Figure S1.

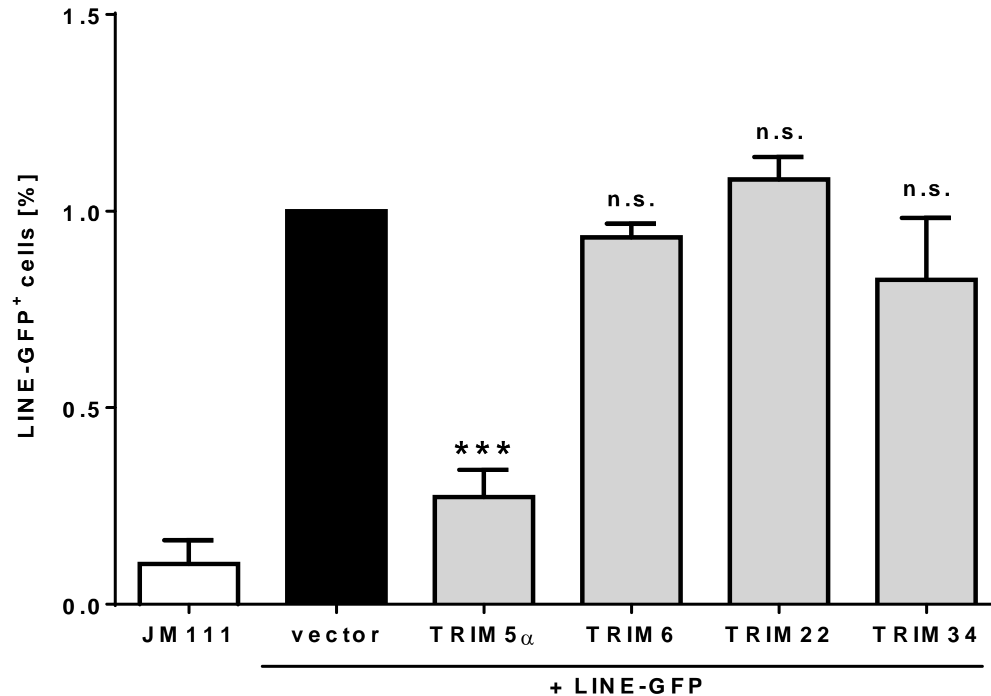

Figure S1. Average of biological replicates of Fig. 1A. Average of three independent reporter assays with LINE-GFP activity in vector-transfected cells set to 1. Error bars indicate standard error. Statistical analysis comparing vector transfected cells with TRIM5/6/22/34 expressing cells was done using one-way ANOVA followed by Tukey's multiple comparison test. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$, n.s. not significant.

www.pnas.org/cgi/doi/10.1073/pnas.1922366117

Figure S2.

