Role of cullin-elonginB-elonginC E3 complex in bovine immunodeficiency virus and maedi-visna virus Vif-mediated degradation of host A3Z2-Z3 proteins

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Abstract

Background: All lentiviruses except equine infectious anemia virus (EIVA) antagonize antiviral family APOBEC3 (A3) proteins of the host through viral Vif proteins. The mechanism by which Vif of human, simian or feline immunodeficiency viruses (HIV/SIV/FIV) suppresses the corresponding host A3s has been studied extensively.

Results: Here, we determined that bovine immunodeficiency virus (BIV) and maedi-visna virus (MVV) Vif proteins utilize the Cullin (Cul)-ElonginB (EloB)-ElonginC (EloC) complex (BIV Vif recruits Cul2, while MVV Vif recruits Cul5) to degrade Bos taurus (bt)A3Z2-Z3 and Ovis aries (oa)A3Z2-Z3, respectively, via a proteasome-dependent but a CBF-β-independent pathway. Mutation of the BC box in BIV and MVV Vif, C-terminal hydrophilic replacement of btEloC and oaEloC and dominant-negative mutants of btCul2 and oaCul5 could disrupt the activity of BIV and MVV Vif, respectively. While the membrane-permeable zinc chelator TPEN could block BIV Vif-mediated degradation of btA3Z2-Z3, it had minimal effects on oaA3Z2-Z3 degradation induced by MVV Vif, indicating that Zn is important for the activity of BIV Vif but not MVV Vif. Furthermore, we identified a previously unreported zinc binding loop [C-x1-C-x1-H-x19-C] in the BIV Vif upstream BC box which is critical for its degradation activity.

Conclusions: A novel zinc binding loop was identified in the BIV Vif protein that is important for the E3 ubiquination activity, suggesting that the degradation of btA3Z2-Z3 by BIV and that of oaA3Z2-Z3 by MVV Vif may need host factors other than CBF-β.

Keywords: E3 ubiquitin ligase, BIV Vif, MVV Vif, CCHC motif

Background

Lentiviruses, a subfamily of retroviruses, cause slow infections in humans and animals. Human immunodeficiency virus type 1 (HIV-1), simian immunodeficiency virus (SIV), caprine arthritis-encephalitis virus (CAEV), feline immunodeficiency virus (FIV), bovine immunodeficiency virus (BIV), maedi-visna virus (MVV) and equine infectious anemia virus (EIAV) are lentiviruses that infect humans, monkeys, goats, cats, cattle, sheep and horses, respectively. Except for EIAV, all lentiviruses require the accessory protein viral infectivity factor (Vif) to establish persistent infection and pathogenesis in vivo [1]. The Vif protein counteracts the antiviral activities of the apolipoprotein B mRNA-editing enzyme, catalytic polypeptide-like 3 (APOBEC3 or A3) proteins of the host [2]. These A3 proteins possess broad antiviral activities for many viruses as natural host restriction factors [3-7]. Among the A3 proteins, A3G is the most intensively studied. In the late stage of viral infection, A3G proteins are packaged into virions and induce dC to dU mutations in newly synthesized minus-strand viral DNA. These mutations cause abnormal expression of viral proteins, resulting in disruptions of the viral life cycle [8-10]. The HIV-1 accessory
factor Vif plays a critical role in maintaining efficient viral replication in non-permissive cell lines [11]. HIV-1 Vif antagonizes the antiviral activity of the cellular protein A3G by recruiting the transcription cofactor CBF-β and ElonginB (EloB)-ElonginC (EloC) to the Culin5 (Cul5)-Rbx complex to degrade A3G [3,12-18]. The functional domains that Vif uses to form the E3 ligase complex have been reported. The main sites involved in the interaction with A3G and CBF-β are in the N-terminal region of Vif [19-23]. The H-x5-C-x17_–18-C-x3_–5-H motif (i.e., HCCH zinc finger) and the PPLx4L motif (also known as the Cul5 box) in the C-terminal region of HIV-1 Vif mediate selective binding to Cul5 [24-26]. Meanwhile, another C-terminal SLQ(Y/F) LA motif (BC box) downstream of the HCCH domain binds with EloC to assemble the E3 ligase complex [12,27,28]. Mechanisms of the degradation of APOBEC3 proteins induced by SIV Vif and FIV Vif also have been well studied. SIVmac239 Vif recruits the transcription cofactor CBF-β and EloB-EloC to the Cul5-Rbx complex, forming the CBF-β-Cul5-EloB-EloC E3 ubiquitin ligase to degrade the cellular antiviral protein A3G [29,30]. FIV Vif interacts with feline Cul5, EloB and EloC to form an E3 complex to induce degradation of fA3s [31].

