Stony Brook University

The Adenovirus E4 ORF3 Protein Functions to Antagonize Aspects of the Interferon-Induced Antiviral State

A Dissertation Presented

by

Amanda Ullman

to

The Graduate School

in Partial Fulfillment of the

Requirements

for the Degree of

Doctor of Philosophy

in

Molecular Genetics and Microbiology

Stony Brook University

May 2008

Stony Brook University

The Graduate School

Amanda Ullman

We, the dissertation committee for the above candidate for the Doctor of Philosophy degree, hereby recommend acceptance of this dissertation.

Patrick Hearing, Ph.D.
Dissertation Director
Professor, Department of Molecular Genetics and Microbiology,
Stony Brook University

Michael Hayman, Ph.D.
Chairperson of Defense
Professor, Department of Molecular Genetics and Microbiology,
Stony Brook University

Carl Anderson, Ph.D.
Senior Geneticist, Chair, Department of Biology
Brookhaven National Laboratory

Janet Hearing, Ph.D
Associate Professor, Department of Molecular Genetics and Microbiology,
Stony Brook University

Nancy C. Reich, Ph.D.
Professor, Department of Molecular Genetics and Microbiology,
Stony Brook University

Rolf Sternglanz, Ph.D.
Distinguished Professor, Department of Biochemistry and Cell Biology
Stony Brook University

This Dissertation is accepted by the Graduate School.

Lawrence Martin
Dean of the Graduate School

Abstract of the Dissertation

The Adenovirus E4 ORF3 Protein Functions to Antagonize Aspects of the IFN-Induced Antiviral State

by

Amanda Ullman

Doctor of Philosophy

in

Molecular Genetics and Microbiology

Stony Brook University

2008

The PML nuclear body (PML-NB) is a common target for disruption by DNA viruses. The adenovirus early protein E4 ORF3 is both necessary and sufficient to rearrange PML-NBs from punctate bodies into nuclear tracks. PML-NB disruption has been correlated with the antagonism of both the innate and intrinsic immune responses. This may reflect the fact that PML, the protein responsible for nucleating PML-NBs, as well as several proteins known to localize to the PML-NB, are products of interferon-stimulated genes. I demonstrate that the E4 ORF3 protein is required for adenovirus DNA replication during the interferon (IFN)-induced antiviral state. When cells are pretreated with either IFN-α or IFN-γ, a mutant virus that does not express E4 ORF3 is severely compromised for replication, suggesting that the functional significance of ORF3 track formation is the inhibition of a PML-NB-mediated antiviral mechanism. Replication of the E4 ORF3 mutant virus is rescued following the introduction of E4

ORF3 from evolutionarily divergent adenoviruses, demonstrating functional conservation for E4 ORF3 inhibition of the IFN-induced antiviral state. Furthermore, E4 ORF3 inhibition of an IFN-induced response is unrelated to the inhibition of adenovirus replication by the Mre11-Rad50-Nbs1 DNA repair complex. I propose that the evolutionarily conserved function of the adenovirus E4 ORF3 protein is the inhibition of an innate response to viral infection mediated by the PML –NB and dependent upon PML and Daxx. Knockdown of either of these proteins is sufficient to rescue replication of a virus deleted for the E4 ORF3 protein during the IFN response. Both the HSV1 ICP0 and HCMV IE1 proteins support DNA replication of adenovirus deleted for the E4 ORF3 protein during the antiviral state, suggesting functional conservation of PML-NB disruption among viruses. I show that this innate response is not based solely on transcriptional repression. While early gene transcription is modestly diminished in the absence of E4 ORF3 protein expression, this reduction does not affect early protein function. I propose that in addition to its abilities to repress gene expression, the PML-NB participates in an alternative innate immune mechanism.

TABLE OF CONTENTS

List of Figures

Abbreviations

2-5A	2'-5' oligoadenylate
aa	amino acid
Ad	adenovirus
Ad*n*	adenovirus serotype n
ADP	adenovirus death protein
Ad Pol	adenovirus DNA polymerase
APL	Acute Promyelocytic Leukemia
ATCC	American Type Culture Collection
BCA	Bicinchoninic acid
bp	base pairs
CAR	coxsackie adenovirus receptor
CPE	cytopathic effects
CR*n*	conserved region *n*
DBP	DNA binding protein
DIC	differential interference contrast
DMEM	Dulbecco's Modified Eagle Medium
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
dsDNA	double stranded DNA
dsRNA	double stranded RNA
DTT	DL-Dithiothreitol
E1A	adenovirus early promoter region 1A

E1B	adenovirus early promoter region 1B
E1B 19K	adenovirus early promoter region 1B, 19 kDa protein
E1B 55K	adenovirus early promoter region 1B, 55 kDa protein
E2	adenovirus early promoter region 2
E3	adenovirus early promoter region 3
E4	adenovirus early promoter region 4
ER	endoplasmic reticulum
FADD	Fas-associated death domain
FITC	fluorescin isothiocyanate
GAS	gamma activated sequence
HCMV	human cytomegalovirus
HDAC	histone deacetylase
HFV	human foamy virus
HP1	heterochromatin protein 1
hr	hour
HSV-1	herpes simplex virus-1
ICP0	HSV-1 infected cell protein 0
IE1	human cytomegalovirus immediate early protein 1
IFN	interferon
IFNAR	IFN-α receptor
IFNGR	IFN-γ receptor
IgG	immunoglobulin G
IRF	IFN regulatory factor

ISG	IFN-stimulated gene
ISRE	IFN-stimulated response element
ITR	inverted terminal repeat
Jak	Janus kinase
kb	kilobase pairs
kDa	kilodaltons
LTR	long terminal repeat
mAb	monoclonal antibody
MAPK	mitogen activated protein kinase
MEFs	mouse embryo fibroblasts
MIEP	Major Immediate Early Promoter
min	minutes
MLP	major late promoter
MRN	Mre11-Rad50-Nbs1
mRNA	messenger RNA
NHEJ	nonhomologous end joining
NLS	nuclear localization signal
ORF-*n*	open reading frame *n*
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate-buffered saline
PML	promyelocytic leukemia protein
PML-NB	PML nuclear body
PML-RARα	promyelocytic leukemia protein-retinoic acid receptor

	alpha fusion protein
PMSF	phenylmethylsulfonylfluoride
pol	polymerase
pTP	preterminal protein
RBCC	RING finger, B-box, coiled-coil domain
RID	receptor internalization and degradation
RNA	ribonucleic acid
SDS	sodium dodecyl sulfate
shRNA	short hairpin RNA
SIM	SUMO interaction motif
ssDNA	single stranded DNA
ssRNA	single stranded RNA
Stat	signal transducer and activator of transcription
SUMO	small ubiquitin-like modifier
TBK-1	tank binding kinase-1
TBP	TATA binding protein
TBS	Tris-buffered saline
TIF1	transcriptional intermediary factor 1a
TNF	tumor necrosis factor
TNFR	tumor necrosis factor receptor
TP	terminal protein
TRIM	tripartite motif
TRITC	Texas red isothiocyanate

UV	ultra violet
VA RNA	virus associated RNA
vDNA	viral DNA
WT	wild type

Chapter 1

General Adenovirus Information and Background

Introduction

The discovery of adenovirus (Ad) occurred as a consequence of studies intended to elucidate the causative agent of the common cold. In 1953, Rowe et al. noted cytopathic effects (CPE) in cell cultures derived from human adenoids (152). Within a year, Hilleman and Werner, in pursuit of an etiologic agent responsible for acute respiratory disease among Army recruits, similarly observed CPE in cells exposed to secretions from infected individuals (78). It was later determined that adenoviruses, aptly named for the tissue of their initial isolation, were not responsible for the common cold, rather they accounted for virtually no respiratory morbidity among healthy individuals and caused only 5-10% of respiratory disease among children; however, the virus may cause severe respiratory distress among the immunocompromised (97).

Although unassociated with the common cold, Ad is responsible for epidemic conjunctivitis, often termed "swimming pool conjunctivitis" or "shipyard eye" (93). In addition, several Ad serotypes are the causative agent of infantile gastroenteritis, causing substantial mortality in the developing world (59).

Within ten years of Ad identification, adenovirus serotype 12 (Ad12) was demonstrated to induce the development of malignant tumors in newborn hamsters, providing the first evidence that a human virus could promote oncogenesis (189). While Ad has not been implicated in human transformation, this virus has proved invaluable as a tool to examine both viral and cellular gene expression, transformation, cell cycle control, as well as the regulation of various cellular processes.

Classification

Adenoviruses comprise the *Adenoviridae* family, which is further divided into two genera: *Aviadenovirus*, which exclusively infects birds, and *Mastadenovirus*, which encompasses human, simian, equine, bovine, porcine, ovine, opossum, and canine viruses (162). At this point >50 human serotypes have been identified on the basis of specific antibody-mediated neutralization. These antigenic epitopes are primarily localized to the hexon protein, as well as the terminal knob of the fiber protein. Serotypes are further categorized into seven subgroups, A-G, on the basis of two criteria: hemagglutanation and the percentage of guanine-cytosine content in the viral DNA genome (162).

Summary of the viral lytic cycle

Ad conducts a lytic cycle, which is initiated, in the case of the vast majority of serotypes, upon the binding of the fiber protein to its cellular receptor, the coxsackie and adenovirus receptor (CAR). To initiate receptor-mediated endocytosis, the Ad penton base interacts with a coreceptor, an α_v integrin, on the cell surface (204). This interaction is facilitated by the presence of RGD peptides in the viral penton protein, mimicking the host extracellular matrix, which is the cellular ligand for these integrins (204). Upon acidification of the endosome, the viral capsid is susceptible to partial degradation (162); however, prior to complete proteolysis, the penton protein, through a poorly understood mechanism, mediates escape from this increasingly hostile subcellular compartment (162). Once the particle has accessed the cytoplasm, it is transported along the microtubule network until it docks at the nuclear pore complex. Subsequently, the viral DNA (vDNA) is translocated into the nucleus (162). Once inside the nucleus, the E1A gene is transcribed exclusively by cellular transcription factors. This region encodes two nearly identical proteins, the larger of which includes an additional exon segment termed conserved region 3. It is this domain, in conjunction with cellular factors, which is responsible for the activation of the other Ad early genes, making E1A the master regulator of the viral transcriptional program (45).

Early gene expression is responsible for executing three major objectives aimed to optimize the intracellular environment for virus replication. Firstly, early genes enable the inhibition of the host innate and acquired immune responses. Secondly, these gene products promote progression of the cell cycle into S-phase. Thirdly, the proteins required for vDNA replication are expressed (45).

Concomitant with the onset of vDNA replication is activation of the adenovirus major late promoter (MLP). Transcription from this locus is accomplished via a two-step cascade. Firstly, a cellular repressor is titrated off of the promoter of the Ad delayed-early gene, IVa2 (88, 112). This titration proceeds as a consequence of the rapidly expanding population of viral genomes generated by robust vDNA replication. Upon expression, the IVa2 protein promotes full activation of the MLP, enabling the transcription of an approximately 30,000 nucleotide long RNA. This primary transcript is subject to extensive alternative splicing, which ultimately results in the genesis of multiple mRNAs. All of these messages contain a complex 5' secondary structural element, termed the tripartite leader (162). It is this structural motif that permits cap-independent translation of MLP derived mRNAs, an essential function, as Ad inhibits cap-dependent translation at late points during infection (162). Electron microscopy studies have revealed that once these proteins have been expressed, viral capsid assembly, DNA packaging, and virion maturation proceed in large intranuclear inclusions (68).

Ad vDNA is packaged in a polar manner, mediated both by viral and cellular factors that are recruited to the 5' terminus of the left end of the genome. This region, termed the packaging sequence, is critical to successful packaging of the viral genome into preformed capsids (70, 71). Following packaging, the Ad protease performs a series of cleavage events to yield infectious virions (196). Although all aspects of cell lysis are not entirely clear, it is known that an early region protein, the E3 death protein, participates in this process, thereby releasing mature, infectious virus (45).

Adenovirus infection is disseminated due, in part, to fiber protein. Both soluble protein and fiber associated with defective particles binds to the CAR, disrupting intercellular contacts, thereby mediating the release of Ad from epithelial junctions to the apical surface where Ad may initiate another cycle of lytic infection (194).

Figure 1-1. Adenovirus genome.

The Ad linear double stranded (ds) DNA genome is represented by the solid black line. Early promoter regions are labeled in purple, while delayed promoter regions are designated by blue. The delayed early genes are colored light blue. Arrows indicate transcriptional directionality.


E1A
E1B
E2b
E2a
E3
E4
Early genes
ITR
pIX
IVa2
ITR
MLP
L1
L2
L3
L4
L5
Late genes

Genomic Organization

The adenovirus genome is a linear, double-stranded DNA (dsDNA) molecule comprised of approximately 36 kilobase pairs (kb). Encapsidated, the genome is extremely condensed and associated with viral chromatin-like proteins V and VII. The viral origins of replication are located at the genomic ends within larger, appropriately named, inverted terminal repeats (ITRs), consisting of roughly 100 base pairs (Fig 1-1). The origin of replication resides within the first 50 bp of the ITRs and the extreme terminal 18 bp of the genome constitutes the core origin, which is the minimal sequence capable of supporting vDNA replication (45).

Replication is initiated by a protein priming event, mediated by the viral pre-terminal protein (pTP) (45). This is preceded by a "jumping back" mechanism, in which the Ad DNA polymerase (Ad Pol)-pTP complex forms a trinucleotide intermediate, pTP-CAT. This is accomplished by means of the Ad Pol utilizing a complimentary sequence located at nucleotides 4-6 at the genomic terminus (45). It is this trinucleotide-protein complex that jumps back to positions 1-3 (97). Finally, Ad Pol dissociates from pTP, facilitating strand elongation (45, 148).

In addition to the three viral E2 proteins required for vDNA replication, pTP, Ad Pol, and the single stranded DNA (ssDNA) binding protein (DBP), the presence of three cellular proteins, NFI/CTF, NFII, and NFIII/Oct-1 increase replication initiation by 200-fold (45). Both NFI/CTF and NFIII/Oct-1, cellular transcription factors, bind to a site adjacent to the core origin, termed the auxiliary region (45). NFI/CTF interacts with the Ad Pol/pTP complex via interaction with Ad Pol, to recruit these proteins to the core origin (45). NFIII/Oct-1 contains a DNA binding domain, the POU. This domain binds a

recognition sequence adjacent to the NFI/CTF binding site and contacts the pTP protein (45). Unlike NFI and NFIII, NFII is a type I DNA topoisomerase. Its presence is required for elongation; however, the reason for its requirement is unknown (45).

The mature counterpart to pTP, TP also executes important functions. Covalently attached to the 5' genomic termini, it facilitates increased transcription from the Ad genome by mediating its attachment to the nuclear matrix (158). Furthermore, TP is hypothesized to protect the genomic ends from nucleolytic assault.

Just downstream of the ITR at what is canonically termed the left end, is located the *cis*-acting packaging sequence (Fig. 1-1). Most proximal to this site is the early promoter region 1A (E1A). Expression of all other early region genes is contingent upon the expression of the E1A protein. Because its expression occurs independently of all other viral genes, it is termed the immediate early gene. Within an hour of Ad infection, the Early Region 4 (E4) proteins become readily detectable. In addition to the above, other early gene promoters, E1B, E2B, and E3 become transcriptionally active due to E1A protein function. These promoters ultimately enable the expression of proteins, which function to inhibit apoptosis, facilitate vDNA replication, and inhibit immune responses, respectively (45). In addition to these genes are two delayed early promoter regions, IX and IVa2, as well as the MLP, discussed in the previous section (162). The aforementioned viral promotes utilize cellular RNA polymerase (pol) II for gene transcription; however, the viral genome also contains at least one gene encoding virus associated (VA) RNA, a RNA pol III product (118). This RNA species is rich in secondary structure, mimicking the dsRNA that is a hallmark of viral infection. Robust VA RNA production occurs during Ad infection to which monomers of the cellular

kinase PKR bind. This inhibits kinase activation, typically a result of PKR monomer dimerization upon low levels of dsRNA. By impeding this interaction, PKR is maintained in an inactive monomeric form; thereby inhibiting PKR-mediated phosphorylation of eIF-2α. This strategy effectively functions to maintain the cellular translational apparatus (118).

Early Region 1 A (E1A)

The Ad genome encodes over 25 early gene products, which are expressed in a coordinated, temporal fashion. Upon infection, the first transcriptional unit expressed is that of the immediate early gene, E1A (Fig. 1-1). Transcription of this promoter region relies exclusively upon cellular factors, enabling the expression of E1A proteins in the absence of other viral proteins (45).

The E1A gene is comprised of two exons, which lead to the production of several polypeptides due to alternate splicing of a primary transcript. Of these, the 243 amino acid (aa) E1A 12S and 289 aa E1A 13S are the most abundant polypeptides expressed. These 12S and 13S proteins function as major regulators of early transcription, as well as modulators of cellular proliferation and gene expression (45).

These proteins share in common two conserved regions, originating from within the 5' exon, termed conserved region (CR) 1 and CR2. The E1A 12S and 13S proteins differ exclusively in an internal exon present in the 13S protein. This region, referred to as CR3, is critical to the transcriptional transactivation properties associated with the E1A 13S protein (3). It is the conserved regions that facilitate interaction of the E1A 13S proteins with a variety of cellular proteins involved in transcriptional regulation. These cellular factors include: a CR1- and CR2-mediated interaction with the retinoblastoma tumor suppressor pRb and its family members, p107 and p130; a CR1- and amino terminal sequence-mediated interaction with transcriptional coactivatiors p300 and CBP; and CR3-directed interactions with an array of transcription factors including TATA-binding protein (TBP) and members of the ATF family, such as ATF-2, Sp1, and c-Jun (3, 41, 60). Of these interactions, most notable is that of the E1A protein with the E2F

family of cellular proteins. In fact, discovery of the E2F family resulted from studies of Ad E2 promoter regulation (102). The Ad E2 promoter region contains binding sites for both ATF and E2F transcription factors. The E2F transcription factors execute important functions in regulating cell cycle progression (41). This is of particular significance as the primary target of Ad infection is terminally differentiated epithelial cells (41). These cells are metabolically quiescent, necessitating that Ad promote cell cycle progression to facilitate macromolecule synthesis (41). Interestingly, the interaction of E1A with p300/CPB also stimulates cellular DNA synthesis (131, 166). This may be partially attributed to the activation of E1A with pRb, facilitating E2F directed transcription (131, 166). In fact, the E1A 13S protein is the major transactivator of early gene expression. This function is primarily executed by the CR3 domain (3, 60). Through this mechanism, the E1A 13S protein may augment transcription by stabilizing the transcription factor complex TFIID at both viral and cellular promoter regions. Transcription initiation proceeds as a result of an ability of the E1A 13S protein to interact with TBP in the TFIID complex (73). It is this ability of E1A to direct transcriptional responses and induce S phase, as well as unscheduled DNA synthesis, that is directly responsible for the oncogenic potential of adenovirus (131).

The E1A protein also functions to induce apoptosis in infected cells. E1A expression has been demonstrated to promote stabilization of the tumor suppressor p53 (30, 203). The presence of p53 elicits two major effects. Firstly, p53 is a potent inducer of cell cycle arrest. Here, p53 transactivates $p21^{WAF\text{-}1/Cip\text{-}1}$, which prevents phosphorylation of pRb family members (171). Secondly, p53 is also a potent inducer of apoptosis. In this case, p53 promotes caspase activation (30, 203).

In addition to interactions with cellular factors, through CR3, the E1A 13S product mediates transcriptional transactivation of the early Ad genes (3, 60). Both p53 activation and the induction of apoptosis would be deleterious to the Ad lytic cycles. Consequently, among the multitude of functions executed by early proteins are included several strategies for counteracting these host responses.

Early Region 1 B (E1B)

The second E1 gene expressed is early region 1 B, which encodes two major mRNA species (Fig. 1-1). These messages yield two distinct proteins, the E1B 19 kDa (E1B 19K) and the E1B 55 kDa (E1B 55K) proteins. These proteins are encoded by alternative reading frames and thus, share no homology. The E1B 19K and 55K proteins function both to inhibit apoptosis and to modulate further the intracellular environment to optimize conditions for viral replication (30, 203).

The E1B 55K protein is essential to the viral lytic cycle. Importantly, it is capable of binding to p53, thereby preventing p53-mediated transcription (116); however, this interaction alone is incapable of inhibiting p53 functions. In this instance, it is unclear whether the E1B 55K protein is itself a transcriptional repressor or if it recruits a repressor to the p53-bound complex on the DNA (117).

In addition to its putative role as a transcriptional repressor or corepressor, the E1B 55K protein functions in concert with another early protein, the E4 ORF6 protein, to promote proteosome-dependent degradation of p53, the components of the Mre11-Rad50-Nbs1 DNA repair complex, and the catalytic subunit of DNA-PK (17, 47, 115, 170, 172, 173). Additionally, a complex containing both the E1B 55K and E4 ORF6 proteins contribute to what is termed host cell shut off. Here, viral mRNAs are selectively stabilized and transported to the cytoplasm while the nuclear export of host mRNAs is inhibited (45).

The E1B 19K protein, like the E1B 55K protein, is involved in the inhibition of apoptosis. This E1B product is a functional homologue of a cellular suppressor of apoptosis, Bcl-2 (30, 203). Like Bcl-2, the E1B 19K protein is capable of binding to the

proapoptotic Bax, thereby maintaining mitochondrial integrity (30, 203). Furthermore, by means of a poorly understood mechanism, the E1B 19K protein is capable of inhibiting TNF-induced apoptotic pathways by preventing the oligomerization of Fas-associated death domain (FADD)-containing complexes (30).

Early Region 2 (E2)

The gene products directly responsible for Ad DNA synthesis are encoded by the E2 transcriptional unit (Fig 1-1). From the E2A locus is expressed DBP, while Ad Pol and pTP are expressed from the E2B unit (175).

The DBP protein is a nuclear phosphoprotein with an apparent molecular weight of 72 kDa. It is synthesized in abundant quantities throughout Ad infection and is involved in numerous functions including vDNA replication, gene expression, transformation, virion assembly, and potentially DNA recombination (77, 147, 191). Utilizing its C-terminus, DBP binds cooperatively to ssDNA to protect it from nuclease digestion. The DBP protein also possesses a helix-destabilizing property, required for unwinding dsDNA in an ATP-independent manner during the elongation phase of vDNA replication (33, 114). Furthermore, DBP is capable of enhancing the renaturation of complimentary strands (220).

The 80 kDa pTP protein is critical to the initiation of vDNA replication. It exists as a stable heterodimer with Ad Pol, with which it exists in a 1:1 stoichiometric ratio (77, 147, 191). Upon initiation of vDNA replication, pTP binds to the core origin in a phosphorylation-dependent manner (77, 147, 191). At late points during infection, the pTP protein is processed by the viral protease to a 55 kDa product (197). While this cleavage event is not required for vDNA replication or virion assembly, it is necessary for full infectivity of mature virus particles (197). The resulting TP protein becomes covalently linked to the 5' genomic termini. Not only is this interaction proposed to protect the integrity genome by inhibiting exonuclease digestion, but it is also responsible for attachment of the viral genome to the nuclear matrix (63, 158).

The Ad Pol is a 140 kDa phosphoprotein that functions in complex with the pTP protein. It is responsible for both initiation and elongation during Ad DNA replication (77, 147, 191). The protein possesses an intrinsic 3' to 5' proofreading activity and two putative zinc finger motifs, believed to important for DNA binding and initiation of vDNA replication (28).

Early Region 3 (E3)

The proteins encoded by the E3 region are typically associated with the inhibition of host immune responses (Fig 1-1). The primary host strategy to counteract Ad infection is the elimination of infected cells. As such, the E3 region encodes several proteins to antagonize the multiple pathways of cell death induced by both the innate and adaptive immune responses (205, 206).

The E3 19K protein is a membrane glycoprotein which localizes to the endoplasmic reticulum (ER). There, it forms a complex with nascent MHC class I molecules, retaining both the antigens and MHC I molecules in the ER membrane. This strategy effectively reduces CTL-mediated killing (92).

The E3 region also encodes proteins capable of inhibiting cell death pathways contingent upon ligand-receptor interactions (205, 206). The E3 RID (receptor internalization and degradation) protein is an integral membrane protein comprised of two E3 products: RIDα (E3 10.4K) and RIDβ (E3 14.5K). This complex localizes to the plasma membrane, Golgi body, and the ER to inhibit TNFR- and Fas-induced apoptosis (67, 185). RID expression promotes Fas clearance from the cell surface and its subsequent degradation in lysosomes. RID also mediates the elimination of TNFR1 from the cell surface, although this process is less efficient than Fas internalization. Furthermore, it remains unknown whether or not TNFR1 is subject to lysosomal degradation (67).

Also involved in the inhibition of apoptosis is the E3 14.7K protein; however, in contrast to many of the other E3 gene products, the E3 14.7K protein is not associated with a cell membrane. Rather, it is present both in the cytosol and the nucleus. Here, it

binds to FIP-3, a protein hypothesized to activate an apoptotic pathway in conjunction with the inhibition of NFκB activation (111). In addition, it has also been reported that the E3 14.7K protein may directly bind caspase 8 to inhibit the caspase proteolytic cascade (111).

In contrast to the E3 proteins, which function to inhibit cell death, the E3 11.6K protein, also termed the adenovirus death protein (ADP), promotes cell death late in the lytic cycle. At this phase of Ad infection, when mature virions have accumulated, the ADP is produced in significant quantities to facilitate cell lysis (187). In fact, cells infected with a virus deleted for E3 11.6K remain viable far longer than those infected by a wild type Ad (186).

Early Region 4 (E4)

While the aforementioned early regions encode proteins of related functions, early region 4 (Fig. 1-1) encodes functionally disparate proteins. Two of these E4 gene products, E4 open reading frame 3 (ORF3) and ORF6, have been exceedingly well characterized and perform critical functions within the infected cell. Thus, they will be discussed extensively in the subsequent section. In contrast, the E4 ORF1 14.3K protein remains relatively uncharacterized; however, it has been demonstrated that the E4 ORF1 14.3K protein is capable of transforming primary rat cells in culture (184). Further, the Ad9 E4 ORF1 protein is capable of inducing mammary carcinomas in rats in an E1A-independent manner (184). In addition, this protein is capable of binding to several cellular proteins containing PDZ domains, such as the tumor suppressor *dlg* (66). The ability to engage in these protein-protein interactions appears to augment the oncogenic capacity of the E4 ORF1 protein (66).

While little is understood about the E4 ORF1 protein, less is known about the E4 ORF2 protein. This is also true for the E4 ORF3/4 7.1K protein (45). In contrast, like E4 ORF3 and E4 ORF6, E4 ORF4 has been the subject of extensive study. This 14 kDa protein has been implicated in several processes during Ad infection. Firstly, the E4 ORF4 protein binds to the Bα subunit of the cellular serine/threonine phosphatase PP2A (99). Upon binding this subunit, the trimeric PP2A is activated and dephosphorylates substrates such as mitogen activated protein kinases (MAPK) (99). Not only does PP2A activation pose consequences for the MAPK-mediated protein-protein interactions necessary to propagate signal transduction cascades, PP2A activity also promotes deposphorylation of transcription factors such as E4F, which are activated by MAPKs

(99). In addition to affecting cellular targets, E4 ORF4 protein expression also results in decreased E1A phosphorylation at MAPK consensus sites, important for E1A transactivation (202). By means of decreasing E1A and E4F activity, the E4 ORF4 protein down regulates expression from early region 4 (14). Secondly, the E4 ORF4 functions to regulate mRNA splicing. Thirdly, the E4 ORF4 protein has been demonstrated to induce p53-independent apoptosis (104). Here, the ability of the E4 ORF4 to both bind to and regulate PP2A is essential for the induction of cell death. Furthermore, this E4 ORF4 protein-dependent apoptosis requires the modulation of Src-family kinases (104).

The E4 ORF6/7 protein is produced from a spliced mRNA encoding the amino terminus of ORF6 linked to the unique E4 ORF7 sequence. The 17 kDa E4 ORF6/7 protein forms stable homodimers that contribute to the efficiency of viral DNA synthesis by enhancing the production of E2 gene products. In addition, the E4 ORF6/7 protein has been demonstrated to induce gene expression from the cellular E2F-1 promoter and is capable of functionally compensating for E1A during Ad infection (137, 159).

The Importance of Early Region 4 Open Reading Frames 3 and 6 During Ad Infection

Adenovirus early region 4 (E4) encodes a variety of proteins responsible for executing both disparate and essential functions within an infected cell. Among them, E4 open reading frame 3 (E4 ORF3) and open reading frame 6 (E4 ORF6) are critical for efficient vDNA replication (18, 87). This requirement was determined upon a mutational analysis of the E4 region. Ablation of the entire region resulted in a virus severely compromised for growth, reflecting a 10^5-fold reduction in plaque formation in comparison to the wild type virus (87). This finding prompted a sequential analysis of the E4 open reading frames (ORFs) and revealed that when either E4 ORF3 or E4 ORF6 were restored to an otherwise E4 deleted virus, either ORF was sufficient to rescue viral production to near wild type levels (87) (Fig 1-2). For this reason, E4 ORF6 and E4 ORF3 were considered redundant in their abilities to promote viral growth; however, upon a more extensive analysis, these proteins were found not only to perform redundant functions, but also to execute unique objectives.