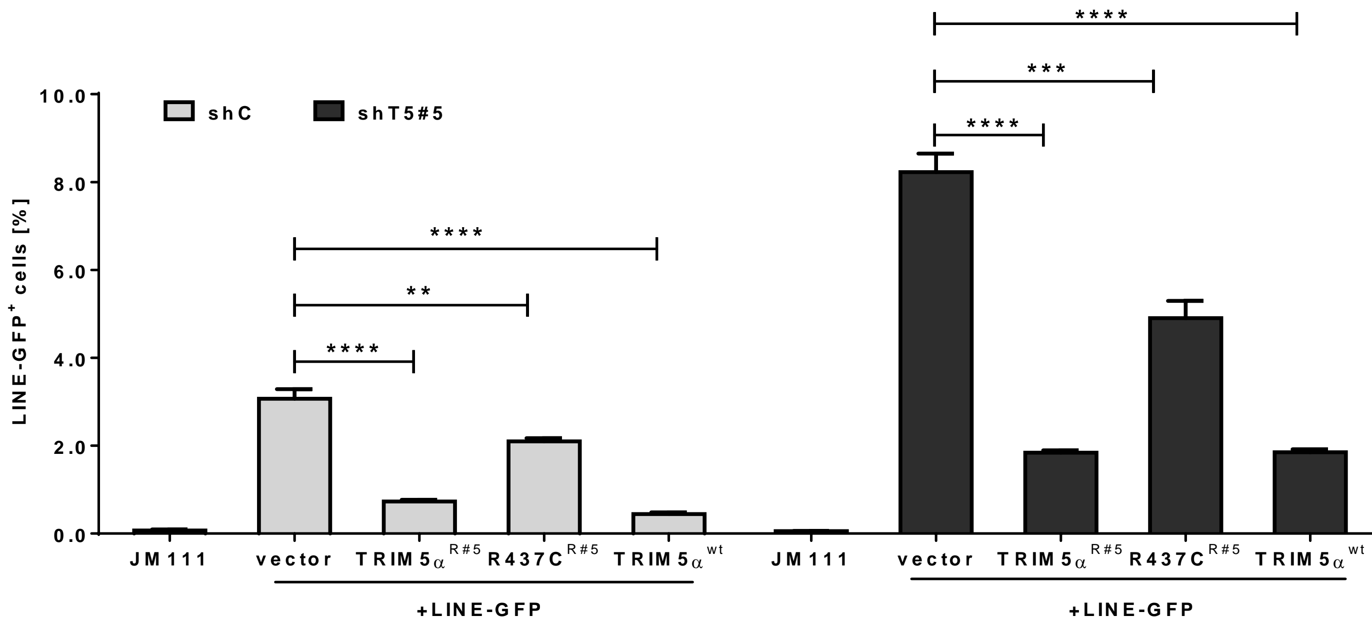

Figure S2. Endogenous TRIM5α restricts LINE-1 in shT5 #5 cells. HEK 293T shC and shT5 #5 cells were transfected with LINE-GFP and plasmids encoding the shRNA #5-resistant proteins TRIM5α$^{R\#5}$ and R437C$^{R\#5}$ or wt TRIM5α. Five days posttransfection, GFP-positive cells were quantified by flow cytometry. The percentage of LINE-GFP positive cells is shown as mean of triplicate transfections. Error bars represent standard deviation. One out of three independent experiments is shown. Statistical analysis comparing vector transfected cells with TRIM5α transfected cells was done using one-way ANOVA followed by Tukey's post-hoc test. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$; n.s. not significant.

Figure S3.

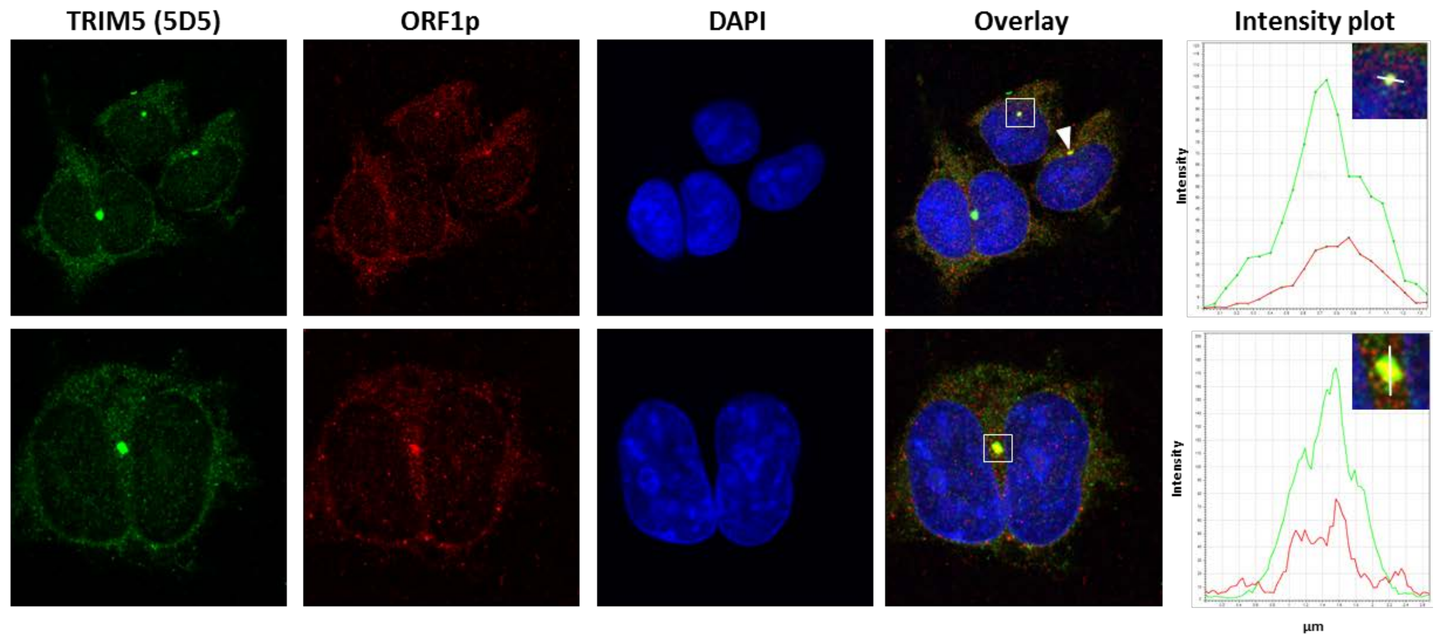

Figure S3. Endogenous TRIM5α and ORF1p colocalize in HEK 293T cells. HEK 293T cells were probed with antibodies targeting endogenous TRIM5α (green) or ORF1p (red). Cellular DNA was stained with DAPI (blue). Samples were analyzed by confocal microscopy. Intensity plots illustrate mean fluorescent signals for defined regions depicted in enlargement. White arrows indicate presence of co-aggregates

Figure S4.

