

SUPPORTING ONLINE MATERIAL (SOM)

**Induction of Ubiquitination and Degradation of APOBEC3G
by HIV-1 Vif-Cul5-SCF**

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Materials and Methods:

Plasmid Constructions. The parental wild-type HIV-1 (HXB2) and Vif mutant (HXB2 Δ Vif) have been described previously (SI) . The HIV-1 Vif-HA fusion construct derived from HXB2 (HXB2VifHA) was generated by PCR amplification and cloning of a 1.1-kb *EcoRI* fragment into HXB2 using the forward primer 5'-gaattcccttacaatccc-3' and reverse primer 5'-ggaattcctacgcgtaatctgggacgtcgtagggtagtggtccattcattgtgtggct-3' containing *EcoRI* sites on both ends. The reverse primer contains the coding sequence for the HA tag and a stop codon after the last amino acid of Vif. HXB2Vif Δ SLQHA was constructed by changing amino acids 144 to 146 of Vif from SLQ to AAA using HXB2VifHA as template. The parental SIVmac was obtained from the AIDS Research Reagents Program, Division of AIDS, NIAID, NIH (Cat. #: 133). The SIVmac Vif mutant was constructed by digestion of *KpnI* sites (nucleotides 5744 and 5751) in the *vif* orf followed by self-ligation, which caused a 7-base deletion and frameshift in the *vif* orf. The VR1012 vector was generously provided by Vical Inc. (San Diego, CA). The human APOBEC3G-HA was amplified by RT-PCR using mRNA from H9 cells with the forward primer 5'-ctcgagaccatgaagcctcactt-3' and reverse primer 5'-gaattctcacgcgtaatctgggacgtcgtagggtagtttctgattctggag-3' containing *XhoI* and *EcoRI* sites, respectively. The PCR product was cloned into pcDNA3.1(-) to generate pAPOBEC3G-HA. The human *RBX1-Myc* was amplified by RT-PCR using mRNA from H9 cells with the forward primer 5'-tctagaacctggcggcagcgatg-3' and reverse primer 5'-ggatccctaaagatcttctctgatgatgattttgtctgcccatactttg-3' containing *XbaI* and *BamHI* sites,

respectively. The PCR product was cloned into VR1012 using the *Xba*I and *Bam*HI sites to generate pRBX1-Myc. The human Cul5-Myc was amplified by RT-PCR using mRNA from H9 cells with the forward primer 5'-tctagaatggcgacgtctaactgtt-3' and reverse primer 5'-ggatccttaaaggctcttcttct gagatgagttttgttctgccatataat-3' containing *Xba*I and *Bam*HI sites, respectively. The human Cul5-HA was amplified by RT-PCR using mRNA from H9 cells with the forward primer 5'- tctagaatggcgacgtctaactgtt-3' and reverse primer 5'- ggatcctcacgcgtaactctgggacgtcgaagggtatgccatataatgaaagt-3' containing *Xba*I and *Bam*HI sites, respectively. The PCR products of Cul5-Myc and Cul5-HA were cloned into VR1012 to generate pCul5-Myc or pCul5-HA. The Myc-tagged Cul5 mutants Cul5 Δ Nedd8, Cul5 Δ Rbx, and Cul5 Δ N2 were generated from pCul5-cmyc by the QuickChangeTM Site-directed Mutagenesis Kit (Stratagene). The following forward and reverse primers were used to amplify and clone Cul5 mutants into VR1012:

Cul5 Δ Nedd8, forward primer 5'-catacaataatggcaatgagagcggcaattagtaactgctcag-3', and reverse primer 5'-ctgagcattactaattgccgctctcattgccattattgtatg-3'; Cul5 Δ Rbx, forward primer 5'-gaagtagaagaattctacaaaaaatacatttaagaatgaagttggtaa-3' and reverse primer 5'-ttgaccaactcattcttaaatgtattttttgtagaattcttctactc-3'; Cul5 Δ N2, forward primer 5'-gcaaaaattcatcaggctattgttgaatggcgaaag-3' and reverse primer 5'-ctttcgccattcaacaatagcctgatgaattttgc-3'.

