Identification of a conserved interface of HIV-1 and FIV Vifs with Cullin 5

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Abstract

The apolipoprotein B mRNA-editing enzyme, catalytic polypeptide-like (APOBEC3, A3) family of DNA cytidine deaminases are intrinsic restriction factors against retroviruses. In felids such as the domestic cat (*Felis catus*), the APOBEC3 (A3) genes encode for the A3Z2s, A3Z3, and A3Z2Z3 antiviral cytidine deaminases. Only A3Z3 and A3Z2Z3 inhibit viral infectivity factor (Vif)-deficient feline immunodeficiency virus (FIV). FIV Vif protein interacts with Cullin (CUL), Elongin B (ELOB), and Elongin C (ELOC) to form an E3 ubiquitination complex to induce the degradation of feline A3s. However, the functional domains in FIV Vif for interaction with Cullin are poorly understood. Here, we found that the expression of dominant-negative CUL5 prevented the degradation of feline A3s by FIV Vif, while dominant-negative CUL2 had no influence on the degradation of A3. In co-immunoprecipitation assays, FIV Vif bound to CUL5 but not CUL2. To identify the CUL5 interaction site in FIV Vif, the conserved amino acids from position 47 to 160 of FIV Vif were mutated, but these mutations did not impair the binding of Vif to CUL5. By focusing on a potential zinc-binding motif (K175–C161–C184–C187) of FIV Vif, we found a conserved hydrophobic region (174IR175) that is important for CUL5 interaction. Mutating this region also impaired the FIV Vif-induced degradation of feline A3s. Based on a structural model of the FIV Vif/CUL5 interaction, residues 52LW53 in CUL5 were identified as mediating the binding to FIV Vif. By comparing our results to the HIV-1 Vif/CUL5 interaction surface (120IR121, a hydrophobic region that is localized in the zinc-binding motif), we suggest that the CUL5 interaction surface in the diverse HIV-1 and FIV Vif is evolutionarily conserved indicating a strong structural constraint. However, the FIV Vif/CUL5 interaction is zinc-independent, which contrasts with the zinc-dependence of HIV-1 Vif.
Importance

Feline immunodeficiency virus (FIV), which is similar to human immunodeficiency virus (HIV), replicates in its natural host in T-cells and macrophages that express antiviral restriction factors APOBEC3 (A3). To escape A3s, FIV and HIV induce degradation of these proteins by building ubiquitination ligase complex using the viral protein Vif to connect to cellular proteins, including Cullin 5. Here, we identified the protein residues that regulate this interaction in FIV Vif and Cullin 5. While our structural model suggests that the diverse FIV and HIV-1 Vifs use conserved residues for Cullin 5 binding, FIV Vif binds Cullin 5 independently of zinc in contrast to HIV-1 Vif.
Introduction

Antiretroviral APOBEC3 (A3) restriction factors are found in clade-specific numbers in placental mammals (1). Interestingly, only this group of animals is a host of lentiviruses, while other retroviruses are also found in animals outside placental mammals, e.g., in fish, reptiles, and birds. Humans encode seven A3 genes (A3A–A3D and A3F–A3H) and cats have four genes (A3Z2a–A3Z2c and A3Z3) (2-4). In addition, cats express alternative splice variants of A3Z2–Z3s by read-through transcription (3, 4). A3 proteins are packaged into nascent lentiviral particles during virus assembly and release, if the virus does not counteract the A3 encapsidation. During the next round of infection, viral core-incorporated A3s deaminate cytidines in the single-stranded (-) DNA generated during reverse transcription, thus causing G-to-A hypermutations in the coding strand (5, 6). The best-characterized mechanism of lentiviruses to prevent this strong restriction is based on a viral protein called viral infectivity factor (Vif). In virus-producing cells, human immunodeficiency virus (HIV)-1 Vif targets, for example, APOBEC3G (A3G) for degradation by forming an SCF (SKP1–cullin/RBX–F-box protein)-like E3 ubiquitin ligase containing Cullin 5, as well as Elongin B and C (CUL5–ELOB–ELOC) through a novel SOCS (suppressor of cytokine signaling) box that binds ELOC (7, 8). The CUL5–SCF E3 ligase is also required for Vif activity against A3s in the related simian immunodeficiency virus of macaques (SIVmac) (8). In these complexes, Vif works as a substrate receptor for A3. In most cases, the interaction of Vif with A3s is species-specific, and the intricate interfaces are still unresolved and a matter of ongoing investigation (9-13).

Feline immunodeficiency virus (FIV) also encodes a vif gene, and Vif is essential for the virus to replicate in cats and cells that express feline A3s (3, 4, 9, 14-17). Similar to primate lentiviruses, FIV Vif interacts with CUL5, ELOB, and ELOC to form an E3 complex to induce degradation of feline A3s (18). However, HIV-1 and SIV Vifs also need CBF-β to stabilize and...
form the E3 ligase complex, whereas FIV and other non-primate lentiviruses (e.g., maedi-
visna virus (MVV), caprine arthritis encephalitis virus (CAEV), and bovine immunodeficiency
virus (BIV)) do not require CBF-β to induce A3 degradation (19-24). Instead, MVV Vif hijacks
cellular cyclophilin A (CYPA) as a cofactor to reconstitute the E3 ligase, while BIV Vif appears
to operate independently of any cofactors (19). Whether FIV Vif recruits any additional
protein to its E3 A3 complex is unclear.

To form an E3 ubiquitin ligase complex, HIV-1 Vif utilizes the BC box (SLQ motif) and the
CUL5 box (HCCH motif) to interact with Elongin B/C and Cullin 5, respectively (7, 8, 25-28).
CUL5 also binds to ELOC and so destabilizing the binding of ELOC to Vif by mutating the SLQ
motif also removes CUL5 from the E3 complex (7, 25, 27). HIV-1 Vif utilizes the HCCH motif
to bind zinc, which in turn is important for HIV-1 Vif-induced A3G degradation and binding to
CUL5 (27-32). In the HIV-1 Vif/CUL5 co-structure, the HCCH motif stabilizes Vif helix 3, which
contains a hydrophobic interface that is involved in the direct CUL5 interaction (33).

