then fixed and analyzed for WDR11 localization via immunofluorescence. Nuclei were identified using Hoechst stain.

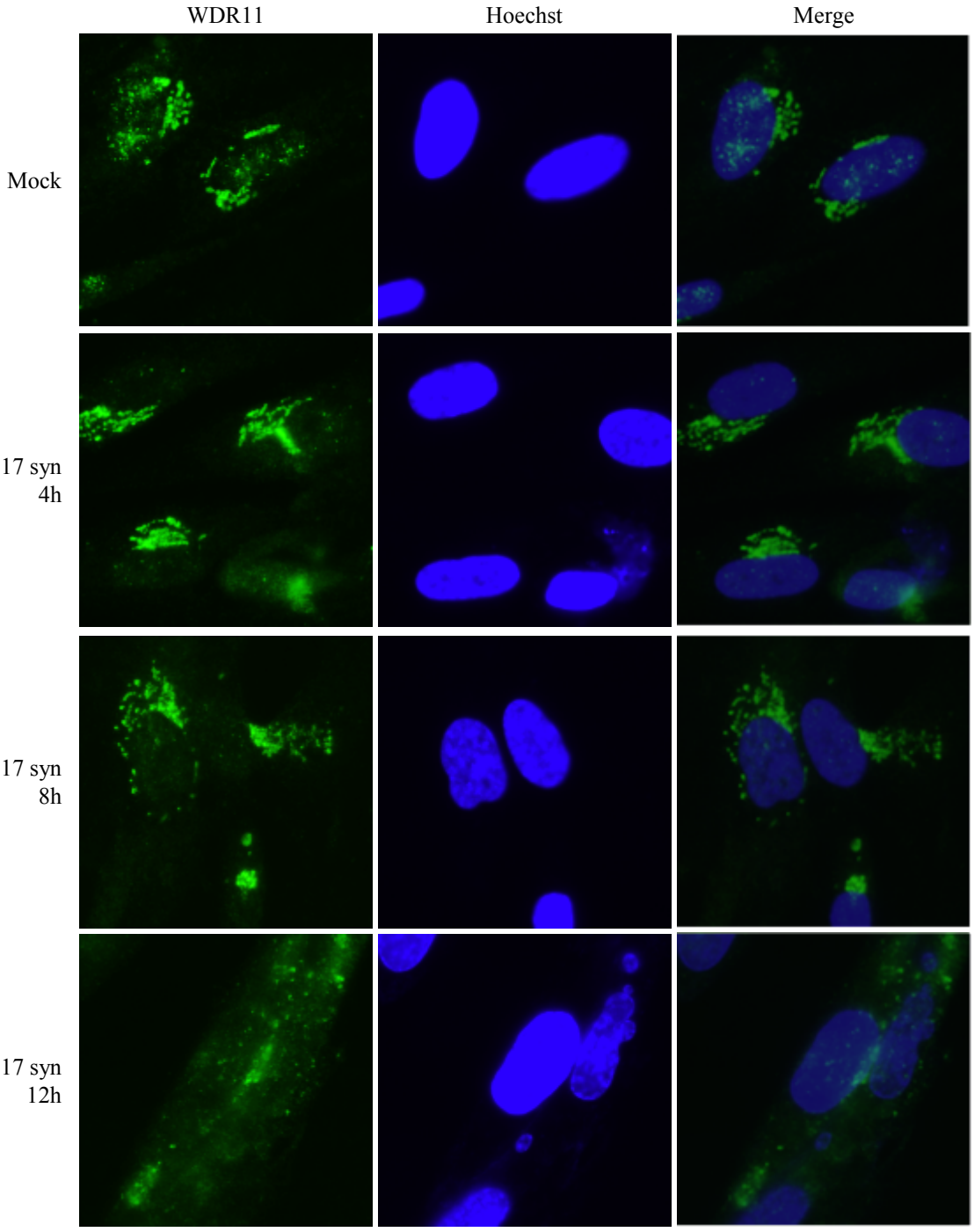


Figure 5.7: The re-localization of WDR11 occurs at late times post-infection. HEL cells were infected with the indicated viruses for the times shown (in hours), then fixed and analyzed for WDR11 localization via immunofluorescence. Nuclei were identified using Hoechst stain.