Antibodies. The following antibodies were used for this study: anti-HA antibody-agarose conjugate (Roche, Cat. #1815016), anti-Vif rabbit polyclonal (AIDS Research Reagents Program, Division of AIDS, NIAID, NIH. Cat. #2221), anti-Elongin B goat

polyclonal (Santa Cruz, Cat.# sc-1558), anti-Elongin C mouse monoclonal (BD Transduction Lab, SIII/P15, Cat. #610760), anti-Rbx1 rabbit polyclonal (Labvision, anti-Roc1, Cat.# RB-069-P), anti-Myc mouse monoclonal (Sigma, Cat.# M5546), anti-HA mouse monoclonal (Covance, Cat. #MMS-101R-10000), and anti-human ribosomal P antigens (Immunovision, Cat. #HP0-0100) antibodies. To generate anti-Cul5 antibody, the cDNA encoding 138 amino acids of the N-terminus of Cul5 was amplified by PCR with the forward primer 5'- ggatcccatggcgacgtctaactg -3' and reverse primer 5'- gaattccctaaagctttcgaacaatactg -3'. The cDNA was inserted into the expression vector pRSETB, and the sequences were confirmed by DNA sequencing. *Escherichia coli* strain BL21(DE3) (Invitrogen) was used as host to express the Cul5-His fusion protein. The soluble fusion protein was obtained by IPTG (0.5mM) induction at 37°C for 3 h and purified using the ProBond™ purification system (Invitrogen). BALB/c mice were immunized with purified protein to produce polyclonal antibodies according to conventional procedures: 100µg of the protein was injected into multiple subcutaneous sites, and mice were boosted twice at 2-week intervals. Seven days after the last injection, anti-Cul5 serum was obtained, and the titer and specificity were determined by immunoblotting using purified Cul5-His protein.

Cell Culture, Transfection, and Virus Purification. 293T, COS-7, and MAGI-CCR5 cells were maintained in Dulbecco's modified Eagle's medium (DMEM, Invitrogen) with 10% fetal bovine serum and penicillin/streptomycin (D-10 medium) and passaged upon confluence. Jurkat and H9 cells were maintained in RPMI 1640 medium (Invitrogen) supplemented with 10% fetal bovine serum with penicillin/streptomycin (R-10 medium).

DNA transfection was carried out using Lipofectamine 2000 (Invitrogen) as described by the manufacturer. To obtain 293T/APOBEC3G cells, the pABOBEC3G-HA plasmid was transfected into 293T cells and selected with 1mg/ml G418 (Invitrogen) for 2 weeks. Expression of APOBEC3G was detected by immunoblotting using the anti-HA monoclonal antibody. COS-7 cells were transfected with HXB2, HXB2VifHA and HXB2 Δ Vif for 48 h. Supernatants of viruses (normalized by p24 level) were used to infect H9 or Jurkat cells. Input viruses were removed by washing cells twice with the Hank's salt solution (Invitrogen), and infected cells were maintained in fresh R-10 medium. HIV-1 replication was determined by measuring the amount of p24 in the culture supernatant using an HIV-1 p24 ELISA kit (PerkinElmer Life Sciences, Inc.). SIVmac p27 antigen was determined by measuring the amount of p27 in the culture supernatant using an SIV p27 ELISA kit (Coulter Corporation).

MAGI assay. Viral infection was determined by MAGI assay: MAGI-CCR-5 cells were prepared in 6-well plates in D-10 medium 1 day before infection, and cells were at 30-40% confluency on the day of infection. Cells were infected by removing medium from each well and adding dilutions of virus in a total volume of 500 μ l of complete DMEM with 20 μ g/ml of DEAE-dextran. After a 2-h incubation at 37 $^{\circ}$ C in a 5% CO₂ incubator, 2 ml of complete DMEM was added to each well. The cells were incubated for 48 h under the same conditions. Supernatants were removed, and 800 μ l of fixing solution (1% formaldehyde, 0.2% glutaraldehyde in PBS) was added. After a 5-min incubation, cells were washed twice with PBS. The staining solution (20 μ l 0.2M potassium ferrocyanide, 20 μ l 0.2M potassium ferricyanide, 2 μ l 1M MgCl₂, 10 μ l 40 mg/ml X-gal) was added.

Cells were incubated for 2 h at 37° C in a non-CO₂ incubator. Staining was stopped by removing the staining solution and thoroughly washing twice with PBS. Positive blue dots were counted, and viral infectivity was determined after normalizing the amount of virus input by p24 antigen.

