Human Merkel Cell Polyomavirus Small T Antigen Is an Oncoprotein Targeting the 4E-BP1 Translation Regulator

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Supplemental data

1) Supplemental figures and legends

   Supplemental Figure 1, related to Figure 1 and Figure 2;

   Supplemental Figure 2, related to Figure 1;

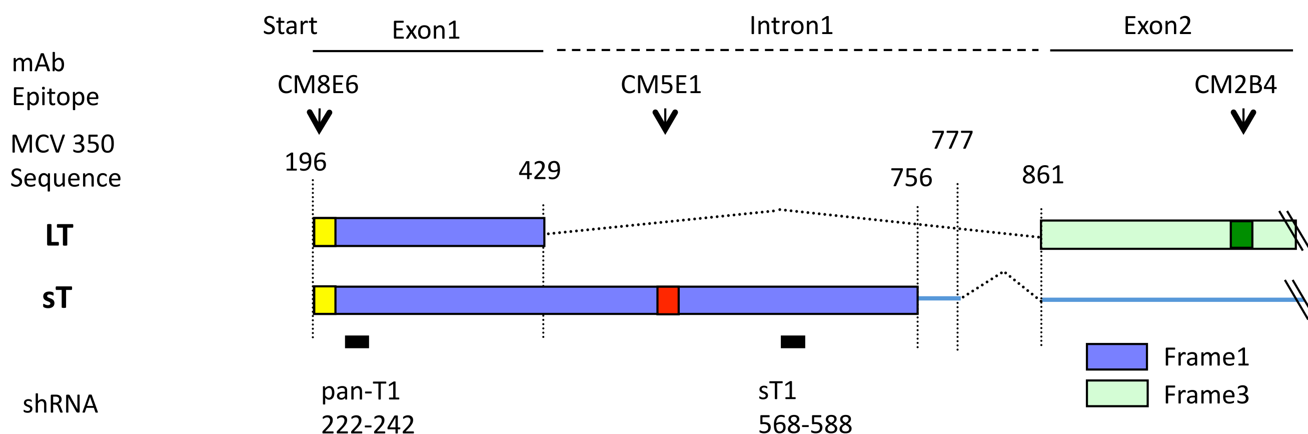
   Supplemental Figure 3, related to Figure 3;

   Supplemental Figure 4, related to Figure 5 and Figure 6;

