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1   Truncation of TRIM5 in Feliformia explains the absence of retroviral restriction

2   in cells of the domestic cat

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4   Running title: Cat TRIM5

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1   ABSTRACT

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3   TRIM5α mediates a potent retroviral restriction phenotype in diverse

4   mammalian species. Here, we identify a TRIM5 transcript in cat cells with a

5   truncated B30.2 capsid binding domain and ablated restrictive function, which,

6   remarkably, is conserved across the Feliformia. Cat TRIM5 displayed no

7   restriction activity but ectopic expression conferred a dominant negative effect

8   against human TRIM5α. Our findings explain the absence of retroviral

9   restriction in cat cells and suggest that disruption of the TRIM5 locus has

10   arisen independently at least twice in the Carnivora, with implications

11   concerning the evolution of host and pathogen in this taxon.

1   TEXT

2

3   One of the major determinants for host restriction of retroviral replication is the

4   longest (alpha) isoform of the host protein TRIM5, a member of the tri-partite

5   motif protein family[34,42]. Human (hu) TRIM5α inhibits pre-integration stages of

6   murine leukaemia virus N-strain (MLV-N) infection while rhesus macaque (rh)

7   TRIM5α inhibits HIV-1 infection[13,16,55]. Recent studies have demonstrated

8   retroviral restriction by TRIM5α species-specific variants from cow (*Bos*

9   *taurus*)[41,56] and rabbit (O*ryctolagus cuniculus*)[38], suggesting a common

10   ancestor for mammalian TRIM5α with antiretroviral properties. Tri-partite motif

11   proteins typically comprise a RING domain with E3-ubiquitin ligase activity

12   capable of auto-ubiquitination[54], a B-Box-2 domain and a coiled-coil domain,

13   collectively know as the RBCC[34]. Additionally, some TRIM proteins including

14   TRIM5α possess a C-terminal B30.2 (PRY-SPRY) domain. TRIM5α blocks

15   reverse transcription in most non-permissive cells[16,42] and evidence suggests

16   that TRIM5α homodimers engage the incoming retroviral capsid in the

17   cytoplasm via the B30.2 domain[18,24,40,44]. The resulting complex is then

18   degraded rapidly by the proteasome[9,47] (proteasomal inhibitors restore

19   reverse transcription but not viral replication[3,53]). Human splice variants

20   TRIM5∂ and TRIM5γ lack a B30.2 domain, disrupting their ability to

21   restrict[30,42]. Moreover, these short TRIM5 isoforms have a dominant negative

22   effect, impairing the activity of full-length TRIM5α presumably by the formation

23   of heterodimers[24,32].

24   Retroviruses have invaded the Felidae on several occasions. Feline leukemia

25   virus (FeLV) and the endogenous retrovirus RD-114 are found in several

26   species within the genus *Felis*, and recently cross species transmissions of

27   FeLV into Iberian lynxes (*Lynx pardinus*) and Florida panthers (*Puma*

28   *concolor*) have been described. In contrast, multiple species of Felidae and

29   one species of Hyenidae have tested seropositive for feline immunodeficiency

30   virus (FIV) and many species harbour monophyletic strains of the virus[8,50].

31   FIV is unique among non-primate lentiviruses in that it infects and depletes

32   CD4+ T-cells, leading to a syndrome analogous to human AIDS[1,31,46].

33   Although FIV and HIV-1 are highly divergent at the nucleotide and protein

level, both viral capsids interact with host factor cyclophilin A (CypA)[20], indicative of shared post-entry interactions between viral and cellular proteins. FIV is susceptible to both primate and non-primate restriction factors[10,14,35,38,39,51] and rhTRIM5α determinants for HIV-1 and FIV restriction overlap, both involving the v1 region of the B30.2 domain[10]. These data suggest shared capsid conformations between the feline and primate lentiviruses. The nature of cat (fe) TRIM5 is unknown, however feline cells are highly permissive to VSV-G pseudotyped retroviral vectors suggesting a TRIM5 null phenotype[6,13,48]. Accordingly, feline cells have been used widely as a negative control permissive cell line in which TRIM5 genes are ectopically expressed and assayed.

