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# Primate TRIM34 is a broadly-acting, TRIM5-dependent lentiviral restriction factor

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## Abstract

Human immunodeficiency virus (HIV) and other lentiviruses adapt to new hosts by evolving to evade host-specific innate immune proteins that differ in sequence and often viral recognition between host species. Understanding how these host antiviral proteins, called restriction factors, constrain lentivirus replication and transmission is key to understanding the emergence of pandemic viruses like HIV-1. Human TRIM34, a paralogue of the well-characterized lentiviral restriction factor TRIM5 $\alpha$ , was previously identified by our lab via CRISPR-Cas9 screening as a restriction factor of certain HIV and SIV capsids. Here, we show that diverse primate TRIM34 orthologues from non-human primates can restrict a range of Simian Immunodeficiency Virus (SIV) capsids including SIV<sub>AGM-SAB</sub>, SIV<sub>AGM-TAN</sub> and SIV<sub>MAC</sub> capsids, which infect sabaues monkeys, tantalus monkeys, and rhesus macaques, respectively. All primate TRIM34 orthologues tested, regardless of species of origin, were able to restrict this same subset of viral capsids. However, in all cases, this restriction also required the presence of TRIM5 $\alpha$ . We demonstrate that TRIM5 $\alpha$  is necessary, but not sufficient, for restriction of these capsids, and that human TRIM5 $\alpha$  functionally interacts with TRIM34 from different species. Finally, we find that both the TRIM5 $\alpha$  SPRY v1 loop and the TRIM34 SPRY domain are essential for TRIM34-mediated restriction. These data support a model in which TRIM34 is a broadly-conserved primate lentiviral restriction factor that acts in tandem with TRIM5 $\alpha$ , such that together, these proteins can restrict capsids that neither can restrict alone.

## Introduction

Restriction factors are a class of cell-intrinsic, germline-encoded host immune factors that can inhibit viral infection and replication. The human genome encodes for approximately 70–100 TRIM (Tripartite Motif) proteins, many of which play a role in host defense [1]. The alpha isoform of TRIM5 (TRIM5 $\alpha$ ) in primates is a well-characterized example of a primate restriction

factor with activity against retroviruses. The activity of TRIM5 $\alpha$  against a given retrovirus depends on both the species from which the TRIM5 $\alpha$  is derived and the capsid (CA) protein of the retrovirus [2–6]. In the context of HIV-1, TRIM5 $\alpha$  acts directly on CA by multimerizing onto the CA lattice [5, 7]. This interaction results in aberrant uncoating of CA, interrupting the viral life cycle [5, 8]. TRIM5 $\alpha$  from rhesus macaques and many other Old World monkeys has much greater antiviral activity against HIV-1 than does human TRIM5 $\alpha$  [2, 5, 9].

TRIM proteins are composed of a common set of N-terminal domains (RING, Bbox, and coiled-coil) followed by one or more variable C-terminal domains [10]. An important characteristic of TRIM5 $\alpha$ , and TRIM proteins more generally, is their ability to oligomerize to form structures with very high binding avidity to CA [8]. Homo-oligomerization of TRIM5 $\alpha$  is essential to its ability to restrict viral CA [11–13]. Furthermore, TRIM

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proteins have also been demonstrated to hetero-oligomerize with each other [10, 14, 15].

Previously, our lab performed a CRISPR-Cas9 screen to identify restriction factors against an HIV-1 strain with a mutation in CA, N74D, that renders it more susceptible to CA-mediated restriction factors [16]. We identified TRIM34 as a restriction factor of the HIV-1 N74D capsid mutant as well as select SIV capsids [17]. TRIM34 is a paralogue of TRIM5 $\alpha$ , sharing a common domain architecture, and human TRIM34 and TRIM5 $\alpha$  have approximately 57% amino acid identity [18]. Moreover, TRIM34-mediated restriction requires TRIM5 $\alpha$ , and TRIM34 and TRIM5 $\alpha$  colocalize with incoming capsids [17].

While TRIM5 $\alpha$  and TRIM34 both can interact with lentiviral CA, TRIM5 $\alpha$ , but not TRIM34, has undergone positive selection [15, 19]. A history of positive selection, in which the rate of nonsynonymous mutations exceeds the rate of synonymous mutations, is characteristic of host proteins that are in evolutionary conflict with pathogens and often occurs at sites of direct physical interface between host and pathogen [20, 21]. Specifically, the v1 loop of TRIM5 $\alpha$ 's C-terminal SPRY domain has been found to have undergone rapid evolution, and this region is responsible for viral recognition [19]. Conversely, the lack of evidence for positive selection on TRIM34 suggests that TRIM34 may not contain sites of evolutionarily-important direct viral interaction.

Although currently there does not exist evidence of evolutionary conflict within TRIM34, here we show that TRIM34 antiviral activity has been broadly conserved across primate species as an antiviral gene against CA of diverse primate lentiviruses. We find that restriction by TRIM34 from primates requires TRIM5 $\alpha$  and that the TRIM5 $\alpha$  SPRY v1 loop is an essential mediator of restriction. These results suggest that TRIM34 relies on the capsid-binding properties of TRIM5 $\alpha$ . However, as the TRIM34 SPRY domain is also required for restriction, our results suggest that both TRIM34 and TRIM5 $\alpha$  contribute to capsid recognition and/or antiviral function. We propose that TRIM34, which has not undergone positive selection, is an antiviral protein that requires TRIM5 $\alpha$ , which has undergone positive selection.

## Results

### Diverse primate TRIM34 orthologues can act as lentiviral restriction factors

Amino acid variation due to positive selection over evolutionary time in the v1 loop of TRIM5 $\alpha$  confers different primate species with varying specificities towards retroviral capsids [19, 22]. Although TRIM34 lacks evidence of positive selection found in TRIM5 $\alpha$ , there is still diversity across different primate TRIM34 alleles.

For example, relative to human TRIM34, chimpanzee, sabaeus monkey, and rhesus macaque orthologues differ by 4, 27, and 26 amino acids, respectively. While we have previously shown that human TRIM34 restricts SIV<sub>AGM-TAN</sub> and SIV<sub>MAC</sub>, here we wished to determine whether amino acid variation in TRIM34 orthologues from divergent primates affects TRIM34's capacity to restrict different primate lentiviral capsids [17]. Specifically, we tested whether a panel of four TRIM34 orthologues from diverse primate species comprising human (*Homo sapiens*), chimpanzee (*Pan troglodytes troglodytes*), sabaeus monkey (*Chlorocebus sabaesus*), and rhesus macaque (*Macaca mulatta*) orthologues would restrict a panel of lentiviral capsids (HIV-1, SIV<sub>CPZ</sub>, SIV<sub>AGM-SAB</sub>, SIV<sub>AGM-TAN</sub>, and SIV<sub>MAC</sub> which arise from humans; chimpanzees; two species of African green monkeys, the sabaeus monkey and the tantalus monkey; and rhesus macaques, respectively).

To test the effects of different primate TRIM34 orthologues in the absence of endogenous TRIM34, we generated a clonal TRIM34 knockout (KO) line in the human THP-1 cell line (Additional file 1: Chromatogram S1). We then introduced codon-optimized primate TRIM34 orthologues (Additional file 2) using a doxycycline-inducible lentiviral vector to generate human, chimpanzee, sabaeus, and macaque TRIM34 over-expressing cell lines. These orthologues were selected because they represent a broad range of primate TRIM34 from both hominid and Old World monkeys for which we also have the corresponding lentiviral capsids. We tested THP-1 cells knocked out for TRIM34 and then transduced with an empty vector as a control for the absence of any TRIM34. We confirmed that expression of each of the TRIM34 orthologues is induced by doxycycline (Fig. 1a), although overall steady-state expression levels varied somewhat with the macaque TRIM34 expressed at higher levels and the chimpanzee TRIM34 at lower levels.