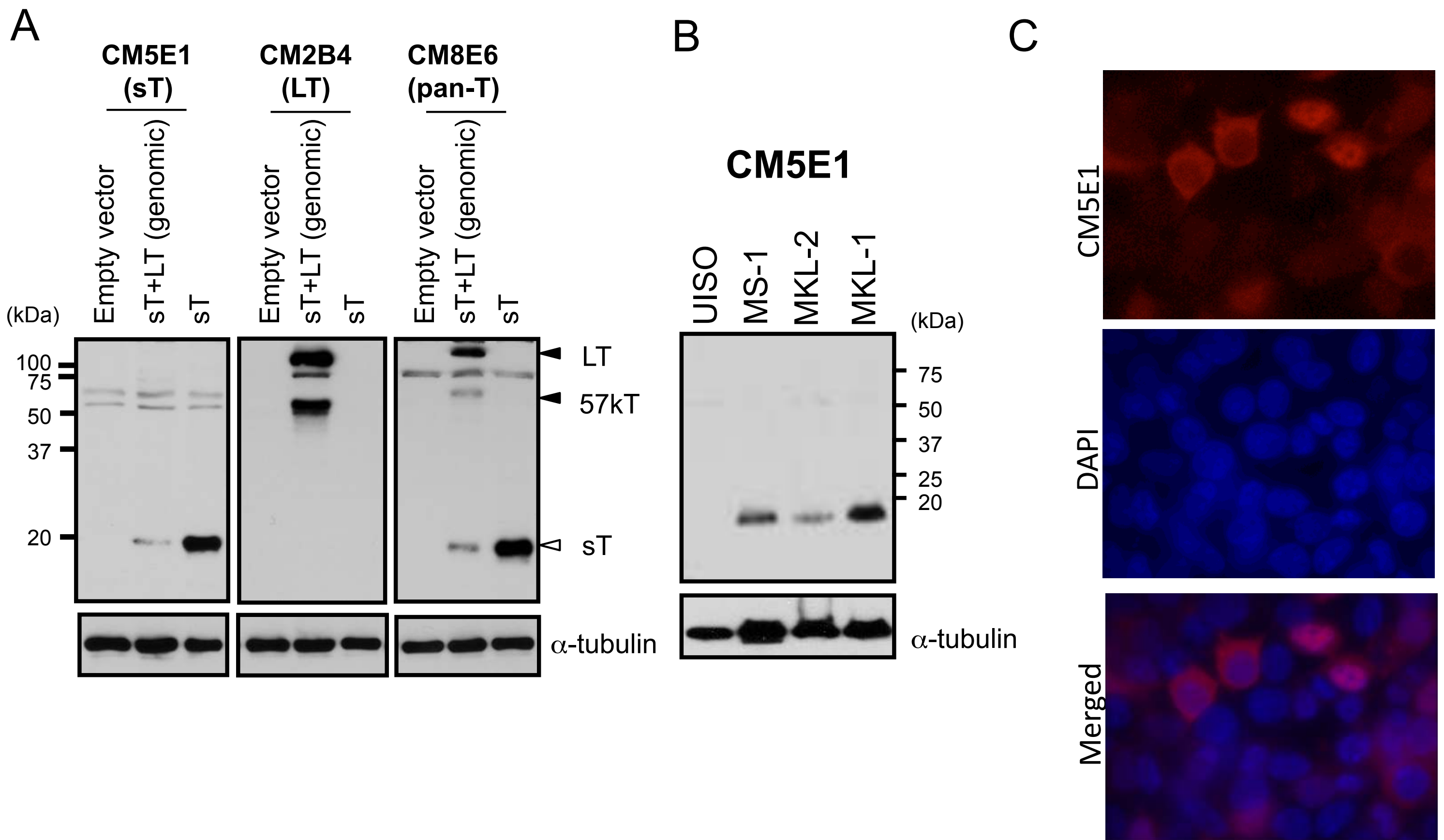
2) Supplemental methods

3) Supplemental references

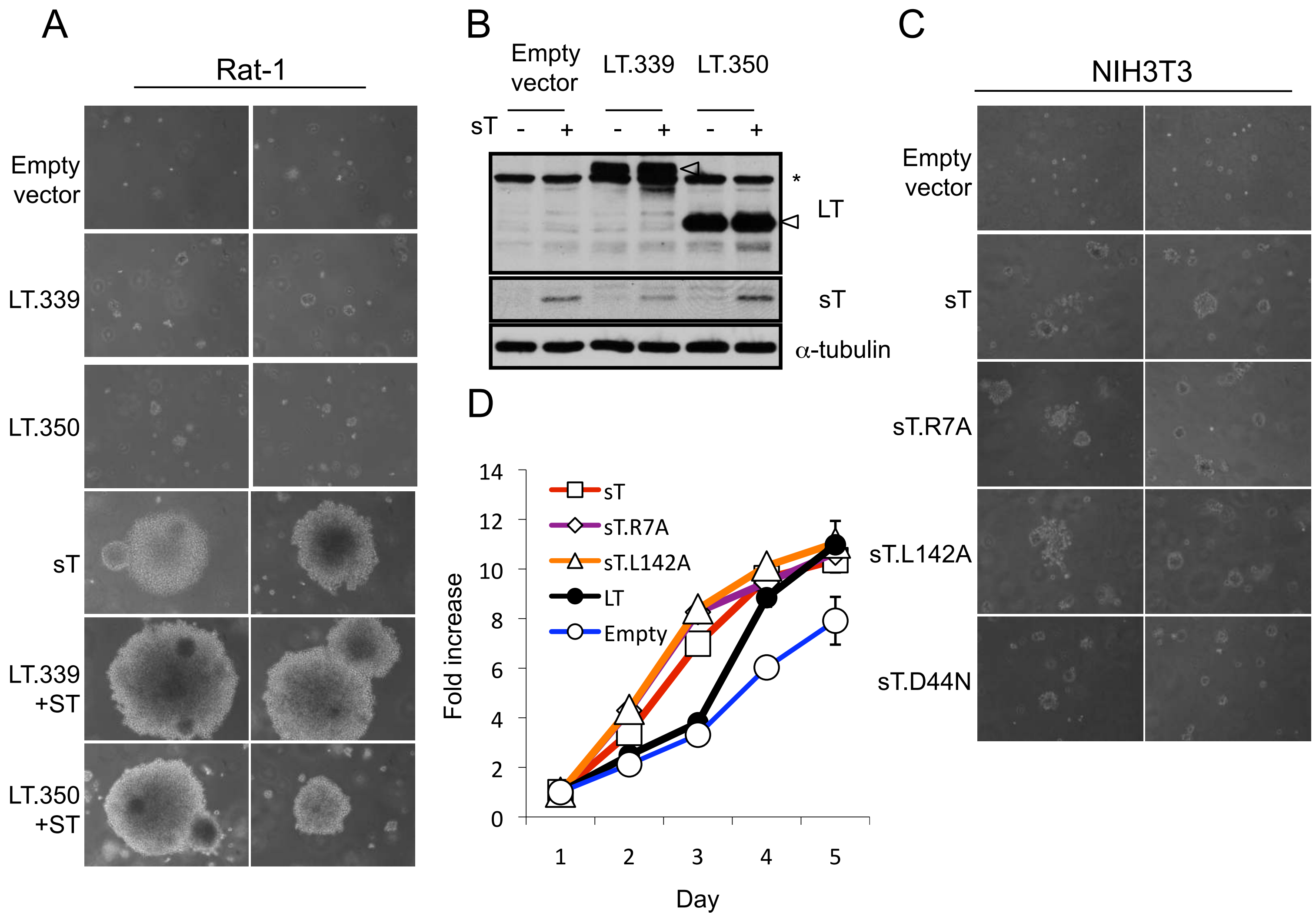
Supplemental figures and legends



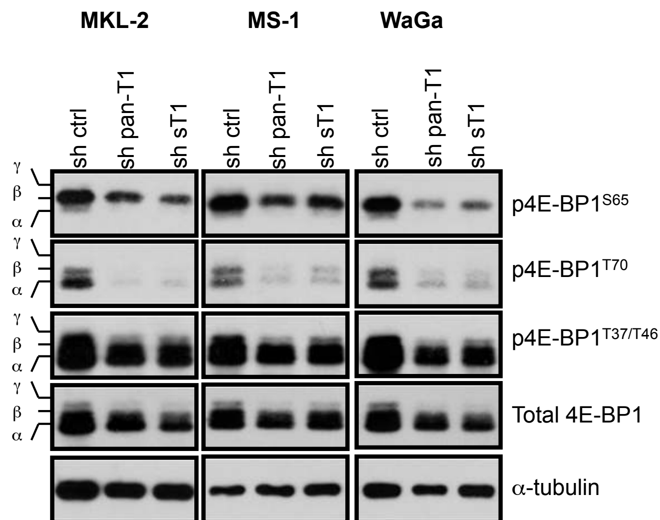
Supplemental Figure 1, related to Figure 2 and Figure 3.
Mapping of MCV antibodies and shRNA sequences. Schematic diagram of the MCV T antigen gene transcript, highlighting sequences targeted for antibodies and shRNAs (black bars) used in this study. The overlapping LT and sT transcripts are encoded by exon1 (frame1) but differential gene expression occurs due to alternative splicing at the exon1-intron1 junction (1, 2). CM5E1 mAb (red) recognizes a unique sT peptide sequence (EEYGTLKDYMQSGYNAR) encoded by sT only. Antibody CM8E6 (yellow) recognizes the N-terminus peptide and detects all isoforms of T antigen encoded by exon1 (3), while CM2B4 (dark green) (4) targets a peptide in exon2 (light green) and does not recognize sT. Black bars indicate the targeting sequences for pan-T1 (nucleotide 222-242) and sT1 (nucleotide 568-588) shRNA, respectively. The pan-T1 shRNA targets all T antigen isoforms (e.g., both LT and sT), while the sT1 shRNA only targets sT expression.

