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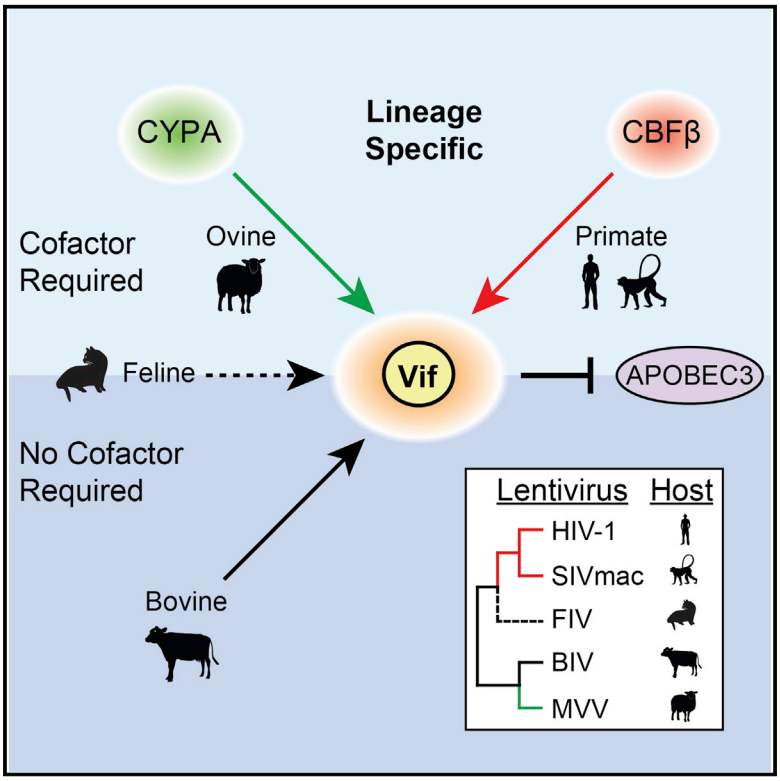
Lineage-Specific Viral Hijacking of Non-canonical E3 Ubiquitin Ligase Cofactors in the Evolution of Vif Anti-APOBEC3 Activity

Graphical Abstract



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In Brief

How viral proteins evolve functions without compromising ancestral ones is not well understood. Kane et al. now report that the lentiviral protein Vif has evolved to hijack host factors in a lineage-specific manner while conserving an ancestral viral-host complex promoting viral infection.

Highlights

- We present a comparative proteomic study of the viral protein Vif across the *Lentivirus* genus
- Only primate lentiviral Vif proteins require cofactor CBFβ
- BIV Vif requires no cofactor; MVV Vif requires a different cofactor, CYPA
- Cofactor use by Vif may serve as a gain-of-function mechanism

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Lineage-Specific Viral Hijacking of Non-canonical E3 Ubiquitin Ligase Cofactors in the Evolution of Vif Anti-APOBEC3 Activity

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SUMMARY

HIV-1 encodes the accessory protein Vif, which hijacks a host Cullin-RING ubiquitin ligase (CRL) complex as well as the non-canonical cofactor CBFβ, to antagonize APOBEC3 antiviral proteins. Non-canonical cofactor recruitment to CRL complexes by viral factors, to date, has only been attributed to HIV-1 Vif. To further study this phenomenon, we employed a comparative approach combining proteomic, biochemical, structural, and virological techniques to investigate Vif complexes across the lentivirus genus, including primate (HIV-1 and simian immunodeficiency virus macaque [SIVmac]) and non-primate (FIV, BIV, and MVV) viruses. We find that CBFβ is completely dispensable for the activity of non-primate lentiviral Vif proteins. Furthermore, we find that BIV Vif requires no cofactor and that MVV Vif requires a novel cofactor, cyclophilin A (CYPA), for stable CRL complex formation and anti-APOBEC3 activity. We propose modular conservation of Vif complexes allows for potential exaptation of functions through the acquisition of non-CRL-associated host cofactors while preserving anti-APOBEC3 activity.

INTRODUCTION

Viruses must overcome host challenges to replicate successfully in an infected host. These challenges include not only the mechanics of viral entry, genome replication, assembly, and budding but also a variety of host-defined replication barriers, both innate and adaptive. During productive infection, viral proteins rewire the host cell through a series of protein-protein interactions (PPIs) to promote viral replication. Systematic and unbiased mapping of these host-pathogen interactions can yield novel information concerning both viral biology and the endogenous functions of hijacked host factors.

An effective method for mapping host-pathogen interactions involves affinity purification of epitope-tagged viral proteins from host cells followed by mass spectrometry (AP-MS) to identify interacting host factors. This approach has been used to map global host-pathogen PPIs for HIV-1 (Jäger et al., 2012a), herpes (Davis et al., 2015), and hepatitis C (Ramage et al., 2015), as well as to study the PPIs of individual viral proteins in HPV (Tan et al., 2012; White et al., 2012a, 2012b), influenza (York et al., 2014), and picornaviruses (Greninger et al., 2012). Historically, these types of proteomic analyses have focused on a single virus or closely related sets of viruses and typically from the same (human) host.

In this study, we devised a strategy for the systematic, comparative analysis of host-pathogen PPIs focusing on the well-characterized lentivirus genus to analyze the complexes formed by representative Vif proteins from different lentiviral clades, including that of HIV-1. HIV-1 Vif is required for pathogenesis in vivo and serves as the virus' defense against host antiviral APOBEC3 (A3) proteins. In the absence of Vif, members of the A3 family of restriction factors package into budding virions, where they interfere with reverse transcription and induce lethal G-to-A hypermutation in viral cDNA (Harris et al., 2003; Iwatani et al., 2007; Mangeat et al., 2003; Zhang et al., 2003). HIV-1 Vif overcomes this replication block by acting as an adaptor between the A3 proteins and an endogenous ubiquitin ligase complex that catalyzes poly-ubiquitylation of the A3 proteins, resulting in their subsequent proteasomal degradation (Hultquist et al., 2011; Sheehy et al., 2002, 2003; Yu et al., 2003).

The HIV-1 Vif E3 ligase complex is composed of the endogenous CRL5 members, including Cullin-5 (CUL5), elongin B (ELOB), elongin C (ELOC), and RING-box protein 2 (RBX2), but also requires the additional Vif-dependent recruitment of a non-canonical cofactor, core-binding factor, beta subunit (CBFβ) (Guo et al., 2014; Jäger et al., 2012b; Zhang et al., 2012). CBFβ heterodimerizes with members of the RUNX family of transcription factors, serving to both stabilize RUNX steady-state levels and to enhance DNA-binding affinity (Huang et al., 2001; Tahirov et al., 2001). Recruitment of CBFβ serves to stabilize HIV-1 Vif and is required for HIV-1 Vif A3 degradation activity in vivo (Hultquist et al., 2012; Jäger et al., 2012b; Kim et al., 2013; Miyagi et al., 2014; Zhang et al., 2012). Recent work has shown that this recruitment alters endogenous RUNX activity through competitive binding of HIV-1 Vif to CBFβ, potentially to the benefit of the virus (Kim et al., 2013; Klase et al., 2014).

We chose to focus our comparative study on Vif for three primary reasons. First, a Vif protein is expressed in four of the five major lentiviral clades, each of which is known to mediate the proteasomal degradation of the cognate host A3 proteins (Larue et al., 2010). Second, unlike ubiquitously conserved lentiviral components such as Gag or Pol, Vif is not known to be required for the mechanics of viral replication and thus is potentially less constrained over the course of virus evolution. Third, whereas the mechanism of HIV-1 Vif-mediated A3 degradation is well characterized, little is known about the requirements for Vif proteins from other clades. Whereas recruitment of ELOC is assumed based on the conserved BC-box motif, it is unknown whether these Vif proteins recruit the same core E3 ligase complex and whether they require recruitment of a non-canonical E3 ligase component such as CBFβ. In fact, recent work has suggested that other factors may be required for Vif stability and function in non-primate lentiviruses (Ai et al., 2014; Zhang et al., 2014a, b).

Here, we report a comparative proteomics strategy for the study of orthologous host-pathogen PPIs, which we subsequently use to analyze the complexes formed by representative Vif proteins from different lentiviral clades, including primate lentiviruses (HIV-1 and simian immunodeficiency virus macaque [SIVmac]), bovine immunodeficiency virus (BIV), feline immunodeficiency virus (FIV), and the ovine-tropic maedi-visna virus (MVV). We find that, whereas all lentiviral Vif orthologs can hijack the CRL5 complex in human cells, only primate lentiviral Vif proteins hijack CBFβ and require it for A3 degradation. Whereas one non-primate lentiviral Vif, from BIV, appears to operate independently of any non-canonical cofactors, another Vif, from MVV, requires a novel non-canonical cofactor, cyclophilin A (CYPA), for in vitro reconstitution and in vivo A3-degrading activity. These results demonstrate an unexpected mechanistic flexibility in viral rewiring of the host cell despite the maintenance of a conserved activity. They furthermore suggest a modular conservation of host-pathogen interactions whereby novel PPIs may be formed with novel partners to serve the same functional purpose yet with potentially new orthogonal roles. We predict that the use of modular conservation to allow for mechanistic flexibility may be a generalizable model for viral protein evolution.

