

ORIGINAL ARTICLE

Sp100A is a tumor suppressor that activates p53-dependent transcription and counteracts E1A/E1B-55K-mediated transformation

J Berscheminski¹, J Brun¹, T Speiseder¹, P Wimmer¹, WH Ip¹, M Terzic², T Dobner¹ and S Schreiner^{1,2}

Human adenoviruses (HAdV) are used as a model system to investigate tumorigenic processes in mammalian cells where the viral oncoproteins E1A and E1B-55K are absolutely required for oncogenic transformation, because they simultaneously accelerate cell cycle progression and inhibit tumor suppressor proteins such as p53, although the underlying mechanism is still not understood in detail. In our present study, we provide evidence that E1B-55K binding to the PML-NB component Sp100A apparently has an essential role in regulating adenovirus-mediated transformation processes. Specifically, when this E1B-55K/Sp100A complex recruits p53, Sp100A-induced activation of p53 transcriptional activity is effectively abolished. Hence, Sp100A exhibits tumor-suppressive activity, not only by stabilizing p53 transactivation but also by depressing E1A/E1B-55K-mediated transformation. E1B-55K counteracts this suppressive activity, inducing Sp100A SUMOylation and sequestering the modified cellular factor into the insoluble matrix of the nucleus or into cytoplasmic inclusions. These observations provide novel insights into how E1B-55K modulates cellular determinants to maintain growth-promoting activity during oncogenic processes and lytic infection.

Oncogene (2016) 35, 3178–3189; doi:10.1038/onc.2015.378; published online 19 October 2015

INTRODUCTION

Sp100 (speckled protein 100 kD) is a major constituent of PML-NB (promyelocytic leukaemia protein/PML nuclear body), involved in chromatin condensation and transcriptional regulation with a stimulatory effect on p53-dependent gene expression.^{1–4} Similar to the *pml* gene, four alternatively spliced mRNAs are expressed from the human *sp100* gene:^{1,2,5,6} Sp100A, Sp100B, Sp100C and Sp100HMG. All of them harbor an N-terminal HSR (homogenous staining region) domain required for oligomerization and PML-NB localization.⁷ Sp100B, Sp100C and Sp100HMG contain additional domains, such as the SAND domain, the bromodomain, the PHD (plant homeodomain) and the HMG domain, all of which have previously been linked to transcriptional regulation, although the detailed function of these Sp100 isoforms are still largely unknown.^{5,8}

Sp100 proteins were also recently shown to have a role during virus infection. Sp100 represses transcription and replication of HPV18 (*human papillomavirus type 18*) only during the initial stages of viral establishment, suggesting that Sp100 acts as a repressor of incoming HPV DNA.⁹ For EBV (Epstein barr virus), Sp100 was suggested to represent a mediator of EBNA-LP coactivation with a role in latent infections.¹⁰ HCMV (human cytomegalovirus) causes the degradation of Sp100 proteins that would otherwise restrict viral gene expression and replication.^{11,12} During Kaposi's sarcoma-associated herpesvirus infection, we recently proposed a model where Sp100 acts as a negative regulator of PRC2 (polycomb repressive complex) recruitment, suggesting that Kaposi's sarcoma-associated herpesvirus actively controls PML-NB proteins to promote establishment of latency.¹³

Recently, we also showed that Sp100A is able to promote HAdV (human adenovirus) gene transcription, whereas Sp100B/C/HMG act as transcriptional repressors. These host restriction factors are potentially inactivated by their active displacement from HAdV-induced PML track-like structures surrounding the newly formed viral replication centers. In contrast, Sp100A is retained to amplify HAdV gene expression.¹⁴

Overexpression of the HAdV oncoprotein E1B-55K (early region 1B 55K) revealed functional association with several PML-NB components, such as PML-IV, PML-V, Daxx (death-associated protein 6) and p53.^{15–17} Intriguingly, these interactions are tightly linked to the transforming potential of the viral factor in non-lytic infections, where E1B-55K contributes to complete cell transformation by antagonizing tumor-suppressor proteins, such as p53. Indeed, E1B-55K uses several mechanisms to inhibit p53 function,¹⁸ such as inhibiting p53-dependent transactivation by directly interacting with the tumor suppressor, accompanied by tethering the E1B-55K C-terminal repression domain to p53 target genes.^{19,20} In addition, to counteract antiviral measures, E1B-55K infiltrates PML-NBs by interacting with PML-IV and PML-V, which causes p53 SUMOylation, again inhibiting p53 transcriptional activity.^{21,22} The E1B-55K leucine-rich nuclear export signal (NES) and the SUMO conjugation motif (SCM) are required for E1B-55K-mediated nuclear-cytoplasmic relocalization of p53, inducing the complete silencing of p53-dependent functions.^{23,24}

Here, we performed mutational analysis to ask whether E1B-55K interacts with the Sp100 component of PML-NBs, and if so, to define an interaction motif within the E1B-55K polypeptide. Our results revealed that E1B-55K specifically interacts with the Sp100A isoform in a SUMOylation-dependent manner. E1B-55K

¹Department of Viral Transformation, Heinrich Pette Institute, Leibniz Institute for Experimental Virology, Hamburg, Germany and ²Institute of Virology, Technische Universität München/Helmholtz Zentrum München, Munich, Germany. Correspondence: Dr T Dobner, Department of Viral Transformation, Heinrich Pette Institute, Leibniz Institute for Experimental Virology, 20251 Hamburg, Germany or Dr S Schreiner, Institute of Virology, Technische Universität München/Helmholtz Zentrum München, Munich, Germany. E-mail: thomas.dobner@hpi.uni-hamburg.de or sabrina.schreiner@tum.de

Received 17 March 2015; revised 3 August 2015; accepted 14 August 2015; published online 19 October 2015

mutants defective in Sp100A binding disrupted both nuclear localization of the viral protein and Sp100 association with the nuclear matrix fraction. Moreover, we observed that E1B-55K efficiently induced the SUMOylation of Sp100A, which relocalized this cellular transcription factor into nuclear aggregates colocalizing with the tumor suppressor p53. Additional data illustrated that E1B-55K facilitates an association between Sp100A and p53 proteins that efficiently counteracts Sp100A-mediated activation of p53-dependent transactivation.

RESULTS

HAdV5 E1B-55K specifically interacts with the human Sp100A isoform

E1B-55K has been shown to interact with different resident PML-NB components such as Daxx/ATRAX, PML-IV and PML-V. To ascertain whether E1B-55K interacts with Sp100, human H1299 cells were transfected with E1B-55K and plasmids encoding human Sp100A, B, C or HMG (Figure 1a). After precipitating Flag-Sp100 and subsequently staining for E1B-55K, we detected an interaction between Sp100A and the viral protein (Figure 1a, left panel, lane 3). We also detected a band at ~ 75 kDa, corresponding

to the size of SUMO-modified E1B-55K (Figure 1a, left panel, lane 3). This observation implies that Sp100A interacts with both E1B-55K and E1B-55K SUMO moieties.

Previous studies have shown that in the course of an infection, E1B-55K initially localizes to PML structures prior to associating with newly formed replication centers upon the expression of HAdV E4orf6.²⁵ Further findings provided data that E4orf6 negatively affects SUMO modification of E1B-55K, which is essential for its localization to the nuclear matrix.²⁶ As the majority of E1B-55K accumulates in a complex with E4orf6, we tested whether expression of this additional viral factor affects E1B-55K binding to endogenously expressed Sp100A (Figure 1b). Again, E1B-55K co-precipitated with endogenous Sp100 (Figure 1b, left panel, lane 2) and the presence of E4orf6 was unable to destabilize this virus-host protein interaction (Figure 1b, left panel, lane 4).

E1B-55K SUMOylation and the C-terminal repression domain promote binding to Sp100A

To map the Sp100A binding domain in E1B-55K, we included different E1B-55K variants (Figure 2A) to our co-immunoprecipitation

