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Short Communication

Vif determines the requirement for CBF-β in APOBEC3 degradation

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APOBEC3 (apolipoprotein B mRNA editing enzyme catalytic polypeptide-like 3) proteins are cellular DNA deaminases that restrict a broad spectrum of lentiviruses. This process is counteracted by Vif (viral infectivity factor) of lentiviruses, which binds APOBEC3s and promotes their degradation. CBF-β (core binding factor subunit β) is an essential co-factor for the function of human immunodeficiency virus type 1 Vif to degrade human APOBEC3s. However, the requirement for CBF-β in Vif-mediated degradation of other mammalian APOBEC3 proteins is less clear. Here, we determined the sequence of feline *CBFB* and performed phylogenetic analyses. These analyses revealed that mammalian *CBFB* is under purifying selection. Moreover, we demonstrated that CBF-β is dispensable for feline immunodeficiency virus Vif-mediated degradation of APOBEC3s of its host. These findings suggested that primate lentiviruses have adapted to use CBF-β, an evolutionary stable protein, to counteract APOBEC3 proteins of their hosts after diverging from other lentiviruses.

Several human APOBEC3 (apolipoprotein B mRNA editing enzyme catalytic polypeptide-like 3) proteins, notably APOBEC3D, APOBEC3F, APOBEC3G and APOBEC3H, have the capacity to restrict human immunodeficiency virus type 1 (HIV-1) replication (Albin & Harris, 2010; Desimmie *et al.*, 2014; Feng *et al.*, 2014; Kitamura *et al.*, 2011; Refsland & Harris, 2013). These restriction factors are incorporated into the progeny virions and enzymically convert viral cDNA cytosines to uracils during reverse transcription, which can debilitate viral function (Albin & Harris, 2010; Desimmie *et al.*, 2014; Feng *et al.*, 2014; Kitamura *et al.*, 2011; Refsland & Harris, 2013). Although original studies focused on human APOBEC3G (Harris *et al.*, 2003; Mariani *et al.*, 2003; Sheehy *et al.*, 2002; Zhang *et al.*, 2003), subsequent work revealed that all placental mammals have APOBEC3 enzymes (Münk *et al.*, 2012), albeit different numbers, and that these enzymes have the potential to attenuate the infectivity of a broad spectrum of viruses, including simian immunodeficiency virus (SIV) (Mariani *et al.*, 2003), feline immunodeficiency virus (FIV) (Münk

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et al., 2008; Zielonka *et al.*, 2010), bovine immunodeficiency virus (BIV) (LaRue *et al.*, 2010) and small ruminant lentiviruses (SRLVs; e.g. Maedi-Visna virus and caprine arthritis encephalitis virus) (LaRue *et al.*, 2010). However, to counteract APOBEC3-mediated restriction, these lentiviruses encode a protein called Vif (viral infectivity factor). Vif recruits a cellular E3 ubiquitin ligase complex including cullin 5 (CUL5) and elongin B/C (ELOB/C), and degrades host APOBEC3 proteins through a ubiquitin/proteasome-dependent pathway (Albin & Harris, 2010; Desimmie *et al.*, 2014; Feng *et al.*, 2014; Kitamura *et al.*, 2011; Refsland & Harris, 2013).

Several studies have demonstrated that CBF-β (core binding factor subunit β) is an essential co-factor for HIV-1 and SIV Vif proteins to form the ubiquitin ligase complex that degrades human and rhesus monkey APOBEC3 enzymes (Hultquist *et al.*, 2012a; Jäger *et al.*, 2012; Zhang *et al.*, 2012). CBF-β normally functions as a transcription factor for haematopoiesis, T-cell differentiation and bone development through binding with Runt-related transcription factors 1, 2 and 3 (RUNX1, 2 and 3) (Adya *et al.*, 2000; de Bruijn & Speck, 2004; Ito, 2008). Structural studies have provided additional compelling evidence that CBF-β is an integral part of the HIV-1 Vif ubiquitin ligase complex (Guo *et al.*, 2014; Kim *et al.*, 2013). In particular, the surface area of the CBF-β/HIV-1 Vif heterodimeric interface is nearly 5000 Å^2, which is larger than that in the CBF-β/RUNX complex (Guo *et al.*, 2014).

