

ORIGINAL ARTICLE

PML isoforms IV and V contribute to adenovirus-mediated oncogenic transformation by functionally inhibiting the tumor-suppressor p53

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Although modulation of the cellular tumor-suppressor p53 is considered to have the major role in E1A/E1B-55K-mediated tumorigenesis, other promyelocytic leukemia nuclear body (PML-NB)/PML oncogenic domain (POD)-associated factors including SUMO, Mre11, Daxx, as well as the integrity of these *nuclear bodies* contribute to the transformation process. However, the biochemical consequences and oncogenic alterations of PML-associated E1B-55K by SUMO-dependent PML-IV and PML-V interaction have so far remained elusive. We performed mutational analysis to define a PML interaction motif within the E1B-55K polypeptide. Our results showed that E1B-55K/PML binding is not required for p53, Mre11 and Daxx interaction. We also observed that E1B-55K lacking subnuclear PML localization because of either PML-IV or PML-V-binding deficiency was no longer capable of mediating E1B-55K-dependent SUMOylation of p53, inhibition of p53-mediated transactivation or efficiently transforming primary rodent cells. These results together with the observation that E1B-55K-dependent SUMOylation of p53 is required for efficient cell transformation, provides evidence for the idea that the SUMO ligase activity of the E1B-55K viral oncoprotein is intimately linked to its growth-promoting oncogenic activities.

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INTRODUCTION

Previous reports demonstrated that human adenovirus (HAdV) genomes preferentially target subnuclear host cell structures called promyelocytic leukemia nuclear bodies (PML-NBs) or PML oncogenic domains (PODs) during initial stages of infection.^{1–3} Intriguingly, PML-NBs exhibit ambivalent characteristics, either acting as an intracellular antiviral defense barrier^{4–6} or having an important role in posttranslational modification and modulation of cellular proteins (for example, p53, Daxx and Mre11) during productive viral infection and/or oncogenic transformation.^{7–10} In fact, dozens of viruses have apparently evolved multiple mechanisms to counteract this cellular antiviral defense barrier or modulate PML/PML-associated proteins.^{11–14}

The PML protein itself was first described as the causal agent in acute promyelocytic leukemia, primarily as a fusion with the retinoic acid receptor alpha receptor generated by the chromosomal translocation t(15;17).^{15–26} Subsequently, it became evident that PML is a general tumor suppressor frequently deregulated in various tumor types,²⁷ as well as being involved in multiple cellular pathways such as protein degradation,²⁸ transcriptional regulation,^{29,30} cellular senescence,^{31–34} tumor suppression,^{35,36} DNA repair,^{37,38} apoptosis^{39,40} and epigenetic regulation.⁴¹

In humans, at least seven PML isoforms (PML-I to PML-VII) are expressed by alternative splicing of a single *pml* gene.^{42,43} Transcription of this gene is tightly controlled by interferons and

p53.^{44,45} All PML isoforms possess the same N-terminal RBCC motif, also known as the tripartite motif (TRIM), comprising the sequential organization of a RING-finger (R), two B-Boxes (B) and a coiled-coil domain (CC); hence these isoforms are grouped as TRIM19 in the TRIM protein family.⁴² This motif is particularly important for dimerization and localization to the PML-NBs.⁴⁶ The C-terminal region of human PML shows remarkable variability among the isoforms and accounts for isoform-specific functions.⁴⁷

In addition, PML function is modulated by SUMO posttranslational modifications,^{42,48–52} as these regulate PML localization⁵³ and nuclear body formation.⁵⁰ Mammalian cells encode four different isoforms, SUMO-1, -2, -3 and -4, although it is not clear whether SUMO-4 is conjugated *in vivo*.⁵⁴ Analogous to ubiquitin, all SUMO proteins are covalently conjugated to substrates via a three-step enzymatic pathway. Considering the impressive number of cellular processes regulated by this posttranslational modification, it is not surprising that many viruses target and exploit the host SUMOylation system.^{12,13,55}

HAdV5, a well-analyzed member of the small DNA viruses, is frequently used to define basic molecular mechanisms in virus/host cell interactions to understand molecular properties of viral oncoproteins and basic principles of virus-induced tumorigenesis. HAdV5 E1A polypeptides mediate the most critical step in cell transformation and are sufficient to promote immortalization and partial transformation of primary rodent cells.^{56,57} However, this

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unprogrammed cell proliferation induces growth arrest and apoptosis through stabilizing the p53 tumor-suppressor protein.⁵⁸ Complete cell transformation requires HAdV5 E1B-55K, the Bcl2-like viral protein E1B-19K^{59,60} or other cooperating oncogenes such as activated Ha-ras.⁶¹ E1B-55K-dependent inactivation of p53-mediated processes induces complete manifestation of a transformed phenotype^{9,62,63} by deregulating p53-dependent tumor-suppressive functions.^{64–67} To do this, the viral polypeptide binds to the amino terminal transactivation domain of p53, blocks its ability to activate transcription of responsive promoters and sequesters the tumor suppressor into large cytoplasmic structures.^{64,66,68}

Besides E1B-55K's role in repressing p53 functions, transformation requires additional anti-apoptotic activities of E1B-55K involving various other protein interactions, especially with PML-NB-associated factors such as Mre11 and Daxx.^{7,10,69} Interestingly, functional inactivation of the E1B-55K leucine-rich nuclear export sequence (NES) not only induces enhanced posttranslational modification of the adenoviral oncogene by SUMO at lysine 104 (SUMO-1 conjugation motif (SCM)) but also elevates transformation of primary rodent cells by mediating accumulation of p53 in subnuclear aggregates.^{70,71} This augments the argument that loss of SUMO conjugation abrogates the ability of E1B-55K to transform in combination with E1A.⁷¹

Recently, Penella *et al.*⁹ reported that E1B-55K is a p53-SUMO1 E3 ligase that mediates SUMOylation of p53, inducing retention of the tumor-suppressor protein in PML-positive nuclear aggregates and thus repressing its functions. In that context, we have previously found that E1B-55K interacts with PML, more specifically with the PML isoforms IV and V.⁸

Here, we analyzed E1B-55K mutants to define a PML interaction motif within the E1B-55K polypeptide. Biochemical analysis revealed that abolished binding of E1B-55K to either PML-IV or PML-V does not affect p53, Mre11 and Daxx interactions, but does restrict efficient E1B-55K SUMO conjugation. Such E1B-55K variants that are unable to bind either PML-IV or PML-V isoforms lost subnuclear PML-NB localization and hence SUMO ligase activity acting on p53. In addition, PML-IV and PML-V non-binding mutants of the viral E1B-55K oncogene lost the ability to transform primary rodent cells together with E1A.

RESULTS

C-terminal mutations in E1B-55K abolish efficient isoform-specific PML-V interaction

We recently showed that E1B-55K physically binds to PML isoforms IV and V in an SCM/K104-dependent manner, and that interaction with PML-IV promotes targeting of E1B-55K to PML-NBs.⁸ To examine this further, we transfected cells with plasmids encoding E1B-55K-wt and different variants of the viral protein (E1B-55K-SCM, E1B-55K-NES, E1B-55K-R443A and E1B-55K-RTR), the latter two affecting isoform-specific PML interactions (Figure 1a). We then screened them via immunoprecipitation analysis for their ability to interact with PML-IV and PML-V (Figure 1b). Consistent with previous results indicating that PML-IV/E1B-55K interactions depend on SUMO, significantly less PML-IV (but not PML-V) co-precipitated with E1B-55K-SCM (Figure 1b, lane 7), whereas E1B-55K-NES shows interaction with equal amounts of both PML isoforms (Figure 1b, lanes 8 and 9). Interestingly, E1B-55K-R443A shows a tendency to interact with higher amounts of PML-V than PML-IV (Figure 1b, lanes 11 and 12) and the C-terminal E1B-55K-RTR mutant was significantly impaired in PML-V precipitation (Figure 1b, lane 14). This indicates that in addition to the SCM of E1B-55K, the C-terminus of the viral oncoprotein has an essential role in PML isoform-specific association (Figure 1a). Interestingly, the pattern of relative PML co-precipitation by the E1B-55K variants illustrates that the two PML isoforms do not

seem to require the same interaction region within the E1B-55K primary sequence (Figure 1a).

