Characterization of red-capped mangabey tetherin: implication for the co-evolution of primates and their lentiviruses

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Primate lentiviruses including human immunodeficiency virus type 1 (HIV-1) and simian immunodeficiency viruses (SIVs) evolved through the acquisition of antagonists against intrinsic host restriction factors, such as tetherin. It is widely accepted that HIV-1 has emerged by zoonotic transmission of SIV in chimpanzee (SIVcpz), and that SIVcpz Nef protein antagonizes chimpanzee tetherin. Although Nef of SIVcpz shares a common ancestor with that of SIVrcm, an SIV in red-capped mangabey (Cercocebus torquatus), it remains unclear whether SIVrcm Nef can antagonize tetherin of its natural host. In this study, we determine the sequence of red-capped mangabey tetherin for the first time and directly demonstrate that SIVrcm Nef is the bona fide antagonist of red-capped mangabey tetherin. These findings suggest that SIVrcm Nef is the functional ancestor of SIVcpz Nef. Moreover, molecular phylogenetic analyses reveal that tetherins of the genus Cercocebus have experienced adaptive evolution, which is presumably promoted by primate lentiviruses.
Nef proteins\textsuperscript{53–56}. On the other hand, vpu is encoded only in seven out of the more than 40 PLVs identified: SIVgsn from greater-spot nosed monkey (Cercopithecus mona), SIVmon from moustached monkey (Cercopithecus cephus), SIVden from Dent’s monkey (Cercopithecus denti), SIVcpz, SIVgor, and HIV-1 from human (Homo sapiens; HU)\textsuperscript{1}. Of note, certain vpu-encoding SIVs from OWMs (SIVgsn, SIVmon, SIVmus, and SIVden) and HIV-1 antagonize tetherins of their own hosts by their Vpu proteins\textsuperscript{10,11,14,17–19}. These findings strongly suggest that PLVs have adapted to their hosts by acquiring the anti-tetherin factors at each adaptation process, and therefore, that the relationship between tetherin and viral antagonists can be a clue to reveal the evolutionary diversification of PLVs.

It is widely accepted that two HIVs, HIV-1 and HIV-2, have respectively emerged from independent zoonotic transmission of SIVs to HU around 100 years ago\textsuperscript{20–22}. HIV-1 has risen out of SIVcpz from CPZ to HU\textsuperscript{23–24}, while HIV-2 has resulted from zoonotic infection of SIVsm from SM\textsuperscript{23}. Also, it has been estimated that SIVcpz has emerged by the recombination of two lineages of SIVs: SIVgsn/mon/mus and SIVrcm from red-capped mangabey (Cercocetus torquatus; RCM)\textsuperscript{25}. Since SIVcpz Nef is phylogenetically similar to SIVrcm Nef, it seems conceivable that the ancestor of SIVcpz Nef is SIVrcm Nef\textsuperscript{14}. Moreover, because SIVcpz Nef is able to counteract CPZ tetherin\textsuperscript{14}, it has been hypothesized that SIVrcm Nef is the antigen of RCM tetherin\textsuperscript{5–7}. However, the sequence of RCM tetherin has not yet determined, and it remains unknown whether SIVrcm Nef is the bona fide antagonist of RCM tetherin. In this study, we determine the sequence of RCM tetherin for the first time and directly demonstrate that SIVrcm Nef counteracts anti-viral activity of RCM tetherin. Moreover, phylogenetic analyses of 47 primate tetherin sequences reveal that tetherins of the genus Cercocetus including RCM and SM are under positive selection.

Results

Phylogeny reconstruction of 47 primate tetherins. In order to directly elucidate the interplay between SIVrcm Nef and RCM tetherin, we set out to determine the sequence of RCM tetherin. The sequencing analyses of genomic DNA isolated from six wild-caught RCMs\textsuperscript{28–29} revealed that five out of the six open reading frames (ORFs) of RCM tetherin were identical, while a RCM tetherin displayed a double peak at nucleotide position 60 (GAC or GAT; synonymous mutation). These results imply the presence of polymorphism in RCM tetherin.