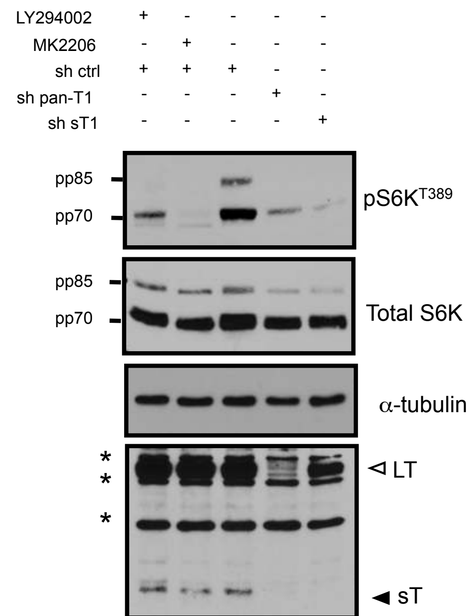


Supplemental Figure 2, related to Figure 2.
CM5E1 antibody specifically detects MCV sT antigen expression. (**A**) CM5E1 detects a specific 18 kDa MCV sT antigen protein. The open arrowhead indicates sT, whereas closed arrowheads indicate LT and 57kT spliced isoforms of MCV LT antigen. 293 cells were transfected with either MCV genomic T antigen (2) or MCV sT expression constructs and harvested at 48 hours after transfection. Protein expression was examined by immunoblotting using CM5E1 (sT), CM2B4 (LT/57kT) (4), and CM8E6 (pan-T) (3) mAbs. α-tubulin was used as loading control. (**B**) CM5E1 detection of MCV sT antigen in MCV-infected MCC cell lines. MCV-infected (MS-1, MKL-2, and MKL-1 (5)) and MCV-negative (UISO (2)) MCC cell lines were lysed and subjected to immunoblot with CM5E1 antibody. (**C**) MCV sT is expressed in both nuclear and cytoplasmic compartments. 293 cells expressing MCV sT were fixed, permeabilized, and stained with CM5E1 to determine the localization of MCV sT by immunofluorescence. Original magnification, x1000.



Supplemental Figure 3, related to Figure 4.
MCV sT, but not LT, induces transformation of rodent fibroblasts. (**A**) Transformation assays for Rat-1 cells expressing MCV sT and/or tumor-derived LTs (LT.339, LT.350). No increase in colony formation by sT was noted when tumor-derived LTs were coexpressed. Two representative fields are shown for each condition. Original magnification, x100. (**B**) Expression of sT and LT proteins in Rat-1 cells used in the soft agar assays. LT and sT proteins were detected with CM2B4 and CM5E1, respectively. Open arrowheads indicate truncated LT bands. Asterisk indicates non-specific reactivity. Coexpression of LT and sT was represented by minus/plus (-, single gene expression; +, coexpression). (**C**) MCV sT induces NIH3T3 colony formation in soft agar. sT-induced soft agar colony formation was unaffected by either PP2A-binding (sT.R7A and sT.L142A) or Hsc70-binding (sT.D44N) mutations. Two representative fields are shown for each condition. Original magnification, x100. (**D**) The effect of sT and LT on Rat-1 cell proliferation. Rat-1 cells stably expressing wild type sT, PP2A-binding mutants of sT (sT.R7A, sT.L142A) or LT were analyzed using a cell proliferation assay in 5% FCS.





Supplemental Figure 4, related to Figure 7 and Figure 8.
(**A**) Knockdown of sT in other MCV-positive cell lines reduces 4E-BP1 phosphorylation as in MKL-1 cells. Pan-T1 and sT1 shRNAs reduce 4E-BP1 hyperphosphorylation in MCV-infected MKL-2, MS-1 and WaGa cells (5). Priming site (T37/T46) phosphorylation in hypophosphorylated forms (α and β) of 4E-BP1 is relatively preserved while phosphorylation of secondary γ forms (S65 and T70) is markedly reduced by sT knockdown. (**B**) sT and pan-T antigen knockdown in MKL-1 cells reduces S6K phosphorylation at residue T389. Protein samples from Figure 6D were used. Knockdown was confirmed by immunoblotting with CM8E6. Asterisks indicate non-specific reactivity.