PML-IV/-V interaction promotes efficient subnuclear targeting of E1B-55K to PML-NBs

To study whether PML-IV/-V-binding-deficient E1B-55K mutants interfere with the intracellular localization of the HAdV5 protein, plasmids encoding wild-type E1B-55K and mutant variants were transfected into human cells and subjected to indirect immunofluorescence analysis. Consistent with previous findings, wild-type E1B-55K protein localizes in the cytoplasm, mostly concentrated in a perinuclear body in all cells examined ($n > 50$; Figure 2, panel a).^{68,72–76} In nearly 90% of the cells, localization of E1B-55K-NES mutant protein differ from wild-type E1B-55K, and numerous E1B-55K containing aggregates colocalizing with PML bodies were distributed throughout the nucleus (Figure 2, panel c). In addition, the observed intracellular distribution of the E1B-55K-R443A polypeptide is similar to the wild-type localization (Figure 2, panel i). However, E1B-55K proteins that poorly interact with either PML-IV (E1B-55K-SCM; Figure 2, panel e) or PML-V (E1B-55K-RTR; Figure 2, panel g) are located exclusively in the cytoplasm forming several aggregates.

Previous experiments have shown that after blocking the CRM1-dependent nuclear export of E1B-55K by treatment with leptomycin B (LMB), the viral protein accumulates within dense nuclear aggregates.^{75,77} We observed in almost all cells (90%) accumulation of E1B-55K wild-type (Figure 2, panel b) and PML-binding E1B-55K variants (E1B-55K-NES, Figure 2, panel d; E1B-55K-R443A, Figure 2, panel j) in nuclear aggregates, colocalizing with PML nuclear bodies. In contrast, PML-binding-deficient mutants E1B-55K-SCM (Figure 2, panel f) and E1B-55K-RTR (Figure 2, panel h) lost the ability to localize to PML-NBs, when artificially restricted to the cell nucleus.

E1B-55K interaction with PML-IV/-V is not required for p53, Mre11 and Daxx binding

As 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, we first tested both PML-binding-deficient E1B-55K mutants for their ability to interact with p53 (Figure 3a) in plasmid-transfected human p53-negative cells. In agreement with previously published data, immunoprecipitation of exogenous p53 resulted in appropriate E1B-55K and p53 signals (Figure 3a, lanes 3–7). In summary, PML binding seems not to be a prerequisite for p53 binding.

Next, the E1B-55K mutants were tested for their ability to interact with already known E1B-55K-associated proteins reportedly having a role in mediating E1B-55K oncogenicity. As expected, immunoprecipitation of Mre11 or Daxx resulted in co-precipitation of E1B-55K protein (Figure 4, lanes 2–6). Taken together, our observations indicated that E1B-55K mutants that lost binding to either PML-IV or PML-V do not restrict Mre11 or Daxx interaction with the viral polypeptide in transfected human cells.

PML-IV/-V interaction is necessary for SUMOylation of p53

Recently, p53 SUMOylation was shown to be regulated by adenoviral E1B-55K,⁷⁸ revealing a p53-SUMO1 E3 ligase function for E1B-55K that mediates SUMOylation of p53 and induces functional inhibition of this pro-apoptotic cellular regulator.⁹ In agreement, we demonstrate here that p53 shows strong SUMOylation after coexpression of E1B-55K wt and E1B-55K-NES (Figure 3b, right panel, lanes 3 and 5). As expected, E1B-55K-SCM showed drastically reduced SUMO-modified p53 protein (Figure 3b, right panel, lane 4). Intriguingly, E1B-55K-RTR, which failed to bind PML-V,

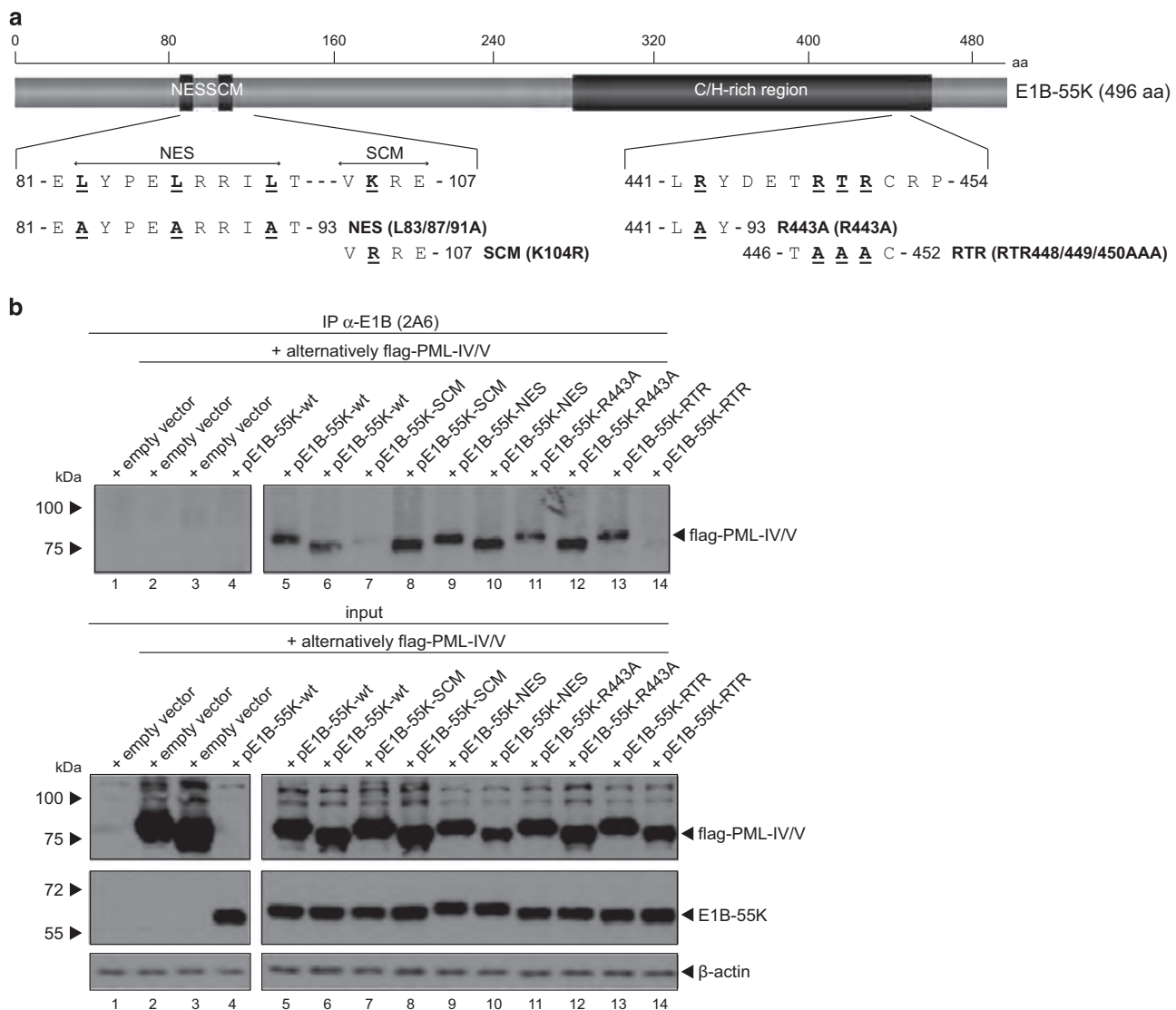


Figure 1. PML-IV/-V binding to E1B-55K oncoprotein. **(a)** The schematic overview shows the coding sequence of E1B-55K including the specific C-terminal mutations influencing isoform-specific PML interactions. Mutations are illustrated according to original aa, position and substituted aa, including abbreviated names in brackets. Amino-acid positions are indicated relative to the E1B-55K sequence and mutations are highlighted. C/H-rich region, cysteine/histidine-rich region. **(b)** Subconfluent H1299 cells were transfected with 10 μ g of pE1B-55K variants plus 10 μ g of different constructs encoding N-terminal flag-tagged human PML isoforms IV or V and harvested after 48 h before preparing total cell extracts. Immunoprecipitation was performed using mAb 2A6 (α -E1B-55K), resolved by 10% SDS-PAGE and visualized by immunoblotting. Co-precipitated proteins (top panel) and input levels 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, whereas corresponding proteins are labeled on the right.

was also unable to promote p53 SUMOylation, because we could not detect SUMO-conjugated p53 moieties (Figure 3b, right panel, lane 7). E1B-55K-R443A was able to maintain SUMO ligase activity toward p53 (Figure 3a, right panel, lane 6). This indicates that although the binding of R443A to PML-IV is impaired (Figure 1b, lane 11), it is adequate enough for efficient PML-NB localization (Figure 2, panel j) and E1B-55K-R443A SUMO ligase capacity toward p53. We also observed less SUMOylated E1B-55K-RTR compared with the wt viral protein or other E1B-55K-PML interacting variants (E1B-55K-NES, E1B-55K-R443A). This supports the idea that besides E1B-55K/PML-IV binding, the viral oncoprotein should interact with PML-V for subnuclear PML-NB localization and posttranslational SUMO modification of E1B-55K, and subsequent efficient SUMOylation of p53. These results are fascinating because interaction with specific PML isoforms has until now not been linked to SUMOylation of the adenoviral oncoprotein.