BIV affects the immune system like many other lentiviruses [32,33], and its name was based on similarities to HIV-1 in genetic, structural, antigenic and biological factors. BIV infects cattle and causes significant but non-persistent infiltrating lymphocytolysis and follicular hyperplasia in the hemolymph nodes [34]. MVV is also a lentivirus which causes slowly progressive meningoencephalomyelitis and pneumonia in sheep [35]. The Vif proteins of BIV and MVV are both indispensable for viral infectivity [36]. The artiodactyl A3 proteins have been reported to have an active N-terminal DNA cytosine deaminase domain, which displays a dinucleotide deamination preference [37]. According to the nonprimate A3 nomenclature, there are four Bos taurus A3 (btA3) proteins: btA3Z1, btA3Z2, btA3Z3, btA3Z2-Z3 and four Ovis aries A3 (oaA3) proteins: oaA3Z1, oaA3Z2, oaA3Z3 and oaA3Z2-Z3. Among the A3 proteins, A3Z2-Z3 is the only double domain protein that displays fully intact levels of antiviral activity similar to HIV-1 Vif. When 293 T cells were co-transfected with HA-tagged btA3Z2-Z3 and cmyc-tagged BIV Vif for 24 h, the expression of A3Z2-Z3 was induced (Figure 1A, lane 4), implying that the degradation of btA3Z2-Z3 by BIV Vif is proteasome-dependent, 293 T cells were co-transfected with HA-tagged btA3Z2-Z3 and cmyc-tagged BIV Vif or VR1012 and treated with the proteasome inhibitor MG132 [39] or DMSO as a negative control. The results showed that btA3Z2-Z3 could be degraded by BIV Vif (Figure 1A, lane 2). However, MG132 blocked the degradation of btA3Z2-Z3 and stabilized the BIV Vif protein (Figure 1A, lane 4), implying that the degradation of btA3Z2-Z3 protein depends on the proteasome activity similar to HIV-1 Vif. When 293 T cells were co-transfected with HA-tagged oaA3Z2-Z3 and cmyc-tagged MVV Vif or VR1012 and treated with the proteasome inhibitor MG132 or DMSO as a negative control, the experimental results were nearly the same as those above (Figure 1B). These findings imply that BIV Vif degrades btA3Z2-Z3 and MVV Vif degrades oaA3Z2-Z3 via a proteasomal pathway, and inhibiting this process would increase the expression of these APOBEC3 proteins to varying degrees.

High levels of btA3Z2-Z3 and oaA3Z2-Z3 in the absence or presence of Vif, the cycloheximide (CHX, protein synthesis inhibitor) stability assay was performed (Figure 1C, D). Two sets of experiments were carried out. 293 T cells were co-transfected with HA-tagged btA3Z2-Z3 (30 ng) or HA-tagged oaA3Z2-Z3 (15 ng) and cmyc-tagged BIV Vif, cmyc-tagged MVV Vif (200 ng) or VR1012. After 18 h of transfection, transfected cells in the first set were treated with CHX, while those in the second set were treated with DMSO as a control. We then checked the expression level of A3Z2-Z3 in the absence or presence of CHX. The function of BIV Vif and its interaction with Cul2 were explored further by mutations of the C-x1-C-x1-H-x19-C (CHHC) motif which may be a novel zinc finger (18, 46). Homology modeling results showed that this CCHC motif is likely a zinc binding loop. Together, results of this study indicate that BIV and MVV Vifs bind with SOCS proteins in a novel manner to form Elo-Cul-SOCS box (ECS) complexes, which may facilitate future studies of virus-host interactions.
of Vif in these two sets of cells at various timepoints up to 24 h after CHX or DMSO treatment. With the addition of CHX, the synthesis of Vif and A3Z2-Z3 was inhibited, and most of the pre-existing A3Z2-Z3 was degraded by Vif. Meanwhile, Vif was partially degraded by the EloB-EloC-Cul E3 ligase. In the DMSO-treated set of cells in which protein synthesis was not inhibited, quantities of A3Z2-Z3 and Vif increased over time (compare A3Z2-Z3 levels at 0 h, 6 h, 12 h and 24 h in the presence of Vif in Figure 1C, D). Under this condition, A3Z2-Z3 was still remarkably degraded by Vif (compare A3Z2-Z3 levels in the absence and presence of Vif in Figure 1C, D). These results revealed that BIV and MVV Vifs could efficiently degrade btA3Z2-Z3 and oaA3Z2-Z3, respectively.

BIV Vif combines with Cul2, EloB and EloC and MVV Vif combines with Cul5, EloB and EloC to induce proteasomal degradation of btA3Z2-Z3 and oaA3Z2-Z3, respectively. HIV-1 Vif interacts with human Cul5, EloB and EloC to form the E3 complex, which degrades human A3G. A recent study showed that CBF-β is involved in this degradation process as well [3,12-17]. Therefore, we wondered...
whether host molecules participating in the degradation of A3 proteins by BIV and MVV Vif are the same or similar to those for HIV-1 Vif. Initially, we investigated the endogenous proteins involved in the degradation process by transfecting HA-tagged BIV Vif or HA-tagged MVV Vif into MDBK or MDOK cells; however, we did not obtain clear and convincing results due to the low transfection efficiency in these cells. Amino acid sequence alignments showed that the homologies of EloB, EloC, Cul2, Cul5 and CBF-β between humans, cattle and sheep exceed 98.0% (Table 1). Therefore, we attempted to study this issue by transfecting HA-tagged BIV Vif and HA-tagged von Hippel-Lindau (VHL) tumor suppressor (as a positive control for binding with Cul2), HA-tagged MVV Vif and HA-tagged HIV Vif (as a positive control for binding with Cul5) or negative vector control VR1012 into 293 T cells to perform a co-immunoprecipitation assay. Cell lysates were immunoprecipitated with HA beads, followed by SDS-PAGE and immunoblot analysis using anti-HA, anti-hCul2 anti-hCul5, anti-hEloB, anti-hEloC and anti-hCBF-β antibodies. The experimental results showed that the BIV Vif protein was capable of binding to endogenous Cul2, EloB and EloC proteins, but not with Cul5 or CBF-β (Figure 2A). Notably, the negative control HA-tagged VHL did not bind with CBF-β (Figure 2B). Meanwhile, the MVV Vif protein was capable of binding to endogenous Cul5, EloB and EloC proteins, but not with Cul2 or CBF-β (Figure 2C). The co-immunoprecipitation assay showed that BIV Vif recruited Cul2, EloB and EloC, while MVV Vif recruited Cul5, EloB and EloC, to form the E3 complex to induce the degradation of btA3Z2-Z3 and OA3Z2-Z3, respectively. Of the various Vif proteins, BIV Vif appears to be unique by recruiting Cul2, and CBF-β was not found to be involved in the degradation of A3Z2-Z3 mediated by either BIV Vif or MVV Vif.