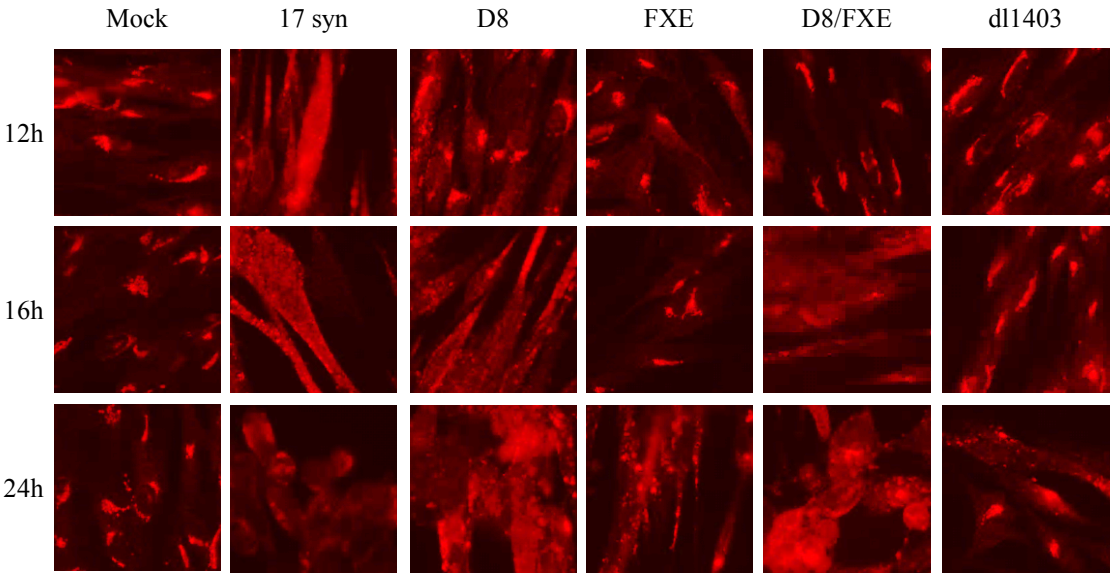


Figure 5.8: Mutation of ICP0 delays but does not prevent the re-localization of WDR11. HEL cells were infected with the indicated viruses at MOI 10 for the times shown (in hours), then fixed and analyzed for WDR11 localization via immunofluorescence.

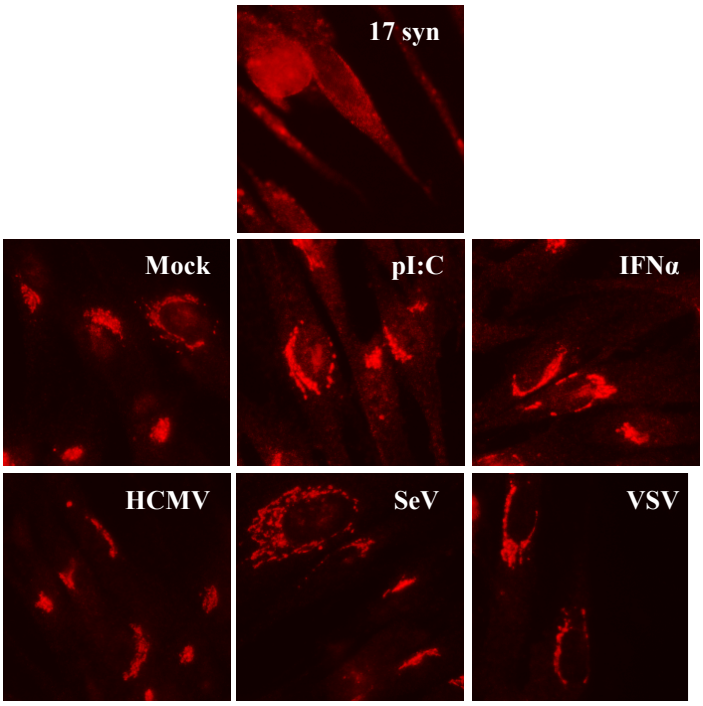


Figure 5.9: WDR11 re-localization is specific to HSV infection. HEL cells were infected with 17 syn (MOI 10), HCMV (MOI 0.5), SeV (80 HAU/10^6 cells) or VSV (MOI 0.1) or treated with pI:C or IFNα for 16 hours, then fixed and analyzed for WDR11 localization via immunofluorescence

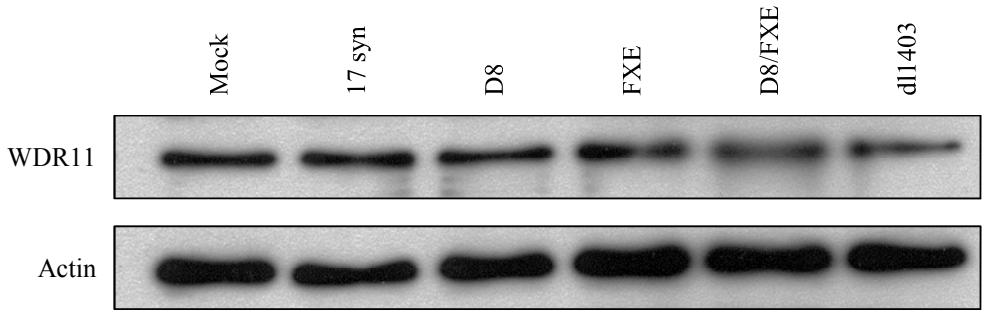


Figure 5.10: WDR11 levels do not change during HSV infection. HEL cells were infected with the indicated viruses for 16 hours, then harvested via RIPA extract and analyzed for WDR11 and actin levels via Western blotting.

