The Role of PML-II in type I Interferon Response and Gene Transcription Regulation

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Yixiang Chen

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Declaration

This thesis is submitted to the University of Warwick in support of my application for the degree of Doctor of Philosophy. It has been composed by myself and has not been submitted in any previous application for any degree.

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Abbreviations

APC	Antigen-presenting cell
APL	acute promyelocytic leukaemia
B-DNA	B-form DNA
β-gal	β-galactosidase
BSA	bovine serum albumin
CARD	caspase recruitment domain
CBP	CREB-binding protein
cDC	conventional dendritic cell
cGAMP/cyclic GMP-AMP	cyclic guanosine monophosphate–adenosine monophosphate
cGAS	cGAMP synthase
ChIP	chromatin immunoprecipitation
CTL	cytotoxic T-lymphocyte
DAI	DNA-dependent activator of IFN-regulatory factors
DAPI	4',6-diamidino-2-phenylindole
DBD	DNA binding domain
DC	dendritic cell
DMEM	Dulbecco's modified Eagle medium
dsRNA	double-stranded RNA
eIF	eukaryotic initiation factor
FBS	foetal bovine serum
FISH	fluorescence in situ hybridization
FRET	fluorescence resonance energy transfer
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GAS	interferon-gamma activated sequence
h.p.i	hours post-infection
HCMV	human cytomegalovirus
HCV	hepatitis C virus
HEK293	human embryonic kidney cell line
HFV	human foamy virus
HMGI(Y)	high-mobility group
HPV	human papilloma viruses
HRP	horseradish peroxidase
HSF1	heat-shock factor 1
Hsp70	heat shock protein 70
HSV-1	herpes simplex virus type-1
IAD	IRF association domain

IFNα/β/γ/λ	interferon alpha/beta/gamma/lambda
IκB	inhibitor of NF-κB
IKK	IκB kinase
IL-2/6/8/12/15/18/28/29	Interleukin 2/6/8/12/15/18/28/29
IP-10	interferon gamma-induced protein 10
IRF-3/7/9	IFN regulatory factor-3/7/9
ISGF3	interferon-stimulated gene factor 3
ISG15/20/54/56	IFN-stimulated gene 15/20/54/56
ISRE	interferon-stimulated response element
JAK	Janus kinase
LPS	lipopolysaccharide
m.o.i	multiplicity of infection
MAPK	mitogen-activated protein kinase
MAVS (cardif/VISA/IPS-I)	mitochondrial anti-viral signalling protein
MDA5	melanoma differentiation-associated gene 5
mDC	myeloid-derived dendritic cell
MEF	mouse embryonic fibroblast
MHC	major histocompatibility complex
MRC5	human fetal lung fibroblast cells
MyD88	myeloid differentiation factor 88
NBS	newborn bovine serum
NF-κB	nuclear factor kappa B
NK cell	natural killer cell
NLR	NOD-like receptor
NLS	nuclear-localization signal
NOD	nucleotide-oligomerization domain
p.f.u	plaque forming unit
p300	E1A binding protein p300
PAGE	Polyacrylamide gel electrophoresis
PAMPs	pathogen-associated molecular patterns
PBMC	peripheral blood mononuclear cell
PCAF	p300/CBP-associated factor
PCR	polymerase chain reaction
pDC	plasmacytoid dendritic cell
PI3K	phosphoinositol 3-kinase
PIV3	parainfluenza virus type 3
PKR	protein kinase R
PML	promyelocytic leukaemia
PML-NBs	promyelocytic leukaemia nuclear bodies
Pol-II	RNA polymerase II
poly(I:C)	polyinosinic:polycytidylic acid
PRD	positive regulatory domain
PRR	pattern-recognition receptor

PV	picornavirus
PTM	post-translational modification
qRT-PCR	quantitative reverse transcriptase polymerase chain reaction
RIG-I	retinoic acid-inducible gene-I
RLA	relative luciferase activity
RLHs	RIG-I like helicases
RNAi	RNA interference
rpm	revolutions per minute
RT-PCR	reverse transcriptase polymerase chain reaction
SAP	shrimp alkaline phosphatase
SB	SDS-PAGE sample buffer
SDS	sodium dodecyl sulphate
SIM	SUMO-interacting motif
siRNA	small interfering RNA
STAT	signal transducer and activator of transcription
STING	stimulator of IFN gene
SUMO	small ubiquitin-like modifier
SYK	spleen tyrosine kinase
TAD	transactivation domain
TAK1	TGF-β-activated kinase 1
TANK	TRAF-associated NF-κB kinase
TBK-I	TANK binding kinase I
TF	transcription factor
TGF-β	transforming growth factor-β
Th1	T lymphocytes of the helper 1
TIR	Toll/interleukin-1 receptor
TIRAP	Toll-IL-1 adaptor protein
TLR	Toll-like receptor
TNTα	Tumour necrosis factor-α
TRAF	TNF receptor-associated factor
TRAM	TRIF-related adaptor molecule
TRIF	TIR-domain-containing adapter-inducing interferon-β
TRIM	tripartite motif
TSS	mRNA transcription start site
VSV	vesicular stomatitis virus
UBL	ubiquitin-like protein

Summary

The promyelocytic leukaemia (PML) gene encodes seven principle protein isoforms due to mRNA alternative splicing; different isoforms have different functions. This study examines the role of PML isoform II (PML-II), in interferon (IFN) and innate immune responses. It was found that siRNA knockdown of PML-II not only inhibited the expression of IFNβ and a large number of IFN-stimulated genes (ISGs) such as ISG15, ISG20, ISG54, ISG56, IP-10 and IRF7, but also affected the induction of many proinflammatory cytokines and chemokines such as IL-6, IL-8, TNFα and RANTES. Signalling to activate these two pathways works principally through regulating interferon regulatory factor 3 (IRF3) and nuclear factor kappa B (NF-κB), respectively. Knockdown of PML-II indeed affected IRF3 and NF-κB activities. However, it had no significant effect on IRF3 and NF-κB nucleus translocation and IRF3 phosphorylation, two features of activation of these factors. These results suggested that PML-II does not affect these signalling pathways, but works downstream of IRF3/NF-κB activation. It was hypothesised therefore that PML-II played a positive role in the assembly of transcription factors at enhancers/promoters.

Using co-immunoprecipitation and chromatin immunoprecipitation experiments, it was found that PML-II could form protein complexes with co-activator CREB-binding protein (CBP) together with IRF3 or NF-κB. Depletion of PML-II reduced IRF3 and NF-κB binding to chromatin at promoters/enhancers of genes such as IFNβ, and also reduced the recruitment of co-activator CBP. PML-II depletion also impaired activation of IFN-responsive genes by signalling from the type I IFN receptor via the JAK-STAT signalling pathway in a similar fashion.

Type III IFNs (IFNλ) are induced and function similarly to IFNα/β while type II IFNγ induces downstream signalling through activating the same transcription factor STAT1 as utilized in the type I IFN response. Extending this study to these IFNs, depletion of PML-II greatly reduced type III IFN expression. It also reduced IFNγ-mediated signalling thus reducing the expression of IFNγ-stimulated genes. PML-II was also identified as a key positive regulatory component of TNFα-mediated NF-κB signalling to stimulate the production of the proinflammatory cytokines such as IL-6, IL-8, RANTES and IP-10 by affecting TFs assembly and CBP recruitment at promoters.

From these data, a model is proposed in which PML-II contributes to the transcription of multiple genes via its association with CBP-transcription factor complexes, which promotes the stable assembly of these complexes at promoters/enhancers of target genes. Through this mechanism, PML-II plays a significant positive role in the development of IFN responses, and hence in the response to virus infection.

Chapter 1 Introduction

1.1 Promyelocytic leukaemia (PML) protein

1.1.1 The discovery and localization of PML protein

The PML gene was originally identified as the chromosomal translocation partner of the retinoic acid receptor RARα, producing an oncogenic fusion protein that was associated with acute promyelocytic leukemia (APL) (de The et al, 1990; Goddard et al, 1991). PML belongs to the RBCC/TRIM protein family (Jensen et al, 2001a) and is covalently modified by the small ubiquitin-like modifier, SUMO. This modification leads to transfer of PML from the nucleoplasm to PML nuclear bodies (PML-NBs) and to the recruitment of other PML-NB-associated proteins. In cells that lack PML, other nuclear body components take on an aberrant localization pattern (Ishov et al, 1999; Zhong et al, 2000a). In contrast, absence of other constitutive nuclear body components such as Sp100 does not affect the localization of PML to nuclear bodies (Zhong et al, 1999b). This suggested that PML is essential for formation of PML-NBs.

1.1.2 PML-NB and components

PML-NBs are small highly dynamic nuclear sub-structures, present in nearly all tissue culture cell lines and normal cells. The composition of PML-NBs changes during the cell cycle, and PML-NBs undergo dramatic rearrangement during mitosis (Dellaire et al, 2006; Everett & Chelbi-Alix, 2007b). An increasing number of proteins have been identified as components of PML-NBs such as SUMO-1, Sp100, Sp140, CREB-binding protein (CBP), BLM, Daxx, pRB and p53 (Figure 1.1) (Doucas et al, 1999; Fogal et al, 2000; Hofmann & Will, 2003; LaMorte et al, 1998; Zhong et al, 2000a). Some component proteins such as

Sp100 and SUMO family members and PML itself, are constitutively present in the PML-NBs (Negorev & Maul, 2001). Other proteins, such as cellular tumour suppressor p53, may be readily detected in or associated with PML-NBs only with particular treatment or under certain circumstances (Salomoni & Pandolfi, 2002). In different cell types, the number, appearance and composition of PML-NBs are varied. This variability may reflect the diverse cell activities with which PML-NBs is associated. The PML-NB is also a target of viral infection. It is reported that upon infection, herpes simplex virus type 1 (HSV-1) early protein ICP0, human cytomegalovirus (hCMV) early proteins IE1 and IE2, Epstein-Barr nuclear antigen EBNA5, the early proteins of papilloma viruses and the adenovirus protein E4 Orf3 can accumulate in the NBs and induce their disruption, which is regarded as a critical event for the expression of viral genes (Ahn & Hayward, 1997; Doucas et al, 1996; Maul & Everett, 1994; Müller & Dejean, 1999; Swindle et al, 1999; Szekely et al, 1996).

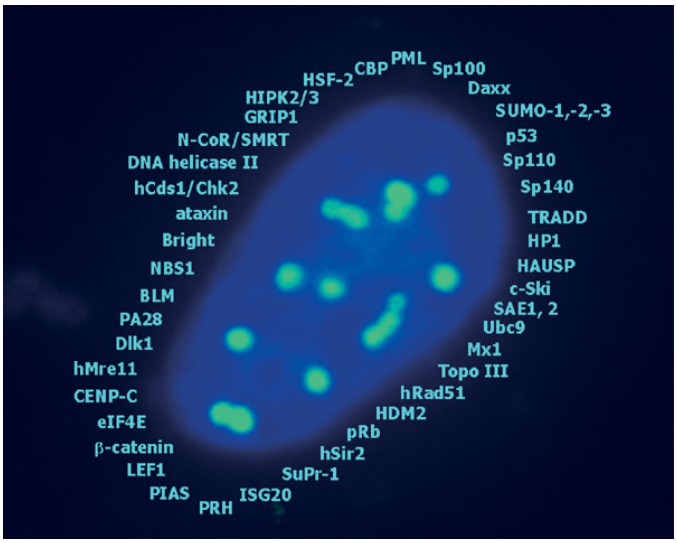


Figure 1.1 Cellular PML-NB components

(Taken from TG Hofmann & H Will, 2003)

1.1.3 PML protein isoforms

The human *pml* gene is located on chromosome 15 and is approximately 35kb in length (Goddard et al, 1991). The *pml* gene consists of nine exons, transcripts from which are alternatively spliced to generate numerous mRNA and hence protein isoforms (Figure 1.2). PML currently has seven principle isoforms, designated as PML I-VII. All isoforms contain an identical N-terminal region that includes RING finger domain, two B-boxes, and a coiled-coil domain (RBCC, also known as the Tripartite Motif, TRIM), and divergent C termini as a result of the different RNA splicing (Borden et al, 1995; Jensen et al, 2001b). Among these seven isoforms, only PML I-VI are known to be incorporated into PML-NBs. PML VII is a cytoplasmic variant of PML because it lacks exon 6 that contains the nuclear localization signal (NLS) and thus is excluded from the nucleus (Reymond et al, 2001). Each of PML I-V may also have several variants depending on the differential splicing pattern of exons 4-6 (Jensen et al, 2001b). All the major PML isoforms are expressed in both primary cells and cell lines, but different cell types may display different patterns of isoform expression (Condemine et al, 2006a). PML I and PML II are the most highly expressed isoforms in normal cells, PML III-V being present at much lower levels, however these become relatively more abundant in tumour cells (Condemine et al, 2006a).

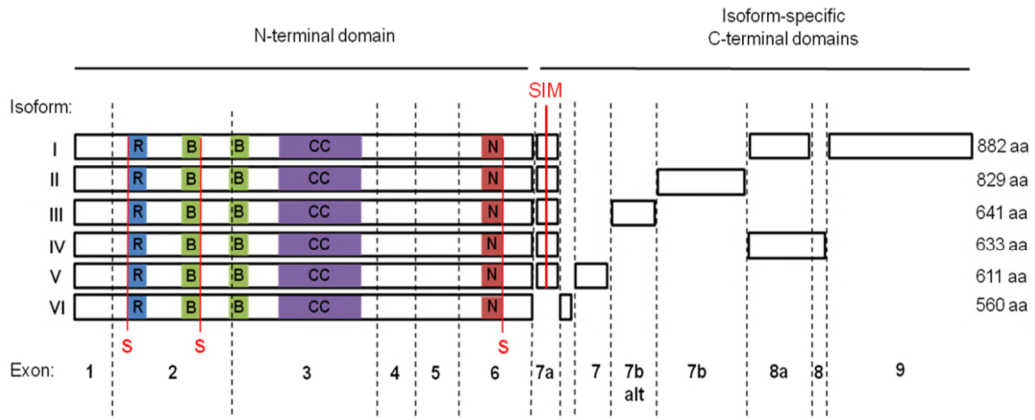


Figure 1.2 Structure of PML gene isoforms

(Taken from Jordan Wright,2010 PhD thesis)

1.1.4 PML post-translational modification

PML proteins can be extensively post-translationally modified in a variety of ways (Nichol et al, 2009). The common known modifications include sumoylation, phosphorylation and acetylation. PML sumoylation is a reversible and dynamic process that is critical for the assembly of PML-NBs (Seeler & Dejean, 2001). Sumoylation has also been shown to regulate the dynamics of exchange of components between PML-NBs and the nucleoplasm (Weidtkamp-Peters et al, 2008). PML sumoylation regulates PML stability by either promoting or inhibiting its degradation depending on the pattern of modification. PML function can also be modified by phosphorylation. During mitosis, PML phosphorylation coincides with PML desumoylation and dispersal of PML-NBs (Everett et al, 1999). Phosphorylation can also regulate PML stability. Increasing evidence suggests that both sumoylation and phosphorylation of PML may "cross-talk", which makes PML structure and functional regulation much more complex. PML may be also subject to acetylation by the histone acetyltransferase (HAT) of E1A binding protein p300 (p300), and this acetylation may enhance its sumoylation (Hayakawa et al, 2008b).

1.1.5 Functions of PML-NBs and PML protein

In recent years a substantial literature reported that PML and PML-NBs are strongly implicated in many areas of cell activity including oncogenesis, DNA damage and subsequent repair processes (Dellaire & Bazett-Jones, 2004), cell apoptosis (Bernardi & Pandolfi, 2003) and senescence (Bischof et al, 2002), ubiquitin pathway (Lallemand-Breitenbach et al, 2001), viral infection and the interferon (IFN) response (Everett & Chelbi-Alix, 2007b; Regad & Chelbi-Alix, 2001a) and various genetic disorders. The following section considers in more detail those aspects of PML function that are relevant to this thesis.

1.2 PML protein functions in interferon responses

Many studies showed that PML protein and PML-NBs confer intrinsic antiviral activity or are linked to innate immunity (Geoffroy & Chelbi-Alix, 2011). The most obvious relationship between PML and antiviral activity is that PML expression can be induced by IFN as the *pml* gene contains both IFN-stimulated response elements (ISREs) and an IFN-gamma (IFNγ) activation site (GAS), thus its transcription can be stimulated by type I and type II IFN respectively (Chelbi-Alix et al, 1995; Stadler et al, 1995). In addition to PML, other components of PML-NBs such as Sp100 are also up-regulated by type I and II IFN (Regad & Chelbi-Alix, 2001a). Moreover, ectopic expression of PML inhibits the growth of IFN-sensitive viruses, for example, influenza A virus replication is significantly repressed by over-expression of some PML isoforms, and correspondingly, depletion of the total PML content leads to an enhancement in viral propagation (Iki et al, 2005; Tavalai & Stamminger, 2008). In addition, PML is a member of the TRIM family, several members of which

perform anti-viral duties (Nisole et al, 2005; Ozato et al, 2008). Thirdly, various viruses encode proteins that disrupt PML and/or PML bodies, including E4 Orf3 of human adenovirus type 5 (HAdVC-5, Ad5) and ICP0 of herpes simplex virus type 1 (HSV1), and viruses lacking these functions are unable to overcome IFN responses (Chelbi-Alix & Wietzerbin, 2007; Leib et al, 1999; Mossman et al, 2000; Ullman & Hearing, 2008; Ullman et al, 2007). Ad5 E4 Orf3 targets PML-II specifically (Hoppe et al, 2006), suggesting that this isoform in particular might have a role in the IFN response, and PML-II is one of two isoforms shown to be inhibitory to HSV1 infection (Cuchet et al, 2011). One recent report showed that overexpression of all nuclear PML isoforms (PML-I-VI) increased IFNγ-induced STAT1 phosphorylation, resulting in higher activation of IFN-stimulated gene (ISGs), and that, conversely, down-regulation of PML by siRNA decreased IFNγ-induced STAT1 phosphorylation, and ISG transcription (El Bougrini et al, 2011).

1.3 Transcriptional role of PML

PML itself is a member of the RING finger family of proteins which also includes other proteins such as BRCA which are involved in cellular transformation and in the regulation of transcription (Miki et al, 1994). More and more evidence have demonstrated the role of PML in transcriptional regulation. PML can interact (directly or indirectly) with transcription factors (TFs) such as Ap-1, RARα, p53, STAT1 and Sp1 and coactivators CBP/p300 or co-repressors such as HDAC, N-coR and mSin3A (Choi et al, 2006; Doucas et al, 1999; Guo et al, 2000a; Khan et al, 2001; Pearson et al, 2000; Shiio et al, 2006; Vallian et al, 1998a; Vallian et al, 1998b; Zhong et al, 1999a; Zhong et al, 2000b). PML does not bind DNA directly, however, it can associate with these transcriptional proteins thus determine

the ability of PML protein in transcription. PML can act both as a transcriptional co-activator and co-repressor. As a co-activator, PML positively regulates RAR-RXR-mediated transcription (Khan et al, 2001) and can also regulate p53 transcriptional activation (Fogal et al, 2000). As a co-repressor, PML interacts with Sp1 and inhibits the transactivation of the epidermal growth factor receptor (EGFR) gene. PML also displays an inhibitory effect on pRB-regulated transcriptional activation of glucocorticoid receptor (Alcalay et al, 1998; Vallian et al, 1998a). However, the mechanisms by which PML acts as a co-repressor need further investigation.

PML-NB may also play an important role in the regulation function of PML in gene transcription because transcriptional regulators such as Sp1, Fos, TIF1α, RXRα and CBP, pRB and p53 are found in the nuclear body (Doucas et al, 1999; Fogal et al, 2000; Vallian et al, 1998b; Zhong et al, 1999a; Zhong et al, 2000b). It is therefore possible that transcription factors and co-factors are transiently recruited to the nuclear bodies, or to their proximity, either to take part in transcription or to be modified (for example by acetylation or Sumoylation) in a way that activates their function.

In recent years, PML protein has been shown to serve as a regulator in IFNγ signalling through affecting STAT-1 DNA binding and transcription activity (Choi et al, 2006; El Bougrini et al, 2011). In addition, the presence of PML was found to enhance IFNγ-induced MHC Class II expression, with PML-II in particular binding the key transcription factor for this gene, CIITA, stabilizing it and causing it and the MHCII gene loci to associate with PML-NBs (Ulbricht et al, 2012). It was reported recently that PML-II also interacts

preferentially with Ad E1A-13S, and thus enhances E1A-mediated transcriptional activation (Berscheminski et al, 2013).

1.4 The innate immune response

The innate immune system is the first line of defence against a variety of pathogens. It not only is essential for early pathogen recognition, but also is involved in the activation and regulating of adaptive immunity (Iwasaki & Medzhitov, 2004; Randall & Goodbourn, 2008). The innate immune response is regulated and mediated by the synthesis and secretion of various inflammatory cytokines, chemokines and type I IFN. When pathogen-associated molecular patterns (PAMP) are recognized by the various pattern recognization receptors (PRR), a multitude of intracellular signalling pathways, including adaptor molecules, kinases and transcription factors, are activated. Signalling cascades ultimately result in the activation of gene expression and the synthesis of a broad range of molecules, including cytokines, chemokines, cell adhesion molecules, intracellular effectors and immunoreceptors (Akira et al, 2006), Thus cells can establish proinflammatory and antimicrobial responses. The transcription factors nuclear factor kappa B (NF-κB) and interferon regulatory factors (IRF) 3 and 7 play pivotal roles in this process due to their capacity to stimulate the production of proinflammatory mediators, particularly the IFNs. Cytokines such as interleukin (IL)-1, IL-6, and tumor necrosis factor alpha (TNFα), as well as chemokines including IL-8 and RANTES are also very important mediators of inflammatory responses.

1.4.1 Pathogen recognition

The innate immune response relies on recognition of PAMPs through different PRRs. Known PAMPs include viral glycoproteins, viral RNA, bacterial endotoxin (lipopolysaccharide), bacterial flagella, or DNA CpG motifs (Randall & Goodbourn, 2008). One pathogen can have various PAMPs and present them in different fashions. For example, viruses may possess several structurally diverse PAMPs, including surface glycoproteins, DNA and RNA species (Jouault et al, 2003; Randall & Goodbourn, 2008).

The repertoire of PAMPs is very extensive, and similarly, the classes of PRRs that recognize pathogens are very diverse. To date, several families of PRR, including Toll-like receptors (TLR), retinoic acid-inducible gene-I (RIG-I)-like receptors (RLR), NOD-like receptors (NLR) and DNA receptors (cytosolic sensors for DNA), have been discovered and characterized (Gürtler & Bowie, 2013; Randall & Goodbourn, 2008). It has become quite obvious that although pathogens may be recognized by distinct cellular receptors and activate different signalling pathways, they all finally lead to the activation of IRF3 or NF-κB, which are critical for the transcriptional activation of type I IFN genes and proinflammatory genes.

1.4.2 TLR signalling pathway

TLRs are the most widely studied PRRs and are considered to be the primary sensors of pathogens. They are type I membrane glycoproteins and consist of extracellular leucine-rich repeats (LRRs) that are required for PAMP recognition, and a cytoplasmic Toll/interleukin-1 receptor (TIR) domain, required for downstream signalling. In humans, 10 TLR family

members have been identified. TLR1, 2, 4, 5 and 6 are primarily expressed on the cell surface and recognize PAMPs derived from bacteria, fungi and protozoa, whereas TLR3, 7, 8 and 9 are expressed within endocytic compartments and primarily recognize nucleic acid PAMPs derived from various viruses and bacteria (Kawai & Akira, 2010; Kumar et al, 2009; Takeuchi & Akira, 2010) . TLR3 also senses a synthetic analog of dsRNA, poly(rI).poly(rC) [poly(I:C)] (Miyake et al, 2009) .

TLR signalling is initiated via the recruitment of adaptor molecules to the intracellular TIR domain (Kawai & Akira, 2010; Kumar et al, 2009; Takeuchi & Akira, 2010) (Figure 1.3). All TLRs, except for TLR3, recruit myeloid deifferentiation factor 88 (MyD88) and the activated MyD88-dependent signalling activates NF-κB and MAP kinase (MAPK). TLR3 directly recruits TRIF to activate NF-κB and IRF3. In the TRIF-dependent signalling pathway, NF-κB and IRF3/7 are activated through the action of IκB kinase (IKK) complex and TBK1/IKKi respectively, to induce the transcription of inflammatory cytokines and type I IFN (Kumar et al, 2011) .

1.4.3 RLR signalling pathways

The RLR family of PRRs includes retinoic acid-inducible gene-I (RIG-I), melanoma differentiation associated gene 5 (MDA5) and Laboratory of genetics and physiology 2 (LGP2) which is required for RIG-I- and MDA5-mediated antiviral responses (Childs et al, 2013; Satoh et al, 2010). Initially, RIG-I was identified as an essential regulator for poly(I:C)-induced signalling (Yoneyama et al, 2004). The related protein MDA-5 was identified as a binding partner for the IFN-induction antagonist protein of many RNA

viruses and has similar properties to RIG-I although it binds less avidly to polyinosinic: polycytidylic acid poly(I:C) (Andrejeva et al, 2004; Yoneyama et al, 2005). However, a more recent study showed RIG-I and MDA5 each recognize distinct features that RIG-I preferentially associates with shorter, 5′ppp containing viral RNA molecules in infected cells by using next-generation RNA sequencing (Baum et al, 2010). These receptors sense the RNA from RNA viruses in the cytoplasm of infected cells and induce inflammatory cytokines and type I IFN. All RLR family members contain an intermediate DExD/H-box RNA helicase domain, which is required for ligand recognition or binding (Yoneyama et al, 2005). RIG-I and MDA5 contain N-terminal tandem caspase recruitment domains (CARD) that are essential for downstream signalling. In addition, RIG-I also contains a repressor domain (RD), which is required for the regulation of RIG-I-dependent downstream signalling. With the cytosolic RLRs RIG-I and MDA-5, both dsRNA origin and length influence recognition and binding. RIG-I preferentially recognizes RNA from a variety of RNA viruses, such as members of the *Paramyxoviridae* and *Flaviviridae* families (Kumar et al, 2011). In addition, RIG-I also recognizes enzymatically synthesized RNA and chemically synthesized double-stranded RNA (dsRNA). MDA5 recognizes RNA from members of the *Picornaviridae* family as well as the synthetic double stranded RNA analog, poly(I:C) (Kato et al, 2006b). Interestingly, enzymatically shortened poly(I:C) is preferentially recognized by RIG-I, rather than by MDA5, suggesting that RIG-I and MDA5 recognize different lengths of dsRNA.

PAMP sensing by RIG-I and MDA5 leads to conformational changes that expose the CARD domains of the receptors, which then interacts with the CARD-containing adaptor protein,

mitochondrial anti-viral signalling protein (MAVS) (also known as IPS-1/Cardif/VISA) (Kawai et al, 2005; Randall & Goodbourn, 2008; Seth et al, 2005) (Figure 1.3). This CARD-CARD interaction is essential for triggering downstream signalling. MAVS acts as a scaffold to recruit signalling components to form cytosolic kinase complexes that activate IRF3/7 and NF-κB.

1.4.4 Cytosolic DNA sensor-dependent signalling

The recognization of intracellular DNA that derived from DNA viruses or pathogenic bacteria by cytosolic DNA sensors is an important step to initiate an effective protective response. Such intracellular DNA might be distinguished from mammalian cell self DNA by its lack of methylation, higher A+T content or cytoplasmic localization (Randall & Goodbourn, 2008). In recent years, several studies have explored the cytosolic DNA sensor-dependent signalling pathway. DAI (DNA-dependent activator of IFN-regulatory factors), also known as DLM1 and ZBP1, was the first discovered cytoplasmic DNA sensor (Takaoka et al, 2007). In vitro studies show that DAI is essential for type I interferon production. The induction of type I IFNs (mainly IFN-β) by DAI/DLM-1/ZBP1 in response to cytoplasmic DNA requires the transcription factor IRF3, which is activated upon phosphorylation by TANK-binding-kinase-1 (TBK1) (Takaoka et al, 2007). NF-κB is also activated via the IKK complex when PAMPs in the cytoplasm of cells are sensed by DAI (Takaoka & Taniguchi, 2008). DNAs from various sources were demonstrated to bind to DAI, thereby inducing DNA-mediated induction of type I IFN and the induction of IFN-responsive genes and proinflammatory chemokines (Takaoka et al, 2007). However, inhibition of DAI by small interfering RNA had little or no effect on the IFN response to different types of DNA (Wang

et al, 2008). This suggested that DAI is probably not the only cytosolic DNA receptor triggering the IFN response.

Recently, an ER-localized transmembrane protein, STING (stimulator of IFN genes), has been shown to be essential for the TBK1-IRF3- dependent induction of IFN-β (Ishikawa & Barber, 2008). In response to cytosolic DNA, STING forms dimers and translocates from the ER to the Golgi; STING interacts with TBK1, leading to the phosphorylation of IRF3, and induces type I interferon production after stimulation by DNA or RNA (Ishikawa & Barber, 2008). However, STING does not interact directly with cytosolic DNA, suggesting that STING acts downstream of a DNA sensor.

STING can be bound and activated by cyclic guanosine monophosphate–adenosine monophosphate (cyclic GMP-AMP, or cGAMP) (Barber, 2014). Most recently, cGAMP synthase (cGAS) has been demonstrated to play a key role in the regulation of IRF3 activation and IFN-β induction in response to DNA transfection or DNA virus infection (Sun et al, 2013). This suggests cGAS is a novel cytosolic DNA sensor that induces interferons by producing the second messenger cGAMP. Besides DAI, STING and cGAS, AIM2 and members of the DExD/H-box helicase superfamily have also been reported to function as cytosolic DNA sensors (Hornung et al, 2009; Zhang et al, 2011).

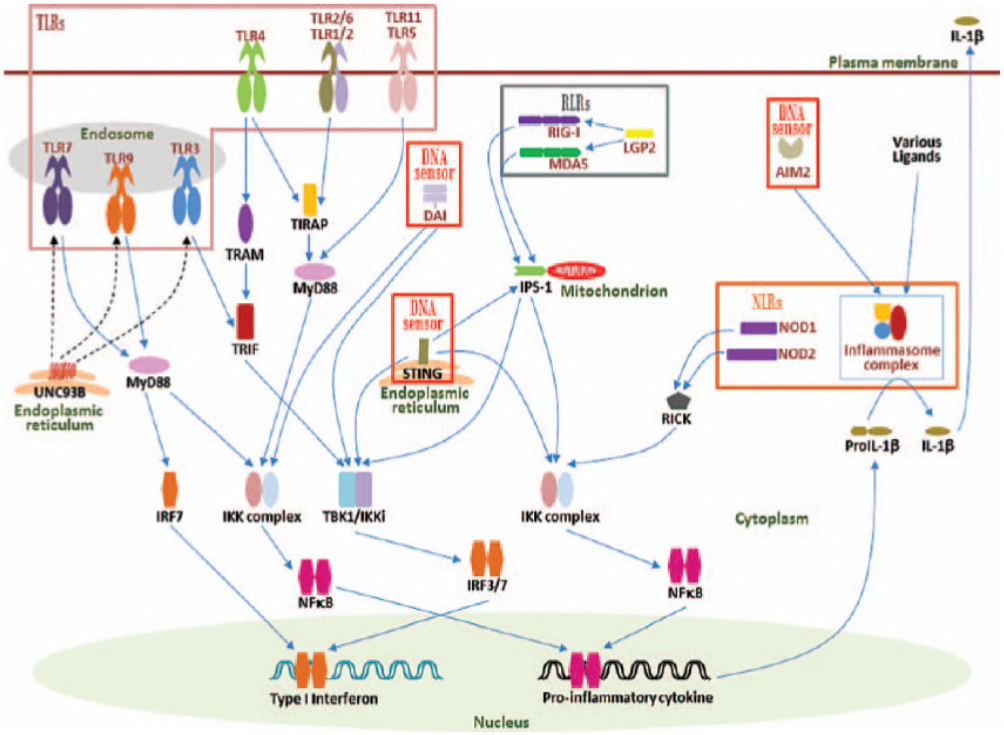


Figure 1.3 Type I IFN and proinflammatory cytokine signalling

(Taken from Himanshu Kumar,et al 2011)

1.5 Interferon

1.5.1 The Interferon family

Interferon (IFN) was first discovered by Isaacs and Lindemann in 1957 (Isaacs & Lindenmann, 1957). Subsequently, more and more IFN family members were identified. IFNs are now classified into three groups: type I, II and III IFNs. Type I IFNs consist of a group of structurally related IFNα protein molecules and a single IFNβ protein, and other proteins such as IFN-*ω*, -*τ*, -*δ* and -*κ* that play less well-defined roles (Randall & Goodbourn, 2008). IFN-α/β can be secreted by many cell types including lymphocytes (NK cells, B-cells and T-cells), macrophages, fibroblasts and endothelial cells. Type II IFN, also called immune IFN, has a single member IFNγ and is mainly secreted by mitogenically activated T lymphocytes of the helper 1 subset (Th1) and NK cells. IFNγ participates in

many aspects of innate and adaptive immunity in cooperation with type I IFN (Stark et al, 1998). Type III IFNs, a more recently discovered group, are encoded by three distinct genes IFNλ1 (IL29), IFNλ2 (IL28A), and IFNλ3 (IL28B) (Sheppard et al, 2003). The IFNλ genes are clustered together on human chromosome 19, and are more closely related in structure and sequence to the IL-10. In untreated cells the mRNA levels of IL-28 and IL-29 are low to undetectable, but upon stimulation with dsRNA or infection with a variety of viruses, the amount of RNA increases significantly (Sheppard et al, 2003). IL-29 and IL-28 can be expressed in variety of cell types including HeLa and A549 cells (Sheppard et al, 2003).

1.5.2 Interferon function

The IFNs have a wide range of biological functions, including antiviral, antiproliferative effect/antitumour activity and immune modulatory properties. Type I IFN (IFNα/β) can be expressed ubiquitously; classical functions of these IFNs comprise antiviral activities including inhibiting protein synthesis, inducing RNA cleavage, and interfering with viral replication (Fensterl & Sen, 2009; Randall & Goodbourn, 2008). In infected cells IFNs act to induce apoptosis and therefore limit the spread of virus. Specifically, IFNs increases p53 activity, which induces rapid apoptosis of virus-infected cells, therefore virus production will be suppressed (Takaoka et al, 2003). The effect of IFNα/β on p53 was also reported to contribute to tumour suppression (Takaoka et al, 2003). In uninfected cells, IFNs act to induce an antiviral state to limit subsequent viral infection. For example, upon virus infection active PKR enzyme is generated which can phosphorylate eukaryotic initiation factor 2 (eIF-2). The phosphorylation of eIF-2 inhibits its activation, the phosphorylated inactive eIF2 forms a stable complex with another protein eIF2B and therefore inhibits virus

protein synthesis. In addition, IFNα/β induces the expression of a large number of ISGs that play an important role in combating viruses. Also, IFN can up regulate the expression of major histocompatibility complex molecules MHC I and MHC II. Higher MHC I and II expression increases the presentation of viral peptides to cytotoxic T cells and NK cells, and helper T cells, respectively. Type I IFN also plays a prominent role in inhibition of cellular growth (Stark et al, 1998).

IFNγ is an important cytokine which plays various roles in different facets of the immune system. IFNγ is critical for adaptive immunity against viral and intracellular bacterial infections and for tumour control. It is also crucial in the establishment of an immediate antiviral state in host cells in response to viral infection. It is reported that IFNγ contributes to protection against some viral infections such as hepatitis B virus, herpes simplex virus, lymphocytic choriomeningitis virus; it directly inhibits viral replication, mainly through the induction of key antiviral enzymes, most notably PKR (Schoenborn & Wilson, 2007).

Type III IFN is functionally similar to type I IFN. It plays a role in the induction and regulation of the immune and inflammatory responses including antiviral activities against a broad range of viruses such as encephalomyocarditis virus (EMCV), vesicular stomatitis virus (VSV), human immunodeficiency virus (HIV1), HSV-1 and hepatitis C virus (HCV) (Diegelmann et al, 2010; Hou et al, 2009; Levy et al, 2011; Sheppard et al, 2003).

1.6 IFN induction and gene transcription

IFNα/β can be induced directly in response to different virus infections. However, IFNα/β is expressed differentially through different signalling pathway in different cell types. In

plasmacytoid dendritic cells (pDCs), which are responsible for the majority of IFN produced during a viral infection, type I IFN is induced through activating TLR7 and TLR9 in a MyD88-dependent manner (Honda et al, 2004; Kawai et al, 2004). Conversely, in macrophages, conventional DCs, and fibroblasts, type I IFN is induced by cytoplasmic RNA primarily via RLR or TLR3/TLR4-mediated signalling by a mechanism dependent on TRIF TIR-domain-containing adapter-inducing interferon-β (TRIF) (Kato et al, 2005; Yamamoto et al, 2003).

1.6.1 IFNβ induction and enhancer assembly

Upon PAMPs being recognized by PRRs, a group of mediator factors at activated which finally leads to the activation of IRF3 and NF-κB (Akira et al, 2006; Kawai & Akira, 2008; Yoneyama & Fujita, 2009). IRF3 is phosphorylated and moves into the nucleus (Lin et al, 1998) while NF-κB is released from its inhibitor IκB and migrates into the nucleus (Baeuerle & Baltimore, 1988). Then, a multi-protein complex containing ATF-2/c-Jun, IRF3 (and IRF7 if present), and NF-κB (p50/RelA) binds cooperatively to a region of the IFNβ promoter to induce the transcription of IFNβ (Figure 1.4).

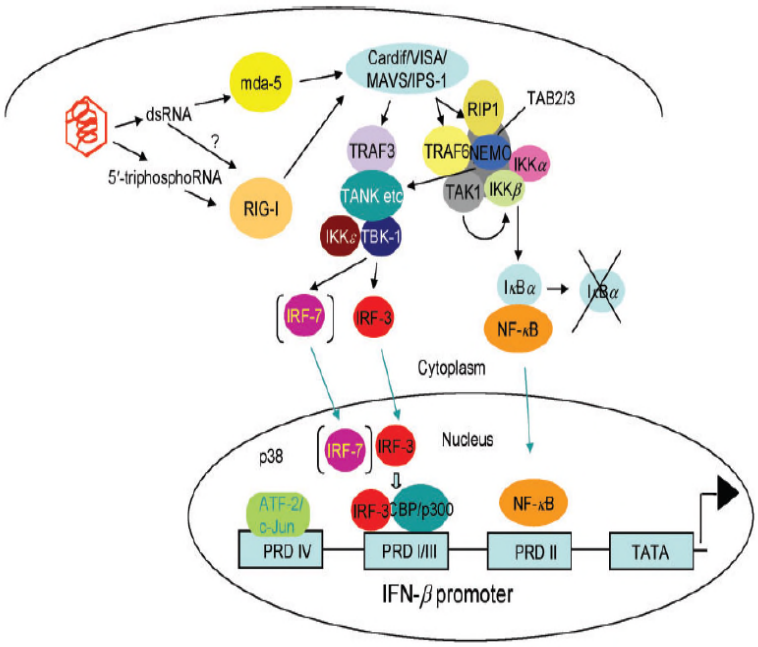


Figure 1.4 Signalling pathway of IFNβ induction

(Taken from Richard E. Randall & Stephen Goodbourn, 2008)

The IFNβ enhancer, spanning the interval from 102 to 47 bp upstream of the mRNA transcription start site (TSS), can be subdivided into four positive regulatory domains (PRDs) (Figure 1.5). A heterodimer of NF-κB p50/Rel A (p65) and an ATF-2/c-Jun heterodimer bind to PRDII and PRDIV, respectively (Maniatis et al, 1998; Thanos, 1996; Thanos & Maniatis, 1995). PRDIII/I are recognized by a protein complex containing IRF3 and/or IRF7 (Panne et al, 2007). Each of these factors can interact with the coactivator, CBP or with its closely related paralog, p300 through distinct protein interaction domains to assemble the IFNβ enhanceosome (Kim et al, 1998; Merika et al, 1998). Two molecules of the high-mobility-group protein I(Y) (HMG-I/Y) also bind the enhancer, one each at PRDII and PRDIV, and bind cooperatively with NF-κB and ATF-2-c-Jun though they do not appear in the final complex (Yie et al, 1997). Studies using ChIP and live cell imaging

techniques showed that these transcription factor assemblies are dynamic and transient with very short half lives *in vivo* (Bosisio et al, 2006). Recent structural studies showed that TFs cooperatively bind to a region of the IFNβ promoter with overlapping nucleotides sites (Bosisio et al, 2006). The extensive overlap of individual TF binding sites at the IFNβ promoter makes it like a composite element that can be regulated as a single unit. Despite TFs tightly packed on the DNA binding domains, there are no/few direct protein-protein interactions between individual components (Panne et al, 2007). The absence of major protein-protein interfaces between the TFs suggests DNA structure changes, which are induced by TFs binding, are required for cooperative binding of TFs at the promoter. In addition, specific interactions between TFs and co-activator proteins such as CBP/p300 are also essential for TFs cooperative binding process (Panne, 2008; Panne et al, 2007)

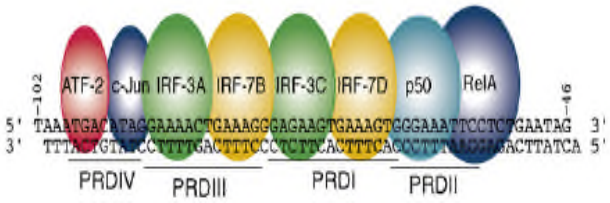


Figure 1.5 The enhancer of IFNβ gene

(Taken from Daniel Panne, 2007)

1.6.2 Type III IFN induction

IFNλ1 (IL-29) and IFNλ2/3 (IL-28A/B) promoters are similar to those of IFNα and IFNβ genes, respectively. Both have functional ISREs as well as NF-κB binding sites. IL-29 is regulated similarly to IFNβ, being dependent on IRF3, IRF7 and NF-κB, while IL-28

regulation resembles IFNα, depending on IRF7 (Figure 1.6) (Onoguchi et al, 2007; Osterlund et al, 2007).

