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Merkel cell polyomavirus (MCV) is the recently discovered cause of most Merkel cell carcinomas (MCCs), an aggressive form of nonmelanoma skin cancer. Although MCV is known to integrate into the tumor cell genome and to undergo mutation, the molecular mechanisms used by this virus to cause cancer are unknown. Here, we show that MCV small T (sT) antigen is expressed in most MCC tumors, where it is required for tumor cell growth. Unlike the closely related SV40 sT, MCV sT transformed rodent fibroblasts to anchorage- and contact-independent growth and promoted serum-free proliferation of human cells. These effects did not involve protein phosphatase 2A (PP2A) inhibition. MCV sT was found to act downstream in the mammalian target of rapamycin (mTOR) signaling pathway to preserve eukaryotic translation initiation factor 4E–binding protein 1 (4E-BP1) hyperphosphorylation, resulting in dysregulated cap-dependent translation. MCV sT–associated 4E-BP1 serine 65 hyperphosphorylation was resistant to mTOR complex (mTORC1) and mTORC2 inhibitors. Steady-state phosphorylation of other downstream Akt-mTOR targets, including S6K and 4E-BP2, was also increased by MCV sT. Expression of a constitutively active 4E-BP1 that could not be phosphorylated antagonized the cell transformation activity of MCV sT. Taken together, these experiments showed that 4E-BP1 inhibition is required for MCV transformation. Thus, MCV sT is an oncoprotein, and its effects on dysregulated cap-dependent translation have clinical implications for the prevention, […]

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Merkel cell polyomavirus (MCV) is the recently discovered cause of most Merkel cell carcinomas (MCCs), an aggressive form of nonmelanoma skin cancer. Although MCV is known to integrate into the tumor cell genome and to undergo mutation, the molecular mechanisms used by this virus to cause cancer are unknown. Here, we show that MCV small T (sT) antigen is expressed in most MCC tumors, where it is required for tumor cell growth. Unlike the closely related SV40 sT, MCV sT transformed rodent fibroblasts to anchorage-and contact-independent growth and promoted serum-free proliferation of human cells. These effects did not involve protein phosphatase 2A (PP2A) inhibition. MCV sT was found to act downstream in the mammalian target of rapamycin (mTOR) signaling pathway to preserve eukaryotic translation initiation factor 4E–binding protein 1 (4E-BP1) and Akt hyperphosphorylation, resulting in dysregulated cap-dependent translation. MCV sT–associated 4E-BP1 serine 65 hyperphosphorylation was resistant to mTOR complex (mTORC1) and mTORC2 inhibitors. Steady-state phosphorylation of other downstream Akt-mTOR targets, including S6K and 4E-BP2, was also increased by MCV sT. Expression of a constitutively active 4E-BP1 that could not be phosphorylated antagonized the cell transformation activity of MCV sT. Taken together, these experiments showed that 4E-BP1 inhibition is required for MCV transformation. Thus, MCV sT is an oncoprotein, and its effects on dysregulated cap-dependent translation have clinical implications for the prevention, diagnosis, and treatment of MCV-related cancers.

Introduction

Polyomavirus research has been central to cancer biology (1). Studies on simian vacuolating virus 40 (SV40) T antigen led to the discovery of p53 and uncovering functions for the retinoblastoma tumor suppressor protein (RB1) in cell cycle regulation (2–4). Research on murine polyomavirus led to the discovery of tyrosine phosphatase 2A (PP2A) inhibition. MCV sT was found to act downstream in the mammalian target of rapamycin (mTOR) signaling pathway to preserve eukaryotic translation initiation factor 4E–binding protein 1 (4E-BP1) inactivation, resulting in dysregulated cap-dependent translation. MCV sT–associated 4E-BP1 serine 65 hyperphosphorylation was resistant to mTOR complex (mTORC1) and mTORC2 inhibitors. Steady-state phosphorylation of other downstream Akt-mTOR targets, including S6K and 4E-BP2, was also increased by MCV sT. Expression of a constitutively active 4E-BP1 that could not be phosphorylated antagonized the cell transformation activity of MCV sT. Taken together, these experiments showed that 4E-BP1 inhibition is required for MCV transformation. Thus, MCV sT is an oncoprotein, and its effects on dysregulated cap-dependent translation have clinical implications for the prevention, diagnosis, and treatment of MCV-related cancers.

Conflict of interest: The authors have declared that no conflict of interest exists.

