Birth, decay, and reconstruction of an ancient *TRIMCyp* gene fusion in primate genomes

Ray Malfavon-Borja[a,b], Lily I. Wu[c], Michael Emerman[b,c], and Harmit Singh Malik[b,d,1]

[a]Department of Genome Sciences, University of Washington, Seattle, WA 98195; and Divisions of [b]Basic Sciences and [c]Human Biology, and [d]Howard Hughes Medical Institute, Fred Hutchinson Cancer Research Center, Seattle, WA 98109

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TRIM5 **is a host antiviral gene with an evolutionary history of genetic conflict with retroviruses. The *TRIMCyp* gene encodes a protein fusion of *TRIM5* effector domains with the capsid-binding ability of a retrotransposed *CyclophilinA* (*CypA*), resulting in novel antiviral specificity against lentiviruses. Previous studies have identified two independent primate *TRIMCyp* fusions that evolved within the past 6 My. Here, we describe an ancient primate *TRIMCyp* gene (that we call *TRIMCypA3*), which evolved in the common ancestor of simian primates 43 Mya. Gene reconstruction shows that *CypA3* encoded an intact, likely active, *TRIMCyp* antiviral gene, which was subject to selective constraints for at least 10 My, followed by pseudogenization or loss in all extant primates. Despite its decayed status, we found *TRIMCypA3* gene fusion transcripts in several primates. We found that the reconstructed "newly born" *TrimCypA3* encoded robust and broad retroviral restriction activity but that this broad activity was lost via eight amino acid changes over the course of the next 10 My. We propose that *TRIMCypA3* arose in response to a viral pathogen encountered by ancestral primates but was subsequently pseudogenized or lost due to a lack of selective pressure. Much like imprints of ancient viruses, fossils of decayed genes, such as *TRIMCypA3*, provide unique and specific insight into paleoviral infections that plagued primates deep in their evolutionary history.**

paleovirology | restriction factor | retroviral capsids

Ancient viruses have selected for changes in host antiviral genes throughout primate evolution (1, 2). Understanding when these adaptive changes occurred, together with how they altered the antiviral specificities of these genes, can lead to strong inferences about the existence of ancient viruses and their consequences on the modern function and specificity of the primate innate immune system. For example, although the TRIM5α protein encodes a retroviral restriction factor that blocks the viral life cycle of several retroviruses (3–8), retroviral specificity varies among primates as a result of ancient selection for changes in antiviral specificity (9–12). These species-specific differences in TRIM5α are due to dramatic variation in both the coiled-coil and B30.2 domains, which are responsible for the interaction with the viral capsid protein of a variety of retroviruses (13, 14). Thus, innovation for capsid-binding specificity has directly resulted in rapid changes in TRIM5α.

An additional form of genetic innovation in the *TRIM5* locus involves novel gene fusions. Such a gene fusion was first identified in owl monkeys (*Aotus trivirgatus*), which encode a fusion protein between the *TRIM5* gene and a retrotransposed *CyclophilinA* (*CypA1*) gene, called the *TRIMCyp* gene fusion (15). The retrotransposition of *CypA* between exons 7 and 8 of owl monkey *TRIM5* (15) occurred 4.5–6 Mya (16, 17). Like TRIM5α, the resulting TRIMCyp protein contains RING, B-box 2, and coiled-coil domains. However, a CypA domain has structurally and functionally replaced the B30.2 domain as the capsid-binding determinant (18, 19). The resulting fusion of *TRIM5* effector domains with the capsid-binding ability of *CypA* in owl monkeys generated a protein with novel antiviral defense activity against HIV-1 (15, 20). This restriction occurs at the same early, post-entry stage before RT as TRIM5α (3, 13, 21–23).

Several macaque species also encode *TRIMCyp*, which is the consequence of another, independent *CypA* retrotransposition (*CypA2* retrogene) downstream of the *TRIM5* gene (24–27). This event is also estimated to have occurred 5–6 Mya (28). Unlike *CypA1* in owl monkeys, the *CypA2*-encoding *TRIM5* allele is found at varying frequencies across macaque species (24, 25, 27, 28). These two *TRIMCyp* gene fusions thus represent a remarkable case of convergent evolution in the generation of novel antiviral specificity in the *TRIM5* locus.

Here, by reconstructing a detailed evolutionary history of *CypA* retrogenes proximal to *TRIM5* across primates, we find two additional currently pseudogenized *CypA* retrogenes that inserted downstream of the *TRIM5* gene 18–43 Mya in primate evolution. One of these (which we refer to as *CypA3* in keeping with prior nomenclature) is still expressed as a novel *TRIMCyp* gene fusion transcript in several Old World monkeys. Our phylogenetic analyses date the origin of *CypA3* to 43 Mya and find that *TRIMCypA3* was maintained as an intact gene for at least 10 My, making it the most ancient *TRIMCyp* yet identified in primates. Although *CypA3* is decayed in all extant primates and the resulting *TRIMCyp* gene fusion is defective, our evolutionary reconstruction and virological assays suggest that *TRIMCypA3* encoded broad and potent restriction activity following its birth. Our findings reveal that convergent evolution has led to at least four independent *CypA* retrogene insertions proximal to *TRIM5* and, consequently, the formation of *TRIMCyp* at least three independent times in primate history. This further reflects the intense, recurrent pressure imposed by ancient viruses. We posit that the currently inactive *TRIMCypA3* gene fusion represents the fossil remnants of an ancient antiviral innovation that points to a retroviral challenge before the common ancestor of all simian primates. Our study highlights the utility of antiviral gene evolution for the study of paleovirology (1, 2).

Results

***CypA* Retrogenes Proximal to *TRIM5*.** The *TRIM5* locus in primates consists of four intact *TRIM* genes: *TRIM22*, *TRIM5*, *TRIM34*, and *TRIM6* (Fig. 1*A*), as well as a *TRIM* pseudogene, *TRIMP1* (Fig. 1*A*, dotted outline). Our analyses revealed the presence of three *CypA* retrogenes proximal to and downstream of *TRIM5* (Fig. 1*A*). The most proximal of these, located ∼1 kb downstream of *TRIM5*

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[1]To whom correspondence should be addressed. E-mail: hsmalik@fhcrc.org.