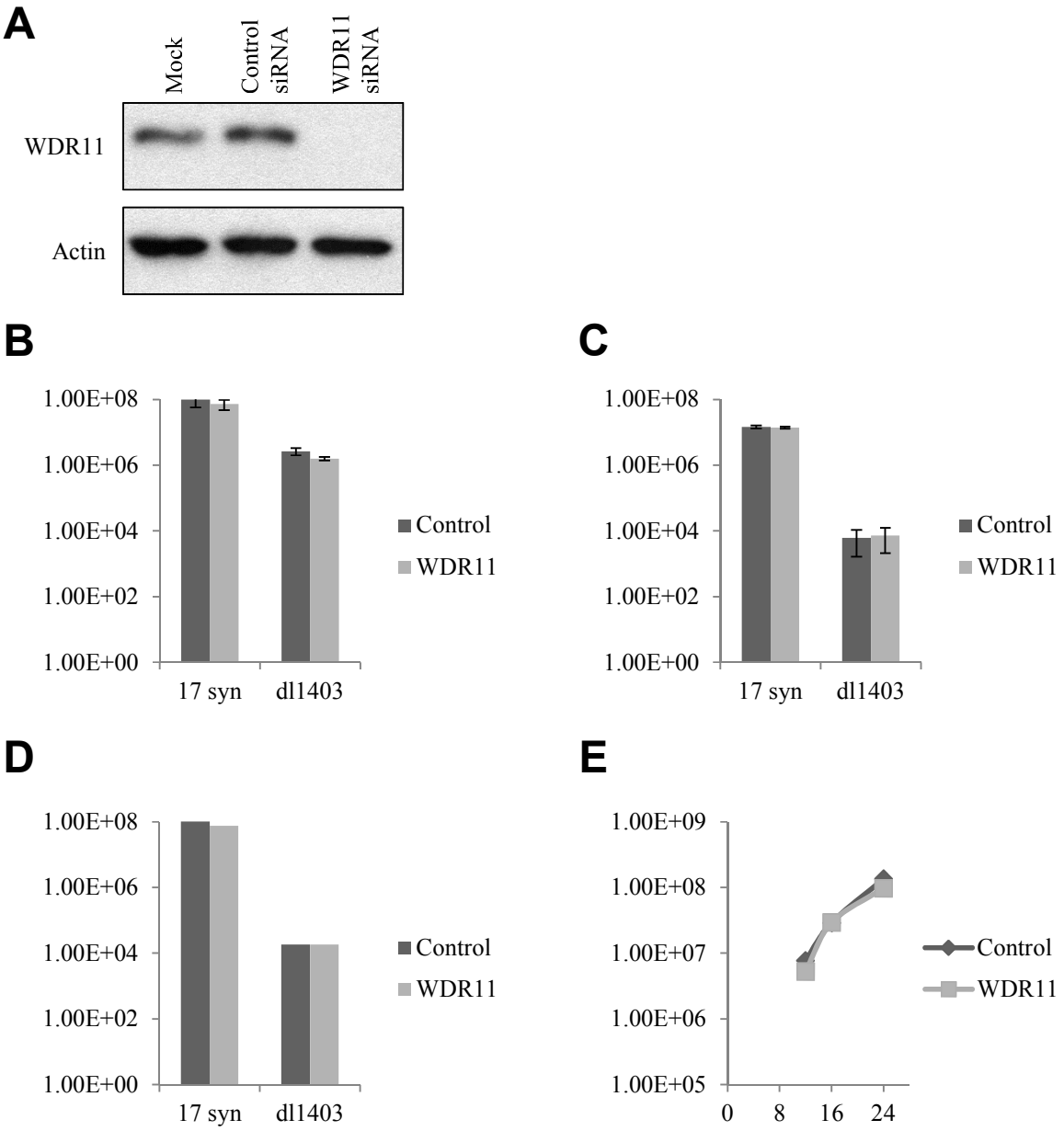


Figure 5.11: WDR11 depletion does not affect HSV replication. HEL cells were treated with WDR11 or control siRNA for 72 hours. In (A), a RIPA harvest was then performed, samples were separated via SDA-PAGE and levels of WDR11 and actin were determined via Western blot. In (B) and (C), cells were then infected with 17 syn or dl1403 at MOI 10 (B) or MOI 0.1 (C) for 24 hours. Cells and supernatant media were then harvested, freeze-thawed three times, and then titered on U2OS cells in the presence of HMBA. In (D), cells were treated with IFNα 64 hours after siRNA transfection, and infected at MOI 10 with the indicated viruses 8 hours later. Cells and supernatant media harvested 24 hours later and were titered as in (B). In (E), cells were infected with 17 syn at MOI 10 and cells and supernatants were harvested at the indicated times post-infection (in hours), then titered as in (B). The data in (B) and (C) are the average of 3 independent replicates.

CHAPTER 6
SUMMARY, IMPACT AND FUTURE DIRECTIONS

Sections of this chapter have been accepted for publication:

Taylor, KE, Mossman, KL. Interference in innate immunity: another dimension in ICP0 multifunctionality. 2014. British Journal of Virology. Accepted June 2 2014.

6.1 Summary of work

It has been reported that 50% of surfaces in an elementary school classroom (847), 53% of objects sampled in daycare facilities (848), 63% of exercise equipment in fitness centers (849), 64% of surfaces in office toilet cubicles (850), and 67% of air samples from airplanes (851) are contaminated by viruses. Given this constant exposure, it is remarkable that the average person is only infrequently stricken with detectable viral disease. The innate antiviral response represents an important first line of defense, with IFN-independent signaling involved in preventing low-level infection from becoming full-fledged disease (9, 103), while the type I IFN response is needed to limit viral replication and protect surrounding cells (852), as well as to launch the adaptive immune response required for the clearance of uncontrolled infections (853). Therefore, early signaling is crucial in determining the ultimate outcome of a virus-host interaction.

In this work, I used a variety of strategies to further characterize the antiviral response. I began by investigating whether the small GTPase Rac1 was involved in the detection of viral entry that activates IFN-independent signaling (Chapter 3) (101, 108, 117). Instead, I found some evidence to suggest that Rac1 plays a role in the negative regulation of ISG production. However, the sensitive nature of the IFNα/β response in primary cells combined with the fact that Rac1 is involved in a tremendous variety of interwoven cellular functions, led to high levels of variability that precluded any definitive conclusions about the role of Rac1 in antiviral signaling. Nevertheless, this experience taught me several key lessons about both study design and technique selection, which were subsequently implemented to improve results in the remainder of this investigation.