However, the interface between FIV Vif and CUL5 is unknown (18). In this study, we first
demonstrate that FIV Vif interacts with CUL5, not CUL2, to induce the degradation of feline
A3s and, secondly, identify specific residues in Vif and CUL5 that are important for this
binding. Our data support a conserved interaction surface of HIV-1 and FIV Vifs with CUL5.
Materials and methods

Plasmids. Domestic cat A3s expressing a carboxy-terminal hemagglutinin (HA) tag, were described previously (3, 34). FIV-34TF10 (codon-optimized) Vif gene was inserted into pcWPRE containing a C-terminal V5-tag (4, 9). All the FIV Vif mutants were produced by fusion PCR and inserted into pcWPRE by using EcoRI and NotI restriction sites (11). HIV-1 Vif expression plasmid with a C-terminal V5 tag was described before (4). Human A3G with a C-terminal HA tag, a gift of Nathanial Landau, was previously described (35). pcDNA3-DN-hCUL5-FLAG (15823) (36), pcDNA3-myc-CUL5 (19895) (37), pcDNA3-DN-hCUL2-FLAG (15819) (36), pcDNA3-myc3-CUL2 (19892) (37), T7-Elongin C-pcDNA3 (19998) (38) and HA-Elongin B-pcDNA3.1(+)Zeo (20000) (39) were obtained from Addgene (Cambridge, USA). The CUL5 mutations were produced by fusion PCR and cloned into pcDNA3-myc-CUL5 by using BamHI and XbaI to replace wildtype CUL5. The replication deficient packaging construct pFP93, a gift of Eric Poeschla (40); the FIV luciferase vector pLinSin (4) and a VSV-G expression plasmid pMD.G were used to produce FIV single-cycle luciferase viruses (FIV-Luc), which were described previously (4).

Cell cultures and transfections. HEK293T cells (293T, ATCC CRL-3216) were maintained in Dulbecco’s high glucose modified Eagle’s medium (DMEM, Biochrom, Berlin, Germany) supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, penicillin (100 U/ml), and streptomycin (100 μg/ml) at 37°C and 5% CO2. The FcaA3Z2bZ3 degradation experiments were performed in 12-well plates, 2×10^5 293T cells were transfected with 300 ng feline A3Z2b or feline A3Z3 or A3Z2bZ3 expression plasmids together with 60 ng codon-optimized FIV Vif expression plasmid, pcDNA3.1(+) was used as control, and 700 ng pcDNA3-
DN-hCUL5-FLAG or pcDNA3-DN-hCUL2-FLAG expression plasmids, pcDNA3.1(+) was used as control. To produce FIV-luciferase viruses, 293T cells were co-transfected with 300 ng FIV packaging construct, 300 ng FIV-luciferase vector, 300 ng A3 expression plasmid, 100 ng VSV-G expression plasmid, 30 ng FIV Vif expression plasmid in 12-well plates; in some experiments pcDNA3.1(+) was used instead of Vif or A3 expression plasmids. The test of interaction between CUL5 and FIV Vif, 293T cells were co-transfected with 1000 ng pcDNA3-myc-CUL5 and 1000 ng FIV Vif-V5 or FIV Vif mutants in 6-well plates; pcDNA3.1(+) was used as control. For the interaction between ELOB, ELOC and FIV Vif, 293T cells were co-transfected with 700 ng T7-Elongin C-pcDNA3, 700 ng HA-Elongin B-pcDNA3.1(+) Zeo and 700 ng FIV Vif-V5 or FIV Vif mutants in 6-well plates; pcDNA3.1(+) was used as control. All the transfections were performed by using Lipofectamine LTX (Thermo Fisher Scientific, Schwerte, Germany) according to manufacturer’s instruction. At 48 h post-transfection, cells and supernatants were collected.

For transfections in the presence of MG132 (474790, Calbiochem) and TPEN (16858-02-9, Calbiochem), the culture medium was replaced with fresh DMEM containing different concentrations of MG132 or TPEN, or dimethyl sulfoxide (DMSO). After the cells were treated for 16 h, the cell lysates were used for immunoprecipitation or immunoblotting as described below.

**Antibodies.** The following antibodies were used for the present study: mouse anti-hemagglutinin (anti-HA) antibody (1:7500 dilution, MMS-101P; Covance, Münster, Germany), mouse anti-V5 antibody (1:4500 dilution, MCA1360, ABDserotec, Düsseldorf, Germany), mouse anti-α-tubulin antibody (1:4000, dilution, clone B5-1-2; Sigma-Aldrich,
Taufkirchen, Germany), mouse anti-T7-tag monoclonal antibody (1:1000 dilution, 69522, mouse monoclonal IgG2b, Merck, Germany), mouse anti-CUL-5 monoclonal antibody (1:1000 dilution, sc-373822, Santa Cruz, USA), mouse anti-myc monoclonal antibody (1:100 dilution, MCA2200, MbD Serotec, Canada), mouse anti-flag M2 monoclonal antibody (1:1000 dilution, F1804, Sigma, USA). The second antibody was horseradish peroxidase-conjugated rabbit anti-mouse antibody (1:10,000 α-mouse-IgG-HRP; GE Healthcare, Munich, Germany).

