# An Evolutionary Screen Highlights Canonical and Noncanonical Candidate Antiviral Genes within the Primate *TRIM* Gene Family

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## Abstract

Recurrent viral pressure has acted on host-encoded antiviral genes during primate and mammalian evolution. This selective pressure has resulted in dramatic episodes of adaptation in host antiviral genes, often detected via positive selection. These evolutionary signatures of adaptation have the potential to highlight previously unrecognized antiviral genes (also called restriction factors). Although the *TRIM* multigene family is recognized for encoding several bona fide restriction factors (e.g., TRIM5alpha), most members of this expansive gene family remain uncharacterized. Here, we investigated the *TRIM* multigene family for signatures of positive selection to identify novel candidate antiviral genes. Our analysis reveals previously undocumented signatures of positive selection in 17 *TRIM* genes, 10 of which represent novel candidate restriction factors. These include the unusual *TRIM52* gene, which has evolved under strong positive selection despite its encoded protein lacking a putative viral recognition (B30.2) domain. We show that *TRIM52* arose via gene duplication from the *TRIM41* gene. Both *TRIM52* and *TRIM41* have dramatically expanded RING domains compared with the rest of the *TRIM* multigene family, yet this domain has evolved under positive selection only in primate *TRIM52*, suggesting that it represents a novel host–virus interaction interface. Our evolutionary-based screen not only documents positive selection in known *TRIM* restriction factors but also highlights candidate novel restriction factors, providing insight into the interfaces of host–pathogen interactions mediated by the *TRIM* multigene family.

**Key words:** *TRIM5*, *TRIM52*, positive selection, dN/dS, restriction factors.

## Introduction

Host-encoded restriction factors confer an intrinsic line of defense that inhibits viruses at various stages of the viral life cycle (Goff 2004; Duggal and Emerman 2012; Yan and Chen 2012). One example of this type of antiviral defense gene is *TRIM5*, which was identified as the block to HIV-1 infection in rhesus macaques (Stremlau et al. 2004). The potent restriction by *TRIM5* is conserved in other mammals, including primates (Yap et al. 2004; Song, Javanbakht et al. 2005; Zhang et al. 2006; Kratovac et al. 2008; Yap et al. 2008; Rahm et al. 2011) and closely related paralogs belonging to glires (Schaller et al. 2007; Tareen et al. 2009; Fletcher et al. 2010) and cows (Si et al. 2006; Ylinen et al. 2006). Restriction activity is attributed to the assembly of a *TRIM5* lattice directly to the surface of the retroviral core (Ganser-Pornillos et al. 2011) that is thought to mediate premature capsid disassembly (Stremlau et al. 2006). Antiviral activity of *TRIM5* has also been attributed to the induction of an inflammatory response (Pertel et al. 2011; Tareen and Emerman 2011). Retroviral specificity of *TRIM5* dramatically differs among primate orthologs due to ancient and ongoing selective pressures reflected by variation in the Coiled-Coil and B30.2 domains, which influence the interaction with viral proteins (Sawyer et al. 2005; Sebastian and Luban 2005; Kirmaier et al. 2010; Maillard et al. 2010).

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*TRIM5* is a member of the *TRIM* multigene family, which encodes as many as 100 genes in humans and is similarly expansive throughout primates (Han et al. 2011). Proteins encoded by the *TRIM* multigene family are characterized by a tripartite motif consisting of a RING domain, one or two B-boxes, and a Coiled-Coil motif, the order and spacing of which are generally conserved (Reymond et al. 2001; Meroni and Diez-Roux 2005; Nisole et al. 2005). Like *TRIM5*, several other *TRIM* genes have been implicated in innate immunity and antiviral defense (reviewed in Nisole et al. 2005; Ozato et al. 2008; Johnson and Sawyer 2009; Kawai and Akira 2011; McNab et al. 2011). However, the majority of *TRIM* genes remain largely uncharacterized, along with their potential for encoding antiviral activities.

Previous studies have used functional characterizations to identify *TRIM* gene family members that encode antiviral activity. For example, a screen of a subset of human and mouse *TRIM* genes highlighted members not previously known to positively or negatively impact retroviral fitness (Uchil et al. 2008). Other functional characterizations have focused on hallmarks of restriction factors, including induction on interferon treatment (Carthagena et al. 2009; Uchil et al. 2013). Although candidate restriction factors were identified from each of these approaches, functional identification of novel restriction factors in the *TRIM* gene family is complicated due to a number of reasons. First, multiple alternatively spliced transcripts are produced from each *TRIM* gene. PML, for instance, is only one of the 11 TRIM19 protein isoforms. TRIM5alpha is the longest of at least nine reported transcripts of the *TRIM5* gene (Reymond et al. 2001; Brennan et al. 2007; Battivelli et al. 2011) but the only protein isoform with antiviral activity. Homodimerization of TRIM5alpha with other TRIM5 isoforms (gamma, delta, and iota) causes dominant negative suppression of the antiviral activity of TRIM5alpha (Stremlau et al. 2004; Passerini et al. 2006; Battivelli et al. 2011), so antiviral activity requires that the correct isoform or combination of isoforms be appropriately expressed in the cells being assayed. Second, viral restriction specificity may further impede identification of antiviral function especially for those restriction factors that act directly at the host–virus interface (like TRIM5alpha) compared with those that may indirectly affect the immune response (like PML); for the former case, detection of antiviral activity would depend on the right combination of *TRIM* genes and viruses. For instance, although rhesus macaque TRIM5 has potent antiviral activity against HIV-1, the human ortholog only has relatively modest effects (Stremlau et al. 2004).

In order to bypass these difficulties associated with a functional screening approach, we have taken a complementary, evolutionary approach to identify candidate antiviral restriction factors in this family. This approach exploits a common feature of restriction genes: the unique selective pressures they are subjected to by virtue of their antagonistic relationship with viral pathogens (Meyerson and Sawyer 2011; Daugherty and Malik 2012). Any mutation that improves the ability of an antiviral gene to recognize the virus is advantageous to the host genome. In contrast, the virus selectively favors mutations that weaken or destroy this interaction. Repeated rounds of mutation in which one party increases affinity while the other party decreases affinity can lead to rapid evolution at the protein–protein binding interface. Specifically, such interactions will result in the rapid accumulation of changes at nonsynonymous (amino acid altering) positions in coding DNA compared with the relatively benign mutations at synonymous sites, a selective regime referred to as positive selection. Such positive selection analysis was successfully used to precisely identify the region of TRIM5alpha that determines its specificity for different retroviral capsids (Sawyer et al. 2005). Importantly, positive selection has also been detected in nearly all other known restriction factors (Duggal and Emerman 2012). Indeed, signals of adaptive evolution are often a hallmark among restriction factors with roles at the direct interface of host–pathogen interactions.

Here, we analyzed members from the *TRIM* gene family for positive selection in primates. Via our evolutionary screen, we recovered two *TRIM* genes previously identified to be under positive selection due to their antiviral role (i.e. *TRIM5* and *TRIM22* [Sawyer et al. 2005, 2007]), five antiviral genes whose evolutionary signatures were previously unknown (i.e., *TRIM15* [Uchil et al. 2008; Uchil et al. 2013], *TRIM21* [Mallery et al. 2010], *TRIM25* [Gack et al. 2007], *TRIM31* [Uchil et al. 2008], and *TRIM38* [Uchil et al. 2008; Xue et al. 2012; Zhao, Wang, Zhang, Yuan et al. 2012; Zhao, Wang, Zhang, Wang et al. 2012]), and ten novel *TRIM* gene antiviral factor candidates. We also present a more detailed analysis of the most intriguing restriction factor candidate revealed by our screen, *TRIM52*. *TRIM52* lacks a C-terminal B30.2 domain but encodes a massively expanded RING domain that we find has been subject to intense positive selection. Our analysis of *TRIM52* evolution reveals its age and birth via a partial duplication of the *TRIM41* gene, followed by independent loss or pseudogenization of *TRIM52* in multiple mammalian and primate lineages. Based both on the strong signatures of adaptive evolution and the recurrent losses, we propose that *TRIM52* represents a novel, noncanonical antiviral *TRIM* gene in primate genomes with unique specificity determined by the rapidly evolving RING domain. Our evolutionary screen to identify novel restriction factors reveals several intriguing candidates that warrant further study to fully elucidate the role played by *TRIM* genes either directly or indirectly in mediating antiviral defense.

