Supporting Information

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SI Materials and Methods

Cell Lines, Cell Culture, and Reagents. HEK293 (Invitrogen) and U2-OS osteosarcoma cells (kind gift of Per S. Holm, Technische Universität München) were cultured in DMEM (Invitrogen) supplemented with 10% FCS and 1% penicillin/streptomycin (PAN-Biotech). Cells (U2-OS) stably expressing the vectors pBABE, pBABE-LT (large tumor antigen), or pBABE-LT$^{\Delta 69–83}$ were generated by retroviral infection followed by antibiotic selection. [^{35}S]Methionine/cysteine was purchased from Hartmann Analytic. All other chemicals were purchased from Sigma-Aldrich, Carl Roth, or AppliChem unless otherwise noted.

Plasmids and siRNA. V5 IRS1, myc-Fbw8, and flag-CUL7 plasmids were described previously (1). All SV40 LT- or LT$^{\Delta 69–83}$-expressing vectors (LT/ΔLT; HA-, V5-tagged) were cloned in pcDNA3.1 and pBABE-puro vectors. DNA sequences of SV40 LT for cloning were kindly provided by Jim Manfredi (Mount Sinai School of Medicine). The CUL7 binding-deficient mutant LT$^{\Delta 69–83}$ was generated by site-directed mutagenesis. The pBABE-puro vector containing CUL7 was a kind gift of James DeCaprio (Harvard Medical School, Cambridge, MA). The HA-tagged full-length and mutant CUL7-expressing plasmids were a kind gift of Azad Bonni (Harvard Medical School, Cambridge, MA). Three different siRNAs directed against Cul7 with comparable efficiencies (5′-GAC UUU GUG CCA CGC UAC U-3′; 5′-CAA UAC CUA UGC UUU GUA U-3′; 5′-GUU GAG UAG UCC UGA UUA U-3′) and a nontargeting control siRNA (siRNA universal negative control 1) were purchased from Sigma-Aldrich. Plasmid DNA and siRNA were transfected using Lipofectamine 2000 reagent (Invitrogen).

Immunoprecipitations, Western Blot Analysis, and Antibodies. Immunoprecipitations and Western blot analyses were performed as described (1, 2). The following antibodies were used: c-myc (9E10; Santa Cruz Biotechnology), HSP90α/β (F-8; Santa Cruz Biotechnology), P-AKT (pS473, 9271; Cell Signaling), AKT (9272; Cell Signaling), P-Erk (pT202/pY204, 9101; Cell Signaling), Erk (4696; Cell Signaling), CUL7 (HPA030095; Sigma-Aldrich), flag (M2, F3165; Sigma-Aldrich), V5 (46-0705; Invitrogen), and HA (clone HA.11; HiSS Diagnostics).

Quantitative Real-Time PCR. Total RNA was extracted using the RNeasy Mini Kit (Qiagen), and reverse transcription (RT-PCR) was carried out with SuperScript II Reverse Transcriptase and oligo dT primers (Invitrogen). Levels of cDNA were quantified using SYBR Green I (Lonza) in an ABI PRISM 7900HT cycler (Applied Biosystems).

1. Xu X, et al. (2008) The CUL7 E3 ubiquitin ligase targets insulin receptor substrate 1 for ubiquitin-dependent degradation. *Mol Cell* 30(4):403–414.

2. Kasper JS, Kuwabara H, Arai T, Ali SH, DeCaprio JA (2005) Simian virus 40 large T antigen's association with the CUL7 SCF complex contributes to cellular transformation. *J Virol* 79(18):11685–11692.

