JOURNAL OF VIROLOGY, Mar. 2008, p. 2089–2096 Vol. 82, No. 5
0022-538X/08/$08.00+0 doi:10.1128/JVI.01828-07

Antiretroviral Activity of Ancestral TRIM5α▽

Valérie Goldschmidt,[1]† Angela Ciuffi,[1]† Millan Ortiz,[1] David Brawand,[2] Miguel Muñoz,[1] Henrik Kaessmann,[2]* and Amalio Telenti[1]*

Institute of Microbiology, University Hospital, 1011 Lausanne,[1] and Center for Integrative Genomics, University of Lausanne, 1015 Lausanne,[2] Switzerland

Received 20 August 2007/Accepted 27 November 2007

The antiretroviral protein TRIM5α is known to have evolved different restriction capacities against various retroviruses, driven by positive Darwinian selection. However, how these different specificities have evolved in the primate lineages is not fully understood. Here we used ancestral protein resurrection to estimate the evolution of antiviral restriction specificities of TRIM5α on the primate lineage leading to humans. We used TRIM5α coding sequences from 24 primates for the reconstruction of ancestral *TRIM5*α sequences using maximum-likelihood and Bayesian approaches. Ancestral sequences were transduced into HeLa and CRFK cells. Stable cell lines were generated and used to test restriction of a panel of extant retroviruses (human immunodeficiency virus type 1 [HIV-1] and HIV-2, simian immunodeficiency virus [SIV] variants SIV_{mac} and SIV_{agm}, and murine leukemia virus [MLV] variants N-MLV and B-MLV). The resurrected TRIM5α variant from the common ancestor of Old World primates (Old World monkeys and apes, ~25 million years before present) was effective against present day HIV-1. In contrast to the HIV-1 restriction pattern, we show that the restriction efficacy against other retroviruses, such as a murine oncoretrovirus (N-MLV), is higher for more recent resurrected hominoid variants. Ancestral TRIM5α variants have generally limited efficacy against HIV-2, SIV_{agm}, and SIV_{mac}. Our study sheds new light on the evolution of the intrinsic antiviral defense machinery and illustrates the utility of functional evolutionary reconstruction for characterizing recently emerged protein differences.

A newly described form of innate immunity, coined "intrinsic immunity," provides a constitutive line of defense, which relies on intracellular obstacles to hinder the replication of pathogens (1). This component of the immune system has gained much attention as a cornerstone of the resistance of mammals against several classes of retroelements and retroviruses (43).

Representative components of this cellular defense system include members of the tripartite motif (TRIM) family (21). The best-studied family member, TRIM5α (31), restricts retroviral infection by specifically recognizing the viral capsid and promoting its premature disassembly (3, 20, 32), and, as recently reported, by blocking viral production at a posttranslational stage (23). Human TRIM5α has limited efficacy against human immunodeficiency virus type 1 (HIV-1), while proteins encoded by some primate *TRIM5*α orthologs can potently restrict this particular lentivirus (18, 28, 29, 33). Longstanding selective pressures exerted by retroviruses and retroelements may have contributed to the generation of diverse patterns of antiretroviral specificity of *TRIM5*α and other host defense genes (18, 28).

To better understand the evolution of antiretroviral specificity patterns in primates, in particular along the lineage leading to humans, we utilized a functional evolutionary genomics approach (34). In the present study, we reconstructed ancestral primate TRIM5α sequences and tested their specificity in vitro against HIV-1 and five other retroviruses. We used these six present day viruses as extant markers to evaluate the functional differences over evolutionary time.

MATERIALS AND METHODS

Determination of ancestral sequences. TRIM5α coding sequences from primates were obtained by amplification and sequencing of genomic DNA or cDNA or downloaded from the National Center for Biotechnology Information database (6, 12, 19, 25, 31, 38, 40): human (*Homo sapiens* AY625000), bonobo (*Pan paniscus* DQ229282), chimpanzee (*Pan troglodytes* AY923177), gorilla (*Gorilla gorilla* AY923178), Bornean orang-utan (*Pongo pygmaeus* AY923179), lar gibbon (*Hylobates lar* AY923180), nomascus (*Hylobates leucogenys* DQ229283), siamang (*Hylobates syndactylus* DQ229284), rhesus monkey (*Macaca mulatta* AY625001), olive baboon (*Papio anubis* AY843505), red guenon (*Erythrocebus patas* AY843514), African green monkey (*Cercopithecus* [*chlorocebus*] *aethiops* AY669399, *Cercopithecus* [*chlorocebus*] *tantalus* AY593973), eastern black-and-white colobus (*Colobus guereza* AY843507), douc langur (*Pygathrix nemaeus* AY843508), Bolivian titi (*Callicebus donacophilus* AY843519), Bolivian squirrel monkey (*Saimiri boliviensis boliviensis* AY928202), pygmy marmoset (*Callithrix pygmaea* AY843512), red-chested mustached tamarin (*Saguinus labiatus* AY843518), cotton-top tamarin (*Saguinus Oedipus* DQ229285), white-faced saki (*Pithecia pithecia* AY843515), Bolivian red howler monkey (*Alouatta sara* AY843511), common woolly monkey (*Lagothrix lagotricha* AY843520), and black-handed spider monkey (*Ateles geoffroyi* AY843516).

*TRIM5*α sequences were aligned by using CLUSTAL W. Coding regions were aligned according to their corresponding amino acid sequences by using the EMBOSS package (22). For the reconstruction of *TRIM5*α ancestral sequences (and for the calculation of posterior probabilities of reconstructed amino acids), we used a maximum-likelihood approach as implemented in the codeml tool (parameter settings according to model M0, i.e., model = 0 and NSsites = 0) of the PAML program package (37), in the framework of the accepted primate phylogeny (5). An alternative model (the free-ratio model, where each branch of the phylogeny may have a different K_A/K_S values) reconstructs the same amino acid variants at all sites and nodes, except for the single site 9 (I instead of V). However, since this site is located in the N terminus, which we demonstrate not

* Corresponding authors. Mailing address for A. Telenti: Institute of Microbiology, CHUV 1011 Lausanne, Switzerland. Phone: 41 21 314 05 50. Fax: 41 21 314 40 95. E-mail: amalio.telenti@chuv.ch. Mailing address for H. Kaessmann: University of Lausanne, Bâtiment Génopode, 1015 Lausanne, Switzerland. Phone: 41 21 692 39 07. Fax: 41 21 692 39 65. E-mail: Henrik.Kaessmann@unil.ch.

† V.G. and A.C. contributed equally to this study.

