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1 # Editing of the Human TRIM5 Gene to Introduce HIV-1 Restrictive
2 # Mutations Using CRISPR-Cas9

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9

10 **ABSTRACT**

11 The type I interferon (IFN-I)-inducible human restriction factor TRIM5α inhibits the infection of
12 human cells by specific nonhuman retroviruses, such as N-MLV and EIAV, but does not
13 generally target HIV-1. However, the introduction of two aminoacid substitutions, R332G and
14 R355G, in the human TRIM5α (huTRIM5α) domain responsible for retroviral capsid recognition
15 leads to efficient HIV-1 restriction. Using a simple DNA transfection-based CRISPR-Cas9
16 genome editing protocol, we successfully mutated *TRIM5* to its HIV-1-restrictive version by
17 homology-directed repair (HDR) in HEK293T cells. Nine clones bearing at least one HDR-
18 edited *TRIM5* allele containing both mutations were isolated (5.6% overall efficiency), whereas
19 another one contained only the R332G mutation. Of concern, several of these HDR-edited clones
20 contained on-target undesired mutations, and none had all the alleles corrected. We observed a
21 lack of HIV-1 restriction in the cell clones generated, even when cells were stimulated with IFN-

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22 I prior to infection. This, however, was partly explained by the low potential for TRIM5α-
23 mediated restriction activity in this cell line as determined in control experiments. Our study
24 demonstrates the feasibility of editing the TRIM5 gene to confer protection from HIV-1 in
25 human cells and identifies the main challenges to be addressed in order to attain that goal.

26

27 **Introduction**

28 Viruses are obligate parasites whose success at infecting a host cell typically requires evasion
29 from antiviral factors. In mammals, many cellular antiviral factors that can potentially interfere
30 with the progression of viral infections have been identified. These factors can often act without
31 external stimulation, but their expression and activity are enhanced by cytokines such as type I
32 interferons (IFN-I) [1]. IFN-I cytokines are a multigene family of small peptides that include IFN-
33 α, IFN-β, IFN-ε, IFN-κ, IFN-ω, IFN-δ and IFN-τ in humans [2]. Whereas these various IFN-I
34 species all interact with the same receptor, they differ in IFN-stimulated gene (ISG)-, pathogen-
35 and cell type-specificity [3].

36 Among the ISGs relevant to retroviruses, the family of viruses to which HIV-1 belongs, is
37 *TRIM5*, which encodes the cytoplasmic protein TRIM5α [4]. In humans, *TRIM5* is transcribed into
38 5 isoforms, among which only TRIM5α possesses antiviral activity [5]. At its C-terminus, a
39 domain called SPRY (PRYSPRY, B30.2) determines the retrovirus targeting specificity. This
40 domain comprises hyper-variable loops that directly interact with the N-terminal domain of
41 capsid proteins early after entry of the retrovirus into the host cell membrane [6]. When such
42 interactions occurs, the retrovirus is inhibited ("restricted") through mechanisms that include
43 destabilization of the capsid core [7], proteasomal degradation of some core components [8] and

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44 sequestration of the viral particle in TRIM5α cytoplasmic bodies [9]. huTRIM5α generally has
45 little-to-no activity against HIV-1, but efficiently inhibits the infectivity of the nonhuman
46 gammaretrovirus "N-tropic" murine leukemia virus (N-MLV) as well as the nonhuman lentivirus
47 equine infectious anemia virus (EIAV) [10]. Those two viruses are typically inhibited ~10-fold
48 (EIAV) and ~100-fold (N-MLV) by endogenous huTRIM5α, with some variation depending on
49 the cellular context.

50 Several groups, including ours, have attempted to harness the antiviral power of TRIM5α in
51 order to interfere with HIV-1. This virus is efficiently restricted (~100-fold) by some orthologs
52 of TRIM5α found in Old World monkeys such as the Rhesus macaque TRIM5α (rhTRIM5α) [4].
53 However, significant sequence variation between the human and macaque orthologs preclude the
54 possibility of using the latter one in gene therapy approaches, as this would increase the risk to
55 elicit an immune response against the transgene in patients. Thus, all the studies have consisted
56 in over-expressing versions of huTRIM5α designed to target HIV-1 through modifications in the
57 SPRY domain. Some of the TRIM5α variants used were chimeric products containing small
58 regions of rhTRIM5α in the SPRY [11,12]. Other teams mapped with further precision the HIV-1
59 restriction determinants in rhTRIM5α that were absent in huTRIM5α, leading to the discovery
60 that mutating the Arg332 residue in huTRIM5α was sufficient to inhibit HIV-1. Although initial
61 observations [13,14] raised the hope that single mutations at this position might inhibit HIV-1 as
62 efficiently as rhTRIM5α did, later work made it clear that this was not the case [15].

63 Our laboratory explored a different approach: generating libraries of TRIM5α SPRY mutants
64 then applying a functional screen to isolate mutants that conferred HIV-1 restriction [16,17]. These
65 studies identified mutations at Arg335 inhibiting HIV-1 by 5- to 10-fold. When we combined a
66 mutation at Arg335 (R335G) with one at Arg 332 (R332G), we obtained restriction levels that

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67 were higher than with either of the single mutants [16,17]. Although not quite as restrictive as
68 rhTRIM5α, R332G-R335G huTRIM5α efficiently inhibited the propagation of a highly
69 pathogenic strain of HIV-1, and cells expressing the transgene had a survival advantage over
70 unmodified cells [15].

71 Although R332G-R335G huTRIM5α is considered a prime candidate in HIV-1 gene therapy
72 approaches to inhibit HIV-1, using lentiviral vectors to overexpress it in human cells is not
73 without caveats. Indeed, the physiological effects of TRIM5α overexpression *in vivo* are not
74 clear, considering that it is involved in innate immune responses [18,19] and possibly in autophagy
75 [20]. In addition, the genotoxicity of lentiviral vectors integrating in the human genome is still
76 poorly predictable. In the longer term, it would thus be desirable to be able to introduce
77 mutations in the endogenous human *TRIM5* by genome editing. Here we describe the use of
78 Clustered Regularly Interspaced Short Palindromic Repeats with Cas9 (CRISPR-Cas9) and of a
79 single-stranded homology-directed repair (HDR) donor DNA to successfully mutate Arg332 and
80 Arg335 in a human cell line.