**Table 1 Homology rates of EloB, EloC, Cul2, Cul5 and CBF-β between humans, cattle and sheep**

| Species          | Protein (% identity of amino acids) | EloB | EloC | Cul2 | Cul5 | CBF-β |
|------------------|------------------------------------|------|------|------|------|-------|
| Homo sapiens/Bos taurus | 98.3 | 100.0 | 99.7 | 100.0 | 100.0 |
| Homo sapiens/Ovis aries | 98.3 | 100.0 | 98.5 | 100.0 | 100.0 |
| Bos taurus/Ovis aries  | 100.0 | 100.0 | 98.3 | 100.0 | 100.0 |

BIV Vif interacts with btCul2 and btEloC directly but not with btCBF-β, and MVV Vif interacts with oACul5 and oAEloC directly but not with oACBF-β

In order to further confirm the cellular proteins involved in BIV Vif-mediated degradation of btA3Z2-Z3, 293 T cells were transiently co-transfected with cmyc-tagged BIV Vif and Flag-tagged btCBF-β or HA-tagged btEloC (or HA-tagged BIV Vif and cmhc-tagged btCul2 or cmhc-tagged btCul5). Subsequently, co-immunoprecipitation experiments were performed to explore the interaction between BIV Vif and btCBF-β, btEloC, btCul2 and btCul5. After 48 h of transfection, cells were immunoprecipitated with HA beads or with an anti-cmyc antibody followed by SDS-PAGE and immunoblot analysis using an anti-HA antibody and an anti-cmyc antibody. The results revealed that BIV Vif could directly interact with btCul2, but not with btCul5 or btCBF-β (Figure 3A, D, G). BIV Vif could also bind with btEloC (data not shown). Of note, the btCBF-β-independent function of BIV Vif has been reported previously [14]. The same experiments were carried out as mentioned above for MVV Vif. The results revealed that MVV Vif could directly interact with oACul5 and oAEloC, but not with oACul2 or oACBF-β (Figure 3B, E, H). MVV Vif could also interact with oAEloC (data not shown). 293 T cells were transiently co-transfected with cmyc-tagged HIV Vif and Flag-tagged hCBF-β (or HA-tagged HIV Vif and cmyc-tagged hCul5), HA-flagged hVHL and cmyc-tagged hCul2 as positive controls (Figure 3C, F, I).

**Mutation of BC box in BIV and MVV Vif or C-terminal hydrophilic residue replacement in btEloC and oAEloC can disrupt the activity of these Vif proteins against btA3Z2-Z3 and oA3Z2-Z3, respectively**

Lentiviral Vif proteins represent substrate receptor proteins that contain relatively conserved BC-box motifs. The known BC-box motif of HIV-1, SIVmac239, BIV and MVV Vifs is SLQ, and that of FIV Vif is TLQ [27,40]. In this study, we replaced the SLQ sequence of BIV Vif and MVV Vif with AAA in order to explore whether the BC box is critical for the degradation of btA3Z2-Z3 and oA3Z2-Z3 induced by BIV Vif and MVV Vif, respectively. To investigate role of the SLQ motif in BIV Vif, 293 T cells were transfected with HA-tagged btA3Z2-Z3 and cmhc-tagged BIV Vif or cmhc-tagged BIV Vif SLQ-AAA. Likewise, the function of the SLQ motif in MVV Vif was examined by transfecting 293 T cells with HA-tagged oA3Z2-Z3 and cmhc-tagged MVV Vif or cmhc-tagged MVV Vif SLQ-AAA. At 48 h after transfection, the cells were harvested for Western blotting using anti-HA, anti-cmyc and anti-tubulin antibodies. The results revealed that BIV Vif and MVV Vif SLQ-AAA respectively lost the ability to degrade btA3Z2-Z3 and oA3Z2-Z3 (Figure 4A, lane 3; Figure 4B, lane 3), suggesting that the BC-box motif of each of these two Vif proteins is critical for the degradation of the corresponding A3Z2-Z3 target. These results are consistent with a previous report showing that the BIV Vif SLQ-AAA and MVV Vif SLQ-AAA mutants have altered function and fail to degrade btA3Z3 and oA3Z2-Z3 proteins, respectively [40].

To further confirm the significance of EloC in the E3 ligase, we constructed btEloC and oAEloC mutants by replacement of critical hydrophobic amino acids A100
and L103 with hydrophilic serine [41]. 293 T cells were transfected with HA-tagged btA3Z2-Z3 and cmyc-tagged BIV Vif or VR1012 with HA-tagged btEloC as a control. In a parallel experiment, 293 T cells were transfected with HA-tagged oaA3Z2-Z3 and cmyc-tagged MVV Vif or VR1012 with HA-tagged oaEloC as a control. At 48 h after transfection, the cells were harvested for Western blotting using anti-HA, anti-cmyc and anti-tubulin antibodies. Addition of EloCΔ2 rescued the levels of both btA3Z2-Z3 and oaA3Z2-Z3 (Figure 4C, lane 4; Figure 4D, lane 4). These results indicated that EloC takes part in the degradation of btA3Z2-Z3 by BIV Vif and of oaA3Z2-Z3 by MVV Vif.