▽ Published ahead of print on 12 December 2007.

to affect anti-HIV-1 specificities of TRIM5α (see below), this ambiguity is not relevant for our conclusions. To validate the predictions from the maximum-likelihood approach, we used a Bayesian approach as implemented in the MrBayes program (7) using the Jones amino acid model and the known species topology as a prior (the topology around node 3 was constrained, i.e., it was fixed to reflect the divergence of the orangutan from the other great apes, as the Bayesian *TRIM5*α gene tree groups the orangutan sequence with gibbons—against the well-established primate phylogeny). Posterior probabilities were calculated on 100 samples representing the last 10,000 generations (sampling frequency 100) of a total of 100,000 generations. We verified that the log likelihoods of the ancestral reconstruction had converged before the last 10,000 generations.

Generation of cells stably expressing TRIM5α variants. pLPCX-TRIM5αhu-HA (NIH AIDS Reagent Program, donated by J. Sodroski), an oncoretroviral vector encoding the human TRIM5α (31), was used to generate the ancestral genes of *TRIM5*α by consecutive rounds of site-directed mutagenesis using the QuikChange protocol (Stratagene). *TRIM5*α from African green monkey was cloned from COS-7 (European Collection of Cell Cultures, ECACC no. 87021302), and *TRIM5*α tamarin was cloned from cells derived from cotton-top tamarin (ECACC no. 85011419). pLPCX-TRIM5αrh-HA (J. Sodroski) encodes the rhesus monkey TRIM5α. Expression of hemagglutinin-tagged TRIM5α proteins was determined by Western blotting.

Oncoretroviral vectors were packaged in 293T cells by cotransfecting the various pLPCX-TRIM5α constructs with the pNB-tropic murine leukemia virus (MLV) Gag-Pol and pVSV-G packaging plasmids (a gift from D. Trono) using the calcium phosphate method. As controls, we also used pLPCX and pLPCX-GFP instead of pLPCX-TRIM5α. Supernatants were concentrated and used to transduce 10^5 HeLa and CRFK (feline renal fibroblasts) cells in the presence of 5 μg of Polybrene/ml. At 72 h after transduction, cells were selected in the presence of 0.5 μg (HeLa cells) and 3 μg (CRFK cells) of puromycin/ml for at least 12 days before testing.

Recombinant virus infections. To produce HIV-1-based reporter vector particles, 293T cells (3×10^6 cells) were cotransfected with four plasmids by the calcium phosphate method (16). Plasmids encoded the vesicular stomatitis virus G protein pantropic envelope (pMD.G), the Gag and Pol proteins (pCMVΔR8.92), and Rev (pRSV-Rev), and the fourth plasmid encoded the HIV vector segment carrying green fluorescent protein (GFP) as the reporter transgene (pSIN.cPPT.EF1.GFP.WPRE). Additional constructs represent N-MLV and B-MLV (a gift from D. Trono), HIV-2 (a gift from A. Lever), and the simian immunodeficiency virus (SIV) variants SIV_{mac} (a gift from F.L. Cosset) and SIV_{agm} (a gift from J. Luban).

The infectivity of recombinant viruses was determined by titration on HeLa and CRFK cells. Single-round infectivity assays with the different recombinant viruses in HeLa and CRFK cells were performed at various multiplicities of infection (MOIs). At 48 h after transduction, cells were analyzed by using a fluorescence-activated cell sorter.

RESULTS AND DISCUSSION

Ancestral gene resurrection. To trace the evolutionary history of *TRIM5*α, we aligned the *TRIM5*α coding region from 24 primate species representing 25 million years of primate evolution. Evolutionary analysis allowed the estimation of the most likely sequence at each ancestral node in the framework of the accepted primate phylogeny (5) (Fig. 1A). We then introduced, by site-directed mutagenesis, all predicted amino acid changes at each ancestral node in a stepwise fashion starting from a cloned human *TRIM5*α (Fig. 1B). The oldest reconstructed sequence represents *TRIM5*α from the last common ancestor of Old World monkeys and apes, ~25 million years ago (mya), and differs by 29 amino acids from human TRIM5α. In addition, we tested *TRIM5*α from selected terminal taxa: Old World (rhesus monkey and African green monkey) and New World (cotton-top tamarin) monkeys. Attempts by our laboratory and other groups (28) to identify a *TRIM5*α ortholog in prosimians (e.g., lemurs and galagos) have been unsuccessful. Thus, a suitable outgroup that would permit us to infer the sequence of the common simian ancestor (i.e., Old World primate-catarrhine/New World monkey-platyrhine, 40 mya, Fig. 1A) is not available.

HIV-1 restriction by ancestral TRIM5α. To test the restriction capacity of the different gene variants, we first stably transduced HeLa cells (a human epithelial carcinoma cell line) with oncoretroviral vectors expressing the various ancestral and modern *TRIM5*α sequences. Multiple independently transduced cell lines were examined for each sequence in order to identify those showing comparable levels of transgene expression (Fig. 2A). We then infected the different cell lines with recombinant viruses—HIV-1, HIV-2, SIV_{agm}, SIV_{mac}, and the N- and B-tropic variants of the murine leukemia virus (N-MLV and B-MLV)—expressing the green fluorescent protein (GFP). The proportion of cells expressing GFP (relative infectivity) was then used as a proxy for the capacity of a particular *TRIM5*α sequence to restrict infection of cells by the respective recombinant virus.

We observed a statistically significant reduction of HIV-1 restriction by reconstructed TRIM5α variants (representing 25 million years of evolution) from the common catarrhine ancestral sequence toward the human variant. Ancestral TRIM5α variants are generally poorly restrictive of HIV-2, SIV_{agm} and SIV_{mac} (Fig. 2B). The pattern of HIV-1 restriction by successive ancestral variants was unique among the various retroviruses tested (Fig. 2C to G). Similar to the reconstructed node 1 sequence, TRIM5α from Old World monkeys displays a high capacity to restrict HIV-1. Some Old World monkey lineages appear to have acquired restriction capacity against other SIVs since the common catarrhine ancestor.

We carefully assessed the amino acid variants included in the reconstruction of each ancestral protein in the context of published experimental data of single and complex mutagenesis of TRIM5α (9, 14, 18, 31, 33, 39). The high HIV-1 restriction capacity of the 25 mya ancestral TRIM5α could not be deduced from the reconstructed sequence based on previous evidence from the literature. In particular, no ancestral construct carries the critical residue proline 332, associated with potent restriction of HIV-1 in previous studies (14, 39). The present study reconstructs position 332 with a glutamine (Q) in nodes 1 to 4 with high probability ($P > 0.95\%$; a substitution from glutamine to arginine occurred in the human-chimpanzee ancestor, between nodes 4 and 5, Fig. 1B). A glutamine at position 332 plays a role in the restriction activity of the gorilla B30.2 domain (18), and the change in restriction of HIV-1 between nodes 4 and 5 may be explained by a substitution from Q to R at this site (10). In general, the ability of human TRIM5α to bind the HIV-1 capsid is modulated by the presence of any charged residue at position 332 (14). However, despite the maintained presence of 332Q between nodes 1 and 4, HIV-1 restriction diminishes between these nodes. It should also be emphasized that the change in restriction occurred without any increase in length of the variable regions of the B30.2 domain (28, 33), since these regions were kept stable in size for the reconstruction of ancestral variants. Residue K389 was built as Q in nodes 4 to 1; however, recent analysis of extant sequences indicates that only nodes 3 to 1 should carry 389Q. Functional analysis indicates that this error is unlikely to modify restriction capacity (see below).

