Supporting Information

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SI Materials and Methods

Cell Lines and Culture Conditions. HeLa, Phoenix, and U2OS cell lines were maintained in Dulbecco's modified eagle medium (DMEM) supplemented with L-glutamine, penicillin, streptomycin, and 10% (vol/vol) FBS (Cambrex). HEK293T and HeLa Tet-on Gam1 WT/mutant inducible cell lines were generated by Flp-recombinase-mediated integration according to the manufacturer's instructions (Invitrogen Flp-In System) and were maintained in DMEM supplemented with 10% (vol/vol) Tet-free FBS (Cambrex), L-glutamine, penicillin, and streptomycin. Hygromycin B and Blasticidin were added to the media for selection. Skin biopsies were collected via standardized operative procedures approved by European Institute of Oncology Ethical Board. Informed consent was obtained from all patients (donors). To obtain adult human epidermal keratinocytes, skin biopsies from donors were digested with Dispase (10 U/mL; Gibco) for 4 h at 37 °C to remove the epidermis, followed by a trypsinization step (Trypsin 500 mg/L) for 30 min at 37 °C to obtain isolated cells. Primary cultures of the isolated cells were then maintained in Keratinocyte Serum-Free Medium (KSFM; Gibco) containing bovine pituitary extract (BPE, 30 μg/mL; Gibco) and epidermal growth factor (EGF, 0.2 ng/mL; Gibco). Cells from passages 2–5 were used for retroviral transduction.

All cells were cultured at 37 °C in a 5% CO_2 buffered incubator.

Luciferase Assay. HeLa cells were transfected with 0.5 μg of HRE-Luciferase reporter plasmid and either with 1 μg of myc Gam1 plasmid or empty vector as control. After 24 h, cells were collected and lysed in Luciferase Cell Culture Lysis Reagent (Promega) according to the manufacturer's instructions. Thirty micrograms of lysates were mixed with 100 μL of Luciferase Assay Reagent (Promega) to proceed with Luciferase assay according to the manufacturer's instructions. Luciferase activity was measured using a luminometer (GDV).

Knockdown Using shRNA. pMX-puroII vector, pMX-puroII-shCul2, and pMX-puroII-shCul5 were kind gifts by K. I. Nakayama, Kyushu University, Fukuoka, Japan (1). To knockdown Cullin2 and Cullin5 in HeLa cells, the shRNA plasmids were transiently transfected in HeLa cells and selected using 1 μg/mL puromycin for a period of 3 d.

Immunoprecipitation, Immunoblotting, and Antibodies. When not differently specified, cells were lysed in Urea-based lysis buffer (8 M Urea, 100 mM NaH_2PO_4, 10 mM Tris·HCl, pH 8.0). For coimmunoprecipitation experiments (Fig. 3*C*), Phoenix cells were lysed in nondenaturing E1A lysis buffer (50 mM Hepes, pH 7.5, 250 mM NaCl, 0.1% Nonidet P-40, 0.2 mM phenylmethylsulfonyl fluoride, 1 mg/mL aprotinin, and 1 mg/mL leupeptin). For immunoprecipitation experiments that investigate protein ubiquitylation (Fig. 2 *B* and *C* and Fig. 6), cells were lysed in SDS-based lysis buffer and diluted with EIA before antibody incubation (2). Deubiquitinating enzyme inhibitor NEM (*N*-ethylmaleimide; SIGMA) was added into the lysis buffer where indicated at the final concentration of 5 mM.

The following antibodies were used: anti-Myc epitope (9E10; Calbiochem), anti-HA epitope (12CA5; Covance), monoclonal anti-GFP (B-2; Santa Cruz), polyclonal anti-GFP (home-made serum), anti-α-Tubulin (Santa Cruz), monoclonal anti-p53 (DO-1; Santa Cruz), anti-Flag epitope and agarose-conjugated anti-Flag epitope (M2; SIGMA), anti-DDDK (Flag) (ab 1162; Abcam), anti-Vinculin (Santa Cruz), anti-Ubiquitin (P4G7; Abcam), anti-Elongin B (FL-118; Santa Cruz), anti-Elongin C (R-20; Santa Cruz), anti-Cullin2 (Zymed), anti-Cullin5 (H-300; Santa Cruz), anti-VHL (Cell Signaling), anti-HIF 1α (BD Bioscience), anti-HSP70 (W-27; Santa Cruz), phospho-Stat3 (9145; Cell Signaling technology), Stat3 (9132; Cell Signaling Technology), anti-Glut1 (H-43; Santa Cruz), and anti-DBP (home-made by R.T.H. laboratory).