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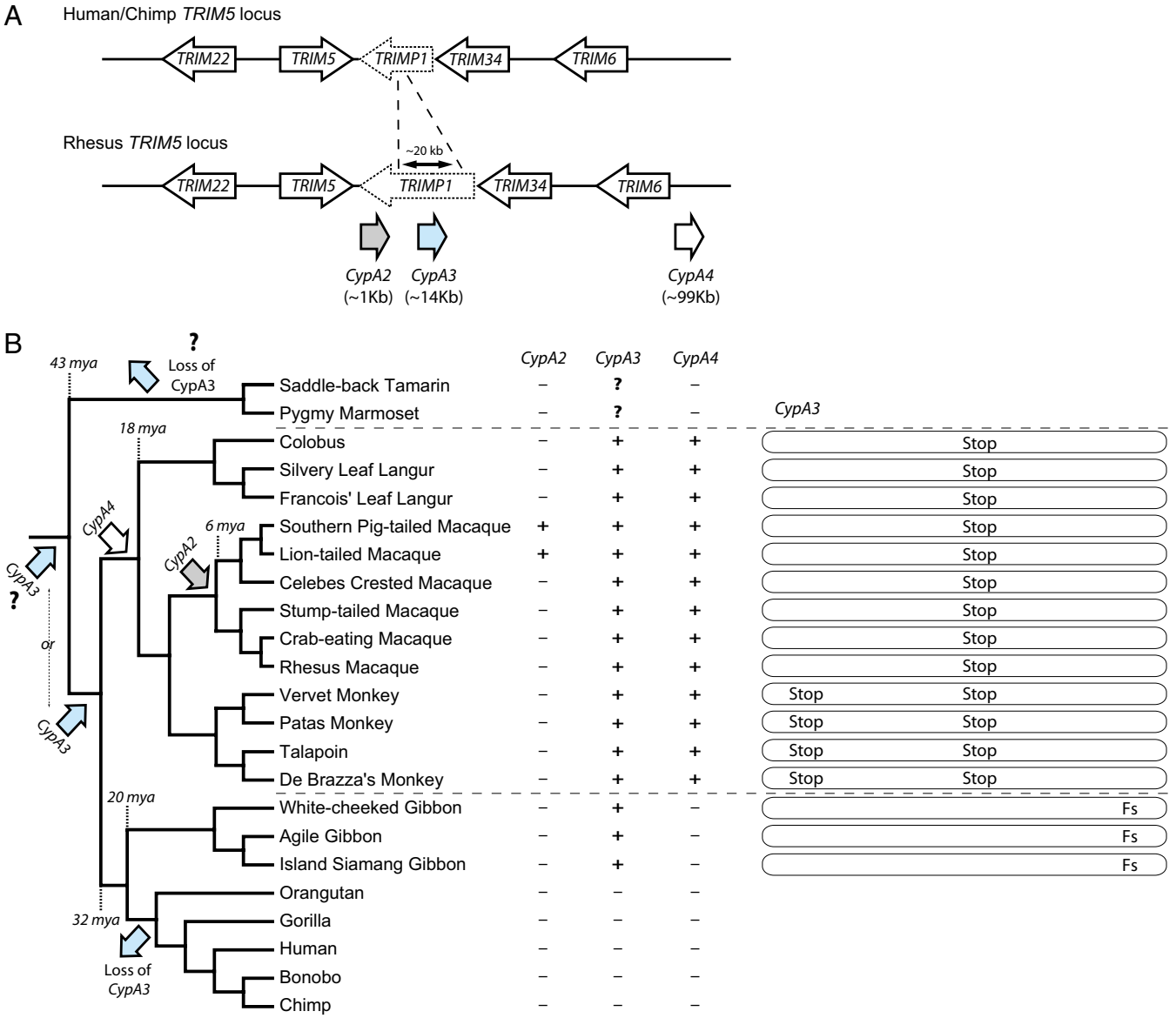


Fig. 1. Summary of *CypA* retrogenes proximal to *TRIM5*. (*A*) Relative locations of the *CypA* retrogenes are displayed below the representations of the *TRIM5* locus. *CypA2* (light gray arrow), *CypA3* (light blue arrow), and *CypA4* (white arrow) retrogenes are ~1 kb, ~14 kb, and ~99 kb downstream of *TRIM5*, respectively. The rhesus macaque *TRIMP1* region contains an additional ~20 kb not present in the human and chimpanzee *TRIMP1*. (*B*) Panel of primates investigated by PCR for *CypA2*, *CypA3*, and *CypA4* is shown in the phylogeny with the notation of retrogene presence or absence indicated to the right. The plus (+) symbol indicates the presence of the *CypA* retrogene. The minus (–) symbol indicates the absence of the *CypA* retrogene. Arrows with labels (*CypA2*, *CypA3*, *Loss of CypA3*, and *CypA4*) indicate the point at which the retrogene was acquired or lost in primate evolution. In our analysis, we recovered *CypA2* from southern pig-tailed and lion-tailed macaques. However, given previous reports of the origin and spread of *CypA2* (28), we could place the date of its acquisition at the root of the macaque lineage. *CypA3* sequences, along with pseudogenizing mutations, are represented for those primates found to encode the retrogene. Stop and Fs denote a stop codon and a frameshift mutation in the *CypA3* sequence, respectively.

(Fig. 1*A* and Fig. S1), is the polymorphic *CypA2* identified in previous studies (24–27, 29) but missing from the reference rhesus macaque genome. We discovered another *CypA*, located ~14 kb downstream of *TRIM5,* which we labeled as *CypA3* (Fig. 1*A* and Fig. S1). *CypA3* lies within *TRIMP1*, which is ~40 kb long in the rhesus macaque genome but ~20 kb shorter in the human and chimpanzee genomes. Finally, we discovered *CypA4,* located ~99 kb downstream of *TRIM5* (Fig. 1*A* and Fig. S1) in the rhesus macaque genome. Neither *CypA3* nor *CypA4* was found in the human and chimpanzee genomes (Fig. 1*B* and Fig. S1).

To determine if the recurrence of *CypA* retrotranspositions into the *TRIM5* locus was greater than what we would expect from random insertions into the genome, we calculated the probability of finding three independent *CypA* retrogenes within 100 kb of rhesus macaque *TRIM5*. We queried available primate genomes for all *CypA* retrogenes and found over 100 *CypA* retrogenes distributed in the human, chimpanzee, and rhesus macaque genomes, consistent with previous analyses of the human genome (30). Based on the number of *CypA* retrogenes and their distribution, we found the probability of the three retrogenes in such close proximity to be highly nonrandom ($P < 0.0233$; *Methods*). We therefore conclude that some recurrently acting selective pressure must have preserved *CypA* retrogenes within the *TRIM5* locus.

Estimating the Age of *CypA2*, *CypA3*, and *CypA4*. We sought to understand the temporal distribution of the *CypA* retrogenes prox-

imal to the *TRIM5* locus among primate species. Using PCR and primers to flanking regions of each retrogene, we genotyped the panel of primate genomes for the presence or absence of *CypA2*, *CypA3*, and *CypA4* (Fig. S1). All *CypA* retrogenes recovered in this analysis were subsequently sequenced to determine their potential to encode a full-length ORF and for phylogenetic analysis. Consistent with previous reports (25, 26), we found *CypA2* to be present in lion-tailed macaque (*Macaca silenus*) and pig-tailed macaque (*Macaca nemestrina*) genomes, each generating an ~2.3-kb band (Fig. S1). We found no evidence of *CypA2* outside of macaques. Our results are consistent with a previous study that showed this retrogene is present only within the macaque lineage that arose 5–6 Mya (25).

In contrast to *CypA2*, we found *CypA3* to be present throughout Old World monkeys as well as in gibbons (Fig. 1*B* and Fig. S1). *CypA3* primers were not expected to generate a PCR product from human and chimpanzee genomes due to an ~20-kb region deletion in *TRIMP1* that corresponds to the genomic region containing *CypA3* (Fig. 1*A*). Results from other hominoids (gorilla and orangutan) suggest that this ~20-kb deletion occurred before the branching of humans and orangutans. We did not observe the presence of *CypA3* in any New World monkey genomes. Investigations of the assembled marmoset genome (WUGSC3.2/calJac3 and GenBank accession no. AC148555) revealed no evidence of *CypA3* within the ~20-kb stretch between *TRIM5* and *TRIM34* (Fig. S2). Additional searches of another New World monkey, Nancy Ma's night monkey (*Aotus nancymaae*; Genbank accession no. AC183999), also did not reveal the presence of *CypA3* in *TRIMP1*. Therefore, based on orthologous *CypA3* retrogenes in gibbons and Old World monkeys (see below), we can estimate that the ancestral *CypA3* retrogene was acquired in primates at least before the Old World monkey/hominoid split (Fig. 1*B*), which occurred 32 Mya (17).

Similar assays revealed that *CypA4* is present in all Old World monkeys assayed but not outside this clade (Fig. 1*B* and Fig. S1). This suggests that *CypA4* retrotransposed before the common ancestor of Old World monkeys, at least 18 Mya (17). Thus, both *CypA3* and *CypA4* considerably predate the macaque-specific *CypA2* retrogene.

