E2F1-3 activate polyomavirus early transcription and replication

Salisbury NJH[1,*], Amonkar S[1], Roman A[1,2], Galloway DA[1,2,*]

1. Fred Hutchinson Cancer Center, Human Biology Division, Seattle, WA, USA
2. University of Washington, Department of Microbiology, Seattle, WA, USA

* To whom correspondence should be addressed: nsalisbu@fredhutch.org, dgallowa@fredhutch.org

Abstract

Polyomaviruses (PyVs) are small, circular double-stranded DNA viruses that establish lifelong persistent infections. PyV infections are typically asymptomatic, but they can cause severe disease particularly in immunosuppressed individuals, including BKPyV-associated nephropathy, and Merkel cell carcinoma (MCC) caused by Merkel cell polyomavirus (MCPyV). While most individuals are infected with PyVs during childhood, it is unclear how these viruses persist in the host and what cellular signals prompt their reactivation. The early regions of PyV genomes encode regulatory proteins that control viral replication and host cell transformation in the case of MCPyV, SV40 and murine polyomavirus (MuPyV). All PyV early regions encode Large and Small Tumor antigens (LT, ST), which are splice variants of a common transcript. PyV LTs bind and inhibit RB1 via a conserved LxCxE motif, deregulating E2F transcriptional activity and cell cycle control to promote viral replication and tumorigenesis. The existing paradigm in the field is during normal PyV infection, LT is expressed and drives the host cell into S phase. However, a recent study revealed that cells infected with BKPyV only express LT and replicate the viral genome when already in S phase, but the mechanisms activating LT expression during S phase are unknown. The Non-Coding Control Region (NCCR), found in all PyVs, contains the viral origin of replication and predicted transcription factor (TF) binding sites, yet only a handful have been experimentally validated and shown to regulate PyV early transcription. Here, we set out to identify host TFs that bind the MCPyV NCCR to regulate early transcription in both MCC cells and in an *in vitro* model of MCPyV infection. We identified E2F1-3 as critical regulators of MCPyV early transcription, which bind to the MCPyV NCCR via a consensus E2 site just upstream of LT/ST transcriptional start site. We discovered E2 sites in the NCCRs of several other polyomaviruses, including MuPyV but not BKPyV. Deletion of the E2 sites in PyV NCCRs downregulates early transcription in luciferase assays. Despite not containing a consensus E2 site, we detected weak E2F binding to the NCCRs of BKPyV and SV40, suggesting that E2Fs also contribute to transcription of their early genes. Overall, our findings challenge the existing paradigm of PyV infection, that LT expression activates E2F signaling via RB1 inhibition, and suggest that E2Fs must already be active in the PyV-infected host cell for LT/ST expression and viral replication.

Introduction

Polyomaviruses (PyVs) are small circular, double-stranded DNA viruses with genomes of approximately 5 kilobases. They infect a wide range of vertebrates, including humans, and establish persistent infections that typically remain asymptomatic in immunocompetent hosts. To date, 16 human polyomaviruses have been identified[1]. In immunosuppressed individuals, human PyVs can reactivate and cause severe disease, such as BKPyV-associated nephropathy and JCPyV-associated progressive multifocal leukoencephalopathy[2]. Merkel cell polyomavirus (MCPyV) causes Merkel cell carcinoma (MCC), a deadly neuroendocrine skin cancer, and is the only human polyomavirus that causes cancer. It was first identified integrated into the cellular genome of MCC tumors but has since been detected in healthy skin and other tissues[3, 4]. Immunosuppressed individuals are at increased risk of developing MCC, but MCC also arises in individuals with no overt immunodeficiency[5]. While most individuals in the population are infected with PyVs during childhood[6, 7], it is unclear how these viruses maintain a persistent infection and what cellular signals prompt their reactivation.

PyV genomes are divided into three regions – the early, late and non-coding control regions (ER, LR, NCCR). The ER encodes regulatory proteins that promote viral replication and oncogenic transformation in the case of SV40, murine polyomavirus (MuPyV), and MCPyV. These three PyVs also characterize three different clades of PyVs, each with a slightly different ER gene structure[8]. All PyVs express the Large and Small Tumor antigens (LT, ST), which are splice variants of a common early transcript. The SV40 clade, which is the largest and includes all the human PyVs except trichodysplasia spinulosa-associated PyV (TSPyV) and MCPyV, express only these two early proteins. SV40 LT can transform and immortalize cells *in vitro* independent of its ST[9]. The MuPyV clade, which includes TSPyV, expresses three early proteins – LT, ST and a Middle Tumor antigen (MT) – three splice variants of a common early transcript. MuPyV MT expression, in the absence of its LT and ST, is sufficient to initiate tumor formation in mice[10], and thus is considered the MuPyV major transforming oncoprotein. MCPyV encodes LT and ST splice variants and a third protein, the Alternate LT Open reading frame (ALTO), which is encoded on the second exon of LT in +1 reading frame[8]. Virus-positive MCC cells express truncated variants of LT (LT-t) and wild-type ST[11], which drive tumorigenesis by inactivating RB1 and p53[12, 13]. We have shown that ALTO is not expressed in MCC and acts as a tumor suppressor by activating NF-κB signaling and downregulating LT/ST transcription[14]. We previously speculated that ALTO is expressed from the LT and ST transcript, but it is currently unknown how its expression is regulated.

LTs have two critical functions in viral replication. Firstly, they bind directly to the PyV origin of replication within the NCCR, unwinds the viral DNA, and recruits host DNA replication machinery to the viral genome[15]. Secondly, LT binds to and inactivates the retinoblastoma protein, RB1, via conserved LxCxE motifs[16, 17]. A key consequence of LT binding to RB1 is deregulation of the E2F family of transcription factors and host cell cycle regulation. In normal quiescent cells, RB1 binds to E2F1-3 and their obligate partner proteins DP1/2, preventing them from activating expression of their target genes that mediate S-phase entry and DNA replication[18]. In response to growth factor stimulation, cyclin-dependent kinases (CDKs) phosphorylate RB1, releasing it from E2F/DP and allowing E2Fs to activate transcription of their target genes[19]. LT binds to RB1 and disrupt the interaction between RB1 and E2F/DP, mimicking RB1 hyperphosphorylation. Thus, the current paradigm in the field is that PyV infection and subsequent expression of LT drives the host cell into S phase by activating E2F signaling. However, a recent study of BKPyV revealed that only cells already

in S phase expressed LT and replicated the viral genome[20], yet how S phase signaling activates BKPyV LT expression is unknown.

PyV NCCRs contain the viral origin of replication, the transcriptional start sites of the early and late viral transcripts and transcription factor (TF) binding sites that regulate early and late transcription. SV40's NCCR has six SP1 binding sites, of which three are important for early transcription[21]. SV40, along with BKPyV and JCPyV, also have NF-κB binding sites that regulate early transcription[22-24]. Little is known about the TFs that regulate MCPyV transcription either in MCC cells or during normal infection. Bioinformatic predictions have been performed to identify TF binding sites in the MCPyV NCCR but these have not been experimentally validated[25, 26]. One study has identified NF-κB as an activator of LT/ST transcription in MCC and 293 cells[27]. We recently showed that MCPyV ALTO activates the NF-κB pathway in MCC cells, leading to NF-κB subunits RelA, RelB, NFKB1 p50 and NFKB2 p52 binding to the MCPyV NCCR and downregulating viral early transcription[14]. Unlike JCPyV and BKPyV, MCPyV's NCCR does not contain a consensus NF-κB binding site (κB site), so how NF-κB binds is unclear. However, the MCPyV transcriptional start sites have been mapped[28], and the early LT/ST transcriptional start site matches the consensus initiator element (Inr, 5'-BBCA$_{+1}$BW)[29]. The MCPyV LT/ST transcriptional start site is 23bp downstream of a consensus TATA box (5'-TATAWAW-3'), which recruits TBP and RNA polymerase II to promoters (Fig. 1A). Here, we set out to identify TFs that bind the NCCR and regulate MCPyV early transcription in MCC cells and in 293A cells, the latter of which supports MCPyV LT/ST expression and replication.

Results

E2F1-3 bind to the MCPyV NCCR in MKL-2 cells and regulate early transcription

To identify TFs that bind to the MCPyV NCCR in MCC cells, we performed DNA pulldowns coupled with mass spectrometry using MKL-2 cell lysates and a dsDNA probe spanning the length of the MCPyV NCCR (464 bp, Fig. 1A&B). As a negative control for non-specific protein-DNA interactions, we used dsDNA probe spanning the 3' end of the ampicillin resistance gene (AmpR, 500 bp). Consistent with the presence of a TATA box, TBP bound to the MCPyV NCCR more strongly than AmpR (FC = 2.64, p = 0.11). We did not detect binding of any NF-κB subunits to either the AmpR or NCCR probes. However, we detected binding of all eight members of the E2F family of transcription factors, E2F1-8, and their obligate partner proteins DP1/2 to the NCCR more strongly than AmpR (Fig. 1B). We validated binding of TBP, E2F1-3, and DP1 to the NCCR probe by Western blot (Fig. 1C). While RelA and NFKB1 p50 are expressed in MKL-2 cells, we still did not detect any binding of RelA and NFKB1 p50 to either probe by Western blot, consistent with our proteomic analysis. E2F/DP complexes bind to a highly conserved E2 site (5'-TTTSSCGC-3') found in the promoters of its target genes[30]. Analysis of the MCPyV NCCR revealed a consensus E2 site 6 bp upstream of the previously identified TATA box (Fig. 1A). To test whether E2F1-3 and DP1 bind to the NCCR via the E2 site, we generated an NCCR probe with the E2 site deleted (NCCR ΔE2). As predicted, E2F1-3 and DP1 binding do not the bind to the NCCR ΔE2 probe (Fig. 1C). In summary we conclude that in MKL-2, absent of any perturbation such as ALTO, E2F/DP complexes, but not NF-κB, bind to the MCPyV NCCR via a consensus E2 site.