Drug and Heat-Shock Treatments. Proteasome inhibitor MG132 (Z-Leu-Leu-Leu-Al) (Sigma-Aldrich) was dissolved in DMSO (dimethyl sulfoxide) and added to culture medium at a final concentration of 10 μM for the indicated time. Cycloheximide (CHX) (SIGMA) was dissolved in water and added to culture medium at a final concentration of 20 μg/mL. Puromycin (SIGMA) was used at the final concentration of 1 μg/mL for selection, where indicated. Doxycycline (SIGMA) was used at final concentration of 1 μg/mL for selection. For Myc Gam1 induction in HeLa Tet-on and HEK293 cells, Doxycycline was used at a concentration of 100 ng/mL and 1 μg/mL, respectively. Heat-shock treatment was carried out by incubating cells at 43 °C for 90 min in a humidified, 5% CO_2 buffered incubator.

Plasmids and Transfections. Constructs expressing myc Gam1 WT and myc Gam1 L258,265A in pSG9M vector were previously described (3). pcDNA3-HA VHL was provided by W. H. Krek, ETH, Zurich (4). pcDNA3.1-Flag Ubiquitin by S. Polo, IFOM, Milan. pMX-puroII vector, pMX-puroII shCul2 and pMX-puroII shCul5 were gifts of K. I. Nakayama (1). pZIP SV40 Large T-antigen was a gift from D. Pasini, European Institute of Oncology, Milan. pcDNA3 EloB and pcDNA3 EloC were generated by subcloning EloB from pGEM3-EloB and EloC from pGEM3-EloC plasmids (provided by M. Pagano, NYU Cancer Institute, New York) respectively into pcDNA3 vector using HindIII/EcoRI (EloB) and HindIII/XbaI (EloC) restriction enzymes. pEF-Flag-I mSOCS1 and pEF-Flag-I mSOCS3 (both of mouse origin) were provided by D. Hilton, The Walter and Eliza Hall Institute of Medical Research, Victoria, Australia (5). pEGFP-C1 SOCS1(172-212) and pEGFP-C1 SOCS3(186-225) were generated by cloning PCR amplified fragments into pEGFP-C1 vector. pcDNA3.1 Flag E4orf6 was generated by sub cloning E4orf6 into pcDNA3.1 Flag frame C, BamHI/EcoRI. pH3SVL-Luciferase reporter plasmid was a generous gift of D. M. Katschinski, Georg-August University of Göttingen, Germany (6). The plasmid pLXSN-HPV16E6E7 was provided by M.T. For primers, see *Primers*.

HeLa, Phoenix, and U2OS cells were transfected overnight by calcium-phosphate method with the indicated plasmids and with empty vectors for control. For shRNA plasmid transfection, Ubiquitin IP, and luciferase assay experiments, transfections were carried out using Lipofectamine 2000 according to instructions (Invitrogen).

Quantitative RT-PCR. RNA was extracted from cells using the QIAGEN RNeasy Protect Mini Kit. cDNAs were generated by reverse transcription-PCR (RT-PCR) using PE Applied Biosystems TaqMan Reverse Transcription Reagents. Relative levels of specific mRNAs were determined by the SYBR Green I detection chemistry system (Applied Biosystems). All quantitative RT-PCR reactions were performed using a 7500 Fast Real-Time PCR System (Applied Biosystems). Quantification was performed by the comparative C_T method as described in the manufacturer procedure manual. The following primers were used: human

Carbonic Anhydrase IX, 5′-GGGTGTCATCTGGACTGTG-TT-3′ and 5′-CTTCTGTGCTGCCTTCTCATC-3′; human VHL, 5′-CAGCTACCGAGGTCACCTTT-3′ and 5′-GCTGTCCGT-CAACATTGAGA-3′; and human SOCS1, 5′-CGCTCGAGT-TGTCCGGCCGCTGCA-3′ and 5′-CCGGGATCCGGTCAG-ATCTGGAAGGGG-3′. Human GAPDH (5′-GAAGGTGAA-GGTCGGAGTC-3′ and 5′-GAAGATGGTGATGGGATTTC-3′) was used as control for normalization.

Infections. For retroviral transduction of human primary keratinocytes, pLXSN constructs were transfected into Ampho cells by calcium-phosphate. The retroviral supernatants were used to transduce the primary human keratinocytes as previously described (7). Twenty-four hours after transduction, the keratinocytes were selected with 0.1 μg/mL G418 (Gibco) for 3 d and then collected for Western blotting.