As shown in Figure 1a, compared with the amino acid sequence of RCM tetherin, HU tetherin displayed the 5-amino acid deletion (\textsuperscript{4}GDIWK\textsuperscript{5}) in the cytoplasmic domain, and HU and CPZ tetherins showed the 2-amino acid insertion (\textsuperscript{59}GTr\textsuperscript{60}) in the transmembrane domain, both of which are in line with the previous observations\textsuperscript{5,14,17,18,27–29}. The phylogenetic tree of 47 primate tetherins reconstructed by using maximum likelihood (ML) method (Figure 1b) revealed that the two RCM tetherins, which are newly sequenced in this study, are clustered with SM tetherins with stronger bootstrap support (87%), suggesting that RCM tetherin is most closely related to SM tetherin. Because only RCM and SM are the monkeys belonging to the genus Cercocetus among the 27 OWMs (the family Cercopithecidae; Table 1), this result is reasonable.

Positive selection detected in the evolution of primate tetherin. The nonsynonymous to synonymous rate ratios (dN/dS) inferred by using a free-ratio model in the PAML package are shown on the ML tree (Figure 1b), which varied among the branches. Since a dN/dS value significantly greater than one is an indicator of positive selection, our result indicates that positive selection probably operated on the tetherin gene episodically during primate evolution. This result is consistent with that obtained in previous studies\textsuperscript{19,21}.

The two pairs of site models in PAML produced similar results and the result obtained from M7 (neutral model) versus M8 (selection model) comparison is shown in Figure 1c. The dN/dS ratio was significantly greater than one for full-length tetherin, cytoplasmic tail, and transmembrane domains. These findings indicate that the functionally important regions of tetherin have evolved under strong positive selection, which agreed with previous reports\textsuperscript{31–33}. The site model analysis also identified three codons, 9, 14, and 17, to be under positive selection with posterior probability greater than 0.95 (Figure 1d). Two of them, codons 9 and 14, were also detected by the random effects likelihood (REL) analysis implemented in the HyPhy package with Bayes factor greater than 50 (Figure 1e; these sites are also indicated with green asterisks in Figure 1a). Although these two sites have been reported by a previous study in which 20 primate tetherins were analyzed\textsuperscript{11}, our results further suggest that they may have experienced positive selection in the evolution of many primate lineages, because more primate tetherins were included in our analysis. Moreover, the codon 41, which has been reported to be a positively selected site in a previous study\textsuperscript{9}, was detected in our analysis as well with posterior probability of 0.841 (Figure 1d).

Furthermore, the branch-site tests in PAML revealed that the likelihood ratio test was significant with P < 0.01 in the analysis of the Cercocetus clade, suggesting that positive selection has most likely operated on Cercocetus tetherins (Figure 1f). On the other hand, no significant positive selection was detected for RCM clade, probably because only two highly similar sequences were included in this clade. In addition, the branch-site analysis identified six codons, 10, 14, 39, 93, 103, and 187, to be positively selected sites in the Cercocetus clade (Figure 1f; these sites are also indicated with pink asterisks in Figure 1a), and three out of the six sites, 93, 103, and 187, are located in the extracellular domain. In this regard, it has been recently reported that human tetherin directly binds to immunoglobulin-like transcript 7 (ILT7), a cellular molecule specifically expressed on plasmacytoid dendritic cells, through its extracellular domain and modulates ILT7-mediated signaling leading to the production of type 1 interferons and proinflammatory cytokines\textsuperscript{34}. Therefore, it is possible that these three sites may associate with the interaction of tetherin and ILT7 in the genus Cercocetus. Taken together, here we firstly demonstrated that Cercocetus tetherins have evolved under stronger positive selection and also identified six codons which may be functionally important.

