Ancestral capture of *syncytin-Car1*, a fusogenic endogenous retroviral *envelope* gene involved in placentation and conserved in Carnivora

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Syncytins **are envelope protein genes of retroviral origin that have been captured for a function in placentation. Two such genes have already been identified in simians, two distinct, unrelated genes have been identified in Muridae, and a fifth gene has been identified in the rabbit. Here, we searched for similar genes in the Laurasiatheria clade, which diverged from Euarchontoglires—primates, rodents, and lagomorphs—shortly after mammalian radiation (100 Mya). In silico search for envelope protein genes with full-coding capacity within the dog and cat genomes identified several candidate genes, with one common to both species that displayed placenta-specific expression, which was revealed by RT-PCR analysis of a large panel of tissues. This gene belongs to a degenerate endogenous retroviral element, with precise proviral integration at a site common to dog and cat. Cloning of the gene for an ex vivo pseudotype assay showed fusogenicity on both dog and cat cells. In situ hybridization on placenta sections from both species showed specific expression at the level of the invasive fetal villi within the placental junctional zone, where trophoblast cells fuse into a syncytiotrophoblast layer to form the maternofetal interface. Finally, we show that the gene is conserved among a series of 26 Carnivora representatives, with evidence for purifying selection and conservation of fusogenic activity. The gene is not found in the Pholidota order and, therefore, it was captured before Carnivora radiation, between 60 and 85 Mya. This gene is the oldest *syncytin* gene identified to date, and it is the first in a new major clade of eutherian mammals.**

endogenous retrovirus | gene capture | cell–cell fusion | endotheliochorial placenta | evolution

Previous studies have identified envelope protein (*env*) genes of retroviral origin that have been independently captured by their host for a function in placentation and that have been named *syncytins*. In simians, *syncytin-1* (1–4) and *syncytin-2* (5, 6) entered the primate genome 25 and >40 Mya, respectively, and retained their coding capacity in all of the subsequent lineages. They display placenta-specific expression and are fusogenic in ex vivo fusion assays, and one of them (*syncytin-2*) displays immunosuppressive activity (7). A pair of *env* genes from endogenous retroviruses (ERVs) was then identified in the mouse, named *syncytin-A* and *-B*, and they share closely related functional properties, although they have a completely distinct origin, showing a divergent sequence and a different genomic location compared with the primate *syncytins* (8). Finally, a *syncytin* gene was identified in a third order of mammals, namely in the lagomorphs, with the rabbit *syncytin-Ory1*, which again, was unrelated to the simian and murine genes but most probably shares with them a similar physiological function (9). Recently, we have unambiguously shown by the generation of *syncytin-A* KO mice that these genes are indeed essential for placentation, with a lack of cell–cell fusion observed in vivo at the level of the syncytiotrophoblast interhaemal layer of the mutant placenta; this lack results in impaired maternofetal exchanges and death of the embryos at midgestation (10). Furthermore, in the sheep, ERV-derived *env* genes, clearly distinct from the *syncytins*, were shown to be involved in periimplantation placental morphogenesis through loss of function experiments (11). Therefore, it seems that, on several occasions in the course of mammalian evolution, *env* genes from endogenous retroviruses have been co-opted by their host to participate in the formation of the placenta.

Interestingly, this stochastic acquisition of genes of exogenous origin might be related to the unexpectedly large diversity observed in placental structures and the physiology of placentation among eutherian mammals (12–15). Three main types of placenta have been identified involving different interfaces between the mother and fetal tissues. In the simplest one, the epitheliochorial placenta, both tissues are simply apposed; in the most intricate one, the hemochorial placenta, the placental tissue is in close contact with the mother's blood. The third type, the endotheliochorial placenta, displays intermediate characteristics. It turns out that the primates, rodents, and lagomorphs in which *syncytin* genes have been identified all belong to the same clade of eutherian mammals, the Euarchontoglires, which diverged from a second major clade of eutherian mammals, the Laurasiatheria, ~100 Mya (Fig. 1) (16). The latter comprises a large number of species belonging to the orders Cetartiodactyla (Ruminantia, Cetacea, Suina, etc.), Perissodactyla, Chiroptera, Insectivora, Pholidota, and Carnivora that display various types of placenta. To determine whether *syncytin* capture is a general process also found in this anciently diverged clade of mammals and whether *syncytins* can be found in animals with a nonhemochorial placentation, we searched

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See Commentary on page 2184.

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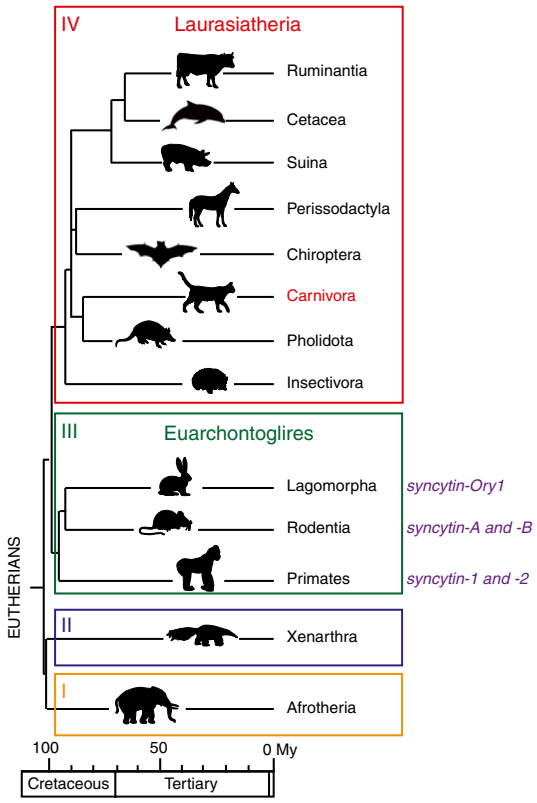

Fig. 1. Phylogeny of Eutherians and previously identified *syncytin* genes. Eutherians can be grouped into four major clades: Afrotheria (I), Xenarthra (II), Euarchontoglires (III), and Laurasiatheria (IV). Adapted from ref. 16. Branch length is proportional to time (in million years), and the orders where *syncytins* have been identified to date are indicated with the corresponding gene name.

for such genes in Carnivora. Carnivora was selected, because the genome from three representatives of this order had been sequenced, namely the dog, the cat, and more recently, the giant panda (17–20). Moreover, at least for the former two representatives, placenta can be easily recovered at various stages of gestation. In addition, placentation in Carnivora is of the endotheliochorial type (13, 15).

An in silico search for full-length *env* genes with an uninterrupted ORF within the dog and cat genomes identified several candidate genes, which were tested for specific expression in the placenta by quantitative RT-PCR on RNA isolated from a large panel of tissues. This test resulted in the identification of a placenta-specific *env* gene belonging to an endogenous retrovirus present in the dog and cat genomes that is also found in the giant panda. This *env* gene is distinct from previously identified *syncytins*, and it corresponds to an independent retrovirus capture by a common ancestor of Carnivora. Functional characterization of the identified placenta-expressed *env* gene, by cloning into a CMV-driven expression vector and transient transfection experiments, showed its fusogenic activity in an ex vivo infectivity assay with viral pseudotypes. Finally, in situ hybridization of dog and cat placenta sections revealed specific expression of this gene at the level of the syncytial junctional zone where the invading placental tissue contacts the maternal myometrium, consistent with a role in the formation of the maternofetal interface. The gene was found at the same genomic position in the 26 Carnivora species that we further investigated, with evidence for purifying selection. This gene that we accordingly named *syncytin-Car1* should, therefore, have entered the Carnivora order before its radiation (i.e., >60 Mya) (16, 21), and as such, it is the oldest *syncytin* found to date that has been conserved in a functional state for a physiological role in placentation.

The identification of a *syncytin* gene within a new major clade of mammals, the Laurasiatheria, strongly supports the notion that, on several occasions, retroviral infections have resulted in the independent capture of genes that have been positively selected for a convergent physiological role. It also shows that genomic and genetic analyses cannot solely rely on the notion that mammalian genes have evolved through a series of mutations/recombinations from primitive genes common to most living species, but they have to take into account stochastic de novo acquisition of exogenous genes of parasitic origin, possibly responsible for dramatic evolutionary transitions. Transition from egg-laying animals to placental mammals might be one illustrative example of such a genomic "coup de force."

