

Hili inhibits HIV replication in activated T cells

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23 **Abstract**

24 Piwil proteins restrict the replication of mobile genetic elements in the germline. They
25 are also expressed in many transformed cell lines. In this report, we discovered that the
26 human piwil 2 (hili) can also inhibit HIV replication, especially in activated CD4+ T cells
27 that are the preferred target cells for this virus in the infected host. Although resting cells
28 did not express hili, it was rapidly induced following T cell activation. In these cells and
29 transformed cell lines, depletion of hili increased levels of viral proteins and new viral
30 particles. Further studies revealed that hili binds to tRNA. Some of them represent rare
31 tRNA species, whose codons are over-represented in the viral genome. Targeting
32 tRNA^{Arg}(UCU) with an antisense oligonucleotide replicated effects of hili and also
33 inhibited HIV replication. Finally, hili also inhibited the retrotransposition of the
34 endogenous intracisternal A particle (IAP) by a similar mechanism. Thus, hili joins a list
35 of host proteins that inhibit the replication of HIV and other mobile genetic elements.

36

37 **Importance**

38 Piwil proteins inhibit the movement of mobile genetic elements in the germline. In their
39 absence, sperm does not form and male mice are sterile. This inhibition is thought to
40 occur via small piRNAs. However, in some species and in human somatic cells, piwil
41 proteins bind primarily to tRNA. In this report, we demonstrate that human piwil proteins,
42 especially hili, not only bind to select tRNA species that include rare tRNAs, but also
43 inhibit HIV replication. Importantly, T cell activation induces the expression of hili in
44 CD4+ T cells. Since hili also inhibited the movement of an endogenous retrovirus (IAP),

45 our finding shed new light on this intracellular resistance to exogenous and endogenous
46 retroviruses as well as other mobile genetic elements.

47

48 **Introduction**

49 The human immunodeficiency virus (HIV) causes the acquired immunodeficiency
50 syndrome (AIDS) (1). It is a lentivirus that originated in primates and jumped to humans.
51 HIV replicates best in activated CD4+ T cells and macrophages. They contain the
52 necessary transcription factors and cellular machineries for the entry, uncoating,
53 integration, transcription, RNA export, translation and assembly of new viral particles
54 (1). However, the virus still has to overcome various restriction factors, such as
55 SamHD1, TRIM5, APOBEC3F/3G, Mx2, Schlafen 11, Tetherin/Bst2 and Serinc3/5 for a
56 successful passage through an infected cell (2-7). Some of these play less important
57 roles in activated CD4+ T cells, and for others, HIV-encoded accessory and regulatory
58 proteins mitigate their effects. Interestingly, HIV has little defense against two interferon
59 stimulated genes (ISGs) Mx2 and Schlafen 11 (2, 6). Moreover, Schlafen 11, which can
60 inhibit translation of viral transcripts, has been implicated in long term suppression of
61 HIV replication in elite controllers (2).

62

63 Another group of host proteins can inhibit the movement of mobile genetic elements,
64 such as endogenous retroviruses and retrotransposons, in the germ line (8-10). They
65 are called P-element induced wimpy like (piwil) proteins (11). As members of the
66 argonaute family of proteins they bind to small piwi-interacting (pi) RNAs, which are
67 slightly larger than miRNAs (12). piRNAs contain sequences that are complimentary to

68 and thus restrict the movement of mobile genetic elements in the germ line (8). In their
69 absence, spermatogenesis is inhibited and male mice are sterile. In humans, there are
70 4 piwil proteins, and they are called hiwi, hili, piwil 3 and hiwi 2 (10, 13). Some of them
71 are also expressed in many tumors, especially those of the reproductive system, e.g.
72 seminomas (14, 15). They are thought to increase malignant transformation by inhibiting
73 p53, which is a tumor suppressor (16). Of interest, in somatic cells, rather than binding
74 to piRNAs, which contain retroviral or retrotransposon sequences, piwil proteins bind to
75 tRNAs (17-19).

76

77 In this report, we discovered that piwil proteins also inhibit the replication of HIV. For
78 example, hili and mili (the mouse piwil 2 protein), decreased levels of viral proteins in
79 cells. They also inhibited the expression of the native green fluorescent protein (GFP)
80 but not of its codon-optimized counterpart (EGFP). We also noted the increased
81 expression of hili upon T cell activation. In all of these scenarios, genetic inactivation of
82 hili increased levels of HIV replication. Since amounts of viral transcripts did not change,
83 we suspected that hili inhibited their translation. Indeed, hili bound to tRNAs. Of these,
84 hili favored rare tRNA^{Arg}(UCU) and tRNA^{Ile}(UAU), whose codons are found abundantly
85 in GFP and HIV transcripts. Importantly, removing the rare tRNA^{Arg}(UCU) via antisense
86 oligonucleotides recapitulated effects of hili. These findings were duplicated with an
87 endogenous retrovirus (IAP). Thus, hili, like Schlafen 11, inhibits HIV replication by
88 interfering with the translation of viral transcripts. Since many of these suboptimal
89 codons are found in areas of RNA secondary structure, HIV cannot easily escape this
90 inhibition.

91 **Materials and Methods**

92 ***Cell culture, plasmids, antibodies and siRNA.***

93 Human embryonic kidney 293 transformed with large T antigen (293T) cells and HeLa
94 cells were maintained at 37 °C with 5% CO₂ in Dulbecco's Modification of Eagle's
95 medium (DMEM) containing 10% fetal bovine serum (FBS) and 100 mM L-glutamine.
96 Jurkat cells were maintained at 37 °C with 5% CO₂ in Roswell Park Memorial Institute
97 (RPMI) medium 1640 containing 10% FBS and 100 mM L-glutamine. 293T cells stably
98 expressing mouse mlii.EGFP fusion protein were a kind gift from Drs. Dubravka Pezic
99 and Alexei Aravin (California Institute of Technology). Trima residuals from healthy
100 donors, from Trima apheresis collection and enriched for PBMC, were obtained from
101 Blood Center of the Pacific (San Francisco, CA). PBMCs were then plated at 5×10⁶
102 cells/ml in 24-well plates, using RPMI 10% human serum AB. After 30 min, non-
103 adherent cells (PBLs) were isolated and cultured in complete RPMI (containing 10%
104 FBS, 100 mM L-glutamine, and 100 µg/ml streptomycin) with 30 units/ml IL-2 (Roche
105 Applied Science). After isolation, PBLs (10⁷) were activated with 5 µg/ml PHA and 30
106 units/ml for 2 days. CD4⁺ T cells were selected from bulk PBMC using negative bead
107 selection (DynaL CD4⁺ untouched beads, Invitrogen). CD4⁺ T cells were maintained for
108 24 hours in RPMI 1640 medium, 10 % FBS, 30 units/ml IL-2 at 37 °C with 5% CO₂
109 before activation and expansion using anti-CD3/anti-CD28 Dynabeads (Invitrogen).

110 The human hili gene was cloned downstream of oligonucleotides corresponding to the
111 flag epitope-tagged peptide, or upstream of an red fluorescent protein (RFP) gene into
112 pcDNA 3.1 plasmid vector. pNL-GFP RRE(SA) and pNL4-3-ΔE-EGFP were a kind gift
113 from Drs. Manqing Li and Michael David (UCSD). Ago2-EGFP plasmid was obtained

114 from Addgene. Reporter pNL4-3.luciferase (Luc) plasmid was obtained from NIH AIDS
115 Reagent Program. pFL was obtained from Kyoji Horie and has been described
116 previously (20). Anti-piwil 2 antibody (H00055124-B03P) was purchased from Abnova.
117 Anti-GFP antibody (A11122) and anti-tubulin (32-2500) were purchased from Life
118 Technologies. Anti-HIV Gag p24 (MAB7360) and anti-HIV Gag p55 (RK-65-014) were
119 purchased from R&D systems and MBL International Corporation, respectively. Anti
120 IAP Gag antibodies were a kind gift for Bryan Cullen (21). Small interfering RNA
121 (siRNA) against hili (4390771) and control siRNA (AM4635) were purchased from
122 Ambion.

123 ***Luciferase and immunofluorescence assays.***

124 293T cells growing in log phase were transfected with the reporter pNL4-3.Luc plasmids
125 with X-tremeGENE HP DNA Transfection Reagent (Roche). Luciferase activity in the
126 cell lysate was determined 24 hours later using the luciferase assay system (Promega)
127 according to the manufacturer's instructions. Data were normalized with the protein
128 concentrations of the cell lysates. Fluorescent microscopic analyses on pFL-expressing
129 cells were performed as described (22).