Extant Transcriptional Expression of a Pseudogenized *TRIMCypA3* Gene Fusion. To determine whether the retrotransposition of *CypA3* or *CypA4* into the *TRIM5* locus led to the formation of novel *TRIMCyp* gene fusions, we probed total mRNA from fibroblasts from 16 primate species by RT-PCR, with the forward primer located in the RING domain of *TRIM5* and the reverse primer designed to either *CypA3* or *CypA4*. We identified four Old World monkeys (vervet monkey, De Brazza's monkey, patas monkey, and talapoin) that expressed *TRIMCyp* transcripts, which included the *CypA3* retrogene on their 3′-end (Fig. 2). We found three distinct isoforms of *TRIM5-CypA3* (*TRIMCypA3*) transcripts. Only one of these, isoform-1, has its *CypA3* in-frame with *TRIM5* exons, where it would be translated as a *TRIMCyp* gene fusion. Isoform-1 encodes *TRIM5* exons 2–7, a short stretch of the upstream region of *CypA3*, and the *CypA3* coding region. The other two isoforms would not result in an in-frame *TRIMCyp* gene fusion (Fig. 2).

Intriguingly, a shared feature of the gene fusions, including those found in the owl monkey and macaque, is the inclusion of a short segment corresponding to the region immediately upstream of the *CypA* retrogene coding region (Fig. 2, labeled CypA upstream region, and Fig. S3*A*). This short DNA segment, which appears to originate from the 5′-untranslated region of the parental *CypA*

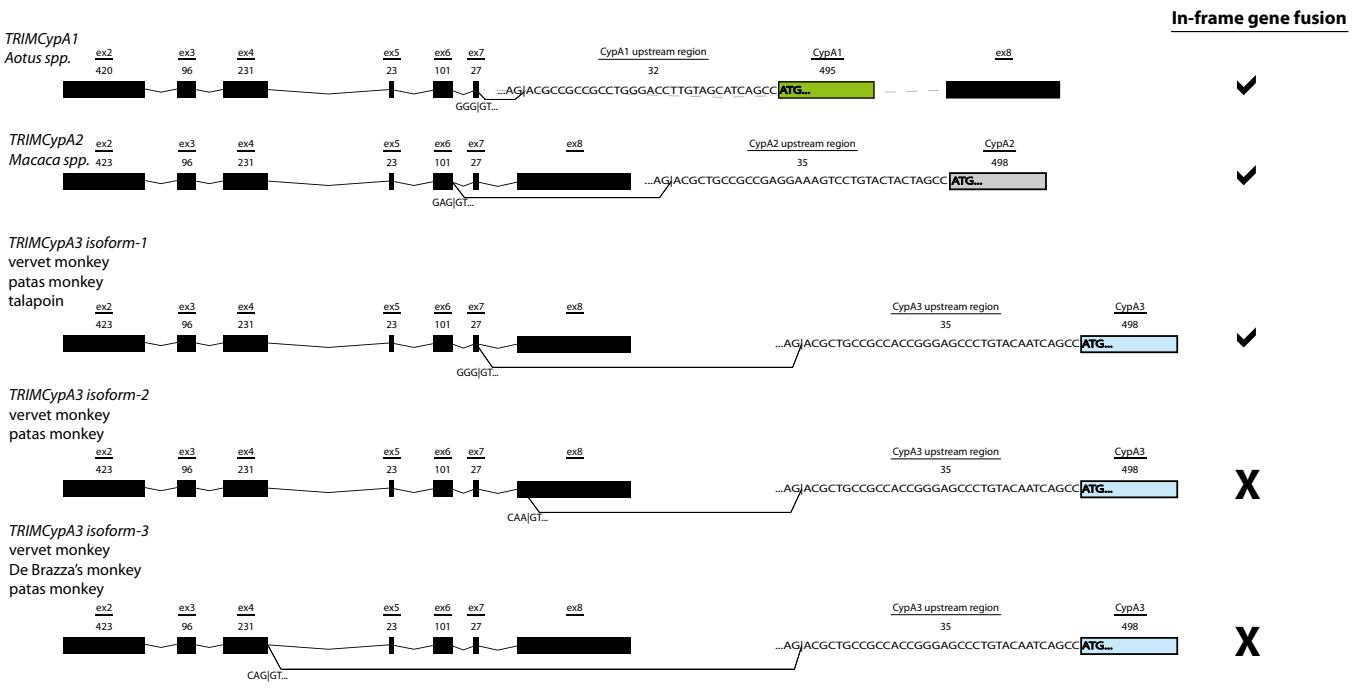


Fig. 2. Structure of *TRIMCyp* transcripts. From a subset of Old World monkeys, we found three *TRIMCypA3* isoforms transcribed. *TRIM5* exons (black blocks) are joined to a stretch of the upstream region of *CypA* and the subsequent *CypA* coding sequence (*CypA1,* green; *CypA2,* light gray; *CypA3,* light blue). We included the sequence of the intron boundaries and the approximate size of each exon. The structures of owl monkey *TRIMCypA1* and macaque *TRIMCypA2* are also presented for comparison (15, 24, 27). The splice acceptor sequence "AGIAC," also present in the *CypA* upstream region, is shown for each retrogene. Isoform-1 encodes *TRIM5* exons 2–7 fused in-frame to the *CypA3* upstream region and *CypA*. In contrast, isoform-2 encodes *TRIM5* exons 2–8 (62 nt of exon 8) to the *CypA3* upstream region and *CypA3* coding region, whereas isoform-3 encodes *TRIM5* exons 2–4 to the *CypA3* upstream region and *CypA3* coding region. To the right, we indicated whether the gene fusion produces a product, where *TRIM5* effector domains are in-frame with *CypA*, with a "check mark," indicating an in-frame product or an "X," indicating that the product would not be in-frame. Both isoform-2 and isoform-3 would result in an "out-of frame" gene fusion with *CypA*.

gene, encodes a cryptic splice acceptor site that appears conserved throughout mammals (Fig. S3*B*). At least among primates, this region provides the splice acceptor site and sequences necessary for an in-frame fusion of the *CypA* retrogene with the *TRIM5* effector domains, thereby facilitating formation of the *TRIMCyp* fusion transcripts.

Sequencing the *CypA3* retrogenes from our PCR survey (Fig. S1) revealed signs of pseudogenization in each case (Fig. 1*B* and Fig. S3), with either a nonsense mutation or a frameshift in their ORF (Fig. 1*B*). A premature stop codon was identified at the 19th codon of *CypA3* in a subset of Old World monkeys (talapoin, patas monkey, and De Brazza's monkey) of the family Cercopithecidae. In addition, all Old World monkey *CypA3* sequences shared a premature stop codon at the site corresponding to the 90th codon of the *CypA* coding sequence. However, neither stop codon is found within the three orthologous, syntenic gibbon *CypA3* sequences from the agile gibbon, island siamang, and white-cheeked gibbon. Instead, all three gibbons encode a frameshift mutation that is predicted to truncate the 3′-end of the *CypA3* coding sequence by 69 nt. Thus, gibbons maintain a large portion of their *CypA3* coding sequence and do not encode the pseudogenizing mutations found in Old World monkeys. Despite encoding a longer intact ORF than Old World monkeys, we did not detect any evidence of a *TRIMCypA3* transcript in gibbons. The *CypA3* found in Old World monkeys was likely pseudogenized in their common ancestor, suggesting that the retrogene has existed as a pseudogene in that lineage of primates for 18 My (17). Likewise, we estimate that gibbon *CypA3* sequences acquired their frame shift mutation 9–20 Mya in either the gibbon or the hominoid common ancestor (17). Thus, although modern *CypA3* sequences are expressed as a *TRIMCyp* gene fusion, the product is likely defective in all extant primates. However, because the pseudogenizing nonsense mutations found in Old World monkey *CypA3* sequences are completely distinct from the frameshift mutation found in gibbon sequences, our analysis strongly implies that *CypA3* in the Old World monkey/hominoid ancestor (*32myoCypA3*) encoded an active ORF (17).