Despite these advances, it is not clear whether CBF-β is required for non-primate lentiviral Vif proteins to degrade the APOBEC3 proteins of their hosts. For instance, although FIV Vif also degrades feline APOBEC3 proteins through a ubiquitin/proteasome-dependent pathway (Wang *et al.*, 2011), a role for CBF-β in this complex has yet to be investigated. Moreover, the feline CBF-β gene (*CBFB*) has yet to be characterized. Here, we sequenced feline *CBFB* and performed molecular phylogenetic analyses to show it is under purifying selection. We further demonstrated that CBF-β was dispensable for FIV Vif function in degrading feline APOBEC3 proteins.

To investigate the conservation of *CBFB* in mammals, including domestic cats, in depth, we set out to determine the sequence of feline *CBFB*. The open reading frame (ORF) of domestic cat (*Felis catus*) *CBFB* was amplified by PCR using a cDNA library of the MYA-1 cell line (Shimojima *et al.*, 2004) as the template and the following primers: fCBFb-F2, 5′-ATGCCGCGCGTCGTGCCCG-3′ and fCBFb-R1, 5′-TTAACGAAGTTTGAGGTCATCACC-AC-3′. PCR was performed by using PrimeStar GXL DNA polymerase (TaKaRa) according to the manufacturer's protocol. The obtained DNA fragment was cloned into pCR-Blunt II-TOPO plasmid by using a Zero Blunt TOPO PCR cloning kit (Life Technologies) according to the manufacturer's protocol. The sequence of domestic cat *CBFB* was determined by a DNA sequencing service (Fasmac) and the data were analysed with Sequencher v5.1 software (Gene Codes). The domestic cat *CBFB* ORF was aligned with 27 mammalian and one avian *CBFB* sequences using MAFFT implemented in the GUIDANCE server (Penn *et al.*, 2010). The sequences used in this study are listed in Table 1 and the resulting alignment was verified manually at the amino acid level. Then the phylogenetic tree of the 29 *CBFB* genes was reconstructed using the maximum-likelihood method with PhyML (Guindon *et al.*, 2010). As shown in Fig. 1(a), domestic cat *CBFB* formed a cluster with those of ferret, Weddell seal and walrus, which is supported by a relatively high bootstrap value (91 %).

We then conducted the analysis to estimate selective pressure acting on *CBFB* genes. The random effects likelihood (REL) method available in the HyPhy package was employed (Pond & Frost, 2005). As shown in Fig. 1(b), 41 sites were found to be under negative selection that had a Bayes factor >50 for $dN<dS$. In addition, the values of E (dN–dS) at all the codons except for codon 180 in *CBFB* were <0 (Fig. 1c). RUNX1-binding sites (Bravo *et al.*, 2001; Tahirov *et al.*, 2001; Yan *et al.*, 2004) and Vif-binding sites (Du *et al.*, 2013) were highly conserved in CBF-β (Fig. 1c). Moreover, although the identity between human *CBFB* and domestic cat *CBFB* is 96.5 % at the nucleotide level, the amino acid sequences were 100 % identical (data not shown). Taken together, these results indicated that mammalian *CBFB* is under strong purifying selection and stably maintained in mammals.

