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

Joy Twentyman[1, 2], Anthony Khalifeh[3], Abby L. Felton [2], Michael Emerman [2], Molly OhAinle [2, 3*]

[1] Department of Global Health, University of Washington, Seattle, WA, United States, [2] Divisions of Human Biology and Basic Sciences, Fred Hutch Cancer Center, Seattle, WA, United States, [3] Department of Molecular and Cell Biology, Division of Immunology and Molecular Medicine, University of California –Berkeley, Berkeley, CA, United States

*Corresponding author, Molly OhAinle, ohainle@berkeley.edu

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 TRIM5α, 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 $SIV_{AGM-SAB}$, $SIV_{AGM-TAN}$ and SIV_{MAC} capsids, which infect sabaeus 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 TRIM5α. We demonstrate that TRIM5α is necessary, but not sufficient, for restriction of these capsids, and that human TRIM5α functionally interacts with TRIM34 from different species. Finally, we find that both the TRIM5α SPRY v1 loop and the TRIM34 SPRY domain are essential for TRIM34-mediated restriction. These data support a model in which TRIM34 is a

35   broadly-conserved primate lentiviral restriction factor that acts in tandem with TRIM5α, such that

36   together, these proteins can restrict capsids that neither can restrict alone.

INTRODUCTION

38   Restriction factors are a class of cell-intrinsic, germline-encoded host immune factors

39   that can inhibit viral infection and replication. The human genome encodes for approximately

40   70-100 TRIM (Tripartite Motif) proteins, many of which play a role in host defense [1]. The

41   alpha isoform of TRIM5 (TRIM5α) in primates is a well-characterized example of a primate

42   restriction factor with activity against retroviruses. The activity of TRIM5α against a given

43   retrovirus depends on both the species from which the TRIM5α is derived and the capsid (CA)

44   protein of the retrovirus [2–5]. In the context of HIV-1, TRIM5α acts directly on CA by

45   multimerizing onto the CA lattice [5,6]. This interaction results in aberrant uncoating of CA,

46   interrupting the viral life cycle [5,7]. TRIM5α from rhesus macaques and many other Old World

47   monkeys has much greater antiviral activity against HIV-1 than does human TRIM5α [2,5,8].

48   TRIM proteins are composed of a common set of N-terminal domains (RING, Bbox, and

49   coiled-coil) followed by one or more variable C-terminal domains [9] . An important characteristic

50   of TRIM5α, and TRIM proteins more generally, is their ability to oligomerize to form structures

51   with very high binding avidity to CA [7]. Homo-oligomerization of TRIM5α is essential to its

52   ability to restrict viral CA [10–12]. Furthermore, TRIM proteins have also been demonstrated to

53   hetero-oligomerize with each other [9,13,14].

54   Previously, our lab performed a CRISPR-Cas9 screen to identify restriction factors

55   against an HIV-1 strain with a mutation in CA, N74D, that renders it more susceptible to CA-

56   mediated restriction factors [15]. We identified TRIM34 as a restriction factor of the HIV-1 N74D

57   capsid mutant as well as select SIV capsids [16]. TRIM34 is a paralogue of TRIM5α, sharing a

58   common domain architecture, and human TRIM34 and TRIM5α have approximately 57% amino

59   acid identity [17]. Moreover, TRIM34-mediated restriction requires TRIM5α, and TRIM34 and

60   TRIM5α colocalize with incoming capsids [16].

61   While TRIM5α and TRIM34 both can interact with lentiviral CA, TRIM5α, but not

62   TRIM34, has undergone positive selection [14,18]. A history of positive selection, in which the

63   rate of nonsynonymous mutations exceeds the rate of synonymous mutations, is characteristic

64   of host proteins that are in evolutionary conflict with pathogens and often occurs at sites of

65   direct physical interface between host and pathogen [19,20]. Specifically, the v1 loop of

66   TRIM5α's C-terminal SPRY domain has been found to have undergone rapid evolution, and this

67   region is responsible for viral recognition [18]. Conversely, the lack of evidence for positive

68   selection on TRIM34 suggests that TRIM34 may not contain sites of evolutionarily-important

69   direct viral interaction.

70   Although currently there does not exist evidence of evolutionary conflict within TRIM34,

71   here we show that TRIM34 antiviral activity has been broadly conserved across primate species

72   as an antiviral gene against CA of diverse primate lentiviruses. We find that restriction by

73   TRIM34 from primates requires TRIM5α and that the TRIM5α SPRY v1 loop is an essential

74   mediator of restriction. These results suggest that TRIM34 relies on the capsid-binding

75   properties of TRIM5α. However, as the TRIM34 SPRY domain is also required for restriction,

76   our results suggest that both TRIM34 and TRIM5α contribute to capsid recognition and/or

77   antiviral function. We propose that TRIM34, which has not undergone positive selection, is an

78   antiviral protein that requires TRIM5α, which has undergone positive selection.

79

80   **RESULTS**

81   **Diverse primate TRIM34 orthologues can act as lentiviral restriction factors**

82   Amino acid variation due to positive selection over evolutionary time in the v1 loop of

83   TRIM5α confers different primate species with varying specificities towards retroviral capsids

84   [18,21]. Although TRIM34 lacks evidence of positive selection found in TRIM5α, there is still

85   diversity across different primate TRIM34 alleles. For example, relative to human TRIM34,

86   chimpanzee, sabaeus monkey, and rhesus macaque orthologues differ by 4, 27, and 26 amino

87   acids, respectively. While we have previously shown that human TRIM34 restricts $SIV_{AGM-TAN}$

88   and SIV_{MAC}, here we wished to determine whether amino acid variation in TRIM34 orthologues

89   from divergent primates affects TRIM34's capacity to restrict different primate lentiviral capsids

90   [16]. Specifically, we tested whether a panel of 4 TRIM34 orthologues from diverse primate

91   species comprising human (*Homo sapiens*), chimpanzee (*Pan troglodytes troglodytes*), sabaeus

92   monkey (*Chlorocebus sabaeus*), and rhesus macaque (*Macaca mulatta*) orthologues would

93   restrict a panel of lentiviral capsids (HIV-1, SIV_{CPZ}, $SIV_{AGM-SAB}$, $SIV_{AGM-TAN}$, and SIV_{MAC}, which

94   arise from humans; chimpanzees; two species of African green monkeys, the sabaeus monkey

95   and the tantalus monkey; and rhesus macaques, respectively).

