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Title

Reduced folate carrier is the receptor for a novel feline leukemia virus subgroup

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Running head: FeLV-E receptor

Abstract

Feline leukemia virus (FeLV) is horizontally transmitted among cats and causes a variety of hematopoietic disorders. Five subgroups of FeLV, A–D and T, each with distinct receptor usage, have been described. Recently, we identified a new FeLV Env (TG35-2) gene as a sixth interference group and proposed its phenotype as FeLV subgroup E (FeLV-E). FeLV-A is the primary virus from which other subgroups have emerged via mutation or recombination of the subgroup A *env* gene. Retrovirus entry into cells is mediated by interaction of envelope protein (Env) with specific cell surface receptors. Here, phenotypic screening of a human/hamster radiation hybrid panel identified SLC19A1, a feline reduced folate carrier (RFC) and receptor for TG35-2 pseudotype virus. RFC is a multipass transmembrane protein. Feline and human RFC cDNAs conferred susceptibility to TG35-2 pseudotype virus when introduced into nonpermissive cells, but did not render these cells permissive to other FeLV subgroups or feline endogenous retrovirus. Moreover, human cells with genomic deletion of RFC were nonpermissive for TG35-2 pseudotype virus infection, but the introduction of feline and human cDNAs rendered them permissive. Mutation analysis of FeLV Env demonstrated that amino acid substitutions within the variable region A altered the specificity of the Env–receptor interaction. We isolated and reconstructed the full-length infectious FeLV-E-phenotypic provirus from a naturally FeLV-infected cat, from which the FeLV Env (TG35-2) gene was previously isolated, and the virus replicated in hematopoietic cell lines compared with FeLV-A 61E. These results provide a tool for further investigation of FeLV infectious disease.

Importance Feline leukemia virus (FeLV) is a member of the genus *Gammaretrovirus*,

which causes malignant diseases in cats. The most prevalent FeLV among cats is FeLV subgroup A (FeLV-A), and specific binding of FeLV-A Env to its viral receptor, thiamine transporter feTHTR1, is the first step of infection. In infected cats, novel subgroups of FeLV have emerged by mutation or recombination of the *env* gene. FeLV subgroup E (FeLV-E) arose from a subtle mutation of FeLV-A Env, which altered the specific interaction of the virus with its receptor. RFC, a folate transporter, is a receptor for FeLV-E subgroup. The perturbation of specific retrovirus–receptor interactions under selective pressure by the host results in the emergence of novel viruses.

Introduction

Retroviral envelope (Env) proteins consist of a trimer of heterodimers formed between the surface subunit (SU) and the transmembrane subunit (TM). Interaction of the retroviral SU with a receptor on the host cell surface is the initial step in viral entry. The specific SU–receptor interaction begins with the fusion of viral and host cell membranes, resulting in viral entry into the host cell. Therefore, viral tropism is determined by whether the target cell expresses a surface receptor protein and can bind to the viral SU protein (1). Infection of the target cell by virus usually prevents successive rounds of infection in the same cell as a result of masking or downregulation of the receptor by the viral Env protein. This phenomenon is known as superinfection interference and this phenomenon identifies whether the virus uses the same or different receptors (2,3). Therefore, elucidation of the molecular basis for the retrovirus–receptor interaction contributes to our understanding of viral entry.

Feline leukemia virus (FeLV) belongs to the genus *Gammaretrovirus* and is transmitted horizontally among domestic cats (*Felis silvestris catus*) (4). A recent epidemiological survey of FeLV infection in Japan detected FeLV in 12.2% of the 1,770 cats tested (5). This virus is known to induce various diseases in domestic cats, such as lymphoma, myelodysplastic syndrome, anemia, acute myelogenous leukemia and immune deficiency (6,7). The mechanisms by which this virus induces the multifarious symptoms of FeLV-associated diseases are still unclear; however, genetic polymorphisms resulting from substitution or recombination have led to changes in FeLV pathogenicity and unexpected symptoms (8-14). Analysis of superinfection interference properties have identified FeLV variants comprising FeLV subgroups A, B, C, D, E and T (15-20). FeLV-A is the primary virus transmitted among cats (21-23) and FeLV subgroups are thought to be generated in cats infected with FeLV-A. FeLV subgroups B and D arise from recombination between FeLV-A *env* and the *env* genes of endogenous FeLV (enFeLV) or endogenous retrovirus of the domestic cat (ERV-DC) (17, 24, 25); subgroups C, E and T possibly arise from mutations in FeLV-A *env* (8-10,18). The cellular viral receptors for FeLV subgroup A, B, C and T have been identified; FeLV-A uses the feline thiamine transporter receptor (feTHTR-1) (26), while FeLV-B uses the phosphate transporter receptors (Pit1/2) (27-30). FeLV-C uses a heme transporter (FLVCR-1/2) as its receptor along with THTR-1 (31-33). FeLV-T, a T-cytopathic FeLV subgroup, also uses Pit1 as a receptor, but it requires a second host protein known as FeLIX, a truncated envelope protein produced by enFeLV for entry (34).

We previously identified the FeLV *env* gene, TG35-2, in a 1-year-old castrated male cat,

TG35, with a bite injury, stomatitis, loss of appetite and FeLV infection, although he had been vaccinated with inactivated FeLV. He eventually died without diagnosis (5,18). The TG35-2 Env is a new interference subgroup of FeLV and shows distinct cell tropism from FeLV-A. Therefore, we proposed naming this FeLV subgroup E (FeLV-E) (18). The *env* sequences of this clone clustered phylogenetically with those of genotype I/clade I FeLV, found mainly in Japan (5). In this study, we used phenotypic screening of radiation hybrid (RH) cell lines (35) to identify SLC19A1, the feline reduced folate carrier (feRFC) as the receptor for FeLV-E. Substitution of a few amino acids within variable region A (VRA) in Env altered the specificity of the Env–receptor interaction, including facilitating the occurrence of a dual tropic virus. Furthermore, we isolated and reconstructed the full length infectious FeLV-E phenotypic provirus from a naturally FeLV-infected cat, from which the FeLV Env (TG35-2) gene had previously been isolated. Our results provide tools for further investigation of FeLV infectious disease.

Results

Identification of RFC as the FeLV-E receptor

FeLV-E phenotypic virus (FeLV 33TGE2), a chimeric infectious virus, infects human but not hamster cells (18), indicating that it might be possible to map the position of the FeLV-E receptor by analyzing the susceptibility of human-hamster RH cell lines to infection by FeLV-E. We used the G3 panel of human RH cell lines from the Stanford Human Genome Center (SHGC) (36) for phenotypic mapping of the FeLV-E receptor. ~~We previously re-genotyped t~~This panel had been previously genotyped using array comparative genomic

hybridization (37,38).