To determine whether E2F binding to the MCPyV NCCR in MKL-2 cells regulates LT/ST transcription, we used CRISPR/Cas9 to delete the E2 site. We screened the NCCR for Protospacer

Adjacent Motifs (PAM sites, 5'-NGG-3') and identified two upstream of the E2 site and two downstream of the TATA box, allowing us to delete both the E2 site and TATA box but not the E2 site or TATA box alone (Fig. 1A). We delivered Cas9 and pairs of sgRNAs, one set annealing to 5' strand (sgF1+F2) and another to 3' strand (sgR1+R2), or a single non-targeting control sgRNA (sgNTC) via lentiviral vectors to MKL-2 cells. Consistent with E2F1-3 and DP1 and TBP binding to the NCCR to activate MCPyV transcription, we observed downregulation of LT/ST mRNA and protein in sgF1+F2 and sgR1+R2 cells compared to either sgNTC or non-transduced control cells (Fig. 1D&E). LT/ST expression was not completely ablated at either the RNA or protein level, suggesting that additional TFs bind the NCCR via other sites to regulate LT/ST transcription in MKL-2 cells. As validation of E2Fs regulating LT/ST transcription in MCC cells, we treated MKL-1 and MKL-2 cells with a small molecule E2F inhibitor (HLM006474) and assessed the effect on LT/ST mRNA and protein expression (Fig. 1F&G). As previously observed[31], HLM006474 treatment significantly downregulated E2F1-3 and DP1 protein levels in both MKL-1 and MKL-2 cells. Consistent with E2F1-3 activating LT/ST transcription, HLM006474 treatment also downregulated LT/ST mRNA and protein levels in both cell lines. Together, our findings identify E2F1-3 as critical activators of LT/ST transcription in MCC cells.

E2F1-3 bind to MCPyV NCCR in 293A cells and regulate early transcription and viral replication

Next, we sought to identify TFs that regulate LT/ST transcription during MCPyV infection. Although dermal fibroblasts are thought to be the natural MCPyV host cell[32], 293 cells have been shown to support MCPyV LT/ST expression and viral replication[33], and are a more experimentally tractable system. Of note, 293 cells and their variants all express adenovirus 5 E1A, which like MCPyV LT binds and inhibits RB1 via its LxCxE motif[34, 35]. Thus, E2F1-3 are constitutively active in 293 cells. We performed DNA pulldowns in 293A cells and detected binding of E2F and DP family members to the NCCR by mass spectrometry and Western blot (Fig. 2A&B). As in MKL-2 cells, E2F1-3 and DP1 binding to the NCCR probe is abolished when the E2 site is deleted. In 293A cells, TBP was not detected by mass spectrometry, but we observed a similar pattern of binding to the AmpR and MCPyV probes as observed in MKL-2 cells by Western blot. As in MKL-2 cells, we did not detect any binding of NF-κB subunits RelA or RelB to either the AmpR or NCCR probe via mass spectrometry. We validated lack of RelA binding to the NCCR by Western blot. In contrast to MKL-2, we could detect some binding of NFKB1 and NFKB2 to the NCCR probe by mass spectrometry. We analyzed NFKB1 binding by Western blot and were able to detect a very faint band in the NCCR wt and ΔE2 pulldowns. As in MKL-2 cells, we conclude that, in the absence of any perturbation such as ALTO, E2Fs, but not NF-κB, bind to the NCCR via a consensus E2 site in 293A cells under normal growth conditions.

As a first step to determine whether E2Fs regulate LT/ST transcription in 293A cells, we mutagenized our previously described MCPyV early NCCR luciferase vector[14] to remove the E2 site (ΔE2), TATA box (ΔTATA) or both (ΔE2+ΔTATA). Loss of the E2 site caused an ~60% reduction in luciferase activity (Fig. 2C). Deleting the TATA box caused an ~80% reduction in luciferase activity. Loss of both the E2 site and TATA box did not significantly reduce luciferase activity below that of the ΔTATA reporter. In all cases, the luciferase activity of the mutant NCCR reporters was higher than that of a negative control luciferase reporter that contained no promoter sequence inserted into the plasmid's multiple cloning site. These data indicate that both E2F and TBP play critical roles in

regulating MCPyV early transcription, consistent with our findings in MKL-2 cells, and suggest that other host TFs found in 293A cells also bind the NCCR via other sites and contribute to early transcription.

Next, we deleted the E2 site, TATA box or both in a recombinant MCPyV minicircle clone[36] and assessed the ability of the mutant MCPyV genomes to express LT/ST and replicate in 293A cells. Loss of either the E2 site or TATA box reduced expression of total LT/ST transcripts by ~90% compared to the wild type at four days post-transfection (Fig. 2D). Loss of both the E2 site and TATA box decreased LT/ST mRNA expression by ~99% at the same time point. We observed a similar trend in LT and ST protein expression at the same time point (Fig 2E). Consistent with decreased LT/ST expression, MCPyV ΔE2 had a ~90% reduction in copies per cell compared to the wild type at four days post-transfection, MCPyV ΔTATA ~75% reduction, and MCPyV ΔE2+ΔTATA ~95% reduction. Overall, our findings suggest that E2Fs are critical regulators of MCPyV LT/ST transcription and replication during the normal productive MCPyV life cycle.

A subset of PyV NCCRs contain E2F binding sites that regulate PyV early transcription

A recent report revealed that BKPyV requires the host cell to be in S phase for robust LT/ST expression and viral replication[20]. Given the well-established role of E2Fs in regulating S phase entry and our findings so far, we postulated that E2Fs might bind the NCCRs of BKPyV and other PyVs to regulate LT/ST expression. We searched the NCCRs from a range of mammalian polyomaviruses, including members of the SV40, MuPyV, and MCPyV clades, for E2 sites (Fig. 3A). Gorilla PyV is closely related to MCPyV and its NCCR resembles that of MCPyV, containing a consensus E2 site (5'-TTTSSCGC-3'), TATA box and Inr element. The NCCR of otomops PyV, more distantly related to MCPyV, contains a consensus TATA box and a variant E2 site (5'-CTTSSCGC-3'). Structural and biochemical studies have shown that that E2F/DP complexes can tolerate mismatches in the TTT part of the E2 site motif, while the central SSCG part of the motif is critical[30, 37]. Murine polyomavirus also contains a previously reported TATA box[38, 39], and a variant E2 site (5'-CTTSSCGC-3'), which has not been previously identified as far as we are aware. MuPyV also contains a consensus Inr element downstream of the TATA box and putative E2 site. While closely related to MuPyV, hamster PyV and TSPyV's NCCRs show a different structure with consensus E2 sites close to the start codon of their respective VP2s. In contrast to our initial prediction, BKPyV's NCCR does not contain a consensus or variant E2 site. The NCCRs of JCPyV and SV40 also do not. In conclusion, E2 sites are not a universal feature of PyV NCCRs but are found in the NCCRs of MT-/ALTO-encoding PyVs.

Next, we examined the ability of E2F3 and DP1 from 293A cells to bind to PyV NCCRs in a DNA pulldown (Fig. 3B). As observed previously, E2F3 and DP1 both bound strongly to MCPyV's NCCR but not to the negative control AmpR probe. As predicted based on the presence of a consensus or variant E2 sites, E2F3 and DP1 bound to the NCCRs of gorilla, otomops and murine PyVs and weakly to TSPyV's. We did not detect any E2F3/DP1 binding to hamster PyV's NCCR, despite the presence of a consensus E2 site. In contrast to the absence of an E2 site, we detected E2F3/DP1 binding to the NCCRs of BKPyV and SV40 but not to JCPyV's. As predicted, deleting the E2 site in the NCCRs of gorilla, otomops, murine and TS PyVs abolished E2F3/DP1 binding (Fig. 3C). Our results suggest that while an E2 site is a strong predictor of E2F/DP binding, E2F/DP can still bind to some PyV NCCRs in the absence of an E2 site.

Finally, to determine whether E2F/DP binding to PyV NCCRs regulates early transcription, we generated PyV early luciferase reporters with either wild-type or ΔE2 NCCRs and compared their luciferase activities when transfected in 293A cells (Fig 3D). As observed previously, deleting the E2 site in MCPyV's NCCR reduced luciferase activity by ~60%. Deleting the E2 site in gorilla PyV NCCR reduced luciferase activity by ~85%, by ~40% in otomops PyV's NCCR, and by ~90% in MuPyV's NCCR. Our TSPyV NCCR luciferase reporter showed no activity above the level of the empty vector negative control and no change with deleting the E2 site, suggesting that E2Fs do not activate TSPyV early transcription. In conclusion, E2F/DP complexes bind to the NCCRs of gorilla, otomops and murine PyV to activate LT/ST transcription.

Discussion

Expression of the PyV early proteins is essential for viral replication and tumorigenesis. Identifying host transcription factors that bind to the NCCR to regulate viral transcription is critical for understanding how PyVs maintain a persistent infection throughout the life of the host and will help identify therapeutic strategies to treat PyV-associated disease. PyV early transcription is thought to be controlled by the NCCR as it contains the early transcriptional start site and TF binding sites. While some host TFs have been identified that bind to the NCCRs for a handful of PyVs, we are still far from a complete picture of how PyV transcription is regulated. In particular, very little is known about the host transcription factors that regulate MCPyV early transcription both during normal infection and in MCC cells. A previous study identified NF-κB as an activator of LT/ST transcription in MCC and 293 cells and suggested that NF-κB binds to the NCCR and activates LT/ST transcription when expressed at low levels and inhibits LT/ST transcription when the pathway is "overstimulated" or RelA is overexpressed[27]. They also showed that RelA binds to the NCCR in a DNA pulldown when overexpressed but not at endogenous levels. We recently showed that MCPyV ALTO activates NF-κB signaling in both MCC cells and 293A cells, downregulating endogenous LT/ST transcription in MCC cells and an MCPyV NCCR early luciferase reporter in 293A cells[14]. We observed binding of endogenous, activated NF-κB to the MCPyV NCCR via a DNA pulldown assay using MKL-2 cells expressing ALTO. Here, we show that NF-κB signaling does not bind to the NCCR in MKL-2 or 293A cells under normal growth conditions and therefore does not appear to activate LT/ST transcription. While NF-κB binding sites have been predicted in the MCPyV NCCR, the predicted sites do not match the consensus κB sequence (5'-GGGRNYYYCC-3') that is found in the BKPyV and JCPyV NCCRs[25, 26]. It remains unclear how NF-κB, once activated by ALTO or overexpressed, binds to the MCPyV NCCR in the absence of a consensus κB site, and how it downregulates LT/ST transcription.