Results

In Silico Search for Retroviral *env* Genes Within the Dog (*Canis lupus familiaris*) and Cat (*Felis catus*) Genomes. To identify putative *env*-derived *syncytin* genes in Carnivora, we made use of the available dog (*Canis lupus familiaris*) and cat (*Felis catus*) genome sequences and the method that we previously devised to screen the human, mouse, and rabbit genomes for such genes (8, 9, 22).

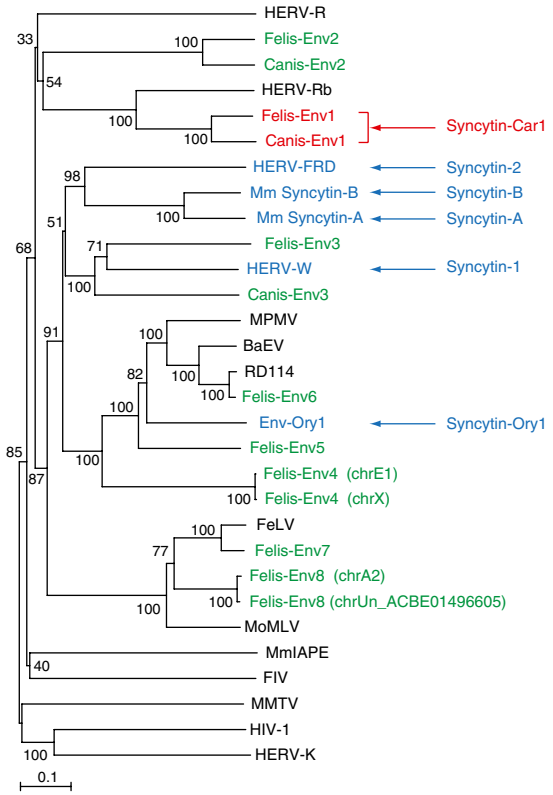

Fig. 2. Retroviral envelope protein-based phylogenetic tree with the identified Canis- and Felis-Env protein candidates. The tree was determined by the neighbor-joining method using envelope amino acid sequences from murine and human endogenous retroviruses and a series of infectious retroviruses. The horizontal branch length is proportional to the percentage of amino acid substitutions from the node (scale bar on the left), and the percent bootstrap values obtained from 1,000 replicates are indicated at the nodes. The two pairs of Felis-Env proteins that were grouped into single families of elements (Felis-Env4 and Felis-Env8) are distinguished by indication of their chromosomal position. BaEV, baboon endogenous virus; FeLV, feline leukemia virus; FIV, feline immunodeficiency virus; HERV, human ERV; MoMLV, moloney murine leukemia virus; MmIAPE, *Mus musculus* intracisternal A-type particle with an envelope gene; MMTV, murine mammary tumor virus; MPMV, Mason–Pfizer monkey virus; RD114, feline endogenous type-C retrovirus.

Basically, it makes use of the CKS17 consensus motif that is associated with the immunosuppressive domain of retroviral envelope proteins and designed to match the majority of *env* genes of exogenous and endogenous origin (22). Dog and cat sequences were screened with a slightly modified CKS17 motif using the BioMotif program, and only coding sequences longer than 1 kb (from start to stop codon) were considered (*Methods*). Accordingly, 3 and 10 coding sequences were identified in the dog and cat genomes, respectively. For the cat genes, two pairs of sequences displayed high identity, and all of the identified coding sequences within the dog and cat genomes could finally be grouped into 11 families that we named *canis-env1* to *-env3* and *felis-env1* to *-env8*, respectively (Figs. 2 and 3). A phylogenetic tree constructed with these sequences and including a series of retroviral *env* genes as well as the known *syncytins* (Fig. 2) showed that the identified genes are distinct from the previously identified *syncytins*, with very short branch lengths observed for both the paired *felis-env4* and *felis-env8* sequences.

In silico analysis of the identified sequences (Fig. 3) also revealed that they possess at least part of the characteristic features of retroviral envelope proteins (23, 24), with a putative furin cleavage site (consensus: R/K-X-R/K-R) delineating the surface (SU) and transmembrane (TM) subunits and a C-X-X-C motif corresponding to a binding domain between the two subunits. Hydrophobicity plots identify a hydrophobic transmembrane domain within the TM subunits, a sequence required for anchoring of the envelope protein within the plasma membrane and membrane fusion, with the exception of Canis-Env2 and Felis-Env2 because of the presence of a premature stop codon. A putative hydrophobic fusion peptide is also identified at the N terminus of the TM subunits as well as a canonical immunosuppressive domain (ISD), which was expected from the screening procedure. In some cases (e.g., Canis-Env1 and -Env2 and Felis-Env1, -Env2, and -Env8), a clear signal peptide is predicted by the Phobius program (http://phobius.sbc.su.se/) at the N terminus of the sequences.

Finally, a BLAST search among the corresponding databases revealed (Fig. 3) that *canis-env1* to *-env3* are present at a low copy number (one to three copies, with only one copy with a full-length ORF in each case), with a similar situation for most of the cat sequences (one to four copies). For *felis-env3* and *-env8*, a much larger number of copies can be identified (up to 50 copies for *felis-env3*) but again, with only one or two containing a complete ORF in each family of elements.

Identification of Placenta-Specific *env* Genes. Quantitative RT-PCR (qRT-PCR) analysis of transcript levels for each candidate *env* gene was then performed using primers that were designed to be specific for the ORF-containing sequences within each family of elements (*Methods*). Accordingly, as shown in Figs. 3 and 4, only *canis-env1* and *felis-env1*, and to a much lesser extent, *felis-env2*, showed the characteristic property of a *syncytin* gene, with high-level expression in the placenta and limited expression in other tissues. Expression is indeed severely restricted to the placenta for *canis-env1* and *felis-env1*, with <2% of the placental level found in only a few other organs (e.g., uterus, cortex, and heart). *Felis-env2* displays up to 20% of the placenta expression level in several tissues, whereas the other candidate genes are found to be expressed at higher levels in nonplacental than placental tissues. Altogether, in silico analyses combined with qRT-PCR assays for the dog and cat retroviral *env* genes clearly identify *canis-env1* and *felis-env1* as putative *syncytin* genes. *Felis-env2*, despite its pattern of expression and its close relationship with *canis-env2* (76% identity in amino acid sequences; see also Fig. 2), was not considered further in the present study, because it lacks the transmembrane domain essential for fusogenic activity.

As already suggested by the relatively short branch length separating *canis-env1* and *felis-env1* (Fig. 2), these two genes are closely related, with 78% identity of their amino acid sequences. Furthermore, a BLAST search using either sequence against the third available Carnivora genome, namely the genome of the giant panda bear (*Ailuropoda melanoleuca*), identified a homologous *env* gene sequence within this species that we consequently named *ailur-env1*. As illustrated in Fig. 5, all three genes are homologous (80% and 78% amino acid sequence identity between *ailur-env1* and *canis-* and *felis-env1*, respectively), with all of the canonical sites and domains characteristic for a retroviral envelope protein being highly conserved. Interestingly, a search using the Comparative Genomic tool of the University of Cal-