For Human Adenovirus infection, HeLa cells were infected with 20 PFU Ad 5 per cell in 50-mm-diameter plates for 24 h and processed as described in ref. 8.

Primers. The following primers were used: pEGFP-C1 SOCS1 (172-212), 5′-CGCTCGAGTTGTCCGGCCGCTGCA-3′ and 5′-CCGGGATCCGGTCAGATCTGGAAGGGG-3′; pEGFP-C1 SOCS3(186-225), 5′-CGCTCGAGTTGTGGCCACCCTC-3′ and 5′-CCGGGATCCGGTTAAAGTGGAGC-3′. Mutagenesis primers for SOCS3 5′-CAGTCTTCCGAAAAAGATGCTGGG-GGGTGGCCAC-3′ and SOCS1 5′- GTGCGGCCGCCGCA-GGAGCTGTTTCGCCAGCGCA-3′ were designed to cover the two desired mutations within the BC box of SOCS1 box 175L→P; 179C→F and SOCS3 box 189L→P; 193C→ F using the Stratagene protocol.

1. Kamura T, et al. (2004) VHL-box and SOCS-box domains determine binding specificity for Cul2-Rbx1 and Cul5-Rbx2 modules of ubiquitin ligases. *Genes Dev* 18(24): 3055–3065.
2. Boggio R, Colombo R, Hay RT, Draetta GF, Chiocca S (2004) A mechanism for inhibiting the SUMO pathway. *Mol Cell* 16(4):549–561.
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4. Lisztwan J, Imbert G, Wirbelauer C, Gstaiger M, Krek W (1999) The von Hippel-Lindau tumor suppressor protein is a component of an E3 ubiquitin-protein ligase activity. *Genes Dev* 13(14):1822–1833.
5. Nicholson SE, et al. (1999) Mutational analyses of the SOCS proteins suggest a dual domain requirement but distinct mechanisms for inhibition of LIF and IL-6 signal transduction. *EMBO J* 18(2):375–385.
6. Linden T, et al. (2003) The antimycotic ciclopirox olamine induces HIF-1alpha stability, VEGF expression, and angiogenesis. *FASEB J* 17(6):761–763.
7. Caldeira S, et al. (2003) The E6 and E7 proteins of the cutaneous human papillomavirus type 38 display transforming properties. *J Virol* 77(3):2195–2206.
8. Webster A, Leith IR, Nicholson J, Hounsell J, Hay RT (1997) Role of preterminal protein processing in adenovirus replication. *J Virol* 71(9):6381–6389.

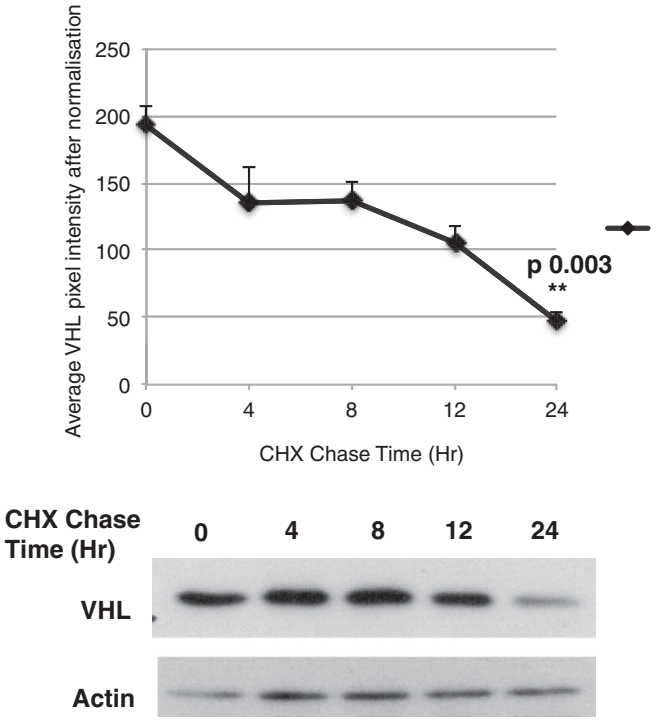

Fig. S1. CHX chase analysis of endogenous VHL protein shows long half-life. HeLa cells were incubated with media containing CHX and harvested at 4, 8, 12, and 24 h after incubation. Untreated cells served as control. Lysates were prepared after the indicated time points and immunoblotted for VHL protein. Loading control: Actin. Average pixel intensity values of VHL protein was calculated after normalization and plotted for each time point. Values are indicated as averages $n = 3 \pm$ SEM. Statistically significant decrease in VHL ($P < 0.003$) protein was seen 24 h after CHX treatment.