We then determined the sensitivity of these cells to infection using a VSV-G pseudotyped lentiviral vector system, in which the CA region of a gag/pol expression construct encoded either HIV-1, SIV<sub>CPZ</sub>, SIV<sub>AGM-SAB</sub>, or SIV<sub>MAC</sub> capsids, whose host species match the species of origin of the TRIM34 orthologues used [23, 24]. These virus particles also encoded a fluorescent reporter that we used as a readout for infection. We also tested a full-length infectious molecular clone of SIV<sub>AGM-TAN</sub> that encodes a luciferase reporter [25]. After induction with doxycycline or media-only control, infectivity was quantified 2 days post-infection (dpi) by luciferase assay (SIV<sub>AGM-TAN</sub>) or flow cytometry (all others). Of note, we found that while none of the TRIM34 orthologues tested restricted HIV-1 or SIV<sub>CPZ</sub> capsids (Fig. 1b, c), all of these same TRIM34 orthologues restricted SIV<sub>AGM-TAN</sub>,

SIV<sub>AGM-SAB</sub>, and SIV<sub>MAC</sub> capsids (Fig. 1d–f). These data suggest that, in contrast to TRIM5 $\alpha$ , the antiviral specificity of TRIM34 does not seem to vary across TRIM34 orthologues. Rather, TRIM34 antiviral activity against the same set of lentiviral capsids is a conserved activity of all primate TRIM34 orthologues we tested. Moreover, the antiviral activity of all primate TRIM34s is specific for the same subset of lentiviral capsids. For example, both hominid lentiviruses tested were largely unaffected by all the TRIM34 orthologues: only HIV-1 was very weakly restricted by sabaeus TRIM34 (Fig. 1b, c). Conversely, the lentiviral capsids that were restricted by human TRIM34—SIV<sub>AGM-TAN</sub>, SIV<sub>AGM-SAB</sub>, and SIV<sub>MAC</sub>—were also restricted by all the other primate TRIM34 orthologues tested (Fig. 1d–f). In sum, these data support the hypothesis that TRIM34 restriction is a conserved activity in primates with shared specificity for certain primate lentiviral capsids.

### TRIM34 requires TRIM5 $\alpha$ for restriction

Previously, we found that human TRIM34 requires TRIM5 $\alpha$  to restrict HIV-1 N74D capsids [17]. Given that the TRIM34 orthologues tested all show selectivity for the same subset of lentiviral capsids (Fig. 1), we reasoned that TRIM5 $\alpha$  might be responsible for the capsid specificity of TRIM34-mediated restriction. Notably, for the experiments described in Fig. 1 in which all TRIM34 orthologues tested restricted the same subset of capsids, these cells expressed endogenous TRIM5 $\alpha$ . Therefore, we next asked whether TRIM5 $\alpha$  was more broadly required for TRIM34-mediated restriction of SIV<sub>AGM-SAB</sub>, which was the most potently restricted capsid of the capsids tested in Fig. 1. Specifically, we assayed for restriction in TRIM34-overexpressing cells in which we removed endogenous TRIM5 $\alpha$  expression. We created pooled knockouts of *TRIM5* in the background of THP-1 *TRIM34* clonal KO cells containing doxycycline-inducible human or macaque TRIM34 (Additional file 1: Chromatograms S2–S5). Although we observed high KO efficiency scores for the pooled KO lines (Fig. 2; legend), these pooled KO cell lines still contained some TRIM5 $\alpha$  expression as KO is not complete at a population level.

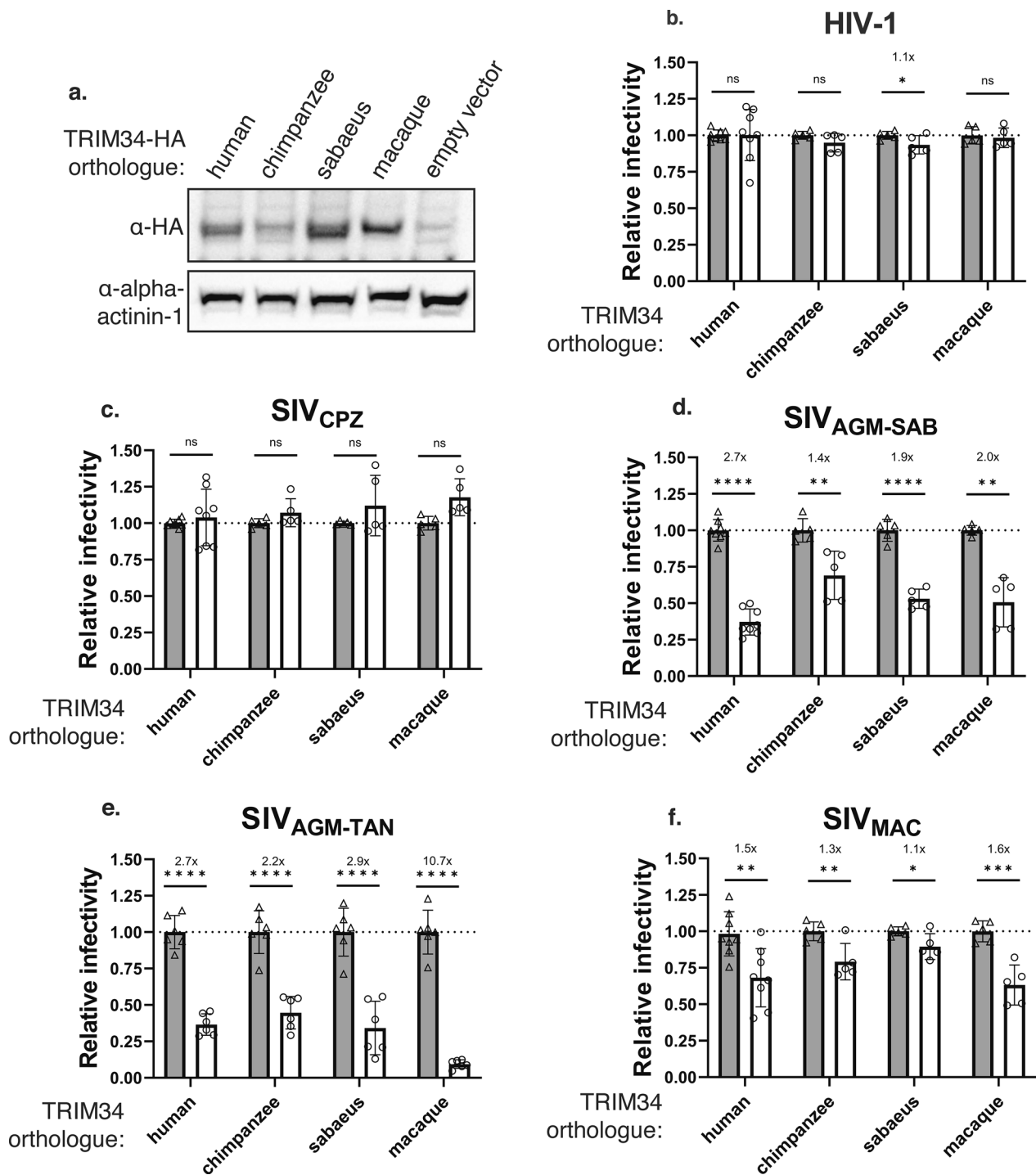
We also generated control cell lines using non-targeting control guides (NTCs); these cells still contained endogenous TRIM5 $\alpha$ . We then infected these cells with SIV<sub>AGM-SAB</sub>. Relative to cells missing both TRIM34 and TRIM5 $\alpha$  (Fig. 2a, b; black symbols), SIV<sub>AGM-SAB</sub> is restricted only in the presence of both TRIM34 and TRIM5 $\alpha$  (Fig. 2a, b; orange symbols). Notably, this was true for both human and rhesus macaque TRIM34 (Fig. 2a—human; Fig. 2b—macaque). That is, human TRIM5 $\alpha$  can fulfill TRIM34's requirement for TRIM5 $\alpha$ , even for a TRIM34 orthologue from a different primate species. Conversely, SIV<sub>AGM-SAB</sub> is not restricted either by cells expressing only TRIM34 that are knocked out for TRIM5 $\alpha$  (Fig. 2a, b; green symbols), nor by cells expressing only endogenous TRIM5 $\alpha$  without induction of TRIM34 (Fig. 2a, b; blue symbols). Although it does appear that there may be a small amount of restriction in the presence of TRIM34 only (Fig. 2a, b; green symbols), we attribute this to the fact that the *TRIM5* KO cells were generated as a KO pool: therefore, at a population level, some cells in this cell pool still express some endogenous TRIM5 $\alpha$ . Overall, these data suggest that primate TRIM34 orthologues broadly require TRIM5 $\alpha$  for restriction of lentiviral capsids.

