Analysis of the Modulation of Nuclear Domains by Human Adenovirus Type 5 Oncoproteins

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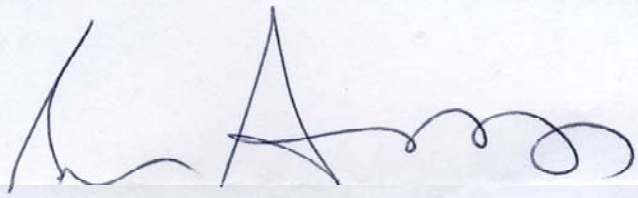

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1 Abstract

Human Adenovirus type 5 (HAdV5) represents a well-studied model system to analyze the transformation capabilities of DNA tumor viruses, the molecular properties of viral oncoproteins and basic principles of virus-induced tumorigenesis. Proteins of the *early region 1* (E1) and *4* (E4) mediate steps in the cellular transformation process, such as initiating unscheduled cell cycle progression, inactivating antiproliferative mechanisms by tumor suppressor proteins (e.g. pRB, p53) and modulating of a complex network of protein interactions involved in transcription, apoptosis, cell cycle control, DNA repair, cell signaling, posttranslational modification and the integrity of nuclear multiprotein complexes known as *PML nuclear bodies* (PML-NBs)/*PML oncogenic domains* (PODs).

The 55-kDa gene product encoded by the *early region 1B* (E1B-55K) is a multifunctional phosphoprotein that mediates essential functions during productive viral infection, as well as transformation of primary rodent cells. Although modulation of the cellular tumor suppressor p53 is considered to play the major role in adenoviral-mediated tumorigenesis, other p53-independent mechansisms have been suggested. Moreover, previous work illustrates that posttranslational modification of E1B-55K by the *small ubiquitin-related modifier 1* (SUMO-1) is absolutely required for efficient cellular transformation. However, the biochemical consequences and functional alterations of 55K by SUMO-1 conjugation have so far remained elusive.

Comprehensive biochemical analyses of functional interactions between E1B-55K and host cell factors identified a tight association between multiple adenoviral proteins and specific components of cellular *PML nuclear bodies*. Moreover, this study provides the first molecular evidence that the adenoviral E1A, E1B-55K and E4orf6 proteins physically interact with different isoforms of the tumor suppressor protein PML. In contrast, isoform-specific interactions of E1B-55K (with PML-IV, PML-V) involve SUMO-dependent and independent mechanisms. Interaction with PML-IV promotes localization of 55K to PML-containing subnuclear structures (PML-NBs) in virus-infected cells. This process is negatively regulated by other viral proteins, indicating that binding to PML is controlled through reversible SUMOylation in a temporally coordinated manner. Furthermore, molecular evidence is presented for E1B-55K being modified by multiple SUMO-isoforms in a phosphorylation-dependent manner, thus drastically expanding E1B-55K´s functional repertoire.

These results together with previous work are consistent with the idea that SUMOylation regulates targeting of E1B-55K to PML-NBs, which are known to control transcriptional regulation, tumor suppression, DNA repair and apoptosis. Furthermore, the data suggest that SUMO-dependent modulation of p53-dependent growth suppression via E1B-55K/PML-IV interaction plays a key role in adenovirus mediated cell transformation.

2 Introduction

2.1 Adenoviruses

2.1.1 Classification and Pathogenesis

Adenoviruses (Ads) were first discovered and isolated in the early 1950s from adenoid tissues and secretions from patients in studies aiming to identify the infectious agent causing the common cold (Rowe *et al.*, 1953; Hilleman & Werner, 1954). Subsequent investigations revealed that although these viruses do not cause the common cold, they generally result in infections of the respiratory tract (Ginsberg *et al.*, 1955; Dingle & Langmuir, 1968), the eye (Jawetz, 1959) or the gastrointestinal tract (Yolken *et al.*, 1982), causing diseases called *acute respiratory disease* (ARD), *adenoid-pharyngeal-conjunctival* (APC), *respiratory illness* (RI) or *adenoid degeneration* (AD). Adenoviruses are widely spread, infecting ~80% of all children by the age of five and mainly causing diarrhea. The family of *Adenoviridae* comprises of about 100 serologically different types and is known to infect a wide range of hosts, including mammalian and other vertebrate species. Consistently, adenoviruses are divided into five genera depending on their host range (Horwitz, 1996; Benkö *et al.*, 1999; Davison *et al.*, 2003):

1.) *Mastadenovirus*: mammalian hosts (Benkö & Harrach, 1998)
2.) *Aviadenovirus*: avian hosts (Benkö & Harrach, 1998)
3.) *Atadenovirus*: avian, reptilian and ruminant hosts (Benkö & Harrach, 1998)
4.) *Siadenovirus*: amphibian hosts (Davison *et al.*, 1993)
5.) *Ichtadenovirus*: fish hosts (Benko *et al.*, 2002)

To date, 54 *Human Adenoviruses* (HAdVs) of the genera *Mastadenovirus* are known and subgrouped into seven species (A-G) according to their sequence homology, hemagglutination and oncogenicity in immunosuppressed rodents (Wadell, 1984; Bailey & Mautner, 1994; Davison *et al.*, 2003; Berk, 2007). HAdV2 and HAdV5 are the most intensively studied types due to their non-oncogenic properties (Fig. 1) (Shenk, 2001).

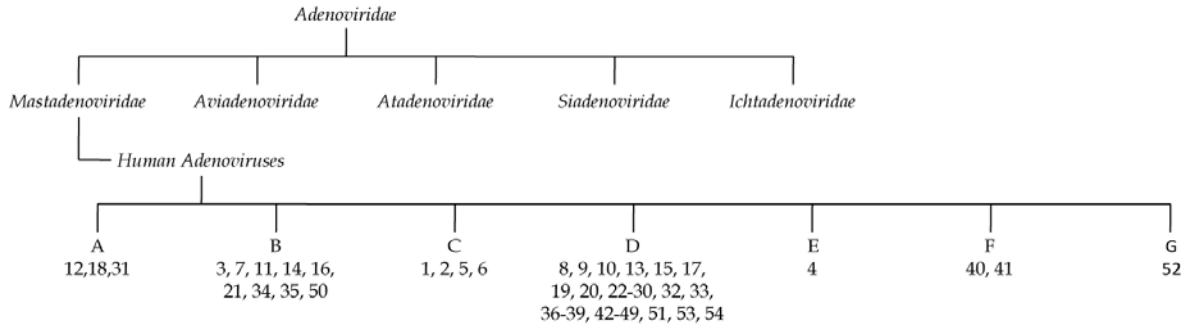

Figure 1. Classification of the family *Adenoviridae*. Schematic representation of *Adenoviridae* classification including HAdV types 1-54**.** The HAdV types 1-52 are classified as published by Davison *et al.* (Davison *et al.*, 2003), whereas type 53 and type 54 are classified by the *International Committee of the Taxonomy of Viruses* (ICTV).

HAdVs are highly prevalent in human populations, causing lytic as well as persistent infections associated with a multitude of different symptoms, although generally respiratory or gastrointestinal. Consequently, these viruses are frequently associated with acute respiratory disease (usually), pneumonia (occasionally), acute follicular conjunctivitis, epidemic keratoconjunctivitis, cystitis and gastroenteritis (occasionally). In infants, pharyngitis and pharyngeal-conjunctival fever are common (Baron, 1996), whereas HAdVs represent the second main causative agent of childhood diarrhea. The symptomatic HAdV infections are usually observed in young children and the subsequent long-term effective immunity represents the major problem in adenoviral-mediated gene therapy. Severe infections may occur in immunosuppressed patients, e.g. HAdVs-induced hemorrhagic cystitis is a recognized cause of morbidity and mortality following allogenic hematopoietic stem cell transplantation (Abe *et al.*, 2003).

2.1.2 Structure and Genome Organization

Adenoviruses are non-enveloped viruses with an icosahedral capsid of ~80-110 nm containing a linear double-stranded DNA genome that is tightly complexed with the core proteins V, VII and μ (Fig. 2) (Shenk, 2001). Furthermore, the genome contains several *inverted terminal repeats* (ITR) at its ends, ranging from 36-200 bp, and both 5´ ends are covalently bound to the 55 kDa *terminal protein* (TP), which is essential for initiating viral DNA replication (Davison *et al.*, 2003). The adenoviral capsid is composed of 252 structural units (*capsomers*) including 240 trimeric *hexon* (II) and 12 *penton* (III) proteins. The capsid protects the viral genome and plays an integral part in receptor-mediated virus entry into the host cell. The *fiber* (IV) proteins, which protrude from the capsid structure (*spikes*), are associated with

the *penton* (III) protein and mediate essential steps in the absorption and internalization of the virus via the *Coxsakie/Adenovirus Receptor* (CAR) (Bergelson *et al.*, 1997). Furthermore, the *penton base* protein facilitates efficient virus uptake by interacting with specific cell surface proteins (integrins) (Wickham *et al.*, 1993; Mathias *et al.*, 1994; Wickham *et al.*, 1994). Additional minor components of the capsid include the proteins IIIa, VI, VIII and IX (Russell & Matthews, 2003; Vellinga *et al.*, 2005; Russell, 2009).

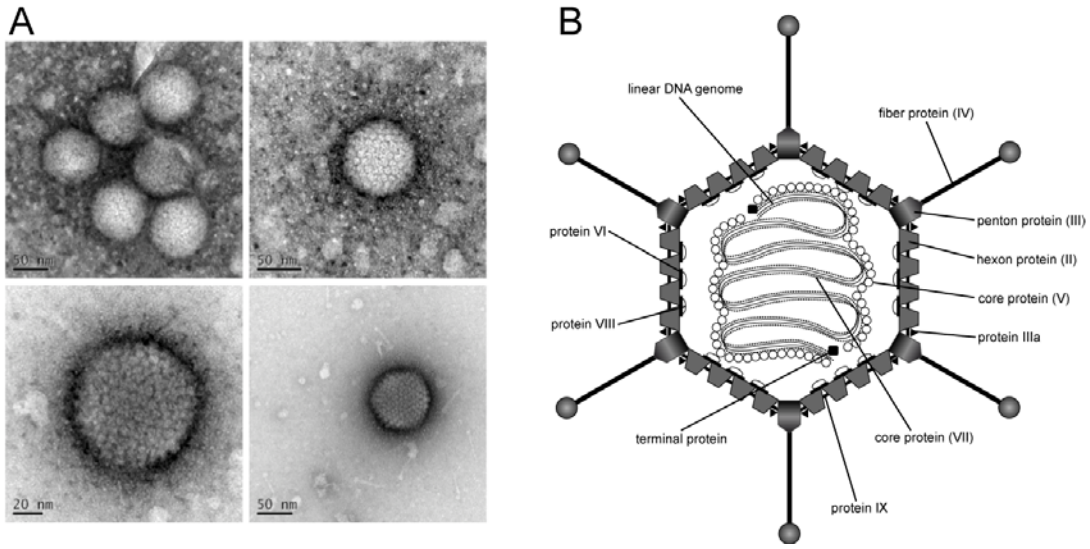

Figure 2. Electron microscope images and schematic representation of HAdV5. The electron microscope images shown in (A) illustrate the icosahedral structure of adenoviral particles as well as the sub-structuration in multiple capsomeres. A schematic structure of HAdV5 is shown in (B) (Stewart *et al.*, 1993; Modrow & Falke, 2002; Russell, 2009).

The genome of the most studied types 2 and 5 comprises nine transcription units encoding approximately 40 regulatory and structural proteins as well as two non-coding RNAs (*virus-associated RNAs*, VA-RNAs) (Fig. 3). The transcription units comprise five *early* (E1A, E1B, E2, E3, E4), two *delayed* (IX, IVa2) and the *major late transcription unit* (MLTU) generating five families of RNAs (L1-L5) upon processing. All of these transcription units are transcribed by RNA polymerase II, whereas the VA-RNAs are synthesized by RNA polymerase III (Shenk, 2001).

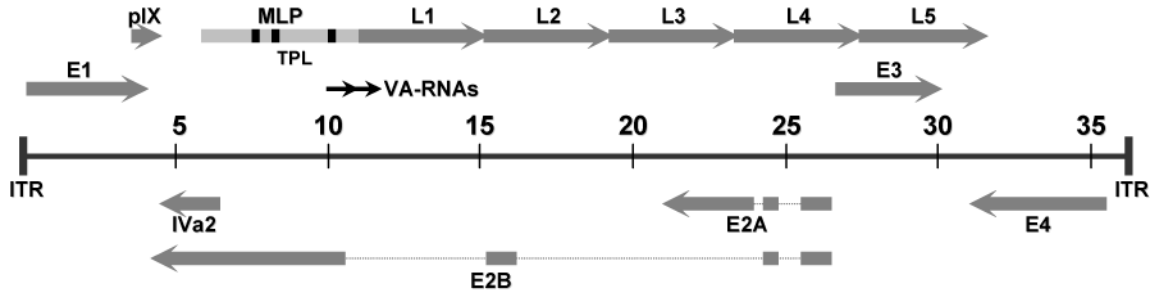

Figure 3. Genome organization of HAdV5. The organization of *early* (E1, E2A, E2B, E3, E4), *delayed* (IX, IVa2) and *late* (L1-L5, MLTU) transcription units on both DNA strands is illustrated by arrows in relation to the 35.9 kbp DNA genome. Early proteins are involved in DNA replication (E2), immune system modulation (E3), transcription/RNA processing (E1, E4) and cell cycle control (E1, E4). Late units (L1-L5) mainly encode for structural proteins, with a few exceptions. Abbr.: E: *early*; ITR: *inverted terminal repeat*; L: *late*; MLP: *major late promoter*; TPL: *tripartite leader*; VA-RNAs: *virus-associated RNAs*.

2.1.3 Productive Infection Cycle

HAdVs can infect a wide range of cell types *in vivo*, generally post-mitotic resting cells, differentiated epithelial cells of the respiratory/gastrointestinal tract and most likely, the central nervous system (Kosulin *et al.*, 2007). Additionally, several established tumor and primary cell lines can be infected by these viruses in tissue culture. Generally, HAdVs cause lytic infection in human cells, whereas infection of animal cells, in particular rodent cells, results in an abortive infection (Liebermann *et al.*, 1996; Shenk, 2001). Recently published observations discuss latent adenovirus infections (Gustafsson *et al.*, 2007; Kosulin *et al.*, 2007; Garnett *et al.*, 2009).

Adenoviral productive infection is divided into two major phases termed *early* and *late* with respect to the onset of viral DNA replication. Upon receptor-mediated internalization of the viral particle, the viral DNA/Core complex is imported into the cell nucleus and subsequently initiates the early phase by transcription/expression of the "*immediate early*" gene E1A (Moran *et al.*, 1986; Schaeper *et al.*, 1998; Avvakumov *et al.*, 2002a; Avvakumov *et al.*, 2002b). This leads to transcription of E1B- to E4-RNAs, which are alternatively spliced to produce the viral early regulatory proteins. These early proteins are multifunctional and establish an optimal environment for virus replication. Hence, these gene products are responsible for the induction of cell cycle progression (E1, E4), inhibition of apoptosis/growth arrest (E1A, E1B), modulation of the immune response (E3) and maintenance of cell viability (E3) (Shenk, 2001). The proteins of the E2 region are mainly involved in viral DNA replication, encoding for the *viral DNA binding protein* (DBP) (E2A), the *viral DNA polymerase* (E2B) and the precursor of the *terminal protein* (pTP) (Shenk, 2001). The E4 region encodes at least six

different products transcribed from several open reading frames, namely E4orf1, E4orf2, E4orf3, E4orf4, E4orf6 and E4orf6/7, mediating essential functions during efficient virus replication (Täuber & Dobner, 2001a; Täuber & Dobner, 2001b).

With the onset of DNA replication, the late phase begins with activation of the *major late promoter* (MLP) and consequently the production of late mRNAs, which are produced by differential splicing of the 29 kbp precursor mRNA (Fig. 3). All of these late mRNAs (L1-L5) contain a common 5´-non-coding sequence of 201 nucleotides (*tripartite leader*; TPL) mainly encoding structural, core and capsid components. During the late phase of infection, host cell mRNA transport and translation pathways are shut-off (*host cell shut-off*), whereas viral late mRNAs are efficiently synthesized, transported to the cytoplasm and preferentially translated. Finally, viral DNA packaging/encapsidation takes place in the nucleus and is orchestrated by late (L4-100K, -33K, -22K) and early regulatory (E1B-55K, E4orf6, E2A-72K) proteins. The viral life cycle is completed after approximately 24-36 hours and up to $1x10^4$ viral particles are released upon host cell lysis (Shenk, 2001).

2.1.4 Oncogenic Potential of HAdVs/Adenoviral Proteins

2.1.4.1 Oncogenic Potential of HAdVs

Since the initial discovery of the oncogenic potential of HAdV12 in newborn rodents by Trentin and co-workers (Trentin *et al.*, 1962), intense research has been carried out on these DNA tumor viruses. Today, most of the genes and their products involved in HAdV transformation have been identified, as well as the differences in oncogenicity among different types (Branton *et al.*, 1985; van der Eb & Zantema, 1992; Williams *et al.*, 1995; Nevins & Vogt, 1996; Endter & Dobner, 2004). According to the frequency and time required to establish tumors in rodents, HAdVs can be subdivided into highly oncogenic, weakly oncogenic and non-oncogenic (Fig. 4). Tumors in rodents induced by the highly oncogenic species A and weakly oncogenic species B develop at the site of injection and vary greatly in type depending on the route of administration, such as neurogenic, neuroepithelial, medulloblastic and adenocarcinomatous tumors (Graham, 1984; Graham *et al.*, 1984; Branton *et al.*, 1985). In contrast, types 9 and 10 (species D) develop estrogen-dependent mammary tumors at 100% frequency within three months of subcutaneous or intraperitonal injection in female rats (Ankerst & Jonsson, 1989; Javier, 1994; Thomas *et al.*, 2001). The differences in oncogenicity among different HAdV species are related to the genetic background of the host animal as

well as the host immune system (van der Eb & Zantema, 1992; Williams *et al.*, 1995). Despite these differential oncogenicities in animals, all tested types are able to transform rodent cells in culture with similar efficiency (McBride & Wiener, 1964; Nevins & Vogt, 1996). In this context, transformed cells loose contact inhibition and grow as multilayered cell colonies (*foci*). However, not all of these transformed cells are capable of tumor induction upon inoculation into rodents. Consistent with the observations for oncogenicity of different HAdV types in rodents (Fig. 4), tumorigenesis depends on different factors such as the thymus-dependent CTL response of the animal (Raska & Gallimore, 1982; Bernards *et al.*, 1983; Cook *et al.*, 1987).

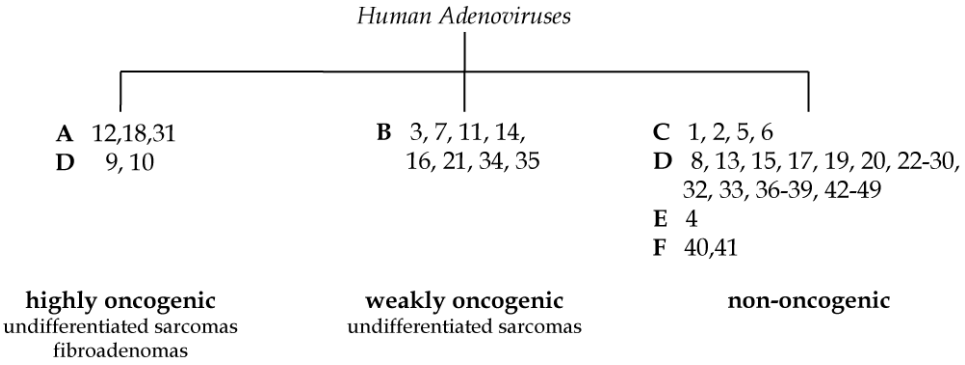

Figure 4. Oncogenic potential of HAdVs in rodents. List of the currently known phenotypes relating to the oncogenicity of HAdVs in rodents as well as the kind of induced tumors. Types 50-54 cannot be included since sufficient studies addressing their oncogenic potential are not yet available.

Contrary to their oncogenic potential in animals and high transforming potential in cultured rodent cells, HAdV-mediated transformation of primary human cells in culture is a very inefficient process (Hahn *et al.*, 1999). This might be explained in part by the fact that HAdVs undergo the full productive replication cycle within human cells, inevitably leading to cell death, whereas rodent cells are not permissive for HAdV infection, which results in an abortive process. To date, HAdVs could never be convincingly associated with malignant diseases in humans (Mackey *et al.*, 1976; Mackey *et al.*, 1979a; Mackey *et al.*, 1979b; Wold *et al.*, 1979; Chauvin *et al.*, 1990). However, rare cases of adenoviral transformed human cell lines in cell culture have been described (Graham *et al.*, 1977; Byrd *et al.*, 1982; Whittaker *et al.*, 1984; Gallimore *et al.*, 1986; Fallaux *et al.*, 1996; Fallaux *et al.*, 1998; Schiedner *et al.*, 2000). Recently two separate studies suggested that adenovirus infections are related to tumorigenesis in brain tissue (Kosulin *et al.*, 2007) and/or in childhood *acute lymphoblastic leukemia* (ALL) (Gustafsson *et al.*, 2007). A possible relationship between supposed long-term, non-harmful adenovirus infecton, adenoviral persistence and human malignancies remains to be established.

2.1.4.2 Oncogenic Potential of Adenoviral Proteins of the E1- and E4-Region

Most HAdV tumors, tumor derived cell lines and transformed cell lines are characterized by the persistence of chromosomally integrated viral DNA and the expression of virus specific antigens (Graham *et al.*, 1984). Correspondingly, HAdV transformation follows the classical concept of viral oncogenesis, where viral genes persist within the transformed cells maintaining the oncogenic phenotype. The E1 region is almost invariably retained in all transformed cells, underlining the importance of the encoded proteins (Branton *et al.*, 1985; van der Eb & Zantema, 1992; Williams *et al.*, 1995; Nevins & Vogt, 1996; Endter & Dobner, 2004). Although intensive studies were carried out to reveal the molecular mechanisms of this process, it still remains elusive why primary rodent cells can be efficiently transformed, whereas transformation of primary human cells is rather inefficient, with a few exceptions (Graham *et al.*, 1977; Byrd *et al.*, 1982; Whittaker *et al.*, 1984; Gallimore *et al.*, 1986; Fallaux *et al.*, 1996; Fallaux *et al.*, 1998; Schiedner *et al.*, 2000).

Initially, permissivity was proposed to be the major determinant for cellular transformation. However, detailed studies using subgenomic DNA fragments suggest rather that it represents one factor in a much more complicated system (Branton *et al.*, 1985; Endter & Dobner, 2004). This assumption appears adequate, since it could be shown recently that the transforming capabilities of adenoviral proteins are not exclusively restricted to rodents, but also include semi-permissive cells of the closely related mammalian order *lagomorpha* (Wimmer *et al.*, 2010).

Although proteins of the E1-region seem to be indispensible for efficient cell transformation, it appears that proteins of the E4-region support this process, although the mode of transformation may differ reflecting the previously described "*Hit & Run Mechanism*" (Nevels *et al.*, 2001). In this context, the E4 gene products operate through a complex network of protein interactions involved in transcription, apoptosis, cell cycle control, DNA repair, cell signaling, posttranslational modification and the integrity of nuclear multiprotein complexes known as *PML nuclear bodies* (PML-NBs)/*PML oncogenic domains* (PODs) (Täuber & Dobner, 2001a; Täuber & Dobner, 2001b).

2.1.4.2.1 Early Region 1A

E1A is the first transcription unit to be expressed after the virus reaches the nucleus of infected cells (Boulanger & Blair, 1991), where two major mRNA species generated by alternative splicing encode for E1A-13S (289 aa) and E1A-12S (243 aa). Additionally, three more

mRNA species of E1A (11S, 10S, 9S) are known to be expressed, but no definitive functions have as yet been assigned to these forms. In principle, E1A proteins represent acidic protein molecules, with a presumed low amount of secondary structure, that are distributed in roughly equal amounts in the cytoplasm and nucleus (Frisch & Mymryk, 2002).

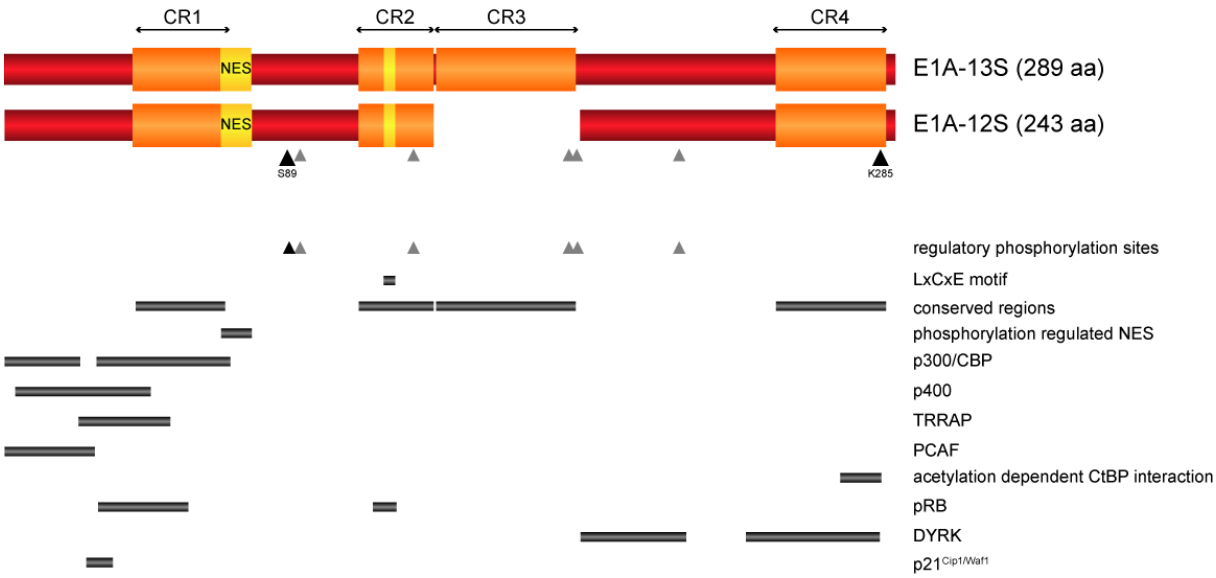

Figure 5. Schematic domain structure of HAdV5 E1A-13S/12S. The schematic domain structure of E1A-13S/12S highlights described regions (orange bars), specifically defined amino acid motifs (yellow bars) and experimentally verified interaction regions (grey bars). Specific phosphorylatable amino acids are illustrated by grey triangles, while S89 and K285 have been shown to regulate phosphorylation-dependent nuclear export and acetylation-dependent CtBP interaction (black triangles). For more detailed explanation/references see the text. Abbr.: CR: *conserved region*; NES: *nuclear export signal*.

E1A proteins mediate the indispensable and most critical step in cell transformation, i.e. initiating unscheduled cell cycle progression by interactions with various different cellular proteins (Fig. 5) (Branton *et al.*, 1985; Frisch & Mymryk, 2002; Endter & Dobner, 2004; Ferrari *et al.*, 2008; Ferrari *et al.*, 2009). This step is essential during viral infection to establish optimal conditions for progeny virus production. In this context, E1A induces immortalization of primary rodent cells by modulating the functions of key regulators controlling cell cycle progression and programmed cell death (Gallimore *et al.*, 1984a; Gallimore *et al.*, 1984b).

Intriguingly, E1A also exhibits anti-oncogenic characteristics by reversing the transformed phenotypes of several human tumor cell lines as well as suppressing human tumor growth (Frisch & Mymryk, 2002). This paradox remains to be solved, since the molecular mechanisms of E1A function are only partly understood due to its modulation of an immense complex network of cellular pathways (Frisch & Mymryk, 2002). However, the most promi-

nent participants known so far are epigenetic as well as transcriptional regulators such as pRB, p300/CBP, PCAF, CtBP, $p21^{Cip1/Waf1}$, $p27^{Kip1}$, DYRKs, p400 and TRRAP (Frisch & Mymryk, 2002; Ferrari *et al.*, 2008; Ferrari *et al.*, 2009), which enable E1A to dynamically, transitorily and temporally modulate approximately 70% of all gene promoters (Ferrari *et al.*, 2008; Ferrari *et al.*, 2009).

The most notable example of such proteins is the *retinoblastoma tumor suppressor* (pRB) (Dyson *et al.*, 1989; Buchkovich *et al.*, 1990; Giordano *et al.*, 1991) as well as its family members p107 and p130 (Classon & Dyson, 2001). E1A binds exclusively to the hypophosphorylated form of pRB via its LxCxE motif in CR2 and partly involving a portion of CR1 (Fig. 5) (Fattaey *et al.*, 1993; Ikeda & Nevins, 1993; Mittnacht *et al.*, 1994). This event mediates the dissociation of pRb from E2F transcription factors, subsequent activation of E2F responsive cellular genes and induction of cell cycle progression (Cress & Nevins, 1996).

In the course of an abortive infection in non-permissive cells, these same growth deregulatory functions can lead to immortalization and/or partial transformation. However, in the majority of E1A-immortalized cells the transformation process is incomplete (Gallimore *et al.*, 1984a; Gallimore *et al.*, 1984b) since E1A additionally induces p53 stabilization (Debbas & White, 1993; Lowe & Ruley, 1993; Grand *et al.*, 1994; Sabbatini *et al.*, 1995b; Samuelson & Lowe, 1997; Turnell *et al.*, 2000) and atypical apoptosis (Mymryk *et al.*, 1994). To counteract these mechanisms and establish a completely transformed phenotype requires the coexpression of E1B (Ruley, 1983).

2.1.4.2.2 Early Region 1B – E1B-19K & E1B-55K

The E1B region encodes the two major proteins E1B-19K and E1B-55K, which are both capable of facilitating complete transformation of pBRK cells in combination with E1A (van der Eb & Zantema, 1992) by distinct but additive mechanisms (Gallimore *et al.*, 1985; McLorie *et al.*, 1991). Additionally, three alternative splice variants and two minor, N-terminal truncated E1B-55K proteins are known, which at least partly seem to share functions with the large E1B-55K protein (Sieber & Dobner, 2007; Kindsmüller *et al.*, 2009).

Although some results suggested that E1B-19K is not definitely needed for efficient transformation (Telling & Williams, 1993), it can be assumed that both E1B proteins contribute to complete cell transformation at least in part by antagonizing apoptosis and growth arrest (Debbas & White, 1993). In this context, E1B-19K functions similarly to the cellular proto-oncogene Bcl2, modulating the apoptosis regulators Bax and CED4, as well as antagonizing

antiproliferative p53 signalling via a so far unknown molecular mechanism (White, 1993; Sabbatini *et al.*, 1995a; White, 1995; White, 1996; White, 1998; White, 2001).

The E1B-55K protein has been studied extensively since it represents a multifunctional protein that mediates its various functions at all stages of cellular transformation and lytic viral lifecycle and on multiple levels such as transcriptional, post-transcriptional, translational and post-translational regulation. Although it is fascinating how this viral 496 aa protein mediates such a variety of functions, it is far beyond the scope here to give a complete overview; however some particular points of special interest are discussed below.

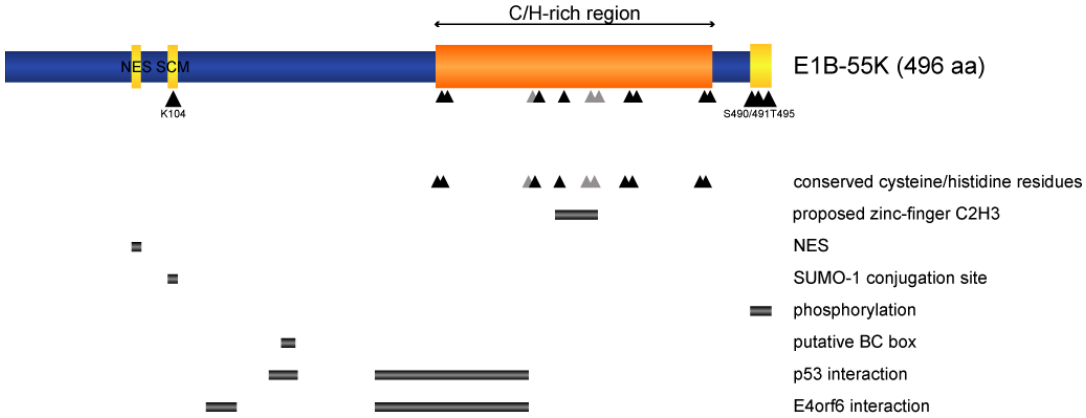

Figure 6. Schematic domain structure of HAdV5 E1B-55K. The schematic domain structure of E1B-55K highlights described regions (orange bars), specifically defined amino acid motifs (yellow bars) and experimentally verified interaction regions (grey bars). The specifically highlighted amino acids K104 and S490/491T495 represent the main residues of posttranslational modification, particularly SUMOylation and phosphorylation. Conserved cysteine (black) and histidine (grey) residues are illustrated by triangles. For more detailed explanation/references see the text. Abbr.: BC box: *Elongin B/Elongin C box*; C/H-rich region: *cysteine/histidine-rich region*; NES: *nuclear export signal*; SCM: *SUMO-1 conjugation motif*.

The E1B-55K protein continuously shuttles between the nucleus and cytoplasm, mediated at least in part by a leucine-rich *nuclear export signal* of the HIV-1 rev-type (NES) (Krätzer *et al.*, 2000; Endter *et al.*, 2005; Kindsmüller *et al.*, 2007) and a *SUMO-1 conjugation motif* (SCM) (Endter *et al.*, 2001; Endter *et al.*, 2005; Kindsmüller *et al.*, 2007). In this context, nuclear export depends on the export factor CRM1, which can be specifically inhibited by the artificial drug leptomycin B. An additional CRM1-independent export pathway has been suggested to play a role during viral infection (Kindsmüller *et al.*, 2007). The involvement of covalently attached SUMO-1 in nucleo-cytoplasmic shuttling appears less clear, however several lines of evidence indicate that this posttranslational modification regulates E1B-55K nuclear localization and subnuclear targeting (Endter *et al.*, 2001; Endter *et al.*, 2005; Kindsmüller *et al.*, 2007). Interestingly, functional inactivation of the E1B-55K NES induces

enhanced posttranslational modification of E1B-55K by SUMO-1, as well as augmenting transformation of pBRK cells through accumulation of p53, E1B-55K and PML in large sub-nuclear aggregates (Endter *et al.*, 2001; Endter *et al.*, 2005).

Vice versa, mutational inactivation of the SCM completely abrogates both E1B-55K sub-nuclear localization and its ability to transform pBRK cells in combination with E1A (Endter *et al.*, 2001; Endter *et al.*, 2005). The pro-tumorigenic functions of E1B-55K are primarily linked to modulation of the tumor suppressor p53 (Farmer *et al.*, 1992; Yew *et al.*, 1994; Nevels *et al.*, 1997; Martin & Berk, 1998; Martin & Berk, 1999; Liu *et al.*, 2000). Subsequent steps involving direct interaction (Sarnow *et al.*, 1982a; Kao *et al.*, 1990), transcriptional re-pression (Yew *et al.*, 1994; Martin & Berk, 1998; Martin & Berk, 1999) and nuclear-cytoplasmic relocalization (Endter *et al.*, 2001; Endter *et al.*, 2005) induce the complete silencing of p53-dependent tumor suppressive functions. Consistently, repression of p53 by E1B-55K ultimately depends on SUMO-1 modification of the viral protein, as revealed by enhanced repression with the E1B-55K-NES mutant and complete loss of repression with the E1B-55K-SCM mutant (Endter *et al.*, 2001; Endter *et al.*, 2005). However, previously described results suggest additional p53-independent mechanisms of E1B-55K induced cellular transformation involving cellular factors such as Mre11 (Härtl *et al.*, 2008) or the transcription factor Daxx (Sieber & Dobner, 2007; Schreiner, 2010).

2.1.4.2.3 Early Region 4 – E4orf3 & E4orf6

The adenoviral E4 region encodes up to seven multifunctional regulatory proteins, which are named E4orf1 to E4orf6/7 according to the arrangement of their open reading frames. These proteins act via an extremely complex network of protein interactions mediating modulation of post-translational modification, signal transduction, transcription, DNA repair, cell cycle control and apoptosis (Täuber & Dobner, 2001a; Täuber & Dobner, 2001b). Two of these early adenoviral proteins, E4orf3 and E4orf6, share at least partially redundant roles (Bridge & Ketner, 1989; Huang & Hearing, 1989) that are particularly important for efficient viral replication, i.e. efficient DNA replication (Bridge *et al.*, 1993), viral late protein synthesis, shut-off of host protein synthesis, late viral mRNA transport (Nordqvist & Akusjärvi, 1990; Nordqvist *et al.*, 1994) and progeny virus production (Huang & Hearing, 1989). Furthermore, E4orf3 and/or E4orf6 have been shown to cooperate with E1A and E1B in transformation of primary BRK cells (Nevels *et al.*, 1999; Nevels *et al.*, 2001). In this context, two observations concerning the functions of these two adenoviral proteins are of significant relevance.