Antagonism of RCM tetherin by SIVrcm Nef. We then assessed the interplay of RCM tetherin and SIVrcm Nef. Western blotting (Figure 2a) and TZM-bl assay (Figure 2b) revealed that increasing concentrations of CPZ, SM and RCM tetherins resulted in a dose-dependent decrease in the release of nascent viral particles. We then investigated whether SIVrcm Nef has the ability to antagonize RCM tetherin. As shown in Figure 2a, the Nefs of SIVcpz, SIVsm, and SIVrcm did not affect the expression levels of tetherins and Gag (Figure 2a), which is consistent with previous reports\textsuperscript{36,37}. Moreover, we revealed that the Nefs of 3 SIVrcm isolates, strains GAB1, NG411, and 02CM8081, enhanced virus release in the presence of RCM tetherin (Figures 2a and 2b). These findings directly demonstrate that SIVrcm Nef is the bona fide antagonist of RCM tetherin, and further support the hypothesis that the anti-tetherin ability of SIVrcp Nef is originated from that of SIVrcm Nef.

In the cytoplasmic tail of Cercocetus tetherins, we found a novel positively selected site positioned at 10 (Arginine in RCM and SM tetherins; Figure 1a) with posterior probability greater than 0.95 (Figure 1f). In this regard, previous papers have reported that human tetherin but not OWm tetherins (e.g., RM and AGM tetherins) has the ability to induce NFκB-dependent proinflammatory signaling, and that certain amino acids in the cytoplasmic tail of human tetherin (Figure 1a, with shading in pale blue) are associated with
Figure 1 | Positive selection detected in primate tetherin. (a) Amino acid sequences of primate tetherin. RCM tetherin (GenBank accession number AB907706; determined in this study), SM tetherin (FJ864713), RM tetherin (FJ943432), African green monkey (Chlorocebus aethiops; AGM) tetherin (FJ943430), CPZ tetherin (NM_001190480), HU tetherin (NM_004335), and night monkey (Aotus vociferans; NM) tetherin (FJ638415) are respectively shown. The numbers indicate the amino acid positions in NM tetherin. The two positively selected sites (positioned at 9 and 14), which are determined by both site model of PAML (Figure 1d) and REL method of HyPhy (Figure 1e), are indicated with green asterisks. The six amino acids (positioned at 10, 14, 39, 93, 103, and 187) inferred to be under positive selection in Cercocebus tetherin (the clade of RCM and SM tetherins) (Figure 1f) are indicated with pink asterisks. The amino acids, which are putatively associated with the ability to induce NFκB-dependent signaling, are indicated with shading in pale blue.

(b) Phylogenetic tree of 47 primate tetherins reconstructed using ML method. The tree was rerooted with the NWM clade. The dN/dS ratios are shown on each branch and the numbers in parenthesis represent nonsynonymous (left) and synonymous (right) changes, respectively. (c) The positive selection detected in different regions of the tetherin gene. The regions inferred to be under positive selection with statistical significance are represented in red. ND, not detected (P<0.000002).

(d and e) Positively selected sites identified in our analyses. In panel d, the codons under positive selection identified by PAML with posterior probability >0.95 are shown in bold. In panel e, the codons under positive selection inferred by HyPhy with Bayes factor >50 are shown in bold.

(f) The result obtained from the twobranch-site analyses for RCM and Cercocebus clades. All PAML analyses were performed under two models of codon usage, F61 and F3x4, and they yield consistent results. * , All nodes/branches within RCM and the Cercocebus clades were respectively designated as the foreground branches. * , The number in parenthesis represents posterior probability.
This activity36–38. It is particularly noteworthy that the site 10 detected (Figure 3b). This may be due to the addition of KGC tag at the N-terminus of tetherin in our study. These results suggest that an arginine residue at the site 10 is not associated with the gain-of-function to induce NFXB-mediated signaling.

**Gain-of-function evolution of SIVcpz Nef.** As described above, phylogenetic analyses has assumed that SIVcpz is the recombinant of two OWM SIV linages, SIVgsn/mon/mus and SIVrcm 26. However, although we detected the induction of NFXB-mediated activation by human tetherin, Cercocebus tethers inclusions RCM and SM tethersins did not elicit this activity (Figure 3b). Although a previous study has shown that CPZ tetherin partially induces this NFXB-mediated signaling36, this effect was not observed in our assay (Figure 3b).