I next adopted an alternative approach to identify novel aspects of the antiviral response by characterizing the mechanism through which the HSV protein ICP0 blocks the activation of IRF3 (Chapter 4). Via the creation of a novel virus expressing mutated ICP0 that localizes to the cytoplasm despite the lack of a functional RING finger domain, I determined that the RING finger, which has E3 ubiquitin ligase activity, has a proteasome-independent role in the cytoplasm to prevent the expression of ISGs, possibly

through tagging a protein involved in antiviral signaling with an atypical ubiquitin chain. This is the first report of a function of the RING finger that does not require the action of the proteasome. I also unexpectedly found that cytoplasmic ICP0 has a RING finger-independent function in promoting viral replication, both in cultured cells and in mice, which is exciting, as almost all the replication-promoting activities of ICP0 are currently thought to occur in the nucleus and require the RING finger.

In the final section of this report, I identified proteins that interact with ICP0 in the cytoplasm, which may be helping it to produce its novel proteasome-independent effects on antiviral responses and RING finger-independent functions in enhancing viral replication (Chapter 5). I identified a novel interaction of ICP0 with WDR11, a poorly characterized cellular protein. I found that WDR11 underwent a dramatic relocation from a distinct perinuclear arrangement to a diffuse cytoplasmic distribution in an HSV and possibly ICP0-specific manner, although the absence of WDR11 did not affect viral replication in cultured cells. Although further study is needed, I propose that WDR11 may have a role in autophagy or viral egress, which may not have been detectable via the techniques used in this investigation. Since little is known about WDR11, particularly in the context of a viral infection, and few cytoplasmic binding partners are known for ICP0, this is an intriguing finding, and a step forward in understanding the activities of both proteins.

6.2 General discussion

6.2.1 The importance of biological relevance

A recurring theme of this thesis is that it is crucial to perform experiments in as biologically relevant a manner as possible. Although a particular technique may produce data, it is important to consider whether these results are a genuine representation of how a process may occur in an undisturbed system. For example, as discussed extensively in Chapter 3, several techniques commonly used for the investigation of Rac1 have serious flaws, because they disrupt the tightly controlled spatio-temporal regulation of Rac1 activation that naturally occurs in cells, and so conclusions drawn using such mechanisms may not be authentic. The absence of straightforward mechanisms to study Rac1 without simultaneously disrupting all of its interconnected functions impaired my ability to characterize the involvement of Rac1 in antiviral immunity, emphasizing the need for more biologically relevant experimental techniques for the study of this protein. Although others have successfully investigated the involvement of this GTPase in a variety of cellular processes, such data is frequently contradictory – for example, directly opposing effects of Rac1 have been reported in proliferation (854, 855), apoptosis (856, 857), E-cadherin-mediated adherens junctions (858), (859), and migration (860, 861), showing the limitations of current strategies.

In the case of ICP0, biological relevance means, first and foremost, considering how various experimental conditions affect the localization of the protein. As discussed in Chapters 4 and 5, a large number of different treatments can result in the nuclear retention of ICP0 (478, 500, 688-692). Although the exact mechanisms through which such conditions prevent ICP0 translocation to the cytoplasm are unclear, it is generally thought that ICP0 cannot exit the nucleus until it has completed all of its functions there (688) – and so preventing the accomplishment of any of its nuclear activities with various interventions will block its transit to the cytoplasm. Despite this, few reports consider the effect of experimental manipulations on ICP0 localization. It has long been known that ICP0 moves between the nucleus and the cytoplasm (478-482), and ICP0 persists in the cytoplasm of most, if not all, cell types at some stage of viral replication, though the exact

rate of translocation differs with the origin and nature of the cell (424, 451, 479, 730). Therefore, the function of ICP0 in this compartment is likely to be biologically relevant. Indeed, as discussed in Chapter 4, neglecting this aspect of ICP0 behaviour has led to some misleading conclusions about the functions of ICP0 in terms of both antiviral evasion and viral replication. Accordingly, some previous work may have to be reinterpreted in light of these findings, as the disruption of a particular function of ICP0 by proteasome inhibition, RING finger disruption, or the absence of other viral proteins may simply mean that cytoplasmic ICP0 is required for that function, as opposed to the direct involvement of the proteasome, RING finger, or additional viral components.

A related consideration in terms of biological relevance concerns the study of ICP0 in the context of an HSV infection. For example, why does expression of ICP0 from a plasmid or in a stable cell line (478, 695) result in its nuclear retention? The answer is not entirely clear, but this likely results from the fact that other viral proteins are involved in regulating the behavior of ICP0 (478). Accordingly, my SILAC results reveal several viral binding partners for ICP0 in the cytoplasm (Chapter 5), and the disruption of translocation that results from blocking viral DNA synthesis (478) implicates a viral late protein, which requires the initiation of DNA replication for its expression, in this process. Indeed, late protein VP22 has been previously reported to have a role in promoting the cytoplasmic localization of ICP0 (726, 862), although immediate-early protein ICP27 has been proposed to be involved in this as well (690, 863). Additionally, ICP0 has been reported to undergo a variety of post-translational modifications (864-867), which are controlled by viral proteins, either directly, in the case of ICP0 phosphorylation by the viral kinase UL13 (866) or indirectly, for instance via the regulation of cellular kinase cdc2 by ICP22 and UL13 (868). Such modifications differ with time post-infection, in addition to subcellular location of ICP0 (864, 869), suggesting that they may have a role in controlling the localization of ICP0. However, nuclear ICP0 is also modified, and phosphorylation of ICP0 has been found to affect the activity of the protein in functions such as transactivation, ND10 disruption, and E3 ubiquitin ligase activity (869, 870). Therefore, even examining nuclear functions of ICP0

