

1 **Entry of the bat influenza H17N10 virus into mammalian cells is enabled by the MHC**
2 **class-II HLA-DR receptor**

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19

20 **Abstract**

21 Haemagglutinin (HA) and neuraminidase (NA) surface glycoproteins of bat influenza H17N10
22 virus neither bind nor cleave sialic acid receptors, indicating this virus employs cell-entry
23 mechanisms distinct from those of classical influenza A viruses. We observed that certain
24 human haematopoietic cancer cell lines and the canine MDCK II cells are susceptible to H17-
25 pseudotyped viruses. We identified the human HLA-DR receptor as an entry-mediator for
26 H17-pseudotypes, suggesting that H17N10 possesses zoonotic potential.

27 **Main**

28 Bats are reservoirs of diverse, potentially zoonotic viruses (*e.g.* *Paramyxoviridae*,
29 *Coronaviridae*, *Filoviridae*), further exemplified by the discovery of the evolutionarily distinct,
30 influenza A-like viruses H17N10 and H18N11 (BatIVs) in asymptomatic bats^{1,2}. This discovery
31 led to concern that bats may be neglected reservoirs of influenza viruses³.

32 The natural reservoir of classical influenza A viruses (IAVs) is aquatic birds, from which they
33 emerge, *via* genome reassortment and mutation, to cause sporadic pandemics in humans and
34 other hosts⁴. The initial cross-species barrier is host cell attachment. The HA mediates virus
35 binding to host-specific sialic acid (SA) moieties⁴. The crystal structures of BatIV HAs exhibit
36 divergence of their protein conformations from those of IAVs and inability to accommodate
37 SA^{2,5,6}. Initial efforts to isolate infectious BatIVs from bats failed, mainly because their
38 receptors were unknown^{1,2,5,7,8}. Synthetic BatIVs were able to infect mammalian cell lines⁹⁻¹¹.
39 Identifying the BatIV receptors is key to assessing their zoonotic risk.

40

41 Lentivirus-derived pseudotypes (PV) with heterologous envelope proteins have facilitated
42 identification of viral receptors¹² and are valuable in assessing H17N10 tropism^{12,13}. We have
43 shown that transduction-competent H17- and H17N10-PV are recovered from HEK293T/17
44 cells only in the presence of proteases (HAT or TMPRSS2) (Fig. 1a)¹³, in keeping with
45 published data¹¹. To study the distribution of the H17N10 receptor(s), a panel of cell lines
46 (Supplementary Table 1) was challenged with H17-PV, assaying transduction *via* the
47 expression of PV-encoded luciferase reporter. PV bearing H5 (H5-PV) or VSV-G (VSV-G-PV)
48 served as positive controls; PV without envelope protein (Δ -env) as negative. H17-PV
49 displayed highly limited species/cell tropism, in agreement with previous work⁹⁻¹¹, suggesting
50 the receptor(s) are not ubiquitous (Fig. 1b). Canine MDCK II (unlike MDCK I) cells are
51 susceptible to H17-PV (Fig. 1c) in agreement with previous studies^{10,11}. MDCK II cells were
52 not susceptible to PV expressing N10 alone while particles bearing H17 alone or both H17 and

53 N10 transduced these cells with high, comparable efficiency, indicating that N10 is
54 dispensable for entry, in keeping with published data^{9,11}. To characterise the H17 putative
55 receptor(s), MDCK II cells were (pre)-treated with enzymatic agents before transduction (Fig.
56 1d). Pre-treatment with neuraminidase, which cleaves surface SA, reduced transduction by
57 H5-PV (by 68-86%) but not H17-PV, further supporting the notion that SA are not the H17-
58 receptors⁸⁻¹¹.

59

60 IAVs primarily enter cells via endocytosis followed by low pH-triggered endosomal fusion.
61 Treatment of cells with ammonium chloride blocked uptake of H5- and H17-PV,
62 demonstrating that entry of H17, like IAVs, is pH-dependent consistent with a previous
63 study⁹. Entry of H17-PV was more affected than H5-PV by pre-treatment of cells with
64 proteases or an inhibitor of *N*-glycosylation (reduced by up to 72 and 78%, compared to 45
65 and 20%, respectively), supporting the supposition⁹ that the H17-receptor(s) is a
66 glycoprotein.

67

68 MDCK cells are heterogeneous, displaying phenotypic variation¹⁴, with MDCK I and II
69 representing early and late passages of the parental NBL-2 cells. Transcriptional differences
70 between early (passage 8) and late (passage 21) MDCKs were investigated using microarrays,
71 identifying 17 differentially regulated transcripts (Fig. 2a). Using transmembrane domain and
72 subcellular localisation prediction algorithms, we identified the dog leukocyte antigen DR α -
73 chain (DLA-DRA) as the only transcript encoding a surface-anchored protein, over-expressed
74 in late, compared to early, passage cells (Supplementary Table 2). Significant over-expression
75 of DLA-DRA (and paralogue DLA-DRB1) was confirmed by qRT-PCR in MDCK II compared to I
76 cells (Fig. 2b).

77

78 DLA-DRA is a well-conserved orthologue of the human MHC-II HLA-DRA (Supplementary
79 Figure 1). MHC-II molecules are heterodimers of two glycosylated, transmembrane
80 polypeptide chains (monomorphic α and polymorphic β), expressed selectively on antigen
81 presenting cells (APCs; Fig. 2c). HLA-DR forms complexes, with endocytosed foreign
82 peptides, which are presented to CD4⁺ T-helper cells for recognition¹⁵.

