Protein-mediated viral latency is a novel mechanism for Merkel cell polyomavirus persistence

Hyun Jin Kwun[a], Yuan Chang[a,1,2], and Patrick S. Moore[a,1,2]

[a]Cancer Virology Program, University of Pittsburgh, Pittsburgh, PA 15213

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Viral latency, in which a virus genome does not replicate independently of the host cell genome and produces no infectious particles, is required for long-term virus persistence. There is no known latency mechanism for chronic small DNA virus infections. Merkel cell polyomavirus (MCV) causes an aggressive skin cancer after prolonged infection and requires an active large T (LT) phosphoprotein helicase to replicate. We show that evolutionarily conserved MCV LT phosphorylation sites are constitutively recognized by cellular Fbw7, βTrCP, and Skp2 Skp-F-box-cullin (SCF) E3 ubiquitin ligases, which degrade and suppress steady-state LT protein levels. Knockdown of each of these E3 ligases enhances LT stability and promotes MCV genome replication. Mutations at two of these phosphoreceptor sites [serine (S)220 and S239] in the full viral genome increase LT levels and promote MCV virion production and transmission, which can be neutralized with anti-capsid antibody. Virus activation is not mediated by viral gene transactivation, given that these mutations do not increase late gene transcription in the absence of genome replication. Mechanistic target of rapamycin inhibition by either nutrient starvation or use of an active site inhibitor reduces Skp2 levels and stabilizes LT, leading to enhanced MCV replication and transmission. MCV can sense stresses in its intracellular environment, such as nutrient loss, through SCF E3 ligase activities, and responds by initiating active viral transmission. Protein-mediated viral latency through cellular SCF E3 ligase targeting of viral replication proteins is a unique form of latency that may promote chronic viral persistence for some small DNA and RNA viruses.

Merkel cell polyomavirus | large T | latency | E3 ligase | transmission

Persistent viral infection is required for viruses to cause cancer (1). During viral latency, the virus genome replicates only in tandem with the host cell, and no infectious particles are produced that might stimulate a host defense response. During escape from latency, both retroviruses and herpesviruses rely on viral RNA transcription to generate infectious virions. For retroviruses, integration of the DNA provirus into the host genome allows silent persistence of the retroviral genome until cellular transactivators initiate RNA transcription to replicate the virus (2). For herpesviruses, viral gene transcription is largely suppressed during latency, and the viral plasmid (episome) uses host cell DNA replication machinery to maintain stable viral copy numbers over time (3). When herpesviruses switch to active replication, viral transactivators induce the expression of viral structural and polymerase mRNAs to initiate viral genome replication and capsid formation. This results in virus amplification, encapsidation, and eventual death of the host cell ("lytic replication"). No comparable latency programs for small DNA viruses or for RNA viruses have been described.

Polyomaviruses are small DNA (~5.3 kb) viruses that have an early gene locus, encoding proteins involved in viral DNA replication, and a late gene locus, encoding structural capsid and accessory proteins. The polyomavirus early large T (LT) protein is highly phosphorylated and specifically recognizes its own viral DNA replication origin to form a double-hexameric helicase complex that initiates genome replication (4). The viral origin found in the noncoding control region (NCCR) also overlaps with bidirectional promoters regulating early and late viral gene expression by LT binding. Expression of LT is known to be autoinhibited in two ways: LT inhibits its own promoter in a negative feedback loop (5, 6), and a virus-encoded miRNA transcribed during early viral gene expression inhibits LT mRNA expression (7–10).

Merkel cell polyomavirus (MCV) is a human polyomavirus that causes ~80% of human Merkel cell carcinomas (MCCs), which are among the most severe skin cancers (11, 12). It was the first human pathogen discovered through nondirected transcriptome sequencing using an approach called digital transcriptome subtraction. MCV infection is nearly ubiquitous among human adults, and MCC tumors arise as rare biological accidents after prolonged infection in which the MCV genome becomes integrated into the host cell genome and mutations to the 3′ MCV LT gene clonally occur that eliminate the carboxyl terminus T antigen helicase domain (12–14). The viral integration and T antigen mutations that occur in tumors are incompatible with transmissible MCV replication, and tumors represent a biological dead end for the virus.

Direct examination of the typical MCV replication lifecycle is technically difficult, because wild-type virus is present in healthy tissues at levels detectable only by PCR or rolling circle amplification (12, 15). Although production of a small amount of virus in persistently infected individuals has not been ruled out, the absence of a viral cytopathic effect and the limited ability to detect infectious virus in infected individuals suggest that MCV persistence largely reflects a true latent state. In support of this idea, MCV infection persists throughout the lifespan, and yet even under conditions of severe immune suppression, no disease syndrome has been described for actively replicating virus (16). In addition, transfection of plasmid viral DNA in cell culture produces scant viral particles even though the viral genome is maintained (15, 17, 18). It has not been possible to achieve serial virion transmission using a variety of cell lines, even when the MCV miRNA locus is mutated (10). Low but detectable transmission to

Significance

Viral latency is required for long-term persistence and allows viruses to evade host immune surveillance. We find that Merkel cell polyomavirus (MCV), a life-long human infection that can cause Merkel cell carcinoma, persists as a nonreplicating, silent infection owing to the continuous cellular turnover of its replication protein. Adverse cellular conditions, such as nutrient starvation, inhibit networks controlling MCV replication protein turnover and signal the virus to spread to uninfected cells or to a new host. This study describes a viral latency mechanism based on phosphorylation-regulated viral replication protein stability that may underlie chronic viral infections for some small genome DNA and RNA viruses.

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[1]Y.C. and P.S.M. contributed equally to this work.

[2]To whom correspondence may be addressed. Email: yc70@pitt.edu or psm9@pitt.edu.

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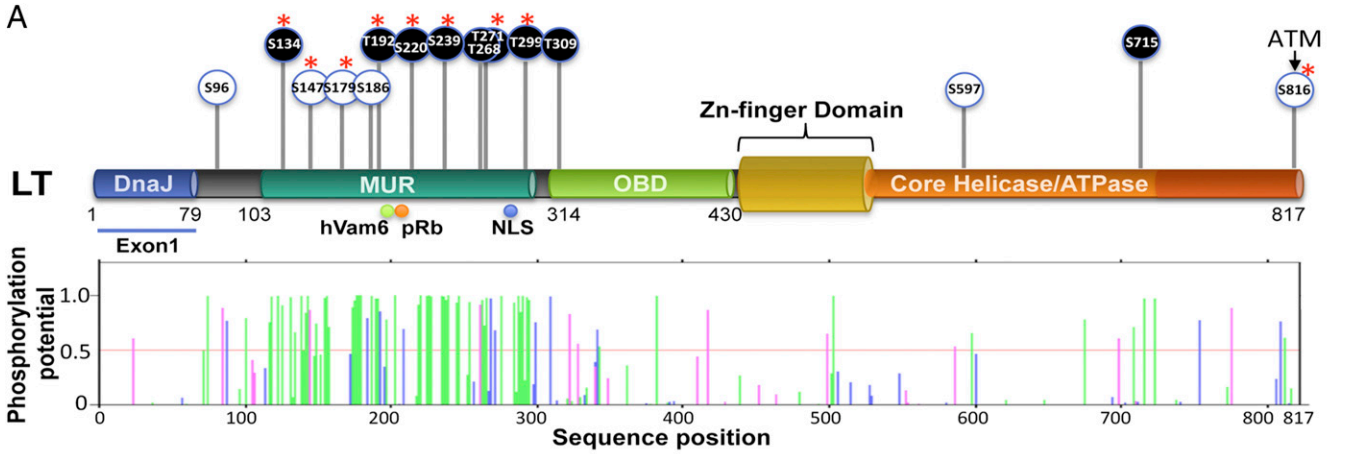
dermal fibroblasts has been reported using a protocol involving in vitro purified virions inoculated onto cells that have undergone serum starvation and later replenishment (19).