It is known that domestic cat (*F. catus*) expresses five kinds of APOBEC3 proteins, APOBEC3Z2a, APOBEC3Z2b, APOBEC3Z2c, APOBEC3Z3 and APOBEC3Z2Z3 (LaRue *et al.*, 2010; Münk *et al.*, 2008; Stern *et al.*, 2010; Zielonka *et al.*, 2010), and that feline APOBECZ3 and APOBEC3Z2Z3 have the ability to restrict FIV infectivity (Zielonka & Münk, 2011). To determine whether CBF-β was required for FIV Vif as a co-factor to degrade feline APOBEC3s, we constructed a FLAG-tagged FIV Vif expression plasmid as follows: the codon-optimized ORF of FIV strain Petaluma with a FLAG-tag at the C terminus (the sequence is available upon request) was obtained from GeneArt Gene Synthesis service (Life Technologies). The obtained DNA was digested with *Bgl*II and *Sal*I, and then was inserted into the *Bam*HI/*Sal*I sites of pDON-AI plasmid (TaKaRa). Expression plasmids for haemagglutinin (HA)-tagged feline APOBEC3Z2b (AY971954), feline APOBEC3Z3 (EU011792), feline APOBEC3Z2Z3 (HM100128) (Münk *et al.*, 2008) and human CBF-β (Jäger *et al.*, 2012) were also used. We co-transfected each feline APOBEC3-HA expression plasmid and FIV Vif-FLAG expression plasmid with or without human CBF-β expression plasmid into HEK293T-sh*CBFB* cells, a HEK293T cell line stably expressing small hairpin RNA targeting the 5′ UTR of endogenous human *CBFB* (Jäger *et al.*, 2012), using Lipofectamine 2000 according to the manufacturer's procedure (Life Technologies). Cells were harvested at 48 h post-transfection, and extracts were analysed by SDS-PAGE and Western blotting as described previously (Kobayashi *et al.*, 2014a, b) with the following antibodies: anti-HA mAb (3F10; Roche), anti-FLAG polyclonal

Table 1. GenBank accession numbers of the *CBFB* genes used in this study

The common name of each primate is identical to that in Fig. 1(a).

Common name	Scientific name	GenBank accession no.
Human	*Homo sapiens*	AK290462
Gorilla	*Gorilla gorilla*	XM_004057790
Chimpanzee	*Pan troglodytes*	GABD01004603
Gibbon	*Nomascus leucogenys*	XM_003262871
Rhesus monkey	*Macaca mulatta*	JU336882
Cynomolgus monkey	*Macaca fascicularis*	XM_005592190
Common marmoset	*Callithrix jacchus*	XM_002761058
Cattle	*Bos taurus*	NM_001191435
Killer whale	*Orcinus orca*	XM_004280877
Bottlenose dolphin	*Tursiops truncatus*	XM_004311020
Thirteen-lined ground squirrel	*Ictidomys tridecemlineatus*	XM_005318290
White rhinoceros	*Ceratotherium simum*	XM_004431701
Ferret	*Mustela putorius furo*	XM_004744336
Domestic cat	***Felis catus***	**LC003231 (this study)**
Walrus	*Odobenus rosmarus*	XM_004393406
Weddell seal	*Leptonychotes weddellii*	XM_006741505
Nine-banded armadillo	*Dasypus novemcinctus*	XM_004460890
Manatee	*Trichechus manatus*	XM_004371510
Cape elephant shrew	*Elephantulus edwardii*	XM_006878708
Cape golden mole	*Chrysochloris asiatica*	XM_006863598
Golden hamster	*Mesocricetus auratus*	XM_005076189
Prairie vole	*Microtus ochrogaster*	XM_005345497
Rat	*Rattus norvegicus*	BC081946
Mouse	*Mus musculus*	D14571
American pika	*Ochotona princeps*	XM_004583922
Naked mole rat	*Heterocephalus glaber*	XM_004843071
Degu	*Octodon degus*	XM_004625892
Chinchilla	*Chinchilla lanigera*	XM_005403704
Chicken	*Gallus gallus*	AF472513

antibody (OctA; Santa Cruz), anti-CBF-β mAb (sc-56751; Santa Cruz) and anti-α-tubulin (TUBA) mAb (DM1A; Sigma). As described in Fig. 2(a), the levels of feline APOBECZ2b, APOBEC3Z3 and APOBEC3Z2Z3 were decreased by FIV Vif regardless of the presence or absence of CBF-β. These results showed that FIV Vif did not require CBF-β for feline APOBEC3 degradation.