## Materials and Methods

### Collecting *TRIM* Orthologs

Human (*Homo sapiens*) *TRIM* gene sequences were obtained from Ensembl (Flicek et al. 2012) and GenBank. Chimpanzee

(*Pan troglodytes*), bonobo (*Pan paniscus*), gorilla (*Gorilla gorilla*), orangutan (*Pongo abelii*), white-cheeked gibbon (*Nomascus leucogenys*), rhesus macaque (*Macaca mulatta*), baboon (*Papio anubis*), squirrel monkey (*Saimiri boliviensis*), marmoset (*Callithrix jacchus*), tarsier (*Tarsius syrichta*), mouse lemur (*Microcebus murinus*), and bushbaby (*Otolemur garnettii*) orthologs were obtained when reported from NCBI by BLAST (Altschul et al. 1990) searches of the "RefSeq RNA" databases with the human *TRIM* sequence as the query and from Ensembl gene orthology/paralogy predictions (Vilella et al. 2009). Additional primate orthologs were collected when available (e.g., African green monkey [*Chlorocebus aethiops*]). Subsequent collection of *TRIM* sequences, specifically *TRIM52* and *TRIM41*, via publically available databases were carried out utilizing Ensembl's genome databases to recover annotated sequences from available animals, including Reptilia, Avian, and Mammalian species.

### Sequencing *TRIM52*

To expand our collection of primate *TRIM52* sequences to improve the power of downstream evolutionary analysis, we amplified *TRIM52* using genomic DNA from the following primates: human, chimpanzee, bonobo, gorilla, orangutan, rhesus macaque, African green monkey, talapoin monkey (*Miopithecus talapoin*), colobus monkey (*Colobus guereza*), Francois' leaf monkey (*Trachypithecus francoisi*), purple-faced langur (*Trachypithecus vetulus*), and silvery langur (*Trachypithecus cristatus*). Exon 1 was amplified and sequenced using the following primer pair: Forward: CCACCG ATCCCAGAGAGAGG and Reverse: CCTCTGGGGAAGCCAAT CTGC. We amplified exon2 by nested PCR with the following primer pairs: Initial primer pair: Forward: GTYGCATGATTT AGAAYTTTACTGACCAA and Reverse: GACAATCCAGGCAT CCAGTTATGC. Second, nested primer pair: Forward: ATWA TGGTTTATTTAATAYARTATACATTATC and Reverse: GAACTC TAACTCATGGGATGGACAAA. The second, nested primer pair was used to sequence exon2. We used PCR Supermix (Invitrogen, Inc.) for amplification reactions. Reactions used 1 µl of each 10 µM forward primer and 10 µM reverse primer and had a final volume of 12.5 µl. Cycling parameters were 94 °C for 3 min; 40 cycles of 94 °C for 15 s, 55 °C for 15 s, 72 °C for 1 min; 72 °C for 10 min; 10 °C thereafter. Sequencing reactions were carried out using BigDye. *TRIM52* sequences have been deposited in the GenBank database (accession numbers JX896135.1–JX896146.1).

### Phylogenetic Analysis

Nonprimate *TRIM52* and *TRIM41* sequences were obtained by Blast (Altschul et al. 1990) analysis with the human *TRIM52* protein as query and psi-blast (Altschul, Madden et al. 1997) analysis with the human *TRIM52* RING expansion as query. Psi-blast of the RING expansion recovers only *TRIM52* and TRIM41 orthologs, suggesting that these are the only *TRIMs* with this expansion. We found no evidence of a protein domain downstream of the B-Box2 domain, with homology to TRIM41, in any of the *TRIM52* orthologs. For instance, there is no identifiable Coiled-Coil domain or B30.2 domain downstream of the human *TRIM52* gene in the human genome assembly. All of the *TRIM41* sequences are predicted to encode a Coiled-Coil and B30.2 domain. Nonprimate and primate *TRIM* sequences (*TRIM52* and *TRIM41*) that we recovered from Blast (Altschul et al. 1990) and Ensembl (Flicek et al. 2012) were aligned using ClustalX (Larkin et al. 2007). We only included the RING (omitting the region containing the RING expansion) and B-Box domain. Using this alignment, we constructed a tree using maximum likelihood methodology (Guindon et al. 2010) and used the program Dendroscope (Huson, Richter et al. 2007) to present a phylogram.

### Delineation of TRIM Protein Domain Boundaries and Secondary Structure

RING, B-box1, and B-box2 domains were identified based on the consensus sequences (Meroni and Diez-Roux 2005). Coiled-Coil domain boundaries were identified by predicting secondary protein structure with PSIPRED (McGuffin, Bryson et al. 2000) and identifying the long alpha helix that is associated with this motif (Lupas 1996). B30.2 or other C-terminal domains were identified by using the CDD (Marchler-Bauer, Anderson et al. 2005) and SMART (Schultz, Copley et al. 2000) domain databases, and the N-terminal boundary of B30.2 domains was aided by secondary structure prediction, as the B30.2 domain consists entirely of sequential tandem beta-strands (Seto, Liu et al. 1999; Masters, Yao et al. 2006).

### Computational Analysis of Positive Selection

Detection of recurrent positive selection by multiple alignment comparisons was carried out using the CODEML program from the PAML package (Yang 1997). Constrained model M7 was tested against unconstrained model M8 under the following parameters: f 61 (codon frequencies of 61 nonstop codons are calculated), starting omega: 0.4 and 1.5. All simulations converged and results are consistent between both codon models (2ln$\lambda$; *P* values were calculated assuming two degrees of freedom). We present the percentage of sites estimated to evolve under positive selection and the average dN/dS for those sites. Posterior probabilities were calculated according to the Naive Empirical Bayes model (Yang 1997). Positive selection, as detected by PAML, was further supported by Fast Unbiased Bayesian AppRoximation (FUBAR) and Random Effects Likelihood (REL), implemented through the Datamonkey suite of phylogenetic analysis tools (Delport et al. 2010). *TRIM* genes were required to exhibit overlapping sites of positive selection by PAML and Datamonkey to be identified as under positive selection. Specific sites of positive selection identified by both PAML and Datamonkey were denoted by underline (table 1).

