



Convergent evolution of antiviral machinery derived from endogenous retrovirus truncated envelope genes in multiple species

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Host genetic resistance to viral infection controls the pathogenicity and epidemic dynamics of infectious diseases. Refrex-1 is a restriction factor against feline leukemia virus subgroup D (FeLV-D) and an endogenous retrovirus (ERV) in domestic cats (ERV-DC). Refrex-1 is encoded by a subset of ERV-DC loci with truncated envelope genes and secreted from cells as a soluble protein. Here, we identified the copper transporter CTR1 as the entry receptor for FeLV-D and genotype I ERV-DCs. We also identified CTR1 as a receptor for primate ERVs from crab-eating macaques and rhesus macaques, which were found in a search of intact envelope genes capable of forming infectious viruses. Refrex-1 counteracted infection by FeLV-D and ERV-DCs via competition for the entry receptor CTR1; the antiviral effects extended to primate ERVs with CTR1-dependent entry. Furthermore, truncated ERV envelope genes found in chimpanzee, bonobo, gorilla, crab-eating macaque, and rhesus macaque genomes could also block infection by feline and primate retroviruses. Genetic analyses showed that these ERV envelope genes were acquired in a species- or genus-specific manner during host evolution. These results indicated that soluble envelope proteins could suppress retroviral infection across species boundaries, suggesting that they function to control retroviral spread. Our findings revealed that several mammalian species acquired antiviral machinery from various ancient retroviruses, leading to convergent evolution for host defense.

antiviral | retrovirus | receptor | restriction factor | evolution

Endogenous retroviruses (ERVs) are the germline remnants of ancestral infections by exogenous retroviruses and are present in all vertebrate genomes (1). ERVs comprise a substantial proportion of the mammalian genome and are transmitted in a Mendelian fashion (2). When retroviruses are inserted into a host genome, they achieve increased copy numbers by repeated reinfection in germline cells (3). ERVs are gradually attenuated or inactivated by mutations that occur during viral replication, as well as by postinsertional mutations that occur during host genome replication. Nucleotide changes in viral genes disrupt or modify the functions of the encoded proteins (4–6). Hosts often control the gene expression of ERVs via epigenetic mechanisms, such as nucleotide CpG methylation or suppressive histone modification, to silence the ERVs (7). It is assumed that such events ensure that the ERVs do not become a threat to the host. Some ERVs are domesticated by their hosts and eventually gain physiological functions [e.g., in processes such as placentation (8), sex-specific muscle development (9), and viral resistance (10–13)], despite the loss of their ancestral viral properties. Domesticated ERVs are strictly regulated, and dysregulation can negatively impact their hosts (14).

Feline leukemia virus (FeLV) belongs to the genus *Gammaretrovirus* and is horizontally transmitted among cats. FeLV has been linked to various diseases, including lymphoma, myelodysplastic syndrome, anemia, and acute myelogenous leukemia, as well as immune suppression (15). Genetic changes resulting from substitutions, recombination, or both have generated viral subgroups defined on the basis of their capacities for viral interference. In particular, changes in FeLV pathogenicity and the occurrence of unexpected symptoms are associated with alterations of viral target cells (16).

ERV-DCs are endogenous gammaretroviruses of domestic cats (DCs) (17–19); they are classified as genotypes I, II, and III according to their phylogenetic relationships (*SI Appendix, Fig. S1*) and receptor interference groups. ERV-DCs were inserted into feline genomes several times within the past few million years. Some ERV-DCs have intact open reading frames (ORFs) that encode *gag*, *pol*, and envelope (*env*) genes; therefore, they are replication-competent in cultured cells and capable of infecting a broad range of cells, including human ones. Furthermore, FeLV-D was generated by recombination of genotype I ERV-DCs in the *env* region (17) and shows distinct

Significance

Endogenous retroviruses (ERVs) are remnants of ancestral viral infections of cells and occupy substantial proportions of mammalian genomes. Most ERVs have numerous mutations, deletions, and insertions in the coding region; truncated envelope (*env*) genes are scattered throughout the genomes. The truncated Envelope (Env) proteins consist of a signal peptide and the N-terminal region of the surface unit, but they lack a transmembrane domain; these proteins are secreted from cells in soluble form and block viral infection via competition with entry receptors. This defense system could function to prevent both intraspecies and interspecies transmission of viral infections. Truncated Envs have been independently acquired by infection with ancient viruses, and they could prevent the spread of infectious diseases.

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tropism, compared with other FeLV subgroups. Importantly, ERV-DCs currently retain the ability to impact their hosts via their potential viral activity and their contribution to the emergence of recombinant viruses. Furthermore, it has been reported that an ERV-DC recombined with Baboon endogenous retrovirus (BaEV) and generated a distinct feline retrovirus, RD-114 (17–19). This past recombination event suggested interspecies retroviral transmission between cats and primates (17–19). Therefore, the interactions between exogenous retroviruses and ERVs have contributed to long-term retroviral diversification and evolution.

Refrex-1 is a feline soluble restriction factor against genotype I ERV-DCs and FeLV-D infection; it is encoded by truncated *env* genes of ERV-DC7 and ERV-DC16 loci, which are classified as genotype II ERV-DCs (20). Thus, Refrex-1 was presumably acquired through ERV domestication as part of an evolutionary arms race between cats and ERV-DCs. Indeed, resistance to viral infection associated with ERV has been discovered in chicken (21) and in mice (22–24), as an evolutionary arms race between hosts and viruses. Refrex-1 consists of a signal peptide and a surface unit (SU) N-terminal domain, also known as the receptor-binding domain (RBD); however, it lacks a transmembrane (TM) domain because of a premature stop codon and lethal mutations in the middle of the *env* ORF. Refrex-1 is efficiently secreted from cells and specifically blocks the infection of genotype I ERV-DCs and FeLV-D, suggesting that its restriction activity is mediated by a mechanism of receptor interference.

In this study, copper transport protein 1 (CTR1) was identified as the entry receptor for infection by FeLV-D and genotype I ERV-DCs. Functional Env proteins from novel primate ERVs were found through a mammalian genome database search, and receptor interference assays revealed that these primate ERVs also used CTR1 as the entry receptor for infection. The restriction capacity of Refrex-1 extended beyond feline retroviruses to primate ERVs, based on the involvement of CTR1. Furthermore, this study showed that endogenous retrovirus truncated envelopes with Refrex-1-like structures are present in primates; they also have antiviral activity toward feline and primate retroviruses. Because genetic analyses showed that these truncated *env* genes were independently endogenized during host evolution, antiviral machinery derived from truncated envelope genes was inferred to have evolved in a convergent manner in multiple mammalian species. This study provides a mechanistic finding that soluble Env proteins broadly suppress retroviruses from different host species through binding interactions with a common entry receptor.