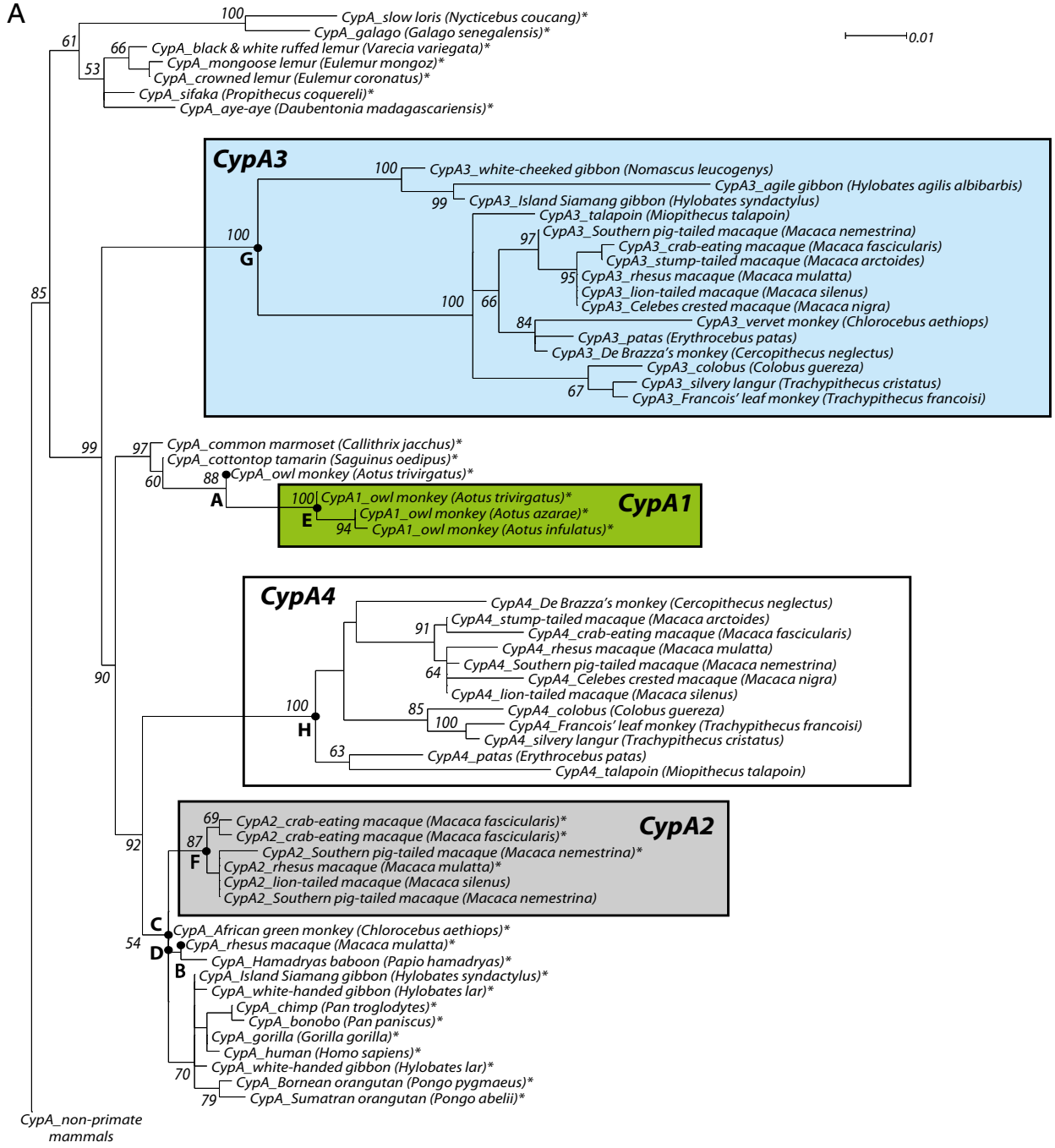
Several attempts to identify a *TRIMCyp* transcript that includes *CypA4* yielded no such product from fibroblast mRNA. It is possible that such a product could be expressed in different tissues. However, it is also likely that *CypA4* never contributed to the formation of a *TRIMCyp* gene fusion, because sequencing of *CypA4* retrogenes revealed a shared indel (2-bp deletion) at the position corresponding to the seventh codon, resulting in a pseudogenizing frameshift. Because the *CypA4* from all Old World monkeys shares this common pseudogenizing mutation, *CypA4* may have become pseudogenized at or shortly after birth.

Evolutionary Analysis of *CypA3*. Despite being decayed in all extant primates, the disparate pattern of pseudogenizing mutations in gibbons vs. Old World monkeys suggests that *TRIMCypA3* might have encoded an active antiviral gene at one time. To test this, we built a phylogeny (Fig. 3*A*) composed of intact functional owl monkey *CypA1* and macaque *CypA2* retrogenes, pseudogenized retrogenes *CypA3* and *CypA4*, as well as primate *CypA* (parental) genes to calibrate the ages of the retrogenes. The phylogeny shows that all four *CypA* retrogenes split into four distinct monophyletic groups and that the tree topology and branch lengths are consistent with the estimated evolutionary origins of the retrogenes. For instance, the closest outgroup to the *CypA* retrogenes that gave rise to *Aotus TRIMCyp* (*CypA1*) is the *Aotus CypA* gene, confirming that *Aotus CypA1* retrogenes are derived from a *CypA* gene within the *Aotus* genus (16). We are unable to gain high resolution within the hominoid and Old World monkey *CypA* (parental) genes due to the very high identity of these sequences, likely the consequence of extremely strong purifying selection (31). However, our phylogenetic analysis also places *CypA2* retrogenes close to the macaque genus, albeit with poor resolution due to the phylogenetic proximity of the Old World monkey and hominoid *CypA* genes.

Our PCR genotyping for the presence or absence of *CypA* retrogenes in primate genomes allowed a tentative dating of their age on a primate phylogenetic tree (Fig. 1). Consistent with our genotyping results, we find that a phylogenetic analysis of the *CypA* sequences themselves shows that *CypA3* and *CypA4* retrogenes are older than *CypA2* retrogenes, with *CypA4* appearing to branch slightly before the common ancestor of the Old World monkeys and hominoids (Fig. 3*A*). Surprisingly, based on the phylogeny, with 99% bootstrap support, we find that *CypA3* was acquired in the simian common ancestor (Fig. 3*A*) at least 43 Mya (17), which is even earlier than the 32 Mya that we had inferred from PCR genotyping (Fig. S1). However, our attempts to detect *CypA3* in New World monkeys were unsuccessful (Fig. S2). This discordance between our genotyping and phylogenetic analysis could be a result of discordance in mutation rates between retrogenes compared with the parental *CypA* genes. However, because we assume that all these retrogenes were the product of a single cycle of retrotransposition (i.e., retrogenes did not give rise to other retrogenes), we do not believe this difference in mutation rates is sufficient to skew our phylogenetic analysis. We also note the consistency between the genotyped and phylogenetic "age" inferences for *CypA1* (16), despite *CypA1* having also gone through a retrotransposition event. Instead, we conclude that the *CypA3* retrogene was independently lost in the lineage of New World monkeys, similar to its loss in some hominoids. Thus, our phylogenetic results clearly reveal *CypA3* as being at least 43 My old, which means that it is, by far, the oldest of the four primate *CypA* retrogenes.