The E4 ORF3 and E4 ORF6 proteins execute complementary functions in genome replication via the inhibition of the cellular Mre11-Rad50-Nbs1 (MRN) DNA repair complex. In the absence of E4 ORF3 and E4 ORF6 expression, the Ad genome induces an MRN-dependent DNA damage response that results in concatenation of viral genomes by the non-homologous end joining (NHEJ) pathway (198), thereby inhibiting viral DNA replication. To counteract this host response, in conjunction with the E1B 55K protein, the E4 ORF6 protein impedes MRN activity by targeting its components for degradation by the ubiquitin-mediated proteasome-dependent pathway (115, 172).

Furthermore, other participants in the NHEJ response are also substrates for viral-directed degradation such as the DNA-PK catalytic subunit (17, 47, 115, 172, 173). In contrast to this strategy, the E4 ORF3 protein of the Group C adenoviruses inhibits MRN activity by sequestering both the nucleoplasmic and PML-NB associated pool of MRN proteins into E4 ORF3-containing track-like structures (47, 115, 172, 173).

In addition to their complimentary roles with respect to MRN complex inhibition, the E4 ORF3 and E4 ORF6 proteins also perform opposing functions. Both proteins are capable of binding the E1B 55K protein; however, as observed by immunofluorescence microscopy, the E4 ORF6 protein eliminates the E1B 55K-E4 ORF3 protein interaction (100, 107). Similarly, the E4 ORF3 and E4 ORF6 proteins behave antagonistically with respect to the modulation of p53 activity. When expressed alone, the E4 ORF6 protein inhibits p53 activity. When expressed in conjunction with E1B 55K, the two proteins promote proteosome-dependent degradation of this tumor suppressor (38, 170). In contrast, when expressed in the absence of the E4 ORF6 protein, the E4 ORF3 protein relieves the p53 repression promoted by the E1B 55K protein (100). When the three viral proteins are expressed simultaneously, the E1B 55K-E4 ORF6 protein functions supercede E4 ORF3 protein function. This complex interplay between the E4 ORF3 and E4 ORF6 proteins and p53 may reflect the critical nature of p53 in promoting cell survival or death. In addition to p53 regulation, both the E4 ORF3 and E4 ORF6 proteins have been demonstrated to utilize alternative mechanisms to affect the degree of alternate splicing in transcripts derived from the MLP (136). While the E4 ORF3 protein promotes the inclusion of a specific exon during splicing, the E4 ORF6 protein facilitates its exclusion (136).

While the above provides evidence of a degree of functional redundancy, both E4 ORF3 and E4 ORF6 perform discrete functions (180) (Fig 1-3). In addition to the MRN complex proteins, as previously mentioned, E4 ORF6 targets a variety of cellular proteins such as p53 for degradation (76, 146) and inhibits the transport of cellular mRNAs from the nucleus (180). Distinct from those of E4 ORF6, several functions have been attributed to E4 ORF3 including cell cycle-independent virus growth, the enhancement of translation of late viral mRNAs, and the targeting of MRN to cytoplasmic aggresomes (5, 68, 136, 138, 163, 164). These structures are cytoplasmic storage compartments that develop adjacent to the microtubule organizing center to accelerate the degradation of misfolded proteins (65, 101).

While the aforementioned activities occur late after infection, E4 ORF3 function is not restricted to this phase of the viral lytic cycle. In the early hours of infection, E4 ORF3 mediates the reorganization of PML nuclear bodies (PML-NBs/PODs/ND10s) into track-like structures within the nucleus (23, 39) and is both necessary and sufficient to execute this process.

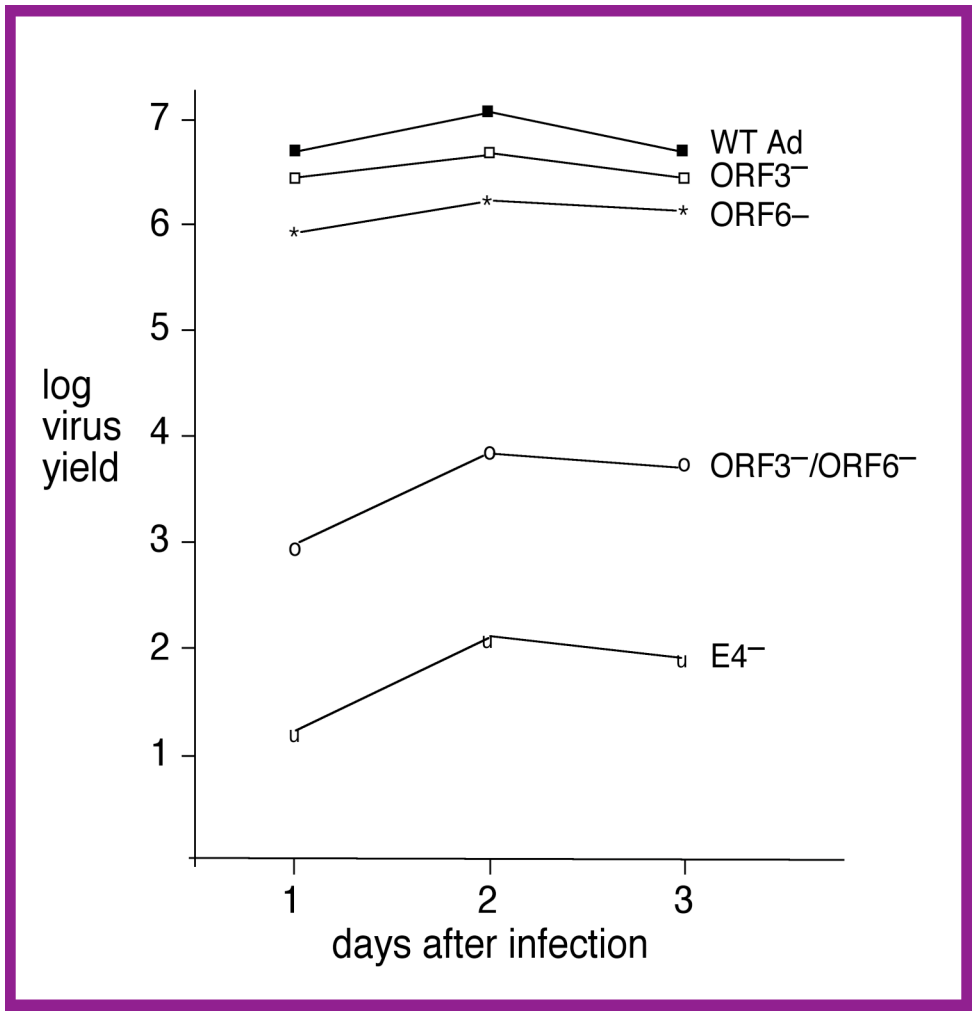


Figure 1-2. Growth Curves of Adenovirus Deletion Mutants.
Single-step growth curves were performed on A549 cells with the wild type (WT) virus and mutant viruses lacking E4 ORF3, ORF6, ORF3 and ORF6, and the entire E4 region. Cells were harvested on day 1, 2, and 3, post-infection. Viral titers were determined by plaque assay on W162 cells. This particular cell line constitutively expresses E4.

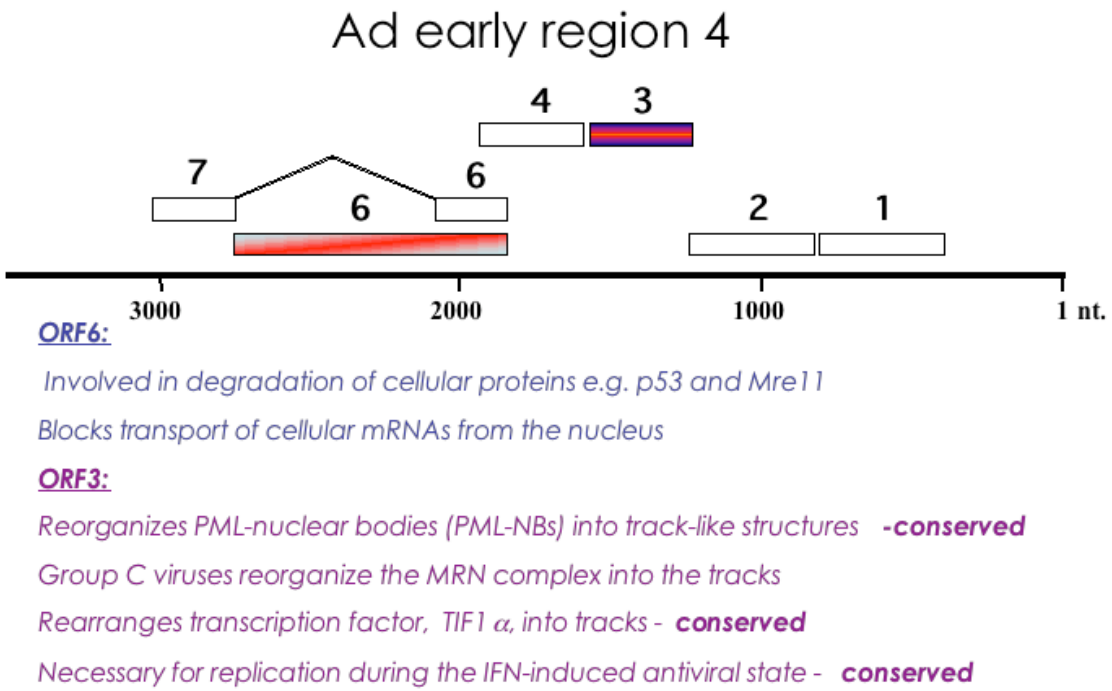


Figure 1-3 The Unique Functions of E4 ORF3 and E4 ORF6.
E4 ORF3 and E4 ORF6 are designated as colored boxes. The genomic location of each ORF is indicated by the number of base pairs from the right end of the genomic terminus. The unique functions of these proteins are designated in blue for E4 ORF6 and purple for E4 ORF3.

PML Nuclear Bodies

PML nuclear bodies (PML-NBs) are electron-dense nuclear subdomains, nucleated by promyelocytic leukemia protein, PML (15, 44). These structures are small, ranging from 0.3-0.5 μm and range between 5-30 per nucleus, depending upon cell type (90). They have been noted to be responsive to cellular stresses such as heat shock and heavy metal treatment (32), as well as to treatment with interferon (IFN) (27).

In cases of Acute Promyelocytic Leukemia (APL), the hematopoietic disorder for which PML was named, a reciprocal translocation event between chromosomes 15 and 17 generates a fusion protein expressing the N-terminus of PML and the C-terminus of the retinoic acid receptor alpha. The resulting PML-RARα product functions as a dominant negative PML protein, disrupting the formation of discrete, punctate PML-NBs, instead generating dispersed micropunctate structures. Furthermore, the PML-RARα product stimulates uncontrolled cellular proliferation and in hematopoetic lineages, cellular differentiation is halted at the promyelocyte stage (174). Remission of the leukemic state occurs upon treatment with either all-*trans* retinoic acid or arsenic trioxide and is associated with restoration of the typical PML localization. As such, PML-NB integrity is correlated with the regulation of cellular proliferation (174).

Both of these therapies promote an increase in the proportion of sumoylated (SUMO, Small Ubiquitin-like Modifier) PML (15, 219). PML sumoylation is required for the recruitment of the host of proteins that, in addition to PML itself, comprise the PML-NB (40). Recent work has identified the existence of a SUMO interaction motif (SIM), enabling a model for PML-NB establishment (161). This hypothesis proposed that PML sumoylation provides a surface for the PML-PML noncovalent interactions

necessary for PML-NB nucleation. Once this event has occurred, additional proteins can be recruited via PML and the SIMs (161). The presence of PML is absolutely required for PML-NB formation and in its absence, PML body resident proteins assume a nucleoplasmically diffuse localization pattern (91, 216, 217).

In addition to its association with proliferation and transformation, PML nuclear bodies have been implicated in a wide variety of cellular processes including transcriptional regulation, apoptosis, the DNA damage response, and post-translational modification (15, 82, 157, 218). This reflects, in part, the diverse population of proteins associated with PML bodies.

Most PML-NB associated proteins are typically present both in the PML-NB and in the nucleoplasm (106, 130). Of these proteins, a vast array have been characterized as transiently residing at the nuclear body and include: Mre11, Rad50, NBS1, BRACA1, pRB, and CBP (133). Proteins that are constitutively localized to the PML-NBs include: Daxx, Sp100, BLM, and SUMO (133). This heterogeneous composition has prompted the association of PML-NBs with nearly every cellular process (36).

There is much evidence in support of PML-NBs as sites of transcriptional regulation (218). Not only are these nuclear bodies proximal to acetylated chromatin, but many transcription factors localize to the PML-NBs (13, 218). Furthermore, nascent RNAs have been detected in the vicinity of PML bodies (13, 179). Initial studies suggested that PML-NBs could regulate transcription by modulating the activity and or availability of transcription factors (218). Indeed, p53 relocalization to the PML-NBs enhances the transactivation functions of this protein (62). PML appears to be required for the posttranslational modifications, specifically acetylation and phosphorylation,

necessary to enhance p53 activity (141). Upon overexpression of PML IV (PML isoforms will be discussed subsequently in greater detail), both p53 and the cellular acetyltransferase CBP have been demonstrated to be recruited to the PML-NBs, promoting an increase in p53 acetylation (141). In addition to participating in transcriptional activation, the PML-NB has also been implicated in transcriptional repression. Both Sp100 and Daxx, proteins known to constitutively localize to PML-NBs, are capable of interacting with transcriptional repressors. While Sp100 associates with HP-1, Daxx is known to interact with HDAC1 and HDAC2 (83, 109, 110).

The association of PML-NBs with p53 is consistent with a role for the PML-NB in the regulation of apoptotic pathways. In addition to mediating the posttranslational modifications of p53, PML also binds to and inhibits MDM2, the primary negative regulator of p53 (8, 34, 176). Further, PML is capable of inducing p53-independent apoptosis. Following DNA damage, PML mediates the autophosphorylation and activation of CHK2, thereby activating apoptotic pathways (212). Furthermore, cells derived from PML-/- mice exhibited defects in Fas, TNF, and IFN-induced apoptosis (195). Here, the induction of apoptosis was reduced, although not abrogated, suggesting that PML indirectly modulates this process (195). Furthermore, PML -/- mice are more sensitive to carcinogenic drugs and more resistant to γ-irradiation as a consequence of these apoptotic defects (195).

PML-NBs have also been postulated to participate in the cellular DNA damage response. Not only do PML-NBs increase in size and number in an ATM- and ATR-dependent manner, but they also colocalize to sites of DNA repair or ssDNA and many DNA repair and checkpoint proteins transiently associate with these nuclear bodies (10,

12, 34, 35). In this instance, the PML-NBs may regulate the DNA damage response by acting as a storage depot for the release of checkpoint and repair proteins upon a damage stimulus (9).

In addition to mediating these diverse and essential cellular processes, PML-NBs have been implicated in the immune response. Among the diverse population of proteins which localize to PML-NBs are the products of Interferon (IFN)-stimulated genes (ISGs) (149).

The Interferon-Induced Antiviral State

IFNs signal through cell surface receptors to effect the activation of specific gene transcription. These proteins are secreted by virally infected cells to induce what is termed the "antiviral state." The presence of double stranded (ds) RNA, a hallmark of viral infection, is sensed by an RNA helicase such as RIG-I (retinoic acid inducible gene I) (215), which activates a series of signaling pathways, culminating in IRF-3 phosphorylation and NFκB activation (58, 80). Alternatively, a recently identified molecule, DAI, is capable both of recognizing cytosolic dsDNA and promoting the association of IRF-3 with TBK-1, the kinase responsible for its activation (177). Upon IRF-3 phosphorylation and NFκB activation, type I IFN is secreted in an autocrine and paracrine manner and binds cell surface receptors (58, 80).

IFNs are classified into two major groups, type I and type II, that bind to distinct receptors and activate both discrete and overlapping pathways (149). Type I IFNs promote dimerization of the type I IFN receptor (IFNAR). This leads to the subsequent tyrosine phosphorylation of Tyk2 and Jak1, Janus kinasaes, associated with the cytosolic domain of the IFNAR. Phosphorylated Janus kinases recruit signal transducers and activators of transcription (STATs). Typically associated with type I IFN responses are Stat1 and Stat2. These molecules are phosphorylated by the Janus kinases, enabling Stat dimerization. Stat1 and Stat2 commonly heterodimerize and subsequently, bind a third protein, IRF-9. This heterotrimer translocates into the nucleus and binds to IFN-stimulated response elements (ISREs) in the promoters of various genes, thereby promoting the expression of ISGs responsive to type I IFNs (31, 143, 169). Analogously, type II IFN binds to its receptor, the IFNGR, promoting receptor dimerization and

phosphorylation of the receptor associated Janus kinases, Jak1 and Jak2. Stat1 is commonly recruited to these activated kinases and is phosphorylated, facilitating Stat1 homodimerization (31, 143, 169). Stat1 homodimers are competent for nuclear translocation and bind to gamma-activated sequences (GAS) in the promoter of various genes, activating ISG transcription (31, 143, 169). The expression of these ISGs collectively constitutes the induction of an intracellular environment hostile to all phases of viral replication, termed the IFN-induced antiviral state.

It is the ability of IFNs to induce the antiviral state that led to their initial discovery (89). All stages of viral replication are subject to inhibition during the IFN response, ranging from entry and uncoating to assembly and release and including all intermediate steps. Among the well-characterized ISGs are PKR, which was discussed previously, the 2-5A system, and Mx proteins.

The 2-5A system is a multienzyme system which in the presence of dsRNA, induces the expression of 2-5A synthetase, which produces a series of short 2'5'-oligoadenylates (2-5A). These, in turn, activate the 2-5A-dependent RNaseL (95). Upon activation, RNaseL is competent for cleavage of ssRNA (22, 61, 208). Mx proteins are GTPases in the dynamin superfamily (6, 86). Mx proteins and dynamins self-assemble into heliacal structures that impede the growth of influenza and other negative-sense RNA viruses at the level of viral transcription (79, 132, 178).

PML Nuclear Bodies as Sites of Antiviral Defense

When cells are treated with type I IFN (e.g., IFN-α) or type II IFN (IFN-γ), there is a dramatic augmentation in both the size and number of PML bodies (72, 103). This is believed to occur because the PML gene itself is an ISG. There are both an ISRE and a GAS located in the 5' untranslated first exon of the PML gene (168). For this reason, PML bodies have been postulated to contribute to innate immunity by participating in the IFN-induced antiviral state (149).

While PML nucleates PML-NB genesis, it is not the only ISG known to localize to the subdomain. Among this population of proteins are Sp100 and Daxx, both of which are ISGs (72, 165). In addition to their putative functions during the IFN-induced antiviral state, these proteins are capable of functioning as constitutively expressed mediators of intrinsic immunity, termed restriction factors, against both HSV-1 and HCMV (145, 154, 155, 181, 207). In this case, the Daxx protein facilitates inhibition of HCMV early gene expression via the recruitment of histone deacetylases (HDACs) to the Major Immediate Early Promoter (MIEP), thereby inhibiting the expression of critical early genes (155, 181).

The Daxx protein was not initially identified as an effector in the innate, nor the adaptive immune response. It was first characterized as a participant in apoptotic responses based on its interaction with the Fas receptor death domain (213); however, several studies suggest that this initial observation may be incorrect as Daxx is present exclusively in the nucleus, even upon the induction of apoptosis by Fas- stimulation (91, 188). This nuclear localization is readily observed by immunofluorescence microscopy or detected by cell fractionation studies, revealing that Daxx is present in the nuclear

fraction (125, 144). Within the nucleus, Daxx localizes to the PML-NBs; however, in the absence of PML, Daxx is known to associate with condensed chromatin (91). This localization is consistent with the observation that Daxx is capable of functioning as a transcriptional corepressor (84). While Daxx is devoid of a canonical DNA binding domain, it exhibits limited homology to the yeast transcriptional corepressor Sin3 (84). When tested, it was demonstrated to repress the transcriptional capacity of Pax3, a transcription factor, by 80% (84). Later studies implicated Daxx in the repression of ETS1-, E2F1-, NFκB-, p53-, and p73- dependent transcription (69, 96, 110, 126). Furthermore, these properties have been attributed to the ability of Daxx to associate with HDAC1 and HDAC2 (83, 109, 110). It is this very association of Daxx with HDACs that mediates cellular restriction of HCMV infection (155, 181).

The concept of intrinsic immunity is not novel, especially with regard to limiting retroviral infection. Constitutively expressed restriction factors include the members of an extensive family of proteins referred to as the TRIM, or tripartite motif, family (135). Most notable among these are TRIM5α, which was recently identified as mediating antiretroviral activities (135). The TRIM family proteins are also known as the RBCC proteins, since they harbor a characteristic RBCC motif composed of a RING finger domain, followed by one or two B-boxes, proceeded by a coiled-coil region. This precise order of these elements is conserved among all family members and they are believed to mediate protein-protein interactions (142, 151). It is intriguing that PML is, in fact, TRIM19. In fact, not only is the SIM critical to the protein-protein interactions essential to PML-NB formation, but also the RING finger domain of the protein (161).

The single PML gene is composed of 9 exons and alternate splicing leads to the production of which result in the generation of several PML isoforms. These are categorized into seven groups, PML I-VII (56, 94). All isoforms share in common their N-terminus, containing the TRIM motif, although they differ in their C-termini (56, 94). These proteins are subject to further classification, a, b, and, c, on the basis of alternative splicing. The b and c variants are devoid of a nuclear localization signal (NLS) and are distributed cytoplasmically (94, 151).

The expression of multiple PML isoforms enables PML isoform-specific protein-protein interactions (94). In addition to its aforementioned interaction with p53, the PML IV isoform has also been shown to interact with HDACs (209). In contrast both PML VI and PML-RARα interact poorly with these transcriptional repressors, suggesting that it is the C-terminus of PML that mediates this interaction (108, 156). In addition to these interactions, both PML II and PML IV have been shown to interact with nonphosphorylated pRb. Here, not only is the C-terminus critical to this interaction, but also the RBCC motif (4).

There exists evidence that PML itself functions as a cellular restriction factor. In the absence of IFN induction, PML III overexpression confers resistance both to vesicular stomatitis virus (VSV), as well as to influenza A virus (27). In these instances, viral gene transcription and protein expression are impeded (27). In addition, nucleoplasmic PML III has been demonstrated to participate in the restriction of a complex retrovirus, human foamy virus (HFV) (150). Here, PML binds the viral transactivator of transcription, tas, preventing it from binding the proviral long terminal repeat (LTR) (150).

Viral Disruption of PML-NBs

In light of their association with both the intrinsic and innate antiviral responses, it is not surprising that numerous viruses target PML bodies for disruption. DNA viruses such as the herpesviruses, papovaviruses, and adenoviruses undergo replication in proximity to PML-NBs and encode proteins that associate with the nuclear bodies (48, 119). This observation is not limited to viruses such as Ad that conduct lytic life cycles. Viruses that persist episomially, such as the herpesviruses, replicate their genomes in proximity to PML-NBs (50). In fact, while the latent state of Epstein-Barr virus, a gammaherpes virus, involves the chromosomal association of its genome, entry into the lytic cycle entails PML-NB association (7).

It has been reported that *de novo* PML nuclear body assembly occurs at the sites of invading viral genomes (52). While those genomes successful in vDNA replication are associated with PML nuclear bodies (167), suggesting that elements of the PML-NB enhance vDNA replicative success, it appears that disruption of PML-NBs serves to antagonize antiviral objectives of these nuclear subdomains. In fact, failure to disrupt PML-NBs during HSV-1 infection inhibits vDNA replication (20). As is the case for HSV-1 ICP0, HCMV IE1, and Ad E4 ORF3, the proteins that mediate POD association also disrupt the structural integrity of these nuclear bodies (48, 119). In the case of both HSV-1 and HCMV, PML was determined to participate in mediating the anti-viral effects of IFN treatment (24, 25, 55, 181). Interestingly, this interference with PML body integrity is not exclusive to DNA viruses, as several RNA viruses have also been found to alter POD structure (11, 37, 150). LCMV, Rabies virus, and VSV have been documented to encode proteins capable of disrupting PML-NBs (150).

Clearly, the adenovirus E4 ORF3 protein is not unique in its ability to disrupt PML bodies (48, 55, 119). Among several virally encoded gene products, the HCMV IE1 protein prompts relocalization of PML from the POD to condensed chromatin (105, 129), while the HSV-1 encoded ICP0 protein is an E3 ubiquitin ligase that directs proteosome-dependent degradation of PML (16, 49, 50). Although these proteins execute mechanisms distinct from that of the Ad E4 ORF3 protein, all three viral proteins accomplish the same objective: to disrupt PML-NBs and to alter the subnuclear localization of a wide variety of proteins known to associate with PML-NBs.

HSV-1, an alphaherpesvirus, encodes the aforementioned E3 ubiquitin ligase, ICP0, a 110 KDa protein. It was initially observed that wild type HSV-1 was capable of promoting PML-NB disruption, while and ICP0 mutant could not (121). The ICP0 protein has been found to mediate degradation of SUMO-1-modified PML and Sp100 (26, 50, 139). This activity is dependent upon the RING finger domain of the protein and it is likely that only low levels of the ICP0 protein are required to mediate PML-NB disruption (50, 81). Later studies revealed that PML degradation is a proteosome-dependent event which affects both sumoylated and unsumoylated PML isoforms (139). While mutational analysis has revealed that the RING finger domain is critical to PML-NB disruption, a distinct region at the C-terminus of the protein mediates localization of the viral protein to the PML-NB (120).

HCMV mutants deficient for IE1 expression exhibit a phenotype similar to HSV-1 ICP0 mutants. These viruses inefficiently enter the lytic cycle, although this defect can be overcome by infection with a high multiplicity of infection (127). While HCMV, a beta herpes virus, does not encode a RING finger protein, the 72 kDa IE1 protein is

capable of promoting PML-NB disruption (1). Here, IE1 promotes the redistribution of PML from the nuclear body to condensed chromatin by virtue of a direct protein-protein interaction (1). This disruption appears to proceed as a consequence of IE1's ability to either prevent the addition of or remove SUMO from PML (105). This activity is dependent upon a central hydrophobic domain of the protein, which is required both for PML association and desumoylation (105).

In contrast to both of these strategies, the small 11 kDa Ad E4 ORF3 protein promotes a dramatic rearrangement of PML-NB structure (Fig .1-4). As previously mentioned, the Ad E4 ORF3 protein is both necessary and sufficient to promote rearrangement of PML bodies from puncate structures into elongated nuclear tracks, which ultimately surround viral replication domains (23, 39). Recent work has demonstrated that the E4 ORF3 protein interacts with a specific PML isoform, PML II (85); however, it appears that PML is not required for track E4 ORF3 track formation, as these structures have been observed in PML -/- mouse embryo fibroblasts (MEFs) (85). While the precise function(s) of track formation has remained elusive, its importance to Ad is underscored by its conservation among evolutionarily divergent serotypes (173). In addition to PML rearrangement, the relocalization of TIF1α, a cellular transcription factor, into tracks is similarly conserved among all serotypes investigated to date (214).

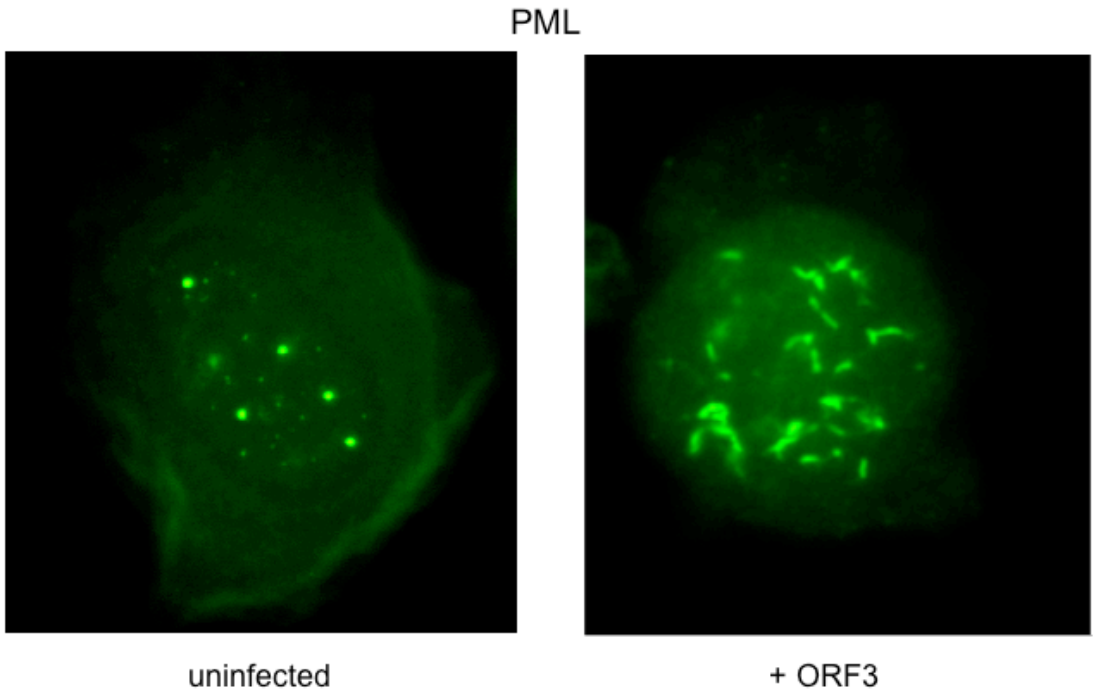


Figure 1-4. PML-NB rearrangement by the E4 ORF3 protein.
A459 cells were uninfected (left panel) or infected with a virus expressing E4 ORF3 (right panel). Cells were fixed approximately 18 h post infection and prepared for indirect immunofluorescence microscopy. Immunostaining was conducted with a mouse anti-PML antibody. The secondary antibody was conjugated to FITC. Each panel corresponds to a single nucleus.

