Investigating the Role of Interferon Regulatory Factor 3 in Response to Genotoxic Stress

By

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ABSTRACT

Interferon regulatory factor 3 (IRF3) plays an important role in activating the innate immune response in a variety of conditions, including viral infection. As well as regulating the immune response to viruses, IRF3 is involved in regulating cellular functions including apoptosis. Apoptosis and the inflammatory response to viral infection are very different; therefore, it is obvious that IRF3 plays dramatically different roles in the cell depending on the conditions. We previously identified a non-activating phosphorylation of IRF3 in response to adenovirus (Ad) in which Serine-173 is phosphorylated. In addition to Ad infection, IRF3-S173 is phosphorylated in response to genotoxic stresses including ultraviolet (UV) irradiation and etoposide. In this study, I show that this phosphorylation event is involved in a variety of processes including protein stability, cell survival and IRF3 regulation. Thus, phosphorylation of IRF3-S173 is a novel and important event in a complex regulatory pathway of an integral protein.

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LIST OF ABBREVIATIONS

A	Alanine
Ad	Adenovirus
AIM1	Absent in Melanoma 1
Akt	Protein-kinase B
AP-1	Activator protein-1
APC	Antigen Presenting Cell
APS	Ammonium Persulfate
ASC	Apoptosis-associated speck-like protein containing a CARD
ATF-2	Activating transcription factor-2
ATM	Ataxia telangiectasia mutated
ATP	Adenosine triphosphate
ATR	Ataxia telangiectasia and Rad3 related
b-ZIP	Basic leucine zipper domain
Bax	Bcl-2-related X protein
BH3	Bcl-2 homology domain
CAR	Coxsackie adenovirus receptor
CARD	Caspase activation and recruitment domain
CAS	Cellular apoptosis susceptibility
CBP	CREB binding protein
CD14	Cluster of differentiation 14
CD46	Cluster of Differentiation 46
cdc42	Cell division control protein 42 homolog
CpG	C-phosphate-G
CsCl	Cesium chloride
D	Aspartic acid
DAMP	Damage associated molecular pattern
DBD	DNA binding domain
DC	Dendritic cell
ddH_2O	Distilled deionized water
DDX41	DEAD box protein 41
DExD/H	DEAD box domain
DMEM	Dulbecco's modified Eagle medium
DNA	Deoxyribonucleic acid
DNA-PK	DNA-dependent protein kinase
DPBS	Dulbecco's phosphate buffered saline
dsDNA	Double-stranded DNA
dsRNA	Double-stranded RNA
E	Glutamic acid
ECL	Enhanced chemiluminescence
EDTA	Ethylenediaminetetraacetic acid
eIF-2α	Eukaryotic initiation factor-2α
EMCV	Encephalomyocarditis virus
ERK	Extracellular signal-regulated kinase
FAK	Focal adhesion kinase-1
FBS	Fetal bovine serum
FLAG	N-DYKDDDDK-C protein tag
HCl	Hydrogen chloride

HMG-I/Y	High Mobility Group -I/Y
hpi	Hours post infection
HRP	Horseradish peroxidase
IAD	IRF association domain
IFN	Interferon
IgG	Immuno-globulin gamma
IKK	IκB kinase
IL	Interleukin
IL-1R	Toll/interleukin-1 receptor
IPAF	ICE-protease activating factor
IPS1	Interferon-β promoter stimulator 1
IRF	Interferon regulatory factor
ISG	Interferon stimulated gene
ISRE	Interferon-stimulated response element
ITR	Inverted tandem repeats
JNK	c-Jun N-terminal kinase
K	Lysine
LGP2	Laboratory of genetics and physiology-2
LPS	Lipopolysaccharide
LRR	Leucine rich repeat
MAC	Membrane attack complex
MAPK	Mitogen-activated protein kinase
MD-2	Lymphocyte antigen 96
MDA5	Melanoma differentiation-associated protein 5
MEK	Mitogen-activated protein kinase kinase
MEKK	MEK kinase
MEM	Minimal essential medium
MeOH	Methanol
MKK	MAP kinase kinase
MKKK	MAP kinase kinase kinase
MOI	Multiplicity of infection
MyD88	Myeloid differentiation primary response gene (88)
NaCl	Sodium chloride
NALP3	NACHT, LRR and PYD domains-containing protein 3
NaOH	Sodium hydroxide
ND10	Nuclear domain 10
NF-KB	Nuclear factor-κB
NLR	NOD-like receptor
ORF	Open reading frame
PAGE	Poly-acrylamide gel electrophoresis
PAMP	Pathogen associated molecular pattern
pDC	Plasmacytoid dendritic cell
PFU	Plaque forming unit
PI3K	Phosphatidyl-inositol-3 kinase
PKR	Protein kinase R
PML	Promyelocytic leukemia
polyI:C	Poly-inosinic:poly-cytidilic acid
Pro	Proline rich domain
PRR	Pattern recognition receptor

PVDF	Polyvinylidene difluoride
RAC	Ras-related C3 botulinum toxin substrate 1
RAF	RAF proto-oncogene serine/threonine-protein kinase
RANTES	Regulated and normal T cell expressed and secreted
RAS	Rat sarcoma protein
RbCl	Rubidium chloride
RD	Repressor domain
RIG-I	Retinoic acid-inducible gene-I
RLR	RIG-like receptor
RLU	Relative luminescence units
RNA	Ribonucleic acid
RNF125	Ring finger protein 125
RNF20	Ring finger protein 20
RSV	Respiratory syncitial virus
S	Serine
S173	Serine 173 (of IRF3)
S396	Serine 396 (of IRF3)
SDS	Sodium dodecyl sulfate
SeV	Sendai virus
ssRNA	Single-stranded RNA
TBK1	TANK-binding kinase 1
TBST	Tris-buffered saline supplemented with 0.1% TWEEN
TEMED	Tetramethylethylenediamine
TIR	Toll/interleukin-1 receptor domain
TLR	Toll-like receptor
Tpl2	Tumor progression locus 2
TRAF	TNF receptor associated factor
TRIF	TIR-domain-containing adapter-inducing interferon-β
TRIM25	Tripartite motif-containing protein 25
UV	Ultraviolet
VAK	Viral activating Kinase
VP16	Etoposide
VSV	Vesicular stomatitis virus
wt	Wildtype
β-gal	Beta-galactosidase

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Chapter 1: INTRODUCTION

Human Adenovirus (Ad) is a significant human pathogen, with 57 identified serotypes causing disease in the form of respiratory infection, conjunctivitis and gastroenteritis. The virus is also a common gene therapy vector with over 23% of gene therapy clinical trials worldwide employing this vector (Edelstein, 2012). The virus was first isolated from the adenoid tissue of patients with respiratory infection in the 1950s (Hilleman and Werner, 1954; Rowe et al., 1953). Some human Ads have been demonstrated to cause tumours in rodents (Trentin et al., 1962; Yabe et al., 1962), which prompted researchers to delve deeper into the biology of these viruses. Ad research has expanded immensely since these early days, gleaning insight to various cell biology mechanisms. Insights into cell biology and virology are immense, yet there is still more to be learned.

Like most viruses, Ads must manipulate the host cell machinery, and avoid detection by the immune system. Studies of the mechanisms used both by the host, to recognize Ad, and by the virus, to evade the host have provided a wealth of novel insight to a variety of areas, including control of host gene expression, the innate, and the adaptive immune responses to viruses. Ad induced innate immunity is one area that continues to be of great interest to researchers, especially the involvement of specific key cellular proteins. Considering the role Ad has played in understanding viruses, infection, and general cell biology, the continued study of this area could mean important discoveries in combating Ad as a pathogen, as well as improving vector

efficacy and safety. Such studies may also allow us to gain a better understanding of other anti-viral responses to other pathogens.

Herein, I explore the interaction of Ad with a key regulator of innate immunity, interferon regulatory factor 3.

1.1. Literature Review

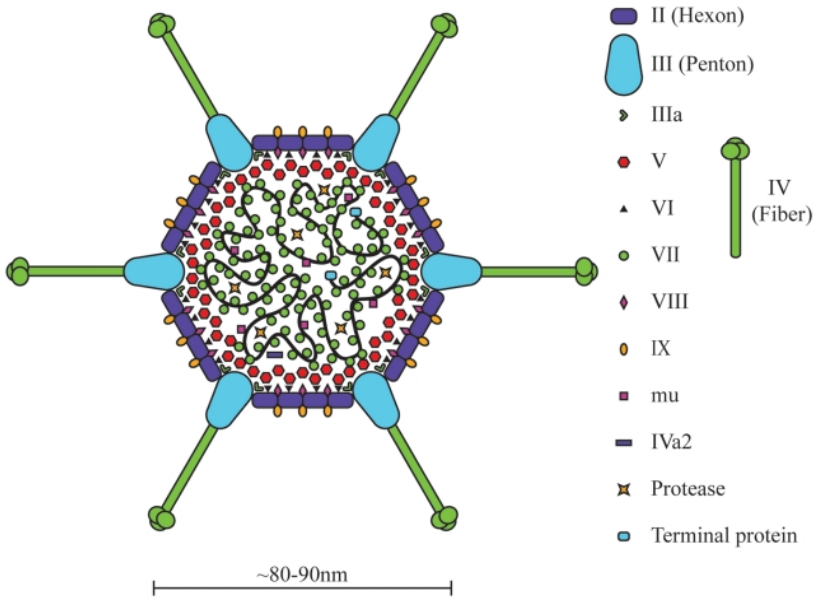
1.1.I Adenovirus Biology

Human Ads have been divided into 6 subgroups (A-F), classified by a number of factors, including DNA homology, hemagglutination properties, oncogenicity in rodents and genomic organization. All human Ads share many structural similarities. The capsid is icosahedral, composed of three major structural proteins: hexon, penton, and fiber; as well as a variety of minor proteins (IIIa, IVa2, VI, VIII and IX) (Christensen et al., 2008; Liu et al., 2010; Reddy et al., 2010; Russell, 2009). The total size of the capsid ranges from ~80-90 nm, and contains a genome of approximately ~30-40 kb of double stranded DNA (Berk, 2007). The 20 facets that make up the icosahedron that is the capsid are composed of hexon protein trimers, arranged in a triangular fashion. At each of the 12 vertices are five penton proteins, which serve as the base for the extending fiber protein (Liu et al., 2010; Reddy et al., 2010). The fiber serves as the main binding protein for the CAR (coxsackie adenovirus receptor), and initiates internalization and infection by the virus (excepting viruses of subgroup B, which uses CD46) (Bergelson et al., 1997; Tomko et al., 1997).

The serotypes 2 and 5 (Ad2 and Ad5) are the most characterized of the human Ads. Ad5 has an ~36 kb genome encoding 39 genes. The genes are organized into the early, and the late genes, based on whether they are expressed before, or after DNA replication (Davison et al., 2003). Predictably, the early transcripts, specifically E1a, E1b, E3 and E4 encode proteins that are integral for modifying the host environment, altering the immune

Figure 1.1 Schematic representation of Ad virion.

((Giberson et al., 2012). Used with permission)


II (Hexon)
III (Penton)
IIIa
V
VI
VII
VIII
IX
mu
IVa2
Protease
Terminal protein
IV
(Fiber)
~80-90nm

response to the virus or transactivating other viral regions. E2 encodes proteins that are necessary for synthesis of the viral DNA. The major late transcripts, organized from L1-L5, are all expressed using the same major late promoter. The late proteins are generated from alternative splicing of a single transcript. It has been recently demonstrated however, that a novel promoter regulates expression of the L4-22K and L4-33K proteins (Morris et al., 2010). The L4 proteins are directly involved in regulation of the major late promoter, so it stands to reason that they would, at least initially, be controlled by another promoter. The late proteins typically include structural proteins. In addition to the proteins generated from the major late promoter, there are an additional four small late transcripts. These other late transcripts include protein IX (pIX), a minor structural protein; IVa2, a protein involved in encapsidating the viral DNA; and VA RNA I and II, viral RNAs that block activation of the interferon response.

The Ad genome is flanked at each end by inverted terminal repeats (ITRs) of ~100 bp. The packaging sequence, of ~150 bp, is located next to the left ITR. The viral DNA does not directly interact with the outer capsid, but rather is associated with three highly basic proteins; VII, V and Mu (m) (Chatterjee et al., 1986; Maizel et al., 1968; Russell et al., 1968). Protein VII wraps and condenses the viral DNA (Mirza and Weber, 1982). A shell of protein V, which in turn also makes contact with the outer capsid, surrounds this complex (Brown et al., 1975; Everitt et al., 1973). Protein V interacts directly with penton and indirectly with hexon. Mu is synthesized as a pre-Mu precursor, which is eventually cleaved by the Ad-encoded proteinase to its final form (Anderson et al., 1989). It is suspected that pre-Mu assists in the tight

condensation of viral DNA within the capsid, and that cleavage to Mu allows the structure to partially relax prior to its introduction to the host nucleus (Perez-Berna et al., 2009). Due to the nature of the interactions between the proteins V, VII and Mu with both the DNA and the capsid, it has been observed that despite no direct interaction, viral DNA serves to add stability to the overall integrity of the virion (Fabry et al., 2005; Kennedy and Parks, 2009; Liu et al., 2010; Silvestry et al., 2009; Smith et al., 2009).

As mentioned above, the primary mode of entry of human Ads is via endocytosis initiated by attachment of the fiber protein with the CAR receptor (Bergelson et al., 1997; Tomko et al., 1997). There is a secondary interaction between penton and a_vb_3 or a_vb_5 integrins, which triggers the internalization (Wickham et al., 1993). Ad5 can use heparin sulfate proteoglycans as an alternative receptor, through an interaction with the fiber shaft (Smith et al., 2003), or bridged through blood factors such as factor IX, factor X or complement component C4-binding protein (Kalyuzhniy et al., 2008; Shayakhmetov et al., 2005a; Waddington et al., 2008). After Ad binding, the virus is internalized via endocytosis and exits the early endosome to escape degradation (Leopold et al., 1998). The microtubule network then serves as a scaffold for the virus to make its way through the cell towards the nucleus (Leopold et al., 1998), being slowly disassembled along the way (Greber et al., 1993). Upon arrival at the nucleus, the DNA enters through the nuclear pore complex, and the remainder of the capsid is degraded (Chatterjee et al., 1986; Greber et al., 1993; Strunze et al., 2011). The entire viral lifecycle, including DNA replication and assembly of new virions occurs within the nucleus

over a period of 24-36 hours and produces ~10^4 daughter virions.

1.1.II. Pattern Recognition Receptors

1.1.II.i. Innate immunity

The body's first line of defense against pathogens and other potentially hazardous, non-self molecules is the innate immune system. The innate immune system provides an immediate, but not long lasting immune response to pathogens, and is an evolutionarily conserved process that is found in many orders of life, from animals and plants, down to insects and fungi (Takeuchi and Akira, 2009). The vertebrate innate immune system is responsible for several key tasks in disposing threats to the organism as a whole. The responsibilities of the innate immune system include: recruiting immune cells and inducing inflammation through secretion of cytokines and chemokines; triggering the complement pathway in order to assist the immune cells as well as activating the membrane attack complex (MAC); and priming of the adaptive immune system through antigen presentation.

The first, and arguably most important task set before the innate immune system, is recognizing pathogens. This is primarily achieved through a set of receptors, known as pattern recognition receptors (PRRs) (Akira et al., 2006; Beutler et al., 2007; Medzhitov, 2007). These receptors are located ubiquitously in the organism and are found in both the intracellular and extracellular compartments. They are sometimes referred to as primitive pattern recognition receptors, due to the fact that they evolved before other facets of the immune system. There are several different classes of PRRs, but for the scope of this study the ones that are most

important are the membrane bound PRRs (Toll-like receptors (TLRs)), and cytoplasmic PRRs (NOD-like receptors (NLRs), RIG-like receptors (RLRs)). PRRs recognize specific molecules that are common to different types of pathogens, but do not retain a “memory” of the pathogens to combat future infections.

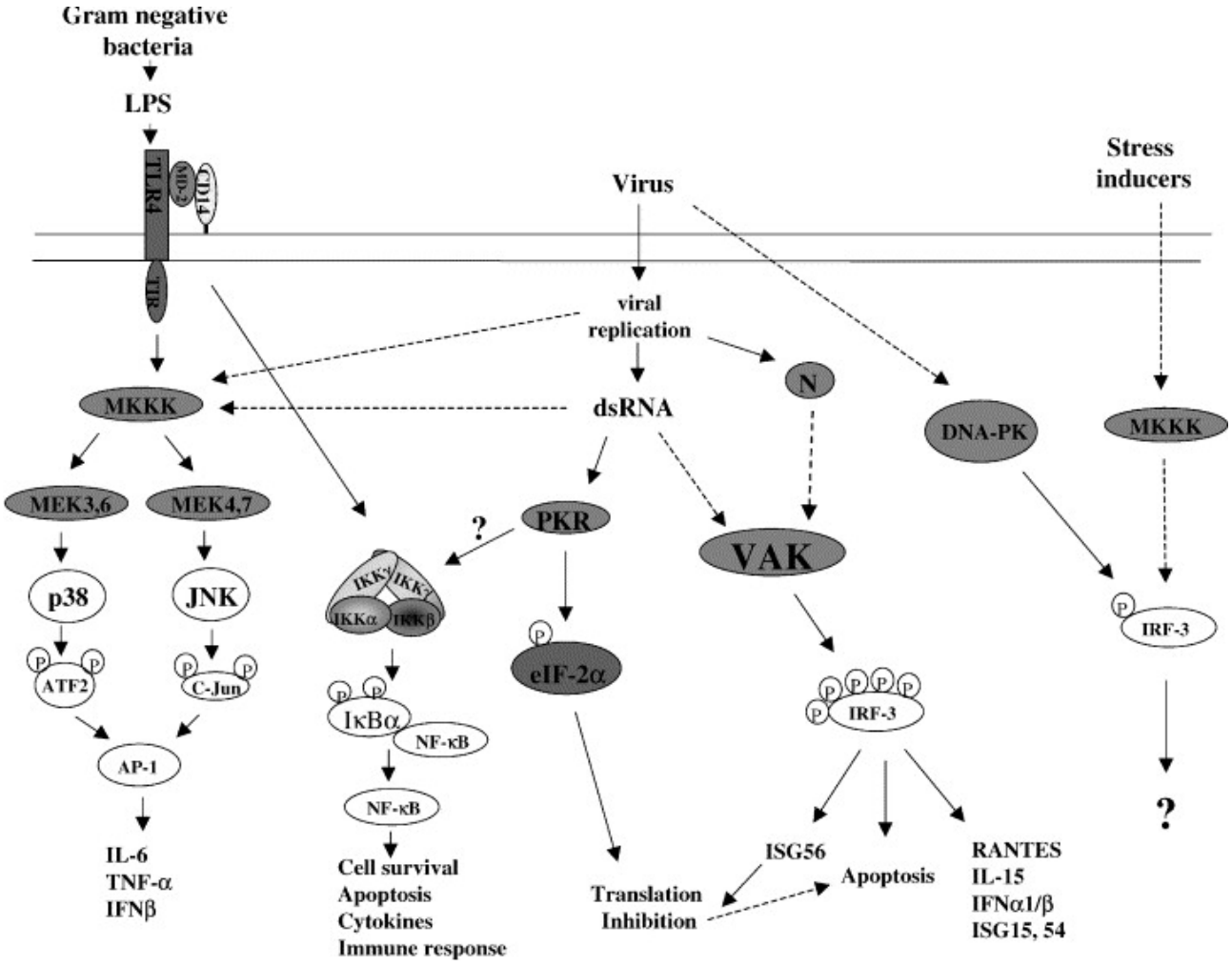
Some PRRs are also capable of recognizing endogenous molecules, in order to stimulate a response (Jeong and Lee, 2011). Some of the cellular molecules that can stimulate an inflammatory response through the PRRs are: the heat-shock proteins and other protein factors associated with infection and cell death, as well as non-protein molecules like ATP, DNA and uric acid. The molecules recognized by PRRs are known as pathogen-associated molecular patterns (PAMPs) or alternatively, in the case of PRRs recognizing endogenous molecules damage-associated molecular patterns (DAMPs). The molecules recognized by PRRs include polysaccharides (i.e. bacterial lipopolysaccharide); nucleic acids (DNA, RNA from bacteria, viruses or damaged cells); and peptides (bacterial or viral proteins). There are other PAMPs that are recognized by PRRs, but they are mainly reserved for bacteria, rather than viruses.

1.1.II.ii. Toll-like receptors

Toll-like receptors (TLRs) are a family of PRRs that are conserved from insects, all the way through to mammals. TLRs are so named, due to their similarity to the Toll gene product, of *Drosophila melanogaster*. Christiane Nusslein-Volhard, Eric Wieschaus and colleagues first discovered toll in 1985 and the protein was cloned in 1988 by Kathryn Anderson’s group (Hashimoto et al., 1988) . Charles

Figure 1.2 Overview of signaling cascades triggered by pathogens

N = Nucleocapsid (Servant et al., 2002). Used with permission.


Gram negative bacteria
LPS
TLR4
MD-2
CD14
TIR
Virus
Stress inducers
viral replication
dsRNA
N
MKKK
DNA-PK
MKKK
MEK3,6
MEK4,7
p38
JNK
ATF2
C-Jun
AP-1
IL-6
TNF-α
IFNβ
?
PKR
VAK
IKKγ
IKK2
IKKα
IKKβ
IκBα
NF-κB
NF-κB
Cell survival
Apoptosis
Cytokines
Immune response
eIF-2α
Translation Inhibition
IRF-3
IRF-3
ISG56
Apoptosis
RANTES
IL-15
IFNα1/β
ISG15, 54
?

Janeway's group first described the TLRs in mammals in 1997 (Medzhitov et al., 1997), while at a similar time, Bruce A. Buetler and colleagues claimed that TLRs are the principal sensors of infection in humans (Poltorak et al., 1998). TLRs recognize pathogens both outside of the cell (receptors bound in the cellular membrane) and within the endosome or lysosome (bound to the membrane of these structures) (Akira et al., 2006). The actual structure of the TLRs is conserved and has three distinct domains: the N-terminal leucine-rich repeats (LRRs), a transmembrane domain, and finally a Toll/IL-1R homology (TIR) domain (Belvin and Anderson, 1996). The LRR is located on the outside of the cell, and the TIR domain is located in the cytoplasm, or in the case of TLRs in the endosome/lysosome, the LRR is located within the vacuole and the TIR is once again located in the cytoplasm.

There have been ten TLRs identified in humans (Chuang and Ulevitch, 2001; Chuang and Ulevitch, 2000; Du et al., 2000; Medzhitov et al., 1997; Rock et al., 1998; Takeuchi et al., 1999). Each TLR has a unique subset of PAMPs that it is capable of detecting; an observation that challenged the archaic concept that innate immunity is non-specific. The different TLRs are also expressed differentially depending on cell type. Phagocytic cells express the highest levels of TLRs as well as the broadest number of different TLRs (Muzio et al., 2000). It appears that nearly all of the cells in the body express some TLRs.

Although there are ten known TLRs, only five of the human TLRs recognize viral components. TLR4 has been extensively studied, and as such, a number of ligands have been identified for it. It was the original TLR that was discovered to be

the receptor for bacterial endotoxin, or lipopolysaccharide (LPS) (Kurt-Jones et al., 2000; Poltorak et al., 1998; Qureshi et al., 1999). LPS is a key component of the gram-negative bacterial cell wall, and the causative agent of septic shock. In addition to recognizing bacterial LPS, TLR4 also recognizes viral components, including the fusion protein of respiratory syncytial virus (RSV) (Haynes et al., 2001; Kurt-Jones et al., 2000). Upon recognition of these ligands, and using myeloid differentiation factor-2 (MD-2), TLR4 forms a homodimer on the surface or cells and initiates the signaling cascade, involving both the NF-κB and IRF3 inflammatory pathways.

The TLRs 3, 7, 8, and 9 represent a subset of TLRs that are responsible for recognition of bacterial and viral nucleic acids, as well as endogenous DNA that is present in pathogenic conditions (e.g. extracellular DNA, signifying lysed cells). Activation of these TLRs leads to inflammation through the type I interferon pathway, as well as through the upregulation of other proinflammatory cytokines. TLR3 recognizes double-stranded (ds) RNA from a variety of viruses, including retroviruses (Chu et al., 1999; Yang et al., 1995). It is also the receptor that is used for stimulating an immune response with the dsRNA mimetic polyinosinic polycytidilic acid (polyI:C). When challenged with polyI:C, mice activate the IFN pathway, as well as a variety of cytokines. TLR3 is not integral in the induction of IFN in response to polyI:C, however, it is necessary for the production of cytokines including IL-12p40; that is, without TLR3 activity, IL-12p40 and other cytokine pathways are not induced (Kato and Inoue, 2006). TLRs 7 and 8 recognize single-stranded (ss) RNA from RNA viruses, as well as from bacteria, within endolysosomes of DCs (Mancuso et al., 2009). Differing from TLRs 3, 7, and 8,

which recognize different forms of RNA, TLR9 recognizes unmethylated DNA, which contains CpG motifs (CpG motifs are DNA oligonucleotides that contain a cytosine followed by a guanine). They are relatively rare in mammalian genomes, and relatively abundant in microbial genomes. When unmethylated, this short DNA sequence serves as a PAMP. Though CpG was originally thought to be integral for TLR9 stimulation, it was soon shown that the backbone of 2' deoxyribose also mediates recognition (Haas et al., 2008). TLRs 7, 8 and 9, but not TLR3 are highly expressed in plasmacytoid dendritic cells (pDCs), immune cells that elicit a strong interferon response to pathogens (Muzio et al., 2000). TLR3 is more ubiquitously expressed, with high levels in epithelial and endothelial cells, as well as immune cells (Matsukura et al., 2006).

1.1.II.iii. RIG-like receptors

The RIG-like receptors (RLRs) are cytoplasmic pathogen sensors that recognize viral RNA in the cytoplasm. There are three members of the RLR family, namely retinoic acid-inducible gene (RIG-I), melanoma differentiation-associated gene 5 (MDA5) and laboratory of genetics and physiology-2 (LGP2) (Takeuchi and Akira, 2009; Yoneyama et al., 2005). Two of the family members, RIG-I and MDA5 possess two N-terminal caspase-recruitment domains (CARDs), a DExD/H box RNA helicase domain and a C-terminal repressor domain (RD). LGP2 lacks a CARD.

Host RNA typically exists in ssRNA form, is turned over quickly, and is capped at the 5' ends. Viral RNA differs from this in that RNA viruses produce large amounts of long dsRNA (not typically present in the cell) and RNAs with uncapped

5'-triphosphate ends. In order to recognize viral RNA, whether it is long dsRNAs or 5'-triphosphate RNA, both the CARD and RD domains are important (Saito et al., 2007; Yoneyama et al., 2004). As LGP2 lacks a CARD, it is believed that it functions as a negative repressor for the other two proteins.

RLRs are integral for mounting a type I interferon response to RNA virus infection. Knockout of any one of the RLRs is enough to make mice susceptible to a subset of RNA viruses (Gitlin et al., 2006; Kato et al., 2005; Kato et al., 2006; Melchjorsen et al., 2005). Both ssRNA viruses and dsRNA viruses are capable of being recognized by either RIG-I or MDA5. RIG-I is the essential sensor for both short dsRNA and 5'-triphosphate ssRNA, and MDA5 is integral for long dsRNA recognition (Hornung et al., 2006; Kato et al., 2006; Pichlmair et al., 2006).

RIG-I signaling is mediated both positively and negatively by ubiquitination. For activation of RIG-I, the CARDs must be ubiquitinated by tripartite motif 25 (TRIM25). TRIM25$^{-/-}$ cells produce a weakened IFN I response during viral infection (Gack et al., 2007). A second ubiquitin ligase, RNF125, also ubiquitinates RIG-I, thereby inducing proteasomal degradation (Arimoto et al., 2007).