The E4orf3 protein has been shown to disrupt cellular PML-bodies and induce the formation of so-called *track-like structures* in the nucleus of cells (Carvalho *et al.*, 1995; Puvion-Dutilleul *et al.*, 1995; Doucas *et al.*, 1996). This reorganization of *PML nuclear bodies* (PML-NBs) is highly conserved among most species of HAdVs (Stracker *et al.*, 2005) and therefore suggests an important function during adenoviral infection, presumably in mediating elimination of intracellular viral defense barriers (2.2.2.3) (Doucas *et al.*, 1996; Everett, 2001; Everett & Chelbi-Alix, 2007; Tavalai & Stamminger, 2008). Interestingly, it appears likely that the PML redistribution exclusively relies on E4orf3 interacting with the PML isoform II (Leppard & Everett, 1999; Hoppe *et al.*, 2006; Leppard *et al.*, 2009). Furthermore, E4orf3 is involved in the modulation of certain transient components of *PML nuclear bodies*, such as the tumor suppressor protein p53 (König *et al.*, 1999) and DNA repair machinery (Weiden & Ginsberg, 1994; Stracker *et al.*, 2002a; Araujo *et al.*, 2005; Liu *et al.*, 2005). This supports the assumption that the transforming potential of E4orf3 (Nevels *et al.*, 1999; Nevels *et al.*, 2001; Täuber & Dobner, 2001a; Täuber & Dobner, 2001b) may be mediated, at least in part, by its interaction with PML and/or associated components. Additionally, the interaction of E4orf3 with the other early proteins E1A, E1B-55K and E4orf6 (König *et al.*, 1999; Stracker *et al.*, 2002a; Araujo *et al.*, 2005; Liu *et al.*, 2005) is a prerequisite for fulfilling its functions during viral infection, as well as for the transformation of primary BRK cells (Nevels *et al.*, 1999; Nevels *et al.*, 2001; Täuber & Dobner, 2001a; Täuber & Dobner, 2001b).

In contrast, the E4orf6 protein has been analyzed in considerably more detail and consequently the known functions are more complex. In principle, E4orf6 protein´s shuttling function is mediated by an arginine-faced amphipathic α–helix that serves as a *nuclear retention signal* (NRS)/*nuclear localization signal* (NLS) (Orlando & Ornelles, 1999) as well as a NES motif (Weigel & Dobbelstein, 2000). The α–helix has been shown to be important for many E4orf6 functions, such as relocalization of E1B-55K from the cytoplasm to the nucleus (Ornelles & Shenk, 1991; Goodrum *et al.*, 1996; Dobbelstein *et al.*, 1997; Orlando & Ornelles, 1999; Orlando & Ornelles, 2002; Marshall *et al.*, 2008). Although it has been repeatedly shown that the α-helix is required for efficient viral replication and late mRNA export (Orlando & Ornelles, 2002; Higashino *et al.*, 2005) the role of nucleo-cytoplasmic shuttling of E4orf6 is still controversial (Rabino *et al.*, 2000; Weigel & Dobbelstein, 2000; Higashino *et al.*, 2005).

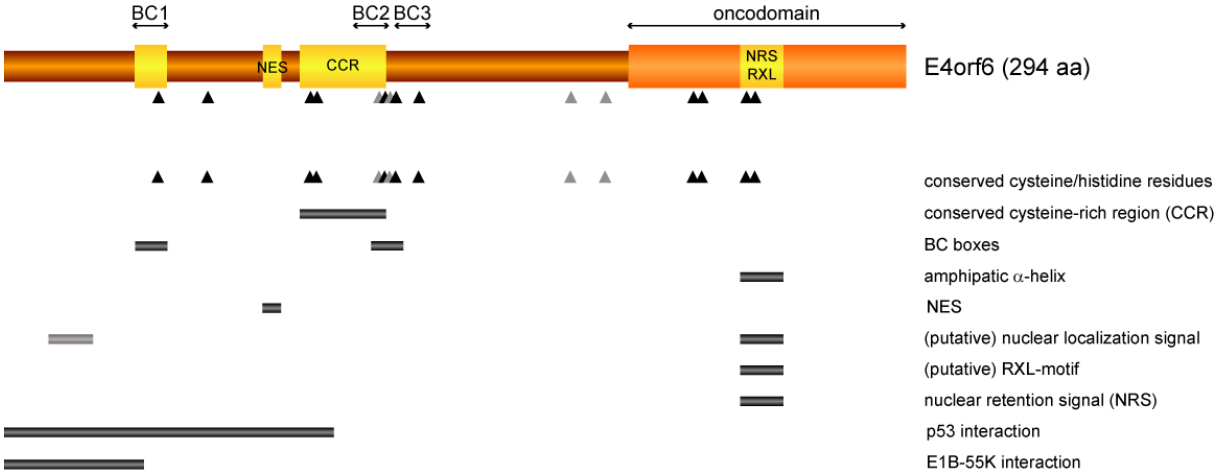

Figure 7. Schematic domain structure of HAdV5 E4orf6. The schematic domain structure of E4orf6 highlights described regions (orange bars), specifically defined amino acid motifs (yellow bars) and experimentally verified interaction regions (grey bars). Conserved cysteine (black) and histidine (grey) residues are illustrated by triangles. For more detailed explanation/references see the text. Abbr.: BC box: *Elongin B/Elongin C box*; CCR: *conserved cysteine-rich region*; NES: *nuclear export signal*; NRS: *nuclear retention signal*.

Interestingly, E4orf6 was first shown to inhibit p53-mediated transcriptional activation and consequently to enhance oncogenic transformation both *in vitro* and *in vivo* by also acting with an additional p53 family member, namely p73 (Dobner *et al.*, 1996). Since many E1B-55K and E4orf6 functions converge and depend upon each other, it has been proposed that these viral proteins form a complex (Querido *et al.*, 2001a; Blanchette *et al.*, 2004). Indeed this has been shown to be critical for most E1B-55K/E4orf6-mediated functions (Blanchette *et al.*, 2004) involving selective degradation of substrate proteins (Querido *et al.*, 2001a; Querido *et al.*, 2001b; Blanchette *et al.*, 2004; Blanchette *et al.*, 2008), proper intracellular localization (Ornelles & Shenk, 1991; Dobbelstein *et al.*, 1997; Orlando & Ornelles, 1999; Dobbelstein, 2000; Orlando & Ornelles, 2002) and late viral mRNA transport (Beltz & Flint, 1979; Babiss *et al.*, 1985; Weigel & Dobbelstein, 2000; Dosch *et al.*, 2001; Higashino *et al.*, 2005; Blanchette *et al.*, 2008). It appears that approximately 60-90% of the viral proteins are associated in this complex, assembling a SCF-like E3 ubiquitin ligase complex comprising of Cullin 5, Rbx 1, Elongin B and C. While E4orf6 mediates the assembly via its BC boxes, E1B-55K provides the cellular substrate proteins (e.g. p53, Mre11, DNA-ligase IV, integrin α3) for the resulting ubiquitination and subsequent proteasomal degradation (Baker *et al.*, 2007, Blanchette *et al.*, 2004, Blanchette *et al.*, 2008, Dallaire *et al.*, 2009a, Dallaire *et al.*, 2009b, Harada *et al.*, 2002, Querido *et al.*, 2001a, Querido *et al.*, 1997, Querido *et al.*, 2001b, Steegenga *et al.*, 1998, Stracker *et al.*, 2002b).

Since some of these proteins mediate important functions in cell cycle regulation and DNA repair, it has been suggested that adenoviral mediated degradation might contribute, at least in part, to the oncogenic properties of the viral proteins. Consequently, compared to E1A/E1B-55K alone, E1A/E1B-55K/E4orf6 synergistically induce malignant transformation of pBRK cells *in vitro* (Moore *et al.*, 1996; Nevels *et al.*, 1997) most likely due to the cooperative modulation/degradation of diverse cellular factors. Interestingly, most of E4orf6´s tumorigenic functions are mediated by a specifically defined C-terminal region appropriately termed "oncodomain" (Nevels *et al.*, 2000). However, so far the detailed molecular mechanism and cellular components involved remain elusive.

2.2 Structure and Functions of Human PML-Bodies

2.2.1 The PML Protein – Structure, Function and Regulation by SUMO

The human PML protein was first described as the causal agent in *acute promyelocytic leukemia* (APL) in the form of a fusion with the retinoic acid receptor generated by the chromosomal translocation t(15;17) (Ascoli & Maul, 1991, Chang *et al.*, 1992, de The *et al.*, 1991, Dyck *et al.*, 1994a, Goddard *et al.*, 1992, Kakizuka *et al.*, 1991, Kastner *et al.*, 1992, Koken *et al.*, 1994, Melnick *et al.*, 1999, Melnick & Licht, 1999, Pandolfi *et al.*, 1992, Weis *et al.*, 1994). Since these initial findings, it has become evident that PML is a general tumor suppressor frequently deregulated in various human tumor types (Koken *et al.*, 1995; Salomoni & Pandolfi, 2002; Gurrieri *et al.*, 2004a; Gurrieri *et al.*, 2004b; Scaglioni *et al.*, 2006; Salomoni *et al.*, 2008) and most likely involving secondary effects of PML-bodies as sites of protein degradation (Lallemand-Breitenbach *et al.*, 2001), transcriptional regulation (Li *et al.*, 2000; Zhong *et al.*, 2000b), cellular senescence (Ferbeyre *et al.*, 2000; Pearson *et al.*, 2000; Bischof *et al.*, 2002; Langley *et al.*, 2002), tumor suppression (Salomoni & Pandolfi, 2002; Salomoni *et al.*, 2008), DNA repair (Bischof *et al.*, 2001; Carbone *et al.*, 2002), apoptosis (Hofmann & Will, 2003; Takahashi *et al.*, 2004) and epigenetic regulation (Torok *et al.*, 2009).

The human PML locus is tightly regulated by various factors such as p53 (Ferbeyre *et al.*, 2000; de Stanchina *et al.*, 2004) and has a size of 53,147 bp with an extremely complicated intron-exon structure (Jensen *et al.*, 2001; Bernardi & Pandolfi, 2007). Due to extensive splicing events, dozens of different mRNAs are produced encoding for several isoforms

(Fagioli *et al.*, 1992; Jensen *et al.*, 2001; Bernardi & Pandolfi, 2007), which in turn have been given extremely complicated names due to different nomenclatures (Jensen *et al.*, 2001). However, based on the sequence of the different gene products, Jensen *et al.* (Jensen *et al.*, 2001) proposed classification of the PML proteins by sorting them into seven major isoforms depending on the amino acid length and exon structure (Fig. 8).

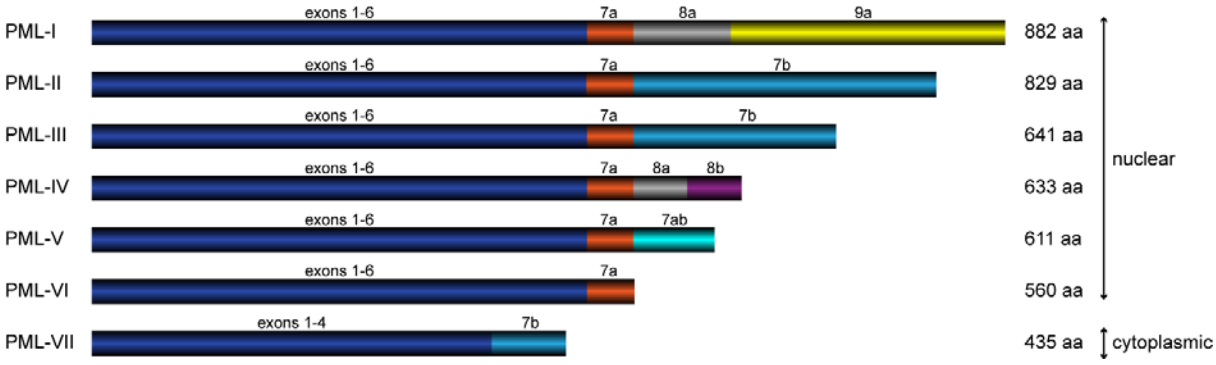

Figure 8. Schematic overview of the seven major human PML-isoforms. The schematic overview of the seven major PML-isoforms is according to the nomenclature of Jensen *et al.* (Jensen *et al.*, 2001). The corresponding exon structure is highlighted by different colors. All human PML-isoforms used in this study are named according to the nomenclature of Jensen *et al.* (Jensen *et al.*, 2001) as PML-I (AAG50180), PML-II (AF230410), PML-III (S50913), PML-IV(AAG50185), PML-V (AAG50181) and PML-VI (AAG50184). The figure represents a compendium of different published results (Fagioli *et al.*, 1992; Jensen *et al.*, 2001; Bernardi & Pandolfi, 2007).

The C-terminal region of human PML shows remarkable variety across its various isoforms, which may represent recent evolutionary diversification compared to other mammalian species (Condemine *et al.*, 2006). Such diversity considerably expands the complexity of regulating distinct proteins by specific PML-isoforms. Furthermore, all PML-isoforms are grouped as TRIM19 in the TRIM protein family, due to a characteristic amino acid motif in the N-terminal region (Jensen *et al.*, 2001; Nisole *et al.*, 2005; Bernardi & Pandolfi, 2007). TRIM proteins are characterized by a *TRIpartide Motif* (TRIM), alternatively called RBCC motif, due to the sequential organization of a RING-finger, two B-boxes and a coiled-coil domain.

Interestingly, a large number of these proteins mediate antiviral activities, especially towards lentiviruses (Nisole *et al.*, 2005). However, the multitude of PML functions is facilitated by extensive modulation of PML itself (Fig. 9), through transcriptional regulation (e.g. interferon) or posttranslational modification (e.g. phosphorylation, SUMOylation) (Borden, 1998; Müller *et al.*, 1998; Everett *et al.*, 1999; Zhong *et al.*, 2000a; Jensen *et al.*, 2001; Condemine *et al.*, 2006; Scaglioni *et al.*, 2006; Bernardi & Pandolfi, 2007).

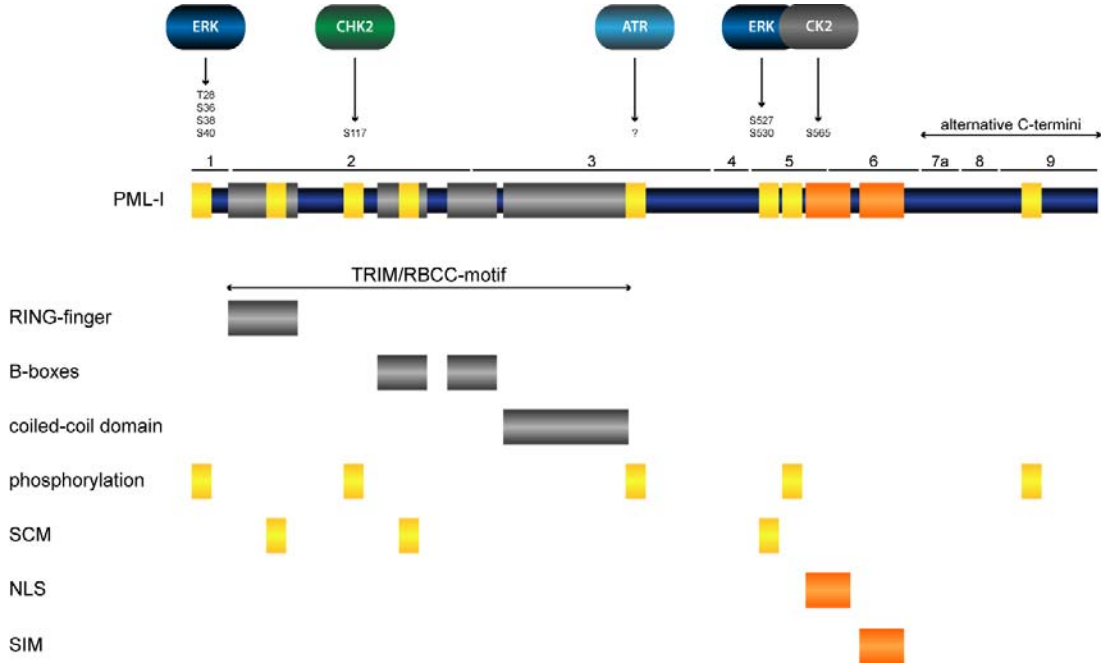

Figure 9. Schematic overview of the domain structure of PML-I. The schematic overview of the domain structure of PML-I represents a compendium of different published results (Bernardi & Pandolfi, 2007), which highlight described regions (orange bars), specifically defined amino acid motifs (yellow bars) and the characteristic parts of the TRIM/RBCC-motif (grey bars). For more detailed explanation/references see the text. Abbr.: NLS: *nuclear localization sequence*; RBCC: *RING-finger, B-box, coiled-coil*; SCM: *SUMO conjugation motif*; SIM: *SUMO interaction motif*; TRIM: *TRIpartide motif*.

In this context, *Casein Kinase 2* (CK2) represents a master regulator of PML function, since phosphorylation by CK2 triggers ubiquitination of PML and subsequent proteasomal degradation (Scaglioni *et al.*, 2006). Consequently, CK2 is frequently up-regulated in human cancers, which induces PML degradation and therefore depletion of PML tumor suppressive functions (Koken *et al.*, 1995; Salomoni & Pandolfi, 2002; Gurrieri *et al.*, 2004a; Gurrieri *et al.*, 2004b; Scaglioni *et al.*, 2006; Salomoni *et al.*, 2008). Additionally, posttranslational modification by the *small-ubiquitin related modifiers* (SUMOs) plays an integral role in PML-NB formation and function (Shen *et al.*, 2006).

Based on sequence similarity to ubiquitin (~18%), tertiary structure and the molecular mechanism of covalent attachment, SUMO is assigned to the family of *ubiquitin-like proteins* (UBLs). Currently, four different isoforms have been identified in mammals and these participate in nearly every cellular pathway known (Seeler & Dejean, 2003; Verger *et al.*, 2003; Hay, 2005; Bossis & Melchior, 2006; Hay, 2006; Kerscher *et al.*, 2006; Geiss-Friedlander & Melchior, 2007; Heun, 2007; Ullrich, 2009). Consequently, the cellular SUMO system is frequently directly or indirectly modulated by various viral proteins (Wilson & Rangasamy, 2001; Boggio & Chiocca, 2006). In this regard, covalent modification of PML at its SCMs, as

well as non-covalent SUMO interaction via its *SUMO interaction motif* (SIM), implements another layer of complexity to the regulation of PML, PML-NBs and PML-associated protein functions (Chang *et al.*, 1997; Shen *et al.*, 2006; Bernardi & Pandolfi, 2007; Reineke & Kao, 2009).

2.2.2 PML-NBs – Structure and Functions

2.2.2.1 The PML-NB – Structure and Associated Proteins

PML nuclear bodies (PML-NBs) resemble nuclear multi-protein complexes with an average size of 0.2 μm-1.0 μm and can be detected in nearly all human cell lines (Chang *et al.*, 1997). However, the abundance, composition, structure and function greatly depend on several factors such as cell cycle and stress (e.g. interferon, irradiation) (Ascoli & Maul, 1991; Dyck *et al.*, 1994; Koken *et al.*, 1994; Weis *et al.*, 1994; Chang *et al.*, 1997; Hodges *et al.*, 1998; Melnick *et al.*, 1999; Melnick & Licht, 1999; Plehn-Dujowich *et al.*, 2000; Negorev & Maul, 2001). Most of the PML functions are mediated via the 166 currently known PML-NB-associated proteins, which not only participate in approximately 615 distinct interactions between one another, but also in nearly every cellular pathway (Van Damme *et al.*, 2010).

Although, the complexity of PML-NB functions are overwhelming, posttranslational modification of PML/PML-associated proteins by the small ubiquitin-like protein SUMO plays an integral role in regulating PML/PML-associated protein functions and PML-NB formation *per se* (Zhong *et al.*, 2000a; Shen *et al.*, 2006; Van Damme *et al.*, 2010). In this context, it was proposed that the *PML nuclear body* is formed by covalently attached SUMO molecules of one PML protein interacting with the non-covalent *SUMO interaction motif* (SIM) of a second PML protein (Zhong *et al.*, 2000a; Zhong *et al.*, 2000b; Shen *et al.*, 2006). However, previously published results support the idea that another constitutive member of PML-NBs, namely Sp100, is also involved in forming the higher three-dimensional hollow sphere-like structure, the integrity of which is based on SUMO SIM interactions (Dellaire & Bazett-Jones, 2004; Ching *et al.*, 2005; Torok *et al.*, 2009; Lang *et al.*, 2010). Interestingly, since most of the enzymes involved in SUMO conjugation/de-conjugation are localized at PML-NBs and 38-56% of PML-associated proteins harbor the intrinsic capacity to be SUMOylated, these nuclear subdomains were proposed to represent the hot spot for regulative SUMOylation (Van Damme *et al.*, 2010). Besides, it remains worth mentioning that PML-NB formation is not exclusively mediated by SUMOylation, but is a complex process requiring additional aspects

such as the different components of the characteristic N-terminal TRIM/RBCC motif (Kastner *et al.*, 1992; Borden *et al.*, 1995; Borden *et al.*, 1996; Borden, 1998).

2.2.2.2 The Role of PML-NBs in Oncogenesis

Obviously, PML has important implications in tumorigenesis (Salomoni & Pandolfi, 2002; Salomoni *et al.*, 2008) and therefore represents a key regulator of multiple cellular proteins´ functions (Van Damme *et al.*, 2010), such as proteins of the DNA repair machinery (e.g. Mre11, rad50), cell cycle regulation (e.g. pRB, p53), telomere metabolism (e.g. TRF), epigenetic regulation (e.g. HDACs) and apoptosis (e.g. Daxx). On the one hand, PML is intensively investigated in the context of leukemogenesis, where it was initially described as the causative agent of *acute promyelocytic leukemia* (APL) (Ascoli & Maul, 1991, Chang *et al.*, 1992, de The *et al.*, 1991, Dyck *et al.*, 1994a, Goddard *et al.*, 1992, Kakizuka *et al.*, 1991, Kastner *et al.*, 1992, Koken *et al.*, 1994, Melnick *et al.*, 1999, Melnick & Licht, 1999, Pandolfi *et al.*, 1992, Weis *et al.*, 1994). On the other hand, PML is frequently down-regulated in non-APL human tumors, for example in 17% of colon adenocarcinomas, 21% of lung tumors, 27% of prostate adenocarcinomas, 31% of breast adenocarcinomas, 49% of CNS tumors (100% medulloblastomas, >90% oligodendroglial tumors), 49% of germ cell tumors and 68% of non-Hodgkin's lymphomas (Koken *et al.*, 1995; Gambacorta *et al.*, 1996; Gurrieri *et al.*, 2004a; Lee *et al.*, 2007). Unfortunately, most of the molecular mechanisms, especially selective regulation of proteins by different PML-isoforms and correlation with human tumorigenesis in different tumor types are so far unknown.

2.2.2.3 The Role of PML-NBs in Antiviral Defense

Despite the multitude of PML-NB functions, these structures have always been proposed to mediate an intracellular antiviral defense mechanism (Everett, 2001; Everett & Chelbi-Alix, 2007; Tavalai & Stamminger, 2008). This idea is based on initial observations that PML/PML-associated proteins are *interferon stimulated genes* (ISGs) and are capable of impairing efficient virus replication. In many aspects this assumption appears adequate, since many viruses encode (immediate) early regulatory proteins that counteract PML-mediated antiviral activities (Chelbi-Alix *et al.*, 1998; Regad *et al.*, 2001). However, growing evidence points to the molecular mechanisms involved being more complicated. Therefore, it has been proposed that some viruses may additionally facilitate specific components of the PML-NBs while simultaneously inactivating others. Although gathering substantial evidence for the involvement of

PML and/or PML-associated factors in viral infection, the relationship between viruses and these nuclear structures remains elusive.

Table 1. Overview of the association of different viruses with PML-NBs. The overview lists the different viruses known to somehow counteract/interact with PML/PML-associated proteins. Note that association/interactions with PML or PML components do not necessarily have a negative effect on virus replication. For more detailed explanation/references see the text or the review articles published by (Everett, 2001; Everett & Chelbi-Alix, 2007; Tavalai & Stamminger, 2008).

VIRUS	VIRAL PROTEIN	CELLULAR PROTEIN	REFERENCE
DNA-Viruses			
Adenoviridae			
HAdV5	E4orf3, E1B-55K	PML-II, Daxx	(Ishov & Maul, 1996; Hoppe *et al.*, 2006; Ullman *et al.*, 2007; Ullman & Hearing, 2008; Leppard *et al.*, 2009; Schreiner *et al.*, 2010)
Herpesviridae			
EBV	BLZF-1, EBNA5	Sp100-A	(Bell *et al.*, 2000; Adamson & Kenney, 2001; Ling *et al.*, 2005; Amon *et al.*, 2006; Sivachandran *et al.*, 2008)
HCMV	pp71, IE1, IE2, IE72	Daxx	(Korioth *et al.*, 1996; Ahn & Hayward, 1997; Ahn *et al.*, 1998; Wilkinson *et al.*, 1998; Ahn *et al.*, 1999; Lee *et al.*, 2004; Everett *et al.*, 2006; Saffert & Kalejta, 2006)
HSV-1	ICP0	Daxx, ATRX, Sp100	(Everett & Maul, 1994; Everett *et al.*, 1995; Meredith *et al.*, 1995; Maul *et al.*, 1996; Burkham *et al.*, 1998; Chelbi-Alix & de The, 1999; Müller & Dejean, 1999; Taylor *et al.*, 2000; Burkham *et al.*, 2001; Chee *et al.*, 2003; Everett & Zafiropoulos, 2004; Everett & Murray, 2005; Everett *et al.*, 2006; Negorev *et al.*, 2006; Everett *et al.*, 2008; Negorev *et al.*, 2009; Nojima *et al.*, 2009; Lukashchuk & Everett, 2010)
KSHV	BLZF-1, LANA2	n.d.	(Szekely *et al.*, 1999; Katano *et al.*, 2001; Wu *et al.*, 2001; Marcos-Villar *et al.*, 2009)
VZV	n.d.	n.d.	(Kyratsous & Silverstein, 2009)
Papillomaviridae			
HPV-16	L2, E2, E4	n.d.	(Florin *et al.*, 2002; Guccione *et al.*, 2004; Bischof *et al.*, 2005; Nakahara & Lambert, 2007; Lin *et al.*, 2009; Louria-Hayon *et al.*, 2009)
Polyomaviridae			
SV40	large TAg	n.d.	(Ishov & Maul, 1996; Jiang *et al.*, 1996; Jul-Larsen *et al.*, 2004)
JCV	VP1, VP2, VP3	n.d.	(Gasparovic *et al.*, 2009)

RNA-Viruses			
Arenaviridae			
LCMV	Z-protein	n.d.	(Borden *et al.*, 1998; Djavani *et al.*, 2001; Asper *et al.*, 2004; Garcia *et al.*, 2010)
Bunyaviridae			
Hantavirus	N-protein	Daxx	(Li *et al.*, 2002; Kaukinen *et al.*, 2005)
Flaviviridae			
Dengue Virus	capsid protein	Daxx	(Netsawang *et al.*, 2010)
HCV	core protein	n.d.	(Herzer *et al.*, 2005)
Filoviridae			
Ebola Virus	n.d.	n.d.	(Bjorndal *et al.*, 2003)
Orthomyxoviridae			
Influenza A	M, NS1, NS2	PML-III/IV/VI	(Chelbi-Alix *et al.*, 1998; Iki *et al.*, 2005)
Retroviridae			
HIV-1	n.d.	n.d.	(Turelli *et al.*, 2001; Berthoux *et al.*, 2003)
HTLV-1	Tax	n.d.	(Desbois *et al.*, 1996)
HFV	Tas	PML-III	(Regad *et al.*, 2001)
Rhabdoviridae			
VSV	n.d.	PML-III	(Chelbi-Alix *et al.*, 1998; Bonilla *et al.*, 2002)
Rabies Virus	P proteins	n.d.	(Blondel *et al.*, 2002)
Picornaviridae			
Poliovirus	n.d.	PML-III	(Pampin *et al.*, 2006)

3 Material

3.1 Cells

3.1.1 Bacteria Strains

STRAIN	GENOTYPE
DH5α	*sup*E44, Δ*lac*U169, (ϕ80d*lacZ*Δ*M15*), *hsd*R17, *rec*A1, *end*A1, *gyr*A96, *thi*-1, *rel*A1 (Hanahan & Meselson, 1983).
XL2-Blue	*rec*A1, *end*A1, *gyr*A96, *thi*-1, *hsd*R17, *sup*E44, *rel*A1, lac, [F'*pro*AB, *lac*I^{q}ZΔM15, *Tn10* (Tet^r), Amy, Cam^r] (Bullock *et al.*, 1987).

3.1.2 Mammalian Cell Lines

CELL LINE	CHARACTERISTICS
pBRK	Primary baby rat kidney cells prepared from 3-5 days old CD rats (Charles River, Kißlegg).
BRK1	Spontaneously immortalized BRK-cells (internal group cell database).
AB115	Established HAdV5-transformed rat cell line stably expressing E1A-12S/13S plus E1B-55K-NES (internal group cell database).
AB120	Established HAdV5-transformed rat cell line stably expressing E1A-12S/13S plus E1B-55K (internal group cell database).
A549	Human lung carcinoma cell line expressing wild-type p53 (Giard *et al.*, 1973).
H1299	Human lung carcinoma cell line, p53 negative (Mitsudomi *et al.*, 1992).
HepaRG	Pseudoprimary human hepatoma cell line (Gripon *et al.*, 2002; Cerec *et al.*, 2007).
HEK-293	Established HAdV5-transformed, human embryonic kidney cell line stably expressing the adenoviral E1A and E1B gene products (Graham *et al.*, 1977).
HEK-293T	HEK-293 derived cell line expressing the SV40 large TAg (DuBridge *et al.*, 1987).
HeLa	Human cervix carcinoma cell line (Gey *et al.*, 1952).
MIO-M1	Human pseudoprimary retinal glia cell line (Limb *et al.*, 2002; Lawrence *et*

	al., 2007; Bull *et al.*, 2008).
U2OS (2T)	Human osteosarcoma cell line (Ponten & Saksela, 1967).
911	Established HAdV5-transformed, human retinoblastoma cell line stably expressing the adenoviral E1A and E1B gene products (Fallaux *et al.*, 1996).
2E2	HEK-293 derived inducible helper cell line expressing the E2 gene products and E4orf6 under control of a tetracycline-dependent promoter (Catalucci *et al.*, 2005).

3.2 Adenoviruses

ADENOVRIUS	CHARACTERISTICS
H5*pg*4100	Wild-type HAdV5 carrying a 1863 bp deletion (nt 28602-30465) in the E3 reading frame (Kindsmüller *et al.*, 2007).
H5*pm*4149	HAdV5 E1B-55K null mutant carrying four stop codons at the aa positions 3, 8, 86 and 88 of the E1B-55K sequence (Kindsmüller *et al.*, 2009).
H5*pm*4101	HAdV5 E1B-55K mutant carrying three aa exchanges (L83/87/91A) within the NES of the E1B-55K sequence (Kindsmüller *et al.*, 2007).
H5*pm*4102	HAdV5 E1B-55K mutant carrying one aa exchange (K104R) within the SCM of the E1B-55K sequence (Kindsmüller *et al.*, 2007).
H5*pm*4154	HAdV5 E4orf6 null mutant carrying a stop codon at aa 66 within the E4orf6 sequence (Blanchette *et al.*, 2004).

3.3 Nucleic Acids

3.3.1 Oligonucleotides

The following oligonucleotides were used as primers for sequencing reactions, PCR amplifications, restriction site insertion and site-directed mutagenesis reactions. All oligonucleotides were ordered from Metabion and numbered according to the internal *Filemaker Pro*

database. Externally provided oligonucleotides are labeled according to the institution providing them (McGill University, Montreal, Canada).

#	NAME	SEQUENCE	PURPOSE
64	**E1B bp2043 fwd**	5`-CGC GGG ATC CAT GGA GCG AAG AAA CCC ATC TGA GC-3`	sequencing
543	**Ad5 3670 rev**	5`-CTG ACG CAC CCC GGC CC-3`	sequencing
592	**E1B K104R fwd**	5`-CTA AAG GGG GTA AGG AGG GAG CGG GGG-3`	mutagenesis
593	**E1B K104R fwd**	5`-CCC CCG CTC CCT CCT TAC CCC CTT TAG-3`	mutagenesis
594	**E1B NES fwd**	5`-GTG GCT GAA GCG TAT CCA GAA GCG AGA CGC ATT GCG ACA ATT ACA-3`	mutagenesis
595	**E1B NES fwd**	5`-TGT AAT TGT CGC AAT GCG TCT CGC TTC TGG ATA CGC TTC AGC CAC-3`	mutagenesis
635	**pcDNA3 fwd**	5`-ATG TCG TAA CAA CTC CGC-3`	sequencing
636	**pcDNA3 rev**	5`-GGC ACC TTC CAG GGT CAA G-3`	sequencing
781	**Seq E1-Box fwd 1582bp**	5`-GAT TGC GTG TGT GGT TAA CGC-3`	sequencing
782	**Seq E1-Box fwd 2454bp**	5`-CAA GGA TAA TTG CGC TAA TGA GC-3`	sequencing
783	**Seq E1-Box rev 3373bp**	5`-CCA CAC TCG CAG GGT CTG C-3`	sequencing
924	**E1B C454S/C256S fwd**	5`-CCA GGT GCA GAC CCT CCG AGT CTG GCG GTA AAC ATA TTA GG-3`	mutagenesis
925	**E1B C454S/C256S rev**	5`-CCT AAT ATG TTT ACC GCC AGA CTC GGA GGG TCT GCA CCT GG-3`	mutagenesis
1318	**Seq E1B bp978-999 fwd**	5`-GGC CTC CGA CTG TGG TTG CTT C-3`	sequencing
1361	**E1B H354 EcoRI-ko fwd**	5`-GCG AGG ACA GGG CTG GGA TTC CAG CCT CTC AGA TGC TGA CC-3`	mutagenesis
1362	**E1B H354 EcoRI-ko rev**	5`-GGT CAG CAT CTG AGA GGC TGG AAT CCC AGC CCT GTC CTC GC-3`	mutagenesis
1489	**Seq E1-Box rev 2454bp**	5`-GCT CAT TAG CGC AAT TAT CCT TG-3`	sequencing
1614	**E1B S490/491D T495D fwd**	5`-GCT GAG TTT GGG GAT GAC GAT GAA GAT GAT GAT TGA GGT ACT GAA TGT GG-3`	mutagenesis
1615	**E1B S490/491D T495D rev**	5`-CCA CAC ATT TCA GTA CCT CAA TCA TCA TCT TCA TCG TCA TCG CCA AAC TCA GC-3`	mutagenesis
1623	**PML-nuc456-rev**	5`-GGA ACC ACT GGT GTG CCT CG-3`	sequencing
1624	**PML-nuc439-fwd**	5`-GCT TG AGT GCG AGC AGC TCC-3`	sequencing
1625	**PML-nuc984-fwd**	5`-GCT GTG CTG CAG CGC ATC CGC ACG-3`	sequencing

1626	**PML-nuc1539-fwd**	5`-GGA GCA GCC CAG GCC CAG CAC C-3`	sequencing
1627	**PML-nuc2135-fwd**	5`-CGG GCT TCC TGG CTG CCC TGC C-3`	sequencing
1643	**E1B R443A-fwd**	5`-GGA AGG TGC TGG CGT ACG ATG AGA CCC-3`	mutagenesis
1644	**E1B R443A-rev**	5`-GGG TCT CAT CCG CCC TCA GCA CCT TCC-3`	mutagenesis
1789	**PML-I-nuc2010 fwd**	5`-CTC CGC TTG CAC CCT CAA TTG C-3`	sequencing
1790	**PML-II-nuc2148 fwd**	5`-CCA TCC TGC CAA TGC CCA GG-3`	sequencing
1811	**EcoRI-E1B fwd**	5`-ACT GAA TTC ATG GAG CGA AGA AAC CCA TCT GAG C-3`	cloning
1812	**BamHI-E1B rev**	5`-ACT GGA TCC TCA ATC TGT ATC TTC ATC GCT AGA GC-3`	cloning
M148	**E1B-RTR448/449/450AAA fwd**	5`-CGA TGA GAC CGC CGC CGC GTG CAG ACC CTG C-3`	mutagenesis
M149	**E1B-RTR448/449/450AAA rev**	5`-GCA GGG TCT GCA CGC GGC GGC GGT CTC ATC G-3`	mutagenesis
M150	**E1B-E446A fwd**	5`-GCT GAG GTA CGA TGC GAC CCG CAC CAG G -3`	mutagenesis
M151	**E1B-E446A rev**	5`-CCT GGT GCG GGT CGC ATC GTA CCT CAG C -3`	mutagenesis
M152	**E1B-E472A fwd**	5`-GCT GGA TGT GAC CGC GGA GCT GAG GCC CG -3`	mutagenesis
M153	**E1B-E472A rev**	5`-CGG GCC TCA GCT CCG CGG TCA CAT CCA GC-3`	mutagenesis

3.3.2 Vectors

The folowing vectors were used for cloning and transfection experiments. All vectors are numbered according to the internal *Filemaker Pro* database.