83 To assess the zoonotic potential of H17N10, we explored the H17-PV tropism using human
84 cell lines with known elevated HLA-DR expression levels¹⁵ (Fig. 2d). Burkitt's lymphoma-
85 derived Raji, Ramos and BJAB B-lymphocytes and the B-lymphoblastoid cells (B-LCL) show
86 decreasing susceptibility, in that order, to H17-PV. Kasumi-1 leukaemic cells showed marginal
87 susceptibility, while Molt-4 and HL-60 leukaemic cells, Jurkat T-cells, pro-monocytic THP-1
88 and U-937, and primary B-cells showed no susceptibility to H17-PV. Differential susceptibility
89 to H17-PV correlated with HLA-DRA expression levels (confirmed by qRT-PCR). Surface
90 expression of the heterodimer was assayed by flow cytometry (Fig. 2e)¹⁶ and showed an
91 association with susceptibility to H17-PV.

92

93 We investigated whether ectopic expression of HLA-DR was sufficient to render non-APC
94 susceptible to H17-PV. HEK293T/17 cells were transiently co-transfected with the DRA and
95 DRB1 expression vectors. Surface expression of the heterodimer was confirmed *via*
96 immunofluorescence (Supplementary Figure 2). Flow cytometry confirmed 47% of cells
97 formed a functional surface heterodimer (Fig. 2f). Over-expression of HLA-DR resulted in
98 significant transduction by H17-PV. Furthermore, transduction was enhanced two-fold (Fig.
99 2g) in cells FACS-enriched for HLA-DR. Similar results were obtained using bat cells
100 (Supplementary Figure 3). Conversely, small interfering RNA targeting HLA-DRA (Fig. 2h) or
101 MHC-II monoclonal antibodies (Fig. 2i; Supplementary Figure 4) drastically reduced Raji cells'
102 susceptibility to H17-PV.

103

104 Together, these findings demonstrate HLA-DR functions as a *bona fide* entry mediator for
105 H17N10. Similarly, Karakus *et al* determined that bat, pig, mouse or chicken HLA-DR
106 orthologues mediate cell entry of H18N11¹⁷. Through efficient binding to HLA-DR, BatIVs
107 might simultaneously access APCs and block T-cell responses. This might explain the viruses'
108 survival and asymptomatic status in carrier bats.

109 With limited functional information available on bat MHC-II, its role in the pathogenesis and
110 transmission of BatIVs remains obscure. Nevertheless, the observation that H17N10 can
111 enter human HLA-DR⁺ cells implies that the virus has zoonotic potential.

112

113 **Methods**

114 **Cell culture and treatment**

115 Cell lines are described in Supplementary Table 1 and were cultured according to standard
116 protocols (www.atcc.org). Bat cell lines were propagated in DMEM supplemented with heat-
117 inactivated 15% fetal bovine serum (Life Technologies), penicillin (100 U/ml) and
118 streptomycin (100 µg/ml; Invitrogen) and maintained at 37°C and 5% CO₂.

119 MDCK II cells were treated as previously⁹ with either the endosomal acidification reagent
120 ammonium chloride (1-100 mM), or pre-treated with neuraminidase from *Clostridium*
121 *perfringens* for 2 h (1-100 mM) or pronase for 30 min (endo-
122 and exoproteases from *Streptomyces griseus* at 5-50 µg/ml; Calbiochem), or an *N*-
123 glycosylation inhibitor for 5 h (tunicamycin at 0.01-1 µg/ml; Sigma-Aldrich). For experiments
124 using glycosidases, Raji and Ramos cells were treated as previously¹⁸ with 0-100 U/ml
125 Peptide:*N*-glycosidase F (PNGase F; Calbiochem) or endoglycosidase H (endo H; New England
126 Biolabs) or a mixture of neuraminidase (50 mM) and *O*-glycosidase (1-20 mU/ml) in Hank's
127 Balanced Salt Solution (HBSS; Sigma) for 2 h at 37°C. Cells were washed twice with HBSS and
128 transduced with H17- and VSV-G-PV for 2 h. The cells were then resuspended in fresh growth

129 medium for 24-48 h before assay. Bafilomycin A1 (Calbiochem) was used at a concentration
130 of 10 nM. Cell viabilities were assessed by a trypan blue exclusion test.

131

132 **Pseudotype production and susceptibility assays**

133 Pseudotypes expressing H17 and N10 (from A/little yellow-shouldered
134 bat/Guatemala/060/2011), H5 (A/Vietnam/1194/2004; H5N1 clade 1) or VSV (Vesicular
135 Stomatitis Virus)-G glycoproteins were produced as described¹³. Briefly, the lentiviral
136 packaging plasmid p8.91, the pCSFLW firefly luciferase vector, the pI.18 expression plasmids
137 for H17 and/or N10 and the protease encoding plasmid pCAGGS-HAT were co-transfected
138 using polyethylenimine (PEI) reagent (Sigma-Aldrich) into HEK293T/17 cells. Filtered
139 supernatants were collected 48-72 h post transfection. To remove viral titer bias between
140 different stocks, PV were concentrated and (re-) titrated by serial dilution. Concentration was
141 carried out by ultra-centrifugation for 2 h at 25,000 rpm at 4°C (Beckman Optima XL-100 K
142 Ultracentrifuge).

143 Two-fold serial dilutions of PV-containing supernatant were performed as previously
144 described¹³ using 96-well plates. Subsequently, 1×10^4 (for adherent) and 3×10^4 cells (for
145 suspension) cells were added in 50 μ l of medium per well. Plates were incubated for 24-48 h,
146 after which 50 μ l of Bright-Glo™ substrate (Promega) was added. Luciferase readings were
147 conducted with a luminometer (FLUOstar OPTIMA, BMG Labtech) after a 5-min incubation.
148 Data was normalized using Δ -env and cell-only measurements and expressed as Relative
149 Luminescence Units (RLU)/ml.