130 ***Viral Infectivity assays***

131 293T cells or 293T stably expressing mili.EGFP (293T.mili) were transfected with the
132 reporter pNL4-3.Luc plasmids (1 μ g) and the empty vector (C) (1 μ g) or the plasmid
133 encoding Flag.hili (1 μ g) with 6 μ l X-tremeGENE HP DMA Transfection Reagent. 24
134 hours after transfection, the culture supernatants were collected. The amounts of HIV
135 viral particles produced from the cells were determined by Gag p24 ELISA(Cell

136 Technologies). The same volume of culture supernatants to TZM-bl cells containing Tat-
137 driven luciferase reporter gene and CD4, and luciferase activities were measured 24
138 hours after the addition of supernatants to determine the amount of infectious viruses.
139 Relative infectivity of HIV produced from cells with or without ectopical expression of hili
140 or mili was determined by calculating the ratio between the luciferase activity and total
141 viral production.

142 ***RNA-IP***

143 293T.mili cells were UV-irradiated (400 J/m^2) to crosslink protein and RNAs. Cells were
144 lysed in RIPA buffer (25mM Tris-HCl pH 7.6, 150mM NaCl, 1% NP-40, 1% sodium
145 deoxycholate, 0.1% SDS) containing protease and RNase inhibitors. Cell lysates were
146 incubated with anti-piwil 2, anti-GFP or normal rabbit IgG pre-coupled with protein A-
147 conjugated dynalbeads (Life Technologies). After extensively washing the beads with
148 RIPA buffer, RNA bounds to the beads were isolated by incubating with 20 μg
149 Proteinase K (New England Biolabs) in the presence of RNase inhibitors for 1 hour at
150 55 °C followed by phenol/chloroform extraction and ethanol precipitation. The purified
151 RNAs were separated in 15% TBE-Urea gel and visualized with Sybr-Gold reagent (Life
152 Technologies). Yeast tRNA and RNA ladder (Biooscientific) were used as size-markers.

153 ***Quantitative reverse-transcriptase PCR (RT-qPCR) analysis***

154 Total RNA was extracted using TRIZOL reagent (Invitrogen) and treated with Turbo
155 DNase (Ambion), and reverse-transcribed using the Superscript III First Strand System
156 (Invitrogen) and random hexamers to produce cDNA. Real-time PCR was performed
157 using the Stratagene Mx3004P real-time PCR system and SensiFAST SYBR Green

158 reagents (Bioline) following the manufacturer's protocol. Results were normalized to the
159 levels of GAPDH. Control reaction mixtures lacking reverse transcriptase (RT-minus)
160 were routinely incorporated and indicated at least 10-fold lower signal in all
161 experiments. The primer sequences used in this study were as follows: hili primers
162 (Forward: 5'-TTGTCTGCTAATCTGGTACGC-3'; Reverse: 5'-
163 CATCTGAACTCCCTCTTCCAAG-3'), GFP primers (Forward: 5'-
164 CTGGAGTTGTCCCAATTCTTG-3'; Reverse: 5'-TCACCCTCTCCACTGACAGA-3'),
165 EGFP primers (Forward: 5'-CAGCAGAACACCCCATC-3'; Reverse: 5'-
166 TGGGTGCTCAGGTAGTGGTT-3'), GAPDH primers (Forward: 5'-
167 CCTGTCCTGTGTGCTGTAAT-3'; Reverse: 5'-GAGAATGTGTATTGGCCTGGA-3') and
168 IAP-gag (F, 5'- ACC CAG GAA GCA GTC AGA GA-3', R, 5'- CCT TTA GGG CTT GAG
169 CAC AG-3'.

170 ***Genetic inactivation of hili***

171 For siRNA knockdown. 293T, HeLa or Jurkat cells were transfected with siRNA against
172 hili or control siRNA (40nM) with Lipofectamine RNAiMax (Invitrogen). 24 hours after
173 transfection, the reporter pNL4-3 Luc plasmid, was transfected with X-tremeGENE HP
174 DNA Transfection Reagent. 24 hours after transfection of the reporter plasmid, culture
175 supernatants were collected and the viral production was quantified by Gag p24 ELISA.
176 RNA or protein expression of hili was measured by RT-qPCR or western blot analysis,
177 respectively.

178 ***UV crosslinking-immunoprecipitation followed by tRNA microarray (Clip-chip)***

179 The tRNA species interacted specifically with hili were determined by Clip-chip
180 experiments as previously described (23). 293T.mili cells were UV-irradiated with 400
181 mJ/cm² at 254 nm. Anti-piwil 2 antibodies were used to immunoprecipitate associated
182 RNA species. RNA species were subjected to tRNA microarray as described before(24-
183 26). Briefly, immunoprecipitated RNAs were released from Protein A beads and the
184 RNAs were recovered by ethanol precipitation. The RNA was 3'-end-³²P-labeled using
185 [5'-³²P]pCp and T4 RNA ligase. The ³²P-labeled mixture was directly analyzed on 10%
186 denaturing PAGE using purified ³²P-labeled yeast tRNA^{Phe} as size control. To analyze
187 the ³²P-labeled RNA by tRNA microarray, the corresponding tRNA sized bands were cut
188 out of the gel and eluted with crush and soak buffer (50 mM KOAc/200 mM KCl, pH 7.0)
189 at 4°C overnight. The eluted RNA was recovered by ethanol precipitation and dissolved
190 in water. tRNA microarray preparation, hybridization and data analysis were performed
191 according to methods described previously (23, 24).

192

193 ***tRNA Sequencing and analysis***

194 Total RNA was extracted from 293T cells and was used to construct transcriptome
195 library using NEBNext Small RNA Library Prep Set for Illumina (Multiplex Compatible)
196 (Catlog No.: E7330S, New England Biolabs).The constructed library was analyzed for
197 small RNA using HiSeq 2000 next generation platform at BGI-Tech. The sequencing
198 read length of the library is 100 bp paired end. Raw FASTQ reads were mapped to
199 human reference genome with *tophat* (v2.0.10). The parameters were set as following:
200 tophat -p 16 --solexa1.3-quals --segment-length 30 --segment-mismatches 2 -r 20 --
201 mate-std-dev 20 --library-type fr-unstranded -o outdir ref.fa in.fq.gz. The mapping rate

202 of clean data is 91.0%. The results were stored in bam format. *cufflinks* (v2.0.2) was
203 used to calculate the refseq gene expression. The parameters were set as following: -u
204 -p 6 -G tRNA.gtf -b hg19.fasta -o outdir sample.bam. The tRNA FPKM results were
205 stored in FPKM Tracking Format. *R software*(R-2.15.3) was used to do tRNA content
206 analysis. Reads mapped to the interested tRNA regions were calculated as percentage
207 of total uniquely mapped reads.

208

209 ***Antisense tRNA assays.***

210 293T cells were grown in 24 well culture plates for 24 hours prior to transfection. Cells
211 were then transfected with the reporter pNL4-3.Luc plasmid (0.5 µg) and 4 µg of
212 antisense oligonucleotide DNA corresponding to the anti-codon region of tRNA^{Arg} (UCU)
213 (TAGAAGTCCAATGCGCTATCCATTGCG) or randomized negative control
214 (AGTCTTATGCTTGCCAGCAGGCTAAA). 24 hours after transfection, virus
215 production in culture supernatants was quantified by Gag p24 ELISA, and luciferase
216 activity in cell lysates were measured as described previously (27). Gag p24 levels were
217 normalized by luciferase activities. Relative targeted tRNA levels after antisense
218 transfection was measured by RTqPCR analysis using specific primers (forward: 5'-
219 TAGAAGTCCAATGCGCTATCCATTGCG; reverse: 5'-
220 TGGGATTCGAACCCACAACCTCTGGAT), and cell viability was measured by counting
221 live cells by trypan blue exclusion.

222

223 ***Reverse transcription assay***

224 Reverse transcription assay was performed as described (22).

225 **Statistical analysis.**

226 For p24 ELISA, luciferase enzymatic assays, GFP assays, microscopic analyses and
227 RT-qPCR measurements, 3 independent experiments were performed in duplicate.
228 Thus, bars represent SEM, n=3. A Student's *t*-test was performed to measure the
229 significance of the data (* P<0.05, ** P<0.01, *** P<0.001).