In the present study, we identified E2F1-3 as critical activators of LT/ST transcription. We identified a consensus E2 site in the MCPyV NCCR, just upstream of a previously reported consensus TATA box and a consensus initiator element (Inr). These three elements appear to form the core promoter regulating LT/ST transcription. As far as we are aware, ours is the first study identifying E2Fs as critical regulators of PyV early transcription and reveal a positive feedback loop regulating LT expression, whereby E2Fs activate LT expression, which binds to and inhibits RB1, sustaining E2F activity (Fig. 4A). Our findings also explain why 293 and 293A cells support robust LT/ST expression as they both express adenovirus 5 E1A that, like LT, contains an LxCxE motif, which mediates RB1 binding and inhibition. Thus, 293(A) cells have constitutive E2F signaling and are primed for MCPyV early transcription (Fig. 4B). Identifying E2Fs as critical regulators of MCPyV LT/ST expression in MCC cells opens the possibility of using E2F inhibitors to treat MCC. Two

previous studies identified the histone acetyltransferases (HATs) CREBBP and EP300 as activators of LT/ST transcription and identified small molecules that downregulate LT/ST transcription[27, 40]. CBP and EP300 bind the transcriptional activation domain of E2F1[41, 42], suggesting that E2Fs recruit CBP and EP300 to the MCPyV NCCR to acetylate NCCR histones and drive LT/ST transcription. Future work ought to assess whether treatment of MCC cells with E2F and CBP/EP300 inhibitors selectively kill MCC cells over normal proliferating cells.

Our results suggest that productive MCPyV infection and replication are intimately tied to the host cell cycle. Dermal fibroblasts have been shown to support MCPyV entry, gene expression and replication *in vitro*[32]. Fibroblasts must be serum-starved for MCPyV entry and then serum-stimulated for robust LT expression and viral replication. Our results explain why serum stimulation, a known activator of E2F signaling, is critical for MCPyV LT expression and replication. In addition to LT expression, E2F signaling might also indirectly regulate MCPyV nuclear entry. In contrast to other PyVs, MCPyV does not enter the nucleus via the nuclear pore complex but appears to take advantage of cell-cycle dependent nuclear envelope breakdown to gain access[43]. Taking all our findings together, it appears that, following MCPyV endocytosis, an infected cell appears to need to pass through mitosis for MCPyV to traffic to the nucleus and then pass through the G_1-S checkpoint again before MCPyV can express its genes and begin replicating.

Currently, the virus-positive MCC cell of origin is unknown. Evidence points to MCC arising from an epithelial cell type or a possibly a cell of neuronal lineage, although fibroblasts and pre-/pro-B cells have also been proposed[44-47]. LT mutation and clonal integration of the mutant MCPyV genome appear to be early events in MCC tumorigenesis[11]. As LT/ST expression appears to depend on the host cell actively dividing, our findings point to the MCC cell of origin being a stem or progenitor cell type, perhaps a basal keratinocyte or a Merkel cell progenitor. In MKL-2 cells, deletion of the E2 site and TATA box significantly downregulated, but did not completely abolish, LT/ST transcription and protein expression (Fig. 1D&E), indicating that other host TFs bind the NCCR in MCC cells and contribute to LT/ST transcription. It is plausible that these factors could be cell lineage transcription factors. MCPyV infection is thought to be relatively promiscuous with MCPyV pseudovirions carrying a CMV-GFP reporter plasmid able to enter a wide variety of different cell types and express GFP[48]. In another study, MCPyV pseudovirus was shown to infect keratinocytes readily and Merkel cells at very low frequencies (1 in 122 KRT20+ cells), LT and VP1 expression from the MCPyV genome was restricted to dermal fibroblasts[32], suggesting that the NCCR contributes to viral tropism. Since E2Fs are essential genes expressed in all cell types, it is reasonable to suspect that mesenchymal lineage TFs bind the NCCR to facilitate MCPyV gene expression in fibroblasts. Further work will be needed to address what these TFs in fibroblasts are and whether these same TFs are expressed and contribute to LT/ST transcription in MCC cells or if other cell lineage TFs plays a role, which could provide insight into the MCC cell of origin.

As BKPyV appears to also require the host cell to be actively replicating[20], we examined E2F/DP binding across a representative collection of PyVs. We identified E2 sites that regulate early transcription in gorilla, otomops, and murine PyVs that are all relatively closely related to MCPyV. We detected very weak bind of E2F/DP to an E2 site in the NCCR of TSPyV, located close to the VP2 start codon, however, a TSPyV early luciferase reporter was not active in 293A cells, suggesting that this TSPyV E2 site does not regulate early transcription. Future work will be required to assess whether E2Fs activate TSPyV late transcription. While we could not identify an E2 site in SV40 or an

archetype strain of BKPyV, we did detect weak binding of E2F/DP to their NCCRs. The NCCRs of BKPyV and JCPyV undergo rearrangements, including deletions and duplications, during pathogenesis[49, 50]. Similarly, SV40 strains with NCCR variants display differences in their ability to replicate in rhesus macaques and in their oncogenic potential in hamsters[51, 52]. It is therefore plausible that the NCCRs of these PyVs contain weak E2F/DP binding sites that get amplified in the NCCRs of pathogenic strains, resulting in increased LT/ST expression and viral replication. In support of this hypothesis, infection of immortalized human renal proximal tubule epithelial (RPTE) cells with an archetype BKPyV strain, followed by long-term passaging, invariably lead to the outgrowth and reactivation of BKPyV clones with rearranged NCCRs and increased LT/ST expression[49], suggesting that continuous RPTE cell division and active E2F signaling selects for BKPyV NCCR mutations that enhance LT/ST and viral replication. Archetype BKPyV does not express LT at detectable levels by Western blot, whereas rearranged variants such BKPyV Dunlop express LT at detectable levels. Of note, the BKPyV Dunlop strain was used in the study that identified the requirement for the host cell to be in S phase for LT expression and viral replication[20]. Further work will be required to examine whether the duplicated sequences in the BKPyV Dunlop NCCR mediate E2F binding.

Overall, our findings support a model whereby PyVs exist in a dormant phase with little to no gene expression if the host cell is in G_0 or G_1 phases of the cell cycle and begins to replicate when the host cell enters S phase. Previously, it was thought that PyV infection and LT expression stimulates host cell S phase entry. Our study of MCPyV and previous findings in BKPyV suggest, in fact, that host cell S phase entry drives PyV replication (Fig. 4C&D). As most PyV infections are asymptomatic, we propose that PyVs spend most of their time in a dormant state in non-dividing host cells. However, if a PyV-infected cell receives a signal to divide, perhaps during a wound healing response following tissue damage, it will also stimulate PyV replication, allowing the virus to spread to other cells and maintain a persistent infection in the regenerating tissue. Furthermore, in a dormant state with little to no viral gene expression, PyVs will be able to evade host immune detection and persistent for the life of the host.

Materials and Methods

Cell lines

MCC cell lines, MKL-1 and MKL-2, were previously obtained from the Chang & Moore lab (U. Pittsburgh) and were maintained in RPMI 1640 HEPES medium (Gibco) supplemented with 10% fetal bovine serum (FBS, Corning), and penicillin-streptomycin solution (Gibco). 293A cells (Invitrogen) were cultured in Dulbecco's modified Eagle's medium (Gibco) supplemented with 10% FBS, glutaMAX (Gibco), non-essential amino acids (Gibco) and penicillin-streptomycin solution. All cells were cultured at 37°C with 5% CO_2 in a humidified atmosphere.

Plasmids and subcloning

MCPyV minicircle genomic DNA was prepared from pMC.BESPX-MCV-HF (kindly provided by the Chang and Moore lab) as previously described[36]. MCPyV minicircle variants were produced using the Q5 site-directed mutagenesis kit (NEB) following manufacturer's instructions. PyV NCCR variants were synthesized (IDT) and cloned into pGL4.11 (Promega) using InFusion HD cloning kit (Takara) following manufacturer's instructions. CMV-renilla plasmid pGL4.75 was obtained from Promega. Lentiviral Cas9+sgRNA vectors were synthesized by VectorBuilder.

DNA pulldown assays

Small scale DNA pulldowns were performed as described previously[14], using 5 µg biotinylated dsDNA probe, 50 µL streptavidin magnetic beads, and 500 µg cell lysate in 500 µL.

Mass spectrometry sample prep and analysis

For large scale DNA pulldowns for mass spectrometry analysis, 30 µg biotinylated dsDNA probe, 300 µL streptavidin magnetic beads, and 3 mg cell lysate was used per replicate. Following washing the beads five times in 20 mM Tris-HCl pH 7.5, 50 mM KCl to remove detergents, the magnetic streptavidin beads and bound proteins were resuspended in 8 M urea in 100 mM Tris pH 8.5. Protein disulfide bonds were reduced with 10 mM tris (2-carboxyethyl) phosphine with mixing at room temperature for 20 min. The reduced proteins were alkylated with 20 mM 2-chloroacetamide with mixing in the dark at room temperature for 30 min. Samples were digested with rLys-C protease (500 ng) with mixing at 37°C for 4 hr. The concentration of urea was reduced to 1 M with 100 mM Tris pH 8.5 and 10 mM calcium chloride was added. Trypsin protease (500 ng) was added and the reaction was mixed overnight at 37°C. Samples were removed from heat, spun, put on a magnetic rack and the supernatant was collected. The beads were washed with 0.1% trifluoroacetic acid and the solutions were combined and dried on a speedvac. Samples were desalted over TopTip (LC Packings), eluted with 15 mM ammonium formate pH 2.8 and dried on a speedvac. The desalted samples were brought up in 2% acetonitrile in 0.1% formic acid prior mass spectrometry analysis.

