Replication of Merkel cell polyomavirus induces reorganization of promyelocytic leukemia nuclear bodies

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Merkel cell polyomavirus (MCPyV) is associated with Merkel cell carcinoma (MCC), a rare but aggressive skin cancer. The virus is highly prevalent: 60–80% of adults are seropositive; however, cells permissive for MCPyV infection are unknown. Consequently, very little information about the MCPyV life cycle is available. Until recently, MCPyV replication could only be studied using a semi-permissive in vitro replication system (Neumann et al., 2011; Feng et al., 2011, Schowalter et al., 2011). MCPyV replication most likely depends on subnuclear structures such as promyelocytic leukemia protein nuclear bodies (PML-NBs), which are known to play regulatory roles in the infection of many DNA viruses. Here, we investigated PML-NB components as candidate host factors to control MCPyV DNA replication. We showed that PML-NBs change in number and size in cells actively replicating MCPyV proviral DNA. We observed a significant increase in PML-NBs in cells positive for MCPyV viral DNA replication. Interestingly, a significant amount of cells actively replicating MCPyV did not show any Sp100 expression. While PML and Daxx had no effect on MCPyV DNA replication, MCPyV replication was increased in cells depleted for Sp100, strongly suggesting that Sp100 is a negative regulator of MCPyV DNA replication.

INTRODUCTION

Merkel cell polyomavirus (MCPyV) is the only human polyomavirus known to be involved in tumorigenesis (Feng et al., 2008). Several lines of evidence suggest that the virus is causally linked to Merkel cell carcinoma (MCC) pathogenesis: (1) frequent detection of viral sequences in the tumour; (2) monoclonal integration of viral DNA in the tumour cell genome as well as subsequent metastasis; and (3) expression of the early viral proteins large T-Antigen (LT) and small T-Antigen (ST) in tumour tissues (Chang & Moore, 2012; Grundhoff & Fischer, 2015; Wendzicki et al., 2015).

Polyomaviruses are highly restricted in tissue tropism and cell type, which greatly complicates the establishment of an in vitro replication system. Consequently, in vitro cell culture systems have been established for only a few polyomaviruses. For example, the life cycle of BK virus can only be studied in primary human renal proximal tubule epithelial cells (RPTE) (Jiang et al., 2009, 2011). Similarly, only human glial cells are fully permissive for JC virus infection in vitro (Radhakrishnan et al., 2003; White & Khalili, 2004). The site of MCPyV persistence and replication in humans is unknown, and no fully permissive replication system has been established to date. We and others recently demonstrated that the MCPyV life cycle can be partially studied in conventionally used cell lines (Feng et al., 2011; Neumann et al., 2011; Schowalter et al., 2011). By using a synthetic MCPyV consensus construct, MCPyVSyn, we demonstrated that three cell lines, 293, PFSK-1 and H1299, efficiently support viral DNA replication (Neumann et al., 2011). Although both early and late gene expression together with viral particles was detected, replication blocks at later stages of the viral life cycle prevented efficient virus particle formation and viral spread. Recently, Liu and colleagues successfully demonstrated the use of human dermal fibroblast or ex vivo skin models, whereby under specific culture conditions these cells can be efficiently infected with MCPyV (Liu et al., 2016). While this system will be highly useful in the future, it still needs formal proof in vivo that human dermal fibroblasts are the natural target cells of MCPyV.
MCPyV particles have been shown to attach to cells using glycosaminoglycan as a primary receptor; subsequently sialylated glycans are required for uptake into the cells (Neu et al., 2012; Schowalter et al., 2011). Due to the lack of fully permissive systems, subsequent steps of the viral lifecycle such as nuclear transport of viral particles, genome delivery, induction of late gene expression, virion assembly and egress have not been studied for MCPyV. Likewise, only a few host cell factors have been shown to positively or negatively regulate MCPyV replication. Two factors that have been described are Vam6p and the bromodomain protein Brd4. The latter, known to be involved in viral replication of herpesviruses and papillomaviruses (McBride & Jang, 2013; Weidner-Glunde et al., 2010), was recently shown to be targeted by the MCPyV LT protein. The interaction releases Brd4 complexes from cellular chromatin and recruits them to the viral episome, thereby serving as a scaffold for the recruitment of cellular DNA replication factors such as RCF (Wang et al., 2012b). In contrast, hVam6p was found to be a negative regulator of MCPyV replication (Liu et al., 2011). The interaction with LT sequesters hVam6p in the nucleus and antagonizes the protein’s ability to induce lysosomal clustering (Liu et al., 2011).

