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Methods

Cell culture and affinity purification. HEK293 cells (ATCC# CRL-1573) were maintained in DMEM high glucose/10% FCS plus Pen/Strep. For AP, $2.5x10^6$ cells were seeded in 15 cm plates and the next day transfected with 3-10 µg plasmid using calcium phosphate. 42 h after transfection, cells were detached and washed with PBS. Jurkat TRex cells (Invitrogen) were cultured in RPMI/10%FBS plus Pen/Strep and 10 µg/ml Blasticidin. Stable Jurkat cell clones were generated by transfection with the linearized vector, selection with 300 µg/ml Zeocin followed by limiting dilution. For AP, $2.5x10^8$ cells were induced with 1 µg/ml doxycyclin for 16 h. In case of the Vif-SF and Vpr-SF clones, 0.5 µM MG132 (Calbiochem) was added 12 h before harvest. Cells were lysed in 1 ml cold lysis buffer (50 mM Tris pH 7.5, 150 mM NaCl, 1 mM EDTA, 0.5% Nonidet P40, complete protease inhibitor (Roche) and phosphostop (Roche)), cells were dounced 20x on ice, and spun at 2800xg for 20 min. The supernatant was incubated with 60 µl preclearing beads (mouse IgG agarose, Sigma or Sepharose 4FF) for 2 h. The precleared lysate was incubated with 30 µl IP beads over night. Flag APs were performed with anti-Flag M2 Affinity Gel (Sigma) and Strep APs with Strep-Tactin Sepharose (IBA). The beads were washed 5x with lysis buffer containing 0.05% Nonidet P40 followed by one wash with lysis buffer without detergent. Proteins were eluted with 40 µl 50 mM Tris pH 7.5, 150 mM NaCl, 1 mM EDTA containing either 100 µg/ml 3xFlag peptide (ELIM) and 0.05% RapiGest (Waters), or 2.5 mM Desthiobiotin (IBA). 4 µl of the eluate was analyzed by 4-20% SDS PAGE (Biorad) and silver staining.

Sample preparation for mass spectrometry. For gel-free MS analysis 10 µl of the IP eluate were reduced with 2.5 mM DTT at 60°C for 30 minutes followed by alkylation with 2.5 mM iodoacetamide for 40 minutes at room temperature. 100 ng sequencing grade modified trypsin (Promega) was then added to the sample and incubated overnight at 37°C. The resulting peptides were concentrated on ZipTip C18 pipette tips (Millipore) and eluted in a final 20 µl solution of 0.1% formic acid. For gel-based analysis, 20 µl IP eluate was separated by 4-20% SDS-PAGE and stained with GelCode Blue (Thermo Scientific). Each lane was cut into 15 pieces. Each gel piece was diced into small (1 mm^2) pieces and washed 3x with 25 mM NH_4HCO_3/50% ACN. Gel pieces were dehydrated and incubated with 10 mM DTT in 25 mM NH_4HCO_3 for 1 hour at 56°C. The supernatant was removed and the gel pieces were incubated with 55 mM iodoacetamide for 40 minutes. Gel pieces were washed with 25 mM NH_4HCO_3, then 25 mM NH_4HCO_3/50% ACN and were then dehydrated. 10 ng/µl trypsin in 25 mM NH_4HCO_3 was then added to the gel pieces and incubated overnight at 37°C. Finally, peptides were extracted from the gel pieces with 50% ACN/5% formic acid and the solvent evaporated. The final peptide sample was resuspended in 20 µl 0.1% formic acid.

Mass spectrometry. All samples were analyzed on a Thermo Scientific LTQ Orbitrap XL mass spectrometer equipped with a nanoACQUITY UPLC (Waters) chromatography system and a nanoelectrospray source. 5 µl of each sample was injected onto a nanoACQUITY Symmetry C18 trap (5 µm particle size, 180 µm x 20 mm) in buffer A (0.1% formic acid in water) at a flow rate of 4 µl/min and then separated over a nanoACQUITY BEH C18 analytical column (1.7 µm particle size, 100 µm x 100 mm) over one hour with a gradient from 2% to 25% buffer B (99.9% ACN/0.1% formic acid) at a flow rate of 0.4 µl/min. The mass spectrometer continuously collected data in a data-dependent manner, collecting a survey scan in the Orbitrap mass analyzer

