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GENETIC DIVERSITY AND EVOLUTION

Capture of a Hyena-Specific Retroviral Envelope Gene with Placental Expression Associated in Evolution with the Unique Emergence among Carnivorans of Hemochorial Placentation in Hyaenidae

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ABSTRACT Capture of retroviral envelope genes from endogenous retroviruses has played a role in the evolution of mammals, with evidence for the involvement of these genes in the formation of the maternofetal interface of the placenta. It has been shown that the diversity of captured genes is likely to be responsible for the diversity of placental structures, ranging from poorly invasive (epitheliochorial) to highly invasive (hemochorial), with an intermediate state (endotheliochorial) as found in carnivorans. The latter recapitulate part of this evolution, with the hyena being the sole carnivoran with a hemochorial placenta. In this study, we performed RNA sequencing on hyena placental transcripts and searched for endogenous retroviral envelope genes that have been captured specifically in the Hyaenidae clade and are not found in any other carnivoran. We identified an envelope gene that is expressed in the placenta at the level of the maternofetal interface, as evidenced by *in situ* hybridization/immunohistochemistry. The gene entry is coincidental with the emergence of the Hyaenidae clade 30 million years ago (Mya), being found at the same genomic locus in all 4 extant hyena species. Its coding sequence has further been maintained during all of Hyaenidae evolution. It is not found in any of the 30 other carnivorans—both Felidae and Canidae—that we screened. This envelope protein does not disclose any fusogenic activity in *ex vivo* assays, at variance with the *syncytin-Car1* gene, which is found in all carnivorans, including the hyena, in which it is still present, transcriptionally active in the placenta, and fusogenic. Together, the present results illustrate the permanent renewal of placenta-specific genes by retroviral capture and *de facto* provide a candidate gene for the endotheliochorial to hemochorial transition of Hyaenidae among carnivorans.

IMPORTANCE The placenta is the most diverse organ among mammals, due in part to stochastic capture of retroviral envelope genes. In carnivorans, capture of *syncytin-Car1* took place 80 Mya. It is fusogenic, expressed at the syncytialized placental maternofetal interface, and conserved among all carnivorans, consistent with their shared endotheliochorial placenta. Hyenas are a remarkable exception, with a highly invasive hemochorial placenta, as found in humans, where disruption of maternal blood vessels results in maternal blood bathing the syncytial maternofetal interface. In this study, we identified a retroviral envelope gene capture and exaptation that took place about 30 Mya and is coincident with the emergence of the Hyaenidae, being conserved in all extant hyena species. It is expressed at the maternofetal interface in addition to the shared *syncytin-Car1* gene. This new *env* gene,

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not present in any other carnivoran, is a likely candidate to be responsible for the specific structure of the hyena placenta.

KEYWORDS endogenous retrovirus, envelope protein, syncytin, placenta, endotheliochorial/hemochorial, RDR/ASCT2/SLC1A5, receptor, hyena, Carnivora

Infection of vertebrate species by retroviruses has been taking place for at least 420 million years (My) (1). Over the course of this vast time span, sequences of retroviral origin have accumulated in animal genomes. In the present day, the fraction of the genome occupied by retroviral sequences represents up to 10%, as is the case in mice for example (8% in humans) (reviewed in references 2 and 3). Since expression of viral proteins has a neutral to deleterious effect for the host, most of these endogenous retroviral (ERV) sequences have since degenerated, presenting disrupted open reading frames (ORFs) or even excision of most of the genome (reviewed in references 2 and 3). While most retroviral genes found in animal genomes are therefore nonfunctional, there exist examples of ERV genes that have been conserved over the course of evolution ever since their insertion. These genes of viral origin conferred an evolutive advantage to their host and now behave as any other cellular gene, contributing to host physiology and being under purifying selection. Syncytin genes are a group of ERV envelope genes that have been conserved as functional ORFs in a number of mammalian clades (reviewed in references 2 and 4). Originally implicated in cellular entry of virions through their fusogenic activity, syncytin genes now play an important role in placental development, in particular in the formation of a fused cell layer that forms the interface between mother and fetus in most types of mammalian placentas. The first identification of syncytin genes occurred in humans, in whom it was shown that *syncytin-1* and *syncytin-2* are ERV *env* genes with placental expression, have been conserved functional since their insertion 25 and 40 million years ago (Mya), respectively, and are fusogenic in *ex vivo* assays (5–7). These three properties have been used to define syncytin genes, and similar genes could thereafter be identified in all mammalian species in which they have been searched for. To this day, homologous genes have been identified in Rodentia (*syncytin-A* and *-B* [8, 9] and *syncytin-Mar1* [10]), Lagomorpha (*syncytin-Ory1* [11]), Carnivora (*syncytin-Car1* [12]), Ruminantia (*syncytin-Rum1* [13]), Afrotherians (*syncytin-Ten1* [14]), and even in the distantly related marsupials (*syncytin-Opo1* [15]) (Fig. 1). Recently we have also discovered a syncytin capture outside the mammalian clade, in the viviparous placental lizard *Mabuya* (*syncytin-Mab1* [16]). Identification of *syncytin-A* and *-B* in mice allowed the establishment of knockout mice which demonstrated the importance of syncytin genes *in vivo*. In the absence of syncytin genes, no fusion was observed at the maternofetal interface, which had deleterious consequences, such as death of the embryos or growth retardation (17, 18). While all identified syncytin genes present similar functional properties, they result from completely independent integration events by distinct retroviruses (Fig. 1) and therefore are a group of diverse sequences expressed from different loci.

As these distinct genes have been independently integrated and adapted for similar essential placental functions during mammalian evolution, we proposed the challenging hypothesis that this stochastic acquisition of genes of exogenous origin has been instrumental in establishing the remarkable structural and functional diversity of the mammalian placenta (reviewed in reference [2]). Indeed, the placenta is the organ displaying the most structural interspecies variation in mammals (19). Two major related criteria by which they can be classified is the invasivity of fetal tissues and the number of layers that separate maternal and fetal blood circulations in the definitive placenta (Fig. 1). The lowest degree of invasion is found in epitheliochorial placentas (e.g., pigs and horses), in which maternal and fetal eptihelia are simply apposed. In more invasive placentas, maternal layers are disrupted and absent from the definitive placenta: in endotheliochorial placentation (e.g., cats and dogs), the fetal epithelium is in direct contact with maternal blood vessel endothelium, and in the most invasive type, hemochorial placentation (e.g., rodents and primates), even this last maternal cell

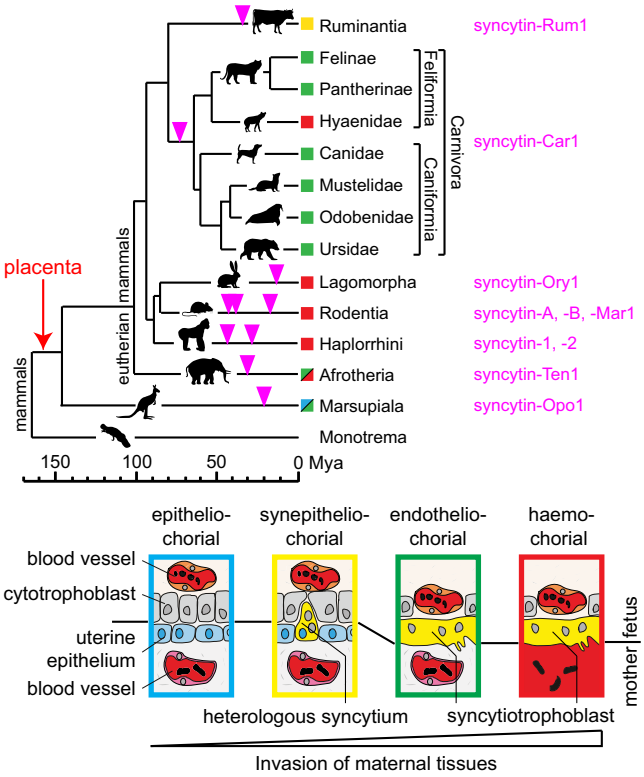


FIG 1 Phylogeny of mammals with emphasis on carnivorans, with placental type and known syncytins indicated. (Top) Mammals comprise the monotremes (e.g., platypus), still laying eggs, and the marsupials and eutherian mammals, which all possess a placenta. For the latter the placental type is indicated using the same color key as the schemes at the bottom. All currently described *syncytin* capture events are indicated by arrowheads in pink, together with the syncytin gene's name. Branch length is proportional to time (expressed in My [15, 43]), as indicated in the scale below the tree. (Bottom) Schemes illustrating the four types of maternofetal interfaces found in mammals, arranged in order of invasiveness of fetal tissues. A line delimits the maternal and fetal sides.

layer is disrupted and the fetal tissue is in direct contact with maternal blood. In the past, we have substantiated the hypothesis linking the diversity of syncytin genes with the diversity of placentas by demonstrating the concomitant acquisition of a syncytin gene and the emergence of a distinct placental organization during mammalian radiation. The emergence of the very specific ruminant synepitheliochorial placenta is concomitant with the acquisition of *syncytin-Rum1* in this clade. The implication of *syncytin-Rum1* in establishing this structure was further substantiated by the observation that the more extensive syncytium formation in ovine species, compared to that in bovine species, is accompanied by a higher level of expression of the captured syncytin (13). The Carnivora clade presents a remarkable and even more challenging situation. Indeed, the carnivoran structural organization of the placenta is of the endotheliochorial type, with only limited variation between species (19). This is consistent with the capture of *syncytin-Car1* being concomitant with the radiation of this clade about 80 Mya and its subsequent conservation in all carnivorans (12). There is a remarkable exception to this rule: the spotted hyena (*Crocuta crocuta*, here referred to as hyena), which belongs to the Feliformia subclade within Carnivora, clearly possesses a hemochorial-type placenta (20–23). Since the hyena presents the same syncytin gene as all other Carnivora, i.e., *syncytin-Car1* (12), we hypothesized that the unique placental structural organization of this species might be associated with a recent *de novo* capture of a hyena-specific syncytin gene, which would be responsible for the observed transition. Accordingly, we searched for such a gene in the hyena and since the genome of this species has not yet been sequenced, we first performed a high-throughput RNA sequencing of placental transcripts to identify putative *env*-related