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Recent studies highlight the importance of cap-dependent translation to tumorigenesis regulated by PI3K-Akt-mTOR signaling (17, 24–26). A key step in cap-dependent translation is the binding of the cap-anchored eIF4E initiation factor to the 5′-methylguanosine cap, which serves as the mRNA recruitment signal for the eIF4G-eIF4A-RNA helicase complex. Inhibitors of this complex, such as the PI3K inhibitor LY294002, or mTOR inhibitors such as rapamycin, inhibit cap-dependent translation and cell growth (31). Phosphorylation of 4E-BP1 by mTOR causes its release from eIF4E, allowing free eIF4E to form the initiation complex (Supplemental Figure 2A). CM5E1 detected endogenous sT protein expression in MCV-infected MCC cell lines (Supplemental Figure 2B), and immunofluorescence showed that it was present in the cytoplasm as well as the nucleus when expressed in 293 cells (Supplemental Figure 2C).

Results

MCV sT protein expression in LT-negative MCC tumors. The CM5E1 mAb, raised against a peptide epitope in sT (Supplemental Figure 1; supplemental material available online with this article; doi:10.1172/JCI46323DS1), distinguished the 18-kDa sT protein from other MCV T antigen isoforms detected by MCV LT–specific (CM2B4; ref. 11) or pan–T antigen–detecting (CM8E6; ref. 14) mAbs (Supplemental Figure 2A). CM5E1 detected endogenous sT protein expression in MCV-infected MCC cell lines (Supplemental Figure 2B), and immunofluorescence showed that it was present in the cytoplasm as well as the nucleus when expressed in 293 cells (Supplemental Figure 2C). MCC sT antigen was expressed in MCC, and it was more commonly detected than MCV LT antigen in MCV-positive tumors. As shown for case MCCR10-123, sT protein was expressed only in MCC tumor cells and not in adjacent stromal cells (Figure 2A), a staining pattern similar to that of MCV LT (Figure 2B and ref. 11). Of 51 consecutively collected, formalin-fixed and cytokeratin 11.0-positive (CK20-positive) MCC tumors, 47 (92%) stained positive for MCV sT expression compared with 38 (75%) positive for MCV LT (p < 0.05, 1-tailed Fisher exact test; Figure 2G). Only 3 of the 51 MCC tumors (6%) were negative for both MCV LT and sT staining. Among MCCs positive for MCV sT, staining intensities with CM5E1 were reproducible but generally lower than those seen for the 2 tumors in Figure 2. This may reflect low levels of sT expression being typical in MCCs.

Figure 2, D and E, illustrated that some MCC tumors that were positive for MCV DNA were negative for MCV LT protein expression, yet retained expression of the MCV sT. The MCC tumor MCC344 was previously described to be negative for MCV LT antigen expression, although it harbors at least 6 MCV viral genome copies per cell that have been partially sequenced and found to retain the MCV LT mAb epitope sequence (9, 11). This tumor had robust MCV sT antigen staining (Figure 2D), but no staining for MCV LT antigen (Figure 2E). Both cases were negative for SV40 LT expression using the anti–SV40 LT antibody Pab419 as a negative control (Figure 2, C and F). Thus, MCV sT antigen was commonly expressed in MCV-positive tumors, even in a substantial fraction of cases in which MCV LT antigen was not detected.
MCV sT is an oncoprotein required for growth of MCV-positive MCC cell lines. We previously showed that shRNA targeting of the common MCV T antigen exon 1 causes pan–T antigen knockdown of both LT and sT and inhibits growth of MCV-infected cell lines (15). As a result of overlapping 3′ coterminal transcripts, LT cannot be readily targeted independent of sT (Supplemental Figure 1). MCV sT mRNA, however, could be knocked down without affecting LT protein expression by targeting the intron 1 sequence (Figure 3A).

A lentiviral shRNA that knocks down only the sT in MCV-positive MKL-1 cells (referred to herein as sT1 shRNA) inhibited MKL-1 cell growth to a similar extent as did shRNA knockdown with an shRNA targeting pan–T antigen exon 1 sequence (referred to herein as pan-T1 shRNA); conversely, control shRNA had no activity (Figure 3B). Proliferation of the virus-negative MCC cell lines UI50 and MCC13 was unaffected by either sT or pan-T antigen knockdown (Figure 3B and data not shown), which indicates that this is not due to an off-target effect. Pan–T antigen knockdown was more efficient in inhibiting cell cycle entry, measured by BrdU incorporation, than was sT knockdown alone, but cell cycle progression was also reproducibly diminished by sT targeting in MCV-positive MCC cells (Figure 3C).

Knockdown of sT did not cause MCC cell death, as measured by LDH release assays (Figure 3D), in contrast to pan-T knockdown (15). Thus, both MCV LT and MCV sT proteins are likely to separately contribute to MCV-positive MCC tumorigenesis.