MCV gene expression and DNA replication have been investigated using origin replicon assays and by transfection of the molecular viral clone DNA into cells (17, 20). These assays have revealed that LT sequesters Vps39 (21), which also inhibits MCV replication through an unknown mechanism (15, 17, 18). In contrast, MCV replication is enhanced by expression of the MCV small T (sT) protein (17), an alternatively spliced form of the T antigen locus that inhibits the cellular Skp-Cullin-F box (SCF) RING Fbw7 (F-box and WD repeat domain-containing 7) E3 ligase and promotes stabilization of the MCV LT protein (22). SCF E3 ligases comprise a large family of multisubunit proteins that bind specific phosphorylated peptide motifs (phosphodegron sites) to initiate substrate degradation after serine/threonine kinase-mediated phosphorylation (23).

Here we describe a unique mechanism for MCV viral latency based on tonic SCF E3 ligase-mediated turnover of phosphorylated LT protein. We identified highly conserved MCV LT phosphoprotein motifs that are recognized by SCF E3 ligases, including Fbw7, Skp2 (S-phase kinase-associated protein 2), and βTrCP (β-transducin repeat-containing protein). Cellular SCF E3 ligase targeting of LT results in low steady-state levels of this protein in



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E3 ligase	Mutation Residue	Motif (SGXXS, T/SPXX)	Half life (t1/2, h)	Replication (Fold)
	WT		4.3	1.0
βTrCP (SGXXS)	S96A	**S**GGFS	4.1	1.5
	S147A	**S**GYGS	**8.2**	-
	S179A	SGSS**S**	4.1	0.74
	S186A	**S**GRES	4.7	1.1
	S597A	**S**GKTS	3.5	3.8
	S816A	SGTF**S**	5.7	1
Fbw7/Skp2 (T/SPXX)	S134A	**S**PPHS	4.3	-
	T192A	**T**PNGT	4.9	1.3
	S220A	**S**PEPP	**9.2**	**7.1**
	S239A	**S**PRQP	**7.5**	**3.1**
	T268A	**T**PKTP	4.4	0.76
	T271A	**T**PPPF	4.8	1.2
	T299A	**T**PPKP	4.2	-
	T309A	**T**PVPT	4.9	1.4
	S715A	**S**PKAN	4.2	1.1

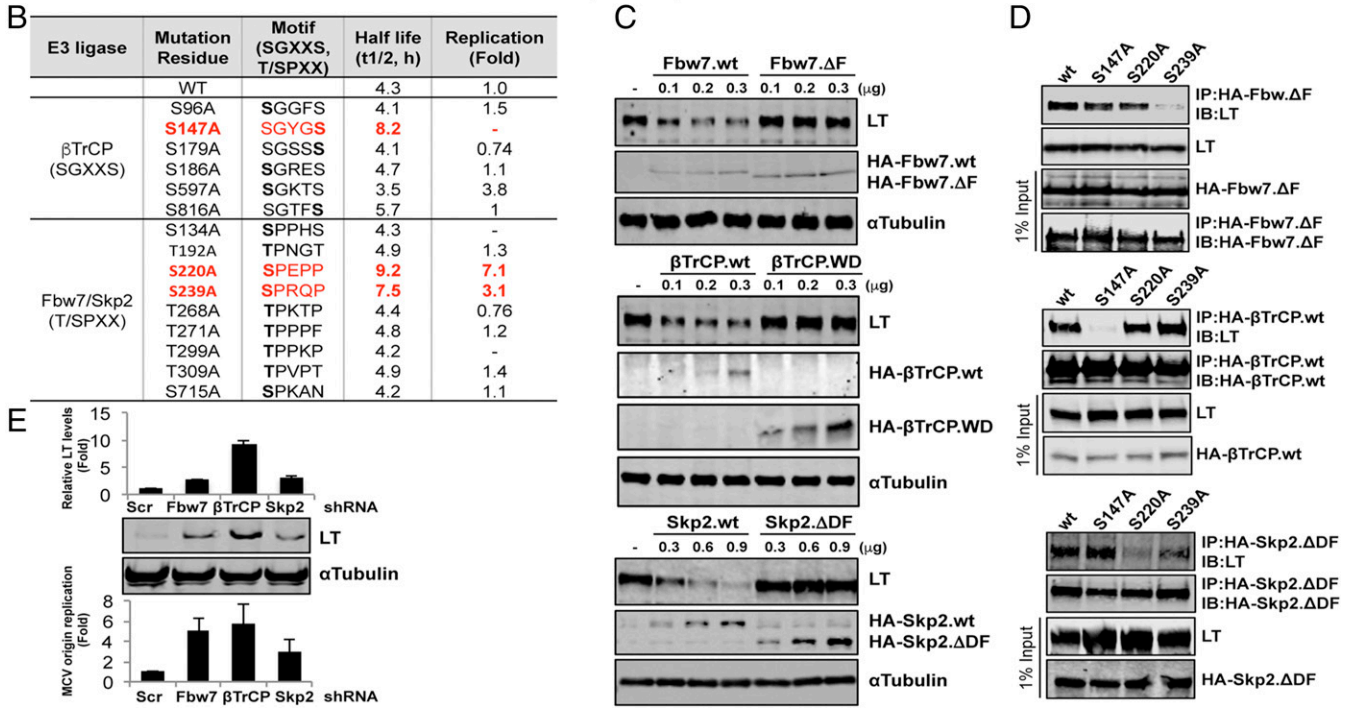

Fig. 1. LT is degraded by SCF E3 ligase recruitment. (*A*) Schematic diagram of MCV LT antigen (817 aa) with predicted Fbw7, Skp2, and βTrCP recognition phosphorylation sites. NetPhos 2.0 predicts 82 potential LT phosphorylation sites (59 Ser, 12 Thr, and 11 Tyr residues) exceeding a 0.5 threshold, including 15 minimal potential Fbw7/Skp2 (black circles) and βTrCP (white circles) recognition motifs. Nine of these are known phosphorylation sites (*) (27–29). (*B*) LT alanine (A) substitution mutants at each of 15 potential Fbw7/Skp2 and βTrCP binding residues were generated and tested for stability by cycloheximide immunoblotting and replication efficiency using an MCV replicon assay. Testing was done in triplicate, and average values are shown. (*C*) Dose-dependent degradation of LT with increasing expression of Fbw7, βTrCP, and Skp2 SCF E3 ligases. LT was expressed in 293 cells with increasing plasmid amounts for each wild-type E3 ligase or its corresponding ubiquitylation-defective mutant (*SI Appendix*, Fig. S2). LT and tubulin were determined on the same blots; SCF proteins were determined on separate blots. (*D*) MCV LT interacts with each E3 ligase at specific phosphodegron sites. LT binding to each E3 ligase was assessed by immunoprecipitation. LT binding to βTrCP was lost with the S147A mutant, binding to Fbw7 was lost with the S239A mutant, and binding to Skp2 was lost with the S220A mutant. (*E*) SCF E3 ligase knockdown increases LT protein steady-state levels and replication of LT-dependent MCV origin replicon (mean ± SEM, $n = 3$).