Here, we analysed the promyelocytic leukemia protein (PML) nuclear bodies (PML-NBs) as an additional candidate host factor that may control MCPyV replication. PML-NBs are subnuclear bodies known to be involved in several cellular processes, including apoptosis, senescence, angiogenesis and DNA repair, as well as innate immunity processes against viral infections (Batty et al., 2008; Bernardi et al., 2008; Geoffroy & Chelbi-Alix, 2011; Torok et al., 2009). PML-NBs are dynamic structures consisting of multiple proteins. More than 150 proteins are presently known to permanently or transiently localize to these nuclear structures (Van Damme et al., 2010). Number and size of PML-NBs vary among different cell types, and the composition of these structures undergoes significant rearrangement during the cell cycle (Everett et al., 1999).

Several viral proteins encoded by nuclear-replicating RNA or DNA viruses have been shown to co-localize with and reorganize PML-NBs, resulting in antagonization of PML-NB antiviral mechanisms. Prominent examples include the ICP0 protein encoded by herpes simplex virus 1 (HSV-1), the E4orf3 gene product of adenovirus and the BLZF gene product of Epstein–Barr virus (EBV). Some of these factors (e.g. ICP0) induce degradation of the PML itself, whereas others target other constitutive PML-NB components. Many viruses have furthermore evolved several proteins to redundantly target multiple components of PML-NBs (Wang et al., 2012a). For example, comprehensive biochemical analyses of functional interactions between the human adenovirus (HAdV)-encoded E1B-55K protein and host cell factors identified a tight association with several components of cellular PML-NBs (Schreiner et al., 2011, 2012).

With regard to polyomavirus infection, conflicting data exist about the role of PML-NBs during replication and viral egress. It is well established that simian virus 40 (SV40) initiates DNA replication juxtaposed to PML-NBs (Ishov & Maul, 1996; Tang et al., 2000). Using a plasmid-based SV40 replication system, it was shown that PML knockdown negatively affects SV40 replication (Boichuk et al., 2011), thereby suggesting that PML might contribute to efficient SV40 replication by providing access to factors specifically localized in these nuclear structures. However, PML-NBs have also been characterized as negative regulators during JC virus infection (Gasparovic et al., 2009), while BK virus infection reorganizes PML-NBs without affecting production of viral progeny (Jiang et al., 2011). In contrast, during the course of mouse polyomavirus infection, no rearrangement of PML-NBs is observed, and virus titres were produced at comparable levels in PML−/− and wt mouse embryonic fibroblasts (Erickson et al., 2012).

In this study, we demonstrated that in cells actively replicating MCPyV, LT protein localizes adjacent to components of PML-NBs, PML, Daxx and Sp100. In cells transfected with a replication-competent viral genome, MCPyV furthermore causes a reorganization of PML-NBs, concomitant with an increase in number and size of these nuclear structures. We showed that reorganization of PML-NBs is strictly dependent on viral DNA replication, since ectopically expressed LT protein in the presence or absence of plasmid carrying the minimal origin of replication did not localize to PML-ND10 nuclear structures. While the lack of a permissive system did not allow us to study the role of PML-NBs in MCPyV particle production, we demonstrated that knockdown of Sp100 increased viral DNA replication and that Sp100 protein level is decreased in cells replicating MCPyV DNA. Hence, we identified Sp100, a component of the PML-ND10 nuclear structure, as a new cellular factor that contributes to the control of MCPyV replication.