at 40,000 resolution with an automatic gain control (AGC) target of 1×10^6 followed by collision-induced dissociation (CID) MS/MS scans of the 10 most abundant ions in the survey scan in the ion trap with an AGC target of 5,000, a signal threshold of 1,000, a 2.0 Da isolation width, and 30 ms activation time at 35% normalized collision energy. Charge state screening was employed to reject unassigned or 1+ charge states. Dynamic exclusion was enabled to ignore masses for 30 s that had been previously selected for fragmentation. Raw mass spectrometric data were converted into peaklists using Bioworks 3.3.1 SP1. The spectra were searched using Prospector v.5.3 (http://prospector.ucsf.edu)[29] against a human-restricted UniProt database (downloaded October 2009) supplemented with HIV protein sequences from 40 strains. Trypsin was specified as the enzyme; one missed cleavage and zero non-specific cleavages at the peptide termini were permitted. Mass accuracy was set to 25 ppm for precursor ions and 0.8 Da for fragment ions. Carbamidomethylation of Cys residues was set as fixed modification, and acetylation of protein N-termini and Met oxidation as variable modifications. Protein Prospector results were filtered by applying a minimum Protein Score of 22.0, a minimum Peptide Score of 15.0, a maximum Protein E-Value of 0.01 and a maximum Peptide E-Value of 0.05.

Cloning of genes for Co-IP confirmation. Coding regions of human cDNA sequences were PCR amplified from HEK293 cDNA and cloned into pcDNA4/TO (Invitrogen) carrying N- or C-terminal 3xFlag tag sequences. Identities of ORFs were confirmed by sequencing.

Co-immunoprecipitation of putative Vif interactors. HEK293T cells were co-transfected with plasmids encoding a human 3xFlag-tagged protein, and EGFP, Nef or Vif fused to a C-terminal 2xStrep-tagII. ~42 h post transfection, cells were lysed in 50 mM Tris pH 7.5, 150 mM NaCl,

1 mM EDTA, 0.5% Nonidet P40, protease inhibitor (Roche). The lysate was clarified by centrifugation (5200xg, 5 min, 4ºC) and incubated with Strep-Tactin sepharose (IBA) for 2 h at 4ºC. After 5 washes in buffer (50 mM Tris pH 7.5, 150 mM NaCl, 1 mM EDTA, 0.1% Nonidet P40), beads were eluted by boiling in SDS sample buffer. Cell lysates and eluates were subjected to SDS-PAGE and transferred to nitrocellulose membranes. Membranes were blocked with 5% milk/TBST and incubated with mouse anti-Flag antibody (SIGMA), and Strep-TagII antibody HRP conjugate (Novagen). Immunoreactive bands were detected by chemiluminescence (ECL kit; Amersham).

Reconstitution of Vif E3 ligase complex. The Vif substrate adaptor or CUL5/RBX2 heterodimer were coexpressed in *E. coli* using Duet vectors harboring HXB2 Vif, HIS_6-tagged CBFβ and ELOBC or His-GB1-CUL5/RBX2. Affinity tags were removed by TEV protease after immobilized metal affinity chromatography and subcomplexes were fractionated by size-exclusion chromatography (SEC). The monodisperse fractions of the Vif substrate adaptor and CUL5/RBX2 were mixed and subjected to a final round of SEC to obtain copurified hexamer. Recombinant A3A- and A3G-myc-His_6 were purified from HEK293T cells as described[22,30]. Ubiquitination assays were performed at room temperature with the ubiquitin activating system containing: 2 mM ATP, human ubiquitin activating enzyme (UBE1) (200 nM), wild-type, methyl-ubiquitin or mutant ubiquitin (75 μM), 4 μM E2 (UBE2R1 or UBCH5b) in addition to 0.625 μM Vif E3 and 200 nM APOBEC3 proteins in buffer containing 30 mM Tris-Cl (pH 7.3), 100 mM NaCl, 5 mM $MgCl_2$, in a total reaction volume of 10 μl. For reactions with UBE2R1, the Vif E3 was NEDD8ylated in conditions that included: 50 mM NaCl, 50 mM Tris-Cl pH 7.6, 2.5 mM $MgCl_2$, 2 mg/ml BSA, 2 mM ATP, 100 nM NEDD8 activating enzyme (NAE), 2 μM