MCV-infected cells that are insufficient to initiate active viral replication. Mutation of these LT phosphodegron sites or knockdown of the corresponding E3 ligases enhances LT stability and promotes MCV origin replication. When mutations to two phosphodegron sites are engineered into an infectious MCV molecular clone, virus replication is activated, and the virus can be transmitted to uninfected cells. We show that inhibition of mechanistic target of rapamycin (mTOR) kinase by nutrient starvation or ATP-site inhibitor treatment leads to Skp2 down-regulation (24, 25), increased accumulation of LT protein, aand increased virus replication. Unlike large DNA viruses, MCV latency is based largely on protein turnover of its major replication protein. These findings may have clinical relevance, because mTOR inhibitors are used in organ transplantation and could be expected to enhance LT oncoprotein expression in transplant recipients with MCC.

Results

SCF E3 Ligases Regulate MCV LT Steady-State Levels. MCV LT is an 817-aa protein with ~82 potential phosphorylation sites (NetPhos 2.0), including multiple consensus F-box binding motifs that might serve as phosphodegron recognition sequences (Fig. 1*A*). To understand the role of ubiquitin ligase activity in MCV replication, we identified six consensus βTrCP (pSGXXpS) and nine consensus Skp2 and Fbw7 (pS/pTPXX) recognition motifs (26). Nine of these 15 residues have been identified as phosphorylation sites by mass spectrometry analysis (27–29).

Each of the 15 potentially phosphorylated residues was individually mutated to alanine (A), and LT protein half-life and LT-dependent replicon replication was determined (20). LT turnover was markedly reduced by alanine mutations at three residues (S147, S220, and S239), and LT-dependent replication was increased over wild-type by mutations at S220 and S239 (Fig. 1*B* and *SI Appendix*, Fig. S1). Alanine substitution at S147 abolished LT-dependent replication despite increasing LT stability, suggesting that phosphorylation at this site is essential to replication enzyme activity. These three residues are conserved in all GenBank-deposited full-length MCV LT sequences.

Overexpression of each SCF E3 ligase (Fbw7, βTrCP, or Skp2, but not their active site-deficient forms, Fbw7.ΔF, βTrCP.WD, or Skp2.ΔDF) (*SI Appendix*, Fig. S2*A*) decreased LT steady-state

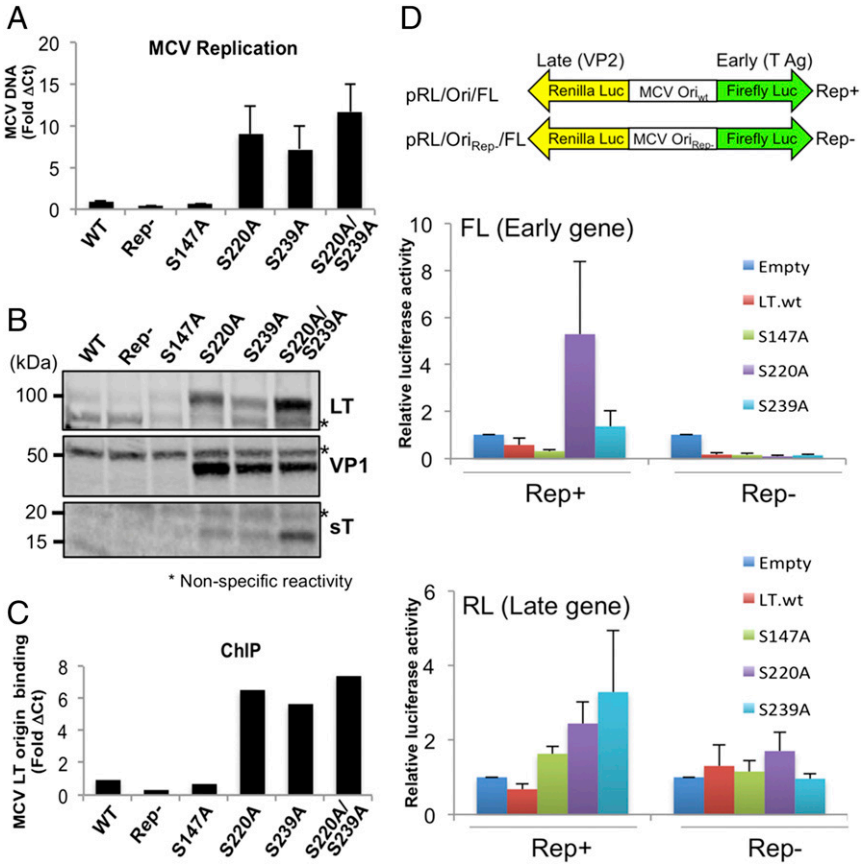

Fig. 2. SCF E3 ligase activity regulates MCV replication in 293 cells. (*A*) MCV genomes with SCF E3 ligase recognition site alanine substitution mutations at Fbw7 (S239)- or Skp2 (S220)- binding sites, or a double mutation (S220A and S239A), have increased replication (qPCR; mean ± SEM, $n = 3$) compared with wild-type (WT) or replication-defective (Rep−) genomes when transfected into 293 cells. Alanine substitution at the βTrCP-binding site (S147A) ablated virus replication. (*B* and *C*) MCV protein expression (*B*) and LT origin binding (by ChIP qPCR) (*C*) after genome transfection into 293 cells. VP1 and sT protein expression was detected only for viruses with Fbw7- or Skp2-binding site mutations. sT expression was prominent for the dual mutation virus MCV-HF$_{LTS220A/S239A}$. (*D*) MCV early and late gene transcription is replication-dependent. The MCV promoter region (nt 4928–195; GenBank accession no. EU375804) (20) was cloned into a bidirectional dual firefly (early) and *Renilla* (late) luciferase reporter, and promoter activity was measured by luciferase activity during cotransfection (0.2 μg) with wild-type or E3 ligase-binding mutant LT DNA plasmids (0.3 μg) into 293 cells. Relative luciferase activity was normalized to empty vector control (mean ± SEM, $n = 3$). Early gene reporter transcription was weakly activated by LT$_{S220A}$ (by fivefold) or LT$_{S239A}$ (by 1.4-fold) compared with wild-type LT (LT.wt) when the replication-competent reporter (Rep+) was used. LT$_{S147A}$ repressed early transcription. When the replication-incompetent reporter (Rep−) was used, all cotransfected LT proteins suppressed early gene transcription to 10–20% of empty vector control. Rep+ late gene expression was not significantly increased by wild-type LT protein coexpression but was increased by twofold to threefold by LT$_{S220A}$ and LT$_{S239A}$. This increase was abolished for the replication-incompetent reporter, consistent with DNA template amplification being responsible for increased late gene expression.