Immunoprecipitation. For Vif-HA or Vif Δ SLQ-HA immunoprecipitation, infected H9 cells were lysed in lysis buffer (50 mM Tris, pH 7.5, with 150 mM NaCl, 0.5% Triton X-100, and complete protease inhibitor cocktail tablets), followed by centrifugation at 10,000 \times g for 30 min. Lysates were applied to anti-HA antibody-conjugated agarose beads (Roche) and washed with washing buffer (20 mM Tris, pH 7.5, with 0.1 M NaCl, 0.1 mM EDTA, 0.05% Tween-20). The beads were eluted with elution buffer (0.1 M glycine, pH 2.0), followed by SDS-PAGE and silver staining or immunoblotting. For Cul5-Myc or Vif-Myc immunoprecipitation, transfected 293T cells were harvested, washed twice with cold PBS, and lysed with PBS containing 0.5% Triton X-100 and protease inhibitor cocktail (Roche, Basel, Switzerland) at 4° C for 1 h. Cell lysates were clarified by centrifugation at 10000 x g for 30 min at 4° C. Anti-Myc agarose (Santa Cruz) was mixed with the pre-cleared cell lysates and incubated at 4° C for 3 h. The reaction mixture was then washed three times with cold PBS and eluted with 0.1M glycine-HCl buffer, pH 3.5. The eluted materials were subsequently analyzed by immunoblotting. For Cul5-HA or APOBEC3G-HA immunoprecipitation, transfected 293T cells were harvested and washed twice with cold PBS, lysed with PBS containing 0.5% Triton X-100 and protease inhibitor cocktail (Roche, Basel, Switzerland) at 4° C for

1 h. Cell lysates were clarified by centrifugation at 10000 x *g* for 30 min at 4°C. Anti-HA agarose (Roche) was mixed with the pre-cleared cell lysates and incubated at 4°C for 3 h. The reaction mixture was then washed three times with cold PBS and eluted with 0.1M glycine-HCl buffer, pH 2.0. The eluted materials were subsequently analyzed by immunoblotting.

Identification of Vif-binding proteins. Vif-containing complexes were purified by immunoprecipitation followed by SDS-PAGE. The gel was fixed in a 50% methanol/10% acetic acid mixture for 10 min, stained with mass spectrometry-compatible colloidal-Coomassie brilliant blue G-250 (Bio-Rad 1610406) staining solution (20% methanol, 8% ammonium sulfate, 1.6% phosphoric acid, 0.08% Coomassie blue G-250) to detect protein bands and de-stained with distilled water. Protein standard markers (Bio-Rad, Cat. #1610314) were used for estimating protein size. Protein bands of interest were cut out of the gel and rinsed twice with 50% methanol (HPLC grade). In-gel digestion was performed on protein bands cut out of colloidal-Coomassie blue-stained SDS-polyacrylamide gels using sequencing-grade modified trypsin (Promega, www.promega.com). Extracted peptides were co-crystallized in 2,5-dihydroxybenzoic acid (DHB) or α -cyano-4-hydroxycinnamic acid (CHCA) (10 mg/ml in 50% acetonitrile/0.3% TFA) and analyzed by matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) spectrometry on a Voyager DE STR (Applied Biosystems, home.appliedbiosystems.com) using Voyager Instrument Control Panel (v 5.1) and Data Explorer (v4.0). Data was acquired in reflector mode, and masses were externally calibrated using a standard peptide mixture to better than 50 ppm error. Proteins were

identified by searching the acquired monoisotopic masses against the NCBI non-redundant or SwissProt databases using the MS-Fit search engine of ProteinProspector (prospector.ucsf.edu).

Immunoblot analysis. Cells were collected 48 h after transfection. Cell lysates and viral lysates were prepared as previously described (*SI*). 1×10^5 cells were lysed in 1x loading buffer (0.08 M Tris, pH 6.8, with 2.0% SDS, 10% glycerol, 0.1 M dithiothreitol, and 0.2% bromophenol blue). Samples were boiled for 10 min, and proteins were separated by SDS-PAGE. For virion lysates, cell culture supernatants were collected 72 h after transfection by removal of cellular debris through centrifugation at 3,000 rpm for 10 min in a Sorvall RT 6000B and filtration through a 0.2- μ m pore-size membrane. Virus particles were concentrated by centrifugation through a 30% sucrose cushion at 100,000 x g for 2 h in a Sorvall Ultra80 ultracentrifuge. Proteins were transferred onto two separate nitrocellulose membranes by passive diffusion for 16 h, producing identical mirror-image blots. Membranes were probed with various primary antibodies against proteins of interest. Secondary antibodies were alkaline phosphatase-conjugated anti-human, anti-rabbit, anti-mouse, or anti-goat IgG (Jackson ImmunoResearch, Inc), and staining was carried out with 5-bromo-4-chloro-3-indolyl phosphate (BCIP) and nitro blue tetrazolium (NBT) solutions prepared from chemicals obtained from Sigma.