**Immunoprecipitation.** 293T cells were transfected with FIV Vif-V5 together with pcDNA3-myc-CUL5 or T7-Elongin C-pcDNA3, HA-Elongin B-pcDNA3.1(+)-Zeo or FcaA3Z2bZ3-HA expression plasmids. At 48h post-transfection, the cells were harvested and lysed in IP-lysis buffer (50 mM Tris/HCl pH 8, 10% Glycerol, 0.8% NP-40, 150 mM NaCl) with protease inhibitor cocktail set III (Calbiochem, Darmstadt, Germany) on ice for 20 min. Cell lysates were clarified by centrifugation at 10,000g for 30 min at 4°C. The supernatant were incubated with 15 µl rabbit anti-c-myc agarose affinity gel antibody beads (A7470, Sigma, USA) or 15 µl rat anti-HA affinity matrix beads (16598600, Roche, USA). After 2 h incubation at 4°C in end-over-end rotation, the samples were washed 4 times with lysate buffer on ice. Bound proteins were eluted by boiling the beads for 5 min at 95°C in SDS loading buffer. The eluted materials were subsequently analyzed by immunoblotting. To detect the interaction of FIV Vif with endogenous CUL5, 5 x 10⁵ 293T cells in 6-well plates were transfected with wildtype FIV Vif or indicated mutant expression plasmids. The cells were harvested like above, and 500 µl cell lysis were incubated with 2 µg mouse anti-V5 antibody (MCA1360, ABDserotec, Düsseldorf, Germany) and 20 µl protein A/G plus agarose (Santa Cruz, Heidelberg, Germany) for 4 h at 4°C in end-over-end rotation. The samples were washed 4 times after 4 h incubation, and bound proteins were analyzed by immunoblotting.
Immunoblotting. Transfected 293T cells were lysed in radioimmunoprecipitation assay (RIPA) buffer (25 mM Tris-HCl [pH 7.6], 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate [SDS], protease inhibitor cocktail set III [Calbiochem, Darmstadt, Germany]) on ice 20 min. Cell lysates were clarified by centrifugation at 10,000g for 30 min at 4°C. Test proteins were boiled for 5 min at 95°C in SDS loading buffer and resolved on a SDS-PAGE gel. Then, proteins were transferred onto immobilon PVDF transfer membrane (IPVH 00010, Merck millipore, Germany) by semidry transfer (Bio-Rad, herclues, CA) at 25 volt for 50 min. After blocking in 5% nonfat milk (A0830, PanReac AppliChem, Germany), the membranes were detected with various primary antibodies against proteins of interest and secondary antibodies were followed, and developed with ECL chemiluminescence reagents (GE Healthcare).

Homology Modeling. We modeled the complex of FIV Vif and Cul5 based on the X-ray crystal structure of the HIV-1 Vif and Cul5 complex as a template (33). Owing to the low sequence identity between FIV and HIV-1 Vif, we performed the modeling in an iterative fashion. We used ClustalW2 (33) to align the sequences, using the (T/S)LQ-BC box and the conserved 174IR175 motif as anchor points to guide the alignment. Next, we modeled the complex employing Modeler v9.10 (41) and, subsequently, manually curated the sequence alignment based on the resulting models: Accounting for a possible zinc dependency of FIV Vif, cysteines in the FIV Vif sequence, which in the models were structurally close to zinc binding cysteine and histidine residues in HIV-1 Vif, were aligned to these zinc binding residues in the HIV-1 sequence (C113, C114, H108, and H139). Furthermore, we used information on the secondary structure of FIV Vif, predicted by PSIPRED (42) to guide the manual curation of the
sequence alignment. The final model was then used to predict interacting residues. The model is accessible at the Protein Model Data Base (PMDB; https://bioinformatics.cineca.it/PMDB/main.php) with accession code: PM0081296.

Nucleotide sequence accession numbers. The FIV Vif sequences were obtained from GenBank, the accession numbers are: FIV C36 (AY600517.1); FIV 34TF10 (M25381.1); FIV PRR (M36968.1); FIV TM-2 (M59418.1); FIV Shizuoka (LC079040.1); FIV Oma (AY713445); FIV Lion B (EU117991); FIV Lion E (EU117992); FIV puma A (U03982), FIV puma B (DQ192583). The feline APOBEC3 and human APOBEC3G GenBank accession numbers are: FcaA3Z2b (AY971954), FcaA3Z3 (EU109281), FcaA3Z2bZ3 (EU109281) and HsaA3G (NM_021822).

Statistical analysis. Data are represented as the mean with SD in all bar diagrams. Statistically significant differences between two groups were analyzed using the unpaired Student’s t-test with GraphPad Prism version 5 (GraphPad software, San Diego, CA, USA). A p value ≤ 0.05 was considered statistically significant: P value < 0.001 extremely significant (**), 0.001 to 0.01 very significant (**), 0.01 to 0.05 significant (*), > 0.05 not significant (ns).
Results

CUL5 and not CUL2 is required for FIV Vif degradation of feline A3s. A previous study reported that FIV Vif interacts with Cullin 5 (CUL5) to form an E3 ubiquitin ligase complex that can induce the degradation of feline A3 proteins by the proteasome (18). However, the mechanism by which FIV Vif binds to CUL5 remains unclear and whether FIV Vif binds to other Cullin family proteins is not known. Thus, we first investigated the interaction of FIV Vif with CUL5 and CUL2. The FIV Vif used in this study was from FIV clone 34TF10, which is derived from domestic cats (*Felis catus*, Fca), here referred to as FIV. To test the Vif/CUL interaction, we co-immunoprecipitated (co-IP) Vif and CUL using lysates of human embryonic kidney 293T cells transfected with expression plasmids for FIV Vif-V5, CUL2-myc, or CUL5-myc. We detected FIV Vif in the CUL5-immunoprecipitated complex, while no FIV Vif was observed in the CUL2 immunoprecipitation (Fig. 1A). CUL2 and CUL5 interact with Rbx by using their C terminus that is critical for E3 ubiquitin ligase activity (See recent review (43)). Dominant-negative (DN) CUL5 or DNCUL2 has a C terminal deletion, which can inhibit SOCS box protein induced substrate degradation (36). Thus, we expressed the indicated feline A3s together with FIV Vif and DNCUL5 or DNCUL2. Immunoblots of protein extracts from transfected 293T cells were used as readout for the degradation of the respective A3 protein. The results showed that FIV Vif efficiently degraded all three tested feline A3s (FcaA3Z2b, FcaA3Z3, and FcaA3Z2bZ3) in the absence of DNCUL5 (Fig. 1B), which is consistent with previous studies (4, 11, 14). The presence of DNCUL5 enhanced the cellular protein level of Vif and abolished the degradation of the A3s (Fig. 1B). In contrast, DNCUL2 did not affect the expression of Vif nor the Vif-dependent FcaA3Z3 and FcaA3Z2bZ3 degradation (Fig. 1C). However, in the presence of high level of FcaA3Z2b, expression DNCUL2 slightly impaired its degradation by FIV Vif, possible due to DNCUL2-dependent
exhaustion of endogenous ElongB/C (Fig. 1D). Additionally, we investigated the impact of the proteasome inhibitor MG132 on Vif-mediated A3 degradation. We found that the degradation of FcaA3Z2bZ3 by FIV Vif was sensitive to MG132 treatment but comparably less sensitive than the HIV-1 Vif-induced degradation of human A3G (Fig. 1E). The reasons for these different responses to MG132 are unclear and might indicate different kinetics of degradation.