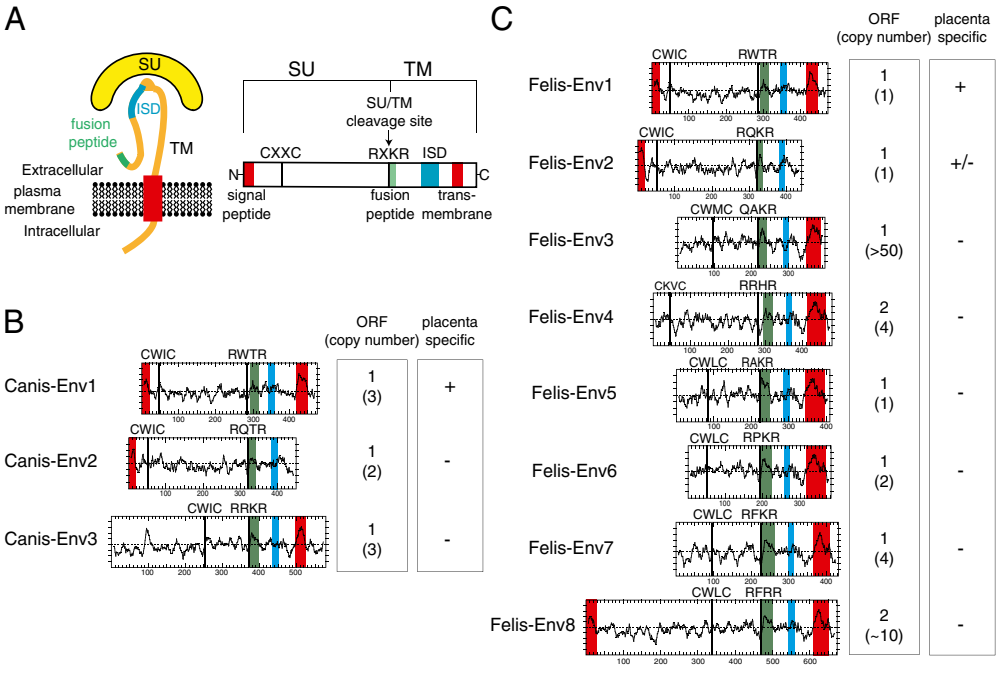

Fig. 3. Structure of a canonical retroviral envelope protein and characterization of the identified dog and cat candidates. (*A*) Schematic representation of a retroviral envelope protein with the SU and TM subunits delineated and the furin cleavage site (consensus: R/K-X-R/K-R) between the two subunits together with the C-X-X-C domain involved in SU–TM interaction indicated; the hydrophobic signal peptide (red), fusion peptide (green), transmembrane domain (red), and putative immunosuppressive domain (ISD; blue) are also indicated. (*B* and *C*) Characterization of the dog and cat candidate envelope proteins, respectively. (*Left*) The hydrophobicity profile for each candidate is shown with the canonical structural features listed in *A* positioned when present (same color code). (*Right*) Number of full-length coding sequences (ORF) of *env* genes within each family of element and total number of genomic copies (in parenthesis) together with indication for specific placental expression (details in Fig. 4).

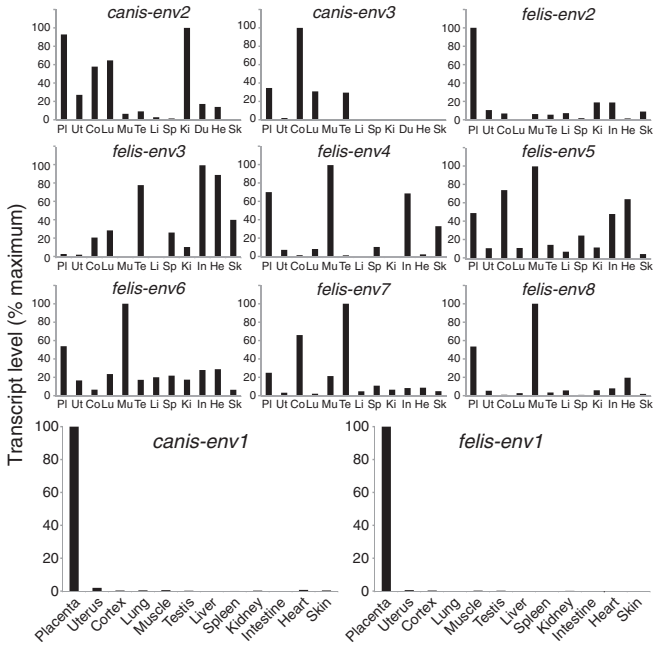

Fig. 4. Real-time qRT-PCR analysis of the candidate *env* gene transcripts from dog and cat. Transcript levels are expressed as percent of maximum and were normalized relative to the amount of a control gene (PPIA) (*Methods*) RNA. The two placenta-specific *canis-env1* and *felis-env1* genes are displayed in enlarged panels in the fourth row; the same tissues (abbreviated and displayed in the same order) for the nine other *env* gene candidates are shown. Values for the placenta and uterus are the means of at least five samples for both dog and cat; RNAs of other tissues are from Zyagen.

ifornia Santa Cruz (UCSC) Genome Browser (http://genome.ucsc.edu) showed that *canis-env1*, *felis-env1*, and *ailur-env1* are positioned at the same syntenic genomic locus: chromosome 3 for the dog, chromosome B1 for the cat, and scaffold GL193130.1 for the giant panda (Fig. 6*A*). Of note, although intrinsically not fusogenic because of a premature stop codon before the transmembrane domain, *canis-env2*, *felis-env2*, and a homologous gene in the giant panda *ailur-env2* (80% and 73% amino acid sequence identity between *ailur-env2* and *canis-env2* and *felis-env2*, respectively) are also positioned at a syntenic genomic locus in these three species.

Orthologous Candidate *syncytin* Genes of Retroviral Origin. Close examination of the *canis-env1*, *felis-env1*, and *ailur-env1* genes shows that they are part of a proviral structure with degenerate but still identifiable flanking LTRs and internal *gag* and *pol* gene sequences (Fig. 6*B*). A primer binding site sequence can also be identified downstream to the 5′ LTR, which is commonly observed for retroviruses (23, 25, 26), where it is used for priming reverse transcription, and found in the present case to be complementary to the Carnivora Arginine tRNA (Fig. 6*C*). The retroviral origin of the *env1* genes is also supported by the occurrence of a retroviral-like mode of generation of the subgenomic *env* transcripts, with a putative donor splice site located close to the 5′ LTR and an acceptor site 5′ to *env*. These sites were predicted by the NetGene2 program (http://www.cbs.dtu.dk/services/NetGene2) and confirmed by RT-PCR analysis—and sequencing—of the *env1* transcripts from the dog and cat placenta using appropriate primers (*Methods* and Fig. 6*B*). Close inspection of the flanking sequences also unambiguously shows that insertion of the ancestral provirus occurred within a LINE retroelement and was of the retroviral type, with evidence for a target site duplication of 4 nt (as shown on the consensus sequence derived from the three loci), a common feature of retroviruses (23, 25, 26) (Fig. 6*C*).

As shown in Fig. 6*A*, the dog, cat, and giant panda proviruses are integrated within an intron of the putative *TBC1D19* gene in the antisense orientation as commonly observed for endogenous retroviruses (25, 26) (Fig. 6*A*). Moreover, high sequence homol-

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Dog           MTLFKPFIFLNLLLSYLSLGKANSPNLFLQWAQHYAESLQKSSCWICGLLPLSSSSGLPW 60
Giant panda   MVLFKSFTFLNLLFPYLYLGKATGSNVFLQWAQHYADSLQKSSCWVCGLLPLSSTSGLPW 60
Cat           MKLLKSFTFLSLLFPYLALGKLNGPNLFLQWAQNYADKLQKSSCWICGLLPLSSTSGLPW 60
              * *:* * ** **: ** ***    *:******:**: *******:********:*****

Dog           WVSPLQGTDWHYLHQYIKDMVIQPNTSIYRDISITNSNVSHWPMVNATWNSPGHRKPFSF 120
Giant panda   WVSPLQGTDWHYLHQYIKDMVFHPNSSIYRDISITNKDVSCWPMVNTTWNSPGHKRHFSY 120
Cat           WVSPLQGTDWHYLCQYIEDMVFSPNTSIYSDISITNRKVSYWPMVNTTWNSPGHERPFSY 120
              ************* ***:***: **:*** ******  ** *****:******* : **:

Dog           LQTAKIMTEFANSKIQSKKALWGISKTSNMRYTEDGYFQVWDEFMWLTPTIGQLNQKALL 180
Giant panda   PQTTKIMTEYANSKIES-KALWGTTKPDNMRITQDGYFQVWDEFMWLTPTIGQLNQKALL 179
Cat           TQTAKIMNEYANANIENKRALWGTMKPNNMRYTDDGYFQVWDEFMWLTPTIGHLNQRALL 180
               **:*** *:**::*:  :****  *  *** *:******************:***:***

Dog           CWEQRNHTYDAWPNSTRELGWLSSRACNHIIILQATDWFATDWIRRPSIRWLAPNGTQWL 240
Giant panda   CWEQRNHTYDAWPNSTRKLGWLSPGACTHIIILQATDWFATDWIRRPGIRWLAPNGTQWL 239
Cat           CWEQRNHTYDAWPNSTRELGWLSQRDCTHTIVLQATDWFATDWFRRPSIRWLAPNGTQWL 240
              *****************:*****   * * *:***********:*** ************