96   To test the effects of different primate TRIM34 orthologues in the absence of

97   endogenous TRIM34, we generated a clonal *TRIM34* knockout (KO) line in the human THP-1

98   cell line. We then introduced primate TRIM34 orthologues using a doxycycline-inducible

99   lentiviral vector to generate human, chimpanzee, sabaeus, and macaque TRIM34 over-

100 expressing cell lines. These orthologues were selected because they represent a broad range

101 of primate TRIM34 from both hominid and Old World monkeys for which we also have the

102 corresponding lentiviral capsids. We tested an empty-vector line as a control for the absence of

103 any TRIM34. We confirmed that expression of each of the TRIM34 orthologues is induced by

104 doxycycline (Figure 1a), although overall steady-state expression levels varied somewhat with

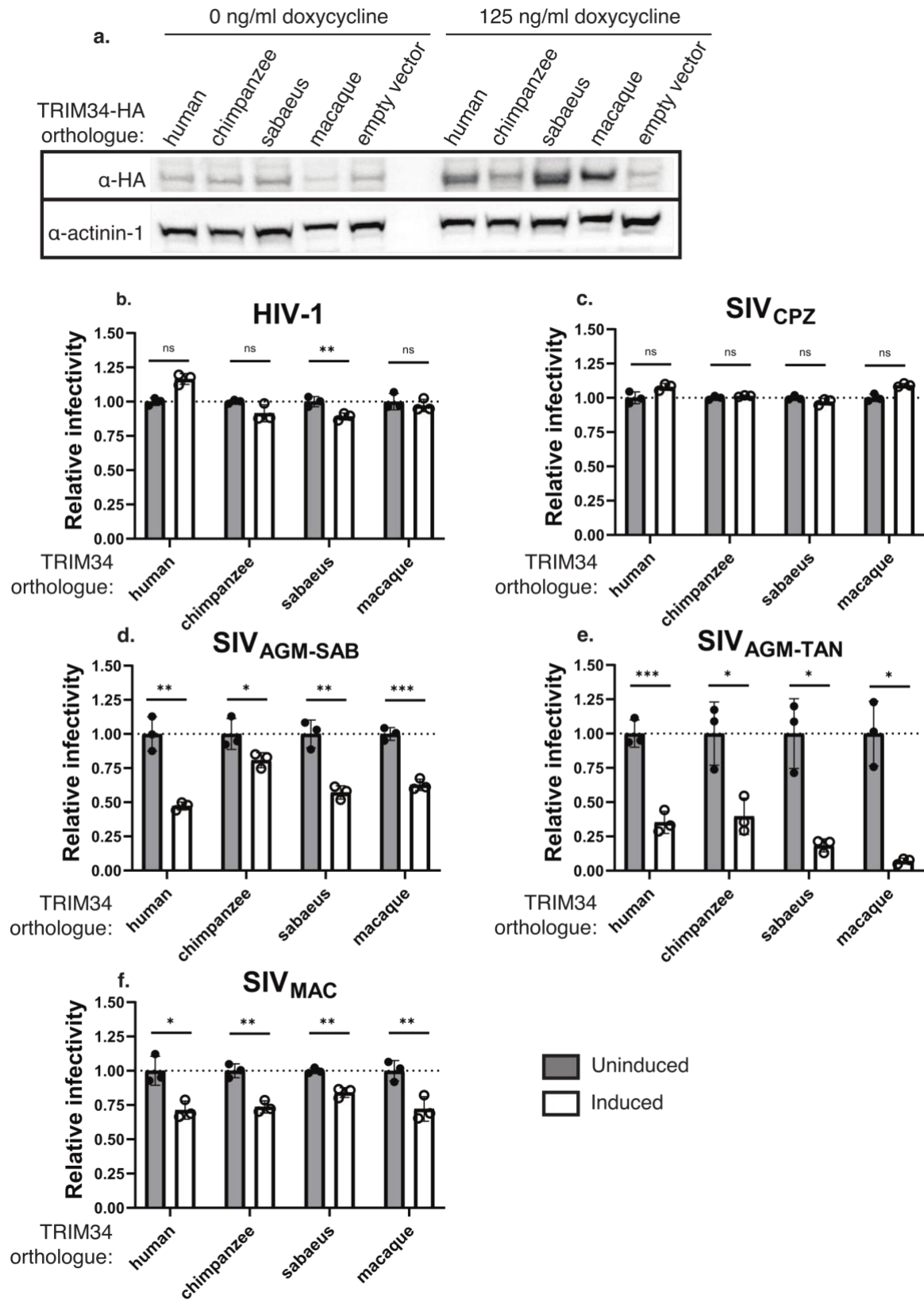105 the macaque TRIM34 expressed at higher levels and the chimpanzee TRIM34 at lower levels.

107 **Figure 1. Diverse primate TRIM34 orthologues restrict SIV$_{AGM-SAB}$, SIV$_{AGM-SAB}$, and SIV$_{MAC}$**
108 **capsids in the presence of TRIM5α**
109 a. THP-1 *TRIM34* clonal KO cells were generated by electroporation of multiplexed sgRNA
110 against *TRIM34* and single cell sorting (ICE KO score = 96%). THP-1 *TRIM34* KO cells were
111 transduced with doxycycline-inducible HA-tagged primate TRIM34 orthologues or empty vector
112 control. Primate TRIM34 expression was induced with doxycline. Expression levels were
113 visualized by immunoblotting 72 h post-induction.
114 b-f. Primate TRIM34 expression was induced in THP-1 *TRIM34* clonal KO cells. 1 day post-
115 induction, cells were either challenged with chimeric virus particles containing HIV-1 capsids co-
116 expressing zsGreen (b) or SIV capsids co-expressing eGFP including SIV$_{CPZ}$ (c), SIV$_{AGM-SAB}$ (d),
117 or SIV$_{MAC}$ (f); or challenged with VSV-G pseudotyped SIV$_{AGM-TAN}$-luc (e). Infection was quantified
118 2 dpi by flow cytometry (b-d, f) or luminometry (e). Relative infectivity in induced cells (white
119 bars) is normalized to average infectivity in uninduced control cells (grey bars). Data are
120 represented as mean +/- s.d. of 3 technical replicates from 1 representative experiment. One-
121 sided p values were calculated by Welch's *t*-test. ns = nonsignificant, * $p ≤ 0.05$, ** $p ≤ 0.01$, ***
122 $p ≤ 0.001$, **** $p ≤ 0.0001$.
123

124 　　　We then challenged these cells to infection using a VSV-G pseudotyped lentiviral vector

125 system, in which the CA region of a gag/pol expression construct encoded either HIV-1, SIV$_{CPZ}$,

126 SIV$_{AGM-SAB}$, or SIV$_{MAC}$ capsids, whose host species match the species of origin of the TRIM34

127 orthologues used [22,23]. These virus particles also encoded a fluorescent reporter. We also

128 tested a full-length infectious molecular clone of SIV$_{AGM-TAN}$ that encodes a luciferase reporter

129 [24]. After induction with doxycycline or media-only control, infectivity was quantified 2 days

130 post-infection (dpi) by luciferase assay (SIV$_{AGM-TAN}$) or flow cytometry (all others). Of note, we

131 found that while none of the TRIM34 orthologues tested restricted HIV-1 or SIV$_{CPZ}$ capsids

132 (Figure 1b-1c), all of these same TRIM34 orthologues restricted SIV$_{AGM-TAN}$, SIV$_{AGM-SAB}$, and

133 SIV$_{MAC}$ capsids (Figure 1d-1f). These data suggest that, in contrast to TRIM5α, the antiviral

134 specificity of TRIM34 does not seem to vary across TRIM34 orthologues. Rather, TRIM34

135 antiviral activity against the same set of lentiviral capsids is a conserved activity of all primate

136 TRIM34 orthologues we tested. Moreover, the antiviral activity of all primate TRIM34s is specific

137 for the same subset of lentiviral capsids. For example neither of the hominid lentiviruses were

138 affected by any TRIM34 orthologue we tested (Figure 1b-c), while the lentiviral capsids that

139 were restricted by human TRIM34—SIV$_{AGM-TAN}$, SIV$_{AGM-SAB}$, and SIV$_{MAC}$—were also restricted by

140 the other primate TRIM34 orthologues. In sum, these data support the hypothesis that TRIM34

141   restriction is a conserved activity in primates with shared specificity for certain primate lentiviral

142   capsids.