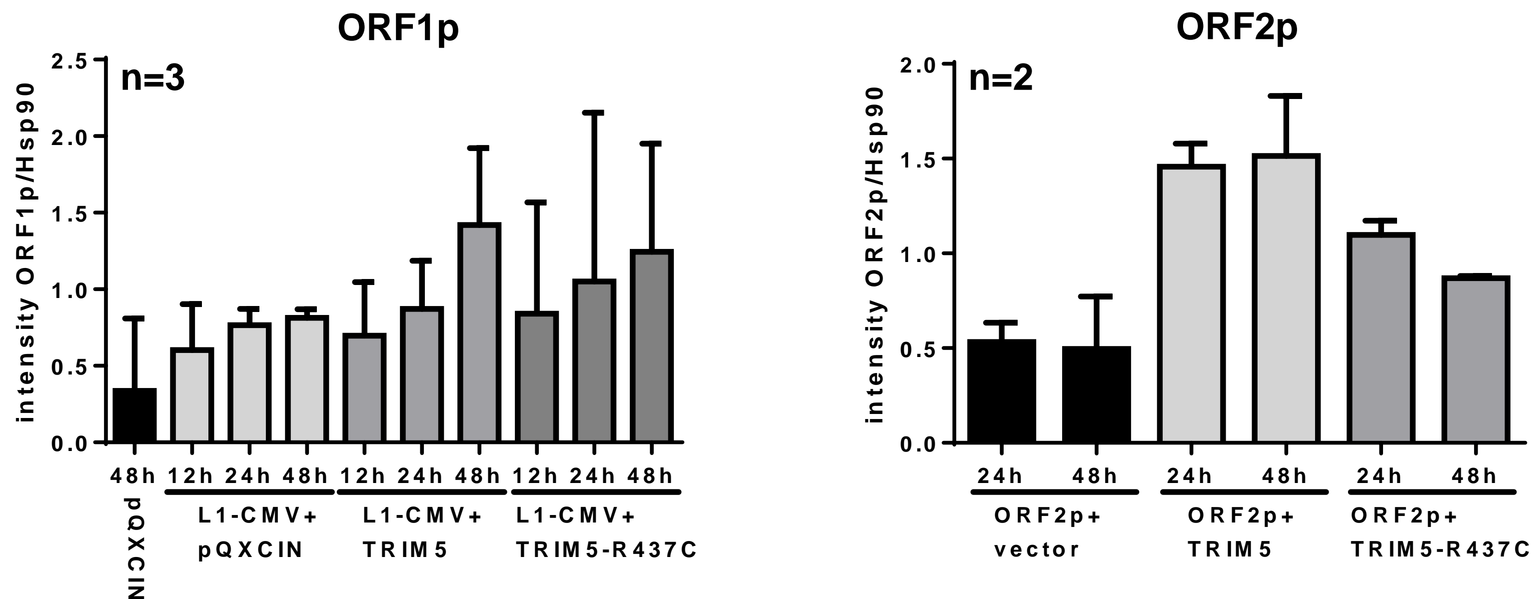

Figure S4. Overexpression of TRIM5α does not affect ORF1p and ORF2p protein levels. Quantifications of biological replicates of the immunoblots presented in Fig. 5A and 5B. Immunoblots were analyzed using ImageJ software. The average of multiple quantifications is shown. Error bars indicate standard error.

Figure S5.

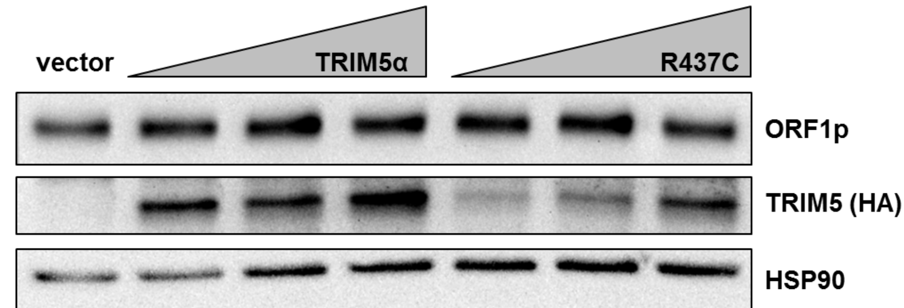

Figure S5. TRIM5α does not degrade endogenous ORF1p. HEK 293T cells were transfected with empty vector or increasing amounts of TRIM5α-HA and R437C-HA. After 24 h, cells were lysed and analyzed by immunoblot. Membranes were probed with ORF1p-specific antibody and HA-specific antibody. HSP90 served as loading control.

Figure S6.

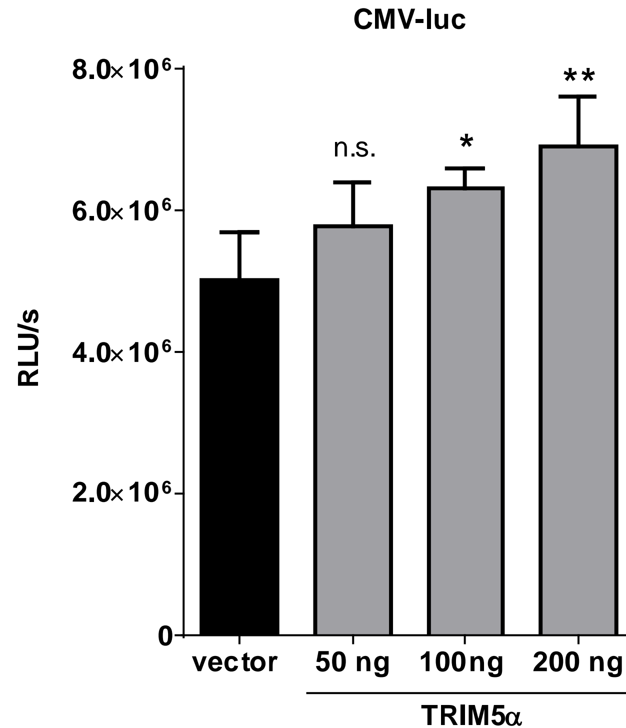

Figure S6. TRIM5α overexpression does not reduce CMV promoter activity. HEK 293T cells were transfected with a reporter plasmid encoding a CMV-driven luciferase reporter gene (CMV-luc) together with empty vector or increasing amounts of human TRIM5α. Two days posttransfection, cells were lysed and luciferase activity was determined in quadruplicates. Error bars represent standard deviation. One out of three independent experiments is shown. Statistics were done using one-way ANOVA followed by Tukey's post-hoc test. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$; n.s. not significant.