Expression of MCV sT, but not MCV LT, results in rodent fibroblast transformation and human fibroblast serum-independent growth. Codon-optimized cDNAs for MCV sT and MCV LT were cloned into lentivirus vectors and expressed in Rat-1 cells. Only sT-expressing cells formed dense foci compared with the empty vector control (32 vs. 0 foci per 60-mm dish; Figure 4, A and B). Truncated, tumor-derived LT cDNAs (LT.339 and LT.350) also did not induce foci formation (Figure 4A). These results were confirmed by an assay for anchorage-independent growth in soft agar (Figure 4C and Supplemental Figure 3A), in which Rat-1 cells were bulk-selected for stable transduction and seeded onto soft agar plates, after which colonies were counted. Rat-1 cells expressing MCV sT readily formed colonies in soft agar, but cells selected for empty vector, full-length LT, or tumor-derived LT remained as nondoning, single cells up to 14 days after plating (Figure 4, C and D, and Supplemental Figure 3, A and B). These results were independently replicated using mouse NIH3T3 cells (Supplemental Figure 3C). Truncated tumor-derived LT proteins induced sparse multicellular aggregates in soft agar, but did not grow into full colonies (Supplemental Figure 3A), which suggests that truncated LT cDNAs have increased cell proliferative capacity compared with the wild-type LT, but, unlike sT, did not fully transform rodent fibroblasts. Dual selection of both sT and tumor-derived LT proteins did not enhance colony formation compared with expression of sT alone (Supplemental Figure 3A). For Rat-1 cells, proliferation was accelerated by expression of MCV sT and, after delay, by LT expression (Supplemental Figure 3D).

MCV sT expression also accelerated human fibroblast proliferation (Figure 5A). BJ fibroblasts immortalized with telomerase reverse transcriptase (BJ-TERT cells) were transduced with MCV sT and grown in 10% or 0% fetal calf serum. In the complete absence of serum, sT expression sustained modest BJ-TERT cell replication, whereas cells infected with empty vector fully arrested and had negligible S-phase entry (Figure 5B). MCV sT expression alone was not able to fully transform human BJ-TERT cells into growth on soft agar (data not shown).

MCV sT-induced cell transformation was independent of PP2A and heat shock protein binding. SV40 sT antigen primarily contributes to cell transformation via targeting of the protein phosphatase PP2A by binding the Act subunit (20, 34). We found that wild-type MCV sT could also be immunoprecipitated by tagged PP2A Act (Figure 4E). To examine PP2A’s role in MCV sT–induced cell transformation, we generated MCV sT mutants with alanine substitutions at 6 residues that are conserved with known SV40 sT PP2A interaction sites (35). Of the 6 mutations, 4 were unsuitable for additional analysis: alanine substitutions at residue histidine 130 or at a previ-
ously described cysteine 109 residue (14) reduced sT protein stability, whereas substitutions at cysteine 104 and proline 107 did not disrupt PP2A-sT interactions (data not shown). 2 proteins, with alanine substitutions at MCV sT arginine 7 (sT.R7A) or leucine 142 (sT.L142A), were expressed at levels comparable to the wild-type MCV sT protein (Figure 4D), but did not interact with PP2A (Figure 4E). The L142A substitution also prevented MCV sT interaction with PP2A subunit C, whereas R7A did not (data not shown).

Lentiviruses expressing the sT.R7A and sT.L142A proteins had equal or greater efficiency compared with the wild-type sT protein in inducing Rat-1 cell focus formation (46 and 36 foci, respectively, per 60-mm dish; Figure 4A) and anchorage-independent colony formation (Figure 4C). We also examined an sT aspartate-to-asparagine substitution in the exon 1–encoded DnaJ domain, sT.D44N, that eliminates Hsc70 binding to MCV LT (14). The MCV sT protein with this substitution was expressed (Figure 4D) and retained rodent cell transformation efficiency similar to that of the wild-type protein (Figure 4, A and C).

For Rat-1 cells, expression of either sT.R7A or sT.L142A proteins accelerated rodent cell growth similarly to the wild-type sT protein (Supplemental Figure 3D). For human BJ-TERT cells grown in 10% FCS (only sT.L142A was examined), both wild-type and PP2A-binding mutant showed accelerated cell growth compared with cells without sT expression (Figure 5A). BJ-TERT cell mitogenesis with sT.L142A was not significantly reduced compared with wild-type sT for cells grown at 0% FCS. Flow cytometry showed that both sT and sT.L142A initiated increased BJ-TERT cell cycle transit, comparable to the empty vector, under serum-free and serum-complete conditions (Figure 5B). Thus, PP2A interaction is not required for MCV sT to initiate rodent cell transformation or serum-independent human cell proliferation.