81 Results

82 **Strategy for the mutagenesis of *TRIM5* by HDR.** Human *TRIM5* is found on chromosome
83 11p15.4 and the region of the gene encoding the SPRY domain is present in exon 8 (Fig. 1A).
84 We searched for DNA loci close to the codons for Arg332 and Arg335 that would be potential
85 targets for CRISPR-Cas9-mediated double-strand cleavage. CRISPR guide RNAs (gRNAs) were
86 designed for the 3 potential target sites that were nearest to the two codons to be mutated (Fig.
87 1B). CRISPR plasmids expressing Cas9 along with one of the designed gRNAs were transfected
88 in human embryonic kidney 293T cells (HEK293T), and a Surveyor assay was performed to test
89 the capacity of the three gRNAs to target the endogenous TRIM5 gene. Results showed that all

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90 three gRNAs were competent (Fig. 1B). Because gRNA1 induces a cleavage that is closest to the
91 targeted codons (right before the first nt of Arg332), the rest of the project was carried out with
92 this gRNA. The HDR donor DNA consisted of a single-stranded oligodeoxynucleotide (ssODN)
93 that was 200 nt long and antisense relative to the gRNA, in keeping with published methods [21,22].
94 The central section of this ssODN containing the mutations introduced is shown in Fig. 1C
95 (depicted in the same orientation as the TRIM5 mRNA for clarity purposes). In addition to the
96 mutations substituting arginine residues into glycine at positions 332 and 335, we included 4
97 silent mutations in the region recognized by the gRNA and 1 more silent mutation in the
98 protospacer adjacent motif (PAM), amounting to a total of 7 substitutions expected to prevent the
99 cleavage of the donor DNA by Cas9. One of the mutations also created a HaeIII cut site for
100 convenient downstream screening of the cell clones obtained.

101 **Isolation of *TRIM5*-edited HEK293T clones.** We transiently transfected a plasmid (pX459)
102 expressing Cas9 and the gRNA1 into HEK293T cells, along with the ssODN. Single-cell clones
103 were then isolated by limiting dilution. We screened 161 clones at random for the presence of
104 HDR-modified alleles by specifically amplifying the mutated TRIM5 sequence using a primer
105 whose sequence is indicated in Fig. 1c. As shown in Supplementary Fig. 1, 14 clones showed a
106 positive signal (of varying intensity) in this assay. One of the clones, F2X, yielded a band whose
107 size seemed bigger compared to another positive clone (C8) analyzed on the same gel. All these
108 clones (minus A12, which did not survive) were re-analyzed using the same specific PCR assay
109 and also using a second assay in which the targeted region is amplified and then digested by
110 HaeIII, which cuts at a site created by one of the silent mutations (see Fig. 1c). In the latter assay,
111 amplification was done using primers that bind outside the 200 nts corresponding to the donor
112 ssODN in order to insure that the ssODN was not inadvertently detected. Figure 2 shows a

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113 positive signal for 10 of these clones in both assays. The three remaining clones were negative in
114 both assays.

115 **Genotype analysis.** The 10 clones showing indications of HDR-mediated editing were then
116 subjected to PCR using the same primers that were also utilized in the HaeIII screen described
117 above. PCR products were analyzed by deep sequencing, and a color-coded alignment of the
118 results is shown in Fig. 3. The HEK293 cells and their derivatives are pseudotriploid [23] and
119 accordingly, we found that two clones had two *TRIM5* alleles and seven were triploid. F2X
120 seemed to possess 6 *TRIM5* alleles, a finding that is discussed below. For each clone, both
121 desired mutations at Arg332 and Arg335 were present on one allele or more, with the exception
122 of F2 which only had the Arg332 mutation. However, only one clone (D11) contained an allele
123 with all 8 substitutions present. The HDR-generated alleles in the other cell clones generally
124 contained the expected mutations in the PAM-proximal side of the cleavage site, with the
125 exception of F2 which lacked the A-to-G mutation required to introduce the R335G change. All
126 HDR-generated alleles had the A-to-C silent mutation creating the HaeIII restriction site at the
127 first nucleotide upstream of the cleavage site on the PAM-distal side. This is consistent with the
128 fact that all clones that were found to be positive in the specific PCR screen were also positive in
129 the HaeIII assay (Fig. 2). Strikingly, for the three other substitution mutations in the PAM-distal
130 region, only one clone (D11) had all of them whereas another one (F11) had only one. It would
131 be tempting to conclude that HDR is biased so that mutations were more likely to be
132 incorporated in the PAM-proximal side of the cut, and other teams have reported such
133 imbalances in the conversion rate [24]. On the other hand, our specific PCR screen requires a
134 successful amplification using a primer whose 3’ half is complementary to the PAM-proximal

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135 region (Fig. 1c), thus creating a bias toward the detection of mutated DNA containing the
136 expected mutations in this PAM-proximal region.

137 Some HDR-generated alleles showed additional, unexpected mutations. In E5, one of the two
138 HDR alleles had a one-nt deletion deletion 16 nts from the cleavage site in the PAM-proximal
139 region. In F11, the HDR allele had a 5 nt (TACCA) duplication in the same region. F2X showed
140 an intriguing genotype: firstly, we found that the HDR allele was present in 17 % (1:6) of the
141 amplicons, whereas 33 % (2:6) were wild-type (WT) and 50% (3:6) contained a TT insertion.
142 HEK293 cells are known to be prone to chromosomal translocations leading to a high level of
143 variation in copy numbers [25], which might explain our findings. The HDR-generated allele in
144 F2X had a surprising structure, with a 21 nt duplication consistent with the slower-migrating
145 bands in Fig. 2 and in Supplementary Fig. 1. The repeated sequence that was closest to the
146 cleavage site had the expected mutations in the PAM and at Arg335 and also contained an
147 additional substitution (G-to-A) that is not present in the donor ssODN, whereas the second
148 repeat of this sequence only had the G-to-C mutation in the PAM. These HDR-generated alleles
149 that also contained unexpected insertions/deletions (indels) are unlikely to encode functional
150 TRIM5α, due to the frameshifts leading to premature termination (E5, F11) or due to the
151 insertion of 7 aminoacids at a region crucial for capsid binding (F2X). The rest of the HDR-
152 generated alleles (in C1, C8, C10, D5, D12 and one of the two HDR alleles in E5), however, may
153 potentially encode proteins that efficiently target HIV-1.