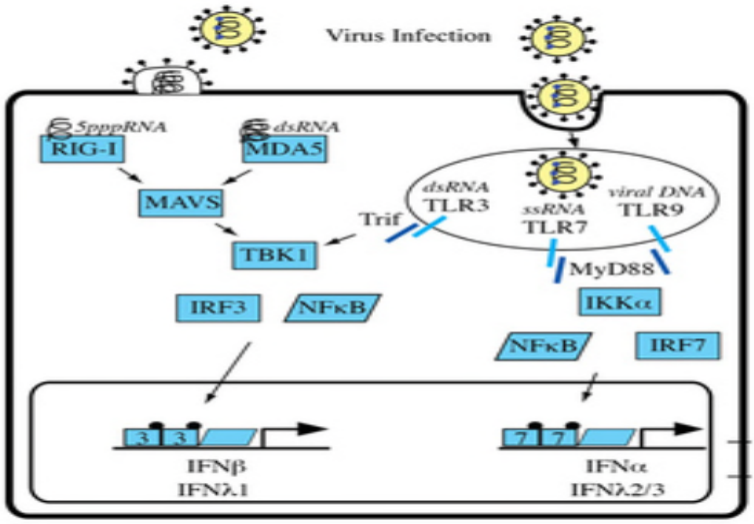


Figure 1.6 The similarity between type I and type III IFN in induction

(Taken from Heim MH, 2012)

1.6.3 Type II IFN production

A number of cytokines such as IL-2, IL-12, IL-15, IL-18 and type I IFNs contribute to the production of IFN-γ in NK cells, while there are some cytokines including transforming growth factor-β (TGF-β) that inhibit the expression of IFN-γ (Li et al, 2006a; Li et al, 2006b; Schoenborn & Wilson, 2007). IL-6 also negatively regulates IFN-γ production in CD4 T cells (Diehl et al, 2000). IFN-γ secretion is controlled by different signalling pathways in different cells. For example, in NK cells, IFN-γ production is heavily dependent on MAPK signalling. Inhibition of spleen tyrosine kinase (SYK) or downstream phosphoinositol 3-kinase (PI3K) and MAPK pathways greatly decreases the ability of NK cells to produce IFN-γ (Tassi & Colonna, 2005; Tassi et al, 2005). The IFN-γ gene comprises four exons spanning ~5.5 kb, upstream of which is a ~600 bp core promoter. A

large number of transcription factors including NFAT, NF-κB, AP-1, T-bet, STAT1-6, ATF2/c-Jun, Ets-1and CREB can bind at the IFN-γ gene promoter and other intronic regulatory elements (Schoenborn & Wilson, 2007).

1.6.4 Interferon inducer poly(I:C)

dsRNA is an extremely efficient inducer of the IFN response (De Clercq, 2006). It is a molecular pattern associated with viral infection because it can be produced by most viruses at some point during their replication (Jacobs & Langland, 1996). Positive- and negative-stranded, and double-stranded RNA viruses can generate dsRNA via transcription/replication, and DNA viruses were proposed to generate dsRNA as a result of convergent transcription (Marcus, 1983).

Poly (I:C) is a synthetic form of dsRNA that can be used experimentally to elicit IFN responses. It is recognized by distinct receptors depending on its location. When added to the culture medium, extracellular poly(I:C) that is internalised is mainly recognized by endosomally localized TLR3 which has been considered to be essential for mediating NF-κB-inducible gene activation (Alexopoulou et al, 2001; Nishiya et al, 2005). However, transfected intracellular dsRNA tends to be sensed by cytosolic MDA-5 and RIG-I (Gitlin et al, 2006; Kato et al, 2006a). Poly(I:C) interacting with these various receptors activates different signalling pathways which involve various adaptor proteins, eventually leading to the activation of TBK-1 and IRF3, and the consequent induction of type I IFNs and many IFN-stimulated genes (ISGs), such as RANTES, ISG15, ISG54 and ISG56 (Grandvaux et al, 2002). Furthermore, poly(I:C) also can induce the activation of NF-κB (Alexopoulou et

al, 2001; Faure et al, 2000), followed by the release of TNFα and the expression of some NK-κB-dependent genes such IL-6 and IL-8 (Collart et al, 1990; Shakhov et al, 1990).

1.7 IFNβ induction related transcription factors

1.7.1 The IRF family

The IRFs play a critical role in many cell activities such as antiviral defence, immune response, cell growth regulation, tumour suppression and apoptosis (Honda et al, 2006). To date, nine IRF family members in human have been identified (Paun & Pitha, 2007; Taniguchi et al, 2001). They share extensive homology in the conserved N-terminal 115 amino acids containing a DNA binding domain (DBD), which specifically recognizes the ISRE motifs (GAAANN and AANNNGAA sequences) in ISGs (Escalante et al, 1998). Each IRF contains an unique C-terminal domain, termed the IRF association domain (IAD); the unique function of a particular IRF is accounted for by interactions of its specific IAD with other members of the IRF family or other transcription factors and co-factors that act as transcriptional activators and/or repressors (Taniguchi et al, 2001). IRF1, IRF2, IRF3 and IRF9 are expressed in most cell types, whereas IRF4, IRF7 and IRF8 are preferentially expressed in the cells of the immune system (Mamane et al, 1999; Taniguchi et al, 2001). IRF3, IRF5, IRF7 and IRF9 are required for type I IFN production and activation of antiviral defence systems, while, IRF1, IRF4 and IRF8 are essential for specific immune responses (Mamane et al, 1999; Taki et al, 1997).

1.7.2 IRF3

Numerous studies have demonstrated that IRF3 plays a critical role in the antiviral response. The IRF3 gene consists of 8 exons and encodes a 427-amino acid protein. IRF3 is a

phosphoprotein and consists of an N-terminal DBD, a C-terminal IAD, and a transactivation domain (TAD) (Lin et al, 1999). It has many splicing variants and ectopic expression of these variants can modulate transactivation capacity of IRF3 on the IFNβ promoter to varying degrees (Karpova et al, 2001; Li et al, 2011). IRF3 is widely expressed in many types of cell lines. It is constitutively expressed at a low level in an autocrine or paracrine pattern to generate IFNβ, which then stimulates the expression of IRF7 and further amplifies the antiviral response (Levy et al, 2002; Marié et al, 1998). Virus and dsRNA can induce phosphorylation of C-terminal serine residues 385 and 386, as well as the serine/threonine cluster between amino acids 396 and 405, which is required for the transcriptional activity of IRF3 (Lin et al, 1998; Yoneyama et al, 2002; Yoneyama et al, 1998). Many viruses prevent IFN induction through targeting the function of IRF3 (Weber et al, 2004).

In un-stimulated cells IRF3 resides predominantly in the cytoplasm, but stimulation with PAMPs induces phosphorylation of its C-terminal serine/threonine residues and leads to exposure of both the DBD and IAD domains (Sharma et al, 2003; Yoneyama et al, 2002). This conformational change result in homo- or hetero- dimerization, and translocation to the nucleus, where IRF3 associates with CBP/p300 coactivator to stimulate the transcription of IFNβ as well as some ISGs (Lin et al, 2000; Lin et al, 1999). After viral infection, the phosphorylated form of IRF3 is rapidly shuttled from nucleus to cytoplasm and degraded via the proteasome pathway, thereby down-regulating the expression of IRF3 targeted genes again (Lin et al, 1998).

1.7.3 IRF7

IRF7, another member of the IRF family, is a multifunctional protein and is also the product of an ISG. Like IRF3, IRF7 also plays an important role in anti-viral responses. Its transcriptional activity, in a similar manner to IRF3, depends on C-terminal phosphorylation during viral infection (Honda et al, 2005; Lin et al, 2000). However, constitutive IRF7 expression is restricted to B cells and dendritic cells; in other cells, IRF7 is virus-inducible and IFN-inducible. IRF7 was thought not to be involved in the initial phase of IFNβ induction as it is expressed at very low levels in most cells in the absence of virus stimulation. IRF7 expression depends on activation of the constitutively expressed IRF3 (with low level IRF7) to activate the IFNβ promoter, the IFNβ so generated in turn leading to IRF7 expression via activated ISGF3 binding at an ISRE element present in IRF7 promoter. The newly produced IRF7 then undergoes activating phosphorylation, similar to IRF3, further activating the IFNβ promoter to achieve massive IFNα/β production through a positive feedback loop (Au et al, 1998; Hata et al, 2001; Levy et al, 2002; Sato et al, 2000). IRF7 is particularly important for the continued expression of IFNα during viral infection, and also contributes to induction of IFNβ by co-operation with IRF3. Subsequent studies by using knockout mice further demonstrated that transcription of both IFNα and IFNβ is dependent on IRF7. Absence of IRF7 or combined absence of IRF3 and IRF7 prevents induction of type I IFN following viral infection (Honda et al, 2005; Sato et al, 2000). This indicated that IRF7 is a master regulator of type I IFNs.

1.7.4 NF-κB

So far, five members of the mammalian NF-κB gene family have been identified: NF-κB1

(p50/p105), NF-κB2 (p52/p100), c-REL, RELA (p65) and RELB (I-REL). A functionally active NF-κB molecule consists of homodimers or heterodimers that contain various combinations of these proteins. The most abundant heterodimer in the majority of cells consists of subunits p65 and p50. In most unstimulated cells, inactive NF-κB is bound by inhibitor of NF-κB (IκB, predominantly IκBα) and sequestered in the cytoplasm. When signalling cascades are activated, IκB proteins are phosphorylated by IκB kinase (IKK) complex followed by ubiquitination and degradation by 26S proteasome (Wullaert et al, 2006). Consequently, dissociation of IκB proteins from NF-κB unmasks the nuclear localization sequence (NLS) of p65. The released p50/p65 NF-κB dimers translocate into the nucleus and bind to specific κB consensus sequences in the chromatin, to regulate a specific group of genes. The transcriptional activity of NF-κB is also regulated by its acetylation state. p65 can be acetylated by both p300 and PCAF on lysines 122 and 123, acetylation of p65 reduces its ability to bind κB-DNA, facilitates its removal from DNA and consequently its IκBα-mediated export from the nucleus. Therefore acetylation of NF-κB modulates its nuclear retention and its interaction with transcriptional coactivators and/or corepressors (Chen & Greene, 2004). The central role of NF-κB is to regulate the expression of hundreds of genes in response to a wide range of stimuli including bacterial and viral pathogens. These NF-κB-inducible genes, including cytokines, cell adhesion molecules, complement factors and a variety of immune receptors, are involved in mammalian immune and inflammatory responses. In addition, NF-κB is also implicated as an important regulator of cellular processes such as apoptosis, cell proliferation and differentiation.

1.8 Interferon downstream signalling

1.8.1 IFN (α/β) JAK-STATs signalling pathway

Type I IFN, secreted from its producer cell, binds to the heterodimeric receptors IFNAR1 and IFNAR2 on this and other cells. This initiates the most well-defined cell signalling pathway Janus kinase-STAT (JAK-STAT) signalling pathway (Figure 1.7) (González-Navajas et al, 2012; Platanias, 2005). Receptor binding leads to signal transducer and activator of transcription 1 (STAT1) and STAT2 being phosphorylated by Tyk2 and Jak1, respectively. The phosphorylated STAT1 and STAT2 form a stable heterodimer that is then transported into nucleus. There the STAT1-STAT2 complex associates with a monomer of IRF9 to form the interferon-stimulated gene factor 3 (ISGF3) heterotrimer (Darnell et al, 1994; González-Navajas et al, 2012; Stark et al, 1998) ISGF3 binds to interferon-stimulated response elements (ISREs) (NGAAANNGAAAG/CN), which usually reside within the promoters of ISGs (Darnell et al, 1994), products of which, together with IFNα/β, establish an antiviral response in target cells (Stark et al, 1998).

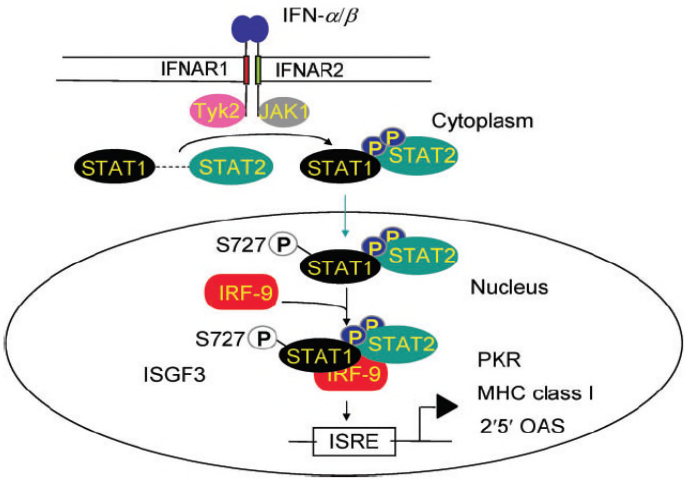


Figure 1.7 JAK-STAT signalling pathway of type I IFN

(Taken from Richard E. Randall1 and Stephen Goodbourn, 2008)

1.8.2 IFNγ-mediated signalling

IFNγ has been studied extensively as a transcriptional regulator, in a pathway that has similarities with the signalling of type I IFN (Figure 1.8). The majority of IFNγ responsive genes are induced through the interaction of STAT1 homodimers and IFNγ activated site (GAS). Briefly, the IFNγ was recognized by its receptor IFNGR1 and IFNGR2, which are then phosphorylated by JAKs. The phosphorylation of IFNGR generates a binding site for STAT1. Phosphorylation of STAT1 on Tyr701 results in the formation of STAT1 homodimers, which translocate to the nucleus, bind to GAS (TTNCNNNAA) and enhance transcriptional activation by recruiting other transcriptional coactivators such as CBP (Sadzak et al, 2008). The STAT1/STAT1 homodimer can also be induced by type I IFN (Platanias, 2005). IFNγ also induces transcription of a subset of genes in a GAS-independent fashion through ISRE elements (Majumder et al, 1998). IFNγ-modulated genes can be classified into primary or secondary responsive genes. Primary responsive genes are induced early due to the binding of STAT1 dimers to GAS elements present in promoters of target genes, e.g. IRF1, CXCL9 (MIG1) and CXCL10. The secondary responsive genes are induced following the binding of IRF1 to ISRE located in promoters of target genes.

In addition to the JAK-STAT pathway, IFNs can activate several other signalling cascades that have crucial roles in their different biological properties, for example, p38 MAPK signalling and phosphatidylinositol 3-kinase (PI3K) signalling (González-Navajas et al, 2012; Li et al, 2004; Platanias, 2005).

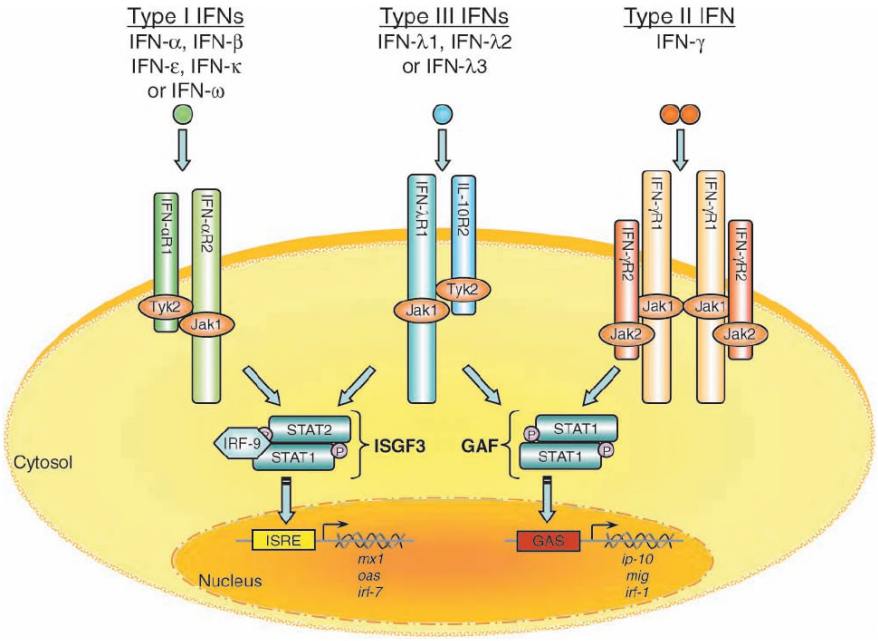


Figure 1.8 The similarity among IFNs downstream signalling

(Taken from Raymond P Donnelly & Sergei V. Kotenko, 2010)

1.8.3 IFNλs downstream signalling

IFNλs signal through a receptor complex comprised of IL-10R2 and a unique subunit, IFNλR1. Although IFNλR1 is constitutively expressed by a broad range of cell lines and tissues, there are many cell types that do not express IFNλ receptors, and, as a consequence, cannot respond to treatment with this cytokine. IL-28 and IL-29 (IFNλ) are able to induce STAT1 phosphorylation, resulting in the activation of STAT1 and STAT2 and the formation with IRF9 of ISGF3 complex which translocates to the nucleus to initiate the induction of target genes (Figure 1.8) (Kotenko et al, 2003). IFNλs also increase mRNA levels for STAT1, STAT2 and IRF9 whose products further enhance the signal of IFNλs and contribute to a prolonged induction of target genes (Maher et al, 2008). Both IL-28 and IL-29 activate mostly identical genes. Among these are numerous genes involved in IFN-

mediated immunity and antiviral defence, such as Mx1, 2', 5'-oligoadenylate synthetase 1 (OAS1), PKR, ISG54, ISG56, ISG60, TAP1 and ISG15 (Diegelmann et al, 2010). Taken together, the IFNλ signal transduction cascade is very similar to that induced by type I IFNs (IFNα/β) (Figure 1.8), and hence they function like type I IFN in biological activities such as antiviral activity.

1.9 Transcription factors utilized in Jak/STATs signalling pathway

1.9.1 IRF9

IRF9 can be expressed in a variety of tissues and is essential for antiviral responses signalled by IFNα/β and IFNγ (Bluyssen et al, 1996; Darnell et al, 1994). IRF9 was originally discovered as a DNA-binding subunit of interferon stimulated gene factor 3 (ISGF3) also termed p48 (Bluyssen et al, 1996; Kessler et al, 1990; Levy et al, 1989). Besides IRF9, ISGF3 also contains STAT1 and STAT2. IRF9 can also form a DNA binding complex with the STAT1 homodimer and with STAT2 alone. These complexes can bind to DNA with the same specificity as ISGF3 (Kraus et al, 2003).

1.9.2 STATs

STAT1 and STAT2 were firstly identified as mediators of the cellular response to IFNs. Since then five more mammalian STAT proteins including STAT3, STAT4, STAT5A, STAT5B and STAT6 were identified (Darnell, 1997; Ihle, 2001; Levy & Darnell, 2002). STAT gene transcripts can be differentially spliced. At least a dozen STAT isoforms are thought to be generated due to STAT mRNA alternative splicing (Dorritie et al, 2014). These proteins have distinct functions in various cellular activities (Dorritie et al, 2014; Subramaniam et al, 2013). In response to distinct stimuli, STAT proteins induce the

transcription of different genes that can elicit various physiological outcomes. STAT1 can be activated by many different ligands. In contrast, STAT2 is activated only by IFNα (Darnell, 1997; Darnell et al, 1994). Upon activation with IFN-α, -β, and -γ, JAKs phosphorylate tyrosine sites on the cytoplasmic tail of the receptor, and these serve as docking sites for the SH2 domains in all the STATs (Fu, 1992) . Tyrosine phosphorylation is required for all STATs activation. Phosphorylation STAT1 residue 701 leads to its homodimerization or heterodimerization with other STATs through SH2-domain interactions (Fu, 1992; Gutch et al, 1992).

Phosphorylated dimers move into the nucleus and that enables STATs to bind to consensus sequences TTCC(C/G) GGAA (or generically TTN5AA, where N represents any nucleotide) in target genes (Horvath et al, 1995; Xu et al, 1996). However, different STAT dimers or heterodimers can bind DNA specifically. In response to particular ligands the natural sites from regulated genes have clear preferential binding affinities for the different STATs (Darnell, 1997). The activation of STAT is transient, like many other transcriptionally active proteins c-Fos, c-Jun and p53 that have short half-lives (Darnell, 1997; Stancovski et al, 1995), which suggests that STATs can either be dephosphorylated by a protein tyrosine phosphatase or be destroyed. Following dephosphorylation in the nucleus, STAT1 and 2 redistribute to the cytoplasm (Banninger & Reich, 2004).

Interaction with co-activator such as CBP/p300 is thought to be an important step for STAT1 and STAT2 mediated-gene activation as CBP/p300 serves as a bridge between STAT1/2 and RNA polymerase II (pol II) (Bhattacharya et al, 1996). The COOH-terminus

of STAT2, a region containing the trans-activation domain, interacts with p300. STAT1 also interacts with CBP or p300 in at least two different sites on each molecule (Zhang et al, 1996).

1.10 IFN-stimulated genes (ISGs) and activation of ISGs

1.10.1 ISG activation and function

Since the first ISGs were discovered 30 years ago (Knight & Korant, 1979; Larner et al, 1984), several hundred ISGs have been identified using genome-scale microarray and knockout techniques (de Veer et al, 2001; Der et al, 1998; Sarasin-Filipowicz et al, 2008). ISGs genes are not expressed or expressed at low level in uninfected cells but they are transcriptionally induced immediately after virus infection (Sarkar & Sen, 2004). ISGs can be induced either by a unique inducer or by multiple inducers (Der et al, 1998; Geiss et al, 2001). ISGs can be induced by dsRNA or virally-induced cytokines such as IFNs (Der et al, 1998; Geiss et al, 2001). In addition, some ISGs are directly induced by viral infection in the absence of IFN production (Sen & Peters, 2007). ISGs also can be activated by IRFs such as IRF1 and IRF7 (Schmid et al, 2010; Schoggins et al, 2011).

ISGs are activated by distinct signalling pathways mainly through ISGF3 and hetero/homodimers of STAT1/2 binding at ISRE and GAS elements (Peters et al, 2002; Sato et al, 2000). ISGs also can be induced through phosphorylated-IRF3-CBP binding at IRFs-E/ISRE elements of certain ISGs promoters.

1.10.2 Induction profiles of ISGs in response to different IFNs

Hundreds of ISGs, that cover many defining functional areas, have been identified in the past decades. However, not all the genes are regulated/induced by all types of IFNs, and the level of induction by different IFNs varies (Ackrill et al, 1991). MHC Class I genes and STAT1 are responsive to all IFNs but 2', 5'-oligoadenylate synthetase 1 (OAS1) is induced selectively by IFNα/β. Among the genes regulated by type I IFN, the induction profiles for specific genes are different; it has been well-established that different IFNα subtypes and IFNβ can also manifest distinct biological responses despite binding the same IFNARs receptor (Hilkens et al, 2003). Compared with the number of genes regulated by either IFNα or IFNγ, IFNβ stimulation resulted in twice as many genes being transcribed (Der et al, 1998); genes preferentially induced by IFNβ over IFNα include PKR, ISG54, ISG56, HIF-1α, STAT1 and GBP-2 (Rani et al, 1996). There is no report of ISGs that are preferentially induced by IFNα but not IFNβ. Some ISGs are preferentially induced by IFNγ include IRF1, IP-10 and CIITA.

1.10.3 Classification of ISGs

The ISGs can also be categorized into distinct groups in terms of the activation of cis-regulatory element/induction mechanism (Nakaya et al, 2001). The first group is "ISGF3 only" group; the expression of these ISGs (including OAS, PKR and IRF7) is totally dependent on the IFNα/β-activated pathway leading to ISGF3 formation. The second group is the "ISGF3/IRF3" group, including ISG15, ISG54, ISG56 and IP-10, which can be activated by both PAMP signalling to IRF3 through the phosphorylated IRF3-CBP pathway and by IFNα/β through the classical JAK-STATs pathway (Daly & Reich, 1995; Weaver et al, 1998). IFNα can be placed into an "IRF7 only" group, although IRF3 may also

participate in the induction of some IFNα family members in collaboration with IRF7 (Au et al, 1998; Marié et al, 1998). Finally, the well-known IFNβ belongs to the fourth group, the "IRF3/IRF7" group.

1.10.4 IRF3-dependent ISGs

In addition to activation by ISGF3, some ISGs also can be activated by IRF family members, particularly IRF3 (Schmid et al, 2010; Schoggins et al, 2011). Thus IRF3 not only cooperates with other transcription factors to regulate expression of IFNβ, but also regulates a subset of type I ISGs independent of the expression of IFN (Daly & Reich, 1993; Daly & Reich, 1995; Honda et al, 2006); examples include ISG15, ISG20, ISG54, ISG56 and CCL5 (RANTES). Phosphorylated IRF3 associates with CBP/p300 and this complex can directly bind promoter sequence (ISRE) of some IRF3-dependent ISGs, leading to induction (Bluyssen et al, 1994). This binding and activation of ISGs directly by IRFs occurs because of the similarity of sequences in particular ISREs to the PRDIII/I element within the IFNβ promoter (Au et al, 1995; Daly & Reich, 1995; Weaver et al, 1998). IRF3-mediated ISG production is an intrinsic antiviral mechanism that allows host cells to rapidly establish an antiviral state before IFN can be produced (Schmid et al, 2010; Schoggins et al, 2011).

1.11 Examples of specific ISGs relevant to this study

1.11.1 ISG15

Among a large number of ISGs, the ubiquitin-like protein (UBL) ISG15 is one of the most strongly induced proteins. Similar to ubiquitin and other UBLs, ISG15 is attached to a variety of target proteins (substrates) on lysine side chains through a C-terminal glycine-glycine motif. The conjugation of ISG15 is a three-step enzymatic cascade, commonly referred to as ISGylation which utilizes a mechanism similar to ubiquitin (Skaug & Chen,

2010). Although ISG15 was the first discovered UBL protein, its biological role and mechanism of action are less well understood than those of most of the other UBLs such as SUMO. Numerous studies have identified nearly 200 ISG15 targeted proteins which span a diverse array of biological processes such as protein translation, cell cycle regulation, signal transduction and immune regulation (Durfee & Huibregtse, 2010; Malakhov et al, 2003; Zhao et al, 2005).

The robust induction of ISG15 in response to IFN treatment or viral infection implies a role for ISG15 in antiviral defense. Over the past few years, a growing body of work strongly suggests a role for ISG15 in defense against many viral pathogens including influenza, vaccinia, Sendai and Japanese encephalitis viruses (Jeon et al, 2010; Lenschow, 2010). Although the precise mechanism of antiviral activity of ISG15 is unknown, recent studies have provided some insight that ISG15 acts mainly through disruption of virus budding, via modification of viral and host proteins (Skaug & Chen, 2010). For example, overexpression of ISG15 in cell culture mimicked the ability of type I IFN to block the release of HIV particles and Ebola virus VP40 VLP from cells. Overexpression of ISG15 inhibited the ubiquitination of both HIV Gag and host Tsg101, disrupting the interaction between these proteins that was known to be critical for efficient viral budding (Okumura et al, 2006). ISG15 also plays an important role in the modification of immunity-related proteins such as STAT1, IRF3, JAK1, RIG-I, ISG56, PKR and MxA (Malakhov et al, 2003; Zhao et al, 2005). Recently, it was reported that ISG15 enhances the innate antiviral response by inhibition of IRF3 degradation (Lu et al, 2006; Shi et al, 2010).

1.11.2 ISG20

Expression of the human ISG20 gene can be modulated by type I and type II IFN (Espert et al, 2004; Gongora et al, 2000). Besides the ISRE element, the TATA-less ISG20 promoter also contains one GAS element and NF-κB and Sp1 binding sites suggesting that it could be induced by other stimuli. Transcriptional signals generated by dsRNA results in a marked increase in ISG20 expression because of the ISRE and NF-κB sites present in the promoter (Espert et al, 2004). However, this is a biphasic process, while the NF-κB site is absolutely required for the first phase of expression, the ISRE element is only involved in the delayed response to dsRNA (Espert et al, 2004). ISG20 protein was reported to closely associate with PML and Sp100 proteins in the PML-NB (Gongora et al, 1997). Biochemical analyses demonstrated that ISG20 is a 3'-5' exonuclease with a strong preference for RNA (Nguyen et al, 2001), making it the second known IFN-regulated RNase along with RNase L. Previous studies have suggested ISG20 was a major effector of innate immune response against various pathogens including viruses, bacteria and parasites (Degols et al, 2007; Espert et al, 2003). Overexpressed ISG20 can restrict infections of many viruses such as EMCV, vesicular stomatitis virus, influenza virus, HIV and Sindbis virus (Espert et al, 2003; Zhang et al, 2007).

1.11.3 ISG56

ISG56 belongs to the ISG56/IFIT1 gene family that encode a cluster of structurally related proteins that are induced in response to outside stresses. In humans, there are three other members of the ISG56 family, ISG60 (protein P60), ISG58 (protein P58), and ISG54 (protein P54) (Fensterl & Sen, 2011; Sarkar & Sen, 2004). In a majority of cell lines, ISG56 does not express at a detectable level in the absence of stimuli. Numerous RNA or DNA

viruses, such as Sendai virus, respiratory syncytial virus, cytomegalovirus and adenovirus, and molecular patterns such as dsRNA or lipopolysaccharides can efficiently induce the transcription of the ISG56 gene (Fensterl & Sen, 2011). The most potent inducers are IFNα/β, whereas IFNγ is a weak inducer (Der et al, 1998). Induction of ISG56 family genes is generally rapid as well as transient in response to IFN treatment (Kusari & Sen, 1986). However, the expression kinetics of ISG56 family genes differ based on the different cell line, the inducer and its signalling pathway. In HT1080 and HEK293 cells, IFN-induced ISG56 and ISG54 mRNA levels are maintained at high level even 24 hour after IFN treatment (Terenzi et al, 2006).

ISG56 inhibits cellular translation by binding to specific subunits of eIF3 and inhibiting the functions of eIF3 during the initiation step of translation (Terenzi et al, 2006; Wang et al, 2003). This function can be considered as part of a nonspecific antiviral program. More specific antiviral activities of ISG56 have been continuously emerging in recent years (Terenzi et al, 2008; Wang et al, 2003). Expression of human ISG56 protein was reported to disrupt the STING/MAVS and STING/TBK1 complexes through binding STING after Sendai virus infection or dsRNA transfection. Consequently, ISG56 inhibits the induction of IRF3 and NF-κB dependent genes and activation of the IFNβ gene in what can be seen as a negative feedback loop (Li et al, 2009).

1.11.4 ISG54

ISG54 is also a prominent member of human ISG56 viral stress-inducible gene family (Levy et al, 1986; Wathelet et al, 1986). ISG54 and ISG56 genes are closely related and located on chromosome 10; the encoded proteins have 42% sequence identity. The two genes are evolutionally related and functionally similar in inhibiting the action of the initiation factor eIF3 (Fensterl & Sen, 2011; Pain, 1996). Both are strongly induced in response to IFN, dsRNA and infection by many viruses (Guo et al, 2000b; Sarkar & Sen, 2004). But the

kinetics of their induction is not fully similar in response to different stimulation (Terenzi et al, 2006). For example, both mRNAs were induced to high level after 6 hours IFN treatment, but ISG54 mRNA level decreased with time, whereas ISG56 mRNA stayed at the same level over 24 hours. ISG54 and ISG56 not only can be induced by ISGF3 complex, but can also be induced in response to viral infection, independent of IFN but dependent on activation of IRF3 (Andersen et al, 2008; Grandvaux et al, 2002; Levy et al, 1986). Thus ISG54 and ISG56 are widely used as marker genes for detecting signalling by the JAK/STAT pathway or the IRF3/IRF7 pathway. Recently, ISG54 was suggested to have a role in the induction of apoptosis via a mitochondrial pathway (Stawowczyk et al, 2011).

1.11.5 PKR

PKR, a dsRNA-dependent serine-threonine protein kinase, plays a critical role in the antiviral defence mechanism of the host (García et al, 2006). PKR has also been implicated in the control of cell growth and proliferation with tumour suppressor function (Meurs et al, 1993). PKR is expressed constitutively in mammalian cells, in response to cellular, viral or synthetic poly(I:C) origin dsRNA. PKR expression can also be activated by a group of other activators such as pro-inflammatory stimuli, growth factors, cytokines and oxidative stress.

A critical role PKR mediates is to phosphorylate the eukaryotic translation initiation factor eIF-2α (Galabru & Hovanessian, 1987; Hovanessian, 1989). Impairing eIF-2 activity inhibits further cellular mRNA translation (Rhoads, 1993). PKR not only affects translation, it also has a role in signal transduction and transcriptional control through influencing various transcriptional factors such as STATs, IRF1, p53, JNK and p38, as well as engaging the NF-

κB pathway (García et al, 2006; Verma et al, 1995). Since these transcription factors regulate the expression of many cellular genes, it is anticipated that PKR controls the expression of multiple genes through engagement of multiple transcription pathways (Guerra et al, 2006; Kazemi et al, 2004).

1.11.6 IP-10

Interferon-γ-inducible protein CXCL10 (IP-10) expression can also be induced by other stimuli during infection or inflammation, such as TNFα, dsRNA or type I IFNs (Clarke et al, 2010; Geiss et al, 2001; Ohmori & Hamilton, 1993; Sarkar & Sen, 2004). The promoter region sequence of human IP-10 contains multiple regulatory elements, including ISRE element, GAS element and NF-κB-binding site (Saha et al, 2010; Spurrell et al, 2005). IFN-induced IP-10 expression is mainly mediated via the ISRE elements or GAS elements bound by IRF1, IRF9 (p48) and STAT-1, whereas in response to IL-1β and TNFα, transcription is mediated through NF-κB (Majumder et al, 1998; Yeruva et al, 2008). IP-10 can be induced by IFNγ alone, and can be synergistically induced by TNFα or IL-1β and IFNγ (Clarke et al, 2010; Majumder et al, 1998).

1.12 Transcription regulation

1.12.1 Transcription factor

In humans, it has been estimated that there are ~1,400 DNA sequence-specific transcription factors (TFs) (Vaquerizas et al, 2009; Venter et al, 2001). TFs can bind proximal promoters and bind enhancers (Lee & Young, 2000). Those binding close to transcriptional start sites (TSS) have been proposed to regulate transcription by stabilizing general transcription factors at the core promoter elements while others bind to enhancers, either upstream or

downstream of a gene, and regulate transcription by mediating, through a looping mechanism, protein-protein contacts between distal complexes and the general transcriptional machinery bound at TSSs (Farnham, 2009). In addition to sequence-specific TFs, there are about 200-300 transcription factors that can bind to core promoter elements consisting of the components of general transcriptional machinery, including pol II and TFIID (Venter et al, 2001). In recent years, large-scale ChIP-chip and ChIP-seq analyses of transcription factor binding has suggested that binding is focused near to CpG islands or within 1-5 kb of the TSS of known genes (Barrera & Ren, 2006; Robertson et al, 2007; Rye et al, 2011; Weinmann et al, 2002). However, transcription factors do not always bind exclusively at proximal promoters. Some TFs bind to diverse regions of the genome, including extragenic regions distant from the TSS and intragenic regions such as GATA1 and zinc finger protein 263, NF-κB and STAT1; some TFs have widespread binding patterns, including p53, p63, forkhead box protein A2 (FOXA2) and transcription factor 4 (TCF4) (Cawley et al, 2004; Farnham, 2009; Satoh & Tabunoki, 2013; Wederell et al, 2008; Wong et al, 2011; Yang et al, 2006).

1.12.2 Promoter

Transcriptional regulation of eukaryotic genes is regulated by *cis* regulatory elements including promoters and enhancers, and by proteins that bind to these elements. Promoters are composed of common sequence elements, such as a TATA box and an initiator sequence, and binding sites for other transcription factors, which work together to recruit the general transcriptional machinery to the TSS. Usually the general transcription factors bind to core promoter but the transcriptional activity is very low. But as an increasing number of

activated site-specific TFs binding to proximal promoter regions the transcription activity is increased. Promoter activity can be further stimulated by the binding of factors to distal enhancer regions (section 1.12.3) and the subsequent recruitment of histone modifying enzymes (section 1.13) that create a more favourable chromatin environment for transcription, or by a kinase that induces a bound initiation complex to begin elongation. Transcriptional activity can also be modulated by repressive factors that bind to repressing sequences and/or silencers far from the TSS, which can interfere with transcription activator or co-activator binding at the promoter or enhancer.

1.12.3 Enhancer

An enhancer is a short region of DNA that can be bound with proteins including a set of TFs to enhance transcription levels of genes whilst not requiring to be in a precise location and orientation with respect to the TSS. It may be located upstream or downstream from the site of transcription initiation of the gene it regulates. Enhancer is bound by combinations of different TFs which can cluster closely to each other to regulate gene transcription cooperatively (Mann & Carroll, 2002). An extensively studied mammalian enhancer is the one associated with the IFNβ gene (Maniatis et al, 1998), on which the IFNβ enhanceosome comprising eight TFs assembles, bound to overlapping elements within a 55 bp region upstream of the IFNβ gene. Several other enhancer regions have also been well studied, including the human growth hormone and major histocompatibility complex II (MHC II) enhancer regions (Dean, 2006).

1.13 Histone, pol II and CBP/p300

1.13.1 Histone modification and acetylation

Eukaryotic chromatin consists of many fundamental unit nucleosomes and genomic DNA. Each nucleosome consists of a protein octamer of two molecules each of histone H2A, H2B, H3 and H4, wrapped by 147 bp of genomic DNA. The amino terminal tails of the histones extend from the core structure and are subject to post-translational modifications (PTMs) such as acetylation, phosphorylation, methylation, ubiquitylation and Sumoylation (Li et al, 2007). These modifications can influence nucleosome stability packing, and facilitate recruiting other TFs; modification of a histone at one amino acid position can influence the type of modification at other positions. Remarkable progress has been made during the past few years in the characterization of histone modifications on a genome-wide scale. The general picture emerging is that promoter regions of active genes have reduced nucleosome occupancy and elevated histone acetylation (Barrera & Ren, 2006; Kim et al, 2005; Wang et al, 2009). The modifications marking either active or inactive genes are highly specific. High levels of histone H3K9 acetylation and H3K4 methylation are detected in promoter regions of active genes (Barski et al, 2007; Bernstein et al, 2005; Kim et al, 2005; Roh et al, 2007), whereas elevated levels of H3K27 methylation correlates with gene repression (Boyer et al, 2006; Lee et al, 2006; Roh et al, 2006).

1.13.2 Histone methylation

Significant progress has also been made in characterizing global levels of histone methylation modifications in mammals. High levels of H3K4me1, H3K4me2, and H3K4me3 are detected surrounding TSSs. In addition to the promoter regions, these

modifications are also detected in intergenic regions. The H3 K4/9acetylation and H3K4 methylation signals outside of promoter regions have been correlated with functional enhancers in various cell types (Heintzman et al, 2007; Roh et al, 2007). The mono methylations of H3K27, H3K9, H4K20, H3K79 and H2BK5 are all linked to gene activation, whereas trimethylations of H3K27, H3K9, and H3K79 are linked to gene repression. Although H3K9 methylation has been implicated in heterochromatin formation and gene silencing, a large-scale analysis suggested that H3K9me3 is enriched in many active promoters. In a recent genome-wide ChIP-seq analysis, a significant dip in the signal was observed between -200 to +50 for H3K4me3, which correlated with the nucleosome loss in active genes. A series of peaks of H4K4me3 signals at +50, +210, and +360 were detected, suggesting similar nucleosome positioning relative to TSS in active genes (Barski et al, 2007)

The levels of H3K4me1 and H3K4me2 positively correlated with transcriptional levels. However, methylation of H3K27 correlated with gene repression (Boyer et al, 2006; Lee et al, 2006; Roh et al, 2006). Indeed, H3K27me3 levels were higher at silent promoters than at active promoters (Barski et al, 2007). Also, high levels of H3K4me1 combined with low levels of H3K4me3 as a signature for predicting enhancers was also observed (Heintzman et al, 2007). In summary, active genes are characterized by high levels of H3K4me1, H3K4me2, H3K4me3 and H3K9me. In contrast, inactive genes are characterized by low or negligible levels of H3K4 methylation at promoter regions, high levels of H3K27me3 and H3K79me3 in promoter and gene-body regions.

1.13.3 RNA Pol II

The synthesis of eukaryotic mRNA is carried out by RNA pol II. During this process, pol II associates transiently with many different factors, including the general transcription factors TFIIB, TFIID, TFIIE, TFIIF and TFIIH, coactivators and elongation factors. Pol II is sufficient to catalyse DNA-directed RNA synthesis, but it is unable to recognize promoter DNA on its own. Thus, general transcription factors TFIIB, -D, -E, -F, and -H, which mediate promoter recognition and opening, are required for transcription initiation.

Although the general transcription factors and pol II are sufficient for accurate transcription initiation *in vitro*, these components alone fail to respond to activator proteins bound to enhancer or upstream activation sequences. Mediator, an enormous complex composed of many subunits, is required for transcription from most pol II promoters. It appears to function as a 'control panel' that integrates regulatory signals from enhancer-bound activators, and transduces this information to pol II and the general transcription factors (Sikorski & Buratowski, 2009). The composition of Mediator complexes is different, depending upon the specific activator, suggesting that Mediator is a dynamic complex that allows for mixing-and-matching of sub complexes in response to different activator or repressor requirements.

1.13.4 CBP

p300 and CBP were originally identified as proteins that bound to the adenoviral E1A and the cAMP-response-element binding protein (CREB), respectively (Chrivia et al, 1993; Eckner et al, 1994a). The human CBP gene is located in chromosomal region 16p13.3.

Interestingly, this region shows extensive homology to a region on chromosome 22 where the p300 gene resides (Eckner et al, 1994b). Thus CBP and its paralog p300 are functionally closely related, both versatile transcriptional co-activators that can influence many physiological processes including proliferation, differentiation and apoptosis (Giordano & Avantaggiati, 1999; Goodman & Smolik, 2000).

CBP and p300 are thought to regulate gene expression acting as adaptor molecules, interacting both with a wide variety of TFs and with components of the basal transcriptional machinery, including TBP, TFIIB, TFIIE and TFIIF (Figure 1.9) (Goodman & Smolik, 2000; Wang et al, 2013). Therefore, p300/CBP is thought provide such a bridge to the transcriptional machinery. Study of the IFNβ enhancer has shown that the surface of p300/CBP provides a scaffold for different components of the transcription apparatus (Kim et al, 1998; Munshi et al, 1998). ATF2/JUN, NF-κB p50/p65 and IRF3/IRF7 can be bound to CBP/p300 to form the IFNβ enhancesome (Munshi et al, 1998).