MCV sT promotes 4E-BP1 hyperphosphorylation. SV40 sT protein contributes to cell transformation by inhibiting PP2A-dependent Akt dephosphorylation (21, 22). To determine whether MCV sT-induced cell proliferation is also dependent on Akt-mTOR signaling, we examined a downstream target for this pathway, 4E-BP1.
Figure 4
MCV stT induces PP2A-binding and DnaJ domain–independent transformation of Rat-1 cells. (A) Lentiviral expression of MCV stT generated dense foci formation in Rat-1 cells compared with empty vector or full-length MCV LT cDNA. Tumor-derived LT antigens (LT.339 and LT.350) also did not increase focus formation. Mutations in the stT PP2A-binding (stT.R7A and stT.L142A) or DnaJ (stT.D44N) domains did not affect focus formation. Number of foci per dish is indicated. (B) Phase-contrast images of foci for empty vector– and MCV stT–expressing Rat-1 cells. Original magnification, ×40. (C) MCV stT, but not LT, induced anchorage-independent growth of Rat-1 cells in soft agar; this was unaffected by PP2A-binding (stT.R7A and stT.L142A) or DnaJ (stT.D44N) domain mutations. Colonies observed in 6-well triplicates were counted to determine average ± SD colonies per well. Colonies in wells (dark spots) and photomicrographs of typical fields are shown for each condition. Original magnification, ×200. (D) LT and stT expression in Rat-1 cells from C were detected by immunoblotting with CM2B4 and CM5E1, respectively. (E) MCV stT interacted with the Aα subunit of PP2A. HA-tagged PP2A Aα subunit was overexpressed in 293 cells, together with wild-type stT cDNA or with PP2A- or DnaJ-binding mutant cDNAs, and immunoprecipitated with HA antibody. Pulldown of stT protein was detected using anti-stT antibody (CM5E1).
As shown in Figure 6A, MCV sT increased 4E-BP1 hyperphosphorylation at S65 (especially for the δ form) in 293 cells but did not markedly change basal phosphorylation (α or β forms, T37/T46). MCV sT expression did not measurably affect raptor expression levels. Long-term raptor (mTORC1) knockdown by selection with a raptor-specific shRNA prevented MCV sT–promoted 4E-BP1 S65δ phosphorylation (Figure 6A). MCV sT only increased steady-state 4E-BP1 phosphorylation once 4E-BP1 was phosphorylated by mTORC1, which suggests it may prevent turnover of hyperphosphorylated 4E-BP1.

mTOR inhibitor studies provided direct evidence in support of this suggestion. Short-term treatment of 293 cells with the mTORC1-specific inhibitor rapamycin resulted in rapid turnover of the hyperphosphorylated 4E-BP1 phospho-S65γ form (Figure 6B). Although rapamycin is not a potent inhibitor of 4E-BP1 phosphorylation, the 4E-BP1 phospho-S65γ form was nearly absent within 15 minutes of the start of rapamycin treatment. mTORC1 phosphorylation, however, prevented turnover of phospho-S65 for up to 1 hour after treatment (Figure 6B). In contrast to S65 phosphorylation, rapamycin treatment or MCV sT expression did not appreciably alter basal T37/T46 phosphorylation.

Treatment with 2 different active site mTORC1/mTORC2 inhibitors, PP242 (36) or Torin1 (37), for 6 hours also resulted in loss of steady-state S65 hyperphosphorylation (Figure 6C). Unlike rapamycin treatment, basal T37/T46 phosphorylation was nearly abolished after PP242 or Torin1 treatment. When MCV sT was expressed in cells and treated with either PP242 or Torin1, the 4E-BP1 δ form (but not γ form) phospho-S65 (32) was mainly preserved (Figure 6C). Taken together, these data confirmed mTORC1 to be the major kinase hyperphosphorylating 4E-BP1 in the δ form at S65. Phosphorylation was rapidly lost when mTORC1 was inhibited, yet was preserved by expression of MCV sT.

To determine whether MCV sT targeting of the phosphatase PP2A is involved in preserving 4E-BP1 hyperphosphorylation (38), we examined the sT.R7A and sT.L142A proteins in 293 cells in the presence or absence of rapamycin for 1 hour. Both of the PP2A-binding defective sT proteins increased steady-state 4E-BP1 hyperphosphorylation comparable to wild-type MCV sT and were equally resistant to rapamycin (Figure 6D). These data indicate that PP2A targeting is not required for sT-promoted 4E-BP1 hyperphosphorylation.