### Table 1 | GenBank accession numbers of primate tetherins used in this study

| Family/infraorder<sup>a</sup> | Common name<sup>b</sup> | Scientific name | Accession number<sup>c</sup> |
|-------------------------------|-------------------------|-----------------|-------------------------------|
| Hominidae [Hominoids]         | Human                   | Homo sapiens    | AK223124                      |
| Human                         | Homo sapiens            | NM_004335       |                               |
| Chimpanzee                    | Pan troglodytes         | NM_00190480     |                               |
| Bonobo                        | Pan paniscus            | HM136907        |                               |
| Bonobo                        | Pan paniscus            | XM_003817802    |                               |
| Gorilla                       | Gorilla gorilla         | GG925926        |                               |
| Gorilla                       | Gorilla gorilla         | HM136906        |                               |
| Gorilla                       | Gorilla gorilla         | XM_004060266    |                               |
| Orangutan                     | Pongo pygmaeus          | HM136908        |                               |
| Orangutan                     | Pongo abelli            | NM_001172587    |                               |
| Gibbon                        | Hylobates agilis        | HM136910        |                               |
| Gibbon                        | Nomascus leucogenys     | HM136909        |                               |
| Night monkey                  | Cercopithecus denti     | HE680870        |                               |
| Red-capped mangabey           | Sooty mangabey          | Cercocebus atys | FJ864713                      |
| Sooty mangabey                | Cercocebus atys         | JF864714        |                               |
| Sooty mangabey                | Cercocebus atys         | FJ943431        |                               |
| Rhesus macaque                | Macaca mulatta          | FJ943432        |                               |
| Rhesus macaque                | Macaca mulatta          | GG304749        |                               |
| Rhesus macaque                | Macaca mulatta          | HM136914        |                               |
| Rhesus macaque                | Macaca mulatta          | HM775182        |                               |
| Rhesus macaque                | Macaca mulatta          | NM_001161666    |                               |
| Pig-tailed macaque            | Macaca nemestrina       | FJ914988        |                               |
| Pig-tailed macaque            | Macaca nemestrina       | FJ914989        |                               |
| Olive Baboon                  | Papio anubis            | XM_003915138    |                               |
| Red-shanked douc langur       | Pygathrix nemaeus       | HM136916        |                               |
| Francois’ leaf monkey         | Trachypithecus francois | HM136917        |                               |
| Francois’ leaf monkey         | Trachypithecus francois | HM136917        |                               |
| Night monkey                  | Colobus guereza         | HM136915        |                               |
| Night monkey                  | Aotus lemurinus         | FJ638414        |                               |
| Night monkey                  | Aotus vociferans        | FJ638417        |                               |
| Night monkey                  | Aotus vociferans        | FJ638418        |                               |
| Night monkey                  | Aotus vociferans        | FJ638415        |                               |
| White-tailed tamarin          | Saginus labiatus        | HM136918        |                               |
| White-faced saki monkey       | Pithecia pithecia      | HM136920        |                               |
| Common woolly monkey          | Lagothrix lagotricha    | HM136922        |                               |
| Bolivian red howler monkey    | Alouatta sara           | HM136921        |                               |

<sup>a</sup>Family (Hominidae and Cercopithecidae) and infraorder (Platyrrhini) are presented in italic. Popular name of each family/infraorder is presented in parenthesis. OWMs, old world monkeys; NWMs, new world monkeys.

<sup>b</sup>The common name of each primate is identical to that in Figure 1B.

<sup>c</sup>The GenBank accession numbers (http://www.ncbi.nlm.nih.gov/genbank/) of tetherins are listed.