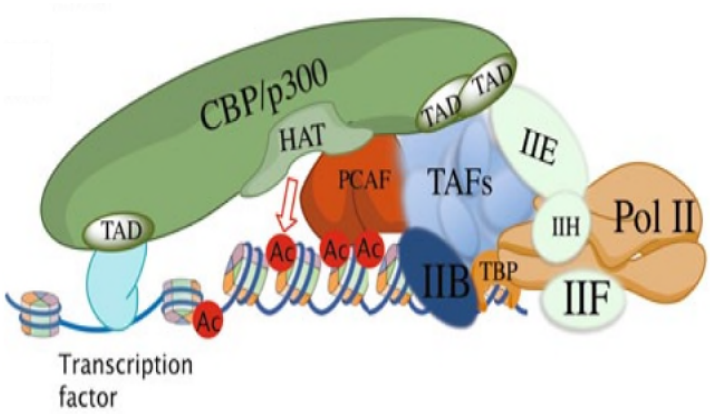


Figure 1.9 CBP and gene transcription

(Taken fromWang Feng, 2013)

Another important function of CBP is to acetylate multiple sites in the core histone tails through its HAT activity (Figure 1.9). Acetylation of lysine residues of histone tails helps transcription factors access the DNA in chromatin (Vettese-Dadey et al, 1996), may also weaken internucleosomal interactions and de-stabilise higher-order chromatin structure (Garcia-Ramirez et al, 1995; Luger et al, 1997; Tse et al, 1998), and may therefore facilitate the processivity of pol II through nucleosome arrays (Nightingale et al, 1998). Besides acetylating all four histones, CBP/p300 HATs have also been shown to modify other proteins (Figure 1.9) (Wang et al, 2013). Examples include tumour suppressor protein p53, acetylation of which results in an enhancement of its DNA binding activity (Gu & Roeder, 1997), NF-κB (RelA/p65) (Huang et al, 2010; Kiernan et al, 2003), STAT1(Zhuang, 2013) and the basal transcription factors TFIIE and TFIIB (Chen et al, 2001).

As discussed (section 1.13.1), specific histone acetylation is closely correlated with transcriptional activity in eukaryotic cells. To date, four mainly multi-gene families of nuclear proteins have been described that possess HAT activity including GCN5 and P/CAF, p300 and CBP (Bedford et al, 2010). GCN5 preferentially acetylates H3 and H4 histones (Kuo et al, 1996) whereas CBP can acetylate all four histones (Bannister & Kouzarides, 1996). CBP and p300 have unique functions that cannot be substituted for by other HATs. For instance, the acetylation of histone H3 lysine 18 is completely dependent on CBP/p300 *in vivo* (Kasper et al, 2010).

1.14 The aim of this research project

Previous studies have demonstrated that PML is linked with transcription regulation through its interaction with TFs, co-activators or co-repressors (Cairo et al, 2005; Doucas et al, 1999; Guo et al, 2000a; Khan et al, 2001; Wu et al, 2001; Wu et al, 2003; Zhong et al, 2000b). In particular, PML protein was shown to regulate IFNγ signalling by affecting STAT-1 DNA binding (Choi et al, 2006; El Bougrini et al, 2011). In addition, PML enhances IFNγ-induced MHC Class II expression, with PML-II in particular binding the key transcription factor CIITA of these genes. (Ulbricht et al, 2012). Most recent, PML-II was reported to preferentially interact with the Ad transactivator E1A-13S, and thus to enhance E1A-mediated transcriptional activation (Berscheminski et al, 2013). These studies imply that PML protein, in particular PML-II, has a regulation function in gene transcription. Previous work had shown that PML-II was targeted specifically during adenovirus infection by a protein responsible for allowing the virus to evade the IFN response (Hoppe et al, 2006), suggesting that this isoform in particular might have a role in the IFN response, PML-II is also one of two isoforms shown to be inhibitory to HSV1 infection (Cuchet et al, 2011). Therefore, the aim of this research project is to explain the role of PML-II in IFN and ISG induction and downstream signalling, and to establish a mechanism for its action in these processes.

Chapter 2 Materials and methods

2.1 Materials

This section includes the collection of biochemical reagents, cell lines, virus strains, bacterial strains, antibodies, plasmids, siRNA sequences, mutagenesis PCR, qPCR, ChIP-qPCR primers and consumer materials that were utilized in this study.

Table 2.1.1 List of reagents and suppliers

Reagents	Supplier
Anti-FLAG M2 Affinity Gel	Sigma
Bright-Glo Luciferase Assay System	Promega
Brilliant III Ultra-Fast SYBR Green qPCR Master Mix	Agilent Technologies
DMEM (41965)	Gibco
ECL prime (WB substrate)	GE healthcare
Etoposide	Sigma
GenElute Mammalian Total RNA Miniprep Kit	Sigma-Aldrich
GFX PCR DNA and Gel Band Purification Kit	GH Healthcare
IFNα	Pbl interferon source
Lipofectamine 2000	Invitrogen
Luciferase Cell Culture Lysis 5X Reagent	Promega
MG132	Sigma-Aldrich
NBCS	Gibco
PageRuler Plus Prestained Protein Ladder	Thermo scientific
Pfu DNA polymerase	Fermentas
poly(I:C)	Sigma
Protein A Sepharose CL-4B beads	GE Healthcare
Proteinase K	Sigma-Aldrich
PureYield Plasmid Midiprep system	Promega
QIAprep Spin Miniprep Kit	QIAGEN
Random Primers	Promoga
Recombinant Human IFNγ	AbDserotec
Recombinant Human TNFα (PHC3015)	Gibco
Restriction enzyme	Invitrogen
Ribonuclease H	Invitrogen
RNase OUT	Invitrogen
RQ1 RNase-Free DNase	Promega
siRNA	Ambion *by Life Technologies*
SuperScript II Reverse Transcriptase	Invitrogen
SYBR green PCR Master Mix	AB Applied Biosystems
T4 DNA ligase	Fermentas
TRI reagent	Sigma

Table 2.1.2 List of cell lines, virus and bacteria

Cell/Virus/Strain	Phenotype/Genotype/Origin
HEK293	Human embryonic kidney cells expressing adenovirus type 5 E1A and E1B genes, supplied by Dr. K. N. Leppard, University of Warwick (Graham et al, 1977).
HeLa	Supplied by Prof. M. A. McCrae, University of Warwick
MRC5	Human fetal lung fibroblast cells (Jacobs et al, 1970), Purchased from Sigma-Aldrich
PIV3	Supplied by Prof. Andrew Easton, University of Warwick
DH5 α	*Escherichia coli* K12, genotype: SupE44, ΔlacU169(φ80lacZΔM15), hsdR17, recA1, endA1, gyrA96, thi-1, relA1

Table 2.1.3 List of antibodies

Antibody	Detail/Supplier
IRF3 (FL-425): sc-9082	Santa Cruz Biotechnology
NF-κB p65(C-20): sc-372	Santa Cruz Biotechnology
CBP(A-22): sc-369	Santa Cruz Biotechnology
STAT1 p84/p91(E-23):sc-346	Santa Cruz Biotechnology
Phospho-IRF3(Ser396)(4D4G) Rabbit mAb	Cell Signalling Technology
Rabbit anti-PML	Prof K.-S. Chang, Houston (Xu et al, 2003)
Rabbit anti-PML-II	Prof K.-S. Chang, Houston (Xu et al, 2003)
Rabbit anti-Flag epitope	Polyclonal F7425 Sigma-Aldrich
Mouse anti-Flag epitope	Monoclonal F3165 Sigma-Aldrich
Goat anti-mouse IgG-HRP	Sigma-Aldrich
Goat anti-rabbit IgG-HRP (sc-2054)	Santa Cruz Biotechnology
Goat anti-mouse Alexa488	Santa Cruz Biotechnology
Goat anti-rabbit Alexa594	Santa Cruz Biotechnology

Table 2.1.4 List of plasmids

Plasmid	Description
pIFNβ-Luc	A firefly luciferase reporter plasmid of the Homo sapiens interferon β promoter. The promoter lies upstream of the firefly luciferase gene. Provided by Professor Stephen Goodbourn.(King & Goodbourn, 1994)
ptk-PRDI/III-Luc	ptk-Luciferase reporter plasmid (Promega) containing the PRDI/III element of the IFN β promoter. Provided by Dr Li Yong, Peking University, China. (Li et al, 2011)
pcDNA3.1-HisA-IRF3	IRF3 expression plasmid. Provided by Dr Li Yong, Peking University, China. (Li et al, 2011)
pPRDII-Luc	A firefly luciferase reporter driven by the NF-κB sites in IFNβ promoter. Provided by Mankouri(Mankouri et al, 2010)
pConA-Luc	A firefly luciferase reporter driven by the NF-κB sites in the Concanavalin A promoter. Provided by Dr Mankouri.(Mankouri et al, 2010)
pNIFTY-Luc	A firefly luciferase reporter driven by five NF-κB repeated transcription factor binding sites (GGGRNNYYCC, R-purine Y=pyrimidine). Provided by Dr Mankouri. (Mankouri et al, 2010)
pISRE-Luc	Luciferase reporter plasmid of five copies interferon-stimulated response element (ISRE) from ISG15 gene. Provided by Prof D Blackbourn, University of Birmingham. (Fuld et al, 2006)
pP53-Luc	A firefly luciferase reporter driven by p53 sites in PIG3 gene promoter. Provided by Dr B. Vogelstein, the Howard Hughes Medical Institute and Sidney Kimmel Comprehensive Cancer Center at The Johns Hopkins University School of Medicine. (Polyak et al, 1997)
pcDNA3.1-HisB::lacZ	β-galactosidase expression plasmid driven by the CMV IE promoter region (Invitrogen)
pCI-neo Flag-PML-I-V	Plasmids for expressing each PML isoforms I-VI tagged with a Flag epitope sequence (MDYKDDDDK). 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-II ΔRBCC	As pCI-neo Flag-PML-II, the RBCC region amino acid residues have been deleted (Leppard et al, 2009)
pCI-neo Flag-PML-II cytoplasmic	As pCI-neo Flag-PML-II, the sequence corresponding to exon-4, 5, 6 of PML-II has been deleted (Wright, 2010)
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)
pCI-neo Flag-PML-II-mut	PML-II siRNA-resistant pCI-neo Flag-PML-II plasmid – this thesis
pCI-neo Flag-PML-II ΔRBCC-mut	PML-II siRNA-resistant pCI-neo Flag-PML-II ΔRBCC plasmid – this thesis
pCI-neo Flag-PML-II cytoplasmic -mut	PML-II siRNA-resistant pCI-neo Flag-PML-II cytoplasmic plasmid – this thesis

Table 2.1.5 List of small interfering RNA

siRNA	Sense 5'-3'	Antisense 5'-3'	References
PML-II(A)	CAUCCUGCCCAGCUGCAAAUU	UUUGCAGCUGGGCAGGAUGUU	(Kumar et al, 2007)
PML-II(B)	GGAAAGCAGAGCCCAGACUUU	AGUCUGGGCUCUGCUUUCCUU	This thesis
PML-V	GUUCAGCCCAGGACUCCUGUU	CAGGAGUCCUGGGCUGAACUU	(Kumar et al, 2007)
Exon-3	GAGCUCAAGUGCGACAUCAUU	UGAUGUCGCACUUGAGCUCUU	(Kumar et al, 2007)
Scramble (A)	GAGCCGGACGCCAAAGAAAUU	UUUCUUUGGCGUCCGGCUCUU	(Wright, 2010)
Scramble (B)	ACGCGAAUAGCGAGCAAGCUU	GCUUGCUCGCUAUUCGCGUUU	This thesis

Table 2.1.6 List of mutagenesis PCR primers

	Sequences 5'→3'	References
Flat PML-II Forward	TGCCACCATGGATTACAAGG	(Wright, 2010)
SBO2 Reverse	GTCTGCTCGAAGCATTAAC	(Wright, 2010)
PML-II Mut Forward	CACCCAGCTCAATTACAGCGGGGCATCAGCCCACC	This thesis
PML-II Mut Reverse	GCTGTAATTGAGCTGGGTGTTCCTGGGCATTGGCA	This thesis

Table 2.1.7 List of qPCR primers

GENES	Forward 5'→3'	Reverse 5'→3'	References
IFNβ	ATTGCCTCAAGGACAGGATG	GGCCTTCAGGTAATGCAGAA	(Shi et al, 2010)
ISG15	CCCTCGAAGGTCAGCCAGA	GGACAAATGCGACGAACCTCT	(Han et al, 2011)
ISG20	CGCAGAGGCAGGCAGCAT	CATGACCCACCACCAGCTT	(Vestergaard et al, 2011)
ISG54	TGCAACCTACTGGCCTATCTA	CAGGTGACCAGACTTCTGATT	(Shi et al, 2010)
ISG56	GCCATTTTCTTTGCTTCCCCTA	TGCCCTTTTGTAGCCTCCTTG	(Yang et al, 2009)
IP-10	GAAATTATTCCTGCAAGCCAATTT	TCACCCTTCTTTTTCATTGTAGCA	(Spurrell et al, 2005)
IRF-7	CAAGTGCAAGGTGTACTGG	CAGGTAGATGGTATAGCGTGG	(de Oliveira et al, 2011)
IRF-1	GCAGCTCAAAAAGGGAAGTG	AAGGCAGGAGTCATGCAAGT	(O'Donnell et al, 2006)
RANTES	TACACCAGTGGCAAGTGCTC	ACACACTTGGCGGTTCTTTC	(Yang et al, 2009)
TNFα	GCCAGAATGCTGCAGGACTT	GGCCTAAGGTCCACTTGTGTCA	(Yun et al, 2011)
IL-8	AGGTGCAGTTTTGCCAAGGA	TTTCTGTGTGGCGCAGTGT	(Yang et al, 2009)
IL-6	AAAGAGGCACTGGCAGAAAA	TTTCACCAGGCAAGTCTCCT	(O'Donnell et al, 2006)
IL-28	AGGGCCAAAGATGCCTTAGA	TCCAGAACCTTCAGCGTCAG	(Shin et al, 2013)
IL-29	GGACGCCTTGGAAGAGTCAC	AGCTGGGAGAGGATGTGGT	(Shin et al, 2013)
Hsp70	AAGTACAAAGCGGAGGACG	GATGGGGTTACACACCTGC	(Zhang et al, 2009)
P21	AGCGGAACAAGGAGTCAG	CGTTAGTGCCAGGAAAGAC	(Wong et al, 2010)
P21	GACTCTCAGGGTCGAAAACG	GGCTTCCTCTTGGAGAAGATCA	(Dehennaut et al, 2013)
PML-II-1+2	AGGCAGAGGAACGCGTTGT	GGCTCCATGCACGAGTTTTC	This study
PML-II-3+4	GAAAACTCGTGCATGGAGCC	GAGGCTCCTTGTGCTCTCAG	This study
MX1	GCCTTCCGATTCCCCATTCA	TGGACTTAGGTGCCTTGTGG	(Mirpuri et al, 2010)
GBP1	CCGCACAGGCAAATCCTACC	TTCTTGGGGTGAGGCACACA	This study
OAS1	TGCGCTCAGCTTCGTACTGA	GGTGGAGTTCTCGCCCTCTT	(Cantaert et al, 2010)
GADPH	GGTCGGAGTCAACGGATTT	CCAGCATCGCCCCACTTG	(Vestergaard et al, 2011)
β-actin	AAAGACCTGTACGCCAACAC	GTCATACTCCTGCTTGCTGAT	(Shi et al, 2010)

Table 2.1.8 List of ChIP-qPCR primers

Genes	Sense 5'-3'	Antisense 5'-3'	Fragment size bp	Reference
IFNβ	TGCTCTGGCACAACAGGTAG	CAGGAGAGCAATTTGGAGGA	82	This study
IFNβ	AACATTAGAAAACCTCACAGTTTGT	TTCCCACTTTCACTTCTCCC	139	This study
PKR	TACCCCAATCCCGTAGCAGA	CGTTTTCCCCTTGGACTCCG	82	This study
ISG15	CGCCACTTTTGCTTTTCCCT	ATAAGCCTGAGGCACACACG	158	This study
ISG56	TTGGGTTTCTGCAGCACTAGA	ACCTAGGGAAACCGAAAGGG	150	This study
IL-8	TGATGACTCAGGTTTGCCCT	AGTGCTCCGGTGGCTTTTTA	131	This study
IP-10	ACAGTTCATGTTTTGGAAAGTGA	CAAAACCTGCTGGCTGTTCC	146	This study
RANTES	ATACCGGCCAATGCTTGGTT	CCACGTGCTGTCTTGATCCT	122	This study

2.2 Methods

2.2.1 Cell culture

Hela cells and HEK293 cells were reseeded at appropriate ratios 1:4 and 1:8 respectively, and were routinely maintained in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% (v/v) newborn bovine serum (NBS) at 37°C in a 5% CO_2 incubator. MRC5 cells were similarly maintained in 10% Eagle's Minimal Essential Medium supplemented with 10% foetal bovine serum, 2mM-L-glutamine and 1% non-essential amino acids.

2.2.2 Separation of cell nuclear and cytoplasmic fractions

Monolayer cells were washed once with cold PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na2HPO4, 2 mM KH2PO4), scraped and collected by centrifugation in a eppendorf centrifuge 5810R at 1300 rpm for 3 min and resuspended in lysis buffer (150 mM NaCl, 1.5 mM MgCl2, 10 mM Tris.HCl pH7.5) plus 0.5-1.0% NP40. After standing on ice for 10 min., nuclei were separated from cytoplasm by centrifugation at 1500 rpm for 3 min at 4 °C. Both fractions were then lysed with SDS-PAGE sample buffer (SB) (2% SDS, 10% (v/v) glycerol, 25mM Tris.HCl (PH6.8). 0.1M DTT, 0.04% (w/v) bromophenol blue).

2.2.3 Cloning

2.2.3.1 Preparation of competent cells

E. coli DH5α were grown in liquid LB (1% (w/v) tryptone, 0.5% (w/v) yeast extract, and 1% (w/v) NaCl) medium containing appropriate antibiotics (100μg/ml of ampicillin) grow at 37°C and shaken vigorously overnight. 2 ml of fresh overnight bacterial culture was inoculated into 200 ml of pre-warmed liquid LB medium and shaken vigorously at 37°C until OD_{600} of the culture reached 0.39. The culture was then put on ice for 5 minutes followed by centrifugation (Beckman Coulter Allegra^{TM} X-12R Centrifuge) at 6,000 rpm for 10 min at 4°C. The pellet was resuspended in 80 ml of ice-cold Transformation Buffer I (10 mM $RbCl_2$, 30 mM potassium acetate, 10 mM $CaCl_2$, 50 mM $MnCl_2$ and 15% (v/v) glycerol, pH 5.8) and then left on ice for 5 minutes. Centrifugation was repeated at 6,000 rpm for

another 10 min at 4°C; the pellet was subsequently resuspended in 8ml of ice-cold Transformation Buffer II (75 mM $CaCl_2$, 10 mM $RbCl_2$, 10 mM MOPS and 15% (v/v) glycerol, pH 6.5) and left on ice for 2 h. The competent cells were transferred into 50/100 μL aliquots for use or frozen with dry ice before storage at -70°C (up to three months).

2.2.3.2 Restriction enzyme digestion/cleave

Normally 1 μg of plasmid DNA or PCR product was digested in a total reaction volume of 20 μL. Restriction enzymes were used with recommended reaction buffers according to the manufacturer's instructions. If a reaction required multiple restriction enzyme digestions, the reaction buffer selected was the most optimal for all enzymes. Usually digestion was carried out at 37 °C for 1-1.5 h.

2.2.3.3 Agarose gel electrophoresis

DNA fragments such as vector, insert and PCR product were typically separated by electrophoresis on 0.8-1% (w/v) agarose in 1 × TBE buffer (89 mM Tris base, 89 mM boric acid and 2 mM EDTA) containing 0.5 μg/ml ethidium bromide (EB). DNA samples were loaded onto the gel with loading buffer (5% (w/v) glycerol, 0.04% (w/v) bromophenol blue and 0.04% (w/v) xylene cyanol). Electrophoresis was carried out at 80-100V for 1 h. DNA fragments were visualized under UV light and imaged using a BioRad Gel/Chemi Doc system with associated software.

2.2.3.4 DNA separation and purification

The desired bands obtained using DNA gel electrophoresis were quickly cut under UV light. DNA was separated and purified by using commercial Gel Band Purification Kit (GE Healthcare). The protocol was obtained from the manufacturers' instructions.

2.2.3.5 De-phosphorylation of vector DNA

To increase the ligation efficiency of vector and insert and to avoid vector self-ligation, 5' phosphate groups from vector DNA were removed by adding 1 Unit of shrimp alkaline phosphatise (SAP) (Fermentas) per μg of DNA, treated according to the manufacturer's

instructions. SAP was subsequently inactivated by incubating the reaction mixture at 65 °C for 15 min.

2.2.3.6 DNA Ligation

Ligation of DNA was performed in a final reaction volume of 20-50 µL. A 3:1 molar ratio of insert DNA to vector DNA was typically used. This ratio was increased in some particular reactions to increase ligation efficiency. Usually at least 100 ng total DNA was required in each reaction. T4 DNA ligase was used according to the manufacturer's instructions with supplied buffers under recommended conditions usually at 22 ℃ for 1 hour.

2.2.3.7 Transformation of competent bacterial cells

Ligation product or plasmid DNA (typically 50 ng) was added into 50-100 µL of thawed competent cells and left on ice for 20-30 min. Cells were heat shocked for 45 s at 42 °C followed by immediate incubation on ice for another 2-3 min. 500-800 µL of pre-warmed liquid LB medium was added to the competent cell mixture and incubated at 37 °C in a shaking incubator for 1 h; the cells were then plated onto LB plates containing appropriate antibiotics and incubated at 37 °C overnight.

2.2.3.8 Extraction of plasmid DNA (Mini Prep and Midi Prep)

Plasmid DNA was extracted using Qiagen MiniprepKit™, or Promega Pureyield™ Plasmid Midiprep System; all the protocols were obtained from the manufacturers' instructions. Before DNA was eluted with Nuclease-free water, the endotoxin, protein, RNA and endonuclease was removed by Endotoxin Removal Wash solution.

2.2.3.10 Quantification of nucleic acid

The concentration of DNA and RNA samples was quantified using a Nanodrop-ND1000 spectrophotometer (Thermo Scientific) by measuring OD_{260}. The purity of DNA and 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.

2.2.3.11 DNA Sequencing

DNA sequences were done by the Molecular Biology Service at University of Warwick using an automated ABI PRISM 3130xl Genetic Analyse. Sequencing data were viewed using Chromas Lite 2.0 and analysed using Clone Manager, SciEd Central, v7.04 software.

2.2.4 PCR-based site-directed mutagenesis

Mutagenesis was carried out by a two-stage overlap PCR method. For first round reactions, plasmid DNA template (approximately 50 ng) was added to a PCR cocktail containing 5 μL 10×Pfu buffer with $MgSO_4$ (200 mM Tris-HCl, 100 mM$(NH_4)_2SO_4$, 100 mM KCl, 1mg/ml BSA, 1% (v/v) Triton X-100, 20mM $MgSO_4$), and 5 μL dNTPs Mix (2mM), forward/reverse primers (mut F/SBO2; Flat PML-II/mut R) 10 μM each, Pfu DNA polymerase 2.5U in total 50 μL volume. The PCR cycling parameters were 94 ℃ 1 min, 45 ℃ 1 min and 72 ℃ 5 min for 6 cycles; followed by 30 cycles of 1 min at 94℃, 1 min at 60℃ and 5 min at 72℃, then 15 min at 72℃.

Second round of PCR was carried out using newly synthesized mutated DNA fragment pairs as template and Flat PML-II and SBO2 as primers under the condition of 1 min at 94 ℃, 1 min at 52 ℃, 5 min at 72 ℃ for 6 cycles; then followed by 30 cycles 94 ℃ 1 min, 61.5 ℃ 1 min, 72 ℃ 5 min, and extra 15 min enlongation at 72 ℃.

Mutated DNA was cloned via its flanking MluI and EcoRI sites into PML-II, RBCC-PML-II, exon-5-PML-II and C-PML plasmids, in place of the corresponding unmutated sequences.

2.2.5 Transfection and stimulation

For transfections performed in 24 and 12-well plates, 2-2.5×10^5 cells or 4-5x10^5 cells respectively were seeded and cultured for 24 h to allow approximately 30-50% cell confluence by the time of transfection. Transfection reagent and siRNA/plasmid DNA/poly(I:C) mixture was prepared in pre-warmed Opti MEM medium to make a total volume of 100 μL per ml culture volume. After incubation for 20 min at room temperature

the complex mixtures were gently pipetted onto the cells in normal growth medium, and gently mixed. Cells were cultured for various periods depending on different requirements of individual experiments.

Lipofectamine 2000 (Invitrogen) was used for transfection of siRNA, plasmid DNA and poly(I:C) throughout the entire study at a ratio of 2 μL of Lipofectamine 2000 to 1 μg plasmid DNA/poly(I:C) or 125 pmol siRNA. HEK293 and Hela cells were transfected with 125 pmol/ml siRNA for 48 h, and stimulated with 1 μg/ml poly(I:C) for 16 h, unless otherwise stated. MRC5 cells were treated with 50 pmol/ml siRNA for 72 h, and stimulated with 1 μg/ml poly(I:C) for 24 h.

For some experiments, cells were alternatively stimulated with 10-20 ng/ml TNFα, 100 or 1000 U/ml IFNα or 50 ng/ml IFNγ, or treated with 100 μM etoposide or 50 μM MG132 for various time courses.

2.2.6 Luciferase reporter assays

HEK293 in 24-well culture plates were transfected with siRNA and co-transfected with 225 ng of luciferase reporter plasmid and 25 ng β-gal control plasmid (pcDNA3.1lacZ::his) using Lipofectamine 2000. Then cells were stimulated with poly(I:C)/IFNα/TNFα/Etoposide as required for various times. Cells were washed once with PBS then incubated with 100 μL 1×passive lysis buffer (Promega) at room temperature on an orbital shaker for 1 h and lysates harvested for analysis.

To determine β-galactosidase activities, 20 μL cell lysate was mixed with 172 μL assay reagent (24 mM 2-nitrophenyl-β-D-galactopyranoside (ONPG), 40 mM β-mercaptoethanol in Z-buffer: 60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 1 mM KCl, 1 mM $MgSO_4$), in a transparent 96-well-plate. Reactions were incubated at 37 °C until yellow colour was seen and then stopped by adding 80 μL 1 M sodium carbonate (Na_2CO_3). Absorbances were read at 405 nm using a plate reader. To determine luciferase activities, 20 μL cell lysate was mixed with 25 μL Bright-Glo substrate in a white 96 well plate. Emitted light was measured

using a luminometer with Ascent software. Raw luciferase activities were normalized to the corresponding β-galactosidase activity to give relative luciferase activities (RLA) to correct for variation in transfection efficiency.

2.2.7 Confocal Immunofluorescence

Cells were grown on coverslips in 12-well culture plates for 24 h, transfected with siRNA and stimulated with poly(I:C) or TNFα for 16 h or 1 h as described for luciferase reporter assays. Cells were processed for immunofluorescence as described (Lethbridge et al, 2003). Briefly, cells were washed with cold PBS, fixed with 4% formaldehyde in PBS for 10 min, washed twice in PBS and then permeablised using 0.5% NP40 in PBS for 10 min. After further PBS washes, 1% bovine serum albumin in PBS was added for 1 h to block non-specific protein binding to the cells and then replaced with 250-300 μL of primary antibody for 1 h. After three washes with PBS, cells were incubated with 250 μL of an appropriate fluorescently labeled secondary antibody. Further antigens were detected in the same samples by repeating these steps. Finally, cells were washed three times with PBS including DAPI (1 μg/ml) in the final wash (5 min incubation) before mounting the coverslips. Samples were viewed by confocal microscopy using a Leica SP2 system and Leica software. All images shown are single images taken in z through the thickest part of the cell.

2.2.8 Western-blotting

Protein samples in SB were boiled for 5-10 min before loading onto SDS polyacrylamide gels and electrophoresis at 120 V for 1.5 h. Proteins were then transferred to a nitrocellulose membrane at 0.35 A for 1.5 h (or sometime 0.08 A overnight) in the cold room. After transfer, NC membranes were blocked with 2% (w/v) milk diluted with PBS containing 0.05% Tween20 (PBS-T) at room temperature for 1 h, or at 4 °C overnight. Membranes were then incubated with specific primary antibody and then the appropriate HRP-conjugated secondary antibody for 1 h each at room temperature with extensive washing in PBS-T between each step. Finally, bound secondary antibody was detected using ECL^{TM}

Advance reagent according to the manufacturer's instructions by exposure to Fuji SuperRX X-Ray film.

2.2.9 Co-immunoprecipiation

Standard Antibody Protocol: Cells cultured in 10 cm dishes (7.5-10 $\times 10^6$), were washed with 10 ml pre-chilled PBS 3 times and lysed with 0.5-1.0 ml pre-chilled NP-40 cell lysis buffer (50 mM Tris.HCl pH8.0, 150 mM NaCl, 1% (v/v) NP-40) and incubated on ice for 10 min, then sonicated 3 times on ice for 5 s each using a Jencons untrasonic processor with microtip. After centrifugation 13,000 rpm for 10 min (microfuge), the whole cell lysate (WCL) supernatant was pre-cleared with 10-30 μL Protein A–Sepharose beads for 1-2 h, then incubated with specific antibody rocking overnight at 4 °C. The antibody-protein complexes were then precipitated with 50 μL Protein A–Sepharose beads for 1-3 h. Collected precipitates were washed with 0.5-1.0 ml IP-washing buffer (10 mM Tris.HCl pH7.4, 150 mM NaCl, 1 mM EDTA, 1% (v/v) Triton X-100, 1 mM EGTA and 0.2 mM Na_3VO_4) five times, then beads were resuspended with 2 × SB and heated at 95-100 °C for 10 min to release bound proteins.

Flag beads Fusion Protein Protocol: Cells were washed twice with pre-chilled PBS, and lysed with 500μl Guccionne buffer (25 mM HEPES (7.0), 0.1% NP-40, 0.5M NaCl, 1mM Na Butyrate) with 1% Protease Inhibitor and 1% phosphatase inhibitor cocktail. Cell lysates were collected and sonicated as above, and supernatants diluted with equal volumes of low salt Guccionne buffer (as above but 50 mM NaCl) to get to a final 275 mM NaCl concentration for Flag bead binding. Flag affinity gel was throughly resuspended and washed with TBS buffer and 0.1 M Glycine (pH 3.5) following product instruction. Lysate supernatants were then precipitated by affinity gel for 3 h or overnight. After extensive washing with TBS buffer, proteins were eluted by 2xSB buffer and detected by WB.

2.2.10 Nucleic acid extraction

2.2.10.1 DNA extraction

Cells from 12-well plate cultures were digested with 100 µg/ml Proteinase K in 5 mM EDTA, 20 mM Tris-HCl (pH6.5) for 1 h at 37-45 °C. DNA was extracted twice with phenol-chloroform (1:1), followed by chloroform extraction. Solutions were made 0.3 M sodium acetate and DNA was precipitated with 2.5 volume of ethanol at -20°C at least 1 h or overnight. The precipitated DNA pellets were washed with 70% ethanol, dried for 15-20 min in a vacuum dessicator, and resuspended in 30-40 µL of dH_2O.

2.2.10.2 RNA extraction

Harvested cells were washed in isotonic buffer (10 mM Tris.HCl pH7.5, 150 mM NaCl, 1.5 mM MgCl2) and re-suspended in 40 µL Isotonic Buffer (10mM Tris-HCl pH7.5, 150mM NaCl, 1.5 mM MgCl2). Cells were then lysed with the addition of 250 µL TRI reagent and RNA recovered according to the manufacturer's instructions. RNA pellets were redissolved in 40 µL sterile H2O then treated using a Promega RNA-free DNAase kit according to the manufacturer's protocol in order to remove genomic DNA contamination. RNA was recovered by phenol-chloroform extraction and ethanol precipitation. Alternatively, RNA was extracted using Sigma-Aldrich Mammalian Total RNA Miniprep Kit, according to the manufacturers' instructions.

2.2.11 Reverse transcription

mRNA was reverse-transcribed to cDNA using Superscript II transcriptase kit. Briefly, 1-3 µg extracted RNA was mixed with 10 mM random primers, and 15mM dNTPs, add H_2O up to 18µl. At 65°C for 5 min, then quick chill on ice, then First-strand buffer, 0.1M DTT and RNase OUT were added to each sample following the instruction of SuperScript II RT kit. After 2 min at 25°C 200U Superscript II reverse transcriptase were added and the reaction incubated successively at 25°C 10 min, 42°C 50 min, and 70°C 15min. Finally, 5U RNase H was added and the reaction further incubated at 37°C for 20 min to remove the RNA complementary to cDNA.

2.2.12 SYBR Green quantitative PCR

For quantitative PCR (qPCR) a SYBR Green qPCR Master Mix was used according to the manufacturer's instructions (ABI or Agilent). Briefly, reactions were 10 μL SYBR green Master Mix, 10 μM each of forward and reverse primers and 50-100 ng DNA or cDNA with H_2O to a total 20 μL volume. qPCR primers used in this study are listed in Tables 2.1.6/7/8. qPCR amplification was carried out in ABI 7000 (BioRad) or Agilent Technologies stratagene Mx3005P. To evaluate qPCR efficiency using specific primers, a standard curve was established by plotting the C(t) values obtained from the qPCR against the log dilution of standard template. All samples, including the no reverse transcriptase and no template controls, were run in triplicate. Reaction conditions were: denaturation at 95 ºC for 10 min, followed by 40-50 cycles of denaturation at 95 ºC 30 s, annealing/extension at 60 ºC for 60 s. The results were analyzed using ABI Prism 7000 system software. Quantification was based on Ct difference performed according to the "delta–delta Ct method" (Livak & Schmittgen, 2001), using the following equation: expression ratio=2-ΔΔCt, where ΔΔCt = (Ct target−Ct reference). Target gene expression was normalized against that of the housekeeping genes glyceraldehyde 3-phosphate dehydrogenase (GAPDH) or β-actin.

2.2.13 Chromatin immunoprecipitation (ChIP) assay

ChIP assays were carried out broadly as described (Weinmann & Farnham, 2002). Briefly, cells cultured in 10-cm dishes were cross-linked with 1% (v/v) formaldehyde for 10 min, followed by addition of glycine to 125 mM (final concentration) to stop the reaction. Fixed cells were washed twice by PBS, harvested and resuspended in Cell Lysis Buffer (20 mM HEPES pH7.9, 25% glycerol, 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA) on ice for 20 min. Nuclei were pelleted by centrifugation at 13,000 rpm (microfuge) for 10 min, and lysed with 100 μL Breaking Buffer (50 mM Tris-HCl pH8.0, 1 mM EDTA, 150 mM NaCl, 1% (w/v) SDS, 2% (v/v) Triton X-100) on ice for 10 min, then sonicated to shear DNA to lengths between 200 and 1000 base-pairs. Cell lysates were then diluted with 1 ml Triton Buffer (50 mM Tris-HCl pH8.0, 1 mM EDTA, 150 mM NaCl, 0.1% (v/v) Triton X-100). At

this point, if necessary, samples were incubated with 20 μL Protein A Sepharose at 4 °C for 1 h to reduce non-specific binding. Cell lysate supernatants were then incubated with 2-6 μg specific antibodies at 4 °C overnight. Antibody/chromatin complexes were washed three times with Triton buffer, and cross-linking then reversed with 200 μL of SDS-NaCl-DTT Buffer (62.5 mM Tris HCl pH6.8, 200 mM NaCl, 2% (w/v) SDS, 10 mM DTT) at 65 °C for 5 h or overnight. The DNA was purified and amplified as described in section 2.10.1. The ChIP–qPCR assay was carried out using SYBR Green qPCR Master Mix and specific primers in an ABI Prism 7000 sequence detection system (Applied Biosystems). The relative ChIP signal of gene in each immunoprecipitate was determined by first subtracting the background signal from a precipitation of the same material by normal IgG and then dividing by input DNA quantified in parallel to give % input specifically precipitated.

Chapter 3 Knockdown of PML-II inhibits IFNβ expression

Type I IFN IFNα/β play a major role in innate antiviral responses. These responses provide a crucial initial defense against invading viruses and also aid in commissioning an effective adaptive response. PML proteins and PML-NBs are strongly implicated in cellular antiviral responses and the interferon response in particular (Everett & Chelbi-Alix, 2007a; Regad & Chelbi-Alix, 2001b; Tavalai & Stamminger, 2008). Various viruses encode proteins that disrupt PML and/or PML bodies and these functions, including the E4 Orf3 protein of human adenovirus type 5 (HAdVC-5, Ad5), are also important for those viruses to overcome IFN responses (Regad & Chelbi-Alix, 2001b; Regad et al, 2001; Ullman & Hearing, 2008). Ad5 E4 Orf3 targets PML-II specifically (Hoppe et al, 2006) suggesting that this isoform might have a role in the IFN response. The experiments presented in this Chapter were therefore designed to address the function of PML-II in the induction of IFNβ.

3.1 PML-II can be successfully depleted by PML-II siRNA-mediated knockdown

3.1.1 The expression of exogenous PML-II protein was down-regulated by PML-II siRNA.

In order to investigate the function of PML-II in interferon expression, the transient knockdown of PML-II by small interfering RNA (siRNA) was first established and optimized. The result showed that the expression of exogenously-expressed PML-II was down-regulated by PML-II siRNA in a dose-dependent manner as compared to cells transfected with control siRNA, a scramble siRNA which has no homology to any gene in the human genome (Figure 3.1.1). The major band at ~120 kDa represents the full length product of the transfected cDNA. It is noticeable that there were some additional higher molecular weight PML-II proteins present in the blot. This can be explained by PML-II protein being subjected to extensive post-translational modification, particularly Sumoylation (Cheng & Kao, 2012b). In addition to Sumoylation, phosphorylation may also contribute to the generating of different molecule weight forms. To avoid unexpected side-

effects of siRNA targeting, 100-125 pmol/ml siRNA was selected as the optimized concentration for subsequent experiments.

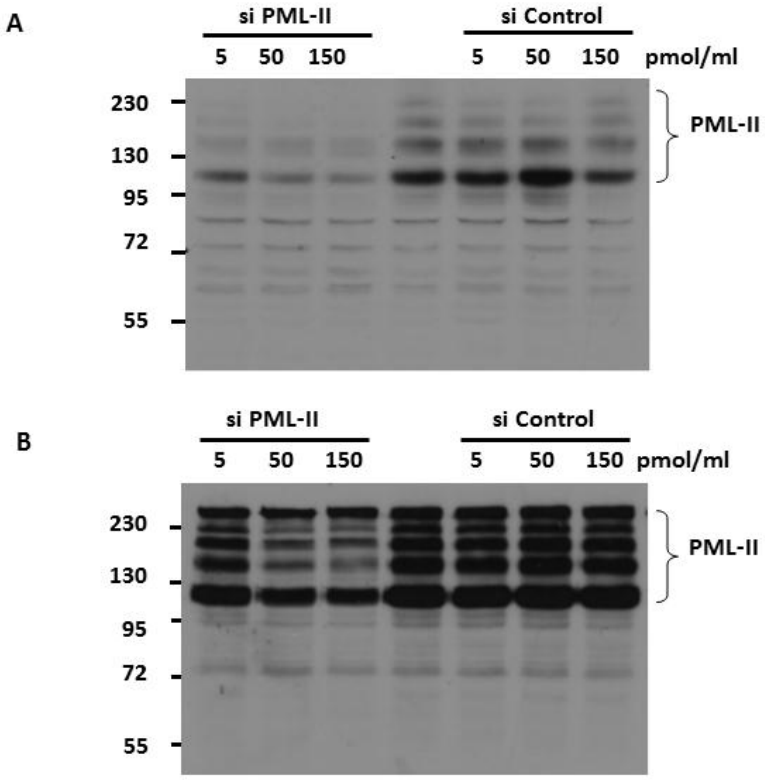


Figure 3.1.1 The expression of exogenous PML-II protein was down-regulated by PML-II siRNA. HEK293 cells were co-transfected with 50 ng/ml FLAG-tagged PML-II expression plasmid plus 450 ng/ml pCI-neo empty vector. 24 h later cells were transfected with 5, 50 or 150 pmol/ml siPML-II or siControl and incubated for 48 h. At this time, a sample was also harvested from untransfected cells (centre lane in each panel). Cells lysates were separated by 8% SDS-PAGE and subjected to western blotting. The blots were probed with anti-PML-II antibody (Panel A) and anti-FLAG antibody (Panel B), respectively. Protein sizes are indicated in kDa on the left side.

3.1.2 Endogenous PML-II can be knockdown by PML-II siRNA

The data presented in the Figure 3.1.1 indicated exogenously-expressed PML-II could be knocked down with PML-II siRNA. To test whether endogenous PML-II could also be depleted, and to what extent, western blotting and immunofluorescence were carried out. By western blotting (Figure. 3.1.2), both nuclear and cytoplasmic forms of endogenous PML-II protein could be knocked down with PML-II siRNA, with observation of a significant

decrease in the amount of the major PML-II bands as compared with control siRNA treatment (compare lanes 1, 2 with 3, 4; and 7, 8 with 9, 10). However, the major band, particularly in cytoplasmic fractions, was not consistent with migration position of full length PML-II as previously tested by exogenous over-expression of PML-II. The possible explanation for this discrepancy is that PML-II protein nuclear localization signal is encoded in exon 6 of the *pml* gene, thus full length PML-II localises to the nucleus. However, exon 6 may be omitted by splicing, creating cytoplasmic variants that have lost the NLS sequence and cannot enter into the nucleus, but they may still retain the unique exons encoding the specific C-terminal of PML-II. In addition, other internal exons such as exon 4 and 5 may be excluded from cytoplasmic or nuclear variants. It is therefore feasible that there are different molecular weight PML-II variant proteins in the cell. The majority of the bands present in the control siRNA-transfected cells were significantly depleted by the PML-II siRNA.