150

151 **Plasmids and transfections**

152 Expression plasmids for HLA-DRA (NM_019111) and HLA-DRB1 (NM_001243965) were
153 purchased from GenScript. HEK293T/17 at sub-confluence in 6-well plates or 100 mm
154 dishes were transfected with HLA-DRA or HLA-DRB1 plasmids or a 1:1 combination of both

155 using Lipofectamine 3000 (Thermo Fisher). 48 h after transfection, cells were used either
156 for immunofluorescence analysis, for FACS analysis or for PV-transduction. The
157 transfection efficiency, as assessed by the GFP expression of a control plasmid, was >70%
158 under microscopic observation. PakiTO3 cells were transfected with HLA-DR plasmids and
159 selected with geneticin (500 µg/ml).

160

161 **Microarray analysis**

162 Total RNA was isolated from triplicates of early and late passage MDCK cells using a Ribopure
163 kit (Ambion). Preparation of samples and hybridization to Affymetrix Canine Genome 2.0
164 GeneChips was performed according to standard Affymetrix protocols. A one-way ANOVA
165 adjusted with the Benjamini–Hochberg multiple-testing correction ($p<0.05$) was performed
166 with Partek Genomics Suite (v6.6) as previously described¹⁹.

167

168 **HLA-DRA knockdown and blocking using siRNA and monoclonal antibodies**

169 A Sigma-Aldrich MISSION endonuclease-derived esiRNA (EHU226621) was used to knock
170 down HLA-DRA expression in Raji cells. Lipofectamine RNAiMAX reagent (Thermo Fisher)
171 was used to transfect exponentially grown cells with 50 nM of siHLA-DRA or of siControl
172 (SIC001). Initial transfection was followed by re-transfection of cells on the following day.
173 Cells were collected after 48 h and either seeded for transduction with PV or processed in
174 order to validate siRNA activity (by qRT-PCR and western blot).

175 To evaluate the interaction of HLA-DR with H17, we used the broadly reactive MHC-II mAb
176 WR18 (Biorad; code MCA477) and anti-HLA-DR mAb (clone 302CT2, Enzo Life Sciences).
177 After 1 h pre-incubation with increasing mAb concentrations of the antibody, Raji cells
178 (3×10^4) were transduced for 2 h with H17-or VSV-G-PV. The cells were then resuspended in
179 fresh growth medium and luciferase was measured after 24 h.

180

181 **Western blot analysis**

182 Washed cells were lysed on ice with lysis buffer [0.5% NP40 in PBS with 10 mM Tris-HCl, pH
183 7.4 supplemented with protease inhibitors (Thermo Fisher)] and protein was quantified by
184 the BCA assay kit (Thermo Fisher). 20-50 µg of protein was electrophoresed on a 4-15%
185 sodium dodecylsulfate polyacrylamide gel, alongside a protein ladder (Bio-Rad) and
186 immunoblotted with the mouse mAb anti-HLA-DRA (1:1000; clone: 302CT2, Enzo Life
187 Sciences) or rabbit mAb α -tubulin (1:2000; Cell signalling Technology). The membranes were
188 washed and incubated with goat anti-rabbit or donkey anti-mouse secondary antibodies (LI-
189 COR) in the dark for 1 h. Scanning was carried out using the Odyssey Imaging system.

190

191 **Immunofluorescence**

192 Transfected cells were seeded onto glass cover slips overnight and were fixed with 4%
193 paraformaldehyde. Fixed cells were then washed and permeabilised with 1% Triton X-100 in
194 PBS for 10 minutes. After washing, the cover slips were incubated with a mouse HLA-DRA
195 mAb (169-1B5.2; Bio-Techne) targeting a monomorphic HLA-DR framework determinant for
196 1 hr, washed and incubated with anti-mouse Alexafluor488 conjugated-Ab (Thermo Fisher)
197 for 30 minutes. Cover slips were mounted using Prolong Gold containing DAPI (Invitrogen).
198 Images were acquired on EVOS fluorescent microscope (Life Technology). Experiments were
199 carried out in duplicate.

200

201 **Flow cytometry**

202 For surface staining, cells were washed twice in ice-cold FACS buffer and stained with a FITC-
203 conjugated anti-human HLA-DR mAb (clone Tü36; BDIS), which binds to a monomorphic
204 epitope of the $\alpha\beta$ complex and not the isolated α or β chains or the HLA-DP and -DQ
205 isotypes¹⁶. The BD LSR Fortessa was used to determine expression of HLA-DR, in
206 combination with the matched isotype control and cells were sorted into HLA-DR⁻ and HLA-

207 DR⁺ subpopulations with a FACS Aria cell sorter (BDIS). The Hoechst 33342 stain was used for
208 cell viability discrimination and the data files were analyzed using FlowJo software v10.0
209 (Tree Star, Inc.). Experiments were carried out in duplicate. Gating strategy: cells were first
210 identified by forward (FSC-Area) versus side scatter (SSC-Area) gating based on cell size and
211 granularity, then the FSC-Area vs FSC-Height gating for single cells. Dead cells were excluded
212 by gating on Hoechst 3342 negative cells. Positive and negative gates for HLA-DR expression
213 were determined by using isotype and unstained controls.

214

215 **qRT-PCR analysis**

216 qRT-PCR was performed using procedures described previously¹⁹. qRT-PCR was conducted in
217 a 384-well plate with the ABI-7900HT system (Applied Biosystems). The following primers
218 were used: DLA-DRA (Forward: 5'-GCTGTGGACAAAGCTAACCTTG-3', Reverse: 5'-
219 TCTGGAGGTACATTGGTGTTCG-3'), DLA-DRB1 (Forward: 5'-AGCACCAAGTTTGACAAGC-3',
220 Reverse: 5-AAGAGCAGACCCAGGACAAAG-3'). Absolute copy numbers of HLA-DRA were
221 calculated using a standard curve of known concentrations of the corresponding expression
222 plasmid. HLA-DRA (Forward: 5'-TCAAGGGATTGCGCAAAGC-3', Reverse: 5'-
223 ACACCATCACCTCCATGTGC-3').

