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Promyelocytic Leukaemia Protein Isoform II and Mild Heat Stress Compromise Human Adenovirus Type 5 Gene Expression

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A thesis submitted for the degree of Doctor of Philosophy in Biological Sciences

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Declaration

I would like to declare that all the work that is presented in this project was achieved by me under the direct supervision of Dr. Keith Leppard and has not been presented in any other work rather than this thesis.

Zeenah

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Dedication

I dedicate this thesis

For my Dad, Mum, brothers and sisters, You were incredibly supportive and understanding all that time. Mum your encouragement and praying made me confident to carry on.

For home, Iraq, land of civilisations and for all who sacrificed themselves to give me a chance to learn abroad and especially those who were killed defending us since June 2014.

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List of Abbreviations

ITR	Inverted terminal repeats
TP	Terminal protein
CAR	Coxsackie and adenovirus receptors
pTP	Pre-terminal protein
NFI/CTFI	CCAAT box-binding transcription factor
NFIII/Oct1	Nuclear factor III
DBP	DNA binding protein
MLTU	Major late transcriptional unit
MLP	Major late promoter
dl321	E1A-deleted mutant of adenovirus 5
CREF cells	cloned rat embryo fibroblast
Bcl2	B-cell lymphoma 2
eIF2 α	Eukaryotic Initiation Factor 2 alpha
PKR	Protein kinase R
CDC25A	M-phase inducer phosphatase 1: encoded by cell division cycle 25 homolog A
p53	Tumor protein p53
IL-6	Interleukin 6
NFI	Nuclear factor I
MHC I	Major histocompatibility complex class I
IL-12	Interleukin 12
NF-KB	nuclear factor kappa-light-chain-enhancer of activated B cells
TLRs	Toll like receptors
P50	DNA binding subunit of the NF-kappaB
CD95	cluster of differentiation 95
TNF- α	Tumor necrosis factor alpha
H3K9me3	Histone 3 Lysine 9 trimethylation
TAFII31	component of the transcription factor IID (TFIID) complex
mTOR	mammalian target of rapamycin
P300	E1A binding protein p300
dl331	adenovirus serotype 5 fails to produce virus-associated (VA) RNAI
TAFI	Template activating factor I
TAF-II-VII	Human transcription factor TFIID contains the TATA-binding protein (TBP) and several TBP-associated factors (TAF _{II} s)
DLXCXE	Sequence motif binds to B domain in cellular proteins
PML	Promyelocytic leukaemia protein
PML-NB	Promyelocytic leukemia protein Nuclear bodies
Sp-100	speckled protein of 100 kDa
HMG	High mobility group box
IFN- β	Interferon beta
IRF3	Interferon regulatory 3
ISGs	Interferon stimulated genes
STAT1	Signal Transducers and Activators of Transcription1
STAT2	Signal Transducers and Activators of Transcription2
Jak	Janus kinase
H2B	Histone protein B
H2B-ub	Histone protein B-ubiquitin

hBre	Human Brefeldin A sensitivity protein 1
CBP	CREB-binding protein
TRAM	Transcription adaptive motif
TFIIB	Transcription Factor II B
SNF2	Sucrose Non Fermenting 2
Srcap	Snf2-related CREBBP activator protein
eIF-2 α	Eukaryotic translation Initiation Factor 2alpha
eIF-4	Eukaryotic translation Initiation Factor 4
eIF4F	Eukaryotic initiation factor F
MnK1	MAP kinase-interacting serine/threonine-protein kinase 1
eIF4G	Eukaryotic translation initiation factor 4G
eIF4E	Eukaryotic translation initiation factor 4E
RAR- α	Retinoic acid receptor- α
Daxx	Death-associated protein 6
SUMO-1	Small ubiquitin-related modifier 1
UBC9	ubiquitin conjugating enzyme E2I
HSV	Herpes simplex virus
EBNA1	Epstein–Barr virus nuclear antigen 1
HAUSP	Herpesvirus-associated ubiquitin-specific protease
HCMV	Human cytomegalovirus
HFFF	Foetal foreskin fibroblasts
IE1	Immediate early HCMV protein
LCMV	Lymphocytic Choriomeningitis Virus
P-TEFb	positive transcription elongation factor b
CDK9	Cyclin-dependent kinase 9
HIV	human immunodeficiency virus
Tat	trans-activating proteins
CHO cells	Chinese Hamster Ovary cells
VSV	Vesicular stomatitis virus
SV40	Simian vacuolating virus 40
RING	Really Interesting New Gene
ATR	ataxia telangiectasia and Rad3-related protein
Mdm2	Mouse double minute 2
fos	proto-oncogene
atRA	all- <i>trans</i> retinoic acid
GAL4	Galactose transcription activator 4
SP-1	specificity protein 1
EGFR	Epidermal growth factor receptor
IFN- γ	Interferon γ
MEF	Mouse embryo fibroblasts
ISGF3	Interferon Stimulated Gene Factor 3
ISRE	Interferon-Stimulated Response Element
p38	mitogen-activated protein
ERK1/2	extracellular signal – regulated kinases
DSBs	double strand breaks
γ H2AX	Phosphorylated histone
Nrf2	nuclear factor erythroid 2- related factor
ROS	reactive oxygen species
RNF4	RING finger protein 4
Hcds chk2	Human Serine/threonine-protein kinase (cds1) checkpoint kinase 2
Hsf1	Heat shock factor 1

HSE	heat shock elements
I κ B- α	nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha
NOS2	nitric oxide synthase 2
TRAF-6	TNF receptor associated factor 6
IKK	inhibitor of nuclear factor kappa kinase subunit (α & β)
TAT	Trans-Activator of Transcription
ICP0	HSV-1 infected-cell protein 0
DCV	Drosophila C virus
S2 cells	Schneider 2 cells
shRNA	Short hairpin RNA
CR2	Complement Receptor Type 2
HFV	human foamy virus
siRNA	Small interfering RNA
F9	teratocarcinoma cell
ES	mouse embryonic stem cells
ATRX	ATP-dependent helicase ATRX
CHIP	Co-chaperone protein
hTERT	Human telomerase reverse transcriptase
AAV	adeno- associated viruses
ICP27	Herpes simplex virus type 1 (HSV-1) regulatory protein ICP27
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
MFI	mean fluorescence intensity
NEMO	NF- κ B essential modifier
PKR	Protein kinase-R
AAV2	Adeno-associated virus 2
HTLV-1	Human T-cell lymphotropic virus type 1
SERPINH2	serpin peptidase inhibitor, clade B (ovalbumin), member
TEBP	Telomere End Binding Protein
FK506	prolyl isomerase binding protein
ATF3	AMP-dependent transcription factor
HLA	Human leukocyte antigen
IFIT1	Interferon-induced protein with tetratricopeptide repeats 1
AP-1	activator protein 1
KSHV	Kaposi's sarcoma-associated herpesvirus
UPR	unfolded protein response
RISC	RNA-interfering silencing complexes
TRBP	Tat-RNA-binding protein

Summary

Promyelocytic Leukaemia proteins (PML), the core component of PML nuclear bodies (NB), are implicated in many critical cellular functions. One of those is confronting virus infection: DNA and RNA viruses target PML-NB and interact with PML proteins and this is thought to facilitate efficient replication by interfering with their antiviral functions. One of those viruses is human adenovirus type 5 (Ad5). Its early E4Orf3 protein interacts specifically with PML isoform II (PML-II), disrupting PML-NB into track-like structures to inhibit PML-antiviral responses. PML-II has been shown to be a positive regulator of type I interferon responses, as are typically induced by virus infection, through regulating the transcription factors that control this innate immune response. Given this role of PML-II and its interaction with Ad5 E4Orf3, the first question that addressed here is how does PML-II affect the progress of Ad5 infection under normal conditions?

PML-NBs are also involved in cellular stress responses, being disrupted by heat shock and other stresses. Thermo-tolerance due to mild heat conditioning protects cells from more aggressive stresses such as higher temperatures through triggering the heat shock response, which helps in re-folding the affected cellular proteins. Interferon and other cellular cytokines are also induced in response to heat shock, suggesting a mechanism whereby heat stress might affect infection, possibly dependent on PML-NBs. The second question addressed here was whether cells became more resistant to infection by pre-stressing them and if so would PML-II have a role in that resistance?

To address these questions, PML-II-depleted cells were first engineered by lentiviral vector delivery of specific shRNA. Physical and functional knock-down was confirmed by measuring mRNA levels for PML-II and for genes normally induced by NF- κ B activation. The biology of Ad5 infection in these cells was then assessed in comparison with control cells.

Ad5 gene expression displayed substantial increases with the transient or permanent depletion of PML-II in HeLa and MRC5 cells. This effect was particularly marked for late gene expression; hexon mRNA and all other late proteins showed substantial increase in PML-II depleted cells compared to several distinct control cell lines. This increase reached up to 100% with PML-II removal, quantified by flow cytometry. Virus yield also showed a 3-fold increase in PML-II depleted cells. This effect was only partly due to the impaired interferon pathway in these cells, which would be expected to augment Ad5 gene expression. The more significant factor in that increase was the overexpression of HSP70 chaperone, a specific member of the heat shock protein family, in PML-II depleted cells. siRNA-mediated HSP70 reduction caused a drastic decrease in Ad5 gene expression in PML-II Kd cells to more or less the same level seen in control cells. This effect was not NF- κ B-dependent but HSP70 depletion did increase expression of interferon response genes such as ISG56.

Pre-exposure of cells to mild heat stress made them significantly more resistant to Ad5 infection: such treatment reduced viral gene expression, in particular hexon and other late mRNA and protein expression, in HeLa cells and MRC5 cells. Ad5 genome replication was also reduced in pre-stressed cells compared to the control conditions. Consistent with previous studies of more extreme heat stress, mild heat stress affected the morphology of PML-NB. It also affected expression of PML protein and specifically showed an increase in PML-II mRNA expression after 3 hours of exposure to 40°C compared to the control conditions. In light of the enhanced infection seen when PML-II was depleted, this increased expression of PML-II following heat stress might play a role in the reduced infection efficiency in such cells. Indeed, PML-II removal reversed the negative effect of the mild heat stress on Ad5 infection: such cells showed more late gene expression after heat stress than control cells.

Chapter 1: Introduction and literature review

Section I: Adenovirus

1.1 Adenovirus Structure and life cycle

Adenovirus was firstly identified as adenoid degeneration agent in 1953 by (Rowe et al., 1953) when this factor showed no ability to be cultivated in bacterial growth media. Adenoviruses were classified according to their ability to haemagglutinate experimental erythrocytes or according to their GC content to six subgroups A to F (Pina and Green, 1965, Bailey and Mautner, 1994). Group C human adenoviruses represent more than 70% of the causative agent of mild upper respiratory tract infection and 14% of the lower respiratory tract infection in young children (Brandt et al., 1969, Avila et al., 1989) and are also reported by another study as the common cause of lower respiratory tract infection reached to 40% as itself or mixed with other viruses (Yun et al., 1995).

Adenovirus type 5 (Ad5) is a human virus within adenovirus species C assigned to the genus *Mastadenovirus*, it has a double-stranded linear DNA genome of 36 kbp in length. Both of the genome ends terminate with 103bp inverted terminal repeats (ITR) where the terminal protein (TP) is covalently linked. The DNA associates with protein V, mu and the histone homologue protein VII to form the virus core which is surrounded by a shell which consists of main (hexon, penton and fibre) and secondary structural proteins (cement proteins). Capsid surfaces consist of hexon (protein II) in its trimeric form (240 capsomers); the other 12 capsomers which form the vertices of the capsid, are pentons (protein III); these serve as an anchor where the fibre (protein IV), the virus protein which is responsible for attachment to the cell surface, is attached. The other minor capsid proteins are IIIa, VI, VIII and IX which participate in stabilising the capsid structure of the virion. The Ad5 also encodes a 23kDa protein (23k protease). Ad5 protease plays a role in virus maturation and then in un-coating upon infection (Fig.1.1) (McConnell and Imperiale, 2004, Leppard, 2008).

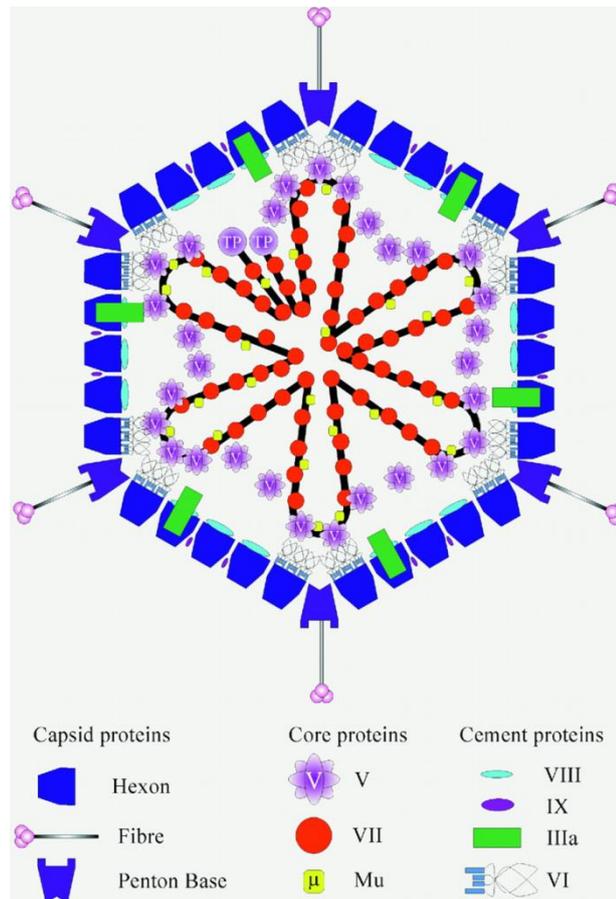


Fig.1.1. Schematic diagram for the structure of adenovirus. Hexon, fiber and penton base compose the virus shell according to results that were gained by cryo-electron microscopy and X-ray crystallographic analyses. At the inside, the core resides and consists of pV, pVII and Mu but its precise location is stylised. Virion stabiliser proteins include pVIII, IX, IIIa and VI bridge between the shown here in capsid and the core and project from that the inner side of the capsid proteins (Russell, 2009).

Ad5 enters the cell through a well-organised process that includes attachment, internalisation and transferring the viral genome to the nucleus where transcription and genome replication take place as well as final assembly after synthesis of the structural proteins in the cytoplasm. Ad5 attaches to the cell through Coxsackie Adenovirus receptors (CAR): expression of such receptors in non-permissive hamster cells increases their susceptibility to infection about 100 times (Bergelson et al., 1997). The virus attaches to CAR via its fibre knobs, the distal ends of fibre structural protein, but is not internalised. Adenovirus internalisation needs another kind of receptor, namely integrins which belong to the class of integrin heterodimer adhesion molecules known as nectin-binding integrins, $\alpha_v\beta_3$ and $\alpha_v\beta_5$. Those two molecules are required for an efficient viral internalisation (Wickham et al., 1993).

Internalisation is followed by series of events which lead to transfer of the viral genome to the nucleus to initiate the viral transcription and replication. Once the virus is internalised within membranous vesicles, endosomes, the virus loses several viral proteins such as fibre and protein VI and is released to the cytoplasm, the latter has a role in the virus entry as it expresses membrane lytic activity (Wiethoff et al., 2005). With more viral proteins continuing to be cleaved and dissociated from the particle, the viral core is then transferred by microtubules to the nucleus to start the next stage of the infectious cycle (Fig.1.2) (Leppard, 2008, Meier and Greber, 2004)

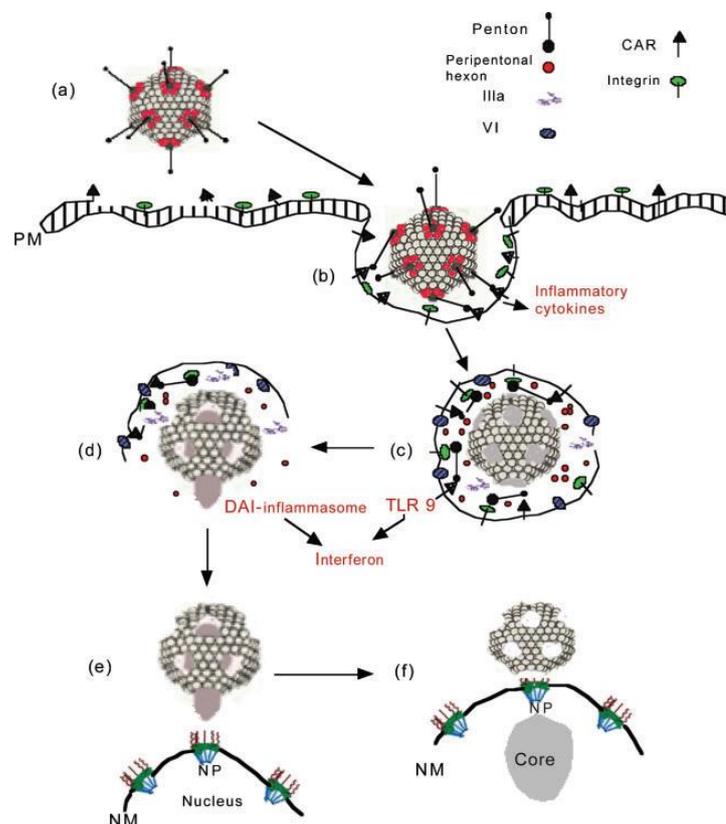


Fig.1.2. Entry pathway stage of adenovirus infection. A. Virus attachment to the plasma membranes through the fiber knob. **B.** Particle internalisation into the cytoplasm through interaction of penton with integrin heterodimer receptors. **C.** Particle uncoating by releasing hexon, IIIa and VI leaving the core intact. **D.** Acidification of lysosome internal environment ruptures the endosomal membrane releasing the hexon shell with the intact core. **E.** Hexon shell and core dock at the nuclear membrane. **F.** The viral core enters the nucleus targeting cellular enzymes to initiate transcription. The innate immune response is represented red (inflammatory and interferon response) (Russell, 2009).

In brief, adenovirus genome replication requires cooperation between virus proteins and cellular proteins. Ad5 DNA polymerase forms a heterodimer with the pre-terminal protein (pTP) which functions as a protein primer for DNA synthesis. Two cellular transcription factors, NF1/CTFI and NFIII/Oct-1, enable the viral DNA polymerase to bind to the origin of DNA replication to initiate replication. Then, with the help of the viral single-stranded DNA binding protein (DBP), replication starts and DNA synthesis continues through a strand displacement mechanism (Liu et al., 2003). At the onset of replication, the major late transcriptional unit (MLTU) is activated to produce most of the packaging proteins such as hexon trimers in the cytoplasm which are transferred back to the nucleus to start the assembly. Viral DNA is encapsidated into pre-formed immature empty particles by a process that requires series of viral proteins including IVa2, L1-52/55K and L4-22k and a specific packaging sequence in the DNA. A further step in maturation is required to make the virus particle infective, in which the viral 23K protease cleaves a series of viral component proteins including IIIa, VI, VII, VIII, X and TP. The mature virions are then released from the infected cells to infect neighbour ones (Leppard, 2008).

1.2 Ad5 gene expression

Ad5 gene expression is highly regulated and is divided into two main stages: early (E) and late (L) which are separated only by the onset of DNA replication and intermediate transitional stage (Fig 1.3). The early comprised 4 regions; E1, E2, E3 and E4. All proteins that are encoded by those 4 regions enable the virus to establish an efficient replication process, modulate late gene expression, confront the antiviral response and shut off cellular translation except the cellular factors that play positive roles in the adenovirus life cycle. The intermediate gene products of the closely related virus Ad2 start to accumulate in the cytoplasm between 10 to 12 hours post infection (Binger and Flint, 1984). This transitional stage is important in switching from the early to the late gene expression and some of its products (for example IVa2) are important in activating the recently described L4 promoter and hence in activating the whole late gene expression (Morris et al., 2010). The other intermediate encoded-protein is L4-22K which is critical for posttranscriptional regulation (Guimet and Hearing, 2013). Protein IX is involved also in transcriptional

regulation since transfection of IX induces the major late promoter activity in a dose- dependent manner (Lutz et al., 1997).

The major late promoter (MLP) which drives adenovirus late gene expression is not shut off completely at early time points of infection, instead it generates L1 52/55K product. This suggests some overlap Ad5 gene expression between the early and the late stages. The whole genomic regions of Ad5 are illustrated in (Fig.1.3). Once DNA replication starts, late mRNAs are expressed that encode viral proteins that make the capsid and are involved in DNA encapsidation and maturation of the virion.

1.2.1 Early gene expression

1.2.1.1 E1 region products

Products of the E1 region are profoundly important in transactivation of all other early regions, transforming certain cell types, DNA replication, efficient late gene expression and protein synthesis. E1A region products are transcribed early in infection within 45 minutes, reaching a maximum at 3 hours post infection then maintaining a steady level at 6 hours post infection (Nevins et al., 1979). E1A protein products are essential for the production of early adenovirus mRNAs. dl312 (E1A deleted mutant)-infected HeLa cells fail to produce mRNAs from the E2, E3 and E4 regions compared to wild type Ad5-infected HeLa cells. This defect is manifest as a massive reduction in the transcription rate of the E1B, E2, E3 and E4 regions in dl312 mutant compared to wild type Ad5 at 5 hours post infection. In contrast, E1A transcription is totally independent of any viral protein synthesis (Nevins, 1981).

Classification of E1A as an intermediate early gene

E1B 19K or (175R) protein has no distinctive role in enhancing the expression of adenovirus early region. This protein displayed no role in enhancing the production of E1A 13S or E1A 12S in non-permissive cells CREF at 10 hours post infection. This suggests that this protein does not have any posttranscriptional impact on E1A expression. Consistent results were obtained in permissive cells like HeLa cells but at shorter time post infection (Herbst et al., 1988) although it shows an important role as anti-apoptotic protein. The function of this viral protein mimics the functional properties of Bcl2. It was found that E1B 19K binds to Bcl2 and sequesters it in cytoplasm in order to block its transcriptional repression function (Kasof et al., 1999).

In contrast, the same protein has a potent role in transforming normal cells (White and Cipriani, 1990). E1B 19K in a specific cooperation with one of the two E1A products, 13S protein, could transform BRK cells. Using flow-cytometry technique, it has been shown that E1B19K interferes with the effects of cisplatin, an anti-tumour agent, by preventing drug-induced apoptosis (Subramanian et al., 1993).

E1B55K has critical functions in viral and cellular gene expression. In the late phase, this protein, in cooperation with another early viral protein, E4Orf6, controls the shuttling of late viral mRNA from the nucleus to the cytoplasm (Bridge and Ketner, 1990). This effect appeared to be through reducing the phosphorylation of eIF2 α and PKR activation during the late phase of adenovirus infection (Spurgeon and Ornelles, 2009). Manipulating the nuclear export signal of E1B 55K causes defects in a series of Ad5 activities including replication, late protein mRNA export and protein synthesis (Schmid et al., 2011). In addition, E1B55K modulates the pattern of late gene expression by changing the ratio of long and short late mRNA splicing for example switching from producing L155/55K to L4100K. This selectivity in E1B55K effect might introduce the idea that this protein is participating in creating favourable conditions for the virus to produce late proteins and guarantee an efficient level of virus yield (Leppard, 1993).

Regarding its effect on the cellular machinery, the protein displays a role in different cellular activities including the cell cycle, apoptosis, transcription and stress and immune responses. In normal human lung cells, it was found that many genes like CDC25A (cell division cycle), hsp70A, hsp706 and IL-6 were modulated by more than 2.5 fold in cells infected with E1B-deleted mutant virus compared to the wild type (Rao et al., 2006). In order to enable adenovirus to replicate successfully, E1B55k in particular shows a specific direct physical interaction with the tumour suppressor p53 and subsequently abolishes its transcription activation function (Martin and Berk, 1998, Sarnow et al., 1982a).

E1B55K shows its effect on p53, in cooperation with another early viral protein, E4Orf6. Both of those proteins reduce the accumulation of p53 that is caused by E1A expression through degrading p53 via the proteasome. Reduction was revealed by a consistent and time-dependent drop in p53 expression post Ad5 infection. The decline started at 10 hours post infection and reached a maximum at 25 hours. The effect of E1B 55K and E4Orf6 is attributed to shortening the half-life of p53 from 2.5 hours to only 20 minutes (Querido et al., 1997). E1B55K/E4Orf6 achieve this by recruiting and retargeting cellular cullin- complexes to ubiquitinate p53 (Querido et al., 2001).

1.2.1.2 E2 region products

Proteins that are produced from the E2 region are principally direct participants in viral DNA replication, but they also participate in many viral activities throughout the infectious cycle; they modulate transcription from E1A and the major late promoter, and inhibit transcription of the E4 region. The E2A protein, also termed the DNA binding protein (DBP) is a protein of a molecular weight of 72K (Lewis et al., 1976), and has a distinctive role in modulating adenovirus type 5 gene expression at the transcription level. This was indicated by the substantial 3-fold increase in E1A and major late promoter (MLP) expression. In contrast, DBP showed an opposite effect on the E4 promoter, which was inhibited with DBP expression. Such an effect was detected at the transcriptional level as the increase with DBP expression was at the rate of transcription initiation with no change in the half-life of RNA (Chang and Shenk, 1990). One of the suggested mechanisms of the effect of DBP on the transcription level is cooperation between DBP and nuclear factor I (NFI). Initiation of Ad2 replication was increased with the addition of purified NFI and this increase was positively regulated by the concentration of DBP in vitro. Cooperation between DBP and NFI was explained by the effect of DBP in increasing the affinity of NFI for Ad origin of replication. In addition, DBP alters the kinetics of NFI binding to a given DNA template, increasing the rate of association and decreasing the dissociation of NFI (Cleat and Hay, 1989).

The other products of the E2 region are the E2B Ad DNA polymerase and terminal protein precursor, pTP. E2B has been extensively studied in the use of adenoviruses in gene therapy. HAdv type 5 and type 2 replicate very efficiently in the infected cell which can produce one million copy/cell within 40 hours (Hoeben and Uil, 2013). Most of the first generation Ad vectors have deletions in the E1A and E3 regions: including an E2B deletion in those vectors has improved their efficiency in terms of carrying capacity and lowering the risk of producing replication competent-adenovirus. One of the remarkable advantages of deleting the Ad DNA polymerase is the defect in producing the late gene expression besides further deficiencies in Ad replication (Amalfitano et al., 1998).

1.2.1.3 E3 region

The early region E3 products are mainly important in counteracting the innate and inflammatory response to infection, inhibiting NF- κ B activation and guaranteeing persistent infection in immune cells. It is well known that this region has no effect on adenovirus replication in standard epithelial cell lines in culture. One of the pieces of early evidence about the function of the E3 region arose from a study in the lungs of cotton rats. The study compared the effect of wild type and E3-deleted mutant adenoviruses infection. Although H2dl801 and H5dl327 (which both lack the E3 region, with a larger deletion in the second mutant) replicated as well as the wild type, the pathological consequences for the mutants were much greater than in the wild type. Such elevated virulence was attributed to the effect of E3-19K glycoprotein. Mutating the region encoding this product increased the expression of MHC class I on the surface of infected cells to levels comparable to those expressed by uninfected cells, allowing greater immune recognition of the infection (Ginsberg et al., 1989). It was also found that E3-19K can interact with MHC I and prevents its expression on the cell surface (Burgert and Kvist, 1985).

The other E3 region product which shows direct inhibitory effect on innate immune response is E3-14.7K. Compared to uninfected mice, Ad5 mice infected with 14.7K showed a substantial reduction in two pro-inflammatory cytokines, IL-6 and IL-12 (Carmody et al., 2006). In contrast, those two cytokines were elevated significantly in mice that were infected with E1/E3 Ad5-deletion mutants. Stimulation of NF- κ B with different Toll-like receptor (TLR) ligands was significantly inhibited by the expression of E3 14.7K. This inhibition in NF- κ B-driven transcription occurs downstream the latter activation and translocation to the nucleus. Specifically, the 14.7 K protein binds to the p50 homodimer (a member of the NF- κ B family) and prevents its DNA binding (Carmody et al., 2006). In another way to establish persistent infection in immune cells, E3-14.7/E3-10.4 complexes work together to block cytotoxic T-cell induced apoptosis through reducing the expression of cell surface Fas (CD95), a TNF- α receptor superfamily member (Shisler et al., 1997), the complex similarly acts to down-regulate NK cell recognition and killing (McSharry et al., 2008).

1.2.1.4 E4 region products

Products that are encoded by the E4 region are critically essential for the viral gene expression at the level of RNA processing, export and stability. Mutants that lack this region showed a defect in mRNA accumulation. In terms of interaction with the cellular machinery, the products of the E4 region are important in silencing host cell translation (Halbert et al., 1985). Protein products for example have an important role in controlling late gene expression, since it is reduced significantly in mutants that lack either E4Orf3 or E4Orf6. This reduction in late protein synthesis was a consequence of lower cytoplasmic mRNA levels; there is no direct effect on DNA replication but there is lower efficiency in forming plaques compared to the wild type (Bridge and Ketner, 1989). E4Orf3, independently of E1B55K/E4Orf6, inactivates p53 responsive genes by forming a blocking network at p53 target promoters. E4Orf3 forms de novo H3K9me3 heterodimer which blocks p53-DNA binding (Soria et al., 2010). E4Orf3 also targets the cellular antiviral response that is mounted by PML and also targets the transcription factor TIF1 α in a step that is highly conserved among different adenovirus serotypes (Ullman et al., 2007, Vink et al., 2012). E4Orf6, has a distinctive role in inactivating the p53 transcriptional activity through a direct interaction with the latter in a TAFII31 independent manner (Dobner et al., 1996). Other E4 region products such as E4Orf1 and E4Orf4 can enhance viral replication through activating the regulator of mRNA translation initiation, mammalian target of rapamycin (mTOR) (O'Shea et al., 2005). The E4Orf2, protein has been detected during the lytic cycle of Ad5 infection but with no functional role or any interaction with any cellular factor yet described (Thomas et al., 2013, Dix and Leppard, 1995). E4Orf4 was found to have a role in p53-independent apoptosis with a small contribution of E4Orf6 protein (Marcellus et al., 1998). E4Orf6/7 is implicated in trans-activation of the E2a promoter through E2F-binding stabilising its interaction with the promoter (Obert et al., 1994).

1.2.2 Adenovirus late gene expression

Although the adenovirus life cycle is clearly distinguished into two stages, early and late phases, there is some overlap between those two stages. One of the early observations about the regulation of Ad2 late gene expression was the early minimal activation level of late mRNA during the early stage of infection. Although this level was less than the level activated late in infection it was as active as early transcription. At this early stage of infection the critical step of the late transcription was termination rather than initiation. It is well-established that at that early stage the mRNA transcripts are only from L1, L2 and L3 and transcription was shown to stop before proceeding to the rest of the late transcription. This could be due to the fact that both the Late transcription unit and the E2 region are located to give convergent transcription and hence the E2 region transcription will block the transcription from the late promoter (Nevins and Wilson, 1981). The intermediate stage products (IX and IVa2) are not expressed until DNA replication happens. However, the late transcription starts 14 hours post infection (Binger and Flint, 1984). The L1 region has an alternative splicing character which enables it to switch from producing its different encoded proteins. This enables the L1 region from shifting the production of L152/55K mRNA to the production of IIIa mRNA as they are encoded by the same 5' splice site but two different 3' splice site (Kreivi et al., 1991).

The cloned full length MLTU expresses all the late adenovirus proteins and at the same relative levels as those produced during wild type infection. Truncated forms of this unit cause deficiency in late gene expression and fail in complete switching from the early to late pattern of adenovirus late gene expression. Plasmids that express regions from L1 to L3 express only very low levels of L1 IIIa, L2 penton and L2 V with undetectable levels of hexon (Farley et al., 2004). MLTU plasmids that failed to express the L4 and L5 regions also failed in switching from early to late phase while the MLTU that fully possessing the L1 to L5 showed a progressive infection represented by two distinctive phases: early and late which contained all

the typically expressed Ad5 late proteins which are normally expressed during Ad5wt300 infection. This difference in late protein expression was very well reflected at the RNA level since the L3 hexon, L1 IIIa and L2 penton were expressed at approximate 50, 10 and 50 fold lower levels respectively. The other factor that affected the switch between those two phases with L1 to L5- plasmid is the loss of Ad5 DNA replication during the natural infection. MLTU plasmids that contained additional sequences from L3 and extended to the L4 polyA site showed very similar levels of all the late proteins: IIIa, penton, V and hexon. In particular, L4-100K and L4-33K were important in such increases in Ad5 gene expression; the L4-33K effect being detected at the posttranscriptional level (Farley et al., 2004). The 55KDa and 52KDa products of the L1 region are selectively produced early during infection while mRNAs encoded by late regions like L2 and L3 were not detectable. Bigger mRNA products could be produced later and those which could be involved in the transition from early to late stage. Alternative and time-dependent production of L1 mRNA was noted, the 55KDa and 52KDa products being detected during the early stage of infection. This abundance was replaced with the significant production of IIIa protein later on infection. Such switching in producing different L1 region products was explained by virus-controlled abundance mechanism which is employed by the virus to manage the levels of each single protein. This mechanism depends on the presence of a U-rich region where transcription of RNA Polymerase III terminates. In the Adenovirus type 2 genome the termination signal of VA RNA1 is located in a site which is separated from the splice junction for 55KDa and 52KDa by 11 nucleotides. VA RNA1 might recognise the U rich region in the acceptor site of 55KDa and 52KDa and then this preferable accumulation of 55KDa and 52KDa mRNA might be explained (Akusjarvi and Persson, 1981).

1.2.2.1 Structural and non-structural late adenovirus proteins

Hexon is the major adenovirus capsid protein with a molecular weight of 109 KDa and there are 240 trimers of it in each mature virion (van Oostrum and Burnett, 1985). In hepatocytes, it was found that an important factor regulating the efficiency of adenovirus vectors in delivering their transgenes is the presence of hexon. Human coagulation factor X binds to the top of hexon trimers in a way that

facilitates the entrance of the virus into the hepatocytes, avoiding the need for an interaction between fibre and CAR. This binding showed 40 fold more affinity than the fiber (Kalyuzhniy et al., 2008). Serologically, it was found that hexon antibodies were the dominant species and secondly dominant antibodies were against fibres (Bradley et al., 2012). Penton is a pentameric structural protein with a molecular weight of 63.2 KDa, it is important in cell surface recognition and endocytosis and postendocytosis events in the virus life cycle (Karayan et al., 1997). The other structural protein is the trimeric fibre with a subunit molecular weight of 61.9 KDa, fibre protein is important as well in cell recognition. Fibre-penton assembly is critical for the for making the infective virion since mutations that cause the accumulation of fibre monomers had been proved to be fatal for adenovirus (Santis et al., 1999). The mature virion contains 74 copies of IIIa with a molecular mass of 63.2 KDa. The rest of the adenovirus polypeptides are V, VI, VII, VIII and IX with 41.6, 23.4, 19.4, 14.5 and 14.3 KDa respectively (van Oostrum and Burnett, 1985).

Three of four non- structural adenovirus proteins are products of the L4 region, L4100 K and L4-33K. Those two proteins play an important role in the full transcription of the late adenovirus products from Major late Transcription unit (MLTU). Another two proteins, L4-22K and L4-33 K are responsible for the complete switch from the early to late phase immediately after DNA replication commences, when the level of MLTU activity increased from very low to about 50 times. The other L4 protein, 100K, is required only for hexon expression which was blocked completely in the absence of this L4 product (Farley et al., 2004, Morris and Leppard, 2009).

The final intermediate-late non-structural protein is L1-52/55K. This protein enables the virus to make mature virions. Although, Ad5 replication and late gene expression were slightly reduced in cells infected with L1-52/55K-deleted viruses, the major effect of the mutation was on virus encapsidation. Analyses of particles manufactured by the wild type and L1-52/55K-deleted mutant revealed that the latter made empty capsids compared to the wild type which produced fully mature virus with fully synthesized DNA (Gustin and Imperiale, 1998).

1.3 Cellular factors that interact with early and late adenovirus products

Although most of the cellular response to the early phase of infection was growth arrest and induced immune response (Granberg et al., 2006), many publications refer to positive roles of some cellular factors in adenovirus replication, transcription and translation. In adenovirus-infected HeLa cells, histones associate with the viral chromatin and viral core protein VII in a chimeric chromatin template to start the early phase transcription. Another cellular factor, Template Activating Factor (TAF-I) is also required to stimulate viral early mRNA expression which was reduced to about 50% in HeLa cells that lacked the TAF-I. Using CHIP analysis, viral-bound TAF-I has been shown to function on the viral chimeric chromatin through binding to acetylated H3 close to E1A promoter. TAF-II plays a role in enhancing the recruitment of transcription factors in the presence of Ad-VII core protein through formation of TAF-II:VII ternary complexes (Komatsu et al., 2011).

Many cellular factors contribute to the enhancement or repression of late adenovirus gene expression. P53, a stress-responsive transcription factor, associates with the adenovirus type 5 L4 promoter at 12 hours post infection. This association enhances L4 promoter activity in cooperation with other viral activators like E4Orf3 and Iva2. In addition, p53 knockdown reduced late gene expression massively which suggests that this cellular factor plays a transient positive role in enhancing the production of proteins that are expressed from this newly discovered promoter. P53 separates from the L4 promoter when the MLTU starts being active (Wright and Leppard, 2013).

Another cellular factor which plays an opposite role for that of p53 in adenovirus gene expression is TFII-I. This multitask transcriptional factor shows an inhibitory effect on adenovirus late gene expression through inhibiting the activity of the L4 promoter. TFII-I binds to an Inr site in the promoter and this binding inhibits L4 promoter activity; this inhibition could be neutralized with the effect of L4P viral activator Orf3 (Wright et al., 2015).

Multi-functional promyelocytic leukaemia proteins (PML) (which will be discussed extensively in section II), have been known for their interaction with adenovirus proteins. Two decades ago, the first observation that connected adenovirus infection with PML was published (Puvion-Dutilleul et al., 1995). In uninfected cells, PML protein is found mainly concentrated in spherical sub-nuclear structures that are now termed PML-NB (other names include PODs or ND10). At early times in adenovirus infection, the structure and morphology of PML remained as normal as in uninfected cells. Later on and at the intermediate stage of infection when viral DNA replication reaches its maximum levels, infection caused PML-NB rearrangement. The nuclear bodies were not detectable as defined structures during the late stage until the end of the infectious cycle. PML-NB were found to be targeted specifically by one of the early region products, E4Orf3. This protein when expressed alone, could target the PML-NB, co-localize with them and change their circular shape to thread-like structures known as PML tracks. E4Orf3 was not the only adenovirus early protein which showed an interaction with PML; the oncoprotein E1A also co-localizes with PML through its CR2 amino acid conserved region (DLXCXE), also conserved among other several viral oncogenic proteins (Carvalho et al., 1995).

PML re-organization by the adenovirus E4Orf3 protein enhances many steps in the adenovirus life cycle. Adenoviruses defective for this protein were unable to rearrange the PML and their replication is significantly reduced. Those E4Orf3 mutants failed in shutting off cellular transcription which might give a chance for the innate immune response to develop (Huang and Hearing, 1989). One of the six PML isoforms, PML-II, co-immunoprecipitated efficiently and directly with E4Orf3 through the C-terminal residue. Mutated Orf3, which fails to interact with PML-II isoform is no longer able to rearrange the PML into track-like structures, hence this Orf3:PML-II interaction is necessary for PML arrangement (Hoppe et al., 2006).

Although Doucas et al. (1996) showed that PML over-expression could suppress Ad replication, one explanation for the reason for targeting PML-II in particular by adenovirus proteins might be to exploit this cellular factor to improve the virus efficiency of virus infection. At the early stage of adenovirus infection, the trans-

activator E1A is very active and enables the virus to start effective transcription of most of the other viral regions. This viral protein has been found to co-immunoprecipitate with PML-II and interact specifically with it. This interaction enhanced the E1A-mediated transcription activation which means that PML-II plays a positive role in the adenovirus life cycle (Berscheminski et al., 2013). In fact, adenovirus does not only interact with PML, the major component of PML-NB, instead it recruits and displaces other PML-NB components to guarantee efficient transcriptional regulation of its own genes. Adenovirus impairs the antiviral activity of some cellular viral repressors such as Sp-100B and C and HMG by displacing them from the nuclear bodies. In contrast, it recruits and keeps the Sp100-A inside the residual PML-NBs in order to benefit from it as a positive transcriptional regulator of the viral early gene (Berscheminski et al., 2014).

1.4 Innate immune response to adenovirus infection: How does adenovirus overcome interferon?

The interferon response is a very well-organised innate immune response and according to the type of the signal transduction pathway, this response is divided into two types, the classical and the Toll-like receptor response. The classical pathway is triggered in cells that have internal compartment receptor which can sense the cytoplasmic components produced upon viral infection and results in IFN- β induction through inducing the type I interferon regulatory factors such as IRF3 and NF- κ B. The other type of interferon response is mounted by plasmacytoid dendritic cells through either surface receptors or endosome receptors to sense the external or the internalised virus compartment respectively (review Haller et al., 2006). In immune cells and early in Ad infection (2 to 6 hours), infection with adenovirus upregulates transcription of IRF3, ISGs (ISG56), IFN- β and phosphorylation of components of IFN secondary signalling pathway (STAT1/STAT2) (Stein and Falck-Pedersen, 2012). In permissive human cell lines such as HeLa cells, infection with adenovirus (dl312: which lacks the E1A coding region) increases the level of ISG54 and ISG56 mRNA level to comparable levels to that induced by interferon treatment and this increase was at the transcription level. This

transcriptional induction of ISGs is suppressed by E1A gene products; E1A 12S but not 13S in particular exerts this property (Reich et al., 1988). The detailed mechanism for interferon induction by adenoviruses was mainly obtained by analysis of the IFN response to adenovirus vectors. E1 and E3 deleted vectors showed dose-dependent IFN response within 6 hours of infection when the virus enters cells, moves to the nucleus and achieves its own effective translation. Adenovirus infection in HeLa cells triggered the direct primary IFN response by phosphorylating the IRF3 which is a significant marker for activation of the early recognition response. This is accompanied by an increase in STING adaptor protein which is degraded at later time points. The primary induction of pattern recognition receptor (PRR) by adenovirus vector infection in HeLa cells was accompanied with inducing the paracrine/autocrine signalling pathway which extended to include other cellular transcription factors. In Ad-infected HeLa cells, STAT1 increased also in a dose dependent manner, thus adenovirus infection induces both an IRF3 primary response and IFN- β receptor (IFNR)/Jak-STAT secondary immune responses (Lam and Falck-Pedersen, 2014).

Infection with human adenovirus type 5 causes a universal increase in monoubiquitination of H2B, this is characteristic for transcriptionally active chromatin. This is specific for E1A deleted viruses which unexpectedly caused such an increase in H2Bub at 8 hours post infection which is then massively decreased in wild type adenovirus infection in different cell types such as A549, murine embryonic fibroblasts and human diploid lung fibroblast line WI38. This H2B-ub increase is due to the elevated level of type interferon (IFN) as a consequence of adenovirus infection. This was verified by the massive increase in H2B-ub in A549 cells treated with IFN- β which was comparable in level to that caused by infection with E1- deleted adenoviruses. Treatment with interferon or infection could not increase the H2B-ub in Vero cells which are deficient for IFN- β . Correspondingly, E1A blocks the increase in H2B-ub and transcription of IFN- β and interferon stimulated genes through its N-terminal region. It is necessary for each virus to overcome the interferon response especially at early stages of infection. Adenovirus achieves that through E1A which targets hBre1 by physical interaction with it,

hence disrupting the latter's function in activating ISG transcription. E1A blocks the interferon response through inhibiting chromatin modification by terminating the existence of H2B-ub within transcriptionally active regions such as the IFN- β promoter region (Fonseca et al., 2012).

Another strategy employed by Ad5 to escape the innate immune response is modulating the activation of NF- κ B transcription by E1 region products. The E1A 13S protein activates the transcription of cytoplasmic NF- κ B that contains p65 which is then followed by translocation to the nucleus. This function of E1A is antagonized by E1B 19 kDa which inhibits NF- κ B activation. Those two opposite actions are important to keep the balance for adenovirus to circumvent the immune response and use the host cell as long as it can to avoid apoptosis (Schmitz et al., 1996).

1.5 Effect of adenovirus infection on cellular transcription

E1A can manipulate most cellular transcription through binding to the transcriptional adaptors CBP/p300 to which many cellular transcription factors bind and which are involved in the transcription of many genes. E1A binds to the transcription adaptive motif (TRAM) of CBP through a region which contains a small peptide that prevents the binding of other cellular transcription factors like P53, E2F and TFIIB. P53 is stabilized by a complex process that depends on interaction with CBP/TRAM which leads to the activation of P53 dependent transcription. Wild type E1A abolishes P53-CBP/TRAM dependent transcription activation (O'Connor et al., 1999). E1B55K SUMO activity is well correlated with the P53 trans-activation function. E1B55K sumoylation of P53 is essential to transformation of rodent cells and to induce the oncogenic growth (Wimmer et al., 2015)

Adenovirus affects and manipulates cellular chromatin modelling through another early gene product, the E2A DBP. DBP interacts with and inhibits one of the SNF2 chromatin family members Srcap (Xu et al., 2001). Another example of the viral control of cellular transcription is the interaction between adenovirus E4-Orf6/7 polypeptide and the cellular factor E2F in an E1A-independent mechanism. This

interaction enhances the transcription activity of E2F specifically on the E2 early promoters and causes a substantial increase in E2 mRNA expression (Marton et al., 1990).

1.6 Blocking cellular translation by adenovirus

The relationship between Ad5 and the cellular translation machinery evolves throughout infection. While the cell responds to Ad5 infection by shutting off cellular translation at early times in infection, this translation is shut down at later time points by the virus to avoid the translation of unwanted immune response effectors (Weitzman and Ornelles, 2005). A well distinguished harmony was revealed between host cellular translation and the kinetics of Ad5 infection. This coincidence was also accompanied by inhibition in CBP phosphorylation. The whole cellular translation was not affected by Ad5 infection at early time points until 12 hours post infection when the whole translation was reduced massively and that went along with reduction in CBP phosphorylation which reached 20 fold less at 24 hours post infection (Huang and Schneider, 1991). Ad5 has evolved to discriminate between viral and host mRNA, such that cellular translation can be shut off whilst carrying on its own viral translation without any delay or disturbance. Adenovirus achieves its own late mRNA translation through a special mechanism called ribosome shunting, when the 5'-capped mRNAs are alternatively jumping the ribosomal scanning. This mechanism provides a selective translation of the viral mRNA and not the cellular translation. The regular translation when the eIF-4 is abundant is achieved through linear ribosome scanning from the 5'-cap, while in conditions when the eIF-4 is inactive or less available, adenovirus late tripartite leader (5'-non coding region) preferentially uses ribosome shunting. This kind of translation also happens when mammalian cells experience heat shock condition, when the need for eIF-4 is reduced too (Yueh and Schneider, 1996). One of the impressive effects of adenovirus during the late phase of infection, is the dephosphorylation of eIF4E by physically blocking the binding of eIF4F kinase (MnK1) to eIF4-G without inactivating the MnK1 activity or changing the cytoplasmic abundance of any of the eIF4 forms. This dephosphorylation step is completely

responsible for preventing the translation of most of the cellular mRNA but not the viral late mRNA, despite adenovirus translation critically needing dephosphorylated eIF4-E. The virus might benefit from de-phosphorylated eIF4-E since the latter provide a weak interaction that is not sufficient to translate the cellular mRNA. As the virus uses the ribosome shunting translation pathway, the less abundant phosphorylated eIF4-F might be the better for such translation mechanism (Cuesta et al., 2000). The L4 product, 100K protein, competes with Mnk1 to bind to the same C-terminal region of eIF4-G, which leads to the phosphorylation of eIF4-E and as a result inhibition of most cellular mRNA translation (Cuesta et al., 2000).

Section II

1.7 PML proteins and PML NB

1.7.1 PML gene and PML isoforms

The promyelocytic leukaemia proteins or PML, were firstly described as a part of a fusion protein with retinoic acid- α receptor (RAR- α) that results from a reciprocal translocation, chromosome 15:17. This translocation was detected in 6 of 8 acute promyelocytic leukaemia patients and RAR- α was connected since that time with leukaemia (de Thé et al., 1991). The PML gene has nine exons that are subject to an array of alternative splicing during gene expression; therefore, different PML protein isoforms are encoded by an enormous number of PML transcripts (Fig.1.4). Variations in the C – terminal region or the length of the central region divide these PML isoforms into two groups (Fagioli et al., 1992). Individual isoforms are expected to have at least some discrete and unique functions.

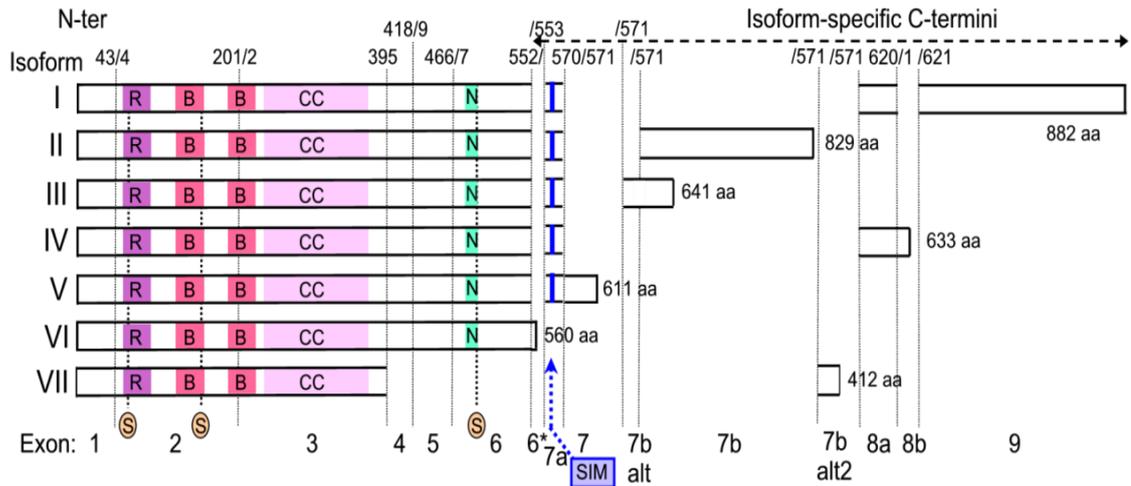


Fig.1.4. Structural diagram for the principal PML isoforms. All known isoforms share the same N-terminal region which comprises the first six exons (1-6), while they differ in their central region, variably including exon 5 and in their C-termini that are responsible for the specific function of each. The RBCC motif consists of R:RING finger, B:B-BOX and CC:Coiled coil. Green N- is the nuclear signal, dark blue is the SUMO-interactive motif (SIM), and yellow S refers to the sumoylated Lysine residues. Number of amino acids of each isoform is indicated at the end of each isoform structure (Wright, 2010).

1.7.2 PML proteins and PML NB function

PML proteins are a principal component of PML nuclear bodies (PML-NB). These are sub-nuclear structures that play crucial roles in many cellular processes. These functions include: tumour suppression, hence some oncogenes developed mechanisms to suppress PML induction as an IFN induced gene (Rego et al., 2001, Buonamici et al., 2005), apoptosis (through interaction of PML with p53)(Guo et al., 2000), and DNA damage repair, when PML co-localizes with repair proteins and stabilizes them as a response to any damage factor (Guo et al., 2000, Xu et al., 2003). One of the main biological roles of PML is in the antiviral response. IFN α , β and γ up-regulate expression of PML proteins in different types of human cells, indicating that PML is involved in the IFN response pathway (Chelbi-Alix et al., 1995). PML-NB are disrupted by many viruses (Everett et al., 1998, Everett et al., 2006, Everett and Orr, 2009, Sivachandran et al., 2008) and some PML-NB components have been shown to be antiviral. One of those proteins is Sp100 which was found to be essential for stabilising NB and protecting the PML from

degradation by ICP0 in order to exert the expected antiviral response since the total knockdown of Sp100 isoforms or PML decreases the constituent ND10 proteins (Negorev et al., 2009). Another NB component is Daxx which mounts an antiviral function in cooperation with PML proteins. E4Orf3 deficient virus restores complete replication activity when the Daxx or PML are antagonised (Ullman and Hearing, 2008). Another component which mediates the SUMO-1 modification of PML is the UBC9 that binds the PML directly or indirectly (Duprez et al., 1999). Opposite to Sp100, CBP is a dynamic component of NB, its accumulation is cell-specific and it could be stimulated to move in and out of the NB according to the stimulus (Boisvert et al., 2001).

1.8 Early intrinsic and innate host response to virus infection and the role of PML in this response

Virus infection triggers innate immune responses which are considered as the first line of defence that counteracts and slows down the infection before the adaptive immune response begins. Viral RNA and DNA and intermediate products can be recognized by Pattern Recognition Receptors (PRR). The latter then initiate an interferon (IFN) response in both the infected cells and other cells (Koyama et al., 2008).

Many studies have pointed to the central role of PML and PML NB in confronting DNA and RNA viruses. This role may be to two main reasons. Firstly, the fact that PML gene is up-regulated by IFN and this is associated with the increased level of PML proteins (Lavau et al., 1995). Secondly, to the targeting of PML and PML NB by many DNA and RNA viruses. Many DNA and RNA viruses cause disruption of PML NB when their proteins accumulate inside these bodies. This accumulation results in a uniform disruption of PML NB throughout the nucleus or change their appearance into track-like structures by a variety of mechanisms (Wilkinson et al., 1998, Ullman et al., 2007, Everett and Chelbi-Alix, 2007).

1.8.1 DNA viruses

As was mentioned earlier, many viruses target PML-NB and disrupt their structure in order to establish efficient replication centres. Herpes simplex virus (HSV) type 1 immediate early protein ICPO can disrupt the PML NB within a few hours post infection. ICPO acts as a ubiquitin ligase to degrade the PML when it co-localises to PML-NB causing their disruption (Everett et al., 1998, Everett, 2006). PML-NB staining disappears at the time of maximal immediate early protein synthesis and at the beginning of early protein synthesis. Furthermore this disappearance is not accompanied by a reduction or difference in PML protein expression, in contrast, the PML isoforms expressed normally (Maul et al., 1993). ICPO, the first causes loss of high molecular weight of PML isoforms. There are two reasons for, firstly, ICPO migration to PML NB. Secondly, the complete C- terminal region of ICPO containing HAUSP (Ubiquitin-specific protease enzyme associated with PML NB) binding and PML NB localisation (Everett et al., 1998). Other herpesviruses also cause PML-NB disruption by various mechanisms. Epstein-Barr virus achieves this through the interaction of EBNA1 protein with a specific PML isoform which results in PML-NB disruption (Sivachandran et al., 2008). Human Cytomegalovirus (HCMV) ensures efficient replication by altering PML NB distribution significantly in human foetal foreskin fibroblasts (HFFF) (Kelly et al., 1995). A transient co-localisation has been revealed between PML and the IE1 protein within a few hours of HCMV infection. IE1 causes PML displacement from PML NB through a direct interaction with the N-terminal RING finger domain (Ahn et al., 1998).