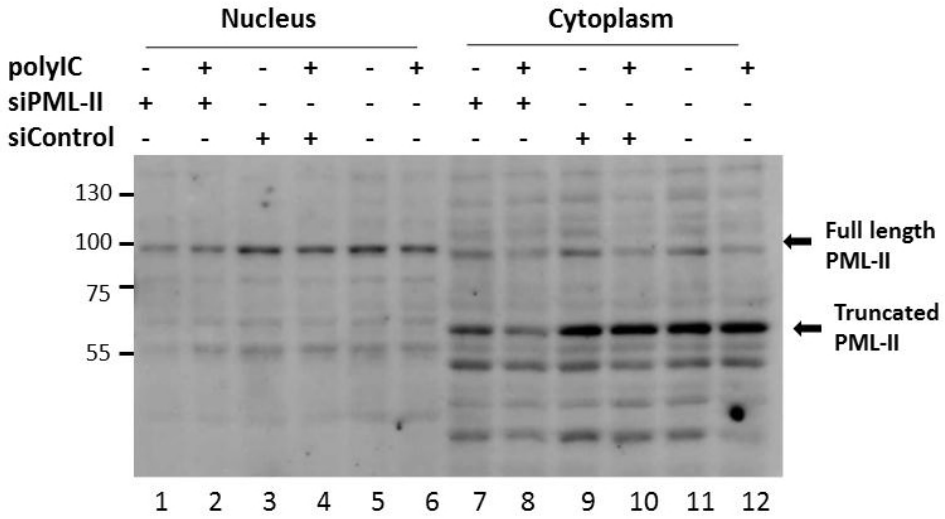


Figure 3.1.2 Endogenous PML-II can be knocked down by PML-II siRNA. HEK293 cells were transfected with 125 pmol/ml siPML-II or siControl for 48 h and stimulated with 1 μg/ml poly(I:C) or not for 16 h before fractionation into nucleus and cytoplasm. Nuclear and cytoplasmic lysates were separated by 8% SDS-PAGE and subjected to western blotting. The blots were probed with anti-PML-II antibody.

To determine the proportion of cells in which PML-II knockdown was being achieved, an immunofluorescence experiment was performed (Figure 3.1.3). The results showed that at

least 80% of cells lost PML-II protein when treated with PML-II siRNA. In contrast, there was still plenty of PML-II protein expressed in the nuclei of cells transfected with control siRNA. It can be concluded that endogenous PML-II can be successfully depleted with PML-II siRNA in HEK293 cells.

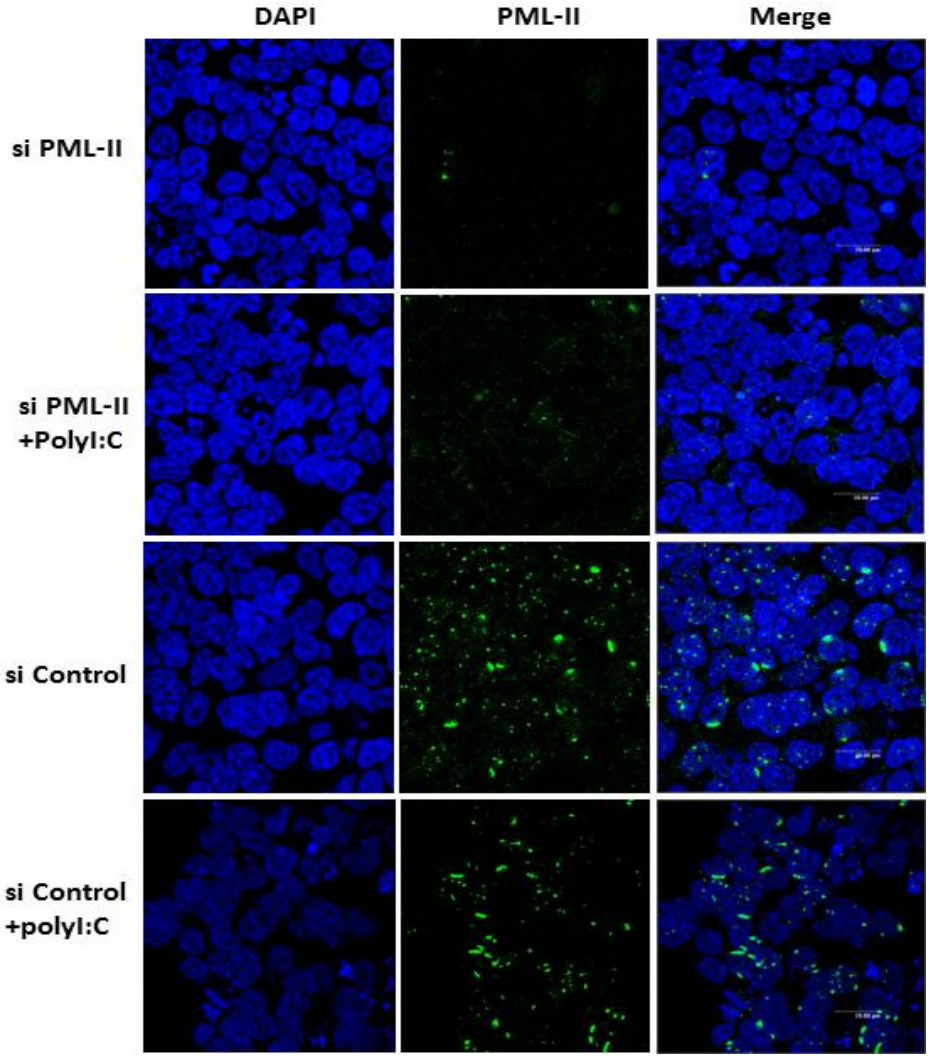


Figure 3.1.3 A **majority of cells lose PML-II protein when treated with PML-II siRNA.** HEK293 cells grown on coverslips were treated with siPML-II or siControl for 48 h, then transfected with poly(I:C). 16 h later cells were fixed and stained for immunofluorescence. Cells were observed with Leica SP2 confocal system; blue-DAPI (DNA) stain; green-PML II. The images displayed above were randomly selected from many views.

3.1.3 The mRNA level of PML-II was reduced by PML-II siRNA

The reduction in PML-II mRNA level achieved by PML-II siRNA was also tested using two different pairs of qRT-PCR primers. The results showed that the basal expression of PML-II mRNA was considerably reduced by PML-II siRNA (Figure 3.1.4). poly(I:C) stimulation significantly increased the expression of PML-II. However, PML-II siRNA still significantly reduced this stimulated mRNA level, taking it back to close to control unstimulated levels (Figure 3.1.4). The result also supported previous studies which showed that PML is an ISG, as the expression of PML-II was increased in response to the stimulation of poly(I:C), an effective inducer of IFN expression.

Taken together, the results presented in this section indicated that PML-II could be successfully knocked down by siRNA at both protein and RNA levels.

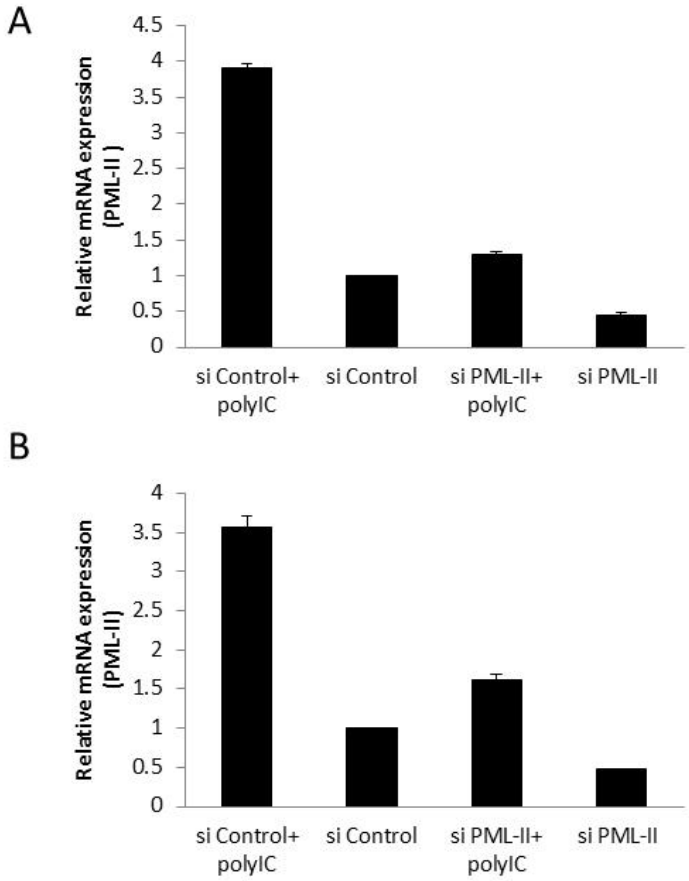


Figure 3.1.4 The mRNA level of PML-II was reduced by PML-II siRNA. HEK293 cells were transfected with siPML-II and siControl for 48 h, and stimulated with poly(I:C) or not for 16 h. Total RNA was extracted and RT-qPCR was performed using two pairs of specific primers (A) and (B) to detect PML-II mRNA, also GAPDH mRNA. The mRNA level was normalized against GAPDH, and values are presented relative to the amount in the control siRNA sample with no polyIC stimulation. Quantification was based on Ct difference performed according to the "delta–delta Ct method". Error bars indicate the standard deviation from the mean of at least three replicates.

3.2 Optimization of IFNβ promoter-driven reporter assay

To study the role of PML-II in the IFNβ response, induction of firefly luciferase by poly(I:C) from a reporter plasmid driven by the IFNβ promoter was used, and the assay was optimized in the following experiments. The results showed that relative luciferase activity (RLA) increased with duration of poly(I:C) treatment from 4 h to 16 h and reached the highest level with 16 h stimulation (Figure 3.2). In cells treated with poly(I:C) for 16 - 24 h,

the RLA was at a plateau, increased more than 100-fold compared to unstimulated cells. The RLA then began to decrease. However considering that once cells were treated with poly(I:C), they grew slowly while cells without poly(I:C) treatment continued to grow, so if cells were stimulated too long time, some cells died and detached which would influence the RLA measure. Cells treated with poly(I:C) for 16 h showed little cytopathology and the β-galactosidase control reporter, used to normalize for transfection efficiency, was not significantly influenced by poly(I:C) treatment. Subsequent confocal immunofluorescence analysis also confirmed that cells treated with poly(I:C) more than 16 h generated much cell debris, suggesting cell death. Extending the plasmid transfection time to 40 h before stimulation made no obvious difference to the signal as compared with 24 treatment (data not shown). Taken together, transfection of plasmid for 24 h, and then poly(I:C) stimulation for 16 h was adopted as a standard optimized reporter system.

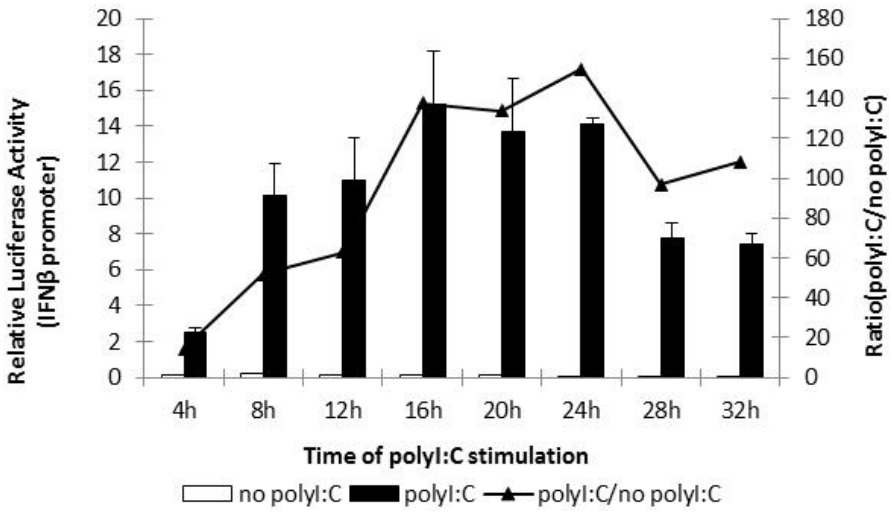


Figure 3.2 Optimization of IFNβ promoter induction by poly(I:C). HEK293 cells transfected with 225 ng/ml pIFNβ-Luc reporter plasmid and 25 ng/ml pcDNA3.1lacZ::his (β-gal) control plasmid were stimulated with poly(I:C), harvested at the indicated times and extracts assayed for firefly luciferase and β-galactosidase activities. Luciferase activities were normalized to the corresponding β-galactosidase values. Error bars indicate the standard deviation of three replicate samples from the mean value.

3.3 Depletion of PML-II affects the activity of IFNβ promoter

The effect of reducing PML-II levels on IFNβ promoter activity was first tested using a luciferase reporter assay (Figure 3.3 A). As expected, upon stimulation with poly(I:C) the activity of the IFNβ promoter was significantly increased but prior depletion of PML-II resulted in a significant decrease in this level of induced IFNβ promoter activity, to levels approximately 25% that of the control.

To determine whether this effect was specific to removal of PML-II, we also tested the effect of a similar knockdown protocol targeting another PML isoform, PML-V, which was previously demonstrated in our lab to be specific for this isoform and to have no effect on the activity of IFNβ promoter (Wright, 2010). In contrast to PML-II, the selective removal of PML-V had no effect on IFNβ promoter activity (Figure 3.3 A and B).

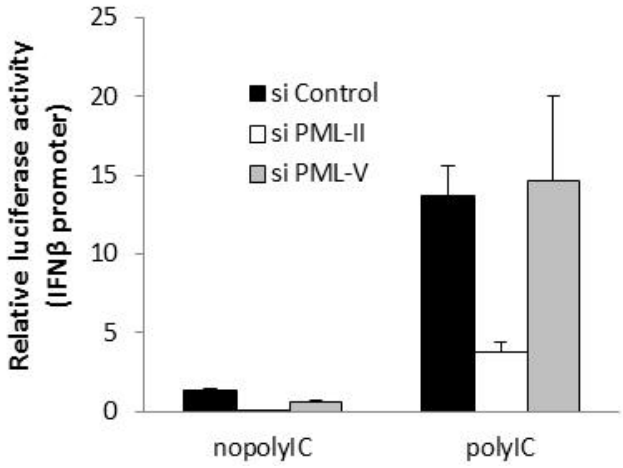


Figure 3.3 Depletion of PML-II affects the activity of IFNβ promoter **(**A) HEK293 cells were transfected in triplicate with 100 pmol/ml siControl or siRNA targeting PML-II or PML-V. 24 h later, cells were co-transfected with IFNβ-Luc and β-gal (pcDNA3.1-HisB::lacZ). Following poly(I:C) treatment for 16 h, cell lysates were assayed for both luciferase and β-galactosidase activities (RLA=luciferase/β-gal activity). (B) HEK 293 cells were transfected with control siRNA or two distinct PML-II siRNAs, co-transfected with reporter plasmids and RLA measured as described in (A). Error bar indicate the standard deviation from the mean of at least three replicates.

3.4 Depletion of PML-II impairs mRNA transcription from the IFNβ gene in various cell lines

Since depletion of PML-II significantly inhibited the activity of IFNβ promoter in reporter plasmid assays, in principle PML-II siRNA should also impair the mRNA transcription of endogenous IFNβ. To test this, a quantitative RT-PCR for detection of mRNA of IFNβ was performed to test the effect of depletion PML-II on IFNβ mRNA induction by poly(I:C). Poly(I:C) achieved a 8000-fold increase of IFNβ mRNA levels in HEK293 cells. However, the amounts of induced mRNA were significantly reduced, to about 20% of this value, by depleting PML-II (Figure 3.4 A). A similar result was obtained in HeLa cells (Figure 3.4 B). Poly(I:C) achieved a 1000-fold stimulation of IFNβ mRNA levels but depletion of PML-II led to a great reduction. In this case, the effect of depleting PML-V was also tested; no reduction in induced IFNβ levels was seen. In these experiments, IFNβ mRNA levels were measured relative to those of housekeeping mRNAs, β-actin or GAPDH. These were judged not to be affected by either PML-II knock-down or poly(I:C) treatment based on the close similarity between the Ct values for these control assays between treated and untreated samples (Appendix 1).

In addition to tumour cell lines, normal human lung fibroblast cells (MRC-5) were also tested to further confirm the effect of PML-II on IFNβ induction (Figure 3.4 C). The level of IFNβ mRNA was greatly increased by poly(I:C) although with extremely low levels in unstimulated cells. Nonetheless, as in immortalized cells, depleting PML-II significantly reduced the induced IFNβ mRNA level whilst PML-V depletion had no effect. This result confirmed very well the data from the luciferase reporter plasmid experiments and also proved that the effect of PML depletion on IFNβ expression was not an artefact of using a naked DNA plasmid. Taken together, these data indicate that expression of the IFNβ gene upon poly(I:C) stimulation is significantly dependent on PML-II.

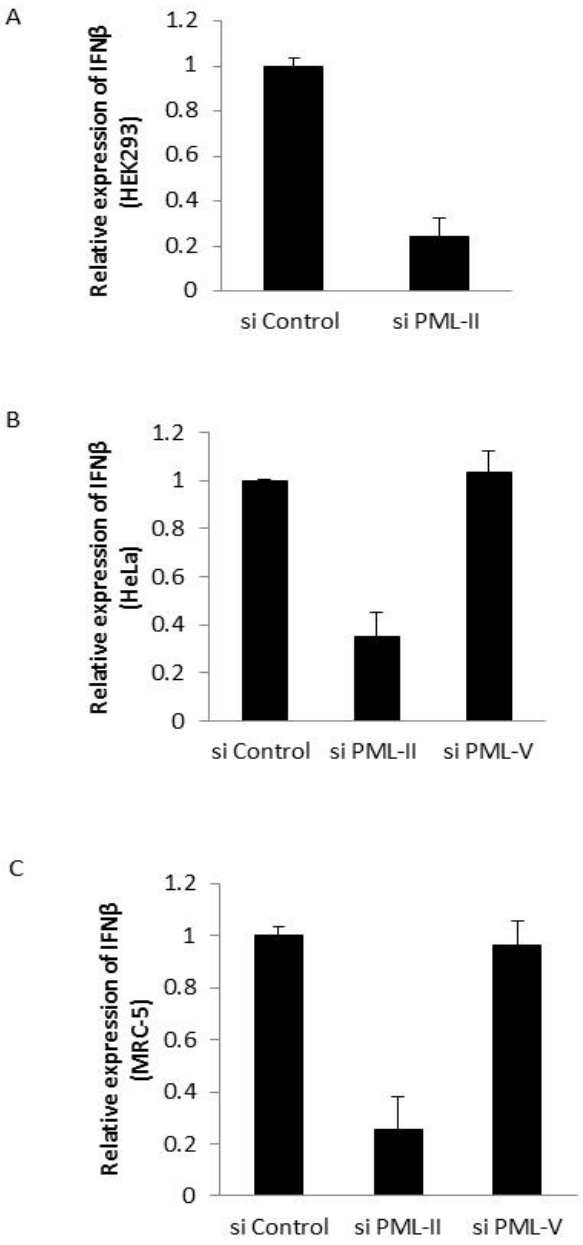


Figure 3.4 Depletion of PML reduces the mRNA expression of IFNβ in various cells. (A) HEK293 cells were treated with the siRNAs indicated for 48 h, then stimulated with poly(I:C). IFNβ mRNA levels in total RNA were determined by qRT-PCR, normalized to β-actin mRNA levels and are shown here relative to values obtained from control siRNA-treated cells. Data are the mean ± SD of three replicate values from one representative of three experiments. (B) Hela cells were treated, assayed for IFNβ mRNA levels and data analysed and presented as in panel A. (C) MRC5 cells were treated with 50 pmol/ml siRNA for 72 h prior to transfection with 1 μg/ml poly(I:C). 24 h later total RNA was analysed for IFNβ expression by qRT-PCR and data analysed as in panel A. Data are the mean ± SD from five replicates in two independent experiments.

3.5 Comparing the effects of two PML-II and Control siRNAs

In order to further confirm the results above and to control for possible off-target effects or other consequences of siRNA transfection, we designed further siRNAs either targeting PML-II via a sequence distinct from that used to this point, or expected not to target any human gene (control siRNA). The two PML-II siRNA target different parts of PML-II gene exon 7b sequence as shown in Figure 3.5.1. Scramble siRNA was designed through online software see website: http://www.genscript.com/ssl-bin/app/scramble.

Homo sapiens promyelocytic leukemia (PML), transcript variant 9, mRNA
NCBI Reference Sequence: NM_033239.2 GI: 67089155

TGCATGGAGCCCATGGAGACCGCCGAGCCACAGTCCTCGCCAGCCCACTCCTCGCCAGCCCACTCCTCGC
CAGCCCACTCCTCGCCAGTCCAGTCTCTGCTGAGAGCACAAGGAGCCTCCAGCCTGCCCTGTGGCACATA
CCACCCCCCAGCTTGGCCTCCCCACCAGCCCGCTGAGCAGGCTGCCACCCCCGATGCTGAGCCTCACAGC
GAGCCTCCTGATCACCAGGAGCGCCCTGCCGTCCACCGTGGGATCCGCTACCTGTTGTACAGAGCACAGA
GAGCCATCCGCCTTCGCCATGCCCTCCGCTTGCACCCTCAATTGCATCGGGCCCCTATTCGGACTTGGTC
TCCCCATGTGGTCCAAGCCAGCACTCCTGCCATCACAGGGCCCCTCAACCATCCTGCCAATGCCCAGGAA
CATCCTGCCCAGCTGCAAAGGGGCATCAGCCCACCCCACCGGATACGAGGGGCTGTGCGATCCCGCAGCC
GCTCCCTCCGGGGCTCCTCCCATTTATCCCAGTGGCTCAACAACTTTTTTGCCCTCCCCTTCTCCTCCAT
GGCTTCCCAGCTTGACATGTCTTCCGTGGTGGGGGCAGGGGAAAGCAGAGCCCAGACTCTTGGAGCAGGT
GTTCCCCCTGGGGACTCTGTCAGAGGCTCCATGGAGGCCTCTCAAGTCCAAGTGCCTCTGGAAGCCTCTC
CAATTACATTCCCACCACCCTGTGCCCCAGAAAGGCCCCCCATCAGCCCAGTCCCAGGCGCCCGTCAAGC
AGGCCTCTGA

Figure 3.5.1 PML variant 9 (PML-II), exon 7b nucleotide sequence. siRNA PML-II(A) highlighted with yellow colour was used for experiments in 3.1 – 3.4; PML-II (B) highlighted with light blue was newly designed for comparison.

The biological effects of siPML-II(A), (B) and siControl(A) and (B) on the IFNβ promoter were compared by both reporter assays (Figure 3.5.2 A) and analysis of endogenous mRNA by qRT-PCR (Figure 3.5.2 B). The result showed that both PML-II-specific siRNAs showed a very similar inhibition of the IFNβ promoter (Figure 3.3 A), and also can significantly inhibit IFNβ expression, though with a little difference. The reason for this may be due to different synthesized batches. Generally, this result shows that both siRNA can be used for knockdown of endogenous PML-II with similar effects on inhibiting IFNβ expression. siPML-II(A) was used for most of the remaining experiments in this thesis.

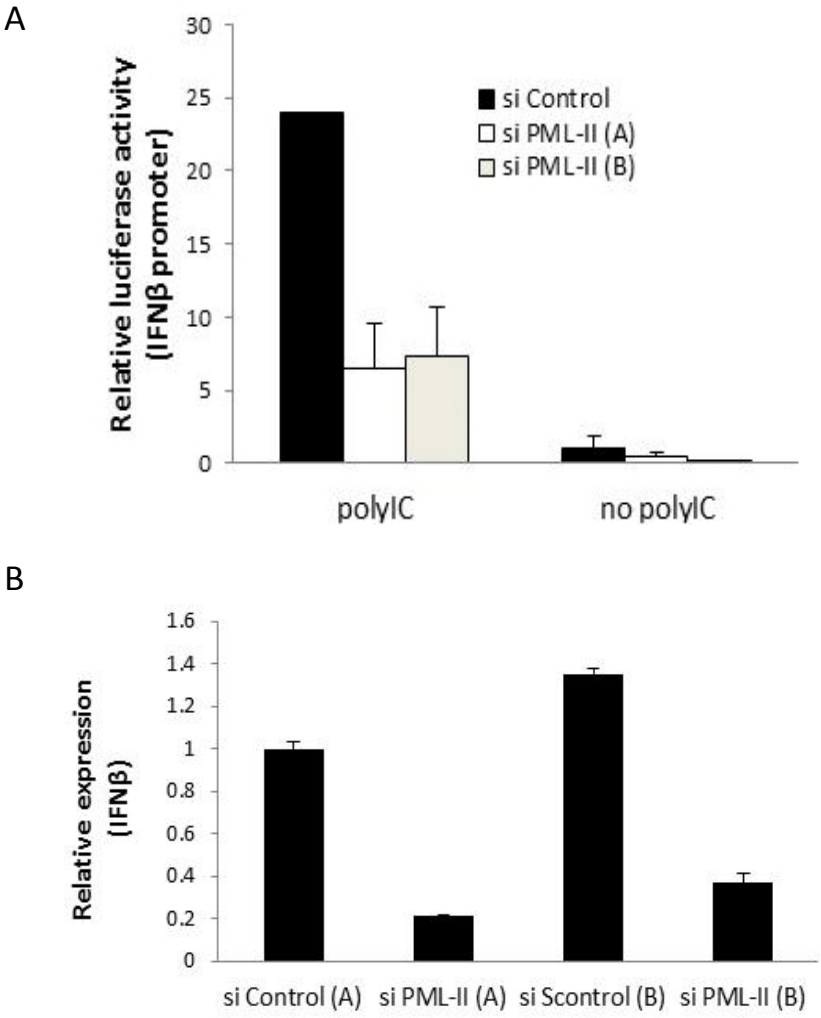


Figure 3.5.2 Compare two designed PML-II siRNA and Scramble siRNA (A) HEK293 cells were transfected in triplicate with 100 pmol/ml siControl or two siRNA targeting PML-II. 24 h later, cells were co-transfected with IFNβ-Luc and β-gal (pcDNA3.1-HisB::lacZ). Following poly(I:C) treatment for 16 h, cell lysates were assayed for both luciferase and β-galactosidase activities (RLA=luciferase/β-gal activity). (B) HEK 293 cells were transfected with either of two PML-II siRNAs or either of two control siRNAs at concentrations of 100 pmol/ml for 40 h, then cells were stimulated with 1μg/ml poly(I:C). 16 h later total RNA was extracted and the effect of knockdown of PML-II on IFNβ mRNA expression was detected by SYBR green qPCR.

3.6 Over-expression of full-length PML II does not significantly increase the expression of IFNβ

Given that depletion of PML-II reduced IFNβ expression, it was of interest to test whether over-expression of PML-II could correspondingly enhance IFNβ expression. The results showed the expression of full length PML-II did not exert a significant positive effect on the transcription of IFNβ and downstream ISG54 (Figure 3.6). The effect of an artificial PML-II form which lacked the N-terminal 360 amino acid residues comprising the RBCC domain was also tested for its effects on IFNβ mRNA (Figure 3.6). As previously observed by reporter assays (Wright, 2010), this PML-II form does increase IFNβ expression. However it does not correspond to a known naturally occurring form of PML and so the biological significance of this effect remains to be determined.

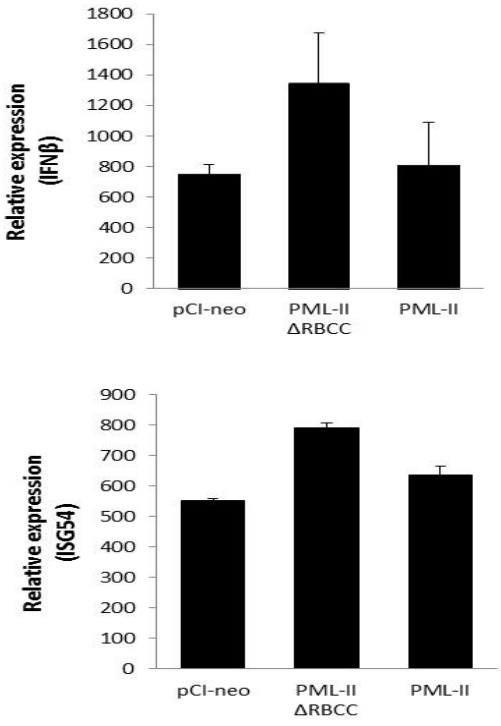


Figure 3.6 Over-expression of full-length PML II did not further enhance the expression of IFNβ HEK 293 cells were transfected 250ng/ml PML-II, PML-II ΔRBCC or pCI-neo (empty vector) plasmids for 24 h, then cells were stimulated with 1 μg/ml poly(IC). 16 h later RNA was extracted and the effect of knockdown of PML-II on IFNβ and ISG54 mRNA expression was detected by SYBR green qPCR.

3.7 Making PML-II siRNA-resistant constructs

I next sought to determine whether over-expression of PML-II could recover the expression of IFNβ in the cells depleted of PML-II. To do this, it was necessary to construct PML-II expression constructs that would be resistant to the siRNA used. Sequence changes were designed that would disrupt the association of the siRNA with its target whilst maintaining the PML-II protein sequence (Table 3.1). This was incorporated into PML-II RNAi-resistant constructs by mutagenesis PCR. Since it was unclear which of the many molecular forms of PML-II might be functionally significant in IFNβ expression, four potentially relevant siRNA-resistant PML-II cDNA variants were constructed using normal PML-II, ΔRBCC-PML-II, Δexon-5-PML-II and C-PML-II as the parent molecules; these were distinguished from their parents by the designation mut-PML. C-PML-II is a cytoplasm variant that excluded exons 4, 5 and 6. All four PML-II siRNA-resistant mutants were sequenced to make sure the inserted fragment contained the expected mutant sequence. Subsequently, the expression level of these mutants were also tested and it was confirmed these mutants can express relevant proteins to levels equal to or better than those from the equivalent parent plasmids (Figure 3.7). Notably, poly(I:C) stimulation enhanced the protein expression of normal and mutant PML-II species. Endogenous PML expression is induced by poly(I:C) as the gene contains GAS and ISRE elements at the promoter. Here PML is expressed from a heterologous promoter that is not known to be induced by poly(I:C). Possibly, PML protein expressed from these constructs is stabilized by interaction with the increased level of endogenous PML that will result from poly(I:C) stimulation. Also, the exogenous PML-II may be post-translationally modified in response to poly(I:C) stimulation in a way that increases its stability so that increased levels of protein are observed.

Table 3.1 Sequence showing the alterations made to confer resistance to siPML-II(A)

si PML-II complementary sequence (3'→5')	GTA	GGA	CGG	GTC	GAC	GTT	T
si PML-II target sequence (5'→3')	CAT	CCT	GCC	CAG	CTG	CAA	A GG
Mutants sequence	CAC	CCA	GCT	CAA	TTA	CAG	C GG
Encode Proteins	His	Pro	Ala	Glu	Leu	Glu	Arg

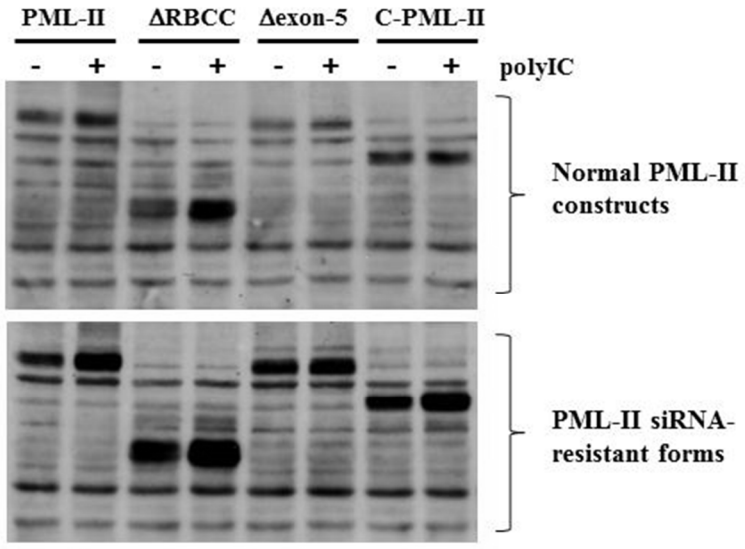
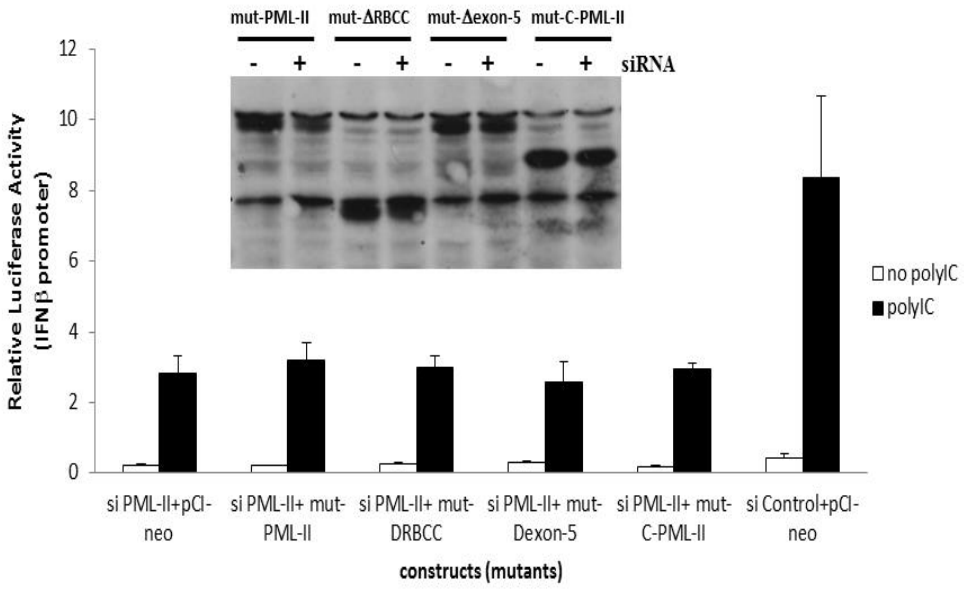


Figure 3.7 PML-II expression from siRNA-resistant constructs. siRNA-resistant PML expression plasmids (lower panel) or the equivalent parent plasmids were transfected into HEK293 cells to express various PML-II-related proteins, either with or without poly(IC) induction. Cells were lysed with SB and subjected to western blot and subsequently protein was detected with anti-Flag antibody.

3.8 PML-II siRNA-resistant mutants could not restore the activity of IFNβ promoter

Using the siRNA-resistant PML-II cDNA clones, a rescue experiment was conducted by reporter assay. As before, depleting endogenous PML-II by siRNA led to a significant decrease of the activity of IFNβ promoter (Figure 3.8 A; siControl vs siPML-II transfected with pCIneo empty vector). However, expressing either full-length resistant PML-II or any other PML-II form, including ΔRBCC, C-PML-II and Δexon-5, had no obvious rescuing effect on the IFNβ promoter activity though these plasmids encoded proteins that largely resisted the knockdown by PML-II siRNA (Figure 3.8A). Similarly, no increase in endogenous IFNβ mRNA level was seen in similar PML-II expressing cells (Figure 3.8B).

A



B

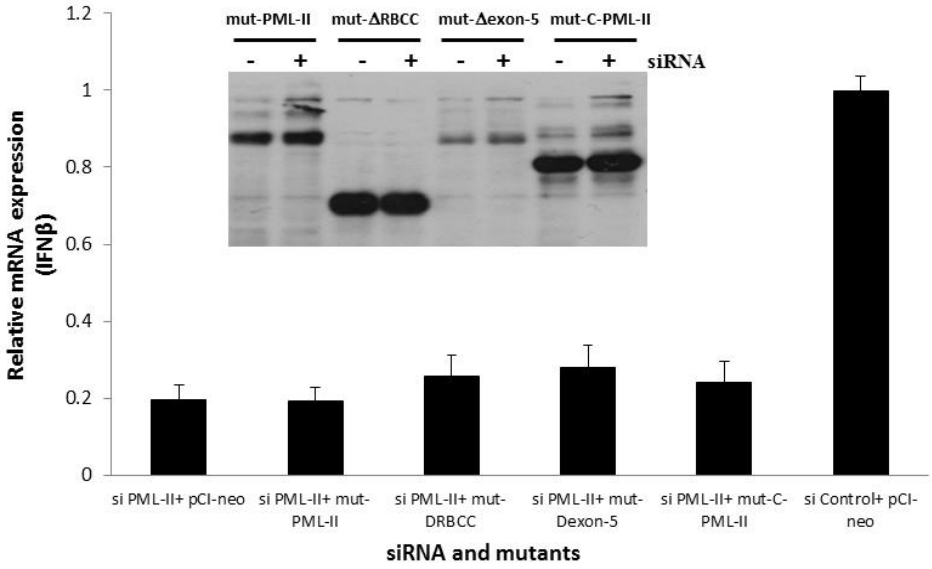


Figure 3.8 PML-II transient expression fails to restore IFNβ expression in PML-II depleted cells (A) HEK293 cells were transfected with 125 pmol/ml siControl or siPML-II for 24 h, then the cells were transfected with 225 ng/ml IFNβ-Luc, 25 ng/ml β-gal and 250 ng/ml different mut-PML-II expression plasmids or control empty vector pCI-neo. 24 h later cells were stimulated with 1μg/ml poly(I:C) for 16 h. Cells were lysed by passive lysis buffer for RLA detection. (B) Cells were treated as in (A) and RNA was extracted and IFNβ expression tested by qPCR.

3.9 Discussion

In this Chapter, PML-II was demonstrated to play an important role in controlling IFNβ expression, using specific knock-down of PML-II expression by siRNA as a tool. RNA interference (RNAi) was first elucidated as a specific endogenous effector which can inhibit gene expression. Subsequently, the ability of synthetic siRNAs to mediate RNA interference in mammalian cells was demonstrated by Elbashir and his colleagues (Elbashir et al, 2001) , leading to the widespread use of synthetic siRNAs as a laboratory tool to selectively knock-down the activity of specific genes. In this study, siRNA-mediated knockdown was demonstrated to successfully inhibit the expression of PML-II at protein and mRNA levels.

Different molecular sizes of endogenous PML-II proteins were observed in this study, which is consistent with previous reports that PML I-V can be post-translationally modified and may have several variants depending on the differential splicing of exon 4, 5 and 6 of PML (Jensen et al, 2001b). A small molecular size PML-II variant was seen particularly in cytoplasmic fractions, suggesting that it lacked exon 6 and the NLS signal it contains, preventing it from entering into the nucleus. All of the major PML-II bands observed were significantly decreased by PML-II siRNA compared with control siRNA treatment. Notably, the over-expressed full-length PML-II was extensively post-translationally modified which generates different molecular weights of PML-II. Again these proteins present in the blot were significantly reduced by PML-II siRNA. It is also known that PTM such as phosphorylation, sumoylation and acetylation have an important role for PML stability, whether these modifications have an effect on the function of PML-II in the expression of IFNβ is unknown. It therefore will be an interesting topic to investigate the relationship between PML post-translational modification and the IFN response.

To avoid possible off-target effects of PML-II siRNA, two pairs of PML-II siRNA were used in this study, one was a previously described PML-II siRNA (Kumar et al, 2007), the other one was designed by ourselves. The results showed that both siRNAs could inhibit PML-II expression and significantly decrease IFNβ promoter activity and mRNA level.

Thus, the effects were very unlikely to be due to off-target effects since these should be unique to one or other of the two siRNAs tested. The fact that endogenous IFNβ mRNA levels behaved as expected based on the promoter reporter assays proved that the effect of PML depletion on IFNβ expression was not an artefact of using a naked DNA plasmid, but was equally observed on experiment from the gene when formed into native chromatin. Taken together, these data indicate that expression of the IFNβ gene upon poly(I:C) stimulation is significantly dependent on PML-II.

To expand the function of PML-II on IFNβ expression, different cell lines were used including cell culture immortalized line HEK293, Hela cells from a cervical carcinoma and normal MRC5 human lung cells. The results showed that loss of PML-II protein in all these cell lines led to a great reduction of IFNβ expression in response to poly(I:C) stimulation. Thus the effect of PML-II depletion on IFNβ expression is not confined to one cell type. It was noted that in the MRC5 cell line, unlike HEK293 or Hela, the basal expression of IFNβ was very low or undetectable. However, upon stimulation the IFNβ mRNA was significantly increased. The relatively elevated levels of IFNβ in transformed cells may reflect their abnormal biology as being in a state of chronic molecular stress.

It is interesting that depletion of PML-II significantly reduced expression of IFNβ, whereas depletion of PML-V had no effect. PML-II is expressed from an mRNA that has been spliced to remove an intron that, when retained as part of an exon, encodes the C-terminus of PML-V. Therefore any siRNA targeted towards the PML-II unique 3' exon necessarily also targets PML-V mRNA within its 3' non-coding region. However, any depletion of PML-V by PML-II siRNA has no role in the biological effect of this knockdown since both the PML-V siRNA for which data are shown and another independent PML-V siRNA had no effect on IFNβ promoter activity. The PML gene also encodes PML-III from an mRNA that is even more similar to PML-II mRNA, differing from it only by the retention of an additional 40 nt of sequence. In this study PML-III alone was not specifically depleted and therefore it cannot formally be excluded that PML-III plays a role in the expression of IFNβ.

However, unlike PML-II, the PML-III unique C-terminus is poorly conserved (Leppard et al, 2009) and PML-III is expressed at very low levels, particularly in normal cells (Condemine et al, 2006b) where a strong negative effect of PML-II siRNA treatment on function was still observed, so it is unlikely that PML-III is significant in this context.

In this Chapter, the expression of PML-II was increased at both mRNA level and protein level in response to the stimulation by poly(I:C). This is because the PML gene itself has ISRE and GAS elements in the promoter, which therefore can be induced by type I and II IFN like other ISGs. Nonetheless, the expression of PML-II could be significantly reduced by PML-II siRNA in both the presence and absence of poly(I:C) stimulation. This suggested that the experiment system used in this study is stable and reliable for carrying out the following experiments.

Depletion of PML-II significantly inhibits IFNβ expression. However, over-expression of full-length PML-II was not observed to correspondingly enhance IFNβ expression in this study. In any process, one factor is necessarily the limiting factor. Possibly, endogenous PML-II levels are already enough to maximize IFNβ expression, ie some other factor is limiting, so that exogenous expression of more PML-II is superfluous. In attempts to restore inducible IFNβ expression to PML-II-depleted cells, overexpression of RNAi-resistant PML-II constructs had no obvious rescuing effect on the IFNβ promoter activity. This result suggested that these cDNAs may not encode the relevant functional PML-II species. It is known that PML mRNA undergoes alternative splicing consequently generating different size functional PML-II proteins, which makes it difficult to determine which form/variant is the real working one. Another reason may be that knockdown of endogenous PML-II impairs the structure of PML NB thus affecting the recruitment of some functional factors which will affect IFNβ transcription, while the newly expressed exogenous protein does not become modified sufficiently rapidly to substitute for it. It therefore will be interesting to look for the real functional PML-II molecular form in the future.