The effects of MCV sT on 4E-BP1 hyperphosphorylation were confirmed in native MCC cell lines. Both MCV sT and pan-T1 knockdown decreased expression of phosphorylated 4E-BP1, particularly hyperphosphorylated forms, in MKL-1 cells but not in UISO cells (Figure 7A). Similar results were seen for 3 other MCC-positive cell lines (Supplemental Figure 4A). Functional consequences of sT-induced 4E-BP1 hyperphosphorylation were examined using a 7mGTP pulldown assay. Knockdown of either pan-T1 or sT1 in MKL-1 cells decreased elf4G binding to 7mGTP-resin, comparable to positive control PP242 treatment (Figure 7B). PP242 reduced both elf4G and elf4E binding to 7mGTP, and we were unable to determine whether differences between shRNA knockdown and PP242 treatment on elf4E cap-binding are due to a partial knockdown effect or reflect other biological relevance.
Effect of MCV sT on other Akt-mTOR pathway proteins. In addition to 4E-BP1, S6K is also an mTORC1 kinase target (39). MCV sT markedly increased steady-state T421/S424 phosphorylation of pp70 S6K in 293 cells (Figure 8A). Rapamycin treatment for 1 hour markedly reduced S6K phosphorylation, which was inhibited by sT expression. However, MCV sT preservation of the S6K phospho-T389 form after rapamycin treatment was much reduced compared with phospho-T421/S424. Mutation of PP2A binding
MCV sT reproducibly formed numerous large, multicellular colonies after 3 weeks of growth in soft agar (Figure 9, A and B). Neither sT.R7A nor sT.L142A PP2A-binding mutants affected Akt phosphorylation (S65 and T70) were markedly reduced. (B) Knockdown of either pan-T antigen or sT (by pan-T1 or sT1, respectively) in MKL-1 cells inhibited elf4G binding to 7mGTP sepharose beads in parallel with loss of 4E-BP1 S65 phosphorylation. Control shRNA–infected MKL-1 cells treated with PP242 are shown as a positive control. Despite partial preservation of 4E-BP1 phospho-S65 (arrowhead) in MKL-1 cells, eIF4E and eIF4G binding was reduced after PP242 treatment. Lanes were run on the same gel but were noncontiguous (white lines).

In MCV-positive MKL-1 cells, Akt was active, as measured by S473 phosphorylation (Figure 8D). Treatment with neither sT.R7A nor sT.L142A PP2A-binding mutants affected Akt phosphorylation (Figure 8C). In MKL-1 cells, Akt was active, as measured by S473 phosphorylation (Figure 8D). Treatment with the PI3K inhibitor LY294002 or the Akt inhibitor MK2206 diminished S473 phosphorylation. T antigen knockdown increased Akt phosphorylation, although this was more pronounced by pan-T antigen knockdown than by knockdown of sT alone. These results indicate that although MCV sT bound some PP2A isoforms (Figure 4E), Akt does not appear to be activated by MCV sT interaction with PP2A, unlike SV40 sT (21, 22).

4E-BP1 hyperphosphorylation is required for MCV sT–induced cell transformation. We expressed sT together with either wild-type 4E-BP1 or 4E-BP1AA (40) in Rat-1 cells and assayed transformation by soft agar assays. 4E-BP1AA is a constitutively active 4E-BP1 with alanine substitutions at the priming T37 and T46 phosphorylation sites. 10 microscopic fields were assessed for each condition by an investigator blinded to the expression conditions.

As was previously found (Figure 4C), Rat-1 cells expressing MCV sT reproducibly formed numerous large, multicellular colonies after 3 weeks of growth in soft agar (Figure 9, A and B). When 4E-BP1 was coexpressed, large colony counts per field were reduced greater than 50%. Large colonies were nearly absent when 4E-BP1AA was expressed together with MCV sT, with most cells remaining as single cells or consisting of clumps of few and scattered cells (Figure 9, A and B). Neither 4E-BP1 nor 4E-BP1AA affected cell viability (data not shown).

Discussion

MCV sT induces cell transformation, anchorage-independent growth, and serum-independent growth. It is expressed in nearly all MCV-positive MCC tumors and required for the proliferation of MCC tumor cells. MCV sT is an oncoprotein in humans and has potential for use as a diagnostic marker and therapeutic target for MCC.

MCV is related to SV40 and shares similar gene structures, but these 2 viruses differ in their carcinogenic mechanisms. SV40 LT induces cell transformation, whereas MCV LT does not. MCV sT alone is sufficient to cause transformation in rodent cell assays, but this is not the case for SV40. PP2A interaction is critical for SV40 sT and murine polyomavirus sT– and middle T–induced proliferation (8, 18, 19, 41, 42), but PP2A interaction can be abolished in MCV sT without affecting rodent cell transformation, human cell serum–independent growth, or 4E-BP1 phosphorylation.