Using both parsimony and likelihood criteria, we were able to reconstruct the sequence of this "intact" Old World monkey/hominoid ancestral *CypA3* (hereafter referred to as *32myoCypA3*) based on extant *CypA3* sequences. Only a single site, residue 144 of *CypA3*, could not be resolved (Fig. S3), and it could encode a proline (P), arginine (R), or histidine (H). To determine whether selective constraints (either diversifying or purifying selection) acted on *CypA3*, we compared the dN/dS ratio of *32myoCypA3* with that of the ancestral parental *CypA* gene from which it likely derived (Fig. 3*B*). Finding a high dN/dS ratio would indicate *CypA3* was under pressure to evolve adaptively, a low dN/dS ratio would be evidence of selective pressure for protein constraint, and a dN/dS ratio ∼1 would indicate an absence of selective pressure. *CypA3* showed evidence suggestive of purifying selection (probability of [dN < dS] = 0.0470–0.0520; Fig. 3*B*). Applying the same analysis to evaluate the modern, functional owl monkey and macaque *TRIMCyps* (Fig. 3*B*), we also find evidence for purifying selection in owl monkey *CypA1* (probability of [dN < dS] = 0.1010) and in macaque *CypA2* (probability of [dN < dS] = 0.0010). In contrast, an analysis of the ancestral version of *CypA4* shows no evidence of selective constraint (probability of [dN < dS] = 0.429). Thus, during the period that it encoded an intact ORF, *CypA3* seems to have evolved under similar selective pressures as both owl monkey and macaque *CypA* retrogenes (Fig. 3*B*).

The dS values of these retrogene-parental gene comparisons are also informative as a rough proxy for their age of divergence (assuming roughly equal rates of evolution at silent sites). Both the owl monkey and macaque *TRIMCyp* gene comparisons have a dS of 0.02 (Fig. 3*B*), consistent with their birth ∼4.5–6 Mya (16, 28). In contrast, the *CypA3* comparison between the parental gene and *32myoCypA3* reveals a dS of 0.04 (Fig. 3*B*), which is twice that of the value estimated for the owl monkey or macaque, suggesting that *CypA3* was preserved as an ORF for at least twice as long as the currently intact *TRIMCyp* gene fusions. This suggests that *CypA3* was preserved as an intact retrogene from the time it was acquired 43 to 32 Mya, when we begin to observe evidence of independent pseudogenization (or loss) events across the primate lineages. The signature of purifying selection (Fig. 3*B*) further suggests the *TRIMCypA3* gene fusion was functional during this period of ∼10 My. Because no extant *CypA3* sequences could be isolated in New World monkeys, the "oldest" version of ancestral *CypA3* that we



B

Pairwise Comparison	dN	dS	Prob. [dN<dS]	Age of retrogene (estimated)
(A) owl monkey CypA gene vs. (E) 'anc' owl monkey CypA1 retrogene	0.011	0.02	0.101	6 million years
(B) macaque CypA gene vs. (F) 'anc' macaque CypA2 retrogene	0.003	0.02	0.001	6 million years
(C) 'anc' OWM-Hominid CypA gene vs. (G) OWM-Hominid 32myo CypA3 retrogene	0.021-0.024	0.04	0.047-0.052	>32 million years
(D) 'anc' OWM CypA gene vs. (H) 'anc' OWM CypA4 retrogene	0.032	0.035	0.429	18 million years

Fig. 3. Phylogeny of *CypA* retrogenes. (*A*) We built a phylogeny of parental *CypA* genes and retrogenes using maximum likelihood methodologies (57). *CypA* gene sequences were collected from rodents (outgroup), prosimians, New World monkeys, Old World monkeys, and hominoids. The *CypA* retrogenes that we included were *CypA1* (green-filled box) from owl monkeys, *CypA2* (light gray-filled box) from macaques, *CypA3* (light blue-filled box), and *CypA4* (white-filled box). Bootstrap support values are shown at the nodes. The phylogeny has been rooted to the rodent parental *CypA* genes: mouse (*Mus musculus*), rat (*Rattus norvegicus*), and squirrel (*Ictidomys tridecemlineatus*). (*B*) We used the K-Estimator program (58) to evaluate the rates of dN and dS for *CypA* retrogenes and computed the probability that dN is significantly different from dS by confidence interval tests.

could faithfully reconstruct represents the version that existed in the last common ancestor of hominoids and Old World monkeys (*32myoCypA3*) 10 My following the birth of *CypA3*.

Testing Ancient and de Novo *TRIMCyp* Proteins for Restriction of Modern or Ancient Lentiviruses. Previous studies have explored the interactions between *CypA* genes and retrogenes with lentiviral capsids (15, 24–27, 32–34). We were therefore interested in assessing whether ancient, potentially active versions of *TRIMCyA3* might have interacted with lentiviral capsids. Both naturally occurring and artificial *TRIMCyp* genes have highlighted the modularity of the *TRIM5-CypA* gene arrangement (35). We therefore designed two synthetic *TRIMCypA3* versions. We elected to use the *TRIM5* effector domains from the owl monkey because the exon structure of *TRIMCypA1* closely resembles *TRIMCypA3*. Thus, the first version (*TRIM5-32myoCypA3)* consisted of owl monkey *TRIM5* effector domains fused to *32myoCypA3* (Fig. 4*A*). Because we were not able to resolve the identity of residue 144 unambiguously from our evolutionary reconstruction, we constructed three separate *TRIM5-32myoCypA3* versions, which encoded a P, R, or H at this site. We also designed a chimera composed of owl monkey *TRIM5* effector domains and the inferred parental *CypA* gene (*TRIM5-parentalCypA*), representing the *CypA* gene from which *CypA3* derived at the time of its birth. Because *CypA* genes have been evolving under strong purifying selection throughout primate history, this approach allows us to evaluate the lentiviral specificity of a de novo *CypA* retrogene unambiguously, representing *TRIMCypA3* immediately following its birth (36).

Consistent with previous results, we observed that owl monkey *TRIMCypA1* was not able to restrict HIV-2 (4) but was able to restrict HIV-1 (15), simian immunodeficiency virus from african green monkeys (SIVagm) (18), and feline immunodeficiency virus (FIV) (37), as well as chimeric viruses encoding the reconstructed capsid of the "paleoviruses" RELIK and pSIV (38) (Fig. 4 *C* and *D*). Remarkably, we found that the *TRIM5-parentalCypA* encodes broad and potent antiviral activity, because it restricts all these lentiviruses tested except a mutant that disrupts the CypA binding site on capsid (HIV-1 G89V) (Fig. 4 *C* and *D*). Thus, *TRIM5-parentalCypA* could restrict all representatives tested from the modern-day lentiviruses and the paleolentiviruses (Fig. 4 *C* and *D*). The variation between the slightly narrowed binding specificity of CypA1 and the broad specificity of parentalCypA is attributed to four amino acid differences that occurred during *CypA1* evolution (Fig. 4*A*). On the other hand, we also found that *TRIM5-32myoCypA* could not restrict any of the paleolentiviruses or modern-day lentiviruses (Fig. 4 *B* and *C*). Depending on the ambiguous residue 144, parentalCypA and 32myoCypA3 differ at seven (or eight) residues (Fig. 4 *A* and *B*), one of which has also independently occurred during *CypA1* evolution. We attempted to explore the loss of restrictive ability by evaluating residues unique to 32myoCypA3 within the parentalCypA backbone using both *32myoCypA3* (*P144*)/*parentalCypA* and *parentalCypA*/*32myoCypA3* (*P144*) chimeras (Fig. S4*A*). We found that the loss of restriction activity in 32myoCypA3 could not be reversed by single residue changes (Fig. S4*B*), suggesting that this observed loss of restrictive ability in *32myoCypA3* is attributable to a combination of multiple residues among the seven (or eight) residues specific to the *32myoCypA3*-encoded protein (Fig. 4 *A* and *B*). These results indicate that the most ancient version of the *Trim5-CypA* fusion gene had the broadest specificity for restriction of retroviruses but that subsequent evolution either narrowed its specificity to (ancient) retroviral capsids that we were not able to test in our assays or destroyed this activity.