In order to explore the permissivity of feline cells to retroviral infection, primers corresponding to conserved regions of huTRIM5α were used to amplify TRIM5 from cDNA derived from Mya-1 primary T-cell line (primers Ts3 directed to exon 2 5'-CATGTGGCCAACATAGTGGAG-3' and Ts16 directed to exon 8 5'-CATAGTCTAGGAAAACTCCAACACG-3'). The resulting product was sequenced and used to identify homologous contigs from the 1.9 fold cat genome[33] using BLAST[2]. Subsequently, primers wam4e from contig AANG01555594 5'- 5'-CAGGGAATTCCTGCT**ATG**GCTTCTGAACTCCTG -3' (start codon in bold) and wam13c against contig AANG01581224 5'-TCATATTTTCGAATCAGTGTGGAATCACGTGAGC-3' were used to amplify and clone the cat TRIM5 into expression vector CXCR. The transcript identified encodes a protein highly related to the primate TRIM5 RBCC (GenBank: GQ183880; Figure S1 in supplementary material). However, the cat transcript bears a stop codon at the proximal exon 8 5' to the v1 region and is thus truncated. Although regions of DNA bearing homology to human and rhesus B30.2 are found 3' to the feTRIM5 stop codon, the sequence bears multiple stop codons in all reading frames. Predicted gene identities on the cat genome browser (GARField, NCI, Frederick, USA) reveal that the TRIM5 gene lies in a region of conserved synteny with TRIM5 paralogues TRIM6, TRIM22 and TRIM34 (Fig. 1A). A Neighbour-Joining tree was constructed using codon-optimised DNA sequences of TRIM5 orthologues and other related TRIM genes (Fig. 1B), revealing domestic cat TRIM5 to be monophyletic with TRIM5 (or TRIM12-2 in mouse, one of an expanded cluster

1 of murine TRIM5 genes[45]) from other mammalian species and was supported
2 by high bootstrap values. Taken with the conserved synteny, these data
3 provide strong evidence that the cat transcript identified is a true TRIM5
4 orthologue.

5 Next we compared TRIM5 expression and identity between members
6 of the order Carnivora. RT-PCR using primers spanning all coding exons
7 shows that expression of the transcript is maintained in all felid species tested
8 (Fig. 2A). To discern the evolutionary history of TRIM5 in the felids, we used
9 degenerate primers to amplify part of TRIM5 exon 8 from genomic DNA of
10 several carnivoran species to discern the point in evolution at which the
11 truncating mutation occurred. Remarkably all species tested from the feliform
12 lineage bore a stop mutation in the same location as cat TRIM5 (Fig. 2B),
13 suggesting that the mutation occurred after the Caniformia/Feliformia
14 divergence but before the Felidae/Hyenidae split estimated at not before 47
15 million years ago (mya)[12] but transcription of the gene has been maintained.
16 The homologous sequence in dog and mink cells was found to lack the stop
17 codon, but has been reported to be disrupted by the insertion of an unrelated
18 gene, PNRC1 in the Boxer breed genome[21,36] and evidence of this insertion
19 exists in the Poodle genome[17] (insertion present in contig AACN010301967).
20 Thus two independent TRIM5 disruption events have taken place during
21 carnivoran evolution. However, although the truncation of cat TRIM5 is
22 compatible with observations that cat cells lack restriction activity, other
23 possibilities such as a read-through transcript with downstream TRIM22, the
24 use of an alternative downstream exon 8 splice acceptor or the splicing of
25 another gene to the 3' end of TRIM5 cannot be excluded. To address this
26 issue, Rapid Amplification of cDNA Ends (3' RACE; Roche, Burgess Hill, UK)
27 was employed using exon 2-specific primer wam4e and an oligo d(T)
28 anchored primer from cDNA derived from cat cell lines Mya-1 and CrFK. The
29 only transcript identified bore an open reading frame identical to that already
30 cloned without 3' modification. Furthermore, given recent findings that TRIM5-
31 CypA fusions have arisen twice during primate evolution, reverse primers
32 directed to feline CypA were used in conjunction with a range of TRIM5
33 forward primers in RT-PCR reactions but no evidence for fusion products was
34 found (data not shown).