Samples were analyzed by LC/ESI MS/MS with a Thermo Scientific Vanquish Neo UHPLC (Thermo Scientific, Waltham, MA) coupled to a tribrid Orbitrap Ascend with FAIMS pro (Thermo Scientific, Waltham, MA) mass spectrometer. In-line desalting was accomplished using a reversed-phase PepMap Neo Trap Cartridge C_{18} (300 µm × 5 mm, 5-µm, 100 Å C_{18} resin; Thermo Scientific, Waltham, MA) followed by peptide separations on a reversed-phase column (75 µm × 270 mm) packed with ReproSil-Pur C_{18}AQ (3-µm 120 Å resin; Dr. Maisch, Baden-Würtemburg, Germany) directly mounted on the electrospray ion source. A 90-minute gradient from 8% to 30% B (80%

acetonitrile in 0.1% formic acid/water) at a flow rate of 300 nL/minute was used for chromatographic separations. LC/MS/MS data were collected using data dependent acquisition methods with the MS survey scans detected in the Orbitrap. MS/MS spectra were detected in the linear ion trap using HCD activation. Selected ions were dynamically excluded for 60 seconds after a repeat count of 1.

Data analysis was performed using Proteome Discoverer 3.1 (Thermo Scientific, San Jose, CA). The data were searched against a Uniprot Human (UP000005640_Human_2023_11_09.fasta), Merkel Cell (NCBI_MerkelCellPolyomavirus_040824.fasta) and common contaminants (cRAPome). Searches were performed with settings for the proteolytic enzyme trypsin. Maximum missed cleavages were set to 2. The precursor ion tolerance was set to 10 ppm and the fragment ion tolerance was set to 0.6 Da. Dynamic peptide modifications included oxidation (+15.995 Da on M). Dynamic modifications on the protein terminus included acetyl (+42.011 Da on N-terminus), Met-loss (-131.040 Da on M) and Met-loss+Acetyl (-89.030 Da on M) and static modification carbamidomethyl (+57.021 on C). Minora was used for peak abundance and retention time alignment. Sequest HT was used for database searching. All search results were run through Percolator for scoring. Raw quantitative results were transformed to $\log_2$ scale and normalized to the median value across samples. Missing data were imputed with half of the global minimum value. P-values for pairwise comparisons were calculated by t-test. Mass spectrometry data sets are available in the Supplementary Data file.

Lentiviral transduction

To generate lentiviral particles, 293T cells were transfected with psPAX2 and pMD2.G (gifts from Didier Trono – Addgene #12260, #12259) and Cas9+sgRNA lentiviral vectors. Supernatants containing lentiviral particles were harvested 48 h post transfection, 0.45 µM filtered, and then supplemented with 6 µg/mL polybrene. MKL-2 cells were incubated with lentiviral supernatants for 24 h followed by 24 h recovery in fresh growth medium. Transduced cells were selected with 0.5 µg/mL puromycin for 4 days and then allowed to recover for 2 days in fresh growth medium lacking puromycin.

RNA extraction and quantitation by qRT-PCR

Total RNA was extracted from cells using RNeasy mini kit (Qiagen) following manufacturer's instruction including DNase I on-column digestion to remove gDNA.

Protein extraction, Western blotting and antibodies

Proteins were extracted and Western blotting performed as previously described[14]. The following antibodies were used: E2F1 (#3742, Cell Signaling Technologies), E2F2 (ab31895, Abcam), E2F3 (ab320731, Abcam), DP1 (ab124678, Abcam), TBP (#44059, Cell Signaling Technologies), RelA (#8242, Cell Signaling Technologies), NFKB1 (#12540, Cell Signaling Technologies), Ab5 for MCPyV LT and ST (a kind gift from the DeCaprio lab, Dana-Farber Cancer Institute), Cas9 (#14697, Cell Signaling Technologies), actin-HRP (#5125, Cell Signaling Technologies).

MCPyV genome transfection

293A cells were seeded at 2.5×10^6 cells per 10 cm plate. The following day, cells were transfected with 5 µg of MCPyV minicircle DNA using TransIT 293 (Mirus Bio) following manufacturer's

instructions. Media was refreshed 2 days post-transfection and cells were harvested 4 days post-transfection for further analysis.

gDNA extraction and quantitation by qRT-PCR.

gDNA was extracted from MKL-2 and 293A cells using PureLink genomic DNA mini kit following manufacturer's instruction. Typically, $2.5x10^6$ cells were used for each extraction. qRT-PCR reactions were performed using SYBR Green PowerUP master mix (Applied Biosystems) and 5 ng of total gDNA as input per reaction. See supplementary data for details of qPCR primers.

Luciferase assays

Luciferase assays were performed using MCPyV NCCR early luciferase reporter previously described[14]. In brief, $2x10^5$ 293A cells were seeded in each well of a 96 well plate. The next day, cells in each well were transfected with 100 ng PyV NCCR luciferase vectors and 100 ng CMV-renilla plasmid. Luciferase and renilla activity was analyzed two days later using the Dual-Glo Luciferase kit (Promega) following manufacturer's instructions. The measured luciferase activity in each well was normalized by the corresponding renilla activity.

Statistics

Statistical analyses were performed using GraphPad Prism 10.

Nucleotides sequences

Sequences for sgRNAs, qPCR primers and PyV NCCRs used for DNA pulldowns and luciferases are found in the Supplementary Tables file.

Acknowledgments

This research was funded by NIH/National Cancer Institute R35 grant to D.A.G. (CA209979), a Brave Fellowship awarded by the Brave Like Gabe Foundation to N.J.H.S, and a Fred Hutch Pathogen Associated Malignancies Integrated Research Center pilot award to D.A.G and N.J.H.S.

Assistance with DNA pulldown mass spectrometry experiments was provided by Lisa Jones, Chenwei Lin, and Phil Gafken of the Fred Hutch Proteomics & Metabolomics Shared Resource, which is funded in part through NIH/NCI Cancer Center Support Grant P30 CA015704. The Orbitrap Ascend mass spectrometer used in this research was acquired through the NIH Office of Research Infrastructure Programs grant S10OD030225.

We are very grateful to Dr. Yuan Chang, Dr. Patrick Moore and Dr. Bizunesh Abere (U. Pittsburgh) for sharing the recombinant MCPyV minicircle genome with us. We would also like to thank Dr. Jim Pipas and Dr. Sunnie Thompson for insightful discussions at the DNA Tumor Virus Meeting 2025 in Madison, WI, USA.

All cartoons and schematics were created using BioRender.com

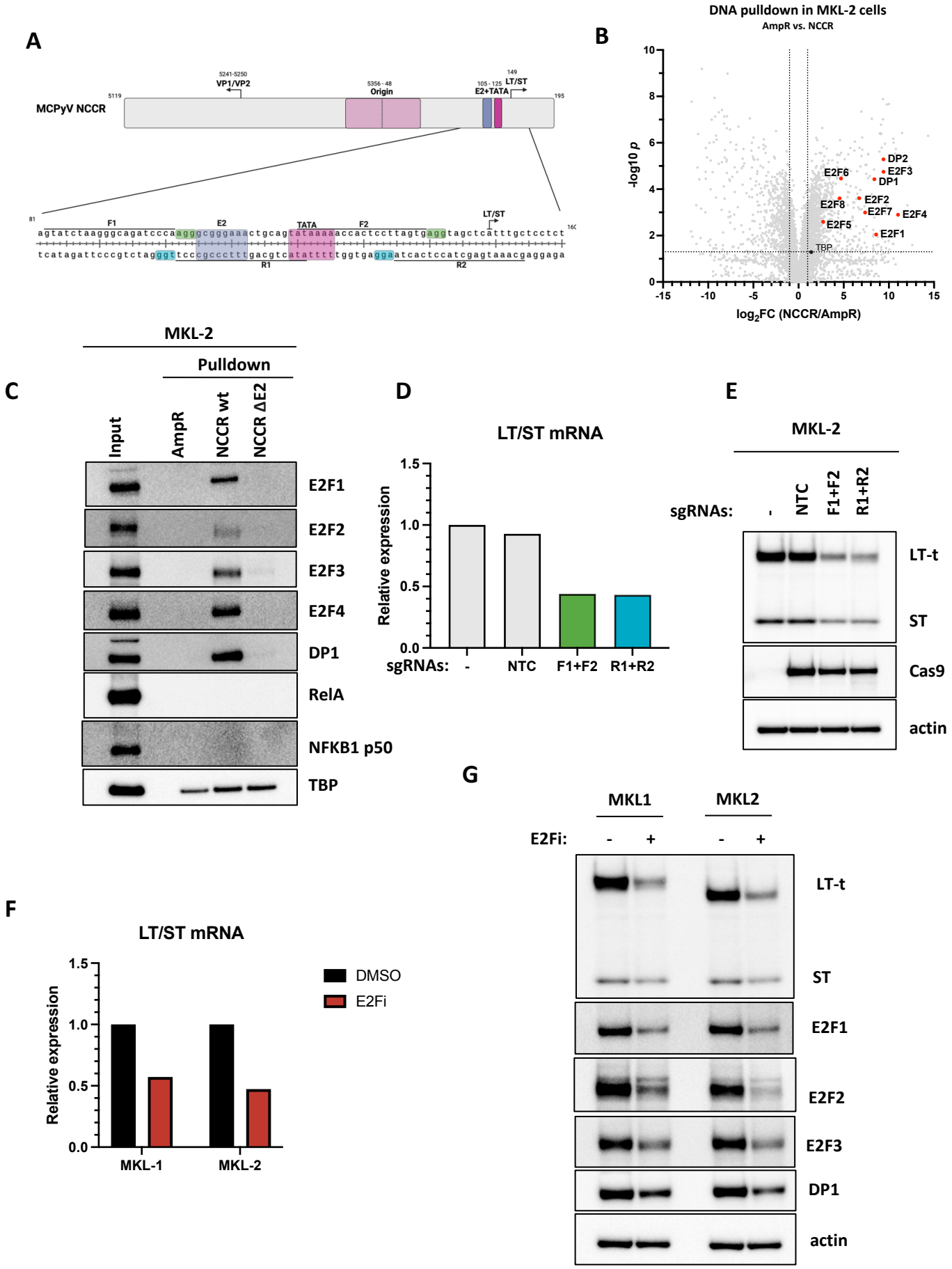
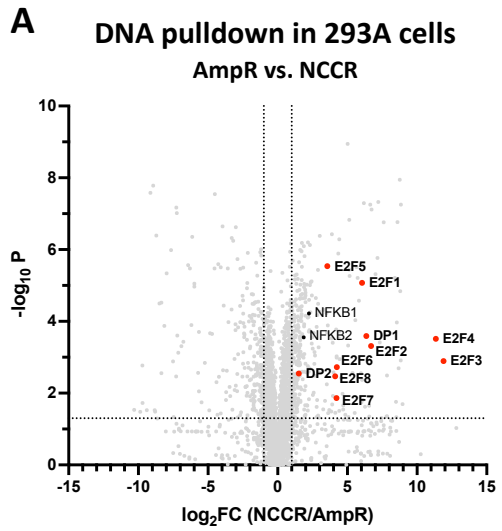
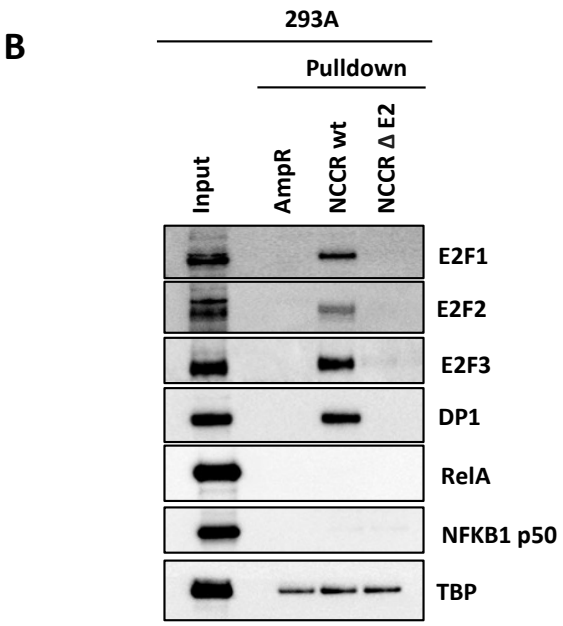
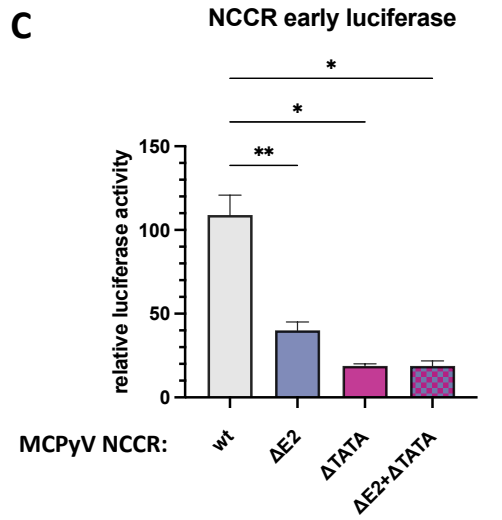
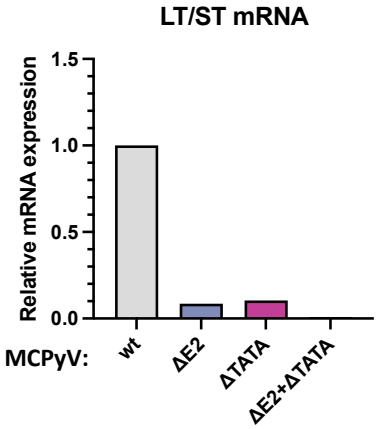