230

231 **Results**

232 **Piwil proteins inhibit HIV replication**

233 Piwil proteins are related to each other within the species and between species (10, 28).
234 For example, mouse and human piwil 2 proteins (mili and hili) are 88% identical in
235 sequence (Fig. 1A). Because piwil proteins are known to inhibit the movement of mobile
236 genetic elements, we investigated if they could also affect HIV (HIV-1_{NL4-3} or pNL4-3.
237 Fig. 1A). To this end, we co-expressed hili and HIV in 293T cells (Fig. 1B, bars 1 and 2).
238 At the same time, we expressed HIV by transfecting pNL4-3 in 293T cells, which stably
239 contained mili (293T.mili, Fig. 1B, bar 3). Supernatants were harvested two days after
240 the transfection. Levels of new viral particles were determined by Gag p24 ELISA. Of
241 note, in the presence of hili or mili, 5- or 10-fold reduced levels of new viral particles
242 were observed from these cells, respectively. Levels of hili and mili were determined
243 with appropriate antibodies by western blotting, where tubulin served as the loading
244 control (Fig. 1B, lower panels, lanes 1 to 3).

245

246 To determine if these piwil proteins affected the infectivity of new viral particles, we used
247 equivalent amounts of viruses from these cells and infected fresh TZM-bl indicator cells.

248 TZM-bl cells express necessary HIV receptors and contain the HIV LTR linked to
249 luciferase and LacZ reporter genes. As presented in Fig. 1C, new viral particles from
250 293T cells transfected with an empty vector (C) and those expressing hili or mili, were
251 equally infectious (Fig. 1C, bars 1 to 3). Thus, these piwil proteins did not affect the
252 infectivity of new viral particles.

253

254 Next, we wanted to know if hili affected levels of viral transcripts or proteins. To this end,
255 we co-expressed hili and the pNL4-3.Luc provirus, which contains the luciferase
256 reporter gene in place of nef, in these cells. We interrogated HIV genomic RNA species
257 using gag-specific primers and RT-qPCR. No differences in levels of HIV genomic
258 transcripts were observed between WT (C) and hili-expressing 293T cells (Fig. 1D, bars
259 1 and 2). In contrast, we found 5-fold lower levels of expression of Gag proteins in hili-
260 expressing cells (Fig. 1E, lanes 1 and 2). This finding was true for the p55 Gag
261 precursor as well as for its p24 (capsid, CA) mature product (Fig. 1E. top panel, lanes 1
262 and 2). The expression of hili was monitored by western blotting, where tubulin again
263 served as the internal control (Fig. 1E, bottom panels, lanes 1 and 2). Finally, despite
264 differences in levels of viral proteins, the luciferase activity was equivalent between
265 these two cells (Fig. 1F, bars 1 and 2). Importantly, this luciferase reporter gene has
266 been optimized for expression in human cells by substituting rare codons for abundant
267 ones found in humans. From these studies, we conclude that hili and mili inhibit HIV
268 replication at the step of translation and that the translation of viral but not codon-
269 optimized transcripts is affected.

270

271 **Hili inhibits the expression of GFP but not codon-optimized EGFP**

272 Since Schlafen 11 also inhibits primarily the translation of HIV transcripts, we next
273 examined the ability of hili to affect additional transcripts from other species that were
274 not codon-optimized. For example, the green fluorescent protein (GFP) from the firefly
275 contains several rare human codons such as Ile-AUA or Arg-AGA (Fig. 2A). They were
276 changed in its humanized version (EGFP). To determine if hili affected the translation of
277 GFP but not EGFP, we co-expressed hili and these GFP transcripts in 293T cells. Two
278 days later, western blotting revealed that hili affected the expression of GFP but not
279 EGFP in these cells (Fig. 2B and C, top panels, lanes 1 to 4). Densitometric analyses of
280 these bands by LiCor are presented in the top bar graphs (Fig. 2B and C, bars 1 to 4).
281 Although 5-fold lower expression of GFP was observed in the presence of hili, levels of
282 GFP and EGFP transcripts remained the same (Fig. 2B and C, lower bar graphs, bars 1
283 to 4). The expression of hili was followed by western blotting, where tubulin again
284 served as the internal control (Fig. 2B and C, lower two western blots, lanes 1 to 4). We
285 conclude that hili affects the expression of only the codon non-optimized version of
286 GFP.

287

288 **Cell activation induces the expression of hili in PBMCs and CD4+ T cells**

289 Thus far, all our studies were carried out in transformed 293T cells, which represent
290 human embryonic kidney cells. Additionally, they express the large T antigen of SV40.
291 To determine if hili plays any role in HIV replication in other transformed indicator and
292 hematopoietic cells as well as primary cells that are the natural hosts of HIV, we
293 examined HeLa, Jurkat, PBMCs and CD4+ T cells. Of these HeLa are the parental

294 TZM-bl cells, Jurkat represent an infectable immature T cell line, and activated PBMCs
295 and CD4+ T cells are the primary target cells in humans. Others already determined
296 that HeLa and Jurkat cells, unlike 293T cells, express hili (29). Thus, we first
297 interrogated if depletion of hili in these cells increases levels of HIV replication. As
298 presented in Fig. 3A and B, RNAi to hili with short interfering RNA species (siRNA)
299 increased the production of new viral particles up to 4-fold in HeLa and Jurkat cells. In
300 these cells, hili was depleted for 24 hours before cells were infected with the VSV-G-
301 pseudotyped HIV-1_{NL4-3}. 48 hours later, supernatants were harvested and levels of new
302 viral particles were determined by Gag p24 ELISA. In Fig. 3A, the lower bar graph
303 contains levels of hili mRNA as measured by RT-qPCR and panels below the bar
304 graphs represent hili and tubulin proteins as determined by western blotting. A similar
305 presentation is given for Fig. 3B. We also examined PBMCs and CD4+ T cells (Fig. 3C).
306 In these cells, levels of hili transcripts were vanishingly low in the resting state, but they
307 increased dramatically following cell activation. For PBMCs, PHA and PMA were
308 administered for two days (Fig. 3C, left bar graph, bars 1 and 2). For purified CD4+ T
309 cells, they were activated with anti-CD3 and anti-CD28 antibodies. Two days later, RT-
310 qPCR and western blotting were performed, which indicated increased levels of hili
311 mRNA and protein (Fig. 3C, bar graph on the right, western blots below this bar graph,
312 bars 1 and 2). Since levels of hili were increased in these activated cells, we also used
313 the same siRNA reagents and observed a similar increase in the production of new viral
314 particles in the absence of hili (Fig. 3D, bar graphs, bars 1 and 2). We conclude that hili
315 inhibits viral replication in transformed cell lines as well as in activated primary CD4+ T
316 cells, which are the primary target for HIV in humans.

317 **Mili binds to some tRNAs in cells**

318 To determine how mili could inhibit HIV translation, we next examined RNA species that
319 bind to mili in cells. 293T stably expressing chimeric mili.EGFP (293T.mili) were UV
320 irradiated and lysed. Anti-piwil 2 and anti-GFP antibodies were then used to
321 immunoprecipitate mili and associated RNA species. RNA was released from
322 immunocomplexes, separated by a 15% TBE-urea gel, and visualized with the Sybr-
323 Gold reagent. Yeast tRNA was used as the molecular size marker for tRNA species
324 (Fig. 4A, lane 5). Other RNA size markers are also present (Fig.4A, lane 1). Importantly,
325 mili bound predominantly to RNAs of the same size as the yeast tRNA marker (Fig. 4A,
326 lanes 3, 4 and 5). There was no binding to the IgG control (Fig. 4A, lane 2). In addition,
327 no smaller RNA species were observed, especially in the 25 to 30 nucleotide range,
328 where one would expect to see piRNAs or degraded tRNA species. Some higher
329 molecular size RNA bands were also observed, but they corresponded to those
330 observed with the yeast tRNA or the IgG negative control (Fig. 4A, lanes 3 to 5). We
331 conclude that mili binds to tRNA in cells.