**Dominant-negative btCul2 and oaCul5 mutants can inhibit the activity of BIV Vif against btA3Z2-Z3 and MVV Vif against oaA3Z2-Z3, respectively, and Zn is important for BIV Vif activity**

All Cullin family members are known to be modulated by the ubiquitin-like small molecule Nedd8, which is critical for E3 ubiquitin ligase activity [42]. To further confirm the participation of btCul2 and oaCul5 in the degradation of btA3Z2-Z3 and oaA3Z2-Z3, we constructed dominant-negative btCul2 and oaCul5 mutants and determined their effect on the degradation of btA3Z2-Z3 and oaA3Z2-Z3 induced by BIV and MVV Vif, respectively. 293 T cells were transfected with HA-tagged btA3Z2-Z3 and cmyc-tagged BIV Vif or VR1012, with cmyc-tagged btCul2ΔNedd8 or with a control vector expressing cmyc-tagged hCul1K720R, which is a dominant-negative hCul1 mutant [43]. In parallel, another set of 293 T cells was transfected with HA-tagged oaA3Z2-Z3 and cmyc-tagged MVV Vif or VR1012, with cmyc-tagged oaCul5ΔNedd8 or with the cmyc-tagged hCul1K720R control vector. As a positive control for the downregulation of hA3G by hCul5ΔNedd8, 293 T cells were transfected with HA-tagged hA3G and cmyc-tagged HIV Vif or VR1012, with cmyc-tagged hCul5ΔNedd8 or the cmyc-tagged hCul1K720R control vector. At 48 h after transfection, the cells were harvested for Western blotting using anti-HA, anti-cmyc and anti-tubulin antibodies. As expected, the expression of btCul2ΔNedd8 and oaCul5ΔNedd8 could up-regulate the amount of btA3G and of oaA3G by 5-10 times, respectively (Figure 5A, lane 4; Figure 5B, lane 4). The results indicated that btCul2 and oaCul5 are recruited to the Cul-E3 complex.

Cellular proteins assemble with Cul-EloB-EloC E3 complexes through a BC box and a downstream Cul box [44]. Some cellular proteins such as the tumor suppressor VHL use a Cul2 box to bind with Cul2, while others such as SOCS3 use a Cul5 box to bind with Cul5 [44,45]. Primate lentiviral (HIV-1/SIV) Vif proteins use a zinc-binding HCCH motif to interact with Cul5 [25]. FIV Vif has neither a Cul5 box nor an HCCH motif, but it still interacts with Cul5 in a novel fashion [31]. Since BIV and MVV Vif have no apparent Cul2 or Cul5 box or an HCCH motif, we wondered whether Zn is significant for its interaction with btCul2 and oaCul5. In order to explore this issue, we used the membrane-permeable zinc chelator TPEN [46] to determine its effects on the BIV and MVV Vif-mediated degradation of btA3Z2-Z3 and oaA3Z2-Z3, respectively. 293 T cells were transfected with HA-tagged btA3Z2-Z3 and cmyc-tagged BIV Vif or VR1012. A parallel set of 293 T cells was transfected with HA-tagged oaA3Z2-Z3 and cmyc-tagged MVV Vif or VR1012. After 36 h of transfection, the cells were treated with TPEN at 3.5 μM (Figure 5D, lanes 3, 4; Figure 5E, lanes 3, 4) or DMSO
At 48 h after transfection, the cells were harvested for Western blotting using anti-HA, anti-cmyc and anti-tubulin antibodies. The results showed that the addition of TPEN blocked the degradation of \textit{btA}3Z2-Z3 induced by BIV Vif (Figure 5D, lane 4), but it had a minimal effect on the degradation of \textit{oaA}3Z2-Z3 induced by MVV Vif (Figure 5E, lane 4). These findings indicated that Zn is important for the activity of BIV Vif, but it is not required for the activity of MVV Vif. We propose that a zinc finger domain is involved in the BIV Vif-mediated degradation of \textit{btA}3Z2-Z3, while MVV Vif may have a yet undefined mechanism for interacting with \textit{oaCul}5, which is similar to FIV Vif.

**CCHC motif is crucial for activity of BIV Vif and its interaction with Cul2**

In order to further explore the mechanism of the interaction between BIV Vif and \textit{btCul}2, we conducted an in-depth analysis of the BIV Vif sequence. A putative zinc binding motif H-x8-C-x1-C-x1-H-x19-C-x14-H was found upstream of the BC box. To explore whether this putative motif is a functional domain for the activity of BIV Vif, we constructed a series of BIV Vif single mutants in which histidines and cysteines (H102, C111, C113, H115, C134, and H149) were replaced individually with leucine or serine and a BIV Vif double mutant containing two amino acids (C111 and C113) replaced with serine. 293 T cells were co-transfected with HA-tagged \textit{btA}3Z2-Z3 and cmyc-tagged BIV Vif, cmyc-tagged BIV Vif H102L, cmyc-tagged BIV Vif C111S, cmyc-tagged BIV Vif C113S, cmyc-tagged BIV Vif H115L, cmyc-tagged BIV Vif C134S, cmyc-tagged BIV Vif H149L or cmyc-tagged BIV Vif C111S/C113S. At 48 h after transfection, the cells were harvested for Western blotting using anti-HA, anti-cmyc and anti-tubulin antibodies. The results revealed that BIV Vif C111S, BIV Vif C113S, BIV Vif H115L and BIV Vif H149L showed a minimal effect on the degradation of \textit{btA}3Z2-Z3 induced by BIV Vif (Figure 5E, lane 4), but it had a minimal effect on the degradation of \textit{oaA}3Z2-Z3 induced by MVV Vif (Figure 5E, lane 4). These findings indicated that Zn is important for the activity of BIV Vif, but it is not required for the activity of MVV Vif. We propose that a zinc finger domain is involved in the BIV Vif-mediated degradation of \textit{btA}3Z2-Z3, while MVV Vif may have a yet undefined mechanism for interacting with \textit{oaCul}5, which is similar to FIV Vif.

