Supporting Information

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SI Materials and Methods

Cells Lines. HEK293T, HeLa, TE671, QT35, and HT1080 were maintained in DMEM supplemented with 10% FBS, 100 U/mL penicillin, 100 μg/mL streptomycin at 37 °C in a humid incubator. 293F cells (Invitrogen) were grown in serum-free Freestyle medium (Invitrogen) in an orbital shaker at 50 rpm at 37 °C. Where appropriate, cells were selected in 1 mg/mL G418 (Invitrogen) or 2 μg/mL puromycin (Sigma-Aldrich).

Virus Production. Adenovirus Ad5-GFP (1) was grown in transcomplementation cell line 293F for 72 h, before three rounds of freeze–thaw to release virus particles and filtration at 0.45 μm. Virus stock was purified by two rounds of ultracentrifugation banding on a caesium chloride gradient, dialyzed into PBS/10% glycerol, and frozen at −80 °C until required. Titers of purified virus were typically 10^8 to 10^9 IU/mL

Generation of Stable Knockdown and Overexpressing Cell Lines. Human tripartite motif-containing 21 (TRIM21) DNA was cloned into pDONAI (Takara) as a NotI/SalI restriction fragment to generate pDON-T21. DNA encoding an shRNA directed to human TRIM21 sequence GCAGCACGCTTGACAATGA was cloned into pSIREN Retro-Q (Clontech) to produce pSIREN-shT21. Control shRNA directed to luciferase was encoded by pSIREN-shLuc. Retroviral transduction particles were produced by transfection of 4×10^6 HEK293T cells with 5 μg of pDON-T21, pSIREN-shT21, empty pDONAI, or pSIREN-Luc along with 5 μg each of MLV gag-pol expression plasmid pCMVi and VSV-G expression plasmid pMDG (2). Supernatant was harvested after 72 h and filtered at 0.45 μm and used to transduce HeLa cells. Stably transduced cells were selected with G418 (pDON-T21, pDONAI) or puromycin (pSIREN-shT21, pSIREN-shLuc). Levels of TRIM21 protein were monitored by Western blotting (sc-25351; Santa Cruz Biotechnology).

Transient siRNA Knockdown. Cells were plated at 1×10^5 cells per well in six-well plates and allowed to adhere overnight. siRNA oligonucleotides T21siRNA1 (UCAUUGUCAAGCGUGCUGC; Dharmacon) and T21siRNA2 (UGGCAUGGAGGCACCUGAAGGUGG; Invitrogen) (150 pmol each) or 300 pmol control oligo (Invitrogen) were transfected into cells using Oligofectamine (Invitrogen). Cells were washed after 3 h and incubated for 72 h before infection. Where indicated, 1,000 U IFN-α (PBL InterferonSource) was added 48 h after knockdown.

Virus Neutralization Assays. HeLa cells were seeded at 1×10^5 cells per well in 2 mL complete DMEM in six-well plates the day before infection. Where stated, cells were incubated with 1,000 U IFN-α. AdV5-GFP [5×10^4 infectious units (IU)] was incubated with antibody in a 10-μL volume for 30 min at room temperature before addition to cells. Cells were incubated for 48 h before washing, trypsinization, and fixing in 4% paraformaldehyde. GFP-positive cells were enumerated by flow cytometry (FACSCalibur; BD Biosciences).

Goat antiadenovirus polyclonal antibody was used in all virus neutralization assay experiments unless otherwise stated (AB1056; Millipore). Other antibodies tested included pooled human serum IgG and IgM (090707 and 090713; Athens Research and Technology), antihexon polyclonal IgG (0151-9004; Abd Serotec), and purified antiadenovirus 5 hexon mouse monoclonal IgG (hybridoma TC31-9C12.C9 obtained from the Developmental Studies Hybridoma Bank, University of Iowa).