Dramatic changes have been detected in the appearance of PML bodies upon Ad5 infection and they increase in number after infection with the wild type virus (Doucas et al., 1996). One of Ad5 protein that arrests the innate immune response is the E4 orf3 protein. This protein plays a crucial role in disruption of PML-NB into track - like structures. Interaction between E4Orf3 and PMLII inhibits the activation of the IFN β promoter (J Wright; Atwan et al., manuscript in preparation). E4Orf3 mutants cannot establish sufficient normal replication centres when the cells are pre-treated with IFNs (Ullman et al., 2007) in contrast to wild type. Furthermore, cells pre-treated with IFN α and infected with wild type or E4Orf3 mutant did show

evidence of viral replication when their PML was disrupted by pre-treatment with shRNA. These results strongly suggest that E4Orf3 blocks an IFN- mediated antiviral effect via its interaction with PML II.

1.8.2 RNA viruses

HIV transcription depends critically on Tat protein- mediated recruitment of pTEFb to the transcription complex (Zhu et al., 1997, Muniz et al., 2010). Both members of the P-TEFb kinase complex CDK9 and cyclinT1 localize inside PML NB. The acetylated and the enzymatically inactive form of CDK9 interacts with PML and binds the integrated and transcriptionally silent HIV-1 viral genome. This suggested an important role for PML NB in HIV-1 repression. These observations had been confirmed when the PML NB were disrupted by arsenic trioxide or by PML knockdown: both treatments caused striking increase in viral transcription (Marini et al., 2011). Recently the mechanism that PML adopts to confer its antiviral response against retrovirus was unraveled. PML inhibits retrovirus reverse transcription indirectly through the stabilization of the cellular repressor Daxx (Dutrieux et al., 2015).

Replication of the (-) strand RNA virus, rabies, has also been shown to be negatively affected by PML. CHO cells stably expressing PML III were infected or not with rabies virus at an M.O.I of 1. The infection resulted in an increase in PML NB size. Electron microscopy showed that these bodies are empty spheres in uninfected cells, while two days post infection they became dense. Rabies P3 colocalizes with PML in the nucleus and reorganises the PML NB in increased size dots (Blondel et al., 2002). Rabies escapes the innate immune response through very well developed mechanisms to inhibit the IFN response through modulating the phosphorylation or the localization of IFN regulatory factors. The other way to avoid cellular resistance is by retaining the PML in the cytoplasm and as a consequence changing the structure of NB (Chelbi-Alix et al., 1998b).

PML proteins regardless of whether they were inside the NB or not and as a part of their antiviral function, they exert an inhibitory effect of the multiplication of VSV and influenza A viruses (Chelbi-Alix et al., 1998a). Seventy hours post infection with

LCMV, a predominant cytoplasmic PML distribution with only minor nuclear staining was detected in NIH3-T3 cells. PML recovered its distribution pattern when the cells were incubated with heat – inactivated virus. PML colocalises with the viral Z protein through a direct interaction which was verified by co-immunoprecipitation and this interaction was necessary for PML re-localization (Borden et al., 1998).

1.9 Effect of PML on cellular transcription

Early evidence implicating PML proteins in regulating transcription was uncovered by transfecting C-terminally truncated GAL4-DNA binding/PML fusion protein constructs in yeast and mammalian cells (Ahn et al., 1998). Unexpectedly, in yeast, the full length constructs which comprised the N-terminal Cys-rich region and the coiled coil domain showed no activity using the β -gal assay. Deleting the coiled coil region but keeping the RING finger plus the Cys/His region improved the transactivation activity of PML constructs. Making the GAL4/PML constructs even shorter, which kept only the intact RING finger domain of PML, still showed higher activity than the full length constructs but 6 fold less than those which retained the Cys/His neighboring region. This means that the coiled coil region may block the transactivation function of intact PML. The finger domain itself plays a crucial role in unmasking the transactivation activity of the intact PML, since mutating the Cys residues within this domain significantly reduced the transactivation activity of the C-terminally truncated PML constructs. The transactivation unmasking property was found to apply also in mammalian cells such as Vero cells. Using the same constructs but driven by a different enhancer (SV40 enhancer derived- vector) and expressing them in Vero cells showed about 22 fold increase in transactivation activity in constructs containing the RING domain and Cys/His rich residues. Again mutating the RING finger domain caused a 3 fold-reduction in the transactivation activity. Altogether, there is enough evidence that the N-terminal region has a role in cellular transcription but it is masked by the α - helical coiled coil domain (Ahn et al., 1998).

1.9.1 PML proteins interact with cellular transcription factors (transcription enhancement)

PML proteins have been discovered to be a positive partner for many cellular transcription factors and thus enhance transcription activity. CBP, a dynamic component of PML-NB (Boisvert et al., 2001) associates with PML-NB through interaction with PML. This association is located between residues 216-331 in the domain common to all PML and residues 331-521 in the N-terminal domain of CBP, PML proteins caused an increase in the transcriptional activity of CBP cofactor (Doucas et al., 1999). Another explanation for the reason for recruitment of CBP to the NB is to keep its level at a steady state inside the cells. Valproic acid, an epilepsy treatment medicine is used to degrade CBP through the ubiquitin proteasome pathway. PML-NB show ubiquitin activity and participate in degrading target proteins to control their levels inside the cells. This might explain why CBP is co-localizing in PML-NB (St-Germain et al., 2008).

PML-NB also show a critical role in tumour suppression after DNA damage by increasing the stability of p53. After DNA damage PML proteins are phosphorylated by ATR and accumulate together with Mdm2 (a negative regulator of p53 levels) in the nucleolus, hence stabilizing p53 by direct interaction between PML and Mdm2 (Bernardi et al., 2004). The transactivation function of p53 is increased with the recruitment of p53 to the nuclear bodies by PML IV in a promoter-dependent manner (Fogal et al., 2000). Another function of PML proteins and in particular PML-IV, is increasing transcription through stabilizing p300 by protecting it from ubiquitin-mediated degradation (Shima et al., 2008).

1.9.2 PML proteins interact with cellular transcription factors (transcription repression)

PML proteins have also been reported to be transcriptional repressors. GAL4/PML fusion proteins inhibited transcription from GAL4-responsive reporter plasmids in HeLa and Cos1 cells (Vallian et al., 1997). Twenty-five years ago, PML protein was depicted as a regulator of the repressive transcriptional function of Daxx. Daxx is an efficient repressor of global cellular transcription: it interacts with Sumo-1-modified PML and is recruited to PML-NB or is recruited to condensed chromatin in PML-

deficient cells. Movement of Daxx between the condensed chromatin and PML-NB reflects the variable equilibrium between the chromatin and NB and how dynamic is this process which is in the same time needs the accumulation of SUMO-1 modified PML to make the basic structure and framework of NB (Ishov et al., 1999). The Daxx-PML interaction therefore determines the correct of this global repressor, keeping it mainly in the nucleus, despite its locations potential to interact with c-fos although a very tiny proportion of Daxx was reported in the cytoplasmic fraction. In the absence of any inducers of the dissociation of co-repressors, such as all-trans-RA (atRA), Daxx can interact with the PML-RAR α complex (Li et al., 2000). This interaction was shown in vivo but could not be confirmed in vitro since it needs highly sumoylated PML, or maybe other cellular factors to associate and facilitate this interaction. Immunofluorescent antibody staining has shown a specific co-localization between Daxx and PML in different tumour cell lines such as HeLa and A549 in addition to normal human fibroblasts. Using reporter gene assays, the ability of PML to inhibit the Daxx repression function in HEK293 cells was measured. Compared to samples which were transfected with GAL4- DNA Binding Domain (DBD) alone, reporter gene expression was greatly reduced when cells were transfected with GAL4-Daxx, and this reduction was rescued in a dose-dependent manner by co-expression of increasing doses of full length PML VI plasmid (Li et al., 2000). This isoform of PML at least is therefore a negative regulator of Daxx repression and its interaction with Daxx may be important for mounting a potent anti-viral response against the invading viruses as it will permit the up-regulation of antiviral response genes.

Many DNA virus proteins, such as Ad5 E4Orf3, disrupt the PML-NB and such disruption is well-correlated with blocking the innate immune response. E4Orf3-deleted viruses do not replicate as well as wild type, but their replication can be rescued by a reduction in PML and Daxx using knockdown approaches (Ullman and Hearing, 2008). Another example that reveals the involvement of PML in regulating cellular transcription in eukaryotic cells is the interaction with the specificity protein 1 (SP1). In vitro co-immunoprecipitation assays showed that SP1 and PML interact directly through the C-terminal DNA binding site of Sp1 and the coiled coil regions

of Sp1 and PML. The physiological significance of such an interaction was demonstrated as an effect of PML on SP1 – dependent gene transcription of genes such as epidermal growth factor receptor (EGFR). In other words, PML represses the transcription of EGFR by inhibiting EGFR'sSp1-dependent activity. PML inhibits transcription from the EGFR promoter through disrupting the DNA binding activity of SP1 and thus PML plays a negative role in regulating the transcriptional activity of the EGFR gene (Vallian et al., 1998a).

1.10 Roles of PML in interferon responses

PML proteins have been implicated in the effective mounting of both type I and type II IFN responses. It was found that PML plays a positive role in regulating the interferon- γ response. Overexpressing the nuclear but not the cytoplasmic PML isoforms caused significant increases in IFN- γ -induced STAT1 phosphorylation and STAT1 DNA binding activity. These effects were reversed by transient siRNA interference of PML. An analogous effect was seen in mouse embryo fibroblasts (MEF), PML(-/-) MEF cells showed a decreased IFN- γ response compared to PML(+/-) cells (El Bougrini et al., 2011).

Similarly, for the type I interferon response, permanent PML knockdown in human fibroblast cells (having lentiviral shRNA transduction) showed less efficient IFN-stimulated gene (ISG) response compared to the control cells; similar results were obtained with the transient siRNA knockdown. This deficiency in interferon response was restored with the re-expression of the most abundant PML isoform. PML increased the level of ISGs through a significant increase in the active form of STAT1 and a slight increase in STAT2. This increase in STAT1 and STAT2 expression arose from a direct association of PML with STAT1 and STAT2 promoters which was accompanied by an increase in ISG promoter activity (Kim and Ahn, 2015).

1.10.1 PML-II regulates the type I interferon response

The involvement of PML in the type I IFN response was further confirmed by a recent report (Chen et al., 2015). Here, PML-II isoform specifically regulated the type I interferon response in multiple cell types. PML-II depletion by transient knockdown caused substantial reductions in IFN- β reporter gene activity and IFN- β mRNA expression measured by RT-qPCR, following stimulation with poly I:C. This effect was specific to PML-II as PML-V transient knockdown showed no such reduction. PML-II effect was mediated through both the IRF3 and NF- κ B regulatory elements of the IFN- β promoter and the major part of that effect seemed to be inside the nucleus since the activation and nuclear translocation of these previously-described cytoplasmic transcription factors was affected only slightly by PML-II removal. In the same way, expression of ISGs following IFN- α treatment was also specifically impaired by PML-II depletion. Poly I:C stimulation normally causes an association between IRF3 and NF- κ B with CBP recruitment to the IFN- β promoter which is known to be important for gene activity (Merika et al., 1998). This association was massively reduced when PML-II mRNA expression was transiently blocked in HEK293 cells. Furthermore, CBP-STAT1 binding was reduced by 62% compared to the control. Those results implicate PML-II in the stability of binding of different transcription factors to CBP. Using Chromatin immunoprecipitation, IRF3, NF- κ B and CBP recruitment to IFN- β promoter was greatly impaired when PML-II was depleted under stimulation conditions, and this depletion similarly reduced the binding of STAT to ISGs promoters which means that PML-II is necessary for the assembly of ISGF3 to ISRE promoters. Mutational analyses showed that PML-II regulates IFN- β expression through its unique C-terminal region as binding to both STAT1 and IFN- β was impaired by two mutants in that region (Chen et al., 2015).

1.11 Effect of PML proteins on general cellular translation

PML proteins show very specific binding to eIF4E, which is necessary for the translation of most of cellular mRNA and hence acts to repress translation. This binding was verified by specific pull-down of PML-RBB-GST which was also accompanied by conformational rearrangement upon binding to eIF4E. PML regulates eIF4E using a different binding site at the dorsal surface of eIF4E rather than the sites that are normally used by other regulators like eIF4G (Volpon et al., 2010).

Mutation of the PML-RING finger reduced the binding to eIF4E and also caused a substantial reduction in repression of cellular translation. This indicates that translation repression requires the fully intact RING domain of PML and its specific interaction with eIF4E since reduced translation did not result from the expected E3 ubiquitination activity of RING containing proteins like PML. PML binding to eIF4E reduced its interaction with the 5' cap structure of mRNA. Depending on fluorescence that was emitted from tryptophan, the fluorescence increased in dose-dependent manner with the increasing m⁷GpppG. Adding PML to the mixture reduced the eIF4E activity by two-fold by reducing the fluorescence of tryptophan. The reduction was similar to that caused by mutating eIF4E and it was rescued when wild type PML was replaced by site I mutated PML (Kentsis et al., 2001).

1.12 PML and Stress

Morphological changes have been detected in PML-NB when cells were exposed to various kinds of environmental and viral protein – induced stress. Heat shock, exposure to Cd²⁺ and Ad5 E1A protein expression all lead to the reversible formation of PML microstructures as PML bodies regained their original sizes after removal of stress (Eskiw et al., 2003). SUMO-1, a ubiquitin-like protein that is post-translationally attached to PML and other proteins in PML- NB, is a key regulator of PML body integrity; its over-expression prevents stress-induced disassembly of PML bodies (Eskiw et al., 2003). Despite the similarity in recovery time of PML-NB after

heat shock or sub-lethal concentrations of Cd^{2+} , each condition makes a different impact on proteins release from PML NB. Upon heat shock, Daxx and SP100 are released from the PML NB, while Cd^{2+} releases additional proteins from PML-NB including PML itself. This release could be blocked by inhibiting activation of p38 mitogen-activated protein (MAP) kinase or extracellular signal – regulated kinases (ERK1/2). In contrast, heat shock induced desumoylation of PML and release of other proteins and PML was unaffected by these inhibitors (Nefkens et al., 2003).

P53 is a central regulator of cell response to stress damage. PML III and IV interact directly through their C terminal regions with the RING finger of the Murine double minute (Mdm2) - a cellular negative regulator of p53 levels, during cellular stress and DNA damage. Over-expression of PML also recruits Mdm2 to the PML-NB following treatment with ultraviolet light, a DNA damaging treatment destruction of PML NB architecture and increased nucleoplasmic PML staining was also detected (Kurki et al., 2003). In response to double strand break (DSB) induction by ionising radiation or chemicals, PML- NB increased in number simultaneously with an increase in γ H2AX (histone protein) phosphorylation, a signature for a cellular DNA repair response. PML-NB numbers reached their peak coinciding with the highest levels of phosphorylated γ H2AX (Dellaire et al., 2006).

Oxidative stress also affects PML- NB. Upon oxidative stress, sumoylated Bach2 (a member of the BTB- basic region leucine zipper factor family) is recruited to PML bodies and forms nuclear foci associated with PML. Bach2 recruitment causes a repression in the transcription activity of PML (Tashiro et al., 2004). Antioxidants such as sulphoraphane particularly target the distribution of PML inside cells in order to regulate the transactivation function of the cellular transcription factor, nuclear factor erythroid 2- related factor (Nrf2). Nrf2 binds to antioxidant response elements (AREs) in promoters in order to promote the cyto-protective response against accumulated reactive oxygen species (ROS), which suggests a role for PML in controlling the anti-oxidative response through regulating Nrf2 activity (Sahin et al., 2014).

Finally, after transfection of plasmid DNAs in HeLa cells, the number of NB increased two- fold, whereas parallel treatment in HEK293 cells did not cause any increase in PML NB number as this cell line shows a deficient response to lethal and sub-lethal ionizing radiation doses. However, stress evoked by transfection preserves NB cargos of associated proteins like SP100, Daxx and SUMO because they still co-localise to NB. IL-6 and IL-8 treatment induced NB numbers, and in addition, IL-6 redistributed NB to the nucleoplasm (Laredj et al., 2008).

Upon UV irradiation, PML regulates apoptosis in a p53-independent manner. Furthermore, there is functional crosstalk between PML and c-jun. This was observed when a dominant negative mutant of c-jun caused a reduction in cell death upon UV radiation. This reduction was also significantly reduced in PML^{-/-} cells. PML- NB components re-localise in micro speckles upon UV-radiation in 60% of MEF cells in a p53 – independent manner. PML NB re-localisation may be triggered by different signals or posttranslational activation (Salomoni et al., 2005).

Non- irradiated 293-T cells showed co-localisation of PML and hcds/chk2 (DNA damage checkpoint kinase) in PML-NB and co-immunoprecipitation showed that there is interaction between Hcds1/chk2 and PML III. This interaction decreased after irradiation. Radiation did not affect the level of these two proteins and kinase activity was required for the separation of hcds1/chk2 from PML within the NB after DNA damage. Hcds1/chk2 phosphorylates PML at Ser 117 and this phosphorylation is important for apoptosis-induction after gamma irradiation (Yang et al., 2002).

In conclusion, different kinds of stresses cause the PML or PML-NB to respond in different ways. The response is often reflected in morphological changes in PML-NB appearance when they form micro speckles or an increase in their numbers and is associated with the release of PML protein partners or the recruitment of others also changes in their sumoylation. PML and PML-NB react to any abnormal condition that the cell experiences. This response might help the cells to overcome or to survive such conditions as moderate stress, and when the stress ends these nuclear structures go back their original morphology and structure.

Section III: Stress responses

1.13 Heat Shock Response

1.13.1 Mechanism of heat shock response

Stress responses are specific protective mechanisms that are elicited in the cell in response to abnormal conditions and are characterized by stress protein synthesis (Liang et al., 2011, Kultz, 2003). The nucleus undergoes dynamic changes upon exposure to abnormal conditions. A stress response includes different cellular activities incorporating re-localization of factors and induction of heat shock gene transcription. There is also cross-talk between different stresses. The purpose of stress response is to retain the normal function of cellular components and allow a return to uncton once the source of stress is removed (Cotto et al., 1997, Saydam et al., 2003).

Heat shock stress causes a global change in cellular activities in both the nucleus and the cytoplasm; the underlying reason for such global response is to help the cell to overcome this abnormal condition. When cells sense any elevation in temperature in their environment they start a highly organised and conservative heat shock response. In mammalian cells, there are three heat shock factors which trigger the heat shock response, hsf1, hsf2 and hsf4 with no very distinct role for the last two factors reviewed by Sonna et al., (2002). Heat shock increases the transcription of a set of heat shock family genes (Table 1.1), which are also induced by other kinds of stress. Heat shock factor 1 (hsf1) is the transcription factor which regulates the production of heat shock family proteins. Cells that are deprived of hsf1 are still able to produce the hsps constitutively but are not able to initiate a proper heat shock response. This steady state level of hsps, that is continually produced without any heat stimulus, cannot provide the cell with cyto-protection or thermo-tolerance, which suggests that hsps produced under heat shock show different functions than those produced under normal conditions (McMillan et al., 1998).

Table1.1. A list of the known human heat-shock family genes (Sonna et al., 2002)

Family	Gene Symbol	Gene Name	Chromosome Location
Small			
HSPs	HSPE1	Heat shock 10-kDa protein 1 (chaperonin 10)	2q33.1
	HSPB1	Heat shock 27-kDa protein 1	7q11.23
	HSPB2	Heat shock 27-kDa protein 2	11q22-q23
	HSPB3	Heat shock 27-kDa protein 3	5q11.2
	HSPB7	Heat shock 27-kDa protein 7 (cardiovascular)	1p36.23-p34.3
	HSPB9	Small heat shock protein B9	17q21.2
	CRYAB	Crystallin, alpha B	11q22.3-q23.1
HSP32	HMOX1	Heme oxygenase-1	22q13.1
HSP40	DNAJA1	DnaJ (Hsp40) homolog, subfamily A, member 1	9p13-p12
	DNAJA2	DnaJ (Hsp40) homolog, subfamily A, member 2	16q11.1-q11.2
	DNAJA3	DnaJ (Hsp40) homolog, subfamily A, member 3	16p13.3
	DNAJA4	DnaJ (Hsp40) homolog, subfamily A, member 4	15q24.1
	DNAJB1	DnaJ (Hsp40) homolog, subfamily B, member 1	19p13.2
	DNAJB2	DnaJ (Hsp40) homolog, subfamily B, member 2	2q32-q34
	DNAJB4	DnaJ (Hsp40) homolog, subfamily B, member 4	1p22.3
	DNAJB5	DnaJ (Hsp40) homolog, subfamily B, member 5	9p12
	DNAJB6	DnaJ (Hsp40) homolog, subfamily B, member 6	11q24.3
	DNAJB9	DnaJ (Hsp40) homolog, subfamily B, member 9	7q31
	DNAJB11	DnaJ (Hsp40) homolog, subfamily B, member 11	3q28
	DNAJB12	DnaJ (Hsp40) homolog, subfamily B, member 12	10q22.1
	DNAJC3	DnaJ (Hsp40) homolog, subfamily C, member 3	13q32
	DNAJC4	DnaJ (Hsp40) homolog, subfamily C, member 4	11q13

	DNAJC6	DnaJ (Hsp40) homolog, subfamily C, member 6	1pter-q31.3
	DNAJC7	DnaJ (Hsp40) homolog, subfamily C, member 7	17q11.2
	DNAJC8	DnaJ (Hsp40) homolog, subfamily C, member 8	1p35.3
HSP47	SERPINH1	Serine (or cysteine) proteinase inhibitor, clade H, member 1	11
	SERPINH2	Serine (or cysteine) proteinase inhibitor, clade H, member 2	11q13.5
HSP56	FKBP5	FK506-binding protein 5 (HSP56)	6p21.3-21.2
HSP60	HSPD1	Heat shock 60-kDa protein 1 (chaperonin)	12q13.2
HSP70	HSPA1A	Heat shock 70-kDa protein 1A	6p21.3
	HSPA1B	Heat shock 70-kDa protein 1B	6p21.3
	HSPA1L	Heat shock 70-kDa protein-like 1	6p21.3
	HSPA2	Heat shock 70-kDa protein 2	14q24.1
	HSPA3	Heat shock 70-kDa protein 3	21
	HSPA5	Heat shock 70-kDa protein 5 (glucose-regulated protein, 78-kDa; grp78; BiP)	9q33-q34.1
	HSPA6	Heat shock 70-kDa protein 6 (HSP70B')	1cen-qter
	HSPA7	Heat shock 70-kDa protein 7 (HSP70B)	1q23.1
	HSPA8	Heat shock 70-kDa protein 8 (HSC70)	11q23.3-q25
	HSPA9A	Heat shock 70-kDa protein 9A (mortalin-1)	Unknown
	HSPA9B	Heat shock 70-kDa protein 9B (mortalin-2)	5q31.1
HSP90	HSPCA	Heat shock 90-kDa protein 1, alpha	1q21.2-q22
	HSPCB	Heat shock 90-kDa protein 1, beta	6p12
	TRAP1	Heat shock protein 75	16p13.3
	HSP105A;		
HSP110	HSP105B	Heat shock 105-kDa protein	13q12.3
	APG-1	Heat shock 110-kDa protein; osmotic stress protein 94	4q28
	HSPA4; APG-2	Heat shock 70-kDa protein 4 (heat shock protein apg-2)	5q31.1-q31.2

Ubiquitins	UBB	Ubiquitin B	17p12-p11.2
	UBC	Ubiquitin C	12q24.3
	UBD	Di-ubiquitin	6p21.3
	UBF-fl	Ubiquitin UBF-fl	19q13.43
Other	HSPABP1; ST13	Suppression of tumorigenicity 13 (colon carcinoma); HSP70-interacting protein	22q13.2
	TOR1B	Torsin family 1, member B (torsin B)	9q34
	TRA1	Tumor rejection antigen 1 (gp96)	12q24.2-q24.3

Heat shock responses are graded in response to elevated temperature and have most often been studied in the context of the acute response to short term exposure of cells to temperatures that are not survivable in the long term. However, even exposure to mildly elevated temperature induces the response. HeLa cells heat shocked for 60 minutes at 42 °C showed consistent co-localization between hsf1-containing nuclear granules and SUMO-1 and a subset of those granules showed a significant co-localization with PML in PML-NB (Hong et al., 2001). In addition, it was found that hsf1 in heat shocked samples was modified by SUMO1 at lysine 298 which showed a strong homology with the modification site of PML by SUMO, which suggests a possible relationship between hsf1 and PML (Hong et al., 2001). SUMO-1 modified hsf2 was found to co-localize with PML in PML-NB and this interaction could be important to bring hsf2 close to other transcription factors that are important to its function to sequester it in order to regulation its function (Goodson et al., 2001).

1.13.2 Transcriptional effect of heat shock

Heat shock transcription activation affects the entire transcription machinery inside the stressed cells. This effect is represented by changes in the subcellular distribution of transcription factors or changes in their expression level or transcriptional activity (Sonna et al., 2002). The heat shock transcription response requires an interaction between the stimulated hsf1 and upstream heat shock elements (HSE) in target promoters. Whether or not this activation needs any protein synthesis is determined by the extent of the elevated temperature. When the cells are heat shocked to extremely high temperature, hsf1-directed transcription happens without any need for protein synthesis, while the protein synthesis becomes a necessity when cells are exposed to mild elevation in environmental temperature (Zimarino and Wu, 1987). In fact hsf-dependent transcription is controlled and repressed by certain chaperones such as hsp70 and hdj1. Both endogenous and co-expressed GAL4-hsf1 transcription activation was reduced by those two chaperones and this was at a transcriptional level as the phosphorylation of hsf1 and DNA-binding activity were not affected by hsp70 expression. Heat shock gene expression is auto-regulated by hsp70 in unstressed conditions due to the association of hsp70 with the hsf1-transactivation domain (Shi et al., 1998). Serine-hyperphosphorylated hsf1 is necessary for the transcription activation in the nucleus and this needs heat shock inducer to greater competent hsf1 (Cotto et al., 1996).

Within a few minutes post heat shock or any other physiological kind of stress, hsf1 which is normally found in a monomeric inactive state in the cytoplasm and nucleus at unstressed conditions, assembles into a trimeric state, acquires DNA binding activity and condenses in the nucleus (Fig1.5). Both immunofluorescence and fractionation protein analysis of cell fractionation showed clear localisation of hsf1 to the nucleus shortly after heat shock which coincides with phosphorylation of hsf1 and acquisition of DNA binding activity in different types of mammalian cells. Human-specific cellular distribution of hsf1 was verified in HeLa cells and several

other human cell lines. In HeLa, but not in mouse 3T3 cells, hsf1 is organised in distinctive bright speckles inside the nucleus shortly after heat shock (Sarge et al., 1993).

This leads to a rapid and highly regulated activation of the heat shock gene response via the interaction of hsf1 with the heat shock elements in the 5'-flanking sequence in heat shock responsive genes. Prolonged exposure to mild heat elevation might attenuate the heat shock transcriptional response by converting the hsf1 trimers to monomers and re-localising them to the cytoplasm, mediated by the increased level of hsp70 as discussed above. In addition, inhibitors of protein synthesis attenuate the heat shock response to the mild heat shock which suggests that the aggregation of folding intermediates or misfolded proteins could be a trigger for initiation of heat shock transcription activation (Morimoto, 1993).

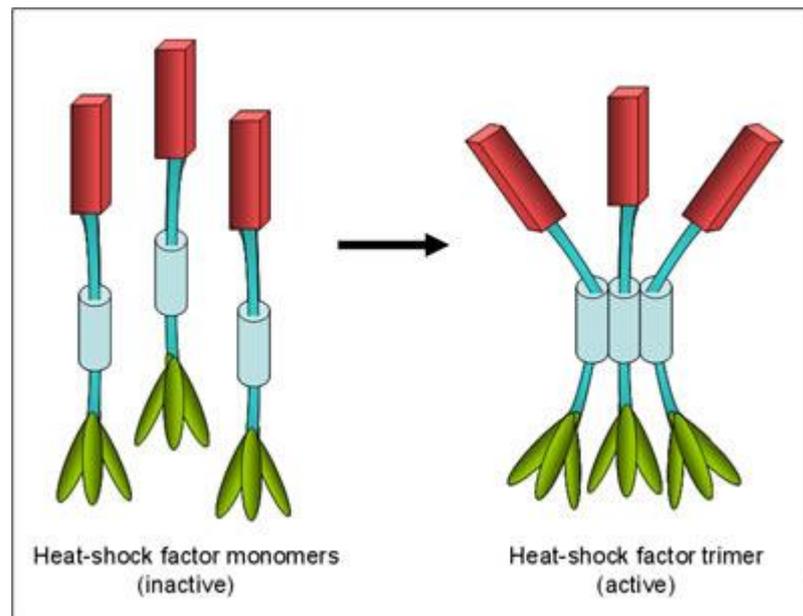


Fig.1.5. HSF1 trimerisation. The left structure shows the monomeric cytoplasmic form of hsf1 at attenuation. Upon heat shock the hsf1 trimerizes and moves to the nucleus to start the transcription of the global heat shock (Liou, 2010)

1.13.3 How do molecular chaperons help the cell to overcome stress?

Mechanisms that explain the protective effect of hsp proteins or what are called sometimes chaperones are still not well understood. Over a wide array of temperatures ranging from 22 to 41°C, hsp70 is one of the most dominant hsps; it can assist the cell under stress conditions by refolding the affected proteins to a native state in the presence of ATP. In contrast, hsp 90 has a different role that is summarized by saying that it keeps the non- native protein in a steady folding state. It has been noticed that unfolded β -galactosidase can recover its activity rapidly after different time periods of pre-incubation with hsp90 even in the absence of ATP (Freeman and Morimoto, 1996).

1.13.4 The protective effect of heat shock

Thermo tolerance, the state established in response to conditioning with heat stress, could have a protective effect against different kinds of stresses. In mitosis, pre-exposure to high temperature for a few minutes protects against heat-induced mitotic division error. The underlying reason for that is the conscription of hsp70, which is one of three HSPs that increase after thermo-tolerance is established, to the mitotic centrosomes (Hut et al., 2005). Stable expression of human hsp70 made rodent cells resistant to thermal stress even when its ATP binding site was deleted (Li et al., 1992). Pre-adapting HT22 mouse hippocampal cells by three days treatment with low concentrations of H₂O₂ protected them against the damaging effect of more extreme oxidative stress. hsp70 is implicated in this protection since this protection was abolished when the cells were treated with quercetin which is an hsp synthesis inhibitor (Khomenko et al., 2007).

Lipopolysaccharide (LPS) –induced apoptosis was mitigated by overexpression of hsp70 in human peritoneal mesothelial cell line (HMrSV5). There is evidence linking this cyto-protection effect of hsp70 with its ability to amplify LPS-stimulated autophagy (Li et al., 2011b, Li et al., 2011a). Another protective role of this protein has been revealed in transgenic mice +hsp72 (which constitutively express hsp72 from multiple organs such as brain, heart, liver, spleen, lung, kidney, skeletal

muscle, and tail). The pre-exposure of such mice to 40 °C for 60 minutes reduced heat-stroke induced hyperthermia, circulatory shock and cerebral ischaemic injury (Lee et al., 2006). Similarly, exposing *Caenorhabditis elegans* to a mild elevation in temperature protected them against heat stroke and heat induced necrosis. This protection was mainly achieved by hsf1 which induces several HSP genes including hsp16.1, hsp16.41 and DNJ-19 (hsp70) regulator (Kourtis et al., 2012).

1.13.5 Effect of heat shock on NF-kB responses

Exposing brain cells to 41°C or 20 minutes was sufficient to induce hsp70 to significant levels after 22 hours. Pre-treatment with mild heat shock reduced NOS2 (reactive oxygen product induced by NO synthetase2) positive staining in most of the brain areas of the treated animal by 90%. Reduced NOS2 staining was accompanied by reduced NF-kB levels through increasing the expression of Ikb- α protein (Heneka et al., 2000). Another route could be exploited by hsp70 to inhibit NF-kB activation through physical binding with TRAF-6, which facilitates the ubiquitination of the latter and results in inhibiting LPS-induced NF-kB activation (Chen et al., 2006). However, other heat shock family members show a completely opposite effect on NF-kB transcription activation. hsp25 or what is known as hspb1 increases the activation of NF-kB through promoting of the degradation of Ikb, which is accompanied by an increase in NF-kB-responsive gene products TNF- α and interleukin 1 beta (Yi et al., 2002). Heat shock protein 27 (Hsp27) regulates NF-kB mediated transcription since siRNA interfering with hsp27 mRNA caused a substantial reduction in and hence in nuclear NF-kB IKK activity (Guo et al., 2009).

1.14 Viral infection and heat shock response

According to the notion that viral infection is another kind of stress, there are many studies that have analysed the relationship between the response to infection and the induction of heat shock proteins. The yield of Influenza virus titre was significantly reduced at 8 hrs post infection when the cells were previously treated with Prostaglandin A PGA1 (which induces the level of hsp70). This is also observed *in vivo*; viral titres in lungs of infected mice treated with TAT-HSP70 (a form which

can enter cells) were substantially reduced in comparison with the controls. It is believed that HSP70 interferes with viral transcription and replication due to translocation of HSP70 from the cytoplasm to the nucleus in the infected cell which associates with the viral RNA and disrupts its binding to the viral RNA polymerase (Li et al., 2011a)

Another example of the inhibitory effect of heat shock on the outcome of viral infection is the blocking effect of accumulated HSPs (90,72 and 70KDa polypeptides) on late HSV gene expression. In human neuroblastoma IMR32 cells which were grown at 40°C, expression of ICP5 (a viral late polypeptide) was obstructed in contrast to the early-expressed genes that were not affected. A plausible explanation here could be a defect in switching from the synthesis of the early to the late polypeptides (Yura et al., 1987). Transfection of hsp70- vector plasmids in HeLa cells also inhibited the cellular arrest that is induced by HIV infection, (Iordanskiy et al., 2004).

On the other hand, some viruses may benefit from HSP expression. HSP40 and HSP70 knockdown decreased viral protein accumulation in hepatitis C virus HCV - infected cells. This suggests a potential role of these two HSPs proteins in translation levels mediated by the virus by the viral IREs. This was further confirmed when the cells were treated with quercetin which showed a significant reduction in virus production which was attributed in partial to reduction of HSP40 and HSP70 (Gonzalez et al., 2009).

1.14.1 Heat shock restricts virus infection in *Drosophila*

Infecting *Drosophila* S2 cells with *Drosophila* C virus (DCV) stimulates the stress response at 8 and 24 hours post infection. Six heat shock gene products were significantly up-regulated upon infection including Hsp70Abm Hsp70Ba, Hsp22, Hsp23, Hsp26, Hsp27. Transcription CHIP enrichment prediction showed substantial enrichment of the transcription factor HSF binding motif at the up-regulated gene promoters. Both RNA viruses like DCV and DNA viruses like DCV iridescent 6(IIV-6) virus caused increases in hsp70m, Hsp23 and Hsp26 8-48 hours post infection. Deficient replication of RNA and DNA viruses when the heat shock response was

impaired was verified by infection of S2 cells with UV-in activated virus. (Merkling et al., 2015).

1.14.2 Heat shock and adenovirus

Heat shock responses to adenovirus infection could be beneficial for viral replication. Some adenovirus proteins, like Gam1 from an avian adenovirus, trigger and re-localize of hsp40 and hsp70 upon infection to ensure a successful DNA replication. Deleting Gam1 in some adenovirus mutant causes impairment in adenovirus replication and this defect was rescued partially by re-expression of hsp40 (Glotzer et al., 2000). Ad5 infection alters the cellular transcriptional machinery by activating or repressing certain cellular factors in a way that enables the virus to establish a successful infection. One of the earliest discovered cellular proteins that is activated by Ad5 is hsp70, which is induced at the transcriptional level. HSP70 mRNA increased specifically with infection and in a regulated manner. Ad5 infection increased cytoplasmic hsp70 mRNA very significantly, with levels reaching about 100 times those before infection at 7.5 hours post infection. The increase declined after 13 hours post-infection. E1A protein played major role in increasing hsp70 mRNA level since the E1A-deleted virus (dl312) caused only a modest increase in hsp70 mRNA level similar to that for mock-infected cells and much less than the wild type (Kao and Nevins, 1983).

To summarize, stress responses are protective mechanisms which induce many drastic events like relocalization of some proteins. There is a cross – talk between different response mechanisms. These responses affect viral infection due to their interaction with the IFN- based immune response through a complex mechanism which is important in controlling IFN induction and also through a direct effects on virus replication. PML NB and PML proteins have pivotal roles in many cellular activities, like tumour suppression, apoptosis, DNA repair and antiviral response. Stress-induced disassembly of PML NB is a characteristic feature of the latter response to different kinds of stresses. This change is accompanied by release of many proteins (including the PML itself) and recruitment of others.

Disruption of PML NB by many DNA and RNA viruses and the fact that the PML proteins are up-regulated by IFN which is also mounted by the stress response motivated us to start this project. For the purpose of this project, the most suitable type of stress for study was heat shock. This was because heat shock is reversible and the recovery starts once the normal temperature returns. Cd²⁺ shock is also reversible but the time of recovery is indeterminate. The time-determined reversible changes due to heat shock were very important for our experiments since we wished to test the sensitivity of the surviving cells to virus infection.

1.15 Aim of Study

Adenovirus infection, PML and PML-NB, and heat shock are interconnected, with the cellular response to adenovirus infection and heat shock having many shared features. Both kinds of effectors, infection and heat stress greatly affect the cellular transcription machinery in order to enable the cell to survive and not to enter apoptosis. If the cell survives then the virus can take advantage of several cellular factors to finish the infectious cycle inside the affected cell and produce progeny that can infect the neighbouring ones. The heat shock response achieves different goals from assisting the cell to survive since it is for keeping cellular proteins in their native form, degrading the affected proteins to avoid any persistent damage to the cell or the whole tissue. PML partners for example Daxx either dissociated or recruited to the NB upon stress, the same proteins whose antiviral activity is antagonized by adenovirus E1b55k (Schreiner et al., 2010). PML or PML-NB and the heat shock response are well connected with the innate immune response, this is another shared feature between both types of stress, which suggests that they might have direct cooperation in triggering the antiviral immune response.

Aim 1: To investigate the effect of mild heat shock conditioning of HeLa cells on PML and PML-NB and on the outcome of adenovirus infection. This was evaluated qualitatively and quantitatively using different approaches in comparison with the control infected samples.

Aim 2: To investigate the role of PML and the specific isoform PML-II in changing the outcome of adenovirus infection in normal and stressed conditions, using a

persistent PML or PML-II knockdown system to be established using lentiviral expression of shRNA.

Chapter 2: Materials and Methods

2.1 Materials

All reagents, prepared or manufactured plasmids, cell types and competent cells that were utilised in this study are listed below:

Table

2.1 List of cell lines, their description and source

Cell type	Description	Source and suppliers
HEK293	Embryonic Kidney immortalized with adenovirus type 5- express E1A and E1B proteins	Supplied by Dr. Keith Leppard, University of Warwick (Graham et al., 1977)
HeLa	Human cervical cancer cells	provided by Prof. M.A. McCrae, University of Warwick
MRC5	Normal diploid human lung fibroblasts	
ATCC No. HB-8117 TM	Hybridoma cells produce anti-Ad5 hexon antibodies	Provided by Dr. Hans-Gerhard Burgert.
293T cells	HEK293 cells; transformed with SV40 to increase protein production	

Table 2.2 List of viruses

Virus	Description	Source and references
Wt300	Wild type Ad5 (Jones and Shenk, 1978)	(Jones and Shenk, 1978)
E4orf3	Implanting of E4 frame-shift (300 backbone)	(Huang and Hearing, 1989)

Table 2.3 Restriction enzymes

Restriction enzymes	Description
AgeI	Promega
EcoRI	Fermentas

Table 2.4 Bacterial cells

Competent cells	Description
DH5 α	<i>supE44, \Delta lacU169(\phi lacZ \Delta M15), hsdR17, recA1, gyrA96, thi-1, relA1</i>

Table 2.5 Plasmids

Plasmid	Description and sources
PLKO.1	Shuttling plasmid with ampicillin bacterial resistance and puromycin as selective marker for production of recombinant lentivirus, (with cloning site 5 AgeI and 3 EcoRI and a stuffer insert of 1900bp.(Addgene)
psPAX2	Packaging plasmids, contains a robust CAG promoter to express packaging proteins for PLKO.1(Addgene)
pMD2	Envelope plasmid for PLKO.1 packaging (Addgene)
PGEM-T	PGEM ^R -T Easy Vector Promega

Table 2.6 PLKO. Sequencing primers

Primers	Sequence	Source
PLKO.1 A	GACTATCATATGCTTACCGT	Addgene
PLKO.1 B	CTTTCCCCTGCACTGTACC	This study
PLKO.1 C	GCTATTATGTCTACTATTC	This study

Table 2.7 ShRNA constructs

shRNA functional products	Sense 5	Antisense 3	Description
PML-II	CATCCTGCCAGCTGCAAATT	TTTGCAGCTGGGCAGGATGTT	Specific sequence targets PML-II within exon 7b (Wright, 2010)
Exon3	GAGCTCAAGTGCACATCATT	TGATGTCGCACTTGAGCTCTT	Targets all PML isoforms (Wright, 2010)
Scrambled DNA	GAGCCGGACGCCAAAGAAATT	TTTCTTTGGCGTCCGGCTCTT	Control shRNA with no target effect of any human gene (Wright, 2010)
GFP	GCAAGCTGACCCTGAAGTTCAT	ATGAACTTCAGGGTCAGCTTGC	Dr. Kirsten Bentley

Table 2.8 siRNA

Target	Sense 5	Antisense 3	Description
HSP70	--	--	Targets HSPA1 and HSPB1 (Ambion)
IRF3	UGGAUGAGUUACUGGGUAATT	UUACCCAGUAACUCAUCCAGA	Targets IRF3, gene ID = 3661 (QIAGEN)
STAT1	GAAAGAGCUUGACAGUAAATT	UUUACUGUCAAGCUCUUUCTG	Targets STAT1 gene ID = 6772 (QIAGEN)
PML-II	CAUCCUGCCCAGCUGCAAUU	UUUGCAGCUGGGCAGGAUGUU	Targets exon 7b (Wright, 2010)
Scramble siRNA "A"	GAGCCCGGACGCCAAAGAAUU	UUUCUUUGGCGUCCGGCUCUU	Control siRNA with no target effect of any human gene (Wright, 2010)
Scramble siRNA "B"	ACGCGAAUAGCGAGCAAGCUU	GCUUGCUCGCUAUUCGCGUUU	(Chen, 2014)

Table 2.9 PCR and qPCR primers

Primer	Forward	Reverse	Description
genomic GAPDH	CCCCACACACATGCACTTACC	CCTAGTCCCAGGGCTTTGATT	(Krog et al., 2007)
PML-II	AGGCAGAGGAACGCGTTGT	GGCTCCATGCACGAGTTTTTC	(Chen, 2014)
PML Exon3	ACTGCCGAGGATGTTCCAAG	TCCTGTCGCTGCTGGATCTC	This study
IL-6	AAAGAGGCACTGGCAGAAAA	TTTACCAGGCAAGTCTCCT	(Chen, 2014)
ISG56	GCCATTTTCTTTGCTTCCCCTA	TGCCCTTTTGTAGCCTCCTTG	(Chen, 2014)
GAPDH	GGTCGGAGTCAACGGATTT	CCAGCATCGCCCCACTTG	(Vestergaard et al., 2011)
B-Actin	AAAGACCTGTACGCCAACAC	GTCATACTCCTGCTTGCTGAT	(Shi et al., 2010)
18S	ACGCTGAGCCAGTCAGTGTA	CTTAGAGGGACAAGTGGCG	(Menager et al., 2009)
HSP70	AGGCCAACAAAGATCACCATC	TCGTCCTCCGCTTGTACTT	(Tanaka et al., 2007)
HSPA1A	GCCGAGAAGGACGAGTTTGA	TCCGCTGATGATGGGGTTAC	www.rtpimerdb.org
HSPA1B	AGGCCAACAAAGATCACCATC	TCGTCCTCCGCTTTGTACTT	www.rtpimerdb.org
Hsp705	GGCCGCACGTGGAATG	ACCTCCAATATCAACTTGAATGTATGG	www.rtpimerdb.org
HSP706	GCCCTTCAGAGATGAACTTT	TGTTAAAGTGCCACACAAGT	www.rtpimerdb.org
HSP27	AAGCTAGCCACGCAGTCCAA	CGACTCGAAGGTGACTGGGA	(Wang et al., 2013)
Daxx	TCTACAACCTTTGGCTGTCACCTC	GTCTCTTCTGTCTCTCGCCCT	(Pan et al., 2013)
hexon	CGCTGGACATGACTTTTGGAG	GAACGGTGTGCGCAGGTA	(Schreiner et al., 2013)
E1A	GTGCCCCATTAAACCAGTTG	GGCGTTTACAGCTCAAGTCC	(Schreiner et al., 2013)

Table 2.10 primary antibodies

Antibody	Description	Dilution			Sources and suppliers
		Western blotting	Immunofluorescence	FACS	
Viral proteins					
Rat-anti E4Orf3 monoclonal	Detects E4Orf3 expression	1/500	--	--	(Nevels et al., 1999)
Mouse anti-E1B55k monoclonal	Detects E1B55K expression	1/250	--	--	(Sarnow et al., 1982b)
Mouse anti DBP	Detects Ad5 DNA binding protein	1/10000		1/5	(Reich et al., 1983)
Mouse anti-Hexon monoclonal antibodies	2Hx-2 IgG2a Adenovirus group specific antigen	--	No dilution	No dilution	Provided by Dr. Hans-Gerhard Burgert
Rabbit poly clonal anti-late (AdJLB1)	Detects all Ad5 late gene expression	1/10000	--	1/500	(Farley et al., 2004)
Cellular proteins					
Anti-Hsp70	Rabbit polyclonal	1/50000	1/300	--	StressMarq (SPC-103C/D)
anti- IRF3	Rabbit polyclonal	1/1000	--	--	Santa Cruz

					(SC-9082X)
Anti PML	Detect total PML (Rabbit polyclonal)	1/50000	1/500	--	(Xu et al., 2003)
Anti- PML-II	Detect PML-II(Rabbit anti-peptide)	1/50000	1/500	--	(Xu et al., 2003)
Anti- PML-V	Detect PML-V(Rabbit anti-peptide)	1/50000	--	--	(Xu et al., 2003)
Mouse anti- GAPDH	Mouse GAPDH(monoclonal Detect)	1/50000	--	--	MA5-15738
Anti-STAT1	Detects STAT1 (Rabbit polyclonal)	1/1000	--	--	Santa Cruz (SC-346)
Anti-Sp1	polyclonal Ads	1/1000	--	--	Santa Cruz (SC-59x)

Table 2.11 Secondary antibodies

Antibody	Description	Dilution			Supplier
		Western blotting	Immunofluorescence	FACS	
Goat anti-Mouse IgG	Horse Radish peroxidase conjugate	1/10000	--	--	Sigma
Goat anti- Rabbit IgG	Horse Radish peroxidase conjugate	1/20000 - 100000	--	--	Santa Cruz (SC-2054)
Goat anti Rat IgG	Horse Radish peroxidase conjugate	1/5000	--	--	Chemicon International
Goat anti- Rabbit IgG(H+L)	AlexaFluor 488(conjugate)	--	1/500	1/50	Life Technology
Goat anti- Rabbit IgG(H+L)	AlexaFluor 594 (conjugate)	--	1/500	--	Life Technology
Goat anti- Mouse IgG (H+L)	AlexaFluor 488(conjugate)	--	1/500	1/50	Life Technology
Donkey anti-Goat IgG	Horse Radish peroxidase conjugate	1/10K	--	--	--

2.12 A list of reagents

Reagent	Supplier
Dulbecco's modified Eagle's medium(DMEM 1X)	Gibco
Fetal bovine serum (FBS)	Sigma
Roswell Park Memorial Institute (RPMI)	Gibco
Minimum Essential Medium(MEM)	Gibco
Puromycin dihydrochloride	Sigma
QNZ, 6-Amino-4(-4-Phenoxyphenylethylamino)quinazolin	Santa Cruz
Recombinant Human Tumor necrosis factor (TNF- α) Lyophilised	Invitrogen
Interferon- α (IFN- α)	Pbl interferon source
Lipofectamine 2000 (LF2000)	Invitrogen
TransIT ^R LT1 transfection reagent (LT1)	Mirus
Dimethyl Sulfoxide(DMSO)	Sigma
GenElute TM Mammalian Total RNA Miniprep kit	Sigma
SV Total RNA Isolation System	Promega
GenElute TM Mammalian Total DNA Miniprep Kit	Sigma
GoScript TM Reverse Transcription System	Promega
QIAquick ^R PCR Purification kit	QIAGEN
QIAprep Spin Miniprep Kit Using a Microcentrifuge	QIAGEN
GeneJET Gel Extraction Kit	Thermo Scientific
Gene detecting primers	Sigma
Brilliant III Ultra-Fast SYBR Green qPCR Master Mix with Low Rox	Agilent
DNase treatment kit	Promega
100bp DNA Ladder	Promega
1kb DNA ladder	Promega
PageRuler Plus Prestained Protein Ladder	Thermo Scientific
Super Signal ^R West Femto Maximum Sensitivity Substrate	Thermo Scientific
Nitrocellulose Blotting Membrane	GE Health Care Amersham
Flow Cytometer Tubes	Elkay

2.2 Methods

2.2.1 Routine cell maintenance

Immortalised human tumour cell lines: human embryonic kidneys (HEK293), HEK293 transformed with T large antigen (293T) and human cervical cancer cells (Hela) were grown at 37 °C, 5% CO₂ in a humidified atmosphere in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% new born bovine or 10% fetal bovine serum (FBS). MRC5 cells were maintained in 10% FCS Eagle's Minimal Essential Medium (MEM) provided with 2mM L- glutamine and 1% non- essential amino acids. For routine work, they were seeded at a ratio of 1:5, 1:6 and 1:8 respectively. For experimental purposes cells were seeded at different densities according to the size of culture that was required. In general, 90 mm dishes were used for routine passaging (twice a week). Routine cell culture was achieved in an aseptic environment. At the time of confluence, cells were washed once with 3 ml versene (0.02% EDTA in PBS), then cells were incubated with 2.5 ml versene containing 0.02% (v/v) of 0.25 mg/ml trypsin and left for 3 minutes. The cells then collected by centrifugation and re-suspended in a suitable medium according to the ratios above. Transduced HeLa cells, were periodically "each 3 days" exposed to puromycin selection and for each experiment the cells were released for 3 days prior any kind of treatment.

2.2.1.1 Long term storage of cells

The cells were plated in the routine way, two days later the sub-confluent dishes were washed, trypsinised and cells collected. The cell pellet was re-suspended in 2 ml 90% (v/v) FBS and 10% (v/v) dimethylsulphoxide DMSO. The suspensions were distributed in small vials as 1 or 2 ml in each. The vials were kept in ice for 20 minutes then they transferred to -80°C freezers for 16 hours and finally relocated to the liquid nitrogen freezers. For 2-Hx2 hybridoma cells, they were frozen in 10% DMSO,30% FBS, 60% RPMI.

2.2.1.2 Making competent cells

DH5 α cells were grown overnight at 37 °C in 5 ml LB broth culture (no antibiotics), Two ml of that culture was used to inoculate a culture size of 100 ml LB broth. The cells were left to grow at 37 °C at 200 rpm shaking and samples were taken every 20 minutes to measure the optical density till it reached to 0.39 after about 2-3 hours. The cells were left on ice for 5 minutes, then a pellet of the bacterial cells was collected by centrifugation at 4 °C, 6000 rpm for 10 minutes in Eppendorf centrifuge 5810. The pellet was re-suspended in 2/5 volume of Transformation Buffer I (TFBI), which consists of 10 mM RbCl₂, 30 mM potassium acetate, 10 mM CaCl₂, 50 mM MnCl₂ 15% (v/v) glycerol, and left on ice for 5 minutes and then collected by centrifugation as before. Transformation Buffer II (TFBII);75 mM CaCl₂, 10 mM RbCl₂, 10 mM 3-N morpholinopropanesulfonic acid (MOPS) and 15 (v/v) glycerol with a pH of 6.5 was pre-chilled for 15 minutes in ice and 4 ml was added to the bacterial pellet and the mixture was incubated for 2 hours on ice. A 200 μ l aliquot of the cell suspension was added to each sterile Eppendorf tubes and moved to -80 C freezers for long term storage.

Transfection

Cells were plated at 1×10^5 /ml in 12- or 24-well plates 24 hours prior to liposome-mediated transfection. For plasmid DNA transfection each 1 μ g of DNA was prepared in 50 or 100 μ l (24- well or 12- well plate)of pre-warmed serum free OptiMEM. An equal volume of the same medium was prepared containing 2 μ l of LT1 transfection reagent. The DNA and LT1 were mixed and left for 20 minutes at room temperature. The transfection mixture was added to each well slowly and drop by drop covering all the surface area of the culture. The cells were incubated for 24 hours (24 or 48 hours for lentiviral production).

Each 125 pmol of siRNA was transfected with 1.2 μ l Lipofectamine2000 transfection reagent. The siRNA was prepared in 50 or 100 μ l of OptiMEM depending on the culture size (0.5 ml or 1 ml respectively), then mixed with the appropriate volume of LF2000 which was also prepared in an equal volume of OptiMEM for 20 minutes. The combination was dropped gently and evenly on cells that were then re-incubated again for 48 hours followed by different kinds of treatments.