TRIM34 requires TRIM5α for restriction

144   Previously, we found that human TRIM34 requires TRIM5α to restrict HIV-1 N74D

145   capsids [16]. Given that the TRIM34 orthologues tested all show selectivity for the same subset

146   of lentiviral capsids (Figure 1), we reasoned that TRIM5α might be responsible for the capsid

147   specificity of TRIM34-mediated restriction. Notably, for the experiments described in Figure 1 in

148   which all TRIM34 orthologues tested restricted the same subset of capsids, the cells used

149   contained endogenous TRIM5α. Therefore, we next asked whether TRIM5α was more broadly

150   required for TRIM34-mediated restriction of SIV$_{AGM-SAB}$, which was the most potently restricted

151   capsid of the capsids tested in Figure 1. Specifically, we assayed for restriction in TRIM34-

152   overexpressing cells in which we remove endogenous TRIM5α expression. We created pooled

153   knockouts of *TRIM5* in the background of THP-1 *TRIM34* clonal KO cells containing

154   doxycycline-inducible human or macaque TRIM34. Although we observed high KO efficiency

155   scores for the pooled KO lines (Figure 2; legend), these pooled KO cell lines still contained

156   some TRIM5α expression as KO is not complete at a population level. We also generated

157   control cell lines using non-targeting control guides (NTCs); these cells still contained

158   endogenous TRIM5α. We then infected these cells with SIV$_{AGM-SAB}$. Relative to cells missing

159   both TRIM34 and TRIM5α (Figure 2a-b; black symbols), SIV$_{AGM-SAB}$ is restricted only in the

160   presence of both TRIM34 and TRIM5α (Figure 2a-b; red symbols). Notably, this was true for

161   both human and rhesus macaque TRIM34 (Figure 2a – human; Figure 2b – macaque). That is,

162   human TRIM5α can fulfill TRIM34's requirement for TRIM5α, even for a TRIM34 orthologue

163   from a different primate species. Conversely, SIV$_{AGM-SAB}$ is not restricted either by cells

164   expressing only TRIM34 that are knocked out for TRIM5α (Figure 2a-b; green symbols), nor by

165   cells expressing only endogenous TRIM5α without induction of TRIM34 (Figure 2a-b; blue

166   symbols). Although it does appear that there may be a small amount of restriction in the

167   presence of TRIM34 only (Figure 2a-b; green symbols), we attribute this to the fact that the

168   *TRIM5* KO cells were generated as a KO pool: therefore, at a population level, some cells in this

169   cell pool still have intact TRIM5α expression. Overall, these data suggest that primate TRIM34

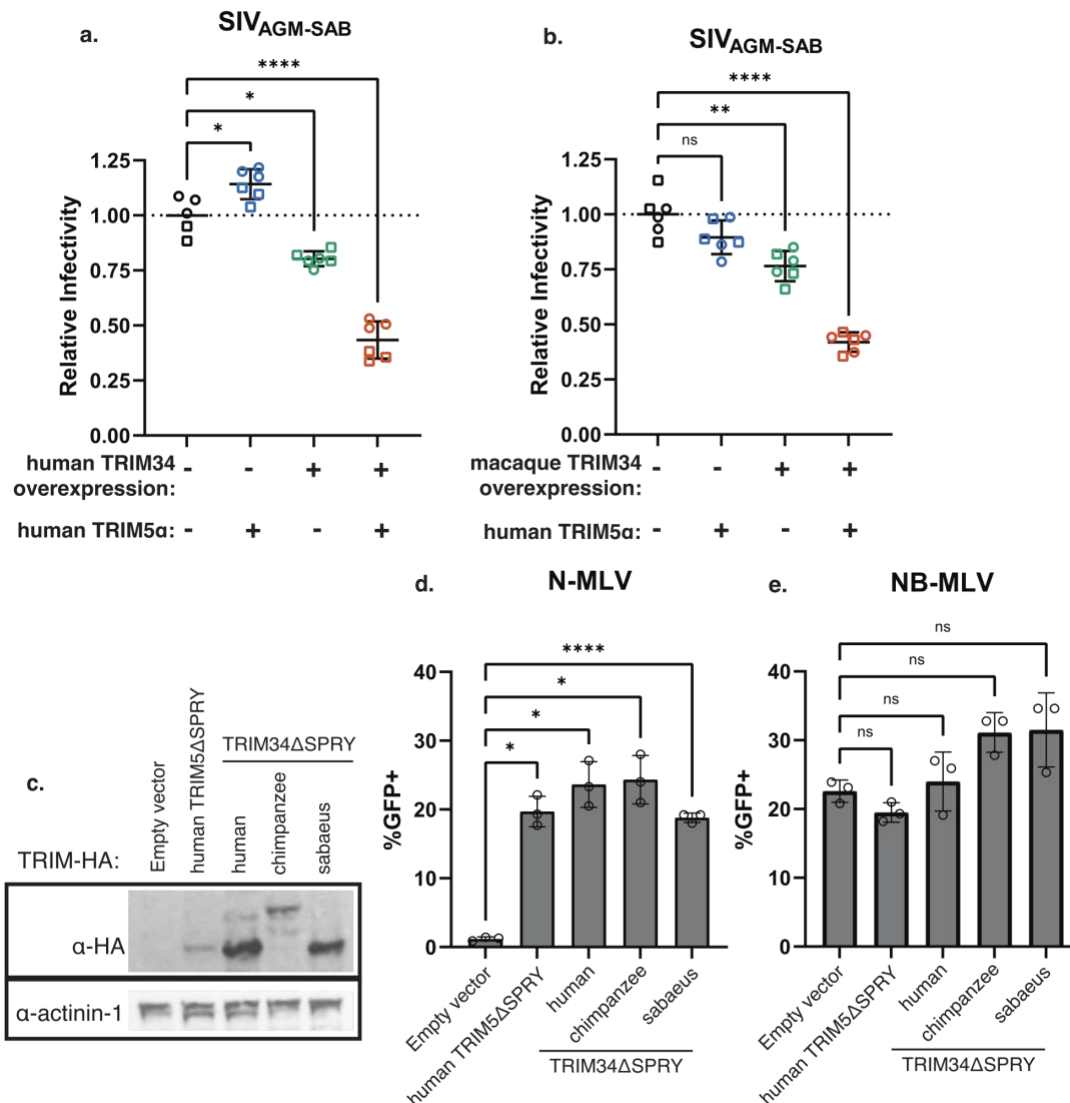170   orthologues broadly require TRIM5α for restriction of lentiviral capsids.