**RESULTS**

**Number and size of PML-NBs are significantly altered in cells transfected with replication-competent MCPyV Syn DNA**

We have previously shown that H1299 and PFSK-1 cells efficiently support early gene expression and DNA replication, as well as low-level late gene expression when transfected with a synthetic MCPyV DNA genome (Neumann et al., 2011). To analyse the consequences of the presence of an intact and replication-competent viral episome on PML nuclear structures, re-ligated MCPyV Syn DNA was transfected into H1299 cells or PFSK-1 cells. At 4 days post-transfection, cells were analysed by confocal laser scanning microscopy immunofluorescence for expression and localization of MCPyV LT and for the major PML-NB components PML and Sp100. As results were nearly identical between both cell lines, we exemplarily present only those for H1299 cells in Figs 1, 2, 3, 4 and 5. As shown in the left-hand panels of Fig. 1, LT staining patterns are reminiscent of viral replication centres, with approximately
4–12 bright, irregular-shaped foci being present per cell nucleus. EdU staining confirmed the foci as sites of active DNA replication, most likely of viral episomes (Fig. S1, centre panels, available in the online Supplementary Material). In Fig. 1, we present co-staining patterns of PML and MCPyV LT in these cultures. In both mock-transfected H1299 cells and the LT protein-negative subpopulation of MCPyVSyn DNA-transfected cultures (Fig. 1, bottom panels), PML staining was indistinguishable from that in the parental cell line. In contrast, in the LT-positive fraction, PML-NBs presented as enlarged, hollow sphere-shaped structures (Figs 1, centre panels, and S2). PML and LT-Ag signals are immediately adjacent but remain distinct, with no extensive co-localization being detectable. Computer-assisted image analysis readily confirmed a statistically significant increase in both the volume and number of PML-NBs in MCPyVSyn- versus mock-transfected H1299 cells (Fig. 2).

We next investigated the localization of Sp100, a PML-NB component previously described as specifically targeted by
several viral proteins, e.g. encoded by herpesvirus saimiri (HVS), adenovirus and BK virus (Berscheminski et al., 2013; Full et al., 2012; Jiang et al., 2009).

As shown in Fig. 3, Sp100 staining patterns remained unchanged in LT-negative cells, whereas 70% of LT-expressing cells again exhibited enlarged, spherical-shaped structures localized in the immediate adjacency of LT-protein signals (see also Fig. S2). Co-staining of PML and Sp100 in LT-positive and -negative cells confirmed co-localization of both proteins, indicating that Sp100 protein was not grossly dispersed from PML-NBs (Fig. 4) in the majority of cells. Again, a significant increase in both volume and number of Sp100 foci in LT/Sp100-positive cells was confirmed by automated image analysis (Fig. 5). However, we also noted that in approximately 30% of MCPyVSyn DNA-transfected LT-Ag-expressing cells, no Sp100 signal was detectable (Fig. 5c), indicating the presence of two distinct subpopulations.

Finally, we investigated the localization of the Daxx protein, which is targeted by the adenovirus E1B-55K protein (Schreiner et al., 2010) or human cytomegalovirus (HCMV) pp71 (Hwang & Kalejta, 2007) for proteasomal degradation. Upon transfection of MCPyVSyn DNA, Daxx was not dispersed from PML-NBs but, similar to PML and Sp100, localized to enlarged, spherical-shaped PML-NBs in LT-protein-expressing cells (Fig. S3). A Daxx-negative subpopulation was not detected.