To asses the ability of cat TRIM5 to restrict retroviruses *in vitro*, human and cat TRIM5 orthologues were over-expressed in *Fv1*-null murine fibroblasts MDTF[5] (Fig. 3A). Expression of human TRIM5α conferred restriction of MLV-N but not MLV-B[48]. However, cat TRIM5 restricted neither MLV-N nor MLV-B, suggesting that as predicted, the truncating mutation ablates antiretroviral activity. Similarly, no restriction of VSV-G pseudotyped lentiviral vectors derived from HIV-1[4] and SIV[27] from rhesus macaques was observed. Next, we investigated whether the truncated cat TRIM5 could act as a dominant negative for human TRIM5α as has been reported for human TRIM5γ[19,24,32]. A stop codon was introduced into huTRIM5α at the same position (P306) as in the cat TRIM5 as a positive control for dominant negative activity. Cat TRIM5 and huTRIM5 P306STOP were expressed in human TE671 cells which express endogenous TRIM5α and restrict MLV-N[16] potently. Expression of either feTRIM5 or huTRIM5 P306STOP resulted in rescue of the MLV-N titre (Fig. 3B), suggesting that like human TRIM5γ and TRIM5δ, feTRIM5 localises to the cytoplasm and is able to heteromultimerise with functional TRIM5α to prevent optimal restriction.

There is evidence of strong positive selection in primate TRIM5α B30.2 sequences and in their bovine and leporine orthologues[38,41,56], presumably driven by a genetic conflict between restriction factors and viruses over at least the past 33 million years[22,37]. The resulting orthologues are highly divergent in the variable regions of their B30.2 domains and each have specific repertoires of restricted virus[26,29,43]. Thus it is likely that ancestral mammalian TRIM5α, as well as the ancestral carnivoran TRIM5α, possessed this ability and positive selection of TRIM5α may be a feature common amongst non-primate mammals. Concomitantly, escaping TRIM5α restriction may be a widespread requirement for zoonosis and population invasion and the loss of antiretroviral TRIM5 in carnivorans has implications for the evolution and cross-species transmission of retroviruses. Although dogs are currently thought to be free of exogenous retroviruses, high titres of replicating FIV can be obtained in canine cells expressing the FIV receptor CD134[52],

suggesting a lack of intracellular defence (and potential vulnerability) to lentiviruses. Molecular phylogenies of FIV from divergent feliforms broadly reflect phylogenies of the host, suggesting that cross-species transmission is relatively rare[49]. However, this may owe more to the lack of interspecies contact than to robust defences, given that cross-species transmission of FIV has now been observed frequently in captive animals[7,50] and transmission of both FIV and FeLV have been observed in isolated examples of free-ranging animals[11,23,28].

The selective pressure to maintain non-antiretroviral TRIM5 alleles in the carnivorans is unclear. One possibility is the acquisition of a novel function of a truncated TRIM5 in cats. In this study, transcripts of the gene were detected in cDNA derived from domestic cat, wildcat, lion and cheetah, indicating maintenance of TRIM5 gene expression. Thus felid TRIM5 may have an alternative role that does not involve the B30.2 domain (analogous to human TRIM5γ and δ). It has been reported that carnivorans have experienced relatively little endogenous retrovirus (ERV) activity compared to other lineages: there are 13,000 LTR/ERV lineage-specific sequences in the dog genome compared to 133,000 in the human and 470,000 in the mouse genomes[21]. Thus the pressure to maintain anti-retroviral TRIM5α in the Carnivora may have been reduced. Nonetheless, it is possible that other antiretroviral factors such as the APOBEC3 family, tetherin, or unidentifed restriction factors may have compensated for the lack of TRIM5α. Indeed the APOBEC3C genes have recently been shown to possess antiretroviral function and are under adaptive selection in the Felidae[25]. Evolving in the absence of antiretroviral TRIM5α presumably has effects on retroviral evolution. FIV is particularly sensitive to TRIM5α-mediated restriction[35,38,51]; and the absence of endogenous TRIM5α-like activity may have permitted the evolution of FIV towards structural optima that would otherwise be strongly restricted.

The lack of post-entry retroviral restriction in the carnivorans contrasts strongly to the primates where TRIM5α can reduce retroviral infectivity by several orders of magnitude in non-permissive cells. Since primates and carnivorans are currently affected by closely related lentiviruses that infect

and deplete similar cell populations, insights may be gained from direct comparisons between these taxa into the comparative role of TRIM5 and other restriction factors in the evolution and cross-species transmission of lentiviruses.