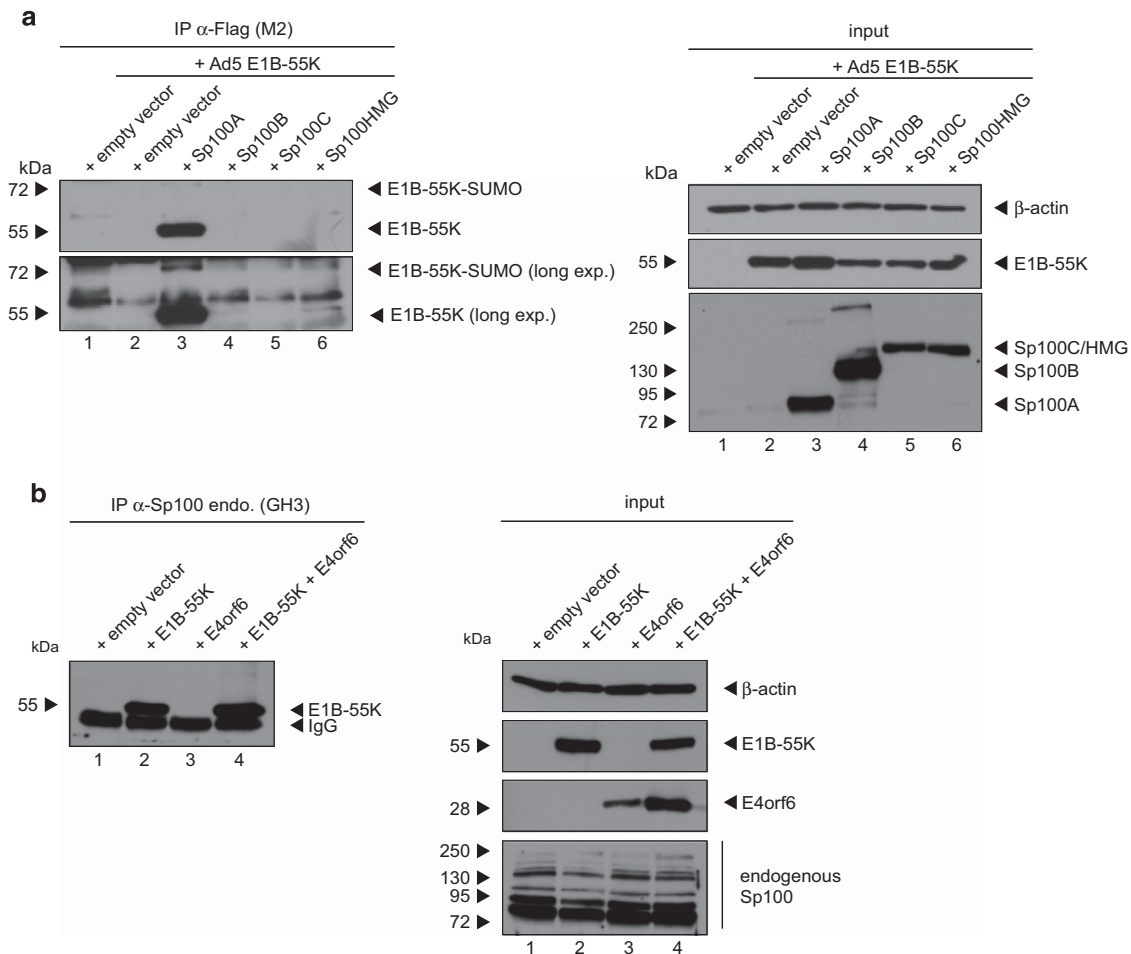
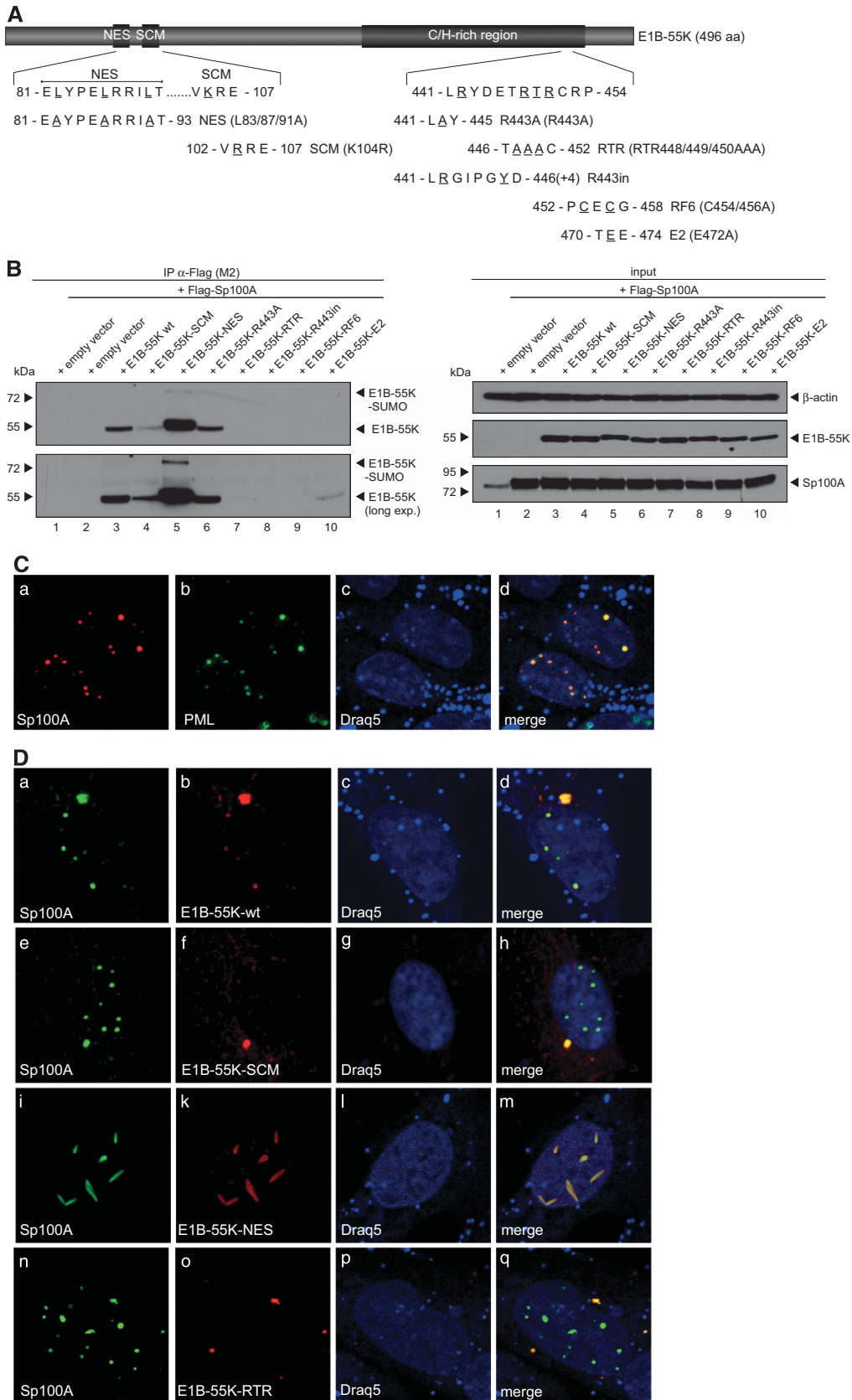


Figure 1. E1B-55K interacts specifically with Sp100A in transiently transfected cells. **(a)** H1299 cells were transfected with 3 μ g of pE1B-55K and 5 μ g of different constructs encoding N-terminal Flag-tagged human Sp100A, B, C and HMG, then harvested after 48 h to prepare total-cell extracts. Flag-Sp100 was immunoprecipitated using mAb Flag-M2 (α -Flag), and proteins resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) were visualized by immunoblotting. Co-precipitated proteins (left panel) and input levels of total-cell lysates (right panel) were detected using mAb 2A6 (α -E1B-55K), pAb GH3 (α -Sp100) and mAb AC-15 (α - β -actin). **(b)** H1299 cells were transfected with 3 μ g of pE1B-55K and/or pE4orf6 and harvested after 48 h to prepare total-cell extracts. Endogenous Sp100 was immunoprecipitated using pAb GH3 and proteins resolved by SDS-PAGE were visualized by immunoblotting. Co-precipitated proteins (left panel) and input levels of total-cell lysates (right panel) were detected using mAb 2A6 (α -E1B-55K), pAb GH3 (α -Sp100), mAb RSA3 (α -E4orf6) and mAb AC-15 (α - β -actin).



analysis (Figure 2B). Although the E1B-55K mutants showed slightly altered gel migration properties, input levels of E1B-55K and Sp100A were comparable (Figure 2B, right panel). Our data provide evidence that E1B-55K with a point mutation in the SCM (K104R) is impaired in Sp100A binding (Figure 2B, left panel, lane 4). E1B-55K with a non-functional NES resulting in nuclear retention of the viral protein, showed strong affinity towards Sp100A (Figure 2B, left panel, lane 5). We also observed that the C-terminal repression domain of E1B-55K seems important for Sp100A interaction, because E1B-55K-RTR, -R4433in, -RF6 and -E2 mutants lacked Sp100A binding (Figure 2B, left panel, lanes 7–10). These mutants have previously been described to exhibit the defects in binding other PML-NB factors such as PML (E1B-55K-RTR), Mre11 (E1B-55K-RF6)²⁷ and Daxx (E1B-55K-E2).¹⁷ Intriguingly, these mutants also failed to be posttranslationally modified with SUMO.²²

We next monitored the intracellular localization of E1B-55K with respect to the Sp100A protein. To exclude unspecific Sp100A pattern due to overexpression of the protein, we first substantiated PML-NB association (Figure 2C, a and d). To then evaluate whether Sp100A co-localizes with E1B-55K, human cells were transfected with the E1B-55K-wt, E1B-55K-SCM, E1B-55K-NES or E1B-55K-RTR together with Flag-tagged Sp100A. Co-staining with monoclonal antibody (mAb) 4E8 (α -E1B-55K) and mAb M2 (α -Flag) revealed that E1B-55K-wt and Sp100A co-localize within the nucleus in dot-like structures and in several cytoplasmic aggregates (Figure 2D, a–d). This was unexpected, because E1B-55K alone is known to localize predominantly in cytoplasmic aggregates. Usually only the expression of additional viral factors such as E4orf3 and/or E4orf6 induce nuclear retention of E1B-55K.²⁸ Our data show that increased abundance of Sp100A is sufficient to retain E1B-55K in the nucleus.

Consistent with our binding assay results, E1B-55K-SCM and E1B-55K-RTR proteins showed exclusively cytoplasmic staining, and they did not colocalize with Sp100A in nuclear dot-like structures. However, Sp100A was still observed within these structures, which presumably represent PML-NBs (Figure 2D, e–h, n–q). Interestingly, expression of the intensively SUMOylated E1B-55K-NES protein resulted in various Sp100A-containing rod-like structures in the nucleus, which efficiently colocalize with the viral protein fraction (Figure 2D, i–m).