**Table 1**
Primate *TRIM* Genes Evolving Under Positive Selection

| *TRIM* Gene | M7vsM8 (2lnλ) | *P* Value | % of Positively Selected Sites | Average dN/dS for Selected Sites | Positively Selected Sites | No. of Primate Taxa |
|---|---|---|---|---|---|---|
| *TRIM2* | 7.31 | 0.026 | 0.64 | 1.54 | 98, 497 | 12 |
| *TRIML2* | 6.03062 | 0.049 | 3.63 | 8.74 | 277 | 8 |
| *TRIM5* | 73.47 | <0.005 | 20.46 | 3.29 | 7, 139, 175, 182, 213, 215, 228, 257, 258, 310, 311, 317, 324, 379, 381, 382, 418, 421, 423, 471, 483 | 22 |
| *TRIM7* | 9.92 | 0.007 | 0.36 | 11.04 | 258 | 10 |
| *TRIM10* | 6.06 | 0.048 | 2.00 | 3.62 | 152, 329 | 11 |
| *TRIM15* | 10.74 | <0.005 | 5.86 | 2.25 | 18, 42, 150, 460 | 11 |
| *TRIM21* | 7.02 | 0.03 | 3.38 | 4.81 | 124, 407 | 10 |
| *TRIM22* | 10.20 | <0.005 | 4.89 | 6.17 | 99, 171, 220, 308 | 13 |
| *TRIM25* | 19.27 | <0.005 | 9.39 | 2.40 | 58, 259, 297, 338, 377, 415, 418, 420, 435 | 10 |
| *TRIM31* | 15.06 | <0.005 | 5.27 | 8.68 | 72, 227, 250 | 7 |
| *TRIM38* | 6.51 | 0.039 | 3.66 | 2.94 | 215 | 12 |
| *TRIM52* | 16.51 | <0.005 | 6.66 | 5.84 | 75, 111, 149, 153, 221 | 7 |
| *TRIM58* | 17.75 | <0.005 | 4.24 | 2.34 | 223, 443, 472, 475, 480 | 10 |
| *TRIM60* | 8.00 | 0.018 | 20.43 | 2.15 | 8, 82, 96, 134, 200, 251, 252, 255, 264, 271, 302, 322, 370, 405, 459 | 11 |
| *TRIM69* | 6.65 | 0.036 | 19.16 | 2.46 | 14, 158, 192, 226, 246, 261, 285, 353, 371, 473 | 10 |
| *TRIM75* | 10.61 | <0.005 | 0.67 | 12.24 | 45, 227 | 10 |
| *TRIM76* | 42.29 | <0.005 | 1.71 | 7.94 | 306, 651, 1507, 2727, 2797, 3314 | 10 |

Note.—PAML model M7 (Ns sites model disallowing positive selection) was directly compared with M8 (Ns sites model permitting one extra category of codons evolving under positive selection) to detect positive selection (Yang 2007). We indicate the category of codons that were found to be in the category of positively selected codons and the average dN/dS associated with those codons. Sites underlined were also found to be under positive selection by Datamonkey (Delport et al. 2010).

### TRIM52 Restriction Assays

We generated CRFK cell lines that stably express HA-tagged human and rhesus *TRIM52* by transduction of a retrovirus vector (LPCX) encoding human and rhesus *TRIM52* as described (Sawyer et al. 2005). Stable cell lines, including a negative control empty vector CRFK cell line, were plated on 12-well plates ($0.8 \times 10^5$ cells/well). These were allowed to incubate overnight and then infected with the following GFP-encoding retroviruses: HIV-1, HIV-2 (ROD9), and FIV. We used a virus titer determined to give us at least 15% infection. Three days after infection, cells were fixed with paraformaldehyde and GFP expression was measured by flow cytometry.

## Results

### Positive Selection Has Acted on Several *TRIM* Genes in Primates

To screen the *TRIM* gene family for signatures of having participated in an evolutionary arms race, we evaluated *TRIM* orthologs from primates for recurrent positive selection via maximum likelihood analyses using the CODEML program from the PAML package (Yang 2007). We compared *TRIM* orthologs from human, chimpanzee, orangutan, rhesus, and marmoset reference genomes. In some instances, we were able to identify additional orthologs from other primate genome sequencing projects that are underway via Ensembl (Vilella et al. 2009) or from previous gene-directed sequencing efforts. For a few *TRIM* genes, we were unable to identify the full complement of orthologs, as the genes were either absent or not intact in the available genome assemblies. Using this collection of orthologs, we identified 17 out of 67 *TRIM* genes as having evolved under positive selection using a *P* value cutoff of 0.05: *TRIM2*, *TRIML2*, *TRIM5*, *TRIM7*, *TRIM10*, *TRIM15*, *TRIM21*, *TRIM22*, *TRIM25*, *TRIM31*, *TRIM38*, *TRIM52*, *TRIM58*, *TRIM60*, *TRIM69*, *TRIM75*, and *TRIM76* (table 1 and supplementary table S1, Supplementary Material online). This list includes *TRIM5* and *TRIM22*, restriction factors that were previously reported to show strong evidence of positive selection (Stremlau et al. 2004; Sawyer et al. 2005; Sawyer et al. 2007; Barr et al. 2008).

Our screen also recovered known restriction factors *TRIM15*, *TRIM21*, *TRIM25*, and *TRIM38* for which no evolutionary analyses had been previously conducted. *TRIM15* was discovered in a knockdown screen to inhibit the release of retroviruses (Uchil et al. 2008) and later found to have a role in the RIG-I sensing pathway (Uchil et al. 2013). We find sites of positive selection within the RING, Coiled-Coil, and B30.2 domains of *TRIM15* (fig. 1). *TRIM21* is able to degrade viruses via an intracellular antibody-mediated mechanism (Mallery et al. 2010). Positive selection was detected within the B-Box and B30.2 domains of TRIM21 (fig. 1). *TRIM25* activates RIG-I signaling via ubiquitination (Gack et al. 2007), and *TRIM25*-mediated signal transduction is known to be inhibited by the direct interaction of influenza A protein NS1 to the

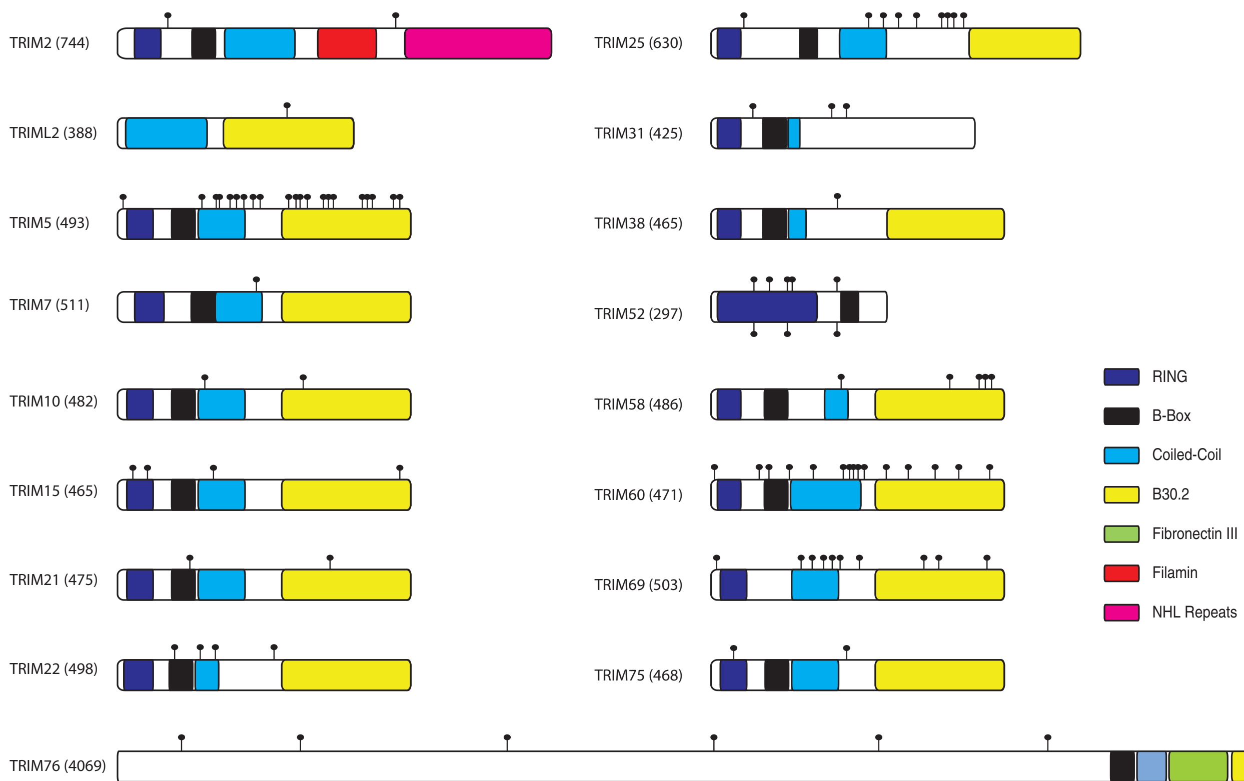



**Fig. 1.**—Architectures of *TRIM* family members exhibiting positive selection in primates. We present a domain schematic for all the proteins with signatures of positive selection in our evolutionary survey (table 1) along with their total length in amino acids (parentheses); in cases of alternate splicing, we represent the largest possible protein isoform encoded by a given *TRIM* gene. The schematized protein domains are based on GenBank and Ensembl reports. Sites of recurrent positive selection are marked with lollipops above the protein representation. The sites identified by a more in-depth analysis of *TRIM52* are shown as lollipops below the protein representation.