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C134S lost almost all of their abilities to degrade btA3Z2-Z3 (Figure 6B, lanes 4, 5, 6, 7, 9), implying that the C-x1-C-H-x19-C motif is critical for the degradation activity of BIV Vif. The BIV Vif CCHC mutant also lost the ability to suppress the antiviral activity of btA3Z2-Z3 (Figure 6C, D), supporting the hypothesis that the CCHC motif of BIV Vif is critical for its activity against btA3Z2-Z3. To further confirm whether the CCHC motif is a critical domain for interacting with Cul2, 293 T cells were transiently transfected with cmyc-tagged btCul2 and VR-BIV Vif C111S/C113S-HA. BIV Vif was used as a positive control. Subsequently, co-immunoprecipitation experiments were performed to explore the interaction between btCul2 and BIV Vif or BIV Vif C111S/ C113S. After 48 h of transfection, the cells were immunoprecipitated with HA beads or with an anti-cmyc antibody, followed by SDS-PAGE and immunoblot analysis using an anti-HA antibody and an anti-cmyc antibody. The results revealed that double mutations in the CCHC motif of BIV Vif could completely block the interaction between BIV Vif and btCul2 (Figure 6E). However, this CCHC motif is different from all previously reported zinc finger structures (46), including the HCCH zinc finger in primate lentiviral Vifs. To further explore whether it is a zinc finger, we built a homology model for this potential zinc binding domain of BIV Vif (Figure 6F, G). The model implies this motif should be able to form a proper zinc coordination site. Although this motif is unlike all previously reported zinc fingers, the sequence and potential zinc coordinated motif of this domain are similar to those of “zinc binding loops”, as described previously [47].

Discussion
Mechanisms of BIV Vif- and MVV Vif-induced degradation of btA3Z2-Z3 and oaA3Z2-Z3 differ by the respective utilization of Cul2 and Cul5
Based on numerous investigations, HIV-1 Vif is known to recruit the transcription cofactor CBF-β and EloB-EloC to the Cul5-Rbx complex, thereby forming the E3 ubiquitin ligase and inducing the degradation of human antiviral proteins [12,17,28,48]. Other studies have explored further the degradation mechanisms of SIV Vif against RhA3G and FIV Vif against feline A3s [29-31]. In the current study, we investigated the effects of BIV and MVV Vifs on
the stability of btA3Z2-Z3 and oaA3Z2-Z3, respectively. First, we found that the proteasome inhibitor MG132 [39] could block the degradation effect of BIV Vif on btA3Z2-Z3 and that of MVV Vif on oaA3Z2-Z3, suggesting that they are proteasome-dependent processes. In addition, BIV and MVV Vifs were found to affect the synthesis rate rather than the stability of btA3Z2-Z3 and oaA3Z2-Z3, respectively. The endogenous immunoprecipitation experiments showed that btEloB, btEloC and btCul2 are involved in the degradation of btA3Z2-Z3 by BIV Vif. Correspondingly, oaEloB, oaEloC and oaCul5 are involved in the degradation of oaA3Z2-Z3 by MVV Vif. Meanwhile, the host CBF-β proteins do not play a role in this function of either BIV or MVV Vif. Co-immunoprecipitation assays further verified the direct interaction of BIV Vif with btEloC and btCul2, but not with btCBF-β or btCul5. MVV Vif also was confirmed to directly interact with oaEloC and oaCul5, but not with oacBF-β or oacCul2. Similarly, we found no interaction between FIV Vif and feline CBF-β (data not shown). CBF-β is reported to regulate Vif-Cul5 ligase by promoting the folding of primate lentiviral Vifs (HIV-1 and SIV) [14,29,48,49]. The fact that other mammalian Vifs do not require the participation of CBF-β for their function implies that BIV/MVV/FIV Vif may utilize a different mechanism for recruiting the Cul-E3 ligase or factors other than CBF-β which have

Figure 5 Dominant-negative btCul2 and oaCul5 mutants block the degradation, while TPEN can inhibit btA3Z2-Z3 degradation. 293 T cells (0.5 × 10⁶) were co-transfected with (A) 30 ng of HA-tagged btA3Z2-Z3 and 200 ng cmyc-tagged BIV Vif or VR1012, adjusted to 500 ng with 300 ng cmyc-tagged btCul2ΔNedd8, a control vector cmyc-tagged hCul1K720R or VR1012. (B) 15 ng HA-tagged oaA3Z2-Z3 and 200 ng cmyc-tagged MVV Vif or VR1012, adjusted to 500 ng with 300 ng cmyc-tagged oaCul5ΔNedd8, a control vector cmyc-tagged hCul1K720R or VR1012. (C) 50 ng HA-tagged hA3G and 600 ng cmyc-tagged HIV Vif or VR1012, adjusted to 900 ng with 300 ng cmyc-tagged btCul2ΔNedd8, a control vector cmyc-tagged hCul1K720R or VR1012. At 48 h after transfection, the cells were harvested for Western blotting using anti-HA, anti-cmyc and anti-tubulin antibodies. The percentages of btA3Z2-Z3, oaA3Z2-Z3 or hA3G in the presence of btCul2ΔNedd8, oaCul5ΔNedd8 or hCul5ΔNedd8 relative to that in the absence of BIV/MVV/HIV Vif (set to 100%) were calculated. 293 T cells (0.5 × 10⁶) were co-transfected with (D) 30 ng HA-tagged btA3Z2-Z3 and 200 ng cmyc-tagged BIV Vif or VR1012, (E) 15 ng HA-tagged oaA3Z2-Z3 and 200 ng cmyc-tagged MVV Vif or VR1012. After 36 h of transfection, the cells were treated with TPEN at 3.5 μM or DMSO as a control. At 48 h after transfection, the cells were harvested for Western blotting using anti-HA, anti-cmyc and anti-tubulin antibodies. Percentages of degradation with DMSO or TPEN treatment were calculated.
Figure 6 (See legend on next page.)
not been discovered in the degradation of btA3Z2-Z3 by BIV Vif and that of oaA3Z2-Z3 by MVV Vif.