Pulse-chase experiments. 293T cells were transfected with VR1012Vif -Myc or empty vector VR1012 and human pAPOBEC3G-HA at a 1:1 ratio. Two days post-transfection, the cells were washed twice with PBS, then starved for 30 min in methionine-cysteine-

free medium supplemented with 2% fetal bovine serum, after which the cells were suspended and labeled for 15 min in medium containing 200 μCi [^{35}S]methionine and 200 μCi [^{35}S]cysteine (PerkinElmer Life Sciences). The pulse was ended by adding prewarmed DMEM supplemented with 10% fetal bovine serum and 0.5mM of cycloheximide (CHX, Sigma) and 5mM of unlabeled methionine and cysteine. After various chase time periods, the cells were transferred to ice and washed twice with ice-cold PBS. For immunoprecipitation, the cells were lysed, and APOBEC3G-HA was immunoprecipitated with anti-HA mAb (Roche). The proteins were separated by SDS-PAGE and quantified on a phosphorimager.

In vivo ubiquitination assay. 293T cells were transfected with expression vectors encoding APOBEC3G-HA, Vif, Myc-tagged ubiquitin, and Cul5 mutants either alone or in combination. Cells were treated with 2.5 μM MG132 from 24 h after transfection for 16 h and lysed in lysis buffer (50 mM Tris, pH7.5, with 150 mM NaCl, 1% Triton X-100, 5 mM iodoacetamide, 10 μM MG132, and complete protease inhibitor cocktail tablets), followed by centrifugation at 10,000 \times g for 30 min. Lysates were applied to anti-HA antibody-conjugated agarose beads (Roche) and washed with washing buffer (20 mM Tris, pH 7.5, with 0.1 M NaCl, 0.1 mM EDTA, and 0.05% Tween-20). The beads were eluted with elution buffer (0.1 M glycine, pH 2.0), followed by SDS-PAGE and immunoblotting with anti-Myc tag antibody.

Supporting Text

Construction and characterization of HXB2VifHA

To efficiently precipitate Vif from HIV-1-infected cells and subsequently identify the cellular protein(s) that interact with Vif, we constructed an infectious HIV-1 clone (HXB2VifHA) in which the end of Vif carried an HA tag. The Vif function of this clone was not affected by the HA tag, and it replicated as efficiently as the parental clone HXB2 (*S1*) in H9 cells, which are not permissive for a Vif deletion, HXB2 Δ Vif (*S1*), mutant virus (Fig. S1A). The pattern of viral protein expression in HXB2VifHA was very similar to that in HXB2 (Fig. S1B), although the HA-tagged Vif in HXB2VifHA was slightly larger than the untagged Vif in HXB2 (Fig. S1C). As expected, HA-tagged Vif could be detected by an HA-specific antibody in HXB2VifHA-infected cells but not in HXB2-infected cells (Fig. S1D).

Construction and characterization of 293T/APOBEC3G

To examine whether the Cul5-containing SCF complex is required for Vif function, we first established 293T cells that expressed the host antiviral factor APOBEC3G (293T/APOBEC3G). APOBEC3G is not normally expressed in 293T cells, and these cells are permissive for the HIV-1 Vif mutant (*S2*). The effect of APOBEC3G expression on wild-type and Vif deletion mutant HIV-1 infectivity was evaluated using viruses from 293T and transfected 293T/APOBEC3G cells in a MAGI-CCR5 cell assay (*S3*). MAGI-CCR5 cells contain one integrated copy of the HIV-1 LTR linked to the β -gal gene, and productive HIV-1 infection will induce the expression of the β -gal gene product. Therefore, HIV-1 infectivity can be measured by counting the number of cells staining