Sp100 oligomerization is dispensable for nuclear retention of E1B-55K

Next, we investigated whether the Sp100A HSR domain, essential for PML-NB localization and oligomerization,⁷ promotes the accumulation of Sp100A and E1B-55K-NES in nuclear rod-like structures. An Sp100A mutant with a deletion in the HSR domain (aa 33–139; Figure 3A) was transfected into HepaRG cells. As expected, this Sp100 isoform showed a diffuse localization pattern and no PML-NB localization (Figure 3B, panel b); however, this Δ HSR mutant protein was still able to interact with E1B-55K in

immunoprecipitation assays (Figure 3C, left panel, lane 6). Co-transfection with E1B-55K led to the accumulation of both proteins in large cytoplasmic aggregates and small nuclear dot-like structures (Figure 3D, panels a–d), although much of Sp100A- Δ HSR was diffusely distributed inside the nucleus (Figure 3D, panel a). As already shown in Figure 2, E1B-55K-SCM assumed a cytoplasmic localization (Figure 3D, panel f). E1B-55K-NES accumulated together with Sp100A in nuclear dot- and rod-like structures (Figure 3D, i–m). These results imply that E1B-55K recruits Sp100A into dot-like structures independent of the HSR domain in the cellular protein. However, E1B-55K-NES-induced elongation of Sp100A structures in the nucleus is dependent on the oligomerization capacity of Sp100A, as without the HSR domain no rod-like structures can be observed. Intriguingly, when cotransfected with either E1B-55K-SCM (Figure 3D, panel h) or E1B-55K-RTR (Figure 3D, panel q), Sp100A- Δ HSR showed some punctate cytoplasmic foci colocalizing with these E1B variants, although the majority of the cellular factor was still diffusely distributed in the nucleus.

E1B-55K induces SUMOylation of Sp100A and recruitment to the insoluble nuclear matrix fraction

To further substantiate these findings, we performed fractionation and western blot analysis after co-expression of Sp100A with E1B-55K-wt, E1B-55K-SCM, E1B-55K-NES or E1B-55K-RTR. Sp100A abundance increased within the nuclear matrix fraction upon E1B-55K-wt and E1B-55K-NES expression (Figure 3E, lanes 2, 4). Sp100A nuclear matrix levels upon expression of E1B-55K-SCM and E1B-55K-RTR remained comparable to the empty vector control (Figure 3E, lanes 1, 3, 5).

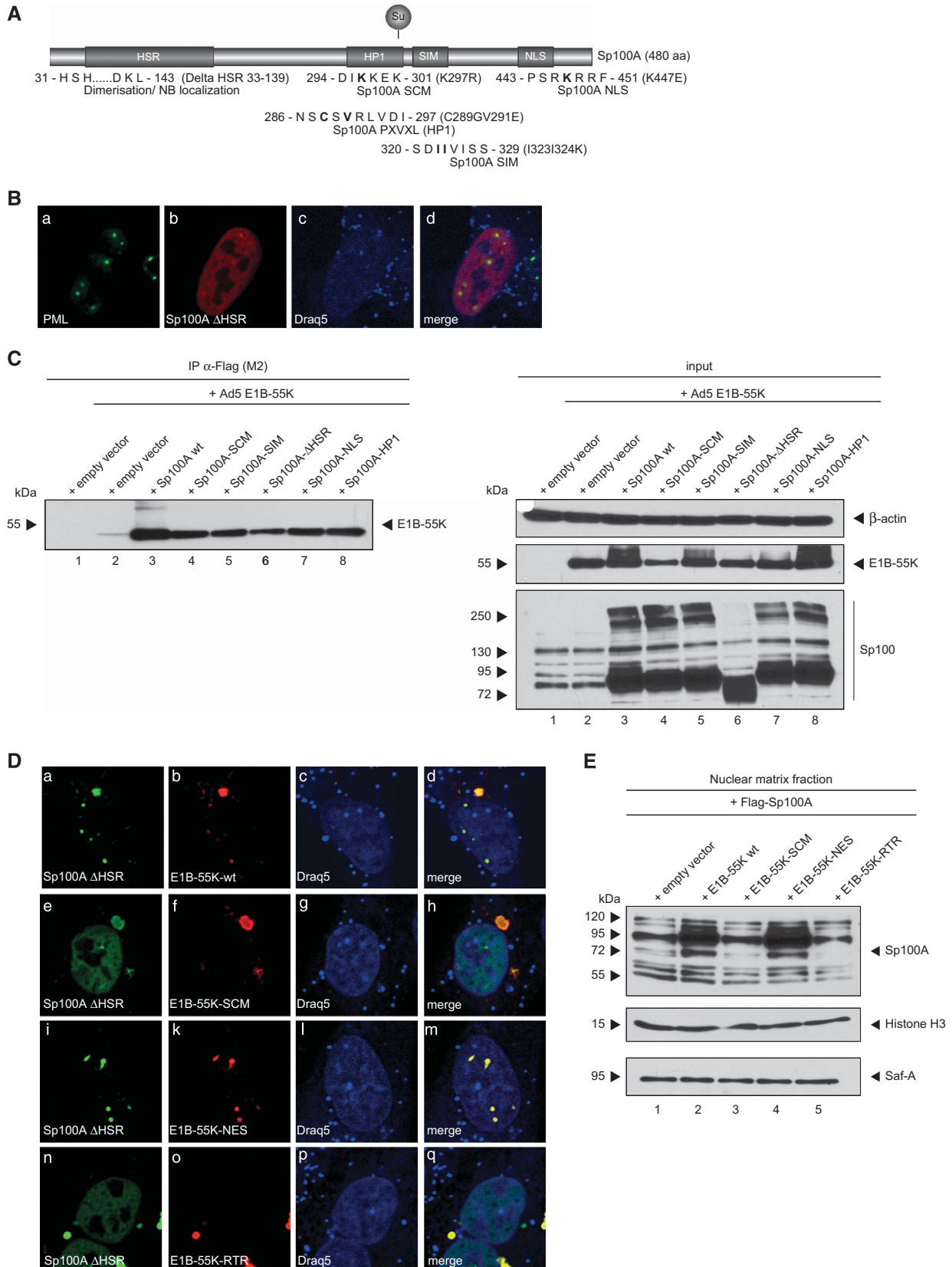
Interaction of E1B-55K with PML-IV and PML-V was recently reported to induce efficient relocalization of p53 to PML-NBs, which in turn induces SUMOylation of p53 at PML-NBs, presumably via the viral protein's SUMO ligase activity.^{21,22,29} As our results imply that E1B-55K recruits Sp100A to nuclear dot-like structures, we tested whether E1B-55K also affects the SUMO modification of Sp100 (Figure 4a). HeLa cells stably expressing His-SUMO-2 were transfected with Sp100A plus empty vector or E1B-55K-wt (Figure 4a). Immunoblotting of Ni-NTA-purified His-SUMO conjugates (Figure 4a, left panel) and crude lysates (Figure 4a, right panel) revealed that Sp100A SUMO-2 modification is increased in the presence of E1B-55K (Figure 4a, left panel, lane 3).

To test whether overexpression of Sp100A affects SUMO modification of p53, we co-transfected Sp100A, p53 and E1B-55K in the combinations indicated (Figure 4b, right panel). As additional controls, we included the E1B-55K variants E1B-55K-SCM, E1B-55K-NES and E1B-55K-RTR. Analysis of Ni-NTA-purified SUMO-2 conjugates revealed that both E1B-55K-wt and E1B-55K-NES, but not E1B-55K-SCM or E1B-55K-RTR, enhanced SUMO-2 modification of Sp100A and p53 (Figure 4b, left panel, lanes 4, 6, 8, 10). Nevertheless, additional co-expression of Sp100A did not

Figure 2. SUMO modification and the C-terminal domain of E1B-55K are important for Sp100A interaction. **(A)** Schematic representation of E1B-55K protein structure, with mutations illustrated beneath, showing previously published E1B-55K mutants as well as the detailed aa substitutions.^{17,19,23,24} **(B)** H1299 cells were transfected with 3 μ g of pE1B-55K mutants and 2 μ g of pFlag-Sp100A, harvested after 48 h, before preparing total-cell extracts. Co-immunoprecipitation assays were performed as described in Figure 1a. **(C)** HepaRG cells were transfected with 1.5 μ g pFlag-Sp100A, fixed with 4% paraformaldehyde 48 h post transfection and double-labeled with mAb M2 (α -Flag) and anti-rabbit PML Ab. Primary antibodies (Abs) were detected with Alexa488 (α -PML; green)- and Cy3 (α -Sp100A; red)-conjugated secondary Abs. For nuclear staining, the DNA intercalating dye Draq5 (Biostatus) was used. Representative α -PML and α -Sp100A staining patterns of at least 50 analyzed cells are shown ($n > 50$). Overlays of single images (merge) are shown in d, (magnification $\times 7600$). **(D)** HepaRG cells were transfected with 1.5 μ g of pE1B-55K-wt, pE1B-55K-SCM, pE1B-55K-NES or pE1B-55K-RTR and 1.5 μ g pFlag-Sp100A, fixed with 4% paraformaldehyde 48 h post transfection and double-labeled with mAb 4E8 (α -E1B-55K) and mAb M2 (α -Flag). Primary Abs were detected with Alexa488 (α -Sp100A; green)- and Cy3 (α -E1B-55K; red)-conjugated secondary Abs. For nuclear staining, the DNA intercalating dye Draq5 (Biostatus) was used. Representative α -E1B-55K (b, f, k, o) and α -Sp100A (a, e, i, n) staining patterns of at least 50 analyzed cells are shown ($n > 50$). Overlays of single images (merge) are shown in d, h, m, q (magnification $\times 7600$).

substantially change p53 SUMOylation (Figure 4b, left panel, compare lanes 4–7 with 8–11), implying that SUMOylation of Sp100A and p53 are not dependent on each other.

