The role of PML proteins in adenovirus type 5 infection and the type I interferon response

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I would like to dedicate this thesis to my grandparents, who I lost during the course of this project. I miss them every day.

Declaration

I hereby declare that, unless explicitly stated in the text, the work described in this thesis was undertaken by myself alone under the supervision of Dr. Keith Leppard. This thesis has not previously been submitted for any other degree.

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Abbreviations

5'ppp – 5' triphosphate
Ad5 – Adenovirus type 5
ALT – Alternative lengthening of telomeres
APB – ALT-associated PML NB
APL – Acute promyelocytic leukaemia
ATM - Ataxia-telangiectasia mutated
ATR - Ataxia telangiectasia and Rad3 related
β-Gal – β-galactosidase
bp – Base pairs
BSA – Bovine serum albumin
CAR – Coxsackie/adenovirus receptor
CARD – Caspase recruiting domain
CK2 – Casein kinase 2
CTL – Cytotoxic T-lymphocyte
cPML – cytoplasmic promyelocytic leukaemia protein
DAPI - 4',6-diamidino-2-phenylindole
DBP – DNA binding protein
DMAT - 2-Dimethylamino-4,5,6,7-tetrabromo-1H-benzimidazole
DMEM – Dulbecco's modified Eagle medium
DMSO – Dimethyl sulfoxide
dNTP - Deoxyribonucleotide
DSB – Double-strand break
dsDNA – Double-stranded DNA
dsRNA – Double-stranded RNA
EBV – Epstein-Barr virus
EDTA – Ethylenediaminetetraacetic acid
EMCV - Encephalomyocarditis virus
ERK - Extracellular-signal-regulated kinase
FBS – Foetal bovine serum
GAPDH - Glyceraldehyde 3-phosphate dehydrogenase
HCMV – Human cytomegalovirus
HDAC – Histone deacetylase
h.p.i. – Hours post-infection
HPV – Human papilloma virus
HSV – herpes simplex virus
ICP – Infected cell polypeptide
IFN – Interferon
IFNAR – Type I interferon recptor
IRF – Interferon regulatory factor
ISG – Interferon-stimulated gene
ISRE – Interferon-stimulated response element
kDA – Kilodalton
LB – Luria broth
LCMV – Lymphocytic choriomeningitis virus
LFV - Lassa fever virus
MAPP – Mitotic accumulation of PML protein
MEF – Mouse embryonic fibroblast

MLP – Major late promoter
m.o.i. – multiplicity of infection
MRN - Mre11-RAD50-NBS1
NBS – Newborn bovine serum
ND10 – Nuclear domain 10
NES – Nuclear export signal
NLS – Nuclear localisation signal
NP40 - Nonyl phenoxylpolyethoxylethanol
OAS – Oligoadenylate synthetase
OD – Optical density
Orf – Open reading frame
PAGE - Polyacrylamide gel electrophoresis
PBS – Phosphate buffered saline
PCR – Polymerase chain reaction
p.f.u. – Plaque forming unit
PLB - Passive lysis buffer
PKR – Protein kinase R
PML – Promyelocytic leukaemia
PML NBs – Promyelocytic leukaemia nuclear bodies
POD – PML oncogenic domain
Poly (I:C) - Polyinosinic:polycytidylic acid
RARα – retinoic acid receptor alpha
RBCC – RING finger, B-Box and coiled-coil
RING – Really interesting new gene
RLA – Relative luciferase activity
RLU – Relative light units
RSV – Respiratory syncytial virus
RT-PCR – Reverse transcriptase polymerase chain reaction
SDS – Sodium dodecyl sulphate
SIM – SUMO-interacting motif
siRNA – short interfering RNA
SR – Serine/arginine rich
SUMO – Small ubiquitin-like modifier
TBE - Tris/Borate/EDTA
TBS – Tris-buffered saline
TERT – Telomere reverse transcriptase
TK – Thymidine kinase
TLR – Toll-like receptor
TP – Terminal protein
TRIM – Tripartite motif
USP – Ubiquitin-specific protease
UTR – Untranslated region
VSV – Vesicular stomatitis virus

Summary

Promyelocytic leukaemia (PML) proteins consist of a collection of related isoforms that are the nucleating components of sub-nuclear structures termed PML nuclear bodies (PML NBs). Numerous functions are attributed to PML and PML NBs, including a role in antiviral responses. Adenovirus type 5 (Ad5) has previously been shown both to disrupt PML NBs and to cause the appearance of a novel PML protein species, termed PML-A, in a manner dependent on the viral E4 Orf3 protein. Here, the interactions between Ad5 and PML proteins were further investigated.

The E4 Orf3-dependent species of PML (PML-A) was found to be related to the full-length PML II isoform. In addition, two previously unknown infection-specific species of PML were detected (PML-B and PML-C). Both PML-B and PML-C were produced independently of Ad5 E4 Orf3, though their appearance required viral DNA replication. PML-C was found to be localised within the cytoplasm of infected cells and to be related to PML I.

To test the hypothesis that these changes in PML were related to virus effects on antiviral responses, the roles of individual PML proteins in type I interferon (IFN) signalling were investigated. Depletion of PML II by siRNA resulted in a 50% decrease in activity of an IFNβ promoter reporter plasmid during stimulation by poly (I:C). Exogenous expression of a ΔRBCC truncated mutant, but not full-length, PML II resulted in enhanced IFNβ promoter activity, suggesting a role of a PML II-related species in type I IFN signalling. Moreover, expression of the Ad5 E4 Orf3 protein was sufficient to inhibit the activity of the IFNβ promoter, an activity that correlated with Orf3's ability to bind PML II. Collectively, these data indicate that a PML II-related molecule is involved in the interferon response and that the E4 Orf3-PML II interaction may therefore facilitate Ad5 in mitigating this activity.

Chapter 1
Introduction

Overview

Upon infection of a cell, a virus faces substantial challenges that must be overcome in order to replicate successfully. For instance, the virus must elude or deactivate antiviral responses initiated by the host cell upon infection. This study primarily concerns an investigation of the nature and function of cellular promyelocytic leukaemia (PML) proteins. As previously stated, PML proteins are the nucleating factor of sub-nuclear structures termed PML nuclear bodies (PML NBs). PML NBs have been implicated in a vast array of cellular functions though, for this study, particular interest lies in their proposed involvement in antiviral mechanisms. Many viruses target and disrupt PML protein and PML NBs, including the adenovirus Ad5, the virus utilised during the course of this study. Alongside this, the roles of PML protein in a specific antiviral response, the type I interferon (IFN) response, are considered.

The following sections provide a more detailed examination of the topics mentioned above. Initially, the basic facets of adenovirus biology are discussed. Attention then moves to the various aspects of IFN responses, including a description of the main signalling cascade as well as the various stimuli that may trigger this response. Finally, the nature of both PML protein and PML NBs are discussed. Whereas the many proposed functions of PML protein are summarised, particular attention and detail are paid to the roles of these proteins in a variety of viral infections, including Ad5. The main aims of this investigation are then stated.

1.1 Adenovirus

1.1.1Classification and structure

Adenoviruses (Ad) are large double stranded DNA viruses that possess a linear genome that may vary from 34-36kbp (Berk, 2007). There are at least 51 human Ad serotypes that may be divided into six subgroups, termed A-F, also now known as species. Subgroup B may be further split into B1 and B2 based on molecular

phylogeny (Berk, 2007). Ad5, the virus used during the course of this study, belongs to subgroup C. Adenoviruses are non-enveloped viruses that possess an icosahedral capsid. The capsid is primarily composed of three major proteins. The hexon protein is the most abundant of these, and forms each of the faces of the icosahedron (Russell, 2009). The hexon protein forms homotrimers, and 240 of these hexon capsomeres are incorporated into the capsid. At each of the 12 vertices of the capsid there is a penton capsomere. This is composed of a homopentameric penton base and the homotrimeric fibre protein, which forms an extension from each vertex (Devaux *et al.*, 1987). Fibre protein is itself divided into three regions; the tail, shaft and knob (Russell, 2009). Proteins IIIa, VI, VIII and IX are minor components of the capsid but are important for its stability. Six proteins, termed V, VII, Mu, IVa2, TP and 23K, form the core of the particle. All but one of these proteins are associated with the viral genomic DNA. 23K is a viral protease that is not associated with viral DNA, and is important both for the maturation of the particle to its infectious form and for the disassembly of the capsid upon infection of a cell (Russell, 2000).

1.1.2 Replication cycle

Ad5 binds to its host receptor, the Coxsackie/adenovirus receptor CAR, through its fibre protein (Russell, 2009). Upon binding, Ad5 may enter the cell through endocytosis in a clathrin-dependent manner (Meier & Greber, 2004). Within the endosome the 23K protease degrades protein VI in order to destabilise the capsid (Greber *et al.*, 1996). Upon contact with the nuclear membrane, the viral genomic DNA enters the nucleus through the nuclear pore (Russell, 2000). Once there, early gene transcription begins with the E1A region. E1A gene products alter the cell to make it more permissive for viral replication and activate transcription of other early gene products, including E1B proteins which also affect the host environment and response to infection (Berk, 2005). The E2 region encodes the machinery required for the replication of viral DNA (Berk, 2007). The protein products of the E3 region are responsible for evasion of the host immune response, and are dispensable for viral propagation in tissue culture (Berk, 2007). The E4 region encodes several

proteins involved in viral mRNA metabolism and the promotion of viral DNA replication (Berk, 2007).

Viral DNA synthesis occurs by initiation from both ends of the linear genome primed by the terminal protein precursor (pTP) that becomes attached to the 5' ends of the newly synthesised DNA strands (Berk, 2007, Russell, 2000). The pattern of viral gene expression changes after the initiation of DNA replication. The IVa2, IX and L4 proteins are expressed and subsequently activate the major late promoter (MLP). The MLP drives expression from the L1-5 regions, which encode proteins involved in the late phase of replication, including structural proteins and proteins involved in assembly (Berk, 2007, Morris & Leppard, 2009, Russell, 2000). Viral assembly occurs within the nucleus, where the viral DNA enters pre-formed viral particles, after which the virus exits the nucleus and the cell altogether (Berk, 2007).

1.1.3 The E4 Region

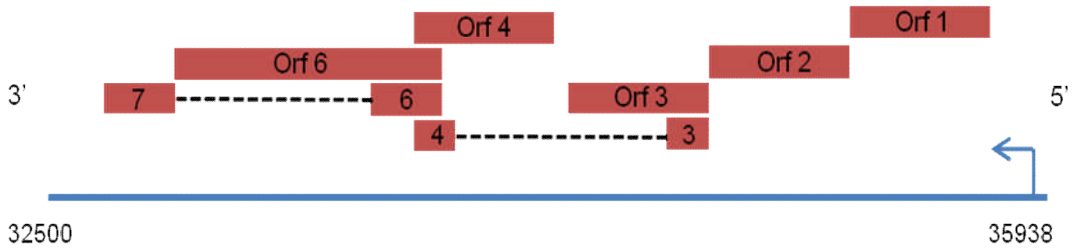


Figure 1.1 The Ad5 E4 region. Diagram showing the relationship of the Ad5 E4 proteins. All E4 proteins are derived from mRNAs generated by splicing from a single transcript. Overlapping boxes of the same number share reading frames, otherwise amino acid sequences are different between proteins. A dashed line indicates a split in the coding regions by intron sequences. The numbers at the bottom represent the postion within the Ad5 genome in base pairs. Adapted with permission from K. Leppard (Leppard, 1997).

The E4 region encodes at least seven protein products that are derived from alternative splicing of a single primary transcript (Leppard, 1997, Fig. 1.1). The E4 region contains one promoter that is activated by E1A during the early phase of infection. At later time points, however, the activity of the promoter decreases due

to an inhibition of E1A by E4 Orf4, and the increasing presence of the E2A repressor (Bondesson *et al*., 1996, Shenk, 1996).

E4 Region Gene Product	Function(s)
Orf1	Unknown; Oncogenic in rats
Orf2	Unknown function
Orf3	Reorganisation of PML NBs Reorganisation of MRN complex Regulation of viral mRNA processing
Orf3/4	Unknown
Orf4	Binds and modulates activity of phosphatase 2A Regulation of viral mRNA processing Inhibits E1A activity
Orf6	Regulation of viral mRNA processing Degradation of p53 and Mre
Orf6/7	Regulates E2F activity Regulation of viral E2 region Regulation of cellular genes

Table 1.1 Function of the E4 region of adenovirus. The known functions of each of the E4 gene products are summarised.

The function of E4 gene products primarily concerns the processing and transport of viral mRNA and overcoming host antiviral responses (Weitzman & Ornelles, 2005). The functions are summarised in Table 1.1. The most intensively studied E4 products are Orf3 and Orf6, as these proteins are largely responsible for the functions mentioned above. The MRN (Mre11-RAD50-NBS1) host cell protein complex poses a significant problem to adenovirus, as it detects the ends of the linear viral genome as it would double-strand breaks (DSBs) in host cell DNA. The end result of activation of the DSB repair pathway is concatemerisation of the viral genome and inhibition of viral replication (Weitzman & Ornelles, 2005). Orf3 expression causes the redistribution of the MRN DNA damage response protein complex away from viral replication centres (Stracker *et al.*, 2002). Orf3 also induces the cytoplasmic localisation of Mre11 into aggregates (Weitzman & Ornelles, 2005). E4 Orf3 also causes the rearrangement of PML NBs into ‘track-like’ structures (Carvalho *et al*., 1995), and this is discussed in detail later.

E4 Orf6 forms a complex with E1B 55K that can recruit cellular Cullin proteins to form an E3 ubiquitin ligase (Berk, 2005, Weitzman & Ornelles, 2005). Orf6 contributes to overcoming the DNA damage response mentioned above by initiating the degradation of Mre11 via this activity (Stracker *et al.*, 2002). The actions of Orf3 and Orf6 on Mre11 and the MRN complex together lead to a decrease in ATM and ATR kinase activitiy. This therefore decreases the phosphorylation and activation of a number of proteins involved in the DNA repair pathway (Carson *et al*., 2009, Weitzman & Ornelles, 2005). The E4 Orf6/E1B 55K ubiquitin ligase complex is also responsible for the ubiquitination and degradation of p53 during adenovirus infection, and hence for blocking the cell cycle arrest/pro-apoptotic activities of this protein (Berk, 2005, Querido *et al.*, 2001).

Finally, both E4 Orf3 and Orf6 act as splicing factors on viral RNA transcribed from the MLP. Orf3 promotes exon inclusion whereas Orf6 promotes exon exclusion (Nordqvist *et al*., 1994). However, despite having opposing functions in this regard, Orf3 and Orf6 are to a degree functionally redundant, as both proteins stabilise viral mRNA, though this is achieved through independent mechanisms (Öhman *et al*., 1993).

Other E4 gene products are less well understood. Orf1 is known to possess oncogenic activity during Ad9 infection of rats, and this ability correlates with high levels of expression (Weiss *et al*., 1996). E4 Orf2 is known to be expressed but has no known function (Dix & Leppard, 1995). E4 Orf4 binds the cellular protein phosphatase 2A and modulates its activity in regulating cellular SR proteins. Through this mechanism Orf4 regulates viral mRNA splicing (Kanopka *et al*., 1998). Orf4 also inhibits the activity of E1A and has been suggested to therefore inhibit viral replication (Bondesson *et al*., 1996). Orf6/7 regulates the cellular transcription factor E2F, and there is evidence that it promotes transcription of the E2 early region of adenovirus (Leppard, 1997).

1.2 The interferon (IFN) response

Interferons were the first cytokines that were discovered in 1957 when they were shown to interfere with viral replication. They form the first line of defence against infection, and are produced in large amounts upon viral infection. IFNs are secreted from infected cells to act in an autocrine and paracrine manner. In infected cells, IFNs act to induce apoptosis and therefore limit the spread of virus. In uninfected cells, IFNs act to induce an antiviral state to limit subsequent viral infection. They also play an important role in modulating the activity of natural cytotoxic T-lymphocytes (CTLs) and macrophages (Stetson & Medzhitov, 2006). Alongside their antiviral properties, interferons also confer anti-proliferative effects and have been proposed to perform functions unrelated to antiviral defence (Taniguchi & Takaoka, 2002).

Interferons may be sub-divided into either type I, II or III groups (Takaoka & Yanai, 2006). Type I IFNs are produced in cells upon virus infection, and they consist of α, β, δ, ω and τ interferons, although only the production of IFN α/β has been well described and is initiated via a complex signalling pathway that is described below. In humans there are at least 13 subtypes of IFNα (Randall & Goodbourn, 2008), though only a single gene exists for IFNβ. IFNγ is the only member of the type II IFNs, and its production is limited to NK cells and activated T-cells (Takaoka & Yanai, 2006). IFNγ is required for macrophage-mediated immune responses against pathogens such as intracellular bacteria (Takaoka & Yanai, 2006). Type III IFNs are the most recent group to be characterised and are also induced upon viral infection of cells. There are three homologous members, IFNλ1-3, and they act in a manner similar to that of type I IFN. However, they differ from type I IFNs as they possess a different structure and utilise an independent membrane receptor for signalling (Kotenko *et al.*, 2003, Sheppard *et al.*, 2003)

1.2.1 The classical type I IFN signalling pathway

The type I IFN signalling pathway is induced upon viral infection (Fig. 1.1). Various indicators of viral infection may be detected by membrane-bound toll-like

receptors (TLRs), or by cytoplasmic receptors such as the RNA helicases RIG-I or MDA5. A summary of the variety stimulants of the IFN response is discussed in Section 1.2.2.

The most well-defined pathway of type I IFN induction is that of the response to viral RNA, which is detected by RIG I and MDA5. Both of these surveillance molecules are ubiquitously expressed and are themselves IFN-inducible, therefore forming part of the IFN positive feedback loop (Haller *et al*., 2006). RIG-I and MDA5 both possess a caspase recruiting domain (CARD) in their N terminus, and it is thought that upon binding to their respective activators the CARD undergoes a conformational change. This change permits the interaction of RIG-I and MDA5 with interferon β promoter stimulator 1 (IPS-1) or mitochondrial antiviral signalling protein MAVS (Haller *et al*., 2006). MAVS localises to the outer membrane of mitochondria, though the reason for this remains unclear. It has been proposed that the mitochondrial localisation keeps the antiviral sensing machinery in close proximity to intracellular membranes, which are often sites for viral replication factories (Haller *et al*., 2006).

Activated IPS-1 and MAVS in turn activate the interferon regulatory factor 3 (IRF3) kinases IKKε (IκB kinase) and TBK-1 (TANK-binding kinase 1). TBK-1 may also be activated by an alternative mechanism. Toll-like receptor TLR3 can detect dsRNA in endocytic compartments, and signals to TBK-1 via TRIF, leading to the phosphorylation of IRF3 (Haller *et al*., 2006). IRF3 is a constitutively expressed transcription factor. In its unphosphorylated form, IRF3 is monomeric, cytoplasmic and inactive (Honda *et al*., 2006, Mercurio *et al*., 1997). However, phosphorylation of the C-terminal domain of IRF3 by its respective kinases results in its dimerisation and migration to the nucleus (Honda *et al*., 2006). Once there, IRF3 binds the IFNβ promoter and recruits transcriptional co-activators p300 and CBP (CREB-binding protein). The DNA-binding transcription factors AP-1 and NF-κB are also recruited in a manner dependent on dsRNA, resulting in the production of an enhanceosome that directs IFNβ mRNA expression (Merika *et al*., 1998, Thanos & Maniatis, 1995, Yie *et al*., 1999). NF-κB normally resides in the cytoplasm in an inactive form due to its binding to IκB. Upon IKKε activation, IκB is phosphorylated and subsequently

degraded, allowing NF-κB to migrate to the nucleus in its active form (Mercurio *et al*., 1997).

IFNα/β is secreted from cells where it acts in an autocrine and paracrine pathway to bind membrane-bound type I interferon receptors (IFNAR). IFNARs are composed of two subunits termed α and β, and both subunits are required for IFN signalling (Bogdan, 2000). Upon IFN binding, IFNARs signal to the Janus kinases Jak-1 and Tyk-2, which in turn phosphorylate and activate the transcription factors STAT-1 and STAT-2 (signal transducing activators of transcription) (Haller *et al*., 2006). Phosphorylated STAT 1/2 recruits IRF9 to form a complex known as IFN stimulated gene factor 3 (ISGF-3) (Haller *et al*., 2006). ISGF-3 translocates to the nucleus and binds interferon response elements (ISREs) that are located in the promoters of interferon stimulated genes (ISGs) (Takaoka & Yanai, 2006).

Amongst these ISGs is IRF7, which upon expression is activated by phosphorylation in a manner dependent on viral infection. IRF7 is considered the 'master regulator' of the IFN response, as it induces the expression of IFNα (Honda *et al.*, 2005) as well as numerous ISGs. Both IRF3 and IRF7 may homo- or hetero-dimerise, and each complex possesses its own promoter specificity (Honda *et al.*, 2005). Induction of IRF7 is important for the positive feedback loop of the IFN response, and it is responsible for the massive amplification in the amount of IFNα/β during virus infection (Honda *et al.*, 2005). IRF7 has a short half-life, and therefore is only present in high concentrations when a viral infection is in progress (Taniguchi & Takaoka, 2002).

1.2.2. Alternative pathways and other stimuli of the IFN response

Mammalian cells are subject to infection by a wide range of viruses that may use either RNA or DNA as their genetic material. The pathway described above demonstrates the classical mechanism of IFN induction by dsRNA. However, until recently it has been unclear as to how cells detect the presence of DNA virus infection. TLRs capable of sensing dsDNA in endosomes were known (Randall & Goodbourn, 2008). However, recent studies have demonstrated that cytosolic

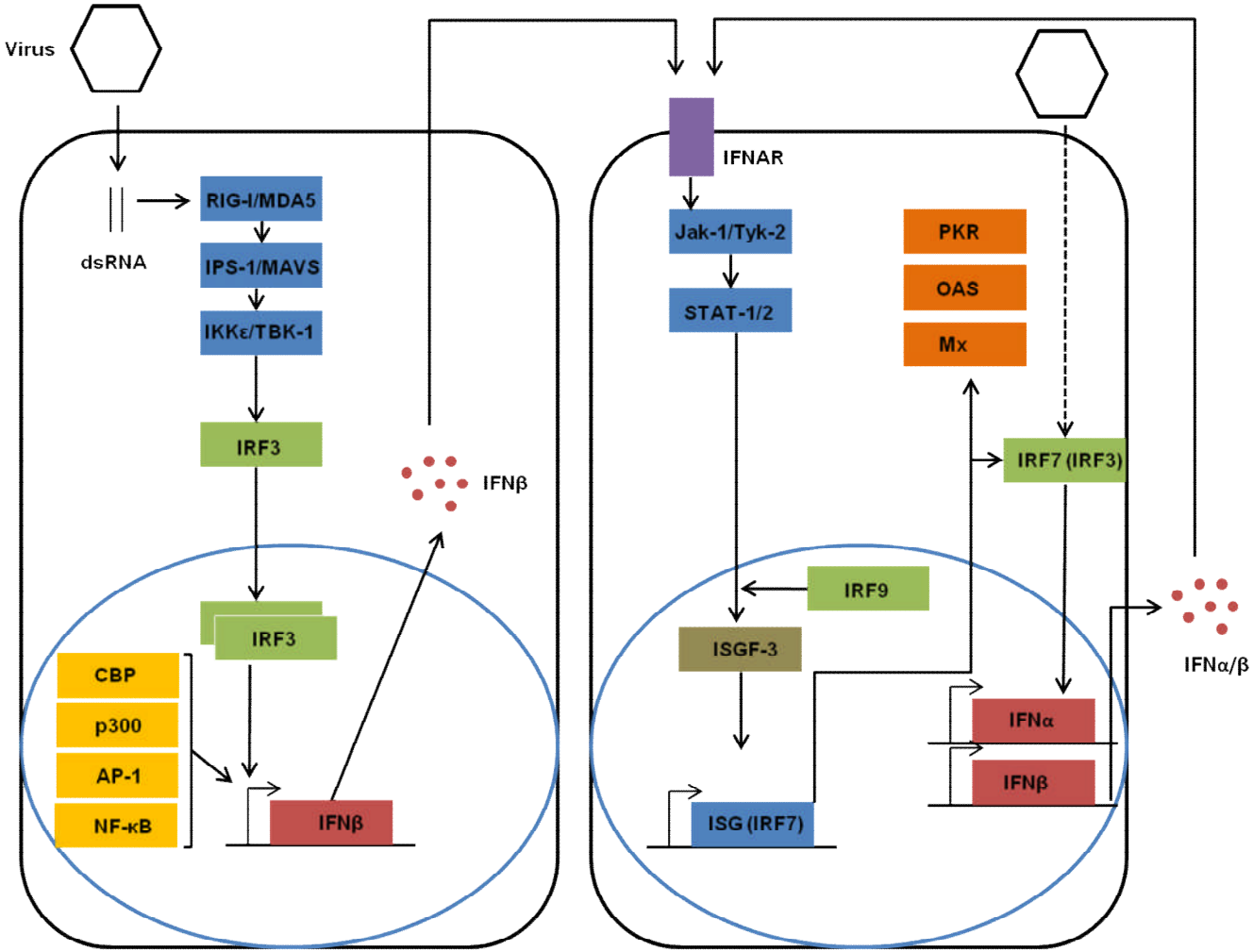


Figure 1.2 The classic type I IFN signalling pathway. Upon infection viral RNA in the cytoplasm is detected by MDA5/RIG-I which leads to a signalling cascade within the cell, ultimately leading to the induction of the IFNβ gene. IFNβ acts through autocrine and paracrine pathways to induce the Jak-STAT signalling pathway. This results in production of IRF7 and ultimately, the amplification of the IFN response through the positive feedback actions of both IFNα and IFNβ. The dashed line in the right hand cell summarises the pathway indicated in the left hand cell.

dsDNA can also activate the IFN response using pathways involved in the response to dsRNA. Transfection of cells with synthetic dsDNA was found to enhance IFNβ promoter activity in a manner dependent on RIG-I (Cheng *et al*., 2007). The mechanism of this was demonstrated in further studies by independent groups. RNA polymerase III was found to transcribe RNA from cytosolic DNA templates. Furthermore, the generated RNA possessed 5' tri-phosphate (5'ppp), which is recognised by RIG-I and leads to its activation (Ablasser *et al*., 2009, Chiu *et al*., 2009). Adenovirus and herpes simplex virus type 1 (HSV-1) genomic DNA were both found to induce IFNβ promoter activity (Cheng *et al*., 2007, Chiu et al., 2009), and this was indeed dependent on RNA Pol III (Chiu *et al*., 2009).

The IFN response may also respond to stimuli other than dsRNA, and this is mediated through the use of various TLRs. TLRs may be found on the plasma membrane as well as endosomal membranes. TLRs 7 and 8 can detect ssRNA, TLR 9 can detect unmethylated DNA and TLR 4 can detect lipopolysaccharide (LPS) (Stetson & Medzhitov, 2006). TLR 3 can signal through to IKKε/TBK-1 via adaptor protein TRIF, whereas TLRs 7 and 9 can activate IRF7 (Kumar *et al*., 2009). Therefore there are numerous molecular stimuli that can alert the IFN response to the presence of a viral infection, and collectively these are termed pathogen-associated molecular patterns (PAMPs) (Akira *et al*., 2006).

1.2.3. Antiviral effects of type I interferons

Interferons have been shown to perform both anti-viral and anti-proliferative functions that are important for survival. Both mice and humans deficient in IFN signalling succumb to viral infections at an early age (Haller *et al*., 2006). There are several hundred ISGs, indicating that an IFN response induces a diverse and complex cellular response. The products of a number of ISGs are antiviral proteins. Three main families of antiviral proteins have been described; the protein kinase R family (PKR), the oligoadenylate synthetase (OAS)/RNase L family and the Mx family. Each family performs a number of antiviral functions and are usually more potent against some viruses more than others (Stetson & Medzhitov, 2006). It should be noted, however, that mouse knockouts of all three of these mechanisms

still possess limited resistance to viral infection that is induced by IFN, reflecting the presence of other cellular antiviral pathways (Haller *et al*., 2006).

The Mx proteins are important in mediating antiviral activity against orthomyxoviruses in mice, and have protective activity against both influenza virus and vesicular stomatitis virus (VSV) (Stetson & Medzhitov, 2006). Although their precise mechanism of action is unknown, Mx proteins are large GTPases that are thought to interfere with intracellular viral assembly and trafficking (Stetson & Medzhitov, 2006). Activation of 2'-5' OAS results in the production of oligoadenylates and subsequent activation of RNase L. RNase L degrades viral RNA as well as host mRNA (Stetson & Medzhitov, 2006). Activation of protein kinase R (PKR) leads to the phosphorylation of the eukaryotic translation initiator factor eIF2α, resulting in the inhibition of translation of both host and viral RNAs known as 'host cell shutoff' (Stetson & Medzhitov, 2006).

The IFN response also leads to general antiviral responses other than those described above. Treatment of cells with type I IFNs sensitizes them to apoptosis upon viral infection (Stetson & Medzhitov, 2006). p53, a tumour suppressor which promotes apoptosis, is directly induced by interferon and is thought to confer antiviral activity through this function (Stetson & Medzhitov, 2006). This hypothesis is supported by the observation that a number of viruses, including poliovirus and adenovirus, actively degrade p53 during the course of infection (Pampin *et al*., 2006, Weitzman & Ornelles, 2005).

1.3 PML protein and PML NBs

The promyelocytic leukaemia (PML) gene was identified by its involvement in the chromosome translocation event responsible for acute promyelocytic leukaemia (APL) (de The *et al*., 1990, Goddard *et al*., 1991). In APL, the PML locus undergoes a t(15;17) translocation with the retinoic receptor α (RARα) gene, resulting in the production of a functional fusion protein which was later identified as PML-RARα (de The *et al*., 1990, Goddard *et al*., 1991). PML protein was shown to possess a distinctive nuclear localisation in the form of nuclear dots, variously

known as ND10, PML oncogenic domains (PODs), Kremer bodies or PML nuclear bodies (PML NBs). PML NBs derive their name from the observation that they do not form in the absence of PML protein, and thus this protein is considered the nucleating factor of these bodies (Ishov *et al*., 1999). As such, many of the functions ascribed to NBs are often also attributed to PML protein. In APL, PML NBs are dispersed, and their reconstitution via specific therapy is associated with regression of the disease (Salomoni *et al*., 2008). Consequently, PML protein and PML NBs have since been the subject of intensive investigation.

1.3.1 Structure of the PML protein

The *pml* gene locus is located on chromosome 15 and is approximately 35kb in length (Goddard *et al*., 1991). The gene consists of nine exons, which are alternatively spliced during gene expression to generate numerous protein isoforms, and is expressed in almost all tissues (Jensen, 2001). All described PML isoforms share exons 1-3, and are thus N-terminally related, though they may differ in composition regarding exons 4-9 (Jensen, 2001). PML proteins can be extensively post-translationally modified, the best studied example of which is sumoylation. The various post-translational modifications of PML protein will be discussed later within this chapter.

Within PML exons 1-3 lies a distinctive domain consisting of three separate elements; a RING finger, two B-boxes and a coiled-coil. This motif is known as an RBCC or a tripartite motif (TRIM). A whole family of TRIM proteins is known to be produced by human cells, and indeed PML protein is also known as TRIM19 (Ozato *et al*., 2008). The RBCC is thought to play a key role in the biological function of PML protein, and is required for the oncogenic activity of the PML-RARα fusion protein. The RING finger, for instance, is crucial for the formation of PML NBs (Shen *et al*., 2006), as a PML RING mutant does not form these substructures. The RING has been hypothesised to possess activity similar to that of an E3 ubiquitin-ligase, since other RING-domain proteins have such activity (Freemont, 2000), though its substrate is thought to be the ubiquitin-like protein SUMO (Ozato *et al*., 2008). Although evidence in mammalian cells is lacking, over-

expression of PML in yeast leads to a general increase in sumoylation (Quimby *et al*., 2006). The coiled-coil of PML is generally thought to allow both homo- and hetero-oligomerisation of PML isoforms, and therefore may also play a role in the assembly of PML NBs (Cao *et al*., 1997, Jensen, 2001). As well as being subject to sumoylation, PMLs I-V possess a SUMO-interacting motif (SIM) that is encoded in exon 7a (Shen *et al*., 2006), which mediates non-covalent associations with other SUMO-modified proteins. The SIM, as well as the RBCC domain, is crucial for the formation of PML NBs.

There are currently seven described isoforms of PML that are defined by their unique carboxyl-terminal sequences. Termed PMLs I-VII, only PMLs I-VI are known to be incorporated into PML NBs (Jensen, 2001). PML VII is a cytoplasmic variant of PML and is thus excluded from the nucleus. With the exception of PML VII, full-length isoforms possess all of exons 1-6, though they differ in their subsequent exon content. However, it should be noted that the structure of the PML gene allows cytoplasmic variants of PML I-V to be produced by splicing from exon 3 to exon 7a (Fig. 1.2). Each of PML I-V may occur as several variants depending on the splicing pattern of exons 4-6 (Jensen, 2001). These exons may be differentially excluded from the primary transcript to generate three possible variants of each of PML I-V. Exon 5 may solely be excluded to create a presumptively nuclear form of each of PML I-VI, exons 5 and 6 may be excluded together, altering the reading frame or all of exons 4, 5 and 6 may be excluded together, retaining the reading frame (Jensen, 2001). Since the nuclear localisation signal (NLS), as well as a sumoylation site, of PML is located in exon 6, PML species lacking this exon are expected to exhibit a cytoplasmic localisation. Indeed cytoplasmic PML VII, an exon 4-7b splice variant that uses an alternative reading frame in exon 7b, lacks exon 6 (Jensen, 2001). PML I is unique among the nuclear isoforms in that it also contains, in its C-terminal domain, a nuclear export signal (NES) (Condemine *et al*., 2006), meaning that it may move between cell compartments.

All PML isoforms are expressed in both primary cells and cell lines, but each cell type displays a distinct and consistent pattern of PML isoform expression (Condemine *et al*., 2006). PML I and PML II were found to be the most heavily expressed isoforms in normal cells, whereas PMLs III-V were expressed to lower

levels. PML VI appeared to be the least abundant PML isoform (Condemine *et al*., 2006).

1.3.2 Post-translational modification of PML protein

The PML protein can be post-translationally modified in a variety of ways. All known post-translational modifications of PML affect regions of the protein that are shared by multiple isoforms. Very little is known about isoform-specific modifications.

Sumoylation

PML may be covalently conjugated with the small ubiquitin-like modifier SUMO at lysines 65, 160 and 490 (Duprez *et al*., 1999). Lysine 490 lies within exon 6 and is therefore absent in cytoplasmic variants of PML (Jensen, 2001). Sumoylation of PML is critical for the assembly of PML NBs, and dispersal of these bodies has been shown to correspond with desumoylation (Bernardi & Pandolfi, 2007). Over-expression of SUMO proteases, for instance, leads to the loss of PML NBs (Nichol *et al*., 2009). Many PML-unrelated components of PML NBs, such as Daxx, are also sumoylated and PML mutants unable to be sumoylated are unable to recruit other NB components (Bernardi & Pandolfi, 2007). The formation of PML NBs is also facilitated through non-covalent interactions between SUMO, and unrelated proteins possessing a SIM. The SIM of PML, for example, is crucial in interacting with unrelated sumoylated proteins in PML NBs (Shen *et al*., 2006).

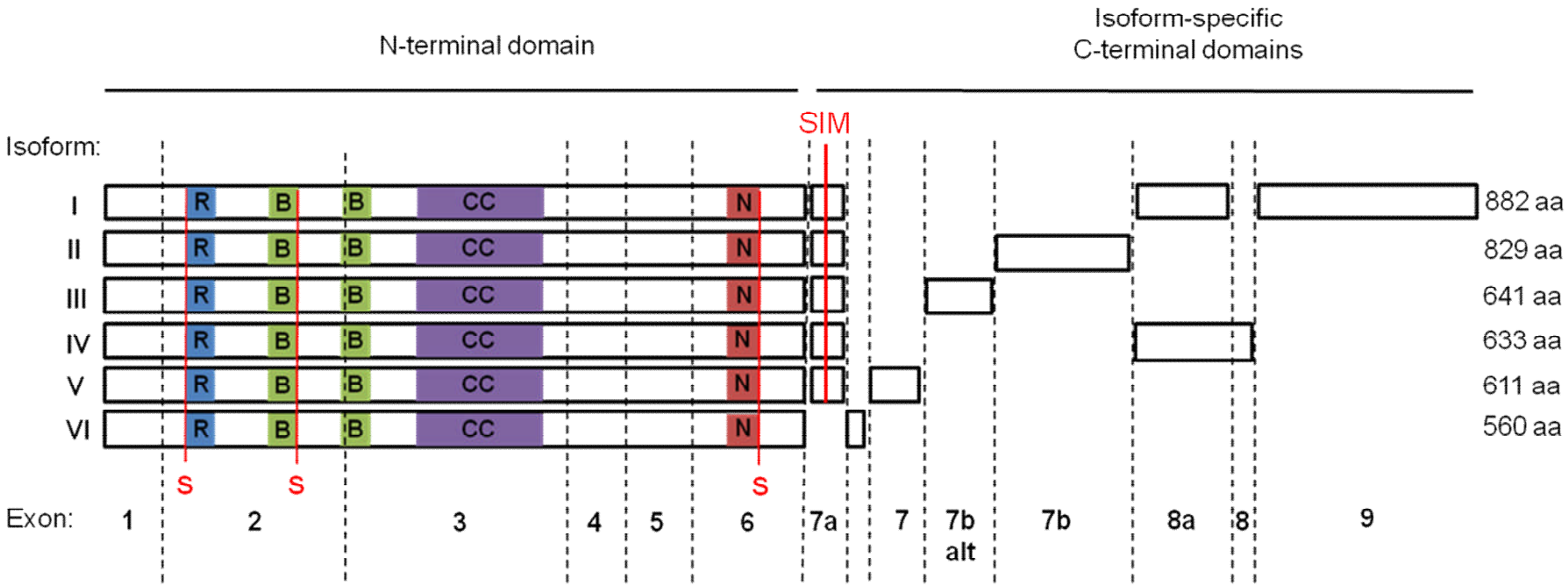


Figure 1.3 The structure of PML isoforms I-VI. All of PML I-VI share a common N-terminal domain (exons 1-6), but differ in composition of exons 7a-9. PML VI is formed by an alternative splice from exons 6 to 7a that alters the reading frame as compared to other isoforms. R – RING finger. B – B-Box. CC – Coiled coil. N – nuclear localisation signal. The position of the three sumoylatable lysine residues (K65, K160, K490) are indicated in red with an S. The SUMO interaction motif (SIM) in exon 7a is also indicated in red. The amino-acid length of each isoform is indicated on the right hand side of the figure.

A further layer of complexity is generated regarding sumoylation modification as there are at least four SUMO homologs described in mammalian cells (Nichol *et al*., 2009). PML protein, for instance, may be sumoylated by each of SUMO 1-3, though the functional significance of each form of modification is incompletely understood (Bernardi & Pandolfi, 2007). For instance, PML may be mono-sumoylated by SUMO 1, which is unable to form polymeric chains, and was the original SUMO protein found to couple with PML. However, it has since been found that poly-sumoylation may occur by SUMO 2 and 3, although SUMO 1 always forms the last subunit of the chain. Poly-sumoylation by SUMO 2 and 3 is required for the formation of PML NBs (Bernardi & Pandolfi, 2007).

Sumoylation of PML has been shown to be mediated by Ubc9, an E2 SUMO-conjugating enzyme (Duprez *et al*., 1999). However, the E3 ligase required for sumoylation of PML is currently unknown, prompting speculation that PML may act as its own E3 ligase through its RING domain (Jensen, 2001). The RING has been shown to possess auto-sumoylation activity in yeast, but as yet no data exist concerning mammalian cells (Quimby *et al*., 2006). Further weight has been lent to this hypothesis by Duprez *et al*, who demonstrated that SUMO modification of PML requires an interaction between Ubc9 and the RING domain (Duprez *et al*., 1999). Ubc 9 is also a potential PML NB component, suggesting that other PML NB components may be recruited to PML NBs in order to be sumoylated (Nichol *et al*., 2009). Sumoylation has also been shown to regulate the dynamics of exchange of components between PML NBs and the nucleoplasm, including PML isoforms I-VI as well as other NB components such as Sp100 (Weidtkamp-Peters *et al*., 2008).

The reciprocal release of SUMO from PML is mediated by at least two members of the SUMO-specific protease family, also known as SENPs. SENP2 is likely to be responsible for de-conjugation of SUMO 1 and SUMO 3 modifications, whereas SENP5 removes all of SUMO 1-3 (Nichol *et al*., 2009). Therefore sumoylation is a reversible and dynamic process that plays an important role in regulating the formation and function of both PML proteins and PML NBs.

Alongside PML, there are numerous reported substrates for SUMO modification, including many factors involved in transcription and genomic stability. The function of sumoylation is very diverse, and varies depending on the substrate being modified (Gill, 2004, Muller *et al*., 2004). For instance, sumoylation regulates the localisation of PML protein to PML NBs, whereas sumoylation of p53 can regulate its ability to function as a transcription factor (Muller *et al*., 2004).

It has recently been shown that sumoylation of PML may either promote or inhibit its degradation depending on the pattern of sumoylation. RNF4/SNURF, an E3 ubiquitin ligase, recognises both mono- and poly-sumoylated PML via its own SIM and promotes the ubiquitination and subsequent degradation of PML (Percherancier *et al*., 2009, Tatham *et al*., 2008). This was somewhat surprising, as sumoylation had previously been thought to protect PML from such degradation. For example, sumoylation has been reported to protect PML from degradation dependent on the activity of the prolyl-isomerase Pin1 (Reineke *et al*., 2008). These conflicting data suggest that the precise pattern of sumoylation on PML may play an important role in regulating the stability of PML protein

Phosphorylation

Transient phosphorylation of PML is known to regulate some of its functions, such as its ability to promote apoptosis in response to arsenic trioxide (Hayakawa & Privalsky, 2004). PML phosphorylation also occurs during the cell cycle. Work by Everett and colleagues demonstrated that, during mitosis, a novel phosphorylated form of PML protein was produced (Everett *et al*., 1999). Furthermore, the appearance of this phosphorylated form coincided with the desumoylation of PML and hence the dispersal of PML NBs (Everett *et al*., 1999). However, the functional significance of this phosphorylated form of PML remains unknown.

A better characterised example of PML phosphorylation is that described by Scaglioni *et al*. They showed that PML is selectively phosphorylated at serine 517 in exon5-deleted PML (Ser565 in full-length PML) by casein kinase 2 (CK2), which is known to be up-regulated in many tumours. Phosphorylation of PML at this residue results in the ubiquitination of PML and its subsequent degradation by the

proteasome (Scaglioni *et al*., 2006). Therefore CK2 is thought to be an important regulator of PML stability. Interestingly, Ser565 is located within exon 7a and is only common to PMLs I-V, and thus PML VI is unlikely to be subject to degradation via this mechanism. Phosphorylation has also been associated with the degradation of PML in a manner dependent on the prolyl isomerise Pin1. Pin1 utilises its WW domain to bind PML IV phosphorylated on Ser527, and initiates its degradation (Reineke *et al*., 2008). SUMO-1 was found to negatively affect this process, as Pin1 was unable to bind sumoylated PML. Since Ser527 is encoded within exon 6, this mechanism may apply to multiple PML isoforms.

There is increasing evidence that suggests that both SUMO and phosphorylation modifications of PML may 'cross-talk', adding a further layer of complexity to the regulation of these proteins. Cells treated with phosphatase inhibitors undergo desumoylation of their PML proteins, suggesting that phosphorylation may negatively regulate sumoylation (Muller *et al*., 1998). However, it is unclear whether this applies to all of SUMO 1-3 or just to individual homologs, and other studies have reported that phosphorylation promotes sumoylation (Hayakawa & Privalsky, 2004). The explanation of this conflict is likely to be that phosphorylation of specific residues may have very different effects on sumoylation. Overall, it is likely that phosphorylation, acting in conjunction with sumoylation, performs an important role in regulating PML function and stability.

Acetylation

It has recently come to light that PML is subject to acetylation on lysines 487 and 515 by the histone acetyltransferase (HAT) p300; both of these residues are encoded in exon 6 (Hayakawa *et al*., 2008). These lysines were acetylated *in vivo* after the addition of trichostatin A (TSA), an inducer of apoptosis. Expression of the acetyl transferase p300 led to an increase in the sumoylation of wild-type PML, but not of mutants substituted with arginine at lysines 487 and 515, suggesting that acetylation may enhance sumoylation. Lysine 487 is located within the NLS of PML, though its acetylation did not affect PML sub-cellular localisation (Hayakawa *et al*., 2008). Since exon 6 is excluded from cytoplasmic PMLs, this acetylation can only occur on nuclear PML proteins.

1.3.3 PML NBs

By immunofluorescent staining, PML NBs appear as small spherical structures within the nucleus that may be up to 1µm in diameter, and may number up to 30 bodies within a single nucleus (Bernardi & Pandolfi, 2007). Examination by electron microscopy had shed light on the structure of PML NBs, as they were initially thought to be composed of a ring-like structure (Bernardi & Pandolfi, 2007). However, recent study has indicated that, during interphase, a PML NB is a hollow shell composed of both PML and Sp100 (Lang *et al*., 2010). PML NBs are therefore proteinaceous structures and, except for those associated with telomeres in cell lines that maintain telomere length in the absence of telomerase (ALT cells), do not contain any detectable levels of nucleic acid, though they do associate with transcriptionally active chromatin. For example, PML NBs associate with the MHC (Major Histocompatibility Complex) locus, where they are thought to indirectly contribute to transcription by altering the higher-order structure of chromatin (Pavan Kumar *et al*., 2007).

Both the size and number of NBs is dependent on numerous factors, reflecting their highly dynamic nature. The number of NBs per nucleus varies between cell types, though this number may further vary within a cell according to various stresses. For example, DNA damage by UV irradiation or chemical agents leads to increase in both the size and number of PML NBs (Dellaire, 2004, Dellaire *et al*., 2006a). This reflects the involvement of these bodies in DNA damage responses, as a number of DNA damage proteins co-localise with PML NBs (Krieghoff-Henning & Hofmann, 2008). Under certain circumstances PML NBs have also been shown to be formed *de novo*. Incoming exogenous DNA, whether it be a viral genome or plasmid, is often associated with the formation of new PML NBs, an observation thought to be a general cellular defence mechanism that initiates gene repression (Bishop *et al*., 2006, Everett & Murray, 2005, Tavalai & Stamminger, 2008).

As mentioned above, PML NBs are primarily composed of PML isoforms I-VI, and indeed PML NBs do not form in the absence of PML protein. Sumoylated PML protein is associated with PML NBs, and subsequent desumoylation leads to the

disassembly of these structures, as seen during mitosis (Everett *et al*., 1999). A main mechanism of PML NB assembly is thought to involve homo- and hetero-oligomerisation of PML isoforms via the RBCC motif alongside interactions between sumoylated proteins and the SIM located in PML exon 7a (Shen *et al*., 2006). Experiments by Weidtkamp-Peters and colleagues have suggested that PML V is likely to be an important scaffold component of PML NBs, since it shows little exchange with the surrounding nucleoplasm in contrast to PMLs I-IV and VI. (Weidtkamp-Peters *et al*., 2008). Interestingly, this study also suggested that the sumoylation status of PML protein was an important regulator of its exchange dynamics. Individual isoforms were also shown to exhibit unique rates of exchange and thus, in terms of PML content, at any one time the composition of PML NBs may vary considerably (Weidtkamp-Peters *et al*., 2008).

Over 50 proteins unrelated to PML have been shown to associate with PML NBs. Such proteins are now thought to pass relatively freely through the PML/Sp100 shell into the hollow interior, which is enriched with polymeric SUMO 2/3 chains (Lang *et al*., 2010). These chains provide possible binding targets for any proteins possessing a SIM, and therefore facilitate association with the PML NB. Therefore, reflecting their diversity in structure and PML content, PML NBs are also highly variable in protein composition (Bernardi & Pandolfi, 2007). It should be noted, however, that the vast majority of proteins shown to co-localise with PML NBs do so only in a transient manner and often only in response to certain stimuli. For example, many factors associated with DNA repair move to PML NBs only in response to DNA damage (Dellaire, 2004, Krieghoff-Henning & Hofmann, 2008). Such observations have implications when trying to understand the functions of PML NBs, as proposing a single function that reconciles the roles of all the protein factors shown to interact with PML NBs is difficult and may not be appropriate.

Other NB components, such as Sp100 and Daxx, are more stably associated with PML NBs. Daxx itself is sumoylated, and is recruited to PML NBs by SUMO-modified PML (Ishov *et al*., 1999). However, such associations are not necessarily exclusive, as Daxx has also been found in both the cytoplasm and nucleoplasm. It is of note that both Daxx and Sp100, as well as numerous other proteins, are

sumoylated at PML NBs, an observation thought to enhance their association with these bodies (Van Damme *et al*., 2010).

1.3.4. Functions of PML and PML NBs

Since the main constituent of PML NBs is PML protein, many of the functions that have been proposed for PML NBs have been attributed to PML protein. Care should be taken with this assumption however, as a multitude of other proteins are known to associate with PML NBs (Bernardi & Pandolfi, 2007). A further difficulty is the diversity within the cellular PML protein population, which may be divided into at least seven protein isoforms (Jensen, 2001). Since each of these has unique sequence features, they may be assumed to possess unique functions. Further functional diversity may also be generated by the extensive post-translational modification that a PML protein may undergo. These considerations must be taken into account when reviewing the functions of PML and PML NBs that are described below.

Roles in the DNA damage response

The observed changes that occur to the PML NB organisation of a cell that receives a DNA-damaging insult prompted investigations into their role in the DNA damage response (Dellaire, 2004). PML NBs localise to sites of DNA damage and co-localise with a number of proteins that are actively involved in DNA repair (Dellaire, 2004). Under normal conditions, a number of proteins involved in DNA repair, such as ataxia telangiectasia-mutated gene product (ATM), localise to PML NBs upon DNA damage (Salomoni *et al*., 2008). In contrast others, including Nijmegen breakage syndrome gene product (NBS1) and Mre11, dissociate from PML NBs upon DNA damage, though re-localise with PML at later time points (Mirzoeva & Petrini, 2001). These somewhat conflicting observations have proved to be obstacles to suggesting a unifying function of PML NBs in the DNA damage response (Bernardi & Pandolfi, 2007). It has therefore been proposed that PML NBs may act as sites of storage for various DNA repair proteins, and also may act as sensors of DNA damage that can link DNA repair responses to signalling responses. This latter

notion stems from observations that various kinases involved in regulating the cell cycle, including ATR, ATM and the checkpoint kinase Chk2, also co-localise with PML NBs (Krieghoff-Henning & Hofmann, 2008).

Role in apoptotic mechanisms and cell cycle control

The characteristic mutation of the *pml* gene in APL patients pointed to PML protein as being a tumour suppressor, though little was known about its biological function. Wang and colleagues later reported that PML protein played an integral role in numerous apoptotic mechanisms and was required for caspase-1 and caspase-3-dependent apoptosis (Wang *et al*., 1998). Indeed, *Pml -/-* mice were less sensitive to multiple apoptotic stimuli such as ionising radiation, interferon treatment and ceramide treatment than wild-type mice and are more susceptible than normal mice to tumours (Wang *et al*., 1998). These activities can account for PML's role as a tumour suppressor. In other studies, caspase-2 and caspase-6 have both been associated with PML NBs, and at least in the case of caspase-6, perform functions within the vicinity of these bodies (Tan *et al*., 2008, Tang *et al*., 2005).

A well-documented example of a transient PML NB localisation is that of the tumour suppressor p53. Upon DNA damage, p53 is recruited to PML NBs where it is subsequently post-translationally modified and activated to perform its pro-apoptotic functions (Fogal *et al*., 2000, Pearson *et al*., 2000). PML protein has been shown to interact with p53 in a number of ways, and is an important regulator of p53 activity. For instance, p53 is recruited, along with the acetyltransferase p300, to PML NBs by PML IV, allowing p53 to be acetylated (Fogal *et al*., 2000). Many factors involved in the stability of p53 are also regulated by PML. For instance, casein kinase 1 activity, a regulator of p53, is enhanced by PML protein (Alsheich-Bartok *et al*., 2008). Also, the E3 ubiquitin ligase MDM2, a negative regulator of p53, is itself inhibited by direct binding to PML, and thus PML prevents p53 degradation (Louria-Hayon *et al*., 2003). Upon DNA damage, PML protein was found to bind MDM2 and sequester it in the nucleolus (Bernardi *et al*., 2004). This interaction was mediated by the region around the coiled-coil domain which is shared by all PML isoforms. Thus all members of the PML protein population may be involved in p53 regulation.

The structure of PML NBs is highly dynamic throughout the cell cycle, varying in size and number depending on the phase. This is thought to be mainly due to modifications in chromatin structure that inevitably cause fission of adjacent nuclear bodies, as represented by the numerous PML NBs during the G2 phase (Dellaire *et al*., 2006b). In contrast, PML becomes dispersed throughout the cytoplasm during mitosis, leading to the formation of PML aggregates termed mitotic accumulations of PML protein (MAPPs) (Dellaire *et al*., 2006c). These, however, are unlike PML NBs as they lack other NB components such as Sp100 and Daxx. Whether or not these changes in PML organisation during mitosis have an active role in the process is not known.

A subset of PML NBs co-localises with telomeric DNA and contains factors required for the alternative lengthening of telomeres (ALT). Termed ALT-associated PML NBs (APBs), they contain factors required for DNA repair as well as telomeric repeat binding factors (TRFs) 1 and 2 (Jiang *et al*., 2007). It has thus been proposed that PML plays a role in maintaining telomere length. PML III directly binds to TRF1 and is required for the formation of APBs, but not PML NBs (Yu *et al*., 2009). In contrast, PML IV has been shown to interact directly with the telomere reverse transcriptase (TERT) and inhibit it (Oh *et al*., 2009), suggesting a complex regulatory role of specific PML isoforms in this subset of PML bodies.

PML isoform-specific functions

Despite the implication of PML in numerous cellular processes, very little is known about specific functions of individual isoforms. However, there are examples where a single PML isoform has been demonstrated to perform a distinct function. PML IV, for example, directly interacts with p53 (Fogal *et al*., 2000). This interaction is mediated by the C-terminus of this isoform, and results in the recruitment of p53 to PML NBs where it is activated. PML relocalisation to the cytoplasm is thought to inhibit p53 activity (Bellodi *et al*., 2006). PML IV has been implicated in causing premature senescence (Bischof *et al*., 2002), an activity which is independent of both the sumoylation state of PML and the presence of PML NBs. Roles for other PML isoforms, however, cannot be discounted, as PML IV was unable to induce

senescence in cells a *pml -/-* genotype (Bischof *et al*., 2002). PML IV also binds the oncoprotein Myc and induces its degradation, a function that is dependent on its RING domain (Buschbeck *et al*., 2007). These observations, in combination with the negative activity of PML IV on TERT (Oh *et al*., 2009), suggest that this isoform has important roles in regulating apoptosis, differentiation and senescence.

Studies on PML III have revealed a possible role in the maintenance of the centrosome (Xu *et al*., 2005). PML III was shown to associate with the centrosome and to inhibit its duplication by inhibiting the Aurora A kinase, over-expression of which leads to centrosome duplication. PML III co-precipitated with Aurora A, and furthermore led to a decrease in the amount of its phosphorylated, active form (Xu *et al*., 2005). This suggests that PML III may bind to and regulate the post-translational modification of Aurora A, though it should be noted that during subsequent studies by another group PML III was not observed to localise to the centrosome (Condemine *et al*., 2006).

Recently, other roles of PML III have been described. The tumour suppressor TIP60 was found to bind a region specific to PML III, and this binding was sufficient to inhibit its MDM2-mediated degradation (Wu *et al*., 2009). This prompted the authors to speculate that PML III may compete with MDM2 for TIP60 binding. Also, as mentioned above, a study by Yu and colleagues demonstrated that PML III was crucial in the recruitment of telomeric repeat binding factor 1 (TRF1) to APBs. It was therefore suggested that PML III may be required for the formation of this particular subset of PML NBs (Yu *et al*., 2009).

Cytoplasmic PML (cPML) may also perform important roles in a number of cell processes. For instance, a cytoplasmic PML IV variant lacking exons 5 an 6 is required for efficient TGF-β signalling in mouse embryonic fibroblasts (MEFs) (Salomoni & Bellodi, 2007) Furthermore, nuclear sequestration of cPML results in a decrease in TGF-β signalling, indicating that its activity may be modulated by its sub-cellular localisation (Salomoni & Bellodi, 2007). TGF-β has roles in proliferation and apoptosis, and through regulating its activity, cPML may exert effects on these processes independently of nuclear PML proteins. cPML has also been shown to bind directly to and suppress the activity M2-type pyruvate kinase

(PKM2), indicating a role in the regulation of glycolysis (Nobukazu *et al*., 2008). However, the biological significance of this is unclear, as the cPML utilised was a NLS mutant of full-length PML VI and not a cytoplasmic splice variant of PML.

1.3.5. Antiviral activity of PML and PML NBs

As well as the functions listed above, PML protein and PML NBs have been extensively implicated in anti-viral functions. There are several lines of evidence to support this hypothesis, though as yet the precise mechanism of this activity remains unknown. The following sections summarise the evidence supporting an anti-viral role for PML proteins. Particular emphasis is placed on documented virus-PML interactions.

1.3.6. PML is induced by interferon

Pml is a primary target gene for interferon activation (i.e. it is an ISG) and has therefore been implicated in performing antiviral functions. The first intron of the *pml* gene contains both ISRE and GAS elements, responsive to type I (α and β) and type II (γ) interferons respectively. Upon interferon treatment, PML transcription is up-regulated, resulting in an increase in both the size and number of PML NBs (Lavau *et al*., 1995). As well as PML, other components of PML NBs are themselves encoded by ISGs, providing further evidence of an antiviral role for these structures. Sp100, for instance, is also up-regulated by type I and II interferons.

However, there is also evidence which suggests that PML may act in a negative feedback mechanism to inhibit the IFN response. Choi *et al* discovered that, in mouse embryonic fibroblasts, PML acts as an inhibitor of IFN γ signalling (Choi *et al*., 2006). A comparison of PML -/- cells with PML +/+ cells revealed that PML explicitly inhibited the DNA binding capacity of STAT-1α, thereby reducing the induction of IFNγ genes (Choi *et al*., 2006) The mechanism through which PML achieves this remains unclear, but it was suggested to be mediated by protein-protein interactions. Interestingly, the PML isoforms examined (I, III, IV) all exhibited this same activity, though to varying degrees, suggesting a role for the common N-

terminal domain of PML. IFNγ is a type II IFN, and so these results do not apply to type I IFN signalling.