UBE2F, 30 μM NEDD8 and 4 μM Vif E3 ligase. After 1 hr, the NEDD8 reaction mixture was diluted ~6 fold upon the addition of the ubiquitin activating system and substrate, with final concentration and buffer conditions identical to the assays done with UBCH5b. The ubiquitination reactions were quenched after 1 hr by the addition of 2x SDS loading dye. UBE1, NEDD8, Ub, Me-Ub, K48R-Ub, and K48-only Ub were purchased from Boston Biochem. GST-NAE (Courtesy of Dr. Brenda Schulman), HIS_6-UBCH5b, HIS_6-UBE2F and HIS_6-UBE2R1 were expressed in *E. coli*, purified by IMAC or GST affinity chromatography. HIS_6 tags were removed by TEV protease. All proteins were subjected to size exclusion chromatography for the final purification. Ubiquitinated Vif or A3 proteins were detected using a polyclonal anti-Vif antibody (#2221 courtesy of Dr. Dana Gabuzda and the NIH AIDS Research and Reference Reagent Program) or monoclonal anti-c-Myc (Sigma) antibodies.

***In vitro* pulldowns.** A3A- and A3G-myc-His_6 were purified from HEK293T cells as described[22,30]. 5 μl Talon Metal Affinity Resin (Clontech) was washed with 20mM HEPES, pH7.4, 0.5 M NaCl, 10% Glycerol and 0.1% Triton X-100, and then blocked with 1 ml of 20mM HEPES, pH7.4, 0.5 M NaCl, 10% Glycerol, 0.1% Triton X-100 and 10% BSA for 2 hrs. The blocked resin was then incubated with 20 pmol of A3G-MycHis or A3A-MycHis protein in 1ml of 20mM HEPES, pH7.4, 0.8 M NaCl, 10% Glycerol, 0.1% Triton X-100 and 5% BSA for 2 hrs with no APOBEC protein as control. These resins were incubated with 10 pmol Vif substrate adaptor complex in 20mM HEPES, pH7.4, 0.5 M NaCl, 10% Glycerol, 0.1% Triton X-100, 10% BSA and 20mM imidazole for 2 hrs. The bound proteins were washed with 1 ml of 20mM HEPES, pH7.4, 0.5 M NaCl, 10% Glycerol, 0.5% Triton X-100, 10% BSA and 20mM imidazole, boiled in 30 μl 1×SDS-PAGE loading buffer, and subjected to SDS-PAGE and

immunoblotting with appropriate primary (anti-Vif, anti-CBFβ, anti-myc (A3A or A3G)) and secondary (goat anti-rabbit IgG-HRP or goat anti-mouse IgG-HRP (Bio-Rad)) antibodies.

Single-cycle HIV-GFP infectivity studies. A non-epitope-tagged CBFβ expression plasmid was constructed by amplifying CBFβ (NM_001755.2) from CEM cDNA using primers NNN NGA ATT CAC CAT GCC GCG CGT CGT GCC CGA CCA and NNN NTC TAG ACT AGG GTC TTG TTG TCT TCT, cleaving with EcoRI/XbaI enzymes, and inserting into pcDNA3.1 (Invitrogen) at the compatible sites. The CBFβ shRNA expression construct and non-silencing control construct (Open Biosystems, catalog number RHS4430-99161432 and RHS4346) were obtained through the Biomedical Genomics Center, University of Minnesota. Knockdown of CBFβ was performed by lentiviral transduction, followed by limiting dilution onto 96-well plates and selection with puromycin at concentration of 0.75 μg/ml. Non-epitope-tagged A3G and HIV-1_{IIIB} Vif expression plasmids were described[31,32]. GFP encoding HIV-1 particles were produced by transiently transfecting HEK293T cells at 50% confluency using 0.7 μg HIV-GFP cocktail [0.3 μg of pCS-CG (LTR flanked GFP), 0.2 μg of pRK5/Pack1(Gag-Pol), 0.1 μg pRK5/Rev, 0.1 μg of pMDG (VSV-G Env)], 140 ng of A3G or empty vector, and 35 ng of codon-optimized Vif or empty vector. 200 ng of CBFβ or empty vector was used for genetic complementation. All reactions were performed in triplicate. After 48 hours, virus-containing supernatants were harvested through PVDF filter with 0.22 μm pores (Millipore) to remove any remaining producer cells. Infectivity of each supernatant was measured by challenging fresh HEK293T cells, incubating 48 hours, and quantifying GFP-positive cells by flow cytometry (FACSCalibur, BD). Sample preparation and immunoblotting for A3G, p24 (capsid), Vif, and