Chapter 4 The mechanism by which PML-II regulates IFNβ transcription and downstream signalling

IFNα/β expression is induced in response to various PAMPs (Mogensen, 2009; Randall & Goodbourn, 2008) including dsRNA, which is produced by many viruses during their replication (Jacobs & Langland, 1996). Transfected dsRNA is mainly recognized by PRRs RIG-I and Mda5, triggering a signalling cascade that leads ultimately to the activation of TFs such as IRF3 and NF-κB (Akira et al, 2006; Kawai & Akira, 2008; Wathelet et al, 1998; Yoneyama & Fujita, 2009). IRF-3 is phosphorylated and moves into the nucleus (Kumar et al, 2000; Lin et al, 1998) while NF-κB is released from its inhibitor IκB and migrates into the nucleus (Wullaert et al, 2006). These nuclear TFs, together with c-Jun/ATF-2, interact with the IFNβ promoter to form an enhanceasome (Maniatis et al, 1998; Thanos, 1996; Thanos & Maniatis, 1995). The assembled TFs recruit co-activator CREB-binding protein (CBP) or its homologue p300, which are histone acetyl transferases whose action promotes the assembly of the basal transcriptional machinery at the promoter (Kim et al, 1998; Merika et al, 1998; Weaver et al, 1998).

Secreted IFNβ stimulates both the producer cells and other cells to produce IFNα, which acts like IFNβ and so amplifies the response, as well as a large number of IFN-responsive gene (ISG) products. Both IFNα and IFNβ are recognized by receptors IFNAR1 and IFNAR2 and activate the JAK-STATs signalling pathway (Platanias, 2005). Phosphorylated STAT1-STAT2 complex then associates with IRF9 to form the ISGF3 heterotrimer which binds to the IFN-stimulated response element (ISRE) located within the promoters of most ISGs (Stark et al, 1998; van Boxel-Dezaire et al, 2006). The ISG products together with IFNα/β establish an antiviral state in target cells (Stark et al, 1998).

The result in Chapter 3 showed that knockdown of PML-II affects the expression of IFNβ. In this Chapter, experiments were designed for investigation of the mechanism by which PML-II affects IFNβ gene expression and downstream signalling.

4.1 Depletion of PML-II inhibits the activity of PRDIII/I promoter

The IFNβ promoter can be subdivided into four domains, PRD I, II, III and IV. Previous studies have demonstrated these four *cis* elements are crucial for virus-induced IFNβ expression (Du et al, 1993). Elements PRDIII/I associate with members of the IRF family regulatory factors, particularly IRF3 and IRF7, while PRDII can be bound by NF-κB (p65/p50) (Honda et al, 2006). These elements can be activated/ enhanced by expressing the appropriate individual transcriptional factors. When tested here, overexpression of IRF3 only modestly increased the unstimulated activity of a PRDIII/I -driven luciferase reporter, while poly(I:C) stimulation greatly increased PRDIII/I promoter activity and IRF3 transfection further increased this activity (Figure 4.1 A).

The effect of depletion of PML-II on IRF3 activity was then tested using this assay. As before, the activity of the IRF3-responsive reporter, PRDIII/I-Luc, was greatly increased upon stimulation with poly(I:C); remarkably, prior depletion of PML-II almost completely abolished this increase (Figure 4.1 B). These results suggested that depletion of PML-II affects IRF3 activity.

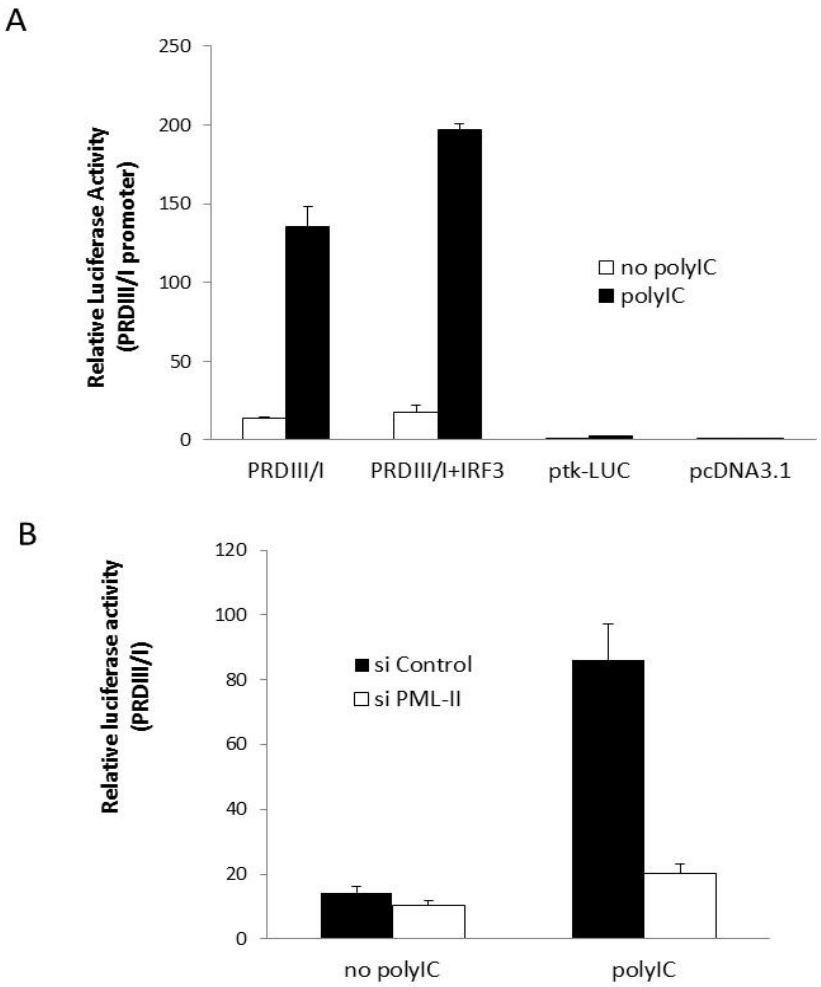


Figure 4.1 Depletion of PML-II affects PRDIII/I promoter activity (A) HEK293 cells were transfected with 225ng/ml PRDIII/I-Luc and 25ng/ml β-gal, and 250ng/ml IRF3, or same amount of empty vector ptk-Luc (PRDIII/I) and pcDNA3.1(IRF3). 24 h later cells were stimulated with poly(I:C) for 16 h, then harvested in passive lysis buffer and relative luciferase activity determined. Mean relative luciferase activities are shown for triplicate cultures/samples. (B) HEK293 cells were transfected with PML-II siRNA or scramble siRNA for 24 h then co-transfected with PRDIII/I-Luc and plasmids as (A). 24 h later cells were stimulated, harvested and assayed as in (A).

4.2 PML-II depletion reduces the expression of IRF3-responsive gene

If PML-II functions in IRF3 transactivation, it should affect expression of the IRF3-dependent/responsive genes that can be activated by IRF3 directly. To further confirm the effect of depletion of PML-II on IRF3 activity, the induction of endogenous mRNAs from

IRF3-responsive genes including ISG15, ISG54 and ISG56 were measured. The results showed that the induction of mRNA expression from these genes was significantly dependent on PML-II. Knockdown of PML-II greatly reduced the mRNA expression (Figure 4.2 A, B and C).

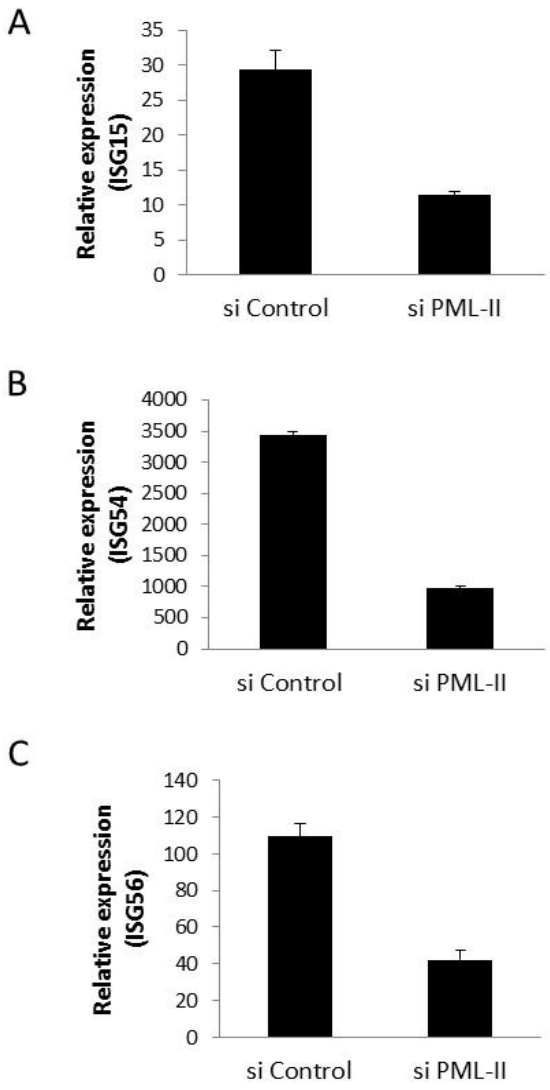


Figure 4.2 Depletion of PML-II affects the expression of IRF3-responsive gene HEK293 cells were transfected with 100 pmol/ml PML-II siRNA or control siRNA; 40 h later, cells were stimulated with 1 μg/ml poly(I:C) for 12-16 h. The mRNA levels of (A) ISG15, (B) ISG54 and (C) ISG56 were measured by SYBR green qPCR. The mRNA level in each sample is presented relative to control siRNA (no stimulation). The expressions were normalized against GAPDH or β-actin. Quantification was based on Ct difference performed according to the "delta-delta Ct method".

4.3 Depletion of PML-II affects NF-κB activity and NF-κB-dependent gene expression

NF-κB is also thought to play an important role in virus-induced IFNβ expression. It interacts with the IFNβ promoter via the PRDII element. The effect of depletion of PML-II on NF-κB activity was determined by using a PRDII-driven luciferase reporter assay. The results showed that depletion of PML-II significantly reduced NF-κB basal activity to 30% in unstimulated cells (Figure 4.3A). Poly(I:C) stimulation did not significantly increase the activity of PRDII promoter over basal levels but the removal of PML-II still reduced substantially its activity, again to 30-40% of levels in control siRNA-treated cells (Figure 4.3A). Since PML-II knockdown inhibited NF-κB activity, it was of interest to investigate whether knockdown of PML-II also affected the induction of endogenous NF-κB-dependent genes. IL-8, IL-6 and TNFα were selected because their expression is largely dependent on NF-κB activity (Alexopoulou et al, 2001; Yun et al, 2011). Similar to IRF3-dependent/responsive genes, the results showed that depletion of PML-II inhibited mRNA expression of these genes in poly(I:C)-stimulated cells to a level ranging from 40% to 70% though with relative lower overall induction level (Figure 4.3B-D). Collectively, these results indicate that depletion of PML-II impairs gene activation by both IRF3 and NF-κB. Notably, many NF-κB-dependent gene products are related to pro-inflammatory response. Therefore this result suggested that PML-II may play a role in the inflammatory response.

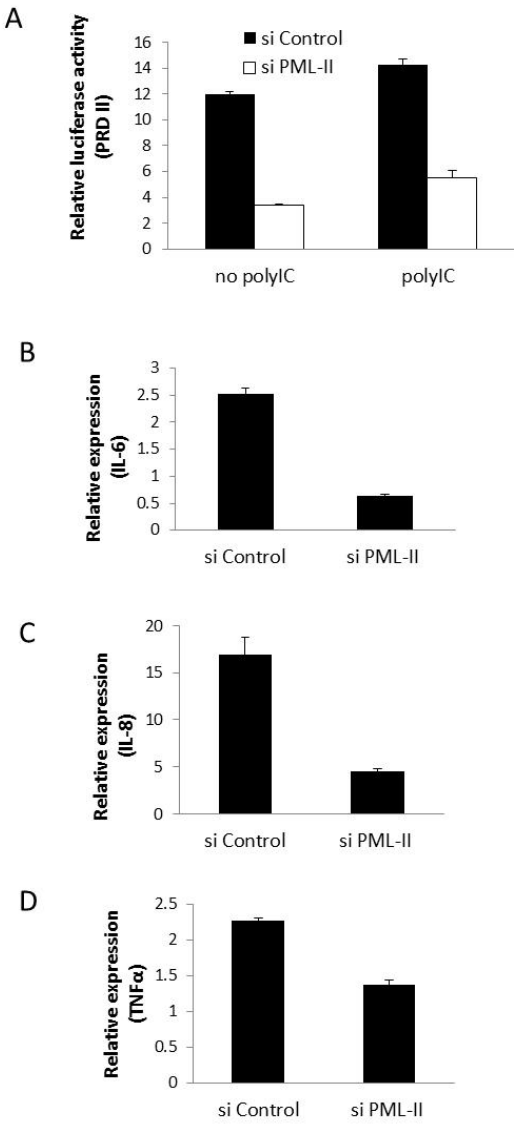


Figure 4.3 Depletion PML-II affects the activity of PRDII promoter and the expression of NF-κB-dependent genes (A) HEK293 cells were transfected with PML-II siRNA or control siRNA, 24 h later, cells were transfected 225ng/ml PRDII-Luc and 25ng/ml β-gal plasmids. 24 h following transfection, cells were transfected with poly(I:C) for 16 h, then harvested for luciferase assay. Mean relative luciferase activities are shown for triplicate cultures/samples. (B-D) HEK293 cells were transfected with PML-II or control siRNAs for 48 h, then stimulated with poly(I:C); 12 h later, mRNA levels of IL-6, IL-8 and TNFα were measured by SYBR green qPCR. Quantification was by Ct difference, normalized against GAPDH or β-actin, performed according to the "delta-delta Ct method".

4.4 The effect of depletion of PML-II on IRF3 phosphorylation and nuclear translocation

In principle, the induction of IFNβ could be modulated by PML-II at any point from PAMPs sensing by PRRs, through the signalling cascade of transduction into the nucleus, to the IFNβ promoter/enhancer assembly process. To investigate the point of PML-II action in this pathway of IFNβ expression, IRF3 phosphorylation and nuclear translocation was firstly examined.

The expression status of IRF3 in HEK293 cells was firstly examined. Western blotting showed that upon stimulation with poly(I:C), the band intensity of IRF3 in the nucleus was greater than that of mock/no stimulation (Figure 4.4 A). Interestingly, IRF3 actually resolves into two bands, and in the poly(I:C) stimulated cells the upper band was more abundant than the lower one, while in mock-stimulated cell lysate the situation was reversed (Figure 4.4 A). This result is consistent with previous reports that, in unstimulated cells, IRF3 is constitutively expressed in two forms (form I and II) and localized in the cytoplasm. Form I is un-phosphorylated whereas form II represents basal IRF3 phosphorylation; on viral infection, IRF3 is phosphorylated within its C-terminus resulting in hyper-phosphorylated forms III and IV that move into nucleus (Servant et al, 2001). Why there was such a small amount of hyper-phosphorylated IRF3 translocated into the nucleus in response to poly(I:C) stimulation is unclear. However, several previous studies have shown that that the nuclear accumulation of IRF3 is not in fact directly correlated with IRF3 hyper-phosphorylation state (Dauber et al, 2006; Spiegel et al, 2005) .

IRF3 phosphorylation and nuclear translocation were described as key steps in signal transduction following PAMP recognition (Lin et al, 1998; Sharma et al, 2003; Wathelet et al, 1998). Upon signalling from PRRs, IRF3 is activated through its phosphorylation and then moves into the nucleus to initiate the expression of IFNβ. The time-course of IRF3 phosphorylation following poly(I:C) stimulation was first tested. As expected, the amount of

phosphorylated IRF3 increased gradually upon poly(I:C) addition in HEK293 cells and HeLa cells (Figure 4.4 B and C). Interestingly, low level phosphorylated IRF3 could also be detected in uninfected cells whereas previous studies have documented that IRF3 C-terminal Ser396 can be phosphorylated only under condition of virus stimulation. This may reflect a requirement to maintain a basal level of ISG expression to give a rapid antiviral response in the initial stages of infection (Basagoudanavar et al, 2011; Taniguchi & Takaoka, 2001); such levels may also be somewhat cell-type specific. Treatment with PML-II siRNA did reduce the accumulation of phospho-IRF3 somewhat (Figure 4.4 B and C) although this effect was modest and was only apparent with longer periods of stimulation, suggesting it was a secondary consequence of the changes in ISG expression observed.

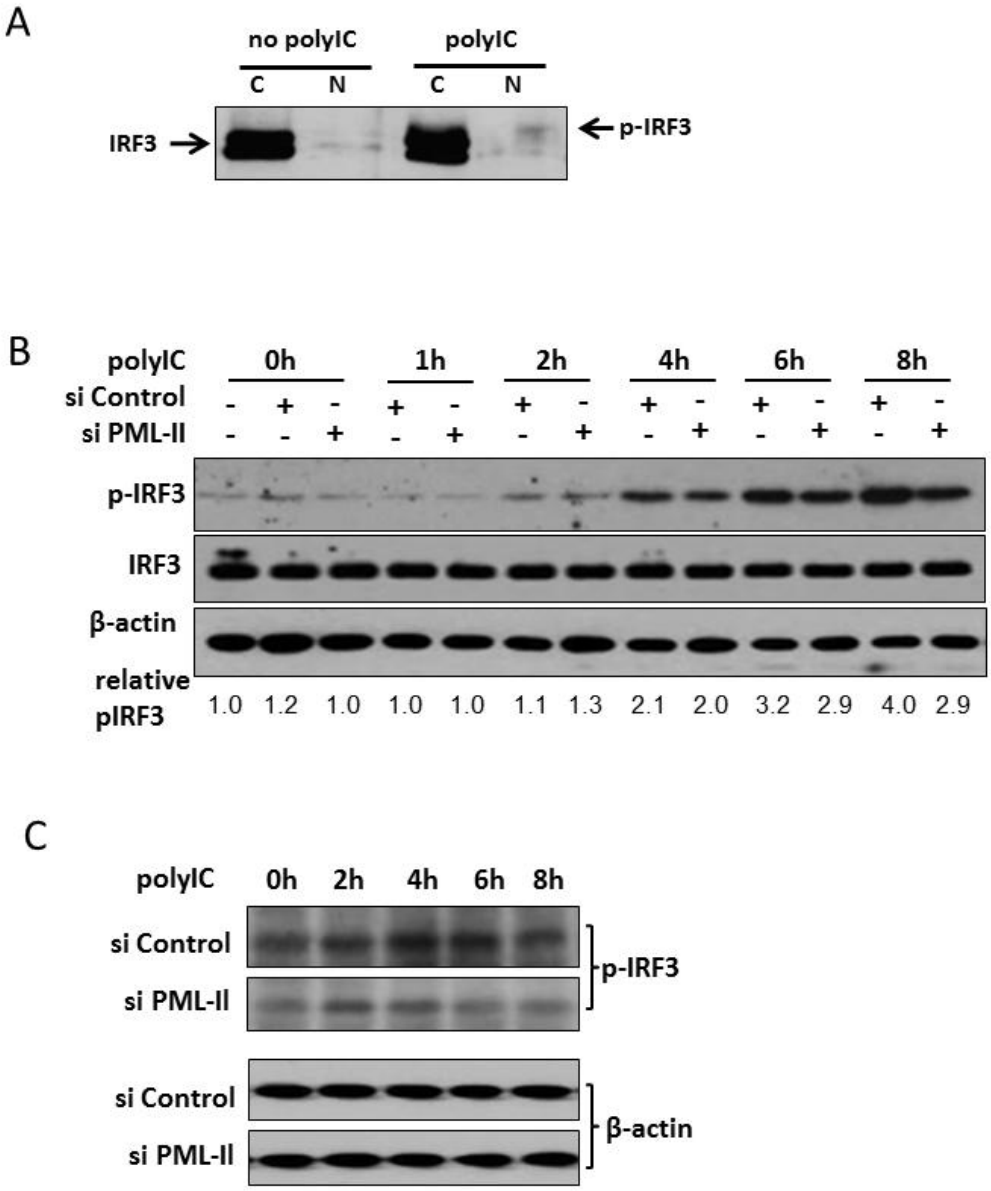
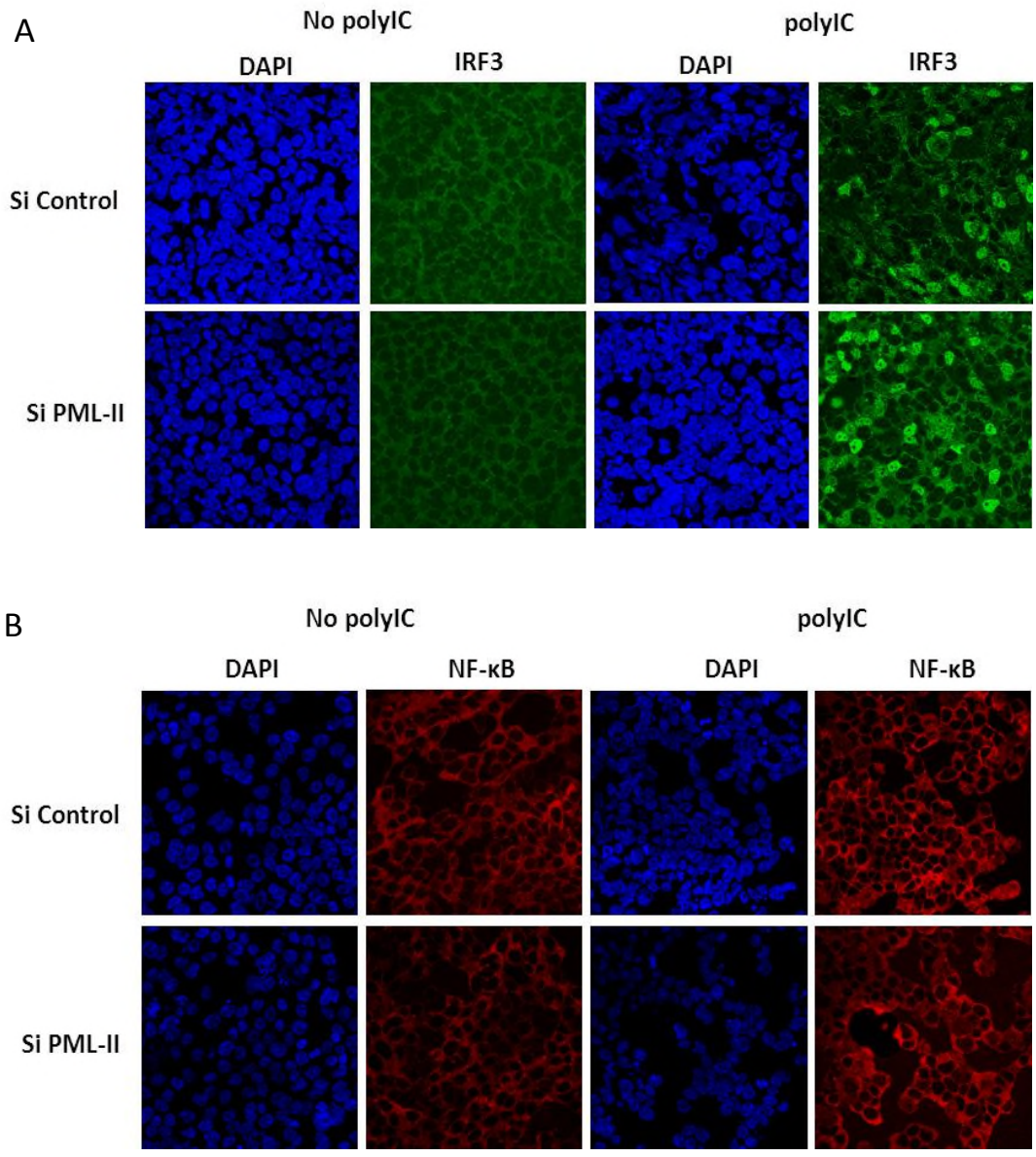


Figure 4.4 IRF3 status and phosphorylation (A) HEK293 cells were fractionated to produce nuclear and cytoplasmic fractions. Samples were then diluted into SB buffer and subjected to 10% SDS-PAGE and western blotting. Blots were probed with IRF3 antibody. The IRF3 was indicated by the arrows on the blots. (B) HEK293 and (C) HeLa cells were transfected with 125 pmol/ml PML-II or control siRNA for 48 h then stimulated by transfection with 1 μg/ml poly(I:C) (t=0) before collection at the indicated time points. Samples were separated by SDS-PAGE, western blotted and probed with antibody to phosphorylated IRF3, or β-actin as a loading control. Band intensities in digitized images were quantified using QuantityOne software (BioRad); the amounts of pIRF3 detected are shown relative to total IRF3.

4.5 Depletion of PML-II does not affect IRF3 and NF-κB nuclear translocation

As an alternative method to study nuclear translocation, confocal immunofluorescence microscopy was utilized to detect IRF3 and NF-κB following poly(I:C) stimulation. IRF3 and NF-κB displayed diffuse cytoplasmic staining patterns and were excluded from the nucleus in the absence of poly(I:C). Upon poly(I:C) treatment, IRF3 moved into nucleus, with the proportion of nuclei staining positive for IRF3 reaching to 30-40% by 16 h stimulation (Figure 4.5A). Like the phosphorylation of IRF3, this nuclear localization response was also unaffected by prior depletion of PML-II. Extending the stimulation to 24 h gave a similar percentage of nuclear IRF3 staining but some cells were broken and detached suggesting cell death was occurring in response to the poly(I:C) treatment. Thus 16 h poly(I:C) exposure was an ideal time for this experimental system.

For NF-κB, the effect of poly(I:C) stimulation was less striking, only very few (<5%) of nuclei staining positive for NF-κB (p65) after 16 h (Figure 4.5B). One possible explanation for this could be NF-κB localization between nucleus and cytoplasm is dynamic which may make it difficult to detect nuclear NF-κB under these conditions. To understand this further, a time-course analysis of NF-κB translocation in HEK293 cells stimulated with poly(I:C) will be required in the future. Alternatively, the low level of nuclear translocation may be due to poly(I:C) not being an efficient inducer in the activation of NF-κB. This is consistent with the lower activity of NF-κB relative to IRF3 when induced by poly(I:C) in the IFNβ promoter subdomain reporter assays. Again, as for IRF3, there was no difference in the extent of NF-κB nuclear translocation in the presence or absence of PML-II (Figure 4.5B). Taken with the findings of subcellular fractionation and p-IRF3 analysis, these results suggested that PML-II does not positively regulate either PAMP recognition or the resulting signal transduction cascade into the nucleus, and hence our attention was focused on the assembly of IRF3 and/or NF-κB at the IFNβ enhancer/promoter as a point in the pathway where the absence of PML-II might cause a reduction in gene expression.


A
No polyIC
polyIC
DAPI
IRF3
DAPI
IRF3
Si Control
Si PML-II
B
No polyIC
polyIC
DAPI
NF-κB
DAPI
NF-κB
Si Control
Si PML-II

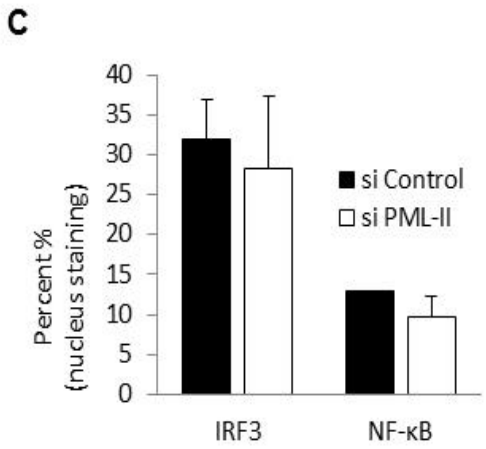


Figure 4.5 Depletion of PML-II does not block IRF3 and NF-κB nuclear translocation. HEK293 cells were transfected with 125 pmol/ml si PML-II or si Control and 48 h later transfected with 1 μg/ml poly (I:C). 16 h later, cells were fixed and stained with (A) IRF3 and (B) NF-κB antibodies. Images were captured with a Leica SP2 confocal system; blue-DAPI (DNA); green-IRF3; Red-NF-κB (p65). (C) Quantification of IRF3 and NF-κB nuclear staining. All nuclei in a field were detected by DAPI staining and the proportion showing positive staining for IRF3 or NF-κB was determined manually over 2-3 fields of view.

4.6 PML-II interacts with IRF3-CBP transcription complex

Previous studies demonstrated that the transcription factors (TFs) assembled at the IFNβ promoter can recruit the histone acetyl transferase co-activators CBP/p300 which act as a scaffold/bridge to contact both transcription factors and basal transcriptional machinery (Lin et al, 1998; Merika et al, 1998; Wathelet et al, 1998; Weaver et al, 1998). It has also been reported that CBP can be bound by PML and recruited into PML-NB (Doucas et al, 1999; Jensen et al, 2001a; Zhong et al, 1999a). These observations suggest that at least some isoforms of PML might form protein complexes with IRF3-CBP and/or NF-κB-CBP and hence regulate the activities of these transcriptional complexes.

Firstly, the association between IRF3 and CBP was evaluated by using co-immunoprecipitation (co-IP). Co-IP is one of the most widely used techniques to identify physiological interactions between proteins. The principle of co-IP is using an antibody

against a known protein to indirectly capture any other proteins that are bound to this specific target protein. After precipitation, western blotting is used to identify these binding partners.

Results in this study showed that in unstimulated cells IRF3 does not binding CBP, but when stimulated with poly(I:C), IRF3 and CBP could be detected from the protein complex precipitated by CBP and IRF3, respectively, which suggested IRF3 binds to CBP under the condition of stimulation (Figure 4.6 A). This result is consistent with previous reports that upon receipt of stimulation signal, activated IRF3 moves into the nucleus, assembles on the IFNβ enhancer and binds CBP (Weaver et al, 1998; Yoneyama et al, 1998).

Next, the association between PML-II and IRF3-CBP transcriptional complexes was tested, A Flag-tagged PML-II (FLAG-PML-II) expression plasmid was transfected, and protein complex was captured by using CBP and IRF3 antibodies separately; precipitated protein complexes were then analysed for CBP and PML-II. Similar to Figure 4.6 A, IRF3 bound to CBP only upon stimulation with poly(I:C) (Figure 4.6 B). Also in agreement with previous reports, PML-II bound to CBP in both the stimulation and un-stimulated conditions (Figure 4.6 B) (Doucas et al, 1999; Zhong et al, 1999a). Importantly, FLAG-PML-II was also pulled down by IRF3 antibody and poly(I:C) stimulation further increased this association (Figure 4.6 B). The interaction between PML-II and IRF3 that is suggested by these results might be direct or indirect. Collectively, these results indicated that PML-II interacts with the IRF3-CBP transcriptional complex and that stimulation further enhances this association.

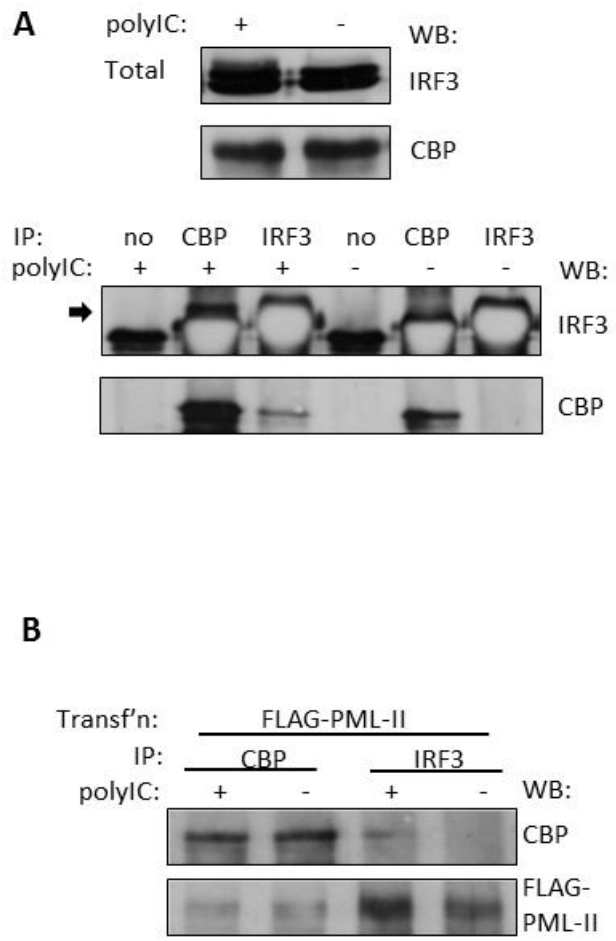


Figure 4.6 PML-II interacts with IRF3-CBP transcriptional complex. (A) HEK293 cells were stimulated with poly(I:C), 16 h later cells were lysed and protein complexes were precipitated by either CBP or IRF3 antibodies for detecting the association between IRF3 and CBP. Upper panel: direct detection of IRF3 and CBP in lysates by western blotting; lower panel: detection of CBP in immunoprecipitates. (B) HEK293 cells were transfected with 250 ng/ml Flag-PML-II plasmid for 24 h, and then cells were stimulated with poly(I:C) for 16 h. Whole cell lysate (WCL) were prepared and incubated with IRF3 or CBP antibodies for detecting any association of these proteins with PML-II.

4.7 PML-II can form a transcriptional complex with NF-κB-CBP

To further test the role of PML-II in the interaction between CBP and transcriptional factors, the association of PML-II and CBP-NF-κB complex was examined. FLAG-PML-II was over-expressed in HEK293 cells and protein complexes were precipitated by Flag-beads. As before, the result demonstrated that PML-II bound CBP in both stimulated and un-stimulated cells (Figure 4.7). Notably, more NF-κB was pulled down by Flag-beads in the poly(I:C) stimulated cells, while in un-stimulated cells there was no significant amount of NF-κB precipitated compared with control sample. Thus association of both IRF3/CBP and NF-κB-CBP with over-expressed PML-II is induced/enhanced by poly(I:C) stimulation.

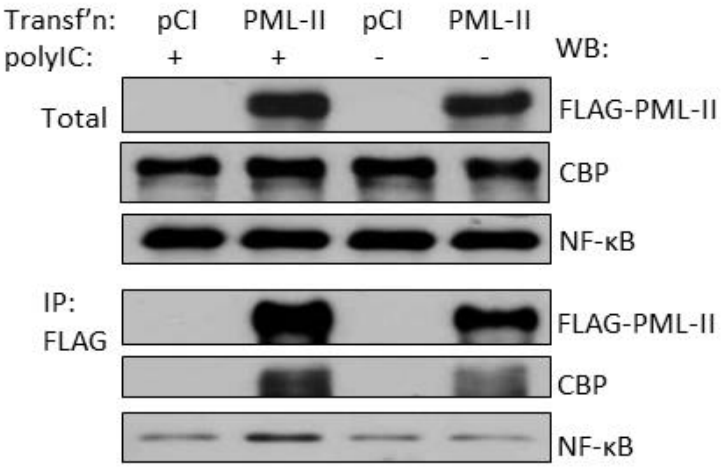


Figure 4.7 PML-II binds NF-κB-CBP transcriptional complex. HEK293 cells were transfected with 250 ng Flag-PML-II plasmid or same amount of pCI-neo empty vector for 24 h. Cells were stimulated with poly(I:C) for 16 h. Total cell lysate were prepared and precipitated with Flag-beads for assaying the formation of the PML-II-CBP-NF-κB protein complex. Samples were subjected to 5-8% SDS-PAGE and western blotting and probed with Flag, CBP and NF-κB (p65) antibodies separately. Upper panels: direct immunoblotting of total cell lysates; lower panels: immunoblotting of Flag-immunoprecipitated proteins.

4.8 Depletion of PML-II affects IRF3 recruitment of CBP

Next, the effect of depleting PML-II on the PML-II-IRF3-CBP complex was investigated.

The result showed the amount of CBP precipitated by IRF3 antibody was less in the PML-II siRNA transfected cell, suggesting depletion of PML-II reduces the association between IRF3 and CBP that is induced by poly(I:C) stimulation (Figure 4.8 A). PML-II depletion also reduced the association between CBP and STAT1 (Figure 4.8 B). Taken together, these data indicate the formation of ternary complexes during poly(I:C) stimulation that involve PML-II, CBP and IRF3/STAT1 and show that PML-II contributes to the stable association of TFs with CBP.

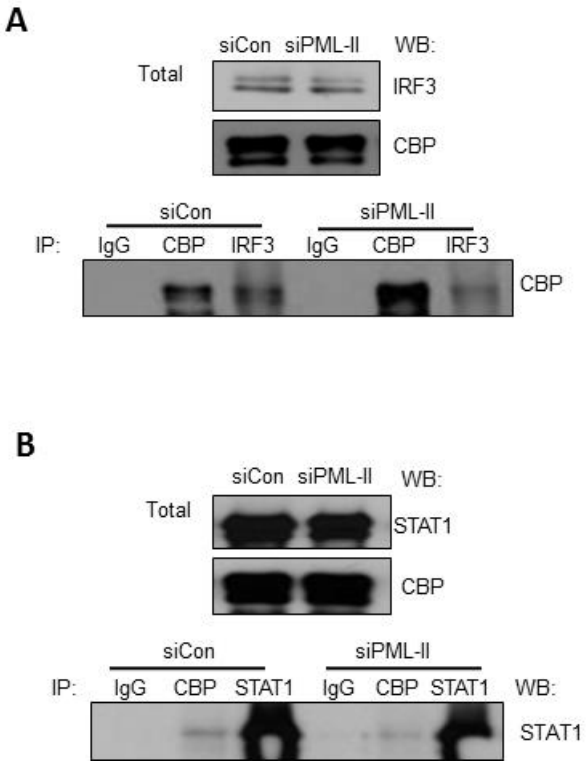


Figure 4.8 Knockdown of PML-II affects the association between IRF3 and CBP. (A) HEK293 were transfected with si PML-II or si Control for 48 h and stimulated with poly(I:C) for 12 h. Cell were lysed with 0.5% NP-40 co-IP buffer, samples were incubated with IRF3 or CBP antibodies overnight, then precipitated with Protein A-sepharose beads for 3-4 h. Following intensive washing, precipitated proteins were displaced from beads with 2x SB buffer and analysed by SDS-PAGE. Proteins were detected by western blotting of either total (top) or immunoprecipitated (bottom) samples. (B) HEK293 cells were treated with siRNA and then stimulated with poly(I:C) as (A). Lysates were prepared, immunoprecipitated with STAT1 or CBP antibodies and precipitates & total lysates analysed for STAT1.

4.9 Knockdown of PML-II affects IRF3, NF-κB binding and CBP recruitment to the IFNβ promoter

To ascertain the effects of depleting PML-II on the assembly of transcription complexes at the IFNβ promoter, chromatin immunoprecipitation (ChIP) was utilized. ChIP is an experimental technique used to investigate protein-DNA interactions that occur inside the nucleus of living cells or tissues. The principle underpinning this assay is to cross-link the DNA in the living material with any bound proteins such as transcription factors. Following crosslinking, the cells are lysed and chromatin is sonicated into 0.2-1.0 kb fragments. Chromatin fragments are then immunoprecipitated by using a specific antibody that targets the specific protein of interest. Finally, the cross-linking between protein and DNA in the immunoprecipitated material is reversed, allowing the DNA to be separated from the proteins. The identity and quantity of the DNA fragments present is determined by PCR/qPCR, genome array hybridization or sequencing.

To analyse the effects of PML-II depletion on protein binding at the IFNβ enhancer, it was first necessary to establish and optimize the ChIP method. Online software (NCBI Primer-designing tool/primer3) was used to design IFNβ-ChIP-qPCR primers. The primers selected are different from those used in Chapter 3 to quantify IFNβ mRNA because they will be used to amplify IFNβ DNA precipitated by IRF3 or other transcription factors such as NF-κB. This requires the targeted sequence should be close to or cover the IFNβ enhancer where transcription factor binding occurs. In addition, the amplified DNA product fragment should not be too long because the chromatin DNA templates are broken into about 200-1000bp fragments after sonication cleavage. If an amplification target is too long, this template fragmentation will certainly affect the efficiency of amplification. Two pairs of primers were designed here for amplification of 82bp and 139bp fragments from the human IFNβ gene, respectively (Figure 4.9.1). Dissociation curve analysis showed that both primers could amplify the specific DNA products with no spurious amplification products see Appendix 3.

Next, the ChIP-qPCR assay, using the (yellow) primer pairs in Figure 4.9.1, was used to compare the assembly of IRF3 and NF-κB on the IFNβ promoter either with or without depletion of PML-II. DNA fragments from the enhancer sequence of the IFNβ genes were amplified successfully from template DNA precipitated by IRF3 and NF-κB antibodies. Normally, two methods were used to analyse ChIP-qPCR data: the Percent Input Method and the Fold Enrichment Method. In this experiment, ChIP-qPCR data was analyzed by using Percent Input Method (relative to input) as this includes normalization for background level and input chromatin going into the ChIP. The result showed that depletion of PML-II led to significant reductions in IRF3 and NF-κB binding at the IFNβ promoter (Figure 4.9.2 A and B).

Given these data, and the fact that PML-II depletion inhibited the association of CBP with both IRF3 and NF-κB (Figure 4.8), it was reasonable that the absence of this PML isoform might also affect CBP binding to the IFNβ promoter. Indeed, there was an almost complete loss of CBP binding at the promoter in the absence of PML-II (Figure 4.9.2 C). Thus it could be concluded that the knockdown of PML-II affects TFs assembly at the IFNβ promoter and prevents CBP recruitment. Given the previously established importance of these factors in inducing transcription of the IFNβ gene these findings can therefore account for the severely reduced expression of the gene when induced under conditions of PML-II depletion.