Finally, to further confirm a direct correlation between PML-IV/-V binding and E1B-55K SUMOylation, we performed SUMOylation analysis *in vitro*. We used *Escherichia coli*-produced, purified human SUMO2, SAE1/SAE2 (human SUMO E1), ubc9 (human SUMO E2) and *in vitro* translated E1B-55K variants. Addition of ubc9 stimulated ligation of SUMO2 to E1B-55K (Figure 5a). As anticipated, E1B-55K-SCM's mutation in the SCM completely abolished posttranslational modification with SUMO2 moieties (Figure 5a). Intriguingly, E1B-55K-RTR, which was not SUMOylated *in vivo*, showed poly-SUMO chains attached to the viral protein *in vitro*. This suggests that the inability of the PML-V-binding-deficient mutant E1B-55K-RTR to localize with PML-NBs is what prevents SUMO conjugation of the viral oncoprotein *in vivo*, not some intrinsic property of the protein, because this mutant can still be posttranslationally modified with SUMO protein as shown *in vitro* (Figure 5a, lane 9). Thus, E1B-55K is apparently SUMOylated

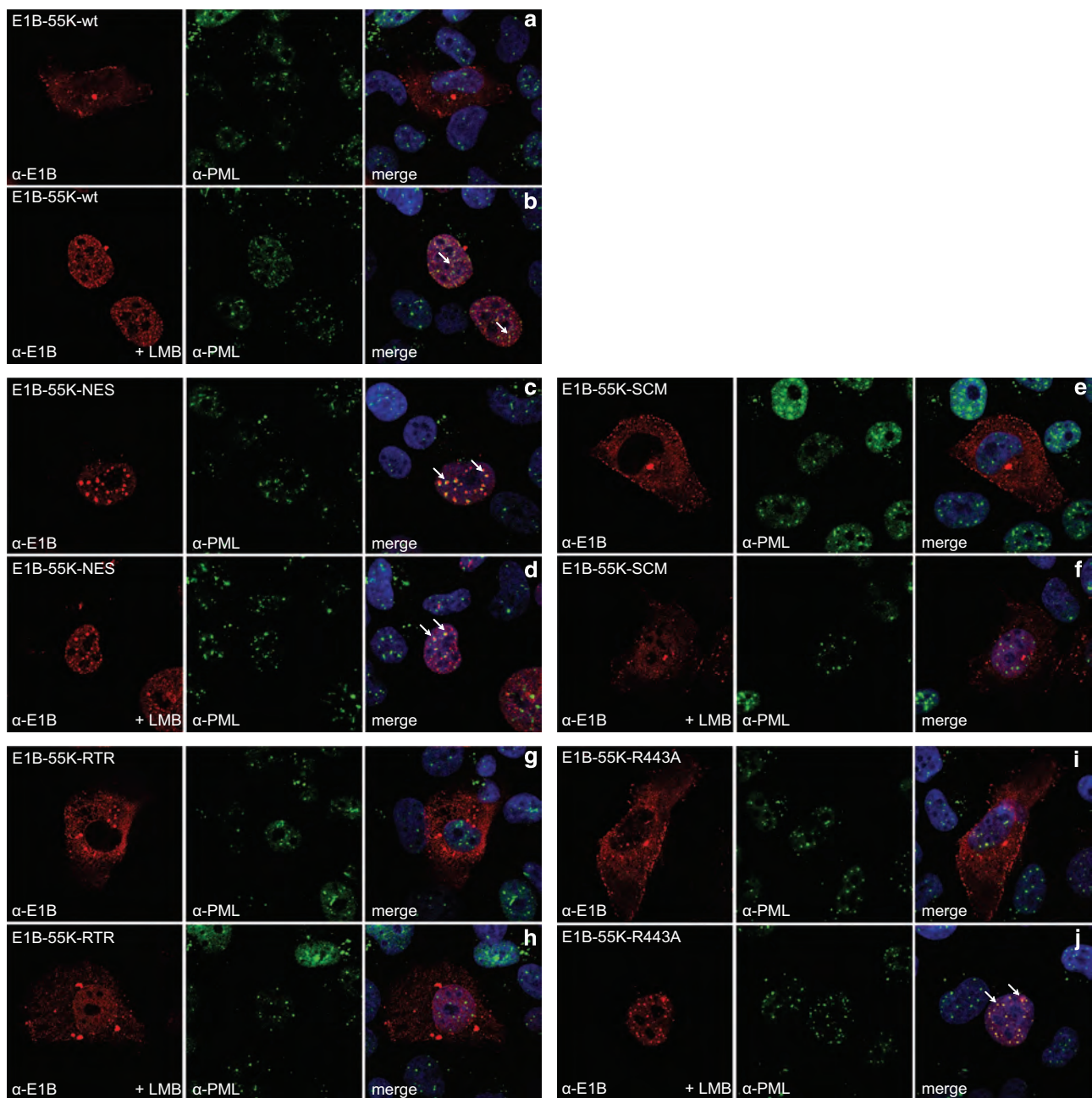


Figure 2. PML-IV/-V-binding targets E1B-55K to PML bodies. H1299 cells were transfected with constructs encoding E1B-55K variants, sometimes treated with +LMB to block E1B-55K nuclear export, and fixed with paraformaldehyde after 24 h, before labeling with mouse mAb 2A6 (E1B-55K) and PML rabbit pAb NB100-59787. Primary Abs were detected with Alexa Fluor 594- and Alexa Fluor 488-conjugated secondary Abs. Representative α -E1B-55K (red) and α -PML (green) staining patterns of at least 50 analyzed cells and overlays of the single images (merge; a–j) are shown. E1B-55K and PML-positive nuclear accumulations are indicated by white arrows.

because of specifically interacting with a PML isoform and hence localizing with PML-NBs.

To exclude any variations in ubc9 (human SUMO E2) association with E1B-55K, we validated binding of the viral proteins to the human SUMO E2 enzyme in plasmid-transfected human p53-negative H1299 cells (Figure 5b); immunoprecipitation of ubc9 resulted in signals for all E1B-55K variant proteins (Figure 5b, upper panel). However, less E1B-55K-SCM mutant viral protein was co-immunoprecipitated (Figure 5b, lane 3, upper panel). In addition, we also observed interaction of all mutants with PML co-immunoprecipitated with an endogenous

PML antibody recognizing both PML-IV and PML-V (Figure 5b, lower panel).

PML isoform binding of E1B-55K is essential for p53 subcellular localization

To study whether PML-IV/-V-binding-deficient E1B-55K mutants interfere with the intracellular localization of the viral protein, plasmids encoding wild-type E1B-55K and mutant variants were transfected into human cells together with human p53 (Figure 6). Consistent with the findings in Figure 2, E1B-55K was mainly