332

333 To investigate further tRNA species that bind to mili, we performed a modified cross
334 linked immunoprecipitation-microarray (Clip-chip) analysis (23). This analysis was
335 preferred to RNA-seq, which would not reveal correctly relative levels of tRNAs of low
336 abundance, i.e. rare tRNA species. 293T.mili cells were first UV-irradiated. RNA bound
337 to mili was immunoprecipitated with anti-piwil 2 antibodies, 3'-end-³²P-labeled and
338 analyzed using purified ³²P-labeled yeast tRNA^{Phe} as the size control. The pattern was
339 identical to the one with mili in Fig. 4A (data not presented). The ³²P-labeled RNA were

340 subjected to tRNA microarray according to methods described previously (30).
341 Microarray data are displayed in the heat blot in Fig. 4B. Progressively redder and
342 greener squares indicate greater and lesser abundance of specific tRNA species. They
343 are quantified further in the bar graph in Fig. 4C. Of note, two rare tRNAs were over-
344 represented on mili. They are tRNA^{Ile}(UAU) and tRNA^{Arg}(UCU) (Fig. 4B and C). They
345 are indicated in red bars (Fig. 4C). We conclude that milis bind to tRNA in cells and that
346 some rare tRNA bind better than expected.

347

348 **Depletion of tRNA^{Arg}(UCU) via an antisense oligonucleotide inhibits HIV**
349 **replication**

350 Both tRNA^{Ile}(UAU) and tRNA^{Arg}(UCU) were over-represented on mili (Fig. 4B and C). To
351 examine their effects on HIV replication, we chose tRNA^{Arg}(UCU). Compared to
352 tRNA^{Ile}(UAU) which represents 14% of all tRNA^{Ile} (30), tRNA^{Arg}(UCU) represents only 5%
353 of all tRNAs^{Arg}. The more abundant ones include tRNA^{Arg}(CCG), tRNA^{Arg}(CCU), and
354 tRNA^{Arg}(ICG) in 293T cells (Fig. 5A)(30). Nevertheless, tRNA^{Arg}(UCU) is encoded by 5
355 loci on different human chromosomes (Fig. 5B). Of interest, HIV_{NL4-3} contains 122 of
356 these rare Arg-AGA codons, many of them in critical double stranded regions, e.g. the
357 RNA frame-shifting site (FS) between gag and pol and the RRE (Fig. 5C).

358

359 Since tRNA^{Arg}(UCU) is encoded on 5 different chromosomes, we could not inactivate all
360 these genes using CRISPR/Cas9 or related technologies. Rather, we synthesized an
361 antisense (AS) oligonucleotide corresponding to tRNA^{Arg}(UCU) sequence [Arg(UCU)].
362 An irrelevant (scrambled) oligonucleotide served as the negative control (C). As

363 presented in Fig. 5D, when Arg(UCU) AS oligonucleotide was expressed in 293T cells,
364 HIV replication was reduced 3-fold (Upper panel, lanes 1 and 2). This finding was
365 validated in activated CD4+ T cells (Fig. 5E, upper panel, lanes 1 and 2). At the same
366 time, we determined that the Arg(UCU) AS oligonucleotide depleted tRNA^{Arg}(UCU)
367 equivalently in these cells (Fig. 5D and E, middle panels, lanes 1 and 2). Moreover,
368 these AS oligonucleotides had no obvious effect on the viability of these cells (Fig. 5D
369 and E, lower panels, lanes 1 and 2). We conclude that the depletion of tRNA^{Arg}(UCU)
370 has the same effect as hili and both decrease the translation as well as replication of
371 HIV.

372

373 **Hili also inhibits the replication of the intracisternal A particles (IAP) in cells**

374 Since piwil proteins block the replication of endogenous mobile genetic elements during
375 the germ line development in the mouse, we also wanted to determine if hili could
376 function similarly in somatic cells. To this end, we employed a well characterized, fully
377 active intracisternal A particle (IAP) marked retrotransposon (pFL). Of note, IAP
378 contains many Arg-AGA codons, 18 in gag, 2 in PR, and 16 in pol. Since hili and mili
379 share 88% sequence identify and 93% sequence similarity, respectively, we also
380 examined effects of hili on pFL in human cells. pFL is diagrammed in Fig. 6A. Into its
381 genome, an interrupted EGFP reporter gene was placed in the opposite orientation.
382 Importantly, pFL-expressing cells turn green only after the complete round of
383 retrotransposition, after the splicing of the introduced intron in the EGFP gene in the IAP
384 sense orientation and transcription of mature EGFP gene in the IAP antisense
385 orientation from the introduced eIF1 α promoter. The number of green cells then reflects

386 the efficiency of retrotransposition (Fig. 6A, IFA). Importantly, we performed our studies
387 in cells that do not contain piRNAs (Fig.4A).

388

389 After co-expressing transiently pFL and hili in 293T cells for 72 hours, and counting over
390 a thousand cells in three independent experiments, 10-fold lower numbers of GFP-
391 expressing cells were found in the presence of hili than in its absence (Fig. 6B, bars 1
392 and 2). The expression of hili is presented in Fig. 6C (top panel). Similar to the situation
393 with HIV, when IAP-specific proteins were examined, greater than 5-fold lower
394 expression of Gag proteins was observed in the presence of hili (Fig. 6C, middle panel,
395 lanes 1 and 2, p73, Gag, p35, MA and p24, CA). Levels of actin served as the loading
396 control (Fig. 6C, bottom panel, lanes 1 and 2). These differences in protein expression
397 occurred despite similar levels of gag transcripts between hili-expressing and parental
398 293T cells transfected with an empty vector (Fig. 6D, lanes 1 and 2), which were
399 quantified using gag-specific primers and RT-qPCR. Finally, reverse transcription was
400 compromised greatly in hili-expressing cells (Fig. 6E, lanes 2 and 3). These data
401 indicate that despite identical levels of IAP transcripts, the expression of IAP proteins is
402 reduced greatly in hili-expressing cells. IAP contains many Arg-AGA codons, 18 in gag,
403 2 in PR, and 16 in pol, representing up to 40% of all Arg codons in the viral genome. We
404 conclude that even in the absence of piRNAs, piwil proteins restrict the movement of
405 mobile genetic elements in cells. Thus, their increased expression in activated T cells
406 could help safeguard the genome in these cells.

407

408

409 **Discussion**

410 In this report, we discovered that hili can inhibit HIV replication, especially in activated
411 CD4+ T cells that are the preferred target cells for this virus in the infected host.
412 Although resting cells do not express hili, it is rapidly induced following T cell activation.
413 Other transformed cell lines express hili constitutively. In them, depletion of hili
414 increased levels of viral proteins and new viral particles. Further studies revealed that in
415 the absence of piRNAs, hili binds to tRNA in these cells. Some of them represent rare
416 tRNA species, whose codons are over-represented in the viral genome. Targeting
417 tRNA^{Arg}(UCU) with an antisense oligonucleotide replicated effects of hili and also
418 inhibited HIV replication in transformed 293T as well as activated primary CD4+ T cells.
419 Importantly, hili also inhibited the retrotransposition of IAP, an active endogenous
420 retrovirus from the mouse, which also contains many Arg-AGA codons. Again, this
421 occurred via the depletion of tRNAs rather than the formation and use of piRNAs. Thus,
422 hili joins a long list of host proteins that complicate the intricate intracellular trafficking of
423 this human retrovirus.

424

425 We found that inducing hili or depleting one of its target rare tRNAs had similar effects
426 on HIV replication. Possibly, AS oligonucleotides targeting both tRNA^{Arg}(UCU) and
427 tRNA^{Ile}(UAU) would have inhibited even more the replication of HIV. However, they
428 should have greater deleterious side effects on host cells as well. In this report, we
429 concentrated on hili because it was induced following T cell activation. Nevertheless,
430 other human piwil proteins such as hiwi, piwil 3 and hiwi 2, when expressed
431 exogenously in cells, also inhibited HIV replication (data not presented). They contain

432 similar PAZ and PIWI domains. Varying expression of hili could also contribute to the
433 elite controller phenotype of some HIV-infected individuals, as has been suggested for
434 Schlafen 11 (31). However, although Schlafen 11 also depletes tRNAs in cells, it is
435 induced by interferon and not by T cell activation (2). Thus, we did not co-express
436 and/or study cooperative effects of hili and Schlafen 11 in our cells.