Dog           CGTNLWPWLPPGWIGRCTLGFPWMQGKLRKEISMPANYPHLLHRWTRSVFHWYDHLVGLF 300
Giant panda   CGTNLWPWLPPGWIGRCTLGFPWMQGTLRNKLPTPANYPYLLHRWTRSVFHWYDHLLGIL 299
Cat           CGANLWPWLPPGWIGRCTLGFPWMQGSWHKEIPKPANYPYLLHRWTRSVFHWYDHLLSIL 300
              **:*********************  ::::  *****:****************: ::

Dog           LPQLGIEDIIWHVEALANYTTHALNDTMHALVLLNSEVALMRKAVLQNRMALDIITAAQG 360
Giant panda   LPQLGMEDIIWHVEALTNYTTRALNDSMHAISLLNSEVALMRMAVLQNRMALDVITAAQG 359
Cat           VPQLGIEDIIWHVEALTNYTTKALNDSMRGLSLLNSEVTLMRKAVLQNRMALDILTAAQG 360
              :****:**********:****:****:*: : ******:*** **********::*****

Dog           GTCAIIKTECCVYIPDNSANVSELLTDMHTQIEAMSDSSGSLNDWISSWYGGNGWSWWKK 420
Giant panda   GTCAIIKSECCVYIPDNSANVSKLLSDMHVQIEGMSDTSSSLNDWISSWFSGNGWSWWKK 419
Cat           GTCAIVKTECCVYIPDSSANISDVLADMRTQVEAMSDASGSLNDWISSWSSGGIWAWWKK 420
              *****:*:********* ***:* :*:**: *:* ***:* *********  *  *:****

Dog           LLLLIVTVVTLGLLLCCGSFCCCVFCINMSEKLSEQVFSGRRSILRIRPEEHL 473
Giant panda   LLLLAVTVVLLGLFLCCGSYCCCILCMNMSEKLTERIFSRRRSII-IRPHEHL 471
Cat           LLLLAIAVVMLAFFLCCGGYCCCMLCMNMSEKLSERVLFRSHFIPRLKPHVHL 473
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signal peptide
SU
furin cleveage site
fusion peptide
immuno-suppressive domain
TM
transmembrane domain

Fig. 5. Primary sequences and alignment of Canis-Env1, Felis-Env1, and Ailur-Env1. Primary amino acid sequence and characteristic structural features of the homologous Env protein from dog, cat, and giant panda. Same color code and abbreviations as in Fig. 3; asterisks indicate amino acid identity, and colons indicate amino acid similarity.

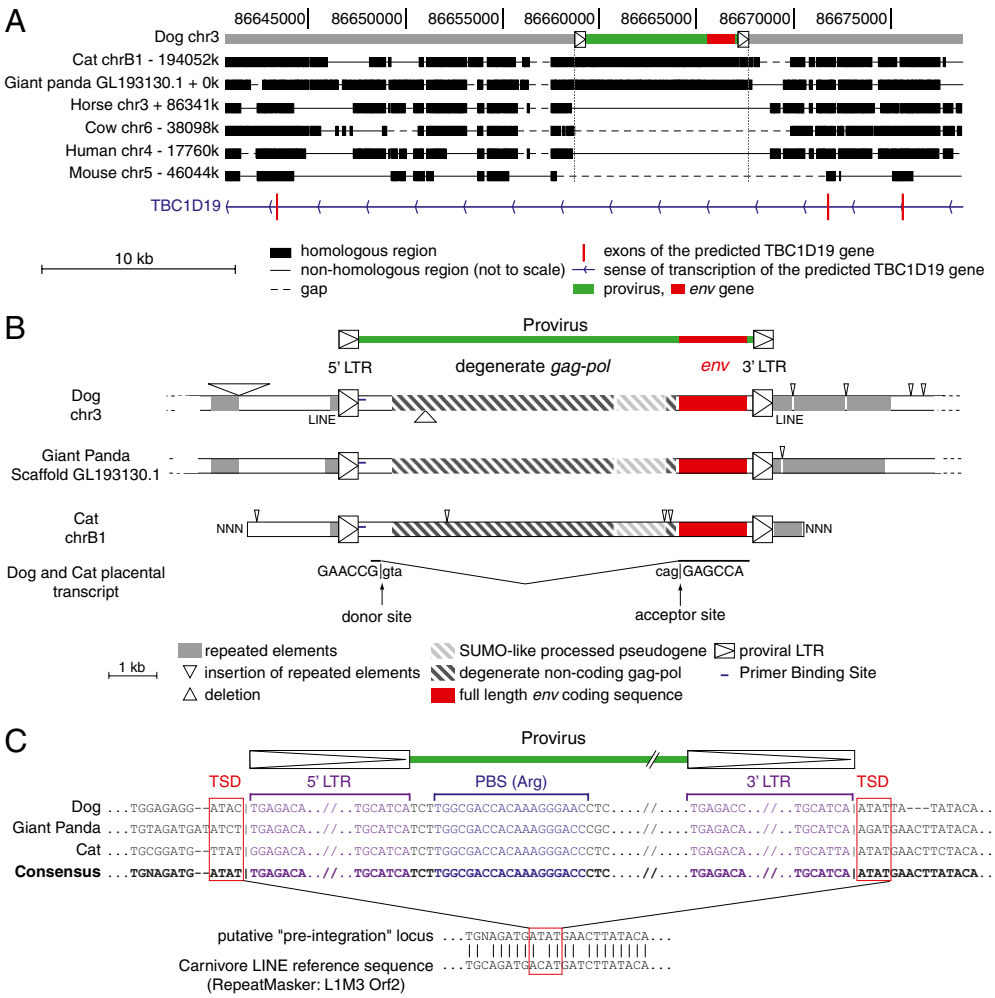


Fig. 6. Characterization of the dog, cat, and giant panda *env1*-containing endogenous retrovirus and its integration site. (*A*) Evidence for orthology of the dog, cat, and panda sequences. The *canis-env1*–containing provirus (shown in green with its LTRs schematized by boxed triangles and the *env* gene shown in red) was used as a reference, and synteny between the dog, cat, giant panda, horse, cow, human, and mouse genomes was determined with the Comparative Genomic tool of the UCSC Genome Browser (http://genome.ucsc.edu/); the position of exons (vertical red lines) of the resident *TBC1D19* gene and the sense of transcription (blue arrows) are indicated. Homologous regions are shown as black boxes, non-homologous regions are shown as thin lines (not to scale), and gaps are shown as dotted lines. (*B*) Structure of the corresponding proviruses and integration sites. Homologous regions common to all three sequences are aligned, with the insertions and deletions represented by triangles placed above and below each sequence, respectively; repeated mobile elements (dark gray) as identified by the RepeatMasker website program are positioned. The proviral LTRs, the degenerate *gag-pol* gene, and the *env* gene coding sequence are indicated together with the SUMO-like processed pseudogene inserted within the provirus (the symbols used are given below the panel). Splice sites for the *env* subgenomic transcript as determined by RT-PCR from dog and cat placental RNA are indicated together with the donor and acceptor site sequences. (*C*) Sequence alignment of proviral and flanking sequences: evidence for retroviral integration. Characteristic sequences are shown, including LTRs (purple with TG . . . CA borders), primer binding site (blue) for an arginine tRNA, and target site duplication (red boxes). Sequence alignment of the reconstituted, putative pre-integration site with the Carnivore LINE L1M3 Orf2 (prototypic reference sequence from the RepeatMasker website) provides evidence for precise proviral integration into a preexisting ancestral LINE.

ogy of the sequences flanking the proviruses (pairwise percentage of identity comprised between 78% and 82% over 1 kb of the 5′ flanking region and between 59% and 67% over 1 kb of the 3′ flanking region for the three proviruses) as well as the presence of a SUMO-like pseudogene inserted at the same position within all three proviruses (Fig. 6*B*) confirm that they are strict orthologous copies of the same gene in the dog, cat, and giant panda genomes. Therefore, the ancestral provirus integrated within the genome of Carnivora before the dog–cat–giant panda divergence. A PCR assay with the forward primer located within the provirus at the 5′ end of the *env* gene and the reverse primer located downstream of the provirus in the 3′ flanking sequence resulted in a single amplification product with a conserved size, confirming orthology of the genes in all three species (see below). Accordingly, the Carnivora gene corresponding to these three orthologous genes will now be referred to as *env-Car1*, whereas *canis-env-Car1*, *ailur-env-Car1*, and *felis-env-Car1* will refer to the dog, giant panda, and cat representatives, respectively.