171

Figure 2. TRIM34 requires TRIM5α to restrict SIV_AGM-SAB.
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. KO efficiency was assessed by ICE analysis. For human TRIM34-expressing cell lines, ICE KO efficiency scores were as follows: *TRIM5* sgRNA 1 (squares) = 76, *TRIM5* sgRNA 2 (circles) = 90. For rhesus macaque TRIM34-expressing cells, ICE KO scores were as follows: *TRIM5* sgRNA 1 (squares) = 83, *TRIM5* sgRNA 2 (circles) = 93. Thus, there existed 4 different cellular conditions: no TRIM34 or TRIM5α expression = black symbols, only

181 endogenous TRIM5α expression = blue symbols, only overexpressed TRIM34 = green symbols,
182 both endogenous TRIM5α and overexpressed TRIM34 = red symbols. 1 day after doxycycline
183 induction, cells were infected with SIV$_{AGM-SAB}$ CA particles; each infection was performed in
184 triplicate. 2 dpi, infectivity was quantified by flow cytometry. Relative infectivity is normalized to
185 mean infectivity in *TRIM5* KO, TRIM34-uninduced cells (black symbols). Data are represented
186 as mean +/- s.d. from 1 representative experiment. Mean represents average across all 6
187 replicates.
188 c. HeLa cells were transduced to stably express primate TRIMΔSPRY orthologues or empty
189 vector control. Expression was visualized by immunoblotting.
190 d-e. HeLa cells stably expressing primate TRIM34ΔSPRY orthologues or empty vector control
191 were infected with N-MLV (d) or NB-MLV (e). Level of infection was quantified 2 dpi by flow
192 cytometry. Data are represented as mean +/- s.d. from 1 representative experiment. Infections
193 were performed in triplicate.
194 a-b, d-e. p values were calculated by Brown-Forsythe and Welch's 1-way ANOVA with
195 Dunnett's T3 test for multiple comparisons. ns = nonsignificant, * p ≤ 0.05, ** p ≤ 0.01, *** p ≤
196 0.001, **** p ≤ 0.0001.
197
198 **Human TRIM5α functionally interacts with TRIM34 from different primates**

199 We next sought to assess whether TRIM34 orthologues and TRIM5α can functionally

200 interact with each other via a TRIM5α restriction assay. Previous work has shown that

201 dimerization and higher-order multimerization are essential for TRIM5α-mediated restriction

202 [14,25–27]. Furthermore, TRIM5α has been shown to associate not only with itself but also with

203 TRIM34 via two-hybrid screen and co-immunoprecipitation [9,13,14]. To assess functional

204 interaction of TRIM34 and TRIM5α more directly, we exploited the fact that TRIM proteins that

205 have been deleted of their SPRY domains (TRIMΔSPRY) exert a dominant-negative effect on

206 restriction of N-tropic MLV (N-MLV) by endogenous TRIM5α constitutively expressed in HeLa

207 cells [13]. Overexpression of human TRIM34ΔSPRY inhibits restriction of N-MLV in HeLa cells

208 by the endogenous TRIM5α [13]. This implies that TRIM34 is able to functionally interact with

209 TRIM5α. Thus, we asked whether primate TRIM34ΔSPRY orthologues could also interact with

210 human TRIM5α in this assay. The human and rhesus macaque TRIM34ΔSPRY constructs were

211 truncated at the start of the SPRY domain, while the chimpanzee TRIM34ΔSPRY construct was

212 truncated partway through the SPRY domain, between the v2 and the v3 loops. We

213 overexpressed the HA-tagged human, chimpanzee, and macaque TRIM34ΔSPRY constructs in

214 HeLa cells together with human TRIM5ΔSPRY as a positive control (Figure 2c). These cells

215   were then infected with N-MLV. We found that relative to empty vector control, overexpression

216   of all three TRIM34ΔSPRY orthologues, as with TRIM5ΔSPRY, relieved restriction of N-MLV by

217   human TRIM5α (Figure 2d). We also infected with NB-tropic MLV (NB-MLV) as a control (Figure

218   2e), since NB-MLV is not restricted by TRIM5α [28,29]. These data further support our model

219   that diverse TRIM34 orthologues and human TRIM5α can functionally interact with each other.

220   **Both TRIM34 and TRIM5α SPRY domains are involved in viral restriction**

221   Since TRIM5α is required for restriction by TRIM34 and does not seem to vary

222   depending on which TRIM34 is used, we hypothesized that capsid recognition comes from

223   TRIM5α, and not from TRIM34. In the case of TRIM5α-mediated restriction, the SPRY domain,

224   and specifically the v1 loop, determines antiviral specificity [18,21,30]. Furthermore, altering a

225   single amino acid in the human TRIM5α SPRY domain (arginine 332) in the v1 loop to match

226   the corresponding macaque residue (proline) is sufficient to confer strong restriction of HIV-1

227   [21,30]. To ask if the TRIM5α SPRY domain is involved in TRIM34-mediated CA recognition, we

228   used cells that were doubly knocked out for endogenous *TRIM5* and *TRIM34* (THP-1 *TRIM34*

229   *TRIM5* clonal KO cells). In these cells, we introduced inducible vectors expressing wild type

230   (WT) human TRIM34 with WT human TRIM5α, TRIM5α in which the v1 loop was deleted

231   (TRIM5α Δv1), and TRIM5α in which the arginine at position 332 was mutated to a proline

232   (TRIM5α R332P). We expressed each TRIM individually as well as in the context of each other

233   and confirmed expression levels by Western blot (Figure 3a). In concordance with the results in

234   Figure 2, we found that SIV$_{AGM-SAB}$ was restricted only in the presence of both human TRIM34

235   and human TRIM5α, but not when either TRIM34 or TRIM5α were expressed in isolation

236   (Figure 3b; grey bars). Furthermore, in agreement with our previous findings [16], HIV-1 N74D,