1.1.II.iv. NOD-like Receptors

NOD-like receptors form a large family of receptors that are responsible for modulating the production of IL-1β. IL-1β is controlled not only transcriptionally, but also by the cleavage of a pro-protein, pro-IL-1β (Kanneganti et al., 2007; Petrilli et al., 2007). This protein is cleaved by caspase-1, contained within the inflammasome, a large complex of proteins that is activated in response to viral infection. The NLRs

Nacht domain-, leucine-rich repeat- and PYD-containing protein 3 (NALP3) and ICE-protease-activating factor (IPAF) are directly responsible for activating caspase-1. Both dsRNA and polyI:C have been demonstrated to activate the inflammasome via NALP-3, although it is unclear whether NALP3 is directly responsible for recognizing dsRNA. Interestingly, NALP3-dependent caspase-1 cleavage has also been shown to activate IL-1β in response to Ad infection. Contrary to this, Akira's group presented data suggesting that the IL-1β production in response to Ad is not due to recognition of genomic DNA. The apoptosis-associated speck-like protein containing a CARD (ASC), an NLR adaptor protein, and caspase-1 are necessary for dsDNA-induced IL-1β production, which leaves the possibility of an unknown NALP functions to recognize cytosolic DNA (Muruve et al., 2008).

1.1.II.v. Interferon Regulatory Factor 3

Interferon regulatory factor 3 (IRF3) is a key component of the innate immune response to microbes, including viruses. The gene itself is constitutively expressed, and is ubiquitous in every tissue of the body (Au et al., 1995). The gene encodes a 427 amino acid protein (Lin et al., 1998; Servant et al., 2002). The protein exists as a monomer in the cytoplasm and when resolved by SDS-PAGE, two isoforms (form I and form II) are typically visible (Servant et al., 2002; Servant et al., 2001). There are four different, distinct regions of the protein that can be phosphorylated (Figure 1.3). Two of the regions are located in the N-terminal region, and the other two in the C-terminal region of the protein (Servant et al., 2002). Upon infection, the C-terminus

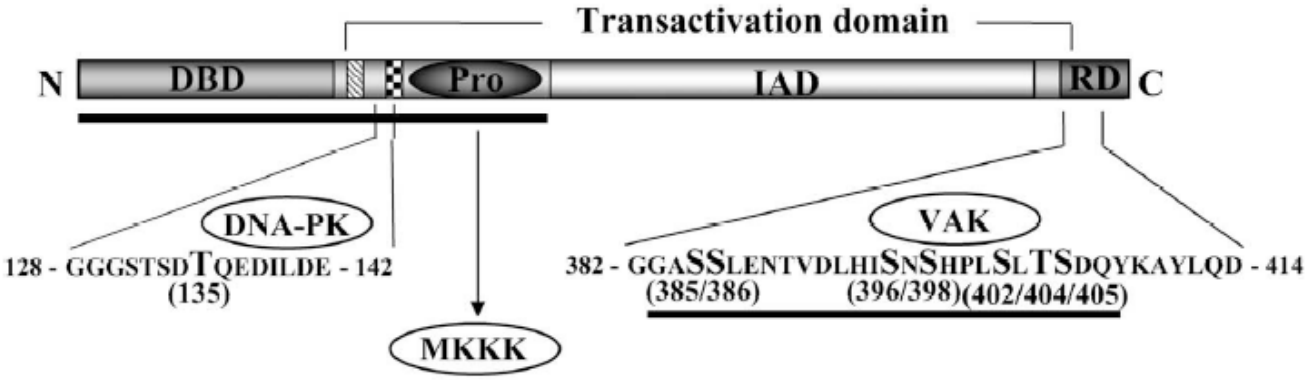
of the protein is hyper-phosphorylated, which leads to a slower migrating protein on an SDS-PAGE (forms III and IV) (Mori et al., 2004; Servant et al., 2001).

Serine 396 (S396) is the canonical activating residue of IRF3 (Servant et al., 2003). IKK-related kinases IKKε and TBK1 form the only known kinase complex that is responsible for activation of IRF3 in response to RNA viruses (Fitzgerald et al., 2003; Hemmi et al., 2004; Perry et al., 2004). The events leading up to and including activation of IRF3 have been very well characterized. There are multiple pathways that recognize viral RNA and lead to IRF3 activation. Both ssRNA viruses and dsRNA viruses lead to activation of IRF3. This is due to the requirement of dsRNA intermediates amongst ssRNA viruses. When the dsRNA is produced, it is recognized by TLR3 (Oshiumi et al., 2003; Yamamoto et al., 2003; Yamamoto et al., 2002), RIG-I (Rothenfusser et al., 2005; Yoneyama et al., 2005; Yoneyama et al., 2004), and MDA5 (Kato et al., 2006). Upon recognition of dsRNA, TLR3 acts through the adapter protein Toll/IL-1 Receptor (IL-1R) homology domain-containing adapter inducing IFN-β (TRIF) (Oshiumi et al., 2003; Yamamoto et al., 2003; Yamamoto et al., 2002). Both RIG-I and MDA5 signal through the interferon-β promoter stimulator-1 (IPS1). Both of these adapter proteins converge on a single kinase complex (the IKKε/TBK1 complex mentioned above) (Kawai et al., 2005; Meylan et al., 2005; Seth et al., 2005; Xu et al., 2005). This complex phosphorylates both IRF3 and IRF7 (another interferon regulatory factor), leading to activation of these proteins.

Phosphorylation of S396 exposes IRF3's DNA binding and transcriptional activation domains via a conformational change

Figure 1.3. Schematic representation of IRF3

IRF3 represented with the four regions of the protein that are phosphorylated; as well as their respective known kinases (Servant et al., 2002). Used with permission.


Transactivation domain
N
DBD
Pro
IAD
RD
C
DNA-PK
128 - GGGSTSDTQEDILDE - 142
(135)
MKKK
VAK
382 - GGASSLENTVDLHISNSHPLSLTSDQYKAYLQD - 414
(385/386)
(396/398)
(402/404/405)

(Lin et al., 1998; Servant et al., 2002; Wathelet et al., 1998). The protein then homodimerizes, translocates to the nucleus and interacts with the IRF3 consensus DNA binding site (Lin et al., 1998; Lin et al., 1999; Servant et al., 2002; Yoneyama et al., 1998). Two extremely similar transcriptional co-activators, the histone acetyltransferases CBP and p300 are recruited and interact with IRF3, preventing it from exiting the nucleus (Kumar et al., 2000; Lin et al., 1998; Lin et al., 1999; Servant et al., 2002; Yoneyama et al., 1998). This complex is known as the IFN-β enhanceosome, and also consists of additional proteins including the NF-κB p50/p65 heterodimer, and the b-ZIP proteins ATF-2 and c-Jun. This structure is assembled on the HMG-I/Y architectural protein (Agalioti et al., 2000). The entire complex binds to positive regulatory domains in type I IFN promoters and interferon-stimulated response elements (ISRE) in target genes (Lin et al., 2000; Lin et al., 1998; Lin et al., 1999; Schafer et al., 1998; Servant et al., 2002; Wathelet et al., 1998; Weaver et al., 1998; Yoneyama et al., 1998). Though this large complex is required for full induction of IRF3 target genes, in response to viral infection, IFN-β transcription can be stimulated by IRF3 alone (Fan and Maniatis, 1989; Fujita et al., 1987; Leblanc et al., 1990). After its activation, IRF3 is degraded via the proteasome pathway (Lin et al., 1998; Ronco et al., 1998; Servant et al., 2002). This final process ensures that as quickly as it came, the inflammatory response subsides.

There have been several reports that have linked IRF3 activation to DNA-PK, a previously unrelated kinase. DNA-PK is a kinase commonly associated with ATM and ATR (ataxia telangiectasia mutated and ataxia telangiectasia and Rad3 related, respectively) (Karpova et al., 2002; Kim et al., 2000; Servant et al., 2001). These

three proteins are well-characterized kinases involved in sensing DNA damage and activating the DNA damage response (Huen and Chen, 2008). In response to genotoxic stress including ultraviolet (UV) irradiation and other genotoxic agents including doxorubicin and etoposide, these kinases become active and initiate cell cycle arrest, which in turn, depending on the extent of the genomic damage, can lead to repair, or alternatively to an apoptotic response (Huen and Chen, 2008). The link of DNA-PK with IRF3, as an initiator to an inflammatory response to not only infection but also genotoxic stress provides interesting implications for investigating previously un-described pathways. Alternatively, as described below, IRF3 has also been linked to the apoptotic response to viral infection. This link between viral and genotoxic-induced apoptosis could uncover a new function for some of the previously unknown regulatory functions of IRF3.

The other phosphorylation sites, as described above, are located in the N-terminal region of the protein, and their functional relevance to the protein has not been fully explored as yet (Servant et al., 2002). There are some proposed mechanisms associated with N-terminal phosphorylation, including the suggestion that it may allow for a conformational change that makes the C-terminal activating residues more readily accessible (Lin et al., 1999). Other suggested implications of the N-terminal region include controlling IRF3 activity prior to nuclear translocation or protein stability (Servant et al., 2002). Further possibilities still, these phosphorylation events may have no effect on IRF3's activity as a transcription factor, but rather hinge on the demonstrated properties of IRF3 to interact with other regulatory proteins, discussed below.

1.1.III. IRF3 and viral-induced apoptosis

A number of recent reports have linked, for the first time, IRF3 and apoptosis. IRF3 has been demonstrated to interact with Bax, a pro-apoptotic member of the Bcl-2 family of proteins. IRF3 has a BH3 domain, located in the C-terminal of the protein, through which it interacts with Bax (Chattopadhyay et al., 2010). Together, these proteins mediate a viral-induced apoptotic event that serves to limit viral replication. Interestingly, the induction of the apoptotic response mediated by IRF3 and Bax requires many of the same proteins that are required for inflammatory activation of IRF3. RIG-I, IPS1, TRAF3 and TBK1 are proteins that overlap both the inflammatory function of IRF3 as well as the apoptotic pathway (Chattopadhyay et al., 2011). Where RIG-I recognizes viral RNA for both the inflammatory and apoptotic pathways mediated by IRF-3, in response to DNA viruses, including Ad, it has been demonstrated that the initial recognition of nucleic acids occurs via RNA-polymerase III. The apoptotic pathway also requires TRAF2 and TRAF6. Additionally, IRF3 that is unable to transactivate target genes still maintains the ability to interact with Bax and mediate virus-induced apoptosis (Chattopadhyay et al., 2011).

Upon interaction with Bax, both proteins translocate to the mitochondria and activate the mitochondrial apoptotic pathway. In this way, it has also been demonstrated recently that this apoptotic response actively serves to limit viral replication, and as such forms another, novel method of combating viral infection (Chattopadhyay et al., 2010; Chattopadhyay et al., 2011).

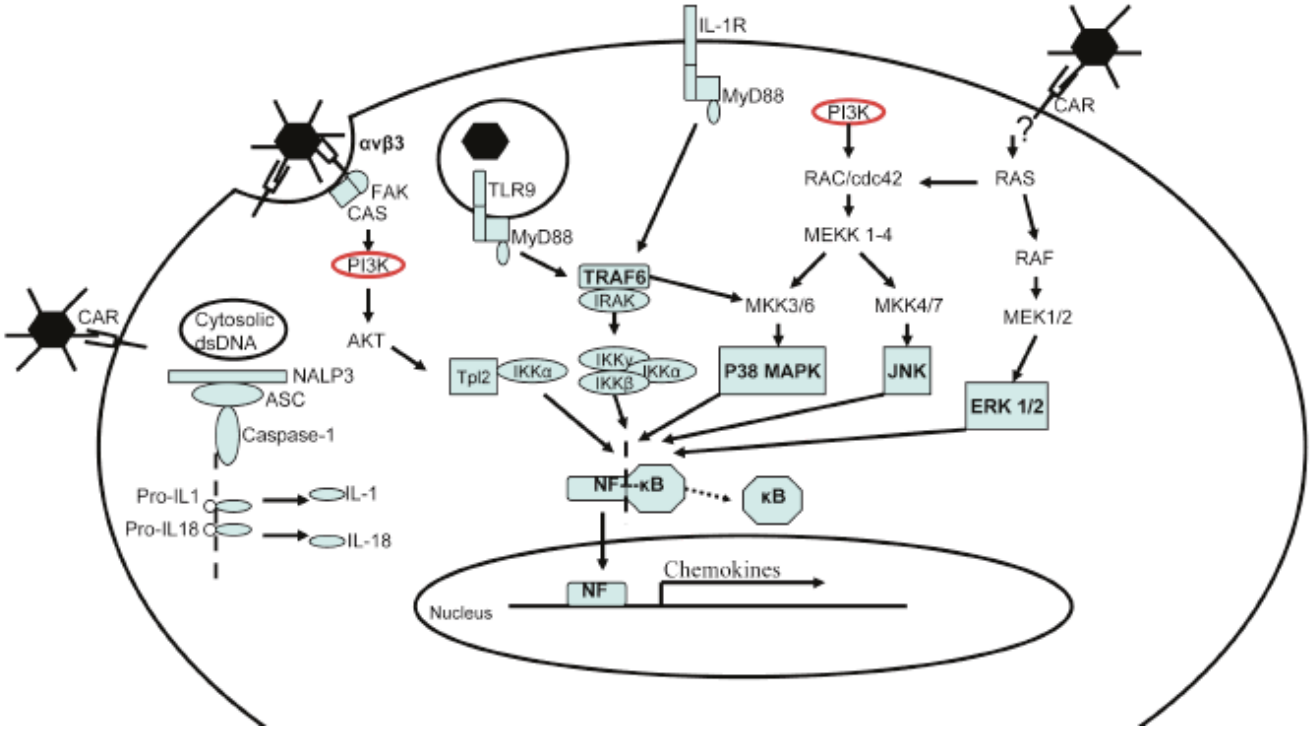
Unlike the C-terminal phosphorylation events that are involved in the activation of IRF3, phosphorylation of the N-terminal regions of the protein do not have a defined function. It is possible that these phosphorylations play a distinct role in the modulation of IRF3 interaction with Bax, or other, as yet undiscovered interacting proteins. Thus, there is much work still to be done towards the characterization of IRF3's involvement in the apoptotic response to viruses.

1.1.IV. IRF3 and Ad infection

In response to Ad, there is a robust innate immune response, involving several pathways (Figure 1.4). The two most important inflammatory pathways involved in blocking viral replication are the interleukin-1 pathway and the type I interferon pathway. In a short period following Ad infection, recognition of the virus leads to full activation of IL-1 (Di Paolo et al., 2009; Shayakhmetov et al., 2005b). A number of kinases are also activated. JNK, ERK1/2 and p38 MAPK make up the kinases that are induced following Ad infection (Gehart et al., 2010). These kinases are also effectors of non-specific stress pathways. Activation of these kinases leads to the induction of the NF-κB pathway and the production of various chemokines, including IL-1. In parallel to this response, is the type I interferon response.

There have been a number of conflicting reports in the literature over the recent past regarding IRF3's involvement in the response to Ad. Some groups have demonstrated an activation of IRF3 in response to Ad infection (Lee et al., 2010; Muruve et al., 2008; Nociari et al., 2009; Nociari et al., 2007; Stein and Falck-Pedersen, 2012; Uematsu and Akira, 2007) ; while other groups, including work from

Figure 1.4. Innate immune pathways activated in response to Ad infection.
(Thaci et al., 2011). Used under terms of Creative Commons Attribution License.


IL-1R
MyD88
PI3K
CAR
?
αvβ3
FAK
CAS
TLR9
MyD88
RAC/cdc42
RAS
MEKK 1-4
RAF
PI3K
TRAF6
IRAK
MKK3/6
MKK4/7
MEK1/2
CAR
Cytosolic
dsDNA
AKT
Tpl2
IKKα
IKKγ
IKKβ
IKKα
P38 MAPK
JNK
ERK 1/2
NALP3
ASC
Caspase-1
NF-κB
κB
Pro-IL1
IL-1
Pro-IL18
IL-18
Chemokines
NF
Nucleus

our own lab have observed modifications to IRF3, but not activation (Fejer et al., 2008; Zhu et al., 2008). What is known for certain is that Ad induces a significant inflammatory response regardless of any modifications that have been made to the viral genome (Ad whole virus, first-generation Ad vectors lacking some viral genes, or Helper-dependent Ad vectors devoid of all viral coding DNA) (Alcorn et al., 2001; Chintakuntlawar and Chodosh, 2009; Di Paolo et al., 2009; Koizumi et al., 2007; Muruve et al., 1999; Philpott et al., 2004; Tamanini et al., 2006; Xing et al., 2000; Zaiss et al., 2002). Ad infection has been demonstrated to invoke all arms of immunity through a variety of pathways (Bruder and Kovesdi, 1997; Cerullo et al., 2007; Hartman et al., 2008; Jiang et al., 2004; Muruve et al., 2008; Tibbles et al., 2002), but there is still a great deal of debate about the involvement of IRF3 in the activation of innate inflammation.

Three related reports from Falck-Pederson's group demonstrated that Ad infection of antigen presenting cells (APCs) leads to activation of IRF3. In the first study, they demonstrated that Ad is recognized through a pathway that is independent of TLR signaling, and recognizes viral dsDNA (Nociari et al., 2007). In addition, intact viral particles and capsid proteins are required for activation of IRF3. In addition to being the first group to demonstrate IRF3 activation in response to Ad infection, this study also showed that the IRF3 pathway is the predominant response in APCs, rather than the Akt/NF-κB pathway (Nociari et al., 2007). The subsequent paper reported that in response to Ad DNA, TBK1 is phosphorylated at serine 172. That phosphorylation event however, is not enough to lead to the activation of IRF3. Ad DNA did not lead to activation of IKKε, the second component of the activating

complex (Nociari et al., 2009). Nociari *et al.* demonstrated that in addition to viral DNA in the cytoplasm, viral capsid leads to activation of Jun N-Terminal Kinase (JNK), and licenses TBK1 to phosphorylate IRF3 S396. The physiological relevance of these reports remains in question due to the high quantities of virus (25,000 virus particles per cell), or alternatively the cell types used for these experiments (dedicated immune cells including plasmacytoid dendritic cells and macrophages).

A third paper from the same group demonstrated activation of IRF3 in RAW 264.7 murine macrophages in response to Ad infection. In this study, the activation was once again TBK1 dependent and was effected via multiple DNA sensors (Stein and Falck-Pedersen, 2012). Knockdown of the absent in melanoma 1 (AIM1) protein or the helicase DDX41 resulted in significantly attenuated IRF3 activation.

Additionally to the studies by Falck-Peterson's group, Muruve *et al.* demonstrated that in response to Ad DNA, or in fact any cytosolic DNA leading to inflammation, the inflammation happens via the interferon pathway, and independent to the inflammasome (Muruve et al., 2008). While there is activation of the inflammasome, leading to IL-1β production, inhibition of the inflammasome did not prevent phosphorylation and activation of IRF3. Once again, these studies were carried out in the macrophage-like cell line THP1 (human acute monocytic leukemia cell line), and as such the results observed may not hold true across all cell types.

Interestingly, a study from Hu's lab that investigated the *in vivo* effects of Ad infection on a co-culture of macrophages and epithelial cells led to the observation that in response to Ad infection, there were signs of macrophage activation, as well as inflammation within 24 hours of infection (Lee et al., 2010). Macrophage

activation was demonstrated by a significant decrease in intracellular pH. Inflammation was observed based on cytotoxicity, release of pro-inflammatory cytokines, and production of nitric oxide and reactive oxygen species. Also of note, Hu's group observed that when cultured alone, the macrophages either were not activated or were activated to a much-attenuated level, as compared to the co-cultures. These results implied a synergistic effect of the cross-talk between epithelial cells and macrophages in response to Ad infection (Lee et al., 2010).

As mentioned above, there have been several studies recently that have linked IRF3 with the pro-apoptotic protein, Bax. Interestingly, in one of these studies, the virus inducing this interaction was in fact, Ad (Chattopadhyay et al., 2011). Upon infection with Ad, as well as the RNA viruses Sendai virus (SeV), vesicular stomatitis virus (VSV), and encephalomyocarditis virus (EMCV), Chattopadhyah and colleagues described a direct interaction, and indeed, a modulation by IRF3 of the Bax regulated pathway of apoptosis (Chattopadhyay et al., 2010; Chattopadhyay et al., 2011).

There are multiple potential explanations for the difference in response between immune and non-immune cells. The most relevant explanations include a viral-encoded strategy to bypass or inhibit the innate sensing of viral components including DNA or proteins; or alternatively, certain pathways that are active in professional immune cells are absent, or inactive in non-professional immune cells. Perhaps most interesting to this particular point is a new study by Fonseca *et al.* that demonstrated active evasion by Ad of the interferon-mediated innate response through an antagonism of a cellar histone posttranslational modifying protein

complex, hBre1/RNF20 (Fonseca et al., 2012). Given that the epithelial cells that line the digestive and respiratory tracts are the first sites of Ad infection, as well as the first lines of defense against Ad and other viruses, the inability of these non-professional immune cells to raise a significant interferon response could have broad-reaching implications regarding pathogenesis of the virus.

1.2. Objective

There is a great deal of information available regarding the inflammatory response to RNA viruses, be they ssRNA or dsRNA viruses. In response to these viruses, the first line of response is the PRRs, including TLRs, RLRs and NLRs. The response to DNA viruses is known to involve the same cellular machinery, yet the mechanisms of response to these viruses are still enshrouded with many questions. IRF3 is a key transcription factor that overlaps as a pro-inflammatory regulatory protein in response to RNA and DNA viruses. In addition to its role as an inflammatory regulator, IRF3 has also been recently implicated in the apoptotic response to viral infection. Apoptosis is a mechanism that is often linked to the DNA-damage response, yet to date, IRF3 has not been implicated as a key protein in the DNA-damage response. Most studies have examined IRF3 as an inflammatory transcription factor and an apoptotic mediator at the protein level. We have attempted in this project, to examine the mechanisms governing IRF3 activity in response to Ad infection. We have investigated a unique post-translational modification that has not previously been associated with Ad infection. We will determine whether our novel modification has implications in antiviral inflammation and/or antiviral apoptosis, as well as investigating novel roles for IRF3 in response to genotoxic stress.

1.3. Hypothesis

Previous studies in our lab have demonstrated a novel phosphorylation event on IRF3 in response to Ad infection. We hypothesize that this phosphorylation event

is key for regulating IRF3 activity in the context of inflammation, as well as apoptosis. Furthermore, we hypothesize that IRF3 is involved in the apoptotic response to DNA damage.

1.4. Approach

Phosphorylation status will be controlled using residue-specific IRF3 mutants and phosphorylation status will be analyzed in infected as well as treated (genotoxic stress) cells. We will use reporter constructs using IRF3 target gene promoters to investigate transcriptional activity. We will use cell viability assays to investigate the importance of IRF3 phosphorylation status in cell survival. Taken together, these data will determine the involvement of IRF3 in the inflammatory and apoptotic pathways in response to both Ad infection, as well as DNA damage.

1.5. Rationale

Despite the ever-growing body of knowledge regarding the innate immune response to Ad, the role of IRF3 in non-immune cells remains elusive. Being a significant pathogen, as well as a promising vector for gene therapy, a greater understanding of the antiviral response is necessary. We believe that there might also be some relevance of our phosphorylation event to the control of IRF3's activity as a mediator of apoptosis.

In order to create a more effective gene therapy vector, as well as to potentially create a better vaccine to the wildtype virus, a greater understanding of the innate immune response to the virus is required. Additionally, insight gained from

this study has a possibility to link the inflammatory response and viral induced apoptosis to the DNA damage response for the first time. Unifying these two pathways has the potential to uncover new, and as yet undiscovered pathways, as well as novel functions for known proteins.

Chapter 2: MATERIALS AND METHODS

2.1. Reagents:

See Appendix A for list of reagents, solutions, and buffers used.

2.2. Cell culture and viral infections.

A549 (ATCC CCL 185) human lung adenocarcinoma cells were maintained in Minimal Essential Media (MEM, Sigma) supplemented with 10% fetal bovine serum (FBS), 1% GlutaMax and 1% Antibiotic/Antimycotic (Invitrogen) at 37^{o} C and 5% CO_2 atmosphere. HeLa (ATCC CCL 2) human epithelium derived cells were maintained under the same conditions in Dulbecco Modified Eagle Medium (DMEM, Sigma) supplemented with 10% FBS, 1% GlutaMax, and 1% Antibiotic/Antimycotic (Invitrogen).

Wild type adenovirus serotype 5 was obtained from J. Bell (Ottawa Hospital Research Institute, Ottawa ON). Ad5 vectors used in the experiments were: AdCA35 (AdΔE1-LacZ) from (Addison et al., 1997), AdRP2825 (AdΔE1-FLAG-wtIRF3), AdRP2762 (AdΔE1-FLAG-IRF3 S173A), AdRP2763 (AdΔE1-FLAG-IRF3 S173D) and AdRP2928 (AdΔE1-FLAG-IRF3 S173E). All Ad infections were performed in Dulbecco-phosphate buffered saline (DPBS, Sigma) at multiplicities of infection (MOI) from 5 to 100 PFU/cell for 1 hour at 37^{o} C. An attenuated Vesicular Stomatitis Virus from Dr J. Bell (OHRI) was used as a positive control for IRF3 activation. VSV Δ51 has a deletion of codon 51 of the M protein. One of the roles of the M protein of VSV is preventing interferon production and this deletion renders the virus highly

susceptible to VSV less able to suppress cellular immunity (Ahmed and Lyles, 1997; Coulon et al., 1990; Stojdl et al., 2003). Infections with VSV were carried out in MEM (for A549s) or DMEM (for HeLas) at MOI of 10 PFU/cell for 1 hour at 37^{o} C.

2.3. DNA damage induction

Cellular DNA damage was induced using either single stranded breaks (UV irradiation) or double stranded breaks (Etoposide). HeLa and A549 cells were irradiated with varying levels of UV using a UVP CL-1000 Ultraviolet Crosslinker, ranging from 5 to 250 J/m^2. The media was aspirated from the cells prior to irradiation to allow for optimal effect. New media was added to the cells and they were allowed to recover before down-stream processing.

The chemotherapeutic drug etoposide (VP16, Sigma) was used at 100 μM and added directly to media. The drug was allowed to act for twenty-four hours before down-stream processing.

2.4. Plasmid cloning

FLAG-tagged human IRF3 was cloned into pcDNA3. The serine residue at position 173 was mutated to alanine, aspartic acid, or glutamic acid in a series of cloning steps involving the insertion of various mutated oligonucleotides (Sigma) into the pcDNA-FLAG-IRF3 vector. Plasmid DNA was transformed into RbCl competent cells (prepared as in (Doyle, 1996)) by heat shock method. Small scale DNA preparation used alkaline lysis per methods described by Birnboim and Doly (Birnboim and Doly, 1979), with large scale preparation performed by alkaline lysis

with purification by CsCl buoyant density centrifugation as described in Sambrook *et al.* (Sambrook et al., 1989). All constructs were sequenced by StemCore Labs (OHRI, Ottawa, ON) to confirm their identity.

The RANTES promoter reporter construct used in this project (-181 RANTES) was provided by Dr D.A. Muruve (University of Calgary, Calgary AB).

2.5. Transient transfections

HeLa cells were seeded for transfection at ~$1.0x10^6$ cells/35 mm dish. Transfection was performed in serum-free DMEM using Lipofectamine 2000 (Invitrogen) and 4 μg of DNA per plate as instructed by the manufacturer.