levels in a dose-dependent fashion (Fig. 1*C*) and increased LT ubiquitylation (*SI Appendix*, Fig. S2*B*) when coexpressed with exogenously-derived LT. Coimmunoprecipitation identified the individual LT phosphorylation-recognition residues as S147 for βTrCP, S239 for Fbw7, and S220 for Skp2 (Fig. 1*D*), and pretreatment with λ phosphatase prevented the binding of these SCF E3 ligases to LT (*SI Appendix*, Fig. S2*C*). Bona fide phosphorylation of LT at these residues was also confirmed by generating a synthetic gene, *npLT330*, encoding the first 330 amino acids of LT but with all serine, threonine, and tyrosines substituted with alanine. Sole-site phosphorylation with revertant mutants of *npLT330* at S147, S220, and S239 was confirmed by immunoprecipitation of and immunoblotting with anti-phosphoserine antibody (*SI Appendix*, Fig. S2*D*). In contrast, knockdown of each SCF E3 ligase (*SI Appendix*, Fig. S3) significantly increased steady-state LT protein expression levels by twofold to ninefold in 293 cells and increased MCV origin replication by threefold to sixfold (Fig. 1*E*). Notably, βTrCP knockdown increased LT expression and origin replication even though its phosphoserine-binding site (S147) is required for replication (Fig. 1*B*).

Effect of SCF E3-Binding Site Mutations on MCV Replication and Transcription. To determine the role of these SCF E3 recognition sites in MCV replication, we examined a whole virus genome clone (MCV-HF) (17). Individual alanine substitution mutations at S147, S220, S239, and a double mutation (S220A/S239A) were engineered into the MCV-HF genome and transfected into 293 cells. As shown in Fig. 2*A*, wild-type MCV-HF had a low level of viral replication after 4 days, which was further reduced for both the MCV-$HF_{rep–}$, a virus with a point mutation in the viral replication origin that ablates replication (20), and the βTrCP-binding site mutant virus MCV-$HF_{LTS147A}$. The amount of viral DNA for the two replication-deficient mutants likely represents residual unreplicated DNA remaining from transfection. In contrast, introduction of mutations at either the Fbw7 (S239)- or the Skp2 (S220)-binding site activated MCV replication by 9- to 12-fold over the wild-type virus. Immunoblotting findings were consistent with these results, showing only low-level expression of LT for the wild-type MCV-HF, MCV-HF_{Rep-} and MCV-$HF_{LTS147A}$ viruses (Fig. 2*B*). In contrast, LT, VP1, and (for the MCV-$HF_{LTS220A/S239A}$ virus) sT proteins were readily detected for the viruses mutated at Fbw7 and/or Skp2 recognition sites. Similarly, LT chromatin immunoprecipitation (ChIP) of the MCV origin was increased by fivefold to sevenfold (Fig. 2*C*) by alanine substitutions at the Fbw7/Skp2 recognition sites in LT, consistent with these mutations stabilizing LT and increasing its assembly on the origin.

To measure the SCF E3 ligase effects on viral transcription, we generated a reporter containing the MCV NCCR in which the *Renilla* luciferase gene was substituted for VP2 at the late gene start site and the firefly luciferase gene was cloned in the opposite, reverse-sense direction at the MCV T antigen early gene start site (Fig. 2*D*). Because the MCV NCCR promoters (similar to those of other polyomaviruses) overlap with the replication origin, a

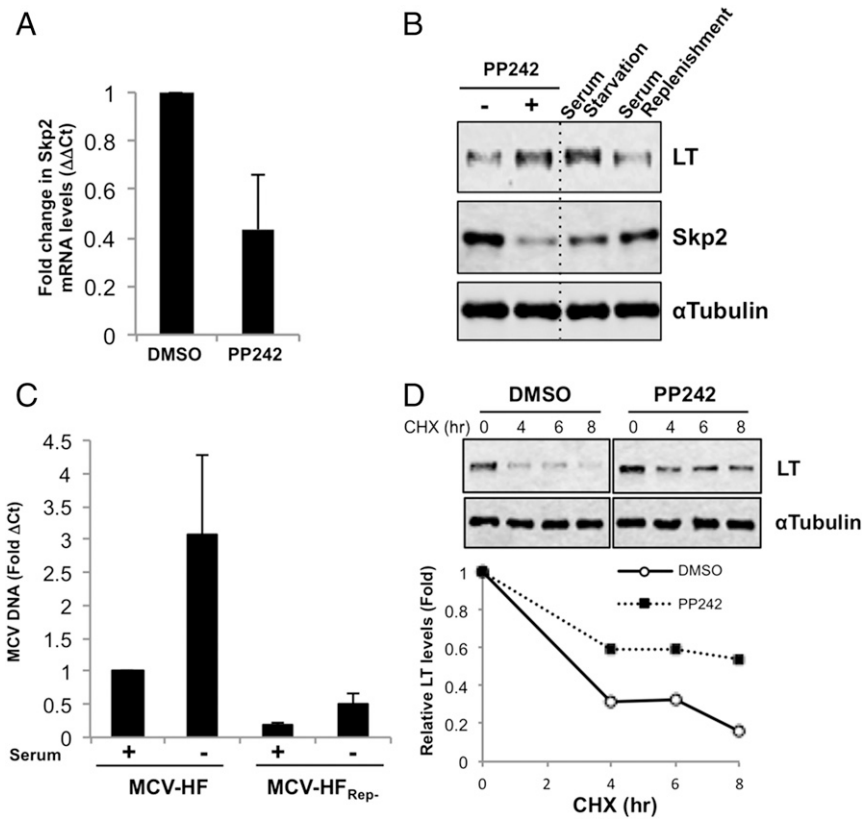

Fig. 3. mTOR inhibition increases MCV virus replication through Skp2 down-regulation. (*A*) 293 cells treated with the mTOR active site inhibitor PP242 (10 μM for 24 h) have down-regulated Skp2 mRNA levels compared with DMSO-treated control cells. Skp2 mRNA levels were standardized to GAPDH mRNA (RT qPCR, mean ± SEM, $n = 3$). (*B*) Levels of Skp2 protein expression decreased, whereas MCV LT protein expression increased, during PP242 treatment as in *A*. mTOR inhibition by serum starvation (24 h, 0.1% FCS) also increased LT and decreased Skp2 protein levels. This was reversed by serum replenishment (24 h, 10% FCS). (*C*) Serum starvation (24 h, 0.1% FCS) activates replication of MCV molecular clone (*Methods*). Viral DNA was determined by qPCR for MCV-HF and MCV-$HF_{Rep–}$ and normalized to MCV-HF in 293 cells cultured with serum (mean ± SEM, $n = 4$). (*D*) mTOR inhibition by 10 μM PP242 stabilizes LT protein. LT stability was determined by quantitative cycloheximide immunoblotting. The graph plots LT expression relative to noncycloheximide-treated samples at time 0. $n = 3$, with a representative blot shown.