A
MCPyV NCCR
5119
5241-5250
VP1/VP2
5356 - 48
Origin
105 - 125
E2+TATA
149
LT/ST
195
F1
E2
TATA
F2
LT/ST
agtatctaagggcagatcccaagggcgggaaactgcagtataaaaaccactccttagtgaggtagctcatttgctcctct
tcatagattcccgtctagggttcccgccctttgacgtcatattttttggtgaggaatcactccatcgagtaaacgaggaga
R1
R2
B
DNA pulldown in MKL-2 cells
AmpR vs. NCCR
-log10 p
log2FC (NCCR/AmpR)
DP2
E2F3
DP1
E2F6
E2F2
E2F7
E2F4
E2F8
E2F5
E2F1
TBP
C
MKL-2
Pulldown
Input
AmpR
NCCR wt
NCCR ΔE2
E2F1
E2F2
E2F3
E2F4
DP1
RelA
NFKB1 p50
TBP
D
LT/ST mRNA
Relative expression
sgRNAs:
-
NTC
F1+F2
R1+R2
E
MKL-2
sgRNAs:
-
NTC
F1+F2
R1+R2
LT-t
ST
Cas9
actin
G
MKL1
MKL2
E2Fi:
-
+
-
+
LT-t
ST
E2F1
E2F2
E2F3
DP1
actin
F
LT/ST mRNA
Relative expression
DMSO
E2Fi
MKL-1
MKL-2

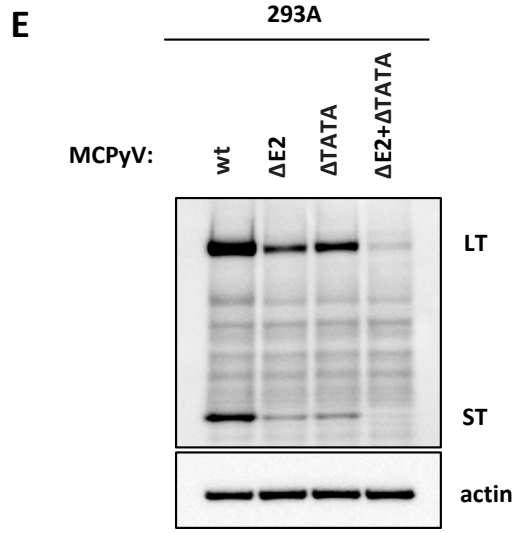
Figure 1. E2Fs bind the MCPyV NCCR and regulate LT/ST transcription in MCC cells. A) Schematic of the MCPyV NCCR highlighting the E2 site (purple), TATA box (pink) and PAM sites (green and blue) and sgRNA sequences (F1+F2, R1+R2) used in (*D)* and (*E)* to delete the E2 site and TATA box. **B)** Mass spectrometry analysis of DNA pulldown samples using MKL-2 cell lysates and AmpR and NCCR probes. **C)** Western blot validation of NCCR-binding transcription factors identified by mass spectrometry. **D)** qRT-PCR analysis of total LT/ST mRNA in MKL-2 cells transduced with lentiviral vectors encoding Cas9 and indicated sgRNAs and then harvested following 4 days of puromycin selection and 2 days of recovery. LT/ST mRNA expression is normalized by the expression of housekeeping gene 36B4. **E)** Western blots from cells analyzed in (*D)*. **F)** qRT-PCR analysis of total LT/ST mRNA expression in MKL-1 and MKL-2 cells were treated with 40 μM HLM006474 for 4 days. **G)** Western blots from cells analyzed in (*F*).


A
DNA pulldown in 293A cells
AmpR vs. NCCR
-log10 P
log2FC (NCCR/AmpR)
E2F5
E2F1
NFKB1
NFKB2
DP1
E2F2
E2F4
E2F3
E2F6
DP2
E2F8
E2F7


B
293A
Pulldown
Input
AmpR
NCCR wt
NCCR Δ E2
E2F1
E2F2
E2F3
DP1
RelA
NFKB1 p50
TBP


C
NCCR early luciferase
relative luciferase activity
MCPyV NCCR:
wt
ΔE2
ΔTATA
ΔE2+ΔTATA


D
LT/ST mRNA
Relative mRNA expression
MCPyV:
wt
ΔE2
ΔTATA
ΔE2+ΔTATA


E
293A
MCPyV:
wt
ΔE2
ΔTATA
ΔE2+ΔTATA
LT
ST
actin

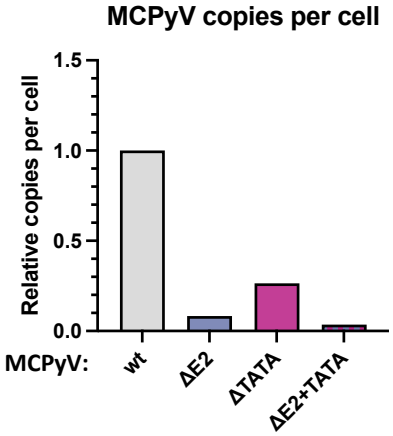
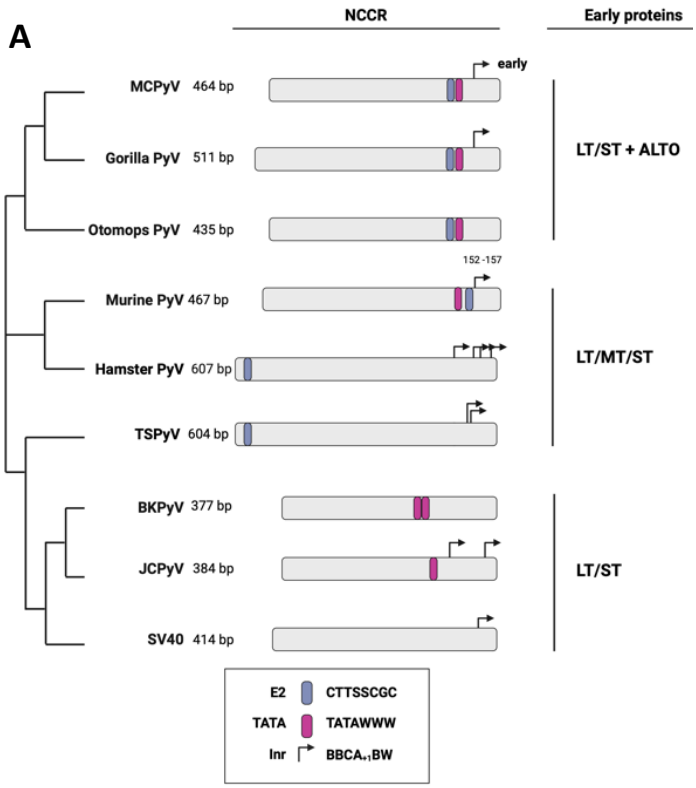
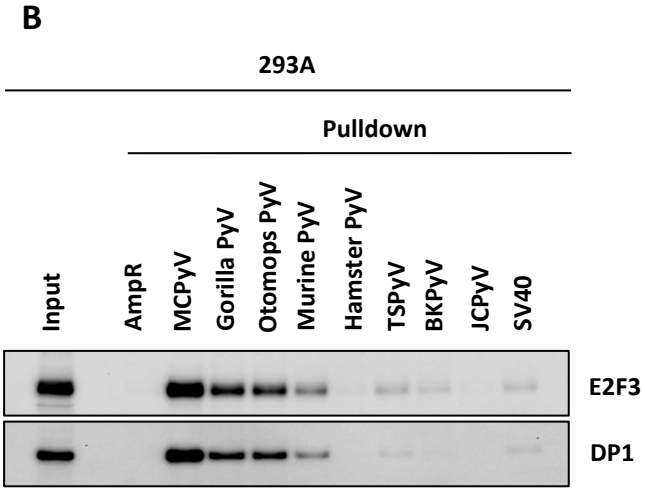
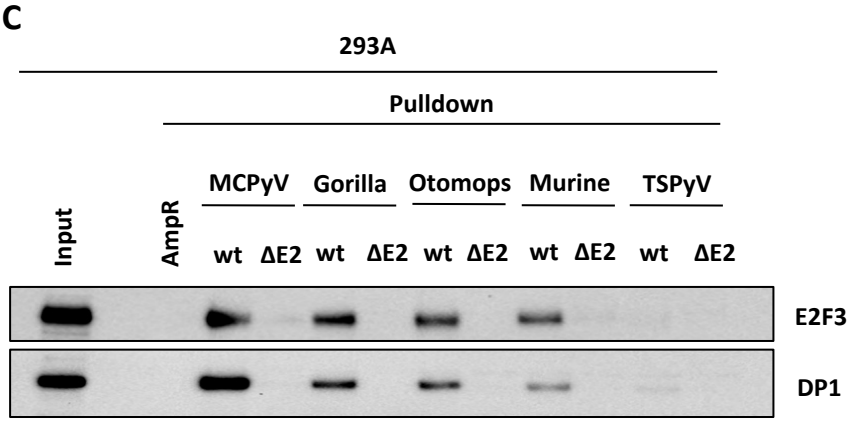