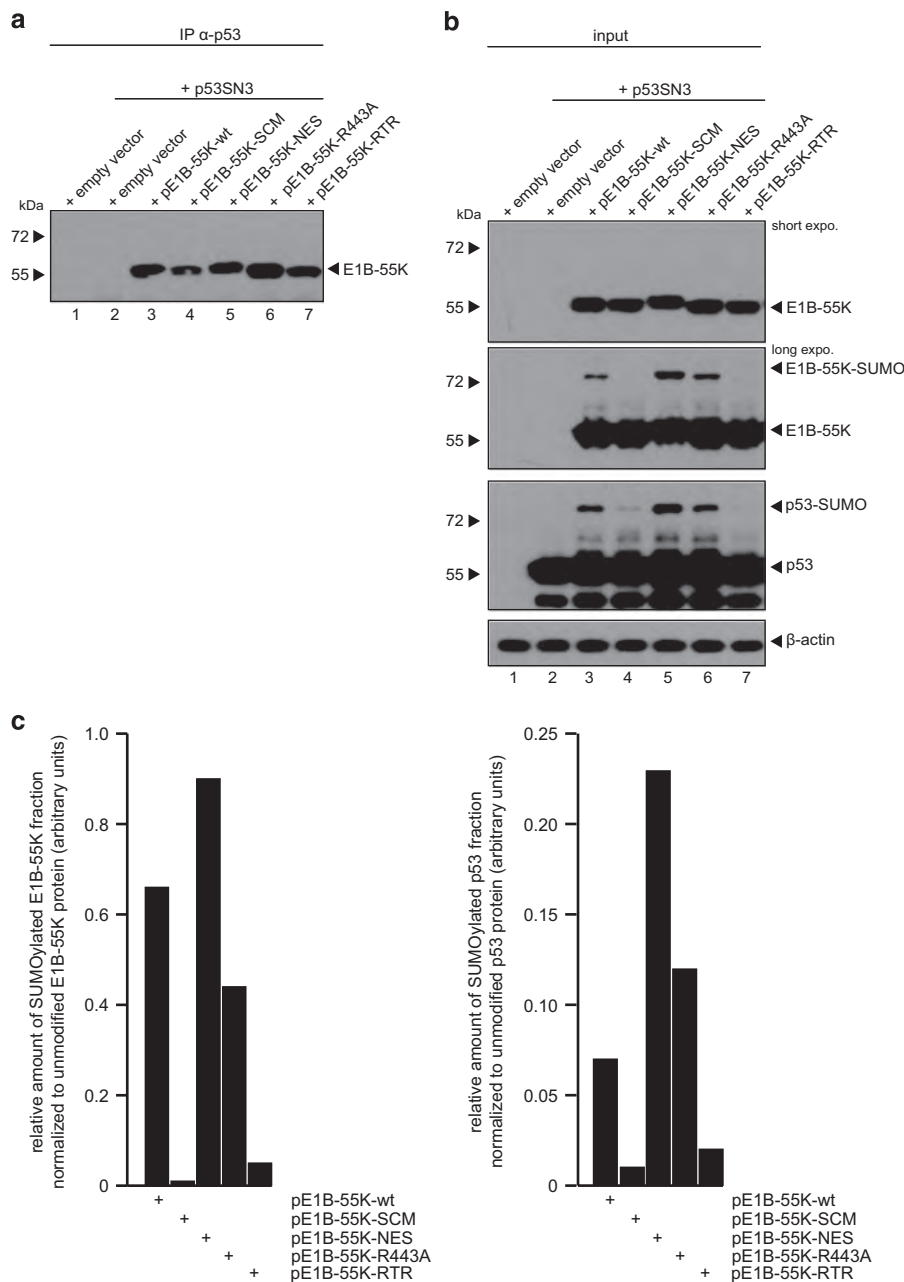


Figure 3. PML-IV/-V-binding E1B-55K induces p53 SUMO conjugation. Subconfluent H1299 cells were transfected with 5 μ g of pE1B-55K variants plus 3 μ g of human p53 (pc53SN3) and harvested after 48 h before preparing total cell extracts. Following immunoprecipitation of p53 using mAb DO-1 (α -p53), proteins were resolved by 10% SDS-PAGE and visualized by immunoblotting. Co-precipitated proteins (**a**) and input levels (**b**) of total cell lysates were detected using mAb 2A6 (α -E1B-55K), mAb DO-1 (α -p53) and mAb AC-15 (α - β -actin). Molecular weights in kDa are indicated on the left, whereas corresponding proteins are labeled on the right. (**c**) Graphs showing the relative amount of SUMOylated E1B-55K fraction normalized to unmodified E1B-55K protein (left panel) and the relative amount of SUMOylated p53 fraction normalized to unmodified p53 protein (right panel). Data were obtained from Figure 3a.

detected in the cytoplasm, mostly concentrated in a perinuclear body ($n > 50$; Figure 6, panel a). E1B-55K localization in these subnuclear aggregates, and thus sequestering p53 (Figure 6, panel a), is known to be directly linked to the transforming potential of the viral oncogene in combination with E1A.^{68,70,71}

After blocking the CRM1-dependent nuclear export of E1B-55K with +LMB, the wt viral protein accumulates with the cellular interaction partner p53 in dense nuclear aggregates (Figure 6, panel b).^{75,77} Similarly, nearly 80% of the cells ($n > 50$) transfected with E1B-NES mutant protein defective in efficient cytoplasmic export also accumulated numerous E1B-55K/p53 aggregates

distributed throughout the nucleus in the absence and presence of LMB (Figure 6, panels c and d). In contrast, we observed an exclusively cytoplasmic localization pattern of both the E1B-55K-SCM and E1B-55K-RTR mutants, in the absence or presence of LMB, whereas p53 is mostly diffusely distributed in the nucleus (Figure 6, panels e, j, g and h). However, these two PML-binding-deficient E1B-55K variants did sequester some p53 into random cytoplasmic aggregates (Figure 6, panels e, g, h and j). In the presence of LMB, which blocks nuclear export in almost all cells (90%), we observed no colocalization of p53 with E1B-55K-SCM and E1B-55K-RTR in nuclear aggregates (Figure 6, panels h and f).

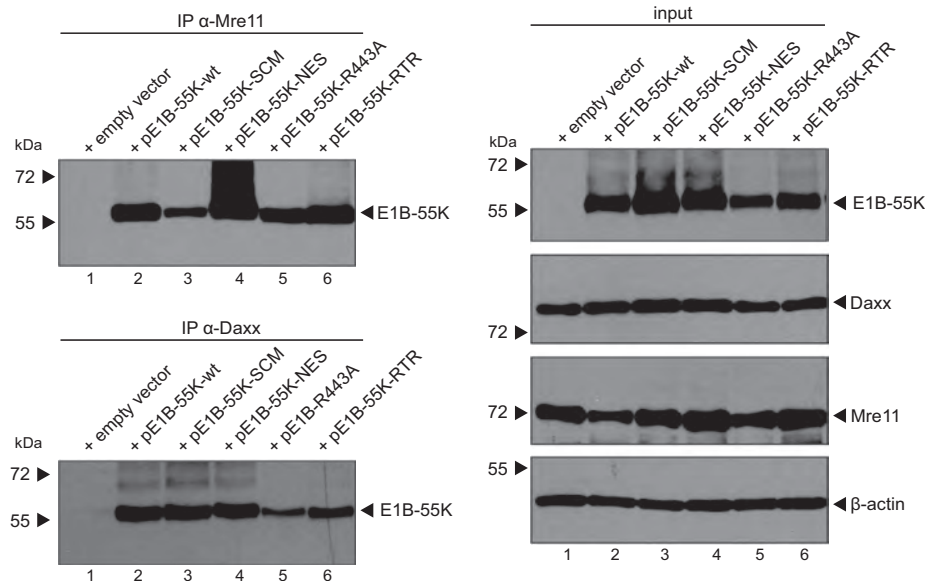


Figure 4. PML-IV/V does not affect E1B-55K binding to p53, Mre11 and Daxx. H1299 cells were transfected with 5 μ g of pE1B-55K variants and harvested after 48 h before preparing total cell extracts. After immunoprecipitation of endogenous Mre11 and Daxx using Mre11 rabbit pAb pNB 100–142 and rabbit pAb α -Daxx, proteins were resolved by 10% SDS–PAGE and visualized by immunoblotting. Co-precipitated proteins (left panel) and input levels (right panel) of total cell lysates were detected using mAb 2A6 (α -E1B-55K), Mre11 rabbit pAb pNB 100–142, rabbit pAb α -Daxx and mAb AC-15 (α - β -actin). Molecular weights in kDa are indicated on the left, whereas corresponding proteins are labeled on the right.

Taken together, one can conclude that inactivating the E1B-55K/PML interaction abolishes the capacity of the viral oncogene to efficiently or specifically relocate human p53.

PML-IV/V interaction promotes p53 repression by the viral oncoprotein E1B-55K

Given the above findings, we performed a transient reporter gene assay to evaluate the PML-binding-deficient E1B-55K variants' ability to repress p53-mediated transcriptional activation (Figure 7a). H1299 cells were transfected with plasmids encoding E1B-55K-wt, E1B-55K-SCM, E1B-55K-NES and E1B-55K-RTR plus a p53 expression plasmid (pC53SN3) and p53-independent, as well as reporter p53-dependent luciferase constructs for a normalized system read-out. The reporter construct contains five artificial p53-binding sites (pRE-Luc) and therefore *Firefly* luciferase expression directly correlates with transcriptional activation by p53, which in turn is directly influenced by the adenoviral E1B-55K protein.^{66,67,79}

As expected, neither transfection of p*Renilla*-Luc (Figure 7a, lane 1) nor p*Renilla*-Luc plus pRE-Luc (Figure 7a, lane 2) shows detectable luminescence because of the lack of endogenous p53 expression in H1299. However, additional co-transfection of pC53SN3 led to maximal activation of p53-dependent luciferase transcription/activity (Figure 7a, lane 3). Gradual addition of E1B-55K-wt drives a progressive drop in luciferase activity to a residual level of $\sim 10\%$ of the positive control (Figure 7a, lanes 4 to 7). Consistent with previous results,⁸⁰ it is interesting that relatively low amounts of E1B-55K (1.0–3.0 μ g) are sufficient to nearly completely repress p53 transcriptional activation.