154 Examination of the *TRIM5* alleles not modified by HDR in these cell clones revealed that they
155 showed clear signs of NHEJ-induced mutations, i.e. indels at the cleavage site. 8 alleles had an A
156 inserted at the cleavage site, whereas one of the F2X alleles had a TT inserted at the same locus.
157 The A insertion was so prevalent that in half of the clones (C1, C10, D11, F2, F11), 2 of the 3

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158 *TRIM5* copies were mutated by NHEJ leading to this particular mutation. Although the nature of
159 NHEJ-directed mutations is known to vary widely depending on the gRNA used [26], such +1
160 insertions have been described to be prevalent as a result of Cas9 editing [27,28]. One D5 allele had
161 a larger, 27 nt-long deletion whereas one D12 allele had a single deletion at the cleavage site.
162 Therefore, and with the exception of F2X, our data strongly suggest that in all cell clones in
163 which one or two of the *TRIM5* alleles were mutated by HDR, the remaining alleles were
164 mutated by NHEJ. This is consistent with findings published by others [21]. Furthermore, these
165 NHEJ-generated *TRIM5* alleles are all expected to encode non-functional TRIM5α due to
166 missense mutations in the SPRY domain and premature termination.

167 **Lack of HIV-1 restriction activity in the *TRIM5*-edited cells.** We challenged the 10 cell
168 clones with HIV-$1_{NL\text{-}GFP}$, an HIV-1-derived vector that was previously used extensively to study
169 TRIM5α [29]. Cells were infected with several different amounts of the GFP-encoding vector, and
170 the % of GFP-positive cells was calculated by FACS as a measurement of infectivity (Fig. 4a).
171 Compared with the parental HEK293T cells, some of the clones showed a slightly increased
172 permissiveness (up to 3-fold) whereas others were slightly less permissive to the infection (up to
173 2-fold). Because of the expected cell-to-cell variation in susceptibility to retroviral infection,
174 irrespective of TRIM5α, we challenged the same cell populations with other retroviral vectors,
175 derived from the nonhuman primate lentivirus SIVmac239, the equine lentivirus EIAV, as well
176 as the huTRIM5α-sensitive murine oncoretrovirus N-MLV and its huTRIM5α-insensitive
177 counterpart B-MLV. In previous studies, we found that compared to the WT huTRIM5α, the
178 R332G-R335G mutant restricted SIVmac and N-MLV at slightly higher levels, whereas EIAV
179 and B-MLV were not affected [16]. Therefore, if HIV-1 was specifically restricted by a TRIM5α
180 mutant in a given cell clone, we would expect the EIAV and B-MLV vectors to be restriction-

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181 negative controls. However, the infectivity profiles obtained in the various cell populations were
182 similar. In particular, C10 was the cell clone least permissive to HIV-$1_{NL\text{-}GFP}$ (Fig. 4a), but it was
183 also poorly permissive toward infection by the 4 other retroviral vectors (Fig. 4b-e). Likewise,
184 C1 was relatively more susceptible to infection by HIV-$1_{NL\text{-}GFP}$, but these cells were also more
185 permissive to infection with the other 4 retroviral vectors. Of interest, however, was the fact that
186 N-MLV_{GFP} was less infectious in the parental cells compared with most of the cell clones,
187 hinting at slightly higher relative levels of restriction in the parental cells for this virus. In
188 conclusion, the modest variations in HIV-1 infectivity observed between cell clones result from
189 cellular factors other than TRIM5α, and instead are probably due to variations in the expression
190 levels of “positive” factors such as the receptor for the viral vector envelope used here.

191 **Interferon treatment does not induce HIV-1 restriction in *TRIM5*-edited cells.** In the
192 infection experiment described above, we were surprised to find that the N-MLV vector was
193 more infectious than is usually observed in human cells. Indeed, N-MLV is usually restricted
194 ~100-fold in human cells such as HeLa cells [30], whereas the restriction level was closer to 3-fold
195 in our HEK293T cells. It is possible that TRIM5α expression levels are lower in this particular
196 cell line. TRIM5α transcription is stimulated by IFN-I, especially IFN-β [31]. Therefore, we
197 reasoned that IFN-I treatment might reveal a restriction activity against HIV-1 and N-MLV by
198 boosting TRIM5α levels. To test this possibility, we challenged the parental HEK293T cells as
199 well as each of the 10 clones with the HIV-1, SIVmac, N-MLV and B-MLV vectors and in the
200 absence or presence of IFN-α, IFN-β and IFN-ω (Fig. 5). In the parental cells, we observed that
201 treatment with IFN-α and IFN-β, but not IFN-ω, slightly inhibited the infectivity of the HIV-1
202 vector (Fig. 5a) but had a relatively bigger effect (2-fold) on the N-MLV vector (Fig. 5c). IFN-β
203 had a very small inhibitory effect on B-MLV, whereas the two other IFN-I species did not affect

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204 the infectivity of this virus (Fig. 5d). These results are consistent with IFN-α and IFN-β (but not
205 IFN-ω) enhancing TRIM5α expression, hence leading to a specific increase in N-MLV
206 restriction. In the absence of IFN-I, the permissiveness of the cell populations to HIV-$1_{NL\text{-}GFP}$
207 infection varied within a ~4-fold window (Fig. 5a), similar to what we observed in the
208 experiment shown in Fig. 4. IFN-I had little-to-no effect on the infectivity of HIV-$1_{NL\text{-}GFP}$ in the
209 10 cell clones analyzed, implying that the treatment did not induce restriction. Interestingly, N-
210 MLV_{GFP} infectivity was similarly not decreased by IFN-I treatment in the 10 edited cell clones
211 except for D5, and in the presence of IFN-α or IFN-β, N-MLV_{GFP} was more infectious in all of
212 the clones than it was in the treated parental cells. These observations suggest that N-MLV
213 restriction was lost in the cell clones due to the mutations introduced in *TRIM5*.

214 **HEK293T cells are suboptimal for TRIM5α-mediated restriction.** Since we had
215 unexpectedly observed that N-MLV was not efficiently restricted by the WT endogenous *TRIM5*
216 in the HEK293T cells used here, we scrutinized its sequence more closely. A published study
217 showed that *TRIM5* in this cell line carries a SNP resulting in the R136Q mutation [25]. The effects
218 of this mutation on retroviral restriction are unclear, with some authors linking it to an increase
219 in susceptibility toward HIV-1 whereas others find the opposite [32]. The presence of this SNP
220 might explain the weak restriction levels observed in this study. In addition, HEK293T cells
221 might provide an inadequate environment for TRIM5α to restrict retroviruses, irrespective of the
222 R136Q mutation in *TRIM5*. To test this possibility, we stably transduced R332G-R335G
223 huTRIM5α into 293s. In other cell lines tested, transduction of this double mutant leads to a ~20-
224 fold decrease in HIV-1 infectivity [16,17,29]. In HEK293T cells, however, we observed restriction
225 levels that were closer to 3-fold (Supplementary Fig. 2). We conclude that the potential for
226 TRIM5α-mediated restriction is abnormally low in HEK293T cells.