1.3.7. PML is a TRIM protein

As mentioned above, the RBCC motif possessed by PML protein is a defining characteristic of the TRIM family of proteins. At present, genes for over 65 members of this protein family have been identified within the human genome, though many of these remain to be characterised (Nisole *et al*., 2005). The role of individual TRIM proteins within the cell varies considerably, though there is increasing evidence to suggest that a number of TRIM proteins perform anti-viral duties (Nisole *et al*., 2005, Ozato *et al*., 2008). For example, TRIM5α acts as a restriction factor against retrovirus replication through an as-yet unknown mechanism (Towers, 2007).

This view of TRIM protein function has been strengthened by a recent study that analysed global expression levels of TRIM proteins in response to interferons. Carthagena *et al* demonstrated that, out of 72 TRIM proteins tested in two cell types, 24 were directly up-regulated by either type I or type II interferons, including PML protein and TRIM5 (Carthagena *et al*., 2009). The activity of individual TRIM proteins may vary depending on the host cell, since TRIM protein expression patterns can vary according to the cell type (Rajsbaum *et al*., 2008).

1.3.8. Viral interactions with PML protein and PML NBs

Perhaps the most compelling line of evidence supporting an anti-viral role of PML and/or PML NBs is that they are subject to targeting and disruption by a number of viruses (Leppard & Dimmock, 2006). A variety of DNA and RNA viruses are known to interact with PML NBs and PML protein, though often the functional consequences remain elusive. Below is a summary of known virus-PML interactions (Table 1.2).

Virus	Effect on PML/PML NBs
Lymphocytic choriomeningitis virus (LCMV) Lassa Fever virus (LFV)	Relocalisation of PML to the cytoplasm
Respiratory syncytial virus (RSV)	Relocalisation of PML and Sp100 to cytoplasm
Influenza A (FLUAV)	Viral proteins M1, NS1 and NS2 localise to PML NBs Viral replication suppressed by PML III, IV and VI
Human foamy virus (HFV)	Viral gene expression suppressed by PML III
Vesicular stomatitis virus (VSV)	Viral gene expression suppressed by PML III
Rabies virus (RABV)	Increase in size of PML NBs
Hepres simplex virus type 1 (HSV-1)	Desumoylation and degradation of PML Infection leads to production of truncated PML Ib
Herpes simplex virus type 2 (HSV-2)	Decreases PML II:PML V ratio
Human cytomegalovirus (HCMV)	Desumoylation of PML and dispersal of PML NBs
Epstein-Barr virus (EBV)	Accumulation of Unsumoylated PML Degradation of PML, possibly via PML IV
Human Papilloma virus (HPV)	Degradation of PML IV Modulation of PML IV activity
Adenovirus (AdV)	Loss of sumoylated PML Production of novel PML species Rearrangement of PML NBs via PML II Formation of inclusion bodies

Table 1.2 Interactions between viruses and PML NBs. Table summarising known interactions between RNA and DNA viruses and PML proteins and PML NBs. Where known, the PML isoform responsible is indicated.

RNA viruses

Proteins from several RNA viruses have been shown to interact with PML NBs, and in some cases cause active disruption of these bodies. PML re-localisation to the cytoplasm was observed during infection by the arenavirus lymphocytic choriomeningitis virus (LCMV) (Borden *et al*., 1998). It was subsequently shown that the arenavirus Z protein was responsible for this phenomenon, as the Z proteins of both LCMV and Lassa fever virus (LFV) target PML NBs through a direct interaction with PML, and subsequently cause its re-localisation to the cytoplasm (Kentsis *et al*., 2001). Once there, PML directly interacts with the translation initiation factor eIF4E and reduces its affinity for the 5' cap of cellular mRNA (Kentsis *et al*., 2001), thus contributing to translational shutoff during these viral infections.

Redistribution of PML to the cytoplasm is also observed during infection with the paramyxovirus respiratory syncytial virus (RSV). RSV replicates exclusively in the cytoplasm. However, upon infection PML protein and the PML NB component Sp100 both dissociate from PML NBs and re-localise to the cytoplasm and by 24 hours post infection exhibit a diffuse staining pattern (Brasier *et al*., 2004).

A number of proteins encoded by the orthomyxovirus influenza A virus co-localise with PML NBs. During infection the M1, NS1 and NS2 proteins migrate to the nucleus and associate with PML NBs, though the reason for doing so remains unclear (Sato *et al*., 2003). Influenza A virus replication is significantly repressed by over-expression of PML IV and PML VI, and correspondingly, depletion of the total PML content leads to an enhancement in viral propagation (Iki *et al*., 2005). This suggests that PML protein exerts an antiviral activity against influenza A. However, there is evidence to suggest that the effectiveness of this antiviral activity varies depending on the subtype of influenza A virus (Li *et al*., 2009). The mechanism underlying this variation remains unknown.

The expression of PML III has been shown to exert an inhibitory effect on some virus infections. Over-expression of PML III during human foamy virus (HFV) infection led to a decrease in viral mRNA and proteins levels (Regad *et al*., 2001).

PML III mediated this effect by binding and sequestering the viral transactivator Tas, preventing it from activating viral gene expression. Interferon-mediated inhibition of HFV was also found to act in a PML-dependent manner, as IFN treatment of wild-type, but not *pml -/-* cells, led to suppression of viral replication (Regad *et al*., 2001). Both influenza A and the rhabdovirus vesicular stomatitis virus (VSV) also showed markedly reduced viral titres in cells over-expressing PML III (Chelbi-Alix *et al*., 1998). However, PML III over-expression had no effect on the replication of the picornavirus encephalomyocarditis virus (EMCV) (Chelbi-Alix *et al*., 1998), or rabies virus, a rhabdovirus (Chelbi-Alix *et al*., 2006). It should be noted though that the rabies virus P protein does interact with PML NBs, causing them to increase in size (Chelbi-Alix *et al*., 2006), and is also responsible for this virus overcoming the IFN response (Chelbi-Alix *et al*., 2006).

PML protein is also implicated in the replication of poliovirus, albeit in a p53-dependent manner. p53 exerts an antiviral effect on viral replication which requires the presence of PML protein (Pampin *et al*., 2006). Upon poliovirus infection, PML NBs become larger due to an influx of nucleoplasmic PML. PML proteins are then phosphorylated by the mitogen activated protein kinase ERK, leading to their subsequent sumoylation. This leads rapidly to p53 recruitment to PML NBs and its activation in order to perform its pro-apoptotic functions. Poliovirus counters this by initiating the degradation of p53 in a manner that is partly dependent on MDM2 (Pampin *et al*., 2006).

DNA viruses

The most intensive study of PML-virus interactions has been performed in the context of infection by DNA viruses. Replication of a number of DNA viruses is closely associated with PML NBs, and often leads to their disruption. Furthermore, PML NBs have been strongly implicated in performing anti-viral functions against these viruses (Everett, 2001, Everett, 2006).

Herpes simplex virus type I and 2 (HSV-1, HSV-2)

The most intensively-studied example of virus-PML interactions is that observed during the course of infection by the alphaherpesvirus HSV-1. Upon infection, new PML NBs are formed around incoming viral DNA (Everett & Murray, 2005), adjacent to which a number HSV-1 proteins accumulate, such as ICP4, VP13/14 and VP22. The most dramatic association, however, occurs upon localisation of ICP0 to PML NBs. After localisation to PML NBs, ICP0 initiates the desumoylation of SUMO-1 modified PML proteins, resulting in their subsequent dispersal and proteasome-mediated degradation (Everett *et al*., 1998), an action dependent on the function of ICP0 as an E3 ubiquitin ligase (Boutell *et al*., 2002). Other PML NB-associated proteins, such as Sp100, are also degraded in this manner. This precedes the onset of viral DNA replication. ICP0 stimulates lytic infection and this ability correlates with the dispersal of PML NBs. However, later in infection PML protein is recruited into replication centres by the viral polymerase, suggesting that the role PML in HSV-1 infection may include functions other than its antiviral activity (Burkham *et al*., 1998).

There is compelling evidence that suggests that ICP0-mediated disruption of PML NBs is closely linked to overcoming cellular antiviral responses. For instance, unlike wild-type virus, ICP0-deficient HSV-1 viruses are sensitive to interferon and grow to lower titres in mice (Everett *et al*., 2006, Leib *et al*., 1999, Mossman *et al*., 2000). This growth defect was partially corrected in transgenic mice defective in IFN I signalling (Leib *et al*., 1999). It was subsequently shown that ICP0 is required by HSV-1 to overcome IFN responses (Härle *et al*., 2002, Mossman *et al*., 2000).
Both wild-type and ICP0 mutant viruses grew to similar titres in *pml* +/+ and *pml* -/- mouse embryonic fibroblasts (MEFs) (Chee *et al*., 2003). However, interferon treatment considerably reduces ICP0 mutant replication in *pml* +/+ MEFs, whilst having no effect in *pml* -/- MEFs. This suggests that the ability of IFN to affect HSV-1 replication is dependent on the PML protein. However, in human fibroblasts (HFs) depleted of PML, both wild-type and ICP0-deficient HSV-1 remained sensitive to IFN-β, though to a much lesser extent than that observed in HFs containing normal levels of PML (Everett *et al*., 2008). The mechanism whereby ICP0 overcomes the IFN response is still unclear, as there are contrasting reports as

to whether it can mitigate IFN signalling pathway and inhibit the expression of ISGs. Initial reports found that ICP0 inhibited the activity of both IRF3 and IRF7, leading to a decrease in the expression of ISGs (Lin *et al*., 2004). However, Everett and colleagues subsequently found that the growth of ICP0-deficient mutants was not improved in IRF3-deficient HFs (Everett *et al*., 2008), and that ICP0 did not interfere with the IRF3-dependent induction of ISGs (Everett & Orr, 2009).

PML knock-down also has no effect on HSV-1 replication, though does result in an increase in viral gene expression of ICP0 mutant HSV-1 (Everett *et al*., 2006), offering a possible explanation for earlier observations where IFN treatment repressed viral gene expression (Taylor *et al*., 2000). Over-expression of a subset of PML isoforms (III, IV and VI), however, had no effect on virus replication, possibly indicating a role for alternative isoforms in inhibiting HSV-1 replication (Tavalai & Stamminger, 2008).

Recent work by McNally has indicated that upon infection with HSV-1, the ratio of cytoplasmic PML proteins is increased amongst the PML population (McNally *et al*., 2008). A novel cytoplasmic variant, termed PML Ib, which lack exons 5 and 6 and possesses a stop codon in exon 7a, was demonstrated to exert a strong antiviral activity against HSV-1. Cells over-expressing the variant displayed reduced expression of ICP0 and Us11 proteins and viral titres were reduced by approximately 10-fold. In contrast, expression of full-length, nuclear PML I had no effect. Interestingly, PML Ib appears to exert its effect through ICP0 by sequestering it within the cytoplasm, thereby reducing expression of viral genes because PML NB disruption is impaired (McNally *et al*., 2008).

Herpes simplex type 2 (HSV-2) also alters the relative ratio of PML isoforms. Upon infection, the ratio of PML V to PML II transcripts is increased (Nojima *et al*., 2009). The viral protein responsible for this was ICP27, which was shown to bind PML pre-mRNA and inhibits splicing from the 3' splice site of exon 7a, resulting in intron retention and read-through to exon 7. The reason why the virus promotes this isoform switching remains unclear, as over-expression of PML II was found paradoxically to enhance viral replication (Nojima *et al*., 2009). The authors

suggested that this may be a mechanism employed by the virus to favour a persistent infection.

Human cytomegalovirus (HCMV)

In the case of HCMV, a betaherpesvirus, PML NBs exert a repressive effect on viral gene activity. For instance, the PML NB component Daxx has been shown to alter the chromatin structure of the viral major immediate early promoter (MIEP) into a repressive state, an activity dependent on histone deacetylases (HDACs) (Saffert & Kalejta, 2006). In order to overcome this, the viral protein pp71, a tegument protein found in the incoming particle, migrates to the nucleus and localises to PML NBs via a direct interaction with Daxx (Cantrell & Bresnahan, 2006, Saffert & Kalejta, 2006). pp71 then initiates the degradation of Daxx and thus relieves repression of the MIEP. Indeed, siRNA-mediated knockdown of Daxx can complement the growth defect of pp71-deficient virus and enhance wild-type virus titres, whereas Daxx over-expression had the opposite effect (Saffert & Kalejta, 2006). PML NB disruption also occurs during HCMV infection, and is maintained by the viral IE1 protein, which initiates the desumoylation and degradation of PML in a manner independent of the proteasome, and hence causes the dispersal of PML NBs (Xu *et al*., 2001). Knock-down of PML proteins can complement the growth of IE1-deficient mutants, emphasising the importance of PML NBs in the limitation of virus replication (Tavalai *et al*., 2006).

Other DNA viruses

Epstein-Barr virus (EBV), a gammaherpesvirus, BZLF-1 protein has been shown to localise to PML NB and consequently to facilitate their dispersal through the desumoylation of PML. Interestingly, the mechanism employed by EBV differs from that of HSV-1 and HCMV as BZLF-1 is itself sumoylated, and is thought to out-compete PML for SUMO binding (Adamson & Kenney, 2001). Unsumoylated PML therefore accumulates, and PML NBs disintegrate into the nucleoplasm. The ability of BZLF-1 to disperse PML NBs correlates with its ability to activate viral gene transcription, thus further suggesting that PML NBs act to repress viral gene expression (Adamson & Kenney, 2001). Whilst BZLF-1 is the key initiator of the

cascade of EBV lytic gene expression, over-expression of the EBNA-1 protein, which is expressed in latently infected cells, also led to the disruption of PML nuclear bodies and a decrease in cellular PML protein levels, which was found to be dependent on the cellular ubiquitin-specific protease USP-7 (Sivachandran *et al*., 2008). EBNA-1 was found to preferentially bind PML IV, indicating that it is through this interaction whereby PML disruption occurs (Sivachandran *et al*., 2008).

A number of proteins encoded by human papilloma virus (HPV) have been associated with PML NBs. The E6 proteins of HPV-11 and HPV-18 were demonstrated to co-localise with PMLs I-IV (Guccione *et al*., 2004). Subsequent experiments showed that the E6 proteins of both virus subtypes interacted with PMLs I, II and IV. The E6 protein of HPV-18, but not HPV-11, induced the proteasome-mediated degradation of PML IV, thereby inhibiting its ability to induce senescence (Guccione *et al*., 2004). The HPV-16 E7 protein was also found to overcome PML-IV induced senescence by inhibiting its ability to activate p53 (Bischof *et al*., 2005).

Upon over-expression, the HPV L2 capsid protein has also been demonstrated to associate with PML NBs through an interaction with Daxx (Day *et al*., 1998, Florin *et al*., 2002, Heino *et al*., 2000). L2 expression causes disruption to PML NBs by causing a decrease in levels of Sp100 (Florin *et al*., 2002). Depletion of Sp100 coincides with the recruitment of another capsid protein, L1, and viral protein E2 to PML NBs. L2 association with PML has therefore been proposed to facilitate viral assembly, though upon lower levels of expression, L2 only seldom associates with PML NBs (Kieback & Müller, 2006). The significance of L2-mediated disruption of PML NBs therefore remains unclear.

Adenovirus (Ad)

PML NBs become disrupted in a unique fashion during the early phase of infection with Ad5. Carvalho *et al* first observed that upon infection PML NBs were redistributed from their native spherical structures into elongated ‘tracks’ (Carvalho *et al*., 1995). Through the use of transient over-expression of individual proteins and viral mutants, this redistribution of PML NBs was found to be dependent on the viral

early gene product E4 Orf3 (Carvalho *et al*., 1995, Doucas *et al*., 1996); this function was conserved across a variety of Ad subtypes (Stracker *et al*., 2005). Indeed, E4 Orf3 has subsequently been shown to directly interact with the C-terminal domain of PML isoform II, and it is through this interaction whereby PML NBs become redistributed (Hoppe *et al*., 2006, Leppard *et al*., 2009).

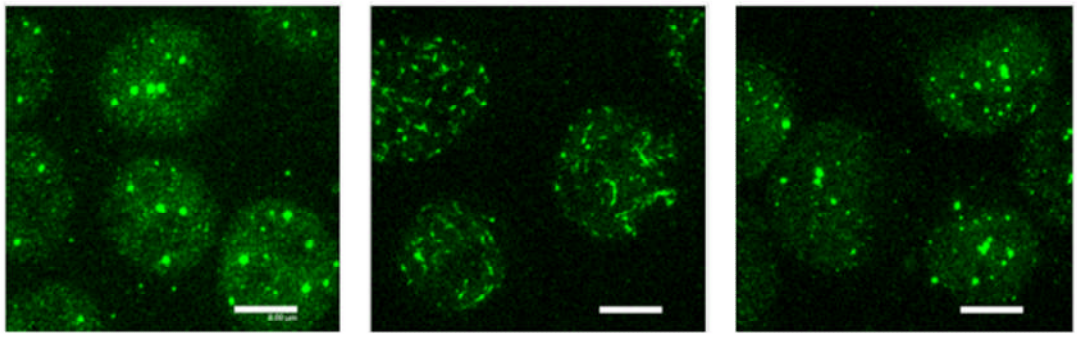


Figure 1.4. Ad5 infection results in reorganisation of PML NBs into tracks. HEp-2 cells were mock-infected or infected with the Ad5 viruses indicated for 18 hours before being fixed and probed with an anti-PML antibody and visualised by confocal microscopy. Images reflect a series of images through the Z-axis. In mock-infected cells (left panel) PML NBs exhibit a spherical appearance that becomes altered upon infection with wild-type Ad5 (centre panel). In contrast, an Ad5 mutant unable to express E4 Orf3 does not reorganise PML NBs, demonstrating the requirement for this protein in this process. Scale bar - 8µm.

Interestingly, E4 Orf3 also exerts two distinct effects on the PML protein population. Leppard and Everett reported that in the later phases of infection with wild-type Ad5, higher molecular weight sumoylated PMLs were lost, and this coincided with the appearance of a novel, infection-specific species of PML (Leppard & Everett, 1999). By 24 hours of infection, sumoylated PMLs were barely detectable by western blot and the novel species of PML was now amongst the most dominant species. Infection with a virus mutant incapable of producing E4 Orf3 failed to cause either loss of sumoylated PMLs or the appearance of this novel PML species (Leppard & Everett, 1999). Intriguingly, the novel species of PML was very similar to a mitosis-

specific species of PML previously observed by Everett and colleagues, which was shown to be a phosphorylated species (Everett *et al*., 1999).

The role of PML NB disruption during the course of Ad5 infection remains elusive, though there is evidence to suggest it may mitigate a cellular antiviral response. (Ullman *et al*., 2007). In cells pre-treated with IFN α or γ, E4 Orf3-mutant Ad5 replication was reduced 15-20 fold compared to untreated cells, in contrast to the minimal effect of IFN on the replication of wild-type virus. The ability of E4 Orf3 to overcome the antiviral state induced by interferon correlated with its ability to rearrange PML NBs, but not with its ability to disrupt the MRN complex (Ullman *et al*., 2007). Ullman then went on to demonstrate that depletion of PML by shRNA could restore the growth of the E4 Orf3 mutant virus in cells pre-treated with IFN (Ullman & Hearing, 2008). Interestingly, depletion of Daxx, but not Sp100 also restored the growth of the E4 Orf3 mutant. Finally, expression of HSV-1 ICP0 and HCMV IE72, two other viral proteins known to disrupt PML NBs (as discussed above), restored the growth E4 Orf3 mutant Ad5 in IFN-treated cells (Ullman & Hearing, 2008). Therefore there is strong evidence that E4 Orf3-mediated disruption of PML NBs is a mechanism employed by Ad5 to overcome cellular antiviral activities that are performed by PML NBs or their components.

At later time points during Ad5 infection other viral proteins begin to associate with PML NBs. Protein IX localises to PML NBs and forms ‘amorphous inclusion bodies’, and it has been proposed that protein IX may act to maintain the disruption of PML NBs, caused initially by E4 Orf3, throughout the later phases of infection when Orf3 expression wanes (Rosa-Calatrava, 2003). Furthermore, impairing the ability of pIX to form the inclusions resulted in reduced viral yields, suggesting this to be a mechanism important for the viral life cycle (Rosa-Calatrava, 2003).

In summary, there is much evidence implicating PML proteins as important mediators of the IFN response, and a large number of viruses selectively disrupt PML protein or PML NBs, including Ad5. Taken together, these facts strongly suggest an anti-viral role of PML proteins, though how PML proteins achieve this remains incompletely understood. As well as inducing the reorganisation of PML NBs into tracks, Ad5 infection leads to the production of a novel, as yet unidentified, species of PML protein through an unknown mechanism. The production of such a species is likely a reflection of the diversity of PML proteins that may be generated by differential splicing and post-translational modifications. The functional significance of the production of this species, as well as PML NB rearrangement, by Ad5 remains unclear, though these effects may form part of a strategy employed by Ad5 to overcome antiviral responses.

Study Aims

This study seeks to expand on the knowledge of Ad5-PML interactions, particularly by identifying PML isoforms that may be altered upon infection. In parallel, the role of individual PML isoforms in the type I IFN pathway is investigated, with an ultimate goal of establishing the functional significance of Ad5-induced changes in the PML population to overcoming innate immune responses. The aims can therefore be stated as:

1. To investigate changes in the PML protein population induced upon Ad5 infection and to characterise such changes by identifying both the Ad5 proteins and PML isoforms responsible, as well as any relevant post-translational modifications that may be involved.

2. To examine the roles played by individual PML isoforms in the type I IFN signalling pathway and, if possible, reconcile this with the changes in PML imposed by Ad5 infection.

Chapter 2

Materials and Methods

2.1 Materials

The following section lists the entire collection of virus strains, cell lines, bacterial strains and additional materials that were utilised during the course of this study.

Table 2.1 Bacterial strains used during the course of this investigation

***Escherichia coli* strain**	**Phenotype**
TG2	K12, *lac-pro supE thi hsdD5* (F' *traD36 proA*$^{+}$*B*$^{+}$ *lacI*^{q} *lacZ* M15) *recA*::Tn*10*
DH5α	*supE*44, Δ*lac*U169(φ80*lacZ*ΔM15), *hsdR*17, *recA*1, *endA*1, *gyrA*96, *thi*-1, *relA*1

Table 2.2 Adenovirus strains used during the course of this investigation

Strain	**Phenotype**
*wt*300	Wild type (Jones & Shenk, 1978)
*in*Orf3	E4 frame-shift insertion (300 background) (Huang & Hearing, 1989)

Table 2.3 Cell lines used during the course of this investigation

Cell Line	**Origin**
A549	Human lung carcinoma (Giard, 1973)
HEK293	Human embryonic kidney (Graham *et al*., 1977)
HEp2	Human epidermoid carcinoma (Moore *et al*., 1955)
U2OS	Human osteosarcoma (Ponten, 1967)
MRC5	Normal diploid human lung fibroblasts (Jacobs J.P., 1970)

Table 2.4 List of primers used during the course of this study.

Primer name	Forward (F) Reverse (R)	Sequence 5'-3'	Application/ Description
Exon 5	F	CCATCAAAGGCCCTTCCTATGGAG	PCR Corresponds to PML exon 5
Exon 1 UTR	F	AGCTTCTCTTCACGCACTCC	PCR Corresponds to 5' UTR of cellular PML mRNA transcript
Exon 3-7a	F	CAGGGGAAAGAGGAACGCGTTGTG	PCR Mutagenesis Overlaps PML exon 3/exon 7a junction
Exon 4-6	F	GTTGACCTGGATGTCTCCAATACA	PCR Mutagenesis Overlaps PML exon 4/ exon 6 junction
Exon 6-4	R	GGAGACATCCAGGTCAACGTCAAT	PCR Mutagenesis Overlaps PML exon 6/ exon 4 junction
Exon 7a-3	R	GCGTTCCTCTTTCCCCTGGGTGAT	PCR Mutagenesis Overlaps PML exon 7a/exon 3 junction
FaltPML	F	TGCCACCATGGATTACAAGG	PCR Mutagenesis Corresponds FLAG sequence of pCI-neo PML I-VI
Exon 3 (1)	F	TGCTGGACATGCACGGTTTC	PCR Corresponds to PML exon 3

Primer name	Forward (F) Reverse (R)	Sequence 5'-3'	Application/ Description
Exon 3 (2)	F	CTTGCATCACCCAGGGGAAA	PCR Corresponds to PML exon 3 (McNally *et al*., 2008)
Orf4 Forward	F	GGATATCGCCACCATGGTTCTTCCAGCTCTTCC	PCR E4 Orf4 coding sequence
Orf4 Reverse	R	GGGATCCCTACTGTACGGAGTGCGCCG	PCR E4 Orf4 coding sequence
PML I Epitope	R	GCAAGTGGGGTGGAGACTC	RT-PCR Corresponds to epitope of anti-PML I monospecific anti-peptide serum
PML II Epitope	R	ATCGCACAGCCCCTCGTATC	RT-PCR Corresponds to epitope of anti-PML II monospecific anti-peptide serum
SB02	R	GTCTGCTCGAAGCATTAAC	PCR Mutagenesis Universal flanking primer

Table 2.5 List of short interfering RNAs used during the course of this study

siRNA	**Sense 5'-3'**	**Antisense 5'-3'**	**Description**
Scramble siRNA	GAGCCGGACGCCAAAGAAAUU	UUUCUUUGGCGUCCGGCUCUU	Control siRNA with no sequence similarity to any *Homo sapiens* coding sequence, according to the Ambion siRNA Target Finder (Ambion website).
siPML exon 3	GAGCUCAAGUGCGACAUCAUU	UGAUGUCGCACUUGAGCUCUU	Sequence corresponding to a region within exon 3 of PML mRNA
siPML exon 5	CGACAGCCCAGAAGAGGAATT	UUCCUCUUCUGGGCUGUCGTT	Sequence corresponding to a region within exon 5 of PML mRNA
siPML I	CGUGAGCUUCAUGGAGCUGUU	CAGCUCCAUGAAGCUCACGUU	Sequence corresponding to a region within exon 9 of PML I mRNA
siPML II	CAUCCUGCCCAGCUGCAAAUU	UUUGCAGCUGGGCAGGAUGUU	Sequence corresponding to a region within exon 7b of PML II mRNA
siPML V	GUUCAGCCCAGGACUCCUGUU	CAGGAGUCCUGGGCUGAACUU	Sequence corresponding to a region within exon 7 of PML V mRNA

Table 2.6 Expression plasmids used during the course of this study

Plasmid	Description
pCI-neo FLAG-PML I-VI	Expression plasmid for each of PML isoforms I-VI that are tagged with a FLAG epitope sequence (MDYKDDDDK) upstream of the PML translation initiation codon. Gene expression is initiated by the human cytomegalovirus (CMV) immediate-early/enhancer region. Constructed by K.J. Lethbridge and N. Killick (Beech *et al*., 2005, Guccione *et al*., 2004)
pCI-neo FLAG-PML ΔRBCC I-VI	As pCI-neo FLAG-PML I-VI, though the sequence from codon position 1-360 of each PML isoform has been deleted by one step PCR. The deleted sequence corresponds to exons 1 and 2, and also the majority of exon 3 and therefore removes the RBCC motif.
pCI-neo FLAG-PML ΔExon5 I-V	As pCI-neo FLAG-PML I-V, though the sequence corresponding to exon 5 of each PML isoform has been deleted by two-step PCR.
pCI-neo FLAG-PML I-V cytoplasmic	As pCI-neo FLAG-PML I-V, though the sequence corresponding to PML exons 4, 5 and 6 of each PML isoform has been deleted by two-step PCR
pCI-neo FLAG-PML II ΔRΔ1	As pCI-neo FLAG-PML IIΔRBCC, though amino acid residues 615 through to 684 of the PML II C-terminal domain have been deleted (Leppard *et al*., 2009)
pCI-neo FLAG-PML II ΔRΔ2	As pCI-neo FLAG-PML IIΔRBCC, though amino acid residues 685 through to 758 of the PML II C-terminal domain have been deleted (Leppard *et al*., 2009)
pCI-neo FLAG-PML II ΔRΔ3	As pCI-neo FLAG-PML IIΔRBCC, though amino acid residues 759 through to 800 of the PML II C-terminal domain have been deleted (Leppard *et al*., 2009)
pCI-neo FLAG-PML II ΔRΔ4	As pCI-neo FLAG-PML IIΔRBCC, though amino acid residues 801 through to 829 of the PML II C-terminal domain have been deleted (Leppard *et al*., 2009)
pCMV-IX	Ad5 pIX expression plasmid driven by the CMV IE enhancer/promoter region (Caravokyri & Leppard, 1995).
pcDNA3.1-*orf4*	E4 Orf4 was amplified from wild-type Ad5 genomic DNA using PCR, and restriction digested before ligation into the *Eco*RV/*Bam*HI site of pcDNA3.1(-).(Invitrogen)
pcDNA3.1-*orf3*	Ad5 wild-type E4 Orf3 expression plasmid (Hoppe *et al*., 2006)
pcDNA3.1-*N82A*	Ad5 E4 Orf3 N82A mutant expression plasmid (Hoppe *et al*., 2006)
pRL-TK	A low to moderate constitutive expression plasmid encoding the *Renilla reniformis* luciferase. Expression is driven by the HSV thymidine kinase (TK) promoter (Promega E2241).
pISRE-Luc	Luciferase reporter plasmid of the Interferon-stimulated response element (ISRE). Five copies of an ISRE lie upstream of the firefly luciferase gene. Provided by David Blackbourn, University of Birmingham. (Stratagene).

Plasmid	Description
pIFNβ-Luc	Luciferase reporter plasmid of the *Homo sapiens* interferon β promoter. The promoter lies upstream of the firefly luciferase gene. Provided by Tony Marriot, University of Warwick. (King & Goodbourn, 1994).
pcDNA3.1-HisB::*lac*Z	β-galactosidase expression plasmid. Driven by the CMV IE promoter region. A poly-histidine region is located upstream of the *lac*Z open reading frame (Invitrogen).
pCMV-22KFLAG	Ad5 L4 22K expression plasmid. A FLAG epitope is adjoined to the C-terminus of 22K (Morris & Leppard, 2009).
pCMV-33KFLAG	Ad5 L4 33K expression plasmid. A FLAG epitope is adjoined to the C-terminus of 33K (Morris & Leppard, 2009).
pCMV-22/33KFLAG	Ad5 expression plasmid that expresses both L4 22K and L4 33K. A FLAG epitope is adjoined to the C-terminus of 33K (Morris & Leppard, 2009).

Table 2.7 List of primary antibodies used during the course of this study

Antibody/ Protein Detected	Details/Reference	Dilution	
		Western Blotting	Immuno-fluorescence
Mouse anti-FLAG epitope	Monoclonal F3165 Sigma-Aldrich	1/100000	1/500
Rabbit anti-FLAG epitope	Polyclonal F7425 Sigma-Aldrich	1/100000	1/500
Mouse anti-PML	Monoclonal 5E10 (Stuurman *et al*., 1992)	1/20	NA
Mouse anti-PML	Monoclonal PG-M3 Santa Cruz Biotechnology	1/250	NA
Rabbit anti-PML	Polyclonal Chang (Xu *et al*., 2003)	1/64000	NA
Rabbit anti-PML I	Rabbit mono-specific Chang (Xu *et al*., 2003)	1/4000	NA
Rabbit anti-PML II	Rabbit mono-specific Chang (Xu *et al*., 2003)	1/4000	NA
Rabbit anti-PML III	Rabbit mono-specific Chang (Xu *et al*., 2003)	1/4000	NA
Rabbit anti-IRF3	Polyclonal FL-425 Santa Cruz Biotechnology	1/500	1/250
Rabbit anti-PML IV	Rabbit mono-specific Chang (Xu *et al*., 2003)	1/4000	NA
Rabbit anti-PML V	Rabbit mono-specific Chang (Xu *et al*., 2003)	1/4000	NA
Rat anti-E4 Orf3	Monoclonal 6A11 T. Dobner; (Nevels *et al*., 1999)	1/500	1/40

Antibody/ Protein detected	Details/Reference	Dilution	
		Western Blotting	Immuno-Fluorescence
Rabbit anti-p53	Polyclonal FL-393 Santa Cruz Biotechnology	1/10000	NA
Rabbit anti-E4 Orf4	Rabbit mono-specific (Shtrichman & Kleinberger, 1998)	1/3000	NA
Rabbit anti-pIX	Polyclonal (Caravokyri & Leppard, 1995)	1/5000	NA
Rabbit anti-Late	Polyclonal (Farley *et al*., 2004)	1/10000	NA
Mouse anti-GAPDH	Monoclonal AB9484 Abcam	1/10000	NA
Mouse anti-DBP B6-8	Monoclonal (Reich *et al*., 1983)	1/5000	1/200

Table 2.8 List of secondary antibodies used during the course of this study

Antibody	Details/Supplier	Dilution	
		Western Blotting	Immuno-fluorescence
Goat anti-Mouse	Horseradish peroxidase conjugate Sigma-Aldrich	1/100000	NA
Goat anti-Rabbit	Horseradish peroxidase conjugate Santa Cruz Biotechnology	1/100000	NA
Goat anti-Rat	Horseradish peroxidase conjugate Chemicon International	1/5000	NA
Goat anti-Mouse Alexa488	Santa Cruz Biotechnology	NA	1/500
Goat anti-Rabbit Alexa594	Santa Cruz Biotechnology	NA	1/500
Goat anti-Rat Alexa 546	Santa Cruz Biotechnology	NA	1/500

Table 2.9 List of suppliers used during the course of this study

Supplier	**Address**
Ambion	2130 Woodward St. Austin, TX 78744-1832 USA
Applied Biosystems	2130 Woodward St. Austin, TX 78744-1832 USA
Fermentas	Opelstrasse 9 68789 St. Leon-Rot Germany
Invitrogen	5791 Van Allen Way Carlsbad, California 92008 USA
Gibco	5791 Van Allen Way Carlsbad, California 92008 USA
Sigma-Aldrich	Sigma-Aldrich Company Ltd. Dorset, U.K.
Promega	Delta House Southampton Science Park Southampton, U.K.
Biorad	Bio-Rad Laboratories Ltd. Bio-Rad House Maxted Road Hemel Hempstead, U.K.
Greiner Bio-one	Greiner Bio-One Ltd. Brunel Way, Stroudwater Business Park U.K.
Melford Laboratories Ltd	Melford Laboratories Ltd. Bildeston Road Ipswich, U.K.
Calbiochem	Boulevard Industrial Park Padge Road, Beeston Nottingham, U.K.
Santa-Cruz Biotechnology	Santa Cruz, Inc. Bergheimer Str. 89-2 69115 Heidelberg, Germany
Falcon	Falcon Laboratories Inc 1305 Pecan St. Colorado Springs, USA
Eppendorf	Endurance House Vision Park Chivers Way U.K.
AFGA	Agfa-Gevaert N.V. Septestraat 27 B-2640 Mortsel Belgium
Fisher scientific	Bishop Meadow Road, Loughborough, Leicestershire, U.K.

Integrated DNA technologies	Integrated DNA Technologies, Inc. 1710 Commercial Park Coralville, Iowa 52241 USA
GE Healthcare	Pollards Wood Nightingales Lane Chalfont St Giles U.K.
Fuji	St. Martins Business Center, St. Martins Way, Bedford MK42 0LF, U.K.
Chemicon International	Suite 3 & 5 Building 6 Croxley Green Business Park U.K.
Thermo scientific	Thermo Fisher Scientific Inc. 81 Wyman Street Waltham, MA 02454
Stratagene	11011 N. Torrey Pines Road La Jolla, CA 92037
Abcam	330 Cambridge Science Park Cambridge CB4 0FL, UK

2.2 Methods

2.21 Manipulation of DNA

Polymerase Chain Reaction (PCR)

PCR amplifications were carried out in a total volume of 25µl in 0.5ml microcentrifuge tubes using an Eppendorf Mastercycler Gradient thermocycler. Typically 50ng of template DNA was used per reaction. Alongside template DNA, the reaction mix would consist of 0.4µM of each primer, 0.2µM of each dNTP, 1X Buffer and 1.25 units *Taq* polymerase (Fermentas). Unless otherwise stated, the $MgCl_2$ concentration was kept constant at 2mM and reactions were cycled as follows: 95^{o}C for 1 min, 29 cycles 94^{o}C for 1 min, 60-66^{o}C for 1 min and 72^{o}C for 2 mins. This was followed by 72^{o}C for 15 mins. The annealing temperature was varied depending on the primer pair being used in order to reduce non-specific amplification.

Colony screen PCR

For screening bacterial colonies for plasmid inserts, PCR reaction mixes were prepared as described above. As a source of template DNA, bacterial colonies to be screened were picked with a sterile pipette tip which was then briefly inserted into the reaction mixture, then scraped onto a master plate. The reaction mixes were heated to 95^{o}C for 3 mins to ensure bacterial cell lysis, and the reaction was then performed as described above. After analysis, positive clones were identified and the corresponding colonies on the master plate were cultured.

Two-step PCR mutagenesis to generate alternative splice variants of PML isoforms

All PCR mutagenesis performed in this study utilised *Pfu* polymerase (Fermentas) rather than *Taq* polymerase. According to the manufacturer's instructions, $MgSO_4$ was used instead of $MgCl_2$ as a source of magnesium. 50ng of template DNA was used in a total reaction volume of 50µl for each primary amplification. The thermocycle was as follows: 6 cycles of (94^{o}C 1 min, 52^{o}C 1 min, 72^{o}C 5 mins), followed by 29 cycles of (94^{o}C 1 min, 61.5^{o}C 1 min, 72^{o}C 5 mins) before a final incubation of 15 mins at 72^{o}C. The sample was then analysed by agarose gel electrophoresis, and the correct band excised from the gel and purified using a GFX PCR DNA and gel band purification kit (GE Healthcare) according to the manufacturer's instructions.

For the second round of amplification, pairs of products generated during the primary round of amplification that possessed complementary sequences at their ends served as a template. 25ng of each template molecule was used to give a total of 50ng of template DNA per 50µl reaction. The thermocycle was initially performed in the absence of primers so as to allow the complementary sequences between template molecules to hybridise and initiate synthesis of full-length template molecule. The cycle was as follows: 6 cycles of (94^{o}C 1 min, 54^{o}C 1 min, 72^{o}C 5 mins), primers were then added and followed by 29 cycles of (94^{o}C 1 min, 61.5^{o}C 1 min, 72^{o}C 5 mins) and finally 72^{o}C for 15 mins.

The product from the second round PCR was analysed by agarose gel electrophoresis and the band corresponding to the correct size was excised and purified using a GFX PCR DNA and gel band purification kit. The product was then ready for use in subsequent applications.

Restriction enzyme digestion

Typically up to 1μg of plasmid DNA was digested in a total reaction volume of 25-50μl. Restriction enzymes were used according to the manufacturer's instructions in the reaction buffer recommended by the manufacturer. In reactions involving multiple restriction enzymes, the reaction buffer used was the most optimal for all enzymes. Where this was not possible sequential digestion reactions were performed, with the DNA being purified after each reaction with a GFX PCR DNA and gel band purification kit according to the manufacturer's instructions. Reactions were inactivated by being heated to 70^{o}C for 10 mins.

Removal of 5' phosphate groups from DNA

Typically 1μg of linear vector DNA was dephosphorylated with FastAP™ Thermosensitive Alkaline Phosphatase (Fermentas) according to the manufacturer's instructions. Phosphatase activity was destroyed by incubating the DNA at 75^{o}C for 10 mins.

DNA ligation

Ligation of DNA molecules was typically performed in a reaction volume of 10-20μl. A 3:1 molar ratio of insert DNA to vector DNA was typically used. T4 DNA ligase was used according to the manufacturer's instructions and reactions were performed in the supplied buffer. Reactions were performed at 16^{o}C for 16h.

Agarose gel electrophoresis of DNA

DNA molecules were typically separated by electrophoresis on 0.7-1.2% (w/v) agarose in 1X TBE containing 0.5μg/ml ethidium bromide. DNA fragments were loaded onto the gel in 1X loading buffer (5% (w/v) glycerol, 0.04% (w/v)

bromophenol blue and 0.04% (w/v) xylene cyanol). Electrophoresis was performed in 1X TBE at 60-120V for 0.5-1h. DNA fragments were visualised under UV light and imaged using a BioRad Gel/Chemi Doc system and the supplied software.

Extraction of DNA from agarose gels

DNA fragments of interest were visualised with UV then excised from the gel using a clean scalpel. The DNA was cleaned and extracted from the agarose using a GFX PCR DNA and gel band extraction kit according to the manufacturer's instructions.

DNA Sequencing

All DNA sequencing was performed in-house by the Molecular Biology Service at the University of Warwick using an automated ABI PRISM 3130xl Genetic Analyser.

2.22 Manipulation of RNA

Extraction of cytoplasmic RNA from cultured HEp-2 cells

Each RNA sample was typically extracted from three 90mm dishes of 80-90% confluent cells. Each dish was washed twice in 3ml of sterile PBS. Cells were then scraped off the dish in 5ml of sterile PBS using a sterile cell scraper. The cell suspension of each dish was then transferred to a 15ml Falcon centrifuge tube. Cells were then collected by centrifugation (Beckman Coulter Allegra^{TM} X-12R Centrifuge) at 1300 r.p.m. for 3 mins, and the supernatant was discarded. The pellet was resuspended in 1.5ml of pre-cooled 1X TBS (50mM Tris, 150mM NaCl, pH7.6)**.** 75µl of 10% NP40 in 1X TBS (Sigma) was added to the cell suspension and the cells were then vortexed and left on ice for 10 mins. The cytoplasmic fraction was then prepared by centrifugation at 1500 r.p.m. for 5 mins at 4^{o}C. 100µl of supernatant was then removed for use in SDS-PAGE, and the remaining cytoplasmic fraction was then dispensed into three 1.5ml micro-centrifuge tubes for RNA preparation. The pelleted nuclei were then resuspended in 2 ml 1 X TBS, treated with 100µl 10% NP40 and 50µl 10% sodium deoxycholate (Sigma), vortexed then

centrifuged again at 1500 r.p.m. for 5 mins at 4^{o}C to collect clean nuclei. Nuclei were then lysed in an appropriate volume of 1X sample buffer for use in SDS-PAGE.

Each micro centrifuge tube containing the cytoplasmic supernatant was treated with 500µl of TRI Reagent (Sigma) and left at room temperature for 1h. 67µl of chloroform was added to each tube and was mixed by inversion. The tubes were then centrifuged at 13, 200 r.p.m. for 15 mins in a micro centrifuge. The upper, aqueous phase of each tube was then removed and placed into a fresh 1.5ml micro centrifuge tube, with care being taken not to disrupt the lower phases. 500µl of isopropanol was then added to each aqueous phase and was placed at –20^{o}C for at least 1h. When RNA was needed, the tubes were centrifuged at 13200 r.p.m. for 15 mins and the supernatant was discarded. The pellets were then washed with 70% ethanol and centrifuged at 13200 r.p.m. for 15 mins. The supernatant was discarded and pellets were left to dry for 15-20 mins in a vacuum dessicator. Pellets were resuspended in 50µl of RNAse-free water and were then immediately used for analysis.

Reverse-Transcription (RT) of RNA

Typically, 1-5µg of RNA was used per reaction for reverse transcription. Reactions were performed in a 50µl reaction using SS-Reverse Transcriptase (Fermentas) according to the manufacturers' instructions. Reactions were left to proceed for 1h at 42^{o}C, before being incubated at 70^{o}C for 15 mins to destroy reverse transcriptase (RT) activity. Finally, 0.5µl RNase H was added to each reaction and incubated at 37^{o}C for 20 mins. Typically 2.5µl of the final reaction volume was used for subsequent PCR analysis.

2.23 Bacteriological techniques

Throughout this study all methods requiring bacteria utilised *E. coli* TG2 cells, or when blue/white screening was required, *E. coli* DH5α cells. Bacteria were grown in

LB at 37°C and shaken vigorously in a sterile vessel approximately five times the volume of the liquid culture.

Preparation of competent cells

100ml of pre-warmed LB was inoculated with 2ml of fresh overnight bacterial culture in LB. The bacteria were then shaken vigorously at 37°C until the OD_{600} (optical density) of the culture was 0.39. The culture was then placed on ice for 5 mins before centrifugation at 6000r.p.m. for 10 mins at 4°C (Beckman Coulter Allegra™ X-12R Centrifuge). The supernatant was discarded and the remaining pellet was resuspended in 40ml transformation buffer I (Tfb I; 10mM $RbCl_2$, 30mM potassium acetate, 10mM $CaCl_2$, 50mM $MnCl_2$, 15% v/v glycerol and adjusted to pH5.8). The resuspended pellet was left on ice for 5 mins then centrifuged at 6000r.p.m for 10 mins at 4°C. The supernatant was again discarded the pellet resuspended in 4ml transformation buffer II (Tfb II; 75mM $CaCl_2$, 10mM $RbCl_2$, 10mM 3[N-morpholino] propanesulphonic acid (MOPS), 15% v/v glycerol, pH 6.5) then left on ice for 2h. The competent cells were then separated into 100µl aliquots and frozen with dry ice before storage at –70°C.

Transformation of competent bacteria

50ng of plasmid DNA or ligation reaction was added to 100µl of thawed competent *E. coli* TG2 cells then left on ice for 30 mins. Cells were then subjected to heat shock for 30s at 42°C and left on ice for 2 mins. 600µl of pre-warmed LB was added and cells were incubated at 37°C with shaking for 1h. Cells were then plated onto LB plates containing100µg/ml ampicillin and left to incubate at 37°C overnight.

Extraction of plasmid DNA from liquid culture

Small quantities of plasmid DNA were typically prepared from 1ml of *E. coli* TG2 overnight culture in LB supplemented with 100µg/ml ampicillin. Plasmid DNA was extracted using a Sigma-Aldrich GenElute™ Plasmid Miniprep Kit according to the manufacturer's instructions.

In order to prepare larger quantities of plasmid DNA for use in transfection experiments a larger culture volume was utilised. 100ml of *E. coli* TG2 overnight LB culture supplemented with 100µg/ml ampicillin was used, and DNA was extracted using a Promega Pureyield^{TM} Plasmid Midiprep System according to the manufacturer's instructions.

Quantification of nucleic acid

The concentration DNA and RNA samples was quantified by measuring the OD_{260} using a Nanodrop-ND1000 (Thermo Scientific) and associated software. An absorbance of 1 is equivalent to 50µg/ml of double-stranded DNA. The purity of DNA or RNA was indicated by the absorbance ratio of OD_{260} to OD_{280}, where a ratio of 1.8 indicates pure DNA, and a ratio of 2 indicates pure RNA.

Blue/white selection of bacterial colonies

Each LB plate containing 100µg/ml ampicillin was aseptically covered with 40µl 20mg/ml X-Gal (Melford Laboratories Ltd.) in dimethylformamide (DMF) and 4µl isopropyl β-D-1-thiogalactopyranoside (IPTG; Melford Laboratories Ltd.) in H_2O using a glass spreader. The plates were then left to dry at 37^{o}C for 1h, after which they were ready for inoculation. 24h after inoculation, plates were examined for white colonies as these were deemed to contain the plasmid carrying the insert of interest. Positive colonies were transferred to 5ml LB containing 100µg/ml ampicillin and were incubated overnight in a shaking incubator at 37^{o}C.

2.24 Mammalian cell culture

Maintenance of cell lines

HEp-2, A549 and MRC5 cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% (v/v) heat-inactivated foetal bovine serum (FBS). HEK293 cells were maintained in DMEM supplemented with 10%

(v/v) newborn bovine serum (NBS). U2OS cells were maintained in McCoy's 5A medium supplemented with 10% FBS.

90mm γ-irradiated tissue culture dishes were used as the standard culture vessels for all cells used in the study. Cells were grown in the dishes at 37^{o}C in a 5% CO_2 atmosphere until confluent. Cells were then passaged by washing the monolayers with 3ml versene (0.02% EDTA in PBS), then incubating the cells with 2.5ml versene, containing 0.02% (v/v) 0.25mg/ml trypsin, for 5 mins. Detached cells were collected in suspension and added to 0.5ml FBS before centrifugation at 1300 r.p.m. for 3 mins. The cell pellet was then resuspended in the appropriate growth medium and seeded into new vessels at the required density.

Transfection, infection and drug treatment of mammalian cells

All cells to be transfected were seeded at the required density into an appropriate culture vessel 24h prior to transfection. Unless otherwise stated, cells were seeded in 10% serum media in a volume suitable for transfection, preventing the need to replace the medium prior to addition of DNA or RNA.

Transfection of plasmid DNA

Lipofectamine 2000 (LF2000; Invitrogen) and Turbofect (Fermentas) were used for liposome-mediated transfection of plasmid DNA throughout this study. For both transfection reagents, a ratio of 2μl reagent for every 1μg plasmid DNA was used, and this was applied to all cell lines used in this study.

All transfections were performed in 12 or 24 well plates. Cells were seeded 24h before the procedure to allow approximately 90% confluence at the time of transfection. Transfection reagent-DNA complexes were prepared in pre-warmed, serum-free OptiMEM to make a volume that corresponded to 100μl (24 well) or 200μl (12 well) per well of cells to be transfected. After incubation for 20 mins at room temperature the complexes were gently pipetted onto the serum media bathing the cells. The cells were then incubated at 37^{o}C for 6h when, where appropriate, the medium was replaced with fresh 10% serum medium.

Transfection of cells by poly (I:C)

Turbofect was found to be an unsuitable reagent for the transfection of cells by poly (I:C), hence only LF2000 was used for this purpose during the course of this study. LF2000 was used at a ratio of 2µl per 1µg of poly (I:C), and this was applied to all cell lines. LF2000/poly (I:C) complexes were prepared in pre-warmed, serum-free OptiMEM to make a volume equivalent to 100µl or 200µl per well, depending on the size of the culture plate. The mixture was left to incubate for 20 mins at room temperature, after which it was gently pipetted into the serum media bathing the cells. Where appropriate, the medium was replaced with fresh serum media after 8h.

Transfection of cells by short interfering RNA (siRNA)

LF2000 and TriFECTin (Integrated DNA Technologies) were used as lipid reagents for the transfection of cells with siRNA. 2µl of transfection reagent was used per well for a 24 well plate. Lipid reagent/siRNA complexes were prepared in pre-warmed, serum-free optiMEM to make a volume equivalent to 100µl per well. The mixture was left to incubate for 20 mins at room temperature, after which it was gently pipetted onto the serum media bathing the cells. For all siRNA experiments, the medium was replaced with fresh 10% serum medium every 24h after the initial transfection.

Infection of cells by Adenovirus

Cells were seeded at least 24h prior to infection. The growth media was removed and cell monolayers were washed once with 0.5ml serum-free medium. Virus stocks were diluted with serum-free DMEM or McCoy's 5A medium as appropriate to the cell type to give the appropriate multiplicity of infection (m.o.i.) of plaque-forming units per cell. U2OS cells were infected at an m.o.i. of 30 whereas all other cell lines used in this study were infected at an m.o.i. of 10. The final volume of diluted virus was typically 100µl per well for a 24 well plate and 1ml per 90mm dish. The diluted virus was added to the cells, which were than kept at 37^{o}C for 1h, being rocked every 15 mins to ensure the medium covered the entire monolayer. After 1h, an

appropriate volume of pre-warmed serum-containing medium was added to the cells and left for the duration of the experiment.

Virus stock titration

All virus titrations were performed in duplicate on sub-confluent HEK293 cells. Cells were seeded onto 6mm plates 24h prior to infection. The growth media was removed and cell monolayers were washed once with 2ml serum-free DMEM. The viral stock was diluted with serum-free DMEM to produce a series of 10-fold dilutions. The dilution range examined was typically 10^{-5}-10^{-10}. 500µl of each dilution was then added to the cells, which were then kept at 37^{o}C for 1h, being rocked every 15 mins to ensure the medium covered the entire monolayer. After 1h, the virus dilutions were aspirated from the cell monolayers, then 4 ml of pre-warmed agar overlay (Stock: 25ml 2X DMEM –$NaHCO_3$, 2ml NBS, 5ml 7.5% (w/v) $NaHCO_3$, 18ml 2.8% noble agar) was added to each well. The cells were then kept at 37^{o}C, and 1ml of pre-warmed agar overlay (Stock: 25ml 2X DMEM –$NaHCO_3$, 0.5ml NBS, 2.5ml 7.5% (w/v) $NaHCO_3$, 18 ml 2.8% noble agar) was added to each well every 3 days until virus plaques were visible. Upon the appearance of plaques, 2ml of pre-warmed staining overlay (Stock: 25ml 2X DMEM, 0.5ml NBS, 2.5ml 7.5% $NaHCO_3$, 18ml 2.8% noble agar, 5ml 0.1% neutral red) was added to each well, and plates were wrapped in aluminium foil and placed at 37^{o}C. After 24h, plaques were counted and the average number of plaques between the two duplicates was calculated. This value was then used to calculate the titre of the undiluted virus stock.

Hydroxyurea treatment

A549 or HEp-2 cells were infected with adenovirus as described above. 1h post infection medium was replaced with 1ml 10% FBS DMEM or 1ml 10% FBS DMEM containing 10mM hydroxyurea (Sigma-Aldrich). Cells were then incubated at 37^{o}C for 23h or until the end of the experiment.

MG132 treatment

A549 or HEp-2 cells were infected by adenovirus as described above. 16h post infection the medium was replaced with 1ml 10% FBS DMEM or 10% FBS DMEM containing 50µM MG132 (Sigma-Aldrich). Cells were left to incubate at 37^{o}C for a further 8h before being harvested.

D-sorbitol treatment

4h prior to harvesting cells were treated with 0.5ml 0.5M D-sorbitol (Sigma-Aldrich) dissolved in 10% FBS DMEM or mock-treated with fresh 10% FBS DMEM alone.

DMAT treatment

To inhibit casein kinase II (CK2) activity the selective inhibitor DMAT (Calbiochem) was used. At the appropriate time the medium was removed from cells and replaced with fresh 10% FBS serum DMEM containing 10µM DMAT (from 10mM stock in DMSO). Cells were mock treated through the addition of 10% serum media containing an equivalent volume of DMSO.

2.5 Analysis of protein expression

Harvesting of total cellular protein

To extract cellular protein for SDS PAGE, cell monolayers were washed once with PBS before incubation with 1X sample buffer (100µl used for every 3 x 10^5 cells unless otherwise stated) at room temperature for 5 mins. The cell lysates were collected and boiled for 10 mins and samples were then used immediately or stored at –70^{o}C.

SDS polyacrylamide gel electrophoresis (SDS-PAGE)

Denatured protein samples were separated by the method described by Laemmli (Laemmli, 1970). Gels consisted of a 5% acrylamide stacking gel and a resolving gel of 8-15% acrylamide (37.5:1 acrylamide:bis-acrylamide, depending on the molecular size(s) of the protein(s) being investigated. Typically, electrophoresis was performed for 1.5-2h at 120V in 1X running buffer. All SDS-PAGE performed in this study utilised the Bio-Rad mini-PROTEAN III systems according to the manufacturer's instructions.

Transfer of proteins onto nitrocellulose membranes

Prior to transfer the stacking gel was removed, and the transfer of the separated proteins from the resolving gel onto nitrocellulose membranes was performed as previously described (Towbin *et al*., 1979). Transfers were performed in 1 X transfer buffer (25mM Tris base, 192mM glycine, 20% methanol) at 350mA for 1.25h at 4^{o}C, or at 80mA for 16h at 4^{o}C using the Bio-Rad Mini Trans-Blot Electrophoretic Transfer Cell system according to the manufacturer's instructions. All transfers utilised Hybond^{TM} ECL^{TM} nitrocellulose membrane.

Protein detection by western blotting

Nitrocellulose membranes were incubated for >1h-overnight in 20ml blocking solution (2% ECL^{TM} Advance blocking agent in PBS containing 0.05% (v/v) Tween-20). Membranes were then probed with 2.5ml of primary antibody against the protein of interest diluted in blocking solution, and then incubated 1h at room temperature on an orbital shaker. The dilution of the primary antibody was dependent on the protein under investigation (Table 2.7). Membranes were then washed for 1h on an orbital shaker with PBS containing 0.05% Tween-20, with the PBS-Tween being replaced every 5 mins. The membranes were then probed with 2.5ml of the appropriate secondary antibody (Table 2.8) diluted in blocking solution and incubated as before. Membranes were then washed in PBS-Tween for 1h as before. The blots were developed using ECL^{TM} Advance according to the manufacturer's instructions and exposed to Fuji SuperRX X-Ray Film then

developed using an AGFA Curix60 developer, or imaged using a Biorad Gel/Chemi Doc system and associated software.

2.6 Luciferase reporter assays

Preparation of cell lysates

All transfections were performed in triplicate on sub-confluent cell monolayers in 24 well plates. Each well was transfected with 500ng total DNA, of which 250ng consisted of the expression plasmid of interest and 250ng consisted of a combination of reporter plasmids listed in Table 2.6 in 9:1 ratio of inducible reporter (pIFNβ-Luc, pISRE-Luc) to autonomous reporter (pRL-TK, pcDNA3.1-hisB::*lac*Z). 16h later the medium was replaced with fresh 10% serum media and cells were transfected with 1µg poly (I:C) per well. Cells were incubated for the appropriate time according to the experiment before being harvested. Lysis was achieved through the addition of 100µl 1X passive lysis buffer (Promega) per well, followed by shaking for 30 mins at room temperature. Cell lysates were then collected and, where necessary, 5µl samples of each triplicate lysate were pooled together for use in SDS-PAGE and Western blot analysis. Remaining lysates were stored at -20^{o}C for short-term storage until analysis.

Dual luciferase assay

10µl of each cell lysate was analysed for both firefly and *Renilla* luciferase activity in an opaque half-volume 96 well plate (Greiner) using the Dual-Luciferase Reporter System (Promega) according to the manufacturer's instructions. Luciferase activity was measured using a Luminoskan Ascent microplate luminometer and the provided software (Thermo Fisher Scientific).

Bright-Glo luciferase assay

All assays were performed in an opaque, half-volume 96 well plate (Greiner) through the addition of 25µl Bright-Glo Luciferase Assay Substrate (Promega) to

10µl of cell lysate. Luciferase activity was measured using a Luminoskan Ascent microplate luminometer and the provided software (Thermo fisher Scientific).

β-Galactosidase Assay

All β-Galactosidase assays were performed in clear 96-well plates (Falcon). 10µl of cell lysate was mixed with 86µl Z-buffer (60mM $Na_2HPO_4{\cdot}7H_20$, 40mM $NaH_2PO_4{\cdot}H_20$, 1mM KCl and 1mM $MgSO_4{\cdot}7H_2O$) containing 40mM β-mercaptoethanol and 24mM ONPG (2-nitrophenyl-β-D-galactopyranoside). The lysates were then incubated at 37°C until reaction mixtures became visibly yellow, at which point reactions were stopped through the addition of 40µl 1M Na_2CO_3. The OD_{405nm} was then recorded using a colorimeter (Labsystems Multiskan RC) and associated software.