Results

Identification of an Entry Receptor for FeLV-D and Genotype I ERV-DCs. FeLV-D Env-pseudotyped virus could infect human cells (Fig. 1A). Therefore, we sought to screen an HEK293 cDNA library for genes encoding receptors used by FeLV-D for entry. *Mus dunni* tail fibroblast (MDTF) cells were resistant to FeLV-D infection (Fig. 1B), and cells transduced with the retroviral HEK293 cDNA library were challenged with FeLV-D (TY26)-enveloped virus carrying a puromycin-resistance gene. Fifty-one puromycin-resistant colonies were replated in culture plates. These cells were subsequently challenged with FeLV-D-enveloped viruses carrying a gene encoding green fluorescent protein (GFP), and 11 of 51 colonies were GFP⁺. Sequence analysis revealed that the cDNAs encoded human CTR1 (huCTR1, solute carrier 31A1 [SLC31A1]) in all GFP⁺ colonies.

Next, to determine whether CTR1 might function as an entry receptor for FeLV-D, we obtained the coding regions of

feline CTR1 (feCTR1) and huCTR1; both cDNAs were then introduced into MDTF cells, termed MDTF-feCTR1 and MDTF-huCTR1, respectively (Fig. 1B). MDTF-feCTR1 and MDTF-huCTR1 cells were permissive to FeLV-D Env-pseudotyped virus infection using four different FeLV-D Env proteins (TY26, ON-T, ON-C, and 44B) derived from leukemia and lymphoma in domestic cats (17) (Fig. 1B). Furthermore, the expression of feCTR1 or huCTR1 rendered MDTF cells permissive to infection by genotype I ERV-DC Env-pseudotyped viruses (loci ERV-DC8, DC14, and DC19) within the same interference group as FeLV-D (Fig. 1B). In contrast, other feline retroviruses (e.g., FeLV-A, FeLV-B, FeLV TG35-2, and genotype III ERV-DC [loci DC6 and DC10] Env-pseudotyped viruses) did not infect MDTF-feCTR1 or MDTF-huCTR1 cells (Fig. 1B). These results indicated that feCTR1 and huCTR1 conferred susceptibility to infection by FeLV-D and genotype I ERV-DC-pseudotyped viruses.

To further investigate whether the infection of HEK293T cells by FeLV-D and genotype I ERV-DCs was mediated by CTR1, we assessed the susceptibility of HEK293T cells transiently transfected with huCTR1-specific small-interfering RNA (siRNA). Silencing of endogenous CTR1 expression caused a 75% reduction in the infective titer of FeLV-D in HEK293T cells and an 88% reduction in the infective titer of ERV-DC14 (genotype I) in HEK293T cells, relative to HEK293T cells transfected with siEnhanced GFP (siEGFP) control (Fig. 1C). However, silencing of CTR1 expression did not affect the infective titers of ERV-DC10 and FeLV-B in HEK293T cells. These results provided further evidence that the infection of HEK293T cells by FeLV-D and genotype I ERV-DCs is CTR1-dependent.

Copper transporter 2 (CTR2) is a second member of the mammalian SLC31 copper transporter family and shares structural similarities with CTR1 (25). To determine the specificity of CTR1 as a viral entry receptor, MDTF cells expressing huCTR2 (MDTF-huCTR2) were generated. As shown in Fig. 1D, none of the viruses (except amphi-MLV [murine leukemia virus]) could infect MDTF-huCTR2 cells. These results indicated the specificity of CTR1 as the entry receptor for FeLV-D and genotype I ERV-DCs.

Expression of CTR1 in Feline and Primate Tissues. FeCTR1, isolated based on the cat genome (*Felis catus* 9.0), was predicted to encode a protein of 180 amino acids (SI Appendix, Fig. S2). The amino acid sequence identity between feCTR1 and huCTR1 was 96.1%. The N-terminal region corresponding to the extracellular domain of CTR1 was most divergent. FeCTR1 mRNA expression was detected by qRT-PCR in total RNA extracted from various feline tissues and feline cell lines (SI Appendix, Fig. S3). Expression of feCTR1 was detected in all feline tissues and cell lines tested; expression levels were highest in the liver, small intestine, and lung.

Primate CTR1 expression levels were also investigated using public RNA-sequencing (RNA-seq) data collected from chimpanzee, crab-eating macaque, and rhesus macaque (26). Primate CTR1 expression was detected in all tissues tested; comparatively high expression levels were observed in kidney and liver (SI Appendix, Fig. S4A). We also detected CTR1 expression in testis and ovary from crab-eating macaque by qRT-PCR (SI Appendix, Fig. S4C).

Effect of Refrex-1 on CTR1. Refrex-1, encoded by the ERV-DC7 and ERV-DC16 envelope genes, is a soluble restriction factor that blocks infection by FeLV-D and genotype I ERV-DCs (20). The ERV-DC7 and ERV-DC16 *env* genes are defective, but