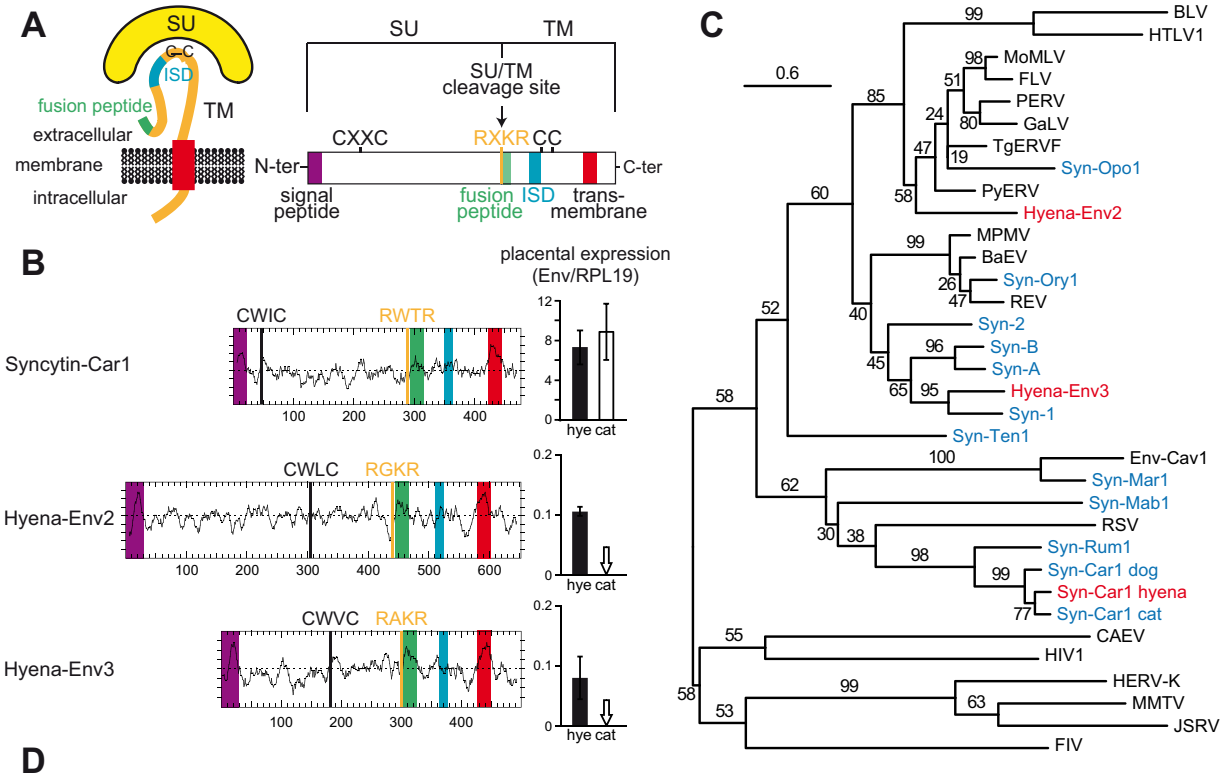
syncytin genes by an *in silico* search, followed by a structural and functional characterization of the identified candidates. These analyses resulted in the identification of a newly captured gene, specific to hyenas and expressed in the placenta, consistent with a role of this gene in the stochastic emergence of the hyena-specific placental structure. The alternative hypothesis involving a severely modified expression profile or fusogenicity of the shared *syncytin-Car1* in the hyena seems to be ruled out, with no significant difference observed between the hyena gene and the other carnivoran orthologs, including that of dog and cat. The results are discussed in the wider context of retroviral gene capture and exaptation as a central driving force generating the observed structural placenta diversity.

RESULTS

High-throughput sequencing and *in silico* search for retroviral *env* genes within the *Crocuta crocuta* placenta transcriptome. Since the hyena genome—at variance with that of some of the other carnivorans—has not been sequenced, we first established the transcriptome of the *Crocuta crocuta* placenta. Transcriptome sequencing (RNA-seq) [using poly(A)$^+$ RNA from a mid-term hyena placenta] was performed in collaboration with the French National Sequencing Center (Genoscope, Evry, France), where assembly of the placental transcripts was also performed using *de novo* assembly methods in the absence of a reference genome (see Materials and Methods). The transcriptome was then screened for putative *env*-derived syncytin genes as described previously for other mammalian genomes. All ORFs corresponding to protein sequences of over 400 amino acids (aa) (Met codon to stop codon) were compared to a set of selected Env sequences, including previously described syncytin genes (see Materials and Methods), using BLAST. As illustrated in Fig. 2, the search yielded 3 sequences, the previously described *syncytin-Car1,* which is conserved among all carnivorans, and two novel *env* sequences that were named *Hyena-Env2* and *Hyena-Env3*. All three contain domains characteristic of canonical gamma-type retroviral Env proteins: a predicted signal peptide at the N terminus, a putative furin cleavage site (R-X-K-R) delimitating the surface (SU) and transmembrane (TM) subunits, a hydrophobic domain of $>$20 aa located downstream of a highly conserved C-X_{5-7}-C motif, and a canonical retroviral Env immunosuppressive domain (ISD) (Fig. 2B and D) (reviewed in reference 24). When placed on a phylogenetic Env tree, Hyena-Env2 does not cluster with previously described syncytin genes (Fig. 2C). Hyena-Env3 shares conserved regions with human syncytin-1 (39% amino acid identities) as well as with the previously described non-syncytin gene Canis-Env3 (66% amino acid identities) (12). A reverse transcription-quantitative PCR (RT-qPCR) analysis of the placental expression of all three envelopes, using primers specific for each gene, showed that s*yncytin-Car1* is very strongly expressed in the hyena placenta, its expression being about 7 times higher than that of the ribosomal protein RPL19 housekeeping gene, at a level similar to that observed in the cat placenta (Fig. 2B). The levels of expression of the two newly identified envelopes are much lower than that of *syncytin-Car1*, namely, about 60-fold lower for *Hyena-Env2* and 90-fold lower for *Hyena-Env3* in hyena, and null in cat placenta (Fig. 2B).

Characterization of the identified hyena candidate genes: are they hyena specific and conserved? *syncytin-Car1* has already been demonstrated to be common to all carnivorans and to be functionally conserved, with evidence for purifying selection. As previously shown, the hyena ortholog did not disclose significant differences following a refined analysis of amino acid identity and nonsynonymous versus synonymous mutation ratios (12), and a thorough BLAST search of the transcriptome did not reveal expression of putative alternative copies in the hyena placenta.

The *Hyena-Env2* gene cannot be found by *in silico* BLAST search within the cat (*Felis catus*), cheetah (*Acinonyx jubatus*), tiger (*Panthera tigris*), dog (*Canis familiaris*), ferret (*Mustela putorius furo*), walrus (*Odobenus rosmarus*), seal (*Leptonychotes weddellii*), polar bear (*Ursus maritimus*), or panda (*Ailuropoda melanoleuca*) genome and thus is a good candidate for being a hyena-specific ERV *env* gene. To verify this hypothesis on a larger



D

Hyena-Env2

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MEGHKEPQKPAQAQAMLLPIFTALLTVCKSNPSSHLPNPHQPTTAKWVLRGPLTTPRDLGRTVQELTLTGPASITFPTFHLDLCSLAGDHWNTNPKICKG 100
QCVDCNTFGCRSGADCQHQNLRQQTFYVCPGTGNFDTCGGIEHFFCGSWGCETIAPWVKQPSNDLITLVRASNQTSPSNRNPISIQLTPRGKTENWSVVK 200
VWGIRLWLTGHDIGFLFSIQKQLVLPPPVAMGPMAASAANHKPRSTPSVPAPTQAAPSLSATDSPLGGVPIQLRPPRSRPVIYSILNLTYSFLNSTNLTN 300
TDCWLCLDSRPPFYVGWAISGQVSRDIEGHCSWGQPPVLTIQEVTGSGLCVLGNGGTLTTFPHLSHLCNQTMTATGSSYLRPPSGAWFACTSGLTPCIHP 400
QVLENDTLCVLVTLFPQVYYQPASSFFEIQPEQKHSRGKRDFRVSAALPTLIVGTGIEAGVGTGTAALIRGNQQFDALAQAIDFDLAQLENSTRHIRGSL 500
DSLAEMALQNRRRLDLILLHQGGLCQALGEQCCFYANNSGIVQDSLAVVRQHLQERAKIREQNKNWYENIFNWSPWLTALITALAGPLALLLLLLTLGPY 600
TLNRLLAFMRERLGAIRLMVLRSQYAQPPADQSEDQYVQLGPLKFQEDP
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Hyena-Env3

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MIGPPRWWAQPNFGWYVLLILVLLHPLVWATECNRCMHGSGQRRYFQSYTYMPKICYEGKTPITCTSTHAPGKTFWASELNAQRTTKSPCNQYPKTGTVC 100
WTYLAQIGLSDGGGVQDQAKHHQTKQMLQNLHTQRQAQTTPTYRGLNLDTLQNFLPTDQLTNTLINSSYNLWQNLTGDRDCWVCFPFSTITNIIGIPIPT 200
TWRLPNITTTNQTVHTGALGKPIPIYNLSSTQDILTFNASSASSSLPYCTPPGIFLSCPEGIYRCLTASDSLNCIFILLSPSTNIYSHTQLLSLLFPTHR 300
AKRAVFVPLLIGAGLATGIATGVAGLGTSINFYYKLSQALNEDMERIADSLTALQTQITSLAAIALQNRRALDLLTAEKGGTCLYLKEECCYYVNQSGIV 400
TSKVRELRDRIQARRQDSHIWGLDPHAWVTWLLPLAGPLCLILLSISVAPCLIRYLWERLQEMSRVSVNQLLLQPYSRLPTSDYPYNDAHPSAGSSRN
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FIG 2 Structure of a canonical retroviral Env protein and characterization of the identified *Crocuta crocuta* envelope candidates. (A) Schematic representation of a retroviral Env protein, delineating the surface (SU) and transmembrane (TM) subunits. The furin cleavage site (consensus: R/K-X-R/K-R [orange]) between the two subunits, the C-X-X-C motif involved in SU-TM interaction, the hydrophobic signal peptide (purple), the fusion peptide (green), the transmembrane domain (red), and the putative immunosuppressive domain (ISD [blue]) along with the conserved C-X_{5-7}-C (CC) motif are indicated. (B) (Left) Characterization of the candidate *Crocuta crocuta* Env proteins. The hydrophobicity profile for each candidate is shown, with the canonical structural features highlighted using the same colors as in panel A. (Right) RT-qPCR analysis of *env* gene expression in the *Crocuta crocuta* and *Felis catus* placentas. Transcript levels are shown as the ratio between expression levels of each *env* gene and those of the *RPL19* control gene (see Materials and Methods). The same specific primer pairs were used in both species. Arrows indicate null values; values shown are the means of triplicates ± SDs. (C) Retroviral envelope protein-based phylogenetic tree with hyena envelope proteins highlighted. The maximum likelihood tree was made using TM subunit amino acid sequences (excluding the cytoplasmic tail) from syncytins and a series of endogenous and exogenous retroviruses. Previously described syncytin genes are indicated in blue. Branch length represents the average number of substitutions per site (scale bar at top left), and the percent bootstrap values obtained from 1,000 replicates are indicated on the branches. (D) Amino acid sequences and characteristic structural features of Hyena-Env2 and Hyena-Env3 (GenBank accession no. MG805956 and MG805959), using the same colors as panel A.

panel of species, a 416-bp internal fragment was tentatively PCR amplified from a panel of genomic DNAs alongside the control amplification of the whole *syncytin-Car1* ORF using the primers described in reference 12 to assay DNA quality (Fig. 3). Amplification of the internal *Hyena-Env2* fragment was observed only in the four extant hyena species and verified by sequencing the PCR products, confirming the results of our *in silico* analysis. Finally, we could amplify and sequence the full-length *Hyena-Env2* ORF (using primers situated on both sides of the reading frame) from all extant hyenas: *Crocuta crocuta*, *Hyaena hyaena*, *Parahyaena brunnea*, and *Proteles cristatus* (Fig. 4), revealing