positive for β -gal expression. The amount of input virus was normalized by p24 antigen concentration, and the infectivity of the wild-type HIV-1 produced from 293T cells was established as 100%. Wild-type HIV-1 and Vif mutant virus produced comparable amount of infectious virions from 293T cells when analyzed in MAGI-CCR5 cells (Fig. S3A). However, when viruses were produced in the presence of APOBEC3G (from 293T/APOBEC3G cells), the virion infectivity of the Vif mutant was reduced by approximately 90% when compared to wild-type virions (Fig. S3A), although virus production by the wild-type or Vif mutant HIV-1 from 293T/APOBEC3G cells was not affected (data not shown). Expression of APOBEC3G had little effect on wild-type virus infectivity (Fig. S3A), suggesting that HIV-1 Vif suppressed the antiviral activity of APOBEC3G. These data are consistent with the recent observations that APOBEC3G is an anti-HIV-1 factor that is suppressed by HIV-1 Vif (S2).

Construction and characterization of Cul5 mutants

Rbx1 has been shown to associate with various cullin SCF complexes (S4). To address more directly whether Cul5-containing SCF complexes are required for Vif function, we studied the effects of Cul5 mutants on HIV-1 infectivity. All cullin family members are known to be modified by the ubiquitin-like small molecule Nedd8 (S5), which shares 60% identity with ubiquitin. Nedd8 modification of cullins is critical for the SCF complex's ubiquitination function (S6,S7). Cul1 is modified by Nedd8 at Lys 720, and the Nedd8 modification region in Cul1 is highly conserved in Cul5 (Fig. 2A). There are three Lys in this region of Cul5, and it is not clear which Lys may be targeted by Nedd8 (Fig. 2A). We therefore made Lys-to-Ala substitutions at amino acids 724, 727, and 728

of Cul5 (Fig. 2A, pCul5 Δ Nedd8). These mutations abolished the Nedd8 modification of mutant Cul5. Both Neddylated and un-Neddylated Cul5 were detected in pCul5-transfected 293T cells (Fig. 2B, lane 1), but only the un-Neddylated Cul5 was detected in pCul5 Δ Nedd8-transfected 293T cells (Fig. 2B, lane 2). The Cul5-containing SCF complex, like that containing Cul1, interacts with Rbx1(S8,S 9). Analysis of the crystal structure of the Cul1-Rbx1-Skp1-Skp2 complex has indicated that the C-terminal domain of Cul1 interacts with Rbx1 (S10), and the regions in Cul1 that are important for interaction with Rbx1 are also conserved in Cul5 (S10). Deletion of one such region, amino acids 566 to 582, in Cul5 (pCul5 Δ Rbx1) significantly reduced mutant Cul5 interaction with Rbx1 (Fig. 2C). Interaction of Rbx1 with wild-type Cul5 (Fig. 2C, lane 2) was detected by co-immunoprecipitation with Cul5-Myc using that anti-Myc tag antibody, but interaction with mutant Cul5 (pCul5 Δ Rbx1) was not detected under the same conditions (Fig. 2C, lane 3). We also observed that the Rbx1-binding mutant of Cul5 had a defect in Nedd8 modification (Fig. 2C, lane 3), which is consistent with the notion that Rbx1 is part of the Nedd8 E3 ligase (S11, S12). Interaction of Rbx1 with Cul5Nedd8 was also detected (Fig. 2C, lane 1), indicating that the abolished Nedd8 modification of this mutant Cul5 was not due to a lack of interaction with Rbx1.