Chapter 2

Materials and Methods

Cells, IFN, viruses, and infections

Vero cells, an African Green Monkey kidney epithelial cell line, responsive to IFNs, but deleted for the type I IFN locus (43), and HT1080 cells, an IFN-responsive human fibrosarcoma cell line (from Nancy Reich, Stony Brook University), were passaged in Dulbecco's modified Eagle Medium. IMR90 cells (ATCC), which are IFN responsive diploid primary fibroblast cells, were propagated in Dulbecco's modified Eagle medium, supplemented with 10% fetal calf serum. The cells were subsequently maintained at 37ºC in 5% CO_2.

Where indicated, cells were pretreated for 24 h with IFN (1000U/ml αIFN, Hoffman LaRoche; or 2000U/ml γIFN, R and D Systems). Following IFN pretreatment, cells were infected with different wild type or mutant viruses. Viruses dl309 (WT Ad5), dl355 (E4 ORF6-minus), and E4inORF3 (E4 ORF3-minus) were described previously (74, 87). In brief, dl309 is phenotypically wild type Ad5, dl355 is derived form dl309 and contains a 14 bp deletion in the E4 ORF6 region, and E4inORF3 is also derived from dl309 and contains an 8 bp insertion in the ORF3 region (74, 87). The Ad-CMV, E1-replacement viruses that express Ad5-HA-ORF3, Ad9-HA-ORF3, and Ad12-HA-ORF3, and mutant viruses dl355/D_{105}A,L_{106}A and dl355-N_{82}A were described previously, as was construction of the Ad-CMV Ad3-HA-ORF3 and Ad4-HA ORF3 expression constructs (46, 47).

Virus particles were purified by CsCl equilibrium centrifugation (46). Once isolated, the virions were quantified by OD_{260} measurements, diluted into a glycerol storage solution and stored at -20ºC until use. Vero cells were infected at 37ºC using 200 virus particles/cell with dl309, dl355 and E4inORF3, or at 1000 particles/cell with the

E1-replacement viruses. Briefly, growth medium was aspirated from the cells and replaced with 1ml of inoculum in the case of 100 mm plates, or 0.5 ml in the case of 24-well trays containing coverslips. After 1 h, the viral suspension was removed and the cells washed once with cold PBS. Dulbecco's modified Eagle medium supplemented with 10% bovine calf serum was replaced and IFN added, where appropriate. The cells were then incubated at 37°C in 5% CO_2.

Immunofluorescence Microscopy

To conduct immunofluorescence microscopy, Vero or HT1080 cells were seeded onto coverslips and infected as described above. At 18 h post infection, the cells were washed with phosphate buffered saline (PBS), fixed with -20°C methanol for 5 min, and then washed with PBS. Cells were blocked in PBS containing 10% goat serum for 1 h at room temperature. Antibodies were diluted in PBS containing 10% goat serum and incubated with fixed cells for 1 h at room temperature. The antibodies and dilutions used were: 1:50 anti-DBP mouse monoclonal antibody B6-8 (from Arnold Levine, Cancer Institute of New Jersey), 1:300 anti-PML rabbit polyclonal antibody H-238 (Santa Cruz Biotechnology), 1:10 anti-ORF3 rat monoclonal 6A-11 (from Thomas Dobner, Regensburg University), 1: 300 anti-HA rabbit polyclonal antibody Y-11 (Santa Cruz Biotechnology), 1:2,500 anti-DBP rabbit polyclonal antibody (from P. Van der Vleit, University Medical Centre Utrecht), 1:150 anti-HSV1 ICP0 mouse monoclonal antibody 11060 (Santa Cruz Biotechnology), 1:300 anti-Daxx rabbit polyclonal antibody M-112 (Santa Cruz Biotechnology), 1:5,000 anti-sp100 rabbit polyclonal antibody (Chemicon), and 1:600 anti-Mre11 rabbit polyclonal antibody (Novus). Subsequently, the cells were

washed with PBS and incubated with 1:300 dilutions of Alexa350-conjugated goat anti-mouse IgG (Molecular Probes), Alexa350-conjugated goat anti-rabbit IgG (Molecular Probes), fluorescin isothiocyanate-conjugated goat anti-rabbit IgG (Zymed), Texas red isothiocyanate-conjugated goat anti-rat IgG (Zymed), or Texas red isothiocyanate-conjugated goat anti-rabbit IgG (Zymed) for 45 min at room temperature in the dark. The cells were then washed with PBS and the coverslips were mounted onto slides with Immu-Mount (Shandon) supplemented with 0.12% PAVA. Microscopy was conducted using a Zeiss Axiovert 200M Digital Deconvolution Microscope, and images captured and analyzed with Axiovision 4.5 software.

Preparation of Whole Cell Extracts

Cells were washed three times with 5 ml cold PBS and scraped into an additional 5 ml cold PBS. The cells were pelleted at 2,000 X g for 10 minutes at 4^{o}C. The resulting cell pellet was lysed in 2X SDS sample buffer (4% SDS, 120 mM TRIS, pH 6.8) supplemented with the protease inhibitors phenylmethylsulfonylfluoride (PMSF), benzamidine, pepstatin, leupeptin, and aprotienin to 10 mM. In addition, phosphatase inhibitors NaF (10 mM) and sodium vanadate (1 mM) were added. Lysates were boiled 10 min and then cooled. 1 μl aliquots were removed for Bicinchoninic Acid (BCA) protein assay (Pierce) prior to the addition of bromophenol blue, to a final concentration of 0.01%, and DL-dithiothreitol (DTT), to a final concentration of 25 mM. Samples were boiled an additional 3 min and frozen at -20^{o}C, if not used immediately. BCA assays to ascertain protein concentration were conducted according to the manufacturer's instructions with at an incubation period of 30 min at 37^{o}C.

Western Blot Analysis

For Western blot analysis, cells were directly lysed in 2X SDS lysis buffer, as described above. Standardized quantities of protein were subjected to 12.5 % SDS-polyacrylamide gel electrophoresis (PAGE). Proteins were transferred to Hybond-P (Amersham). Membranes were probed with 1:10 anti-DBP mouse monoclonal antibodies A1-6 and B6-8 (from Arnold Levine) or 1:300 anti-E1A rabbit polyclonal antibody 13S-5 (Santa Cruz Biotechnology). E1A and DBP were detected using alkaline phosphatase reagent (Promega) and scanned on an ABI Storm 680 Phosphorimager (Molecular Dynamics). To visualize γ-tubulin, 1:2,000 anti-γ-tubulin rabbit polyclonal antibody (Sigma) was utilized and detected by enhanced chemiluminescent HRP substrate (Millipore).

Analysis of viral DNA replication

To determine the extent of viral DNA replication, high molecular weight DNA was prepared from Vero cells infected with 200 particles/cell of dl355 or E4inORF3. Nuclei were isolated at 18 h after infection. In brief, cells were washed three times with 5 ml cold PBS and scraped into an additional 5 ml cold PBS. The cells were pelleted at 2,000 x g for 10 minutes at 4^{o}C. The resulting pellets were resuspended in 1 ml isotonic buffer (150 mM NaCl, 10 mM Tris pH 7.4, 1.5 mM $MgCl_2$). NP40 was added to a final concentration of 0.6% and the samples were subsequently vortexed for 10 seconds and incubated 10 min on ice. The samples were subjected to a 5 minute 3,000 rpm spin at 4^{o}C. The supernatant was removed and the nuclei stored a minimum of 18 h at -80^{o}C.

Once thawed, the nuclei were vortexed at low speed and resuspended in 200 μl 10 mM Tris pH 8. Following the addition of 200 μl solution 1 (for 10 ml: 800 μl dH_20, 4 ml 1 M Tris pH 8, 4 ml 0.25 M EDTA, 1 ml 10% SDS, 200 μl 10 mg/ml proteinase K), the samples were incubated overnight at 60°C. Samples were subsequently subjected to phenol/chloroform extraction. The total DNA was diluted 1:50, denatured in 0.3 N NaOH for 1 hour at 65° C, then neutralized in 2 M ammonium acetate. Samples were applied to Hybond N^+ (Amersham) with a slot blot apparatus. The DNAs were crosslinked to the membrane using a Stratalinker (Stratagene), then probed overnight by Southern hybridization at 68°C. An Ad5 whole genome probe labeled with ^{32}P-dATP by random priming (57) was utilized to visualize the extent of vDNA replication (46). The results were visualized on an ABI Storm 680 Phosphorimager and quantified using Image Quant software (Molecular Dynamics).

Expression vectors, plasmid transfection, and shRNA expressing constructs

All transfections using protein and shRNA expression vectors were performed upon subconfluent monolayers of cells using Lipofectamine 2000 transfection reagent (Invitrogen), according to the manufacturer's protocol.

1.6 μg/ml of the pICP0 construct (from Sandra Weller, University of Connecticut Health Center) (153) or pCGN-IE1 construct (from Thomas Shenk, Princeton University) (134) was used in the transfection assays. These studies were performed in Vero cells.

All shRNA sequences were cloned (Patrick Hearing, Stony Brook University) into a 64 bp shRNA sequence as per the manufacturer's instructions (OligoEngine). A pSuper vector (from Dafna Bar-Sagi, New York University), consisting of sequences

from pSuper (OligoEngine) and pCDNA (Invitrogen) was used for shRNA expression. Xu et al. (211) described PML- 2 (5'-AAGCACGAAGACAGACCTCTGG-3') and PML-3 (5'-AACGACAGCCCAGAAGAGGAA-3') shRNA targets previously. Everett et al. (55) previously published the PML-4 target sequence (5'-CAATACAACGACAGCCCAGAA-3'). Likewise, Michaelson and Leder (126) published the Daxx shRNA target sequence (5'-GGGAGGGTGTGTGGAGAGGAA-3'). Sp100-1 (5'-CTGTGAAACAGAACAGATA-3') and Sp100-2 (5'-GCTGCTCTATGACATTGTA-3') sequences were obtained from Open Biosystems.

1.6 μg/ml of the shRNA expressing constructs were utilized to transfect HT1080 cells. 24 h post-transfection, cells expressing the shRNAs were selected with 10 μg/ml blasticidin. Blasticidin was dissolved in dH_2O to a final concentration of 10 mg/ml, and stored at -80^{o}C. Selection conditions were maintained for 48 h, in the case of PML and Daxx shRNA, or 72 h, in the case of Sp100 shRNA. These cells were stimulated with IFN, as described above, in conjunction with blasticidin selection, in the case of PML and Daxx knockdown. In the instance of Sp100 knockdown, IFN stimulation was conducted 24 h post blasticidin application. 24 h post-stimulation, cells were infected, as described above, with either dl309 or E4inORF3. After an additional 24 h had elapsed, cells were prepared for immunofluorescence microscopy, also described above.

Reverse Transcription and Quantitative PCR analysis

Twenty four hours post infection by either dl309 or E4inORF3, cells were washed three times with 5 ml cold PBS. Cells were then scraped into 5 ml cold PBS and pelleted

at 2,000 x g at 4°C for 10 min. Subsequently, total RNA was isolated using an RNeasy kit and Qiashredder (Qiagen) according to the manufacturer's protocol.

5μg RNA was reverse transcribed by 1 μl (200 units) SuperScript II (Invitrogen) for 1 h at 37°C. The reaction was primed by 0.5 μg/ml oligo-$(dT)_{12-18}$ with the first strand synthesis kit (Invitrogen), following the manufacturer's instructions. In addition, 1 μl (40 units) RNaseOut (Invitrogen) was added to the reaction. Enzymes were inactivated by heating the samples to 72°C for 5 min and cooled prior to using as template for Quantitative-polymerase chain reaction (Q-PCR).

Q- PCR primers were designed using Primer3 software to contain roughly 50% GC content (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) and to amplify specific viral genes as follows: E1A forward (5'-TCCGGTCCTTCTAACACA-3'); E1A reverse (5'-GGCGTTTACAGCTCAAGT-3'); E1B forward (5'-GGCTTGTGAGGCTACAGAGG-3'); E1B reverse (5'-TGCGCCTTTTACTGCTGCTG-3'); E2B forward (5'-CGCGCGTCGAAGTAGTCTAT-3'); E2B reverse (5'-CGGTGGAAGATGCTACCCTA-3'); DBP forward (5'-CCGTAGTGGCATCAAAAGCT-3'); DBP reverse (5'-GTCTCGCAAGGCCAAGAT-3'); E4 ORF6 forward (5'-TCCCGCGTTAGAACCATATC-3'); E4 ORF6 reverse (5'-CCATTTGGCATGACACTACG-3'); GAPDH forward (5'-GTCAGTGGTGGACCTGACCT-3'); GAPDH reverse (5'-TGACCAAGTGGTCGTTGAGG-3').

Q-PCR analysis was performed on a LightCycler (Roche) utilizing the cDNA templates obtained and above mentioned primer pairs. The reaction mixture consisted of

FastStart DNA Master SYBR Green 1 (Roche), 25 mM $MgCl_2$, and 10 μM of each primer. In brief, the PCR program consisted of 95° C for 5 minutes, proceeded by 38 amplification cycles of 95° C for 5 seconds, 58°-60° C, depending upon the primer set, for 5 seconds, followed by 72° C for 10 seconds. The identities of products obtained were confirmed by melting curve analysis. Results were normalized to GAPDH levels for each sample. Here, the calculated concentration of each viral cDNA was divided by the calculated concentration of GAPDH cDNA in that particular sample. The final results represent the average of three independent experiments.

Chapter 3

Results

E4 ORF3 is necessary for efficient viral DNA replication following IFN treatment.

Vero cells were utilized in these analyses as they are efficiently infected with adenovirus, respond well to IFN treatment, and lack the endogenous type I IFN locus, thus preventing autocrine activation (43, 199). Cells were pretreated with either IFN-α or IFN-γ for 24 h, or left untreated. Subsequently, cells were infected with dl309 (wild type Ad5), dl355 (E4 ORF6-minus), or E4inORF3 (E4 ORF3-minus). After 18 h, the cells were fixed and immunostained with an antibody against the viral DNA binding protein (DBP; Fig. 3-1). The presence of circular, DBP-positive viral DNA replication domains is indicative of active viral DNA replication (193) and was apparent in untreated cells infected with all three viruses (panels A–C), signifying the replication competence of the viruses in Vero cells under standard culture conditions. dl309 and dl355 were capable of significant viral DNA replication following infection of cells treated with either IFN-α or IFN-γ (panels D, E, G and H). In contrast, only diffuse nuclear DBP staining was evident in cells infected with mutant E4inORF3 and treated with either IFN-α or IFN-γ (panels F, I), indicative of a replication defect (193).

To confirm this observation biochemically, a viral DNA replication assay was performed to quantify the levels of replication. Cells were pretreated with either IFN-α or IFN-γ for 24 h, or left untreated, and infected with dl355 or E4inORF3. Total nuclear DNA was isolated 18 h after infection, slot-blotted, and probed by Southern blot analysis. Viral DNA at the 18 h time point corresponds to newly replicated DNA. These results recapitulated the immunofluorescence data and demonstrated

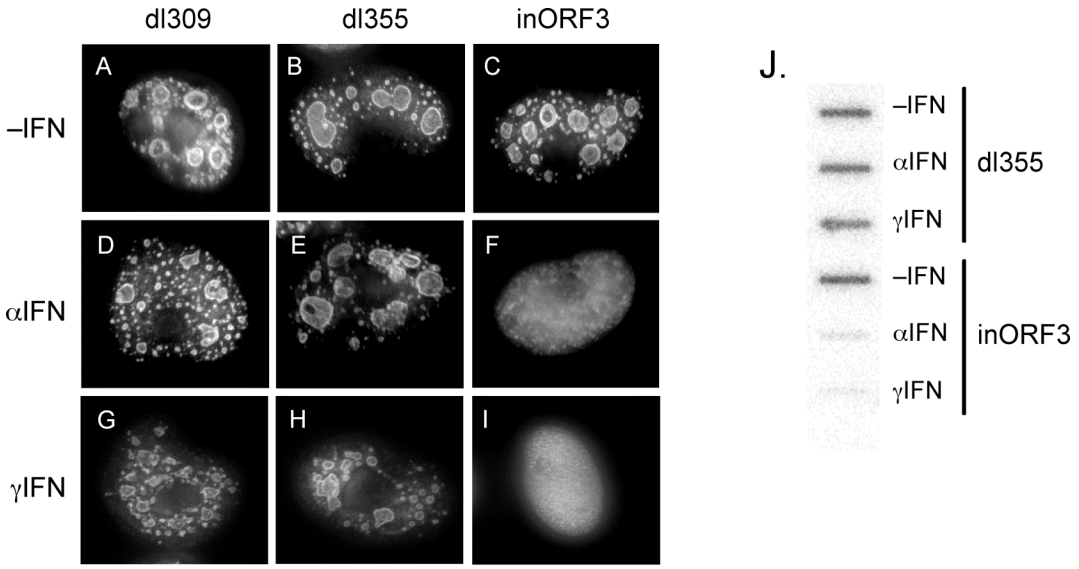


Figure 3-1. E4 ORF3 is required for efficient Ad DNA replication following IFN treatment. Vero cells were grown on coverslips and were untreated (–IFN, A–C), or pretreated with αIFN (D–F) or γIFN (G–I) for 24 h. Subsequently, the cells were infected with dl309 ($ORF3^+/ORF6^+$) (A, D, G), dl355 ($ORF3^+/ORF6^-$) (B, D, H), or E4inORF3 ($ORF3^-/ORF6^+$) (C, E, I). At 18 h after infection, cells were immunostained for Ad DBP. The images represent compressed, deconvolved Z-stacks. **J**. Vero cells were untreated (–IFN), or pretreated with αIFN or γIFN for 24 h. Subsequently, the cells were infected with dl355 ($ORF3^+/ORF6^-$) or E4inORF3 ($ORF3^-/ORF6^+$). Total nuclear DNA was isolated at 18 h after infection, slot-blotted, and probed by Southern hybridization.

that mutant E4inORF3 exhibited a marked reduction in viral DNA replication in cells treated with either IFN-α or IFN-γ (Fig. 3-1 panel J). Mutant E4inORF3 replication was reduced 15- to 20-fold in the presence of IFN. In contrast, dl355 replicated efficiently either in the presence or absence of IFN treatment. These results demonstrate that E4 ORF3 is necessary for Ad DNA replication during an IFN response and contributes a unique function to the process, which is not provided by other Ad5 proteins, including E4 ORF6.

To investigate more thoroughly the functional significance of E4 ORF3 during the IFN-induced antiviral state, infection of IMR90 cells, a human primary diploid fibroblast cell line, was conducted. Here, cells were pretreated with either IFN-α or IFN-γ for 24 h and subsequently infected with dl309, dl355, or E4inORF3, or were mock infected (Fig. 3-2). They were then fixed and immunostained with antibodies against either PML or DBP 72 h post-infection. Here, PML nuclear bodies became more numerous in both IFN-α and IFN-γ-stimulated cells, signifying that IMR90 cells are responsive to IFN (72, 103, 168) (panels A, E, I). Upon infection under standard culture conditions, DBP staining revealed the presence of viral replication domains in those cells infected with dl309, dl355, and E4inORF3 alike (panels B-D). Just as observed in Vero cells, both dl309 and dl355 were capable of replicating in cells that had been subjected to either IFN-α or IFN-γ pretreatment (panels F, G, J, K). In contrast, E4inORF3 was incapable of replicating in cells stimulated with either IFN-α or IFN-γ, as indicated by diffuse DBP staining (panels H, L). This data further strengthens the assertion that E4 ORF3 is necessary for Ad vDNA replication during

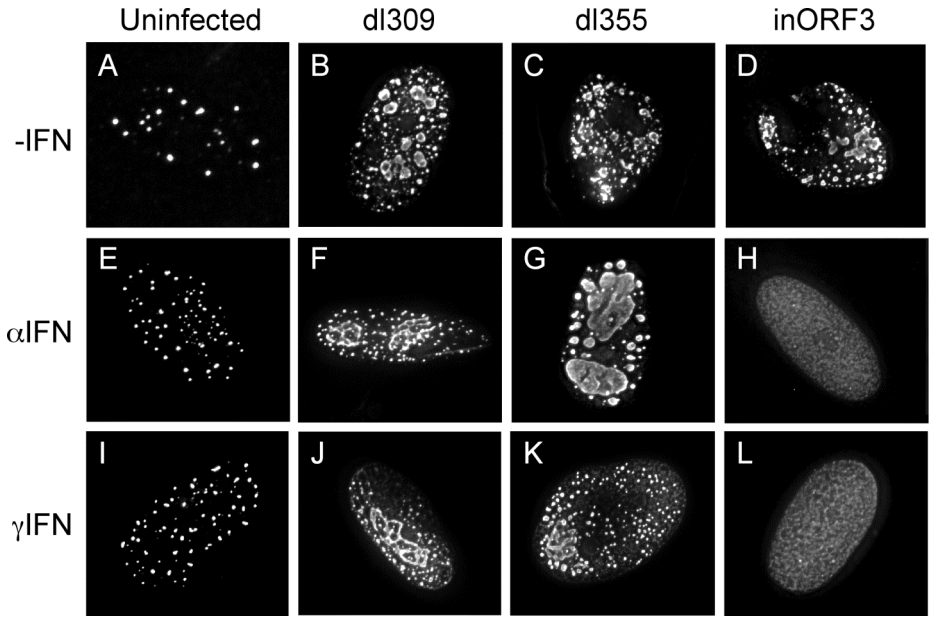


Figure 3-2. E4 ORF3 is required for efficient Ad DNA replication following IFN treatment in human primary cells. IMR90 cells were grown on coverslips and were untreated (–IFN, A–D), or pretreated with αIFN (E–H) or γIFN (I–L) for 24 h. Subsequently, the cells were infected with dl309 ($ORF3^+/ORF6^+$) (B, F, J), dl355 ($ORF3^+/ORF6^-$) (C, G, K), or E4inORF3 ($ORF3^-/ORF6^+$) (D, H, L). At 72 h after infection, cells were immunostained for either PML (A, E, I) or Ad DBP (B-D, F-H, J-L). The images represent compressed, deconvolved Z-stacks.

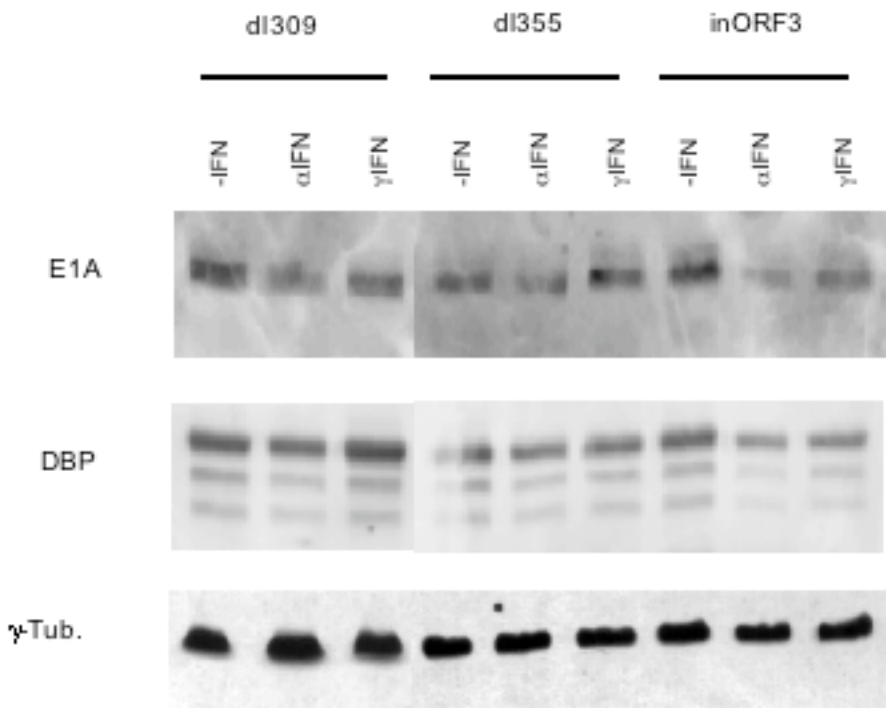


Figure 3-3. IFN treatment does not significantly reduce the expression of Ad early proteins. Vero cells were untreated (–IFN), or pretreated with αIFN or γIFN for 24 h. Subsequently, the cells were infected with dl309 ($ORF3^+/ORF6^+$), dl355 ($ORF3^+/ORF6$), or E4inORF3 ($ORF3^-/ORF6^+$). Total cell lysates were prepared 18 h after infection and analyzed by Western blot using antibodies against E1A, DBP, or γ-tubulin.

the IFN-induced antiviral state and demonstrates its functional significance in human cells.

To determine if this replication defect was fully attributable to the absence of E4 ORF3 and not a consequence of reduced synthesis of other early viral proteins essential to viral DNA replication, expression of early viral genes was examined. Vero cells were pretreated with IFN-α, IFN-γ, or left untreated, and infected with dl309, dl355, or E4inORF3. Cell lysates were prepared, and E1A and DBP protein levels were measured by Western blot analysis (Fig. 3-3). All three viruses, with or without IFN treatment, synthesized comparable levels of these viral proteins. Therefore, the replication defect observed with mutant E4inORF3 following IFN treatment does not occur as a consequence of appreciably reduced synthesis of these early viral proteins.

E4 ORF3 rearranges PML nuclear bodies into tracks following IFN treatment.

PML is an IFN-stimulated gene and both the size and number of PML-NBs are augmented by IFN treatment (72, 103, 168). As such, this nuclear subdomain has been implicated in cellular antiviral activities (149). For this reason, the ability of the E4 ORF3 protein to rearrange PML-NBs into track-like structures in the cellular environment of increased PML synthesis associated with IFN stimulation was examined. Vero cells were pretreated with IFN-α or IFN-γ for 24 h, or left untreated. Subsequently, the cells were infected with either dl309, dl355, E4inORF3, or mock infected. The cells were then fixed and immunostained with antibodies against PML

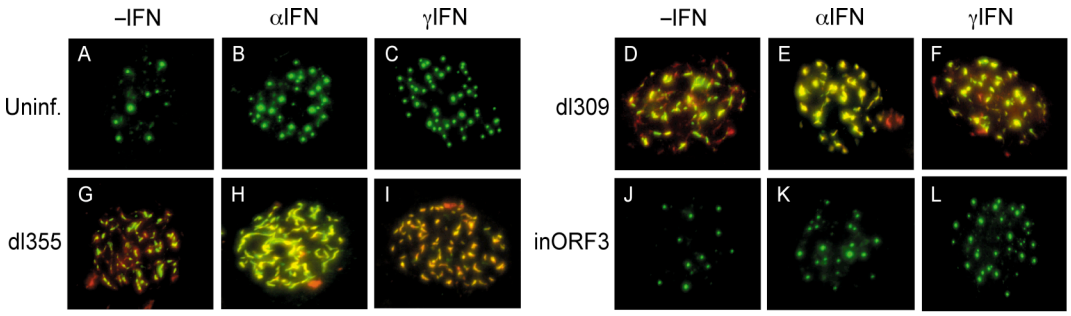


Figure 3-4. E4 ORF3 rearranges PML during an IFN response. Vero cells seeded onto coverslips and were untreated (A, D, G, J), or pretreated with αIFN (B, E, H, K) or γIFN (C, F, I, L) for 24 h. Subsequently, the cells were left uninfected (A–C) or infected with dl309 (ORF3^{+}/ORF6^{+}, D–F), dl355 (ORF3^{+}/ORF6^{-}, G–I), or E4inORF3 (ORF3^{-}/ORF6^{+}, J–L). At 18 h after infection, the cells were immunostained for PML (mAb PG-M3, FITC-coupled secondary antibody) and ORF3 (mAb 6A11, TRITC-coupled secondary antibody). The images represent a merge of compressed, deconvolved Z-stacks.

and E4 ORF3 (Fig. 3-4). As previously reported (72, 103, 168), both the size and number of PML nuclear bodies were increased as a result of treatment with either IFN-α or IFN-γ (panels A–C). Both dl309 and dl355 were fully capable of rearranging PML into characteristic track-like structures that contain E4 ORF3 following IFN treatment in a manner indistinguishable from PML body rearrangement in virus-infected, untreated cells (panels D–I). Rearrangement of IFN-induced PML was not observed when cells were infected with mutant E4inORF3 (panels J–L). Because E4 ORF3 is known to be both necessary and sufficient for PML rearrangement (23, 39) and the deletion of E4 ORF3 elicits a dramatic replication defect during an IFN response (Fig. 3-1, Fig. 3-2), these data strongly indicate that E4 ORF3-induced PML track formation during an IFN response inhibits an IFN-induced, antiviral function of the PML-NB.