RESULTS

Divergent Vif Proteins Interact with a Conserved Host CRL Complex

All known lentiviruses express a Vif protein except for equine infectious anemia virus and the extinct rabbit endogenous lentivirus RELIK (Katzourakis et al., 2007; Kawakami et al., 1987). The Vif protein from each lentivirus is known to perform at least one conserved function, degradation of the restrictive A3 proteins from the cognate host (Hultquist et al., 2011; Larue et al., 2010; Sheehy et al., 2003; Yu et al., 2003). Despite this conservation in function, the primary sequence of these proteins is highly divergent, sharing no more than 25% identity of alignable residues between any pair (Figures 1A and S1A). The only obvious conserved motif is the ELOC-binding BC-box, which is known to be essential for A3 degradation. This has led to the hypothesis that each Vif protein mediates A3 degradation by recruitment of the same CRL5 complex, but a systematic characterization of each lentivirus' Vif complex had not previously been carried out.

In order to determine which host factors are physically bound to Vif proteins across the lentivirus phylogeny, we employed an unbiased proteomic approach using AP-MS to study five divergent Vif proteins from MVV, BIV, FIV, SIVmac, and HIV-1 (Table S1). Affinity tags comprising 2×Strep or 3×Flag were fused to either the amino (N) or carboxy (C) terminus of the Vif proteins, and tagged Vif constructs were either transiently expressed in HEK293T cells or used to make stable, doxycycline-inducible Jurkat T cell lines (Figure 1B). Tagged Vif proteins were affinity purified and eluates subjected to SDS-PAGE and silver staining (Figure 1C) and then analyzed by liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) to identify co-purified host factors (Jäger et al., 2012a). Putative interactions identified by AP-MS were scored using the significance analysis of interactome (SAINT) algorithm (Choi et al., 2011), and interactions with a SAINT probability score $\geq$0.9 in at least one Vif data set were included (Table S2). Prey scores were then organized by cell line and hierarchically clustered by correlation (Figure S2A). Within this data set, we observe expected interactions with CRL complex proteins, including CUL2, CUL5, ELOB, ELOC, RBX2, and RBX1, strongly suggesting a generally conserved mechanism for A3 proteasomal degradation among Vif proteins (Figure 1D). We observe within the data set a terminus-specific effect on Vif-CULLIN specificity between CUL2 and CUL5, particularly with the non-primate lentivirus Vif proteins (Figures 1D, 1E, and S2B–S2D).

The association of Vif with CBFβ was only observed for primate lentivirus Vif proteins (SIVmac and HIV-1) in the AP-MS data sets for both HEK293T cells and Jurkat T cells (Figure 1D), an observation confirmed by immunoblot analysis (Figures 1E and S2B). To test whether Vif proteins not observed to physically interact with CBFβ were still functionally regulated by the factor, we employed a single-cycle HIV-1 infectivity assay testing for Vif-mediated A3 degradation in the presence and absence of CBFβ (Figure 2A). We observed that HIV-1 and SIVmac require CBFβ for Vif-mediated rescue of HIV-1 infectivity from A3 restriction (Figures 2B and 2C), as previously reported (Hultquist et al., 2012; Jäger et al., 2012b). Conversely and consistent with the AP-MS results, the non-primate lentivirus Vif proteins

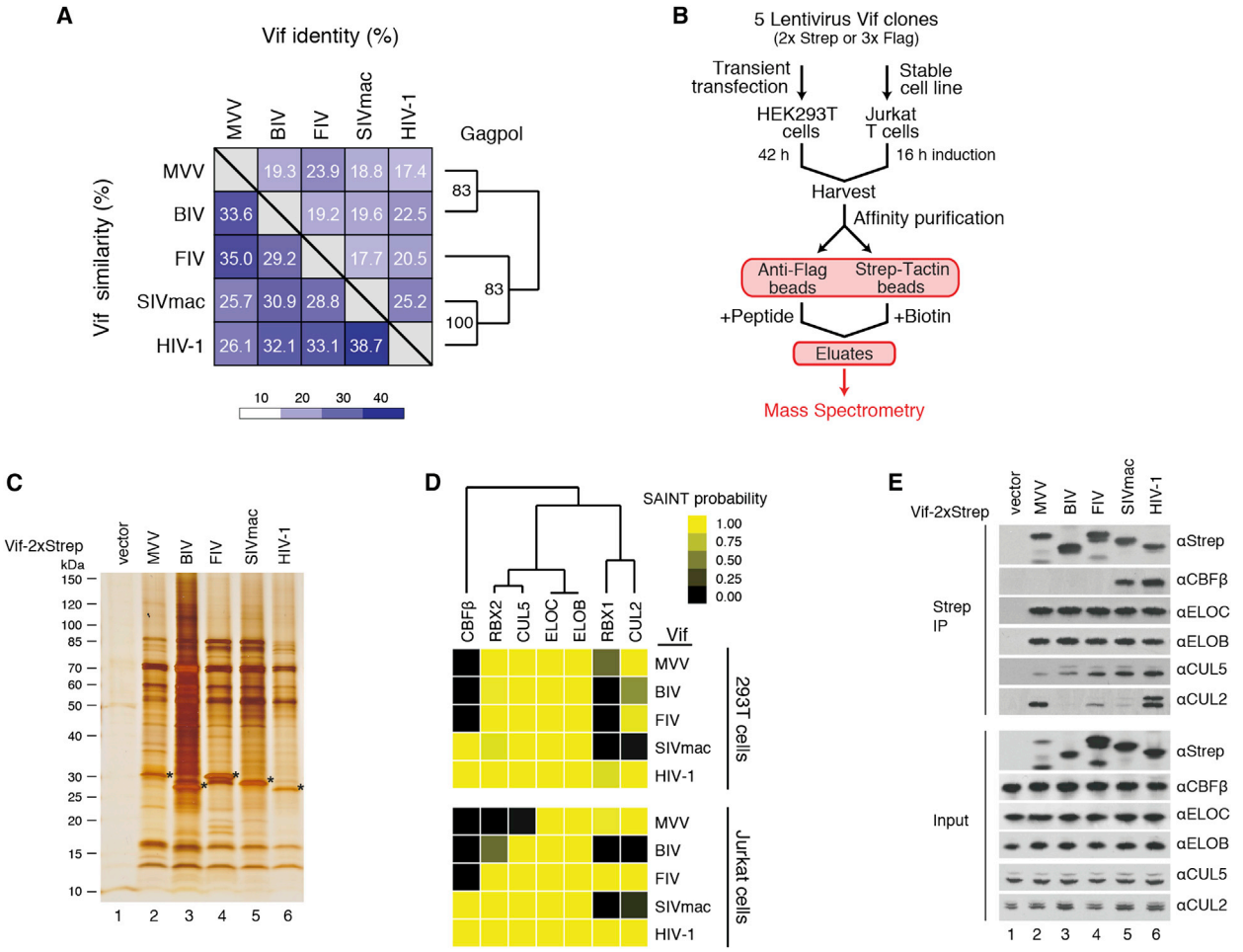

Figure 1. Proteomic Analysis of Lentiviral Vif Proteins

(A) Percent identity and percent similarity matrix of Vif proteins used in this study. The distance tree was generated from Gagpol protein sequence of viruses, with bootstrap support values.

(B) Flow chart of affinity purification-mass spectrometry (AP-MS) pipeline used to identify Vif-interacting host proteins.

(C) Representative silver-stained SDS-PAGE of eluates from a Vif-Strep purification in transiently transfected HEK293T cells. Asterisks indicate Vif proteins.

(D) Cullin ubiquitin ring ligase (CRL) complex proteins identified in AP-MS experiments, colored by SAINT score and clustered hierarchically by correlation.

(E) Immunoblot of a Strep affinity purification of Strep-tagged Vif proteins transiently expressed in HEK293T cells, probing for CRL proteins highlighted in (D).

See also Figures S1 and S2 and Tables S1 and S2.

(MVV, BIV, and FIV) showed no dependence on CBFβ for activity, eliciting equivalent ability to rescue HIV-1 infectivity from A3 restriction both in the presence and in the absence of CBFβ (Figures 2D–2F). This finding agrees with recently reported data for MVV, BIV, and FIV Vif proteins (Ai et al., 2014; Han et al., 2014; Zhang et al., 2014a, 2014b).

BIV Vif Assembles a CRL Complex without a Non-canonical Cofactor

Having observed no dependence of the non-primate Vif proteins on CBFβ for the in vivo degradation of cognate A3 proteins, we asked whether the corresponding Vif-CRL complexes might reconstitute in vitro without CBFβ or other additional host factors. We have shown previously that HIV-1 Vif requires CBFβ to form a stable complex with ELOB and ELOC and CUL5/RBX2 in vitro (Figures 2G and 2H; Jäger et al., 2012b). Unexpectedly, BIV Vif formed a stable trimer with ELOB and ELOC alone with no other cofactors required as assessed by size-exclusion chromatography (Figure 2G). This trimer readily associated with the CUL5/RBX2 scaffold (Figures 2G and 2H) to form an active complex capable of poly-ubiquitylating Myc-tagged bovine A3Z3 (BtA3Z3) (Figure 2I). The level of ubiquitylation was comparable to HIV-1 Vif-CRL-mediated ubiquitylation of Myc-tagged human A3F CTD domain (HsA3F-CTD) (Figure 2I). Reconstitution of active BIV Vif-CRL5 complex thus confirmed that only the endogenous CRL5 complex is required for BIV Vif activity, in stark contrast with the primate lentiviral Vif proteins that require the additional recruitment of CBFβ for equivalent activity.