in the absence of other viral proteins could potentially produce misleading results, as ICP0 may not be appropriately modified under these conditions. The generation of ICP0 mutant viruses is not a simple task, given the high GC content of HSV DNA (871-873), which makes many routine techniques more difficult. In addition, the unusual structure of the HSV genome, which consists of stretches of unique sequences flanked by inverted repeats (874), means that ICP0 is present in two copies in the viral genome (875, 876), making the manipulation of ICP0 even more complex. Despite these difficulties, in this work I have only employed various ICP0 mutant-expressing viruses. This strategy was used not only in the investigation of the antiviral-blocking and replication-enhancing effects of ICP0 (Chapter 4), but also in the identification of cytoplasmic binding partners for ICP0 (Chapter 5), which made use of untagged ICP0 in the context of an HSV infection, combined with quantitative mass spectrometry that does not disturb cellular behavior. This allowed this investigation to occur in a biologically relevant manner, and so the proteins identified have a greater probability of being true interaction partners for ICP0 as opposed to artifacts. However, a limitation of the immunoprecipitation technique is that it disrupts the precise compartmentalization that normally exists in intact cells, potentially allowing proteins to come into contact that would never have access to one another in an undisturbed system. Such complexes cannot be discounted by SILAC or the PFL, as they represent specific interactions that are simply not biologically relevant, and so must be excluded by further characterizing the function of a potential binding partner.

It is also important to note that all of the viruses used in this investigation were produced from the same parental strain. Different laboratory strains of HSV-1, derived from independent clinical isolates, are commonly used in the investigation of this virus, with unique mutations in ICP0 available in distinct backgrounds. However, these various strains have long been known to show differences in antigenic properties, virulence and genome (877, 878), and we have observed subtle variations in the abilities of even UV-inactivated viruses from assorted strains to induce ISGs (P. Paladino and K. Mossman, unpublished observation). Comparing the behavior of the different ICP0 mutants in the

background of the same strain ensures that my results are not produced by differences unrelated to ICP0.

However, the study of ICP0 in the context of mutant viruses is not without limitations – indeed, it has been suggested that such a strategy is fraught with complication (476, 695, 748). One such difficulty is that the lack of ICP0 has been suggested to lead to decreased expression of downstream genes, meaning that rather than resulting from the absence of ICP0 itself, a particular phenotype could instead be caused by the reduced level of an E or L protein (370, 401). However, the defect in ICP0-null viruses is generally thought to consist of a decreased probability that a particular viral genome will initiate the lytic replication cycle – but once initiated, replication occurs normally (reviewed in (666)). In other words, there are fewer productively infected cells, but each expresses normal levels of viral proteins, as opposed to a greater number of cells all expressing lower levels of E and L proteins – making it unlikely that the effects observed are due to the loss of a protein other than ICP0. Other concerns are that differences in particle to pfu ratio means that more particles of ICP0-null viruses are applied compared to the wildtype at a particular MOI (686), confounding data interpretation, as more virion-associated components will be present in one case versus the other (476). However, since I was most interested in comparing D8 and D8/FXE to one another or to dl1403, and since all of my mutant viruses had similar particle to pfu ratios (Chapter 4), this was not a major concern. Titering my viruses on U2OS cells, which complement the ICP0 deficiency (879), in the presence of HMBA, which stimulates IE gene expression through an unknown mechanism (880-882), minimizes the difficulty in obtaining an accurate titer for ICP0-deficient viruses (748). Finally, it has been proposed that the slower rate of progression through the lytic replication cycle of ICP0-null versus wildtype viruses can lead to misleading results (476). This is less problematic here, because the mutant ICP0 viruses, which have less profound differences in their replication, were again compared amongst themselves.

A caveat of the ICP0 mutants used in this study is that they contain relatively large deletions, of 74 amino acids for the D8 mutation and 45 amino acids for the FXE

deletion (438), which may have impacts on ICP0 structure or function beyond the disruption of the RING finger or the NLS. Since D8 continues to both block antiviral signaling and promote viral replication, the deletion in this ICP0 construct does not disrupt the functions of interest. On the other hand, it is possible that a protein involved in the regulation of the antiviral response has a binding site in ICP0 that is disrupted by FXE mutation, which could explain the RING-dependent but proteasome-independent effect observed, in a mechanism that does not involve atypical ubiquitin modifications. Less disruptive mutations do exist – for example, site-directed mutagenesis of three amino acids between residues 500-506 of ICP0 blocks the action of the NLS (473), while single (K144E) (694) or double (C116G/C156A) (693) amino acid changes produce an ICP0 mutant similar to FXE. However, we were limited in the use of such mutants because we generated the D8/FXE mutant by homologous recombination, which requires screening for a very rare recombination event. Since the growth of D8/FXE is intermediate to both the wildtype and the ICP0-null strains, identifying successful recombinants based on plaque size was difficult, and the growth advantage provided by D8/FXE was not sufficient to allow for the recombinant virus to efficiently outcompete the parental strain during purification. The large D8 and FXE deletions produced a smaller ICP0 protein that could easily be distinguished from wildtype or either single mutant in a Western blot, allowing for the efficient simultaneous screening of a large number of plaques. This could not have been performed with the point mutants, which are the same size as the wildtype ICP0 – requiring instead PCR amplification and sequencing, which is more time-consuming, challenging and expensive. Alternative strategies have been described, such as inserting the full-length HSV genome into a bacterial artificial chromosome (BAC) (883). These constructs can be grown, manipulated and screened in *Escherichia coli*, with infectious virus produced only after the desired mutation has been made and verified (reviewed in (884)). Although such constructs are too large for conventional cloning techniques to be used, targeted mutagenesis can be performed using the more easily controlled prokaryotic recombination machinery, and because virus does not have to be made and grown up prior to screening, this theoretically makes the generation of

recombinant viruses easier. However, we have had great difficulty with the practical use of this technique, possibly due to the high GC content of the HSV genome, and were unable to successfully produce the D8/FXE virus using this strategy.