Inhibition of an IFN response is a conserved function of E4 ORF3 among evolutionarily divergent Ad serotypes.

The ability of E4 ORF3 to rearrange PML nuclear bodies from punctate nuclear domains into track-like structures is a common function of E4 ORF3 of all Ad serotypes examined to date (173). This observation implies that E4 ORF3-mediated rearrangement of PML performs an essential function, however such a function has not yet been elucidated. Due to PML-NB association with innate immune responses, PML rearrangement could potentially relate to Ad replicative success during the IFN-induced antiviral state. To examine if the E4 ORF3 proteins of different Ad serotypes are capable of blocking the effect of IFN on Ad replication, Vero cells were pretreated with IFN-α, IFN-γ, or left untreated, and then coinfected with two viruses: mutant E4inORF3 and one

of several E1-replacement viruses that express E4 ORF3 proteins of different Ad serotypes. The E1-replacement viruses carry a deletion of E4 open reading frames 1–3, so they do not express E4 ORF3 from the natural E4 context. Mutant E4inORF3 expresses the E1A and E1B proteins missing in the E1-replacement viruses, and the E1-replacement viruses express E4 ORF3 proteins missing with mutant E4inORF3. Ad DBP and E4 ORF3 were visualized after infection by immunofluorescence (Fig. 3-5). The presence of circular, DBP-positive viral DNA replication domains is indicative of active viral DNA replication whereas diffuse DBP staining is indicative of a replication defect (193). As described above, mutant E4inORF3 replicated in the absence of IFN treatment, but not following treatment of cells with either IFN-α or IFN-γ (panels A, F, K). No E4 ORF3 expression was observed, as expected, due to mutation of the E4 ORF3 protein. The same results were observed in cells coinfected with E4inORF3 and an E1-replacement virus that does not express the E4 ORF3 protein (Ad-CMV; panels B, G, L). Coinfection of IFN-treated cells with mutant E4inORF3 and E1-replacement viruses that express E4 ORF3 proteins of Ad5 (panels C, H, M), Ad9 (panels D, I, N), or Ad12 (panels E, J, O) resulted in the restoration of replication competence following IFN pretreatment to levels comparable to that of mutant E4inORF3 without IFN treatment. Similarly, E4inORF3 infection in conjunction with transfection of constructs expressing Ad3 (Fig. 3-6 panels E, H) or A4 HA-E4 ORF3 (panels F, I) yielded comparable results. E4 ORF3 was evident in track-like structures with all

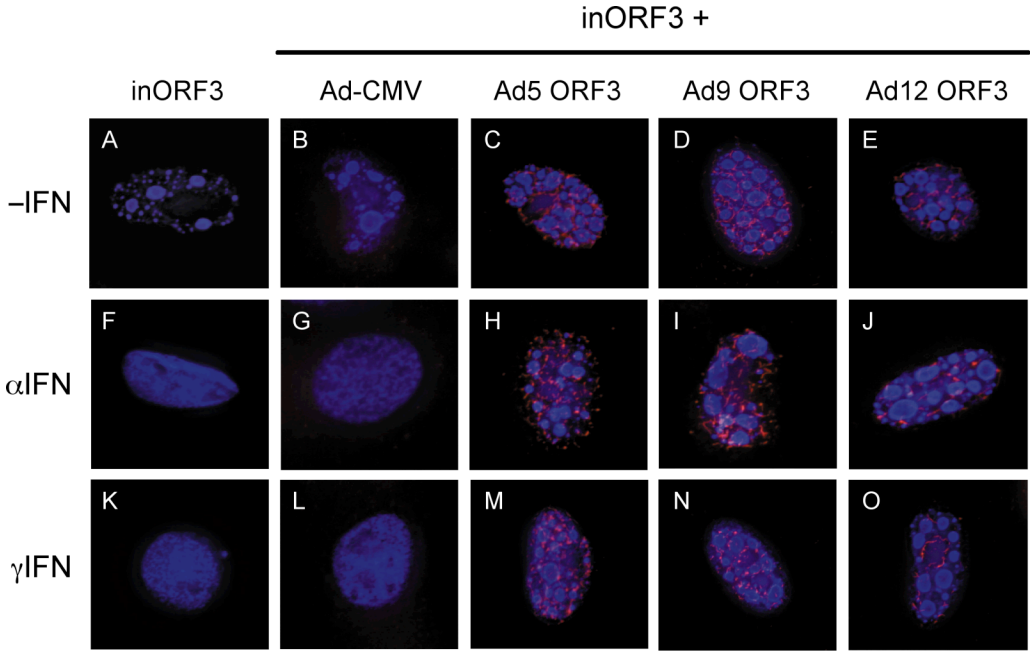


Figure 3-5. E4 ORF3 proteins of different Ad serotypes block the effect of IFN on Ad replication. Vero cells were seeded onto coverslips and were untreated (A–E), or pretreated with αIFN (F–J) or γIFN (K–O) for 24 h. Subsequently, the cells were infected with mutant E4inORF3 alone (A, F, K), or coinfected with E4inORF3 and an E1-replacement virus that does not express any E4 ORF3 protein (Ad-CMV; B, G, L) or E1-replacement viruses that express E4 ORF3 proteins of Ad5 (C, H, M), Ad9 (D, I, N), and Ad12 (E, J, O). At 18 h after infection, cells were immunostained for DBP (mAb B6-8, Alexa350-coupled secondary antibody) and HA (rabbit antibody Y-11, TRITC-coupled secondary antibody). The images represent a merge of compressed, deconvolved Z-stacks.

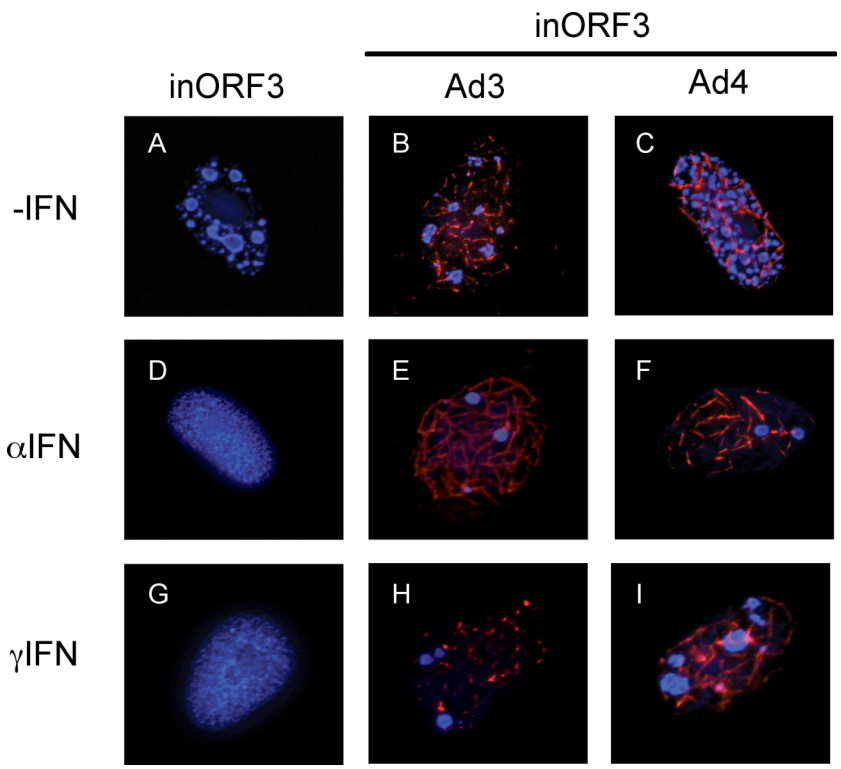


Figure 3-6. E4 ORF3 proteins of different Ad serotypes inhibit the effect of IFN on Ad replication. Vero cells were seeded onto coverslips and were untreated, or pretreated with IFN–α (D–F) or IFN-γ (G–I) for 24 h. Subsequently, the cells were transfected with constructs expressing Ad3 **(**B, E, H) or A4 HA-E4 ORF3 (C,F, I), or were not transfected (A, D, G). At 18 h after infection, cells were immunostained for DBP (mAb B6-8, Alexa350-coupled secondary antibody) and HA (rabbit antibody Y-11, TRITC-coupled secondary antibody). The images represent a merge of compressed, deconvolved Z-stacks

five serotypes. Replication was not detected in any cells infected with the E1-replacement viruses alone (data not shown). The quantification of these results is shown in Fig. 3-7. In the absence of IFN treatment, Ad replication domains were observed in >50% of cells infected with mutant E4inORF3. Similar results were observed in cells coinfected with E4inORF3 and Ad-CMV. In both cases, IFN treatment reduced the number of cells showing replication domains to <10%. When cells were coinfected with mutant E4inORF3 and E1-replacement viruses expressing E4 ORF3 of different Ad serotypes without IFN treatment, the number of cells exhibiting the formation of replication domains increased to 70-80%. This result is consistent with the increase in virus growth observed when E4 ORF3 is expressed under ordinary culture conditions (18, 87). When cells were treated with either IFN-α or IFN-γ, the expression of E4 ORF3 by the E1-replacement viruses rescued the replication defect observed with mutant E4inORF3 such that ≥60% of cells expressing HA-ORF3 tracks exhibited viral DNA replication domains, in contrast to the <10% of cells infected with mutant E4inORF3 alone or E4inORF3 in conjunction with Ad-CMV. These results demonstrate that the inhibition of the IFN-induced antiviral state by the E4 ORF3 protein is a function that is conserved among Ad serotypes.

The MRN complex is not responsible for the inhibition of Ad replication during an IFN response.

The E4 ORF3 proteins of subgroup C adenoviruses are capable of rearranging the MRN complex into track-like structures, but E4 ORF3 proteins produced by

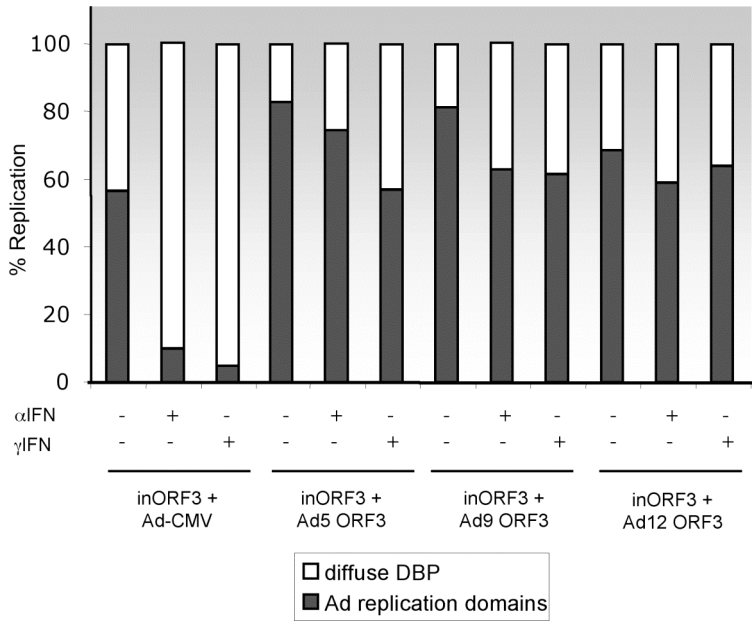


Figure 3-7. Quantification of the analysis of E4 ORF3 proteins of different Ad serotypes. The percentage of Vero cells exhibiting viral replication centers is presented in cells, with or without IFN pretreatment, infected with mutant E4 inORF3 alone or coinfected with mutant E4 inORF3 and E1-replacment viruses that express no E4 ORF3 protein (Ad-CMV) or E4 ORF3 proteins of Ad5, Ad9, and Ad12. Replication was quantified by counting cells that exhibited viral replication centers in comparison to cells that displayed diffuse nuclear DBP staining.

adenoviruses of other subgroups are not (173). This function is hypothesized to protect the linear double-stranded DNA genomes from nucleolytic cleavage by the endo- and exonuclease activities of Mre11 (201). While Mre11, Rad50, and Nbs1 do not appear to be ISGs, as assessed by protein levels in the presence or absence of IFN treatment (data not shown), the possibility remained that the enzymatic activities of MRN are enhanced due to IFN treatment. To determine if the MRN complex is involved in the IFN-induced replication defect of mutant E4inORF3, Vero cells were coinfected with mutant E4inORF3 and one of two mutant viruses carrying specific point mutants in the E4 ORF3 reading frame in the natural E4 context: dl355-$D_{105}A/L_{106}A$ or dl355-$N_{82}A$. The $D_{105}A/L_{106}A$ mutations in E4 ORF3 prevent rearrangement of the MRN complex into track-like structures, but do not affect the ability of the mutant protein to rearrange PML (47), thereby segregating the ability of E4 ORF3 to rearrange PML from its ability to rearrange the MRN complex. The $N_{82}A$ mutation in E4 ORF3 blocks both PML and MRN reorganization (172). Both E4 ORF3 mutants are in the genomic background of dl355—a virus that does not express the E4 ORF6 protein. Both of these mutant viruses are completely defective for viral growth due to the mutations in the E4 ORF3 and E4 ORF6 proteins. Thus, in these coinfection experiments, functional E4 ORF6 is provided by the virus E4inORF3 and the $D_{105}A/L_{106}A$ or $N_{82}A$ mutant E4 ORF3 proteins are provided by the viruses dl355-$D_{105}A/L_{106}A$ or dl355-$N_{82}A$.

Vero cells were pretreated with either IFN-α or IFN-γ, or left untreated, and infected individually or in combination with these viruses. Viral DNA replication domains and the E4 ORF3 proteins were visualized after infection by

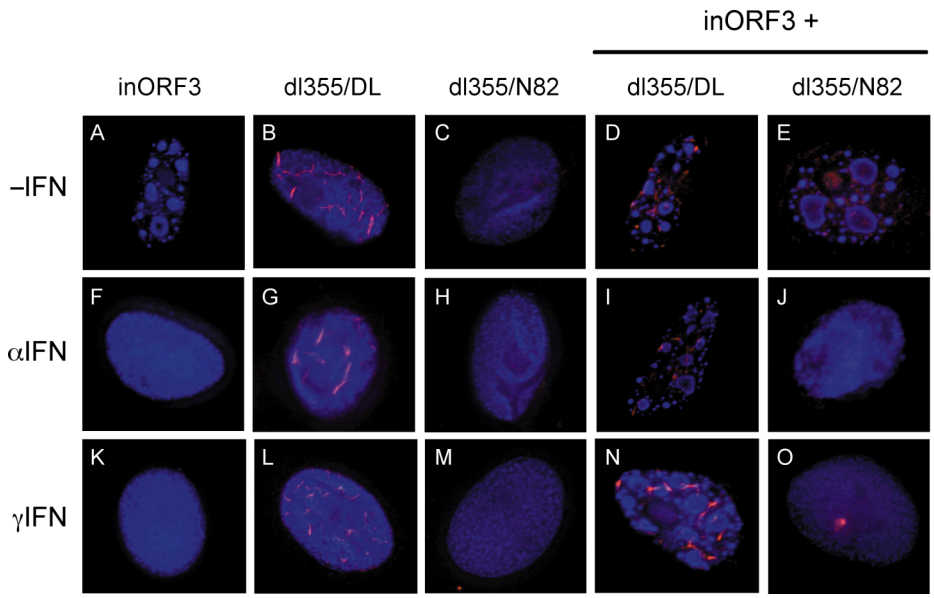


Figure 3-8. The MRN complex does not contribute the effect of IFN on Ad replication. Vero cells were seeded onto coverslips and were untreated (–IFN, A–E), or pretreated with αIFN (F–J), or γIFN (K–O). Subsequently, the cells were infected with mutant E4 inORF3 alone (A, F, K), dl355/$D_{105}L_{106}$ alone (B, G, L), dl355/N_{82} alone (C, H, M), or coinfected with E4 inORF3 and dl355/$D_{105}L_{106}$ (D, I, N) or dl355/N_{82} (E, J, O). At 18 h after infection, the cells were immunostained DBP (Alexa350-coupled secondary antibody) and HA (Texas red-coupled secondary antibody). The images represent a merge of compressed, deconvolved Z-stacks.

immunofluorescence (Fig. 3-8). Mutant E4inORF3 exhibited a significant number of replication domains in the absence of IFN treatment, as described above. In single infections, mutants dl355-$D_{105}A/L_{106}A$ and dl355-$N_{82}A$ were defective for replication due to the mutations in the E4 ORF3 and E4 ORF6 proteins (panels A–C), as previously described (172). None of these three viruses replicated in single infections when cells were pretreated with IFN-α or IFN-γ (panels F–H, K–M). In the absence of IFN treatment, efficient viral replication was evident in cells coinfected with E4inORF3 and either dl355-$D_{105}A/L_{106}A$ or dl355-$N_{82}A$, as indicated by the formation of active viral DNA replication domains (panels D and E), as observed with E4inORF3 infection alone (panel A). When cells were pretreated with either αIFN or γIFN, coinfection of cells with E4inORF3 and dl355-$D_{105}A/L_{106}A$ rescued the replication defect observed with E4inORF3 infection alone in the presence of IFNs (panels F and K. vs. I and N). In contrast, coinfection of cells with E4inORF3 and dl355-$N_{82}A$ did not rescue the replication defect when cells were pretreated with either IFN-α or IFN-γ, as indicated by diffuse DBP nuclear staining (panels J and O). Quantification of these results is presented in Fig. 3-9. Because the E4 ORF3 mutant $D_{105}A/L_{106}A$ is capable of inhibiting the IFN-induced antiviral state and is able to reorganize PML but not MRN, we conclude that the IFN-induced replication defect does not relate to inhibition of the MRN complex. This conclusion is consistent with the replication defect observed in IFN-treated cells infected with mutant E4inORF3 alone where the E1B 55K-E4 ORF6 complex is present and would be expected to inhibit MRN activity by degradation, yet IFN-treatment still ablated viral DNA replication.

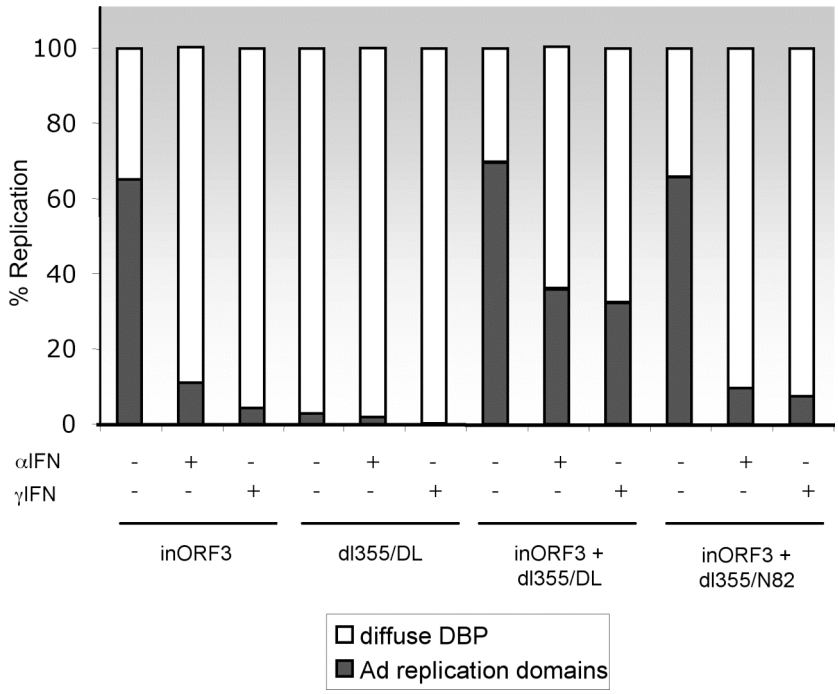


Figure 3-9. Quantification of the analysis of E4 ORF3 mutants $D_{105}L_{106}$ and N_{82}. The percentage of Vero cells exhibiting viral replication centers is presented in cells, with or without IFN pretreatment, infected with mutant E4 inORF3 alone, mutant dl355/$D_{105}L_{106}$ alone, or coinfected with mutant E4 inORF3 and dl355/$D_{105}L_{106}$ or dl355/N_{82}. Replication was quantified as described for Figure 3-7.

The E4 ORF3 protein antagonizes a PML-dependent antiviral effect.

This work has demonstrated that the E4 ORF3 protein is required for efficient Ad vDNA replication during the interferon-induced antiviral state (190). This viral protein is both necessary and sufficient to rearrange PML bodies from punctate nuclear bodies into nuclear track structures (23, 39). To test whether or not PML is implicated, either directly or indirectly, in the antiviral response antagonized by the E4 ORF3 protein, shRNAs were employed to reduce the expression of PML in HT1080 cells, a human fibrosarcoma cell line responsive to IFN. To confirm efficacy of the shRNA constructs, cells grown on coverslips were transfected with the shRNA expression vectors PML-2, PML-3, and PML-4. After 24 h, cells were placed under blasticidin selection to select for transfected cells. After an additional 24 h, the cells were fixed and immunostained with an antibody against PML, revealing the absence of PML bodies in those nuclei successfully transfected with the shRNA expression vectors (Fig. 3-11 panels A to C). Transfection of control vector alone did not affect PML-NB integrity (Fig. 3-10 panel D).

To ascertain whether or not the E4 ORF3 protein antagonizes a PML-dependent antiviral phenomenon, HT1080 cells grown on coverslips were transfected with the aforementioned PML shRNA expression vectors or empty vector alone. 24 h after transfection, the cells were placed under selection and stimulated with either IFN-α or IFN-γ. After an additional 24h, the cells were infected with either dl309, or mutant E4inORF3. After 24 h, the cells were fixed and immunostained with antibodies against PML and the viral DBP. The presence of circular, DBP-positive viral replication domains in the nuclei of infected cells is indicative of robust Ad

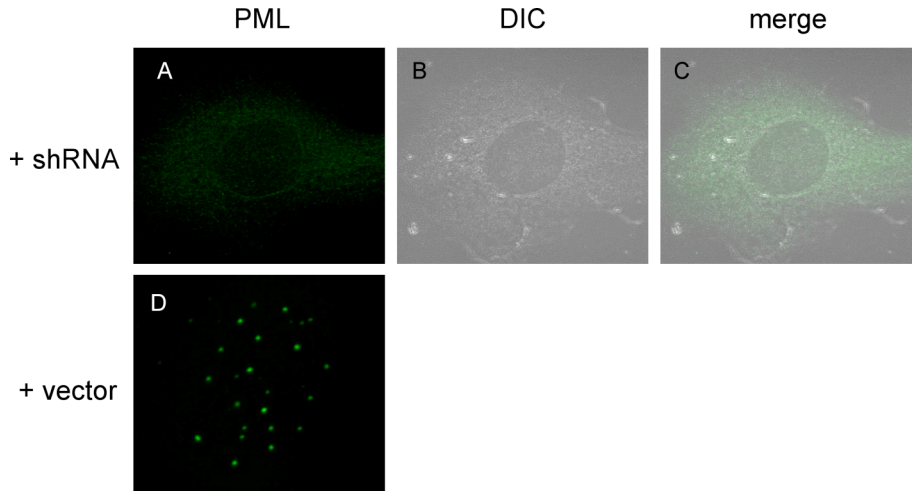


Figure 3-10. PML knockdown by shRNAs. HT1080 cells grown on coverslips were transfected with plasmids expressing shRNAs against PML (A-C) or with control vector alone (D). Subsequently, the cells were fixed and immunostained with an antibody against PML (FITC-coupled secondary antibody). The images represent deconvolved, compressed Z-stacks.

vDNA replication, whereas diffuse nucleoplasmic localization of the protein signifies replication incompetence (193). vDNA replication was evident in the case of untreated cells infected with both dl309 and mutant E4inORF3 (Fig. 3-11 panels D and G). As previously reported (190), dl309 exhibited active replication in cells pretreated with either IFN-α or IFN-γ (panels E and F).

The E4 ORF3 protein is required for active vDNA replication during the antiviral state induced by either IFN-α or IFN-γ (190). Here, when PML-NBs were disrupted using shRNAs, mutant E4inORF3 exhibited vDNA replication in cells pretreated with either IFN-α or IFN-γ (panels H and I). Infection of cells transfected with the control vector alone did not affect replication of dl309 nor did it rescue replication of E4inORF3 during the IFN response (data not shown). The percentage of cells supporting active vDNA replication was quantified in three independent experiments. Infected cells exhibiting substantial PML-NB disruption were scored for the presence of vDNA replication domains or diffuse DBP expression. This analysis revealed that PML-NB disruption effectively restored replication competence to the E4 ORF3 mutant virus during the antiviral state induced by either IFN-α or IFN-γ to an extent comparable to that observed in unstimulated cells (Fig 3-12 B). This is in contrast to the significant reduction in vDNA replication with this mutant in IFN-treated cells in the absence of PML-NB disruption (Fig 3-12 A). These results clearly implicate PML as a participant in an innate antiviral defense antagonized by E4 ORF3 protein expression.

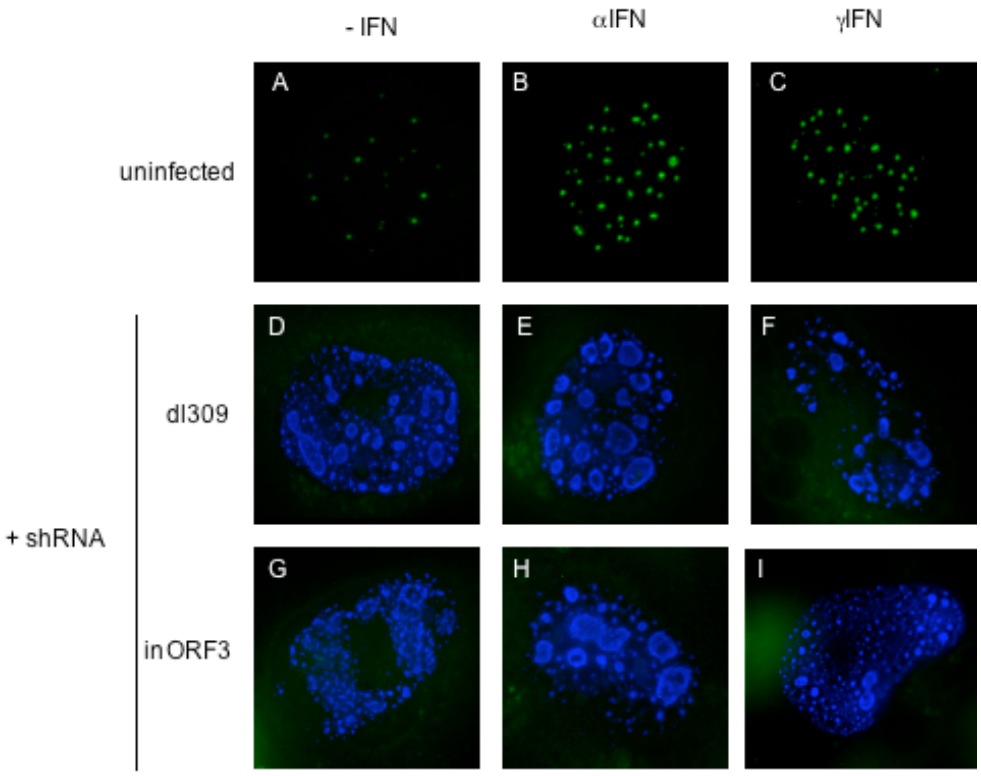


Figure 3-11. The E4 ORF3 protein antagonizes a PML-dependent antiviral effect. HT1080 cells grown on coverslips were transfected with constructs expressing PML shRNAs (D-I) and were either untreated (A, D, G), or stimulated with IFN-α (B, E, H) or IFN-γ (C, F, I). The cells were subsequently infected with dl309 (E4 ORF3^{+}/E4 ORF6^{+}; D-F) or E4inORF3 (E4 ORF3^{-}/E4 ORF6^{+}; G-I), or were not subjected to Ad infection (A-C). The cells were then fixed and immunostained for PML (FITC-coupled secondary antibody) and DBP (Alexa350-coupled secondary antibody). The final images represent deconvolved, compressed Z-stacks.