Figure S7.

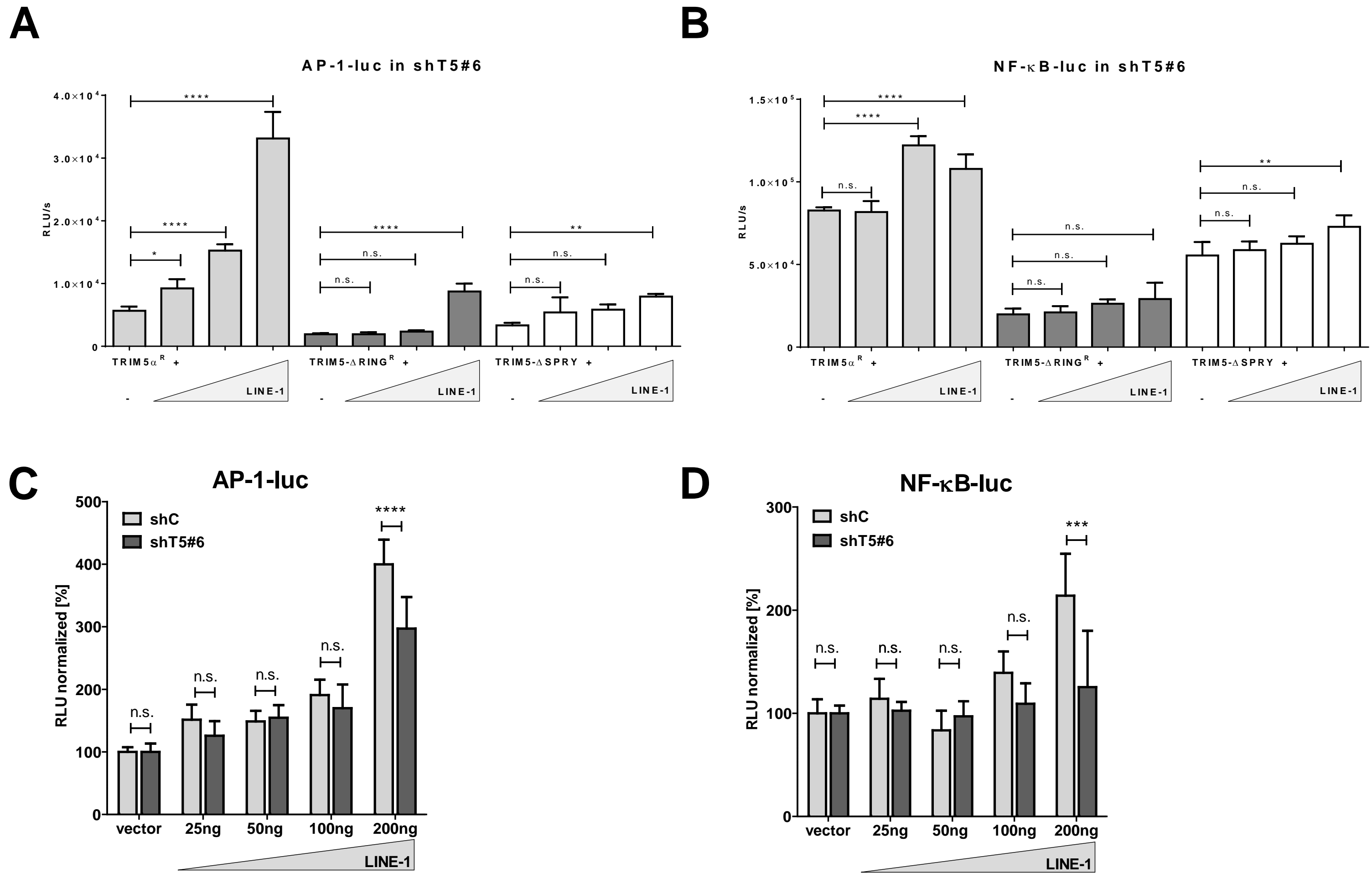

Figure S7. Endogenous TRIM5α induces innate signaling upon LINE-1 interaction. (A, B) HEK 293T shT5 #6 cells were transfected with (A) AP-1-luc or (B) NF-κB-luc together with shRNA resistant TRIM5α^R, TRIM5α^R lacking the RING domain (TRIM5-ΔRING^R), or TRIM5α^R lacking the SPRY domain (TRIM5-ΔSPRY), and increasing amounts of LINE-1. (C, D) HEK 293T shT5 #6 or shC cells were transfected with (C) AP-1-luc or (d) NF-κB-luc and increasing amounts of LINE-1. Statistical analysis was done using two-way (A, B) or one-way (C, D) ANOVA followed by Bonferroni post-hoc test. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$; n.s. not significant.

Figure S8.