### Human TRIM5 $\alpha$ functionally interacts with TRIM34 from different primates

We next sought to assess whether TRIM34 orthologues and TRIM5 $\alpha$  can functionally interact with each other via a TRIM5 $\alpha$  restriction assay. Previous work has shown that dimerization and higher-order multimerization are essential for TRIM5 $\alpha$ -mediated restriction [15, 26–28]. Furthermore, TRIM5 $\alpha$  has been shown to associate not only with itself but also with TRIM34 via two-hybrid screen and co-immunoprecipitation [10, 14, 15]. To assess functional interaction of TRIM34 and TRIM5 $\alpha$  more directly, we exploited the fact that TRIM proteins that have been deleted of their SPRY domains (TRIM $\Delta$ SPRY) exert a dominant-negative effect on restriction of N-tropic MLV (N-MLV) by endogenous TRIM5 $\alpha$  constitutively expressed in HeLa cells [14]. Overexpression of human TRIM34 $\Delta$ SPRY inhibits TRIM5 $\alpha$ -mediated restriction of N-MLV in HeLa

(See figure on next page.)

**Fig. 1** Diverse primate TRIM34 orthologues restrict SIV<sub>AGM-SAB</sub>, SIV<sub>AGM-SAB</sub>, and SIV<sub>MAC</sub> capsids in the presence of TRIM5 $\alpha$ . **a**. THP-1 *TRIM34* clonal KO cells were generated by electroporation of multiplexed sgRNA against *TRIM34* and single cell sorting (ICE KO score = 96%, Additional file 1: Chromatogram S1). THP-1 *TRIM34* KO cells were transduced with doxycycline-inducible HA-tagged primate TRIM34 orthologues or empty vector control. Primate TRIM34 expression was induced in the presence of 125 ng/mL doxycycline, and expression levels were visualized by immunoblotting 72 h post-induction. **b–f**. Primate TRIM34 expression was induced in THP-1 *TRIM34* clonal KO cells. 1 day post-induction, cells were either challenged with chimeric virus particles containing HIV-1 capsids co-expressing zsGreen (**b**) or SIV capsids co-expressing eGFP including SIV<sub>CPZ</sub> (**c**), SIV<sub>AGM-SAB</sub> (**d**), or SIV<sub>MAC</sub> (**f**); or challenged with VSV-G pseudotyped SIV<sub>AGM-TAN</sub>-luc (**e**). Infection was quantified 2 dpi by flow cytometry (**b–d,f**) or luminometry (**e**). Relative infectivity in induced cells (white bars, circles) is normalized to average infectivity in uninduced control cells (grey bars, triangles). Fold inhibition is indicated where applicable. Infection of each cell line with each virus was performed 5–6 times across at least 2 different occasions. Combined data are represented as mean  $\pm$  s.d., where each point represents a unique infection. One-sided p values were calculated by Welch's t-test. ns nonsignificant, \*p  $\leq$  0.05, \*\*p  $\leq$  0.01, \*\*\*p  $\leq$  0.001, \*\*\*\*p  $\leq$  0.0001



**Fig. 1** (See legend on previous page.)

cells (Fig. 2d) [14]. This implies that TRIM34 is able to functionally interact with TRIM5 $\alpha$ . Thus, we asked whether primate TRIM34 $\Delta$ SPRY orthologues could also interact with human TRIM5 $\alpha$  in this assay. We generated TRIM $\Delta$ SPRY constructs that were truncated at

the start of the SPRY domain. We overexpressed the HA-tagged human, sabaeus, chimpanzee, and macaque TRIM34 $\Delta$ SPRY constructs in HeLa cells together with human TRIM5 $\Delta$ SPRY as a positive control (Fig. 2c). These cells were then infected with N-MLV and NB-MLV,

which is not restricted by TRIM5 $\alpha$  [29, 30]. While there was no effect observed of TRIM $\Delta$ SPRY constructs on NB-MLV infection (Fig. 2d, white bars), we found that relative to empty vector control, overexpression of all four TRIM34 $\Delta$ SPRY orthologues, as with TRIM5 $\Delta$ SPRY, relieved restriction of N-MLV by human TRIM5 $\alpha$  (Fig. 2d, grey bars). These data further support our model that diverse TRIM34 orthologues and human TRIM5 $\alpha$  can functionally interact with each other.

### Both TRIM34 and TRIM5 $\alpha$ SPRY domains are involved in viral restriction

Since TRIM5 $\alpha$  is required for restriction by TRIM34 and does not seem to vary depending on which TRIM34 is used, we hypothesized that capsid recognition comes from TRIM5 $\alpha$ , and not from TRIM34. In the case of TRIM5 $\alpha$ -mediated restriction, the SPRY domain, and specifically the v1 loop, determines antiviral specificity [19, 22, 31]. Furthermore, altering a single amino acid in the human TRIM5 $\alpha$  SPRY domain (arginine 332) in the v1 loop to match the corresponding macaque residue (proline) is sufficient to confer strong restriction of HIV-1 [22, 31]. To ask if the TRIM5 $\alpha$  SPRY domain is involved in TRIM34-mediated CA recognition, we used cells that were doubly knocked out for endogenous *TRIM5* and *TRIM34* (THP-1 *TRIM34 TRIM5* clonal KO cells) (Additional file 1: Chromatograms S6–S10). In these cells, we introduced inducible vectors expressing wild type (WT) human TRIM34 with WT human TRIM5 $\alpha$ , TRIM5 $\alpha$  in which the v1 loop was deleted (TRIM5 $\alpha$   $\Delta$ v1), and TRIM5 $\alpha$  in which the arginine at position 332 was mutated to a proline (TRIM5 $\alpha$  R332P). We expressed each TRIM individually as well as together and confirmed expression levels by Western blot (Fig. 3a). Corresponding to the results in Fig. 2, we found that SIV<sub>AGM-SAB</sub> was restricted only in the presence of both human TRIM34 and human TRIM5 $\alpha$ , but not when