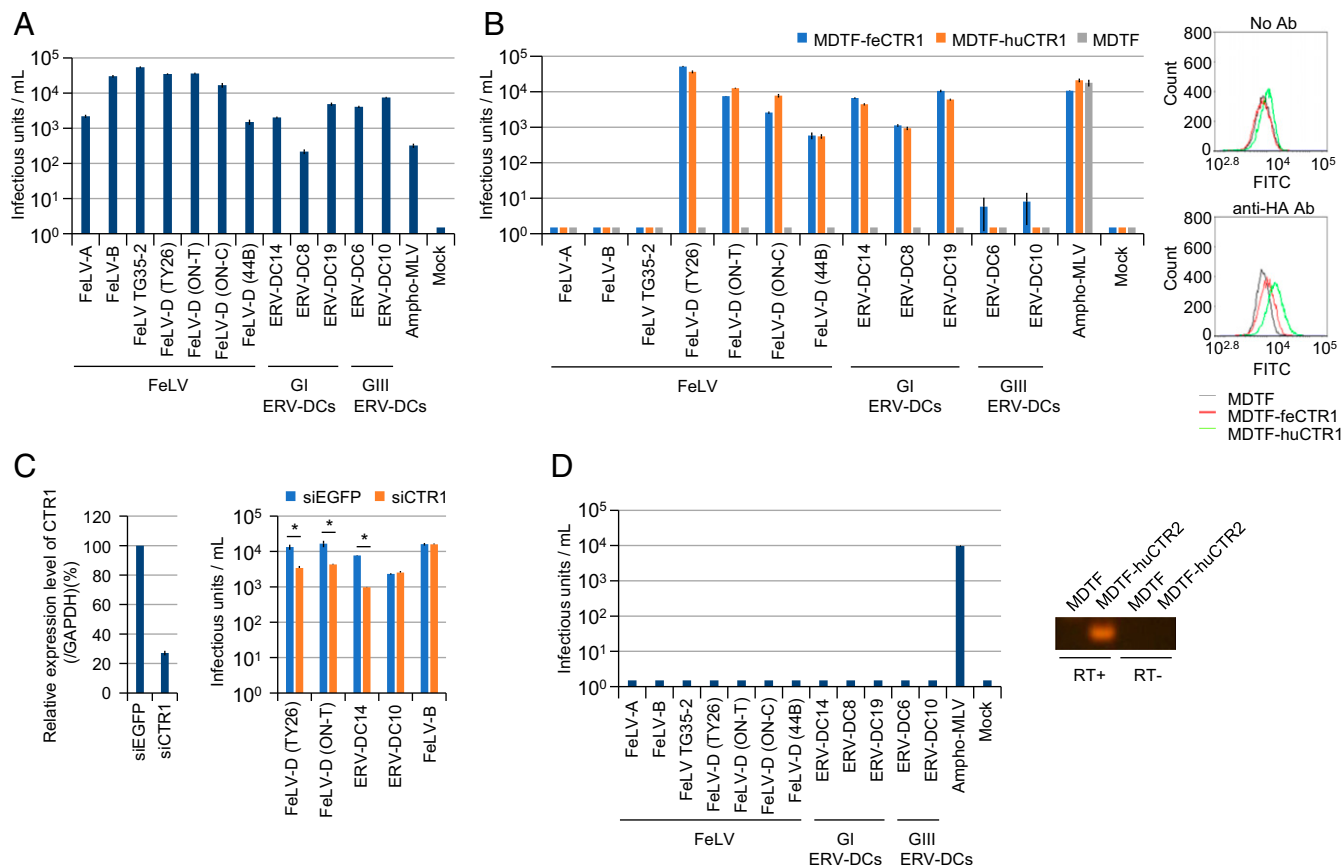


Fig. 1. CTR1 as the entry receptor for FeLV-D and Genotype I ERV-DCs. Viruses pseudotyped with the Envs shown in the x axis were tested in the following cell lines: (A) HEK293T cells, (B) MDTF cells, MDTF cells expressing human CTR1 (MDTF-huCTR1), and MDTF cells expressing feline CTR1 (MDTF-feCTR1) (Left). The expression of CTR1 was monitored by flow cytometry (Right). (C) Infection assay using HEK293T cells with endogenous CTR1 knockdown using siRNA. Expression of human CTR1 was analyzed by quantitative real-time RT-PCR and normalized by the expression level of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (Left). Infective titers of the indicated LacZ-coding Env-pseudotyped viruses in HEK293T cells with CTR1 siRNA (Right). Enhanced GFP (EGFP) siRNA was used as a negative control. Comparisons with siEGFP control were performed using Student's *t* test ($*P < 0.01$). (D) Env-pseudotyped viruses shown in A were tested in MDTF cells expressing human CTR2 (MDTF-huCTR2) (Left). Expression of CTR2 was monitored by RT-PCR (Right). The infectious units were assessed via log₁₀ β -galactosidase (LacZ)⁺ cells per milliliter of virus. Infective titers with SDs were means of three independent infection experiments. GI ERV-DCs, genotype I ERV-DCs; GIII ERV-DCs, genotype III ERV-DCs.

their reconstructed sequences (ERV-DC7rec and ERV-DC16rec) have been restored to their infectious origin belonging to the same interference group as genotype I (27) (Fig. 2A). We used ERV-DC7rec and ERV-DC16rec to determine whether the ancestor of Refrex-1 infects cells via the CTR1 entry receptor. ERV-DC7rec and ERV-DC16rec Env-pseudotyped viruses infected MDTF-feCTR1 and MDTF-huCTR1 cells, but they did not infect MDTF cells (Fig. 2B). These results suggest that CTR1 was used as an entry receptor for infection by the ancient retrovirus from which Refrex-1 originated.

Because Refrex-1 is secreted into the supernatant by feline cells and specifically suppressed FeLV-D and genotype I ERV-DCs, its antiviral mechanism was expected to involve receptor interference (20). Therefore, we investigated whether the antiviral effect of Refrex-1 depended on the viral entry receptor, CTR1. Refrex-1 was prepared from the supernatants of HEK293T cells that had been transfected with expression vectors encoding ERV-DC7 Env or ERV-DC16 Env. The supernatants were incubated with MDTF-feCTR1 and MDTF-huCTR1 cells; viral challenge experiments were then conducted. Refrex-1 blocked infection of MDTF-feCTR1 and MDTF-huCTR1 cells by ERV-DC7rec and ERV-DC16rec Env-pseudotyped viruses in a concentration-dependent manner (Fig. 2C). In contrast, the supernatants of HEK293T cells transfected with an empty vector could not block

infection by these viruses. These results suggested that Refrex-1 restricts viral infection via competitive binding with the CTR1 entry receptor.

Identification of Functional Envs in Primates and FeLV-D Interference Subgroup. Human CTR1 is reportedly a receptor for extinct chimpanzee and rhesus macaque ERVs (i.e., CERV2 and RhERV2-A, respectively) (28). CERV1 was identified as a phylogenetically distinct virus from CERV2 and did not use CTR1 as an entry receptor. Although a previous study reported that these primate ERVs were extinct and described artificial reconstruction of their Envs (28), we sought to identify intact *env* genes related to CERV2 and RhERV2-A. Our genomic database search identified six *env* sequences encoding intact Env protein related to RhERV2-A in rhesus macaques and crab-eating macaques (i.e., RmRV1, and CmRV1, CmRV1_1, and CmRV1_2, respectively) (SI Appendix, Fig. S5 and Table S1), but the CERV-related ERV Env sequences were not found in the human genome. The amino acid sequences of Env proteins were identical in RmRV1 and CmRV1, but not in CmRV1_1 and CmRV1_2; as a result, three different amino acid sequences were revealed (SI Appendix, Fig. S5). The infectivity of RmRV1 (CmRV1), CmRV1_1, and CmRV1_2 Env-pseudotyped viruses with MLV Gag-Pol was found in