The mechanism of MCV sT maintenance of 4E-BP1 hyperphosphorylation may provide new insights into Akt-mTOR signaling. Determining how MCV sT does this can only remain speculative until its cellular partners have been more fully described. MCV sT has no recognizable kinase domains and it acts on phosphorylation sites in both 4E-BP1 and S6K, the proximal substrates for mTORC1 kinase. MCV sT preserved 4E-BP1 hyperphosphorylation, particularly of the phospho-S65 δ form; our raptor knockdown revealed that mTORC1 must be active for MCV sT to achieve this effect. It is unlikely, for example, for sT to activate a non-mTORC1 kinase to phosphorylate 4E-BP1.

Figure 7

MCV sT knockdown in MCV-infected MCC cells reduces 4E-BP1 hyperphosphorylation and inactivates cap-dependent translation initiation complex formation. (A) sT and pan-T antigen knockdown reduced 4E-BP1 γ S65 hyperphosphorylation in MCV-positive MKL-1 cells, but not MCV-negative UISO cells. Priming site T37/T46 phosphorylation was preserved after sT antigen knockdown, whereas secondary sites of phosphorylation (S65 and T70) were markedly reduced. (B) Knockdown of either pan-T antigen or sT (by pan-T1 or sT1, respectively) in MKL-1 cells inhibited elf4G binding to 7mGTP sepharose beads in parallel with loss of 4E-BP1 S65 phosphorylation. Control shRNA–infected MKL-1 cells treated with PP242 are shown as a positive control. Despite partial preservation of 4E-BP1 phospho-S65 (arrowhead) in MKL-1 cells, elf4E and elf4G binding was reduced after PP242 treatment. Lanes were run on the same gel but were noncontiguous (white lines).
Hyperphosphorylated forms of 4E-BP1, however, demonstrated quick turnover when cells were treated with mTOR inhibitors, and this turnover was prevented by MCV sT expression (Figure 6C). It is therefore also unlikely that MCV sT activates mTOR complex kinase activity. We do not find a direct interaction between MCV sT and mTOR or 4E-BP1 by coimmunoprecipitation experiments (data not shown).

Our results point toward MCV sT inhibiting 4E-BP1 dephosphorylation or turnover and so elevating steady-state levels of hyperphosphorylated 4E-BP1 (Figure 10). Experiments using Torin1 and PP242 suggested that MCV sT preferentially preserves 4E-BP1 S65 phosphorylation, even when other 4E-BP1 sites are dephosphorylated. We are unaware of a phosphatase or other metabolic protein that regulates both 4E-BP1 and S6K, and so identifying the cellular partner for MCV sT may more generally help to explain how Akt-mTOR signaling is normally turned off. PP2A cannot be responsible for this effect, since PP2A-binding mutations did not affect steady-state 4E-BP1 levels induced by MCV sT.

Our results indicate that deregulated cap-dependent translation through 4E-BP1 hyperphosphorylation may contribute to Merkel cell carcinogenesis. Constitutively active 4E-BP1 that cannot be inactivated by MCV sT prevented sT-induced cell transformation. Whether increased phosphorylation of S6K and other 4E-BP proteins by MCV sT is also required for transformation remains to be examined. These findings have potential therapeutic implications. Akt-mTOR activation is common for other tumor viruses that target upstream components of the Akt-mTOR signaling cascade (43) and are highly sensitive to mTOR inhibitors (44). MCV sT, in contrast, was active downstream of mTOR. Consistent with this, rapamycin had little activity on MCC cell line survival or proliferation at micromolar concentrations (R. Arora, Y. Chang, and P.S. Moore, unpublished observations).

Although MCV sT is necessary for MCC, it is not the only viral factor sufficient to cause the tumor. Loss of sT expression does not fully recapitulate pan–T antigen knockdown (15), and MCV sT did not induce full human BJ cell transformation. We found that LT did promote cell proliferation, and in the setting of human tumors, MCV sT was likely to act in combination with other MCV T antigens, immune suppression, and possibly host cell mutations to promote MCC outgrowth.

Viral tumorigenesis is an uncommon, incidental, and accidental consequence of viral infection, and so sT targeting of 4E-BP1 must promote the replication fitness of nontumorigenic MCV. Viruses commonly induce cap-dependent translation through Akt-mTOR manipulation (17). Adenovirus, for example, activates Akt-mTOR signaling to induce 4E-BP1 phosphorylation early in infection (45), and human herpesviruses, including Kaposi sarcoma–associated herpesvirus (46), herpes simplex 1 (40), and cytomegalovirus (47), induce 4E-BP1 phosphorylation during lytic cycle initiation. MCV sT may similarly target 4E-BP1 to augment viral replication and transmission (48), but in doing so, it places the infected cell at risk for carcinogenic transformation (49).
Methods

Further information can be found in Supplemental Methods.