We first confirmed that the FeLV-E phenotypic virus (FeLV 33TGE2) does not infect the recipient A23 hamster cells used in the construction of the G3 panel. We then correlated the genotypes of the RH clones with their susceptibility to FeLV-E infection. The ~~overall~~ combined narrow sense (additive) heritability, h^2, of this phenotype was indistinguishable from 1 (0.99 ± 0.12 s.d.), suggesting a simple monogenic architecture (39). Consistent with this observation, we identified a single genome-wide significant locus with a logarithm of the odds (LOD) score of 16.3 on chromosome 21q22.3, with a peak marker at 46,822,915 bp (Figures 1A and 1B). The mean $\log_{10}$(IU+1) (infectious units/ml supernatant + 1) was 3.6 ± 0.5 s.e.m. for RH clones with a peak marker and 0.3 ± 0.1 s.e.m. for clones without (Figure 1C). The additive heritability for the locus was 0.63 ± 0.13 s.d., explaining the majority of the overall narrow sense heritability, and consistent with a monogenic trait. The 2LOD critical region of the chromosome 21 locus extended from 46,677,060 bp to 47,058,655 bp, or from 146 kb to the left of the peak marker (in the direction of the centromere) to 236 kb to the right (in the direction of the q telomere) (Figure 1D). ~~Careful~~ ~~e~~Examination of this region of 21q22.3 showed that none of the previously mapped retroviral receptors localized to the same position. Thus, the FeLV-E receptor most likely represents a new retroviral receptor. The gene closest to the peak marker was COL18A1, which was 52.5 kb to the right. The second closest gene was the reduced folate carrier (RFC) gene (SLC19A1), which was 95.2 kb in the same direction.

To determine whether RFC might function as the FeLV-E receptor, we isolated human RFC (huRFC) and feRFC cDNAs from HEK293T cells and feline peripheral blood mononuclear

cells (PBMCs), respectively. We generated retroviral expression vectors expressing the cDNAs encoding huRFC or feRFC and introduced them into MDTF cells~~, because~~. MDTF cells ~~were~~ are resistant to infection with Env-pseudotyped FeLV-A and FeLV-E (TG35-2) (18). MDTF cells carrying huRFC (MDTF-huRFC) and feRFC (MDTF-feRFC) were tested for permissiveness to Env-pseudotyped viruses of FeLV-A (FeLV-A/Clone33), FeLV-B (FeLV-B Gardner–Arnstein), FeLV-C (FeLV-C Sarma), FeLV-D (FeLV-D Ty26), FeLV-E (TG35-2), ERV-DC10 and ampho-MuLV (MuLV 4070A) carrying a LacZ reporter gene, which were prepared in GPLac cells. Ampho-MuLV ~~was used as a positive control because it~~ is known to infect mouse and human cells and was used as a positive control (18). MDTF-huRFC and MDTF-feRFC cells were susceptible to FeLV-E-pseudotyped virus infection with $>10^3$ infectious units (Figure 2). However, MDTF cells carrying an empty vector were not susceptible to FeLV-E-pseudotyped virus infection (Figure 2). Other feline retroviruses, FeLV-A, FeLV-B, FeLV-C and FeLV-D, and ERV-DC10-pseudotyped viruses, could not infect MDTF-huRFC or MDTF-feRFC cells. FeLV-A, FeLV-B, FeLV-C, FeLV-D, FeLV-E, and ERV-DC10-pseudotyped viruses could successfully infect HEK293T cells. These results indicated that huRFC and feRFC conferred susceptibility to FeLV-E-pseudotyped virus infection.

Expression of human or feline RFC renders HeLa-R5 cells susceptible to FeLV-E pseudotyped virus. We previously showed that FeLV-E phenotypic virus (FeLV 33TGE2), a chimeric infectious virus, could infect HeLa cells (18). HeLa-R5 cells, a derivative of HeLa cells, are characterized by the genomic deletion of RFC as a result of exposure to methotrexate (MTX) (40). As shown in Figure 3A, we confirmed that human RFC was not

151 expressed in HeLa-R5 cells, but was expressed in HeLa cells by RT-PCR. Therefore, we
152 used HeLa-R5 cells to determine susceptibility to FeLV-E infection. FeLV-A, FeLV-B,
153 FeLV-C, FeLV-D and FeLV-E Env-pseudotyped viruses were prepared in GPLac cells and
154 tested in the cell lines indicated below. As expected, HeLa-R5 cells were non-permissive for
155 FeLV-E Env-pseudotyped virus infection, while FeLV-E Env-pseudotyped virus
156 successfully infected the parent HeLa cells (Figure 3B). FeLV-B, FeLV-C and FeLV-D Env-
157 pseudotype viruses could infect HeLa and HeLa-R5 cells, while FeLV-A Env-pseudotype
158 virus could not infect HeLa cells or HeLa-R5 cells. Next, a retroviral expression vector
159 expressing huRFC or feRFC was introduced into HeLa-R5 cells, to generate R5-huRFC and
160 R5-feRFC cells (Figure 3A), and the cells were tested for infectivity with FeLV-E Env-
161 pseudotype virus as well as FeLV-A, FeLV-B, FeLV-C and FeLV-D Env-pseudotype viruses.
162 As shown in Figure 3B, both R5-huRFC and R5-feRFC cells were permissive for FeLV-E
163 Env-pseudotype virus infection with $>10^4$ infectious units, as well as FeLV-B, FeLV-C and
164 FeLV-D-pseudotype virus infection. However, R5-huRFC and R5-feRFC cells were non-
165 permissive for FeLV-A Env-pseudotype virus infection. A retroviral expression vector
166 expressing the cDNA encoding mouse RFC was introduced into HeLa-R5 cells, to generate
167 R5-mRFC cells (Figure 3A), and these cells were tested for infectivity with FeLV-E Env-
168 pseudotype virus. As shown in Figure 3C, R5-mRFC cells were not permissive for FeLV-E-
169 pseudotype virus infection, consistent with the data from the mouse cell line MDTF (Figure
170 2). These results indicated that transduction of huRFC and feRFC into HeLa-R5 cells
171 rendered them susceptible to viral entry and FeLV-E infection. Because HeLa and HeLa-R5
172 cells were not permissive for FeLV-A infection, we conducted the following experiment. A

retroviral expression vector expressing feline THTR1, which was known to be the receptor for FeLV-A (26), was introduced into HeLa-R5 cells, termed R5-feTHTR1, and the cells were tested for FeLV-A Env-pseudotype viruses from FeLV-A clone 33 (41), FeLV-A Glasgow-1 (42) and FeLV-A TG35-4 from a TG35 case (18), and FeLV-E Env-pseudotype virus. As shown in Figure 4A, all FeLV-A Env-pseudotype viruses could infect R5-feTHTR1 cells, but FeLV-E Env-pseudotype virus could not. FeLV-B Env-pseudotype virus was used as a positive control. The results indicated that transduction of feTHTR1 into HeLa-R5 cells could not render cells susceptible to FeLV-E infection. We next conducted an interference assay to determine whether FeLV-E subgroup classification depends on feRFC receptor. R5-feRFC cells pre-infected with FeLV 33TGE2 (R5-feRFC/33TGE2 cells) were tested for FeLV-E infection and the FeLV-E (TG35-2) Env-pseudotyped virus could not infect R5-feRFC/33TGE2 cells, but could infect R5-feRFC cells (Figure 4B).
Taken together, these results indicated that both feline and human RFC are receptors for FeLV-E and that viral interference of FeLV-E depends on the RFC receptor.