Immunofluorescence. HeLa cells (2.5×10^4) were seeded onto coverslips in 24-well plates and allowed to adhere overnight. Cells were washed twice in DMEM before infection. AdV5-GFP (5×10^4 IU) was incubated with polyclonal or monoclonal antihexon adenovirus antibody (e.g., 500 ng of mouse monoclonal IgG in a 20-μL volume for 30 min at room temperature before addition of 230 μL DMEM). Cells were infected with 250 μL of this mixture for 30 min at 37 °C. Cells were washed three times with PBS, fixed with 4% paraformaldehyde, permeabilized with 0.5% Triton X-100 in PBS, and blocked with PBS–BSA (5% BSA, 0.1% Tween in PBS) for 1 h. Immunostaining for TRIM21 was performed with a rabbit 50-kDa Ro/SSA primary antibody 20960 (Santa Cruz Biotechnology) and for ubiquitin with a goat primary 6085 (Santa Cruz Biotechnology) at 1:200 dilution in PBS–BSA. Alexa Fluor-conjugated secondary antibodies (Invitrogen) were used to detect primary antibodies at 1:200 dilution. Streptavidin-coated 0.25-μm latex beads (Sigma-Aldrich) were incubated with rabbit antistreptavidin polyclonal serum S6390 (Sigma-Aldrich) overnight at 4 °C. Beads were washed three times with PBS and transfected into cells using Oligofectamine. Cells were washed with PBS 3 h after transfection and fixed as above. Immunostaining for TRIM21 was performed with immune serum raised in mouse against recombinant TRIM21 RING, B Box and coiled-coil domains (RBCC) and for conjugated ubiquitin as above, both at 1:200 dilution in PBS–BSA. Alexa Fluor-conjugated secondary antibodies (Invitrogen) were used to detect primary antibodies at 1:500 dilution. Confocal images were taken using a Zeiss 63× lens on a Jena LSM 710 microscope (Carl Zeiss MicroImaging).

Fate-of-Capsid Assay. HeLa cells were plated at 2×10^5 cells per well in a six-well plate in 2 mL DMEM and left overnight to attach. A proportion of the wells were treated with 8 μM MG132 (Boston Biochem) for 4 h. Untreated cells were exposed to an equivalent quantity of DMSO for the duration of the treatment. Ad5-GFP (4×10^7 IU) was mixed with 6 μg Ad5 monoclonal antibody and incubated at room temperature for 30 min, then added onto the cells in 1 mL complete media. Infections were incubated at 37 °C for 1 h before infection mixtures were removed and replaced with DMEM. Cells were harvested at indicated time points after initial infection and boiled in 100 μL sample buffer with reducing agent (Invitrogen). Virus was detected with goat antihexon Ad5 (1:1,000, AB1056; Millipore) and HRP-conjugated anti-goat IgG (1:5,000, sc-2056; Santa Cruz Biotechnology). Antibody was detected with donkey anti-mouse IgG (1:500, AP192; Millipore) and protein A-HRP (1:2,000, 610438; BD Biosciences). TRIM21 was detected with TRIM21 RBCC immune sera (1:2,000) and protein A HRP to avoid cross-reaction to the mouse antibody on the gel.

Immunoblotting. Cells from a single well of a six-well plate were scraped off, resuspended, and heated at 98 °C for 5 min in 100 μL 1× LDS sample buffer with reducing agent (Invitrogen). Equal volumes were loaded onto a 4–12% NuPAGE gel and electrophoresed in 1× Mops buffer (Invitrogen). Proteins were transferred onto Protran nitrocellulose membrane (Whatman) and immunoblotted with the indicated antibodies. In all cases blots were incubated with antibody in PBS containing 5% milk and 0.1% Tween and washed with PBS–Tween. Visualization was carried out using a ECL Plus Western Blotting Detection System (GE Healthcare). Western blots were stripped for reprobing as per manufacturers instructions with 1× Re-Blot Plus Strong So-

lution (2504; Millipore). Loading control blots were carried out with rabbit polyclonal β-actin (1:1,000, #4967; Cell Signaling).

Fluorescence Titration. Full-length and ΔRING-Box recombinant TRIM21 was expressed as maltose binding protein (MBP)-fusion proteins in *Escherichia coli* and purified using amylose resin and size-exclusion chromatography (SEC). The MBP tag was removed via tev protease cleavage, and cleaved TRIM21 was dialyzed into 20 mM Tris (pH 8), 100 mM NaCl, and 1 mM DTT. Steady-state fluorescence titration experiments were performed at 20 °C using a Cary Eclipse fluorescence spectrophotometer (Varian) with excitation at 296 nm and emission at 335 nm, using 15-nm slit widths and a PMT voltage of 850. The quenching in intrinsic TRIM21 tryptophan fluorescence upon titration of IgG was measured with an averaging time of 5 s. Each titration was fit using Kaleidagraph (Synergy Software) to the quadratic expression $F = F_{TR} + f'((-(I_0 - TR_0 + K_d) \pm (((I_0 - TR_0 + K_d)^2) + (4K_dTR_0))^{1/2}))/2$; where F is the observed fluorescence, F_{TR} is the molar TRIM21 fluorescence, f' is the molar change in fluorescence, (TR_0) is the total TRIM21 concentration, (I_0) is the total antibody concentration, and K_d is the dissociation constant.