224

225 **Bioinformatic and statistical analyses**

226 Phobius²⁰ and TMHMM v2.0²¹ software were used to predict the existence of transmembrane
227 domains and Deeploc-1.0²² was used to determine sub-cellular protein localisation. The
228 amino acid sequences of DLA-DRA (NP_001011723.1), HLA-DRA (NP_061984.2), and their bat
229 orthologues [(*Pteropus alecto* (XP_006907484.1); *Desmodus rotundus* (XP_024413747.1))]
230 were subjected to multiple alignment using CLC workbench 7. Graphical representation and
231 statistical analyses were performed using Prism 8 (GraphPad). Unless otherwise stated,
232 results are shown as means \pm SEM from three independent experiments. Selection of

233 statistical analysis was based on the data distribution. Data distributions were tested
234 for normality using the Shapiro–Wilk normality test. $p < 0.05$ was considered significant unless
235 otherwise stated.

236

237 **Ethics statement:** The buffy coat residues for the isolation of CD19⁺ primary B-cells were
238 purchased from the UK Blood Transfusion Service from anonymous volunteer blood donors.
239 Therefore, no ethical approval is required.

240

241 **Competing Interests**

242 The authors declare no competing interests.

243

244 **Data Accessibility**

245 Microarray data are available at the GEO repository under series record number GSE14837.
246 All other data supporting the findings of this study are available from the authors on
247 request.

248

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Figure Legends

276 **Figure 1: a.** PV-production schematic. **b.** H17-PV transduction efficiencies in cell lines ($n=6$).
277 Relative Luminescence Units (RLU)/ml are plotted as box-plots (Upper/lower bounds: 25%
278 and 75% quantiles; middle band: median; whiskers: minimum-maximum values). **c.** PV-
279 transduction efficiencies (means \pm SEM, $n=8$) in MDCK II cells. Statistical significance was
280 determined by Kruscal-Wallis and Dunn's *post-hoc* tests. **d.** Left Y-axis: % PV-transduction
281 efficiencies in (pre-)treated MDCK II cells (means \pm SEM, $n=4$). Right Y-axis: % cell viability
282 (means \pm SEM, $n=3$).

283 **Figure 2: a.** Up-/down-regulated (green/red) transcripts identified by microarrays in late-
284 versus early-passaged MDCKs. Red box: membrane protein-encoded transcript. **b.** mRNA
285 expression levels (means \pm SEM, $n=4$) of DLA-DRA/-DRB1. Significance was calculated by
286 one-way ANOVA and Tukey's tests ($***p=0.0006$; $****p<0.0001$). **c.** HLA-DR schematic
287 (Motifolio). **d.** Left Y-axis: H17-PV transduction efficiencies in human cell lines [$n=4$; box-plot
288 (top/bottom: min/max values; middle band: median)]. Right Y-axis (line): HLA-DRA mRNA
289 copies ($n=4$). **e.** FACS analysis of HLA-DR cell surface expression (representative of two
290 independent experiments). Blue and red peaks represent HLA-DR⁻ and -DR⁺ subpopulations. **f.**
291 FACS analysis of HLA-DR expression in HEK293T/17 cells transfected with HLA-DR $\alpha\beta$
292 plasmids ($n=2$). Right top corner: immunofluorescence confirming HLA-DR expression ($n=2$;
293 Scale bar=10 μ m). **g.** PV-transduction efficiencies (means \pm SEM, $n=4$) in FACS-sorted DR⁻,
294 unsorted (DR⁺ & DR⁻) and FACS-sorted DR⁺ HEK293T/17 cells. Significance was calculated by
295 one-way ANOVA and Tukey's tests ($****p<0.0001$). **h.** Left: PV-transduction efficiencies
296 (means \pm SD, $n=3$) in Raji cells transfected with siControl or siHLA-DRA. Significance was

297 calculated with a *t*-test (Holm-Sidak-adjusted) and right: corresponding western blot and
298 qRT-PCR data showing HLA-DRA protein and mRNA levels (means \pm SD, $n=3$). qRT-PCR
299 significance was calculated by two-sided *t*-test ($p=0.0065$). **i.** % PV-transduction levels
300 (means \pm SD, $n=3$) in Raji cells pre-incubated with MHC-II monoclonal antibody. Significance
301 was calculated by one-way ANOVA and Dunnett's tests ($***p=0.0003$, $****p<0.0001$).