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  1 AGTCAGTAGAATCCACGGATACAGAACCTATGGATAGGAAGGACCAACTGTATCTTTTAG
 61 TGTTTTGAGGTTCTTGAATTCTCAGGTCGTTTGCTTTCCTTTGCTTTCTCCCAAGTCTTG
121 TTTTACAATTTGCTTTAGTCATTCACTGAAACTTTAAAAAACATTAGAAAACCTCACAGT
181 TTGTAAATCTTTTTCCCTATTATATATATCATAAGATAGGAGCTTAAATAAAGAGTTTTA
241 GAAACTACTAAAATGTAAATGACATAGGAAAACTGAAAGGGAGAAGTGAAAGTGGGAAAT
301 TCCTCTGAATAGAGAGAGGACCATCTCATATAAATAGGCCATACCCATGGAGAAAGGACA
361 TTCTAACTGCAACCTTTCGAAGCCTTTGCTCTGGCACAACAGGTAGTAGGCGACACTGTT
421 CGTGTTGTCAACATGACCAACAAGTGTCTCCTCCAAATTGCTCTCCTGTTGTGCTTCTCC
481 ACTACAGCTCTTTCCATGAGCT
```

Figure 4.9.1 IFNβ gene enhancer and designed primers. DNA sequence come from proximal promoter genome sequence of human IFNβ gene. Purple background with red characters is enhancer sequence of IFNβ gene; green and yellow highlighted sequences indicate the positions of two pairs of ChIP-qPCR primers.

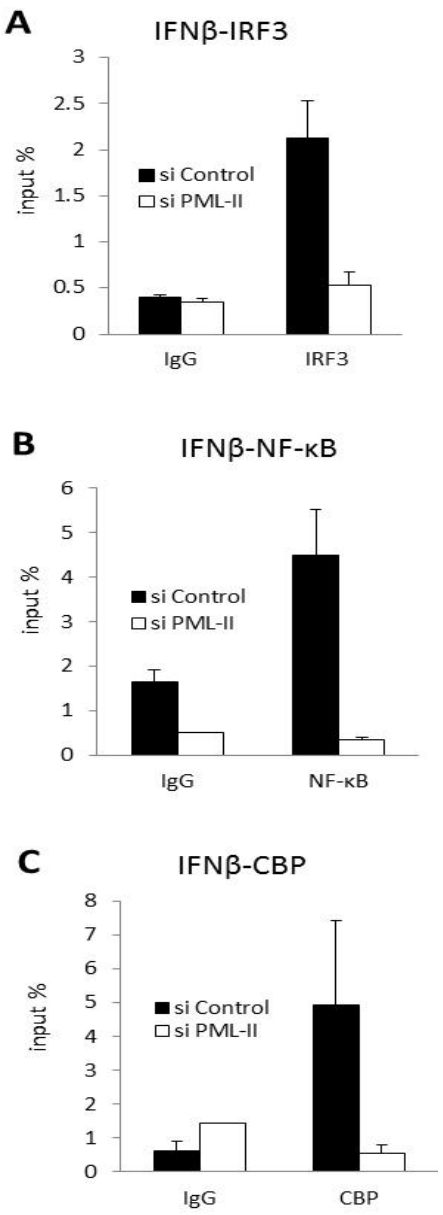


Figure 4.9.2 Knockdown of PML-II affects IRF3/NF-κB/CBP binding to the IFNβ promoter. HEK293 cells were transfected with PML-II or control siRNA for 48 h and stimulated with poly(I:C) for 16 h for IRF3 (A); 4-10 h for NF-κB (B) and 10 h for CBP (C), then fixed and subjected to ChIP with (A) IRF3, (B) NF-κB or (C) CBP antibodies. Precipitated DNA was assayed for IFNβ promoter sequences by SYBR green qPCR. In each case, signals obtained from the ChIP are divided by signals obtained from an input sample. This input sample represents the amount of chromatin used in the ChIP. Typically, 1% or 5% of starting chromatin is used as input. Results shown are the means ± SD from one representative of at least two experiments, each performed in triplicate.

4.10 The activity of ISRE promoter was affected by depleting PML-II

To analyse the effect of PML-II on IFNβ downstream signalling, the activity of a luciferase reporter plasmid driven by an interferon-stimulated response element (ISRE) promoter was measured. Both poly(I:C) and IFNα were used as inducers to test the effect of depleting PML-II on different stages of the IFN response; poly(I:C) works indirectly, via stimulation of IFNβ expression which then activates the Jak/STAT pathway, while IFNα acts directly to stimulate the Jak/STAT pathway. The results showed that, as expected, both IFNα and poly(I:C) could significantly activate the ISRE promoter in HEK293 cells (Figure 4.10A), the relative luciferase activities increasing more than 20-fold and 60-fold, respectively. Upon knockdown of PML-II, the activity of the ISRE reporter induced in response to poly(I:C) was significantly inhibited, being reduced to <50% of the activity seen with control siRNA. More surprisingly, IFNα stimulation of the ISRE reporter was also strongly inhibited by PML-II depletion (Figure 4.10A). Whilst the effect of PML-II depletion on ISRE activation by poly(I:C) might be due in part to an indirect effect of impaired IFNβ expression already demonstrated, this latter result indicates that PML-II plays a direct role in the Jak/STAT1 signalling pathway in addition to its role in IFNβ expression.

To exclude any possibility that IFNα affects ISRE activity in part indirectly, through activating IFNβ expression, the activity of an INFβ promoter reporter during IFNα stimulation was tested under conditions of PML-II depletion. The results showed that, in comparison with poly(I:C) stimulation, IFNα stimulation could not activate IFNβ expression in this system (Figure 4.10B). As a further control, the effect of depletion of PML-V on ISRE promoter activity was also tested, because it was already known that PML-V had no effect on IFNβ activity. The result showed that PML-V siRNA also had no apparent inhibitory effect on ISRE activity, in contrast to PML-II siRNA (Figure 4.10C). Therefore it can be concluded that knockdown of PML-II not only impairs IFNβ induction, but also has a direct effect on the Jak/STATs signalling pathway.

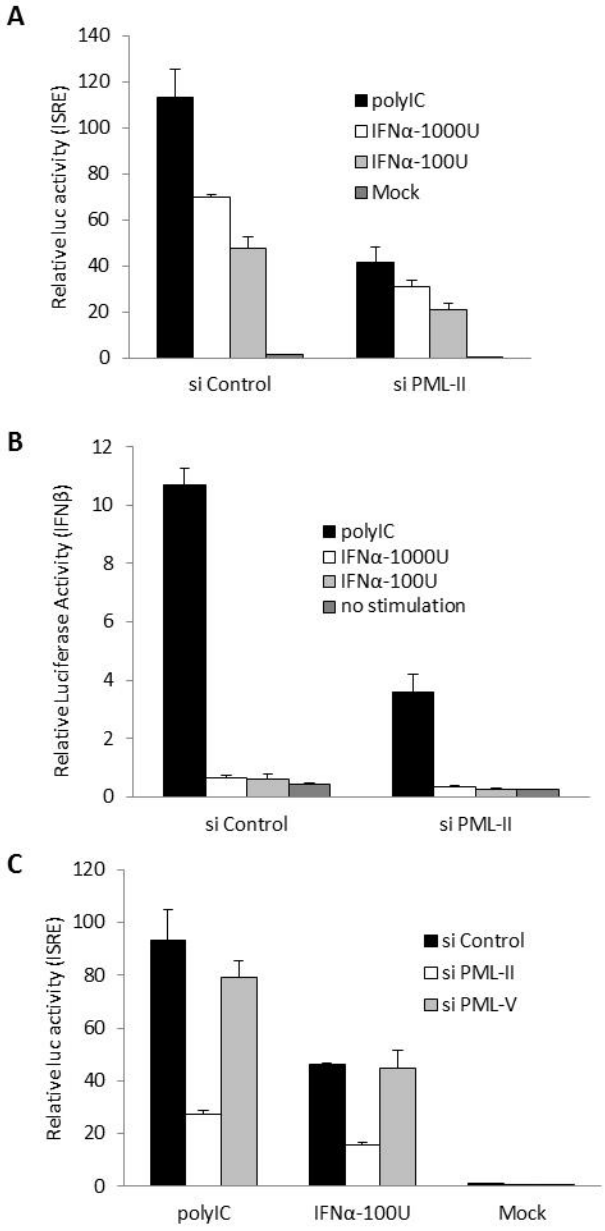


Figure 4.10 Knockdown of PML-II inhibits the activity of an ISRE promoter (A) HEK293 cells were incubated with PML-II siRNA or Control siRNA for 48 h, then co-transfected with 225 ng/ml ISRE-Luc reporter plasmid and 25 ng/ml β-gal (pcDNA3.1-HisB::lacZ). 24 h later cells were mock-transfected or transfected with 1 μg/ml poly(I:C) or treated with 100 U/ml or 1000 U/ml IFNα for 16 h. Cells were harvested and lysed with 1× Passive Lysis Buffer. Both firefly luciferase and β–gal activities of lysates were assayed to allow calculation of relative luciferase activity (RLA). (B) As in panel A, but replacing ISRE-Luc with 225ng/ml IFNβ-Luc. (C) HEK293 cells were transfected with PML-II, PML-V or control siRNAs for 48 h, then treated and analysed as in panel A. Error bars indicate the standard deviation of three replicate samples from the mean value.

4.11 Knock-down of PML-II affects the expression of ISGs in response to poly(I:C) stimulation

The finding that knockdown of PML-II inhibited ISRE promoter activity suggested that PML-II must play a role in the induction of the numerous IFN-stimulated genes (ISGs). To further investigate the importance of PML-II to IFNβ downstream signalling, HEK293 cells were stimulated with poly(I:C), and the mRNA levels of a series of ISGs measured by qPCR. Data for three genes that are responsive to IRF3 as well as to ISGF3 (ISG15, ISG54 and ISG56) were shown in Figure 4.2 while those for other ISGs including ISG20, RANTES (CCL5), IP-10 (CXCL10), IRF7 and OAS1 are presented here (Figure 4.11). The results showed that the mRNA levels of all these genes were significantly reduced by depleting PML-II.

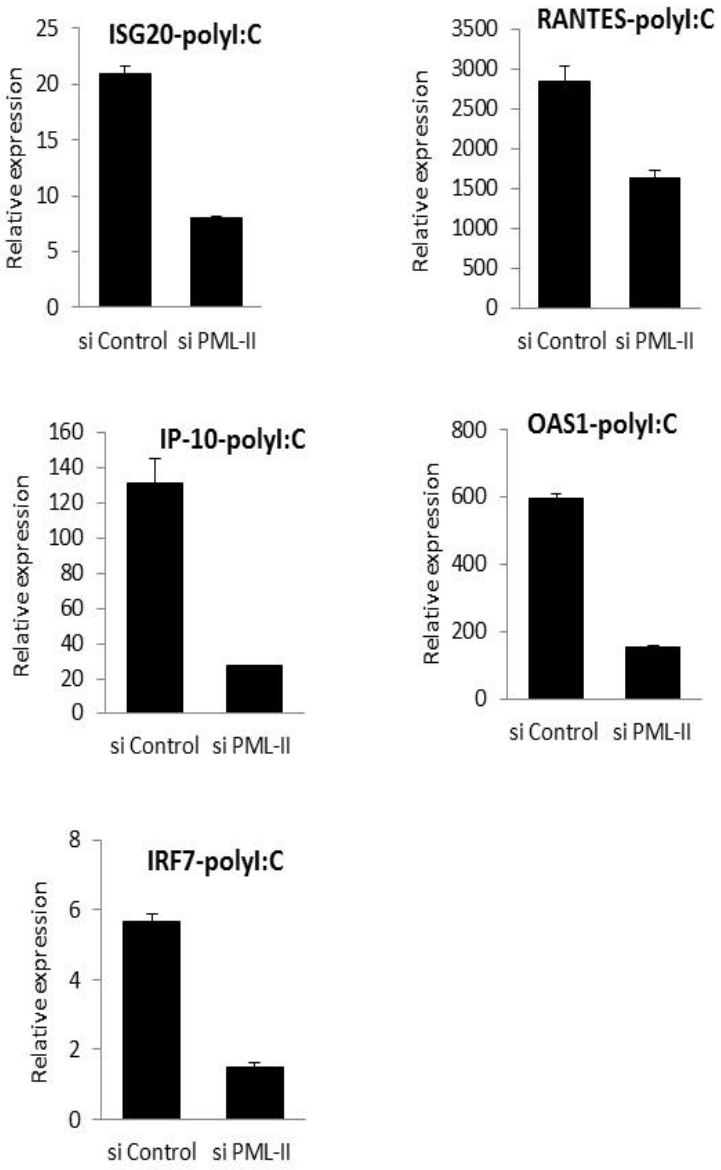


Figure 4.11 Knockdown of PML-II reduces the expression of poly(I:C)-stimulated ISGs. HEK293 cells were transfected with PML-II siRNAs or Control siRNA; 48 h later cells were stimulated with poly(I:C) for 16 h. The mRNA levels of ISGs were measured by SYBR green qPCR. The mRNA level in each sample is presented relative to control siRNA uninduced cells. The expressions were normalized against GAPDH or β-actin. Quantification was based on Ct difference performed according to the "delta-delta Ct method".

4.12 The loss of PML-II affects the expression of ISGs stimulated by IFNα.

To further test the direct effect of depletion of PML-II on the Jak/STATs signalling pathway, HEK293 cells were treated with 1000U/ml IFNα, the mRNA levels of selected ISGs: ISG20, RANTES, IP-10, IRF7, ISG15, ISG54 and ISG56 were tested (Figure 4.12). The result showed that the mRNA levels of all these genes were significantly reduced by depletion of PML-II when induced by IFNα. This result further confirmed that PML-II has a direct effect on gene induction via the Jak/STATs signalling pathway.

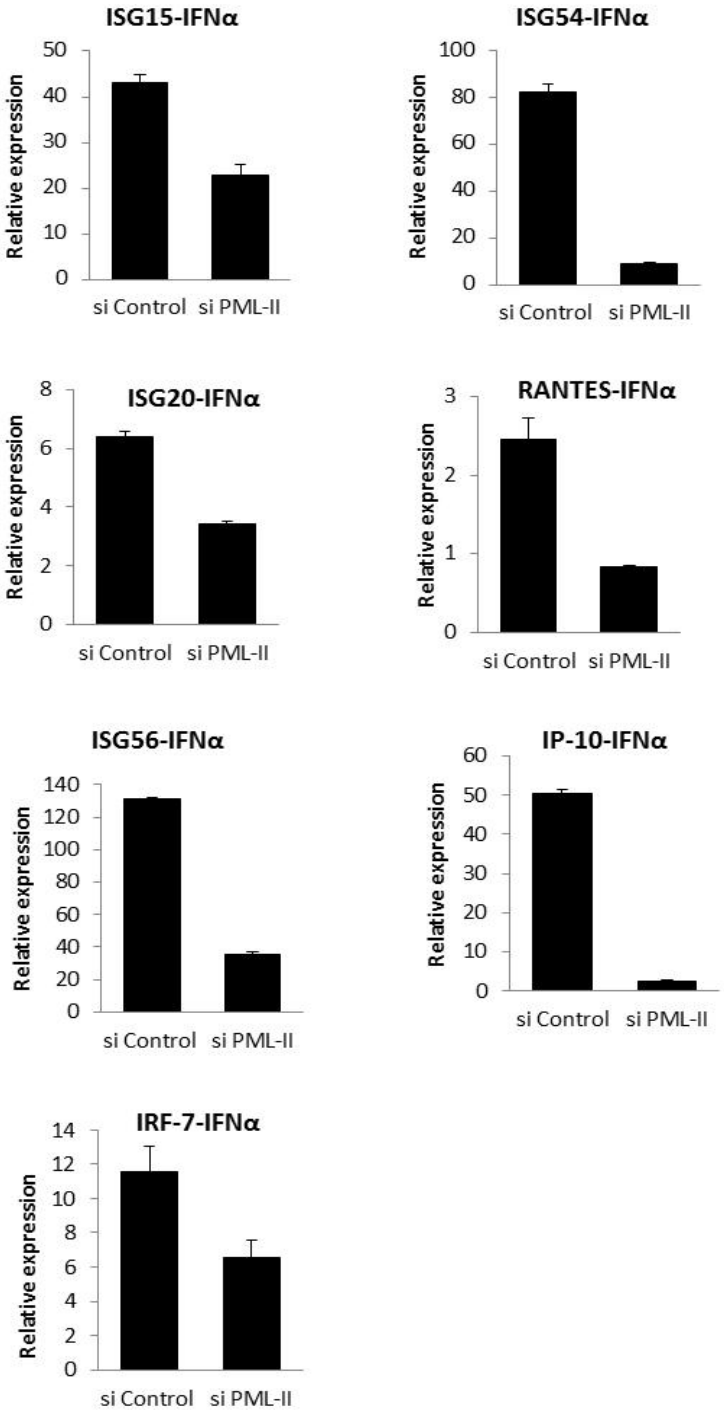


Figure 4.12 Knockdown of PML-II decreases the expression of IFNα-stimulated ISGs
HEK293 cells were transfected with PML-II siRNA or control siRNA; 48 h later, cells were stimulated with 1000U/ml IFNα for 16 h. The mRNA expression of ISGs was measured and analyzed as described in Figure 4.11.

4.13 PML-II can form protein complex with CBP-STAT1

The interaction of PML-II between CBP and IRF3 or NF-κB have been demonstrated in Figure 4.6 and 4.7, whether PML-II can also bind transcriptional complex ISGF3 in Jak/STAT1 signalling pathway was examined in this experiment. The result showed that PML-II binds to CBP, whereas in un-stimulated cells PML-II did not bind to the components of ISGF3 as there was no STAT1 precipitated, while STAT1 was precipitated by Flag-beads in the poly(I:C) stimulated cells. This result indicated that PML-II associates with CBP-STAT1 transcription complex under the condition of stimulation.

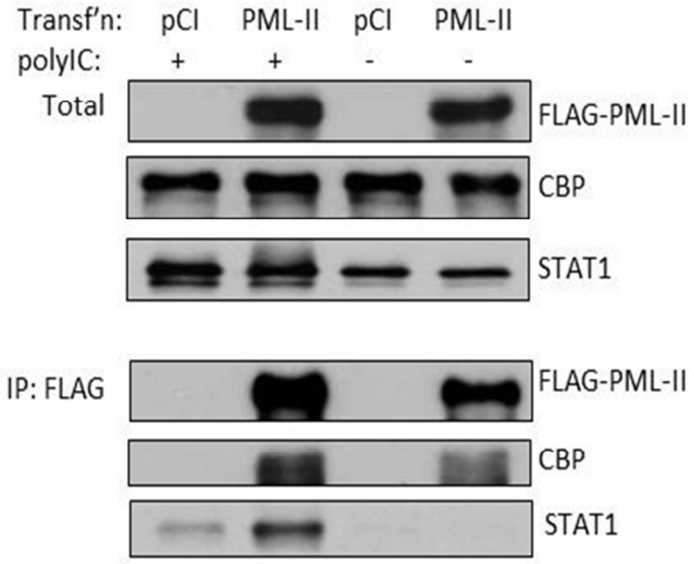


Figure 4.13 PML-II binds STAT1-CBP transcriptional complex. HEK293 cells were transfected with 250 ng Flag-PML-II plasmid or same amount of pCI-neo empty vector for 24 h. Cells were stimulated with poly(I:C) for 16 h. Total cell lysate were prepared and precipitated with Flag-beads for assaying the formation of the PML-II-CBP-CBP protein complex. Samples were subjected to 5-8% SDS-PAGE and western blotting and probed with Flag, CBP and STAT1 antibodies separately. Upper panels: direct immunoblotting of total cell lysates; lower panels: immunoblotting of Flag-immunoprecipitated proteins.

4.14 Depletion of PML-II affects STAT1 binding at the promoter of ISG

The expression of ISGs requires ISGF3 activation and its binding to ISRE elements. Therefore, in light of the findings for IFNβ regulation, PML-II might also affect the binding of ISGF3 components, which include STAT1, STAT2 and IRF9, to the ISRE. To test this, STAT1 DNA binding to the promoters of representative ISGs: ISG15, ISG56 and PKR, was measured. In this experiment, to avoid higher and variable background signal due to normal IgG and beads non-specific binding, ChIP data was analyzed by the Fold Enrichment Method. With this method, the ChIP signals are divided by the no-antibody signals, representing the ChIP signal as the fold increase in signal relative to the background signal. To further optimize this method, the fold- enrichment was normalized to input to correct for any differences in the amount of input DNA used for precipitation. As predicted, in all cases STAT1 binding was somewhat or considerably inhibited in the absence of PML-II (Figure 4.14). This result indicates that PML-II does positively regulate TF binding at ISRE elements, similar to its effect on TF binding at the IFNβ promoter.

Since the expression of ISGs requires the recruitment of CBP/p300 to ISGF3 components (Bhattacharya et al, 1996; Zhang et al, 1996), the effect of PML-II depletion on CBP recruitment at the ISG15, ISG56 and PKR promoters was tested. Binding of CBP to all these promoters was significantly impaired under conditions of PML-II depletion (Figure 4.14), although the levels of CBP binding to the PKR promoter were very low, even under control conditions. These results indicate that PML-II affects activation of ISGs via the JAK-STATs signalling pathway in a manner similar to its role in activation of the IFNβ gene, via positive effects on TF and co-activator association with ISG promoters.

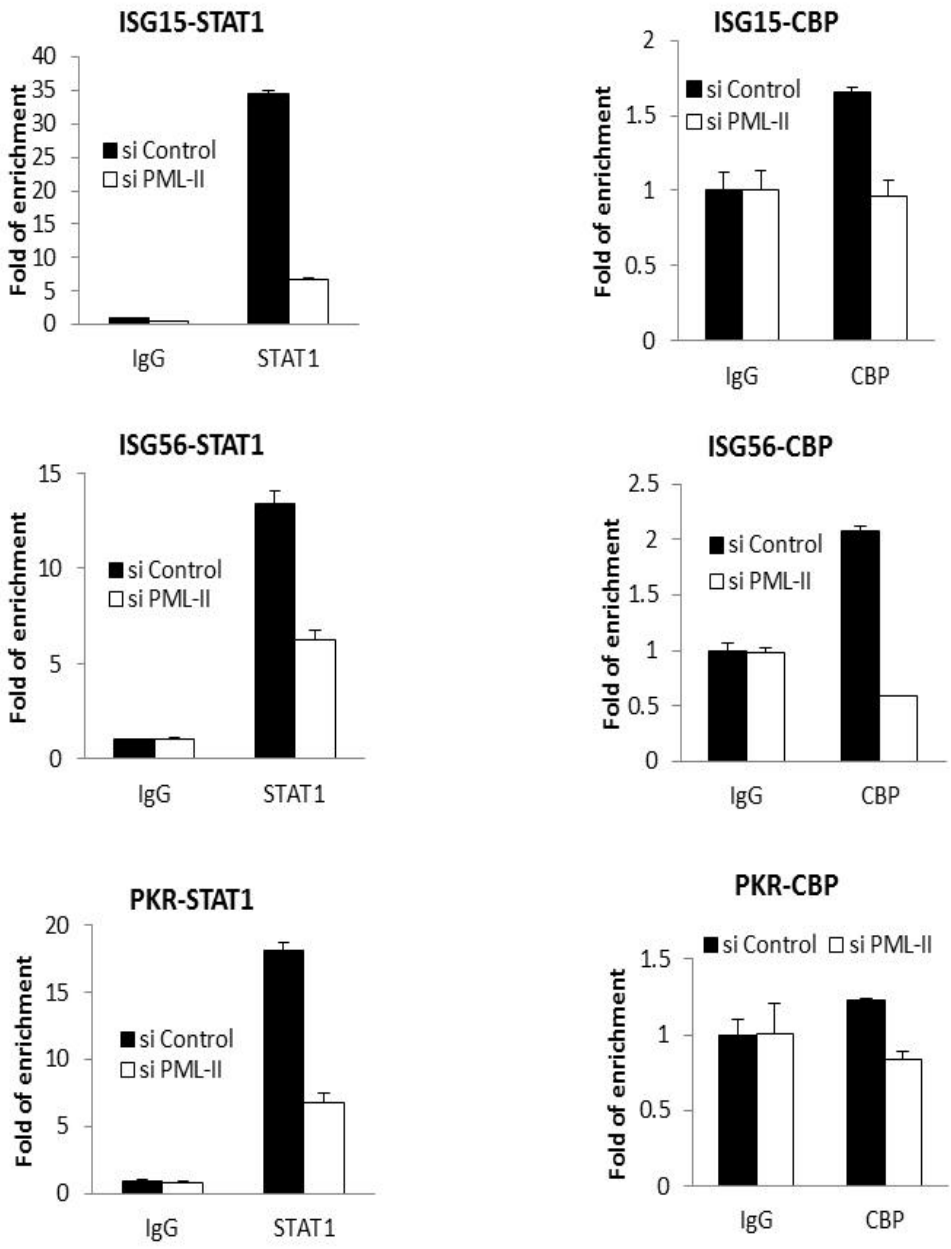


Figure 4.14 Depletion of PML-II affects STAT1 and CBP binding at the promoters/(ISREs) of ISGs HEK293 cells were transfected with PML-II or control siRNA and 48 h later, cells were transfected with poly(I:C) for 16 h. Cells lysates were subjected to ChIP precipitation with STAT1 or CBP antibody. Precipitated chromatin DNA was assayed by SYBR green qPCR for ISG15, ISG56 and PKR promoter sequences. The relative specific promoter DNA binding signal was determined by subtracting non-specific binding (normal IgG) and normalization to the input signal. Results are presented as mean ± SD from one representative of at least two experiments, each performed in triplicate

4.15 Discussion

Gene transcription can be regulated by multiple mechanisms including regulating the activity of DNA-binding proteins termed specific transcription factors (TFs). PML proteins have been shown previously to regulate the activity of various TFs including STAT1, NF-κB and p53 (Cheng & Kao, 2012a; Choi et al, 2006; El Bougrini et al, 2011; Pearson et al, 2000). In this Chapter, the activities of IRF3 and NF-κB were demonstrated to be severely impaired by depleting PML-II. And loss of PML-II was found also to reduce the expression of numerous IRF3-dependent genes, such as ISG15, ISG54 and ISG56, and NF-κB-dependent genes including IL-6, IL-8, RANTES and TNFα.

In the process of investigating the mechanism by which PML-II regulated IFNβ and downstream signalling, it was notable that poly(I:C) stimulation increased PRDIII/I (IRF3)-mediated activity in reporter assays to 80 fold, however, the activity of PRDII (NF-κB) was not obviously increased. The mRNA expression of IRF3-dependent genes was also significantly increased by poly(I:C) stimulation, from 15-fold (ISG15) to 3500-fold (ISG54). However, the mRNA levels of NF-κB-dependent genes, including IL-6, IL-8 and TNFα, were increased only from 2-fold (TNFα) to 15-fold (IL-8). This result can be explained by a recent study which showed that upon infection, IRFs take over IFNβ production, thus IFNβ-stimulated genes, and NF-κB, particularly Rel A (p65), is instead responsible for the induction of proinflammatory gene expression (Basagoudanavar et al, 2011). It is also interesting to notice that depletion of PML-II significantly reduced the basal activity of NF-κB, which is consistent with a recent microarray analysis which showed that the knockdown of all PML suppressed the expression of a group of NF-κB-dependent genes such as IL-6 and IP-10 in un-stimulated cells (Cheng & Kao, 2012a), though this study did not reveal any mechanism for this effect.

Among the ISGs tested, the expression of IRF7 was also reduced. IRF7 is a member of the

IRF family that is involved in the induction of IFN, but is also produced from an ISGF3-dependent promoter. In most cells, IRF3 is constitutively expressed while IRF7 is present at very low levels. Initial expression of IFNβ is therefore largely dependent on the activation of IRF3, but the secreted IFNβ subsequently acts on neighboring cells to induce expression of IRF7. This IRF7, together with IRF3, further induces IFNα/β expression, so IRF7 is an ISG and is essential in the positive feedback loop of IFNβ expression (Au et al, 1998; Hata et al, 2001; Levy et al, 2002; Sato et al, 2000). The impaired IRF7 expression that results from the absence of PML-II will certainly affect IFNα/β expression and thus contribute to the further depression of downstream ISGs expression.

Previous studies have demonstrated that the expression of IFNβ, RANTES and IP-10 can be regulated by both NF-κB and IRF3 in response to dsRNA stimulation (Rudd et al, 2005; Yamamoto et al, 2003). IL-6 is also a target of both NF-κB and the IRF family such as IRF5, although whether NF-κB and IRF synergize at this promoter is less clear (Colonna, 2007). In this Chapter, the expression of IFNβ, RANTES and IL-6 in response to poly(I:C) stimulation was significantly reduced by depleting PML-II. This suggests that PML-II may have a synergistic effect on these genes, via its effects on transactivation by both IRF3 and NF-κB.

IRF-3 phosphorylation and nucleus translocation were also tested in this Chapter; the results showed that poly(I:C) stimulation activated IRF3, and subsequently the level of phosphorylated-IRF3 (p-IRF3) was increased with time. Knockdown of PML-II has only a small effect on the the amount of p-IRF3 produced. Confocal microscopy showed that about 30-40% of nuclei displayed IRF3 staining in response to poly(I:C) stimulation, and in the PML-II siRNA transfected cells, the percentage of IRF3 staining was similar to that in control siRNA treated cells (Figure 4.5A). These results indicated that phosphorylation and nucleus translocation of IRF3 were largely independent of PML-II status. Although IRF3 movement into the nucleus was also observed by western blotting, it was unexpected that only a small fraction of total IRF3 was involved. Reasons for this discrepancy with the immunofluorescence data are unclear, but it is possible that a considerable part of the nuclear

IRF3 seen by IF is not anchored there and is extracted into the cytoplasmic fraction during cell lysis.

Nuclear accumulation of NF-κB (p65) in response to poly(I:C) was much weaker, only a very limited number of nuclei (5%) being stained with NF-κB p65 antibody. This result is consistent with the result of NF-κB -driven luciferase activity in response to poly(I:C) stimulation (Figure 4.1B) which showed only a small increase in NF-κB activity. The observation of a strong induction of IFNβ despite weak activation of NF-κB is explicable since NF-κB (p65) is not essential for IFNβ induction by poly(I:C) (Peters et al, 2002; Wang et al, 2007). This weak NF-κB response may also reflect the effects of the endogenous E1A proteins present in HEK293 cells on NF-κB activity (Schmitz et al, 1996).

An association between PML-II and CBP was also detected, which is consistent with previous report that PML isoforms including PML-II bind to CBP even in the absence of poly(I:C) stimulation (Doucas et al, 1999; Zhong et al, 1999a). PML-II was found also to interact with transcription complexes and poly(I:C) stimulation enhanced the interaction between CBP and NF-κB or IRF3. This suggests that PML-II can form complexes with these transcription factors, perhaps through its association with CBP. It is also interesting to note that knockdown of PML-II affects CBP's interaction with IRF3, and the binding of these factors and NF-κB to the IFNβ promoter, indicating that PML-II contributes to the formation or stability of this transcription complex at the promoter.

The effect of depletion of PML-II on ISRE promoter activity and mRNA expression from numerous ISGs in response to poly(I:C) and IFNα was also tested in this Chapter. The results showed that both were significantly affected by depleting PML-II suggesting that PML-II indeed plays an important role in the expression of ISGs through affecting the Jak/STATs signalling pathway. Results in this Chapter also demonstrate that not all the PML isoforms are functionally similar in the type I IFN response. PML-V reduction had no effect on IFNβ induction or ISGs expression in contrast to the strong positive role for PML-II in

these processes as already discussed.

PML proteins have been shown previously to regulate the activity of various transcription factors (El Bougrini et al, 2011; Fogal et al, 2000; Vallian et al, 1998a; Wu et al, 2003). In investigating the mechanism of PML-II regulated Jak/STATs signalling, depletion of PML-II was found to affect the binding of transcription factor STAT1 at ISGs promoters such as ISG15, ISG56 and PKR. Knockdown of PML-II also affects binding of CBP to the IFNβ promoter during poly(I:C) stimulation and its recruitment/binding at the ISRE of various ISGs such as ISG15, ISG56 and PKR. Based on the results obtained in this Chapter, a scenario can be proposed that knockdown of PML-II affects recruitment of CBP at promoters, destabilizing the interaction of specific transcription factors at these promoters, and consequently attenuates the transcription ability of many CBP-targeted genes.

Chapter 5 PML-II functions in TNFα-mediated NF-κB signalling

NF-κB has a crucial role in the regulation of many genes involved in mammalian immune and inflammatory responses, such as cytokines, cell adhesion molecules, complement factors and a variety of immune receptors. The observation that depletion of PML-II inhibits activity of a PRDII (NF-κB-responsive element of IFNβ) reporter plasmid (Figure 4.2) suggests that PML-II may function in NF-κB signalling or can in some way regulate NF-κB-relevant transcription. In this Chapter, the effect of depletion of PML-II on the induction of NF-κB by a variety of stimuli was firstly tested, then a series of experiments were performed to further explain the mechanism of PML-II in NF-κB signalling.

5.1 Knock-down of PML-II affects the basal activityof NF-κB

NF-κB has been detected in most cell types and regulates the induction of many genes. Specific NF-κB binding sites with the general consensus sequence: GGGRNNYYCC (R=purine Y=pyrimidine) have been identified in promoters and enhancers of a large number of these inducible genes. To further test the effect of PML-II on NF-κB activity, experiments were performed in HEK293 cells using two alternative NF-κB-responsive luciferase reporter plasmids, ConA-LUC (3 binding sites) and NIFTY-Luc, which has five NF-κB sites (GGGGACTTTCC), in comparison with PRDII (Figure 5.1). The activity levels were markedly increased with increasing numbers of NF-κB binding site sequences in the promoters. In both cases, depletion of PML-II significantly inhibited activity by 50-60% in both un-stimulated cells and poly(I:C) stimulated cells. However, poly(I:C) stimulation did not have a significant effect on the enhancement of NF-κB activity, as was found previously for the PRDII reporter in this cell type.

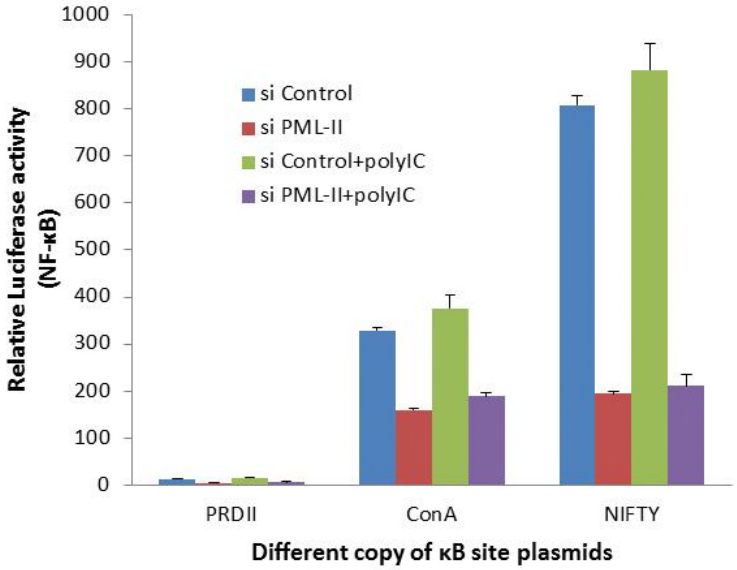


Figure 5.1 Knock-down of PML-II affects NF-κB activity. HEK293 cells were transfected with 100 pmol/ml PML-II or control siRNA, and 24 h later cells were transfected with NF-κB-Luc reporter plasmids (PRDII, ConA or NIFTY) together with β-gal used as a transfection control. After 24 h, cells were transfected with 1μg/ml poly(I:C) for 12-16 h. Cells extract were harvested and assayed for luciferase and β-gal and RLAs calculation. Data are presented as mean ±SD from one representative of three experiments, each performed in triplicate.

5.2 Depletion of PML-II affects NF-κB activity stimulated with PIV3 virus

Results in 5.1 indicated that poly(I:C) could not further stimulate NF-κB activity in HEK293 cells. To further test the function of PML-II on NF-κB inducible activity, an alternative stimulus was tested. Sendai virus, a paramyxovirus, is a well-known, effective and widely used inducer of NF-κB signalling. Here, parainfluenza virus type 3 (PIV3) was used because it is classified in the same virus family as Sendai virus (Figure 5.2 A). The results showed that NF-κB signalling was activated upon PIV3 infection, and that the activity of NF-κB increased gradually with the time of PIV3 stimulation. Also, the activity of NF-κB was inhibited to 50% in cells transfected with PML-II siRNA compared to the cells transfected with control siRNA. Thus PML-II potentiates NF-κB activity induced in response to PIV3 infection as well as being important for basal activity. The reason why PIV3 could

effectively induce NF-κB activation while poly(I:C) could not, is probably because PIV3 virus generates more PAMPs that then stimulate cells through a variety of receptors and signalling pathways.

To demonstrate the specificity of the PML-II effect, a comparison with PML-V siRNA, which has no known effect on the induction of IFNβ was also conducted (Figure 5.2 B). The result showed that PML-V siRNA had no effect on the activity of NF-κB stimulated by PIV3, while PML-II siRNA markedly inhibited this induction.

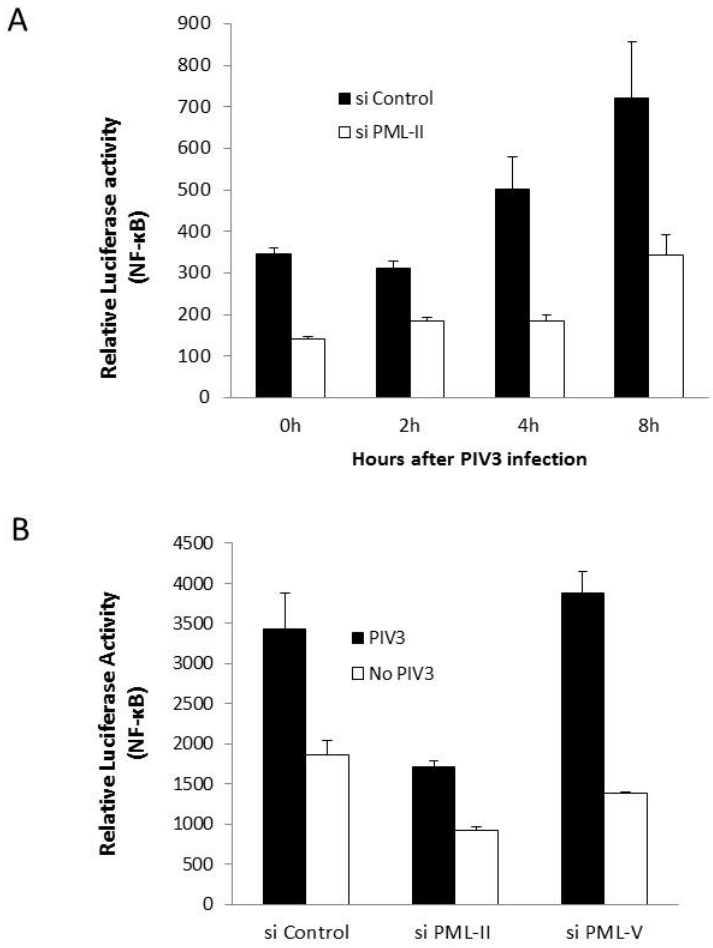


Figure 5.2 Depletion of PML-II affects the activity of NF-κB induced in response to PIV3 infection

(A) HEK293 cells were transfected with 100 pmol/ml si Control or si PML-II, 24 h later cells were transfected with NF-κB reporter plasmid NIFTY plus β-gal for 24h, then cells were stimulated by infection with 10 PFU/cell PIV3, harvested at the indicated times and extracts assayed for firefly luciferase and β-galactosidase activities. Error bars indicate the standard deviation of three replicate samples from the mean value. (B) HEK293 cells were transfected with 100 pmol/ml control, PML-II or PML-V siRNAs, 24 h later transfected with NF-κB-Luc reporter plasmid NIFTY together with β-gal. 24 h following plasmid transfection, cells were challenged with 10 PFU/cell PIV3 for 8 h, and then harvested for luciferase assay. Mean relative luciferase activities (firefly luciferase vs. Renilla luciferase) are shown for triplicate cultures, mean ±SD from at least three triplicates.

5.3 Depletion of PML-II affects TNFα-stimulated NF-κB activity

TNFα is a very effective inducer of many proinflammatory factors. The signalling pathways induced by TNFα mainly operate by activating NF-κB. To further determine the role of PML-II in the activation of NF-κB, the effect of PML-II depletion on TNFα-mediated NF-κB activity was tested. As shown in Figure 5.3 A, upon stimulation with TNFα, the activity of NF-κB was greatly increased and the relative activity of NF-κB in control siRNA treated cells was similar to that in cells that underwent only mock transfection. Thus siRNA itself has no inducer activity in the system. However, depletion of PML-II by siRNA resulted in a significant decrease in TNFα-induced NF-κB-Luc activity. This result further indicates that PML-II has a positive role in the up-regulation of NF-κB activity by different inducers.

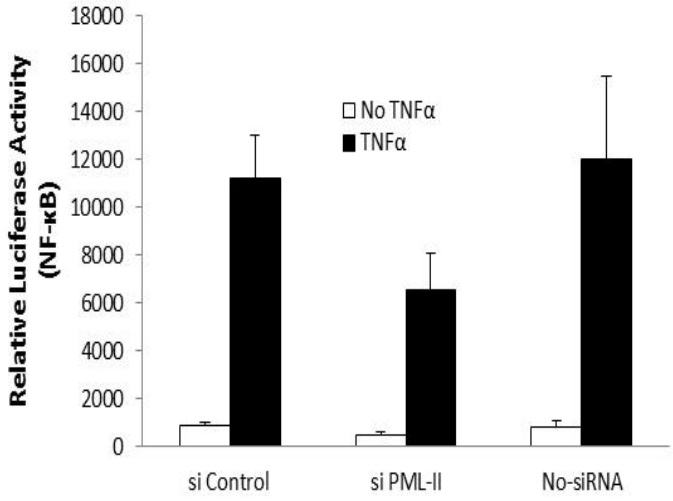


Figure 5.3 Depletion of PML-II affects the activity of NF-κB induced by TNFα in HEK293 cells. (A) HEK 293 cells were transfected with 100 pmol/ml control or PML-II siRNA, 24 h later co-transfection was performed with 225 ng/ml NF-kB-Luc and 25 ng/ml β-gal. 24 h following transfection, cells were untreated or treated with 20 μg/ml TNFα for an additional 12 h before NF-κB activity was measured in cell extracts by luciferase assay. The results were indicated as relative luciferase activity from triplicate samples, and the error bars represents SD from mean values.

5.4 Knockdown of PML-II affects expression of NF-κB-dependent genes

The function of PML-II on NF-κB-Luc activity led me to further investigate the effects of PML-II on the expression of TNFα-mediated NF-κB-dependent genes. Given that HEK293 cells lack TLR receptors (Flo et al, 2002) and that only modest induction of NF-κB dependent genes was observed in response to TNFα stimulation in these cells, an alternative cell line Hela was used and the expression of some well-characterized NF-κB dependent genes IL-8, IL-6, RANTES and IP-10 (Alexopoulou et al, 2001; Yun et al, 2011) was measured. NF-κB-dependent genes can be divided into three classes: early, intermediate, and late genes. IL-6 and IL-8 are the early genes, whereas RANTES and IP-10 belongs to the intermediate/late class (Tay et al, 2010).

The results showed that the expression level of IL-8 and IL-6 was up-regulated greatly and immediately in response to TNFα stimulation, whereas RANTES and IP-10 belongs to the intermediate/late class, whose expressed level builds up slowly during TNFα constant stimulation, unlike IL-6 and IL-8 which reaches its peak only 30 min after the stimulation, and then decreases (Figure 5.4 A and C). Knockdown of PML-II decreased TNFα-stimulated IL-8 and RANTES mRNA levels (Figure 6.4 A and B). Similarly, IL-6 and IP-10 mRNA level was also reduced (Figure 5.4 C and D). Therefore, these results confirmed that PML-II must play a role in the NF-κB activity and hence in the expression of NF-κB-dependent genes. Interestingly, many NF-κB-dependent gene products are related to inflammatory responses. Therefore this result suggests that PML-II plays a role in the inflammatory response.

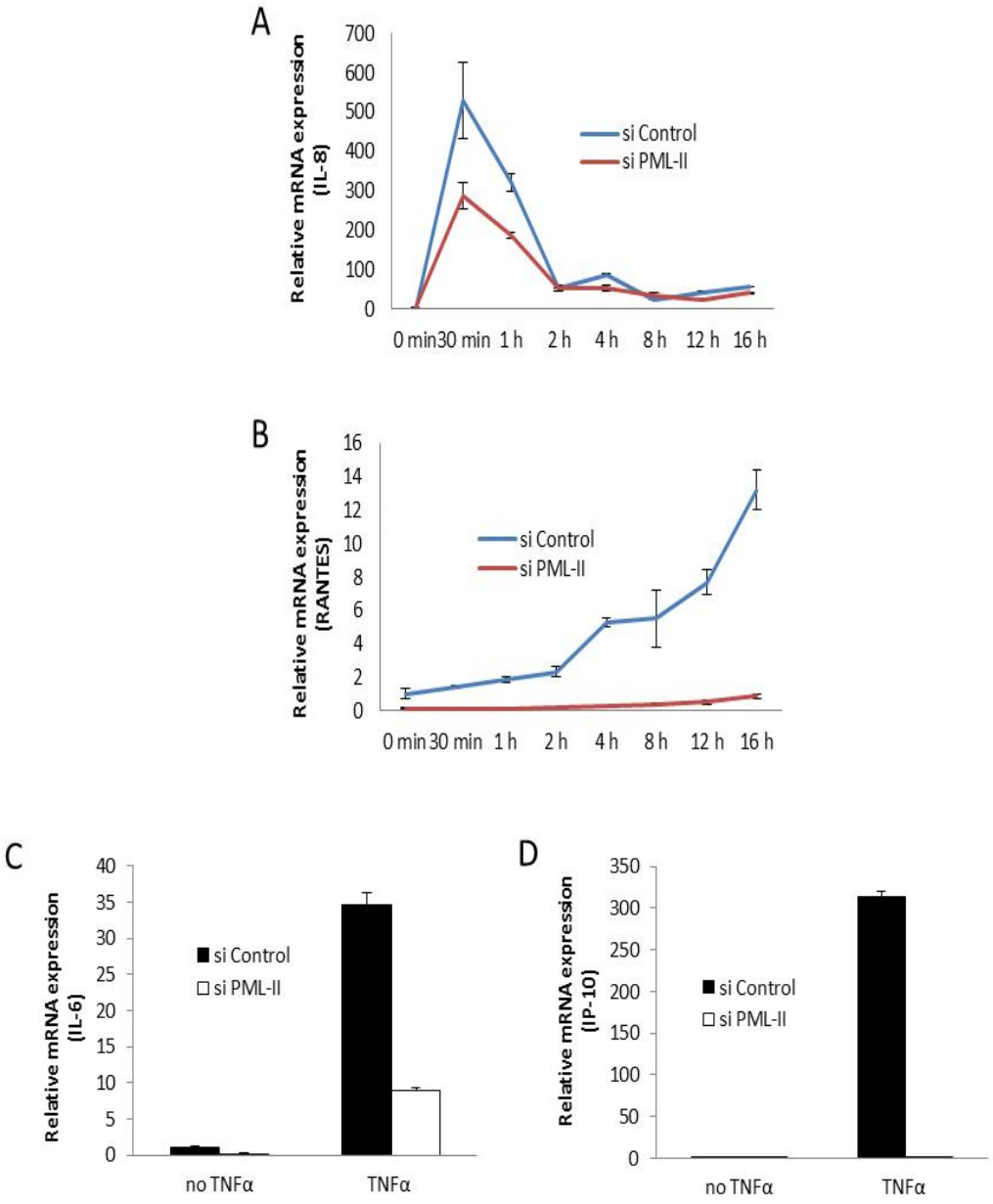


Figure 5.4 Knockdown of PML-II affects the expression of NF-κB-dependent gene stimulated with TNFα in Hela cells. Hela cells were transfected with 100 pmol/ml control siRNA or PML-II siRNA, 48 h later cells were untreated or treated with 10 ng/ml TNFα, (A) and (B) at different time points 0 min, 30 min, 1 h, 2 h, 4 h, 8 h, 12 h, 16 h; (C) and (D) for 1 h and 16 h respectively before harvest of total RNA. Levels mRNA expression were analyzed by SYBR green qPCR and are represented relative to amounts detected in si Control treated cells with no stimulation. Data were expressed as the mean± SD from triplicate samples.

5.5 Over expression of PML-II modestly increases the activation of NF-κB

Since depletion of PML-II decreases the activity of NF-κB, it was of interest to evaluate whether over-expression of PML-II could cause a corresponding increase the activity of NF-κB. The result showed that expression of full-length PML-II increased to a modest degree the basal activity of NF-κB (Figure 5.5 A) though, during stimulation by TNFα when the activity of NF-κB was increased significantly, over expression PML-II could not further enhance NF-κB activity; this is probably because endogenous PML-II is already sufficient to exert its function in response to TNFα stimulation. Shortened variants of PML-II either lacking the internal sequences encoded by exons 4-6 (C-PML-II) or lacking the N-terminal RBCC motif (residues 1-360), lacked the stimulatory activity observed for full-length PML-II in unstimulated cells and are included here as controls. Mapping of sequences in PML-II responsible for its activities in transcription was not pursued in this project.