CYPA Implicated as an MVV Vif Host Cofactor

Whereas we successfully reconstituted the BIV Vif-CRL5 complex without a non-canonical host cofactor, we were unable to

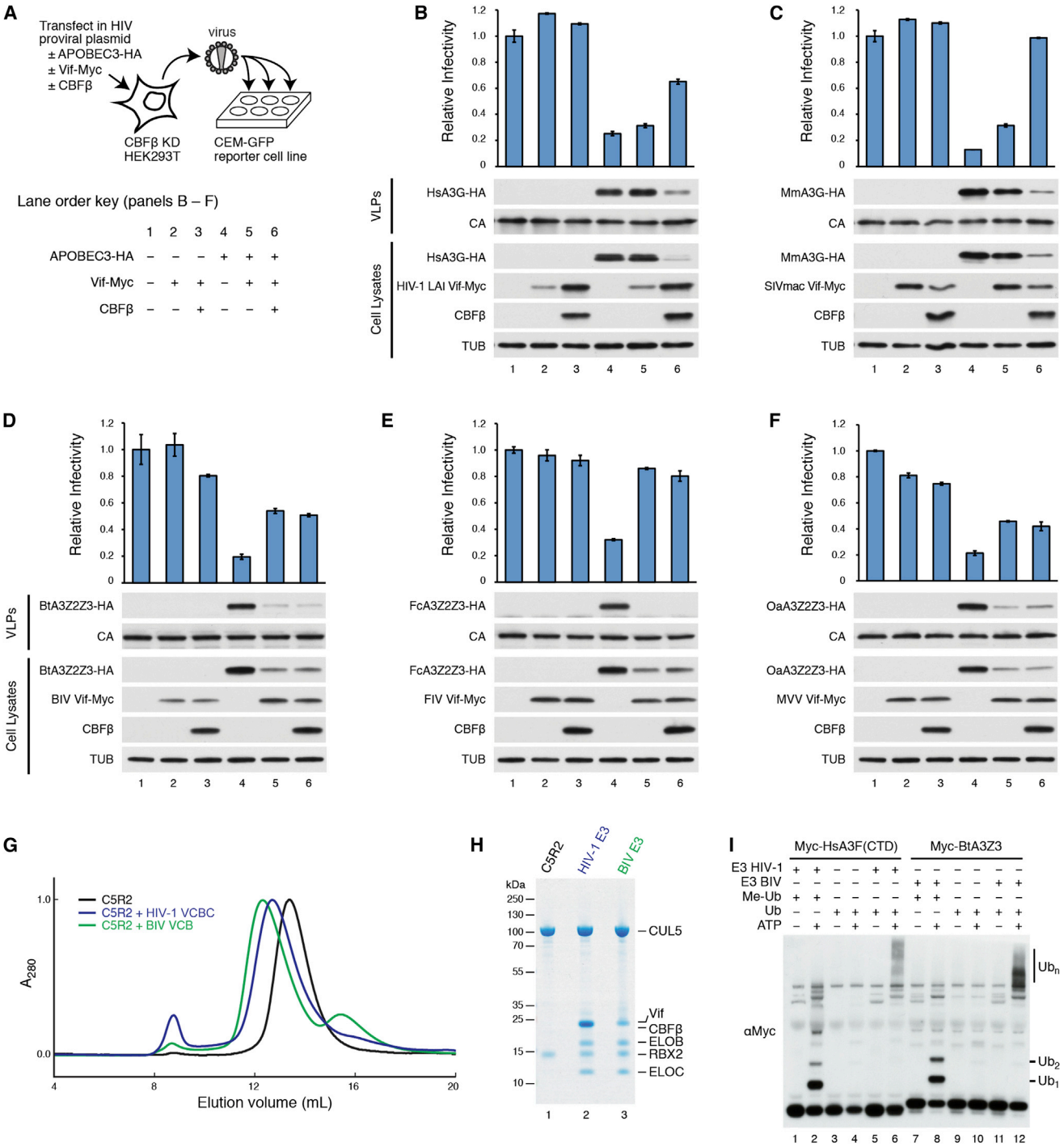

Figure 2. Vif Dependence on CBFβ Is Primate Lentivirus Specific

(A) Schema of single-round infection assay.

(B–F) Infectivity assays described in (A), using Vif proteins and cognate A3 proteins. Bars represent mean ± SE of GFP expression from virus reporter lines. Viral, A3, and CBFβ proteins are detected by immunoblot. VLPs, virus-like particles.

(G) UV absorbance curves are shown for gel filtration of CUL5-RBX2 (C5R2) alone or mixed with an excess of indicated Vif complexes (BIV VCB, Vif-ELOB-ELOC; HIV-1 VCBC, Vif-ELOB-ELOC-CBFβ). Peaks are observed at earlier elution volumes when C5R2 is mixed with Vif complexes, indicating E3 complex formation.

(H) Coomassie-blue-stained SDS-PAGE of peak fractions collected from gel filtration runs shown in (G).

(I) Immunoblot of ubiquitylation reactions with either HIV-1 Vif or BIV Vif E3, using myc-tagged C-terminal domain of human A3F (Myc-HsA3F CTD) or bovine A3Z3 (Myc-BtA3Z3) as substrate, respectively. Me-Ub, methylated ubiquitin; Ub, ubiquitin.

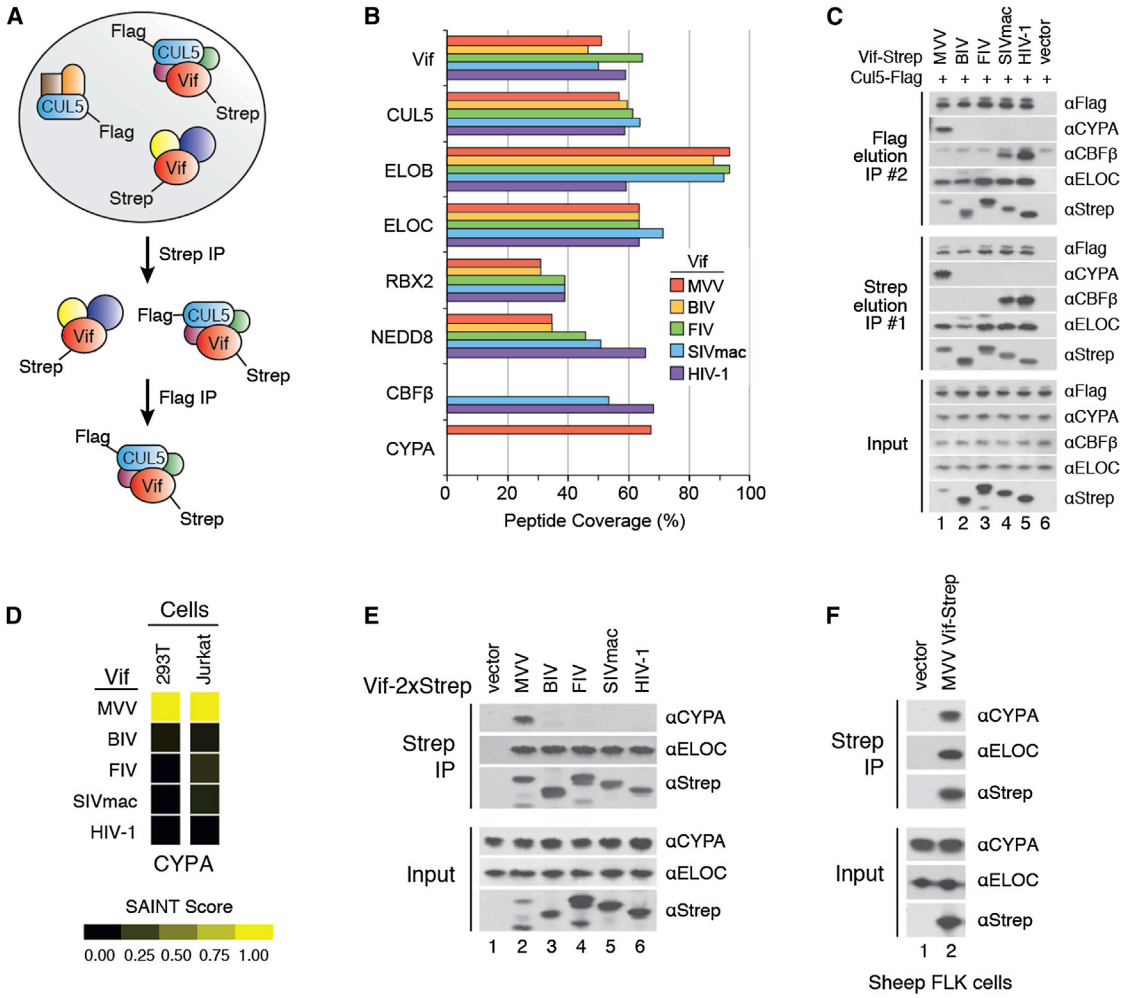

Figure 3. CYPA Is Tightly and Uniquely Associated with MVV Vif

(A) Cartoon of double affinity purification experiment.