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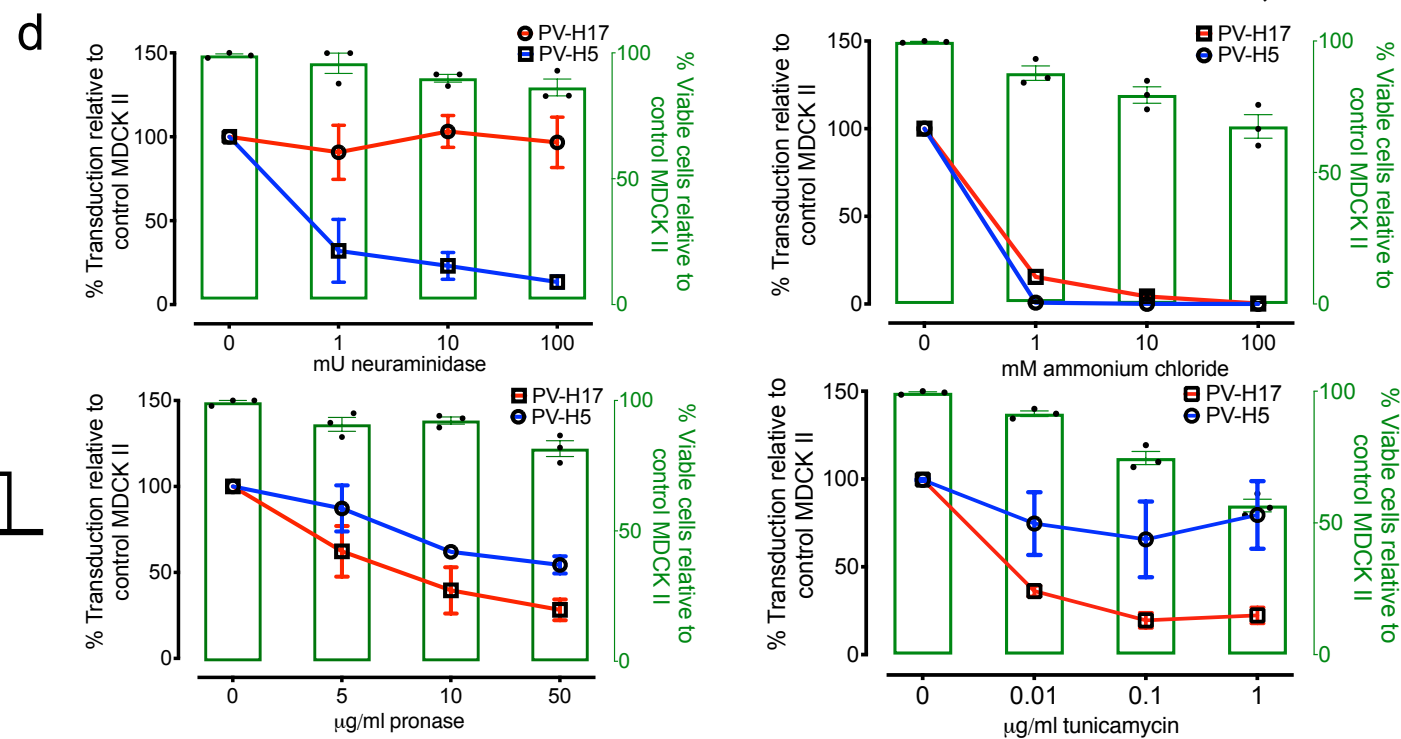
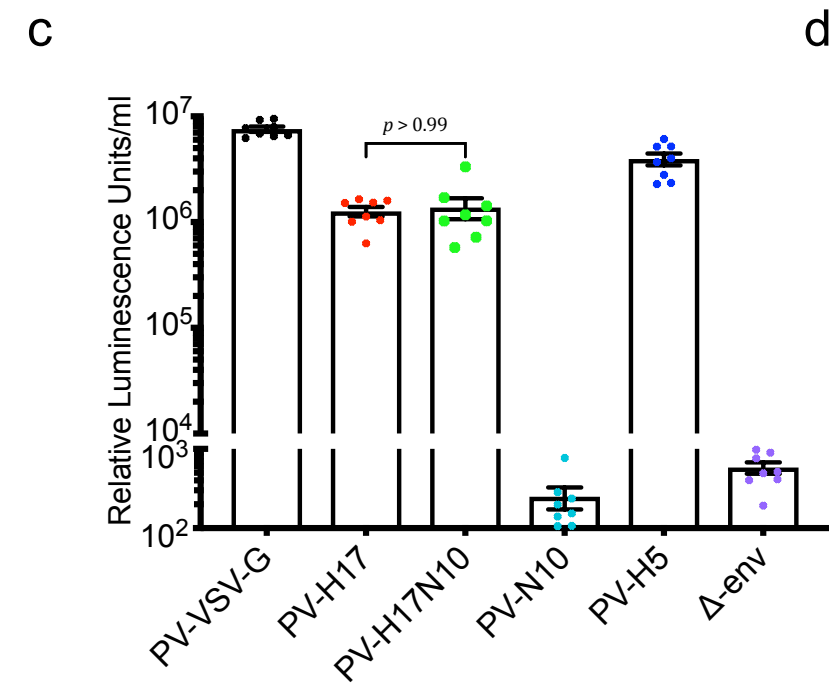