Analysis of luciferase assay data

For each sample the Relative Luciferase Activity (RLA) was calculated in order to normalise luciferase activity to the general transfection efficiency of the cells. This was achieved by dividing the value of firefly luciferase activity by the activity of the autonomous reporter (*Renilla* luciferase or β-Galactosidase) for a given sample. The RLA was calculated independently for control cells left untreated or stimulated with poly (I:C). All RLAs were then normalised to the respective control, with control samples having a value of 1. All RLAs were therefore expressed as a ratio to this. Within an experiment the standard deviation from the mean of the ratios obtained from each set of triplicates was calculated. Where experiments were repeated a minimum of three separate times, therefore generating at least nine values, two-tailed Student's t-test analysis was performed with two sample unequal variance to test whether or not the two datasets were significantly different.

2.25 Immunofluorescence Microscopy

Fixing of cells to cover slips

Cells were seeded at an appropriate density into a 12 well plate containing a single cover slip per well. At the end-point of the experiment, cells were washed twice with 0.5ml PBS then 0.5ml of 10% (v/v) formalin in PBS was added to each well and left at room temperature for 10 mins. Cells were then washed with 0.5ml PBS prior to the addition of 0.5ml 10% (v/v) NP40 in PBS per well. Cells were incubated at room temperature for 10 mins then the cells were washed twice with 0.5ml PBS. Cells were then left in 1ml PBS and stored at 4^{o}C in air-tight conditions for up to two weeks.

Staining cells

Cells were blocked for 1h in the presence of 0.5ml 1% (w/v) bovine serum albumin (BSA, Sigma) in PBS, then washed three times with 0.5ml PBS and incubated with 0.25ml primary antibody (Table 2.7**)** in 1% BSA in PBS for 1h. The primary antibody was removed, then the cells were washed three times in PBS and 0.25ml of the appropriate secondary antibody in 1% BSA in PBS was added per well (Table 2.8). Cells were then incubated for 1h in darkness then washed twice with 0.5ml PBS. If staining by multiple primary antibodies was required then this process was repeated, but all antibody incubations were performed in darkness. After incubation with the final secondary antibody, cells were washed twice with 0.5ml PBS then incubated in darkness with 0.001% (w/v) 4',6' –diamidino-2-phenylindole (DAPI) in PBS for 5 mins. The cover slips were then removed, dried and inverted onto microscope slides containing 5µl of mounting medium (Vectorshield, Vector Laboratories Inc.). The edges of the cover slip were then sealed to the slide using nail varnish.

Immunofluorescence analysis

Microscope images were generated using either a Leica SP2 or SP5 confocal system. Images were generated by using sequential scanning for multiply stained samples

using settings which had been optimised to eliminate fluorescence crossover between channels. Images typically represent a single focal plane, though where indicated images may consist of a maximal projection through a z-series. Nuclei were visualised by staining the DNA with DAPI. Images were assembled using the Leica confocal software.

Chapter 3
Adenovirus infection alters the population of PML proteins

3.1 Introduction

PML protein is the major component of sub-nuclear structures termed PML nuclear bodies (PML NBs). The major role performed by PML and PML NBs is the subject of much debate, as PML protein has been implicated in numerous cellular processes, including anti-viral responses. Much evidence for this role stems from observations where viruses specifically interact with PML proteins and therefore PML NBs. In some cases, perturbation of PML NBs has been shown to enhance viral replication (Chee *et al*., 2003, Härle *et al*., 2002, Mossman *et al*., 2000, Tavalai *et al*., 2006).

Adenovirus type 5 (Ad5) is one virus which has been shown to alter both PML NBs and the PML protein population. Infection with Ad5 leads to reorganisation of PML NBs, and their constituents, from their native spherical appearance into track-like structures (Carvalho *et al*., 1995). This observation has been recorded in a number of cell types and is mediated by the viral E4 Orf3 protein, which interacts specifically with the unique C terminus of PML II isoform (Hoppe *et al*., 2006). PML NB disruption is maintained at later times during infection by the viral intermediate gene product protein IX (pIX), which sequesters PML into 'inclusion bodies' (Rosa-Calatrava, 2003).

Ad5 infection has also been shown to alter the PML protein population, as infection with a wild type Ad5 leads to the production of a novel, infection-specific species of PML that becomes a dominant species within the population by 24 hours post infection (Leppard & Everett, 1999). This species was not produced upon infection with a virus lacking the early gene products E4 Orf1-3, and it has subsequently been found that the viral protein responsible is E4 Orf3 (K. Leppard, unpublished). The number of higher molecular weight, sumoylated forms of PML has also been observed to decrease upon infection with Ad5. As yet there is no clear reason why adenovirus disrupts PML NBs and alters the PML population, though it is hypothesised that adenovirus targets PML to overcome its antiviral properties and thus make the cell more conducive to viral replication.

The experiments performed in this chapter explore further the interaction between Ad5 and the endogenous PML proteins. By using a polyclonal anti-PML antibody

raised against a GST-PML fusion protein containing the entire N-terminal domain of PML (Vallian *et al.*, 1998, Xu *et al.*, 2003), it is assumed that the majority of the PML population of cells could be detected and a comparison made between mock-infected and infected cells. Emphasis was initially placed on the novel E4 Orf3-dependent species of PML described above, termed species A throughout the course of this study, and the extent to which it was produced in various cell lines. During this work, two other novel infection-specific species of PML were also detected that were independent of E4 Orf3. The sub-cellular localisation of these infection-specific species and the requirements for their production were then investigated.

3.2 Infection-specific changes in PML proteins can be detected in various cell lines

PML species A was previously detected during wild-type Ad5 infection of both HEp-2 and HeLa cells (Leppard & Everett, 1999), though its presence in other cell lines has never been investigated. In order to more fully understand the nature of species A several commonly used cell lines were examined for their capability to produce this PML species upon Ad5 infection.

HEp-2, HEK293, U2OS and A549 cells were chosen to investigate any infection-specific changes in the PML population of immortalised cell lines. Normal diploid MRC5 cells were also investigated as these represent typical cells that are not immortalised. Such a comparison would ensure that any changes observed were biologically relevant in the context of a normal diploid cell and not just an artefact of any transformation processes. Each cell type was mock infected or infected with wild-type Ad5 strain 300 or, where indicated, E4 Orf3-deficient mutant inOrf3. Cells were harvested 24 hours post infection (h.p.i.) and the protein content of the lysates was separated by SDS-PAGE and subjected to Western blotting. The blots were probed with the polyclonal anti-PML antibody that detected all isoforms of PML, allowing changes in the entirety of the PML population to be investigated (Fig. 3.1).

With the exception of U2OS cells, species A was detected in all of the cell lines tested upon infection with wild-type Ad5. However, its abundance relative to the PML population varied depending on cell type. Of the cell lines tested, HEp-2 cells

consistently appeared to express species A to a level comparable with the endogenous PML population, consistent with the earlier report (Leppard & Everett, 1999). HEK293 cells were also found to produce a significant amount of species A, though it was not expressed at a level comparable to the major endogenous species of PML. The presence of species A in MRC5 cells confirmed that the appearance of this PML form was independent of the transformation state of the cell line.

Between experiments, the level of expression of species A was found to vary even within a single cell type. In particular A549 cells were capable of producing species A, but often to only low levels that made detection difficult. For instance, in Fig. 3.1 species A is barely visible in Ad5 300-infected A549 cells. After repeated attempts, species A was never detected in U2OS cells despite being infected at an increased multiplicity of infection, suggesting that U2OS cells are intrinsically unable to support the production of this PML species.

In all the cell lines examined the production of two novel, infection-specific species of PML was observed (arrows B and C). These species did not clearly co-migrate with any known species of PML, as determined through over-expression of PML from cDNA expression plasmids (Beech *et al*., 2005), and were found to be independent of the presence of E4 Orf3. Species B in particular was found to be strongly expressed in all cell lines tested, and indeed was amongst the major bands detected after infection, particularly in 293 cells. Species C was more variable in its relative abundance, and was observed to be either strongly (HEK293 cells) or weakly (MRC5 cells) expressed depending on the cell type being examined.

Thus infection by wild-type adenovirus leads to the production of at least three infection-specific PML species that can be detected with a polyclonal anti-PML antibody in most or all of a panel of cell lines, including normal MRC5 cells. Whilst the appearance of species A was dependent on the viral E4 Orf3 protein, two novel species B and C were independent of the viral E4 Orf3 protein. Thus these data indicate that adenovirus infection causes several distinct changes in the cellular PML population.

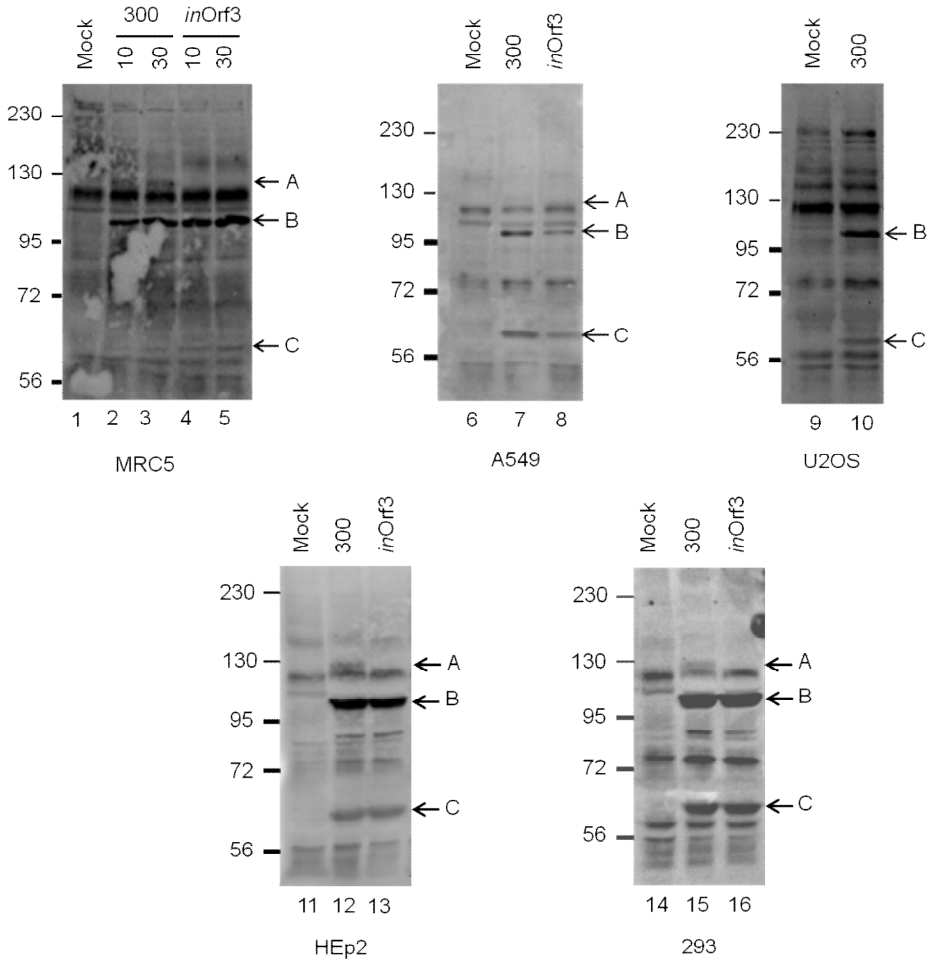


Figure 3.1. Adenovirus type 5 infection induces multiple changes in the PML population. 3 x 10^5 cells of the type indicated were mock infected or infected with wild type Ad5 (300) or, where indicated, an E4 Orf3-deficient virus (*in*Orf3) at a multiplicity of infection (m.o.i.) of 10pfu/cell, except U2OS cells and MRC5 cells (lanes 3, 5), which were infected at an m.o.i. of 30 pfu/cell. 24 hours post infection (h.p.i.) all cells were harvested and the protein content of the lysates was separated by sodium dodecyl sulfate 8% polyacrylamide gel electrophoresis (SDS-PAGE) and subjected to western blotting. Blots were probed with a polyclonal anti-PML antibody (Xu *et al.*, 2003) which recognises all isoforms of PML protein. The infection-specific species of PML are indicated by the arrows to the right of each blot. Protein sizes are indicated in kDa to the left hand side of each blot.

3.3 Detection of infection-specific species of PML by monoclonal pan-PML specific antibodies

It was important to ensure that the infection-specific species of PML B and C were PML related, and not the product of cross-reactivity of the antibody to proteins unrelated to PML. To address this problem, alternative monoclonal anti-PML antibodies were employed to investigate the PML population in infected cells. PG-M3 is a commercially available monoclonal antibody that targets an epitope overlying the the exon 1/2 boundary of PML. Since all PML isoforms are thought to possess the RBCC domain encoded by exons 1-3, PG-M3 is an excellent candidate antibody to use when attempting to detect the entire population of PML species within a cell. It is however reputed not to detect sumoylated PML well. Species A has previously been detected in infected cells by the alternative monoclonal anti-PML antibody 5E10. However, the 5E10 antibody recognises an epitope that has been mapped to PML exon 5 (Stuurman *et al*., 1992). Exon 5 lies within the central domain of PML proteins and is thought to be excluded from both cytoplasmic and a subset of nuclear PML transcripts by differential splicing (Jensen, 2001). When using this antibody this information must be taken into account, as it is likely that 5E10 does not detect the entire population of PML proteins.

HEp-2 cells were mock infected or infected with wild type Ad5 300 or E4 Orf3-deficient mutant inOrf3, and after 24 hours the cells were harvested and multiple aliquots of extract were separated in parallel by SDS-PAGE and subjected to Western blotting to generate three replica blots. Each blot was then probed with a single pan-PML antibody, either a rabbit polyclonal antibody (Xu *et al*., 2003) or mouse monoclonal antibodies 5E10 (Stuurman *et al*., 1992) or PG-M3 (Santa Cruz) (Fig. 3.2).

PML species A was detected by both the rabbit polyclonal and 5E10 antibodies, but not by monoclonal antibody PG-M3. PG-M3 did not appear to detect any bands that correspond with the higher molecular weight endogenous PML I and II isoforms that were detected by both the polyclonal and 5E10 antibodies (Beech *et al*., 2005).

As in Fig 3.1, species B was clearly detected by the polyclonal antiserum as it formed the most dominant species within the PML population. PG-M3 did not

detect a species of similar molecular weight to B in infected lanes, and resembled the blotting pattern detected by the 5E10 antibody, which also did not detect a species which clearly co-migrated with species B. This suggests that these antibodies do not recognise this particular molecule.

As expected from figure 3.1, the polyclonal antibody detected species C only in the infected lanes. A band co-migrating with species C was also detected by both 5E10 and PG-M3. However, this band was present in both mock and virus infected lanes, though in the PG-M3 blot this band was expressed more strongly in virus-infected lanes, which would be expected for species C. It is therefore possible that PG-M3 but not 5E10 detects species C, while both antibodies also detect an unrelated molecule or unchanging PML species of a similar molecular weight that is present equally in uninfected and infected cells.

Both monoclonal antibodies 5E10 and PG-M3 detected an alternative infection-specific species which did not co-migrate with any of species A, B or C. This species, labelled X, was not detected by the polyclonal anti-PML antibody but may represent an additional infection-specific species of PML. However, in order to limit the scope of this study this species was not examined any further.

It is pertinent to note that none of the single infection-specific species was detected by all three antibodies utilised. Indeed, each antibody produced a unique banding pattern of endogenous PML, possibly resulting from a combination of unique antibody-epitope binding kinetics and various post-translationally modified PML proteins. Curiously, PG-M3 seemed unable to detect either species A or higher molecular weight species of endogenous PML that correspond to the migration positons of PML I and PML II (Beech *et al*., 2005), though the pattern of lower molecular weight PML isoforms detected resembled that seen by both the polyclonal and 5E10 antibodies. This observation makes interpretation somewhat difficult when using western blotting to examine endogenous levels of PML proteins, and one must consider that when using a particular anti-PML antibody for western blotting the true population of PML proteins may not be fully reflected.

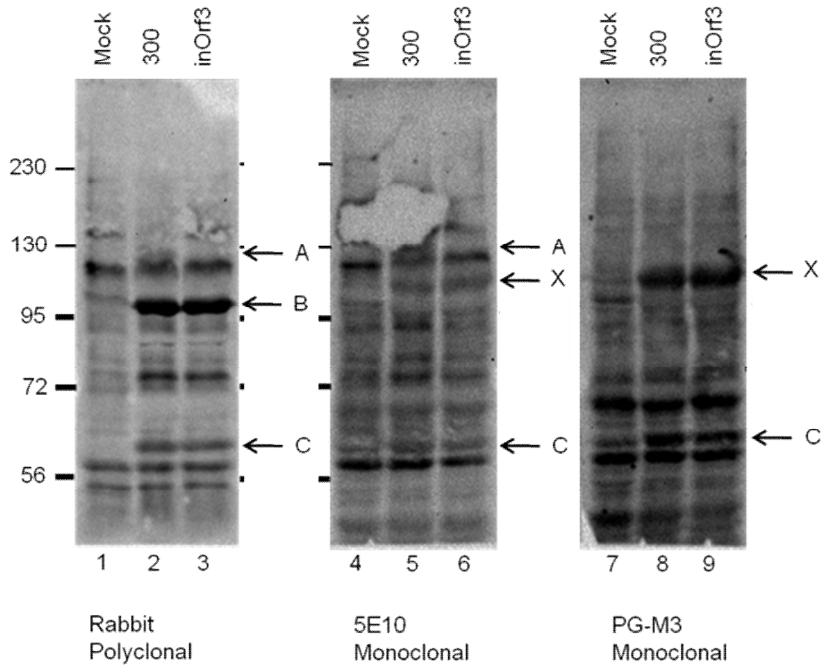


Figure 3.2. The infection-specific species of PML can be detected by multiple anti-PML antibodies. 3 x 10^5 HEp-2 cells were mock-infected or infected with Ad5 wild-type 300 or *in*Orf3 at an m.o.i. of 10 pfu/cell. 24 h.p.i. cells were harvested and aliquots of extract were separated in parallel by 8% SDS-PAGE and subjected to western blotting in order to generate three replica blots. Each blot was probed with a single anti-PML antibody, as indicated beneath each blot. The arrows to the right hand side indicate the migration positions of the infection-specific species A, B and C. An additional infection-specific species is marked as X. Protein sizes are indicated in kDa to the left hand side of the blots.

3.4 All infection-specific species of PML can be depleted using siRNA targeting PML

No infection-specific species of PML was readily detected by all three antibodies employed, thus not totally eliminating the possibility that either one or more of these species could be an artefact of non-specific antibody cross-reactivity. To overcome this, an alternative technology was utilised with the aim of confirming that the infection-specific species detected by western blotting were PML-related. siRNA technology was used to deplete PML proteins in HEp-2 cells prior to subsequent infection by wild-type adenovirus. A suitable siRNA target within exon 3 of the PML transcript was designed using appropriate software (Ambion siRNA target finder). Exon 3 was chosen as a target sequence as it is thought not to be excluded by differential splicing and is therefore possessed by all isoforms of PML (Jensen, 2001). Software validation was used to ensure that the siRNA target sequence was not found elsewhere in either the adenovirus genome or the human genome, except for the PML transcript (NCBI/BLAST).

HEp-2 cells were mock-transfected or transfected with up to 200 picomoles of siRNA targeting exon 3 of the PML primary transcript. As a negative control, 200 picomoles of a scrambled siRNA with no homology to any gene in the human genome were used. 48 hours after transfection with siRNA, cells were mock infected or infected with wild-type Ad5 300, and cells were harvested 24 hours post infection. Aliquots of the lysates were separated in parallel by SDS-PAGE and subjected to western blotting to generate two replica blots. One blot was probed with a polyclonal anti-PML antibody, and the other probed for the housekeeping gene product glyceraldehyde 3-phosphate dehydrogenase (GAPDH) with a monoclonal anti-GAPDH antibody. This served as a control for equal protein loading of the samples and also ensured that any knockdown by PML siRNA was not due to a global decrease in translation (Fig. 3.3).

The endogenous PML population was successfully depleted in a dose-dependent manner by transfection of the siRNA targeting PML exon 3, resulting in a significant reduction in PML protein (lanes 3-7) as compared to cells transfected with a control siRNA (lane 8). Infection-specific species A, B and C were all observed in the

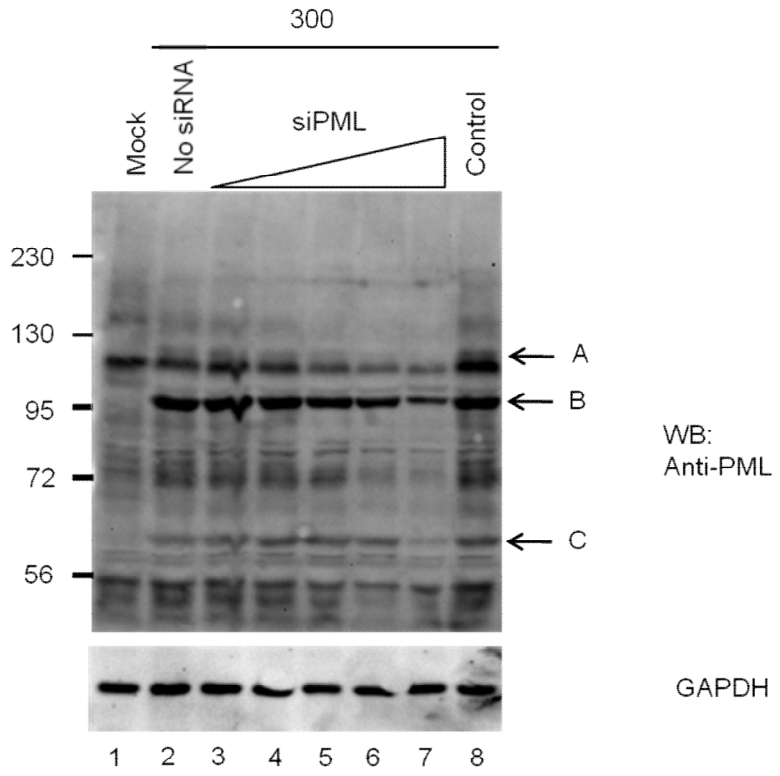


Figure 3.3. The infection-specific species of PML can be depleted by transfection with PML-specific siRNA. 2 x 10^5 HEp-2 cells were mock transfected or transfected with 1, 5, 10, 50 or 200 picomoles of PML siRNA, or 200 picomoles of control scrambled siRNA. 48 hours after transfection, cells were mock-infected or infected with Ad5 300 at an m.o.i. of 10 pfu/cell. 24 h.p.i. the cells were harvested and the protein content was separated in parallel by 8% SDS-PAGE and subjected to western blotting in order to generate two replica blots. Blots were probed with either a polyclonal anti-PML antibody (Xu *et al*., 2003) or a monoclonal anti-GAPDH antibody, which acted as a loading control. The arrows to the right of the blot mark the migration positions of infection-specific species A, B and C, and the loading control GAPDH. Protein sizes are indicated in kDa to the left hand side of the blot.

mock-transfected, Ad5 300 infected lane (lane 2), though species A in particular was poorly expressed in this experiment. Pre-treatment with PML siRNA strongly depleted all of these infection specific species in contrast to transfection with the control siRNA (lane 8). Protein loading was comparable across the lanes, as indicated by the level of GAPDH, thus confirming that PML had been selectively

knocked down and its depletion was not the result of a general down-regulation of translation. This, in conjunction with the data presented in figure 3.2, provided strong evidence that all of species A, B and C are PML species that are produced upon infection with wild-type Ad5.

Virtually all of the bands present in the control siRNA-transfected cells were significantly depleted by the PML siRNA. This suggested that the vast majority of proteins detected by the polyclonal antibody are PML-related, reflecting the complex diversity of the PML population generated by alternative splicing and various post-translational modifications.

3.5 Sub-cellular localisation of infection-specific species of PML

The most strongly expressed, and therefore best characterised, PML proteins all contain a nuclear localisation signal that is encoded in exon 6 of the *pml* gene (Jensen, 2001). Consequently, isoforms possessing this sequence localise to the nucleus and are incorporated into PML nuclear bodies. However, cytoplasmic variants of PML are known to exist, such as PML VII (Jensen, 2001). These variants must exclude exon 6 and thus lose the NLS sequence encoded within this region, though they may still retain the unique exons encoding the carboxyl termini of specific PML isoforms. Other internal exons such as exon 4 and 5 may also be excluded from cytoplasmic variants. It is therefore feasible that for every nuclear isoform PML I-VI, there exists a cytoplasmic splice variant of smaller molecular weight. Cytoplasmic PML proteins remain relatively poorly understood relative to their nuclear counterparts.

Infection-specific species C migrates on a polyacrylamide gel to a size that is notably smaller than the six major nuclear isoforms of PML (Beech *et al*., 2005). One explanation for this is that species C represents a splice variant of PML protein that lacks the internal exons, including exons 6, which define full-length PML proteins. Such a variant of PML would be expected to show a cytoplasmic localisation within the cell. To investigate this possibility, the sub-cellular localisation of the infection-specific species of PML was examined in Ad5-infected A549 cells.

A549 cells were mock-infected or infected with wild-type Ad 5 for 24 hours. After this time, the cells were separated into the respective nuclear and cytoplasmic fractions before the addition of denaturing SDS-PAGE loading buffer. Equivalent amounts of protein from each fraction were then separated by SDS-PAGE and subjected to western blotting. The blot was probed with a polyclonal anti-PML antibody in order to detect the infection-specific species of PML (Fig. 4).

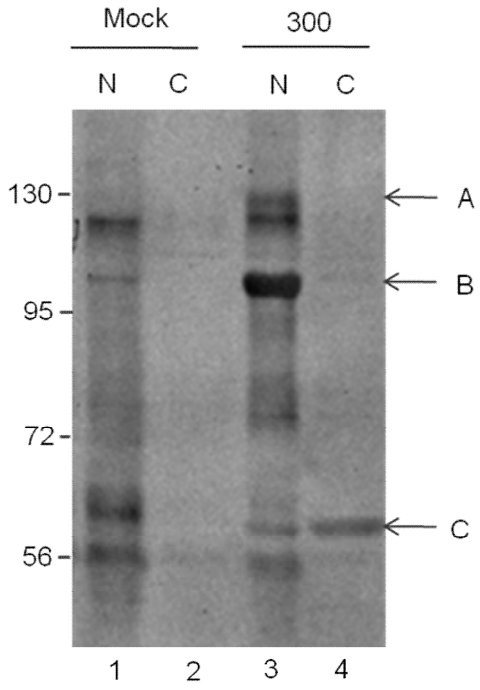


Figure 3.4. Infection-specific PML species C is abundant in the cytoplasm. 1 x 10^7 A549 cells were mock infected or infected with Ad5 300 at a multiplicity of 10 pfu/cell. At 24 h.p.i., the cells were fractionated into nuclear (N) and cytoplasmic (C) fractions, and the protein content was separated by 8 % SDS-PAGE then subjected to western blotting. The blot was probed with a polyclonal anti-PML antibody (Xu *et al.*, 2003). The arrows to the right hand side of the blot indicate the migration positions of the infection-specific species of PML. Sizes are indicated in kDa to the left hand side of the blot.

All the PML proteins detected in the mock-infected cells were predominantly in the nuclear fraction, with barely detectable levels being detected in the cytoplasmic fraction. Higher molecular weight PML proteins are assumed to be full-length and hence possess exon 6 and the NLS, and therefore are expected to show a nuclear

localisation. Surprisingly, the lower-molecular weight PML proteins, assumed to be cytoplasmic variants, also localised to the nuclear fraction. The reason for this is unclear, but it is possible that some nominally cytoplasmic species of PML are still incorporated into PML nuclear bodies by both homo- and hetero-oligomerisation of PML isoforms through coiled-coil mediated interactions, thus maintaining a nuclear localisation.

Upon infection by Ad5, expression of all three infection-specific species of PML (arrows A, B, C) could be detected at levels comparable to that of endogenous PML. Importantly, a distinct difference in banding pattern could be observed between nuclear and cytoplasmic lanes, ruling out the possibility of spill-over between these fractions by experimental error. Species A and B were confined solely to the nuclear fraction of infected cells, possibly suggesting that these represent nuclear species of PML. However, species C was consistently detected in both the nuclear and cytoplasmic fractions of infected cells, though it was always found to be more abundant in the cytoplasmic fraction. This suggests that species C is a cytoplasmic variant of PML, a scenario made more likely when one considers the relatively small size of this protein. A small amount of species C could be detected in the nuclear fraction of infected cells, suggesting that this species may either be free to diffuse across the nuclear envelope or, more likely, indicates cytoplasmic contamination of the nuclear fraction.

3.6 Infection-specific PML species are not protected from proteasome-mediated degradation by Ad5 infection

The complexity of the PML protein population within a cell is reflected by the large number of bands that may be detected by western blot using a panel of anti-PML antibodies and correspondingly depleted by PML siRNA (Figs. 3.2 and 3.3). The underlying cause of this complexity is two-fold; PML is a differentially spliced gene thought to give rise to at least 11 protein isoforms, and PML proteins are subject to a number of different post-translational modifications, including sumoylation, phosphorylation and acetylation (Duprez *et al*., 1999, Hayakawa *et al*., 2008, Hayakawa & Privalsky, 2004, Scaglioni *et al*., 2008). It is easy to see how various

isoforms of PML, or indeed various modified states of an isoform, may vary in their susceptibility to host cell processes. In particular, it is possible that the stability of PML species may be highly variable, where certain modified PMLs or isoforms may be less or more stable than other species. It is therefore possible that infection specific-species A, B and C may represent unusual species of PML that are rapidly turned over in uninfected cells, yet are specifically stabilised upon infection with adenovirus. Often, cellular proteins targeted for degradation are sent to the proteasome, a multi-protein complex, where they are proteolytically cleaved. Therefore the possible role of the proteasome in the degradation of species A, B and C in uninfected cells was considered.

A549 and HEp-2 cells were mock-infected or infected with Ad5 wild-type 300 or an E4 Orf3-deficient virus. 16 hours post-infection cells were mock treated or treated with MG132, a selective proteasome inhibitor. At 24 h.p.i., all cells were harvested and the protein content of the lysates was separated by SDS-PAGE and subjected to western blotting. Blots were probed with either a polyclonal anti-PML antibody, or a polyclonal anti-p53 antibody. P53 is a cellular protein that is targeted for proteasome-mediated degradation by the E1B 55K and E4 Orf6 proteins of Ad5 (Querido *et al*., 2001), and thus should be protected from degradation by the addition of MG132. Inhibition of the proteasome was achieved, as indicated by the stabilisation of p53 in both cell types upon the addition of MG132 (Fig 3.5 lower panels, lanes 4-6, 10-12). The addition of MG132 did not lead to the production of any species of PML in mock-infected cells of either cell type that co-migrated with any of species A, B and C from infected cells. This suggests that these do not represent rare species of PML that are normally subject to rapid proteasome-mediated degradation in uninfected cells. Compared to other experiments, infection of both cell types resulted in only low amounts of species C being produced. The reason for this is unclear, but species C levels are unlikely to vary due to proteasome-mediated degradation as the addition of MG132 did not result in an increase in the amount of this species in either of the cell lines examined.

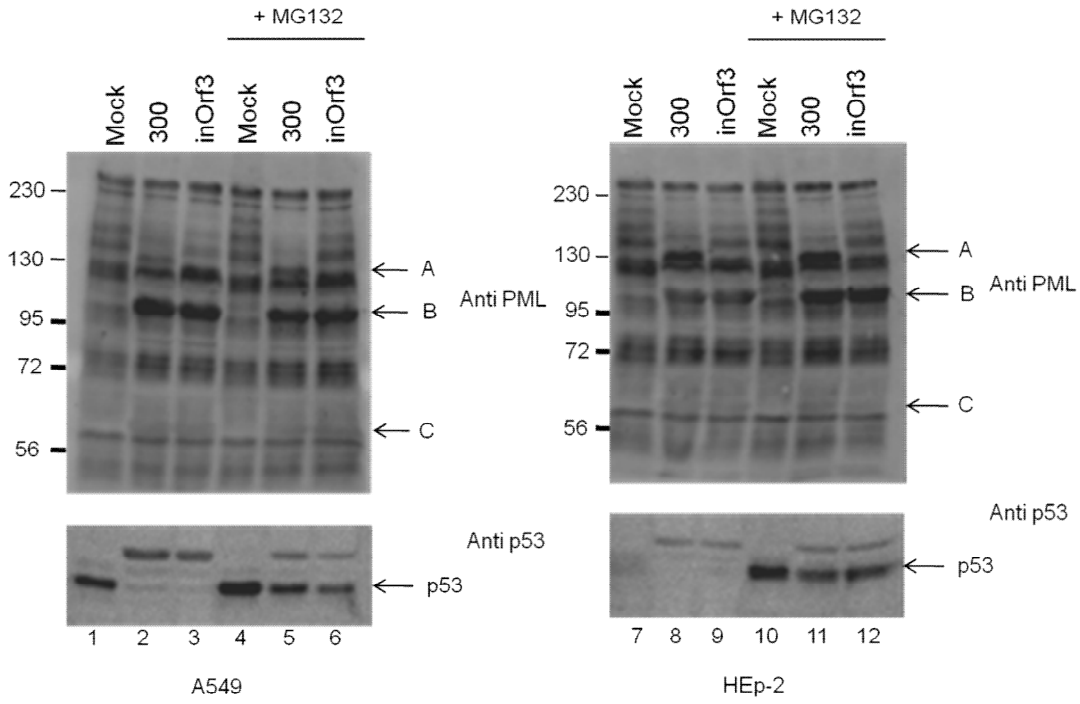


Figure 3.5. The infection-specific species of PML are not stabilised in uninfected cells by a proteasome inhibitor. 3 x 10^5 A549 cells or HEp-2 cells were mock infected or infected with Ad5 300 or Ad5 mutant *in*Orf3 at an m.o.i. of 10 pfu/cell. 16 h.p.i. cells were mock treated with DMSO, or treated with DMSO containing MG132 to a final concentration of 50µM. At 24h.p.i. all cells were harvested the protein content of the lysates was separated in parallel by 8% SDS-PAGE and subjected to western blotting to generate two replica blots per cell line. Each blot was probed with either a polyclonal anti-PML antibody (Xu *et al*., 2003) or a polyclonal anti-p53 antibody as indicated. The arrows to the right of the blots indicate the migration positions of infection-specific PML species A, B and C. Protein sizes are indicated in kDa to the left hand side of the blots.

PML species A and B were expressed strongly upon infection of both A549 and HEp-2 cells, though with notable differences in their relative amounts. Species A was clearly detected in A549 cells, but its relatively weaker expression was characteristic of more minor species of the PML population. This is in contrast to species B, which in A549 cells is the most abundant species of PML present during infection. Addition of MG132 led to an increase in the amount of species A to levels comparable to the major species of PML, though the amount of species B remained unchanged.

The opposite pattern was observed in infected HEp-2 cells, where species A in untreated cells was the most dominant species of PML. This is an observation similar to that previously reported (Leppard & Everett, 1999). Species A again was stabilised by the addition of MG132, as indicated by the stronger band in cells treated with the inhibitor. Species B, however, was a relatively minor species of PML in untreated infected HEp-2 cells but was stabilised by MG132, resulting in species B being produced at levels similar to that of species A.

Thus species A, B and C could not be stabilised in uninfected cells by the proteasome inhibitor, indicating that they are truly infection-specific. In infected cells species A and B displayed some stabilisation by MG132, suggesting that they are subject to proteasome-mediated degradation. The apparent difference in their stability between the two cell types may indicate differences in the cells' ability to regulate PML proteins, and may explain the difficulty in detecting species A in A549 cells.

3.7 Infection-specific PML species are not produced in response to interferon

PML species A, B and C are produced upon infection of cells by adenovirus. Whereas the appearance of species A is dependent on the viral protein E4 Orf3, it is unclear whether species B and C are the direct result of a specific viral activity, or rather a cellular response to virus infection. The best-characterised of such responses is the interferon response. Type I interferons are cytokines that are produced via a complex signalling pathway upon infection of a cell by a virus and exert numerous effects on the cell through both autocrine and paracrine pathways, resulting in cells adopting a state that is less permissive for both virus infection and virus replication. PML proteins have been shown to be up-regulated in response to interferon (Lavau *et al*., 1995), though it is not known whether interferon alters the splicing of the PML primary transcript to promote the production of novel variants of PML. Herpes simplex virus type I infection has previously been shown to result in the production of such a PML splice variant, and this species could be also induced through the addition of interferon (McNally *et al*., 2008). It is therefore

possible that species B and C could be interferon-inducible forms of PML that are only produced upon infection with Ad5. The effect of interferon on the PML population, and the possibility that this could account for the infection-specific species of PML, was therefore examined.

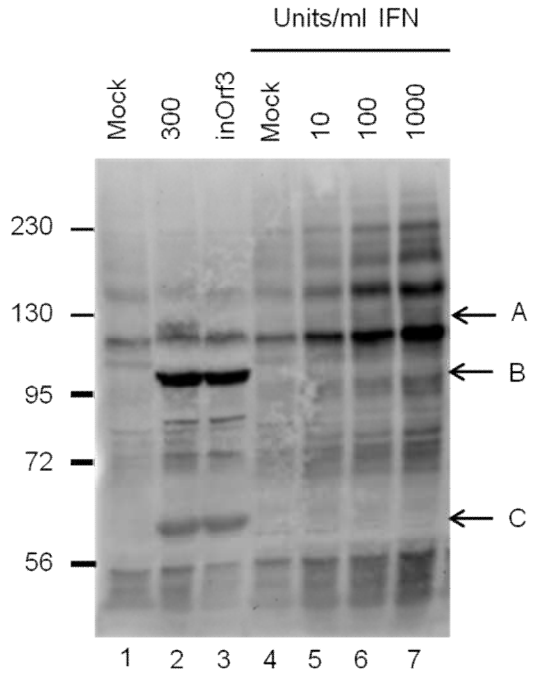


Figure 3.6. The infection-specific species of PML are not induced by the addition of exogenous interferon. 3 x 10^5 HEp-2 cells were mock infected or infected with Ad5 300 or Ad5 *in*Orf3 at an m.o.i. of 10 pfu/cell. In parallel, 3 x 10^5 uninfected HEp-2 cells were mock-treated or treated with 10, 100 or 1000 units/ml of interferon α in 10% serum media. All cells were harvested at 24 h.p.i. and the protein content of the lysates was separated by 8% SDS-PAGE and subjected to western blotting. The blot was probed with a polyclonal anti-PML antibody (Xu *et al*., 2003). The arrows to the right of the blot indicate the migration positions of infection-specific species A, B and C as detected in lane 2. Protein sizes are indicated in kDa to the left hand side of the blot.

HEp-2 cells were mock infected, or infected with Ad5 wild-type 300 or E4 Orf3-deficient virus (inOrf3) for 24 hours prior to harvest. In parallel, uninfected HEp-2 cells were mock-treated or treated with increasing amounts of recombinant interferon α (IFNα) for 24 hours before being harvested. The protein content of all cells was then separated by SDS-PAGE and subjected to western blotting. The blot was probed with a polyclonal, anti-PML antibody (Fig.3.6).

The addition of IFNα to the cells had, as expected, a strong dose-responsive effect, as higher concentrations led to more PML proteins being produced. Interferon treatment increased the amount of the entire PML protein population proportionately, as no difference in the relative amounts of any species was observed. The large majority of the species detected by this antibody were increased in response to interferon, providing evidence that all of the species observed are PML related. This conclusion agrees also with the data in figure 3.3, where an siRNA targeting all PML species resulted in a clear loss of the majority of bands on a western blot, as detected by an anti-PML antibody. Clearly, interferon treatment did not appear to alter the ratios of species of PML, suggesting that it has little effect on the splicing of the PML primary transcript. Importantly, no novel species were produced upon interferon treatment that co-migrated with any of species A, B and C. This suggests that the infection-specific PML species A, B and C are likely to be produced as a direct result of Ad5 functions, and do not represent part of a host anti-viral response mediated by interferon.

3.8 PML species B and C require Ad5 DNA replication for their production

Production of the infection-specific PML species A has been shown to require the viral E4 Orf3 protein in order to be produced (Leppard & Everett, 1999). E4 Orf3 is expressed early in the course of an adenovirus infection, and precedes the onset of viral DNA replication. The viral determinant for the production of infection-specific species B and C however remains unknown. In order to further understand species A, B and C, the role viral DNA replication plays in their production was investigated. HEp-2 cells and A549 cells were mock infected or infected with Ad5 wild type 300 or E4 Orf3-deficient (inOrf3) adenovirus. At 1 h.p.i., cells were mock treated or treated with 10mM hydroxyurea. Hydroxyurea is an inhibitor of ribonucleotide reductase and thus prevents the production of deoxyribonucleotides, therefore inhibiting DNA synthesis. 24 h.p.i. cells were harvested and the protein content was separated by SDS-PAGE and western blot. Blots were probed with a polyclonal anti-PML antibody, or antibodies to various adenovirus proteins (Fig. 3.7).

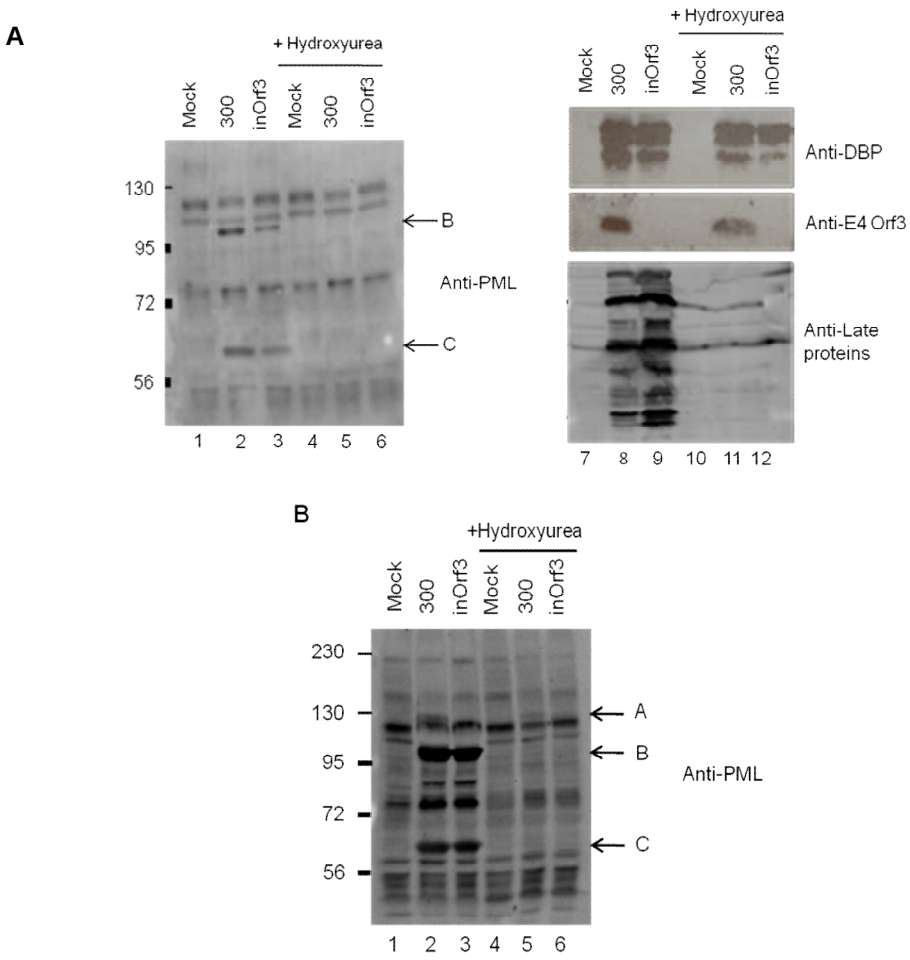


Figure 3.7. Production of infection-specific PML species B and C require Ad5 late phase functions. A 3 x 10^5 A549 were mock infected or infected with Ad5 300 or Ad5 *in*Orf3 at an m.o.i. of 10 pfu/cell. 1h.p.i. the culture medium was replaced with fresh 10% serum DMEM alone or medium containing 10mM hyrdoxyurea. All cells were harvested at 24 h.p.i. and the protein content of the lysates was separated in parallel by 8%, 10% and 15% SDS-PAGE and subjected to western blotting in order to generate four blots. The blot representeing 8 % PAGE separation was probed with a polyclonal anti-PML antibody (Xu *et al*., 2003). The blots representing 10% PAGE separation was probed with a polyclonal antibody against late structural proteins of Ad5 (AdJLB), and blots representing 15% PAGE separation were probed with either a monoclonal anti-DBP antibody or an anti-E4 Orf3 antibody (6A11). The arrows to the right of the anti-PML blot indicate the migration position of infection-specific species A, B and C. Protein sizes are indicated in kDa to the left hand side of each blot. **B** 3 x 10^5 HEp-2 cells were treated as in **A,** though the protein content of the lysates was separated by 8% SDS-PAGE alone. The blot was probed with a polyclonal anti-PML antibody. The arrows to the right of the blot indicate the migration positions of infection-specific species A, B and C. Protein sizes are indicated in kDa to the left hand side of the blot.

The addition of hydroxyurea in A549 clearly inhibited the production of adenoviral late proteins, which are produced after viral DNA replication, but had no effect on two early gene products, Orf3 and DBP, suggesting that viral DNA replication had been sufficiently inhibited (Fig 3.7A, right hand panels). Species A was particularly poorly expressed in infected A549 cells, even in the absence of hydroxyurea, making any analysis of this species impossible within this cell type. HEp-2 cells did produce detectable amounts of species A (Fig. 3.7B), though the amount relative to endogenous PML appeared to decrease after the addition of hydroxyurea, suggesting that species A may not be solely dependent on E4 Orf3, and indeed may require an additional factor produced by DNA replication or subsequently to it.

In both HEp-2 and A549 cells the production of species B and C was completely inhibited by hydroxyurea, suggesting that these species are produced upon, during or after viral DNA replication, and directly require a factor produced by this process or subsequent to it. Without viral DNA replication, Ad5 is unable to produce the proteins characteristic of the late phase of infection. It is possible that a late phase protein or proteins of Ad5 may be directly responsible for the production of species B and C, or else the altered cellular environment of a late phase infection may generate these new PML forms.

3.9 Investigating viral determinants required for the production of PML species A, B and C

Each of PML species A, B and C is only produced when infection by Ad5 occurs. In the case of species A, E4 Orf3 is necessary for its production, though any viral determinants required for the production of B and C remains unknown. In order to investigate what viral proteins may be required, a selection of adenoviral proteins were chosen and exogenously expressed in several cell lines. The effects on the PML population could then be monitored by use of an anti-PML antibody. The viral proteins were selected according to their known functions and how these might influence the PML population through either a post-translational modification or by altering the splicing pattern of PML mRNA.

E4 Orf3 and E4 Orf4

Ad5 E4 Orf3 has been shown to be required for the production of species A, as viruses deficient in this protein do not produce this species. Unlike species B and C, species A does not require viral DNA replication or any viral late proteins for its production. However, exogenous expression of E4 Orf3 alone is unable to induce changes in the PML population of transfected cells (K. Leppard, unpublished). It is therefore possible that E4 Orf3 functions in concert with an additional Ad5 early gene protein to alter the PML population. Previous studies have indicated that E1B-55K and E4 Orf6 are not required for the production of species A (Leppard & Everett, 1999), so an alternative early gene product was considered in E4 Orf4. This protein is known to modulate viral transcript splicing through dephosphorylation of cellular SR proteins, which is achieved by Orf4-induced activation of protein phosphatase 2A (Kanopka *et al*., 1998). It was considered that Orf4 may alter post-translational modifications of PML proteins, and this possibility was therefore investigated.

Wild-type E4 Orf4 was amplified by PCR from Ad5 genomic DNA and cloned into the expression vector pcDNA 3.1(-) (see Appendix). In order to confirm expression of Orf4 from two plasmid clones, HEK 293 cells were mock-transfected or transfected by either E4 Orf3 or E4 Orf4 expression plasmids. Two different clones of the Orf4 expression plasmid were used (Cl.1 and Cl.2). Cells were harvested 24 hours later and SDS-PAGE and western blotting was used to analyse the protein content for PML, Orf3 and Orf4 expression (Fig. 3.8). Strong Orf3 and Orf4 expression was confirmed using antibodies specific to each of these proteins.

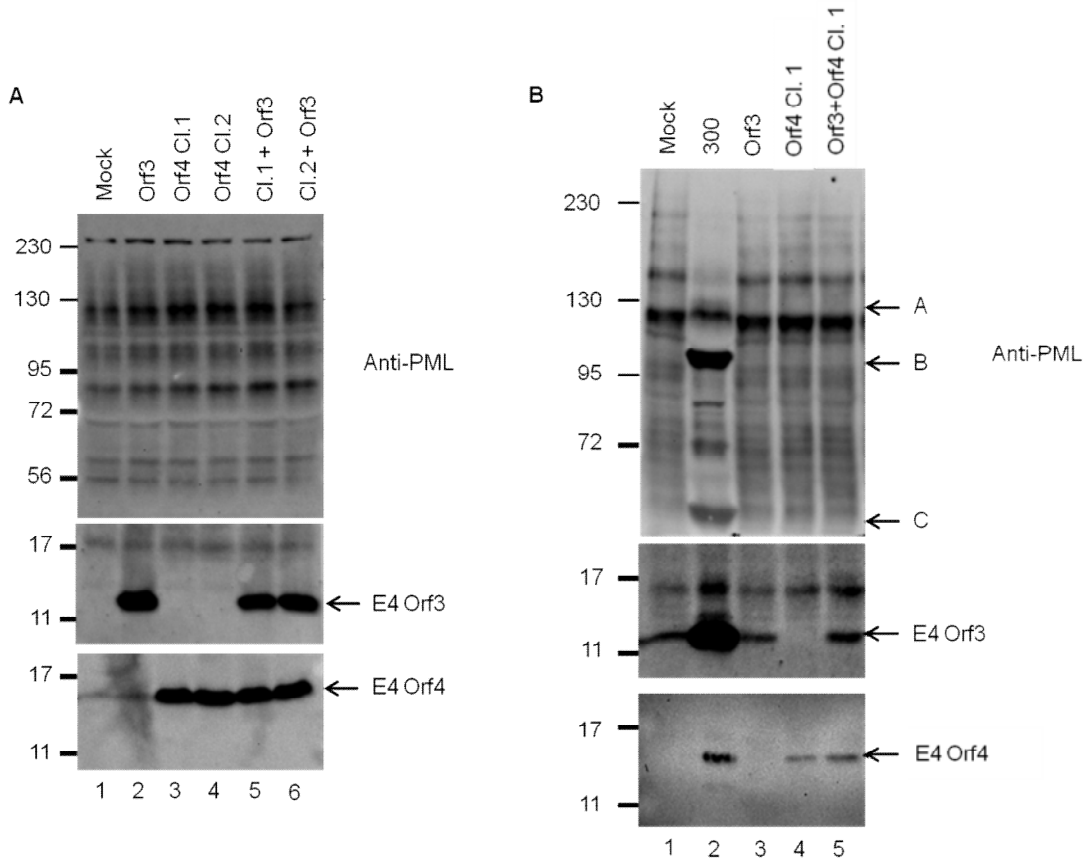


Figure 3.8. Expression of E4 Orf3 or Orf4 does not alter the PML protein population. **A** 2.5 x 10^5 HEK293 cells were transfected with 500ng empty vector (pcDNA3.1 (-), lane 1)) or transfected with 250ng pcDNA3.1-*orf3* alongside 250ng empty vector pcDNA3.1 (lane 2). 250ng of two clones of pcDNA3.1-*orf4* were used to transfect cells alongside 250ng of empty vector pcDNA3.1 (lanes 3 and 4) or pcDNA3.1-*orf3* (lanes 5 and 6). 24 hours later cells were harvested and the protein content of the lysates was separated by 8% (for PML) or 15% (Orf3, Orf4) SDS-PAGE and subjected to western blotting. Replica blots were probed with polyclonal anti-PML (Xu *et al*., 2003), anti-E4 Orf3 (6A11) or anti-E4 Orf4. The arrows to the right of lower panels indicate the presence of E4 Orf3 or Orf4 respectively. Proteins sizes are indicated in kDa to the left of the blot. **B** 2.5 x 10^5 HEp-2 cells were mock infected or infected with wild-type Ad5 300 at an m.o.i. of 10 (lanes 1 and 2). In parallel, 2.5 x 10^5 HEp-2 cells were co-transfected with 250ng pcDNA3.1-o*rf3* or pcDNA3.1-*orf4* alongside 250ng empty vector pcDNA3.1 (lanes 3 and 4) or pcDNA3.1-o*rf3* and pcDNA3.1-o*rf4* together (lane 5). Cells were harvested 24 hours post infection or 24 hours after transfection, and the protein content of the lysates analysed as in panel A. The infection-specific species of PML are indicated to the right of the blots by arrows marked A, B and C. The arrows to the right of the lower panels indicate E4 Orf3 and Orf4 respectively. Protein sizes are indicated in kDa to the left of the blot.

However, expression of these proteins had little effect on the PML population, which remained unchanged from that of mock-transfected cells.

HEp-2 cells were then mock transfected or transfected with E4 Orf3 or the E4 Orf4 clone 1 plasmid, then harvested 24 hours later. In parallel HEp-2 cells were mock-infected or infected with wild type Ad5 300, and harvested 24 h.p.i. The protein content of the lysates was then analysed by SDS-PAGE and western blotting. Orf3 and Orf4 expression was confirmed using the antibodies mentioned above, though the level of expression in transfected cells was significantly weaker than that seen in 293 cells, probably due to a lower transfection efficiency.

Species A, B and C were detected in the Ad5 300 infected HEp-2 cells, though no corresponding changes were observed in any of the cells transfected with Orf3, Orf4 or both of these proteins. Thus none of the infection-specific species of PML can be recreated by exogenous expression of these two proteins.

L4 22K and 33K

A possible mechanism underlying the production of species A, B and C is an alteration in splicing of the PML primary transcript that is induced by Ad5. Ad5 is known to use alternative splicing to generate various mRNAs, and has been shown to encode proteins that can alter the cellular splicing machinery. In particular, the L4 22K and 33K proteins have been shown to act as splicing factors which regulate the production viral mRNAs (Farley *et al*., 2004, Morris & Leppard, 2009, Törmänen *et al*., 2006). These proteins are produced at the onset of the late phase of infection, and are good candidates as viral factors responsible for producing infection-specific changes in the PML population (Fig. 3.9).

HEp-2 cells were mock transfected or transfected with pCMV-22KFLAG, pCMV-33KFLAG or pCMV-22/33KFLAG, which expresses both L4 22K and L4 33K from their overlapping reading frames. 24 hours later cells were harvested and the protein content was analysed by SDS-PAGE and western blotting. An anti-FLAG antibody was used to confirm expression of the epitope-tagged proteins, and a polyclonal anti-PML antibody was used to probe the PML protein population.

Expression of 22K and 33K was detected only when expressed from the plasmids encoding one of these proteins. When expressed in *cis* alongside 22K, 33K was

poorly expressed in comparison to cells transfected with 22K and 33K in *trans*. The 22K of pCMV-22/33KFLAG is not FLAG-tagged, and cannot be detected by the antibody used in this instance. However, expression of 22K or 33K, either alone

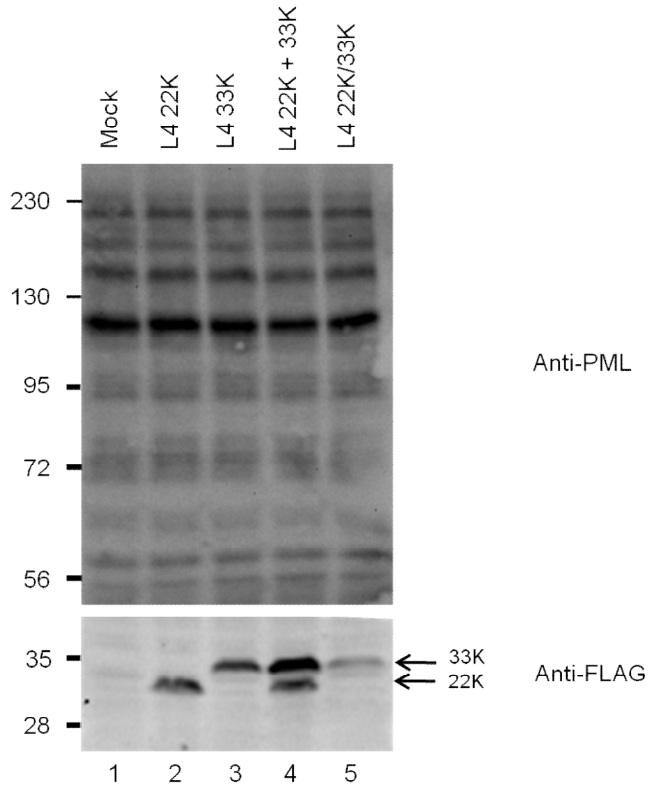


Figure 3.9 L4 22K and 33K do not induce the production of any infection-specific species of PML. 2.5 x 10^5 HEp-2 cells were mock transfected with 500ng empty vector pCI-neo (lane 1) or transfected with 250ng of empty vector alongside 250ng L4 22K expression plasmid pCMV-22KFLAG (lane 2) or L4 33K expression plasmid pCMV-33KFLAG (lane 3). Where simultaneous 22K and 33K expression was required, cells were transfected with 250ng each of pCMV-22KFLAG and pCMV-33KFLAG (lane 4), or with 500ng of pCMV-22/33KFLAG (lane 5). 24 hours later cells were harvested, separated by 8% SDS-PAGE and western blotting in order to generate two replica blots. Each blot was probed with either a polyclonal anti-PML antibody (Xu *et al*., 2003) (upper panel) or a monoclonal anti-FLAG antibody (M2, lower panel). 22K and 33K are indicated by arrows to the right hand side of the blot. Protein sizes are indicated in kDa to the left hand side.

or together in *trans*, did not produce any detectable changes in the PML population, and did not lead in the production of species A, B or C. These data therefore suggest

that 22K and 33K are not the viral proteins responsible for the production of the infection-specific species of PML.

Protein IX (pIX)

pIX is an intermediate gene product of adenovirus that is thought to stabilise the capsid of the virus, as well as to act as a transcriptional activator (Lutz *et al*., 1997, Rosa-Calatrava *et al*., 2001). Expression of pIX leads to the appearance of novel nuclear structures, termed clear amorphous inclusions that do not co-localise with viral RNA or capsid proteins. PML protein has been shown to be recruited to and confined within these inclusions (Rosa-Calatrava, 2003), leading the authors to hypothesise that pIX maintains the disruption of PML protein throughout late stagesof infection in order to enhance the growth of the virus.