#	NAME	PURPOSE	REFERENCE
36	**pcDNA3**	Expression vector for mammalian cells, CMV promoter.	Invitrogen
181	**pRL-TK**	*Renilla*-Luciferase-*Assay*.	Promega
232	**LeGO-iVLN2**	Lentiviral expression vector containing a neomycine resistance as well as an IRES triggered ORF for Venus.	(Weber *et al.*, 2008)
234	**LeGO-iBLB2**	Lentiviral expression vector containing a neomycine resistance as well as an IRES triggered ORF for BFP.	(Weber *et al.*, 2008)

3.3.3 Recombinant Plasmids

The folowing recombinant plasmids were used for cloning and transfection experiments. All vectors are numbered according to the internal *Filemaker Pro* database.

#	NAME	VECTOR	INSERT	REFERENCE
2	**pc53SN3**	pCMV/neo	human p53 cDNA	group database
499	**pRE-Luc**	uknown	reporter gene construct with five p53 binding sites within the CMV-promoter	N. Horikoshi
1022	**pE1B-55K-K104R**	pcDNA3	HAdV5 E1B-55K	group database
1023	**pE1B-55K-NES**	pcDNA3	HAdV5 E1B-55K	group database
1174	**pE1B-55K-V103D**	pcDNA3	HAdV5 E1B-55K	group database
1319	**pE1B-55K**	pcDNA3	HAdV5 E1B-55K	group database
1496	**pE1A-12S**	pcDNA3	chimeric HAdV2/5 E1A-12S	group database
1497	**pE1A-13S**	pcDNA3	chimeric HAdV2/5 E1A-13S	group database
1521	**pE1B-55K-SST490/491/495AAA**	pcDNA3	HAdV5 E1B-55K	group database
1730	**pE1B-55K-C454S/C456S**	pcDNA3	HAdV5 E1B-55K	group database
1765	**pE1B-55K-H354**	pcDNA3	HAdV5 E1B-55K	group database
2055	**flag-PML-I**	pLKO.1.neo	human N-terminal flag-tagged PML-I	provided by R. Everett
2056	**flag-PML-II**	pLKO.1.neo	human N-terminal flag-tagged PML-II	provided by R. Everett
2057	**flag-PML-III**	pLKO.1.neo	human N-terminal flag-tagged PML-III	provided by R. Everett
2058	**flag-PML-IV**	pLKO.1.neo	human N-terminal flag-tagged PML-IV	provided by R. Everett
2059	**flag-PML-V**	pLKO.1.neo	human N-terminal flag-tagged PML-V	provided by R. Everett
2060	**flag-PML-VI**	pLKO.1.neo	human N-terminal flag-tagged PML-VI	provided by R. Everett

2068	**pE1B-55K-SST490/491/495DDD**	pcDNA3	HAdV5 E1B-55K	this work
2076	**pE4orf6-HA**	pcDNA3	N-terminal HA-tagged HAdV5 E4orf6	group database
2103	**pE1A**	LeGO-iVLN2	HAdV5 E1A (genomic)	group database
2104	**pE1B-55K**	LeGO-iBLB2	HAdV5 E1B-55K	group database
2141	**pE1B-55K-R443A**	pcDNA3	HAdV5 E1B-55K	group database
2192	**pE1B-55K-E446A**	pcDNA3	HAdV5 E1B-55K	group database
2193	**pE1B-55K-RTR448/449/450AAA**	pcDNA3	HAdV5 E1B-55K	group database
2194	**pE1B-55K-E472A**	pcDNA3	HAdV5 E1B-55K	group database
2279	**pE1B-55K-E472A**	LeGO-iBLB2	HAdV5 E1B-55K	this work
2281	**pE1B-55K-C454S/C456S**	LeGO-iBLB2	HAdV5 E1B-55K	this work
2283	**pE1B-55K-RTR448/449/450AAA**	LeGO-iBLB2	HAdV5 E1B-55K	this work

3.4 Antibodies

3.4.1 Primary Antibodies

NAME	PROPERTIES
2A6	Monoclonal mouse antibody raised against the N-terminus of HAdV5 E1B-55K (Sarnow *et al.*, 1982b).
3F10	Monoclonal rat antibody raised against the HA-tag (Roche).
4E8	Monoclonal rat antibody raised against the central region of HAdV5 E1B-55K (Kindsmüller *et al.*, 2009).
5E10	Monoclonal mouse antibody raised against the human PML protein (Stuurman *et al.*, 1992).
610	Polyclonal rabbit mouse antibody raised against the HAdV5 E1A (kindly provided by R. Grand).

7C11	Monoclonal rat antibody raised against the C-Terminus of HAdV5 E1B-55K (Kindsmüller *et al.*, 2009).
α–PML	Polyclonal rabbit antibody raised against aa 375-425 of human PML (NP_150241.2) (Novus Biologicals).
α-SAFA	Polyclonal rabbit antibody raised against the human SAF-A protein (Herrmann *et al.*, 2005).
β-actin (AC-15)	Monoclonal mouse antibody raised against β-actin (Sigma Aldrich).
B6-8	Monoclonal mouse antibody raised against HAdV5 E2A-72 kDa protein (Reich *et al.*, 1983).
DO-I	Monoclonal mouse antibody raised against the N-terminal aa 11-25 of human p53 (Santa Cruz).
flag-M2	Monoclonal mouse antibody raised against the flag-tag (Sigma Aldrich).
H238	Polyclonal rabbit antibody raised against aa 157-394 of human PML (Santa Cruz).
M58	Monoclonal mouse antibody raised against HAdV5 E1A-12S and -13S (Harlow *et al.*, 1985).
M73	Monoclonal mouse antibody raised against HAdV5 E1A-12S and -13S (Harlow *et al.*, 1985).
RSA3	Monoclonal mouse antibody raised against the N-terminus of HAdV5 E4orf6 and E4orf6/7 (Marton *et al.*, 1990).

3.4.2 Secondary Antibodies

The following antibodies were used for *Western Blot* or immunofluorescence analysis.

NAME	PROPERTIES
HRP-Anti-Mouse IgG	HRP (*horseradish peroxidase*)-coupled antibody raised against mouse IgGs in sheep (Amersham Life Science).
HRP-Anti-Rat IgG	HRP (*horseradish peroxidase*)-coupled antibody raised against rat IgGs in sheep (Amersham Life Science).
HRP-Anti-Rabbit IgG	HRP (*horseradish peroxidase*)-coupled antibody raised against rabbit IgGs in sheep (Amersham Life Science).

NAME	PROPERTIES
Cy™3-Anti-Rat IgG	Affinity purified, Cy™3-coupled antibody raised against rat IgGs in

	donkey (H + L; Dianova).
Cy™5-Anti-Mouse IgG	Affinity purified, Cy™5-coupled antibody raised against mouse IgGs in donkey (H + L; Dianova).
FITC-Anti-Mouse IgG	Affinity purified, fluorescein-isothiocyanat (FITC)-coupled antibody raised against mouse IgGs in donkey (H + L; Dianova).
FITC-Anti-Rabbit IgG	Affinity purified, fluorescein-isothiocyanat (FITC)-coupled antibody raised against rabbit IgGs in donkey (H + L; Dianova).
***Texas Red*-Anti-Mouse IgG**	Affinity purified, *Texas Red*-coupled antibody raised against mouse IgGs in donkey (H + L; Dianova).
***Texas Red*-Anti-Rat IgG**	Affinity purified, *Texas Red*-coupled antibody raised against rat IgGs in donkey (H + L; Dianova).
Alexa™ 488 Anti-Mouse IgG	AlexaTM 488 antibody raised against mouse IgGs in goat (H + L; $F(ab`)_2$ Fragment; Molecular Probes).

3.5 Standards and Markers

Size determination of DNA fragments on agarose gels was based on a *1 kp* and *100 bp* DNA ladder (New England Biolabs), whereas the molecular weight of proteins on SDS-poly-acrylamid gels was determined by *PageRuler™ Prestained Protein Ladder Plus* (Fermentas).

3.6 Commercial Systems

The following commercial systems were used:

PRODUCT	COMPANY
Dual-Luciferase® Reporter Assay System	Promega
Protein Assay	BioRad
Quiagen Plasmid Mini, Midi und Maxi Kit	Qiagen
QIAquick Gel Extraction Kit	Qiagen
***QuikChange™ Site-Directed Mutagenesis* Kit**	Agilent
SuperSignal® West Pico Chemiluminescent Substrate	Pierce

3.7 Animals

ANIMALS	COMPANY
CD rats	Charles River (Kißlegg)

3.8 Chemicals, Enzymes, Reagents and Equipment

Chemicals, enzymes and reagents used in this study were purchased from Agilent, Applichem, Biomol, Merck, New England, Biolabs Roche and Sigma Aldrich. Cell culture materials, general plastic material as well as equipment were obtained from BioRad, Biozym, Brand, Engelbrecht, Eppendorf GmbH, Falcon, Gibco BRL, Greiner, Hartenstein, Hellma, Nunc, Pan, Sarstedt, Protean, Schleicher & Schuell, VWR and Whatman.

3.9 Software and Databases

SOFTWARE	PURPOSE	SOURCE
Acrobat 9.0 Pro	PDF data processing	Adobe
BioEdit 7.0.5.2	sequence data processing	Open Software (provided by *Ibis Therapeutics Carlsbad*)
CLC Main Workbench 5.0	sequence data processing	CLC bio
Endnote 9.0	reference management	Thomson
Filemaker Pro 8.5	data base management	FileMaker, Inc.
Gene Tools	quantification of DNA/protein bands	SynGene
Illustrator CS4	layout processing	Adobe
Microsoft Office XP 2003	text processing	Microsoft
Photoshop CS4	image processing	Adobe

PubMed	literature database, open sequence analyis software	Open Software (provided by NCBI)

4 Methods

4.4 Bacteria

4.1.1 Culture and Storage

Solid Plate Culture

Bacteria were plated on solid LB media containing 15 g/l agar with the appropriate antibiotics (100 µg/ml ampicillin; 50 µl/ml kanamycin) and incubated at 30°C/37°C for 16-20 hours. Solid plate cultures can be stored for several weeks at 4°C sealed with *Parafilm* (Pechiney Plastic Packaging).

Liquid Culture

For liquid culture of *E. coli*, single colonies were picked from plates and inoculated into sterile LB media containing 100 µg/ml ampicillin or 50 µg/ml kanamycin. Cultures were incubated at 30°C/37°C for 16-20 hours in an *Inova 4000 Incubator* (New Brunswick). If necessary, bacteria concentrations were determined by meassuring the optical density (OD) of the cultures at 600 nm wavelength (*SmartSpec™ Plus*; BioRad) against plain media (1 OD_{600}= $8x10^8$ cells/ml).

Storage

Liquid cultures of single colony bacteria were centrifuged briefly at 4,000 rpm for 10 min (*Multifuge 3 S-R*; Heraeus) at RT. The bacteria pellets were then resuspended in 1.0 ml LB media containing 50% sterile glycerol and transferred into *CryoTubes™* (Nunc). These glycerol cultures can be stored at -80°C for years.

LB Media	Trypton	10 g/l
	Yeast Extract	5 g/l
	NaCl	5 g/l
	• autoclaving	
Antibiotic Solutions	Ampicillin (500x)	50 mg/ml
	Kanamycin (200x)	10 mg/ml
	• filter sterilization	
	• storage at -20°C	

4.1.2 Transformation of *E. coli*

4.1.2.1 Chemical Transformation of *E. coli*

Between 50–100 µl of chemically treated competent XL2-Blue/DH5α bacteria were transferred into a 15 ml *Falcon 2059* tube. Afterwards 2 µl of β-Mercaptoethanol (1.2 M) and 1-10 µl of diluted plasmid DNA (~200 ng) were added to the cells and incubated on ice for 30 min. This mixture was kept at 42°C for 45 s and subsequently transferred to ice for 2 min. After applying this heat shock, 1 ml of NZCYM media was added to the cells, followed by incubation for 1 hour at 37°C in an *Inova 4000 Incubator* (New Brunswick). After centrifugation the bacteria pellet was resuspended in 100 µl LB, plated on LB agar containing appropriate antibiotics and incubated at 30°C/37°C.

NZCYM Media

NZ Amine	10 g/l
NaCl	0.5 g/l
Yeast Extract	0.5 g/l
$MgSO_4 \times 6H_2O$	0.2 g/l
Casamino acids	1 g/l
• pH 7.0	
• autoclaving	

4.1.2.2 Electroporation of *E. coli*

Electrocompetent *E. coli* cells were produced as described by Sharma & Schimke (Sharma & Schimke, 1996). 10 ml of an overnight culture of *E. coli* were inoculated into 1 l of YENB media and incubated at 37°C until reaching an OD_{600} of 0.5-0.9. Afterwards, the cells were cooled for 5 min on ice and centrifuged for 10 min at 6,000 rpm at 4°C (*Avanti J-E*; Beckman & Coulter). The bacteria pellet was washed twice by resuspending in 100 ml of ice-cold ddH_2O and once with 20 ml of 10% glycerol. The pellet was resuspended in a final volume of 3 ml of 10% glycerol, divided into 50 µl aliquots, frozen in liquid nitrogen and stored at -80°C. For transformation the electrocompetent cells were thawed on ice, mixed with 1-10 µl of plasmid DNA and placed in a pre-cooled electroporation cuvette (Bio-Rad) with an electrode gap of 1 mm. Electroporation was performed in a *GenePulser* machine (Bio-Rad; 1,25 kV, 25 µF, 200 Ω) for a time of approximately 5 ms. After the pulse, the cells were resuspended immediately in 1 ml of SOC media, transferred into 1.5 ml reaction tubes and incubated for 1 hour at 37°C in an *Inova 4000 Incubator* (New Brunswick). After centrifugation the bacteria pellet was resuspended in 100 µl LB, plated on LB agar containing appropriate antibiotics and incubated at 30°C/37°C.

YENB Media	Bacto Yeast Extract	7.5 g/l
	Bacto Nutrient Broth	8 g/l
	• autoclaving	
SOC Media	Trypton	20 g/l
	Yeast Extract	5 g/l
	NaCl	10 mM
	KCl	2.5 mM
	$MgCl_2$	10 mM
	$MgSO_4$	10 mM
	Glucose	20 mM
	• autoclaving	

4.2 Mammalian Cell Lines

4.2.1 Maintenance and Passage of Cell Lines

Adhesive mammalian cells were cultured as a monolayer in polystyrene cell culture dishes (6-well, 100 mm or 150 mm diameter dishes) with *Dulbecco's Modified Eagle Media* (DMEM; PAA) containing 0.11 g/l sodium pyruvate, 10% FCS (Pan) and 1% of penicillin/streptomycin solution (1,000 U/ml penicillin & 10 mg/ml streptomycin in 0.9% NaCl; Pan). Cultured cells were incubated at 37°C in a Heraeus incubator in a 5% CO_2 atmosphere. To split confluent cells, the media was removed, cells were washed once with sterile PBS solution and incubated with trypsin/EDTA solution (Pan) for approximately 5 min at 37°C. Trypsin activity was inhibited by adding standard culture media and the solution was transferred into 15 ml/50 ml tubes for 5 min of centrifugation at 1,500 rpm (*Multifuge 3S-R*; Heraeus). The supernatant was removed and cells were resuspended in an appropriate amount of culture media. Depending on the experimental conditions, cells were counted as described in 4.2.4 or split in a constant ratio of 1:2 to 1:20.

PBS	NaCl	140 mM
	KCl	3 mM
	Na_2HPO_4	4 mM
	KH_2PO_4	1.5 mM
	• pH 7.0–7.7	
	• autoclaving	

4.2.2 Preparation of Primary Baby Rat Kidney Cells

To establish a primary BRK cell line, kidneys of 4-6 days old CD rats (Charles River) were extracted under semi-sterile conditions and mechanically shredded by scalpel. The cell mixture was incubated for 3 hours at 37°C in PBS solution (1 ml/kidney) containing 0.05 mg/ml collagenase/dispase (Roche). The cells were vigorously shaked every 15-20 min to achieve homogenous dispersion. Subsequently, the cell solution was washed three times in 50 ml of sterile PBS to remove the added enzymes. Afterwards, the cell pellet was re-suspended in an appropriate volume and plated on 150 mm culture dishes distributing approximately four kidneys per dish. Due to the limited growth capacity of these primary cells, the culture media was changed every day to remove cell debris and keep the cells viable.

4.2.3 Storage of Mammalian Cell Lines

For long-time storage of mammalian cell lines, subconfluent cultures were trypsinized and pelleted as described previously (4.2.1). The cells were resuspended in pure FCS and transferred to *CryoTubes*™ (Nunc). The samples were frozen stepwise (-20°C, 2 hours; -80°C, 2 hours) or slowly using "*Mr. Frosty*" (Nalgene Labware) before storage in liquid nitrogen. To recultivate frozen cells, the *CryoTube*™ (Nunc) was rapidly thawed at 37°C and cells were immediately resuspended in 15 ml of pre-warmed culture media. Cells were pelleted once by centrifugation to remove the DMSO containing media, resuspended in 1 ml of culture media, seeded in an appropriate cell culture dish and incubated at standard conditions (4.2.1).

4.2.4 Determination of Total Cell Number

The total number of viable cells was determined by a *Neubauer cell counter* (C. Roth). Cells were harvested by trypsination, pelleted by centrifugation and resuspended in an appropriate volume (4.2.1). Afterwards, 50 µl of cell suspension was mixed with 50 µl of trypan blue solution and placed in the *Neubauer counter* (C. Roth). The mean value of two manual counts (16 squares) under the light microscope (Leica DMIL) was multiplied by the dilution factor and the factor 10^4 to obtain the concentration of cells per ml. Viable cells were determined by trypan blue exclusion and dead cells were excluded during counting.

Trypan Blue Solution	Trypan Blue	0.15%

NaCl	0.85%

4.2.5 Transfection of Mammalian Cells by Polyethylenimine

For efficient transfection of mammalian cell lines, linear 25 kDa polyethylenimine (PEI; Polysciences) was used in general since it offers a suitable, reproducible and easily adaptable system. PEI was dissolved in ddH_2O at a concentration of 1 mg/ml, neutralized with 0.1 M HCl (pH of 7.2), filter sterilized (0.2 µm), aliquoted and stored at -80°C. 1 hour before transfection the culture media of the cells was replaced by fresh DMEM media without supplements to ensure a neutral pH. The transfection solution was prepared by incubating a mixture of DNA, PEI and DMEM in a ratio of 1:10:100 for 30 min at RT. After application of transfection solution, cells were incubated for 8 hours at standard conditions (4.2.1) before replacing the transfection media with standard culture media. Since PEI exhibits some toxicity to mammalian cells, the absolute amount of transfection reagent used was adjusted to the cell lines, where tumor cell lines in general tolerated a much higher and longer exposure compared to primary cell lines.

4.2.6 Harvest of Mammalian Cells

Transfected or infected adherent mammalian cells were harvested using cell scrapers. Collected cells were transferred into 15 or 50 ml tubes and centrifuged at 2,000 rpm for 3 min at RT (*Multifuge 3 S-R*; Heraeus). After removing the supernatant, the cell pellet was washed once with PBS and stored at –20°C for subsequent experiments.

4.3 Adenovirus

4.3.1 Propagation and Storage of High-Titer Virus Stocks

In order to produce high-titer virus stocks, several 150 mm dishes were infected with established laboratory stocks at a moi of 5 as described (4.3.3). After 3-5 days of incubation, cells were harvested and centrifuged at 2,000 rpm for 5 min at RT (*Multifuge 3 S-R*; Heraeus). The virus-containing cell pellet was washed once with PBS and resuspended in an appropriate volume DMEM (~1 ml/150 mm dish) without FCS and antibiotics. The viral par-

ticles were released by repeated freezing/thawing cycles, centrifuged at 4,500 rpm for 10 min (*Multifuge 3 S-R*; Heraeus) and virus-containing supernatant was mixed with 87% glycerol (sterile; 10% final concentration) for preservation at -80°C.

4.3.2 Titration of Virus Stocks

The titer of virus stocks was determined in terms of *fluorescent forming units* (ffu) by immunofluorescence staining using the adenoviral E2A-72K *DNA binding protein* (DBP) specific antibody B6-8 (Reich *et al.*, 1983). $5x10^5$ HEK-293 cells were seeded in six-well dishes (4.2.1, 4.2.4) and infected with 1 ml of virus dilution (4.3.3) ranging from 10^{-2} to 10^{-6}. These infected cells were fixed 20 h p.i. with 2 ml of ice-cold methanol for 15 min at -20°C (4.5.7), air dried at RT and partially rehydrated by adding 2 ml of PBS-Triton for 15 min. Subsequently, PBS-Triton was removed and each well was blocked with 2 ml TBS-BG for 1 hour at RT on an *orbital shaker* (GFL). Afterwards, each well was incubated for 2 hours in an antibody solution containing the E2A-72K (DBP) specific antibody B6-8 (1:10 in TBS-BG), washed three times for 15 min with TBS-BG before adding *Alexa Fluor® 488*-coupled secondary antibody (Invitrogen) for 2 hours at RT. Finally, the secondary antibody was removed, each well was washed three times for 15 min with TBS-BG and infected cells were counted using a fluorescence microscope (Leica). The total number of infectious particles was calculated as the mean value of at least three independent countings taking into account the infected cell numbers, virus dilutions and microscope magnification.

PBS-Triton	*Triton® X-100* • in PBS	0.5 mM
TBS-BG	Tris/HCl, pH 7.6	20 M
	NaCl	137 mM
	KCl	3 mM
	$MgCl_2$	1.5 mM
	Tween 20	0.05% (v/v)
	Sodium Azide	0.05% (w/v)
	Glycine	5% (w/v)
	BSA	5% (w/v)

4.3.3 Infection with Adenovirus

Mammalian cells were seeded in appropriate dishes 6-16 hours before infection, resulting in a confluency of 60-80%. Growth media was removed and cells were washed once with PBS. According to the size of the dish, virus dilutions were prepared in an appropriate vo-

lume of DMEM without any supplements and added to the cell culture plates. Virus infection was carried out for 2 hours at 37°C in an incubator (Heraeus) gently shaken every 15 min to achieve efficient and homogenous virus absorption. Finally, infection media was replaced with standard culture media and infected cells were harvested at indicated time points post infection (h p.i.) according to the experimental procedure.

4.4 DNA Techniques

4.4.1 Preparation of Plasmid-DNA from *E. coli*

For large-scale plasmid preparation, 500-1000 ml of liquid culture was inoculated with 200-500 µl of pre-cultured liquid culture derived from a single bacteria clone. After 16-20 hours of incubation at 30°C/37°C in an *Inova 4000 Incubator* (New Brunswick), the bacteria pellet was prepared by centrifugation at 6,000 rpm for 10 min (*Avanti J-E*; Beckman & Coulter) and plasmid DNA was extracted according to the manufacturer´s instructions using a *MaxiKit* (Quiagen).

For analytical purposes, a small volume of liquid culture (1-5 ml) was prepared by a modified protocol of Beck and co-workers (Sambrook *et al.*, 1989). The bacteria pellet was prepared from 1-5 ml of liquid culture by centrifugation (*Eppendorf 5417R*) and resuspended in 300 µl of Buffer P1. The bacteria suspension was gently mixed with 300 µl of Buffer P2 to lyse the cells. After 5 min of incubation, the solution was mixed with 300 µl of Buffer P3 and incubated for 5 min on ice for neutralization and precipitation. Finally, salts and cellular debris were pelleted by centrifugation at 14,000 rpm for 15 min (*Eppendorf 5417R*), the supernatant was transferred into a new 1.5 ml reaction tube and DNA was precipitated by centrifugation at 14,000 rpm for 30 min (*Eppendorf 5417R*) after adding 1 vol isopropanol and 0.1 vol 3 M NaAc. The clear supernatant was removed and the DNA pellet was washed in 1 ml 75% ethanol, again pelleted at 14,000 rpm for 5 min (*Eppendorf 5417R*), dried in a vacuumed centrifuge and rehydrated in an appropriate volume of ~20-50 µl 10 mM Tris (pH of 8.0) solution.

Buffer P1

Tris/HCl; pH 8.0	50 mM
EDTA	10 mM
RNAse A	100 µg/ml
• storage at 4°C	

Buffer P2	NaOH	200 mM
	SDS	1% (w/v)
Buffer P3	Ammonium Acetate	7.5 M
	• storage at 4°C	

4.4.2 Quantitative Determination of DNA Concentrations

DNA concentrations were determined by using an automatic *NanoDrop* spectrophotometer (PEQLAB) at a wavelength of 260 nm. As described previously (Sambrook *et al.*, 1989), an optical desity of 1 corresponds to an absolute concentration of 50 µg/ml for double-stranded DNA or 33 µg/ml for single-stranded DNA. In parallel, DNA purity was determined by the ration of OD_{260}/OD_{280} and should be above 1.8 to assure high DNA purity.

4.4.3 Agarose Gel Electrophoresis

Analytical and preparative agarose gels were prepared by dissolving agarose (*Seakem® LE agarose*; Biozym) in TBE buffer to a final concentration of 0.6-1.0% (w/v). After boiling the solution in a microwave oven (Moulinex) the solution was cooled down, ethidium bromide was added to a final concentration of 0.05 µg/ml and the solution poured in an appropriate gel tray. DNA samples were mixed with 6x*Loading Buffer* and subjected to agarose gel electrophoresis at a voltage of 5-10 V/cm gel length for 1-2 hours. DNA was visualized by an UV transilluminator system at 312 nm (*G:BOX*; SynGene). To minimize harmful UV irradiation for preparative purposes, agarose gels were supplemented with 1 mM guanosine and gel slices were prepared under minimal exprosure to UV light at 365 nm. Extraction of DNA from gel slices was performed by centrifugation (20,000 rpm, 2 hours; *RC 5B Plus*; Sorvall) and subsequent DNA precipitation from the obtained supernatant, as described previously (Groitl *et al.*, 2005).

5xTBE	Tris	450 mM
	Boric Acid	450 mM
	EDTA	10 mM
	• pH 7.8	
6xLoading Buffer	Bromphenol Blue	0.25% (w/v)
	Xylen Cyanol	0.25% (w/v)
	Glycerol	50% (v/v)
	EDTA	10 mM
Ethidium Bromide	Ethidium Bromide	10 mg/ml

Stock Solution	• storage at 4°C, light protected

4.4.4 Polymerase Chain Reaction

4.4.4.1 Standard PCR Protocol

For standard amplification of a DNA template, a 50 µl reaction mixture was prepared by mixing 25 ng of DNA template, 125 ng of forward primer, 125 ng of reverse primer, 1 µl of dNTP mixture (dATP, dTTP, dCTP, dGTP; each 1 mM), 5 µl of 10xPCR reation buffer and 5 U of *Taq*-polymerase (Roche) in a 0.2 ml PCR reaction tube. The thermocycler (*Flexcycler*; Analytic Jena) was programmed as follows:

1 min	95°C	DNA denaturation
45 sec	55°–70°C	primer annealing
1 min/kb	72°C	extension

After 25-30 cycles, samples were further incubated once at 72°C for 10 min and stored at 4°C. To determine PCR efficiency 5 µl of PCR reaction was analyzed by gel electrophoresis (4.4.3).

4.4.4.2 Site-Directed Mutagenesis

Mutations were inserted into target plasmids via *in vitro QuikChange™ Site-Directed Mutagenesis* (Agilent) according to the manufacturer´s instructions. The desired mutations were designed in appropriate forward and reverse primers and ordered from Metabion. Depending on the introduced mutation the PCR program was as follows:

1 min	95°C	DNA denaturation
45 sec	55°C	primer annealing
45 sec/kb	68°C/72°C	extension

After 12-16 cycles, samples were further incubated once at 68°C/72°C for 10 min and stored at 4°C. To determine PCR efficiency 10 µl of PCR reaction was analyzed by gel electrophoresis (4.4.3). Subsequently, the PCR mixture was incubated with *DpnI* for 3 hours at 37°C to remove unmutated template DNA and 10 µl were transformed into chemical competent DH5α (4.1.2.1). Finally, single clones were picked, cultured in 5-10 ml LB media (4.1.1) and prepared plasmid DNA (4.4.1) was analysed by restriction digest (4.4.5.1), agarose gel electrophoresis (4.4.3) and sequencing (4.4.6) before storage (4.1.1).

4.4.5 Cloning of DNA Fragments

4.4.5.1 Enzymatic DNA Restriction

Restriction enzymes were used according to the manufacturer´s instructions in appropriate reaction buffers (New England Biolabs; Roche). For analytical restriction digests, 1 µg of DNA was incubated with 3-10 U of enzyme for 2 hours at 37°C, unless indicated otherwise. For preparative restriction digests, 20 µg of DNA was incubated with 50 U enzyme for at least 3 hours at 37°C. In general, multiple steps of enzymatic restriction were carried out sequentially, following separation by preparative agarose electrophoresis (4.4.3) and/or isopropanol/ethanol precipitation (4.4.1).

4.4.5.2 Ligation and Transformation

Enzymatically restricted DNA fragments were ligated using 5 U of *antarctic phosphatase* (New England Biolabs) at 37°C for 30 min/13°C overnight and if required, dephosphorylated with *shrimp alkaline phosphatase* (SAP; Roche) at 65°C for 45 min. Before ligation, the DNA fragments were purified by agarose gel electorphoresis (4.4.3) and/or isopropanol/ethanol precipitation (4.4.1). For a standard ligation 20-100 ng of vector DNA was mixed with 3-5 times more insert DNA in a final volume of 20 µl, including 2 µl of 2xligation buffer and 1 U of *T4 DNA ligase* (Roche). Finally, the ligation product was transformed into chemical competent *E. coli* (4.1.2.1), single clones were picked, cultured in 5-10 ml LB media (4.1.1) and prepared plasmid DNA (4.4.1) was analysed by restriction digest (4.4.5.1), agarose gel electrophoresis (4.4.3) and sequencing (4.4.6) before storage (4.1.1).

4.4.6 DNA Sequencing

For DNA sequencing 0.5-1.0 µg of DNA and 20 pmol of sequencing primer were mixed with ddH_2O to reach a total volume of 7 µl. Sequencing reactions were performed by Seqlab (Göttingen).

4.5 Protein Techniques

4.5.1 Preparation of Total Cell Lysates

In general, all protein analysis steps were carried out on ice or at 4°C to preserve highly instable posttranslational modifications (e.g. SUMO modification). Furthermore, in all experiments highly stringent RIPA lysis buffer was used to eliminate unspecific and/or weak interactions of proteins, as well as to ensure proper solubilisation of matrix-associated proteins. Initially, cell pellets were resuspended in an appropriate volume of lysis buffer supplemented with 1 mM DTT, 0.2 mM PMSF, 1 mg/ml pepstatin A, 5 mg/ml aprotinin, 20 mg/ml leupeptin, 25 mM iodacetamide and 25 mM N-ethylmaleimide. After incubation for 30 min on ice and subsequent vortexing steps every 10 min, complete cell disruption as well as genomic DNA shearing was facilitated by sonification (40 pulses; output 0.60; 0.8 Impulse/s; *Branson Sonifier 450*). Subsequently, cellular debris and insoluble components were pelleted by centrifugation (14,000 rpm, 5 min, 4°C; *Eppendorf 5417R*) and the protein concentration of the supernatant was determined by spectrophotometry (4.5.3).

RIPA

Tris/HCl, pH 8.0	50 mM
NaCl	150 mM
EDTA	5 mM
Nonidet P-40	1% (v/v)
SDS	0.1% (w/v)
Sodium Desoxycholate	0.5% (v/v)

4.5.2 Preparation of Fractionated Cell Lysates

For preparation of the fractionated cell lysates by a modified protocol of Lee and co-workers (Lee *et al.*, 1995), cell pellets from 150 mm culture dishes were resuspended in 400 µl of Buffer A directly after harvest (4.2.6), incubated for 15 min on ice and lysed by passing through a 28.5 gauge needle for five times using a 1 ml syringe. The cytoplasmic fraction was generated by centrifugation at 12,000xg for 30 sec at 4°C (*Eppendorf 5417R*) and transferred to a new 1.5 ml reation tube. Afterwards, the pelleted nuclei were subjected to cell lysate preparation as described previously (4.5.1).

Buffer A

HEPES, pH 8.0	10 mM
$MgCl_2$	1.5 mM
KCl	10 M
DTT	1 mM
PMSF	0.5 mM

4.5.3 Quantitative Determination of Protein Concentrations

Protein concentrations in samples were determined using *Protein-Assays* (BioRad) accoring to Bradford (Bradford, 1976) by meassuring the 595 nm absorption of proteins bound to chromogenic substrates. 1 µl of protein solutions were mixed with 800 µl of PBS and 200 µl of *Bradford Reagent* (BioRad), incubated for 5 min at RT and measured in a *SmartSpec™ Plus* spectrophotometer (BioRad) at 595 nm against a blank of 800 µl of PBS and 200 µl of *Bradford Reagent*. Protein concentrations were determined by calculation based on a concurrently derived standard curve with BSA (concentrations of 1-16 µg/µl; New England Biolabs).

4.5.4 Immunoprecipitation

For immunoprecipitation equal amounts (0.1 mg-3 mg) of total cell lysates (4.5.1; 4.5.2; 4.5.3) were precleared by addition of protein A- or protein G-sepharose (Sigma-Aldrich) for 1 hour at 4°C in a rotator (GFL). Simultaneously, 1 µg of purified antibody or 100 µl/mg sepharose for hybridoma supernatant was coupled to 3 mg of sepharose/IP. Antibody-coupled sepharose beads were washed three times with 15 ml of lysis buffer before being added to the precleared protein lysate, which has been transferred to a new 1.5 ml reation tube after clearing by centrifugation (600xg, 5 min, 4°C; *Eppendorf 5417R*). Immunoprecipitation was performed at 4°C in a rotator (GFL) for 2 hours to preserve short-lived posttranslational protein modifications (e.g. SUMO modification). The protein A/protein G immune complexes were pelleted by centrifugation (600xg, 5 min, 4°C; *Eppendorf 5417R*) and washed three times with 1.5 ml RIPA lysis buffer. Finally, the samples were mixed with an appropriate volume of *2xSDS sample buffer* (Sambrook *et al.*, 1989), boiled for 3 min at 95°C to elute proteins and stored at -20°C until detailed analysis (4.5.5; 4.5.6).

2xSDS Sample Buffer		
	Tris/HCl, pH 6.8	100 mM
	SDS	4% (w/v)
	DTT	200 mM
	Bromphenol Blue	0.2% (w/v)
	Glycerol	20%

4.5.5 SDS Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Protein samples of cell lysates (4.5.1; 4.5.2) or immunoprecipitation (4.5.4) were separated by SDS-PAGE according to their molecular weights. Polyacrylamide gels were made using a

30% acrylamide/bisacrylamide solution (37.5:1 *Protogel*; National Diagnostics) diluted to the final concentration of 8%-15% by ddH_20. Protein samples were concentrated between the lower percentage stacking and the higher percentage separating gel via an artificial pH discrepancy (Weber *et al.*, 2008). Acrylamide polymerization was initiated by addition of APS (c_{final}=0.1%) and TEMED (c_{final}=0.01%). All gels were prepared by the SDS-PAGE system of Biometra according to the manufacturer´s instructions and run at 15 mA/gel in TGS-buffer. In general, protein samples were prepared for SDS-PAGE by addition of *2xSDS sample buffer* (Sambrook *et al.*, 1989) and boiling at 95°C for 3 min in a thermoblock (*Thermomixer Comfort*; Eppendorf). *PageRuler™ Prestained Protein Ladder Plus* (Fermentas) was used to determine protein weights. Afterwards, separated proteins were transferred onto nitrocellulose membranes (*Protran®*; Whatman) by Western blotting (4.5.6).