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227 **Discussion**

228 Genome editing has the potential to considerably expand the range of genetic interventions
229 available to treat disease. Although the majority of the applications pursued at present concern
230 monogenic inheritable diseases [33] and cancer [34], chronic infectious diseases may also be
231 addressed by genome editing, especially in cases where pharmacological treatments are
232 nonexistent or noncurative [35]. Regarding HIV-1, many genome editing-based approaches have
233 aimed at knocking out its co-receptor CCR5 using zinc finger nucleases (ZFNs), transcription
234 activator-like effector nucleases (TALENs) or CRISPR-Cas systems [28,36,37]. Although CCR5-
235 targeting is at a more advanced state of development compared with other approaches, it is not
236 without caveats. For instance, the frequent presence of CXCR4 tropism may decrease the
237 usefulness of CCR5 knockout in some patients [38]. In addition, reports have linked the loss of
238 CCR5 function with an increased sensitivity to several serious pathologies [39], suggesting that
239 CCR5 depletion might not be as innocuous as initially thought. Thus, there is impetus to pursue
240 alternative strategies for a genetic intervention targeting HIV-1. The objective of the research
241 program of which this study constitutes the first step is to introduce coding substitutions in
242 *TRIM5* in order to modify its target specificity and enable HIV-1 restriction. Compared with
243 CCR5 knockout, our approach presents advantages and some technical challenges. Among the
244 benefits are the fact that no cellular gene is depleted; in fact, it is expected that R332G-R335G
245 TRIM5α would retain all of its functions, including the restriction of some nonhuman
246 retroviruses [16]. Furthermore, TRIM5α-mediated restriction might provide the added benefit of
247 inducing a cellular antiviral state through the activation of NF-κB and AP-1 [40]. Also, this
248 approach does not include the expression of a foreign protein, and the changes introduced in
249 *TRIM5* are minimal; therefore, immunogenicity should not be an issue.

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250 To our knowledge, this is the first attempt at using genome editing to mutate a restriction factor
251 and confer an innate antiviral function in human cells. Although we did not detect any antiviral
252 activity following successful *TRIM5* editing events, we identified the likely reasons for this, of
253 which the major one was the cellular model used here (HEK293T cells). Another factor likely
254 impeding HIV-1 restriction was the co-presence of indel-containing *TRIM5* alleles in all cell
255 clones in which an allele had been modified by HDR. It is predicted that the SPRY-truncated
256 TRIM5α proteins resulting from the presence of indels will interact with the full-length TRIM5α
257 and will interfere with its targeting of incoming retroviruses, similar to the activity of natural,
258 shorter TRIM5 isoforms [5,41]. Therefore, in addition to increasing the overall percentage of edited
259 cells, future research will need to address the difficulty of achieving bi-allelic HDR-mediated
260 editing. Recent technological advances, including the development of a marker-free system to
261 enrich cells in which HDR occurred [42], are likely to enhance editing efficiency as well as bi-
262 allelic editing. Also affecting the therapeutic potential of *TRIM5* editing in our study was the
263 occurrence of unwanted on-target indels in 3 out of 9 alleles bearing the two therapeutic
264 mutations. Therefore, future studies will also need to identify the determinants of fidelity in HDR
265 repair in order to minimize the incidence of such indels. Despite the absence of an antiviral
266 effect, it is encouraging that we could relatively easily obtain cells in which one or two alleles of
267 *TRIM5* had the desired therapeutic mutations using a simple DNA transfection-based protocol.
268 This study paves the way and identifies the pitfalls toward the goal of efficient, bi-allelic,
269 scarless *TRIM5* editing in order to confer HIV-1 resistance in human cells.

270 Methods

271 **Cells and *TRIM5* genotyping.** HEK293T cells were maintained in Dulbecco's modified Eagle's
272 medium (DMEM; HyClone). All culture media were supplemented with 10% fetal bovine serum

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273 (FBS) and penicillin/streptomycin (HyClone). To analyze the sequence of the targeted genomic
274 region, cellular DNA was prepared using the Bioline genomic DNA kit (London, UK), and the
275 *TRIM5* region encompassing the targeted locus was PCR-amplified using primers
276 T5a_Surveyor_fwd (5'GTCCGACGCTACTGGGGTAAG) and T5a_Surveyor_rev
277 (5'ATAATCACAGAGAGGGGCACA). The PCR product was Sanger-sequenced using the
278 same primers. We found no variation in this region compared to the consensus sequence (NCBI
279 Gene ID: 85363).

280 **Design of gRNAs and Surveyor assay.** The lentiviral expression vector pLentiCRISPRv2
281 (pLCv2) was a gift from Feng Zhang (Addgene plasmid # 52961) [43]. Three gRNAs targeting
282 *TRIM5* were designed using the Zhang lab online software available at crispr.mit.edu. The
283 sequences targeted are 5'AGATAATATATGGGGCACGA (gRNA1),
284 5'CCGAAACCACAGATAATATA (gRNA9) and 5'AATTGAAATTCACAAATGTC
285 (gRNA19). The ODNs needed for the generation of pLCv2-based constructs targeting those
286 sequences were designed exactly as described in published protocols [26,43]. Sense/antisense pairs
287 of primers were annealed and cloned into pLCv2 cut with BsmBI. To evaluate the capacity of the
288 constructed plasmids to induce on-target indels in *TRIM5*, a surveyor nuclease assay was
289 performed. HEK293T cells were transfected with either pLCv2-gRNA1, -gRNA9 or -gRNA19
290 using polyethyleneimine [44]. 3 d later, the genomic DNA was extracted from the transfected cells
291 using the Bioline genomic DNA kit. The targeted *TRIM5* region was PCR-amplified using
292 primers T5a_Surveyor_fwd and T5a_Surveyor_rev. PCR amplicons were heat-denatured at 95
293 °C, and re-annealed by slow cooling to promote formation of dsDNA heteroduplexes. The
294 heteroduplexes were then cleaved by Surveyor nuclease S provided as part of the Transgenomic

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295 Surveyor mutation detection kit (Integrated DNA Technologies, Coralville, IA), according to the
296 manufacturer's instructions. Digestion products were visualized by agarose gel electrophoresis.