(B) LC-MS/MS mass spectrometry results from the double purification of 2×Strep-tagged Vif proteins and 3×Flag-tagged CUL5. Bars indicate peptide percent coverage of proteins identified in eluates after second purification step.

(C) Immunoblot of input lysates and first and second purification eluates used for MS analysis in (B).

(D) Heatmap of AP-MS data for CYPA in HEK293T and Jurkat T cell lines. Color indicates SAINT score.

(E) Re-probing of immunoblot of Strep affinity purification shown in Figure 1F; ELOC is shown as a control for CRL complex interaction.

(F) Strep purification of MVV Vif from transient transfection of ovine FLK cells. CYPA, ELOC, and Vif are detected by immunoblot.

do so with the other non-primate lentiviral Vif complexes (MVV and FIV). Both MVV Vif-ELOB-ELOC and FIV Vif-ELOB-ELOC complexes aggregated during size-exclusion chromatography after purification, mimicking behavior observed with HIV-1 Vif in the absence of CBFβ (Figures S3A and S3B; Kim et al., 2013). These data suggested additional host cofactors are required for stable complex formation of the MVV Vif and FIV Vif CRL5 complexes.

To identify the missing components of the non-primate Vif-CRL5 complexes, we utilized a double affinity-tag purification approach (He et al., 2010; Jäger et al., 2012b). We co-transfected HEK293T cells with 2×Strep-tagged Vif proteins and 3×Flag-tagged CUL5 and then performed two-step tandem affinity purification, initially purifying Vif then CUL5 (Figure 3A). Eluates were then subjected to LC-MS/MS analysis. In each sample, we identified high peptide coverage of the Vif and CUL5 baits as well as of proteins associated with the CRL5 complex (ELOB, ELOC, RBX2, and NEDD8; Figures 3B and 3C). Consistent with our previous data, peptides from CBFβ were only observed in the SIVmac and HIV-1 Vif samples. Although we failed to identify likely cofactors in the FIV Vif sample, we did observe a highly abundant non-CRL5 complex host factor in the MVV sample: the peptidyl-prolyl isomerase CYPA (Fischer et al., 1984, 1989; Takahashi et al., 1989; Figures 3B and 3C). CYPA has been reported previously to interact with HIV-1 Capsid (Luban et al., 1993; Thali et al., 1994) but has never before been implicated to play a direct role in Vif biology. Re-examining our Vif single-purification AP-MS data set (Table S2), we

observed that CYPA has a specific and highly scoring interaction with only MVV Vif in both HEK293T cells and Jurkat T cells (Figures 3D and 3E). To verify that the CYPA-MVV Vif interaction is not an artifact of expression in human cells, we performed an affinity purification of MVV Vif in natural host sheep (ovine) FLK cells and observed a strong association with endogenous ovine CYPA (Figure 3F).

Identification of MVV Vif Residues P21, P24 as a Likely CYPA Binding Site

After identifying CYPA as a likely member of the MVV Vif-hijacked CRL5 complex, we performed a limited alanine scan of MVV Vif focusing on proline residues, the substrate of CYPA peptidyl-prolyl isomerase activity. We co-expressed Strep-tagged MVV Vif constructs with 3×Flag-tagged CYPA in HEK293T cells and performed a Flag immunoprecipitation, assaying mutants for co-purification of CYPA (Figure 4A). We identified two MVV Vif mutants that were deficient in CYPA binding: P21A/P24A and P192A. In addition, a BC-box mutant (SLQ::AAA) unable to interact with ELOC also was deficient in CYPA binding, suggesting that at least partial assembly of the Vif-hijacked ubiquitin ligase complex is required for stable interaction between MVV Vif and CYPA. We focused on the P21/P24 region as the site of CYPA binding as the sequence is unique within sheep (MVV) and the closely related goat (CAEV)-infecting lentivirus Vif proteins (Figure 4B), whereas the P192 residue is potentially conserved across the broader genus (Figure S1). Indeed, we observe that CAEV Vif is able to co-purify CYPA in an analogous manner to MVV (Figure S4A), confirming our suspicion that the MVV/CAEV clade-specific P21/P24 region is important for CYPA binding.

MVV Vif $_{21}$PxxP$_{24}$ Region Binds CYPA Active Site

To determine whether the P21/P24 region of MVV Vif is directly responsible for binding CYPA, we performed NMR shift experiments on CYPA in the presence of a Vif peptide. 2D ^{15}N-^{1}H chemical shift correlation spectroscopy was performed on labeled CYPA in the unbound state and in the presence of increasing concentrations of a MVV Vif peptide containing both P21 and P24 (MVV Vif$^{17–26}$). The addition of the Vif peptide (up to 300 μM) induced significant chemical shift perturbations on the protein spectrum, although saturation was not reached even at the highest Vif concentration and a binding constant could not be determined (Figure 4D). Mapping of the resonances that disappeared upon addition of the MVV Vif peptide indicated that the peptide binds to the active site of CYPA (Figures 4C and 4D). Additional residues surrounding the active site were observed to enter slow exchange mode or displayed peak broadening (Figure S3C).

To independently verify that MVV Vif interacts with CYPA via the active site of the enzyme, we treated cells expressing 2× Strep-tagged MVV Vif with the CYPA inhibitor cyclosporine A (CsA), which binds to the active site of CYPA and competes for substrate binding (Figure 4C; Takahashi et al., 1989; Thériault et al., 1993). Performing a titration of CsA treatment followed by affinity purification of MVV Vif, we observed a loss of MVV Vif binding to CYPA in the 5–10 μM range, with corresponding weakening of ELOC binding (Figure 4E). The CsA concentration required to disrupt CYPA interaction with MVV Vif is about an order of magnitude greater than our observed concentration required for disrupting the interaction between CYPA and HIV-1 Capsid (Figure S4B). Whereas HIV-1 Capsid binds to CYPA, we find that MVV Capsid does not (Figure S4C). From these experiments, we conclude that MVV Vif binds directly to the CYPA binding site via the $_{21}$PxxP$_{24}$ motif.

CYPA Is Critical for MVV Vif-CRL5 Reconstitution

After observing the CYPA-MVV Vif interaction in vivo, we revisited reconstitution of the MVV Vif-CRL5 complex with the addition of CYPA, in a manner analogous to HIV-1 Vif and CBFβ (Jäger et al., 2012b). CYPA rescued MVV Vif-ELOB-ELOC complex stability (Figures 4F and 4G) and allowed for the formation of a stable complex with CUL5-RBX, capable of ubiquitylating ovine A3Z3, but not human A3H, in vitro (Figure 4H).

To determine whether MVV Vif-CYPA-CRL5 complex conforms to a similar macromolecular organization as the HIV-1 Vif-CBFβ-CRL5 complex, small-angle X-ray scattering (SAXS) analysis was performed on the reconstituted MVV Vif-CYPA-CRL5 complex as well as HIV-1 Vif$^{1–174}$-CBFβ-CRL5. Both complexes were monodisperse and well folded under the SAXS experimental conditions, therefore suitable for envelope generation (Figures S5A–S5C). The pairwise distribution function revealed that both HIV-1 and MVV complexes have similar maximal dimensions (Dmax), with values of 190 Å and 200 Å, respectively (Figures 4I; Table S3). Analysis of the resulting envelopes revealed that, despite different complex constituents, the overall surface of the macromolecular assemblies is quite similar with an overall elongated E3 ring-ligase conformation. To determine how well the SAXS envelopes fit the available structure data, a model of HIV-1 Vif-CRL5 was generated. Whereas the HIV-1 Vif-CRL5 model was relatively well fit into its experimental SAXS envelope, with a chi value of 1.89 (Figure S5D), there was a slightly poorer fit into the experimentally determined MVV Vif-CRL5 envelope, possibly due to differences in substrate receptor structure (Figure 4J). This likely reflects different folding of CYPA and CBFβ and possibly of MVV and HIV-1 Vif proteins.

Correlated Deficiencies in CYPA Binding and A3 Antagonism in MVV Vif Mutants

We next asked whether CYPA also affected MVV Vif A3 degradation activity. Focusing our analysis on the P21, P24 putative CYPA binding site, we co-expressed 3×HA-tagged ovine A3Z2Z3 (OaA3Z2Z3) with 2×Strep-tagged wild-type, P21A, P24A, P21A/P24A, and SLQ::AAA MVV Vif mutants and observed that the loss of CYPA binding to these mutants correlated with reduction in OaA3Z2Z3 degradation activity (Figure 5A). To determine whether the loss-of-function MVV Vif mutants were deficient in forming active CRL complexes or merely unable to bind substrate due to structurally compromising mutations, we performed co-affinity purification experiments with Strep-tagged MVV Vif mutants in the presence of HA-tagged OaA3Z2Z3. We observed that both mutants deficient in A3 degradation activity (P21A/P24A and SLQ::AAA) bound OaA3Z2Z3 (Figure 5B), indicating that the mutations prevent proper assembly of a functional CRL complex without disrupting substrate binding.