A

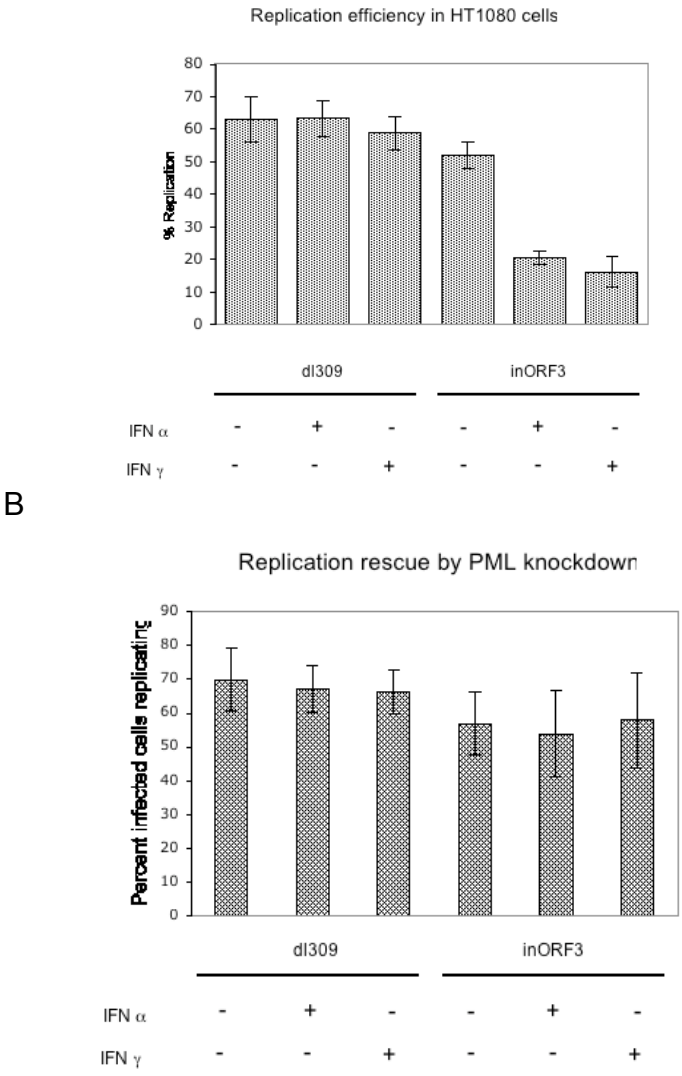


Figure 3-12 A. Quantification of vDNA replication in HT1080 cells. The percentage of cells exhibiting viral replication centers is presented in cells, with or without IFN pretreatment, infected with dl309 or mutant E4inORF3. Replication was quantified by counting cells that exhibited viral replication centers in comparison to cells that displayed diffuse nuclear DBP staining. **B. Quantification of E4 ORF3 replication following PML knockdown.** The percentage of HT1080 cells exhibiting viral replication domains is presented in cells both infected with dl309 or E4inORF4 and demonstrating PML knockdown, with or without IFN pretreatment. Replication was quantified by counting cells that exhibited viral replication centers in comparison to cells that displayed diffuse nuclear DBP staining.

The disruption of PML-NBs by HSV-1 ICP0 and HCMV IE1 is functionally equivalent to disruption by E4 ORF3 during the IFN-induced antiviral state.

The adenovirus E4 ORF3 protein is not unique in its ability to disrupt PML bodies (48, 55, 119). Among several virally encoded gene products, the HCMV IE1 protein prompts relocalization of PML from the PML-NB to condensed chromatin (105, 129), while the HSV-1 ICP0 protein is an E3 ubiquitin ligase that promotes proteosome-dependent degradation of PML, and consequently PML-NB disruption (16, 49, 50). While these proteins execute mechanisms distinct from that of the Ad E4 ORF3 protein, all three viral proteins accomplish the same objective: to disrupt PML-NBs and to alter the subnuclear localization of a wide variety of proteins known to associate with these structures. For this reason, whether or not the HSV-1 ICP0 and HCMV IE1 proteins were capable of substituting for the E4 ORF3 protein to promote Ad vDNA replication during the IFN-induced antiviral state was investigated.

Vero cells grown on coverslips were transfected with either pICP0 or pIE1 expression vectors. 6 h post-transfection, the cells were stimulated with either IFN-α or IFN-γ. After an additional 24 h, the cells remained uninfected or were subjected to infection by either dl309 or E4inORF3. The ICP0 protein assumed a punctate staining pattern within the nuclei of cells (Fig. 3-13 panel A), as previously described (51, 120, 121). The ICP0 has been established to associate with PML bodies and promote proteosome-dependent degradation of PML, thereby causing dispersal of PML body-associated proteins throughout the nucleoplasm (16, 49, 50). When ICP0-expressing cells were infected with dl309, the virus demonstrated replication competence under both uninduced and IFN-induced conditions (panels B, C, D). Wild type Ad5 is known to

replicate under both IFN stimulated or unstimulated conditions (190). Of interest, ICP0 was relocalized from punctate dots into E4 ORF3-induced nuclear tracks following infection with dl309 (panel B).

When quantified, 70% of dl309-infected cells were observed to replicate vDNA, irrespective of IFN treatment (Fig. 3-14 panel A). In the absence of IFN treatment, roughly 60% of cells infected with mutant E4inORF3 supported vDNA replication (panel A). When cells were stimulated with IFN-α and subsequently subjected to infection by E4inORF3, 31% of infected cells exhibited robust vDNA replication, as indicated by multiple viral replication domains. Ninety one percent of these cells were found to express the ICP0 protein to a level detectable by immunofluorescence microscopy (panel B). Of the cells infected with E4inORF3 that did not support vDNA replication and exhibited diffuse DBP staining, 82% did not express the ICP0 protein (panel B). Similar to what was observed in the case of cells induced with IFN-α, those cells subjected to IFN-γ treatment prior to infection by mutant E4inORF3 supported vDNA replication in 25% of infected cells. Of this population, 89% of cells demonstrating vDNA replication expressed the ICP0 protein to a level detectable by immunofluorescence microscopy (panel B). Those cells incapable of permitting vDNA replication accounted for 75% of the population infected with E4inORF3. Of this group, 95% of the cells did not express the ICP0 protein. These data strongly suggest that the HSV-1 ICP0 protein is capable of

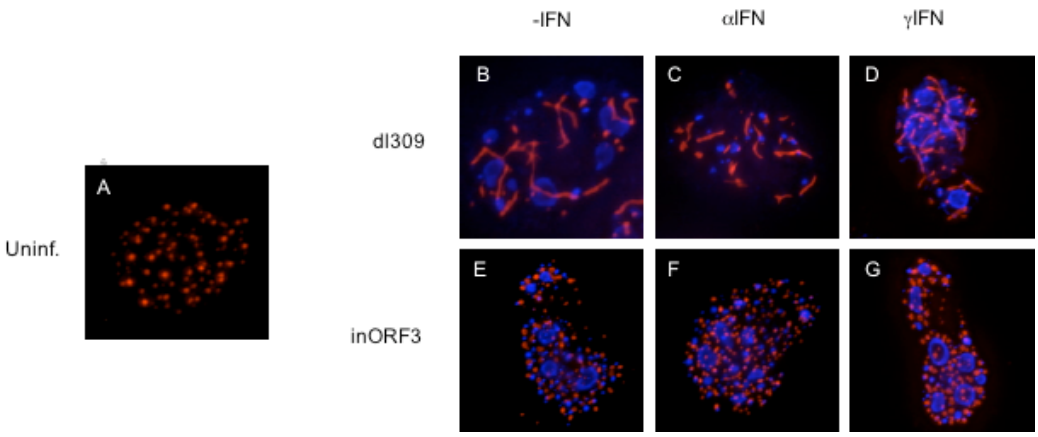
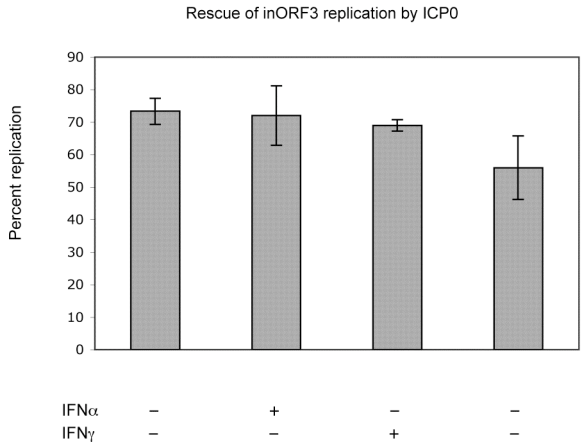


Figure 3-13. HSV1 ICPO supports replication of E4inORF3 during the IFN induced antiviral state. Vero cells were transfected with a construct expressing HSV1 ICP0 (A-G) and remained untreated (A, B, E) or were stimulated with either IFN-α (C, F) or IFN-γ (D, G). Cells were then infected with dl309 (B-D), E4inORF3 (E-G), or remained uninfected (A). Cells were subsequently fixed and immunostained with antibodies against ICP0 (TRITC-coupled secondary antibody) and DBP (Alexa350-coupled secondary antibody). The images represent deconvolved compressed Z-stacks.

A



B

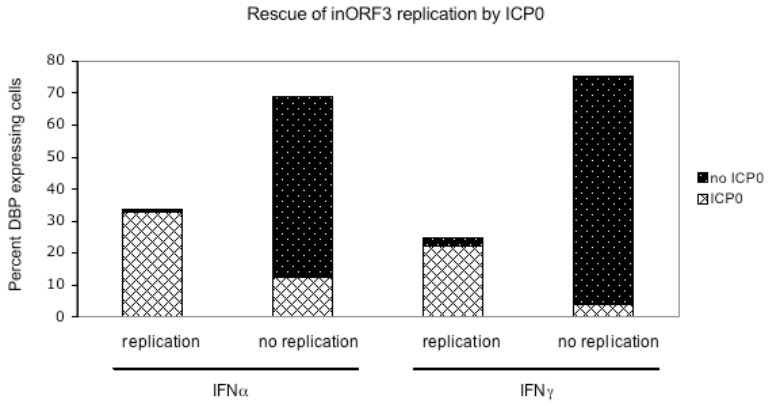


Figure 3-14 A. Expression of ICP0 does not hinder vDNA replication. The percentage of Vero cells exhibiting viral replication domains is presented in cells both transfected with a construct expressing HSV1 ICP0 and infected with dl309 in the presence or absence of IFN stimulation, as well as in untreated cells infected with E4inORF3. Replication was quantified by counting cells that exhibited viral replication centers in comparison to cells that displayed diffuse nuclear DBP staining. **B. HSV1 ICP0 supports replication of E4inORF3 during the IFN-induced antiviral state.** 100 Vero cells infected by E4inORF3 were counted and scored based upon the expression of viral replication domains or diffuse DBP expression. Of those cells exhibiting replication domains, cells were scored for the presence or absence of ICP0 expression. Of the population exhibiting diffuse DBP immunostaining, cells were also scored for the presence of ICP0 expression. The proportion of ICP0-expressing to non-expressing cells within each population was then compared. The data represents the average of three independent experiments.

complementing for the absence of the E4 ORF3 protein to promote Ad vDNA replication during the antiviral states induced by IFN-α and IFN-γ.

Upon its expression, the HCMV IE1 protein assumes diffuse nucleoplamic localization (Fig. 3-15 panel A). When IE1-expressing cells were infected with wild type Ad5, vDNA replication domains were observed regardless of whether or not the cells had been pretreated with either IFN-α or IFN-γ (panels B, C, D). As anticipated, mutant E4inORF3 was incapable of replicating during the antiviral state induced by either IFN-α or IFN-γ (data not shown). In contrast, when cells expressing the IE1 protein were infected with E4inORF3, vDNA replication domains were observed under both IFN-uninduced as well as induced conditions (panel E, F, G). When quantified, 75% of dl309-infected cells exhibited vDNA replication under both IFN stimulated and unstimulated conditions (Fig. 3-16 panel A). Similarly, roughly 60% of unstimulated cells infected with mutant E4inORF3 supported robust vDNA replication (panel A). When subjected to IFN-α pretreatment, 33% of E4inORF3-infected cells demonstrated vDNA replication domains. Of this population, 87% expressed the HCMV IE1 protein. Eighty nine percent of the infected cells that were deficient for inORF3 replication did not show detectable IE1 expression (panel B). Consistent with these observations, in cells pretreated with IFN-γ, 24% of infected nuclei displayed vDNA replication domains and 87% of these cells expressed the IE1 protein (panel B). Those cells incapable of permitting vDNA replication accounted for 76% of the population infected with E4inORF3. Of this group, 85% of the cells did not express the IE1 protein.

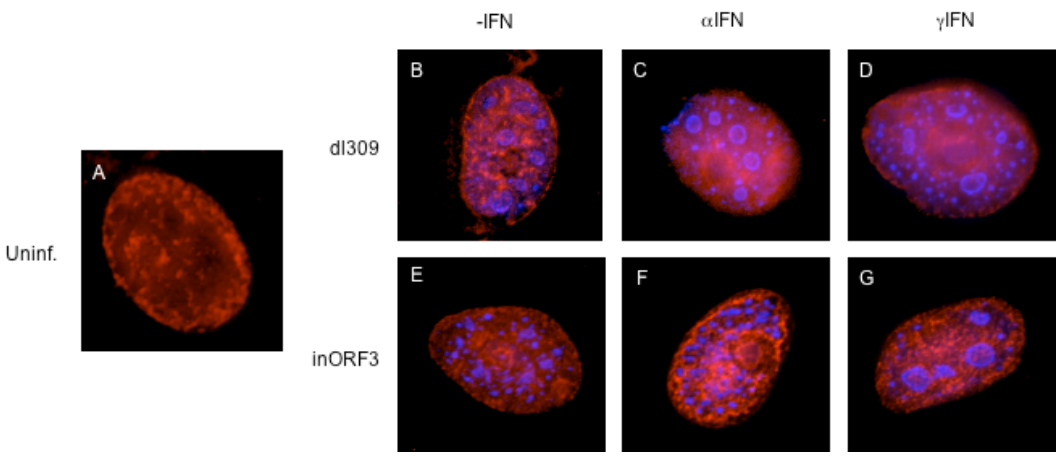
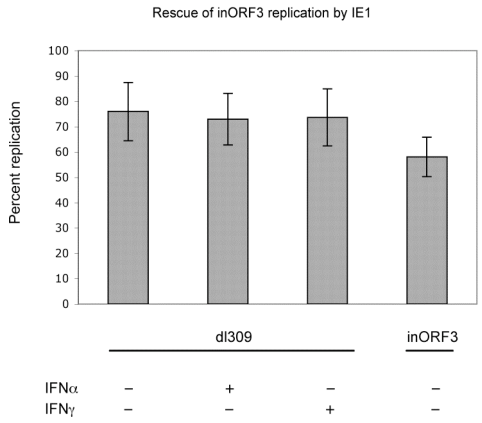


Figure 3-15. HCMV IE1 supports replication of E4inORF3 during the IFN-induced antiviral state. Vero cells were transfected with a construct expressing HCMV HA-tagged IE1 (A-G) and remained untreated (A, B, E) or were stimulated with either IFN-α (C, F) or IFN-γ (D, G). Cells were then infected with dl309 (B-D), E4inORF3 (E-G), or remained uninfected (A). Cells were subsequently fixed and immunostained with an anti-HA antibody (TRITC-coupled secondary antibody) as well as an antibody against DBP (Alexa350 –coupled secondary antibody). The images represent deconvolved compressed Z-stacks.

A



B

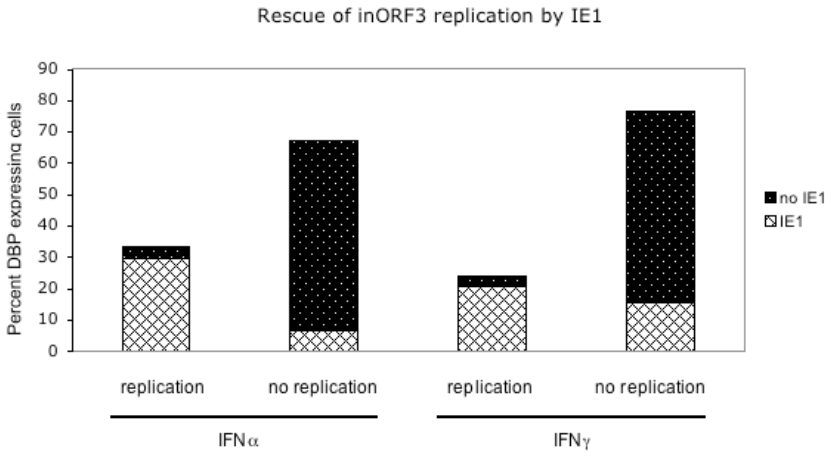


Figure 3-16 A. Expression of IE1 does not hinder vDNA replication. The percentage of Vero cells exhibiting viral replication domains is presented in cells both expressing HA-tagged HCMV IE1 and infected with dl309 in the presence or absence of IFN stimulation. In addition, the percentage of cells both expressing HA-IE1 and infected with E4inORF3 in the absence of IFN treatment were scored. Replication was quantified by counting cells that exhibited viral replication centers in comparison to cells that displayed diffuse nuclear DBP staining. **B. HCMV IE1 supports replication of E4inORF3 during the IFN-induced antiviral state.** 100 Vero cells infected by E4inORF3 were counted and scored based upon the expression of viral replication domains or diffuse DBP expression. Of those cells exhibiting replication domains, cells were scored for the presence or absence of IE1 expression. Of the population exhibiting diffuse DBP immunostaining, cells were also scored for the presence of IE1 expression. The proportion of IE1-expressing to non- expressing cells within each population was then compared. The data represents the average of three independent experiments.

Taken together, these data assert that the Ad E4 ORF3, HSV-1 ICP0, and HCMV IE1 proteins are capable of promoting efficient Ad vDNA replication during the antiviral state induced by either IFN-α or IFN-γ. While these viral proteins employ distinct mechanisms to promote PML-NB disruption, the data suggest functional conservation in their ability to alter PML-NB-associated antiviral activity.

PML body disruption by the E4 ORF3 protein antagonizes the antiviral properties of Daxx.

Although the above data implicate PML in the antiviral response induced by IFNs, it remained unclear whether or not PML functioned as the direct antiviral effector or if PML indirectly functions by mediating the function of another protein. A variety of proteins localize to PML-NBs via protein-protein interactions (9, 133). For this reason, the potential involvement of additional PML-NB-associated proteins in this cellular antiviral strategy was investigated. Among the proteins known to regularly reside within PML-NBs is Daxx, a protein involved in both apoptosis and transcriptional repression (83, 109, 110). Daxx expression is induced by type I IFN and confers intrinsic antiviral properties against HCMV (21, 145, 155, 207). Intriguingly, Daxx degradation is required for transcription from the HCMV major immediate early promoter (MIEP) and in the absence of this proteolysis, HCMV DNA replication is inhibited (155, 181). For these reasons, the participation of Daxx in the antiviral response antagonized by the E4 ORF3 protein was examined.

To address this question, Daxx expression was reduced in HT1080 cells by shRNA expression using the approach described above for PML.

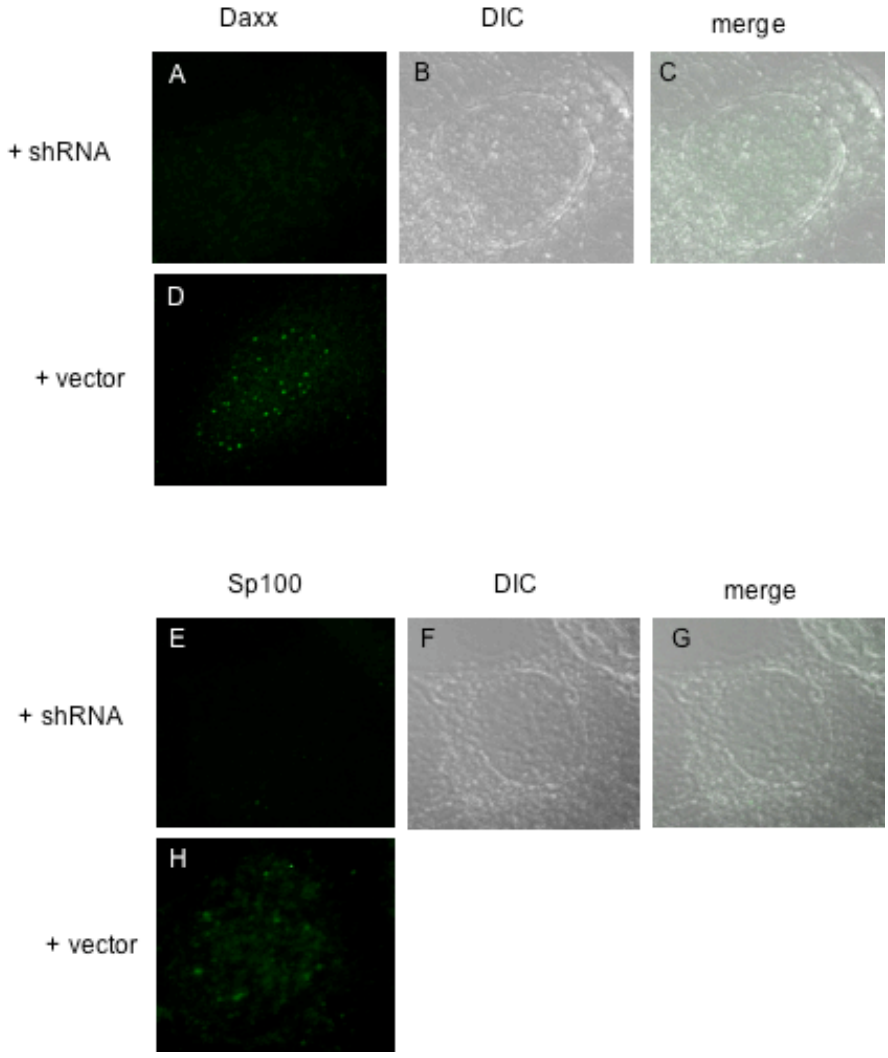


Figure 3-17. Daxx and Sp100 knockdown by shRNAs. HT1080 cells grown on coverslips were transfected with plasmids encoding shRNAs against Daxx (A-C), sp100 (E-G), or with control vector alone (D, H). Subsequently, the cells were fixed and immunostained with an antibody against Daxx (A-D; FITC-coupled secondary antibody) or Sp100 (E-H; FITC-coupled secondary antibody). The images represent deconvolved, compressed Z-stacks.

Immunofluorescence microscopy revealed the expected punctate nuclear Daxx localization in those cells transfected with vector alone (Fig. 3-17 panel D). In contrast, the cells subjected to Daxx shRNA expression exhibited a marked absence of Daxx nuclear foci (panels A-C) (53). Notably, and consistent with other reports, Daxx knockdown did not abrogate PML body formation in HT1080 cells (data not shown). As anticipated, in the absence of shRNA expression, Daxx localized to punctate nuclear bodies, known to colocalize with PML (Fig. 3-18 panel A). When cells were stimulated with either IFN-α or IFN-γ, a slight augmentation in the size of Daxx punctae was observed (panels B and C). This is consistent with the fact that Daxx is an ISG (165).

To test the consequences of Daxx reduction during the IFN-induced antiviral state, HT1080 cells were transfected with the Daxx shRNA expression vector, and subsequently placed under blasticidin selection followed by treatment with either IFN-α or IFN-γ. After 24 h, the cells were infected with either wild type Ad5 (dl309) or mutant E4inORF3. When an additional 24 h had elapsed, cells were fixed and immunostained with antibodies to Daxx and DBP. Upon Daxx reduction, cells infected with dl309 displayed replication competence under both unstimulated and IFN-stimulated conditions (panels D and F). Furthermore, replication domains were observed when cells exhibiting Daxx reduction were infected with E4inORF3 in the absence of IFN treatment (panel G). Importantly, in those cells both demonstrating Daxx reduction and induced with either IFN-α or IFN-γ, replication of mutant E4inORF3 vDNA was rescued, as indicated by the presence of replication

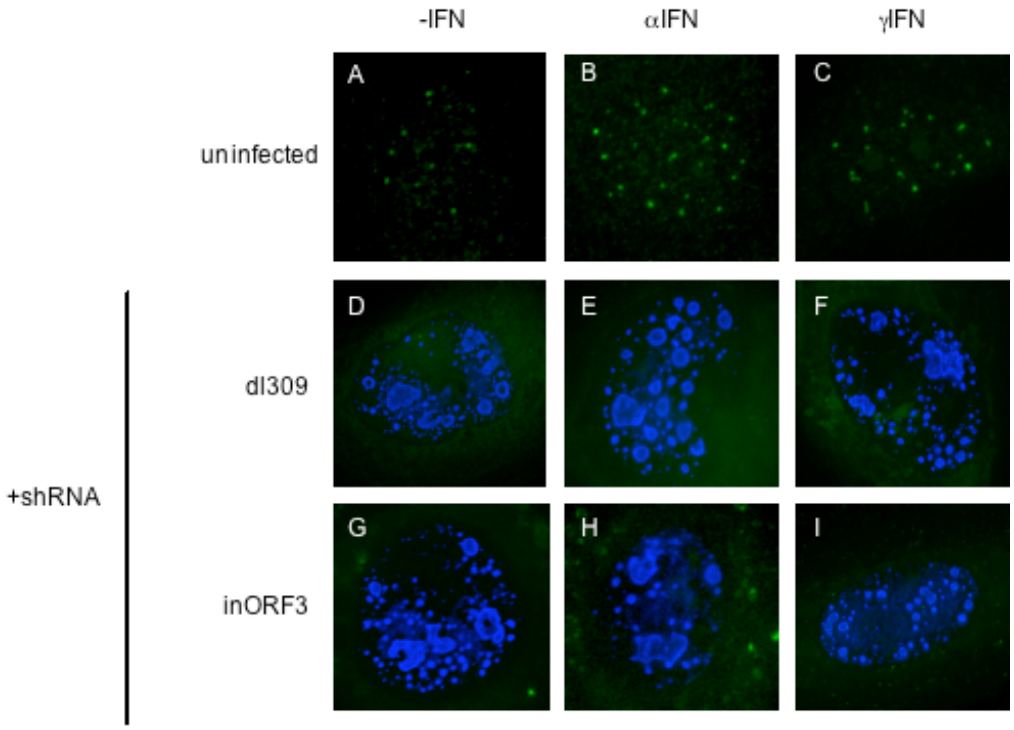


Figure 3-18. The E4 ORF3 protein antagonizes a Daxx-dependent antiviral effect. HT1080 cells grown on coverslips were transfected with constructs expressing Daxx shRNAs (D-I) and were either untreated (A, D, G), or stimulated with IFN-α (B, E, H) or IFN-γ (D, F, I). The cells were subsequently infected with dl309 (D-F) or E4inORF3 (G-I) or were not subjected to Ad infection (A-C). The cells were then fixed and immunostained for PML (FITC-coupled secondary antibody) and DBP (Alexa350-coupled secondary antibody). The final images represent deconvolved, compressed Z-stacks.

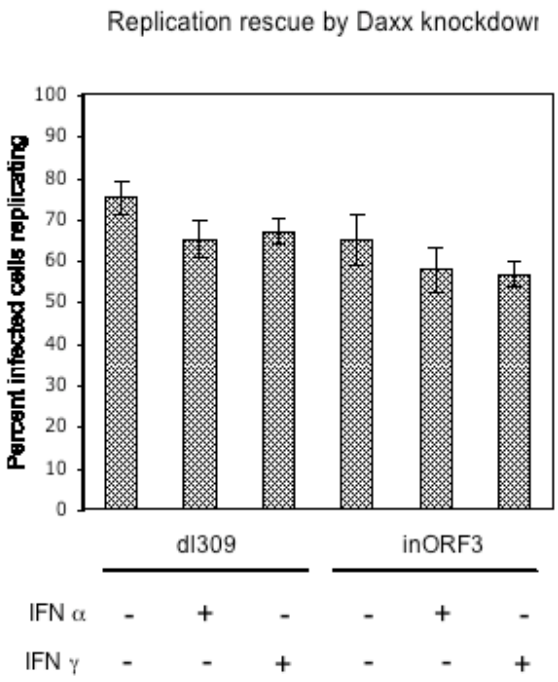


Figure 3-19. Quantification of E4 ORF3 replication following Daxx knockdown. The percentage of HT1080 cells exhibiting viral replication domains is presented in cells both infected with dl309 or E4inORF3 and demonstrating Daxx knockdown, with or without IFN pretreatment. Replication was quantified by counting cells that exhibited viral replication centers in comparison to cells that displayed diffuse nuclear DBP staining.

domains (panels H and I). When quantified, this analysis confirmed that a reduction in Daxx expression stimulated vDNA replication of mutant E4inORF3 when the antiviral state had been induced by IFN stimulation (Fig. 3-19). This dramatic rescue suggests that the Daxx protein plays a critical role in inhibiting mutant E4inORF3 replication during the IFN-induced antiviral state, thereby suggesting that the E4 ORF3 protein functions to antagonize the antiviral effects of Daxx. By extension, PML rearrangement by E4 ORF3 may serve to promote Daxx sequestration into the E4 ORF3 tracks.