2.6. Immunoblot analysis

HeLa or A549 cells in 35 mm dishes were harvested using 200 μL of 2x denaturing protein buffer containing β-mercaptoethanol. Twenty microlitres of these lysates were processed by electrophoresis on 10% acrylamide gels. Proteins were electrophoretically transferred onto PVDF membranes (Millipore) using semi-dry transfer (BioRad). The membranes were blocked in Tris-buffered saline with 0.1% Tween 20 (TBST) containing 5% powdered skim milk for 1 hr at room temperature with shaking, or at 4^o C overnight. Primary and secondary antibodies were diluted in the blocking solution and incubated with membranes as instructed by the manufacturers. After incubation with each antibody, membranes were washed (with shaking) three times with TBST for 5 min at room temperature. The primary antibodies used in these studies were anti-IRF3 mouse monoclonal (550428, BD

Pharmingen) (1:5000), anti-FLAG M2 mouse monoclonal (200-301-383, Rockland) (1:5000), anti-IRF3-phospho-396 rabbit monoclonal (4947, Cell Signaling) (1:1000), anti-αtubulin mouse monoclonal (CP-06, CalBiochem) (1:5000), and anti-pan-actin rabbit monoclonal (8456, Cell Signaling) (1:1000). Secondary antibodies used in these studies were IgG (H+L)-HRP-conjugated goat anti-rabbit (BioRad) (1:5000) and goat anti-mouse (BioRad) (1:5000). Membranes were developed with with Pierce ECL Western Blotting Substrate (Pierce) according to the manufacturer's instructions. To remove bound antibody from membranes for re-probing, membranes were washed with TBST for 5 min at room temperature and incubated in Restore Western Blot Stripping Buffer (Pierce) for 10-20 min at 37^{o} C. Membranes were washed with TBST for 5 min at room temperature then blocked in blocking solution for 1 hr at room temperature with shaking, or at 4^{o} C overnight.

2.7. Densitometry analysis

Short exposures of immunoblots, with emphasis on unsaturated signal, were scanned using standard computer scanning software. The images were analyzed using ImageJ software (NIH) in order to quantify relative signal levels on the immunoblots. Relative signal level was determined by subtracting the background intensity from the actual signal, and normalizing the corrected IRF3 signal to the corrected tubulin signal.

2.8. Luciferase assays

After removal of culture medium, HeLa cells were harvested with 200 μL of

Reporter Lysis Buffer (E397A, Promega) and frozen at -80^{o} C. Cell lysates were thawed and cell supernatant (10 μL) was added to 100 μL of Luciferase Assay Substrate (E1501, Promega), and luciferase activity was measured in relative luciferase units using a Promega GloMax 20/20 luminometer.

2.9. Cytotoxicity assays

Media was aspirated from A549 cells on 35 mm dishes and they were washed with DPBS. Cells were incubated with 150 μL of DPBS containing ~2 μM of Calcein AM (Ex. 494 nm; Em. 517 nm) and ~4 μM of ethidium homodimer (Ex. 528 nm; Em. 617 nm) for 30 min in the dark using a LIVE/DEAD Cell Viability Assay kit (Invitrogen). After 30 minutes, samples were observed using a Zeiss fluorescence microscope.

2.10. Crystal violet assays

A549 cells were aspirated, washed with DPBS, and fixed using β-gal fixative for 30 min. The fixative was removed and the cells were washed again with DPBS. The plates were incubated with 1 mL of 0.1% crystal violet with shaking for 30 min at room temperature. Excess crystal violet was washed off with gentle flowing water. Cells were then left to dry overnight. Next day, bound crystal violet was reconstituted using 1 mL of 10% acetic acid and absorbance at 590 nm was taken using a microplate reader.

2.11. Statistical Analysis

Microsoft Excel was used for statistical analysis using two-tailed Student's t-Test assuming two-sample equal variance. Results were considered statistically significant when p-values of $p \leq 0.05$ was achieved.

Chapter 3: RESULTS

3.1. IRF3 is phosphorylated differentially during Ad and VSV infection

There have been a variety of studies that have explored the phosphorylation state of IRF3 during the course of infection by a variety of RNA viruses (Lin et al., 1998; Servant et al., 2001; Yoneyama et al., 1998). The hyper-phosphorylation associated with IRF3 activation leads to an electrophoretic shift which can be visualized by immunoblot. The phosphorylation of IRF3 results in a more slowly migrating band by SDS-PAGE. In uninfected cells and at steady-state, IRF3 exists predominantly in form I (the fastest migrating form; unphosphorylated IRF3) and form II (slower migrating; minimally phosphorylated). In response to activating stimulus, including VSV, IRF3 is hyper-phosphorylated to forms III/IV (Servant et al., 2001).

We conducted preliminary studies to recapitulate this migration shift in response to VSV, as well as to investigate whether infection with Ad induced the same shfit, indicative of IRF3 activation. A549 cells were mock infected or infected with either Ad or VSV (MOI 100 PFU/cell for Ad, 10 PFU/cell for VSV). Due to the respective life-cycle lengths of the two viruses, protein was harvested in protein-loading buffer at 24 hours post infection (hpi) for Ad, or 0, 3, and 6 hpi for VSV. IRF3 was resolved by a combination of SDS-PAGE and immunoblot. Under mock infection conditions, IRF3 was observed to be in the baseline state of form I. As early as 3 hpi with VSV, there was an observable shift from form I to form II. By 6 hpi, the shift was higher, to forms III and IV (Figure 3.1A). After 24 hrs of Ad infection, there

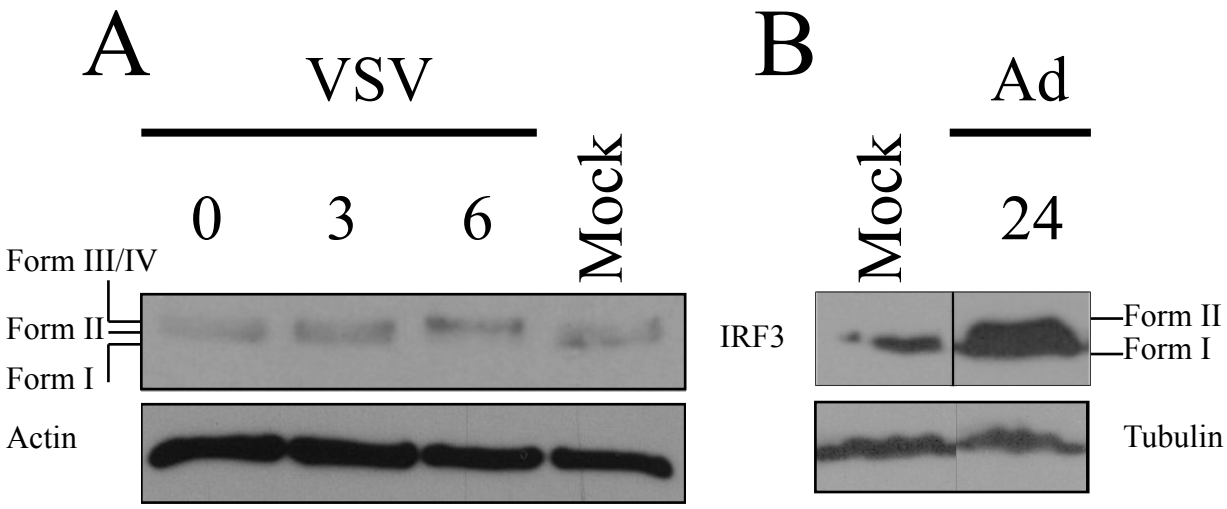
was a shift of IRF3 from form I to form II (Figure 3.1B). These results corroborated previous experiments completed in our lab which showed that under normal conditions in non-immune cells (primarily epithelial cells), there is an absense of IRF3 activation in response to Ad infection. Further to this fact, previous studies in our lab have used a series of point mutants to determine that the shift observed in response to Ad infection is due to a phosphorylation of IRF3 at serine 173 (S173) (K. Powell and R. Parks, unpublished). In addition to the observation of an S173 phosphorylation being the primary response of IRF3 to Ad infection, it was observed that this phosphorylation event was dependent on Ad genome replication (K. Chaisson and R. Parks, unpublished). As mentioned previously, all known activating events occur via the phosphorylation of carboxy-terminal residues, therefore this phosphorylation may regulate some other aspect of IRF3 infection.

3.2. IRF3 is phosphorylated similarly in response to genotoxic stress as in response to Ad infection

As mentioned above, previous studies in our lab determined that the phosphorylation responsible for the difference between IRF3 form I and II is at S173. We hypothesized that since the observation of this phosphorylation event in the context of Ad infection is dependent on the production of new Ad genomes, this phosphorylation could be a cellular response to what is perceived to be damaged DNA. In order to explore this phenomenon, we irradiated A549s with ultraviolet light (UV) at an intensity of 5 J/m^2. Protein was harvested at different timepoints and resolved by immunoblot to examine the phosphorylation status of IRF3. In response

Figure 3.1. VSV and Ad induced IRF3 phosphorylation.

(A) A549 cells were mock-infected, or infected with VSV (MOI 10 PFU/cell). (B) A549 cells were mock-infected, or infected with wtAd (MOI 100 PFU/cell). In panel A, cells were harvested with 2X denaturing protein buffer at 0, 3, and 6 hpi. In panel B, cells were harvested with 2X denaturing buffer at 24 hpi. Proteins were resolved by 10% SDS-PAGE, transferred to a PVDF membrane, and probed using anti-IRF3 antibody (BD Pharmingen). IRF3 forms I, II, and III/IV are indicated on each panel. Blots were re-probed with anti-actin (Cell Signaling) or anti-tubulin (CalBioChem) to ensure equal loading.


A
VSV
0
3
6
Mock
Form III/IV
Form II
Form I
Actin
B
Ad
Mock
24
IRF3
Form II
Form I
Tubulin

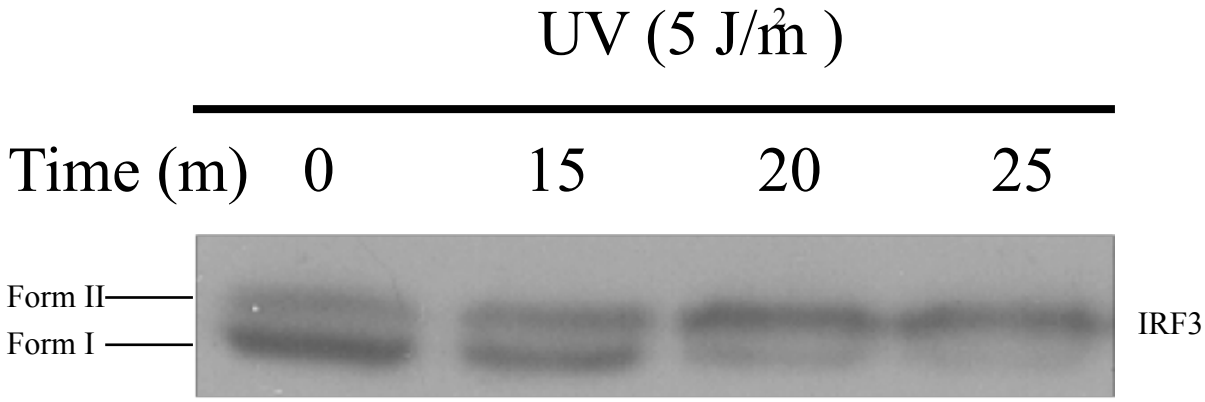
to UV irradiation, we observed a similar shift of IRF3 from form I to form II (Figure 3.2). This shift was both interesting, and significant, as it was the first major piece of corroborating evidence connecting IRF3 regulation in immunity and DNA damage. The phosphorylation of S173 after Ad infection, is directly reliant on Ad DNA replication, and occurs between 18-21 hpi in A549 cells. Phosphorylation of S173 occurs after a much shorter time in response to UV irradiation; In response to a modest dose of UV (5 J/m^2), a noticeable shift from form I to form II occurs within 15 minutes. By 20 minutes, the predominant form is form II. Thus, UV irradiation of cells caused a rapid phosphorylation of IRF3 S173.

In addition to UV irradiaton, which leads to single-stranded DNA breaks due to the formation of thymine dimers in the DNA structure, we examined the effects of the chemotherapeutic drug etoposide on IRF3 phosphorylation. Etoposide acts through inhibition of topoisomerase II activity, thus preventing re-ligation of the DNA strand, and eventually, double-stranded breaks. We observed a similar phosphorylation of IRF3, with a shift from form I to form II (data not shown). Unlike UV irradiation however, this shift did not occur immediately after treatment, and much like Ad infection, took twenty-four hours to elicit a response, at a dose of 100 μg/mL, making it another, albeit less convenient method for further study of IRF3 S173 phosphorylation.

While low level UV irradiation (5 J/m^2) appeared to provide a good paradigm for studying the significance and function of IRF3 S173 phosphorylation, it seemed possible that the shift observed from form I to form II may have simply been an intermediary observed on the way to a higher level of phosphorylation (signifying

Figure 3.2. UV-induced IRF3 phosphorylation.

A549 cells were irradiated with 5 J/m^2 UV light. Cells were harvested in 2X denaturing protein buffer, resolved by 10% SDS-PAGE, transferred to a PVDF membrane, and probed using an anti-IRF3 antibody (BD Pharmingen). IRF3 phosphorylation forms I and II are indicated.


UV (5 J/m²)
Time (m) 0 15 20 25
Form II
Form I
IRF3

activation of IRF3). We next determined whether a higher level of UV irradiation would lead to further phosphorylation of IRF3 (to forms III and IV) and the associated activation of the molecule. To investigate this, we irradiated cells with different levels of UV (5-250 J/m^2). As previously, protein was harvested in protein loading buffer and resolved using SDS-PAGE and immunoblot. In response to varying doses of irradiation, all doses gave rise to the same shift to predominantly form two (Figure 3.3). There was no further shift up to forms III or IV.

To confirm that UV did not lead to activation of IRF3, we examined the phosphorylation status of IRF3 S396. S396 is the canonical residue associated with IRF3 activation, and is involved in the relaxation of the IRF3 structure that is required for DNA binding and transactivation of target genes. Using much the same procedure as the experiment described above, we irradiated A549 cells with different levels of UV (5 or 250 J/m^2), harvested the protein 15 minutes later, and used SDS-PAGE and immunoblot with a phospho-specific antibody to IRF3 S396 to examine whether UV irradiation activates IRF3. VSV infection, but not UV irradiation at either low or high levels, leads to phosphorylation of S396 (Figure 3.4). Thus, although UV irradiation leads to a change in phosphorylation status of IRF3, it does not lead to phosphorylation of the canonical activating residue.

3.3. Phosphorylation of S173 influences IRF3 stability

Once it was determined that IRF3 undergoes a phosphorylation event in response to genotoxic stress similar to infection with Ad, we next attempted to discern a function of S173 phosphorylation. Phosphorylation, like other post-

Figure 3.3. High-dose UV-induced phosphorylation of IRF3.

A549 cells were irradiated with 0, 5, 25, 50, or 250 J/m^2 UV light. Cells were harvested in 2X denaturing protein buffer, resolved by 10% SDS-PAGE, transferred to a PVDF membrane, and probed using an anti-IRF3 antibody (BD Pharmingen). IRF3 phosphorylation forms I and II are indicated. Blots were re-probed with anti-tubulin (CalBioChem) to ensure equal loading.

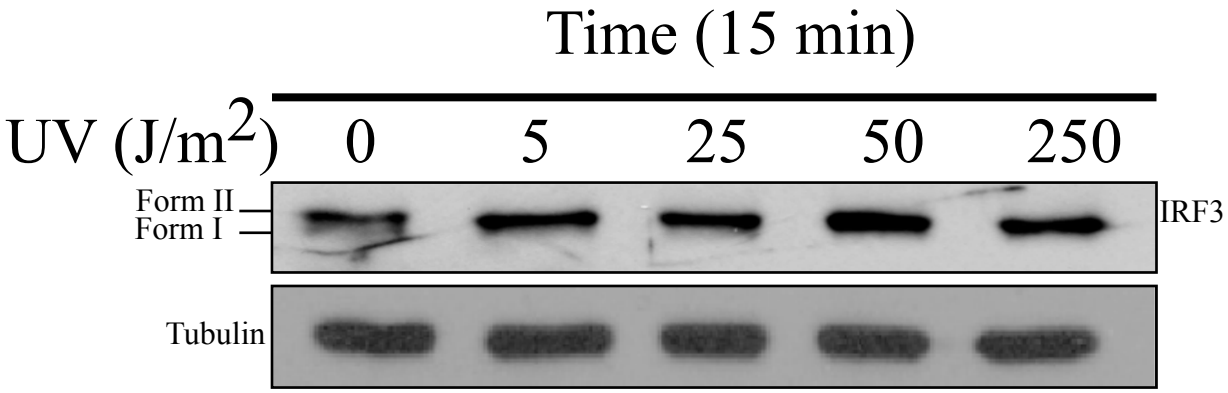
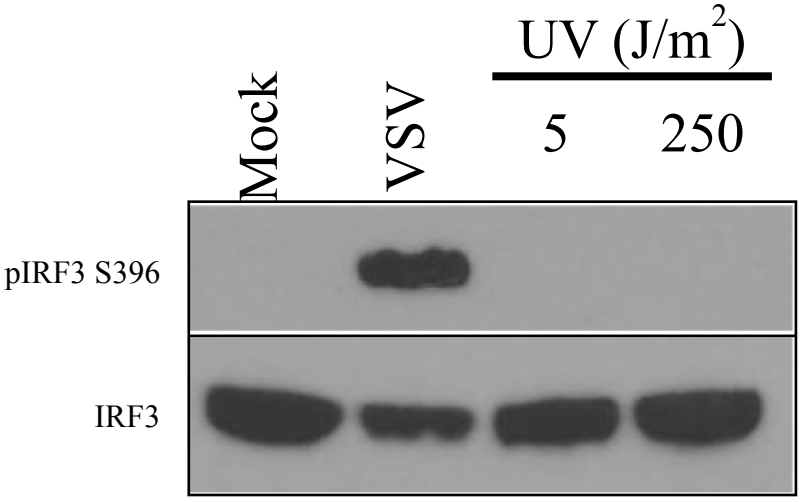

Time (15 min)
UV (J/m2)
0
5
25
50
250
Form II
Form I
IRF3
Tubulin

Figure 3.4. IRF3 S396 is not phosphorylated in response to UV.

A549 cells were mock-infected, infected with VSV (10 PFU/cell), or irradiated with UV (5 or 250 J/m^2). Cells were harvested in 2X denaturing protein buffer, resolved by 10% SDS-PAGE, transferred to a PVDF membrane, and probed using an anti-IRF3 antibody (BD Pharmingen). Blots were re-probed with a phospho-specific antibody for IRF3 S396 (Cell Signaling) to examine activation status.


UV (J/m2)
Mock
VSV
5
250
pIRF3 S396
IRF3

translational modifications, often influences protein stability. Phosphorylation targets some proteins for ubiquitination by E3-ligases, enhancing their degradation by the ATP-dependent ubiquitin/proteasome pathway (Ciechanover, 1994), while other phosphorylated proteins are less able to interact with other proteins (including the same E3-ligases). These are just two of the many different ways phosphorylation can modify the function or stability of a protein, and just one of the pathways by which proteolysis can occur. Given that S173 phosphorylation has not been linked to activation of IRF3, we investigated whether S173 phosphorylation altered IRF3 stability.

We transfected HeLa cells with plasmid constructs expressing wildtype IRF3, or IRF3 mutants with S173 mutated to either an alanine (S173A; cannot be phosphorylated) or glutamic acid (S173E; phosphomimetic). Twenty-four hours later, the cells were infected with VSV (chosen because it is known that VSV targets IRF3 for degradation by SUMOylation regardless of IRF3 activation status) and protein was harvested over a six hour time course. The resulting protein was resolved by SDS-PAGE, transferred to a membrane and probed by immunoblot. Relative levels of IRF3 or the respective mutants were analyzed by densitometry.

At steady state, before stimulation by VSV, IRF3 S173A appeared to accumulate to a higher relative level. When stimulated with VSV however, the wtIRF3 and IRF3 S173E both accumulated to a significantly higher level than steady state over the first two hours of infection. After this point, the drop in protein was rapid and the level of IRF3 was reduced to nearly undetectable by 6 hpi (Figure 5A & 5C). IRF3 S173A was also nearly completely degraded by 6 hours. In contrast to the

other mutants, IRF3 S173A did not initially accumulate to the same level, suggesting that inability to phosphorylate S173 results in lowered protein stability (Figure 5B).

3.4. IRF3 S173A leads to lowered IRF3 target gene (RANTES) expression

In addition to affecting protein stability, phosphorylation is also responsible for affecting the activity of many proteins, as exemplified by the C-terminal phosphorylations (especially S396) of IRF3. Although many studies have shown that C-terminal phosphorylation leads to activation, it is possible the N-terminus may also affect the ability of IRF3 to activate target genes (reviewed in: (Servant et al., 2002)).

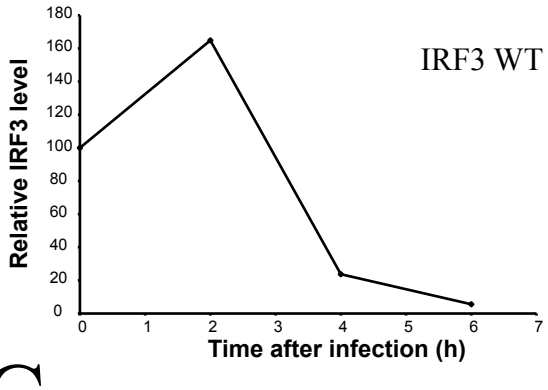
We used constructs expressing a firefly-luciferase protein controlled by a RANTES (IRF3 target gene) promoter, co-transfected with an empty vector (cDNA3), wtIRF3 or IRF3 S173A into HeLa cells to investigate the transactivating activity of IRF3. Forty-eight hours after transfection, cell lysates were harvested in luciferase buffer. These samples were measured for relative luminescence using a luminometer.

In comparing expression from the RANTES promoter relative to the protein level per sample, it appears that IRF3 WT expressing cells show similar transactivating activity compared to IRF3 S173A (Figure 3.6A). This result suggests that S173 phosphorylation does not affect IRF3's transactivating abilities, at least at baseline. After further study, the harvested protein was quantified by Bradford assay (Figure 3.6B), demonstrating that there was less total protein harvested from the cells transfected with IRF3 S173A. This suggested that there were less cells available on the plate when harvested. As

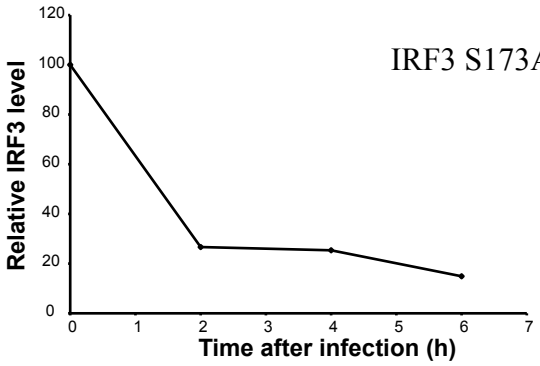
Figure 3.5. IRF3 stability following VSV infection.

FLAG-tagged IRF3 mutants ((A) IRF3 WT, (B) IRF3 S173A, and (C) IRF3 S173E) were transfected into HeLa cells. Twenty-four hours later, cells were infected with VSV (MOI 10 PFU/cell). Cells were harvested in 2X denaturing protein buffer, resolved by 10% SDS-PAGE, transferred to a PVDF membrane, and probed using an anti-FLAG antibody (Rockland). Blots were re-probed with anti-tubulin (CalBioChem). Signal intensities were analysed using ImageJ software and graphed in Excel. FLAG signal intensity was standardized to tubulin signal intensity, and normalized with t=0 being assigned the arbitrary value of 100. One experiment is shown, but results reflect 4 independent experiments.

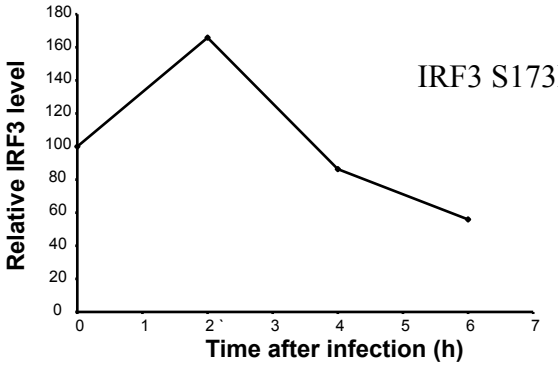
A


IRF3 WT
Relative IRF3 level
180
160
140
120
100
80
60
40
20
0
0 1 2 3 4 5 6 7
Time after infection (h)

B


IRF3 S173A
Relative IRF3 level
120
100
80
60
40
20
0
0 1 2 3 4 5 6 7
Time after infection (h)

C


IRF3 S173E
Relative IRF3 level
180
160
140
120
100
80
60
40
20
0
0 1 2 3 4 5 6 7
Time after infection (h)

equal numbers of cells were transfected, with equal quantities of DNA, the lower protein level could be due to cell death or reduced growth of S173A treated cells over the two days. Upon further investigation, this result suggests that S173 phosphorylation does not affect IRF3's transactivating abilities.

These data suggest that IRF3 S173 phosphorylation does not affect target gene activation. However, these studies suggest that overexpression of IRF3 S173A may adversely affect cell growth or survival.

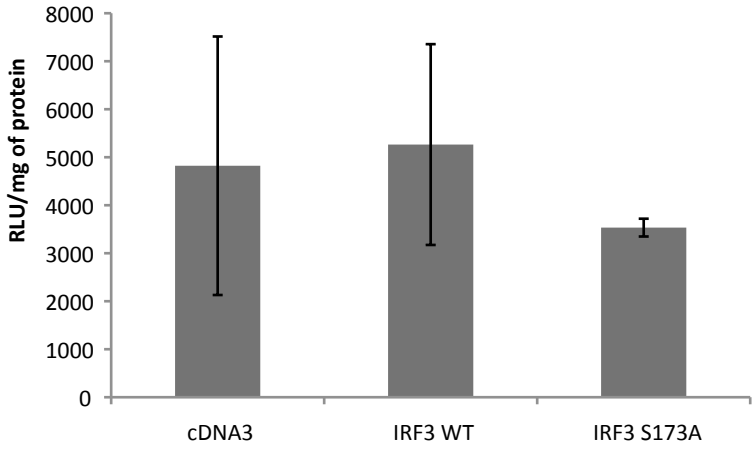
Drawing from the suggestion that IRF3 S173A could somehow be detrimental to the survival of cells, we investigated whether IRF3 S173 plays a role in cell growth, survival, or death. We infected A549 cells with Ad vectors over-expressing wtIRF3, IRF3 S173A, or IRF3 S173E. Twenty-four hpi we assayed the viability of the cells using a Live/Dead assay kit. Using this kit, dead cells are stained using ethidium homodimers to appear red by fluorescence microscopy, while live cells appear green, due to green fluorescent calcein-AM to show intracellular esterase activity.

From the images in Figure 3.7, there are more red cells present in samples treated with Ad-IRF3 S173A than any of the other IRF3 expressing vectors (Figure 3.7). It also appears that the density of the IRF3 S173A appears lower, and the cells appear more rounded, and therefore cell death is increased, despite identical plating conditions for all cells.

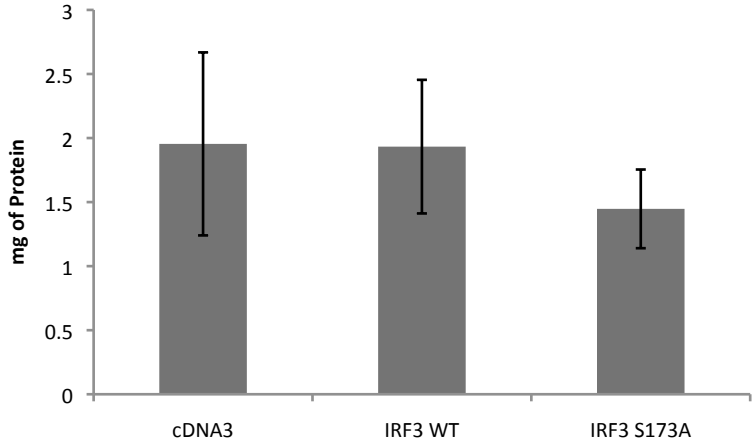
Figure 3.6. IRF3 mutants effect on RANTES promoter activity.