tubulin were done as described[33]. CBFβ was detected with an anti-CBFβ mouse monoclonal antibody (Santa Cruz, sc-56751).

Replication-competent HIV-1 infectivity studies. A stable HEK293T-shCBFβ clone (as described above) was used for all the replication-competent HIV-1 infectivity studies. For Fig. 3b, cells were transfected in triplicate with 1 μg HIV-1_{IIIB} molecular clone in the presence or absence of 100 ng A3G expression plasmid and 100 ng of CBFβ expression plasmid (both untagged). For Fig. 3d, cells were transfected in triplicate with 1 ug Vif-deficient HIV-1 molecular clone in the presence or absence of 100 ng human or rhesus A3G-HA expression plasmid, 25 ng HIV or SIV Vif-Myc, and 25 ng of CBFβ expression plasmid. After 48 hrs, virus-containing supernatants were harvested through PVDF filters with 0.45 μm pores (Millipore) to remove any remaining producer cells. Infectivity of each supernatant was measured by challenging CEM-GFP reporter cells, incubating 48 hours, and quantifying GFP-positive cells by flow cytometry (FACSCalibur, BD) [33,34]. The remaining supernatant was used to collect viral particles. Sample preparation and immunoblotting for A3G, p24 (capsid), Vif, and tubulin were done as described[33].

Methods References

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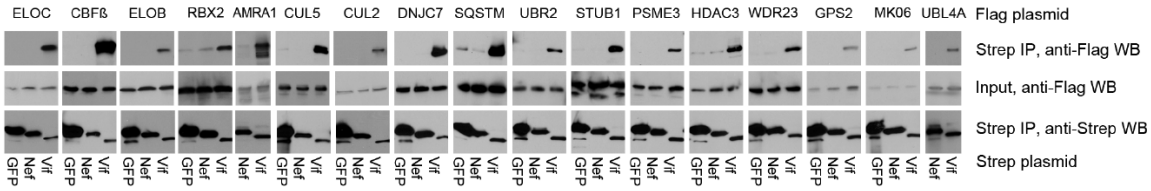

Fig. S1. Co-immunoprecipitation confirmation of Vif interactors. Seventeen Flag-tagged human proteins were co-expressed with Strep-TagII-tagged HIV Nef, Vif or GFP in HEK293T cells, and a Strep-Tactin pulldown was performed. Eluates were analyzed by SDS-PAGE and anti-Flag (upper blots), as well as anti-Strep-TagII (lower blots) western blotting. Cell lysates were probed against Flag (middle blots).