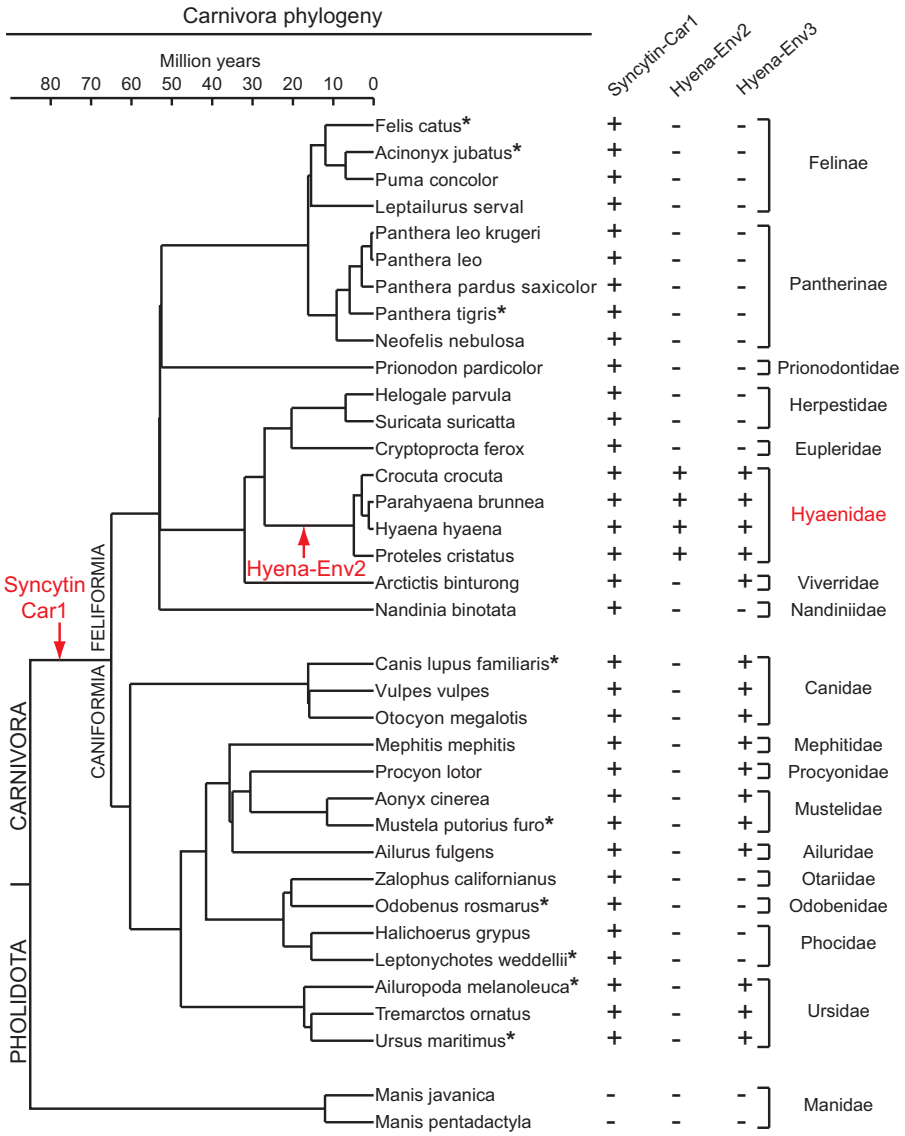


FIG 3 Entry date and conservation of *env* genes in *Carnivora*. (Left) *Carnivora* phylogenetic tree with the *Pholidota* outgroup indicated (43). Horizontal branch length is proportional to time (scale bar at the top). The names of the 34 *Carnivora* species (from both the Feliformia and Caniformia suborders) tested for the presence of the three *env* genes are indicated together with the names of their corresponding families. Species for which a sequenced genome is available are indicated by an asterisk. Approximate capture dates of *syncytin-Car1* and *Hyena-Env2* are represented by red arrows. (Right) Presence or absence of the three *env* genes in each species. For syncytin-Car1, a plus sign indicates the presence of the full-length coding ORF and a minus sign indicates the absence of the gene. For Hyena-Env2, a plus sign indicates amplification of the full-length coding ORF and a minus sign indicates the absence of the gene, both by *in silico* search when available or PCR of a 416-bp internal fragment. For Hyena-Env3, a plus sign indicates the presence of this gene as seen by *in silico* search or PCR amplification of a 674-bp internal fragment or the entire coding ORF, while a minus sign indicates its absence. The presence of *Hyena-Env2* is restricted to Hyaenidae (red).

that they share a very high sequence similarity (>98.4% nucleotide identities). Although the small data set and low number of variable sites are statistical limitations, the ratio of nonsynonymous to synonymous mutations using the Nei-Gojobori method is well below unity (0.20 to 0.67), as is the case for the functional and conserved carnivoran (<0.77) or human (0.28 to 0.85) syncytin genes (12, 25), indicating that the Hyena-Env2 gene is under selective pressure. Together these results indicate that *Hyena-Env2* endogenization occurred after the separation of the Hyaenidae family from the rest of carnivorans about 30 Mya and that the gene has been conserved since the emergence of this clade.

FIG 4 Alignment of Hyena-Env2 proteins from all four extant hyena species. Functional domains are highlighted using the same color code as in Fig. 2, and the SU and TM subunits are delineated. Asterisks indicate amino acid identities and colons indicate amino acid similarities. GenBank accession numbers are MG805956 and MG805960 to MG805962.

Interestingly, multiple sequences bearing high similarity to *Hyena-Env3* can be found *in silico* within many Caniformia genomes (e.g., *Canis familiaris*, *Ailuropoda melanoleuca*, *Ursus maritimus*, and *Mustela putorius furo*), at a variable number of copies, but not Feliformia genomes (e.g., *Felis catus*, *Acinonyx jubatus*, and *Panthera tigris*). The dog genome presents a full-length protein-coding copy, previously identified as *Canis-Env3* (12), alongside a large number of noncoding copies. However, in *Canis-Env3* the codon for the methionine which precedes the signal peptide in the *Hyena-Env3* ORF is not present, suggesting that *Canis-Env3* is not a functional copy of this gene, lacking a membrane addressing signal. A dog provirus (canFam3 genome assembly, chr3: 82194219 to 82202071) associated with a noncoding *Canis-Env3* copy presents two 100% identical long terminal repeats (LTRs) and almost intact *gag*, *pol*, and *env* ORFs, suggesting that this family was active until very recently. In addition, most conserved copies in the dog genome are not present in the orthologous regions of the panda or ferret genome, indicating that numerous insertions occurred after separation of the three lineages, about 60 Mya (Fig. 3). To assay whether this family of *env* genes could have entered the Caniformia branch after the Feliformia/Caniformia split about 65 Mya and still be specific to hyenas within Feliformia, we used primers located in conserved regions to attempt to amplify a 674-bp fragment internal to the *Hyena-Env3* ORF from genomic DNA of the species in our panel. As indicated in Fig. 3, PCR fragments of the expected size were obtained in many Caniformia species tested and sequencing confirmed them being closely related to *Hyena-Env3*. In Feliformia the PCR assay revealed the presence of *Hyena-Env3* in all four extant hyena species as well as in

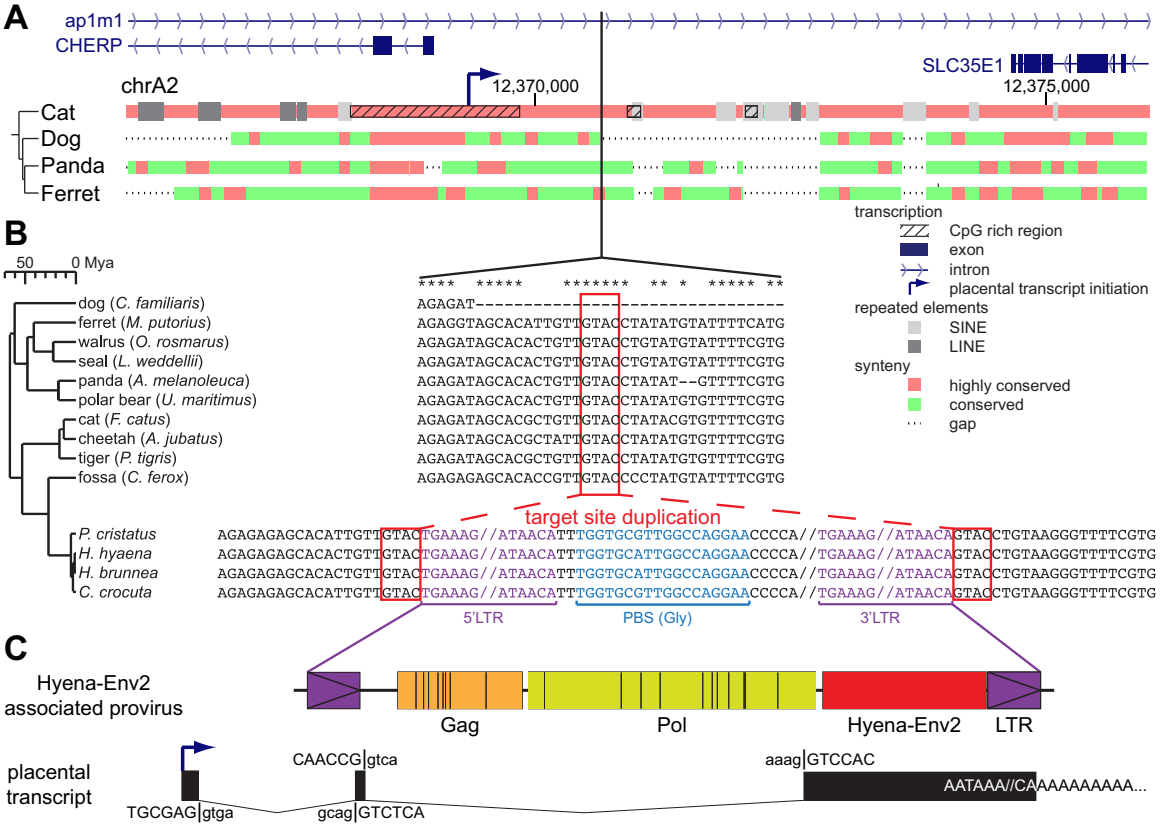


FIG 5 Characterization of the *Hyena-Env2*-associated provirus and its genomic location. (A) *Hyena-Env2* insertion occurred in a relatively conserved and transcriptionally active region. (Top) Predicted transcripts at the syntenic cat locus with exons and introns delineated. (Bottom) Ten-kilobase region of the cat genome containing the ortholog site to the *Hyena-Env2* insertion locus, with repeated sequences (as indicated by RepeatMasker [http://www.repeatmasker.org/]) and CpG islands marked, as well as conservation of this region in other carnivorans. The ortholog region to the hyena transcript initiation site is also indicated. See key on the bottom right. (B) The insertion site of the *Hyena-Env2*-associated provirus is empty in other *Carnivora*. Shown are a timed phylogenetic tree and nucleotide alignment of the syntenic loci of sequenced *Carnivora*, the four extant Hyaenidae, as well as *Cryptoprocta ferox*, the closest relative to Hyaenidae. The target site duplication is highlighted by red boxes, showing that insertion occurred only in Hyaenidae. For the latter the nucleotide sequence of the extremities of both LTRs as well as the PBS are shown. Asterisks indicate sequence identities in the locus alignment, excluding the dog sequence. (C) Structure of the *Hyena-Env2*-associated provirus (MG805957) and its placental transcript (MG805958) in *Crocuta crocuta*. LTRs are indicated in purple; Gag is indicated in orange, Pol in yellow-green and *Hyena-Env2* in red. Vertical bars indicate stop codons or frameshifts in the three reading frames. The spliced placental transcript is drawn to scale at the bottom, with the exons aligned to their corresponding proviral regions. The sequences of the splicing sites are indicated.