Isolation of cDNA encoding feline RFC

RFC (SLC19A1) transports folates, but not thiamine (41). Feline RFC has not been isolated previously. In this study, feline cDNA isolated from feline PBMCs was sequenced and predicted to encode a protein of 522 amino acids. The similarity between feline and human RFC and between feline and mouse RFC were 92.1% and 90.4%, respectively. Alignment of the predicted amino acid sequences of the proteins encoded by the feline and human RFC genes is shown in Figure 5. The amino acid sequence of human RFC obtained from

HEK293T cells was used in this alignment. There were 69 amino acid differences between the feline and human proteins. Phylogenetic analysis of RFC sequences and related sequences including FeLV receptors indicated that our clones were likely to be feRFC (Figure 6). We examined mRNA expression by RT-PCR using total RNA extracted from various feline tissues. Feline RFC was detected in all feline tissues tested (Figure 7). The feline RFC transcript was detected in the CRFK feline kidney cell line (44), AH927 feline embryo fibroblasts (45), Fet-J feline T-cells, MCC feline large granular lymphoma (46), 3201 (47) feline T-cell lymphoma, and MS4 feline B-cell lymphoma (48) (Figure 7).

Determination of the amino acids in the Env protein that are required for the FeLV-E phenotype

We have previously shown that a subtle change in the VRA altered the interference patterns of the FeLV-E and FeLV-A phenotypes (18). In this study, a series of Env mutants (Figure 8A) were tested for receptor usage using MDTF-feTHTR1 and MDTF-feRFC cells. FeLV-A TG35-4 isolated in a cat infected with FeLV-E TG35-2 was used for the construction of mutants. As shown in Figure 7, FeLV-A (TG35-4) Env-pseudotype virus could infect MDTF-feTHTR1 cells, but not MDTF-feRFC cells. However, FeLV-E (TG35-2) Env-pseudotype virus could infect MDTF-feRFC, but not MDTF-feTHTR1 cells. Chimeras 1 and 2, which contained the VRA of TG35-2 and the backbone of TG35-4, could infect MDTF-feRFC, but not MDTF-feTHTR1 cells, while chimera 3, which comprised the VRA of TG35-4 and the backbone of TG35-2, could infect MDTF-feTHTR1, but not MDTF-feRFC. These results indicated that the VRA conferred specific receptor usage to FeLV-A and FeLV-E.

A further 12 Env mutants with substituted amino acids in the VRA with the TG35-4 backbone were tested for infectivity in MDTF-feTHTR1 and MDTF-feRFC cells. Some mutants (mt2(K96P), mt4(i99T100L), mt5(R100H) and mt4,5) exhibited infectivity in both MDTF-feTHTR1 and MDTF-feRFC cells. The infectious unit measurements of mt2, mt4 and mt5 were higher in MDTF-feTHTR1 than MDTF-feRFC cells, while the mt4,5 mutant infected MDTF-feTHTR1 and MDTF-feRFC cells to a similar extent. These Env mutants showed a dual tropic phenotype combining that of FeLV-A and FeLV-E. Thus, one or three amino acid substitutions in the VRA of FeLV-A (mt2 or mt4,5 mutants, respectively) effectively altered the FeLV-A-specific phenotype to a FeLV-A and FeLV-E dual phenotype. The mt2,3,4,5, mt2,3,4, mt3,4,5 and mt3,4 mutants demonstrated infectivity in MDTF-feRFC cells, but not MDTF-feTHTR1, indicating that they were of the FeLV-E phenotype. The mt3,4 mutant, which was newly constructed in this study, had only three amino acid substitutions in the FeLV-A VRA. These results indicated that subtle mutation of the FeLV VRA alters the specificity of infection via the viral receptor, feTHTR1 or feRFC.

Isolation and construction of infectious FeLV provirus

We isolated the FeLV provirus from the genome of a cat TG35, in which the TG35-2 Env clone was detected. PCR primers designed in the U3 region of the 5′LTR and 3′LTR were used for amplification of the provirus. The infectious provirus was reconstructed as described in the Materials and Methods and was termed TP2R clone. The amino acid sequence of Env from FeLV TP2R was almost the same as that of TG35-2 Env (Figure 9). Phylogenetic analysis classified FeLV TP2R as belonging to Genotype I/ Clade 1, which is often observed in Japanese FeLV strains (data not shown) (5). The LTRs of TP2R did not

contain tandem repeats in the enhancer. The 293T cells were transfected with FeLV TP2R and FeLV-A 61E (49) plasmids and the supernatants of the cells were prepared as a virus stock. AH927 cells infected with FeLV TP2R persistently produced the virus at high titer, as well as FeLV-A 61E when the supernatant from the cells was measured using two methods: quantitative real-time RT-PCR and the determination of tissue culture infectious doses ($TCID_{50}$) (Figure 10A). FeLV Env and Gag proteins were detected in AH927 cells infected with FeLV TP2R by western blot analysis and the molecular weight of FeLV TP2R Env was slightly higher than that of FeLV-A 61E (Figure 10B). To determine the viral interference group of FeLV TP2R, AH927 cells infected with FeLV TP2R (AH927/ TP2R cells) were tested with the FeLV-A, -B, -C, -D and -E (TG35-2) Env-pseudotyped viruses. FeLV-A, FeLV-B, FeLV-C and FeLV-D Env-pseudotyped viruses could infect AH927/ TP2R cells, whereas FeLV-E(TG35-2) Env-pseudotyped virus could not. By contrast, FeLV-A, -B, -C, -D and -E (TG35-2) Env-pseudotyped viruses could infect AH927 cells (Figure 10C). Next, to determine the receptor of FeLV TP2R, FeLV-A 61E and FeLV TP2R viruses were prepared from 293Lac cells that contained the LacZ-coding retroviral vector and viral infection of MDTF-feRFC and MDTF-feTHTR1 cells was analyzed. As shown in Figure 10D, FeLV TP2R could infect MDTF-feRFC cells, but not MDTF or MDTF-feTHTR1 cells. By contrast, FeLV-A 61E could infect MDTF-feTHTR1 cells, but not MDTF or MDTF-feRFC cells. These results demonstrated that FeLV TP2R could be classified as FeLV subgroup E and used feRFC as its receptor.