We constructed an EloC mutant in which the key hydrophobic amino acids (A100 and L103) were substituted with hydrophilic serine, which disrupted the interaction between the EloC and the BC box of the cellular proteins [41]. These mutations disrupted the degradation of btA3Z2-Z3 induced by BIV Vif and that of oaA3Z2-Z3 induced by MVV Vif, implicating the involvement of EloC in the process. Although the sequence homology of lentiviral Vifs is poor, they share a highly conserved S/TLQY/RLA motif, which is a BC box essential for the binding of EloC [12,27,28]. The SLQ mutations in BIV and MVV Vifs could block the degradation of btA3Z2-Z3 and oaA3Z2-Z3, confirming that the BC-box motifs in these Vifs are essential for the recruitment of the E3 complex. These two experiments both showed that EloC is a member of the E3 ligase complex. The participation of btCul2 in the degradation induced by BIV Vif and that of oatCul5 in the degradation induced by MVV Vif was further verified by use of dominant-negative btCul2 and oatCul5 mutants. The disruption of BIV Vif-induced degradation of btA3Z2-Z3 and MVV Vif-induced degradation of oatA3Z2-Z3 by the corresponding mutants demonstrated that btCul2 and oatCul5 are required for the BIV and MVV Vif activity against btA3Z2-Z3 and oatA3Z2-Z3, respectively.

**CCHC motif is crucial for BIV Vif activity and predicted to be a zinc binding loop**

Primate lentiviral Vifs contain a zinc coordination site $\text{H-x$_4$-C-x$_{17}$-H-x$_{19}$-C}$ (HCCH) [24, 26, 48], which determines the selective recruitment of Cul5 by HIV-1 and SIV Vif. Although FIV Vif does not contain an HCCH domain, it can still recruit Cul5 [31]. Previous research has indicated that FIV Vif may utilize a new non-zinc finger dependent mechanism for interacting with Cul5 [31]. These findings suggest that the Cul2 box and Cul5 box downstream of the BC box or HCCH domain are not absolutely necessary for the recruitment of Cul2 or Cul5. In this study, we found a potential zinc finger, the $\text{C-x$_1$-C-x$_1$-H-x$_{19}$-C}$ motif, which was critical for the degradation activity of BIV Vif and the interaction with a Cul protein. This motif is different from all previously reported zinc finger structures, including the HCCH zinc finger in primate lentiviral Vifs, but the sequence and potential zinc coordinated motif of this domain are almost identical to those of “zinc binding loops” (45), except the distance between the 3rd (H115) and 4th (C134) residues is much longer in our protein. We then further analyzed the sequence of this potential zinc binding domain and found only one hit covering the full-length sequence of this domain, the crystal structure of tatD DNase of *Escherichia coli*, which is also a metallic ion binding protein [50]. Using this structure as a template, we built a homology model for the potential zinc binding domain of BIV Vif. The model suggests that the entire domain likely forms an alpha-beta-alpha super secondary structure. The 4th residue of this potential zinc domain folds back through this structure and is distally close to the other three residues. Thus, they should be able to form a proper zinc coordination site, which is also consistent with our mutagenesis studies.

**Various mechanisms of lentiviral Vif-mediated degradation of A3 proteins are slightly different**

Slight differences in various mechanisms of lentiviral Vif-mediated degradation of A3 proteins (Table 2) may be attributed to different factors involved. First, the host proteins involved in the degradation are different. Primate lentiviral Vifs require CBF-$\beta$ as a regulator of the folding of Vif to neutralize the A3 proteins, but non-primate lentiviral Vifs, including those of FIV, BIV and MVV, do not require CBF-$\beta$ to neutralize the A3 proteins. All lentiviral Vifs recruit Cul5 except for BIV Vif, which was found here to recruit Cul2. Second, the mechanisms of interaction between the Vif protein and Cul protein are different. Primate lentiviral Vifs utilize a highly conserved HCCH zinc-binding motif to bind with Cul5. By contrast, BIV Vif may utilize the CCHC domain to bind with Cul2, while FIV and MVV may use a novel method for recruiting Cul5.
Conclusions
Overall, this study supplements our knowledge of the mechanism of degradation of host antiviral proteins induced by BIV Vif and MVV Vif. Our work described the interaction between BIV Vif and btCul2 through a previously unreported zinc binding loop [C-x1-C-x1-H-x19-C] which may provide a foundation for further studies on similar protein-protein interactions. The CBF-β-independent degradation pathway suggests that the degradation of btA3Z2-Z3 by BIV Vif and that of oaA3Z2-Z3 by MVV Vif may require factors different from CBF-β. These viruses and their hosts have co-evolved various mutually antagonistic proteins, which over the long evolutionary process have facilitated viral entry into new hosts. Thus, our work may shed light on the course of disease in cows and sheep, as well as the potential for cross-species transmission of BIV or MVV. Further studies to identify these factors may provide new insights into the molecular mechanism(s) of Vif-mediated neutralization of host innate immune defenses.