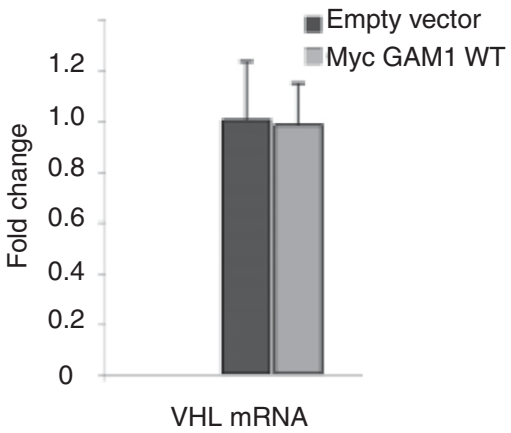

Fig. S2. Gam1-dependent VHL protein decrease is not due to reduced VHL mRNA synthesis. Quantitative RT-PCR analysis of VHL mRNA upon myc Gam1 wild-type (WT) expression does not display any change compared with the control condition (empty vector). Total RNA was extracted from HeLa cells transfected with either empty vector or myc Gam1 plasmid. Results are presented as fold induction of VHL mRNA amount relative to the control (empty vector) and normalized to GAPDH mRNA. Results represent the average of three independent experiments, $n = 3 \pm$ SD.

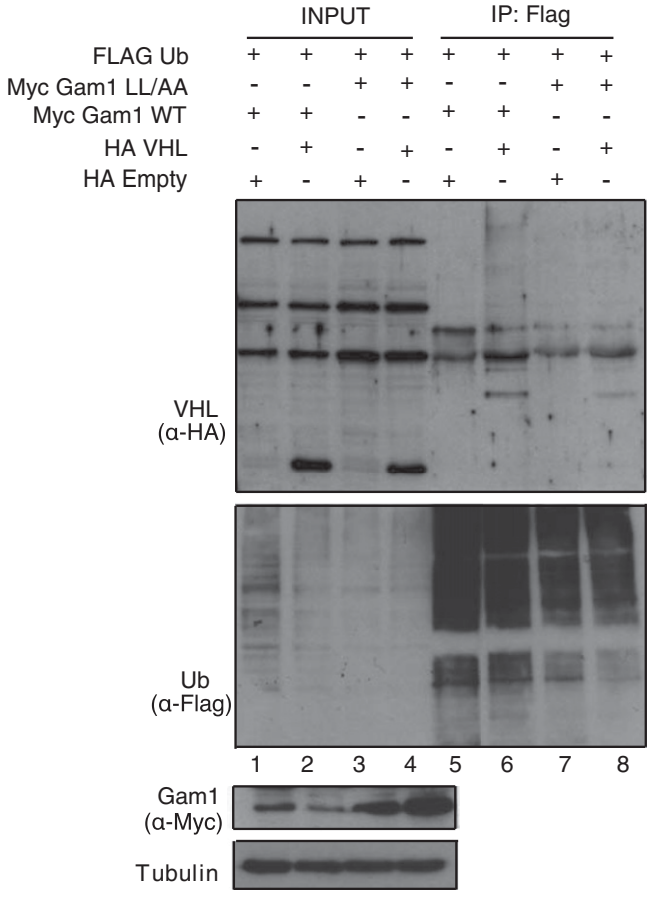

Fig. S3. Gam1 WT and not Gam1 LL/AA causes ubiquitylation and proteasome-dependent decrease of VHL. HeLa cells were transiently transfected with HA VHL, Flag Ub, and myc Gam1 (WT or mutant LL/AA). Cells were harvested 48 h later and lysed in a denaturing SDS-lysis buffer. The 10 μM MG132 was added to the culture medium 2 h before lysis. One milligram of total protein was immunoprecipitated with Flag Ub and immunoblotted against HA tag. Loading control: Tubulin.