Ectopic expression of MCPyV LT alone or in combination with an ori-containing plasmid is not sufficient to induce reorganization of PML-NBs

In order to determine whether the interactions of ectopically expressed MCPyV LT observed resulted in quantitative or qualitative alteration of PML-NBs, we performed confocal laser scanning microscopy to detect LT protein, PML or Sp100 in H1299 or PFSK-1 cells transfected with either a eukaryotic expression vector for MCPyV YFP-LT or a mock control. The results obtained were consistent between H1299 and PFSK-1 cells and uniformly indicated that LT expression alone did not alter the structure or abundance of PML-NBs. In Fig. 6, we present exemplary images of YFP-LT and Sp100 immunofluorescence staining in H1299 cells at 2 days post-transfection. YFP-LT was diffusely distributed throughout the nucleoplasm, with cytoplasm and nucleoli being exempt from staining (Fig. 6, left-hand panels). Regardless of the presence or absence of YFP-LT, PML (Fig. S4a) and Sp100 (Fig. 6, centre panels, and Fig. S4b) exhibited typical staining in small, compact, dot-like structures, with approximately 3–10 dots being present per cell nucleus. Automated image analysis, using the Volocity software package (see Methods for details), confirmed the absence of significant changes regarding either the average number or volume of PML or Sp100 signals in YFP-LT-transfected versus mock-transfected cultures (Figs 7 and S4c, d). Identical results were obtained upon expression of an untagged MCPyV LT, indicating that failure to modify PML-NB architecture was not due to the presence of the N-terminal tag (Fig. S4). We repeated these experiments with ectopically expressed sT protein and the major structural protein VP1 (here a codon-optimized expression construct was used; Pastrana et al., 2009). While we did not observe a significant change in PML-NB structure in cells overexpressing VP1 protein (Fig. S5), we noticed a slight increase in the volume of PML-NBs in cells overexpressing sT protein (Fig. S6). This increase was significantly smaller when compared with the results obtained in cells actively replicating MCPyV genomes and positive for LT protein expression.

![Graph](http://jgv.microbiologyresearch.org) **Fig. 2.** PML-NBs are altered in size and number in H1299 cells transfected with replication-competent MCPyVSyn DNA. The number (a) and size (b) of the PML-NBs were determined using Volocity as described in Methods. Fifty-six cell nuclei were included in the analysis. P values as calculated using an unpaired t-test are shown. *P<0.05, **P<0.01 and ***P<0.001. Results are shown for cells actively replicating MCPyVSyn (here cells positive and negative for LT staining were included) and mock-transfected cells.
In a second series of experiments, we also co-transfected our LT expression constructs with a construct containing the MCPyV origin of replication (ori) in a bacterial plasmid backbone. Although these constructs underwent low-level LT-dependent DNA replication (Fig. S7) (Borchert et al., 2014), we did not observe significant alteration in staining patterns of MCPyV LT, PML or Sp100. Hence, at least under the conditions used and in the cell lines employed here, ectopically expressed MCPyV LT is unable to remodel PML-NBs, regardless of the presence or absence of an artificial ori-containing plasmid.

**Sp100 knockdown leads to increased replication of MCPyV genomes**

To investigate potential functional consequences of knockdown of PML-NB components, we transduced H1299 or PFSK-1 cells with retroviruses expressing short-hairpin RNAs (shRNAs) targeting PML, Sp100 or Daxx. An shRNA directed against GFP was used as a negative control. All cultures were bulk selected with puromycin prior to analysis. As shown in Fig. 8(a), PML knockdown cells did not contain any detectable amounts of PML protein as judged by immunoblotting, whereas residual amounts of some Sp100 and

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**Fig. 3.** Sp100 protein localization in H1299 cells transfected with replication-competent MCPyV Syn DNA. Cultures were analysed for LT and Sp100 expression and localization by immunofluorescence staining and confocal microscopy at 4 days post-transfection. Shown are images of LT-positive and -negative cells from the same cultures. Bars: 30 µm (top panel); 5 µm (centre and lower panels); 1 µm (enlarged images).
Daxx were still present. All cell lines were also analysed for target protein expression by immunofluorescence staining, with none of the different shRNA cell lines showing detectable speckles of the depleted protein (Fig. S8). Measurement of proliferation rates by MTT assay furthermore confirmed that there were no significant differences between the growth rates of the different shRNA transductants (Fig. S9).