Arctictis binturong, which forms a sister clade of Hyaenidae. This *env* gene seems therefore to have been captured on multiple occasions and at different time points in evolution but is clearly not specific to hyenas.

Structure of the *Hyena-Env2*-associated provirus and placental transcript. To further characterize the hyena-specific *Hyena-Env2* gene, we searched for its insertion locus by inverse PCR. Genomic DNA from *Crocuta crocuta* was digested, using restriction enzymes with only one cutting site in the *Hyena-Env2* gene, and self-ligated to generate circular genomic DNA fragments. PCR was performed on these circular fragments using divergent primers located within the *Hyena-Env2* gene fragment, and the obtained PCR products were sequenced. One of them contained the expected part of the *env* gene and extended 3′ to it for >1,000 bp, with evidence for the presence of an LTR and flanking genomic sequence. The identified flanking sequence mapped to a locus of the cat genome (the closest sequenced carnivoran genome), most probably corresponding to the ortholog of the integration site of the *Hyena-Env2*-associated provirus (Fig. 5A). Primers were designed, using the cat genome sequence for the 5′ side, to tentatively amplify the whole provirus from *Crocuta crocuta* genomic DNA and to assay its presence in closely related species, as well as in cat and dog as controls. A

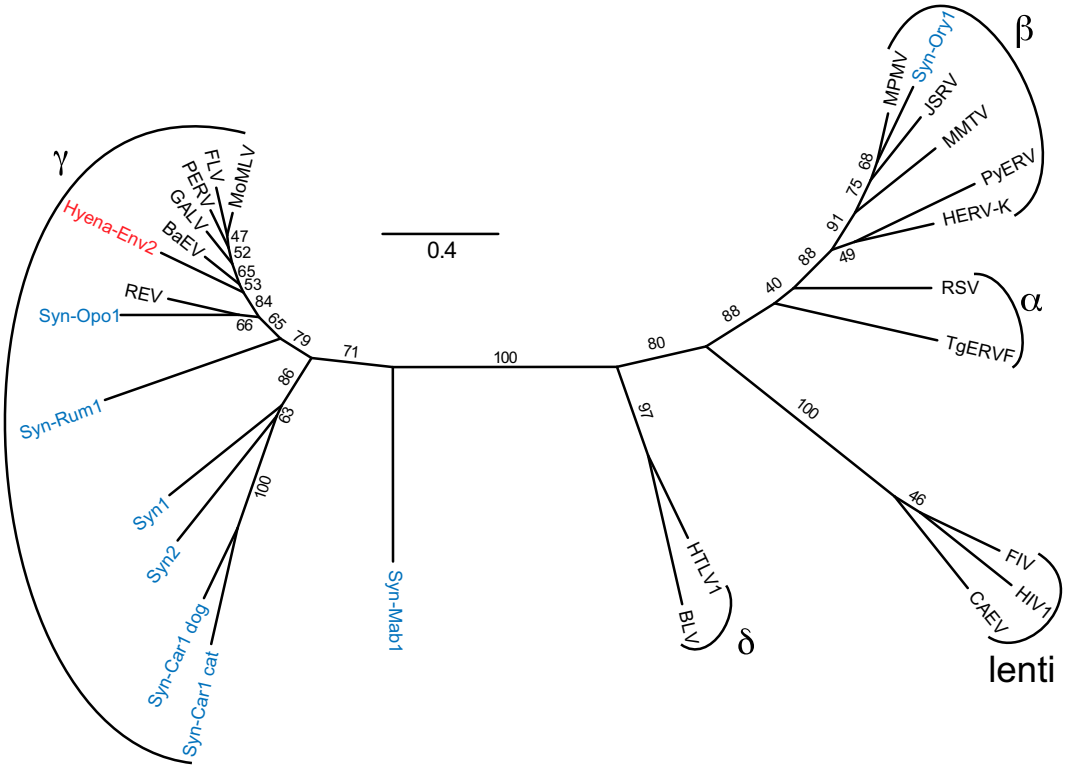


FIG 6 *Hyena-Env2*-associated virus is a gammaretrovirus. Shown is an unrooted PhyML tree showing the position of retroviral endogenous and exogenous RT domains using the same data set as in Fig. 2C when available. The *Hyena-Env2*-associated RT is in red, and those associated with previously described syncytin genes are shown in blue. Major retroviral families are indicated on the tree. Bootstraps were calculated after 1,000 replicates and are indicated on each branch. Branch length is proportional to the average number of substitutions per site (see scale).

large fragment, of about 9 kb, could be amplified from *Crocuta crocuta* genomic DNA and, once sequenced, allowed characterization of the *Hyena-Env2*-containing provirus, bordered by clearly recognizable 5′ and 3′ LTRs (89% identity) and inserted within the locus identified above (Fig. 5B and C). At the junctions between LTRs and genomic DNA we observed a 4-bp target site duplication, as expected for a proviral integration. The provirus contains the full-length *Hyena-Env2* gene and recognizable *gag* and *pol* genes, although with severely altered, noncoding sequences. A putative tRNA primer binding site (PBS) could be identified by BLAST using a tRNA database, the sequence matching a glycine tRNA (Fig. 5). Comparing the catalytic RT domain of *pol* with a set of other, endogenous as well as exogenous, retroviral catalytic RT domains classifies this provirus as a gammaretrovirus (Fig. 6).

PCR amplification and sequencing using the same set of primers and genomic DNA from cat, dog, and the four species closest to *Hyaenidae* (*Arctictis binturong*, *Cryptoprocta ferox*, *Suricata suricatta*, and *Helogale parvula*) showed that the ortholog sites in these species are indeed empty, confirming the results obtained with primers internal to *Hyena-Env2*. This was further confirmed by *in silico* analyses of the ortholog site in all sequenced carnivoran genomes (Fig. 5B), which disclosed an empty site in all cases. Of note, the dog genome presents a 2-kb deletion in this region.

In order to characterize further the sequence of the placental *Hyena-Env2* transcript, we performed rapid amplification of cDNA ends (RACE) using total placenta RNA samples, followed by PCR to amplify the 5′ or 3′ end of the cDNA. A single major band was obtained and sequenced for each end, providing the complete sequence of the *Hyena-Env2* placental mRNA. By aligning the obtained transcript with the *Hyena-Env2*-associated provirus, we were able to identify the canonical retroviral envelope transcript splicing sites, with a donor site just after the 5′ LTR and an acceptor site before the envelope start codon (Fig. 5C). The transcript ends in the 3′ LTR with a polyade-

nylation signal, as classically observed for retroviral transcripts. However, initiation does not occur in the 5′ LTR as expected for retroviral transcripts; instead, another splicing event takes place since the 5′ end of the transcript does not match the sequence of the proviral LTR. Since no hyena genome has been published, we used the BLAT algorithm to try to position the 5′ end of the transcript in the cat genome (felCat8 assembly). The sequence matches a region about 1 kb upstream of the site orthologous to the insertion locus of the *Hyena-Env2*-associated provirus. In the cat genome, this region presents a predicted splice donor site at the expected position and appears to be in a CpG island. This is reminiscent of the recently described human HEMO *env* transcript which is initiated in a cellular CpG island upstream of the provirus insertion site (25). Several other transcripts are predicted to be initiated nearby (e.g., CHERP and SLC35E1), while others seem to present this region as an intron (e.g., ap1m1) (Fig. 5A). This suggests that expression of the placental *Hyena-Env2* transcript, a gene of retroviral origin, is driven by a promoter of cellular origin.

***In situ* hybridization of the identified *Hyena-Env* genes on placenta sections.** The hyena is the only carnivoran with a placenta of the hemochorial type (20–22). In all other carnivorans, the placenta is of the less invasive endotheliochorial type, in which the fetal tissues break down the maternal epithelium but leave the maternal blood vessels intact (Fig. 7A, top) (19). In the definitive hemochorial hyena placenta, maternal blood vessels have been destroyed and the fetal tissues form villi that are in direct contact with maternal blood (Fig. 7A, bottom) (20–22). Each primary villus contains fetal blood vessels and is covered by a layer of fused cells: the syncytiotrophoblast (22, 23). Under this layer are the cytotrophoblasts, which in the hyena do not form a continuous layer and have a cytoplasm filled with numerous lipid droplets (22, 23). The villi present frequent ramifications (not schematized in Fig. 7A) forming a complex labyrinthine organization with numerous linked maternal blood spaces. The surface of these ramifications is also covered by syncytiotrophoblast (23).

To determine the expression pattern of the three hyena envelopes in the placenta, we performed *in situ* hybridization (ISH) on placenta tissue sections (gestation day 62, about mid-pregnancy) using three specific digoxigenin-labeled antisense RNA probes for each envelope, as well as the corresponding sense probes as negative controls.

As previously described for the cat and dog placenta (12), hyena *syncytin-Car1* is expressed specifically in the syncytiotrophoblast and not in the underlying cytotrophoblast, indicating that the hyena s*yncytin-Car1* expression locus does not differ from that observed in other carnivorans (Fig. 7B).

Unexpectedly, for *Hyena-Env2* the sense probe used as a negative control resulted in a stronger signal than the antisense probe detecting the sense envelope transcript (Fig. 7B). Sense/antisense specific RT-PCR on placental RNA with the primer pairs used to synthesize the 3 ISH probes revealed the existence of an antisense transcript that covered at least all 3 regions detected by the probes, since all pairs allowed amplification from sense and antisense cDNA. This antisense transcript might be initiated downstream from the proviral insertion site, at a CpG island (by analogy with the cat genome [Fig. 5A]) that could drive antisense transcription. Nevertheless, the signal observed using the antisense probes is differential between the different placental cell layers: staining is observed only in the layer that contains the cytotrophoblasts, just below the syncytiotrophoblast (Fig. 7B). This localization of expression is clearly different from that of hyena *syncytin-Car1* and suggests a distinct role for *Hyena-Env2*. To confirm this ISH result, we generated anti-Hyena-Env2 antibodies by immunizing mice with bacterium-synthesized polypeptides derived from the *Hyena-Env2* SU and performed immunohistochemistry (IHC) with the postimmunization sera, using the corresponding preimmune sera as negative controls (see Materials and Methods). As illustrated in Fig. 7B and consistent with the ISH, a strong staining was observed at the level of the cytotrophoblasts and not of the syncytiotrophoblast.

Finally, no conclusive result could be obtained by ISH for *Hyena-Env3* due to low signal strength. Since this envelope is not hyena specific (Fig. 3), the placental expression profile was not investigated further.