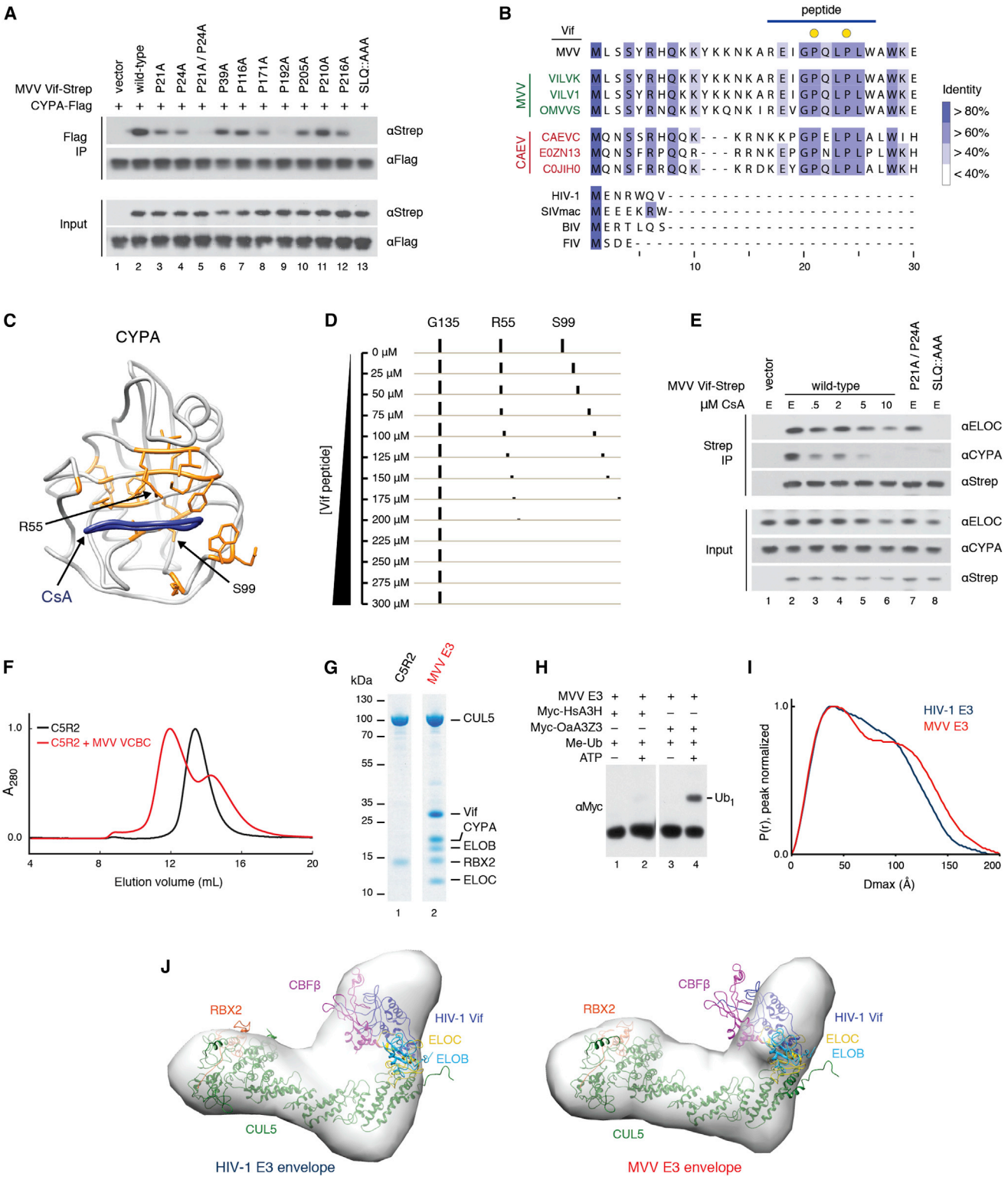

Figure 4. CYPA Is a Component of the MVV Vif-Hijacked CRL Complex

(A) Co-purification testing in vivo interaction between CYPA and either wild-type or mutant MVV Vif. CYPA-Flag and various MVV Vif-Strep constructs are co-transfected, followed by a Flag immunoprecipitation. Co-purification of MVV Vif constructs is assayed by immunoblot.

(B) Multiple sequence alignment of MVV, CAEV, and the other Vif proteins used in this study (BIV, FIV, SIVmac, and HIV-1) referenced to the first 30 amino acids of MVV Vif. Residues P21 and P24 are highlighted, as well as the region used for the CYPA-binding assay in (D). Residues are colored by percent identity.

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To test the importance of the MVV Vif-CYPA interaction for viral infectivity in vivo, we performed a spreading infection with MVV strain KV1772 in primary sheep macrophages with wild-type, P21A, P24A, P21A / P24A, or *vif*-null strains. Whereas all mutants showed reproducibly diminished spreading kinetics compared to wild-type virus, the P21A/P24A mutant showed a greater spreading defect than the individual P21A and P24A mutations, copying the severe spreading defect observed with the SLQ::AAA and *vif*-null viruses (Figure 5C). The assay was also performed in primary sheep choroid plexus (SCP) cells with similar results (Figure S6E). After spread, we cloned and sequenced the integrated proviruses in each infection to determine whether viral restriction correlated with G-to-A mutational load indicative of A3 antiviral activity. We observed significant increases in G-to-A mutations in P21A/P24A ($p = 1.41 \times 10^{-4}$), SLQ::AAA ($p = 5.27 \times 10^{-5}$), and Δvif ($p = 1.84 \times 10^{-5}$) MVV compared to wild-type using a one-sided Wilcoxon rank-sum test (SCP cell results; Figure 5D). No significant increase in mutations was observed for either the P21A or the P24A single mutations relative to wild-type (Figures 5D and S7A–S7C). The tri-nucleotide sequence preferences of the G-to-A mutations were similar across all conditions (G(G/A)A), again strongly indicative of A3-mediated mutation (Figures 5E and S7D).

CYPA Is Required for Ovine A3 Degradation by MVV Vif

We tested MVV Vif dependence on CYPA for A3 degradation activity using a CYPA knockdown in human cells but were unable to achieve a knockdown sufficient to prevent either OaA3 degradation or MVV Vif-CRL complex formation using polyclonal lines (data not shown). Titration experiments using CsA showed limited but reproducible inhibition of OaA3Z2Z3 degradation by MVV Vif, increasing with CsA concentration until about 5 μM when toxicity became apparent (Figure S4B). Importantly, the inhibition was not observed with HIV-1 Vif and its cognate substrate, human A3G (HsA3G) (Figure S4C).

Due to lack of efficacy of either CYPA knockdowns or CsA treatments individually, we attempted to inhibit MVV Vif by combining both protocols, treating a monoclonal CYPA knockdown line with 2 μM CsA and comparing MVV Vif A3 degradation activity to a non-targeting control line. We observed a modest inhibition of Vif-mediated OaA3Z2Z3 degradation in the knockdown line compared to the control line without CsA treatment. In contrast, we observe near-complete inhibition of MVV Vif degradation activity in CYPA knockdown cells treated with CsA (Figure 6A, lanes 9 and 10). HIV-1 Vif A3 degradation activity was not affected by CsA treatment in either the knockdown or the control line (Figure S4D), indicating the combination of CYPA knockdown and CsA treatment was specifically and cooperatively interfering with the MVV Vif-CYPA interaction.

Given that drug and shRNA treatments can have off-target effects, we next performed ovine A3 degradation assays in an isogenic system using a Jurkat T cell line and $CYPA^{-/-}$ knockout (KO) line (Braaten and Luban, 2001). Immunoblots confirmed the lack of *CYPA* expression in the Jurkat $CYPA^{-/-}$ KO line compared to the parental Jurkat E6-1 $CYPA^{+/+}$ line (Figure 6B). The KO line or the parental line was then nucleofected with an HA-tagged OaA3Z2Z3 expression construct in the presence or absence of Strep-tagged MVV Vif and Flag-tagged CYPA complementation (Figure 6B). We observe a complete loss of MVV Vif-mediated degradation of OaA3Z2Z3 in the $CYPA^{-/-}$ KO line (Figure 6C, top, lanes 5 and 6), which can be rescued upon complementation with exogenous CYPA (Figure 6C, top, lanes 7 and 8). We were unable to detect expression of MVV Vif in the $CYPA^{-/-}$ KO line without exogenous CYPA complementation, suggesting MVV Vif stability depends on CYPA presence in vivo. This closely mimics the stability requirements of HIV-1 Vif on CBFβ and is consistent with our in vitro reconstitution results with MVV Vif. We did not observe any dependence on exogenous CYPA expression for MVV Vif A3 degradation activity in the parental Jurkat E6-1 $CYPA^{+/+}$ line (Figure 6C, bottom) and did not observe any dependence on CYPA for A3 degradation activity for HIV-1 Vif and HsA3G in either cell line (Figure 6D).