Reconstructed ancestral sequences also reveal amino acid substitutions in the other protein domains of TRIM5α. How-

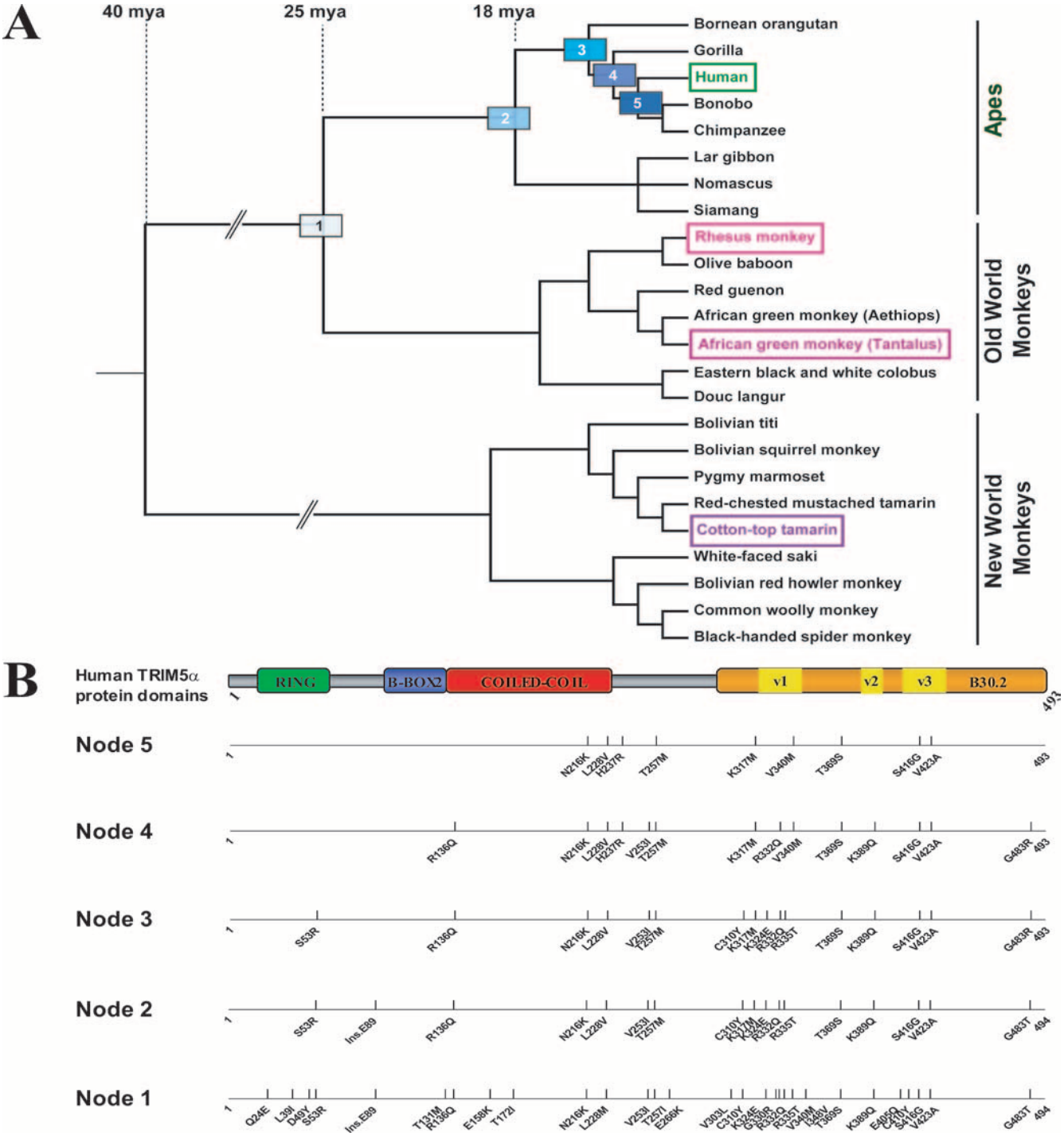

FIG. 1. Reconstruction of ancestral TRIM5α. (A) primate phylogenetic tree. Investigated nodes and species are color-coded. Approximate divergence times in millions of years (mya) are shown. (B) Identity and location of amino acid variants in ancestral nodes. Substitutions are shown relative to the human sequence. Although residue K389 is built as Q in nodes 4 to 1, recent analysis of extant sequences indicates that only nodes 3 to 1 should carry 389Q.

ever, single-amino-acid mutagenesis data from published functional studies (9) do not include any of the positions mutated here, with the exception of the R136Q substitution in the coiled-coil domain. This variant was shown not to modify TRIM5α restriction of HIV-1 (4, 24, 30), while in a study by Javanbahkt et al. the 136Q variant of human TRIM5α was associated with protection from HIV-1 infection in African Americans and with a weak restriction phenotype in vitro (8).

Restriction of other retroviruses. In contrast to the pattern seen for HIV-1, restriction of the murine oncoretrovirus N-