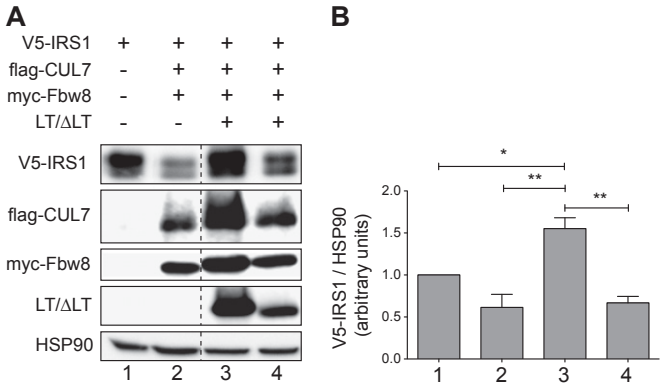

Fig. S1. CUL7/Fbw8-mediated degradation of IRS1 is impaired by SV40 LT in HEK293 cells. (*A*) HEK293 cells were transfected with empty vector or plasmids encoding V5-tagged IRS1, flag-CUL7, myc-Fbw8, LT (lane 3), and LT$^{\Delta 69–83}$ (lane 4; designated ΔLT). Lysates were separated by SDS/PAGE and subjected to Western blot analysis. (*B*) Quantification of IRS1 protein level. $n = 3$; $^{*}P < 0.05$, $^{**}P < 0.01$. Data are presented as means ± SEM.

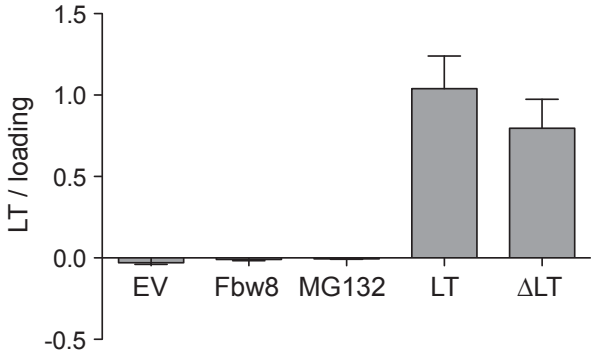

Fig. S2. Protein expression level of LT and LT$^{\Delta 69-83}$ in HEK293 cells. HEK293 cells were transfected with empty vector (EV) or plasmids encoding V5-IRS1, myc-Fbw8, HA-LT, and HA-CUL7 binding-deficient mutant LT$^{\Delta 69-83}$. Where indicated, cells were treated with the proteasomal inhibitor MG132 (10 μM) for 8 h. Lysates were separated by SDS/PAGE and subjected to Western blot analysis. $n = 8$. Data are presented as means ± SEM.

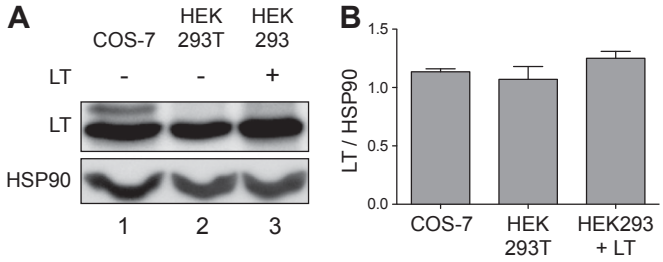

Fig. S3. Comparison of LT protein expression levels in different cell lines. (*A*) Whole-cell lysates of COS-7 cells, HEK293T cells, or HEK293 cells transiently expressing LT were subjected to SDS/PAGE and immunoblot analysis. (*B*) Quantification of LT protein level. Data are presented as means ± SEM.

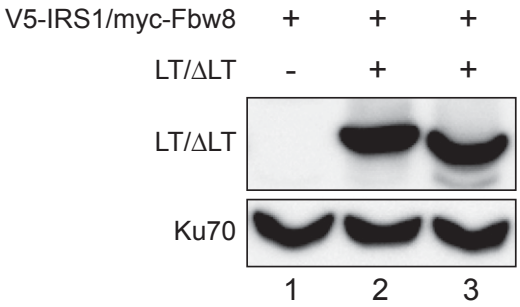

Fig. S4. Protein expression level of LT and LT$^{\Delta 69-83}$ in ^{35}S pulse–chase experiments. HEK293 cells were transfected with plasmids encoding V5-IRS1, myc-Fbw8, HA-LT, and HA-LT$^{\Delta 69-83}$. Whole-cell lysates were prepared immediately before ^{35}S labeling, separated by SDS/PAGE, and subjected to Western blot analysis.