Coiled-Coil domain of TRIM25 (Gack et al. 2009). We find that *TRIM25* exhibits a number of sites under positive selection, clustered between the Coiled-Coil and B30.2 domains (fig. 1). *TRIM38* is known to negatively regulate innate immunity by targeting TRIF (Xue et al. 2012), NAP1 (Zhao, Wang, Zhang, Yuan et al. 2012), and TRAF6 (Zhao, Wang, Zhang, Wang et al. 2012) for ubiquitination and degradation. *TRIM38* has also been shown to improve the fitness of HIV-1 during entry by an unknown mechanism (Uchil et al. 2008). Only a single site of positive selection residing between the Coiled-Coil and B30.2 domains was detected (fig. 1).

The *TRIM* genes with known antiviral activity that we recovered in our positive selection screen (*TRIM5*, *TRIM15*, *TRIM21*, *TRIM22*, *TRIM25*, *and TRIM38*) belong to the C-IV family of *TRIM* genes based on their domain structure (Ozato et al. 2008). Our screen highlighted six additional genes from the C-IV family that have not been previously implicated in antiviral defense: *TRIM7*, *TRIM10*, *TRIM58*, *TRIM60*, *TRIM69*, and *TRIM75*. We found modest signatures of positive selection for three of these genes, *TRIM7*, *TRIM10*, *and TRIM75*. A single site was found to be under positive selection in *TRIM7* in the Coiled-Coil domain (fig. 1). Similarly, *TRIM75* has a single site of positive selection in its RING domain associated with E3 ubiquitin ligase activity. For *TRIM10*, positive selection was found in the Coiled-Coil and B30.2 domains, which may reflect changes in target recognition, similar to *TRIM5* (Sawyer et al. 2005; Maillard et al. 2010). We further found evidence of robust positive selection for *TRIM58*, *TRIM60*, and *TRIM69*, which have multiple sites with high dN/dS values (fig. 1). Indeed, among all known primate *TRIM* genes, the signature of positive selection for these three genes appears to be on par with what we see for the bona fide restriction factor *TRIM5*. For *TRIM58*, we find a cluster of positively selected sites in the B30.2 domain and a single sight highlighted in the Coiled-Coil domain. *TRIM60* has positively selected sites in each of the RING, B-Box, Coiled-Coil, and B30.2 domains (with significant clustering in the latter two domains). *TRIM69*, similarly, exhibits a cluster of sites under positive selection in the Coiled-Coil and B30.2 domains. Thus, this evolutionary screen of primate *TRIM* genes identified multiple new candidate restriction factors belonging to the same subfamily (C-IV) already known to harbor known antiviral genes.

We also identified candidate restriction factors outside of the C-IV family of TRIM genes: *TRIM2* (C-VII), *TRIML2* (UC), *TRIM31* (C-V), *TRIM52* (C-V), and *TRIM76* (UC). *TRIM2* contains a Filamin-type immunoglobulin domain and array of NHL

repeats. Two sites of positive selection were found in *TRIM2*, with neither of these residing within the known domains of *TRIM2* (fig. 1). *TRIML2* is a highly unusual *TRIM*-like gene. It lacks canonical RING and B-box domains, being solely composed of Coiled-Coil and B30.2 domains. Formally, it does not meet the criteria of being an RBCC-type *TRIM* gene. However, given the propensity of *TRIM* proteins to homo- and heterodimerize, we also included such noncanonical genes within our analysis. We find a single site of positive selection within the C-terminus B30.2 domain of *TRIML2* (fig. 1). *TRIM31* is a suspected retroviral restriction factor that acts at the stages of entry and release for HIV-1 and MLV, respectively (Uchil et al. 2008). We identified three sites exhibiting signatures of positive selection in *TRIM31*; two of these sites are in the C-terminal region, which is not homologous to any known *TRIM*-associated domains but may represent an analogous virus-interacting domain (fig. 1). *TRIM52* is unique among the restriction factor candidates as it only encodes the RING and B-Box domains. We identified the majority of positive selection within the RING domain and a single site immediately upstream of the B-Box domain. Intriguingly, the RING domain of *TRIM52* has expanded and is the largest among the genes recovered by our screen (fig. 1); this expansion also appears to contain the positively selected sites. *TRIM76* is the final candidate identified. It encodes for a large ~3,500 amino-acid protein that contains B-Box, Coiled-Coil, Fibronectin III, and B30.2 domain in its C-terminus region. The remainder of the protein does not contain homology to any annotated domains but contains the six sites of positive selection we identified.

Thus, our evolutionary screen for novel restriction factors among the *TRIM* gene family identified 15 members not previously known to be under positive selection. Most excitingly, our screen identifies as many as 10 novel candidates for antiviral function. Although all of these *TRIM* genes may not participate in host–pathogen interactions, several exciting canonical candidates (e.g.,*TRIM58* and *TRIM60*) emerged from our screen. In addition, we identified several noncanonical candidates (e.g., *TRIML2* and *TRIM52*) that may have otherwise been overlooked as potential restriction factors because they are missing some of the key RBCC domains. We decided to pick one of these noncanonical candidates, *TRIM52*, for a more in-depth analysis to confirm and expand the findings of our initial screen.

### Rapid Evolution of the *TRIM52* RING Domain in Primates

We decided to evaluate *TRIM52* in more detail because it structurally deviated the most from the canonical *TRIM* restriction factors (i.e., *TRIM5* and *TRIM22*). For example, *TRIM52* lacks the viral recognition (B30.2) domain and displays signatures of rapid evolution within the RING domain. Moreover, *TRIM52* appears to lack an intact Coiled-Coil domain within its coding region (fig. 1). Thus, *TRIM52* is comprised solely of the RING and B-Box2 domains, making it a highly unusual member of the TRIM multigene family. Even the RING domain of *TRIM52* is highly unusual. RING domains of the *TRIM* family are generally defined by the consensus sequence Cx2Cx9-45Cx1-3Hx2-3Cx2Cx4-48Cx2[C/D], where eight cysteine, histidine, or aspartic acid residues coordinate two zinc atoms (Meroni and Diez-Roux 2005). The region between the sixth and the seventh coordinating residues is referred to as the "loop 2" region of the RING tertiary structure using the precedent of the human c-cbl RING-containing E3 ubiquitin ligase (Zheng et al. 2000). The majority of *TRIM* genes encode between 4 and 48 amino acids in their loop 2 region, with the mode being 13 amino acids (fig. 2). However, several *TRIM* genes were found to deviate from the consensus range. Most notably, *TRIM52* encodes 139 amino acids in its loop 2 region (fig. 2). Thus, *TRIM52* encodes the largest RING domain of any human *TRIM* gene. BLAST (Altschul et al. 1990) analysis of this region reveals similarity only to mammalian *TRIM52* and *TRIM41* genes, both of which have exceptionally large RING domain expansions.

In order to elucidate the evolutionary relationship between *TRIM52* and *TRIM41* and to deduce when this large loop 2 RING expansion occurred, we carried out phylogenetic analyses of *TRIM52* and *TRIM41* sequences that were obtained from Blast (Altschul et al. 1990) searches of vertebrate genomes. Our analyses revealed that *TRIM52* and *TRIM41* are close paralogs that are found in close proximity to each other in most mammalian genomes (fig. 3). We found that the reptile (anole lizard), avian (chicken and wild turkey), and marsupial (Tasmanian devil and opossum) genomes have only single *TRIM41*-like genes, which are phylogenetic outgroups to both the *TRIM52* and *TRIM41* clades from eutherian mammals (fig. 3). This suggests that *TRIM52* was born in eutherian mammals ~190 Ma via a partial duplication of *TRIM41*, having lost both the Coiled-Coil and B30.2 domains at birth (Meredith et al. 2011).