FLAG-tagged IRF3 mutants (IRF3 WT, IRF3 S173A) and a RANTES-luciferase plasmid were transfected into HeLa cells. Forty-eight hours later, cells were harvested in 1X luciferase buffer. (A) Luciferase activity was assayed using a Promega kit and a Promega GloMax 20/20 luminometer. Luciferase activity, as represented by relative light units (RLU) was normalized to protein levels as determined in panel B. (B) Protein content of samples was determined using Bradford reagent (BioRad). 2.5 μL of sample was mixed with 1 mL of 1X Bradford reagent. Absorbance at 595 nm was taken and compared to a standard curve of BSA to determine protein level per sample. Error bars represent the range of values observed in 2 replicates. Results reflect 2 independent experiments.

A


8000
7000
6000
5000
4000
3000
2000
1000
0
RLU/mg of protein
cDNA3
IRF3 WT
IRF3 S173A

B


3
2.5
2
1.5
1
0.5
0
mg of Protein
cDNA3
IRF3 WT
IRF3 S173A

3.5. Over-expression of IRF3 S173A leads to greater cell death than wtIRF3 or phosphomimetic IRF3

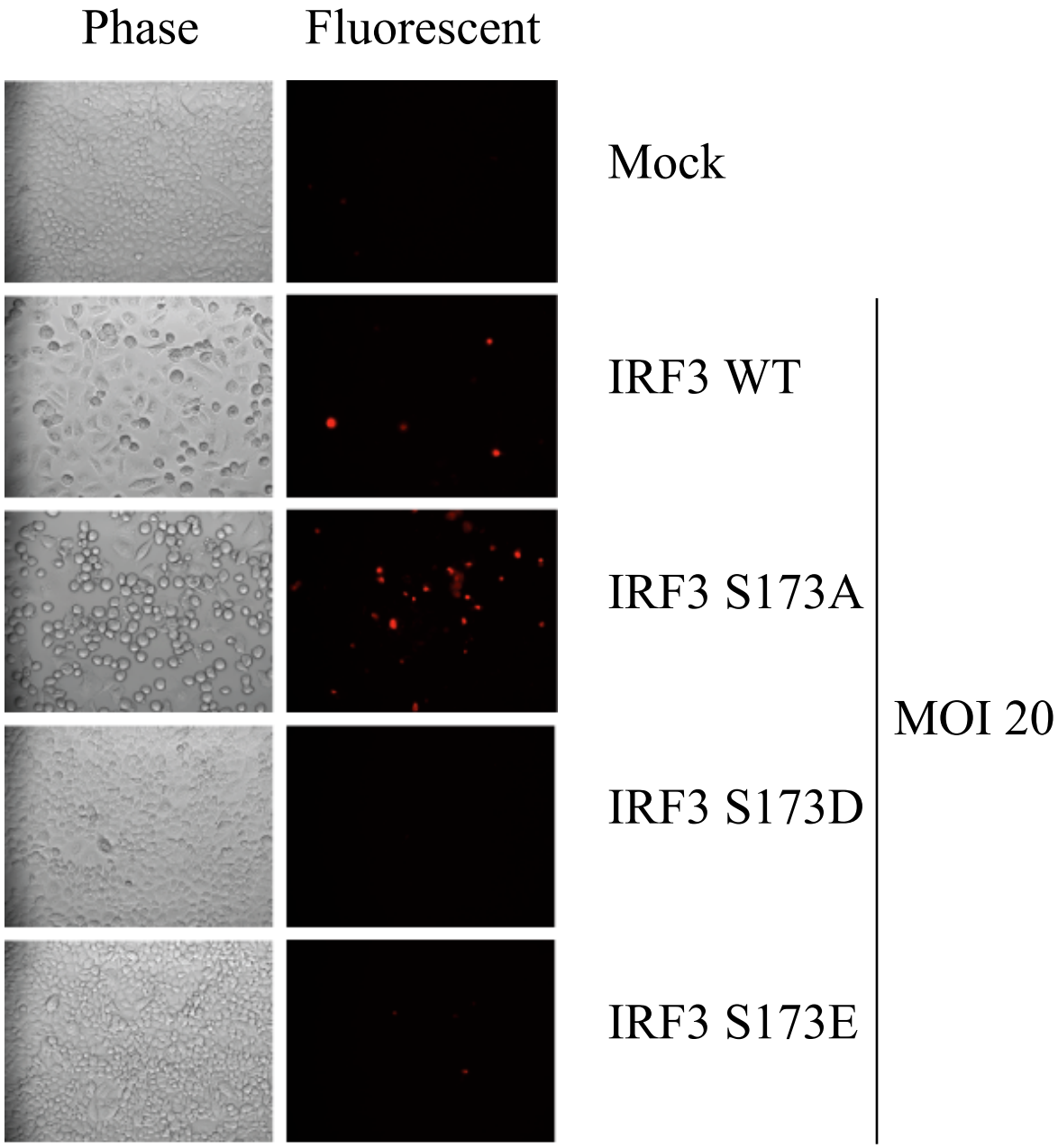
We explored cell survival after treatment of cells with the various Ad-IRF3 by crystal violet assay. In this assay, cells that remain attached to the plate are stained with crystal violet. Cells were infected with wtIRF3, IRF3 S173A and IRF3 S173E expressing Ad vectors. In generating these vectors, the E1 region of the viral genome is deleted, thus rendering the vectors replication incompetent. Replication incompetent vectors are appropriate for this study, since as mentioned above, we have linked Ad-induced phosphorylation of IRF3 S173 to viral DNA replication. Replication incompetent vectors do not induce this phosphorylation. Twenty-four hours later, cells were fixed and stained with crystal violet. After extensive rinsing, the bound crystal violet was collected in 10% acetic acid and assayed colourimetrically to determine the relative intensity, which correlates to cell survival.

From the crystal violet assay, it was determined that cells infected with Ad-IRF3 S173A showed a trend towards less cells on the plate than the other IRF3 mutants (Figure 3.8). This result lends strength to the previous observations that expression of IRF3 S173A reduces cell viability, whether the observed phenomenon can be attributed to decreased cell growth in addition to active cell death remains to be determined, but it is apparent that the inability to phosphorylate IRF3 S173 is detrimental to cell viability.

To glean additional insight on cell survival to add to the previous experiment, we repeated the crystal violet assay, but examined several time points. As previously, A549 cells were infected with Ad vectors expressing the

Figure 3.7. Cell death in response to IRF3 over-expression.

A549 cells were mock-infected or infected with IRF3 mutant expressing Ad-vectors (IRF3 WT, IRF3 S173A, IRF3 S173D, IRF3 S173E) at MOI 20 PFU/cell. Twenty-four hours later, cells were stained using a LIVE/DEAD cytotoxicity kit (Invitrogen) and viewed using phase contrast and fluorescence microscopy.


Phase
Fluorescent
Mock
IRF3 WT
IRF3 S173A
IRF3 S173D
IRF3 S173E
MOI 20

panel of IRF3 mutants, a vector expressing LacZ, or alternatively mock infected. Each of these infections was carried out in a single dish, in order to ensure an equal efficiency of infection. Twenty-four hours later, the treated cells were trypsinized, replated at low density and checked for expression of IRF3 to verify similar levels of protein expression (Figure 3.9A). We assayed cell growth over four days by the crystal violet assay. As expected, the mock-infected cells grew most rapidly, with any over-expression of IRF3 slowing growth below that of an unrelated Ad-vector (Ad-LacZ). WT IRF3, and phospho-mimetic IRF3s showed very similar growth rates, while IRF3-S173A was significantly slower than any of the other conditions (Figure 3.9B).

Upon closer inspection, the graph demonstrates that S173A over-expression leads not only to delayed cell growth, but the intensity of the colourimetric readings goes down, suggesting dying cells. Though it is clear that over-expressing any of the IRF3 mutants delays growth beyond either mock or Ad-vector infected cells, the levels of IRF3 expression appear similar and the increased cell death associated with IRF3 S173A expression is not simply a collateral effect of a greater level of IRF3 expression.

In returning to data previously collected in the LIVE/DEAD assay from Figure 3.7, using cell counts, we quantified the number of red (dead) cells per field of view as a means of better quantifying the level of cell death apparent in each of the conditions. Consistent with the previously reported data in Figures 3.8, 3.9, and 3.10, IRF3 S173A over-expression resulted in the highest number of dead cells (Figure 3.10). IRF3 WT, IRF3 S173D, and IRF3 S173E lead to similar (lower)

Figure 3.8. Cell survival in response to IRF3 over-expression.

A549 cells were mock-infected or infected with FLAG-tagged IRF3 mutant expressing Ad-vectors (IRF3 WT, IRF3 S173A, IRF3 S173D, IRF3 S173E) at MOI 20 PFU/cell. Twenty-four hours later, cells were fixed and stained using 0.1% crystal violet. Twenty-four hours later, the crystal violet was collected in 10% acetic acid and absorbance at 590 nm was taken. Absorbances were converted to relative crystal violet concentrations and plotted using Excel. Error bars represent the range of values observed in duplicate samples. One experiment is shown, but is representative of 4 independent experiments.

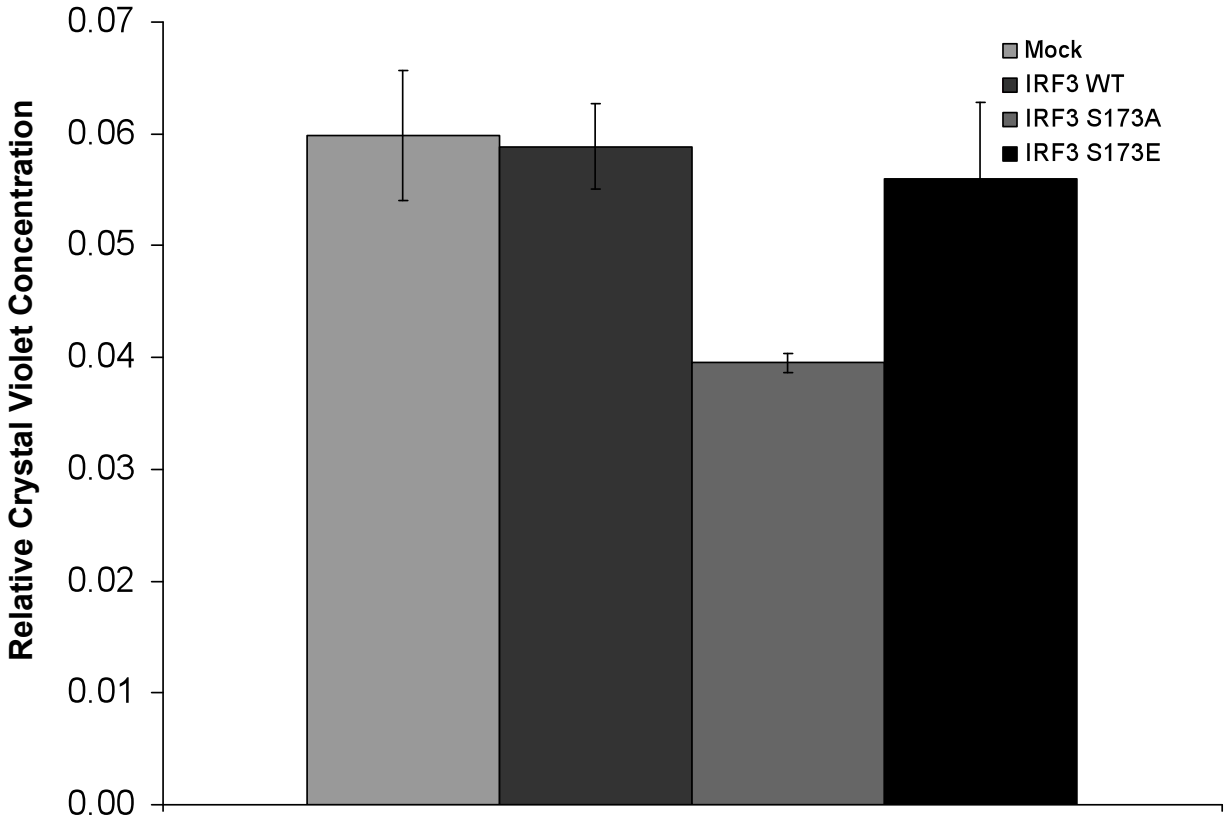

Relative Crystal Violet Concentration
0.07
0.06
0.05
0.04
0.03
0.02
0.01
0.00
Mock
IRF3 WT
IRF3 S173A
IRF3 S173E

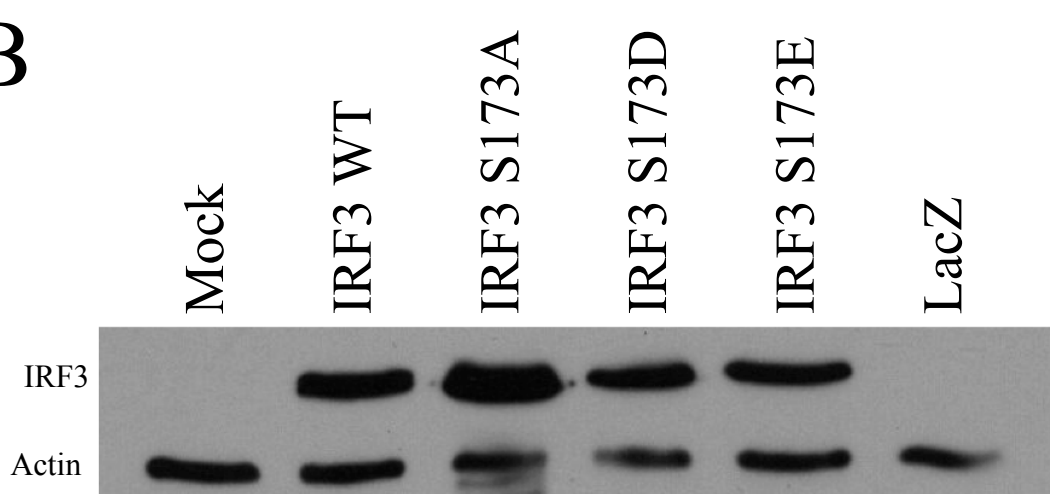
Figure 3.9. Growth of cells over-expressing IRF3.

A549 cells were mock-infected or infected with FLAG-tagged IRF3 mutant expressing Ad-vectors (IRF3 WT, IRF3 S173A, IRF3 S173D, IRF3 S173E), or LacZ expressing Ad-vectors at MOI 20 PFU/cell in duplicate. Twenty-four hours later, one set of plates were treated with trypsin and re-plated at low density. The other set of plates was lysed using 2X denaturing protein buffer. (A) Cells harvested in 2X denaturing protein buffer were resolved by 10% SDS-PAGE, transferred to a PVDF membrane, and probed using an anti-FLAG antibody (Rockland). Blots were re-probed with anti-actin (Cell Signaling) to ensure equal loading. (B) Cells were fixed and stained with 0.1% crystal violet over 4 days. Twenty-four hours later, the crystal violet was collected in 10% acetic acid and absorbance at 590 nm was taken. Absorbances were converted to relative crystal violet concentrations and plotted using Excel. Error bars represent standard deviation of the data points. Means at a single time point without a common letter differ by two-tailed Student's t-test, $P < 0.05$. n=4.

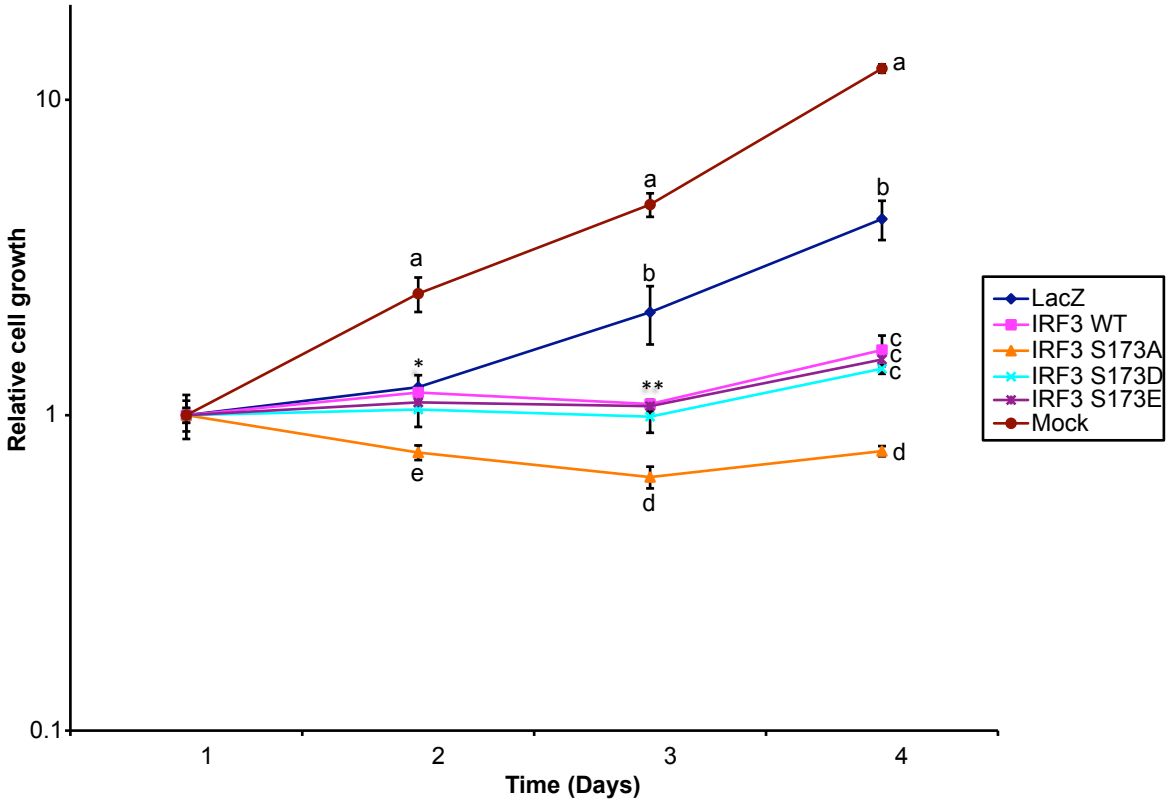
* - LacZ=bc, IRF3 WT=bcd, IRF3 S173D=cd, IRF3 S173E=bcd, IRF3 S173A=e.

** - IRF3 WT=c, IRF3 S173D=c IRF3 S173E=c

B


Mock
IRF3 WT
IRF3 S173A
IRF3 S173D
IRF3 S173E
LacZ
IRF3
Actin

A

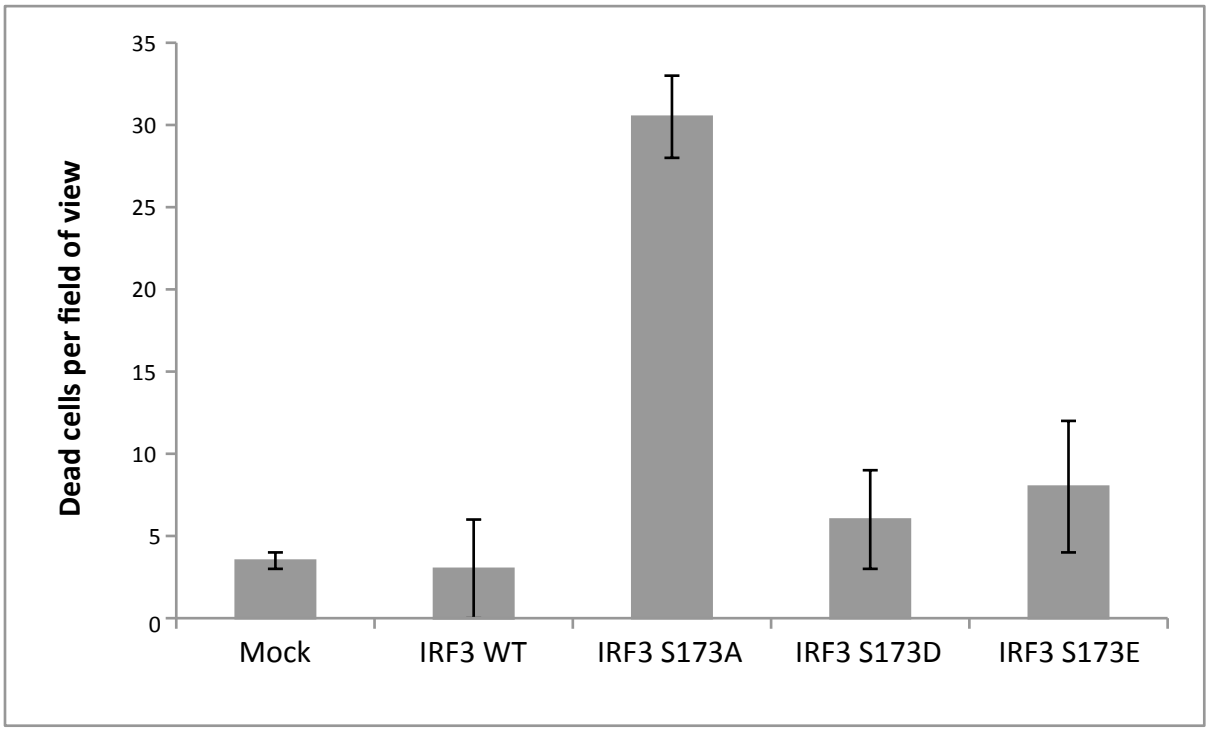

Relative cell growth
10
1
0.1
1
2
3
4
Time (Days)
LacZ
IRF3 WT
IRF3 S173A
IRF3 S173D
IRF3 S173E
Mock

levels of cell death. Once again, it was readily apparent that there was a greater level of cell death in the cells over-expressing any form of IRF3 compared to mock infected cells.

In summary, these data demonstrate that in response to UV irradiation, as in response to Ad infection, IRF3 is phosphorylated, but not activated. The phosphorylation of IRF3 S173 stabilizes IRF3 and prevents its degradation. Phosphorylation of IRF3 S173 does not affect target gene expression. Finally, phosphorylation of IRF3 S173 affects cell viability, in that expression of a mutant that cannot be phosphorylated at residue 173 leads to a lower level of viable cells.

Figure 3.10. Cell death in response to IRF3 over-expression.

A549 cells were mock-infected or infected with IRF3 mutant expressing Ad-vectors (IRF3 WT, IRF3 S173A, IRF3 S173D, IRF3 S173E) at MOI 20 PFU/cell. Twenty-four hours later, cells were stained using a LIVE/DEAD cytotoxicity kit (Invitrogen) and viewed using phase contrast and fluorescence microscopy. Dead cells were counted and graphed using Excel. Error bars represent the range of data observed over 2 independent experiments. Similar numbers were observed in experiments using MOIs of 5 and 10 PFU/cell (data not shown).


Dead cells per field of view
35
30
25
20
15
10
5
0
Mock
IRF3 WT
IRF3 S173A
IRF3 S173D
IRF3 S173E

Chapter 4: DISCUSSION

In response to viral infection, one of the key components of the innate immune response is IRF3. This transcription factor is activated early on during infection by RNA viruses, and in some conditions, during infection by DNA viruses. The order of events leading up to and including the activation of IRF3 are well defined, however, there are aspects of the protein that have only just emerged. IRF3 has recently been demonstrated to be a more diverse protein than simply a pro-inflammatory transcription factor. The protein also has newly described activity as a key modulator of apoptosis during the anti-viral response. To date, this modulatory role is apparent in the apoptotic response to viruses, but it is quite possible that this novel function of IRF3 could impact other facets of cell survival and death.

The goal of this research was to determine what effect, if any, a novel phosphorylation of IRF3 in response to Ad infection had on protein function. We hypothesized that the novel phosphorylation of IRF3 in the N-terminal region would have a direct effect not only on the transcription factor activity of the protein, but also on the ability of IRF3 to modulate apoptosis. Our data indicates that phosphorylation of IRF3 S173 may have some previously undescribed impact on IRF3, as well as the pathways it is involved in. It is interesting to note that IRF3 S173 phosphorylation may also have multiple effects on the protein. This is important given the versatile nature, and multiple roles of IRF3. Taken together, our data suggest new emerging roles for an often-overlooked regulatory region of IRF3.

4.1. IRF3 phosphorylation in response to Ad

It has been well established in the literature that VSV infection leads to IRF3 activation (tenOever et al., 2004). Additionally, by immunoblot, forms III and IV of IRF3 have been identified, signifying a hyper-phosphorylated C-terminal region of the protein. As such, we used VSV infection as a positive control for IRF3 activation (Figure 3.1A). In the case of Ad infection, we observed a change in IRF3 migration, but unlike VSV infection, we only see a shift to form II (Figure 3.1B). This result suggests that in our hands, unlike VSV, Ad infection of epithelial cells does not lead to activation of IRF3.

As mentioned previously, there have been a number of studies that have linked Ad infection to IRF3 activation (Lee et al., 2010; Muruve et al., 2008; Nociari et al., 2009; Nociari et al., 2007; Stein and Falck-Pedersen, 2012; Uematsu and Akira, 2007). In these studies, IRF3 was activated in response to Ad infection through a cooperation of TBK1 and JNK. Instead of the typical activating complex of TBK1/IKKε that has become the canonical kinase complex for activating IRF3 in response to RNA viruses, Nociari *et al.* demonstrated that recognition of Ad DNA alone via TBK1 was not sufficient to activate IRF3 (Nociari et al., 2009). Instead, JNK mediated recognition of Ad capsid leads to licensing of TBK1 for phosphorylation of IRF3 S396. We believe that these events observed may have to do with the usage of PDCs, as they are dedicated immune cells, or alternatively due to the high MOI used in these studies (25,000 PFU/cell). Our results, coupled with previous experiments from our lab have demonstrated that IRF3 is indeed phosphorylated in response to Ad infection, but this phosphorylation is not part of the

hyper-phosphorylation that is associated with activation of IRF3. This observation supports previous work that has suggested that IRF3 is activated in a dichotomous manner, activated in some cell types, but not in others. To expand on this, work from John Hu's lab demonstrated an integral link between epithelial cells and macrophages for the activation of IRF3 (Lee et al., 2010). Without epithelial cells in the co-culture, many of the aspects of the interferon response were absent. Though our results conflict with those observed by Falck-Pedersen's group (Nociari et al., 2009; Nociari et al., 2007; Stein and Falck-Pedersen, 2012), it is becoming more obvious that the cellular molecules involved in the inflammatory response vary greatly depending on the experimental system used.

There is no doubt that there is a describable difference between the IRF3 responses to Ad and VSV infection. The involvement of IRF3 in response to RNA viruses is well described (reviewed in (Takeuchi and Akira, 2009)). Signaling through TLRs 3, 7, and 8 have long been studied as the PRRs responsible for RNA recognition, with TLRs 7 and 8 signaling leading to robust interferon-responses (Kawagoe et al., 2007; Kawagoe et al., 2008; Suzuki et al., 2002). As such, the anti-viral response to RNA viruses is fairly well characterized. The requirement for active Ad DNA replication led us to hypothesize that rather than being recognized via traditional pathogen recognition systems, Ad DNA, being double-stranded and structurally similar to mammalian DNA, may be recognized through alternative methods, such as the DNA damage response.

This hypothesis opened the door for not only a new connection between innate immunity and the DNA damage response, but also provided a new paradigm for the study of the function of IRF3.