E1B-55K nucleates p53 and Sp100A in a SUMO-dependent manner To test whether p53 interacts with Sp100A, human cells were transfected with Sp100A, p53 and E1B-55K variants as indicated in



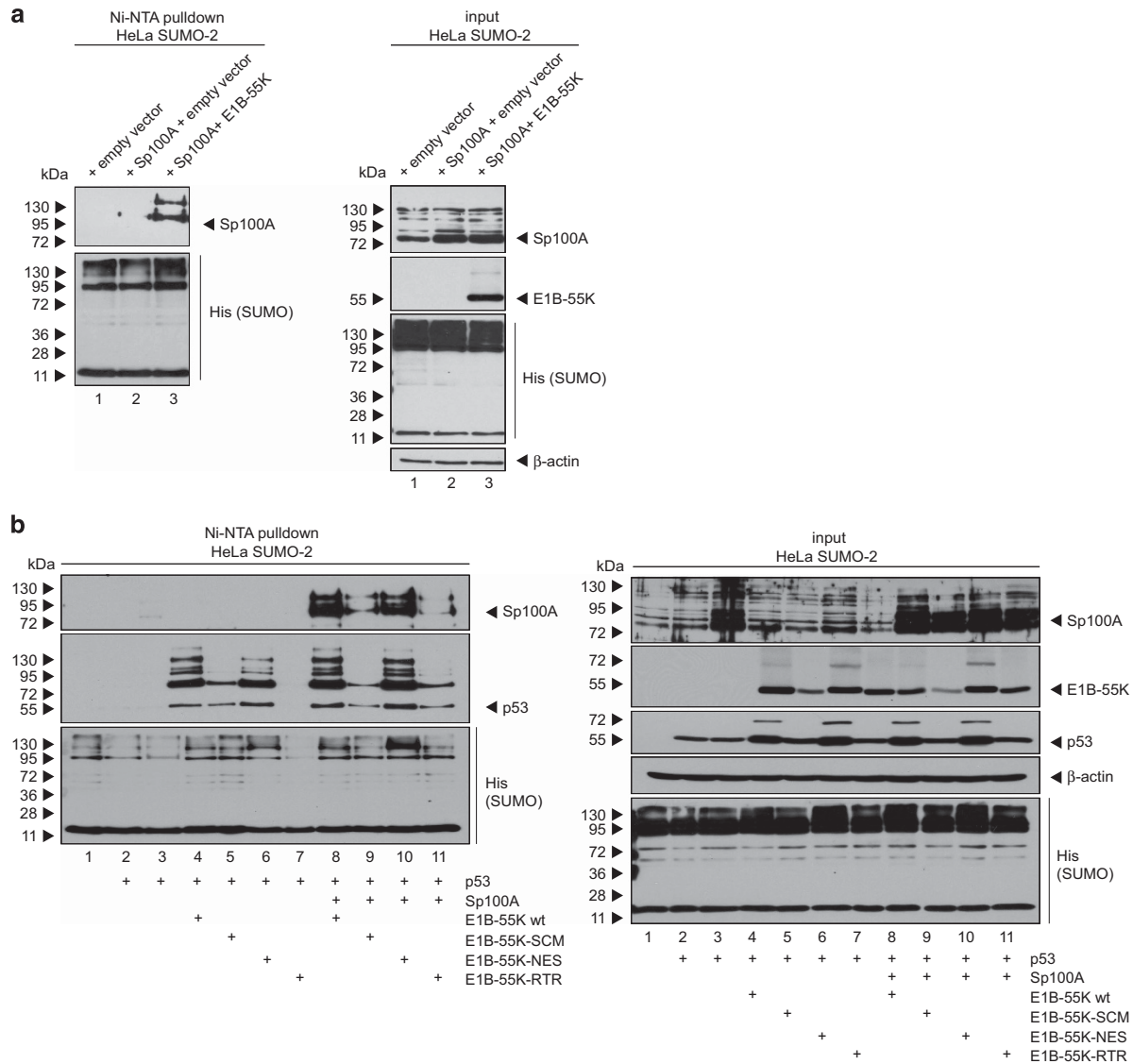


Figure 4. E1B-55K-induced SUMO-2 modification of Sp100A does not affect p53 SUMOylation. **(a)** HeLa cells stably expressing 6His-SUMO-2 were transfected with 3 μ g of pFlag-Sp100A in combination with 3 μ g of pcDNA3 (empty vector) or E1B-55K. Whole-cell lysates were prepared with guanidinium chloride buffer, subjected to Ni-NTA purification of 6His-SUMO conjugates before immunoblot analysis. Ni-NTA-purified proteins and input levels of total-cell lysates were detected using mAb 2A6 (α -E1B-55K), mAb M2 (α -Flag-Sp100A), mAb 6xHis (SUMO) and mAb AC-15 (α - β -actin). **(b)** HeLa cells stably expressing 6His-SUMO-2 were transfected with 2 μ g of p53 or pcDNA3, 3 μ g of pFlag-Sp100A and E1B-55K variants (wt, SCM, NES, RTR) in the indicated combinations (+). Whole-cell lysates were prepared with guanidinium chloride buffer, subjected to Ni-NTA purification of 6His-SUMO conjugates before immunoblot analysis. Ni-NTA-purified proteins and input levels of total-cell lysates were detected using mAb 2A6 (α -E1B-55K), mAb DO-1 (α -p53), mAb M2 (α -Flag-Sp100A), mAb 6xHis (SUMO) and mAb AC-15 (α - β -actin).

Figure 3. Sp100 recruitment by E1B-55K to nuclear matrix associated dot-like structures. **(A)** Schematic representation of Sp100A protein structure and the deletion mutation within the HSR domain. HP1, heterochromatin protein interaction region; SIM, SUMO interaction motif; NLS, nuclear localization signal. **(B)** HepaRG cells were transfected with 1.5 μ g of pFlag-Sp100A Δ HSR, fixed with 4% paraformaldehyde 48 h post transfection and double-labeled with mAb pAb NB 100-59787 (α -PML) and mAb M2 (α -Flag). **(C)** HepaRG cells were transfected with 1.5 μ g of pE1B-55K-wt, pE1B-55K-SCM, pE1B-55K-NES and pE1B-55K-RTR and 1.5 μ g of pFlag-Sp100A Δ HSR, fixed with 4% paraformaldehyde 48 h post transfection and double-labeled with mAb 4E8 (α -E1B-55K) and mAb M2 (α -Flag). **(D)** Primary antibodies (Abs) were detected with Alexa488 (green) and Cy3 (red) conjugated secondary Abs. Draq5 was used for nuclear staining. Representative α -Sp100A (**D**, a, e, i, n) and α -E1B-55K (**D**, b, f, k, o) staining patterns of at least 50 analyzed cells are shown ($n > 50$). Overlays of single images (merge) are shown in d, h, m, q (magnification $\times 7600$). **(E)** H1299 cells were transfected with 3 μ g of pcDNA3, pE1B-55K-wt, pE1B-55K-SCM, pE1B-55K-NES or pE1B-55K-RTR in combination with 3 μ g pFlag-Sp100A, harvested after 48 h and cell fractionation was performed as described in Materials and Methods before analysis of the nuclear matrix fractions by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting using pAb α -Histone H3, pAb GH3 (α -Sp100) and mAb α -Saf-A as an internal control for the nuclear fraction.

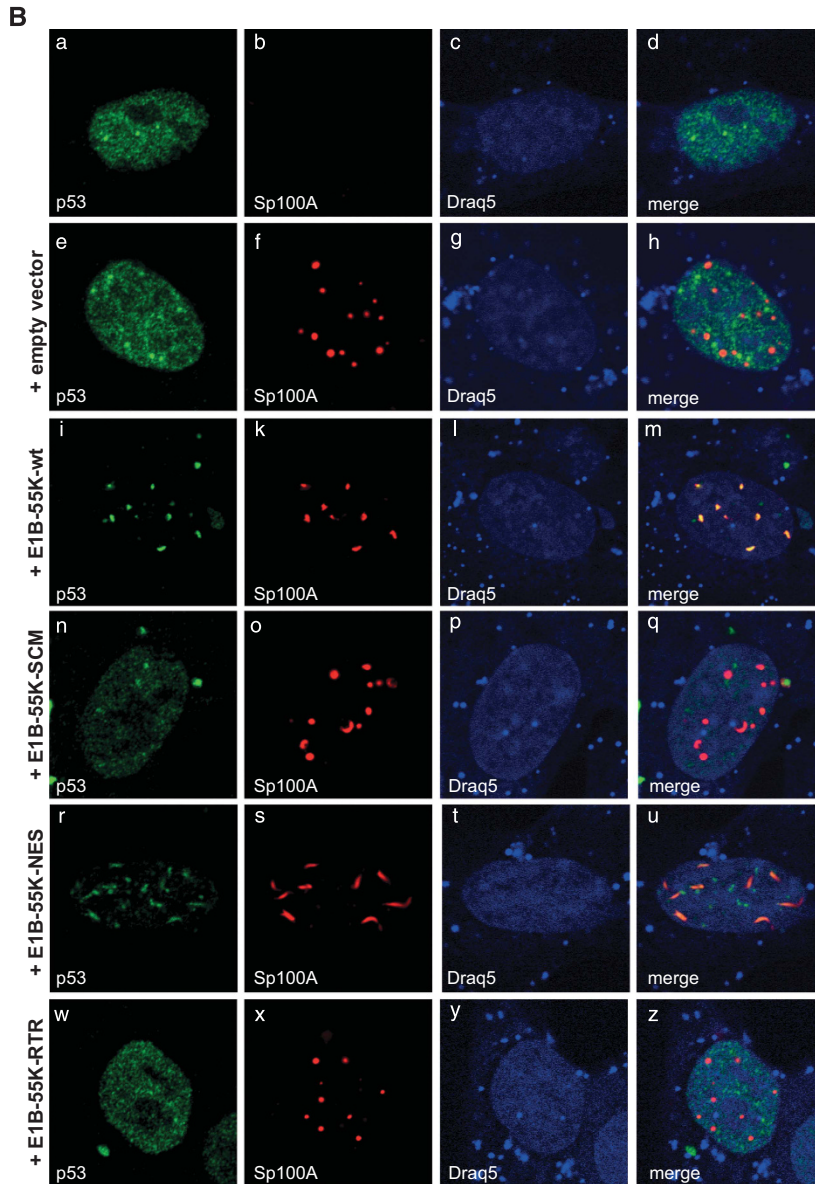
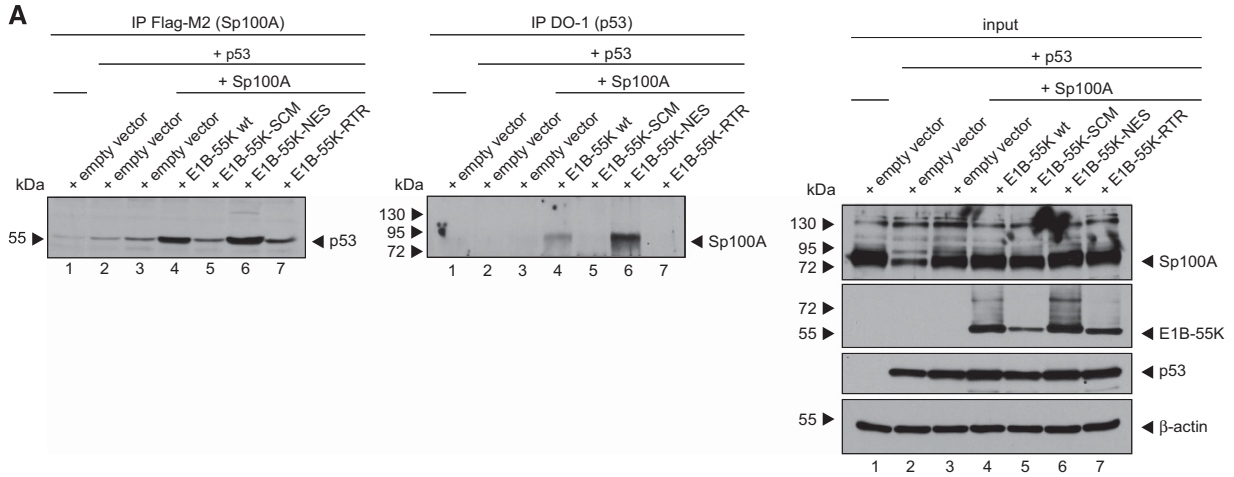