FeLV-E TP2R and FeLV-A 61E viruses were prepared from AH927 cells and viral replication in different cell lines (CRFK, Fet-J, MCC, 3201 and MS4 cells) was tested by

determining the viral copy number at 10 days post-infection. As shown in Figure 11, FeLV-E TP2R and FeLV-A 61E viruses exhibited replication with high copy numbers in CRFK cells. In particular, the copy number of FeLV-A 61E was significantly higher than that of FeLV-E TP2R in CrFK cells ($P<0.01$). Both viruses could replicate in hematopoietic cells and FeLV-E TP2R virus exhibited significantly higher viral copy numbers compared with FeLV-A/61E in Fet-J feline T-cells, MCC feline large granular lymphoma cells and MS4 cells (Figure 11). These results indicated that FeLV-E TP2R could replicate with high virus titer, similar to FeLV-A 61E, in cultured cell lines.

Discussion

FeLV is transmitted among domestic cats at high prevalence in Japan and shows high genetic diversity due to mutation or recombination of viral genes (4, 67). Mutation of the FeLV Env sequence, especially in the VRA, may lead to a change in the viral receptor interference group. Our previous study identified a novel FeLV interference group based on FeLV Env and proposed FeLV-E subgroup as a new FeLV interference group. In this study, we report that the receptor of FeLV-E is RFC, which is classified as SLC19A1. We mapped the receptor for FeLV-E to within region q22.3 of chromosome 21 using phenotypic screening of RH cell lines (Figure 1) and further analyses demonstrated that RFC confers susceptibility to FeLV-E infection. Expression of feline and human RFC cDNA, but not mouse RFC cDNA, in non-permissive MDTF cells rendered these cells susceptible to FeLV-E infection. Sequence similarity and phylogenetic analysis indicated that the feline receptor is an orthologue of huRFC and is most likely a folic acid transport protein. Further functional experiments clarified that it is a folic acid transporter.

Analysis of the amino acid sequence encoded by feRFC revealed gene polymorphisms encoded by Ala or Gly at position 249 and both cDNAs can function as the FeLV-E receptor (data not shown). Genetic variants in the huRFC gene locus have been found and are reported to be associated with differences in folate homeostasis (51). Two huRFCs were isolated from HEK293T cells and were found to contain polymorphisms when compared with the huRFC sequence with gene accession numbers AAC50180 and NM_194255, and both function as FeLV-E receptors (data not shown). The feRFC revealed high amino acid similarity (more than 90%) with huRFC and moRFC. Forced expression of huRFC rendered cells permissive for FeLV-E infection (Figure 2). However, despite high amino acid similarity with mRFC, FeLV-E could not infect mouse cells (MDTF cells) or cells ectopically expressing mRFC (Figure 2 and Figure 3C). FeLV subgroup was classified by viral interference and its *in vitro* host range properties, and we demonstrated here that the FeLV-E subgroup required RFC (Figure 4B).

The utilization of transport proteins for cell entry is a common feature of gammaretroviruses, including extinct retroviruses (1, 52, 53). For example, ecotropic murine leukemia virus utilizes mCAT, the cationic amino acid transporter (54). To date, all known receptors for feline and murine gammaretroviruses have been multi-transmembrane receptors (4,55). The discovery of RFC is a receptor for FeLV-E follows this pattern of multi-pass membrane transport molecules acting as retroviral receptors. RFC was recently reported to be the receptor for murine endogenous retrovirus (56). Because some gammaretroviruses are known to share a receptor, such as GaLV, FeLV-B, KoRV-A and 10A1-MuLV, which all use Pit1 (27, 28, 29, 57, 48), it is plausible that other known viruses

may also use RFC as a receptor.

FeLV-A is transmitted among domestic cats and novel subgroups of FeLV are usually generated *in vivo*. In other words, FeLV-A evolves into FeLV-B, FeLV-C, FeLV-D, FeLV-E and FeLV-T subgroups in FeLV-A infected cats, and each of these subgroups display altered tropisms because of their differential receptor use. The RFC transporter belongs to the SLC19 family of reduced folate transporters, of which there are three members: two thiamine transporters, THTR1 and THTR2, and RFC. It is known that huRFC is ubiquitously expressed in tissues (43), and as shown in Figure 7, feRFC is also ubiquitous in feline tissues. FeLV-E utilizing RFC as its receptor may have the potential to be endogenized in cats, as seen for murine endogenous retrovirus (56). In experiments using Env mutants, the VRA region of FeLV-A and FeLV-E determined the specificity of viral receptors. A slight change in the amino acid sequence altered the tropism from FeLV-A to FeLV-E (Figure 8).

In other words, receptor usage was changed from feTHTR1 to feRFC. The amino acid residues ETL in the VRA partly contribute to this specific shift to FeLV-E tropism. Interestingly, some Env mutants with point mutations in the VRA utilized both feTHTR1 and feRFC. This indicates that the structure of the VRA of Env determines the interaction with the receptor, and this interaction leads to the specificity of viral infection. Although Env mutants, mt 3,4,5 and mt 4,5, infected MDTF cells expressing the receptor (Figure 8B), these mutants did not infect AH927 cells (18). Therefore, this suggests that additional factors may influence viral entry and infection.

Since FeLV-E was detected in FeLV-A-vaccinated cats, this may indicate that selection

pressure may have led to the emergence of FeLV-E. Dual tropic FeLVs with different receptor usages have been reported (33, 59, 60), and dual tropic mutants that use both feTHTR1 and feRFC may occur as an intermediate phenotype, but this phenotype may not be sufficiently stable. Koala retrovirus is known to use THTR1 and Pit1 as receptors (1). Therefore, KORV may evolve to a novel virus that uses RFC as a acceptor, due to the subtle mutation in the Env.

Methotrexate (MTX) is a chemotherapeutic agent and immune system suppressant that is transported by RFC (43, 51). In a similar way, FeLV-E Env pseudotype virus carrying retroviral expression vector can be utilized in transduction via RFC.

The FeLV-E TP2R provirus isolated from cat case TG35 was reconstructed as infectious provirus. FeLV-E TP2R was characterized as belonging to the FeLV-E phenotype (Figure 10) and was able to preferentially replicate in hematopoietic cells compared with FeLV 61E (Figure 11), which may be due to FeLV TP2R promoter activity or viral receptor usage.

In this study, we identified the cellular receptor for FeLV-E and isolated FeLV-E-phenotypic provirus. However, the prevalence and pathogenicity of FeLV-E in cats remain to be determined. Identification of the FeLV-E receptor may therefore help establish a strategy to detect FeLV-E infection in domestic cats. This study provides a tool for further investigations into FeLV-induced diseases.