This observation suggested a possible interaction of pIX with the PML population, and pIX was therefore considered as a candidate protein responsible for the induction of species B and C. HEp-2 or U2OS cells were mock transfected, or transfected with either empty vector plasmid or a pIX expression plasmid (pCMV-IX) (Caravokyri & Leppard, 1995). 24 hours later the cells were harvested and the protein content of the lysates was subjected to SDS-PAGE and western blotting (Fig. 3.10). pIX expression in both cell lines was confirmed using an anti-pIX antibody (Caravokyri & Leppard, 1995), though expression was far weaker in HEp-2 cells. However, no changes in the PML population were detected using a polyclonal anti-PML antibody in either cell type. Thus pIX is unlikely to be responsible for the production of species B and C, though the possibility that it works in concert with other viral proteins cannot be ruled out.

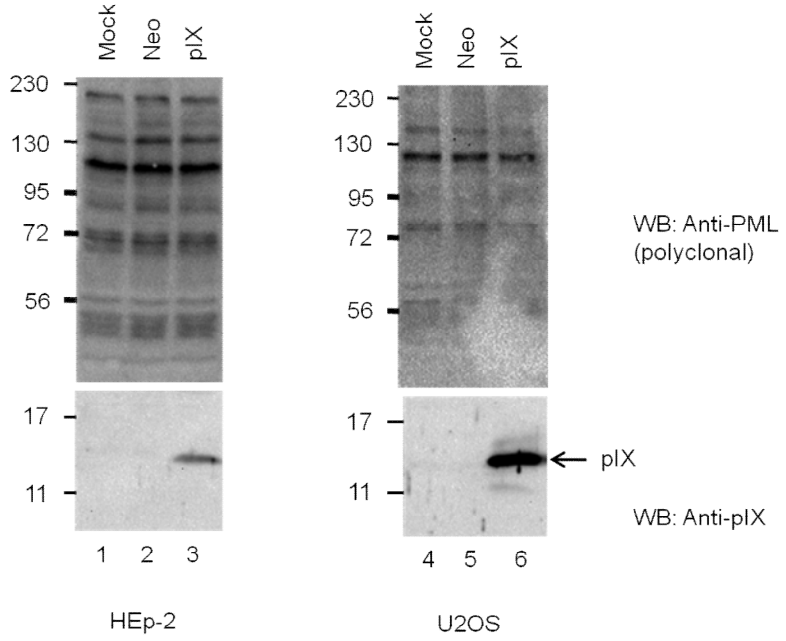


Figure 3.10 Expression of protein IX does not induce changes in the PML protein population. 2.5 x 10^5 HEp-2 (lanes 1-3) or U2OS (lanes 4-6) cells were mock transfected (lanes 1-4), or transfected with 500ng of empty vector DNA pCI-neo (lanes 2, 5) or 500ng pCMV-IX (lanes 3 and 6). 24 hours later cells were harvested and the protein content of the lysates was separated by 8% (PML) or 15% (pIX) SDS-PAGE and subjected to western blotting. Blots were probed with a polyclonal anti-PML antibody (Xu *et al*., 2003)or an anti-pIX antibody. The arrow to the right of the lower panels marks the migration position of pIX. Protein sizes are indicated in kDa to the left hand side of the blots.

3.10 Discussion

The diversity of the PML protein population of a cell is generated by two principle mechanisms. Firstly, the PML primary transcript can be differentially spliced to generate mRNAs encoding at least 11 protein isoforms consisting of both nuclear and cytoplasmic variants of PML (Jensen, 2001). Second, PML proteins can be extensively post-translationally modified in a number of ways, including sumoylation, phosphorylation and acetylation (Hayakawa *et al*., 2008, Kamitani *et al*., 1998, Scaglioni *et al*., 2006). With these two processes combined, it is easy to see how a multitude of PML species may be generated within the cell that each possesses its own unique biochemical properties and functions. Thus, by altering the constitution of the PML population a cell may adjust to suit various external or internal stimuli, and these processes may be exploited by adenovirus to make the cell more conducive to viral replication.

The data presented in this chapter describe changes induced in the PML population upon infection by adenovirus. Notably, three infection-specific species of PML were detected upon infection of a variety of cell types. These species were validated as PML species by the use of three independent anti-PML antibodies and through the use of siRNA-mediated depletion of PML. One of these species, termed species A, has previously been described and requires the viral E4 Orf3 protein for its production. The two other species B and C are previously unrecorded and therefore represent novel species of PML. The production of these species was independent of the E4 Orf3 status of the infecting virus, but required viral DNA replication. Species C is of a lower molecular weight than that of known nuclear PML isoforms, and accordingly was shown to be localised within the cytoplasm of infected cells. In uninfected cells, species A, B and C could not be detected even after inhibition of proteasome-mediated degradation, suggesting that these species are produced only upon infection by adenovirus. The mechanism employed by adenovirus to generate these species remains unknown, but it is likely to involve alterations to the processes mentioned above.

Infection-specific species A was detected by the polyclonal anti-PML antibody and, as previously described, by the monoclonal antibody 5E10 (Leppard & Everett, 1999). Species A was not detected by the monoclonal PG-M3 antibody. The reason for this is unknown, but it was noted that PG-M3 did not detect any bands corresponding to the migration positions of full-length PML I and PML II and their sumoylated forms. This may suggest that PG-M3 has difficulty detecting the entire PML population on a western blot. One possible explanation is that the epitope targeted by this antibody may be the subject of a post-translational modification in a subset of PML species, masking them from the antibody. The epitope of PG-M3 lies 15 amino acid residues from the first sumoylation site of PML (Santa Cruz Biotechnology), and antibody recognition may be disrupted when this particular site is sumoylated.

The 5E10 antibody did not detect species B and C in infected cells for two possible reasons. Firstly, they may be post-translationally modified at or in close proximity to the amino acid sequence recognised by this antibody, thus preventing recognition by 5E10. Alternatively, they may represent alternative splice variants of PML that lack exon 5, which encodes the 5E10 epitope (Stuurman *et al*., 1992), and it is possible that adenovirus is altering cellular splicing to favour the production of these species. Species C in particular is likely to arise from a novel differentially spliced transcript representing a cytoplasmic variant of PML. Indeed species C was found to be strongly expressed in the cytoplasm of infected A549 cells. Cytoplasmic variants of PML exclude exon 6 (which contains the NLS) in conjunction with exon 5 alone or with both exons 4 and 5. Therefore these variants would not possess the epitope detected by 5E10. Species B, however, showed exclusively nuclear localisation and is of a molecular weight similar to that of other nuclear PMLs. It may elude detection by 5E10 because it possesses a post-translational modification within the epitope, though it is possible that it too lacks exon 5. PML variants are known to exist that lack exon 5, but retain all the other exons typical of nuclear isoforms (Jensen, 2001), and these would be nuclear in location.

The PG-M3 antibody did detect infection-specific species that co-migrated with species C, supporting a PML protein basis for this species. However, it was apparent that the relative amounts detected varied from the polyclonal anti-PML antibody, though care has to be taken when comparing relative amounts of a protein detected

by two different antibodies. The binding kinetics between each antibody and its respective epitope(s) will differ according to multiple factors, including post-translational modifications, sample preparation and experimental method.

All of the infection-specific PML species were depleted by siRNA targeting exon 3 of PML, though only species A was detected by the 5E10 antibody which recognises an epitope encoded within exon 5 of the PML protein. These observations, taken together with the relatively large size of species A, suggest that it is based on a full-length nuclear form of PML. Species A was solely confined to the nuclear fraction of infected cells alongside other larger PMLs. Indeed the migration position of species A on a PAGE gel corresponds to a molecular weight that is slightly larger than PML isoforms I and II. Given that E4 Orf3 has been shown to directly interact with the unique C terminus of PML II (Hoppe *et al*., 2006), this isoform is the most likely candidate as the basis of this species. A post-translational modification of PML II induced by E4 Orf3 may be responsible for the increase in molecular weight which could account for species A. This possibility is addressed in the following chapter.

Notably, PML species A was the only species that was not detected in every cell type examined. After repeated attempts, U2OS cells were found to be incapable of producing this species, yet still supported the appearance of species B and C. This suggests either that this cell type possesses an unusual PML population or that it lacks the required cellular machinery to support the production of species A. U2OS cells have previously been recorded to possess unusual properties that may be linked to an aberrant PML population. For example, the HSV-1 early gene product ICP0 has been shown to target PML NBs and cause de-sumoylation, and the subsequent proteasome-mediated degradation, of PML isoforms (Everett *et al*., 1998). ICP0 mutants grow poorly in Vero cells, but to levels similar to wild-type in U2OS cells (Mossman *et al*., 2000). Furthermore, while IFNα inhibits ICP0$^-$ virus growth, it has no effect on the growth of the virus in U2OS, suggesting that this cell type is somehow deficient in the IFN pathway (Mossman *et al*., 2000). Given that PML proteins are implicated in the IFN response pathway, it is possible that U2OS cells lack an isoform of PML normally responsible for exerting an antiviral effect. One may then speculate that it is this isoform that is usually targeted by Ad5 E4 Orf3, resulting in the production of species A. In support of this, exogenously-expressed

ICP0 has been shown to function analogously to E4 Orf3 within the context of an Ad5 inOrf3 infection (Ullman & Hearing, 2008). However further experimentation would be required to test this hypothesis. Specifically, it would be of interest to compare the spectrum of individual PML isoforms in U2OS cells to that of a cell line capable of producing species A. If U2OS cells were found to be deficient in a particular PML isoform then this would be a good candidate as the basis of species A.

None of the infection-specific PML species were induced through the addition of exogenous IFNα to the culture medium. As previously described, addition of IFNα led to a marked increase in PML protein expression, further supporting the notion that PML can act as an antiviral effector. Such an increase in PML may pose a particular problem to Ad5, as increasing amounts of an antiviral protein will inhibit viral replication. Therefore, Ad5 may possibly alter the PML population to overcome the antiviral effects of PML, and these alterations exhibit themselves as the infection-specific species observed. As mentioned above, the two most plausible mechanisms whereby Ad5 may generate novel PML species is either novel post-translational modification or by interfering with the splicing process that generates different PML species. It is worth noting that these two mechanisms are not mutually exclusive, as a novel splice variant of PML may still be post-translationally modified exclusively upon virus infection.

The possibility that post-translational modification in the form of proteolytic cleavage by the adenovirus 23K protease was considered, though comparison of the amino acid sequence of each of the PML protein isoforms revealed no similarity to the 23K target site consensus sequence (M/I/L XGG-X or M/I/L XGX-G (Webster *et al.*, 1989)). This indicated that this protease is not responsible for the production of the infection-specific species. Nonetheless, this would need to be confirmed experimentally and does not discount the possibility of a role of cellular proteases that may be induced upon infection in the production of the infection-specific forms of PML protein.

The population of PML mRNA transcripts has previously been shown to be altered during HSV-1 infection. The amount of a cytoplasmic variant of PML, termed PML Ib, was shown to be disproportionately increased (McNally *et al.*, 2008). This

species of PML excludes exons 5 and 6, resulting in a frameshift that ultimately terminates the protein prematurely in exon 7a. While this species was up-regulated in response to IFN-γ, its ratio relative to the total PML population remained unchanged.

Herpes simplex virus type 2 (HSV-2) has recently been shown also to alter the PML population. The viral ICP27 protein was shown to influence the splicing of the PML primary transcript to favour production of PML V over PML II (Nojima *et al*., 2009). Whether Ad5 exerts similar effects on PML splicing to produce any of the infection-specific species of PML is unknown.

None of the infection-specific species can be recreated in uninfected cells by inhibiting proteasome-mediated degradation, supporting the hypothesis that these are specific changes induced by the virus. The possibility that adenovirus may selectively target and interact with rare splice variants or post-translationally modified PML molecules in order to protect them from degradation, can therefore be discounted. Thus all three of the infection-specific species are likely to be produced only upon the action of a specific viral protein or proteins, or indirectly via virus infection-induced effects on the cell. Interestingly, species A and B were somewhat stabilised by addition of MG132 in A549 and HEp-2 cells respectively. This indicates that once these species are produced, they are subject to regulation and proteasome-mediated degradation that may vary according to cell type. This may explain the relative difficulty in detecting species A within infected A549 cells as compared with HEp-2 cells, and suggests that the infection-specific species of PML may be regulated in differential ways depending on the cell type.

The precise viral determinants required for the production of these infection-specific PML species remains incompletely understood. E4 Orf3 is known to be a viral factor required for species A, yet is unable to induce this species alone by exogenous expression. However, this methodology has limitations, the most important of which is the efficiency of transfection. HEp-2 cells were shown to express Orf3 poorly by transient transfection when compared to that produced during a viral infection, indicating a poor transfection efficiency. Poor efficiency reduces the ability to detect any potential changes in the PML population induced by the protein being over-expressed, and thus may lead to a false negative result. This problem was somewhat

overcome by examining the effects of Orf3 expression in HEK293 cells, which are readily transfectable and expressed Orf3 to high levels. That no change was detected in either cell type suggests that E4 Orf3 may require an additional viral protein in order to produce species A. Since the production of species A is not inhibited by the addition of hydroxyurea and hence does not seem to require any late proteins, it is likely that the additional protein required is an early or intermediate Ad5 gene product. Adenovirus is known to use early viral proteins in concert in order to achieve specific functions, for instance E4 Orf6 and E1B 55K act together to target p53 for proteasomal degradation (Querido *et al*., 2001). E4 Orf6 and E1B 55K deficient viruses are still capable of producing species A, however, and are unlikely to be the proteins responsible (Leppard & Everett, 1999). In both HEp-2 and HEK293 cells, no evidence could be found that exogenous expression of Orf3 alone, or in concert with the early protein E4 Orf4, could alter the PML population. Therefore it is likely that species A is produced by an action of Orf3 working in tandem with another unknown viral protein.

Production of PML species B and C was completely inhibited by treatment of infected cells with hydroxyurea, indicating a late protein or proteins are responsible for their appearance. However, expression of the splicing factors L4 22K, 33K, or the intermediate protein pIX, did not result in the production of any species resembling B or C, though as mentioned above, the success of this methodology is dependent on obtaining a sufficiently high transfection efficiency, so any role of these proteins cannot be completely ruled out. Nonetheless, significant expression of these viral proteins was detected and no corresponding change in the PML population was observed, suggesting that these proteins do not induce any infection-specific species of PML alone.

Chapter 4
Characterisation of the infection-specific species of PML

4.1 Introduction

Infection of a number of cell types by Ad5 results in the production of three novel species of PML protein (Chapter 3), henceforth termed PML- A, -B and -C. The basis and nature of these species remained unknown and these questions were therefore addressed by the experiments presented in this chapter. PML-A is dependent on the viral E4 Orf3 protein and is not produced upon infection of U2OS cells. In contrast, PML-B and -C are E4 Orf3-independent and are produced in U2OS cells. Based on these observations, it is likely that the production of species A is a separate phenomenon from that of PML-B and -C, and therefore the following experiments were designed with this is mind.

The experiments initially focused on determining the PML isoform basis of each of PML- A, -B and- C. The primary tool utilised to achieve this was a set of PML isoform-specific antibodies. These antibodies recognise the unique C terminus of their respective isoform. The use of antibodies was complemented by studies using various siRNAs against different PML isoforms. Preliminary studies were then made to characterise the differences between infection-specific PML species and those present in uninfected cells. Experiments focused on the two primary mechanisms that Ad5 might employ to generate novel species of PML; by alternative splicing or by targeted post-translational modification of existing isoforms.

4.2 Validation of PML isoform-specific antibodies

The primary transcript of the *pml* gene is alternatively spliced to give rise to mRNAs encoding at least 11 protein isoforms. Until recently, anti-PML antibodies have tended to be pan-isoform specific and therefore detect most, if not all, of the PML protein isoforms produced by the cell. In order to study individual PML isoforms it is necessary to utilise antibodies that recognise the unique C-terminal region of the respective isoforms. Such antibodies were used during the course of this study, though only antisera to PML isoforms I-V were available (Xu *et al*., 2005), as the C-terminal region unique to PML VI is too short for an epitope to produce an isoform-specific antibody. Prior to experimental use, it was important that each isoform-specific antibody was confirmed as specific for the relevant PML isoform. In particular, it was important to verify that the antibodies were specific to one PML isoform only, and did not cross-react with other PML isoforms.

HEp-2 cells were transiently transfected with one of the expression plasmids pCI-neoFLAG PML I-VI or empty vector pCI-neoFLAG. 24 hours later the cells were harvested to generate a set of lysates that possessed an abundance of a single PML isoform. The protein content of these lysates was separated multiple times in parallel by SDS PAGE to allow a set of replica blots that could then be subjected to western blot analysis using either anti-FLAG or one of the isoform-specific anti-PML antibodies (Fig. 4.1).

The presence of each of the exogenously expressed PML proteins was confirmed by using the anti-FLAG antibody, as the FLAG epitope is present on the N-terminus of these proteins (Fig. 4.1). All proteins were expressed, though PML IV expression was weak. Replica blots, independently probed with each of the isoform-specific antibodies anti-PML II-V (Fig. 4.1), clearly detected a single band only in the expected lane that corresponded to the correct size of the respective PML. With each of these antisera, few non-specific bands were observed in any of the other lanes, indicating that the antibodies are highly specific and produce little non-specific background. However, anti-PML II, IV and V detected some bands within the relevant lane that were of a lower molecular weight than the isoform being detected. These bands possibly represent degradation products arising from the

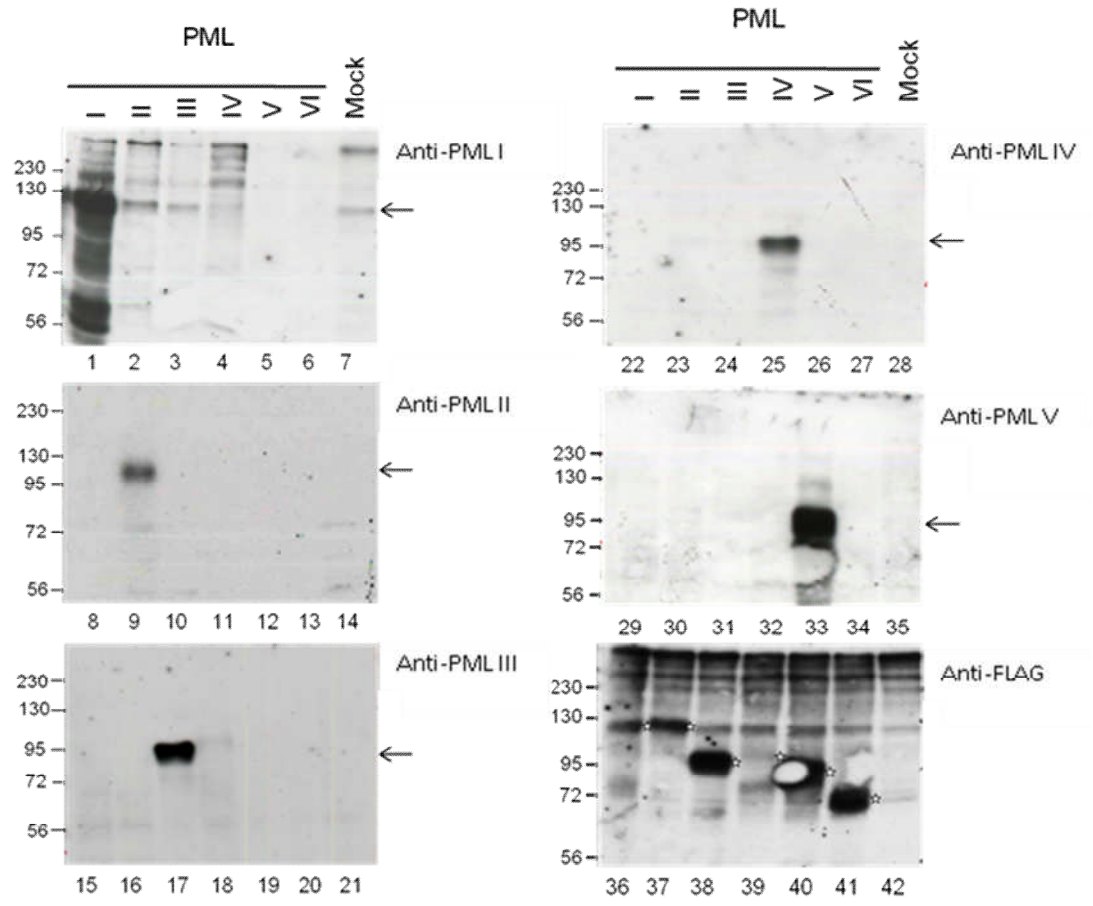


Figure 4.1 Validation of PML isoform-specific antibodies. 3 x 10^5 HEp-2 cells were transfected with 500ng each of expression plasmids pCI-neo FLAG-PML I-VI or empty vector pCI-neo (mock). 24 hours later cells were lysed and proteins were separated by 8% SDS-PAGE then subjected to western blotting. Each lysate was loaded 6 times in parallel to allow the protein content to be independently probed by each of Anti PML I-V or Anti FLAG (indicated to the right of each blot). For blots Anti-PML I-V the correct migration position of the protein being detected is indicated by an arrow. For lanes 36-41 an asterisk is adjacent to the band that corresponds to the relevant PML protein. Protein sizes are indicated in kDa to the left-hand side of each blot.

exogenously-expressed protein that still possess the C-terminal epitope of the isoform in question.

In contrast, the anti-PML I antibody clearly detected numerous bands in all lanes, though the strongest band seen appeared in the correct lane and was of the expected size for full-length PML I. The abundance of lower molecular weight proteins in the PML I lane may be the degradation products of the exogenously expressed PML

I protein. This explanation is strengthened when one considers the epitope of the anti-PML I antiserum, which corresponds to the last 20 amino acids of the C terminus of PML I. With this extreme C-terminal epitope, it is clear how this antiserum could detect a multitude of PML I-related fragments that are the result of various cleavage reactions along the length of this protein.

Relative to the other blots, anti-PML I also produced a relatively strong banding pattern in lanes other than those representing PML I-transfected cells. This banding pattern was similar to that of PML-I transfected cells and occurred both in cells transfected with empty vector (mock) and PML expression plasmids, ruling out cross-reactivity with other PML isoforms. It is therefore likely that the anti-PML I antibody was detecting the endogenous PML I and its higher molecular weight, sumoylated forms (lanes 2-4 and 7), an explanation that is supported by the fact that PML I is amongst the most strongly expressed PML isoforms (Condemine *et al*., 2006). Taken together, it was therefore judged that all of the isoform-specific antibodies were sufficiently specific for use in subsequent experiments.

Differences were observed in endogenous levels of PML I when PMLs IV, V and VI were over-expressed (lanes 4-6), as compared to mock-transfected cells. Over-expression of PML IV caused an increase in sumoylated forms of PML I, whereas expression of PMLs V and VI caused a decrease in total PML I content. These observations suggested a possible auto-regulatory role of PML isoforms on the total PML population, but investigation of this phenomenon was outside the scope of this study.

4.3 Attempted detection of PML species A using PML isoform-specific antibodies

Infection-specific PML-A has been shown to be dependent on the presence of the Ad5 early gene product E4 Orf3. The molecular basis of this novel PML species is unknown, as it does not co-migrate with any known PML isoforms by SDS-PAGE separation. In order to identify this species, the validated PML isoform-specific antibodies were employed to probe lysates from cells infected with either wild-type or an E4 Orf3-deficient mutant Ad5 (Fig. 4.2).

In the case of anti-PMLs I, II, IV and V a band of significant intensity was seen in both infected and mock-infected cells that corresponded to the expected size for that full-length isoform. It is notable that anti-PML III did not detect a species that co-migrated with PML III expressed from cDNA. A possible explanation may be that PML III has been shown to be the most weakly expressed of PML isoforms (Condemine *et al*., 2006), making detection of the low levels of endogenous PML III by this anti-serum particularly difficult.

As expected, infection by Ad5 wild type 300 (lane 2) resulted in the production of a species of PML detected by the pan-PML antibody of approximately 130kDa in size (PML-A) that was absent in mock infected cells (lane 1) and mutant inOrf3-infected cells (lane 3) (Leppard & Everett, 1999). Infection by wild type Ad5 also caused a reduction in the amount of high-molecular weight, sumoylated PMLs over 130kDa. It is of note that the pattern of higher molecular weight, SUMOylated PMLs above 130kDa also differed from that in both mock-infected and Ad5 300-infected cells, suggesting that E4 Orf3 may be in part, but not completely, responsible for the changes seen in these species during infection by Ad5 300.

None of the isoform-specific anti-sera were able to detect PML-A in this experiment or three other repeat experiments (data not shown), despite its relative abundance in the PML population (as indicated by the pan-PML antibody). However, other infection-specific changes were seen that were independent of E4 Orf3. In particular, the antisera against PMLs I and II detected significant differences (*, Fig.4.2) in the cellular PML content between cells that were infected or mock infected.

Anti-PML I detected changes in both infections (lanes 5 and 6), as there was less high-molecular weight, SUMOylated PML present above 130kDa in size than mock-infected cells (lane 4). This is unlikely to be a consequence of Ad5 infection causing a general decrease in the total amount of PML I, as equal amounts of the unmodified protein were observed in infected and mock-infected cells (indicated by the arrow). Second, an infection-specific species that was not dependent on E4 Orf3 is present at

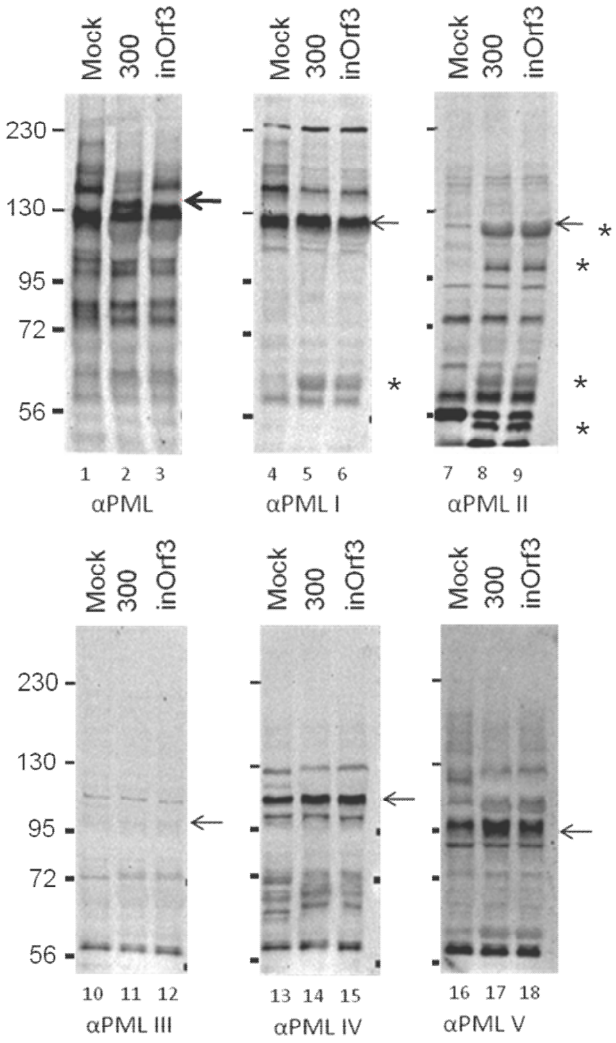


Figure 4.2. PML isoform-specific antibodies do not detect the infection-specific, E4 Orf3-dependent species of PML. 3 x 10^5 HEp-2 cells were mock-infected or infected with either Ad5 wt 300 or E4 Orf3 mutant *in*Orf3 at an M.O.I. of 10 p.f.u./cell. Cells were lysed at 24h.p.i. and lysates were separated by 8% SDS-PAGE then subjected to western blotting. Each lysate was loaded 6 times in parallel to allow the protein content to be independently probed by each antibody (indicated below each blot). The bold arrow adjacent to the anti-PML blot indicates the migration position of PML-A. The arrows adjacent to the blots probed with isoform-specific antibodies indicate the expected migration positions of the respective full-length PML isoforms that are being detected, as determined from over-expression of cDNA PML isoform clones. Protein sizes are indicated in kDa to the left hand side of each blot. The anti-PML antibody used here was 5E10, a monoclonal antibody that recognises an epitope common to all PML proteins (including PML VI). Asterisks indicate infection specific bands.

approximately 60kDa. It is interesting to note that this species was not detected by the pan-PML monoclonal anti-serum (lanes 1-3). This suggested that this species may lack exon 5, as the epitope for the 5E10 pan-PML antibody used in this instance is located within this exon (Stuurman *et al*., 1992).

The anti-PML II anti-serum detected numerous infection-specific changes in the PML II population that were independent of E4 Orf3. Strikingly, several apparent infection-specific PML species were detected (asterisks). These infection-specific species, although relatively strong, do not appear to be detected by the 5E10 antibody, but do resemble infection-specific species detected by the polyclonal anti-PML antiserum in HEp-2 cells (Fig. 3.1). It is possible that these species are not detected by 5E10 because they may lack exon 5, in which lies the epitope for the this antibody (Stuurman *et al*., 1992).

4.4 Super-infection of PML-transfected HEp-2 cells

The E4-Orf3 dependent species PML-A was not detected by any of the isoform-specific anti-sera. One possible explanation for this is that the isoform that forms the basis of this novel species is post-translationally modified at a site which corresponds to, or is adjacent to, the epitope of the relevant antibody. It is feasible that such a modification could 'mask' the epitope from the antibody, thereby preventing detection by western blotting. It could also not be discounted that PML-A was an extensively post-translationally modified form of PML VI, for which no specific antibody was available. To investigate these possibilities, an alternative strategy was devised in which cells were infected that were expressing FLAG-tagged PML isoforms (Fig. 4.3). The FLAG epitope is located in each case at the N terminus upstream of the first exon of PML. Thus any post-translational modifications that these exogenously expressed PML proteins undergo should not interfere with the detection of the FLAG epitope.

Transfection of HEp-2 cells with empty vector did not prevent the appearance of the E4 Orf3 dependent species of PML, as it is quite clearly detected by the polyclonal anti-PML antibody in infected cells (Fig. 4.3, lane 2, arrow). However, although

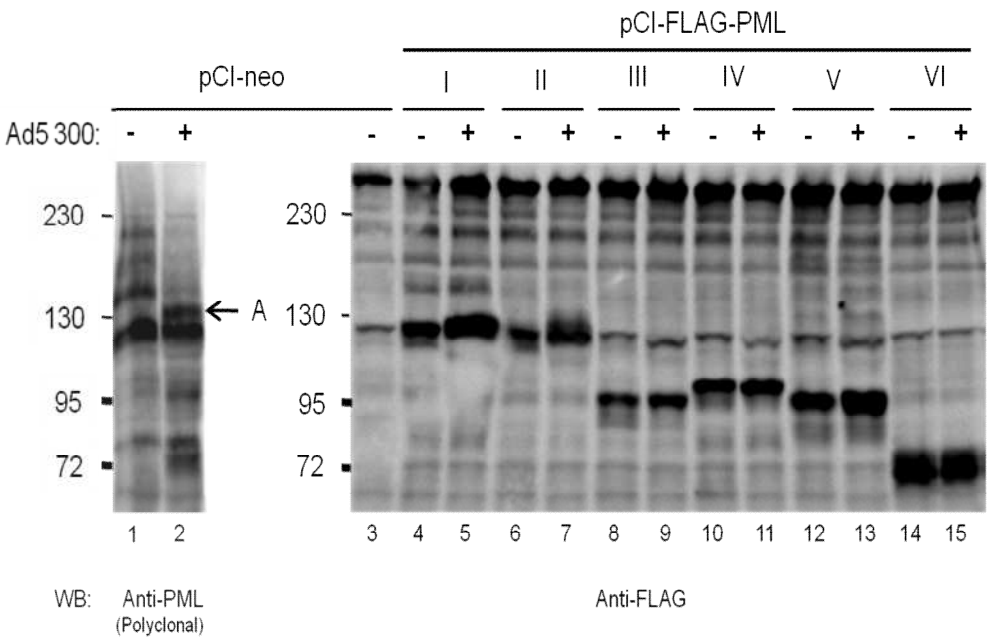


Figure 4.3 PML-A cannot be detected upon super-infection of HEp-2 cells expressing FLAG-tagged PML proteins. 2.5 x 10^5 HEp-2 cells were transfected with 500ng empty vector pCI-neo alone (lanes 1-3), or co-transfected with 410ng pCI-neo plus 90ng pCI-neoFLAG-PMLI-VI (lanes 4-15). 16 hours later, cells were mock infected or infected with wild-type Ad5 300 at an m.o.i. of 10. At 16 h.p.i. the medium bathing the cells was replaced with fresh medium containing 50µM MG132. At 24 h.pi. the cells were harvested and subjected to 8% SDS-PAGE and western blotting. The blot was separated into two pieces, and probed with either a polyclonal anti-PML antibody (lanes 1 and 2) or a polyclonal anti-FLAG antibody (F7425, lanes 3-15). Species A, as detected by the anti-PML antibody, is indicated by an arrow to the right of the anti-PML blot. Protein sizes are indicated in kDa to the left hand side of the blots.

expression of FLAG-PMLs I-VI was confirmed in both uninfected and infected cells (lanes 4-15), in no case was a FLAG-tagged band co-migrating with PML-A clearly detected in any of the PML-transfected/Ad5-infected cells. Despite this, a subtle difference was noticed in the banding pattern of PML II-transfected cells upon infection that could represent the conversion of FLAG-PML II into PML-A (lane 7). Infection with Ad5 appeared to alter the mobility of the PML II band detected by the anti-FLAG antibody, and caused the band to migrate to a slightly higher position than that of uninfected cells (lane 6).

This possibly indicated that PML II was undergoing post-translational modification upon infection with wild-type Ad5. However, it was unclear if a new species of PML II was genuinely being produced, as a complication of the approach was that infection by Ad5 tended to result in increased expression of all of the FLAG-tagged PML isoforms. Therefore it was difficult to determine whether PML II was being selectively modified, or whether the apparent difference in banding pattern between infected and uninfected cells was an artefact of differences in expression.

4.5 siRNA mediated knockdown of the E4 Orf3-dependent PML-A

It was previously shown that PML-A was not produced in cells that had been previously depleted of their total PML content by PML siRNA prior to infection (Fig. 3.3). In order to identify the PML isoform forming the basis of PML-A, siRNA was used to deplete the cells of individual PML isoforms before infection with Ad5. Two previously described isoform-specific siRNAs were employed that target either PML I or PML II mRNA species (Pavan Kumar *et al*., 2007). PML I and II were chosen as the main candidates because they migrate to a similar position as PML-A by SDS-PAGE and were therefore considered more likely to be the basis of this species. Also, PML II has been shown to directly interact with the Ad5 E4 Orf3 protein, which is necessary for the production of PML-A (Hoppe *et al*., 2006, Leppard *et al*., 2009, Leppard & Everett, 1999).

Infection by wild-type Ad5 led to the production of infection-specific PML-A in both mock transfected and control siRNA transfected cells (Fig. 4.4, lanes 2 and 3). Only a moderate decrease in PML expression was observed upon transfection with the pan-PML siRNA, but PML-A was clearly depleted in comparison to both mock transfected and control siRNA transfected cells (lane 4). Upon transfection with increasing amounts of PML I siRNA, PML-A was unaffected. However, upon transfection of PML II siRNA PML-A was depleted in a dose-dependent manner, while total cellular protein levels remained equivalent, as indicated by GAPDH levels.

Thus knock-down of all PML II species within the cell results in a loss of PML-A, implying that this species is a novel variant of PML II. PML-A may be a novel

splice variant of PML II that retains the carboxyl terminal region, or it may represent a novel post-translational modification of this isoform that is induced by infection with an adenovirus possessing the E4 Orf3 protein.

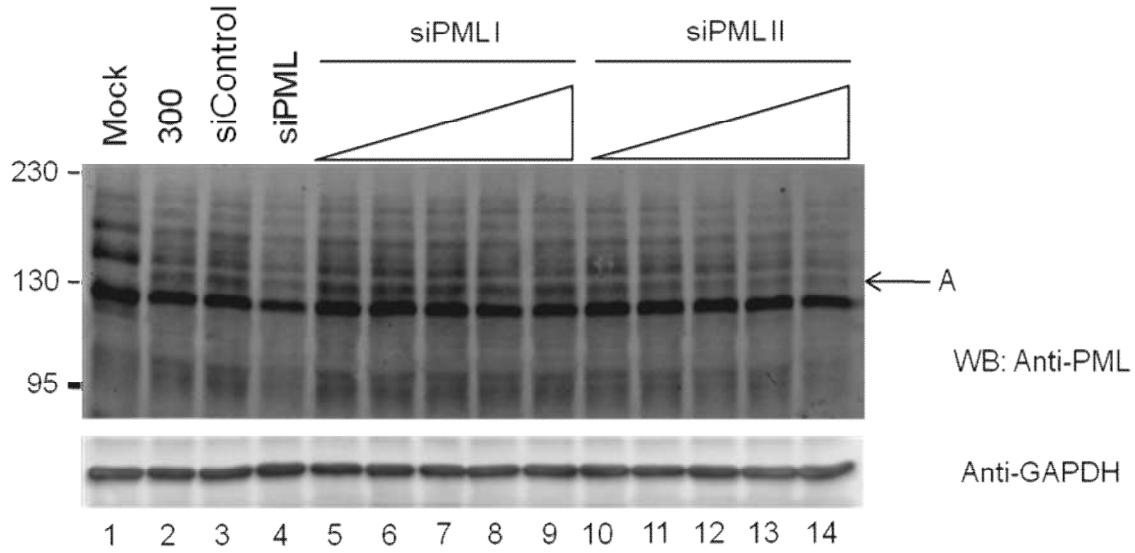


Figure 4.4 PML-A is depleted by siRNA targeting PML II. 2.5 x 10^5 HEp-2 cells were mock transfected (lanes 1 and 2) or transfected with 200pmol of control siRNA (lane 3) or pan-PML siRNA (lane 4), or with 0.25, 1, 50 or 200pmol of siRNA targeting PML I (lanes 5 to 9) or PML II (lanes 10 to 14). 48h later cells were mock infected (lane 1) or infected with wild type Ad5 300 at an m.o.i. of 10 (lanes 2-14). 24 h.p.i. the cells were harvested and the protein content of the lysates was separated in parallel by 8% SDS-PAGE and western blotting in order to generate two replica blots. The blots were probed with monoclonal anti-PML antibody 5E10 or monoclonal anti-GAPDH antibody (ab9484). The arrow to the right hand side of the blots indicates the migration position of PML-A. Protein sizes are indicated in kDa to the left hand side of the blots.

4.6 Can expression of E4 Orf3 complement an inOrf3 infection for PML-A production?

E4 Orf3 directly interacts with a region located in the carboxyl terminus of PML II, and it is through this interaction that PML NBs become reorganised into 'tracks' (Carvalho *et al*., 1995, Hoppe *et al*., 2006, Leppard *et al*., 2009). E4 Orf3 is required for the production of PML-A, which above has been showed to be related to PML II (Fig. 4.4). Consideration was therefore given to the possibility that PML-A arises as

a consequence of the direct interaction between PML II and E4 Orf3 (Hoppe *et al*., 2006, Leppard *et al*., 2009). To investigate this, E4 Orf3 mutant N82A was utilised alongside wild-type E4 Orf3. N82A cannot interact with PML II and hence does not cause rearrangement of PML NBs (Hoppe *et al*., 2006). The capability of this and wild-type E4 Orf3 proteins to cause the appearance of PML-A during infection by an Ad5 E4 Orf3 mutant, and specifically their ability to complement the Orf3 defect and allow the production of PML-A was examined in HEp-2 cells (Fig. 4.5).

PML-A was produced upon infection with the wild-type Ad5 (lane 2), but was absent as expected from inOrf3-infected cells (lane 3). Exogenous expression of either wild-type or N82A E4 Orf3 alone led to an increase in the amount of PML detected. This was likely to be an artefact of the exogenous protein expression, as expression of other proteins unrelated to Orf3 led to similar observations (data not shown). Upon super-infection of E4 Orf3-transfected cells by inOrf3 infection specific PML-B and PML-C were produced (lanes 4 and 5), suggesting that the infections were successful. However, no band was observed that co-migrated with PML-A. This indicated that the inOrf3 infections had been inefficiently complemented despite expression of wild-type E4 Orf3. The failure of complementation may be explained by the relatively poor amounts of E4 Orf3 expressed from transfected cells (lanes 4-7) as compared to a wild-type infection (lane 2). This is likely to be a consequence of poor transfection efficiency of HEp-2 cells so that few cells in the infected population could be expected to produce PML-A. In order to overcome this, the same experiment was performed in HEK293 cells which support high transfection efficiencies. However, transfection followed by super-infection appeared to cause high levels of cell death and therefore it was not possible to use this cell type. The question of whether the formation of PML-A by infection in the presence of Orf3 is linked to PML-II binding cannot therefore be answered at this time.

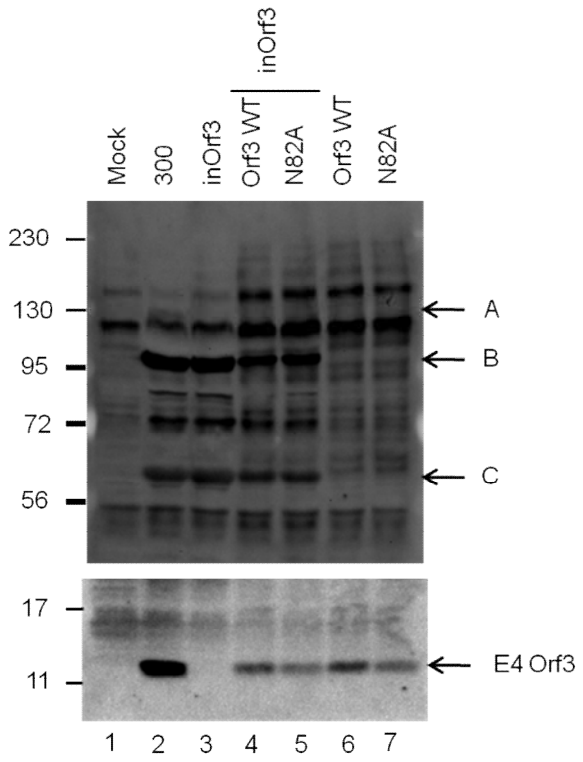


Figure 4.5 Can expression of E4 Orf3 complement an inOrf3 infection for PML-A production? 2.5 x 10^5 HEp-2 cells were transfected with 500ng of empty vector pCI-neo (lanes 1-3), pcDNA3.1-*orf3* (lanes 4 and 6) or pcDNA3.1-*N82A* (lanes 5 and 7). 16 hours later, cells were mock infected, or infected with Ad5 wild-type 300 or E4 Orf3-deficient mutant *in*Orf3 at an m.o.i. of 10. Cells were harvested at 24 h.p.i. and aliquots of the samples were separated in parallel by 8% and 15% SDS-PAGE and subjected to western blotting. One blot was probed with a polyclonal anti-PML antibody (upper panel), the other with an antibody recognising E4 Orf3 (6A11, lower panel). The migration position of the three infection-specific species of PML and E4 Orf3 are indicated to the right of the blots. Protein sizes are indicated in kDa to the left of the blots.

4.7 PML-A is stabilised by the Casein Kinase 2 inhibitor DMAT

The apparent molecular weight, by SDS-PAGE, of PML-A is greater than full-length PML II, as determined from experiments utilising PML II cDNA (Fig. 4.1). PML-A may therefore represent a novel post-translationally modified form of PML II that is produced upon infection by wild-type Ad5. PML proteins are subject to numerous post-translational modifications (Hayakawa *et al*., 2008, Kamitani *et al*., 1998,

Scaglioni *et al*., 2006), and it is possible that Ad5, through a function of its E4 Orf3 and an additional viral factor (Fig. 3.8), alters the modification state of PML II. The small size difference between PML-A and full-length PML II suggested this might be a phosphorylation event, and thus this possibility was considered.

PML proteins are subject to phosphorylation, the best characterised example of which involves phosphorylation of serine residue 517 in exon 7a of Δexon5 PML, Ser565 in full-length PML, by casein kinase 2 (CK2) (Scaglioni *et al*., 2006). Phosphorylation of this residue leads to ubiquitination, and subsequent proteasome-mediated degradation of PML. Exon 7a is present in all of PMLs I-V, and thus a modification of Ser517 does not constitute an isoform-specific modification. However, it was possible that Ad5 was inducing selective phosphorylation of Ser517 of PML II via a mechanism involving E4 Orf3. This was a possibility made more plausible by the observation that PML-A is stabilised by the addition of MG132, a proteasome inhibitor (Fig. 3.5). The role of CK2 in the production of species A was therefore assessed using a CK2-specific inhibitor, DMAT.

HEp-2 cells were mock-infected or infected with Ad5 wild-type 300 or E4 Orf3 mutant inOrf3. 20 h.p.i. the medium was replaced with medium containing sorbitol, to de-stabilise cellular PML protein, allowing any subsequent stabilisation of PML to be more easily observed (Scaglioni *et al*., 2006). At 22 h.p.i. cells were mock-treated or treated with DMAT, MG132, or DMAT and MG132, before analysis at 24 h.p.i. (Fig. 4.6).

Upon infection with wild-type Ad5, the levels of the major uninfected cell PML protein decreased significantly, and little PML-A was present (lane 6). The addition of DMSO appeared to cause a small increase in PML levels (lane 7), though the addition of DMAT alone or in combination with MG132 (lanes 9 and 10) led to a stronger increase in both endogenous PML and PML-A. A similar pattern was observed for inOrf3-infected cells (lanes 11-15).

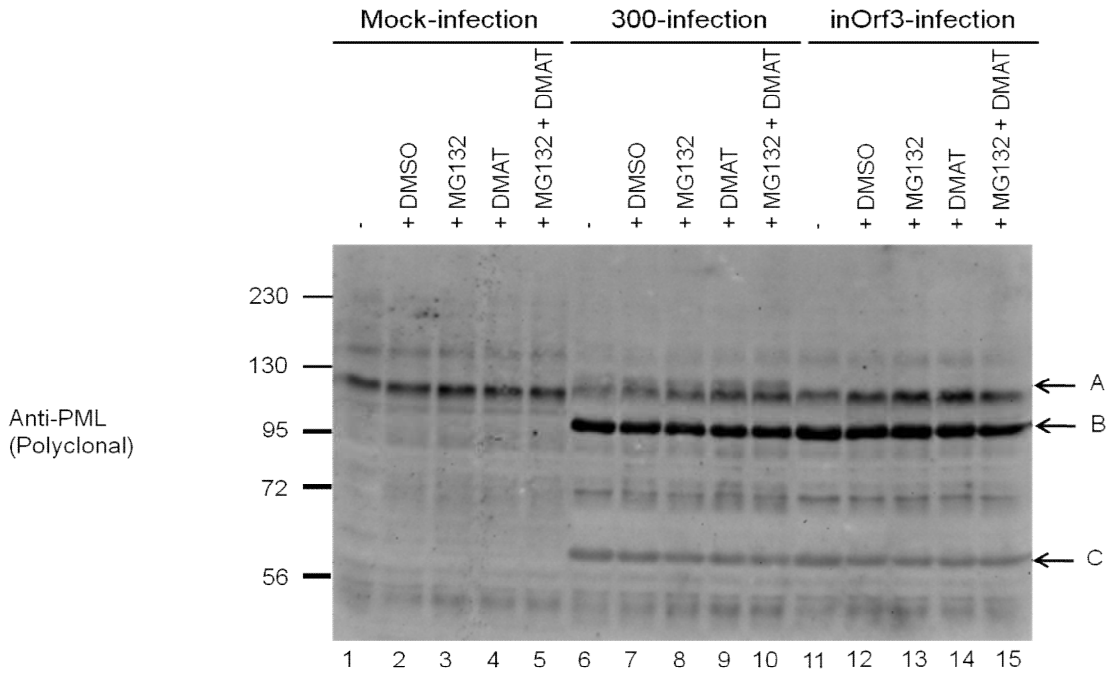


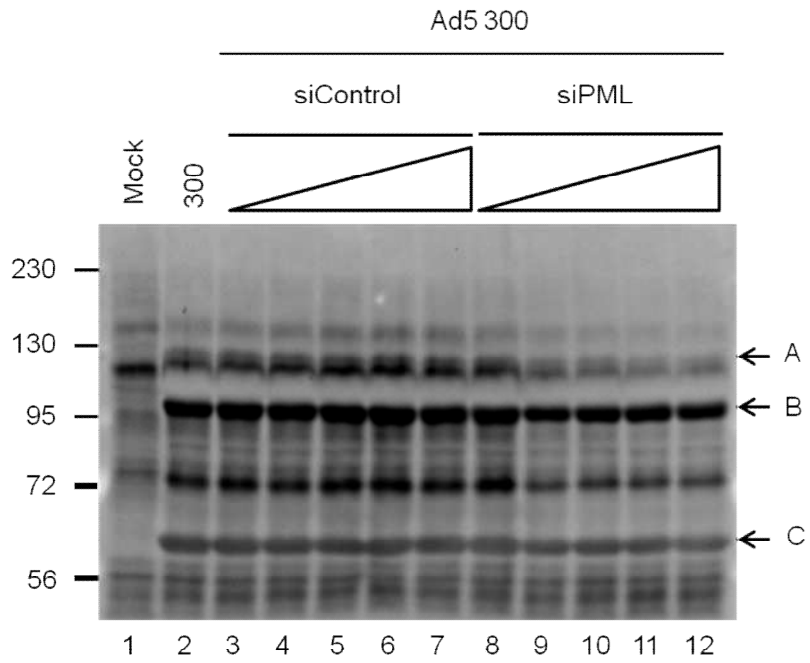
Figure 4.6 PML-A is not dependent on Casein Kinase II. 3 x 10^5 HEp-2 cells were mock-infected or infected by Ad5 wild-type 300 or E4 Orf3-deficient *in*Orf3 at an m.o.i. of 10. At 20 h.p.i., the medium bathing the cells was replaced with fresh 10% FCS DMEM containing sorbitol to a final concentration of 0.5M. At 22 h.p.i. the medium was replaced with 10% FCS DMEM containing DMSO, DMAT to a final concentration of 10µM, MG132 to a final concentration of 50µM, or DMAT and MG132. At 24 h.p.i. the cells were harvested and the samples were analysed by 8% SDS-PAGE and western blotting. The blot was probed with a polyclonal anti-PML antibody (Xu *et al*., 2003). The migration positions of the three infection-specific species of PML are indicated by arrows to the right of the blot. Protein sizes are indicated in kDa to the left hand side.

The addition of DMAT, particularly when the proteasome was inhibited, did not lead to a decrease in the amount of PML-A, but rather appeared to enhance its accumulation. This suggests that the post-translational modification responsible for species A is not dependent on CK2-mediated phosphorylation. The enhancing effect of DMAT on PML-A may be caused by a general stabilisation of PML proteins, which is due to inhibition of proteasome-mediated degradation that is normally initiated upon phosphorylation of Ser517 by CK2 (Scaglioni *et al*., 2006).

4.8 PML-B and PML-C are not diminished by siRNA targeting PML exon 5

PML-B and PML -C were previously shown to be depleted in a dose-dependent manner with siRNA corresponding to exon 3 of PML mRNA (Fig. 3.3). In order to investigate further the nature of these species, an alternative siRNA was designed corresponding to exon 5 of the PML transcript. Exon 5, as previously mentioned, is spliced out of cytoplasmic variants of PML as well as a subset of nuclear variants (Jensen, 2001). Neither PML-B nor PML-C was detected with the monoclonal antibody 5E10, the epitope of which corresponds to exon 5. This suggested that these species lack this exon, and this possibility was investigated using siRNA technology (Fig. 4.7).

Infection with Ad5 led to the production of all three infection-specific species of PML (lane 2). The transfection of control siRNA did not alter the abundance of either the standard PML species or any of the infection-specific species (lanes 3-7). Upon transfection of PML siRNA targeting exon 5, PML-A was clearly depleted in a dose-dependent manner alongside higher molecular weight standard PMLs (lanes 8-12). PML-B and PML-C showed no such depletion and their levels remained constant across all transfections, indicating equivalent protein loading. Thus, PML-B and PML-C cannot be depleted by transfection of siRNA targeting PML exon 5, suggesting that these species lack this exon. This is in accordance with the data presented in figures 3.2 and 4.1, where antibody 5E10 was unable to detect these species.



4.7 PML- B and PML-C are not depleted by siRNA targeting PML exon 5. 2.5 x 10^5 HEp-2 cells were mock transfected (lanes 1 and 2) or transfected with 0.25, 1, 10, 50 or 200pmol of control siRNA or siRNA targeting PML exon 5 (Pavan Kumar et al., 2007). 48 hours after transfection cells were mock infected or infected with wild type Ad5 300 at an m.o.i. of 10 (lanes 2 to 12). 24 h.p.i. the cells were harvested and the protein content of the lysates was separated by 8% SDS-PAGE and subjected to western blotting. The blot was probed with the polyclonal anti-PML antibody. PML- A, -B and -C and a novel species are indicated to the right of the blot by arrows. Protein sizes are indicated in kDa to the left hand side of the blot.

4.9 PML-C is a variant of PML I

PML-A was diminished upon Ad5 infection of HEp-2 cells previously depleted of PML II, suggesting this isoform as the basis of this PML species (Fig. 4.4). However, the basis of PML-B and PML-C was yet to be determined. Unlike PML-A, PML-B and PML-C lack exon 5, as determined from PML antibody and PML siRNA studies (Figs. 3.2, 3.3, 4.1 and 4.7).

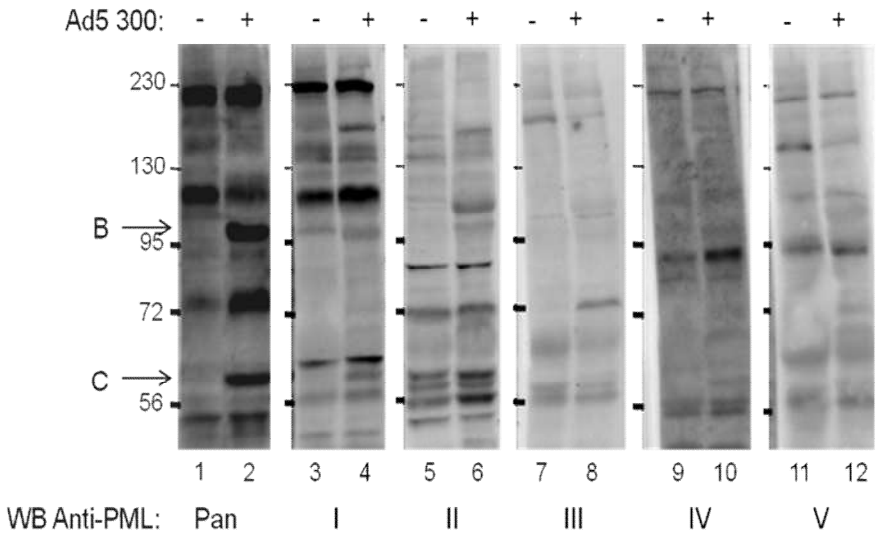


Figure 4.8 PML-C co-migrates with an infection specific species detected by anti-PML I. 2.5 x 10^5 A549 cells were mock infected or infected with wild-type Ad5 300 at an m.o.i. of 10. 24 h.p.i. cells were harvested and the protein content of the lysates was separated multiple times in parallel by 8% SDS-PAGE and subjected to western blotting. The blot was cut to produce 6 direct replicates, each of which was probed with a single antibody. The polyclonal anti-PML antibody was used to detect all species of PML, and individual isoforms were detected using anti-PML I-V. PML-B and PML-C are indicated by arrows to the left of the blot. Protein sizes are indicated in kDa.

In order to determine whether infection-specific PML-B and PML-C carried an identifiable PML C-terminus, the isoform-specific antibodies described above were employed in an analysis of infected A549 cells. A549 cells were chosen as this cell line was repeatedly found to support strong expression of PML-B and PML-C (Fig. 4.8). Infection with Ad5 led to strong expression of both PML-B and PML-C (lane 2), and poor expression of PML-A. After repeated attempts, no infection-specific band was detected by any of the isoform-specific antisera that co-migrated with PML-B. However, an infection-specific band detected by anti-PML I was repeatedly found to co-migrate with PML- C, suggesting that this species may represent a truncated variant of PML I.

4.10 Attempted knockdown of PML-C by siRNA targeting PML I

By western blot, PML-C co-migrates with an infection-specific band of PML that is detected by anti-PML I (Fig 4.8). This suggested that this species may be a truncated PML I variant that is induced upon infection with Ad5. In an attempt to confirm this hypothesis, siRNA targeting PML I was used to attempt to deplete PML-C (Fig. 4.9).

Infection with wild-type Ad5 led to the production of all three infection-specific species of PML (Fig 4.9 A, lane 2), that were unaffected by transfection of control siRNA (lane 3). Upon infection of cells depleted of PML species containing exon 5, PML-A was decreased though both PML-B and PML-C remained unaffected as before (lane 4). As expected, transfection of increasing amounts of siPML I had no effect on the abundance of PML-A. However, both species B and C also remained unaffected, suggesting that neither of these species were related to PML I. This is in contrast to figure 4.8, where species C was detected by the anti-PML I antibody.

It was noted that transfection of siPML I did not lead to any noticeable decrease of any PML species as detected by the polyclonal anti-PML antibody. Therefore, in order to determine the efficiency of PML I knock-down, selected samples from figure 4.9 A were analysed for their PML I content using the anti-PML I antibody (Fig. 4.9 B). PML I knockdown was observed and was proportionate the use of increasing amounts of siPML I (lanes 3-7). However, significant amounts endogenous PML I and PML-C remained even after treatment with the highest concentration of PML I siRNA. This suggested that either PML I could be intrinsically more stable than other PML isoforms, and hence more difficult to deplete, or that the siRNA targeting PML I may be less efficient at depleting its respective isoform than other PML isoform siRNAs, such as PML II siRNA (Fig. 4.4). Consequently, it was difficult to verify whether the infection-specific species of PML detected by anti-PML I was the same as infection-specific PML-C.