The PML-NB-associated Sp100 protein does not contribute to the antiviral effect antagonized by the E4 ORF3 protein.

To determine whether the PML-NB-mediated antiviral response was specific to PML and Daxx, or whether it reflected a global ability of proteins, which localize to the PML-NB to inhibit Ad vDNA replication, expression of another PML-NB component, Sp100, was knocked down. Sp100 was found to restrict the replication of an HSV-1 ICP0 mutant virus and to repress HSV-1 immediate-early protein expression during an IFN response (53, 54). HT1080 cells grown on coverslips were transfected with vectors expressing shRNAs directed against Sp100 following the approach described for PML and Daxx. Transfected cells were selected with blasticidin and after 72 h, the coverslips were fixed and immunostained with an antibody against Sp100. Immunofluorescence microscopy revealed both a nucleoplasmic population of Sp100, as well as Sp100 localized to nuclear foci in those cells transfected with vector alone (Fig. 3-17 panel H). In stark contrast, those cells transfected with the shRNA expression vector demonstrated a marked absence of Sp100 immunostaining (panels E-G).

To ascertain the effects of Sp100 reduction on Ad DNA replication during the IFN-induced antiviral state, HT1080 cells were transfected with the Sp100 shRNA expression vectors. Following blasticidin selection for 48 h, the cells were stimulated with either IFN-α or IFN-γ for 24 h. Subsequently, the cells were infected with either dl309 or mutant E4inORF3. Twenty-four hours post-infection, the cells were fixed and immunostained with antibodies to DBP and Sp100. In those cells both deficient for Sp100 expression and infected with dl309, vDNA replication domains could be observed irrespective of whether or not the cells had been stimulated with either IFN-α or IFN-γ (Fig. 3-18 panels D-F). Dissimilar from the instances in which either PML or Daxx expression were reduced and replication of mutant E4inORF3 was rescued during an IFN response, replication of mutant E4inORF3 was observed only in untreated cells, not in cells pretreated with either IFN-α or IFN-γ (panels G-I). When quantified, this analysis confirmed that a reduction in Sp100 expression did not stimulate vDNA replication of mutant E4inORF3 when the antiviral state had been induced by IFN stimulation (Fig. 3-21). This data demonstrates that Sp100, while implicated in an intrinsic immune response against HSV-1 (55) does not participate in the IFN response antagonized by the E4 ORF3 protein. Furthermore, the data indicates that the observed results may be attributed to specific functions of both PML and Daxx.

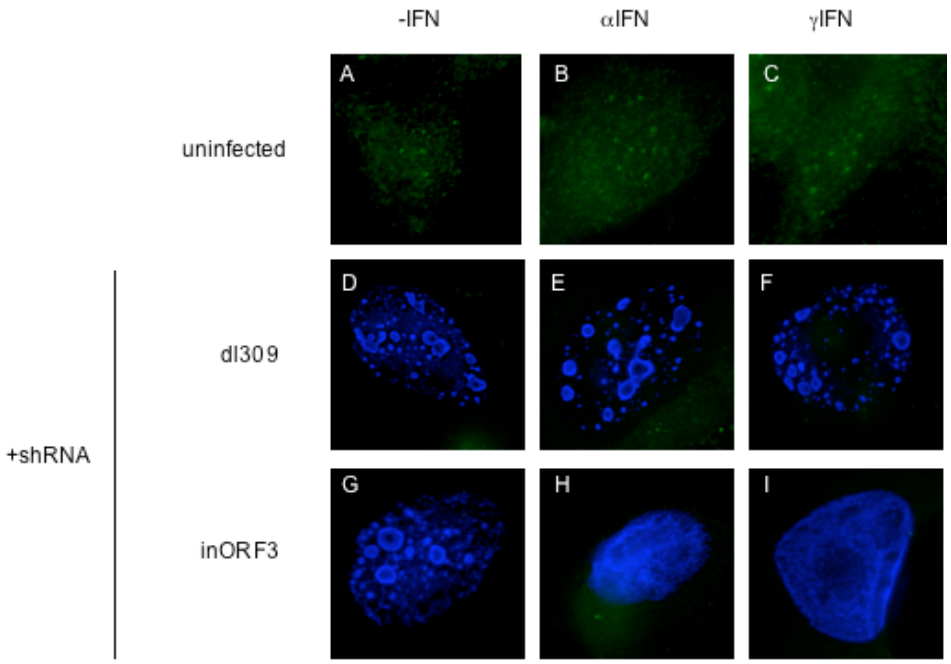


Figure 3-20. Sp100 does not contribute to the antiviral defense antagonized by the E4 ORF3 protein. HT1080 cells grown on coverslips were transfected with constructs expressing Daxx shRNA (D-I) and were either untreated (A, D, G), or stimulated with IFN-α (B, E, H) or IFN-γ (D, F, I). The cells were subsequently infected with dl309 (D-F) or E4inORF3 (G-I) or were not subjected to Ad infection (A-C). The cells were then fixed and immunostained for PML (FITC-coupled secondary antibody) and DBP (Alexa350-coupled secondary antibody). The final images represent deconvolved, compressed Z-stacks.

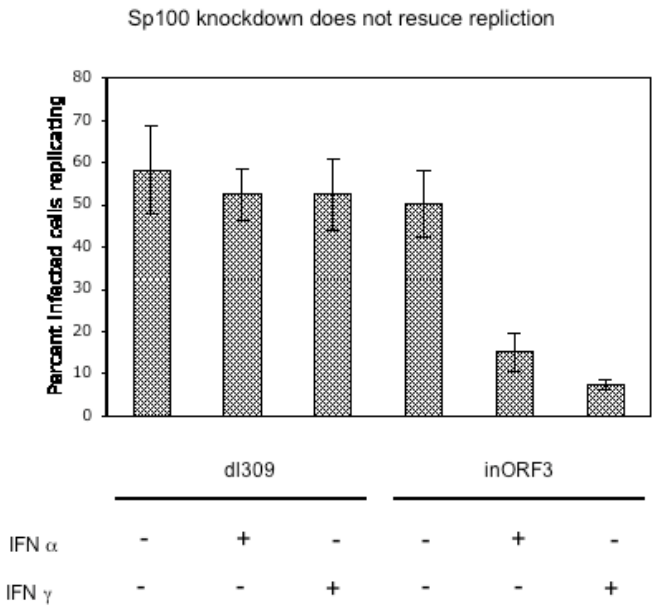


Figure 3-21. Quantification of E4 ORF3 replication following Sp100 knockdown. The percentage of HT1080 cells exhibiting viral replication domains is presented in cells both infected with dl309 or E4inORF3 and demonstrating Sp100 knockdown, with or without IFN pretreatment. Replication was quantified by counting cells that exhibited viral replication centers in comparison to cells that displayed diffuse nuclear DBP staining.

IFN stimulation induces a modest decrease in Ad early mRNA expression but it does not impede early protein function.

Daxx is a known transcriptional corepressor which has been shown to recruit both HDAC1 and HDAC2 (83, 110). Its association with the HCMV MIEP promotes transcriptional repression of the viral immediate early genes, thereby inhibiting progression of the viral lytic cycle (155, 181). Since the data described above implicates Daxx in an antiviral defense mediated by the E4 ORF3 protein, the consequence of the absence of the E4 ORF3 protein during the IFN-induced antiviral state would be transcriptional repression of early gene expression was investigated.

Ad vDNA replication is dependent upon the expression of six early gene products: E1A, E1B 55K, terminal protein, Ad DNA polymerase, E4 ORF3, E4 ORF6, and DBP. The levels of mRNA transcripts that correspond to these gene products in Vero cells infected with wild type Ad5 (dl309) or E4inORF3, with or without pretreatment with either IFN-α or IFN-γ were determined. Twenty-four hours post infection, total RNA was harvested and reversed transcribed. The resulting cDNAs were subjected to quantitative-PCR (Q-PCR) analysis to assess expression levels of these six critical early genes (Fig. 3-21). Lack of DNA template in the RNA samples was confirmed by Q-PCR (data not shown).

In those cells infected with dl309, treatment with either IFN-α or IFN-γ promoted a negligible to modest (2- to 3-fold) decrease in transcript levels for all six early genes. Because this virus is known to exhibit replication competence during the IFN-induced antiviral state (190), it is probable that a reduction in early transcript expression of this

magnitude exerts negligible effects upon the viral lytic cycle. Similarly, modest reductions in the expression of these early genes were evident in IFN-treated cells infected with E4inORF3.

To validate this conclusion, the levels of Mre11, in the presence or absence of IFN treatment, were examined in cells infected with dl309 or E4inORF3. The E1B 55K and E4 ORF6 proteins act in concert to recruit an E3 ubiquitin ligase complex to promote the proteosome-dependent degradation of a variety of cellular proteins including Mre11 and p53 (146, 170, 172). To assay E1B 55K and E4 ORF6 protein function, Vero cells were stimulated with either IFN-α or IFN-γ, or remained untreated. Twenty-four hours later, the cells were infected with either dl309 or E4inORF3. After an additional 24 h had elapsed, the cells were fixed and immunostained with antibodies against the viral DBP and Mre11. In this instance, immunofluorescence microscopy was utilized to assay Mre11 levels as roughly 20-30% of Vero cells are infected by Ad. Due to this fact, it would be difficult to assay protein degradation by Western blot analysis since Mre11 levels would be unaffected by E4 ORF6 and E1B55K proteins in 70-80% of the Vero cell population.

In uninfected cells, Mre11 assumes a nucleoplamic staining pattern with punctae known to colocalize with PML (Fig. 3-23 panels A, D, G). In contrast, in those cells infected with either dl309 or E4inORF3, Mre11 signal is lost under all conditions, thereby signifying degradation of the protein, irrespective of IFN-α or IFN-γ treatment (panels B, C, E, F, H, I). Taken together, this data suggest that while stimulation by either IFN-α or IFN-γ induces a modest decrease in Ad early mRNA transcription, this reduction does not reduce early protein expression to an extent capable of compromising

protein function. Therefore, while Daxx and PML execute inhibitory effects upon Ad vDNA replication during the IFN-induced antiviral state, the mechanism of this antiviral strategy is not based solely upon transcriptional repression.

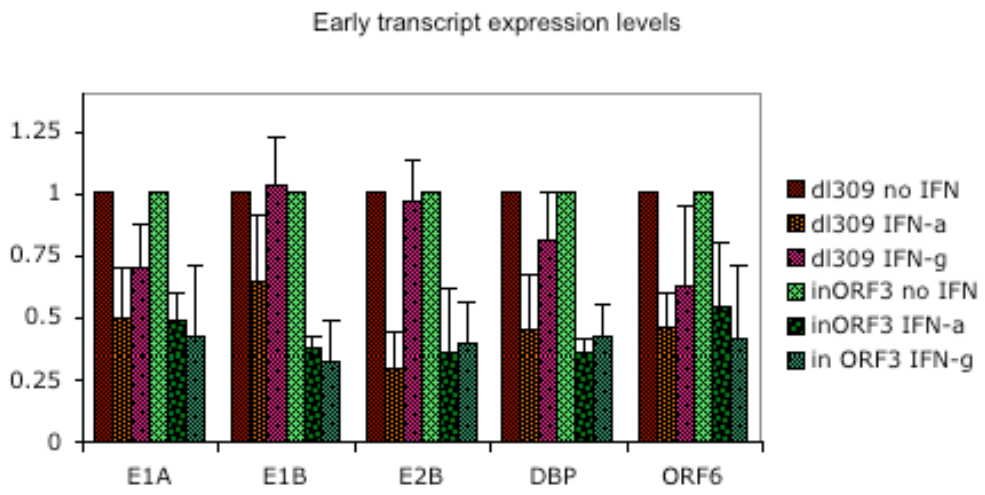


Figure 3-22. IFN stimulation induces a very modest decrease in Ad early transcript expression. Vero cells were untreated or stimulated with either IFN-α or IFN–γ. The cells were then infected by dl309 or E4inORF3. 24 h post-infection, total RNA was harvested and cDNAs were generated. cDNA levels were quantified by Q-PCR analysis using primers specific to E1A, E1B, E2B, DBP and E4 ORF6. The data was normalized to GAPDH levels and the final result reflects the average of three independent experiments.

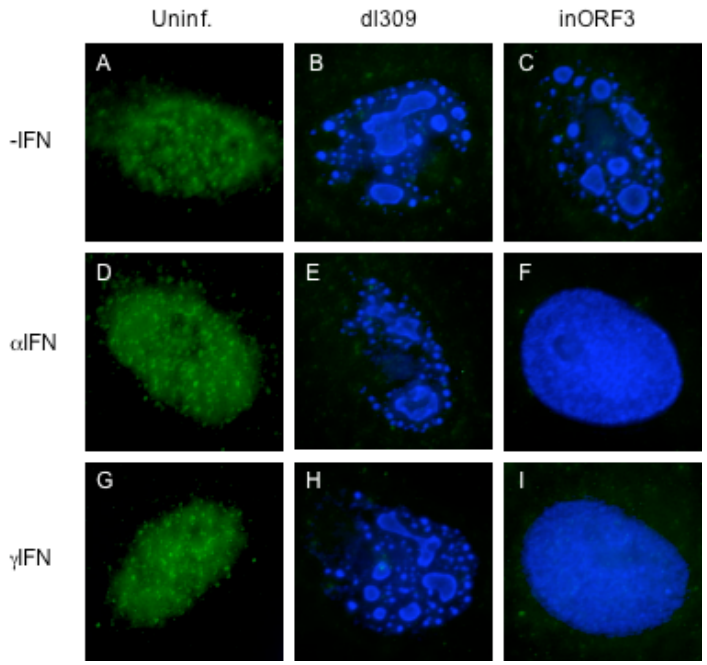


Figure 3-23. IFN-induced decreases in Ad early mRNA levels do not impede early protein function. Vero cells grown on coverslips were either untreated (A-C), or stimulated with IFN-α (D-F) or IFN-γ (G-I). The cells were subsequently infected with dl309 (B, E, H) or E4inORF3 (C, F, I), or were not subjected to Ad infection (A, D, G). The cells were then fixed and immunostained for Mre11 (FITC-coupled secondary antibody) and DBP (Alexa350-coupled secondary antibody). The final images represent deconvolved, compressed Z-stacks.

Chapter 4

Discussion

The PML-nuclear body has been implicated in the both the intrinsic and innate immune response to viral infection and as such, is a target of virus-directed inactivation (2, 24, 48, 55, 128, 134, 154, 155, 181-183, 190). It has been noted that upon treatment with either a type I or type II IFN, both the size and number of PML-NBs undergo a dramatic augmentation (72, 103). This is a likely consequence of the fact that the PML protein itself is an ISG (72, 103). Furthermore, a variety of proteins known to localize to these nuclear structures are also ISGs, suggesting a function for the PML-NB as a depot of antiviral effectors (149). Reorganization of PML bodies into track-like structures is a conserved function of the Ad E4 ORF3 protein (173), yet the consequence(s) of this activity has not been elucidated. For this reason, the potential function of E4 ORF3-mediated PML-NB rearrangement during the IFN-induced antiviral state was examined.

This work demonstrated that when cells have mounted either a type I or a type II IFN response, the E4 ORF3 protein is required for efficient Ad DNA replication. This phenomenon is illustrated by the severe replication defect exhibited by cells that have been prestimulated with either IFN-α or IFN-γ and subsequently infected with E4 inORF3, a mutant virus that does not express E4 ORF3 (Fig. 3-1, 3-2). Interestingly, this IFN-induced replication defect mimics the phenotype associated with deletion of the entire E4 region. Here, vDNA replication is severely compromised (74, 200); however, under standard culture conditions, restoration of either E4 ORF3 or E4 ORF6 rescues viral genome replication to levels comparable to that of wild type virus (18, 87). In contrast, this IFN-induced replication defect is not abrogated by the presence of E4 ORF6. These results demonstrate that E4 ORF3 is necessary for efficient vDNA

replication during the IFN-induced antiviral state and define a novel function for this viral protein.

To ensure that E4 ORF3 was directly responsible for permitting vDNA replication in IFN-treated cells, experiments were conducted to determine if loss of E4 ORF3 impeded production of the Ad gene products required for vDNA replication. Six viral early proteins are essential for Ad DNA replication in the absence of E4 ORF3: E1A, E1B 55K, DBP, Ad pol, Ad pTP, and E4 ORF6. Levels of E1A, the immediate early gene required for the inset of the Ad transcriptional program, and DBP, one of the essential E2 gene products, were quantified by Western blot analysis (Fig. 3-3). This assay confirmed that relatively comparable levels of these proteins were, indeed, synthesized by wild type, ORF6-deleted, and ORF3-deleted viruses irrespective of IFN prestimulation. Thus, it was ascertained that IFN treatment did not significantly alter either E1A nor DBP protein expression in the absence of E4 ORF3 expression.

Substantial colocalization of E4 ORF3 with PML in track-like structures was evident following pretreatment of cells with either IFN-α or IFN-γ (Fig. 3-4). Here, E4 ORF3 elicited PML body rearrangement despite the increased PML synthesis associated with IFN treatment (168). This characteristic PML-NB reorganization by E4 ORF3 is a conserved function among all Ad serotypes investigated (Figs. 3-5, 3-6, 3-7), whereas the reorganization of the MRN complex is not (173). Such results demonstrate that the E4 ORF3 proteins of evolutionarily divergent Ad serotypes are capable of inhibiting the IFN response, thereby facilitating efficient vDNA replication. Furthermore, an Ad5 E4 ORF3 mutant ($D_{105}A/L_{106}A$) that is competent for PML, but not MRN rearrangement, rescued the inhibitory effect of IFN treatment (Figs. 3-8 and 3-9). Together, these data strongly

indicate that MRN complex activity does not participate in the IFN-mediated inhibition of Ad DNA replication in the absence of E4 ORF3 expression. This conclusion is consistent with the fact that the E1B 55K/E4 ORF6 complex would be expected to inhibit MRN activity under standard tissue culture conditions (115, 172). In contrast, here, E4 ORF6 was incapable of facilitating vDNA replication during the IFN-induced antiviral state.

The DNA virus strategy of PML body rearrangement facilitates viral genome replication by an unknown mechanism. Conversely, inhibition of PML-NB reorganization diminishes the process, suggesting that elements of the PML-NB enhance successful vDNA replication. It is well established that the genomes of DNA viruses localize in proximity to PML-NBs early after infection (90, 119, 122). Indeed, recent results have demonstrated that *de novo* PML body assembly occurs at the location of infecting viral genomes (52) and that viral genomes that successfully enter the DNA replication program are primarily those that localized at PML-NBs following infection (167). It seems plausible, then, that a shared objective of multiple viruses would be to simultaneously access desirable PML-NB functions while antagonizing antiviral elements of these nuclear subdomains. This very principle may account for the evolution E4 ORF3 as a multifunctional protein. Not only is the E4 ORF3 protein capable of inhibiting the antiviral functions of the PML-NB (190), but it is also capable of interacting with a cellular transcription factor TIF1α (214). Furthermore, among the group C viruses, the E4 ORF3 protein mediates sequestration of the MRN complex into nuclear tracks (47, 172, 173).

Here, evidence is presented that PML-NB rearrangement by the E4 ORF3 protein antagonizes an innate defense mediated by the PML protein itself (Fig. 3-11). When PML expression is knocked down by shRNA, mutant E4inORF3, otherwise replication-deficient during the IFN-mediated antiviral response, regained its replicative capacity during the antiviral state induced by either IFN-α or IFN-γ. In this instance, levels of E4inORF3 replication after stimulation by either IFN-α or IFN-γ were restored to levels comparable to those typical for vDNA replication in the absence of IFN stimulation (Fig. 3-11, 3-12).

While the PML protein has been documented to exhibit antiviral effects against HCMV and HSV-1 deleted for the ICP0 protein in an intrinsic capacity (182). The fact that Ad deleted for E4 ORF3 is capable of sustaining near wild type levels of vDNA synthesis under standard tissue culture conditions suggests that if PML, or any other PML-NB associated protein, executes an intrinsic defense against Ad, it is overcome. This work has provided compelling evidence that during the IFN-induced antiviral state the E4 ORF3 protein antagonizes a deleterious cellular response; however, the E4 ORF3 protein is not unique in its ability to disrupt PML nuclear bodies. A variety of viruses express proteins that compromise POD integrity. Herpesviruses such as HSV-1, HCMV and EBV encode gene products capable of disrupting this nuclear subdomain (48, 55). Similarly, RNA viruses such as LCMV, rabies virus, and VSV have been documented to produce functional homologues (150). In several instances, the viral gene products responsible for mediating POD disruption inhibit IFN-stimulated antiviral activities. In the case of HSV-1, the ICP0 protein is an E3 ubiquitin ligase that directs PML protein degradation (48, 55) and an ICP0 null virus is sensitive to the effects of IFN treatment in

a PML-dependent manner (24, 128). Further, a reduction in PML protein levels enhances the growth properties of an ICP0 mutant virus (55). These results support both the intrinsic antiviral role of PML under normal growth conditions and the amplification of such activity in response to IFN. This idea is consistent with the observation that failure to disrupt PML bodies in HSV-1-infected cells inhibits viral DNA replication (20). Comparable results have also been described for HCMV IE1 mutant viruses (48, 55, 181). Similar to both HSV1 ICP0 and Ad E4 ORF3, the HCMV IE1 protein engenders PML-NB disruption. In this instance, IE1-mediated PML desumoylation promotes the loss of PML-NB integrity (105, 134).

It is for this reason that the question of whether or not the PML-NB disruptors encoded by HSV-1 and HCMV were capable of facilitating vDNA replication of Ad deleted for E4 ORF3 expression during the antiviral state was posed. Here, when cells expressing either HSV1 ICP0 or HCMV IE1 were infected with mutant E4inORF3, vDNA replication was rescued during the IFN-induced antiviral state (Fig. 3-13-16). These data suggest that all three viral proteins behave in a functionally analogous manner with respect to antagonizing antiviral defenses during the IFN-induced antiviral state. While all three viral proteins employ mechanistically distinct strategies to execute PML-NB disruption, their abilities to do so must represent a critical component of the DNA virus strategy for innate immune evasion. This evidence further supports the hypothesis that PML-NBs contribute to nuclear defenses against viral assault. Nevertheless, it remained unclear whether or not this antiviral response was mediated directly by the PML protein itself, or if PML at the nuclear body mediated critical protein-protein interactions capable of facilitating the antiviral effects of another cellular protein.

Preliminary evidence suggested that the PML protein was not the sole mediator of these antiviral effects. When A549 cells or HeLa cells were treated with either IFN-a or IFN-g, PML-NB augmentation was readily observed by immunofluoresce microscopy (data not shown); however, upon infection with the E4 ORF3 mutant virus, replication domains were evident. These data suggest that although IFN induced an upregulation of PML expression, this event alone in insufficient to mediate an antiviral response. To investigate the question of whether or not additional cellular factors were involved in this antiviral response more extensively, the consequences of Daxx knockdown were examined.

Daxx is a transcriptional corepressor, known to associate with HDAC1 and HDAC2 (83, 109, 110). Not only does this protein mediate the silencing of cellular genes, it also has been reported to interact with the HCMV Major Immediate Early Promoter (MIEP) to repress HCMV early gene transcription (154, 155, 182). Furthermore, it has been demonstrated recently to be a component of the intrinsic antiviral response directed against HCMV (53, 182). While the emphasis of these studies have been the subversion of PML-NB-mediated innate immunity, it is conceivable that like PML, Daxx could exhibit enhanced antiviral properties during the IFN-induced antiviral state. Indeed, Daxx is an ISG whose expression is responsive to treatment with type I IFNs (165).

Upon Daxx knockdown by shRNA, replication of the virus deleted for E4 ORF3 during the IFN-α or IFN-γ induced antiviral state was rescued to levels comparable to those observed in unstimulated cells (Fig. 3-18, 3-19). These results support the hypothesis that Daxx participates in the antiviral response antagonized by the E4 ORF3

protein. This is conclusion is somewhat intuitive, as Daxx is known to be targeted to PML-NBs through its SUMO interaction motif, thereby promoting Daxx enrichment at the nuclear body (9). Upon the upregulated expression of these two proteins, known to occur as a consequence of IFN stimulation, it is plausible that as PML-NBs become augmented by increased PML expression and a more substantial pool of Daxx becomes available, this corepressor may be recruited to the PML-NB in increasing quantities. Under these conditions, Daxx may exert its antiviral effects in an amplified capacity. It is logical to assume that HCMV IE1 protein-directed desumoylation of PML would effectively antagonize this interaction. In fact, it has been noted that in addition to promoting rescue of E4inORF3 vDNA replication during the IFN-induced antiviral state, expression of both the HCMV IE1 and HSV-1 ICP0 proteins alters the subnuclear localization of Daxx (data not shown). When either of these proteins are expressed, the typical punctate nuclear localization of Daxx is lost (data not shown). This result is consistent with the fact that in the absence of PML expression, or upon PML-NB disruption, Daxx has been reported to associate with condensed chromatin (91). This disruption of the typical nuclear localization of Daxx may function to sequester it away from vDNA replication centers, thereby preventing the protein from executing its deleterious antiviral effects.

While it is tempting to speculate that the E4 ORF3 protein antagonizes an antiviral effect dependent exclusively upon PML and Daxx, numerous proteins reside within the PML-NB (133). Recently, Sp100, a constitutive resident of PML-NBs, which interacts with HP-1 to facilitate gene silencing (83, 109, 110), was reported to inhibit ICP0 deleted HSV1 in an intrinsic capacity by diminishing early gene expression (53,

54). To determine whether or not the inhibitory effects of both PML and Daxx were specific to these proteins and not simply attributable to knockdown of a PML-NB component, Sp100 expression was knocked down by shRNA expression. In contrast to what had been observed in the case of both PML and Daxx knockdown, Sp100 knockdown did not support DNA replication of the virus deleted for E4 ORF3 during a type I or type II IFN response (Fig. 3-20, 3-21). These data, therefore, suggest that the E4 ORF3 protein functions during the IFN response to antagonize a Daxx-dependent innate immune response contingent upon PML-NB integrity. Together, the results demonstrate that interference with either of these components is sufficient to antagonize this antiviral response. Thus, PML-NB disruption by Ad E4 ORF3 protein-mediated track formation, HSV-1 ICP0 protein-directed degradation, or desumoylation promoted by HCMV IE1 protein antagonize the innate antiviral response, thereby rescuing vDNA replication of E4inORF3 during the antiviral state induced by either IFN-α or IFN-γ.

The inhibition of HSV-1 infection by both IFN and PML is associated with the down regulation of early gene expression, as are the intrinsic effects of Daxx against HCMV infection (2, 24, 48, 128, 134, 154, 155, 181-183). It was, then, critical to investigate whether or not in the absence of the E4 ORF3 protein, early gene transcription was compromised during the IFN response. When mRNA levels of the five critical early genes critical to vDNA replication, E1A, E1B, E2B, DPB, and E4 ORF6, were assayed by quantitative-PCR analysis, only modest decreases in early gene expression were observed during the IFN response (Fig. 3-22). In these experiments, the reduction in viral mRNA levels was on the order of two- to three-fold. This situation was true both for wild type as well as virus deleted for E4 ORF3 (Fig 3-22). This result is consistent with

the earlier observation that E1A and DBP protein expression by the virus deleted for E4 ORF3 were not appreciably compromised during the IFN response (Fig. 3-3). While these effects resulted in a very modest reduction in early gene transcription, it remained imperative to ascertain whether or not these effects resulted in functional inhibition of early protein activity. To address this question, the extent of Mre11 degradation was monitored during infection by both wild type and virus deleted for E4 ORF3 subsequent to stimulation with either IFN-α or IFN-γ, or in the absence of IFN treatment.