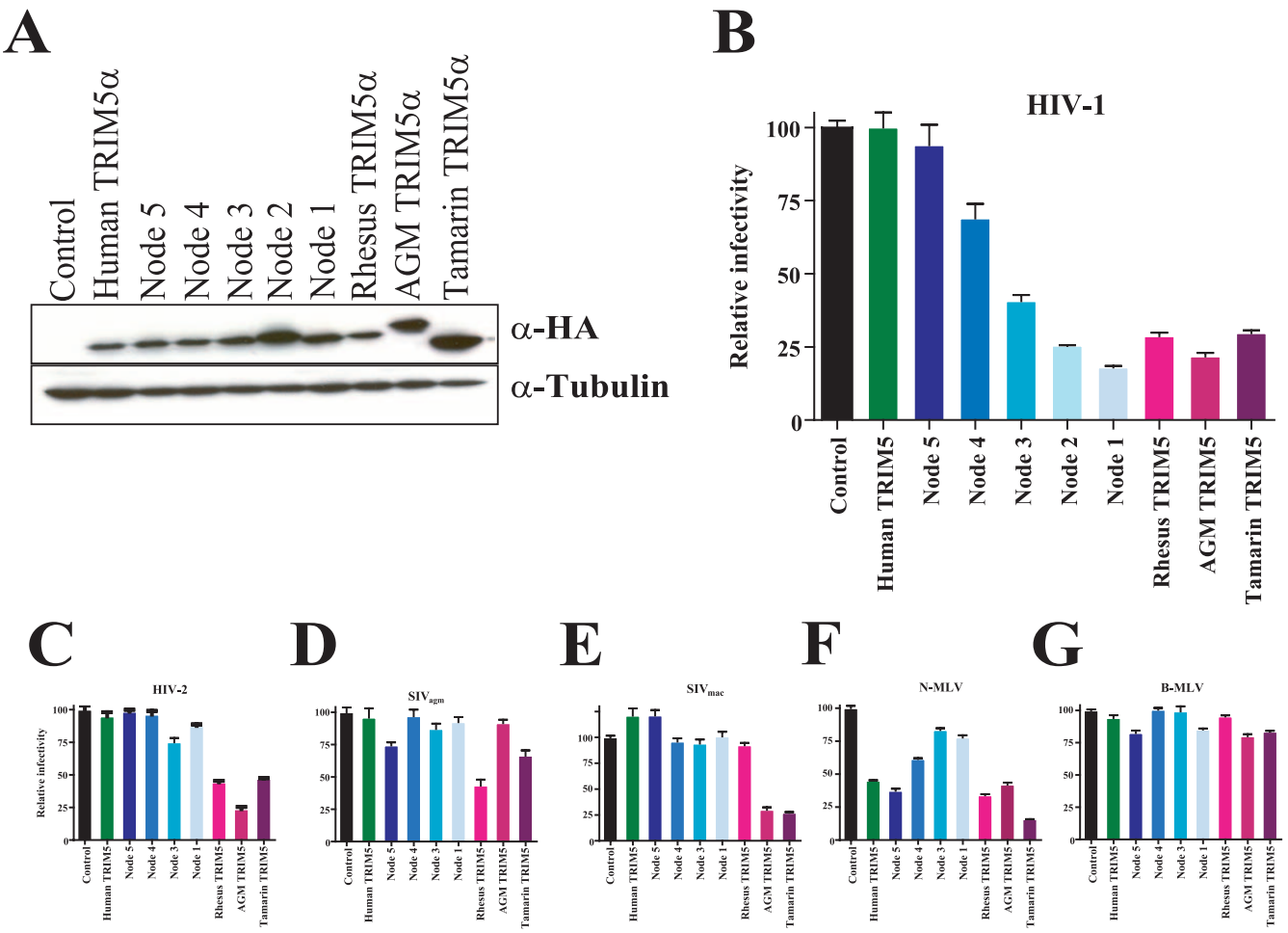

FIG. 2. Functional assessment of antiretroviral activity of ancestral and modern TRIM5α in HeLa cells. (A) Western blot assessment of stable expression of various TRIM5α in HeLa cells. (B) Restriction of HIV-1 recombinant virus in HeLa cells expressing the various reconstructed ancestral *TRIM5*α, as well as *TRIM5*α from humans, Old World monkeys (rhesus monkey, African green monkey [AGM]), and a New World monkey (cotton-top tamarin). Statistical analysis reveals significant restriction differences (one-way analysis of variance, $P < 10^{-3}$). Pairwise comparisons of nodes show significant restriction differences between nodes 1 to 5 ($P > 0.05$, Tukey's post hoc test), except for the node 1 and 2 comparison. Note that the restriction difference between node 1 and macaque, AGM, and tamarin TRIM5α variants is not statistically significant. (C to G) Restriction of five additional retroviruses in HeLa cells expressing reconstructed and extant *TRIM5*α. Node 2, which differs by two amino acids from node 3, is not shown. Shown are representative normalized results from infection at the optimal MOI for each virus. The error bars represent the standard error of the mean on three replicates.

MLV was stronger for reconstructed TRIM5α of extant taxa than for more ancestral TRIM5α constructs (Fig. 2F). The functional change was observed without changes in the RING domain, previously reported to be relevant for N-MLV restriction (24) (Fig. 1B). Specifically, ancestral nodes do not carry the substitution H43Y, a frequent human TRIM5α allele that may negatively affect its putative E3 ubiquitin ligase activity and is associated with impaired restriction of N-MLV (4, 24). The detrimental allele dates back to before the emergence of the African diaspora and is found at a frequency of 43% in indigenous Central and South Americans (24).

Ancestral TRIM5α showed various patterns of restriction capacities against other retroviruses tested (HIV-2, SIV_{agm}, SIV_{mac}, B-tropic variants of the MLV, B-MLV) (Fig. 2C to E and G). Overall, the 25-mya ancestral TRIM5α (node 1) exhibited limited efficacy against HIV-2, SIV_{agm}, and SIV_{mac}. Extant TRIM5α variants from Old World monkeys display higher restriction capacity against HIV-2 than the reconstructed TRIM5α from the common catarrhine ancestor (suggesting a gain in restriction capacity), whereas ancestral hominoid TRIM5αs all show little restriction efficiency with respect to this virus.

To rule out cell line-specific effects, we assessed the antiretroviral activities of selected ancestral and modern TRIM5α in another cell line (Fig. 3), CRFK (feline renal fibroblasts, no intrinsic retroviral restriction [6]). The CRFK series was less complete than that for HeLa cells, because we failed to generate stable cell lines carrying node 3 or cotton-top tamarin TRIM5α variants, whereas all tested clones carrying node 2 TRIM5α were massively overexpressing the protein. However, the overall pattern was confirmed: (i) decreasing restriction of modern HIV-1 from nodes 1 to 5, (ii) more restriction of N-MLV in humans and the human-chimpanzee ancestral TRIM5α, and (iii) greater restriction of HIV-2 by TRIM5α