Supplemental methods

Constructs and transfections. We synthesized codon-optimized (DNA 2.0, Inc) LT and sT that deleted potential splicing donor and acceptor sites in the sequence. To generate pcDNA6 sTco, codon-optimized sT sequence was amplified using primers (5′- GGG CGG CGA TAT CAC CAT GGA CTT GGT CCT TAA CAG G-3′ and 5′- GGG CCG GCT CGA GTT ATC AGA AGA GAT GCA AGT GAA GCA AGC -3′) and cloned into pcDNA6 vector (Invitrogen) with EcoRV and XhoI restriction sites. sT.R7A, sT.L142A, and sT.D44N were generated by site-directed mutagenesis, with pcDNA6 sTco as a template, using the QuickChange Lightning Site-Directed Mutagenesis kit (Stratagene) and the following primer pairs: sTco.R7A.F (5′-TTG GTC CTT AAC GCG AAA GAA CGA GAG-3′)/sTco.R7A.R (5′-CTC TCG TTC TTT CGC GTT AAG GAC CAA-3′), sTco.L142A.F (5′- CAG AAG AAT TGC GCC ACA TGG GGA GAA-3′)/sTco.L142A.R (5′- TTC TCC CCA TGT GGC GCA ATT CTT CTG-3′), and sT.co.D44N.F (5′- CTC AAA CAT CAC CCA AAT AAG GGT GGC AAC CC-3′)/sTco.D44N.R (5′- GGG TTG CCA CCC TTA TTT GGG TGA TGT TTG AG -3′). The codon-optimized LT sequence, generated with flanking EcoRV and XhoI sites, was cloned with these sites into a pcDNA6 vector to generate pcDNA6 LTco. Expression of codon-optimized LT in 293 cells did not produce 57 kT protein, a spliced isoform of LT antigen (2) (data not shown). DNA transfection was carried out using Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer's instructions.

Lentivirus construction, production and infection. pLVX puro lentiviral vector (Clontech) was modified by replacing the CMV promoter with the elongation factor-1 (EF1) promoter using ClaI and AfeI restriction sites to generate pLVX EF MCS vector. The EF promoter was amplified by PCR using primers (5′- GGG CGG CAT CGA TGG CTC CGG TG CCC GTC AGT G-3′ and 5′- GGG CGG CCC GGG CGC GCC TCG AGC CTG CAG GTG TAC AGC GCT TGT CTA TAT GCA GAA GGA GTT TGC -3′) from pEF1 vector (Invitrogen). Codon-optimized MCV LT and sT sequences were amplified

and cloned into the pLVX EF MCS vector described above using AfeI and SmaI sites. Tumor-derived LTs, LT.339 and LT.350 were amplified from pcDNA6 LTco using forward primer (5′- GGG CGG CCG GCC AAC ATG GAT CTG GTA CTG AAT CGC -3′) and two reverse primers (5′- GGG CGG CTT AAT TAA CTA GTC AAG TTC ATA TTC GCA TGC -3′ and 5′-GGG CGG CTT AAT TAA CTA GTC TGT AAA CTG GGA CGA CG -3′ for LT.350 and LT.339, respectively). These two fragments were cloned into the pSMPUW-hygro lentiviral vector (Cell Biolabs, Inc) using FseI and PacI restriction sites. For lentiviral shRNA expression, 6 shRNA sequences were designed to target T antigen intron1: sT1 (568-588; sense strand, 5′-CCG GAA GTT GTC TCG CCA GCA TTG TTC AAG AGA CAA TGC TGG CGAGAC AAC TTT TTT TG-3′), sT2 (720-740; sense strand, 5′-CCG GAA CTG ACT ACT GCT TAC TGC ATC AAG AGT GCA GTA AGC AGT AGT CAG TTT TTT TG -3′), sT3 (498-520; sense strand, 5′-CCG GTA GAT TTT GCA GAG GTC CTG GGT CAC GAG ACC CAG GAC CTC TGC AAA ATC TAT TTT TTT G -3′), sT4 (568-590; sense strand, 5′-CCG GAA GTT GTC TCG CCA GCA TTG GAG CTC GAG CTC CAA TGC TGG CGA GAC AAC TTT TTT TTT G -3′), sT5, (613-635; sense strand, 5′-CCG GAA CTG TCT GAC GTG GGG AGG GTG CTC GAG CAC CCT CCC CAC GTC AGA CAG TTT TTT TTT G -3′) and sT6 (660-682; sense strand, 5′-CCG GTT GGT TTG GAT TTC CTC CTG CTT CTC GAG AAG CAG GAG GAA ATC CAA ACC AAT TTT TTT G -3′). These were cloned at AgeI and EcoRI sites of pLKO.1 lentiviral vector to generate sT1, sT2, sT3, sT4, sT5, and sT6 shRNAs. Of them, only sT1 knocked down MCV sT expression efficiently in MCV-infected cell lines. An shRNA sequence to target raptor (sense strand, 5′-CCG GGA CAA CGG CCA CAA GTA CTT CTC GAG AAG TAC TTG TGG CCG TTG TCC TTT TTG -3′) was also cloned into pLKO.1. The control pLKO.1 plasmid, possessing a control non-targeting short-hairpin RNA sequence (sh ctrl), was obtained from Addgene. We previously described two puromycin-selectable lentiviral shRNAs (shT1.puro and shT2.puro) that target the common T antigen