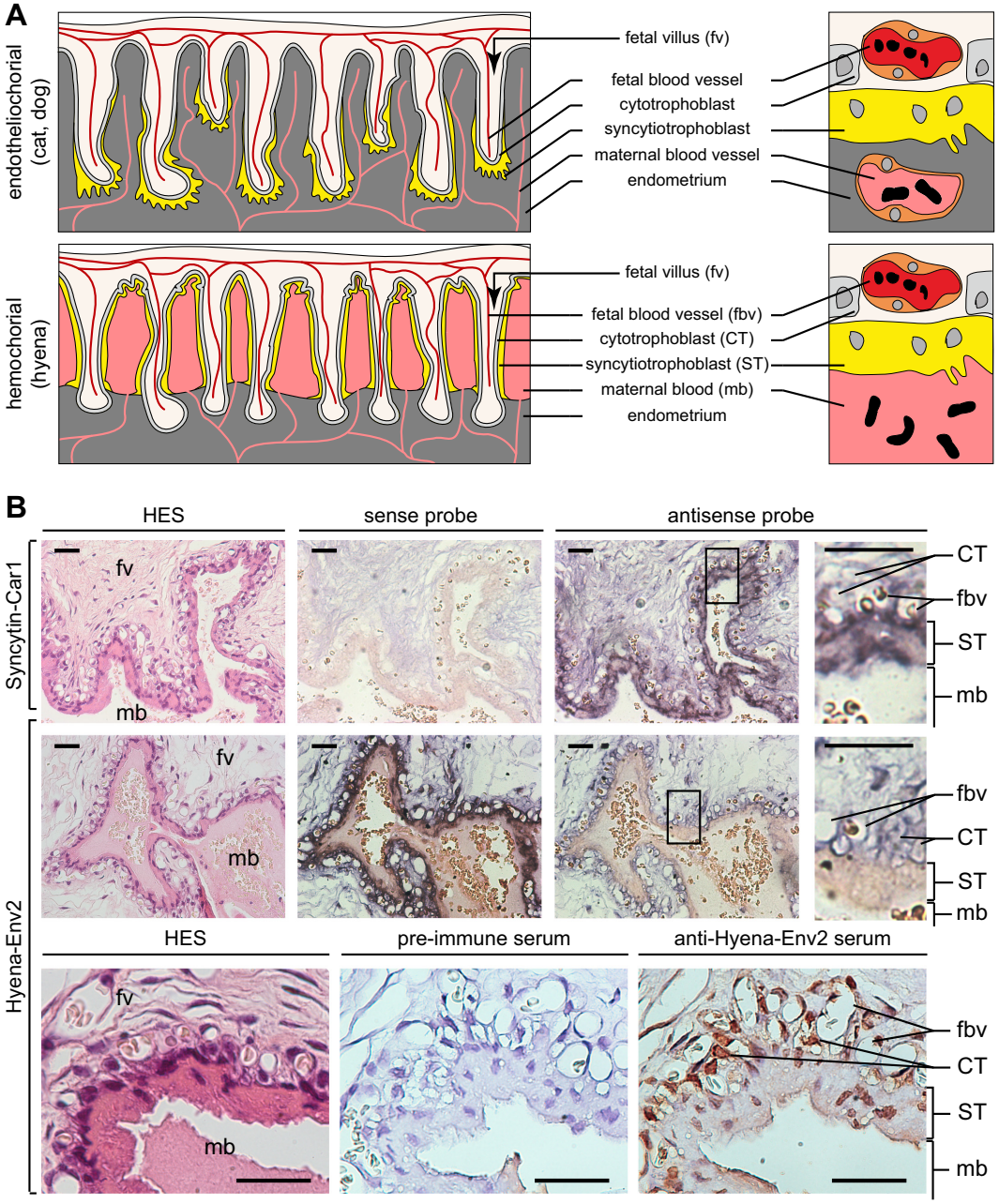


FIG 7 Expression pattern of *syncytin-Car1* and *Hyena-Env2* in *Crocuta crocuta* placenta. (A) Schematic representation of the mid-gestation carnivoran endotheliochorial placenta (top row) and hemochorial hyena placenta (bottom row). The drawing on the right presents the cellular organization of the syncytial interface. In the hemochorial hyena placenta, the maternal blood vessels have been disrupted resulting in the presence of large maternal blood spaces. Only the primary villi are represented for both placental types. (B) (Top rows) Hematoxylin-eosin-saffron (HES) staining and *in situ* hybridizations on serial sections of *Crocuta crocuta* placenta using specific digoxigenin-labeled antisense probes for *syncytin-Car1* and *Hyena-Env2* (and corresponding sense probes as a control). Specific staining is observed in the syncytiotrophoblast for *syncytin-Car1* and in the cytotrophoblasts for *Hyena-Env2*. Strong staining with the *Hyena-Env2* sense probes indicates existence of an antisense transcript. Areas marked by a rectangle are enlarged on the right; all scale bars represent 50 μm. (Bottom row) HES staining and immunohistochemistry on serial sections of *Crocuta crocuta* placenta using an anti-Hyena-Env2 mouse serum (and preimmune serum as a control). The region shown is equivalent to that of the *Hyena-Env2* ISH. Specific staining is again observable in the cytotrophoblasts (CT). Scale bars: 50 μm.

Fusogenicity of the identified *Hyena-Env* genes in *ex vivo* assays. The fusogenicity of the identified hyena envelopes was first assayed using a pseudotyping assay, which tested whether the envelope proteins could render recombinant *env*-less murine leukemia virus (MLV)-derived viral particles infectious. 293T cells were cotransfected

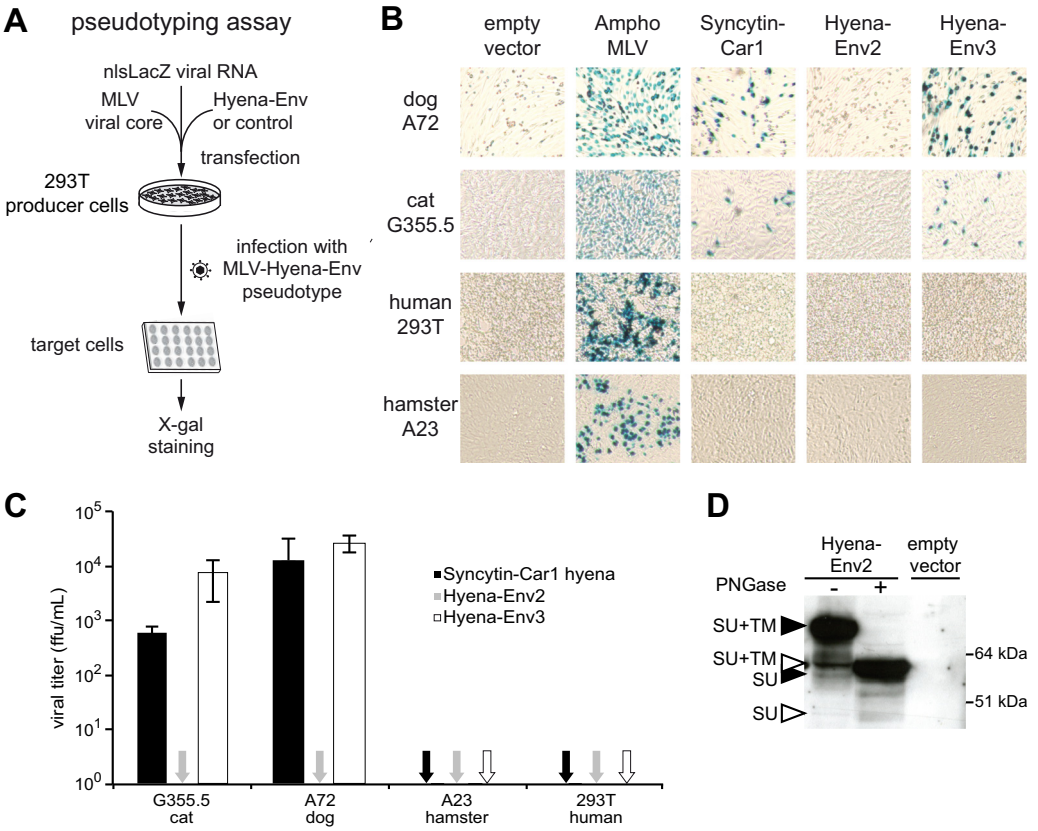


FIG 8 Fusion assay for the three hyena envelope proteins. (A) Schematic representation of the pseudotyping assay with Hyena-Env-pseudotyped viral particles. Pseudotypes are produced by cotransfecting 293T cells with expression vectors for the MLV core, a β-galactosidase encoded by an nls-LacZ-containing retroviral transcript, and either a vector expressing Hyena-Env proteins or a control vector. Supernatant of the transfected cells is then added to the indicated target cells, which are X-Gal stained 3 days postinfection to reveal viral entry. (B) X-Gal-stained target cells for particles without Env or pseudotyped with hyena syncytin-Car1, Hyena-Env2, Hyena-Env3, or the Env protein from the amphotropic murine leukemia virus (Ampho-MLV) as a positive control. Target cells were cat (G355.5), dog (A72), human (293T), or hamster (A23) cells. Infection foci were detected when using hyena syncytin-Car1 or Hyena-Env3 and targeting carnivoran cell lines, indicating species tropism. Hyena-Env2 tested negative in this assay. (C) Quantification of viral titers expressed in focus-forming units per milliliter after infection with syncytin-Car1- or Hyena-Env-pseudotyped MLV virions. An arrow indicates values below background staining. Values shown are the means from three independent experiments ± SDs. (D) Western blotting using the anti-Hyena-Env2-SU mouse serum used for Fig. 7 and lysates of MCF10A human cells transduced with a control empty vector or a Hyena-Env2 expression vector (see Materials and Methods). Lysates were deglycosylated (or not) using PNGase F. Black arrowheads point at the glycosylated and white arrowheads at the unglycosylated full-length SU plus TM and cleaved SU.

with a plasmid containing the *env* gene under the control of a cytomegalovirus (CMV) promoter, a plasmid expressing the MLV *gag-pol* genes, and a plasmid expressing a defective MLV genome containing an nls-LacZ reporter gene. Viral particles were then used to infect various target cell lines and infection was assayed by counting 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal)-stained infection foci (Fig. 8A).

Hyena *syncytin-Car1* has been shown previously to behave like other Feliformia *syncytin-Car1* genes in this test: it is able to mediate viral infection and displays a strong tropism for carnivoran cell lines (12) (Fig. 8B and C).

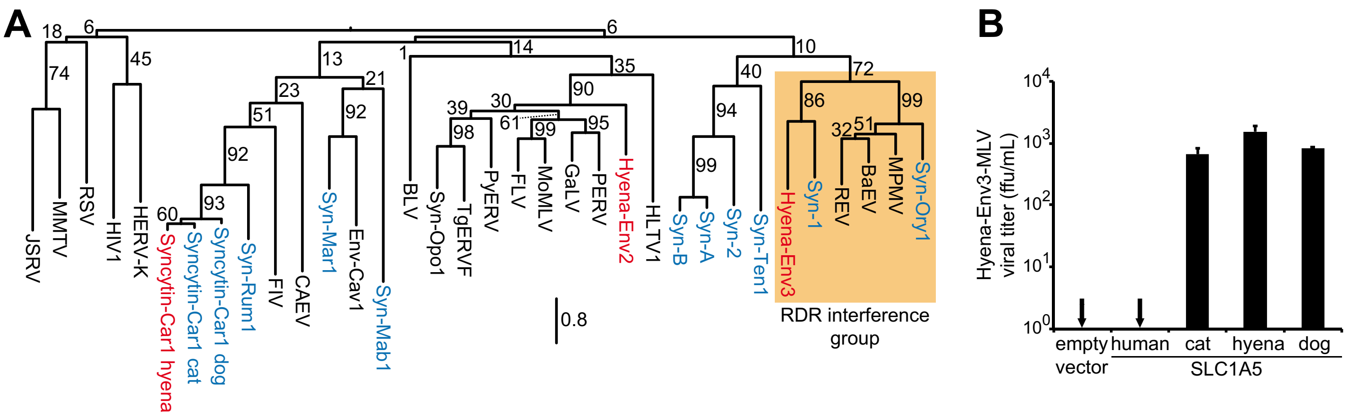
Hyena-Env2 tested negative in the pseudotyping assay (see Fig. 8B and C). It is unknown whether this is due to the absence of the proper receptor in all cell lines tested, though we tested several cell lines from cat and dog which should present proteins closely related to hyena proteins, or if an intrinsic loss of fusogenic activity is the cause of this result. Of note, similar results were obtained with human immunodeficiency virus (HIV)- and simian immunodeficiency virus (SIV)-pseudotyped particles. These negative results might also be due to a lack of incorporation of Hyena-Env2 into the viral particles. We therefore performed a more straightforward cell-cell fusion assay

in which cat G355.5 or human 293T cells were transfected with a Hyena-Env2-expressing plasmid and put into contact with a diverse panel of nontransfected target cells, including cat and dog cells, 24 h posttransfection. No syncytium formation was observed for any combination of cells used in this assay. Since a lack of fusogenicity could also be due to incorrect posttranslational processing of this hyena protein, we investigated its glycosylation and SU-TM cleavage status by Western blotting using the antibodies raised for IHC. As illustrated in Fig. 8D, when lysates of cells expressing Hyena-Env2 are deglycosylated using peptide-*N*-glycosidase F (PNGase F), the anti-SU antibodies detect a major band at roughly the expected size for SU+TM (ca. 65 kDa) and a faint band for cleaved SU (ca. 45 kDa). Both bands are shifted and display a higher molecular weight in the absence of PNGase F treatment, indicating that they are glycosylated, as expected. Although the Hyena-Env2 furin cleavage site is canonical, the amount of cleaved SU is low, but a similar pattern is observed for most functional syncytins in similar *in vitro* assays (26).