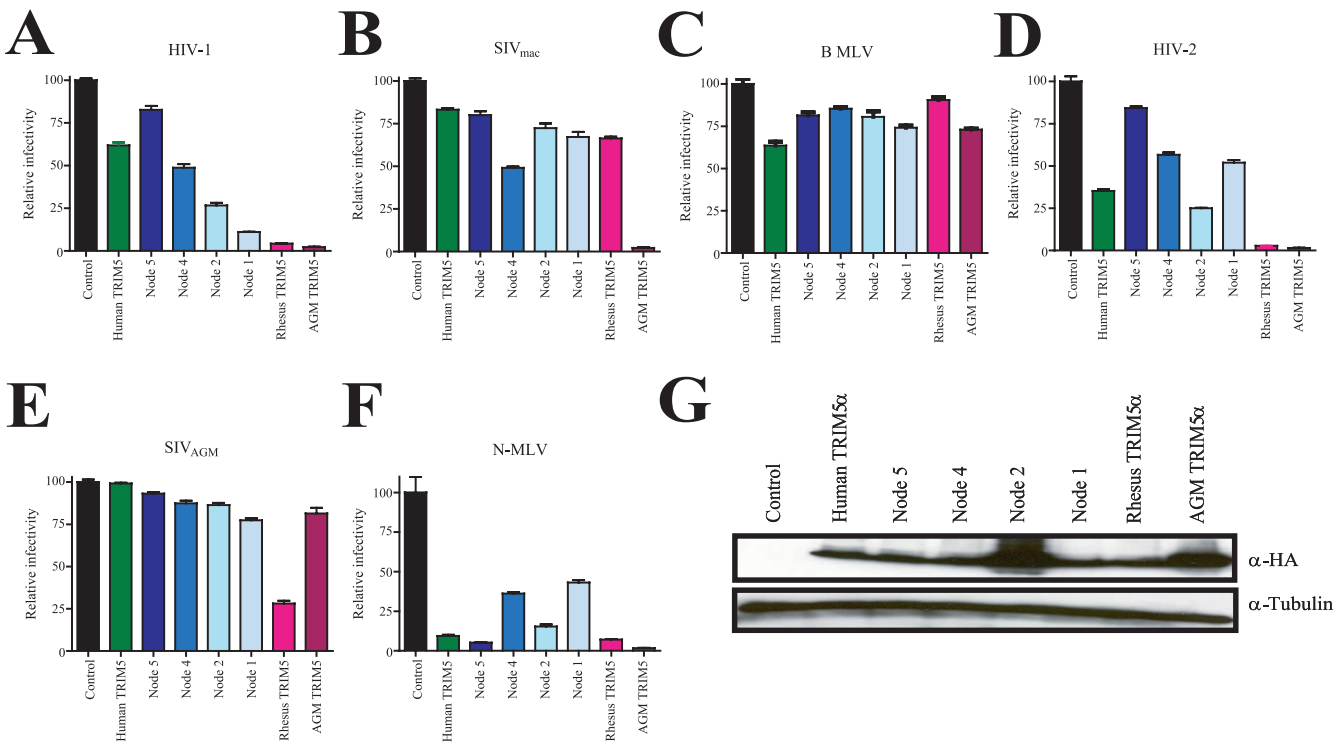

FIG. 3. Functional assessment of antiretroviral activity of selected ancestral and modern TRIM5α in CRFK cells. (A) Restriction of HIV-1 recombinant virus in CRFK cells expressing selected reconstructed ancestral *TRIM5*α, as well as *TRIM5*α from humans, rhesus monkey, and African green monkey (AGM). (B to F) Restriction of five additional retroviruses in CRFK cells expressing reconstructed and extant *TRIM5*α. Shown are representative normalized results from infection at optimal MOI. for each virus. The error bars represent the standard error of the mean on three replicates. (G) Western blot analysis. Node 2 was massively overexpressed in all tested clones; node 3 could not be expressed in stable CRFK cells.

from Old World monkeys. Consistent with previous reports (6, 42), we found that the expression of human TRIM5α in CFRK is associated with partial restriction of HIV-1 and HIV-2, which explains the different results for the human TRIM5α between the two cell lines tested. We note that we used vesicular stomatitis virus-pseudotyped particles for our challenge experiments. While the characteristic of the envelope protein is reported to not significantly change restriction by TRIM5α, human TRIM5α restricts primary HIV-1 isolates to variable degrees (11).

Reliability of ancestral reconstructions. A potential concern with respect to the functional reconstructions in our study is the accuracy of the inferred ancestral TRIM5α sequences. The sequences studied are from closely related species, an approach which, in principle, facilitates ancestral inference. However, some regions of TRIM5α may have been affected by multiple (parallel and convergent) substitutions at the same site due to positive selection that has been driving its evolution (17), which may render reconstruction of ancestral variants potentially less reliable. Nevertheless, we believe that the ancestral proteins tested here are of adequate quality, for the reasons detailed below.

The posterior probabilities obtained for the reconstruction of key residues using the maximum-likelihood procedure are generally high (the proportions of reconstructed amino acids with a P of >0.95 were 92% [node 1], 97% [node 2], 98% [node 3], 98% [node 4], and 99% [node 5]), and the reconstructions are robust with respect to different models and parameters used. Furthermore, J. W. Thornton (34) indicates that errors in ancestral sequence reconstruction are generally expected to bias resurrected genes toward less functional variants. However, in our study, ancestral sequences exhibit greater efficacy against HIV-1 than modern TRIM5α. In contrast to J. W. Thornton, Williams et al. (35) suggest that the assumption that reconstruction errors can be considered similar to random (i.e., generally deleterious) mutations is questionable. In their view, extant variants used for reconstructions are necessarily acceptable in the functional context, which cannot be assumed for random mutations (35).

Williams et al. (35) compared the properties of ancestral proteins inferred by using maximum-parsimony, maximum-likelihood, and Bayesian methods. These researchers concluded that these approaches offered various benefits. To address the possibility of systematic error and to accommodate reasonable alternative scenarios in ancestral reconstruction, we used an alternative Bayesian approach for reconstruction, in addition to the maximum-likelihood approach (see Materials and Methods for details). Key amino acid positions, for which the maximum-likelihood procedure yielded potentially ambiguous reconstructions (i.e., relatively low posterior probabilities for reconstructed variants) and for which a different amino acid was reconstructed by the Bayesian approach, were incorporated into the functional analyses. For the 29 residues that were found to differ between human TRIM5α and node 1

TABLE 1. Identification of potentially ambiguous reconstructions and of residues responsible for restriction by ancestral TRIM5a[a]

Residue	Human TRIMα amino acid	Node 1 amino acid (probability)[b]		Positive selection	Node 1 amino acid (alternative amino acid selected for functional assays)
		ML	Bayes		
24	Q	E (0.93)	E (1.00)	–	None
39	L	I (0.94)	I (1.00)	–	None
49	D	Y (0.59)	H (1.00)	–	H
53	S	R (1.00)	R (1.00)	–	None
89		Insertion		–	Not assessed
131	T	M (0.84)	V (1.00)	–	V
136	R	Q (1.00)	Q (1.00)	–	None
158	E	K (0.79)	K (1.00)	–	None
172	T	I (0.83)	I (1.00)	–	None
216	N	K (1.00)	Q (0.99)	–	None
228	L	M (0.82)	M (0.54)	–	None
253	V	I (0.83)	V (0.99)	–	V (failure to build)
257	T	I (0.94)	I (1.00)	–	None
266	E	K (0.83)	K (0.99)	–	None
303	V	L (0.95)	L (1.00)	–	None
310	C	Y (0.94)	Y (0.76)	–	None
324	K	E (0.77)	E (0.98)	+	K
330	G	R (0.95)	P (0.85)	–	None
332	R	Q (0.98)	V (0.99)	+	R (failure to build)
335	R	T (0.96)	T (0.91)	+	R
340	V	M (0.42)	V (0.74)	+	V
348	I	V (0.85)	V (0.99)	–	None
369	T	S (1.00)	S (0.98)	–	None
389	K	Q (0.94)	G (0.90)	+	K
405	E	Q (0.95)	R (0.91)	–	None
410	C	Y (0.85)	Y (1.00)	–	None
416	S	G (0.73)	G (1.00)	–	None
423	V	A (1.00)	A (1.00)	–	None
483	G	T (0.72)	E (1.00)	+	E and G

[a] Among the 29 residues that differ between node 1 and human TRIM5α, we identified amino acid positions for which the maximum-likelihood procedure and an alternative Bayesian approach for TRIM5α reconstruction yielded potentially ambiguous reconstructions (i.e., relatively low posterior probabilities for reconstructed variants). Five residues showed low codeml probabilities (<95%), and an alternative amino acid was supported by Bayesian analysis. In addition, a number of residues that differed from human TRIM5α and were under positive selective pressure were identified as possibly responsible for the HIV-1 restriction capacity of the node1 ancestral TRIM5α.