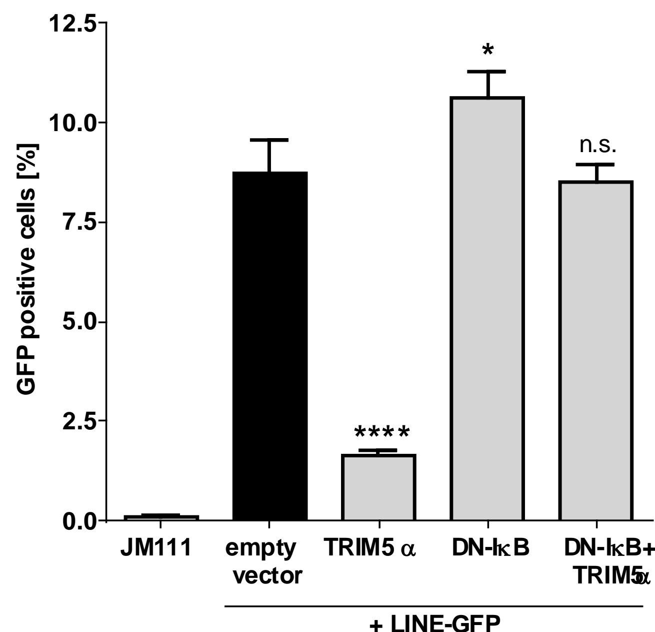

Figure S8. Inhibition of NF-κB signaling relieves TRIM5α-mediated LINE-1 restriction. HEK 293T cells were transfected with LINE-GFP together with TRIM5α or empty vector in combination with the dominant-negative NF-κB inhibitor DN-IκB. Five days posttransfection, GFP-positive cells were quantified by flow cytometry. The percentage of GFP-positive cells is shown as mean of triplicate transfections. Error bars indicate standard deviation. One out of three independent experiments is shown. Statistical analysis comparing vector transfected cells with TRIM5/DN-IkB transfected cells was done using one-way ANOVA followed by Tukey's multiple comparison test. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$, n.s. not significant

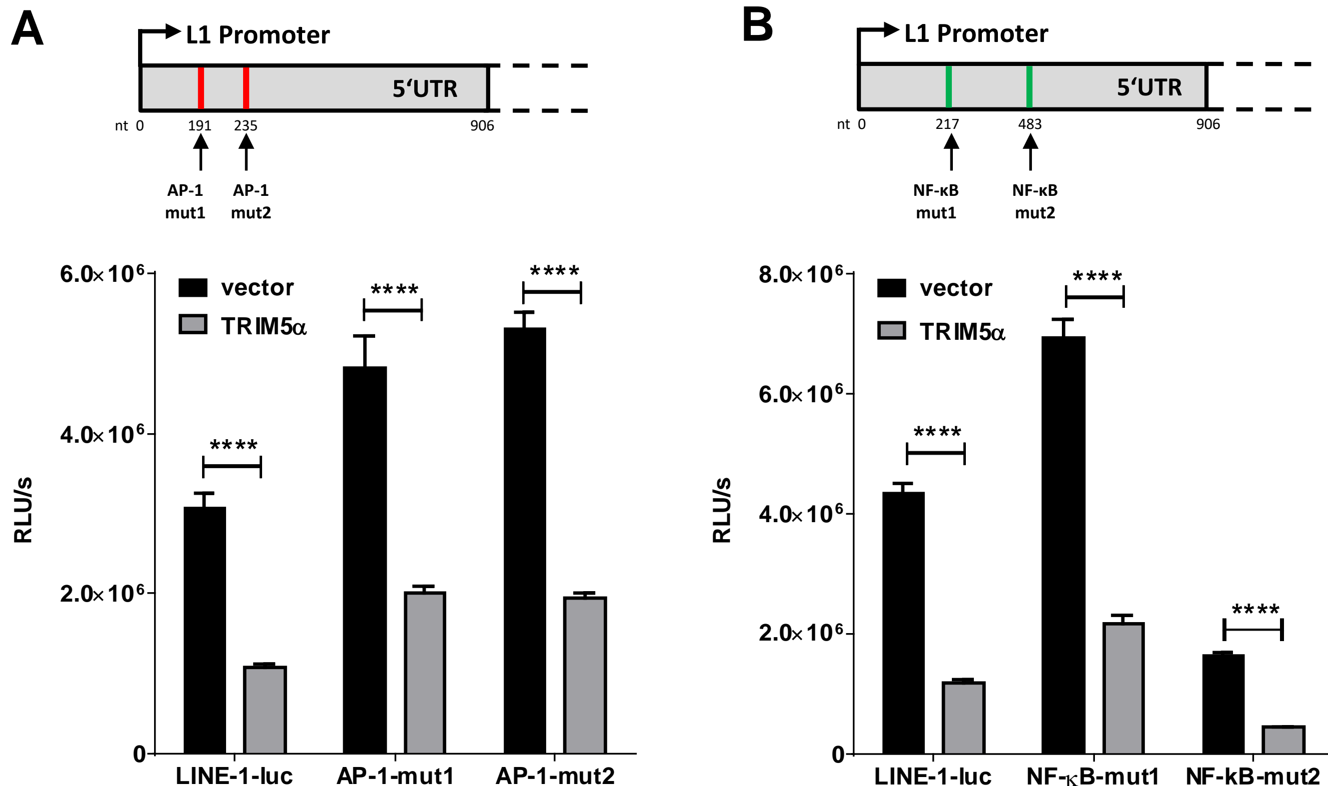

Figure S9. Putative AP-1 and NF-κB binding sites in the LINE-1 promotor region do not regulate TRIM5α-mediated restriction. (A) HEK 293T cells were transfected with LINE-1 promoter reporter (LINE-1-luc) variants containing mutated putative AP-1 binding sites, AP-1-mut1 and AP-1-mut2, or (B) mutated putative NF-kB binding sites, NF-kB-mut1 and NF-kB-mut2, together with either empty vector or 200 ng human TRIM5α. Cells were lysed two days posttransfection and luciferase activity was determined in quadruplicates. Error bars indicate standard deviation. One out of two independent experiments is shown. Illustrations indicate nucleotide position of mutated transcription factor binding sites in LINE-1 5' UTR. Statistical analysis comparing vector transfected cells with TRIM5α transfected cells was done using two-way ANOVA followed by Bonferroni post-hoc test. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$; n.s. not significant.