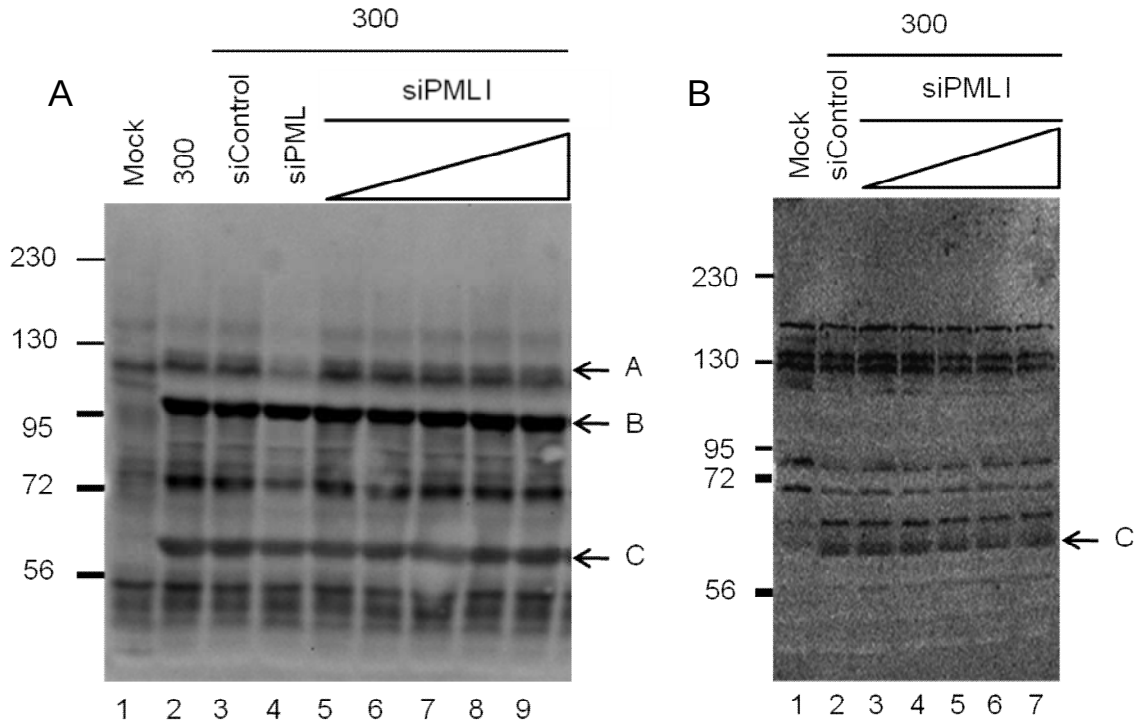


Fig 4.9 PML-C is depleted by siRNA targeting PML I. **A** 2.5 x 10^5 HEp-2 cells were mock- transfected or transfected with 200pmoles of control siRNA or siRNA to PML exon 5, or transfected with 0.25, 1, 10, 50 or 200pmoles of siRNA targeting PML I (Pavan Kumar et al., 2007). 48 hours later cells were mock infected or infected with wild-type Ad5 300 at an m.o.i. of 10. At 24 h.p.i. the cells were harvested and the protein content of the lysates was separated by 8% SDS-PAGE and subjected to western blotting. The blot was probed with a polyclonal anti-PML antibody. PML- A, PML-B and PML-C are indicated by arrows to the right of the blot. Protein sizes are indicated in kDa on the left hand side of the blot. **B** The indicated lysates from **A** were subjected to 10% SDS-PAGE and western blotting. The blot was probed with the anti-PML I antiserum. The arrow to the right of the blot indicates the migration position of PML-C. Protein sizes are indicated in kDa to the left hand side of the blot.

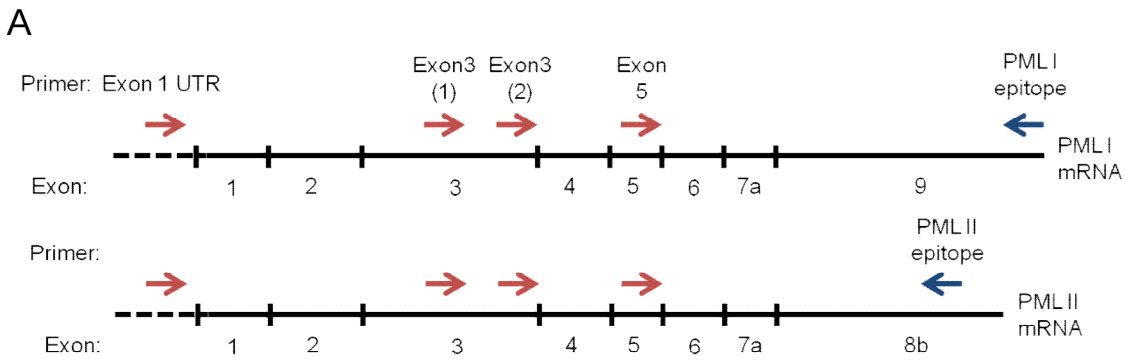
4.11 Detection of PML splice variants during Ad5 infection

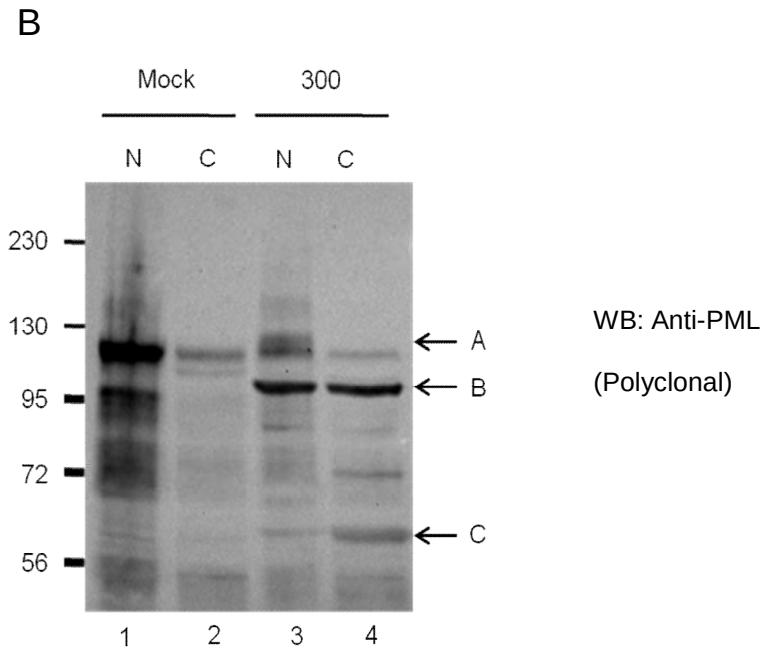
A wide range of viruses have been shown to interact with or disrupt PML protein and PML NBs. Whereas most of these interactions are thought to occur at a post-translational level, infection by both HSV-1 and HSV-2 causes changes in the PML protein population that are mediated by altering the splicing of PML mRNA (McNally *et al*., 2008, Nojima *et al*., 2009). HSV-1 leads to the production of a

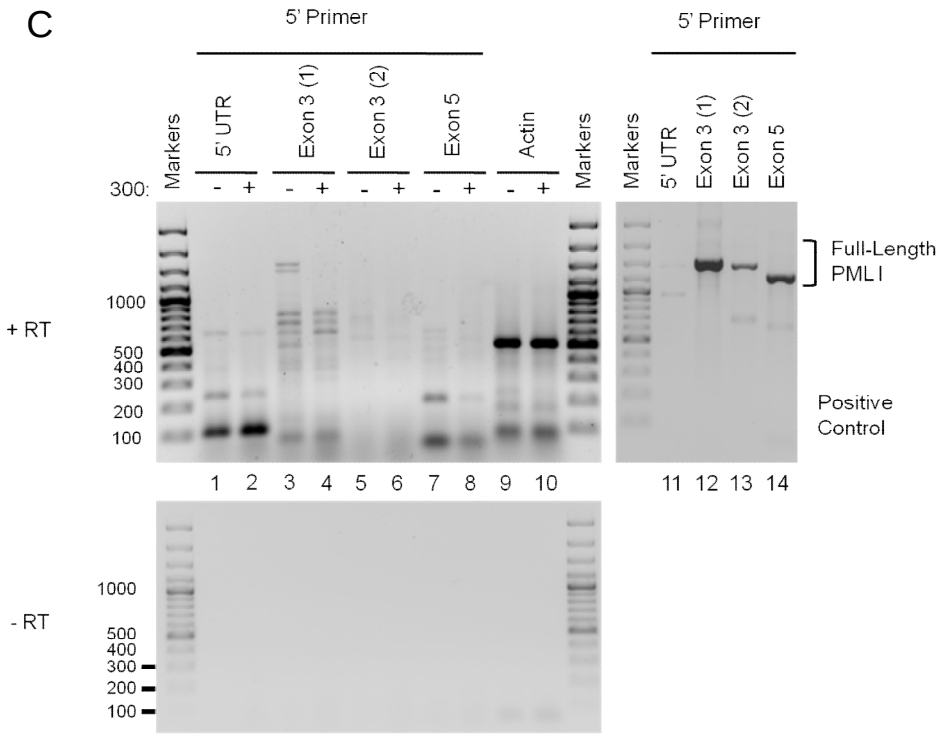
truncated form of PML that terminates in exon 7a (McNally *et al*., 2008), whereas HSV-2 alters PML splicing to promote the production of PML V over PML II (Nojima *et al*., 2009). Infection by Ad5 leads to the production of three novel species of PML that do not co-migrate with known isoforms, raising the possibility that Ad5 may also interact with the cellular splicing machinery to favour the production of novel variants of PML. The population of PML mRNAs in infected cells was therefore examined. Particular focus was paid to PML-C, as this is smaller than all of the full-length PML isoforms. It is possible that PML-C is a splice variant of PML I, as this species can be detected by an anti-PML I antibody. PML-C is also detected with the pan-PML antibody PG-M3. The epitope for this antibody overlaps with the exon 1/2 boundary of PML. Taken together, these facts suggest PML-C must be derived from a novel splice variant. Only PML I and PML II species were investigated, as anti-sera targeting these isoforms detect numerous lower-molecular weight bands that migrate to a similar position as PML-C in both HEp2 and A549 cells (figs. 4.2 and 4.8).

HEp-2 cells were mock-infected or infected with Ad5 wild-type 300. At 24 h.p.i. the cytoplasmic RNA content of the cells was harvested and subjected to reverse-transcription (RT) into cDNA. Prior to the harvesting of RNA, aliquots of the nuclear and cytoplasmic fractions of the cells were prepared for protein analysis using a polyclonal anti-PML antibody. The RT reactions included actin 3', PML I 3' and PML II 3' primers. The PML I and PML II reverse primers were designed so that they corresponded with the epitopes recognised by their respective isoform-specific anti-sera. Equivalent amounts of cDNA were then used as template for subsequent polymerase chain reactions (PCRs). The PML I and PML II reverse primers were each used in combination with one of four different forward primers that corresponded to various regions along the PML gene (Fig. 4.10A). One primer corresponded to the 5' untranslated region (UTR) of PML, two primers corresponded to different positions approximately 100 base pairs (bp) apart within exon 3, and one primer corresponded to exon 5. The 5' UTR primer was utilised, as in principle, it allowed the entire length of the PML I or II transcripts to be amplified, including any internally deleted splice variants of these isoforms. Primers corresponding to exon 3 were used to account for the possibility of splicing out of exon 3 to exon 7a. The exon 5 forward primer would allow analysis of full-length transcripts of PML I or

PML II. As a positive control, PML I or PML II expression plasmids served as templates in PCRs containing each of these primer combinations.



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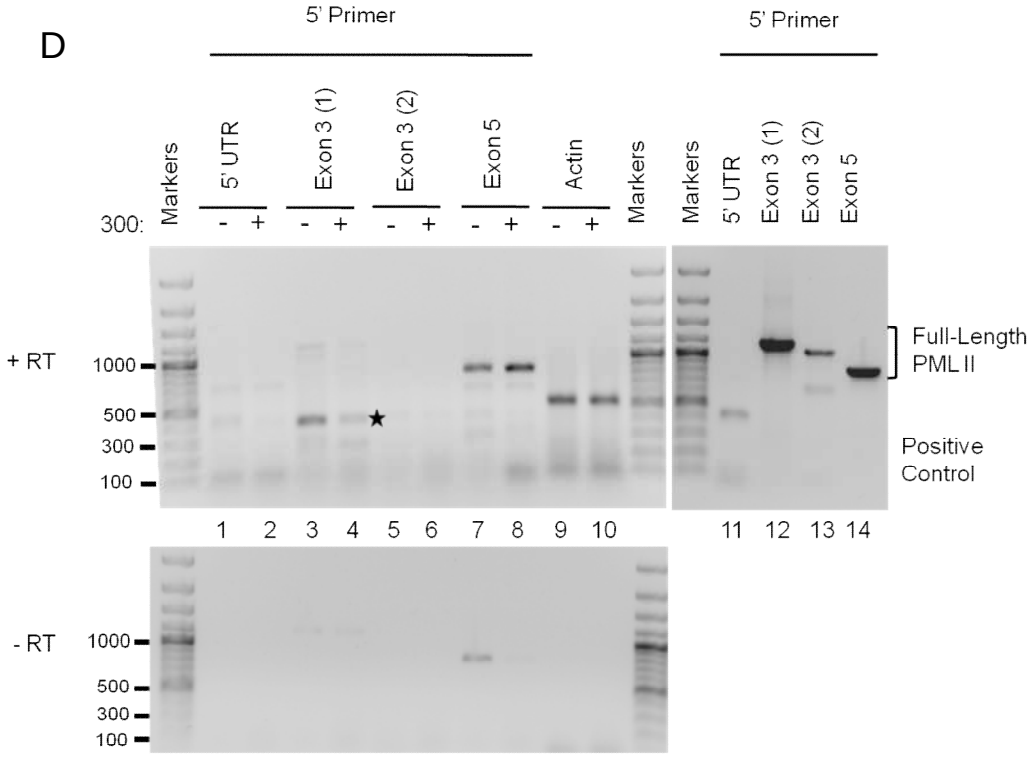
PML I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Exon 3 (2)
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+ RT
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Full-Length
PML II
Positive
Control
1 2 3 4 5 6 7 8 9 10
11 12 13 14
- RT
1000
500
300
100

PML II

Figure 4.10 No novel splice variants of PML induced by adenovirus infection can be detected by RT-PCR. 1 x 10^7 HEp-2 cells were mock infected or infected with wild-type Ad5 300 at an m.o.i. of 10. At 24 h.p.i. the cells were separated into nuclear and cytoplasmic fractions and cytoplasmic RNA was extracted (see methods). **A.** Schematic diagram showing the respective positions of the primers used in the RT-PCR assay. The dashed portion of each mRNA indicates the 5' untranslated region (UTR). **B.** Immediately after fractionation, the entire nuclear fraction and 100µl of the cytoplasmic were harvested in 1 X Sample Buffer. For each sample, volumes of the cytoplasmic and nucleoplasmic fractions corresponding to equal amounts of protein was separated by 8% SDS-PAGE and subjected to western blotting. The blot was probed with a polyclonal anti-PML antibody. PML- A, PML-B and PML-C are indicated by arrows to the right of the blot, and protein sizes are indicated in kDa to the left of the blot. N = Nuclear fraction, C = Cytoplasmic fraction. **C** 5µg of total cytoplasmic RNA used as template for reverse-transcription (RT) using 3' reverse primers specific for PML I and actin in the presence (+RT) or absence (-RT) or reverse transcriptase. 2.5µl of the resulting cDNA was used as template in a polymerase chain reaction using the actin 5' and 3' primers (lanes 9 and 10) or the PML I 3' primer in combination with each of the 5' primers indicated (lanes 1-8, 11-14). 50ng of pCI-neoFLAG-PMLI was used as template for the reactions indicated in lanes 11-14, and served as a positive control. PCR products were mixed with separated by electrophoresis through a 1% agarose gel containing 0.5µg/ml ethidium bromide, and imaged under UV light. Full-length PML I PCR products are indicated to the right of the upper panels, and the DNA size are indicated in base-pairs (bp) to the left hand side of the panels. **D** As in **C,** except the PML I 3' primer was replaced with the PML II 3' primer. Full-length PML II products are indicated to the right hand side of the upper panels, and the DNA size is indicated in bps to the left hand side of the panels. The excised band is indicated by an asterisk.

As indicated in figure 4.10 B, infection of HEp-2 cells with wild-type Ad5 led to the production of all three infection-specific species of PML. As seen in A549 cells (fig 3.4), PML species A was confined to the nucleus whereas PML-C was abundant in the cytoplasmic fraction. PML-B, however, was equally present in both nuclear and cytoplasmic fractions, which was in contrast to the exclusively nuclear localisation observed in A549 cells (Fig 3.4). It was a point of interest that in both uninfected and Ad5 infected cells, a higher-molecular weight endogenous species of PML was also present in both fractions. The possibility that this was the result of contamination between fractions appeared unlikely, as PML-A retained a solely

nuclear localisation. This suggested that in HEp-2 cells a subset of full-length PML species are localised in the cytoplasm.

In testing for PML-I related mRNAs, all of the RT-PCR products observed in figure 4.10 C (upper left panel) were ultimately amplified from RNA, as no corresponding bands were seen in the reactions lacking reverse transcriptase (lower left panel). Equivalent amounts of actin PCR product were seen in both mock-infected and Ad5 infected cells (lanes 9 and 10), suggesting that equivalent amounts of RNA were used as template for RT-PCR. A product of expected size was observed for each of the positive control reactions using PML I 3' reverse primers (lanes 12-14). The different sizes of these bands result from the different positions of the 5' forward primers. The reaction including the 5' UTR primer could not produce any PML product as the 5' UTR sequence is not present in the PML expression plasmids used (lane 11).

No infection-specific PML I mRNA variants were detected with any of the primer combinations used (Fig. 4.10 C, lanes 1-8). The 5' UTR primer reactions produced three bands in both uninfected and Ad5 infected cells. As well as not being infection-specific, these were unlikely to represent PML-B or PML-C mRNA as this particular primer combination amplified the entire coding region of any PML I transcripts. Therefore the products observed were too small to account for these infection-specific species.

The PML reaction containing the PML I reverse primer in combination with the Exon 3 (1) primer produced a DNA product of a similar size (lane 3) to that observed in the corresponding positive control reaction (lane 12), though it was not observed in Ad5-infected cells (lane 4). Interestingly, many smaller bands were observed in both mock-infected and infected cells when this primer combination was used, possibly indicating the presence of internally deleted splice variants of PML I (lanes 3 and 4, between 600-800 bp). In contrast, very little product was made when the Exon 3 (2) primer was used in conjunction with the PML I reverse primer. The relatively weak amount of product produced from the reactions using the exon 3 (2) primer may due to an intrinsic property of this primer, as relatively little product was made in the reaction using pCI-neoFLAGPML I as template as compared with the

other positive control reactions. Consequently, it was difficult to determine whether the difference between the products of the two exon 3 5' primers was significant.

The exon 5 forward primer reaction did not yield any products that corresponded to full-length PML I (lanes 7 and 8), as indicated by the appropriate positive control (lane 14), suggesting that primer combination was insufficient or that the majority of PML I transcripts within the cell lack exon 5. However, an RT-PCR product of about 250bp was found to disappear upon infection (lanes 7 and 8).

When testing for PML II-related mRNAs, the blank RT-negative controls (Fig. 4.10 D, lower left panel) suggest that the majority of bands observed in figure 4.10 D (upper left panel) were amplified from cDNA, the exception being a product that was detected in lane 7 of the RT-negative reaction. Also, the PCR positive controls gave the expected products (lanes 11-14). The 5' UTR primer reaction did not produce any significant products when the PML II 3' reverse primer was used (lanes 1 and 2). This suggests that there are no splice variants of PML II with large internal deletions. In contrast, the exon 3 (1) primer produced a small amount of product corresponding to full-length PML II (lanes 3, 4 and 12), though the major product was a species too small to be full-length PML II that became weaker in Ad5-infected cells (indicated by an asterisk), despite equivalent amounts of actin mRNA being detected (lanes 9 and 10). This band was excised and cloned into a plasmid vector before being sequenced (see Appendix). Sequence analysis of the clones generated revealed that this band was not PML related and was the product of non-specific priming. The exon 3 (2) primer reaction yielded little product (lanes 5 and 6), though the respective positive control reaction (lane13) also produced relatively little product. This suggests that the exon 3 (2) primer was not performing optimally under the conditions used, or that it is intrinsically less efficient than the other primers used. The exon 5 forward primer reaction produced a product that was larger than full length PML II, as determined by the positive control reaction (lanes 7, 8 and 14). This suggested that this species was not a variant of PML II and was a result of non-specific priming.

Hence no obvious infection-specific species of PML I or PML II mRNA were detected by RT-PCR, despite several primer combinations being used. This indicated that Ad5 infection does not initiate the production of alternative splice

variants of these isoforms, and therefore this could not account for the formation of PML-B or PML-C. Caution was needed with this interpretation, however, as very few PCR products were observed that corresponded with full-length PML I or PML II, meaning that the efficiency of detection might not have been sufficient. Also, the possibility that PML-B and/or PML-C are novel splice variants derived from an alternative PML C-terminal isoform not investigated here cannot be discounted.

4.12 Discussion

The E4 Orf3-dependent species of PML, termed PML-A, is depleted in cells transfected with either siRNA targeting PML isoform II, or siRNA targeting exon 5 of all PML isoforms. This finding suggests that PML-A is a post-translationally modified variant of full-length PML II that is induced upon infection. However, it was difficult to verify this finding through other experimental techniques. An antibody solely targeting PML II was unable to detect PML-A (Fig. 4.2), despite previously being validated against exogenously-expressed PML II (fig. 4.1). It is unclear why this antiserum did not detect PML-A. One possibility is that the infection-specific modification responsible for PML-A interferes with the ability of the PML II antiserum to recognise its epitope, with the effect of 'masking' this species from the antibody.

However, it was noted that full-length endogenous PML II was also detected poorly by the PML II antiserum. This was a somewhat surprising result, as PML II, alongside PML I, was found by Condemine *et al* to be amongst the most strongly expressed isoforms (Condemine *et al*., 2006). In the aforementioned study, independent isoform-specific antibodies were used and the majority of PML II was found to be the higher molecular weight, full-length form. This is in contrast to the data presented here, as the dominant PML II species detected by the isoform-specific antibody are of a lower molecular weight than full-length PML. This disparity may be due to intrinsic differences of the cell lines being used, as Condemine used COS cells, a simian kidney-derived cell line, whereas HEp-2 cells were investigated here. Nonetheless, HEp-2 cells are thought to express high amounts of endogenous PML protein (Leppard & Everett, 1999), so it was unexpected that comparatively little

full-length PML II was detected. It is possible that the anti-PML II antibody used here is inefficient at detecting endogenous levels of PML II species. Although this antibody was validated, this was against lysates that were over-expressing PML II, so it is possible that any inefficiency of this antibody may have been overlooked due to the increased amount of antigen present.

Replicating the modification responsible for PML-A upon full-length FLAG-tagged PML II proved difficult, as no clear species co-migrating with PML-A was detected by the anti-FLAG antibody (Fig. 4.3). The ability to detect PML-A in this way depends on the proportion of cells that are simultaneously transfected with the relevant expression plasmid and infected by Ad5. If only a small proportion of cells meet these criteria then the ability to detect PML-A with an anti-FLAG antibody will be limited.

The post-translational modification that is responsible for PML-A is unlikely to be the CK2-mediated phosphorylation previously described by Scaglioni and colleagues (Scaglioni *et al*., 2006), as inhibition of CK2 did not reduce the amount of this species (Fig. 4.6). Instead, inhibition of CK2 led to an increase in the amount of PML-A, possibly through inhibiting its proteasome-mediated degradation as MG132 had a similar effect (Fig. 4.6). Therefore PML-A may represent an alternative post-translational modification of PML II that is as yet unknown.

E4 Orf3 rearranges PML NBs via a direct interaction with the carboxyl terminus of PML II (Hoppe *et al*., 2006, Leppard *et al*., 2009), and is necessary, but not sufficient, for the production of a novel variant of PML II during the course of infection (Leppard & Everett, 1999). It remains unknown whether these two phenomena are linked, although it is tempting to speculate that E4 Orf3 exerts both of these effects through a single interaction with PML II. However, attempts to address this issue found no evidence that this is the case (Fig. 4.5). Exogenous expression of E4 Orf3 is sufficient to cause rearrangement of PML NBs, as determined by confocal microscopy (Hoppe *et al*., 2006), yet is unable to complement an Ad5 *in*Orf3 infection in HEp-2 cells (Fig. 4.5). The approach used to investigate this, however, is limited by the transfection efficiency of the cells in question, as it was difficult to obtain a level of E4 Orf3 expression that was comparable to a wild-type Ad5 infection. Therefore Orf3 expression may not have

been sufficiently high to fully complement *in*Orf3, and hence the propensity to produce, and therefore detect, PML-A would have been limited.

The amount of PML- B was decreased in cells depleted of PML proteins possessing exon 3 (Fig. 3.3), but not when depletion targeted exon 5 (Fig. 4.7). This, in combination with the observation that PML-B is not detected with the 5E10 antibody, supports the hypothesis that it is a variant of PML that lacks exon 5. The nature of any unique C terminus on PML-B remains unknown. No infection-specific band was detected by PML isoform-specific antibodies that co-migrated with PML-B in A549 cells (Fig. 4.8). It must be noted that this does not necessarily indicate that species B is unrelated to PML I-V, as PML-A was depleted by siRNA targeting PML II yet was not detected by the relevant antiserum. However, PML-B could not be depleted by siRNA targeting PML I, and no novel splice variants of PML I and II could be detected in Ad5-infected cells by RT-PCR. Based on these observations in this chapter, PML-B is unlikely to be a variant of PML I, and is probably related to one of the remaining PML isoforms (II-VI). Further experimentation would be required to confirm this.

As predicted, the production of PML-C was unaffected by the depletion of PMLs possessing exon 5 (Fig. 4.7). PML-C is, in any case, unlikely to possess exon 5 as it is of a molecular weight that is too small to be any full-length PML isoform and is a cytoplasmic variant. Indeed, a band co-migrating with PML-C was detected in infected cells by the PML I antibody, suggesting that it may be related to PML I (Fig. 4.8). Attempts to deplete endogenous PML I and PML-C using siRNA targeting PML I were successful and led to modest decreases in PML I expression, as determined by detection with the anti-PML I antibody. In contrast, detection with the pan-PML polyclonal antibody did not display any such decrease in these PML species. This may be because, by SDS-PAGE, both PML I and PML-C co-migrate with other PML isoforms that are also detected by this antibody. Since other isoforms should remain unaffected by siRNA targeting PML I, the depletion of PML I and PML-C may therefore be more difficult to detect. For instance, PML II co-migrates to a similar position to PML I. Also, in HEp-2 cells, the anti-PML II antibody detected an infection-specific band of a similar size to PML-C (third asterisk down, Fig. 4.2), indicating that this band may also be heterogenous with regard to PML protein content.

It must be noted, however, that both endogenous PML I and PML-C expression could not totally be abolished. PML I is the most strongly expressed PML isoform (Condemine *et al*., 2006) , perhaps explaining why this was the case. It may also be particularly slowly turned-over in the cell, making siRNA-mediated depletion more difficult. The siRNA targeting PML I could also be less efficient at achieving knock-down, in which case a stronger knock-down effect may be obtained by using an alternative PML I siRNA either alone or in conjunction with the PML I siRNA used in this study.

Despite using a combination of primer sets, no infection-specific PML I or PML II transcripts could be detected that could account for PML-C, suggesting that Ad5 infection does not result in the production of novel splice variants of these PMLs. This is in conflict with other data presented (Figs. 3.2 and 4.8), as PML-C can be detected by both the pan-PML PG-M3 antibody and the anti-PML I antibody. The epitope recognised by PG-M3 lies over the exon 1/exon 2 junction of PML, whereas the anti-PML I epitope lies in the last 20 amino acids of the C terminal end of PML I. This suggests that, if PML-C is related to PML I then it must be a legitimate internal splice variant of this isoform, and not the product of a cleavage reaction of a larger species. There is reason to suggest that the RT-PCR assay presented in this chapter was of insufficient sensitivity to detect any infection-specific transcripts of PML. Very few bands were observed that would correspond to full-length PML I or II mRNAs which might be expected to be at least as abundant as any novel mRNAs. This indicates that PML mRNA was inefficiently amplified, resulting in very small amounts of product being produced. For further investigation, it may be necessary to utilise a visualisation technique more sensitive than ethidium bromide staining when detecting PCR amplification products from total PML I and PML II RNA species. For example, southern blotting using probes directed against PML I and II could be used.

Chapter 5
Optimisation of interferon reporter assays

5.1 Introduction

PML protein exists as a collection of at least seven amino-terminally related isoforms that differ from one another in their carboxyl terminal domains by up to 300 amino acids. Therefore individual PML isoforms are likely to possess functions that are separate from the rest of the PML protein population. This is likely to account for the fact that, despite intensive investigation, a definitive role of PML proteins remains elusive.

There are several lines of evidence to suggest that one function of PML proteins is to exert an antiviral activity. First, the *pml* gene is directly inducible by type I interferon α (Lavau *et al*., 1995), a major agonist of the innate antiviral response of cells. Type I interferons are produced upon stimulation of a signalling pathway by double-stranded RNA (dsRNA) or other molecular signatures of pathogens. dsRNA is often a product of viral replication, and interferons act by inducing the expression of a range of proteins that limit viral replication (Takaoka & Yanai, 2006). Second, a number of different viruses actively target and disrupt PML protein and PML NBs by either relocalising PML protein or targeting it for proteasome-mediated degradation (Carvalho *et al*., 1995, Doucas *et al*., 1996, Everett *et al*., 1998). Mutant viruses unable to make the normal interactions with PML NBs are often more sensitive to interferon treatment than wild-type viruses (Mossman *et al*., 2000, Ullman *et al*., 2007).

During the course of this investigation the role of PML proteins in the interferon pathway was examined through the use of reporter gene assays. Two firefly luciferase reporter plasmids, driven by either the interferon β promoter or an interferon-stimulated response element (ISRE), allowed analysis of different stages of the IFN response. The IFNβ promoter is activated by a signalling cascade that is induced upon the detection of double-stranded RNA (dsRNA) within the cell. IFNβ is produced and secreted from cells, where it acts in an autocrine or paracrine manner to induce a further signalling cascade that ultimately results in the activation of ISRE-driven interferon-stimulated genes (ISGs).

In order to study the role of PMLs in the IFN response it was necessary to optimise both reporter assays. In particular, it was first necessary to identify a cell line that could support both IFNβ and ISRE reporter activation through the addition of poly (I:C), which is an analogue of dsRNA and induces cells to produce IFNβ. Through this action, poly (I:C) may then stimulate ISRE-driven genes in an indirect manner.

The work presented in this chapter outlines the efforts that were made to identifying a suitable cell line as a basis of IFN reporter assays. Particular emphasis was placed on cell lines that had been shown to support the Ad5-induced changes in the PML protein population (Chapters 3, 4).

5.2 HEp-2 cells do not support activation of the interferon β promoter

HEp-2 cells were first analysed to determine whether they could support induction of the IFNβ reporter plasmid by poly (I:C). Cells were transfected with an IFNβ promoter reporter plasmid and 24 hours later the cells were mock-transfected or transfected with poly (I:C). Cells were harvested at various times, and the lysates analysed for firefly luciferase activity (Fig. 5.1).

Transfection with poly (I:C) did not cause a significant increase in reporter activity over basal levels at any of the time points tested. This suggested that the HEp-2 cells were unable to respond to poly (I:C). Overall, the general IFNβ reporter activity of the lysates decreased as the incubation time with poly (I:C) increased. The reason for this is unclear, though it may reflect a general stress response to transfection, as both treated and untreated cells displayed this pattern.

In order to confirm this result, HEp-2 cells were compared with A549 and Vero cells in parallel for their ability to support IFNβ promoter activation. A549 cells have previously been shown to support IFNβ-promoter reporter assays (Hayman *et al*., 2007).

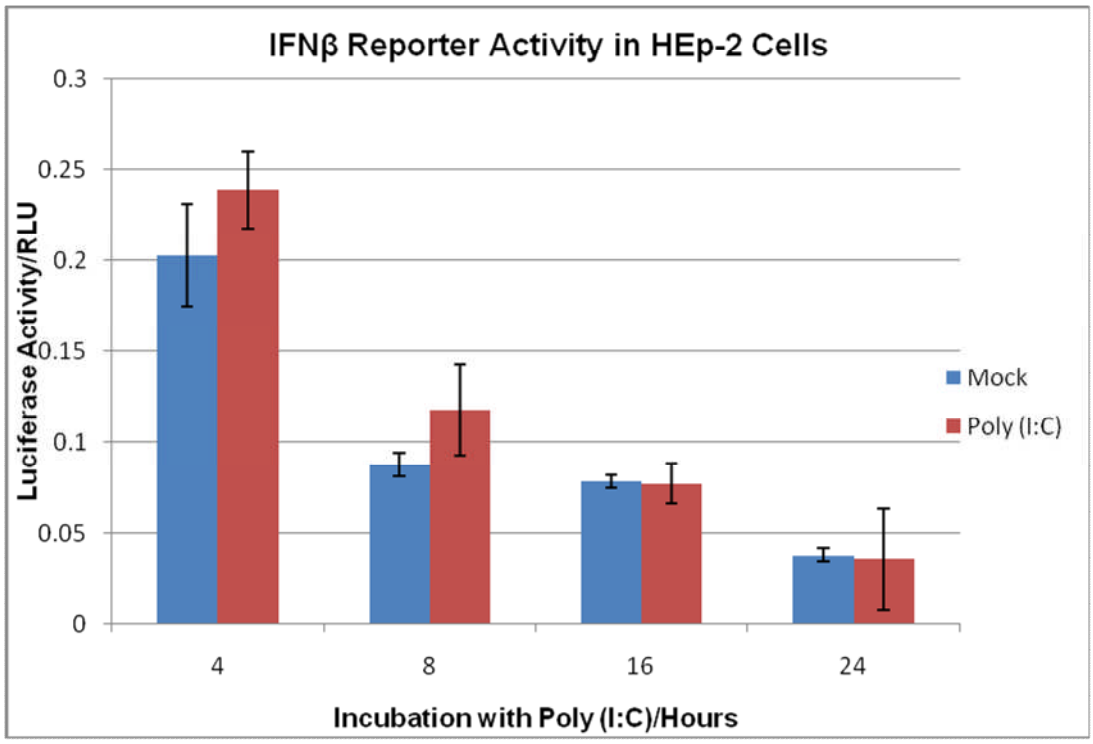


Figure 5.1 Time-course of IFNβ activity in HEp-2 cells. 2.5 x 10^5 HEp-2 cells were transfected in triplicate with 500ng of IFN β reporter plasmid pIFNβ-Luc. 24 hours later cells were mock-transfected or transfected with 1µg poly (I:C). Cells were incubated with poly (I:C) for 4, 8, 16 or 24 hours prior to harvest. 50µl of each lysate was then assayed for firefly luciferase activity. The light intensity was recorded using a luminometer and appropriate software. Luciferase activity is expressed as arbitrary Relative Light Units (RLU). Error bars indicate the standard deviation from the mean of three replicate samples. Blue bars: mock-transfected cells. Red bars: poly (I:C)-transfected cells.

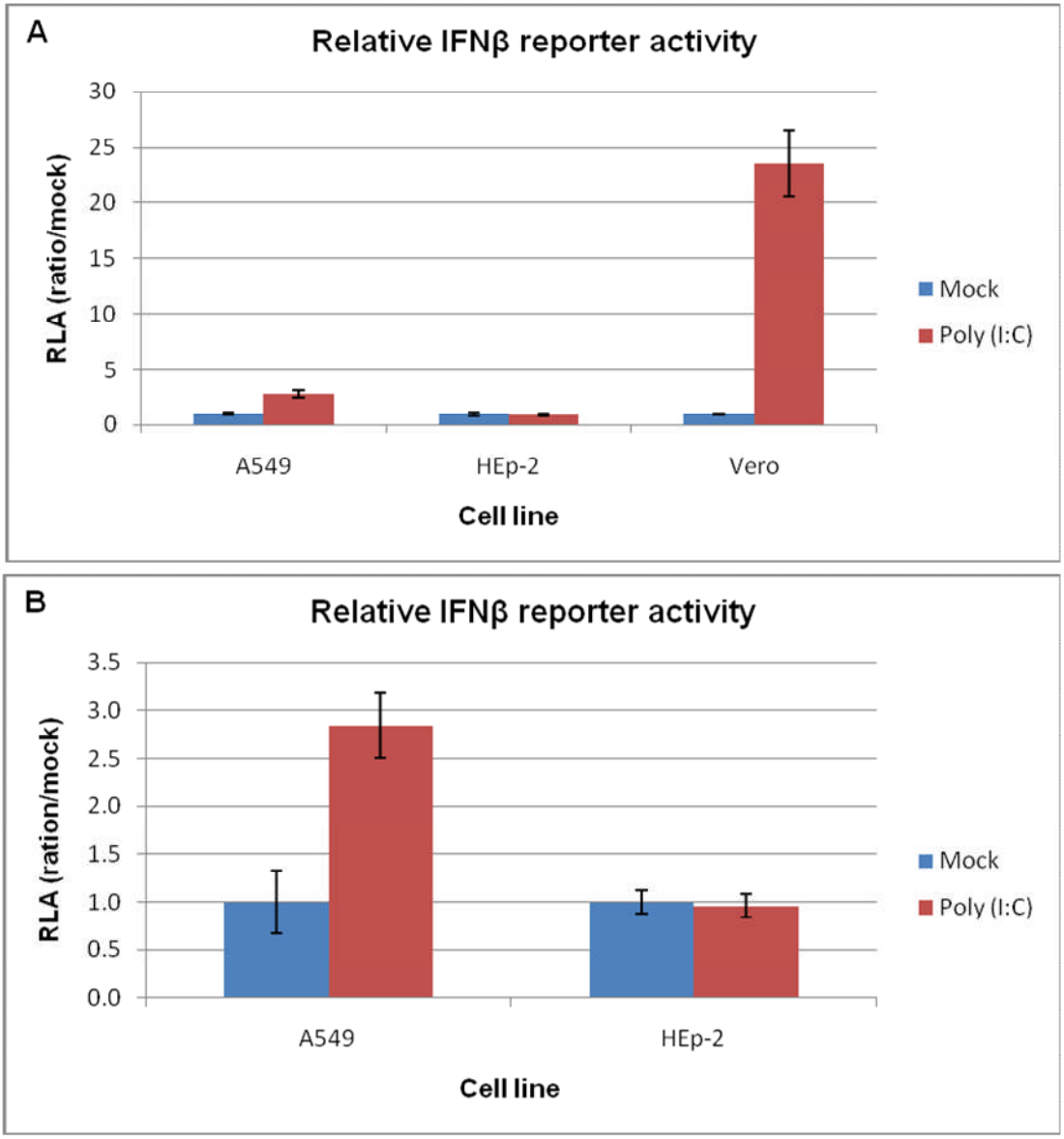


Figure 5.2 The IFNβ reporter is not stimulated by poly (I:C) in HEp-2 cells. 2.5 x 10^5 A549, HEp-2 or Vero cells were transfected in triplicate with 500ng of IFN β reporter plasmid pIFNβ-Luc. 24 hours later cells were mock-transfected or transfected with 1µg poly (I:C). All cells were harvested 24 hours later and lysates were analysed for luciferase activity. All values for each cell line were normalised to the mean value of the mock-treated samples of the respective cell line to generate the Relative Luciferase Activity (RLA), which is expressed here as a ratio relative to mock-transfected cells. Blue bars: mock-transfected cells. Red bars: poly (I:C)-transfected cells. Error bars indicate the standard deviation from the mean value of three replicate samples. **A.** A comparison of all cell lines tested. **B.** The same values as represented in **A.** on a smaller scale.

Upon incubation with poly (I:C), luciferase activity increased by approximately 30 fold in Vero cells (Fig. 5.2A). This suggested that the IFNβ reporter was strongly induced, and so confirmed that the assay system was working. The reporter was also induced in A549 cells, though the level of induction was low compared to Vero cells, (Fig. 5.2B). In contrast, in HEp-2 cells the IFNβ promoter did not show any induction. This suggested that HEp-2 cells are unable to respond to poly (I:C), and therefore were discarded as a candidate cell line for subsequent assays. Vero cells were also not utilised any further as they are not a human cell line, and have also been suggested to be deficient in IFN production, which is assumed to be due to a defect in or absence of the IFNβ gene (Emeny & Morgan, 1979).

5.3 A549 cells do not respond to exogenous interferon α

A549 cells were shown to support to the induction of the IFNβ reporter by poly (I:C), placing them as a candidate cell line for reporter assay experimentation (Fig. 5.2). Therefore, their ability to support the induction of the ISRE reporter plasmid was examined to determine whether downstream aspects of the IFN signalling cascade were also functional in these cells (Fig. 5.3A). When performing reporter assays based on transient transfection methodologies it is important to control for variation in the transfection efficiency. Reporter readings are quantitative and will largely depend on the number of cells successfully transfected with the respective reporter plasmid. To account for this, a control reporter plasmid is used alongside the experimental reporter plasmid. The control reporter activity is autonomous and should not be influenced by any of the experimental variables. In order to control for transfection efficiency, an autonomous *Renilla* luciferase reporter plasmid, driven by the HSV thymidine kinase (TK) promoter, was used alongside the ISRE reporter plasmid.

Transfection with poly (I:C) stimulated only a two-fold increase in ISRE reporter activity, though poly (I:C) is not a direct stimulant of the ISRE and this may explain why the level of induction was poor. Surprisingly, the addition of IFN α to the cells

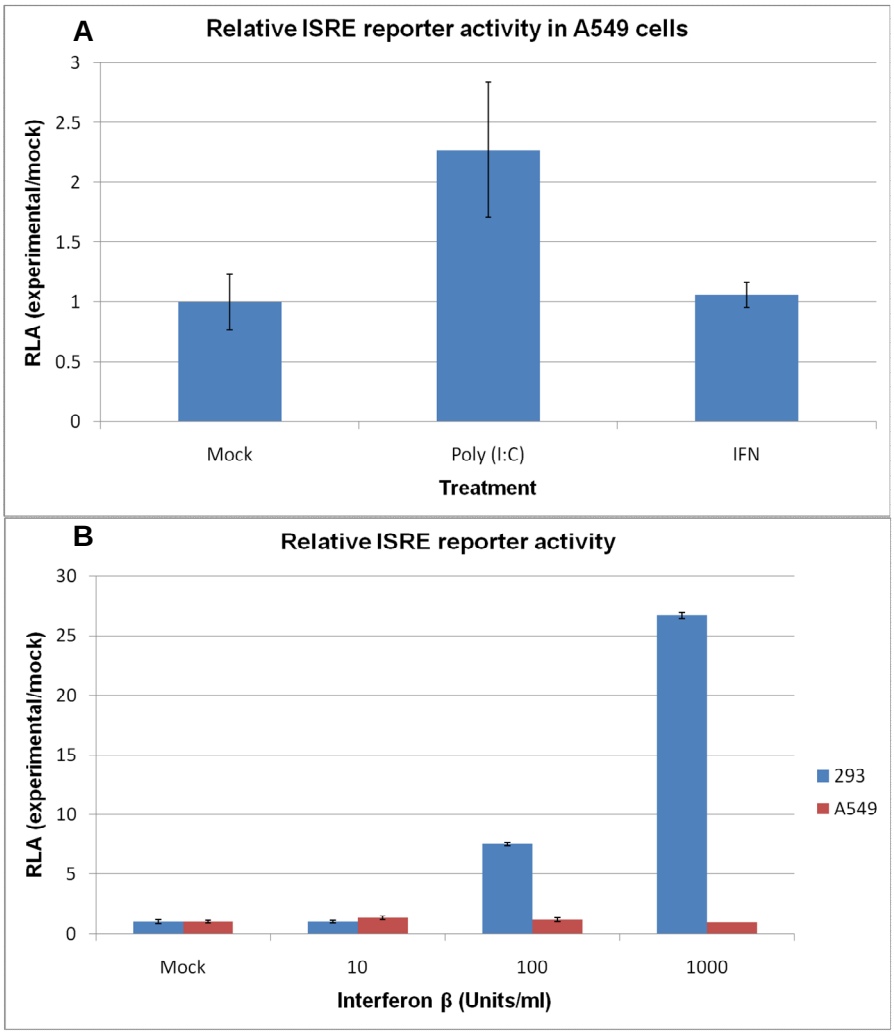


Figure 5.3 The pISRE-Luc reporter plasmid responds poorly to the addition of exogenous IFN α in A549 cells. **A.** 2.5 x 10^5 A549 cells were co-transfected in triplicate with 270ng pISRE-Luc alongside 30ng *Renilla* luciferase reporter plasmid pRL-TK**.** 24 hours later cells were mock-transfected, transfected with 1µg poly (I:C), or the medium was replaced with fresh 10% FBS DMEM containing IFN α to a final concentration of 100 units/ml. 24 hours later all cells were harvested and both the firefly and *Renilla* luciferase activity for each lysate was recorded. Firefly luciferase activity was normalised to *Renilla* luciferase activity in order to generate a relative luciferase activity (RLA) value for each sample. RLA values were expressed as a ratio compared to mock-treated cells. Error bars indicate the standard deviation of three replicate samples from the mean value. **B.** 2.5x10^5 A549 or HEK293 cells were co-transfected with 270ng pISRE-Luc alongside 30ng pRL-TK. 24 hours later cells were mock-treated or treated with IFN α to a final concentration of 10, 100, 1000 units/ml. Other details as panel A. Blue bars – 293 cells. Red bars – A549 cells.

(intended as a positive control) did not lead to an increase in reporter activity. IFN α is a direct stimulant of the ISRE and its addition would be expected to strongly induce the ISRE reporter. To examine this observation further, the ability of A549 cells to respond to increasing concentrations of IFN α was compared to that of an alternative cell line, 293 cells (Fig. 5.3B). 293 cells showed a strong activation of the ISRE by IFNα, which was dose-responsive. No induction was seen at 10 Units IFN α /ml, but at concentrations of 100 and 1000 U/ml the ISRE reporter was induced by 7-fold and >25-fold respectively. This strong induction was in contrast to that observed in A549 cells, where no increase in reporter activity was observed at any of the concentrations of IFN α tested. These results indicated that A549 cells are incapable of responding to exogenous IFN α. Although both the IFNβ and ISRE reporter plasmids responded to poly (I:C) in A549 cells, the levels of induction by poly (I:C) were considered too poor to warrant further use of this cell line.

5.4 U2OS cells support both IFNβ and ISRE promoter activation

The focus of investigation then shifted to an alternative cell line, U2OS cells, which are derived from human osteosarcoma. In these cells, both reporters were strongly induced by the addition of poly (I:C) (Fig.5.4A). IFNβ reporter activity was stimulated by approximately 7-fold, whereas the ISRE reporter was stimulated by approximately 12-fold. However, after 24h incubation with poly (I:C) a large amount of cell death was observed. This suggested that the experiment needed to be further optimised to reduce toxicity to the cells. Therefore to address this, the same experiment was repeated, but all cells were harvested 8 hours after transfection with poly (I:C) (Fig. 5.4B). Relatively little cell death was observed after 8 hours incubation time with poly (I:C), yet the activity of both the IFNβ and ISRE reporters was induced, by 6-fold and 8-fold respectively.

The difference in the amount of stimulation of these reporters reflects the fact that they represent different stages of the interferon pathway. Therefore it was not to be expected that the fold-inductions would necessarily be similar to one another. U2OS cells could therefore support the stimulation of both reporter plasmids by poly (I:C), and were therefore utilised in initial reporter studies.

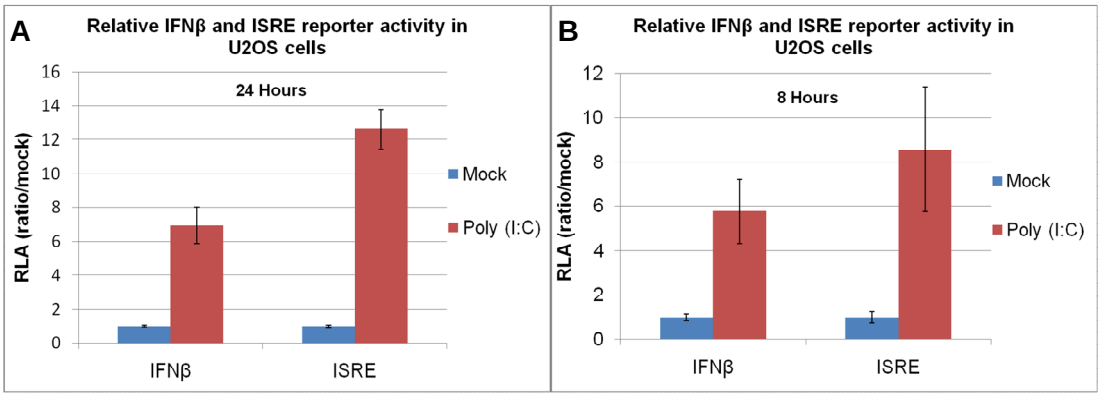


Figure 5.4 U2OS cells support activation of both pIFNβ-Luc and pISRE-Luc reporters. **A.** 2.5 x 10^5 U2OS cells were co-transfected in triplicate with 250ng empty vector pCI-neo, 25ng pRL-TK (*Renilla* luciferase reporter) and 225ng pIFNβ-Luc or 225ng pISRE-Luc. 16 hours later cells were mock-transfected or transfected with 1µg of poly (I:C). Cells were harvested 24 hours later and the firefly and *Renilla* luciferase activities of the lysates were assayed. Firefly luciferase activity was normalised to *Renilla* luciferase activity in order to generate an RLA, which was expressed as a ratio relative to mock-transfected cells. Blue bars represent pIFNβ-Luc reporter activity, red bars indicate pISRE-Luc reporter activity. Error bars indicate the standard deviation of three replicate samples from the mean value. **B.** as in **A.** but cells were harvested 8 hours after transfection with poly (I:C).

5.5 The effect of PML protein expression on ISRE reporter activity

The precise role of PML proteins in the interferon response remains unclear, and it is unknown whether specific PML isoforms can modulate the interferon response. To address this, the effect of over-expressing full-length PML isoforms on ISRE reporter activity was investigated. To do this, individual PML cDNA expression plasmids were included with the reporter plasmids in the initial transfection of U2OS cells, which were then stimulated by poly (I:C) transfection as before.

In cells untreated by poly (I:C), expression of all PML isoforms was strong (Fig. 5.5B, lanes 9-14). However, upon treatment with poly (I:C) PML expression was

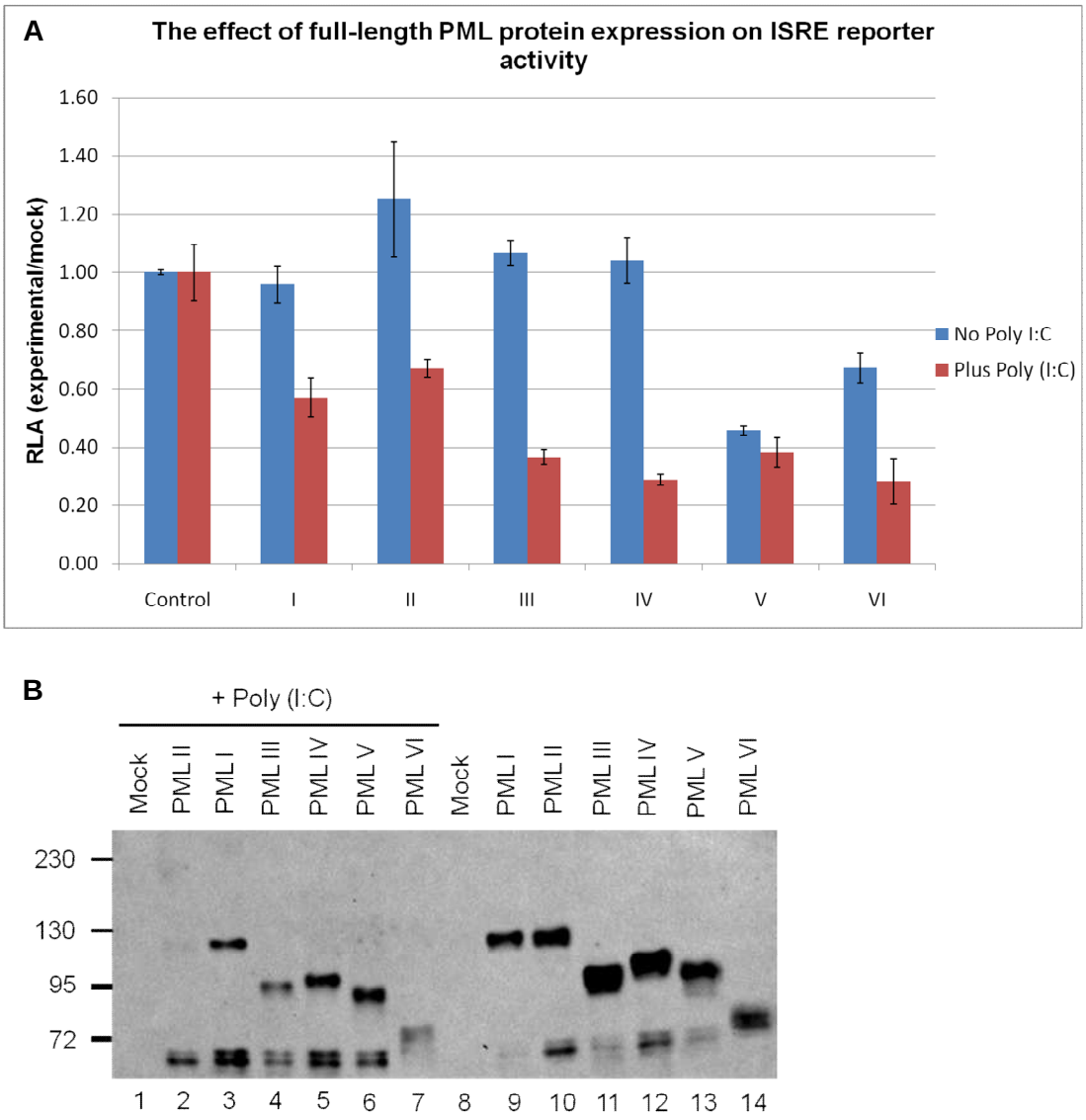


Figure 5.5 Expression of PML proteins influences pISRE-Luc reporter activity. **A.** 2.5 x 10^5 U2OS cells were co-transfected in triplicate with 225ng pISRE-Luc, 25ng pRL-TK and 250ng empty vector pCI-neo or pCI-neo FLAG-PML I-VI. 16 hours later cells were mock-transfected or transfected with 1µg poly (I:C). All cells were harvested 8 hours later and both the firefly and *Renilla* luciferase activity of the lysates was assayed. Firefly luciferase activity was normalised to *Renilla* luciferase activity to generate an RLA, which was expressed as a ratio relative to mock transfected cells. **B.** Samples from the replicate extracts used for assay in **A.** were pooled together and prepared for SDS-PAGE by the addition of sample buffer. Samples were separated by 8% SDS-PAGE and subjected to western blotting. A mouse anti-FLAG antibody was used to detect all exogenously-expressed PML proteins. Lanes 1-7 represent cells that were transfected with poly (I:C), lanes 8-14 represent mock-transfected cells. Protein sizes are indicated in kDa to the left hand side of the blot.

considerably reduced. It is unclear whether this was a result of translational shut-off, a known effect of the interferon response (Randall & Goodbourn, 2008), or due to cell death. PMLs II and III showed particular sensitivity to poly (I:C), as their expression was significantly reduced (lanes 2 and 4) compared to untreated cells (lanes 10 and 11).

Expression of PMLs I, II, III and IV had little effect on the basal activity of the ISRE reporter when compared to cells transfected with empty vector (Fig. 5.5A). In contrast, PMLs V and VI both exerted strong inhibitory effects on the basal activity of the ISRE reporter, as activity was reduced to approximately 40% and 60% of the control respectively. All these basal activities were however low compared to the activity of the reporter upon poly (I:C) stimulation (approximately 46-fold activation in this experiment). Upon stimulation with poly (I:C), all PML isoforms inhibited the activity of the ISRE reporter. Expression of PMLs III-VI was particularly inhibitory, as ISRE reporter activity was reduced to less than 40% of that in the poly (I:C)-stimulated control.

These data would suggest that expression of all full-length PML proteins, especially PML V and VI, inhibits the ability of the cell to mount an effective interferon response when challenged with dsRNA. However, the activity of the *Renilla* reporter was found also to vary depending on the PML protein being expressed, and whether cells had been treated with poly (I:C) (Table 5.1). PML II, IV, V and VI were found to increase *Renilla* luciferase activity by up to five fold, whereas poly (I:C) treatment decreased luciferase expression by 3- to 4-fold. There were two possible explanations for this observation. It was possible that the reporter plasmids, when mixed with PML III-VI expression plasmids, had a much higher transfection efficiency than when mixed with any of the other plasmids tested, though this seemed unlikely. Instead, it was more likely that the *Renilla* control plasmid was being influenced by expression of various PML proteins. There was also a systematic decrease in *Renilla* luciferase activity upon poly (I:C) treatment. One possible explanation for this could be that the *Renilla* luciferase-expressing cells were being killed off. However, no corresponding cell death was observed within the culture, so this possibility was unlikely. Again, it could not be discounted that *Renilla* luciferase activity was being influenced by external factors, although it was

	Expression vector	Sample	pISRE-Luc activity (RLU)	pRL-TK Activity (RLU)	Ratio	Average
Mock		1	0.0575	0.7689	0.0748	
	Empty vector	2	0.0964	1.2970	0.0743	0.0749
		3	0.0931	1.2320	0.0756	
		4	0.1608	2.1050	0.0764	
	PML I	5	0.1383	2.0630	0.0670	0.0718
		6	0.1123	1.5590	0.0720	
		7	0.0882	1.0990	0.0803	
	PML II	8	0.0909	0.9986	0.0910	0.0936
		9	0.1286	1.1730	0.1096	
		10	0.2497	3.1350	0.0796	
	PML III	11	0.2819	3.3840	0.0833	0.0800
		12	0.2100	2.7300	0.0769	
		13	0.3369	4.7390	0.0711	
	PML IV	14	0.4293	5.2350	0.0820	0.0779
		15	0.4253	5.2870	0.0804	
		16	0.1172	3.5020	0.0335	
	PML V	17	0.1189	3.5510	0.0335	0.0341
		18	0.1008	2.8440	0.0354	
		19	0.1250	2.7120	0.0461	
	PML VI	20	0.1599	3.0080	0.0532	0.0503
		21	0.1168	2.2590	0.0517	
Poly (I:C)		22	0.2154	0.2791	0.7718	
	Empty vector	23	0.2440	0.3823	0.6382	0.7001
		24	0.2792	0.4044	0.6904	
		25	0.2341	0.6067	0.3859	
	PML I	26	0.1878	0.5218	0.3599	0.3988
		27	0.1564	0.3470	0.4507	
		28	0.1465	0.3062	0.4784	
	PML II	29	0.1512	0.3396	0.4452	0.4688
		30	0.1616	0.3348	0.4827	
		31	0.1924	0.7822	0.2460	
	PML III	32	0.3016	1.2250	0.2462	0.2563
		33	0.1767	0.6388	0.2766	
		34	0.2023	1.0840	0.1866	
	PML IV	35	0.2533	1.1940	0.2121	0.2007
		36	0.2400	1.1810	0.2032	
		37	0.0860	0.3439	0.2501	
	PML V	38	0.2771	1.1370	0.2437	0.2672
		39	0.2263	0.7351	0.3078	
		40	0.2038	0.8595	0.2371	
	PML VI	41	0.0925	0.5789	0.1598	0.1527
		42	0.1347	0.6098	0.2209	

Table 5.1 The pRL-TK reporter plasmid is influenced by external stimuli. The luciferase values are those from the experiment represented in figure 5.5. RLU – Relative light units

plausible that the decrease in activity reflected a general shutoff of translation by the IFN response. Therefore *Renilla* expression could not be safely used as a transfection control.