[b] The maximum-likelihood (ML) posterior and Bayesian probabilities are given in parentheses.

based on the maximum-likelihood procedure, 5 showed low codeml probabilities (<95%), and an alternative amino acid was supported by Bayesian analysis (Table 1). We successfully tested four of the alternative amino acids (49Y/H, 131 M/V, 340 M/V, and 483T/E) by introducing them into the original node 1 sequence and failed to build 253V. None of the ambiguous-position variants modified the restriction of HIV-1 (Fig. 4), which ensures that potentially erroneously reconstructed residues likely do not account for the efficient restriction of HIV-1 by the node 1 TRIM5α sequence.

Another issue relates to the recent finding of long-term persistence of multiple TRIM5α alleles at critical positions due to balancing selection (17). Thus, reconstruction of specific nodes may be complicated by the simultaneous presence of key alleles in multiple lineages. Humans present features of balancing selection at position R136Q (4), while sooty mangabeys and rhesus monkeys (17) present multiple alleles at positions 182, 194, 213, 331, 332, indel 337-338, and 339 (human numbering). However, we analyzed the accuracy of reconstruction for these positions (except for indel 337-338, which is not present in all species and is thus not included in the analysis) and found that the ancestral inference at these sites appears to generally be unambiguous (with high posterior probabilities, >98%, for the reconstructed amino acid variant) and not affected by the putative trans-species polymorphisms. In addition, we searched novel TRIM5α submissions to GenBank, aligned available entries to the sequences from our study, and screened critical residues under positive selective pressure for the presence of polymorphism within the species. We re-estimated node 1 under the premise of balancing selection and found the original prediction unchallenged on the basis of currently available data on intraspecies polymorphism. Only residue 253 could have been built as 253V in addition to 253I at node 1, due to the fact that gorilla, orangutan, and nomascus primates are now known to be polymorphic at this position.

Identifying residues responsible for restriction by ancestral TRIM5α. After confirming the reliability of reconstruction of node 1, we sought to describe domains and residues that could contribute to node 1 restriction. For this, we generated reciprocal chimeric constructs of the N^{1-284}- and $C^{285-493}$-terminal portions of the node 1 and node 4 sequences. We then assessed their restriction capacities using CRFK cells (Fig. 4). This analysis demonstrates that only substitutions in the C-terminal domain appear to have affected restriction of HIV-1 by reconstructed TRIM5α on the human lineage (Fig. 4), since it is the hybrid construct with the node 1 C terminus that displays high restriction capacity (as high as that of the intact node 1 sequence).

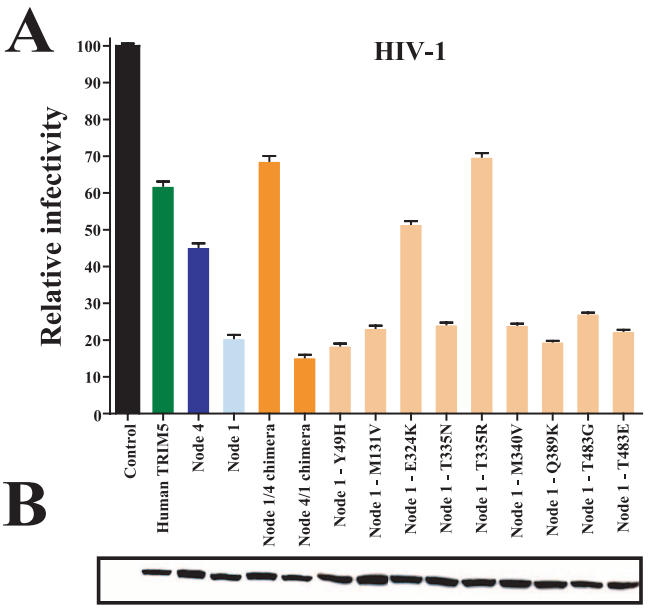

FIG. 4. Functional assessment of ambiguous ancestral variants and identification of residues responsible for restriction by ancestral TRIM5 α. (A) Restriction of HIV-1 recombinant virus in CRFK cells expressing *TRIM5*α from humans, selected reconstructed ancestral *TRIM5*α, N-terminal and C-terminal chimeras of nodes 4 and 1, and key mutants of node 1. Mutants 49H, 131V, 340V, and 483E test alternative reconstructions derived from Bayesian analysis. Mutants 324K, 335R, 340V, 389K, and 483G test the role of critical residues under positive selection by reintroducing the human TRIM5α amino acid variant into the node 1 sequence. Mutant 335N tests an alternative amino acid seen in African green monkeys at this position. Shown are normalized, representative results from infections at an MOI of 5. The error bars represent the standard error of the mean on three replicates. (B) Anti-HA immunoblot of the different TRIM5α-CRFK cell lines.

We then selected residues in the C-terminal region of node 1 that (i) differed from human TRIM5α and (ii) were under positive selective pressure (Table 1). Using site-directed mutagenesis experiments, we demonstrate that changing the ancestral 324E to the more recent 324K (a substitution occurring on the lineage leading to the last common African ape ancestor, node 4) or changing the ancestral 335T residue to 335R (originating in the common human/chimpanzee ancestor on the lineage leading to node 5) strongly reduces the ability of the 25-mya ancestral TRIM5α to restrict HIV-1. These positions have been previously investigated as 324N and 335L and found to be associated with restriction of HIV-1 (14, 33). In addition, a sequence context that includes 324E/332Q/335T in variable region v1 and 389Q in the v2 region was associated with effective restriction in a recent study (18). For amino acid residue 335, we tested another amino acid (335N) found in Old World monkeys. The resulting sequence variant maintains the high restriction capacity seen for the original node 1 sequence (Fig. 4), which underscores that the loss of restriction capacity is specific to reversion to 335R. Thus, the present study reveals novel functionally relevant amino acid variants at positions 324 and 335 and generally confirms the importance of these sites for virus restriction by TRIM5α. It is noteworthy, however, that although this sequence context is maintained from nodes 1 to node 3, restriction efficacy is gradually diminishing between these nodes. This suggests that other substitutions and/or sites (potentially in combination) explain the diminishing restriction capacity of reconstructed TRIM5α compared to reconstructed TRIM5α of the last common Old World primate ancestor (node 1). We individually tested three additional residues (340V, 389K, and 483G) that were previously shown to have evolved under positive selection (Table 1) in this domain, but none of these substitutions appear to reduce the restriction of the original node 1 TRIM5α protein when changed individually (Fig. 4). We did not assess the ambiguous position C385, which corresponds to the V2 region of length polymorphism. Substitution of tyrosine with cysteine at position 385 does not modify the restriction of HIV-1 (18).