Clinical samples and immunohistochemistry. Human MCC tissues were obtained through a cohort study at the University of Pittsburgh Medical Center (11). Immunohistochemical staining of paraffin-embedded tissues were performed as previously described with mAbs CM2B4 (1:100) and CM5E1 (1:1,000) (11). SV40 T antigen mAb (PAb419) was used as a control for staining. Staining protocols were as described previously (11). Negative (non-MCC human tumor) and positive (sT- and LT-positive MCC tumor) tissue controls were used. Immunohistochemical results were scored in a blinded fashion by 2 different readers, including an American Association of Pathology board-certified pathologist. sT and LT staining were graded from 0 to 3, with 0 being no staining, 1 being weak (<20% cell positivity), 2 being intermediate (20–50% cell positivity), and 3 being strong (>50% cell positivity). The number of high-power fields (HPF) stained was determined. (B) Phase-contrast images of sT-induced Rat-1 cell colonies in soft agar. Original magnification, ×40.

Figure 9
Role of 4E-BP1 phosphorylation in sT-induced transformation. (A) Rat-1 cells were stably transduced with empty vector, wild-type 4E-BP1, or constitutively active 4E-BP1AA. Empty vector or MCV sT were then cotransduced into the cells without selection, and they were grown in soft agar for 3 weeks. Colonies were stained with crystal violet, and the colony number per high-power field was determined. (A) Colonies (per field) Empty vector sT Colonies (per field) Empty vector 4E-BP1 4E-BP1AA

Lentiviral/retroviral infection. pLVX EF.puro was modified from pLVX-puro vector (Clontech) by replacing CMV promoter with elongation factor-1 (EF) promoter. Codon-optimized MCV LT and sT sequences were then inserted using AfeI and SmaI sites. MCV LT and sT were then cloned into the pSMPUW-hygro vector (Cell Biolabs Inc.) using FseI and PacI restriction sites. Tumor-derived LTs LT.339 and LT.350 were amplified using primers (see Supplemental Methods). For shRNA knockdown, 6 shRNAs targeting sT (introns 1 and 1) and 1 shRNA targeting raptor (shRaptor) were designed and cloned into pLKO.1 (Addgene) using AgeI and EcoRI. A control shRNA was obtained from Addgene (shRaptor3). The shRaptor4 lentiviral shRNA targeting pan–T antigen exon 1 sequence, was previously described (15). For lentivirus production, 293FT (Invitrogen) cells were used for induction according to the manufacturer’s instructions. Lentivirus infection was performed in the presence of 1 μg/ml polybrene. For retrovirus production, 12 μg of retrovirus constructs pBabe empty.puro, pBabe 4E-BP1.wt, or 4E-BP1.AA were transfected to HEK293T phoenix-ampho cells by standard calcium phosphate transfection method. MCK-1 cells infected with control shRNA, pan-T1 shRNA, or sT1 shRNA were selected with puromycin (1 μg/ml) for 4 days after infection. Knockdown was evaluated at day 6 after shRNA transduction. For raptor knockdown experiments, 293 cells transduced with shRaptor were selected with G418 (0.9 μg/ml) for 3 days, seeded in 6-well plates for transfection, and used for DNA transfection experiments. Rat-1 cells infected with Babo retroviral vectors were selected with 2 μg/ml puromycin, followed by LVX lentiviral infection encoding MCV sT for transformation assay.

Immunoblotting, antibodies, and inhibitors. Cells were lysed in buffer (10 mM Tris-HCl, pH 8.0; 0.6% SDS, 2 mM NaF; 2 mM NaVO$_4$) containing protease inhibitors (Roche). The lysate was electrophoresed in SDS-PAGE and transferred to nitrocellulose membrane (Amersham). Primary antibodies were incubated overnight at 4°C, followed by anti-mouse IgG–HRP (Amersham) or anti-rabbit IgG–HRP (Amersham) for 1 hour at room temperature. Signals were detected using Western Lightning plus-ECL reagent (Perkin Elmer). The mouse mAb CM5E1 was generated using standard methods of immunizing mice with the KLH-conjugated peptide (EYEGLTCKDYMQSQYNAR) from intron 1 of MCV T antigen (Epitope Recognition...
Immunoreagent Core facility, University of Alabama). The following antibodies were used in this study: CM5E1 (1:1,000), CM2B4 (1:2,500) (11), CM8E6 (1:250) (14), PAB419 (50), 4E-BP1, phospho-4E-BP1S70/T46, phospho-4E-BP1S65, phospho-4E-BP1T37/T46, phospho-4E-BP1T37/S46, phospho-4E-BP1S473, S6K, phospho-S6KThr24/S24, phospho-S6KThr389, S6, phospho-S6Thr23/S24 (Cell Signaling), and α-tubulin (Sigma-Aldrich). Rapamycin (Sigma-Aldrich), the mTOR active site inhibitor PP242 (Chemdea), Torin1 (provided by D. Sabbatini, Massachusetts Institute of Technology, Cambridge, Massachusetts, USA) and the Akt inhibitor MK2206 (Selleck) were dissolved in DMSO.