Env-Car1 Is a Fusogenic Retroviral Envelope Protein. The functionality of Env-Car1 as an ancestral, retrovirally derived fusogenic envelope protein was assayed ex vivo as previously described for other syncytins (6, 9). Basically, it was tested whether this envelope protein added *in trans* could render a recombinant retrovirus—deprived of its own native *env* gene—able to mediate infection of test target cells (Fig. 7*A*, scheme). To do so, the amplification products of *canis-env1* and *felis-env1* were cloned into a CMV promoter-containing expression vector (*Methods*), and plasmids with a full-length *env* gene ORF 100% identical to the genomic sequences were assayed. As expected for an envelope protein of retroviral origin, both Canis-Env1 and Felis-Env1 can produce infectious particles. As illustrated in Fig. 7*B*, pseudotypes generated in human 293T cells with an HIV core are able to infect the neuroblastoma-derived G355.5 cat cell line and the epithelial kidney-derived A-72 dog cell line, in both cases with a high infectious titer ($4.4 \pm 1.9 \times 10^4$ focus forming unit [ffu] /mL in G355.5 and $1.8 \pm 1.1 \times 10^5$ ffu/mL in A-72 for Canis-Env1 and $1.1 \pm 0.7 \times 10^5$ ffu/mL in G355.5 and $2.2 \pm 0.7 \times 10^5$ ffu/mL in A-72 for Felis-Env1). Of note, as classically observed for most retroviral envelope proteins, not all cells could be infected with identical efficiency, even among cells from the same animal species (Fig. 7*C*). This finding is illustrated, for instance, by the embryonic kidney-derived MDCK dog cell line, which shows a close to 100-fold reduction in infectivity of both the Canis-Env1 and Felis-Env1 pseudotypes, possibly associated with poor expression of the cognate receptor for this ancestral

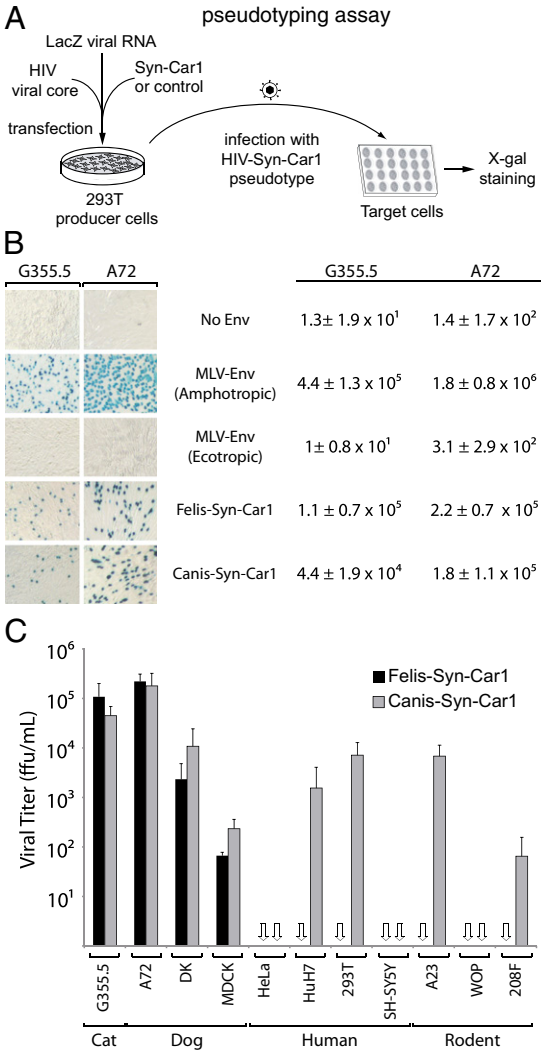


Fig. 7. Syncytin-Car1 is a fusogenic retroviral envelope protein. (*A*) Schematic representation of the assay for cell infection with Syncytin-Car1–pseudotyped virus particles. Pseudotypes are produced by cotransfection of human 293T cells, with expression vectors for the HIV-1 core, Syncytin-Car1 proteins (or control retroviral envelope proteins or an empty vector), and β-Gal encoded by a *LacZ*-containing retroviral transcript. Productive cell supernatants are then assayed for infection of the indicated target cells, which are X-gal–stained 3 d postinfection. Syn-Car1: Syncytin-Car1. (*B*) X-gal–stained target cells (*Left*) and viral titers (*Right*) for particles pseudotyped with Felis-Syn-Car1, Canis-Syn-Car1, or control Env proteins from murine leukemia viruses of either the ecotropic (infecting only murine cells) or the amphotropic (infecting both murine and nonmurine cells) subtype. Target cells were cat (G355.5) and dog (A72) cells. Values (focus forming unit per milliliter ± SD) are the means from three independent experiments. (*C*) Viral titers for particles pseudotyped with Felis-Syn-Car1 or Canis-Syn-Car1 assayed on a panel of target cells from cat (G355.5), dog (A72, MDCK, and DK), human (HeLa, HuH7, 293T, and SH-SY5Y), or rodent (A23, WOP, and 208F). Titers are corrected for the background values of control particles without an envelope protein, and they are the means from at least two independent experiments (focus forming unit per milliliter ± SD; open arrows indicate null values).

retroviral envelope protein on this cell line. Interestingly, it can also be observed that the human HeLa and SH-SY5Y as well as the murine WOP cell lines cannot be infected by either pseudotype, whereas other cell lines—such as human 293T and HuH7 or rodent A23 and 208F—can be infected, at least by Canis-Env1. This finding strongly suggests that a human or rodent receptor—with variable levels of expression depending on the cell type—can substitute to some extent to the carnivore receptor to mediate pseudotype cell entry, although it has to be noted that it seems not to be efficient in mediating Felis-Env1 pseudotype cell entry. This difference could be because of a subtle divergence in the expected parallel coevolution of the captured *env-Car1* gene (that can now be named *syncytin-Car1*) and its cognate—but still unknown—receptor.

In Situ Hybridization on Placenta Sections. The placenta of Carnivora is of the endotheliochorial type (27–29). As schematized in Fig. 8*A*, *Left*, it is composed, from fetus to mother, of the labyrinthine zone where fetal and maternal villi are interspersed, the junctional zone where the fetal villi are invading the maternal tissue, and the maternal uterine glandular zone and myometrium. At the tip of the invasive fetal villi, cytotrophoblast cells migrate and differentiate into a multinucleated syncytial layer, the syncytiotrophoblast, by cell–cell fusion (Fig. 8*A*, *Right*). This syncytiotrophoblast is highly invasive and phagocytic: it destroys the maternal epithelium and surrounds the maternal vessels, thus forming the endotheliochorial pattern of the maternofetal interface. At variance with human and mice, the syncytiotrophoblast in Carnivora does not disrupt the maternal vessels.

To further assess the physiological relevance of the specific *syncytin-Car1* expression in the placenta, which is revealed by the qRT-PCR assays in Fig. 4, in situ hybridization experiments were performed on paraffin sections of dog and cat placentae. Specific digoxigenin-labeled antisense riboprobes were synthesized for the detection of the *syncytin-Car1* transcripts from each species as well as the corresponding sense riboprobes to be used as negative controls. As illustrated in Fig. 8, specific labeling was observed only with the antisense probes and not with the control probes. In the dog, *syncytin-Car1* expression is restricted to the junctional zone at the tip of the villi in areas where placental cells invade the maternal tissues (Fig. 8 *C* and *E*). In the cat, the junctional zone is thinner, and specific labeling is observed on the entire villi (Fig. 8 *I* and *K*). Higher magnification images also show that *syncytin-Car1* is expressed, for both the dog and cat, at the surface of the fetal villi. Expression is detected at the level of the multinucleated syncytiotrophoblast interface between the fetal mononucleated cytotrophoblasts and the maternal vessels (Fig. 8 *G* and *M*). Such a labeling is consistent with a role for the fusogenic Syncytin-Car1 in the formation of the syncytiotrophoblast.