We have previously shown that in H1299 or PFSK-1 cells transfected with MCPyVSyn DNA, replicated viral genomes accumulated over an 8-day period whereas levels of LT protein typically peaked at day 2 and then declined (Neumann et al., 2011). Fig. 8(b) shows a DpnI resistance assay used to detect replicated MCPyV DNA in H1299 cells. In this assay, Hirt DNA extracts prepared from transfected cultures were subjected to digestion with the restriction enzyme DpnI, which degrades Dam+-methylated input DNA but leaves newly replicated episomes intact. Southern blotting then allowed the detection and quantitation of full-length replicated DNA (see Fig. 8b, upper bands) as well as restriction fragments from the input DNA. The latter can be used to normalize for transfection efficiency. Fig. 8(c) shows the results of three independent replication assays performed in H1299 cells, all normalized to input signal at day 2. The results indicated that PML knockdown has little influence on replication efficiency, whereas knockdown of Sp100 resulted in approximately threefold higher levels of replicated DNA at 8 days post-transfection. Collectively, these results indicate that Sp100 negatively influences replication of MCPyV genomes.

**DISCUSSION**

PML-NBs have been described as playing a role in the life cycle of many DNA and RNA viruses (Everett, 2001; Everett & Chelbi-Alix, 2007; Tavalai & Stamminger, 2008). For
some of these viruses PML-NB components are beneficial for viral replication whereas for others these nuclear structures have to be degraded or reorganized to counteract their anti-viral activities (Chelbi-Alix et al., 1998; Regad et al., 2001). Here, we show that MCPyV LT reorganizes PML-NBs to enlarged, hollow sphere-shaped structures when viral genomes are present.

Interestingly, we found Sp100 to be absent from PML-NB structures in 30% of the cells expressing MCPyV LT protein during MCPyV replication. In contrast, in the majority of cells the protein was still detectable and localized to enlarged PML-NB spherical structures. At present, we do not know the reasons for this difference, or its functional implications. Likewise, we do not know whether the minor cell fraction Sp100 is simply being dispersed from PML-NBs, or whether the protein is also being actively degraded. The fact that overall levels of Sp100 (in contrast to PML or Daxx) were slightly decreased in bulk protein preparations (Fig. S10), however, would seem to argue for the latter. While we observed a decrease in Sp100 protein in the soluble fraction in cells over-expressing the complete early region of MCPyV (with sT protein stabilizing LT protein and subsequent high LT protein expression), protein levels of PML and Daxx remained unchanged (Fig. S10). Furthermore, in applying Western blot analysis we did not detect any significant change in Sp100, PML or Daxx protein levels in cells individually over-expressing LT, sT or VP1, although LT in the absence of sT protein was expressed at significantly lower amounts. However, confocal microscopy revealed that cells over-expressing sT protein showed slightly enlarged PML-NB structures and absence of Sp100 in cells highly expressing sT protein (Fig. S6); we would like to point out that overexpression of sT protein does not reflect physiological sT protein levels expressed during the MCPyV life cycle, and that this highly toxic to the cells.