As anticipated, SUMO-less pE1B-55K-SCM coexpression fails to efficiently repress luciferase activity (Figure 7a, lanes 8 and 9), whereas hyper-SUMOylated E1B-55K-NES represses p53 transactivation even more efficiently than the wt viral oncoprotein (Figure 7a, lanes 10 and 11).^{8,70,81} Intriguingly, PML-IV/V binding seems to be directly linked to the p53 repression abilities of the different E1B-55K mutants, because E1B-55K-RTR (PML-V-binding deficiency) did not efficiently repress p53 transcriptional activation

as E1B-55K-SCM (Figure 7a, lanes 12 and 13; schematically depicted in Figure 7b).

E1B-55K association with PML-IV/V is essential for efficient transforming potential in combination with E1A

To more closely evaluate whether modulating endogenous PML-IV/V is necessary for the transformation potential of E1B-55K, freshly prepared primary baby rat kidney cells were transfected simultaneously with plasmids encoding E1A and the newly identified PML-binding mutants. After incubation under standard conditions, *focus* formation was visualized by crystal violet staining and quantified relative to E1A plus E1B-55K-wt (Figure 8a). As expected, expression of E1A alone resulted in low *focus* formation activity (Figure 8a, lane 2), whereas simultaneous co-transduction with E1B-55K-wt significantly enhanced cellular transformation in combination with E1A (Figure 8a, mix 3). Interestingly, all combinations containing the PML-binding mutants pE1B-55K-SCM and pE1B-55K-RTR (Figure 8a, lanes 4 and 6) show a significant reduction in *focus*-forming activity compared with the positive control, suggesting that PML-IV/V interaction with E1B-55K is important for efficient cellular transformation.

To validate this hypothesis in another rodent system, we also performed transformation assays in primary baby mouse kidney cells with a stable genetic knock-out of the PML gene (PML^{-/-}).⁸² Consistent with the rat model system, we observed significantly lower cellular transformation levels with E1B-55K mutants defective in PML-IV/V binding in combination with E1A (Figure 8b, lanes 4 and 6).

Strikingly, the *focus*-forming activity of all E1B-55K variants in combination with E1A was drastically reduced in primary rodent cells genetically depleted for PML expression, implying that E1B-55K/PML cooperation is a prerequisite for efficient cell transformation (Figure 8b). As our results above show that E1B-dependent SUMOylation of p53 also depends on PML-IV/V interactions and is required for efficient cell transformation, this supports the idea that the SUMO ligase activity of E1B-55K is

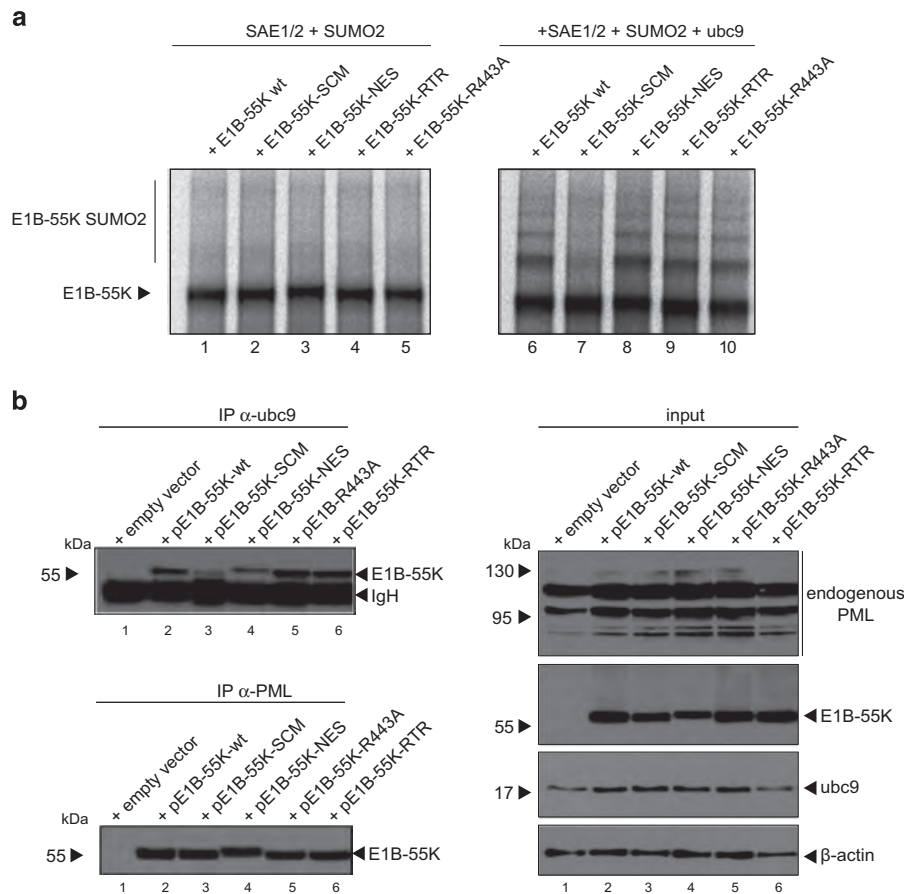


Figure 5. PML-NB association is a prerequisite for E1B-55K SUMO conjugation. **(a)** E1B-55K wt and mutants were produced using the wheat germ *in vitro* translation system (TNT) as described in Materials and Methods section. The reactions were incubated 4 h at 37 °C, then stopped by adding 2x SDS buffer, boiled and separated by SDS-PAGE. ³⁵S-labeled proteins were visualized by autoradiography. **(b)** Subconfluent H1299 cells were transfected with 5 µg of pE1B-55K variants and harvested after 48 h before preparing total cell extracts. Immunoprecipitation of endogenous Ubc9 or PML was performed using mAb α-Ubc9 or rabbit pAb α-PML, resolved by 10% SDS-PAGE and visualized by immunoblotting. Co-precipitated proteins (left panel) and input levels (right panel) of total cell lysates were detected using mAb 2A6 (α-E1B-55K), mAb α-Ubc9, rabbit pAb α-PML and mAb AC-15 (α-β-actin). Molecular weights in kDa are indicated on the left, whereas corresponding proteins are labeled on the right.

intimately linked to its growth-promoting oncogenic activities (Figure 8c).

DISCUSSION

Our results show that E1B-55K derivatives defective in binding either PML-IV or PML-V, and hence unable to localize with subnuclear PML, are no longer capable of mediating E1B-55K-dependent SUMOylation of p53. These mutated E1B-55K proteins are also defective in inhibiting p53-mediated transactivation and have greatly reduced transforming potential in primary rodent cells.

HAdVs encode several multifunctional proteins that initiate transformation of primary rodent cells.^{83–86} The oncogenic determinants of this viral model system are located within E1 and E4 region of the HAdV genome. The E1A and E1B proteins are absolutely required for oncogenic transformation,^{83–86} because they simultaneously accelerate cell cycle progression⁸⁷ and inhibit tumor-suppressor proteins such as p53.^{66,67,79}

As the initial description of adenoviral tumorigenicity⁸⁸ it is still unclear why HAdVs are oncogenic in rodents, but not as far as we know, in humans. With very few exceptions,^{89–96} attempts to transform primary human cells in culture using different types of HAdV subgenomic DNA fragments have proved extremely

inefficient. In contrast, primary rat, hamster or mouse cells can be transformed efficiently.^{83,84,97,98} Although purely speculative at this point, species-specific expression patterns of PML isoforms IV and V in humans and their orthologues in rodent tissue may represent the primate-specific factor as a prerequisite for efficient E1A/E1B-55K-mediated oncogenesis that mainly occurs in rodent cells, unlike cells of human origin. Although it is difficult to draw conclusions as yet, because no data are available on the expression of PML in rats, recent data on murine *pml* locus organization underline the possibility of species-specific PML expression with partially homologous, but nevertheless considerably different PML isoforms.^{52,99} Moreover, as PML expression patterns also differ considerably because of extensive regulation in different mammalian tissues, oncogenic transformation may only occur in a specific subset of human cells.