Methods

Cell lines and cell culture

HEK 293T and 2102EP cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum, 100 U/ml penicillin, 10 µg/ml streptomycin, and 1 mM glutamine. HEK 293T cells and 2102 EP cells expressing shRNA targeting TRIM5α or control shRNA were generated by lentiviral transduction. Three days postinfection, cells were selected with 2.5 µg/ml puromycin. HEK 293T cells expressing the various shRNAs were cultivated in DMEM containing 2.5 µg/ml puromycin.

Plasmids and Cloning

Plasmids encoding amino-terminal HA-tagged human and rhesus TRIM5α alleles *Mamu1-5* and *Mamu7* (TRIMCyp) were a kind gift of Welkin Johnson (Boston College) and were generated by insertion of the alleles into pQCXIN via the *Age*I and *Eco*RI restriction sites (1). TRIM5α variants and truncated versions were generated by overlapping PCR mutagenesis and inserted into pQCXIN via *Age*I/*Eco*RI. To generate HA-tagged NLS-TRIM5α, the nuclear localization signal (NLS) "PAAKRVKLD" was inserted in frame at amino acid position 6. For immunoprecipitation studies, myc-tagged TRIM5α was generated by replacing the amino-terminal HA-tag. Expression plasmids encoding carboxy-terminal HA-tagged TRIM6, TRIM22, and TRIM34, as well as chimeric TRIM5α and TRIM6 proteins containing heterologous SPRY (SPRY) or Linker2-SPRY domains (L2-SPRY), were generated by inserting PCR amplicons into pcDNA3.1 via the *NotI and XhoI*. All constructs were confirmed by nucleotide sequencing. ShRNA targeting TRIM5α (#5: AACCTGATGCAATGTGTAATA; #6: GGTTAGAGGAAGGAGTTAAAT) or a scrambled RNA (AGGAAAGTAGGATTGTAGGTA) were cloned into the lentiviral vector pLKO.1-puro (Addgene #8453) via *Age*I and *Eco*RI. Lentiviral particles were generated by cotransfection with the packaging plasmid pCMVΔR8.91 and a VSV glycoprotein expression plasmid, pVSV-G. ShRNA-resistant TRIM5α variants (TRIM5α^R, TRIM5α-ΔRING^R, R437C^R) were generated by overlapping PCR mutagenesis. The retrotransposition-competent reporter plasmid 99 PUR RPS EGFP (LINE-GFP) and the inactive control construct 99 PUR JM111 EGFP (JM111) were a kind gift of John Goodier (John Hopkins University) (2). Full–length LINE-1 expression plasmid encoding T7-tagged ORF1 (pAD2TE1) has been described previously (3, 4). A LINE-1 construct lacking functional ORF1p due to a 330bp deletion within ORF1, L1$_{RP}$ΔneoΔORF1, was a kind gift of Gerald Schumann (5). To generate the LINE-1-luc promoter reporter plasmid, the promoter sequence of 99 PUR RPS EGFP (LINE-1$_{RP}$) was amplified by PCR and inserted into the pGL3 Basic vector (Promega), similar to previously generated constructs (6). LINE-1-luc with altered AP-1 consensus sites at position bp 191-194 (AP-1-mut1) or position bp 235-238 (AP-1-mut2) or

with altered NF-κB consensus sites at position bp 217-228 (NF-κB -mut1) or position bp 483-494 (NF-κB -mut2) were generated by overlapping PCR mutagenesis and inserted into pGL3 basic via *Xho*I/*Hind*III. To generate the CMV luciferase reporter plasmid (CMV-luc), the CMV promoter sequence was amplified from pEGFP-N1 (Clontech) and cloned into pGL3 basic via the *Xho*I/*Hind*III. The expression plasmid for Mov10 was obtained from Addgene (#10976) (7). pTMO2F3 encoding 3xFlag-tagged ORF2p was a kind gift of John Moran (University of Michigan) (8). AP-1 and NF-κB activity was monitored using the luciferase reporter plasmids AP1-luc (9), which was a kind gift of Reinhard Voll (Freiburg), and NF-κB-Luc (Stratagene). Expression plasmids for MEK1, the constitutively active MEK1-DD, the inactive MEK1-K97M (10), and for IKK2, the constitutively active IKK2-EE, the inactive IKK2-KM (11), as well as dominant-negative IkB, pEF1-DN-IkB (12), have been described previously.