Materials and Methods

Cells

The CRFK feline kidney cell line (44), AH927 feline embryo fibroblasts (45), 3201 (47) feline T-cell lymphoma cells, HEK293T, and *Mus dunni* tail fibroblasts (MDTF) (61) were

cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS) and 1× penicillin/streptomycin. Fet-J feline T-cells (ATCC CRL-11967) and MCC feline large granular lymphoma cells (46) were cultured in RPMI1640 with 10% FCS and 1× penicillin/streptomycin. MS4 feline B-cell lymphoma cells (48) were cultured in RPMI1640 with 20% FCS and 1× penicillin/streptomycin. HeLa cells and RFC-null HeLa (R5) cells (40) (kindly provided by Dr. I. David Goldman (Albert Einstein College of Medicine)), were cultured in DMEM with 10% FCS and 1× penicillin/streptomycin. MDTF and R5 cells expressing feline, human and mouse RFC, MDTF-feRFC, MDTF-huRFC, R5-feRFC, R5-huRFC and R5-mRFC, MDTF cells expressing feline THTR1 (MDTF-feTHTR1) (18), and R5 cells expressing feline THTR1 (R5-feTHTR1) were cultured in DMEM with 10% FCS and 1× penicillin/streptomycin and 0.6 mg/mL G418. PLAT-E and PLAT-A retroviral packaging cells (62), GPLac cells (5), an *env*-negative packaging cell line containing β-galactosidase (LacZ)-coding pMXs retroviral vector (5, 62), and 293Lac cells (63) containing LacZ-coding pMXs retroviral vector, were cultured in DMEM with 10% FCS and 1× penicillin/streptomycin.

Viruses

Feline retroviruses were prepared from the supernatants of AH927 cells infected with FeLV-B Gardner-Arnstein (64), FeLV-A 61E (49) and FeLV 33TGE2 (18), a replication-competent virus (33TGE2) containing the TG35-2 *env* gene and the *LTR*, *gag* and *pol* genes of FeLV-A clone 33 (41). Ampho-MuLV was prepared from the supernatants of NIH3T3 cells infected with ampho-MuLV (18).

The retroviral vector pMXs encoding LacZ with FeLV-A 61E, FeLV-E TP2R and Ampho-

MuLV were harvested from 293Lac cells infected with each replication-competent virus. Virus-containing cell supernatants were filtered through 0.22-μm-pore filters and stored as viral stocks at -80°C until use.

Isolation and reconstruction of FeLV TP2R provirus

Genomic DNA was isolated from the blood of case TG35 (5) using the QIAamp DNA Blood kit (QIAGEN, Venlo, Netherlands). The FeLV provirus was amplified by PCR using KOD FX Neo (Toyobo, Osaka, Japan) with a primer pair designed to the FeLV 5′ LTR and 3′LTR: Fe-227S (5′-TTACCCAAGTATGTTCCCRTGAGATANAAGGAAGT-3′, nucleotide position 67–101 of FeLV-A 61E; GenBank M18247) and Fe-7R (5′-GTCAACTGGGGAGCCTGGAGAC-3′, nucleotide position 8174–8195 of FeLV-A 61E). The resulting PCR products of 8–10 kbp were cloned into pCR-Blunt II-TOPO vectors (Invitrogen, Carlsbad, CA, USA) and sequenced by dye terminator cycle sequencing carried out by Fasmac Co., Ltd., Kanagawa, Japan. Two clones, TG35LL1 and TP1, were isolated and clone TG35LL2 was used for further experiments. The 5′LTR U3 and the 3′ LTR R-U5 of the TG35LL2 clone were repaired based on the LTR sequences of TG35LL2 using the In-Fusion HD Cloning Kit (Takara, Shiga, Japan). The 1.5 kb restriction fragment generated by excision at the *Nru*I restriction enzyme site located upstream of the *pol* gene and the BspT104I restriction site located in the *pol* gene, was replaced with that of the TP1 clone. The results indicated that the phenylalanine at amino acid position 384 of Pol was changed to isoleucine, and the isoleucine at amino acid position 485 of Pol was changed to leucine. The reconstructed provirus was designated as TP2R. The nucleotide sequence was deposited in the GenBank database under accession number OOO.

HEK293T cells were seeded in six-well plates one day prior to transfection and the TP2R expression plasmid was transfected using Lipofectamine 3000 (Invitrogen). Two days post-transfection, the supernatant was filtered through 0.22-μm-pore filters and was used to infect AH927 cells in the presence of 10 μg/ml polybrene (Santa Cruz Biotechnology, Santa Cruz, CA, USA). The cells were cultured for more than 14 days and the virus-containing cell supernatant was filtered through 0.22-μm-pore filters and stored as a viral stock at -80°C until use.

Screening of the G3 RH panel

The RH cell lines from the human/hamster G3 panel were initially obtained from Dr A. Dusty Miller (Fred Hutchinson Cancer Research Center, Seattle, WA, USA). The cells were maintained in *α* minimum essential medium with 10% fetal bovine serum (FBS), 1× penicillin/streptomycin (Wako Pure Chemical Industries, Osaka, Japan) and 1× hypoxanthine-aminopterin-thymidine (HAT; 100 μM hypoxanthine, 0.4 μM aminopterin and 16 μM thymidine; Life Technologies, CA) (36). A total of 79 clones were available, of which 75 were tested in our experiment. Prior to the infection assay, the cells were weaned from HAT by growing in HAT medium for 1 week, then for 2 weeks in HT medium lacking aminopterin, and then for 1 week in non-supplemented medium (38).

The RH cell lines were plated at 10^4 cells per well in a 24-well plate and exposed to FeLV-E pseudotype virus carrying LacZ (33TGE2-LacZ) on the next day. Two days after infection, the cells were stained with X-gal (5-bromo-4-chloro-indolyl-β-D-galactopyranoside; Wako Pure Chemical Industries). Viral titers were determined as infectious units (IU)/ml by counting the blue-stained nuclei.

The 75 hybrid cell lines were RH1 to RH83, omitting RH36, RH38, RH48, RH49, RH69, RH71, RH76 and RH78. The viral titers for the cell lines (IU/ml) were 0, 0, 0, 0.70, 0, 1.54, 0, 0, 0, 0, 1.48, 0, 0, 4.92, 0.85, 0.95, 0, 1.49, 0, 0, 0, 2.50, 1.11, 0, 0, 1.63, 2.00, 0, 1.79, 0, 0, 0, 0, 0, 0, 4.24, 0, 4.16, 0, 0, 5.37, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 1.80, 0, 0, 3.41, 0, 0, 0, 0.48, 0, 0, 3.28, 0, 3.46, 0.48, 3.17, 0, 3.47, 0.18, 0, 0 and 0, respectively. The cell lines had been previously genotyped using 235,789 markers by array comparative genomic hybridization (37,38).

Human markers were binned into 0 or 1 extra copies (diploid or triploid) using coordinates from the GRCh37/hg19 genome assembly (https://genome.ucsc.edu). Markers were discarded if they possessed ~~with identical~~ the same genotype vector~~genotypes across the clones~~, were ~~or~~ present in four or fewer clones, or present in all clones~~., were discarded, leaving a~~ A total of 54,560 markers remained. The phenotype used for mapping in each RH clone was log10 of the IU/ml value plus one. The LOD scores were computed and genome-wide significance levels were set by permutation as described previously (38). A 5% family-wise error rate (FWER) was used as the threshold for genome-wide significance.