![Figure 5](https://example.com/figure5.png)

**Fig. 5.** Sp100-positive NB components are altered in number and size in H1299 cells. The number (a) and size (b) of the Sp100-positive spots in cells transfected with recircularized MCPyVSyn DNA or mock transfected were determined as described in Methods. Thirty-two cell nuclei were included in the analysis. Statistics were performed according to Methods. (c) Calculation of number of Sp100-positive spots in LT-positive and -negative cells, including cells not showing any Sp100 expression. *P≤0.05, **P≤0.01 and ***P≤0.001.
Specific degradation of Sp100 has recently been reported for HVS (Full et al., 2012), where the orf3 gene product specifically targets Sp100 for proteasomal degradation, while both Daxx and PML protein levels are not affected. Given that Sp100 knockdown leads to enhanced MCPyV DNA replication, it is possible that viral replication will proceed more efficiently in the minor fraction of cells seemingly devoid of Sp100. It is also possible that in a system that is fully permissive for MCPyV, Sp100 will be ultimately evicted from all PML-NBs and that further enlargement of PML-NB components to hollow sphere-shaped structures may be an intermediate of fully dispersed PML-NB. Disassembly of PML-NBs into PML-microstructures either with or without Sp100 been previously been described for

![Image: MCPyV LT Sp100 Overlay](image)

**Fig. 6.** Sp100 protein localization to PML-NB structures in H1299 cells overexpressing LT. H1299 cells overexpressing N-terminal YFP-tagged MCPyV LT protein were stained by immunofluorescence for endogenous Sp100 protein. LT-protein-positive and -negative cells were analysed 2 days post-transfection for Sp100 localization applying confocal microscopy. Bars: 30 µm (top panel); 5 µm (centre and lower panels); 1 µm (enlarged images).
different cellular stress conditions. For example, PML microstructures generated upon environmental stress are devoid of Sp100 (Dellaire & Bazett-Jones, 2004; Eskiw et al., 2003) whereas DNA damage produces microstructures with a protein composition identical to that of parental PML NBs (Dellaire & Bazett-Jones, 2004; Eskiw et al., 2004). Furthermore, PML-NB composition undergoes profound changes during the cell cycle. While Sp100 separates from the PML-NB structures during mitosis, it has been shown that Sp100 is tightly localized to these structures during the entire interphase (Everett et al., 1999). Post-translational modification, phosphorylation and conjugation to the ubiquitin-like protein SUMO-1 during interphase are involved in localization of PML-NB components to these nuclear structures. Taken together with findings on the effects of infection by DNA viruses and PML-NB structure and PML modification, it is believed that viral factors that have been shown to modify PML NB structures may do so by interfering with the mechanisms regulating post-translational modification of PML-NB components during the cell cycle.

Reorganization of PML-NBs into enlarged spherical structures has been observed previously in other viral systems, for example as HSV-1 (Everett & Chelbi-Alix, 2007) or BK virus infection (Jiang et al., 2011). Given the absence of efficient particle production in our system, LT protein expression and DNA replication appear to be the major drivers of this reorganization. Nevertheless, low-level late gene expression and particle production may also be contributory factors. In this context, it is noteworthy that focal localization of LT protein and PML-NB reorganization were observed in the presence of authentic viral genomes, but did not occur in LT-expressing cells harbouring a plasmid with the minimal MCPyV ori. While it is possible that PML-NB reorganization requires a particular stoichiometry between LT protein levels and origin abundance, we deem it more likely that other cis- or trans-acting features of full-length genomes are required, such as, for example, active transcription at or close to the origin, or expression of capsid proteins. Interestingly, another study has previously found LT protein to localize to punctate nuclear foci in the presence of a minimal ori plasmid in a portion of transfected cells (Li et al., 2015). Since this plasmid differed from ours in that it contained a CMV promoter upstream of the origin, it seems possible that forced transcription through the ori region may at least in part explain the different results.