437

438 HIV is a very compact retrovirus, whose different transcripts must fold into complicated
439 RNA secondary structures that are required for its optimal replication. The choice of
440 tRNA codons is thus mandated by the species where the virus originated, frequent
441 cytosine deamination during reverse transcription by APOBEC3 proteins and demands
442 of these secondary structures. For example, HIV full length or genomic RNA contains
443 TAR, packaging, Lys³ tRNA priming, frame-shifting, and RRE sequences and structures.
444 They are required for transcription, nuclear export, translation and packaging of RNA
445 into new viral particles. Even more complex HIV RNA structures were revealed by
446 SHAPE analyses (32). To this end, it is not surprising that HIV_{NL4-3} contains 122 rare Arg
447 AGA codons, 5 and 7 of which are found in the frame shifting site, which is required for
448 the translation of the Gag-Pol polyprotein precursor protein, and in the RRE,
449 respectively. Thus, the virus would have a difficult time to substitute these rare codons
450 for more abundant ones. HIV transcripts also overwhelm the infected cell. It has been
451 estimated that the virus usurps up to 20% of the translational machinery in activated
452 CD4+ T cells (33). Thus, viral rather than host transcripts would be more sensitive to the
453 relative abundance of rare tRNA species. Small perturbations in levels of these rare
454 tRNA would thus be expected to have greater effects on HIV than on host cell proteins.

455 tRNAs were observed in all studies of piwil proteins. Indeed, tRNA degradation products
456 predominate with tetrahymena piwil proteins (17, 18). In another study, human piwil
457 proteins (hiwi 2) also associated with tRNAs in somatic cells (19). Indeed, hiwi 2 bound
458 preferentially to 9 specific tRNAs in a breast cancer cell line (19). Although in these
459 tumor cells, most tRNAs were degraded or processed tRNA fragments, in our study we
460 found intact tRNAs. Selected tRNAs are double stranded at their 5' and 3' ends.
461 Distances between them are not sufficient to fit into the two separated RNA-binding
462 pockets of the recently crystalized piwil protein from silkworm (34). Nevertheless, hili
463 could potentially unwind the secondary structure of selected tRNAs to separate their 5'
464 and 3' ends. Alternatively, hili could bind these tRNAs simply due to strong electrostatic
465 interactions that would favor some but not other tRNAs. Importantly, these tRNAs do not
466 contain optimal 5' U residues and 2'-O-methyl ribose modifications at appropriate
467 positions (17, 19, 35, 36). Thus, further structural studies will be required to determine
468 how some tRNAs bind selectively to hili.

469

470 Although piRNAs are critical for restricting the movement of mobile genetic elements in
471 the germline, depleting rare tRNA species could also play a significant role. Most
472 endogenous mobile genetic elements originated from different species and contain rare
473 codons. If they are not translated, they also do not jump and replicate. Thus, depleting
474 rare tRNAs would have similar effects to piRNAs with these mobile genetic elements.
475 To this end, it is of interest that hili also restricted the movement of IAPs, which are
476 intact mouse endogenous retroviruses, in our cells. This finding not only informs studies
477 of piwil proteins in organisms, where piRNAs are not found, but also adds another level

478 of regulation to these restriction factors. Thus, transcriptional and translational effects of
479 piwil proteins could synergize for more efficient protection of the genome from these
480 mobile genetic elements in the germline. Furthermore, their expression following cell
481 activation could also protect the genome of rapidly dividing cells from such inadvertent
482 expression and movement of endogenous retrotransposons and retroviruses.

483

484 Finally, manipulating the balance of rare tRNAs might represent an attractive anti-viral
485 strategy (37). Should lower levels of these rare tRNA be sufficient for host cell
486 homeostasis but not support optimal viral replication, one could envision engineering a
487 slower progression of disease, leading to more robust anti-viral immune responses. This
488 could result in a creation of new elite controllers, who would be able to go for long
489 periods of time without aggressive anti-retroviral therapies. Possibly, introducing AS
490 oligonucleotides or genetically inactivating some but not all these tRNAs would achieve
491 these goals.

492

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497

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503

504 **Author Contribution**

505 BMP, TP and KF, conception and design, acquisition of the data, analysis and
506 interpretation, drafting and revising of the manuscript; PL, XW, GL, DC, WS, ML, TH
507 acquisition of the data, analysis and interpretation, drafting of the manuscript.

508

509 **Competing interests**

510 The authors declare no competing financial interests.

511

512

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- 612
613

614 **Figure Legends**

615

616 Fig. 1. Piwil proteins inhibit HIV replication.

- 617 A. Schematic representation of effectors and targets. Human and mouse Piwil 2
618 proteins (hili and mili, respectively) are 88% identical and 93% similar. They
619 contain PAZ (Piwi, argonaute and zwili) and PIWI (P-element induced wimpy)
620 domains that bind to RNA. They contain 973 and 971 residues, respectively.
621 pNL4-3 and pNL4-3.Luc plasmid encode the WT HIV-1_{NL4-3} provirus and the
622 mutant provirus, where the luciferase reporter gene was inserted into the Nef ORF
623 (open reading frame), respectively. The env gene was inactivated by introducing a
624 stop codon. Other ORFs are intact, including those coding for Gag, Pol, Vif (f), Vpr
625 (r), Vpu (u), Tat and Rev proteins. Their transcription is regulated by 5' and 3' long
626 terminal repeats (LTRs) of HIV. The luciferase reporter gene was codon-optimized
627 for expression in human cells.
- 628 B. The expression of hili and mili inhibits HIV replication in 293T cells. Equivalent
629 amounts of HIV-1_{NL4-3} were transfected in 293T cells. Virus production was
630 monitored with p24 ELISA in supernatants of infected cells. For experiments in
631 bar 2, hili was expressed transiently in 293T cells (hili). For experiments in bar 3,
632 293T cells expressed stably mili.EGFP (293T.mili). Supernatants were harvested
633 2 days after the infection. Values were normalized to those of viruses produced in
634 WT cells, which were 293T cells transfected with an empty vector (bar 1, C). The
635 expression of hili and mili was monitored with anti-FLAG (hili) and anti-GFP (mili)
636 antibodies by western blotting. Tubulin represented the loading control. Error bars

637 represent standard errors of the mean from 3 independent experiments [SEM (n =
638 3)], which were performed in duplicate. A Student's *t*-test was used to measure
639 the significance of the data (* P<0.05, ** P<0.01, *** P<0.001).

640 C. Viruses produced by hili- and mili-expressing cells are equally infectious in TZM-bl
641 cells. Supernatants from panel B were normalized to levels of p24 and used to
642 infect TZM-bl cells, which express receptors for HIV-1 and contain luciferase and
643 LacZ reporter genes under the control of the HIV-1 LTR. Values were normalized
644 to those of viruses produced from WT cells (bar 1, C). Error bars are as in panel
645 B.

646 D. HIV RNA levels are not affected by hili. Hili was co-expressed with pNL4-3.Luc in
647 293T cells. 2 days later, total RNA was extracted. Using primers to gag, RT-qPCR
648 quantitated levels of viral transcripts.

649 E. Hili inhibits the expression of Gag. Cell lysates from panel D were also examined
650 for production of viral proteins. Full-length Gag protein (p55) and its proteolytic
651 product (capsid, CA, p24) were examined by western blotting. Ratios of these
652 proteins between WT (C) and hili-expressing 293T cells are presented to the right
653 of western blots.

654 F. Luciferase levels are not affected by hili. In contrast to levels of viral proteins,
655 luciferase activity was not affected by hili.

656

657 Fig. 2. Hili inhibits expression of GFP but not codon-optimized EGFP proteins.

- 658 A. Codon usage of GFP and EGFP genes. Of two rare codons analyzed, GFP
659 contains one Ile-AUA and 5 Arg-AGA codons. In EGFP, they were all optimized
660 for abundant human Ile and Arg codons.
- 661 B. Hili inhibits the expression of GFP. GFP was co-expressed transiently with an
662 empty vector (C) or hili (hili) in 293T cells. Western blots reveal levels of co-
663 expressed proteins and were normalized to tubulin. Densitometry of western blots
664 (using LiCor) revealed relative expression of GFP in these cells (bar graph below
665 the western blots). At the same time, RNA levels of GFP transcripts were
666 determined by RT-qPCR.
- 667 C. Hili does not affect the expression of EGFP. The codon-optimized EGFP was co-
668 expressed transiently with an empty vector (C) or hili (hili) in 293T cells. The
669 quantitation of western blots and EGFP transcripts were performed as in panel A.
670
- 671 Fig. 3. Hili is expressed constitutively in HeLa and Jurkat cells, is induced in PMBCs
672 and CD4+ cells by cell activation and inhibits HIV replication in these cells.
- 673 A. Hili is expressed constitutively in HeLa cells. Its depletion increases HIV
674 replication in these cells. Western blots and RT-qPCR revealed the presence of
675 hili in HeLa cells. Cells were transfected with appropriate siRNAs and one day
676 later, infected with VSV-G-pseudotyped HIV-1_{NL4-3}. Two days later, cells were
677 examined for levels of hili and virus production. Using hili siRNA (siHil) but not
678 scrambled siRNA (C), levels of hili were reduced (lower bar graph and western
679 blot) and those of HIV (top panel) were increased.