30% Acrylamide Stock Solution	Acrylamide	29% (w/v)
	N,N'Methylenbisacrylamide	1% (w/v)
Stacking Gel 5%	Acrylamide Stock Solution	17% (v/v)
	Tris/HCl, pH 6.8	120 mM
	SDS	0.1% (w/v)
	APS	0.1% (w/v)
	TEMED	0.1% (v/v)
Separating Gel 8%	Acrylamide Stock Solution	27% (v/v)
	Tris/HCl, pH 8.8	250 mM
	SDS	0.1% (w/v)
	APS	0.1% (w/v)
	TEMED	0.06% (v/v)
Separating Gel 10%	Acrylamide Stock Solution	34% (v/v)
	Tris/HCl, pH 8.8	250 mM
	SDS	0.1% (w/v)
	APS	0.1% (w/v)
	TEMED	0.04% (v/v)
Separating Gel 12%	Acrylamide Stock Solution	40% (v/v)
	Tris/HCl, pH 8.8	250 mM
	SDS	0.1% (w/v)
	APS	0.1% (w/v)
	TEMED	0.04% (v/v)
Separating Gel 15%	Acrylamide Stock Solution	50% (v/v)
	Tris/HCl, pH 8.8	250 mM
	SDS	0.1% (w/v)
	APS	0.1% (w/v)
	TEMED	0.04% (v/v)
TGS-Buffer	Tris	25 mM
	Glycine	200 mM
	SDS	0.1% (w/v)

4.5.6 Western Blot

Protein Transfer onto Nitrocellulose Membranes

For immunoblotting, equal amounts of protein samples (4.5.1; 4.5.2; 4.5.4) were separated by SDS-PAGE and transferred onto nictrocellulose membranes (*Protran®*; Whatman) using *Trans-Blot® Electrophoretic Transfer Cell* (BioRad) in *Towbin*-buffer accoring to the manufacturer´s instructions. Gels and membranes were soaked in *Towbin*-buffer, placed upon one another between two soaked blotting papers (Whatman) and two blotting pads in a plastic cassette. The electric transfer was performed in "full wet" mode in a blotting chamber with *Towbin*-buffer at 400 mA for 90 min. Subsequently, nitrocellulose membranes were incubated for at least 2 hours at RT or overnight at 4°C in PBS-Tween containing 5% non-fat dry milk (Frema) on an *orbital shaker* (GFL) to saturate unspecific antibody binding areas on the nitrocellulose membrane.

Antibody Staining

Afterwards, the blocking solution was discarded, membranes were washed briefly to remove residual blocking solution and incubated for 2 hours at RT in PBS-Tween containing the primary antibody. The dilutions of primary antibodies as well as the added non-fat dry milk (Frema) to achieve optimal results were determined for each individual antibody. After primary staining, the antibody solution was removed, the nitrocellulose membranes were washed three times for 15 min and incubated for 2 hours at RT in PBS-Tween with the HRP-coupled secondary antibody (1:10,000; Amersham) containing 3% non-fat dry milk (Frema).

Protein Visualization by Enhanced Chemiluminescence

Protein bands were visualized by enhanced chemiluminescence using *SuperSignal® West Pico Chemiluminescent Substrate* (Pierce) according to the manufacturer´s instructions and detected by X-ray films (*RP New Medical X-Ray Film*; CEA) using *GBX Developer* (Kodak), after discarding the secondary antibody solution and three times washing in PBS-Tween for 15 min. X-ray films were scanned, cropped using *Photoshop CS4* (Adobe) and figures were prepared using *Illustrator CS4* (Adobe)

***Towbin*-Buffer**	Tris/HCl, pH 8.3	25 mM
	Glycine	200 mM
	SDS	0.05% (w/v)
	Methanol	20% (v/v)
PBS-Tween	Tween 20	0.1% (v/v)
	• in PBS	

4.5.7 Immunofluorescence

Methanol Fixation

$3x10^5$ cells were seeded in six-well dishes with glass coverslips and fixed at certain time points after transfection (4.2.5), infection (4.3.3) or during standard cultivation (4.2.1). Media was removed and ice-cold methanol was added. After incubation (15 min; -20°C) the methanol was removed and the plates were air dried before being frozen at -20°C until further use.

Antibody Staining and Image Aquisition

For immunofluorescence staining six-well dishes with glass coverslips were thawed for 10 min at RT and partially rehydrated by adding 2 ml of PBS-Triton for 15 min. Subsequently, PBS-Triton was removed and samples were blocked with 2 ml TBS-BG for 1 hour at RT on an *orbital shaker* (GFL) before adding 50 µl of appropiate antibody dilutions in PBS-Tween directly onto the glass coverslips. The primary staining was performed in a damp plastic chamber for 1 hour. After washing the coverslips three times with PBS-Tween for 15 min, cells were further incuated for 1 hour with fluorescence-coupled secondary antibodies (dilution 1:100 in PBS-Tween) supplemented with 0.5 µg/ml DAPI at RT in a damp plastic chamber (light protected). Finally, the samples were washed three times with PBS-Tween for 15 min, air dried and mounted in *Glow Mounting Media* (EnerGene). Digital images were acquired using a *DM6000 fluorescence microscope* (Leica) with a charge-coupled device camera (Leica), cropped and decoded by *Photoshop CS4* (Adobe) and assembled with *Illustrator CS4* (Adobe).

PBS-Triton	*Triton® X-100* • in PBS	0.5 mM
TBS-BG	Tris/HCl, pH 7.6	20 M
	NaCl	137 mM
	KCl	3 mM
	$MgCl_2$	1.5 mM
	Tween 20	0.05% (v/v)
	Sodium Azide	0.05% (w/v)
	Glycine	5% (w/v)
	BSA	5% (w/v)

4.6 Reporter Gene Assay

For quantitatively determining of promoter activities, the *Dual-Luciferase® Reporter Assay System* (Promega) was used according to the manufacturer´s instructions. Promoter activity was determined by the expression of firefly luciferase (*Photinus pyralis*) under the control of a promoter of interest and normalized to consitutively expressed renilla luciferase (*Renilla reniformis*) that served as internal transfection control. Adherent cells were seeded (4.2.1; 4.2.4) and transfected in six-well plates according to the experimental setting as described previously (4.2.5). Afterwards, samples were harvested 48 hours post transfection by directly adding 150 µl of *passive lysis buffer* (Promega) to the cells and incubating for 15 min at RT on an *orbital shaker* (GFL). Finally, 5 µl of lysate was subjected to sequential meassuring of *Firefly* (10 sec) and *Renilla* luciferase activity (10 sec) in a *Lumat LB 9507 luminometer* (Berthold Technologies).

4.7 Lentiviruses and Transformation Assay

4.7.1 Generation of Lentivial Particles Encoding Adenoviral Oncogenes

Cloning of Lentiviral Plasmids

Lentiviral particles were generated based on the system previously described by Weber and co-workers (Weber *et al.*, 2008). The coding sequences of different adenoviral oncogenes were amplified by PCR introducing a 5´-BamHI site and a 3´-EcoRI site (4.4.4.1), purified by agarose gel electrophoresis (4.4.3), sequentially digested using the appropriate enzymes (4.4.5.1) and ligated into LeGO-iVLN2/iBLB2 (4.4.5.2). Mutations within these coding sequences were introduced subsequently via *QuikChange™ Site-Directed Mutagenesis* (Agilent) (4.4.4.2).

Generation and Storage of Lentiviral Particles

To generate a suitable amount of lentiviral particles, HEK-293T cells were seeded on 150 mm dishes to reach a confluency of 70-80% for transfection (4.2.1). Subseqeuntly, a total amount of 18 µg of DNA/plate (8.5 µg LeGO-construct; 2.5 µg pRSV-Rev; 2.5 µg pCMV-VSV-G; 4.5 µg pMDLg/pPRE) was transfected using PEI (4.2.5), incubated for 8 hours at standard conditions (4.2.1) and the transfection media was replaced afterwards with 15 ml of standard culture media additionally supplemented with 20 mM sterile filtrated HEPES buffer. Two

days after transfection, the supernatant was collected, centrifuged shortly (4,500 rpm, 3 min; *Multifuge 3S-R*; Heraeus) and sterile filtrated (0.45 µm). Finally, the clear supernatant was aliquoted, frozen in liquid nitrogen and stored at -80°C.

4.7.2 Titration of Lentiviral Particles

To determine the exact virus titers, $5x10^4$ HEK-293T cells were seeded in 24-well dishes and infected with lentivirus after 6-10 hours. Cells were infected with either 10 µl or 100 µl of lentivirus in a total volume of 500 µl of standard culture media additionally supplemented with 8 µg/ml polybren (Sigma-Aldrich) and the media was replaced after virus absorption overnight. Cells were harvested by trypsination 48 h p.i. (4.2.1), resuspended in a volume of 500 µl and stored on ice until subsequent analyis in a *FACS CantoII* (BD Biosciences). Since all of the LeGO constructs express different fluorescent markers via an IRES sequence, infected cells were determined by fluorescence against a mock infected control. To ensure precise titer determination, all titrations were performed at least in duplicate with two different concentrations and calculated according to the formula shown below. Finally, the mean value of all settings was calculated and used in the respective experiments.

$$T = N \times P/100 \times V$$

T: titer [particles/µl]
N: seeded HEK-293T cells [number of cells]
P: fluorescence positive cells [%]
V: used virus supernatant [µl]

4.7.3 Transformation Assay

To test the transformation potential of HAdV5 E1B-55K in combination with E1A, $3x10^3$ pBRK cells were seeded in six-well dishes (4.2.1; 4.2.2; 4.2.4) and transduced with lentiviral particles encoding the respective adenoviral proteins. The cells were simultaneously infected with $1x10^5$ particles for each particle type. For efficient transduction, absorption was performed overnight and the media was additionally supplemented with 8 µg/ml polybrene. After transduction the media was replaced by standard culture media and each well was expanded onto a 150 mm culture dish 48 hours post plating (4.2.1). The culture media was replaced every 3-4 days and multilayered cell accumulations (*foci*) were visualized by crystal violet staining after 2-3 weeks of incubation.

Crystal Violet Solution	Crystal Violet	1% (w/v)
	Methanol	25% (v/v)

5 Results

5.1 The Adenoviral E1B-55K Protein is Associated with PML in Transformed Rat Cell Lines

Over the past decades, primary rodent cells have been shown to represent a suitable tool for analyzing the transformation potential of adenoviral oncogenes (Branton *et al.*, 1985; Endter & Dobner, 2004). Although the obtained results are not always completely transferable to the human system, experiments performed in the heterologous rodent system have provided significant insights into the underlying molecular mechanisms of adenoviral transformation. Therefore, stably transformed rodent cell lines expressing the HAdV5 E1A and E1B-55K were used in the initial experiments to determine the intranuclear distribution of E1B-55K and the association of the adenoviral protein with PML-NBs.

5.1.1 E1B-55K Colocalizes with Endogenous Rat PML

The HAdV5 E1B-55K protein continuously shuttles between the nucleus and the cytoplasm, mediated partly by its *SUMO-1 conjugation motif* (SCM) and leucine-rich *nuclear export signal* (NES) (Endter *et al.*, 2001; Endter *et al.*, 2005; Kindsmüller *et al.*, 2007). The equilibrium in rodent cells favors the cytoplasmic side, inducing formation of a large cytoplasmic aggregate (Zantema *et al.*, 1985a; Zantema *et al.*, 1985b). As published previously (Endter *et al.*, 2001; Endter *et al.*, 2005), pBRK derived transformed cell lines AB120/AB115 continuously express high levels of the adenoviral E1A plus E1B-55K-wt/E1A plus E1B-55K-NES protein, recommending this as an adequate model system to analyze subnuclear E1B-55K localization.

Therefore, both cell lines were prepared for immunofluorescence analysis (Fig. 10), where AB120 cells were alternatively treated with 20 nM leptomycin B (LMB) to irreversibely block CRM1-mediated nuclear export via the E1B-55K NES.

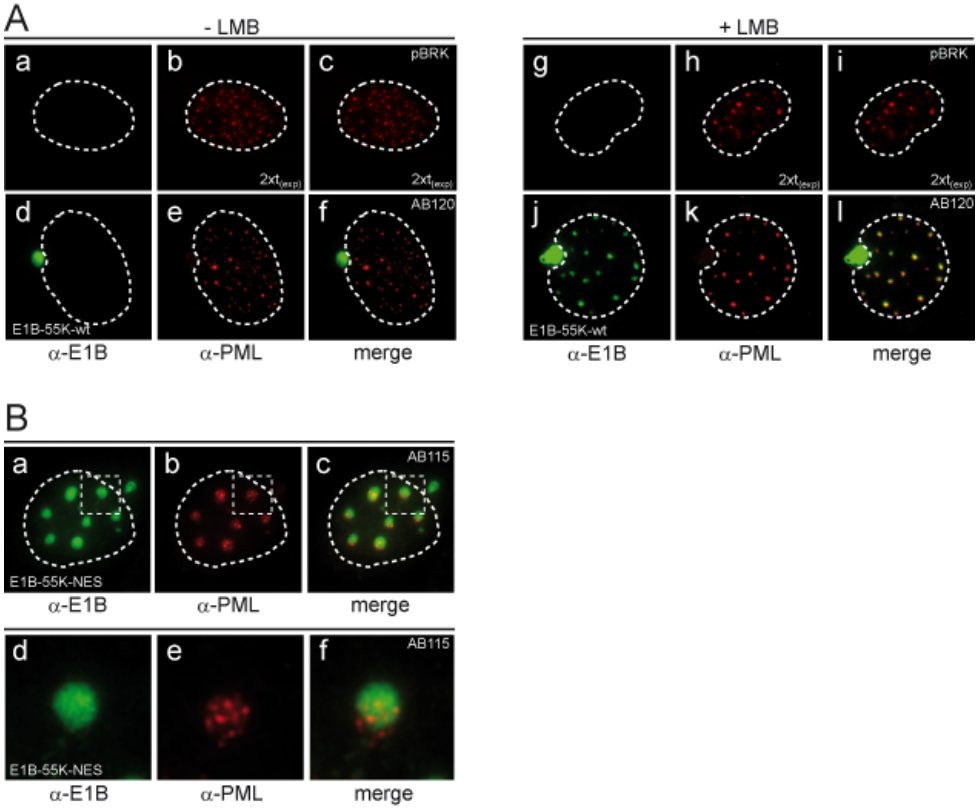

Figure 10. E1B-55K colocalizes with endogenous rat PML. The indicated cell lines (pBRK, AB120, AB115) were double labeled *in situ* with mAb 7C11 (α-E1B-55K) and mAb 5E10 (α-PML) (4.5.7). Prior to methanol fixation, pBRK/AB120 cells were alternatively treated with 20 nM LMB for 2 hours. Primary antibodies were detected with FITC- or Texas Red-conjugated secondary antibodies. The dotted box areas in Ba-Bc were enlarged 5-fold to illustrate the organization of E1B-55K-NES/PML positive nuclear accumulation (Bd-Bf). Representative α-E1B-55K (green; Aa, Ad, Ag, Aj, Ba, Bd) and α-PML (red; Ab, Ae, Ah, Ak, Bb, Be) staining patterns of at least 50 analyzed cells are shown. Due to lower expression levels of PML in pBRK cells, the images Ab, Ac, Ah and Ai are documented at double exposure times for Texas Red ($2xt_{(exp)}$). Overlays of the single images (merge) are shown in Ac, Af, Ai, Al, Bc and Bf. In all panels, nuclei are indicated by a dotted line. (Magnification x7600)

Consistent with published results, E1B-55K-wt localizes in a large cytoplasmic accumulation in close proximity to the nucleus of rodent cells (Fig. 10; Ad) (Zantema *et al.*, 1985a; Zantema *et al.*, 1985b), whereas PML exhibits punctuated nuclear staining (Fig. 10; Ae) (Endter *et al.*, 2005). However, upon inhibition of CRM1-mediated nuclear export by LMB, E1B-55K-wt additionally accumulates in several brightly stained nuclear aggregates, clearly colocalizing with endogenous PML in ~70% of all cells examined (Fig. 10; Aj-Al). Consistently, a similar phenotype was observed in the majority of AB115 (>90%) (Fig. 10; B) as these cells constitutively express the E1B-55K-NES mutant protein. Correlating with the amount of E1B-55K-NES nuclear accumulation, PML is sequestered in brightly stained subnuclear aggregates (Fig. 10; Ba-Bc) exhibiting a punctuated PML surrounding globular E1B-55K-NES (Fig. 10; Bd-Bf; 5x enlarged boxed areas).

Interestingly, expression of the HAdV5 E1A and E1B-55K proteins also seems to induce stabilization of endogenous PML, since both AB cell lines exhibit remarkably higher PML levels than pBRK as judged by the necessary exposure times to visualize subnuclear PML. Taken together, E1B-55K apparently associates with endogenously expressed rat PML, inducing relocalization of PML-NBs upon accumulation within the nucleus.

5.1.2 E1B-55K Interacts with a Specific Subset of Endogenous Rat PML

To further confirm the association of E1B-55K and PML (Fig. 10), coimmunoprecipitation assays to detect interaction of the viral protein with endogenous PML were performed in these transformed rat cells (Fig. 11).

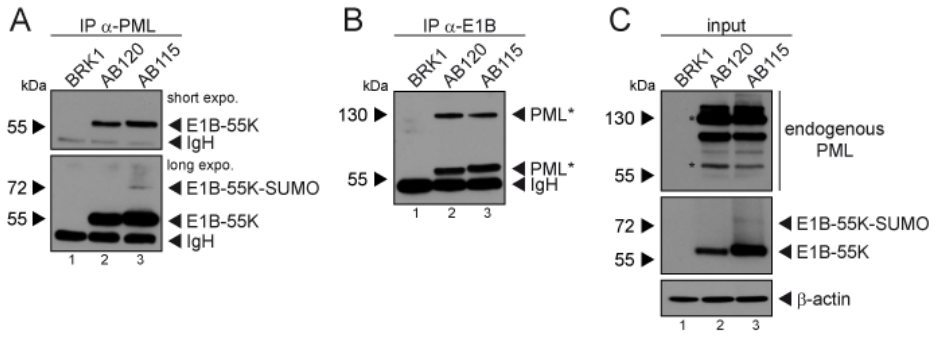

Figure 11. E1B-55K interacts with a specific subset of endogenous rat PML. Immunoprecipitation of PML/E1B-55K was performed using mAb 5E10 (α-PML)/mAb 2A6 (α-E1B-55K), resolved by 10% SDS-PAGE and visualized by immunoblotting (4.5). Coprecipitated proteins (A/B) and input levels (C) of total cell lysates were detected by using mAb 2A6 (α-E1B-55K), mAb 5E10 (α-PML) and mAB AC-15 (α-β-actin). All the monoclonal rodent cell lines (BRK1, baby rat kidney; AB120/AB115, transformed pBRK cell lines stably expressing HAdV5 E1A and E1B-55K-wt/NES) were derived from freshly prepared pBRK cells as described previously (Nevels *et al.*, 1997). Specific PML bands are highlighted (*) and discussed in more detail in the text. Molecular weights in kDa are indicated on the *left*, while corresponding proteins are labeled on the *right*.

As described previously (Stuurman *et al.*, 1992), immunoblotting for rat PML using mAb 5E10 shows a set of PML-specific bands with sizes ranging from 60 kDa–130 kDa (Fig. 11; C; panel 1). Intriguingly, it appears that concurrent expression of E1A plus E1B-55K and/or transformation *per se* alters the PML expression pattern compared to BRK1, leading to remarkably higher levels of endogenous protein (Fig. 11; C; panel 1). This is consistent with the observations from the immunofluorescence analysis (Fig. 10). Although E1B-55K-wt and PML show no, or only partial colocalization (Fig. 10; A), E1B-55K-wt as well as E1B-55K-NES possess the intrinsic capacity to coprecipitate with endogenous rat PML in total cell lysates (Fig. 11; A), supporting the assumption that colocalization of E1B-55K with PML (Fig. 10) is

mediated by direct or indirect interaction. Interestingly, the reverse experiment reveals coprecipitation of only two PML-bands corresponding to about 60 kDa and 130 kDa (Fig. 11; B; *), indicating preferential binding of E1B-55K to a specific subset of PML proteins.

Taken together, these data show for the first time that viral E1B-55K colocalizes (Fig. 10) and interacts (Fig. 11) with endogenous rat PML. This, together with the observation that E1B-55K-NES significantly reorganizes PML-NBs (Fig. 10; B) and augments transformation of primary rodent cells (Endter *et al.*, 2001; Endter *et al.*, 2005), further indicates that binding of E1B-55K to the tumor suppressor protein PML may be critical for its transforming potential. Although the results of these experiments may not be completely transferable to the human system, they provide further implications for the multifunctional character of E1B-55K during viral infection of human cells, which may at least in part be orchestrated via manipulation of the PML protein network (2.2.2) (Van Damme *et al.*, 2010).

5.2 Adenoviral Manipulation of PML-NBs is Mediated by the Interaction of Multiple Viral Proteins with Distinct PML-Isoforms

The observed PML expression pattern varies considerably as a result of extensive regulation (2.2.1) and within cells from different tissues or mammalian species. Although human cell lines exhibit a complicated system expressing at least seven distinguishable PML-isoforms (2.2.1) (Jensen *et al.*, 2001), the ability of E1B-55K to interact with the human orthologs of rodent PML was analyzed, since very little is known about the expression of distinct PML-isoforms in rats. Moreover, recent data concerning the organization of the murine *pml* locus underline the possibility of species specific PML expression with partially homologous, but also quite different PML-isoforms (Goddard *et al.*, 1995; Condemine *et al.*, 2006). Furthermore, analysis of human PML in a corresponding system could help elucidate the putative role of PML during productive adenoviral infection.

5.2.1 Endogenous PML Expression is Dependent on the Cell Type and Species Origin

As shown previously, PML expression is frequently deregulated in human tumor cell lines and/or during oncogenesis (Koken *et al.*, 1995; Salomoni & Pandolfi, 2002; Gurrieri *et al.*, 2004a; Gurrieri *et al.*, 2004b; Scaglioni *et al.*, 2006; Salomoni *et al.*, 2008; Lukashchuk & Everett, 2010) indicating that certain tumor cell lines may not represent a suitable tool to evaluate the role of PML or its various isoforms during adenoviral infection and/or transformation.

Therefore, several available cell lines were screened for endogenous PML expression (Fig. 12; A). Additionally, isoform specific expression constructs were transiently transfected into H1299 cells to allow comparative analyses and assignment of the different endogenously expressed PML-isoforms (Fig. 12; B).

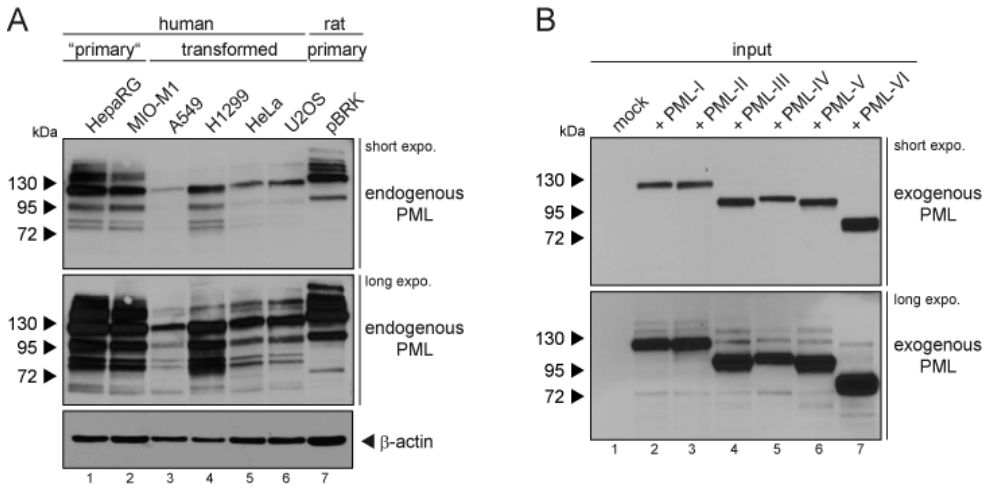

Figure 12. Endogenous PML expression is dependent on cell type and species origin. Total cell extracts from different cell lines/transiently transfected H1299 cells were prepared, resolved by 10% SDS-PAGE and visualized by immunoblotting (4.5). Endogenous PML levels (A)/exogenous overexpressed flag-PML (B) was detected by using mAb 5E10 (α-PML)/mAb flag-M2 (α-flag) and mAB AC-15 (α-β-actin). Molecular weights in kDa are indicated on the *left*, while corresponding proteins are labeled on the *right*.

Consistent with published results, PML expression varies considerably in the different cell lines (Fig. 12; A). All human tumor cell lines investigated (Fig. 12; A; lanes 3-6) show significantly reduced levels of PML compared to the HepaRG and MIO-M1 cell lines (Fig. 12; A; lanes 1/2). Both human hepatocellular HepaRG (Gripon *et al.*, 2002; Cerec *et al.*, 2007) and retinal MIO-M1 (Limb *et al.*, 2002; Lawrence *et al.*, 2007; Bull *et al.*, 2008) exhibit several characteristics of primary cell lines, such as metabolic markers (e.g. glutamine synthetase) and

the capability to differentiate into the respective tissue components. However, they show an immortalized phenotype and can therefore be kept in cell culture without undergoing senescence. The obvious reduction in PML expression in transformed cells (Fig. 12; A; lanes 3-6) may correspond with upregulation of the PML regulator CK2, described as occurring frequently in human tumors and/or tumor cell lines (2.2.1) (Scaglioni *et al.*, 2006). Since H1299 cells represent a well-established system in the field of adenovirology, these were used in addition to the "primary" cells in subsequent experiments. Moreover, expression in H1299 cells resembled the most physiological expression pattern of PML (Fig. 12; A; lane 4), which is characterized by predominant expression of PML-isoforms I and II, with significant lower levels of the smaller isoforms III-VI compared to the "primary" HepaRG/MIO-M1 cells (Fig. 12; A; lane 1/2) (Condemine *et al.*, 2006). However, the observed endogenous PML (Fig. 12; A) revealed a much more complicated pattern, since most of the isoforms are extensively posttranslationally modified, most predominantely by the *small ubiquitin-related modifiers* as can be seen in Fig. 12 (B) (2.2.1) (Jensen *et al.*, 2001; Eskiw *et al.*, 2004; Fu *et al.*, 2005; Bernardi & Pandolfi, 2007; Lallemand-Breitenbach *et al.*, 2008).

5.2.2 E1B-55K Interacts Specifically with PML-IV/V in Transiently Transfected H1299 Cells

Unfortunately, very little is known about the diverse functions of different PML-isoforms *in vivo*, although all six nuclear isoforms are apparently necessary to form a physiologically functional PML-NB (Condemine *et al.*, 2006). In addition, detailed microscopic (Weidtkamp-Peters *et al.*, 2008; Brand *et al.*, 2010; Lang *et al.*, 2010) and *in vivo* analysis (Fogal *et al.*, 2000; Oh *et al.*, 2009; Wu *et al.*, 2009; Yu *et al.*, 2010) showed that the different isoforms vary considerably in their subnuclear localization and interaction/modulation of cellular proteins, indicating isoform-specific functions despite their obvious sequence similarities (2.2.1).

Consequently, since E1B-55K seems to associate with a distinct subset of endogenous rat PML (Fig. 11), H1299 cells were transiently transfected with constructs expressing human flag-tagged PML-isoforms plus E1B-55K-wt and subjected to immunoprecipitation analysis (Fig. 13).

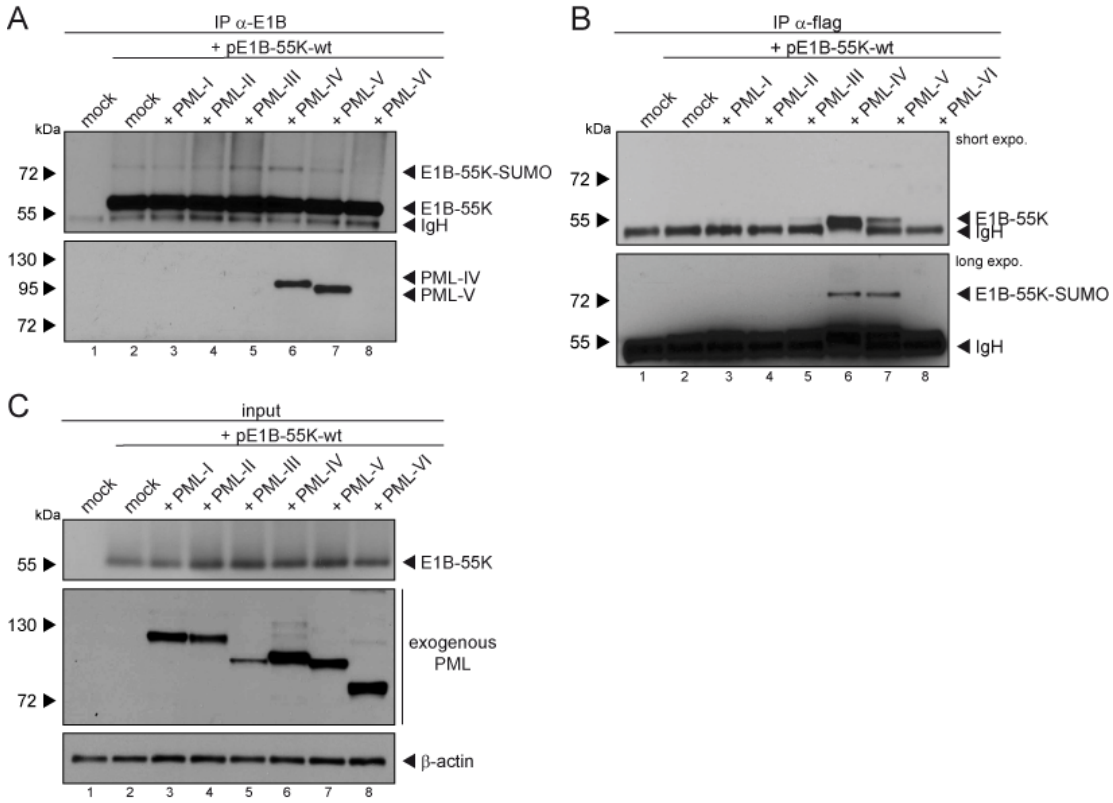

Figure 13. E1B-55K interacts specifically with PML-IV/V in transiently transfected H1299 cells. Subconfluent H1299 cells ($7.0x10^6$) were transfected with 10 µg of pE1B-55K-wt plus 10 µg of different lentiviral constructs encoding N-terminal flag-tagged human PML-isoforms I-VI and harvested after 48 hours before preparing total cell extracts (4.5). Immunoprecipitation of E1B-55K/flag-PML was performed by using mAB 2A6 (α-E1B-55K)/mAB flag-M2 (α-flag), resolved by 10% SDS-PAGE and visualized by immunoblotting (4.5). Coprecipitated proteins (A/B) and input levels (C) of total cell lysates were detected using mAb 2A6 (α-E1B-55K), mAb flag-M2 (α-flag) and mAb AC-15 (α-β-actin). Molecular weights in kDa are indicated on the *left*, while corresponding proteins are labeled on the *right*.

Direct immunoprecipitation of E1B-55K shows sufficient and comparable amounts of the adenoviral protein in all transfections (Fig. 13; A; panel 1). Another band of ~75 kDa was also evident, which presumably corresponds to covalently SUMO-1-modified E1B-55K (Fig. 13; A; panel 1) (Endter *et al.*, 2001; Endter *et al.*, 2005). Interestingly, the analysis of coprecipitated flag-PML reveals a highly specific interaction between E1B-55K and PML-isoforms IV and V (Fig. 13; A; panel 2; lanes 6/7), although some minor interactions with isoforms I and III are visible in the long exposure (data not shown). Corresponding observations were made by precipitating flag-tagged PML and subsequent staining for E1B-55K (Fig. 13; B; panel 1; lanes 6/7). Interestingly, it appears that covalently SUMOylated E1B-55K could also be co-immunoprecipitated by PML-IV/V, implying interaction with both E1B-55K species (Fig. 13; B; panel 2; lanes 6/7).

All these findings were confirmed in at least two independent experiments, including validation of the established system by reproducing already published data on the binding of E4orf3 to PML-II (Hoppe *et al.*, 2006; Leppard *et al.*, 2009), as well as several negative test adenoviral proteins such as E2A-72K (data not shown). Taken together, these results show that E1B-55K exhibits high affinity to only two human PML-isoforms.

5.2.3 Viral Infection Alters the PML Binding Capabilities of E1B-55K in H1299 Cells

Previously published data have shown that the adenoviral protein E4orf3 is necessary and sufficient to disrupt cellular PML-bodies during adenoviral infection (Carvalho *et al.*, 1995; Puvion-Dutilleul *et al.*, 1995; Doucas *et al.*, 1996). Furthermore, detailed studies have revealed an astonishing amount of cross-talk between adenoviral proteins in the modulation of host cell factors, as demonstrated for the transcriptional repression of p53 by E1B-55K in the presence of E4orf3 and/or E4orf6 (König *et al.*, 1999).

To evaluate whether other viral proteins interfere with, or alter the interaction between E1B-55K and PML-IV/V (Fig. 13), H1299 cells were transiently transfected with constructs expressing human flag-tagged PML-isoforms, superinfected with wild-type virus (H5*pg*4100) and subjected to immunoprecipitation analysis (Fig. 14).

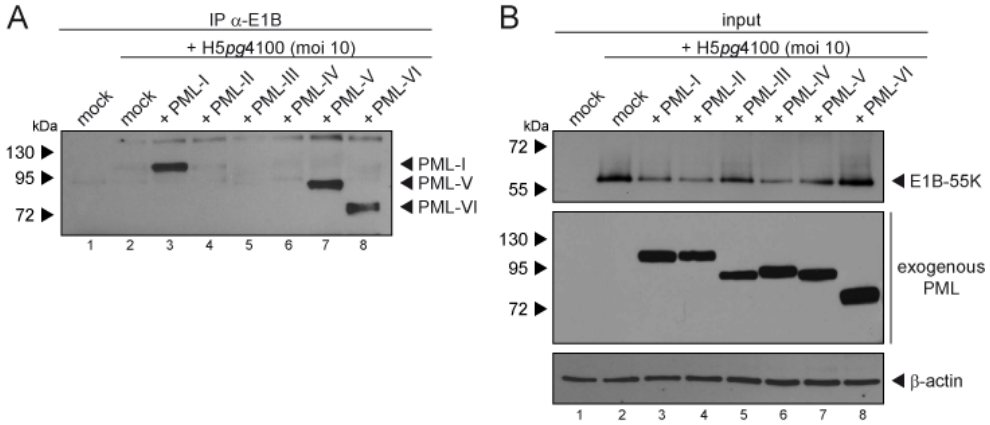

Figure 14. Viral infection alters the PML binding capabilities of E1B-55K in H1299 cells. Subconfluent H1299 cells ($7.0x10^6$) were transfected with 10 µg of different lentiviral constructs encoding N-terminal flag-tagged human PML-isoforms I-VI, infected 10 hours after transfection with wt virus (H5*pg*4100) at a multiplicity of 10 ffu per cell and harvested 36 h p.i. before preparing total cell extracts (4.5). Immunoprecipitation of E1B-55K was performed by using mAb 2A6 (α-E1B-55K), resolved by 10% SDS-PAGE and visualized by immunoblotting (4.5). Coprecipitated proteins (A) and input levels (B) of total cell lysates were detected using mAb flag-M2 (α-flag), mAb 2A6 (α-E1B-55K) and mAB

AC-15 (α-β-actin). Molecular weights in kDa are indicated on the *left*, while corresponding proteins are labeled on the *right*.

As suspected, the E1B-55K PML binding pattern (Fig. 14) differs considerably from transfected H1299 cells (Fig. 13). E1B-55K still interacts specifically with PML–V, whereas binding to PML-IV is completely abolished (Fig. 14; A; lanes 7/6). Interestingly, binding to PML-I and PML-VI seems to be exclusively present in virus superinfected cells (Fig. 14; A; lanes 3/8) strongly indicating that the interaction of E1B-55K with specific PML-isoforms during infection most likely involves other posttranslational modifications and/or the timely regulated expression of additional viral proteins such as E4orf6, which is known to regulate localization (Goodrum *et al.*, 1996) and SUMOylation of E1B-55K (Lethbridge *et al.*, 2003). In fact, E4orf3 seems the likely prime candidate responsible for triggering the observed change in E1B-55K binding behavior (Fig. 14), since it was reported to interact with both E1B-55K (Leppard & Everett, 1999) and PML-II (Hoppe *et al.*, 2006; Leppard *et al.*, 2009). However, it remains elusive as to how these properties could induce the observed changes in PML coprecipitation of E1B-55K (Fig. 14).

5.2.4 E4orf6 Modulates the PML Binding Capabilities of E1B-55K during Viral Infection of H1299 Cells

Over the last few years, it has been well established that E1B-55K and E4orf6 physically interact (Sarnow *et al.*, 1984), forming an SFC-like E3 ubiquitin ligase complex (Querido *et al.*, 2001a; Blanchette *et al.*, 2004; Blanchette *et al.*, 2008) that mediates multiple functions during productive viral infection (2.1.4.2.3). This, together with the fact that E4orf6 has been shown to modulate localization (Ornelles & Shenk, 1991; Goodrum *et al.*, 1996; Dobbelstein *et al.*, 1997; Orlando & Ornelles, 1999; Orlando & Ornelles, 2002; Marshall *et al.*, 2008) and posttranslational modification (Lethbridge *et al.*, 2003) of E1B-55K, implicates E4orf6 as the causative component triggering the shift in PML binding of E1B-55K during transfection/infection (Fig. 14) compared to transient transfection (Fig. 13).

Consequently, H1299 cells were transiently transfected with constructs expressing human flag-tagged PML-isoforms, superinfected with an E4orf6 null virus (H5*pm*4154) (Blanchette *et al.*, 2008) and subjected to immunoprecipitation analysis (Fig. 15).