297 **Design of the HDR donor DNA and *TRIM5* editing.** The following *TRIM5* minus strand-
298 derived HDR DNA was synthesized by Integrated DNA Technologies:
299 5'CGTCTACCTCCCAGTAATGTTTCCCTGATGTGATACTTTGAGAGCCCAGGATGCCA
300 GTACAATAATTGAAATTCACAAATGTCTGGTATCCTGTGCCGCCGGCCCCGTAGATT
301 ATTTGTGGTTTCGGAGAGCTCACTTGTCTCTTATCTTCAGAAATGACAGCACATGAA
302 ATGTTGTTTGGAGCCACTGTCACATCAACT. Residues mutated compared to the WT
303 *TRIM5* sequence are underlined. pX459-gRNA1 was constructed in a manner similar to pLCv2-
304 gRNA1, by ligating the corresponding annealed gRNA1 ODN duplex into pX459
305 (pSpCas9(BB)-2A-Puro; Addgene #62988) [45] digested with BbsI. HEK293T cells were plated in
306 6-well plates at 2.7 x 10^5 per well and transfected the next day using polyethyleneimine, with 2.5
307 µg of pX459-gRNA1 together with 5 µl of the HDR DNA prepared at 20 µM. When cells
308 reached confluence, they were trypsinized and plated at 0.5 cell per well in 96-well plates, using
309 conditioned medium. To screen the colonies for HDR-mediated *TRIM5* editing, part of the cells
310 were lyzed in the DirectPCR Lysis reagent (Viagen Biotech, Los Angeles, USA) diluted 1:1 in
311 proteinase K-containing water as recommended by the manufacturer. Lysis was allowed to
312 proceed overnight at 55˚C followed by heating at 85˚C for 90 min to deactivate proteinase K.
313 For the specific PCR-based screening, 5 µl of the lysed cells were subjected to PCR using
314 primers T5a_mut_fwd (5'-AAATAATCTACGGGGCCGGCGGCACAG) and T5a_qPCR_rev
315 (5'- CCAGCACATACCCCCAGGAT). PCR was performed for 30 cycles using the following
316 conditions: 30 sec at 94˚C, 30 sec at 61.5˚C, 30 sec at 68˚C. The 157-bp expected PCR product
317 was resolved on agarose gels. For the HaeIII-based screening, lysed cells were subjected to PCR

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318 using primers T5a_Surveyor_fwd and T5a_Surveyor_rev, similar to the Surveyor assay. 10 µl of
319 the PCR product were digested by HaeIII for 60 min at 37˚C. The reaction products were
320 analyzed using agarose gels in order to reveal the 307-bp and 372-bp bands corresponding to
321 digested products. For MiSeq sequencing, cellular DNA was submitted to PCR using the
322 following ODNs, which bind to DNA sequences located 10 nt outside the 200 nt-long region
323 corresponding to the HDR: huTR5aGG_seq_FOR,
324 5'ACACTGACGACATGGTTCTACAATCCCTTAGCTGACCTGTTA, and
325 huTR5aGG_seq_REV,
326 5'TACGGTAGCAGAGACTTGGTCTCCCCCAGGATCCAAGCAGTT. The underlined
327 sequences are barcodes. MiSeq sequencing results were analyzed using the online tool
328 Integrative Genomics Viewer (http://software.broadinstitute.org/software/igv/).

329 **Retroviral vectors production and viral challenges.** To generate the HEK293T cells stably
330 expressing WT and R332G-R335G huTRIM5α, cells were transduced with the corresponding
331 pMIP-huTRIM5α vectors followed by puromycin selection as described previously [16]. To
332 produce GFP-expressing retroviral vectors, HEK293T cells were seeded in 10 cm culture dishes
333 and transiently co-transfected with the following plasmids: pMD-G, pCNCG and pCIG3-B or
334 pCIG3-N to produce B-MLV$_{GFP}$ and N-MLV$_{GFP}$, respectively; pMD-G and pHIV-1$_{NL\text{-}GFP}$ to
335 produce HIV-1$_{NL\text{-}GFP}$; pMD-G and pSIV$_{mac239\text{-}GFP}$ to produce SIV$_{mac\text{-}GFP}$; or pONY3.1, pONY8.0
336 and pMD-G to produce EIAV$_{GFP}$ (see [46,47] and references therein). For retroviral challenges, cells
337 were seeded into 96-well plates at 10,000 cells per well and infected the following day with
338 multiple doses of the GFP-expressing retroviral vectors. Cells were trypsinized at 2 d post-
339 infection and fixed in 2.5% formaldehyde (Fisher Scientific, MA, USA). The percentage of GFP-
340 positive cells was then determined by analyzing 1×10^4 cells on a FC500 MPL cytometer

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341 (Beckman Coulter, CA, USA) using the CXP Software (Beckman Coulter). For infections done
342 in presence of IFN-I, recombinant human IFN-α, IFN-β or IFN-ω (PeproTech, Rocky Hill, NJ)
343 was added to cell cultures 16 h prior to infection and at a final concentration of 10 ng/ml.

344

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352

353 **Author contributions**

354 CD and LB designed the study, interpreted results, prepared figures and wrote the main
355 manuscript. CD, AC, NJ, TM and MBP performed experiments. AC, NM and MBP helped
356 design specific portions of the study. All authors reviewed the manuscript.

357

358 **Competing financial interests**

359 The authors declare no competing financial interests.

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361 **Availability of materials and data**

362 The datasets generated during the current study are available in the FigShare repository,

363 https://figshare.com/projects/Dufour_et_al_2017/24445. Biological materials are available for

364 sharing.

365

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485

486

487 FIGURE LEGENDS

488 **Fig. 1. Design of the gRNA and donor ssODN for the HDR-mediated editing of *TRIM5*.** (**a**)
489 *TRIM5* localization on chromosome 11 (top), and Arg332-Arg335 localization in exon 8 of the
490 gene (bottom). (**b**) Top panel: position of the three gRNAs (gRNA1, 9 and 19) designed to target
491 the Arg332-Arg335 region. The two arginine codons are underlined and in bold. Bottom panel:
492 Surveyor assay following the transfection of HEK293T cells with CRISPR-Cas9 plasmids
493 expressing one of the three gRNAs. WT DNA from nontransfected cells was used as a control.
494 (**c**) HDR donor DNA mutagenesis strategy. 8 substitutions were present, including three

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495 nonsilent substitutions to mutate Arg332 and Arg335 into Gly (green), one silent mutation to
496 disrupt the PAM sequence (pink), and four silent mutations in the sequence targeted by gRNA1
497 (orange). The HaeIII restriction site created as a result of one of the silent substitutions is
498 indicated, as is the position of the primer used in specific PCR screening.