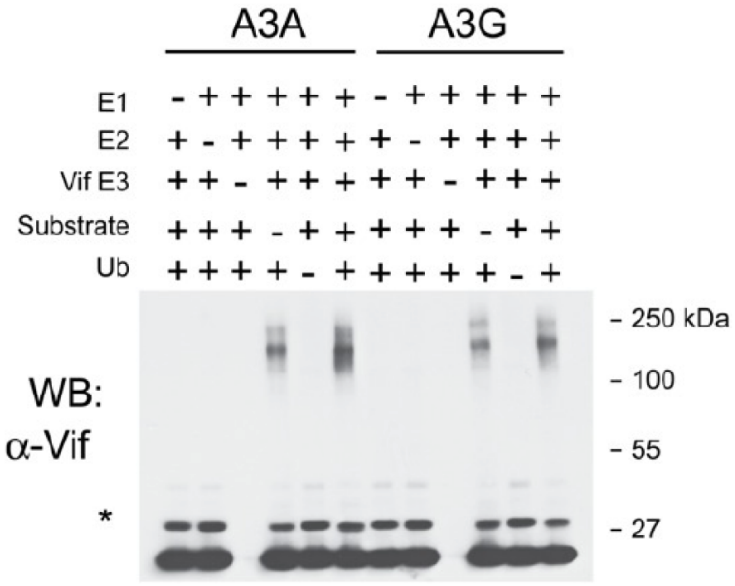

Fig. S2. The presence of A3 proteins does not significantly affect autoubiquitination activity of CRL5-Vif-CBFβ. Reactions products of experiment in Fig. 2d were probed with a polyclonal anti-Vif antibody. In addition to unmodified Vif and poly-Ub Vif, a small fraction of Nedd8ylated Vif was detected (indicated by the asterisk) under our assay conditions (documented further in Fig. S3).

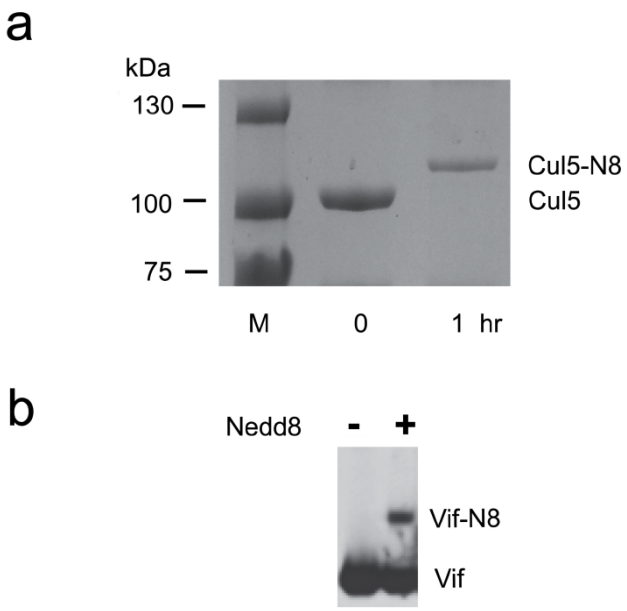

Fig. S3. Nedd8ylation of the ubiquitin ligase complex by the NEDD8 conjugating enzyme UBE2F. **a**, Coomassie stained SDS-PAGE showing a NEDD8 "pulse" reaction at time 0 and 1 hr after addition of ATP, NEDD8, NEDD8 E1, and UBE2F indicate complete modification of CUL5. See methods for reaction conditions. M designates molecular weight standards. **b**, Immunoblot of a "pulse" reaction with and without NEDD8 sampled after 1 hour of initiation, indicating a small (<5%) fraction of NEDD8ylated Vif, quantified by a fluorescent secondary antibody. UBE2F was chosen because it was previously shown to promote NEDD8ylation of CUL5/RBX2 but not complexes between RBX1 and CUL1-4 *in vivo*[17]. Consistent with this observation and our proteomic data, knockdown of UBE2F blocks HIV infectivity by reducing CUL5 NEDD8ylation in HIV producing cells, abrogating Vif mediated ubiquitination and degradation of A3G (D.S., J.D.G. *et al*., in preparation).