Obviously, the cellular PML protein represents a key regulator of multiple cellular functions,¹⁰⁰ because it has important implications in tumorigenesis.^{35,36} It was described as a general tumor-suppressor protein frequently deregulated in human tumor types: 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. In our study, we demonstrate that cellular

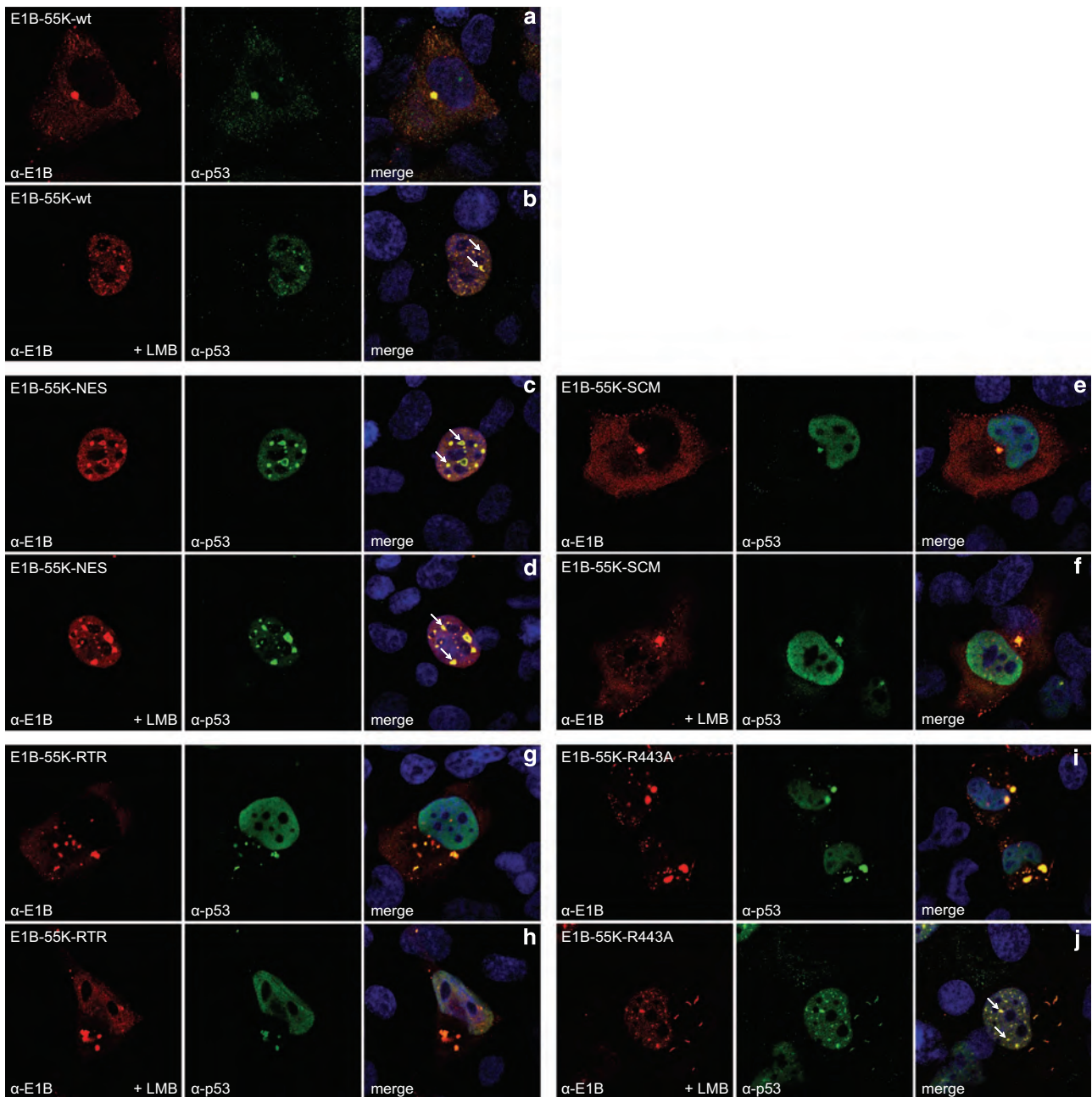


Figure 6. PML-IV/V-binding affects E1B-55K-dependent p53 subcellular localization. H1299 cells were transfected as described in Figure 2 before labeling with mouse mab 2A6 (E1B-55K) and DO-1 (α -p53). Primary Abs were detected with Alexa Fluor 594- and Alexa Fluor 488-conjugated secondary Abs. Representative α -E1B-55K (red) and α -p53 (green) staining patterns from at least 50 analyzed cells and overlays of the single images (merge; a–j) are shown. E1B-55K and p53-positive nuclear accumulations are indicated by white arrows.

transformation by adenoviral oncoproteins E1B-55K and E1A orchestrates a novel pathway, as the data clearly show that PML expression (PML-IV, PML-V) is essential for sustaining the oncogenic potential of E1B-55K. Presumably, the anti-oncogenic functions of PML involve secondary effects of PML-NBs as sites of a huge PML-regulated network with over 150 different proteins involved in over 750 separate interactions and various biological processes.¹⁰⁰

However, most of the molecular mechanisms, especially selective regulation of proteins by isoforms of PML (seven major isoforms so far), and any correlation with human tumorigenesis in different tissue types are still unknown. There are rare descriptions

about the different functions of each isoform, or the molecular mechanisms of how they fulfill different functions despite close sequence similarities. In principle, the functions of all PML isoforms may differ because of the biochemical behavior of the isoform and the number/kind of specific cellular interaction partners. In addition, 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, whereas the exchange rate for PML-V takes nearly an hour. Thus, it is assumed that PML-V represents the major scaffold protein of PML-NBs.^{101–103} Within our study, we showed for the first time that PML-IV and PML-V

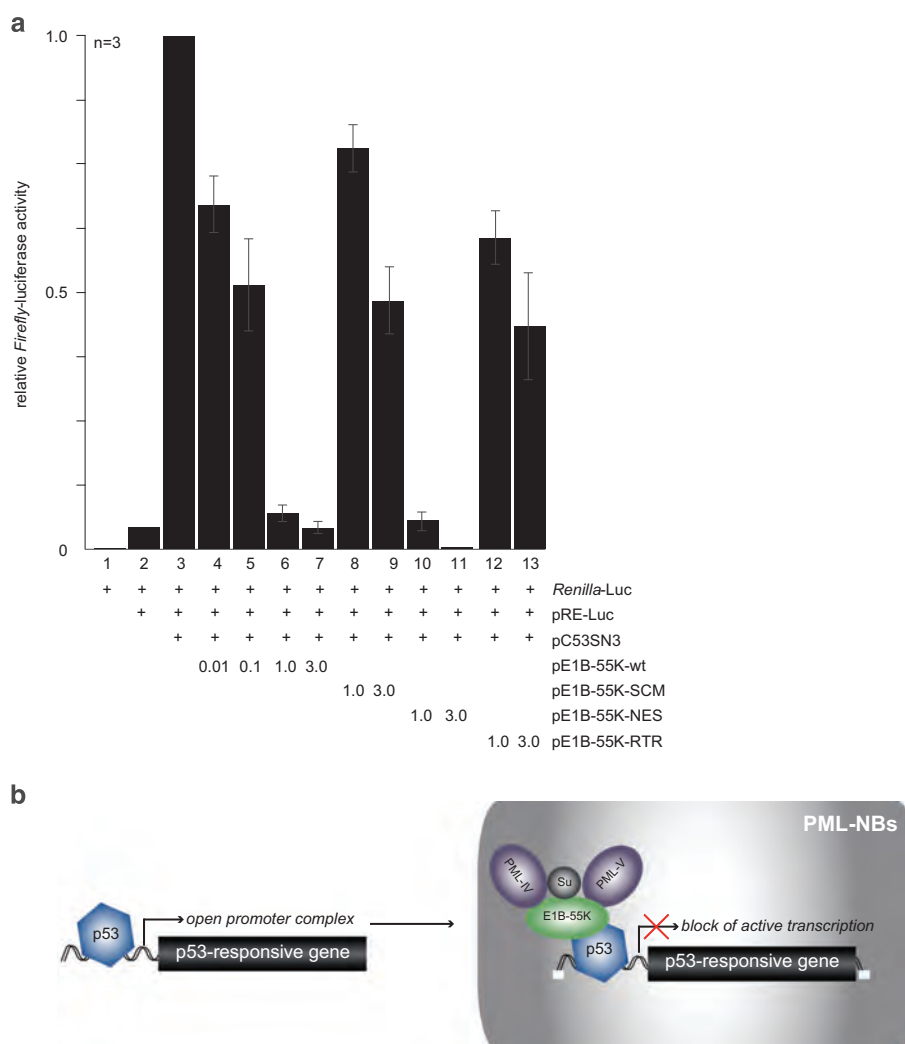


Figure 7. PML-IV/-V interaction promotes p53 repression by E1B-55K in transiently transfected H1299 cells. **(a)** Subconfluent p53-negative H1299 cells were transfected with 1.0 μg of pRenilla-Luc, 0.25 μg of pRE-Luc, 0.02 μg of pC53SN3, pE1B-55K-wt/-SCM/-NES/-RTR plus empty vector pcDNA3 to a total amount of 2.5 μg DNA. The amount of transfected E1B-55K expression plasmid is shown in μg . The cells were harvested after 48 h according to the manufacturer's instructions using the Dual-Luciferase Reporter Assay System (Promega) and subsequently measured in a Lumat LB 9507 luminometer (Berthold Technologies, Bad Wildbad, Germany). Luciferase activity was determined by correlating p53-dependent Firefly luciferase activity with p53-independent Renilla luciferase activity to account for transfection efficiency. The luciferase activities were plotted relative to the positive control (mix 3) and are based on the average of three independent experiments. **(b)** Model of the interplay between E1B-55K and p53-dependent transcriptional repression.

expression is highly implicated in oncogenic transformation of primary mammalian cells by adenoviral proteins E1A and E1B-55K.