**Immunoprecipitation.** For immunoprecipitation, 293 cells were cotransfected with 5 μg of PP2A α subunit (51) and either empty vector or various sT antigen expression vectors (sT, sT.R7A, sT.L142A, and sT.D44N) using Lipofectamine 2000 (Invitrogen). Samples were immunoprecipitated using a previously described protocol (14) with anti-HA mAb (Covance). Immunoblotting was performed using CM5E1 to detect sT.

**Cell proliferation assay and cell cycle analysis.** Rat-1 (2.0 × 10^4 cells/well) and BJ-TERT (2.5 × 10^4 cells/well) were seeded in 96-well plates, and Wst-1 cell proliferation assay was performed as described previously (15). OD values were divided by the OD value of day 1 for normalization, and fold increase was used to evaluate cell proliferation. Assays were performed in triplicate. For cell cycle analysis, cells were fixed with 70% ethanol, resuspended in buffer (1% FCS, 0.05 mg/ml propidium iodide, 0.1 mg/ml RNase A in PBS) and incubated for 1 hour at 37°C. For BrdU incorporation studies, MKL-1 cells were labeled with 10 μM BrdU for 3 hours on day 8 after shRNA transduction. BrdU incorporation was detected using Alexa Fluor 488–conjugated mouse anti-BrdU antibody (BD Biosciences — Pharmingen) followed by propidium iodide staining for cell cycle analysis.

**Lactate dehydrogenase (LDH) release assay.** After lentiviral infection and selection with puromycin, supernatant collected from infected MKL-1 cells with or without addition of Triton X-100 was subjected to LDH release assay (Roche). Percentage of LDH release activity was determined according to the manufacturer’s instructions. Results represent the average of 3 independent experiments.

**Foci formation assay and soft agar colony formation assay.** Rat-1 cells were infected with 100 μl recombinant lentiviruses and grown for 3 weeks for foci formation assay. To determine viral titer and establish stable cell lines, cells were selected with puromycin (2 μg/ml) and hygromycin (300 μg/ml) for pLVX EF and pSMPUW-Hygro lentiviral vectors, respectively. Rat-1 stably expressing MCV T antigens were trypsinized to single cells, counted, suspended in complete medium containing 0.3% agarose (Sigma-Aldrich), and seeded over a 0.6% agar layer in 60-mm dishes (5 × 10^4 cells/dish). After 3 weeks, colonies were stained with crystal violet (0.025% in PBS), and plates were photographed for soft agar colony formation assay.

**Cap-binding assay.** MKL-1 cells were lysed in buffer (50 mM Tris-HCl, pH 7.4; 0.15 M NaCl; 1% Triton X-100) supplemented with protease inhibitor (Roche). Lysates (250 μg) were incubated with 50 μl 7-mGTP sepharose 4B (GE Healthcare) for 2 hours at 4°C. Beads were collected, washed, and subjected to immunoblotting. MKL-1 cells treated with PP242 at 5 μM for 6 hours were used as a control.

**Statistics.** Fisher exact test was performed to determine the significance of differences between sT expression and LT expression in MCC tissues. 2-tailed Student’s t test was calculated for LDH release assay. P values less than 0.05 were considered significant. All data presented in cell proliferation assay and LDH cell death assay are mean ± SD. Representative results of more than 2 independent experiments are shown for cell proliferation assay.

**Study approval.** All specimens were obtained with patients’ informed consent and tested under guidelines approved by the University of Pittsburgh Institutional Review Board (protocol no. 07110141).

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**Figure 10**
Proposed mechanism of action for MCV sT in cell transformation. MCV sT preserves 4E-BP1 hyperphosphorylation, most likely by preventing hyperphosphorylation of 4E-BP1 turnover, which increases cap-dependent protein translation in Merkel cell cancers. The MCV protein does not markedly affect priming 4E-BP1 phosphorylation at residues T37 and T46 and does not directly induce 4E-BP1 phosphorylation.
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