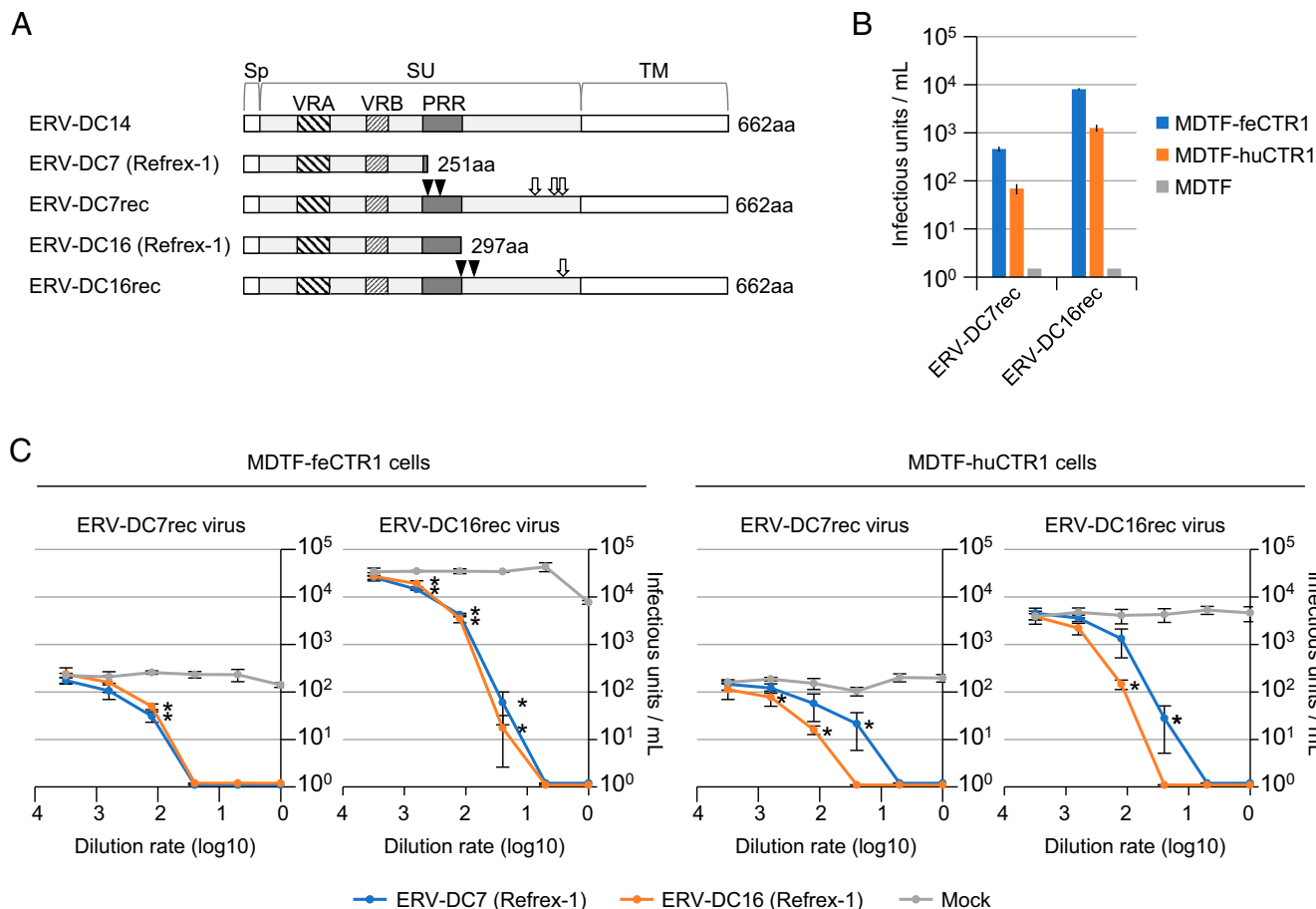


Fig. 2. Refrex-1 inhibition of ancestral virus infection via CTR1. (A) Schematic representation of the structures of Refrex-1 and reconstructed ancestral Env proteins (ERV-DC7rec and ERV-DC16rec). *Env* genes of ERV-DC7rec and ERV-DC16rec were reconstructed by the removal of stop codons (triangles) and amino acid substitutions (arrows) based on ERV-DC7 and ERV-DC16 *Env* sequences, respectively. The amino acid substitutions were R407G, I427N, and T429A in ERV-DC7rec, and D431Y in ERV-DC16rec. PRR, proline-rich region; Sp, signal peptide. The number of amino acids (aa) is indicated on the right. (B) Infection of MDTF-feCTR1, MDTF-huCTR1, and MDTF cells by LacZ-coding Env-pseudotyped ERV-DC7rec and ERV-DC16rec viruses. The y axis indicates infectious units per milliliter of supernatant. (C) Dose-dependent inhibitory effect of Refrex-1 on viral infection. Supernatants of HEK293T cells transfected with expression vectors encoding ERV-DC7 Refrex-1 (blue), ERV-DC16 Refrex-1 (orange), or empty vector (Mock; gray) were diluted with medium; 250 μ L of diluted supernatant was added to the culture when MDTF-feCTR1 or MDTF-huCTR1 cells were infected with ERV-DC7rec or ERV-DC16rec pseudotyped viruses. Infection titers with SDs are means of three independent infection experiments. Comparisons with Mock control were performed using Student's *t* test (**P* < 0.01).

human and feline cells (Fig. 3A and *SI Appendix*, Fig. S6A), and these pseudotyped viruses infected MDTF-feCTR1 and MDTF-huCTR1 cells, but not MDTF cells (Fig. 3B and *SI Appendix*, Fig. S6B). RmRV1 (CmRV1) Env-pseudotyped virus broadly infected cell lines from various mammalian species (*SI Appendix*, Table S2). These results indicated that CERV2- and RhERV2-A-related functional *Env* genes are present in primates and the pseudotyped viruses infect cells using feCTR1 and huCTR1 as entry receptors.

Next, we performed viral interference assays using Env-pseudotyped viruses to determine whether these feline and primate retroviruses belong to the same interference group. RmRV1, CERV2, and RhERV2-A interfered with FeLV-D and a genotype I ERV-DC (ERV-DC14), but they did not interfere with a genotype III ERV-DC (ERV-DC10) (Fig. 3C). These results indicated that CERV2, RhERV2-A, RmRV1, FeLV-D, and genotype I ERV-DCs belong to the same viral interference group. In contrast, CERV1, ERV-DC10, and FeLV-B belong to distinct interference groups. Taken together, these experiments showed that intact ERV *env* sequences capable of generating infectious viral proteins, which belong to the FeLV-D interference group, are present in the genomes of crab-eating macaques and rhesus macaques.

Antiviral Effect of Refrex-1 against Primate ERV Infection.

The common usage of CTR1 for infections by FeLV-D, feline ERVs, and primate ERVs implied that Refrex-1 could block these primate retroviral infections. To test this hypothesis, we challenged feline and primate ERV Env-pseudotyped viruses with HEK293T cells that had been treated with Refrex-1 (Fig. 4A). Refrex-1 corresponding to ERV-DC7 Env and ERV-DC16 Env, blocked infection by CERV2, RhERV2-A, RmRV1, FeLV-D, and ERV-DC14; it did not block infection by FeLV-B (Fig. 4A). These results suggested that Refrex-1 has broad inhibitory activity toward feline and primate retroviral infections.

Antiviral Effect of Truncated Envs in Primate Genomes against Retroviral Infection.