Figure Legends

Figure 1. Conserved synteny and phylogenetic clustering indicate that cat TRIM5 is a true TRIM5 orthologue. A) Paralogues TRIM6/34/5/22 are found in a cluster in human and dog genomes and in the 1.9x cat genome project. In all cases, the cluster is surrounded by olfactory genes. Exons 2 and 8 of the pseudogenised dog TRIM5 are shown. Other genes in cat, as well as dog TRIM6 are predicted genes or regions of homology only and may not be expressed or functional. B) Neighbour-Joining tree of TRIM5 orthologues from several mammalian species. Cat TRIM5 clusters with other TRIM5 orthologues in preference to closely related paralogues TRIM34, 6 22 and more distantly related TRIM21. Moreover, the TRIM5 phylogeny reflects established mammalian evolutionary relationships. Numbers indicate bootstrap values after 1000 iterations and branch length reflects base substitutions per site. Nucleic acid sequences were codon optimised and aligned using ClustalW with manual adjustment. All positions containing gaps were eliminated from the dataset.

Figure 2. TRIM5 expression is maintained in the felids and the truncation is conserved across the Feliformia. A) TRIM5 transcripts are present in cells from diverse members of the Feliformia. In addition to the *Felis catus* cell lines CrFK and Mya-1, exon 2 to exon 8 TRIM5 transcripts were amplified from cDNA derived from T-cells of cheetah (*A. jubatus*), lion (*P. leo*) and European wildcat (*F. sylvestris*). B) The 5' end of TRIM5 exon 8 was sequenced from genomic DNA from several species to discern at which point in carnivoran evolution the truncation occurred. The stop codon TGA was found in all feliforms examined, but absent from both dog and mink sequences. Imposing the findings on an established phylogenetic tree of the carnivorans[12,15], the truncation of TRIM5 is seen to have taken place before the split of the Felidae and Hyenidae lineages. An independent TRIM5 disruption is proposed to have take place in the Caniformia lineage after the Feliformia-Caniformia split 53.8 mya and is present in modern dogs.

1 **Figure 3. Biological function of cat TRIM5.** A) Mouse fibroblasts which lack
2 restriction activity were transduced with domestic cat (fe) TRIM5 or human
3 (hu) TRIM5α. While huTRIM5α specifically restricts N-tropic but not B-tropic
4 MLV, feTRIM5, which lacks a B30.2 domain restricts neither MLV-N nor MLV-
5 B. Nor is feTRIM5 able to restrict lentiviral vectors derived from HIV-1 and SIV
6 from rhesus macaques. B) Cat TRIM5 acts as a dominant negative against
7 human TRIM5α-mediated restriction. The TE671 cell line, which expresses
8 endogenous TRIM5α, was stably transduced with feTRIM5, or with huTRIM5
9 P306STOP which bears a stop codon at the corresponding residue to
10 feTRIM5. Expression of feTRIM5 or huTRIM5 P306STOP resulted in a rescue
11 of infectivity of MLV-N. Error bars represent mean +/- standard error (n=3).
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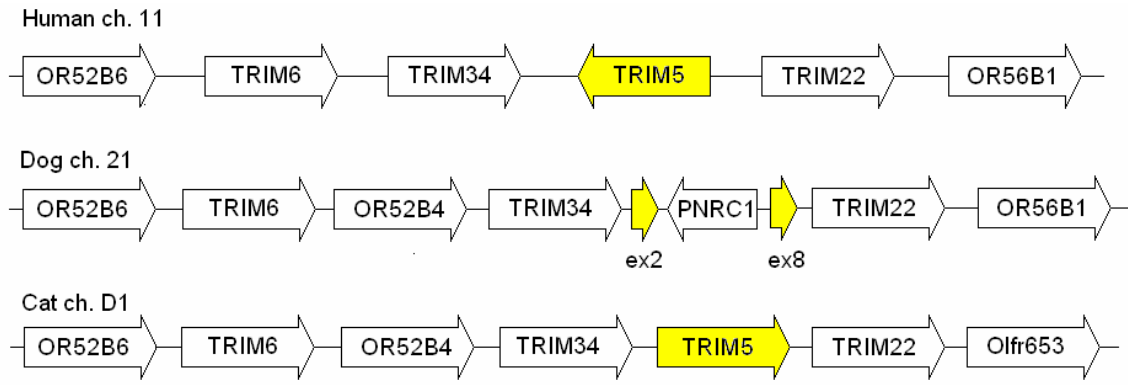
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