Fig.S1

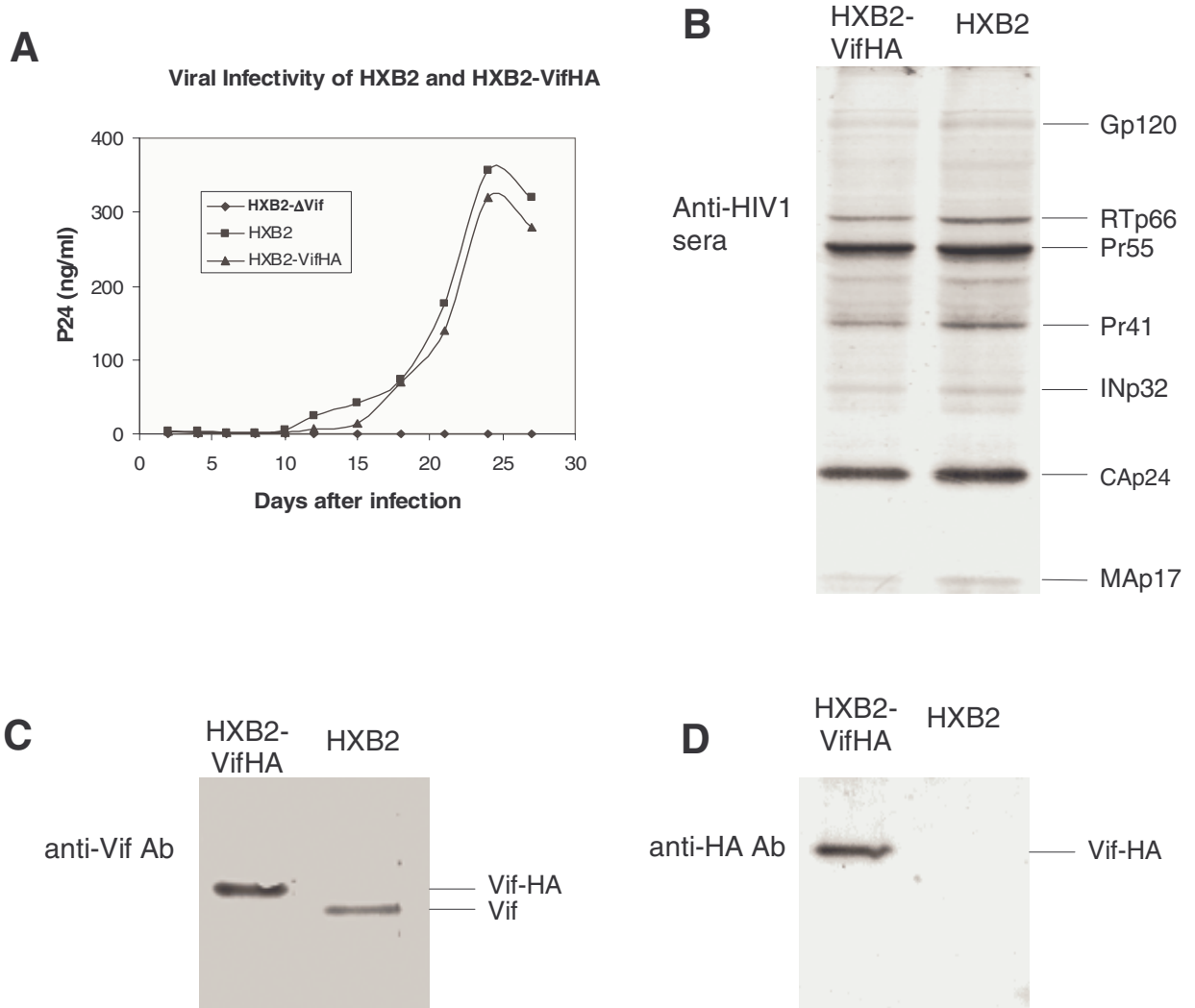


Fig. S2

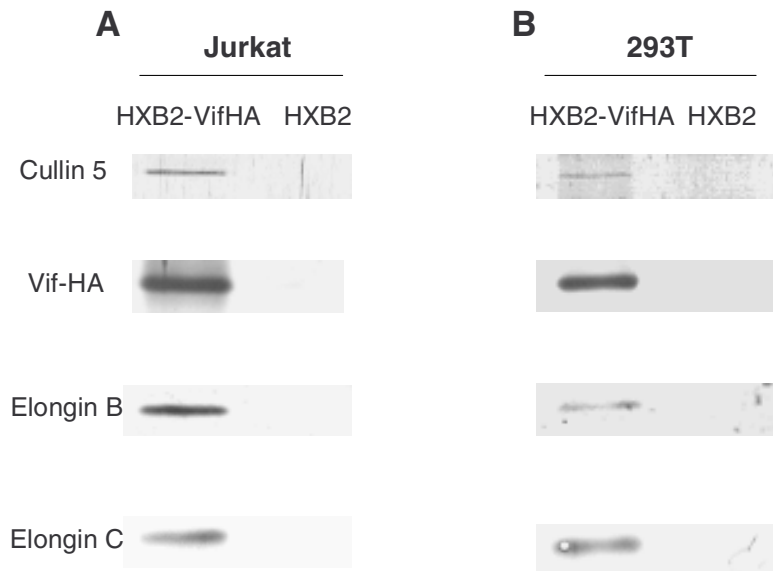


Fig.S3

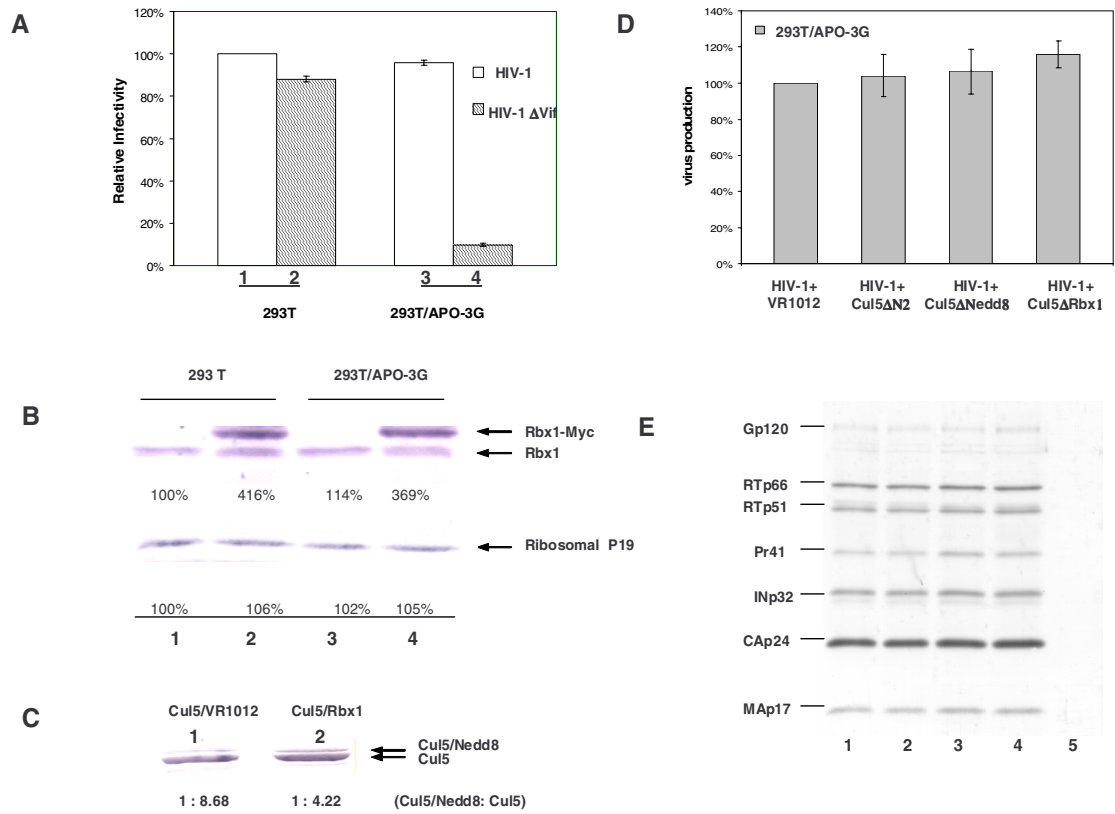


Fig.S4

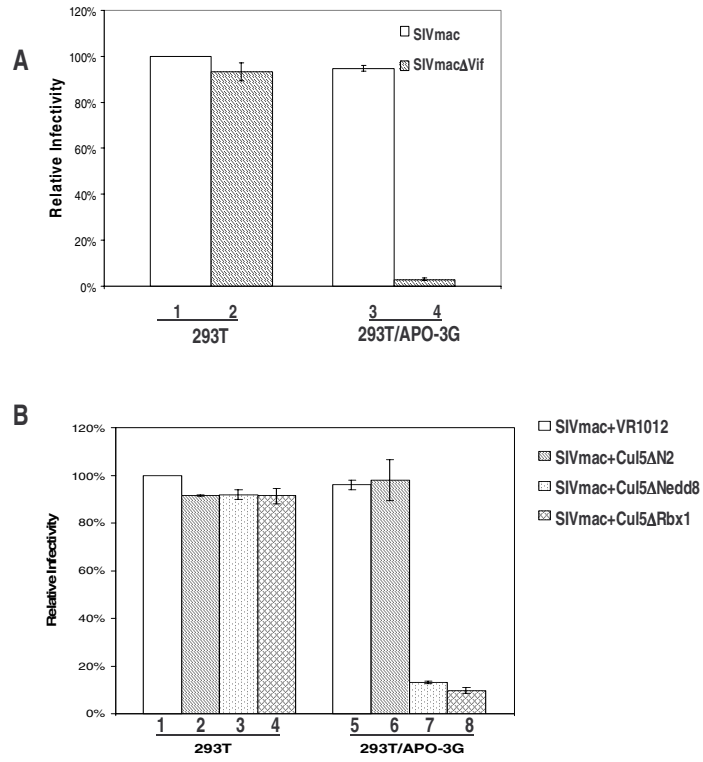


FIGURE LEGENDS

Fig. S1. (A) Viral replication of HXB2, HXB2VifHA, and HXB2 Δ Vif in H9 cells. Virus input was normalized by the level of p24. Virus replication was monitored by the level of p24 in the supernatants of infected cells. (B) Comparison of viral protein expression in HXB2 and HXB2VifHA-infected H9 cells by immunoblotting using HIV-1+ human sera. (C) Immunoblotting of cell lysates using anti-Vif antibody. (D) Immunoblotting of cell lysates using anti-HA antibody.

Fig. S2. Immunoblotting of precipitated samples from HXB2 and HXB2VifHA-infected Jurkat cells (A) or transfected 293T cells (B). Cell lysates were precipitated with the anti-HA antibody, and the precipitated samples were separated by SDS-PAGE, transferred to nitrocellulose membranes and reacted with antibodies to Cul5, HIV-1 Vif, Elongin B, or Elongin C.