exon1 sequence (5). In the current study, shT1.puro is used and is renamed pan-T1 for the sake of clarity since it targets all T antigen isoforms. For Lentivirus production, 293FT cells (Invitrogen) were transfected with lentiviral constructs and packaging plasmids, psPAX2 and pMD2.G (Addgene) by Lipofectamine 2000 (Invitrogen). One day after transfection, medium containing plasmids and transfection reagent was replaced with fresh 10% FBS DMEM medium. 72 hours after transfection, culture supernatant was collected as infectious lentivirus, aliquoted, and stored at -80°C. Rat-1 or NIH3T3 cells seeded in 60 mm dishes were infected with lentivirus in the presence of 1 μg/ml polybrene.

Immunoblot analysis. Cells transfected with expression constructs or lentiviruses were lysed in buffer [10 mM Tris-HCl (pH 8.0), 0.6% SDS] containing protease inhibitor cocktail (Roche) and phosphatase inhibitors (2 mM NaF and 2 mM $NaVO_3$). Total protein was quantified using BioRad Protein Assay kit (BioRad). The lysates were electrophoresed in SDS-PAGE and transferred to nitrocellulose membranes (Amersham). Blots were blocked in 5% (w/v) non-fat dry milk in PBS-T (PBS with 0.05% Tween 20) for 1 hour. 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. Peroxidase activity was detected using Western Lightning plus-ECL reagent (Perkin Elmer). The following primary antibodies were used: CM5E1 (1:1000), CM2B4 (1:2500) (4), CM8E6 (1:250) (3), 4E-BP1, phospho 4E-BP1$^{T37/T46}$, phospho 4E-BP1^{T70}, phospho 4E-BP1^{S65}, S6K, phospho S6K^{T389} (1:1000, Cell Signaling). A monoclonal antibody to α-tubulin (1:2500, Sigma) was used for the protein loading control.

Cell proliferation Assay. Rat-1 cells expressing T antigens were seeded in a 96 well plate ($2.0x10^3$ cells per 96 well), and cell proliferation was monitored the following day by measuring optical densities.

To normalize, OD values were divided by the value of day 1. Fold-increase was calculated to evaluate cell proliferation.

Immunofluorescence. 293 cells were spotted on glass slides by Cytospin3 (Shandon), fixed with 10% buffered formalin for 20 min and permeabilized with phosphate-buffered saline (PBS) with 0.1% Triton X-100. After blocking, cells were incubated with CM5E1 (1:500 dilution) followed by secondary antibody (Alexa Fluor 568-conjugated anti-mouse, 1:1000, Invitrogen) for 1 hour at room temperature. Stained cells were mounted in aqueous medium containing DAPI (Vector Laboratories, CA). Cells were analyzed using an Olympus AX70 epifluorescence microscope equipped with a Spot RT digital camera.

Soft agar colony formation assay. Rat-1 or NIH3T3 cells stably expressing MCV T antigens were trypsinized to single cells and counted, suspended in complete medium containing 0.3% agarose (Noble agar; Sigma), and seeded on a 0.6% agar layer in 60 mm dishes (5×10^4 cells) or 6 well (2.5×10^4 cells) plate. After 3 weeks, colonies were stained with crystal violet (0.025% in PBS) and plates were photographed. Stable Rat-1 cell lines expressing truncated LT alone (LT.339 and LT.350) (1) or both sT and truncated LT (LT.339+sT and LT.350 +sT) were established by dual infection, followed by selection with two different selection drugs (puromycin or/and hygromycin).

Supplemental References

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