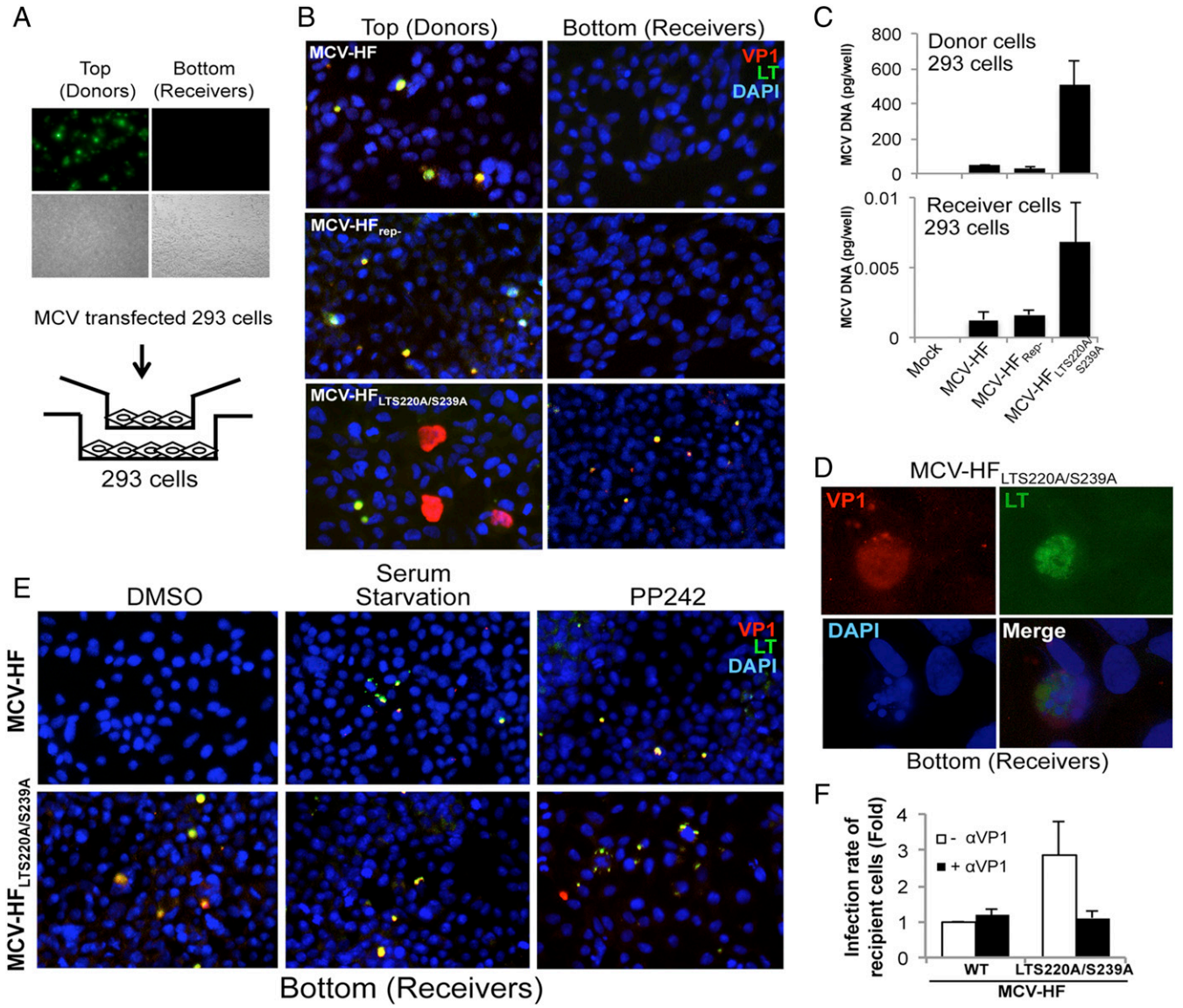

Fig. 4. Inhibition of SCF E3 ligase targeting of LT causes a switch from latency to lytic MCV replication and permits virus transmission. (*A*) For transwell transmission assays, the integrity of transwell 0.4-μ membranes was monitored by placing eGFP-expressing 293 cells in the upper donor wells and untransfected 293 cells in the bottom receiver wells. (*Upper*) No fluorescence was detected among bottom recipient cells. (*Lower*) Phase-contrast microscopy. (*B* and *C*) Mutations to LT SCF E3 ligase recognition sites permit MCV encapsidation and transmission. Wild-type (MCV-HF), replication-deficient (MCV-$HF_{Rep–}$), or MCV-$HF_{LTS220A/S239A}$ viruses were transfected into donor cells, and transmission to receiver cells was detected by LT (green) and capsid VP1 (red) immunofluorescence (n = 3, with representative result shown) (*B*) and by qPCR analysis (mean ± SEM, n = 3) (*C*). Virus DNA was determined per well. (*D*) MCV-$HF_{LTS220A/S239A}$ replication in recipient cells induces apoptotic cell death. $VP1^+$ virions (red) and LT (green) protein were detected in recipient cells. MCV-$HF_{LTS220A/S239A}$ induced apoptotic nuclear fragmentation shown by DAPI (blue) staining at 24 h postinfection. (*E*) Transmissibility of MCV to cells in bottom of transwells was significantly increased by mTOR inhibition (serum starvation and PP242 treatment) compared with DMSO vehicle control. (*F*) MCV neutralization assay. Donor cells (*Upper*) and receiver cells (*Lower*) were seeded into the upper transwell insert and lower six-well plate, respectively. Receiver target cells were incubated for 24 h with VP1 antibody (CM9B2; 5 μg/mL), followed by collection for qPCR analysis. The relative infection rate was determined as the fold change qPCR inverse ΔΔCt values for MCV-HF and MCV-$HF_{LTS220A/239A}$ in recipient cells (mean ± SEM, n = 4).

nonreplicating mutant origin was used to prevent amplified expression owing to reporter plasmid DNA replication. We used a tumor-derived nonreplicating origin (MCV350) with a single point mutation in one pentanucleotide sequence (20). The bidirectional reporter was then cotransfected together with LT-expressing plasmids, and simultaneous expression of early and late gene was determined.