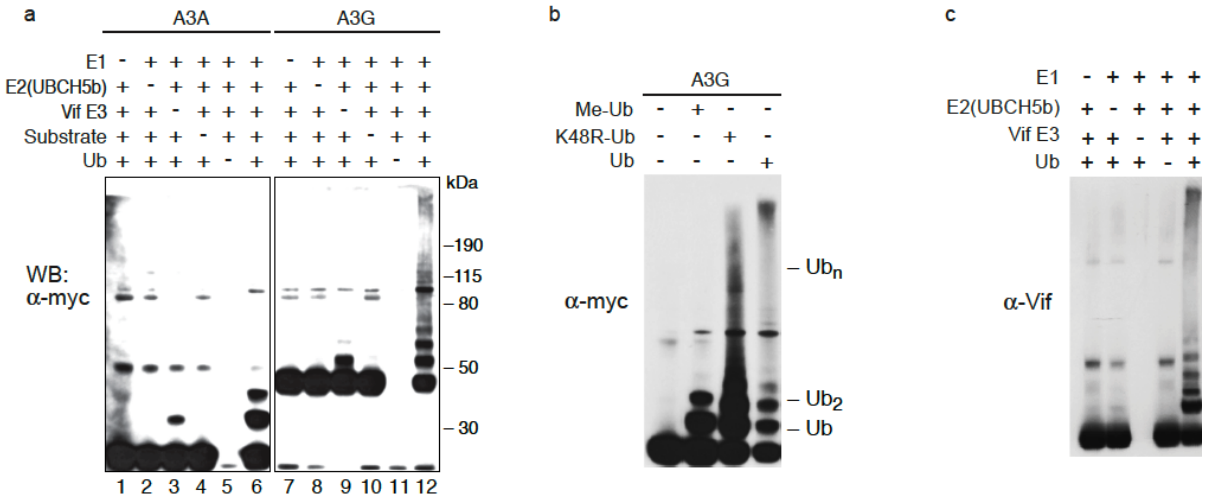

Fig. S4. CRL5-Vif-CBFβ catalyzes polyubiquitin chain formation on A3G in presence of ubiquitin E2 UBCH5b. **a**, Immunoblots of substrate subject to ubiquitination in presence of ATP, E1 (UBE1), UBCH5b and CRL5-Vif-CBFβ. **b**, Immunoblots of A3G in reactions lacking Ub, containing Me-Ub, K48R Ub or wildtype Ub. As with UBE2R1 (Fig. 2e), two sites are modified on A3G. However, the chain is not K48-linked, consistent with previous reports that UBCH5b generates heterogeneous chain linkages[20]. Me-Ub experiments on A3A show a single modified site (data not shown); the di-ubiquitin chain observed in panel (a) is unlikely to trigger proteasomal degradation because tetra-ubiquitin chains are the minimal chain length required for efficient recognition by the proteasome[12]. **c,** Immunoblot showing CRL5-Vif-CBFβ autoubiquitination activity in presence of UBCH5b as E2.

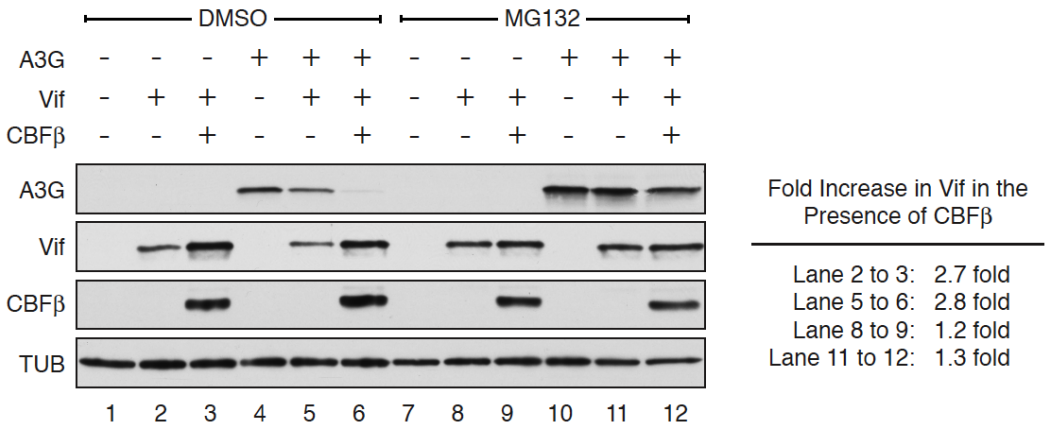

Fig. S5. CBFβ is essential for Vif expression and/or stability. A HEK293T-shCBFβ clone was transfected with plasmids expressing 50ng A3G, 50ng CBFβ, and either 1μg Vif^{+} or Vif^{-} HIV-1_{IIIB} molecular clone, as indicated. 24 hrs post-transfection, cells were treated with either 2.5uM proteasome inhibitor MG132 or with a volumetric equivalent of DMSO. 16 hrs later, cells were processed for immunoblotting. The percent increase in Vif steady-state levels was determined as the amount of Vif (relative to tubulin) in the $CBFβ^{+}$ lane over the amount of Vif (relative to tubulin) in the $CBFβ^{-}$ lane. Quantification was done using Image J.