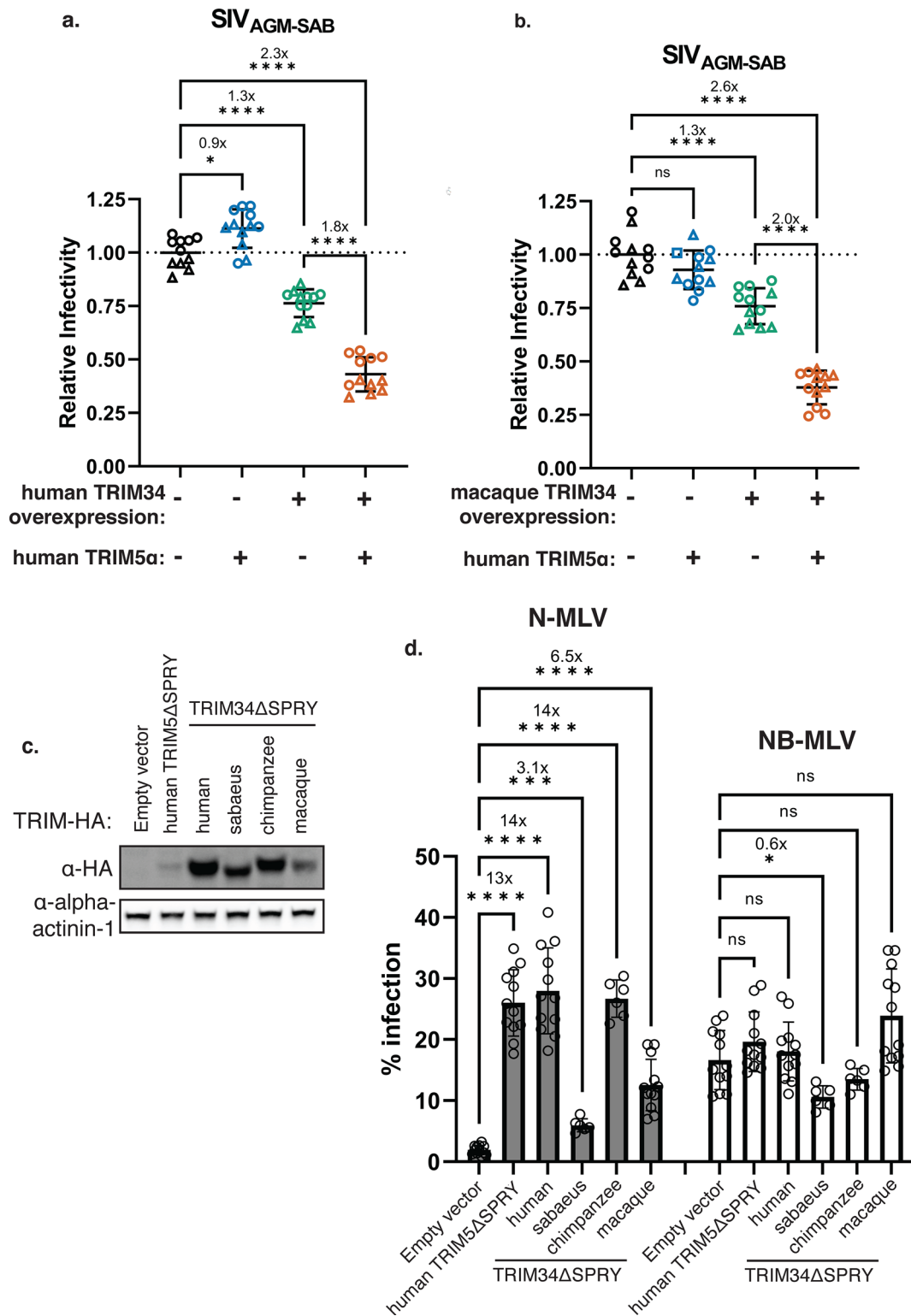
either TRIM34 or TRIM5 $\alpha$  were expressed in isolation (Fig. 3b; grey bars). Furthermore, in agreement with our previous findings [17], HIV-1 N74D, but not WT HIV-1, was restricted only in the presence of both TRIM34 and TRIM5 $\alpha$  (Fig. 3c, d; grey bars).

Given that TRIM5 is required for TRIM34-mediated restriction, we next asked whether the TRIM5 $\alpha$  v1 loop, known to be important for capsid specificity by TRIM5 $\alpha$ , was essential for restriction by TRIM34. We found that unlike co-expression of TRIM34 with full-length TRIM5 $\alpha$ , co-expression of TRIM34 with TRIM5 $\alpha$   $\Delta$ v1 was not sufficient for restriction of either SIV<sub>AGM-SAB</sub> and HIV-1 N74D (Fig. 3b, c; orange bars). This suggests that the TRIM5 $\alpha$  v1 loop is required for TRIM34-mediated restriction.

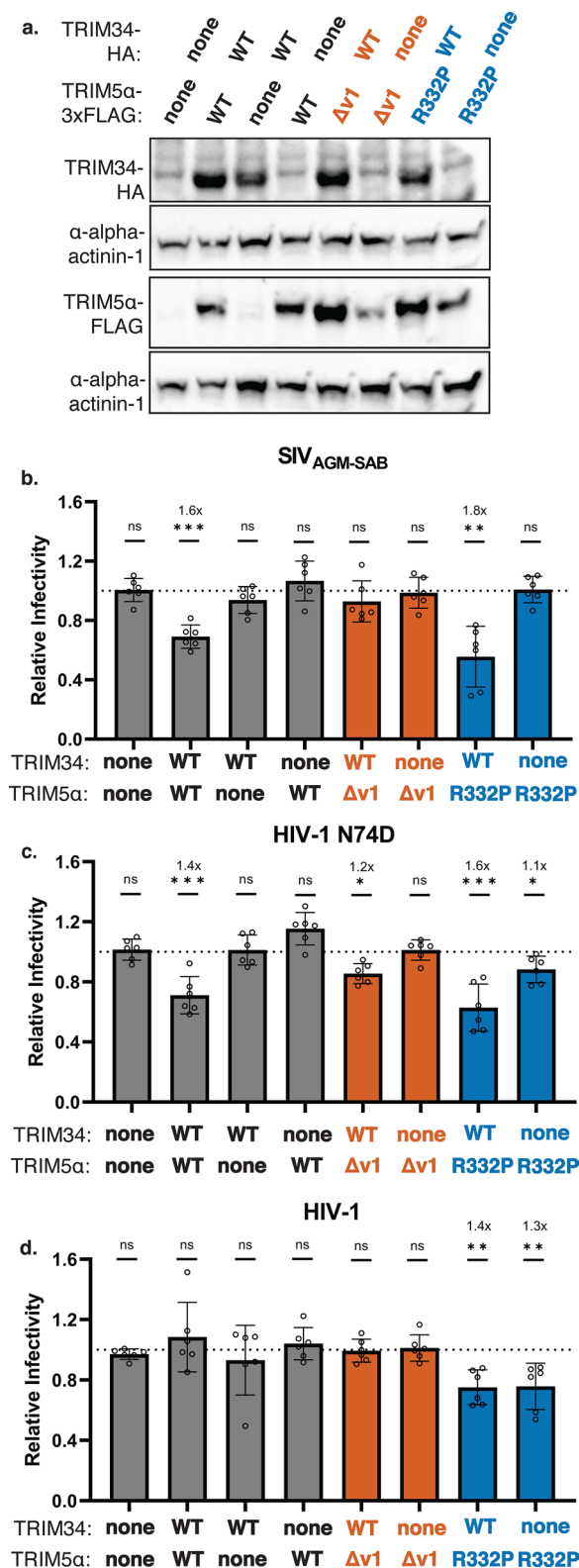
We then asked whether changing the identity of the TRIM5 $\alpha$  v1 loop, but not deleting it entirely, could also alter viral specificity. To assess this question, we generated the mutant TRIM5 $\alpha$  R332P, which converts the human residue at position 332 to the rhesus macaque residue and has been shown to have enhanced antiviral inhibition of HIV-1 [22, 31]. We co-expressed these cells with TRIM34 and infected with SIV<sub>AGM-SAB</sub>, HIV-1 N74D, or HIV-1. In accordance with the literature, TRIM5 $\alpha$  R332P was able to restrict HIV-1 while WT TRIM5 $\alpha$  did not (Fig. 3d; blue bars) [22, 31]. Furthermore, restriction of HIV-1 by TRIM5 $\alpha$  R332P was agnostic to the presence of TRIM34; that is, TRIM34 did not appear to augment restriction of HIV-1 by TRIM5 $\alpha$  R332P (Fig. 3d; blue bars). Similar to wild type TRIM34 and TRIM5 $\alpha$ , TRIM34 co-expression with the mutant TRIM5 $\alpha$  R332P allele maintained restriction of the N74D CA mutant virus (Fig. 3c; blue bars). Interestingly, TRIM34 appears to be the major factor contributing to restriction of HIV-1 N74D, whereas TRIM5 $\alpha$  R332P alone only weakly restricted HIV-1 N74D, (Fig. 3c, d; blue bars). This suggests that TRIM34 is critical for restriction of HIV-1

(See figure on next page.)