Finally, Hyena-Env3 was found to confer infectivity to MLV-derived viral particles for some target cell lines. Among tested cells, only carnivoran cell lines were permissive to viral infection and presented significant numbers of infection foci 72 h postinfection, reaching approximately 10^4 focus-forming units/ml (Fig. 8C). These results suggest that Hyena-Env3 confers a carnivoran-specific tropism to the pseudotyped particles. Sequence comparison further showed that the SU of Hyena-Env3 seems to be related to the SUs of human syncytin-1, of rabbit syncytin-Ory1, and of other Env from retroviruses of the RDR interference group (Fig. 9A), a group of viruses all using the membrane transporter SLC1A5, previously known as ASCT2 or RDR, as a common receptor (27, 28). Alignments reveal that Hyena-Env3 and RDR group Envs share conserved domains that are essential for interaction with the receptor (29). Transfection of nonpermissive human HEK293T cells with expression vectors for SLC1A5 that we cloned from cat, dog, or hyena placental cDNA (see Materials and Methods) rendered the cells permissive to infection by Hyena-Env3-pseudotyped MLV particles, while transfection with human SLC1A5 did not allow infection, confirming both the role of SLC1A5 as a receptor and the tropism of Hyena-Env3 observed in the pseudotyping assay (Fig. 9B). Some of the amino acids conserved among the different envelopes of the RDR interference group and implicated in protein conformation or receptor binding (29) appear to be missing or shifted by one or two positions in Hyena-Env3, which might explain the peculiar carnivoran tropism (Fig. 9C).

DISCUSSION

In this study, we have performed a high-throughput analysis of the hyena placenta transcriptome, which revealed a newly captured gene of retroviral origin that is unique to the Hyaenidae family. It is present in all hyena species and is not found in any other carnivoran. Its entry date is coincident with the emergence of the Hyaenidae within Feliformia, about 30 Mya. It is present at the same integration site in all hyena species, with an intact coding sequence. This time-resolved retroviral *env* gene capture is an additional illustration that endogenization of retroviruses is a permanent process that accounts for the presence of remnant proviral structures in up to 10% of the vertebrate genome (reviewed in reference 3). Such captures are still ongoing, with, for instance, koala leukemia retrovirus (KoRV), which is presently being endogenized in the Australian koala population after the infectious exogenous retrovirus spread across the whole country (3, 30). In most cases endogenization of retroviruses is neutral to the host physiology, but there are a series of exceptions to this rule. They fall into two major categories, exaptation of either (i) the retroviral transcription promoters and enhancers (LTR sequences) or (ii) the retroviral protein-coding sequences *per se* (most commonly the *env* gene). The former has been demonstrated to be responsible for the coordinated expression of genes located near the ERV integration site, with special emphasis on their role in development (reviewed in reference 31). The latter includes the syncytin envelope genes, which proved to be involved in placenta formation (reviewed in references 2 and 4). Their impact is directly linked to the canonical properties of



C

SU subunit

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Syn-1        MALP 13 TLTAPPPC-RCMTS 37 MPRNCYHSATLCMHANTHYWTGKM  1 NPSCP-GGLGVTVCWTYFTQTGMSDGGGVQDQAREKHVKEVI 180 RNKR
BaEV         MGFT 26 QKRYGRPC-DCSGG 54 VHSSCYTSYQQCRSGNKTYYTATL 21 QSPC-NGIKGQSICWSTTAPIHVSDGGGPLDTTRIKSVQRKL 189 RPKR
SRV-1        MNFN 30 QQKHGKPC-DCAGG 57 VHATCYNHYQQCTIGNKTYLTATM 24 IAGCPENKKGQVVCWNSQPSVHMSDGGGPQDKVREIIVNKKF 197 RPKR
SRV-2        MTVK 30 HQQHGKPC-DCAGG 54 VHSSCYTTYQECFFGNKTYYTAIL 24 SAGC-TGTVGQHICWNPKAPVHISDGGGPQDKAREIAVQKRL 188 RARR
MPMV         MNFN 30 QQKHGKPC-DCAGG 57 VHASCYNHYQQCNIGNKTYLTATI 24 TAGCPNGKKGQVVCWNSRPSVHISDGGGPQDKARDIIVNKKF 196 KAKR
SRV-4        MNFE 30 QNKHGKPC-ECKGG 57 VHSTCYSSYQQCTIGNKTYFTATI 24 SAGCPKDELGKTACWSATPPVHVSDGGGPQDKVREILVQKKF 192 RTKR
SMRV         MLCI 25 TALFGAPC-DCKGG 68 MHSSCYSSFSQCTQGNNTYFTAIL 22 QAGC-DGTVGKSVCWNQQAPIHVSDGGGPQDAVRELYVQKQI 185 RQKR
SNV          MDCL 35 QQLWGLPC-DCSGG 70 MHSTCYEKTQECTLLGKTYFTAIL 16 QASC-TGTVGKPVCWDPVAPVYVSDGGGPTDMIREESVRERL 191 RHKR
Syn-Ory1     MALS 31 QQHH--PC-TCSGG 65 VHSSCYTQYQTCNKGNQRLLFAVI 18 SPSCP--TKGQVACWYPIAPVGVSDGGGPQDTLTKLKTQKVL 186 RQKR
Dasy-Env1.1  MHSF 29 KTLYGTPC-ECRGG 65 MHVHCYSEVQSCTRNNKTYYTAIL 25 QAGC-YGTIGDTVCWNKIPPAHISDGGGPQDQARQIIVQERM 184 RSKR
TvERV(D)     MIST 23 HALFGAPC-DCKGG 64 LHSLCYTSTQQCTGKSGTYLTSRQ 24 QASCDKINIGKNVCWSLHAPIHVSDGGGPTDQIREMEVKERV 189 RPKR
REV-A        MDCL 35 QQLWGLPC-DCSGG 68 MHSTCYEKAQECTLMGKTYFTAIL 16 QASC-TGIIGKPVCWDPAAPVYVSDGGGPTDMIREESVRERL 191 RHKR
DrERV        MFSQ 27 DALFGPPC-DCQGG 75 HSACCYYKVYTCSKGGQTYFTARI 28 PDKCPG-EIGKPACWKLDSPLG-SDGGGPQDKRRTERVRIQI 179 RHKR
RD114        MKLP 26 QKQHGKPC-ECSGG 55 MHSSCYTEYRQCRANNKTYYTATL 21 QSPC-RGSINQPVCWSATAPIHISDGGGPLDTKRVWTVQKRL 190 RPKR
Hyena-Env3   MIGP 21 PLVWATECNRCMHG 12 MPKICYEGKTPITCTSTHAPGKTF 12 KSPCNQYPKTGTVCWTYLAQIGLSDGGGVQDQAKHHQTKQML 171 RAKR
             *              *  *           **                         *         **        *****  *                *  *
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FIG 9 Hyena-Env3 belongs to the RDR interference group of envelopes. (A) The Hyena-Env3 SU is closely related to envelopes previously described as using SLC1A5 (also known as RDR and ASCT2) as a receptor. Shown is a maximum likelihood tree using SU subunit amino acid sequences from syncytins and a series of endogenous and exogenous retroviruses. Previously described syncytin genes are in blue, and the RDR interference group is in orange. Branch length represents the average number of substitutions per site (see scale), and the percent bootstrap values obtained from 1,000 replicates are indicated on the branches. (B) Carnivoran but not human SLC1A5 allows Hyena-Env3-mediated infection of nonpermissive cells. 293T cells were transfected with either an empty vector or expression vectors for SLC1A5 from human, cat, hyena (MG805963), or dog. Transfected target cells were then infected using Hyena-Env3-pseudotyped MLV particles, and viral titer in focus forming units per milliliter were quantified after LacZ staining. Arrows indicate titers below background. Values shown are the means from three independent experiments ± SDs. (C) Sequence alignment of SU protein subunits, from the methionine to the putative furin cleavage site, of endogenous and exogenous envelopes using SLC1A5 as a receptor as well as Hyena-Env3 (in bold). Variable regions are represented by the number of omitted residues; dashes correspond to deletions/insertions. The conserved motif shown to be essential for the syncytin-1/SLC1A5 interaction ($SDGGGX_2DX_2R$) is in orange, and the three cysteine-containing motifs (PCXC, CYX_5C, and $CX_{7-9}CW$) and amino acids that are highly conserved among retroviral members of the RDR interference group are in yellow (29). Amino acid mismatches in conserved regions are in red; asterisks indicate amino acid identities.

envelope genes of exogenous retroviruses, which include a fusogenic activity to mediate membrane fusion during virus entry and an immunosuppressive activity to inhibit the host antiviral immune response. Fusion activity has unambiguously been exapted through syncytin genes and is directly responsible for cell-cell fusion and formation of the syncytiotrophoblast cell layer at the maternofetal interface in many mammalian species, with an unambiguous demonstration of this role in mice with knockouts of these genes (17, 18). More recently it has been shown that in some rare nonmammalian species with a placenta, the establishment of a fused cell layer at the maternofetal interface is also associated with the capture of a syncytin gene (16). The immunosuppressive activity also carried by captured syncytins is likely to play a role—at least in part—for the taming of the maternal immune response against the semiallogeneic graft of the fetus and placenta during pregnancy (reviewed in reference 2). Along these lines, the *Hyena-Env2* capture falls within the now well-established set of data showing that the present-day syncytin genes, found in all placental mammals in which they have been searched for, simply correspond to independent retroviral gene captures that have taken place over the course of mammalian evolution, resulting in different syncytin genes present in the major clades of placental mammals. To account for the apparent convergent evolution of such gene captures, we have

previously suggested that the emergence of placental mammals was initiated by the primitive capture of an ancestral retroviral envelope gene, about 150 Mya, which was followed by reiterated captures of new retroviral *env* genes. These new genes, depending on their fitness with regard to fulfilling placental functions, were selected in place of the previously captured envelopes, replacing them following a classical Darwinian selection process (reviewed in reference 2). This model, first proposed by our group (32, 33), accounts in a simple manner for the diversity of the syncytin genes found today. It was recently further sustained by the discovery that the primate Env-V gene that shows all the characteristic features of a *bona fide* syncytin gene in Old World monkeys has lost its fusogenic activity in higher primates—including humans—in which two other syncytins are present and active (34). However, this *env* gene is still under purifying selection, thus suggesting that its fusogenic activity might not be the sole property for which it has been and/or still is selected for (see below). Along this line, we had previously identified a captured retroviral *env* gene in the *Cavia* genus that shows only part of the features characteristic of syncytins and thus named it *syncytin-like env-Cav1* (35). This envelope is conserved under purifying selection and expressed specifically in the "syncytial streamer" of the *Cavia* placenta, suggesting that it plays a role in placentation, but we could not demonstrate any fusogenic activity *ex vivo*. In sheep, nonfusogenic endogenous Jaagsiekte sheep retrovirus (JSRV) Envs are expressed in the placenta and their inhibition *in utero* leads to a reduction in trophoblast proliferation, preventing implantation and leading to embryo death (36, 37). Finally, two additional examples of retroviral gene captures in humans with strong expression in the placenta and conservation for $>$100 Mya of evolution, with evidence for strong purifying selection, are worth mentioning, as these genes clearly cannot be fusogenic since they are lacking cell membrane attachment in the case of ERV-3 (due to a conserved premature stop codon [2, 15]) or are actively shed after they are exported to the cell membrane in the case of HEMO (25). Together these data reinforce the notion that fusion activity is not the only function exapted through capture of *env* genes with placental expression. Other classically described (and non-mutually exclusive) roles include immunosuppression or protection from viral infection through receptor interference, as well as roles in cell differentiation and proliferation (reviewed in references 2 and 4).