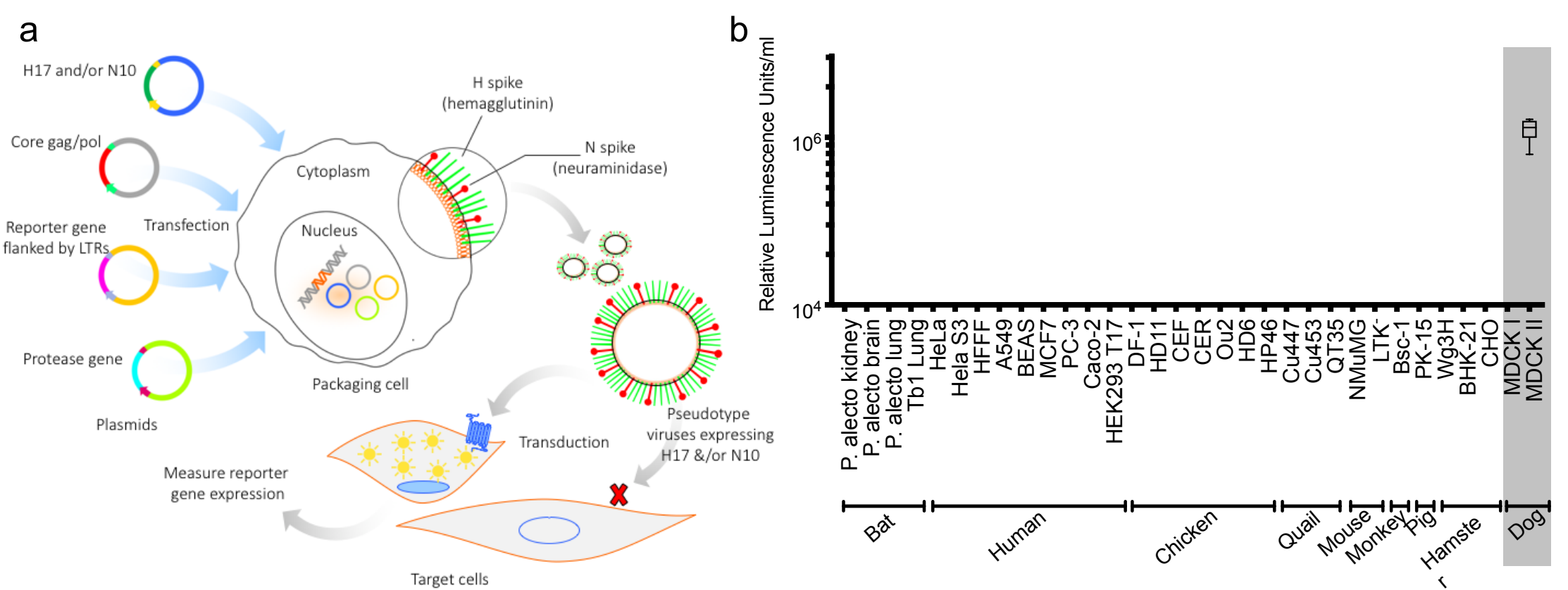
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