4.2. IRF3 S173 phosphorylation occurs in response to various stimulation

There is a large body of knowledge regarding the DNA damage response, and it has been estimated that cells must repair upwards of 10,000 DNA lesions per day (Lindahl, 1993). Recently, several DNA viruses have been shown to trigger proteins of the DNA damage response (Weitzman et al., 2004). For some, these signaling cascades serve as antiviral responses (Boutell and Everett, 2004; Dahl et al., 2005; Kudoh et al., 2005; Lilley et al., 2005; Shi et al., 2005; Shirata et al., 2005; Takaoka et al., 2003; Wilkinson and Weller, 2004). Other viruses appear to hijack proteins of the DNA damage response for their own purposes (Shi, Dodson et al. 2005; Shirata, Kudoh et al. 2005). Rather than activating or hijacking the DNA damage response, Ad has been demonstrated to inhibit several DNA damage response proteins. Adenovirus DNA could potentially be recognized as broken dsDNA strands, and using the double-stranded repair machinery, be ligated to form unpackageable concatamers (Stracker et al., 2002). The complex responsible for this repair function, consisting of Mre11, Rad50 and NBS1 is effectively inhibited by the Ad-E4orf3 protein and tagged for degradation by E4orf6 and E1b55K (Araujo et al., 2005; Evans and Hearing, 2005; Liu et al., 2005; Stracker et al., 2002).

There are other DNA damage response proteins as well that are inhibited by Ad during infection. E4orf3 and E4orf6 both bind DNA-PK, which is thought to further

prevent concatamerization (Boyer et al., 1999). Ad also interacts with promyelocytic leukemia (PML) nuclear bodies or ND10. These small subnuclear structures are common to transcriptionally active regions of the host genome, and several DNA damage proteins have been observed to localize with these structures. The genomes of several viral genomes associate with ND10 in what is suspected to be an anti-viral response (Everett et al., 2006; Tavalai et al., 2006). To counter this, Ad causes a mislocalization of PML and effectively disrupts this activity (Hoppe et al., 2006).

Adenovirus is one of a group of viruses that first activates, and then limits activity of p53 as a means to bypass anti-viral apoptosis (Querido et al., 2001a; Querido et al., 1997a; Querido et al., 2001b; Querido et al., 1997b). Interaction of E1A with the gene product of the Retinoblastoma (Rb) gene also prevents cell cycle arrest, thereby preventing or attenuating apoptosis (Bandara and La Thangue, 1991).

In contrast to these viral strategies used to prevent the DNA damage response, recent work by Chattopadhyah and colleagues has linked Ad infection with the pro-apoptotic protein Bax (Chattopadhyay et al., 2010; Chattopadhyay et al., 2011). What is most interesting about this finding is that it appears that this anti-viral apoptotic event is mediated by IRF3. Taken together, these previous studies suggest an important link between Ad infection and the DNA damage response.

Upon our discovery of IRF3 S173 phosphorylation in response to Ad infection, coupled with the emergence of a link between IRF3 and the apoptotic program, we examined whether we could replicate the phosphorylation of IRF3 S173 using

genotoxic stress. We irradiated A549 cells with a low level of UV (5 J/m^2), and examined the phosphorylation status of IRF3 over a short time course (25 minutes). Within 15 minutes of irradiation, there is a notable shift from form I to form II, consistent with the observed shift in response to Ad infection (Figure 3.2). Next, we used a varied dose of UV irradiation (5 J/m^2 to 250 J/m^2). Even at high levels of irradiation, we did not see a shift beyond form II, suggesting that IRF3 is not being activated in response to low or high doses of UV (Figure 3.3). Likewise, there was no phosphorylation of IRF3 S396 in response to UV irradiation, unlike VSV infection (Figure 3.4).

This result significantly strengthened the case for a connection between IRF3 phosphorylation in the context of Ad infection and in the context of DNA damage. It also suggests that UV irradiation leads to down regulation of protein synthesis, it could be a useful system for studying S173 phosphorylation in existing protein, as the phenomenon occurs much faster and without the confounding factors of relying on not only a viral lifecycle, but other cellular metabolic events that could complicate the study of S173 function and significance.

4.3. IRF3 S173A is less stable than IRF3 WT

VSV has been shown to circumvent the interferon response by tagging IRF3 for degradation, via SUMOylation (Kubota et al., 2008). For this reason, the virus is a useful tool for inducing protein turnover in a rapid fashion. We transfected HeLa cells with plasmids expressing one of three IRF3 variants. FLAG-tagged wildtype IRF3, IRF3 S173A or IRF3 S173E (glutamic acid acts as a phospho-mimetic) were over-

expressed before the cells were infected with VSV. Protein levels were followed using immunoblot and analyzed by densitometry. In cells expressing wildtype IRF3 or IRF3 S173E, there is an immediate spike in IRF3 following infection, with protein levels peaking around two hpi (Figure 3.5A & C). Soon after, there is a drastic drop in relative IRF3 level over the next four hours. In cells expressing IRF3 S173A, the original spike in IRF3 protein is absent, and instead the protein level declines sharply almost immediately (Figure 3.5B). This result suggests that the inability to phosphorylate S173 due to the alanine residue leads to decreased protein stability, and by association, that S173 phosphorylation leads to increased protein stability.

As mentioned above, VSV infection leads to a decrease in IRF3 stability via SUMOylation (Kubota et al., 2008). This SUMOylation occurs on Lys152 and Lys406. The relative proximity of K152 and S173 to one another means that it is possible that phosphorylation of IRF3 S173 could actively inhibit SUMOylation and thereby prevent the protein from being tagged for degradation.

4.4. IRF3 S173A trans-activation is reduced compared to wildtype IRF3

Inflammation in response to UV irradiation has been well studied and documented, but we do not believe that this inflammation is mediated through IRF3. As mentioned previously, in response to both low and high doses of UV irradiation, we did not observe phosphorylation of IRF3 S396. Instead, the phosphorylation happened at S173. We believe that there is a definite, and important role to IRF3 S173 phosphorylation, but it does not appear to be analogous to the C-terminal phosphorylation associated with IRF3 activation. As such, IRF3 S173

phosphorylation is not an activating event, but may still be involved in such functions as regulating or fine-tuning IRF3 activity, influencing protein stability and turn-over, or alternatively, regulate a function of IRF3 that has yet to be described.

Phosphorylation often affects protein activity, so we examined whether S173 phosphorylation could be modulatory to the trans-activating activity of the protein. We co-transfected HeLa cells with plasmids expressing an empty vector, wildtype IRF3, or IRF3 S173A; as well as plasmids expressing a firefly luciferase protein controlled by a RANTES promoter and examined the trans-activation in each. We were not able to notice a discernable difference in the trans-activating ability between wildtype IRF3 and IRF3 S173A when normalized to amounts of protein (Figure 3.6A), upon further inspection by Bradford assay, it appeared that there was less protein per sample in the samples transfected with IRF3 S173A (Figure 3.6B).

From this result we hypothesized that IRF3 S173 phosphorylation does not affect trans-activating capability of the protein, but may instead affect the interaction of IRF3 with another protein. This interaction could be responsible for increased cell growth, cell survival, or decreased cell death. Any of these potential methods could lead to a higher number of cells on the plate.

With the recent discovery of IRF3 as a modulator of Bax activity, it seemed plausible that IRF3 S173 phosphorylation could prevent this interaction, which would lead to a decreased level of apoptosis, therefore accounting for the decreased cell density in plates that over-express IRF3 S173 that cannot be phosphorylated.

4.5. IRF3 S173 phosphorylation protects against cell death

To investigate the mechanism by which IRF3 S173A over-expression reduced cell density, we first used a cytotoxicity assay on A549 cells infected with Ad vectors over-expressing wildtype IRF3, IRF3 S173A, IRF3 S173D (aspartic acid, like glutamic acid acts as a phospho-mimetic), and IRF3 S173E. In this assay, living cells are stained with calcein AM, which emits green fluorescence when viewed by fluorescence microscopy. Dead cells are stained with ethidium homodimers which appear red. Using this assay, there are significantly more red cells over-expressing IRF3 S173A than any of the other IRF3 mutants, or mock infected cells (Figure 3.7). This piece of evidence supports the hypothesis that IRF3 S173 phosphorylation is protective against cell death, and is consistent with the hypothesis that IRF3 S173 phosphorylation prevents interaction with Bax.

To provide a more quantitative analysis of cell survival, we used a crystal violet cell viability assay to determine cell viability, twenty-four hours after infection with the Ad-IRF3 vectors described above. Once again, there was a significantly lower level of viable cells upon IRF3 S173A over-expression than with the other IRF3 variants (Figure 3.8). Taken with the previous result, it is apparent that IRF3 S173A is detrimental to cell survival; cells over-expressing IRF3 S173A die more readily than cells over-expressing an IRF3 that can be phosphorylated at S173.

Using the same assay, we explored cell growth and survival over a period of four days. While it was interesting to note that any over-expression of IRF3 leads to a decreased growth rate of A549 cells when compared to a vector expressing a LacZ construct rather than IRF3, the cells expressing wtIRF3, IRF3 S173D and IRF3

S173E showed statistically identical growth over the four days (Figure 3.9A). This result suggests that the neither the ability to phosphorylate residue 173, nor the constitutive phosphorylation of the residue affects cell growth negatively. In contrast, IRF3 S173A caused not only delayed growth, but active cell death. By immunoblot we also analyzed cell extracts to ensure that the levels of IRF3 expression was equal (Figure 3.9B).

In order to confirm the observations we have made about IRF3 S173A instigating cell death, we once again used a cytotoxicity assay, and quantified the number of dead cells per field of view over multiple experiments. There was a striking difference between IRF3 S173A and the other IRF3 constructs, with IRF3 S173A over-expression leading to a much higher rate of cell death. These results, taken together, suggest that IRF3 S173 phosphorylation is an important event in the survival and growth of epithelial cells.

There is still much to be uncovered about the importance of IRF3 S173 phosphorylation, but with the data available, we can hypothesize that S173 phosphorylation is an important function of the cell that increases the stability of the protein, perhaps in an effort to maintain an “at-the-ready” condition, in order to await further signal, be it activating or inhibiting. This would be beneficial in the context of infection, where more of the anti-viral protein would be accumulated, and thus the immune response would be more robust. Additionally, as well as a noted effect at the protein level, it appears that IRF3 S173 phosphorylation has important implications towards overall cell survival. IRF3 S173 phosphorylation is protective

and the ability to phosphorylate that residue is intimately tied with preventing apoptosis.

4.6. Future Directions

Our preliminary findings, that IRF3 is not activated in response to Ad infection are in direct contrast to the work of several other groups. In order to determine whether IRF3 is indeed activated in a dichotomous fashion, we should repeat the experiments that we have used epithelial cell lines (A549, HeLa) for, using either pure or co-cultures of professional immune cells. In performing these experiments, we could validate whether we are able to replicate activation of IRF3 in response to Ad, but additionally, we would be able to determine whether IRF3 S173 is phosphorylated in these professional immune cells, like it is in epithelial cells. It is possible that although it does not appear that activation of IRF3 is ubiquitous to all cell types in response to Ad infection, phosphorylation of IRF3 S173 may be.

Our data suggest that IRF3 S173 phosphorylation improves the stability of the protein. In order to directly confirm these results, different methods of exploring IRF3 stability should be employed. Pulse-chase experiments using radio-labelled IRF3 would be useful for accurately determining IRF3 protein levels. Pulse-chase experiments would lead to more reliable quantification of the absolute levels of IRF3 throughout the experiment. Using cyclohexamide to prevent de novo protein synthesis of IRF3 would also be a useful method, rather than an outside stimulus such as VSV infection to induce turnover of the protein. With cyclohexamide, it would be possible to accurately determine the half-life of each of the IRF3 variants.

Once we have fully proven that IRF3 S173 phosphorylation increases the stability of the protein, we could use a proteasome inhibitor in order to investigate whether the phosphorylation of S173 protects from proteasome degradation, or whether the turnover is happening via a lysosome dependent pathway.

We have suggested based on our observations of IRF3 S173's effect on cell survival and apoptosis that IRF3 S173 phosphorylation could impact on the recently described interaction of IRF3 and Bax. To confirm this, it would be relatively easy to perform co-immunoprecipitation assays using the different IRF3 mutants with the hypothesis that IRF3 S173A would be more likely to interact with Bax than wildtype IRF3. Additionally, phospho-mimetic IRF3 (IRF3 S173D and IRF3 S173E) should interact even more sparingly than the wildtype protein. Performing these assays in the presence and absence of apoptotic stress would provide adequate evidence to conclude whether the impact IRF3 S173 phosphorylation has on cell survival is due to modulation of the interaction with Bax.

To determine a physiological endpoint, it would be interesting to explore both ends of the spectrum regarding IRF3's involvement in both Ad infection and genotoxic stress. Using a cell line expressing a dominant-active IRF3, we could explore the impact of IRF3 in its active state on limiting viral replication. Conversely, knocking out IRF3 would be an easy system to investigate whether IRF3 is indeed necessary for managing genotoxic stress.

Once we have determined the endpoint of IRF3 S173 phosphorylation, it would be interesting to further explore the upstream pathway that leads to this phosphorylation event. We suspect that phosphorylation of IRF3 occurs via the DNA

damage response, and as such it would be interesting to investigate what molecules are involved in processes such as the recognition of Ad DNA, or damaged DNA in this pathway, as well as the kinase that phosphorylates IRF3 S173. With these molecules identified, we could move forward with identifying any intermediary proteins involved. To determine the DNA sensor, we could use knockout cell lines to explore whether IRF3 S173 is phosphorylated when important DNA sensors are absent. It is possible that some of the previously identified, and mentioned above sensors that have been demonstrated to recognize Ad DNA are involved in this pathway, or it could be previously undescribed pathways. To determine the kinase responsible for phosphorylating IRF3 S173, we could use a high through-put kinase screen employing a panel of human kinases. Elucidating a pathway leading to the phosphorylation of IRF3 would be an important step in consolidating the anti-viral response with the DNA damage response.

4.7. Conclusions

Our study provides the first data towards joining previously un-related pathways. We have demonstrated a link between Ad infection and genotoxic stress. IRF3 is phosphorylated at S173 in response to both of these forms of stress stimulus. Furthermore, we have demonstrated that in epithelial cells at least, IRF3 is not activated in response to Ad. This observation goes against a variety of evidence, but as mentioned previously, the experimental system used in our study varies greatly from those previously used. We believe that our system demonstrates a more physiologically relevant system, complete with more reasonable infectious

doses, as well as relevant cell types for the initial point of infection. Our results suggest that IRF3 S173 phosphorylation improves the stability of IRF3, which we suggest may mean that in the context of infection, S173 phosphorylation acts as a priming event, allowing for greater protein build up in anticipation of a more robust inflammatory signal once activated. Finally, we have demonstrated an increased cell survival that is linked to IRF3 S173 phosphorylation. We believe that this survival may be due to a decreased tendency of phosphorylated IRF3 to interact with and induce Bax mediated apoptosis. This interaction between IRF3 and Bax has previously only been described in the viral apoptotic response. Our data provides the first data that could potentially reconcile the anti-viral apoptotic response with the DNA damage response.

REFERENCES

Addison, C.L., Hitt, M., Kunsken, D., and Graham, F.L. (1997). Comparison of the human versus murine cytomegalovirus immediate early gene promoters for transgene expression by adenoviral vectors. J Gen Virol *78 (Pt 7)*, 1653-1661.

Agalioti, T., Lomvardas, S., Parekh, B., Yie, J., Maniatis, T., and Thanos, D. (2000). Ordered recruitment of chromatin modifying and general transcription factors to the IFN-beta promoter. Cell *103*, 667-678.

Ahmed, M., and Lyles, D.S. (1997). Identification of a consensus mutation in M protein of vesicular stomatitis virus from persistently infected cells that affects inhibition of host-directed gene expression. Virology *237*, 378-388.

Akira, S., Uematsu, S., and Takeuchi, O. (2006). Pathogen recognition and innate immunity. Cell *124*, 783-801.

Alcorn, M.J., Booth, J.L., Coggeshall, K.M., and Metcalf, J.P. (2001). Adenovirus type 7 induces interleukin-8 production via activation of extracellular regulated kinase 1/2. J Virol *75*, 6450-6459.

Anderson, C.W., Young, M.E., and Flint, S.J. (1989). Characterization of the adenovirus 2 virion protein, mu. Virology *172*, 506-512.

Araujo, F.D., Stracker, T.H., Carson, C.T., Lee, D.V., and Weitzman, M.D. (2005). Adenovirus type 5 E4orf3 protein targets the Mre11 complex to cytoplasmic aggresomes. J Virol *79*, 11382-11391.

Arimoto, K., Takahashi, H., Hishiki, T., Konishi, H., Fujita, T., and Shimotohno, K. (2007). Negative regulation of the RIG-I signaling by the ubiquitin ligase RNF125. Proc Natl Acad Sci U S A *104*, 7500-7505.

Au, W.C., Moore, P.A., Lowther, W., Juang, Y.T., and Pitha, P.M. (1995). Identification of a member of the interferon regulatory factor family that binds to the interferon-stimulated response element and activates expression of interferon-induced genes. Proc Natl Acad Sci U S A *92*, 11657-11661.

Bandara, L.R., and La Thangue, N.B. (1991). Adenovirus E1a prevents the retinoblastoma gene product from complexing with a cellular transcription factor. Nature *351*, 494-497.

Belvin, M.P., and Anderson, K.V. (1996). A conserved signaling pathway: the Drosophila toll-dorsal pathway. Annu Rev Cell Dev Biol *12*, 393-416.

Bergelson, J.M., Cunningham, J.A., Droguett, G., Kurt-Jones, E.A., Krithivas, A., Hong, J.S., Horwitz, M.S., Crowell, R.L., and Finberg, R.W. (1997). Isolation of a

common receptor for Coxsackie B viruses and adenoviruses 2 and 5. Science *275*, 1320-1323.

Berk, A.J. (2007). FAdenoviridae: the viruses and their replication, 5 edn (Philadelphia, PA, Lippincott Williams & Wilkins).

Beutler, B., Eidenschenk, C., Crozat, K., Imler, J.L., Takeuchi, O., Hoffmann, J.A., and Akira, S. (2007). Genetic analysis of resistance to viral infection. Nat Rev Immunol *7*, 753-766.

Birnboim, H.C., and Doly, J. (1979). A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucleic Acids Res *7*, 1513-1523.

Boutell, C., and Everett, R.D. (2004). Herpes simplex virus type 1 infection induces the stabilization of p53 in a USP7- and ATM-independent manner. J Virol *78*, 8068-8077.

Boyer, J., Rohleder, K., and Ketner, G. (1999). Adenovirus E4 34k and E4 11k inhibit double strand break repair and are physically associated with the cellular DNA-dependent protein kinase. Virology *263*, 307-312.

Brown, D.T., Westphal, M., Burlingham, B.T., Winterhoff, U., and Doerfler, W. (1975). Structure and composition of the adenovirus type 2 core. J Virol *16*, 366-387.

Bruder, J.T., and Kovesdi, I. (1997). Adenovirus infection stimulates the Raf/MAPK signaling pathway and induces interleukin-8 expression. J Virol *71*, 398-404.

Cerullo, V., Seiler, M.P., Mane, V., Brunetti-Pierri, N., Clarke, C., Bertin, T.K., Rodgers, J.R., and Lee, B. (2007). Toll-like receptor 9 triggers an innate immune response to helper-dependent adenoviral vectors. Mol Ther *15*, 378-385.

Chatterjee, P.K., Vayda, M.E., and Flint, S.J. (1986). Adenoviral protein VII packages intracellular viral DNA throughout the early phase of infection. EMBO J *5*, 1633-1644.

Chattopadhyay, S., Marques, J.T., Yamashita, M., Peters, K.L., Smith, K., Desai, A., Williams, B.R., and Sen, G.C. (2010). Viral apoptosis is induced by IRF-3-mediated activation of Bax. EMBO J *29*, 1762-1773.

Chattopadhyay, S., Yamashita, M., Zhang, Y., and Sen, G.C. (2011). The IRF-3/Bax-mediated apoptotic pathway, activated by viral cytoplasmic RNA and DNA, inhibits virus replication. J Virol *85*, 3708-3716.

Chintakuntlawar, A.V., and Chodosh, J. (2009). Chemokine CXCL1/KC and its receptor CXCR2 are responsible for neutrophil chemotaxis in adenoviral keratitis. J Interferon Cytokine Res *29*, 657-666.

Christensen, J.B., Byrd, S.A., Walker, A.K., Strahler, J.R., Andrews, P.C., and Imperiale, M.J. (2008). Presence of the adenovirus IVa2 protein at a single vertex of the mature virion. J Virol *82*, 9086-9093.

Chu, W.M., Ostertag, D., Li, Z.W., Chang, L., Chen, Y., Hu, Y., Williams, B., Perrault, J., and Karin, M. (1999). JNK2 and IKKbeta are required for activating the innate response to viral infection. Immunity *11*, 721-731.

Chuang, T., and Ulevitch, R.J. (2001). Identification of hTLR10: a novel human Toll-like receptor preferentially expressed in immune cells. Biochim Biophys Acta *1518*, 157-161.

Chuang, T.H., and Ulevitch, R.J. (2000). Cloning and characterization of a sub-family of human toll-like receptors: hTLR7, hTLR8 and hTLR9. Eur Cytokine Netw *11*, 372-378.

Ciechanover, A. (1994). The ubiquitin-proteasome proteolytic pathway. Cell *79*, 13-21.

Coulon, P., Deutsch, V., Lafay, F., Martinet-Edelist, C., Wyers, F., Herman, R.C., and Flamand, A. (1990). Genetic evidence for multiple functions of the matrix protein of vesicular stomatitis virus. J Gen Virol *71 (Pt 4)*, 991-996.

Dahl, J., You, J., and Benjamin, T.L. (2005). Induction and utilization of an ATM signaling pathway by polyomavirus. J Virol *79*, 13007-13017.

Davison, A.J., Benko, M., and Harrach, B. (2003). Genetic content and evolution of adenoviruses. J Gen Virol *84*, 2895-2908.

Di Paolo, N.C., Miao, E.A., Iwakura, Y., Murali-Krishna, K., Aderem, A., Flavell, R.A., Papayannopoulou, T., and Shayakhmetov, D.M. (2009). Virus binding to a plasma membrane receptor triggers interleukin-1 alpha-mediated proinflammatory macrophage response in vivo. Immunity *31*, 110-121.

Doyle, K. (1996). Protocols and Applications Guide, P. Corporation, ed. (Madison, Wisconsin, Pormega Corporation), pp. 45-46.

Du, X., Poltorak, A., Wei, Y., and Beutler, B. (2000). Three novel mammalian toll-like receptors: gene structure, expression, and evolution. Eur Cytokine Netw *11*, 362-371.

Edelstein, M. (2012). Gene Therapy Clinical Trials Worldwide (John Wiley and Sons Ltd.).

Evans, J.D., and Hearing, P. (2005). Relocalization of the Mre11-Rad50-Nbs1 complex by the adenovirus E4 ORF3 protein is required for viral replication. J Virol *79*, 6207-6215.

Everett, R.D., Rechter, S., Papior, P., Tavalai, N., Stamminger, T., and Orr, A. (2006). PML contributes to a cellular mechanism of repression of herpes simplex virus type 1 infection that is inactivated by ICP0. J Virol *80*, 7995-8005.

Everitt, E., Sundquist, B., Pettersson, U., and Philipson, L. (1973). Structural proteins of adenoviruses. X. Isolation and topography of low molecular weight antigens from the virion of adenovirus type 2. Virology *52*, 130-147.

Fabry, C.M., Rosa-Calatrava, M., Conway, J.F., Zubieta, C., Cusack, S., Ruigrok, R.W., and Schoehn, G. (2005). A quasi-atomic model of human adenovirus type 5 capsid. EMBO J *24*, 1645-1654.

Fan, C.M., and Maniatis, T. (1989). Two different virus-inducible elements are required for human beta-interferon gene regulation. EMBO J *8*, 101-110.

Fejer, G., Drechsel, L., Liese, J., Schleicher, U., Ruzsics, Z., Imelli, N., Greber, U.F., Keck, S., Hildenbrand, B., Krug, A.*, et al.* (2008). Key role of splenic myeloid DCs in the IFN-alphabeta response to adenoviruses in vivo. PLoS Pathog *4*, e1000208.

Fitzgerald, K.A., McWhirter, S.M., Faia, K.L., Rowe, D.C., Latz, E., Golenbock, D.T., Coyle, A.J., Liao, S.M., and Maniatis, T. (2003). IKKepsilon and TBK1 are essential components of the IRF3 signaling pathway. Nat Immunol *4*, 491-496.

Fonseca, G.J., Thillainadesan, G., Yousef, A.F., Ablack, J.N., Mossman, K.L., Torchia, J., and Mymryk, J.S. (2012). Adenovirus evasion of interferon-mediated innate immunity by direct antagonism of a cellular histone posttranslational modification. Cell Host Microbe *11*, 597-606.

Fujita, T., Shibuya, H., Hotta, H., Yamanishi, K., and Taniguchi, T. (1987). Interferon-beta gene regulation: tandemly repeated sequences of a synthetic 6 bp oligomer function as a virus-inducible enhancer. Cell *49*, 357-367.

Gack, M.U., Shin, Y.C., Joo, C.H., Urano, T., Liang, C., Sun, L., Takeuchi, O., Akira, S., Chen, Z., Inoue, S.*, et al.* (2007). TRIM25 RING-finger E3 ubiquitin ligase is essential for RIG-I-mediated antiviral activity. Nature *446*, 916-920.

Gehart, H., Kumpf, S., Ittner, A., and Ricci, R. (2010). MAPK signalling in cellular metabolism: stress or wellness? EMBO Rep *11*, 834-840.

Giberson, A.N., Davidson, A.R., and Parks, R.J. (2012). Chromatin structure of adenovirus DNA throughout infection. Nucleic Acids Res *40*, 2369-2376.

Gitlin, L., Barchet, W., Gilfillan, S., Cella, M., Beutler, B., Flavell, R.A., Diamond, M.S., and Colonna, M. (2006). Essential role of mda-5 in type I IFN responses to polyriboinosinic:polyribocytidylic acid and encephalomyocarditis picornavirus. Proc Natl Acad Sci U S A *103*, 8459-8464.

Greber, U.F., Willetts, M., Webster, P., and Helenius, A. (1993). Stepwise dismantling of adenovirus 2 during entry into cells. Cell *75*, 477-486.

Haas, T., Metzger, J., Schmitz, F., Heit, A., Muller, T., Latz, E., and Wagner, H. (2008). The DNA sugar backbone 2' deoxyribose determines toll-like receptor 9 activation. Immunity *28*, 315-323.

Hartman, Z.C., Appledorn, D.M., and Amalfitano, A. (2008). Adenovirus vector induced innate immune responses: impact upon efficacy and toxicity in gene therapy and vaccine applications. Virus Res *132*, 1-14.

Hashimoto, C., Hudson, K.L., and Anderson, K.V. (1988). The Toll gene of Drosophila, required for dorsal-ventral embryonic polarity, appears to encode a transmembrane protein. Cell *52*, 269-279.

Haynes, L.M., Moore, D.D., Kurt-Jones, E.A., Finberg, R.W., Anderson, L.J., and Tripp, R.A. (2001). Involvement of toll-like receptor 4 in innate immunity to respiratory syncytial virus. J Virol *75*, 10730-10737.

Hemmi, H., Takeuchi, O., Sato, S., Yamamoto, M., Kaisho, T., Sanjo, H., Kawai, T., Hoshino, K., Takeda, K., and Akira, S. (2004). The roles of two IkappaB kinase-related kinases in lipopolysaccharide and double stranded RNA signaling and viral infection. J Exp Med *199*, 1641-1650.

Hilleman, M.R., and Werner, J.H. (1954). Recovery of new agent from patients with acute respiratory illness. Proc Soc Exp Biol Med *85*, 183-188.

Hoppe, A., Beech, S.J., Dimmock, J., and Leppard, K.N. (2006). Interaction of the adenovirus type 5 E4 Orf3 protein with promyelocytic leukemia protein isoform II is required for ND10 disruption. J Virol *80*, 3042-3049.

Hornung, V., Ellegast, J., Kim, S., Brzozka, K., Jung, A., Kato, H., Poeck, H., Akira, S., Conzelmann, K.K., Schlee, M.*, et al.* (2006). 5'-Triphosphate RNA is the ligand for RIG-I. Science *314*, 994-997.