2.2.1.3 Cell culture treatment

Cells were treated with 1000 U/ml with IFN- α for 24 hours prior infection. To stimulate NF- κ B activity, cells were treated with 100 ng/ml of TNF- α for 1 hour. In order to block the NF- κ B signalling pathway, cells were treated with 10 μ M N4-[2-(4-phenoxyphenyl)ethyl]-4,6-quinazolinediamine (QNZ) for 45 minutes. To study heat stress, cells were grown in 35 mm dishes and plated at 10⁶ cell/dish. After 24 hours cells were exposed to 40^o C and individual cultures harvested at 30 minute intervals. Then the temperature was shifted again to 37C and further samples were harvested.

2.2.2 Generation of PML shRNA cell lines

2.2.2.1 Generation of PML shRNA lentiviral constructs

PLKO.1TRC cloning vector (Addgene) was used to produce lentiviral particles following the Addgene protocol. ShRNA-coding sequences for insertion into the vector were purchased as pairs of complementary oligonucleotides each having the form: 5' CCGG- sense-CTCGAG- antisense-TTTTTG 3' (forward) with the sense and antisense elements of each oligonucleotide being as described; and 5' AATTCAAAAA- sense-CTCGAG- antisense 3' (reverse). The forward and reverse oligos were annealed to give dsDNA with overhangs compatible with AgeI and EcoRI-cut DNA by mixing 5 μ l of each with 5 μ l of 10x NEB buffer 2 and the volume completed to 50 μ l using ddH₂O. The mixture was incubated at 95^oC for 4 min at 70^o C for 10 min followed by a slow cooling to room temperature for 5 hrs.

PLKO.1 vector digestion was achieved using AgeI and EcoRI, 6 μ l of the vector digested firstly with AgeI, for 2 hrs at 37C purified using QIAquick PCR extraction kit, and then secondly digested with EcoRI in each case using manufacturer's recommendations. The double digested vector was then electrophoresed on a 0.8% agarose 1x TBE gel and purified using Thermo scientific gel extraction kit according to the manufacturer's instructions. Ligation was performed by mixing 60 ng of the double digested PLKO.1 TRC vector with either diluted or ten- fold diluted of the annealed shRNA oligos and 4 μ l of 5xNEB T4 DNA ligase buffer, 1 μ l of NEB T4 DNA

ligase was added and the volume was completed to 20 μ l with ddH₂O. The mixture was incubated overnight at 16 °C. Two microliters of each ligation mixture were mixed with 25 μ l of competent DH5- α *E. coli*, plated out on LB agar containing 100 μ g/ml ampicillin and incubated for 16 hrs at 34 °C. The selected colonies were grown in 5 ml of LB broth containing 100 μ g/ml ampicillin for 16hrs at 37 °C. The plasmids were purified using QIAprep Spin MiniprepKit and sent for sequencing at GATC Biotech using PLKO.1 TRC vector primers. Another pair of reverse primers was designed to overcome the difficulty in sequencing through the secondary structure of the shRNA-encoding DNA hairpin.

2.2.2.2 Preparing Lentiviral particles

HEK-293T cells were counted and seeded at a density of 7×10^5 in 3ml in 60mm dishes overnight. Growing cells were transfected for 15 hrs with 1 μ g of each PLKO.1 sh plasmid (Addgene) mixed with 750ng of psPAX2 packaging plasmid (Addgene) and 250 ng of pMD2 envelope plasmid (Addgene) usingTransi LT-1 (Mirus) in a ratio of 2 μ l of LT-1 to 1 μ g of DNA, 80 μ l of LT-1 in OPTI-MEM was added to the 20 μ l plasmid mixture, incubated for 20 minutes at room temperature and then added to HEK-293T cells gently and dispersed evenly throughout the plate. The plates were incubated for 15 hrs. The transfection reagents were removed and replaced with fresh DMEM with 10% FBS and left at 37 °C for 24 hours. The medium was harvested as a lentivirus stock, stored at 4 °C and new fresh medium was added to the same plates and the cells were left for another 24 hours before harvesting the medium again. Both harvests were mixed and centrifuged at 1250 rpm for 5 min to remove any cells from the lentiviral suspension. For long term storage the lentivirus was kept at -20 °C.

2.2.2.3 Lentiviral transfection and selection

Prior to attempts to transduce cells, the intended target cells (HeLa) were tested for their puromycin sensitivity, cells were plated out at a density of 1×10^5 /ml in 35 mm dishes and incubated for 24 hrs at 37 °C. Puromycin was prepared in a wide range of concentrations (1 μ g/ml to 10 μ g/ml) and each concentration was added to a different plate and the level of killing was monitored every day. For transduction

HeLa cells were plated at a density of 4×10^5 / ml in a 6- well plate, 24 hrs later cells were infected with 0.1, 0.5 and 1ml of the prepared lentiviral particles and incubated at 37°C for 24 hrs. The medium was replaced with DMEM plus 10% FBS containing the pre-determined concentration of puromycin. Death was monitored every day. Efficiency of knockdown in puromycin-resistant cell population was analysed by RT-qPCR, western blotting and confocal imaging at 3, 9 and 14 days post transduction.

2.2.3 RNA and DNA analyses

2.2.3.1 DNA isolation

Mammalian DNA samples were extracted by a column based purification technique which was provided by Sigma-Aldrich GenElute™ Mammalian Genomic DNA Miniprep Kit (G1N70). DNA samples were extracted by following the manufacturer's instructions and the eluted DNA was stored at -20°C. Viral and cellular gene expression and viral DNA copy number were quantified by RT-qPCR assay using the specific primers for each target gene.

2.2.3.2 RNA extraction

Acquiring the total cellular RNA in all experiments relied on using the purification columns kits provided by Sigma-Aldrich (GenElute™ Mammalian RNA Miniprep Kit (RTN70) or Promega (SV Total RNA Isolation System (z3100)). Purification steps were accomplished according to the manufacturer's instructions; the purified eluted RNA was immediately DNase-treated and converted to the complementary DNA by reverse transcription as described below.

2.2.3.3 Routine DNaseI treatment

Using the Promega reagent kit, a 10 µl reaction was prepared by mixing 200ng of the extracted RNA with 1 µl of DNaseI and 1 µl of DNase buffer and the volume adjusted to 10 µl with sterile water. The reaction was incubated for 30 minutes at 37°C and then stopped by adding 1 µl of DNase stop solution and incubated for 10 minutes at 65°C.

2.2.3.4 Reverse transcription reaction

The DNase treated RNA samples were reverse transcribed to complementary DNA using GoScript™ reverse transcription System A5000. Mixed oligo-dT and random hexamers were used as primers for first strand cDNA synthesis according to the manufacturer's protocol. The thermal cycle of the reverse transcription was as follow:

Step	Temperature	Period of time (minutes)
Annealing	25°C	5
Extension	42°C	60
Reverse transcriptase Inactivation	70°C	15

2.2.3.5 Real Time Quantitative Polymerase Chain reaction

The reaction was prepared containing 0.1 µl of 20µM Forward and Reverse primers (Table 2.9), 10 µl of SYBR Green reaction mix and 0.3 µl ROX (1:500 dilution) or SYBR green pre-mixed with low Rox as a reference dye(Agilent). For DNA copy number analysis 1 ng and 5ng of total DNA were used per reaction for Ad5 and GAPDH plasmids respectively. For early and late viral and cellular gene expression, cDNA samples were uses as templates at concentrations of 5 to 10 ng/ well. All qPCR experiments were performed on a Stratagene MX3005P light cycler (Agilent Technologies) using the following cycling conditions: 95 °C for 3 mins, then cycles of 1 min at 95°C, 30 sec at 55°C and 30 sec at 95°C. Cycle threshold and baseline values were determined using the machine software and all PCR products were subjected to dissociation curve analysis to ensure primer specificity. Viral and cellular gene expression were calculated as Δ Cts by subtracting house-keeping gene Ct values while viral DNA copy number was quantified from standard curves of viral genome and GAPDH plasmids, the latter to quantify cellular DNA as an internal control (see below).

2.2.3.6 Construction of GAPDH DNA plasmid

HeLa cells were used as a source of genomic GAPDH DNA for PCR amplification. Genomic GAPDH was amplified using GAPDH genomic specific primers to give a 97 bp product. The reaction was performed using an Eppendorf Master Cycler Gradient Thermo-cycler and Taq DNA polymerase. The concentration of the template was adjusted to 50 ng/μl and mixed with 1 μl of 20mM of forward and reverse primers and dNTPs. MgCl₂ was added at a concentration of 50mM. The volume was completed to 50 μl. The reaction was set as follow: 94°C for 3.5 minutes, 30 sec at 60°C and 7 minutes at 70°C. The amplified gene was then decontaminated by QIAquick PCR purification kit and according to the manufacturer's directions. The PCR product was ligated to pGEMT using 2X rapid ligation buffer (PGEM^R-T Easy Vector Promega) at a ratio of 3:1 insert to vector in a total volume of 10 μl, according to the manufacturer's instructions. Half of this was used to transform 100 μl of *E. coli* competent DH5α cells as described in section 2 and plated on. XGAL/IPTG LB ampicillin plates prepared by mixing 40 μl of 20mg/ml XGAL with 40 μl of 100mM IPTG and spreading the mixture on the surface of the sterile LB-amp agar plates. Potential insert-containing colonies (white) were grown in 5 ml of LB broth containing 100μg/ml ampicillin for 16hrs at 37C. The plasmids were purified using QIAprep Spin Miniprep Kit and sent to GATC Biotech to determine correct DNA sequence.

2.2.4 Qualitative and quantitative assays of proteins

2.2.4.1 Cells extraction and Western-blotting

Total cell extracts were made by lysing cell monolayers in 1x sample buffer (1x SB) which was prepared from a stock of Laemmli sample buffer2x (4 ml of 10% SDS, 2 ml of 20% glycerol, 1.2 ml of 1 M Tris-HCl pH 6.8 2.8 ml of H₂O and 0.02% w/v of bromophenol blue). Samples were boiled for 10 minutes and loaded onto 8%, 10% or 12% polyacrylamide gels and electrophoresed at 70 V for 3.5 hours. Proteins were then transferred to nitrocellulose membrane (10 cim ×7 cim) at 0.35 A for 1.5 h (or 0.08 A overnight) in a cold room. After blocking for 1h at room temperature or overnight at 4°C with 2% blocking reagent in PBS/0.05% Tween 20 (PBS-T),

membranes were incubated with the appropriate antibodies (Table 2.10) for 1 h. Membranes were washed 7 times for 13 minutes with PBS-T, then incubated with the appropriate secondary antibodies (Table 2.11). Finally, the reaction was detected by ECLT^M Advance reagent according to the manufacturer's instructions by exposure to Fuji Super RX X-Ray film. Different loading controls were used in this study. Depending on the molecular mass of the protein (GAPDH, β -actin or α -tubulin) the loading control was either acquired by cutting the lower part of the same membrane or loading the same samples on a different gel.

2.2.4.2 Western blotting stripping

Nitrocellulose membranes which were previously probed to evaluate the expression of certain genes were used again to display expression of other genes by stripping and re-probing. The membranes were stripped with 50 ml reagent consisting of 2% SDS, 62.5 mM Tris-HCl pH 6.8 and the last component is 350 μ l of β -mercaptoethanol, all those contents were mixed together and the volume was adjusted to 50 ml by distilled water. The solution was pre-warmed for 10 minutes at 50 °C and then added to the membranes and warmed again for 15 minutes at 50 °C. The membranes were washed 3 times for 15 minutes with PBS-T, then re-blocked and probed using the same steps of membrane blotting as the first time.

2.2.4.3 Cellular fractionation

To prepare separate cytoplasmic and nuclear extracts, cells were washed once with pre-chilled PBS, scraped and collected in 1.5 ml microfuge tubes by centrifugation at 1300 rpm for 3 minutes. Cells were lysed using lysis buffer (10mM NaCl, 1.5 mM MgCl₂, 10 mM Tris-HCl pH 7.5, 0.67% NP40). The suspension was left in ice for 10 minutes and followed by separating the nuclei from cytoplasm by centrifugation at 1500 rpm for 3 minutes at 4 °C; cytoplasm (supernatant) and nucleus (pellet) samples then were mixed with 1xSB.

2.2.4.4 Confocal Immunofluorescence

Cells were plated at 2×10^5 /well or 1×10^5 / well culture in 12- well containing sterile coverslips to analyse the outcome of infection and effect of knockdown experiments respectively. At the end of the experiments, cells were fixed with 10% formalin and permeabilised with 0.5% NP40 in PBS with washing after each step. Blocking was achieved by 1% bovine serum albumin (BSA) in PBS for 45 minutes. After 3 washes with PBS, appropriate dilutions primary antibodies (Table 2.10) were added and left for 45 minutes. After further washes, the primary antibodies were replaced with diluted 250 μ l of Alexafluor 488 or 594-labelled secondary antibodies (Table 2.11) for 45 minutes. Coverslips were washed twice with PBS. Cells were washed for a third time with PBS included 1 μ g/ml DAPI for 5 minutes. Coverslips were mounted with Vectasheld mounting medium. The images were taken as single optical section by a Leica SP2 or Leica SP5 system and Leica software.

2.2.4.5 Fluorescence- Activated Cell Sorting (FACS analysis)

HeLa Cells were seeded at a density of 0.8×10^6 or 2.2×10^6 per dish in 60 and 100 mm^2 dishes respectively; 24 hours later cells were heat shocked or not and then infected with Ad5 wt300 at a multiplicity of infection (m.o.i) of 5 or 10 fluorescence foci units (f.f.u.) per cell. Cells were then washed once with pre-warmed PBS. The cells were then trypsinized, re-suspended in 10 ml DMEM 10% FBS, then pelleted by centrifugation at 1280 rpm for 7 minutes, supernatant was removed to leave a final volume of 0.5 ml, 8 ml of PBS was added and the cells were gently re-suspended. Then the cells were pelleted by centrifugation for 7 min at 1280 rpm and re-suspended in 450 μ l or 810 μ l of PBS for 60 mm^2 and 100 mm^2 dishes respectively. Cells were fixed by adding by formaldehyde to give a final concentration of 4% and left to incubate at room temperature for 20 minutes with vortexing each 5 mins, then cells were incubated with 600/900 μ l of 50 mM NH_4Cl for 30 minutes at room temperature. PBS (500 or 900 μ l) was then added to the tubes followed by centrifugation for 3 min at 3000 rpm. The pelleted cells were

permeabilized with 500 or 900 μ l of 0.5% NP40 for 10 minutes, then were centrifuged 3 minutes at 3000 rpm and blocked with 1% BSA in PBS for 45 minutes.

To analyse for specific antigens, they were re-suspended to a concentration of 1.67×10^7 / ml in FACS Buffer (PBS containing 3% FBS and 0.07% NaN_3). Anti- Hexon monoclonal, anti-late polyclonal and DBP monoclonal antibodies (Table 2.10) were used in a 96- well plates; 100 μ l of Abs were mixed with 30 μ l of the fixed cells and left for 45 minutes at 4 °C. Cells were then removed by centrifugation of the plate and washed twice with FACS buffer. The process was then repeated with appropriate AlexaFluor 488- labelled secondary antibodies (Table 2.11). Cells were recovered by plate centrifugation and washed three times with FACS buffer.

2.2.4.6 FACS Analysis

Stained cells were re-suspended in 100 μ l of FACS buffer and transferred to FACS tubes containing 300 μ l of FACS buffer. The suspension was mixed by pipetting and the readings were acquired by FACSCAN (BECTON DICKINSON) and data were analysed by WinMDI software.

2.2.5 Virus titration

Ad5 wt300 was titered by counting DBP a fluorescence-positive cells as Fluorescent Focus Assay (FFA). HeLa cells were plated at 1×10^5 / well in 24- well plates for 24 hours. The virus to be titred was prepared in serial ten-fold dilution (10^{-1} to 10^{-10}) in serum-free DMEM free serum. The medium was removed from the cells in each well and 50 μ l of each dilution was added to the cells in duplicate. Cells were incubated with the virus for 1 hour, then the virus was discarded and the cells were covered again with a fresh DMEM 10% FBS and incubated for 16 hours. Cells were then fixed and stained with anti-DBP antibodies and AlexaFluor 4888 secondary antibody as for confocal microscopy. The DBP foci were counted using UV-microscopy.

Chapter 3: Characterising PML Kd and PML-II Kd cells

3.1 Introduction

PML and PML-NB display an antiviral function against wide range of viral infections, for example decreased human foamy virus (HFV) and rabies gene expression and replication have been found following PML and PML-IV overexpression in different cell lines (Regad, 2001a, Blondel et al., 2010). Also permanent removal of PML results in increased replication and gene expression in HSV and HCMV infections (Everett et al., 2006, Tavalai et al., 2006). In a broader aspect, this function has been verified even in immune cells when HIV proviruses were reactivated in about 90% of latently infected primary CD4⁺ T- cells with stable shRNA PML knockdown (Lusic et al., 2013). In addition, PML has been described as an enhancer of HPV mRNA transcription (Day et al., 2004). This project is concerned with the role of PML and PML-II in Ad5 infection, for which there is already evidence to correlate viral infection and PML proteins. Adenovirus infection causes PML reorganisation into track-like structures and this has been particularly attributed to a direct interaction between Ad5 E4Orf3 protein and the PML-II isoforms (Carvalho et al., 1995, Hoppe et al., 2006). In addition, the viral E1A oncoprotein co-localises to PML through its conserved CR2 amino acid domain that is homologous to sequences found in other oncogenic viral proteins (Carvalho et al., 1995).

It seems that Adenovirus interaction with PML and in particular PML-II is of a distinctive importance and the latter in particular has been suggested to play a decisive role. Hence, there are good reasons to investigate the role of PML and PML-II in the adenovirus life cycle. To facilitate this, a permanent PML knockdown system was established as described in this Chapter. This would generate consistent conditions among different experiments. Other important reasons for establishing this system include the ease and cheapness of achieving quantitative analyses in cell populations using FACS assays and the saving of time from avoiding the 48 hour incubation for the transient knockdown. The technique that was employed to meet the requirements for permanent knockdown was shRNA that targets the gene of interest, delivered using lentiviral vectors to infect and select the target cells.

3.2 Isolation and characterisation of PML- Knockdown cells

The early attempts that were achieved in this work to transiently knock down total PML or the PML-II isoform specifically created many variations among the individual experiments, especially when applying heat stress (chapter 5). The ultimate aim was to quantify the outcome of infection in the presence and absence of PML or PML-II at both the normal temperature and after mild heat shock. Hence, growing large size cultures which show consistent knockdown throughout the culture was critical to achieve this and transient knockdown was not appropriate.

The shRNA interfering technique was the robust approach chosen to meet the above goals. This technique is characterised by the ability to transduce both dividing and non- dividing cells, and express the designed shRNA by stable integration of the sequence into the cellular genome, based on puromycin resistance which allows selection to differentiate transduced from non- transduced cells (Naldini et al., 1996, Zufferey et al., 1997). The shuttling plasmid expresses the cloned shRNA from an RNA polymerase III (POL III) promoter derived from the U6 gene and terminates with the Pol III signal of a 5T repeat sequence cloned at the U6 5' end (Stewart et al., 2003).

The shRNAs which were made in this study were designed to mimic siRNA templates that were designed by Wright (2010). It is well known that the PML gene is an alternatively spliced human gene; it consists of 9 exons which encode for different nuclear and cytoplasmic PML protein isoforms (PML I to PML VII) (Jensen, 2001). Total PML mRNA was targeted by a 21 nucleotide fragment which hybridises to exon3 RNA, common to all human PML mRNAs (Fig 3.1). PML-II mRNA expression was interfered by a siRNA fragment which uniquely targeted exon 7b (Fig 3.1). The efficiency of those siRNAs has already been verified by (Wright, 2010)) proving their ability to deplete total and PML-II transcripts.

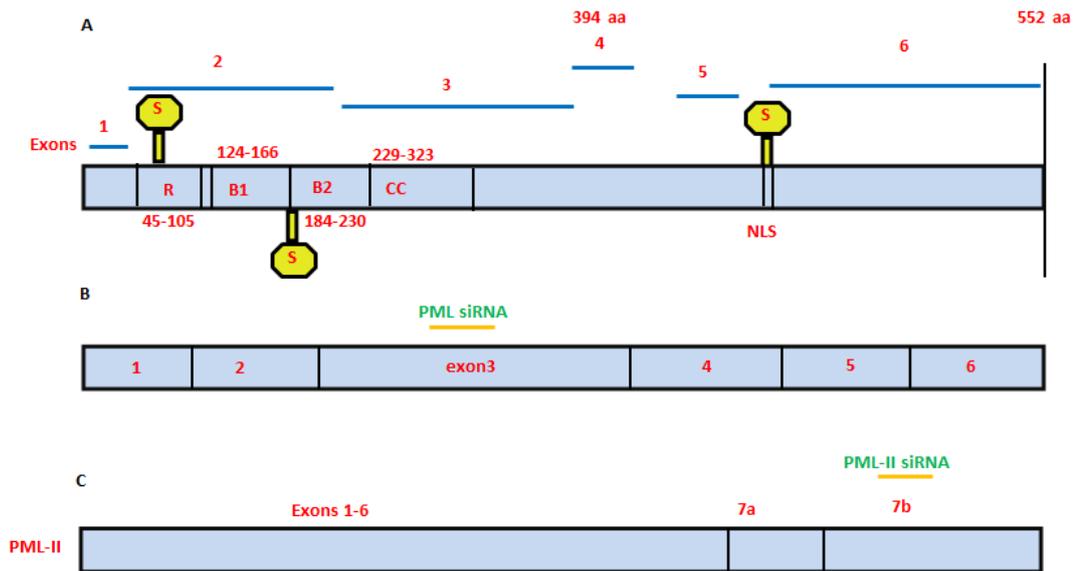


Fig.3.1. A schematic diagram illustrates PML gene structure and targets of siRNA interference. A. The PML gene cartoon which shows (1-6) shared exons among PML I to PML V with recognised protein elements RING (R), B-boxes (B1,B2) and coiled coil (CC) known collectively as the RBCC motif. Nuclear localisation signal (NLS), SUMO modification site (S). **B.** Exon3 siRNA sketch targets all PML mRNA. **C.** PML-II siRNA that was designed to target exon 7b, the unique identifier of PML-II mRNA.

The plan was to permanently knock down PML or PML II using lentiviral transduction of shRNA constructs and to explore the outcome of infection in HeLa cells that lack PML or PML-II compared to HeLa cells in control and stressed conditions. The system in general includes ligating the shRNA constructs as cDNAs with the plasmid vector (PLKO.1). The control constructs were either scrambled (Ctrl.DNA –shRNA) which do not interfere with any human sequences, verified using Ambion siRNA target finder (Wright, 2010), shRNA for GFP constructs or PLKO.1 empty vector. Then, the recombinant PMLKO TRC.1 was transfected with the second generation packaging vector psPAX2 and the second generation envelope vector pMD2.G, encoding the vesicular stomatitis virus (VSV) G protein, into 293T cell to produce the lentiviral particles and these particles were used to infect target HeLa cells.

HeLa cells were chosen to be the target of the lentiviral transduction for two main reasons. Firstly, those cells are amenable for human adenovirus type 5 infection.

Secondly, HeLa cells show a good infectivity level using HIV lentiviral particles; they display a 60% transduction efficiency when they are challenged with those vectors at MOI of 1 (Ikeda et al., 2002). The physical and functional knockdown in these cells was investigated at both RNA and protein levels using RT-qPCR, WB and IF.

3.2.1 Constructing the shRNA- PLKO.1 plasmids

The U6 human promoter which is transcribed by RNA polymerase III is a feature of the cloning vector PLKO.1 (Kunkel et al., 1986). This short small nuclear RNA promoter in all kinds of human cells provides substantial expression of short hairpin RNA transcripts which consist of 2x 19 nucleotides with an intermediate loop and 4U overhangs at the antisense strands (Paul et al., 2002). Another important feature of this promoter which facilitates the transcription of short hairpin small interfering RNA sequences is the lack of the non- coding DNA sequences or what is called intragenic sequences, which is unusual for the promoters that are transcribed by Pol III. This characteristic feature is crucial for the purpose of transcribing the desired gene as the promoter will drive expressing of the shRNA rather than its own intragenic sequences (Das et al., 1988, Kunkel and Pederson, 1989). U6- driven-shRNAs exhibit substantial and specific knockdown of the target gene, in addition to which when they are delivered into cells they form duplexes and are thus well protected from intracellular nucleases (Miyagishi and Taira, 2002).

3.2.2 Digestion of PLKO.1 TRC cloning vector and construction of shRNA – PLKO.1 recombinant vectors

The two restriction enzyme sites *AgeI* and *EcoRI* were the target sites where the shuttle vector PLKO.1 TRC was double digested. The puromycin resistance property allows a positive selection for the transduced cells (Fig.3.2.A). The constructs were ordered with CCGG at the sense end and TTTTGG at the antisense end, with a short sequence (CTCGAG) separating the sense and anti-sense elements (Fig.32.B).

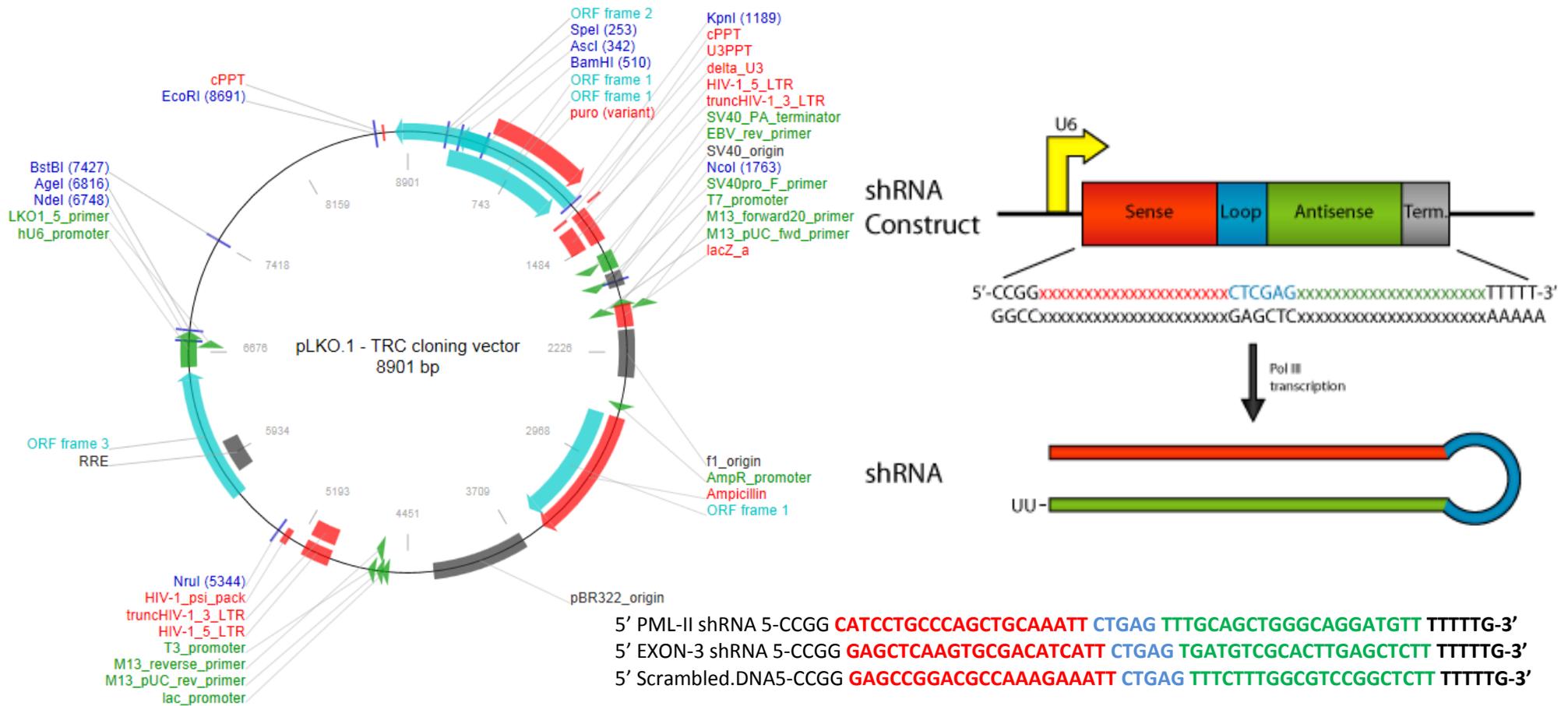


Fig.3.2 PLKO.1 TRC vector diagram and sequence – definite knockdown of PML-II, EXON3 and scrambled DNA. A. Cartoon presentation of PLKO.1 TRC cloning vector with its genetic properties. Those characters are mainly; the human U6 promoter to operate the shRNA transcription via RNA polymerase III; the Amp and Puro resistance genes to allow successful selection in bacterial cells and mammalian cells correspondingly. B. Upper diagram : the stem loop which incorporates the specific sense and anti-sense RNA . The lower panel : the knockdown- specific sequences; top, PML-II shRNA; middle, total PML, bottom, control DNA sequence (Wright 2010)

Following Agel digestion the product was extracted using PCR extraction kit then was digested again with EcoRI (Fig.3.3.A). The second digestion was to remove the stuffer and leaving the blunt ends which would facilitates ligating the shRNA constructs into the blunt ends. Two bands were visualised by UV light, the small and large bands were very well distinguished from each other and from the undigested plasmid which appeared as one big smear. The larger one (7kb) was removed and then was ligated with annealed oligonucleotides and used to transform DH5 α *E. coli*. Positive clones containing PML-II shRNA, exon-3 shRNA and Ctrl. Potentially positive shRNA plasmids were identified by size in agarose gel (about 9 kb) and confirmed by DNA sequence using PLKO.1 TRC vector primers (Fig.3.3.B and C).

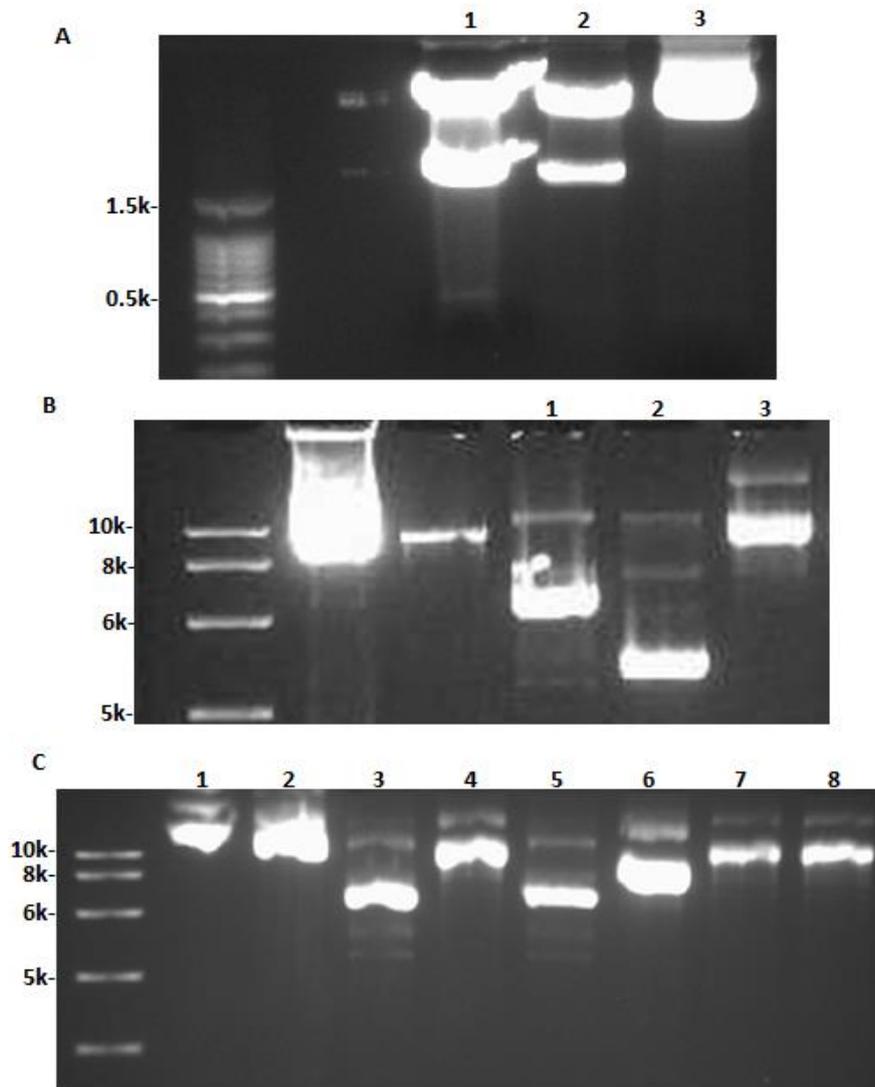


Fig.3.3. Agarose gel electrophoresis of the intact, double digested and recombinant PLKO.1 cloning vector. **A.** lanes 1 and 2 are the linearized PLKO.1 two separate double digestion reactions with *AgeI* and *EcoRI*, lane 3: the whole circular PLKO.1 plasmid, markers indicated are 1.5 and 0.5 kb. **B.** From left to right: 1kb ladder, the undigested PLKO.1, the large segment of double digested PLKO.1, 1 and 2 positive selected clones and lane 3 is the PML-II shRNA clone. **C.** From left to right: 1 kb ladder; 1 and 2 uncut plasmid; 3, 4, 5 and 6 are different positive selected clones, 7 and 8 are Exon3 and Ctrl. shRNA clones respectively.

3.2.3 Characterising Exon 3 stable knockdown cells

In order to determine the concentration of puromycin that would give effective selection, standard HeLa cells were plated and exposed to an increasing dose of puromycin (1 to 10 $\mu\text{g/ml}$), the percentage of killing was monitored each 24 hours. The minimal killing concentration was 3 $\mu\text{g/ml}$ as it was the dose that killed the cells within 2 days. This concentration was adopted as the standard to differentiate the lentiviral infected (transduced) cells from the un-transduced cells for all the upcoming experiments. Samples were collected at 3, 9 and 14 days after transduction with PML, PML-II and Ctrl shRNAs to analyse the efficiency of knockdown by RT-qPCR and western blotting. The 3 day analysis didn't show any physical or functional sign of PML removal as the mRNA and protein accumulation was equal in the exon3 and the Ctrl. DNA transduced cells (data not shown). However, quantitative RNA analysis by RT-qPCR after 9 days of selection showed more or less 80 % reduction in the total PML mRNA level (Fig.3.4A). Another control cell line was constructed by transducing the target HeLa cells with an shRNA targeting an irrelevant gene (GFP) and the knockdown effect analysis was repeated with the newly selected cells and at the same time points after selection, when the knockdown started to emerge. PML expression was knocked down to about 50% in PML Kd cells compared to the new control cells (Fig 3.4. B).

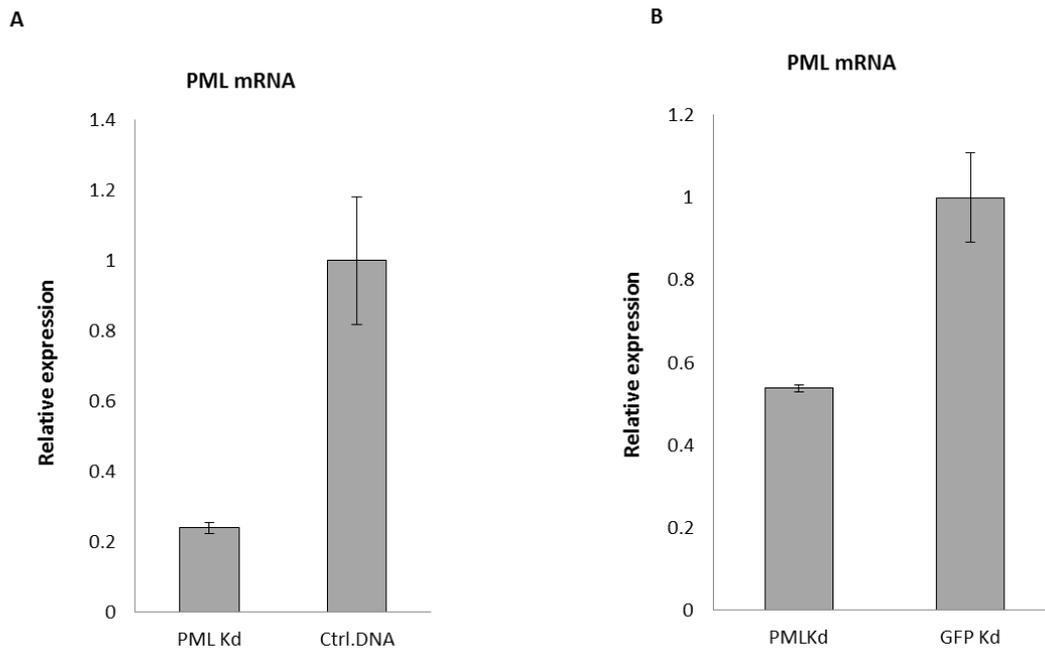


Fig.3.4. Characterising the physical removal of PML RNA in PML Kd cells in comparison with Ctrl.DNA and GFP Kd control cells by RTqPCR. PML Kd, Ctrl.DNA and GFP Kd cells were seeded at a density of 1×10^5 /ml for 24 hours. RNA samples were extracted from each cell type, total PML mRNA was quantified by RT-qPCR using 10 ng/well of complementary analysed DNA (cDNA) as a template. Data were calculated by the $\Delta\Delta C_t$ method, using GAPDH as internal control, and then normalised to the control cell value. All PCR products were subjected to dissociation curve analysis to ensure primer specificity. **A.** Total PML depletion in PML Kd in comparison with Ctrl.DNA control cells. **B.** Total PML removal in PML Kd in comparison to GFP Kd control cells.

3.2.3.1 PML depletion affects the NF-kB signalling pathway

Treating cells with TNF- α can induce the NF-kB signalling pathway and IL-6 mRNA expression (Kurokouchi et al., 1998). That means NF-kB induction by TNF- α treatment could induce IL-6 expression and other cytokines in the cells, thus removing total PML would decrease the expression of IL-6 which is one of the functional effects of removing total PML proteins. To analyse the functional effect of PML removal on the response to TNF- α treatment, PMLKd and Ctrl.DNA cells were treated with 100 ng/ ml of TNF- α for 1 hour, then the effects of PML removal was determined by comparing the level of IL-6 by RT-qPCR. As expected the level of IL-6 mRNA was reduced significantly in PML Kd in comparison with Ctrl.DNA (Fig 3.5.A)

The level of NF- κ B response has been also measured in comparison with a different control cell line, GFP Kd. This control cell line was made by using the same lentiviral transduction approach to express an shRNA targeting GFP. There was a drastic decrease in IL-6 mRNA expression in PML Kd cells at both stimulated and unstimulated levels (Fig.3.5.B).

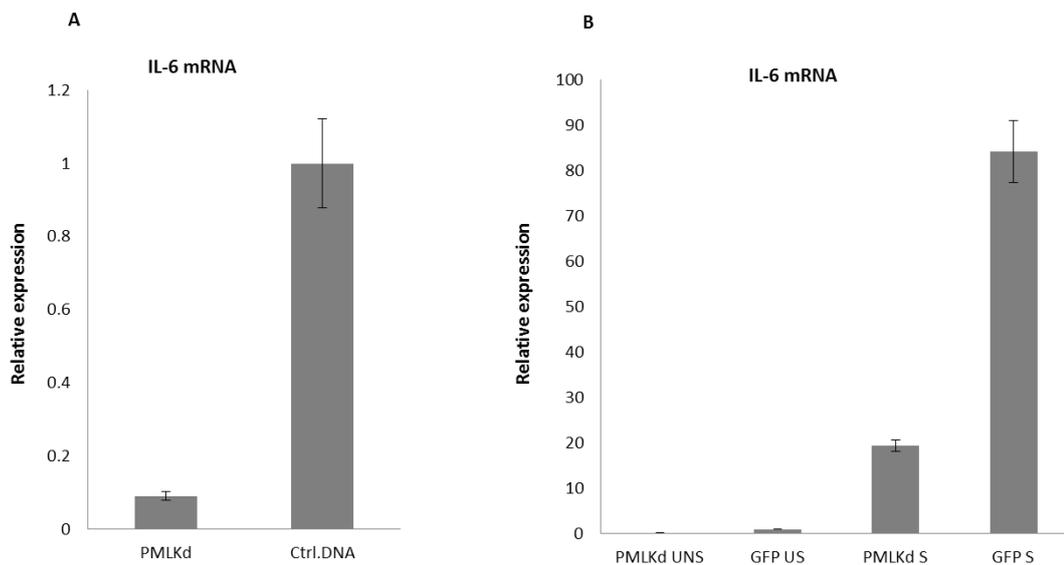


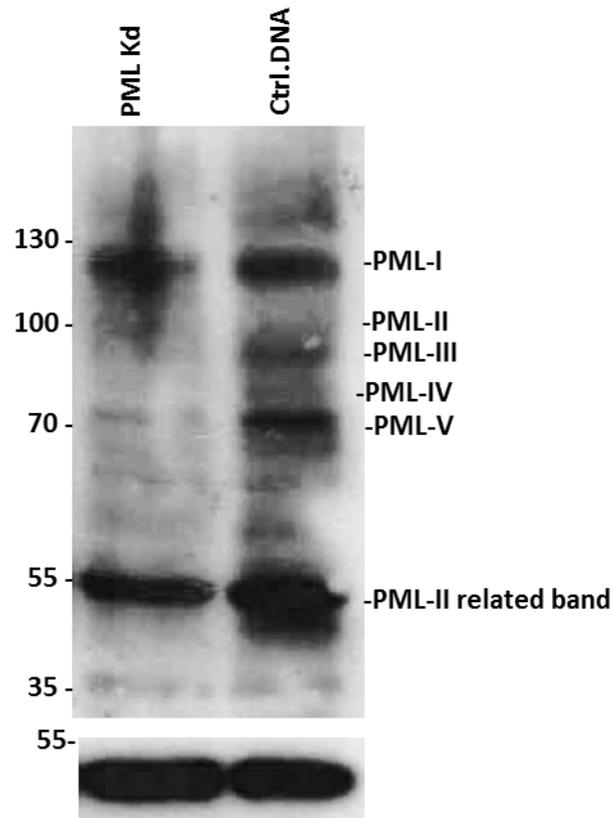
Fig.3.5. Characterising the functional effect of removal of PML on NF- κ B. PML Kd , Ctrl.DNA and GFP Kd cells were seeded at a density of 1×10^5 /ml for 24 hours. Prior to harvesting RNA samples, cells were treated with 100ng /ml of TNF- α for 1 hour . RNA samples were extracted from each cell type, total IL-6 mRNA was quantified by RT-qPCR using 10 ng/well of complementary DNA (cDNA) as a template. Data were analysed by the $\Delta\Delta$ Ct method, using β -actin as the internal control. All PCR products were subjected to dissociation curve analysis to ensure primer specificity **A.** IL-6 mRNA level in PML Kd in comparison with Ctrl.DNA control cells; the values were normalised to the control cells. **B.** IL-6 mRNA level in PML Kd in comparison to GFP Kd control cells; values were normalised to the unstimulated GFPKd cells value.

3.2.3.2 Total PML protein expression was affected by exon3 permanent knockdown

At the protein level and to further confirm the removal of total PML proteins, two weeks post puromycin selection PML Kd and Ctrl.DNA cells were either lysed for WB or stained to visualise the PML by IF techniques. WB analysis showed a level of reduction in expression of all PML-related bands, however this was not dramatic (Fig.3.6.A). The knockdown affected all the PML isoforms to some extent. The effect was weakest on the major PML band (PMLI/II) which is around 130 kDa and increased in strength with the lower molecular PML isoforms, including smallest one (around 55 kDa), which is believed to be a PML-II related band. This could be expected as the 130 kDa bands are the most abundant ones and they are expressed higher level than the rest of the PML isoforms. PML-I in particular is the most abundant and conserved isoform which might be essential for PML's various functions in the cell (Condemine et al., 2006). This is consistent with a study by (Chang et al., 2013), who showed a significant level of total PML knockdown using two different siRNAs when they normalised their data to the loading control. However one siRNA showed efficient knockdown though a significant level of detectable PML expression. The other possible reason here is the single target shRNA within exon 3 region was used rather than a pool of sequences, this could possibly give less efficient binding. Many companies recommend using more than one target within the same gene sequence to PML mRNA ensure an effective reduction in the target gene mRNA (Smith, 2006). Levels of RNA and proteins in the cells were previously considered as reflective of each other. However, recently using proteomic and next generation sequencing techniques, it has been found that there can be a big difference in the ratio of RNA and protein levels inside the cells. One reason for that is the half- life of mRNA and protein may be totally different as the latter is typically 5 times more stable than the RNA. Although there is a high correlation between mRNA and protein levels, on average there are approximate 2800 times more protein copies than its corresponding transcripts. The average rate of translation in mammalian cells also exceeds the rate of mRNA synthesis. It has been found that a protein is translated at a rate of 140 per mRNA per hour while mRNA is transcribed in a rate of two molecules per hour in cells

(Schwanhausser et al., 2013). However, confocal imaging of a population of PML Kd and Ctrl.DNA cells showed a relatively efficient decrease and diffuse staining of PML proteins inside the nucleus (Fig.3.6.B). The number of PML –NB was apparently greater, brighter and well distinguished in the Ctrl.DNA cell compared to the PML Kd cells.

A



B

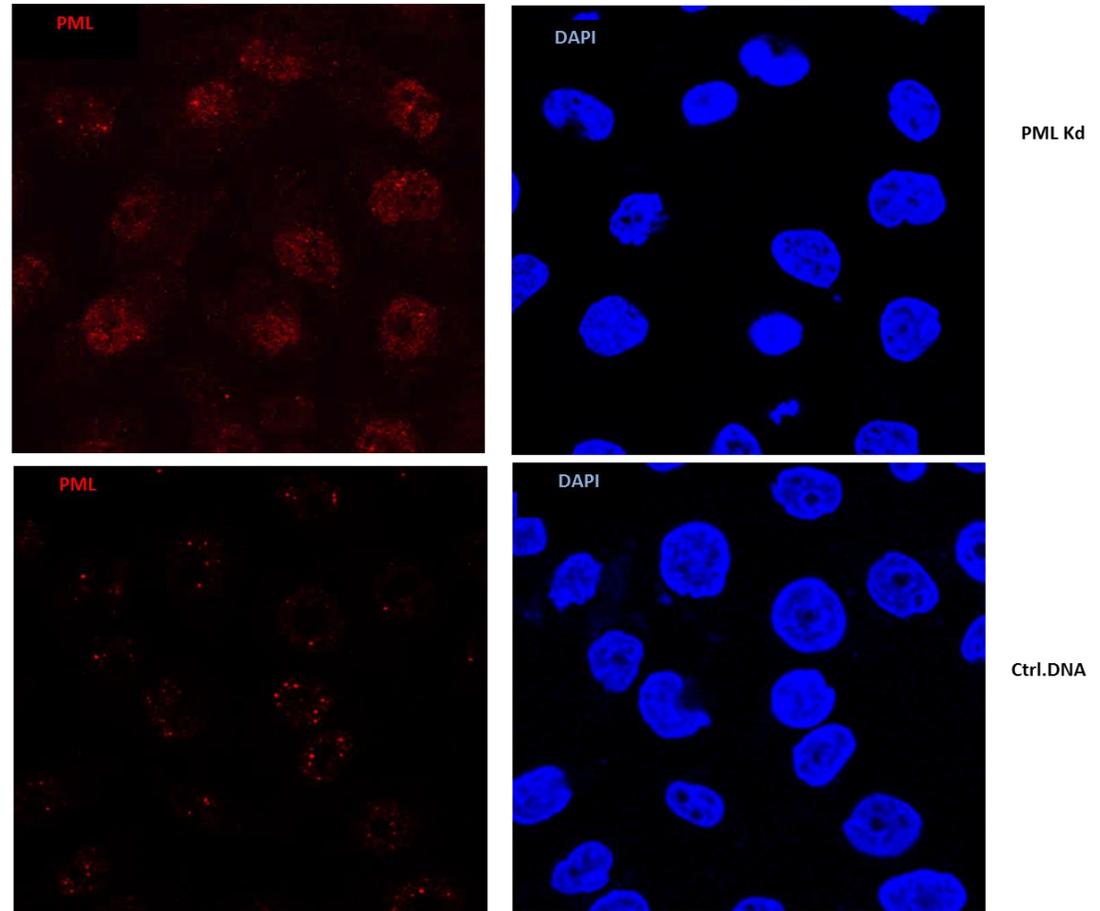


Fig 3.6. Characterising the removal of PML at the protein level. A. PML Kd and Ctrl.DNA cells were seeded at a density of 1×10^5 /ml for 24 hours. Protein samples were collected by 1xSB, total PML proteins were probed using specific anti-PML antisera. The protein sizes were indicated relying on migrating known- molecular sizes protein (kDa) on the left. B. PML Kd and Ctrl.DNA cells were grown at a density of 2.5×10^5 /ml for 24 hours, fixed with 10 % formaldehyde and then stained with anti-PML polyclonal antibodies (primary) and detected with Alexafluor 594 goat anti-rabbit IgG (H+L) (red). Nuclei (blue) were visualised with DAPI. Images were taken by confocal immunofluorescence microscopy; those shown are representative of around 8 photos taken for each field.

3.2.3 Characterising stable PML-II knockdown cells

In parallel with the creation of PML Kd cells, experiments were also done to generate and to characterise the depletion of PML-II from HeLa cells. Cells were infected with shPML-II lentiviral particles for 24 hour and then subjected to puromycin selection. The same selective concentration of puromycin was applied to select PML-II Kd cells. The analyses of effectiveness of knockdown were accomplished at the same time interval (3, 9 and 14 days) post selection when samples were collected for both RNA and protein analysis. The best RNA physical and functional knockdown was again at 9 days while the protein effect was detected at 14 days post selection. PML-II expression level showed an approximately 60% decrease in comparison with its level in Ctrl.DNA cells (Fig.3.7.A). This level of decline in PML-II expression was very consistent with the results that were obtained from the transient knockdown in human lung fibroblast cells (MRC5), using the same sequence of shRNA as used in siRNA for transient knockdown to interfere with PML-II expression (Fig.3.7.B). This constancy in level of knockdown between two different cell lines validates the knockdown effect in the newly constructed PML-II Kd cells. As a consequence of that reduction in PML-II expression, the NF- κ B activity was diminished to about 10% of its normal level. This was evaluated by measuring IL-6 after stimulating the cells with 100ng/ml TNF- α for 1 hour (Fig.3.7C).

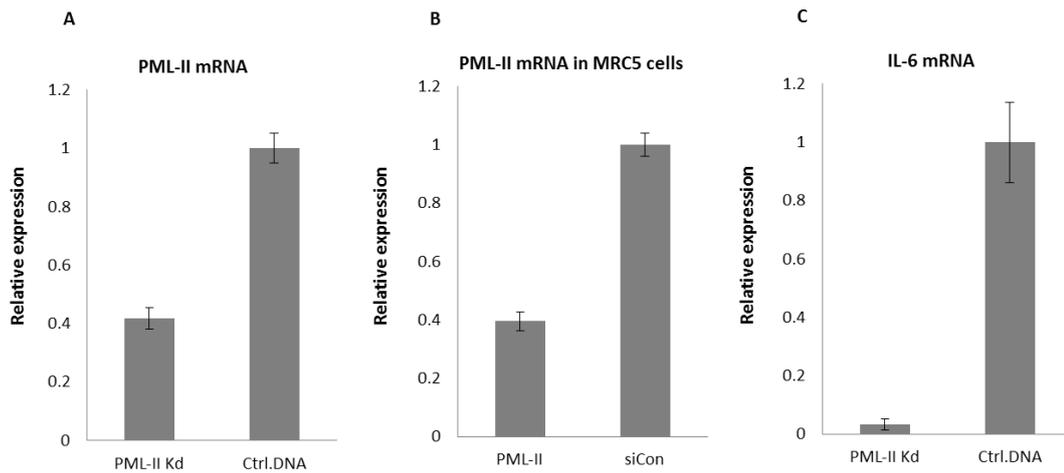


Fig.3.7. Characterising the physical and functional effect of PML-II removal quantitatively by Real Time qPCR. PML-II kd, Ctrl.DNA and MRC5 cells were seeded at a density of 1×10^5 /ml for 24 hours. In characterising the knockdown effect on NF-kB signalling pathway, prior to harvest RNA samples, cells were treated with 100ng /ml of TNF- α for 1 hour to quantify IL-6 mRNA. RNA samples were extracted from each cell type, total PML-II and IL-6 mRNA were quantified by RT-qPCR using 10 ng/well of complementary DNA (cDNA) as a template. Data were presented by the $\Delta\Delta C_t$ method and then normalised to the control cell value. All PCR products were subjected to dissociation curve analysis to ensure primer specificity. **A.** PML-II mRNA level in PML-II Kd in comparison with Ctrl.DNA control cells, data were normalised to GAPDH. **B.** Transient knockdown in MRC5 cells, PML-II level in MRC cells in comparison to siC, data were normalised to 18S RNA as internal control. **C.** IL-6 mRNA expression in PML-II Kd and Ctrl.DNA and results were normalised to GAPDH.

To further confirm the RNA quantitation data, PML-II protein expression in PML-II Kd cells was measured by western blotting and IF techniques. Consistent with the RNA data there was an obvious reduction in a PML-II related protein band by Western blotting (Fig.3.8.A) and the appearance of bright stained nuclear PML- foci, detected by PML-II specific antibody, was also reduced (Fig.3.8.B).