Methods
Plasmid construction
The genes encoding btEloB, btEloC, btCBF-β, btCul2, btCul5, oaEloB, oaEloC, oaoCBF-β, oaoCul2 and oaoCul5 were obtained by RT-PCR. Bovine total RNA was extracted from MDBK cells, and ovine total RNA was extracted from MDOK cells using TRIzol (Invitrogen, Carlsbad, CA) separately. The following primers were used to amplify the genes by RT-PCR: btCul2, forward (5'-ATGGACG TGTTCCT CATGATC-3') and reverse (5'-CTAGGGTCTTGTCTTCTT-3'); btCul5, forward (5'-GAGTCTAAGTTGAAGGAACATG-3') and reverse (5'-ATTGTCCATGATATTCAAAATTA-3'); btCul5-cmyc, forward (5'-ATGGACG TGTTCCT CATGAT-3') and reverse (5'-TCACTGCACAGCCTGTTCGT-3'); oaoCBF-β, forward (5'-ATGCCGCGCTGTCGTGGCCCC-3') and reverse (5'-CTAGGGTCTTGTCTTCTT-3'); oaoCul2, forward (5'-ACACTAAACTGAAATACCTTCT-3') and reverse (5'-TCAGGCCACGTAG CTGTACTCATCT-3'); oaoCul5, forward (5'-ATGGACG TGTTCCT CATGAT-3') and reverse (5'-TCACTGCACAGCCTGTTCGT-3'); oaoCul5-cmyc, forward (5'-ATGCCGCGCTGTCGTGGCCCC-3') and reverse (5'-CTAGGGTCTTGTCTTCTT-3').

Table 2 Comparison of various mechanisms of lentiviral Vif-mediated degradation of A3 proteins

| Lentivirus | E3 complex | Method of binding with EloC | Cul2/5 | Method of binding with Cul | CBF-β involved |
|------------|------------|-----------------------------|--------|---------------------------|----------------|
| HIV        | Y          | BC box (SLQ)                | Cul5   | zinc finger               | Y              |
| SIV        | Y          | BC box (SLQ)                | Cul5   | zinc finger               | Y              |
| FIV        | Y          | BC box (TLQ)                | Cul5   | unknown                   | N              |
| BIV        | Y          | BC box (SLQ)                | Cul2   | zinc binding loop         | N              |
| MVV        | Y          | BC box (SLQ)                | Cul5   | unknown                   | N              |
codon-optimized BIV \textit{vif} gene and then subcloned into the VR1012 vector at the \textit{Bam}HI and \textit{Not}I restriction sites. VR-BIV \textit{Vif}-HA was derived from VR-BIV \textit{Vif}-cmyc with primers that added an HA tag in frame at its C-terminus. VR-btCul2-HA, and VR-btCul5-HA were similarly obtained from corresponding cmyc-tagged plasmids (VR-btCul2-cmyc and VR-btCul5-cmyc, respectively).

Via site-directed mutagenesis, VR-BIV \textit{Vif} SLQ-AAA-cmyc, VR-BIV \textit{Vif} H102L-cmyc, VR-BIV \textit{Vif} C111S-cmyc, VR-BIV \textit{Vif} C113S-cmyc, VR-BIV \textit{Vif} H115L-cmyc, VR-BIV \textit{Vif} C134S-cmyc and VR-BIV \textit{Vif} H149L-cmyc and VR-BIV \textit{Vif} C111S/ C113S-cmyc were derived from VR-BIV \textit{Vif}-cmyc. Meanwhile, VR-BIV \textit{Vif} C111S/ C113S-HA was derived from VR-BIV \textit{Vif}-HA. The btCul2, btCul5 and btEloC mutants were engineered based on the corresponding plasmids (VR-btCul2-cmyc, VR-btCul5-cmyc and VR-btEloC-HA, respectively). These mutant constructs were made using the QuickChange mutagenesis system (Stratagene, La Jolla, CA) and confirmed by sequencing.

VR-MVV \textit{Vif-cmyc} is a eukaryotic plasmid expressing the codon-optimized MVV Icelandic strain 1514 \textit{vif} gene. It was generated by adding a cmyc tag to the C-terminus of the codon-optimized MVV \textit{vif} gene and then subcloned into the VR1012 vector at the \textit{Sal}I and \textit{Not}I restriction sites.

VR-MVV \textit{Vif} SLQ-AAA-cmyc was derived from VR-MVV \textit{Vif-cmyc} via site-directed mutagenesis, and the oaCul2, oaCul5 and oaEloC mutants were engineered based on the corresponding plasmids (VR-oaCul2-cmyc, VR-oaCul5-cmyc and VR-oaEloC-cmyc, respectively). VR1012 clone using the QuickChange mutagenesis system and confirmed by sequencing.

The expression vectors VR-HIV \textit{Vif-cmyc}, PC-hA3G-HA, VR-hec-HA, VR-hCBF-\beta-Flag and the infectious molecular clone pNL4-3 were described previously [12,16,51]. The expression vectors VR-hCul5-cmyc, VR-hCul5 \Delta Nedd8-cmyc and VR-hCul1K720R-cmyc were derived from VR-hCul5-HA, VR-hCul5 \Delta Nedd8-HA and VR-hCul1K720R-HA, respectively, as described previously [43,52].

**Cells and transfections**

HEK293T (CRL-11268) cells, MDBK (CCL-22) cells and MDOK (CRL-1633) cells were purchased from the American Type Culture Collection (ATCC, Manassas, VA). MAGI-CCR5 cells (catalog number 3522) were obtained from the NIH AIDS Research and Reference Reagent Program (NIH-ARRRP). HEK293T and MAGI-CCR5 cells were cultured in Dulbecco’s Modified Eagle’s Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) at 37°C with 5% CO₂. MDBK cells were cultured in DMEM supplemented with 10% horse serum at 37°C with 5% CO₂. MDOK cells were cultured in Minimum Essential Medium (MEM) supplemented with 10% FBS at 37°C with 5% CO₂. Transfections of 293 T cells were performed using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions.