Discussion

Recurrent *TRIM5-CypA* Gene Fusions Across the Primate Phylogeny. Retrotransposition of *CypA* retrogenes proximal to the *TRIM5* locus has the instantaneous effect of creating a new restriction factor, potentially expanding the restrictive range of primate genomes (24–27). Including the present study, at least three such instances of *TRIMCyp* gene fusion are now documented in primate genomes. In addition to the still active *CypA1* and *CypA2* retrogenes that were born in the owl monkey and macaque species 4.5–6 Mya, we have identified a third, much more ancient retrogene that is still present as a fusion transcript and likely encoded a putative restriction factor 43 Mya in primate history. This remarkably convergent retrotransposition proximal to *TRIM5*, in contrast to the frequent but otherwise random pattern of *CypA* retrogene insertions elsewhere in primate genomes, strongly suggests that the *CypA* retrogene-bearing haplotype must have had a strong enough selective advantage to sweep through populations and species. Based on the potent antiviral activity of TRIMCyp fusion proteins, we posit that it is most likely that this selective advantage was conferred by protection against an ancient viral infection.

Previous studies have suggested that *CypA* fusions function in concert with a variety of *TRIM* genes (35, 39); however, only *TRIM5* has recurrently been revealed to accommodate a functional gene fusion with *CypA* naturally. It may be that the expression patterns of other *TRIM* genes could not accommodate a functional antiviral gene fusion without compromising endogenous function. Alternatively, given that *TRIM* genes homomultimerize via their B-box and coiled-coil domains, homomultimerization of a *TRIM* gene with *CypA* might have had deleterious consequences that would only be tolerated when this involved a canonical restriction factor like *TRIM5* but perhaps not a *TRIM* gene that plays an essential housekeeping function in the cell. Consistent with this hypothesis, our survey of rhesus macaque, chimpanzee, and human genomes has not revealed any other *TRIMCyp* candidates in which a *CypA* retrogene was found within 20 kb of a *TRIM* gene.

The retention pattern of *TRIMCyp* genes could pose a cost to antiviral defense and the cell. In the case of *CypA1* and *CypA2*, it precludes the production of a B30.2-containing TRIM5α from that allele, providing a tradeoff in terms of restrictive potential. This would explain why *CypA2* has variably swept through to fixation in macaque species (40), likely as a result of balancing selection, as seen previously in the *TRIM5* locus of macaque populations (41). An additional explanation could be that the fusion of the TRIM5 E3 ubiquitin ligase domain to a CypA protein that may bind numerous client proteins in the primate proteome increases the toxic burden of such gene fusions, or leads to aberrant cell signaling (42). In light of this "cost," if the restriction activity of the evolved *TRIMCyp* is obviated, either because the restricted viral capsid is eliminated or evolves away from *TRIMCyp* recognition, the advantage to retain the *TRIMCyp* gene fusion diminishes greatly. It is also possible that rapid evolution of the B30.2 domain of TRIM5α to recognize the target retroviral capsid might obviate the need to maintain *TRIMCyp*. Thus, it is not surprising that the evolution of these gene fusions is recurrent and dynamic, both remarkably convergent but also relatively short-lived in evolutionary time. This would also help explain why *TRIMCypA3*, which may have encoded an active antiviral protein in primate history, is now an extinct gene. Such "extinct" *TRIMCyp* gene fusions have also been identified outside primates. Indeed, such a pseudogenized *TRIMCyp* gene fusion (*ftr52*) was recently identified in fish genomes (43). Finally, even though the CypA domain of *TRIMCypA3* has decayed, it is formally possible that the *TRIMCypA3* fusion transcript in some primates still serves the same function as some of the alternate TRIM5 isoforms, to attenuate TRIM5α function (44).

***CypA3* as a Potential Paleoviral Marker in Primate Evolution.** Based on a relatively abundant record of endogenization revealed by sequencing and bioinformatic efforts, retroviral lineages have been shown to date back many millions of years (45, 46). Indeed, lentiviruses have been estimated to be at least 4 My old in primates (47) and ~12 My old in other mammals based on the presence of endogenous copies within the host genome, although these dates are likely vast underestimations of the true age of lentiviruses (48–51).