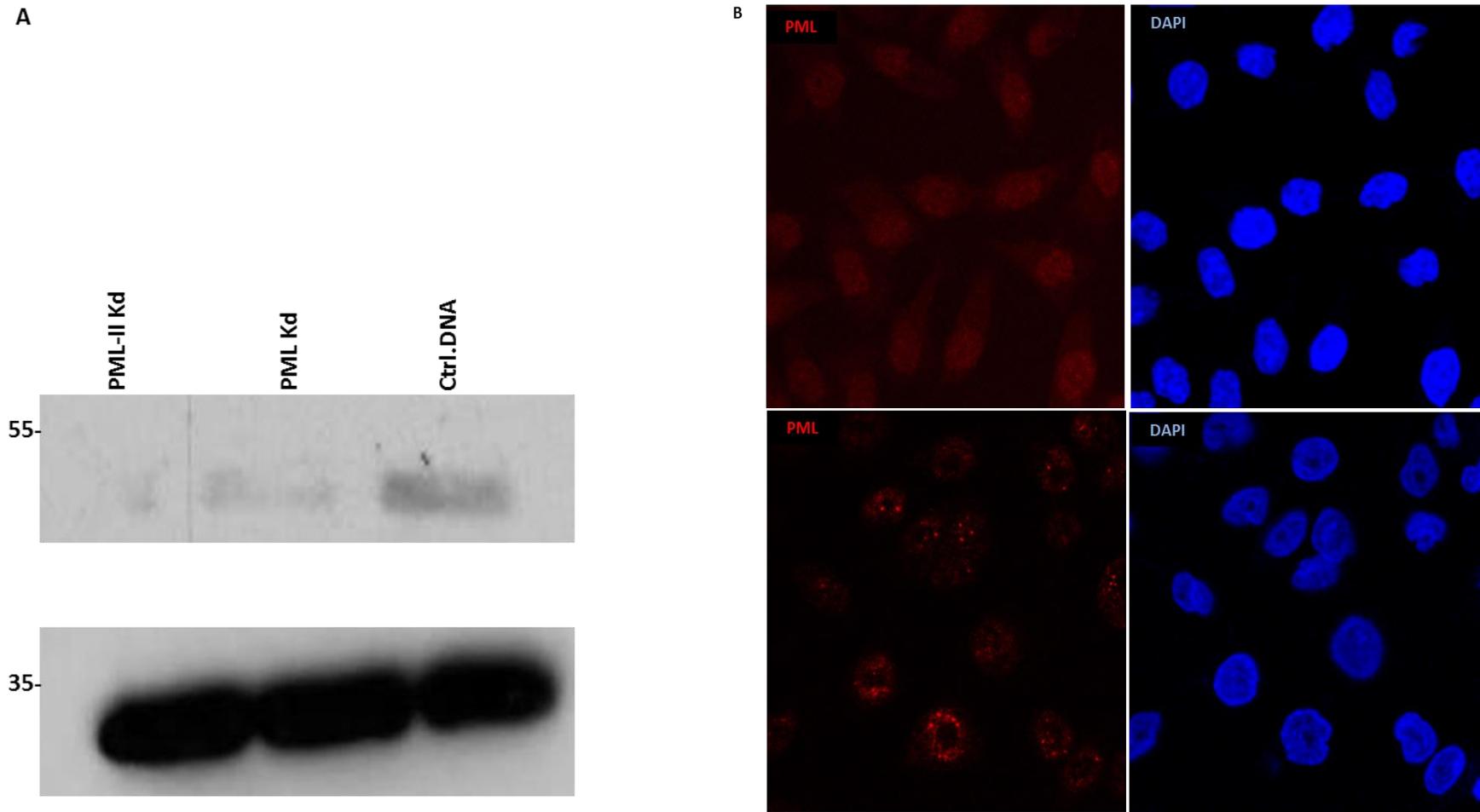


Fig 3.8. Characterising PML-II removal at the protein level. **A.** PML-II Kd and Ctrl.DNA cells were seeded at a density of 1×10^5 /ml for 24 hours. **A.** Total protein extracts were prepared and analysed by Western blotting using specific anti-PML peptide antisera. The positions to which proteins of known molecular mass migrated are indicated on the left (kDa). **B.** PML Kd and Ctrl.DNA” cells were grown at a density of 2.5×10^5 for 24 hours and then processed for immunofluorescence using anti-PML polyclonal antibodies and Alexafluor 594 goat anti-rabbit IgG (H+L) (red). Nuclei (blue) were visualised with DAPI. Images were taken by confocal immunofluorescence microscopy an average of 8 photos were taken for each field.

3.2.3.1 Effect of PML-II depletion on type I interferon

It is well known that PML is an IFN- up-regulated gene. Type I and type II IFN treatment increases the mRNA and protein level of PML in HeLa S3 cells after 2 hours of treatment. This augmentation has been detected at the transcriptional level and is regulated by the transcription factor ISGF3 (Grotzinger et al., 1996). Actually this transcriptional connection between IFN and PML expression is of special importance in achieving the antiviral response that is exhibited by PML. PML physically fixes the HFV-trans-activator in a way that blocks the latter from binding with the viral DNA, here reducing viral replication. A similar decrease in HFV replication could be obtained only by treating the cells with IFN but not in PML-null cells, which emphasizes the role of PML in the IFN antiviral function (Regad, 2001b). Very recent data have established a role for PML proteins in IFN transcription. PML- null human fibroblast cells show down-regulated ISGs expression when pre-treated with IFN- β or UV attenuated HCMV (Kim and Ahn, 2015). Among PML isoforms, PML-II has a role in the reduced transcription in a type I interferon response. It was displayed that this isoform particularly participated in the active binding of NF-kB, STAT1 and CBP with IRF3 to the IFN- β promoter (Chen et al., 2015). Hence PML-II Kd cells should show a decrease in ISG56 expression and in order to verify this, both PML-II kd and Ctrl.DNA cells were plated for 24 hours and stimulated with 1 μ g of Poly I:C to increase the level of interferon in both types of cells. As expected, the level of ISG56 mRNA, one of the Interferon stimulated genes (ISGs), was reduced to about 50% in PML-II Kd cells in comparison with the control cells (Fig.3.9).

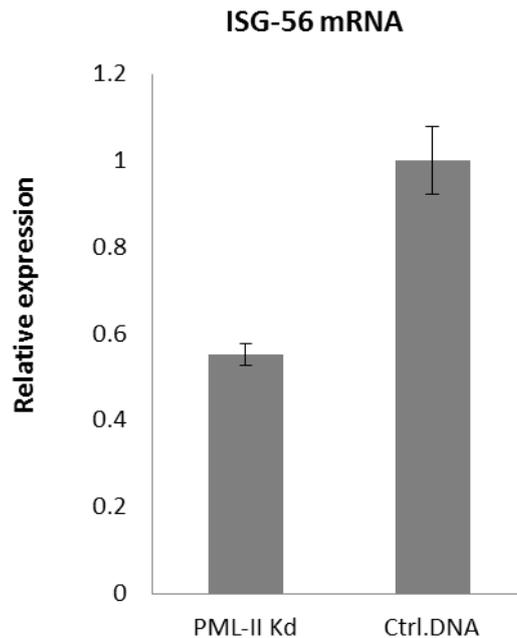


Fig.3.9. ISG56 mRNA level in PML-II Kd and Ctrl.DNA cells. PML-II Kd and Ctrl.DNA cells were seeded at a density of 1×10^5 /ml for 24 hours. Cells were treated with $1 \mu\text{g/ml}$ of Poly I:C for 16 hours. RNA samples were extracted from each cell type, total ISG mRNA was quantified by RT-qPCR using 10 ng/well of complementary DNA (cDNA) as a template. Data were presented by the $\Delta\Delta\text{Ct}$ method and then normalised to the control cell value. All PCR products were subjected to dissociation curve analysis to ensure primer specificity. Results were normalised to GAPDH as an internal control.

To summarize, and from all RNA and protein analyses that were accomplished, PML Kd and PML-II Kd cells exhibited a good level of physical and functional knockdown of the target genes. This was the base to start in the next part of work by investigating the sensitivity of these cells to be infected by adenovirus.

3.3 Assessing Ad5 wt300 infection in the presence and absence of PML

A key objective was to study the effect of PML depletion on Ad5 infection. All the cells (PML Kd, PML-II kd and Ctrl.DNA) were plated out, infected with wt300 for 20 hours with multiplicity of infection of 5 and analysed for the outcome of the infection. Unexpectedly, since this moi is sufficient to infect standard HeLa cells efficiently, the Ctrl.DNA cells showed no sign of infection while the other two cell types showed an acceptable level of infection. Hexon, which is the main late structural viral protein, was completely absent from the Ctrl.DNA sample while it was expressed well in PML-II Kd cells and to a lesser extent in PML Kd cells (Fig.3.10.A). On the other hand GAPDH, which was the loading control in this

experiment, was expressed from all samples indicating that the deficiency in expression was particular to the viral proteins not because of an error in loading process or less protein in this particular harvest. This situation suggested increasing the moi to 50 to boost the level of infection and in fact this high dosage did infect the cells to a level that could be acceptable to compare the outcome of the infection among cells (data not shown). However, this high level of infection could also interfere with the accuracy of the analyses as many phenomena could disappear or be altered with the high dose of particles that the cell would receive. The overall conclusion from this was that the Ctrl.DNA cells were not amenable to be the control cell line with which to compare the outcome of infection.

After the discovery of gene silencing by siRNA or shRNA there were many publications that pointed to siRNA or shRNA interference with different cellular pathways and in particular the innate immune response. Some siRNAs and gene-target shRNAs could increase the innate immune response to similar levels as the stimulation with poly I:C does. It has been proved in certain kinds of cells like porcine fetal fibroblasts that the scrambled siRNA and shRNA increased the expression of IFN- β and 2'-5'-oligoadenylate synthetase 1 (OAS1) to about 10 to 15 times more in quantitative PCR assay respectively (Stewart et al., 2003). To investigate the possibility of Ctrl.DNA cell having a high level innate immune response they were stimulated with 1 μ g/ml of Poly I:C for 16 hours in comparison with standard HeLa cells then the level of ISG-56 was quantified by RT-qPCR. It was obvious that the innate immune response was nearly twice higher in Ctrl.DNA cells compared to the standard HeLa cells (Fig3.10.B). This could be a reason for the poor infectivity of the Ctrl.DNA cells as the interferon response is mainly mounted by the cells to suppress the viral infection.

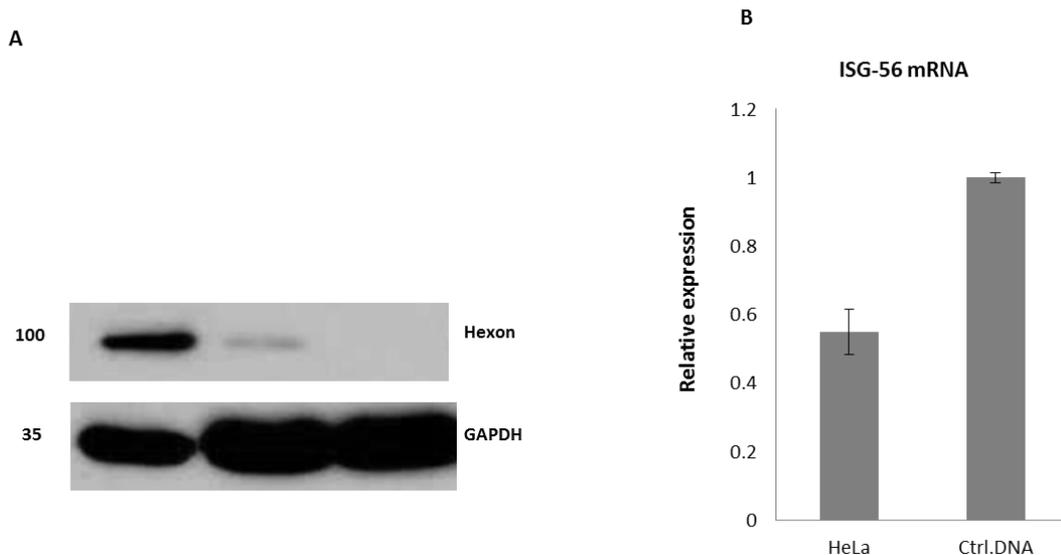


Fig.3.10. Evaluating the infectivity level of the transduced cells and level of interferon stimulated cells in control cell lines. **A.** Western blotting analysis of Adenovirus late gene expression in PML-II Kd, PML Kd and Ctrl.DNA cells. Cells were seeded at a density of 1×10^5 /ml for 24 hours, infected with wt300 at moi=5 and protein samples were collected with 1SD Laemmli buffer. Samples were probed with anti- late adenovirus polyclonal antisera from which the hexon while GAPDH is shown below as a loading control. **B.** ISG56 expression in PML-II kd and Ctrl.DNA cells. Prior to collecting samples, cells were treated with $1 \mu\text{g/ml}$ poly I:C for 16 hours. RNA samples were extracted from each cell type, total PML-II and IL-6 mRNA were quantified by RT-qPCR using 10 ng/well of complementary DNA (cDNA) as a template. Data were presented by the $\Delta\Delta\text{Ct}$ method and then normalised to the control cell value. All PCR products were subjected to dissociation curve analysis to ensure primer specificity. **A.** PML-II mRNA level in PML-II Kd in comparison with Ctrl.DNA control cells, data were normalised to GAPDH.

This poor infectivity of Ctrl.DNA cells was the reason to start to make new control cell lines which could show a comparable level of infection to the standard HeLa cells, in order to allow a study of the role of PML and PML-II in the viral life cycle under normal and stress conditions. GFP Kd and EV cells were made using the GFP shRNA insert and the empty PLKO.1 vector respectively.

The level of late viral gene expression was compared in those three control cell lines with moi of 5. The two new control cell types showed good levels of late viral protein while Ctrl.DNA cells didn't show any late protein expression (Fig.3.11.A). Whilst late protein was detected in both GFP Kd and EV cells, expression was greater in GFP Kd cells. To determine which of these cell types matched best the infectivity of standard HeLa cells, viral gene expression was compared in standard HeLa, GFP Kd and EV vector. As expected, GFP Kd and EV cells showed an

acceptable level of infection (Fig.3.11.B). Although EV cells showed roughly similar expression in standard HeLa cells, GFP Kd cells showed more late gene expression (Fig.3.11.C). Finally, to assess relative growth ability the different cell lines were counted frequently and both EV and HeLa cells showed faster level of growth than all the rest cell types despite HeLa cells showed the quickest growth rate among them. However, PML-II Kd in particular had showed the slowest level of division although the cells appearing healthy (Fig.3.11.C and D). In a separate experiment and in the context of optimising the conditions before starting in the next stage of work, for the purposes of transient knockdown of other genes, two control siRNAs were evaluated in their ability to trigger the NF-KB signalling pathway in EV cells. The basal level of IL-6 was measured in EV RNAs, which were left without treatment or treated with siC1 or siC2 for 48 hours. It was clear that siC1, the sequence of which was used in making Ctrl.DNA cells, has the ability to trigger NF-kB activity compared to the other control and the untreated samples (Fig.3.11.E).

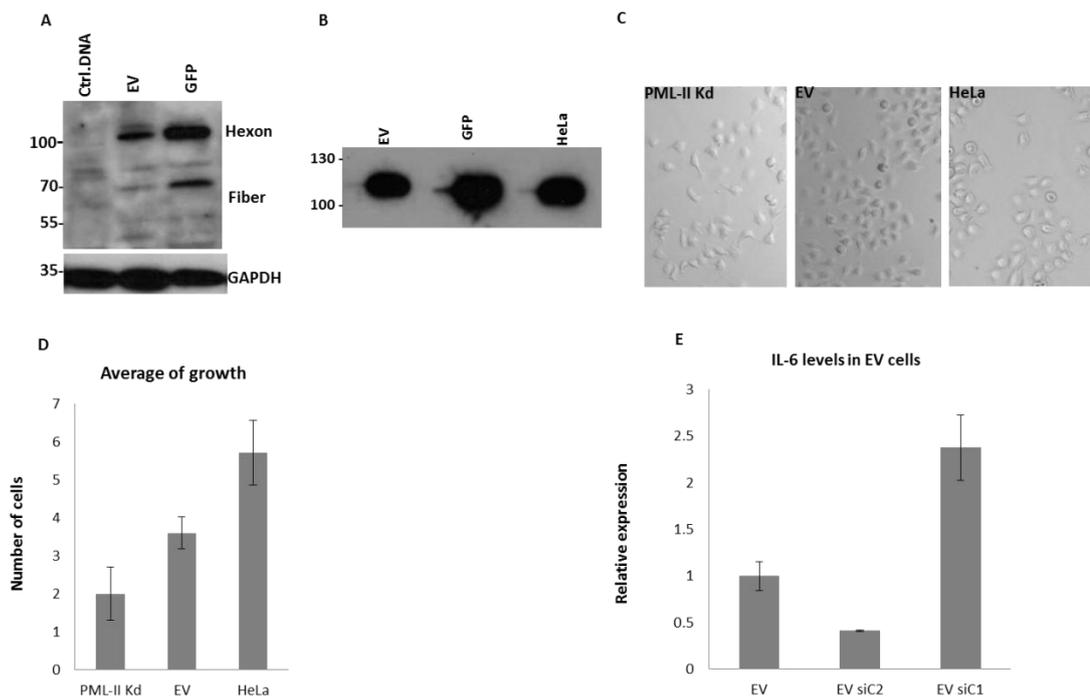


Fig.3.11. Comparing the viral late gene expression in control cell lines. **A.** Ad5 wt300 late gene expression in Ctrl.DNA EV and GFP Kd cells. Cells were seeded at a density of 1×10^5 /ml for 24 hours, infected with wt300 at moi=5 and protein samples were collected with 1xSB Laemmli buffer. Samples were probed with anti- late adenovirus polyclonal antisera. The “upper” hexon and fiber expression while “lower” is GAPDH expression and a loading control. **B.** Ad5 wt300 late gene expression in EV, GFP Kd and standard HeLa cells PML-II Kd, EV and HeLa morphology under microscopy. **C.** PML-II Kd, EV HeLa cells morphology under microscopy. **D.** Average of PML-II, EV and HeLa cell growth over repeated experiments. **E.** Levels of IL-6 mRNA in EV cells which were transfected with two types of scrambled DNA C1 and C2.

3.4 Evaluation of PML-II Kd cells in comparison with GFPKd and EV control cells

To evaluate the state of PML knockdown in PML-II Kd cells in comparison with the newly selected control cells, PML-II and IL-6 mRNA expression were evaluated in PML-II Kd, GFP Kd and EV cells. Consistent with the previous analyses PML-II Kd cells again showed physical loss of PML-II and its functional effects. The knockdown efficiency was about 60% and 50% in comparison with GFP Kd and EV cell lines respectively (Fig.3.12.A&B) but the functional effect in comparison with the EV was not as strong as when PML-II kd was compared to the Ctrl.DNA and GFP Kd cells (Fig3.12.C&D and Fig.3.7.C).

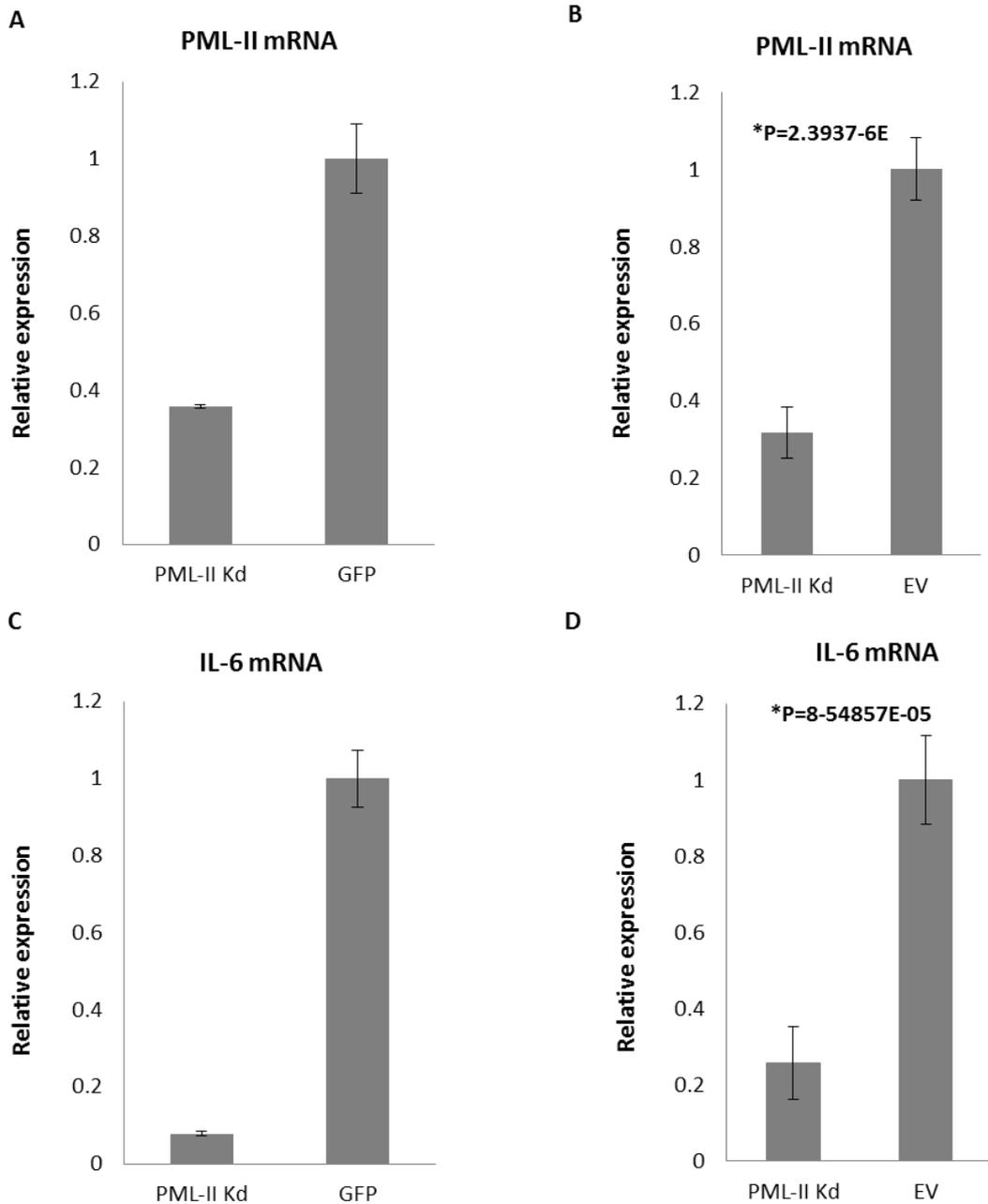


Fig.3.12. Characterising the physical and the functional effect of PML-II removal I in PML-II Kd cells in comparison with the GFP and EV cells. PML-II Kd, GFP Kd and EV cells were seeded at a density of 1×10^5 /ml for 24 hours. For characterising the effect on NF-kB signalling pathway, prior to harvest RNA samples, cells were treated with 100ng /ml of TNF- α for 1 hour. RNA samples were extracted from each cell type, total PML-II and IL-6 mRNA were quantified by RT-qPCR using 10 ng/well of complementary DNA (cDNA) as a template. Data were analysed by the $\Delta\Delta C_t$ method and then normalised to the control cell value. All PCR products were subjected to dissociation curve analysis to ensure primer specificity. **A&B.** PML-II expression in PML-II Kd, GFP Kd and EV. **C&D.** IL-6 expression in PML-II Kd, GFP Kd and EV. Data normalised to GAPDH in case of GFP Kd and to β -Actin in case of EV cells as house-keeping genes. Data show a significant decrease in PML-II mRNA and IL-6 mRNA in PML-II Kd cells (*P<0.05, n=3).

The most likely explanation for the difference in effect is the difference in the number of passages between those two analyses. The first analysis with GFP cells was achieved many passages earlier than the other one with EV, alongside the same PML-II Kd cell batch. This could create a difference in the knockdown efficiency in terms of knockdown itself or the functionality of it. In general, cells may gradually lose the knockdown with increasing number of passages. Cells were selected as a mixed population and not as single clones, and with this type of selection and especially if the down-regulated gene causes growth defects (in this case the PML-II Kd cells showed typically a 30-40% slower growth rate, then the knockdown cells will be easily outgrown by any cells that evolve to lose knock-down whilst retaining puromycin resistance. Within the same heterogeneous population it is possible to find cells which show the antibiotic resistance but no functional shRNA expression as the promoters that drive the two characters are not firmly linked (Schuck et al., 2004).

As was mentioned before, transient PML-II removal substantially decreased the IFN- β response (Chen et al., 2015). The level of interferon response in PML-II Kd cells in both mock and infected conditions was therefore measured. As expected, the PML-II Kd cells showed a lower basal level of ISG56 mRNA in comparison with the EV cells and at two time points, 8 and 20 hours post infection with HAdv5 wt300, PML-II Kd cells displayed a deficient interferon response (Fig.3.13).

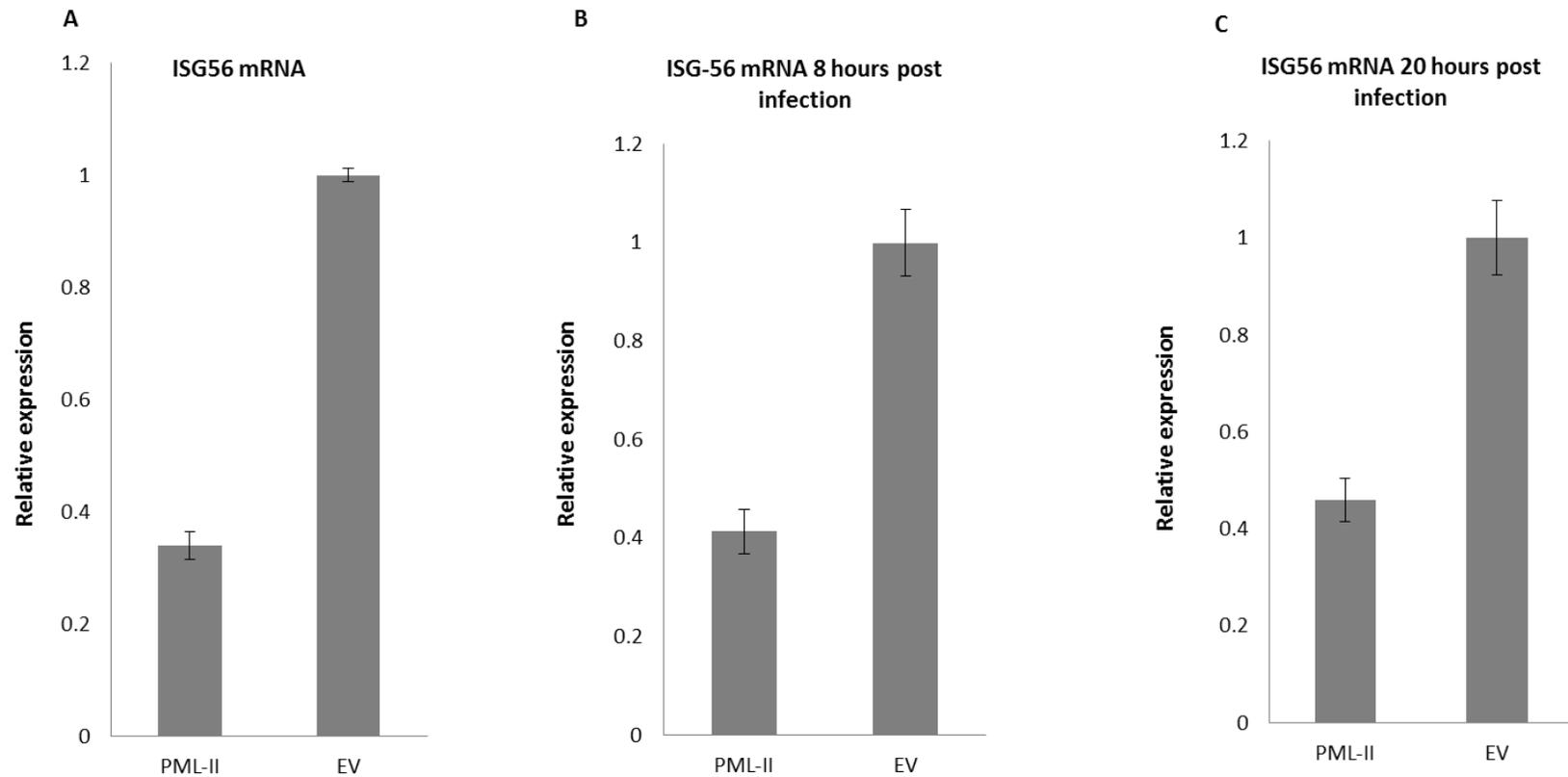


Fig. 3.13. ISG56 expression level in mock and infected PML-II Kd and EV. RNA samples were extracted from each cell type, total ISG56 and IL-6 mRNA were quantified by RT-qPCR using 10 ng/well of complementary DNA (cDNA) as a template. Data were analysed by the $\Delta\Delta C_t$ method and then normalised to the control cell value. All PCR products were subjected to dissociation curve analysis to ensure primer specificity. **A.** ISG56 in PML-II Kd mock infected and EV. **B.** ISG56 mRNA levels in PML-II Kd and EV 8 hours post infection. **C.** ISG56 mRNA levels in PML-II Kd and EV 20 hours post infection.

3.5 HSP70 expression in all cell lines

HSP70 is one of the heat shock protein family which is induced under different chemical or physiological stresses. HSP70 helps in folding the mis-folded proteins that arise. One of the early observations that were made about the profile of gene expression in PML-II Kd cells was the elevated level of hsp70 (Fig.3.14 A&B).

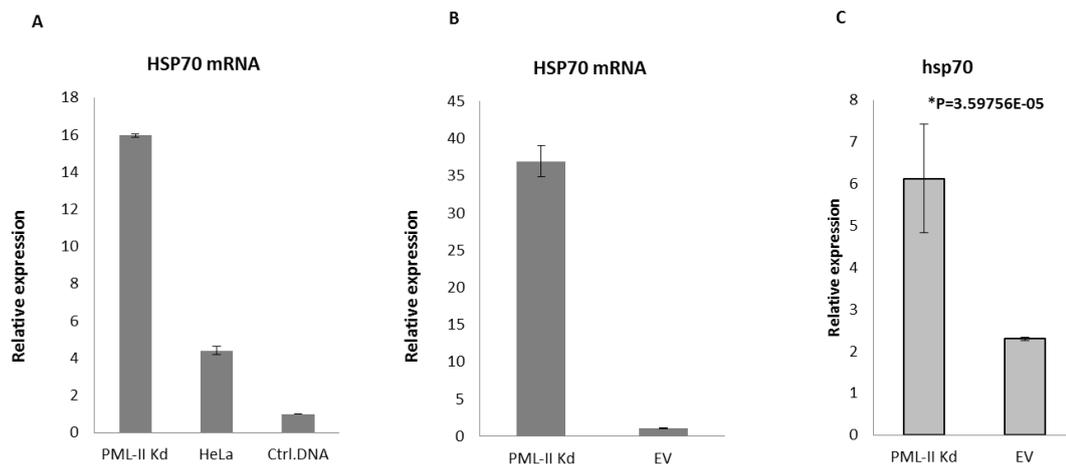
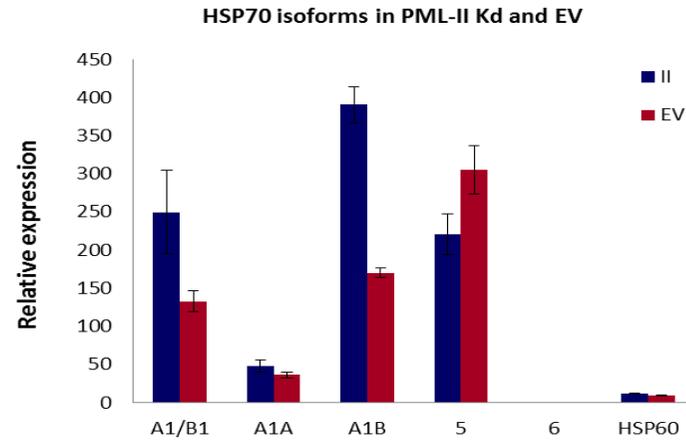


Fig.3.14. HSP70 expression in PML-II Kd, HeLa, Ctrl.DNA and EV cell lines. PML-II Kd, HeLa, Ctrl.DNA and EV cells were seeded at a density of 1×10^5 /ml for 24 hours. RNA samples were extracted from each cell type, total PML-II and IL-6 mRNA were quantified by RT-qPCR using 10 ng/well of complementary DNA (cDNA) as a template. Data were presented by the $\Delta\Delta Ct$ method and then normalised to the control cell value. All PCR products were subjected to dissociation curve analysis to ensure primer specificity. **A.** HSP70 expression in PML-II Kd HeLa and Ctrl.DNA, data were normalised to GAPDH as an internal control. **B.** HSP70 expression in PML-II Kd and EV cells, data were normalised to 18S RNA. Primers are specifically detecting the heat shock 70 kDa 1A/1B. Data shows significant increase in hsp70 mRNA in PML-II Kd cells (* $P < 0.05$, $n = 3$).

This increase was specific for the hsp70 protein and its related isoforms, highly heat shock inducible genes HSPA1A/1B, that were not distinguished by qPCR primer pair used, since other members of HSP70 (HSPA) family, A5 and A6 were not induced by absence of PML-II and neither was a member of another HSP family, HSP60. Using primers that distinguished HSPA1A and HSPA1B, it was clear that the induction by PML-II loss was highly specific for HSPA1B. However, in Ad5 wt300 infected PML-II Kd cells for 20 hours and at moi of 5, PML-II Kd cells showed again significant elevated levels of HSP70 and HSP70A1B was expressed much higher than the other two tested isoforms of HSP70 (Fig.3.15B). Presenting the data as fold induction of

each hsp70 isoform in PML-II Kd and EV cells showed that hsp70A1b had the highest level of induction in PMI-II Kd cells compared to its level in the control cells.

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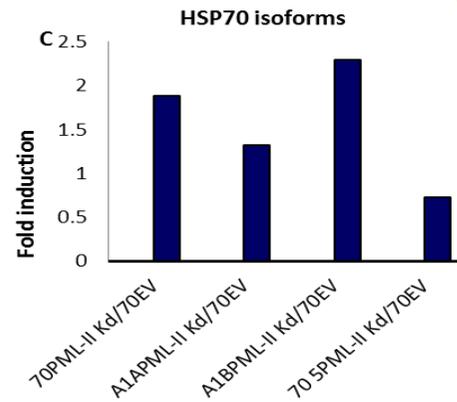
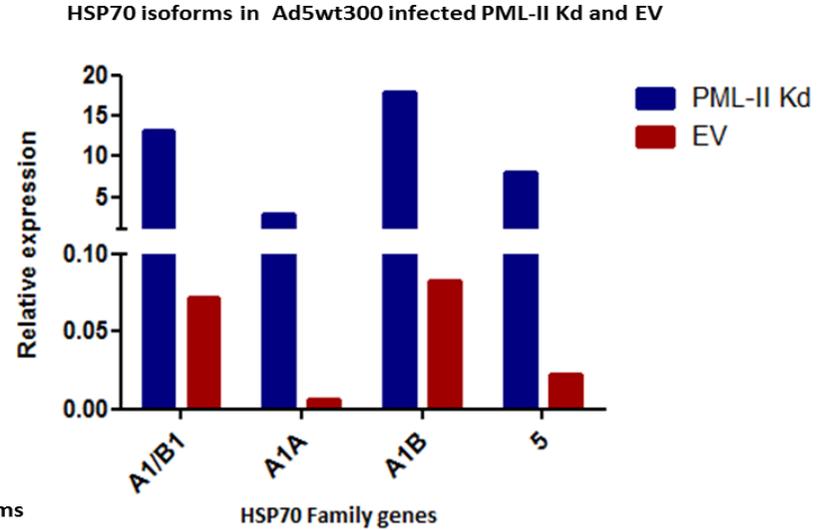


Fig.3.15. Expression of heat shock mRNAs in PML-II Kd and EV cell. PML-II Kd, and EV cells were seeded at a density of 1×10^5 /ml for 24 hours. RNA samples were extracted from each cell type, total PML-II and IL-6 mRNA were quantified by RT-qPCR using 10 ng/well of complementary DNA (cDNA) as a template. Data were presented by the $\Delta\Delta C_t$ method and then normalised to the control cell value. All PCR products were subjected to dissociation curve analysis to ensure primer specificity. **A.** HSPA/B1, HSPA1A, HSPA1B and HSP60 expression in un-infected cell Data were normalised to GAPDH. **B.** HSPA/B1, HSPA1A, HSPA1B and HSP5 in RNA from cells infected for 20 hours with wt300 at moi of 5, data were normalised to β -actin. **C.** Fold change induction of the increase in each gene in PML-II Kd / EV cells.

Levels of HSP70 vary according to the cell line under the study. HeLa cells have more expression of hsp70 than many other cells under induced or un-induced conditions except HEK 293 (Imperiale et al., 1984) which expresses even more hsp70 due to that fact that they were immortalised by adenovirus E1A which is a known inducer of HSP70 transcription (Nevins, 1982). This level of un-induced hsp70 is controlled by the stage of the cell cycle which is also well reflected in adenovirus gene expression. For example in mouse F9 teratocarcinoma cell line (F9 stem cells), upon differentiation the cells restrict E1A expression which is accompanied by shut off hsp70 expression (Imperiale et al., 1984). Another study which showed the induction of hsp70 by Ad5 infection of HeLa cells, showed a high level of 70K heat shock protein which was induced in harmony with the expression of E1A since it disappeared in E1A-deficient Ad5 mutant dl312 – infected HeLa cell. The increased hsp70 reached up to 100 fold greater levels than its level pre-infection, while it increased only two fold in the absence of E1A expression. This hsp70 induction was at the transcriptional level and was controlled by E1A (Kao and Nevins, 1983, Nevins, 1981)

A possible explanation of the elevated levels of hsp70 is the chaperone function of PML and HSP70. In mouse embryonic stem cells (ES) it has been found the PML represents the required platform to enable the DAXX / α -thalassemia mental retardation X-linked (ATRX) complex to function in maintaining telomere chromatin integrity. Simply, PML achieves that via being the physical podium where all the proteins that are involved in histone methylation form (Chang et al., 2013). As a way to compensate that deficiency in chaperone function inside the cells in which total PML is removed, the cell starts to up-regulate hsp70 expression because of the importance of the latter in preserving the correct function of the telomeres. Hsp70 and co-chaperone protein CHIP (C- terminus of HSC interaction protein) bind to hTERT(human telomerase reverse transcriptase) to ubiquitinate it and thus direct its proteasomal degradation (Lee et al., 2010).

3.6 PML- II stable knockdown in 293 HEK cells

In a trial to transduce another cell line with lentiviral shRNA vectors, Human Embryonic Kidney (HEK) 293 cells were chosen as the target for PML-II removal. This transduction was accomplished mainly to further confirm the effect of PML-II on the innate immune response. The same PML-II shRNA lentivirus transduction as applied to HeLa cells was used here. The 5- day analysis of the knockdown and its functional consequences showed a substantial decrease in PML-II mRNA, reaching about 90% (Fig.3.16 A), accompanied by a similar reduction in interferon response which is represented by a decrease in ISG56 mRNA (Fig.3.16.B). Thus lentiviral shRNA transduction can successfully disrupt PML-II function in multiple cell types. This could be a good reason to generalize the role of PML-II in the innate immune response and to conclude the real function of this gene in the adenovirus life cycle.

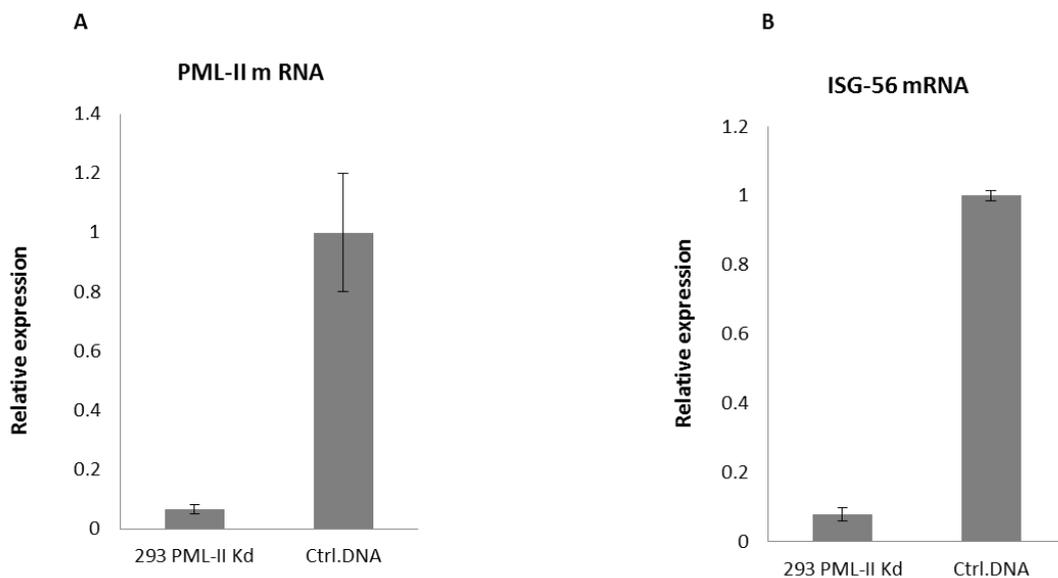


Fig.3.16. PML-II and ISG56 expression in 293PML-II Kd and 293Ctrl.DNA cells. 293PML-II Kd and Ctrl.DNA 293 cells were seeded at a density of 1×10^5 /ml for 24 hours. RNA samples were extracted from each cell type, total PML-II and ISG56 mRNA were quantified by RT-qPCR using 10 ng/well of complementary DNA (cDNA) as a template. Data were analysed by the $\Delta\Delta C_t$ method and then normalised to the control cell value. All PCR products were subjected to dissociation curve analysis to ensure primer specificity and normalised to GAPDH as house-keeping gene. **A.** PML-II expression. **B.** ISG56 expression

As this study is concerned with defining the role of PML-II in the Ad5 life cycle, HEK 293 cells were excluded from this study despite the fact they support Ad growth

efficiently. HEK293 cells are immortalised by the Ad5 E1A oncoprotein which has a critical role in viral early and late gene expression and might therefore compromise the results. Thus the next part of this work will focus on using HeLa cells as fully competent cells in supporting Ad5 infection.

3.7 Discussion

Lentiviral shRNA transduction which was employed in this study has allowed establishment of the HeLa cells in which target genes (PML and PML-II) were permanently knocked down. This means that the main goal of this part of the project was achieved. The stable PML-II Kd and PML Kd, with EV cells, as control cells are suitable and completely amenable to analyse the outcome of Ad5 infection and to define the suggested role of PML and PML-II in virus life cycle. The knockdown system and its significant functional effects on the innate immune response was efficient and it was used in the transient knockdown in HeLa cells and in other tumour and normal cells like HEK293 and MRC5 cells which have been tested in this study or in different projects by other current and previous members in the group.

The more profitable aspects from this kind of knockdown alongside its functional effects is the consistency over different experiments. Treating a bulk of cells which display the same knockdown characteristics reduced the probability of transient knockdown failure in one experiment as compared to another one. In addition, the experiments were achieved in less time (2 days quicker) than the transient knockdown. In addition as this project includes working with stress, this system reduced the consequences that could arise from adding the transient knockdown reagents (siRNA and lipofectamine) to the cells which is another kind of stress (Fig.3.11 D&E). Finally, and in order to attain quantitative data, it was easier to grow the cells to any culture size to analyse quantitatively by FACS.

Experience from working with such kinds of cells and knockdown system suggested many considerations and further steps which should be incorporated during experiments. An important observation was that knockdown cells grew at a slower

rate than the control cells. This is possible and expected as many genes are important for cell growth, division and proliferation and PML in particular interacts with other proteins which are important in cellular proliferation. In order to make a fair comparison in all analyses it was necessary to compare an equal number of knockdown and control cells. In each single experiment, knockdown and control cells were counted at the day of plating out, then at infection and harvesting and the number of viral particles used for infection corrected according to the number of cells in each sample, measured in parallel wells that were made for purposes of counting only. Slower growth was a source of another critical consideration, the gradual loss of knockdown efficiency over number of passages. Over a wide range of selection experiments it was noticed that the knockdown starts to weaken over a number of passages that ranged from 10 to 22, with some exceptions which showed shorter or longer duration of sustained knockdown. This might depend on the percentages of the transduced cells that show a real functional knockdown in a bulk of selected cells, or could be attributed to the amount of the viruses that the cells were infected with. As mentioned before, removing PML-II caused a slowdown of growth in the knockdown cells and this resulted in a reduced number of cells that showed a real functional knockdown compared to those that displayed puromycin resistance only.

Shown below (Fig.3.17) is an example of this gradual decline in the knockdown phenotype of cells were selected on 22/02/2015 and grown until 18/05/2015. This bulk of selected cell showed 70% knockdown at the first analysis after puromycin selection. Gradually, the efficiency of knockdown started to drop over time and reached 30% after 8 weeks since the first selection. This weakening was well reflected in the biological effect and also in terms of the effects of the knockdown genes in the virus life cycle. This situation was the reason to establish a periodic knockdown analysis at the RNA level to make sure that there was a reasonable knockdown at the time of doing any experiment.

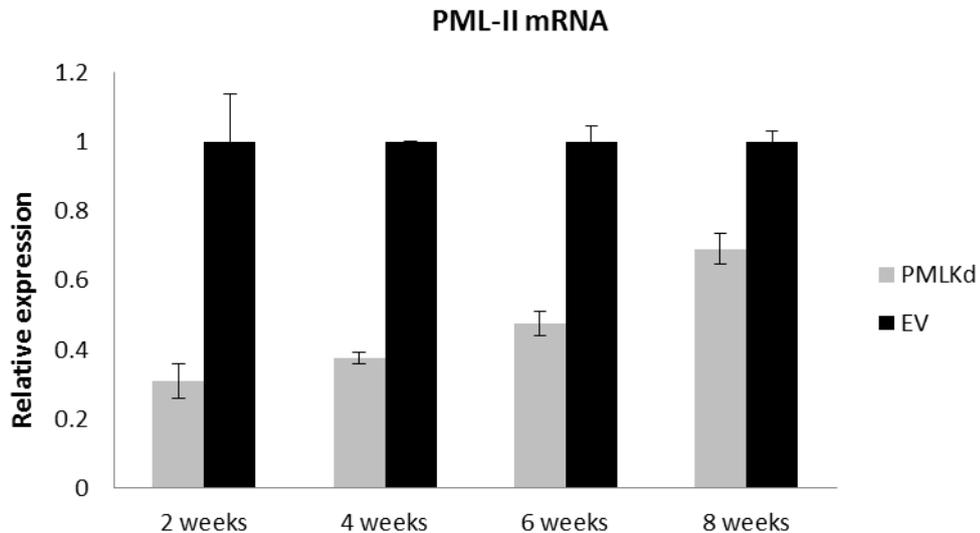


Fig.3.17. PML-II expression in PML-II Kd and EV cells over 8 weeks. Cells were seeded at a density of 1×10^5 /ml for 24 hours. RNA samples were extracted from each cell type, total PML-II were quantified by RT-qPCR using 10 ng/well of complementary DNA (cDNA) as a template. Data were analysed by the $\Delta\Delta C_t$ method and then normalised to the control cell value. All PCR products were subjected to dissociation curve analysis to ensure primer specificity and normalised to GAPDH as house-keeping gene.

In order to keep the work continuously effective and to provide a good platform to do the corresponding analyses, large amounts of the shRNA-lentiviral particles were prepared as stock and when the level of knockdown started to decline a new parallel selection was made. This substantial supply of shRNA-lentiviral selection was not only required because of the declining level of knockdown during passage but also to avoid the sudden loss of knockdown for some batches of cells when they were grown after storage in liquid nitrogen.

In general, freshly selected knockdown cells did not readily show the knockdown effect; it seems they need time to build up and express the shRNA to produce its effect and in this study the time required was about 9 days. Also the cells needed to be released from puromycin selection at least 24 hours before doing any biological experiment with them. The best results were obtained by releasing cells for 3 days before infecting, or transfecting cells or applying any other biological treatment. It is worth mentioning here that these cells were very sensitive to even mild changes in their environment. Thus, it was necessary to keep the conditions stable as much as it is possible.

As controls are critical for any biological experiment, the challenge was to choose the right control cell type for comparison with PML-depleted cells. The required control was required to behave very similarly to the standard HeLa cells while at the same time they are genetically modified. In this project, empty vector cells, which were made from filling the overhangs in PLKO.1 vector, were the best choice as they showed closer growth rate and comparable level of virus infection to HeLa cells.

Despite all these difficulties which were mentioned earlier, the substantial biological effect of the knockdown of PML that arose from heterogeneous population of selected cells was very convincing and provided a solid start for verifying the role of PML-II in the Ad5 life cycle at both normal and stressed conditions.

Chapter4: Role of PML-II in the adenovirus life cycle at 37°C

4.1 Introduction

PML-II is a human PML isoform, the unique part of which is encoded by exon 7b, with a molecular weight around 130 kDa. PML-II contributes to PML-NBs but has a very distinctive pattern of localisation inside the nucleus when it is overexpressed, being relocated in the area very close to the inner nuclear membrane. PML-II has been found to play an important role in the cellular immune response as its removal by siRNA interference reduces the IFN- β levels produced in response to stimuli (Chen et al., 2015). It was also shown that this particular isoform restricts the growth of some viruses. For example, PML-II overexpression inhibits adeno-associated virus (AAV) transduction about 5 fold in comparison with the other overexpressed PML isoforms which succeeded in reducing this transduction only 2 fold (Mitchell et al., 2014).

In another example, PML-II and PML-I overexpression in PML-depleted cells can restrict ICPO-null mutant HSV replication efficiency. ICPO is the viral protein that disrupts PML function and ICPO-null mutant HSV plaque formation was increased significantly in PML-depleted cells and overexpressing PML-I and PML-II reduced plaque formation to 50%. This suggests that PML-II might interact with other cellular repressive factors to prevent infected cells from entering the lytic cycle (Cuchet et al., 2011). Another interesting observation linking PML-II with antiviral effects is that HSV specifically down regulates its expression. After 3 hours of HSV infection, it was noted that the levels of PML-II mRNA and protein foci were reduced. This reduction was occurred in parallel with increased retention of PML intron 7a in mRNA. This change switches the encoded protein isoform from PML-II to PML-V (Nojima et al., 2009). This effect was attributed to the expression of ICP27 which works as an alternative splicing regulator. This alteration in PML splicing pattern is of crucial importance in ensuring a successful viral infection especially since ICP27 is an immediate early protein and this could be a strategy developed by the virus to overcome the immediate innate immune response and interferon

response (Nojima et al., 2009). This proposal is supported by the fact that the PML-II isoform is necessary to induce the interferon response (Chen et al., 2015).

4.1.1 PML-II and Adenovirus infection

There is a clear evidence for a link between Ad5 infection and expression of the PML-II isoform. It is well-known that Ad5 infection causes disruption in PML –NB morphology as they redistribute into thread- like structures rather than spherical or dot shapes upon infection (Carvalho et al., 1995). This interaction was found to be specific for the PML-II isoform and is achieved by one of the early viral products E4 Orf3. This interaction between the viral and cellular proteins is direct and critical to change the PML-NB morphology since mutating the Orf3 caused a failure in Orf3-PML-II interaction and PML rearrangement (Hoppe et al., 2006). Overexpressing PML-II Δ RBCC truncated mutants increased interferon promoter activity, which is reduced again when Orf3 protein is overexpressed (Wight, 2010). This suggests that Ad5 overcomes the innate immune response by expressing Orf3 protein which decreases the IFN response that is mounted by PML-II related isoforms (Wright, 2010)This suggests a negative role for the PML-II isoform in the Ad5 life cycle.

In contrast, it was found that PML-II plays a positive role in Ad5 early transcription. E1A-13S was selectively bound to the PML-II isoform resulting in augmented E1A-mediated transcriptional activation. Virus growth was also enhanced in PML-null cells by expressing PML-II, suggesting a positive role for PML-II during the early viral life cycle (Berscheminski et al., 2013).

4.1.2 Aim of work described in this chapter

PML-II has a positive role in the innate immune response represented by its regulation of interferon and NF-kB responses. PML-II is specifically disrupted and antagonized by Ad5 E4Orf3, an example of targeting PML-NB by various viruses This suggests that PML-II should be a negative regulator of adenovirus growth. On the other hand the augmented E1A transcriptional activation and Ad5 replication with the re-expression of PML-II in PML-null cells points to how viruses might benefit from the proteins that are involved in the immune response to promote their own

transcription and replication. These apparently conflicting possibilities motivated us to analyse the effect of PML-II in Ad5 gene expression and replication.

Work described in the previous chapter established a very convenient system in which to study the role of PML-II in the Ad5 life cycle. Well characterised PML-II depleted and suitable control cell lines that provided reliable and persistent physical and functional knockdown provided useful reagents with which to study the role of PML-II in the progress of adenovirus infection.

The experimental design was to infect the knockdown and control cells with Ad5wt300 at moi of 5 for 20 hours. The cells were then harvested and RNA and proteins analysed by RT-qPCR, Western blotting and FACS. Virus replication was assayed by infecting the knockdown and control cells for different time intervals (24 and 48 hours), samples were harvested and infectious virus titres determined by the fluorescent focus assay (FFA). In transient knockdown experiments, the same systems of infection and analyses were used except 48 hours of IRF3 or hsp70 knockdown were performed prior to infection.

4.2 The role of PML-II in the Ad life cycle

4.2.1 Effect of transient removal of PML-II on adenovirus gene expression and genomic copy number

In order to investigate the specific role of PML-II in the Ad5 life cycle, a pilot study was carried out using PML-II or Ctrl siRNAs for transient knockdown in standard HeLa cells. Cells were plated for 24 hours and transfected with PML-II siRNA or siC for 48 hours before Ad5 wt300 infection for 20 hours at moi of 5. It is clear that PML-II knockdown augmented hexon mRNA expression by approximately 2- fold in comparison with cells transfected with siRNA control (Fig.4.1A). A similar effect was noticed in Ad5 genomic copy number per cell. There was a 3-fold increase in Ad5 genomic copies following PML-II knockdown compared with the control sample (Fig.4.1B).