Retrotransposition assays

The *in vitro* analysis of LINE-1 retrotransposition using a GFP reporter construct has been described previously(2). Briefly, HEK 293T cells were transfected with the reporter construct 99 PUR RPS EGFP (LINE-GFP) or JM111, and the indicated plasmid at a molecular ratio of 3:1 using the calcium phosphate. After two days, 2.5 µg/ ml puromycin was added to the medium. Five days posttransfection, GFP expression as surrogate for successful retrotransposition was quantified by flow cytometry. Oxozeaenol (VWR) was diluted in DMSO and added 6 h posttransfection to a final concentration of 300 nM. To assess the impact of AP-1 and NF-κB signaling on the TRIM5α-mediated inhibition, 250 ng DN-IκB, 750 ng MEK1, or 250 ng IKK2 encoding vectors were cotransfected.

Immunoblot analysis

Transiently transfected HEK 293T cells were lysed at the indicated time points posttransfection using NP-40 lysis buffer (10 mM Tris-HCl pH 7.5, 150 mM NaCl, 2 mM EDTA, 0.5 % NP-40, Halt Protease Inhibitor). In general, 30 µg of the lysates were separated by SDS-PAGE, transferred onto a PVDF membrane (Merck), blocked with 5 % low-fat milk, and probed with anti-HA antibody (16B12, Biolegend), anti-myc antibody (9B11, Cell Signaling), anti-Flag M2 (F3165, Sigma), or anti-T7 antibody (69522, Novagen). Endogenous TRIM5α was detected using the monoclonal antibodies 3F1-1-9 and 5D5-1-1 (NIH AIDS Reagent Program). LINE-1 ORF1p was visualized using anti-ORF1 antibody (4H1, MerckMillipore). Membranes were incubated with anti-mouse or anti-rabbit horseradish peroxidase (HRP)-labelled secondary antibodies (Cell Signaling) and incubated in a HRP luminescence reagent. Signals were visualized using the Intas Advanced Fluorescence Imager. Band intensities were quantified with the AIDA image analyzer tool.

Digital droplet PCR

To quantify LINE-GFP integration events, cells were transfected with 99 PUR RPS EGFP or JM111 and the indicated TRIM proteins by calcium phosphate. Five days posttransfection, genomic DNA was extracted using QIAamp DNA Blood Mini Kit (Quiagen). LINE-GFP insertions were quantified in PCR reactions included 100ng of genomic DNA, 2x ddPCR Supermix (No dUTP, BioRad), 900 nM 5' oligo targeting GFP (5'-TGTTCTGCTGGTAG-3'), 900 nM 3' oligo targeting GFP (5'-GGCATCAAGGTGAAC-3'), as well as 250 nM of a FAM-labeled probe spanning across the splice junction of the LINE-GFP reporter construct (FAMTcggccagctgcac-BHQ1). To normalize LINE-GFP copy numbers to host genomes, ribonuclease P subunit p30 (RPP30) gene-specific primers and probe (HEX) were used (Bio-Rad). Droplets were generated using the QX200 Droplet Generator (BioRad). PCRs were analyzed on a QX200 Plate Reader using the Quanta Soft Analysis Software (BioRad).

LEAP assay

The effect of TRIM5α on LINE-1 reverse transcription *in vitro* was analyzed using the well-established LEAP assay as described previously(13). Here, HEK 293T cells were transfected with the LINE-1-ORF1-T7 together with huTRIM5α-myc or empty vector at a molecular ratio of 1:1 using calcium phosphate. Two days posttransfection, cells were lysed in CHAPS lysis buffer (10 mM Tris-HCl pH 7.5, 0.5 % CHAPS, 1 mM MgCl2, 1 mM EGTA, 10 % Glycerol, 1 mM DTT, and Halt Protease Inhibitor). LINE-1 ribonucleoprotein complexes (RNPs) were isolated by ultracentrifugation (178.000 x g for 1 h at 4 °C) through a 17 % sucrose cushion. RNPs were resuspended and total protein concentrations were adjusted to 1.5 mg/ml. Samples were split and used in immunoblot analyses, MLV RT PCR, and LEAP reaction. RNA was isolated using the Nucelospin RNA isolation Kit (Macherey-Nagel), treated with DNaseI and reverse transcribed using a 3'-RACE primer (5'-GCGAGCACAGAATTAATAC-GACTCACTATAGGTTTTTTTTTTTTVN) and M-MLV RT (Promega). To analyze ORF2p activity, the 3'-RACE primer and RNP precipitates were added to the RT reaction. The generated cDNA was amplified using the Phusion DNA polymerase (Thermo Scientific) with a 3'-RACE outer primer (5'-GCGAGCACAGAATTAATACGACT-3') and a 5′-LINE-1 primer (5'-GGGTTCGAAATCGATAAGCTTGGATCCAGAC-3'). PCR products were separated on 2 % agarose gels and visualized with the QUANTUM ST5 imaging system (Peqlab).