Interestingly, it has been reported that the Vif protein of SIVmac can degrade feline APOBEC3Z2Z3 (Stern *et al.*, 2010). This raises the possibility that feline APOBEC3Z2Z3 degradation may be governed directly by the APOBEC3–Vif interaction (i.e. SIVmac Vif may be able to degrade feline APOBEC3Z2Z3 without CBF-β). To address this, we constructed a FLAG-tagged SIVmac239 Vif expression plasmid as follows: PCR was performed by using *PfuUltra* High-Fidelity DNA Polymerase (Agilent Technologies) with pSIVmac239 (M33262) (kindly provided by Dr Tomoyuki Miura) as the template and the following primers: forward, 5′-TTTTTTTTGGATCCGCCACCATG-GAGGAGGAAAAGAGG-3′ and reverse, 5′-TTTTTTTT-TTGTCGACTCACTTATCGTCGTCATCCTTGTAATCT-GCCAGTATTCCCAAGAC-3′. The obtained SIVmac239 Vif-FLAG ORF fragment was digested with *Bam*HI and *Sal*I, and then was inserted into the *Bam*HI/*Sal*I site of pDON-AI plasmid. Plasmid integrity was confirmed by DNA sequencing, as described above. We co-transfected the feline APOBEC3Z2Z3-HA expression plasmid and either a FIV Vif-FLAG or a SIVmac Vif-FLAG expression plasmid with or without a human CBF-β expression plasmid into HEK293T-sh*CBFB* cells (Jäger *et al.*, 2012). As shown in Fig. 2(b), we found that CBF-β was essential for SIVmac Vif to degrade feline APOBEC3Z2Z3. These results showed that CBF-β was essential for the Vif protein of SIVmac, a primate lentivirus, to degrade APOBEC3, regardless of the origin of the APOBEC3 protein.

Thus, we have shown that *CBFB* is highly conserved in mammals, including the hosts of lentiviruses such as human, gorilla, chimpanzee, rhesus monkey, cattle, and domestic cat (Fig. 1). We have also demonstrated that FIV Vif does not use CBF-β for feline APOBEC3 degradation (Fig. 2). These data corroborate recent reports suggesting that not only FIV Vif but also BIV and SRLV Vif proteins do not require CBF-β to degrade APOBEC3 proteins of their hosts (Ai *et al.*, 2014; Zhang *et al.*, 2014). These observations indicate that only primate lentiviruses require CBF-β for APOBEC3 degradation and further suggest that