Lastly, we assessed whether or not various active-site mutants of CYPA could also rescue MVV function in the KO background. We tested three CYPA mutants—R55K, F113W, and H126A—all located within the active site of CYPA and previously reported to affect activity (Figure S7A; Bosco et al., 2010). We found that two mutants, R55K and F113W, failed to rescue MVV Vif A3 degradation activity in the $CYPA^{-/-}$ KO line and that a third mutant, H126A, was able to rescue activity as efficiently as wild-type CYPA (Figure 6E). Similar results were obtained in the monoclonal CYPA knockdown line (Figure S7B). We additionally tested the CYPA mutants for MVV Vif binding through in vitro reconstitution and found that all three were capable of forming a stable MVV Vif-ELOB-ELOC-CYPA complex (Figures S7C and S7D). Whereas it is possible that the lack of R55K and F113W rescue of MVV Vif activity is due to a weakening of binding in vivo, the in vitro binding data suggest

(C) 2D ^{15}N-^{1}H chemical shift mapping of CYPA in the presence of MVV $Vif^{17–26}$ peptide. The resonances of Y48, R55, I56, I57, F60, M61, C62, Q63, G65, G72, L98, S99, A101, Q111, F112, E120, W121, and K125 (orange sticks) shift and then disappear upon addition of the Vif peptide. CsA is labeled in blue. PDB: 1CWA.
(D) Column 1: representative example of a CYPA residue (G135) that is not affected by the presence of the MVV Vif peptide. Column 2: by comparison, R55 and S99 undergo significant chemical shift and intensity reduction. The bars are scaled to the intensity of the HSQC peak at the corresponding Vif concentration.
(E) Affinity purification of MVV Vif in the presence of a titration of CsA. Co-purification of endogenous Vif interactors is assayed by immunoblot. BC-box and proline mutants are used as controls for ELOC and CYPA binding, respectively. E, ethanol.
(F) UV absorbance curves are shown for gel filtration of CUL5-RBX2 (C5R2) alone or mixed with an excess of indicated Vif complexes (MVV VCBC, Vif-ELOB-ELOC-CYPA). Peaks are observed at earlier elution volumes when C5R2 is mixed with Vif complexes, indicating E3 complex formation.
(G) Coomassie-blue-stained SDS-PAGE of peak fractions collected from gel filtration runs shown in (F).
(H) Immunoblot of methyl-ubiquitylation reactions with MVV E3 and either myc-tagged human A3H (Myc-HsA3H) or ovine A3Z3 (Myc-OaA3-Z3).
(I) Pair distance distribution function, P(r), calculated from SAXS intensity data.
(J) Molecular envelopes of HIV-1 $Vif^{1–174}$-CBFβ-CRL5 (left) and MVV Vif-CYPA-CRL5 (right) calculated from P(r). An HIV-1 E3 model was superimposed into both envelopes.
See also Figures S3 and S5 and Table S3.

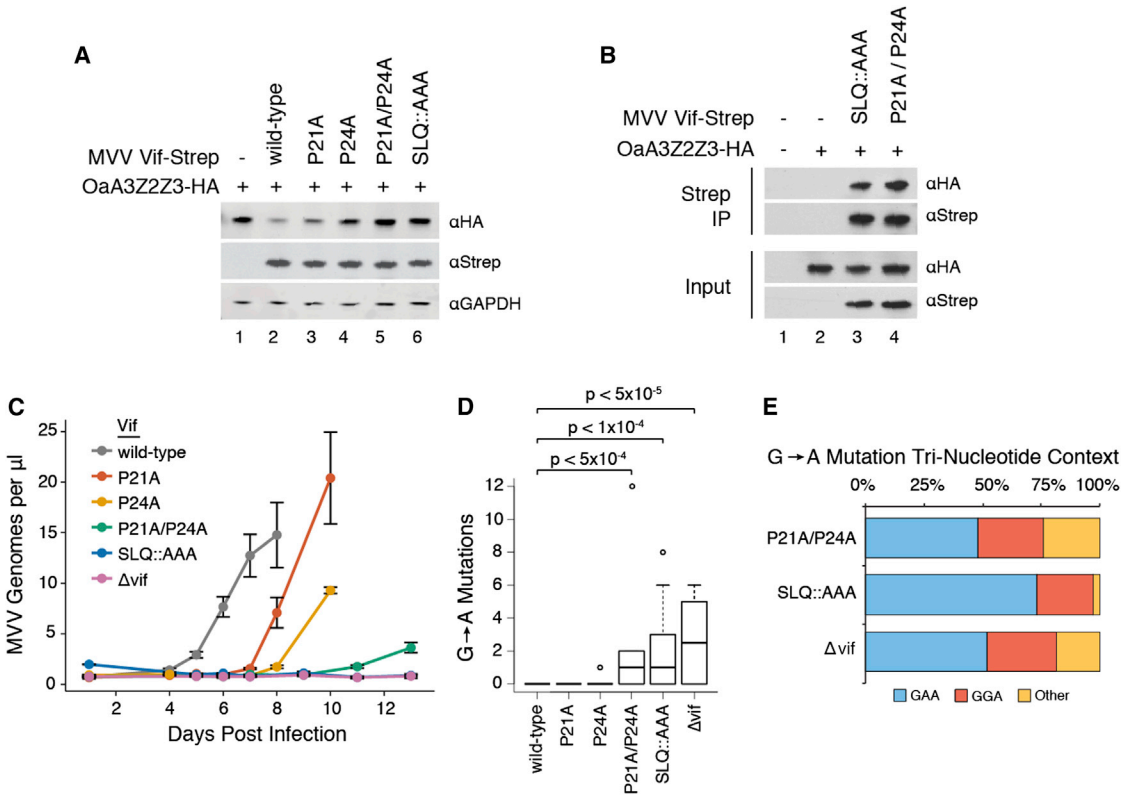

Figure 5. MVV Vif Mutants Deficient in CYPA Binding Are Deficient in A3 Antagonism and Cannot Promote MVV Infectivity In Situ

(A) Co-transfection of HA-tagged ovine A3Z2Z3 (OaA3Z2Z3-HA) and either wild-type or proline mutant MVV Vif. A3 stability in the presence of Vif is assayed by immunoblot.

(B) Co-affinity purification between OaA3Z2Z3 and MVV Vif constructs that were deficient in OaA3Z2Z3 destabilization in (A). Interaction between A3 and Vif proteins is assayed by immunoblotting.

(C) MVV spreading assay in ovine primary macrophage cells. Lysates were harvested at various time points post-infection, and virus genome copies were quantified using TaqMan-based real-time PCR, mean ± SE (n = 3).

(D) Hypermutation assay of MVV strain KV1772. MVVs with either wild-type or mutant *vif* were subjected to a single-cycle infection assay in primary sheep choroid plexus (SCP) cells, and produced viruses were then used to infect SCP cells, generated pro-viruses cloned, and assayed for A3-mediated G-to-A mutations. Wild-type, P24A: n = 20; P21A: n = 16; P21A/P24A: n = 17; SLQ::AAA: n = 19; Δvif: n = 10. Significance values were determined by a one-sided Wilcoxon rank-sum test compared to wild-type; no annotated p value indicates p value > 0.05.

(E) Tri-nucleotide context of G-to-A mutations measured in (C). Other refers to any GNN tri-nucleotide other than GGA or GAA.

See also Figure S6.

CYPA isomerase activity is important for MVV Vif A3 degradation activity.

DISCUSSION

The retrovirus family infects a diverse set of mammalian hosts, with each viral lineage required to evolve a mechanism to overcome the host challenge to infection presented by A3 proteins. Solutions to this A3 host challenge by retroviruses include the lentivirus protein Vif that induces the proteasomal degradation of A3 proteins (Larue et al., 2010; Sheehy et al., 2002; Yu et al., 2003), sequestration of A3 proteins away from virions by the foamy virus protein Bet (Löchelt et al., 2005), or preventing A3 binding to virion proteins in HTLV-1 (Derse et al., 2007). Even within lentiviruses, the equine infectious anemia virus has evolved a Vif-independent, non-degrading mechanism of escaping A3 restriction (Bogerd et al., 2008). Through a series of proteomic, virological, biochemical, and structural approaches targeting five evolutionarily distinct lentiviral Vif proteins from HIV-1, SIVmac, MVV, BIV, and FIV, we present data consistent with a model of high biochemical plasticity at the molecular level but "modular conservation" due to their conserved function of ubiquitylation and subsequent proteasomal degradation of host A3 restriction factors (Figure 7).