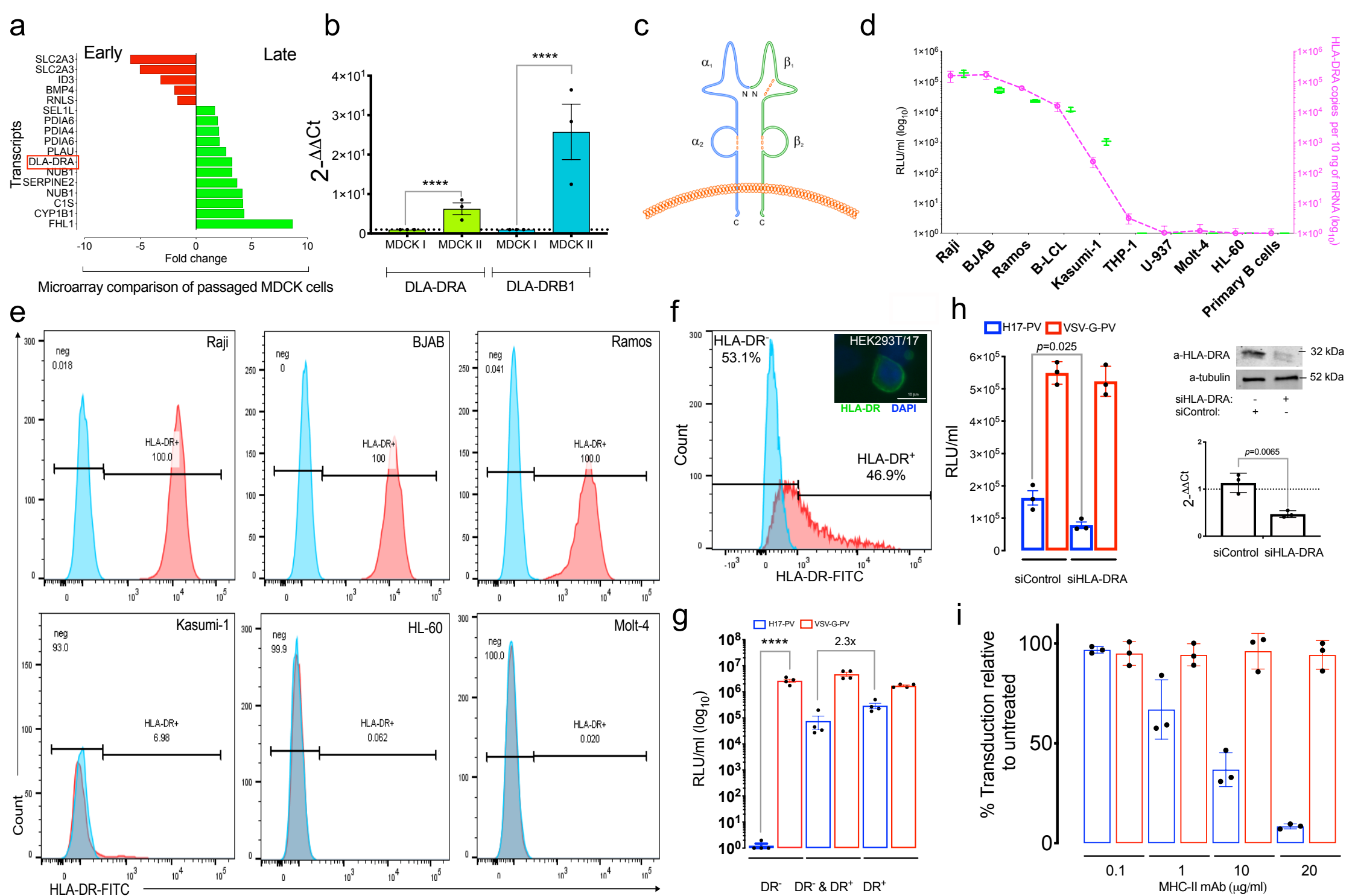
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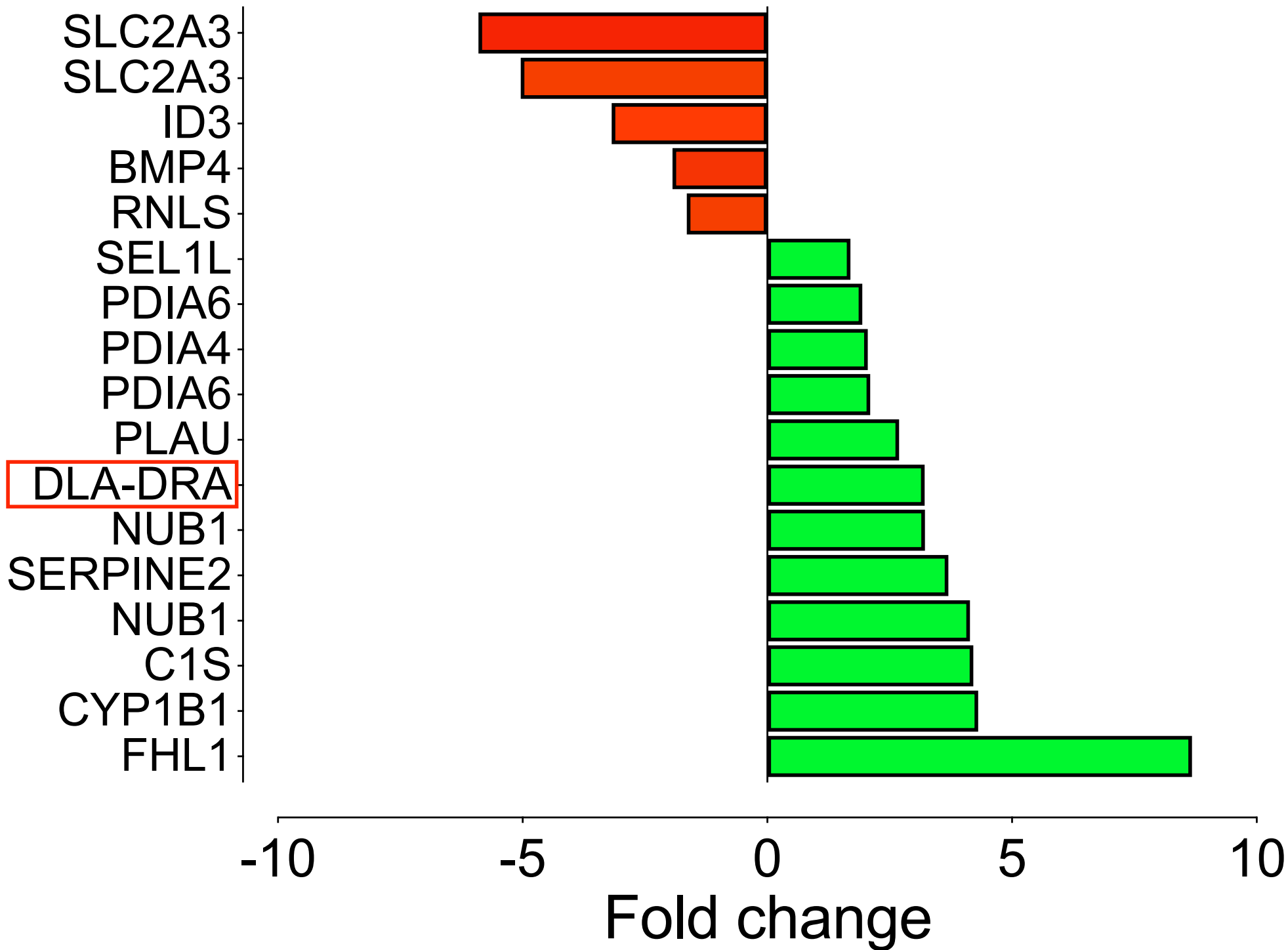
316