Isolation of RFC and construction of an RFC expression vector

Total RNA was isolated from feline PBMCs (65) and HEK293T cells using RNAiso Plus (Takara), and the extracted RNA was treated with recombinant DNase I (RNase-free) (Takara). cDNA was synthesized with a PrimeScript II first-strand cDNA synthesis kit (Takara) using oligo(dT) primers. FeRFC cDNA was amplified by PCR using the primers fRFC-1S (CCGCCCGCCCGCCCGGGTACCTGGGGAG) and fRFC-1R

(GCCAGCCCGCAGTGCCCCAGCAGGCAGCGGGAT) and was then cloned into pCR-Blunt II-TOPO vectors (Invitrogen) and sequenced. FeRFC and huRFC were amplified by PCR using the primers fRFCEI (5′-GCGAATTCACAGCAAGCATGGTGCCCTCCGGCCAGGTGGCGG-3′) and fRFCBII (5′-GGAGATCTTCACAGGTCTTCTTCAGAGATCAGTTTCTGTTCGGCTTTGGCCTCGGGCTGCTGGTTCTGTT-3′; underlining indicates the myc tag sequence) for feRFC, and huRFC-S (5′-CGCTCGAGATGGTGCCCTCCAGCCCAGCGGTGGAG-3′) and huRFC-R (5′-CGAGATCTTCACAGGTCTTCTTCAGAGATCAGTTTCTGTTCCTGGTTCACATTCTGAACACCGT-3′; underlining indicates the myc tag sequence) for human RFC.

Mouse RFC (mRFC) cDNA (clone H4025H01) was obtained from Riken BRC (National Research and Development Institute of RIKEN Bioresource Center). Mouse RFC was amplified by PCR using a specific primers pair: mRFC-F (5′-CTGGGCACCATGGTGCCCACTGGCCAGGTGGCAG-3′) and mRFC-R (5′-AGAGATCTAGATCTTCACAGATCCTCTTCTGAGATGAGTTTTTGTTCCTGGTTCACATTCTGAACACCGTCGCTTGGAAGACA-3′; underlining indicates the myc tag sequence).

PCR reactions were conducted with KOD FX Neo DNA polymerase (Toyobo). PCR products were digested with *Eco*RI and *Bgl*II for feRFC and mRFC, and with *Xho*I and *Bgl*II for huRFC, and then each fragment was inserted into pMSCVneo retroviral vector (Takara).

Generation of cell lines

The feRFC, huRFC and feTHTR1(26) retroviral expression vectors were transfected into PLAT-E (ecotropic MuLV packaging) cells or PLAT-A (amphotropic MuLV packaging) cells (58) using Lipofectamine 3000 (Invitrogen). The pMSCV-neo empty vector was used as a control. Two days post-transfection, supernatants were collected and filtered through a 0.22-μm filter, and 1 ml of the filtrate was used to infect MDTF and RFC-null HeLa (R5) cells which were then seeded into 12-well plates. Polybrene, at a concentration of 8 μg/μl, was added to the infection. Cells were maintained in complete medium containing G418 at a concentration of 0.6 mg/ml.

Preparation of LacZ-carrying Env-pseudotyped viruses

GPLac cells, an *env*-negative packaging cell line containing a LacZ-coding retroviral vector, were seeded in 6-well plates one day prior to transfection and were transfected with *env* expression plasmids to produce LacZ-carrying Env-pseudotyped virus. After 48 h, cell culture supernatants were collected, filtered through a 0.22-μm filter and stored at -80°C.

Env expression plasmids for pseudotyped virus preparations: pFUΔss clone33 (FeLV-A Clone 33 *env*), pFUΔss GB (FeLV-B Gardner–Arnstein *env*), pFUΔss SC (FeLV-C sarma *env*), pFUΔss Ty2.0 (FeLV-D Ty26 *env*), pFUΔss TG35-2 (FeLV-E TG35-2 *env*), pFUΔss TG35-4 (FeLV-A TG35-4 *env*), pFUΔss DC10 (ERV-DC10 *env*) and pFUΔss 4070A (amphotropic MuLV 4070A *env*), have been described previously (17,18).

Env expression plasmids for the mutant FeLV *env* genes, constructed in either TG35-2 or TG35-4 *env* were: chimera 1, chimera 2, chimera 3, mt1, mt2, mt3, mt4, mt5, mt6,

mt2,3,4,5, mt2,3,4, mt3,4,5, mt2,3 and mt4,5, as previously described (18). In this experiment, the *env* expression plasmid, mutant mt3,4, was newly generated by site-directed mutagenesis with Fe-602S (CTAGCAATGTAAAACATGAAACCCTCGCTCGTTATCC) and its complimentary sequence in the pFUΔss vector. The sequences of the Env mutants are shown in Figure 8A.

Viral infection and titration by a LacZ assay. Target cells seeded in 24-well plates were inoculated with 250 μl of Env-pseudotyped viruses and cultured in medium containing 10 μg/ml of polybrene. After 48 h, cells were stained with X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside), and single-cycle infectivity was titrated by counting blue-stained nuclei under the microscope.

Detection of RFC by RT-PCR

Feline tissues were obtained from a specific-pathogen-free (SPF) cat (Kyoto-SPF1) described in our previous study (65). Total RNA was extracted from the tissues with an RNAiso Plus kit (Takara) and from cell lines using miRNeasy (QIAGEN) and recombinant DNase I. cDNA was synthesized with the PrimeScript II first-strand cDNA synthesis kit (Takara). PCR for detecting RFC in the feline tissues and cell lines was performed using KOD One PCR Master Mix -Blue- (Toyobo) with primer set Fe-626S (5′-CACCGACTACCTGCGCTACA-3′) and Fe-601R (5′-CGTAGTTGACCGTGGAGAAGG-3′). Thermal cycling conditions were 35 cycles of 98°C for 10 s, 60°C for 5 s and 68°C for 1 s. PCR for detecting RFC in HeLa, R5, R5-huRFC and R5-feRFC cells was performed using the KOD One PCR Master Mix -Blue- (Toyobo) with primer set Fe-649S (5′-AGAGCTTCATCACCCCCTAC-3′) and Fe-625R (5′-GCTGTAGAAGAGCTCCATGA-3′).

Thermal cycling conditions were 35 cycles of 98°C for 10 s, 55°C for 5 s and 68°C for 1 s. PCR for detecting β-actin was performed using the same master mix kit and a previously reported primer set (17). Thermal cycling conditions were 30 cycles of 98°C for 10 s, 52°C for 5 s and 68°C for 1 s.