**Fig. 2** TRIM34 requires TRIM5 $\alpha$  to restrict SIV<sub>AGM-SAB</sub>. **a–b.** THP-1 *TRIM34* clonal KO cells containing doxycycline-inducible TRIM34 from humans (**a**) or rhesus macaques (**b**) were transduced with lentiviral vectors encoding Cas9 and 1 of 2 independent sgRNA against *TRIM5* or a non-targeting control to generate pooled *TRIM5* knockout or NTC cell lines. Pooled KO efficiency was assessed by ICE analysis (Additional file 1: Chromatograms S2–S5). For human TRIM34-expressing cell lines, ICE KO efficiency scores were as follows: *TRIM5* sgRNA 1 (triangles) = 76%, *TRIM5* sgRNA 2 (circles) = 90%. For rhesus macaque TRIM34-expressing cells, ICE KO scores were as follows: *TRIM5* sgRNA 1 (triangles) = 83%, *TRIM5* sgRNA 2 (circles) = 93%. Thus, there existed 4 different experimental conditions: no TRIM34 or TRIM5 $\alpha$  expression (black symbols), only endogenous TRIM5 $\alpha$  expression (blue symbols), only overexpressed TRIM34 (green symbols), both endogenous TRIM5 $\alpha$  and overexpressed TRIM34 (orange symbols). 1 day after doxycycline induction, cells were infected with SIV<sub>AGM-SAB</sub> CA particles. 2 dpi, infectivity was quantified by flow cytometry. Relative infectivity is normalized to mean infectivity in *TRIM5*KO, TRIM34-uninduced cells (black symbols). Infection of each cell line with each virus was performed a total of 6 times across 2 different occasions. Combined data are represented as mean  $\pm$  s.d., where each point represents a unique infection. Fold inhibition is indicated where applicable. **c.** HeLa cells were transduced to stably express primate TRIM $\Delta$ SPRY orthologues or empty vector control. Expression was visualized by immunoblotting. **d.** HeLa cells stably expressing primate TRIM34 $\Delta$ SPRY orthologues, human TRIM5 $\Delta$ SPRY, or empty vector control were infected with N-MLV (grey bars) or NB-MLV (white bars). Level of infection was quantified 2 dpi by flow cytometry. Infection of each cell line with each virus was performed a total of 6 times across at least 2 different occasions. Combined data are represented as mean  $\pm$  s.d., where each point represents a unique infection. Fold rescue relative to empty vector control cells is indicated where applicable. **a–b, d.** p values were calculated by Brown-Forsythe and Welch's 1-way ANOVA with Dunnett's T3 test for multiple comparisons. ns nonsignificant, \* p  $\leq$  0.05, \*\* p  $\leq$  0.01, \*\*\* p  $\leq$  0.001, \*\*\*\* p  $\leq$  0.0001



**Fig. 2** (See legend on previous page.)



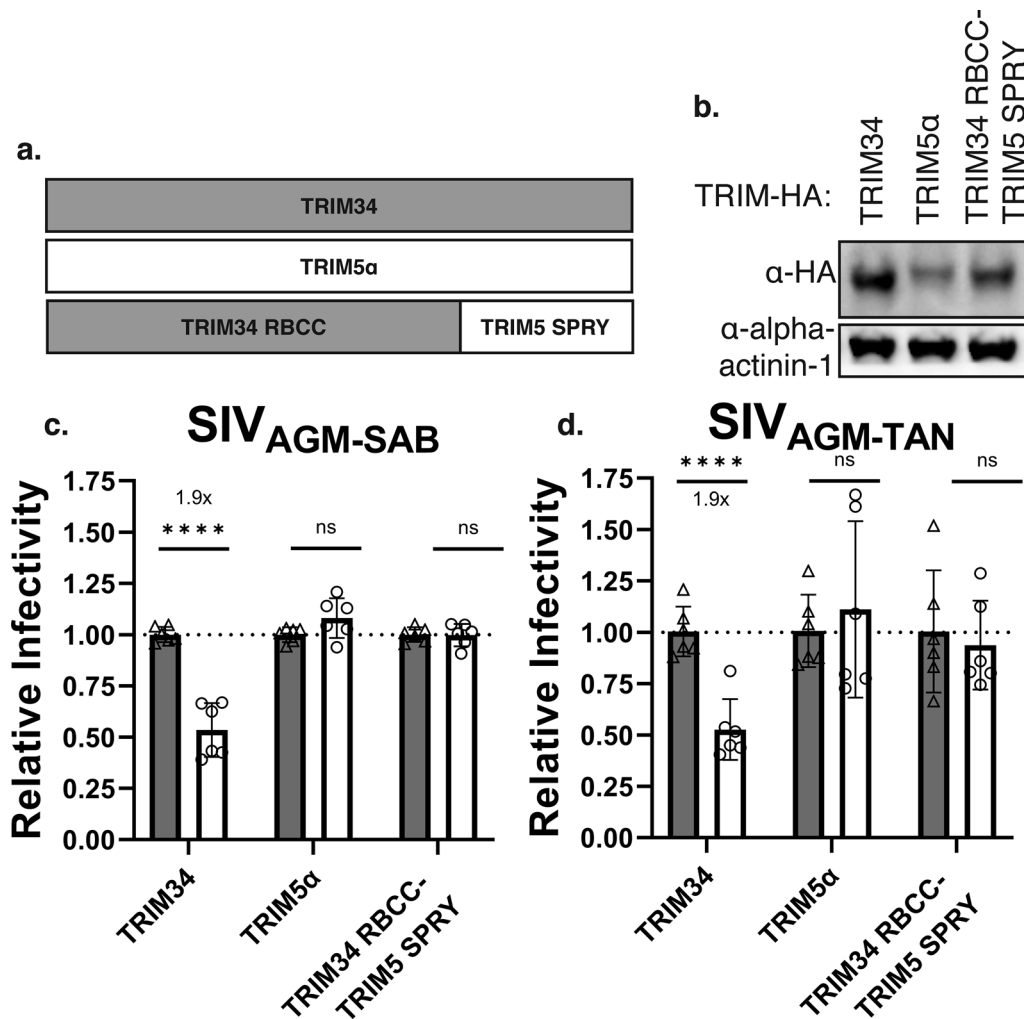
**Fig. 3** TRIM5α v1 loop is necessary for restriction of SIV<sub>AGM-SAB</sub>. **a.** THP-1 *TRIM34* clonal KO cells were electroporated with multiplexed sgRNA against *TRIM5*. Single cell clonal lines were generated by limiting dilution to generate a THP-1 *TRIM34* *TRIM5* double KO clonal cell line. KO efficiency was assessed by ICE analysis (ICE KO score = 72%, Additional file 1: Chromatograms S6–S10). Cells were doubly transduced with doxycycline-inducible, HA-tagged human TRIM34 or empty vector control in tandem with doxycycline-inducible, FLAG-tagged human TRIM5α, TRIM5Δv1, TRIM5 R332P, or empty vector control. Expression was induced in the presence of 125 ng/mL doxycycline, and expression levels were visualized by immunoblotting 72 h post-induction. **b–d.** THP-1 *TRIM34* *TRIM5* double KO clonal cells co-expressing doxycycline-inducible human TRIM34 or empty vector control and human TRIM5α (grey bars), TRIM5Δv1 (orange bars), TRIM5 R332P (blue bars), or empty vector control were infected with SIV<sub>AGM-SAB</sub> (**b**), HIV-1 N74D (**c**), or HIV-1 (**d**) CA 1 day post-induction. Levels of infection were quantified 2 dpi by flow cytometry. Relative infectivity in induced cells (solid bars) is normalized to average infectivity in uninduced control cells (not shown). Fold inhibition is indicated where applicable. Infection of each cell line with each virus was performed a total of 6 times across 2 different occasions. Combined data are represented as mean +/- s.d., where each point represents a unique infection. One-sided p values were calculated by Welch’s t-test. ns nonsignificant, \* p ≤ 0.05, \*\* p ≤ 0.01, \*\*\* p ≤ 0.001, \*\*\*\* p ≤ 0.0001

N74D, even in the presence of a TRIM5α mutant that is more potent against WT HIV-1. Finally, TRIM34 co-expressed with TRIM5α R332P restricted SIV<sub>AGM-SAB</sub> CA, whereas TRIM5α R332P alone did not (Fig. 3b; blue bars). These findings support a role for both the TRIM5α v1 loop and for TRIM34 in viral recognition and specificity.