F
MCPyV copies per cell
Relative copies per cell
MCPyV:
wt
ΔE2
ΔTATA
ΔE2+TATA

Figure 2. E2F1-3 bind the MCPyV NCCR to regulate LT/ST transcription and MCPyV replication in 293A cells. A) Mass spectrometry analysis of DNA pulldown samples using 293A cell lysates and AmpR and NCCR probes. **B)** Western blot validation of NCCR-binding transcription factors from mass spectrometry. **C)** Relative luciferase activity of MCPyV NCCR variant reporters transfected in 293A cells harvested two days later. Firefly luciferase activity was normalized by CMV-renilla activity. The reported luciferase activity is relative to cells transfected with an empty vector firefly luciferase reporter and CMV-renilla. Error bars represent the standard deviation of three technical replicates. Statistical significance was calculated by one-way ANOVA. * $p < 0.033$, ** $p < 0.002$. This experiment has been performed twice and representative results from one repeat are shown. **D)** qRT-PCR analysis of total LT/ST mRNA in 293A cells transfected with MCPyV variants and harvested four days later. LT/ST mRNA expression is normalized by the expression of housekeeping gene 36B4. **E)** Western blots from cells analyzed in (*D)*. **F)** qRT-PCR analysis of relative MCPyV copy number four days post-transfection, normalized to GAPDH DNA content.


A
NCCR
Early proteins
early
MCPyV 464 bp
Gorilla PyV 511 bp
Otomops PyV 435 bp
LT/ST + ALTO
152-157
Murine PyV 467 bp
Hamster PyV 607 bp
TSPyV 604 bp
LT/MT/ST
BKPyV 377 bp
JCPyV 384 bp
SV40 414 bp
LT/ST
E2 CTTSSCGC
TATA TATAWWW
Inr BBCA.,1BW


B
293A
Pulldown
Input
AmpR
MCPyV
Gorilla PyV
Otomops PyV
Murine PyV
Hamster PyV
TSPyV
BKPyV
JCPyV
SV40
E2F3
DP1


C
293A
Pulldown
Input
AmpR
MCPyV
Gorilla
Otomops
Murine
TSPyV
wt ΔE2 wt ΔE2 wt ΔE2 wt ΔE2 wt ΔE2
E2F3
DP1

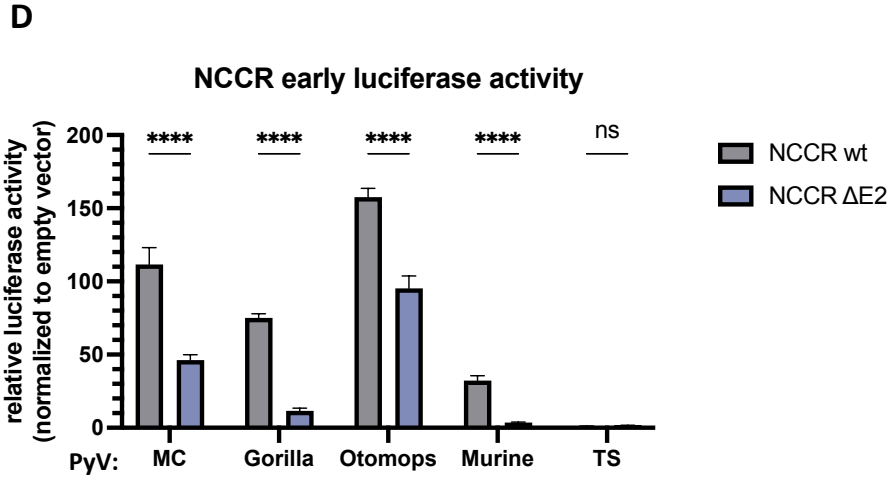

D
NCCR early luciferase activity
relative luciferase activity
(normalized to empty vector)
200
150
100
50
0
****
****
****
****
ns
PyV:
MC
Gorilla
Otomops
Murine
TS
NCCR wt
NCCR ΔE2

Figure 3. A subset of PyV NCCRs contain E2 sites that regulate early transcription. A) Schematic of PyV NCCRs highlighting known and putative promoter elements including E2 sites, TATA boxes, and initiator (Inr) elements. **B)** Western blots of DNA pulldowns using PyV NCCRs as bait and 293A cells lysates as prey. **C)** DNA pulldowns using wild-type (wt) PyV NCCRs and a variant with the putative E2 sites deleted (ΔE2) as bait and 293A cell lysates as prey. **D)** Relative luciferase activity of PyV NCCR variant reporters transfected in 293A cells harvested two days later. Firefly luciferase activity was normalized by CMV-renilla activity. The reported luciferase activity is relative to cells transfected with an empty vector firefly luciferase reporter and CMV-renilla. Statistical significance was calculated by one-way ANOVA. **** $p < 0.0001$. This experiment has been performed twice and representative results from one repeat are shown.

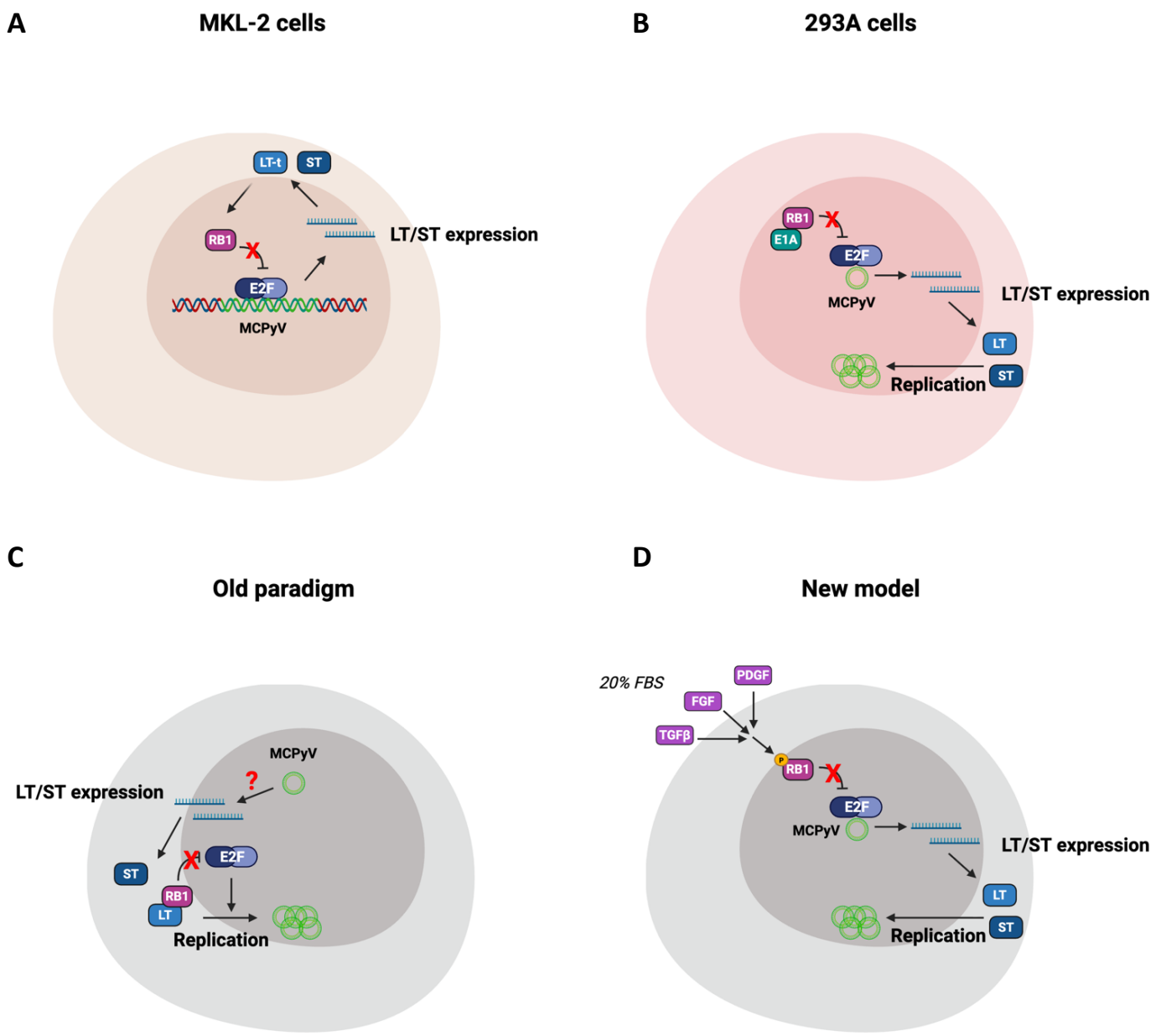


Figure 4. E2F signaling activates MCPyV LT/ST expression and viral replication. A) In MCC cells, mutant MCPyV genome is integrated in the host cellular genome that encodes truncated LT-t, which still retains the LxCxE motif for RB1 inhibition. E2F binds to the E2 site in the MCPyV NCCR and drives LT/ST transcription. Together, LT/E2F signaling creates a positive feedback loop that deregulates E2F activity, sustains LT/ST expression, and promotes tumorigenesis. **B)** In 293A cells, Ad5 E1A is expressed, which binds and inhibits RB1 via its LxCxE motif. Thus, 293A cells are primed for MCPyV LT/ST expression and replication. **C)** In the old paradigm, PyV infection drives a quiescent host cell into S phase via expression of LT, which binds and inhibits RB1 leading to activated E2F signaling that promotes viral replication. **D)** We propose that growth factors in serum stimulate RB1 phosphorylation and E2F activation, leading to S phase entry, LT/ST expression and MCPyV replication in dermal fibroblasts.