The novelty in the present study resides in the experimental approach used to characterize recently emerged protein differences (reconstruction of ancestral host sequences). The strategy was able to identify and experimentally test new variants despite the fact that this protein has been very thoroughly investigated over the past 3 years. Thus, evolutionary genetics and ancestral reconstruction could be of interest in future investigation of less-well-characterized proteins. Interestingly, as for TRIM5α, evolutionary analysis of a second antiretroviral protein, APOBEC3G (27), would also predict shifts in antiretroviral specificity on the human lineage since the catarrhine ancestor, because the ancestral APOBEC3G likely carried the amino acid variant K128 that governs sensitivity of this protein to modern HIV-1 Vif-mediated inhibition (2, 15, 26, 36). There is clearly a great level of complexity in imputing an evolutionary direction of restriction of modern viruses by reconstructed TRIM5α variants. However, ancestral proteins can in the future be tested against resurrected infectious agents (10, 13).

Such various restriction capacities might be explained by lineage-specific pandemics that shape and redirect the intrinsic defense mechanisms. For example, the recent retroviral infection of two great ape lineages—chimpanzees and gorillas—by horizontal transmission from an exogenous source of PtERV1 has not affected the human genome (41). Recent data indicate that the R332 mutation in human and chimpanzee TRIM5α improves the ability of this protein to restrict PtERV1 while resulting in increased susceptibility to HIV-1 (10). Thus, as a result of trade-offs in the ability to restrict different retroviruses, humans might have been exposed to SIV_{cpz} and/or HIV-1 at a time when one or several intrinsic defense proteins lacked the appropriate specificity to avert its transmission from chimpanzees.

ACKNOWLEDGMENTS

We thank D. Trono, A. Lever, F. L. Cosset, J. Luban, and G. Towers for reagents.

A.T. and H.K. conceived the study. A.T., V.G., and A.C. designed the experiments. V.G., A.C., M.O., and M.M. performed the experiments. MO., D.B., and H.K. performed the evolutionary analysis. A.T., H.K., V.G., and A.C. wrote the report.

This study was funded by the Swiss National Science Foundation and a grant for interdisciplinary research from the Faculty of Biology and Medicine of the University of Lausanne.

REFERENCES

1. **Bieniasz, P. D.** 2004. Intrinsic immunity: a front-line defense against viral attack. Nat. Immunol. **5:**1109–1115.
2. **Bogerd, H. P., B. P. Doehle, H. L. Wiegand, and B. R. Cullen.** 2004. A single amino acid difference in the host APOBEC3G protein controls the primate species specificity of HIV type 1 virion infectivity factor. Proc. Natl. Acad. Sci. USA **101:**3770–3774.
3. **Chatterji, U., M. D. Bobardt, P. Gaskill, D. Sheeter, H. Fox, and P. A. Gallay.** 2006. Trim5α accelerates degradation of cytosolic capsid associated with productive HIV-1 entry. J. Biol. Chem. **281:**37025–37033.
4. **Goldschmidt, V., G. Bleiber, M. T. May, R. Martinez, M. Ortiz, and A. Telenti.** 2006. Role of common human TRIM5alpha variants in HIV-1 disease progression. Retrovirology **3:**54.
5. **Goodman, M.** 1999. The genomic record of Humankind's evolutionary roots. Am. J. Hum. Genet. **64:**31–39.
6. **Hatziioannou, T., D. Perez-Caballero, A. Yang, S. Cowan, and P. D. Bieniasz.** 2004. Retrovirus resistance factors Ref1 and Lv1 are species-specific variants of TRIM5α. Proc. Natl. Acad. Sci. USA **101:**10774–10779.
7. **Huelsenbeck, J. P., and F. Ronquist.** 2001. MRBAYES: Bayesian inference of phylogenetic trees. Bioinformatics **17:**754–755.
8. **Javanbakht, H., P. An, B. Gold, D. C. Petersen, C. O'Huigin, G. W. Nelson, S. J. O'Brien, G. D. Kirk, R. Detels, S. Buchbinder, S. Donfield, S. Shulenin, B. Song, M. J. Perron, M. Stremlau, J. Sodroski, M. Dean, and C. Winkler.** 2006. Effects of human TRIM5α polymorphisms on antiretroviral function and susceptibility to human immunodeficiency virus infection. Virology **354:** 15–27.
9. **Javanbakht, H., F. az-Griffero, M. Stremlau, Z. Si, and J. Sodroski.** 2005. The contribution of RING and B-box 2 domains to retroviral restriction mediated by monkey TRIM5α. J. Biol. Chem. **280:**26933–26940.
10. **Kaiser, S. M., H. Malik, and M. Emerman.** 2007. Restriction of an extinct retrovirus by the human TRIM5α antiviral protein. Science **316:**1756–1758.
11. **Kaumanns, P., I. Hagmann, and M. T. Dittmar.** 2006. Human TRIM5α mediated restriction of different HIV-1 subtypes and Lv2 sensitive and insensitive HIV-2 variants. Retrovirology **3:**79.
12. **Keckesova, Z., L. M. Ylinen, and G. J. Towers.** 2004. The human and African green monkey TRIM5α genes encode Ref1 and Lv1 retroviral restriction factor activities. Proc. Natl. Acad. Sci. USA **101:**10780–10785.
13. **Lee, Y. N., and P. D. Bieniasz.** 2007. Reconstitution of an infectious human endogenous retrovirus. PLoS. Pathog. **3:**e10.
14. **Li, Y., X. Li, M. Stremlau, M. Lee, and J. Sodroski.** 2006. Removal of arginine 332 allows human TRIM5α to bind human immunodeficiency virus capsids and to restrict infection. J. Virol. **80:**6738–6744.
15. **Mangeat, B., P. Turelli, S. Liao, and D. Trono.** 2004. A single amino acid determinant governs the species-specific sensitivity of APOBEC3G to Vif action. J. Biol. Chem. **279:**14481–14483.
16. **Naldini, L., U. Blomer, P. Gallay, D. Ory, R. Mulligan, F. H. Gage, I. M. Verma, and D. Trono.** 1996. In vivo gene delivery and stable transduction of nondividing cells by a lentiviral vector. Science **272:**263–267.
17. **Newman, R. M., L. Hall, M. Connole, G. L. Chen, S. Sato, E. Yuste, W. Diehl, E. Hunter, A. Kaur, G. M. Miller, and W. E. Johnson.** 2006. Balancing selection and the evolution of functional polymorphism in Old World monkey TRIM5α. Proc. Natl. Acad. Sci. USA.
18. **Ohkura, S., M. W. Yap, T. Sheldon, and J. P. Stoye.** 2006. All three variable