Figure 5A. Precipitation of Flag-Sp100A and subsequent detection of p53 showed an interaction of both proteins only in the presence of E1B-55K-wt and E1B-55K-NES (Figure 5A, left panel, lanes 4 and 6). Unfortunately, a non-specific band at the size of ~55 kDa, presumably the heavy antibody chain, was present in all lanes including the negative control. We therefore precipitated p53 prior to detection of Sp100A (Figure 5A, middle panel). These co-immunoprecipitation experiments confirmed that Sp100A only interacts with p53 in the presence of E1B-55K-wt and even more efficiently with E1B-55K-NES (Figure 5A, middle panel, lanes 4 and 6). No binding was detected with E1B-55K-SCM and E1B-55K-RTR, the E1B variants that failed to localize to PML-NBs (Figure 5A, middle panel, lanes 5 and 7). This suggests that Sp100A does not interact directly with p53, but rather through the intermediary partner E1B-55K protein.

We further monitored the localization of Sp100A together with endogenously expressed p53 in combination with the E1B-55K variants (Figure 5B). Staining of p53 alone revealed its diffuse nuclear localization with more intensively stained dot-like structures (Figure 5B, panels a and d). These dots did not coincide

with PML-NBs because transiently expressed Flag-Sp100A localized to different dot-like structures (Figure 5B, panels f and h) from those observed for p53 (Figure 5B, panels e and h). Consistent with the co-immunoprecipitation data, co-transfection of E1B-55K-wt induced the co-localization of p53 and Sp100A within dot-like structures (Figure 5B, panel m). Notably, p53 was also recruited to the rod-like structures induced by Sp100A and E1B-55K-NES (Figure 5B, panel u). Co-localization of Sp100A and p53 was not observed in the presence of E1B-55K-SCM or E1B-55K-RTR (Figure 5B, panels q and z).

Sp100-activated p53-dependent transcription is counteracted by E1B-55K

We recently reported that Sp100A is able to promote adenoviral gene expression.¹⁴ This is consistent with the ability of Sp100A to recruit histone acetyl transferases, thereby inducing chromatin decondensation.³ To address whether Sp100A is able to affect p53-dependent gene expression, we performed luciferase reporter assays with p53-dependent Cyclin G and Mdm2 promoters,

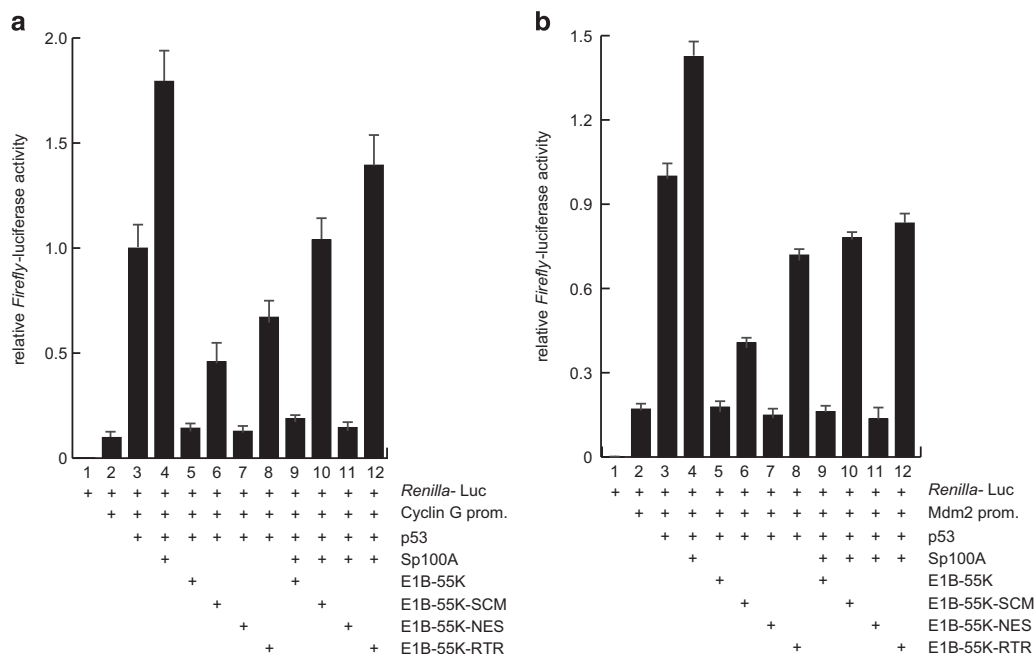


Figure 6. Sp100A activates p53-dependent transcription that is efficiently repressed by E1B-55K. **(a)** H1299 cells were transfected with 0.5 µg of p*Renilla*-Luc, 0.5 µg of pCyclinG-promoter and 0.015 µg pP53, 0.5 µg pSp100A and/or 0.5 µg E1B-55K-wt, -SCM, -NES or RTR in the combinations indicated (+). Total-cell extracts were prepared and luciferase activity was determined 24 h post transfection 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 (sample 3) and are based on the average of three independent experiments with triplicates ($n = 3$) **(b)** H1299 cells were transfected as in **(a)**, together with 0.5 µg of pMdm2-promoter plasmid in the combinations indicated (+). Luciferase activity was determined 24 h post transfection 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 (sample 3) and are based on the average of three independent experiments with triplicates ($n = 3$).

Figure 5. E1B-55K complexes p53 with Sp100A in nuclear dot-like structures. **(A)** H1299 cells were transfected with 2 µg of p53, 3 µg pFlag-Sp100A and 3 µg of different constructs encoding the different E1B-55K variants (wt, SCM, NES, RTR), and harvested after 48 h to prepare total-cell extracts. Flag-Sp100 was immunoprecipitated using mAb Flag-M2 (α-Flag), p53 was immunoprecipitated using pAb FL-393 (α-p53), and proteins resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) were visualized by immunoblotting. Co-precipitated proteins and input levels of total-cell lysates were detected using pAb FL-393 (α-p53), pAb GH3 (α-Sp100), mAb 2A6 (α-E1B-55K) and mAb AC-15 (α-β-actin). **(B)** HepaRG cells were transfected with 1.5 µg pFlag-Sp100A and 1.5 µg of pcDNA or pE1B-55K-wt, pE1B-55K-SCM, pE1B-55K-NES or pE1B-55K-RTR, fixed with 4% paraformaldehyde 48 h post transfection and double-labeled with mAb FL-393 (α-p53) and mAb M2 (α-Flag). Primary antibodies (Abs) were detected with Alexa488 (green)- and Cy3 (red)-conjugated secondary Abs. Draq5 was used for nuclear staining. Representative α-p53 (a, e, i, n, r, w) and α-Flag-Sp100A (b, f, k, o, s, x) staining patterns of at least 50 analyzed cells are shown ($n > 50$). Overlays of single images (merge) are shown in d, h, m, q, u, z (magnification × 7600).

combined with expression of human p53, Sp100A and the E1B-55K variants (Figure 6). Renilla luciferase was included to control for transfection efficiency. Co-expression of Sp100A and p53 significantly stimulated luciferase activity from both promoters (Figures 6a and b, lane 4).