237   but not WT HIV-1, was restricted only in the presence of both TRIM34 and TRIM5α (Figure 3c-
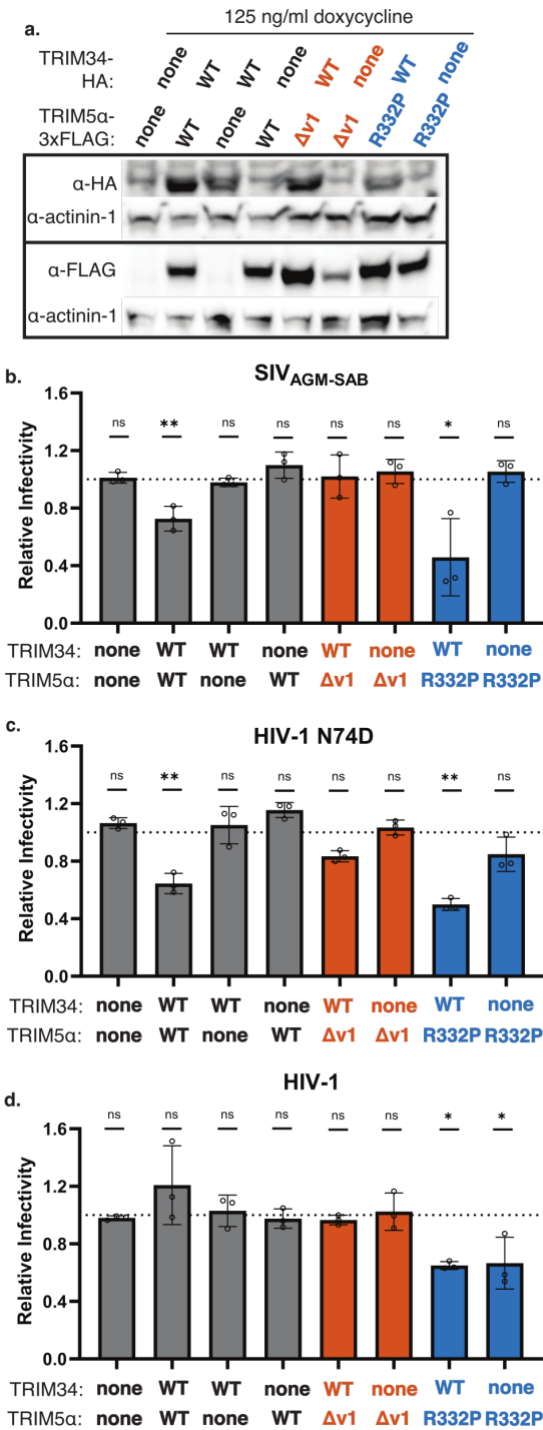
238   d; grey bars).

239

Figure 3. TRIM5α v1 loop is necessary for restriction of SIV$_{AGM-SAB}$.
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. 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 levels were visualized by immunoblotting 72 h post-induction.

247   b-d. THP-1 *TRIM34 TRIM5* double KO clonal cells co-expressing doxycycline-inducible human
248   TRIM34 or empty vector control and human TRIM5α (grey bars), TRIM5Δv1 (red bars), TRIM5
249   R332P (blue bars), or empty vector control were infected with SIV$_{AGM-SAB}$ (b), HIV-1 N74D (c), or
250   HIV-1 (d) CA 1 day post-induction. Levels of infection were quantified 2 dpi by flow cytometry.
251   Relative infectivity in induced cells (solid bars) is normalized to average infectivity in uninduced
252   control cells (not shown). Data are represented as mean +/- s.d. of 3 technical replicates from 1
253   representative experiment. One-sided p values were calculated by Welch's *t*-test. ns =
254   nonsignificant, * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$.
255
256   Given that TRIM5 is required for TRIM34-mediated restriction, we next asked whether

257   the TRIM5α v1 loop, known to be important for capsid specificity by TRIM5α, was essential for

258   restriction by TRIM34. We found that unlike co-expression of TRIM34 with full-length TRIM5α,

259   co-expression of TRIM34 with TRIM5α Δv1 was not sufficient for restriction of either SIV$_{AGM-SAB}$

260   and HIV-1 N74D (Figure 3b-c; red bars). This suggests that the TRIM5α v1 loop is required for

261   TRIM34-mediated restriction.

262   We then asked whether changing the identity of the TRIM5α v1 loop, but not deleting it

263   entirely, could also alter viral specificity. To assess this question, we generated the mutant

264   TRIM5α R332P, which converts the human residue at position 332 to the rhesus macaque

265   residue and has been shown to have enhanced antiviral inhibition of HIV-1 [21,30]. We co-

266   expressed these cells with TRIM34 and infected with SIV$_{AGM-SAB}$, HIV-1 N74D, or HIV-1. In

267   accordance with the literature, TRIM5α R332P was able to restrict HIV-1 while WT TRIM5α did

268   not (Figure 3d; blue bars) [21,30]. Furthermore, restriction of HIV-1 by TRIM5α R332P was

269   agnostic to the presence of TRIM34; that is, TRIM34 did not appear to augment restriction of

270   HIV-1 by TRIM5α R332P (Figure 3d; blue bars). Similar to wild type TRIM34 and TRIM5α,

271   TRIM34 co-expression with the mutant TRIM5α R332P allele maintained restriction of the N74D

272   CA mutant virus (Figure 3c; blue bars). Interestingly, TRIM5α R332P alone did not restrict HIV-

273   1 N74D, in contrast to WT HIV-1 (Figure 3c-d; blue bars). This suggests that TRIM34 is critical

274   for restriction of HIV-1 N74D, even in the presence of a TRIM5α mutant that is more potent

275   against WT HIV-1. Finally, TRIM34 co-expressed with TRIM5α R332P restricted SIV$_{AGM-SAB}$ CA,

276   whereas TRIM5α R332P alone did not (Figure 3b; blue bars). These findings support a role for

277   both the TRIM5α v1 loop and for TRIM34 in viral recognition and specificity.

278   **TRIM34 SPRY Domain is Required for Restriction**

279   To more directly test whether the TRIM34 SPRY domain plays a role in antiviral activity

280   and specificity, we generated a chimeric protein that encodes the TRIM34 RING, Bbox and

281   coiled-coil domains in frame with the TRIM5 SPRY domain (TRIM34 RBCC-TRIM5 SPRY)

282   (Figure 4a). We hypothesized that if the TRIM34 SPRY domain was required for restriction, the

283   chimera would lose restriction relative to full-length TRIM34. We transduced this chimera into

284   THP-1 *TRIM34* clonal KO cells and confirmed expression by Western blot (Figure 4b). We

285   assayed this construct for restriction in our THP-1 cells that lack TRIM34 expression but do

286   contain endogenous, full-length TRIM5α. We found that in the presence of endogenous