Another important aspect of biological relevance was the choice of cell type used to perform my studies. Throughout this investigation, a primary human fibroblast cell line was used – despite their low transfection efficiency, minimal tolerance of toxicity, requirement for careful culturing and finite number of doublings. Although immortalized or transformed cell types are generally less problematic in all of these aspects, such cell lines are abnormal in their behavior, raising concerns about the relevance of data obtained in these cells. This is particularly crucial when studying the innate antiviral response, because it has been found that the immortalization process frequently results in the dysregulation of genes involved in IFNα/β signaling (587, 885), possibly because IFNα/β genes are normally upregulated during senescence (587). Additional defects may occur during transformation, as several ISGs also have tumor suppressive effects (reviewed in (886)). In fact, the defect in immune signaling found in tumor cells has been exploited to make oncolytic viruses for cancer therapy, as viruses lacking proteins involved in antiviral evasion are attenuated in healthy cells with an intact IFNα/β response, but can productively infect and kill tumor cells that cannot protect themselves via the production of ISGs (reviewed in (887)). Indeed, ICP0-null viruses have been found to be effective oncolytics (585). Therefore, the use of many common cell lines was not appropriate for this study. Additionally, fibroblasts in general represent one of first obstacles that must be overcome during many viral infections, making them a relevant cell type for the study of early innate antiviral responses, as this is likely a role they play during natural infections. Finally, in terms of the study of HSV, although the initial site of infection is thought to be epithelial cells, the underlying fibroblasts are expected to encounter the virus during the development of HSV lesions *in vivo*, and may help to spread the virus to the terminal nerve endings found below the epidermal-dermal junction (888, 889), and so this choice is, once again, biologically relevant.

However, it is noteworthy that the natural host of HSV is not, of course, cultured cells, but rather human beings. The study of a single cell type in a dish does not truly represent the complexity of what occurs *in vivo*, particularly in the case of HSV, for which a key part of the replication cycle occurs in neurons. This is especially relevant in the case of WDR11 – although this protein was not found to be necessary for the replication of HSV in fibroblasts, the strong interaction of this protein with ICP0 is unlikely to have arisen by chance (Chapter 5). Therefore, the relocation of WDR11 that occurs during infection may well have an important function in other cell types. Various methods can be used for the study of HSV infection of cultured neurons (reviewed in (890)), which may clarify whether WDR11 is involved in latency or reactivation. However, such techniques are again not fully representative of what occurs *in vivo*. Therefore, my results showing that cytoplasmic ICP0 can promote viral replication in mice are significant (Chapter 4), because this confirms my earlier observations were not an artifact of studying a virus in a single cell type in culture. Conversely, although a mouse model is useful, and tests viral replication in a situation with multiple cell types, including a nervous system for latency and an immune system for viral clearance, it is not strictly biologically relevant, as HSV does not naturally infect mice. Indeed, no murine alphaherpesvirus has been identified (891). In addition, HSV infections in mice differ from what is observed in humans, as the virus does not spontaneously reactivate in mice (892), and mucosal infections in mice often lead to fatal encephalitis (893), while this is very rare in humans (894). However, the experimental infection of humans is clearly not possible, and alternative animal models, such as rabbits, also have limitations (reviewed in (895)), making the caveats of *in vivo* studies in mice difficult to overcome.

6.2.2 Contributions to the field and future directions

The excessive variability encountered in the investigation of the potential role of Rac1 in IFN-independent signaling means that the major contribution of this study may simply be this: to clarify that the investigation of signaling nodes with important functions in diverse cellular activities should be avoided in future. However, any potential

involvement of Rac1 in antiviral responses would seem to be in the negative regulation of ISG production, as opposed to the activation of IRF3 in response to the entry of an enveloped virus – raising the question of how to better identify prospective effectors involved in IFN-independent signaling. There are currently over 23 million records from the biomedical literature found in the PubMed database – much of it contradictory, with significant differences in cell types and methodology making conclusions increasingly difficult. Selecting potential targets by searching the literature, as I did to choose Rac1, is thus a challenging path. However, during my tenure as a graduate student, three new proteins have been implicated in the IFN-independent response – STING, Trex1, and peroxisome-localized MAVS (113, 116). If the involvement of these proteins in IFN-independent signaling can be validated in our cell type, likely through siRNA-mediated knockdown, they may represent ideal downstream proteins for further study. This could be accomplished via the quantitative mass spectrometry technique that I have successfully pioneered in our cell type (Chapter 5). RNA interference screens to identify components required for the production of ISGs in the absence of IFNα/β could present a way forward as well (reviewed in (896)).

In contrast to the study of Rac1, my characterization of the cytoplasmic activities of ICP0 is of clear significance, impacting not only our understanding of the role of ICP0 in antiviral signaling, but also having repercussions for the entire ICP0 field. My results demonstrate how the reliance on RING finger mutants that predominantly localize to the nucleus overlooks the importance of the localization of ICP0 to its function, which is something that should be considered in all future studies using RING-deficient ICP0 mutants. Additionally, my data showing both proteasome-independent effects of the RING finger, as well as RING finger-independent effects of ICP0, add new dimensions to the study of this protein, clarifying that not all functions of ICP0 are necessarily achieved by targeting a particular cellular protein for degradation. These findings are likely to be controversial – which is valuable, because this stimulates discussion, further investigation and ultimately, progress. Additionally, the importance of considering how experimental manipulations affect the localization of a protein of interest is not specific to ICP0 –

instead, this is a general matter that applies to any investigation, meaning that this study has a message that is relevant to all researchers.