**TRIM34 SPRY domain is required for restriction**

To more directly test whether the TRIM34 SPRY domain plays a role in antiviral activity and specificity, we generated a chimeric protein that encodes the TRIM34 RING, Bbox, and coiled-coil domains in frame with the TRIM5 SPRY domain (TRIM34 RBCC-TRIM5 SPRY) (Fig. 4a). We hypothesized that if the TRIM34 SPRY domain was required for restriction, the chimera would lose restriction relative to full-length TRIM34. We transduced this chimera into THP-1 *TRIM34* clonal KO cells and confirmed expression by Western blot (Fig. 4b). Although protein expression of the chimeric construct is lower than that of WT TRIM34 (Fig. 4b, compare third lane to first lane), expression is higher than that of WT TRIM5α. We assayed this construct for restriction in our THP-1 cells that lack TRIM34 expression but do contain endogenous, full-length TRIM5α. We found that in the presence of endogenous TRIM5α, the TRIM34 RBCC-TRIM5α SPRY chimera was not able to restrict SIV<sub>AGM-SAB</sub> CA nor SIV<sub>AGM-TAN</sub> (Fig. 4c, d). Therefore, the TRIM34





**Fig. 4** TRIM34 SPRY domain is necessary for restriction of SIV<sub>AGM-SAB</sub>. **a.** Schematic of TRIM34 RBCC-TRIM5 SPRY chimera. **b.** THP-1 TRIM34 clonal KO cells were generated by electroporation of multiplexed sgRNA against TRIM34 and single cell sorting. Cells were transduced with doxycycline-inducible HA-tagged human TRIM34, TRIM5α, or TRIM34-TRIM5α chimeras. Expression was induced in the presence of 125 ng/mL doxycycline, and expression levels were visualized by immunoblotting 72 h post-induction. **c.** THP-1 TRIM34 clonal KO cells expressing doxycycline-inducible human TRIM34, TRIM5α, or TRIM34 RBCC-TRIM5 SPRY were infected with SIV<sub>AGM-SAB</sub> CA or SIV<sub>AGM-TAN</sub>-luc particles 1 day post-induction. Level of infection was quantified 2 dpi by flow cytometry or luminometry, respectively. Relative infectivity in induced cells (white bars, circles) is normalized to average infectivity in uninduced control cells (grey bars, triangles). Fold inhibition is indicated where applicable. Infection of each cell line with each virus was performed a total of 6 times across 2 different occasions. Combined data are represented as mean +/- s.d., where each point represents a unique infection. One-sided p values were calculated by Welch's t-test. ns nonsignificant, \* p ≤ 0.05, \*\* p ≤ 0.01, \*\*\* p ≤ 0.001, \*\*\*\* p ≤ 0.0001

SPRY domain, in addition to the TRIM5α SPRY domain, is involved in restriction.

### Discussion

Human TRIM34 was recently identified as a restriction factor of the CA mutant HIV-1 N74D and certain SIVs [17]. Here we show that this antiviral phenotype is a broadly conserved function of primate TRIM34 genes. We find that the same subset of lentiviral capsids is restricted by a diverse panel of TRIM34 orthologues,

irrespective of TRIM34 species of origin. Moreover, we find that although antiviral specificity does not seem to vary with the identity of TRIM34, both the TRIM34 and TRIM5α SPRY domains are critical to restriction, suggesting that TRIM5 confers a level of specificity to the TRIM34 restriction.

Despite the lack of evidence for positive selection on TRIM34, primate TRIM34 alleles have broadly maintained the ability to restrict certain primate lentiviruses. This is surprising in light of the fact that positive

selection is a marker of critical regions of direct host-viral interaction. We propose that although TRIM34 lacks this characteristic, it utilizes TRIM5 $\alpha$ , which is a rapidly-evolving gene in primates, to restrict lentiviruses. Prior work has shown that TRIM34 and TRIM5 $\alpha$  are capable of multimerizing in vitro in addition to binding capsid [10, 14, 15]. In this work we demonstrate that TRIM5 $\alpha$  can functionally interact with a number of primate TRIM34 orthologues. Indeed, we found that overexpression of TRIM34 $\Delta$ SPRY was able to abrogate the restrictive capacity of TRIM5 $\alpha$  towards N-MLV, suggesting a functional interaction between the two proteins. Human TRIM5 $\Delta$ SPRY as well as human and chimpanzee TRIM34 $\Delta$ SPRY had the strongest phenotype, whereas sabaeus and macaque TRIM34 $\Delta$ SPRY had weaker phenotypes (Fig. 2d). One possible explanation for this observation is that the sabaeus and macaque constructs were expressed at relatively lower levels (Fig. 2c). Another possibility is that human TRIM5 $\alpha$  interacts most readily with itself and next most well with human TRIM34. Nonetheless, this supports a model in which TRIM5 $\alpha$  and TRIM34 could hetero-multimerize to form a higher-order structure, similar to how TRIM5 $\alpha$  forms homo-dimers and homo-multimers with itself mediated by the coiled-coil and Bbox domains [8]. This then could enable both SPRY domains to interact with the viral capsid. Thus, we propose that despite the lack of an overt, positively-selected site of viral interface on TRIM34, TRIM34 restricts viral infection through interaction with the rapidly-evolving TRIM5 protein.

It is also possible that physical interaction between TRIM34 and TRIM5 $\alpha$  alters the half-life of one or both proteins, affecting turnover rate or stability. Prior work has demonstrated that different TRIMs have different half-lives, and that alterations to the RING or Bbox domains of TRIM proteins can affect protein turnover [15, 32, 33]; thus, it is possible that interactions between different TRIMs might lead to changes in stability and restrictive capacity.

Another possibility is that TRIM34 could be filling the role of an effector molecule. Previously, the TRIM5 $\alpha$  RING domain has been implicated as an E3 ubiquitin ligase that can both contribute to restriction directly and act as a signal transducer to initiate innate immune activation subsequent to CA sensing by the TRIM5 $\alpha$  SPRY domain [11, 33–37]. In the case of TRIM34, it is possible that TRIM5 $\alpha$  is responsible for recognizing CA, while TRIM34's E3 ligase domain contributes to downstream signaling, capsid degradation, or recruitment of other molecules that aid restriction. Although previous work found that TRIM34 does not activate AP-1, it remains possible that it is acting by another mechanism [35].

Notably, these models are not mutually exclusive and could function in tandem with TRIM34 aiding TRIM5 $\alpha$  in more than one capacity.

Our findings suggest that the SPRY domains of both TRIM34 and TRIM5 $\alpha$  are important for restriction. On its own, human TRIM5 $\alpha$  is a relatively weak restriction factor for most retroviral capsids besides N-MLV. We propose that TRIM34 can assist human TRIM5 $\alpha$  to restrict some—but not all—viral capsids. For example, human TRIM5 $\alpha$  in the presence of TRIM34 is able to restrict SIV<sub>AGM-SAB</sub>; conversely, even in the presence of TRIM34, TRIM5 $\alpha$  is still unable to potently restrict HIV-1. One possibility is that the TRIM34 SPRY domain also contributes to viral recognition. Previous work has shown that TRIM34 is able to bind HIV-1 CA-NC complexes in vitro, even though it does not restrict HIV-1, raising the possibility that TRIM34 may still contribute to capsid binding [15, 38]. Although the v1 loop of TRIM34 has not undergone positive selection and is only 6 amino acids long, we cannot exclude the possibility that it, or one of the other variable loops, might contribute to capsid recognition. Given the relatively high conservation of the variable loops of the primate TRIM34 alleles that we assessed (no amino acid differences in v1, v3, or v4; 1 amino acid difference in each allele in v2), this could explain the observation that all the TRIM34 alleles tested restricted the same subset of viruses. Furthermore, the observation that TRIM5 $\alpha$  R332P—which gains restriction of HIV-1 relative to WT TRIM5 $\alpha$ —poorly restricts HIV-1 N74D in the absence of TRIM34, supports the idea that TRIM34 could be assisting in capsid recognition beyond the capacity of TRIM5 $\alpha$  alone. That is, TRIM34 may be contributing an additional measure of specificity for HIV-1 N74D CA beyond what is sufficient for TRIM5 $\alpha$  R332P to restrict WT HIV-1 CA. Thus, as many TRIM5 $\alpha$  alleles can act as potent restriction factors of a few very specific viral capsids, but a poor restriction factor of many others, we speculate that TRIM34 might act as a cofactor to enable TRIM5 $\alpha$  to restrict viral capsids that TRIM5 $\alpha$  is not able to restrict on its own. Of interest, we previously did not find any evidence that TRIM34 is required for TRIM5 restriction of the CypA-binding deficient P90A capsid mutants, suggesting that both TRIMs are required for restriction of only a subset of capsids [17].