<sup>d</sup>This studye

<sup>e</sup>This studyd
counteraction efficacy of OWM SIV Nefs including SIVsm, SIVrcm and SIVgsn/mon/mus was significantly lower than that of SIVcpz Nef (Figures 4b and 4c). These results suggest that the difference in anti-tetherin activity (Figure 2) is not related to Nef but is due to the differences between RCM and CPZ tetherins. On the other hand, it was of interest that SIVcpz Nef counteracted RCM tetherin in comparable to SIVrcm Nefs (Figures 4d and 4e). Because SIVcpz Nef is able to antagonize RCM tetherin in addition to CPZ tetherin, these findings suggest that SIVcpz Nef has accomplished a gain-of-function during evolution.

Discussion
In this study, we have determined the sequence of RCM tetherin. In addition, we have demonstrated that RCM tetherin has the ability to impair the release of nascent viral particles and that SIVrcm Nef is the bona fide antagonist against RCM tetherin. Although the two concepts: (i) SIVrcm Nef counteracts RCM tetherin; and (ii) this ability is succeeded to SIVcpz Nef, have been proposed elsewhere, no direct evidence has been shown so far. Therefore, to our knowledge, this is the first report directly elucidating the interplay between SIVrcm Nef and RCM tetherin (Figure 2). Moreover, we here firstly performed the phylogenetic analysis of primate tetherins with Cercocebus tetherins including SM and RCM tetherins and revealed that stronger positive selection occurred in the evolution of Cercocebus tetherins (Figure 1).

To better understand the Red Queen dynamics in the co-evolution of PLVs and primates, our findings should be compared with the evolutionary interplay between Vpx and simian SAMHD1. To antagonize the anti-viral ability of simian SAMHD1, certain SIVs including SIVrcm degrade SAMHD1 by a viral protein, Vpx. In this regard, Etienne et al. have recently demonstrated that vpx, which is located at the recombination site of SIVrcm and SIVgsn/mon/mus, was lost during the emergence of SIVcpz and that SIVcpz does not possess any anti-CPZ SAMHD1 factor(s). When compared to the evolutionary interplay between SIV Vpx and SAMHD1, our findings on the relationship between SIV Nef and tetherin suggest that anti-tetherin ability was more crucial for PLVs, at least for SIVcpz, to adapt and expand in the new host because SIVcpz Nef has successfully inherited its anti-tetherin ability from SIVrcm Nef. Moreover, SIVcpz Nef has acquired the ability to antagonize CPZ tetherin without the loss of anti-RCM tetherin activity, indicating that SIVcpz Nef has made the way through a gain-of-function evolution (Figure 4). To our knowledge, here we demonstrated that SIVcpz Nef is able to antagonize OWM tetherin for the first time. Our findings can be a milestone to decipher the complicated co-evolutionary process between PLVs and primates.
As described in the beginning of this paper, HIV-2 has resulted from the zoonotic infection of SIVsm from SM25, while SIVrcm in RCM is one of the ancestral viruses of SIVcpz giving rise to HIV-1.26. These insights imply that the ancestors of these two HIVs have passed through the genus Cercocebus, namely SM or RCM. In this study, we firstly performed molecular phylogenetic analyses of primate tetherins with the genus Cercocebus and revealed that positive selection had operated on Cercocebus tetherins (Figure 1). Since it has been estimated that the genus Cercocebus has evolutionary diversified around 4–4.85 million years ago45,46, the observed positive selection probably occurred after the divergence of the genus Cercocebus. In fact, a recent paper has suggested that PLVs had already existed at least 12 million years ago47. Therefore, it is plausible that a Nef-like protein encoded by ancient PLVs (s) can be regarded as a selective pressure against Cercocebus tetherins. Moreover, it is of interest that the 2 types of genetic deletion in CCR5, CCR5Δ3244 and CCR5Δ3326, have been observed in the population of genus Cercocebus including RCM and SM, and that the homoyzomous mutants are resistant to CCR5-tropic SIV infections. To circumvent the restriction at the entry step of infection, some SIVrcm and SIVsm have acquired the ability to use alternative coreceptors for their infections presumably as a result of genetic conflict between viruses and hosts45,46. Although SIVsm is nonpathogenic in SM, it is conceivable that the ancestors of the genus Cercocebus have been subjected to stronger selective pressure from pathogenic PLVs and that the genetic conflict between pathogenic PLVs and the genus Cercocebus, including ancient Nef and Cercocebus tetherins, might have occurred in their evolutionary history.