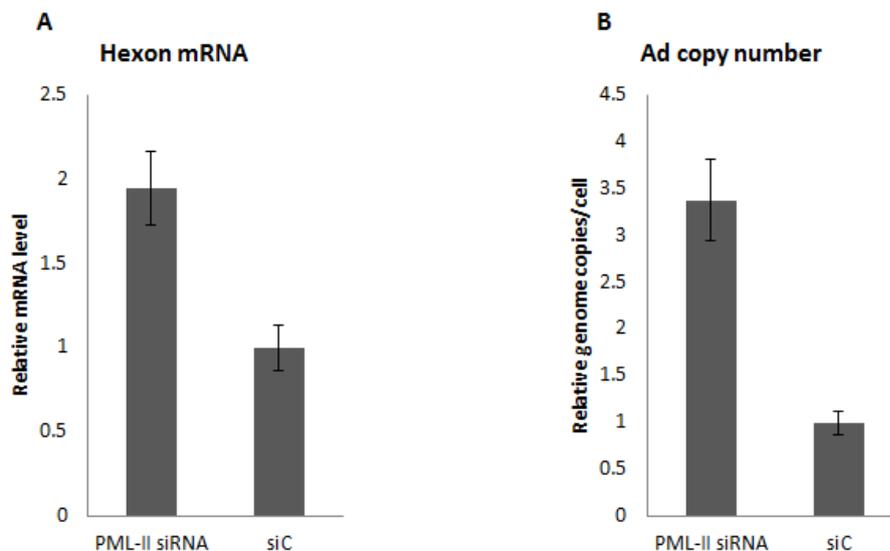


Fig.4.1. Ad5 late expression and genome replication. Standard HeLa cells were seeded at a density of 1×10^5 /ml for 24 hours in 24-well cultures. Cells were transfected with 125 pmol of either PML-II siRNA or siC for 48 hour then samples were infected with Ad5 wt300 at moi of 5 for 20 hours. RNA or DNA samples were extracted from each sample. Total hexon mRNA was quantified by RT-qPCR using 10 ng/well of complementary DNA (cDNA) as a template. Ad5 genomic copy number was calculated from standard curves of Ad5 and genomic GAPDH plasmids made by 10 fold serial dilutions and then normalised to the control sample value. mRNA data were calculated by the $\Delta\Delta C_t$ method, using β -actin as internal control. All PCR products were subjected to dissociation curve analysis to ensure primer specificity. **A.** Hexon mRNA expression in PML-II depleted and control samples. **B.** Ad5 relative copy number in PML-II depleted and control samples.

Adenovirus gene expression and replication in stable PML-II knockdown cells

One of the main goals of making permanent PML-II knockdown cells was to extensively explore the role of PML-II in the Ad5 life cycle and how infection progressed in the presence or absence of PML-II under normal and stress conditions (stress conditions will be discussed in the next chapter). Cells were plated for 24 hours at 37 °C and infected with Ad5wt300 at moi of 5 and samples were harvested for either RNA or protein analysis. Compared to different control cell lines, early and late Ad5 gene expression was significantly elevated in PML-II Kd cells than in control cells. The increase in late gene expression was more drastic than the early gene expression; it was about 7- fold greater compared to the GFPKd control and more than 30-fold when compared to the Ctrl.DNA cells (Fig.4.2B). On the other hand, the increase in E1A expression was not as big as for hexon; it displayed 3 and 9 times more expression compared to GFP Kd and Ctrl.DNA cells respectively (Fig.4.2A). In Chapter 3, EV cells showed Ad5 infection characteristics that were most similar to standard HeLa cells among the three potential control cell lines available for comparison with PML-II Kd cells. Using EV cells as control, the results were very consistent with the previous comparison. Hexon mRNA expression was about 5-fold greater in PML-II Kd cells in comparison to the EV cells (Fig.4.2C). Protein analysis verified the RNA analysis; it was clear that there was more DNA binding protein (DBP), E1B55K and hexon protein in PML-II Kd cells when compared to the EV cells (Fig.4.2D).

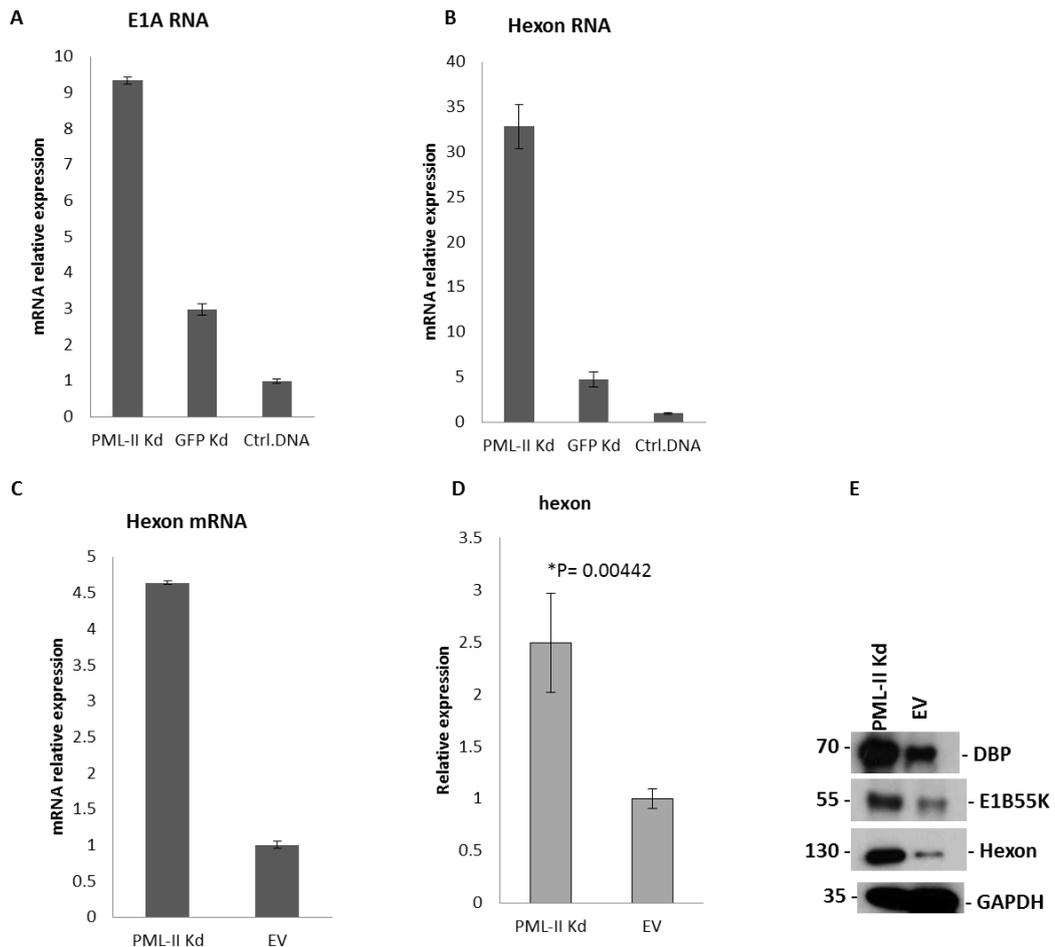


Fig.4.2 Ad5 gene expression in PML-II knockdown and control cell lines. PML-II Kd, GFP Kd, Ctrl.DNA and EV cells were seeded at a density of 1×10^5 /ml for 24 hours. Cultures were infected with Ad5 wt300 at moi of 5 for 20 hours. RNA samples were extracted from each cell type, viral gene expression was quantified by RT-qPCR using 10 ng/well of complementary DNA (cDNA) as a template. Data were calculated by the $\Delta\Delta C_t$ method, using 18S RNA as internal control, and then normalised to the control cell value. All PCR products were subjected to dissociation curve analysis to ensure primer specificity. **A.** E1A RNA expression in PML-II Kd, GFP Kd and Ctrl.DNA control cells. **B.** Hexon RNA expression in PML-II Kd, GFP Kd and Ctrl.DNA control cells. **C.** Hexon RNA expression in PML-II Kd and EV cells. **D.** For WB analysis, samples were harvested with 1xSB, the membranes were blotted against the appropriate Abs: (anti-DBP mouse monoclonal Abs (upper panel), anti-E1B55K mouse monoclonal Abs (second panel), anti-late polyclonal rabbit Abs (third panel) and anti-GAPDH mouse monoclonal Abs (bottom panel). Data show a significant increase in hexon expression in PML-II Kd cells (*P<0.05, n=3).

4.2.2 Ad5 gene expression at different time points post infection

With the intention of investigating the progress of the Ad5 infection in the presence or absence of PML-II, cells were subjected to Ad5 infection at moi of 5, and samples were harvested at different time intervals (6, 12, 18 and 24 hours) post infection. At all these time points, protein samples were harvested from both PML-II Kd and EV cells and the were analysed for early and late gene expression. Over the time course, there was an evidently greater increase in early and late viral proteins in PML-II Kd cells than the EV cell in all time intervals, having accounted for loading differences (GAPDH), both for E1B 55K (Fig.4.3A) and for late gene products, at 18 h and 24 when late protein begin to accumulate (Fig.4.3B). However, the difference appeared more significant for late than early proteins. These findings are consistent with the results of infection following transient knockdown. In conclusion, it is clear that PML-II plays a negative role in the progress of Ad5 infection.

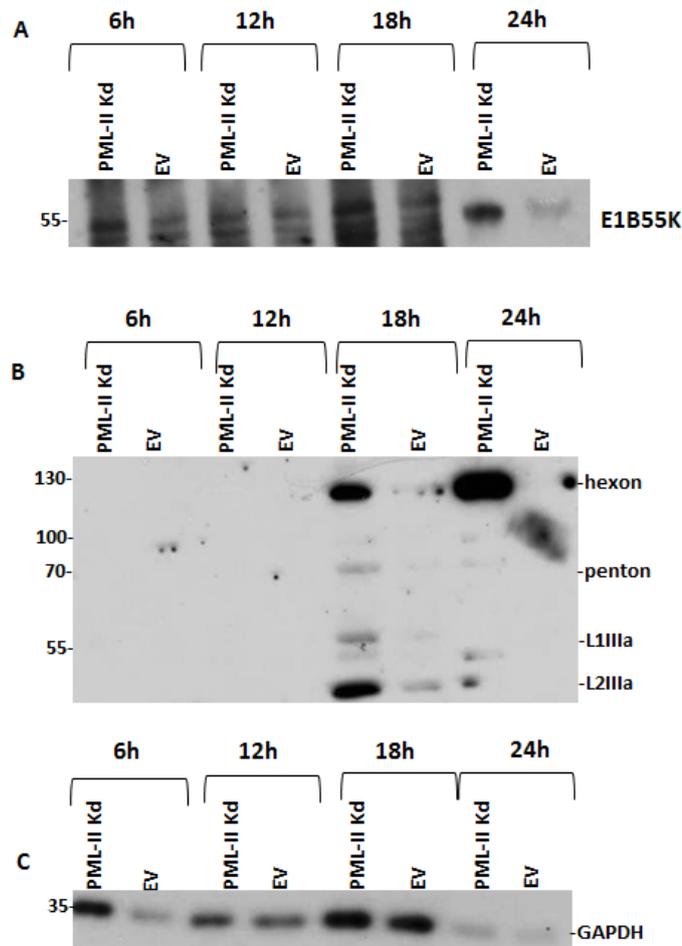


Fig.4.3. Ad5 gene expression in PML-II knockdown and control cell lines. PML-II Kd and EV cells were seeded at a density of 1×10^5 /ml for 24 hours. Cells were infected with Ad5 wt300 at moi of 5 for 24 hours. Protein samples were harvested for WB analysis at the times indicated, analysed by SDS-PAGE and western blotting. The membranes were probed with the appropriate Abs. **A.** anti-E1B55K mouse monoclonal abs; **B.** Anti-late polyclonal rabbit Abs; **C.** Anti-GAPDH mouse monoclonal Abs. The last two samples at 24 hours were diluted 100 times relative to the others as the expression was very high and as a consequence, the GAPDH band was very faint for those two samples in particular.

4.2.3 Quantitative estimation of early and late gene expression in PML-II Kd and EV cells

All the previous RNA or protein analyses showed more viral gene expression in PML-II kd cells compared to different control cell lines. This increase could be attributed to an increase in the number of cells that showed viral gene expression or to the same numbers of cells showing more gene expression within the same population of cells or in comparison with other cell types. In order to make a quantitation that could count the number of cells that expressed early and late Ad5 proteins and to give an idea about the intensity of expression of a given protein in

the population of positive cells, we used FACS for that purpose. Using this technique it was possible to distinguish the percentage of positive (infected) cells from the negative uninfected cells in each sample, which allows a fair comparison by obtaining the fraction of infected cells in each culture. At the same time and within a population of positive cells it was possible to detect the amount of each viral protein by measuring the intensity of the mean of fluorescence (MFI). Cells were plated for 24 hours and infected with Ad5wt300 at moi of 5 and samples were fixed and stained to measure the DBP and hexon expression in PML-II Kd and EV cells. The results further confirmed the previous RNA/protein analyses through showing more or less 100% increase in the population of cells that showed late Ad5 protein expression (Fig.4.4B, the black curves) PML-II Kd cells showing 26.53% late expressing cells compared to only 13.58% in EV cells. The MFI of hexon expression within the positive cells was also higher in PML-II Kd cells, 1493.45 compared to its value in EV cells which was 965.16 (Fig 4.4B). However, and consistent with the previous results, the DBP fluorescence was only slightly higher in PML-II Kd cells compared to the EV cells. The percentage of cells that showed DBP positive staining was 50.79% in PML-II Kd cells in comparison with 44.1% in EV cells. A greater difference was noticed in the MFI of DBP between the two types of cells, PML-II Kd cells showed an MFI value of 1636.79 while EV cells showed 1160 (Fig 4.4.A). This increase is likely to reflect largely the continuing expression of DBP in the late phase. To summarise, it is clear that both the proportion of infected cells and the intensity of viral gene expression was higher in PML-II Kd cells than the EV cells, particularly in the late phase.

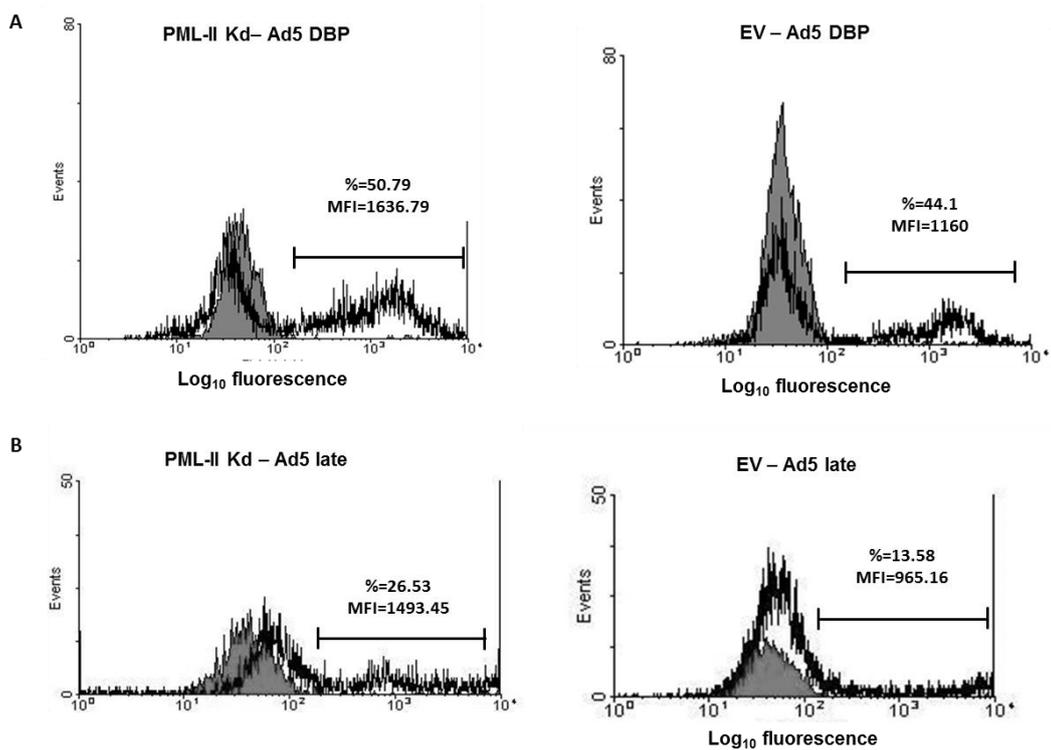


Fig.4.4 FACS analysis of early and late gene expression in PML-II Kd and EV cells. Cells were plated at a density of 0.8×10^6 or 2.2×10^6 per dish in 60 and 100 mm² dishes respectively for 24 hours. Cells were infected at moi of 5 with Ad5wt300 for 20 hours. Cells were then fixed, permeabilized and stained with either anti-DBP monoclonal Abs or anti-hexon monoclonal Abs as primary antibodies, followed by Alexa-Fluor 488 Gt anti-Mouse Abs staining. **A.** DBP fluorescence in PML-II Kd cells (left) in EV cells (right). **B.** Hexon fluorescence in PML-II Kd cells (left) in EV cells (right). The grey curves represent the mock infected cells (background) while the black curves represent the infected cells.

4.2.4 Virus yield in PML-II Kd and EV cells

Differences in viral gene expression may or may not be translated into effects on the yield of infection progeny. To determine whether loss of PML-II affected the virus yield, PML-II Kd and EV cells were infected with Ad5wt300 at moi of 5 for 24 and 48 hours, total cell cultures were harvested and freeze-thaw extracts titered on HeLa cells by fluorescence focus assay. There was a noticeable increase of about 3-fold in virus yield in PML-II Kd when compared with control cells at both time points (Fig 4.5A&B).

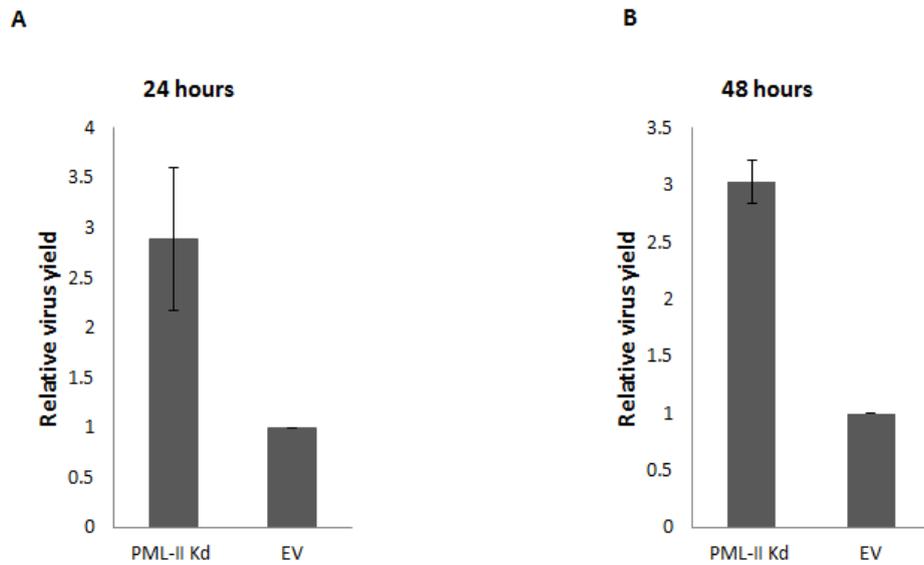


Fig.4.5. Ad5 yield in PML-II Kd and EV cells. PML-II Kd and EV cells were plated at a density of 1×10^5 /ml for 24 hours, infected at moi of 5 with Ad5wt300 for 24 and 48 hours. Samples were harvested and subjected to freeze-thaw cycles to release virus and then serial dilutions were prepared from each sample and used to infect standard HeLa cells for 16 hours. Cells were fixed and stained using anti-DBP monoclonal antibodies as primary Abs and 488 Alexafluor goat anti mouse Abs as secondary antibodies. Samples were examined using UV- microscopy and DBP foci were by eye. Numbers from duplicate cultures were averaged and divided by cell numbers in the producedr culture, then normalised to the yield in EV cells. **A.** Virus yield at 24 hours. **B.** Virus yield at 48 hours.

As an overall conclusion, it is clear that PML-II has a negative impact on Ad5 gene expression and virus replication. This could be related to the fact that this protein in particular is antiviral, either in promoting the IFN- β response or other functions, and although it is targeted by E4Orf3 in a way that could result in blocking its specific function, this may not completely abrogate its negative effect (Hoppe et al., 2006, Chen et al., 2015). Inhibiting the innate immune response may facilitate the virus's mission in establishing efficient replication and the progress of infection.

4.3 Mechanisms underlying the increased Ad5 gene expression and replication in the absence of PML-II

4.3.1 Decreased level of Interferon response

PML-II is known to facilitate a robust IFN response and at the same time it is upregulated significantly from basal level with stimulation by IFN- α or β (Der et al., 1998, Chen et al., 2015). Hence, the straightforward mechanism that could be proposed here is that the reduced level of IFN response in PML-II Kd cells allows Ad5 to grow better, even though Ad5 expresses E4Orf3 that presumably inhibits this factor. To test this, the idea was to block the interferon response before Ad5 infection in both PML-II Kd and EV cells by another means, then to compare the outcome of Ad5 infection. IRF3 is a transcriptional factor that is retained in an inactive form in the cytoplasm due to a constitutive nuclear export signal. When a cell senses a pathogen, IRF3 becomes activated and is transported to the nucleus where it starts its transcription function in triggering the type I interferon response (Yoneyama et al., 1998). Adenovirus infection in particular induces interferon type I in an IRF3-dependent manner soon after infection which results in a direct stimulation of ISG56 and other ISGs (Stein and Falck-Pedersen, 2012).

IRF3 siRNA treatment was optimised by using a series of concentrations: 31.25, 62.5 and 125 pmol (data not shown); the best concentration was 62.5 pmol which showed about 50% reduction in ISG56 mRNA (Fig4.6A) which was used later as the standard for IRF3 knockdown experiments.

To confirm the functional effect of IRF3 knockdown EV cells were treated with control or IRF3 siRNA, stimulated with poly I:C and ISG56 mRNA was quantified as one of the directly IRF3-stimulated genes (Grandvaux et al., 2002). In both poly I:C stimulated and un-stimulated cells, IRF3 knockdown caused the IFN response to decline to about 50 and 40% respectively (Fig.4.6B). IRF3 knockdown did not block the response completely, either because IRF3 loss was not complete or because there is another transcription factor, NF- κ B, that is also activated and contributes to the IFN response within this same classical induction pathway (Haller et al., 2006)

Next, the effect of IRF3 knockdown on Ad5 infection was tested. PML-II Kd and EV cells were transfected with IRF3 siRNA or siC for 48 hours, then infected with Ad5wt300 for 20 hours. Late viral proteins was analysed and compared in both cell lines (Fig.4.6C). The level of late viral proteins was considerably greater in PML-II Kd cells than in EV cells when treated with siC, which is consistent with results that were obtained in these cells without siRNA treatment. IRF3 knockdown in PML-II Kd cells had no effect on the already high level of hexon being expressed. This reflects the fact that the interferon response is already strongly inhibited in these cells through the absence of PML-II.

In EV cells the effect of IRF3 knockdown was to increase hexon expression somewhat compared to the control. Significantly though, this hexon expression was still substantially lower than was observed in PML-II kd cells (Fig 4.6C). Thus, blocking of type I interferon responses by IRF3 knockdown in EV cells does not make them equivalent to PML-II Kd cells in terms of their hexon expression. This sort of effect suggests that there might be other mechanisms in addition to loss of the IFN response that contribute to increased viral gene expression in PML-II Kd cells.

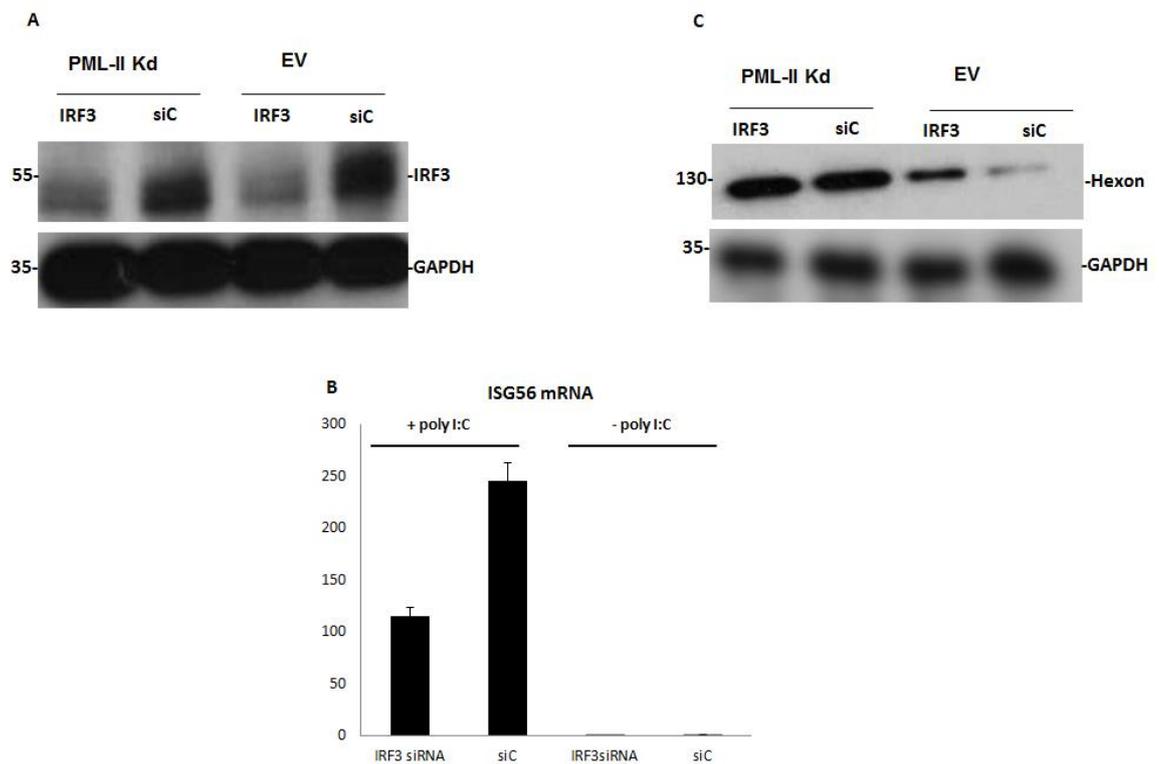


Fig.4.6. Ad5 late gene expression in PML-II Kd and EV cell lines with or without IRF3 knockdown. PML-II Kd and EV cells were seeded at a density of 1×10^5 /ml for 24 hours in 24-well cultures. **A.** IRF3 expression in PML-II Kd and EV cells. Cells were transfected with 62.5 pmol of either IRF3 siRNA or siC for 48 hours. Cultures were harvested for SDS-PAGE analysis and membranes were reacted with rabbit polyclonal anti- IRF3 Abs. **B.** ISG56 mRNA in EV cells with or without stimulation. Cells were transfected with 62.5 pmol of either IRF3 siRNA or siC for 48 hours then either stimulated with poly I:C for 16 hours or left without treatment. Total ISG56 mRNA was quantified by RT-qPCR using 10 ng/well of complementary DNA (cDNA) as a template. Data were presented by the $\Delta\Delta Ct$ method, using β -actin as an internal control. All PCR products were subjected to dissociation curve analysis to ensure primer specificity. **C.** Ad5 hexon expression in PML-II Kd and EV cells. Cells were transfected with 62.5 pmol of either IRF3 siRNA or siC for 48 hours, then infected with Ad5 at moi of 5 for 20 hours. Cultures were harvested for SDS-PAGE analysis and membranes were probed with polyclonal anti-late rabbit Abs or monoclonal anti-GAPDH mouse Abs.

To reach to a final conclusion about the effect of IFN response on Ad5 infection and because of the clear relationship between PML-II, IFN and E4Orf3 (Chen et al., 2015, Ullman et al., 2007), infection was evaluated in PML-II Kd and EV cells in the presence and absence of E4Orf3 with and without IFN treatment. The idea was that E4Orf3 deletion, as it counteracts the interferon antiviral effect, would make the mutant virus more sensitive to interferon treatment than the wild type, especially in the EV cells where the level of interferon response would be normal. PML-II Kd and EV cells were both treated with 1000 U/ml of IFN- α for 24 hours prior to

infection with Ad5wt300 or inOrf3 viruses at a moi of 5 for 20 hours. In essence, there was a clear increase in late viral gene expression in PML-II Kd cells for both viruses compared with the EV cells, this is consistent with all previous results as PML-II plays a negative role in the Ad5 life cycle (Fig.4.7A). The inOrf3 mutant displayed less gene expression in EV cells than the wild type and this is also expected as it lacks E4Orf3, the protein that compromises the interferon activity (Fig.4.7A). The wt300 was affected to a lesser extent with the interferon treatment than the mutant in both cell lines despite it was more striking in EV cells which is also the expected scenario as the control cells express more interferon than PML-II Kd cells (Fig4.7B). The IFN treatment had more effect on the mutant virus in both cell lines, especially in the control cells and it showed a more significant effect than the wild type as shown by the hexon band quantitation (Fig.4.7B). Hence, the mutant showed a greater level of late gene expression in PML-II Kd cells despite the interferon treatment because these cells are deficient in the interferon response due to the lack of PML-II protein. As an overall conclusion from this part of the work, the mutant was more vulnerable to the interferon treatment in the EV cells than in PML-II Kd cell, which suggests that the E4Orf3-PML-II interaction is necessary to overcome the antiviral function of interferon since the mutant was affected to a lesser extent when the interferon response was low due to PML-II removal.

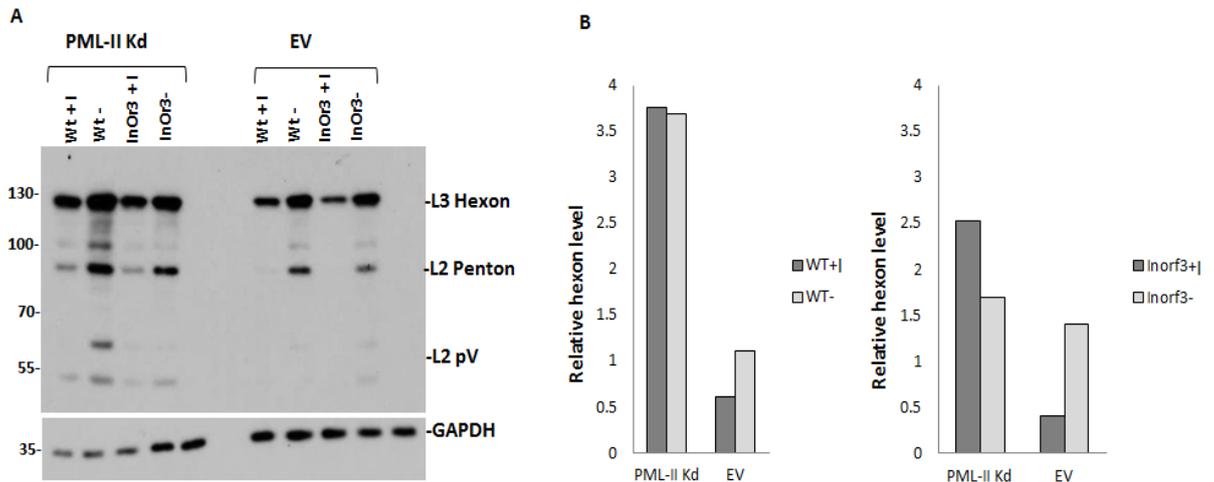


Fig.4.7. Late gene expression in PML-II Kd and EV cells with and without IFN type I treatment. **A.** PML-II Kd and EV cells were plated for 24 hours, treated or not with 1000 U of IFN - α for another 24 hours (+I,-) and infected with either wt300 or InOrf3 mutant at moi of 5 for 20 hours. Protein samples were harvested for WB analysis; the membranes were probed with polyclonal anti-late rabbit Abs or monoclonal anti-GAPDH mouse Abs. **B.** Hexon band density. The upper main band with a molecular weight of about 100 kDa was quantified using Quantity one software to compare its intensity in all 8 samples. The background of the negative film was subtracted and then the results were normalised to the related GAPDH bands, and then further normalised to the value for wt300 in EV cells with no treatment

4.3.2 Role of heat shock protein 70 (hsp70) in adenovirus gene expression during PML-II knockdown

It was mentioned in Chapter 3 that the level of hsp70 expression was significantly higher in PML-II Kd cells than in any of the control cell lines or standard HeLa cells. In addition, hsp70 is significantly induced during adenovirus infection (Kao and Nevins, 1983, Nevins, 1982) which suggests that it might be beneficial to the infection, since Ad5 down-regulates many host genes that are inhibitory (Dorn et al., 2005). It was therefore tested whether the increase in hsp70 levels in PML-II Kd cells was correlated with an increase in hexon expression in these cells. The aim was to do parallel transient knockdown of hsp70 by siRNA in both PML-II Kd and EV cells to investigate late gene expression in the presence and absence of hsp70. Cells were transfected with hsp70 siRNA or siC for 48 hours and infected with Ad5wt300 at a moi of 5 for 20 hours. The level of hexon mRNA expression, encoding the major late adenoviral structural protein, was significantly reduced following hsp70

knockdown in PML-II Kd cells, reducing it to more or less the same level seen in EV cells in the presence of hsp70 (Fig4.8A). Hsp70 knockdown also further reduced the already low level of hexon expression in EV cells. To further confirm the RNA results, protein analysis was carried out to compare the DBP and hexon expression by Ad5wt300 with or without hsp70, applying the same conditions as in Fig.4.8A in PML-II Kd cells. Consistent with the RNA analysis, removing hsp70 reduced to some extent the DBP expression compared to the siC sample (Fig.4.8B). A greater effect was detected on the level of hexon protein which was reduced to a very low level in comparison with the siC sample (Fig.4.8C). The overall conclusion is that the elevated hsp70 level in PML-II Kd cells is a significant factor in enhancing Ad5 infection in these cells.

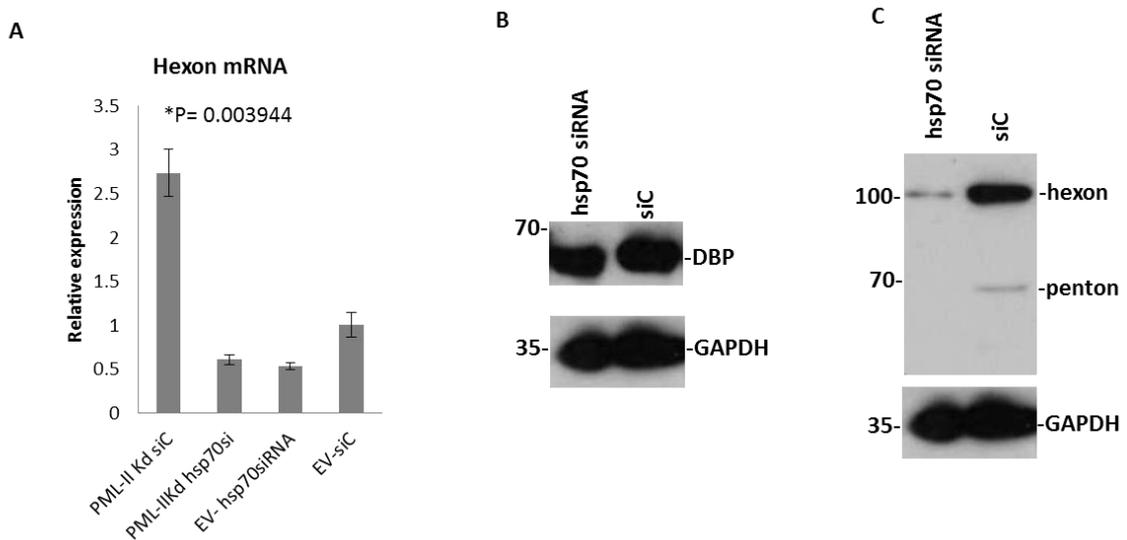


Fig.4.8. Hsp70 effects on Ad5 hexon expression. PML-II Kd and EV cells were seeded at a density of 1×10^5 /ml for 24 hours. Cultures were transfected with hsp70 siRNA or siC for 48 hours then infected with Ad5 wt300 at moi of 5 for 20 hours. **A.** RNA samples were extracted from each cell type, hexon mRNA expression was quantified by RT-qPCR using 10 ng/well of complementary DNA (cDNA) as a template. Data were calculated by the $\Delta\Delta C_t$ method, using β -actin as internal control, and then normalised to the control cell value (siC sample). All PCR products were subjected to dissociation curve analysis to ensure primer specificity. **B & C.** Protein samples were harvested for WB analysis, the membranes were probed with monoclonal anti-DBP mouse Abs, anti-late polyclonal rabbit Abs or anti-GAPDH mouse monoclonal. **B.** DBP expression **C.** Late protein expression.

4.3.2.1 HSP70 and the NF- κ B signalling pathway

NF- κ B is a transcription factor which is retained in the cytoplasm by I κ B α until it is activated by certain stimuli, which lead to phosphorylation and ubiquitination of I κ B α . These modifications lead to I κ B α degradation by the 26S proteasome (Chen et al., 1995). Hsp70 within the cell was found to preferentially bind the I κ B kinase IKK γ , preventing NF- κ B activation and thus abolishing its anti-apoptotic function (Ran et al., 2004). However hsp72 or hsp70A1A secreted from cells or applied externally could induce the NF- κ B transactivation and binding to DNA via TLR signaling (Chase et al., 2007).

Many facts were associated with this part of work. First of all, the elevated level of hsp70 in PML-II Kd cells, secondly the down-regulating effect of hsp70 on NF- κ B signaling (Cao et al., 2012, Ran et al., 2004, Senf et al., 2008) and finally the low level of NF- κ B response in PML-II Kd cells which was monitored by measuring the basal and inducible levels of IL-6 mRNA. Thus, the idea was to test whether the reduced level of hexon expression obtained following hsp70 removal belongs to the effects of the latter on TNF- α induced NF- κ B activation during Ad5 infection, since NF- κ B is generally thought to be inhibitory to virus replication (Brown et al., 2003). TNF- α potentially stimulates NF- κ B signalling and hence it was expected to oppose the effect of any hsp70 inhibition of the pathway.

Before starting the extensive experiment it was necessary first to determine the effect of TNF- α treatment on Ad5 gene expression at both RNA and protein levels. EV cells were transfected with hsp70 siRNA or siC for 48 hours and parallel samples were treated with 50ng/ml of TNF- α for 1 hour prior to infection with Ad5 wt300 for 20 hours at moi of 5. Surprisingly, the overall effect of TNF- α treatment was an increase in hexon mRNA expression (Fig.4.9A).

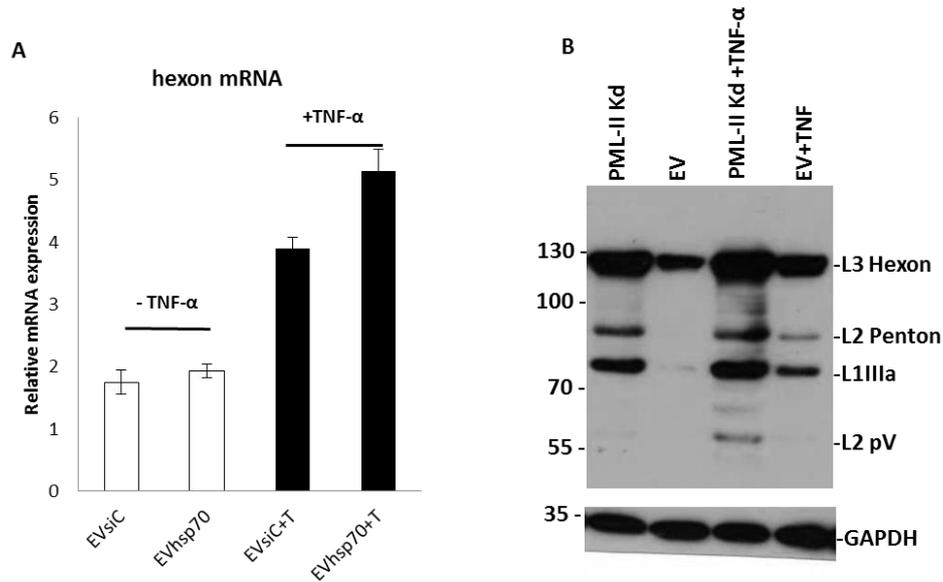


Fig.4.9. Ad5 hexon expression in PML-II Kd cells and EV cells with or without TNF- α treatment. PML-II Kd and EV cells were seeded at a density of 1×10^5 /ml for 24 hours. **A.** Cultures of EV cells were transfected with hsp70 siRNA or siC for 48 hours and either treated with 50 ng/ml of TNF- α for 1 hour or not prior to infection. All samples were then infected with Ad5wt300 at moi of 5 for 20 hours. RNA samples were extracted from each cell type, hexon mRNA expression was quantified by RT-qPCR using 10 ng/well of complementary DNA (cDNA) as a template. Data were calculated by the $\Delta\Delta C_t$ method, using β -actin as internal control, and then normalised to the control cell value (siC sample). All PCR products were subjected to dissociation curve analysis to ensure primer specificity. The white bars are the non- treated samples while the black bars are the TNF- α treated samples. **B.** Cultures of PML-II Kd and EV cells were treated or not with 50 ng/ml of TNF- α for 1 hour prior to infection. Protein samples were harvested for WB analysis, the membranes were probed with polyclonal anti-late rabbit Abs or monoclonal anti-GAPDH mouse Abs.

At the protein level, without hsp70 transient knockdown, the result was consistent with the RNA experiment; TNF- α treated samples in both cell lines showed more late protein expression than in untreated samples (Fig.4.9B). Thus although hsp70 interacts with NEMO to prevent NF- κ B activation, which was reduced in PML-II Kd cells (Ran et al., 2004), the hypothesized role of hsp70 in downregulating the TNF- α induced NF- κ B activation was excluded as the effect of TNF- α treatment on infection was totally the opposite of expectation.

Effect of NF- κ B blocking on Ad5 protein expression

As a further test of the role of NF- κ B activation in the adenovirus life cycle, the NF- κ B signalling pathway was abolished with the inhibitor QNZ. This chemical inhibits NF- κ B activation and TNF- α production in Jurkat cells with an IC₅₀ of 2nM and 3 nM respectively (Tobe et al., 2003). PML-II Kd cells and EV cells were either treated with 10 μ M of QNZ for 45 minutes or left without treatment then infected with Ad5 at moi of 5 for 20 hours. NF- κ B blockage by QNZ decreased the viral late gene expression in both cell lines (Fig.4.10). This is in agreement with the effect of TNF- α (Fig.4.9A&B): both treatments demonstrate a positive role of NF- κ B activation in adenovirus gene expression. These results suggest that the high hsp70 in PML-II Kd cells must favour Ad5 infection through mechanisms other than inhibiting the NF- κ B pathway.

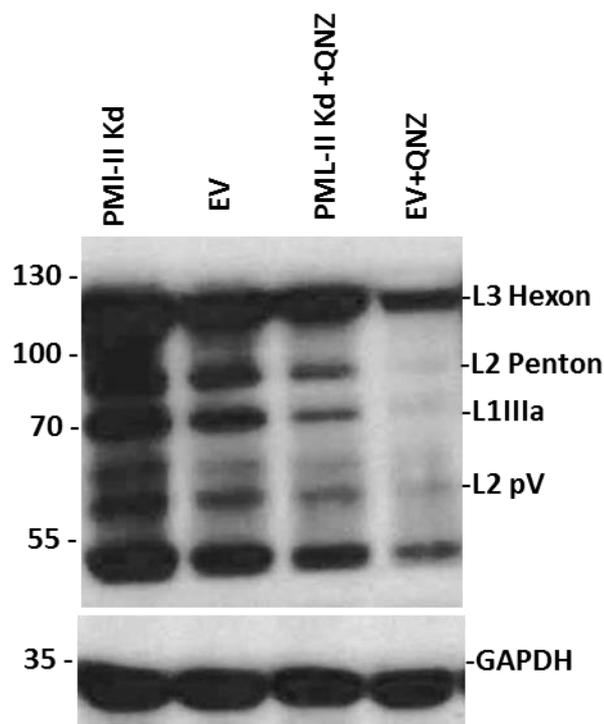


Fig.4.10. Late Ad5 gene expression in PML-II Kd and EV cells with blockage of the NF- κ B signalling pathway. PML-II Kd and EV cells were plated for 24 hours, cultures were treated with QNZ for 45 minutes or not and infected with Ad5 wt300 at moi of 5 for 20 hours. Samples were collected for Western blott analysis, the membranes were probed with polyclonal anti-late rabbit Abs or monoclonal anti-GAPDH mouse Abs.

In the light of these results it is not possible to conclude that increased expression of hsp70 caused an increase in Ad5 gene expression through down-regulating TNF- α induced NF- κ B activation. The results shown in figures 4.9 and 4.10 all come together to suggest a positive role of NF- κ B in the adenovirus life cycle and hence enhancing its gene expression. The fact that there was an increase in the level of late viral gene expression with TNF- α treatment in EV cells or with NF- κ B blocking in PML-II Kd and EV cells possibly indicates that the virus can benefit from the interaction with the innate immune response for its own transcription and replication. This is surprising given the virus encodes E3 functions that inhibit TNF- α signaling (Gooding et al., 1990), however the negative effect of TNF- α may only be manifest *in vivo* in the presence of a functional immune response. The virus might have evolved a mechanism by which it could take advantages from the cellular immune response, hence it can augment its own gene expression. This is actually consistent with a study performed by Korner et al., (1992). This study showed that treating 293 HEK cells with low doses of TNF- α for a short time increased the expression of E3/19K mRNA and protein which led to a reduction in HLA molecules on the cell surface (Korner et al., 1992). During adenovirus infection, the effect of TNF- α could be shifted to be through stimulating expression of the viral proteins that might neutralize the lytic effect of TNF- α which was used to treat the cells, as in this experiment, or the TNF- α that is already induces because of adenovirus infection (Pahl et al., 1996).

4.3.2.2 How does hsp70 affect Ad5 replication?

Within a short time post adenovirus infection, or upon heat shock or when the cell enters S phase, hsp70 is transported to the peri-nuclear and nuclear areas. This is probably as a part of hsp70 function in stabilising and re-folding abnormal or unfamiliar proteins. It was also found that hsp70 associates with the hexon of incoming virus particles (Milarski and Morimoto, 1986, Niewiarowska et al., 1992). Therefore, it was considered whether a part of the benefit to Ad5 of high hsp70 might relate to the localisation of late proteins. Cells were transfected with hsp70 siRNA or siC and then infected with Ad5wt300 for 16 hours. The cells were

lysed and the cytoplasmic and nuclear fractions were separated from each other. The general effect of hsp70 removal on the late gene expression seen earlier was replicated here since the hsp70 knockdown samples showed fainter hexon protein bands (Fig.4.11A). The hexon protein was found in reduced levels in the nuclear fraction, this conclusion was supported by band quantitation when the data were normalised to the internal cytoplasmic and nuclear loading controls. In contrast, hsp70 removal had little effect on DBP levels or distribution (Fig4.11B). GAPDH and Sp1, known cytoplasmic and nuclear proteins serve to show effective and equivalent fractionation between the two cell types.

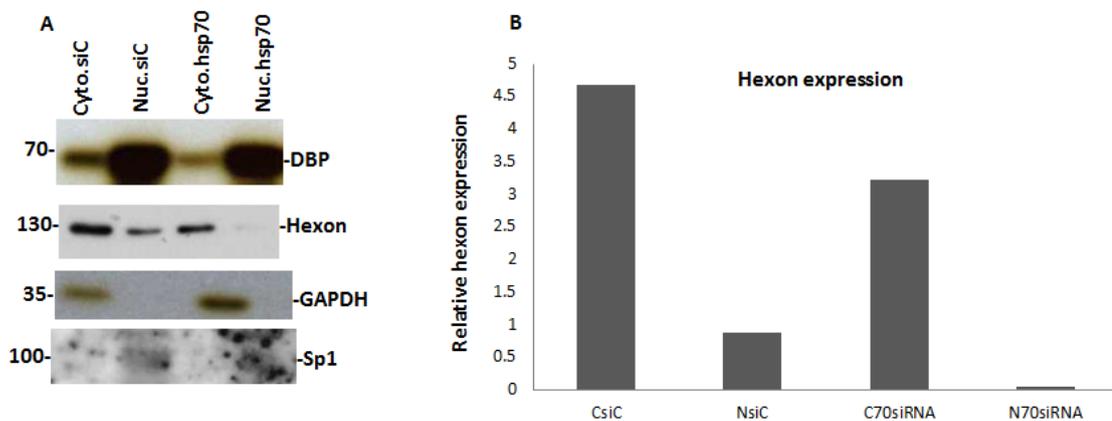


Fig.4.11. Cytoplasmic and nuclear distribution of hexon in correlation with hsp70 knockdown. PML-II Kd cells were plated for 24 hours at a density of 1×10^5 /ml, transfected with either hsp70 or siC for 48 hours, then infected with Ad5wt300 at moi of 5 for 16 hours. Cells were collected, lysed and cytoplasmic and nuclear fractions were separated and then lysed with 1x SB buffer, WB membranes were blotted against anti-late polyclonal rabbit Abs, anti-GAPDH or anti-Sp1 mouse monoclonal Abs. **A.** WB analysis. **B.** Hexon band quantitation from panel A, data were normalised to the cytoplasmic and nuclear proteins, GAPDH and Sp1.

This reduction in hexon movement into the nucleus as a result of hsp70 removal could be explained if absence of the latter prevents viral assembly. It is well-known that adenovirus assembly happens in the nucleus where the viral genome is replicating. So the removal of hsp70 might cause a defect in the assembly process. More plausibly, hsp70 promotes either trimerization of hexon polypeptides in the cytoplasm (which also needs L4 100K) or the association of hexon trimers with the

viral protein pVI, which is needed for their nuclear import (Matthews and Russell, 1995, Wodrich et al., 2003). These effects of hsp70 cannot however explain its significant effect on the level of late protein synthesis.

4.3.2.3 Effect of hsp70 on type I interferon response

It has been found that the removal of the highly expressed hsp70 during Dengue virus infection could increase the type I interferon response (Padwad et al., 2010). In order to investigate any broader effect of hsp70 on cellular cytokine expression, the idea was to analyse how hsp70 could affect the level of type I interferon response in mock and Ad5wt300 infected cells. PML-II Kd or EV cells were transfected with hsp70 siRNA or siC and then infected with Ad5wt300 for 20 hours or left as mock. In mock infected cells, PML-II cells showed much lower ISG56 expression than the EV cells (Fig.4.12A) which is very consistent with what was noticed before regarding the functional effect of PML-II isoform removal (Chen et al., 2015). Interference with hsp70 expression by siRNA increased the level of ISG56 expression in both cell lines with a noticeably greater increase in the control EV cells (Fig.4.12A). In a separate experiment with infected EV cells, the trend was similar since hsp70 removal strongly augmented the ISG56 expression (Fig.4.12B).

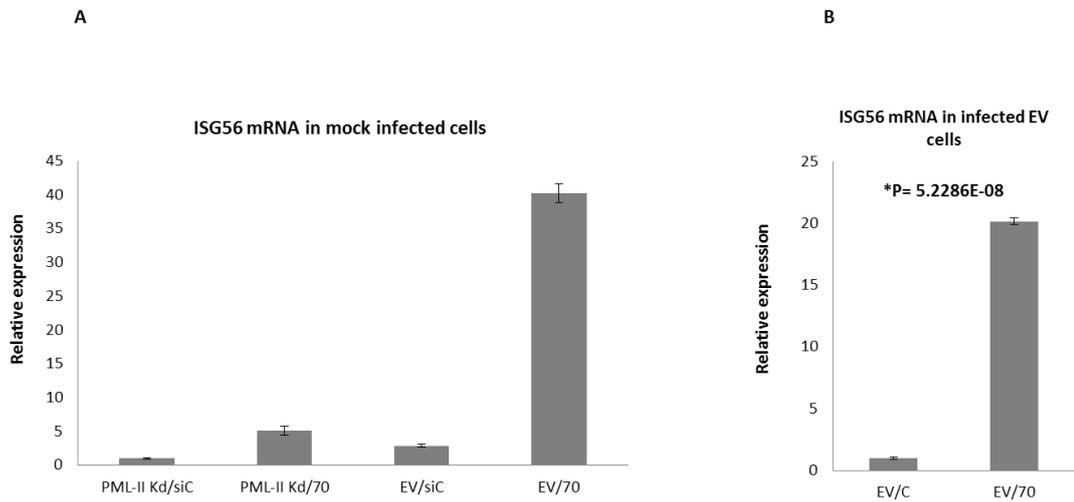


Fig.4.12 ISG56 expression in PML-II Kd and control cell lines with or without hsp70 knockdown. PML-II Kd and EV cells were seeded at a density of 1×10^5 /ml for 24 hours. Samples were transfected with hsp70 siRNA or siC for 48 hours. Samples were either infected with Ad5 wt300 at moi of 5 for 20 hours **B** or left as mock **A**. RNA samples were extracted from each, and ISG56 mRNA expression was quantified by RT-qPCR using 10 ng/well of complementary DNA (cDNA) as a template. Data were calculated by the $\Delta\Delta C_t$ method, using GAPDH or 18S RNA as internal controls, and then normalised to the siC-treated PML-II Kd value **A**. or the siC-treated EV value **B**. All PCR products were subjected to dissociation curve analysis to ensure primer specificity. Data show significant increase with hsp70 removal (*P<0.05, n=2).

This result agrees with the study of Padwad et al., (2010), in which Dengue infection triggered high level of hsp70 expression, and viral replication was reduced significantly with hsp70 knockdown associated with an increased level of IFN- α and protein kinase R (PKR) expression. Another possibility which could be suggested in that context is that the infection-induced type I interferon might suppress hsp70 expression. In Human T-Cell Leukemia virus type 1 (HTLV-I) infected primary cord blood –derived mononuclear cells, it was noted that viral infection could also increases hsp70 expression which could persist for at least 4 weeks. This increase in hsp70 expression was totally suppressed by treatment with IFN (D'Onofrio et al., 1993). In the present study, the decreased level of interferon response in PML-II Kd cells could result in an increased level of hsp70 expression as a consequence, and because of the importance of hsp70 in enhancing viral gene expression, there was much more viral gene expression in those knockdown cells. Consequently with hsp70 removal viral gene expression was diminished significantly and thus the antiviral response which is represented by the interferon response would dominate again.

4.4 Ad5 gene expression in PML-Kd cells

Having shown the negative effect of PML-II on Ad5 infection, it was important to test whether the presence or absence of other PML isoforms modified the effect of PML-II, in order to investigate the role of total PML in the adenovirus life cycle, PML Kd and control cells were infected at moi of 5 for 20 hours. Samples were harvested for either RNA or protein analysis. PML and PML-II mRNA was reduced in PML Kd cells compared to the control cell line (Fig.4.13A). The functional knockdown was significant since the interferon response, measured by ISG56 mRNA levels, was reduced to about 50% in PML Kd cells in comparison with the GFPKd and Ctrl.DNA cells (Fig.4.13A). In terms of the progress of adenovirus infection, it was obvious that hexon mRNA expression was significantly higher in PML Kd cells than in the control cells (Fig.4.13.C).