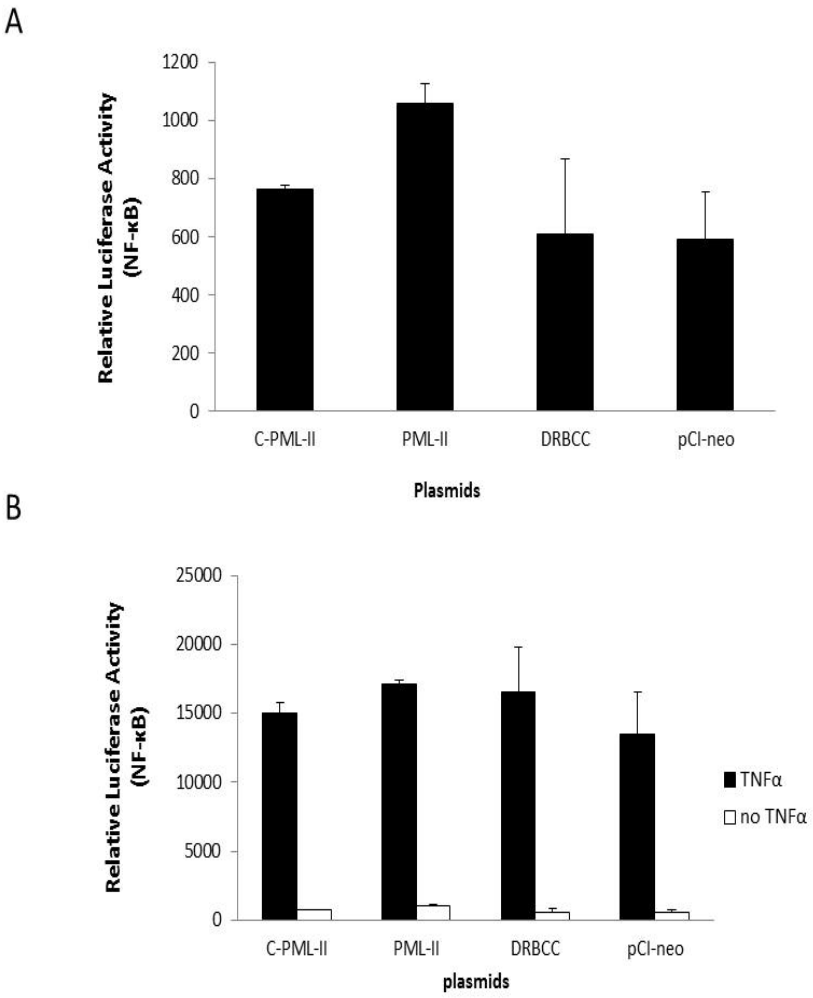


Figure 5.5 Expression of full-length PML proteins increases the activity of NF-κB. HEK293 cells were co-transfected in triplicate with NF-κB-Luc plasmid plus PML-II or pCI-neo. β-gal was also included in each transfection and β-gal activity was determined to normalize transfection efficiencies. 40 h later cells were unstimulated (A) or stimulated (B) with 20 ng/ml TNFα. 12 h later cells were lysed and assayed for luciferase and β-gal activity.

5.6 Depletion of PML-II does not affect NF-κB nuclear translocation

To further investigate the molecular mechanism by which PML-II functions in NF-κB activation, NF-κB nuclear translocation was observed by using immunofluorescence for the Rel A (p65) subunit. The result showed that in unstimulated cells there was abundant NF-κB (p65) located in the cytoplasm (Figure 5.6 A). Upon stimulation with TNFα, p65 quickly moved into the nuclei of the cells, however knockdown of PML-II had no effect on this (Figure 5.6 B).

Meanwhile, whether PML-II had an effect on IκBα degradation was also tested because proteasome-dependent degradation of IκBα is another central step in NF-κB activation. To do this, MG132 (a proteosome inhibitor) was applied. The result showed that NF-κB nuclear translocation could be largely inhibited by adding MG132 (Figure 5.6 C and D). But still there were no obvious differences between PML-II knockdown cells and control siRNA transfected cells (Figure 5.6 C and D). Thus the effect of PML-II depletion on NF-κB activity, like the effect on IRF3 activity (Chapter 4), is manifested after the factor moves into the nucleus.

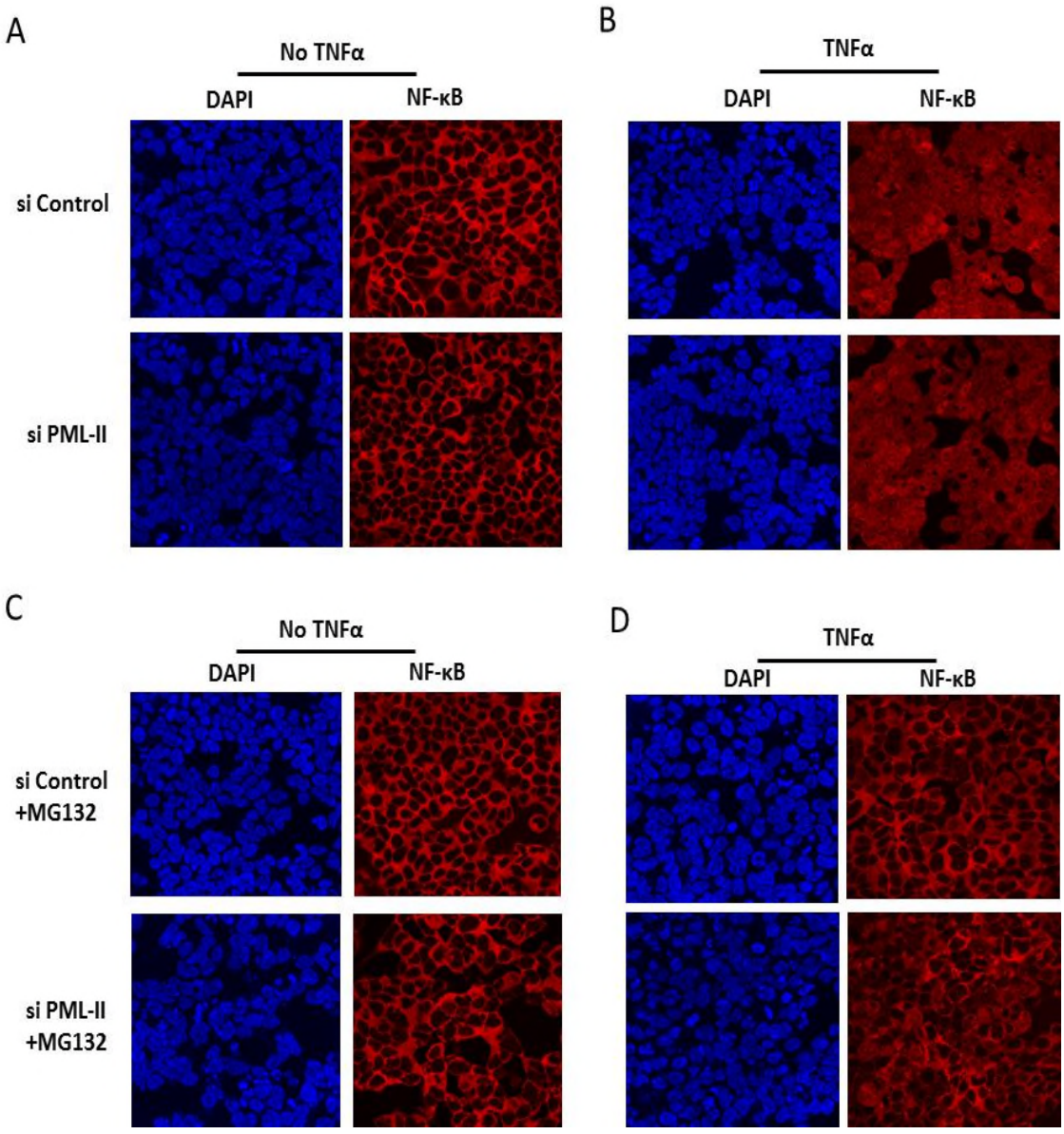


Figure 5.6 Depletion of PML-II does not affect NF-κB nuclear translocation. (A and B) HEK293 cells were transfected with 100 pmol/ml si Control or si PML-II for 40 h, and cells were then stimulated with 20 ng/ml TNFα for 30 min. Immunofluorescence staining was performed using rabbit anti-NF-κB (p65) Ab and goat-anti-rabbit secondary Abs. The observation was performed under a confocal fluorescent microscope Leica SP2 system. Blue: DAPI, red: NF-κB p65. (C and D) After 40 h treatment with siRNA, HEK293 cells were treated by adding 50 μM MG132. Following 8 h treatment, immunofluorescence staining was performed as described for panels A and B.

5.7 PML-II can bind NF-κB and CBP forming a multi-protein complex.

The physical interaction of PML-II with NF-κB and CBP was also tested by co-IP, using Flag-PML-II expressed from a plasmid. Results showed that control anti-Flag precipitations from extracts of empty vector pCI-neo transfected cells contained some non-specifically bound NF-κB, but similar precipitations from Flag-PML-II transfected cells contained significantly more NF-κB, indicating that PML-II and NF-κB/p65 associate (Figure 5.7). Furthermore, CBP was found only in the precipitates from the Flag-PML-II cells, indicating that it too is associated with the complex. Thus, NF-κB and CBP are specifically associated with PML-II protein.

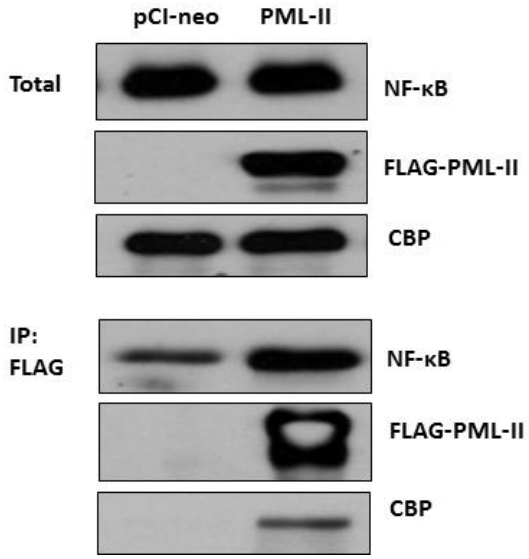


Figure 5.7 PML-II can bind NF-κB and CBP/p300 forming a multi-protein complex. 5 x 10^6 HEK293 cells were transfected with empty vector pCI-neo or PML-II. 24 h later, cells were stimulated with 10 ng/ml TNFα for 1 h, lysed with NP-40 lysis buffer, and sonicated cell lysate supernatant collected for precipitation with Flag-beads. Following an intensive washing, precipitated protein was displaced from beads with 2×SDS sample buffer. Proteins were detected by WB with appropriate antibodies; upper panels: proteins in samples of total cell lysates; lower panels: proteins recovered from Flag-immunoprecipitated samples.

5.8 PML-II has a stronger ability to bind NF-κB than other PML isoforms

To examine the question of whether PML-II was unique among PML isoforms in its associations with transcription factors, the binding ability of other PML isoforms with NF-κB and CBP was also examined. As shown in Figure 5.8 A (upper panels), all six of the well-characterised full-length PML isoforms expressed very well and their overexpression had no obvious effect on endogenous NF-κB expression. When precipitated on Flag-beads, the amounts of PML-II recovered were actually lower than for the other isoforms but, despite this, the amount of NF-κB pulled down was greater than for the other isoforms (Figure 5.8 A, lower panel). When co-precipitated p65 amounts were quantified by densitometry and expressed relative to the amounts of each PML isoform recovered, PML-II has a considerably greater ability to associate with NF-κB than other isoforms. Taken together with the results shown in section 5.7, these data suggest that PML-II has a particular ability to associate with NF-κB to form a multi-protein complex.

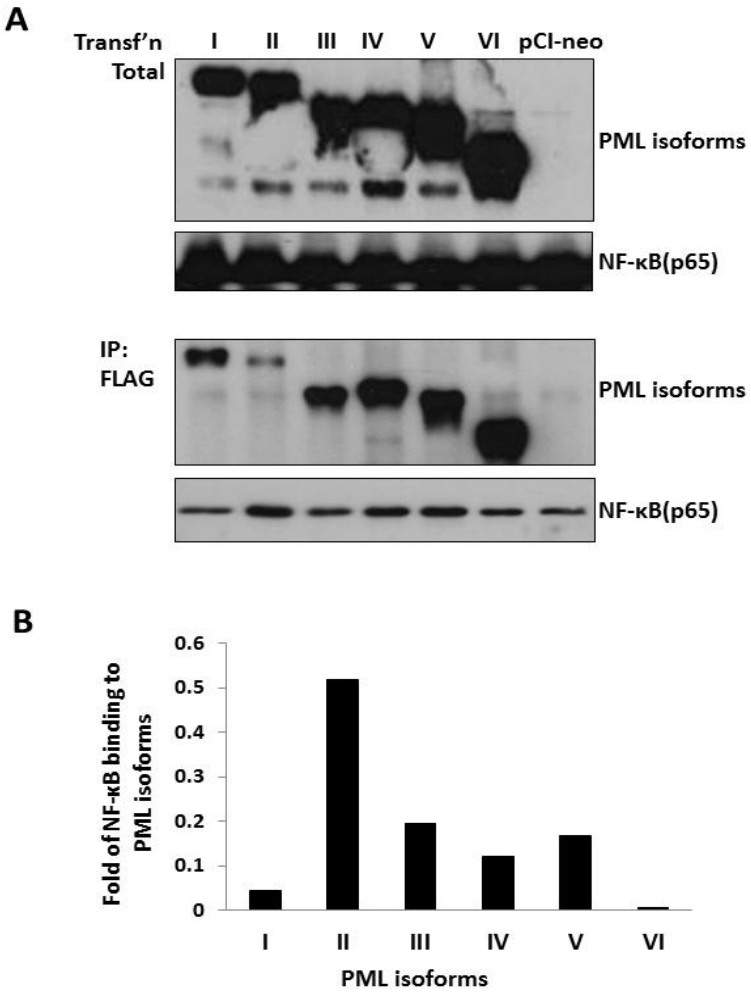


Figure 5.8 PML-II can bind NF-κB and CBP/p300 forming a multi-protein complex

5 x 10^6 HEK293 cells were transfected with empty vector pCI-neo or PML isoforms I-VI. 24 h later, cells were stimulated with 10 ng/ml TNFα for 1h and then treated as described in Fig 6.7. (B) Western blotting bands of NF-κB and PML proteins in panel A were analyzed according to their intensity. Amounts of precipitated NF-κB were normalized by subtracting the amount precipitated from empty vector pCI-neo transfected cells, which can be regarded as non-specific binding, and then further normalized by dividing by the amount of precipitated PML protein in that sample.

5.9 Depletion of PML-II affects NF-κB binding and CBP recruitment at promoters of NF-κB-dependent genes

The transcriptional activation of a gene requires the assembly of relevant transcription factors and basal transcriptional apparatus at its promoter. For NF-κB-dependent genes, appropriate signals activate NF-κB to enter into the nucleus and bind to κB site motifs present in NF-κB-dependent genes. NF-κB also interacts with other DNA-associated factors as well as the general transcriptional apparatus, e.g. with TBP, TFIIB or CBP/p300 (Clarke et al, 2010; Merika et al, 1998). Previous promoter studies revealed that usually NF-κB acts in synergy with other transcription factors such as c-Jun, Sp1 and STAT, in order to mediate an effective transcriptional activation. To advance our understanding of the role of PML-II in the regulation of NF-κB-dependent genes, the effect of depleting PML-II on NF-κB and CBP assembly at the promoters of NF-κB-responsive genes IL-6, RANTES and IP-10 was tested. Primer pairs were designed to amplify κB site regions in each of these promoters and the effects of knockdown of PML-II on NF-κB binding to the promoters were determined by using ChIP-qPCR. Data were analysed by the Fold-Enrichment method and normalized to input (see Section 4.14).

The results showed that the binding of both NF-κB/p65 and CBP at the IL-6 gene promoter was moderately reduced in PML-II siRNA-transfected HeLa cells, while NF-κB and CBP binding at the promoters of RANTES and IP-10 was significantly affected (Figure 5.9). This difference may stem from the different kinetics of response of these genes. RANTES and IP-10 are delayed NF-κB-dependent genes (Tay et al, 2010); the longer duration 16 h TNFα stimulation makes transcriptional complex more stable. In contrast, IL-6 is a rapidly-responsive early NF-κB-dependent gene (Tay et al, 2010), so CBP and NF-κB association with the promoter is transient leading to binding that may be unstable. Although individual genes may have different transcription characteristics, the results here demonstrated that

PML-II regulates TNFα-mediated NF-κB downstream signalling pathways in a similar manner to that in IFN signalling, mainly through affecting TFs and CBP recruitment to the promoters.

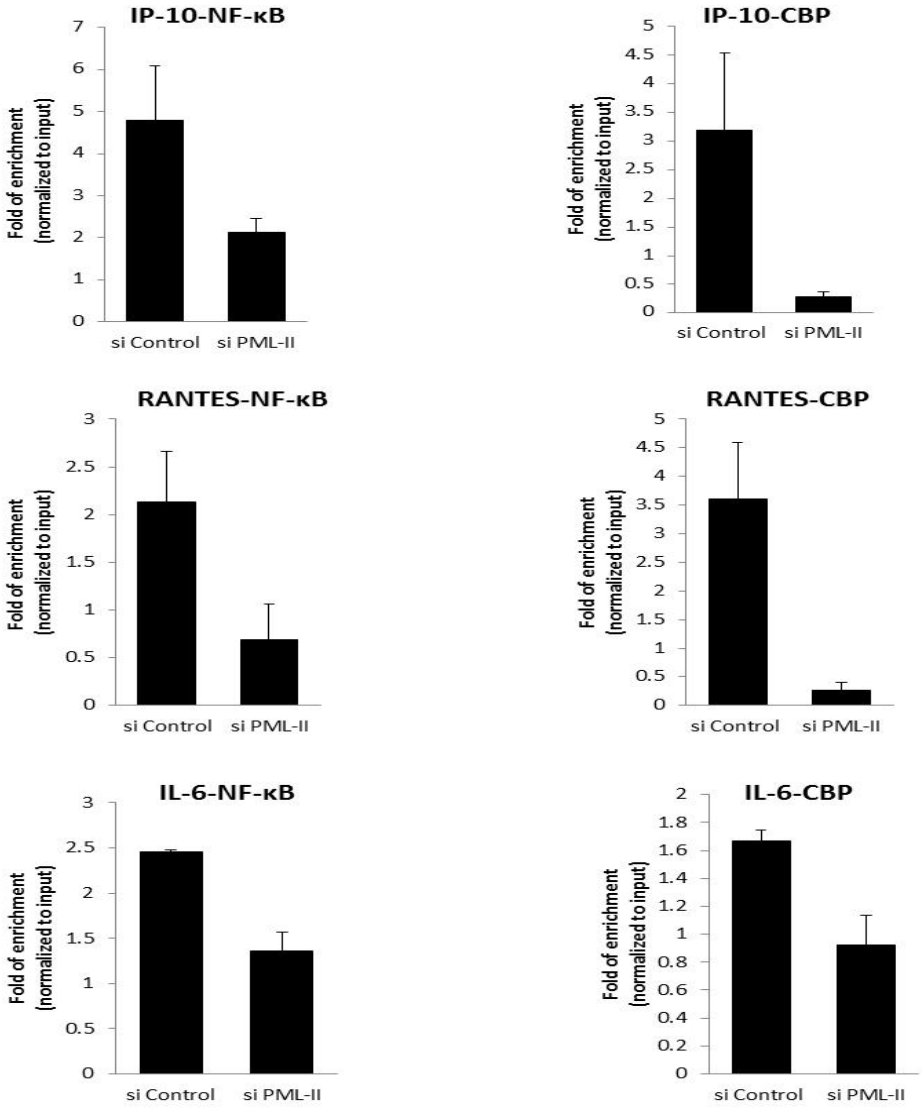


Figure 5.9 Depletion of PML-II affects NF-κB and CBP binding at responsive gene promoters. HeLa cells were transfected with 100 pmol/ml control or PML-II siRNA. At 48 h after transfection, cells were treated with 10 ng/ml TNFα 1 h for IL-6, and 16 h for IP-10 and RANTES, after which ChIP-enriched DNAs were prepared, immunoprecipitation were conducted by using mock IgG (normal rabbit IgG) or NF-κB/p65and CBP antibodies. The enriched DNA was analysed by using SYBR-green qPCR. The binding of NF-κB and CBP at promoter was analysed by fold of enrichment method.

5.10 Discussion

In this Chapter, the effect of PML-II on NF-κB activity was firstly tested by using poly(I:C) stimulation. However, poly(I:C) could not further increase kB site-driven luciferase activity in HEK293 cells, probably because poly(I:C) is not an efficient inducer to activate an NF-κB-mediated signal. In addition, the fact that HEK293 cells lack Toll-like receptors that are important in the NF-κB signalling (Flo et al, 2002) may also affect the activation of NF-κB by poly(I:C), although intracellular transfected poly(I:C) tends to be sensed by Mda5/RIG-I rather than TLRs. Another possible reason for the minimal response might be the presence of the Ad5 E1A gene in HEK293 cells, which partially inhibits the NF-κB activity (Schmitz et al, 1996). However, the mRNA levels of some NF-κB-dependent genes such IL-6, IL-8 and TNFα were increased by poly(I:C) stimulation although the scale of this effect was small in comparison with effects on IRF3-dependent genes.

Unlike poly(I:C), PIV3 virus infection effectively activated NF-κB and consequently increased the expression of NF-κB-dependent genes in HEK293 cells. This difference may be because PIV3 infection generates various PAMPs which can be recognized by more PRRs and through various signalling pathways to activate various TFs including NF-κB. These TFs may synergize to regulate NF-κB-dependent gene transcription. Also, different stimuli may induce distinct post-translational modifications of NF-κB (p65), which affect p65-mediated transcription.

TNFα plays a variety of roles in inflammatory responses and infection. TNFα can induce the inflammatory cascade, which results in the production of proinflammatory factors including cytokines and chemokines, adhesion molecules, destructive enzymes, and angiogenic factors (Baud & Karin, 2001; Feldmann et al, 2001; Ghosh et al, 1998). The distinct signalling pathways induced by TNFα act mainly through activating NF-κB and MAPKs (Aggarwal, 2003; Wajant et al, 2003). In this study, the activity of NF-κB was significantly increased by

TNFα, and the transcription of its target genes such as RANTES, IL-8 and IL-6 was also promoted.

NF-κB plays a vital role in innate and adaptive immunity. In unstimulated cells, NF-κB is sequestered in the cytoplasm as an inactive transcription factor, associated with its inhibitor IκBα. Upon stimulation, NF-κB induction involves the rapid activation of IKKb- and NEMO-dependent phosphorylation and subsequent degradation of IκBα, after which NF-κB translocates into the nucleus (Ghosh et al, 1998; Pahl, 1999). Nucleus translocation of NF-κB is therefore regarded as one of the markers of NF-κB activity. In this Chapter, it was observed that nearly all HeLa cell nuclei were stained with NF-κB (p65) after stimulating with 10-20 ng/ml TNFα for 30 min. This is different from poly(I:C) stimulation in HEK293 cells, in which only a very limited numbers of nuclei become NF-κB-positive when stimulated with 1 μg/ml transfected poly(I:C) for 16 h. However, in both cases, knockdown of PML-II caused a significant reduction in the expression level of NF-κB-dependent genes whilst it did not affect NF-κB nucleus translocation in response to TNFα stimulation in Hela cells. MG132 was also used to inhibit IκBα degradation and so to prevent NF-κB nucleus translocation. There was no difference between the PML-II siRNA transfected cells and control siRNA treated cells during MG132 treatment. These results suggest that, as found in Chapter 4 for IRF3 activation, PML-II does not affect NF-κB signalling pathways in the cytoplasm and instead affects events in the nucleus.

The interactions of PML-II, NF-κB/p65 and CBP were tested by co-IP. It was previously described (Chapter 4), that poly(I:C) stimulation provokes an association between PML-II, IRF3 and CBP. Similarly, it was found here that PML-II could form a protein complex with CBP and NF-κB, and that TNFα stimulation enhanced this association. When other PML isoforms were investigated, PML-II had a uniquely strong ability to associate with NF-κB compared to other PML isoforms. The mechanism for this is unclear so far; probably the unique functional C-terminal region of PML-II contributes to this ability to bind NF-kB. The interaction between PML-II and NF-κB provides the basis for PML-II to regulate NF-κB -

mediated signalling. The fact that some other PML isoforms also showed some association with this factor may be attributed to the ability of PML molecules to oligomerize (Wu et al, 2003), such that an introduced Flag-PML-III molecule, for example, might bind to an endogenous PML-II and hence show some association with proteins that bind specifically with PML-II.

Data in this study has demonstrated that knockdown of PML-II decreases the expression of many NF-κB-dependent genes. Subsequently, the mechanism study showed that PML-II depletion did reduce NF-κB binding at promoters of IL-6, RANTES and IP-10, and the recruitment of CBP at these genes' promoters was also severely impaired. This suggests that PML-II regulates TNFα-mediated NF-κB signalling in a similar manner as in IFN signalling, mainly by affecting TFs binding and CBP recruitment to the promoters. The results here further support the mode of PML-II action in the regulation of gene transcription proposed in Chapter 4.

NF-κB bound to a κB motif requires to interact with other DNA-associated TFs as well as the general transcriptional apparatus, e.g. with TBP, TFIIB or CBP/p300. This suggested a possibility that PML-II may also regulate other factors and thus affect NF-κB dependent gene expression. It is known that NF-κB acts in synergy with other transcription factors such as IRFs, c-Jun or Sp1 in order to mediate effective transcriptional activation, with distinct combinations of binding sites for different transcription factors within individual gene promoters that contribute to the selective regulation of gene expression. A recent report showed that p300-mediated acetylation of p65 regulates the specificity of NF-κB dependent gene expression (Buerki et al, 2008; Rothgiesser et al, 2010b). Given the results in this study that PML-II affects CBP recruitment (function), it therefore is possible that PML-II also plays a role in NF-κB p65 post-translational modification which determines the outcome of p65-mediated gene activation.

In summary, in this Chapter PML-II was identified as a key positive regulatory component of TNFα-mediated NF-κB signalling to stimulate the production of the proinflammatory cytokines such as IL-6, IL-8, RANTES and IP-10. The results here suggest that PML-II controls NF-κB-dependent gene expression by affecting nuclear events including TFs assembly and CBP recruitment at promoters.

Chapter 6 Does PML-II have a general function in gene transcription?

The results in Chapter 4 demonstrate that depletion of PML-II significantly impairs the expression of IFNβ and ISGs by affecting IRF3, NF-κB or STAT1 binding and CBP recruitment to the promoters. Type III IFNs, a relatively recently discovered group of interleukin 10 (IL-10) -like cytokine family proteins, comprise the products of three distinct genes IFNλ1 (IL29), IFNλ2 (IL28A) and IFNλ3 (IL28B) (Sheppard et al, 2003). Type III IFNs have demonstrated similar activities to type I IFN (α/β) in the induction and regulation of the immune and inflammatory response (Diegelmann et al, 2010; Hou et al, 2009; Levy et al, 2011; Sheppard et al, 2003). IL-29 and IL-28 can be induced by various viruses and dsRNA in signal cascades similar to the induction of IFNβ and IFNα genes, respectively. Namely, the expression of IL-29 is regulated largely by IRF3, IRF7 and NF-κB, while the regulation of IL-28 depends only on IRF7 (Onoguchi et al, 2007; Osterlund et al, 2007). Thus, whether PML-II also positively regulates type III IFN induction was investigated in this Chapter. Type II IFNγ induces a downstream signal through activating the same transcription factor STAT1 as utilized in the type I IFN response. It is therefore possible that PML-II also plays a role in IFNγ-mediated signalling. To further address the hypothesis that PML-II has a general function in gene transcription, the responses of a wide range of inducible genes were investigated in this Chapter.

6.1 Depletion of PML-II affects type III IFN induction

In light of the similarity between the induction of type I and type III IFNs, and given the role of PML-II in the expression of type I IFN and downstream signalling, it was necessary to test the function of PML-II in the induction of type III IFN. As before, siRNA was used to reduce PML-II expression prior to induction, in comparison with control siRNA-treated cells. The results showed that depletion of PML-II significantly affected IL-28 and IL-29 expression in both the cell line HEK293 (Figure 6.1 A) and normal MRC5 human fibroblast cells (Figure 6.1 B).

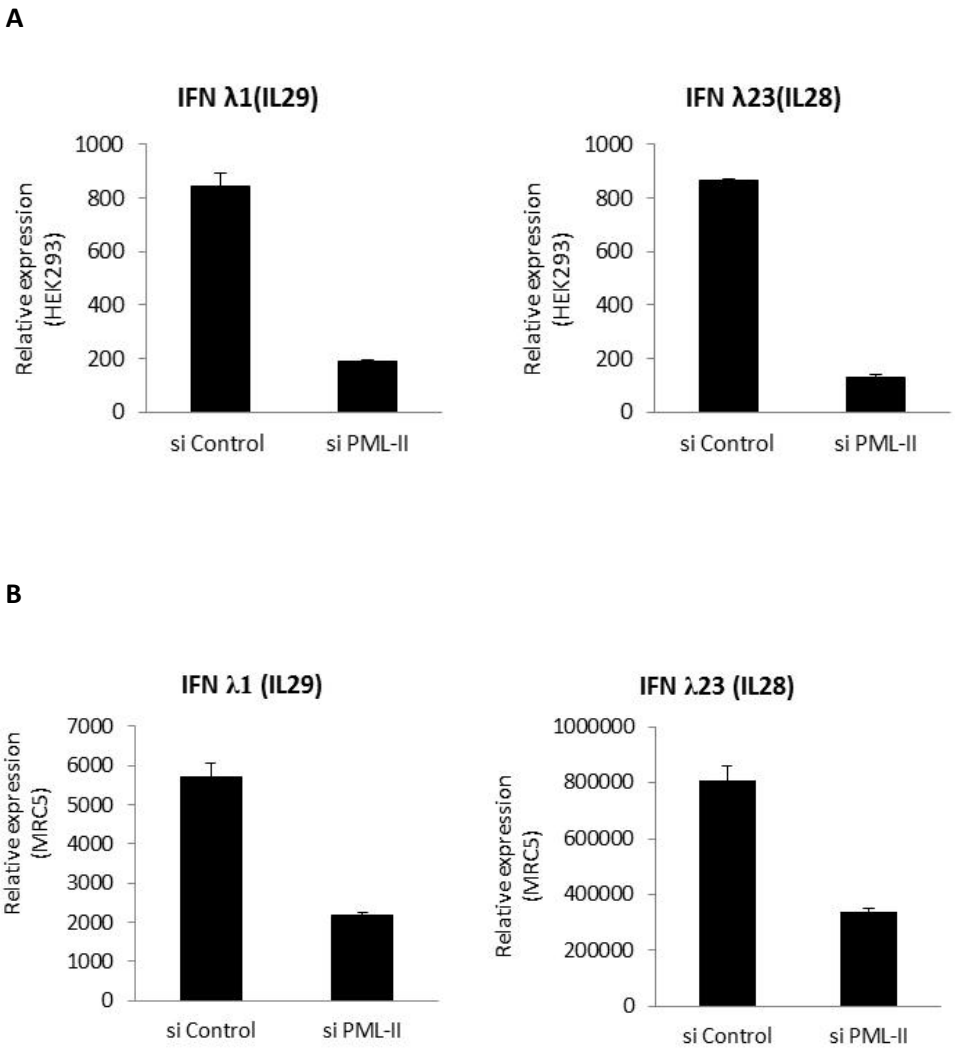


Figure 6.1 Depletion of PML-II reduces the mRNA expression of IL28/29 (A) HEK293 cells were treated with 125 pmol/ml PML-II or Control siRNA for 48 h, and stimulated with poly(I:C) for 16 h. mRNA expression levels were measured by SYBR Green qPCR. The values presented are relative to control siRNA with no-stimulation and are the mean +/- standard deviation of five replicates performed across two independent experiments. (B) MRC5 cells were treated with 50 pmol/ml siRNA for 72 h and RNA harvested after 24 h poly(I:C) stimulation. Other details as in panel A.

6.2 PML-II regulates type II IFN (IFNγ)-mediated signalling

Given the fact that transcription factor STAT1 and recruited CBP are utilized to activate the downstream signalling in type II IFN responses, as they are also in type I IFN responses, it was of interest to test whether knockdown of PML-II affected the expression of type II IFN-

mediated ISGs similar to its effects on type I and type III IFN pathways. Stimulation with IFNγ for 6 h significantly induced the expression of IFNγ-stimulated gene IP-10 and GBP1, as expected. Depletion of PML-II prior to stimulation considerably reduced the mRNA expression of IP-10 and GBP1 (Figure 6.2). Notably, that depletion of PML-II considerably reduced the basal expression of both genes again suggests that PML-II has a role in gene basal transcription mechanism.

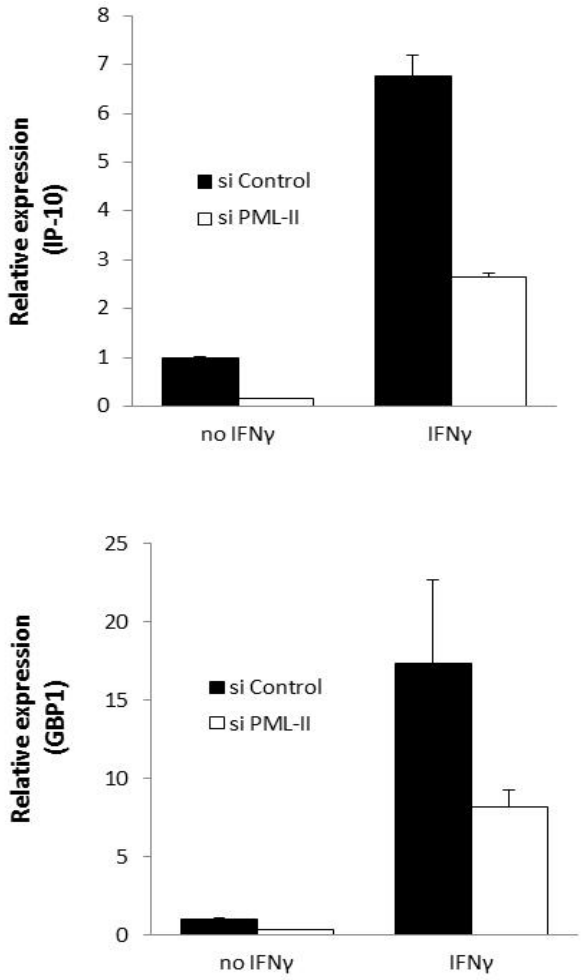


Figure 6.2 Knockdown of PML-II inhibits the expression of IFNγ-stimulated gene. HEK293 cells were transfected with PML-II siRNA or control siRNA; 48 h later, cells were stimulated with 50 ng/ml IFNγ for 6 h. The mRNA levels of (A) IP-10 and (B) GBP1 were measured by SYBR green qPCR..

6.3 How widely does PML-II regulate gene expression?

The results presented in this thesis so far have demonstrated that knockdown of PML-II severely impairs CBP recruitment at the promoters of IFNβ, various ISGs and other NF-κB-dependent promoters, and in response to induction by poly(I:C), IFNα, IFNγ and TNFα stimulation. It is therefore proposed that, among the family of PML protein isoforms, PML-II is of particular significance in regulating transcription of a wide range of inducible genes that are dependent on CBP. CBP plays an important role in the activation of a huge number of genes (Ramos et al, 2010). The loss of CBP affects the ability to recruit components of general transcriptional machinery including RNA pol II and consequently affects the mRNA expression of these genes. To further test this hypothesis, mRNA expression from two other genes that can be induced in response to different stresses was tested. Heat shock protein 70 (Hsp70), inducible by transient heat stress was selected as a further known CBP-dependent gene to compare with a CBP- independent gene, the cell cycle regulator gene p21 that is induced by DNA damage induction of p53 activitiy.

6.3.1 PML-II regulates the transcription of heat shock protein Hsp70

Hsp70s are a family of conserved, structually similar, ubiquitously expressed heat shock proteins that exist in virtually all living organisms. The Hsp70s are important components for protein folding catalysis and also are central components of the cellular network of molecular chaperones (Kim et al, 2013; Mayer & Bukau, 2005). Hsp70s have housekeeping functions in the cell in which they are built-in components of folding and signal transduction pathways; they have a wide range of protein quality control functions including protein folding, refolding of stress denatured proteins, protein transport, membrane translocation and protein degradation (Kim et al, 2013).

The expression of Hsp70 gene is regulated through a conserved transcription response. Briefly, upon heat shock, heat-shock factor 1 (HSF1), which requires phosphorylation and trimerization, is activated and binds to heat-shock elements (HSEs) located in the promoters of the Hsp70 gene (Morimoto, 2002). Then transcription factors including HSF1 recruit

p300/CBP to modulate transcriptional activity (Ghosh et al, 2011; Li et al, 1998; Xu et al, 2008). A heat-inducible chromatin-associated protein complex involving HSF1 and p300 coincides with enhanced chromatin acetylation in Hsp genes probably through a mechanism that involves the HAT activity of p300/CBP (Ghosh et al, 2011; Xu et al, 2008). This suggests an important role of p300 in Hsp70 transcription.

The effect of PML-II depletion on Hsp70 expression was tested in HEK293 cells. The result showed that immediately after heat shock in control siRNA transfected cells, the expression of Hsp70 had increased 2-fold (Figure 6.3.1). After return to 37°C for recovery, Hsp70 mRNA continued to increase, reaching a level 3-fold more than the non-heat-treated control after 2 hr. In contrast, for cells transfected with PML-II siRNA, heat shock only slightly increased the expression of Hsp70 and after cells were returned to 37 °C, these levels still did not increase, final expression being equivalent to the basal expression. Thus after 120 min recovery from heat shock, the mRNA expression of Hsp70 in control siRNA transfected cells was 6-fold more than in PML-II siRNA treated cells. Notably, PML-II siRNA treatment also inhibited the basal mRNA expression of Hsp70 to 50%, suggesting that the same PML-II-dependent mechanism involved in heat induction is also involved in maintaining this basal level of expression.

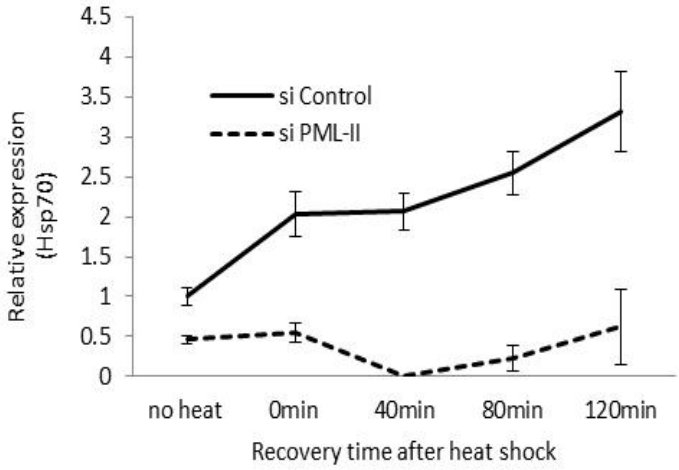


Figure 6.3.1 Knockdown of PML-II affects Hsp70 expression HEK293 cells were transfected with PML-II or control siRNAs and, 48 h later, were heat-shocked at 42 °C for 20 min and then returned to 37 °C for different time intervals (0, 40, 80, 120 min) before harvest of total RNA. Hsp70 mRNA expression was then detected by SYBR Green RT-qPCR. Each reaction was done in triplicate, and the mean signal reading was normalized to that of GAPDH. Data from different time points were finally expressed relative to the value for the non-heat-treated control siRNA cells.

6.3.2 Depletion of PML-II did not affect the expression of p21 but affected PIG3 promoter activity

To further test the hypothesis that PML-II has a general regulation function in inducible gene transcription, the function of PML-II in gene expression induced by DNA damage was tested. In response to etoposide treatment, transcription factor p53 activates transcriptional programs to either allow repair of DNA or to kill the damaged cells by apoptosis (Vousden & Prives, 2009). The expression of cell cycle regulatory genes such as p21 and proapoptotic genes such as PUMA and PIG3 are tightly regulated by p53. I therefore first tested the effect of PML-II on the activity of the PIG3 promoter using a luciferase reporter assay. In this experiment, the effect of etoposide was minimal, however depletion of PML-II reduced the basal activity of the PIG3 promoter by about 50% (Figure 6.3.2A). It is possible that the

short time period in which etoposide was able to affect gene expression was not sufficient to allow significant accumulation of reporter product in comparison with the background level that had accumulated from the transfection of the plasmids.

HATs are essential for p53-dependent transcription. Among HATs known to act on p53 and enhance its transcriptional activity are p300, CBP, p300/CBP-associated factor (PCAF) and GCN5 (Lee et al, 2010; Love et al, 2012; Teufel et al, 2007) of which the involvement of p300 and CBP is the best characterized. However, most recently, the expression of p21 and Mdm2 was reported not to require CBP/p300 in response to etoposide stimulation (Kasper et al, 2011). I therefore measured p21 mRNA levels before and after etoposide treatment in cells with or without PML-II. Depletion of PML-II had only a minimal effect on p21 basal expression. Upon stimulation with etopside, the mRNA level increased significantly but, in this case, the level of induction was not affected by knockdown of PML-II. In the context of the global hypothesis that PML-II affects gene expression via CBP, this result is consistent with the previous report that there is no absolute requirement for CBP and/or p300 in the p53-dependent expression of p21.

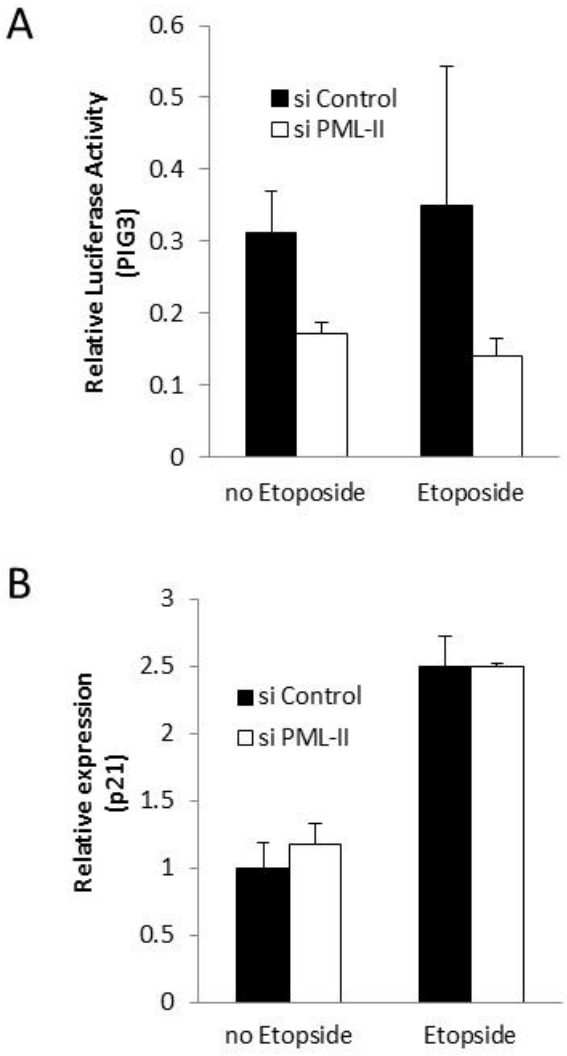


Figure 6.3.2 Knockdown of PML-II does not affect p21 expression. (A) HEK293 cells were transfected with PML-II or control siRNAs, and 48 h later cells were transfected with 250 ng PIG3-Luc plasmid together with 50 ng β-gal for 24 h. Cells were treated with 100 μM etoposide for 3 h, before cell extracts were harvested and relative luciferase activity measured. (B) HEK293 cells wre transfected with siRNA and stimulated with etopside as described in (A), total RNA was harvested and the p21 mRNA expression was detected by SYBR Green RT-qPCR. The mRNA level of p21 is presented relative to control siRNA without stimulation.

6.4 Discussion

The purpose of this Chapter was to explore how broadly acting was the effect of PML-II in gene expression. The function of PML-II in type III IFN induction was tested and the result showed that depletion of PML-II greatly reduced type III IFN gene IL-29/28 expression in both immortalized cell lines and normal cells. It is known that IL-29 and IL-28 promoters are similar to IFNβ and IFNα genes, respectively. Both of these promoters have functional ISRE and NF-κB binding sites. IL-29 is regulated similarly to IFNβ, dependent on IRF3, IRF7 and NF-κB, while IL-28 regulation is similar to IFNα, depending on IRF7 (Onoguchi et al, 2007; Osterlund et al, 2007). The results in Chapter 4 suggested that knockdown of PML-II affects the binding of TFs IRF3 and NF-κB and recruitment of CBP at the IFNβ promoter, therefore it can be proposed that PML-II most likely affects these events at IL-29 and IL-28 promoters. IL-28 and IL-29 are able to induce STAT1 phosphorylation, resulting in the activation of STAT1 and STAT2 which, together with IRF9, form the ISGF3 complex which translocates to the nucleus to initiate the induction of target genes (Kotenko et al, 2003). Since STAT1 and CBP binding to ISG promoters following IFNα stimulation was already shown to be dependent on PML-II (Chapter 4), it is likely that PML-II also affects this same pathway when stimulated by IFNλs.