Methods

Sequencing PCR. Blood was collected from six wild-caught RCMs in Nigeria according to the Guide for the Care and Use of Laboratory Animals under a NIAID Animal Care and Use Committee-approved protocol.28,29. RCM genomic DNA was isolated from cryopreserved peripheral blood mononuclear cells, and PCR was performed by using PfuUltra High Fidelity DNA polymerase (Agilent Technologies) and the following primers: 5′-CAG CTA GAG GGG AGA TCT GGA TG-3′; 5′-CTC CTC CTT TGC TCC CAA AA-3′; 5′-TTC AGG GTC TGG TGA AGT CC-3′. The sequencing PCR was performed by using ABI Prism 3130x genetic analyzer (Applied Biosystems), and the data was analyzed by Sequencher v5.1 software (Gene Codes Corporation).

Phylogenetic analyses. The two RCM tetherins newly sequenced in this study (note that five out of the six analyzed RCM tetherin sequences were identical) was aligned with 45 primate tetherins (listed in Table 1) by using ClustalW implemented in MEGA5. The resulting alignment was verified manually at amino acid level. Then the phylogenetic tree of 47 primate tetherins was reconstructed using both neighbor-joining (NJ) method49 with MEGA5 and ML method with PhyML50. Since the two methods yield almost identical topology, the unrooted ML tree was used for further analyses.

The detection of positive selection was conducted as follows: first, to detect the positive selection across various primate lineages, two pairs of site models implemented in the PAML package v 4.751 were used to conduct the likelihood ratio tests: M1 (neutral model) versus M2 (selection model) and M7 (neutral model) versus M8 (selection model). The REL model in HyPhy was also employed to this analysis. Second, to calculate the dN/dS ratio of each primate tetherin, a free-ratio branch model in PAML was performed, which assumes an independent dN/dS ratio for each branch of the tree. Third, since we were particularly interested in whether the RCM and Cercocebus clades have evolved under positive selection, the branch-site model in
PAML was further applied to our analyses. This model allows dN/dS ratio vary among sites and branches, which is useful for detecting positive selection along a particular lineage or clade (pre-specified as foreground branches) with positively selected site identified by 40-50. We conducted two branch-site tests in which RCM and the Cercocebus clades were designated as the foreground branches, respectively. SIV Nef sequences were aligned by using ClustalW implemented in MEGAS8. The phylogenetic tree of SIV Nef was reconstructed using NJ method with MEGAS5.