Despite their evolutionary relationship, our screen for positive selection in primates highlighted *TRIM52* but not *TRIM41*. To further evaluate the evolutionary history of *TRIM52*, we repeated our analysis of recurrent, site-based positive selection via maximum likelihood analyses using primate *TRIM52* orthologs obtained by our additional sequencing efforts. From this in-depth analysis, we refined the sites of recurrent, codon-based positive selection (figs. 1 and 4*B*). The sites of positive selection reside primarily within the expanded "loop 2" region of *TRIM52*. This rapid evolution of the RING domain is especially evident in an evolutionary comparison focused on the "loop 2" expansion unique to *TRIM41* and *TRIM52*, which highlights the dramatic acceleration of amino acid replacements in *TRIM52* (fig. 4*A* and *B*). In contrast to *TRIM52*, we found no evidence of positive selection having acted on the *TRIM41* using available primate sequences from databases (fig. 4*A*). Thus, in stark contrast to *TRIM41* and its RING domain that has been evolving under constraint, we find

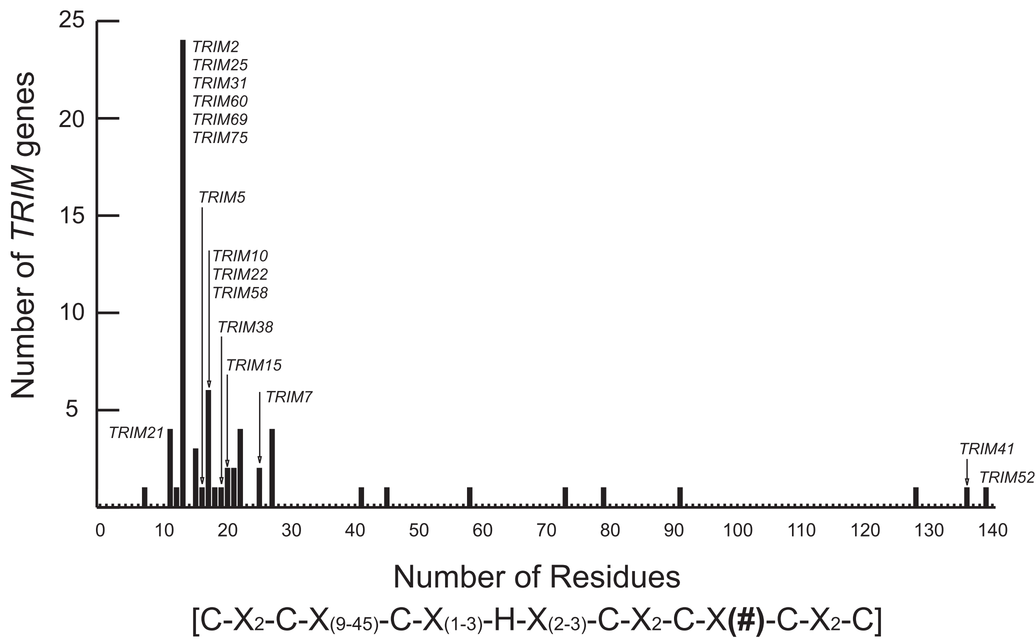



**Fig. 2.**—Variability in the length of the RING domain. The RING domains from 67 annotated human *TRIM* genes were collected from Ensembl (Flicek et al. 2012) and GenBank and evaluated to determine the length of the variable loop 2 region located within the domain. Alignments of homologous regions were built using ClustalX (Larkin et al. 2007) and the number of residues residing in the variable region were counted. The predicted length of this variable region ranges from 4 to 48 amino acids. *TRIM52* and *TRIM41* have the largest expansion of their RING domains.

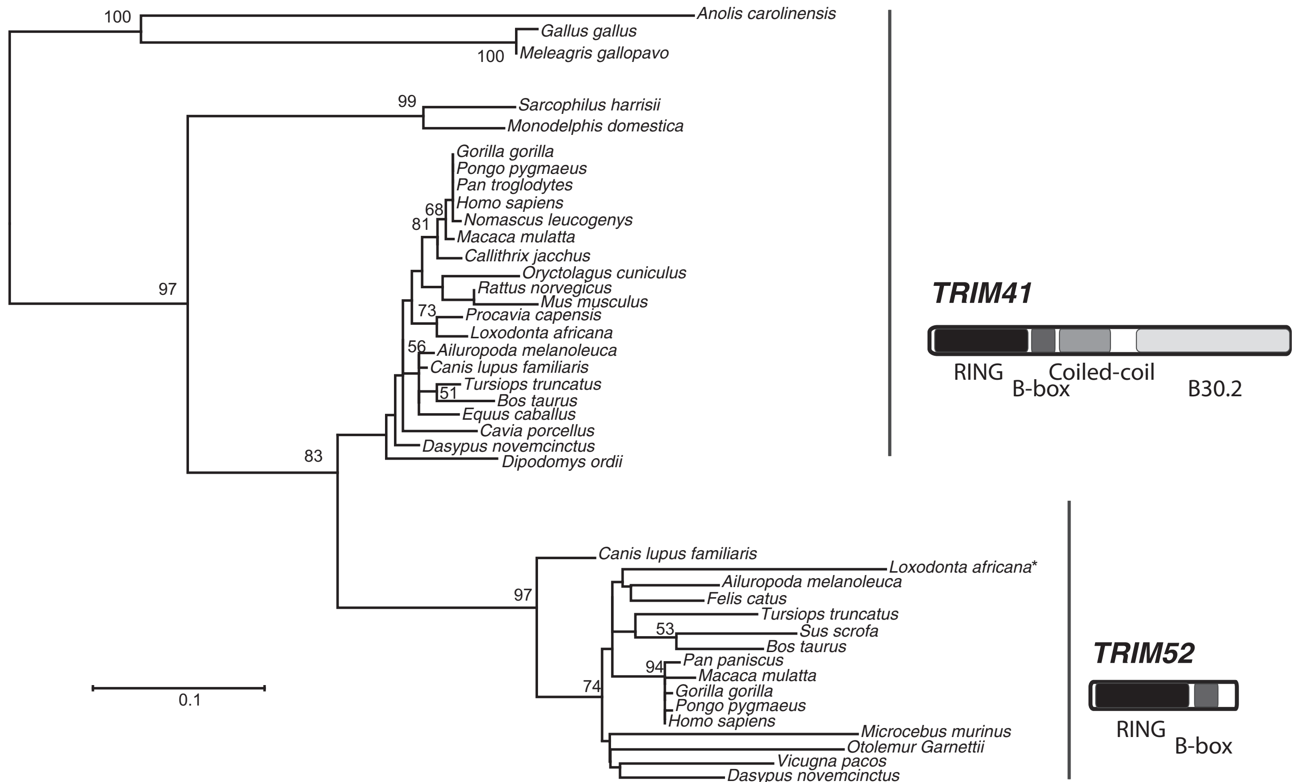
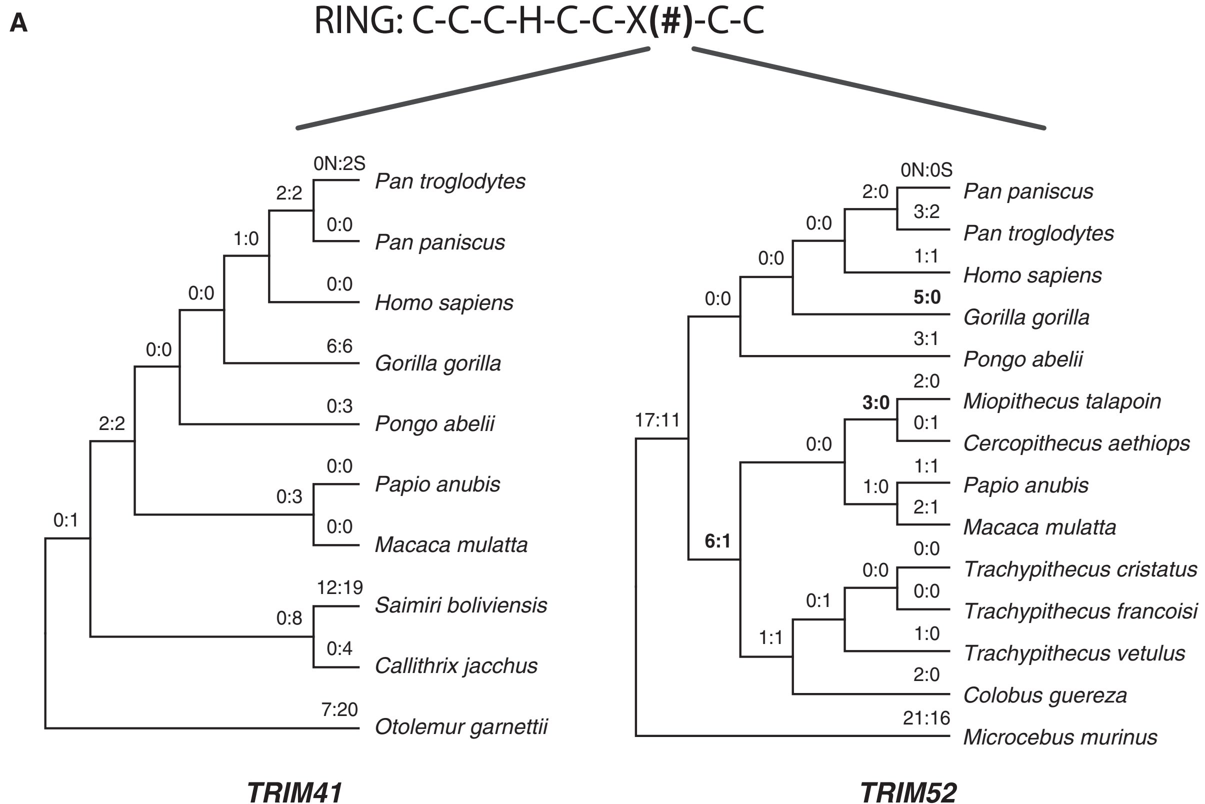