Increased early gene transcription for LT_{S220A} compared with wild-type LT expression occurred only with the replication-competent reporter (Fig. 2*D*). In contrast, LT_{S147A}, which cannot activate replication, was a strong repressor in this assay. Examination of the replication-incompetent reporter identified the wild-type and SCF E3 ligase-binding mutant LTs as strong and equal repressors of MCV early gene transcription to ∼20% of the empty vector control. SCF E3 ligase-binding mutations increased late gene expression for the replication-competent reporter, but there was no significant increased late gene expression for any of the mutant LT proteins using the replication-deficient reporter. These data indicate that wild-type LT and SCF E3 ligase-binding mutants are early gene transcriptional repressors and do not promote late gene transcription except through genome amplification from DNA replication. These results were confirmed by qRT-PCR for

wild-type LT mRNA in the context of whole viral transcription (*SI Appendix*, Fig. S4).

Viral Intracellular Sensing Based on Cellular SCF E3 Ligase Activity. Skp2 is regulated by mTOR kinase (24, 25). mTOR inhibition, by treatment with the mTOR active site inhibitor PP242 (30) or by serum starvation (31, 32), reduces Skp2 expression and increases LT protein expression (Fig. 3 *A* and *B*). With serum replenishment, Skp2 levels rebound and LT levels decline (Fig. 3*B*). Whole-virus MCV replication was similarly activated not only by SCF E3 ligase phosphorylation site mutations in LT (Fig. 2*B*), but also by serum starvation (Fig. 3*C*). This is consistent with the virus initiating replication under conditions (e.g., serum starvation) that will activate an antiviral, proautophagic state in the cell (33). This effect was related to protein stability, as seen in Fig. 3*D*, in which 293 cells expressing LT were treated with cycloheximide in the presence or absence of the specific mTOR inhibitor PP242. Furthermore, knockdown of both mTORC1 (raptor) and mTORC2 (rictor) increased steady-state LT protein levels (*SI Appendix*, Fig. S5*A*), consistent with mTOR inhibition leading to loss of Skp2 and increased stability of MCV LT.

We surveyed MCV LT steady-state level responses to eight different kinase inhibitors. Treatment with the five PI-3K/Akt/mTOR pathway small-molecule inhibitors that down-regulate mTOR activity (LY294002, MK2206, Torin1, PP242, and rapamycin) significantly increased steady-state LT protein levels, whereas treatment with other kinase inhibitors (MEK1/2, CDK1/2, and GSK) did not (*SI Appendix*, Fig. S5*B*).

Effect of SCF E3 Ligase Activity on MCV Cell-to-Cell Transmission. Because serial transmission of MCV has not been achieved to date, we assessed the role of SCF E3 ligase targeting of LT in virus transmission using a transwell assay. MCV-HF–transfected 293 cells (donors; top wells) were cocultured with 293 cells (receivers; bottom wells) separated by 0.4-μm membranes. No eGFP fluorescence was detected in the bottom wells after coincubation with eGFP-expressing 293 cells in the top wells, confirming the integrity of the membranes separating donor and recipient cells (Fig. 4*A*). Neither wild-type MCV-HF nor MCV-HF_{rep-} transfections into donor cells resulted in significant levels of viral protein detection by immunofluorescence in the bottom receiver cells, whereas both LT and VP1 proteins were readily detected in bottom well cells exposed to MCV-$HF_{LTS220A/S239A}$ virus (Fig. 4*B*, *Right*). Of note, only the SCF-recognition mutant virus generated abundant MCV VP1 protein, the self-assembling capsid protein required for virion transmission. In addition, MCV-$HF_{LTS220A/S239A}$-transfected donor cells generated a sevenfold increase in MCV DNA (7 fg) in receiver well cells (Fig. 4*C*) compared with that detected with both MCV-HF and MCV-HF_{rep-} viruses (1 fg). Enhanced transmission also was seen for the MCV-$HF_{LTS220A/S239A}$ virus compared with wild-type MCV-HF when human foreskin dermal fibroblasts were used as the recipient cell line (*SI Appendix*, Fig. S6), showing that these results are not 293 cell-dependent.

The receiver cells infected by MCV-$HF_{LTS220A/S239A}$ that became abundantly positive for capsid protein had DNA fragmentation and annexin A1 positivity typical for an apoptotic cytopathic effect occurring during lytic viral replication (Fig. 4*D* and *SI Appendix*, Fig. S7). The transmissibility of wild-type MCV was significantly increased when MCV-HF–transfected 293 cells were treated with PP242 or were serum-starved (Fig. 4*E*), consistent with Skp2 suppression resulting in replication permissivity. MCV transmission was neutralized by an anti-VP1 monoclonal antibody (Fig. 4*F*), demonstrating that MCV transmission requires encapsidation, as would be expected for a productive polyomavirus infection.

Discussion

This study reveals that MCV relies on SCF E3 ligases to maintain latent persistence in cells as a nonreplicating viral plasmid. This is a different mechanism from herpesvirus and retrovirus latency, which rely on viral transactivator proteins to initiate lytic replication. The major MCV replication protein, LT, is continually transcribed and translated, but is also rapidly degraded by constitutive SCF E3 ligase activities in most cell lines so that LT levels do not surpass the threshold concentration required to assemble the multimeric LT helicase complex on the viral origin (Fig. 5). Furthermore, LT protein autoinhibits its own transcription, establishing a negative feedback loop that further buffers against LT accumulation to levels sufficient to activate viral genome replication. Nonetheless, point mutations that prevent Fbw7 or Skp2 recognition of LT allow the accumulation of LT protein,

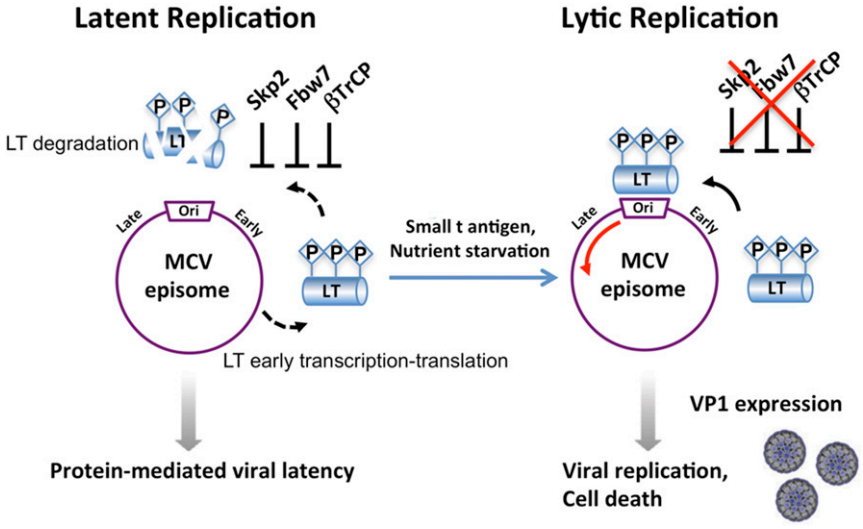

Fig. 5. Model for MCV protein-mediated viral latency. MCV LT retains highly conserved phosphorylation sites recognized by cellular Fbw7, βTrCP, and Skp2 E3 ubiquitin ligases that cause degradation of LT, establishing viral latency. Cellular stresses, such as nutrient starvation, can reduce SCF E3 ligase activity, allowing LT accumulation to levels that permit assembly of the replication complex on the viral origin, which initiates virus DNA synthesis, capsid protein expression, and lytic replication. MCV sT protein, a replication accessory factor, enhances LT-dependent replication (17) by targeting SCF E3 ligases such as Fbw7 (22).

which can assemble on the origin to allow full genome MCV replication. We found no evidence indicating that LT protein specifically transactivates the transcription of late virion protein genes (VP1, VP2), but instead observed increased expression of these genes as the viral DNA genome was amplified by DNA replication.