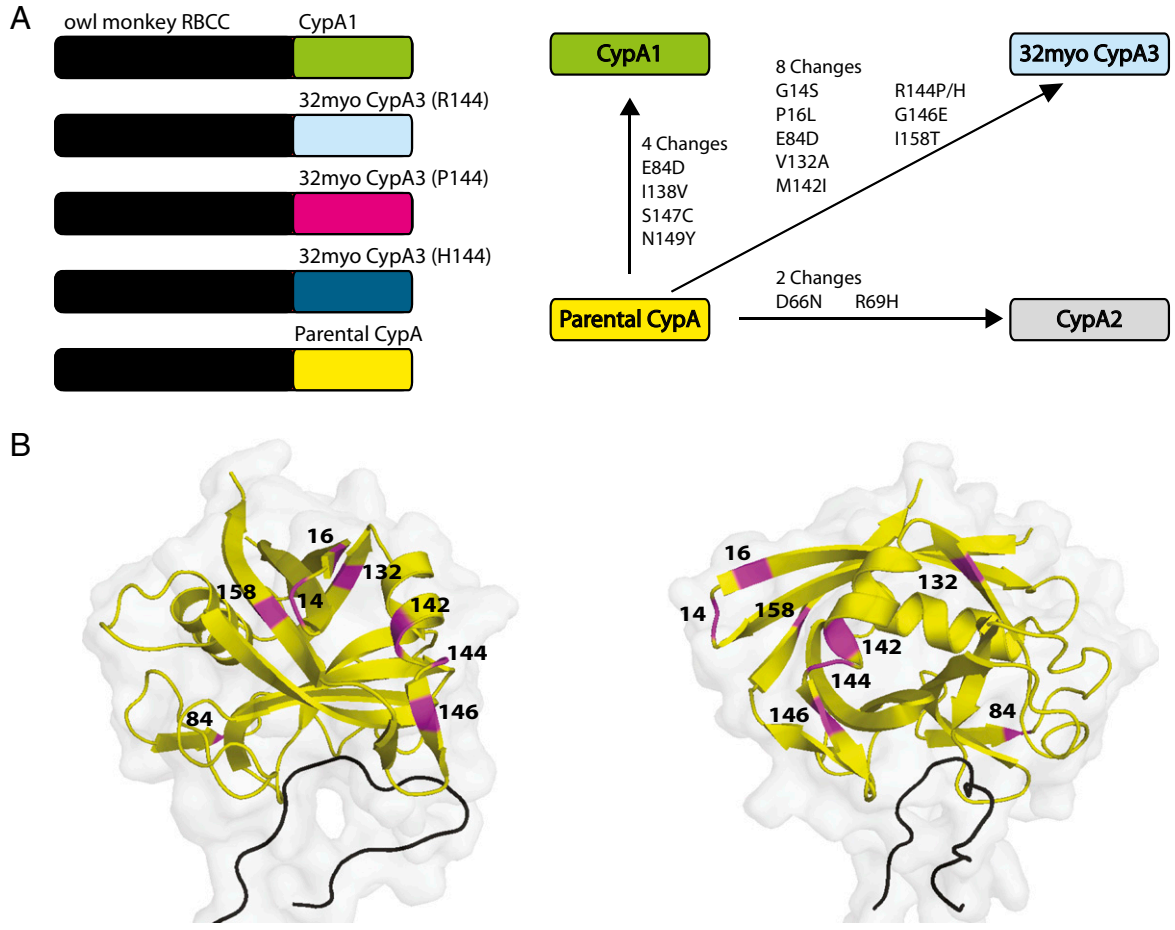


Fig. 4. Reconstructed ancestral *CypA3* vs. modern and extinct (reconstructed) lentiviruses. (*A*) Cartoon representations of the owl monkey TRIMCypA1 (CypA1, green), owl monkey TRIM5-32myoCypA3 [32myoCypA3 (R144), light blue; 32myoCypA3 (P144), magenta; 32myoCypA3 (H144), teal], and owl monkey TRIM5-parental CypA (yellow) gene fusions, with the owl monkey TRIM5 effector domains (RING, B-box, coiled-coil) represented as a black block. Differences in residues encoded by CypA1, parental CypA, CypA2 (light gray), and 32myoCypA3 have been identified and listed according to the direction of the arrow. (*B*) Eight residues unique to 32myoCypA3 (P144) (magenta) can be mapped onto a structure of parental CypA (yellow) interacting with capsid (black) using PyMOL (The PyMOL Molecular Graphics System, Version 1.5.0.4 Schrödinger, LLC). (*C*) Stable CRFK cell lines encoding an empty vector (black box), owl monkey *TRIMCypA1* (green box), *TRIM5-32myoCypA3 (R/P/H144)* (light blue, magenta, and teal boxes, respectively), and owl monkey *TRIM5-parentalCypA* (yellow box) were assayed against chimeric EIAV encoding the ancient (resurrected) capsid of paleolentiviruses RELIK and pSIV (38) and the modern lentiviruses HIV-1 (LAI strain), HIV-1 G89V0, HIV-2 (ROD9 strain), and FIV (9, 56). (*D*) SIVagm. Viruses are listed along the *x* axis. The *y* axis reflects virus infectivity, determined by the percentage of cells infected with GFP-expressing virus, normalized to 100% for infections against CRFK cells encoding an empty vector. The virus inoculums were standardized to give the absolute percentage of GFP between 15% and 30%. In the case of SIVagm, this system used a luciferase reporter. Shown is a representative experiment that was repeated three times. We confirmed the stable expression of TRIMCyp proteins by Western blot analysis, using 30 μg of protein extract for each sample (lane 1, owl monkey TRIMCypA1; lane 2, TRIM5-32myoCypA3 (R144); lane 3, P144; lane 4, H144; lane 5, TRIM5-parental CypA; lane 6, CRFK with an empty LPCX vector).

In response to these retroviral challenges faced throughout their evolution, primates encode a number of intrinsic mechanisms with the capability of inhibiting viral replication. Positive selection of such restriction factors is a potent mechanism for primate genomes to respond to novel or adapted viral pathogens (9), but it is not the only mode of adaptation. Primate genomes also use other mechanisms, such as gene duplications (52), and in the case of *TRIMCyp*, recurrent gene fusions, to respond to new viral challenges.

TRIMCyp evolution not only serves to belie the traditional view that retrotransposed genes are evolutionary dead ends but suggests that *CypA* retrogenes are highly labile modules that can be gained and lost throughout primate history. Although whole-gene dN/dS analyses strongly suggest that *CypA3* evolved under purifying selection for 10 My following its birth, we identified seven (or eight) residues within *32myoCypA3* that differentiate a loss of capsid-binding from broad-range capsid-binding (as exhibited by *parentalCypA*). Similarly, only four residues separate the broad binding of *parentalCypA* to the narrowed binding specificity documented from *CypA1*. Macaque *CypA2* further demonstrates this trajectory of narrowed binding specificity (40). In the cases of *CypA1* and *CypA2*, deviation from broad capsid-binding evolved within 6 My. Therefore, although broad capsid binding appears as a predisposed feature of *CypA*, the specificity that each *CypA* retrogene evolves is determined by minor changes that have a great impact on the capsid-binding trajectory (28, 33, 40). Based on our results with *TRIM-parentalCypA*, we predict that *TRIMCypA3* was also capable of interacting with a broad range of lentiviral capsids on birth. Similar to the specificity-narrowing changes that occurred during *TRIMCypA1* and *TRIMCypA2* evolution, we posit that in the 10 My after its birth, *TRIMCypA3* narrowed its specificity to restrict only ancient retroviruses rather than any of the retroviruses we tested. Finally, after the utility of *TRIMCypA3* as a retroviral restriction factor was exhausted ~32 Mya, the *TRIMCypA3* gene decayed in all extant primates.

From a paleovirology perspective, even currently inactive or pseudogenized *CypA* retrogenes may represent remnants of antiviral genes that were active at an earlier time in primate evolution. We propose that ancient *TRIMCypA3* arose in response to a pathogen encountered by evolutionarily successful ancestors. Although it is formally possible that the true target of *TRIMCypA3* was a nonlentiviral or even a nonretroviral pathogen, there is little precedent for this conjecture. It is also unlikely that *TRIMCypA3* was a genomic innovation due to some other "housekeeping" adaptation, based both on the intrinsic costs of *TRIM5-CypA* gene fusions and the recurrent pseudogenization/loss of *TRIMCypA3* in extant primates. Instead, we propose that the birth and demise of *TRIMCypA3* are more consistent with the model wherein it helped protect host genomes against viral invasions for at least 10 My of primate history. Thus, "fossil" antiviral genes like *CypA3* provide unique paleoviral insight into viral challenges encountered by primate ancestors 43 Mya and complement the incomplete fossil record of retroviral imprints in animal genomes.

Methods

Identifying *CypA* Retrogenes Proximal to *TRIM5*. The human *CypA* gene (NC_000007) and mRNA (NM_021130) sequences were used as query sequences in a BLAST-like alignment tool (BLAT) analysis to identify *CypA* homologs (53). BLAT searches were performed from the University of California, Santa Cruz Genome Browser on the human (*Homo sapiens*), chimpanzee (*Pan troglodytes*), and rhesus macaque (*Macaca mulatta*) genomes (54). For each of the primate genomes, the BLAT search results from the two query sequences were combined to assemble a comprehensive list of *CypA* homologs that was evaluated and compiled into a catalog of *CypA* retrogenes. *CypA* retrogenes were mapped back to their respective primate genome, and *CypA* retrogenes proximal to *TRIM5* were identified (Fig. 1). *CypA* retrogenes were named according to their distance from *TRIM5* and based on previously established nomenclature. These *CypA* retrogenes were then evaluated for an ORF, indels, and premature stop codons. We also catalogued the distribution of *CypA* retrogenes and organized these based on the number of *CypA* retrogenes found in a random stretch of 100 kb of the evaluated primate genomes. To calculate the probability of multiple *CypA* insertions within a given distance, 100 kb in this case, we counted the number of 100-kb stretches that contained 0–10 *CypA* retrogenes. We focused on rhesus macaques because the largest number of events in which multiple *CypA* retrogenes could be found in any 100-kb stretch of its genome was reported in this species. We identified 116 cases of only finding 1 *CypA* retrogene within 100 kb. In addition, we identified four cases of finding 2 *CypA* retrogenes and one case of finding 3 *CypA* retrogenes within 100 kb of each other in the rhesus macaque genome. Thus, of 129 total *CypA* retrogenes in the rhesus macaque genome, only 3 *CypA* retrogenes could be found within 100 kb of each other (proximal to the *TRIM5* locus), which we can calculate as a probability ($P = 0.02325$).

Determining the Presence or Absence of Proximal *CypA* Retrogenes. Genomic DNA (gDNA) was isolated from primate fibroblast cells purchased from Coriell Cell Repositories. The primate panel was composed of human, chimpanzee (ID no. 3448), bonobo (*Pan paniscus*, ID no. 5253), gorilla (*Gorilla gorilla*, ID no. 5251), orangutan (*Pongo pygmaeus*, ID no. 5252), island siamang gibbon (*Hylobates syndactylus*, PR00722), agile gibbon (*Hylobates agilis albibarbis*, PR00773), rhesus macaque (ID no. 7098), crab-eating macaque (*Macaca fascicularis*, ID no. 3446), celebes-crested macaque (*Macaca nigra*, ID no. 7101), pig-tailed macaque (*M. nemestrina*, ID no. 8452), stump-tail macaque (*Macaca arctoides*, ID no. 3443), lion-tailed macaque (*M. silenus*, OR1890), silvery leaf langur (*Trachypithecus cristatus*, bl.4381), Francois' leaf langur (*Trachypithecus francoisi*, PR01099), colobus (*Colobus guereza*, PR00980), talapoin (*Miopithecus talapoin*, PR00716), patas monkey (*Erythrocebus patas*, ID no. 6254), De Brazza's monkey (*Cercopithecus neglectus*, PR01144), pygmy marmoset (*Callithrix pygmaea*, OR690), and saddle-back tamarin (*Saguinus fuscicollis nigrifrons*, OR621) species. gDNA from this diverse primate panel, representing New World monkeys, Old World monkeys, and hominoids, was used to determine the presence or absence of *CypA2*, *CypA3*, and *CypA4* throughout primates in a PCR survey. All PCR reactions were performed using 25-μL reaction volumes and the PCR SuperMix High Fidelity (Invitrogen) reagent. The thermocycler parameters were 94 °C for 3 min; 39 cycles at 94 °C for 15 s, 60 ° for 15 s, and 72 °C for 2 min; and a final extension step at 72 °C for 10 min. All products were directly sequenced using BigDye sequencing (Applied Biosystems).