- 680 B. Hili is expressed constitutively in Jurkat cells. Its depletion increases HIV
681 replication in these cells. Similar experiments to those in panel A were performed
682 in Jurkat cells.
- 683 C. Hili is induced following the activation of PBMCs with PHA and PMA and CD4+ T
684 cells following the activation by anti-CD3 and anti-CD28 antibodies. In the bar
685 graph on the left, PBMCs from anonymous donors were stimulated with PHA and
686 PMA for 2 days and levels of hili transcripts were measured by RT-qPCR. Bar 1,
687 no stimulation, Bar 2, the addition of PHA and PMA. Similar experiments were
688 performed with CD4+ T cells, which were activated with anti-CD3 and anti-CD28
689 antibodies. RT-qPCR and western blots reveal the induction of hili in these cells.
- 690 D. Depletion of hili in activated CD4+ T cells increases HIV replication. Activated
691 CD4+ T cells co-expressed siScr (C) or siHil RNAs. siHil RNA decreased the
692 expressed of hili transcripts (bottom bar graph) and increased the production of
693 new viral particles (top bar graph).
- 694 For all bar graphs, error bars represent standard errors of the mean from 3
695 independent experiments [SEM (n = 3)], which were performed in duplicate. A
696 Student's *t*-test was used to measure the significance of the data (* P<0.05, **
697 P<0.01, *** P<0.001).

698

699 Fig. 4. Mili binds preferentially to some tRNAs in cells.

- 700 A. Mili binds to tRNA in cells. 293T.mili cells were UV-irradiated and lysed. Anti-GFP
701 and anti-piwil 2 antibodies were used to immunoprecipitate associated RNA
702 species (lanes 3 and 4). RNA species were separated by 15% TBE-urea gel and

703 stained with the Sybr-Gold reagent. Bands were visualized with the LiCor
704 instrument. Yeast tRNA was used as the marker for tRNA (lane 5). The additional
705 RNA size marker is presented in lane 1. IgG served as the negative control (lane
706 2). Note the co-immunoprecipitation of tRNAs with mili, but no smaller RNA
707 species were observed (lanes 3 and 4).

708 B. Mili binds preferentially to some tRNAs in cells. UV-irradiated (cross-linked) and
709 anti-piwil2 immunoprecipitated RNA was 3' end-labeled with ^{32}P , separated by
710 10% TBE-urea gels and submitted to autoradiography. The band corresponding to
711 tRNA was cut out of the gel, eluted and hybridized to a human tRNA microarray.
712 The heat map of this microarray is presented. Relative abundances of these tRNA
713 species on mili are compared to those in cells. Increasing red and green colors
714 signify greater and lesser abundances of these species, respectively.

715 C. Data on up- or down-regulated tRNAs from panel B are presented in this bar
716 graph with arrows highlighting rare tRNAs. Note that the rare tRNA^{Ile}(UAU) and
717 tRNA^{Arg}(UCU) are over-represented on mili and are highlighted as red bars. Fold
718 increased and decreased abundance is given below the bar graph.

719 A Student's *t*-test was performed to measure the significance of the data (*
720 $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$).

721

722 Fig. 5. Levels of tRNA^{Arg}(UCU) are low in cells and antisense Arg(UCU)
723 oligonucleotides inhibit HIV replication.

- 724 A. tRNA^{Arg}(UCU) represents 5% of all tRNA^{Arg} in human cells. tRNA^{Arg}(CGU)
725 represents the vast majority of tRNA^{Arg} in cells, determined by a tRNA-sequencing
726 analysis in 293T cells. The pie chart represents these ratios.
- 727 B. 5 loci on different human chromosomes encode tRNA^{Arg}(UCU). The location of
728 these loci and their relative abundance are presented in this pie chart.
- 729 C. HIV-1_{NL4-3} contains 122 Arg-AGA codons. They are distributed in all reading
730 frames and abundant in the gag-pol frame-shifting site (FS) and the RRE. The
731 distribution in various ORFs of the virus is presented in this table.
- 732 D. Antisense Arg(UCU) oligonucleotides inhibit HIV replication in 293T cells. Specific
733 and scrambled oligonucleotides were expressed in 293T cells for 24 hours and
734 cells were then transfected with HIV-1_{NL4-3}. 48 hours later, supernatants were
735 harvested for levels of Gag p24 (top bar graph) and cells were examined for levels
736 of tRNA^{Arg}(UCU) by RT-qPCR (middle graph) and viability (bottom graph). C,
737 scrambled oligonucleotide, AS, Arg(UCU) oligonucleotide. Error bars represent
738 SEM (n = 3).
- 739 E. Antisense Arg(UCU) oligonucleotides inhibit HIV replication in CD4+ T cells.
740 Specific and scrambled oligonucleotides were expressed in activated CD4+ T
741 cells for 24 hours. Cells were then infected with HIV-1_{NL4-3} and examined as in
742 panel C. Error bars are as in panel C.
- 743 For panels D and E, error bars represent standard errors of the mean from 3
744 independent experiments [SEM (n = 3)], which were performed in duplicate. A
745 Student's *t*-test was used to measure the significance of the data (* P<0.05, **
746 P<0.01, *** P<0.001).

747 Fig. 6. Hili inhibits the replication of IAP.

748 A. Schematic representation of pFL, which contains the full coding IAP genome,
749 where an interrupted EGFP reporter gene was inserted in the opposite
750 orientation at the 5' end. The 5' LTR contains the CMV enhancer/chicken β -
751 actin promoter for higher levels of expression in cells. The EGFP gene in the
752 antisense orientation was interrupted by an intron in the sense orientation
753 (arrow). It is transcribed from an eIF1 α promoter. Other notations are as
754 follows: PBS, primer binding site; PPT, poly-purine tract. Upon transcription of
755 IAP, the intron in EGFP is removed and the new integrated provirus expresses
756 EGFP. EGFP-positive cells were then counted. The white arrow points to a
757 green cells. The white bar represents 10 μ m.

758 B. Hili inhibits IAP retrotransposition in 293T cells. The number of EGFP-positive
759 cells in the presence of hili relative to such cells transfected with an empty
760 vector is presented (bar 2).

761 C. Hili inhibits the expression IAP proteins in 293 T cells. Below the hili western
762 blot is presented the expression of IAP Gag proteins. A greater than 5-fold
763 decrease in IAP proteins was observed in the presence of hili in these cells.
764 The expression of actin served as the internal control