References

1. Fazeli MM, Heydari Sirat S, Shatizadeh Malekshahi S. Novel Human Polyomaviruses Discovered From 2007 to the Present: An Update of Current Knowledge. Rev Med Virol. 2025;35(2):e70017. doi: 10.1002/rmv.70017. PubMed PMID: 40000590.
2. Wiedinger K, Bitsaktsis C, Chang S. Reactivation of human polyomaviruses in immunocompromised states. J Neurovirol. 2014;20(1):1-8. Epub 20140131. doi: 10.1007/s13365-014-0234-x. PubMed PMID: 24481784; PMCID: PMC4000910.
3. Feng H, Shuda M, Chang Y, Moore PS. Clonal integration of a polyomavirus in human Merkel cell carcinoma. Science. 2008;319(5866):1096-100. Epub 20080117. doi: 10.1126/science.1152586. PubMed PMID: 18202256; PMCID: PMC2740911.
4. Pastrana DV, Tolstov YL, Becker JC, Moore PS, Chang Y, Buck CB. Quantitation of human seroresponsiveness to Merkel cell polyomavirus. PLoS Pathog. 2009;5(9):e1000578. Epub 20090911. doi: 10.1371/journal.ppat.1000578. PubMed PMID: 19750217; PMCID: PMC2734180.
5. Engels EA, Frisch M, Goedert JJ, Biggar RJ, Miller RW. Merkel cell carcinoma and HIV infection. Lancet. 2002;359(9305):497-8. doi: 10.1016/S0140-6736(02)07668-7. PubMed PMID: 11853800.
6. Rockett RJ, Bialasiewicz S, Mhango L, Gaydon J, Holding R, Whiley DM, Lambert SB, Ware RS, Nissen MD, Grimwood K, Sloots TP. Acquisition of human polyomaviruses in the first 18 months of life. Emerg Infect Dis. 2015;21(2):365-7. doi: 10.3201/eid2102.141429. PubMed PMID: 25626138; PMCID: PMC4313659.
7. Kean JM, Rao S, Wang M, Garcea RL. Seroepidemiology of human polyomaviruses. PLoS Pathog. 2009;5(3):e1000363. Epub 20090327. doi: 10.1371/journal.ppat.1000363. PubMed PMID: 19325891; PMCID: PMC2655709.
8. Carter JJ, Daugherty MD, Qi X, Bheda-Malge A, Wipf GC, Robinson K, Roman A, Malik HS, Galloway DA. Identification of an overprinting gene in Merkel cell polyomavirus provides evolutionary insight into the birth of viral genes. Proc Natl Acad Sci U S A. 2013;110(31):12744-9. Epub 20130711. doi: 10.1073/pnas.1303526110. PubMed PMID: 23847207; PMCID: PMC3732942.
9. Chang LS, Pan S, Pater MM, Di Mayorca G. Differential requirement for SV40 early genes in immortalization and transformation of primary rat and human embryonic cells. Virology. 1985;146(2):246-61. doi: 10.1016/0042-6822(85)90008-x. PubMed PMID: 2996219.
10. Guy CT, Cardiff RD, Muller WJ. Induction of mammary tumors by expression of polyomavirus middle T oncogene: a transgenic mouse model for metastatic disease. Mol Cell Biol. 1992;12(3):954-61. doi: 10.1128/mcb.12.3.954-961.1992. PubMed PMID: 1312220; PMCID: PMC369527.
11. Shuda M, Feng H, Kwun HJ, Rosen ST, Gjoerup O, Moore PS, Chang Y. T antigen mutations are a human tumor-specific signature for Merkel cell polyomavirus. Proc Natl Acad Sci U S A. 2008;105(42):16272-7. Epub 20080923. doi: 10.1073/pnas.0806526105. PubMed PMID: 18812503; PMCID: PMC2551627.
12. Hesbacher S, Pfitzer L, Wiedorfer K, Angermeyer S, Borst A, Haferkamp S, Scholz CJ, Wobser M, Schrama D, Houben R. RB1 is the crucial target of the Merkel cell polyomavirus Large T antigen in Merkel cell carcinoma cells. Oncotarget. 2016;7(22):32956-68. doi: 10.18632/oncotarget.8793. PubMed PMID: 27121059; PMCID: PMC5078066.
13. Kim YJ, Kim M, Park HK, Yu DB, Jung K, Song K, Choi YL. Co-expression of MDM2 and CDK4 in transformed human mesenchymal stem cells causes high-grade sarcoma with a dedifferentiated liposarcoma-like morphology. Lab Invest. 2019;99(9):1309-20. Epub 20190603. doi: 10.1038/s41374-019-0263-4. PubMed PMID: 31160689; PMCID: PMC6760642.
14. Salisbury NJH, Amonkar S, Landazuri Vinueza J, Carter JJ, Roman A, Galloway DA. Polyomavirus ALTOs, but not MTs, downregulate viral early gene expression by activating the NF-kappaB pathway. Proc Natl Acad Sci U S A. 2024;121(34):e2403133121. Epub 20240814. doi: 10.1073/pnas.2403133121. PubMed PMID: 39141346; PMCID: PMC11348336.
15. Wan L, Toland S, Robinson-McCarthy LR, Lee N, Schaich MA, Hengel SR, Li X, Bernstein KA, Van Houten B, Chang Y, Moore PS. Unlicensed origin DNA melting by MCV and SV40 polyomavirus

LT proteins is independent of ATP-dependent helicase activity. Proc Natl Acad Sci U S A. 2023;120(30):e2308010120. Epub 20230717. doi: 10.1073/pnas.2308010120. PubMed PMID: 37459531; PMCID: PMC10372695.
16. DeCaprio JA, Ludlow JW, Figge J, Shew JY, Huang CM, Lee WH, Marsilio E, Paucha E, Livingston DM. SV40 large tumor antigen forms a specific complex with the product of the retinoblastoma susceptibility gene. Cell. 1988;54(2):275-83. doi: 10.1016/0092-8674(88)90559-4. PubMed PMID: 2839300.
17. Dyson N, Bernards R, Friend SH, Gooding LR, Hassell JA, Major EO, Pipas JM, Vandyke T, Harlow E. Large T antigens of many polyomaviruses are able to form complexes with the retinoblastoma protein. J Virol. 1990;64(3):1353-6. doi: 10.1128/JVI.64.3.1353-1356.1990. PubMed PMID: 2154613; PMCID: PMC249255.
18. Sellers WR, Rodgers JW, Kaelin WG, Jr. A potent transrepression domain in the retinoblastoma protein induces a cell cycle arrest when bound to E2F sites. Proc Natl Acad Sci U S A. 1995;92(25):11544-8. doi: 10.1073/pnas.92.25.11544. PubMed PMID: 8524800; PMCID: PMC40438.
19. Kato J, Matsushime H, Hiebert SW, Ewen ME, Sherr CJ. Direct binding of cyclin D to the retinoblastoma gene product (pRb) and pRb phosphorylation by the cyclin D-dependent kinase CDK4. Genes Dev. 1993;7(3):331-42. doi: 10.1101/gad.7.3.331. PubMed PMID: 8449399.
20. Needham JM, Perritt SE, Thompson SR. Single-cell analysis reveals host S phase drives large T antigen expression during BK polyomavirus infection. PLoS Pathog. 2024;20(12):e1012663. Epub 20241205. doi: 10.1371/journal.ppat.1012663. PubMed PMID: 39636788; PMCID: PMC11620372.
21. Gidoni D, Kadonaga JT, Barrera-Saldana H, Takahashi K, Chambon P, Tjian R. Bidirectional SV40 transcription mediated by tandem Sp1 binding interactions. Science. 1985;230(4725):511-7. doi: 10.1126/science.2996137. PubMed PMID: 2996137.
22. Macchi M, Bornert JM, Davidson I, Kanno M, Rosales R, Vigneron M, Xiao JH, Fromental C, Chambon P. The SV40 TC-II(kappa B) enhanson binds ubiquitous and cell type specifically inducible nuclear proteins from lymphoid and non-lymphoid cell lines. EMBO J. 1989;8(13):4215-27. doi: 10.1002/j.1460-2075.1989.tb08607.x. PubMed PMID: 2556265; PMCID: PMC401618.
23. Gorrill T, Feliciano M, Mukerjee R, Sawaya BE, Khalili K, White MK. Activation of early gene transcription in polyomavirus BK by human immunodeficiency virus type 1 Tat. J Gen Virol. 2006;87(Pt 6):1557-66. doi: 10.1099/vir.0.81569-0. PubMed PMID: 16690919.
24. Ranganathan PN, Khalili K. The transcriptional enhancer element, kappa B, regulates promoter activity of the human neurotropic virus, JCV, in cells derived from the CNS. Nucleic Acids Res. 1993;21(8):1959-64. doi: 10.1093/nar/21.8.1959. PubMed PMID: 8388103; PMCID: PMC309438.
25. Prezioso C, Obregon F, Ambroselli D, Petrolo S, Checconi P, Rodio DM, Coppola L, Nardi A, Vito C, Sarmati L, Andreoni M, Palamara AT, Ciotti M, Pietropaolo V. Merkel Cell Polyomavirus (MCPyV) in the Context of Immunosuppression: Genetic Analysis of Noncoding Control Region (NCCR) Variability among a HIV-1-Positive Population. Viruses. 2020;12(5). Epub 20200504. doi: 10.3390/v12050507. PubMed PMID: 32375383; PMCID: PMC7291121.
26. Abdulsalam I, Rasheed K, Sveinbjornsson B, Ehlers B, Moens U. Promoter activity of Merkel cell Polyomavirus variants in human dermal fibroblasts and a Merkel cell carcinoma cell line. Virol J. 2020;17(1):54. Epub 20200419. doi: 10.1186/s12985-020-01317-x. PubMed PMID: 32306957; PMCID: PMC7168875.
27. Yang JF, Liu W, You J. Characterization of molecular mechanisms driving Merkel cell polyomavirus oncogene transcription and tumorigenic potential. PLoS Pathog. 2023;19(8):e1011598. Epub 20230830. doi: 10.1371/journal.ppat.1011598. PubMed PMID: 37647312; PMCID: PMC10468096.
28. Theiss JM, Gunther T, Alawi M, Neumann F, Tessmer U, Fischer N, Grundhoff A. A Comprehensive Analysis of Replicating Merkel Cell Polyomavirus Genomes Delineates the Viral Transcription Program and Suggests a Role for mcv-miR-M1 in Episomal Persistence. PLoS Pathog. 2015;11(7):e1004974. Epub 20150728. doi: 10.1371/journal.ppat.1004974. PubMed PMID: 26218535; PMCID: PMC4517807.