**Fig. 3.**—Phylogenetic relationship of *TRIM52* and *TRIM41*. A phylogram of homologous regions of the RING and B-Box2 domains from *TRIM52* and *TRIM41* orthologs was built using a maximum likelihood based approach via PhyML (Guindon et al. 2010). Statistical support is represented by bootstrap values, collected from 100 iterations. The * symbol denotes the presence of nonsense mutations that result in pseudogenization.



Fɪɢ. 4.—Positive selection within the RING domain of *TRIM52*. (*A*) More than half the sites predicted to be evolving under positive selection (fig. 1 and table 1) are located within the RING domain of *TRIM52*. To further highlight this, we identified the number of synonymous (S) and nonsynonymous (N) substitutions that have occurred in the expanded loop 2 region of *TRIM52* in primate evolution (the equivalent domain of *TRIM41* is shown for comparison). Examples of dramatic episodes of lineage-specific positive selection in *TRIM52's* RING domain are highlighted in bold. (*B*) The differences in evolutionary signals are further demonstrated by an alignment of a 90 amino acid-long stretch of the loop 2 region from primate *TRIM41* and *TRIM52*. Sites of positive selection are highlighted with a star and boldface.

that *TRIM52* has been rapidly evolving throughout primate history, with much of that selection acting on the expanded RING domain.

### Repeated Loss/Pseudogenization of *TRIM52* in Mammals

Our sequencing survey also revealed at least two instances of *TRIM52* loss or pseudogenization over the course of primate evolution (fig. 5). For instance, within marmoset and other New World monkey genomes, we were only able to identify exon 2 using a combination of Blast (Altschul et al. 1990) and BLAT (Kent 2002) searches (supplementary fig. S1, Supplementary Material online) and our own polymerase chain reaction (PCR) analyses. These analyses suggested that *TRIM52* is present but pseudogenized throughout the New World monkey lineage. We were also unable to detect *TRIM52* from gibbon genomes (*N. leucogenys*, *Hylobates agilis*, and *Symphalangus syndactylus*) via PCR with genomic DNA, despite using PCR primers that amplified *TRIM52* from all other Hominoids and Old World monkeys. Blast (Altschul et al. 1990) and BLAT (Kent 2002) analyses support the absence of *TRIM52* from publicly available gibbon genomes.

This pattern of stochastic *TRIM52* loss was also evident in other mammalian orders. We identified *TRIM52* pseudogenization or loss in African elephant (*Loxodonta africana*), horse (*Equus caballus*), microbat (*Myotis lucifugus*), and megabats (*Pteropus vampyrus*) (supplementary fig. S1, Supplementary Material online). We were also unable to identify *TRIM52* throughout the glires (Rodentia and Lagomorpha) lineage of mammals, suggesting that it has been deleted early within this lineage. However, utilizing UCSC (Kent et al. 2002) and Ensembl (Flicek et al. 2012) predictions, we were able to recover *TRIM52* from the genomes of the mouse and rat. Sequence analysis of these predicted mouse and rat *TRIM52* revealed that they do not encode a B-Box domain. Therefore, the annotated mouse and rat *TRIM52* comprised only a RING domain. Furthermore, the *TRIM52* orthologs we did identify in mouse and rat genomes were not located proximal to *TRIM41* and are therefore the only nonsyntenic *TRIM52* orthologs in mammals (supplementary fig. S2, Supplementary Material

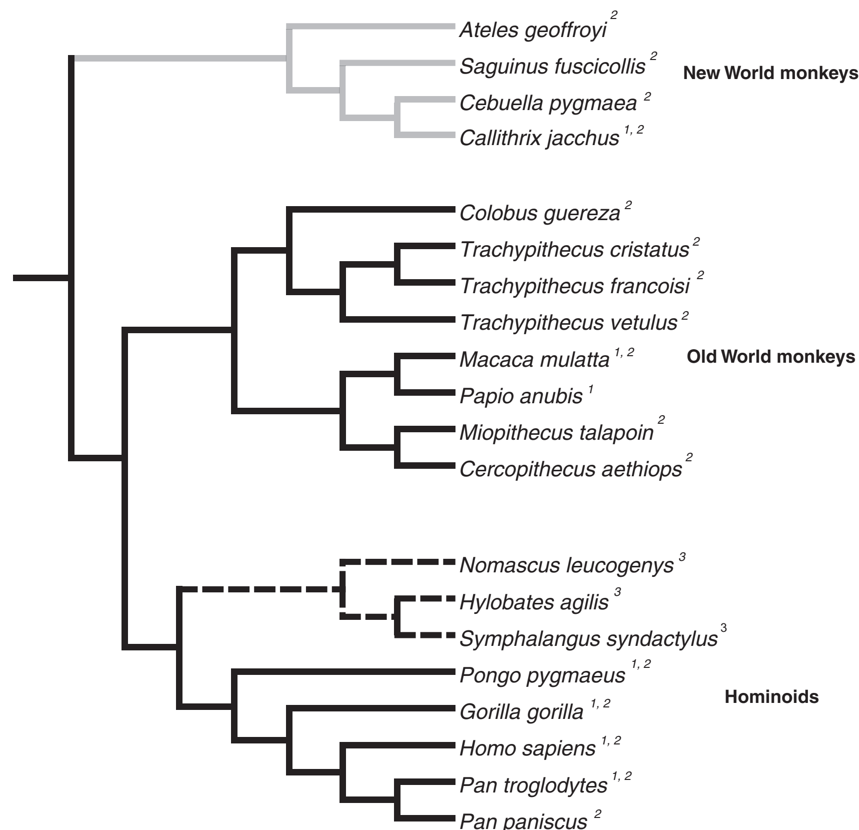



**Fig. 5.**—Presence/absence of *TRIM52* in primates. We evaluated *TRIM52* from a range of Hominoids, Old World monkeys, and New World monkeys, using sequences collected from 1) Ensembl (Flicek et al. 2012) and GenBank and via 2) PCR. Primates surveyed by our analysis are presented in a guide tree of the well-accepted primate phylogeny (Perelman et al. 2011). 3) We were unable to amplify *TRIM52* from the gibbon lineage of Hominoids (represented by dotted branches), despite the use of primers that we used to amplify orthologs from other Hominoids and Old World monkeys. Grayed branches represent lineages where we observed *TRIM52* to be pseudogenized.