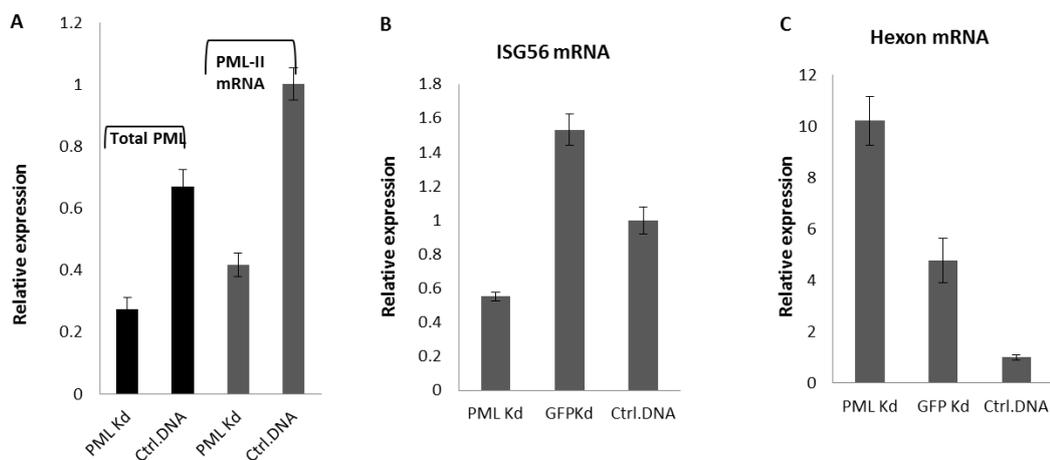


Fig.4.13. Late Ad5 gene expression in the presence and absence of total PML. PML Kd, Ctrl.DNA and GFP Kd cells were seeded at a density of 1×10^5 cell/ml for 24 hours. Cultures were left as mock for PML and ISG56 levels or infected with Ad5 wt300 at moi of 5 for 20 hours to detect hexon mRNA. RNA samples were extracted from each cell type, and mRNAs were quantified by RT-qPCR using 10 ng/well of complementary DNA (cDNA) as a template. Data were analysed by the $\Delta\Delta C_t$ method, using GAPDH or 18S RNA as internal control, and then normalised to the control cell value. All PCR products were subjected to dissociation curve analysis to ensure primer specificity. **A.** Total PML and PML-II mRNA depletion in PML Kd in comparison with Ctrl.DNA cells: black bars- exon3 specific primers (total PML); grey bars –PML-II specific primers. **B.** ISG56 mRNA in PML Kd in comparison to GFP Kd and Ctrl.DNA cells. **C.** Hexon mRNA expression in PML Kd in comparison to GFP Kd and Ctrl.DNA cells infected with Ad5wt300.

To confirm this effect, early and late viral protein expression were evaluated by western blotting. DBP expression was slightly increased in PML Kd cells compared with its level in Ctrl.DNA cells (Fig.4.14.A). Late proteins, which are represented by the major protein, hexon, and the vertex protein, penton, were expressed to significantly higher levels in PML Kd cells in comparison with the control cells (Fig.4.14.B). When the multiplicity of infection was increased to 50 to increase the infectability of Ctrl.DNA cells, the same trend of change in DBP expression was noticed (Fig.4.14.C). However, although protein expression had increased to some extent in PML Kd cells, it was not as dramatic as when analysed at a moi of 5 (Fig.4.14.D). This may be explained as high multiplicity of infection can overcome/overwhelm cellular defects. This could be related to the fact that adenovirus infection alters the distribution and morphology of PML-NB. At the later stage of Ad5 infection and when mature viruses started to be synthesized, the PML started to disappear from the NB bodies and inter-chromatin granule-associated zones where it was located initially in mock or early infected nuclei. In fact, this may suggest that this virus targets those proteins to abolish their antiviral function as they are interferon up-regulated proteins (Puvion-Dutilleul et al., 1995).

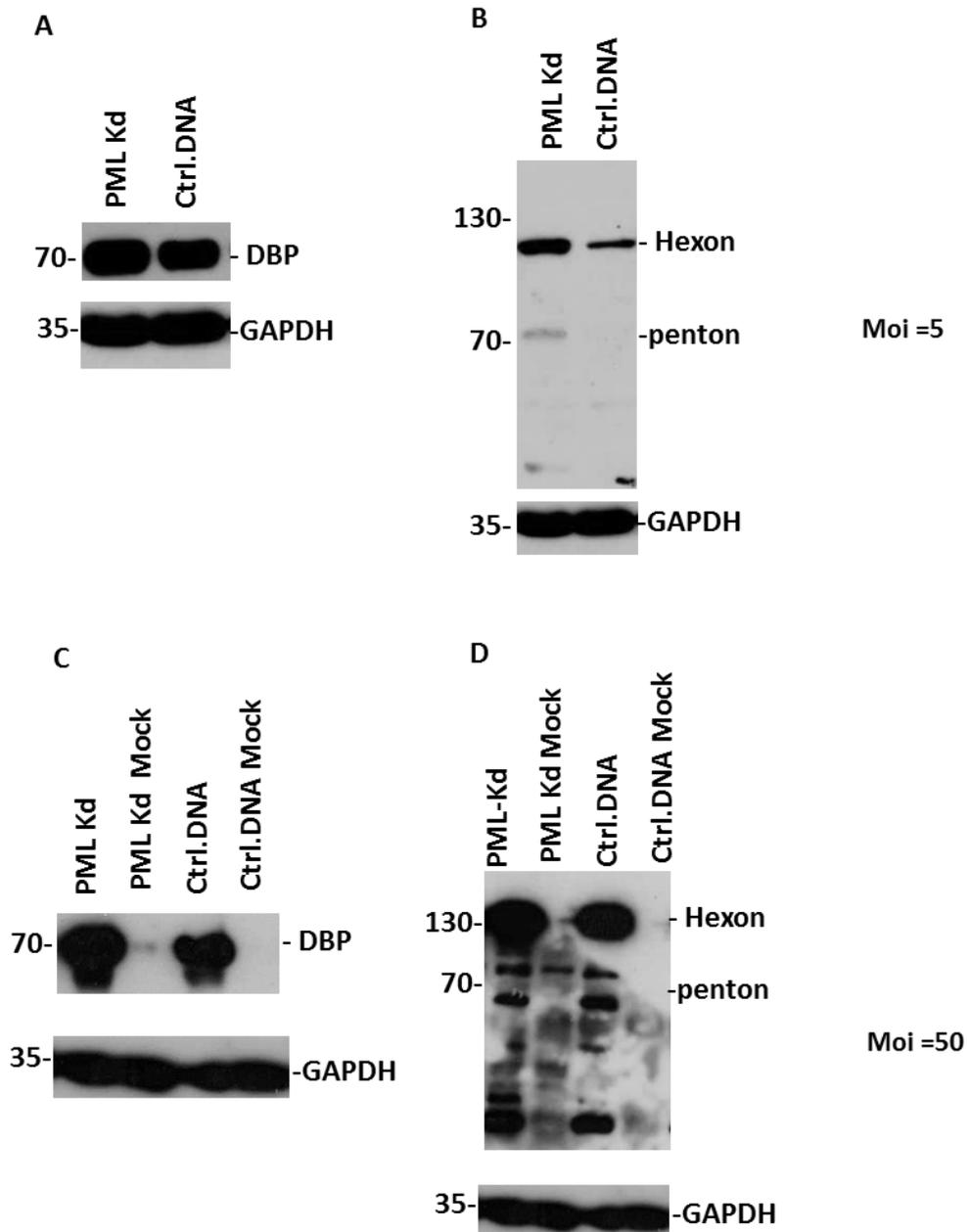


Fig.4.14. Late Ad5 gene expression in the presence and absence of all PML isoforms. PML Kd and Ctrl.DNA cells were plated at density of 1×10^5 cell/ml for 24 hours then either left as mock or infected with Ad5wt300 for 20 hours at moi of 5 or 50. Cultures were harvested for WB analysis with 1xSD buffer and blotted with either monoclonal anti DBP-antibodies, polyclonal anti- late antibodies or monoclonal anti-GAPDH mouse monoclonal Ab. **A.** DBP expression at moi of 5. **B.** Late gene expression at moi of 5. **C.** DBP expression at moi of 50. **D.** Late gene expression at moi of 50.

4.4.1 Quantitative analysis of early gene expression in PML Kd and Ctrl.DNA cells

In order to quantify early gene expression in PML Kd and Ctrl.DNA cells, FACS analysis was used. Cells were plated for 24 hours, infected with Ad5 at moi of 5 and samples were fixed and stained to measure DBP expression in both cell types. Consistent with the RNA and protein results, FACS analysis showed a small increase in DBP expression in PML Kd cells compared to Ctrl.DNA cells: percentages of cells that expressed DBP were 32.56% in the PML Kd population in Ctrl.DNA cells (Fig4.15A&B).

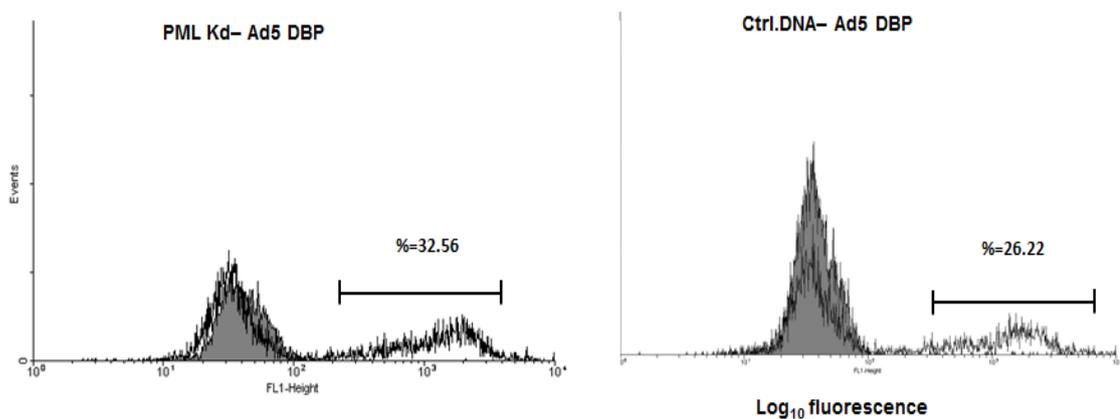


Fig.4.15. FACS analysis of early gene expression in PML Kd and EV cells. Cells were plated at a density of 0.8×10^6 or 2.2×10^6 per dish in 60 and 100 mm² dishes respectively for 24 hours. Cells were infected at moi of 5 with Ad5wt300 for 20 hours. Cells were then fixed, permeabilized and stained with anti-DBP monoclonal Abs as primary antibodies, followed by Alexa-fluor488 Gt anti-Mouse Abs staining. **A.** DBP fluorescence in PML Kd cells . **B.** DBP fluorescence in Ctrl.DNA cells. The grey curves represent the mock infected cells (background) while the black curve represent the positive infected cells.

This is consistent with the analysis of DBP fluorescence in PML-II Kd cells compared to EV cells (Fig4.4) in which the percentage of cells that showed DBP positive staining was 50.79% in PML-II Kd cells in comparison with 44.1% in EV cells. To summarize, it seems that total PML proteins play a negative role in Ad5 late gene

expression as viral gene expression increases with PML removal. This removal of course includes removal of PML-II, shown earlier to be important in determining the productivity of infection.

4.5 Discussion

The functional knockdown of all PML isoforms via the common PML exon3 target displayed a clear increased level of hexon RNA and protein expression compared to the control cells. However, this increased viral gene expression with total PML removal was less dramatic than the effect of PML-II removal alone. This suggests that some aspects of PML function lost in an exon 3 knockdown are beneficial to the virus and potentially offset the negative influence of PML-II. This result is very consistent with what is mentioned in the literature about the interrelation between PML & PML-NB and DNA & RNA viruses. One of the major functions of PML proteins is an involvement in antiviral responses against the invading viruses. PML proteins comprise some of many proteins that are upregulated with the interferon treatment (α , β and γ) in different human cell types and they could mediate their antiviral effect through IFN-induced responses (Chelbi-Alix et al., 1995, Regad, 2001b). Those proteins are involved in confronting the virus infection invitro and in vivo. For example, it was found the replication rate of influenza virus is decreased with the overexpression of either PML-IV or PML-VI in human adenocarcinoma Caco-2 cells. This was further confirmed by the enhanced replication of influenza virus with the transient knockdown of PML in Caco-2 cells (Iki et al., 2005). It was also shown PML^{-/-} mice were more susceptible to infection with LCMV than normal mice: PML suppressed viral multiplication, since virus titers were 50 times more in PML^{-/-} mice than the control group (Bonilla et al., 2002). PML-II knockdown showed consistent effects on the Ad5 life cycle in both transient and permanent knockdown scenarios. In transient interference with PML-II mRNA in HeLa cells, there was greater hexon expression and increases in viral DNA copy number. This was further confirmed by experiments in stably knocked-down cells in which early and late viral RNA and protein expression were increased significantly and consistently in repeated experiments. The yield of infectious progeny virus was affected in a

parallel way and showed significant increase over two time points (24 and 48 hours) with PML-II removal.

Quantitatively, PML-II Kd cells showed a greater percentage of cells expressing late proteins following infection than the control cells. These results agree with a number of studies that have pointed to the involvement of PML-II in restricting viral gene expression or replication. For example, in HuH7 cells both transient interference with PML-II or over-expressing of PML-II suggested a negative role for PML-II in the AAV life cycle. While knockdown of PML-II increased transduction by recombinant AAV, PML-II overexpression resulted in inhibition of vector production, genome replication and second strand synthesis. Furthermore, the production and infectivity of wild type AAV2 was inhibited by over-expression of PML-II (Mitchell et al., 2014). However, the conclusion that PML-II is a negative regulator of the Ad5 life cycle is totally contradictory to the work of Berscheminski et al., (2013), who defined PML-II as a coactivator of E1A-13S transactivation (Berscheminski et al., 2013). In fact there are many basic differences between this present study and the work of Berscheminski et al., (2010). The latter study mainly depended on exogenous over-expression of E1A13S or PML-II or both. Even when its ability to activate adenovirus transcription was analysed in cooperation with endogenous PML, PML-II was not able to activate the transcription of E2 region when it was overexpressed alone. The increase in transcription was detected only when an E1A 13S plasmid was coexpressed with PML-II.

It was necessary to understand how PML-II could affect Ad5 gene expression and replication. The straightforward explanation for the greater progress of infection in the absence of PML-II would be a decreased level of the type I interferon response (Chen et al., 2015). However this was not the only reason since blocking the interferon response by IRF3 knockdown in EV cells did not mimic the effect of PML-II removal, i.e. it did not reduce the gap in viral gene expression between PML-II Kd and EV cells significantly. This factor was also demonstrated to be an effective agent when both wt300 and InOrf3 mutants were treated with IFN- α 24 hours prior to infection as the mutant was less affected in PML-II Kd cells where the interferon response was lower.

The other conceivable factor that might have contributed here is the greatly increased level of hsp70 in PML-II Kd cells when compared with control cells. Removal of this factor had a greater contribution in increasing viral gene expression than the interferon blocking state. The hsp70 chaperone seems important in translocating the hexon from the cytoplasm to the nucleus and hence its removal probably reduced the rate of virus assembly and maturation. However, whilst potentially of some significance in the productivity of infection, this cannot explain the positive effect of PML-II removal on hexon expression.

The increased level of hsp70 expression, the decreased level of NF- κ B response in PML-II Kd cells and finally the published role of hsp70 in down regulating TNF- α induced NF- κ B activation, collectively, motivated us to attempt to correlate the hsp70 increase with the decreased TNF- α induced NF- κ B activation. To test this idea, it was important to define the role of an activated or blocked NF- κ B response in the Ad5 life cycle. In fact, this signalling pathway proved to be beneficial to the virus given that viral gene expression was increased with TNF- α treatment or decreased with NF- κ B blockage, a finding which discounted a beneficial effect of hsp70 via inhibition of NF- κ B. This result could be explained by the observation that NF- κ B could negatively regulate the interferon response and IFN-stimulated genes. However, it is important to recall that the weight of evidence is for NF- κ B acting positively in the IFN response.

It was possible that PML-II might regulate the IFN response through elevating the level of hsp70 expression, i.e. that the reduced IFN response was due to elevated hsp70. However, hsp70 knockdown actually increased the level of ISG56 mRNA in both cell lines under either mock or infection conditions. This result disagrees with a study that pointed to a positive correlation between IFN and the hsp70 response. In genetically modified mice (stably expressing hsp70 in neurons), a high level of IFN response was detected in comparison with the non-modified mice (Kim et al., 2013). However, most of these studies were achieved in mouse immune cells and others were performed by using recombinant hsp70 prepared in *E.coli*. This preparation of hsp70 in bacteria is likely to be contaminated with lipopolysaccharide which is recognised as a cytokine inducer through a Toll-like receptor 4-mediated signal transduction pathway and it was shown that TNF- α

induction was completely due to this contaminating factor (Gao and Tsan, 2003). In contrast, the results presented here are consistent with those of D'Onofrio et al.,(1993), who found that increased hsp70 expression in HTLV-1 infected primary cord blood-derived mononuclear cells was suppressed IFN treatment.

Regardless of which mechanism could dominate in this augmented gene expression or replication, the data produced here shown and robust evidence supporting the idea that PML-II plays a negative role in the Ad5 life cycle at the normal growth temperature (37°C).

To summarize, both the reduced interferon response and the increased hsp70 expression are contributing to enhanced Ad5 gene expression in the absence of PML-II. Despite the fact that both IFN and NF-kB responses cooperate with each other against the viral infection (Hiscott, 2007), and both of them were low in PML-II Kd cells, it is possible, that in Ad5 infection, NF-kB activity was altered and started to work in favour of productive virus infection.

Chapter 5 : Role of PML-II and mild heat stress in adenovirus gene expression

5.1 Introduction

5.1.1 Heat shock upregulated genes in HeLa cells

Exposing HeLa cells to high temperature (42°C) can increase the expression of a large set of genes, especially the heat shock genes that encode for chaperones which assist in protein folding and their cofactors. These highly expressed proteins which show about 10 fold upregulation from their normal level include: hsp70, hsp40, hsp105, hsp110, hsp47 (SERPINH2), and a modulator of hsp70 activity (BAG3). Another group of proteins, induced between 4 to 10 fold by heat stress, comprise regulators of chaperone function such as Hsp70-interacting protein ST13, Hsp90 co-chaperone TEBP, and FK506 binding protein 4. All other groups of genes that are specifically elevated by heat shock to levels similar to the chaperone regulators are listed in Table 5.1.

Table 5.1. List of genes that are up-regulated to a similar level of HSPs regulators (fold change 4 to 10) (Murray et al., 2004)

	Function	Gene name
1	Post translational modification	transglutaminase and proline 4-hydroxylase α 1 and 2
2	Protein degradation pathways	proteasomal subunit PSMD10 and Siah-interacting protein
3	Signal transduction	G protein GNAS, transcription factor GABPB2 and GADD45 α
4	Membrane transport and signalling	phospholamban, monoamine transporter SLC22A3, and leukocyte receptor cluster 4
5	Metabolic processes	ArylamineN-methyltransferase and a cytochrome b ₅ reductase

In a unique pattern for cancer cells and not primary cells, heat-shocked HeLa cells display no evidence for any cell cycle arrest. They showed more accumulated transcripts of signal transduction molecules and transcription factors such as c-fos

and c-jun between 8 and 24 hours after heat shock (Murray et al., 2004). Activating transcription factor 3 (ATF3) was one of the most up-regulated genes in 43°C heat-shocked hepatocytes which were left to recover for 2 hours (Kus-Liskiewicz et al., 2013). This factor has an important role in protecting cells from UV- induced apoptosis and has a suggested role in cell cycle arrest as it helps fibroblasts transition from a growth phase to another (Lu et al., 2006).

5.1.2 Cellular response to adenovirus infection

Adenovirus infection leads to a regular decline in cellular transcription and translation while the virus keeps its own transcription increasing, ultimately dominating cellular activity. In a DNA microarray experiment that dissected viral and cellular gene activities during Ad12 infection, one of the remarkable negative impacts of infection was the diminished expression of MHC I genes (Dorn et al., 2005). This effect was noticed earlier when MHC I expression was reduced in cells in cells infected with Ad12 or cells that are expressing E3/19k protein. This reduction is due to the binding of E3/19k to HLA in a mechanism by which adenovirus evades the immune response through affecting the cytotoxic T cells response (Burgert and Kvist, 1985). Expression of MHC I is further inhibited with TNF- α stimulation since the latter increases E3/19k transcription (Deryckere and Burgert, 1996).

Time post Ad12 infection was critical to determine which group of genes was up-regulated or down-regulated and how. Regarding the immune response, the related genes: IFN- α inducible protein (GIP2), IFN- α inducible protein with tetratricopeptide (IFIT2) and IFN- α induced protein with tetratricopeptide 1 (IFIT1) all were down-regulated at later time points, 24, 32 and 48 hours post-infection. In contrast, at an earlier time point (12 hours post infection), GIP2, IFTI2 and IFTI1 were strongly up-regulated to 10 to 30 fold. IL-6 expression was up-regulated after 24 hours post-infection and increased about two-fold from its original level at 12 hours post infection (Dorn et al., 2005). This explains how adenovirus manipulates the cellular machinery in order to exploit the advantageous factor and escape the innate immune response.

5.1.3 The shared features between the cellular response to adenovirus infection and heat shock

In fact, both stimuli of adenovirus infection and heat shock cause up-regulation of certain transcription factors which eventually modulate the production of interferon and other cellular cytokines. For example, cells respond to these two stresses by increasing the level of c-fos transcription within about 8 hours post infection or stress. This transcription factor helps to enhance the viral ability in transformation and at the same time promotes the interferon response through an AP-1 dependent mechanism (Murray et al., 2004, Su et al., 1995)

5.1.4 PML, c-fos and AP-1 activation

It has been reported that PML and c-fos can cooperate with each other to enhance the AP-1 transcriptional activity in Cos-1 cells (Vallian et al., 1998b). This correlates PML and c- fos with a probable role in enhancing the interferon response through AP-1 transcriptional activation.

5.2 Aims of the chapter work described in this Chapter

Linking together the similarities in the cellular response to both heat shock and adenovirus infection could be beneficial in preparing the cell to counter the viral infection. If stress renders cells to become more resistant to virus infection, would PML-II have a role in that alteration in susceptibility to infection? The approach to test this hypothesis was to expose HeLa cells to a slightly elevated temperature and then to analyze the outcome of infection after such stress and normal conditions in presence and absence of PML or PML-II.

The experimental design was to heat shock HeLa cells to 40 °C for 3 hours, then return them to 37 °C before infecting them with Ad5wt300 for 20 hours and finally analyzing viral gene expression. In parallel, the effect of heat shock on the morphology and expression of PML-NB and PML proteins respectively was investigated to confirm that mild heat stress did affect these components. The last part of the chapter is to investigate the effect of heat shock on virus infection in the

the presence and absence of PML or PML-II and to determine whether any effects correlate with effects on the innate immune response.

5.3 Elevated temperature affects the progress of adenovirus infection

In order to compare the development of adenovirus infection in cells that had experienced high or normal growth temperature, confocal microscopy analysis was used to compare the stages of infection achieved by a standard Ad5 dose in stressed and control cells. Adenovirus infection proceeds through characteristic stages whereby DBP is initially diffuse in the nucleus then forms increasingly numerous replication centres before finally late proteins accumulate in the cytoplasm. Two groups of cultures were either stressed for 3 hrs at 40 °C or left as controls at 37°C. Both cultures were infected at 37°C with Ad5wt300 at moi 10 and incubated for 20 hrs, then cells were fixed and reacted with antibodies against the DBP and late adenovirus proteins. The effect of pre-exposure to elevated temperature was more significant for late gene expression than the early stage. Immunofluorescent antibody staining showed more cells were in the late stage with more late protein expression (red staining) in each cell in the representative fields (Fig 5.1).

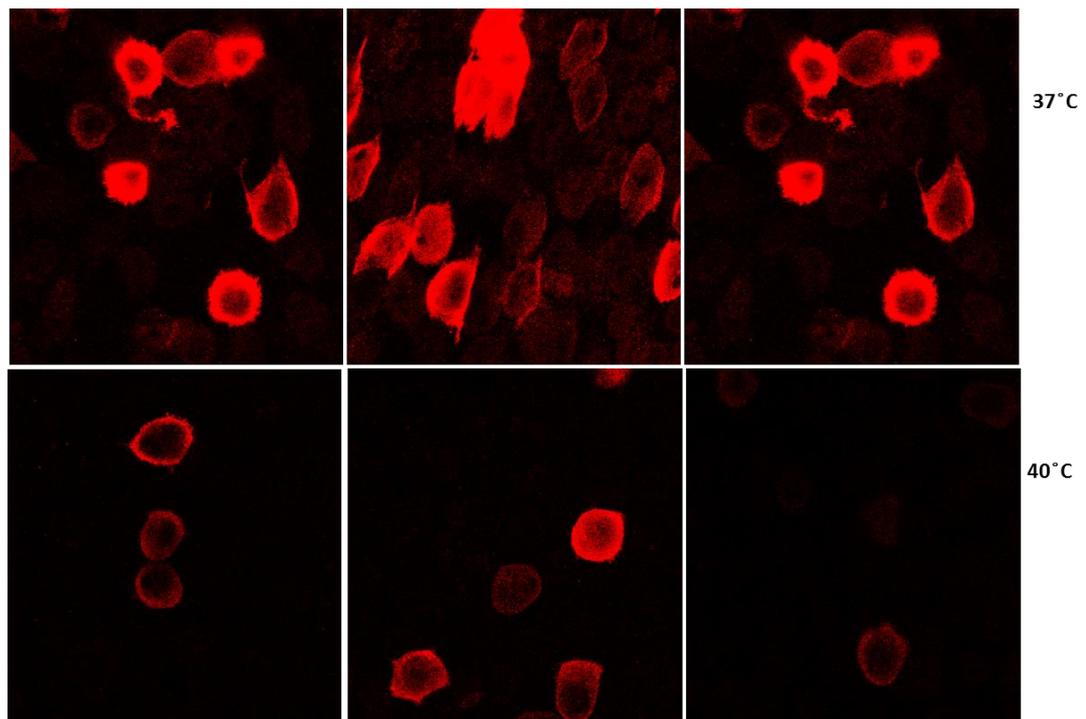


Fig.5.1. Ad5 late gene expression in control and heat shocked HeLa cells. Equivalent cultures (1×10^5 cell/ml) of HeLa cells grown on cover-slips in 12- well plates were either stressed at 40°C for 3 hrs, or maintained at 37°C throughout (control). All cultures were then infected at 37 °C with Ad5 wt at moi of 10, fixed at 20 hrs post-infection, permeabilized and stained with anti-late polyclonal Abs as primary Abs followed by staining with Alexa-fluor 594 Gt α Rb Abs. Representative Fields is shown for each condition.

In contrast only, a slight decrease was noticed in the number of cells showing DBP expression in the previously stressed cells compared to those which had been grown throughout at the normal growth temperature. To some extent there were more cells that showed more and larger replication centres in the control cultures in comparison with the stressed cultures (Fig.5.2).

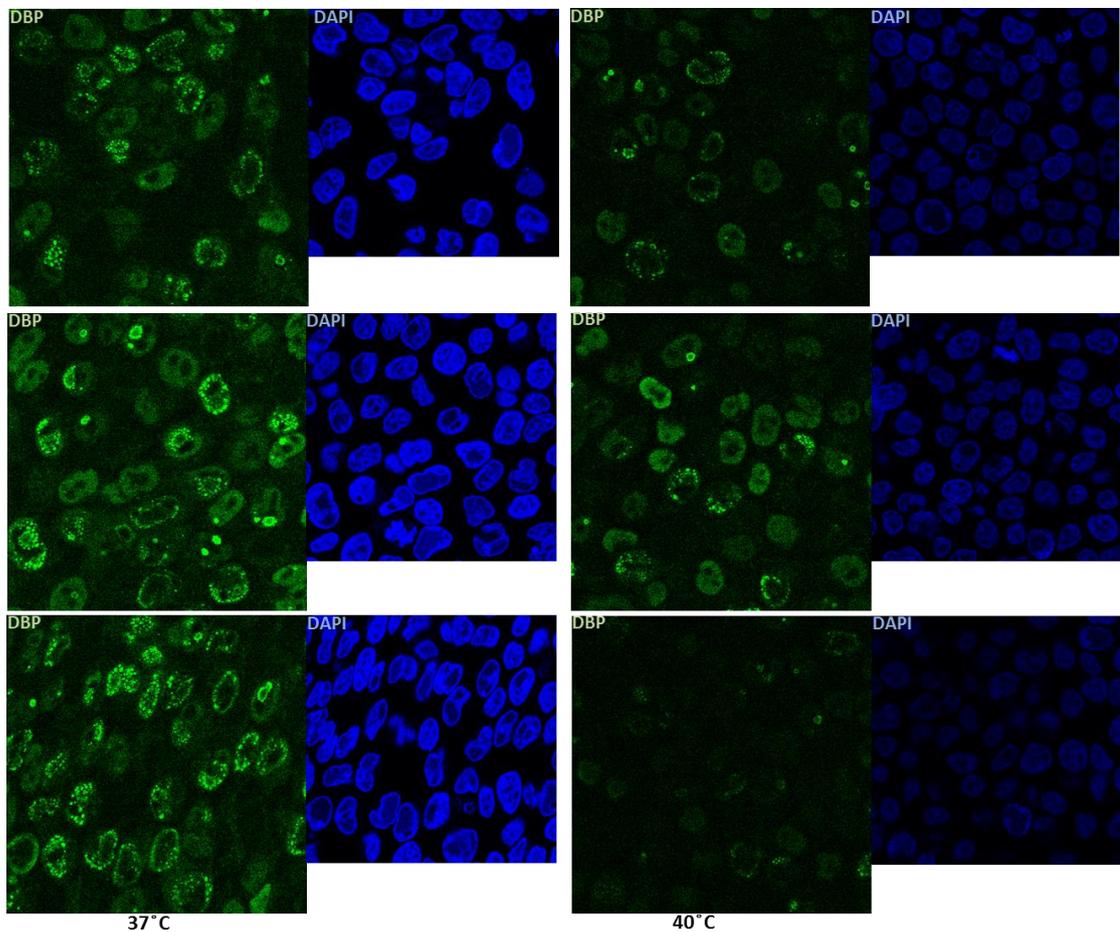


Fig.5.2. Ad5 DBP expression in control and heat shocked HeLa cells. Corresponding cultures (1×10^5 cell/ml) of HeLa cells grown on cover-slips in 12- well plates were either stressed at 40°C for 3 hrs, or maintained at 37°C throughout (control). All cultures were then infected at 37°C with Ad5 wt at moi 10, fixed at 20 hrs post-infection, permeabilized and stained with anti-DBP monoclonal Abs as primary Abs followed by staining with Alexa-fluor 488 Gt α M. Abs. Three representative field is shown for each condition.

To substantiate the effect of prior heat stress on Ad5 gene expression and since the effect of treatments and infections were clearer and more consistent at lower multiplicities, a lower multiplicity of infection was used and Western blotting analysis was implemented as another approach to verify the immunofluorescence data. Comparable cultures were either heat shocked or left to grow at normal conditions, then infected at 37°C with Ad5 at moi of 2.5 for 20 hours. Both immunofluorescence and WB data were consistent with the previous results (Fig.5.3 A and

B). Random fields from stressed or control samples were selected to count the percentage of cells that show late gene expression. Percentages of cells that showed late gene expression was more in the control cells while it reduced to 30% of its value in the stressed cells. The WB result supported the IF data: hexon and penton bands had diminished with the stress compared to their expression in the control cells (Fig 5.3B).

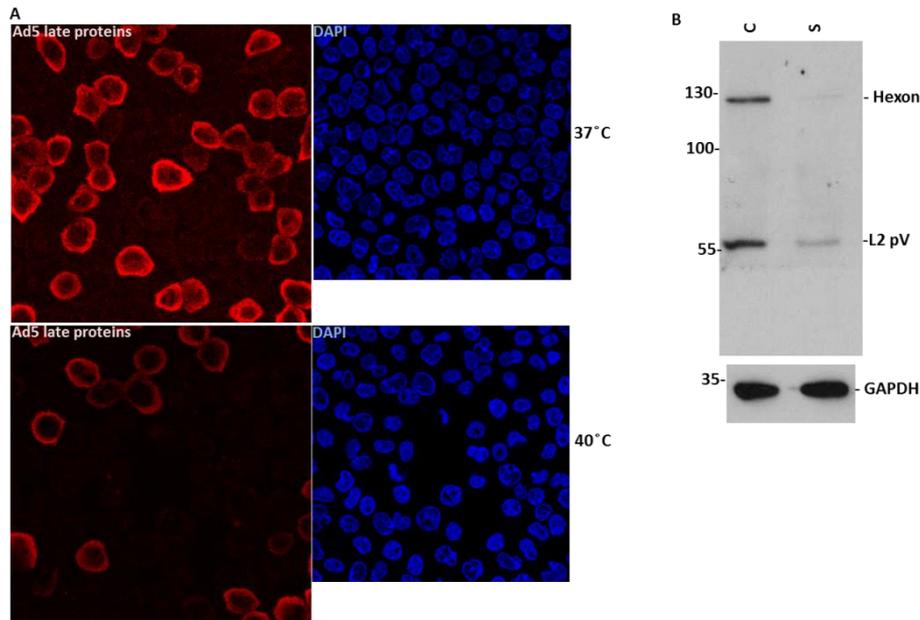


Fig.5.3. Ad5 gene expression in stressed and normal conditions. HeLa cells were seeded at a density of 1×10^5 /ml for 24 hours either direct to the bottom of plate or on cover-slips. Cultures were either heat shocked for 3 hours or left to grow at normal temperature. Cultures were infected with Ad5 at moi of 2.5 for 20 hours. **A.** Cells were fixed at 20 hrs post-infection, permeabilized and stained either with anti-late polyclonal Abs as primary Abs followed by staining with Alexa-fluor 594 Gt α Rb Abs. **Upper panels:** culture were infected after incubation throughout at 37 °C, **Lower Panels:** were infected at 40 °C. **B.** Protein samples were harvested for WB analysis, analysed by SDS-PAGE and western blotting. The membranes were probed with the appropriate Abs. **Upper panel.** Anti-late polyclonal rabbit Abs; **lower panel:** anti-GAPDH mouse monoclonal Abs.

5.3.1 Hexon mRNA expression at normal and stress condition

With the intention of analysing the effect of heat stress on the development of Ad5 infection in cells previously grown at normal or elevated temperature, hexon mRNA expression was measured at two time points post-infection. Two parallel cultures of HeLa cells were prepared, one was stressed for 3 hours at 40 °C while the other was incubated as control at 37 °C. Both cultures were infected with at 37 °C Ad5 at

moi of 5 and samples were harvested at 12 and 16 hours post-infection. At 12 hours post infection in stressed cells there was a reduction in hexon mRNA to 65% of its level in non-stressed cells. The reduction continued at 16 hours post infection but with a less dramatic difference, since hexon mRNA was reduced by stress by about 40% (Fig.5.4).

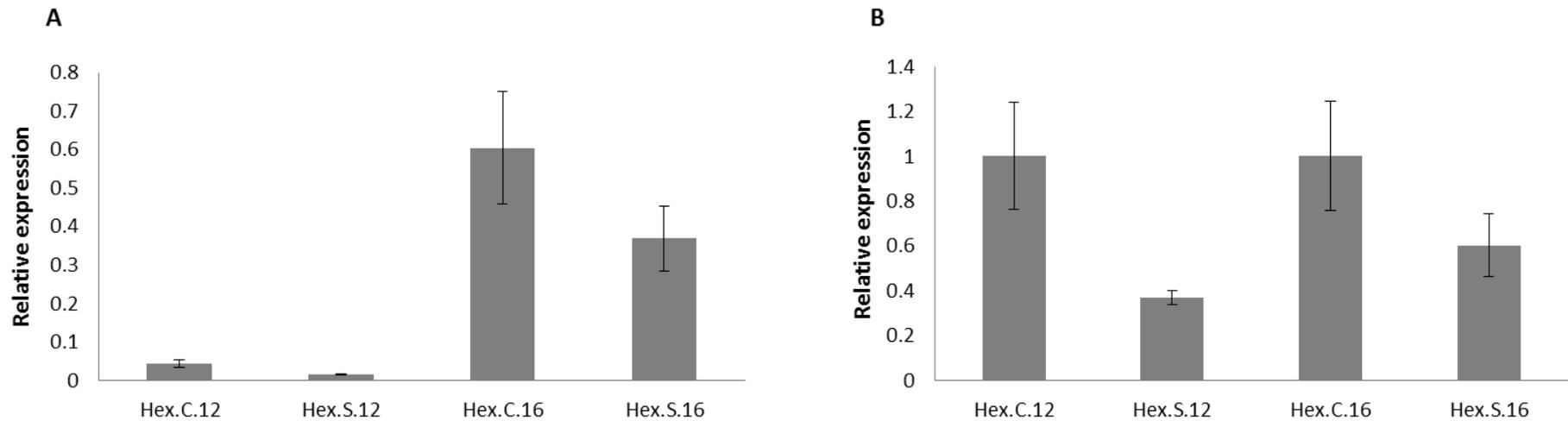


Fig.5.4. Ad5 hexon expression in control and heat shocked HeLa cells. . Parallel cultures (1×10^5 cell/ml) of HeLa cells were plated in 12- well plates and either stressed at 40°C for 3 hrs, or maintained at 37°C throughout (control). All cultures were then infected at 37 °C with Ad5 wt at moi and RNA was harvested at 12 and 16 hours post infection. Hexon expression was quantified by RT-qPCR using 10 ng/well of complementary DNA (cDNA) as a template. Data were calculated by the $\Delta\Delta C_t$ method, using GAPDH as internal control, and then normalised to the control cell value. All PCR products were subjected to dissociation curve analysis to ensure primer specificity. **A.** Hexon expression in cells previously cultured at 37 °C (C) and 40 °C (S). **B.** Data from panel A normalised to the control value at each time point.

5.3.2 Progress of Ad5 infection in MRC5 cells in normal and stress conditions

To test whether the reduced Ad5 gene expression observed after heat shock was a cell-line specific phenomenon or was a broader effect that could be generalised to normal cells, the same stress and infection conditions were applied on MRC5 human fibroblasts. Heat treatment reduced the level of hexon mRNA by around 80% of the level seen in infected cells maintained throughout at normal temperature (Fig 5.5A). The same trend of effect, but less dramatic, was noticed on early gene expression: E1A mRNA was reduced by 45% in the stressed cultures compared to the control (Fig 5.5B). This result is consistent with the previous results which were obtained with HeLa cells after exposing them to the higher temperature

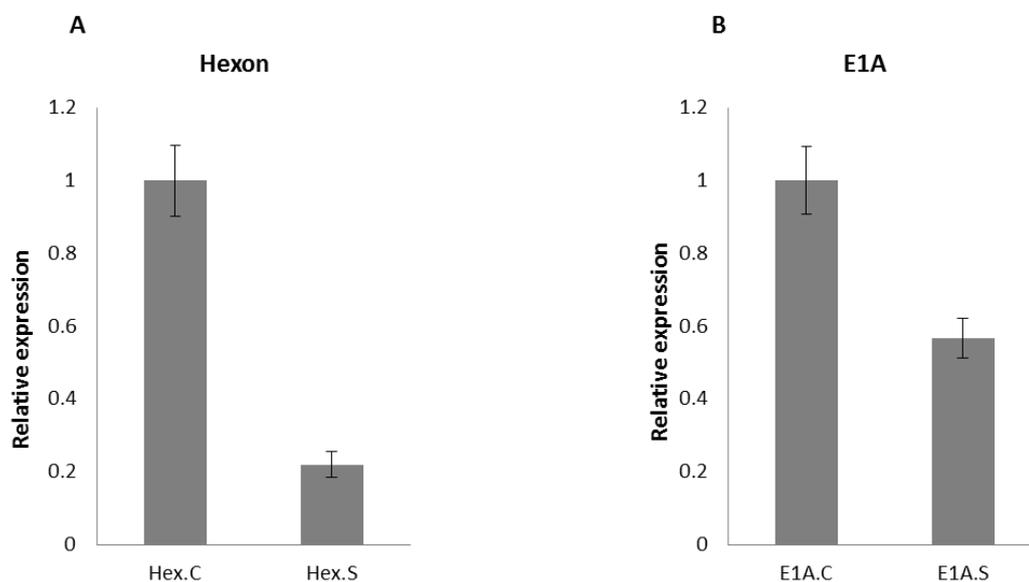


Fig.5.5. Ad5 hexon and E1A expression in control and heat shocked MRC5 cells. Parallel cultures (1×10^5 cell/ml) of MRC5 cells were plated in 12- well plates and either stressed at 40°C for 3 hrs, or maintained at 37°C throughout (control). All cultures were then infected at 37°C with Ad5 wt at moi 5 for 20 hours. Hexon and E1A expression were quantified by RT-qPCR using 10 ng/well of complementary DNA (cDNA) as a template. Data were presented by the $\Delta\Delta C_t$ method, using 18S RNA as internal control, and then normalised to the control cell value. All PCR products were subjected to dissociation curve analysis to ensure primer specificity. **A.** Hexon expression after prior culture at 37°C (C) and 40°C (S). **B.** E1A expression after prior culture at 37°C (C) and 40°C (S).

5.3.3 Quantitative analysis of late gene expression in stressed and unstressed cells

All the previous results showed that mild heat shock decreases Ad5 late gene expression. It was therefore necessary to quantify the difference in viral gene expression to verify these qualitative results. To quantify the IF data previously shown in Fig 5.3, percentages of cells that displayed Ad5 late expression were counted in heat-shocked cultures or the control. The number of infected cells was divided by the total number (DAPI stained cells) in each experiment. The previously stressed cultures showed a slight decrease in percentages of cells that express the late protein (14%) compared to the control which reached to 22% (Fig.5.6 A). However, this assessment was rather subjective, based on assigning individual cells to be either positive or negative.

The use of more robust approach (FACS) which could count thousands of infected cells in a few seconds and which provides an idea about the intensity of the viral protein expression in the population of positive cells, would give an accurate comparison between the heat shocked and the normal cell populations. The infected cells (black curves) are distinguished from the mock infected (the grey filled background curves), this separation allows the acquisition of absolute numbers of each positive (infected) and negative (mock) populations.

Initially, the appropriateness of the Abs was tested with fixed and unfixed cells. At moi 10 and 20 hr post infection, there was a good separation between the background (uninfected) and the infected fixed cells. The DBP and late protein expressing cells reached to 89.5% and 74.5% respectively (data not shown).

Standard stress and infection conditions were applied in this and subsequent experiments. The cells were plated out into two groups, the first was exposed to 40 °C for 3 hrs and other one was assigned as a control maintained at 37 °C throughout. Both groups were then infected at 37 °C at the same time with moi=5 of wt Ad5 for 20 hrs. The proportion of cells that showed late expression (positive cells) was reduced in the stressed group, being 79%, 30% and 14% compared to the

control group in three different experiments. This drop in viral late expression is represented by the noticeable shifting of the gated infected cells towards the uninfected cells in stressed cells compared to the control in representative experiments (Fig 5.6 B).

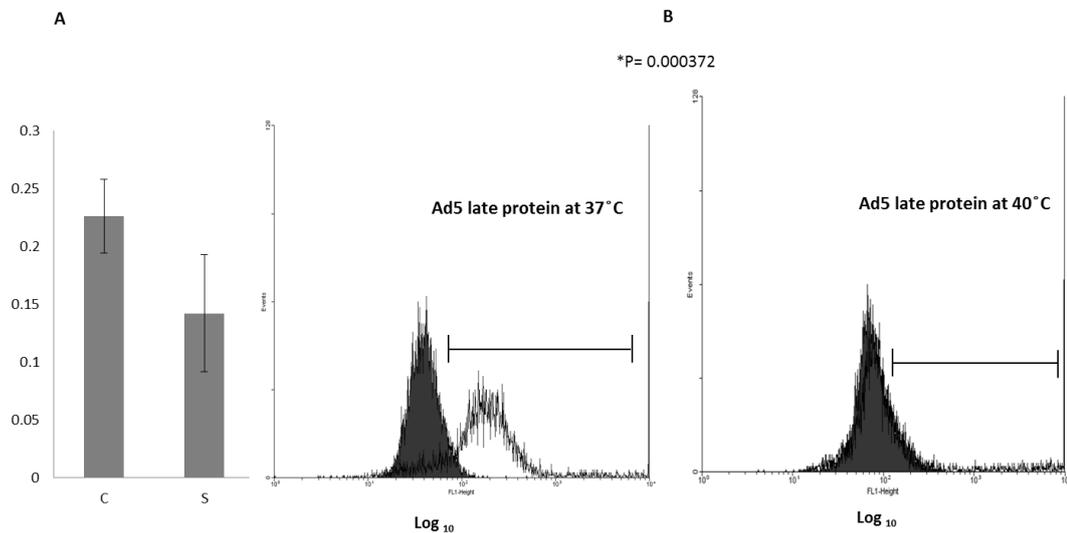


Fig.5.6. IF & FACS analyses of late gene expression in control and heat shocked cells. A. Equivalent cultures (1×10^5 cell/ml) of HeLa cells grown on cover-slips in 12- well plates were either stressed at 40°C for 3 hrs, or maintained at 37°C throughout (control). All cultures were then infected with Ad5 wt at 37°C at moi 5, fixed at 20 hrs post-infection, permeabilized and stained with anti-late polyclonal Abs as primary Abs followed by staining with Alexa-fluor 594 Gt α Rb Abs. Numerical data were extracted from counting the numbers of late infected cells and dividing that by the total number of cells in randomly selected stained fields. **B.** Cells were plated at a density of 0.8×10^6 or 2.2×10^6 per dish in 60 and 100 mm^2 dishes respectively for 24 hours, then stressed or not as in **A**. Cells were then infected at 37°C with moi of 5 with Ad5wt300 for 20 hours. Cells were then fixed, permeabilized and stained with anti-late polyclonal Abs as primary antibodies, followed by Alexa-fluor 594 Gt α Rb Abs staining. **Left.** Late gene expression in cells previously cultured at normal temperature. **Right.** Late gene expression in cells previously cultured under conditions of stress. The black curves represent the mock infected cells (background) while the grey curve represents the positive infected cells. FACS data show that the late gene expression decreased significantly in stressed cells compared with those which were grown at normal temperature (* $P < 0.005$, $n = 4$).

5.3.3.1 Hexon protein expression at stress and normal conditions

To quantify the alteration in the pattern of hexon expression, more specific quantitation was achieved to count the cells that expressed this capsid protein. The

same stress and infection conditions were applied here and the results were consistent with the alteration in total late gene expression due to the heat shock. The number of hexon- expressing cells declined in the stressed sample compared to the control and this was obvious with the complete shift in the peak of infected cells to be indistinguishable from the background (mock) (Fig5.7).

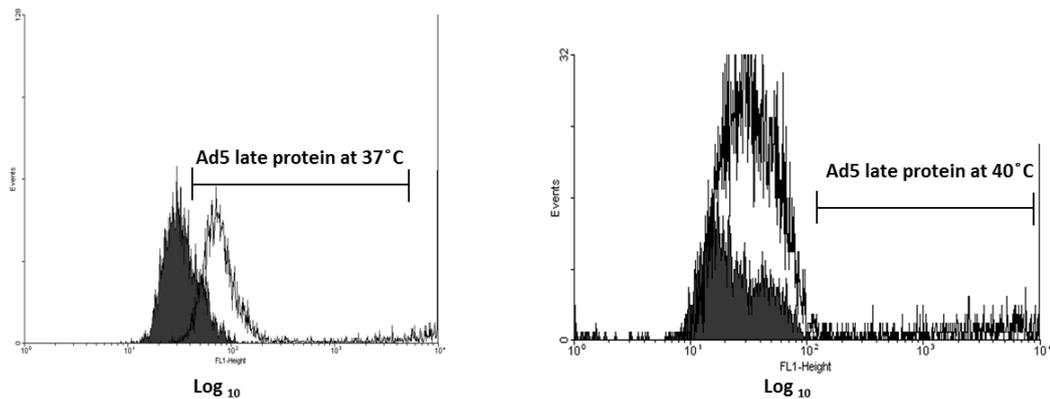


Fig.5.7. FACS analysis of hexon expression in control and heat shocked cells. Cells were plated at a density of 0.8×10^6 or 2.2×10^6 per dish in 60 and 100 mm² dishes respectively for 24 hours. Cells were infected at moi of 5 with Ad5wt300 for 20 hours. Cells were then fixed, permeabilized and stained with anti-hexon monoclonal Abs as primary antibodies, followed by Alexa-fluor 488 G α M Abs staining. **Left.** Hexon expression at normal temperature. **Right.** Hexon expression at stress. The grey curves represent the mock infected cells (background) while the black curve represents the positive infected cells.

5.3.4 Effect of higher temperature on Ad5 gene expression

Many previous publications analysed the effects of extreme heat shock on cellular transcription and much of such work was to expose cell lines to mild temperature earlier and then the higher temperature. Heat shocking cells to higher temperatures makes cells respond in different ways in correspondence with the how extreme is the elevation in temperature. For example 42°C or 44 °C, exposing to 44 °C inhibits cellular protein synthesis when cells start to synthesize nothing except limited amounts of hsp70, while a classical heat shock response was observed at 42 °C (Margulis et al., 1991). In order to evaluate the effect on adenovirus gene expression of higher temperatures than the mild stress employed above, exposure to higher temperature was applied but for a shorter time. Cells were either exposed to 42°C for 1 hour or left to grow at normal temperature and

then infected with Ad5 at moi of 10 for 20 hours. Consistently, shorter exposure to higher temperature caused a clear reduction in the number of cells that displayed late gene expression compared to the control conditions (Fig 5.8).

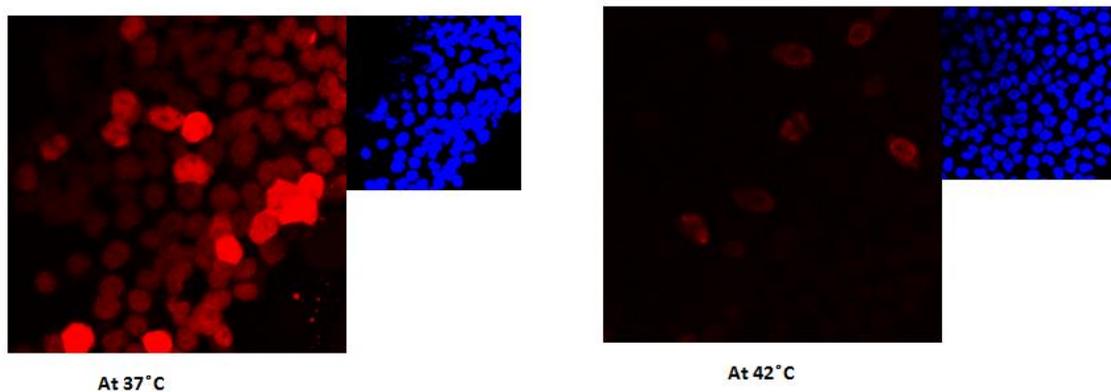


Fig 5.8 Ad5 late gene expression in control and 42 °C heat shocked HeLa cells. Equivalent cultures (1×10^5 cell/ml) of HeLa cells grown on cover-slips in 12- well plates were either stressed at 42°C for 1 hour, or maintained at 37°C throughout (control). All cultures were then infected with Ad5wt300 at 37 °C with moi 10, fixed at 20 hrs post-infection, permeabilized and stained with anti-late polyclonal Abs as primary Abs followed by staining with Alexa-fluor 594 Gt α Rb Abs. Left panel; culture were infected at 37 °C, right Panel were infected at 37 °C after incubation at 42 °C.

5.3.5 Effect of heat stress on adenovirus genome replication

The reduction in late gene expression in previously stressed cells led to the question of whether Ad5 genome replication might be affected by prior heat stress, since late gene expression depends on replication.

5.3.5.1 Standard Curves

The intention of this experiment was to compare the copy number of Ad5 genomic DNA in pre-heat-stressed and control cultures. To permit absolute quantitation, standard curves for both cellular and viral genomes were generated. A genomic GAPDH plasmid was constructed using PGEM-T and HeLa cells as source of template

DNA for PCR (Fig.5.9 A and B), while Ad5 plasmid pTG3602 (pTG) was made by (Finnen et al., 2001). These two plasmids were used to make standard curves to detect the copy number of Ad5 per cell. The efficiency of detection of the plasmids was tested and they presented accurate standard curves that make them meet the requirements of this experiment (Fig 5.10).

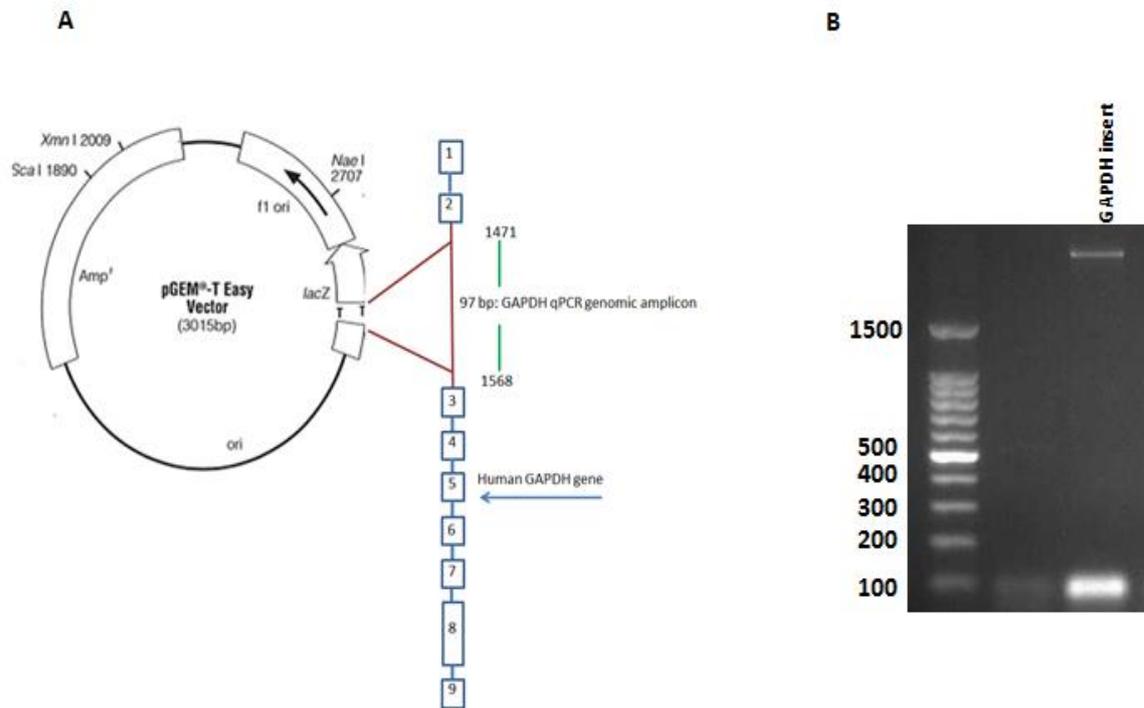


Fig 5.9. Schematic diagram of GAPDH plasmid. A. pGEMT- easy vector was used to build genomic GAPDH plasmid, 97 bp- piece of genomic GAPDH in the intron between exons 2 and 3. DNA piece was ligated to pGEMT-easy vector (Promega) and the product was sequenced using GATC sequence service. **B.** Agarose gel shows the GAPDH insert in PGMT-easy vector, DNA ladder to the left and the insert is the second sample at he right side.