The three phosphodegron residues that we identified are conserved in all MCV sequences examined to date. It may seem counterintuitive for MCV to reduce its own replication efficiency unless one considers latency to be a powerful mechanism in the life cycle of viruses to maintain a more fit state for long-term persistence and eventual transmission. Unlike the Skp2 (S220)- and Fbw7 (S239)-binding sites, mutation to the βTrCP-binding motif at S147 eliminates virus replication. Evidence that βTrCP serves a similar function as Fbw7 and Skp2 comes from βTrCP knockdown experiments using the wild-type replicon in which replication is amplified. Thus, activated phosphorylation at S147 is required for LT-initiated replication, and also serves as a marker for βTrCP to initiate LT degradation. More extensive mapping of these sites may refine the phosphodegron motifs; we surveyed only SCF E3 ligases targeting 3 of the ∼82 phosphorylation sites on LT. Furthermore, which kinases target these sites is unknown; it is likely that other phosphorylation-SCF E3 ligase pairs that we have not examined similarly regulate MCV replication.

This mechanism for viral latency might not be restricted to MCV. Hepatitis C virus (HCV), a positively stranded RNA virus that is also notoriously difficult to grow in the laboratory, has a conserved Fbw7 recognition motif in its NS5B RNA polymerase, and knockdown of Fbw7 amplifies replication of the JFH1 HCV strain in tissue culture (34). Further study is needed to determine whether replication protein-mediated viral latency is a common feature of chronic RNA and DNA virus infections, or whether HCV and MCV are unique in hijacking cellular kinase-E3 ligase networks to regulate their own replication. Continuous viral protein degradation could be expected to enhance immune peptide presentation. It is unknown if MCV LT has features that inhibit this process, as occurs with latent proteins from herpesviruses (35).

Our results have direct clinical importance, given that rapamycin and related rapalogs are commonly used as immunosuppressants and are widely assumed to have both antiviral and anticancer activities (36). For transplant recipients with MCV-positive MCC, however, the use of mTOR inhibitors will increase LT oncoprotein levels in tumors through Skp2 inhibition even though the virus is defective and cannot replicate. In support of this idea, Clarke et al. (37) reported a twofold greater risk of MCC in transplant recipients receiving mTOR inhibitor therapy compared with those on other immunosuppressant therapies, suggesting that mTOR inhibition may promote MCC carcinogenesis beyond its immunosuppressant effects alone. If other viruses are regulated through Skp2-mediated latency, then mTOR inhibition can be expected to increase the activated replication for these infections.

The kinetics of polyomavirus replication and transmission (particularly for SV40) have been thoroughly examined, but the mechanisms used by these viruses to persist in their host cells are less well understood (4, 38–40). It is possible that replication protein-mediated viral latency allows a quicker shift to lytic virus replication in response to a changing cellular environment compared with a mechanism relying on replication protein transcription and translation. One unexpected finding of our study is that MCV uses SCF E3 ligase signaling as a sensor of the intracellular environment. mTOR inhibition generates an antiviral autophagic response (41), and thus the initiation of replication when Skp2 levels decline may serve as a signal for the viral episome to initiate virion replication to escape an unfavorable cellular environment. Other persistent small genome viruses could be regulated by different sets of E3 ligases than those regulating MCV, given that each virus needs to be highly attuned to its own cellular niche and to maintain the capability for reactivated lytic replication.

This study also clarifies a role for MCV sT as an MCV replication protein (22). MCV sT is generated from alternative splicing of the T antigen encoding gene to form an iron-sulfur protein with an unclear function for the virus (20, 42). Similar to other polyomavirus sT proteins, MCV sT binds and inactivates some PP2A isoforms (43); however, it is transforming in rodent cells (44) and induces cell proliferation and tumorigenicity in transgenic mice (45–47). Its transforming activity has been mapped to a domain called the LT stabilization domain (LSD) that is separate from its PP2A-binding motif, and the LSD promiscuously binds SCF and anaphase-promoting complex E3 ligases (22, 48). MCV sT protein inhibits MCV LT turnover, at least in part, by sequestering Fbw7 (22), which led us to search for Fbw7 phosphodegron motifs encoded by LT protein. In tumors, in which MCV replication is abrogated by integration and mutation, MCV sT serves as an oncoprotein required for cell proliferation (49); however, in the natural setting of the virus, it is likely to be a replication accessory protein that promotes LT stabilization (22) and genome replication (17). The factors controlling alternative splicing to generate LT or sT mRNAs remain unknown, but this reifies the importance of LT stability in controlling MCV replication.

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1. Moore PS, Chang Y (2010) Why do viruses cause cancer? Highlights of the first century of human tumour virology. *Nat Rev Cancer* 10:878–889.
2. Blankson JN, Persaud D, Siliciano RF (2002) The challenge of viral reservoirs in HIV-1 infection. *Annu Rev Med* 53:557–593.
3. Moore PS, Chang Y (2003) Kaposi's sarcoma-associated herpesvirus immunoevasion and tumorigenesis: Two sides of the same coin? *Annu Rev Microbiol* 57:609–639.
4. Fanning E, Zhao K (2009) SV40 DNA replication: From the A gene to a nanomachine. *Virology* 384:352–359.
5. Cogen B (1978) Virus-specific early RNA in 3T6 cells infected by a tsA mutant of polyoma virus. *Virology* 85:222–230.
6. Fenton RG, Basilico C (1982) Changes in the topography of early region transcription during polyoma virus lytic infection. *Proc Natl Acad Sci USA* 79: 7142–7146.
7. Broekema NM, Imperiale MJ (2013) miRNA regulation of BK polyomavirus replication during early infection. *Proc Natl Acad Sci USA* 110:8200–8205.
8. Seo GJ, Chen CJ, Sullivan CS (2009) Merkel cell polyomavirus encodes a microRNA with the ability to autoregulate viral gene expression. *Virology* 383:183–187.
9. Sullivan CS, Grundhoff AT, Tevethia S, Pipas JM, Ganem D (2005) SV40-encoded microRNAs regulate viral gene expression and reduce susceptibility to cytotoxic T cells. *Nature* 435:682–686.
10. Theiss JM, et al. (2015) 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* 11:e1004974.
11. Chang Y, Moore PS (2012) Merkel cell carcinoma: A virus-induced human cancer. *Annu Rev Pathol* 7:123–144.
12. Feng H, Shuda M, Chang Y, Moore PS (2008) Clonal integration of a polyomavirus in human Merkel cell carcinoma. *Science* 319:1096–1100.
13. Sastre-Garau X, et al. (2009) Merkel cell carcinoma of the skin: Pathological and molecular evidence for a causative role of MCV in oncogenesis. *J Pathol* 218:48–56.
14. Shuda M, et al. (2008) T antigen mutations are a human tumor-specific signature for Merkel cell polyomavirus. *Proc Natl Acad Sci USA* 105:16272–16277.
15. Schowalter RM, Pastrana DV, Pumphrey KA, Moyer AL, Buck CB (2010) Merkel cell polyomavirus and two previously unknown polyomaviruses are chronically shed from human skin. *Cell Host Microbe* 7:509–515.
16. Tolstov YL, et al. (2011) Asymptomatic primary Merkel cell polyomavirus infection among adults. *Emerg Infect Dis* 17:1371–1380.
17. Feng H, et al. (2011) Cellular and viral factors regulating Merkel cell polyomavirus replication. *PLoS One* 6:e22468.
18. Neumann F, et al. (2011) Replication, gene expression and particle production by a consensus Merkel Cell Polyomavirus (MCPyV) genome. *PLoS One* 6:e29112.