online). When we included mouse and rat *TRIM52* in our phylogenetic analysis (supplementary fig. S3, Supplementary Material online), branch support at the node separating the *TRIM41* and *TRIM52* clades was lowered (even though mouse and rat *TRIM52* genes localized within the *TRIM52* clade). Due to the apparent loss of *TRIM52* throughout glires, the truncated structure of mouse and rat *TRIM52*, and their ambiguous phylogenetic placement, we therefore cannot confidently assign these mouse and rat *TRIM* genes as bona fide *TRIM52* orthologs, labeling them *TRIM52*-like instead (supplementary fig. S1, Supplementary Material online). Given this uncertainty, we had omitted the mouse and rat *TRIM52*-like sequences from our phylogenetic analysis (fig. 3). Additional genome sequencing within eutherian mammals may reveal still additional instances of *TRIM52* loss or pseudogenization, suggestive of episodes of relaxed selective pressure among individual lineages.

### Human and Rhesus *TRIM52* Do Not Restrict Lentiviruses

The history of positive selection uncovered among primate *TRIM52* orthologs indicates that its function has been adaptively evolving. Although many members of the *TRIM* family positively and negatively impact retroviruses (Uchil et al. 2008), *TRIM52* was not tested in previous analyses. Indeed, the degree of adaptive evolution within the RING domain of *TRIM52* suggests that the role of viral recognition has shifted in the absence of the B30.2 domain to the RING domain. Thus, we evaluated human and rhesus *TRIM52* orthologs for antiviral activity against a limited panel of lentiviruses (supplementary fig. S4, Supplementary Material online). Although many of these viruses are restricted by other *TRIM* proteins, we found no evidence of restriction by either human or rhesus *TRIM52*. Thus, although the evolutionary patterns of positive selection and episodic loss strongly implicate *TRIM52* in some form of host defense, the targets of this activity are still unknown and likely not retroviral.

## Discussion

### *TRIM52*, A Candidate Antiviral Gene

A screen for positive selection identified 15 new members of the primate *TRIM* gene family. Of these, *TRIM52* was the most unusual. Indeed, in the absence of these evolutionary analyses, *TRIM52* might not draw attention as a candidate antiviral factor because it lacks a canonical virus-interaction domain (fig. 1). Although *TRIM52* lacks B30.2 and Coiled-Coil domains, the gene bears an ancient expansion of the RING domain that exhibits positive selection. TRIM52 is not the first candidate antiviral TRIM gene that lacks a canonical viral capsid-binding domain, however. In a previous analysis of rodent *TRIM5* paralogs, we identified mouse (*Mus musculus*) *TRIM12*, which only encodes RING, B-Box2, and Coiled-Coil domains (Tareen et al. 2009). Similar to *TRIM52*, mouse *TRIM12* exhibits signatures of positive selection despite the absence of a recognized interaction interface (i.e., B30.2 domain). Indeed, our finding of positive selection within the RING domain leads to the intriguing model whereby the antiviral interaction interface of *TRIM52* may have now shifted to within its RING domain. This is an unusual exception to the highly modular arrangement of the mammalian and fish *TRIM* gene family in which the target interaction interface is usually restricted to the Coiled-Coil or B30.2 domains, which are also the hotspots for positive selection (Reymond et al. 2001; Meroni and Diez-Roux 2005; Nisole et al. 2005; Song, Gold et al. 2005; Yap et al. 2005; Sawyer et al. 2007; van der Aa et al. 2009).

Despite the strong signature of positive selection, we identified at least six independent losses of *TRIM52* within mammals, including two events in primates. The absence of *TRIM52* from gibbon genomes may reflect its genomic position, proximal to the telomeric region in Hominoids and Old World monkeys. However, this genomic positioning is not shared in other mammals (supplementary fig. S2, Supplementary Material online) and therefore cannot account for the multiple loss events we have observed. Furthermore, we found no evidence for either loss or pseudogenization of the proximally located *TRIM41* gene. This suggests that the parental gene is under strong functional constraint, whereas the episodes of *TRIM52* loss strongly suggest that this *TRIM* gene does not carry out a conserved, housekeeping function in mammalian genomes, further supporting its proposed role as an antiviral factor. Intriguingly, the recurrent loss of *TRIM52* is reminiscent of the dynamic evolutionary history observed by other *TRIM* genes with antiviral function. For instance, the dog *TRIM5* ortholog is pseudogenized (Sawyer et al. 2007), whereas cats encode a truncated form of *TRIM5* with a disrupted B30.2 domain; both lineages are unable to express TRIM5alpha (McEwan et al. 2009). Both rodent (mouse and rat) and cow genomes lack *TRIM22* orthologs but contain expanded sets of *TRIM5* paralogs (Sawyer et al. 2007; Tareen et al. 2009). Expansions are not unique to *TRIM5*. Han et al (2011) identified several *TRIM* genes that are copy number variable in human genomes. Similar dynamics have also been observed in several teleost species, where unique *TRIM* genes (fintrims) have expanded and diversified in each lineage (van der Aa et al. 2009). We have previously suggested that positive selection and the expansion of *TRIM* genes is driven by new or continuous selective pressure, likely provided by viral pathogens (Sawyer et al. 2007; Tareen et al. 2009; Han et al. 2011). Similarly, the loss or relaxation of such a selective pressure could result in the loss of a *TRIM* gene (Sawyer et al. 2006; Sawyer et al. 2007). Thus, considering the dynamic history of *TRIM52* and our evidence of positive selection, we posit that this unusual *TRIM* gene is involved in genome defense but can be lost either due to relaxed selection or because of the high costs borne by encoding such a defense (Sawyer et al. 2006).

It is also possible that *TRIM52* is under positive selection not because of antiviral activity but instead to maintain its interaction with a host target substrate that is also adaptively evolving. However, we find this coevolutionary scenario unlikely because such host–host interaction surfaces are not typically found to evolve under positive selection unless they are challenged by a pathogenic influence (Koyanagi et al. 2010; Daugherty and Malik 2012). Furthermore, this scenario would posit that the many incidences of *TRIM52* loss we have documented would have to coincide with the simultaneous loss of the target substrate or the requirement to maintain the interaction.