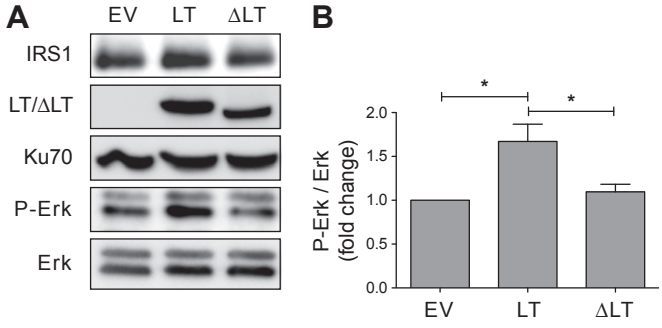

Fig. S5. Activation of the Erk MAPK signaling pathway upon LT expression in HEK293 cells. (*A*) HEK293 cells were transfected with empty vector or plasmids encoding LT or LT$^{\Delta 69-83}$. Forty-eight hours posttransfection, lysates were subjected to SDS/PAGE and Western blot analysis. (*B*) Quantification of Erk MAPK activation (as evidenced by Erk$^{pT202/pY204}$ phosphorylation). $n = 4$; $^*P < 0.05$. Data are presented as means ± SEM.

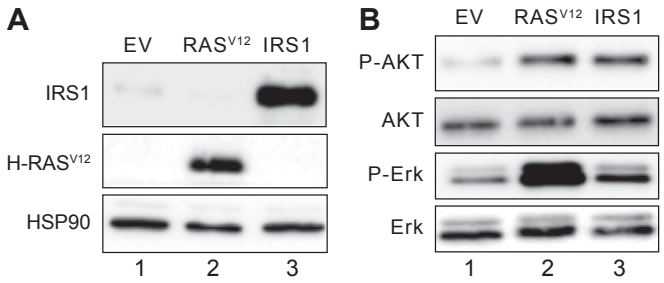

Fig. S6. Enhanced activation of the phosphatidylinositol-3-kinase (PI3K)/AKT and Erk MAPK signaling pathways upon ectopic IRS1 expression. Primary human diploid fibroblasts (IMR90 cells) were infected with amphotropic retroviruses (pBabe-puro) expressing empty vector, IRS1, or H-RAS^{V12}. Cell lysates were subjected to SDS/PAGE and immunoblot analysis. (*A*) Protein expression levels of IRS1 and H-RAS^{V12}. (*B*) Activation of PI3K/AKT and Erk MAPK signaling pathways (as evidenced by AKT^{pS473} and Erk$^{pT202/pY204}$ phosphorylation).

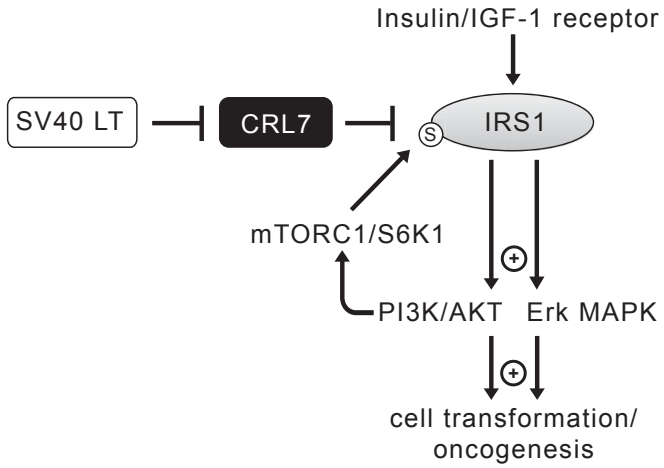

Fig. S7. Model for the role of LT interaction with CUL7. Cullin-RING E3 ubiquitin ligase 7 (CRL7) regulates PI3K/AKT and Erk MAPK signaling pathways via ubiquitin-mediated degradation of IRS1 that is dependent on a negative feedback loop via mTORC1/S6K1. Binding of LT to CUL7 inhibits CRL7 ubiquitin ligase function, resulting in desuppression and activation of IRS1 downstream signaling pathways. This may contribute to cell transformation and oncogenesis by SV40.