It is intriguing that all the TRIM34 orthologues tested restricted the same subset of viruses: SIV<sub>AGM-SAB</sub>, SIV<sub>AGM-TAN</sub>, and SIV<sub>MAC</sub>. Notably, only viruses originating from Old World monkeys were restricted, whereas the hominid viruses, HIV-1 and SIV<sub>CPZ</sub>, were not restricted. It is possible that this is driven by the identity of TRIM5 $\alpha$ : HIV-1 and SIV<sub>CPZ</sub> are better evolved to evade restriction by human TRIM5 $\alpha$  compared to the

Old World monkey viruses [24]. The three restricted viruses share only 70.6% pairwise amino acid identity across their CA regions, making it difficult to identify critical residues or motifs that determine restriction. We explored the possibility that restriction might be dependent on CypA binding, as CypA can modulate infectivity in both restriction-dependent and restriction-independent fashions [39–46]. However, CypA binding capacity varied across the restricted viruses. For example, SIV<sub>AGM-TAN</sub> does bind CypA, while SIV<sub>MAC</sub> and SIV<sub>AGM-SAB</sub> do not [14, 47–49]. Furthermore, while HIV-1 is well-established to bind and incorporate CypA into virions, the CypA binding capacity of HIV-1 N74D is controversial [16, 50, 51]. Thus, at minimum, restriction does not depend exclusively on CypA binding. Instead, restriction of these capsids by TRIM34 and TRIM5 $\alpha$  may be dependent on other unknown features that distinguish these capsids. A number of blocks to HIV infection that are capsid-dependent have been characterized—for example, *Lv2*, *Lv3*, and *Lv4*—for which the responsible cellular components have either not been identified or have only been partly identified [52–56]. It is possible that TRIM34 could contribute to one or more of these blocks.

We find that TRIM34 alleles from a broad spectrum of primates, when paired with TRIM5 $\alpha$ , are able to restrict capsids that neither TRIM is able to restrict on its own. Despite lacking signs of positive selection characteristic of many restriction factors, TRIM34—acting in tandem with TRIM5 $\alpha$ —can act as a barrier to cross-species transmission events. It is possible that these proteins have co-evolved such that TRIM34 can enhance or modify TRIM5 $\alpha$ 's antiviral potential. Indeed, not only do host immune proteins evolve in the context of the pathogens they counteract, but they also evolve in the context of other, complementary host proteins. Thus, our data suggest that restriction factors evolve not only in isolation in response to evolutionary pressures exerted by viral pathogens but may also co-evolve with each other resulting in more powerful antiviral activity than either could achieve on its own.

## Materials and methods

### Cell culture

All cells were cultured at 37 °C and 5% CO<sub>2</sub>. THP-1 monocytic cells (American Type Culture Collection, Manassas, VA, #TIB-202) were cultured in Roswell Park Memorial Institute 1640 Media (RPMI 1640) (Gibco, Grand Island, NY, #118875-093) supplemented with 10% v/v fetal bovine serum (FBS) (GE Cytiva, Marlborough, MA #SH30541.03), 100 U/mL penicillin-streptomycin (Gibco #15140-122), 10mM HEPES (Gibco #15630-080), 1mM sodium pyruvate (Gibco #11360-070), 2 g/L

D-Glucose (Gibco #A24940-01), and 1X GlutaMAX supplement (Gibco #35050-061). HEK 293T/17 cells (American Type Culture Collection #CRL-11268) and HeLa cells (American Type Culture Collection #CCL-2) were cultured in Dulbecco's Modified Eagle Medium (DMEM) (Gibco #11965-092) supplemented with 10% fetal bovine serum, and 100 U/mL penicillin-streptomycin.

### Cloning, plasmids, and virus production

All transfections were performed on HEK 293T/17 cells in the presence of serum-free DMEM and Trans-IT transfection reagent (Mirus Bio, Madison, WI, #MIR 2305). All transductions were performed by spinoculation at 1100 x g for 30 min at 30 °C.

Fluorescent reporter viruses were generated by transfection of a three-plasmid system: pMD2.G (Addgene, Watertown, MA, #12259, a gift from Didier Trono) for expression of VSV-G envelope; a variable plasmid for expression of a chimeric gag/pol containing an NL4-3 backbone and HIV-1 [23], SIV<sub>CPZ</sub>, SIV<sub>AGM-SAB</sub>, or SIV<sub>MAC</sub> CA (gifts from Theodora Hatzioannou) [24]; and either pALPS-eGFP (Addgene #101323, a gift from Jeremy Luban) [57] or pHIV-zsGreen (Addgene #18121, a gift from Bryan Welm and Zena Werb) [58]. SIV<sub>CPZ</sub>, SIV<sub>AGM-SAB</sub>, or SIV<sub>MAC</sub> chimeric virus particles were made with pALPS-eGFP, and HIV-1 virus particles were made with pHIV-zsGreen. The SIV<sub>MAC</sub> CA construct contained the mutation A77V. Luciferase reporter viruses were generated by transfection of a two-plasmid system: SIV<sub>agmTAN</sub> E- R- luc [25] and L-VSV-G (VSV glycoprotein expression) [59]. Particles were harvested 2 days post-transfection, syringe-filtered through 0.22  $\mu$ m PES membranes, and frozen at -80 °C. N-MLV reporter viruses were generated by transfection of a three-plasmid system: pCIG3-N for expression N-MLV gag/pol (Addgene #132941, a gift from Jeremy Luban) [40], pQCXIP-eGFP (a gift from Jeannette Tenthorey) [22] for expression of a fluorescent reporter, and pMD2.G for expression of VSV-G envelope. NB-MLV reporter viruses were generated by transfection of a four-plasmid system: JK3 for expression of NB-MLV gag/pol [59], L-VSV-G for expression of VSV-G envelope [59], CMV-tat for transactivation [59], and pQCXIP-eGFP for expression of a fluorescent reporter.

pLentiCRISPR-v2 (Addgene #52961, a gift from Feng Zhang) constructs were generated by BsmBI (New England BioLabs, Ipswich, MA, #R0580) restriction cloning of TRIM5 guides into pLentiCRISPR-v2, a lentiviral vector encoding for Cas9 [60]. pLentiCRISPR-v2 containing guides were transfected with pSPAX2 (Addgene #12260, a gift from Didier Trono) and pMD2.G to generate lentiviral particles. Particles were harvested 2 days

post-transfection, syringe-filtered through 0.22  $\mu$ M PES membranes, and frozen at  $-80^{\circ}\text{C}$ .

HA-tagged TRIM34 and 3xFLAG-tagged TRIM5 $\alpha$  codon-optimized expression constructs (Additional file 2) were synthesized by Twist Bioscience (South San Francisco, CA). TRIM5 $\alpha$  mutant constructs (TRIM5 $\alpha$  v1 loop and TRIM5 $\alpha$  R332P) were a gift from Jeannette Tenthorey [22]. TRIM constructs were cloned into pLKO (a gift from Melissa Kane) [61] by SfiI (New England Biolabs #R0123S) restriction enzyme digest. pLKO-TRIM constructs were transfected along with pMD2.G and pSPAX2 to generate particles. Particles were harvested at 2 and 3 days post-transfection, cell pellets spun down at 300  $\times$  g, and supernatants frozen at  $-80^{\circ}\text{C}$ .

HA-tagged TRIM34 $\Delta$ SPRY constructs were cloned in pQCXIP (TaKaRa Bio, San Jose, CA, #631516) by SbfI (New England Biolabs #R3642S) and NotI (New England Biolabs #R3189S) restriction digest. pQCXIP-TRIM34 $\Delta$ SPRY constructs were transfected along with pJK3 (MLV gag/pol), L-VSV-G (VSV-G envelope), and CMV-tat to generate particles. Particles were harvested 2 days post-transfection.