Next, we hypothesized that the antiviral mechanism derived from truncated *env* genes is also present in primates (i.e., it is not limited to Refrex-1 in cats). To test this hypothesis, we performed a sequence search for genes encoding only the SU domain of RmRV1-related Envs lacking TM regions. Consequently, we found seven truncated Envs (CmRV-trunc1, RmRV-trunc1, PNRC2-ChRV, CERV2 provirus [ChrY], PNRC2-BoRV, BoRV-7, and GoRV-trunc1) in crab-eating macaque, rhesus macaque, chimpanzee, bonobo, and

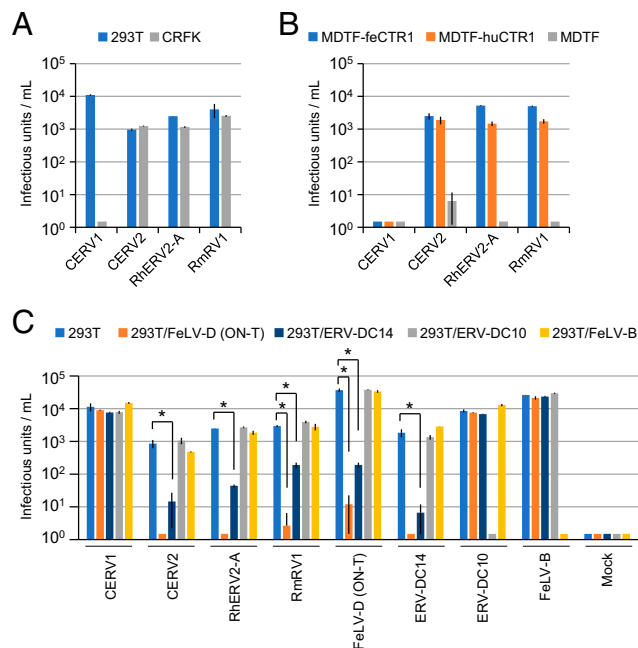


Fig. 3. Infection of primate Env-pseudotyped viruses belonging to FeLV-D interference groups. Infection of LacZ-coding primate Env-pseudotyped viruses in (A) HEK293T and CRFK cells and (B) MDTF-feCTR1, MDTF-huCTR1, and MDTF cells. (C) Infection of HEK293T cells and HEK293T cells preinfected with FeLV-D (ON-T), ERV-DC14, ERV-DC10, or FeLV-B by Env-pseudotyped viruses indicated in the x axis. Comparisons with 293T control were performed using Student's *t* test (**P* < 0.01). The y axis indicates infectious units per milliliter of virus. Infective titers with SDs are means of three independent infection experiments.

gorilla genomes (Fig. 4B and *SI Appendix*, Fig. S7 and Table S1). The expression plasmids of primate truncated Envs were transfected into HEK293T cells. Following immunoprecipitation of the supernatants, Western blot analysis detected Myc-tagged Env proteins and Refrex-1. These results indicated that the primate truncated Env proteins except for CERV2 provirus (ChrY) were proteins secreted in a manner similar to Refrex-1 (Fig. 4C). The supernatants containing soluble Env proteins were incubated with HEK293T cells prior to viral challenge experiments to evaluate their inhibitory effects against viral infection. CmRV-trunc1, RmRV-trunc1, PNRC2-ChRV, and BoRV-7 specifically blocked infection by feline and primate retroviruses within the same interference group: FeLV-D, ERV-DC14, CERV2, RhERV2-A, and RmRV1 (Fig. 4D). Furthermore, GoRV-trunc1, which is larger than the other truncated Envs in terms of molecular weight, showed a reduced ability to block infection by RmRV1, FeLV-D, and ERV-DC14 (Fig. 4D); similar results were observed after viral infection of cells transfected with GoRV-trunc1 (*SI Appendix*, Fig. S8A).

Furthermore, PNRC2-ChRV inhibited FeLV-D, ERV-DC14, CERV2, RhERV2-A, and RmRV1 (Fig. 4D), while the PNRC2-ChRV ortholog, PNRC2-BoRV, inhibited only FeLV-D infection; PNRC2-BoRV did not block infection by CERV2, RhERV2-A, RmRV1, ERV-DC14, or FeLV-B, despite abundant expression of this Env in HEK293T cell supernatants (Fig. 4C). Only one amino acid at position 82 differed between PNRC2-ChRV (isoleucine) and PNRC2-BoRV (threonine) (*SI Appendix*, Fig. S7), indicating that this isoleucine in PNRC2-ChRV exerts an inhibitory effect on viral infection. There are seven amino acid differences between PNRC2-BoRV and BoRV-7 (isoleucine at position 82) (*SI Appendix*, Fig. S7); BoRV-7 Env blocked infection by FeLV-D, ERV-DC14, CERV2, RhERV2-A, and RmRV1 (Fig. 4D).

Notably, the truncated Env protein (188 amino acids in length) encoded by the CERV2 provirus (ChrY) was not secreted extracellularly (Fig. 4C), but expressed intracellularly (*SI Appendix*, Fig. S8B). Likewise, artificial CERV2-trunc (188 amino acids), which has a sequence identical to amino acid positions 1 to 188 of full-length CERV2 Env, was not secreted extracellularly (Fig. 4C), but expressed intracellularly (*SI Appendix*, Fig. S8B). There are five amino acid differences between CERV2 provirus (ChrY) Env and CERV2-trunc Env (*SI Appendix*, Fig. S7). The expression of CERV2 provirus (ChrY) Env and CERV2-trunc Env in 293T cells did not result in an inhibitory effect against viral infection (*SI Appendix*, Fig. S8B).

These results indicated that the antiviral mechanism derived from truncated *env* genes is also present in primates, and primate truncated Envs characterized as secreted proteins have inhibitory activity toward feline and primate retroviral infections. Truncated Env proteins with lengths of 230 to 439 amino acids are efficiently secreted from cells and exhibited antiviral activity. A subtle amino acid change in SU variable region A (VRA), which could be the RBD (*SI Appendix*, Fig. S7) (29), affected antiviral activity.