287   TRIM5α, the TRIM34 RBCC-TRIM5α SPRY chimera was not able to restrict SIV$_{AGM-SAB}$ CA

288   (Figure 4c). Therefore, the TRIM34 SPRY domain, in addition to the TRIM5α SPRY domain, is
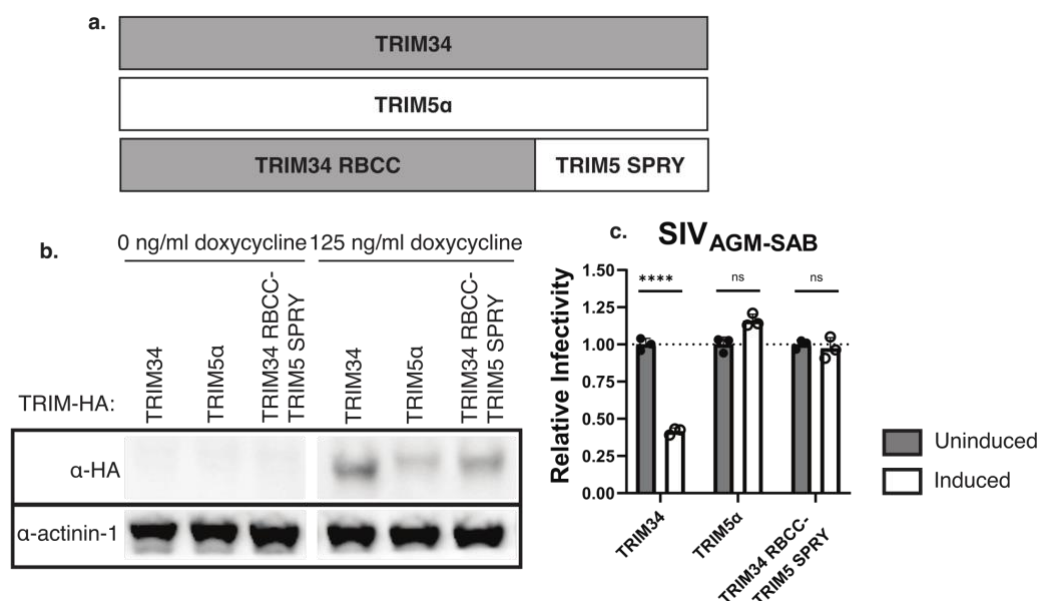
289   involved in restriction.

290

291   **Figure 4. TRIM34 SPRY domain is necessary for restriction of SIV$_{AGM-SAB}$.**
292   a. Schematic of TRIM34 RBCC-TRIM5 SPRY chimera

293 b. THP-1 *TRIM34* clonal KO cells were generated by electroporation of multiplexed sgRNA
294 against *TRIM34* and single cell sorting. Cells were transduced with doxycycline-inducible HA-
295 tagged human TRIM34, TRIM5α, or TRIM34-TRIM5α chimeras. Expression levels were
296 visualized by immunoblotting 72 h post-induction.
297 c. THP-1 *TRIM34* clonal KO cells expressing doxycycline-inducible human TRIM34, TRIM5α, or
298 TRIM34 RBCC-TRIM5 SPRY were infected with SIV$_{AGM-SAB}$ CA particles 1 day post-induction.
299 Level of infection was quantified 2 dpi by flow cytometry. Relative infectivity in induced cells
300 (white bars) is normalized to average infectivity in uninduced control cells (grey bars). Data are
301 represented as mean +/- s.d. from 1 representative experiment comprising 3 technical
302 replicates. One-sided p values were calculated by Welch's *t*-test. ns = nonsignificant, * p ≤ 0.05,
303 ** p ≤ 0.01, *** p ≤ 0.001, **** p ≤ 0.0001.
304

DISCUSSION

306   Human TRIM34 was recently identified as a restriction factor of the CA mutant HIV-1

307 N74D and certain SIVs [16]. Here we show that this antiviral phenotype is a broadly conserved

308 function of primate *TRIM34* genes. We find that the same subset of lentiviral capsids is

309 restricted by a diverse panel of TRIM34 orthologues, irrespective of TRIM34 species of origin.

310 Moreover, we find that although antiviral specificity does not seem to vary with the identity of

311 TRIM34, both the TRIM34 and TRIM5α SPRY domains are critical to restriction, suggesting that

312 TRIM5 confers a level of specificity to the TRIM34 restriction.

313   Despite the lack of evidence for positive selection on TRIM34, primate TRIM34 alleles

314 have broadly maintained the ability to restrict certain primate lentiviruses. This is surprising in

315 light of the fact that positive selection is a marker of critical regions of direct host-viral

316 interaction. We propose that although TRIM34 lacks this characteristic, it utilizes TRIM5α,

317 which is a rapidly-evolving gene in primates, to restrict lentiviruses. Prior work has shown that

318 TRIM34 and TRIM5α are capable of multimerizing *in vitro* in addition to binding capsid [9,13,14].

319 In this work we demonstrate that TRIM5α can functionally interact with a number of primate

320 TRIM34 orthologues. This supports a model in which TRIM5α and TRIM34 multimerize to form

321 a higher-order structure that enables both SPRY domains to interact with the viral capsid.

322 Therefore, despite the lack of an overt, positively-selected site of viral interface on TRIM34,

323 TRIM34 restricts viral infection through interaction with the rapidly-evolving TRIM5 protein. It is

324   also possible that physical interaction between TRIM34 and TRIM5α alters the half-life of one or

325   both proteins, affecting turnover rate or stability. Prior work has demonstrated that different

326   TRIMs have different half-lives, and that alterations to the RING or Bbox domains of TRIM

327   proteins can affect protein turnover [14,31,32]; thus, it is possible that interactions between

328   different TRIMs might lead to changes in stability.

329   Another possibility is that TRIM34 could be filling the role of an effector molecule.

330   Previously, the TRIM5α RING domain has been implicated as an E3 ubiquitin ligase that acts as

331   a signal transducer to initiate innate immune activation subsequent to CA sensing by the

332   TRIM5α SPRY domain [33–35]. In the case of TRIM34, it is possible that TRIM5α is responsible

333   for recognizing CA while TRIM34's E3 ligase domain contributes to downstream signaling,

334   capsid degradation, or recruitment of other molecules that aid restriction. Although Lascano *et*

335   *al.* found that TRIM34 does not activate AP-1, it remains possible that it is acting by another

336   mechanism [34]. Notably, these models are not mutually exclusive and could function in tandem

337   with TRIM34 aiding TRIM5α in more than one capacity.

338   Our findings suggest that the SPRY domains of both TRIM34 and TRIM5α are important

339   for restriction. On its own, human TRIM5α is a relatively weak restriction factor. We propose that

340   TRIM34 can assist human TRIM5α to restrict some—but not all—viral capsids. For example,

341   TRIM5α in the presence of TRIM34 is able to restrict $SIV_{AGM-SAB}$; conversely, even in the

342   presence of TRIM34, TRIM5α is still unable to restrict HIV-1. One possibility is that the TRIM34

343   SPRY domain is contributing to viral recognition. Previous work has shown that TRIM34 is able

344   to bind HIV-1 CA-NC complexes *in vitro*, even though it does not restrict HIV-1, raising the

345   possibility that TRIM34 may still contribute to capsid binding [14,36]. Although the v1 loop of

346   TRIM34 has not undergone positive selection and is only 6 amino acids long, we cannot

347   exclude the possibility that it, or one of the other variable loops, might contribute to capsid

348   recognition. Given the relatively high conservation of the variable loops of the primate TRIM34

349   alleles that we assessed (no amino acid differences in v1, v3, or v4; 1 amino acid difference in

350   each allele in v2), this could explain the observation that all the TRIM34 alleles tested restricted

351   the same subset of viruses. Furthermore, the observation that TRIM5α R332P—which gains

352   restriction of HIV-1 relative to WT TRIM5α—does not restrict HIV-1 N74D alone and only

353   restricts it in the presence of TRIM34, supports the idea that TRIM34 could be assisting in

354   capsid recognition beyond the capacity of TRIM5α alone. Thus, as many TRIM5α alleles can act

355   as potent restriction factors of a few very specific viral capsids, but a poor restriction factor of

356   many others, we speculate that TRIM34 might act as a cofactor to enable TRIM5α to restrict

357   viral capsids that TRIM5α is not able to restrict on its own.