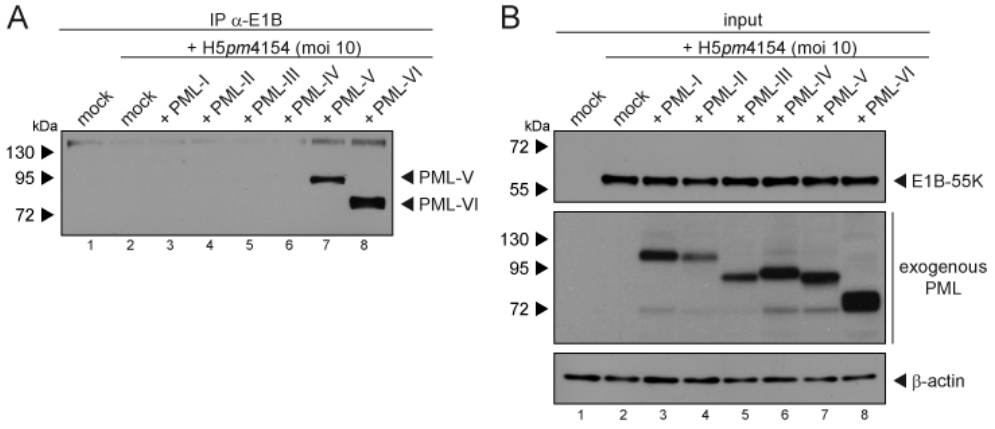

Figure 15. E4orf6 modulates the PML binding capabilities of E1B-55K during viral infection of H1299 cells. Subconfluent H1299 cells ($7.0x10^6$) were transfected with 10 µg of different lentiviral constructs encoding N-terminal flag-tagged human PML-isoforms I-VI, infected 10 hours after transfection with an E4orf6 null virus (H5*pm*4154) at a multiplicity 10 ffu per cell and harvested 36 h p.i. before preparing total cell extracts (4.5). Immunoprecipitation of E1B-55K was performed using mAB 2A6 (α-E1B-55K), resolved by 10% SDS-PAGE and visualized by immunoblotting (4.5). Coprecipitated proteins (A) and input levels (B) of total cell lysates were detected using mAb flag-M2 (α-flag), mAb 2A6 (α-E1B-55K) and mAb AC-15 (α-β-actin). Molecular weights in kDa are indicated on the *left*, while corresponding proteins are labeled on the *right*.

Interestingly, superinfection with the E4orf6 null mutant H5*pm*4145 changed the coprecipitation capacity of E1B-55K towards the different PML-isoforms (Fig. 15; A). Interaction of E1B-55K with PML-V and VI (Fig. 15; A; lanes 7/8) was retained in amounts comparable to H5*pg*4100 infected cells (Fig. 14), whereas PML-I binding appears to be completely abolished (Fig. 15; A; lane 3), strongly indicating that the viral E4orf6 protein actively participates in establishing a robust E1B-55K PML-I interaction. This observation further substantiates the assumption that the shift of PML binding E1B-55K during viral infection (Fig. 14) compared to transient transfection experiments (Fig. 13) is at least in part mediated by other viral proteins such as E4orf6. Although the results here point to E4orf6 as a prime candidate in this process (Fig. 15), they do not explain the efficient coprecipitation of PML-VI by E1B-55K during infection, since E1B-55K *per se* was shown to exclusively interact with isoforms IV and V (Fig. 13). This suggests that even more viral proteins are involved.

5.2.5 E4orf6 Interacts Specifically with PML-I/II/IV/V in Transiently Transfected H1299 Cells

Intriguingly, E4orf6 was described previously to reduce E1B-55K posttranslational modification by SUMO and subsequently facilitates release of E1B-55K from the nuclear matrix

(Lethbridge *et al.*, 2003). This may in part be linked to the impact of E4orf6 on E1B-55K´s capacity to bind PML during infection (Fig. 14; Fig. 15). Since PML-NBs have been proposed to represent the nuclear SUMOylation hotspot (2.2.2.1) (Van Damme *et al.*, 2010), it is therefore tempting to speculate that E4orf6 itself might facilitate the functions of PML and/or PML-associated proteins.

Therefore, H1299 cells were transiently transfected with constructs expressing human flag-tagged PML-isoforms plus E4orf6-wt and subjected to immunoprecipitation analysis (Fig. 16).

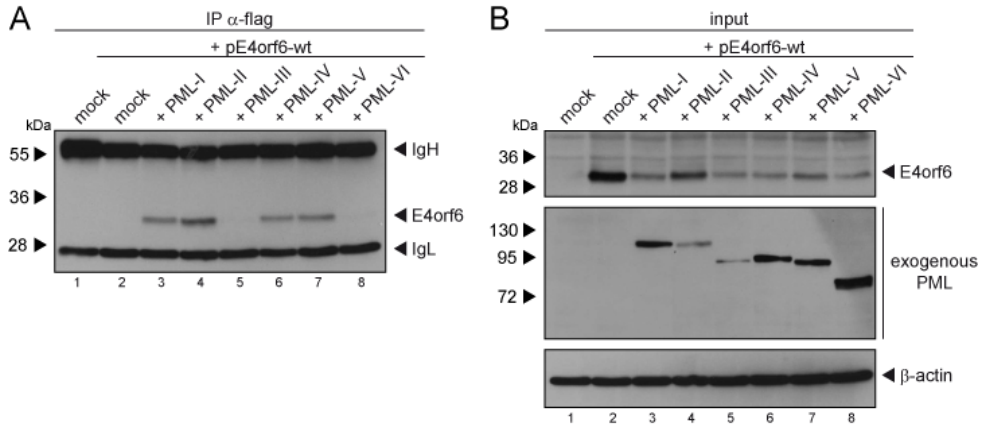

Figure 16. E4orf6 interacts specifically with PML-I/II/IV/V in transiently transfected H1299 cells. Subconfluent H1299 cells ($7.0x10^6$) were transfected with 10 µg of pE4orf6-wt plus 10 µg of different lentiviral constructs encoding N-terminal flag-tagged human PML-isoforms I-VI and harvested after 48 hours before preparing total cell extracts (4.5). Immunoprecipitation of flag-PML was performed using mAB flag-M2 (α-flag), resolved by 12% SDS-PAGE and visualized by immunoblotting (4.5). Co-precipitated proteins (A) and input levels (B) of total cell lysates were detected using mAb RSA3 (α-E4orf6), mAb flag-M2 (α-flag) and mAb AC-15 (α-β-actin). Molecular weights in kDa are indicated on the *left*, while corresponding proteins are labeled on the *right*.

As suspected, the adenoviral E4orf6 protein itself is intrinsic capable of specifically coprecipitating the PML-isoforms I, II, IV and V (Fig. 16; A; lanes 3/4/6/7), whereas no interaction could be detected with the isoforms III and VI (Fig. 16; A; lanes 5/8). These observations might also partially explain the previous results concerning E4orf6-dependent PML-I coprecipitation by E1B-55K during infection (Fig. 14; Fig. 15), since E4orf6 *per se* is intrinsic able to bind PML-I (Fig. 16; A; lane 3) in contrast to E1B-55K (Fig. 13). Since E4orf3 is the only adenoviral protein known so far to interact with PML, more specifically PML-II (Hoppe *et al.*, 2006; Leppard *et al.*, 2009), it is fascinating that most of the adenoviral pro-tumorigenic proteins (2.1.4.2) seem to interact with the cellular tumor suppressor PML.

5.2.6 Viral Infection Alters the PML Binding Capabilities of E4orf6 in H1299 Cells

Due to the shift in E1B-55K binding to PML during infection (Fig. 14), it seeems plausible that the same phenomenon may appear in the context of E4orf6 PML interactions. To evaluate whether the PML binding pattern of E4orf6 is altered during infection, H1299 cells were transiently transfected with constructs expressing human flag-tagged PML-isoforms, superinfected with wild-type virus (H5*pg*4100) and subjected to immunoprecipitation analysis (Fig. 17).

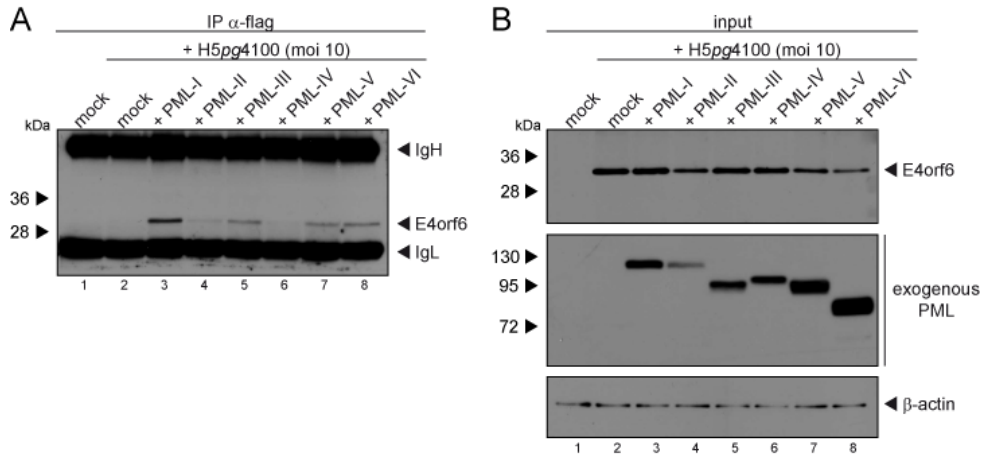

Figure 17. Viral infection alters the PML binding capabilities of E4orf6 in H1299 cells. Subconfluent H1299 cells ($7.0x10^6$) were transfected with 10 µg of different lentiviral constructs encoding N-terminal flag-tagged human PML-isoforms I-VI, infected 10 hours after transfection with wt virus (H5*pg*4100) at a multiplicity of 10 ffu per cell and harvested 36 h p.i. before preparing total cell extracts (4.5). Immunoprecipitation of flag-PML was performed using mAB flag-M2 (α-flag), resolved by 12% SDS-PAGE and visualized by immunoblotting (4.5). Coprecipitated proteins (A) and input levels (B) of total cell lysates were detected by using mAb RSA3 (α-E4orf6), mAb flag-M2 (α-flag) and mAb AC-15 (α-β-actin). Molecular weights in kDa are indicated on the *left*, while corresponding proteins are labeled on the *right*.

As anticipated, the E4orf6 PML binding pattern differs significantly from the pattern in transiently transfected H1299 cells (Fig. 16). Although E4orf6 retains the ability to coprecipitate with the PML-isoforms I and V (Fig. 17; A; lanes 3/7), interactions with the isoforms II and IV (Fig. 17; A; lanes 4/6) are drastically reduced compared to the transient transfection experiments (Fig. 16). Furthermore, the conserved PML-I interaction appears to be enhanced in the context of virus infection (Fig. 17; A; lane 3), which fits well with previous observations that the E1B-55K PML-I interaction during infection (Fig. 14) depends on the presence of E4orf6 (Fig. 15). Interestingly, E4orf6 also coprecipitated with PML-III and VI (Fig. 17; A;

lanes 5/8) in contrast to transient transfection (Fig. 16), indicating that these interactions rely on further viral proteins as seen before for E1B-55K (Fig. 14). Although both viral proteins E1B-55K and E4orf6 show a tendency to interact with PML-VI during infection (Fig. 14; Fig. 17), interaction with PML-III is exclusively detected with E4orf6 (Fig. 17; A; lane 5). It remains elusive how this can be achieved since neither viral protein possesses the intrinsic capability to coprecipitate these isoforms *per se* (Fig. 13; Fig. 16).

5.2.7 E1A-13S Interacts Specifically with PML-I/II/V in Transiently Transfected H1299 Cells

Interestingly, almost 15 years ago Carvalho and co-workers (Carvalho *et al.*, 1995) described an association of HAdV5 E1A and E4orf3 with nuclear matrix-associated PML-bodies, but subsequent studies have exclusively focused on E4orf3 PML interactions. In this context, it could be shown that E1A colocalizes with PML-NBs in HeLa cells during early stages of infection, although no direct interaction could be detected via immunoprecipitation using the E1A specific mouse antibody M73. More importantly, it appears that the CR2 region within both E1A isoforms (2.1.4.2.1) somehow participates in mediating the subcellular colocalization of PML and the viral protein. Although antibody M73 is frequently used in various studies to detect E1A, other more sophisticated antibodies have been developed, namely M58 (Harlow *et al.*, 1985) and 610 (kindly provided by R. Grand; University of Birmingham). In the light of previously shown results (Fig. 14; Fig. 15; Fig. 17), it is therefore tempting to speculate that E1A might represent another viral factor involved in modulating PML nuclear domains.

To closer evaluate whether E1A shows an interaction with specific PML-isoforms, H1299 cells were transiently transfected with constructs expressing human flag-tagged PML-isoforms plus chimeric HAdV2/5 E1A-12S or E1A-13S and subjected to immunoprecipitation analysis (Fig. 18).

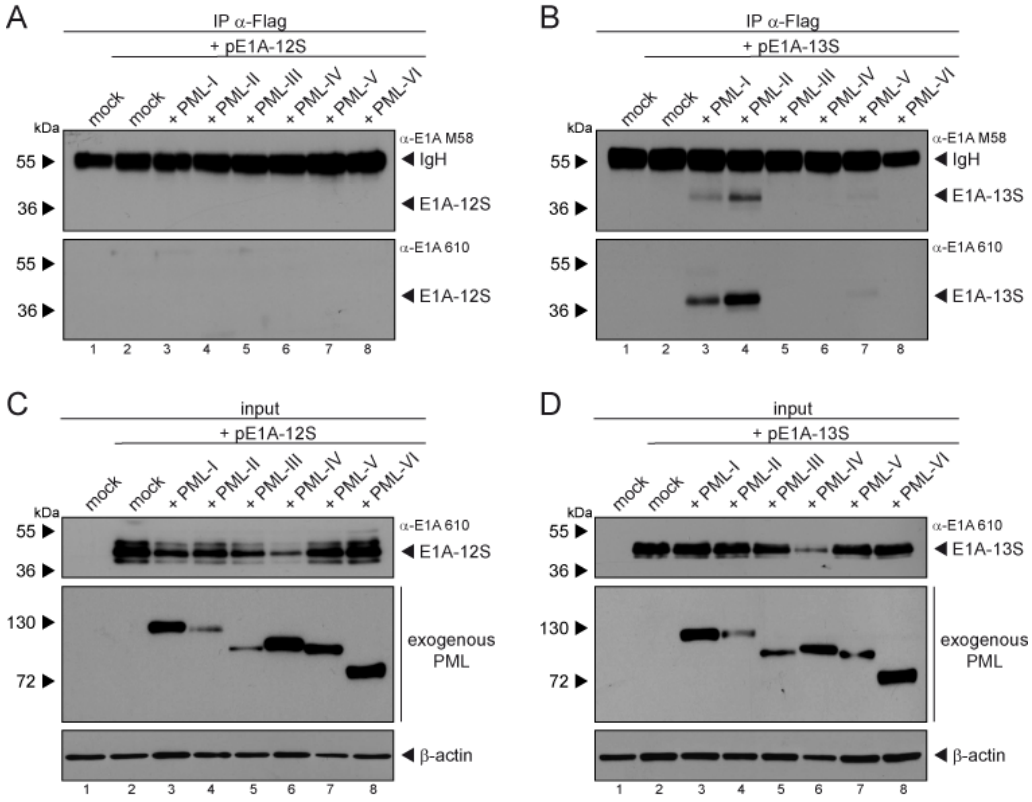

Figure 18. E1A-13S interacts specifically with PML-I/II/V in transiently transfected H1299 cells. Subconfluent H1299 cells ($7.0x10^6$) were transfected with 10 µg of HAdV2/5 chimeric pE1A-12S/13S plus 10 µg of different lentiviral constructs encoding N-terminal flag-tagged human PML-isoforms I-VI and harvested after 48 hours before preparing total cell extracts (4.5). Immunoprecipitation of flag-PML was performed using mAB flag-M2 (α-flag), resolved by 12% SDS-PAGE and visualized by immunoblotting (4.5). Coprecipitated proteins (A/B) and input levels (C/D) of total cell lysates were detected using mouse mAb M58 (α-E1A), rabbit pAb 610 (α-E1A), mAb flag-M2 (α-flag) and mAb AC-15 (α-β-actin). Representative E1A-12S/13S input levels are shown using rabbit pAb 610 (α-E1A); staining by mouse mAb M58 (α-E1A) shows comparable results (data not shown). Molecular weights in kDa are indicated on the *left*, while corresponding proteins are labeled on the *right*.

As implied by previous results (Carvalho *et al.*, 1995), the adenoviral E1A proteins show highly specific interactions with several isoforms of the human PML protein (Fig. 18). In this regard, it is fascinating that only E1A-13S (Fig. 18; B/D) could coprecipitate in significant amounts with PML-I and II (Fig. 18; B; lanes 3/4), although minor amounts were visible at longer exposure times with PML-V (data not shown). Due to using different antibody species for E1A detection (mouse mAb M58, rabbit pAb 610) after immunoprecipitation with mouse mAb flag-M2, another band representing the heavy immunoglobulin chain was also evident in Fig. 18 (B; panel 1 versus panel 2). Although the input levels show some variations for E1A-12S and exogenously expressed flag-PML (Fig. 18; C), it was not possible to detect any appreciable coprecipitation of E1A-12S with the different PML-isoforms (Fig. 18; A). However, maximal exposure (~1 hour) reveals some minor coprecipitation of E1A-12S with PML-

I and II (data not shown), strongly indicating considerably reduced affinity of E1A-12S for the same PML-isoforms as E1A-13S (Fig. 18; B). These results are in part consistent with already published data and may provide the molecular evidence for E1A PML colocalization during early stages of infection in HeLa cells (Carvalho *et al.*, 1995). Interestingly, the sequence differences between E1A-12S and E1A-13S further suggest that CR3 (Fig. 18; A/B) (2.1.4.2.1), as well as to a lesser extent CR2 (Fig. 18; A/B) (Carvalho *et al.*, 1995), is important for efficient interaction with certain PML-isoforms.

So far, E4orf3 has been the only adenoviral protein described to physically interact with PML, more precisely PML-II (Leppard & Everett, 1999; Hoppe *et al.*, 2006; Leppard *et al.*, 2009), inducing the formation of so-called *track-like structures* in the nucleus of cells (2.1.4.2.3) (Carvalho *et al.*, 1995; Puvion-Dutilleul *et al.*, 1995; Doucas *et al.*, 1996). This reorganization of *PML nuclear bodies* is highly conserved among most species of HAdVs (Stracker *et al.*, 2005), therefore suggesting an important function during adenoviral infection, presumably by mediating elimination of intracellular viral defense barriers (2.2.2.3) (Doucas *et al.*, 1996; Everett, 2001; Everett & Chelbi-Alix, 2007; Ullman *et al.*, 2007; Tavalai & Stamminger, 2008; Ullman & Hearing, 2008). Although it remains elusive as to whether the interaction of E1A (Fig. 18), E1B-55K (Fig. 13) and E4orf6 (Fig. 16) is also conserved between different human adenovirus species, it is fascinating that all adenoviral proteins described as being essential and/or supportive for cellular transformation of primary rodent cells do in fact interact with specific isoforms of the tumor suppressor protein PML.

5.3 Adenoviral Oncogenes of the E1 and E4 Region Associate with Endogenous PML during Viral Infection

Taken together, the results here indicate that adenoviral proteins may modulate PML as well as PML-associated proteins on multiple levels to facilitate efficient virus progeny production *in vivo* and presumably to induce cellular transformation of primary rodent cells *in vitro*. Since it was previously reported that PML expression is frequently deregulated in human tumor cell lines and/or during oncogenesis (Fig. 12) (Koken *et al.*, 1995; Salomoni & Pandolfi, 2002; Gurrieri *et al.*, 2004a; Gurrieri *et al.*, 2004b; Scaglioni *et al.*, 2006; Salomoni *et al.*, 2008), we decided to use "primary" human hepatocytic cells (HepaRG) (Gripon *et al.*, 2002; Cerec *et al.*, 2007). These cells express PML in well-defined physiological amounts

(Condemine *et al.*, 2006) and represent a suitable system to analyze endogenous PML components during viral infection (Everett *et al.*, 2008; Lukashchuk & Everett, 2010).

5.3.1 E1B-55K and E4orf6 Interact with Endogenous PML during Viral Infection of HepaRG Cells

To further evaluate the association of adenoviral proteins with exogenously expressed PML-isoforms, as well as putative involvement of SUMOylation of E1B-55K, immunoprecipitation analysis of total cell lysates was performed to determine whether the viral proteins interact with endogenous PML during virus infection of HepaRG cells (Fig. 19). Human HepaRG cells were infected with wt virus (H5*pg*4100), E1B-55K null mutant H5*pm*4149 (Kindsmüller *et al.*, 2009), H5*pm*4102 (Kindsmüller *et al.*, 2007), H5*pm*4101 (Kindsmüller *et al.*, 2007) and the E4orf6 null mutant H5*pm*4154 (Blanchette *et al.*, 2008) before being subjected to immunoprecipitation analysis (Fig. 19).

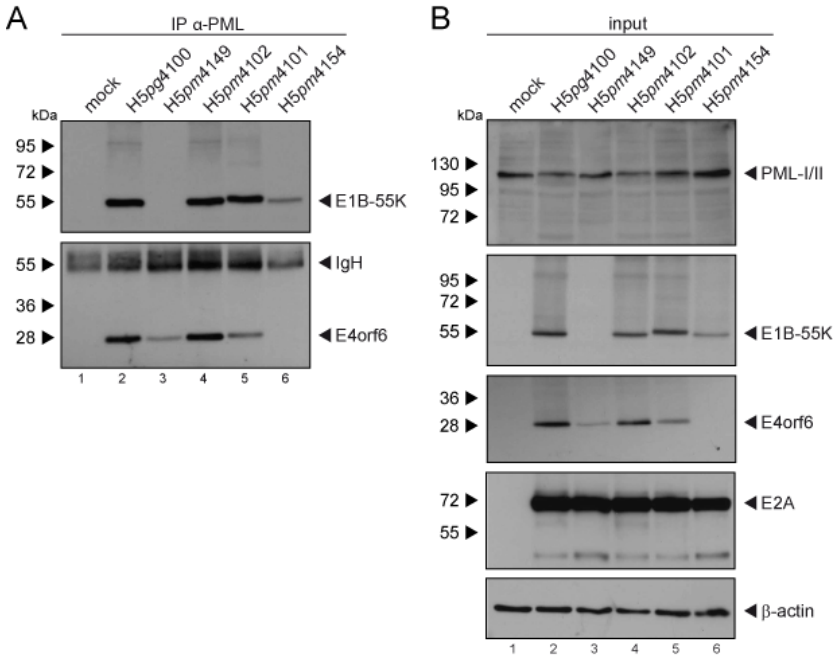

Figure 19. HAdV5 E1B-55K and E4orf6 interact with endogenous PML during viral infection of HepaRG cells. Human HepaRG cells (15.0×10^6) were infected with wt (H5*pg*4100) or mutant (H5*pm*4149, H5*pm*4102, H5*pm*4101, H5*pm*4154) viruses at a multiplicity of 200 ffu per cell and harvested 36 h p.i. before preparing total cell extracts (4.5). Immunoprecipitiation of PML was performed using pAb α-PML (Novus Biologicals) resolved by 10% SDS-PAGE/12% SDS-PAGE for E4orf6 and visualized by immunoblotting (4.5). Coprecipitated proteins (A) and input levels (B) of total cell lysates were detected using mAb 2A6 (α-E1B-55K), mAB RSA3 (α-E4orf6), pAb H-238 (α-PML), mAb B6-8 (α-DBP) and mAB AC-15 (α-β-actin). Molecular weights in kDa are indicated on the *left*, while corresponding proteins are labeled on the *right*. Abbr.: H5*pg*4100: wt virus; H5*pm*4149: E1B-55K null

mutant virus; H5*pm*4102: E1B-55K-K104R virus; H5*pm*4101: E1B-55K-NES virus; H5*pm*4154: E4orf6 null mutant virus (3.2).

Visualization of the *viral DNA binding protein* (DBP; E2A-72K) shows comparable expression levels in all infected samples, indicating equal infection with the different virus mutants (Fig. 19; B; panel 4). The β-actin loading control documents equal amounts of analyzed protein *per se* (Fig. 19; B; panel 5). Thus, the reduced amounts of E1B-55K/E4orf6 can be attributed to the corresponding mutations within the different virus mutants (Fig. 19; B; panel 2; lane 5)/(Fig. 19; B; panel 3; lanes 3/5). In comparison, coimmunoprecipitation of E1B-55K and E4orf6 by human endogenous PML reveals interactions in all different virus mutants except their respective null mutants (Fig. 19; A). Correlating with the respective input levels (Fig. 19; B; panels 2/3), lower amounts of E1B-55K or E4orf6 could be coprecipitated using H5*pm*4154 (Fig. 19; A; panel 1; lane 6) or H5*pm*4149/H5*pm*4101 (Fig. 19; A; panel 2; lanes 3/5), whereas no differences were observed between E1B-55K-SCM and E1B-55K-NES (Fig. 19; A; panel 1; lanes 4/5). In summary, E1B-55K and E4orf6 interact with endogenous PML during infection of HepaRG cells and mutations within the SCM and NES of E1B-55K do not influence the intrinsic capability of E1B-55K to interact with PML.

5.3.2 Subnuclear Colocalization of E1B-55K with Endogenous PML during Infection of HepaRG Cells Is Dependent on a Functional SCM

Inhibition of CRM1-mediated nuclear export by LMB, or equivalently, mutational inactivation of the E1B-55K NES results in enhanced SUMO-1 modification and relocalization to PML containing nuclear bodies, whereas functional inactivation of E1B-55K SCM exhibits completely the opposite phenotype (2.1.4.2.2) (Endter *et al.*, 2001; Endter *et al.*, 2005; Kindsmüller *et al.*, 2007). It is thus tempting to speculate that E1B-55K SUMOylation might play an integral role in subnuclear localization during infection.

Therefore, HepaRG cells were infected with wt virus (H5*p*g4100), E1B-55K null mutant H5*pm*4149 (Kindsmüller *et al.*, 2009), or previously described H5*pm*4102/H5*pm*4101 virus harboring mutations within the SCM/NES of E1B-55K (Kindsmüller *et al.*, 2007).

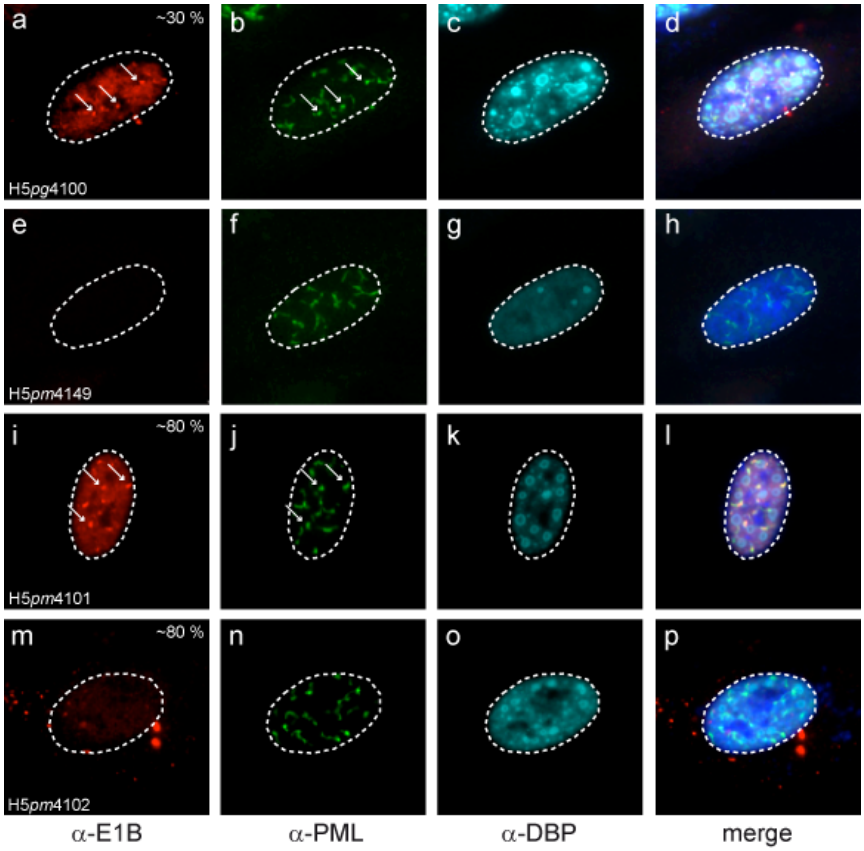

Figure 20. Subnuclear Colocalization of E1B-55K with endogenous PML during infection of HepaRG cells is dependent on a functional SCM. Human HepaRG were infected with wt (H5*pg*4100) or mutant (H5*pm*4149, H5*pm*4102, H5*pm*4101) viruses at a multiplicity of 50 ffu per cell and fixed with methanol 24 h p.i.. Samples were triple labeled *in situ* with mAb 4E8 (α-E1B-55K), mAB B6-8 (α-DBP) and pAb H-238 (α-PML) (4.5). Primary antibodies were detected with Cy3-, Cy5- or FITC-conjugated secondary antibodies (4.5.7). Representative α-E1B-55K (red; Aa, Ae, Ai, Am), α-DBP (turquoise; Ac, Ag, Ak, Ao) and α-PML (green; Ab, Af, Aj, An) staining patterns of at least 50 analyzed cells are shown. Overlays of the single images (merge) are shown in Ad, Ah, Al and Ap. In all panels, nuclei are indicated by a dotted line. E1B-55K and PML positive nuclear accumulations are indicated by white arrows. (Magnification x7600)

As expected, PML is relocalized in virus infected cells (Fig. 20; b, f, j, n) by E4orf3 into the respective *track-like structures* (Carvalho *et al.*, 1995; Puvion-Dutilleul *et al.*, 1995; Doucas *et al.*, 1996). E1B-55K shows a more complex localization pattern (Ornelles & Shenk, 1991); however a subpopulation of about 30% of infected HepaRG cells exhibit small nuclear accumulations of the viral protein (Fig. 20; a). These E1B-55K aggregates colocalize with a fraction of redistributed PML protein in globular accumulations that do not form the same *track-like structure* (Fig. 20; a-d; white arrows). In contrast, infection with H5*pm*4101 resulted in complete accumulation of E1B-55K-NES within the nucleus of infected cells and exhibited a comparable phenotype in terms of localization of nuclear E1B-55K accumulations and PML in >80% of the cells (Fig. 20; i-l; white arrows). Interestingly, functional inactivation of the E1B-

55K SCM completely abolished the formation of E1B-55K positive nuclear accumulations (Kindsmüller *et al.*, 2007) and also colocalization with PML (Fig. 20; m-p). Therefore, it can be concluded that proper localization of E1B-55K mediated by the NES and SCM is essential for nuclear colocalization with endogenous PML during adenoviral infection of human HepaRG cells. In contrast, no comparable results could be obtained for E4orf6, since this viral protein is diffusely distributed throughout the nucleus and thus shows no nuclear accumulations in standard immunofluorescence analysis (4.5.7).

Taken together, it appears that both E1B-55K and E4orf6 can be coprecipitated with human endogenous PML during viral infection using highly stringent immunoprecipitation conditions (Fig. 19) (4.5), which furthermore underlines the reliability of the previously transient transfection experiments (Fig. 13; Fig. 16). Since E1B-55K-K104R and E1B-55K-NES could be coprecipitated in comparable amounts to E1B-55K-wt from total cell lysates (Fig. 19; A), it appears that mutations within these regions do not necessarily abolish the intrinsic capability of E1B-55K to bind endogenous PML during infection (Fig. 19; A) despite their influence on nuclear localization of the viral protein (Fig. 20). Unfortunately, these results do not illustrate specific endogenous coprecipitated PML-isoforms, since respective experiments remain inconclusive so far due to insufficiently strong PML antibodies. However, these results together with the observations concerning interactions of E1B-55K with endogenous rat PML (Fig. 11) strongly suggest an important role of PML during adenoviral cell transformation and/or infection. Moreover these processes might somehow involve posttranslational modification of E1B-55K by SUMO (Fig. 10; Fig. 11; Fig. 20).

5.4 E1B-55K PML Interaction is Mediated by Distinct Mechanisms Involving Posttranslational Modification by SUMO

The adenoviral E1B-55K protein facilitates multiple functions during productive viral infection *in vivo* and/or during cellular transformation of primary rodent cells *in vitro* (2.1.4.2.2). Although many of these aspects require additional viral factors such as E4orf6 (2.1.4.2.3) and/or E1A (2.1.4.2.1) for achievment of optimal efficiency, the detailed molecular mechanisms as well as the cellular components involved remain largely unknown. In this context, it appears likely that access to the PML protein network via modulation of specific isoforms and subsequently corresponding downstream factors, might offer a new perspec-

tive to deconstructing E1B-55K functions (Van Damme *et al.*, 2010). Therefore, the interaction of viral proteins with specific PML-isoforms and especially the binding regions involved represent important questions to be answered.

5.4.1 Posttranslational Modification of E1B-55K by SUMO Regulates Interaction with PML-IV in Transiently Transfected H1299 Cells

Since it is well know that SUMOylation plays an essential role in the formation of PML-NBs and recruitment/regulation of PML-NB-associated proteins (Shen *et al.*, 2006; Van Damme *et al.*, 2010), besides the obvious involvement of SUMOylation for E1B-55K function (2.1.4.2.2) (Endter *et al.*, 2001; Endter *et al.*, 2005), it appears plausible that this posttranslational modification of the viral protein might also influence E1B-55K PML interaction *per se*.

Therefore, H1299 cells were transfected with plasmids encoding E1B-55K-wt, E1B-55K-V103D, E1B-55K-K104R, E1B-55K-NES and either human PML-isoform IV or isoform V before being subjected to immunoprecipitation analysis (Fig. 21).

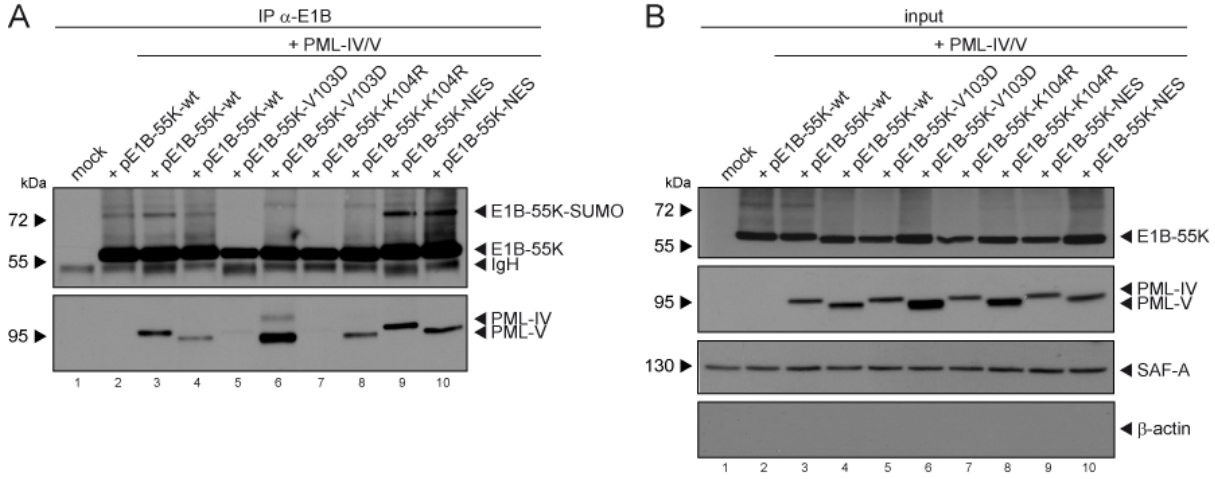

Figure 21. Posttranslational modification of E1B-55K by SUMO regulates binding to PML-IV in transiently transfected H1299 cells. Subconfluent H1299 cells ($7.0x10^6$) were transfected with 10 µg of pE1B-55K-wt/V103D/-K104R/-NES plus 10 µg of different lentiviral constructs encoding N-terminal flag-tagged human PML-isoforms IV (lanes 3/5/7/9) or V (lanes 4/6/8/10) and harvested after 48 hours before preparing total cell extracts (4.5). Immunoprecipitation of E1B-55K from the nuclear fraction (Lee *et al.*, 1995) was performed using mAB 2A6 (α-E1B-55K), resolved by 10% SDS-PAGE and visualized by immunoblotting (4.5). Coprecipitated proteins (A) and input levels (B) of total cell lysates were detected using mAb 2A6 (α-E1B-55K), mAb flag-M2 (α-flag), pAb α-SAF-A and mAB AC-15 (α-β-actin). Molecular weights in kDa are indicated on the *left*, while corresponding proteins are labeled on the *right*.