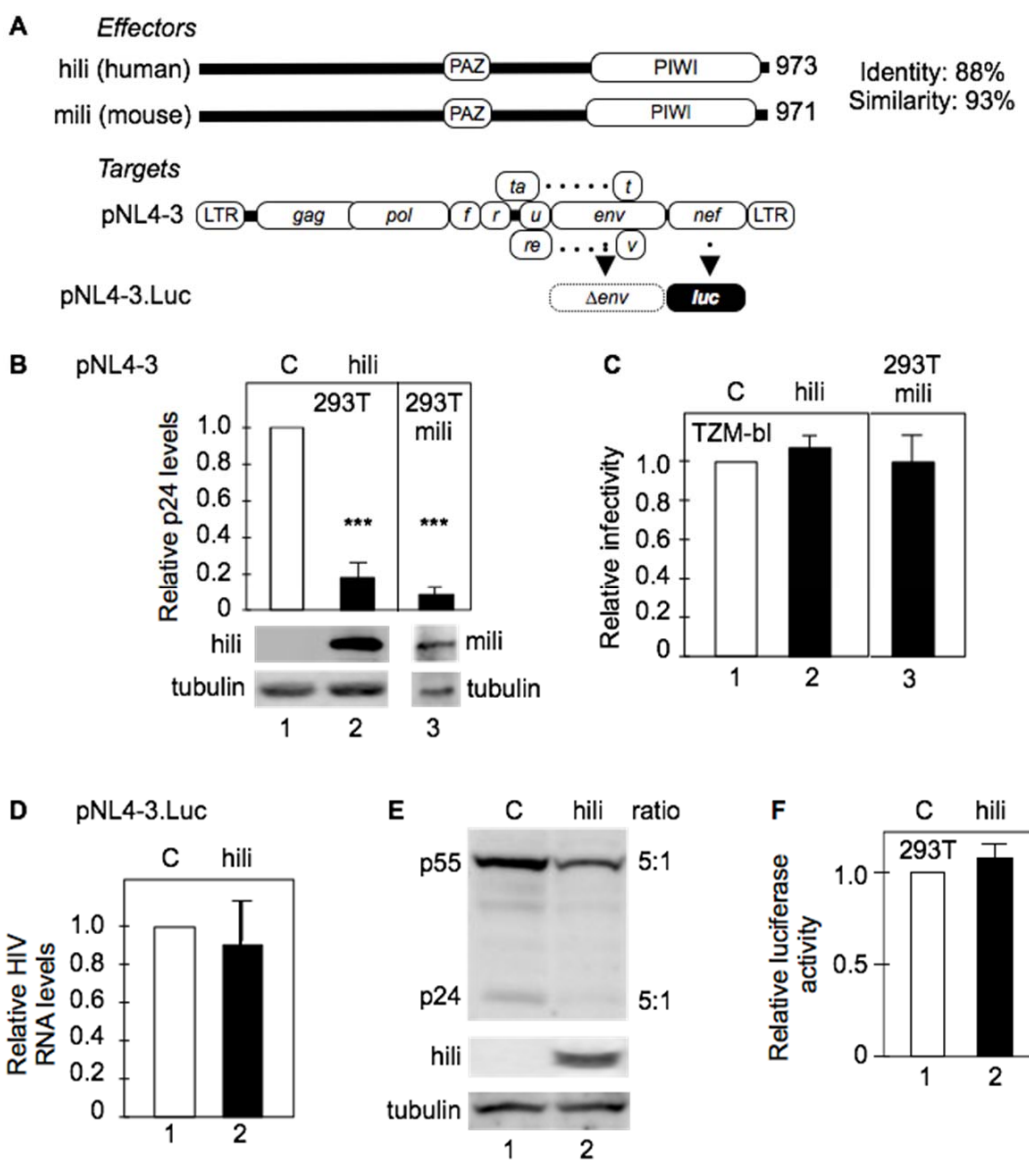
765 D. Levels of IAP transcripts are equivalent in 293T cells that do or do not express
766 hili. Relative levels of IAP gag RNA in the presence of hili are presented in bar
767 2. Primers used for RT-qPCR are presented in Fig. 1A.

768 E. Reverse transcription of IAP is decreased in the presence of hili. A 200 nt
769 product of RT was detected in control 293T cells transfected with an empty
770 vector, but not hili-expressing 293T cells (lanes 2 and 3).

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Fig. 1

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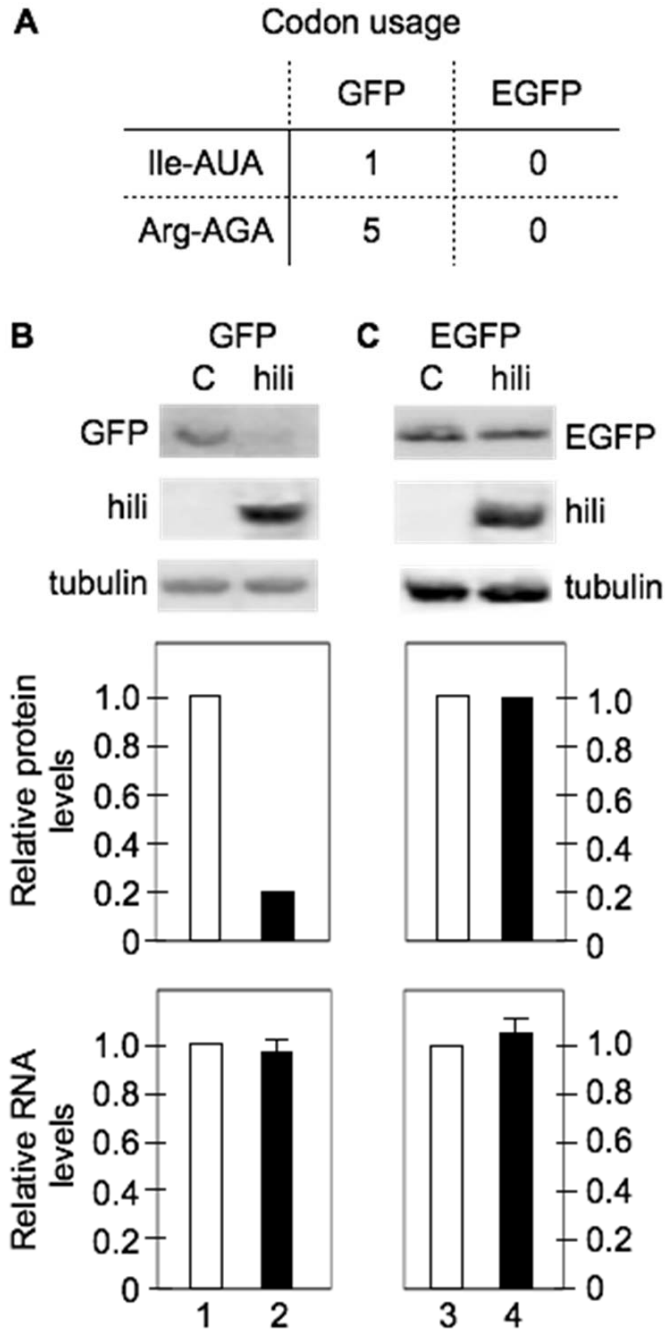
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Fig. 2

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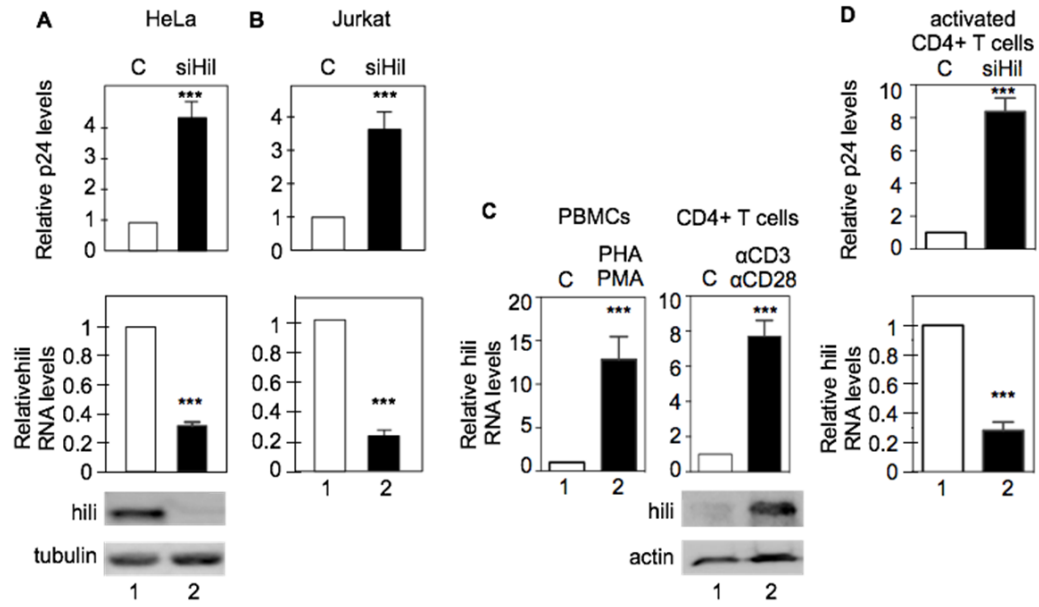
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Fig. 3