358   It is intriguing that all the TRIM34 orthologues tested restricted the same subset of

359   viruses: $SIV_{AGM-SAB}$, $SIV_{AGM-TAN}$, and SIV_{MAC}. Notably, only viruses originating from Old World

360   monkeys were restricted, whereas the hominid viruses, HIV-1 and SIV_{CPZ}, were not restricted. It

361   is possible that this is driven by the identity of TRIM5α: HIV-1 and SIV_{CPZ} are better evolved to

362   evade restriction by human TRIM5α compared to the Old World monkey viruses [23]. The three

363   restricted viruses share only 70.6% pairwise amino acid identity across their CA regions, making

364   it difficult to identify critical residues or motifs that determine restriction. We explored the

365   possibility that restriction might be dependent on CypA binding. However, CypA binding

366   capacity varied across the restricted viruses. For example, $SIV_{AGM-TAN}$ does bind CypA, while

367   SIV_{MAC} and $SIV_{AGM-SAB}$ do not [13,37–39]. Furthermore, while HIV-1 is well-established to bind

368   and incorporate CypA into virions, the CypA binding capacity of HIV-1 N74D is controversial

369   [15,40,41]. Thus, at minimum, restriction does not depend exclusively on CypA binding. Instead,

370   restriction of these capsids by TRIM34 and TRIM5α may be dependent on other unknown

371   features that distinguish these capsids.   A number of blocks to HIV infection that are capsid-

372   dependent have been characterized—for example, Lv2, Lv3, and Lv4—for which the

373   responsible cellular components have either not been identified or have only been partly

374   identified [42–46]. It is possible that TRIM34 could contribute to one or more of these blocks.

375   We find that TRIM34 alleles from a broad spectrum of primates, when paired with

376   TRIM5α, are able to restrict capsids that neither TRIM is able to restrict on its own. Despite

377   lacking signs of positive selection characteristic of many restriction factors, TRIM34—acting in

378   tandem with TRIM5α—can act as a barrier to cross-species transmission events. It is possible

379   that these proteins have co-evolved such that TRIM34 can enhance or modify TRIM5α's

380   antiviral potential. Indeed, not only do host immune proteins evolve in the context of the

381   pathogens they counteract, but they also evolve in the context of other, complementary host

382   proteins. Thus, our data suggest that restriction factors evolve not only in isolation in response

383   to evolutionary pressures exerted by viral pathogens but may also co-evolve with each other

384   resulting in more powerful antiviral activity than either could achieve on its own.

385

395

396   **AUTHOR CONTRIBUTIONS**

397   Conceptualization, methodology, writing, and funding acquisition were performed by J.T., M.E.,

398   and M.O. Investigation was conducted by J.T., A.K., and A.L.F. Validation, formal analysis, and

399   visualization were performed by J.T. and A.K. Supervision was provided by M.E. and M.O.

400

MATERIALS AND METHODS

Cell culture

All cells were cultured at 37°C and 5% CO_2. 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 [22], SIV_{CPZ}, $SIV_{AGM-SAB}$, or SIV_{MAC} CA (gifts from Theodora Hatziioannou) [23]; and either pALPS-eGFP (Addgene #101323, a gift from Jeremy Luban) [47] or pHIV-zsGreen (Addgene #18121, a gift from Bryan Welm and Zena Werb) [48]. SIV_{CPZ}, $SIV_{AGM-SAB}$, or SIV_{MAC} chimeric virus particles were made with pALPS-eGFP, and HIV-1 virus particles were made with pHIV-zsGreen. The SIV_{MAC} CA construct contained the mutation A77V. Luciferase reporter viruses were generated by transfection of a two-plasmid system: SIVagmTAN E- R- luc [24] and L-VSV-G (VSV glycoprotein expression) [49]. Particles were harvested 2 days post-

427   transfection, syringe-filtered through 0.22uM PES membranes, and frozen at -80°C. N-MLV

428   reporter viruses were generated by transfection of a three-plasmid system: pCIG3-N for

429   expression N-MLV gag/pol (Addgene #132941, a gift from Jeremy Luban) [50], pQCXIP-eGFP

430   (a gift from Jeannette Tenthorey) [21] for expression of a fluorescent reporter, and pMD2.G for

431   expression of VSV-G envelope. NB-MLV reporter viruses were generated by transfection of a

432   four-plasmid system: JK3 for expression of NB-MLV gag/pol [49], L-VSV-G for expression of

433   VSV-G envelope [49], CMV-tat for transactivation [49], and pQCXIP-eGFP for expression of a

434   fluorescent reporter.

435   pLentiCRISPR-v2 (Addgene #52961, a gift from Feng Zhang) constructs were generated

436   by BsmBI (New England BioLabs, Ipswich, MA, #R0580) restriction cloning of TRIM5 guides

437   into pLentiCRISPR-v2, a lentiviral vector encoding for Cas9 [51]. pLentiCRISPR-v2 containing

438   guides were transfected with pSPAX2 (Addgene #12260, a gift from Didier Trono) and pMD2.G

439   to generate lentiviral particles. Particles were harvested 2 days post-transfection, syringe-filtered

440   through 0.22 μM PES membranes, and frozen at -80°C.

441   HA-tagged TRIM34 and 3xFLAG-tagged TRIM5α codon-optimized expression

442   constructs (Supplemental data) were synthesized by Twist Bioscience (South San Francisco,

443   CA). TRIM5α mutant constructs (TRIM5α v1 loop and TRIM5α R332P) were a gift from

444   Jeannette Tenthorey [21]. TRIM constructs were cloned into pLKO (a gift from Melissa Kane)

445   [52] by SfiI (New England BioLabs #R0123S) restriction enzyme digest. pLKO-TRIM constructs

446   were transfected along with pMD2.G and pSPAX2 to generate particles. Particles were

447   harvested at 2 and 3 days post-transfection, cell pellets spun down at 300 x g, and supernatants

448   frozen at -80°C.

449   HA-tagged TRIM34ΔSPRY constructs were cloned in pQCXIP (TaKaRa Bio, San Jose,

450   CA, #631516) by SbfI (New England BioLabs #R3642S) and NotI (New England Biolabs

451   #R3189S) restriction digest. pQCXIP-TRIM34ΔSPRY constructs were transfected along with

452   pJK3 (MLV gag/pol), L-VSV-G (VSV-G envelope), and CMV-tat to generate particles. Particles

453   were harvested 2 days post-transfection.

CRISPR Knockouts

455   Clonal knockout lines in THP-1 cells were generated by electroporation of multiplexed

456   small guide RNA (sgRNA) from Gene Knockout Kit v2 (Synthego, Redwood City, CA) against

457   *TRIM34* (guide sequences = CTTGCTTAACGTACAAG, CCACAGTCTAGACTCAA,

458   GCAGTGACCAGCATGGG) or *TRIM5* (guide sequences = GGUAACUGAUCCGGCACACA,

459   ACUUCUUGUGGUUUGCAGUG, CCUGGUUAAUGUAAAGGAGG). Single cell clonal lines

460   were generated by single cell sorting (*TRIM34*) or limiting dilution (*TRIM5*). Knockout efficiency

461   was validated by Interference of CRISPR Edits (ICE) analysis (Synthego) [53].