Direct immunoprecipitation of E1B-55K shows a sufficient and comparable amount of the viral protein precipitated in all samples (Fig. 21; A; panel 1). As expected, SUMOylation of

E1B-55K appears most prominent upon mutation of the NES (Fig. 21; A; panel 1; lanes 9/10), whereas no covalent modification was detected with E1B-55K harboring the V103D or K104R mutation within the ψ-K-x-E motif (Fig. 21; A; panel 1; lanes 5/6/7/8) (Endter *et al.*, 2001; Endter *et al.*, 2005). Since the SUMOylation deficient mutants are substantially impaired in coimmunoprecipitating PML-IV (Fig. 21; A; panel 2; lanes 5/7), it can be assumed that SUMOylation of E1B-55K is a major determinant of binding to PML-IV. Intriguingly, E1B-55K PML-IV interaction does not exclusively depend on this posttranslational modification, since longer exposures revealed some residual amounts of coprecipitated PML-IV (data not shown), indicating at least one other minor determinant is involved in this process.

In contrast, PML-V coprecipitation seems to be mostly independent of E1B-55K SUMO modification (Fig. 21; A; panel 2; lanes 4/6/8/10), although some variations can be seen correlating with the protein input levels. These results are consistent with previous data (Fig. 19) showing no observable difference in E1B-55K-K104R/E1B-55K-NES coprecipitation by endogenous PML, since PML-IV binding is not evident during infection (Fig. 14), presumably due to E4orf6 expression (Lethbridge *et al.*, 2003). In summary, it appears that the interaction of E1B-55K with PML-IV depends on SUMOylation of the viral protein, whereas binding to PML-V mainly occurs in a SUMO-independent manner.

5.4.2 Mutations within the C-Terminus of E1B-55K Abolish Efficient Isoform-Specific PML Interaction in Transiently Transfected H1299 Cells

In parallel, more than thirty different E1B-55K mutants were screened via immunoprecipitation for their ability to interact with PML-IV/V in transiently transfected H1299 cells (data not shown). Although, the E1B-55K PML-IV interaction seems to be linked to SUMOylation of the viral protein (Fig. 21), it is worth noting that this posttranslational modification is not the only determinant involved. In fact, several mutants mainly clustering at the C-terminus of E1B-55K could be identified as being significantly impaired in PML-IV/V interaction (Fig. 23), whereas the previously described mutation H354 is exceptional since it is localized in the central region of E1B-55K (Fig. 22) (Yew *et al.*, 1990; Yew & Berk, 1992).

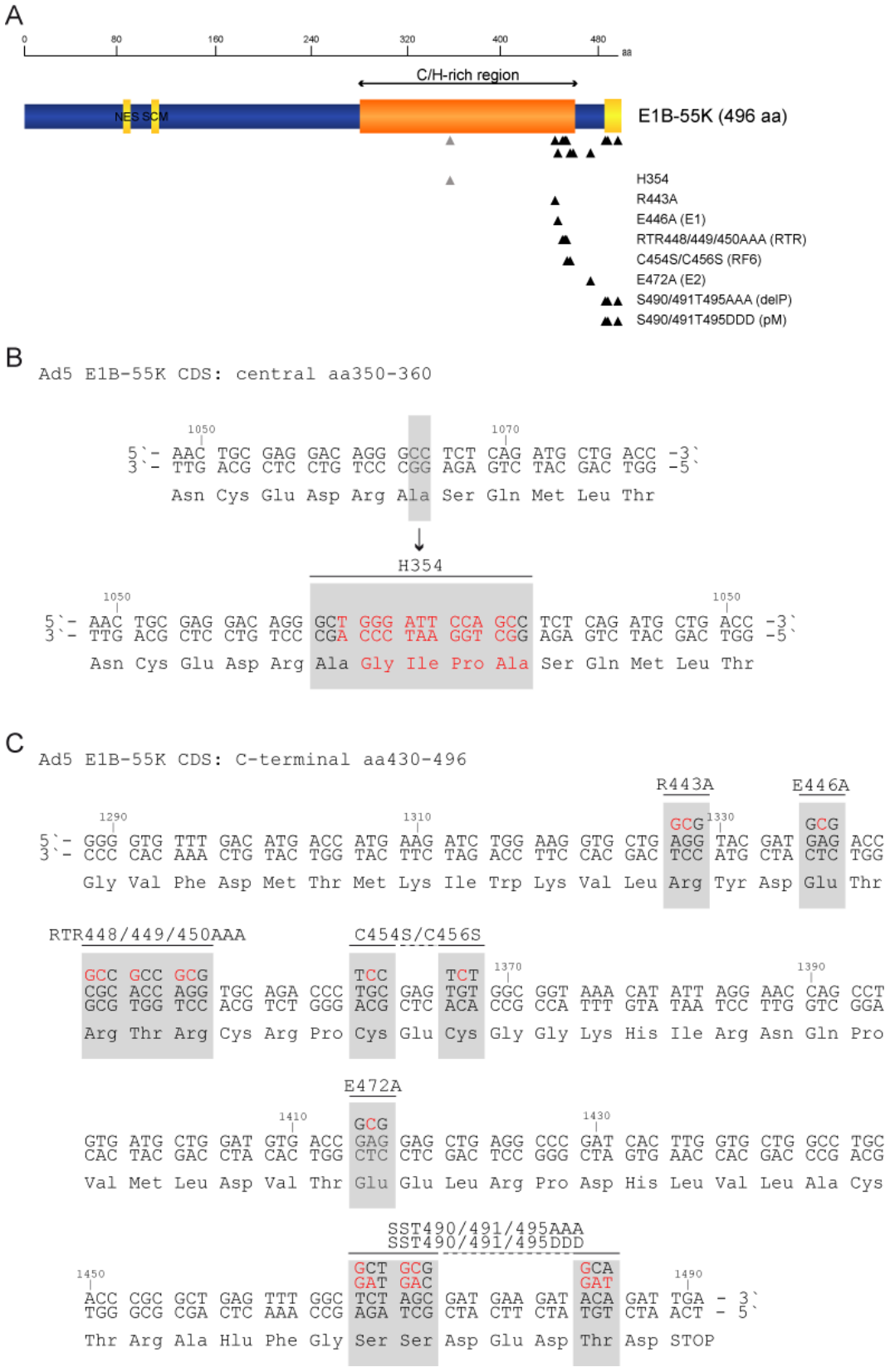

Figure 22. Mutations within HAdV5 E1B-55K influencing isoform-specific PML interaction. (A) The schematic overview shows the coding sequence of HAdV5 E1B-55K (AY339865) including the specific mutations influencing isoform-specific PML interactions. Insertion (grey) and point (black) mutations are illustrated by triangles and mutation names are given according to original aa, position

and substituted aa, including abbreviated names in brackets. Section B illustrates the previously published insertion mutant H354 (Yew *et al.*, 1990; Yew & Berk, 1992) within the central region of E1B-55K (aa354). Section C illustrates previously published point mutants C454S/C456S (abbr. RF6) (Härtl *et al.*, 2008), SST490/491/495AAA (abbr. delP) (Teodoro *et al.*, 1994; Teodoro & Branton, 1997) plus newly established point mutants R443A, E446A (abbr. E1), RTR448/449/450AAA (abbr. RTR), E472A (abbr. E2) and SST490/491/495AAA (abbr. pM). Nucleotide/amino acid positions are indicated relative to the E1B-55K sequence and mutations are highlighted in red color. Abbr.: C/H-rich region: *cysteine/histidine-rich region*; NES: *nuclear export signal*; SCM: *SUMO-1 conjugation motif*.

Some of the E1B-55K mutants in Fig. 22 have been published previously, due to specific phenotypes, in particular their ability to repress p53 and oncogenic potential in combination with E1A. Whereas results concerning the transformation potential of E1B-55K-H354 are conflicting (Yew *et al.*, 1990; Yew & Berk, 1992), E1B-55K-delP was constructed to completely abolish C-terminal phosphorylation of E1B-55K (Fig. 6) and subsequently the ability to repress p53-mediated transcriptional activation as well as transformation of primary rodent cells (Teodoro *et al.*, 1994; Teodoro & Branton, 1997). In contrast, the E1B-55K-pM mutant was generated in this study to mimic constitutive phosphorylation at the far C-terminus of E1B-55K and so far remains uncharacterized (see 5.5). Both mutants influencing E1B-55K phosphorylation were included in subsequent experiments, since phosphorylation is suspected to possibly influence SUMOylation of E1B-55K (see 5.5) and therefore PML interaction (Fig. 21).

Although E1B-55K-RF6 has been shown to exhibit a phenotype comparable to E1B-55K-wt concerning repression of p53, it has been proposed that other mechanisms of transformation must exist, since the RF6 mutation eliminates E1B-55K transformation potential, despite retaining the potential to repress p53 transactivation – a function believed to be the prerequisite for efficient cellular transformation by the viral oncogene (Härtl *et al.*, 2008). Interestingly, mutations R443A, E1, RTR and E2 were initially constructed to disrupt E1B-55K Daxx interaction, since they fit previously described Daxx interaction motifs (Schreiner, 2010). However, subsequent studies have shown that only the E2 mutation is sufficient to abolish Daxx interaction (Schreiner, 2010). Taken together, four mutants influencing posttranslational modification (phosphorylation: delP, pM; SUMOylation: K104R, NES) and six mutants described as being linked to PML-NB-associated proteins (H354, R443A, E1, RTR, RF6, E2) were used in subsequent experiments to determine the explicit requirements for PML-IV/V specific interactions of E1B-55K.

Therefore, H1299 cells were transfected with plasmids encoding E1B-55K-wt, E1B-55K-K104R, E1B-55K-NES, E1B-55K-delP, E1B-55K-pM, E1B-55K-H354, E1B-55K-R443A, E1B-

55K-E1, E1B-55K-RTR, E1B-55K-RF6, E1B-55K-E2 and either human PML-isoforms IV or isoform V before being analyzed by immunoprecipitation (Fig. 23).

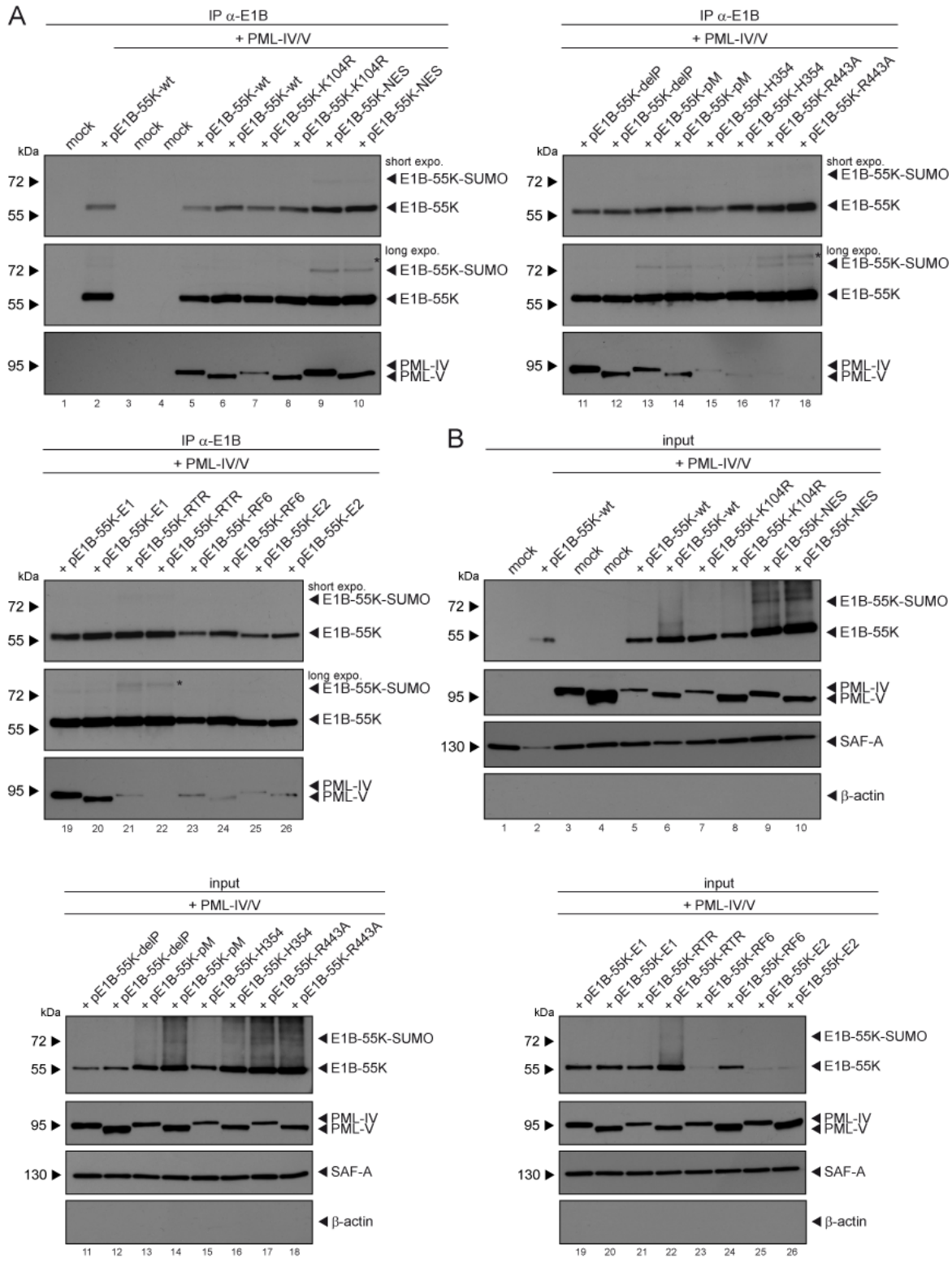

Figure 23. Mutations within the C-terminus of E1B-55K abolish efficient isoform-specific PML interactions in transiently transfected H1299 cells. Subconfluent H1299 cells ($7.0x10^6$) were trans-

fected with 10 µg of pE1B-55K-wt/-K104R/-NES/-delP/-pM/-H354/-R443A/-E1/-RTR/-RF6/E2 (Fig. 22) plus 10 µg of different lentiviral constructs alternatively encoding N-terminal flag-tagged human PML-isoforms IV (odd numbered lanes) or V (even numbered lanes) and harvested after 48 hours before preparing total cell extracts (4.5). Immunoprecipitation of E1B-55K from the nuclear fraction (Lee *et al.*, 1995) was performed using mAB 2A6 (α-E1B-55K), resolved by 10% SDS-PAGE and visualized by immunoblotting (4.5). Coprecipitated proteins (A) and input levels (B) of the nuclear fraction were detected using mAb 2A6 (α-E1B-55K), mAb flag-M2 (α-flag), pAb α-SAF-A and mAB AC-15 (α-β-actin). Specific E1B-55K bands are highlighted (*) and discussed in more detail in the text. Molecular weights in kDa are indicated on the *left*, while corresponding proteins are labeled on the *right*.

Mutational analysis of E1B-55K is frequently hampered by considerably reduced protein stability even upon the slightest change in selected amino acids within the primary sequence. It is therefore fortunate that most of the E1B-55K mutants, such as E1B-55K-delP (Fig. 23; B; panel 1; lanes 11/12), exhibit only slight reductions in protein stability compared to the wt prtein (Fig. 23; B; panel 1; lanes 2/5/6) considering the variability of transient transfection experiments. However, E1B-55K-RF6 as well as E1B-55K-E2 (Fig. 23; B; panel 1; lanes 23-26) show significantly reduced amounts of steady state protein expression in this experiment, which might be due to low transfection efficiency and/or reduced protein stability. As expected, no significant amounts of the cytoplasmic marker β-actin could be detected in the nuclear fraction, whereas staining for the nuclear SAF-A protein as well as transfected PML-IV/V indicate comparably uniform amounts of protein in all samples (Fig. 23; B; panels 3/4).

To eliminate the flaw of differentially expressed E1B-55K protein levels, E1B antibody-coupled protein A-beads were used in substoichiometric amounts to enforce complete saturation of the beads and therefore equal amounts of directly precipitated E1B-55K. As shown in Fig. 23 (A; panels 1/2) comparable amounts of E1B-55K could be directly precipitated in all samples and only minimal variations were seen with the E1B-55K-RF6 or E1B-55K-E2 mutants discussed above (Fig. 23; A; panels 1/2; lanes 23-26). Interestingly, these results further confirm previous experimental observations (data not shown) revealing another E1B-55K specific band migrating close to posttranslationally SUMOylated E1B-55K (Fig. 23; A; panels 1/2; lanes 10/18/22; *), which can be seen in almost all mutants at longer exposures (data not shown). This observation is fascinating since it may suggest the existence of another so far unknown posttranslational modification of E1B-55K.

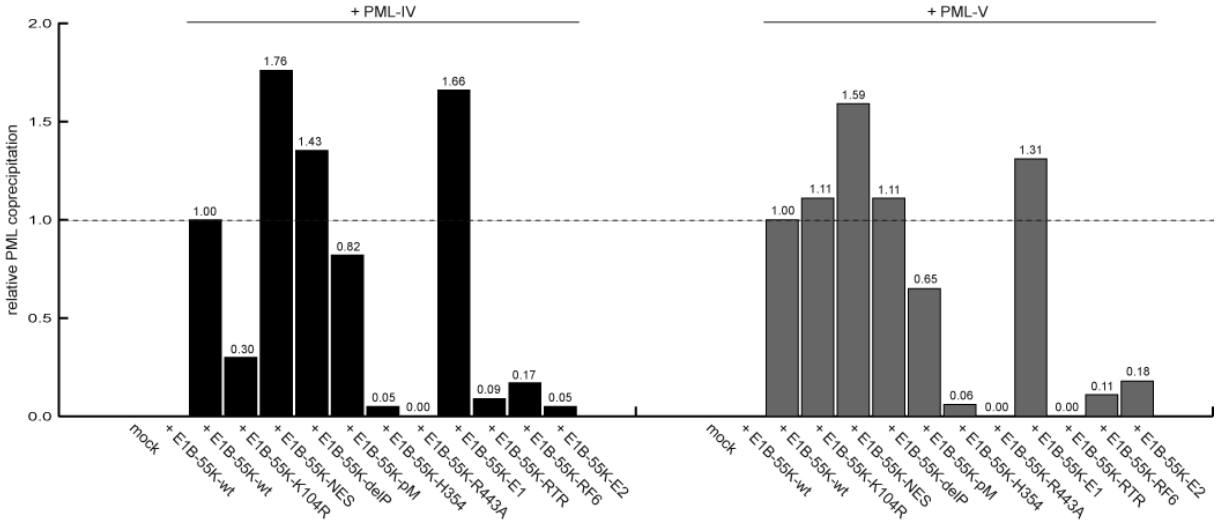

Figure 24. Mutations within the C-terminus of E1B-55K abolish efficient isoform-specific PML interaction in transiently transfected H1299 cells. The band intensities of coprecipitated PML-IV (left panel) and PML-V (right panel) from Fig. 23 (A) were quantified using the *GeneSnap™* software (SynGene) and normalized to E1B-55K-wt coprecipitated PML-IV (Fig. 22, A; panel 3; lane 5) or PML-V (Fig. 22, A; panel 3; lane 6) indicated by a dotted line across the bar graphs.

Although these results clearly highlight several PML binding deficient mutants of the viral E1B-55K protein, interpretation of the results is complicated, since multiple factors must be taken in account. First, consistent with previous results, PML-IV coprecipitation of E1B-55K shows a correlation with posttranslational SUMO modification of the viral protein (Fig. 21). In this context, PML-IV coprecipitation of E1B-55K-K104R (Fig. 23; A; panel 3; lane 7) (Fig. 24) is significantly reduced, whereas E1B-55K-NES shows a tendency to interact with higher amounts of PML-IV (Fig. 23; A; panel 3; lane 9) (Fig. 24) correlating with the enhanced SUMOylation of the viral protein (Fig. 23; A; panel 2; lane 9). Despite the obvious caveats of quantifying immunoprecipitation results in fluctuating transient transfection experiments *per se*, these observations repeatedly underline that PML-IV interaction with E1B-55K is not exclusively dependent on E1B-55K SUMOylation, but also involves additional so far unknown determinants (5.4.1).

Second, several mutants including E1B-55K-H354, E1B-55K-R443A, E1B-55K-RTR, E1B-55K-RF6 and E1B-55K-E2 are significantly impaired in PML-IV as well as PML-V coprecipitation (Fig. 23; A; panel 3) (Fig. 24), indicating that PML binding occurs in the C-terminal region of E1B-55K (Fig. 22; A). Intriguingly, most of these mutations cluster between aa 443-474, strongly suggesting that the C-terminus of E1B-55K is essential for efficient E1B-55K PML interaction. However, in this context E1B-55K-H354 is exceptional since the mutation is quite distant from the identified C-terminal region harboring the mutations between aa 443-

447 (Fig. 22; A). Therefore, it is possible that multiple regions within the C/H-rich region of E1B-55K (Fig. 6) participate in PML binding. However, the fact that H354 represents the only insertion mutant introducing four additional amino acids (Fig. 22; B) additionally suggests possible conformational changes within the C/H-rich region and/or the proposed zinc finger (Fig. 6) (Flint & Gonzalez, 2003) and subsequent loss of efficient PML binding (Fig. 23; A; panel 3; lanes 15/16) (Fig. 24). Interestingly, the compendium of relative PML coprecipitaion by E1B-55K (Fig. 24) illustrates that both PML-isoforms seem to require the same interaction region within the E1B-55K primary sequence, despite the SUMO dependency of E1B-55K PML-IV interactions.

Third, previously shown results concerning the binding of the PML-NB-associated protein Mre11/Daxx, which is completely abrogated by the RF6/E2 mutation (Härtl, 2005; Härtl *et al.*, 2008; Schreiner, 2010), add another layer of complexity to the results here. In this context, it remains elusive as to how and to what extent E1B-55K PML interactions are additionally modulated/facilitated by these previously described protein interactions occurring at the C-terminus of the viral protein.

5.4.3 E1B-55K Interaction with PML-IV/V is Dispensable for p53 Interaction and Repression in Transiently Transfected H1299 Cells

Cellular transformation of primary rodent cells by the adenoviral oncogenes E1A and E1B-55K is a complex multi-step process predominantly involving modulation of the cellular tumor suppressor protein p53 (2.1.4.2) (Farmer *et al.*, 1992; Yew *et al.*, 1994; Nevels *et al.*, 1997; Martin & Berk, 1998; Martin & Berk, 1999; Liu *et al.*, 2000). Direct interaction (Sarnow *et al.*, 1982a; Kao *et al.*, 1990), transcriptional repression (Yew *et al.*, 1994; Martin & Berk, 1998; Martin & Berk, 1999) and nuclear-cytoplasmic relocalization (Endter *et al.*, 2001; Endter *et al.*, 2005) of p53 by the adenoviral E1B-55K protein induces complete silencing of p53-dependent tumor suppressive functions. Interestingly, previously published data show the necessity of a so far unknown cellular corepressor for efficient p53-repression by E1B-55K (Yew *et al.*, 1994; Martin & Berk, 1998; Martin & Berk, 1999; Punga & Akusjärvi, 2000). Although p53 inactivation by E1B-55K is believed to play the major role in cellular transformation, other p53-independent mechanisms have been proposed (Nevels *et al.*, 2001; Sieber & Dobner, 2007; Härtl *et al.*, 2008; Schreiner, 2010).

Therefore, the identified E1B-55K mutants shown to be impaired in PML-IV/V binding (Fig. 22; Fig. 23; Fig. 24) were analyzed in a transient reporter gene assay for their capability to repress p53-mediated transcription (Fig. 25). H1299 cells were transfected with plasmids encoding E1B-55K-wt, E1B-55K-K104R, E1B-55K-NES, E1B-55K-delP, E1B-55K-pM, E1B-55K-H354, E1B-55K-R443A, E1B-55K-E1, E1B-55K-RTR, E1B-55K-RF6 and E1B-55K-E2 plus a p53 expression plasmid and a p53-independent as well as p53-dependent luciferase construct for a normalized system read-out (4.6).

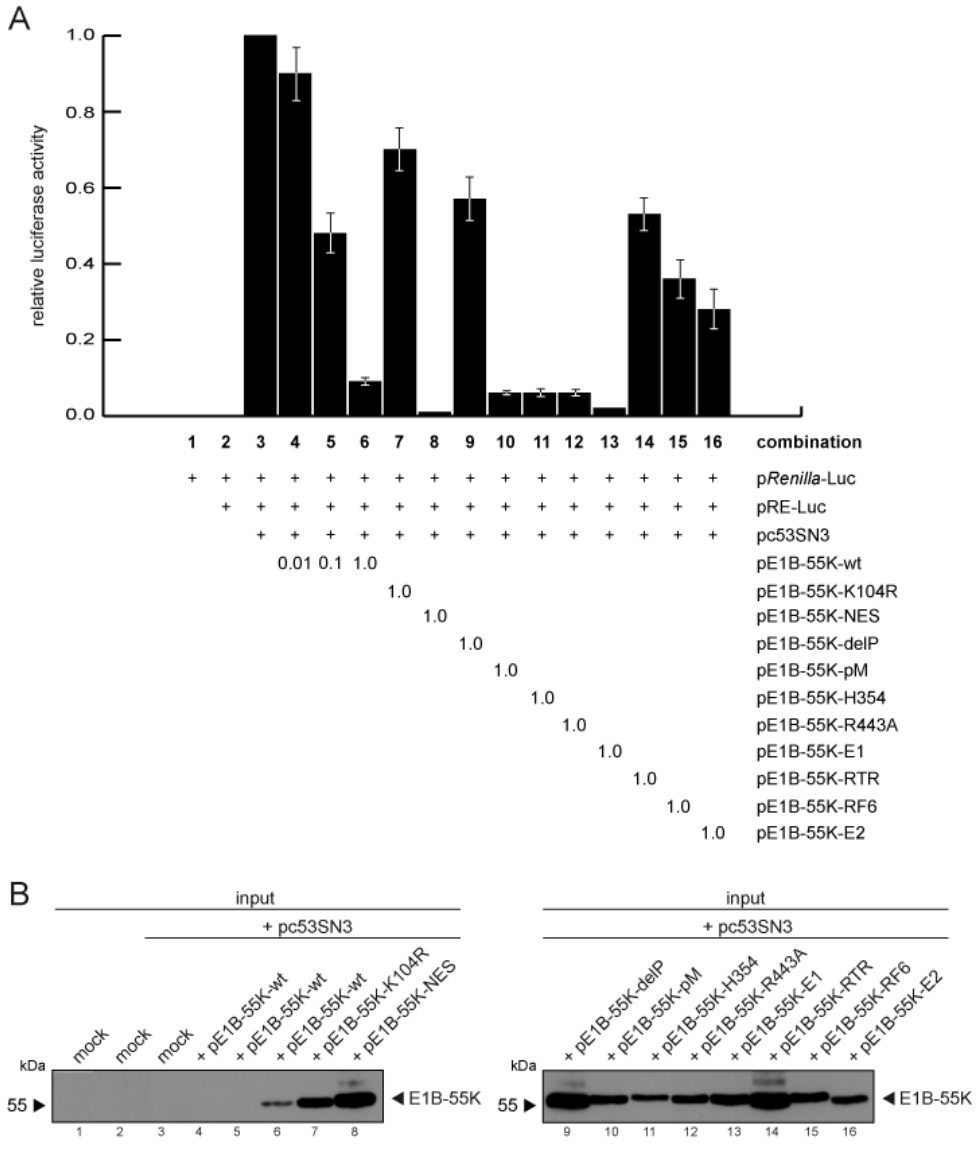

Figure 25. E1B-55K interaction with PML-IV/V is dispensible for p53-repression in transiently transfected H1299 cells. Subconfluent H1299 cells ($3.5x10^5$) were transfected with 1.0 µg of p*Renilla*-Luc, 0.25 µg of pRE-Luc, 0.02 µg of pc53SN3, pE1B-55K-wt/-K104R/-NES/-delP/-pM/-H354/-R443A/ -E1/-RTR/-RF6/E2 (Fig. 22) plus empty vector pcDNA3 to a total amount of 2.5 µg DNA. The amount of transfected E1B-55K expression plasmid is shown in µg. The cells were harvested after 48 hours according to the manufacture´s instructions using the *Dual-Luciferase® Reporter Assay System*

(Promega) and subsequently measured in a *Lumat LB 9507 luminometer* (Berthold Technologies) (4.6). (A) Luciferase activity was determined by correlating p53-dependent *Firefly* luciferase activity and p53-independent *Renilla* luciferase activity for transfection efficiency. The luciferase activities were plotted relative to the positive control (combination 3) and are based on the average of three independent experiments. (B) To determine E1B-55K protein expression/stability, 35 µl of total cell lysates were prepared, resolved by 10% SDS-PAGE and visualized by immunoblotting (4.5). E1B-55K protein levels were detected using mAb 2A6 (α-E1B-55K). Molecular weights in kDa are indicated on the *left*, while corresponding proteins are labeled on the *right*.

The reporter construct in this assay contains five artificial p53-binding sites (pRE-Luc) and therefore *Firefly* luciferase expression/activity directly correlates with transcriptional activation by p53, which in turn is directly influenced by the adenoviral E1B-55K protein (Yew *et al.*, 1994; Martin & Berk, 1998; Martin & Berk, 1999). As expected, neither transfection of pRenilla-Luc (Fig. 25; A; combination 1) nor pRenilla-Luc plus pRE-Luc (Fig. 25; A; combination 2) show detectable luminescence due to the lack of endogenous p53 expression in H1299, whereas additional cotransfection of pc53SN3 leads to maximal activation of p53-dependent luciferase transcription/activity (Fig. 25; A; combination 3).

Also as expected, gradual addition of E1B-55K-wt drives a progressive drop in luciferase activity to a residual activity of ~10% of the positive control (Fig. 25; A; combinations 4-6). Consistently with previous results (Kindsmüller *et al.*, 2009), it is interesting that relatively low levels of E1B-55K are sufficient for nearly complete repression of p53 transcriptional activation as seen in Fig. 25 (A; combination 6), although the protein levels are barely detectable in *Western Blot* (Fig. 25; B; lane 6). Furthermore, these results again underline the dependency of E1B-55K´s repression function on posttranslational modification by SUMO (Endter *et al.*, 2001; Endter *et al.*, 2005). For example, E1B-55K-K104R completely fails to significantly lower luciferase activity (Fig. 25; A; combination 7), whereas hyper-SUMOylated E1B-55K-NES represses p53 transactivation even more efficient than the E1B-55K-wt protein (Fig. 25; A; combinations 8/6).

Although mutations within the C-terminal phosphorylation motif of E1B-55K (Fig. 22) do not significantly alter the PML-IV/V binding capability (Fig. 23; Fig. 24), it appears that mimicking constitutive phosphorylation (E1B-55K-pM) reverts the previously described phenotype of the phosphorylation deficient E1B-55K-delP mutant (Teodoro *et al.*, 1994; Teodoro & Branton, 1997) leading to significantly reduced luciferase activity (<10%) (Fig. 25; A; combinations 10/9). Obviously, it is not possible to link PML-IV/V binding (Fig. 23; Fig. 24) to the p53-repression abilities (Fig. 25; A) of the different mutants E1B-55K-H354/-R443A/-RTR/-RF6/-E2 (Fig. 22). Whereas some of the PML binding deficient mutants (E1B-55K-RTR/-

RF6/-E2) moderately repress p53 transactivation (Fig. 25; A; combinations 14-16), others (E1B-55K-H354/-R443A) are still fully capable of efficient p53 transcriptional inactivation (Fig. 25; A; combinations 11/12) comparable to E1B-55K-wt (Fig. 25; A; combination 6).

In this context, it is possible that mutations within E1B-55K lead to abolishing p53 interaction and subsequent loss of transcriptional repression despite retaining the intrinsic capability to do so. To test this, H1299 cells were transfected with plasmids encoding for E1B-55K-wt, E1B-55K-K104R, E1B-55K-NES, E1B-55K-delP, E1B-55K-pM, E1B-55K-H354, E1B-55K-R443A, E1B-55K-E1, E1B-55K-RTR, E1B-55K-RF6, E1B-55K-E2 and human p53 before being subjected to immunoprecipitation analysis (Fig. 26).

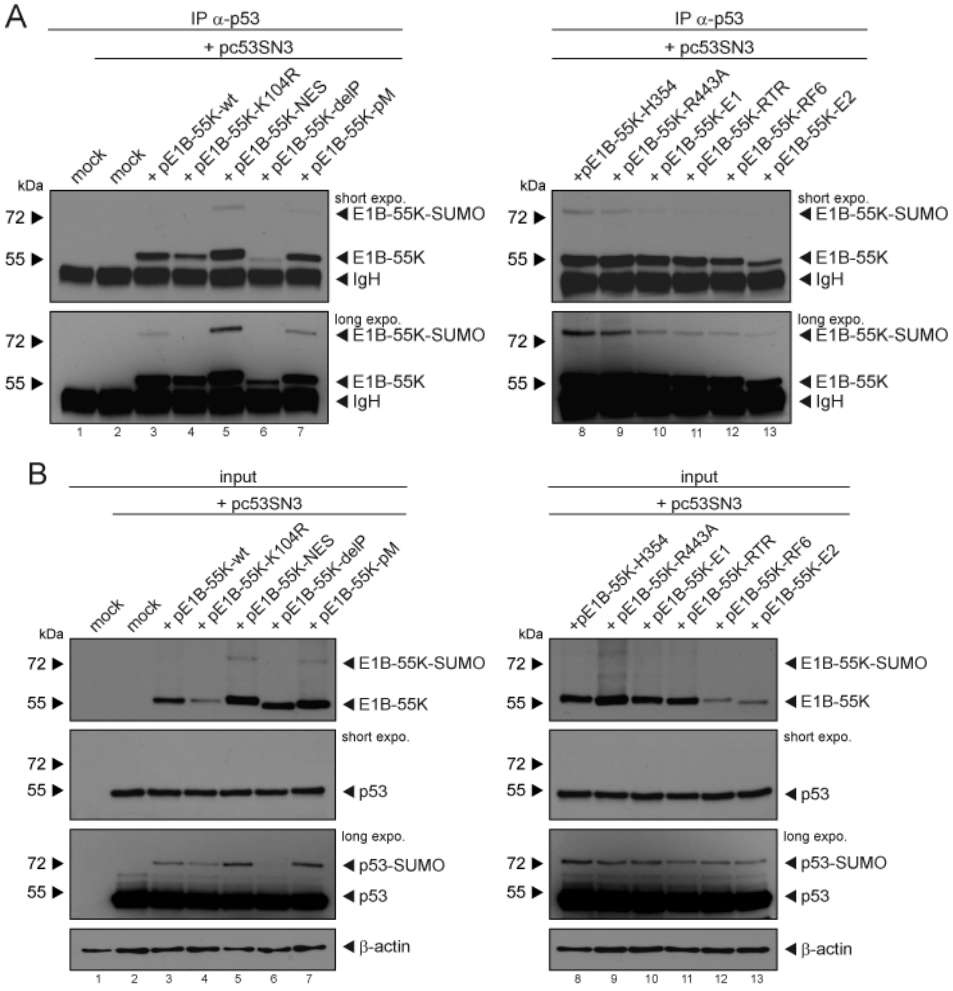

Figure 26. E1B-55K interaction with PML-IV/V is dispensible for p53 E1B-55K interaction in transiently transfected H1299 cells. Subconfluent H1299 cells ($3.0x10^6$) were transfected with 5 µg of pE1B-55K-wt/-K104R/-NES/-delP/-pM/-H354/-R443A/-E1/-RTR/-RF6/E2 (Fig. 22) plus 3 µg of pc53SN3 and harvested after 48 hours before preparing total cell extracts (4.5). Immunoprecipitation of p53 was performed by using mAb DO-I (α-p53), resolved by 10% SDS-PAGE and visualized by immunoblotting (4.5). Coprecipitated proteins (A) and input levels (B) of total cell lysates were

detected by using mAb 2A6 (α-E1B-55K), mAb DO-I (α-p53) and mAB AC-15 (α-β-actin). Molecular weights in kDa are indicated on the *left*, while corresponding proteins are labeled on the *right*.

In general, all analyzed E1B-55K mutants could be coprecipitated with human p53 (Fig. 26; A). Only E1B-55K-delP shows some reduced affinity to the tumor suppressor protein (Fig. 26; A; panel 1; lane 6) despite comparably high input levels (Fig. 26; B; panel 1; lane 6). Interestingly, all E1B-55K mutants show some degree of SUMOylation (Fig. 26; A; panel 2) with exception of E1B-55K-K104R (Fig. 26; A; panel 2; lane 4) and E1B-55K-delP (Fig. 26; A; panel 2; lane 6). These results are fascinating since phosphorylation of E1B-55K has never before been linked to SUMOylation of the adenoviral protein, although both mutants E1B-55K-K104R and E1B-55K-NES show similar phenotypes with respect to subcellular localization, p53 transcriptional repression and cellular transformation of primary rodent cells (Teodoro *et al.*, 1994; Teodoro & Branton, 1997; Endter *et al.*, 2001; Endter *et al.*, 2005). Intriguingly, these results also correlate with SUMOylation of p53 (Fig. 26; B; panel 3), which was previously shown to be regulated by the adenoviral E1B-55K protein strongly depending on SUMOylation of E1B-55K *per se*, whereas concurrently no evidence has been found that E1B-55K functions as an E3 SUMO ligase (Müller & Dobner, 2008).