**FIV Vif N-terminal residues are not essential for CUL5 binding.** A previous study suggested that FIV Vif may utilize a novel mechanism for binding CUL5 (18). To investigate which domain of FIV Vif is involved in the CUL5 interaction, we analyzed FIV Vif sequences from different FIV strains. We found several conserved residues at the N-terminal protein region (residues 53 to 132). Thus, we replaced the N-terminal conserved residues (53FI54, 57LR58, 61EGI63, 65WSF67, 68HTR70, 71DYY73, 74IGY76, 77VRE79, 81VAG83, 92MY93, 95YI96, 99PLW101, 105YRP107, 128MED130, and 132IEK134) of FIV Vif by alanines (Fig. 2A). All FIV Vif mutants were co-expressed with CUL5-myc in 293T cells. FIV Vif with a TLQ-AAA mutation served as a control because destroying ELOC binding destabilizes the CUL5 interaction (18). Co-immunoprecipitation assays followed by immunoblots were used to evaluate the binding of FIV Vif mutants to CUL5. The results showed that FIV Vif with mutations in 53FI54, 57LR58, and 77VRE79 bound CUL5, but the binding affinity appeared to be weaker compared to the wildtype FIV Vif (Fig. 2B). These three FIV Vif mutants had similar binding affinity to FcaA3Z2bZ3 (Fig. 2C) and ELOB/C (Fig. 2D). Consistent with a reduced CUL5 interaction, these Vif mutants partially lost the function to induce FcaA3Z2bZ3 degradation (Fig. 2E). All other Vif mutants showed no change in CUL5 binding (Fig. 2B). Overall, it
appears that the residues in the N-terminus (53 to 132) of FIV Vif are not important for the CUL5 interaction.

Identification of determinants in the C-terminus of FIV Vif that regulate binding to CUL5. It has been shown that in HIV-1 Vif, CUL5 interacts with the C-terminal HCCH box (27, 28, 44). Thus, we analyzed the C-terminal region of Vifs from different FIV strains. As in HIV-1, all FIV Vifs contained a TLQ-BC box that is essential for ELOB/C binding (Fig. 3A). In addition, a KCCC motif that is similar to the HCCH motif of HIV-1 Vif is found in FIV Vif. In the KCCC motif, we identified a conserved hydrophobic domain (172MIIRGE177; Fig. 3A). Despite the low sequence identity of only 13% in this region between FIV Vif and HIV-1 Vif, this hydrophobic domain matched when the C-terminal regions of both Vifs were aligned (Fig. 3A). In the complex structure of HIV-1 Vif/CUL5 (33), residues 120IR121 of HIV-1 Vif are involved in the direct interaction with CUL5 (Fig. 3B). To investigate whether the equivalent hydrophobic domain 172MIIRGE177 of FIV Vif is also involved in CUL5 binding, we replaced 172MI173, 174IR175, or 176GE177 with alanines (Fig. 3C). The binding activities of these FIV Vif mutants with CUL5 were evaluated by co-IP assays. The results showed that FIV Vif 176177GE-AA bound CUL5 similar to the wildtype FIV Vif and 172MI173, 174IR175, or 176GE177 impaired the FIV Vif/CUL5 interaction, while 174175IR-AA impaired binding (Fig. 3C). Additionally, single point mutations were introduced at 174IR175 and the I174A and R175A mutations of FIV Vif also decreased the CUL5 interaction (Fig. 3C). Taken together, we conclude that the conserved 174IR175 motif of FIV Vif is the main CUL5 binding site.

Next, we tested the degradation activity of FIV Vif 174175IR-AA towards feline A3. The results showed that FIV Vif 174175IR-AA did not induce degradation of co-expressed...
FcaA3Z2bZ3 (Fig. 4A) and lost the ability to inhibit the anti-FIV activity of FcaA3Z2bZ3 (Fig. 4B). These data support the model that CUL5 binding of FIV Vif is essential for its antagonism of feline A3s. Furthermore, we asked whether impairing the CUL5 binding sites of FIV Vif affects its interaction with feline A3 and ELOB/C. To address this question, 293T cells were co-transfected with expression plasmids for wildtype FIV Vif or alanine mutations of TLQ or 174IR175 together with expression plasmids for FcaA3Z2bZ3 or ELOB and ELOC. The immunoprecipitation results indicate that alanine mutations of FIV Vif TLQ and 174IR175 variants did not lead to a loss of binding to FcaA3Z2bZ3 (Fig. 4C). However, the FIV Vif TLQ-AAA variant lost its interaction with ELOB/C, as shown previously (18). The Vif 174175IR-AA variant bound to ELOB/C similar to the wildtype FIV Vif (Fig. 4D). Together, these results support that residues 174IR175 of FIV Vif are required for the interaction with CUL5.

Modeling the FIV Vif/CUL5 complex structure. To identify further interacting residues in the FIV Vif/CUL5 complex, we built a homology model of the complex to guide mutational analyses. As described in the methods section, we incorporated information of the predicted secondary structure of FIV Vif and our results about the importance of residues 174IR175 and the TLQ-BC box for CUL5 binding when generating the sequence alignment of FIV Vif to HIV-1 Vif. We did so to ameliorate the fact that the sequence identity between FIV and HIV-1 Vif is only 5.2%. In this way, residues corresponding to the FIV 174IR175 and TLQ-BC box motifs were identified in HIV-1. The homology model of the FIV Vif/CUL5 complex showed a contact of the TLQ-BC box with CUL5 and revealed a close proximity of residues 174IR175 of Vif to residues 52LWDD55 of CUL5 (Fig. 5A and B). Thus, we mutated 52LW53 and 54DD55 of CUL5 to alanines and tested these CUL5 variants for interaction with FIV Vif. The results showed that CUL5 52LW53-AA no longer bound to FIV Vif; however, the CUL5 54DD55-AA
variant still bound Vif similar to the wildtype CUL5 (Fig. 5C). Additionally, we tested whether the CUL5 52LW53-AA mutant effects FIV Vif induced feline A3s degradation. We expressed the indicated feline A3s together with FIV Vif and the CUL552LW53-AA mutant in 293T cells. Immunoblots of protein extracts from transfected cells were used as readout for the degradation of the respective A3 protein. The results showed that FIV Vif degraded feline A3s with an unchanged efficiency in the presence of this CUL5 mutant (Fig. 5D). A model representing the FIV Vif E3 complex is shown in Fig. 5E.