462   Pooled knockout lines were generated by transduction of THP-1 cells with lentiviral

463   preps containing guides delivered by pLentiCRISPR-v2. *TRIM5* guide sequences =

464   TCACCACACGTTCCTCACAG and GTTGATCATTGTGCACGCCA. NTC guide sequences =

465   GGGCCCGCATAGGATATCGC and TAGACAACCGCGGAGAATGC. Cells were spinoculated

466   in the presence of 20 µg/mL DEAE-Dextran (Pharmacia Fine Chemicals, Uppsala, Sweden,

467   #17-0350-01) and then selected in 10 µg/mL blasticidin S HCl (Gibco #A11139-03). Knockout

468   efficiency was validated by ICE analysis [53].

Inducible Overexpression

470   Doxycycline-inducible expression of TRIM34 and TRIM5α was achieved by transduction

471   of lentiviral preps containing pLKO TRIM constructs in THP-1 cells. Cells were spinoculated in

472   the presence of 5 µg/mL polybrene (EMD Millipore, Burlington, MA, #TR-1003-G) and then

473   selected in 0.5 µg/mL puromycin (TRIM34) (Sigma-Aldrich, St. Louis, MO, #P8833-25MG) or 10

474   µg/mL blasticidin (TRIM5α). Protein expression was induced by the addition of 125 ng/mL

475   doxycycline hyclate (Sigma-Aldrich #D9891-5G) to cells cultured in complete RPMI 1640

476   containing tetracycline-approved FBS (Sigma-Aldrich #F0926-50ML).

477   Stable expression of TRIM34ΔSPRY was achieved by transduction of lentiviral preps

478   containing pQCXIP constructs expressing TRIMΔSPRY constructs. HeLa cells were

479   spinoculated in the presence of 20 µg/mL DEAE-Dextran. Selection was performed in the

480   presence of 1 µg/mL puromycin.

Restriction assays

482   1 day after induction of TRIM expression, THP-1 cells were infected with chimeric CA

483   virus particles expressing a fluorescent reporter. Cells were spinoculated in the presence of 20

484   µg/mL DEAE-Dextran. 2 dpi, relative infectivity was quantified by flow cytometry using a

485   FACSCelesta Analyzer (BD Biosciences, San Jose, CA) or Bright-Glo luciferase assay reagent

486   (Promega, Madison, WI #E2620) using a LUMIstar Omega luminometer (BMG Labtech,

487   Ortenberg, Germany).

N-MLV Restriction assay

489   HeLa cells that had been transduced to stably express TRIM34ΔSPRY constructs were

490   infected with N-MLV particles expressing an eGFP reporter by spinoculation in the presence of

491   20 µg/mL DEAE-Dextran. Infectivity was assessed 2 dpi by flow cytometry using an

492   LSRFortessa Cell Analyzer (BD Biosciences).

Western blotting

494   3 days after induction of TRIM expression, THP-1 cells were harvested; 1 day prior to

495   infection, HeLa cells were harvested. Cells were washed once with PBS. Cells were then lysed

496   on ice for 30 min in 20 mM HEPES (Fisher Scientific, Fair Lawn, NJ, #BP310-1) containing 8 M

497   urea (Sigma-Aldrich #U-6504), 50 mM DL-Dithiothreitol (DTT) (Gold Biotechnology, St. Louis,

498   MO, #DTT10), 0.1% w/v SDS (Fisher Scientific #BP166-500), 1.5 mM $MgCl_2$ (Sigma-Aldrich

499   #208337-100G), 0.5 mM $CaCl_2$ (Sigma-Aldrich #C-3306), 50 µg/mL DNAse I (Roche

500   Diagnostics, Mannheim, Germany, #10104159001), and 1X EDTA-free protease inhibitor

501   cocktail (Roche Diagnostics #11836170001). 6X SDS-PAGE sample loading buffer (G

502   Biosciences, St. Louis, MO #785-701) containing 5% v/v 2-mercaptoethanol (Sigma-Aldrich

503   #M3148-100ML) was added to lysates. Lysates were acid treated with 50 mM HCl (Sigma-

504   Aldrich #320331-300ML) and then boiled at 95°C for 7 min. After cooling, lysates were

505   neutralized with 50 mM NaOH (Sigma-Aldrich #72068-100ML). Products were resolved by

506   SDS-PAGE on NuPage Bis-Tris 4-12% acrylamide gels (Invitrogen, Carlsbad, CA,

507   #NP0335BOX) and transferred onto nitrocellulose membranes (Bio-Rad Laboratories, Hercules,

508   CA, #1620115). Membranes were blocked with 2% w/v milk (Research Products International,

509   Mt. Prospect, IL, #M17200-500.0) and 2% w/v bovine serum albumin (Sigma-Aldrich #A7906-

510   100G) in tris-buffered saline (Fisher Scientific #BP152-5) containing 1% v/v Tween-20 (Fisher

511   Scientific #BP152-1) (TBS-T). Primary antibodies were incubated overnight at 4°C: rabbit anti-

512   HA high affinity at 1:500 (Roche Diagnostics #ROAHAHA), mouse anti-FLAG M2 at 1:500

513   (Sigma-Aldrich #F1804), and rabbit anti-alpha actinin-1 at 1:1000 (Bio-Rad Laboratories

514   #VPA00889). Membranes were washed with TBS-T and then incubated with HRP-conjugated

515   secondary antibodies for 1 h at RT: goat anti-rat IgG at 1:2000 (Abcam, Waltham, MA

516   #ab97057), sheep anti-mouse IgG at 1:2000 (GE Cytiva #NA931), and donkey anti-rabbit IgG at

517   1:2000 (GE Cytiva #NA934V). Membranes were washed again and then incubated with

518   SuperSignal West Pico substrate (HA and alpha actinin-1 blots) (Thermo Fisher Scientific,

519   Waltham, MA, #34580) or Supersignal West Femto substrate (FLAG blots) (Thermo Fisher

520   Scientific #34095) for 3 min. Membranes were imaged with a ChemiDoc MP imaging system

521   (Bio-Rad Laboratories).

522   **Statistical methods**

523   For comparisons of 2 independent variables, one-tailed p values were computed by

524   Welch's *t*-test. For comparisons of 3 or more independent variables, p values were computed by

525   Brown-Forsythe and Welch's 1-way ANOVA with Dunnett's T3 test for multiple comparisons. All

526   calculations and data visualizations were performed using GraphPad Prism version 9.3

527   (GraphPad Software, San Diego, CA).

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ADDITIONAL FILES

657 Additional File 1 (Additional File 1.txt). TRIM34 and TRIM5a sequences used in this study,

658 codon-optimized for expression in human cells where applicable.

COMPETING INTERESTS

660 The author(s) declare(s) that they have no competing interests.

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