29. Vo Ngoc L, Cassidy CJ, Huang CY, Duttke SH, Kadonaga JT. The human initiator is a distinct and abundant element that is precisely positioned in focused core promoters. Genes Dev. 2017;31(1):6-11. Epub 20170120. doi: 10.1101/gad.293837.116. PubMed PMID: 28108474; PMCID: PMC5287114.
30. Tao Y, Kassatly RF, Cress WD, Horowitz JM. Subunit composition determines E2F DNA-binding site specificity. Mol Cell Biol. 1997;17(12):6994-7007. doi: 10.1128/MCB.17.12.6994. PubMed PMID: 9372931; PMCID: PMC232556.
31. Ma Y, Kurtyka CA, Boyapalle S, Sung SS, Lawrence H, Guida W, Cress WD. A small-molecule E2F inhibitor blocks growth in a melanoma culture model. Cancer Res. 2008;68(15):6292-9. doi: 10.1158/0008-5472.CAN-08-0121. PubMed PMID: 18676853; PMCID: PMC3615411.
32. Liu W, Yang R, Payne AS, Schowalter RM, Spurgeon ME, Lambert PF, Xu X, Buck CB, You J. Identifying the Target Cells and Mechanisms of Merkel Cell Polyomavirus Infection. Cell Host Microbe. 2016;19(6):775-87. Epub 20160519. doi: 10.1016/j.chom.2016.04.024. PubMed PMID: 27212661; PMCID: PMC4900903.
33. Neumann F, Borchert S, Schmidt C, Reimer R, Hohenberg H, Fischer N, Grundhoff A. Replication, gene expression and particle production by a consensus Merkel Cell Polyomavirus (MCPyV) genome. PLoS One. 2011;6(12):e29112. Epub 20111227. doi: 10.1371/journal.pone.0029112. PubMed PMID: 22216177; PMCID: PMC3246459.
34. Graham FL, Smiley J, Russell WC, Nairn R. Characteristics of a human cell line transformed by DNA from human adenovirus type 5. J Gen Virol. 1977;36(1):59-74. doi: 10.1099/0022-1317-36-1-59. PubMed PMID: 886304.
35. Whyte P, Williamson NM, Harlow E. Cellular targets for transformation by the adenovirus E1A proteins. Cell. 1989;56(1):67-75. doi: 10.1016/0092-8674(89)90984-7. PubMed PMID: 2521301.
36. Abere B, Zhou H, Shuda M, Stolz DB, Rapchak K, Moore PS, Chang Y. Replication Kinetics for a Reporter Merkel Cell Polyomavirus. Viruses. 2022;14(3). Epub 20220225. doi: 10.3390/v14030473. PubMed PMID: 35336880; PMCID: PMC8950423.
37. Zheng N, Fraenkel E, Pabo CO, Pavletich NP. Structural basis of DNA recognition by the heterodimeric cell cycle transcription factor E2F-DP. Genes Dev. 1999;13(6):666-74. doi: 10.1101/gad.13.6.666. PubMed PMID: 10090723; PMCID: PMC316551.
38. Kamen R, Jat P, Treisman R, Favaloro J, Folk WR. 5' termini of polyoma virus early region transcripts synthesized in vivo by wild-type virus and viable deletion mutants. J Mol Biol. 1982;159(2):189-224. doi: 10.1016/0022-2836(82)90493-4. PubMed PMID: 6292432.
39. Jat P, Novak U, Cowie A, Tyndall C, Kamen R. DNA sequences required for specific and efficient initiation of transcription at the polyoma virus early promoter. Mol Cell Biol. 1982;2(7):737-51. doi: 10.1128/mcb.2.7.737-751.1982. PubMed PMID: 6100910; PMCID: PMC369855.
40. Rapchak K, Yagobian SD, Moore J, Khattri M, Shuda M. Merkel cell polyomavirus small T antigen is a viral transcription activator that is essential for viral genome maintenance. PLoS Pathog. 2022;18(12):e1011039. Epub 20221227. doi: 10.1371/journal.ppat.1011039. PubMed PMID: 36574443; PMCID: PMC9829177.
41. Trouche D, Cook A, Kouzarides T. The CBP co-activator stimulates E2F1/DP1 activity. Nucleic Acids Res. 1996;24(21):4139-45. doi: 10.1093/nar/24.21.4139. PubMed PMID: 8932363; PMCID: PMC146244.
42. Lee CW, Sorensen TS, Shikama N, La Thangue NB. Functional interplay between p53 and E2F through co-activator p300. Oncogene. 1998;16(21):2695-710. doi: 10.1038/sj.onc.1201818. PubMed PMID: 9652736.
43. Wang K, Eady AN, Amaya I, Stanczak A, Spriggs CC. Temporal gating of nuclear import: How Merkel cell polyomavirus exploits the cell cycle for nuclear entry. PLoS Pathog. 2025;21(5):e1013217. Epub 20250530. doi: 10.1371/journal.ppat.1013217. PubMed PMID: 40445976; PMCID: PMC12157840.
44. Kervarrec T, Aljundi M, Appenzeller S, Samimi M, Maubec E, Cribier B, Deschamps L, Sarma B, Sarosi EM, Berthon P, Levy A, Bousquet G, Tallet A, Touze A, Guyetant S, Schrama D, Houben R. Polyomavirus-Positive Merkel Cell Carcinoma Derived from a Trichoblastoma Suggests an Epithelial

Origin of this Merkel Cell Carcinoma. J Invest Dermatol. 2020;140(5):976-85. Epub 20191122. doi: 10.1016/j.jid.2019.09.026. PubMed PMID: 31759946.
45. Harold A, Amako Y, Hachisuka J, Bai Y, Li MY, Kubat L, Gravemeyer J, Franks J, Gibbs JR, Park HJ, Ezhkova E, Becker JC, Shuda M. Conversion of Sox2-dependent Merkel cell carcinoma to a differentiated neuron-like phenotype by T antigen inhibition. Proc Natl Acad Sci U S A. 2019;116(40):20104-14. Epub 20190916. doi: 10.1073/pnas.1907154116. PubMed PMID: 31527246; PMCID: PMC6778204.
46. Sunshine JC, Jahchan NS, Sage J, Choi J. Are there multiple cells of origin of Merkel cell carcinoma? Oncogene. 2018;37(11):1409-16. Epub 20180111. doi: 10.1038/s41388-017-0073-3. PubMed PMID: 29321666; PMCID: PMC5854515.
47. Zur Hausen A, Rennspiess D, Winnepenninckx V, Speel EJ, Kurz AK. Early B-cell differentiation in Merkel cell carcinomas: clues to cellular ancestry. Cancer Res. 2013;73(16):4982-7. Epub 20130410. doi: 10.1158/0008-5472.CAN-13-0616. PubMed PMID: 23576560.
48. Schowalter RM, Reinhold WC, Buck CB. Entry tropism of BK and Merkel cell polyomaviruses in cell culture. PLoS One. 2012;7(7):e42181. Epub 20120731. doi: 10.1371/journal.pone.0042181. PubMed PMID: 22860078; PMCID: PMC3409148.
49. Zhao L, Imperiale MJ. A Cell Culture Model of BK Polyomavirus Persistence, Genome Recombination, and Reactivation. mBio. 2021;12(5):e0235621. Epub 20210902. doi: 10.1128/mBio.02356-21. PubMed PMID: 34473564; PMCID: PMC8546605.
50. Ciardi MR, Zingaropoli MA, Iannetta M, Prezioso C, Perri V, Pasculli P, Lichtner M, d'Ettorre G, Altieri M, Conte A, Pietropaolo V, Mastroianni CM, Vullo V. JCPyV NCCR analysis in PML patients with different risk factors: exploring common rearrangements as essential changes for neuropathogenesis. Virol J. 2020;17(1):23. Epub 20200211. doi: 10.1186/s12985-020-1295-5. PubMed PMID: 32046748; PMCID: PMC7014659.
51. Sroller V, Vilchez RA, Stewart AR, Wong C, Butel JS. Influence of the viral regulatory region on tumor induction by simian virus 40 in hamsters. J Virol. 2008;82(2):871-9. Epub 20071031. doi: 10.1128/JVI.01626-07. PubMed PMID: 17977966; PMCID: PMC2224577.
52. Lednicky JA, Arrington AS, Stewart AR, Dai XM, Wong C, Jafar S, Murphey-Corb M, Butel JS. Natural isolates of simian virus 40 from immunocompromised monkeys display extensive genetic heterogeneity: new implications for polyomavirus disease. J Virol. 1998;72(5):3980-90. doi: 10.1128/JVI.72.5.3980-3990.1998. PubMed PMID: 9557685; PMCID: PMC109625.