Using AP-MS, we identified a conserved core ubiquitin ligase complex—CUL5-ELOB-ELOC-RBX2—involved in Vif-mediated degradation of host A3 proteins. For a functional complex, HIV-1 and SIVmac Vif proteins additionally require the host cofactor CBFβ for both complex assembly and activity (Han et al., 2014; Hultquist et al., 2012; Zhang et al., 2012). Our data both identify the CBFβ interaction with HIV-1 and SIVmac Vif proteins and demonstrate their dependence on this cofactor for A3 degradation activity. The interaction with CBFβ was not observed by AP-MS nor was it functionally required for A3

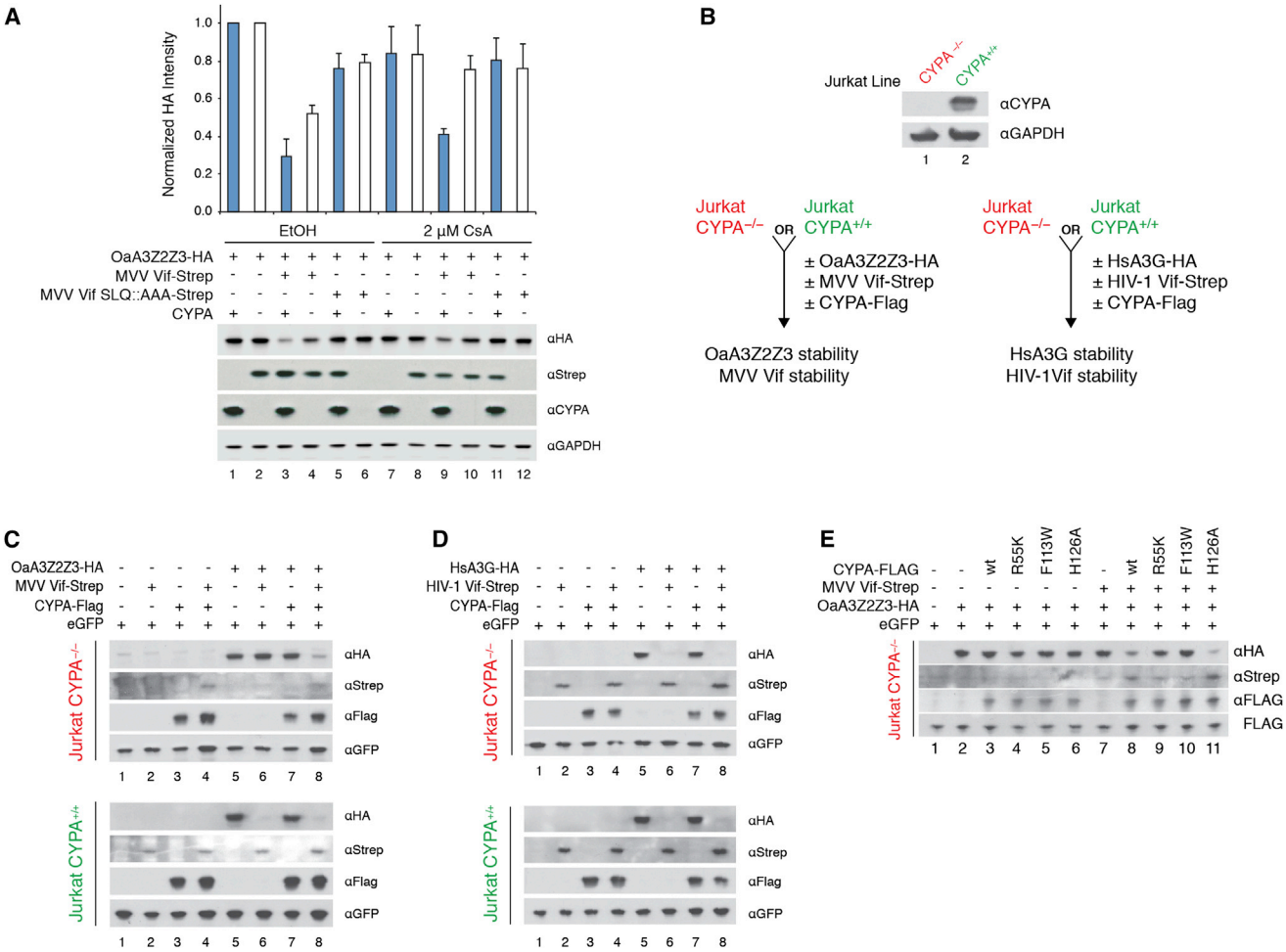

Figure 6. CYPA Is Required for MVV Vif A3 Degradation Activity

(A) Comparison of Vif A3 degradation activity in monoclonal CYPA knockdown versus control cells in the presence or absence of CsA. Cells were transiently transfected with HA-tagged ovine A3Z2Z3 (OaA3-Z2Z3) and either wild-type or BC-box mutant (SLQ::AAA) Strep-tagged MVV Vif and then treated 6 hr later with either ethanol (E) or 2 μM CsA overnight. Bars represent HA immunoreactivity normalized first by GAPDH loading control and then to no Vif control for each cell line; mean ± SE (n = 3). Proteins are detected by immunoblotting.

(B) CYPA immunoblot in Jurkat E6-1 *CYPA*$^{+/+}$ "parental" line and derived E6-1 *CYPA*$^{-/-}$ knockout (KO) line.

(C) Top: Jurkat *CYPA*$^{-/-}$ KO cells are transiently transfected with HA-tagged OaA3Z2Z3, Strep-tagged MVV Vif, and Flag-tagged CYPA. eGFP is used as transfection control. Bottom: identical experiment performed in E6-1 *CYPA*$^{+/+}$ control line.

(D) Top: Jurkat *CYPA*$^{-/-}$ KO cells are transiently transfected with HA-tagged human A3G (HsA3G), Strep-tagged HIV-1 Vif, and Flag-tagged CYPA. eGFP is used as transfection control. Bottom: identical experiment performed in E6-1 *CYPA*$^{+/+}$ control line.

(E) MVV Vif activity rescue assay using mutants of CYPA. Strep-tagged MVV Vif, HA-tagged OaA3Z2Z3, and various Flag-tagged CYPA constructs are transfected into Jurkat *CYPA*$^{-/-}$ KO line and Vif activity assessed through A3 stability.

See also Figures S4 and S7.

degradation activity in the non-primate lentiviral Vif proteins, consistent with recent observations (Ai et al., 2014; Han et al., 2014; Zhang et al., 2014a, 2014b). We identified one non-primate lentivirus, BIV, with a Vif protein that requires no non-canonical host cofactor for both complex assembly and activity in vitro and identified another, MVV, with a Vif protein that requires a novel non-canonical host cofactor, CYPA, to play a CBFβ-like role in regulating ligase assembly and activity both in vitro and in vivo. Though no FIV Vif cofactor was identified, we cannot exclude the possibility that it requires an as of yet unidentified one.

Interaction of MVV Vif with the Non-canonical Cofactor CYPA

The interaction between MVV Vif and CYPA appears to be unique among the lentiviruses examined in this study. Through a combination of targeted mutagenesis and NMR spectroscopy, we identified a di-proline motif in the N terminus of MVV Vif, $_{20}$GPQLP$_{24}$, which directly binds the CYPA active site. This site is uniquely found in MVV and the closely related CAEV Vif proteins (Figure 4B). The motif bears some resemblance to the CYPA binding site of HIV-1 Capsid, $_{89}$GPIAP$_{93}$ (Gamble et al., 1996), a GPxxP motif. However, the strength of the interaction

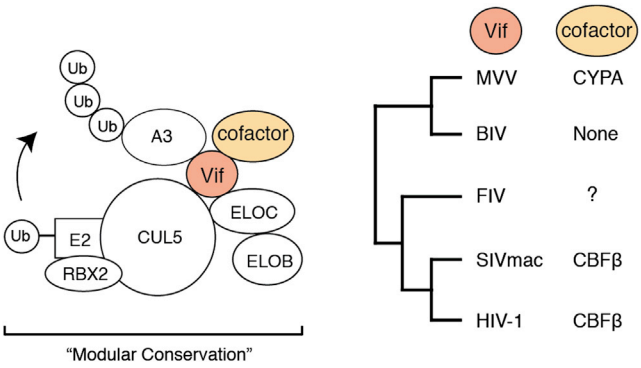

Figure 7. Modular Conservation of CRL Hijacking and Non-canonical Cofactor Recruitment by Vif

The host CRL complex hijacked by Vif represents a conserved host-pathogen interaction module. Vif proteins recruit non-canonical host cofactors in a lineage-specific manner within the lentivirus genus. Tree from Figure 1A.

between MVV Vif and CYPA appears to be much greater than that of CYPA and HIV-1 Capsid as assessed by interaction disruption by CsA treatment (Figures 4E and S5A). Further to this point, the relatively low affinity measured by NMR spectroscopy between CYPA and MVV Vif$^{17–28}$ peptide is incompatible with the observed stability of the reconstituted MVV Vif-ELOB-ELOC-CYPA complex. These data suggest the existence of binding surfaces on MVV Vif in addition to the identified di-proline motif and these surfaces are likely outside of the active site of CYPA as evidenced by the inefficacy of CsA treatment in disrupting MVV Vif-CYPA interaction or inhibiting MVV Vif anti-A3 activity without complementary knockdown of CYPA (Figure 6A).

Cellular chaperones interact with a large contingent of clients to catalyze their folding and often interact with their substrates via general features, such as hydrophobic patches, rather than specific PPI surfaces or domains (Jaya et al., 2009; Spiess et al., 2006). The relatively promiscuous interactions of CYPA combined with a high cellular concentration may have eased the evolution of MVV Vif to capture CYPA. If the interaction between the two proteins is mechanistically unrelated to perturbing endogenous CYPA activities, this may explain the selection of CYPA by the virus. The capture of CYPA by MVV Vif would involve transition of the CYPA interaction from a potentially catalytic one in aiding Vif folding to a stoichiometric one in forming a stable complex. The importance of CYPA catalytic function for MVV Vif appears to be retained, as two CYPA active-site mutants appear to bind MVV Vif without forming an active A3-degrading complex (Figure 6E). The one CYPA mutant that was not deficient in this activity, H126A, had been previously shown to be active with an HIV-1 Capsid substrate (Bosco et al., 2010). This model of capture would likely not apply to the interaction between primate lentiviral Vif proteins and CBFβ, as the interaction falls in a specific PPI surface evolved by CBFβ to interact with the runt domain of RUNX transcription factors (Guo et al., 2014).