The FIV Vif/CUL5 interaction is zinc-independent. Our FIV Vif/CUL5 complex model suggests that helix 3 of FIV Vif-CTD regulates the CUL5 binding (Fig. 5 and 6A). There are two positively charged lysines (K181 and K182) and several potential zinc-binding cysteines (C138, C161, C184, C187, C192, and C209) at the FIV Vif CTD (Fig. 6B). To further address the FIV Vif/CUL5 interaction properties, we mutated K181 and K182 to alanines and several potential zinc-binding residues to serine (C138S, C161S, C184S, C187S, C192S, and C209S; Fig. 6B). The immunoprecipitation results revealed that the C184S and C184C187-SS variants decreased the CUL5 interaction, but Vif mutations K181A, K182A, C187S, C138S, C161S, C192S and C209S did not affect CUL5 binding (Fig. 6C). Additionally, we tested the interaction of several FIV C-S mutants (C161S, C184S, C187S and C209S) with endogenous CUL5. The result showed that only C184S mutant lost CUL5 binding activity (Fig. 6D). We further investigated the binding property of FIV Vif C184S mutant to feline A3 and ELOB/C. The immunoprecipitation results indicate that FIV Vif C184S mutant has weaker binding affinity to FcaA3Z2bZ3 (Fig. 6E), compared with FIV Vif TLQ-AAA mutant and both, FIV Vif TLQ-AAA and C184S, lost interaction with ELOB/C (Fig. 6F). We further found that FIV Vif C161S, C184S and C187S mutants completely lost FcaA3Z2bZ3 degradation function, but the
other cysteine mutants (C138S, C192S and C209S) efficiently degraded FcaA3Z2bZ3 (Fig. 6G).

Taken together, these data indicate that C184 of FIV Vif is not specific for CUL5 interaction, and we speculate that C184 may regulate the integral structure of FIV Vif.

To test the zinc dependency of the FIV Vif/CUL5 interaction, the cell-permeable zinc chelator TPEN (N,N,N',N'-tetrakis (2-pyridylmethyl) ethane-1,2-diamine) was applied in the following experiments. We first tested the effect of TPEN on FIV Vif’s ability to induce the degradation of feline A3s. 293T cells were co-transfected with FIV Vif or HIV-1 Vif and FcaA3Z2bZ3 or HsaA3G and were treated with increasing amounts of TPEN. The immunoblotting results obtained from lysates of these cells indicated that FIV Vif efficiently degraded FcaA3Z2bZ3 in the presence of low concentrations of TPEN (2–4 µM), while higher concentrations of TPEN (5–8 µM) blocked the FIV Vif-induced FcaA3Z2bZ3 degradation (Fig. 7A). A similar observation was found in the HIV-1 Vif-HsaA3G degradation assay; however, 4 µM TPEN blocked the HIV-1 Vif-induced HsaA3G degradation, while this concentration of TPEN had no influence on FcaAZ2bZ3 degradation by FIV Vif (Fig. 7A and B). Because higher concentrations of TPEN may nonspecifically influence the A3 degradation, we tested the interaction of HIV-1 Vif or FIV Vif with CUL5 by co-IP assays using lysates of cells treated with 5 µM TPEN. The results showed that 5 µM TPEN repressed the HIV-1 Vif/CUL5 interaction, thus supporting the previous model that HIV-1 Vif/CUL5 interaction is regulated by zinc (Fig. 7C) (27-29, 44). In stark contrast, treatment with 5 µM TPEN did not impair the FIV Vif/CUL5 binding. These data suggest that the FIV Vif/CUL5 interaction is zinc-independent.

Discussion
FIV Vif interacts with CUL5, ELOB, and ELOC to form an E3 complex to induce degradation of feline A3s (18). The interaction properties of FIV Vif with feline A3s and ELOB/C were previously identified (9, 11, 18). However, the interface between FIV Vif and CUL5 was not characterized.

Feline CUL5 and human CUL5 only differ in one amino acid, and this mutation does not affect its interaction with FIV Vif (18). It was also shown that FIV Vif can induce the degradation of feline A3s in both feline CRKF and human 293T cells, thus supporting that FIV Vif can interact with human and feline CUL5 (11, 18). In this study, we first identified that three FIV Vif N terminal mutants (53FI54, 57LR58, and 77VRE79) partially lost CUL5 binding, compared to wildtype FIV Vif (Fig. 2B). These Vif mutants showed the capacity to induce significant amounts of feline A3, however clearly less efficient than wildtype Vif. According to our FIV Vif/CUL5 structural model, does the N terminus of FIV Vif not directly bind CUL5 (Fig. 5A). Thus, we speculate that these residues rather interact with an unknown factor that regulates the FIV Vif/CUL5 binding. Indeed, a previous study suggested that FIV Vif requires an unknown factor to stabilize the FIV Vif/CUL5/ELOB/C complex (19).