#### CRISPR knockouts

Clonal knockout lines in THP-1 cells were generated by electroporation of multiplexed small guide RNA (sgRNA) from Gene Knockout Kit v2 (Synthego, Redwood City, CA) against *TRIM34* (guide sequences=CTTGCTTAA CGTACAAG, CCACAGTCTAGACTCAA, GCAGTG ACCAGCATGGG) or *TRIM5* (guide sequences=GGU AACUGAUCCGGCACACA, ACUUCUUGUGGU UUGCAGUG, CCUGGUUAAUGUAAAGGAGG). Single cell clonal lines were generated by single cell sorting (*TRIM34*) or limiting dilution (*TRIM5*). Knockout efficiency was validated by Interference of CRISPR Edits (ICE) analysis (Synthego) [62] (Additional file 1: Chromatograms S1, S6–S10).

Pooled knockout lines were generated by transduction of THP-1 cells with lentiviral preps containing guides delivered by pLentiCRISPR-v2. *TRIM5* guide sequences=TCACCACACGTTCCCTCACAG and GTTGATCATTGTGCACGCCA. NTC guide sequences=GGGCCCGCATAGGATATCGC and TAG ACAACCGCGGAGAATGC. Cells were spinoculated in the presence of 20  $\mu$ g/mL DEAE-Dextran (Pharmacia Fine Chemicals, Uppsala, Sweden, #17-0350-01) and then selected in 10  $\mu$ g/mL blasticidin HCl (Gibco #A11139-03). Knockout efficiency was validated by ICE analysis [62] (Additional file 1: Chromatograms S2–S5).

#### Inducible overexpression

Doxycycline-inducible expression of TRIM34 and TRIM5 $\alpha$  was achieved by transduction of lentiviral preps

containing pLKO TRIM constructs in THP-1 cells. Cells were spinoculated in the presence of 5  $\mu$ g/mL polybrene (EMD Millipore, Burlington, MA, #TR-1003-G) and then selected in 0.5  $\mu$ g/mL puromycin (TRIM34) (Sigma-Aldrich, St. Louis, MO, #P8833-25MG) or 10  $\mu$ g/mL blasticidin (TRIM5 $\alpha$ ). Protein expression was induced by the addition of 125 ng/mL doxycycline hyclate (Sigma-Aldrich #D9891-5G) to cells cultured in complete RPMI 1640 containing tetracycline-approved FBS (Sigma-Aldrich #F0926-50ML).

Stable expression of TRIM34 $\Delta$ SPRY was achieved by transduction of lentiviral preps containing pQCXIP constructs expressing TRIM $\Delta$ SPRY constructs. HeLa cells were spinoculated in the presence of 20  $\mu$ g/mL DEAE-Dextran. Selection was performed in the presence of 1  $\mu$ g/mL puromycin.

#### Restriction assays

1 day after induction of TRIM expression, THP-1 cells were infected with chimeric CA virus particles expressing a fluorescent reporter. Cells were spinoculated in the presence of 20  $\mu$ g/mL DEAE-Dextran. 2 dpi, relative infectivity was quantified by flow cytometry using a FACSCelesta Analyzer (BD Biosciences, San Jose, CA) or Bright-Glo luciferase assay reagent (Promega, Madison, WI #E2620) using a LUMIstar Omega luminometer (BMG Labtech, Ortenberg, Germany).

#### N-MLV restriction assay

HeLa cells that had been transduced to stably express TRIM34 $\Delta$ SPRY constructs were infected with N-MLV particles expressing an eGFP reporter by spinoculation in the presence of 20  $\mu$ g/mL DEAE-Dextran. Infectivity was assessed 2 dpi by flow cytometry using an LSRFortessa Cell Analyzer (BD Biosciences).

#### Western blotting

3 days after induction of TRIM expression, THP-1 cells were harvested; 1 day prior to infection, HeLa cells were harvested. Cells were washed once with PBS. Cells were then lysed on ice for 30 min in 20 mM HEPES (Fisher Scientific, Fair Lawn, NJ, #BP310-1) containing 8 M urea (Sigma-Aldrich #U-6504), 50 mM DL-Dithiothreitol (DTT) (Gold Biotechnology, St. Louis, MO, #DTT10), 0.1% w/v SDS (Fisher Scientific #BP166-500), 1.5 mM MgCl<sub>2</sub> (Sigma-Aldrich #208337-100G), 0.5 mM CaCl<sub>2</sub> (Sigma-Aldrich #C-3306), 50  $\mu$ g/mL DNase I (Roche Diagnostics, Mannheim, Germany, #10104159001), and 1X EDTA-free protease inhibitor cocktail (Roche Diagnostics #11836170001). 6X SDS-PAGE sample loading buffer (G Biosciences, St. Louis, MO #785-701) containing 5% v/v 2-mercaptoethanol (Sigma-Aldrich #M3148-100ML) was added to lysates. Lysates were acid treated

with 50 mM HCl (Sigma-Aldrich #320331-300ML) and then boiled at 95 °C for 7 min. After cooling, lysates were neutralized with 50 mM NaOH (Sigma-Aldrich #72068-100ML). Products were resolved by SDS-PAGE on NuPage Bis-Tris 4–12% acrylamide gels (Invitrogen, Carlsbad, CA, #NP0335BOX) and transferred onto nitrocellulose membranes (Bio-Rad Laboratories, Hercules, CA, #1620115). Membranes were blocked with 2% w/v milk (Research Products International, Mt. Prospect, IL, #M17200-500.0) and 2% w/v bovine serum albumin (Sigma-Aldrich #A7906-100G) in tris-buffered saline (Fisher Scientific #BP152-5) containing 1% v/v Tween-20 (Fisher Scientific #BP152-1) (TBS-T). Primary antibodies were incubated overnight at 4 °C: rabbit anti-HA high affinity at 1:500 (Roche Diagnostics #ROAHAHA), mouse anti-FLAG M2 at 1:500 (Sigma-Aldrich #F1804), and rabbit anti-alpha actinin-1 at 1:1000 (Bio-Rad Laboratories #VPA00889). Membranes were washed with TBS-T and then incubated with HRP-conjugated secondary antibodies for 1 h at RT: goat anti-rat IgG at 1:2000 (Abcam, Waltham, MA #ab97057), sheep anti-mouse IgG at 1:2000 (GE Cytiva #NA931), and donkey anti-rabbit IgG at 1:2000 (GE Cytiva #NA934V). Membranes were washed again and then incubated with SuperSignal West Pico substrate (HA and alpha actinin-1 blots) (Thermo Fisher Scientific, Waltham, MA, #34580) or Supersignal West Femto substrate (FLAG blots) (Thermo Fisher Scientific #34095) for 3 min. Membranes were imaged with a ChemiDoc MP imaging system (Bio-Rad Laboratories). Images of full membranes are available in Additional file 3.

### Statistical methods

For comparisons of 2 independent variables, one-tailed *p* values were computed by Welch's *t*-test. For comparisons of 3 or more independent variables, *p* values were computed by Brown-Forsythe and Welch's 1-way ANOVA with Dunnett's T3 test for multiple comparisons. All calculations and data visualizations were performed using GraphPad Prism version 9.3 (GraphPad Software, San Diego, CA).

### Supplementary Information

The online version contains supplementary material available at <https://doi.org/10.1186/s12977-023-00629-4>.

**Additional file 1.** ICE chromatograms.

**Additional file 2.** Sequences used in this study.

**Additional file 3.** Full Western blot membranes.

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### Author contributions

Conceptualization, methodology, writing, and funding acquisition were performed by JT, ME and MO. Investigation was conducted by JT, AK and ALF. Validation, formal analysis, and visualization were performed by JT and AK. Supervision was provided by ME and MO.

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### Availability of data and materials

All data generated or analyzed during this study are included in this published article and its supplementary information files.

### Declarations

#### Ethics approval and consent to participate

Not applicable.

#### Consent to publish

Not applicable.

#### Competing interests

The authors declare no competing interests.

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