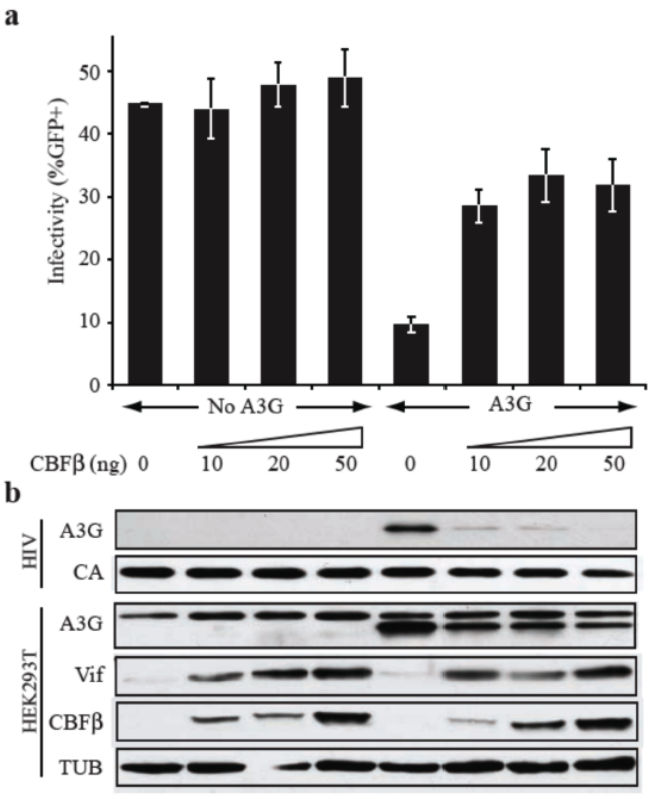

Fig. S6. CBFβ titration experiment. a, The infectivity of replication-competent Vif^{+} HIV-1_{IIIB} produced using a HEK293T-shCBFβ clone in the presence of 100 ng control plasmid or 100 ng untagged A3G expression plasmid and the indicated amount of untagged CBFβ expression plasmid. Immunoblots were performed as described in the methods.