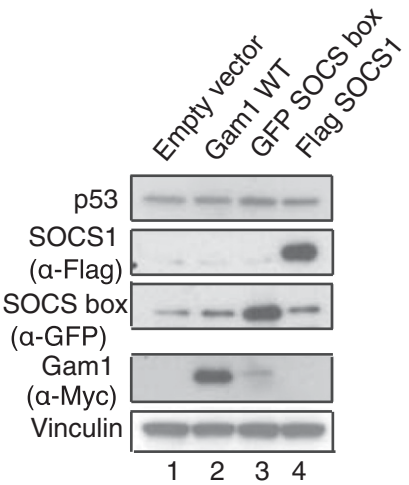

Fig. S4. Transient expression of the SOCS domain does not affect cellular levels of p53. U2OS cells were transiently transfected with Gam1 WT, SOCS box, and SOCS1. Cell lysates were probed for p53 levels. Loading control: Vinculin.

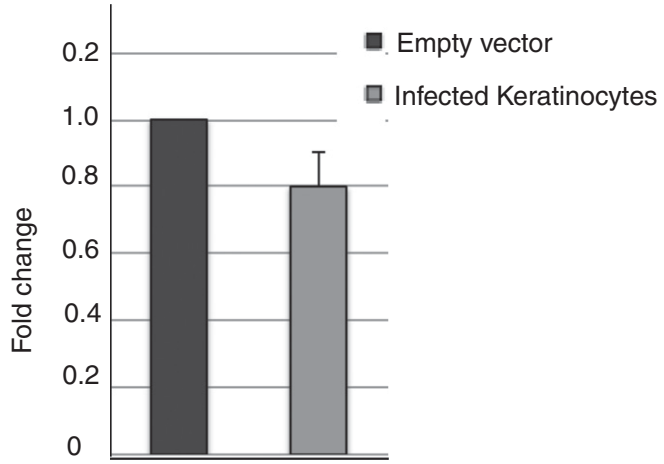

Fig. S5. HPV16 E6/E7-dependent VHL protein decrease is not due to reduced VHL mRNA synthesis. Quantitative RT-PCR analysis of VHL mRNA upon transduction of human primary keratinocytes with recombinant retrovirus expressing E6 and E7 oncoproteins from human papillomavirus type 16 (HPV16). Expression does not display any significant change compared with the control condition (empty vector). Total RNA was extracted from primary keratinocytes either expressing HPV16 E6/E7 or empty control. Results are presented as fold induction of *VHL* mRNA amount relative to the control (empty vector) and normalized to GAPDH mRNA. Results represent the average of six independent experiments ($n = 6$, P value > 0.05 using paired t test; error bar denotes SD).

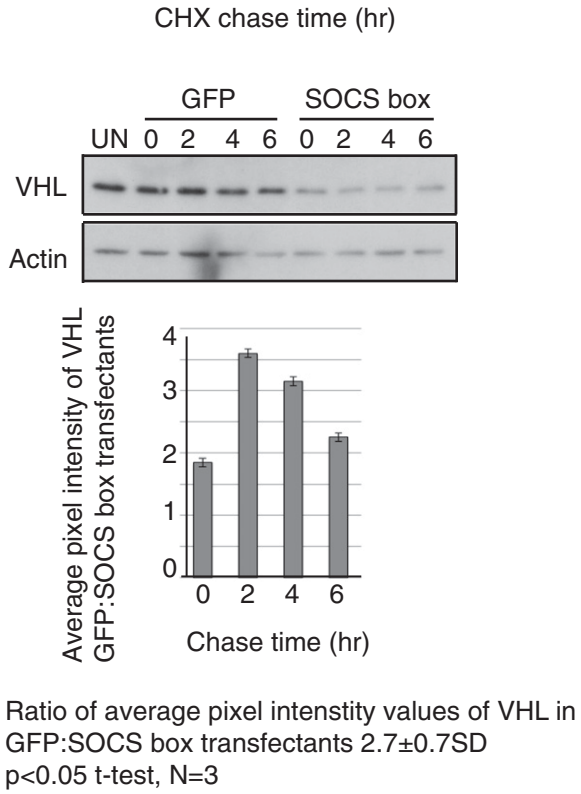

Fig. S6. Transient expression of SOCS box in HeLa cells rapidly decreases VHL half-life. HeLa cells were transiently transfected with GFP control or SOCS box plasmids. Twenty-four hours after transfection, CHX was added to the medium, and cells were lysed at 0, 2, 4, and 6 h after treatment. Lysates were then immunoblotted for VHL. Loading control: Actin. Pixel intensity values from VHL immunoblots were calculated using ImageJ and plotted to quantify the results. A significant and reproducible ($n = 3$) decrease in VHL protein levels was observed as early as the 0 h time point. Ratio of VHL protein in GFP transfectants versus SOCS box was 2.7 $\pm$ 0.7 SD with $P < 0.05$ using the t test statistic.