Without Sp100A, addition of E1B-55K-wt was sufficient to nearly completely repress p53 transcriptional activation (Figures 6a and b, lane 5). Agreeing with previous data, E1B-55K-SCM or E1B-55K-RTR co-expression failed to efficiently lower the luciferase activity to basal levels (Figures 6a and b, lanes 6 and 8), whereas

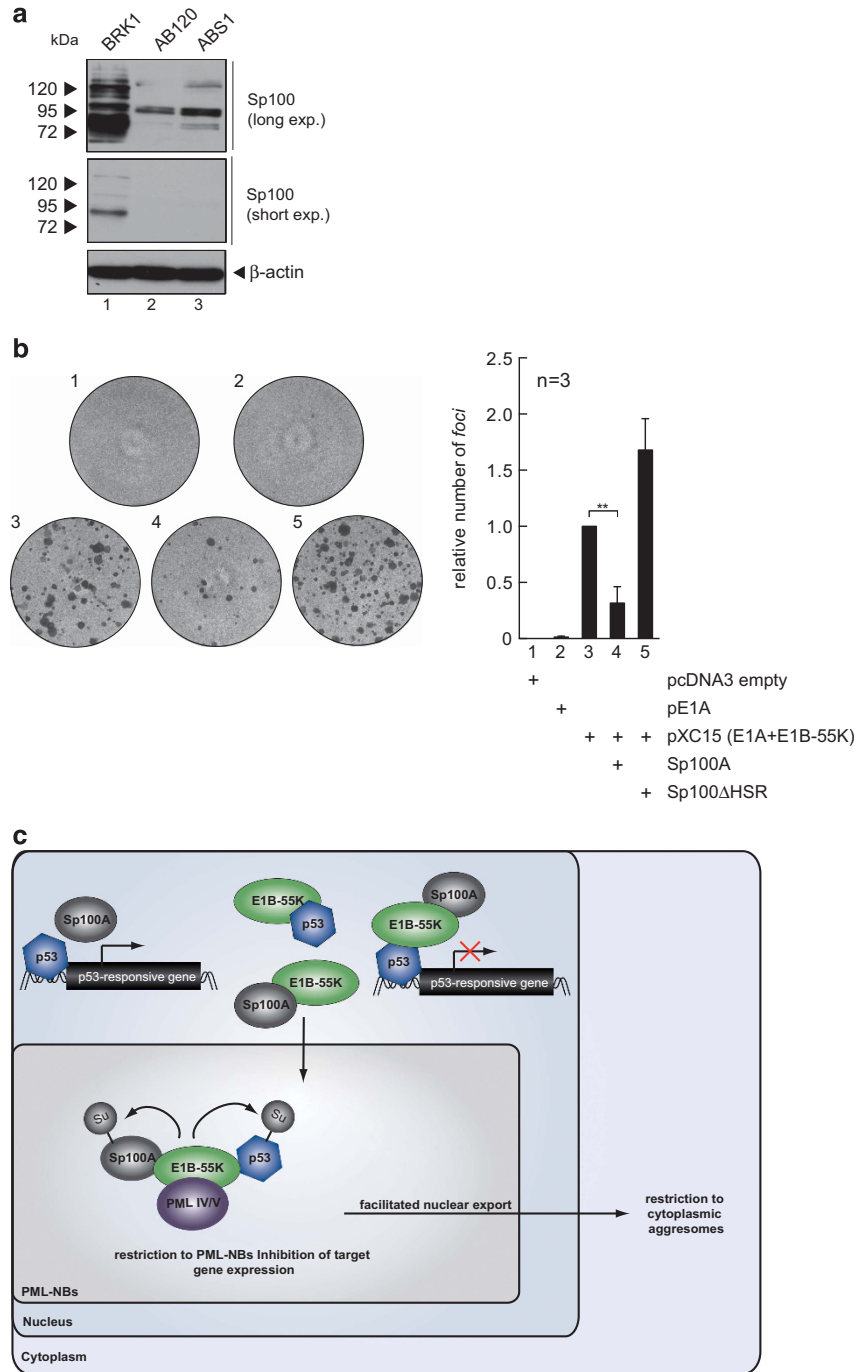


Figure 7. Sp100A exhibits tumor-suppressive capacity by counteracting E1A/E1B-55K-mediated transformation. **(a)** Total-cell lysates of BRK1, AB120 and ABS1 cell lines were prepared and analyzed by immunoblotting using antibodies pAb GH3 (α -Sp100) and mAb AC-15 (α - β -actin). **(b)** Primary baby rat kidney (pBRK) cells were simultaneously transfected with a combination of plasmids encoding for E1A alone, E1A and E1B-55K (pXC15) and Sp100 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 (pXC15) were plotted based on the average of three independent experiments with triplicates ($n=3$). Representative crystal violet-stained plates showing foci from transfections with plasmids encoding empty vector, plate 1; pE1A, plate 2; pE1A+E1B-55K, plate 3; pE1A+E1B-55K+Sp100A, plate 4 and pE1A+E1B-55K+ Sp100A- Δ HRSR, plate 5. **(c)** Schematic model of how E1B-55K recruits the two factors p53 and Sp100A from the nucleoplasm and complexes both proteins within PML-NBs, thereby shutting down target gene expression. Within PML-NBs, p53 and Sp100A are SUMOylated, which induces nuclear export of the modified cellular proteins and their restriction to aggregates in the cytoplasm.

hyper-SUMOylated E1B-55K-NES repressed p53 transactivation as efficiently as E1B-55K-wt (Figures 6a and b, lane 7). Upon co-expression of Sp100A, luciferase activity was further elevated in cells expressing E1B-55K-SCM or E1B-55K-RTR (Figures 6a and b, lanes 10 and 12); however, Sp100A co-expression could not counteract the transcriptional repression mediated by E1B-55K-wt and E1B-55K-NES (Figures 6a and b, lanes 9 and 11). Taken together, our results suggest that Sp100A can activate p53-dependent transcription and may therefore orchestrate tumor-suppressive functions, which are efficiently counteracted by E1B-55K.

Sp100A represses E1A/E1B-55K-mediated transformation of primary baby rat kidney cells

We next screened for endogenous Sp100 expression in spontaneously immortalized baby rat kidney cells (BRK1;²³) compared with E1A/E1B-55K-transformed BRK cells (AB120;³⁰) (Figure 7a). We also included ABS1 cells because these are stably transformed by a combination of E1A, E1B-55K and E4orf6.³⁰ Immunoblot analysis of whole-cell lysates indicated that compared with BRK1 cells, E1A/E1B-55K transformed cells have much lower levels of endogenously expressed Sp100 (Figure 7a, lanes 1 and 2). Co-expression of E4orf6 did not additionally alter Sp100 protein levels (Figure 7a, lane 3).

To further evaluate whether modulation of the Sp100A protein is necessary for the transformation potential of HAdV E1B-55K, we transfected primary BRK cells with plasmids encoding E1A alone or in combination with E1B-55K (pXC15) and/or Sp100A and Sp100A- Δ HSR. After incubation under standard conditions, *focus* formation was visualized by crystal violet staining and quantified relative to E1A plus E1B-55K-wt (Figure 7b). As expected, the expression of E1A alone resulted in low *focus* formation activity compared with the positive control, E1A+E1B-55Kwt (pXC15) cellular transformation frequencies (Figure 7b, lane 2, 3; plate 2, 3). However, combining E1A, E1B-55K and Sp100A significantly reduced *focus*-forming activity compared with the positive control (Figure 7b, lane 4; plate 4).

As our data above indicated that Sp100 oligomerization is dispensable for nuclear retention of E1B-55K (Figure 3), we also included the Sp100A mutant deleted in the HSR oligomerization domain in the co-expression transformation analysis. Unexpectedly, Sp100A- Δ HSR completely lost the ability to repress, and even enhanced the transforming potential of E1A+E1B-55K on their own (Figure 7b, lane 5; plate 5). Thus, Sp100A inhibition of E1A/E1B-55K-mediated transformation processes seems to depend on Sp100 oligomerization and efficient PML-NB localization.

DISCUSSION

Together with the observation that E1B-55K-dependent SUMOylation of Sp100A abrogates transcriptional activation of cellular p53-responsive promoters, we provide novel insights into how E1B-55K modulates key host regulatory factors during lytic infection cycles, which in turn is intimately linked to E1B-55K's growth-promoting activity in cooperation with E1A during oncogenic transformation processes. Here, we show that Sp100A represents one of these key host factors involved in depressing E1A/E1B-55K-mediated transformation. This information, combined with the fact that Sp100A supports p53-dependent transactivation, infers that Sp100A possesses tumor-suppressive activity.

Our notion that Sp100A is a tumor-suppressor protein is supported by a few other studies, although these studies only focused on the role of Sp100 during cellular transformation in general, and did not distinguish between the different isoforms A, B, C and HMG. For example, a recent clinical study linked low Sp100 expression with laryngeal cancer, correlating with reduced

cell differentiation, suggesting that Sp100 expression, availability and/or location is inversely linked to progression of such carcinomas. Indeed, Sp100 displays a predominantly nuclear pattern in well-differentiated cancer cells and a cytoplasmic distribution in poorly differentiated cancer cells.³¹

Here, for the first time, we demonstrate that the Sp100A isoform counteracts HAdV E1A/E1B-55K-mediated transformation processes in primary rodent cells, as long as Sp100 oligomerization and efficient PML-NB localization are functional. Conversely, the adenoviral E1B-55K oncoprotein binds to Sp100A, as long as the E1B-55K SUMOylation and C-terminal repression domains are intact. As a way to combat host defense strategies, the E1B-55K oncoprotein induces Sp100A SUMOylation and either sequesters Sp100A into the insoluble nuclear matrix or restricts it to cytoplasmic inclusion bodies.

E1B-55K inhibits p53 function, that is, transactivation, via a direct protein interaction, accompanied by tethering of the E1B-55K C-terminal repression domain to p53 target genes.^{19,20,32} Intriguingly, we found that this C-terminal repression domain of the viral oncoprotein also mediates Sp100A binding. In addition, E1B-55K possesses SUMO E3 ligase activity towards p53, which also inhibits transcriptional activity.^{21,29}

On the basis of the above results, it seems likely that E1B-55K applies mechanisms similar to p53 restriction on Sp100A, as depicted in Figure 7c. We show that E1B-55K is also able to repress Sp100A-mediated coactivation of p53-dependent promoters, possibly by recruiting Sp100A from the nucleoplasm and restricting it to the nuclear matrix. The repression of Sp100A depends partially on the SUMO modification of E1B-55K and the C-terminal transcriptional repression region of E1B-55K, described above. Similar to p53, we observe Sp100A together with E1B-55K in cytoplasmic inclusion bodies.