However, unanswered questions remain. In particular, the possibility that ICP0 is producing its effects through the generation of atypical ubiquitin chains is highly intriguing, but requires further study. As the importance of proteasome-independent functions of ubiquitin is just beginning to be fully appreciated (reviewed in (415)), reagents for the investigation of atypical modifications are still fairly limited, making this no simple task. One interesting technique involves a cell line in which the endogenous ubiquitin genes can be inducibly silenced and simultaneously replaced by the expression of a mutated ubiquitin protein, where a specific lysine has been replaced by an arginine – for example, Lys 63, so that the most common atypical lysine modification is prevented (897). This could be used, for example, to determine whether Lys 63 modifications are required for the ability of ICP0 to block antiviral signaling. However, this cell line was derived from U2OS cells, which have an abnormal antiviral response and are thus commonly used to complement ICP0-null viruses, which grow normally in these cells (377, 898), making them unsuitable. Additionally, even if this construct were introduced into a more appropriate cell line, this general strategy is, once again, a relatively "blunt tool," disrupting the ubiquitination of a vast number of proteins involved in a wide variety of cellular functions – which as was shown in Chapter 3, is not an ideal approach. A less disruptive strategy could be to use SILAC to compare proteins precipitated using the Lys 63-specific TUBE described in Chapter 4 (710) in cells infected with D8 versus D8/FXE. A protein enriched in the D8 sample may be a target of ICP0-mediated atypical ubiquitination. However, reagents specific for linkages other than Lys 63 (or Lys 48) are still fairly scarce, and so if ICP0 produces atypical chains that do not involve Lys 63, this investigation may have to be postponed until such components become available.

My discovery of WDR11 as an interaction partner for ICP0 is also an intriguing contribution to the field. This is a very poorly understood protein, with most of the publications mentioning it simply reporting its identification in some form of screen, as opposed to a molecular characterization of its function. However, these studies have

implicated WDR11 in diseases such as cancer (749, 751) and idiopathic hypogonadotropic hypogonadism (750, 752), suggesting that an understanding of the activities of this protein is relevant to human health. Further study is required to clarify why WDR11 is targeted by ICP0. Interestingly, my data demonstrating that WDR11 levels do not decrease during HSV infection supports my observations that some functions of ICP0 are unrelated to its ability to target proteins to the proteasome for degradation, and so it is possible that this interaction is involved in the ability of cytoplasmic ICP0 to promote viral replication in the absence of the RING finger. Indeed, as proposed in Chapter 5, WDR11 may have a role in autophagy or egress, possibilities that are worthy of being pursued further. However, since no difference in viral replication was observed in human fibroblasts, such an investigation would likely have to consider the role of WDR11 in alternative cell types, such as neurons. Finally, the importance of WDR11 to viral replication may only be detectable *in vivo* – but a full characterization of this possibility will have to wait for the development of a WDR11 knockout mouse.

In addition to WDR11, several other intriguing potential cytoplasmic partners were found for ICP0 in my SILAC results, which represent a valuable resource for future investigations. For example, Ras GTPase-activating protein-binding protein 2 (G3BP2) was identified as possibly interacting with ICP0. This protein, along with the closely related G3BP1 (often collectively referred to as G3BP) is important for the assembly of stress granules (SGs) (899, 900), which are non-membranous cytoplasmic structures formed under conditions of cellular stress that result in obstructed translation initiation (reviewed in (901)). SGs consist of accumulations of translationally silent mRNAs and aggregated cellular RNA-binding proteins such as G3BP and T cell internal antigen 1 (TIA-1) (901), and may act as storage sites that allow for the rapid resumption of translation when conditions improve (902-904). SGs are thought to play an antiviral role, with effects ranging from simply sequestering factors involved in translation initiation, to potentially activating IFNα/β signaling, though the exact role of these structures in antiviral signaling is unclear (reviewed in (902, 903)). Interestingly, blocking SG formation by knockdown of G3BP inhibited IFNα/β expression (905), but disrupting SGs

via a number of alternative strategies did not (906). Also, G3BP strongly co-localizes with RIG-I (905), and knockdown of G3BP1 decreases IFNα/β production after encephalomyocarditis virus infection (907). Therefore, it is possible that G3BP may have a role in IFNα/β signaling that is distinct from its functions in SG formation. In support of this, many viruses have mechanisms to inhibit G3BP via relocation (908-913) or cleavage (907, 914).

The ICP0-G3BP2 interaction could be responsible for blocking SG formation, thus improving viral replication. Indeed, inducing SGs resulted in decreased expression of late genes in a wildtype HSV infection (915), and HSV replicates to higher titers in mouse embryonic fibroblasts defective in SG formation (916). However, wildtype HSV has been found to block the formation of SGs (917, 918), via the actions of ICP34.5, US11, gB (388, 397, 919-923) and possibly vhs (915, 917, 924). This may occur indirectly, through the ability of these proteins to counteract translational inhibition induced by the phosphorylation of eIF2α (918, 924) (915). Therefore, the ICP0-G3BP2 complex may not be necessary for the blockage of SG formation. However, the possibility of an SG-independent role for G3BP2 in IFNα/β production makes it of interest to investigate this further, both to determine whether there is an increased accumulation of SGs after infection with an ICP0-null virus, and also to see whether viral replication, along with ISG accumulation, is affected in G3BP2-deficient cells. Although G3BP2 interacts with both D8 and D8/FXE ICP0, this protein could serve as a target for the RING-dependent atypical ubiquitination required for the ability of ICP0 to block antiviral signaling.