In the present case, as in the cases listed above, Hyena-Env2 was not found to possess fusogenic activity. In hyena placenta, this function is most probably carried by the shared *Syncytin-Car1*, as in other carnivorans. The hyena *Syncytin-Car1* ortholog does not show any deviation from the normal behavior of a gene under purifying selection (12), and we could further show that its fusogenic activity and pattern of placental expression are similar to those in cat and dog. The acquisition of *Hyena-Env2* is concomitant with the radiation of Hyaenidae and the associated structural transition of their placenta, and accordingly we propose that *Hyena-Env2* is a recently captured add-on accomplishing a role distinct from that of *Syncytin-Car1* and directly involved in the transition from an endotheliochorial carnivoran placenta to the hemochorial hyena placenta. The latter differs from the former mainly by the disruption of maternal blood vessel walls by invading fetal cells, resulting in maternal blood bathing the syncytial maternofetal interface, as observed in all hemochorial placentas, including human placenta (19). In this respect it is worth mentioning that a recent analysis of the properties of the envelope protein of the human endogenous retrovirus K (HERV-K) family of human endogenous retroviruses has provided hints that this envelope could promote an epithelial-mesenchymal phenotypic transition (EMT) as well as cellular invasion (38) and that the recently described *syncytin-Mab1* seems able to modulate cell migration phenomena through interaction with its cognate receptor (16). Although preliminary assays carried out with *Hyena-Env2* could not demonstrate a similar effect, a more refined experimental setup, or even better, identification of the *Hyena-Env2* receptor, should now be carried out to understand in molecular terms how the captured envelope can contribute to the transition in placentation type.

MATERIALS AND METHODS

Animals, tissues, and ethical agreement. Genomic DNAs of dog (*Canis familiaris*) and cat (*Felis catus*) were extracted by phenol-chloroform extraction from nitrogen frozen organs taken from pregnant females and supplied by the ENVA-INRA (Maisons-Alfort, France). Genomic DNAs of spotted hyena (*Crocuta crocuta*), fossa (*Cryptoprocta ferox*), binturong (*Arctictis binturong*), meerkat (*Suricata suricatta*), and mongoose (*Helogale parvula*) were extracted from blood samples obtained from B. Mulot and R. Potier (Zooparc de Beauval, France) using the DNA blood kit II (PaxGene). Genomic DNA of striped hyena (*Hyaena hyaena*) was obtained from G. Veron (Muséum National d'Histoire Naturelle, France, sample identifier MNHN-AM60). Genomic DNA of brown hyena (*Parahyena brunnea*) was extracted from blood samples provided by the Aspinall Foundation (Lympne, England). Genomic DNA of aardwolf (*Proteles cristata*) was provided by F. Delsuc (Université Montpellier 2, France; sample identifier TS307). Total RNA extracts and paraffin-embedded tissue samples of *Crocuta crocuta* placenta were provided by A. Conley and S. Glickman (University of California Veterinary School, USA).

This study was carried out in strict accordance with the French and European laws and regulations regarding animal experimentation (directive 86/609/EEC regarding the protection of animals used for experimental and other scientific purposes).

***Crocuta crocuta* placentome transcriptome high-throughput sequencing and *de novo* assembly.** Total RNA was purified from *Crocuta crocuta* placenta before sequencing using the Illumina HiSeq 2000 sequencing system. cDNA library construction, sequencing, and transcript assembly were performed by the French National Sequencing Center (Genoscope, Evry, France). Briefly, poly(A)$^+$ RNA was selected with oligo(dT) beads, chemically fragmented, and converted into single-stranded cDNA using random hexamer priming according to the Illumina TruSeq protocol. The second strand was then generated to create double-stranded cDNA. Next, the paired-end library was prepared following Illumina's protocol: fragments were end repaired and then 3′ adenylated, and Illumina adapters were added by using NEBNext sample reagent set (New England BioLabs); ligation products were purified, and DNA fragments (>200 bp) were PCR amplified using Illumina adapter-specific primers. After library profile analysis (showing a typical library size of 200 to 600 bp) with an Agilent 2100 bioanalyzer (Agilent Technologies) and qPCR quantification (MxPro; Agilent Technologies), the library was sequenced using 100-base-length read chemistry in a paired-end flow cell on an Illumina HiSeq 2000 instrument (Illumina). To maximize the number of reconstructed transcripts, several independent assemblies were performed using two different *de novo* assembly software programs (Oases and Trinity) as well as two k-mer lengths (55 and 59), and only transcripts of >100 bp were conserved.

Database screening and sequence analyses. Retroviral endogenous *env* gene sequences were searched for in each transcriptome assembly. Sequences containing an ORF corresponding to a sequence longer than 400 amino acids (from start to stop codon) were extracted from the transcriptomes using the getorf program from the EMBOSS package (http://emboss.sourceforge.net/apps/cvs/emboss/apps/getorf.html) and translated into amino acid sequences. These sequences were compared with the TM subunit amino acid sequences of 35 retroviral envelope glycoproteins (from representative ERVs—among which known syncytin genes—and infectious retroviruses), using the BLASTp program of the National Center for Biotechnology Information (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Putative envelope protein sequences were then selected based on the presence of a hydrophobic domain (transmembrane domain) located 3′ to a highly conserved C-X_{5-7}-C motif.

Multiple alignments of amino acid sequences were carried out by using the AliView program (39) and the MUSCLE protocol. Maximum likelihood phylogenetic amino acid trees were constructed with PhyML 3.0 (40), with bootstrap percentages computed after 1,000 replicates. Substitution models were selected using the SMS program (41): LG + Γ+I for TM sequences, WAG + Γ+I + F for SU sequences, and RtREV + Γ for RT sequences. Nei-Gojobori ratios of nonsynonymous to synonymous substitution rates (*dN/dS* ratios) were obtained using PAML4. For all genomes available at http://genome-euro.ucsc.edu/index.html, orthology and other genome-wide *in silico* studies were performed on the same site using the BLAT algorithm. Assemblies used were *Felis catus* felCat8 (2014), *Canis familiaris* canFam3 (2011), *Mustela putorius furo* musFur1 (2011), and *Ailuropoda melanoleuca* ailMel1 (2009). The BLASTn algorithm (https://blast.ncbi.nlm.nih.gov/Blast.cgi) was used to screen the genomes of *Panthera tigris* PanTig1.0, *Ursus maritimus* UrsMar_1.0, *Acinonyx jubatus* aciJub1, *Odobenus rosmarus divergens* Oros_1.0, and *Leptonychotes weddellii* LepWed1.0, all of them available at https://www.ncbi.nlm.nih.gov/genome/?term=carnivora.

Entry date in the hyena species. For both *Hyena-Env2* and *-3*, presence or absence was assayed by PCR of an internal fragment in all species indicated in the carnivoran family tree in Fig. 3. PCR was performed on 100 ng of genomic DNA, using conserved primers (Table 1) and Accuprime polymerase (Invitrogen), for 50 cycles (30 s at 94°C, 30 s at 55°C, 1 min at 68°C). For species for which a sequenced genome is available, BLASTn was used to assay the presence of either of the envelopes. Finally, for *Hyena-Env2* the full-length reading frame of all four species of Hyaenidae and the empty locus in cat, dog, and the 4 species closest to Hyaenidae according to our tree (*Arctictis binturong*, *Cryptoprocta ferox*, *Suricata suricatta*, and *Helogale parvula*) were amplified by PCR using the above-described protocol. In all sequenced carnivores, the sequence of the empty locus was determined by BLASTn search.

Identification and sequencing of the *Hyena-Env2*-associated provirus and integration site. Inverse PCR was performed by digesting 2 μg of *Crocuta crocuta* genomic DNA with NcoI (New England BioLabs) and inactivating the enzyme at 65°C for 30 min before DNA purification using the gel and PCR cleanup kit (Macherey-Nagel) and self-ligation using T4 DNA ligase (New England BioLabs). PCR amplification was performed with divergent primers (Table 1) and Accuprime DNA polymerase (Invitrogen) for 50 cycles (30 s at 94°C, 30 s at 55°C, and 7 min at 68°C), followed by a nested PCR using the same

TABLE 1 List of primers used in this study[a]