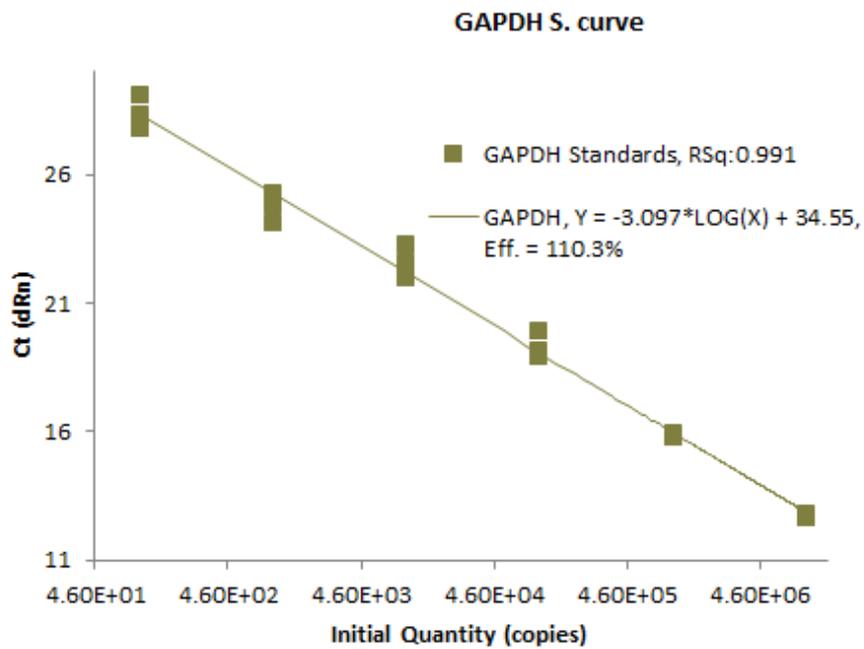
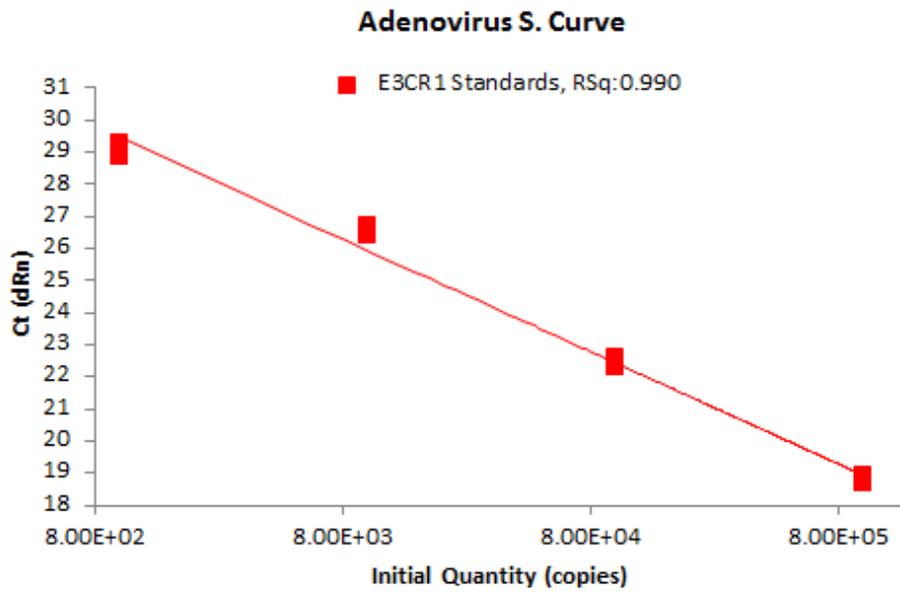


Fig 5.10 GAPDH and Ad5 plasmids qPCR amplification standard curves. A. Standard curve of Ad5 (PTG) plasmid copies. **B.** Standard curve of GAPDH plasmid copies.

5.3.5.2 Ad5 Genome Copy Number

In order to compare Ad5 genome replication in control and previously stressed cells, cells were either stressed for 3hrs or left at 37°C as a control and then infected at 37°C; samples were collected 20 hrs post infection. Over repeated experiments, variation arose which interfered with drawing conclusions from this part of the work. This variation came mainly from the inconsistency in Ad5 genome copy number in biologically equivalent samples between different experiments. Thus an average of repeated experiments could be obtained only with big error bars (data not shown).

To clarify whether this variation was due to a technical problem or to true biological variation in the cellular response to heat stress in equivalent cultures on different occasions, two different approaches were used. The first approach was to test the efficiency of DNA purification columns. The other was to make duplicates of the same experiment in the same day and processing them independently.

5.3.5.3 Efficiency of purification columns

This experiment was intended to try to find the source of variation in Ad5 copy number over repeated experiments. The thought was that the efficiency of the purification columns in recovering the viral genome might be affected by saturation of column capacity. DNA was extracted from two infected samples 20 hrs post infection, the first sample was divided into two portions (10% and 90%) and the second was left to be extracted in total. If column saturations were an issue, then these samples would give different values for Ad5 copy number. However, all the samples were recovered with similar efficiency by the columns since, after calculating the copy number from each determination, all gave equivalent values (Fig.5.11). Thus the conclusion is the viral DNA was extracted equally from the treated and untreated cultures.

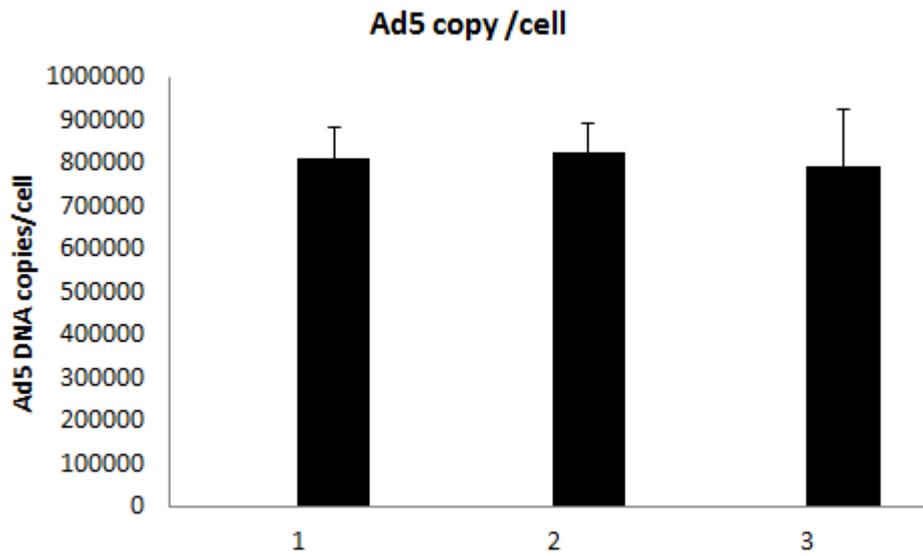


Fig 5.11. Efficiency of DNA purification columns in recovering Ad5 genomes. Parallel cultures (1×10^5 cell/ml) of HeLa cells were plated in 12- well plates. Cultures were then infected with Ad5 wt at moi 5 for 20 hours. DNA was extracted from each individual sample as follow: one sample was divided into two parts (10% and 90%) which are referred by 1 and 2 the second sample was extracted as a total bulk. Ad5 copy number was assessed using Ad (pTG) and GAPDH plasmids as standards. 2 or 10 ng/well were used to quantify the Ad5 and GAPDH respectively. Data were calculated by dividing the GAPDH copies by 2 (diploid genome), then dividing Ad5 copies by GAPDH and then multiplying the result by 5 to correct for the input difference.

5.3.5.4 Differences in the biology of the cells

Given the sensitivity of real time PCR analysis, which could detect any tiny variation in the cellular activity and gene expression over time, virus genome copy number was detected in duplicate samples of equivalent cultures prepared in parallel at the same time. The same stress conditions were applied to those two different cultures in the same 12-well plate and then they were infected with Ad5 at moi of 5 for 20 hours. Infection was stopped at the same time and DNA was extracted from each sample to quantify the Ad5 copy number. The results showed a massive reduction in DNA copy number in previously stressed cells compared to the control cultures. Despite that, the copy number was significantly different between the duplicates, one of the control cell samples showed approximately 10^5 Ad5DNA copy/cell (Fig 5.12A), while the other gave about 1.6×10^5 Ad5DNA copy/cell (Fig 5.12B). This difference, though substantial, was consistently less than was observed between equivalent experiments carried out at different times.

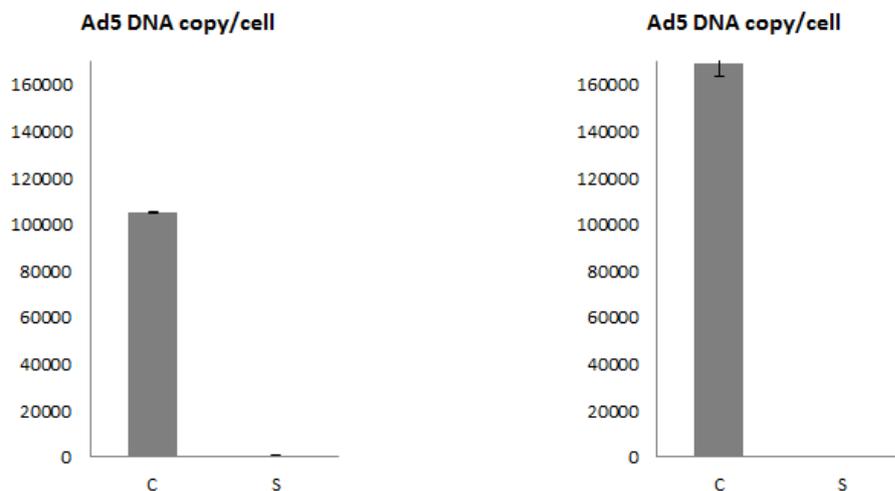


Fig 5.12. Ad5 genome replication at stress and normal conditions. Parallel cultures (1×10^5 cell/ml) of HeLa cells were plated in 12-well plates as duplicate and either stressed at 40°C for 3 hrs, or maintained at 37°C throughout (control). All cultures were then infected with Ad5 wt at 37°C with moi 5 for 20 hours. DNA was extracted from each individual sample and Ad5 copy number was assessed "as in Fig 5.10. **A.** First replicate. **B.** Second replicate

5.3. 6 Effect of heat stress on adenovirus genome replication at moi of 1

Based on the fact that the reduction in Ad5 late gene expression was more pronounced at lower multiplicities of infection, and the sensitivity of qPCR assay to detect small amounts of Ad5 genome, cells were stressed for 3 hrs or incubated at 37°C and then infected with Ad5 at a lower moi of 1. The results in repeated experiments showed a clear pattern and less variability than seen at higher moi. The previously stressed cells displayed a significant reduction in Ad5 DNA copy number compared to the control (Fig.5.13).

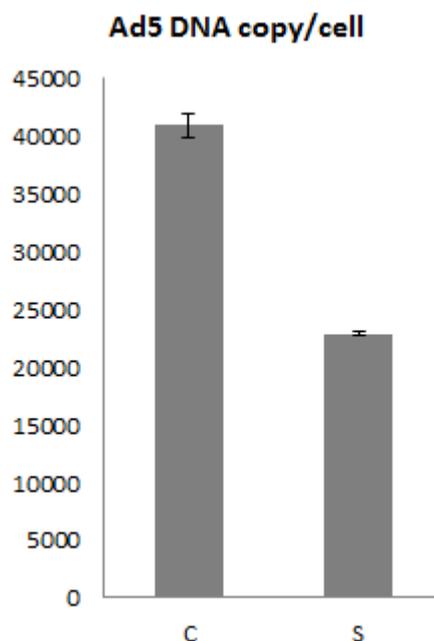


Fig 5.13. Ad5 genomic replication at stress and normal conditions at moi of 1. Parallel cultures (1×10^5 cell/ml) of HeLa cells were plated in 12- well plates and either stressed at 40°C for 3 hrs, or maintained at 37°C throughout (control). All cultures were then infected with Ad5 wt at moi 1 for 20 hours. DNA was extracted from each individual sample and Ad5 copy number was assessed using Ad(PTG) and GAPDH plasmids. 2 or 10 ng/well were used to quantify the Ad5 and GAPDH respectively. Data were calculated by dividing the GAPDH copies by 2, then dividing Ad5 copies by GAPDH and then multiplying the result by 5.

Statistics showed significant decrease in Ad5 genome replication with stress compared to the control conditions at $*P < 0.05$ (Fig.5.14).

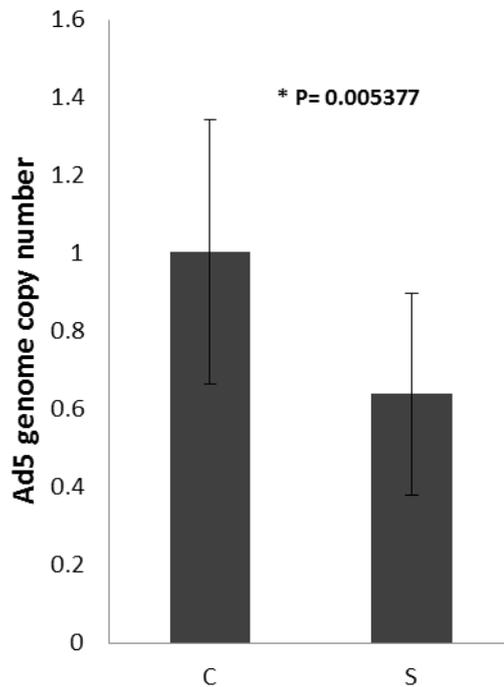


Fig 5.14. Statistical analysis of Ad5 replication at stress and normal conditions. Data show a significant decrease in Ad5 replication in pre-stressed cells ($*P < 0.05$, $n=5$)

5.4 Proposed mechanisms of stress-induced restriction of Ad5 infection

5.4.1 Role of PML-II in reduced Ad5 gene expression

PML is an IFN up-regulated gene and PML-II isoform positively regulates the type I interferon response. PML is also mobilised in response to heat stress and other stresses, so the aim was to investigate the role of PML and PML-II in the reduction of adenovirus gene expression in pre-stressed cells and to explore any possible relationship between PML-II and Ad5 infection, heat shock and the innate immune response. The first step was to address the response of PML and PML-II to the mild elevation in temperature employed as a stress throughout this study then to

analyse the heat-mediated restriction of Ad5 in the presence and absence of PML and PML-II.

5.4.1.1 Effect of high temperature treatment on PML NB

The first objective was to characterise the response of PML proteins and PML NB to stress. Initially, a stress condition of exposure to elevated temperature of 40 °C for 3 hrs was chosen for analysis. Depending upon the outcome of initial analyses, the intention was to adjust these conditions to define an optimal scenario for investigating the effects of cell stress upon virus infection. A series of replicate cultures were either exposed to 40°C or incubated as control for 3hrs at 37 °C and individual cultures were harvested every 30 minutes either for western blotting or immunofluorescence. The main PML band that is around 130 KDa (which comprises PML-I and PML-II) started to reduce after 1.5 hr (Fig.5.15A). In contrast, the 90 KDa PML band (isoforms III-V) showed an expression similar to the control after the same period of exposure. By immunofluorescence, PML NB as previously reported (Eskiw et al., 2003) manifested bright focused staining in control samples while in treated cells they became fewer in number and there was an increased diffuse staining (Fig 5.15B). Diffused staining was characteristic for the heat treatment which was more obvious after 90 minutes of exposure to 40°C then the bright staining starts to appear again after removing the stress.

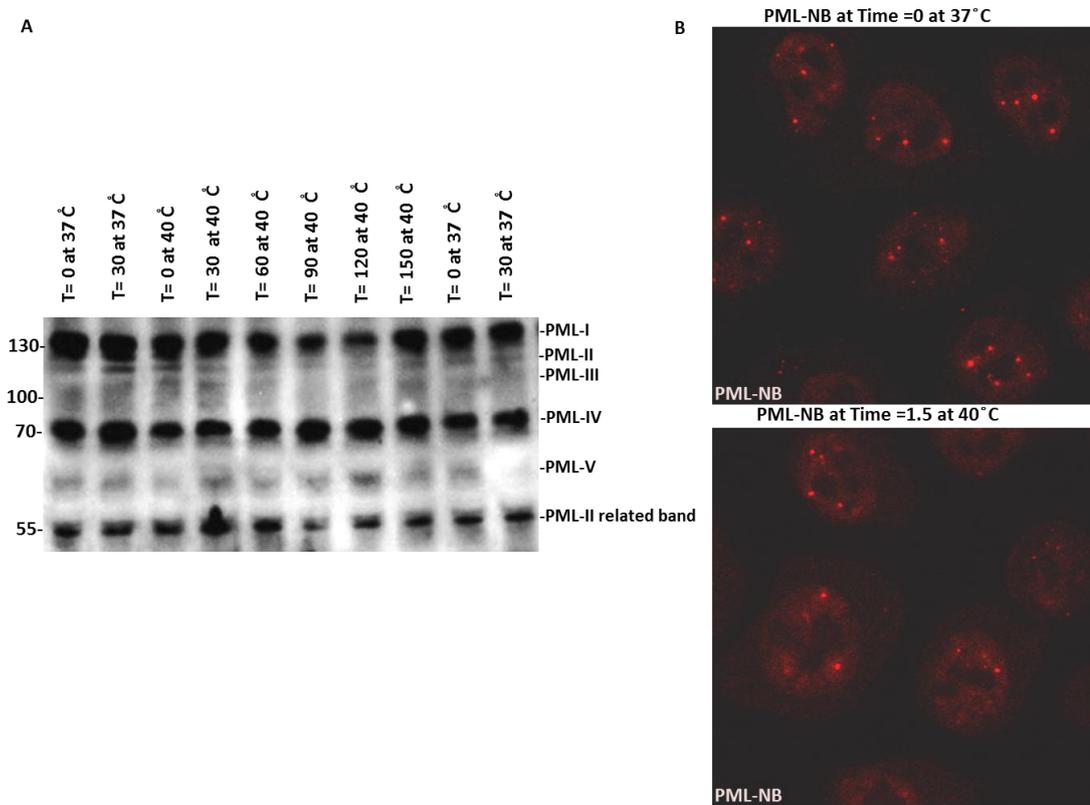


Fig.5.15. Effect of 40°C treatment on PML protein expression and organization in PML NB in HeLa cells. Replica cultures were subjected to sequential periods at 37 °C and 40 °C and harvested at the times indicated. **A.** PML proteins were detected by Western blotting analysis of control and heat treated samples using a polyclonal anti- PML antiserum. **B.** Confocal images of cells stained with anti- PML (primary antibodies) and Alexa fluor 594 goat anti-rabbit IgG (H+L), either at 37°C or after heat stress.

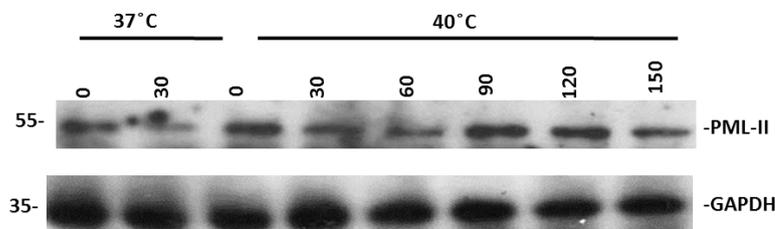
5.4.1.2 Effect of elevated temperature treatment on PML-II

In a more specific and detailed investigation, the effects of heat stress on three PML isoforms were investigated, PML I, PML V in addition to PML-II were analysed. In fact every individual band responded to the elevated temperature in a unique way. In time that PML-I oscillated with the change in temperature, PML-V showed a steady decrease pattern with the increased temperature (results not shown). while a PML-II related band showed an increase with the increased temperature. In fact, anti-PML-II antibodies detect predominantly a lower band (around the 55k in protein marker ladder) in HeLa cells, this band is believed to be another small

variant of PML-II. The evidence that we have that this band is PML-II related rather than background is that it disappeared with PML-II knockdown (exon7b-shRNA), it could be detected only by anti-PML-II antibodies but not anti-PML or anti-PML-I with the induction with IFN α (unpublished data). The band we detected was around 55K which we think is a truncated or cleaved form of PML II.

PML II protein was also detected by immunofluorescence to study whether its location was altered in response to the stress. In contrast to the control, PML II increased in amount and NB numbers and showed increased diffuse staining directly after 30 min of temperature elevation.

A



B

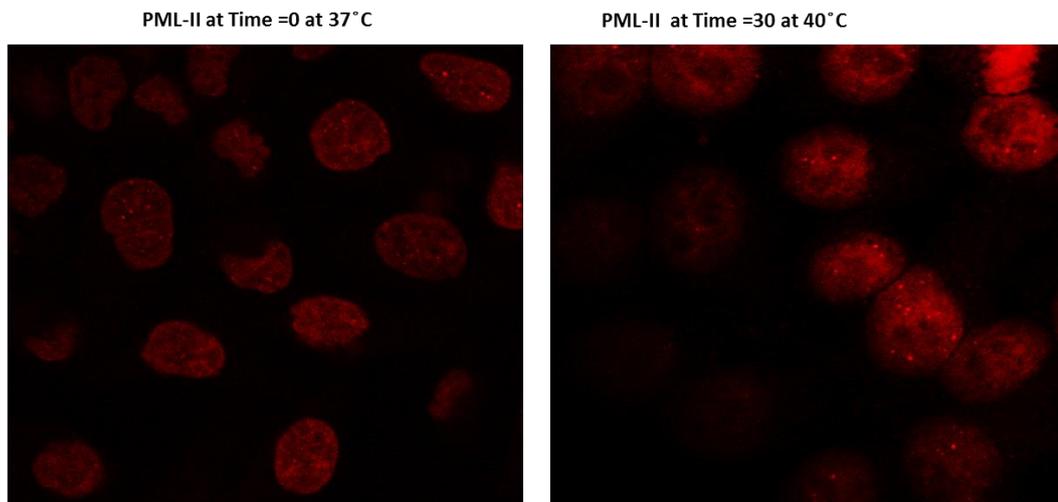


Fig.5.16. Effect of 40°C treatment on PML-II protein expression and organization in PML NB in HeLa cells. Replica cultures were subjected to sequential periods at 37 C and 40 C harvested at the times indicated (A) PML proteins were then detected by western blotting analysis of PML bands in control and heat treated samples using a polyclonal anti- PML-II antiserum. (B) Confocal image of cells stained with anti- PML-II (primary antibodies) and Alexa fluor 594 goat anti-rabbit IgG (H+L).

5.4.1.3 Effect of heat stress on viral gene expression in presence and absence of PML-II

To explore whether PML-II has any role in the observed reduction of viral gene expression following stress, the idea was to analyse the progress of Ad5 infection in HeLa cells in presence or absence of PML-II. PML-II Kd cells and EV cells (Chapter3) were used as a well-characterised cell line system that provides conditions where PML-II is stably present or absent. PML-II Kd and EV cells were either heat-shocked at 40 °C for 3 hours or left to grow normally at 37 °C, then both types of cells were infected at 37 °C at the same time with Ad5 at moi of 5 for 20 hours. In control, unstressed cells, PML-II-Kd cells showed more hexon mRNA expression compared to Ctrl.DNA cells (Fig.5.17); this result is consistent with the previous results (Chapter 4), since PML-II Kd cells support Ad5 gene expression better.

The general effect of heat stress on hexon expression observed in standard HeLa cells didn't change in Ctrl.DNA cells since they showed less hexon mRNA level with the heat shock. In contrast, PML-II Kd cells expressed more hexon mRNA with the stress conditions compared to the control temperature (Fig.5.17).

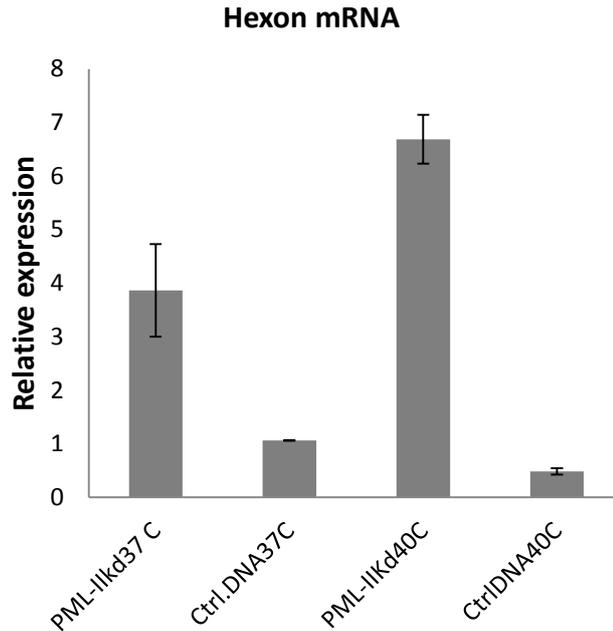


Fig.5.17. Ad5 hexon expression at control and previously heat PML-II Kd and Ctrl.DNA cells. Parallel cultures (1×10^5 cell/ml) of PML-II Kd and Ctrl.DNA cells were plated in 12- well plates and either stressed at 40°C for 3 hrs, or maintained at 37°C throughout (control). All cultures were then infected at 37°C with Ad5 wt300 at moi 5 for 20 hours post infection. Hexon was quantified by RT-qPCR using 10 ng/well of complementary DNA (cDNA) as a template. Data were presented by the $\Delta\Delta C_t$ method, using 18S RNA as internal control, and then normalised to the control cell value. All PCR products were subjected to dissociation curve analysis to ensure primer specificity.

To confirm and support the above results about the role of PML-II in reduced Ad5 gene expression and replication in previously heat-stressed cells, the effect of heat shock on viral gene expression in the presence and absence of PML-II was analysed at the protein level. Cells were stressed and infected as above and extracts prepared for western blot analysis. Similar to the mRNA analysis, EV cells showed more or less the same effect of reduced Ad5 late gene expression after heat stress (Fig.5.18; lane 4 at 37 °C & 40 °C). However, a stress-induced difference was observed in the pattern of Ad5 gene expression in PML-II Kd cells; they showed more hexon expression with the stressed conditions (Fig.5.18; lane1 at 37 °C & 40 °C), while the other late proteins in the same samples, penton and L1IIIa showed less expression with the stress. Thus these results show that PML-II does play a role in the stress-induced effect on Ad5 infection as its absence alters Ad5 late gene expression.

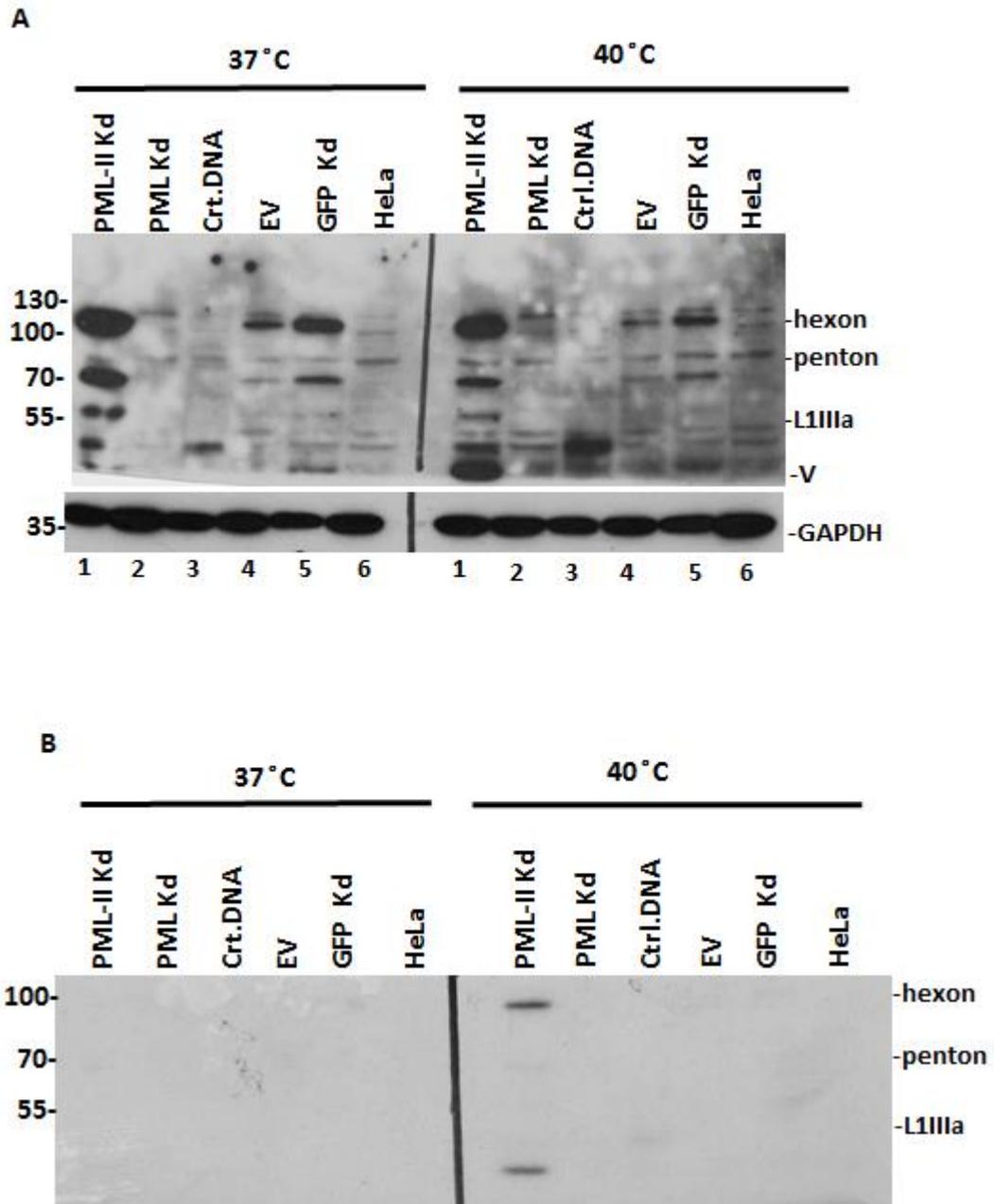


Fig.5.18. Ad5 gene expression in pre-stressed or control PML-II knockdown cells and control cell lines. PML-II Kd, PML-Kd, Ctrl.DNA, EV, GFP and HeLa cell lines were either heat stressed at at 40°C for 3 hours or left to grow at normal temperature. Cultures were then infected at 37°C with Ad5 at moi of 5 for 20 hours. Protein samples were harvested for WB analysis, analysed by SDS-PAGE and western blotting. The membranes were probed with the appropriate Abs; anti-late polyclonal rabbit Abs (1:10K) or anti-GAPDH mouse monoclonal Abs (1:25k). **A.** Long exposure. **B.** Short exposure

5.4.1.4 Characterising heat shock protein response to mild heat stress

With the aim of analysing the effect of mild heat treatment on the heat shock protein family response, a small selection of HSP mRNAs were chosen to be investigated (hsp60, hsp90 and hsp70). Comparing the expression of those HSPs was of special importance since each of them has a specific function during the virus life cycle.

Hsp60, produced from the HSPD1 gene, is critical in inducing the IFN- β response during virus infection. It was found that hsp60 is an IRF3-interacting protein and that it facilitates IRF3 dimerization and activation. This activation by hsp60 was proved by studying the effect of both over-expression and knockdown of hsp60 on IFN- β induction in HEK293 cells (Lin et al., 2014). To understand whether hsp60 expression was induced with the increased temperature, HeLa cells were either heat stressed for 3 hours at 40 °C or left as control at 37 °C. Hsp60 mRNA showed a modest 1.5-fold increase (Fig.5.19A).

HSP90, another highly conserved chaperone, has been shown to regulate type I and II interferon responses. In HeLa cells, interfering with hsp90 synthesis using siRNA as well as some cancer treatments will block the phosphorylation of STAT1 which is crucial to both types of response. In the absence of hsp90, JAK1/2 kinases are degraded since JAK interacts with hsp90 and CDC37 and this interaction is destabilised by hsp90 inhibitors (Shang and Tomasi, 2006). Due to this link between hsp90 expression and type I and II interferon responses, hsp90 expression was analysed as for hsp60 above. The same stress and control conditions were applied and hsp90 mRNA expression was detected by RT-qPCR. Elevated temperature increased the expression of hsp90 about 2-fold (Fig.5.19B). To summarise, mild heat shock was sufficient to modestly increase the levels of two hsp that are known to have positive roles in the antiviral interferon response.

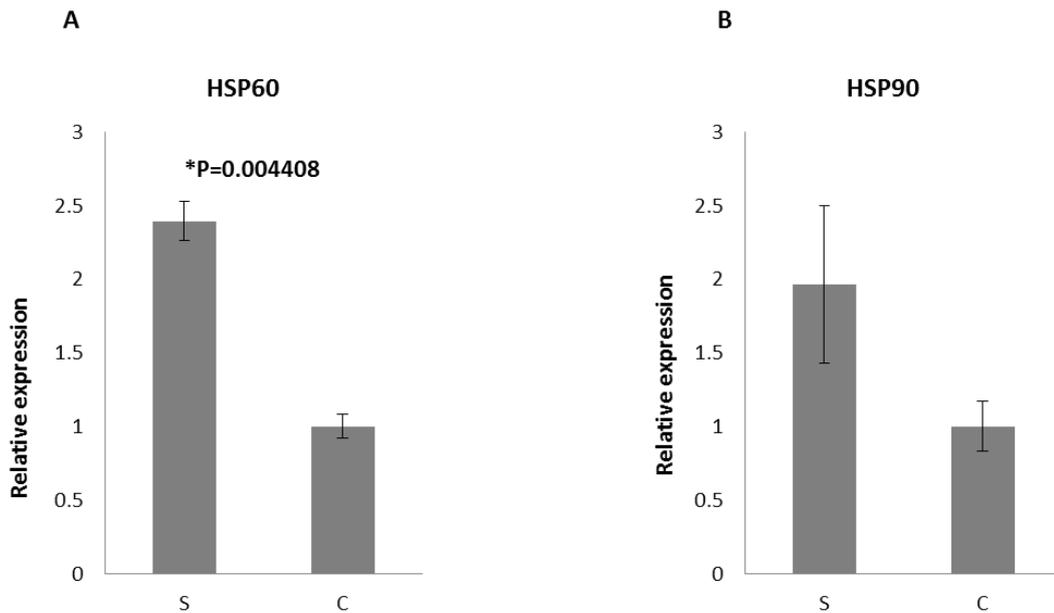


Fig.5.19. HSPs expression in control and heat shocked standard HeLa cells. Parallel cultures (1×10^5 cell/ml) of HeLa cells were plated in 12- well plates and either stressed at 40°C for 3 hrs (S), or maintained at 37°C throughout (C). HSPs were quantified by RT-qPCR using 10 ng/well of complementary DNA (cDNA) as a template. Data were calculated by the $\Delta\Delta C_t$ method, using 18S RNA as internal control, and then normalised to the control cell value. All PCR products were subjected to dissociation curve analysis to ensure primer specificity. **A.** Hsp60 mRNA expression at stress and normal conditions. **B.** Hsp90 mRNA expression at stress and normal conditions. Data show significant increase in hsp60 expression with the heat shock compared to the control conditions (* $P < 0.05$, $n = 2$).

5.4.2 Effect of heat shock on Interferon stimulated genes (ISGs)

The major shared features between stress and Ad5 infection is the early innate immune response which is represented by the early induced interferon response.

The protective effect of heat shock against Ad5 infection might be attributed to an IFN response pre-triggered by stress. Hence, it was logical to measure the level of ISG56, chosen as a marker for the activation of this response under both stress and normal conditions. After stressing cells or leaving them at 37 °C, ISG56 was measured by RT-qPCR. ISG56 mRNA expression was increased in stressed cultures to about 1.7 fold compared to the control conditions (Fig 5.20). This is a very small increase when compared with the induction seen during an active interferon response.

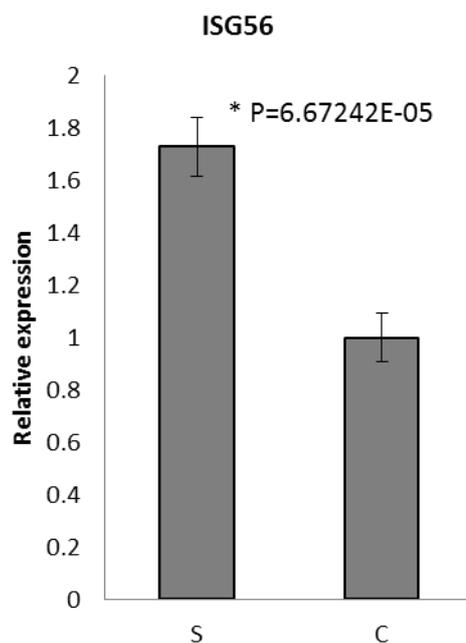


Fig.5.20. ISG56 expression at control and heat shocked standard HeLa cells. Parallel cultures (1×10^5 cell/ml) of HeLa cells were plated in 12- well plates and either stressed at 40°C for 3 hrs (S), or maintained at 37°C throughout (C). ISG56 mRNA was quantified by RT-qPCR using 10 ng/well of complementary DNA (cDNA) as a template. Data were presented by the $\Delta\Delta C_t$ method, using 18S RNA as internal control, and then normalised to the control cell value. All PCR products were subjected to dissociation curve analysis to ensure primer specificity.

5.5 Discussion

The overall conclusion of this chapter is that pre-stressing tumour or normal cells makes them more resistant to Ad5 infection. This was clear with the substantial reduction in viral gene expression observed at both RNA and protein levels, and with the reduction in genomic DNA replication. The stress conditions compromised viral gene expression to about 80% in some FACS experiments. These findings agree with publications that refer to the protective role of a heat shock response against more extreme stress conditions or infections. In *Drosophila*, absence of heat shock factor (HSF) makes the adults more sensitive to virus infection while re-activating the same factor could reduce the viral replication to undetectable levels (Merkling et al., 2015). In general, mild hyperthermia protects against more severe stress

conditions. It was reported that mild heat shock could protect from subsequent heat-induced necrosis that is caused by heat stroke (Kourtis et al., 2012). This happens when *C. elegans* was pre-exposed to mild heat stress, when HSF1 and hsp16.1 regulate a Ca⁺⁺-homeostasis response under heat stroke conditions. Thus this preconditioning response could protect cells from stimuli which could cause necrosis (Kourtis et al., 2012).

Heat shock can cause an accumulation of heat shock proteins, only a few of which were measured in this study. Certain hsp proteins could have a direct relationship with the innate immune response elements. In this study the reduced Ad5 gene expression and replication might be attributed partially to the increased level of hsp90 after exposing cells to mild hyperthermia. This protein has been found to be a regulator of NF-κB activation. In human lung endothelial cells, chemical inhibition of hsp90 inhibits the NF-κB activation through preventing the recruitment of the latter co-activators from the target (Thangjam et al., 2014). Alternatively, hsp90 and cell surface hsp90 (cshp90) were found to serve as a direct cofactor for NF-κB activation by Kaposi's sarcoma-associated herpesvirus (KSHV) in primary human cells (Defee et al., 2011)

Considering Ad5 infection, it was reported that adenovirus infection causes endoplasmic reticulum (ER) overload independently from MHC-I, due to the accumulation of E319K protein which is retained inside the ER. This ER overload has a direct regulatory effect on NF-κB activation through ER-nuclear signal transduction. This mechanism differs from the unfolded protein response (UPR) and it is an important cellular response to trigger the innate immune response against adenovirus infection (Pahl et al., 1996). In the context of the mild heat treatment used in the present study it is possible that the increased hsp90 caused a prior activation of NF-κB which in turn prepared the cells to counteract the virus infection instantly.

As previously mentioned in the introductory part of this chapter, there are shared features between the cellular response to ad5 infection and to elevated temperature, especially at the transcriptional level. C-fos is one of those shared

transcriptional regulators; it is elevated post-adenovirus infection and post-heat shock at roughly similar times. This factor has an impact on triggering the innate immune response like type I interferon. In cold-sensitive host-range type 5 adenovirus mutant (5hr1) infected cloned rat embryo fibroblast (CREF) cells, levels of c-fos transcripts increased within 8 to 12 hours (Terris et al., 1995). This increase was accompanied by an increase in other host immediate early response genes like c-jun and jun-b. The increase in c-fos is necessary in the very early stages of carcinogen enhancement of viral transformation, CET (Su et al., 1995). This transcription factor is also induced and its transcripts accumulate in heat shocked HeLa cells (Murray et al., 2004) (Fig 5.21).

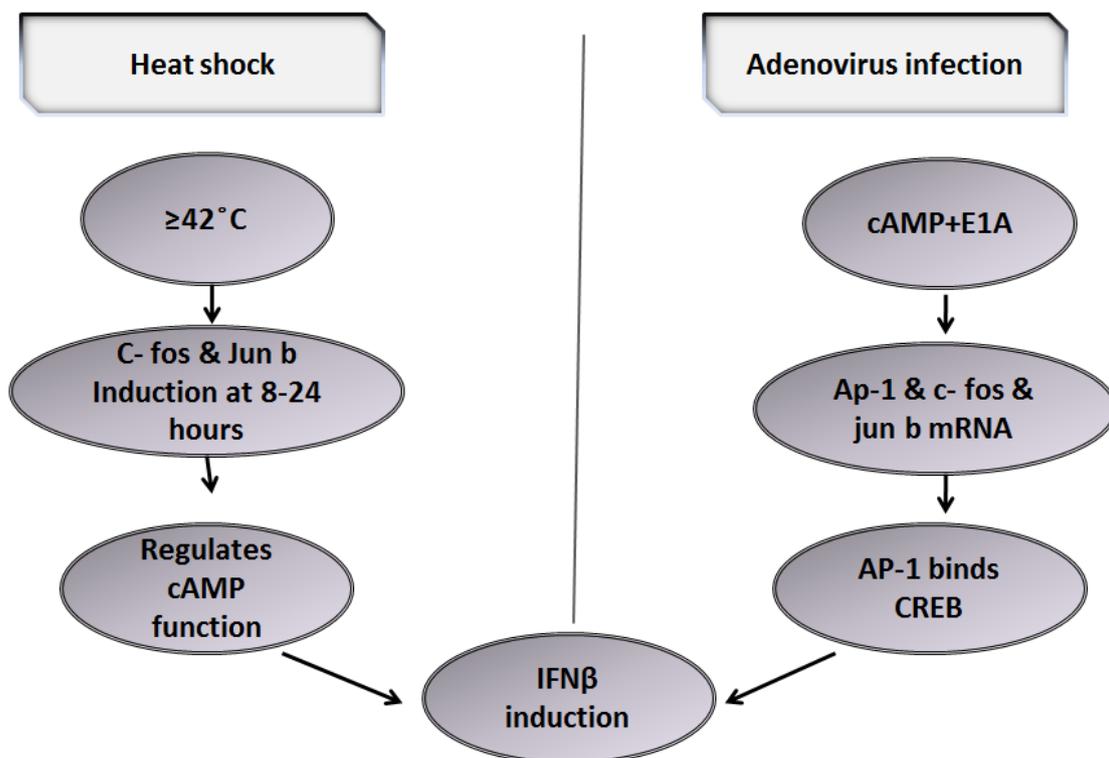


Fig 5.21. Schematic diagram of the shared features of the responses to heat shock and to adenovirus infection. Both responses induce the level of c-fos transcripts which regulates the cAMP function. As a consequence, AP-1 is activated leading to increase the IFN- β response

C-fos is also critical for cAMP function in suppressing the TNF- α response in RAW264.7 cells, a macrophage cell line (Koga et al., 2009). cAMP is of a special importance in triggering adenovirus early transcription: both cAMP and E1A cooperate to initiate the transcription of many genes located in the early regions. It was found that treating adenovirus-infected S49 cells with cAMP considerably induced the level of AP-1 transcription factor and cytoplasmic mRNA levels of c-fos and jun b. Subsequently, AP-1 bound CREB binding sites in E1A-inducible promoters to activate transcription (Muller et al., 1989). AP-1 is one of the three transcription factors that comprise the enhancesome that assembles on the IFN- β promoter to induces ISGs transcription (Fig.5.22) (Yarilina and Ivashkiv, 2010)

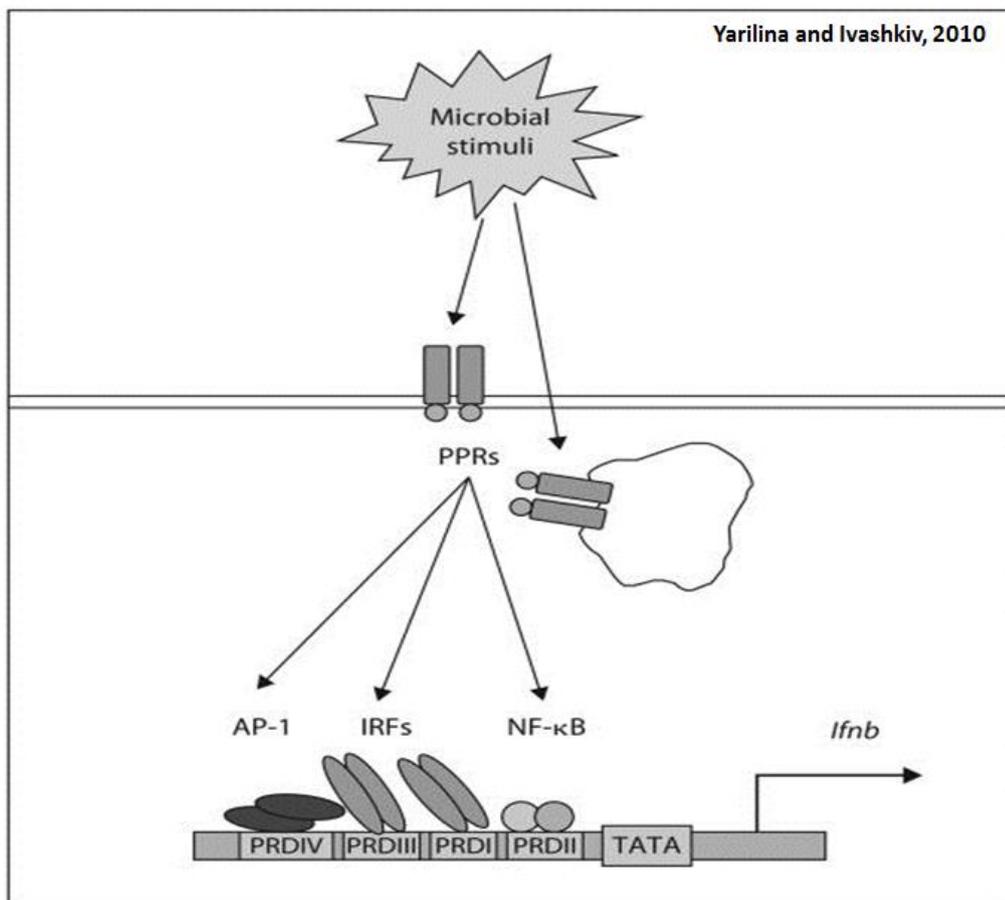


Fig.5.22. Schematic diagram of the role of AP-1 in interferon beta induction. AP-1 is one of three transcription factors that comprise the IFN- β enhancesome. Within a cell, when the stimulus (e.g virus) induces signal transduction (extra or intra cellular), the signal induces the level of AP-1 which binds at IFN- β promoter and subsequently increases in ISGs expression.

Another possibility to explain the reduced level of viral gene expression after stress could be through the c-jun pathway. This transcription factor in cooperation with other transcription factors like IRF3 regulate IFN- α/β to achieve the activation as a result of the host-virus interaction (Malmgaard, 2004). Since c-jun was also found to be part of the cellular response to heat shock, then its elevation in advance could contribute to an enhanced innate immune response against Ad5 infection. Whether that suggests any role for PML or PML-II is unclear as yet. However, it is known that, as a part of their function as a tumour suppressor, PML proteins displays a pivotal role in the transcriptional activation of c-jun upon exposure to DNA damage stimuli like UV-radiation. PML interacts physically with c-jun and both accumulate leading to an apoptotic response to UV-radiation in a p53-independent manner (Salomoni et al., 2005)

Turning away from the IFN response, another potential reason for the decreased late viral gene expression observed following heat stress is the increase in synthesis of p32 protein; an RNA splicing and transcription regulator. This cellular protein was found to be increased about two-fold when cells were exposed to 45°C for 10 minutes (Hiwasa and Sakiyama, 1986). This protein in particular has an important role in controlling the adenovirus major late transcription unit (MLTU) activity. P32 over-expression substantially prevents late gene expression from the MLTU at both mRNA and protein levels. This effective blockage occurs through a disturbance in Pol II function, with a failure in transition from initiation to elongation (Ohrmalm and Akusjarvi, 2006). It is also possible that p32 affects the late viral gene expression through retaining MLTU products inside the nucleus. One of the p32 functions that were discovered recently is its ability to retain some cellular proteins such as U2AF homologue inside the nucleus which suggests that this might be of importance in RNA alternative splicing (Heyd et al., 2008); such regulation is crucial for proper Ad late gene expression Adenovirus exploits p32 to deliver its own genome to the nucleus since confocal imaging showed coincidence in the cellular localisation between p32 and adenovirus pV as well as co-immunoprecipitation (Matthews and Russell, 1998).

If p32 worked through a similar mechanism on viral late gene expression then it would be through retaining L4-100K product inside the nucleus. The consequence of such retention will dramatically reduce the late viral gene expression and eventually the virus yield. Abolishing the ability of L4-100K to move from the nucleus to the cytoplasm would intensely reduce structural protein synthesis and as a result cause an extreme decline in virus yield without any obvious effect on Ad5 early gene expression or DNA replication (Koyuncu and Dobner, 2009).

Regarding the role of PML-II in stress-reduced Ad5 gene expression and replication, the progress of Ad5 infection in the presence and absence of PML-II suggested a role of the latter in altering the pattern of late stage gene expression. Quantitative RNA data suggested a possible role of PML-II in that reduced gene expression after stress, protein analyses further verified that removing PML-II altered the relative expression of some late proteins with increased levels of hexon in previously stressed PML-II Kd cells. The absence of PML-II during stress increased L3 hexon and L2pV protein expression during subsequent infection which is the opposite effect of stress alone when all late expression was reduced. However, the restrictive effect of stress overcame the beneficial effect of PML-II removal when considering the expression of L2 penton and IIIa. Protein L4 22K and L4 33K have been shown to regulate the alternative splicing of MLTU RNA (Törmänen et al., 2006, Morris and Leppard, 2009, Farley et al., 2004) and this effect could be due to an impact of heat stress on the expression of these proteins from the dedicated promoter. The L4-22K product also has an important role in activating transcription from the major late promoter (MLP) and its overexpression results in two opposite effects on L2 and L3 proteins. L4-22K overexpression causes a decrease in L3 hexon expression and a totally opposite effect on L2 penton expression as the latter substantially increased in expression (Backstrom et al., 2010, Morris and Leppard, 2009). Such differences might be correlated with the differences in effects of PML-II removal or stress or both.

In summary, prior exposure to mild heat stress can help cells resist Ad5 infection. All evidence suggests a role for a pre-induced innate immune response in this effect through different channels; type I interferon and induction of NF- κ B through

ER-overload or hsp90 mechanisms. Alteration in the pattern of late gene expression in the absence of PML-II could suggest a role for the latter and this role may be through modulating the IFN- β response as PML-II promotes the IFN response (Chen et al.,2015).

Chapter 6: Discussion and proposed mechanisms

6.1 The experimental system

The overall aim of the work was to investigate the role of PML-II and mild heat shock in the adenovirus life cycle and whether there was a correlation between those two factors. Determining the function of any cellular protein can be achieved by analysis of certain markers in the presence and absence of the concerned protein. Cytoplasmic interference by siRNA with the messenger RNA of any protein is the best biologically optimised technique that was suggested to be used in this piece of work to silence PML or PML-II isoform expression. This powerful technique has its own theoretical and practical limitations and so it was eventually decided to move to an shRNA stable knockdown system. To understand the reasons for preferring the permanent knockdown rather than the transient system, it is necessary to explain how both systems work. In mammalian cells to ensure an efficient specific gene down regulation, the concerned siRNA is delivered to the cytoplasm either quickly using the direct microinjection technique or the slower way using lipofection and in this case a long continuous contact with the siRNA should be guaranteed. Either way, the intact siRNA is concentrated shortly at the nucleus, then after 4 hours the intact and the dissociated siRNA could be detected in the cytoplasm where it basically interferes with the mature target mRNA and this continues typically for 48 hours after which the effect diminishes as the siRNA is lost (Jarve et al., 2007).

In fact, siRNA kinetics is accompanied by the presence of two different RNA-interfering silencing complexes (RISC) which contain an essential RNA processor protein Ago2. According to their distribution they are either large cytoplasmic molecule or 20x smaller nuclear component and the latter is loaded with the siRNA and transferred to the cytoplasm (Ohrt et al., 2008). Gene silencing by siRNA in mammalian cells is achieved by the cooperation between the human RNA III endonuclease to digest the double stranded RNA to 21-32 nucleotide siRNA product and Tat-RNA-binding protein (Paddison et al., 2002) (TRBP) or PKR activating protein (PACT). The Ago2 splits the duplex which leads to removal the passenger siRNA and liberating of the guide siRNA which in presence of RISC will achieve repeated mRNA interference (Matranga et al., 2005). In contrast, shRNA is

endogenously transcribed as a slightly larger hairpin duplex, but eventually it is processed through the same route that siRNA goes through. shRNAs of less than 30bp were first described as RNA gene regulators in *Drosophila melanogaster* with less silencing efficiency in mammalian cells (Paddison et al., 2002).