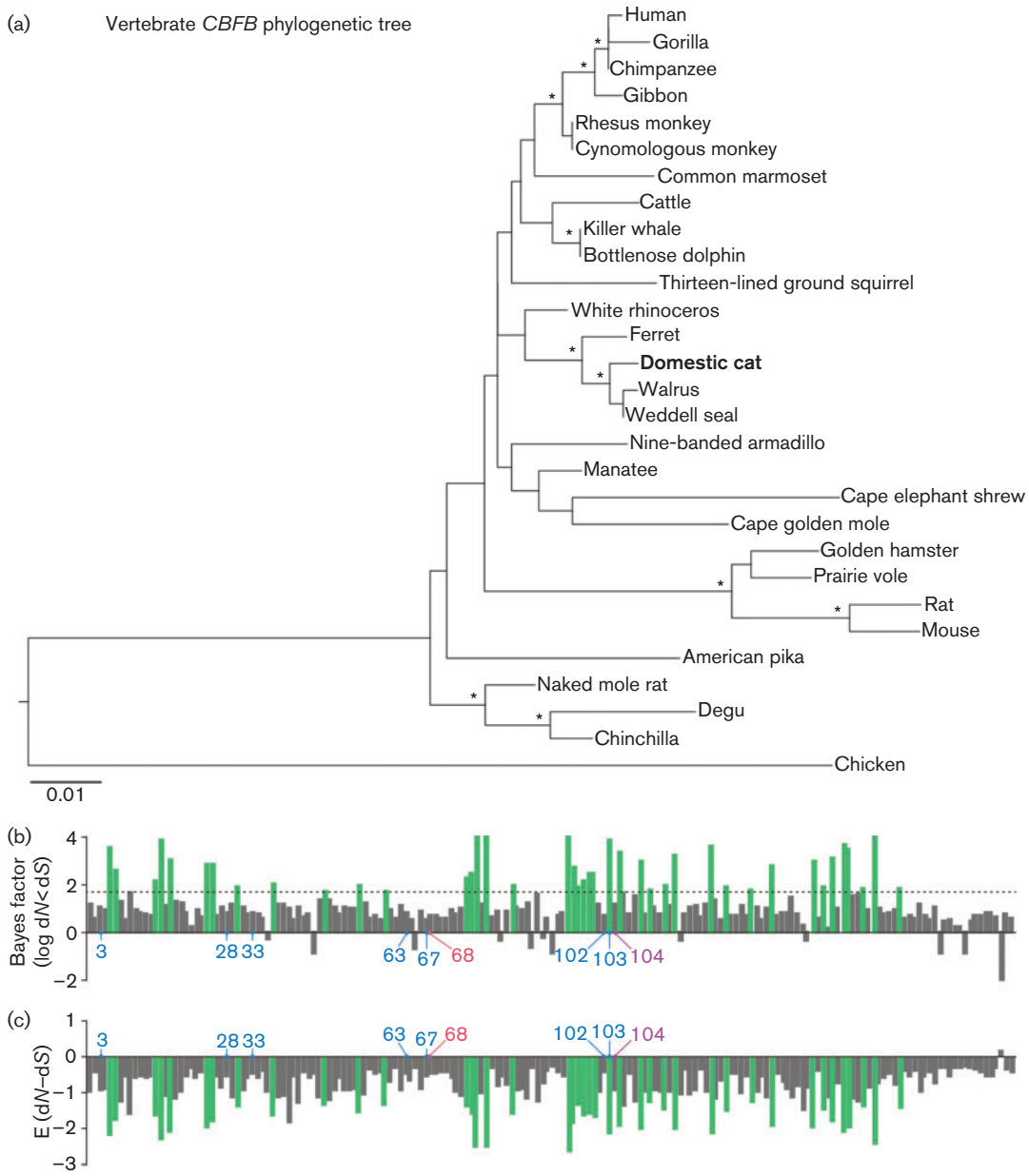

Fig. 1. Molecular phylogenetic analyses of *CBFB*. (a) Phylogenetic tree of 29 *CBFB* genes reconstructed using the maximum-likelihood method. Chicken *CBFB* was used as an outgroup. Nodes with >70 % bootstrap values are indicated with asterisks. (b, c) Negative selection in 29 *CBFB* genes inferred by the REL method in HyPhy. The Bayes factor for d*N*<d*S* (negative selection) (b) and the *E* (d*N*–d*S*) value (c) are shown. RUNX1-binding sites (aa 3, 28, 33, 63, 67, 102 and 103; blue) (Bravo *et al*., 2001; Tahirov *et al*., 2001; Yan *et al*., 2004), Vif-binding site (aa 68; red) (Hultquist *et al*., 2012b), and the site binding to both RUNX1 and Vif (aa 104; purple) are indicated. The green bars represent the 41 negatively selected sites identified with Bayes factor >50. The dotted line in (b) indicates the Bayes factor threshold of 50 specified for the REL analysis.

non-primate lentiviruses have either maintained an ancestral CBF-β-independent mechanism or have evolved to use another co-factor in the same way to degrade APOBEC3 proteins of their ancestral hosts. Interestingly, it has been recently reported that HIV-1 Vif can utilize CBF-β proteins from flies (*Drosophila melanogaster*) and worms (*Saccoglossus kowalevskii*) as the co-factor for human APOBEC3 degradation (Han *et al*., 2014). This further suggests that the usability of CBF-β is not dependent on the hosts and that primate lentiviral Vif has adapted to use CBF-β as an essential co-factor during its evolution.