Several of the other SILAC hits are intriguing as well. For example, of the proteins interacting with both D8/FXE and D8, ERC1 (ELKS/RAB6-interacting/CAST family member 1) has been proposed to be a regulatory subunit of IκB kinase involved in the activation of NFκB in response to inflammatory cytokine stimulation (925), or genotoxic stress (926, 927), though it has not yet been implicated in IFNα/β signaling. Also, the Core protein from hepatitis C virus (HCV) has been found to bind to solute carrier family 25 member 5 (SLC25A5) – the depletion of which produces a mild

improvement in virus replication (928). Another interesting possibility is heterogeneous nuclear ribonucleoprotein K (HNRNPK), a multifunctional protein involved in promoting the replication of HCV (929), VSV (930), influenza virus (931), HCMV (932), Chikungunya virus (933), enterovirus 71 (934, 935), HIV (936, 937), dengue virus (938), and hepatitis B virus (939, 940), and bound by viral proteins from HCV (941), Epstein-Barr virus (942), Chikungunya virus (933), dengue virus (938, 943), HIV (937), African swine fever virus (944), Sindbis virus (945), KSHV (946), and human herpesvirus 6 (947). Particularly relevant is the fact that although HNRNPK has been previously suggested to have an early nuclear function in HSV replication by binding to ICP27 (948), a late cytoplasmic action in HSV egress for HNRNPK has been more recently suggested as well (949).

Proteins interacting exclusively with D8 ICP0, even though the D8/FXE mutant accumulates to much higher levels, are also intriguing. One such protein is Serpin E1, which was found to be upregulated after infection with an HSV mutant defective in ICP34.5 (950), which as discussed in Chapter 1, has been implicated in disrupting antiviral responses (392, 393). Additionally, Serpin E1 can be induced by type II IFN (IFNγ) under some conditions (951), and a related protein, Serpin B2, has been found to form a complex with IRF3 (952). Other interesting proteins include C14orf166, which binds to the Core protein from HCV and has a cytoplasmic role in promoting acute viral replication, possibly in entry or egress (953). This protein has also been implicated in promoting influenza virus replication via interaction with the viral RNA polymerase (954). UACA is a pro-apoptotic protein involved in the suppression of NFκB signaling via its cytoplasmic retention (955, 956). Fragile X mental retardation protein 1 (FXR1) shuttles between the cytoplasm and nucleus and has functions in RNA transport and translation (reviewed in (957, 958)) and is required for influenza virus replication (959), while ubiquitin-associated protein 2-like (UBAP2L) is a poorly-characterized ubiquitin-associating protein (960). IFNα has been found to induce the expression of a microRNA that targets insulin-like growth factor 2 mRNA binding protein 1 (IGF2BP1), a protein involved in translation, resulting in the inhibition of HCV replication (961). Finally, to

come full circle, Trio is a GEF for Rac1 (962). Overall, my SILAC results represent an excellent starting place for further investigation of the cytoplasmic functions of ICP0.

6.3 Concluding remarks

Influenza, measles, AIDS, rabies, hepatitis and even Ebola hemorrhagic fever – what do these varied conditions have in common? All are caused by enveloped viruses, which must enter the cell via fusion of the viral envelope with a cellular membrane. The IFN-independent response exploits this commonality, recognizing the initial penetration event and launching a protective response, preventing a low-level infection from escalating without requiring the potentially harmful infiltration of immune cells. The characterization of this response not only improves our understanding of a remarkable cellular phenomenon, but could ultimately be used in the design of therapies that target broad classes of human pathogens. Although my investigation of the involvement of Rac1 in this pathway did not prove fruitful, further study of this system using more targeted tools would be well worth the effort.

While the cold sores commonly associated with HSV may sometimes be dismissed as trivial, this is, in fact, an important human virus. The economic burden of genital herpes alone has been estimated to be as high as $984 million in the United States (963), with an additional $17.7 million spent on ocular diseases caused by this virus (964). Despite much effort, an effective vaccine for HSV remains elusive (722), and although antiviral therapies are useful in managing the symptoms of HSV, they cannot eradicate the virus, while viral drug resistance is mounting (reviewed in (965)). Improving our knowledge of HSV is crucial, not only in order to improve our ability to combat it, or even to exploit it via oncolytic viral therapy (585) – but also to learn from it, as characterizing the masterful way in which this virus manipulates the host cell reveals new insights into the workings of these processes. Indeed, my findings of the potential negative regulation of IRF3 by ICP0-mediated atypical ubiquitination represents a novel concept in innate immunology, the implication of WDR11 in ICP0 function brings new insight into a poorly understood cellular protein, and the identification of several previously uncharacterized interaction partners for ICP0 provides an exciting foundation for future research.

Although my work brings insight to the function of HSV, it also has a more broadly applicable message. My discovery of cytoplasmic RING finger-independent replication-promoting effects of ICP0 highlights two important lessons – first, to consider localization when studying protein function, and second, to be unafraid to challenge the currently accepted dogma – in this case, the long-held assumption that the RING finger is crucial for all functions of ICP0. In the words of Nobel Prize winner Albert Szent-Györgyi: "Research is to see what everybody else has seen, and to think what nobody else has thought."

CHAPTER 7
LIST OF REFERENCES

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