### Positive Selection within the *TRIM* Gene Family

Based on the unbiased approach of our screen, we predicted the recovery of several known restriction factors. In particular, there was an expectation of identifying *TRIM5* and *TRIM22*, both previously highlighted for their positive selection (Sawyer et al. 2005; Sawyer et al. 2007). In addition to these, we recovered other known or suspected restriction factors: *TRIM15*, *TRIM21*, *TRIM25*, and *TRIM38*. We detected positive selection occurring all along *TRIM25*, in particular within the Coiled-Coil and B30.2 domains (fig. 1). *TRIM25* plays a role in influenza infection, where its activity is critical for the activation of the RIG-I-dependent signaling cascade (Gack et al. 2007). Specifically, influenza A encodes protein NS1 that directly interacts and inhibits *TRIM25* at the Coiled-Coil domain inhibiting the ubiquitination and activation of RIG-I. This is reminiscent of adaptive evolution in other known restriction factors, such as in the case of MAVS to evade protease cleavage by hepatitis C virus (Patel et al. 2012) or in tetherin to evade lentivirus Nef or Vpu (Lim et al. 2010). In both cases, positive selection highlights regions of the host-encoded protein targeted by viral antagonists and provided insight into mechanisms of host evasion. Thus, it is likely that the sites of positive selection exhibited by *TRIM25* reveal adaptation during primate history to evade NS1 or NS1-like antagonists. *TRIM15* similarly plays a role regulating innate immune signaling, and its positive selection may reflect a similar constraint as *TRIM25*. In contrast, *TRIM21* is able to target cytosolic antibodies bound to viruses and auto-ubiqutinate, leading to the proteasomal degradation of the *TRIM21*-bound complex (Mallery et al. 2010). As this complex forms via *TRIM21* binding to the invariant region of antibodies (James et al. 2007), it is unlikely that the interaction between host-encoded products is responsible for the positive selection we detected. Instead, it is much more likely that a novel viral antagonist targets TRIM21 and that positive selection is reflective of evasion from such an antagonist. Unique among these recovered *TRIM* genes, *TRIM38* has been found to assist HIV-1 during entry (Uchil et al. 2008). *TRIM38* has recently been recognized for having a role in negatively regulating innate immunity by targeting components of innate immunity for degradation (Xue et al. 2012; Zhao, Wang, Zhang, Yuan et al. 2012; Zhao, Wang, Zhang, Wang et al. 2012). Positive selection on *TRIM38* may therefore also reflect its escape from viral-mediated antagonism of innate immunity. Thus, our analysis of positive selection provides insight into the interface and nature of host–pathogen interactions in cases of known restriction factors (fig. 6). Specifically, we expect these sites of positive selection to be affecting structure, either indirectly altering the host–pathogen interface or at the direct interface contacting viral proteins. In specialized cases like *TRIM5*, this direct interaction is predicted of a rapidly evolving antiviral *TRIM* gene. Alternatively, as we predict of *TRIM25*, positive selection is reflective of evading viral antagonism.

One *TRIM* gene that did not show a signature of positive selection at all is *TRIM19/PML*. This is in agreement with an extended sequencing of primate *TRIM19/PML* orthologs which concluded that there was no evidence for positive selection of this gene (Ortiz et al. 2006). This may be surprising in light of the evidence that PML functions in antiviral defense (reviewed in Nisole et al. 2005). However, positive selection would only be expected to act on genes encoding proteins that directly interact with viral proteins, and so any upstream or downstream effector may not present such a signal. It is interesting that *TRIM1* (MID2) also shows no adaptive signature, given that the human *TRIM1* protein has been shown to have anti-MLV activity (Yap et al. 2004). This may reflect a retroviral restriction that is not currently being utilized by humans or chimpanzees, because our screen was especially focused on this lineage of mammals. Nonetheless, it is important to point out that the absence of positive selection does not preclude *TRIM* genes from being candidate restriction factors, but those *TRIM* genes that have evolved under positive selection represent the most likely candidates for having an antiviral role via direct interaction (fig. 6).

As many of the *TRIM* genes remain largely uncharacterized, our evolutionary screen is able to highlight candidate restriction factors based on exhibition of positive selection, a hallmark of antiviral genes at the direct interface of the host–viral pathogen arms race (Daugherty and Malik 2012). Based on the extent of rapid evolution observed among them, we propose that *TRIM58*, *TRIM60*, and *TRIM69* represent the best uncharacterized candidates for novel restriction factors within the primate *TRIM* multigene family and should therefore be intensively investigated for antiviral function (fig. 6).

Based on previous studies with APOBEC and *TRIM5* restriction genes, it is informative to identify antiviral restriction factors even if they are not currently active against modern viral pathogens. Restriction factors honed against evolutionarily "recent" viral infections might protect us against future viruses or viral variants or might be artificially enhanced to be active against current forms. Genes with partial activity might vary in potency within the human population. Furthermore, such genes serve as barriers to animal models of viral infection (Hatziioannou et al. 2006; Kirmaier et al. 2010). To this end,

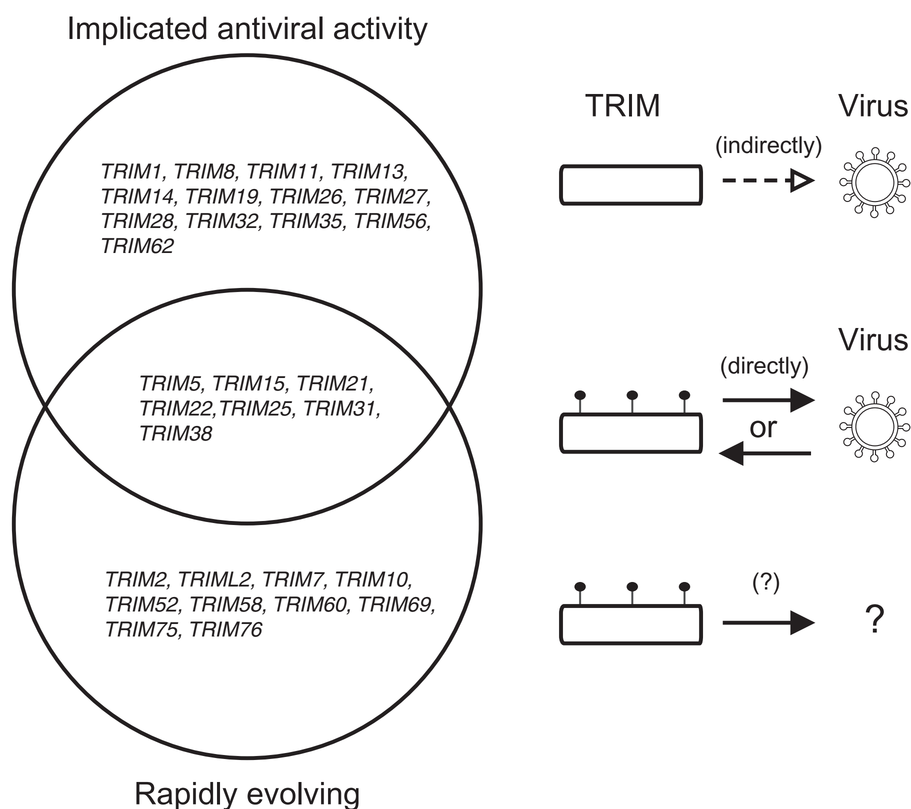



FIG. 6.—Implicated restriction factors. Of 67*TRIM* genes, several members have been implicated as restriction factors, either positively or negatively impacting viral fitness. In many of these cases, direct interactions with viral proteins has not been detected (top) (reviewed in Ozato et al. 2008; Kawai and Akira 2011). Seven of these genes have evolved under positive selection in primates—two that were previously published, *TRIM5* and *TRIM22* (Sawyer et al. 2007; Sawyer et al. 2005), in addition to *TRIM25* (Gack et al. 2009), *TRIM21* (Mallery et al. 2010), *TRIM15*, *TRIM31*, and *TRIM38* (Uchil et al. 2008) (Overlap). We believe these restriction factors likely act via a direct interaction interface to recognize or evade viral proteins. In addition, we found ten *TRIM* genes to be rapidly evolving that represent novel restriction factor candidates which may also act via direct host–virus interactions (bottom).

our evolutionary approach to identify potential restriction factors in the *TRIM* family has revealed ten canonical and noncanonical members of this gene family that bear previously unrecognized signatures of recent positive selection. These primate *TRIM* genes are therefore primate candidates to be investigated as novel restriction factors against viruses.

## Supplementary Material

Supplementary figures S1–S4 and table S1 are available at *Genome Biology and Evolution* online (http://www.gbe.oxfordjournals.org/).

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