Cofactor Acquisition as Gain-of-Function Adaptations

Whereas we cannot definitively infer the ancestral form of Vif-hijacked CRL complex, parsimony suggests the ancestral form would resemble the BIV Vif-hijacked complex. BIV Vif most resembles an endogenous BC-box E3 substrate adaptor, which binds CRL2/5 complexes without the need of non-canonical cofactors. The parsimony model would imply that the interactions between Vif and non-canonical host cofactors (MVV-CYPA; HIV-1/SIVmac-CBFβ) are derived interactions that occurred independently during the evolution of modern lentiviruses. Why these interactions evolved or what selective advantage they may grant to the viruses remains unclear. We previously suggested that the interaction between HIV-1 Vif and CBFβ may disrupt the endogenous activities of RUNX transcription factors (Jäger et al., 2012b) and have shown Vif-mediated interference of RUNX-controlled genes in T-lymphocytes (Kim et al., 2013). This "dual-hijacking" may also exist for the MVV-CYPA interaction, although it is worth noting that CYPA is a highly abundant protein in virtually every cell type, and therefore the secondary effect would necessarily be one that required relatively small changes in cellular CYPA abundance or involve local effects proximate to MVV Vif activity.

Another driving force in the evolution of novel Vif cofactors is the evolution of the viral proteins' substrates, the A3 family. The number of *A3* genes in a given mammalian genome varies widely, from only a single gene in mice to seven members in primates (Bogerd et al., 2008; Jarmuz et al., 2002; LaRue et al., 2008; Münk et al., 2008). The acquisition of CBFβ as a Vif cofactor by primate lentiviruses coincides with the expansion of A3 family of proteins in primates, and it is possible that the cofactor acquisition enabled Vif to preserve viral fitness without the need to evolve another, non-CRL mechanism to recognize and degrade the larger A3 substrate repertoire (Ai et al., 2014). A recent structure of the SIV/HIV-2 accessory factor Vpx showed the co-crystallized Cullin-4A adaptor protein DCAF1 directly contacts the viral substrate SAMHD1 (Schwefel et al., 2014); this example may be generalizable to a model of host factors extending the viral E3 surface for substrate recognition. The A3 expansion-driving cofactor acquisition model would fail to explain the interaction between MVV Vif and CYPA, as the ovine genome shows no increase in the number of A3 proteins compared with the bovine genome and one less than the feline genome (LaRue et al., 2008; Münk et al., 2008). Interestingly, MVV Vif is observed to have a high promiscuity for A3 proteins, showing activity against many non-cognate A3s (Larue et al., 2010), potentially supporting a model of cofactor-mediated substrate expansion.

An intriguing possibility is that non-canonical cofactors act to increase the evolvability of their Vif partner, enabling Vif proteins to retain activity with otherwise lethal mutations during transitions to more-fit states. Restriction factors are a major barrier to zoonosis (Sharp and Hahn, 2011), and a Vif protein able to adapt more quickly to a new host's A3 proteins should grant the lentivirus a competitive advantage in zoonotic transmission, as well as in populations with diverse restriction factor haplotypes (Binka et al., 2012; Ooms et al., 2013). Whether gaining an interaction partner makes a viral protein more robust to mutation is debatable, as the benefits of stabilization via the interaction may be outweighed by the mutational constrains imposed by the interaction itself. HIV-1 Vif must maintain direct interactions with ELOC, CUL5, multiple A3 proteins, and a large surface with CBFβ to be functional, yet even after the establishment of

the interaction between CBFβ and primate lentivirus Vif, it has recently undergone dramatic sequence changes (Etienne et al., 2013).

CYPA Potentially Bridges Vif Antagonism of A3 with TRIM5 Escape

We explored the idea of whether or not the interaction between CYPA and MVV Vif may be related to the genetic conflict between lentiviruses and the host restriction factor TRIM5 (Stremlau et al., 2004). In HIV-1, CYPA interaction with the viral capsid is involved in mitigating the antiviral actions of TRIM5 (Sokolskaja and Luban, 2006), and some old world primate hosts have evolved a TRIM5-CYPA fusion protein, TRIMCyp, which uses its CYPA domain to recruit the TRIM5 domain to the viral core (Nisole et al., 2004; Sayah et al., 2004). MVV is restricted by sheep TRIM5 when overexpressed in cell culture, but the mechanism by which MVV avoids this restriction in vivo is unknown (Jáuregui et al., 2012). It remains possible that the interaction between CYPA and MVV Vif is to allow crosstalk of Vif between two different host restriction pathways—A3 and TRIM5—particularly if there exists an as of yet unidentified TRIMCyp gene in the sheep genome.

In addition to its role in TRIM5-mediated restriction, the interaction of CYPA with HIV-1 Capsid has recently been found to be essential for the virus to evade detection by the innate immune sensor cGAS and to avoid subsequent activation of the innate immune response (Lahaye et al., 2013; Rasaiyaah et al., 2013). Intriguingly, a pair of mutants in MVV Vif and Capsid (P205S and L120R, respectively) results in a restricted growth phenotype, whereas neither mutant alone impacts replication (Gudmundsson et al., 2005). Whether or not this is related to the MVV Vif interaction with CYPA is under investigation.

Modular Conservation and Lineage-Specific Interactions

We have shown that, despite a generally conserved mechanism of mediating host A3 degradation by ubiquitylation and subsequent proteasomal degradation, lentiviruses demonstrate surprising plasticity in the biochemical requirements for this activity through their Vif proteins. Whereas other lentiviruses have been observed to change viral protein-host substrate interactions (Lim et al., 2012; Sauter et al., 2009) or the sites of substrate recognition (Fregoso et al., 2013), Vif is unique in that the viral protein substrate and the core machinery required for activity appear to be conserved and yet cofactor interaction partners are varied and lineage specific. We have previously noted this concept of modular conservation where the function of biochemical modules is conserved but the underlying molecular interactions between the modules have changed significantly. For example, we have shown that, although genetic interactions between different species have evolved rapidly, the genetic relationships within protein complexes, or modules, are highly conserved (Beltrao et al., 2010; Roguev et al., 2008; Ryan et al., 2012). In this vein, it remains to be seen whether or not the Vif paradigm is generalizable, but it is clear that viral proteins, even in the context of "stable" interactions with host factors, can still undergo rapid and surprisingly dramatic changes over the course of virus evolution.

EXPERIMENTAL PROCEDURES

Detailed experimental procedures are available in the Supplemental Experimental Procedures.

Expression Constructs

HIV-1_{LAI}, SIV_{mac239}, BIV, FIV, and MVV *vif* constructs in pVR1012 (Vical) have been reported previously (Larue et al., 2010).

Ovine Cells and MVV Infections

SCP cells and sheep-blood-derived macrophages were infected with RT-normalized wild-type and mutant viruses for spreading infection assays. Samples were taken daily for Taqman qPCR.

Affinity Purifications

Affinity purifications (AP) were generally performed as described previously (Jäger et al., 2012a).

Mass Spectrometry Data and Analysis

Digested peptide mixtures were analyzed on either a Thermo Scientific Velos Pro or a Thermo Scientific LTQ XL ion trap mass spectrometry system. Data were searched against a database containing SwissProt human protein sequences. Interactions were scored using the SAINT algorithm (Choi et al., 2011) with prey-identified spectral counts.

In Vitro Reconstitution and Ubiquitylation Assays

Vif complexes for in vitro work were produced by co-expression in BL21-Star(DE3)pLysS cells. CUL5-RBX2 and all ubiquitin and Nedd8 pathway components used in ubiquitylation assays were obtained as previously described (Stanley et al., 2012).

CYPA Knockdown and Cyclosporine Treatment

CYPA knockdown lines were generated in HEK293T cells using pLKO.1-derived lentiviruses encoding an shRNA-targeting CYPA (TRCN0000049277). For assays involving CsA (no. 9973; Cell Signaling Technology), cells were treated with 2–5 μM CsA 4–6 hr post-transfection to prevent potential loss in transfection efficiency.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, seven figures, and three tables and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2015.04.038.

AUTHOR CONTRIBUTIONS

J.R.K. conceived the project with N.J.K. and R.S.H., performed affinity purifications, scored mass spectrometry data, created stable cell lines, and performed CsA assays, knockdown assays, and Jurkat nucleofection experiments. D.J.S. performed in vitro reconstitution of Vif-CRL complexes and ubiquitylation assays. J.F.H. performed single-cycle HIV-1 infectivity assays and assisted with Jurkat nucleofection experiments. J.R.J., B.W.N., T.L.J., and K.E.F.-S. performed MS sample preparation, machine runs, and data searching. N.M., S.R.J., H.I.G., and A.A. performed MVV virology assays. J.M.B. generated SAXS envelopes of Vif-CRL5 complexes. S.B. performed NMR experiments. M.L. purified A3 proteins for in vitro ubiquitylation assays. W.L.B. sequenced MVV proviral sequences for hypermutation assays. J.R.K. wrote the manuscript with assistance from J.F.H. and N.J.K. Manuscript editors were J.R.K., D.J.S., J.F.H., J.M.B., S.B., J.S.F., R.S.H., V.A., J.D.G., and N.J.K.

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