A function for PML protein in IFNγ signalling has been reported previously by two groups. However, both groups demonstrated contrary/conflicting results. One study showed that in PML-deficient *pml*-/- mouse embryonic fibroblasts (MEF) cells, IFNγ-induced STAT1α transcriptional activity was enhanced, and DNA binding activity at a target promoter was also increased with numerous IFNγ-regulated genes being up-regulated (Choi et al, 2006). However, a more recent report showed that knockdown of PML reduced IFNγ-induced STAT1 phosphorylation, and subsequently affected the binding of STAT1 to DNA and thus decreased the activation of IP-10 expression (El Bougrini et al, 2011). The reason for this discrepancy remains unknown. PML protein has 7 principal isoforms, and in different cell lines the relative expression of these isoforms is different. Since it is clear that different

isoforms are functionally distinct, these functions may synergise with or antagonize each other depending on the relative levels of the isoforms. In this study, knockdown of all PML protein isoforms by exon-3 siRNA had no effect on IP-10 expression in response to IFNγ stimulation while it marginally increased the expression of IRF-1 (see Appendix 4). In contrast, knockdown of PML-II alone significantly inhibited IP-10 expression. These results demonstrate that PML-II does play a role in the IFNγ-mediated signalling, but that other isoforms must have inhibitory effects. This result may be helpful to explain the previous contrary results from different research groups.

The function of PML-II in regulation of co-activator CBP/p300 during the cellular response to various forms of stress was also tested in this Chapter. The results showing that depletion of PML-II reduced Hsp70 expression further support the hypothesis that PML-II has general effect on transcription of inducible genes. However, the mechanism by which PML-II regulates Hsp70 expression remains uninvestigated in my thesis. Given the association between HSF1 and p300/CBP in the control of Hsp70 transcription (Ghosh et al, 2011; Li et al, 1998; Xu et al, 2008), it can be proposed that PML-II may also form a chromatin-bound protein complex with HSF1 and p300/CBP, to activate transcription of the Hsp70 promoter. It is therefore most possible that PML-II participates in regulating through its ability to stabilize transcription complex p300/CBP at promoters as in the model of PML-II action on IFNβ expression. The data here indicate that PML-II could take on a similar role during the heat-shock response, by interacting with HSF1 and recruiting p300/CBP.

Previous studies demonstrated that the transcription of two classical p53 target genes, p21 and Mdm2, is less CBP/p300 dependent (Kasper et al, 2011). To further test the hypothesis that PML exerts function through regulating CBP, the mRNA expression of p21 was measured. As expected, the loss of PML-II, which impairs the function of CBP/p300, did not reduce the expression of p21 (Figure. 6.3.2 B). Why the transcription of p53-dependent genes does not require CBP/p300 is probably because the loss of CBP/p300 function increases the availability or activity of other potential p53-coactivators, for example, non-

HAT co-activator CRTC which can buffer the loss of CBP/p300 for at least some CREB target genes (Kasper et al, 2010).

Previous studies showed that overexpression of PMLIV significantly increased PIG3 promoter activity while have no effect on p21 promoter activity (Fogal et al, 2000) suggesting p21 is different from PIG3, as was also found in the present study. Overexpression of PML-IV increases CBP function thus will certainly increase CBP-dependent gene PIG3 promoter activity, while have no effect on CBP-independent gene p21 promoter activity. Most recently, it was demonstrated that PCAF, but not CBP and p300, is a critical regulator of p21 expression in response to multiple p53-activating stresses, including DNA damage reagents (Love et al, 2012). Although p21 promoter occupancy by p53 was not affected by PCAF knockdown, activation of p21 transcription required an intact PCAF HAT domain (Love et al, 2012). This suggested that PML-II may have a specific function regulating HAT activity of CBP/p300 rather than the activity of HATs generally. Taken together, these results support the hypothesis that PML-II may have a regulation function in the transcription of inducible genes that use CBP/p300.

Chapter 7 Final discussion

In this study, the role of PML-II in the control of transcription of IFNβ and ISGs was investigated. The underlying mechanism was found to be the participation of PML-II in the stable association of TFs and particularly the co-activator CBP with the promoters of these genes. Induction of IFN responses is crucial in the initial defence against virus infection and so this new understanding of how PML-II is involved in this process is important in broader understanding of infection. This work also gives a new mechanistic insight into the previously suggested role of PML and PML-NB in regulation of gene expression more generally.

7.1 PML-II protein and variants

The PML gene is expressed to give a diverse array of protein isoforms due to alternative splicing. All 7 principal isoforms contain an identical N-terminal region and divergent C termini (Borden et al, 1995; Jensen et al, 2001b). Each isoform may also have several variants depending on the differential splicing pattern (Jensen et al, 2001b). The majority of PML proteins are located in the nucleus, while various splicing PML isoforms devoid of an NLS have also been found in the cytoplasm (Bernardi & Pandolfi, 2007; Jensen et al, 2001a; McNally et al, 2008). The cellular functions of cytoplasmic PML (cPML) have been well reviewed by two recent papers (Giorgi et al, 2010; Jin et al, 2013). In this study, various molecular size PML-II variants including nuclear full length ~107 kD PML-II, and another ~50 kD cytoplasmic truncated PML-II variant were observed. The expression level of both nuclear and cytoplasmic endogenous PML-II forms could be significantly reduced by PML-II siRNA. Depletion of PML-II significantly inhibits induction of IFNβ, while over expressing full-length PML-II could not obviously increase the activity of IFNβ promoter or mRNA expression from the IFNβ gene. This suggested that PML-II may have more than one functional variant, but which one plays an important role in the IFN response remains unclear.

Defining functions unique to PML-II is complicated because the PML-II N terminal contains the homo-multimerization domain RBCC structure which can interact with other PML isoforms that may have an opposite function to PML-II in IFNβ response. The dimerization of PML-II with other isoforms makes it hard to exclude the possibility that other PML isoforms are involved in this function of PML-II in type I IFN response, but it is clear that PML-II must play an important role in this function. Overexpression of PML-II-ΔRBCC plasmid, which expresses a protein that lacks the N terminal RBCC structure, thus reducing the homo-interaction with other PML isoforms, increased the activity of the IFNβ promoter and endogenous mRNA levels of IFNβ and ISGs. This suggested that the C-terminal of PML-II is also essential for PML-II function. However, the specific sequence requirements for PML-II function in type I IFN response was not investigated further, so it will be very interesting to conduct such work in the future.

In the subsequent experiments, overexpressed PML-II siRNA resistant-mutants including full-length PML-II, ΔRBCC, C-PML-II and Δexon-5-PML-II could not restore the induction of IFNβ that was inhibited due to prior knockdown of PML-II. This is probably because depletion of PML-II affects dimerization of PML-II and other isoforms which affect PML-NB assembly as PML-II is essential for PML-NB formation, and the recruitment of some other components which may also participate in the transcription function of PML-II. In addition, it is possible that the smaller molecular size cytoplasmic PML-II variant is the functional one that participates in IFNβ gene transcription. Actually, previous studies have suggested that cytoplasm accumulated PML plays a critical role in cytokine signalling, antiviral response and apoptosis (Giorgi et al, 2010; Lin et al, 2004; McNally et al, 2008). If it really is the cytoplasmic PML-II variant that contributes to this function in IFNβ expression, how the cytoplasmic variants shuttles from cytoplasm to nucleus and regulates TFs binding and CBP recruitment in the nucleus remains unclear so far. Therefore, further research is needed to identify the role of this endogenously expressed cytoplasmic PML-II variant.

7.2 PML-II intrinsic antiviral activity and innate immune response

PML proteins have been involved with a very wide range of biological functions in cells. Among these is a role in antiviral defence (Everett & Chelbi-Alix, 2007a; Jensen et al, 2001a). In recent years a substantial literature has demonstrated that PML protein and PML-NBs confer intrinsic antiviral activity (Geoffroy & Chelbi-Alix, 2011). Many viruses express viral proteins that target PML for survival. For example, it was shown previously that Ad5 E4 Orf3 rearranges PML NB through an interaction with PML-II (Hoppe et al, 2006). Ad5 E4 Orf3 is also necessary for the virus to replicate in cells with an established IFN response (Ullman & Hearing, 2008; Ullman et al, 2007). Other viruses also rearrange/disrupt PML-NBs in various ways, leading to the concept that viruses target PML to evade antiviral activities in which PML participates (Leppard & Wright, 2012). HSV1 causes global degradation of PML protein including PML-II, which is one of two isoforms that are inhibitory to HSV1 infection (Cuchet et al, 2011), while HSV2 alters PML RNA splicing to favour PML-V expression over PML-II (Nojima et al, 2009).

In this study, the direct relationship between PML and interferon responses was demonstrated. It was found that PML-II positively regulates IFNβ induction and the downstream expression of a large number of ISGs. These ISGs have been implicated in a wide range of cellular activities including antiviral activity, transcriptional regulation, immune modulation, inflammatory responses, apoptosis, and cell signalling. Some ISGs exert multiple functions under different conditions. This may be the reason why PML proteins are linked with numerous different cellular activities, perhaps through the regulation of the activity of these ISGs. Also, the results presented here show that PML-II has an important role in the innate immune response.

The activity of IRF3 was demonstrated to be regulated by PML-II. IRF3 plays a critical role in the antiviral response. Many viruses prevent IFN through targeting the function of IRF3 (Weber et al, 2004). IRF3 not only regulates expression of the IFNβ gene cooperatively with

other transcription factors, but also regulates a subset of type I ISGs such as ISG20, ISG54, ISG56, RANTES and ISG15, independent of the expression of IFN (Daly & Reich, 1993; Daly & Reich, 1995; Honda et al, 2006). IRF-mediated transcriptional cascades may be intrinsic antiviral mechanisms that allow rapid ISGs expression before IFN itself can be produced (Schmid et al, 2010; Schoggins et al, 2011). The role of PML-II in regulating IRF3 transactivation further supports the function of PML-II in antiviral activity.

In this study PML-II was found to positively regulate not only the type I IFN response but also type II IFN-mediated signalling. IFNs are key cytokines in the establishment of a multifaceted antiviral response in cells. Although all IFNs are important mediators of antiviral protection, their roles in antiviral defence are different. Type I IFNs possess strong intrinsic antiviral activity, and are able to induce a potent antiviral state in a wide variety of cells (Levy & García-Sastre, 2001; Samuel, 2001). In contrast to type I IFN, antiviral activity is not the primary biological function of type II IFN (Dorman et al, 2004; Novelli & Casanova, 2004). Type II IFN plays a central role in the development of adaptive immune responses, and it can also amplify the induction of antiviral activity by IFNα/β. Therefore, type I and type II IFNs often work together to activate a variety of innate and adaptive immune responses.

PML-II was also demonstrated to affect the expression of IL-28 and IL-29 in this study. Type III IFNs IFN-λs are usually coexpressed together with type I IFNs following viral infection (Sheppard et al, 2003). IFNλ can be induced by many different viruses, including encephalomyocarditis virus, vesicular stomatitis virus, and hepatitis B virus (HBV) (Ank et al, 2006; Kotenko et al, 2003; Robek et al, 2005; Sheppard et al, 2003). Type III IFNs and type I IFNs induce similar biological activities in cells (Iversen & Paludan, 2010; Onoguchi et al, 2007; Osterlund et al, 2007). Taken together, these results support the idea that PML-II has a critical function in antiviral activity and also provide further evidence that PML-II functions in IFN responses.

7.3 The association between PML-II and IRF3, NF-κB, STAT1 and coactivator CBP

The implication is that PML isoforms are involved in various cellular activities due to their ability to interact with different partners. More than a hundred proteins have been found that can be physically and/or functionally linked to PML protein including transcription factors p53 and NF-κB, and co-activator CBP (Doucas et al, 1999; Guo et al, 2000a; Pearson et al, 2000; Wu et al, 2003; Zhong et al, 2000b). In this study, PML-II was demonstrated to form transcriptional protein complex with multiple TFs including IRF3-, NF-κB- and STAT1-CBP under the condition of stimulation. Notably, depletion of PML-II considerably affects IRF3 recruitment of CBP. Knockdown of PML-II also significantly affects IRF3, NF-κB and CBP binding at the IFNβ promoter. These results suggest that PML-II contributes to the stability of transcriptional protein complexes at promoters. Similarly, STAT1 and CBP binding at ISG15 and ISG56 promoters was also impaired by depleting PML-II.

IRF3 is an essential transcription factor that not only controls expression of the IFNβ gene, but also regulates a group of ISGs (Daly & Reich, 1993; Daly & Reich, 1995; Honda et al, 2006). In this study, the activity of IRF3 was severely impaired by the depletion of PML-II thereby down-regulating the expression of IRF-3-targeted genes. The role of PML-II in regulating IRF3 transactivation further supported the function of PML-II in antiviral activity.

NF-κB is a ubiquitous transcription factor which exists in most cell types. NF-κB regulates the transcription of a variety of genes, including cytokines and growth factors, adhesion molecules, immune receptors, and is thus involved in many biological processes, such as inflammation, immunity, cell activation, apoptosis, proliferation, differentiation and survival (Vallabhapurapu & Karin, 2009). Data in the present study demonstrated that PML-II can also form protein complexes with NF-κB and CBP in response to TNFα stimulation, and that PML-II has a stronger ability to bind to NF-κB compared to other PML isoforms. The activity of NF-κB in response to various different stimuli, including poly(I:C), PIV3 and

TNFα, was reduced by depleting PML-II. Knockdown of PML-II also impaired mRNA expression from many NF-κB-dependent genes including IL-6, IL-8, RANTES and IP-10. Previously a study showed that PML is transcriptional repressor of NF-κB by interfering with its binding to the NF-κB-target gene in $PML^{-/-}$ mouse embryo fibroblasts in response to TNFα stimulation (Wu et al, 2003). This suggests there is at least one other specific isoform of PML that functions diffently from PML-II in gene transcription, at least in TNFα-mediated NF-κB signalling. In fact, a specific isoform of PML was demonstrated to interact with histone deacetylases for transcriptional repression (Wu et al, 2001). Collectively, the effect of PML-II on the activity of IRF3 and NF-κB provides the mechanistic basis for, and highlights the importance of, PML-II in numerous cellular biological activities.

The interaction of CBP with PML has been reported previously to involve PML sequences that are present in all the principal nuclear PML isoforms (Doucas et al, 1999). Therefore it might be expected that CBP binding would not be restricted to PML-II. Equally it is possible that the differing C-terminal domains affect the conformation or availability of this CBP-binding sequence so that its activity is only manifest in certain isoforms. Although a full range of isoforms were not tested in this study, the data show that PML-II has a functional role in CBP activity. Given the evidence that PML-II participates in a ternary complex and influences both TF and CBP binding to target promoters, it can be suggest that the PML-II unique C-terminus is important for bringing specific TFs into this complex.

7.4 Post translational modification on PML-II protein

More and more evidence indicates that PTM of PML, such as Sumoylation, phosphorylation, ubiquitination and the newly identified acetylation, affect the ability of PML to interact with various partners and the regulation of PML function in various stress responses (Cheng & Kao, 2012b). PML can also interact non-covalently with small ubiquitin-like modifiers (SUMOs) including SUMO1/2/3 through a SUMO-interacting motif (SIM) at the C-terminus of PML (Lin et al, 2006; Shen et al, 2006). PML can be modified by SUMO at

multiple Sumoylation sites (Cheng & Kao, 2012b). Various extracellular or intracellular stimuli can induce SUMO conjugation of PML and mediate PML degradation (Cheng & Kao, 2012b), for example arsenic trioxide (As_2O_3) (Müller et al, 1998); DNA damage also triggers PML Sumoylation (Gresko et al, 2009). In contrast, heat shock causes a de-Sumoylation of PML (Nefkens et al, 2003). Some viral infections can also abolish PML Sumoylation (Everett et al, 2013).

Sumoylation regulates PML's ability in apoptosis and PML's anti-viral activities. Sumoylation of PML also directly or indirectly regulates gene transcription, through sequestration of or dissociation of transcription factors from PML-NBs (Gao et al, 2008; Lehembre et al, 2001; Ohbayashi et al, 2008; Pearson & Pelicci, 2001). Sumoylation of PML is required for the recruitment of components of PML-NBs (Zhong et al, 2000a). Phosphorylation is another common modification that has effects on PML protein biological functions. The phosphorylation of PML can occur in several regions of PML, such as N-terminal region, the RBCC domain, NLS and the C-terminal SIM (Cheng & Kao, 2012b). PML phosphorylation can modulate its Sumoylation (Percherancier et al, 2009; Scaglioni et al, 2006; Stehmeier & Muller, 2009). PML phosphorylation also coordinates with other post-translational modifications in response to different cellular stimuli, although the mechanism of this remains incompletely clear. Acetylation of PML by p300 was also reported to promote its Sumoylation (Hayakawa et al, 2008a). PTM adds a complex layer of regulation to the physiological function of PML-II. A recent study showed that Sumoylation of PML was required in the process of PML regulating IFNγ-mediated signalling (El Bougrini et al, 2011). Given the implication of PTM on PML protein and the results in the present study, particularly the regulatory function of PML-II on CBP, it therefore will be interesting to investigate whether PTM is also required for PML-II exerting its function in type I IFN responses and in gene transcription regulation.

7.5 PML-II functions in posttranslational modification of TFs

PTM of specific TFs is one of the factors that influence their transcriptional activation. In terms of NF-κB, several PTMs have been described, particularly phosphorylation and acetylation (Perkins, 2006). RelA/p65 can be acetylated by CBP/p300 but some lysines also can be acetylated by PCAF (Buerki et al, 2008; Rothgiesser et al, 2010b). HDACs were reported also to mediate p65 deactivation by removal of the acetyl group from lysine residues (Rothgiesser et al, 2010a). Acetylation of p65 regulates diverse functions of NF-κB, including its DNA-binding capacity, transcriptional activity and subcellular localization (Chen et al, 2001; Huang et al, 2010; Kiernan et al, 2003). A cross-interaction between acetylation and other modifications has also been reported. For example, phosphorylation of p65 at serines S276 and S536 has been shown to enhance the binding of p300/CBP (Chen et al, 2005), which mediates the acetylation of RelA. Given the effect of PML-II on NF-κB activity and CBP function, it is therefore possible that PML-II plays a role in p65 modification which determines the outcome of p65-mediated gene activation.

Increasing evidence showed that PTM of STAT1 is also important for STAT1 to efficiently activate the transcription of STAT1-targeted genes (Hu & Ivashkiv, 2009; Platanias, 2005). For example, phosphorylation of STAT1 at Y701 is pivotal for stable association with chromatin during IFNγ-driven transcriptional activation (Sadzak et al, 2008). Phosphorylation is required for STAT1 to move into nucleus and to interact with the CBP/p300. A recent study showed that overexpression of PML increased IFNγ-induced STAT1 phosphorylation, and thus promoted STAT1 DNA binding at a promoter resulting in higher activation of IFNγ-ISGs, while knockdown of PML decreased IFNγ-induced STAT1 phosphorylation and subsequently affected the binding of STAT1 to DNA and thus decreased the expression of ISGs (El Bougrini et al, 2011). In my study, PML-II was demonstrated to associate with STAT1 and knockdown of PML-II affected STAT1 binding at the ISRE element of ISGs. Taken together, this suggests that PML-II may also affect

STAT1 phosphorylation in response to type I IFNs signalling. Acetylation of STAT1 and STAT2 is another critical modification involved in IFNs initiated Jak/STATs signalling. In fact all three components of ISGF3 can be acetylated by CBP, which is critical for the ISGF3 complex activation and gene regulation (Zhuang, 2013). The function of PML-II on CBP suggests a possibility that PML regulates gene transcription through affecting STATs acetylation.

7.6 A model for PML-II involvement in regulation of gene transcription

In this study, PML-II was demonstrated to positively regulate IFNβ induction and its downstream signalling. PML-II interacts with multiple transcriptional complexes such as IRF3-, NF-kB- and STAT1-CBP and contributes to their stability at promoters. Knockdown of PML-II impairs TFs binding and CBP recruitment at promoter. Together with other results regarding the function of PML-II regulating CBP/p300 during the cellular response to various forms of stress, it can be proposed that PML-II has a general function in the transcription of inducible genes, particularly CBP-dependent/required genes (Figure 7.1).

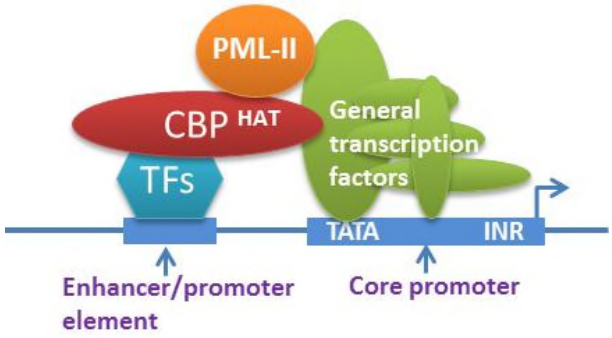


Figure 7.1 Model of PML-II actions in gene transcription regulation Upon activation, TFs translocate into the nucleus and bind to promoter/enhancer elements. CBP is recruited by TFs to the promoter where it acts as a bridge to contact both TFs and basal transcription factors/apparatus. It is proposed that PML-II is also recruited to these transcriptional complexes through an association with CBP and/or TFs and so stabilizes transcriptional complex assembly at promoters. Consequently, PML-II regulates the transcription of inducible genes, particularly CBP-targeted/dependent genes.

7.7 Future work

Results in this study provide evidence of PML modulating IFNβ gene and ISGs transcription, and also suggested that PML-II maybe a master regulator in inducible gene transcription in response to various stress responses. However, to further dissect this transcription regulation function of PML-II, there is still much more work needed in the future.

PML-II bound multiple transcriptional complexes under the condition of stimulation in this study. Stimulation of cells leads to rapid recruitment of p300/CBP and pol II to target promoters (Byun et al, 2009). This suggests that PML-II should also be recruited to the basal transcription complex and that it should interact with basal transcription factors including pol II at activated gene promoters, directly or indirectly. Therefore, it will be very interesting to detect whether PML protein is binding at promoter/enhancer sequences of regulated genes, and whether depletion of PML-II will affect pol II recruitment to these promoters.

Histone modifications influence nucleosome stability and facilitate recruiting other transcriptional factors (Li et al, 2007). Previous studies have revealed the relationship between gene expression and histone modifications. Generally, high levels of histone H3K9 acetylation and H3K4 methylation (H3K4me3) are detected in promoter regions of active genes (Bernstein et al, 2002; Bernstein et al, 2005; Roh et al, 2006), whereas elevated levels of H3K27 methylation correlate with gene repression (Boyer et al, 2006; Lee et al, 2006; Roh et al, 2006). This dynamic acetylation correlates with pol II association and gene activation (Crump et al, 2011). Therefore, it will be interesting to test the effect of PML-II on histone modification, particularly acetylation and methylation.

The function of PML-II on the induction of IFNβ and a large number of ISGs suggests PML-II has an important role in innate immune responses. In the past few years, remarkable progress in gene deep sequencing makes it possible to conduct investigation on the PML-II targeted genome-wide gene network which contributes to antiviral activity. ChIP-sequencing

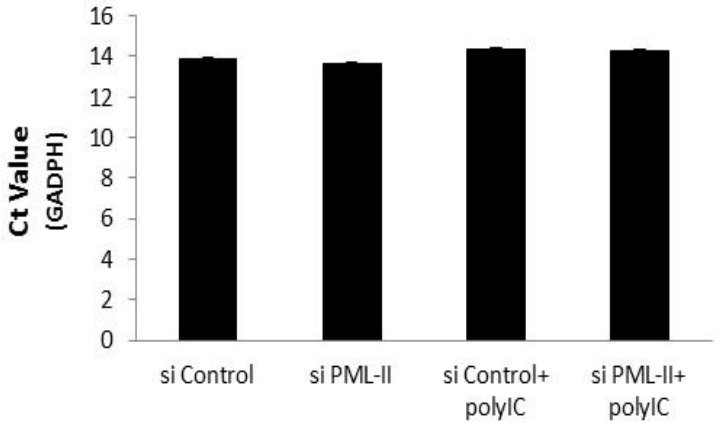
(ChIP-seq) is a newly developed technique, which combines chromatin immunoprecipitation (ChIP) with massively parallel DNA sequencing, for analysis of the interaction pattern of any protein with DNA, or the pattern of any epigenetic chromatin modifications. Results in this study have demonstrated that depletion of PML-II affects IRF3 activity and the expression of many IRF3-dependent genes. Therefore, it is also of significance to conduct a genome-wide investigation into the effect of PML-II on IRF3, CBP and pol II binding at gene promoters in response to various stimuli. Such a study will further expand our understanding of the PML-II-targeted gene network and its involvement in the innate immune response, and contribute greatly to the explanation of why PML proteins are involved in many cellular activities.

Appendix

Appendix 1 qPCR house keeping gene Ct value

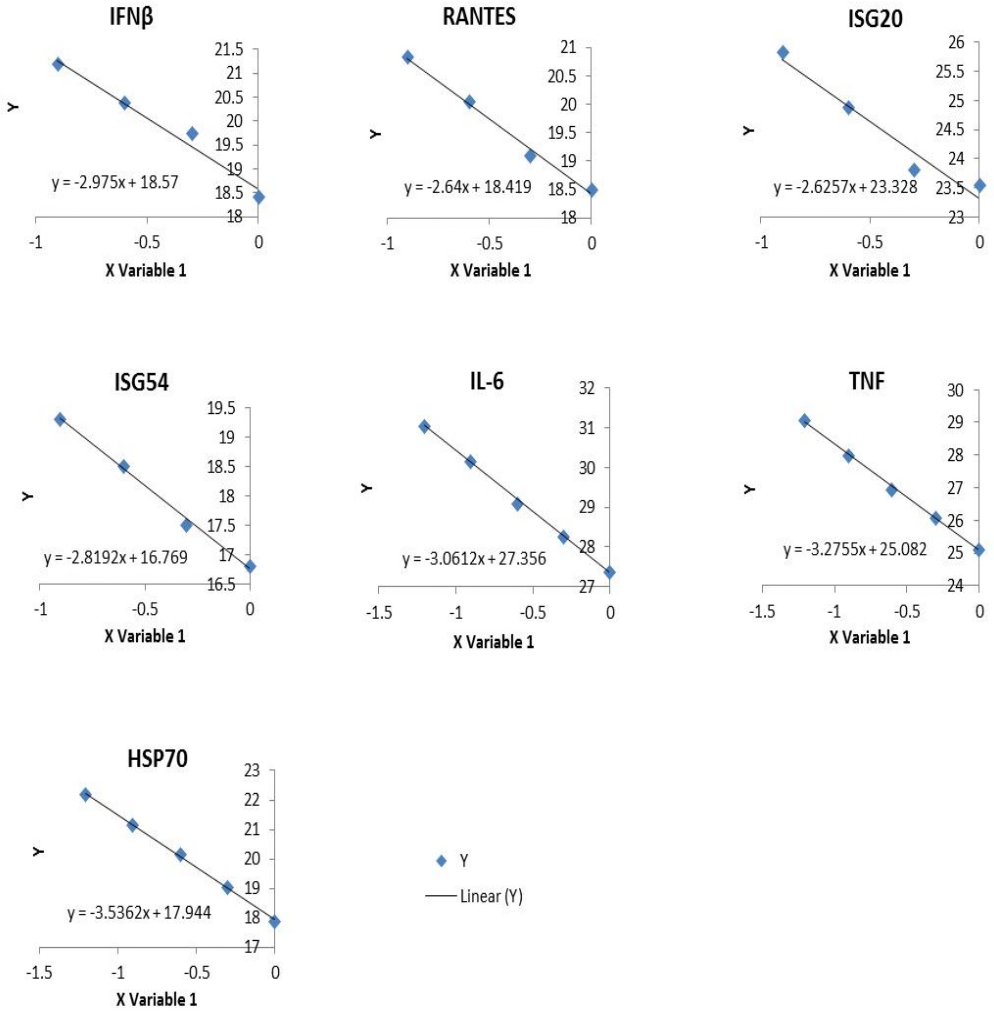
qPCR Ct value of GADPH gene

	Ct value	STDEV
si Control	13.8387	0.092042
si PML-II	13.66413333	0.065456
si Control+ polyIC	14.36153333	0.066887
si PML-II+ polyIC	14.27996667	0.050325

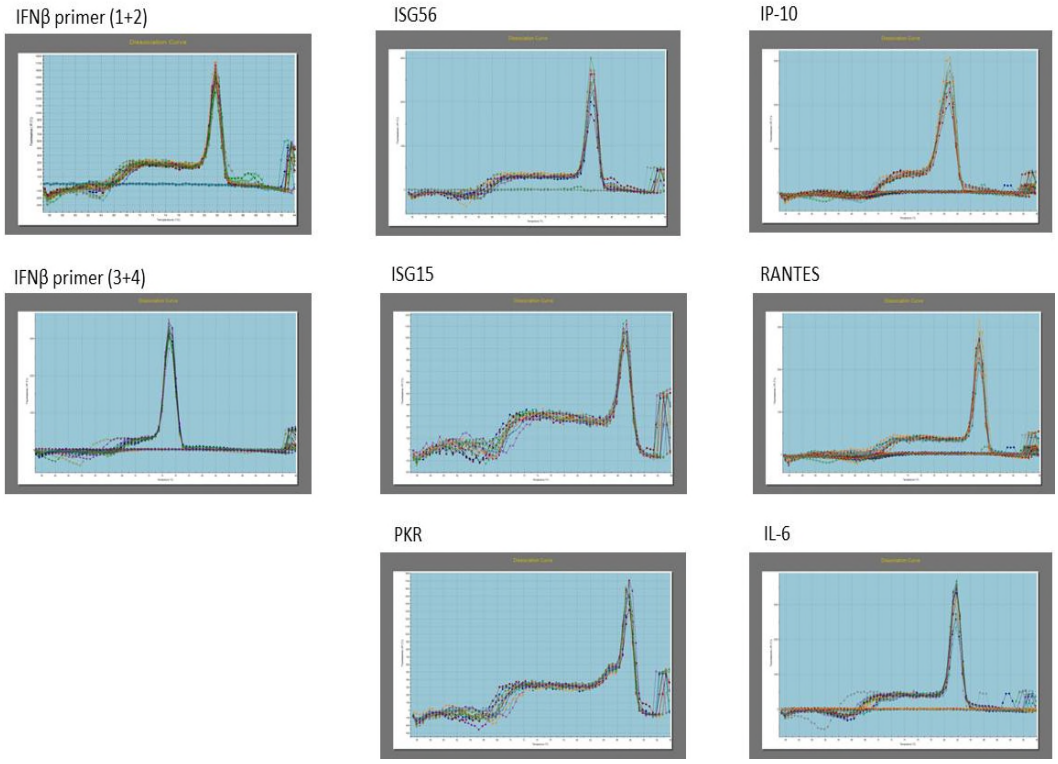


Appendix 2 qPCR primer efficiency

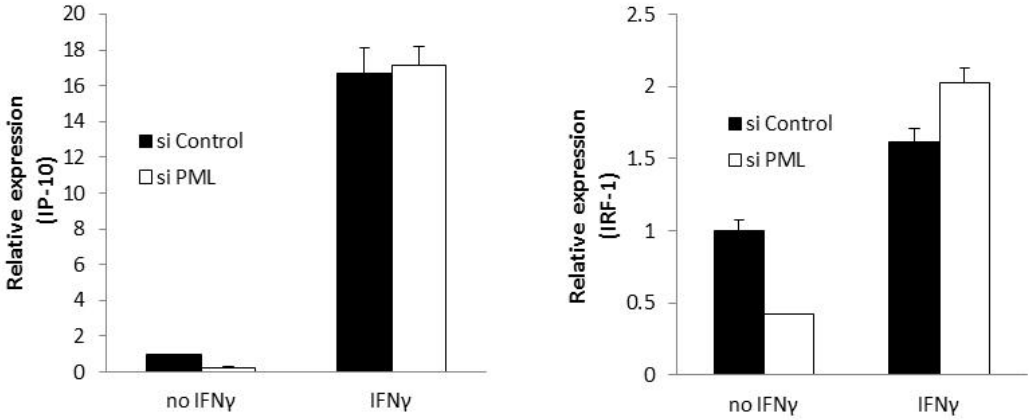
To evaluate qPCR efficiency using specific primers, a standard curve was established by plotting the C(t) values (Y) obtained from the qPCR against the log of control siRNA cDNA (X variable). To be acceptable for the study, primer pairs had to show a good correlation and a qPCR efficiency (calculated from standard curves, E = [10-1/slope]-1) of >90%. Some of qPCR primers efficiency was provided as following.



Appendix 3 Dissociation curve of ChIP-qPCR primers



Appendix 4 mRNA expression of IFNγ-ISGs



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