In comparison with BIV and SRLVs, the feline lentivirus FIV is phylogenetically closer to primate lentiviruses (Gifford, 2012). Therefore, our findings strongly suggest

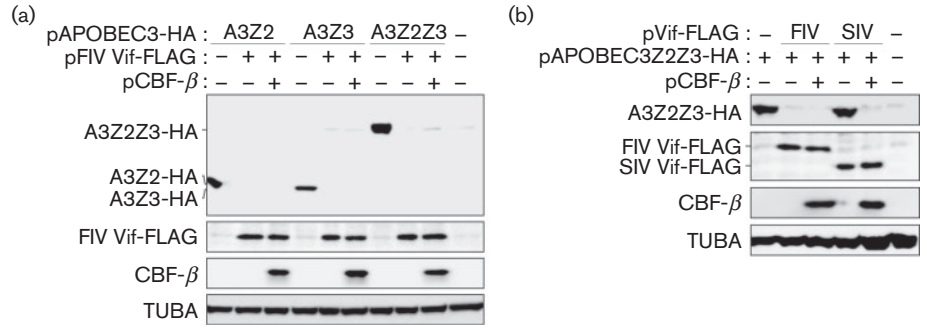

Fig. 2. Functional analyses of the requirement for CBF-β. (a) Feline APOBEC3-HA (150 ng) and FIV Vif-FLAG expression plasmids (500 ng) were co-transfected with or without human CBF-β expression plasmid (400 ng) into HEK293T-sh*CBFB* cells, and the transfected cell lysates were analysed by SDS-PAGE and Western blotting. As negative controls, pDON-AI and pcDNA3.1 plasmids were used in place of FIV Vif-FLAG and CBF-β expression plasmids, respectively. A3Z2, feline APOBEC3Z2b; A3Z3, feline APOBEC3Z3; A3Z2Z3, feline APOBEC3Z2Z3. (b) Feline APOBEC3Z2Z3-HA expression plasmid (150 ng) and either FIV Vif-FLAG or SIV Vif-FLAG expression plasmid (500 ng) were co-transfected with or without human CBF-β expression plasmid (400 ng) into HEK293T-sh*CBFB* cells, and the transfected cells were analysed by SDS-PAGE and Western blotting. As negative controls, pDON-AI and pcDNA3.1 plasmids were used in place of Vif-FLAG and CBF-β expression plasmids, respectively. FIV, FIV Vif-FLAG; SIV, SIVmac Vif-FLAG.

that the requirement of CBF-β for primate lentiviral Vif arose after the divergence with the FIV lineage. As CBF-β is under purifying selection in mammals (Fig. 1), it would be difficult for CBF-β to change in order to evade primate lentiviral Vif-mediated hijacking. This may be advantageous for the virus because CBF-β is an evolutionarily stable protein.

FIV Vif is very different from HIV/SIV Vif. For instance, FIV Vif has ~60 extra amino acids compared with HIV/SIV Vif, and the identity and the similarity of FIV Vif (strain Petaluma) to HIV-1 Vif (strain NL4-3) are only 16.6 and 64.7 %, respectively. These characteristics imply that FIV Vif is structurally dissimilar to HIV/SIV Vif. Additionally, Ai *et al.* (2014) have shown that the conserved CBF-β interaction sequences in SIV/HIV Vif are not present in FIV Vif. Furthermore, Wang *et al.* (2011) have reported that the FIV Vif has neither a CUL5 box nor HCCH zinc-binding motif, despite a functional requirement for CUL5 in APOBEC3 degradation. Due to these extensive differences with primate lentiviral Vif proteins, it is not clear whether FIV Vif has adapted to use another cellular protein(s) or whether it is capable of functioning without a CBF-β-like co-factor. More work will be needed to distinguish between these possibilities.

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