499

500 **Fig. 2. Identification of HDR-edited clones.** Following the isolation of HEK293T clones,
501 HDR-edited clones were identified by a dual PCR screen. The figure shows the analysis of 13
502 clones that passed a pre-screen step (see Supplementary Information). (**a**) PCR using a primer
503 specific for the mutated *TRIM5*. Untransfected HEK293T cells were used as a control. M,
504 molecular weight marker. (**b**) Non-specific PCR of the targeted region followed by HaeIII
505 digestion. The expected sizes of the digested PCR products are shown on the right.

506

507 **Fig. 3. Deep sequencing analysis of *TRIM5* editing in 10 screened clones.** The ~200-nt HDR-
508 targeted *TRIM5* region was amplified by PCR and the PCR products were then analyzed by
509 Illumina MiSeq sequencing. The alignment shown includes the targeted locus for each allele of
510 the 10 clones, in comparison with the WT sequence and with the expected HDR-mutated
511 sequence (top 2 lines). Expected substitutions are shown in white. The star indicates the position
512 of an unexpected substitution within a duplicated region in one allele of F2X. The color code for
513 duplications/insertions and for deletions is explained at the bottom of the alignments. The Cas9
514 cleavage site on the WT sequence is shown at the top. On the left is a table summarizing the
515 results obtained for each clone: presence of the two therapeutic mutations R332G/R335G,
516 proportion of *TRIM5* alleles modified by HDR and the proportion of the expected substitution

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517 mutations in the HDR-edited alleles. Note that only one clone (D11) has an allele containing all
518 the desired mutations and that most of the non-HDR-edited alleles contain indels at the cleavage
519 site.

520

521 **Fig. 4. Retrovirus restriction ability of HDR-edited clones.** The 10 HDR-edited clones were
522 challenged with increasing doses of GFP-expressing retroviral vectors based on HIV-1_{NL4-3} (**a**),
523 SIV_{mac239} (**b**), N-MLV (**c**), B-MLV (**d**) or EIAV (**e**). Non-transfected HEK293T cells were used
524 as a control. Infected cells were quantified by FACS for GFP expression.

525

526 **Fig. 5. Retrovirus restriction ability following IFN-I treatment.** HDR-edited clones and the
527 non-transfected WT control cells were treated with IFN-α, IFN-β or IFN-ω for 16h prior to a
528 single-dose infection with HIV-1_{NL-GFP} (**a**), $SIV_{mac-GFP}$ (**b**), N-MLV_{GFP} (**c**) and B-MLV_{GFP} (**d**).
529 The percentage of infected cells was determined by FACS.

530

531

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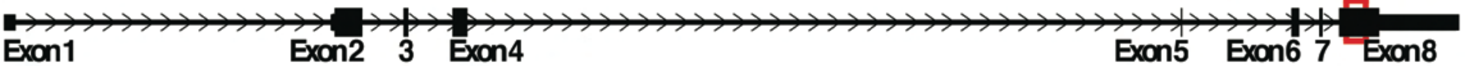
a

***TRIM5* genomic position**

(minus strand)



b

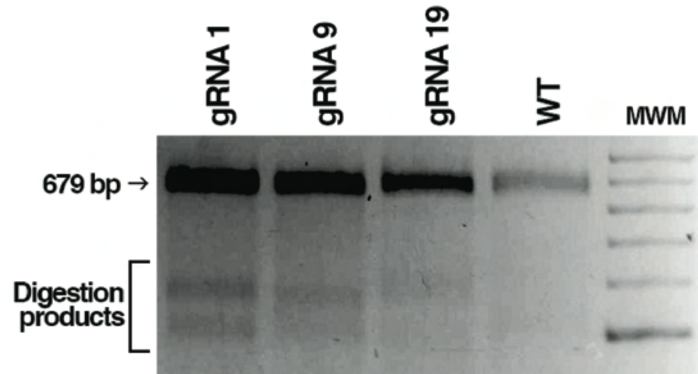
gRNA1: 5'-**AGATAATATATGGGGCACGA**-3'

gRNA9: 5'-**CCGAAACCACAGATAATATA**-3'

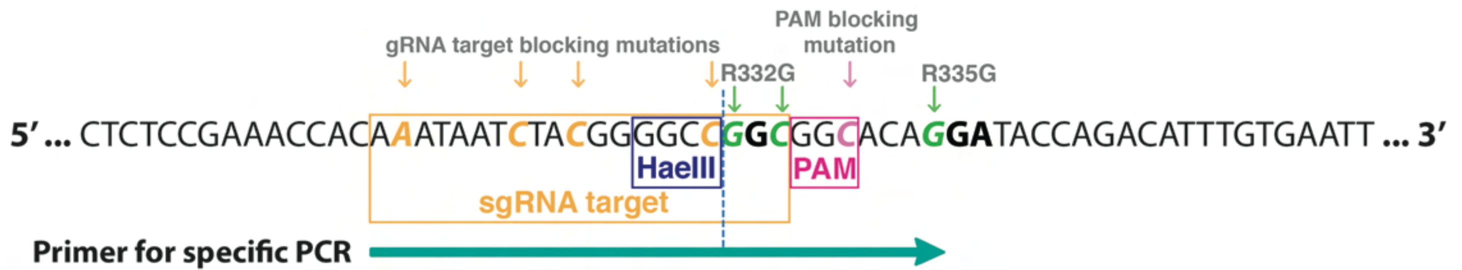
5' ... CTCTCCGAAACCACAGATAATATATGGGGCA**CGA**GGGACA**AGA**TACCAGACATTTGTGAATTTCAATTAT ... 3'

3' ... GAGAGGCTTTGGTGTCTATTATATACCCCGT**GCT**CCCTGT**TCT**ATGGTCTGTAAACACTTAAAGTTAATA ... 5'