Phylogenetic analysis

A phylogenetic tree was constructed using the following sequences: human RFC (AAC50180 and NM_194255), mouse RFC (NP_112473.1), human THTR1 (NP_008927), feline THTR1 (ABD61002.1), human THTR2 (NP_079519), feline THTR2 (AFV75033), human FLVCR1 (NP_054772), feline FLVCR1 (NP_001009302), porcine FLVCR2 (NP_001136312), human FLVCR2 (NP_060261), human Pit1 (NP_005406), feline Pit1 (NP_001009840), human Pit2 (NP_006740) and feline Pit2 (NP_001009839). The MEGA7 program package was used for the phylogenetic analysis (66). The alignments for each phylogenetic tree were conducted using MUSCLE software (67). The phylogenetic tree was constructed using 341 positions, the neighbor-joining method (68) and the JTT matrix-based method (69), and the robustness of each tree was evaluated by the bootstrap method (1000 times).

Viral titration

AH927 cells were seeded into 24-well plates one day prior to infection. Then, 250 μl of diluted virus stock (10-fold serial dilutions) was added in the presence of 10 μg/ml polybrene in quadruplicate. Eight hours post-infection, 250 μl of medium was added to each well. Three days post-infection, the cells were fixed with 3.7% formaldehyde solution,

permeabilized with 0.2% Triton-X 100 and then blocked with 1% BSA. The cells were stained overnight at 4°C with a FeLV Gag p27 antibody, then stained for one hour at room temperature with horseradish peroxidase (HRP)-conjugated anti-mouse IgG antibody (Cell Signaling, Danvers, MA, USA). The cells were added with DAB-peroxidase substrate solution (Nacalai Tesque, Kyoto, Japan), and colonies of brown cells were counted under a microscope. The viral titer was calculated as the 50% tissue culture infectious dose ($TCID_{50}$) according to the Reed–Muench method (70).

Quantification of viral RNA by quantitative real-time RT-PCR

Fet-J, MCC, MS4 and 3201 cells were seeded at 4×10^4 cells/well into 24-well plates and infected with 2×10^3 $TCID_{50}$ of virus stock in the presence of 10 μg/ml polybrene in a total volume of 500 μl. Twenty-four hours post-infection, the culture medium was changed and the cells were cultured for 10 days. Then the culture supernatants were collected after centrifugation at 300 × *g* at 4°C for 5 min.

The culture supernatants (200 μl) were treated for 40 min at 37°C with 10 mM of $MgCl_2$ and 20 μg/mL of DNaseI, and total RNA was extracted using the High Pure Viral RNA kit (Roche, Basel, Switzerland). cDNA was generated from 8 μl of RNA using the PrimeScript II 1st Strand cDNA Synthesis kit (Takara) with random 6 mers in a total volume of 20 μl. For real-time RT-PCR of FeLV 61E, a probe (FeLV_U3-probe) and primers (Forward: FeLV_U3-exo-f, Reverse: FeLV_U3-exo-r) against FeLV LTR were used as previously reported by Tandon *et al*. (71). For real-time RT-PCR of FeLV TP2R, a reverse primer was designed (FeLV_U3-exo-r2: 5′-TTATAGCAAAAAGCGCGGG-3′). The probe was labelled at the 5′-end with the fluorescent reporter dye FAM (6-carboxyfluorescein) and at the 3′-end

with the fluorescent quencher dye TAMRA (5,(6) carboxytetramethylrhod-amine). Then, 2 µl of cDNA were amplified in a total volume of 25 µl using Premix Ex Taq (Takara) with a final concentration of 300 nM of forward primer, 300 nM of reverse primer and 200 nM of probe. Reactions were performed using CFX96 Touch (Bio-Rad, Hercules, CA, USA). Thermal cycling conditions were 95°C for 30 s, then 45 cycles of 95°C for 5 s and 60°C for 30 s. Plasmid p61E (a gift from Dr. Edward Hoover), which contains the full-length FeLV-A 61E provirus subcloned into pUC18, and TP2R, were used as standards for PCR quantification. The plasmid standard copy number was calculated from optical density measurements at 260 nm. A 10-fold dilution series of the plasmid standard template DNA was made in 10 mM Tris-Cl, pH 8.5. Quantification of the sample amplicon was achieved by comparing the threshold cycle value of the sample with the standard curve of the co-amplified standard template DNA.

Western blot analysis

Cell lysates were prepared by resuspending the cells in lysis buffer (20 mM Tris-HCl [pH 7.5], 150 mM NaCl, 10% glycerol, 1% Triton X-100, 2 mM EDTA, 1 mM Na_3VO_4 and 1 µg/ml each of aprotinin and leupeptin) followed by incubation on ice for 20 min. Insoluble components were removed by centrifugation, and the protein concentrations were determined using a protein assay kit (Bio-Rad). Proteins were separated by electrophoresis on 7.5% or 10%–20% gradient Tris-glycine mini gels (Oriental Instruments, Kanagawa, Japan) under reducing conditions (3.5×10^{-2} M 2-mercaptoethanol) and then transferred electrophoretically to nitrocellulose filters for western blotting using goat anti-FeLV gp70

and goat anti-FeLV p27 primary antibodies (NCI-Frederick), and a HRP-conjugated anti-goat IgG secondary antibody (Cell Signaling). Detected proteins were visualized using 20× LumiGLO (Cell Signaling).

Ethical approval

Animal studies were conducted following the guidelines for the Care and Use of Laboratory Animals of the Ministry of Education, Culture, Sports, Science and Technology, Japan. All experiments were approved by the Genetic Modification Safety Committee of Yamaguchi University, Yamaguchi, Japan.

Accession numbers

The nucleotide sequences reported in this study were deposited in the DDBJ, EMBLE and GenBank databases under accession numbers LC223819 and LC223820.

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796 **Figure Legends**

797 **Figure 1. Mapping of a locus for FeLV-E infectivity.** (**A**) A genome-significant locus
798 resides on chromosome 21. LOD, logarithm of the odds. (**B**) The locus is located near the
799 telomere on the long arm of chromosome 21 at 21q22.3. (**C**) $\log_{10}$(IU+1) for RH clones
800 containing the peak marker (genotype = 1) and for clones without (genotype = 0). IU,
801 infectious units/ml supernatant. (**D**) The peak marker is close to the RFC gene (red).

802 **Figure 2. Infection of cell lines by LacZ-carrying Env-pseudotyped viruses.** Env-
803 pseudotyped viruses of FeLV-A (FeLV-A Clone33), FeLV-B (FeLV-B Gardner–Arnstein),
804 FeLV-C (FeLV-C Sarma), FeLV-D (FeLV-D Ty26), FeLV-E (TG35-2), ERV-DC10 and
805 ampho-MuLV (MuLV 4070A) were tested in the cell lines, HEK293T, MDTF, MDTF

806 expressing human RFC (MDTF-huRFC), and MDTF expressing feline RFC (MDTF-
807 feRFC) shown on the x axis. The y axis indicates the infectious units using the $\log_{10}$ of β-
808 galactosidase (LacZ) positive cells per ml of supernatant. Data were obtained from three
809 independent experiments in triplicate and represent the averages of nine results and the
810 standard deviation.