Taken together, it can be concluded that all tested E1B-55K mutants (Fig. 22) show little reduction of p53 binding in transiently transfected H1299 cells (Fig. 26). This strongly suggests that the previous results concerning the p53-repression capabilities (Fig. 25) are not due to a lack of p53 E1B-55K interaction *per se*.

5.4.4 E1B-55K Interaction with PML-IV/V Might be a Key Regulatory Event in Adenoviral Induced Cell Transformation of Primary Rodent Cells

It has been repeatedly shown that primary rodent cells represent a suitable tool to analyze the transformation potential of human adenoviral oncogenes (Branton *et al.*, 1985; Endter & Dobner, 2004). Therefore, to more closely evaluate whether the modulation of endogenous PML is a necessary determinant for the transformation potential of E1B-55K, freshly prepared primary BRK cells were transduced simultaneously with lentiviruses encoding E1A and the newly established PML-binding mutants E1B-55K-RF6/-RTR/-E2 (Fig. 22; Fig. 23; Fig. 24). After 21 days of incubation under standard conditions *focus* formation was visualized by crystal violet staining and quantified relative to E1A plus E1B-55K-wt (Fig. 27).

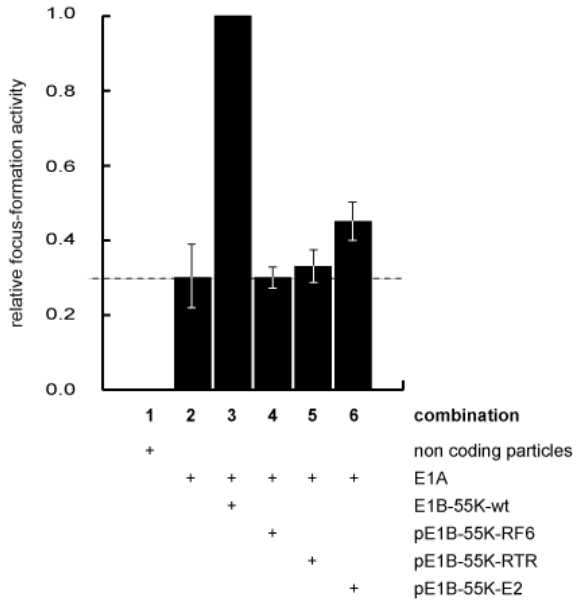

Figure 27. E1B-55K interaction with PML-IV/V may be a key regulatory event in adenoviral induced cell transformation of primary rodent cells. Freshly prepared primary baby rat kidney (pBRK; 4.2.2) cells ($3.0x10^5$) were simultaneously transduced with a combination of two different lentiviruses encoding for E1A and individually E1B-55K-wt/-RF6/-RTR/-E2 ($1x10^5$ particles/lentivirus), split after 24 hours of absorption and cultivated for 21 days under standard conditions (4.7). *Focus* formation was visualized by crystal violet staining and the absolute numbers were counted. Representative results normalized to the positive control (E1A plus E1B-55K-wt) were plotted based on the average of three independent experiments. *Focus* formation by E1A alone (combination 2) is indicated by a dotted line across the graph.

As expected (2.1.4.2), transduction by E1A alone resulted in low *focus* formation activity (Fig. 27; combination 2), whereas simultaneous cotransduction of E1B-55K significantly enhanced cellular transformation in combination with E1A (Fig. 27; combination 3). Intriguingly, all combinations containing the relevant PML-binding mutants (Fig. 27; combinations 4-6) show a significant reduction in *focus* forming activity compared to the positive control (Fig. 27; combination 3), suggesting that PML E1B-55K interactions may be important for efficient cellular transformation.

Although the results presented so far indicate a general affinity of E1B-55K for human PML-IV/V (Fig. 13; Fig. 21; Fig. 23; Fig. 24) as well as a specific subset of rat PML (Fig. 11), possibly the rat orthologs of these human isoforms, it is premature to draw a final conclusion about the exchangeability of results obtained in either of these two systems. This particularly includes the impact of E1B-55K PML interaction on cellular transformation since no data is available concerning the expression of PML in rats. In this context, recent data concerning the organization of the murine *pml* locus underline the possibility of species-specific PML expression with partially homologous, nevertheless considerably different PML-isoforms

(Goddard *et al.*, 1995; Condemine *et al.*, 2006). Apparently, mice encode three different PML-isoforms with high similarity to human PML-I/IV/V, which in part might explain previous results concerning the PML expression pattern as well as isoform-specific binding of E1B-55K in transformed rat cell lines (Fig. 11). However, with no available data on endogenous expression of defined PML-isoforms in rats, although the results here are interesting and may link PML to the transforming potential of E1B-55K (Fig. 27), these data need to be interpreted carefully.

5.5 E1B-55K is Modified by All Three Isoforms of the *Small Ubiquitin-Related Modifiers*

Since early on in molecular biology posttranslational modifications of proteins have always been recognized as one of the principle mechanisms for achieving substantial control of protein stability and function. As mentioned previously (2.2.1), dozens of viral proteins have been described so far to directly or indirectly modulate the cellular SUMOylation machinery, including downstream factors such as PML/PML-associated proteins. Since already published data highlight the importance of SUMO-1 conjugation in subcellular localization, intranuclear targeting, p53-repression and cellular transformation of the adenoviral E1B-55K protein (Endter *et al.*, 2001; Endter *et al.*, 2005; Kindsmüller *et al.*, 2007) as well as the influence of SUMOylation on E1B-55K PML interactions (Fig. 21; Fig. 23; Fig. 24), more effort was put into closer analysis of E1B-55K SUMOylation.

5.5.1 E1B-55K is Modified by All Three Isoforms of SUMO in Transiently Transfected H1299 Cells

Currently, four different isoforms of SUMO have been identified in mammalian cells, although molecular evidence for SUMO-4 functions remain elusive, since it seems to lack the possible means of covalent attaching to target molecules (Melchior, 2000; Verger *et al.*, 2003; Hay, 2005; Bossis & Melchior, 2006; Kerscher *et al.*, 2006; Geiss-Friedlander & Melchior, 2007; Heun, 2007; Ullrich, 2008; Zhu *et al.*, 2008; Ullrich, 2009). To more closly evaluate whether and to what extent E1B-55K is modified by SUMO-1/2/3, H1299 cells were transfected with

plasmids encoding E1B-55K-wt, E1B-55K-K104R, E1B-55K-NES and human N-terminal HA-tagged versions of SUMO-1/2/3 before being subjected to *Western Blot* analysis (Fig. 28).

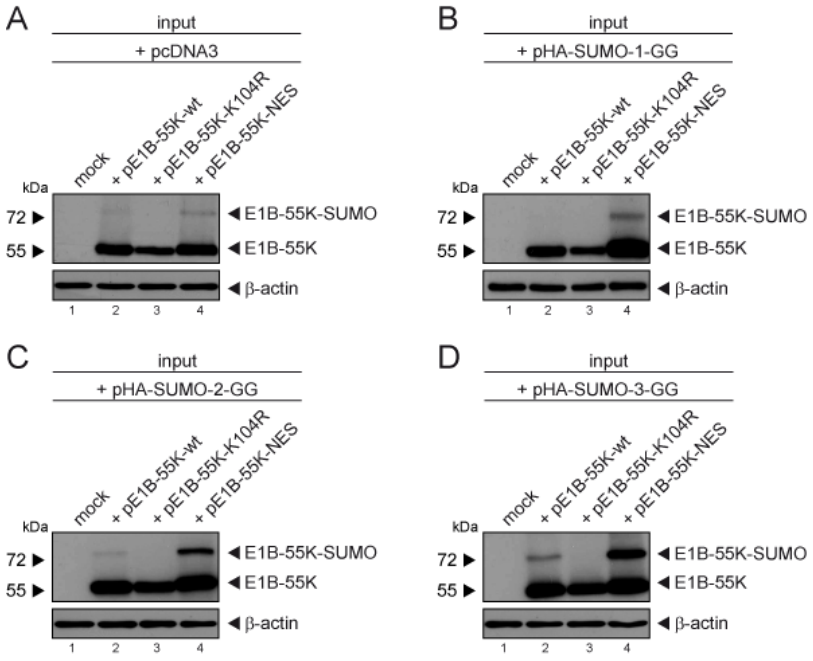

Figure 28. E1B-55K is modified by all three isoforms of SUMO in transiently transfected H1299 cells. Subconfluent H1299 cells ($7.0x10^6$) were transfected with 10 µg of pE1B-55K-wt/-K104R/-NES plus 5 µg of empty vector/pHA-SUMO-1/2/3-GG and harvested after 48 hours before preparing total cell extracts (4.5). Input levels (A-D) of total cell lysates were detected by using mAb 2A6 (α-E1B-55K) and mAB AC-15 (α-β-actin). All SUMO-isoforms were expressed as N-terminal HA-tagged versions in an already mature form with a directly accessible C-terminal GG-motif (pHA-SUMO-1/2/3-GG). Molecular weights in kDa are indicated on the *left*, while corresponding proteins are labeled on the *right*.

SUMO molecules are conjugated via a complex enzymatic machinery that in principle resembles the same mechanism as ubiquitin conjugation (Melchior, 2000; Verger *et al.*, 2003; Hay, 2005; Bossis & Melchior, 2006; Kerscher *et al.*, 2006; Geiss-Friedlander & Melchior, 2007; Heun, 2007; Ullrich, 2008; Zhu *et al.*, 2008; Ullrich, 2009). The initial step in SUMO conjugation is mediated by specific proteases called SENPs that facilitate SUMO maturation by C-terminal cleavage and exposure of the conjugatable double glycine motif (Mukhopadhyay & Dasso, 2007). To achieve relative high levels of SUMOylation *per se*, all SUMO molecules used in these experiments were expressed as N-terminal HA-tagged versions in an already mature form with a directly accessible C-terminal GG-motif (pHA-SUMO-1/2/3-GG).

Consistent with previous results (Endter *et al.*, 2001; Endter *et al.*, 2005; Kindsmüller *et al.*, 2007), E1B-55K-wt shows low levels of SUMOyation (Fig. 28; A/B; lane 2), whereas inactivation of the SCM by the K104R mutation completely abolished posttranslational SUMO mo-

dification (Fig. 28; A/B; lane 3). In parallel, enhanced E1B-55K SUMOylation directly correlated with nuclear retention, as revealed by mutating the leucine-rich NES motif (Fig. 28; A/B; lane 4). Interestingly, it appears that separate overexpression of SUMO-2/3 triggers considerably enhanced posttranslational modification of the viral protein compared to SUMO-1 (Fig. 28; C/D/B). Moreover, repeated done approaches showed significantly enhanced amounts of modified E1B-55K by SUMO-3 compared to SUMO-2 (Fig. 28; D/C). Since corresponding bands cannot be seen with E1B-55K-K104R, it can be concluded that the major posttranslational modification of E1B-55K by all three SUMO-isoforms takes place at lysine 104 (Fig. 28; C/D; lane 3), although preferences for SUMO-1/2/3 conjugation are different.

5.5.2 Multiple Modification of E1B-55K Induces SUMO Chain Formation at Lysine 104

In principle, all SUMO-isoforms share similar amino acid sequences with an overall similarity of 42-98% and SUMO-2/3 only differ in a total of three residues. However, these differences appear to be sufficient to allow isoform-specific conjugation and/or deconjugation *in vivo*, leading to different functions according to the conjugated isoform (Matic *et al.*, 2008; Ullrich, 2008; Zhu *et al.*, 2008). In contrast to SUMO-1, SUMO-2 and SUMO-3 are characterized by an internal SCM that enables multiple SUMO conjugation and formation of SUMO chains (Melchior, 2000; Verger *et al.*, 2003; Hay, 2005; Bossis & Melchior, 2006; Kerscher *et al.*, 2006; Geiss-Friedlander & Melchior, 2007; Heun, 2007; Ullrich, 2008; Zhu *et al.*, 2008; Ullrich, 2009).

To more closely evaluate whether E1B-55K is multiply modified by SUMO moieties, H1299 cells were transfected with plasmids encoding E1B-55K-wt, E1B-55K-K104R, E1B-55K-NES and human N-terminal HA-tagged versions of SUMO-1/2/3 before being subjected to immunoprecipitation analysis (Fig. 29).

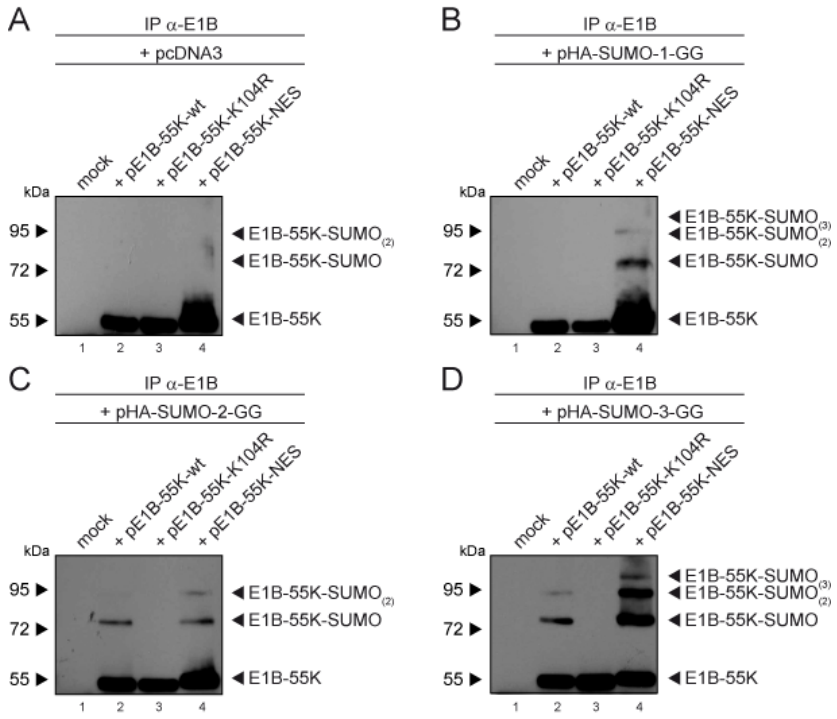

Figure 29. Multiple modification of E1B-55K induces SUMO chain formation at lysine 104. Subconfluent H1299 cells ($7.0x10^6$) were transfected with 10 µg of pE1B-55K-wt/-K104R/-NES plus 5 µg of empty vector/pHA-SUMO-1/2/3-GG and harvested after 48 hours before preparing total cell extracts (4.5). Immunoprecipitation of E1B-55K was performed using mAb 2A6 (α-E1B-55K), resolved by 10% SDS-PAGE and visualized by immunoblotting (4.5). Directly precipitated E1B-55K protein (A-D) from total cell lysates was detected using mAb 2A6 (α-E1B-55K). Corresponding input levels are shown in Fig. 28. Molecular weights in kDa are indicated on the l*eft*, while corresponding proteins are labeled on the *right.*

As expected, total SUMOylation *per se* gradually increases in the different immunoprecipitation experiments (Fig. 29) using the three SUMO-isoforms, which perfectly fits the corresponding input levels shown before (Fig. 28). Directly correlating with this observation, multiple modified forms of E1B-55K can be observed in the different coprecipitations, where enhanced SUMOylation upon mutational inactivation of the E1B-55K NES motif leads to the most obvious results (Fig. 29; A-D; lane 4). Typically, conjugation of a ~10 kDa SUMO molecule led to a shift of ~20 kDa in denaturating SDS-PAGE, although the weight differences obviously appear smaller towards higher molecular weight complexes in the gel due to a lack of sufficient separation. Although only SUMO-2/3 harbor the necessary internal SCM within their primary sequence, some minor amounts of multiply modified E1B-55K can be detected during exogenous overexpression of SUMO-1 (Fig. 29; B; lane 4) since H1299 cells express all isoforms endogenously. Due to the lack of an internal SCM, it has been proposed that

SUMO-1 may serve as a kind of SUMO-chain terminator *in vivo*, although there is no direct molecular evidence.

5.5.3 SUMOylation of E1B-55K is Regulated by Phosphorylation in Transiently Transfected H1299 Cells

While many of the basic molecular mechanisms involved in regulating SUMOylation remain elusive, SUMOylation of substrate proteins has frequently been shown to be at least partly linked to phosphorylation (Bossis & Melchior, 2006; Matic *et al.*, 2008; Stehmeier & Müller, 2009). Furthermore, initial observations concerning the SUMOylation of E1B-55K-delP (Fig. 23; Fig. 26) provide putative evidence for a similar regulation mechanism of E1B-55K posttranslational modification by SUMO.

To test this, H1299 cells were transfected with plasmids encoding E1B-55K-wt, E1B-55K-K104R, E1B-55K-NES, E1B-55K-delP, E1B-55K-pM (Fig. 22) and human N-terminal HA-tagged versions of SUMO-1/2/3 before being subjected to *Western Blot* analysis (Fig. 30).

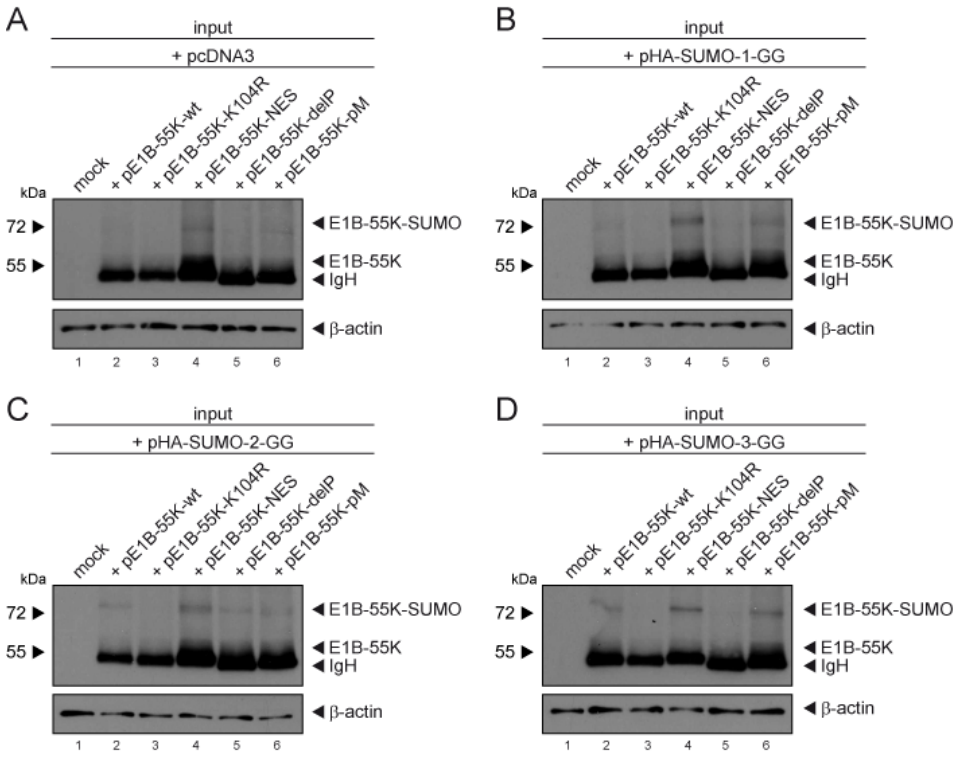

Figure 30. SUMOylation of E1B-55K is partly regulated by phosphorylation in transiently transfected H1299 cells. Subconfluent H1299 cells ($7.0x10^6$) were transfected with 10 µg of pE1B-55K-wt/-K104R/-NES/-delP/-pM plus 5 µg of empty vector/pHA-SUMO-1/2/3-GG and harvested after 48 hours before preparing total cell extracts (4.5). Input levels (A-D) of total cell lysates were detected using mAb 2A6 (α-E1B-55K) and mAB AC-15 (α-β-actin). All SUMO-isoforms are expressed as N-ter-

minal HA-tagged versions in an already mature form with a directly accessible C-terminal GG-motif (pHA-SUMO-1/2/3-GG). Molecular weights in kDa are indicated on the *left*, while corresponding proteins are labeled on the *right*.

As before, E1B-55K-NES shows the highest levels of covalently modified protein in all different experimental set-ups (Fig. 30; A-D; panel 1; lane 4), whereas no SUMOylation could be observed in the corresponding negative controls (Fig. 30; A-D; panel 1; lane 3). Intriguingly, the mutants either abolishing (E1B-55K-delP) or constitutively mimicking (E1B-55K-pM) phosphorylation show significant alterations in the amount of posttranslationally modified protein (Fig. 30; A-D; panel 1; lanes 5/6). Upon cotransfection of any SUMO-isoform E1B-55K-pM elicits higher SUMOylation levels than E1B-55K-wt (Fig. 30; B-D; panel 1; lanes 6/2), whereas E1B-55K-delP (Fig. 30; B-D; panel 1; lane 5) shows significantly lower levels of posttranslationally modified E1B-55K. Since precise estimation of E1B-55K SUMO modification levels is challenging in this context, the corresponding total cell lysates were subjected to immunoprecipitation to purify the adenoviral protein for detailed *Western Blot* analysis (Fig. 31).

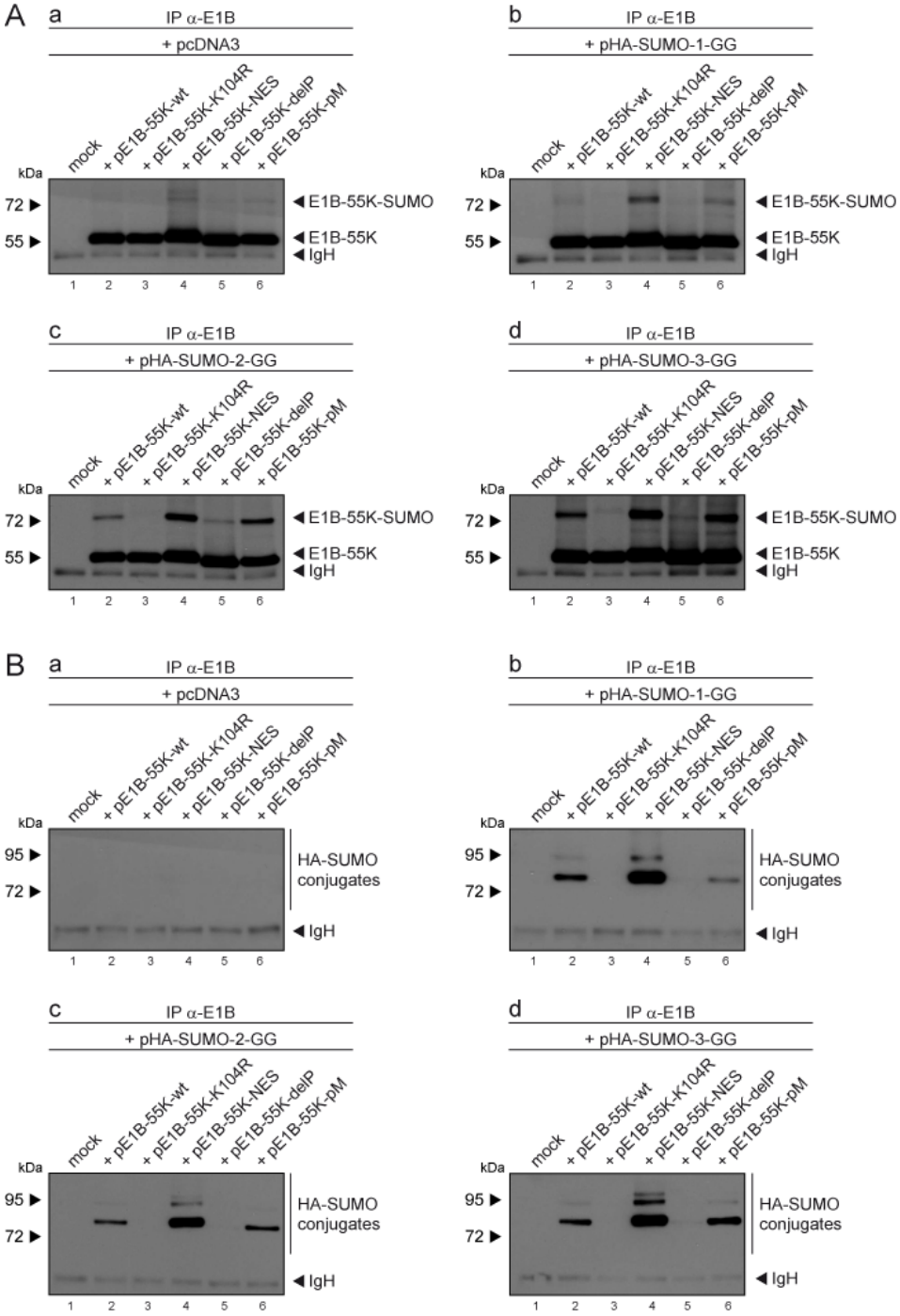

Figure 31. SUMOylation of E1B-55K is regulated by phosphorylation in transiently transfected H1299 cells. Subconfluent H1299 cells ($7.0x10^6$) were transfected with 10 µg of pE1B-55K-wt/-K104R/-NES plus 5 µg of empty vector/pHA-SUMO-1/2/3-GG and harvested after 48 hours before preparing total cell extracts (4.5). Immunoprecipitation of E1B-55K was performed using mAb 2A6 (α-E1B-55K), resolved by 10% SDS-PAGE and visualized by immunoblotting (4.5). Directly precipitated E1B-55K protein (a-d) from total cell lysates was detected using mAb 2A6 (α-E1B-55K) (A)/mAb 3F10 (α-HA) (B). Corresponding input levels are shown in Fig. 30. Molecular weights in kDa are indicated on the *left*, while corresponding proteins are labeled on the *right*.

The immunoprecipitation results further substantiate the assumption that C-terminal phosphorylation and SUMOylation of E1B-55K are ultimately linked (Fig. 31; A), explaining the similar phenotypes of E1B-55K-K104R and E1B-55K-delP. In contrast, E1B-55K-NES and E1B-55K-pM concurrently show completely opposite characteristics in terms of subcellular localization, p53-repression and cellular transformation of primary rodent cells (Fig. 25; Fig. 27) (Teodoro *et al.*, 1994; Teodoro & Branton, 1997; Endter *et al.*, 2001; Endter *et al.*, 2005; Kindsmüller *et al.*, 2009). Thus, it appears reasonable to assume that phosphorylation of E1B-55K occurs as a prerequisite for efficient SUMOylation and not *vice versa* since the SUMOylation deficient mutant E1B-55K-K104R still shows a considerable amount of phosphorylation (Ching W.; unpublished results). Although clearly speculative at this point, it is tempting to assume that E1B-55K SUMOylation directly correlates with the C-terminal tethered negative charges, since the E1B-55K-pM mutant (3x aspartic acid; three negative charges) seemingly does not achieve the same levels of covalently modified protein as E1B-55K-NES (3x phosphorylation; nine negative charges) (Fig. 31; B-D; lanes 6/4). Parallel immunoprecipitation experiments visualizing HA-tagged SUMO molecules (Fig. 31; B) additionally confirmed previous results, illustrating multiple modification of E1B-55K (Fig. 29). Correlating with the total amount of SUMOylated protein (Fig. 31; A), E1B-55K-wt/-NES/-pM show several HA-cross-reactive bands differing by ~20 kDa in molecular weight, strongly implying multiple modifications of E1B-55K (Fig. 31; B; lanes 2/4/6). Although the experimental design allows coprecipitation of unknown SUMOylated E1B-55K binding partners that might also be detected by the HA-specific antibodies, it appears unlikely in the light of previous results (Fig. 29). Since the detected bands perfectly correlate with the total amount of SUMOylated viral protein (Fig. 31; A) and fit the exact molecular weight of multiple modified E1B-55K, they most likely do not represent any proteins other than the different E1B-55K mutants.

Taken together, it could be shown for the first time that adenoviral E1B-55K is not exclusively modified by SUMO-1 (Endter *et al.*, 2001; Endter *et al.*, 2005; Kindsmüller *et al.*, 2007), but is also modified by SUMO-2 and 3, consequently enabling SUMO chain formation and multiple modification of E1B-55K (Fig. 28; Fig. 29; Fig. 30; Fig. 31). Although many questions remain to be answered, the putative correlation between E1B-55K posttranslational modification by phosphorylation and SUMOylation (Fig. 30; Fig. 31) may provide the first insights into the molecular mechanism and establish a link between previously described but unrelated observations concerning E1B-55K´s transformation potential (Teodoro *et al.*, 1994; Teodoro & Branton, 1997; Endter *et al.*, 2001; Endter *et al.*, 2005; Kindsmüller *et al.*, 2007).

6 Discussion

6.1 PML-NBs May Play an Integral Role in Adenoviral Mediated Transformation

Human Adenoviruses encode several multifunctional proteins that support and/or can transform primary rodent cells (Graham, 1984; Branton *et al.*, 1985; Ricciardi, 1995; Nevins & Vogt, 1996). The oncogenic determinants of the model system HAdV type 5 are located within the viral genome´s *early region 1* (E1) and *early region 4* (E4) (2.1.4.2). Whereas the HAdV5 E1A and E1B proteins are absolutely required for oncogenic transformation (Graham, 1984; Branton *et al.*, 1985; Ricciardi, 1995; Nevins & Vogt, 1996), mediating intensive cell cycle progression (Gallimore & Turnell, 2001) and concurrent inhibition of tumor suppressor proteins such as p53 (Yew *et al.*, 1994; Martin & Berk, 1998; Martin & Berk, 1999), additional proteins expressed in the E4 region substantially increase the transforming potential. They achieve this by operating through a complex network of cellular regulatory components involved in transcription, apoptosis, cell cycle control, DNA repair and cell signaling (Täuber & Dobner, 2001a).

Since the initial description of HAdV12 induced tumorigenicity in newborn rodents by Trentin, Yabe and Taylor (Trentin *et al.*, 1962), most of the viral genes involved in adenovirus transformation are now known. Yet it remains unclear why HAdVs are oncogenic in rodents, but not as far as we know, in humans. With very few exceptions (Graham *et al.*, 1977; Byrd *et al.*, 1982; Whittaker *et al.*, 1984; Gallimore *et al.*, 1986; van den Heuvel *et al.*, 1992; Fallaux *et al.*, 1996; Fallaux *et al.*, 1998; Schiedner *et al.*, 2000), attempts to transform primary human cells in culture using different types of adenovirus/subgenomic DNA fragments have proved unsuccessful or extremely inefficient, whereas primary rat, hamster or mouse cells can be efficiently transformed (Graham, 1984; Graham *et al.*, 1984; Branton *et al.*, 1985; Endter & Dobner, 2004). In this context, identifying new interaction partners of the adenoviral oncoproteins might not only provide insights into the fundamental differences between human and rodent cells, but also into the basic principles of tumorigenesis.

6.1.1 The Cellular Tumor Suppressor Protein PML Might be Involved in Adenoviral Mediated Cell Transformation

The PML protein was first described as the causal agent in *acute promyelocytic leukemia* (APL) (2.2), making PML a general tumor suppressor frequently deregulated in various tumor types (Koken *et al.*, 1995; Salomoni & Pandolfi, 2002; Gurrieri *et al.*, 2004a; Gurrieri *et al.*, 2004b; Scaglioni *et al.*, 2006; Salomoni *et al.*, 2008). Presumably such antioncogenic functions involve secondary effects of PML-bodies as sites of protein degradation (Lallemand-Breitenbach *et al.*, 2001), transcriptional regulation (Li *et al.*, 2000; Zhong *et al.*, 2000b), cellular senescence (Ferbeyre *et al.*, 2000; Pearson *et al.*, 2000; Bischof *et al.*, 2002; Langley *et al.*, 2002), tumor suppression (Salomoni & Pandolfi, 2002; Salomoni *et al.*, 2008), DNA repair (Bischof *et al.*, 2001; Carbone *et al.*, 2002), apoptosis (Hofmann & Will, 2003; Takahashi *et al.*, 2004) and epigenetic regulation (Torok *et al.*, 2009). Most of these functions are mediated by a huge PML-regulated network of over 166 different proteins, which are involved in over 781 separate interactions (2.2.2) (Van Damme *et al.*, 2010).

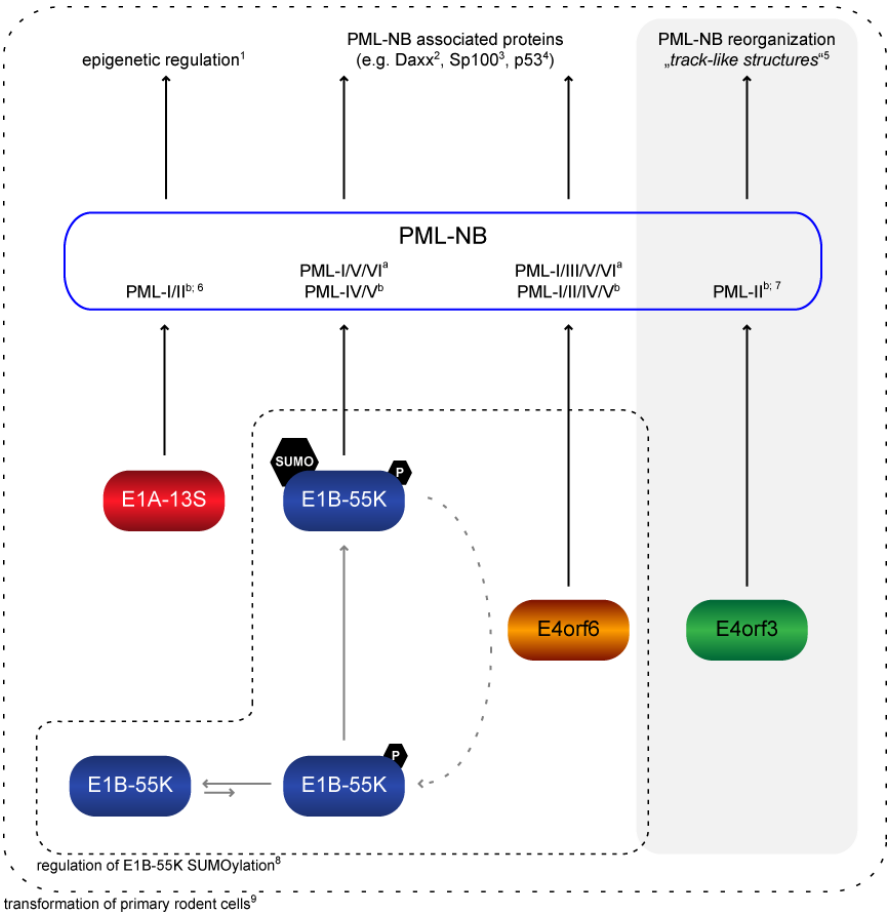

Figure 32. Overview highlighting newly identified adenoviral oncoproteins interacting with PML-NBs. The overview illustrates the previously shown association of E4orf3 (green) and PML-II

(Leppard & Everett, 1999; Hoppe *et al.*, 2006; Leppard *et al.*, 2009) as well as newly identified interactions of E1A-13S (red), E1B-55K (blue) and E4orf6 (orange). Isoform-specific interactions during infection ([a]; Fig. 14; Fig. 15; Fig. 17) and transient transfection ([b]; Fig. 13; Fig. 16; Fig. 18) are indicated by arrows. Putative functional implications are described in the upper panel, substantiated by previously published results and referred to in more detail in the text. References: (1) (Butler *et al.*, 2009; Torok *et al.*, 2009); (2) (Schreiner *et al.*, 2010); (3) (Doucas *et al.*, 1996; Ishov & Maul, 1996); (4) (Fogal *et al.*, 2000); (5) (Carvalho *et al.*, 1995; Puvion-Dutilleul *et al.*, 1995; Doucas *et al.*, 1996); (6) (Carvalho *et al.*, 1995); (7) (Leppard & Everett, 1999; Hoppe *et al.*, 2006; Leppard *et al.*, 2009); (8) (Lethbridge *et al.*, 2003); (9) (Bernards & Van der Eb, 1984; Branton *et al.*, 1985; Endter & Dobner, 2004).

Interestingly, with regard to the results presented in this study, it appears that most so far known interaction partners of adenoviral oncoproteins, as well as the described phenoltypes and/or mediated functions can be conclusively linked with PML-NB functions and/or PML-associated proteins (Fig. 32) (Van Damme *et al.*, 2010).