CUL5-type ubiquitin ligases have a variety of adaptors that induce degradation of different cellular substrates (see recent review (43)). The adaptors of CUL5 share one common domain, i.e., the SOCS box, which consists of a BC box and a CUL5 box (43). The CUL5 box has a common sequence, -LPΘP-Θ-YL, in which Θ represents a hydrophobic residue, and CUL5 box is localized downstream of the BC box (43). Such a CUL5-like box is also found in HIV-1 Vif (PPLP motif), but this region does not interact with CUL5 (26, 45). In fact, HIV-1 Vif uses a hydrophobic region of helix 3 to interact with CUL5 (28, 33, 44). In FIV Vif, a typical CUL5 box is also missing (18). However, we found that FIV Vif, similar to HIV Vif, uses a hydrophobic region of helix 3 upstream of the BC box, position 174 and 175, to interact with CUL5 (Fig. 3
and 5). Vif proteins are not unique in applying unusual CUL5 boxes. For example, the adenovirus serotype 5 (Ad5) protein E4orf6 does not have a typical CUL5 box either, but it still interacts with CUL5 and forms an E3 ligase complex to degrade p53 (46).

Three HIV/SIV accessory proteins (Vpr, Vpx, and Vif) bind zinc, which is essential for the assembly of their E3 ligase complexes (47-49). Zinc is also required for BIV Vif/CUL2 binding, while MVV Vif/CUL5 interaction does not need zinc (50). In this study, we used the cell-permeable zinc chelator TPEN to investigate whether this chelator impairs the function of FIV Vif or HIV-1 Vif in A3 degradation. We found that TPEN inhibited both FcaA3 and HsaA3 degradation induced by FIV Vif and HIV-1 Vif, respectively (Fig. 7A and B). It is important to point out that high concentrations of TPEN will repress cellular pathways, such as those of the cellular lysosome and autophagy (51). Thus, it is possible that high concentrations of TPEN may impact upon many cellular degradation pathways. However, we found that 4 µM TPEN inhibited the HIV-1 Vif-induced HsaA3G degradation, while this concentration of TPEN had no influence on FcaAZ2bZ3 degradation by FIV Vif (Fig. 7A and B). In addition, the presence of 5 µM TPEN did not allow the isolation of HIV-1 Vif/CUL5 complexes, whereas FIV Vif/CUL5 complexes could still be detected (Fig. 7). Thus, our data support that zinc is not important for the FIV Vif/CUL5 interaction, as discussed previously (18). Other studies have demonstrated that TPEN treatment blocks the function of HIV-1 Vif, Vpr, and SIVmac Vpx (47). These findings indicate that, in the group of lentiviruses, there are zinc-dependent (e.g., HIV-1, BIV) and zinc-independent (e.g., FIV, MVV) Vif proteins. The inter-Vif diversity is high, and HIV-1 Vif shares only around 16% and 5.2% identical residues with BIV or FIV Vif, respectively. Thus, the requirement and structural consequences of zinc binding in Vifs are currently unclear. However, whether other metals (Mg$^{2+}$ or Ca$^{2+}$) bind FIV or MVV Vif needs more investigation. The SOCS3/CUL5 and E4orf6/CUL5 interactions are also zinc-
independent (46, 52), which, together, indicates that zinc is not necessary for binding of CUL5.

As the sequence of FIV Vif is 59 amino acids longer than that of HIV-1 Vif and no other structural template is available, an unstructured loop is found in our model between helices 2 and 3 (Fig. 5A). This loop could be involved in the binding of CUL5 or other cofactors; however, without a structural template, its function remains unknown. Whether this loop is specific for FIV Vif requires further investigation. Despite the low sequence identity between FIV and HIV-1 Vif and their difference in sequence length, our homology model of the FIV Vif/CUL5 complex is apparently accurate enough to predict interacting residues between these proteins. Our data from the CUL5 alanine variant of 52LW53 (Fig. 5C) and our homology model suggest that the interaction between Vif and CUL5 is also mediated by a hydrophobic contact. This is similar to the importance of the hydrophobic motif in the CUL5 BC box. In our homology model, helix 3 of Vif, which interacts with CUL5, is followed by C184. This cysteine was shown to be important for CUL5 interaction, and also for feline A3 or ELOB/C interaction, which differs from a previous study (18). As C184 is neither located in the Vif/CUL5 interface according to our homology model nor involved in zinc binding, we speculate that it might contribute to stabilizing the integral structure of FIV Vif. Overall, while our model certainly cannot be expected to be a perfect structural representation of the FIV Vif/CUL5 complex given the low sequence identity between FIV and HIV-1 Vif, we were able to successfully predict interacting residues between the two proteins in those regions with a higher sequence similarity.

Our data suggest that the Vif/CUL5 interaction in FIV structurally mirrors the one in HIV-1, even if the zinc dependency of both interactions is different. After cross-species transmissions of lentiviruses, the lentiviruses adapt to rather variable A3 proteins, which are
under positive selection. Positive selection is not seen in mammalian CUL5 and, thus, it makes sense that the mechanism by which Vif interacts with CUL5 shows evolutionarily preserved interfaces.

Acknowledgement

We thank Wioletta Hörschken for excellent technical assistance. We thank Eric Poeschla for plasmid pFP93. QG and ZZ are supported by China Scholarship Council. CM is supported by the Heinz-Ansmann foundation for AIDS research. We are grateful for computational support by the “Zentrum für Informations und Medientechnologie” at the Heinrich Heine University Düsseldorf.
Figure legends:

Figure 1. CUL5 is required for FIV Vif induced degradation of feline APOBEC3s. (A) FIV Vif interacts with CUL5, but not CUL2. myc-CUL5 or myc-CUL2 expression plasmids were co-transfected with FIV Vif-V5 expression plasmid. Cell lysates were immunoprecipitated with anti-myc beads and then analyzed by immunoblotting with anti-V5 antibody for FIV Vif and anti-myc antibody for CUL2 and CUL5. (B, C) Dominant-negative (DN)-CUL5, but not DN-CUL2, disrupts the degradation of feline A3s induced by FIV Vif. 293T cells were co-transfected with 300 ng expression plasmids for FcaA3Z2b-HA, FcaA3Z3-HA or FcaA3Z2bZ3-HA; 700 ng of DN-CUL5-FLAG or DN-CUL2-FLAG with 30 ng of FIV Vif-V5. pcDNA3.1 was used as control plasmid to replace the FIV Vif or dominant negative CUL5/2 expression plasmids. Cells were analyzed by immunoblotting using anti-HA, anti-V5, anti-CUL5, anti-Flag and anti-tubulin antibodies, respectively. (D) 293T cells were co-transfected with 50, 100 or 200 ng expression plasmids for FcaA3Z2b-HA, 700 ng of DN-CUL2-FLAG with 30 ng of FIV Vif-V5. The immunoblotting was performed as (B). (E) FIV Vif induces the degradation of FcaA3s in a proteasome-dependent manner. 293T cells were transfected with HsaA3G-HA or FcaZ2bZ3-HA; HIV-1 Vif-V5 or FIV Vif-V5 expression plasmids, pcDNA3.1 was used as empty plasmid control. The transfected cells were treated with the proteasome inhibitor MG132 (2.5, 5, 7.5 or 10 μM) or DMSO as control 36 h post transfection. Cells were harvested 12 h later (48 h after transfection) and then analyzed by immunoblotting with anti-HA, anti-V5 and anti-tubulin antibodies. The percentage of FcaA3 and HsaA3G were calculated relative to that in the absence of FIV Vif or HIV-1 Vif (set as 1.00).