811 **Figure 3. Infection of cell lines by LacZ-carrying Env-pseudotyped viruses.** (A) The
812 expression of RFC was tested in the cell lines, HeLa, HeLa-R5 (R5), R5 expressing human
813 RFC (R5-huRFC), R5 expressing feline RFC (R5-feRFC) and R5 expressing mouse RFC
814 (R5-mRFC) by RT-PCR. β-*actin* was used as a control. (B) The Env-pseudotyped viruses of
815 FeLV-A (FeLV-A Clone33), FeLV-B (FeLV-B/Gardner–Arnstein), FeLV-C (FeLV-C
816 Sarma), FeLV-D (FeLV-D Ty26) and FeLV-E (TG35-2) viruses were tested for infection in
817 the cell lines shown on the x axis. (C) The Env-pseudotyped virus of FeLV-E (TG35-2) was
818 tested in the cell lines shown on the x axis. The y axis indicates the infection units by log10
819 of β-galactosidase (LacZ) cell positives per ml of supernatant. Data represent the averages
820 from three independent experiments with the standard deviation shown.

821 **Figure 4. Infection and interference assay of LacZ-carrying Env-pseudotyped viruses.**
822 (A) The Env-pseudotyped viruses of FeLV-A Clone 33, FeLV-A Glasgow-1, FeLV-A
823 (TG35-4), FeLV-B (FeLV-B Gardner–Arnstein) and FeLV-E (TG35-2) were tested for
824 infection in R5-expressing feTHTR1 cells (R5-feTHTR1). (B) The Env-pseudotyped FeLV-
825 B (FeLV-B/Gardner–Arnstein) and FeLV-E (TG35-2) viruses were tested for infection in
826 the R5 cells expressing feline RFC (R5-feRFC) and R5-feRFC cells pre-infected with FeLV

827 33TGE2 (R5-feRFC/33TGE2). The infection units were indicated by log10 of β-
828 galactosidase (LacZ) cell positives per ml of supernatant. Data represent the averages from
829 three independent experiments with the standard deviation shown.

830 **Figure 5. Alignment of the predicted amino acid sequences of feRFC and huRFC.** The
831 alignment in single letter amino acid code was conducted using MUSCLE software (67).
832 Dots indicate conserved amino acid residues, and positions where there are differences
833 between feline and human RFC sequences are shown as letters. A hyphen (–) indicates a
834 gap in the amino acid sequence. Transmembrane domains (gray boxes) based on huRFC
835 were predicted using the constrained consensus topology prediction (72).

836 **Figure 6. Phylogenetic analysis of RFC and related proteins with FeLV receptors.** A
837 neighbor-joining tree was generated from the amino acid sequences of human RFC, feline
838 RFC and mouse RFC with proteins indicated from the FeLV-A, FeLV-B and FeLV-C
839 receptors. The scale bar indicates evolutionary distance in amino acid substitutions per site.

840 **Figure 7. RFC expression in feline tissues and feline cell lines.** Detection of RFC by RT-
841 PCR using total RNA isolated from the indicated tissues and cell lines (AH927, CRFK, Fet-
842 J, MCC, 3201 and MS4). A representative 2% agarose gel with electrophoresed PCR
843 product (133 bp) is shown. The gels were stained by ethidium bromide. RT(+) and RT(-)
844 controls were included during cDNA synthesis.

845 **Figure 8. Determination of the amino acids in the Env protein that are required for**
846 **FeLV-E receptor usage.** (A) The indicated mutant FeLV *env* genes, constructed in either

the TG35-2 (FeLV-E) or TG35-4 (FeLV-A) *env* gene, were generated with site-directed mutagenesis or recombination of the VRA (18). The mutant FeLV *env* gene, mt3,4, was newly generated in this study. The *env* sequences other than the VRA, derived from TG35-2 or TG35-4 *env*, are referenced on the right side. (B) The indicated Env-pseudotyped viruses were tested for infection in the MDTF, MDTF expressing feline THTR1 (MDTF-feTHTR1) and MDTF expressing feline RFC (MDTF-feRFC) cell lines. The infection units were indicated by log10 of β-galactosidase (LacZ) cell positives per ml of supernatant. Data represent the averages from three independent experiments with the standard deviation shown.

Figure 9. Alignment of the amino acid sequence of FeLV Env. Surface subunit (SU), transmembrane subunit (TM), receptor-binding domain (RBD), proline-rich region (PRR) and C domain of the Env protein are shown for FeLV-A 61E (49), FeLV-A clone 33 (41) and FeLV TG35-2 env clone (18) compared with FeLV TP2R. The variable regions, VRA and VRB, are also shown. Dots indicate identical residues, and dashes indicate spaces that were introduced for the amino acid alignment. Boxes indicate the positions of the PCR primers (18). The Env sequences were aligned with the Genetyx program (Genetyx Corporation, Tokyo, Japan).

Figure 10. Characterization of FeLV-E TP2R. (A) Viral copies and titers of FeLV-A 61E and FeLV TP2R harvested from the supernatants of FeLV-infected AH927 cells were shown as copies per ml and 50% tissue culture infectious doses ($TCID_{50}$) per ml. (B) FeLV proteins were detected in AH927 cells infected with FeLV-A 61E (61E) or FeLV TP2R (TP2R) using anti-FeLV gp70 Env and anti-FeLV p27 Gag antibodies by western blot

analysis. (C) Interference assay of FeLV TP2R. Env-pseudotyped viruses of FeLV-A (FeLV-A clone 33), FeLV-B (FeLV-B Gardner-Arnstein), FeLV-C (FeLV-C Sarma), FeLV-D (FeLV-D Ty26) and FeLV-E(TG35-2) were tested for infection in AH927 cells and AH927 cells pre-infected with FeLV TP2R (AH927/TP2R). (D) The replication-competent viruses of FeLV-A 61E, FeLV TP2R and ampho-MuLV carrying LacZ were tested for infection in MDTF, MDTF-feTHTR1 and MDTF-feRFC cells. The infection units by log10 of β-galactosidase (LacZ) cell positives per ml of supernatant. Data represent the averages from three independent experiments with the standard deviation shown.

Figure 11. FeLV replication in different cell lines. The cells (CRFK, Fet-J, MCC, 3201 and MS4) were infected with 2×10^3 $TCID_{50}$ of FeLV TP2R or FeLV-A 61E virus. The viral copy number was measured in the culture supernatants at 10 days post-infection by **quantitative real-time RT-PCR**. The y axis indicates the viral copy number. ** $P < 0.01$, * $P < 0.05$ (Student's *t* test). Data represent the averages from three independent experiments with the standard deviation shown.