A previous study reported that Refrex-1 was expressed in various feline tissues, with greatest expression in the blood (30). To determine whether the truncated *env* genes are currently expressed in primate tissues, we analyzed the expression levels of primate *env* genes using publicly available RNA-seq data collected from chimpanzee, crab-eating macaque, and rhesus macaque (26). Notably, we identified abundant expression of truncated *env* genes (i.e., PNRC2-ChRV, CmRV-trunc1, and RmRV-trunc1) in various tissues (*SI Appendix*, Fig. S4B), including thymus and bone marrow; their expression levels were much higher than the expression levels of full-length *env* genes (i.e., CmRV1_1, CmRV1_2, and RmRV1) (*SI Appendix*, Fig. S9) in each species. We also detected the CmRV-trunc1 expression in testis and ovary from crab-eating macaque, as determined by qRT-PCR (*SI Appendix*, Fig. S4C). Expression of CERV2 provirus (ChrY) *env* gene was not clearly detected in tissues (*SI Appendix*, Fig. S4B). These results suggested that RNA expression of truncated *env* genes in reproductive tissues and immune-related tissues may contribute to prevent endogenous and exogenous retroviral infection and endogenization of ERVs. Additionally, the mapping pattern of RNA-seq reads to CmRV-trunc1, RmRV-trunc1, and PNRC2-ChRV suggested that the spliced transcripts encode Env proteins (*SI Appendix*, Fig. S9), although we do not know the protein levels in vivo. These results suggest that truncated *env* genes intrinsically function as antiviral factors in primates. Expression of truncated *env* genes, but not full-length *env* genes, may have evolutionarily important implications for the host.

Genetic Analyses of Feline and Primate ERVs. The evolutionary relationships of feline and primate ERVs were investigated by genetic analyses. Phylogenetic trees constructed using *gag* and *pol* genes indicated that ERV-DC and CERV-related viruses were located in the same clade (*SI Appendix*, Fig. S10 A and B). However, *env* genes of ERV-DC-related viruses and CERV-related viruses were distinct (*SI Appendix*, Figs. S7 and S10C). As an example, comparison of amino acid sequences between ERV-DC14 Env and RmRV1 Env showed 43.8% identity. These results suggest that the backbones of feline and primate ERVs are derived from a common ancestor, while the *env* genes have a different origin.

A previous study reported that the timing of integration of ERV-DCs was 2.8 million y ago (Mya) (17). The timing of

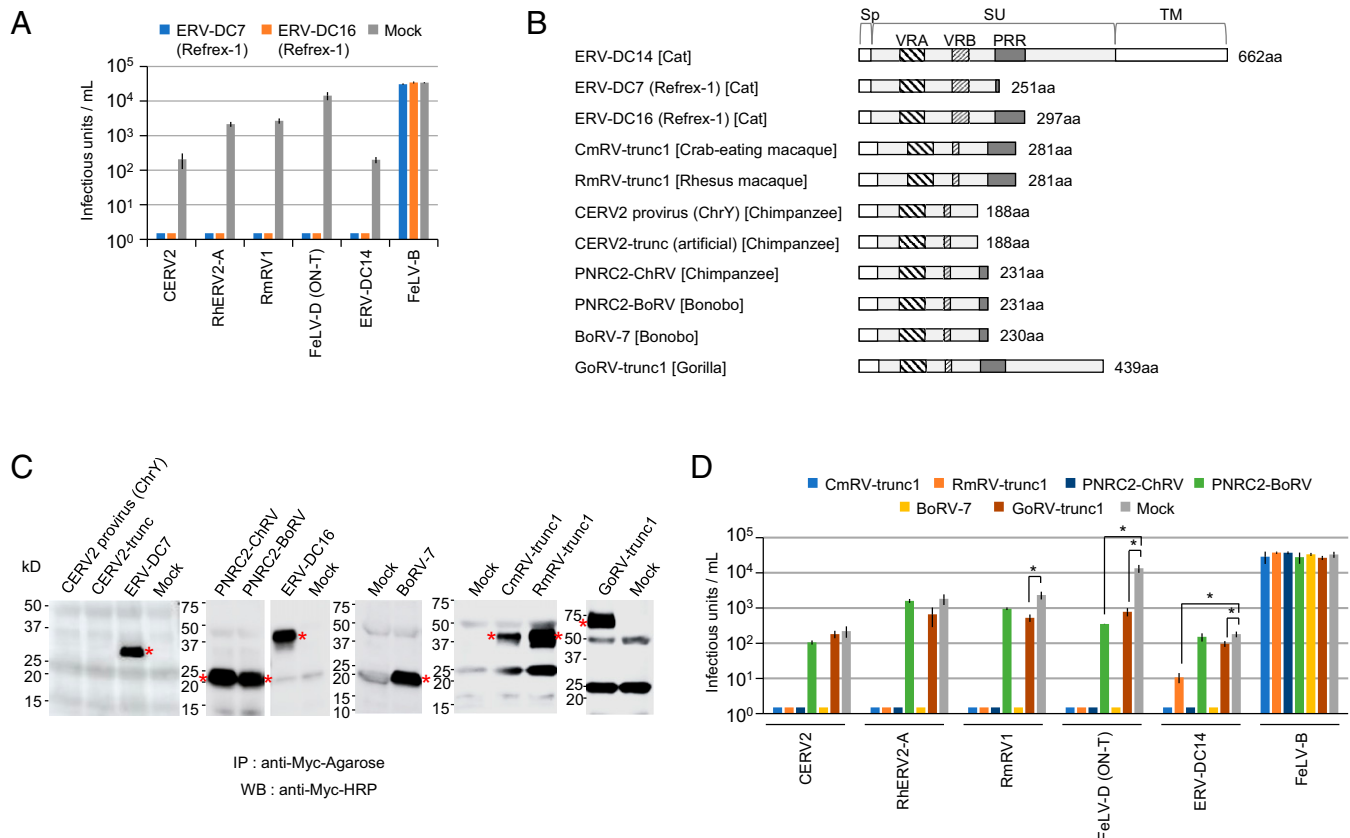


Fig. 4. Effects of Refrex-1 and primate soluble Env proteins on retroviral infection. (A) Supernatants were collected from HEK293T cells transfected with expression vectors encoding ERV-DC7 Refrex-1, ERV-DC16 Refrex-1, or empty vector (Mock); HEK293T cells preincubated with the supernatant were then infected with pseudotyped viruses indicated in the x axis. (B) Schematic representation of Env structures. Number of amino acids (aa) is indicated on the right side. (C) Immunoprecipitation (IP) and Western blotting (WB) with anti-c-Myc antibody of supernatants from HEK293T cells transfected with indicated plasmids. Asterisks indicate truncated Env proteins. (D) Supernatants were collected from HEK293T cells that had been transfected with expression vectors encoding primate truncated Env proteins or empty vector (Mock); HEK293T cells preincubated with the supernatant were then infected with Env-pseudotyped viruses indicated in the x axis. The bar colors correspond to the truncated Env protein contained in the supernatant. The infectious units were assessed via log₁₀ LacZ⁺ cells per milliliter of virus. Infection titers with SDs are means of three infection experiments. Comparisons with Mock control were performed using Student's t test (**P* < 0.01).

integration of CERV-related viruses has been calculated using the identification of ortholog *env* genes in sister species (31). For example, the integration timings of CmRV-trunc1 and RmRV-trunc1 were estimated to be ~3.69 to 4.53 Mya; the integration timings of PNRC2-ChRV and PNRC2-BoRV were estimated to be ~2.82 to 6.55 Mya, corresponding to the diversification of crab-eating macaque (*Macaca fascicularis*) and rhesus macaque (*Macaca mulatta*), and of chimpanzee (*Pan troglodytes*) and bonobo (*Pan paniscus*), respectively (SI Appendix, Fig. S11). Each locus was identified as the true ortholog (SI Appendix, Figs. S11–S14). These results suggested that ancestral primate retroviruses might have spread their infections in host lineages several million years ago; moreover, the primate ERV *env* genes were independently acquired in the ancestral genome of macaque monkeys, that of chimpanzee and bonobo, or in a species-specific manner.