Huen, M.S., and Chen, J. (2008). The DNA damage response pathways: at the crossroad of protein modifications. Cell Res *18*, 8-16.

Jeong, E., and Lee, J.Y. (2011). Intrinsic and extrinsic regulation of innate immune receptors. Yonsei Med J *52*, 379-392.

Jiang, H., Wang, Z., Serra, D., Frank, M.M., and Amalfitano, A. (2004). Recombinant adenovirus vectors activate the alternative complement pathway, leading to the binding of human complement protein C3 independent of anti-ad antibodies. Mol Ther *10*, 1140-1142.

Kalyuzhniy, O., Di Paolo, N.C., Silvestry, M., Hofherr, S.E., Barry, M.A., Stewart, P.L., and Shayakhmetov, D.M. (2008). Adenovirus serotype 5 hexon is critical for virus infection of hepatocytes in vivo. Proc Natl Acad Sci U S A *105*, 5483-5488.

Kanneganti, T.D., Lamkanfi, M., and Nunez, G. (2007). Intracellular NOD-like receptors in host defense and disease. Immunity *27*, 549-559.

Karpova, A.Y., Trost, M., Murray, J.M., Cantley, L.C., and Howley, P.M. (2002). Interferon regulatory factor-3 is an in vivo target of DNA-PK. Proc Natl Acad Sci U S A *99*, 2818-2823.

Kato, A., and Inoue, H. (2006). Growth defect and mutator phenotypes of RecQ-deficient Neurospora crassa mutants separately result from homologous recombination and nonhomologous end joining during repair of DNA double-strand breaks. Genetics *172*, 113-125.

Kato, H., Sato, S., Yoneyama, M., Yamamoto, M., Uematsu, S., Matsui, K., Tsujimura, T., Takeda, K., Fujita, T., Takeuchi, O*., et al.* (2005). Cell type-specific involvement of RIG-I in antiviral response. Immunity *23*, 19-28.

Kato, H., Takeuchi, O., Sato, S., Yoneyama, M., Yamamoto, M., Matsui, K., Uematsu, S., Jung, A., Kawai, T., Ishii, K.J*., et al.* (2006). Differential roles of MDA5 and RIG-I helicases in the recognition of RNA viruses. Nature *441*, 101-105.

Kawagoe, T., Sato, S., Jung, A., Yamamoto, M., Matsui, K., Kato, H., Uematsu, S., Takeuchi, O., and Akira, S. (2007). Essential role of IRAK-4 protein and its kinase activity in Toll-like receptor-mediated immune responses but not in TCR signaling. J Exp Med *204*, 1013-1024.

Kawagoe, T., Sato, S., Matsushita, K., Kato, H., Matsui, K., Kumagai, Y., Saitoh, T., Kawai, T., Takeuchi, O., and Akira, S. (2008). Sequential control of Toll-like receptor-dependent responses by IRAK1 and IRAK2. Nat Immunol *9*, 684-691.

Kawai, T., Takahashi, K., Sato, S., Coban, C., Kumar, H., Kato, H., Ishii, K.J., Takeuchi, O., and Akira, S. (2005). IPS-1, an adaptor triggering RIG-I- and Mda5-mediated type I interferon induction. Nat Immunol *6*, 981-988.

Kennedy, M.A., and Parks, R.J. (2009). Adenovirus virion stability and the viral genome: size matters. Mol Ther *17*, 1664-1666.

Kim, T., Kim, T.Y., Lee, W.G., Yim, J., and Kim, T.K. (2000). Signaling pathways to the assembly of an interferon-beta enhanceosome. Chemical genetic studies with a small molecule. J Biol Chem *275*, 16910-16917.

Koizumi, N., Yamaguchi, T., Kawabata, K., Sakurai, F., Sasaki, T., Watanabe, Y., Hayakawa, T., and Mizuguchi, H. (2007). Fiber-modified adenovirus vectors

decrease liver toxicity through reduced IL-6 production. J Immunol *178*, 1767-1773.

Kubota, T., Matsuoka, M., Chang, T.H., Tailor, P., Sasaki, T., Tashiro, M., Kato, A., and Ozato, K. (2008). Virus infection triggers SUMOylation of IRF3 and IRF7, leading to the negative regulation of type I interferon gene expression. J Biol Chem *283*, 25660-25670.

Kudoh, A., Fujita, M., Zhang, L., Shirata, N., Daikoku, T., Sugaya, Y., Isomura, H., Nishiyama, Y., and Tsurumi, T. (2005). Epstein-Barr virus lytic replication elicits ATM checkpoint signal transduction while providing an S-phase-like cellular environment. J Biol Chem *280*, 8156-8163.

Kumar, K.P., McBride, K.M., Weaver, B.K., Dingwall, C., and Reich, N.C. (2000). Regulated nuclear-cytoplasmic localization of interferon regulatory factor 3, a subunit of double-stranded RNA-activated factor 1. Mol Cell Biol *20*, 4159-4168.

Kurt-Jones, E.A., Popova, L., Kwinn, L., Haynes, L.M., Jones, L.P., Tripp, R.A., Walsh, E.E., Freeman, M.W., Golenbock, D.T., Anderson, L.J.*, et al.* (2000). Pattern recognition receptors TLR4 and CD14 mediate response to respiratory syncytial virus. Nat Immunol *1*, 398-401.

Leblanc, J.F., Cohen, L., Rodrigues, M., and Hiscott, J. (1990). Synergism between distinct enhanson domains in viral induction of the human beta interferon gene. Mol Cell Biol *10*, 3987-3993.

Lee, B.H., Kushwah, R., Wu, J., Ng, P., Palaniyar, N., Grinstein, S., Philpott, D.J., and Hu, J. (2010). Adenoviral vectors stimulate innate immune responses in macrophages through cross-talk with epithelial cells. Immunol Lett *134*, 93-102.

Leopold, P.L., Ferris, B., Grinberg, I., Worgall, S., Hackett, N.R., and Crystal, R.G. (1998). Fluorescent virions: dynamic tracking of the pathway of adenoviral gene transfer vectors in living cells. Hum Gene Ther *9*, 367-378.

Lilley, C.E., Carson, C.T., Muotri, A.R., Gage, F.H., and Weitzman, M.D. (2005). DNA repair proteins affect the lifecycle of herpes simplex virus 1. Proc Natl Acad Sci U S A *102*, 5844-5849.

Lin, R., Genin, P., Mamane, Y., and Hiscott, J. (2000). Selective DNA binding and association with the CREB binding protein coactivator contribute to differential activation of alpha/beta interferon genes by interferon regulatory factors 3 and 7. Mol Cell Biol *20*, 6342-6353.

Lin, R., Heylbroeck, C., Pitha, P.M., and Hiscott, J. (1998). Virus-dependent phosphorylation of the IRF-3 transcription factor regulates nuclear translocation, transactivation potential, and proteasome-mediated degradation. Mol Cell Biol *18*, 2986-2996.

Lin, R., Mamane, Y., and Hiscott, J. (1999). Structural and functional analysis of interferon regulatory factor 3: localization of the transactivation and autoinhibitory domains. Mol Cell Biol *19*, 2465-2474.

Lindahl, T. (1993). Instability and decay of the primary structure of DNA. Nature *362*, 709-715.

Liu, H., Jin, L., Koh, S.B., Atanasov, I., Schein, S., Wu, L., and Zhou, Z.H. (2010). Atomic structure of human adenovirus by cryo-EM reveals interactions among protein networks. Science *329*, 1038-1043.

Liu, Y., Shevchenko, A., and Berk, A.J. (2005). Adenovirus exploits the cellular aggresome response to accelerate inactivation of the MRN complex. J Virol *79*, 14004-14016.

Maizel, J.V., Jr., White, D.O., and Scharff, M.D. (1968). The polypeptides of adenovirus. II. Soluble proteins, cores, top components and the structure of the virion. Virology *36*, 126-136.

Mancuso, G., Gambuzza, M., Midiri, A., Biondo, C., Papasergi, S., Akira, S., Teti, G., and Beninati, C. (2009). Bacterial recognition by TLR7 in the lysosomes of conventional dendritic cells. Nat Immunol *10*, 587-594.

Matsukura, S., Kokubu, F., Kurokawa, M., Kawaguchi, M., Ieki, K., Kuga, H., Odaka, M., Suzuki, S., Watanabe, S., Takeuchi, H.*, et al.* (2006). Synthetic double-stranded RNA induces multiple genes related to inflammation through Toll-like receptor 3 depending on NF-kappaB and/or IRF-3 in airway epithelial cells. Clin Exp Allergy *36*, 1049-1062.

Medzhitov, R. (2007). Recognition of microorganisms and activation of the immune response. Nature *449*, 819-826.

Medzhitov, R., Preston-Hurlburt, P., and Janeway, C.A., Jr. (1997). A human homologue of the Drosophila Toll protein signals activation of adaptive immunity. Nature *388*, 394-397.

Melchjorsen, J., Jensen, S.B., Malmgaard, L., Rasmussen, S.B., Weber, F., Bowie, A.G., Matikainen, S., and Paludan, S.R. (2005). Activation of innate defense against a paramyxovirus is mediated by RIG-I and TLR7 and TLR8 in a cell-type-specific manner. J Virol *79*, 12944-12951.

Meylan, E., Curran, J., Hofmann, K., Moradpour, D., Binder, M., Bartenschlager, R., and Tschopp, J. (2005). Cardif is an adaptor protein in the RIG-I antiviral pathway and is targeted by hepatitis C virus. Nature *437*, 1167-1172.

Mirza, M.A., and Weber, J. (1982). Structure of adenovirus chromatin. Biochim Biophys Acta *696*, 76-86.

Mori, M., Yoneyama, M., Ito, T., Takahashi, K., Inagaki, F., and Fujita, T. (2004). Identification of Ser-386 of interferon regulatory factor 3 as critical target for inducible phosphorylation that determines activation. J Biol Chem *279*, 9698-9702.

Morris, S.J., Scott, G.E., and Leppard, K.N. (2010). Adenovirus late-phase infection is controlled by a novel L4 promoter. J Virol *84*, 7096-7104.

Muruve, D.A., Barnes, M.J., Stillman, I.E., and Libermann, T.A. (1999). Adenoviral gene therapy leads to rapid induction of multiple chemokines and acute neutrophil-dependent hepatic injury in vivo. Hum Gene Ther *10*, 965-976.

Muruve, D.A., Petrilli, V., Zaiss, A.K., White, L.R., Clark, S.A., Ross, P.J., Parks, R.J., and Tschopp, J. (2008). The inflammasome recognizes cytosolic microbial and host DNA and triggers an innate immune response. Nature *452*, 103-107.

Muzio, M., Bosisio, D., Polentarutti, N., D'Amico, G., Stoppacciaro, A., Mancinelli, R., van't Veer, C., Penton-Rol, G., Ruco, L.P., Allavena, P.*, et al.* (2000). Differential expression and regulation of toll-like receptors (TLR) in human leukocytes: selective expression of TLR3 in dendritic cells. J Immunol *164*, 5998-6004.

Nociari, M., Ocheretina, O., Murphy, M., and Falck-Pedersen, E. (2009). Adenovirus induction of IRF3 occurs through a binary trigger targeting Jun N-terminal kinase and TBK1 kinase cascades and type I interferon autocrine signaling. J Virol *83*, 4081-4091.

Nociari, M., Ocheretina, O., Schoggins, J.W., and Falck-Pedersen, E. (2007). Sensing infection by adenovirus: Toll-like receptor-independent viral DNA recognition signals activation of the interferon regulatory factor 3 master regulator. J Virol *81*, 4145-4157.

Oshiumi, H., Matsumoto, M., Funami, K., Akazawa, T., and Seya, T. (2003). TICAM-1, an adaptor molecule that participates in Toll-like receptor 3-mediated interferon-beta induction. Nat Immunol *4*, 161-167.

Perez-Berna, A.J., Marabini, R., Scheres, S.H., Menendez-Conejero, R., Dmitriev, I.P., Curiel, D.T., Mangel, W.F., Flint, S.J., and San Martin, C. (2009). Structure and uncoating of immature adenovirus. J Mol Biol *392*, 547-557.

Perry, A.K., Chow, E.K., Goodnough, J.B., Yeh, W.C., and Cheng, G. (2004). Differential requirement for TANK-binding kinase-1 in type I interferon responses to toll-like receptor activation and viral infection. J Exp Med *199*, 1651-1658.

Petrilli, V., Dostert, C., Muruve, D.A., and Tschopp, J. (2007). The inflammasome: a danger sensing complex triggering innate immunity. Curr Opin Immunol *19*, 615-622.

Philpott, N.J., Nociari, M., Elkon, K.B., and Falck-Pedersen, E. (2004). Adenovirus-induced maturation of dendritic cells through a PI3 kinase-mediated TNF-alpha induction pathway. Proc Natl Acad Sci U S A *101*, 6200-6205.

Pichlmair, A., Schulz, O., Tan, C.P., Naslund, T.I., Liljestrom, P., Weber, F., and Reis e Sousa, C. (2006). RIG-I-mediated antiviral responses to single-stranded RNA bearing 5'-phosphates. Science *314*, 997-1001.

Poltorak, A., He, X., Smirnova, I., Liu, M.Y., Van Huffel, C., Du, X., Birdwell, D., Alejos, E., Silva, M., Galanos, C*., et al.* (1998). Defective LPS signaling in C3H/HeJ and C57BL/10ScCr mice: mutations in Tlr4 gene. Science *282*, 2085-2088.

Querido, E., Blanchette, P., Yan, Q., Kamura, T., Morrison, M., Boivin, D., Kaelin, W.G., Conaway, R.C., Conaway, J.W., and Branton, P.E. (2001a). Degradation of p53 by adenovirus E4orf6 and E1B55K proteins occurs via a novel mechanism involving a Cullin-containing complex. Genes Dev *15*, 3104-3117.

Querido, E., Marcellus, R.C., Lai, A., Charbonneau, R., Teodoro, J.G., Ketner, G., and Branton, P.E. (1997a). Regulation of p53 levels by the E1B 55-kilodalton protein and E4orf6 in adenovirus-infected cells. J Virol *71*, 3788-3798.

Querido, E., Morrison, M.R., Chu-Pham-Dang, H., Thirlwell, S.W., Boivin, D., and Branton, P.E. (2001b). Identification of three functions of the adenovirus e4orf6 protein that mediate p53 degradation by the E4orf6-E1B55K complex. J Virol *75*, 699-709.

Querido, E., Teodoro, J.G., and Branton, P.E. (1997b). Accumulation of p53 induced by the adenovirus E1A protein requires regions involved in the stimulation of DNA synthesis. J Virol *71*, 3526-3533.

Qureshi, S.T., Lariviere, L., Leveque, G., Clermont, S., Moore, K.J., Gros, P., and Malo, D. (1999). Endotoxin-tolerant mice have mutations in Toll-like receptor 4 (Tlr4). J Exp Med *189*, 615-625.

Reddy, V.S., Natchiar, S.K., Stewart, P.L., and Nemerow, G.R. (2010). Crystal structure of human adenovirus at 3.5 A resolution. Science *329*, 1071-1075.

Rock, F.L., Hardiman, G., Timans, J.C., Kastelein, R.A., and Bazan, J.F. (1998). A family of human receptors structurally related to Drosophila Toll. Proc Natl Acad Sci U S A *95*, 588-593.

Ronco, L.V., Karpova, A.Y., Vidal, M., and Howley, P.M. (1998). Human papillomavirus 16 E6 oncoprotein binds to interferon regulatory factor-3 and inhibits its transcriptional activity. Genes Dev *12*, 2061-2072.

Rothenfusser, S., Goutagny, N., DiPerna, G., Gong, M., Monks, B.G., Schoenemeyer, A., Yamamoto, M., Akira, S., and Fitzgerald, K.A. (2005). The

RNA helicase Lgp2 inhibits TLR-independent sensing of viral replication by retinoic acid-inducible gene-I. J Immunol *175*, 5260-5268.

Rowe, W.P., Huebner, R.J., Gilmore, L.K., Parrott, R.H., and Ward, T.G. (1953). Isolation of a cytopathogenic agent from human adenoids undergoing spontaneous degeneration in tissue culture. Proc Soc Exp Biol Med *84*, 570-573.

Russell, W.C. (2009). Adenoviruses: update on structure and function. J Gen Virol *90*, 1-20.

Russell, W.C., Laver, W.G., and Sanderson, P.J. (1968). Internal components of adenovirus. Nature *219*, 1127-1130.

Saito, T., Hirai, R., Loo, Y.M., Owen, D., Johnson, C.L., Sinha, S.C., Akira, S., Fujita, T., and Gale, M., Jr. (2007). Regulation of innate antiviral defenses through a shared repressor domain in RIG-I and LGP2. Proc Natl Acad Sci U S A *104*, 582-587.

Sambrook, J., Fritsch, E.F., and Maniatis, T. (1989). Molecular Cloning: A Laboratory Manual (Cold Spring Harbor, Cold Spring Harbor Laboratory Press).

Schafer, S.L., Lin, R., Moore, P.A., Hiscott, J., and Pitha, P.M. (1998). Regulation of type I interferon gene expression by interferon regulatory factor-3. J Biol Chem *273*, 2714-2720.

Servant, M.J., Grandvaux, N., and Hiscott, J. (2002). Multiple signaling pathways leading to the activation of interferon regulatory factor 3. Biochem Pharmacol *64*, 985-992.

Servant, M.J., Grandvaux, N., tenOever, B.R., Duguay, D., Lin, R., and Hiscott, J. (2003). Identification of the minimal phosphoacceptor site required for in vivo activation of interferon regulatory factor 3 in response to virus and double-stranded RNA. J Biol Chem *278*, 9441-9447.

Servant, M.J., ten Oever, B., LePage, C., Conti, L., Gessani, S., Julkunen, I., Lin, R., and Hiscott, J. (2001). Identification of distinct signaling pathways leading to the phosphorylation of interferon regulatory factor 3. J Biol Chem *276*, 355-363.

Seth, R.B., Sun, L., Ea, C.K., and Chen, Z.J. (2005). Identification and characterization of MAVS, a mitochondrial antiviral signaling protein that activates NF-kappaB and IRF 3. Cell *122*, 669-682.

Shayakhmetov, D.M., Gaggar, A., Ni, S., Li, Z.Y., and Lieber, A. (2005a). Adenovirus binding to blood factors results in liver cell infection and hepatotoxicity. J Virol *79*, 7478-7491.

Shayakhmetov, D.M., Li, Z.Y., Ni, S., and Lieber, A. (2005b). Interference with the IL-1-signaling pathway improves the toxicity profile of systemically applied adenovirus vectors. J Immunol *174*, 7310-7319.

Shi, Y., Dodson, G.E., Shaikh, S., Rundell, K., and Tibbetts, R.S. (2005). Ataxia-telangiectasia-mutated (ATM) is a T-antigen kinase that controls SV40 viral replication in vivo. J Biol Chem *280*, 40195-40200.

Shirata, N., Kudoh, A., Daikoku, T., Tatsumi, Y., Fujita, M., Kiyono, T., Sugaya, Y., Isomura, H., Ishizaki, K., and Tsurumi, T. (2005). Activation of ataxia telangiectasia-mutated DNA damage checkpoint signal transduction elicited by herpes simplex virus infection. J Biol Chem *280*, 30336-30341.

Silvestry, M., Lindert, S., Smith, J.G., Maier, O., Wiethoff, C.M., Nemerow, G.R., and Stewart, P.L. (2009). Cryo-electron microscopy structure of adenovirus type 2 temperature-sensitive mutant 1 reveals insight into the cell entry defect. J Virol *83*, 7375-7383.

Smith, A.C., Poulin, K.L., and Parks, R.J. (2009). DNA genome size affects the stability of the adenovirus virion. J Virol *83*, 2025-2028.

Smith, T.A., Idamakanti, N., Rollence, M.L., Marshall-Neff, J., Kim, J., Mulgrew, K., Nemerow, G.R., Kaleko, M., and Stevenson, S.C. (2003). Adenovirus serotype 5 fiber shaft influences in vivo gene transfer in mice. Hum Gene Ther *14*, 777-787.

Stein, S.C., and Falck-Pedersen, E. (2012). Sensing adenovirus infection: activation of interferon regulatory factor 3 in RAW 264.7 cells. J Virol *86*, 4527-4537.

Stojdl, D.F., Lichty, B.D., tenOever, B.R., Paterson, J.M., Power, A.T., Knowles, S., Marius, R., Reynard, J., Poliquin, L., Atkins, H.*, et al.* (2003). VSV strains with defects in their ability to shutdown innate immunity are potent systemic anti-cancer agents. Cancer Cell *4*, 263-275.

Stracker, T.H., Carson, C.T., and Weitzman, M.D. (2002). Adenovirus oncoproteins inactivate the Mre11-Rad50-NBS1 DNA repair complex. Nature *418*, 348-352.

Strunze, S., Engelke, M.F., Wang, I.H., Puntener, D., Boucke, K., Schleich, S., Way, M., Schoenenberger, P., Burckhardt, C.J., and Greber, U.F. (2011). Kinesin-1-mediated capsid disassembly and disruption of the nuclear pore complex promote virus infection. Cell Host Microbe *10*, 210-223.

Suzuki, N., Suzuki, S., Duncan, G.S., Millar, D.G., Wada, T., Mirtsos, C., Takada, H., Wakeham, A., Itie, A., Li, S.*, et al.* (2002). Severe impairment of interleukin-1 and Toll-like receptor signalling in mice lacking IRAK-4. Nature *416*, 750-756.

Takaoka, A., Hayakawa, S., Yanai, H., Stoiber, D., Negishi, H., Kikuchi, H., Sasaki, S., Imai, K., Shibue, T., Honda, K.*, et al.* (2003). Integration of interferon-alpha/beta signalling to p53 responses in tumour suppression and antiviral defence. Nature *424*, 516-523.

Takeuchi, O., and Akira, S. (2009). Innate immunity to virus infection. Immunol Rev *227*, 75-86.

Takeuchi, O., Kawai, T., Sanjo, H., Copeland, N.G., Gilbert, D.J., Jenkins, N.A., Takeda, K., and Akira, S. (1999). TLR6: A novel member of an expanding toll-like receptor family. Gene *231*, 59-65.

Tamanini, A., Nicolis, E., Bonizzato, A., Bezzerri, V., Melotti, P., Assael, B.M., and Cabrini, G. (2006). Interaction of adenovirus type 5 fiber with the coxsackievirus and adenovirus receptor activates inflammatory response in human respiratory cells. J Virol *80*, 11241-11254.

Tavalai, N., Papior, P., Rechter, S., Leis, M., and Stamminger, T. (2006). Evidence for a role of the cellular ND10 protein PML in mediating intrinsic immunity against human cytomegalovirus infections. J Virol *80*, 8006-8018.

tenOever, B.R., Sharma, S., Zou, W., Sun, Q., Grandvaux, N., Julkunen, I., Hemmi, H., Yamamoto, M., Akira, S., Yeh, W.C.*, et al.* (2004). Activation of TBK1 and IKKvarepsilon kinases by vesicular stomatitis virus infection and the role of viral ribonucleoprotein in the development of interferon antiviral immunity. J Virol *78*, 10636-10649.

Thaci, B., Ulasov, I.V., Wainwright, D.A., and Lesniak, M.S. (2011). The challenge for gene therapy: innate immune response to adenoviruses. Oncotarget *2*, 113-121.

Tibbles, L.A., Spurrell, J.C., Bowen, G.P., Liu, Q., Lam, M., Zaiss, A.K., Robbins, S.M., Hollenberg, M.D., Wickham, T.J., and Muruve, D.A. (2002). Activation of p38 and ERK signaling during adenovirus vector cell entry lead to expression of the C-X-C chemokine IP-10. J Virol *76*, 1559-1568.

Tomko, R.P., Xu, R., and Philipson, L. (1997). HCAR and MCAR: the human and mouse cellular receptors for subgroup C adenoviruses and group B coxsackieviruses. Proc Natl Acad Sci U S A *94*, 3352-3356.

Trentin, J.J., Yabe, Y., and Taylor, G. (1962). The quest for human cancer viruses. Science *137*, 835-841.

Uematsu, S., and Akira, S. (2007). Toll-like receptors and Type I interferons. J Biol Chem *282*, 15319-15323.

Waddington, S.N., McVey, J.H., Bhella, D., Parker, A.L., Barker, K., Atoda, H., Pink, R., Buckley, S.M., Greig, J.A., Denby, L.*, et al.* (2008). Adenovirus serotype 5 hexon mediates liver gene transfer. Cell *132*, 397-409.

Wathelet, M.G., Lin, C.H., Parekh, B.S., Ronco, L.V., Howley, P.M., and Maniatis, T. (1998). Virus infection induces the assembly of coordinately activated transcription factors on the IFN-beta enhancer in vivo. Mol Cell *1*, 507-518.

Weaver, B.K., Kumar, K.P., and Reich, N.C. (1998). Interferon regulatory factor 3 and CREB-binding protein/p300 are subunits of double-stranded RNA-activated transcription factor DRAF1. Mol Cell Biol *18*, 1359-1368.

Weitzman, M.D., Carson, C.T., Schwartz, R.A., and Lilley, C.E. (2004). Interactions of viruses with the cellular DNA repair machinery. DNA Repair (Amst) *3*, 1165-1173.

Wickham, T.J., Mathias, P., Cheresh, D.A., and Nemerow, G.R. (1993). Integrins alpha v beta 3 and alpha v beta 5 promote adenovirus internalization but not virus attachment. Cell *73*, 309-319.

Wilkinson, D.E., and Weller, S.K. (2004). Recruitment of cellular recombination and repair proteins to sites of herpes simplex virus type 1 DNA replication is dependent on the composition of viral proteins within prereplicative sites and correlates with the induction of the DNA damage response. J Virol *78*, 4783-4796.

Xing, Z., Zganiacz, A., Wang, J., Divangahi, M., and Nawaz, F. (2000). IL-12-independent Th1-type immune responses to respiratory viral infection: requirement of IL-18 for IFN-gamma release in the lung but not for the differentiation of viral-reactive Th1-type lymphocytes. J Immunol *164*, 2575-2584.

Xu, L.G., Wang, Y.Y., Han, K.J., Li, L.Y., Zhai, Z., and Shu, H.B. (2005). VISA is an adapter protein required for virus-triggered IFN-beta signaling. Mol Cell *19*, 727-740.

Yabe, Y., Trentin, J.J., and Taylor, G. (1962). Cancer induction in hamsters by human type 12 adenovirus. Effect of age and of virus dose. Proc Soc Exp Biol Med *111*, 343-344.

Yamamoto, M., Sato, S., Hemmi, H., Hoshino, K., Kaisho, T., Sanjo, H., Takeuchi, O., Sugiyama, M., Okabe, M., Takeda, K.*, et al.* (2003). Role of adaptor TRIF in the MyD88-independent toll-like receptor signaling pathway. Science *301*, 640-643.

Yamamoto, M., Sato, S., Mori, K., Hoshino, K., Takeuchi, O., Takeda, K., and Akira, S. (2002). Cutting edge: a novel Toll/IL-1 receptor domain-containing adapter that preferentially activates the IFN-beta promoter in the Toll-like receptor signaling. J Immunol *169*, 6668-6672.

Yang, Y.L., Reis, L.F., Pavlovic, J., Aguzzi, A., Schafer, R., Kumar, A., Williams, B.R., Aguet, M., and Weissmann, C. (1995). Deficient signaling in mice devoid of double-stranded RNA-dependent protein kinase. EMBO J *14*, 6095-6106.

Yoneyama, M., Kikuchi, M., Matsumoto, K., Imaizumi, T., Miyagishi, M., Taira, K., Foy, E., Loo, Y.M., Gale, M., Jr., Akira, S.*, et al.* (2005). Shared and unique functions of the DExD/H-box helicases RIG-I, MDA5, and LGP2 in antiviral innate immunity. J Immunol *175*, 2851-2858.