CypA2 reactions were performed using primers 105 (forward: 5′-CTGTGCTCACCAAGCTCTTGAAC-3′) and 103 (reverse: 5′-TCCCACATAATTCAGTTTGTTTGATAAA-3′), and *CypA4* reactions were performed using primers 108 (forward: 5′-AATCTGCTGGCACCTTGTTTTGTAC-3′) and 110 (reverse: 5′-TAGCTTTTGGGCAGCTAGGAGG-3′). We used nested PCR analysis to amplify *CypA3* from primates, with the first-round primers being 87 (forward: 5′-GAACTACTTGAATCCAGGAGGCAGA-3′) and 101 (reverse: 5′-TATCCTCTTTTTGAATCAATTCCTTTGTCA-3′) and the second round primers being 100 (forward: 5′-GCAGGAGTAAGTCCTCACCTATC-3′) and 84 (reverse: 5′-TTATTCGAGTTGTCCACAGTCAGCAG-3′).

Detecting TRIM5-*CypA3* and TRIM5-*CypA4* Transcripts. A two-step RT-PCR/seminested PCR-based method was used to amplify *TRIMCyp* from primate RNA. The primates used were human, chimpanzee, island siamang gibbon, agile gibbon, talapoin, patas monkey, De Brazza's monkey, vervet monkey (*Cercopithecus aethiops*, PR01190), Francois' leaf monkey, colobus, rhesus macaque, woolly monkey (*Lagothrix lagotricha*, ID no. 5356), spider monkey (*Ateles belzebuth*, KB6701), titi monkey (*Callicebus donacophilus*, OR1522), and owl monkey (*Aotus trivirgatus*, CRL-1556). Total RNA was isolated from fibroblast cells purchased from Coriell Cell Repositories.

The initial RT-PCR step was performed using a primer designed to the start of the coding region of the *TRIM5* gene (primer 80) and an oligo-dT reverse primer. This primer combination was used to amplify all products encoded by the *TRIM5* gene. Next, we used either a *CypA3*- or *CypA4*-specific reverse primer in combination with primer 80 to confirm the transcription of a *TRIMCyp* gene fusion. RT-PCR reactions were performed using SuperScript III Reverse Transcriptase with Platinum Taq (Invitrogen) in 12.5-μL volume reactions. The RT-PCR parameters were an initial RT step at 50 °C for 30 min; followed by 34 cycles at 94 °C for 15 s, 60 °C for 15 s, and 68 °C for 3 min; and a final extension at 72 °C for 10 min. A 1:300 dilution of the RT-PCR product was then prepared for the subsequent seminested PCR step. This was performed using primers 80 and 73 (reverse: 5′-TTATTMGAGTTGTCCACAGTCAGCARTRGTGA-3′) to amplify *TRIMCyp* without targeting a specific *CypA* retrogene. The PCR parameters were kept unchanged. All products were TOPO TA (Invitrogen) cloned and BigDye sequenced using M13 universal primers.

Construction of *CypA* Phylogeny, Alignment, and *32myoCypA3*. The nucleotide sequences of modern *CypA* genes and *CypA1–4* retrogenes were used to build an alignment of all *CypA* sequences using Clustal W2 (55). This was done for *CypA* sequences at the nucleotide and protein levels. The nucleotide alignment was used in reconstructing the 32-My-old form of *CypA3* (*32myoCypA3*). We were able to use a parsimony-based approach to reconstruct the sequence of *32myoCypA*, which was in agreement with a maximum likelihood reconstruction.

Phylogenetic trees were generated using Mr. Bayes (version 3.1) in the construction of the *CypA* phylogeny. We performed 1 million Markov chain Monte Carlo generations with a sampling every 1,000th generation and discarded the first 250 samplings as run-in.

Assessing Ancient and de Novo *TRIMCyp* Proteins for Antiviral Activity. To test *32myoCypA3* for the ability to interact with viral capsid protein, we used "stitch-PCR" to join the *TRIM5* effector domains (RING, B-box, and coiled-coil) from owl monkey *TRIMCypA1* to *32myoCypA*. All PCR parameters were as previously mentioned. The first-round set of stitch-PCR used primers 144 (forward: 5′-GCGCTTCTCGAGGCCACCAT-3′) and 134 (reverse: 5′-GGGG-TTGACCATGGCTGATGCTAC-3′) to amplify the *TRIM5* region of owl monkey *TRIMCypA1* and primers 133 (forward: 5′-GTAGCATCAGCCATGGTCAACCCC-3′) and 177 (reverse: 5′-GCGCGCTTATCGATGAATTCTTATTC-3′) to amplify *32myoCypA*. Dilutions of the first-round products were combined and stitched together by PCR using primers 144 and 177. This PCR product was sequenced to verify the successful construction of the *TRIM5-32myoCypA3* gene fusion. Owl monkey *TRIMCypA1* and *TRIM5-32myoCypA3* were cloned into the expression vector pLPCX and then transduced into Crandell-Rees feline kidney (CRFK) cell lines to establish the following stable cell lines: CRFK (owl monkey *TRIMCypA1*) and CRFK (*TRIM5-32myoCypA*). *TRIM5-32myo-CypA3* P144R and P144H were generated using a QuikChange II Site-Directed Mutagenesis Kit (Agilent). A CRFK cell line containing pLPCX without an insert was also established to serve as a negative control. The gene fusion of owl monkey *TRIM5* effector domains and *parentalCypA* (*TRIM5-parentalCypA*) was built by first amplifying the mRNA from the rhesus macaque *CypA* gene with primers: 262 (forward: 5′-CTGGGACCTTGTAGCATCAGC-CATGGTCAACCCCACCGTGTTCTTC-3′) and 264 (reverse: 5′-GCGCGCTTATC-GATGAATTATTCGAGTTGTCCACAGTCAGCAATG-3′). Next, the owl monkey *TRIM5* amplicon was combined with the rhesus macaque *CypA* gene using primers 177 and 264. We confirmed TRIMCyp protein expression in the appropriate cell lines by Western blot analysis.

We used the following viruses in assessing our cell lines: HIV-1 (LAI strain), HIV-2 (ROD9), FIV, RELIK, and pSIV, and we used no virus as a control. RELIK and pSIV were prepared by cotransfection of 293T cells with pL-vesicular stomatitis virus-G, pCMV-tat, equine infectious anemia virus (EIAV) GFP 6.1 (encoding the genome of EIAV with a GFP expression cassette), and either pEIAV-RELIK or pEIAV-pSIV (38) (a kind gift from Melvyn Yap, MRC National Institute for Medical Research, London). Viruses were harvested by collecting supernatant and titered on CRFK cells to determine the dose of virus that would infect between 15% and 30% of the cells in a 12-well plate based on flow cytometry for GFP expression. Other viruses were similarly constructed and assayed (9, 56). For infection assays, cell lines were seeded onto 12-well plates and subsequently infected with the aforementioned viruses using the predetermined viral titers. Three days postinfection, cells were collected from the 12-well plates and suspended in fixing agent for immediate analysis by flow cytometry. SIVagm infections were performed using 96-well plates. We did not need to fix RELIK- or pSIV-infected samples before flow cytometry. In all experiments, mock-infected cells were used to set the GFP gate.

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