3'-**CTGTAAACACTTAAAGTTAA**-5' : gRNA19



c



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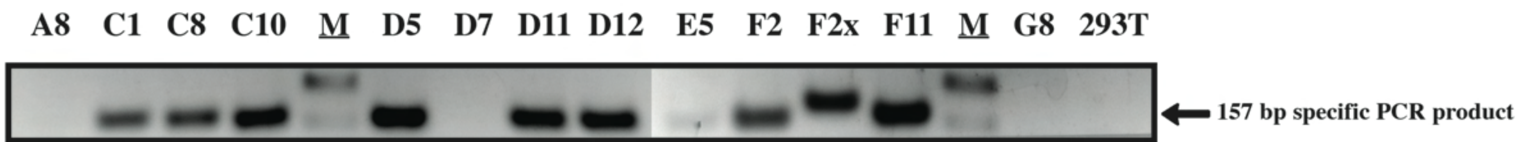
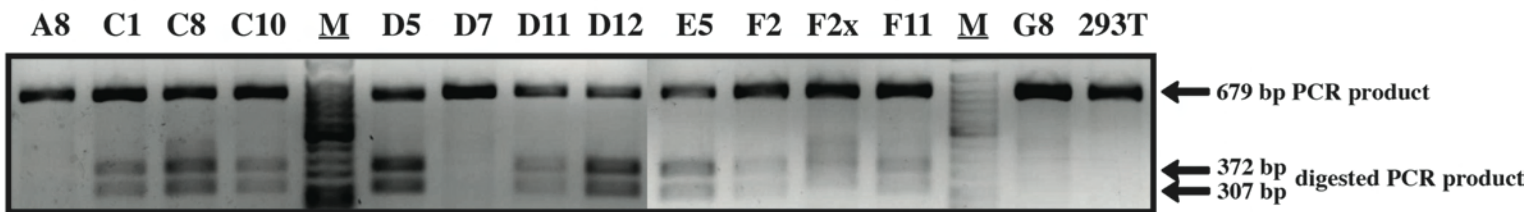
a



b



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WT 5' AAACCACAGATAATATATGGGGCA...CGAGGGACAAGATACCAG 3'

Donor 5' AAACCACAAATAATCTACGGGGCC...GGCGGCACAGGATACCAG 3'

	R332G-R335G	HDR-mutated alleles	N mutations	Sequences
C1	✔	1/3	5/8	AAACCACAGATAATATATGGGGCC...GGCGGCACAGGATACCAG AAACCACAGATAATATATGGGGCAACGAGGGACAAGATACCAG
C8	✔	1/2	5/8	AAACCACAGATAATATATGGGGCC...GGCGGCACAGGATACCAG AAACCACAGATAATATATGGGGCAACGAGGGACAAGATACCAG
C10	✔	1/3	5/8	AAACCACAGATAATATATGGGGCC...GGCGGCACAGGATACCAG AAACCACAGATAATATATGGGGCAACGAGGGACAAGATACCAG
D5	✔	1/2	5/8	AAACCACAGATAATATATGGGGCC...GGCGGCACAGGATACCAG A...GGGACAAGATACCAG
D11	✔	1/3	8/8	AAACCACAAATAATCTACGGGGCC...GGCGGCACAGGATACCAG AAACCACAGATAATATATGGGGCAACGAGGGACAAGATACCAG
D12	✔	1/3	5/8	AAACCACAGATAATATATGGGGCC...GGCGGCACAGGATACCAG AAACCACAGATAATATATGGGGCAACGAGGGACAAGATACCAG AAACCACAGATAATATATGGGGC...CGAGGGACAAGATACCAG
E5	✔	2/3	5/8	AAACCACAGATAATATATGGGGCC...GGCGGCACAGGATACCAG AAACCACAGATAATATATGGGGCC...GGCGGCACAGGATACAG AAACCACAGATAATATATGGGGCAACGAGGGACAAGATACCAG
F2	R332G	1/3	4/8	AAACCACAGATAATATATGGGGCC...GGCGGCACAAGATACCAG AAACCACAGATAATATATGGGGCAACGAGGGACAAGATACCAG
F2x	✔	1/6	5/8	AAACCACAGATAATATATGGGGCC...GGCGGCACAGGATACCAAACATTTGTCACAAGATACCAG AAACCACAGATAATATATGGGGCTTACGAGGGACAAGATACCAG AAACCACAGATAATATATGGGGCA...CGAGGGACAAGATACCAG
F11	✔	1/3	6/8	AAACCACAGATAATCTATGGGGCC...GGCGGCACAGGATACCATACCAG AAACCACAGATAATATATGGGGCAACGAGGGACAAGATACCAG

= deletion
GATC = duplication

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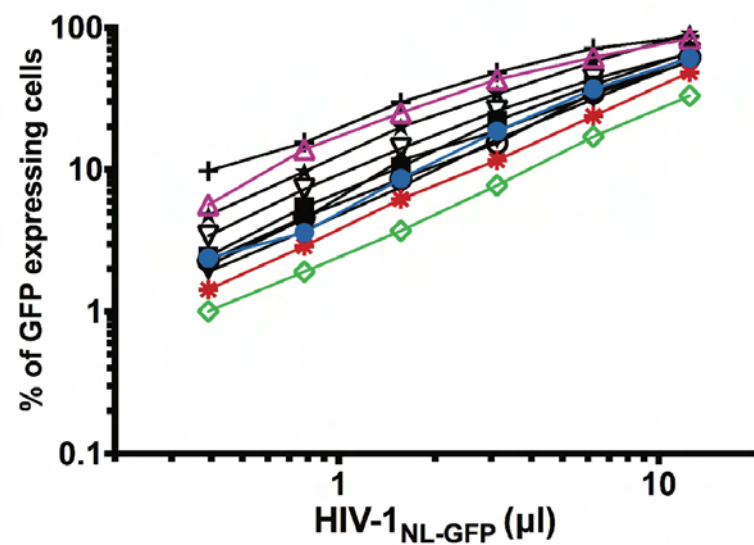
a