Degradation of Mre11 is mediated by a complex of two Ad early gene products E1B 55K and E4 ORF6 (146, 170). Together, these proteins recruit an E3 ubiquitin ligase complex, capable of promoting ubiquitin-mediated, proteosome-dependent degradation of several cellular substrates including components of the Mre11-Rad50-Nbs1 (MRN) DNA repair complex (47, 146, 170, 173). When Mre11 degradation was examined by immunofluorescence analysis, it was evident that both wild type and virus deleted for E4 ORF3 were capable of facilitating Mre11 degradation irrespective of IFN treatment (Fig. 3-23). The data clearly demonstrates that early protein function was not compromised as a result of the modestly diminished early gene transcription observed during the antiviral state induced by either IFN-α or IFN-γ. While these slight decreases in gene expression were more pronounced in cells stimulated with IFN and subsequently infected with the virus deleted for E4 ORF3, these effects appear to be negligible. For this reason, it is proposed that in addition to their participation in intrinsic immune responses (53, 54, 154, 155, 181, 182), both PML and Daxx, as a function of the PML-NB, actively contribute to an innate antiviral immune response that is not based solely on transcriptional repression. This is in contrast to the case for both HSV-1 and HCMV. In

these instances, the major antiviral effects of PML and IFN involve down-regulation of immediate early gene expression and are reversed upon inhibition of PML activity (24, 55, 128, 134, 181, 183, 210). With respect to the aforementioned RNA viruses, LCMV, rabiesvirus, and VSV, the antiviral effects of PML are executed via inhibition of viral RNA and protein synthesis (150). Of particular interest is the observation that the role of the Ad E4 ORF3 in the inhibition of an IFN response appears to be distinct from the previous HSV-1 and HCMV illustrations, since substantial reduction in both protein synthesis mRNA levels were not observed following IFN treatment in the absence of E4 ORF3 expression (Fig. 3-3, 3-22).

It is plausible that while Daxx may execute transcriptional repression for certain DNA viruses during the intrinsic immune response (53, 54, 154, 155, 181, 182), upon the increased ISG expression, which is a hallmark of IFN-induction, the antiviral functions of the PML-NB are enhanced. While Ad appears immune to an antiviral strategy involving transcriptional repression during the IFN response (Fig 3-21), in the absence of the E4 ORF3 protein, increased Daxx association with the PML-NB, the site proximal to Ad vDNA replication domains, or potentially with the viral genome itself could potentially impede Ad vDNA synthesis. It is possible that the E4 ORF3 protein-mediated track formation could prevent the accumulation of the 120 kDa Daxx protein at the PML-NB (98, 144, 213), thereby inhibiting the antiviral objectives of this cellular protein. Alternatively, while Daxx-mediated transcriptional repression has not been observed in the case of Ad early genes, Daxx has been well established to inhibit the function of a variety of cellular transcription factors including Pax3, ETS1, E2F1, NFκB, p53, and p73 (69, 84, 96, 110, 126). This inhibitory property of Daxx might be relevant not to early

gene transcription, but to Ad vDNA replication itself. The presence of two transcription factors, NFI/CTF and NFIII/Oct-1, which bind to the auxiliary region, a site adjacent to the core origin of replication, increase replication initiation by 200-fold (45). At this site, NFI/CTF interacts with the Ad Pol/pTP complex via its interaction with Ad Pol to recruit these proteins to the core origin while NFIII/Oct-1 contains a DNA binding domain, the POU domain. This region binds a recognition sequence adjacent to the NFI/CTF binding site and contacts the pTP protein (45). While speculative, it is certainly conceivable that if Daxx were to bind NF1/CTF and/or NFIII/Oct-1, it might impede these critical contacts with Ad Pol and pTP to elicit a replication defect of the magnitude observed here. In this instance, consistent with these reported results, E4inORF3 early gene expression would proceed to a comparable extent irrespective of whether or not antiviral state had been induced by either IFN-α or IFN-γ. As such, this is a mechanistically attractive hypothesis that warrants further investigation.

It is apparent that PML body rearrangement by E4 ORF3 inhibits the deleterious effects of IFN on Ad replication. Clearly, the E4 ORF3 protein is capable of circumventing aspects of the IFN-induced antiviral state to permit successful viral genome replication. Therefore, it would seem unlikely that E4 ORF3 interferes with the induction of upstream IFN signaling pathways. Rather, it is intuitive that E4 ORF3 counteracts the antiviral effect of PML overexpression following IFN stimulation and/or the function of a distinct IFN-stimulated gene product(s) such as Daxx; however, the ability of viral proteins to both mediate PML-NB disruption and to antagonize aspects of the IFN signal transduction cascade is not unprecedented.

The ring finger domain of HSV-1 ICP0 has been demonstrated to inhibit both IRF-3- and IRF-7-mediated transcriptional responses (113, 124). In fact, the ICP0 protein is sufficient to inhibit prevent the induction of IFN-β (25, 42, 123). While the exact mechanism of ICP0-directed IRF-3 inhibition has not been fully elucidated, it is known that the ICP0 protein appears not to prevent the phosphorylation and dimerization of this protein. Instead it has been demonstrated to form a complex with IRF-3, as well as with CPB/p300, prior to promoting proteosome-dependent degradation of IRF-3 (124). Furthermore, there is evidence implicating the ICP0 protein in inhibition of Jak/Stat signaling downstream of IFN induction. Replication of HSV-1 deleted for ICP0 expression is rescued *in vivo* by both IFNAR and Stat1 knockout mice (75). Similarly, the HCMV IE1 protein has been demonstrated to antagonize Stat1 dependent signaling (140). Here, the IE1 protein complexes with both Stat1 and Stat2 in the nuclei of infected cells and thereby inhibits the association of these proteins with both ISRE and GAS sites within promoters of ISGs (140). As is the case for ICP0 protein-mediated inhibition of IRF3, the IE1 protein does not interfere with phosphorylation or nuclear translocation of either Stat1 or Stat2, rather it impedes nuclear binding, promoting the downregulation of ISG transcription (140). The ability of viral proteins both to disrupt PML-NB integrity and to antagonize IFN responses is not limited to the DNA viruses. The rabiesvirus P protein is a noncatalytic cofactor and regulatory protein that participates in viral transcription and RNA replication (29, 64, 160). While the P protein is capable of sequestering PML in the cytoplasm, an N-terminally truncated variant of the P protein, P3, localizes to the nucleus where it mediates PML-NB disruption via nuclear aggregation (11). Furthermore, P expression interferes with the kinase upstream of

IRF-3, TBK-1, thereby inhibiting phosphorylation, dimerization, and the subsequent nuclear localization of IRF-3 (19). The P protein is also capable of deregulating the signaling cascade downstream of IFN induction. The Stat1 protein has been identified a cellular binding partner of the P protein (192). This interaction was demonstrated to prevent the nuclear accumulation of phosphorylated Stat1 homodimers as well as Stat1-Stat2-IRF-9 heterotrimers, termed ISGF3, following stimulation with both IFN-γ and IFN-α, respectively (192).

Due to the association of viral protein-directed PML-NB disruption with the antagonism of IFN signal transduction, it is logical that the E4 ORF3 protein might execute similar functions with respect to deregulating these cellular pathways. In addition to promoting PML-NB track formation and antagonizing a PML- and Daxx-dependent antiviral response, the E4 ORF3 protein may compromise the induction of IFN-β or Jak/Stat signal transduction. The examination of potential for Ad interference with these critical cellular antiviral responses has been seemingly neglected. Thus, the understanding of the E4 ORF3 protein's ability to antagonize aspects of the innate immune response gained as a result of these studies would provide the foundation necessary to further investigate adenovirus antagonism of the innate immune response.

Regardless of the precise mechanism, it is apparent that the PML-NB participates in critical immune responses to viral infection. Its integrity is essential to facilitate both the effects of intrinsic restriction factors and innate immune effectors, underscoring its complex functions as a site of host immune defense and as a critical nuclear target for antagonism by the E4 ORF3 protein.

References

1. **Ahn, J. H., E. J. Brignole, 3rd, and G. S. Hayward.** 1998. Disruption of PML subnuclear domains by the acidic IE1 protein of human cytomegalovirus is mediated through interaction with PML and may modulate a RING finger-dependent cryptic transactivator function of PML. Mol Cell Biol **18:**4899-913.
2. **Ahn, J. H., Y. Xu, W. J. Jang, M. J. Matunis, and G. S. Hayward.** 2001. Evaluation of interactions of human cytomegalovirus immediate-early IE2 regulatory protein with small ubiquitin-like modifiers and their conjugation enzyme Ubc9. J Virol **75:**3859-72. s
3. **Akusjarvi, G.** 1993. Proteins with transcription regulatory properties encoded by human adenoviruses. Trends Microbiol **1:**163-70.
4. **Alcalay, M., L. Tomassoni, E. Colombo, S. Stoldt, F. Grignani, M. Fagioli, L. Szekely, K. Helin, and P. G. Pelicci.** 1998. The promyelocytic leukemia gene product (PML) forms stable complexes with the retinoblastoma protein. Mol Cell Biol **18:**1084-93.
5. **Araujo, F. D., T. H. Stracker, C. T. Carson, D. V. Lee, and M. D. Weitzman.** 2005. Adenovirus type 5 E4orf3 protein targets the Mre11 complex to cytoplasmic aggresomes. J Virol **79:**11382-91.
6. **Arnheiter, H., M. Frese, R. Kambadur, E. Meier, and O. Haller.** 1996. Mx transgenic mice--animal models of health. Curr Top Microbiol Immunol **206:**119-47.
7. **Bell, P., P. M. Lieberman, and G. G. Maul.** 2000. Lytic but not latent replication of epstein-barr virus is associated with PML and induces sequential release of nuclear domain 10 proteins. J Virol **74:**11800-10.
8. **Bernardi, R., and P. P. Pandolfi.** 2003. Role of PML and the PML-nuclear body in the control of programmed cell death. Oncogene **22:**9048-57.
9. **Bernardi, R., and P. P. Pandolfi.** 2007. Structure, dynamics and functions of promyelocytic leukaemia nuclear bodies. Nat Rev Mol Cell Biol **8:**1006-16.
10. **Bischof, O., S. H. Kim, J. Irving, S. Beresten, N. A. Ellis, and J. Campisi.** 2001. Regulation and localization of the Bloom syndrome protein in response to DNA damage. J Cell Biol **153:**367-80.
11. **Blondel, D., T. Regad, N. Poisson, B. Pavie, F. Harper, P. P. Pandolfi, H. De The, and M. K. Chelbi-Alix.** 2002. Rabies virus P and small P products interact directly with PML and reorganize PML nuclear bodies. Oncogene **21:**7957-70.
12. **Boe, S. O., M. Haave, A. Jul-Larsen, A. Grudic, R. Bjerkvig, and P. E. Lonning.** 2006. Promyelocytic leukemia nuclear bodies are predetermined processing sites for damaged DNA. J Cell Sci **119:**3284-95.
13. **Boisvert, F. M., M. J. Hendzel, and D. P. Bazett-Jones.** 2000. Promyelocytic leukemia (PML) nuclear bodies are protein structures that do not accumulate RNA. J Cell Biol **148:**283-92.
14. **Bondesson, M., K. Ohman, M. Manervik, S. Fan, and G. Akusjarvi.** 1996. Adenovirus E4 open reading frame 4 protein autoregulates E4 transcription by inhibiting E1A transactivation of the E4 promoter. J Virol **70:**3844-51.
15. **Borden, K. L.** 2002. Pondering the promyelocytic leukemia protein (PML) puzzle: possible functions for PML nuclear bodies. Mol Cell Biol **22:**5259-69.

16. **Boutell, C., S. Sadis, and R. D. Everett.** 2002. Herpes simplex virus type 1 immediate-early protein ICP0 and is isolated RING finger domain act as ubiquitin E3 ligases in vitro. J Virol **76:**841-50.
17. **Boyer, J., K. Rohleder, and G. Ketner.** 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:**307-12.
18. **Bridge, E., and G. Ketner.** 1989. Redundant control of adenovirus late gene expression by early region 4. J Virol **63:**631-8.
19. **Brzozka, K., S. Finke, and K. K. Conzelmann.** 2005. Identification of the rabies virus alpha/beta interferon antagonist: phosphoprotein P interferes with phosphorylation of interferon regulatory factor 3. J Virol **79:**7673-81.
20. **Burkham, J., D. M. Coen, C. B. Hwang, and S. K. Weller.** 2001. Interactions of herpes simplex virus type 1 with ND10 and recruitment of PML to replication compartments. J Virol **75:**2353-67.
21. **Cantrell, S. R., and W. A. Bresnahan.** 2006. Human cytomegalovirus (HCMV) UL82 gene product (pp71) relieves hDaxx-mediated repression of HCMV replication. J Virol **80:**6188-91.
22. **Carroll, S. S., E. Chen, T. Viscount, J. Geib, M. K. Sardana, J. Gehman, and L. C. Kuo.** 1996. Cleavage of oligoribonucleotides by the 2',5'-oligoadenylate-dependent ribonuclease L. J Biol Chem **271:**4988-92.
23. **Carvalho, T., J. S. Seeler, K. Ohman, P. Jordan, U. Pettersson, G. Akusjarvi, M. Carmo-Fonseca, and A. Dejean.** 1995. Targeting of adenovirus E1A and E4-ORF3 proteins to nuclear matrix-associated PML bodies. J Cell Biol **131:**45-56.
24. **Chee, A. V., P. Lopez, P. P. Pandolfi, and B. Roizman.** 2003. Promyelocytic leukemia protein mediates interferon-based anti-herpes simplex virus 1 effects. J Virol **77:**7101-5.
25. **Chee, A. V., and B. Roizman.** 2004. Herpes simplex virus 1 gene products occlude the interferon signaling pathway at multiple sites. J Virol **78:**4185-96.
26. **Chelbi-Alix, M. K., and H. de The.** 1999. Herpes virus induced proteasome-dependent degradation of the nuclear bodies-associated PML and Sp100 proteins. Oncogene **18:**935-41.
27. **Chelbi-Alix, M. K., F. Quignon, L. Pelicano, M. H. Koken, and H. de The.** 1998. Resistance to virus infection conferred by the interferon-induced promyelocytic leukemia protein. J Virol **72:**1043-51.
28. **Chen, M., and M. S. Horwitz.** 1989. Dissection of functional domains of adenovirus DNA polymerase by linker-insertion mutagenesis. Proc Natl Acad Sci U S A **86:**6116-20.
29. **Chenik, M., K. Chebli, Y. Gaudin, and D. Blondel.** 1994. In vivo interaction of rabies virus phosphoprotein (P) and nucleoprotein (N): existence of two N-binding sites on P protein. J Gen Virol **75 (Pt 11):**2889-96.
30. **Chinnadurai, G.** 1998. Control of apoptosis by human adenovirus genes. Semin Virol **8:**505-513.
31. **Darnell, J. E., Jr., I. M. Kerr, and G. R. Stark.** 1994. Jak-STAT pathways and transcriptional activation in response to IFNs and other extracellular signaling proteins. Science **264:**1415-21.

32. **de Graaf, A., A. M. Meijne, A. J. van Renswoude, B. M. Humbel, P. M. van Bergen en Henegouwen, L. de Jong, R. van Driel, and A. J. Verkleij.** 1992. Heat shock-induced redistribution of a 160-kDa nuclear matrix protein. Exp Cell Res **202:**243-51.
33. **Dekker, J., P. N. Kanellopoulos, A. K. Loonstra, J. A. van Oosterhout, K. Leonard, P. A. Tucker, and P. C. van der Vliet.** 1997. Multimerization of the adenovirus DNA-binding protein is the driving force for ATP-independent DNA unwinding during strand displacement synthesis. Embo J **16:**1455-63.
34. **Dellaire, G., and D. P. Bazett-Jones.** 2004. PML nuclear bodies: dynamic sensors of DNA damage and cellular stress. Bioessays **26:**963-77.
35. **Dellaire, G., R. W. Ching, K. Ahmed, F. Jalali, K. C. Tse, R. G. Bristow, and D. P. Bazett-Jones.** 2006. Promyelocytic leukemia nuclear bodies behave as DNA damage sensors whose response to DNA double-strand breaks is regulated by NBS1 and the kinases ATM, Chk2, and ATR. J Cell Biol **175:**55-66.
36. **Dellaire, G., R. Farrall, and W. A. Bickmore.** 2003. The Nuclear Protein Database (NPD): sub-nuclear localisation and functional annotation of the nuclear proteome. Nucleic Acids Res **31:**328-30.
37. **Djavani, M., J. Rodas, I. S. Lukashevich, D. Horejsh, P. P. Pandolfi, K. L. Borden, and M. S. Salvato.** 2001. Role of the promyelocytic leukemia protein PML in the interferon sensitivity of lymphocytic choriomeningitis virus. J Virol **75:**6204-8.
38. **Dobner, T., N. Horikoshi, S. Rubenwolf, and T. Shenk.** 1996. Blockage by adenovirus E4orf6 of transcriptional activation by the p53 tumor suppressor. Science **272:**1470-3.
39. **Doucas, V., A. M. Ishov, A. Romo, H. Juguilon, M. D. Weitzman, R. M. Evans, and G. G. Maul.** 1996. Adenovirus replication is coupled with the dynamic properties of the PML nuclear structure. Genes Dev **10:**196-207.
40. **Duprez, E., A. J. Saurin, J. M. Desterro, V. Lallemand-Breitenbach, K. Howe, M. N. Boddy, E. Solomon, H. de The, R. T. Hay, and P. S. Freemont.** 1999. SUMO-1 modification of the acute promyelocytic leukaemia protein PML: implications for nuclear localisation. J Cell Sci **112 (Pt 3):**381-93.
41. **Dyson, N.** 1998. The regulation of E2F by pRB-family proteins. Genes Dev **12:**2245-62.
42. **Eidson, K. M., W. E. Hobbs, B. J. Manning, P. Carlson, and N. A. DeLuca.** 2002. Expression of herpes simplex virus ICP0 inhibits the induction of interferon-stimulated genes by viral infection. J Virol **76:**2180-91.
43. **Emeny, J. M., and M. J. Morgan.** 1979. Regulation of the interferon system: evidence that Vero cells have a genetic defect in interferon production. J Gen Virol **43:**247-52.
44. **Eskiw, C. H., and D. P. Bazett-Jones.** 2002. The promyelocytic leukemia nuclear body: sites of activity? Biochem Cell Biol **80:**301-10.
45. **Evans, J. D., and P. Hearing.** 2002. Adenovirus Replication.39-70.
46. **Evans, J. D., and P. Hearing.** 2003. Distinct roles of the Adenovirus E4 ORF3 protein in viral DNA replication and inhibition of genome concatenation. J Virol **77:**5295-304.

47. **Evans, J. D., and P. Hearing.** 2005. Relocalization of the Mre11-Rad50-Nbs1 complex by the adenovirus E4 ORF3 protein is required for viral replication. J Virol **79:**6207-15.
48. **Everett, R. D.** 2001. DNA viruses and viral proteins that interact with PML nuclear bodies. Oncogene **20:**7266-73.
49. **Everett, R. D.** 2000. ICP0 induces the accumulation of colocalizing conjugated ubiquitin. J Virol **74:**9994-10005.
50. **Everett, R. D., P. Freemont, H. Saitoh, M. Dasso, A. Orr, M. Kathoria, and J. Parkinson.** 1998. The disruption of ND10 during herpes simplex virus infection correlates with the Vmw110- and proteasome-dependent loss of several PML isoforms. J Virol **72:**6581-91.
51. **Everett, R. D., and G. G. Maul.** 1994. HSV-1 IE protein Vmw110 causes redistribution of PML. Embo J **13:**5062-9.
52. **Everett, R. D., and J. Murray.** 2005. ND10 components relocate to sites associated with herpes simplex virus type 1 nucleoprotein complexes during virus infection. J Virol **79:**5078-89.
53. **Everett, R. D., J. Murray, A. Orr, and C. M. Preston.** 2007. Herpes simplex virus type 1 genomes are associated with ND10 nuclear substructures in quiescently infected human fibroblasts. J Virol **81:**10991-1004.
54. **Everett, R. D., C. Parada, P. Gripon, H. Sirma, and A. Orr.** 2007. Replication of ICP0 null mutant herpes simplex virus type 1 is restricted by both PML and Sp100. J Virol.
55. **Everett, R. D., S. Rechter, P. Papior, N. Tavalai, T. Stamminger, and A. Orr.** 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.
56. **Fagioli, M., M. Alcalay, P. P. Pandolfi, L. Venturini, A. Mencarelli, A. Simeone, D. Acampora, F. Grignani, and P. G. Pelicci.** 1992. Alternative splicing of PML transcripts predicts coexpression of several carboxy-terminally different protein isoforms. Oncogene **7:**1083-91.
57. **Feinberg, A. P., and B. Vogelstein.** 1983. A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. Anal Biochem **132:**6-13.
58. **Fitzgerald, K. A., S. M. McWhirter, K. L. Faia, D. C. Rowe, E. Latz, D. T. Golenbock, A. J. Coyle, S. M. Liao, and T. Maniatis.** 2003. IKKepsilon and TBK1 are essential components of the IRF3 signaling pathway. Nat Immunol **4:**491-6.
59. **Flewett, T. H., A. S. Bryden, and H. Davies.** 1973. Letter: Virus particles in gastroenteritis. Lancet **2:**1497.
60. **Flint, J., and T. Shenk.** 1997. Viral transactivating proteins. Annu Rev Genet **31:**177-212.
61. **Floyd-Smith, G., E. Slattery, and P. Lengyel.** 1981. Interferon action: RNA cleavage pattern of a (2'-5')oligoadenylate--dependent endonuclease. Science **212:**1030-2.
62. **Fogal, V., M. Gostissa, P. Sandy, P. Zacchi, T. Sternsdorf, K. Jensen, P. P. Pandolfi, H. Will, C. Schneider, and G. Del Sal.** 2000. Regulation of p53 activity in nuclear bodies by a specific PML isoform. Embo J **19:**6185-95.

63. **Fredman, J. N., and J. A. Engler.** 1993. Adenovirus precursor to terminal protein interacts with the nuclear matrix in vivo and in vitro. J Virol **67:**3384-95.
64. **Fu, Z. F., Y. Zheng, W. H. Wunner, H. Koprowski, and B. Dietzschold.** 1994. Both the N- and the C-terminal domains of the nominal phosphoprotein of rabies virus are involved in binding to the nucleoprotein. Virology **200:**590-7.
65. **Garcia-Mata, R., Y. S. Gao, and E. Sztul.** 2002. Hassles with taking out the garbage: aggravating aggresomes. Traffic **3:**388-96.
66. **Glaunsinger, B. A., S. S. Lee, M. Thomas, L. Banks, and R. Javier.** 2000. Interactions of the PDZ-protein MAGI-1 with adenovirus E4-ORF1 and high-risk papillomavirus E6 oncoproteins. Oncogene **19:**5270-80.
67. **Gooding, L. R., L. Aquino, P. J. Duerksen-Hughes, D. Day, T. M. Horton, S. P. Yei, and W. S. Wold.** 1991. The E1B 19,000-molecular-weight protein of group C adenoviruses prevents tumor necrosis factor cytolysis of human cells but not of mouse cells. J Virol **65:**3083-94.
68. **Goodrum, F. D., and D. A. Ornelles.** 1999. Roles for the E4 orf6, orf3, and E1B 55-kilodalton proteins in cell cycle-independent adenovirus replication. J Virol **73:**7474-88.
69. **Gostissa, M., M. Morelli, F. Mantovani, E. Guida, S. Piazza, L. Collavin, C. Brancolini, C. Schneider, and G. Del Sal.** 2004. The transcriptional repressor hDaxx potentiates p53-dependent apoptosis. J Biol Chem **279:**48013-23.
70. **Grable, M., and P. Hearing.** 1990. Adenovirus type 5 packaging domain is composed of a repeated element that is functionally redundant. J Virol **64:**2047-56.
71. **Grable, M., and P. Hearing.** 1992. cis and trans requirements for the selective packaging of adenovirus type 5 DNA. J Virol **66:**723-31.
72. **Grotzinger, T., T. Sternsdorf, K. Jensen, and H. Will.** 1996. Interferon-modulated expression of genes encoding the nuclear-dot-associated proteins Sp100 and promyelocytic leukemia protein (PML). Eur J Biochem **238:**554-60.
73. **Hagmeyer, B. M., P. Angel, and H. van Dam.** 1995. Modulation of AP-1/ATF transcription factor activity by the adenovirus-E1A oncogene products. Bioessays **17:**621-9.
74. **Halbert, D. N., J. R. Cutt, and T. Shenk.** 1985. Adenovirus early region 4 encodes functions required for efficient DNA replication, late gene expression, and host cell shutoff. J Virol **56:**250-7.
75. **Halford, W. P., C. Weisend, J. Grace, M. Soboleski, D. J. Carr, J. W. Balliet, Y. Imai, T. P. Margolis, and B. M. Gebhardt.** 2006. ICP0 antagonizes Stat 1-dependent repression of herpes simplex virus: implications for the regulation of viral latency. Virol J **3:**44.
76. **Harada, J. N., A. Shevchenko, A. Shevchenko, D. C. Pallas, and A. J. Berk.** 2002. Analysis of the adenovirus E1B-55K-anchored proteome reveals its link to ubiquitination machinery. J Virol **76:**9194-206.
77. **Hay, R. T., A. Freeman, I. Leith, A. Monaghan, and A. Webster.** 1995. Molecular interactions during adenovirus DNA replication. Curr Top Microbiol Immunol **199 (Pt 2):**31-48.
78. **Hilleman, M. R., and J. H. Werner.** 1954. Recovery of new agent from patients with acute respiratory illness. Proc Soc Exp Biol Med **85:**183-8.

79. **Hinshaw, J. E., and S. L. Schmid.** 1995. Dynamin self-assembles into rings suggesting a mechanism for coated vesicle budding. Nature **374:**190-2.
80. **Hiscott, J., N. Grandvaux, S. Sharma, B. R. Tenoever, M. J. Servant, and R. Lin.** 2003. Convergence of the NF-kappaB and interferon signaling pathways in the regulation of antiviral defense and apoptosis. Ann N Y Acad Sci **1010:**237-48.
81. **Hobbs, W. E., D. E. Brough, I. Kovesdi, and N. A. DeLuca.** 2001. Efficient activation of viral genomes by levels of herpes simplex virus ICP0 insufficient to affect cellular gene expression or cell survival. J Virol **75:**3391-403.
82. **Hofmann, T. G., and H. Will.** 2003. Body language: the function of PML nuclear bodies in apoptosis regulation. Cell Death Differ **10:**1290-9.
83. **Hollenbach, A. D., C. J. McPherson, E. J. Mientjes, R. Iyengar, and G. Grosveld.** 2002. Daxx and histone deacetylase II associate with chromatin through an interaction with core histones and the chromatin-associated protein Dek. J Cell Sci **115:**3319-30.
84. **Hollenbach, A. D., J. E. Sublett, C. J. McPherson, and G. Grosveld.** 1999. The Pax3-FKHR oncoprotein is unresponsive to the Pax3-associated repressor hDaxx. Embo J **18:**3702-11.
85. **Hoppe, A., S. J. Beech, J. Dimmock, and K. N. Leppard.** 2006. Interaction of the adenovirus type 5 E4 Orf3 protein with promyelocytic leukemia protein isoform II is required for ND10 disruption. J Virol **80:**3042-9.
86. **Horisberger, M. A.** 1995. Interferons, Mx genes, and resistance to influenza virus. Am J Respir Crit Care Med **152:**S67-71.
87. **Huang, M. M., and P. Hearing.** 1989. Adenovirus early region 4 encodes two gene products with redundant effects in lytic infection. J Virol **63:**2605-15.
88. **Iftode, C., and S. J. Flint.** 2004. Viral DNA synthesis-dependent titration of a cellular repressor activates transcription of the human adenovirus type 2 IVa2 gene. Proc Natl Acad Sci U S A **101:**17831-6.
89. **Isaacs, A., and J. Lindenmann.** 1957. Virus interference. I. The interferon. Proc R Soc Lond B Biol Sci **147:**258-67.
90. **Ishov, A. M., and G. G. Maul.** 1996. The periphery of nuclear domain 10 (ND10) as site of DNA virus deposition. J Cell Biol **134:**815-26.
91. **Ishov, A. M., A. G. Sotnikov, D. Negorev, O. V. Vladimirova, N. Neff, T. Kamitani, E. T. Yeh, J. F. Strauss, 3rd, and G. G. Maul.** 1999. PML is critical for ND10 formation and recruits the PML-interacting protein daxx to this nuclear structure when modified by SUMO-1. J Cell Biol **147:**221-34.
92. **Jackson, M. R., T. Nilsson, and P. A. Peterson.** 1993. Retrieval of transmembrane proteins to the endoplasmic reticulum. J Cell Biol **121:**317-33.
93. **Jawetz, E.** 1959. The story of shipyard eye. Br Med J **1:**873-6.
94. **Jensen, K., C. Shiels, and P. S. Freemont.** 2001. PML protein isoforms and the RBCC/TRIM motif. Oncogene **20:**7223-33.
95. **Kerr, I. M., and R. E. Brown.** 1978. pppA2'p5'A2'p5'A: an inhibitor of protein synthesis synthesized with an enzyme fraction from interferon-treated cells. Proc Natl Acad Sci U S A **75:**256-60.
96. **Kim, E. J., J. S. Park, and S. J. Um.** 2003. Identification of Daxx interacting with p73, one of the p53 family, and its regulation of p53 activity by competitive interaction with PML. Nucleic Acids Res **31:**5356-67.