Negorev and coworkers³³ previously reported that overexpression of N-terminal fragments of Sp100 significantly redistributed PML-NB-associated proteins, resulting in elongated rods containing only Sp100 at the ends. The authors argued that removing the globular C-terminal domain of Sp100 exposed self-aggregating parts of the molecule, resulting in large rod-like structures. Therefore, posttranslational modifications possibly alter the three-dimensional structure of Sp100, exposing the oligomerization domain usually hidden in the non-modified protein. The fact that E1B-55K enhances Sp100A SUMOylation supports this hypothesis. Another possibility is that the interaction with E1B-55K conformationally changes the Sp100A protein, resulting in interconnected free Sp100 dimers within the observed rod-like structures.

As yet, we have not identified the region of Sp100A that interacts with E1B-55K, although we can exclude the Sp100 HSR, SCM and SIM domains as well as the HP1 interaction region (Figure 3c). As Sp100A shares 477 amino acids with Sp100B/C/HMG isoforms, which do not co-precipitate with E1B-55K, one can speculate that the SAND domains of those Sp100 variants mask potential interaction regions.

In conclusion, together with already published work, we provide evidence that Sp100 is involved in early and late events of cellular transformation/oncogenesis. Our findings suggest that Sp100A exhibits tumor-suppressive properties, as long as its oligomerization function is intact. Sp100A therefore represents a novel host determinant that can oppose the pro-tumorigenic activity of HAdV oncoproteins in primary rodent cells, as exposed by the smoking gun of HAdV E1B-55K evolution to efficiently counteract this factor.

MATERIALS AND METHODS

Cell culture

HepaRG,³⁴ H1299 (ATCC Number CRL-5803;³⁵), pBRK³⁶ and HeLa cells stably expressing 6His-SUMO-1 and -2³⁷ 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. For HepaRG cells, the medium was additionally supplemented with 5 µg/ml of bovine insulin and 0.5 µM of hydrocortisone. HeLa 6His-SUMO cell lines were maintained under 2 µM puromycin selection. All cell lines are frequently tested for mycoplasma contamination.

Plasmids, transient transfections and reporter gene assays

pXC15,³⁸ containing the whole HAdV5 E1 region (bp 1–5790) (originally constructed by J. Logan and G. Winberg), pE1A (E1B region (bp 1916–5187) cut out from the pXC15 vector), Human p53,³⁹ HAdV E1B-55K (wt, SCM,²⁴ NES,²³ RTR, R443A,¹⁷ R443in,⁴⁰ RF6,²⁷ E2¹⁷) and E4orf6 were expressed from pcDNA3. N-terminal Flag-tagged human Sp100 isoform constructs were used as recently described.⁴¹ Mutations were introduced into the *sp100A* gene by site-directed mutagenesis using oligonucleotides (Sp100A-SIM: fwd primer 5'-CAGGCATCTGACAAAAAGTCATCAGCAG-3', rev primer 5'-CTGCTGATGACT TTTTGTGATGATGCCTG-3', Sp100A-SCM: fwd primer 5'-CTGGTGGATATA AGAAAGGAAAGCC-3', rev primer 5'-GGCTTTCTCTTC TTATATCCACCAG-3', Sp100A-HP1-C289GV291E: fwd primer 5'-CCAAA TTAATTCGGTCTGAGC GACTGGTGG-3', rev primer 5'-CCACCAGT CGCT CAGAACCGGAATTAATTGG-3'; Sp100A-NLS-K447E fwd primer 5'-GAAT ACCCAGCAGGAGAGACGTTT CAGC-3', rev primer 5'- GCTGAAACGTCT CTCCTGCTGGGTATTC-3'; Sp100ΔHSR fwd primer 5'-GTGGCTGTGTGCA GGAAGATGG-3', rev primer 5'-GACAAATGCTCTCCAAGAAAG-3'). For transient transfection, subconfluent cells were treated with a mixture of DNA and 25 kDa linear polyethylenimine (Polysciences, Eppelheim, Germany) as described previously.⁴²

For dual luciferase assays, subconfluent cells were transfected as described above, using 0.5–1 µg of reporter (pGL-CyclinG-promoter, pGL-Mdm2-promoter), 1 µg of pRL-TK (Promega, Madison, WI, USA), which expresses *Renilla* luciferase under the control of the HSV-TK (herpes simplex virus thymidine kinase) promoter, and 0.5–1 µg of effector plasmids (Sp100A, Sp100B, Sp100C, Sp100HMG, E1B-55K, p53). Cell extracts were prepared, measured and normalized as described recently.⁴³

To test the influence of Sp100A on the oncogenic potential of E1B-55K in combination with E1A, freshly prepared primary BRK (pBRK) cells were prepared from 3- to 5-day-old CD rats (Charles River, Wilmington, MA, USA).³⁶ Subsequently, primary cells were transfected with the respective plasmids using calcium phosphate according to the manufacturer's instructions (ProFection Mammalian Transfection System, Promega). 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 antibodies specific for viral proteins included E1B-55K mouse mAb 2A6⁴⁴ and E4orf6 mouse mAb RSA3.⁴⁵ Primary antibodies specific against cellular and ectopically expressed proteins included PML rabbit pAb NB100-59787 (Novus Biologicals, Inc., Littleton, CO, USA), Sp100 rabbit pAb GH3 (kindly provided by H. Will), mouse mAb Flag-M2 (A2220; Sigma-Aldrich, Inc., St. Louis, MO, USA), Histone H3 (ab1791; Abcam, Cambridge, MA, USA), β-actin mouse mAb AC-15 (A5441; Sigma-Aldrich, Inc.), 6His mouse mAb (631213; Clontech, St. Germain en Laye, France), Saf-A rabbit pAb NB100-2135 (Novus Biologicals, Inc.), p53 mouse mAb DO-1 (sc-126; Santa Cruz, Heidelberg, Germany) and p53 rabbit pAb FL-393 (FL393; Santa Cruz). All protein extracts were prepared in RIPA lysis buffer as described recently.⁴⁶ Precipitated proteins were boiled for 3 min at 95 °C in 2× Laemmli buffer and analyzed by immunoblotting exactly as described recently.⁴² Denaturing purification and analysis of SUMO conjugates was performed as described recently.⁴⁷ Cells were fractionated based on a modified protocol described by Leppard et al.,⁴⁸ which we reported recently.¹³

Indirect immunofluorescence

Cells were prepared and analyzed as described recently.²² Images were cropped using Adobe PhotoshopCS6 and assembled with Adobe Illustrator CS.

Statistical analysis

Data are presented as mean, error bars as standard deviation (s.d.). Statistical analysis was carried out using paired students *t*-test (Figure 6 and 7). The *P*-values are indicated as * < 0.05 and ** < 0.01.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGEMENTS

We thank Ron Hay, Ellis Jaffray, Roger Everett, Hans Will, Thomas Sternsdorf and Nicole Fischer for providing reagents and scientific discussion. The Heinrich Pette Institute, Leibniz Institute for Experimental Virology is supported by the *Freie und Hansestadt Hamburg* and the *Bundesministerium für Gesundheit* (BMG). JB was supported by grant from the *Deutsche Forschungsgemeinschaft* (DFG) to TD (DO 343/7-1). MT was supported by the *Dräger Stiftung*. TD is supported by the DFG and the *Wilhelm Sander-Stiftung*. SS was supported by the *Else Kröner-Fresenius-Stiftung* and the *Deutsche Krebshilfe e. V.* Part of this work was supported by the *B. Braun Stiftung* and the *Fonds der Chemischen Industrie*.