Purpose	Primer name	Sequence
RT-qPCR	Syncytin-Car1-F	5′-CAGACTGCCAAAATCATGA
	Syncytin-Car1-R	5′-AGGAGTAAGCCACATAAATTC
	Hyena-Env2-F	5′-ACGACCTAATTACCCTTGTTC
	Hyena-Env2-R	5′-GCCCCACACCTTCACTACT
	Hyena-Env3-F	5′-AGACCAAGCTAAACACCATCA
	Hyena-Env3-R	5′-CAGTGGGGAGAAAATTTTGTA
	Carnivora-RPL19-F	5′-ACCGTGAATCTAAGAAGATTG
	Carnivora-RPL19-R	5′-TCCATGAGAATCCGCTTGT
Conservation	Hyena-Env2-Int-F	5′-GGCTGCAAATCATAAACCT
	Hyena-Env2-Int-R	5′-CTGTAGCCGTCATGGTCTG
	Hyena-Env2-ORF-F	5′-AACCCCGACAGCCCTGAAA
	Hyena-Env2-ORF-R	5′-AGTGAAGCCCCGAACAATA
	Hyena-Env3-cons-F	5′-CTCCTGGAATATTTCTCTCATG
	Hyena-Env3-cons-R	5′-GTGGGCAGGCGAGAGTA
Locus amplification	Hyena-Env2-Locus-F1	5′-CRGGGYTTTAATTTGTGATC
	Hyena-Env2-Locus-R1	5′-TGCATTTTACTGGTTTCCTTA
In situ hybridization	Hyena-Env2-HIS-F1	5′-ATTGACATTAACCGGACCA
	Hyena-Env2-HIS-R1	5′-AAAAGCCTATGTCGTGACC
	Hyena-Env2-HIS-F2	5′-GGCTGCAAATCATAAACCT
	Hyena-Env2-HIS-R2	5′-CTGTAGCCGTCATGGTCTG
	Hyena-Env2-HIS-F3	5′-CCTAGTCACCCTCTTTCCA
	Hyena-Env2-HIS-R3	5′-GGGGGACCAGTTAAAGATG
	Hyena-Env3-HIS-F1	5′-CAAACCTTACTCCCTGGAGAC
	Hyena-Env3-HIS-R1	5′-ATTAAGTTCAGAGGCCCAAAA
	Hyena-Env3-HIS-F2	5′-GCCCAAATAGGACTCTCTGA
	Hyena-Env3-HIS-R2	5′-TTCCAGGAGGGGTACAGTAG
	Hyena-Env3-HIS-F3	5′-GTTTTCGTTCCCCTTCTCA
	Hyena-Env3-HIS-R3	5′-GATTAAGCATAGGGGTCCC
Inverse PCR	Hyena-Env2-InvPCR-F1	5′-GTAAAAACCCCGCTCACTAT
	Hyena-Env2-InvPCR-F2	5′-CTTCACTCTGCTGCCGC
	Hyena-Env2-InvPCR-R1	5′-GCACCAAATGTTATGTCTGAA
	Hyena-Env2-InvPCR-R2	5′-CCCCTCAGGTGTCATTCC
RACE	Hyena-Env2-3′RACE-F1	5′-CAACCCCGACAGCCCTGAAA
	Hyena-Env2-3′RACE-F2	5′-CTTACTCCTTGCCTCCACCCTCA
	Hyena-Env2-5′RACE-R1	5′-CCCAGGAGCCACAAAAGAAATG
	Hyena-Env2-5′RACE-R2	5′-TGGGGTTTAGGCAAGAGGTGAG
Antibody production	Hyena-Env2-SU-F-BspHI	5′-ATACATTCATGAATCATAAACCTAGATCCACCCC
	Hyena-Env2-SU-R-XhoI	5′-ATACATCTCGAGAGAGTGTTTTTGTTCAGGCTG
Cloning	Hyena-Env2-F-EcoRI	5′-ATACATGAATTCCAAAGAGGACTACCGGGCA
	Hyena-Env2-R-MluI	5′-ATACATACGCGTAGTGAAGCCCCGAACAATA
	Hyena-Env3-F-XhoI	5′-ATACATCTCGAGATCAATAACCTTAAGCGGG
	Hyena-Env3-R-MluI	5′-ATACATACGCGTTTTTACTGTATGCACCCTT
	Carni-SLC1A5-F-EcoRI	5′-NNNNNNGAATTCGCCACCATGGTGGCCGATCCSCC
	Carni-SLC1A5-R-MluI	5′-NNNNNNACGCGTTTACATAACYGAYTCCTTCTCGCAGGG

[a]Underlined sequences correspond to the restriction enzyme site indicated in the primer name.

conditions with nested divergent primers (Table 1). Fragments of >1 kb were gel purified and cloned into a pGEM-T Easy vector (Promega) before sequencing. Sequences were checked for presence of the *Hyena-env* gene, and BLAT analysis was performed to identify the syntenic region of the *Felis catus* genome. Locus primers were designed based on the syntenic region (Table 1) and used to amplify the locus in hyenas and other carnivora.

The full *Hyena-Env2*-associated provirus was amplified from 100 ng of *Crocuta crocuta* genomic DNA using Long Expand DNA polymerase (Roche) with buffer 2, a 0.32 μM concentration of each primer, and the following program: 10 cycles of 10 s at 94°C, 30 s at 55°C, and 8 min at 68°C, followed by 30 cycles of 15 s at 94°C, 30 s at 55°C, and 8 min 20 s/cycle at 68°C. The PCR product was purified and sequenced using primers designed to anneal about every 600 bp.

Characterization of the placental *Hyena-Env2* transcript. Rapid amplification of cDNA ends (RACE) was performed using the SMARTer RACE cDNA amplification kit (Clontech). cDNA was generated from 100 ng of *Crocuta crocuta* placental RNA, and the placental *Hyena-Env2* transcript 5′ and 3′ ends were

amplified using the RACE primers in Table 1 and the Advantage 2 polymerase kit (Clontech) with the following program: 5 cycles of 30 s at 94°C and 5 min at 72°C, followed by 5 cycles of 30 s at 94°C, 30 s at 70°C, and 5 min at 72°C and, finally, 30 cycles of 30 s at 94°C, 30 s at 68°C, and 5 min at 72°C.

Real-time RT-qPCR. *Hyena-env* RNA expression was determined by RT-qPCR. Reverse transcription was performed with 100 ng of DNase-treated RNA as described in reference 42. PCR was carried out with 10 μl of diluted (1:10) cDNA in a final volume of 25 μl using the FastSYBR green PCR master mix (Qiagen) in an ABI PRISM 7000 sequence detection system. Transcript levels were normalized relative to the amount of the housekeeping RPL19 gene (ribosomal protein L19). Samples were assayed in triplicate; see Table 1 for primer sequences.

***In situ* hybridization.** Freshly collected *Crocuta crocuta* placentas were fixed in 4% paraformaldehyde at 4°C and were embedded in paraffin. Serial sections (4 μm) were either stained with hematoxylin and eosin (H&E) or used for *in situ* hybridization. Three PCR-amplified fragments of *Hyena-Env2* (452 bp, 416 bp, and 496 bp) and three fragments of *Hyena-Env3* (415 bp, 448 bp, and 414 bp) (primers are listed in Table 1) were cloned into pGEM-T Easy (Promega) for *in vitro* synthesis of the antisense and sense riboprobes generated with SP6 RNA polymerase and digoxigenin-11-UTP (Roche Applied Science) after cDNA template amplification. *In situ* hybridization of hyena *syncytin-Car1* transcripts was performed using two sets of sense and antisense riboprobes directed against cat *syncytin-Car1* (12). Sections were processed, hybridized at 42°C overnight with the pooled riboprobes, and incubated further at 4°C overnight with alkaline phosphatase-conjugated anti-digoxigenin antibody fragments (Roche Applied Science). Staining was revealed with nitroblue tetrazolium and 5-bromo-4-chloro-3-indoyl phosphate alkaline phosphatase substrates, as indicated by the manufacturer (Roche Applied Science).

Antibody production and immunohistochemistry. A DNA fragment coding for 197 amino acids of the *Hyena-Env2* envelope SU subunit (amino acids 240 to 437; for primers, see Table 1) was inserted into the pET28b (Novagen) prokaryotic expression vector and expressed in BL21(DE3) bacteria. The recombinant C-terminally His-tagged protein was purified from bacterial lysates by nickel affinity chromatography. Mouse immunization was performed in accordance with standard procedures, using female 8-week-old BALB/c mice. Preimmunization sera as well as sera containing polyclonal antibodies were recovered independently from 10 mice and were tested by Western blot analyses using lysates of 293T cells transiently transfected with a Hyena-Env2 expression vector. The most specific sera of the Western blot experiment were then tested for signal strength and absence of background staining by performing immunohistochemistry on 293T cells transfected with an empty vector or a Hyena-Env2 expression vector. For immunohistochemistry, paraffin sections were processed for heat-induced antigen retrieval (Tris EDTA, pH 9; Abcam) and incubated overnight with anti-Hyena-Env2 serum or the corresponding preimmune serum. Staining was visualized using the peroxidase/diaminobenzidine mouse PowerVision kit (ImmunoVision Technologies). All slides were immunostained in cover plates on the same day to obtain a standardized intensity of staining.

Hyena-Env2 Western blot assay. MCF10A human cells were transduced using vesicular stomatitis virus glycoprotein (VSV-G)-pseudotyped HIV particles and a modified Hyena-Env2-expressing CSGW genome as described previously (16). Postselection, cells were lysed using Dulbecco's phosphate-buffered saline (DPBS)–1% NP-40 supplemented with Halt phosphatase inhibitor mixture (Thermo Fisher). Proteins were deglycosylated using PNGase F (New England BioLabs) according to the manufacturer's protocol, separated by SDS-PAGE, and transferred to nitrocellulose membranes. Phosphate-buffered saline–0.1% Tween–5% milk buffer was used for blocking and antibody binding. Anti-Hyena-Env2-SU sera were obtained as described above.

Expression vectors and fusogenicity assays. PCR fragments containing the *Hyena-Env2* and *Hyena-Env3* ORFs were amplified from *Crocuta crocuta* genomic DNA by PCR using the cloning primers (Table 1). They were digested with EcoRI (*Hyena-Env2*) or XhoI (*Hyena-Env3*) and MluI and cloned into the phCMV vector (GenBank accession AJ318514; gift from F.-L. Cosset, Ecole Normale Supérieure de Lyon, France). Cat, dog, and *Crocuta crocuta* SLC1A5 was amplified from placental cDNA using conserved primers (Table 1), and PCR fragments were digested using EcoRI and MluI and cloned into the same phCMV vector as mentioned above.

Hyena-env-pseudotyped MLV particles were produced by cotransfecting 10^6 293T cells with 1 μg of CMVi (MLV *gag-pol* expression vector), 1.5 μg of MFG-nlsLacZ (retroviral genome expressing nls-LacZ), and 0.5 μg of hyena syncytin-Car1, Hyena-Env2, Hyena-Env3, or amphotropic murine leukemia virus (A-MLV) Env expression vectors using the JetPRIME transfection kit (Polyplus transfection). Forty-eight hours posttransfection, virus-containing supernatants were harvested, filtered (polyvinylidene difluoride [PVDF] membranes, pore size, 0.45 μm; Millipore), and transferred to target cells in 24-well plates (5×10^4 to 8×10^4 cells/well) supplemented with Polybrene (final concentration, 8 μg/ml). Spinoculation was performed at 1,200 $\times$ *g* for 2.5 h at room temperature. X-Gal staining was performed 72 h postinfection, and infection foci were counted under microscope. When relevant, target cells were transfected using Lipofectamine LTX (Life Technologies) 48 h before infection.

For coculture cell-cell fusion assays, 2×10^6 HEK293T or G355.5 cells were cotransfected with 2.75 μg of *Hyena-env* expression vector and 2.75 μg of R9SA (an nls-LacZ-expressing reporter plasmid) using Lipofectamine LTX (Life Technologies). Four hours posttransfection, cells were resuspended and seeded alongside target cell lines in 24-well plates at a ratio of transfected cells to target of 20:80, 30:70, or 50:50 and at 200,000 cells/well total. X-Gal staining was performed 24 or 48 h after start of coculture, and the presence or absence of syncytia was assayed under microscope.

All cell lines are described in reference 13 and were grown in Dulbecco modified Eagle medium (DMEM) supplemented with 10% fetal calf serum (FCS; Invitrogen), 100 mg/ml of streptomycin, and 100 U/ml of penicillin at 37°C and 6% CO_2.

Accession number(s). *Crocuta crocuta* placental transcriptome data were deposited at the European Nucleotide Archive under accession number PRJEB23040 (https://www.ebi.ac.uk/ena/data/view/PRJEB23040). DNA sequences of the *Hyena-Env2* ORFs of all extant hyenas, as well as the associated *Crocuta crocuta* provirus and placental transcript, the *Crocuta crocuta Hyena-Env3* ORF, and the *Crocuta crocuta SLC1A5* ORF, were deposited in GenBank (https://www.ncbi.nlm.nih.gov/genbank/) under accession numbers MG805956 to MG805963.

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