Fluorescence Anisotropy. The PRYSPRY domain of TRIM21 was expressed and purified as previously described (3, 4). The protein was labeled with Alexa Fluor 488 5-SDP ester (Invitrogen) and dialyzed into 50 mM Tris (pH 8) with 200 mM NaCl. Anisotropy experiments were performed using a Cary Eclipse fluorescence spectrophotometer (Varian) with excitation at 488 nm and emission at 530 nm, using 10-nm slit widths and a photomultiplier voltage of 600. IgM (Athens Research and Technology) was titrated into 50 nM PRYSPRY and the polarized fluorescence averaged over 5 s. The dissociation constant (K_d) was determined by fitting the change in anisotropy to the quadratic expression given above using Kaleidagraph (Synergy Software).

SEC MALS. SEC multiangle light scattering (MALS) was performed using a Wyatt Heleos II 18-angle light-scattering instrument coupled to a Wyatt Optilab rEX online refractive index detector. Samples were prepared as described above and resolved on a Superdex S-200 analytical gel filtration column running at 0.5 mL/min before passing through the light-scattering and refractive index detectors in a standard SEC MALS format. Protein concentration was determined from the refractive index based on 0.186 ΔRI for 1 mg/mL and combined with the observed scattered intensity to calculate absolute molecular mass using Wyatt's ASTRA analysis software. The major species in TRIM21 has a mass of 107 kDa averaged across the indicated region of the peak. The predicted mass of monomeric TRIM21 is 54 kDa, making TRIM21 a dimer in solution and not a trimer as previously reported. SEC MALS of IgG gives the expected mass of 154 kDa with small (<10%) levels of dimer mass 325 kDa, which is typical for IgG. TRIM21–IgG complex resolves as multiple peaks, corresponding to excess IgG with mass and elution volume as previously and a peak with mass ≈280 kDa. The 280-kDa peak is consistent with a 1:1 complex of TRIM21:IgG, in which each protein is a homodimer.

Complementation Neutralization Assay Using Exogenous TRIM21. HeLa cells were seeded at 1×10^5 cells per well in 2 mL complete DMEM in six-well plates the day before infection. AdV5-GFP (5×10^4 IU) was incubated with 4 μg goat antiadenovirus polyclonal antibody (AB1056; Millipore) for 15 min before addition of 200 μg of appropriate recombinant TRIM21 protein, 100 μL total volume, and incubation for a further 15 min at room temperature. Media on the cells were exchanged for this mixture made up to 1 mL with complete DMEM. Cells were incubated at 37 °C in a humid incubator for 48 h and then treated as in a virus neutralization assay (see above).

In Vitro Ubiquitination Assays. In vitro assays were carried out largely as previously described (5). Reactions were carried out in 1× ubiquitination buffer [50 mM Tris-HCl (pH 7.4), 2.5 mM $MgCl_2$, and 0.5 mM DTT] with the addition of 2 mM ATP, 300 ng His-Uba1, 300 ng His-UbcH5c, anbd 1 μg ubiquitin (Sigma) and 50 ng MBP-TRIM21 or MBP-TRIM21 ΔRing-Box as indicated. Human Uba1 and UbcH5c were expressed in bacteria and purified using Ni-NTA resin (Qiagen) as previously described (5). Antibody adenovirus mixtures were made by incubating 5×10^4 IU AdV5-GFP per 150 ng goat polyclonal antihexon (Millipore) for 30 min, whereby 1 μL mix contains 3.6×10^4 IU and 106 ng antibody. Increasing amounts were added into the reaction mixture as indicated. Controls with either just Ad5 or antihexon antibody contained 1.25×10^5 IU and 150 ng antibody, respectively. Reaction mixtures were incubated at 37 °C for 1 h then stopped by addition of LDS sample buffer and heating to 98 °C for 5 min. Samples were run on a gel and Western blotted for TRIM21 (1:500, sc-25351; Santa Cruz Biotechnology), Ad5 hexon (donkey anti-goat IgG HRP 1:5,000, sc-2056; Santa Cruz Biotechnology), or ubiquitin (1:1,000, FK-2; Enzo Life Sciences) as indicated.

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Fig. S1. TRIM21 neutralizes infection independent of cell type and antibody. (*A*) Neutralization of virus by TRIM21 is reversed by knockdown, independent of siRNA sequence or siRNA vs. shRNA, in the presence of antibody. (*B*) TRIM21 neutralizes adenovirus infection in different cell lines. Neutralization is enhanced by IFN-α and reversed by TRIM21 depletion. (*C*) TRIM21 neutralizes adenovirus infection when using different polyclonals or an antihexon monoclonal IgG (Poly#1 is Millipore AB1056; Poly#2 is Abd Serotec 0151-9004; Mono is hybridoma TC31-9C12.C9).