5.6 HEK293 cells support both IFNB and ISRE reporter assays

HEK293 cells were analysed for their ability to activate both IFNβ and ISRE reporters when stimulated with poly (I:C). These cells had shown a strong response to exogenous IFNα (Fig. 5.3). CMV-β-Gal was used as a control for transfection efficiency, since its activity was less sensitive than pRL-TK to external variables.

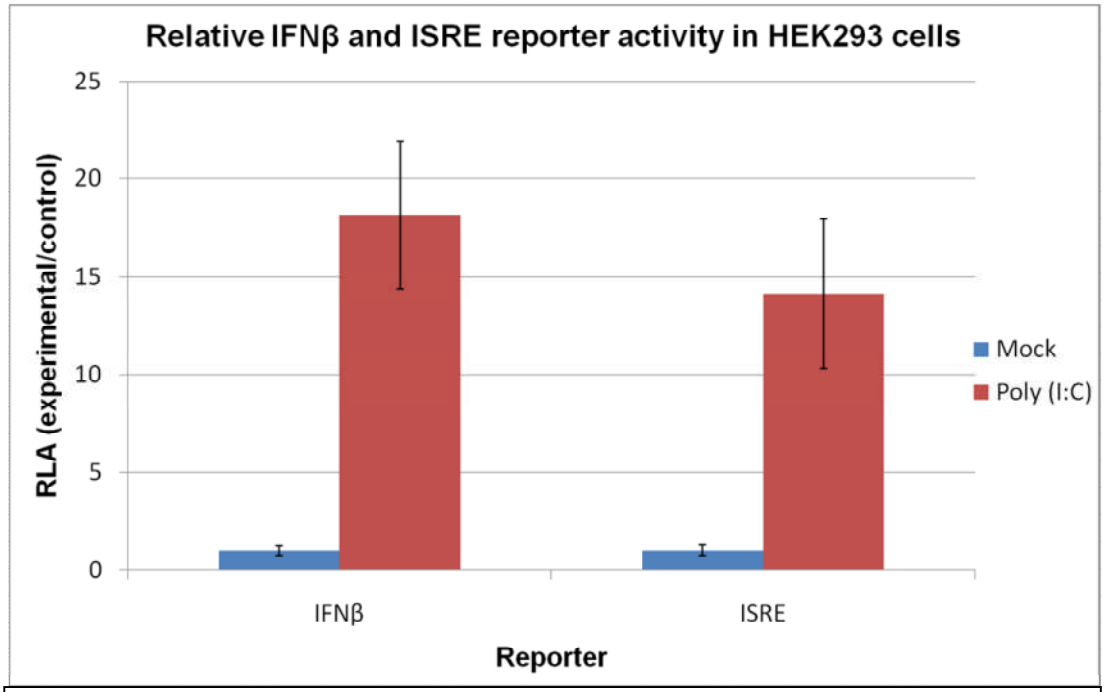


Figure 5.6 HEK 293 cells support both IFNβ and ISRE reporter assays. 2.5 x10^5 HEK293 cells were co-transfected in triplicate with 250ng pCI-neo, 25ng pcDNA3.1-HisB::*lacZ* and 225ng pLuc-IFNβ or 225ng pLuc-ISRE. 16 hours later cells were mock-transfected or transfected with 1µg poly (I:C). All cells were harvested 8 hours later, and the firefly luciferase and β-galactosidase activity of the lysates were measured. Luciferase activity was normalised to β-galactosidase activity to generate an RLA for each sample. RLAs are expressed as a ratio to mock-transfected cells. Error bars indicate the standard deviation of three replicate samples from the mean value. Blue bars: mock-transfected cells. Red bars: poly (I:C)-transfected cells.

Reporter	Treatment	Sample	Luciferase reporter activity (RLU)	Adjusted β-Gal activity (OD_{405nm})	Ratio	Average
IFNβ-Luc	Mock	1	0.013	0.139	0.091	0.116
		2	0.012	0.115	0.108	
		3	0.013	0.088	0.148	
	Poly (I:C)	4	0.145	0.060	2.408	2.140
		5	0.116	0.072	1.604	
		6	0.153	0.066	2.300	
ISRE-Luc	Mock	7	0.054	0.130	0.417	0.616
		8	0.053	0.075	0.701	
		9	0.048	0.065	0.729	
	Poly (I:C)	10	0.383	0.053	7.175	8.686
		11	0.437	0.038	11.387	
		12	0.348	0.046	7.494	

Table 5.2 HEK 293 cells support both IFNβ and ISRE reporter assays. Values shown are from the experiment represented in figure 5.7. RLU: Relative light units. β-galactosidase activity is represented as the optical density at a wavelength of 405nm, and is adjusted to exclude the cellular background.

Transfection of cells with poly (I:C) led to 18-fold and 15-fold increases in IFNβ and ISRE reporter activities, respectively (Fig. 5.6). Most of the induction observed was due to a direct increase in luciferase reporter activity, as β-galactosidase activity only varied by approximately 2-fold between mock-treated and poly (I:C)-treated cells (Table 5.2). This was in contrast to the 4-fold decrease in pRL-TK activity observed upon poly (I:C) treatment of U2OS cells (Table 5.1). This indicated that the β-galactosidase reporter was not significantly influenced by poly (I:C) treatment and could be deemed as a reliable indicator of transfection efficiency.

5.7 Discussion

The assays performed in this chapter were designed to identify a cell line that would respond effectively to the IFN inducer, poly (I:C). They were based upon transient transfection methodology, and therefore their success is highly dependent on the transfection efficiency of the cell line being used. If a cell line has a poor efficiency of transfection then general reporter activity across the cell population will be low. Consideration must also be taken that the stimulant used in these assays, poly (I:C) was also delivered to cells by transient transfection. Therefore, within a population of cells of poor transfection efficiency only a tiny subset of cells would be transfected with both the necessary reporter and the poly (I:C) required to induce it. In such a scenario only a small, if any, increase in reporter activity would be recorded. This could possibly explain why the IFNβ reporter responded relatively poorly to poly (I:C) in both HEp-2 and A549 cells (Fig. 5.2).

U2OS cells were initially found to support significant increases in both IFNβ and ISRE reporter activity, in contrast to that of either HEp-2 or A549 cells. However, upon closer inspection, much of the apparent induction seen was due to decreasing *Renilla* luciferase activity after poly (I:C) treatment that was over 5-fold in some cases; this created an increase in corrected ISRE activity. Cell death could not account for this decrease, though could have reflected host cell translational shutoff induced by the IFN response. *Renilla* luciferase activity was also found to vary depending on the PML protein being expressed, suggesting that it was not independent of the experimental variables. Subsequent analysis of 293 cells, where a β-galactosidase reporter was used as a transfection control, revealed that poly (I:C) treatment again decreased control reporter activity, though in this case the decrease was typically only <2-fold. Uncorrected IFNβ and ISRE reporter activity, however, gave strong responses to poly (I:C), suggesting that the relative induction was not strongly dependent on decreasing β-gal reporter activity in response to poly (I:C). Furthermore, the β-gal reporter was found not to be strongly affected by PML protein expression (Chapter 6), suggesting that it is truly independent of the experimental variables.

The mechanism underlying the differences between *Renilla* luciferase activity is unclear, though one possibility is that the autonomous HSV-TK promoter used to drive this reporter was being selectively down-regulated upon poly (I:C) treatment. The β-galactosidase reporter, in contrast, utilised a CMV immediate early promoter and therefore would not be subjected to the same regulatory mechanisms. Such a scenario could also explain why the *Renilla* luciferase activity was altered upon expression of various PML proteins.

As well as affecting translation, interferon has also been shown to exert anti-tumour properties (Gresser & Belardelli, 2002), and to enhance the p53 apoptotic response (Takaoka *et al*., 2003) . This has implications when performing IFN reporter assays in immortalised cell lines, as normal IFN responses may be intrinsically impaired. For example, Vero cells are known to lack the ability to produce IFN α due to a mutation in the IFN β promoter (Emeny & Morgan, 1979). It is therefore not inconceivable that the poor response to poly (I:C) observed in the majority of cell lines tested here may be due to deficiencies in the IFN signalling pathway upstream of IFNβ promoter activation. Indeed, U2OS cells have been suggested to possess deficiencies in IFN signalling (Mossman *et al*., 2000), which may be a contributing factor to their poor response to poly (I:C).

Aberrant IFN signalling pathways may also be responsible for the apparent lack of reporter induction by poly (I:C) in both A549 and HEp-2 cells. In particular, A549 cells were unable to induce ISRE reporter activity in the presence of exogenous IFN α., though they did support induction of the IFNβ promoter by poly (I:C). Altogether, this suggested that A549 cells may possess deficiencies in IFN signalling downstream of IFNβ production. It is of interest that a small induction of the ISRE reporter was observed in A549 cells upon transfection with poly (I:C), indicating that the cells may still respond to endogenously-produced IFNβ, but have lost the ability to respond to exogenous IFNα. In contrast, the IFNβ reporter did not show any induction upon transfection of HEp-2 cells with poly (I:C). This would indicate a failure of IFN signalling upstream of IFNβ induction. However, further work would be required to confirm these hypotheses.

HEK293 cells are an immortalised cell line, yet both IFNβ and ISRE activity increased markedly upon stimulation with poly (I:C). Unlike A549 cells, 293 cells

also responded in a dose-dependent manner to the addition of exogenous IFN α, indicating that the IFN signalling pathway is functional within these cells. 293 cells are routinely reported to give very high transfection efficiencies, and in accordance with this, they also supported relatively strong expression of exogenous proteins, as observed in Fig. 3.8A. Altogether, this suggests that 293 cells are an ideal cell line for an analysis of the effect of different PML proteins on the IFN response that utilises reporter assays.

Chapter 6
Investigation of the roles of individual PML isoforms in type I IFN signalling

6.1 Introduction

PML proteins have been extensively implicated in exerting anti-viral activity against a number of viruses. Furthermore, they have also been implicated in the interferon response, though no clear role of PML proteins in this pathway has been defined. Therefore, the experiments presented in this chapter were designed to address this utilising the reporter assay system set up in HEK293 cells, as described in Chapter 5. The assays performed all utilised the IFNβ promoter reporter plasmid, which is activated by both IRF3 and IRF7. This reporter is therefore activated upon induction of MDA5/RIG-1, TLR and JAK/STAT signalling pathways by cytosolic dsRNA.

The primary aim of the experiments was to identify any functions that individual PML isoforms might perform in the IFNβ signalling pathway. The principle approach was to over-express both full-length and selected truncated PML variants and to examine the effect on reporter activity. These studies were complemented with studies utilising siRNA-mediated depletion of various PML species, and studies using Ad5 E4 Orf3 to perturb the PML protein population.

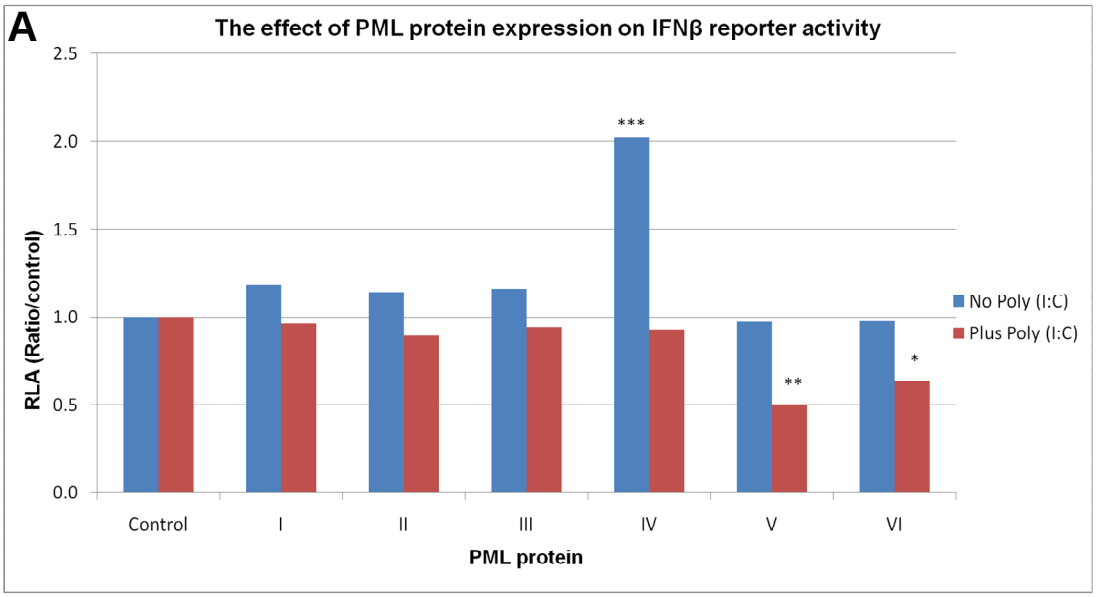
6.2 Over-expression of full-length PML IV, V and VI alters the activity of the IFNβ promoter

To investigate any potential role of PML proteins in the interferon response, IFNβ promoter reporter assays were performed in cells over-expressing each of six full-length PML isoforms (Fig. 6.1A). Essentially uniform expression of all PML proteins was achieved, as indicated by western blotting (Fig. 6.1B), though in each case a slight decrease in expression was observed in poly (I:C) treated cells (lanes 9-14) when compared to the equivalent untreated cells (lanes 2-7). The reason for this was unclear, though it may have been due to small amounts of cell death induced by poly (I:C). The decrease was unlikely to be due to the effects of host translational shutoff, a known function of interferon (Haller *et al*., 2006), as the control β-galactosidase reporter activity in these cells remained comparable to cells untreated with poly (I:C).

Expression of PMLs I-III, V or VI did not exert any significant effect on basal IFNβ reporter activity in the absence of stimulation by poly (I:C), as compared to the appropriate control. (Fig. 6.1A, blue bars). In stark contrast, expression of PML IV led to a significant increase in IFNβ promoter activity, suggesting a regulatory role of this isoform in the IFN response.

Upon stimulation with poly (I:C), IFNβ promoter activity in control cells was stimulated by an average of 15-fold across the three assays. This stimulation was similar to that in cells over-expressing PMLs I-III. Unexpectedly, expression of PML IV did not further enhance the activity of the IFNβ promoter in response to poly (I:C), in contrast to its effect in the absence of stimulation, perhaps suggesting that its capability to enhance reporter activity in the already activated state was saturated. Expression of both PML V and VI, however, significantly inhibited IFNβ promoter activity to approximately 50% and 60% of the control respectively, suggesting that these isoforms may play negative roles in IFN signalling.

Thus, altering the balance of PML isoforms by exogenous expression of full-length PML proteins clearly exerts differential effects on the activity of the IFNβ promoter depending on the isoform in question, suggesting that the unique C-termini of these proteins may perform different functions within the IFN response.



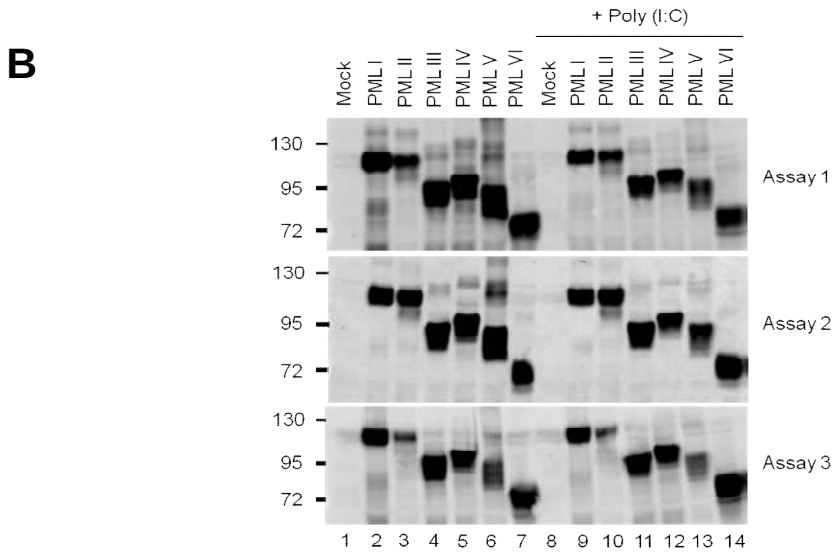
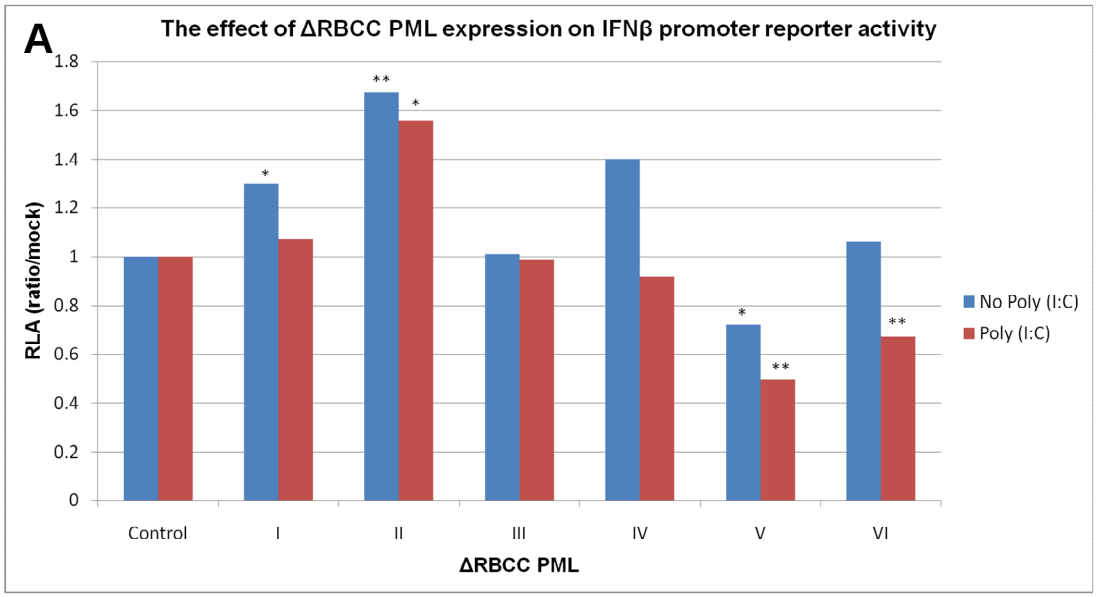


Figure 6.1 Expression of full-length PML proteins alters expression of the IFNβ reporter. **A** HEK293 cells were co-transfected in triplicate with pIFNβ-luc plus pcDNA3.1hisB::*lac*Z and pCI-neo or pCI-neo FLAG-PML I-VI. 16 hours later cells were mock-transfected or transfected with poly (I:C). 8 hours later cell lysates were assayed for both β-galactosidase and luciferase activity. Luciferase activity was normalised to β-galactosidase activity to generate the relative luciferase activity (RLA), which was expressed as a ratio to that of pCI-neo control transfected cells. The data presented are the average of three separate experiments (i.e. 9 values). Student's T-test analysis was performed on PML-transfected cell data against the equivalent control data. An asterisk above a bar indicates a significant difference from the control (pCI-neo). * - P <0.05, ** - P<0.005, *** - P<0.001. **B.** Samples of the three replicate lysates from each assay performed in **A** were pooled together and separated by 8% SDS-PAGE and subjected to western blotting. Blots were probed with a rabbit anti-FLAG antibody. Protein sizes are indicated in kDa on the left side of each blot.

6.3 PML ΔRBCC isoforms exert different effects on the IFNβ promoter to that of their full-length counterparts

The RBCC domain is found in all described PML proteins, and allows homo- and hetero-oligomerisation between various PML isoforms through interactions via the coiled-coil motif (Fig. 1.2). Therefore, exogenous expression of any full-length PML isoform may indirectly affect the activity of other endogenous isoforms through this interaction. This is a consideration that needs to be accounted for when studying the function of individual PML isoforms. Although individual expression of full-length PMLs IV, V and VI altered the activity of the IFNβ reporter in different ways (Fig. 6.1), it cannot be discounted that some of the effects observed were mediated through interactions with other endogenous PML isoforms. To try to separate out the effects of the unique C-terminal regions of PML from such indirect effects, PML isoform mutants lacking the N-terminal 361 residues, and therefore the RBCC motif, were utilised in experiments similar to those with full-length PMLs in Fig. 6.1. These ΔRBCC mutants do not localise to PML NBs and show a diffuse nuclear localisation (Leppard *et al*., 2009).

Consistent expression of PML I-VIΔRBCC was observed in all but one of the four assays performed (Fig 6.2B), no expression being observed for PMLs III, V and VI in assay 4. Consequently, the reporter activity of these samples was discarded from the analysis in Fig. 6.2A. Unlike full-length PMLs, no decrease in expression upon poly (I:C) treatment was observed. Also, in contrast to the full-length PMLs, expression of PML IΔRBCC and PML IIΔRBCC led to significant increases in the basal activity of the IFNβ promoter (Fig. 6.2A). PML II ΔRBCC consistently exerted the strongest activating effect, increasing reporter activity approximately 1.6-fold. In contrast to its full-length counterpart, PML IV ΔRBCC did not significantly increase activity of the IFNβ promoter. Taken together, these assays suggest that the RBCC domain is required for the positive activity of PML IV. PML V ΔRBCC expression led to a significant decrease in IFNβ promoter basal activity, an effect that was also not observed upon expression of full-length PML V (Fig. 6.1A).



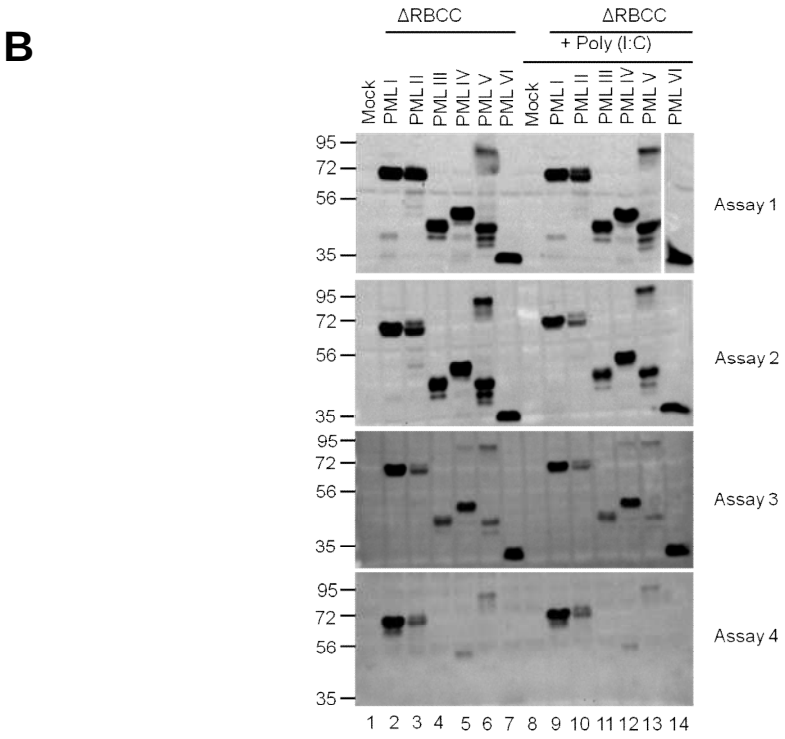


Figure 6.2 PML ΔRBCC isoforms have different effects on the IFNβ reporter to full-length PML proteins. A. As Fig. 6.1 panel A except PML ΔRBCC plasmids were used and four independent experiments were done. The data presented are the average of either three (PML III, V and VI) or four (Control, PML I, II and IV) separate experiments. Student's T-test analysis was performed on PML-transfected cell data against the equivalent control cell data. An asterisk above a bar indicates a significant difference from the control (pCI-neo). * - P <0.05, ** - P<0.005. **B.** Samples of the three replicate lysates within each assay performed in **A.** were pooled together and separated by 8% SDS-PAGE and subjected to western blotting. Blots were probed with a rabbit anti-FLAG antibody. Protein sizes are indicated in kDa on the left side of each blot.

In accordance with data from full-length PMLs, none of ΔRBCC mutant PMLs I, III-VI significantly enhanced the activity of the IFNβ promoter during stimulation by poly (I:C). Expression of PML II ΔRBCC, however, caused a significant (1.5-fold) increase in activity, and indeed was the only PML species found to enhance promoter activity in response to poly (I:C). In line with their full-length counterparts, both PML VΔRBCC and VIΔRBCC significantly inhibited poly (I:C)-mediated stimulation of promoter activity, to 50% and 70% of the control respectively. This suggested that the C-terminal domains of these isoforms are sufficient to mediate their inhibitory actions.

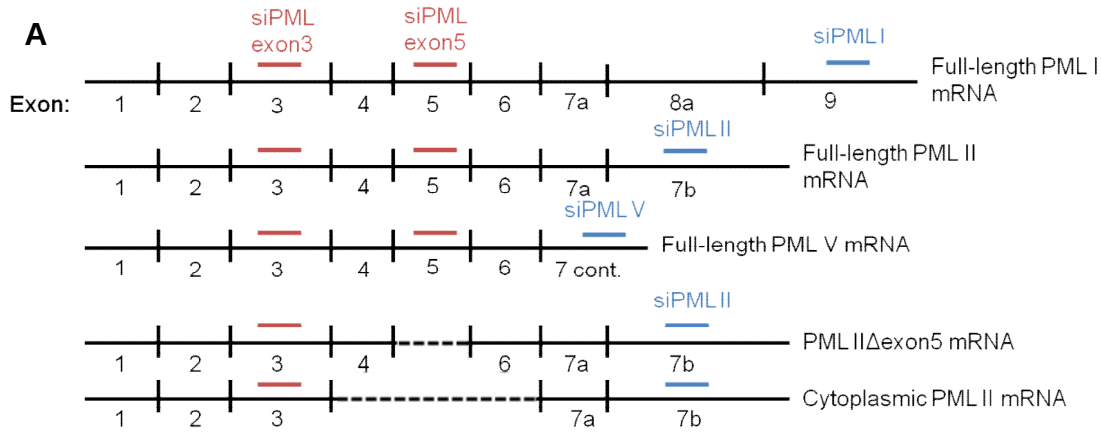
6.3 Depletion of various PML species alters the activity of the IFNβ promoter

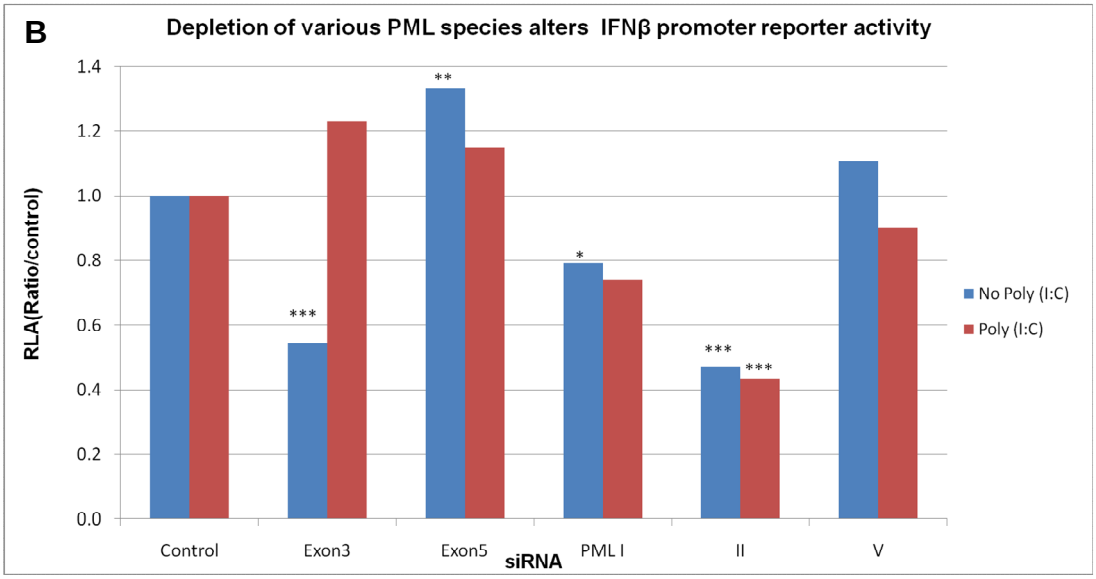
The data presented in Figures 6.1 and 6.2 collectively indicated a role of specific PML proteins in regulating the IFNβ promoter. However, these studies utilised transient over-expression methodology and the use of PML ΔRBCC mutants that are not known to occur naturally. It was therefore possible that the effects that were observed on the activity of the IFNβ promoter were due to stress responses to high-concentrations of exogenously-expressed protein or to the presence of unnatural proteins (ΔRBCC mutant PMLs). To address this, cells were depleted of various endogenous PML species using siRNA technology prior to being assayed for IFNβ promoter activity.

Initially, knockdown of total PML was tested. Two different siRNAs targeting all PML isoforms were used independently in order to account for the possibility that internally-deleted PML proteins could differ from full-length PML proteins in their ability to influence IFNβ promoter activity. siRNA targeting PML exon 3 would deplete all known PML mRNA species, including those encoding cytoplasmic variants or internally truncated species that arise from skipping of exons 4, 5 and/or 6. siRNA targeting PML exon 5 would deplete mRNA encoding nuclear PML proteins that retain this exon, though it should be noted that nuclear variants of PML protein can exist that lack exon 5 (Jensen, 2001) (Fig. 6.3A**)**. Isoform-specific knockdown was also developed for PML isoforms I, II and V, since these had been implicated in IFNβ promoter regulation (Figs. 6.1 and 6.2).

Successful knockdown of the total PML protein content of cells was achieved in all three assays performed using the siRNAs targeting PML exon 3 and exon 5 (Fig. 6.3 C). No obvious difference was apparent between the PML species depleted by siRNA targeting exon 3 or exon 5 although, with the exception of assay 2, the pan-PML anti-serum only detected a major PML species of approximately 120kDa that was depleted by both exon 3 and exon 5 siRNA. It was therefore impossible to determine the extent of knockdown of different PML isoforms. Subsequent attempts to detect PML I, II and V protein levels in these lysates using isoform-specific anti-sera also proved extremely difficult, and therefore their knockdown was investigated separately (see section 6.4).

Depletion of PML proteins possessing exon 3 resulted in a significant decrease in basal IFNβ promoter activity, to levels of approximately 50% to that of the control (Fig. 6.3B). Depletion of PML proteins containing exon 5, however, had the opposite effect, as it led to a significant increase in IFNβ promoter activity (~1.3 fold). Depletion with either siPML I or siPML II also led to significant inhibition of the IFNβ promoter, to levels equivalent to 80% and 40% of the control respectively. In contrast, depletion with siPML V had no significant effect. Upon stimulation with poly (I:C), only depletion using siPML II significantly affected the activity of the IFNβ promoter, where stimulation was again reduced to approximately 40% of control cells, a result that fits with the observed increase in activity caused by over-expressing PMLIIΔRBCC depletion with siPML V had no effect. This contrasts with the effect of PML V over-expression, which inhibited reporter activity after treatment with poly (I:C) (Figs. 6.1 and 6.2).


A
siPML exon3
siPML exon5
siPML I
Exon:
1 2 3 4 5 6 7a 8a 9
Full-length PML I mRNA
siPML II
1 2 3 4 5 6 7a 7b
Full-length PML II mRNA
siPML V
1 2 3 4 5 6 7 cont.
Full-length PML V mRNA
siPML II
1 2 3 4 6 7a 7b
PML IIΔexon5 mRNA
1 2 3 7a 7b
Cytoplasmic PML II mRNA


B
Depletion of various PML species alters IFNβ promoter reporter activity
RLA(Ratio/control)
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
**
***
*
***
***
No Poly (I:C)
Poly (I:C)
Control
Exon3
Exon5
PML I
II
V
siRNA

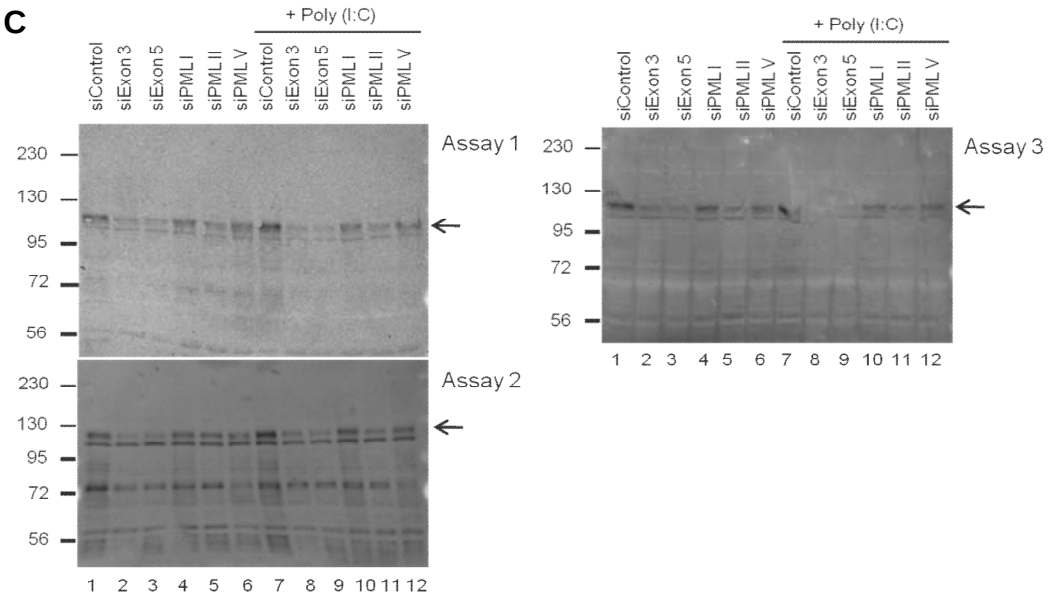

C
+ Poly (I:C)
siControl
siExon 3
siExon 5
siPML I
siPML II
siPML V
Assay 1
Assay 2
Assay 3
230
130
95
72
56
1 2 3 4 5 6 7 8 9 10 11 12

Figure 6.3 Depletion of various PML species exhibits different effects on the IFNβ reporter. **A.** Diagram showing the positions of PML siRNA. PML mRNA lacking exon5 or exons 4, 5 and 6 will not be depleted by siPML exon5, though will be depleted by siPML exon 3. **B.** HEK293 cells were transfected in triplicate with 150pmol of control siRNA, or siRNA targeting PML exon 3, PML exon 5, PML I, PML II or PML V. 32 hours later cells were co-transfected in triplicate with the reporter plasmids and pCI-neo control. 16 hours later cells were mock-transfected or transfected with poly (I:C). 8 hours later the cell lysates were assayed for reporter activity, and data analysed as before (Fig. 6.1, panel A). The data presented are the average of three separate experiments. Student's T-test analysis was performed on siPML knockdown data against control siRNA cell data. An asterisk above a bar indicates a significant difference from the control (pCI-neo). * - P <0.05, ** - P<0.005, *** - P<0.001. **B.** Three replicate lysates from each assay performed in **A.** were pooled together and separated by 8% SDS-PAGE and subjected to western blotting. Blots were probed with a polyclonal anti-PML antibody. Protein sizes are indicated in kDa on the left side of each blot. The arrows to the right hand side of the blots indicate successful depletion of the major PML species.

6.4 siRNA-mediated depletion of PMLs I, II and V in 293 cells.

Western blot analysis of 293 cell lysates prepared for reporter assay analysis revealed low levels of PML protein, as detected by a pan-PML antibody (Fig. 6.3B), as compared with those seen in chapters 3 and 4. This was likely to be a consequence of using lysates prepared in Passive Lysis buffer (PLB), suitable for enzyme activity assays, for SDS-PAGE. These lysates will be more dilute than lysates prepared directly in SDS gel sample buffer. Also, because it lacks ionic detergent, PLB is unlikely to solubilise the entire PML content of the cell, hence making subsequent detection difficult. This was indeed the case for another nuclear matrix-associated protein, E4 Orf3, which is demonstrated in figure 6.8B.

In order to confirm that PML I, II and V were being successfully knocked-down by siRNA in HEK293 cells, the extent of depletion of these isoforms in cells lysed directly in sample buffer was investigated. After 48 hours of incubation with siRNA, PML I species were not visibly depleted (Fig. 6.4, lanes 2-5). This suggests that either PML I is intrinsically difficult to knock-down due to a high degree of stability, or the siRNA used to deplete this isoform is inefficient. In contrast,

endogenous PML V was clearly detected by its respective anti-serum (lane 11), and was depleted by siPML V in a dose dependent manner (lanes 12-15).

Depletion with siPML II led to a decrease in the amount of the major species of PML detected with a pan-PML antibody (Fig. 6.3B, arrow). This band was consistent with the migration position of PML I and II, as determined previously by exogenous over-expression of PML (Fig. 4.1), and therefore suggested PML II was being successfully depleted. However, analysis of siPML II-mediated depletion with an anti-PML II antibody was inconclusive, as no endogenous PML II was observed in the control lane with which to draw comparison (Fig. 6.4, lane 6). One possible explanation for this discrepancy is that 293 cells possess low levels of endogenous full-length PML II, which may below the level of detection by anti-PML II. Interestingly, this mirrored an observation previously noticed in HEp-2 cells (Fig. 4.2).

An alternative strategy was devised to assess PML I and II knockdown in HEK293 cells. siRNA knockdown of each of these isoforms was performed on cells over-expressing the isoform in question (Fig. 6.5). Again, PML I knockdown was found to be very poor, with only a modest decrease in expression at the highest concentration of siPML I used as compared to siControl-transfected cells (Fig. 6.5A, lane 6). In contrast, PML II was found to be strongly depleted at all the siPML II concentrations used, but not by the control siRNA (Fig. 6.5B). This suggested that either PML II is more rapidly turned over, and hence more readily depleted than PML I, or that siPML II is highly efficient. Therefore, after 48 hours of incubation with the relevant siRNA, PML II and V can be successfully depleted by siRNA. PML I, however, only exhibits very modest knock-down by siRNA at the concentration used in the reporter assays described in Fig. 6.3. Although the number of cells and the concentrations of siRNA used were the same as for the assay described in Fig. 6.3A, the incubation time with siRNA was 8 hours shorter in this experiment. Therefore the knockdown of PML during the assays performed in Fig. 6.3A is likely to have been greater than that observed in Figs. 6.4 and 6.5. It may be concluded however, that PML I knock-down was considerably weaker than knock-down of PML II and PML V.

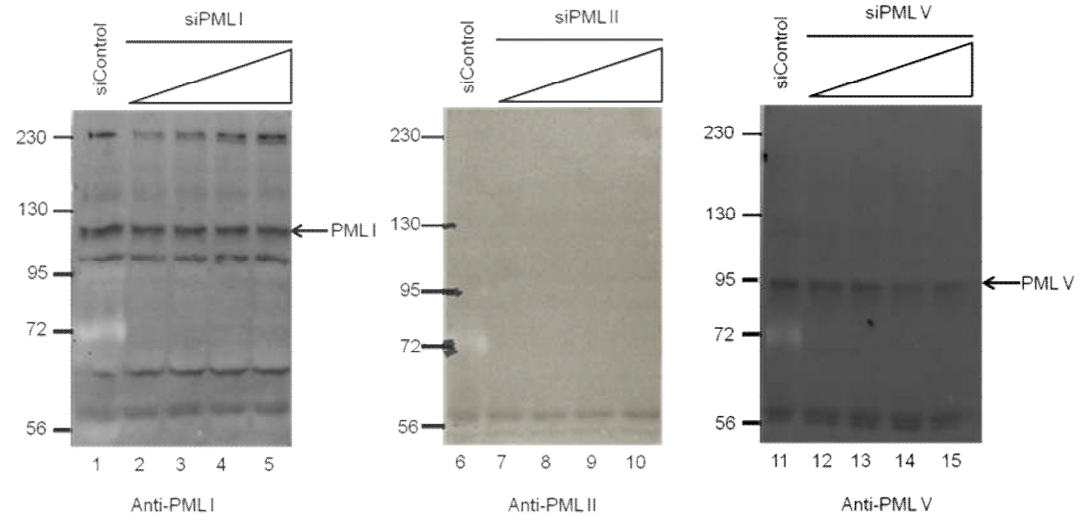


Figure 6.4 Attempted depletion of endogenous PMLs I, II and V in HEK293 cells. 2.5 x 10^5 HEK293 cells were transfected with 1, 5, 50 or 150pmol siRNA targeting PML I, II or V, or 150pmol of control siRNA. 48 hours later cells were harvested and the lysates separated by 8% SDS-PAGE and subjected to western blotting. The blots were probed with either anti-PML I, anti-PML II or anti-PML V antibodies. The migration positions of PML I and PML V are indicated by arrows to the right of the respective blots. Full-length PML II would be similar in size to PML I. Protein sizes are indicated in kDa to the left of each blot.

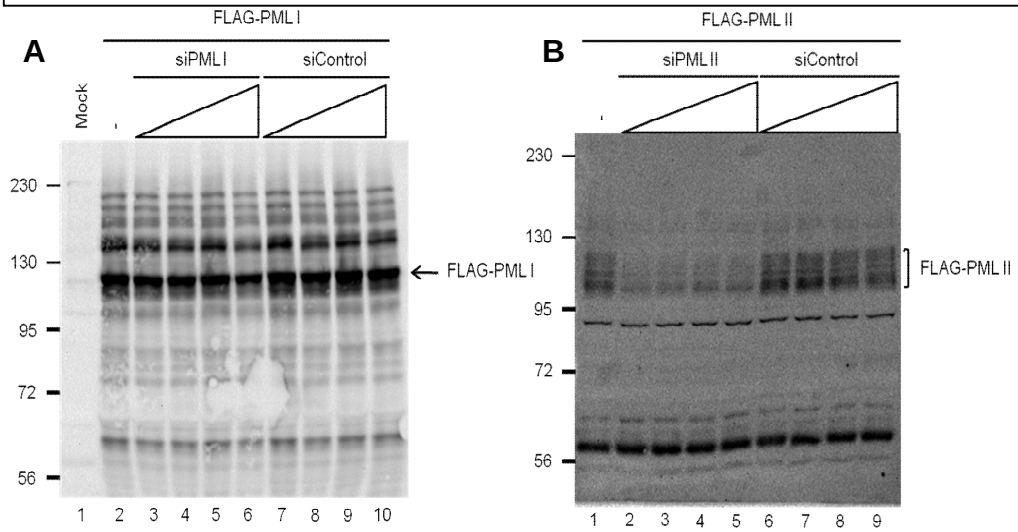


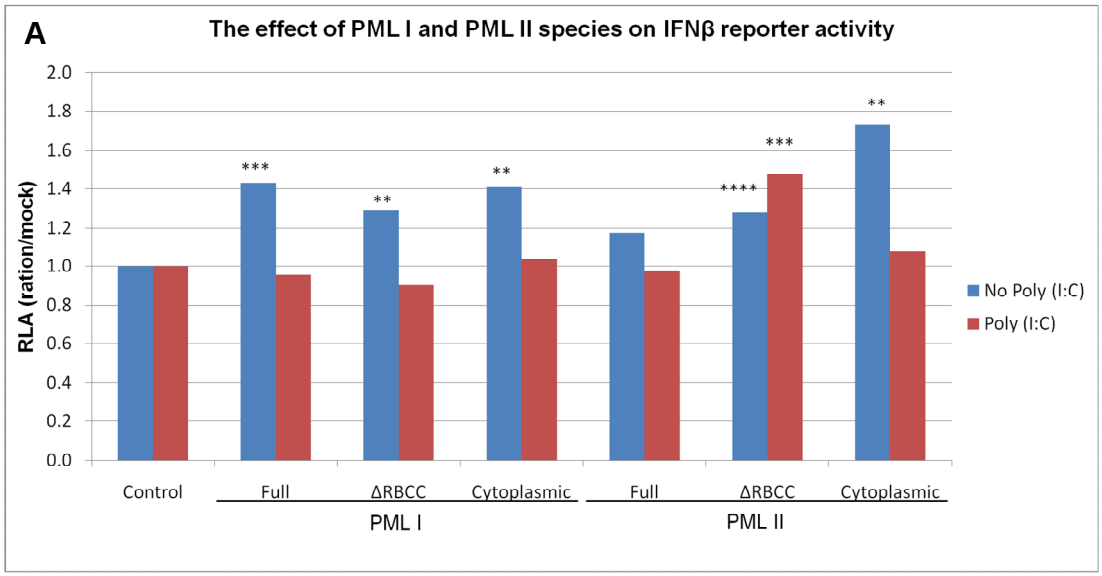
Figure 6.5 Knockdown of exogenously-expressed FLAG-PML I and II in HEK293 cells. A. HEK293 cells were mock-transfected or co-transfected with 450ng pCI-neo plus 50ng pCI-neo FLAG-PML I. 16 hours later cells were transfected with 1, 5, 50 or 150pmol control siRNA or PML I siRNA. 48 hours later cells were harvested and the lysates were separated by 8% SDS-PAGE and subjected to western blotting. The blot was probed with an anti-PML I antibody. The migration position of PML I is indicated to the right hand side of the blot, and protein sizes are indicated in kDa on the left. **B.** As panel A, except FLAG-PML II, PML II siRNA and anti-PML II were used instead.

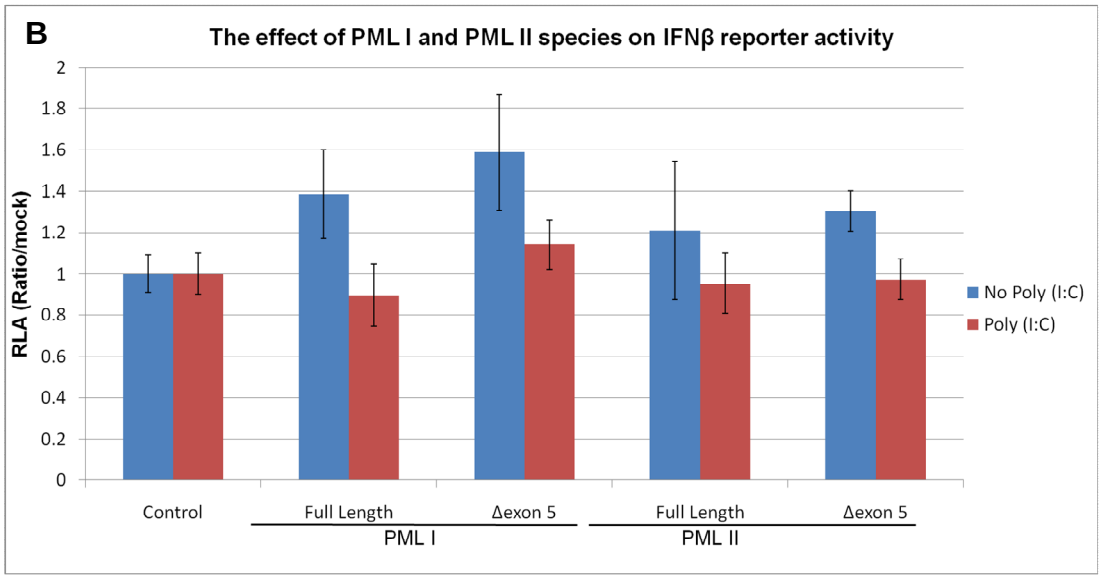
6.5 Cytoplasmic and ΔExon 5 PML II do not enhance IFNβ promoter activity

The data presented in Figures 6.1, 6.2 and 6.3 suggested that PML II plays a role in regulating the activity of the IFNβ promoter. Expression of a ΔRBCC mutant PML II enhanced IFNβ activity, whereas depletion of all endogenous PML II species led to a significant decrease in activity. However, expressing additional full-length PML II exerted no effect on the IFNβ promoter, raising the possibility that a splice variant of PML II rather than full-length PML II may be responsible for enhancing IFNβ promoter activity. Therefore, cDNAs encoding natural alternative splice variants of PML II were created and their effect on the IFNβ promoter was investigated. cDNA clones for two naturally occurring PML splice variants (Jensen, 2001) were constructed by PCR mutagenesis using full-length PML II or, as a control, full-length PML I as the parent molecule. Exons 4, 5 and 6 were excluded from both PML I and PML II to create cytoplasmic variants of these isoforms (see Appendix). In parallel, clones of PML I and PML II were generated that just lacked exon 5 (see Appendix). Exclusion of these exons in both types of construct left the resulting sequence in frame, and hence the PML I and PML II C-termini were maintained.

Upon transfection of these new plasmids alongside the previously analysed full-length and ΔRBCC isoforms, uniform expression was observed of all PML I and PML II species (Fig. 6.6C). In the absence of stimulation with poly (I:C), all PML I variants significantly enhanced IFNβ promoter activity to similar extents (Fig. 6.6 A, B). This result accords with the previous data for PML I ΔRBCC (Fig. 6.2) but contrasts with that for full-length PML I (Fig. 6.1), in which only a slight and not significant increase was seen. As seen in earlier experiments, PML II ΔRBCC but not full-length PML II significantly enhanced IFNβ promoter activity. The cytoplasmic variant of PML II mimicked this activity, and appeared to exert an even stronger effect. However, PML IIΔexon5 behaved similarly to full-length PML II.

Upon stimulation with poly (I:C), neither the cytoplasmic PML I variant nor PML I Δexon5 significantly enhanced or decreased IFNβ promoter activity, again resembling in activity both full-length and PML IΔRBCC. As demonstrated above, PML IIΔRBCC again significantly enhanced reporter activity, while full-length PML


A
The effect of PML I and PML II species on IFNβ reporter activity
RLA (ration/mock)
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
***
**
**
****
***
**
Control
Full
ΔRBCC
Cytoplasmic
Full
ΔRBCC
Cytoplasmic
PML I
PML II
No Poly (I:C)
Poly (I:C)


B
The effect of PML I and PML II species on IFNβ reporter activity
RLA (Ratio/mock)
2
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
Control
Full Length
Δexon 5
Full Length
Δexon 5
PML I
PML II
No Poly (I:C)
Poly (I:C)

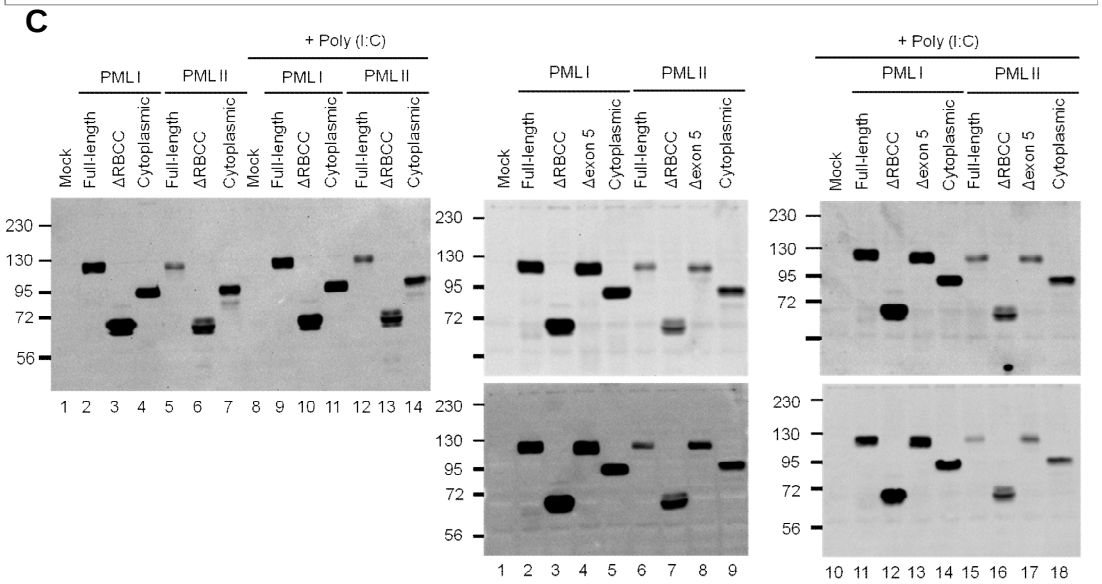

C
+ Poly (I:C)
PML I
PML II
Mock
Full-length
ΔRBCC
Δexon 5
Cytoplasmic
230
130
95
72
56
1 2 3 4 5 6 7 8 9 10 11 12 13 14
1 2 3 4 5 6 7 8 9
10 11 12 13 14 15 16 17 18

Figure 6.6 Cytoplasmic and Δexon5 PML I and PML II do not enhance the response of the IFNβ reporter to poly (I:C). **A**. HEK293 cells were co-transfected in triplicate with the reporter plasmids and pCI-neo FLAG-PML I-II, pCI-neo FLAG-PML ΔRBCC I-II or pCI-neo FLAG-PML I-II cytoplasmic. 16 hours later cells were mock-transfected or transfected with poly (I:C). All cells were harvested 8 hours later and lysates were assayed for both luciferase and β-galactosidase activity. Luciferase activity was normalised to β-galactosidase activity to obtain the RLA, which was expressed as a ratio to that of the Mock control. The data presented are the average of three separate experiments. Student's T-test was performed on data from PML-transfected cells against pCI-neo-transfected (control) cells. An asterisk above a bar indicates a significant difference from the mock control. **- P <0.005, *** - P<0.0005, **** - P<0.0001. **B**. As A, except FLAG-PML I Δexon5 and IIΔexon5 were used instead of the cytoplasmic constructs, and the data presented are the average of two separate experiments. Error bars indicate the standard deviation from the mean of at least five replicates. **C.** Replicate lysates from the assays shown in **A** and **B** were pooled together and separated by 8% SDS-PAGE and subjected to western blotting. Blots were probed with a rabbit anti-FLAG antibody. Protein sizes are indicated in kDa to the left hand side of each blot.

II did not. However, neither cytoplasmic PML II nor PML IIΔexon5 species significantly altered reporter activity. Therefore, during poly (I:C) stimulation of the IFNβ promoter, cytoplasmic and Δexon5 variants of both PML I and PML II behave in similar ways to their full-length counterparts, and the enhancing activity of PML II ΔRBCC on IFNβ promoter activity does not mimic the properties of either of these naturally occurring variants.

6.6 The sub-cellular localisation of PML I and II splice variants upon poly (I:C) stimulation

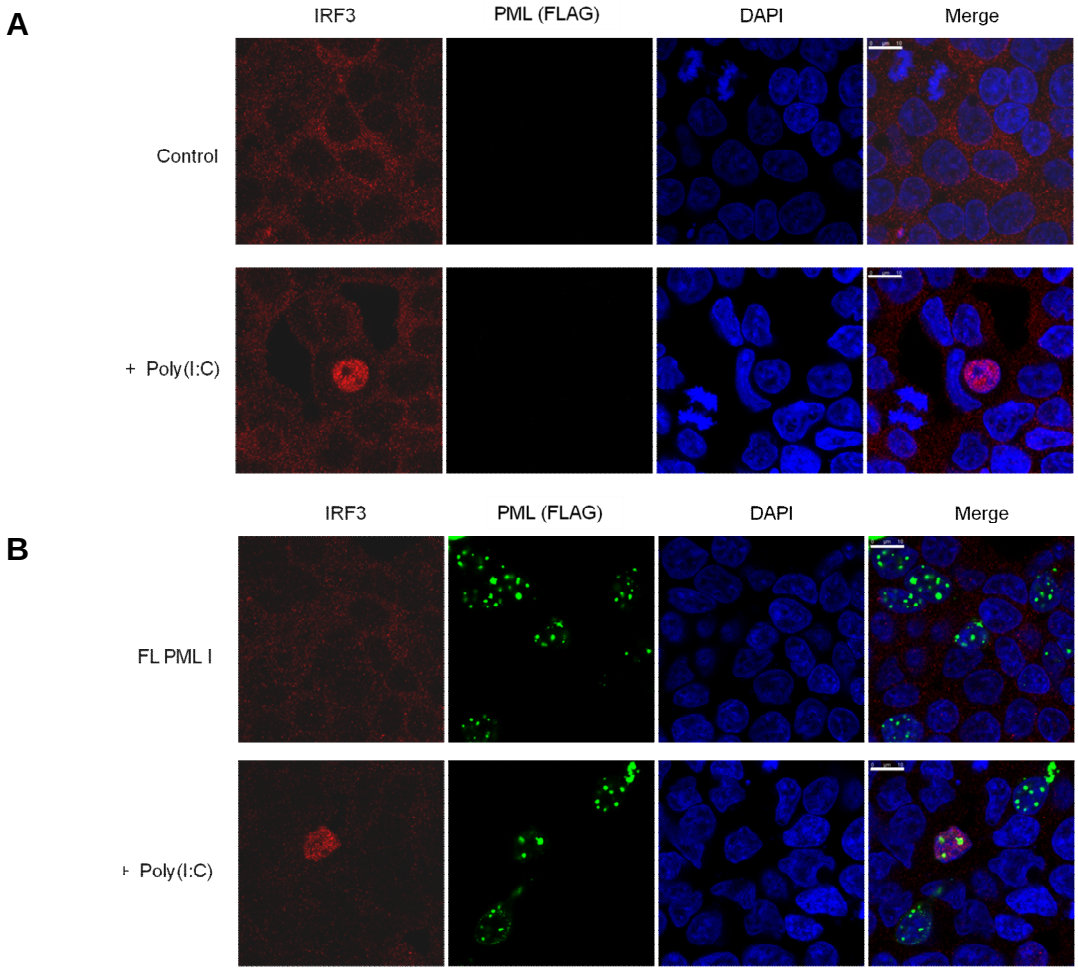
PML IIΔRBCC was the only PML-related molecule found to enhance IFNβ promoter activity during stimulation with poly (I:C), but the mechanism by which this molecule achieved this remained unclear. This protein has been shown to have a diffuse nuclear localisation (Leppard *et al*., 2009). It was therefore of interest to determine whether PML IIΔRBCC underwent any re-localisation during poly (I:C) stimulation which could contribute to its positive activity on the IFNβ reporter. To test this, PML IIΔRBCC was over-expressed and visualised in HEK293 cells undergoing stimulation of an IFN response by poly (I:C), and its location was

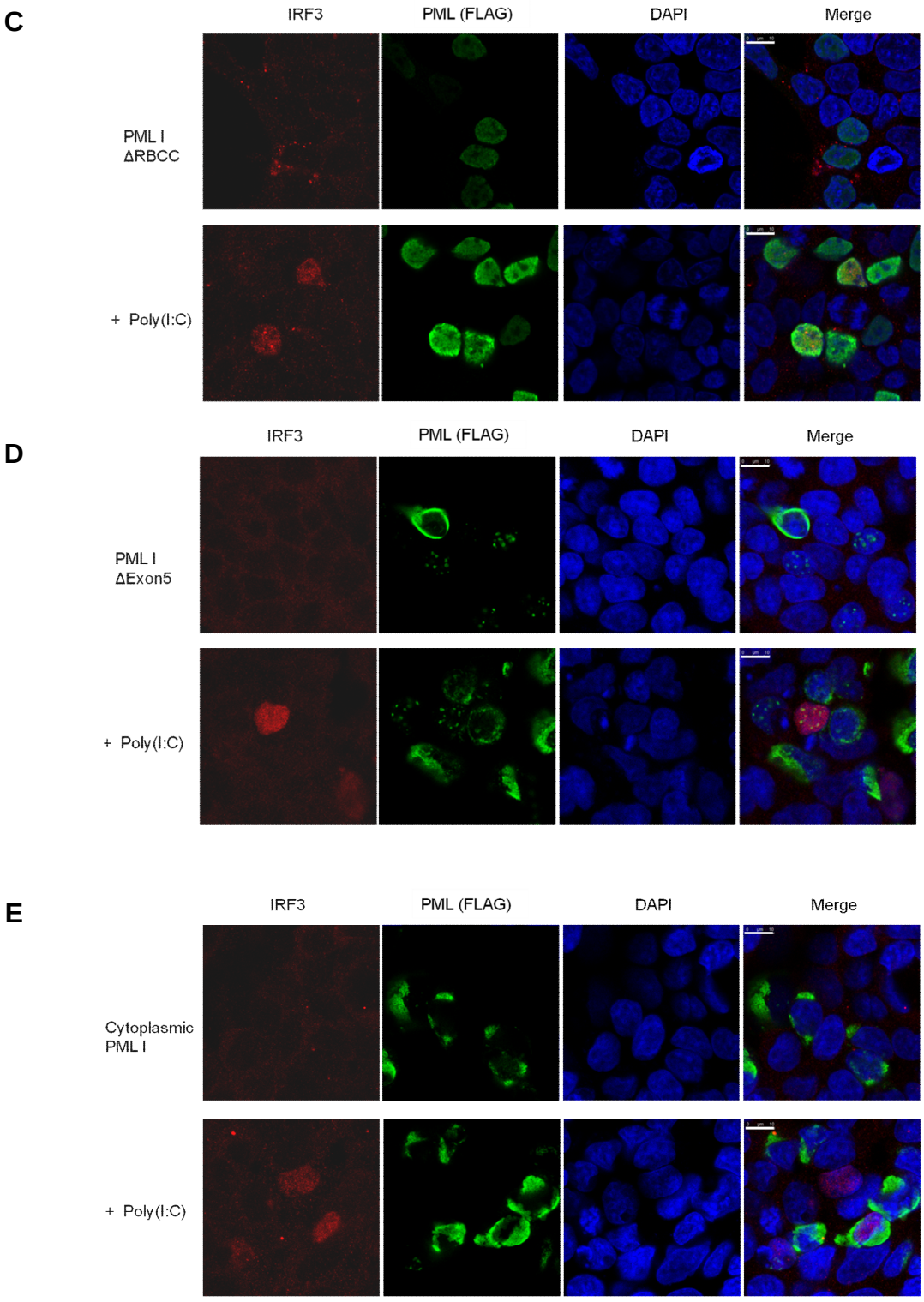
compared to that of a variety of other PML I and PML II proteins expressed from cDNA constructs (Fig. 6.7). In order to identify cells responding to poly (I:C) and hence undergoing an IFN response, cells were co-stained with an anti-IRF3 antibody. During an IFN response, IRF3 migrates to and accumulates in the nucleus, where it acts as a transcription factor (Haller *et al*., 2006) (Fig. 1.1). Therefore, cells that had been transfected with the PML construct and poly (I:C) could be distinguished from the rest of the population.