REFERENCES

- Guldner HH, Szostecki C, Grotzinger T, Will H. IFN enhance expression of Sp100, an autoantigen in primary biliary cirrhosis. *J Immunol* 1992; **149**: 4067–4073.
- Grotzinger T, Sternsdorf T, Jensen K, Will H. Interferon-modulated expression of genes encoding the nuclear-dot-associated proteins Sp100 and promyelocytic leukemia protein (PML). *Eur J Biochem* 1996; **238**: 554–560.
- Newhart A, Negorev DG, Rafalska-Metcalf IU, Yang T, Maul GG, Janicki SM. Sp100A promotes chromatin decondensation at a cytomegalovirus-promoter-regulated transcription site. *Mol Biol Cell* 2013; **24**: 1454–1468.
- Moller A, Sirma H, Hofmann TG, Staeger H, Gresko E, Ludi KS et al. Sp100 is important for the stimulatory effect of homeodomain-interacting protein kinase-2 on p53-dependent gene expression. *Oncogene* 2003; **22**: 8731–8737.
- Seeler JS, Marchio A, Losson R, Desterro JM, Hay RT, Chambon P et al. Common properties of nuclear body protein SP100 and TIF1alpha chromatin factor: role of SUMO modification. *Mol Cell Biol* 2001; **21**: 3314–3324.
- Guldner HH, Szostecki C, Schroder P, Matschl U, Jensen K, Luders C et al. Splice variants of the nuclear dot-associated Sp100 protein contain homologies to HMG-1 and a human nuclear phosphoprotein-box motif. *J Cell Sci* 1999; **112**: 733–747.
- Sternsdorf T, Jensen K, Reich B, Will H. The nuclear dot protein sp100, characterization of domains necessary for dimerization, subcellular localization, and modification by small ubiquitin-like modifiers. *J Biol Chem* 1999; **274**: 12555–12566.
- Seeler JS, Marchio A, Sitterlin D, Transy C, Dejean A. Interaction of SP100 with HP1 proteins: a link between the promyelocytic leukemia-associated nuclear bodies and the chromatin compartment. *Proc Natl Acad Sci USA* 1998; **95**: 7316–7321.
- Stapp WH, Meyers JM, McBride AA. Sp100 provides intrinsic immunity against human papillomavirus infection. *MBio* 2013; **4**: e00845–13.
- Ling PD, Peng RS, Nakajima A, Yu JH, Tan J, Moses SM et al. Mediation of Epstein-Barr virus EBNA-LP transcriptional coactivation by Sp100. *EMBO J* 2005; **24**: 3565–3575.
- Kim YE, Lee JH, Kim ET, Shin HJ, Gu SY, Seol HS et al. Human cytomegalovirus infection causes degradation of Sp100 proteins that suppress viral gene expression. *J Virol* 2011; **85**: 11928–11937.
- Adler M, Tavalai N, Muller R, Stamminger T. Human cytomegalovirus immediate-early gene expression is restricted by the nuclear domain 10 component Sp100. *J Gen Virol* 2011; **92**: 1532–1538.
- Gunther T, Schreiner S, Dobner T, Tessmer U, Grundhoff A. Influence of ND10 components on epigenetic determinants of early KSHV latency establishment. *PLoS Pathog* 2014; **10**: e1004274.
- Berscheminski J, Wimmer P, Brun J, Ip WH, Groitl P, Horlacher T et al. Sp100 isoform-specific regulation of human Adenovirus type 5 (Ad5) gene expression. *J Virol* 2014; **88**: 6076–6092.
- Wimmer P, Schreiner S, Everett RD, Sirma H, Groitl P, Dobner T. SUMO modification of E1B-55K oncoprotein regulates isoform-specific binding to the tumour suppressor protein PML. *Oncogene* 2010; **29**: 5511–5522.
- Sieber T, Dobner T. Adenovirus type 5 early region 1B 156R protein promotes cell transformation independently of repression of p53-stimulated transcription. *J Virol* 2007; **81**: 95–105.
- Schreiner S, Wimmer P, Groitl P, Chen SY, Blanchette P, Branton PE et al. Adenovirus type 5 early region 1B 55K oncoprotein-dependent degradation of

- cellular factor Daxx is required for efficient transformation of primary rodent cells. *J Virol* 2011; **85**: 8752–8765.
- 18 Endter C, Dobner T. Cell transformation by human adenoviruses. *Curr Top Microbiol Immunol* 2004; **273**: 163–214.
- 19 Yew PR, Berk AJ. Inhibition of p53 transactivation required for transformation by adenovirus early 1B protein. *Nature* 1992; **357**: 82–85.
- 20 Teodoro JG, Branton PE. Regulation of p53-dependent apoptosis, transcriptional repression, and cell transformation by phosphorylation of the 55-kilodalton E1B protein of human adenovirus type 5. *J Virol* 1997; **71**: 3620–3627.
- 21 Pennella MA, Liu Y, Woo JL, Kim CA, Berk AJ. Adenovirus E1B 55-kilodalton protein is a p53-SUMO1 E3 ligase that represses p53 and stimulates its nuclear export through interactions with promyelocytic leukemia nuclear bodies. *J Virol* 2010; **84**: 12210–12225.
- 22 Wimmer P, Berscheminski J, Blanchette P, Groitl P, Branton PE, Hay RT *et al*. PML isoforms IV and V contribute to adenovirus-mediated oncogenic transformation by functional inhibition of the tumor suppressor p53. *Oncogene* e-pub ahead of print 16 March 2015.
- 23 Endter C, Hartl B, Spruss T, Hauber J, Dobner T. Blockage of CRM1-dependent nuclear export of the adenovirus type 5 early region 1B 55-kDa protein augments oncogenic transformation of primary rat cells. *Oncogene* 2005; **24**: 55–64.
- 24 Endter C, Kzhyshkowska J, Stauber R, Dobner T. SUMO-1 modification required for transformation by adenovirus type 5 early region 1B 55-kDa oncoprotein. *Proc Natl Acad Sci USA* 2001; **98**: 11312–11317.
- 25 Ornelles DA, Shenk T. Localization of the adenovirus early region 1B 55-kilodalton protein during lytic infection: association with nuclear viral inclusions requires the early region 4 34-kilodalton protein. *J Virol* 1991; **65**: 424–429.
- 26 Lethbridge KJ, Scott GE, Leppard KN. Nuclear matrix localization and SUMO-1 modification of adenovirus type 5 E1b 55K protein are controlled by E4 Orf6 protein. *J Gen Virol* 2003; **84**: 259–268.
- 27 Härtl B, Zeller T, Blanchette P, Kremmer E, Dobner T. Adenovirus type 5 early region 1B 55-kDa oncoprotein can promote cell transformation by a mechanism independent from blocking p53-activated transcription. *Oncogene* 2008; **27**: 3673–3684.
- 28 Goodrum FD, Shenk T, Ornelles DA. Adenovirus early region 4 34-kilodalton protein directs the nuclear localization of the early region 1B 55-kilodalton protein in primate cells. *J Virol* 1996; **70**: 6323–6335.
- 29 Muller S, Dobner T. The adenovirus E1B-55K oncoprotein induces SUMO modification of p53. *Cell Cycle* 2008; **7**: 754–758.
- 30 Nevels M, Spruss T, Wolf H, Dobner T. The adenovirus E4orf6 protein contributes to malignant transformation by antagonizing E1A-induced accumulation of the tumor suppressor protein p53. *Oncogene* 1999; **18**: 9–17.
- 31 Li W, Shang C, Guan C, Zhang Y, Sun K, Fu W. Low expression of Sp100 in laryngeal cancer: correlation with cell differentiation. *Med Sci Monit* 2010; **16**: BR174–BR178.
- 32 Martin ME, Berk AJ. Adenovirus E1B 55K represses p53 activation in vitro. *J Virol* 1998; **72**: 3146–3154.
- 33 Negorev D, Ishov AM, Maul GG. Evidence for separate ND10-binding and homo-oligomerization domains of Sp100. *J Cell Sci* 2001; **114**: 59–68.
- 34 Gripon P, Rumin S, Urban S, Le Seyec J, Glaise D, Cannie I *et al*. Infection of a human hepatoma cell line by hepatitis B virus. *Proc Natl Acad Sci USA* 2002; **99**: 15655–15660.
- 35 Mitsudomi T, Oyama T, Gazdar AF, Minna JD, Okabayashi K, Shirakusa T. [Mutations of ras and p53 genes in human non-small cell lung cancer cell lines and their clinical significance]. *Nihon Geka Gakkai Zasshi* 1992; **93**: 944–947.
- 36 Nevels M, Dobner T. Determination of the transforming activities of adenovirus oncogenes. In: Wold WS, Tollefson AE. (eds). *Adenovirus Methods and Protocols Methods in Molecular Medicine*. 2nd ed. Humana Press Inc: Totowa, NJ, 2006, pp 187–195.
- 37 Tatham MH, Rodriguez MS, Xirodimas DP, Hay RT. Detection of protein SUMOylation in vivo. *Nat Protoc* 2009; **4**: 1363–1371.
- 38 Logan J, Pilder S, Shenk T. Functional analysis of adenovirus type 5 early region 1B. *Cancer Cells* 1984; **2**: 527–532.
- 39 Berscheminski J, Groitl P, Dobner T, Wimmer P, Schreiner S. The adenoviral oncogene E1A-13 S interacts with a specific isoform of the tumor suppressor PML to enhance viral transcription. *J Virol* 2012; **87**: 965–977.
- 40 Yew PR, Liu X, Berk AJ. Adenovirus E1B oncoprotein tethers a transcriptional repression domain to p53. *Genes Dev* 1994; **8**: 190–202.
- 41 Guldner HH, Szostecki C, Schroder P, Matschl U, Jensen K, Luders C *et al*. Splice variants of the nuclear dot-associated Sp100 protein contain homologies to HMG-1 and a human nuclear phosphoprotein-box motif. *J Cell Sci* 1999; **112**: 733–747.
- 42 Muller D, Schreiner S, Schmid M, Groitl P, Winkler M, Dobner T. Functional cooperation between human adenovirus type 5 early region 4, open reading frame 6 protein, and cellular homeobox protein HoxB7. *J Virol* 2012; **86**: 8296–8308.
- 43 Schreiner S, Martinez R, Groitl P, Rayne F, Vaillant R, Wimmer P *et al*. Transcriptional activation of the adenoviral genome is mediated by capsid protein. *PLoS Pathog* 2012; **8**: e1002549.
- 44 Sarnow P, Sullivan CA, Levine AJ. A monoclonal antibody detecting the adenovirus type 5-E1b-58Kd tumor antigen: characterization of the E1b-58Kd tumor antigen in adenovirus-infected and -transformed cells. *Virology* 1982; **120**: 510–517.
- 45 Marton MJ, Baim SB, Ornelles DA, Shenk T. The adenovirus E4 17-kilodalton protein complexes with the cellular transcription factor E2F, altering its DNA-binding properties and stimulating E1A-independent accumulation of E2 mRNA. *J Virol* 1990; **64**: 2345–2359.
- 46 Wimmer P, Blanchette P, Schreiner S, Ching W, Groitl P, Berscheminski J *et al*. Cross-talk between phosphorylation and SUMOylation regulates transforming activities of an adenoviral oncoprotein. *Oncogene* 2013; **32**: 1626–1637.
- 47 Berscheminski J, Wimmer P, Brun J, Ip WH, Groitl P, Horlacher T *et al*. Sp100 isoform-specific regulation of human Adenovirus type 5 (Ad5) gene expression. *J Virol* 2014; **88**: 6076–6092.
- 48 Leppard KN, Shenk T. The adenovirus E1B 55 kd protein influences mRNA transport via an intranuclear effect on RNA metabolism. *EMBO J* 1989; **8**: 2329–2336.