Yoneyama, M., Kikuchi, M., Natsukawa, T., Shinobu, N., Imaizumi, T., Miyagishi, M., Taira, K., Akira, S., and Fujita, T. (2004). The RNA helicase RIG-I has an essential function in double-stranded RNA-induced innate antiviral responses. Nat Immunol *5*, 730-737.

Yoneyama, M., Suhara, W., Fukuhara, Y., Fukuda, M., Nishida, E., and Fujita, T. (1998). Direct triggering of the type I interferon system by virus infection: activation of a transcription factor complex containing IRF-3 and CBP/p300. EMBO J *17*, 1087-1095.

Zaiss, A.K., Liu, Q., Bowen, G.P., Wong, N.C., Bartlett, J.S., and Muruve, D.A. (2002). Differential activation of innate immune responses by adenovirus and adeno-associated virus vectors. J Virol *76*, 4580-4590.

Zhu, J., Huang, X., and Yang, Y. (2008). A critical role for type I IFN-dependent NK cell activation in innate immune elimination of adenoviral vectors in vivo. Mol Ther *16*, 1300-1307.

CONTRIBUTIONS OF COLLABORATORS

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APPENDIX A: REAGENTS

Tissue Culture

- MEM (Minimal Essential Medium) (Sigma)
- DMEM (Dulbecco Modified Eagle Medium) (Sigma)
- DPBS (Dulbecco Phosphate Buffered Saline) (Sigma)
- FBS (Fetal Bovine Serum) Sigma

- MEM/DMEM with 10% FBS:

    500 mL MEM/DMEM (Sigma)

    50 mL FBS (10%) (Sigma)

    5 mL antibiotic/antimycotic (1%) (Invitrogen)

    5 mL GlutaMax (1%) (Invitrogen)

- 100X (2.5%) Trypsin (Invitrogen)

- 0.025% Trypsin:

    5 mL 2.5% Trypsin (Invitrogen)

    up to 500 mL DPBS (Invitrogen)

- Lipofectamine 2000 (Invitrogen)

Western Blotting

- 2X Protein Denaturing Buffer:

  2.5 mL 0.5M Tris-HCl pH 6.8

  25 mL Glycerol (25%)

  10 mL 20% SDS (2%)

  0.01 g bromophenol blue

  up to 100 mL H_2O

  Supplemented with 5% β-mercaptoethanol

- 10% APS:

  0.1 g ammonium persulfate

  up to 1 mL H_2O

- 2X Separating Gel Buffer:

  18.164 g Tris (750 mM)

  0.4 g SDS (0.2%)

  up to 200 mL H_2O

  adjust pH to 8.8

- Separating Gel Solution:

  2.0 mL ddH_2O

  5.0 mL 2X Separating Gel Buffer

3.0 mL 30% Acrylamide

100 µL 10% APS

10 µL TEMED

- 2X Stacking Gel Buffer:

  6.056 g Tris (250 mM)

  0.4 g SDS (0.2%)

  up to 200 mL H_2O

  adjust pH to 6.8

- Stacking Gel Solution:

  2 mL H_2O

  3 mL 2X Stacking Gel Buffer

  1 mL 30% Acrylamide

  100 µL 10% APS

  10 µL TEMED

- 1X Running Buffer:

  6.08 g Tris (50 mM)

  28.8 g Glycine (1.44%)

  2.0 g SDS (0.1%)

  up to 2 L H_2O

- 1X Transfer Buffer:

  5.82 g Tris (48 mM)

  2.93 g Glycine (0.293%)

  3.75 mL 10% SDS (0.0375%)

  200 mL MeOH (20%)

  up to 1 L H_2O

- 0.1% TBST (Tris Buffered Saline with Tween20):

  30 mL 5 M NaCl (150 mM)

  10 mL 1M Tris-HCl pH 8.0 (10 mM)

  1 mL TWEEN-20 (0.1%)

  up to 1 L H_2O

- 5% Milk Blocking Solution:

  1 g skim milk powder

  up to 20 mL with 0.1% TBST

<u>General Chemicals and Reagents</u>

- 1 M Tris-HCl pH 8.0:

  121.14 g Tris

  up to 1L H_2O

  adjust to pH 8.0 with HCl

- 0.5M EDTA:

  146.13 g EDTA (ethylenediaminetetraacetic acid)

  up to 1L H_2O

  adjust to pH 8.0 with HCl

- TE: Tris-EDTA:

  10 mM Tris-HCl pH 8.0

  1 mM EDTA pH 8.0

- 10 M NaOH:

  39.978 g NaOH

  up to 500 mL H_2O

- 5 M NaCl:

  146.11 g NaCl

  up to 500 mL H_2O

- 20% SDS:

  100 g SDS (sodium dodecyl sulfate)

  up to 500 mL H_2O

APPENDIX B: PUBLISHED MANUSCRIPT

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SURVEY AND SUMMARY

Chromatin structure of adenovirus DNA throughout infection

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ABSTRACT

For more than half a century, researchers have studied the basic biology of Adenovirus (Ad), unraveling the subtle, yet profound, interactions between the virus and the host. These studies have uncovered previously unknown proteins and pathways crucial for normal cell function that the virus manipulates to achieve optimal virus replication and gene expression. In the infecting virion, the viral DNA is tightly condensed in a virally encoded protamine-like protein which must be remodeled within the first few hours of infection to allow for efficient expression of virus-encoded genes and subsequent viral DNA replication. This review discusses our current knowledge of Ad DNA–protein complex within the infected cell nucleus, the cellular proteins the virus utilizes to achieve chromatinization, and how this event contributes to efficient gene expression and progression of the virus life cycle.

INTRODUCTION

Human Adenovirus (Ad) was first isolated from adenoid tissue in the 1950s as novel viral agents associated with respiratory infections (1,2). Over 100 Ad family members have been identified and characterized in a wide range of host organisms, from a variety of mammals and birds, to reptiles and amphibians (3). In the early 1960s, researchers showed that some human Ads can cause tumours in rodents (4,5), which led to a surge in studies of the molecular biology, genetics and physiology of Ads which continues to this day. Since Ads must manipulate the host cell to promote a microenvironment conducive to virus replication, studies of basic Ad biology have contributed a great deal of novel insight into all fields of cellular biology, including DNA replication, tumourigenesis and control of gene expression in the host cell.

While the pool of knowledge regarding the Ad lifecycle is immense, few studies have investigated the structure and protein association of Ad DNA within the infected cell nucleus. Considering the fundamental importance of chromatin in regulating gene expression in host cells, it is surprising that, until recently, it remained unclear whether Ad DNA interacted with cellular histones or assembled into chromatin. This review summarizes our current knowledge of the nucleoprotein structure of the Ad genome within the infected cell.

ADENOVIRUS BIOLOGY

All Ads have the same general structural characteristics. The virion is a non-enveloped icosahedral capsid with a diameter of ~80–90 nm, containing a linear double stranded DNA genome of ~30–40 kb (Figure 1) (3). Of the human Ads, serotype 2 (Ad2) and 5 (Ad5), both of subclass C, are the most extensively characterized. The Ad5 genome is ~36 kb in size and encodes ~39 genes, which are classified as either early or late depending on whether they are expressed before or after DNA replication (Figure 1A) (6). Four early transcription units (E1a, E1b, E3 and E4) encode proteins that are required for transactivating other viral regions, modifying the host cellular environment or altering the immune response. E2 encodes proteins directly involved in viral DNA replication. All major late proteins, organized in the transcription units L1 to L5, are expressed from a common major late promoter and are generated by alternative splicing of a single transcript. However, recent work has shown that the L4-22K and L4-33K proteins, which are themselves involved in regulation of the major late promoter, are initially expressed from a novel promoter (7). The late transcripts generally encode virion structural proteins. Four other small late transcripts

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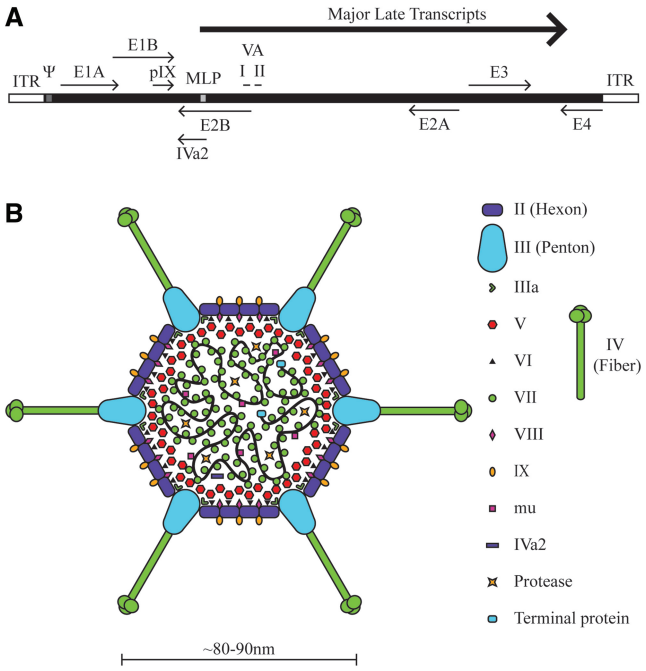

Figure 1. Schematic of the adenovirus genome and virion. (**A**) A simplified map of the Ad5 genome showing the early genes (E1–E4) and the region from which the major late transcript is produced (the extensively spliced L1–L5 transcripts produced from alternative splicing of the major late transcript are not shown). The relative position of pIX, VA RNA I and II and IVa2 are indicated. Also shown are the viral inverted terminal repeats (ITR) located at each end of the genome, the viral packaging element (Ψ) located adjacent to the left ITR, and the position of the major late promoter (MLP). Please note that these features are not drawn to scale. (**B**) Model of the Ad5 virion, adapted from (9), with modifications based on additional data provided by (8,10,11).

are also produced: protein IX (pIX, encoding a minor structural protein), IVa2 (encodes a protein involved in encapsidating the viral DNA into the immature virion) and VA RNA I and II (the RNA itself blocks activation of the interferon response). Inverted terminal repeats (ITR) of ~100 bp flank both ends of the viral DNA and contain the origins of replication. Directly adjacent to the left ITR is the viral packaging sequence (~150 bp). The genome organization is relatively conserved through all Ad species.

The Ad5 capsid is composed of three major (II, III and IV) and five minor (IIIa, IVa2, VI, VIII and IX) polypeptides (Figure 1B) (8–11). The facets are composed primarily of hexons (trimers of protein II) with pentons (five molecules of protein III) capping each vertex. The latter is the base from which extends fibre (trimer of protein IV), the distinctive projections at the Ad capsid vertices. Within the capsid, the viral DNA is associated with three highly basic proteins, core proteins VII, V and Mu (μ) (12–14). Protein VII is a protamine-like protein and is responsible for wrapping and condensing the viral DNA (15). The protein VII-DNA nucleoprotein complex is organized into a central dense core with 12 large spherical nucleoprotein projections, termed adenosomes, which extend into each vertex (16,17). A shell of protein V is thought to coat the protein VII-DNA complex (16,18). Protein V is believed to make contact with the outer capsid in several different ways; protein V interacts directly with penton, and indirectly with peripentonal hexon and the remainder of hexon bridged through IIIa and protein VI, respectively (10,11,19–22). Mu is synthesized as a 79 amino acid precursor protein, pre-Mu, which is cleaved by the Ad-encoded proteinase to its final 19 amino acid, highly basic mature form (23). Pre-Mu is speculated to interact with and aid in tightly condensing the viral DNA within the capsid, and cleavage of pre-mu may serve to partially relax this structure prior to its entry into the nucleus (24). Although the viral DNA does not interact directly with the major capsid proteins (10,25,26), the DNA still appears to contribute to the physical stability to the virion; packaging of subgenomic sized DNA [<90% of the wild-type (wt) genome length] results in virions that are less stable than wtAd (27,28).

Many of the details of Ad5 infection of cells have been elucidated. Initially, the Ad fibre protein binds to the Coxsackie-Adenovirus receptor (CAR), which is the primary receptor for both Ad5 and Coxsackie B virus (29,30). This binding is followed by a secondary interaction between Ad penton and $\alpha_v\beta_3$ or $\alpha_v\beta_5$ integrins (31). Recent studies have shown that Ad5 can enter cells using heparin sulfate proteoglycans as an alternative receptor, either through direct binding to the Ad fibre shaft (32), or bridged through interaction of Ad with blood factors such as factor IX, factor X or complement component C4-binding protein (33–35). Ad is internalized by receptor-mediated endocytosis and evades degradation by escaping from the early endosome (36). The virion is transported through the cytoplasm to the nucleus along the microtubule network (36), and the capsid is slowly disassembled en route (37). Upon reaching the nuclear pore complex, the protein VII-wrapped Ad DNA enters the nucleus (14,37,38), while the rest of the capsid remains at the nuclear membrane and is subsequently degraded. Viral DNA replication and assembly of progeny virions occur entirely within the nucleus of infected cells. The life cycle takes 24–36 h, although the time for completion of the lifecycle is slightly extended in primary cells. A single cell infected with a single virus can produce ~10^4 daughter virions.

EARLY EVENTS WITHIN THE INFECTED CELL NUCLEUS

An overview of our current understanding of Ad DNA chromatin state in the infected cell is shown in Figure 2. Although a number of Ad capsid proteins reach the nucleus, it is only the protein VII-wrapped DNA that enters the nucleus (14). Histone H1 (H1) escorts the Ad DNA–protein complex through the nuclear pore (39); however, this function appears to be independent of any structural role for H1 on the viral DNA. During this phase of infection, protein VII protects the viral DNA from activating the DNA damage response (40). The ultimate fate of protein VII after entry into the nucleus is currently a topic of debate. Some studies suggest that

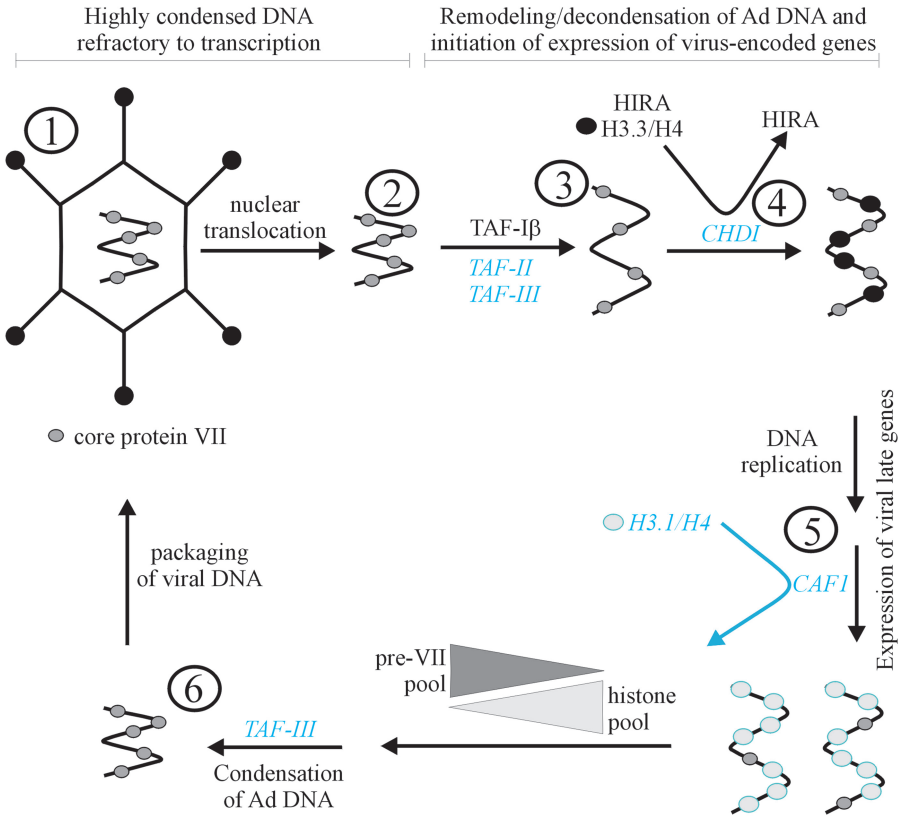

Figure 2. Adenovirus DNA chromatin structure throughout infection. In black—elements that we know. In blue and italics—speculation/areas of future research. DNA in the Ad capsid is highly condensed with core protein VII (**1**), along with protein V and μ (not shown). The protein VII-DNA complex transits to the nucleus (**2**), and undergoes remodeling to decondense the core before transcription of early genes can begin (**3**). Remodeling may involve loss of at least some VII. At the time when viral gene expression is first detected, histones can be found bound to the viral DNA along with VII. Histone variant H3.3 is preferentially deposited on the viral DNA, through the action of HIRA and CHD1 (**4**). Onset of viral DNA replication may be accompanied by a shift to deposition of H3.1 by the CAF1 complex on the DNA, as is observed for HSV1 (**5**). As the histone pool is depleted and the intracellular levels of newly synthesized pre-protein VII increase, there is a transition of Ad DNA association from nucleosomes to pre-protein VII, possibly mediated by TAF-III (**6**). The viral DNA condensed with pre-protein VII is packaged into the Ad capsid (1).

protein VII stably associates with Ad DNA throughout the early phase of infection (14,41), while other groups suggest that VII is removed gradually from at least certain regions of the genome during this same time period (42,43). Other reports have shown that the overall level of VII within the infected cell declines rapidly within the first few hours of infection with a concomitant decline in VII association with viral genomes (44). Whether the eviction of protein VII requires active transcription of the Ad genome is also in dispute (41,44,45).

Cell-free systems developed to study Ad DNA replication have shown that the compacted nature of the VII-wrapped viral DNA allows for only limited transcription and DNA replication (46,47). This observation suggests that the core/DNA structure must be remodeled to allow these processes to proceed with greater efficiency. Three cellular proteins have been identified that can remodel the Ad core in these cell-free systems: template activating factor Iβ (TAF-Iβ) [also known as SET (46)], TAF-II [NAP-1 (48)] and TAF-III [B23/nucleophosmin (49)]. Using the cell-free system, all three TAF's were shown to stimulate replication from the Ad core, while TAF-Iβ and TAF-II were also shown to enhance transcription. TAF-1β, the best characterized TAF in the context of Ad core remodeling, forms a tertiary complex with the VII-wrapped DNA (41,50,51), which results in increased accessibility of the viral DNA to nucleases and restriction enzymes and, presumably, transcriptional activators (47). It is not clear if increased accessibility was due to actual removal or only shifting of VII on the DNA template. siRNA-mediated knockdown of TAF-Iβ in infected cells delayed virus gene expression, DNA replication and virus yield (42), although the effect was relatively modest. Knockdown of TAF-Iβ did not affect the binding level of protein VII on viral DNA as assessed by chromatin immunoprecipitation (ChIP), at least at 4 hpi (43). Thus, additional proteins are likely involved in preparing the Ad core for efficient gene expression and DNA replication within the infected cell nucleus.

Several lines of evidence suggest that VII remains associated with Ad DNA during the early stage of infection. First, VII can be cross-linked to the viral DNA at virtually all stages of infection (52). Second, based on immunofluorescence analysis, foci of VII can be observed in the nucleus of infected cells, which represent

the VII-wrapped viral DNA (40,41,45,53). Third, ChIP studies have shown directly that VII is bound to the viral DNA up to at least ~10 hpi (41,43,44,51,54). There is some disagreement in these studies regarding the level of VII association over time; some studies indicate that VII association is constant and does not change throughout the early stage of infection (41,45,52), while others suggest a gradual (or more rapid) decline in VII association with the viral DNA during this time period (40,43,44,51). Based on ChIP experiments by Komatsu *et al.* (43), it appears that the degree and timing of VII association with the viral genome during the early phase of infection can vary depending on the region of the genome that is analysed. For example, between 1 and 10 hpi, VII remains stably associated with the late-gene hexon coding region, but shows declining association over time with the major late promoter (43). This observation somewhat rationalizes the previous disparate studies. In plasmid-based *in vitro* assays, addition of small amounts of protein VII with the DNA actually enhanced transcription over naked DNA, suggesting that small quantities of protein VII may function in part to keep repressive histone/chromatin features from forming on certain promoter regulatory elements (43). Taken together, these results suggest that dynamic regulation of protein VII is necessary for optimal viral growth; sufficient protein VII must be removed or remodeled to decondense the viral DNA–nucleoprotein complex to allow access to the transcription machinery, but some protein VII must remain to stimulate transcription.

It is unclear whether transcription through the Ad DNA template is required for removal or remodeling of VII. Inhibition of transcription has been correlated with prolonged retention of VII on the Ad genome (40,45), although in other studies inhibition of transcription or transcription elongation did not affect loss of VII (43,44). Although it has been suggested that *de novo* expressed E1A (the first viral gene product expressed within the infected cell) associates with protein VII and is involved in stimulating transcription on the viral genome which subsequently strips VII from the viral DNA (45,54), this function is likely not completely necessary since VII is still removed in the absence of E1A or active transcription (43,44). It is possible that other proteins within the cell can perform this function in the absence of E1A; indeed, E1-deleted Ad can complete a full replication cycle in certain cell types, although the time required to complete the replication cycle is extended (55), suggesting that compensating proteins may exist.

AD DNA ASSOCIATES WITH CELLULAR HISTONES IN THE INFECTED CELL NUCLEUS

In eukaryotic cells, the basic unit of chromatin is the nucleosome, with 147 bp of DNA wrapped around a histone octamer, composed of two sets of H2A–H2B and H3–H4 dimers. The notion that nucleosomes are simply 'beads on a string' has been challenged by the realization that histones and nucleosomes play key roles in gene regulation (56). The post-translationally modified N-terminal tails of histones serve as docking/recognition sites for other regulatory proteins (57), providing the epigenetic information governing gene expression, as dictated by the 'histone code' (58).

Conflicting data from the 1980s suggested that Ad DNA is or is not associated with cellular histones within the infected cell (59–63). With the development of more sensitive techniques, the subject of the Ad nucleoprotein structure within the infected cell has been revisited recently. Based on ChIP analysis, Ad and its derivative vectors (either E1-deleted, replication defective Ad or helper-dependent Ad [hdAd—devoid of all Ad protein coding sequences (64)]) do interact with cellular histones within a few hours of infection (43,44,65). Histones can be detected on the Ad DNA as early as 1-h post-infection, and ChIP/re-ChIP experiments show that both protein VII and histones can be found associated with the same DNA molecule in the cell (43). Since the histones almost certainly bind directly to the Ad DNA, at least some VII must be removed from the viral DNA at these time points to allow for binding of histones. The mechanism by which VII is removed, and the cellular protein(s) involved in this process, have yet to be determined.

Within the cell, deposition of cellular histones can occur either through a replication-coupled or replication-independent mechanism, and there are specific histone variants and chaperones associated with each mechanism (66). Histone variant H3.1 is expressed exclusively during S-phase and is deposited on *de novo* synthesized DNA by the Chromatin Assembly Factor I (CAF1) complex in what is considered a replication-coupled mechanism (67). In contrast, the replacement histone variant H3.3, which differs from H3.1 by only five amino acids, is expressed at all phases of the cell cycle, and is deposited through a replication-independent mechanism (66). H3.3 is deposited on actively transcribed genes by the histone chaperone HIRA, or on specific regions of the chromosome [such as pericentric DNA repeats and on telomeres (68–70)] by the H3.3 chaperone DAXX (68). The H3.3 variant is also deposited on incoming male pro-nuclear DNA shortly after fertilization utilizing the histone chaperone HIRA (71). Although TAF-Iβ can act as a chaperone to transfer histones to DNA templates (47), it does not appear to perform this function during Ad infection (43).

As Ad can infect both dividing and non-dividing cells (and only induces cell cycle progression after viral gene expression has initiated), it suggests that Ad DNA is likely to be chromatinized by exploiting a mechanism independent of DNA replication. Chromatin immunoprecipitation (ChIP) experiments have recently demonstrated hdAd and E1-deleted Ad (44), and wtAd (our unpublished data) DNA preferentially associates with H3 variant H3.3 as early as 4 hpi, suggesting that chromatinization does indeed occur by a replication-independent mechanism. A preferential association with H3.3 was also found with Herpes Simplex Virus 1 (HSV1) DNA during the early phase of infection (72). siRNA-mediated knockdown of HIRA reduced the total association of H3 with the hdAd and HSV1 DNA, as well as reducing expression of virally encoded genes for both viruses, suggesting that

chromatinization was necessary for efficient expression. The involvement of the H3.3 chaperone HIRA, and not DAXX, is consistent with the observation that DAXX is actively degraded during normal Ad infection (73). As deposition of H3.3 on Ad (44) and on HSV1 (72) was dependent on HIRA, it suggests a common mechanism for deposition of histones on the genomes of invading dsDNA nuclear viruses. Moreover, the similarity between the chromatinization of sperm DNA and nuclear virus DNA suggests that both use a similar pathway to achieve chromatinization in the absence of cellular DNA replication. *In vitro* observations suggest that histone chaperones, such as HIRA, either do not assemble nucleosomes or assemble them at a greatly reduced rate in the absence of ATP-utilizing factors (74). In the male pronucleus, HIRA is necessary for delivery of H3.3 to the site of nucleosome formation (71,75), but it is the ATP-dependent chromatin remodeling complex CHD1 that is required for H3.3 deposition (74). In HeLa extracts, HIRA interacts directly with CHD1 (74). Thus, it is likely CHD1 that is directly involved in deposition of histones on the Ad DNA, although this has yet to be formally proven.

Work by Komatsu *et al*. (43) showed that wtAd can be found associated with all members of the nucleosome, H2A–H2B and H3–H4, as early as 1 hpi which, together with studies showing Ad DNA in the nucleus is wrapped in a repeating ~200 bp structure, suggests that the DNA may be wrapped in complete, physiologically spaced nucleosomes (44,59–62,76). The chaperone responsible for deposition of H2A/H2B is unknown. It has been estimated that up to 40% of infecting wtAd DNA is contained in nucleosomes at 3 hpi, and all regions of the genome are represented in micrococcal nuclease-protected fractions (59). The observation that both protein VII and histones can be found bound to the same viral DNA molecule at the same time suggests that the viral chromatin may not completely resemble that of the host cell (43). Interestingly, HSV1 genomic DNA associates with regularly spaced nucleosomes in a latent infection, but the spacing becomes 'unstable' during a lytic infection and generates heterogeneously sized fragments upon MNase digestion (77). Whether wtAd assembles into stable or unstable chromatin during a productive infection remains to be determined. Electron microscopy analysis of viral genomes isolated during late time points of infection (16–18 hpi) showed irregularly spaced nucleosome-like particles at approximately one-tenth the density of cellular chromatin in HeLa cells [3 versus 26 nucleosomes per μm of DNA, respectively (61)]. However, it is not clear whether this is due to 'unstable' chromatin or the limited quantities of histones that are available late in Ad infection. During the replicative phase of the HSV1 lifecycle, there is a switch from early association with H3.3 to deposition of H3.1 (72). Whether a similar phenomenon occurs with wtAd has yet to be determined. The observation that Ad-induced shut-off of host protein synthesis results in a reduction in histone gene expression (78,79) suggests that the virus may simply switch from association of nucleosomes containing H3.3 to re-deposition of pre-protein VII in preparation for DNA packaging into the viral capsid at the final stage of the virus lifecycle (discussed below).