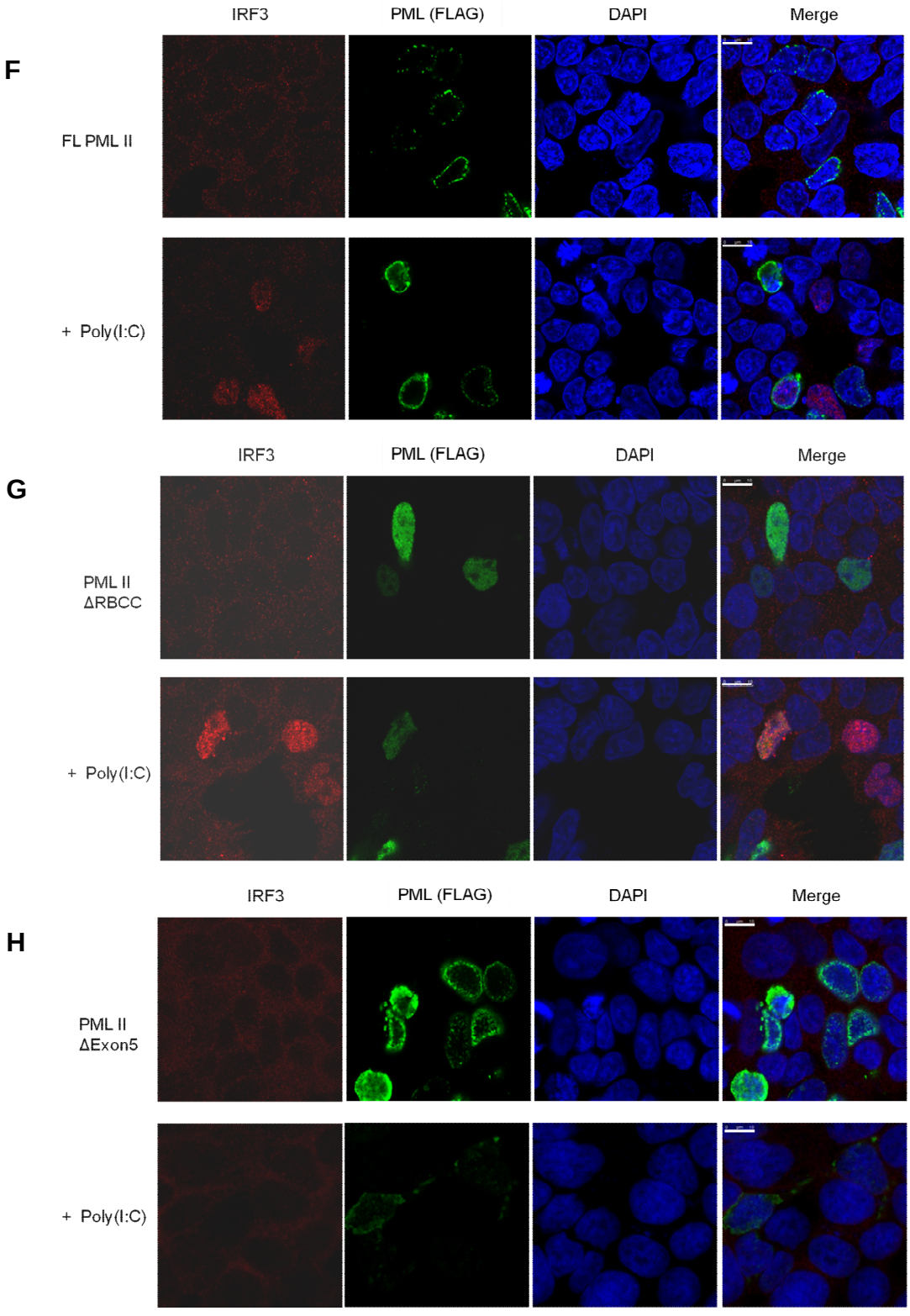
As expected, in the absence of poly (I:C) stimulation, IRF3 displayed a diffuse cytoplasmic staining pattern and was excluded from the nucleus (Fig. 6.7A). Upon poly (I:C) treatment, nuclear localisation of IRF3 was observed, however, surprisingly, this was only seen in a small fraction of cells. This suggested a relatively low transfection efficiency of poly (I:C), which is contrary to the normal experience in HEK293 cells, which are generally considered to be highly supportive of transient transfection (Parham *et al*., 1998).

As expected, full-length PML I localised to PML NBs (Beech *et al*., 2005), and this localisation was unaffected in cells undergoing an IFN response, as indicated by the nuclear localisation of IRF3 (Fig. 6.7B). PML IΔRBCC displayed a diffuse nuclear localisation (Fig 6.7C), as described previously (Leppard *et al*., 2009). This pattern of localisation was also unaffected by induction of the IFN response. Of the novel forms of PML I, PML IΔexon5 localised to PML NBs, but a subset of cells were also found to display alternative localisation patterns around the nuclear membrane and within the cytoplasm (Fig. 6.7D). This was not wholly unexpected, as PML I contains a nuclear export sequence (NES) allowing it migrate to the cytoplasm (Jensen, 2001), but the same was not seen for full-length PML I, which contains the same NES. However none of these alternative patterns were exclusive to poly (I:C)-transfected cells, indicating that the localisation is not influenced by the IFN response (Fig. 6.7D). Cytoplasmic PML I was restricted to the cytoplasm and hence did not associate with PML NBs (Fig. 6.7E). As with the other PML I constructs, induction of the IFN response did not alter its localisation (Fig. 6.7E), further suggesting that PML I is inert regarding the IFN response.

In contrast to full-length PML I, full-length PML II did not co-localise with PML NBs in the absence of poly (I:C), but instead localised to the periphery of the nucleus (Fig. 6.7F). This was unexpected, as previous studies have shown that exogenously expressed PML II localises to PML NBs in other cell types (Beech *et al*., 2005). This pattern of localisation was independent on the IFN response, as it was similarly observed in poly (I:C)-transfected cells (Fig. 6.7F). Similar patterns of full-length PML II staining have been observed in other cell types in a proportion of cells, particularly those with high levels of expression (K. Leppard, personal communication). PML IIΔexon5 staining resembled that of full-length PML II in that it did not form PML NBs, but instead associated with the periphery of the nucleus (Fig. 6.7H). No cells were found that exhibited strong nuclear staining of IRF3 alongside PML IIΔexon5 expression, making it impossible to determine whether its localisation is altered during an IFN response (Fig. 6.7H).




C
IRF3
PML (FLAG)
DAPI
Merge
PML I
ΔRBCC
+ Poly(I:C)
D
IRF3
PML (FLAG)
DAPI
Merge
PML I
ΔExon5
+ Poly(I:C)
E
IRF3
PML (FLAG)
DAPI
Merge
Cytoplasmic
PML I
+ Poly(I:C)


F
IRF3
PML (FLAG)
DAPI
Merge
FL PML II
+ Poly(I:C)
G
IRF3
PML (FLAG)
DAPI
Merge
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ΔRBCC
+ Poly(I:C)
H
IRF3
PML (FLAG)
DAPI
Merge
PML II
ΔExon5
+ Poly(I:C)

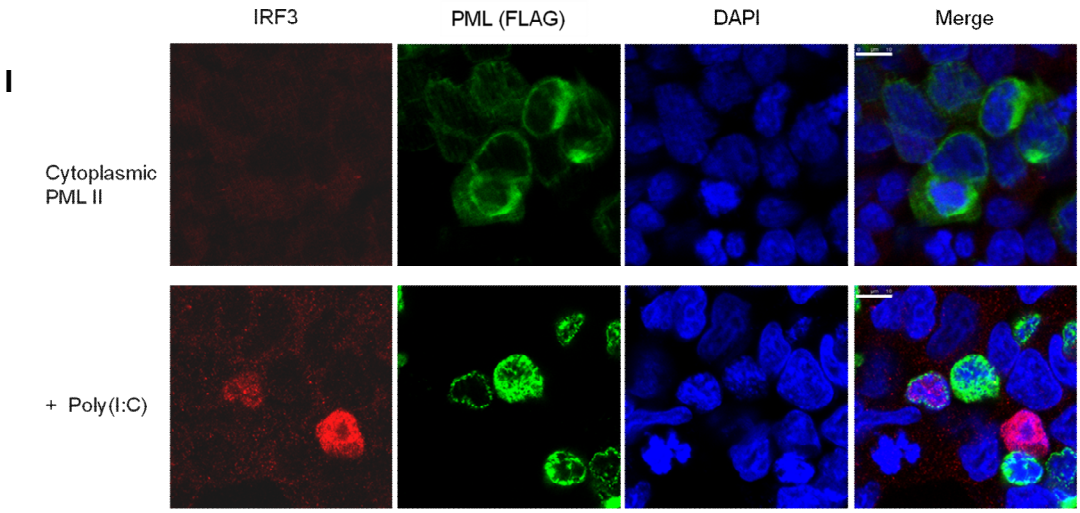


Figure 6.7. Investigating the sub-cellular localisation of various exogenous PML I and PML II species during an IFN response. HEK293 cells were transfected with empty vector pCI-neo or variants of either PML I or PML II. 16 hours later cells were mock transfected or transfected with poly (I:C), and then fixed and stained 4 hours later. The cells were then treated with primary antibodies to IRF3 and the FLAG epitope, and then treated with the appropriate fluorophore-conjugated secondary antibodies. Nuclei were visualised by DAPI staining. For each of A-I, the upper row of images represent control cells untreated with poly (I:C), while the lower row represent poly (I:C) treated cells. All images are single slices on the Z-axis, and are representative of the cell population. Scale bar - 10µM. **A.** pCI-neo control; **B.** pCI-neo FLAG-PML I; **C.** pCI-neo FLAG PML IΔRBCC; **D.** pCI-neo FLAG-PML IΔexon5; **E.** pCI-neo FLAG-PML I cytoplasmic; **F.** pCI-neo FLAG-PML II; **G.** pCI-neo FLAG-PML IIΔRBCC;. **H.** pCI-neo FLAG-PML IIΔExon5; **I.** pCI-neo FLAG-PML II cytoplasmic.

Cytoplasmic PML II displayed a distinct cytoplasmic localisation in untreated cells (Fig. 6.7I). Upon poly (I:C) treatment, however, cytoplasmic PML II localisation was altered as it displayed a nuclear localisation pattern. It must be noted though that this nuclear localisation also occurred in cells displaying cytoplasmic IRF3 localisation, suggesting that an induction of the IFN response was not responsible. Alternatively, more cells may be responding to poly (I:C) than are displaying prominent nuclear IRF3 staining (see below), in which case this location change could be relevant. As with PML IΔRBCC, PML IIΔRBCC displayed a diffuse nuclear localisation in both the presence and absence of poly (I:C) (Fig. 6.7G). This suggested that the localisation of PML II ΔRBCC is unchanged during an IFN

response, and therefore is not associated with its positive effect of IFNβ promoter activity.

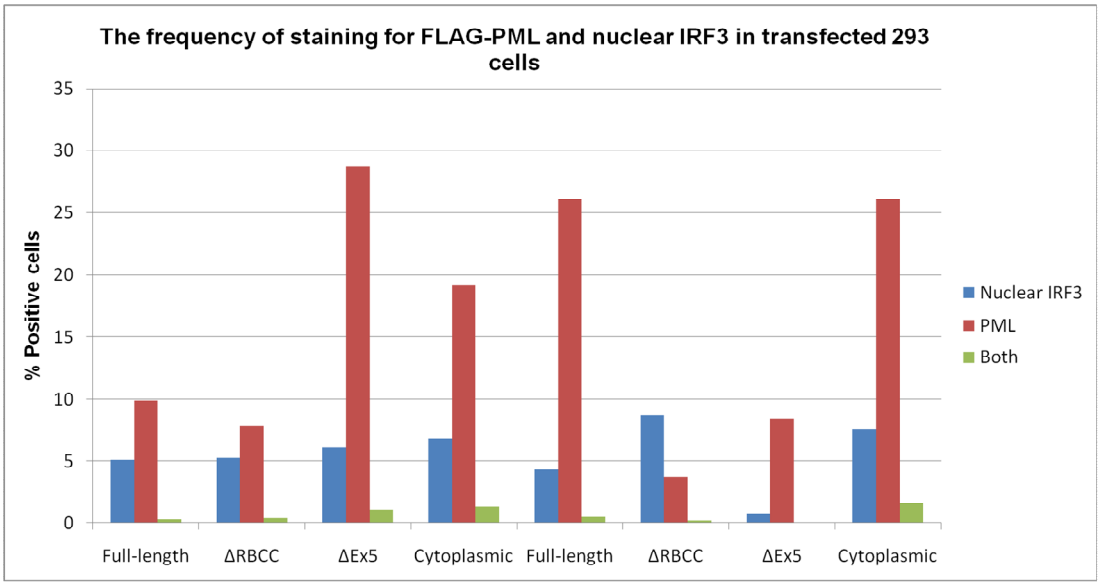
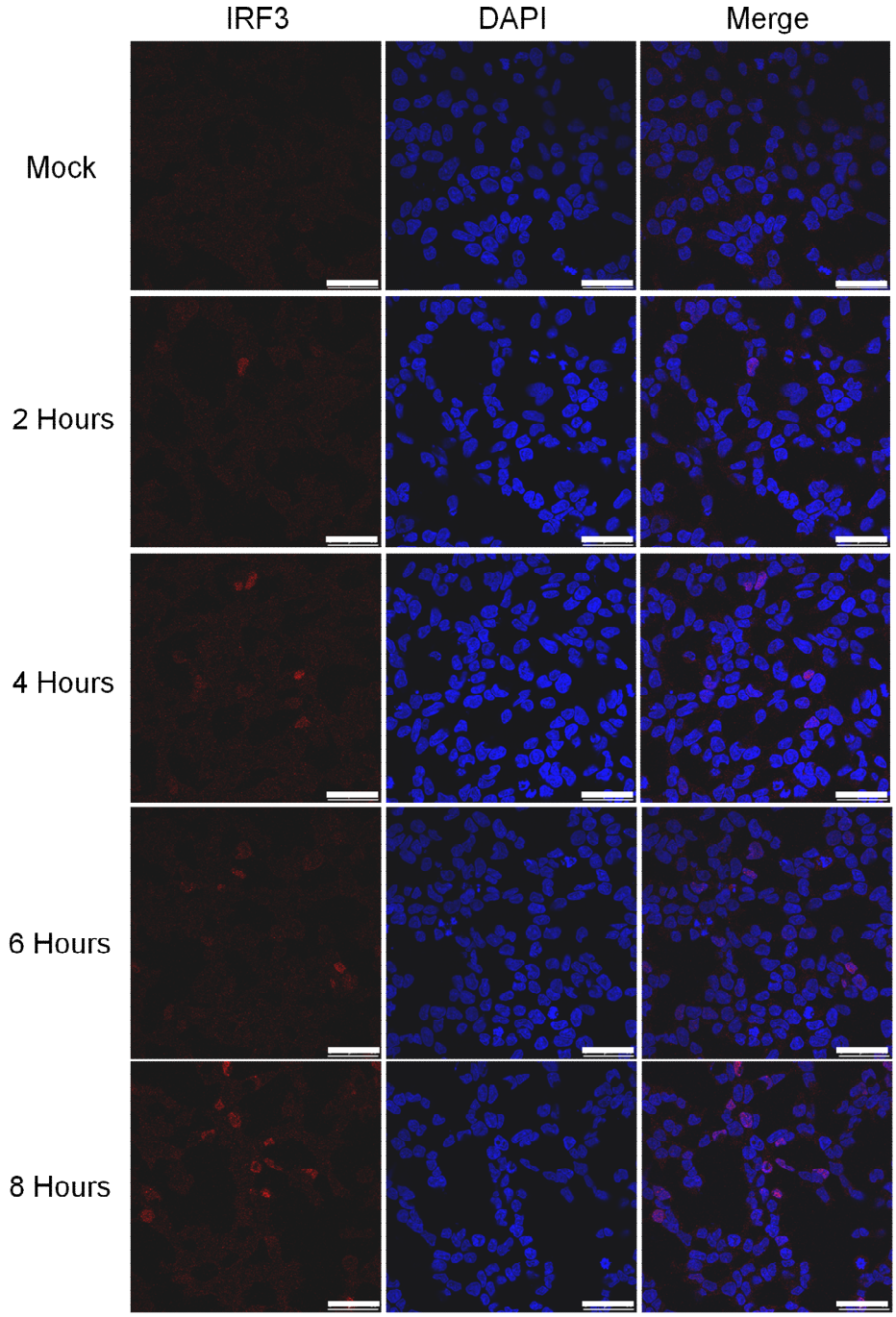


Fig. 6.8 The co-transfection efficiency of poly (I:C) with PML I and PML II plasmids. The images obtained from the experiment described in Fig. 6.7 were subjected to quantification analysis. Only cells treated with poly (I:C) were analysed. Cells from fields of view chosen at random were scored for individual and simultaneous nuclear IRF3 localisation and exogenous PML expression. Numbers are expressed as a percentage of the total number of cells present, as counted by DAPI-stained nuclei. For each plasmid, a minimum of 130 cells were counted in total.

The proportion of cells exhibiting both expression of FLAG-PML and a nuclear IRF3 localisation was very low, making analysis of the experiment very difficult; quantification revealed this was no more than 2% of cells (Fig. 6.8). The transfection efficiency of the PML expression plasmids varied between 5% and 30%, depending on the plasmid used (Fig. 6.8). Poly (I:C) transfection was found to be more consistent, as the proportion of cells displaying nuclear IRF typically varied between 5% and 10%. This was surprisingly lower than anticipated, and prompted a subsequent time-course analysis of IRF3 localisation in HEK293 cells following poly (I:C) treatment (Fig. 6.9).

A



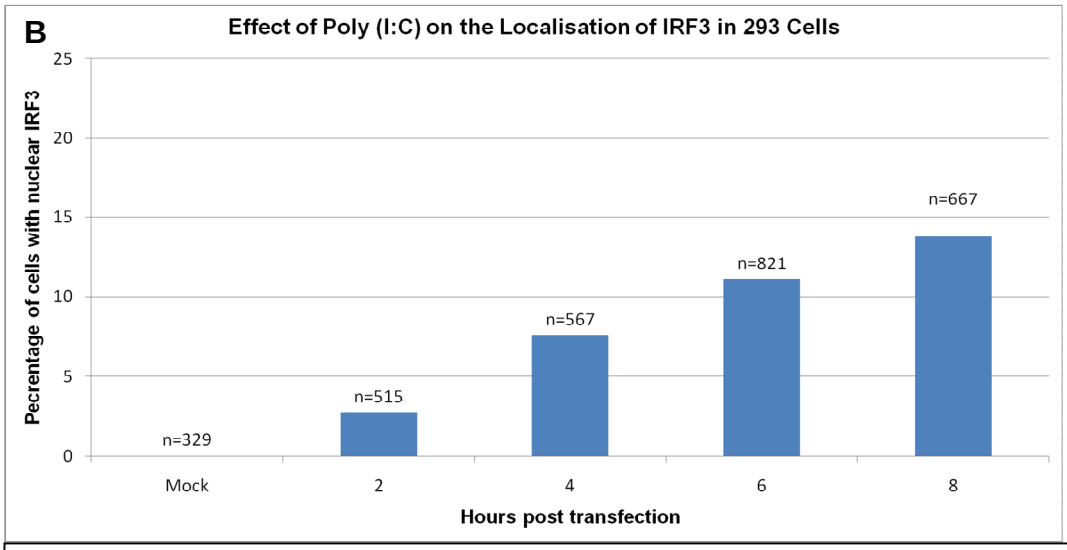


Figure 6.9 Time course of IRF3 nuclear localisation upon stimulation with poly (I:C). **A.** HEK293 cells were mock transfected or transfected with poly (I:C). The cells were fixed at the indicated time points post-transfection. Cells were probed with an anti-IRF3 antibody and appropriate fluorophore-conjugated secondary antibody, then stained with DAPI. Images represent a single slice through the Z-axis. Scale bar - 50µm. **B.** Cells from fields of view (**A.**) chosen at random were scored for nuclear localisation of IRF3, and are expressed as a percentage of the total number of cells counted (n).

Approximately 2.5% of cells display a nuclear IRF3 localisation after two hours of incubation with poly (I:C). As expected, this proportion increased upon longer incubation times, reaching 14% after 8 hours of incubation. Note that the proportion of 7.5% observed after 4 hours' incubation is similar to that observed in the experiment quantified in Fig. 6.8, which also used 4 hours incubation time. This provided evidence that the prior transfection of plasmid DNA, such as that used in Fig. 6.7 as well as in the reporter assays described in this chapter, does not interfere with the induction of the IFN response by poly (I:C). Also, the consistency between experiments indicated that this was a reliable figure which could be applied to the reporter assays described in this chapter, which utilised HEK293 cells and an 8 hour incubation time with poly (I:C). The figure of 14% after eight hours incubation with poly (I:C) was still lower than anticipated, given the propensity of HEK293 cells to support transfection. It must be noted, however, that this method involved the visual detection of IRF3 within the nucleus, and it is therefore possible that low levels of nuclear IRF3 accumulation would not be scored. This would result in an under-estimation of the percentage of cells transfected with poly (I:C).

6.7 Endogenous PML II localises to PML NBs in HEK293 cells

In the experiment described in Fig. 6.7, full-length, ΔRBCC and cytoplasmic PML II tended to localise to the periphery of the nuclear membrane, rather than in PML NBs. This was both unexpected and in contrast to that observed in PML I-transfected cells, which localised to PML NBs. In U2OS cells, exogenously-expressed PML II localised to PML NBs (Beech *et al*., 2005). It must be noted that a subset of cells with high-levels of exogenous over-expression of PML II displayed localisation patterns similar to that observed in Fig. 6.7 (K. Leppard, personal communication). However, in HEK293 cells, no cells displayed exogenously-expressed PML II association with PML NBs, which therefore prompted an investigation to determine the localisation of endogenous PML II in this cell line (Fig. 6.10).

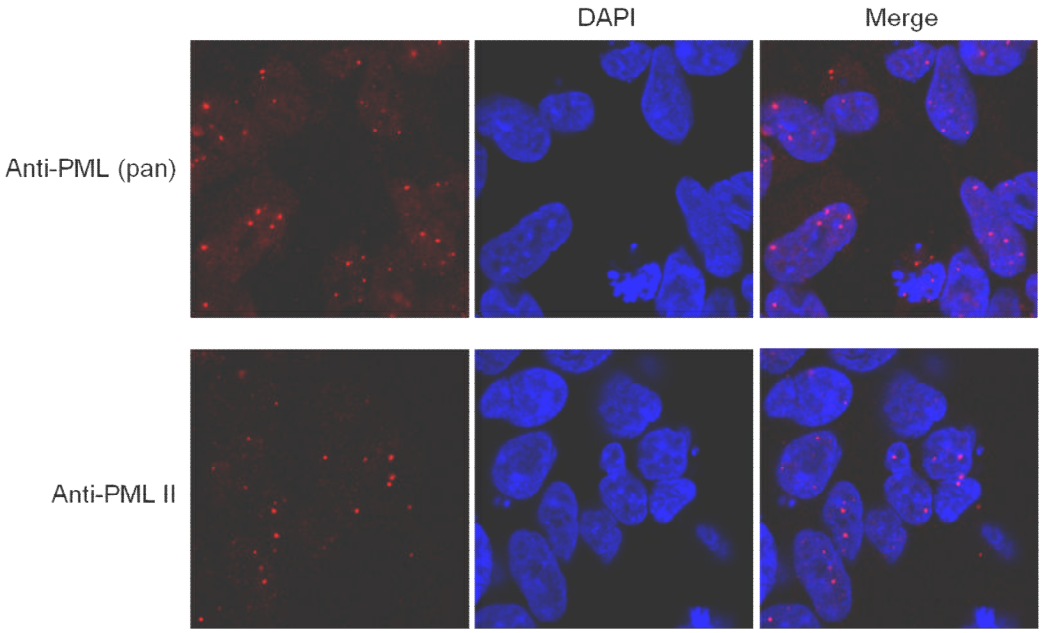


Figure 6.10 Endogenous PML II localises to PML NBs in HEK293 cells. HEK293 cells were fixed 24 hours after seeding and probed with pan-PML or anti-PML II antibodies, and an appropriate fluorophore-conjugated secondary antibody. Nuclei were visualised by DAPI staining. The images represent maximum projections of images made in the Z-axis.

Upon staining with a pan-PML antibody, HEK293 cells displayed normal PML NB structures. Staining with an anti-PML II antibody revealed that this isoform shared a similar localisation. There was no evidence of localisation to the nuclear membrane. These results therefore suggest that exogenously-expressed PML II variants possessing an RBCC domain display an atypical pattern of localisation in HEK293 cells, probably associated with the high levels of expression achieved in transient assays.

It was therefore clear that the localisation of PML IIΔRBCC during an IFN response remained unchanged, and therefore a change in localisation was unable to account for its positive effect on the activity of the IFNβ promoter.

6.8 Analysis of PML IIΔRBCC C-terminal mutants

It has previously been described that the Ad5 E4 Orf3 protein initiates rearrangement of PML NBs into tracks via a direct interaction with the C-terminus of PML II (Hoppe *et al*., 2006, Leppard *et al*., 2009). Furthermore, E4 Orf3 has been shown to be essential for efficient virus growth during an interferon response (Ullman *et al*., 2007), and to overcome an intrinsic antiviral activity of PML protein (Ullman & Hearing, 2008). Given the results just described which suggest a role for PML II in the activation of the IFNβ promoter, these findings raised the possibility that E4 Orf3 could interfere with this activity of PML II through its documented interaction with this isoform. In order to investigate this, four clones of PMLIIΔRBCC that each carry unique deletions in the carboxyl terminus, termed IIΔRΔ1-4, were utilised in IFNβ promoter reporter assays. Orf3 directly interacts with the region deleted in IIΔRΔ1, and will therefore only bind IIΔRΔ2-4 (Leppard *et al*., 2009).

The effect of each IIΔRΔ mutant was compared to 'wild-type' IIΔRBCC in the presence or absence of Orf3 (Fig. 6.11). Expression of PML IIΔRBCC and its mutants IIΔR1-4 was detected in all of the relevant assays (Fig. 6.11B-E), though IIΔR3 and IIΔR4 expression was always found to be considerably lower than that of

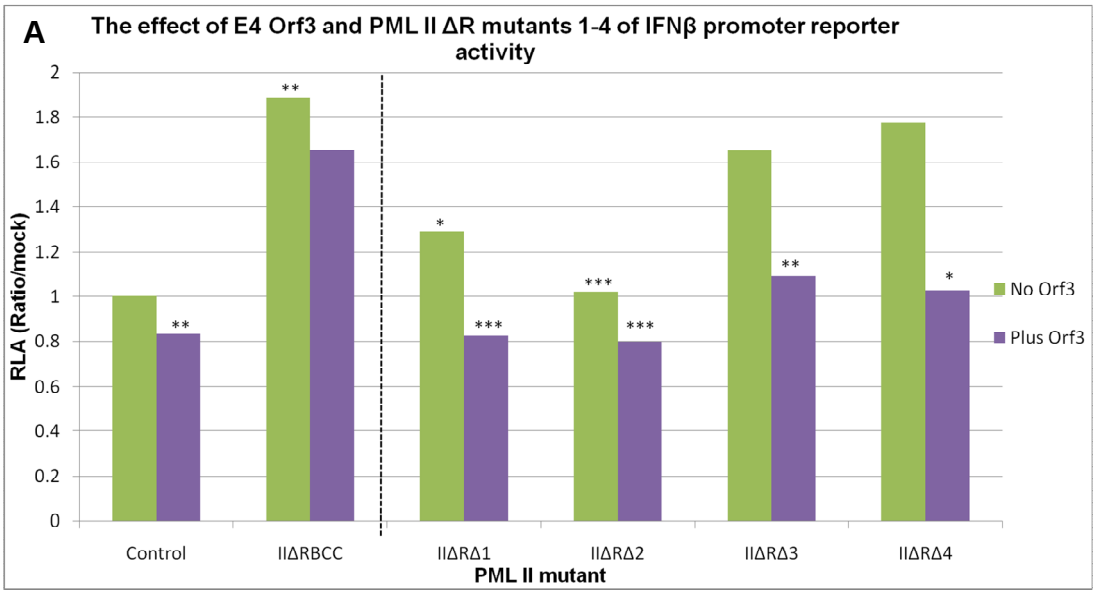
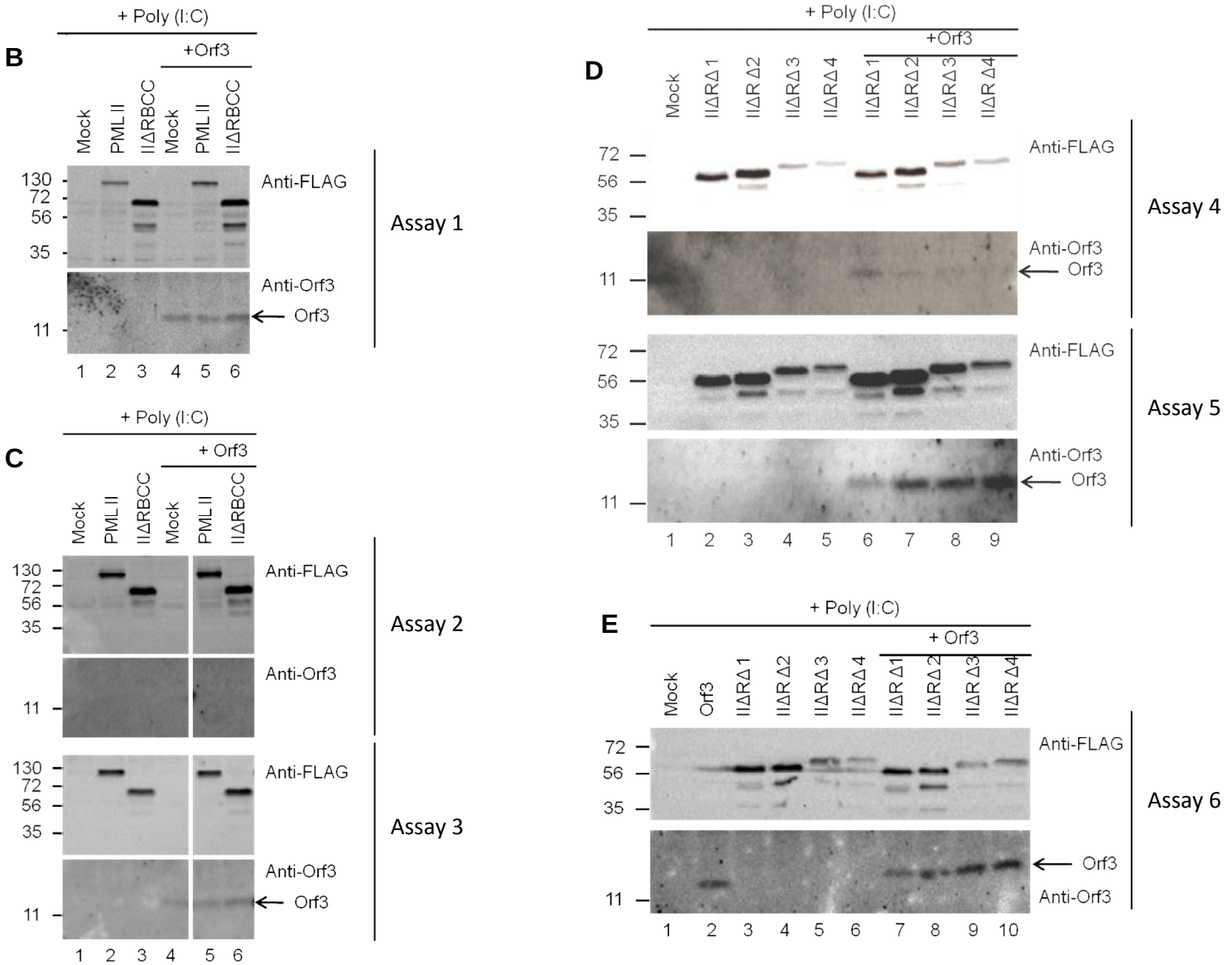


Figure 6.11. PML II mutants ΔRΔ1 and ΔRΔ2 are unable to enhance IFNβ promoter activity after stimulation with poly (I:C). **A**. 2.5 x 10^5 HEK293 cells were co-transfected in triplicate with 25ng pcDNA3.1-HisB::*lacZ*, 225ng pIFNβ-Luc and 125ng pcDNA3.1-*Orf3* alone or with pCI-neo FLAG-PML ΔRBCC II, pCI-neo FLAG-PMLII ΔRΔ1-4. All DNA mixes were made to a total of 500ng with pCI-neo. 16 hours later cells were transfected with 1µg poly (I:C). All cells were harvested 8 hours later and lysates were assayed for both luciferase and β-galactosidase activity. Luciferase activity was normalised to β-galactosidase activity to obtain the RLA, which was expressed as a ratio to that of the control. The data presented represent the activity of poly (I:C)-treated cells only, and are the average of at least three separate experiments. For the control+Orf3 and ΔRBCC PML II, Student's T-test was performed against the control –Orf3. ΔRBCC PML II+Orf3 was compared to ΔRBCC PML II-Orf3. For ΔR1-4 +/- Orf3, Student's T-test analysis was performed against IIΔRBCC +/- Orf3. * - P <0.05, ** - P <0.005, ***-P <0.001. Green bars – activity in the absence of Orf3. Purple bars – activity in the presence of Orf3. **B-E.** Replicate extracts from each assay used in **A.** were pooled together and prepared for SDS-PAGE by the addition of sample buffer. Samples were separated by 15% SDS-PAGE and subjected to western blotting. Blots were probed with either rabbit anti-FLAG or rat anti-Orf3 antibodies. Where appropriate, Orf3 expression is indicated with an arrow to the right of the blot. Protein sizes are indicated in kDa to the left of each blot.


B
+ Poly (I:C)
+Orf3
Mock
PML II
IIΔRBCC
Mock
PML II
IIΔRBCC
130
72
56
35
Anti-FLAG
Anti-Orf3
11
Orf3
1 2 3 4 5 6
Assay 1
C
+ Poly (I:C)
+ Orf3
Mock
PML II
IIΔRBCC
Mock
PML II
IIΔRBCC
130
72
56
35
Anti-FLAG
Anti-Orf3
11
Orf3
1 2 3 4 5 6
Assay 2
Assay 3
D
+ Poly (I:C)
+Orf3
Mock
IIΔRΔ1
IIΔRΔ2
IIΔRΔ3
IIΔRΔ4
IIΔRΔ1
IIΔRΔ2
IIΔRΔ3
IIΔRΔ4
72
56
35
Anti-FLAG
11
Anti-Orf3
Orf3
Assay 4
Assay 5
1 2 3 4 5 6 7 8 9
E
+ Poly (I:C)
+ Orf3
Mock
Orf3
IIΔRΔ1
IIΔRΔ2
IIΔRΔ3
IIΔRΔ4
IIΔRΔ1
IIΔRΔ2
IIΔRΔ3
IIΔRΔ4
72
56
35
Anti-FLAG
11
Orf3
Anti-Orf3
Assay 6
1 2 3 4 5 6 7 8 9 10

IIΔR1 and IIΔR2 (Fig. 6.11D and E). E4 Orf3 expression was found to be highly variable across the assays. This was a consequence of the lysis procedure, which was inefficient at solubilising all cellular material. However, the use of passive lysis buffer was necessary in order to perform luciferase assays. In later experiments, this was addressed by using sample lysis buffer on an additional replicate of each DNA treatment (Fig. 6.12B). Under conditions where Orf3 was not detected in the lysates used for reporter analysis, it was readily detected in a complete lysate, indicating adequate levels of Orf3 expression were occurring. Therefore, Orf3 expression across all these assays was assumed to be successful.

Consistent with earlier results, PML IIΔRBCC significantly enhanced IFNβ promoter activity in response to poly (I:C) to almost twice of that of the control (Fig. 6.7A). In contrast, Orf3 expression alone significantly inhibited IFNβ promoter activity during poly (I:C) stimulation, which suggested that Orf3 expression was successful and was acting to inhibit the IFN response pathway. However, Orf3 expression did not significantly reduce the potentiation due to PML IIΔRBCC, as IFNβ promoter activity when PML IIΔRBCC and Orf3 were expressed together remained comparable to that observed when IIΔRBCC was expressed in the absence of Orf3.

The effect of each of IIΔR1-4 was compared to that of 'wild-type' PML IIΔRBCC. In the absence of Orf3, only IIΔR3 and IIΔR4 enhanced IFNβ promoter activity to levels comparable with PML IIΔRBCC. Indeed, their effects did not significantly differ from that of the intact C-terminal domain. Upon IIΔRΔ1 and IIΔRΔ2 expression, however, IFNβ promoter activity was significantly lower compared to PML IIΔRBCC. Co-expression of Orf3 with IIΔR1-4 caused a significant decrease in IFNβ promoter activity, in contrast to the limited effect which it had on activity in the presence of PML IIΔRBCC. Orf3 expression with IIΔR1 and IIΔR2 reduced activity of the IFNβ promoter to levels similar to Orf3 alone, which was to be expected as these mutants alone did not enhance IFNβ promoter activity. Orf3 expression alongside IIΔR3 and IIΔR4 reduced IFNβ promoter activity to levels similar to that of the control in the absence of E4 Orf3.

6.9 E4 Orf3 inhibits the ability of PML IIΔRBCC to enhance the IFNβ promoter

The data presented in Fig. 6.7 suggested that Orf3 could inhibit the potentiating activity of PML IIΔRΔ3 and IIΔRΔ4 on the IFNβ promoter, though surprisingly PML IIΔRBCC activity was not significantly affected by the presence of E4 Orf3. Since the hypothesised mechanism of Orf3 action here is via its binding to the PML II C-terminal domain, one possible reason for this may be that the level of Orf3 expression achieved in these experiments was insufficient to inactivate the over-expressed PML. To test this possibility, the activity of PML IIΔRBCC on IFNβ promoter activation was analysed in the presence of increasing amounts of Orf3 (Fig. 6.12). Expression of PML IIΔRBCC was consistent across all samples, as determined by western blotting (Fig. 6.12). E4 Orf3 was previously found to be poorly solubilised in the buffer used for luciferase analysis (Fig. 6.11), and so one transfection replicate of each Orf3 plasmid input was lysed directly in sample buffer to allow accurate analysis of Orf3 expression levels. As expected, E4 Orf3 expression was detected at levels corresponding to the amount of input DNA (lanes 11-13).

During poly (I:C) stimulation, expression of PML IIΔRBCC enhanced reporter activity by ~1.5-fold, consistent with earlier experiments. Moreover this enhancement was reduced by expression of E4 Orf3 in a dose-responsive manner. At an Orf3:PML II DNA ratio of 3:1, IFNβ promoter activity was reduced to control levels. However, at a ratio of 5:1, the activity of the IFNβ promoter was reduced to less than 80% of the control. Therefore this suggested that E4 Orf3 expression can inhibit the stimulation of the IFNβ promoter caused by expression of PML IIΔRBCC.

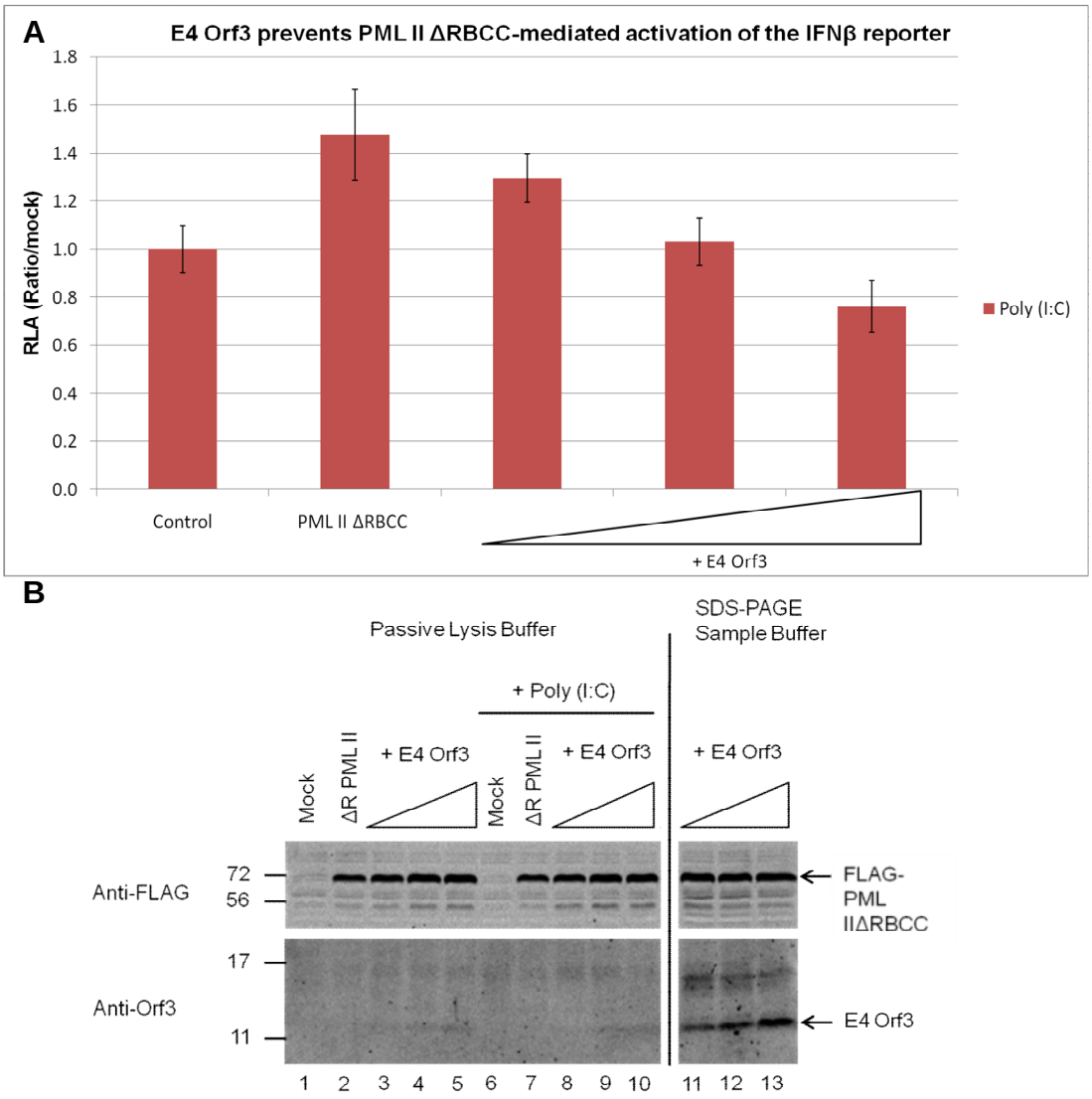
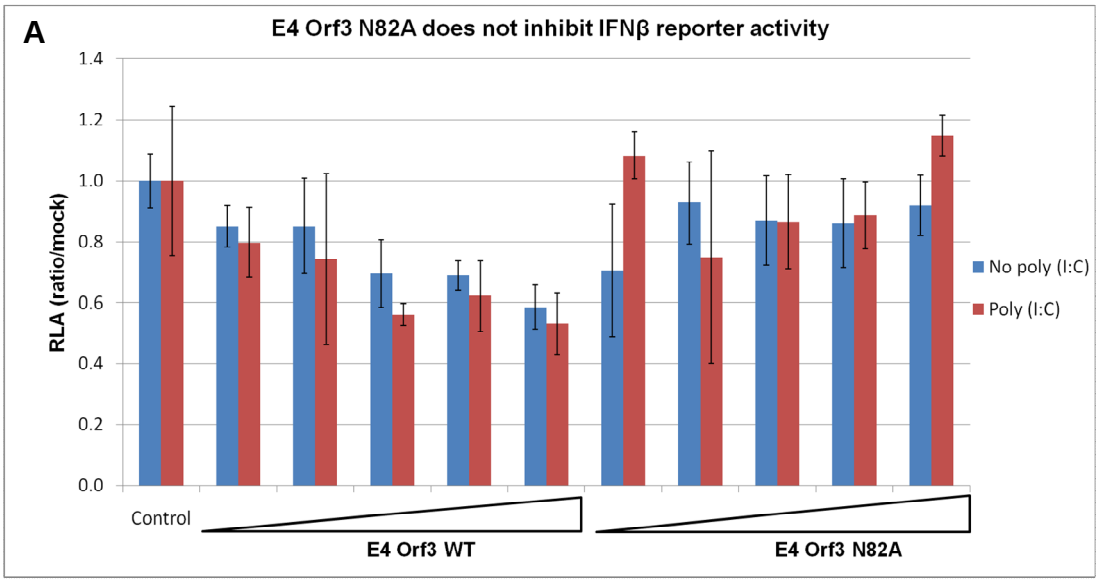


Figure 6.12. Expression of wild-type E4 Orf3 inhibits the ability of ΔRBCC PML II to activate the IFNβ reporter. A. HEK293 cells were co-transfected in triplicate with 25ng pcDNA3.1-HisB::*lacZ*, 225ng pIFNβ-Luc, 125ng pCI-neo FLAG-PML ΔRBCC II and 125ng, 375ng or 625ng pcDNA3.1-*Orf3*. Each transfection mix was made up to 1µg total DNA with the appropriate amount of pCI-neo. 16 hours later cells were mock-transfected or transfected with 1µg poly (I:C). 8 hours later all cells were harvested and the lysates were assayed for both luciferase and β-galactosidase activity. The RLA was calculated as before and expressed as a ratio to the RLA of control cells (pCI-neo). Error bars indicate the standard deviation from the mean of three replicate values. **B.** Replicate lysates from the assay represented in **A.** were pooled together. One replicate well was lysed directly in Sample Buffer. Lysates were separated by 8% (FLAG) and 15% (Orf3) SDS-PAGE and subjected to western blotting. The blots were probed with either a rabbit anti-FLAG or rat anti-E4 Orf3 antibody. Protein sizes are indicated in kDa to the left of each blot.

6.10 Expression of wild-type E4 Orf3, but not the N82A mutant, is sufficient to inhibit IFNβ promoter activity.

Expression of wild-type E4 Orf3 overcame the enhancing activity of PML IIΔRBCC on the IFNβ promoter, and at higher concentrations decreased promoter activity to less than that of the control (Fig. 6.12). This raises the possibility that E4 Orf3 might also inhibit IFNβ promoter activity in the absence of PML IIΔRBCC. One possible mechanism whereby Orf3 could achieve this is by interacting with, and inhibiting, endogenous PML II species that are required for normal IFNβ promoter function. Such a model would fit the observation that depletion of all PML II-related species by siRNA significantly reduced the responsiveness of the promoter to poly (I:C) (Fig. 6.3). To test this model, the abilities of wild-type E4 Orf3 and E4 Orf3 mutant N82A to modulate IFNβ promoter activity were compared in the absence of exogenous PML. N82A does not interact with PML II, and thus cannot rearrange PML NBs (Hoppe *et al*., 2006). Interestingly, N82A does not appear to possess the same antagonistic properties of E4 Orf3 on the IFN response (Ullman *et al*., 2007), suggesting that the interaction with PML II may be important for this particular function.

Expression of both wild-type and N82A E4 Orf3 increased in a dose-dependent manner (Fig. 6.13), and expression levels of the two proteins at equivalent DNA inputs were similar. Increasing expression of wild-type E4 Orf3 further reduced the low basal activity of the IFNβ promoter to approximately 60% of the control. Expression of the N82A mutant, however, did not exhibit any dose-dependent effects on IFNβ promoter activity. During stimulation with poly (I:C), expression of wild-type E4 Orf3 again inhibited the activity of the IFNβ promoter in a dose-responsive manner, with promoter activity reduced to 50% of the control at the highest amount of Orf3 used. N82A, despite similar expression levels to wild-type E4 Orf3, did not affect the activity of the stimulated promoter in a dose-dependent manner. IFNβ promoter activity in the presence of N82A remained broadly comparable with that of the control, suggesting that this mutant exerts no effects on the promoter.



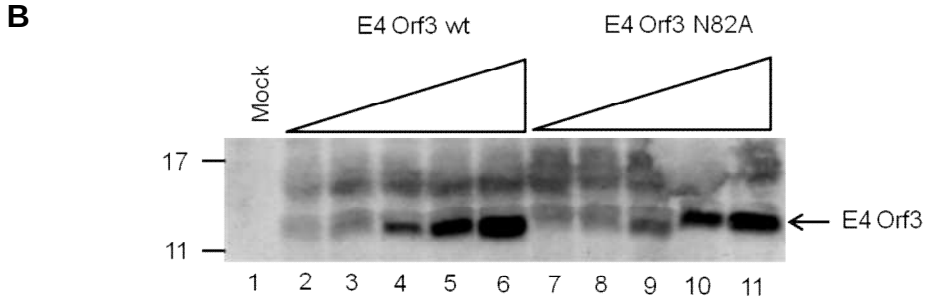


Figure 6.13. Wild-type E4 Orf3, but not the N82A mutant, inhibits the activity of the IFNβ reporter. A. 2.5 x 10^5 HEK293 cells were co-transfected in triplicate with 25ng pcDNA3.1-HisB::*lacZ*, 225ng pIFNβ-Luc and 150ng, 300ng, 450ng, 600ng or 750ng pcDNA3.1-*Orf3*, or pcDNA3.1-*N82A*. Each transfection mix was made up to 1µg total DNA with the appropriate amount of pCI-neo. 16 hours later cells were mock-transfected or transfected with 1µg poly (I:C). 8 hours later all cells were harvested and the lysates were assayed for both luciferase and β-galactosidase activity. The RLA was calculated as before and expressed as a ratio to the RLA of control cells (pCI-neo). Error bars indicate the standard deviation from the mean of three replicate values. **B.** One replicate well from the assay represented in **A.** was lysed directly in Sample Buffer. Lysates were separated by 15% SDS-PAGE and subjected to western blotting. The blot was probed with a rat anti-E4 Orf3 antibody. Protein sizes are indicated in kDa to the left of the blot.

6.11 Discussion

The data presented in this chapter provide evidence that various PML isoforms play roles in modulating the activity of the IFNβ promoter. A somewhat surprising finding was that, depending on the isoform being expressed, IFNβ promoter activity could be enhanced or inhibited by over-expression of PML protein. This was apparent when full-length PML isoforms were expressed (Fig. 6.1). Expression of PML IV significantly enhanced the basal activity of the promoter. PML IV has been shown to bind the tumour suppressor p53 via a direct interaction in its C-terminus and subsequently to recruit it to PML NBs, where activating modifications of p53 are promoted (Bischof *et al*., 2002, Fogal *et al*., 2000). p53 is also an IFN-inducible gene, and has been proposed to exert antiviral activities of its own via the IFN pathway (Porta *et al*., 2004). It is possible therefore that p53 recruitment to PML NBs by PML IV could support the normal induction of the IFNβ promoter. This could explain why PML IVΔRBCC failed to exert a similar effect, as it is unable to localise to PML NBs and, unlike full-length PML IV, cannot activate a p53-responsive promoter (K. Leppard, personal communication). In contrast to these effects on basal IFNβ promoter activity, neither full-length nor PML IVΔRBCC enhanced IFNβ promoter activity during stimulation by poly (I:C), suggesting that the capability of this isoform to the enhance IFNβ promoter activity is saturated under a normal IFN response. It would be interesting to ascertain whether depletion of PML IV by siRNA can reciprocally inhibit IFNβ promoter activity.

One must use caution when considering changes in the basal values of IFNβ promoter reporter activity brought about by over-expression of exogenous proteins. By implication, these values are very low in cells not stimulated by poly (I:C), and hence only slight variations in activity will cause a large change in relative activity which may not be genuine effects. For instance, in Figs. 6.1 and 6.2 expression of PML I and PML IΔRBCC respectively had no effect on basal IFNβ reporter activity, though in Fig. 6.6A expression of these proteins led to small (<1.4 fold) but significant increases in activity. Such discrepancies highlight the inconsistency of basal reporter activity, and indicate that interpretation of any changes may be somewhat unreliable. It is far more prudent to concentrate on IFNβ reporter activity during poly (I:C) stimulation, where reporter values are much larger and hence any

variations are more likely to be significant. It should be noted that in Figs. 6.1, 6.2 and 6.6, over-expression of PML I and PML IΔRBCC had no effect on IFNβ promoter activity during stimulation.

Expression of both full-length and ΔRBCC versions of PML V and PML VI significantly inhibited IFNβ promoter activity during activation by poly (I:C). The fact that full-length and ΔRBCC forms were equivalent in this respect suggests that the ability to localise to PML NBs is dispensable for these actions. One model to explain these activities is that the C-terminal domains of these isoforms can bind and sequester factors involved in activating the IFNβ promoter, and hence limit the activation observed when they are over-expressed. Depletion of PML V by siRNA did not lead to a corresponding increase in IFNβ promoter activity, supporting the notion that over-expression of PML V sequesters a factor involved in IFNβ promoter induction that is not limiting for IFNβ activation under normal levels of PML expression.

Of all the PML isoforms tested, only over-expression of PML II-related species was sufficient to enhance the activity of the IFNβ promoter during poly (I:C) stimulation. Over-expression of PML IIΔRBCC, but not-full-length PML II, caused a significant increase in promoter activity. Since no naturally occurring species of PML II lacking the RBCC domain have been described, the physiological significance of this result is hard to determine. One possibility is that full-length PML II negatively regulates the activity of an activator of the IFNβ promoter, perhaps binding it via its C-terminus and with inhibition requiring the RBCC domain, and PMLIIΔRBCC merely acts as a dominant negative. If this were the case, one would expect depletion of endogenous PML II to enhance IFNβ promoter activity. However, upon depletion with siPML II siRNA, a significant and substantial decrease in promoter activity was observed during poly (I:C) stimulation. This provides evidence that an endogenous PML II-related species plays a role in activating the IFNβ response, and suggests that PMLIIΔRBCC may not act as a dominant negative but instead plays an active role in regulating IFNβ promoter activity that mimics the properties of a natural PML II variant. It should be noted, however, that the isoform-specific siRNA targets all mRNAs containing the target exon, regardless of whether this exon is ultimately translated. For example, a PML II mRNA containing a stop codon

upstream of exon 7b could not produce a PML II-related protein, yet would still be depleted by PML II siRNA.

The exact nature of the PML II-related species that is required for normal IFNβ promoter activity remains unknown. Expression of either cytoplasmic or PML II Δexon 5, two known splice variants of full-length PML II (Jensen, 2001), could not replicate the activity of PML IIΔRBCC during poly (I:C) treatment. In contrast to other constructs, PML IIΔRBCC exhibits a diffuse nuclear localisation, possibly allowing it to freely interact with soluble nucleoplasmic factors. The localisation of PML IIΔRBCC was unaltered during poly (I:C) stimulation, ruling out the possibility that the positive activity of this molecule on IFNβ reporter activity is due to an increased ability to mobilise during an IFN response.