RNA immunoprecipitation assay

For analysis of TRIM5α interaction with LINE-1 mRNA, HEK 293T cells were transfected with LINE-1 expression plasmid encoding T7-tagged ORF1p (pAD2TE1) or a LINE-1 construct lacking ORF1p expression ($L1_{RP}$ΔneoΔORF1) (5) together with the indicated HA-tagged protein or empty vector control at a molecular ratio of 1:1. After 24 h, cells were lysed in RNA

IP lysis buffer (160 mM NaCl, 50 mM Tris-HCl pH7.5, 1 mM EDTA, 0.25 % NP-40) supplemented with Halt Protease Inhibitor Cocktail, 1 mM PMSF (Sigma), and RNaseOut (Life Technologies). HA-tagged TRIM5α proteins were immunoprecipitated with anti-myc antibody (Cell Signaling) coupled to magnetic Dynabeads (Thermo Fisher). Precipitated protein lysates were partially used in immunoblots to control for protein expression and for cDNA synthesis by MLV RT-mediated reverse transcription. To control for $L1_{RP}$ΔneoΔORF1 expression, RNA was extracted and reverse transcribed from lysates of cells transfected with pAD2TE1 and $L1_{RP}$ΔneoΔORF1. For reverse transcription, lysates were incubated at 95 °C for 10 min and then supplemented with 10mM dNTPs, RNaseOut, MLV-RT Buffer and MLV RT (Promega) and 10 µM 3'-LEAP primer and incubated at 42 °C for 1 h. cDNA was amplified via PCR using Phusion DNA polymerase and HF-Buffer (Thermo Scientific), 10 mM dNTP mix together with the 3'-LEAP outer primer and a 5'-LINE-1 primer described previously(13). PCR products were separated on a 2% agarose gel and visualized with the QUANTUM ST5 imaging system (Peqlab).

Quantitative RT-PCR

Total RNA of 293T cells cotransfected with the LINE-GFP reporter plasmid and the indicated plasmids was isolated 8 h posttransfection using a NucleoSpin RNA kit (Macherey-Nagel) according to the manufacturer´s instructions. RNA concentration and purity were evaluated by spectrophotometry (NanoDrop 1000, Thermo Fisher Scientific). RNA (200ng) was reverse-transcribed using an oligo-dT primer and Superscript II reverse transcriptase (Life Technologies). Quantitative PCR was performed in triplicates using the Maxima SYBR Green qPCR Mastermix (Life Technologies) on an ABI Prism 7500 cycler (Applied Biosystems). Values for LINE-1 transcript were normalized to expression levels of GAPDH using the $2^{-\Delta\Delta Ct}$ method. Primers used for quantification of transcripts by RT-qPCR were indicated as follows: GAPDH, 5´-AACAGAGTGAGCCCTTCTTCA-3´ (forward) and 5´-GGAGGCTGCATCATCGTACT-3´ (reverse); LINE1 (targeting the intron sequence of GFP reporter gene), 5´-CAGGCTGGAGCTCTCAGCTCAC-3´ (forward) and 5´-CCGACAAGCAGAAGAACGGC-3´ (reverse).

Immunofluorescence analysis

2102EP cells and HEK 293T cells were grown on poly-lysine (Millipore) coated coverslips. Cells were fixed with ice-cold methanol for 10 min at 4 °C and permeabilized with PBS containing 3 % BSA (Sigma) and 0.05 % Tween-20 for 30 min at 37 °C. Polyclonal rabbit ORF1p antibody (1:200) (5) and the monoclonal mouse TRIM5α antibodies (3F1-1-9, 5D5-1-1; 1:100) were diluted in PBS-Tween and incubated with the slides for 1 h at 37 °C. After washing, cells were incubated with anti-mouse-IgG-Alexa-488 (NEB, 1:1000 in PBS) and anti-

rabbit-IgG-Alexa-555 (NEB, 1:1000) in PBS for 1 h, mounted with Vectashield including DAPI (Linaris) and analyzed using a Leica Confocal SP5 microscope. To analyze endogenous ORF1p in the presence of TRIM5α, HEK 293T cells were transfected with HA-TRIM5α or empty vector (pQCXIN) and transferred onto poly-lysine-coated coverslips 24 h later. After 24 h, cells were fixed, permeabilized and probed with monoclonal mouse ORF1p antibody (Millipore, 1:500) and polyclonal rabbit HA antibody (Proteintech, 1:500) for 1 h at 37 °C. After washing with PBS-Tween, cells were incubated with a secondary anti-mouse-IgG-Alexa-555 (NEB, 1:1000) and an anti-rabbit-IgG-Alexa-488 antibody (NEB, 1:1000) for 1 h at 37 °C. Coverslips were mounted with Vectashield including DAPI (Linaris) and analyzed with a Leica Confocal SP5 microscope using the LAS Software. Quantification of ORF1p foci was done using the ImageJ2 software.

Luciferase reporter assays

To asses LINE-1 promotor activity, HEK 293T cells were transfected in 96 wells with 100 ng LINE-luc together with increasing amounts of TRIM5α, R437C, or empty vector using Lipofectamine2000 (Life Technologies). Additional CMV promoter-containing LINE-1, pAD2TE1 (75 ng – 300 ng), was added to 75 ng of TRIM5α or empty vector to test for the impact of LINE-RNPs on TRIM5α-mediated inhibition. Oxozeanol (VWR) was diluted in DMSO and added 6 h posttransfection to a final concentration of 300 nM. For AP-1 and NF-κB signaling assays, HEK 293T, shC, or shT5α #6 cells were transfected with 10 ng of AP1-luc or 2.5 ng of NF-κB-luc reporter plasmid together with increasing amounts of TRIM5α, TRIM5α-$\Delta RING^{R}$, TRIM5α-ΔSPRY or empty vector using Lipofectamine2000 (Life Technologies). In all experiments, empty vector was added to adjust for equal DNA amounts. Two days posttransfection, cells were lysed with 5x Cell Culture Lysis reagent (Promega) and luciferase activity was quantified using Promega Luciferase Assay Kit (Promega) on a Berthold microplate reader.

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