**Fig. 7.** Number and size of PML-NB components is unchanged in H1299 (a, b) or PFSK-1 (c, d) cells overexpressing the MCPyV LT protein. (a, c) Number of Sp100-positive PML-NBs. (b, d) Volume of Sp100-positive PML-NBs. Images were taken applying confocal microscopy in cells transfected with MCPyV YFP LT or mock transfected as described in Methods. *P<0.05, **P<0.01 and ***P<0.001.
Fig. 8. Sp100 knockdown increases MCPySyn DNA replication. (a) H1299 cells stably transduced with shRNAs targeting PML, Daxx and Sp100 and bulk selected were analysed for protein expression of the individual PML-NB components at the individual time points of the replication assay. 2d, 4d and 8d: 2 days, 4 days and 8 days, respectively, post-transfection (p.t.). (b) MCPyVSyn replication assays. One microgram low-molecular mass DNA isolated at the indicated time points was digested with DpnI and EcoRI, separated on an agarose gel, and the transferred DNA was probed using a random-prime-labelled PCR fragment corresponding to LT-Ag region 126–429. The blot was exposed for 24 h and scanned using a Fuji phosphoimager FLA7000. M, mock. (c) The MCPyVSyn replication assay described in (b) was performed three times; exposed Southern blots were scanned and quantified applying MultiGauge software. Graph illustrates relative replication compared with time point 2d normalized against the input DNA levels at the time point 2d.
While our data indicate that MCPyV DNA replication can be limited by PML-NB components, we were unable to investigate their potential impact on virion production due to the lack of a fully permissive system. Very recently, an improved in vitro cell culture system to study the MCPyV life cycle was published by Liu et al. (2016). Human dermal fibroblasts were cultured under specific conditions (epidermal growth factor and fibroblast growth factor treatment combined with stimulators of the β-catenin WNT signalling pathway) to stimulate MCPyV entry and MCPyV replication. Future studies will address the role of PML-NBs and Sp100 in this improved MCPyV replication system in dermal fibroblasts.

In summary, our experiments provide evidence that MCPyV LT counteracts antiviral effects of PML-NBs during MCPyV replication, and suggest Sp100 as another host factor that is able to limit MCPyV replication. Future experiments will address the precise molecular mechanisms that lead to MCPyV-mediated remodelling of PML-NBs, e.g. with regard to the involvement of SUMOylation pathways that are utilized by other viruses to regulate PML-NB functions (Adamson & Kenney, 2001).

METHODS

Cell lines. H1299 cells (Mitsudomi et al., 1992) were grown as a monoculture in Dulbecco’s modified Eagle’s medium (DMEM technologies) supplemented with 10% FCS and 5% penicillin/streptomycin in a 5% CO2 atmosphere at 37°C. PFSK-1 cells (ATCC CRL-2060) were grown in RPMI medium supplemented with l-glutamine, 10% FCS and 5% penicillin/streptomycin. Knockout cells for Daxx, PML and Sp100 were bulk selected after transduction with lentiviral supernatant expressing shRNAs against Daxx, Sp100 and PML (Everett et al., 2011) as a template and primers flanked by XhoI and restriction sites.

Plasmids and transient transfections. The YFP-MCPyV T region (coding for all MCPyV T-antigens) is described elsewhere (Borchert et al., 2014). Retroviral plasmids expressing sT or LT were purchased from Addgene, plasmids #37861 and #32862 (Rozenblatt-Rosen et al., 2012). YFP-MCPyV LT was cloned from pADL* (Addgene plasmid #32097) with primers containing a XhoI restriction site: LT-F: AAGGGGCGCCGTTATGGAGAAAAAGTACCAGAATC and LT-R: AAGCGGCCGCTTATTGAGAAAAAGTACCAGAATC and assembled with Microsoft PowerPoint. For quantification, transfected cells were recorded with >2 zoom as stacks every 0.5 µm, and the measurement tool of Volocity was used. Nuclei and PML-NBs were processed as objects. The object size of nuclei was set to 1250 µm², and objects smaller than 600 µm² were excluded. For PML and Sp100 the size parameter was set to 0.5 µm³ and objects smaller than 0.1 µm³ were excluded.

To investigate viral DNA replication by immunofluorescence, a Click-iT EdU Alexa Fluor 594 Imaging kit from Molecular Probes was used according to the manufacturer’s instructions with the following modifications: (I) cells were incubated for 5–20 min with EdU 3 days post-transfection and (II) the Click-iT reaction was performed 1 day after incubation followed by immunofluorescence for MCPyV LT.