Discussion

Refrex-1, a soluble restriction factor for FeLV-D and genotype I ERV-DCs, is secreted from feline cells (20). This antiviral machinery is driven by truncated Env proteins encoded by ERV-DC7 and ERV-DC16, which are classified as genotype II ERV-DCs (SI Appendix, Fig. S1). In this study, we found that CTR1 was the entry receptor for FeLV-D and genotype I ERV-DCs; moreover, the antiviral effect of Refrex-1 was found to depend on

CTR1 (Figs. 1 and 2). These results imply that receptor interference is the mechanism underlying Refrex-1 activity. Furthermore, we determined that Refrex-1 could inhibit primate ERV infections using CTR1 as an entry receptor; likewise, we also found the antiviral machinery derived from truncated Envs in primate species (Fig. 4), suggesting that they could function as a barrier to cross-species transmission of ancient retroviruses. These results suggest that truncated Envs might constitute a convergently evolving antiviral system in feline and primate species. Our findings provide insight into the coevolutionary history between retroviruses and their hosts.

Evolutionary Arms Race between ERV-DC-Related Viruses and Refrex-1

ERV-DCs are classified into three genotypes (SI Appendix, Fig. S1). Genotype II ERV-DCs comprise the most ancient ERV-DC group, and the integration time was estimated to be ~2.8 Mya (17); this is consistent with the observation that genotype II ERV-DCs exhibit early phylogenetic divergence from the common ancestor of genotype I and genotype III ERV-DCs (SI Appendix, Fig. S1). Genotype I and II ERV-DCs belong to the same interference group, but genotype III ERV-DCs do not (27). This study revealed that genotype I and II ERV-DCs can use CTR1 as the entry receptor, but ERV-DC6 and ERV-DC10 (genotype III) cannot (Fig. 1B). These results suggest that genotype III ERV-DCs may have been generated by changes of entry receptor usage to escape

host pressure (e.g., host immunity and antiviral effect of Refrex-1). Similarly, the occurrence of RD-114 might have resulted from selective pressure by the host because RD-114 is a recombinant virus that consists of an ERV-DC-like backbone and a BaEV-related *env* gene (17–19, 32). In fact, host resistance genes provide important defenses against viral infection (33, 34), but also produce selective pressures that favor the evolution of virus variants that subvert those blocks (35–37). Therefore, we assume that the antiviral machinery, such as truncated Envs, has driven an evolutionary arms race against viral infection.

Antiviral Activity by Feline and Primate Truncated Envelopes.

The truncated Env proteins with antiviral activity comprise the 5' region of the envelope, which contains the signal sequence, VRA, and variable region B (VRB) with or without the proline-rich region. These proteins are extracellularly released because they lack the TM region (20). VRA and VRB correspond to the RBD; thus, they mediate the first step of the interaction between the viral envelope and its receptors for viral entry (29). The mechanism by which truncated Envs exhibit antiviral activity was shown by analyzing Refrex-1 and its ancestral viruses, ERV-DC7rec and ERV-DC16rec, both of which use CTR1 as the entry receptor. Here, we suggested that these truncated Envs compete for viral binding with the entry receptor, CTR1 (Fig. 2C).

Refrax-1 has been shown to protect against FeLV-D and genotype I ERV-DCs (20); it also effectively restricts infections by primate-derived ERVs (e.g., RmRV1/CmRV1, CERV2, and RhERV2-A) (Fig. 4A), despite their divergence from feline viruses (*SI Appendix, Fig. S10*). Truncated Envs with structures similar to Refrex-1 were identified in crab-eating macaque, rhesus macaque, chimpanzee, bonobo, and gorilla genomes (Fig. 4B); some of these Envs also effectively prevented infections by primate and feline retroviruses. Ten truncated Env proteins analyzed in this study exhibited lengths ranging from 188 to 439 amino acids; eight truncated Env proteins with lengths of 230 to 439 amino acids were efficiently secreted from cells (Fig. 4C) and exhibited antiviral activity (Fig. 4A and D).

It was revealed that threonine at position 82 of the VRA amino acid sequence in PNRC2-BoRV influenced protection against infection in comparison with its ortholog PNRC2-ChRV (isoleucine at position 82) (Fig. 4D and *SI Appendix, Figs. S7 and S11*). In total, five amino acid differences (isoleucine, valine, threonine, glutamic acid, and leucine) at position 82 in ERV Envs were found in primate genomes, and three amino acid differences (isoleucine, valine, and threonine) at this position were found in primate truncated Envs analyzed in this study (*SI Appendix, Fig. S7*). These findings suggest that this position may be subject to selection pressure for viral entry of exogenous as well as endogenous viruses. Alternatively, these changes may target distinct viruses because a subtle mutation within the VRA of Env SU alters receptor usage (38).

Overall, characteristic soluble Env protein structure, lengths, or specific amino acid sequences may be necessary for truncated Envs to efficiently suppress viral infection. The precise mechanism behind the antiviral activity is unknown, but it is inferred to be due to the difference in the binding affinity, strength, or stability between CTR1 and truncated Env.

Two truncated Envs, BoRV-7 and PNRC2-BoRV, were identified in the bonobo genome; BoRV-7 showed broad antiviral activity, while PNRC2-BoRV showed limited inhibitory potential (Fig. 4D). Interestingly, the PNRC2-BoRV ortholog, PNRC2-ChRV, showed broad antiviral activity. These findings

suggest that functional evolution has occurred between PNRC2-ChRV and PNRC2-BoRV after their speciation. It is speculated that the functional limitation of PNRC2-BoRV may have been replaced or complemented by BoRV-7, as it is suggested that the captured syncytins can be replaced in the divergent lineage by the *env* gene, which has a selective advantage in placental formation (39).

The finding that the mRNA expression of truncated *env* genes was detected in many tissues from primates (*SI Appendix, Figs. S4 B and C and S9*) and cats (20, 30), and the differences of those expression levels in each tissue may suggest the role of truncated Env in particular tissues (e.g., hematopoietic tissues, immune-related tissues). It should be noted that protein and RNA expression levels are not always correlated, and protein expression could not be investigated in this study.

Further elucidation of the precise mechanism behind the antiviral activity of the truncated Env proteins in vitro and in vivo is needed, which should provide insights for controlling various viral diseases.