Inconsistently, many reports illustrate the importance of PML deregulation and/or loss during tumorigenesis.^{27,35,36,104,105} On the contrary, PML is obviously stabilized by simultaneous expression of viral protein E1A,¹⁰⁶ as well as E1B-55K in stably HAdV transformed rodent cells,⁸ implying some function during adenoviral cell transformation. Similarly, p53 is stabilized by E1A,^{58,107–112} whereas the pro-tumorigenic functions of adenoviral E1B-55K are primarily linked to repression of the tumor-suppressor p53. Subsequent direct interactions,^{64,65} transcriptional repression^{66,67,79} and nuclear-cytoplasmic relocalization^{70,71} induce the complete silencing of p53-dependent tumor-suppressive functions.

Here, we show that the oncogenic functions of E1B-55K are tightly linked to binding of particular PML isoforms and efficient recruitment to PML-NBs. SUMOylation of E1B-55K at PML-NBs is a prerequisite for E1B-dependent modulation of p53, and thus closely linked to the growth-promoting activities of this viral oncoprotein. Although clearly speculative at this point, it appears

that some of the other factors such as TERT and TRF1 described to interact with PML-IV are implicated in telomere maintenance. This then may represent one of the previously suggested alternative pathways of E1B-55K-mediated cellular transformation.⁷

Neither PML-IV nor PML-V harbor identical protein sequences that are unique compared with the remaining isoforms, thereby ruling out a specific E1B-55K-binding region. Both harbor the exons 1–6, which are present in all PML proteins except PML-VII, exon 7a, which again is also present in PML-I/-II/-III and their own unique C-termini. 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 may contain an essential determinant for SUMOylation-dependent E1B-55K PML-IV interaction.

Conversely, efficient interaction with PML-V requires a highly conserved motif in the C-terminal region within E1B-55K. The sequence organization of this part of the viral oncoprotein seems to resemble some degree of organized tertiary structure comparable to a zinc-finger and/or RING domain. However, owing to the immense number of PML-NB-associated proteins¹⁰⁰ and

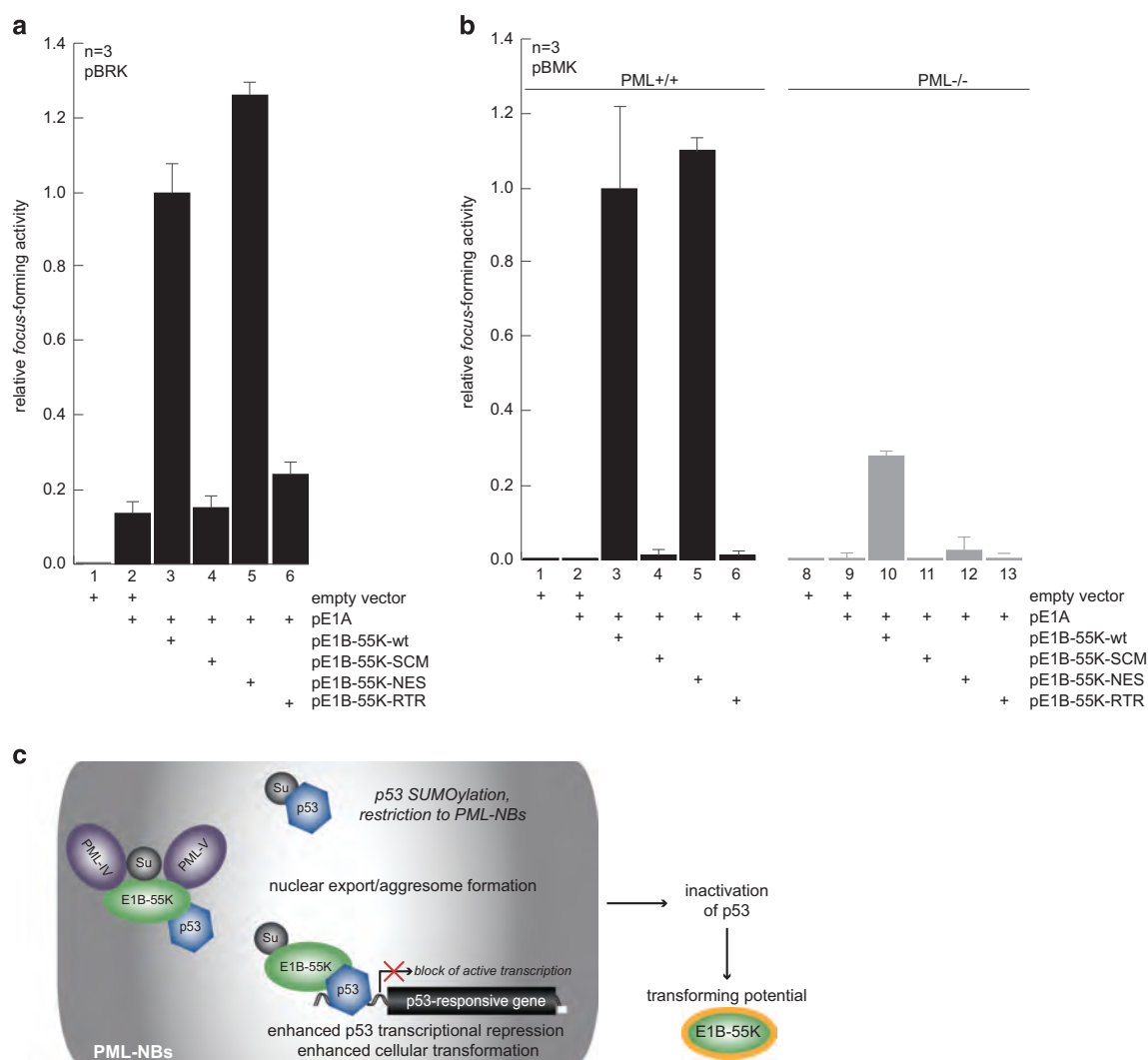


Figure 8. E1B-55K binding to PML-IV/-V promotes efficient transformation in combination with E1A. Freshly prepared primary (a) baby rat kidney (pBRK) and (b) baby mouse kidney cells (BMK/PML +/+, PML -/-) were simultaneously transfected with a combination of two plasmids encoding E1A and the individual E1B-55K variants. Cells were split after 48 h of absorption and cultivated for 6–8 weeks under standard conditions. Focus formation was visualized by crystal violet staining and absolute numbers were counted. Representative results normalized to the positive control (pE1A plus pE1B-55K-wt) were plotted based on the average of three independent experiments. (c) Model of the interplay between E1B-55K and the functional inhibition of the tumor-suppressor p53 mediated by the viral oncoprotein.

interactions of E1A and E1B-55K with various cellular components, it is also important to consider that specific binding of certain PML isoforms possibly occurs indirectly.

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 the pro-tumorigenic functions of E1B-55K through modulating p53. 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 augmented transformation of primary rodent cells through the accumulation of p53, E1B-55K and PML in subnuclear aggregates.^{70,71,113}

In contrast, mutational inactivation of the SCM domain abrogates E1B-55K SUMOylation, PML-IV interaction, p53 transcriptional repression and the oncogenic potential of E1B-55K in combination with E1A.^{78,114} Certainly, inactivation of PML-V binding to E1B-55K adds another layer of complexity to adenoviral-mediated p53 modulation; such a mutation also abrogates E1B-55K SUMOylation, p53 inhibition and the transforming capacity of E1B-55K in combination with E1A.⁷⁸

p53 itself is SUMOylated at an apparently unique C-terminal lysine residue (K386), resulting in modulated transcriptional activity *in vivo*.^{115–119} Although the modulation of p53 activity by SUMO conjugation remains controversial, this observation again underlines the difficulty in assigning particular functions to SUMOylation. However, simultaneous expression of E1B-55K and p53 leads to enhanced posttranslational modification of the tumor-suppressor protein in an E1B-55K-dependent manner, and here SUMO modification of E1B-55K itself is absolutely essential.^{9,78}

Besides the hypothesis that E1B-55K serves as an E3 SUMO ligase,⁹ a so far unknown factor may also facilitate p53 SUMOylation in the presence of SUMOylated E1B-55K. In this context, our results in general offer a new perspective to not only unravel the complexity of E1B-55K/PML cooperation, but also HAdV-mediated transcriptional repression of the tumor-suppressor protein p53.^{66,67,79} In addition, E1B-55K's oncogenic potential may also involve the proper localization of E1B-55K in subnuclear aggregates sequestering p53 and PML, which is regulated at least in part by the viral protein's binding capacity to PML-IV and PML-V.