19. Liu W, et al. (2016) Identifying the target cells and mechanisms of Merkel cell polyomavirus infection. *Cell Host Microbe* 19:775–787.
20. Kwun HJ, et al. (2009) The minimum replication origin of Merkel cell polyomavirus has a unique large T-antigen loading architecture and requires small T-antigen expression for optimal replication. *J Virol* 83:12118–12128.
21. Liu X, et al. (2011) Merkel cell polyomavirus large T antigen disrupts lysosome clustering by translocating human Vam6p from the cytoplasm to the nucleus. *J Biol Chem* 286:17079–17090.
22. Kwun HJ, et al. (2013) Merkel cell polyomavirus small T antigen controls viral replication and oncoprotein expression by targeting the cellular ubiquitin ligase SCFFbw7. *Cell Host Microbe* 14:125–135.
23. Cardozo T, Pagano M (2004) The SCF ubiquitin ligase: Insights into a molecular machine. *Nat Rev Mol Cell Biol* 5:739–751.
24. Shapira M, Kakiashvili E, Rosenberg T, Hershko DD (2006) The mTOR inhibitor rapamycin down-regulates the expression of the ubiquitin ligase subunit Skp2 in breast cancer cells. *Breast Cancer Res* 8:R46.
25. Totary-Jain H, et al. (2012) Rapamycin resistance is linked to defective regulation of Skp2. *Cancer Res* 72:1836–1843.
26. Welcker M, Clurman BE (2008) FBW7 ubiquitin ligase: A tumour suppressor at the crossroads of cell division, growth and differentiation. *Nat Rev Cancer* 8:83–93.
27. Diaz J, Wang X, Tsang SH, Jiao J, You J (2014) Phosphorylation of large T antigen regulates Merkel cell polyomavirus replication. *Cancers (Basel)* 6:1464–1486.
28. Li J, Diaz J, Wang X, Tsang SH, You J (2015) Phosphorylation of Merkel cell polyomavirus large tumor antigen at serine 816 by ATM kinase induces apoptosis in host cells. *J Biol Chem* 290:1874–1884.
29. Schrama D, et al. (2016) Serine 220 phosphorylation of the Merkel cell polyomavirus large T antigen crucially supports growth of Merkel cell carcinoma cells. *Int J Cancer* 138:1153–1162.
30. Feldman ME, et al. (2009) Active-site inhibitors of mTOR target rapamycin-resistant outputs of mTORC1 and mTORC2. *PLoS Biol* 7:e38.
31. Kim DH, et al. (2002) mTOR interacts with raptor to form a nutrient-sensitive complex that signals to the cell growth machinery. *Cell* 110:163–175.
32. Zoncu R, Efeyan A, Sabatini DM (2011) mTOR: From growth signal integration to cancer, diabetes and ageing. *Nat Rev Mol Cell Biol* 12:21–35.
33. Rey-Jurado E, Riedel CA, González PA, Bueno SM, Kalergis AM (2015) Contribution of autophagy to antiviral immunity. *FEBS Lett* 589:3461–3470.
34. Chen J, et al. (2016) Ubiquitin ligase Fbw7 restricts the replication of hepatitis C virus by targeting NS5B for ubiquitination and degradation. *Biochem Biophys Res Commun* 470:697–703.
35. Kwun HJ, et al. (2011) The central repeat domain 1 of Kaposi's sarcoma-associated herpesvirus (KSHV) latency associated-nuclear antigen 1 (LANA1) prevents cis MHC class I peptide presentation. *Virology* 412:357–365.
36. Blagosklonny MV (2013) Immunosuppressants in cancer prevention and therapy. *OncoImmunology* 2:e26961.
37. Clarke CA, et al. (2015) Risk of merkel cell carcinoma after solid organ transplantation. *J Natl Cancer Inst* 107:dju382.
38. Sowd GA, Fanning E (2012) A wolf in sheep's clothing: SV40 co-opts host genome maintenance proteins to replicate viral DNA. *PLoS Pathog* 8:e1002994.
39. McKay R, DiMaio D (1981) Binding of an SV40 T antigen-related protein to the DNA of SV40 regulatory mutants. *Nature* 289:810–813.
40. Luo Y, et al. (2016) Interaction between simian virus 40 major capsid protein VP1 and cell surface ganglioside GM1 triggers vacuole formation. *MBio* 7:e00297–e00216.
41. Schmeisser H, et al. (2013) Type I interferons induce autophagy in certain human cancer cell lines. *Autophagy* 9:683–696.
42. Tsang SH, et al. (2015) The oncogenic small tumor antigen of Merkel cell polyomavirus is an iron-sulfur cluster protein that enhances viral DNA replication. *J Virol* 90:1544–1556.
43. Kwun HJ, et al. (2015) Restricted protein phosphatase 2A targeting by Merkel cell polyomavirus small T antigen. *J Virol* 89:4191–4200.
44. Shuda M, Kwun HJ, Feng H, Chang Y, Moore PS (2011) Human Merkel cell polyomavirus small T antigen is an oncoprotein targeting the 4E-BP1 translation regulator. *J Clin Invest* 121:3623–3634.
45. Shuda M, et al. (2015) Merkel cell polyomavirus small T antigen induces cancer and embryonic Merkel cell proliferation in a transgenic mouse model. *PLoS One* 10: e0142329.
46. Spurgeon ME, Cheng J, Bronson RT, Lambert PF, DeCaprio JA (2015) Tumorigenic activity of Merkel cell polyomavirus T antigens expressed in the stratified epithelium of mice. *Cancer Res* 75:1068–1079.
47. Verhaegen ME, et al. (2015) Merkel cell polyomavirus small T antigen is oncogenic in transgenic mice. *J Invest Dermatol* 135:1415–1424.
48. Shuda M, et al. (2015) CDK1 substitutes for mTOR kinase to activate mitotic cap-dependent protein translation. *Proc Natl Acad Sci USA* 112:5875–5882.
49. Houben R, et al. (2010) Merkel cell polyomavirus-infected Merkel cell carcinoma cells require expression of viral T antigens. *J Virol* 84:7064–7072.