Identification of Syncytin-Car1 in All Carnivora Suborders. Phylogenetic relationships in the Carnivora order are illustrated in Fig. 9 (adapted from refs. 16, 21, and 30). This order includes two major suborders, the Caniformia and the Feliformia, with the dog and cat as reference species, respectively. Each group comprises a series of families as indicated in Fig. 9. To further characterize *syncytin-Car1* and determine its status and evolution in Carnivora, DNA from at least one representative species within each family was recovered to tentatively determine the presence, sequence, and functional properties of the gene. Locus-specific pairs of PCR primers (forward primer in the provirus upstream of *syncytin-Car1* and reverse primer downstream of the provirus in the 3′ flanking sequence) (Table S1) were used to amplify genomic DNA from 26 representative species. In all cases, PCR amplification showed a single amplification product with a conserved size, strongly suggesting the presence of the orthologous *syncytin-Car1* in all carnivores. This finding was confirmed by sequencing the PCR products (sequences were deposited in GenBank, and accession numbers are listed in Fig. 9), which revealed the presence of a *syncytin-Car1* gene encoding a full-length ORF (471–476 aa long) in all of the carnivores tested.

The closest outgroup for Carnivora is the Pholidota order (comprising the pangolin species), but no representatives from this group have been sequenced to date. The presence of *syncytin-Car1* in this order was, therefore, similarly assayed by PCR with the genomic DNA from two distinct pangolin species

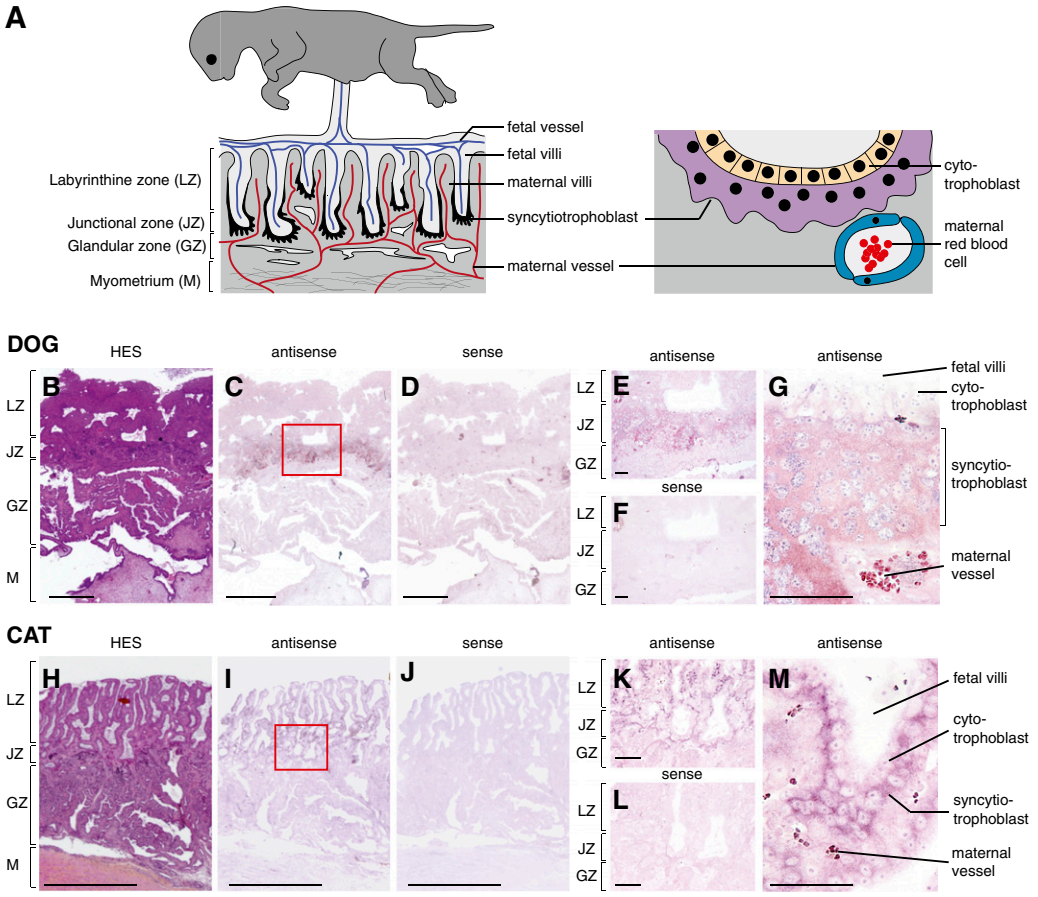
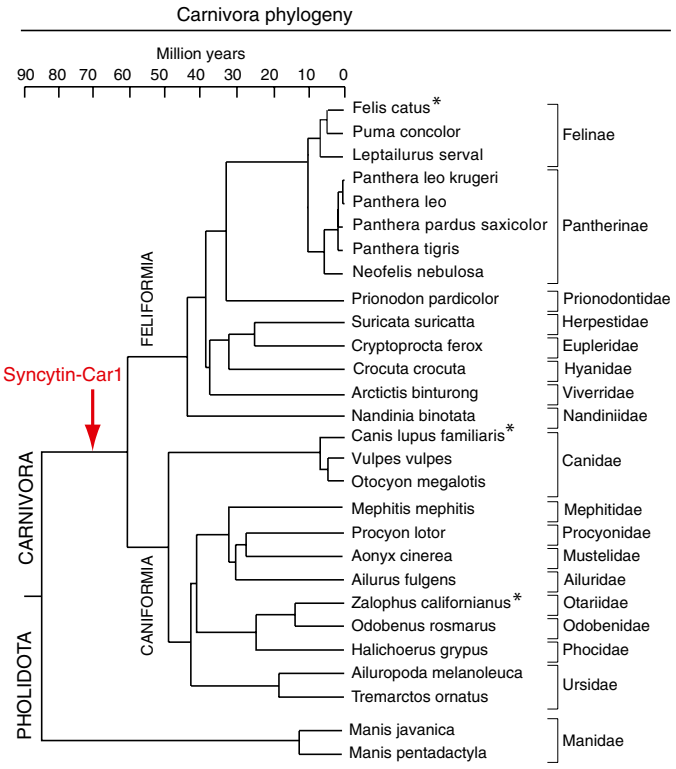


Fig. 8. Structure of the Carnivora placenta and in situ hybridization for *syncytin-Car1* expression on dog and cat placental sections. (*A*) Schematic representation of the Carnivora placenta (*A Left*) with, from mother to fetus, the myometrium (M), the glandular zone (GZ), the junctional zone (JZ), and the labyrinthine zone (LZ). The maternal and fetal vessels are schematized, with the invasive syncytiotrophoblast at the fetomaternal interface colored in black at the tip of the invading fetal villi. An enlarged view (*A Right*) shows the multinucleated syncytiotrophoblast (purple) generated by fusion from the underlying mononucleated cytotrophoblast cells (yellow) forming the interface between the fetal compartments and the maternal vessels (with the red blood cells schematized). Sections of placenta from dog (*B–G*) and cat (*H–M*) at midgestation. (*B* and *H*) Hematoxylin–eosin–saffron staining (HES) of the placental section with the four layers indicated. (*C–G* and *I–M*) In situ hybridization on serial sections observed at different magnifications using digoxigenin-labeled antisense (*C*, *E*, *G*, *I*, *K*, and *M*) or sense (negative control; *D*, *F*, *J*, and *L*) riboprobes revealed with an alkaline phosphatase-conjugated antidigoxigenin antibody. (*E*, *F*, *K*, and *L*) Intermediate magnification of the junctional domain delineated in *C* and *I* (red boxed areas). (*G* and *M*) Higher magnification with the fetal villi, the labeled syncytiotrophoblast, and the maternal vessels clearly visible. (Scale bars: *B–D* and *H–J*, 1 mm; *E*, *F*, *K*, and *L*, 100 μm; *G* and *M*, 50 μm.)

(*Manis javanica* and *Manis longicaudata*). Both were found negative (Fig. 9). The absence of *syncytin-Car1* was confirmed using PCR primers internal to the *syncytin-Car1* ORF placed at positions where all previously sequenced Carnivora genes showed a strictly identical nucleotide sequence. Again, PCRs with the pangolin DNA were negative under conditions where DNAs from all of the carnivores in Fig. 9 were positive. Although we cannot formally exclude that pangolin sequences may be too divergent to allow primers annealing and PCR amplification and despite the fact that we could not amplify the empty locus in this species (a rather expected issue taking into consideration that integration in Carnivora was within a LINE mobile element, not necessarily present at the same locus in pangolins), the data strongly suggest that *syncytin-Car1* entered the identified locus after the divergence between Carnivora and Pholidota (i.e., 85 Mya). Analyses using the comparative tool from the UCSC Genome browser (http://genome.ucsc.edu/) confirmed the absence of *syncytin-Car1* at the syntenic locus of the available horse, cow, human, and mouse genomes. Taking into consideration that the radiation of Carnivora occurred 60 Mya, the presence of a proviral integration at the same locus in all of the carnivores tested indicates that the provirus entered the genome of the common Carnivora ancestor between 60 and 85 Mya.