In summary, a growing body of evidence suggests that repression of p53 by E1B-55K in isolation during transient transfection, involves additional determinants such as posttranslational modification, which mainly occur at PML-NBs.^{31,70,71,113,120–124} E1B-55K exploits certain PML isoforms and associated proteins to mediate its multiple functions during oncogenic processes, in particular conditional transcriptional repression of p53 (refs 65,125–133) and control proteins involved in transcriptional regulation, tumor suppression, DNA repair and apoptosis.^{6,7,10,69}

Our study provides novel insight into the molecular mechanism regulating subnuclear localization of E1B-55K via PML-IV/PML-V interactions and PML-NB recruitment. Furthermore, isoform-specific interaction of E1B-55K and/or the remaining adenoviral oncoproteins represents a general mechanism of HAdV5 to gain access to the PML regulatory network and modulate the functions of specific PML-associated proteins. Identifying new interaction partners of the adenoviral oncoproteins may not only provide insights into the fundamental differences between human and rodent cells, but also into the basic principles of tumorigenesis, and potentially illuminate oncogenic processes in human tissue.

MATERIALS AND METHODS

Cell lines and culture conditions

H1299,¹³⁴ HEK293⁸⁹ and primary baby rat kidney cells¹³⁵ were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, 100 U of penicillin, 100 µg of streptomycin per ml in a 5% CO₂ atmosphere at 37 °C.

Plasmids, transient transfections and reporter gene assays

HAdV5 E1B-55K-wt, the E1B mutant proteins E1B-55K-NES (L83/87/91 A), E1B-55K-SCM (K104R),⁷¹ E1B-55K-R443A (R443A),¹⁰ E1B-55K-RTR (RTR448-/449/450AAA)¹⁰ and human p53¹³⁶ were expressed from pcDNA3. Subconfluent cells were transfected using 25 kDa polyethylenimine (Polysciences, Eppelheim, Germany) as described before.⁸ For luciferase assays, H1299 cells were transfected with indicated amounts of reporter and effector plasmids (see figure legend) and 1.0 µg of pRL-TK (Promega, Madison, WI, USA) expressing *Renilla* luciferase under control of the HSV-TK promoter. The p53-dependent (pRE-Luc) reporter constructs for expressing *Firefly* luciferase and p53 have been described previously.^{71,137} Total cell extracts were prepared 36 h after transfection and *Firefly* activity was assayed in an automated luminometer measuring relative light units (luminescence) using *Dual-Luciferase Reporter Assay* (Promega). All samples were normalized for transfection efficiency by measuring *Renilla* activity.

Transformation assays

To test the oncogenic potential of E1B-55K in combination with E1A, freshly prepared primary baby rat kidney or baby mouse kidney cells were prepared from 3 to 5-day-old CD rats (Charles River, Wilmington, MA, USA)¹³⁷ or mice.⁸² Subsequently, primary cells were transfected with the respective plasmid¹³⁸ or altered versions with the respective mutations in the E1B-55K. Cells were expanded after 48 h, cultivated for 4–6 weeks changing the medium every 3–4 days, and multilayered cell accumulations (*foci*) were visualized by crystal violet staining.

Antibodies and protein analysis

Primary Abs for HAdV5 proteins included E1B-55K mouse mAb 2A6.¹³⁹ Primary Abs for detecting of cellular and etopically expressed proteins used, included p53 mouse mAb DO-1 (Sigma-Aldrich, Inc., St Louis, MO, USA), PML rabbit pAb NB100-59787 (Novus Biologicals, Inc., Littleton, CO, USA), Mre11 rabbit polyclonal antibody pNB 100-142 (Novus Biologicals, Inc.), PML rabbit pAb NB100-59787 (Novus Biologicals, Inc.), mAb α-Ubc9 (BD Transduction, Heidelberg, Germany), rabbit pAb α-Daxx (Millipore, Darmstadt, Germany), β-actin mouse mAb AC-15 (Sigma-Aldrich, Inc.) and mouse mAb Flag-M2 (Sigma-Aldrich, Inc.). All protein extracts were prepared in RIPA buffer (50 mM Tris-HCl/pH 8.0, 150 mM NaCl, 5 mM EDTA, 1 mM DTT, 0.1% SDS, 1% NP-40, 0.1% Triton X-100, 0.5% sodium deoxycholate) containing 1% (v/v) phenylmethylsulfonyl fluoride, 0.1% (v/v) aprotinin, 1 µg/ml leupeptin, 1 µg/ml pepstatin, 1 mM DTT, 25 mM

iodoacetamide and 25 mM N-ethylmaleimide. For IP, proteinA-sepharose was coupled with primary Ab for 1 h at 4 °C. The Ab-coupled beads were added to precleared extracts and rotated for 2 h at 4 °C. Proteins bound to the beads were precipitated by centrifugation, washed three times and boiled for 3 min at 95 °C in 2xLaemmli buffer. For immunoblotting of protein, input levels/IP solutions equal amounts were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE), transferred to nitrocellulose (Schleicher & Schüll/Whatman, Freiburg, Germany) and incubated as described previously.¹⁰⁶ Bands were visualized by ECL as recommended by the manufacturer (Pierce, Rockford, IL, USA) on X-ray films (CEA RP, Hamburg, Germany; medical X-ray film). Autoradiograms were scanned, cropped and prepared using Adobe PhotoshopCS6 and Adobe IllustratorCS6.

Indirect immunofluorescence

Cells grown on glass coverslips in six-well dishes were transfected for 24 h with up to 1 µg of plasmid DNAs expressing E1B-55K or mutants. Some wells were treated with 20 nM LMB for 6 h. Cells were then fixed for 15 min with 4% paraformaldehyde (Electron Microscopy Science, Hatfield, PA, USA), and permeabilized for 15 min with 0.5% Triton X-100 in phosphate-buffered saline. Following washes in phosphate-buffered saline, immunofluorescence was performed with appropriate antibodies for 2 h at room temperature in a humidity chamber. The secondary antibodies used were antibodies conjugated to Alexa 594 and Alexa 488 dyes (Molecular Probes, Darmstadt, Germany). 4,6-Diamidino-2-phenylindole dye was added with the secondary antibodies. Coverslips were washed and mounted in Immumount (Thermo Scientific, Darmstadt, Germany). In all cases, we have analyzed cells expressing low-to-intermediate levels of protein to avoid potential localization artifacts related to overexpression. Images were obtained using an LSM4 confocal microscope (Zeiss, Jena, Germany) with a 63x objective using the Zen 2011 Image browser software (Jena, Germany). Images were cropped using Adobe PhotoshopCS6 and assembled with Adobe IllustratorCS6.

In vitro translation and *in vitro* SUMOylation

E1B-55K wt and mutants were produced using the wheat germ *in vitro* translation system (TNT, Promega) and 5 µCi L-[³⁵S]methionine (43 TBq/mMol, 10 µCi/µl; Perkin-Elmer, Waltham, MA, USA) according to the supplier's protocol. *In vitro* SUMOylation reactions contained 1.5 µl ³⁵S-labeled substrate, 1.5 µg SUMO-1, 10 ng Ubc9, 100 ng SAE1/2, 50 mM Tris-HCl, pH 7.6, 5 mM MgCl₂, 2 mM ATP, 10 mM creatine phosphate, 3.5 U/ml creatine kinase and 0.6 U/ml inorganic pyrophosphatase. All *in vitro* SUMOylation reagents were kindly provided by Ron Hay. The reactions were incubated 4 h at 37 °C, then stopped by adding 2x SDS buffer, boiled and separated by SDS–PAGE. ³⁵S-labeled proteins were visualized by autoradiography.

CONFLICT OF INTEREST

PW is currently an employee of Novartis. The remaining authors declare no conflict of interest.

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