Functional Full-Length Env in Primate ERVs. To our knowledge, this study is unique in showing that two primates (crab-eating macaque and rhesus macaque) possess intact Envs capable of viral infection through viral receptor CTR1 (Fig. 3 and *SI Appendix, Fig. S6*). The existence of intact full-length *env* genes in the genome suggests the possibility of a viral threat, such as the formation of infectious particles due to the generation of recombinant viruses [e.g., FeLV-B and FeLV-D (17, 40, 41)], endogenous MLV (42, 43), or activation of infectious ERV provirus [e.g., ERV-DC (17, 30)].

In addition to making an infectious virus with the consequent risks of retroviral insertional mutagenesis, the TM domain of the Env protein has immunosuppressive effects with negative influences on the host (44), and ERV Env, which is known to have cell fusion potential in the placenta (8), can also pose a risk to the host (45).

Reconstruction of full-length *env* genes, DC7rec and DC16rec from Refrex-1, revealed that there are additional gene mutations with some stop codons that can result in an inability to make infectious particles (Fig. 2A) (27). Furthermore, expression analysis of the primate ERV *env* genes (*SI Appendix, Figs. S4 B and C and S9*) and ERV-DC *env* genes (20, 30) showed the suppression of intact full-length ERV *env* genes in primates and cats. In contrast, the truncated *env* genes were abundantly expressed in various tissues in primates (*SI Appendix, Figs. S4 B and C and S9*) and cats (20, 30). Combined with our previous studies (27, 30), it can be speculated that truncated *env* genes pose less risk to the host than full-length *env* genes.

Trade-off for Truncated Env between Antiviral Effect and Copper Metabolism.

There may be a functional trade-off of truncated Envs regarding their benefits to cats and primates; for example, CTR1 is characterized as a high-affinity copper uptake transporter (25), while the truncated Envs have antiviral activity against viral infection depending on CTR1. Therefore, it cannot be ruled out that the interaction between truncated Env and CTR1 perturbs copper metabolism. The effects of full-length and truncated Envs on copper metabolism are currently unknown. Elucidation of the effects of ERV Env–receptor interactions on copper metabolism would be a worthwhile topic for future study.

Receptors for FeLV. A viral interference experiment is a powerful way of identifying viral receptors and interference groups (46). Cells persistently infected by retroviruses display resistance to the

same or related retroviruses due to the interaction of viral envelope protein with the receptor by masking or down-regulating the receptor in the cell (46). We have revealed the FeLV-D interference group and its receptor CTR1 via a viral interference experiment. Likewise, thiamine transporter 1 (THTR1), sodium-dependent phosphate transporters (Pit-1 and Pit-2), feline leukemia virus subgroup C receptor (FLVCR), FeLV infectivity X-essory protein (FeLIX), and reduced folate carrier have been identified to be involved in FeLV entry (38, 47–54). In other words, the substitutions of the nucleotide sequence as well as recombination in *Env* genes have led to a change in the usage of the virus receptor, and variants of FeLV accompanied by changes in viral entry receptors have frequently emerged in domestic cats (16).

The expression of viral receptors in germline cells may contribute to the formation of ERVs (55). Although CTR1 appears to be ubiquitously expressed (*SI Appendix, Figs. S3 and S4 A and C*), it is unknown whether it is expressed in germline cells. However, the expression of CTR1 in the ovary and testis (*SI Appendix, Figs. S3 and S4 A and C*) suggests a risk of viral transmission (e.g., exogenous or endogenous virus) to offspring, while the presence of viral interference, including that due to truncated *Env* or Refrex-1, may contribute to prevention of viral infection or additional viral endogenization (46).

Viruses that can infect a wide range of species, such as the FeLV-D interference group, can cause epizootics in multiple species. Against such viruses, hosts may acquire truncated *Env* proteins as a common mechanism for preventing interspecies and intraspecies viral infection. Even under epizootics of infectious diseases, the acquisition of such resistant molecules appears to be effective. Convergent acquisition of these antiviral systems by multiple species could prevent epizootics of infectious diseases and greatly contribute to viral extinction. This study provides mechanistic insights into antiretroviral factors derived from truncated *Envs* and broad suppression of retroviruses across different species by targeting a common viral entry receptor.

Materials and Methods

Receptor Screening. MDTF cells were transduced with a retroviral HEK293 cDNA library constructed in the pMX retroviral vector. FeLV-D (TY26) Env-pseudotyped viruses were used to challenge cDNA-transduced MDTF cells in eight 10-cm culture dishes. Cells were selected with 6 µg/mL puromycin. Chromosomal DNA from puromycin-resistant cells was extracted and served as a template for PCR amplification using primers annealing in the pMX vector. The PCR products were directly sequenced (*SI Appendix, Supplementary Materials and Methods*).

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cDNA Cloning and Establishment of Cell Lines. FeCTR1 and huCTR1 were PCR-amplified using cDNA from FT-1 cells and HEK293 cells as templates; they were cloned into the pFUΔss and pMSCVneo vectors, respectively. A DNA construct encoding huCTR2 was synthesized and cloned into the pMSCVneo vector. MDTF cells expressing these genes were termed MDTF-feCTR1, MDTF-huCTR1, and MDTF-huCTR2 (*SI Appendix, Supplementary Materials and Methods*).

Env-Pseudotyped Virus Preparation. GLac cells, an Env[−] packaging cell line containing a LacZ-coding retroviral vector, were transfected with Env expression plasmids to produce LacZ-carrying Env-pseudotyped viruses. Cell culture supernatants were collected, filtered, and stored at −80 °C (*SI Appendix, Supplementary Materials and Methods*).

Infection Assay. Cells inoculated with viruses were cultured for 2 d postinfection. The LacZ assay was conducted by counting blue-stained nuclei (*SI Appendix, Supplementary Materials and Methods*).

RNA Interference. MISSION esiRNA was used to knock down huCTR1 expression. Cells transfected with siRNA were infected with the pseudotyped viruses and CTR1 expression was monitored by qRT-PCR (*SI Appendix, Supplementary Materials and Methods*).

Genetic Analyses. Primate ERV sequences were searched in the National Center for Biotechnology Information database (<https://www.ncbi.nlm.nih.gov/>) and in the University of California, Santa Cruz, genome browser (<https://genome.ucsc.edu/>). Phylogenetic trees were constructed based on the maximum-likelihood method and the neighbor-joining method (*SI Appendix, Supplementary Materials and Methods*).

Data Availability. The nucleotide sequence reported in this study was deposited in the DNA Data Bank of Japan database (accession no. LC705724)(56). The relevant codes and data are available in GitHub at https://github.com/Junna-Kawasaki/Refrax_2021 (57). All other study data are included in the main text and supporting information.

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