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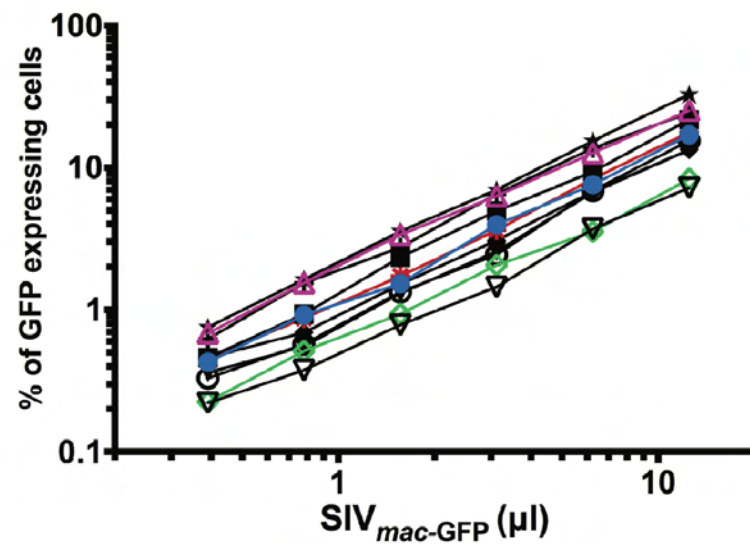

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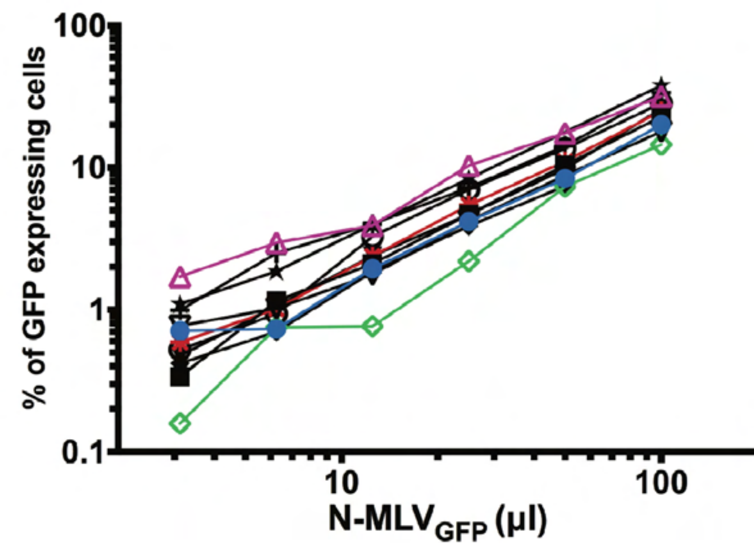
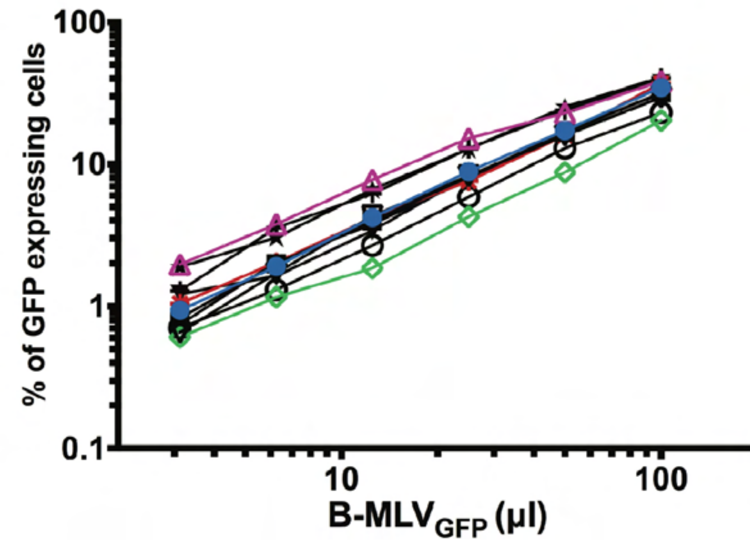
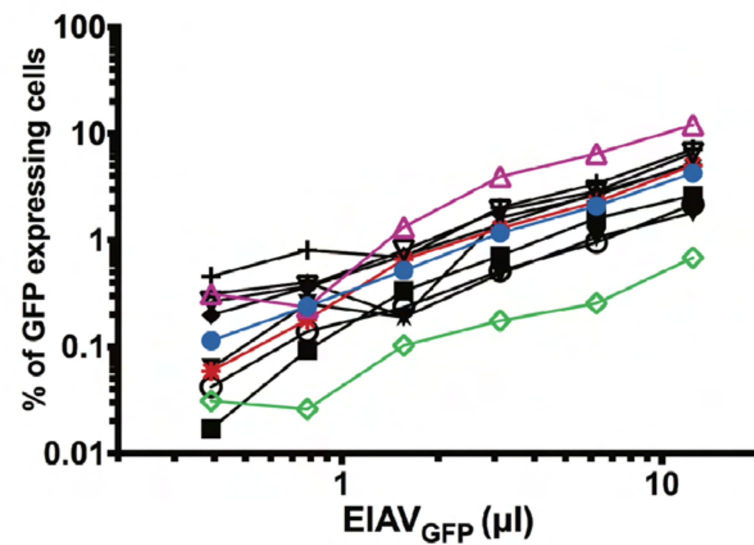


d



e



293T
C1
C8
C10
D5
D11
D12
E5
F2
F2x
F11

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2 **Editing of the Human TRIM5 Gene to Introduce HIV-1 Restrictive**

3 **Mutations Using CRISPR-Cas9**

4

5 SUPPLEMENTARY INFORMATION

6

7 **Suppl. Fig. 1. Pre-screening of 161 isolated clones by specific PCR.** Following 20 days of
8 growth, 161 isolated HEK293T clones were screened for HDR-edited TRIM5 gene by mutation-
9 specific PCR. 14 clones that passed this pre-screen step are indicated by their names. MWM,
10 molecular weight marker.

11

12 **Suppl. Fig. 2.** Levels of HIV-1 restriction in HEK293T cells transduced with R332G-R335G
13 huTRIM5α. HEK293T cells were retrovirally transduced with WT huTRIM5α, R332G-R335G
14 huTRIM5α or with the "empty" vector as indicated. Untransduced cells were eliminated, and the
15 cell populations were then challenged with increasing amounts of the HIV-$1_{NL\text{-}GFP}$ vector. The
16 percentage of cells expressing GFP was then determined by FACS.

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MWM A12 C1
200 bp →
100 bp →
MWM D7
MWM C10 MWM D12
MWM E5 F2 MWM
MWM
MWM D11 F11
MWM D5 MWM
200 bp →
150 bp →
100 bp →
MWM MWM G8
MWM A8 MWM
MWM MWM
MWM
MWM
MWM C8 MWM F2x

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a

empty vector
huTRIM5α
R332G-R335G

% of GFP expressing cells

100
10
1
0.1
0.01

0.1
1
10

HIV-1$_{NL-GFP}$ (µl)