97. **King, J. C., Jr.** 1997. Community respiratory viruses in individuals with human immunodeficiency virus infection. Am J Med **102:**19-24; discussion 25-6.
98. **Kiriakidou, M., D. A. Driscoll, J. M. Lopez-Guisa, and J. F. Strauss, 3rd.** 1997. Cloning and expression of primate Daxx cDNAs and mapping of the human gene to chromosome 6p21.3 in the MHC region. DNA Cell Biol **16:**1289-98.
99. **Kleinberger, T., and T. Shenk.** 1993. Adenovirus E4orf4 protein binds to protein phosphatase 2A, and the complex down regulates E1A-enhanced junB transcription. J Virol **67:**7556-60.
100. **Konig, C., J. Roth, and M. Dobbelstein.** 1999. Adenovirus type 5 E4orf3 protein relieves p53 inhibition by E1B-55-kilodalton protein. J Virol **73:**2253-62.
101. **Kopito, R. R.** 2000. Aggresomes, inclusion bodies and protein aggregation. Trends Cell Biol **10:**524-30.
102. **Kovesdi, I., R. Reichel, and J. R. Nevins.** 1986. Identification of a cellular transcription factor involved in E1A trans-activation. Cell **45:**219-28.
103. **Lavau, C., A. Marchio, M. Fagioli, J. Jansen, B. Falini, P. Lebon, F. Grosveld, P. P. Pandolfi, P. G. Pelicci, and A. Dejean.** 1995. The acute promyelocytic leukaemia-associated PML gene is induced by interferon. Oncogene **11:**871-6.
104. **Lavoie, J. N., C. Champagne, M. C. Gingras, and A. Robert.** 2000. Adenovirus E4 open reading frame 4-induced apoptosis involves dysregulation of Src family kinases. J Cell Biol **150:**1037-56.
105. **Lee, H. R., D. J. Kim, J. M. Lee, C. Y. Choi, B. Y. Ahn, G. S. Hayward, and J. H. Ahn.** 2004. Ability of the human cytomegalovirus IE1 protein to modulate sumoylation of PML correlates with its functional activities in transcriptional regulation and infectivity in cultured fibroblast cells. J Virol **78:**6527-42.
106. **Lehembre, F., S. Muller, P. P. Pandolfi, and A. Dejean.** 2001. Regulation of Pax3 transcriptional activity by SUMO-1-modified PML. Oncogene **20:**1-9.
107. **Leppard, K. N., and R. D. Everett.** 1999. The adenovirus type 5 E1b 55K and E4 Orf3 proteins associate in infected cells and affect ND10 components. J Gen Virol **80 (Pt 4):**997-1008.
108. **Li, H., and J. D. Chen.** 2000. PML and the oncogenic nuclear domains in regulating transcriptional repression. Curr Opin Cell Biol **12:**641-4.
109. **Li, H., C. Leo, J. Zhu, X. Wu, J. O'Neil, E. J. Park, and J. D. Chen.** 2000. Sequestration and inhibition of Daxx-mediated transcriptional repression by PML. Mol Cell Biol **20:**1784-96.
110. **Li, R., H. Pei, D. K. Watson, and T. S. Papas.** 2000. EAP1/Daxx interacts with ETS1 and represses transcriptional activation of ETS1 target genes. Oncogene **19:**745-53.
111. **Li, Y., J. Kang, J. Friedman, L. Tarassishin, J. Ye, A. Kovalenko, D. Wallach, and M. S. Horwitz.** 1999. Identification of a cell protein (FIP-3) as a modulator of NF-kappaB activity and as a target of an adenovirus inhibitor of tumor necrosis factor alpha-induced apoptosis. Proc Natl Acad Sci U S A **96:**1042-7.
112. **Lin, H. J., and S. J. Flint.** 2000. Identification of a cellular repressor of transcription of the adenoviral late IVa(2) gene that is unaltered in activity in infected cells. Virology **277:**397-410.

113. **Lin, R., R. S. Noyce, S. E. Collins, R. D. Everett, and K. L. Mossman.** 2004. The herpes simplex virus ICP0 RING finger domain inhibits IRF3- and IRF7-mediated activation of interferon-stimulated genes. J Virol **78:**1675-84.
114. **Lindenbaum, J. O., J. Field, and J. Hurwitz.** 1986. The adenovirus DNA binding protein and adenovirus DNA polymerase interact to catalyze elongation of primed DNA templates. J Biol Chem **261:**10218-27.
115. **Liu, Y., A. Shevchenko, A. Shevchenko, and A. J. Berk.** 2005. Adenovirus exploits the cellular aggresome response to accelerate inactivation of the MRN complex. J Virol **79:**14004-16.
116. **Martin, M. E., and A. J. Berk.** 1998. Adenovirus E1B 55K represses p53 activation in vitro. J Virol **72:**3146-54.
117. **Martin, M. E., and A. J. Berk.** 1999. Corepressor required for adenovirus E1B 55,000-molecular-weight protein repression of basal transcription. Mol Cell Biol **19:**3403-14.
118. **Mathews, M. B., and T. Shenk.** 1991. Adenovirus virus-associated RNA and translation control. J Virol **65:**5657-62.
119. **Maul, G. G.** 1998. Nuclear domain 10, the site of DNA virus transcription and replication. Bioessays **20:**660-7.
120. **Maul, G. G., and R. D. Everett.** 1994. The nuclear location of PML, a cellular member of the C3HC4 zinc-binding domain protein family, is rearranged during herpes simplex virus infection by the C3HC4 viral protein ICP0. J Gen Virol **75 (Pt 6):**1223-33.
121. **Maul, G. G., H. H. Guldner, and J. G. Spivack.** 1993. Modification of discrete nuclear domains induced by herpes simplex virus type 1 immediate early gene 1 product (ICP0). J Gen Virol **74 (Pt 12):**2679-90.
122. **Maul, G. G., A. M. Ishov, and R. D. Everett.** 1996. Nuclear domain 10 as preexisting potential replication start sites of herpes simplex virus type-1. Virology **217:**67-75.
123. **Melroe, G. T., N. A. DeLuca, and D. M. Knipe.** 2004. Herpes simplex virus 1 has multiple mechanisms for blocking virus-induced interferon production. J Virol **78:**8411-20.
124. **Melroe, G. T., L. Silva, P. A. Schaffer, and D. M. Knipe.** 2007. Recruitment of activated IRF-3 and CBP/p300 to herpes simplex virus ICP0 nuclear foci: Potential role in blocking IFN-beta induction. Virology **360:**305-21.
125. **Michaelson, J. S., D. Bader, F. Kuo, C. Kozak, and P. Leder.** 1999. Loss of Daxx, a promiscuously interacting protein, results in extensive apoptosis in early mouse development. Genes Dev **13:**1918-23.
126. **Michaelson, J. S., and P. Leder.** 2003. RNAi reveals anti-apoptotic and transcriptionally repressive activities of DAXX. J Cell Sci **116:**345-52.
127. **Mocarski, E. S., G. W. Kemble, J. M. Lyle, and R. F. Greaves.** 1996. A deletion mutant in the human cytomegalovirus gene encoding IE1(491aa) is replication defective due to a failure in autoregulation. Proc Natl Acad Sci U S A **93:**11321-6.
128. **Mossman, K. L., H. A. Saffran, and J. R. Smiley.** 2000. Herpes simplex virus ICP0 mutants are hypersensitive to interferon. J Virol **74:**2052-6.

129. **Muller, S., and A. Dejean.** 1999. Viral immediate-early proteins abrogate the modification by SUMO-1 of PML and Sp100 proteins, correlating with nuclear body disruption. J Virol **73:**5137-43.
130. **Muller, S., M. J. Matunis, and A. Dejean.** 1998. Conjugation with the ubiquitin-related modifier SUMO-1 regulates the partitioning of PML within the nucleus. Embo J **17:**61-70.
131. **Mymryk, J. S.** 1996. Tumour suppressive properties of the adenovirus 5 E1A oncogene. Oncogene **13:**1581-9.
132. **Nakayama, M., K. Yazaki, A. Kusano, K. Nagata, N. Hanai, and A. Ishihama.** 1993. Structure of mouse Mx1 protein. Molecular assembly and GTP-dependent conformational change. J Biol Chem **268:**15033-8.
133. **Negorev, D., and G. G. Maul.** 2001. Cellular proteins localized at and interacting within ND10/PML nuclear bodies/PODs suggest functions of a nuclear depot. Oncogene **20:**7234-42.
134. **Nevels, M., W. Brune, and T. Shenk.** 2004. SUMOylation of the human cytomegalovirus 72-kilodalton IE1 protein facilitates expression of the 86-kilodalton IE2 protein and promotes viral replication. J Virol **78:**7803-12.
135. **Nisole, S., J. P. Stoye, and A. Saib.** 2005. TRIM family proteins: retroviral restriction and antiviral defence. Nat Rev Microbiol **3:**799-808.
136. **Nordqvist, K., K. Ohman, and G. Akusjarvi.** 1994. Human adenovirus encodes two proteins which have opposite effects on accumulation of alternatively spliced mRNAs. Mol Cell Biol **14:**437-45.
137. **O'Connor, R. J., and P. Hearing.** 2000. The E4-6/7 protein functionally compensates for the loss of E1A expression in adenovirus infection. J Virol **74:**5819-24.
138. **Ohman, K., K. Nordqvist, and G. Akusjarvi.** 1993. Two adenovirus proteins with redundant activities in virus growth facilitates tripartite leader mRNA accumulation. Virology **194:**50-8.
139. **Parkinson, J., and R. D. Everett.** 2000. Alphaherpesvirus proteins related to herpes simplex virus type 1 ICP0 affect cellular structures and proteins. J Virol **74:**10006-17.
140. **Paulus, C., S. Krauss, and M. Nevels.** 2006. A human cytomegalovirus antagonist of type I IFN-dependent signal transducer and activator of transcription signaling. Proc Natl Acad Sci U S A **103:**3840-5.
141. **Pearson, M., R. Carbone, C. Sebastiani, M. Cioce, M. Fagioli, S. Saito, Y. Higashimoto, E. Appella, S. Minucci, P. P. Pandolfi, and P. G. Pelicci.** 2000. PML regulates p53 acetylation and premature senescence induced by oncogenic Ras. Nature **406:**207-10.
142. **Peng, H., G. E. Begg, D. C. Schultz, J. R. Friedman, D. E. Jensen, D. W. Speicher, and F. J. Rauscher, 3rd.** 2000. Reconstitution of the KRAB-KAP-1 repressor complex: a model system for defining the molecular anatomy of RING-B box-coiled-coil domain-mediated protein-protein interactions. J Mol Biol **295:**1139-62.
143. **Platanias, L. C.** 2005. Mechanisms of type-I- and type-II-interferon-mediated signalling. Nat Rev Immunol **5:**375-86.

144. **Pluta, A. F., W. C. Earnshaw, and I. G. Goldberg.** 1998. Interphase-specific association of intrinsic centromere protein CENP-C with HDaxx, a death domain-binding protein implicated in Fas-mediated cell death. J Cell Sci **111 (Pt 14):**2029-41.
145. **Preston, C. M., and M. J. Nicholl.** 2006. Role of the cellular protein hDaxx in human cytomegalovirus immediate-early gene expression. J Gen Virol **87:**1113-21.
146. **Querido, E., P. Blanchette, Q. Yan, T. Kamura, M. Morrison, D. Boivin, W. G. Kaelin, R. C. Conaway, J. W. Conaway, and P. E. Branton.** 2001. Degradation of p53 by adenovirus E4orf6 and E1B55K proteins occurs via a novel mechanism involving a Cullin-containing complex. Genes Dev **15:**3104-17.
147. **Ramachandra, M., and R. Padmanabhan.** 1995. Expression, nuclear transport, and phosphorylation of adenovirus DNA replication proteins. Curr Top Microbiol Immunol **199 (Pt 2):**50-88.
148. **Rawlins, D. R., P. J. Rosenfeld, R. J. Wides, M. D. Challberg, and T. J. Kelly, Jr.** 1984. Structure and function of the adenovirus origin of replication. Cell **37:**309-19.
149. **Regad, T., and M. K. Chelbi-Alix.** 2001. Role and fate of PML nuclear bodies in response to interferon and viral infections. Oncogene **20:**7274-86.
150. **Regad, T., A. Saib, V. Lallemand-Breitenbach, P. P. Pandolfi, H. de The, and M. K. Chelbi-Alix.** 2001. PML mediates the interferon-induced antiviral state against a complex retrovirus via its association with the viral transactivator. Embo J **20:**3495-505.
151. **Reymond, A., G. Meroni, A. Fantozzi, G. Merla, S. Cairo, L. Luzi, D. Riganelli, E. Zanaria, S. Messali, S. Cainarca, A. Guffanti, S. Minucci, P. G. Pelicci, and A. Ballabio.** 2001. The tripartite motif family identifies cell compartments. Embo J **20:**2140-51.
152. **Rowe, W. P., R. J. Huebner, L. K. Gilmore, R. H. Parrott, and T. G. Ward.** 1953. Isolation of a cytopathogenic agent from human adenoids undergoing spontaneous degeneration in tissue culture. Proc Soc Exp Biol Med **84:**570-3.
153. **Sacks, W. R., and P. A. Schaffer.** 1987. Deletion mutants in the gene encoding the herpes simplex virus type 1 immediate-early protein ICP0 exhibit impaired growth in cell culture. J Virol **61:**829-39.
154. **Saffert, R. T., and R. F. Kalejta.** 2007. Human cytomegalovirus gene expression is silenced by Daxx-mediated intrinsic immune defense in model latent infections established in vitro. J Virol **81:**9109-20.
155. **Saffert, R. T., and R. F. Kalejta.** 2006. Inactivating a cellular intrinsic immune defense mediated by Daxx is the mechanism through which the human cytomegalovirus pp71 protein stimulates viral immediate-early gene expression. J Virol **80:**3863-71.
156. **Saha, V., B. D. Young, and P. S. Freemont.** 1998. Translocations, fusion genes, and acute leukemia. J Cell Biochem Suppl **30-31:**264-76.
157. **Salomoni, P., and P. P. Pandolfi.** 2002. The role of PML in tumor suppression. Cell **108:**165-70.

158. **Schaack, J., W. Y. Ho, P. Freimuth, and T. Shenk.** 1990. Adenovirus terminal protein mediates both nuclear matrix association and efficient transcription of adenovirus DNA. Genes Dev **4:**1197-208.
159. **Schaley, J., R. J. O'Connor, L. J. Taylor, D. Bar-Sagi, and P. Hearing.** 2000. Induction of the cellular E2F-1 promoter by the adenovirus E4-6/7 protein. J Virol **74:**2084-93.
160. **Schoehn, G., F. Iseni, M. Mavrakis, D. Blondel, and R. W. Ruigrok.** 2001. Structure of recombinant rabies virus nucleoprotein-RNA complex and identification of the phosphoprotein binding site. J Virol **75:**490-8.
161. **Shen, T. H., H. K. Lin, P. P. Scaglioni, T. M. Yung, and P. P. Pandolfi.** 2006. The mechanisms of PML-nuclear body formation. Mol Cell **24:**331-9.
162. **Shenk, T.** 1996. Adenoviridae: The Viruses and Their Replication. Field's Virology.
163. **Shepard, R. N., and D. A. Ornelles.** 2004. Diverse roles for E4orf3 at late times of infection revealed in an E1B 55-kilodalton protein mutant background. J Virol **78:**9924-35.
164. **Shepard, R. N., and D. A. Ornelles.** 2003. E4orf3 is necessary for enhanced S-phase replication of cell cycle-restricted subgroup C adenoviruses. J Virol **77:**8593-5.
165. **Shimoda, K., K. Kamesaki, A. Numata, K. Aoki, T. Matsuda, K. Oritani, S. Tamiya, K. Kato, K. Takase, R. Imamura, T. Yamamoto, T. Miyamoto, K. Nagafuji, H. Gondo, S. Nagafuchi, K. Nakayama, and M. Harada.** 2002. Cutting edge: tyk2 is required for the induction and nuclear translocation of Daxx which regulates IFN-alpha-induced suppression of B lymphocyte formation. J Immunol **169:**4707-11.
166. **Somasundaram, K., and W. S. El-Deiry.** 1997. Inhibition of p53-mediated transactivation and cell cycle arrest by E1A through its p300/CBP-interacting region. Oncogene **14:**1047-57.
167. **Sourvinos, G., and R. D. Everett.** 2002. Visualization of parental HSV-1 genomes and replication compartments in association with ND10 in live infected cells. Embo J **21:**4989-97.
168. **Stadler, M., M. K. Chelbi-Alix, M. H. Koken, L. Venturini, C. Lee, A. Saib, F. Quignon, L. Pelicano, M. C. Guillemin, C. Schindler, and et al.** 1995. Transcriptional induction of the PML growth suppressor gene by interferons is mediated through an ISRE and a GAS element. Oncogene **11:**2565-73.
169. **Stark, G. R., I. M. Kerr, B. R. Williams, R. H. Silverman, and R. D. Schreiber.** 1998. How cells respond to interferons. Annu Rev Biochem **67:**227-64.
170. **Steegenga, W. T., N. Riteco, A. G. Jochemsen, F. J. Fallaux, and J. L. Bos.** 1998. The large E1B protein together with the E4orf6 protein target p53 for active degradation in adenovirus infected cells. Oncogene **16:**349-57.
171. **Steegenga, W. T., T. van Laar, N. Riteco, A. Mandarino, A. Shvarts, A. J. van der Eb, and A. G. Jochemsen.** 1996. Adenovirus E1A proteins inhibit activation of transcription by p53. Mol Cell Biol **16:**2101-9.

172. **Stracker, T. H., C. T. Carson, and M. D. Weitzman.** 2002. Adenovirus oncoproteins inactivate the Mre11-Rad50-NBS1 DNA repair complex. Nature **418:**348-52.
173. **Stracker, T. H., D. V. Lee, C. T. Carson, F. D. Araujo, D. A. Ornelles, and M. D. Weitzman.** 2005. Serotype-specific reorganization of the Mre11 complex by adenoviral E4orf3 proteins. J Virol **79:**6664-73.
174. **Strudwick, S., and K. L. Borden.** 2002. Finding a role for PML in APL pathogenesis: a critical assessment of potential PML activities. Leukemia **16:**1906-17.
175. **Swaminathan, S., and B. Thimmapaya.** 1995. Regulation of adenovirus E2 transcription unit. Curr Top Microbiol Immunol **199 (Pt 3):**177-94.
176. **Takahashi, Y., V. Lallemand-Breitenbach, J. Zhu, and H. de The.** 2004. PML nuclear bodies and apoptosis. Oncogene **23:**2819-24.
177. **Takaoka, A., Z. Wang, M. K. Choi, H. Yanai, H. Negishi, T. Ban, Y. Lu, M. Miyagishi, T. Kodama, K. Honda, Y. Ohba, and T. Taniguchi.** 2007. DAI (DLM-1/ZBP1) is a cytosolic DNA sensor and an activator of innate immune response. Nature **448:**501-5.
178. **Takei, K., P. S. McPherson, S. L. Schmid, and P. De Camilli.** 1995. Tubular membrane invaginations coated by dynamin rings are induced by GTP-gamma S in nerve terminals. Nature **374:**186-90.
179. **Tashiro, S., A. Muto, K. Tanimoto, H. Tsuchiya, H. Suzuki, H. Hoshino, M. Yoshida, J. Walter, and K. Igarashi.** 2004. Repression of PML nuclear body-associated transcription by oxidative stress-activated Bach2. Mol Cell Biol **24:**3473-84.
180. **Tauber, B., and T. Dobner.** 2001. Adenovirus early E4 genes in viral oncogenesis. Oncogene **20:**7847-54.
181. **Tavalai, N., P. Papior, S. Rechter, M. Leis, and T. Stamminger.** 2006. Evidence for a role of the cellular ND10 protein PML in mediating intrinsic immunity against human cytomegalovirus infections. J Virol **80:**8006-18.
182. **Tavalai, N., P. Papior, S. Rechter, and T. Stamminger.** 2008. Nuclear domain 10 components promyelocytic leukemia protein and hDaxx independently contribute to an intrinsic antiviral defense against human cytomegalovirus infection. J Virol **82:**126-37.
183. **Taylor, J. L., D. Unverrich, W. J. O'Brien, and K. W. Wilcox.** 2000. Interferon coordinately inhibits the disruption of PML-positive ND10 and immediate-early gene expression by herpes simplex virus. J Interferon Cytokine Res **20:**805-15.
184. **Thomas, D. L., S. Shin, B. H. Jiang, H. Vogel, M. A. Ross, M. Kaplitt, T. E. Shenk, and R. T. Javier.** 1999. Early region 1 transforming functions are dispensable for mammary tumorigenesis by human adenovirus type 9. J Virol **73:**3071-9.
185. **Tollefson, A. E., T. W. Hermiston, D. L. Lichtenstein, C. F. Colle, R. A. Tripp, T. Dimitrov, K. Toth, C. E. Wells, P. C. Doherty, and W. S. Wold.** 1998. Forced degradation of Fas inhibits apoptosis in adenovirus-infected cells. Nature **392:**726-30.
186. **Tollefson, A. E., A. Scaria, T. W. Hermiston, J. S. Ryerse, L. J. Wold, and W. S. Wold.** 1996. The adenovirus death protein (E3-11.6K) is required at very late

stages of infection for efficient cell lysis and release of adenovirus from infected cells. J Virol **70:**2296-306.

187. **Tollefson, A. E., A. Scaria, S. K. Saha, and W. S. Wold.** 1992. The 11,600-MW protein encoded by region E3 of adenovirus is expressed early but is greatly amplified at late stages of infection. J Virol **66:**3633-42.
188. **Torii, S., D. A. Egan, R. A. Evans, and J. C. Reed.** 1999. Human Daxx regulates Fas-induced apoptosis from nuclear PML oncogenic domains (PODs). Embo J **18:**6037-49.
189. **Trentin, J. J., Y. Yabe, and G. Taylor.** 1962. The quest for human cancer viruses. Science **137:**835-41.
190. **Ullman, A. J., N. C. Reich, and P. Hearing.** 2007. Adenovirus E4 ORF3 protein inhibits the interferon-mediated antiviral response. J Virol **81:**4744-52.
191. **Van der Vliet, P. C.** 1995. Adenovirus DNA replication. Curr Top Microbiol Immunol **199:**1-30.
192. **Vidy, A., M. Chelbi-Alix, and D. Blondel.** 2005. Rabies virus P protein interacts with STAT1 and inhibits interferon signal transduction pathways. J Virol **79:**14411-20.
193. **Voelkerding, K., and D. F. Klessig.** 1986. Identification of two nuclear subclasses of the adenovirus type 5-encoded DNA-binding protein. J Virol **60:**353-62.
194. **Walters, R. W., P. Freimuth, T. O. Moninger, I. Ganske, J. Zabner, and M. J. Welsh.** 2002. Adenovirus fiber disrupts CAR-mediated intercellular adhesion allowing virus escape. Cell **110:**789-99.
195. **Wang, Z. G., D. Ruggero, S. Ronchetti, S. Zhong, M. Gaboli, R. Rivi, and P. P. Pandolfi.** 1998. PML is essential for multiple apoptotic pathways. Nat Genet **20:**266-72.
196. **Weber, J. M., F. Cai, J. Horvath, and J. G. Guillemette.** 1995. Predicted structure of the adenovirus DNA binding protein. Virus Genes **9:**171-5.
197. **Webster, A., I. R. Leith, J. Nicholson, J. Hounsell, and R. T. Hay.** 1997. Role of preterminal protein processing in adenovirus replication. J Virol **71:**6381-9.
198. **Weiden, M. D., and H. S. Ginsberg.** 1994. Deletion of the E4 region of the genome produces adenovirus DNA concatemers. Proc Natl Acad Sci U S A **91:**153-7.
199. **Weinberg, D. H., and G. Ketner.** 1983. A cell line that supports the growth of a defective early region 4 deletion mutant of human adenovirus type 2. Proc Natl Acad Sci U S A **80:**5383-6.
200. **Weinberg, D. H., and G. Ketner.** 1986. Adenoviral early region 4 is required for efficient viral DNA replication and for late gene expression. J Virol **57:**833-8.
201. **Weitzman, M. D., C. T. Carson, R. A. Schwartz, and C. E. Lilley.** 2004. Interactions of viruses with the cellular DNA repair machinery. DNA Repair (Amst) **3:**1165-73.
202. **Whalen, S. G., R. C. Marcellus, A. Whalen, N. G. Ahn, R. P. Ricciardi, and P. E. Branton.** 1997. Phosphorylation within the transactivation domain of adenovirus E1A protein by mitogen-activated protein kinase regulates expression of early region 4. J Virol **71:**3545-53.

203. **White, E.** 1998. Regulation of apoptosis by E1A and E1B. Semin Virol **8:**505-513.
204. **Wickham, T. J., P. Mathias, D. A. Cheresh, and G. R. Nemerow.** 1993. Integrins alpha v beta 3 and alpha v beta 5 promote adenovirus internalization but not virus attachment. Cell **73:**309-19.
205. **Wold, W. S., K. Doronin, K. Toth, M. Kuppuswamy, D. L. Lichtenstein, and A. E. Tollefson.** 1999. Immune responses to adenoviruses: viral evasion mechanisms and their implications for the clinic. Curr Opin Immunol **11:**380-6.
206. **Wold, W. S., A. E. Tollefson, and T. W. Hermiston.** 1995. E3 transcription unit of adenovirus. Curr Top Microbiol Immunol **199 (Pt 1):**237-74.
207. **Woodhall, D. L., I. J. Groves, M. B. Reeves, G. Wilkinson, and J. H. Sinclair.** 2006. Human Daxx-mediated repression of human cytomegalovirus gene expression correlates with a repressive chromatin structure around the major immediate early promoter. J Biol Chem **281:**37652-60.
208. **Wreschner, D. H., J. W. McCauley, J. J. Skehel, and I. M. Kerr.** 1981. Interferon action--sequence specificity of the ppp(A2'p)nA-dependent ribonuclease. Nature **289:**414-7.
209. **Wu, W. S., S. Vallian, E. Seto, W. M. Yang, D. Edmondson, S. Roth, and K. S. Chang.** 2001. The growth suppressor PML represses transcription by functionally and physically interacting with histone deacetylases. Mol Cell Biol **21:**2259-68.
210. **Xu, Y., J. H. Ahn, M. Cheng, C. M. apRhys, C. J. Chiou, J. Zong, M. J. Matunis, and G. S. Hayward.** 2001. Proteasome-independent disruption of PML oncogenic domains (PODs), but not covalent modification by SUMO-1, is required for human cytomegalovirus immediate-early protein IE1 to inhibit PML-mediated transcriptional repression. J Virol **75:**10683-95.
211. **Xu, Z. X., A. Timanova-Atanasova, R. X. Zhao, and K. S. Chang.** 2003. PML colocalizes with and stabilizes the DNA damage response protein TopBP1. Mol Cell Biol **23:**4247-56.
212. **Yang, S., J. H. Jeong, A. L. Brown, C. H. Lee, P. P. Pandolfi, J. H. Chung, and M. K. Kim.** 2006. Promyelocytic leukemia activates Chk2 by mediating Chk2 autophosphorylation. J Biol Chem **281:**26645-54.
213. **Yang, X., R. Khosravi-Far, H. Y. Chang, and D. Baltimore.** 1997. Daxx, a novel Fas-binding protein that activates JNK and apoptosis. Cell **89:**1067-76.
214. **Yondola, M. A., and P. Hearing.** 2007. The adenovirus E4 ORF3 protein binds and reorganizes the TRIM family member transcriptional intermediary factor 1 alpha. J Virol **81:**4264-71.
215. **Yoneyama, M., M. Kikuchi, T. Natsukawa, N. Shinobu, T. Imaizumi, M. Miyagishi, K. Taira, S. Akira, and T. Fujita.** 2004. The RNA helicase RIG-I has an essential function in double-stranded RNA-induced innate antiviral responses. Nat Immunol **5:**730-7.
216. **Zhong, S., P. Hu, T. Z. Ye, R. Stan, N. A. Ellis, and P. P. Pandolfi.** 1999. A role for PML and the nuclear body in genomic stability. Oncogene **18:**7941-7.
217. **Zhong, S., S. Muller, S. Ronchetti, P. S. Freemont, A. Dejean, and P. P. Pandolfi.** 2000. Role of SUMO-1-modified PML in nuclear body formation. Blood **95:**2748-52.

218. **Zhong, S., P. Salomoni, and P. P. Pandolfi.** 2000. The transcriptional role of PML and the nuclear body. Nat Cell Biol **2:**E85-90.
219. **Zhu, J., M. H. Koken, F. Quignon, M. K. Chelbi-Alix, L. Degos, Z. Y. Wang, Z. Chen, and H. de The.** 1997. Arsenic-induced PML targeting onto nuclear bodies: implications for the treatment of acute promyelocytic leukemia. Proc Natl Acad Sci U S A **94:**3978-83.
220. **Zijderveld, D. C., M. H. Stuiver, and P. C. van der Vliet.** 1993. The adenovirus DNA binding protein enhances intermolecular DNA renaturation but inhibits intramolecular DNA renaturation. Nucleic Acids Res **21:**2591-8.