THE IMPORTANCE OF AD DNA CHROMATINIZATION

Since Ad DNA is chromatinized, this suggests that epigenetic regulation may be as important for expression of Ad-encoded genes as is it for expression of genes encoded by the host cell. ChIP analysis showed that there was an increase over time in the level of association of acetylated H3 at all Ad promoters tested (43). Since acetylated histones are commonly associated with actively transcribed genes, it suggests that as these promoters become active, they adopt an epigenetic status similar to cellular genes, which may aid in recruiting appropriate co-factors for optimal gene expression. Interestingly, the cell also uses an epigenetic approach in an attempt to down-regulate expression from some foreign, invading DNAs, including Ad. Indeed, DAXX can act as an anti-Ad defense factor and down-regulate gene expression during wtAd infection (73,80). Thus, Ad-induced degradation of DAXX at late times during infection may be a mechanism that the virus uses to evade down-regulation of its expressed genes (73). A similar phenomenon occurs for vectors based on Ad (65), and this can affect vector function *in vitro* and *in vivo* (81). In these latter studies, the vector chromatin was preferentially associated with deacetylated histones, which is a marker of transcriptionally inactive chromatin (65); thus the DNA was 'marked' to reduce expression of vector-encoded genes. These observations clearly illustrate the ongoing battle between host and pathogen, and the importance of epigenetic regulation of viral DNA at the chromatin level.

LATE STAGE PROTEIN VII REPLACEMENT

During the final stage of virus replication, the viral DNA must be condensed once again into the compact structure required for packaging within the viral capsid. The histones must therefore be displaced from the Ad DNA and replaced with pre-protein VII, the precursor of the mature protein VII [the N-terminus of pre-protein VII is cleaved by the viral-encoded protease during virion maturation (3)]. Little is known about how this switch occurs.

In eukaryotic cells, expression and synthesis of new histones is tightly regulated to coincide with cellular DNA replication (82). Interestingly, however, there is a dramatic decline in histone synthesis at late times during Ad infection (78,79). This puts forth the hypothesis that at the late stage of infection, the decline of available histones relative to the increased levels of pre-protein VII leads, by default, to the deposition of pre-VII on the newly synthesized viral DNA (60,62,83). Experiments in cell free systems have shown that simply mixing Ad DNA and purified pre-protein VII leads to the formation of an insoluble complex, suggesting that a specific cellular chaperone(s) mediates the placement of pre-protein VII

on Ad DNA (84). Based on co-immunoprecipitation studies using extracts from infected cells, TAF-III/nucleophosmin was shown to have a greater affinity for pre-protein VII than the mature protein VII, suggesting that TAF-III may be involved in placing pre-protein VII on the viral DNA (85). In a cell free system, TAF-III was able to transfer pre-VII onto DNA, suggesting that TAF-III is indeed a pre-VII chaperone. However, additional studies are required to further support the role for TAF-III as a chaperone during normal Ad infection of a cell.

CONCLUSION

Insight into the mechanism of Ad chromatinization has the potential to impact three specific areas of research. First, Ad is widely used as a gene delivery system for basic studies and gene therapy applications (64), and improved understanding of the parameters that aid in establishing gene expression within the host cell will improve vector efficacy and safety. Second, Ad is a significant and often overlooked human pathogen (86), and understanding early events in the cell that permit expression of viral genes may lead to the identification of new therapeutic targets to limit or prevent wtAd-induced morbidity and mortality. Finally, numerous studies of basic aspects of Ad biology have contributed significantly to our understanding of how the host cell works (3). Undoubtedly, delineation of the proteins and pathways involved in Ad DNA chromatinization will also improve our understanding of this process within the host cell.

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REFERENCES

1. Rowe,W.P., Huebner,R.J., Gilmore,L.K., Parrott,R.H. and Ward,T.G. (1953) Isolation of a cytopathogenic agent from human adenoids undergoing spontaneous degeneration in tissue culture. *Proc. Soc. Exp. Biol. Med.*, **84**, 570–573.
2. Hilleman,M.R. and Werner,J.H. (1954) Recovery of new agents from patients with acute respiratory illness. *Proc. Soc. Exp. Biol. Med.*, **85**, 183–188.
3. Berk,A.J. (2007) Adenoviridae: the viruses and their replication. In: Knipe,D.M. and Howley,P.M. (eds), *Fields Virology*, 5th edn. Lippincott Williams & Wilkins, Philadelphia, PA, pp. 2355–2394.
4. Trentin,J.J., Yabe,Y. and Taylor,G. (1962) The quest for human cancer viruses. *Science*, **137**, 835–841.
5. Yabe,Y., Trentin,J.J. and Taylor,G. (1962) Cancer induction in hamsters by human type 12 adenovirus. Effect of age and of virus dose. *Proc. Soc. Exp. Biol. Med.*, **111**, 343–344.
6. Davison,A.J., Benko,M. and Harrach,B. (2003) Genetic content and evolution of adenoviruses. *J. Gen. Virol.*, **84**, 2895–2908.
7. Morris,S.J., Scott,G.E. and Leppard,K.N. (2010) Adenovirus late-phase infection is controlled by a novel L4 promoter. *J. Virol.*, **84**, 7096–7104.
8. Christensen,J.B., Byrd,S.A., Walker,A.K., Strahler,J.R., Andrews,P.C. and Imperiale,M.J. (2008) Presence of the adenovirus IVa2 protein at a single vertex of the mature virion. *J. Virol.*, **82**, 9086–9093.
9. Russell,W.C. (2009) Adenoviruses: update on structure and function. *J. Gen. Virol.*, **90**, 1–20.
10. Liu,H., Jin,L., Koh,S.B., Atanasov,I., Schein,S., Wu,L. and Zhou,Z.H. (2010) Atomic structure of human adenovirus by cryo-EM reveals interactions among protein networks. *Science*, **329**, 1038–1043.
11. Reddy,V.S., Natchiar,S.K., Stewart,P.L. and Nemerow,G.R. (2010) Crystal structure of human adenovirus at 3.5 A resolution. *Science*, **329**, 1071–1075.
12. Maizel,J.V. Jr, White,D.O. and Scharff,M.D. (1968) The polypeptides of adenovirus. II. Soluble proteins, cores, top components and the structure of the virion. *Virology*, **36**, 126–136.
13. Russell,W.C., Laver,W.G. and Sanderson,P.J. (1968) Internal components of adenovirus. *Nature*, **219**, 1127–1130.
14. Chatterjee,P.K., Vayda,M.E. and Flint,S.J. (1986) Identification of proteins and protein domains that contact DNA within adenovirus nucleoprotein cores by ultraviolet light crosslinking of oligonucleotides 32P-labelled in vivo. *J. Mol. Biol.*, **188**, 23–37.
15. Mirza,M.A. and Weber,J. (1982) Structure of adenovirus chromatin. *Biochim. Biophys. Acta*, **696**, 76–86.
16. Brown,D.T., Westphal,M., Burlingham,B.T., Winterhoff,U. and Doerfler,W. (1975) Structure and composition of the adenovirus type 2 core. *J. Virol.*, **16**, 366–387.
17. Newcomb,W.W., Boring,J.W. and Brown,J.C. (1984) Ion etching of human adenovirus 2: structure of the core. *J. Virol.*, **51**, 52–56.
18. Everitt,E., Sundquist,B., Pettersson,U. and Philipson,L. (1973) Structural proteins of adenoviruses. X. Isolation and topography of low molecular weight antigens from the virion of adenovirus type 2. *Virology*, **52**, 130–147.
19. Everitt,E., Lutter,L. and Philipson,L. (1975) Structural proteins of adenoviruses. XII. Location and neighbor relationship among proteins of adenovirion type 2 as revealed by enzymatic iodination, immunoprecipitation and chemical cross-linking. *Virology*, **67**, 197–208.
20. Chatterjee,P.K., Vayda,M.E. and Flint,S.J. (1985) Interactions among the three adenovirus core proteins. *J. Virol.*, **55**, 379–386.
21. Stewart,P.L., Fuller,S.D. and Burnett,R.M. (1993) Difference imaging of adenovirus: bridging the resolution gap between X-ray crystallography and electron microscopy. *EMBO J.*, **12**, 2589–2599.
22. Matthews,D.A. and Russell,W.C. (1998) Adenovirus core protein V is delivered by the invading virus to the nucleus of the infected cell and later in infection is associated with nucleoli. *J. Gen. Virol.*, **79(Pt 7)**, 1671–1675.
23. Anderson,C.W., Young,M.E. and Flint,S.J. (1989) Characterization of the adenovirus 2 virion protein, mu. *Virology*, **172**, 506–512.
24. Perez-Berna,A.J., Marabini,R., Scheres,S.H., Menendez-Conejero,R., Dmitriev,I.P., Curiel,D.T., Mangel,W.F., Flint,S.J. and San Martin,C. (2009) Structure and uncoating of immature adenovirus. *J. Mol. Biol.*, **392**, 547–557.
25. Fabry,C.M., Rosa-Calatrava,M., Conway,J.F., Zubieta,C., Cusack,S., Ruigrok,R.W. and Schoehn,G. (2005) A quasi-atomic model of human adenovirus type 5 capsid. *EMBO J.*, **24**, 1645–1654.
26. Silvestry,M., Lindert,S., Smith,J.G., Maier,O., Wiethoff,C.M., Nemerow,G.R. and Stewart,P.L. (2009) Cryo-electron microscopy structure of adenovirus type 2 temperature-sensitive mutant 1 reveals insight into the cell entry defect. *J. Virol.*, **83**, 7375–7383.

27. Smith,A.C., Poulin,K.L. and Parks,R.J. (2009) DNA genome size affects the stability of the adenovirus virion. *J. Virol.*, **83**, 2025–2028.
28. Kennedy,M.A. and Parks,R.J. (2009) Adenovirus virion stability and the viral genome: size matters. *Mol. Ther.*, **17**, 1664–1666.
29. Bergelson,J.M., Cunningham,J.A., Droguett,G., Kurt-Jones,E.A., Krithivas,A., Hong,J.S., Horwitz,M.S., Crowell,R.L. and Finberg,R.W. (1997) Isolation of a common receptor for Coxsackie B viruses and adenoviruses 2 and 5. *Science*, **275**, 1320–1323.
30. Tomko,R.P., Xu,R. and Philipson,L. (1997) HCAR and MCAR: the human and mouse cellular receptors for subgroup C adenoviruses and group B coxsackieviruses. *Proc. Natl Acad. Sci. USA*, **94**, 3352–3356.
31. Wickham,T.J., Mathias,P., Cheresh,D.A. and Nemerow,G.R. (1993) Integrins alpha v beta 3 and alpha v beta 5 promote adenovirus internalization but not virus attachment. *Cell*, **73**, 309–319.
32. Smith,T.A., Idamakanti,N., Rollence,M.L., Marshall-Neff,J., Kim,J., Mulgrew,K., Nemerow,G.R., Kaleko,M. and Stevenson,S.C. (2003) Adenovirus serotype 5 fiber shaft influences in vivo gene transfer in mice. *Hum. Gene Ther.*, **14**, 777–787.
33. Shayakhmetov,D.M., Gaggar,A., Ni,S., Li,Z.Y. and Lieber,A. (2005) Adenovirus binding to blood factors results in liver cell infection and hepatotoxicity. *J. Virol.*, **79**, 7478–7491.
34. Waddington,S.N., McVey,J.H., Bhella,D., Parker,A.L., Barker,K., Atoda,H., Pink,R., Buckley,S.M.K., Greig,J.A., Denby,L. *et al.* (2008) Adenovirus serotype 5 hexon mediates liver gene transfer. *Cell*, **132**, 397–409.
35. Kalyuzhniy,O., Di Paolo,N.C., Silvestry,M., Hofherr,S.E., Barry,M.A., Stewart,P.L. and Shayakhmetov,D.M. (2008) Adenovirus serotype 5 hexon is critical for virus infection of hepatocytes in vivo. *Proc. Natl Acad. Sci. USA*, **105**, 5483–5488.
36. Leopold,P.L., Ferris,B., Grinberg,I., Worgall,S., Hackett,N.R. and Crystal,R.G. (1998) Fluorescent virions: dynamic tracking of the pathway of adenoviral gene transfer vectors in living cells. *Hum. Gene Ther.*, **9**, 367–378.
37. Greber,U.F., Willetts,M., Webster,P. and Helenius,A. (1993) Stepwise dismantling of adenovirus 2 during entry into cells. *Cell*, **75**, 477–486.
38. Strunze,S., Engelke,M.F., Wang,I.H., Puntener,D., Boucke,K., Schleich,S., Way,M., Schoenenberger,P., Burckhardt,C.J. and Greber,U.F. (2011) Kinesin-1-mediated capsid disassembly and disruption of the nuclear pore complex promote virus infection. *Cell. Host Microbe*, **10**, 210–223.
39. Trotman,L.C., Mosberger,N., Fornerod,M., Stidwill,R.P. and Greber,U.F. (2001) Import of adenovirus DNA involves the nuclear pore complex receptor CAN/Nup214 and histone H1. *Nat. Cell. Biol.*, **3**, 1092–1100.
40. Karen,K.A. and Hearing,P. (2011) Adenovirus core protein VII protects the viral genome from a DNA damage response at early times after infection. *J. Virol.*, **85**, 4135–4142.
41. Xue,Y., Johnson,J.S., Ornelles,D.A., Lieberman,J. and Engel,D.A. (2005) Adenovirus protein VII functions throughout early phase and interacts with cellular proteins SET and pp32. *J. Virol.*, **79**, 2474–2483.
42. Haruki,H., Okuwaki,M., Miyagishi,M., Taira,K. and Nagata,K. (2006) Involvement of template-activating factor I/SET in transcription of adenovirus early genes as a positive-acting factor. *J. Virol.*, **80**, 794–801.
43. Komatsu,T., Haruki,H. and Nagata,K. (2011) Cellular and viral chromatin proteins are positive factors in the regulation of adenovirus gene expression. *Nucleic Acids Res.*, **39**, 889–901.
44. Ross,P.J., Kennedy,M.A., Christou,C., Risco Quiroz,M., Poulin,K.L. and Parks,R.J. (2011) Assembly of helper-dependent adenovirus DNA into chromatin promotes efficient gene expression. *J. Virol.*, **85**, 3950–3958.
45. Chen,J., Morral,N. and Engel,D.A. (2007) Transcription releases protein VII from adenovirus chromatin. *Virology*, **369**, 411–422.
46. Matsumoto,K., Nagata,K., Ui,M. and Hanaoka,F. (1993) Template activating factor I, a novel host factor required to stimulate the adenovirus core DNA replication. *J. Biol. Chem.*, **268**, 10582–10587.
47. Okuwaki,M. and Nagata,K. (1998) Template activating factor-I remodels the chromatin structure and stimulates transcription from the chromatin template. *J. Biol. Chem.*, **273**, 34511–34518.
48. Kawase,H., Okuwaki,M., Miyaji,M., Ohba,R., Handa,H., Ishimi,Y., Fujii-Nakata,T., Kikuchi,A. and Nagata,K. (1996) NAP-I is a functional homologue of TAF-I that is required for replication and transcription of the adenovirus genome in a chromatin-like structure. *Genes Cells*, **1**, 1045–1056.
49. Okuwaki,M., Iwamatsu,A., Tsujimoto,M. and Nagata,K. (2001) Identification of nucleophosmin/B23, an acidic nucleolar protein, as a stimulatory factor for in vitro replication of adenovirus DNA complexed with viral basic core proteins. *J. Mol. Biol.*, **311**, 41–55.
50. Gyurcsik,B., Haruki,H., Takahashi,T., Mihara,H. and Nagata,K. (2006) Binding modes of the precursor of adenovirus major core protein VII to DNA and template activating factor I: implication for the mechanism of remodeling of the adenovirus chromatin. *Biochemistry*, **45**, 303–313.
51. Haruki,H., Gyurcsik,B., Okuwaki,M. and Nagata,K. (2003) Ternary complex formation between DNA-adenovirus core protein VII and TAF-Ibeta/SET, an acidic molecular chaperone. *FEBS Lett.*, **555**, 521–527.
52. Chatterjee,P.K., Vayda,M.E. and Flint,S.J. (1986) Adenoviral protein VII packages intracellular viral DNA throughout the early phase of infection. *EMBO J.*, **5**, 1633–1644.
53. Walkiewicz,M.P., Morral,N. and Engel,D.A. (2009) Accurate single-day titration of adenovirus vectors based on equivalence of protein VII nuclear dots and infectious particles. *J. Virol. Methods*, **159**, 251–258.
54. Johnson,J.S., Osheim,Y.N., Xue,Y., Emanuel,M.R., Lewis,P.W., Bankovich,A., Beyer,A.L. and Engel,D.A. (2004) Adenovirus protein VII condenses DNA, represses transcription, and associates with transcriptional activator E1A. *J. Virol.*, **78**, 6459–6468.
55. Nelson,J.E. and Kay,M.A. (1997) Persistence of recombinant adenovirus in vivo is not dependent on vector DNA replication. *J. Virol.*, **71**, 8902–8907.
56. Luger,K. (2006) Dynamic nucleosomes. *Chromosome Res.*, **14**, 5–16.
57. Kouzarides,T. (2007) Chromatin modifications and their function. *Cell*, **128**, 693–705.
58. Jenuwein,T. and Allis,C.D. (2001) Translating the histone code. *Science*, **293**, 1074–1080.
59. Sergeant,A., Tigges,M.A. and Raskas,H.J. (1979) Nucleosome-like structural subunits of intranuclear parental adenovirus type 2 DNA. *J. Virol.*, **29**, 888–898.
60. Daniell,E., Groff,D.E. and Fedor,M.J. (1981) Adenovirus chromatin structure at different stages of infection. *Mol. Cell. Biol.*, **1**, 1094–1105.
61. Beyer,A.L., Bouton,A.H., Hodge,L.D. and Miller,O.L. Jr (1981) Visualization of the major late R strand transcription unit of adenovirus serotype 2. *J. Mol. Biol.*, **147**, 269–295.
62. Dery,C.V., Toth,M., Brown,M., Horvath,J., Allaire,S. and Weber,J.M. (1985) The structure of adenovirus chromatin in infected cells. *J. Gen. Virol.*, **66(Pt 12)**, 2671–2684.
63. Wong,M.L. and Hsu,M.T. (1988) Psoralen-cross-linking study of the organization of intracellular adenovirus nucleoprotein complexes. *J. Virol.*, **62**, 1227–1234.
64. Amalfitano,A. and Parks,R.J. (2002) Separating fact from fiction: assessing the potential of modified adenovirus vectors for use in human gene therapy. *Curr. Gene Ther.*, **2**, 111–133.
65. Ross,P.J., Kennedy,M.A. and Parks,R.J. (2009) Host cell detection of noncoding stuffer DNA contained in helper-dependent adenovirus vectors leads to epigenetic repression of transgene expression. *J. Virol.*, **83**, 8409–8417.
66. Tagami,H., Ray-Gallet,D., Almouzni,G. and Nakatani,Y. (2004) Histone H3.1 and H3.3 complexes mediate nucleosome assembly pathways dependent or independent of DNA synthesis. *Cell*, **116**, 51–61.
67. Smith,S. and Stillman,B. (1989) Purification and characterization of CAF-I, a human cell factor required for chromatin assembly during DNA replication in vitro. *Cell*, **58**, 15–25.
68. Drane,P., Ouararhni,K., Depaux,A., Shuaib,M. and Hamiche,A. (2010) The death-associated protein DAXX is a novel histone

chaperone involved in the replication-independent deposition of H3.3. *Genes Dev.*, **24**, 1253–1265.
69. Lewis,P.W., Elsaesser,S.J., Noh,K.M., Stadler,S.C. and Allis,C.D. (2010) Daxx is an H3.3-specific histone chaperone and cooperates with ATRX in replication-independent chromatin assembly at telomeres. *Proc. Natl Acad. Sci. USA*, **107**, 14075–14080.
70. Goldberg,A.D., Banaszynski,L.A., Noh,K.M., Lewis,P.W., Elsaesser,S.J., Stadler,S., Dewell,S., Law,M., Guo,X., Li,X. *et al.* (2010) Distinct factors control histone variant H3.3 localization at specific genomic regions. *Cell*, **140**, 678–691.
71. Loppin,B., Bonnefoy,E., Anselme,C., Laurencon,A., Karr,T.L. and Couble,P. (2005) The histone H3.3 chaperone HIRA is essential for chromatin assembly in the male pronucleus. *Nature*, **437**, 1386–1390.
72. Placek,B.J., Huang,J., Kent,J.R., Dorsey,J., Rice,L., Fraser,N.W. and Berger,S.L. (2009) The histone variant H3.3 regulates gene expression during lytic infection with herpes simplex virus type 1. *J. Virol.*, **83**, 1416–1421.
73. Schreiner,S., Wimmer,P., Sirma,H., Everett,R.D., Blanchette,P., Groitl,P. and Dobner,T. (2010) Proteasome-dependent degradation of Daxx by the viral E1B-55K protein in human adenovirus-infected cells. *J. Virol.*, **84**, 7029–7038.
74. Konev,A.Y., Tribus,M., Park,S.Y., Podhraski,V., Lim,C.Y., Emelyanov,A.V., Vershilova,E., Pirrotta,V., Kadonaga,J.T., Lusser,A. *et al.* (2007) CHD1 motor protein is required for deposition of histone variant H3.3 into chromatin in vivo. *Science*, **317**, 1087–1090.
75. Bonnefoy,E., Orsi,G.A., Couble,P. and Loppin,B. (2007) The essential role of Drosophila HIRA for de novo assembly of paternal chromatin at fertilization. *PLoS Genet.*, **3**, 1991–2006.
76. Tate,V.E. and Philipson,L. (1979) Parental adenovirus DNA accumulates in nucleosome-like structures in infected cells. *Nucleic Acids Res.*, **6**, 2769–2785.
77. Lacasse,J.J. and Schang,L.M. (2009) During lytic infections, herpes simplex virus type 1 DNA is in complexes with the properties of unstable nucleosomes. *J. Virol.*, **84**, 1920–1933.
78. Hodge,L.D. and Scharff,M.D. (1969) Effect of adenovirus on host cell DNA synthesis in synchronized cells. *Virology*, **37**, 554–564.
79. Tallman,G., Akers,J.E., Burlingham,B.T. and Reeck,G.R. (1977) Histone synthesis is not coupled to the replication of adenovirus DNA. *Biochem. Biophys. Res. Commun.*, **79**, 815–822.
80. Ullman,A.J. and Hearing,P. (2008) Cellular proteins PML and Daxx mediate an innate antiviral defense antagonized by the adenovirus E4 ORF3 protein. *J. Virol.*, **82**, 7325–7335.
81. Parks,R.J., Bramson,J.L., Wan,Y., Addison,C.L. and Graham,F.L. (1999) Effects of stuffer DNA on transgene expression from helper-dependent adenovirus vectors. *J. Virol.*, **73**, 8027–8034.
82. Robbins,E. and Borun,T.W. (1967) The cytoplasmic synthesis of histones in hela cells and its temporal relationship to DNA replication. *Proc. Natl Acad. Sci. USA*, **57**, 409–416.
83. Brown,M. and Weber,J. (1980) Virion core-like organization of intranuclear adenovirus chromatin late in infection. *Virology*, **107**, 306–310.
84. Burg,J.L., Schweitzer,J. and Daniell,E. (1983) Introduction of superhelical turns into DNA by adenoviral core proteins and chromatin assembly factors. *J. Virol.*, **46**, 749–755.
85. Samad,M.A., Okuwaki,M., Haruki,H. and Nagata,K. (2007) Physical and functional interaction between a nucleolar protein nucleophosmin/B23 and adenovirus basic core proteins. *FEBS Lett.*, **581**, 3283–3288.
86. Wold,W.S. and Horwitz,M.S. (2007) Adenoviruses. In: Knipe,D.M. and Howley,P.M. (eds), *Fields Virology*, 5th edn. Lippincott Williams & Wilkins, Philadelphia, PA, pp. 2396–2436.

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CURRICULUM VITAE

Adam Davidson

Academic Qualifications

McMaster University
2012 – Present
MSc. Physiotherapy

University of Ottawa
2010 – 2012
MSc. Microbiology and Immunology

Master's Thesis Title: Investigating the role of IRF3 in response to genotoxic stress

Lakehead University
2006 – 2010
HBSc Applied Bio-Molecular Science

Honours Thesis Title: Lambda Red Mediated Labelling of *Escherichia coli* (pathogenic and non-pathogenic)

Sir Winston Churchill C&VI
2002 – 2006
International Baccalaureate Diploma Graduate

Research Experience

Virology and Immunology
2010 – Present
Master's Research

Examine the innate immune response of human cells to infection with adenovirus gene therapy vectors

Environmental Microbiology
2009 – 2010
Honours Research

Investigated the genomic labelling of *Escherichia coli* using the Lambda Red Recombinase system

Plant Physiology
2008 – 2009
Research Internship

Studied the ethnobotany of grapes and the survival of cold-hardy mutant strains in Thunder Bay, Ontario

Technical Skills

Proficient in laboratory techniques, including:

- Tissue culturing and experimental techniques
- Bacterial culturing and plating techniques
- Bacterial transformation
- Genetic cloning
- Slide preparation and staining techniques
- PCR
- Polyacrylamide and agarose gel electrophoresis
- Western Blotting
- DNA and protein extraction and purification methods
- Gel filtration and paper chromatography methods

Teaching Experience

Superior Science
2010
Instructor

Instructed children aged 6-14 in multiple facets of science. Presented workshops to elementary school classrooms. Travelled to remote aboriginal reserves to present workshops as part of an aboriginal outreach program

Lakehead University
2009 – 2010

Teaching Assistant – Biology Department
Prepared laboratory sessions, evaluated written presentations and administered quizzes and exams

Thunder Bay Rowing Club
2009
Head Coach

Provided instruction and coaching for athletes of all ages and skill levels. Completed administrative work in preparation for the season as well as upcoming competitions

Thunder Bay Rowing Club
2006 – 2009
Assistant Coach

Assisted the Head Coach with program delivery as well as athlete development and technical improvements

Publications and Presentations

Giberson AN **Davidson AR** Parks RJ (2012) Chromatin structure of adenovirus DNA throughout infection. *Nucleic Acids Research*. 40**(6)**:2369-2376.

Davidson AR Parks RJ (2012) Investigating the role of IRF3 in response to genotoxic stress. Biochemistry, Microbiology, and Immunology Department, University of Ottawa, Ottawa

- Seminar presented at the BMI Seminar Day

Davidson AR Parks RJ (2011) Investigating the role of IRF3 in response to genotoxic stress. The Ottawa Hospital Research Institute, Ottawa

- Research poster presented at the BMI Poster Day

Davidson AR Kennedy MA Screaton R, Parks RJ (2010) Characterization of the innate immune response to adenovirus vectors. The Ottawa Hospital Research Institute, Ottawa

- Research poster presented at the OHRI Research Day

Scholarships and Awards

- 2011 – Queen Elizabeth II Graduate Scholarship in Science and Technology
- 2011 – University of Ottawa Excellence Scholarship
- 2010 – Ontario Graduate Studies in Science and Technology Scholarship
- 2010 – University of Ottawa Excellence Scholarship
- 2010 – University of Ottawa Admission Scholarship
- 2006 – 2010 – Dean's List, Lakehead University
- 2006 – 2010 – Recipient of Lakehead University Academic Entrance Scholarship
- 2007 – Recipient of Webster Silver Jubilee Scholarship
- 2006 – Graduate – International Baccalaureate Program
- 2006 – Ontario Scholar
- 2006 – Recipient of Sir Winston Churchill High School Alumni Award

Volunteer Experience

Lakehead University
2009 – 2010
Biology Department representative

Gave biology-based interactive demonstrations and answered questions to prospective students. Participated in Biology Department departmental review panel

Volunteer for various charity groups.
2008 – present

Easter Seals
St. Joseph's Care Group
Special Olympics
Canadian Blood Services

Athlete's Representative. Thunder Bay Rowing Club.
2004 – 2008

Competitive athletes' representative on the Board of Directors

Memberships/Certifications

- Member – American Society for Human Genetics
- WHMIS
- Accessibility Training
- BioSafety Training
- Standard First Aid and CPR-C
- Pleasure Craft Operator's Certificate
- Canadian Firearm Safety Course
- Hockey Canada – HCOP Level IV Certification
- Hockey Canada – Speak Out! Certification
- NCCP Level 1 Theory/Technical Rowing Certificate
- Ontario Hunter Education Course
- Alpine Club of Canada
- Rowing Canada