**Plasmid construction.** To construct tetherin expression plasmids tagged with Kusabira green C (KGC) at N-termini, tetherin ORFs were inserted into the KpnI-Xhol site of pmkKGC-MC vector (Medical and Biological laboratories, Inc.). The ORFs of HU tetherin (GenBank accession number NM_004335), AGM tetherin (AF635165), Cercocebus tugurialis (XM_512491), Cercocebus mitis (NM_198095) were used in our previous report44, CPZ tetherin (XM_512491), GOR tetherin (GQ925926), RM tetherin (FJ943342), and SM tetherin (FJ864713), and rhesus (AB907076), determined in this study were generated by overlap extension PCR using artificially synthesized oligonucleotides (Greiner bio-one). The ORF of the 5 amino acid ("GDIWK")-inserted HU tetherin (designated "HU +5 aa") was prepared by overlap extension PCR using HU tetherin expression plasmid as the template and the following primers: 5'-GGG GTA CCC CCA TGT CAT CTA GTG ATTG-3'; 5'-TTT CCT CCA AAT GTC ATC GAC GGC TCT GCA ATAT-3'; 5'-GAT GAC ATT TGG AAA GAC GGC GAT AAG CGG TGTC-3'; 5'-CCG CCT CGG CAG GTC ATC TCT GAG CGG AGC CTG GA-3'. To construct SIV Nef expression plasmids tagged with KGC at N-termini, HA was inserted at the C-termini of Nef ORFs by PCR and the Nef-HA fragments were then inserted into the XhoI-MluI site of pCGCG-IRES-EGFP vector (kindly provided by Dr. Frank Kirchhoff). ORFs of the SIVs of Nam (strain FY1 [D02927], 203c Nef ORFs (strains NG411 [AF349680] and 02CM8081 [HM803689]), SIV sgNef (strain M99C [AF688289]), AGM Nef (strains NM_004335 and GNG [GQ925927]), and 2 SIV mus ORFs (strains 01CM1085 [AY340709] and 01CM1239 [EF070330]) were obtained from GeneArt Gene Synthesis service (Life Technologies). The ORF of SIVrcm Nef (strain GAB1 [AF382890]) was generated by overlap extension PCR using artificially synthesized oligonucleotides (Greiner bio-one). The ORF of SIV Nef ORF (strain MB97 [EF035994]) was prepared by overlap extension PCR using pMB97 (an infectious molecular clone of SIVpze strain MB97 [IN835461]; kindly provided by Dr. Beatrice Hahn) as the template and the following primers: 5'-TTA ATA CCT AGT CTA GAC TAG ATG GGA AAC AAA TGG TCA AAA AGC AGC-3'; 5'-TAG CCA CGA GTG GCC GAG GAT TCT ACG CTA GCT CTG CTC AGT CTA GCG AGC GAT ACC AGT CCT GGC ATT GAT CCT GCT GAT GC-3'. To construct pNL3-3-ApenvInf, an HIV-1-producing plasmid with defects in vpu and nef, pNL43-Uel (a vpu-deficient HIV-1-producing plasmid, based on HIV-1 strain NL4-3 [M19921]; kindly provided by Dr. Klaus Strebel) was digested with XhoI, blunted, and then self-ligated. The sequencing PCR was performed by using ABI Prism 3130xl genetic analyzer (Applied Biosystems), and the data were analyzed by Sequencer v5.1 software (Hitachi).

**Cell culture and transfection.** 293T cells and TZM-bl cells (obtained through NIH AIDS Research and Reference Reagent Program) were maintained in Dulbecco's modified Eagle medium (Sigma) containing FCS and antibiotics. Transfection was performed by using Lipofectamine 2000 (Life Technologies) according to the manufacturer's protocol. Various amounts of KGC-tagged tetherin expression plasmids (0.2, 0.4, 0.8, and 1.6 ng) of pNL3-3-ApenvInf (1,500 ng) were cotransfected with or without the expression plasmids of SIV Nefs of their natural hosts (400 ng) into 293T cells. At 48 hours post-transfection, the culture supernatants harvested at 48 hours post-transfection were centrifuged and then filtered through a 0.45-

**Viral budding and Western blotting.** The culture supernatant harvested at 48 hours post-transfection was centrifuged and then filtered through a 0.45-

**NFXB reporter assay.** One hundred nanogram of either respective tetherin expression plasmid or parental empty vector (pPKGC-MC) was cotransfected with 25 ng of pNL3-3-ApenvInf reporter plasmid, kindly provided by Dr. Takashi Fujita55, 250 ng of a vpu-deficient HIV-1-producing plasmid (pNL3-4-Uel; kindly provided by Dr. Klaus Strebel)56, and 125 ng of pUC19 plasmid into 293T cells. At 48 hours post-transfection, the cells were harvested and used for Western blotting and luciferase assay. Luciferase assay was performed as previously described56.
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**Author contributions**

T.K., J.S.T., F.R., K.M., K.S., H.T., V.M.H. and Y.Koyanagi wrote the main manuscript text; J.S.T. and F.R. performed molecular phylogenetic analyses and prepared Figure 1; T.K., J.S.T., K.S., Y.Kimura, N.M., R.Y., Y.N. and E.Y. performed the experiments and prepared Figures 2–4; K.M. and V.M.H. contributed to the samples of RCM genomic DNA; K.S. conceived and designed the experiments. All authors reviewed the manuscript.

**Additional information**

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