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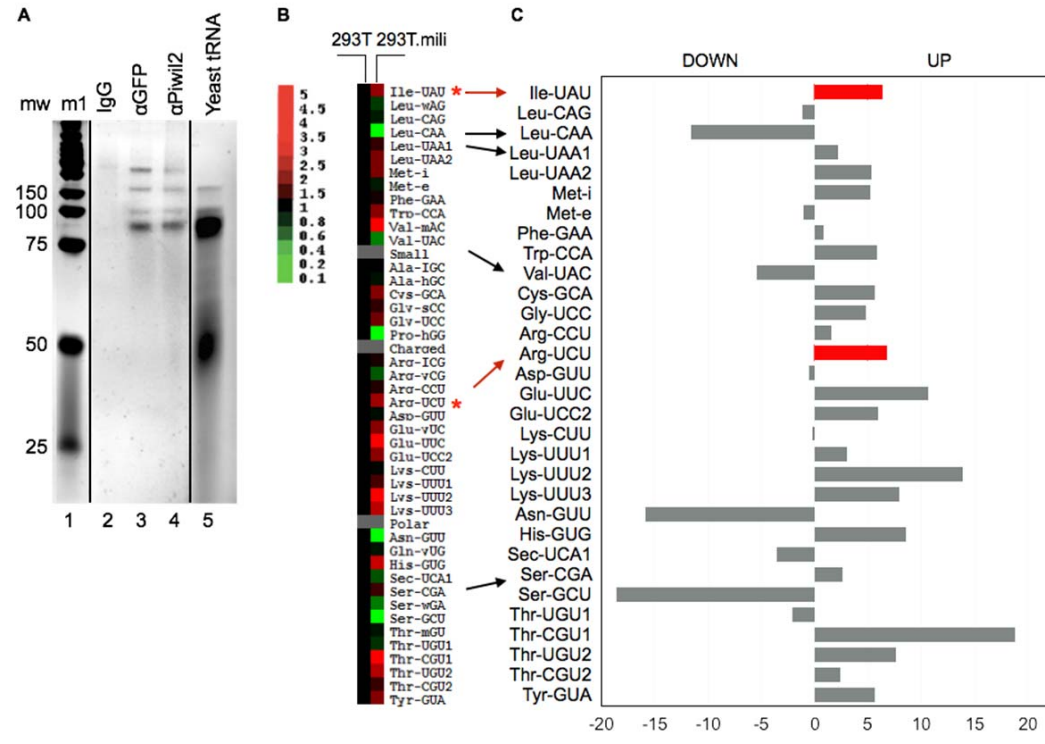


Fig. 4

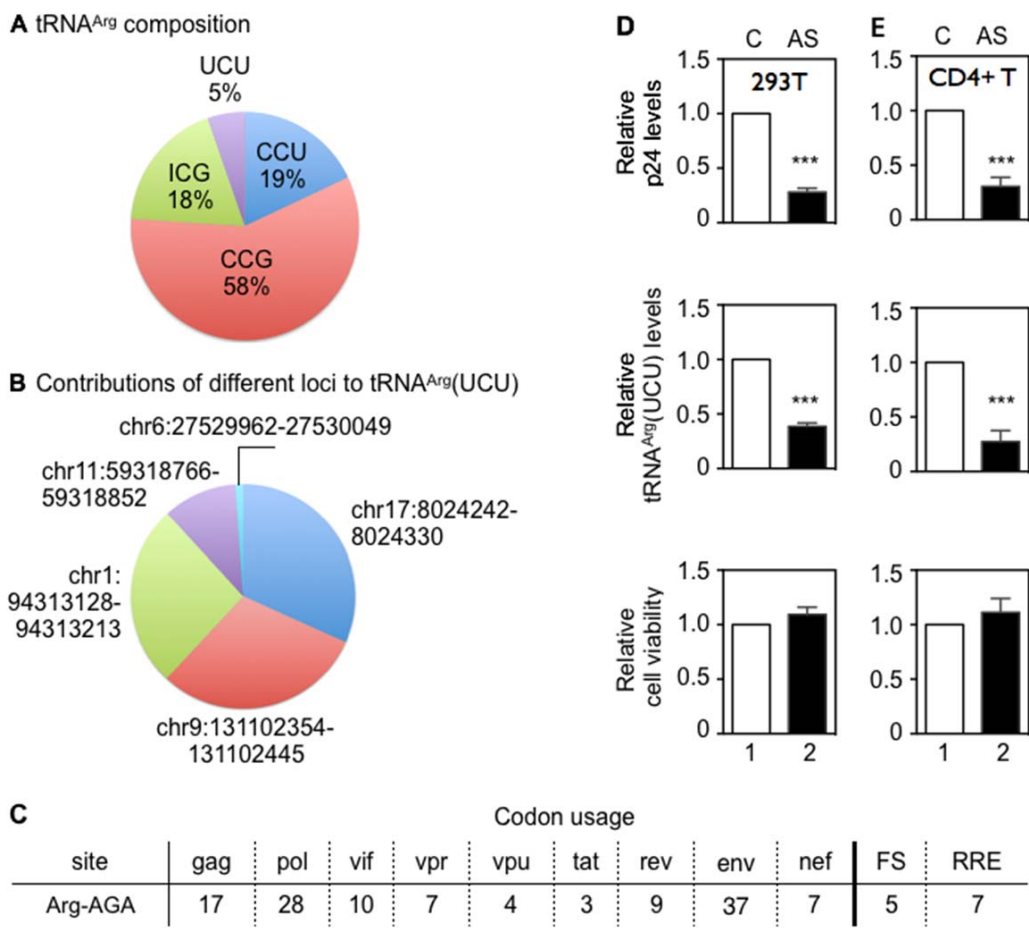
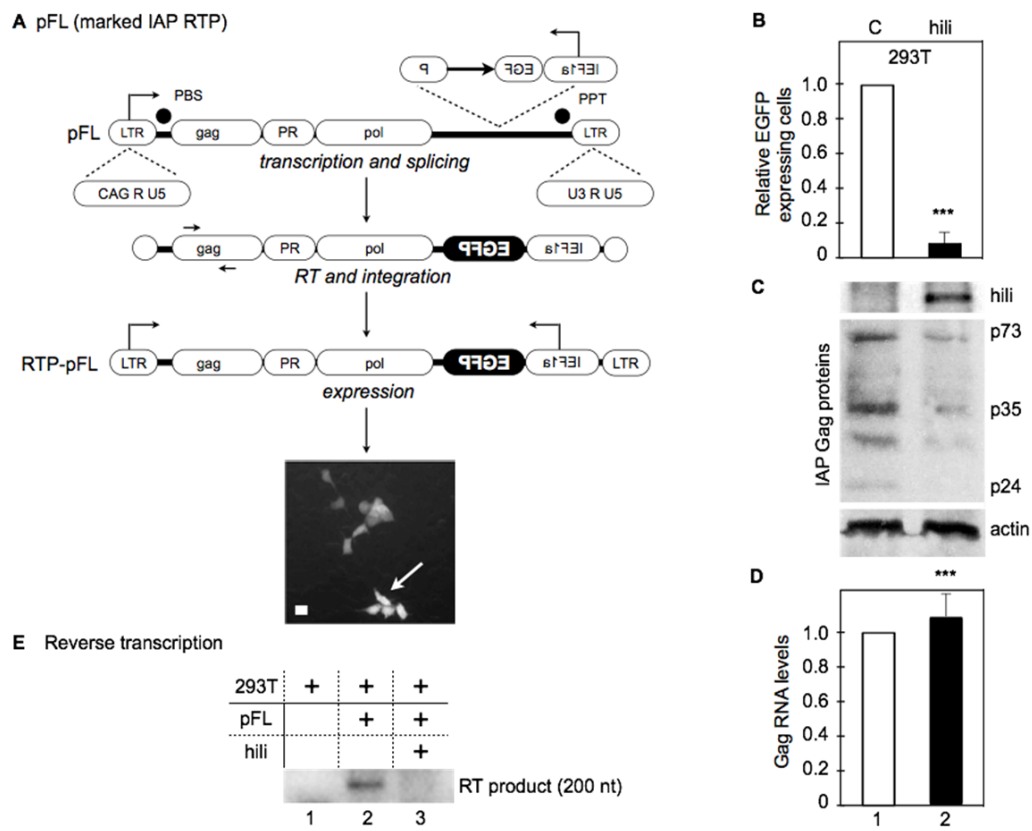


Fig. 5

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Fig. 6