regions of the TRIM5α B30.2 domain can contribute to the specificity of the retrovirus restriction. J. Virol. **80:**8554–8565.
19. **Ortiz, M., G. Bleiber, R. Martinez, H. Kaessmann, and A. Telenti.** 2006. Patterns of evolution of host proteins involved in retroviral pathogenesis. Retrovirology **3:**11.
20. **Perron, M. J., M. Stremlau, M. Lee, H. Javanbakht, B. Song, and J. Sodroski.** 2007. The human TRIM5α restriction factor mediates accelerated uncoating of the N-tropic murine leukemia virus capsid. J. Virol. **81:**2138–2148.
21. **Reymond, A., G. Meroni, A. Fantozzi, G. Merla, S. Cairo, L. Luzi, D. Riganelli, E. Zanaria, S. Messali, S. Cainarca, A. Guffanti, S. Minucci, P. G. Pelicci, and A. Ballabio.** 2001. The tripartite motif family identifies cell compartments. EMBO J. **20:**2140–2151.
22. **Rice, P., I. Longden, and A. Bleasby.** 2000. EMBOSS: the European molecular biology open software suite. Trends Genet. **16:**276–277.
23. **Sakuma, R., J. A. Noser, S. Ohmine, and Y. Ikeda.** 2007. Rhesus monkey TRIM5α restricts HIV-1 production through rapid degradation of viral Gag polyproteins. Nat. Med. **13:**631–635.
24. **Sawyer, S. L., L. I. Wu, J. M. Akey, M. Emerman, and H. S. Malik.** 2006. High-frequency persistence of an impaired allele of the retroviral defense gene TRIM5α in humans. Curr. Biol. **16:**95–100.
25. **Sawyer, S. L., L. I. Wu, M. Emerman, and H. S. Malik.** 2005. Positive selection of primate TRIM5α identifies a critical species-specific retroviral restriction domain. Proc. Natl. Acad. Sci. USA **102:**2832–2837.
26. **Schrofelbauer, B., D. Chen, and N. R. Landau.** 2004. A single amino acid of APOBEC3G controls its species-specific interaction with virion infectivity factor (Vif). Proc. Natl. Acad. Sci. USA **101:**3927–3932.
27. **Sheehy, A. M., N. C. Gaddis, J. D. Choi, and M. H. Malim.** 2002. Isolation of a human gene that inhibits HIV-1 infection and is suppressed by the viral Vif protein. Nature **418:**646–650.
28. **Song, B., B. Gold, C. O'Huigin, H. Javanbakht, X. Li, M. Stremlau, C. Winkler, M. Dean, and J. Sodroski.** 2005. The B30.2(SPRY) domain of the retroviral restriction factor TRIM5α exhibits lineage-specific length and sequence variation in primates. J. Virol. **79:**6111–6121.
29. **Song, B., H. Javanbakht, M. Perron, D. H. Park, M. Stremlau, and J. Sodroski.** 2005. Retrovirus restriction by TRIM5α variants from Old World and New World primates. J. Virol. **79:**3930–3937.
30. **Speelmon, E. C., D. Livingston-Rosanoff, S. S. Li, Q. Vu, J. Bui, D. E. Geraghty, L. P. Zhao, and M. J. McElrath.** 2006. Genetic association of the antiviral restriction factor TRIM5α with human immunodeficiency virus type 1 infection. J. Virol. **80:**2463–2471.
31. **Stremlau, M., C. M. Owens, M. J. Perron, M. Kiessling, P. Autissier, and J. Sodroski.** 2004. The cytoplasmic body component TRIM5α restricts HIV-1 infection in Old World monkeys. Nature **427:**848–853.
32. **Stremlau, M., M. Perron, M. Lee, Y. Li, B. Song, H. Javanbakht, F. az-Griffero, D. J. Anderson, W. I. Sundquist, and J. Sodroski.** 2006. Specific recognition and accelerated uncoating of retroviral capsids by the TRIM5α restriction factor. Proc. Natl. Acad. Sci. USA **103:**5514–5519.
33. **Stremlau, M., M. Perron, S. Welikala, and J. Sodroski.** 2005. Species-specific variation in the B30.2(SPRY) domain of TRIM5α determines the potency of human immunodeficiency virus restriction. J. Virol. **79:**3139–3145.
34. **Thornton, J. W.** 2004. Resurrecting ancient genes: experimental analysis of extinct molecules. Nat. Rev. Genet. **5:**366–375.
35. **Williams, P. D., D. D. Pollock, B. P. Blackburne, and R. A. Goldstein.** 2006. Assessing the accuracy of ancestral protein reconstruction methods. PLoS. Comput. Biol. **2:**e69.
36. **Xu, H., E. S. Svarovskaia, R. Barr, Y. Zhang, M. A. Khan, K. Strebel, and V. K. Pathak.** 2004. A single amino acid substitution in human APOBEC3G antiretroviral enzyme confers resistance to HIV-1 virion infectivity factor-induced depletion. Proc. Natl. Acad. Sci. USA **101:**5652–5657.
37. **Yang, Z.** 1997. PAML: a program package for phylogenetic analysis by maximum likelihood. Comput. Appl. Biosci. **13:**555–556.
38. **Yap, M. W., S. Nisole, C. Lynch, and J. P. Stoye.** 2004. Trim5α protein restricts both HIV-1 and murine leukemia virus. Proc. Natl. Acad. Sci. USA **101:**10786–10791.
39. **Yap, M. W., S. Nisole, and J. P. Stoye.** 2005. A single amino acid change in the SPRY domain of human Trim5α leads to HIV-1 restriction. Curr. Biol. **15:**73–78.
40. **Ylinen, L. M., Z. Keckesova, S. J. Wilson, S. Ranasinghe, and G. J. Towers.** 2005. Differential restriction of human immunodeficiency virus type 2 and simian immunodeficiency virus SIVmac by TRIM5α alleles. J. Virol. **79:**11580–11587.
41. **Yohn, C. T., Z. Jiang, S. D. McGrath, K. E. Hayden, P. Khaitovich, M. E. Johnson, M. Y. Eichler, J. D. McPherson, S. Zhao, S. Paabo, and E. E. Eichler.** 2005. Lineage-specific expansions of retroviral insertions within the genomes of African great apes but not humans and orangutans. PLoS. Biol. **3:**e110.
42. **Zhang, F., T. Hatziioannou, D. Perez-Caballero, D. Derse, and P. D. Bieniasz.** 2006. Antiretroviral potential of human tripartite motif-5 and related proteins. Virology **353:**396–409.
43. **Zheng, Y. H., and B. M. Peterlin.** 2005. Intracellular immunity to HIV-1: newly defined retroviral battles inside infected cells. Retrovirology **2:**25.