Fig. S3. (A) Expression of APOBEC3G in 293T cells inhibits the infectivity of the HIV-1 Vif mutant. Wild-type HIV-1 (HXB2) and Vif mutant (HXB2 Δ Vif) viruses were produced from 293T cells or 293T/APOBEC3G cells, and their infectivity was examined using MAGI-CCR5 cells. Virus input was normalized by the level of p24. The infectivity of HXB2 produced from 293T cells was set as 100%. (B) Expression of Rbx1 proteins. 293T cells or 293T/APOBEC3G cells were transfected with VR1012 or pRbx1, and cell lysates were prepared 48 h after transfection and analyzed by immunoblotting using anti-Rbx1 antibody. The Rbx1-c-myc and endogenous Rbx1 are indicated by

arrows. Ribosomal P19 antigen was used as total protein loading control. (C). Overexpression of Rbx1 enhances Cul5 modification by Nedd8. 293T cells were transfected with pCul5-Myc plus VR1012 or pRbx1, and cell lysates were prepared 48 h after transfection and analyzed by immunoblotting using anti-Myc tag antibody for the detection of Cul5-Myc. (D) Analysis of virus production. Virus-containing supernatants were collected from 293T/APOBEC3G cells transfected with HXB2 plus VR1012, pCul5 Δ N2, pCul5 Δ Nedd8, or pCul5 Δ Rbx1. Virus production was monitored by the level of p24 in the supernatants. (E) Comparison of viral protein patterns from 293T/APOBEC3G cells transfected with HXB2 plus VR1012, pCul5 Δ N2, pCul5 Δ Nedd8 or pCul5 Δ Rbx1 by immunoblotting using HIV-1 positive human sera.

Fig. S4. Cul5 mutants block the Vif function of SIVmac in the presence of APOBEC3G.

(A) Expression of APOBEC3G in 293T cells inhibits the infectivity of the SIVmac Δ Vif mutant. Wild-type SIVmac and Vif mutant (SIVmac Δ Vif) viruses were produced from 293T cells or 293T/APOBEC3G cells, and their infectivity was examined using MAGI-CCR5 cells. Virus input was normalized by the level of p27. The infectivity of SIVmac produced from 293T cells was set as 100%. (B) Cul5 mutants render SIVmac wild-type virus sensitive to the antiviral activity of APOBEC3G. SIVmac viruses were produced from 293T cells or 293T/APOBEC3G cells co-transfected with control vector VR1012, pCul5 Δ N2, pCul5 Δ Nedd8, or pCul5 Δ Rbx1. Virus input was normalized by the level of p27. The infectivity of SIVmac produced from 293T cells co-transfected with VR1012 was set as 100%. Results are the average of 3 independent experiments.

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