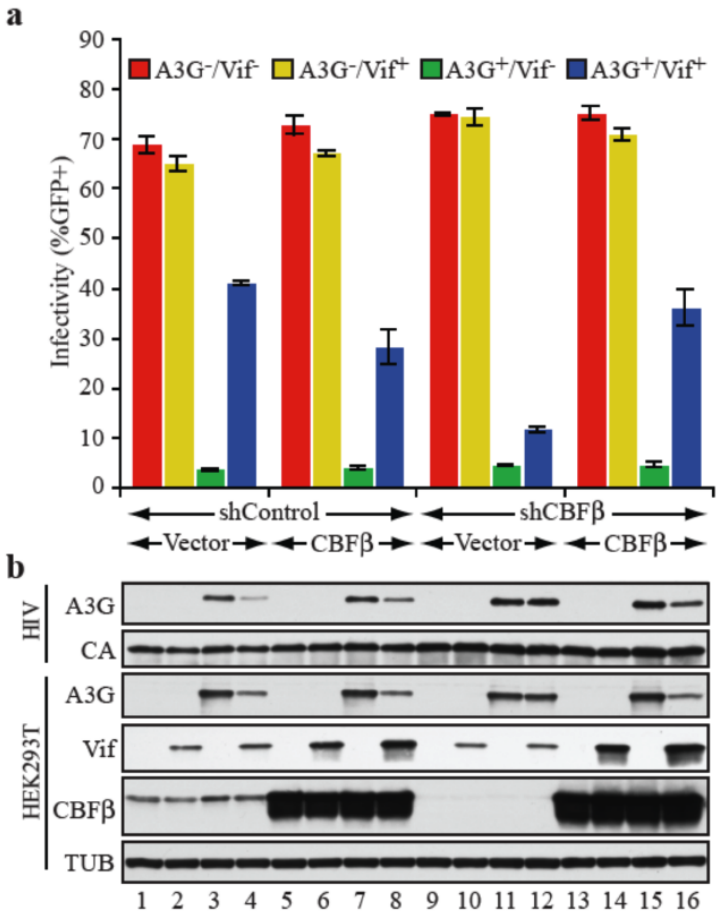

Fig. S7. CBFβ and Vif collaborate to degrade APOBEC3G and enable HIV-1 infectivity. a, Infectivity of Vif-deficient HIV-GFP produced in clonal HEK293T stably transduced with control shRNA or CBFβ-specific shRNA constructs and transiently expressing A3G, Vif, and/or CBFβ as shown. This experiment is an independent replicate of the one shown in Fig. 4C. **b**, Immunoblots of A3G and CA in viral particles (top 2 panels) and A3G, Vif, CBFβ, and tubulin in the producer cell lysates (bottom 4 panels).

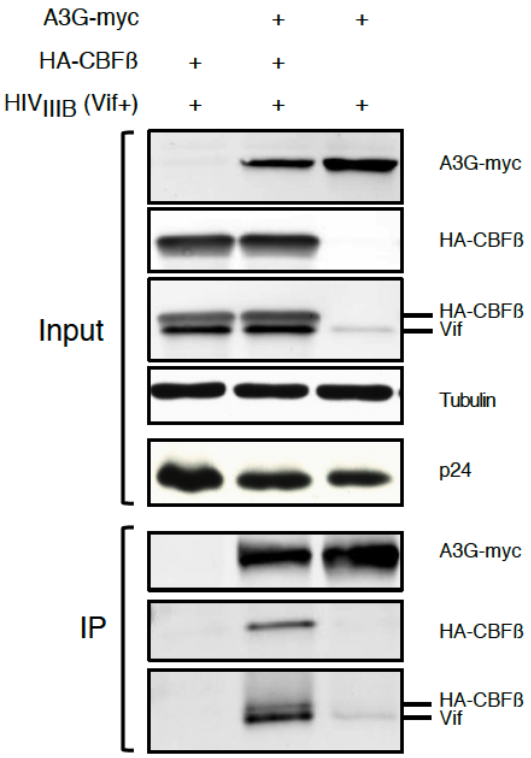

Fig. S8. Co-immunoprecipitation of CBFβ and Vif in HIV-1 producing cells. An HA-tagged CBFβ expression construct was co-transfected with infectious Vif^{+} HIV-1$_{IIIB}$ and A3G-myc expression plasmids using TransIT-LT1 (Mirus) into HEK293T-shCBFβ knockdown cells. 24 hrs post-transfection, 2.5 μM MG132 was added to inhibit the proteosome and 16 hrs later lysates were prepared [25mM HEPES pH7.4, 150mM NaCl, 1mM EDTA, 1mM $MgCl_2$, 1mM $ZnCl_2$, 0.1% Triton-X100, 10% glycerol, and complete EDTA free protease inhibitor (Roche)], treated with DNase I and RNase A to break apart potential nucleic acid-mediated interactions, and used for anti-myc (A3G) immunoprecipitation [anti-c-myc 9E11 (Thermo Scientific)]. Input and co-IP samples were fractionated by SDS-PAGE, transferred to Immobilon-FL PVDF (Millipore), probed with a rabbit anti-myc polyclonal antibody (Sigma C3956) to detect A3G-myc, a rabbit anti-HA polyclonal antibody (Sigma H6908) to detect HA-CBFβ, a rabbit anti-Vif polyclonal antibody (NIH AIDS Reagent Program #2221) to detect HIV-1 Vif, a mouse anti-p24 monoclonal antibody (NIH AIDS Reagent Program #3537) to detect HIV-1 capsid, and a mouse anti-α-tubulin monoclonal antibody (Covance MMS-407R) to detect endogenous tubulin. Primary rabbit antibodies were detected with goat anti-rabbit IgG-Alexa Fluor 680 (Molecular Probes - Invitrogen), and primary mouse antibodies were detected with goat anti-mouse IgG HRP conjugate (Bio-Rad 170-6516). Similar results were obtained in analogous experiments with untagged CBFβ in place of HA-tagged CBFβ (data not shown).