Figure 2. Relevance of FIV Vif N-terminal residues for interaction with CUL5. (A) Schematic structure of FIV Vif. The numbers indicate the positions of amino acids mutated to alanines.
The relative positions of the feline A3Z2 and A3Z3 interaction sites (Z2 box, Z3 box) and the Elongin B/C interaction site (BC box) are represented. (B) Co-immunoprecipitation of FIV Vif wildtype and mutants with CUL5. myc-CUL5 or pcDNA3.1 empty plasmid were co-transfected with expression plasmids for FIV Vif-V5 wildtype or FIV Vif mutants. Immunoprecipitated complexes (IP) were analyzed by immunoblotting with anti-V5 for FIV Vif and anti-myc for CUL5. (C, D) 293T cells were transfected with expression plasmids for FcaZ2bZ3-HA, FIV Vif-V5 wildtype, indicated FIV Vif mutants or pcDNA3.1 empty plasmid (C); or FIV Vif-V5 wildtype, indicated FIV Vif mutants, T7-ELOC or HA-ELOB and pcDNA3.1 empty plasmid (D). Cells were harvested at 48 h after transfection, protein of cell lysates (input) and immunoprecipitated complexes (IP) were analyzed by immunoblots stained with anti-V5 antibody for FIV Vif, anti-HA antibody for FcaZ2bZ3-HA and HA-ElonginB and anti-T7 antibody for T7-ElonginC. (E) 293T Cells were transfected with FcaA3Z2bZ3-HA and FIV Vif-V5 wildtype or indicated FIV Vif mutant expression plasmids or pcDNA3.1 empty plasmid. Cells were harvested and analyzed by immunoblotting with anti-HA, anti-V5 and anti-tubulin antibodies, respectively.

Figure 3. Identification of determinants in the C-terminus of FIV Vif that regulate binding to CUL5. (A) Top: Sequence alignment of FIV Vifs from different FIV strains including 34TF10, C36, TM2, PRR, Shizuoka, Oma (Pallas’s cats), and lion subtype E. Bottom: Sequence alignment of C-terminal residues of FIV Vif (clone 34TF10) and HIV-1 Vif (clone NL4-3). KCCC: a motif of FIV Vif similar to the HIV-1 Vif Zinc interaction region HCCH. Boxed region: a conserved hydrophobic motif. Non-similar: black with no background; Conservative: blue with cyan background; Block of similar: black with green background; identical: Ren with yellow background; weakly similar: green with no background. (B) The structure of the HIV-1
Vif-CUL5 complex (Vif, orange; CUL5, green) (PDB entry 4N9F). A close-up view of the HIV-1 Vif-CUL5 interface is shown. The residues that are involved in the HIV-1 Vif-CUL5 interaction are indicated. Red dashed lines represent hydrogen bonds. (C) FIV Vif residues 174IR175 are important for FIV Vif interaction with CUL5. myc-CUL5 or pcDNA3.1 empty plasmids were co-transfected with FIV Vif-V5 wildtype or indicated FIV Vif mutant expression plasmids. Immunoprecipitated complexes (IP) were analyzed by immunoblotting with anti-V5 for FIV Vif and anti-myc for CUL5.

Figure 4. Mutating residues 174IR175 in FIV Vif does not impair interaction with FcaA3s, ELOB and ELOC. (A) FIV Vif 174175IR-AA mutant lost degradation activity against FcaA3Z2bZ3. Cells were transfected with FcaA3Z2bZ3-HA and FIV Vif-V5 wildtype or indicated FIV Vif mutant expression plasmids or pcDNA3.1 empty plasmid. Cells were harvested and analyzed by immunoblotting with anti-HA, anti-V5 and anti-tubulin antibodies, respectively. (B) FIV Vif 174175IR-AA mutant does not antagonize FcaA3Z2bZ3 antiviral activity. Single round FIVΔvif luciferase reporter virions were produced in the presence of feline A3 expression plasmids (FcaA3Z2bZ3) with FIV Vif wildtype or Vif mutants, pcDNA3.1(+) was added as a control for feline A3 (control) and FIV Vif (vector). Infectivity of reporter vectors was determined by quantification of luciferase activity in 293T cells transduced with normalized amounts of viral vector particles. (C, D) FIV Vif 174175IR-AA mutant still keep binding capability to FcaA3s, ELOB and ELOC. 293T cells were transfected with expression plasmids for FcaZ2bZ3-HA, FIV Vif-V5 wildtype, indicated FIV Vif mutants or pcDNA3.1 empty plasmid (C); or FIV Vif-V5 wildtype, indicated FIV Vif mutants, T7-ELOC or HA-ELOB and pcDNA3.1 empty plasmid (D). Cells were harvested at 48 h after transfection, protein of cell lysates (input) and immunoprecipitated complexes (IP) were analyzed by
western blots stained with anti-V5 antibody for FIV Vif, anti-HA antibody for FcaZ2bZ3-HA and HA-ElonginB and anti-T7 antibody for T7-ElonginC.