The whole idea of gene silencing arose from the fact that mammalian cells potentially mount a substantial antiviral response against viral dsRNA intermediates which is represented by PKR which represses the global cellular translation reviewed by Rao et al., 2009. The same mechanism could be a limitation for gene silencing if the small interfering RNA was of a larger size (> 30bp) as cell will direct the innate immune response against the incoming dsRNA (Paddison et al., 2002). Small siRNA and shRNA escape such responses through their small sizes and the secondary structure of the hairpin. In this study the whole length of a single shRNA strand was 30 bp including the hairpin sequence and thus would not trigger such responses.

After transfecting the three lentiviral plasmids, each one of them achieves its own role to eventually integrate the target sequence into the cellular genome. The modified 3' LTR-shuttle or transfer plasmid provides the genome to deliver the target DNA (PLKO-1 in this study); pPAX2 provides the viral protein to produce the mature virion, and the envelope plasmid pMD2 provides an envelope attachment protein VSV-G envelope in this study to give the particles infectivity. Once the transducing virions succeed in integrating the target piece of DNA inside the cellular genome then this DNA will be endogenously transcribed by RNA Polymerase III (Paddison et al., 2002). Similar to miRNA, shRNA is processed by Drosha and Dicer to transform it from pri-miRNA to mature miRNA (Lee et al., 2003). All the rest of the steps will be similar to siRNA processing steps apart from taking off the hairpin loop by Dicer/TRBP/PACT complex to liberate the double stranded siRNA with 2 bp overhangs reviewed by (Rao et al., 2009). The shRNA gene silencing pathway follows the ordinary RNAi channels: Dicer is an essential RNAi component to process the shRNA since the transient knockdown of Dicer inhibits gene down-regulation that is induced by shRNA but not siRNA. shRNA, expressed by transduction of mammalian cells, is seen for several weeks at least

(Paddison et al., 2002) and shows up to 10-fold more efficiency in loading on Dicer than siRNA due to its asymmetrical relative stability of the overhangs (UU) in this study (Rao et al., 2009, Sano et al., 2008). The reduced off-target effect of shRNA, a consequence of it functioning at lower peak concentrations than transfected siRNA, is also another significant reason to decide to use shRNA in this project since off-target effects on the transfected cells could mean the results will be not very specific (Rao et al., 2009).

Other factors that might constrain siRNA interference are the variable transfectibility of certain types of mammalian cells which makes delivering the siRNA sometimes difficult, duration of knockdown and limitations of studying a wide range of genes (Paddison et al., 2002). For the purpose of this project, HeLa cells were chosen as was mentioned earlier because of their amenability for adenovirus infection. In addition to that they express a good level of PML proteins and also they are suitable for doing IF assays because they adhere very well to coverslips and resist loss during the staining steps which are aggressive for other cells such as HEK293 cells. Early experiments which were achieved in this project in silencing PML or PML-II transiently with siRNAs brought to the surface some obstacles, firstly the stress and inconsistent transfection efficiencies over experiments and secondly the difficulty in infecting the previously siRNA-transfected HeLa cells with adenovirus or its mutants. Since part of this work was concerned with the effect of mild stress on adenovirus life cycle, prior transient knockdown represented an additional factor which could contribute to the consequences of the mild heat treatment and created variation. Also, the level of adenovirus gene expression and replication was too low when the pre-transfected cells were infected with adenovirus in early stage of this work. The other reasons for moving from siRNA transfection to stable shRNA expression which were mentioned earlier in Ch3 were the ease, cheapness, homogeneity and consistency over repeated experiments, all of which prompted the work in Chapter 3 to stably transduce HeLa cells to be deficient for PML or PML-II to study their function inside the cell and when it faces a challenge such as adenovirus infection.

What is noticed in this study, is that the functional effect of long term stable knockdown was more consistent and potent than the equivalent transient knockdown. This agrees with the fact that at the molar level (apart from the maximal concentrations of siRNA and shRNA), shRNA shows higher potency to knockdown the target gene than the siRNA (McAnuff et al., 2007). This is due to the fact that Dicer substrates (shRNA) which consist of 29 bp can produce more potent RNAi than the smaller siRNAs without triggering an unwanted interferon response since shRNA is subjected to the endogenous RNA processing system which produce less immunogenic 5' ends (Siolas et al., 2005, Kim et al., 2005, Rao et al., 2009). However, the PML knockdown system in this project was consistent especially the first few weeks after selection and showed comparable knockdown compared to different control cell lines ranges between the 40 to 50 % physically and 10% to 25 % functionally at the RNA level.

6.2 PML-II knockdown

The PML-II isoform was specifically chosen for study for many reasons. In addition to its interaction with the early adenovirus protein E4Orf3 and its role in regulating the interferon response (Chen et al., 2015) it has a unique structure which might implicate it in fighting virus infection. The PML-II isoform has common features with the other isoforms in addition to unique characteristic features that make its function different to the others. This protein is encoded by the six shared exons (1 to 6), the first three of which encode for the common N-terminal region (cysteine cluster rich-394 amino acids) while the other three (4 to 6) are alternatively spliced and are hence partially responsible for the variable length of each isoform. The unique sequence that defines PML-II is encoded by exon 7b where our siRNA or shRNA are targeting to ensure the specificity of knockdown of this particular isoform but not any other. The C-terminal region of this isoform is rich in serine/proline segments which comprise 13 to 14 % of a total of 258 amino acids (Fagioli et al., 1992). The C-terminal region of PML-II has 8 serine-proline motifs which serine/threonine kinases may identify and modify (Vulliet et al., 1989). These kinases are of special importance in regulating the innate immune response and type I interferon induction. For instance, tumour progression locus 2 (are such

serine-threonine kinase) regulates the production of type I and type III interferon and restricts influenza virus replication through type I interferon signal transduction. Thus such kinases are involved in sensing virus infection and mounting a proper antiviral response that limits the consequences of the virus infection (Kuriakose et al., 2015). This actually links the PML-II isoform clearly with the innate immune response through sensing adenovirus infection in HeLa cells and interacting with the concerned cellular factors that raise the antiviral response.

Designing siRNA that specifically interferes with PML-II mRNA had already been achieved by (Wright, 2010). This siRNA interferes with the mRNA that is encoded by PML-II unique exon 7b and thus the knockdown target should be the PML-II isoform. The sequence that encodes for the C-terminus of PML-V is excised when PML-II mRNA is generated by removing the 7a-7b intron. However, the PML-II siRNA target sequence is retained within the 3' UTR of PML-V mRNA so it might be expected that targeting PML-II could affect the level of PML-V mRNA. This possibility was countered by Chen et al., (2015) when the functional knockdown of PML-II was evaluated in comparison with that of PML-V and a clear result showed that regulating type I interferon was specific for PML-II but not PML-V.

In characterising HeLa cells lacking PML-II, a long duration of PML-II knockdown was successfully achieved with levels of physical knockdown comparable to the transient knockdown achieved by siRNA in HeLa cells or MRC5 cells. At the protein level, the PML-II related band showed comparable physical absence compared to transient knockdown at a concentration reached to 125nM of PML-II siRNA or siC but with a better consistency. The effect of PML-II removal on NF-kB, as a mean of functional knockdown ranged between less than 60-90% considering the variation among selected batches of the knockdown or control cells. PML-II Kd cells showed very consistent reductions in their interferon response which agrees with the conclusion of Chen et al., (2015) about the effect of PML-II on such innate immune responses.

6.3 PML-II and Ad5 infection

The characterised PML Kd or PML-II Kd cells produced by shRNA formed a consistent basis for investigating the role of PML-II in the adenovirus life cycle under normal and stress conditions. This line of study was prompted by very early experiments when Ad5 late gene expression and replication were evaluated in the presence and absence of PML-II, and reduced transiently. It was obvious that hexon expression and Ad5 genome copy number were increased significantly with the removal of PML-II in HeLa cells. Compared to different control cell lines (Ctrl.DNA cells, GFP Kd cells and EV cells) the viral early and late gene expression increased substantially at both RNA and protein levels. More extensive data were obtained over a time course with clearer and sharper increase in the late gene expression in particular. Quantitatively, the increase in late gene expression reached up to 100% ie 2-fold in PML-II Kd cells compared to the EV control cells. Consequently, there was a corresponding effect of PML-II removal on Ad5 yield, which increased 3- fold. Given the role of PML-II in regulating type I interferon responses, an obvious hypothesis was Ad5 increased its gene expression upon PML-II removal due to the reduced level of interferon response in PML-II Kd cells compared to EV cells. Testing that idea showed only a partial role of the reduced interferon response in that substantial increase in Ad5 gene expression. This could instead be attributed either to other IRF3-independent mechanisms acting to keep a substantial level of interferon response or to the loss of other roles of PML proteins inside the cell. A role of the partially reduced interferon response in elevating Ad5 gene expression was suggested since this was affected more when PML-II Kd and EV cells were infected with Orf3-deficient virus than with wt virus. This agrees with Ullman et al., (2007), who showed that pre-treating cells with IFN- α or IFN- β compromised the replication of Ad5 that lacks E4Orf3, which is necessary for rearranging PML-NB into track-like structures and thus disrupting their antiviral function, but not the wt virus. The fact that E4Orf3 is essential to establish successful Ad5 replication in an IFN antiviral state and this happens after the over-expression of PML as a result of IFN treatment suggests that the effect of this viral protein is not upstream of the IFN signalling pathway (Ullman et al., 2007). The effect of interferon treatment was

mainly on replication (Ullman et al., 2007) which might explain the more robust and drastic effect of PML-II depletion on late gene expression in particular than the early stage; the major switch to initiate the expression of Ad5 late proteins occurs immediately after the DNA replication starts.

The implication of PML with interferon and virus infection is not restricted solely to Ad infection, it extends to infections with other DNA and RNA viruses such as HSV (Chee et al., 2003) or influenza A virus (Chelbi-Alix et al., 1998b). Other regulators of type I interferon response for example the cytoplasmic latent transcription factors STAT1 and STAT2, when they are phosphorylated by JAK1 and TYK2 move to the nucleus to bind to IRF9 forming ISGF3 to bind to ISREs reviewed by (Ivashkiv and Donlin, 2014). This channel of type I interferon transcription activation in addition to other IRF family members may keep some of the mounted interferon response at a reasonable level inside EV cells but not the PML Kd cells (since this phenotype already shows reduced interferon response). Thus concluding the role of decreased IFN response in the enhanced Ad5 gene expression would not be easy given this enormous number of regulatory factors, since it is impossible to eliminate all of them from the cells simultaneously.

6.4 Hsp70 and Ad5 infection

The more effective factor that participated in increased Ad5 gene expression when PML-II was removed was hsp70, since its level was very high at both mock and infection conditions. The extensive analysis of the PML-II Kd stable cell line showed an increase in hsp70 chaperone but not other HSPs, with the highest increase -at the mRNA level - in hsp70B one of a pair, with hsp70A, of highly sequence- related, strongly heat shock-inducible hsp70 genes. This was not an artefact of transducing those cells or due to the selection system since transient depletion of PML-II caused the same specific increase in hsp70B. Hsp70B was increased by 50% with PML-II transient knockdown, this increase was not as drastic as that detected with the permanent knockdown system; this could be due to the differences in the percentage of cells that showed the knockdown between the transient and permanent removal of PML-II. Alternatively, long term PML-II knockdown many

cause an accumulative effect, leading to progressive elevation in hsp70. In any case, removing hsp70 by siRNA transient knockdown in PML-II depleted cells massively decreased Ad5 late gene expression back to comparable levels to those seen in EV control cells, demonstrating its importance to efficient Ad5 infection. This result is similar to the role of hsp70 in rabies virus gene expression and replication. Rabies infection increases hsp70 by 4 hours post infection and removing hsp70 with siRNA inhibits the virus life cycle (Lahaye et al., 2012).

The significant increase in hsp70 mRNA expression in PML-II Kd cells was specific and not a general response triggered by the removal of PML-II, either in mock conditions or infection with Ad5. This was clear since the other hsp family proteins like hsp60 did not increase and this agrees with (Phillips et al., 1991). This suggests a critical function of hsp70 specifically in Ad5 gene expression. Previously it had been shown that there is specific and selective induction of hsp70 (but not other hsp family members) in Ad5 or HSV-1 infected HeLa cells compared to mock samples. In Ad5- infected cells, there is a detectable induced level of hsf1 transcription factor and this induced hsf was specifically increased the transcription of hsp70 and peaks it at 12 hours post infection since the levels of hsp90 (another hsf- responsive gene) did not increase by the activated hsf1. This means that there is a specificity and selectivity during adenovirus infection to induce the expression of hsp70 rather than other hsp family proteins.

An extensive analysis was achieved by Phillips et al., (1991) to detect the precise interaction between Ad5 E1A (the main virus encoded trans-activator) and the hsp70 promoter. An interesting finding was revealed during the analysis of 120 bp of the hsp70 promoter in mock and Ad5 infected cells. Hsf1 binding to the promoter can be measured by techniques such as DNase I foot printing. This detects hsf1 binding on heat shock but not (or much less) after Ad5 infection even though the promoter is induced. Hence, Ad5 induction of hsp70 promoter is considered not to be dependent on hsf1. Instead, E1A activates the hsp70 promoter and further experiments showed that it works via the CCAAT box in the promoters.

Since hsp70 is enhanced selectively and massively, Ad5 should benefit from that to carry on its own transcription and translation. One of the suggested roles of hsp70 in such enhancement of Ad5 gene expression is the induction of global virus transcription through the interaction with E1A. A CCAAT sequence was identified to be the initiation site of hsp70 transcription when was identified to be an enhancer of the global E1A- dependent transcription (Lum et al., 1992).

Over different and numerous experiments a high level of hsp70 expression was noticed in PML-II Kd cells especially hsp70A1B. This increase might be explained as was mentioned in Chapter 3 by a kind of compensation that the cells initiate to achieve the hsp70 chaperone function enabling them to overcome abnormal conditions. An indirect explanation could also be employed here: Daxx-hsf1 interaction might be altered in a way that enhances the latter's trans-activation of hsp70 expression. Thus when PML-II was removed, the Daxx was liberated from its nuclear boundary by PML-II removal, this gave the chance to Daxx to interact with hsf1 trimer and interfere with the latter's repression by different cellular chaperones (Boellmann et al., 2004).

6.5 Hsp70 and PML-II

Hsp70 up-regulation due to removal of PML-II might be explained in an indirect way. PML proteins has been described as regulators of the TNF- α signalling pathway (Cheng and Kao, 2012) and analysis of TNF- α responsive genes (IL-6) in this study showed an agreement with Cheng and Kao (2012) since IL-6 level was reduced with PML-II removal. In addition, it was found that treatment with TNF- α increases the sensitivity of cells to apoptosis due to the transient inhibition of hsf1 activation and as a consequence decreased hsp70 production (Schett et al., 2003). The other possible explanation for the increase in hsp70 could be through Daxx protein which is sequestered in PML-NB where its repressive function is inhibited by PML proteins (Li et al., 2000). Since Daxx is a modulator of hsp70 since it's release from the NB blocks the repression of hsf1 (Boellmann et al., 2004). Hence the scenario that might happen here is that removing PML-II released Daxx, so ending the inhibitory effect that is exerted by PML on Daxx repressive function, and as a

result the synthesis of hsp70 is up-regulated. This hypothesis needs extensive and more robust data in order to test it but the initial RNA quantitation shows that Daxx mRNA expression is increased about 4-fold with PML-II depletion compared to the control (Fig. 6.1), suggesting that increased Daxx activity may indeed underlie the elevation in hsp70 expression upon PML-II removal.

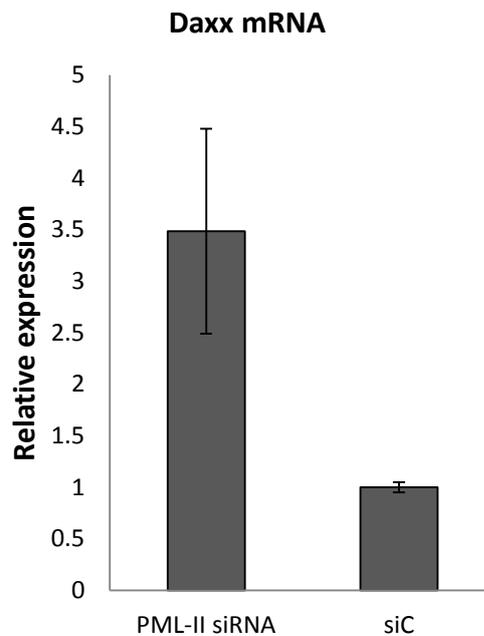


Fig.6.1. Daxx mRNA expression during transient depletion of PML-II. HeLa cells were seeded at a density of 1×10^5 /ml for 24 hours, then transfected with 125 nMsiPML-II or siC for 48 hours. RNA samples were extracted, total Daxx mRNA was quantified by RT-qPCR using 10 ng/well of cDNA as a template. Data were calculated by the $\Delta\Delta C_t$ method, normalised to 18s internal control rRNA and then normalised to the control sample value. All PCR products were subjected to dissociation curve analysis to ensure primer specificity and.

6.6 Ad5 infection of pre-stressed cells

In order to analyse the effect of pre-stressing HeLa cells via a mild elevation in growth temperature on the progress of subsequent Ad infection, various parameters of infection were monitored in pre-stressed and control cells. Initially the progress of infection was followed by confocal imaging and those experiments formed the basis of the rest of the work described in Chapter 5. The mildly elevated temperature affected Ad5 late gene expression significantly at the mRNA level but showed much lower and inconsistent effects on the early stages of Ad5 infection

when DBP expression was influenced only mildly. This was also confirmed by analysis of the early and late proteins using the same moi or even less since lower moi makes counting the infected cells in a confocal-stained field easy and fruitful. RNA and protein data were consistent in showing that hexon was expressed at lower level in cells that had been stressed pre-infection and at different time points. This phenomenon was not cell-specific since pre-stressed MRC5 cells also expressed lower hexon protein and mRNA compared to the control conditions.

Quantitatively and over repeated FACS experiments, the pre-stressed cells presented less Ad5 gene expression than the controls, with more obvious effects on late gene expression and in particular hexon expression. Consistently, counting the number of cells that showed late gene expression in confocal imaging gave the same results. Cells exposed for shorter periods to elevated temperature also showed lower gene expression. Ad5 genome replication was also analysed in both pre-stressed and control cells. Given the sensitivity of such analysis, the system was very sensitive to any tiny variation in Ad5 genome copy number. Consequently the statistics of the repeated experiments did not give any significant difference between the stress and normal conditions at moi of 5. However, lower multiplicity (moi of 1), where the genome copy number was lower, showed statistically significant differences in Ad5 replication in pre-stressed cells compared to the control. Several possible explanations can be found for why pre-stressing cells impairs Ad5 infection.

As previously mentioned mild heat shock triggered expression of a series of HSP family members, several of which have direct or indirect positive relationships with innate immune and inflammatory responses, inducing hsp90 and hsp60. At the same time, innate immune responses such as type I interferon are already triggered in different cell lines that that were pre-treated at elevated physiological temperature. The other cellular factor that might have a role in this particular reduction in Ad5 late gene expression is the RNA splicing factor, p32. This protein has a direct effect on reducing transcription from the MLTU and is at the same time one of the few proteins that continue to be expressed during heat shock (Hiwasa and Sakiyama, 1986). Hence, it is possible that the elevated temperature has

caused an increase in p32 expression which results in reducing late gene expression.

The mild heat treatment employed to pre-stress showed an impact on the PML protein expression and morphology of PML-NB, as they showed disruption and increased diffused staining when examined by confocal microscopy. A PML-II related protein band in particular showed an increase in expression, which reached a maximum after 90 minutes of stress as also did the mRNA level of PML-II, which showed a 3-fold increase after 3 hours of mild heat stress. Such increases in PML-II expression encouraged us to extensively analyse Ad5 gene expression in the presence and absence of PML-II in pre-stressed and control cells. Initial experiments which compared hexon mRNA expression in PML-II Kd and Ctrl.DNA cells showed an obvious increase in hexon mRNA expression following stress in PML-II Kd cells compared to control cells which showed more or less the same level of expression at both condition. This result was further confirmed by protein analysis. Connecting these results together suggests a role for PML-II in the reduced Ad5 gene expression in pre-stressed cells. The over-expressed PML-II due to the treatment with high temperature, which reaches to 3 times more (Fig.6.2), might trigger an increased or accelerated innate immune response since PML proteins are already IFN-upregulated genes (Lavau et al., 1995). The other possible explanation is that PML-II over-expression could up-regulate the expression of ASF/SF2 regulator, p32 (Petersen-Mahrt et al., 1999), which in turn would negatively affect late gene expression from the Ad5 MLTU. The integrated roles of PML-II in up-regulating type I interferon and pre-triggered innate immune responses could all work together to provide previously stressed cells with a mechanism which reduces Ad5 gene expression.

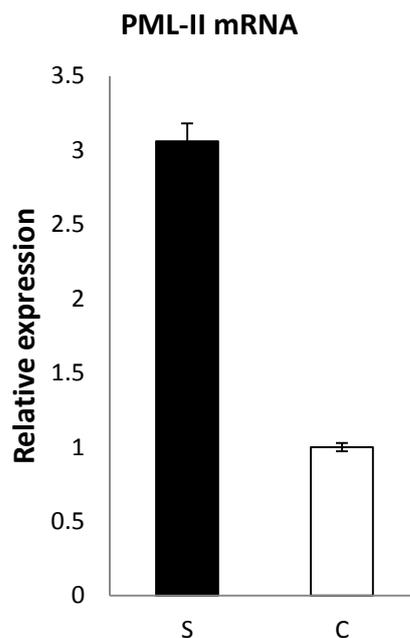


Fig.6.2. PML-II mRNA expression in pre-stressed and control cells. HeLa cells were seeded at a density of 1×10^5 /ml for 24 hours, then transfected with 125 nM siPML-II or siC for 48 hours. RNA samples were extracted, total Daxx mRNA was quantified by RT-qPCR using 10 ng/well of cDNA as a template. Data were calculated by the $\Delta\Delta C_t$ method, normalised to 18s internal control rRNA and then normalised to the control sample value. All PCR products were subjected to dissociation curve analysis to ensure primer specificity and.

6.7 How can heat-stress inhibit Ad5 while hsp70 elevation enhances its replication?

The effect of PML-II depletion in increasing viral gene expression and yield according to this study was attributed to the elevated hsp70 chaperone in PML-II Kd cells. This was the possible explanation since the elevation was very specific to hsp70 and hsp70B but not other HSPs such as hsp60, hence depleting the cells of PML-II did not initiate a general non-specific heat shock response. This specific elevation in hsp70 chaperone is consistent with the specific elevation of hsp70 that is triggered shortly after Ad infection (Phillips et al., 1991). This might explain the contradictory role of hsp70 in the Ad5 life cycle under normal and stressed conditions, since the latter initiates a global cellular response that includes all HSP family members. Within this study, expression of several hsp members was evaluated in parallel with hsp70 after mild stress; hsp60, hsp90, hsp27 and hsp100 (Fig 6.3). All of them showed an elevation compared with unstressed control cells

which ranged from moderate for the first two to very dramatic for the rest (the study was extended to include further genes at a late stage in order to further validate the conclusion). Thus the pre-stress conditions studied here were materially different from from the PML-II depleted unstressed conditions in that a full range of HSPs was induced. Universal promotion in HSP members and other cellular factors such as p32 might mitigate or equilibrate or even outweigh the beneficial effect of hsp70 which would promote Ad5 gene expression if it was specifically stimulated.

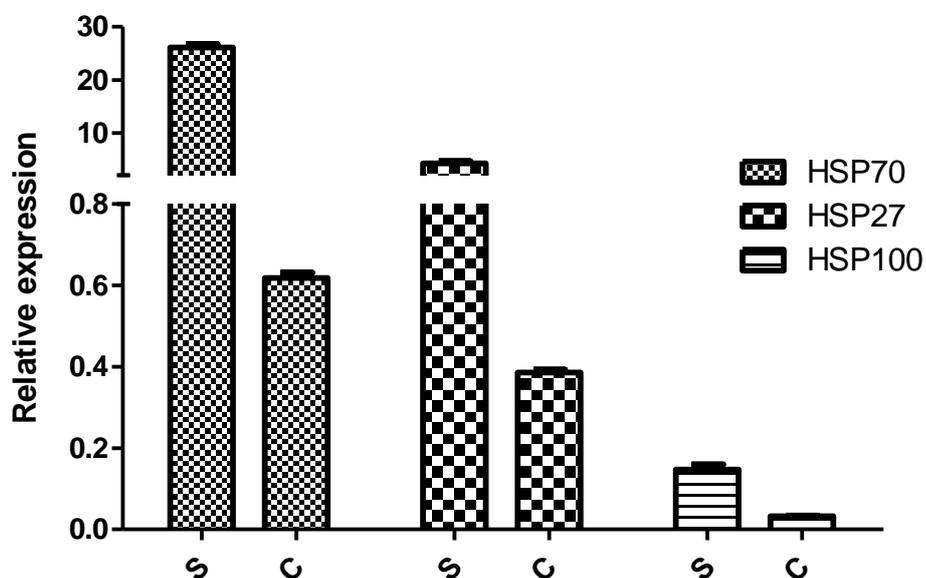


Fig.6.3. HSPs RNA expression under stress and control conditions. HeLa cells were seeded at a density of 1×10^5 /ml for 24 hours, either exposed to 40°C for 3 hours or left to grow at 37°C . RNA samples were extracted, total hsp70, 27 and 100 mRNA was quantified by RT-qPCR using 10 ng/well of complementary DNA (cDNA) as a template. Data were presented by the $\Delta\Delta Ct$ method and then normalised to the control sample value. All PCR products were subjected to dissociation curve analysis to ensure primer specificity and normalised to 18s.

6.8 Conclusions and future directions

In summary, PML-II showed an antiviral function against Ad5 which was eliminated to the benefit of the virus upon its permanent removal. This beneficial effect was due in large part to the elevated hsp70 that followed from PML-II removal since removal of hsp70 by siRNA in PML-II depleted cells reduced Ad5 late gene expression back to its normal level. Loss of type I interferon responses had only a partial role in the elevated gene expression in the absence of PML-II, because blocking the type I interferon response in control cells only elevated hexon expression somewhat to levels that did not approach those seen in PML-II Kd cells. Possibly there is crosstalk between the heat stress and IFN pathways since removing hsp70 by siRNA interference caused a substantial increase in ISG56 expression in control EV cells following Ad5 infection (Fig.6.4). Thus hsp70 induction during Ad5 infection normally opposes the innate response and may benefit the infection for that reason.

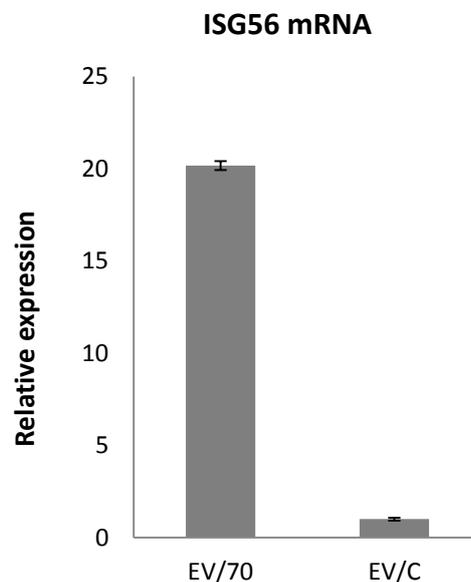


Fig.6.4.ISG56 mRNA expression at transient depletion of hsp70. HeLa cells were seeded at a density of 1×10^5 /ml for 24 hours, either exposed to 40 °C for 3 hours or left to grow at 37 °C . RNA samples were extracted, total hsp70, 27 and 100 mRNA was quantified by RT-qPCR using 10 ng/well of complementary DNA (cDNA) as a template. Data were calculated by the $\Delta\Delta C_t$ method and then normalised to the control sample value. All PCR products were subjected to dissociation curve analysis to ensure primer specificity and normalised to 18s.

So it is possible that the progression of Ad5 infection was affected by those two factors; IFN and hsp70 since both of them were either reduced or up-regulated with PML-II removal (Fig.6.5). Moreover, PML-II has a possible role in reducing Ad5 gene expression after mild heat stress since PML-II depletion prior to heat stress showed an increase in Ad5 late gene expression compared to non-depleted stressed cells.

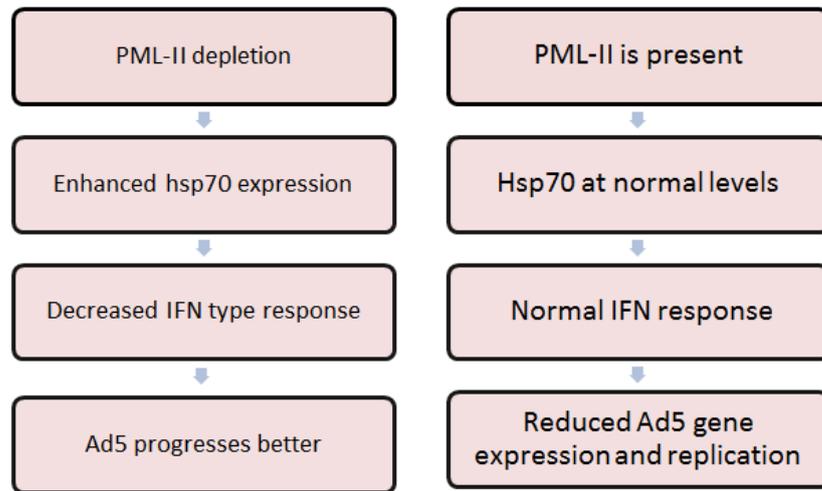


Fig.6.5. Schematic diagram for the proposed effect of PML-II removal on Ad5 infection.

This study has demonstrated two important phenomena: that Ad5 progresses better with the absence of PML-II and worse when it infects pre- stressed cells. Hence, future work would need to investigate the mechanism that explains how PML-II removal promoted hsp70 expression and why. The other open question is how the mild stress compromises the Ad5 gene expression, which pathway exactly was involved in that reduction and how. More extensive analysis is also needed to identify the PML-II related band of 55KDa molecular weight and its role in the Ad5 life cycle. Finally, further investigation should explore the effect of pre-stressing cells depleting PML-II on other types of DNA or RNA viruses and in different cell lines.

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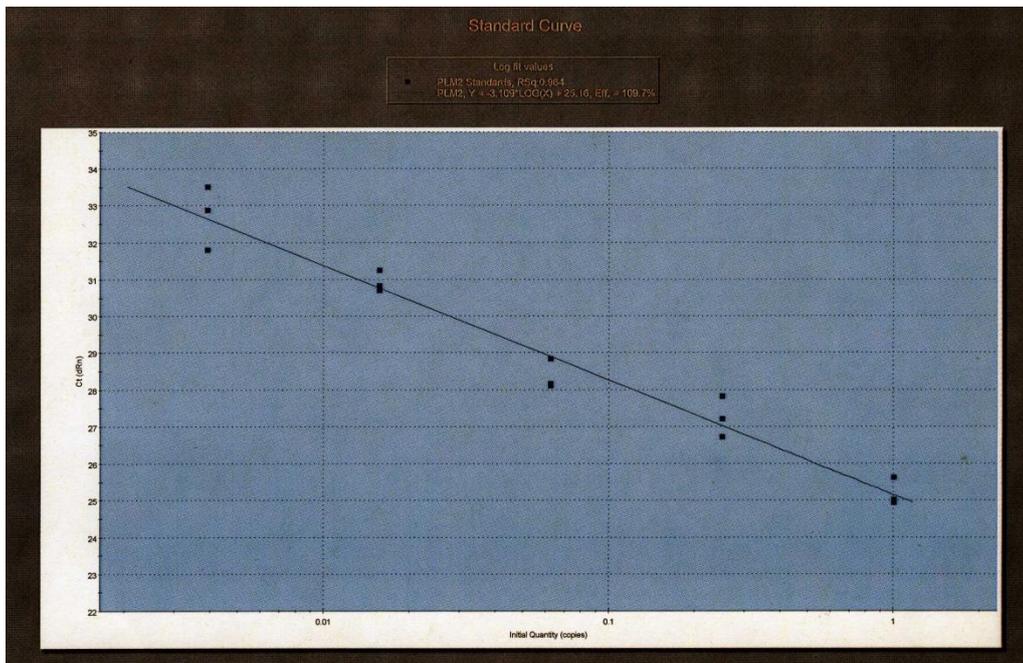
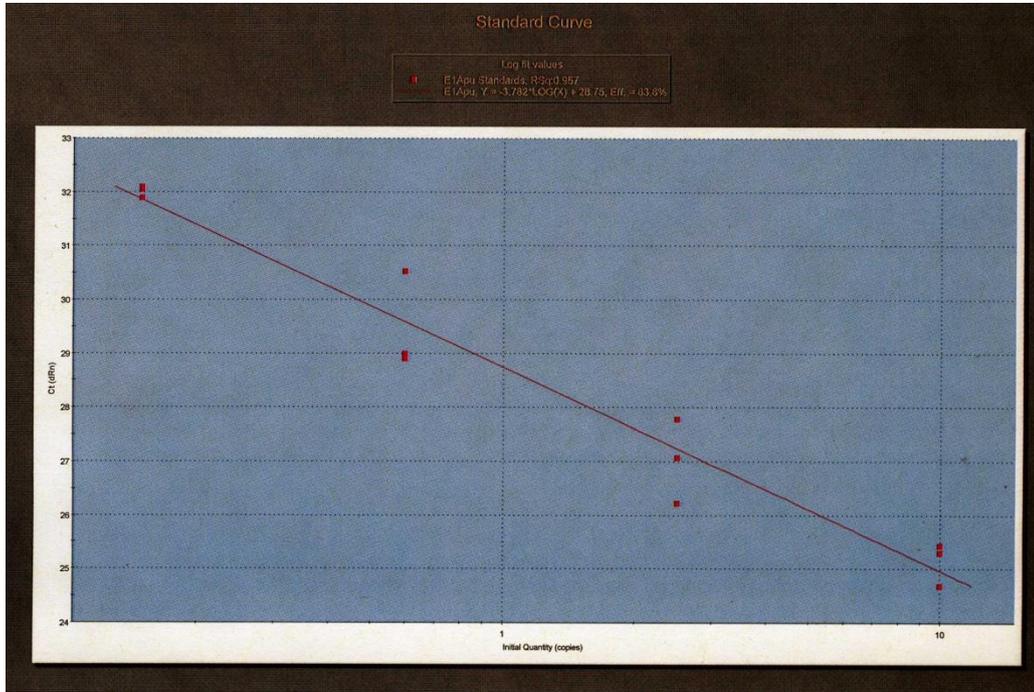
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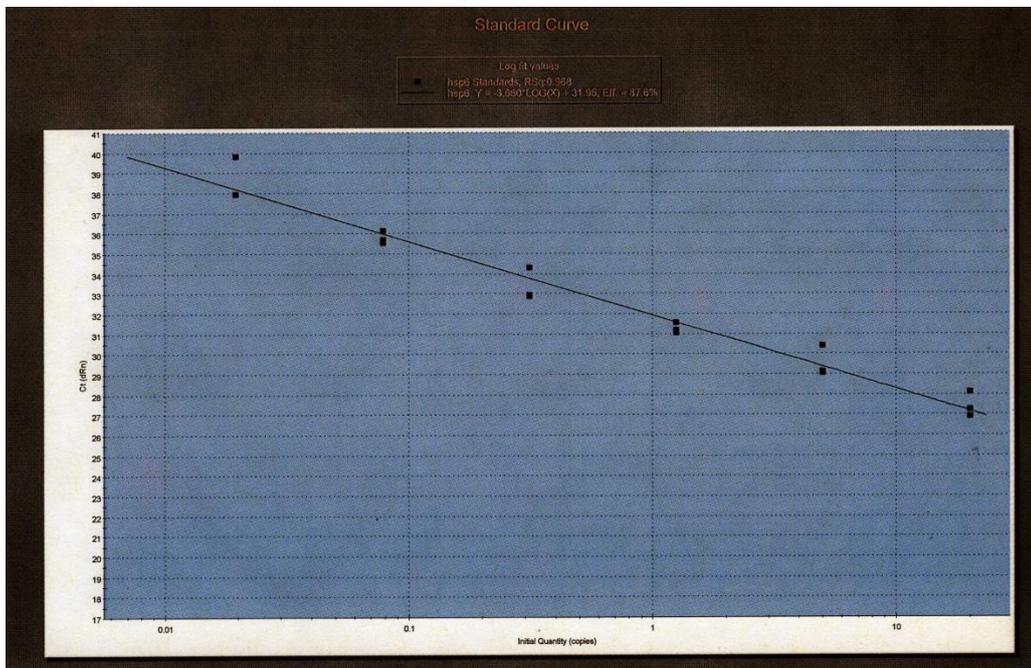
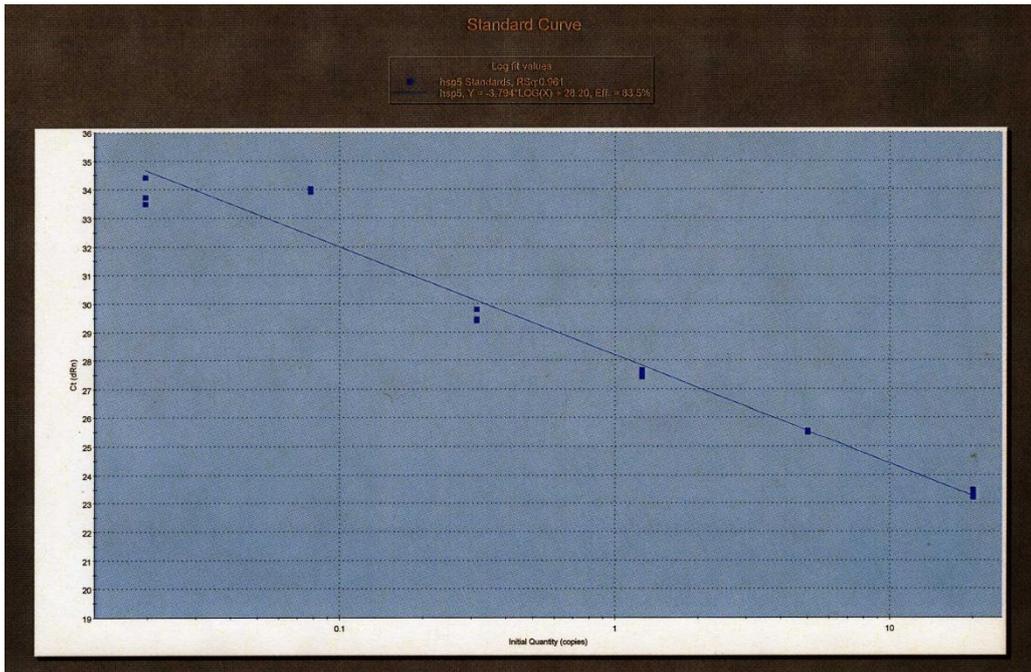
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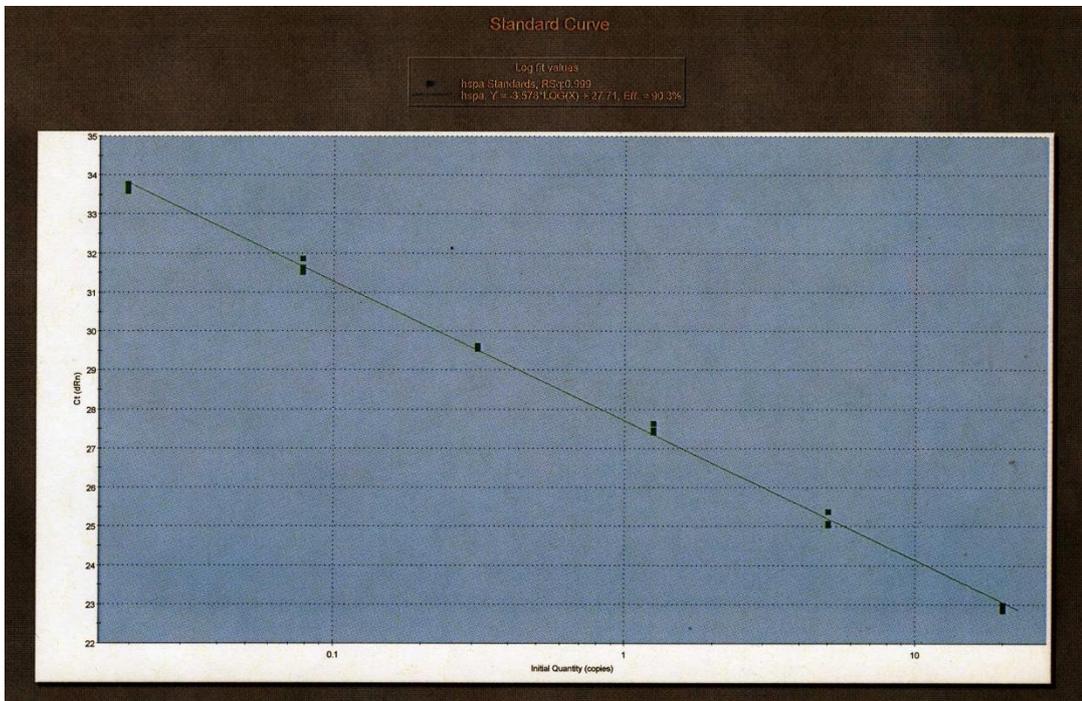
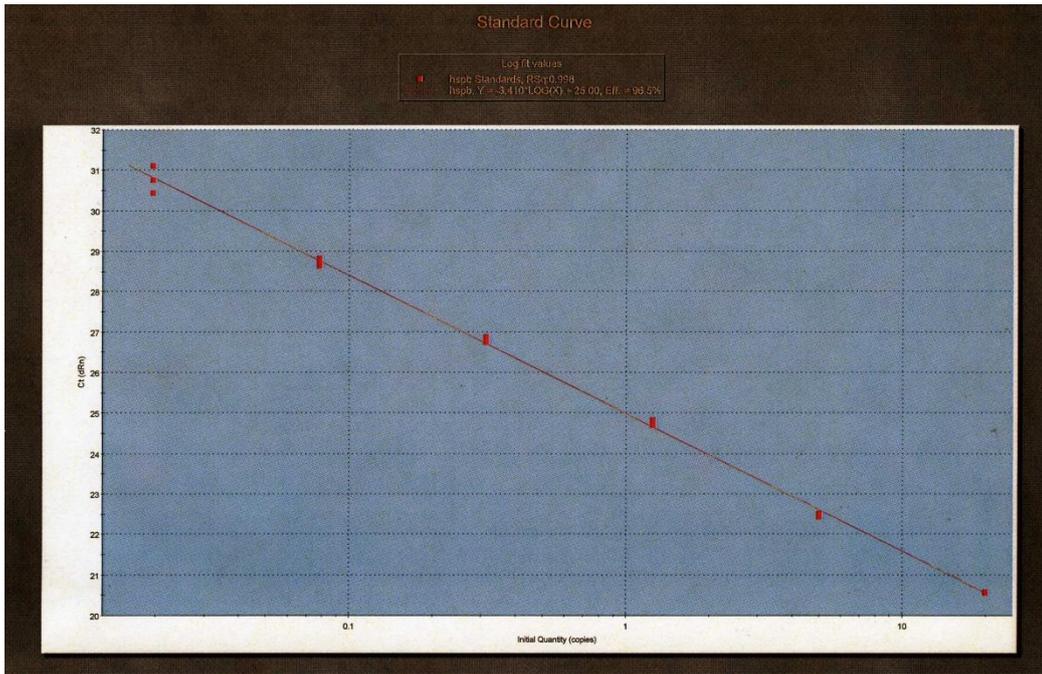
Appendix 1

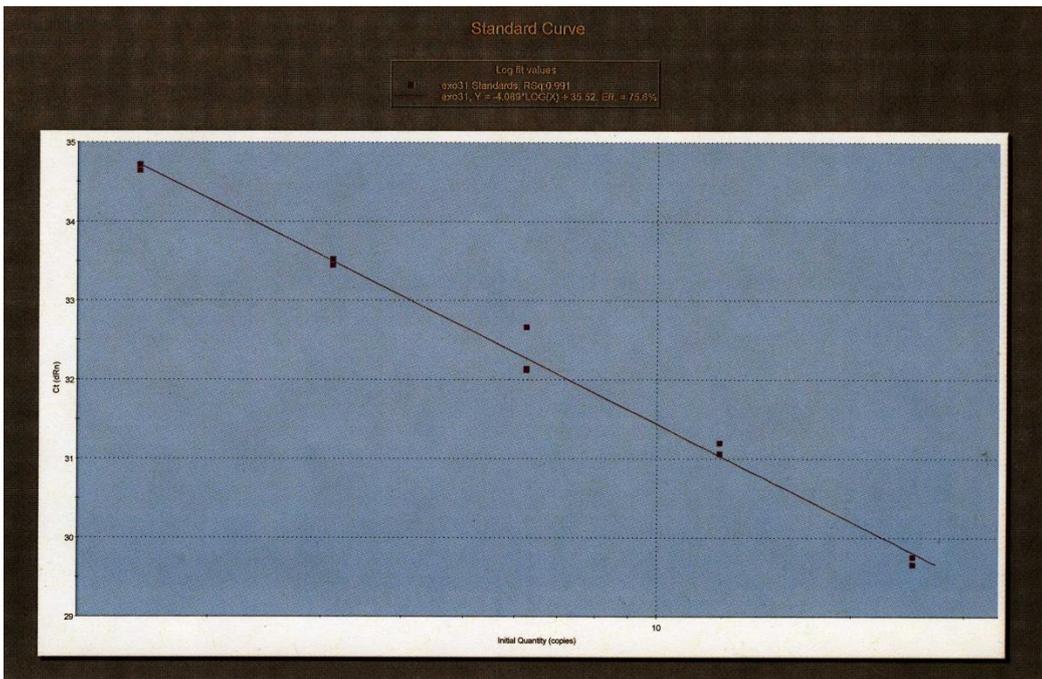
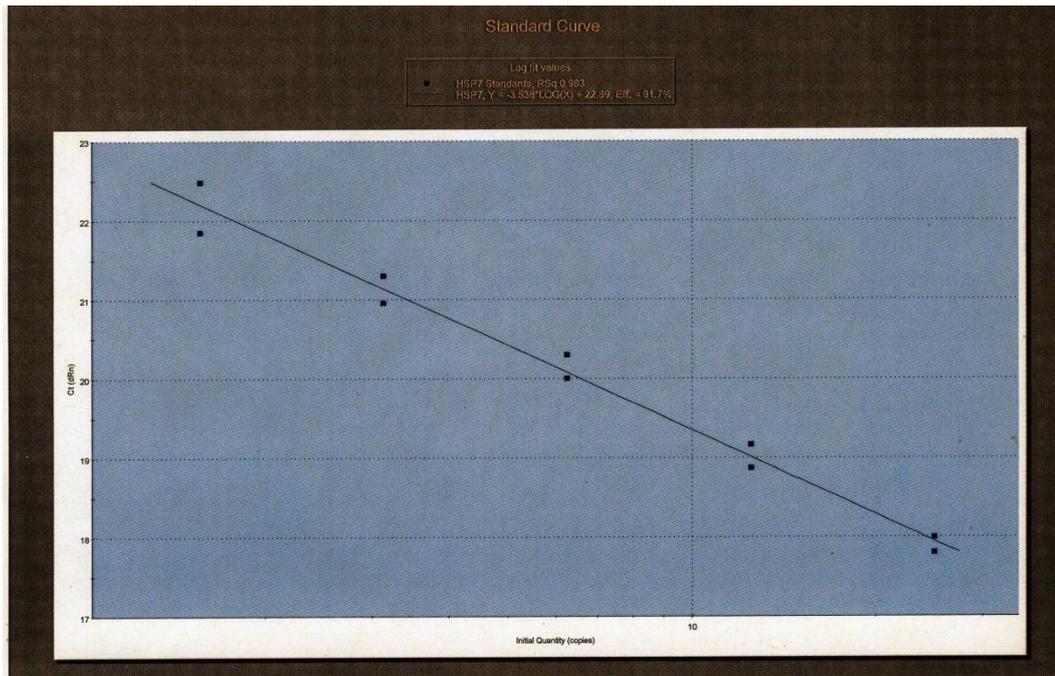
Standard curves of qPCR primers

NB: Efficiency of HSP70 was mentioned in Chen 2014 thesis



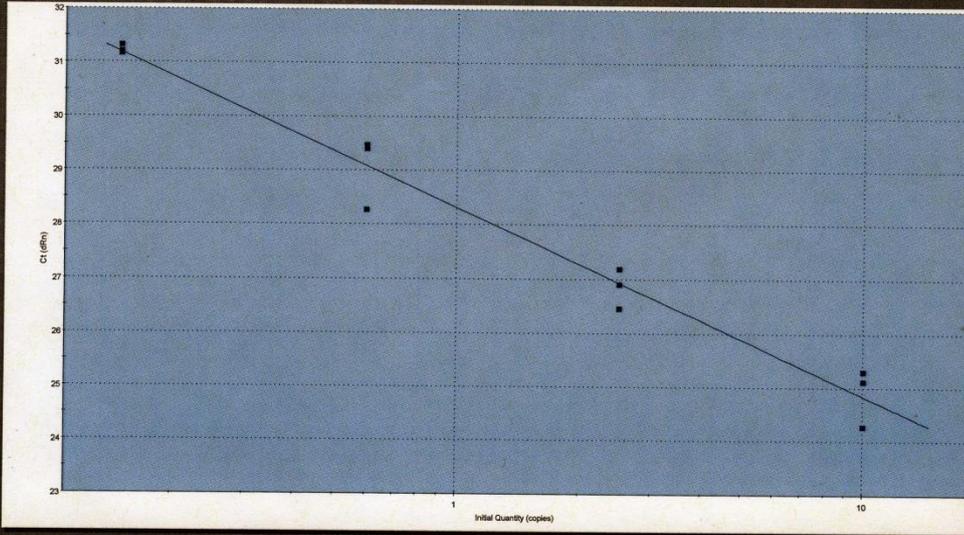






Standard Curve

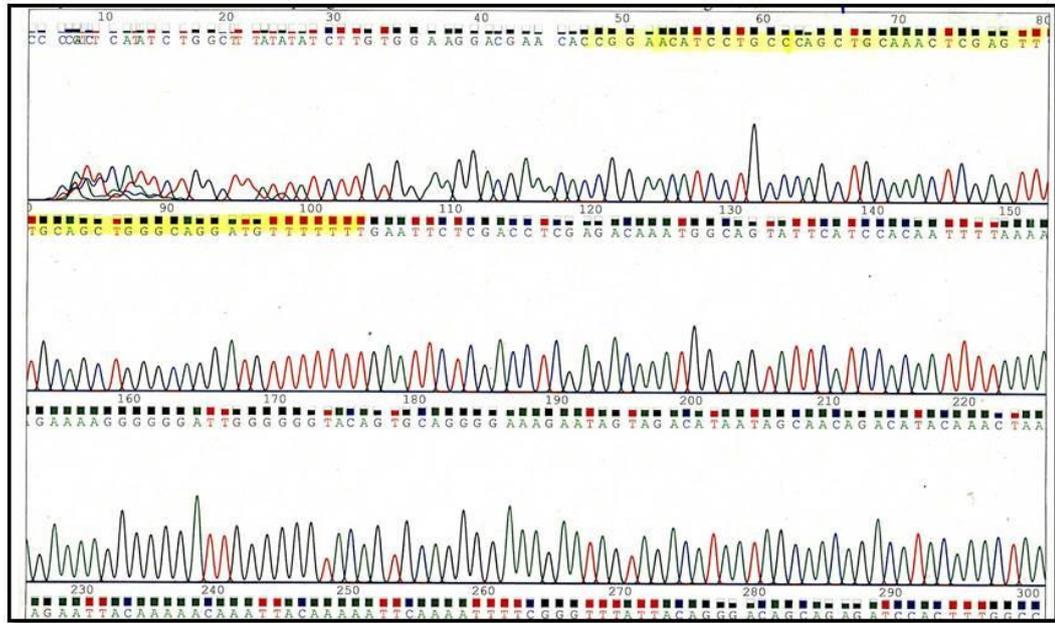
Log fit values:
hex Standards, Rsq: 0.973
hex, Y = -3.482 LOG(X) + 28.32, Eff. = 93.7%



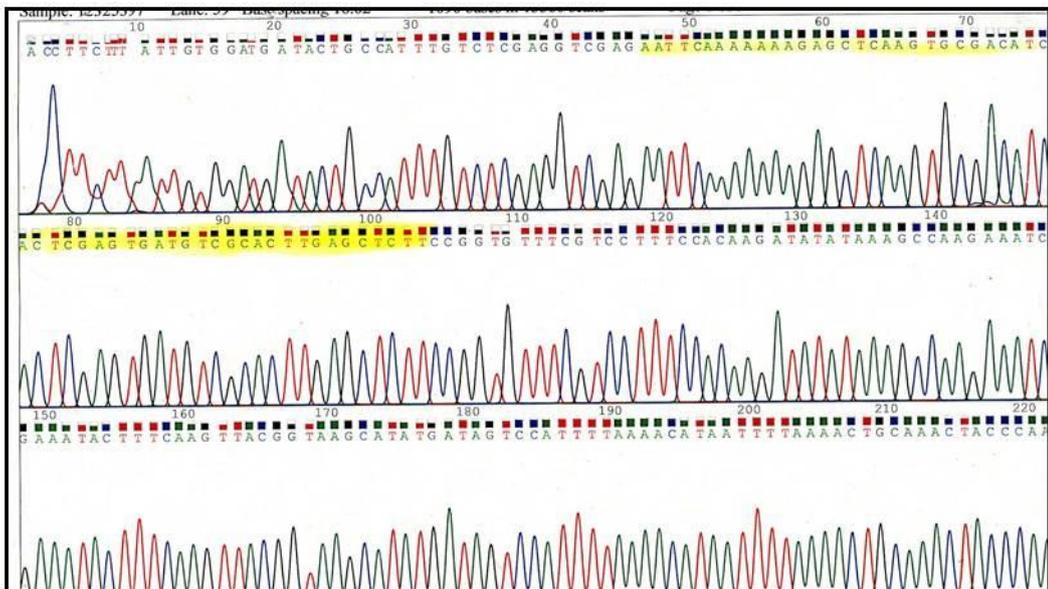
Appendix 2

shRNA sequences

PML-II-shRNA



PML-shRNA



Ctrl.DNA