**Antibodies**

The following antibodies were used in the present study: anti-HA mouse monoclonal antibody (mAb; Covance, Emeryville, CA), anti-cmyc mouse mAb (Millipore, Billerica, MA), anti-Flag mouse mAb (Sigma, St. Louis, MO), anti-tubulin mouse mAb (Covance), anti-Cul2 rabbit polyclonal antibody (pAb; Santa Cruz Biotechnology, Santa Cruz, CA), anti-Cul5 rabbit pAb (Santa Cruz Biotechnology), anti-CBF-\beta mouse mAb (Santa Cruz Biotechnology), anti-EloB rabbit pAb (Santa Cruz Biotechnology) and anti-EloC mouse mAb (Santa Cruz Biotechnology).

**Western blotting**

Cells were collected 48 h post-transfection and lysed with sodium dodecyl sulfate (SDS) sample buffer. The samples were boiled for 20 min and separated by SDS-polyacrylamide gel electrophoresis (PAGE) and then transferred onto nitrocellulose membranes (Whatman, Kent, UK). After blocking in 5% nonfat milk, the membranes were probed with various primary antibodies against proteins of interest. Secondary antibodies were alkaline phosphatase-conjugated anti-rabbit, anti-mouse IgG (Jackson ImmunoResearch, West Grove, PA). Immunoreactions were detected with 5-bromo-4-chloro-3-indolylphosphate (BCIP) and nitro blue tetrazolium chloride (NBT) solutions.

**MG132 inhibition assay**

To determine if the degradation of btA3Z2-Z3 by BIV \textit{Vif} or that of oaA3Z2-Z3 by MVV \textit{Vif} is proteasome-dependent, 293 T cells were treated with the proteasome inhibitor MG132 (Sigma–Aldrich) at 10 \(\mu\)M and DMSO as negative control at 36 h after transfection with indicated plasmids. At 48 h after transfection, 293 T cells were harvested and analyzed by Western blotting.

**TPEN inhibition assay**

To explore if Zn is significant for the degradation of A3Z2-Z3 induced by \textit{Vif}, 293 T cells were treated with TPEN at 3.5 \(\mu\)M and DMSO as negative control at 36 h after transfection with indicated plasmids. At 48 h after transfection, 293 T cells were harvested and analyzed by Western blotting.

**CHX-treated A3Z2-Z3 stability assay**

At 36 h after transfection with indicated plasmids, 293 T cells were treated with CHX (Sigma–Aldrich) at the final concentration of 100 \(\mu\)g/ml for 0, 6, 12, 24 h and then harvested and analyzed by Western blotting.
Immunoprecipitation assay
At 48 h after transfection with indicated plasmids, 293 T cells were obtained and dissociated in lysis buffer (50 mM Tris, pH 7.5, with 150 mM NaCl, 1% Triton X-100 and complete protease inhibitor cocktail tablets) at 4°C for 1 h, followed by centrifugation at 10,000 × g for 10 min at 4°C to pellet the cell debris. The pre-cleared supernatants were collected and then mix with anti-HA Ab-conjugated agarose beads (Roche, Mannheim, Germany), followed by incubation at 4°C for 3 h. Alternatively, the pre-cleared supernatants were collected and incubated with mouse anti-cmcY (Millipore) for 1 h and then mix with Protein G-agarose (Roche), followed by incubation at 4°C for 3 h. The beads were washed three times with wash buffer (20 mM Tris, pH 7.5, with 100 mM NaCl, 0.1 mM EDTA and 0.05% Tween 20), and the pellet was resuspended in 30 μl glycine HCl (pH 2.0) elution buffer. The eluted materials were subsequently analyzed by Western blotting.

Viral infectivity assay
Viral infection was determined by a multineuronal activation of a galactosidase indicator (MAGI) assay as described previously [12]. Briefly, MAGI-CCR5-5 cells were seeded in 24-well plates 1 day before infection. The MAGI-CCR5 cells were infected at 20 MOI in 96-well plates 1 day before infection. The MAGI-CCR5-5 cells were inoculated with medium with infected supernatant (150 mM NaCl, 1% Triton X-100 and complete protease inhibitor cocktail tablets) at 4°C for 1 h, followed by centrifugation at 10,000 × g for 10 min at 4°C to pellet the cell debris. The pre-cleared supernatants were collected and then mix with Protein G-agarose (Roche), followed by incubation at 4°C for 3 h. The beads were washed three times with wash buffer (20 mM Tris, pH 7.5, with 100 mM NaCl, 0.1 mM EDTA and 0.05% Tween 20), and the pellet was resuspended in 30 μl glycine HCl (pH 2.0) elution buffer. The eluted materials were subsequently analyzed by Western blotting.

Sequence analysis and modeling
The homology model of BIV Vif was built by Discovery Studio 2.1 software package using the crystal structure of the homology model of BIV Vif was built by Discovery Studio 2.1 software package using the crystal structure of Torpedo californica metalloproteinase as the template. Modeling was performed at the medium optimization level with refined loop parameters, and no additional restraints were used. Ten models were built, and the model with the best score was chosen.

Competing interests
The authors declare that they have no competing interests.

Authors’ contributions
JYZ, XY and WK conceived and designed the experiments and wrote the paper. JYZ, BY and WRW performed the experiments. JYZ, JW and HW analyzed the data. JWV, MYL, KDDW and HZH helped design the study and provided reagents. All authors read and approved the final manuscript.

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