**Figure 5. FIV Vif-CUL5 3D structure model.** (A) Homology model of the FIV Vif (orange)-CUL5 (green) complex in cartoon representation. Helices α3 and α4 of Vif are interacting with CUL5. The model contains two unstructured loops (navy) before and after helix α2, as no structural template is available for these regions. These loops might be important for binding other parts of the complex. Residues that were subjected to mutational analysis are shown in spheres representation. The region of the close up shown in B is indicated by a light plum rectangle. (B) Close-up view of the homology model of the FIV Vif (orange)-CUL5 (green) complex in cartoon representation, with interacting residues shown in sticks representation. (C) The myc-CUL5 wildtype or indicated mutants expression plasmids were co-transfected with FIV Vif-V5 wildtype expression plasmid into 293T cells. Cell lysates were immunoprecipitated with anti-myc beads and then analyzed by immunoblotting with anti-V5 antibody for FIV Vif and anti-myc antibody for CUL5. (D) 293T cells were co-transfected with expression plasmids for FcaA3Z2b-HA, FcaA3Z3-HA or FcaA3Z2bZ3-HA; CUL5-52LW53-AA mutant with FIV Vif-V5. pcDNA3.1 was used as control plasmid to replace the FIV Vif or CUL5 expression plasmids. Cells were analyzed by immunoblotting using anti-HA, anti-V5, anti-CUL5, anti-Flag and anti-tubulin antibodies, respectively. (E) Model of FIV Vif with E3 ligase complex. The CUL5 interaction sites of FIV Vif (174IR175) are shown as red star.

**Figure 6. C184 of FIV Vif is essential for Vif-CUL5, Vif-FcaA3 and Vif-ELOB/C interaction** (A) Close-up view of the homology model of the FIV Vif (orange)-CUL5 (green) complex in
cartoon representation, with residues that underwent mutational analysis shown in sticks 
representation. These residues are: K181, K182, C184, C187 and C192 in the loop following 
helix 3 of FIV Vif; C138 that forming a disulfide bond with C192 is also shown. (B) Sequence 
representation of FIV Vif C terminal domain (CTD). (C) myc-CUL5 or pcDNA3.1 empty plasmid 
was co-transfected with expression plasmids for FIV Vif-V5 wildtype or indicated FIV Vif 
mutants. Immunoprecipitated complexes (IP) were analyzed by immunoblotting with anti-V5 
for FIV Vif and anti-myc for CUL5. (D) 293T cells were transfected with expression plasmids 
for FIV Vif-V5 wildtype or indicated FIV Vif mutants. Immunoprecipitated complexes (IP) 
were analyzed by immunoblotting with anti-V5 for FIV Vif and anti-CUL5 for CUL5. (E, F) FIV 
Vif C184S mutant lost binding capability to FcaA3s, ELOB and ELOC. 293T cells were 
transfected with expression plasmids for FcaZ2bZ3-HA, FIV Vif-V5 wildtype, indicated FIV Vif 
mutants or pcDNA3.1 empty plasmid (E); or FIV Vif-V5 wildtype, indicated FIV Vif mutants, 
T7-ELOC or HA-ELOB and pcDNA3.1 empty plasmid (F). Cells were harvested at 48 h after 
transfection, protein of cell lysates (input) and immunoprecipitated complexes (IP) were 
analyzed by western blots stained with anti-V5 antibody for FIV Vif, anti-HA antibody for 
FcaZ2bZ3-HA and HA-ElonginB and anti-T7 antibody for T7-ElonginC. (G) 293T cells were 
transfected with FcaA3Z2bZ3-HA and FIV Vif-V5 wildtype or indicated FIV Vif mutant 
expression plasmids or pcDNA3.1 empty plasmid. Cells were harvested and analyzed by 
immunoblotting with anti-HA, anti-V5 and anti-tubulin antibodies, respectively.

**Figure 7. FIV Vif binding to CUL5 is zinc independent.** (A, B) 293T cells were transfected with 
expression plasmids for FcaZ2bZ3-HA (A) or HsaA3G-HA (B); HIV-1 Vif-V5 or FIV Vif-V5, 
pcDNA3.1 was used as empty plasmid control. The transfected cells were treated with zinc 
chelator TPEN (2, 3, 4, 5, 6, 7 or 8 μM) or DMSO as control 36 h post transfection. Cells were
harvested 12 h later (48 h after transfection) and then analyzed by immunoblotting with anti-HA, anti-V5 and anti-tubulin antibodies. (C) myc-CUL5 expression plasmid was co-transfected with FIV Vif-V5 or HIV-1 Vif-V5 expression plasmids into 293T cells. The transfected cells were treated with 5 µM TPEN or DMSO 36 h post transfection. Cell lysates were immunoprecipitated with anti-myc beads and then analyzed by immunoblotting with anti-V5 antibody for FIV Vif and HIV-1 Vif, anti-myc antibody for CUL5.


Fig. 1

A

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Input

IP-myc

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α-HA

α-CUL5

α-V5

α-Tubulin

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α-HA

α-Flag

α-V5

α-Tubulin

D

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α-HA

α-Flag

α-V5

α-Tubulin

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α-HA

α-V5

α-Tubulin
Fig. 2

A

12-19
24-27

NTD

CUL5

CTD

B

FIV Vif-V5
CUL5-myc
FIV Vif-V5
CUL5-myc

WT
TLQ
S964
51386
77497

Input
IP-myc

C

FcaA3Z2bZ3

FIV Vif-V5
FcaA3Z2bZ3-HA
FIV Vif-V5
FcaA3Z2bZ3-HA

WT
TLQ
S964
51386
77497

Input
IP-HA

D

Elongin B+Elongin C

WT
WT
S964
77497

FIV Vif-V5
Input
Elongin C-T7
Elongin B-HA
FIV Vif-V5
Elongin C-T7
Elongin B-HA

FIV Vif
Input
FcaA3Z2bZ3

IP-HA

α-HA

α-HA

α-V5

α-Tubulin
Fig. 4

A

B

C

D

Relative Infectivity (%)

Control

Vector

WT

TLQ

17AIR175

***

***
Fig. 6
Fig. 7

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HIV-1 Vif

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α-HA, α-V5, α-Tubulin