The localisation pattern of full-length and PML II Δexon5 were remarkably similar, in that they all tended to associate with the nuclear periphery. This was unexpected, as exogenously-expressed full-length PML II has previously been confirmed to associate with PML NBs (Beech *et al*., 2005), raising the possibility that PML II localisation in HEK293 cells was unusual. Analysis of endogenous PML II in this cell type, however, revealed it to be largely confined to PML NBs similar to other cell types. It is therefore possible that the unusual localisation of exogenous full-length and PML IIΔexon5 in HEK293 cells is due to an artefact of the experimental method, most likely the level of over-expression. The precise mechanism underlying this remains elusive, as exogenously-expressed full-length PML I and PML IΔexon5 were still able to form PML NBs. Another unexpected result was the apparent nuclear localisation of the 'cytoplasmic' PML II upon poly (I:C) stimulation, which was also observed in HEp-2 cells (see Appendix), though in this case cytoplasmic PML II clearly localised to PML NBs and the cells were not treated with poly (I:C). A similar phenomenon was seen with a cytoplasmic PML III construct (see Appendix), but not with cytoplasmic constructs of PML I, IV and V. This suggests that PML II and PML III possess additional sequences within their unique C-terminal domains that can promote nuclear accumulation, at least in some cell types.

Pan-isoform depletion of PML proteins using siRNA targeting exons 3 and 5 did not lead to a decrease in IFNβ activity after stimulation with poly (I:C), in contrast to

what was observed upon PML II-depletion. There are two possible explanations why this is the case. Knockdown of total PML protein by targeting exon 3 or exon 5 may deplete PMLs that perform both inhibitory and positive roles during promoter activation, with the net effect being no difference in promoter activity. This is supported by the data presented in this chapter that specific PML proteins do indeed exert both positive and negative effects on the promoter. Alternatively, the PML II species responsible for enhancing IFNβ promoter activity may lack both exons 3 and 5 and therefore evade knock-down. Although there is no direct evidence for such a truncated variant of PML II, anti-PML II anti-serum detects many lower-molecular weight bands by western blot (Fig. 4.2), suggesting that such molecules may exist. However, further experimentation would be required to determine that this is the case.

Examination of four PML II ΔRBCC deletion mutants found that only two of these constructs were able to activate the IFNβ promoter. Neither IIΔRΔ1 nor IIΔRΔ2 could significantly enhance promoter activity, whereas mutants IIΔRΔ3 and IIΔRΔ4 displayed similar activity to that of the 'wild-type' PML IIΔRBCC. This suggests that the regions deleted in IIΔRΔ1 and IIΔRΔ2 are responsible for enhancing the activity of the promoter. Since the IIΔR1 deletion covers the region that serves as a target for E4 Orf3 binding (Leppard *et al*., 2009), this provides a possible mechanism whereby Orf3 may inhibit the activity of any PML II-related species by direct binding competition with the natural interaction partner of this region of PML II. However, E4 Orf3 is able to bind the IIΔRΔ2 mutant, yet this construct also lacks the ability to enhance IFNβ promoter activity. The region absent in the IIΔRΔ2 mutant interacts with at least one as yet unidentified protein (K. Leppard, unpublished observations), which could possibly be required to enhance IFNβ promoter activity. Further work would be required to identify the precise regions absent in IIΔRΔ1 and IIΔRΔ2 that are required for enhancing IFNβ promoter activity.

When cells were transfected with equivalent amounts of PML IIΔRBCC and Orf3 DNA, Orf3 expression was too low to significantly overcome the PML IIΔRBCC-mediated enhancement of IFNβ activity (Fig. 6.11). However, the same amount of Orf3 was able to successfully inhibit the activating ability of both the IIΔRΔ3 and

IIΔRΔ4 deletions. This may be explained by two observations. First, although expression of IIΔRΔ3 and IIΔRΔ4 could not be directly compared to IIΔRBCC expression, they were consistently expressed at much lower levels than IIΔRΔ1 and IIΔRΔ2 (Fig. 6.11D-E). Therefore, their biological effects may have been more readily overcome by limited amounts of Orf3 expression than those of highly-expressed IIΔRBCC. Second, both IIΔRΔ3 and IIΔRΔ4 were shown to co-precipitate Orf3 more strongly than IIΔRBCC, IIΔRΔ1 or IIΔRΔ2 (Leppard *et al*., 2009). This suggests that these constructs may more readily bind Orf3 and therefore be inhibited by it more effectively than IIΔRBCC.

Many viruses have been demonstrated to encode proteins that mitigate the interferon response (Haller *et al*., 2006, Levy & Garcia-Sastre, 2001, Randall & Goodbourn, 2008). In some cases, the proteins responsible have also been demonstrated to alter PML protein and PML NBs (Everett *et al*., 1998, Mossman *et al*., 2000, Ullman *et al*., 2007). The Ad5 E4 Orf3 protein has been implicated in overcoming the cellular IFN response, and also disrupts PML NBs through a direct interaction with PML II (Hoppe *et al*., 2006, Leppard *et al*., 2009, Ullman & Hearing, 2008, Ullman *et al*., 2007). Here it has been demonstrated that exogenous expression of wild-type E4 Orf3 inhibits IFNβ promoter activity in a dose-responsive manner. Furthermore, expression of an Orf3 mutant unable to interact with PML II, N82A, does not exhibit similar activity. Expression of Orf3 is also sufficient to overcome the enhancement in IFNβ promoter activity upon expression of PML IIΔRBCC. This suggests that the Orf3-PML II interaction, and subsequent PML NB disruption, may be a viral mechanism employed to negate the positive influence on the IFN response of any PML II-related species present within the cell. However, further experimentation would be required to confirm this, as the Orf3 N82A mutant also lacks functions other than the PML-binding capability of wild-type Orf3 (Stracker *et al*., 2002).

Chapter 7
Final Discussion

It has long been established that Ad5 causes disruption to PML NBs by redistributing PML protein into nuclear 'tracks' (Carvalho *et al*., 1995). This phenomenon is dependent on the direct interaction between the viral E4 Orf3 protein and the carboxyl terminal domain of PML II (Hoppe *et al*., 2006). Ad5 infection also results in the production of a novel species of PML protein, termed PML-A throughout this investigation (Leppard & Everett, 1999). Like PML NB redistribution, the production of PML-A is dependent on the viral E4 Orf3 protein. PML proteins have been extensively implicated in exerting antiviral effects, though the mechanism by which they achieve this is incompletely understood. Furthermore, very little is known about the roles of individual PML isoforms in established antiviral pathways. This study sought to understand further the relationship between Ad5 and PML protein by examining the the changes in the PML protein population induced upon infection. Also, the roles of individual PML isoforms in the type I IFN response were investigated, with the ultimate goal of linking Ad5-induced changes in the PML protein population to the modulation of the IFN response.

It was hypothesised that PML-A would be related to one of the known PML protein isoforms, though initial attempts at identifying PML-A using isoform-specific antibodies proved to be unsuccessful. However, PML-A was successfully depleted by siRNA targeting PML isoform II, indicating that it shares the same C-terminal domain as this isoform. This was not entirely unexpected because, as described above, E4 Orf3 directly interacts with the C-terminal domain of PML II (Hoppe *et al*., 2006, Leppard *et al*., 2009). The significance of PML-A and its role in Ad5 infection remains unclear.

E4 Orf3 is responsible for both the production of PML-A and the rearrangement of PML NBs, with both phenomena involving PML II. This initially suggested that these observations could be directly related and possibly dependent on one another. However, over-expression of E4 Orf3, even at relatively high levels, did not result in the production of PML-A in HEK293 nor HEp-2 cells (Fig. 3.8A). In contrast, previous studies have demonstrated that E4 Orf3 expression alone is sufficient to induce rearrangement of PML NBs (Carvalho *et al*., 1995, Hoppe *et al*., 2006).

Taken together, these observations indicate that the production of PML-A may require a viral or cellular infection-specific factor in addition to E4 Orf3.

As well as its depletion by siRNA targeting PML II, PML-A was also successfully depleted independently by siRNAs targeting PML exon 3 or exon 5. These observations indicate that PML-A is derived from an mRNA that possesses all of these exons, showing it to be related to full-length PML II. Indeed, by SDS-PAGE, PML-A migrates to a position that corresponds to a molecular weight that is slightly larger than endogenous PML II. The most likely explanation for this size discrepancy is post-translational modification of PML II that is induced upon infection with an E4 Orf3-competent Ad5, leading to the accumulation of PML-A.

As yet, there are no known post-translational modifications to PML that are isoform-specific. However, PML-A shows a distinct similarity in size to a phosphorylated, mitosis-specific form of PML that was described by Everett and colleagues (Everett *et al.*, 1999), prompting the consideration of PML-A as a phosphorylated form of PML II. The possibility that PML-A is a phosphorylated variant of PML II led to the question as to whether additional viral proteins are involved in its production. However, upon co-expression of E4 Orf3 with E4 Orf4, a protein known to affect cellular phosphatase activity, no changes in the PML population were detected. Therefore Orf4 is unlikely to be the cooperating factor with Orf3 responsible for the production of PML-A, and the nature of this factor remains unknown. It is possible that alternative viral proteins which were not examined in this study could be jointly responsible with E4 Orf3 for the production of PML-A. Alternatively, the additional factor required for PML-A may instead be a cellular protein that is induced or activated during viral infection.

The possibility that alternative post-translational modifications, such as sumoylation, may play a role in PML-A production cannot be discounted. For instance, infection with wild-type Ad5 leads to the loss of higher molecular weight, sumoylated forms of PML (Leppard & Everett, 1999). This activity, like the presence of PML-A, positively correlates with the presence of E4 Orf3. The appearance of PML-A has been shown to precede the loss of sumoylated PMLs, suggesting that it may be the cause of this phenomenon (Leppard & Everett, 1999). In this study, U2OS cells, which are incapable of supporting the production of PML-A, display no such loss of

sumoylated PML species (Fig. 3.1). This observation further suggests that the loss of sumoylated PML and the production of PML-A may be directly related.

The mechanism whereby this link between PML-A and desumoylation could be achieved is unclear, though there are several described examples where phosphorylation can regulate the sumoylation status of a PML protein. For instance, early studies showed that treatment with phosphatase inhibitors led to a decrease in sumoylated PML species (Muller *et al*., 1998), suggesting that phosphorylation may inhibit sumoylation. Interestingly, the appearance of the mitosis-specific PML species also correlates with the loss of higher molecular weight PML species (Everett *et al*., 1999). These observations bear striking similarities to the circumstances under which PML-A occurs, and may imply that it can also be produced in uninfected cells, though only under certain circumstances. It is tempting to speculate that E4 Orf3 acts in conjunction with an additional protein to hijack a cellular pathway, perhaps the same one responsible for the mitosis-specific species of PML, which results in a phosphorylation on PML II which subsequently initiates desumoylation of PML proteins. This model would fit with previous observations, as PML-A is produced prior to the loss of sumoylated PML species (Leppard & Everett, 1999). It is unclear, however, how phosphorylation of PML II alone could mediate desumoylation of other PML isoforms.

Alongside PML-A, two other infection-specific PML species were detected (PML-B and PML-C). These species were previously unreported and, unlike PML-A, were produced upon infection with either wild-type or E4 Orf3-deficient Ad5, indicating that the production of PML-B and PML-C does not require E4 Orf3. Both of these PML forms are produced after the onset of DNA replication, suggesting that a viral late protein is responsible for their production. Over-expression of pIX, a viral protein previously been shown to interact with PML at late time points of infection (Rosa-Calatrava, 2003), or the L4 22K and 33K proteins, which regulate late mRNA formation post-transcriptionally, did not lead to any alterations in the PML population. This suggests that these proteins are not responsible for the production of PML-B and -C, though it cannot be discounted that one of them may act in conjunction with other viral proteins to produce PML-B and PML-C. However, as with PML-A, the factor responsible for the production of PML-B and PML-C may also be a cellular protein that is induced by viral infection.

The precise nature of PML-B and PML-C remains elusive. Both are depleted by siRNA targeting exon 3 of PML, but not by siRNA targeting exon 5. This is in contrast to PML-A, which was depleted by both siRNAs. This indicates that these proteins are encoded by mRNAs that lack exon 5. PML-B was not detected by any PML isoform-specific antibodies, and could not be depleted with siRNA targeting PML I. RT-PCR analysis of PML I and PML II mRNA did not reveal any infection-specific mRNA that could account for PML-B. It is possible that PML-B may represent a splice variant or an infection-specific post-translational modification of one of PML III-VI.

An infection-specific band co-migrating with PML-C was detected using an anti-PML I antibody, and this protein was correspondingly depleted by siRNA targeting PML I. This strongly indicates PML-C to be a PML I-related molecule, though its low molecular weight and sub-cellular localisation indicate it to be a cytoplasmic variant of PML I. However, RT-PCR analysis of mRNA from Ad5-infected cells did not yield any infection-specific product that was PML I-related. This suggests that PML-C may not be an infection-specific splice variant of PML I but instead may represent a novel Ad5-induced post-translational modification of an existing PML I splice variant.

In order to understand the functions of PML-A, -B and –C it will first be necessary to identify and characterise these species in terms of their PML exon composition as well as any post-translational modifications that distinguish them from the rest of the PML population. Once understood, these species may then be artificially recreated and their roles in the cell may be elucidated. For instance, in the case of PML-A, mass spectrometry could be used to identify the post-translational modification that is likely to be responsible for its appearance. This, however, would require it to be successfully identified out the entire cellular protein content, and given the variable levels of PML-A production observed during the course of this study, this approach could be problematic. Ideally, targeted mutations to residues within PML protein that are known to be post-translationally modified could be used to identify the modification of PML II that is responsible for PML-A. Again, this approach would not be without difficulty, as the post-translational modification responsible for PML-A could not be replicated on exogenously-expressed PML II, an essential prerequisite for such an approach (Fig. 4.3).

For PML-B and PML-C, a more thorough analysis of PML mRNA splice variants during infection may reveal the nature of these proteins. The RT-PCR analysis performed in this study focused on PML I and PML II-related mRNA only, so it remains possible, for instance, that PML-B may be derived from a splice variant of one of PMLs III-V. PML-C on the other hand, is related to PML I though its exact composition remains unknown. More detailed analysis of PML I splice variants during infection, perhaps incorporating more sensitive methods of specific PCR product detection, may reveal an infection-specific splice variant mRNA responsible for PML-C. If RT-PCR analysis yields no such infection-specific PML mRNAs, then mass spectrometry could reveal the nature of PML-B and PML-C. Both of these PML species were relatively strongly expressed and, especially in the case of PML-C, are likely to be more soluble than PML-A. This could allow the identification of these proteins by this methodology somewhat more easily than would be the case for PML-A, which was the least abundant of the infection-specific species.

As mentioned above, PML proteins have been proposed to possess antiviral activities, though the nature of their action remains largely unknown. This study sought to shed light on this issue by investigating the effects of altering the balance of individual PML isoforms during a type I interferon response. The data generated suggest that the role of PML proteins in type I interferon signalling is complex, as IFNβ promoter activity was decreased or enhanced depending on the PML isoform being altered. For instance, over-expression of PML V and PML VI was sufficient to significantly decrease the activity of the IFNβ promoter after stimulation with the dsRNA analogue poly (I:C). On the other hand, expression of PML IV led to a significant 2-fold increase in basal reporter activity. However, out of all of the PML isoform constructs tested, only expression of PML IIΔRBCC was found to significantly enhance IFNβ promoter reporter activity during stimulation with poly (I:C). This was in contrast to expression of full-length PML II, which had no significant effect on reporter activity. It may be argued that PML IIΔRBCC represents an unnatural protein, and that its positive effect on IFNβ promoter reporter activity is due to a non-specific cellular stress response to the presence of a misfolded or damaged protein. However, ΔRBCC variants of PML I, III-VI did not

display such an effect, and in the case of PML VΔRBCC and VIΔRBCC, actually inhibited IFNβ promoter activity, making the above scenario unlikely.

An alternative explanation may be that PML IIΔRBCC acts as a dominant negative over full-length PML II, inhibiting the normal function of endogenous protein. For instance, the lack of the RBCC domain may render PMLIIΔRBCC biochemically inactive, but free to interact with any proteins that normally bind to the C-terminal domain of PML II. In such a scenario, over-expressed PMLIIΔRBCC could out-compete full-length PML II in binding proteins that are normally regulated by this isoform, resulting in a loss of regulation. If, for example, such a protein was a regulator of the type I IFN response, over-expression of PML IIΔRBCC could conceivably alter the activity of the IFNβ promoter. On the other hand, over-expression of full-length PML II would not necessarily have a significant effect, as under normal conditions this supposed regulatory mechanism may already be saturated. In order to determine the nature of PML IIΔRBCC-mediated enhancement, a construct of this molecule could be made that was resistant to knockdown by PML II siRNA. If PML IIΔRBCC possesses its own activating ability, then the siRNA-resistant mutant would still enhance promoter activity in cells depleted of endogenous PML II. However, if PML IIΔRBCC acts as a dominant negative then its ability to enhance IFNβ promoter activity in such cells would be diminished.

The study of PML IIΔRBCC-mediated enhancement of IFNβ promoter activity was complemented through protein depletion studies. Depletion with siRNA targeting exon 7b, which encodes the PML II-specific C-terminal domain, led to a considerable decrease in IFNβ promoter activity upon stimulation with poly (I:C) that was not observed during depletion of PML I or PML V. This provides direct evidence of a role for an endogenous PML II-related protein in type I IFN signalling, though the nature of the protein responsible remains unclear. Depletion of total endogenous PML with siRNAs targeting exon 3 or exon 5 did not lead to any such decrease in IFNβ activity. It is possible that, given the apparently opposing effects of PML II, PML V and VI on IFNβ activity, pan-isoform PML knock-down leads to no net effect on IFNβ promoter activity. Alternatively, the PML II-related species responsible for enhancing IFNβ promoter activity may lack both exons 3 and 5. It must be noted, however, that no such variant of any PML isoform has been recorded.

Like full-length PML II, expression of internally spliced PML II variants known to occur naturally (PML IIΔexon5 and cytoplasmic PML II) did not replicate the enhancing effect of PML IIΔRBCC on IFNβ promoter activity. This suggests that none of these variants of PML II are responsible for its positive effect on IFNβ promoter activity. However, exogenously expressed full-length PML II and PML IIΔexon5 displayed an atypical localisation pattern in HEK293 cells, when compared to that of endogenous PML II. Therefore it cannot be discounted that, in HEK293 cells at least, exogenously-expressed PML II does not function in the same way as endogenous PML II. In such a case, any effect of endogenous PML II on the activity of the IFNβ promoter may not be replicated by expression of exogenous PML II. This offers a possible explanation for why expression of full-length PML II did not enhance IFNβ promoter activity in HEK293 cells. It would be interesting to compare the effect of PML II expression on IFNβ promoter activity in alternative cell types where the localisation of exogenous PML II mirrors that of endogenous PML II.

Depletion of the total PML content with siRNA targeting exon 3 or exon 5 led to a decrease in PML protein expression, yet had no significant effect on stimulated IFNβ reporter activity. Although not demonstrated in this investigation, previous studies have demonstrated that knock-down of the cellular PML content leads to a decrease in the size and number of PML NBs (Ullman & Hearing, 2008). Thus, the absence of an effect of PML depletion on IFNβ promoter activity suggests that the structural integrity of PML NBs is not required for IFN signalling. This is interesting for two reasons. First, as described in Section 1.3.5, a number of viruses disrupt PML NBs and initiate their dispersal. Such observations have been taken to indicate that PML NBs exert antiviral effects. The data presented in this study would suggest that such effects may not be directly related to blocking the IFN response to virus infection. Second, PMLIIΔRBCC was the only molecule used in this study that could enhance IFNβ promoter activity during stimulation with poly (I:C). PML IIΔRBCC does not localise to PML NBs, but instead displays a diffuse nuclear localisation. Therefore it is possible that the endogenous PML II-related species responsible for enhancing IFNβ promoter activity may also not associate with PML NBs. Although there is no direct evidence to suggest such a PML II molecule exists, the anti-PML II antibody does detect numerous lower-molecular weight species in HEp-2 cells (Fig. 4.2).

Depletion with siRNA targeting PML II could determine whether these are genuine PML II-related molecules or artefacts of antibody cross-reactivity.

Analysis of four PML IIΔRBCC C-terminal deletion mutants identified two regions, defined by the Δ1 and Δ2 deletions, which were required for PML IIΔRBCC to enhance the response of the IFNβ promoter to poly (I:C). It is therefore possible that the sequences deleted in these mutants may contain binding sites for other proteins that are involved in IFN signalling. For instance, IIΔRΔ2 binds an as-yet unidentified protein (K. Leppard, personal communication) which could be such a factor. E4 Orf3 has been shown to bind a site deleted in IIΔRΔ1 (Leppard *et al*., 2009), providing at least two possible mechanisms whereby E4 Orf3 might overcome the enhancing effect of PML IIΔRBCC on the IFNβ promoter. E4 Orf3 binding to PML IIΔRBCC may disrupt its binding to the unknown factor described above, thereby preventing an interaction that may be necessary for enhancing IFNβ promoter activity. Alternatively, E4 Orf3 may directly out-compete any cellular factors for binding to PML IIΔRBCC, with the ultimate result of limiting its ability to enhance promoter activity.

It is likely that any such mechanism would also apply to an endogenous PML II-related species involved in IFNβ promoter activation. Indeed, expression of the E4 Orf3 mutant N82A, which is unable to bind PML II, in the absence of exogenously-expressed PML II had no significant effect on IFNβ promoter activity while wild-type E4 Orf3 expression led to a decrease in promoter activity in a dose-responsive manner. The N82A mutant, however, is also deficient in reorganisation of the MRN complex. Whereas there is evidence that suggests Ad5-mediated reorganisation of the MRN complex is not required for overcoming the IFN response (Ullman *et al*., 2007), this possibility was not tested directly during this study. Therefore, it would be of interest to determine if an Ad5 Orf3 mutant that is unable to bind PML II, yet still retains the ability to reorganise the MRN complex, could replicate the results presented in this study.

The effects of wild-type E4 Orf3 on IFNβ promoter activity, alongside the evidence presented that supports a role for PML II in IFN signalling, imply that E4 Orf3 may act on an endogenous PML II species in a manner that reduces the response of this promoter to inducers such as poly(I:C). It is likely that this activity of E4 Orf3

would apply during Ad5 infection, and may indicate a mechanism whereby Ad5 can 'dampen' the IFN response. In order to confirm this, further work comparing the effect on IFNβ promoter activity of wild-type Ad5 and E4 Orf3-deficient Ad5 would be required. According to the data presented in this study, infection with an E4 Orf3-deficient virus would be predicted to lead to an increase in IFNβ promoter activity that is greater than that caused by infection with the wild type virus.

A possible model that accounts for the observations made in this study is presented in Figure 7.1. PML IIΔRBCC may mimic the activity of a natural nucleoplasmic, PML II-related molecule by increasing the nucleoplasmic concentration of a regulatory factor involved in type I IFN signalling, with the ultimate result of increasing IFNβ promoter activity. siRNA targeting PML II would deplete the cell of this enhancing nucleoplasmic species, resulting in a loss of IFNβ promoter induction. Full-length PMLII-mediated localisation of this regulatory factor to PML NBs, however, sequesters it and renders it inactive. An interaction between Ad5 E4 Orf3 and the nucleoplasmic PML II species or PML IIΔRBCC could then prevent their binding to the regulatory factor, thus biasing its distribution to sequestration and so inhibiting IFNβ promoter activity.

This model makes several predictions which require confirmation experimentally. For instance, the model assumes that the endogenous PML II species responsible for enhancing IFNβ promoter activity is not full-length PML II, but instead a novel, nucleoplasmic version of this protein that is required for the full induction of the IFNβ promoter under normal circumstances. The model also assumes that such a species may compete with full-length PML II for binding to a protein that performs regulatory functions in the IFN response, with the nucleoplasmic localisation of this protein resulting in a positive effect on IFNβ promoter activity.

It is currently impossible to determine the stage in IFN signalling whereby the nucleoplasmic PML II-related species acts to enhance IFNβ promoter activity. For example, it is possible that it directly affects the availability of a component of the enhanceosome that directs expression from the IFNβ promoter, such as NF-κB or AP-1. Alternatively, this nucleoplasmic PML II-related species may interact with and modulate the activity of factors involved in sensing dsRNA or signalling proteins upstream of IFNβ induction. PML proteins are known to interact with such

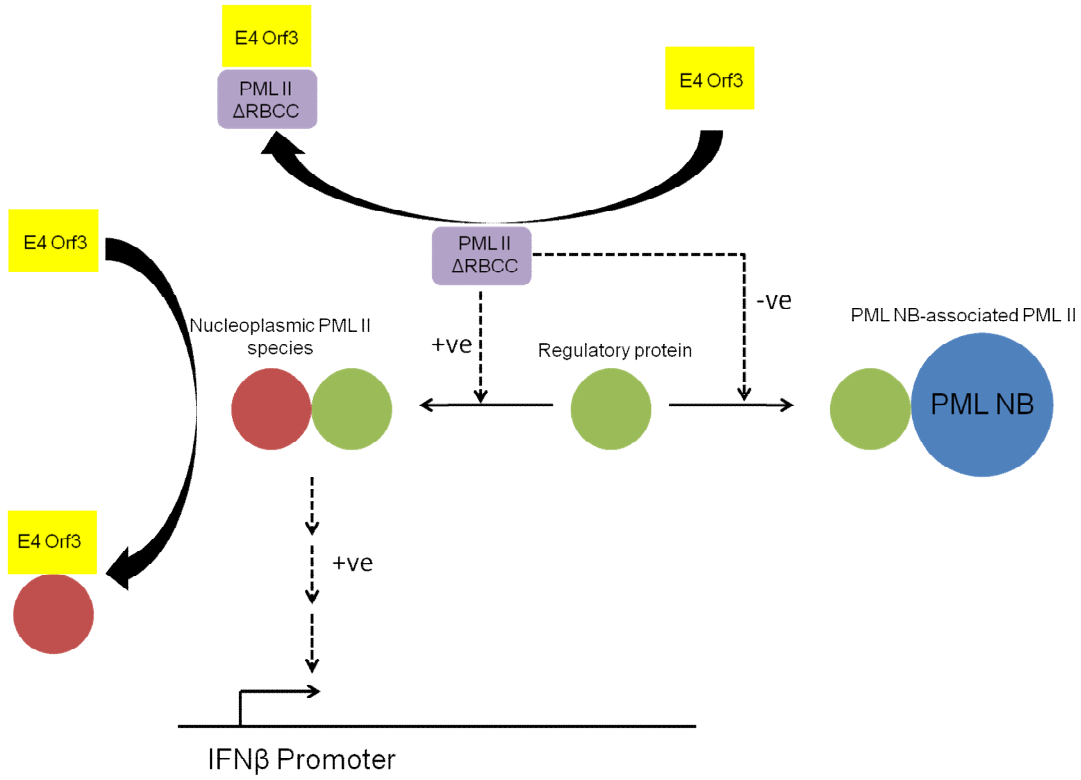


Figure 7.1 A model of PML II-related regulation of the IFNβ promoter. This diagram outlines a possible mechanism of action of nucleoplasmic PML II-related species in IFN signalling. A hypothetical nucleoplasmic PML II-related species interacts with a regulatory protein involved in IFN signalling and promotes its nucleoplasmic localisation, resulting in induction of the IFNβ promoter. Addition of PML IIΔRBCC promotes further the nucleoplasmic localisation of this regulatory protein, whereas addition of E4 Orf3 results in the sequestration of PML IIΔRBCC and the nucleoplasmic species of PML II, with the ultimate effect of preventing IFNβ promoter induction.

factors. For instance it is known that that PMLs I, II and IV can reduce the transcriptional activity of phosphorylated STAT-1, resulting in a decrease in IFNγ signalling (Choi *et al*., 2006). In the case of PML IV, the C-terminal domain was required for the interaction with STAT-1. Thus, it is conceivable that the endogenous PML II-related species can also interact with such a factor through its unique C-terminal domain, but instead promote its activity, resulting in an increase in type I IFN signalling. It would therefore be of particular interest to identify cellular interaction partners with the C-terminal domain of PML II, as this may provide a clue to the stage of IFN signalling at which the PML II-related molecule may act.

As stated above, the C-terminal domain of PML II is known to directly interact with at least one unidentified cellular protein. Once this protein is identified it would be possible to examine other cellular interaction partners of this protein, including any variants of PML II, by co-immunoprecipitation. This could therefore be used as a possible strategy to test for the presence of the hypothesised alternative form of PML II that is responsible for enhancing IFNβ promoter activity.

It remains unclear whether E4 Orf3-induced rearrangement of PML NBs, or indeed the production of PML-A, is related to the ability of Orf3 to inhibit IFNβ promoter activity. As shown by Ullman and colleagues, PML rearrangement correlates with the ability of Ad5 to replicate during an IFN response (Ullman *et al*., 2007). However, it is difficult to distinguish between E4 Orf3-induced rearrangement of PML and its effects on endogenous PML II species. Therefore it is possible that PML rearrangement is not a necessity for replication during an IFN response, but rather it is an interaction of Orf3 with the nucleoplasmic form of PML II implied by this study that is required.

One may speculate that the E4 Orf3-dependent modification of PML II responsible for PML-A is part of a mechanism of Ad5 to overcome the antiviral effect of PML II. As described above, PML-A is likely to be related to full-length PML II, expression of which exerted no effect of IFNβ promoter activity. However, it is possible that the modification responsible for PML-A also occurs on an undetected variant of PML II-related protein that does perform a role regulating IFNβ promoter activity. In such a case, modification of this species may result in a loss of ability to promote IFNβ activity. Further experimentation would be required to establish if this is the case, though it does provide a particularly interesting avenue of investigation. For example, once the modification responsible for PML-A is elucidated it would be possible to examine the effects of this species on the IFN response. This approach could similarly be applied to PML-B and PML-C, though both of these proteins are E4 Orf3-independent and their production is thus unlikely to represent a mechanism for overcoming PML II-mediated enhancement of the IFNβ promoter. Given the involvement of PML proteins in processes important to the cell and the virus, such as the cell cycle, it is possible that these infection-specific species promote viral replication in other ways.

It is clear that the relationship between Ad5 and PML proteins is incompletely understood. This study has provided evidence that Ad5 induces additional changes in the PML protein population within infected cells that were previously unrecorded. It has also provided strong evidence that a PML II-related molecule is important in regulating the type I IFN response, and that Ad5 may selectively target this isoform in order to overcome innate immune responses. However, it remains to be established if these changes in the PML population lead directly to alterations in the ability of PML proteins to regulate the type I IFN response.

Appendix

Appendix A

Construction of an Ad5 E4 Orf4 expression plasmid

E4 orf4 was amplified from wild-type Ad5 genomic DNA by PCR using primers that corresponded to 20 base-pairs at the 5' and 3' ends of the open reading frame. In order to facilitate subsequent cloning, the forward and reverse primers incorporated EcoRI and BamHI restriction sites respectively. The forward primer also incorporated a Kozak sequence around the AUG initiation codon to allow efficient translation of Orf4 after insertion into the vector plasmid (Kozak, 1987). PCR products were separated by agarose gel electrophoresis in order to confirm that Orf4 had been successfully amplified (Fig A1 A). A band corresponding to the expected size (341bp) of the correct PCR product was excised and ligated into pcDNA3.1(-). The ligation products were used to transform *E. coli* DH5α, and successfully transformed colonies were selected by ampicillin selection. 10 colonies were picked and screened for the correct Orf4 plasmid by colony screen PCR using the primers described above. PCR products were separated by agarose gel electrophoresis, and all were found to possess an insert of the correct size (Fig. A1 B). Clones 2 and 8 were selected and confirmed to express E4 Orf4 (Fig. 3.8).

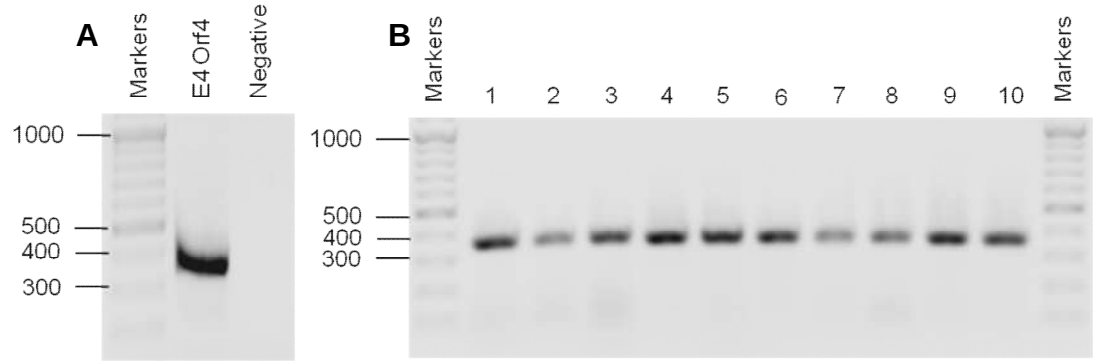


Figure A1. Generation of an E4 Orf4 expression plasmid. A. Orf4 was amplified by PCR from wild-type Ad5 genomic DNA, and PCR products were separated by 0.7% agarose gel electrophoresis. A negative control PCR was used to account for the possibility of contamination. DNA size in base-pairs is indicated to the left. **B** The PCR product in **A** was excised and ligated into pcDNA3.1 (-). Ligation mixes were used to transform *E. coli* DH5α, and subsequent colonies were screened by PCR. PCR products were then separated by 0.7% agarose gel electrophoresis. DNA size is indicated in base pairs to the left.

Appendix B

Sequencing of RT-PCR products

The RT-PCR product under investigation (Section 4.11) was excised and ligated into a pGEM-T Easy vector (Promega). The ligation mix was used to transform DH5α *E. coli*, which were subsequently plated on selective media and subjected to blue/white selection. After incubation, positive colonies were picked and screened by PCR using PML exon 3 5' and PML II 3' primers (Table 2.4). At least 4 different PCR products were detected (3, 4, 5, 8, 9, and 10 being similar to 1) (Fig. A2), and one of each was sequenced using a T7 primer that corresponded to the sequence upstream of the multiple cloning site in pGEM-T Easy. Sequences were compared against the NCBI BLAST database; none of the inserts was found to be related to PML.

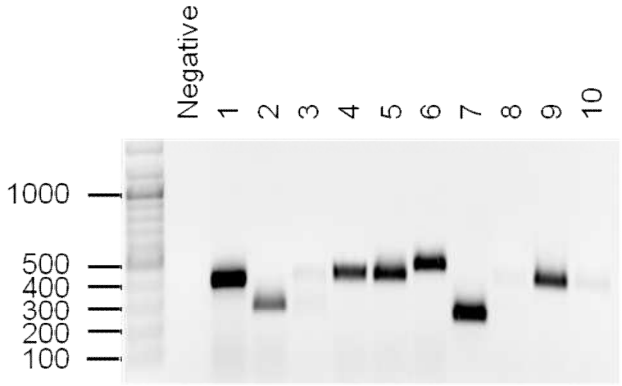


Figure A2 Identification of RT-PCR products. The RT-PCR product under investigation (section 4.11) was cloned into pDEM-T Easy and used to transform DH5α *E. coli.* Subsequent colonies were screened by PCR, and products were separated by 0.7% agarose gel electrophoresis. DNA size is indicated in base-pairs to the left.

BLAST results:

1 - *Homo sapiens* oxoglutarate (alpha-ketoglutarate) dehydrogenase (lipoamide)
2- *Homo sapiens* RNA, 18S ribosomal 1 (RN18S1), ribosomal RNA
6 - *Homo sapiens* transketolase (TKT), transcript variant 3, mRNA
7 - *Homo sapiens* chaperone, ABC1 activity of bc1 complex homolog (*S. pombe*) (CABC1)

Appendix C

Construction of cytoplasmic PML I-V expression plasmids

A two step PCR methodology using pCI-neo FLAG-PML I as the initial template was implemented. In the first round a combination of four primers was used to amplify two regions of PML, which would later serve as templates for the second round (Fig. A3).

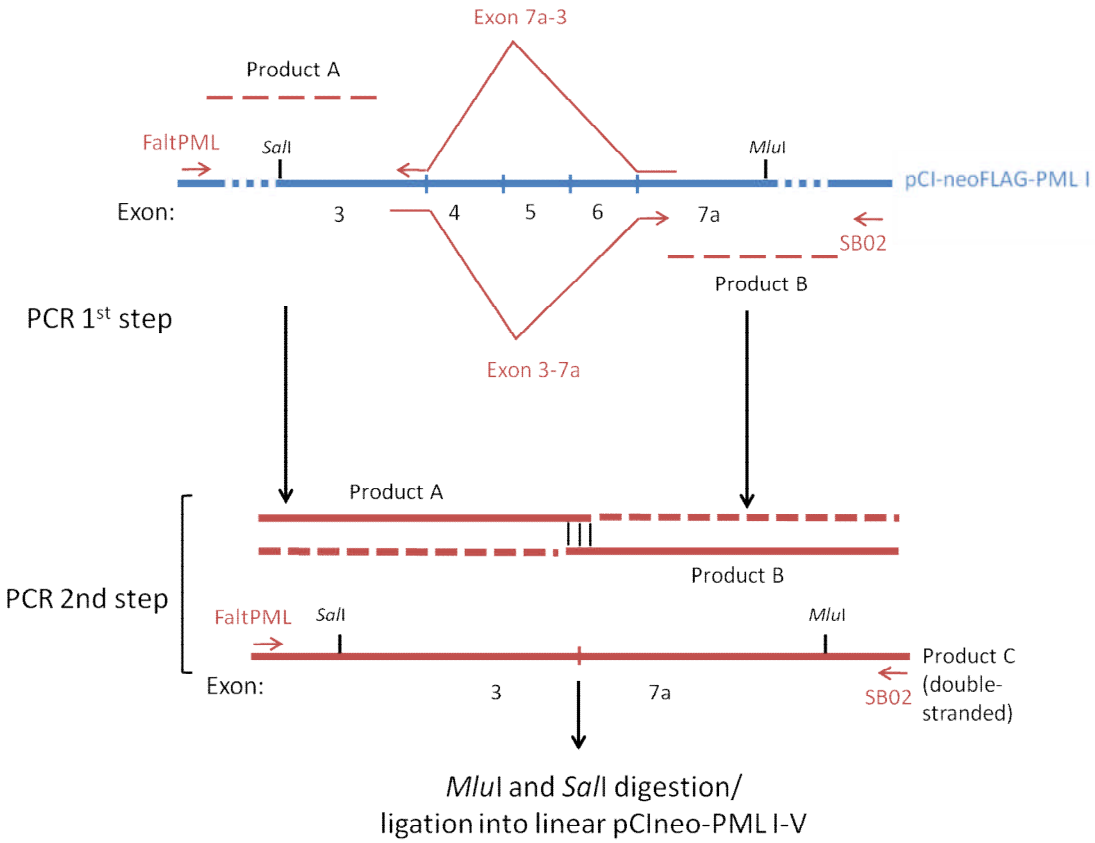


Figure A3. Diagram outlining the PCR strategy employed to generate cPML expression plasmids. The first round of PCR generates 2 overlapping products (A and B), which can then reciprocally act as primers in the second round of reactions to generate product C. Addition of 5' and 3' terminal primers further amplifies product C. Product C contains the required exon3-7a splice junction, and can be cloned into the appropriate plasmid using the described restriction sites.

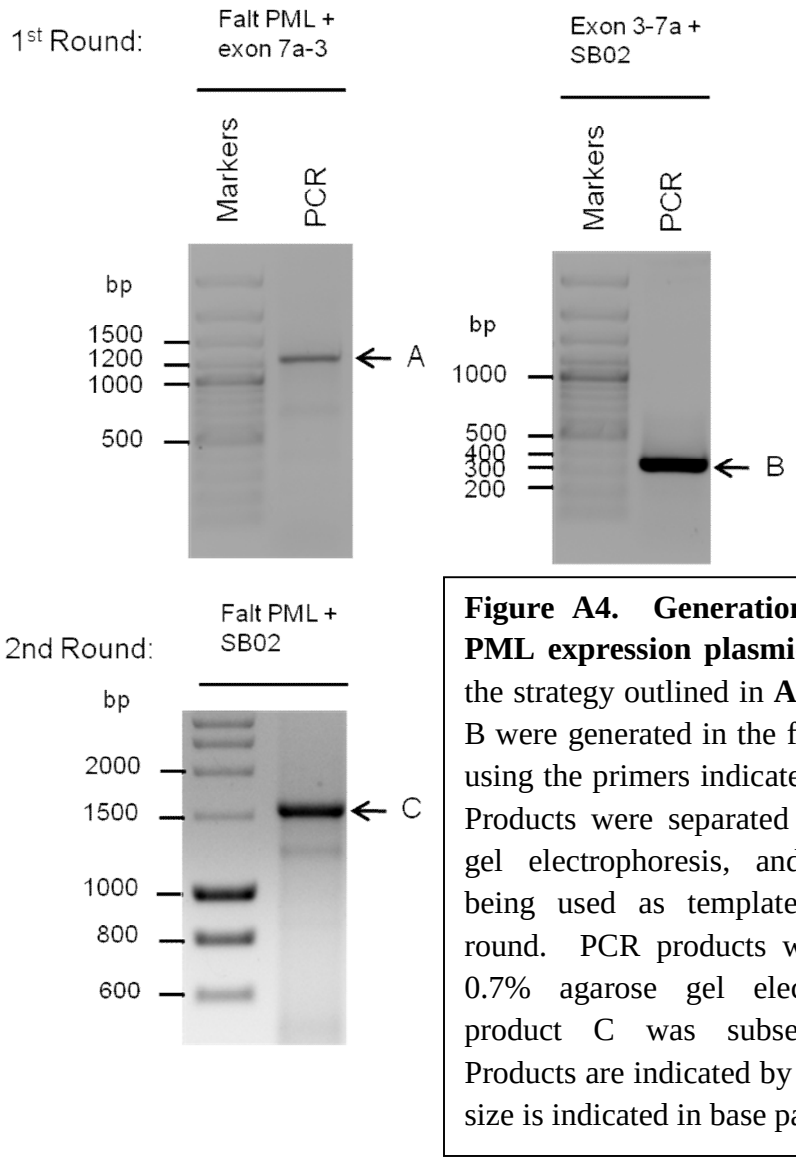


Figure A4. Generation of cytoplasmic PML expression plasmids. According to the strategy outlined in **A3**, products A and B were generated in the first round of PCR using the primers indicated above each gel. Products were separated by 0.7% agarose gel electrophoresis, and excised before being used as template for the second round. PCR products were separated by 0.7% agarose gel electrophoresis, and product C was subsequently excised. Products are indicated by arrows, and DNA size is indicated in base pairs.

Second round PCR products were digested with *Mlu*I and *Sal*I, then ligated into pCI-neo FLAG PML I-V that had previously been cut with *Mlu*I and *Sal*I (Fig. A4). The ligation mixes were then used to transform DH5α *E. coli*, and colonies were screened for the correct insert by PCR, and confirmed by DNA sequencing. pCI-neo FLAG-PML I-Vcytoplasmic plasmids were then used to transfect HEp-2 cells, and exogenous PML localisation was examined using an anti-FLAG antibody (Fig. A5).

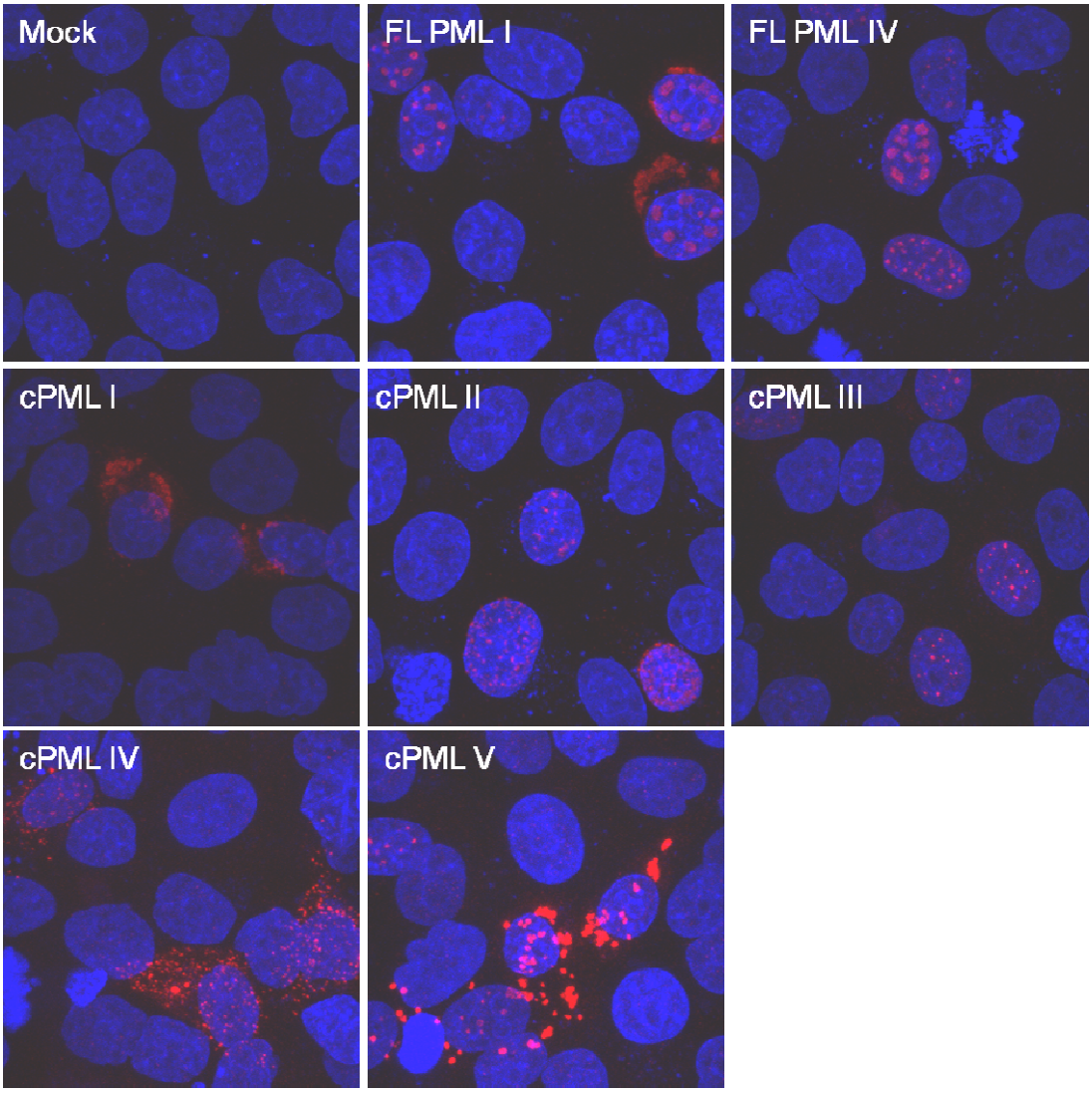


Figure A5. Localisation of cPML I-V. HEp-2 cells were mock-transfected or transfected with full-length FLAG-tagged PML I and PML IV, or FLAG-tagged cytoplasmic PML I-V expression plasmids. 24 hours later cells were fixed and probed with rabbit anti-FLAG and an anti-rabbit fluorophore-conjugated secondary antibody. Nuclei were stained with DAPI. Images represent maximum projections of images made in the Z-axis.

Appendix D

Construction of Δexon5 PML I-VI expression plasmids

A two step PCR methodology using pCI-neo FLAG-PML I as the initial template was implemented. In the first round a combination of four primers were used to amplify two regions of PML, which would later serve as templates for the second round (Fig. A6).

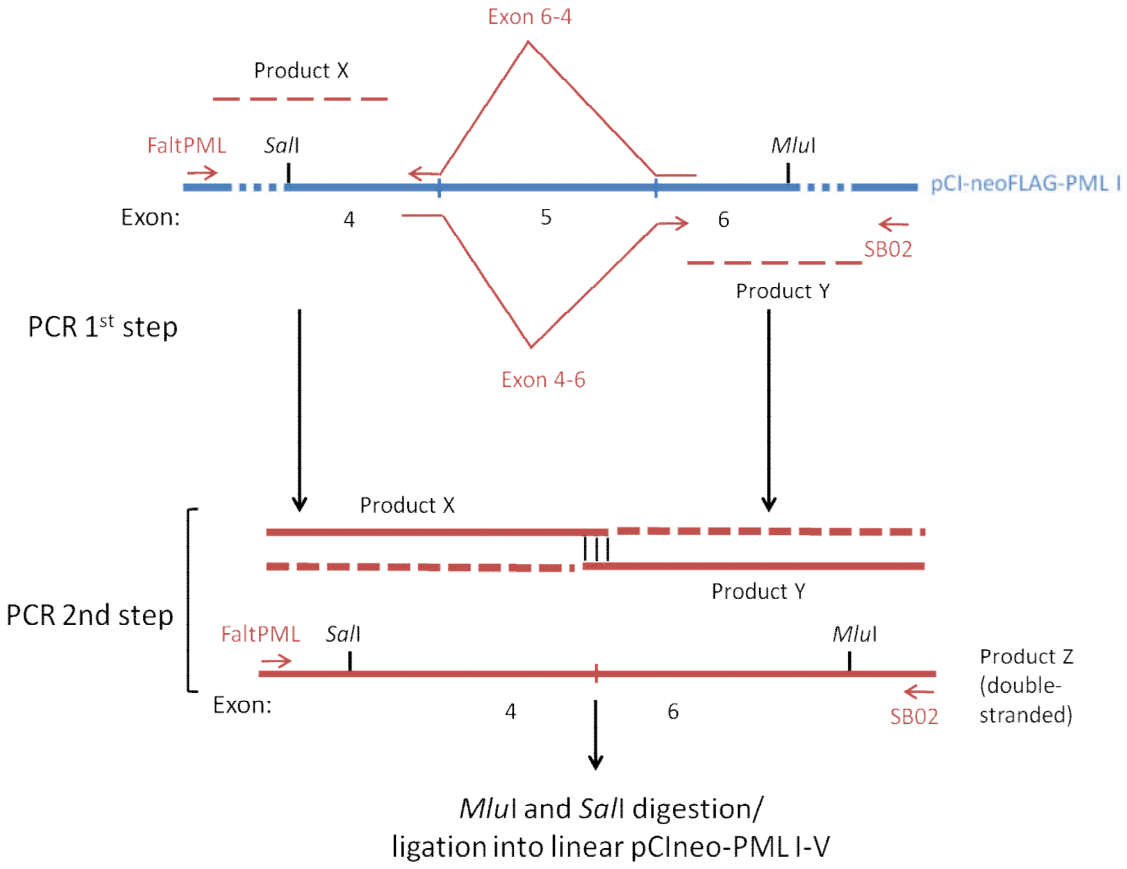


Figure A6. Diagram outlining the PCR strategy employed to generate cPML expression plasmids. The first round of PCR generates 2 overlapping products (X and Y), which can then reciprocally act as primers in the second round of reactions to generate product Z. Addition of 5' and 3' terminal primers further amplifies product Z. Product Z contains the required exon 4-6 splice junction, and can be cloned into the appropriate plasmid using the described restriction sites.

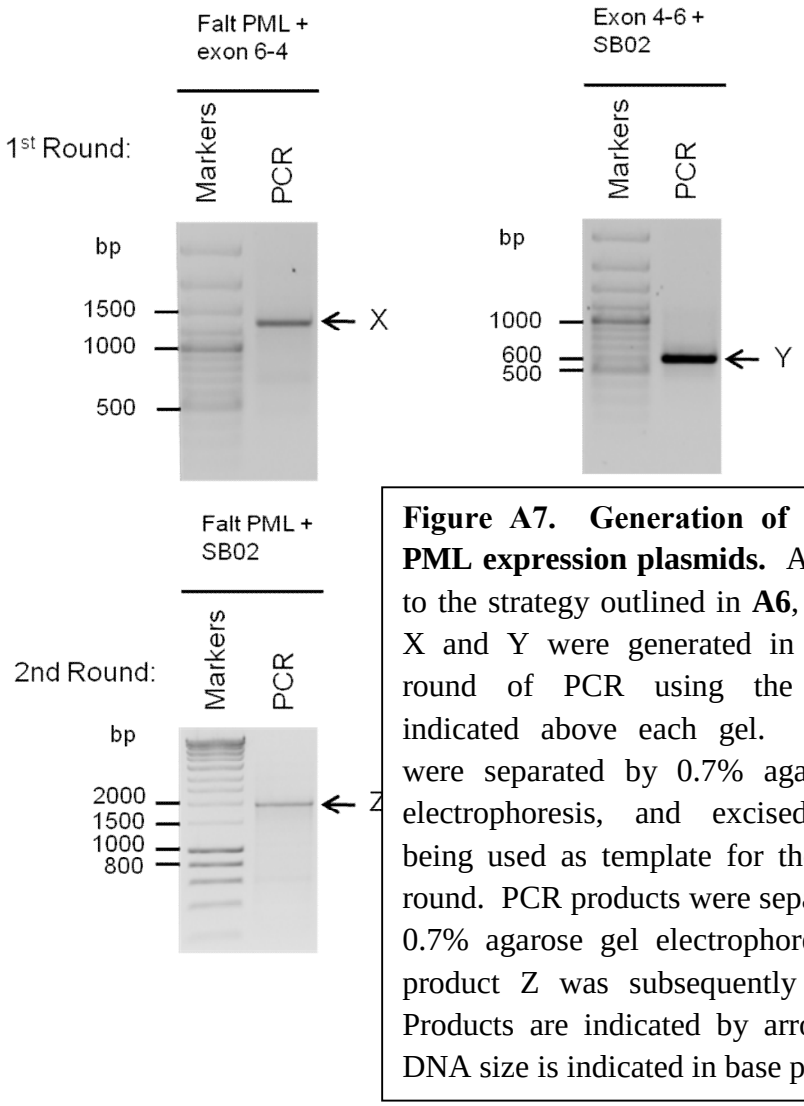
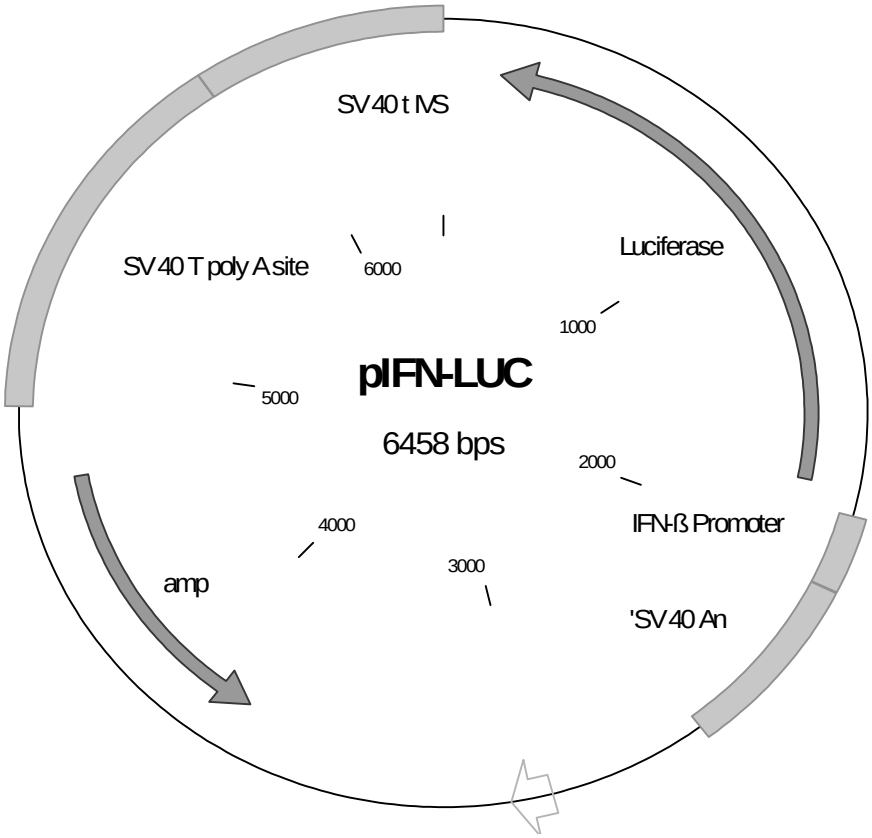


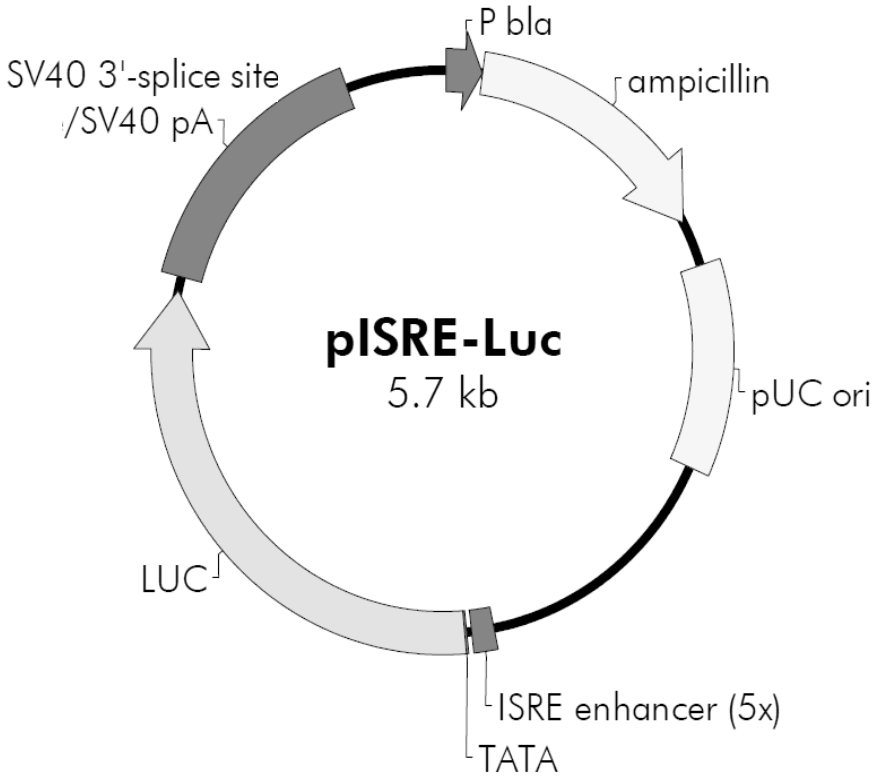
Figure A7. Generation of Δexon 5 PML expression plasmids. According to the strategy outlined in **A6**, products X and Y were generated in the first round of PCR using the primers indicated above each gel. Products were separated by 0.7% agarose gel electrophoresis, and excised before being used as template for the second round. PCR products were separated by 0.7% agarose gel electrophoresis, and product Z was subsequently excised. Products are indicated by arrows, and DNA size is indicated in base pairs.

Second round PCR products were digested with *Mlu*I and *Sal*I, then ligated into pCI-neo FLAG PML I-V that had previously been cut with *Mlu*I and *Sal*I (Fig. A7). The ligation mixes were then used to transform DH5α *E. coli*, and colonies were screened for the correct insert by PCR, and confirmed by DNA sequencing.

Appendix E

Plasmid maps of IFN reporter plasmids used during the course of this study




P bla
ampicillin
SV40 3'-splice site
/SV40 pA
pISRE-Luc
5.7 kb
pUC ori
LUC
ISRE enhancer (5x)
TATA

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