E1A proteins mediate the indispensable and most critical step in cell transformation by initiating unscheduled cell cycle progression through interactions with various different cellular proteins (2.1.4.2.1) (Gallimore *et al.*, 1984a; Gallimore *et al.*, 1984b; Branton *et al.*, 1985; Frisch & Mymryk, 2002; Endter & Dobner, 2004; Ferrari *et al.*, 2008; Ferrari *et al.*, 2009). Although dozens of interaction partners for E1A are known, it appears acceptable to simplify E1A functions as primarily modulating host cell transcription via epigenetic modification, chromatin remodeling and transcription deregulation (Gallimore & Turnell, 2001; Frisch & Mymryk, 2002; Ferrari *et al.*, 2008; Ferrari *et al.*, 2009). Interestingly, over 50% of the PML-NB-associated proteins are transcription factors and epigenetic regulators (Van Damme *et al.*, 2010) and E1A was shown to transiently colocalize with PML positive subnuclear structures (Carvalho *et al.*, 1995). In parallel to E4orf3 induced reorganization of PML-NBs into so-called *track-like structures* (Carvalho *et al.*, 1995; Puvion-Dutilleul *et al.*, 1995; Doucas *et al.*, 1996), E1A colocalizes with PML in fibrillous subnuclear structures during virus infection (Carvalho *et al.*, 1995). Consistently, E1A alone colocalizes with PML in spherical nuclear accumulations in transiently transfected cells (Carvalho *et al.*, 1995).

Although the results of this study (Fig. 18) further substantiate these previous observations, it is premature to draw an overall conclusion since many points remain to be clarified. Due to the overall binding efficiency of E1A-13S/12S to PML-I/II (Fig. 18), it is tempting to speculate that the CR3 region is essential for efficient PML interaction, which would explain the difference in binding efficiencies between E1A-12S and E1A-13S (2.1.4.2.1). However, the results of Carvalho and co-workers (Carvalho *et al.*, 1995) are somehow conflicting with this observation, since they concluded that although E1A PML colocalization is independent of pRB binding, it still depends on the LxCxE motif within E1A CR2, which in turn is present in

both E1A isoforms. However, it is possible that the LxCxE motif is essential for proper subnuclear localization of both E1A isoforms, while the CR3 region is necessary for physical interaction. Ongoing studies are more closely evaluating the E1A PML binding region as well as the putative impact on global reprogramming of host cell functions (Ferrari *et al.*, 2008; Ferrari *et al.*, 2009).

Currently, the pro-tumorigenic functions of E1B-55K are primarily linked to the modulation of the tumor suppressor p53. Subsequent steps of direct interaction (Sarnow *et al.*, 1982a; Kao *et al.*, 1990), transcriptional repression (Yew *et al.*, 1994; Martin & Berk, 1998; Martin & Berk, 1999) and nuclear-cytoplasmic relocalization (Endter *et al.*, 2001; Endter *et al.*, 2005) induce the complete silencing of p53-dependent tumor suppressive functions. However, previously described results suggest additional p53-independent mechanisms of E1B-55K induced cellular transformation involving cellular factors such as Mre11 (Härtl *et al.*, 2008) and/or the transcription factor Daxx (Sieber & Dobner, 2007; Schreiner, 2010). Interestingly, most so far known interaction partners of E1B-55K are transient components of nuclear *promyelocytic leukemia* (PML)-bodies (Van Damme *et al.*, 2010) and concurrent expression of E4orf6 induces proteasomal degradation of these target proteins via the E1B-55K E4orf6 ubiquitin ligase complex (2.1.4.2.3) (Querido *et al.*, 2001a; Querido *et al.*, 2001b; Blanchette *et al.*, 2004; Blanchette *et al.*, 2008). Thus, the synergistic functions of E1B-55K and E4orf6 are tightly linked, including their ability to interact with certain PML-isoforms during infection (Fig. 14; Fig. 15; Fig. 17) compared to their intrinsic interaction profile during transient transfection (Fig. 13; Fig. 16). Taken together, observations concerning the transient subnuclear localization of E1A, E1B-55K, E4orf6 and E4orf3 during infection as well as transfection highlight the importance of PML-NBs or specific subnuclear domains for proper localization and presumably viral protein function.

Unfortunately, the results of this study do not provide sufficient evidence to answer the most interesting question of whether PML *per se* and/or certain PML-isoforms themselves represent positive or negative factors during adenoviral mediated cell transformation. On the one hand, many published data illustrate the importance of PML deregulation/loss during tumorigenesis (Fig. 12) (Koken *et al.*, 1995; Salomoni & Pandolfi, 2002; Gurrieri *et al.*, 2004a; Gurrieri *et al.*, 2004b; Salomoni *et al.*, 2008). On the other hand, PML is obviously stabilized by simultaneous expression of E1A and E1B-55K in stably transformed rodent cells, implying some function during adenoviral cell transformation (Fig. 10; Fig. 11). However, PML stabilization might also reflect a comparable phenomenon observed for p53. In this context, p53 is

stabilized by E1A (Debbas & White, 1993; Lowe & Ruley, 1993; Grand *et al.*, 1994; Sabbatini *et al.*, 1995b; Samuelson & Lowe, 1997; Turnell *et al.*, 2000), but at the same time inactivated by E1B-55K-mediated p53 transcriptional repression (Yew *et al.*, 1994; Martin & Berk, 1998; Martin & Berk, 1999) and nucleocytoplasmic relocalization (Endter *et al.*, 2001; Endter *et al.*, 2005). However, although a large amount of data are available concerning the different biochemical/modulatory functions of PML-NBs, but very little concerning the modulation of specific cellular proteins by unique PML-isoforms, it remains elusive at this point as to how and to what extent certain PML-isoforms influence the transformation capabilities of E1A, E1B-55K and/or E4orf6.

6.1.2 Interaction of the Adenoviral Oncoproteins with Specific PML-Isoforms Might Facilitate Access to the PML-NB Regulatory Network and Subsequently Modulation of Cellular Molecular Mechanisms

Currently, seven major isoforms of PML have been described according to their exon structure (Table 2) (Jensen *et al.*, 2001; Bernardi & Pandolfi, 2007). PML-I to PML-VI are mainly localized in the nucleus due to a NLS in exon 5/6 (Fig. 8; Fig. 9). Unfortunately, very little is known about the different functions of certain isoforms, or the molecular mechanisms of how they fulfill their differential functions despite relatively high organization/sequence similarities (Table 2).

Table 2. Exon structure of the main nuclear PML-isoforms (Jensen *et al.*, 2001).

PML ISOFORM	EXON STRUCTURE
PML-I	1 – 2 – 3 – 4 – 5 – 6 – 7a – 8a – 9 (882 aa)
PML-II	1 – 2 – 3 – 4 – 5 – 6 – 7a – 7b (829 aa)
PML-III	1 – 2 – 3 – 4 – 5 – 6 – 7a – 7ab retained intron – 7b (641 aa)
PML-IV	1 – 2 – 3 – 4 – 5 – 6 – 7a – 8a – 8b (633 aa)
PML-V	1 – 2 – 3 – 4 – 5 – 6 – 7a – 7ab retained intron (611 aa)
PML-VI	1 – 2 – 3 – 4 – 5 – 6 – intron sequ. – 7a (560 aa)

In principle, the respective functions of all PML-isoforms may differ in two basic aspects: First, the biochemical behavior of the isoform *per se* mediated at least in part by the domain/exon structure and second, the amount/kind of specific cellular interaction partners. Obviously, due to the very similar primary sequence of all isoforms (Table 2), it seems unlikely that this fact reflects a major determinant of PML regulation/activity. However, it has

been repeatedly shown that indeed the PML-isoforms exhibit remarkable differences in their biochemical and subcellular characteristics, despite nearly identical primary sequences. In the context of normal cell metabolism, PML-NBs show high subnuclear mobility, coupled with continuous exchange of PML-isoforms and associated components. Interestingly, PML-I/II/III/IV/VI exhibit a relatively high exchange rate (~2-10 min), whereas the exchange rate for PML-V takes nearly an hour (~50 min-1 hour). This leads to the assumption that PML-V represents the major scaffold protein of PML-NBs (Weidtkamp-Peters *et al.*, 2008; Brand *et al.*, 2010). However, the molecular mechanisms of this discrepancy is a mystery, keeping in mind that PML-V´s exchange rate is significantly different despite an almost identical exon arrangement (Table 2). A comparable puzzle is the binding of the adenoviral proteins to certain PML-isoforms during transient transfection and infection (Table 3).

Table 3. Overview of the interactions of HAdV5 proteins with specific PML-isoforms during transient transfection and infection.

ADENOVIRAL PROTEIN	TRANSIENT TRANSFECTION	INFECTION	REFERENCE
E1A	PML-I/II	n.d.	this work (Fig. 18)
E1B-55K	PML-IV/V	PML-I/V/VI	this work (Fig. 13; Fig. 14)
E4orf3	PML-II	n.d.	(Hoppe *et al.*, 2006; Leppard *et al.*, 2009)
E4orf6	PML-I/II/IV/V	PML-I/III/V/VI	this work (Fig. 16; Fig. 17)

Although many examples for the described phenomena are listed in the overview (Table 3), the most complicated is represented by the binding behavior of E1B-55K. Neither PML-IV nor PML-V harbor identical, but unique protein sequences compared to the remaining isoforms, thereby ruling out a specific PML E1B-55K binding region (Table 2). Both harbor the exons 1-6, which are actually present in all PML proteins except PML-VII, the exon 7a, which again is also present in PML-I/II/III and their own unique C-terminus. Interestingly, PML-IV is characterized by the unique amino acid sequence of exon 8b and although sequence analysis has so far identified no defined domain of highly similar sequence motifs, this exon might contain an essential determinant for SUMOylation-dependent E1B-55K PML-IV interaction (Fig. 21).

Vice versa, efficient interaction with both PML-IV and PML-V requires the same C-terminal region within E1B-55K (Fig. 22; Fig. 23; Fig. 24). This domain seems to resemble some degree of organized tertiary structure comparable to a zinc-finger and/or RING domain due to

the sequence composition/organization (Fig. 6). A similar phenomenon has been described for the exclusive interaction of HPV E6 with a distinct subset of PML-isoforms, although no conclusions can be drawn on the basis of the PML-isoform sequences (Guccione *et al.*, 2004). However, due to the immense number of PML-NB-associated proteins (Van Damme *et al.*, 2010) and interactions of E1A, E1B-55K and/or E4orf6 with various cellular components, it is also important to consider that specific binding of certain PML-isoforms possibly occurs in an indirect fashion.

In fact, the results presented here indicate a general affinity of E1B-55K for human PML-IV/V as well as a specific subset of rat PML, which may represent the rat orthologs of these isoforms. However, it seems premature to draw a final conclusion about the exchangeability of results obtained in either of these two systems, since no data are available on the expression of PML in rats. In this context, recent data on the organization of the murine *pml* locus underline the possibility of species specific PML expression with partially homologous, but nevertheless considerably different PML-isoforms (Goddard *et al.*, 1995; Condemine *et al.*, 2006). Ongoing studies aim to reveal the molecular background of the isoform specific E1B-55K/E4orf6 PML interaction during infection (Fig. 13/16) since E1B-55K/E4orf6 *per se* only seems capable of binding to a certain subset of PML-isoforms (Fig. 14/17).

To date, only a few examples of specific PML isoforms regulating cellular processes/proteins have been described; however, the potential is fascinating since many functions so far assigned to PML in general might be facilitated by certain isoforms (Table 4).

Table 4. Overview of specific PML isoform regulated cellular processes/proteins.

PML ISOFORM	CELLULAR PROCESS/PATHWAY	REFERENCE
PML-I	regulation of apoptosis induction via survivin expression	(Xu *et al.*, 2004)
PML-I	involvement in nucleolus metabolism, senescence and proteolysis	(Condemine *et al.*, 2007)
PML-I	induction of cell differentiation via c-Myc destabilization	(Buschbeck *et al.*, 2007)
PML-IV	regulation of p53 transcriptional activity	(Fogal *et al.*, 2000) (see 6.2.2)
PML-IV	regulation centrosome duplication and genome stability via modulation of Aurora A kinase activity	(Xu *et al.*, 2005)
PML-IV	suppression of TERT activity	(Oh *et al.*, 2009)
PML-IV	regulation of protein stability of the histone acetyltransferase TIP60	(Wu *et al.*, 2009)

PML-IV	regulation of *alternative lengthening of telomers* (ALT) via TRF1	(Yu *et al.*, 2010)

Based on available data, it is still premature to draw final conclusions about the impact of adenoviral proteins on different PML-isoforms and the corresponding downstream factors. However, since E1B-55K´s pro-tumorigenic functions are predominantely linked to modulating of the tumor suppressor protein p53 (Sarnow *et al.*, 1982a; Kao *et al.*, 1990), it is fascinating that PML-IV so far represents the only described PML isoform modulating p53 functions *in vivo* (Fogal *et al.*, 2000). In turn, PML-IV interacts with E1B-55K in a SUMOylation-dependent manner (Fig. 21; Fig. 23; Fig. 24). Although clearly speculative at this point, it appears promising that some of the additional factors described to interact with PML-IV (TERT, TRF1) are implicated in telomere maintenance. This then might represent one of the previously suggested alternative pathways of E1B-55K-mediated cellular transformation (Härtl, 2005; Wimmer, 2007; Härtl *et al.*, 2008).

Taken together, the results of this study provide the first insights into the molecular mechanism regulating subnuclear localization of E1B-55K via SUMO-dependent interaction with PML-IV (Fig. 10; Fig. 20; Fig. 21) (Endter *et al.*, 2001; Endter *et al.*, 2005; Kindsmüller *et al.*, 2007). Furthermore, isoform-specific interaction of E1B-55K and/or the remaining adenoviral oncoproteins (Fig. 32; Table 3; Table 4) might represent a general mechanism of HAdV5 to gain access to the PML regulatory network and modulate the functions of specific PML-associated proteins.

6.2 SUMOylation Regulates Integral Aspects of the Interaction of Adenoviral Proteins with PML

6.2.1 E1B-55K is Modified by All Three SUMO-Isoforms in a Phosphorylation-Dependent Manner

Detailed studies have shown that most of E1B-55K functions rely not only on the protein´s biochemical properties mediated by the primary sequence structure including various regions/motifs (2.1.4.2.2), but also include proper localization mostly facilitated via post-translational modification involving phosphorylation and SUMOylation (Teodoro *et al.*, 1994; Teodoro & Branton, 1997; Endter *et al.*, 2001; Endter *et al.*, 2005; Kindsmüller *et al.*, 2007).

Interestingly, mutational inactivation of either posttranslational modification motif within E1B-55K results in similar phenotypes: subcellular mislocalization, loss of p53-repression function and abrogation of cellular transformation of primary rodent cells in combination with E1A.

Dozens of publications highlight the cellular SUMOylation system as a prime target for a whole multitude of different viruses, which can result in modulating nearly every cellular pathway known. Due to the immense number of SUMO-regulated processes (Melchior, 2000; Verger *et al.*, 2003; Hay, 2005; Bossis & Melchior, 2006; Kerscher *et al.*, 2006; Geiss-Friedlander & Melchior, 2007; Heun, 2007; Ullrich, 2008; Zhu *et al.*, 2008; Ullrich, 2009), it is complicated to draw any overall conclusion concerning the relationship of E1B-55K and the cellular SUMOylation system.

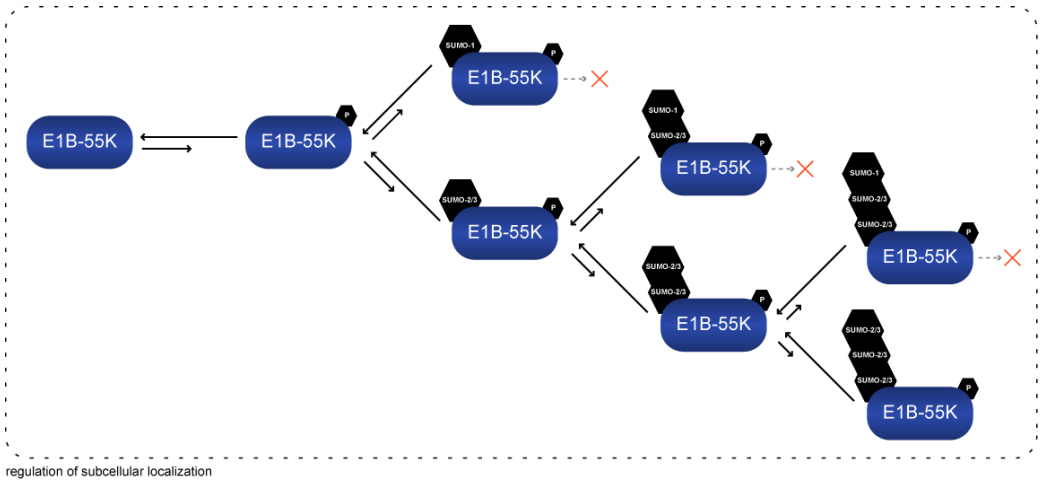

Figure 33. Putative modification cascade of the adenoviral E1B-55K. The overview schematically illustrates the putative posttranslational modification cascade of the adenoviral E1B-55K protein that involves phosphorylation and SUMOylation (Fig. 28; Fig. 29; Fig. 30; Fig. 31). Modification by SUMO-2/3 allows SUMO chain formation based on an internal SCM, whereas chain elongation is terminated upon SUMO-1 conjugation (red) (Melchior, 2000; Verger *et al.*, 2003; Hay, 2005; Bossis & Melchior, 2006; Kerscher *et al.*, 2006; Geiss-Friedlander & Melchior, 2007; Heun, 2007; Ullrich, 2008; Zhu *et al.*, 2008; Ullrich, 2009). Since SUMOylation *per se* only occurs on a small subfraction of the total protein, chain formation is biochemically unstable, as indicated by the shortener arrows.

On the one hand, E1B-55K itself is clearly a substrate for SUMOylation and this modification is essential for many functions of the adenoviral protein (Endter *et al.*, 2001; Endter *et al.*, 2005; Kindsmüller *et al.*, 2007). This observation is further substantiated by the fact that E1B-55K appears to be modified by all three SUMO-isoforms (Fig. 33), with SUMO-3 modification apparently most prominent (Fig. 28; Fig. 30). Although SUMO-1/2/3 share some redundant characteristics, research into SUMO-isoform-specific functions is just at the beginning and only rare results give a hint of the enormous potential (Mukhopadhyay & Dasso, 2007;

Ullrich, 2008; Zhu *et al.*, 2008). In this context, it is fascinating that E1B-55K seems to be preferentially modified by SUMO-3, when both SUMO-2 and SUMO-3 share exactly the same tertiary structure, just differing in three amino acid residues (Fig. 28; Fig. 30). However, clearly speculative at this point, SUMO-3 phosphorylation at serine 2 might be the essential determinant for discriminating between these two isoforms *in vivo*, since SUMO-2 cannot be phosphorylated due to an alanine residue at position 2 (Matic *et al.*, 2008). Equally elusive at this point is whether and how SUMO-2/3 modification and the consequent SUMO chain formation (Fig. 29; Fig. 31) modulate/facilitates E1B-55K function.

The observation that multiple cellular pathways, as well as the functions of the adenoviral E1B-55K protein are extensively regulated by posttranslational modification via different SUMO-isoforms, despite only low amounts of detectable modified effector proteins, currently represents one of the most impenetrable questions and is perceptively called the SUMO enigma (Hay, 2005). The current model suggested by Hay proposes that SUMOylation triggers the assembly of stable multi-protein complexes that continuously persist until reactivation, whereas SUMO is immediately deconjugated after the complexes are established (Hay, 2005). Consequently, SUMOylation seems to represent the molecular switch to target E1B-55K to the nuclear matrix and/or certain PML containing subnuclear domains (Fig. 10; Fig. 20) (Lethbridge *et al.*, 2003) and thereafter SUMO is immediately deconjugated by the corresponding SUMO proteases (SENPs) (Mukhopadhyay & Dasso, 2007).

On the other hand, E1B-55K might also modulate/facilitate the cellular SUMOylation system to induce modification or demodification of cellular substrate proteins. In this context, another early adenoviral protein, GAM-1 from avian CELO (*chicken embryonic lethal orphan*) adenovirus (Chiocca *et al.*, 1997), has been found to mediate extensive modulation of the cellular SUMOylation system. Interestingly, GAM-1 forms an E3 ubiquitin ligase complex containing mostly the same components (Elongin B, Elongin C, Roc 1, Cullin 5, Cullin 2) as shown for HAdV5 E1B-55K/E4orf6. This GAM-1 complex induces proteasomal degradation of the E1 activating enzymes (SAE1/SAE2) of the SUMO conjugation machinery, leading inevitably to the inhibition of cellular SUMOylation and concurrently dispersion of the cellular *PML nuclear bodies* (Boggio *et al.*, 2004; Boggio & Chiocca, 2005; Boggio *et al.*, 2007; Chiocca, 2007; Pozzebon *et al.*, 2009). Although so far there is no direct evidence for E1B-55K and/or E1B-55K/E4orf6 triggering a comparable mechanism, together with the currently unique observation that E1B-55K utilizes the cellular SUMOylation system to induce SUMOylation of the tumor suppressor protein p53 (see 6.2.2), it seems quite likely that

adenoviral proteins are immersed in complex interactions with this cellular posttranslational modification system.

Taken together, the results of this study (Fig. 28; Fig. 29; Fig. 30; Fig. 31) and previously published results (Endter *et al.*, 2001; Endter *et al.*, 2005; Kindsmüller *et al.*, 2007) highlight the promiscuity of the adenoviral E1B-55K protein to fulfill its functions during infection/transformation, as well as provide the basic findings for more detailed investigations into modulation of cellular regulatory mechanisms.

6.2.2 SUMOylation-Dependent Interaction of E1B-55K and PML-IV May Reflect a Key Regulatory Event in p53 Regulation

Previously published results convincingly link the pro-tumorigenic functions of E1B-55K to modulating the tumor suppressor p53. The subsequent steps of direct interaction (Sarnow *et al.*, 1982a; Kao *et al.*, 1990), transcriptional repression (Yew *et al.*, 1994; Martin & Berk, 1998; Martin & Berk, 1999) and nuclear-cytoplasmic relocalization (Endter *et al.*, 2001; Endter *et al.*, 2005; Kindsmüller *et al.*, 2007) are thought to induce complete silencing of p53-dependent tumor suppressive functions. Although the molecular mechanisms have so far remained elusive, it appears that posttranslational modification of the viral E1B-55K protein is an essential determinant in this process. Indeed, functional inactivation of the E1B-55K NES induces enhanced posttranslational modification of E1B-55K by SUMO at lysine 104, in addition to transcriptional repression of the tumor suppressor protein p53 and augmenting transformation of primary rat cells through the accumulation of p53, E1B-55K and PML in subnuclear aggregates (Fig. 10; Fig. 20) (Endter *et al.*, 2001; Endter *et al.*, 2005; Kindsmüller *et al.*, 2007). *Vice versa*, mutational inactivation of the SCM completely abrogates E1B-55K SUMOylation, p53 transcriptional repression and the oncogenic potential of E1B-55K in combination with E1A (Fig. 25; Fig. 27) (Lethbridge *et al.*, 2003; Müller & Dobner, 2008). However, recently published observations add another layer of complexity to adenoviral-mediated p53 modulation (Fig. 34) (Müller & Dobner, 2008).

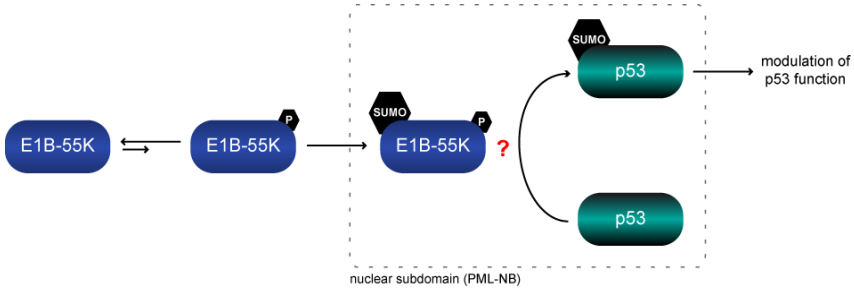

Figure 34. Model of the interplay between E1B-55K and the tumor suppressor p53. The model illustrates the interplay between the adenoviral protein E1B-55K and the cellular tumor suppressor protein p53. As already shown (Müller & Dobner, 2008), p53 is SUMOylated in the presence of E1B-55K. This activity is exclusively dependent on E1B-55K SUMOylation itself and thus a functional SCM. An additional unknown factor has been proposed to link E1B-55K-SUMO and p53 SUMOylation (red) (Müller & Dobner, 2008).

The protein p53 itself is SUMOylated at a so far unique C-terminal lysine residue (K386), leading to enhanced transcriptional activity *in vivo* (Gostissa *et al.*, 1999; Rodriguez *et al.*, 1999; Müller *et al.*, 2000; Kahyo *et al.*, 2001; Schmidt & Müller, 2002). Although the modulation of p53 activity by SUMO conjugation remains controversial, this observation again underlines the difficulty in assigning certain functions to SUMOylation. For example, post-translational modification of p53 induces enhanced transcriptional activity (Gostissa *et al.*, 1999; Rodriguez *et al.*, 1999), whereas the complete opposite phenotype is observed upon modification of the transcription factor c-Jun (Müller *et al.*, 2000).

However, simultaneous expression of E1B-55K and p53 leads to enhanced posttranslational modification of the tumor suppressor protein in an E1B-55K-dependent manner, where SUMO modification of E1B-55K itself is absolutely essential (Fig. 26) (Müller & Dobner, 2008). Although repeatedly hypothesized that E1B-55K may serve as an E3 SUMO ligase, all experiments so far have failed to detect any SUMO conjugating activity by the viral protein, implying that a so far unknown factor facilitates p53 SUMOylation in the presence of SUMOylated E1B-55K. In this context, the results of this study in general offer a new perspective to unraveling the complexity of E1B-55K-dependent SUMOylation and in parallel, adenoviral mediated transcriptional repression of the tumor suppressor protein p53 (Yew *et al.*, 1994; Martin & Berk, 1998; Martin & Berk, 1999). To date, PML-IV is the only isoform so far described to recruit/modulate endogenous p53 (Fogal *et al.*, 2000), so one could speculate that interaction between E1B-55K, p53 and PML-IV is a key regulatory event in modifying/repressing p53 functions. Since it has been already proposed that PML itself might act as an E3 SUMO ligase due to its structural properties (TRIM-motif; Fig. 9), PML-IV might represent the unknown factor linking E1B-55K, p53 and SUMOylation (Fig. 34).

In addition, E1B-55K´s oncogenic potential might also involve the proper localization of E1B-55K in subnuclear aggregates sequestering p53 and PML, which is at least in part regulated by the viral protein´s SCM and NES motifs (Endter *et al.*, 2001; Endter *et al.*, 2005; Kindsmüller *et al.*, 2007). Unfortunately, it remains unclear whether the interaction of E1B-55K with PML is conserved among other human adenovirus types, as it is the case for

E4orf3-mediated PML reorganization (Stracker *et al.*, 2005). This possibility is tantalizing since it would offer a possible explanation for the different transformation capabilities of the large adenovirus E1Bs, as well as previous suggestions linking nuclear localization of HAdV5 E1B-55K (Endter *et al.*, 2001; Endter *et al.*, 2005; Kindsmüller *et al.*, 2007), and presumably subgroup HAdV12 E1B-54K (Krätzer *et al.*, 2000), to their oncogenic potential.

Taken together, it is fascinating that the adenoviral protein strongly represses p53 transcriptional activation via its C-terminal repression domain (Yew *et al.*, 1994; Martin & Berk, 1998; Martin & Berk, 1999), yet in parallel facilitates p53 SUMOylation and consequently a prerequisite signal for transcriptional activation (Gostissa *et al.*, 1999; Rodriguez *et al.*, 1999).

In the context of virus infection, matters seem even more complicated, since E1B-55K apparently triggers transcriptional repression of genes involved in innate and adaptive antiviral defense mechanisms, whereas repression of p53-regulated genes occurs in an E1B-55K-independent fashion during later stages of infection (Miller *et al.*, 2007; Miller *et al.*, 2009). In summary, a growing body of evidence suggests that activation and/or repression of p53 by E1B-55K during infection, as well as in isolation during transient transfection, involves additional determinants such as precise subcellular localization and posttranslational modification, which mainly occurrs at PML-NBs (Fig. 35) (Fogal *et al.*, 2000; Guo *et al.*, 2000; Pearson *et al.*, 2000; Endter *et al.*, 2001; Pearson, 2001; Bernardi & Pandolfi, 2003; Gostissa *et al.*, 2003; Endter *et al.*, 2005; Bernardi & Pandolfi, 2007; Kindsmüller *et al.*, 2007).

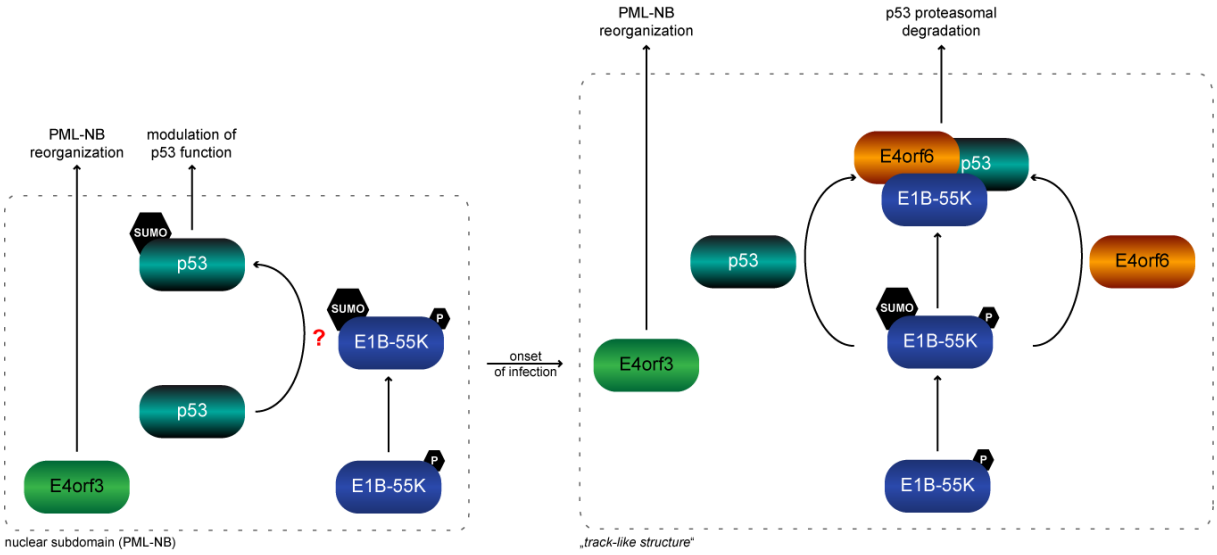

Figure 35. Two-step model of selective p53 modulation by temporally regulated expression of adenoviral early regulatory proteins. Depending on experimental conditions, the adenoviral proteins E1B-55K and E4orf3 are expressed in detectable amounts about 6-10 hours post infection. E1B-55K is localized in the nucleus exhibiting a granular distribution (Ornelles & Shenk, 1991), E4orf3-induced reorganization of PML-NBs is initiated (Carvalho *et al.*, 1995; Puvion-Dutilleul *et al.*, 1995; Doucas *et*

al., 1996; La Cour *et al.*, 2003; Hoppe *et al.*, 2006; Leppard *et al.*, 2009) and p53 transcriptional activity is elevated (König *et al.*, 1999). During the onset of infection (~6 hours later), E4orf6 is expressed and facilitates nuclear matrix release of E1B-55K (Lethbridge *et al.*, 2003), transcriptional repression of p53 via E1B-55K (Yew *et al.*, 1994; Martin & Berk, 1998; Martin & Berk, 1999) and proteasomal degradation of p53 via the SCF-like E3 ubiquitin ligase complex (Sarnow *et al.*, 1982a; Sarnow *et al.*, 1984; Querido *et al.*, 1997; Querido *et al.*, 2001a; Blanchette *et al.*, 2004).

The assumptions above appear adequate since collectively available data in combination with the results in this study imply a two-step model of p53 modulation during adenoviral infection in a temporally regulated manner. During the first 6-10 hours of infection the regulatory proteins of the E1 region (E1A, E1B) as well as E4orf3 are expressed, where E1A predominantly orchestrates the temporally regulated expression of viral proteins (Shenk, 2001). Initially, E4orf3 induces the reorganization of *PML nuclear bodies* by interacting with PML-isoform II, which leads to the formation of the so-called *track-like structures* (Carvalho *et al.*, 1995; Puvion-Dutilleul *et al.*, 1995; Doucas *et al.*, 1996; La Cour *et al.*, 2003; Hoppe *et al.*, 2006; Leppard *et al.*, 2009). In parallel, SUMOylated E1B-55K is localized to the PML-NBs presumably by interacting with PML-IV (Fig. 13; Fig. 20). Interestingly, the presence of E4orf3 and/or E1B-55K synergistically enhances p53 transcriptional activity (König *et al.*, 1999), which might come about through abusing PML-IV function and consequently inducing E1B-55K-dependent SUMOylation of the p53 protein (Gostissa *et al.*, 1999; König *et al.*, 1999; Rodriguez *et al.*, 1999; Fogal *et al.*, 2000). During the onset of viral infection, E4orf6 is expressed with a relative delay of 6 hours compared to E1B-55K/E4orf3 and dominantly alters p53 regulation (König *et al.*, 1999). In this context, E4orf6 facilitates nuclear matrix release of E1B-55K via deSUMOylation (Lethbridge *et al.*, 2003), consequent loss of PML-IV binding (Fig. 21; Fig. 23; Fig. 24) and transcriptional repression of p53 (Yew *et al.*, 1994; Martin & Berk, 1998; König *et al.*, 1999; Martin & Berk, 1999). Finally, E1B-55K/E4orf6 complex formation induces proteasomal degradation of p53 (Sarnow *et al.*, 1982a; Sarnow *et al.*, 1984; Querido *et al.*, 1997; Querido *et al.*, 2001a; Blanchette *et al.*, 2004).

However, it remains a subject for further studies to evaluate whether and how E1B-55K exploits certain PML-isoforms and/or associated proteins to mediate its multiple functions during productive infection, in particular conditional transcriptional repression of p53 (Sarnow *et al.*, 1982a; Sarnow *et al.*, 1984; Querido *et al.*, 1997; Dobner & Kzhyshkowska, 2001; Querido *et al.*, 2001a; Querido *et al.*, 2001b; Stracker *et al.*, 2002b; Blanchette *et al.*, 2004; Baker *et al.*, 2007; Dallaire *et al.*, 2009), selective protein degradation and viral mRNA transport in combination with E4orf6 (Endter *et al.*, 2005; Kindsmüller *et al.*, 2007).

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Publications

I. Publications in Scientific Journals

Schindler M, Rajan D, Banning C, **Wimmer P**, Koppensteiner H, Iwanski A, Specht A, Sauter D, Dobner T, Kirchoff F.
Vpu serine 52 dependent counteraction of tetherin is required for HIV-1 replication in macrophages, but not in ex vivo human lymphoid tissue. (2010). Retrovirology Jan 15;7:1.

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Proteasome-dependent degradation of Daxx by the viral E1B-55K protein in human adenovirus-infected cells. (2010). J Virol. Jul 84(14): 7029-38.

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SUMO Modification of E1B-55K Oncoprotein Regulates Isoform-Specific Binding to the Tumour Suppressor Protein PML. (2010). Oncogene. [Epub ahead of print]

Wimmer P, Schreiner S, Müller D, Everett R, Sirma H, Groitl P, Dobner T.
The Adenoviral E4orf6 Protein Interacts with Various Isoforms of the Tumor Suppressor Protein PML Mediated by its C-terminal Oncodomain. [in preparation]

Wimmer P, Schreiner S, Everett R, Sirma H, Groitl P, Dobner T.
Mutations within the C-terminus of E1B-55K Abolish Interaction with the Tumor Suppressor Protein PML. [in preparation]

Wimmer P, Schreiner S, Groitl P, Ching W, Dobner T.
SUMO Modification of HAdV5 E1B-55K Is Regulated by C-terminal Phosphorylation – A Model System to Study the Link of SUMOylation and Phosphorylation. [in preparation]

II. Oral Presentations at Scientific Meetings

Wimmer P, Härtl B, Dobner T.
Adenovirus type 5 early region E1B-55kDa oncoprotein promotes cell transformation by a mechanism independent from blocking p53-activated transcription.
GfV Annual Meeting; Heidelberg, Germany. (2008).

Wimmer P, Dobner T.
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9th International Adenovirus Meeting; Dobogókő, Hungary. (2009).

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Wimmer P, Schreiner S, Everett RD, Sirma H, Groitl P, Dobner T.
SUMO Modification of E1B-55K Oncoprotein Regulates Isoform-Specific Binding to the Tumor Suppressor Protein PML.
Molecular Biology of DNA Tumor Viruses Conference; Madison, Wisconsin, USA. (2010).

III. Poster Presentations at Scientific Meetings

Wimmer P, Härtl B, Dobner T.
Adenovirus type 5 early region E1B-55kDa oncoprotein promotes cell transformation by a mechanism independent from blocking p53-activated transcription.
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A 49K isoform of the adenovirus type 5 early region 1B 55K protein is sufficient to support viral replication.
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IV. Participation in Scientific Meetings/Workshops

GBM Conference for Molecular Life Sciences; Hamburg, Germany. (2007.)

DNA Repair; Charitè Berlin, Germany. (2008).

Adenovirus Workshop; Hamburg, Germany. (2009).

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