Purifying Selection and Functional Conservation of *syncytin-Car1* in Carnivora. Sequence analysis of the 26 *syncytin-Car1* Carnivora genes shows high sequence similarities ranging from 74% to 99% (Fig. 10, *Right*), which was expected for a bona fide cellular gene. Interestingly, the *syncytin-Car1*–based phylogenetic tree that can be generated from the aligned sequences using the neighbor-joining method (Fig. 10, *Left*) is strongly congruent with the Carnivora phylogenetic tree in Fig. 9 (comparison in Fig. S2), with the Feliformia and Caniformia sequences branching into two distinct monophyletic groups. Closer examination only reveals minor differences within these groups, which actually correspond to nodes with low bootstrap values.

To further characterize the conservation/evolution of the *syncytin-Car1* gene among Carnivora, analysis of the non-synonymous to synonymous mutation (dN/dS) ratio between all pairs of species sequences was performed using the method in the work by Nei and Gojobori (31). Accordingly, the entire *env* gene shows purifying selection between all pairs of species, with dN/dS ratios comprised between 0.02 and 0.77 (Fig. 10, *Right*).



ORF (aa)	Fusion activity	Accession Number
473	+	JN587095
473	+	JN587107
473	+	JN587097
473	+	JN587104
473	+	JN587103
473	+	JN587105
473	+	JN587106
473	+	JN587100
473	+	JN587108
475	+	JN587110
474	n.d.	JN587094
474	+	JN587093
473	+	JN587091
476	+	JN587099
473	+	JN587092
473	+	JN587112
473	+	JN587102
473	+	JN587098
473	+	JN587109
472	+	JN587090
472	+	JN587089
473	+	JN587113
473	+	JN587101
473	n.d.	JN587096
471	+	JN587088
471	+	JN587111
—	n.r.	n.r.
—	n.r.	n.r.

Fig. 9. Entry date and conservation of *syncytin-Car1* in the Carnivora radiation. (*Left*) Carnivora phylogenetic tree with the Pholidota outgroup indicated. Adapted from refs. 16, 21, and 30. Horizontal branch length is proportional to time (scale bar at the top). The names of the 26 Carnivora species (from both the Feliformia and Caniformia suborders) tested for the presence of the *syncytin-Car1* gene are indicated together with the names of their corresponding families. (*Right*) The length (in amino acids) of the Syncytin-Car1 proteins that were identified for each species and the accession number for the sequences that were deposited in GenBank are indicated; the fusogenic activity for each cloned gene, as determined by the pseudotyping assay described in Fig. 7, is provided (n.d., not determined). For the two *Manidae* tested (from the Pholidota Order), *syncytin-Car1* was not detected (n.r., not relevant), thus dating back acquisition of the gene between 60 and 85 Mya. Asterisks indicate species where placenta-specific expression could be shown (Figs. 4 and 8 for dog [*C. lupus familiaris*] and cat [*F. catus*], and Fig. S1 for sea lion [*Zalophus californianus*]).

This finding is what is classically found for cellular genes with a physiological role in which nonsynonymous mutations are strongly selected against. However, because *syncytin-Car1* has a retroviral origin, it could be asked whether, as observed, for instance, for the HIV *env* gene (32), some specific domains of the *syncytin-Car1* gene could still display some variability (i.e., be under positive selection). To answer this question, we performed a more refined analysis of the sequences using methods to assay site-specific selection (33, 34). These methods use a continuous time Markov chain process, allowing the dN/dS ratio to vary between codons. Such an analysis, using the PAML package (35), provided support for a model (model M8 vs. model M7: χ^2 = 12.2, degree of freedom = 2, *P* value = 2.2e-3) in which most of the codons are under strong purifying selection (1e-4 ≤ dN/dS ≤ 0.82, 77% of the codons), a few codons are under nearly neutral selection (dN/dS = 0.97, 8.5% of the codons), and some codons are under weak positive selection (dN/dS = 1.6, 14.5% of the codons). However, no definite amino acid but one (Fig. S3) and no specific domain can be identified with a dN/dS value significantly higher than unity, suggesting that positive selection, if any, is weak and that *syncytin-Car1* is mainly under strong purifying selection. Analyses using the HyPhy package (34) with slightly different site-specific models (random effect likelihood and fixed effect likelihood) or a branch-specific model (GA branch), which allowed the dN/dS value to vary among the branches, lead to similar conclusions (Fig. S3). *Syncytin-Car1* genes are, therefore, mainly under strong purifying selection, although one cannot definitely exclude that few events of positive selection may have occurred. In the case of HIV, several domains of the *env* gene have been shown to be subject to positive selection (e.g., the variable regions of the SU subunit), with mutations in these domains favoring virus escape from the host immune response. Clearly, *syncytin-Car1* behaves differently, a result consistent with this gene having acquired a cellular gene status and consequently, having lost the high mutation rate of a replicating retrovirus. Altogether, the data strongly suggest that *syncytin-Car1* is now a bona fide cellular gene co-opted by Carnivora for a physiological role in placentation.

To determine whether the strong selective pressure shown for the *syncytin-Car1* gene among Carnivora indeed correlates with conservation of its functional properties, an ex vivo assay for its fusogenic activity, which is illustrated in Fig. 7 for the dog and cat representatives, was finally performed. The PCR-amplified *syncytin-Car1* genes in Fig. 9 were cloned into the same eukaryotic expression vector, and pseudotypes were similarly assayed using either cat (G355.5 neuroblastoma cells) or dog (A-72 epithelial kidney cells) cells as the target. As indicated in Fig. 9, all of the 24 tested *syncytin-Car1* genes were found positive.

Discussion

Here, we have identified *syncytin-Car1*, the *env* gene from an endogenous retrovirus that has integrated into the genome from a common ancestor of Carnivora before the radiation of this order more than 60 Mya (16, 21), as the most ancient gene of the *syncytin* group identified to date. This gene has been maintained as a functional retroviral *env* gene since that time, being conserved in all of the 26 species tested, which were selected among the known Carnivora families. This gene displays all of the canonical characteristics of a *syncytin* gene. (*i*) It exhibits fusogenic activity, because it can functionally replace a present day retroviral *env* gene within a recombinant infectious retrovirus. (*ii*) It has been subject to purifying selection in the course of evolution, displaying low rates of nonsynonymous to synonymous substitutions and full conservation of its fusogenic property. (*iii*) It is specifically expressed in the placenta, which was evidenced by both RT-PCR analyses and in situ hybridization of cat and dog placental tissue sections. The in situ hybridization experiments using *syncytin-Car1* sequences as a probe clearly show that expression takes place at the level of the invading fetal villi, consistent with a direct role of this fusogenic *syncytin* gene in syncytiotrophoblast formation. *Syncytin-Car1* adds to the two primate *syncytin-1* and *-2* genes first identified (1, 2, 5), the two *syncytin-A* and *-B* genes later found in Muridae (8), and the