MTT assay. Proliferation of the cell lines was determined by MTT growth assay following the manufacturer’s instructions (Chemicon, Millipore).

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MCPyV replication assay. Generation of a consensus MCPyV genome, MCPyVSyn, and its use in DNA replication assays has been described recently (Neumann et al., 2011, 2015). DNA replication assays using a MCPyV ori-containing plasmid and the YEP-MCPyV LT protein have been published previously (Borchert et al., 2014).

Antibodies and Western blot analysis. Cells were resuspended in lysis buffer (50 mM Tris pH 8.0, 150 mM NaCl, 1% NP40, 0.5% sodium deoxycholate, 5 mM EDTA, 0.1% SDS) supplemented with 2.5 mM Na3 VO4, 5 mM NaF, 1 mM PMSF and protease inhibitors (complete, GE Healthcare). Proteins were separated by SDS PAGE and blotted to a PVDF membrane. Primary antibodies specific for polyomavirus proteins used in this study included mouse monoclonal MCPyV LT-Ag antibody Cm2B4 (Shuda et al., 2009; Santa Cruz Biotechnologies). FLAG-M2 antibody was purchased from SIGMA-Aldrich, PML rabbit pAb NB100-95787 from Novus Biochemicals, PML mouse-mAb 36.1-104 from Millipore, Daxx rabbit polyclonal Ab 07-471 from Upstate/Millipore, and Daxx mouse monoclonal Ab ALX-804-404-C100 from Enzo Life Science. The Sp100 rabbit polyclonal serum was a kind gift from Hans Will, Hamburg. MCPyV VP1 rabbit serum and sT 2t2 hybridoma cells were kindly provided by Chris Buck, NIH, USA (Pastrana et al., 2009; Wang et al., 2012b). Actin mouse mAb was applied in Western blot analyses to ensure that equal protein amounts loaded (Chemicon).

Secondary antibodies conjugated to horseradish peroxidase (HRP) for detection of proteins by immunoblotting were anti-mouse IgG and anti-rabbit IgG (Santa Cruz Biotechnologies). Secondary antibodies used in confocal microscopy were purchased from Santa Cruz Biotechnologies (anti-mouse IgG and anti-rabbit IgG conjugated to TRITC or FITC) and from Life Technologies (anti-rabbit IgG conjugated to Cy5).

Immunofluorescence staining, EdU staining and confocal microscopy. For indirect immunofluorescence, cells were grown on glass coverslips coated with 0.2% gelatine (Sigma). Cells were fixed in 4% paraformaldehyde in PBS and permeablized in PBS, 1% Triton X-100 and 0.1% sodium citrate. After 1h of blocking in Ca2+/Mg2+-free PBS containing 1% Triton X-100, 0.5% Tween and 3% BSA (albumin fraction) buffer, coverslips were treated for 2h with the primary antibody diluted in blocking buffer and washed in PBS followed by 2h of incubation with the corresponding secondary antibodies. Coverslips were mounted in vectashield medium containing DAPI (Vectashield), and digital images were acquired with a confocal laser-scanning microscope (Leica DM IRE2 with a Leica TCS SP5AOBS confocal point scanner) equipped with an oil-immersion plan Apo 63 NA 1.4 objective. Image processing was performed applying Volocity, ImageJ, Adobe Photoshop CS5 and assembled with Microsoft PowerPoint. For quantification, transfected cells were recorded with >2 zoom as stacks every 0.5 µm, and the measurement tool of Volocity was used. Nuclei and PML-NBs were processed as objects. The object size of nuclei was set to 1250 µm², and objects smaller than 600 µm² were excluded. For PML and Sp100 the size parameter was set to 0.5 µm³ and objects smaller than 0.1 µm³ were excluded.

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