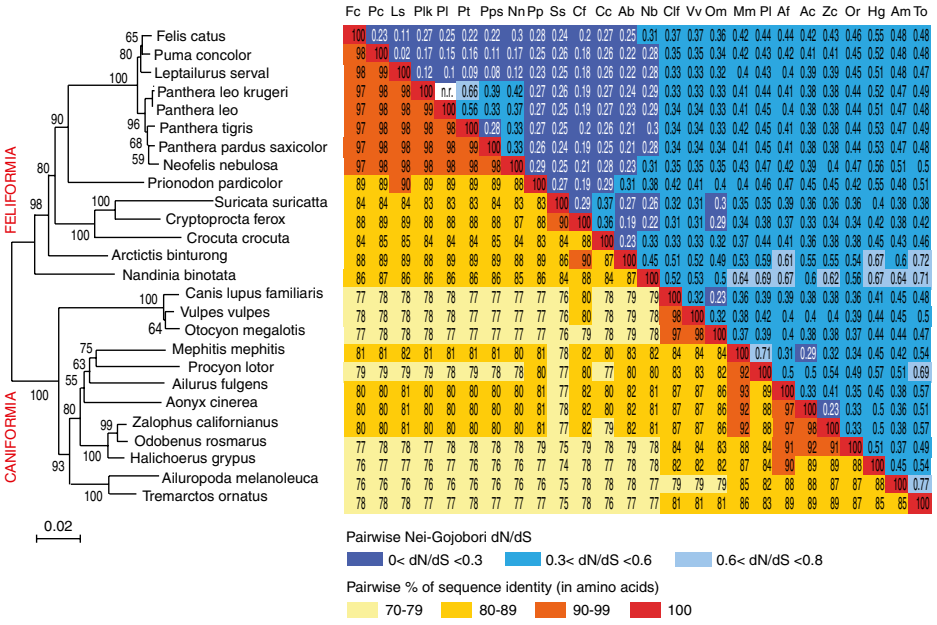


Fig. 10. Sequence conservation and evidence for purifying selection of *syncytin-Car1* in Carnivora. (*Left*) Syncytin-Car1–based phylogenetic tree determined using amino acid alignment of the Syncytin-Car1 proteins identified in Fig. 9 by the neighbor-joining method. The horizontal branch length and scale indicate the percentage of amino acid substitutions. Percent bootstrap values obtained from 1,000 replicates are indicated at the nodes. (*Right*) Double-entry table for (lower triangle) the pairwise percentage of amino acid sequence identity between the *syncytin-Car1* gene among the indicated species and (upper triangle) the pairwise Nei–Gojobori (30) dN/dS; dN/dS between *Panthera leo* and *P. leo krugeri* could not be determined (only one nonsynonymous and no synonymous mutation between both species; n.r. not relevant). A color code is provided below the table for both series of values.

syncytin-Ory1 gene recently described in Leporidae (9). Importantly, all six *syncytins* are unrelated and correspond to independent captures in separate mammalian lineages of genes of retroviral origin. In the case of the *syncytin-A* gene, KO mice unambiguously showed that it is absolutely required for placentation, with evidence for a defect in syncytiotrophoblast formation causing embryonic lethality in the mutants (10). It, therefore, can be proposed that the other identified *syncytins* as well as the newly discovered Carnivora *syncytin-Car1* are likely to play a similar role in placentation by being involved in syncytiotrophoblast formation. This finding would be consistent with the Primate, Muridae, and Leporidae placentae being of the hemochorial type, where an extended multinucleated syncytial layer forms the interface between maternal blood spaces and fetal vessels (36–38). In the case of Carnivora, placentation is of the endotheliochorial type, and syncytiotrophoblast formation also takes place at the level of the invading fetal trophoblast cells, although in that case, invasion does not result in disruption of the maternal vessels (27–29).

An important outcome of the present investigation is that the discovery of *syncytin-Car1* extends the presence of *syncytin* genes outside the Euarchontoglire clade of placental mammals, where previously identified *syncytins* had been found, to include members from the highly diverse Laurasiatheria. This clade contains—among others and in addition to Carnivora—the Ruminantia (cow and sheep), the Suina (pig), and the Perissodactyla (horse), and it diverged from the Euarchontoglires 100 Mya (Fig. 1) (16). It, therefore, clearly shows that *syncytin* gene capture has been a widespread process, which finally turns out to have taken place in several widely separate lineages in the course of eutherian evolution. In this respect, it should be emphasized that, in the course of our search for *syncytins* in mammals, in every species investigated up to date, at least one such gene could be found. It is, therefore, likely that *syncytins* are a common feature of placental mammals, and search for their presence in other Laurasiatheria orders, as well as in the two other Xenarthra and Afrotheria clades, is of prime interest. A specific issue should concern (among other Laurasiatheria) the Suina and Perissodactyla, because they display a third type of placentation—the epitheliochorial type—where the fetal and maternal tissues are simply apposed (39–41) without evidence for syncytiotrophoblast formation, thus raising the question of the presence and possible role of *syncytins* in these species besides cell fusion. In the case of ruminants, an intermediate type of placentation, the synepitheliochorial type, is also observed, with syncytiotrophoblast formation occurring only to a limited extent (42, 43). Accordingly, it is tempting to hypothesize that the large variability in placental structures that can be observed simply results from the diversity of the *syncytin* genes that have been stochastically captured in the course of mammalian evolution and that parameters, such as the intrinsic level of fusogenicity of the Env proteins, the presence of the receptor required for Env-mediated fusion at the surface of the right neighboring cell, and the specific regulation of *syncytin* expression in the appropriate tissues, control and finely tune the placentation process. In this respect, the presently identified Carnivora *syncytin* with its cloning in a large number of carnivore species should help in understanding the subtle differences in placental structures observed among them, especially after the cognate receptor for the Syncytin-Car1 protein will be identified and analyzed for its species-specific interactions.

Methods

Database Screening and Sequence Analyses. Retroviral *env* gene sequences were searched by using the BioMotif program (http://www.lpta.univ-montp2.fr/users/menes/bioMotif_html_doc/ref_Run.html) with the degenerate modified CKS17 consensus motif [(LIYIFIPIWIAISIM) (QINIEID) N XX (GIAIDIMIVIC) (LIPIIV) (DIHINIGIV) X (LIFIPISIVITII) XXXX (GIDIKIEIS) (GIEISIRIH) X C] as a query and the getorf program from the EMBOSS package (http://emboss.sourceforge.net/apps/cvs/emboss/apps/getorf.html) to identify coding sequences (from start to stop codons) >1 kb. We made use of the available domestic dog and cat genome sequences (7.6× coverage assembly of the *C. lupus familiaris* genome [UCSC Broad/canFam2, May of 2005] and 2.8× coverage assembly of the *F. catus* genome [UCSC, catChrV17e, December of 2008], respectively). The identified *env* coding sequences (Fig. 3) coordinates are listed in *SI Methods*.

The dog, cat, and giant panda genomes were screened with the identified envelope glycoprotein sequences using the BLAST programs from the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/BLAST). Multiple alignments of amino acid sequences were carried out by using the Seaview program under ClustalW protocol. Phylogenetic trees were constructed from manually corrected alignments by neighbor-joining with 1,000 replicates using the Seaview program (44).

PAML4 (35) was used to run site-specific selection tests and obtain dN/dS ratios for all *syncytin-Car1* sequences. PAML models analyzed assumed no molecular clock (clock = 0) and a single dN/dS for all tree branches (model = 0), and we used likelihood ratio tests to compare the improvement in likelihood for a model (M8), allowing for positive selection compared with a model (M7; NS site = 7–8) that does not. Each analysis ran until conver-

gence (Small_Diff = 0.5e-6), and the control file is available on request. HyPhy (34) was used on the datamonkey webserver (www.datamonkey.org) to run site-specific Random Effect Likelihood (REL) and Fixed Effect Likelihood (FEL), and branch-specific GA-branch selection tests. The best branch-specific model was selected using the Akaike Information Criterion.

The horse genome (*Equus caballus*, 6.79× coverage, UCSC Broad/equCab2, 2007), the cow genome (*Bos taurus*, 7.1× coverage, UCSC Baylor 4.0/bosTau4, 2007), the human genome (*Homo sapiens*, UCSC GRCh37/hg19, 2009), and the mouse genome (*Mus musculus*, UCSC NCBI37/mm9, 2007) assemblies were also screened for the presence of the identified *syncytin-Car1* containing provirus sequence using syntenic genomic regions from the UCSC genome browser (http://genome.ucsc.edu/).

Search for *syncytin-Car1* in Other Species. PCRs were performed on 100 ng genomic DNA using Accuprime Taq DNA Polymerase (Invitrogen) for 40 cycles (30 s at 94 °C, 30 s at 50 °C, and 2 min at 68 °C). All genomic DNAs from carnivores were amplified with primers upstream to the syncytin-Car1 ORF within the provirus and downstream to the 3′ LTR of the provirus within the flanking region. All genes could be amplified with the same primers except for *Ailurus fulgens* and *Mephitis mephitis*, where the reverse primer was different (all primers listed in Table S1). *Manis longicaudata* and *Manis javanica* genomic DNAs were tentatively amplified with primers conserved in the dog and cat sequences either close to the start and stop codons of the *syncytin-Car1* ORF (within the provirus) (Table S1, ORF primers) or external to the provirus (Table S1, locus primers) together with primers internal to the ORF (Table S1, internal primers) and conserved among all sequenced *syncytin-Car1* genes. PCR products were directly sequenced without cloning to avoid low-level mutations introduced by PCR.

Biological Samples, RT-PCR, In Situ Hybridization, and Infection Assays. See *SI Methods*.

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