317 **Author Contributions:** ESG conceived, designed and performed the experiments, analysed
318 data and wrote the manuscript. GC generated luciferase reporter plasmids, produced
319 pseudotype viral stocks and wrote the manuscript. EFY, SG and PS performed microarray
320 work. WSB, MAS, and NT designed experiments and wrote the manuscript. L-FW provided
321 materials and engaged in discussion of the project.

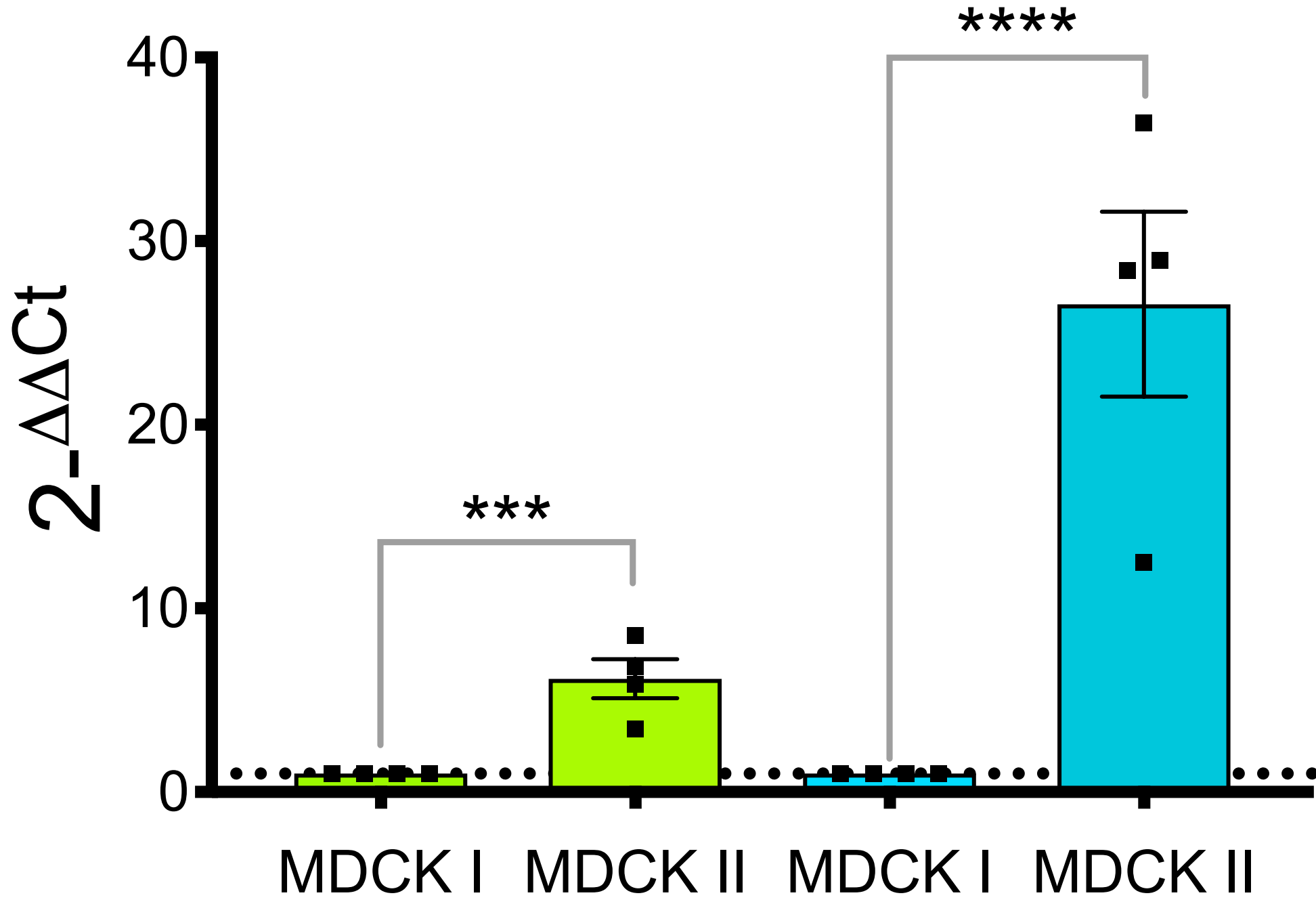




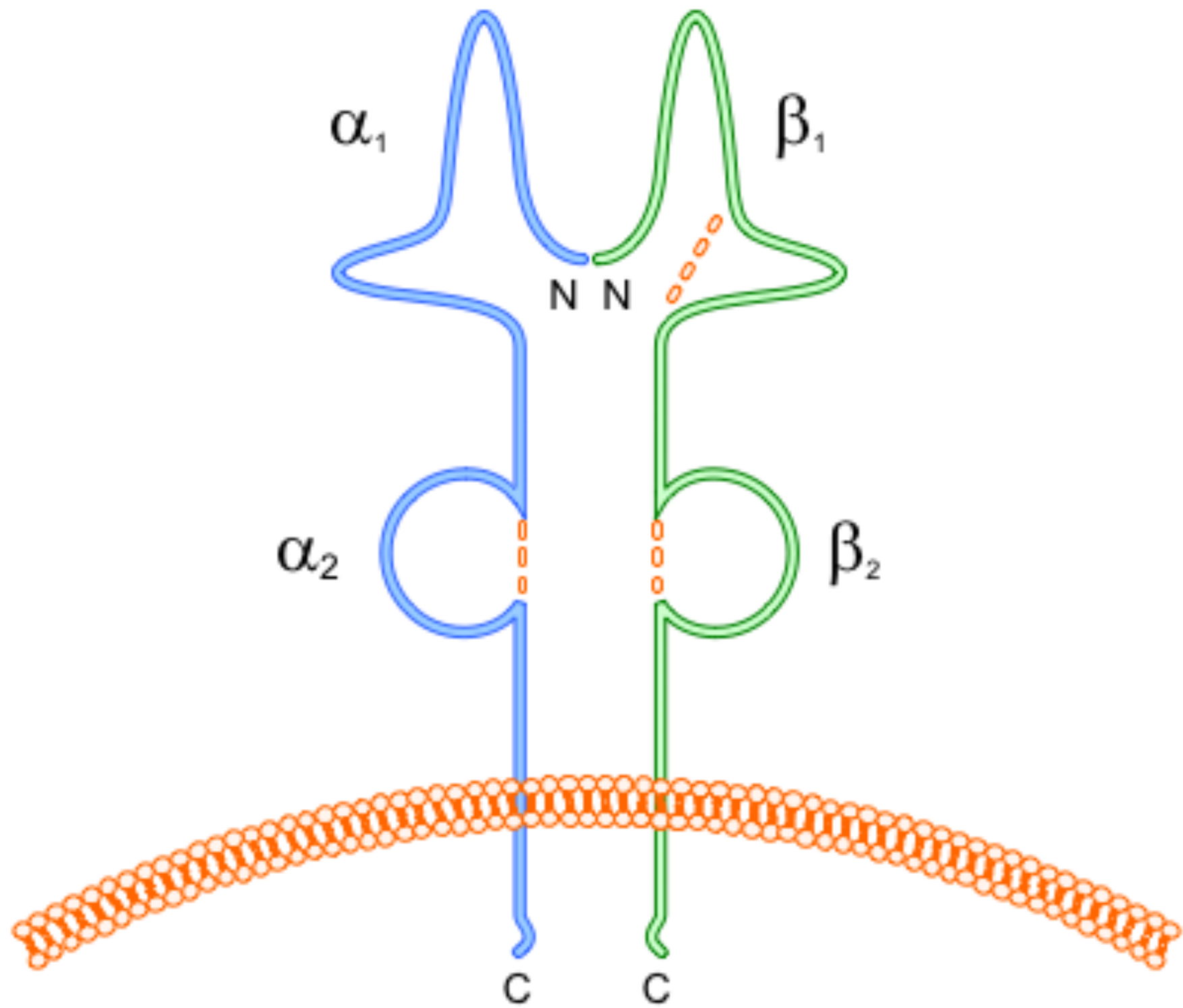
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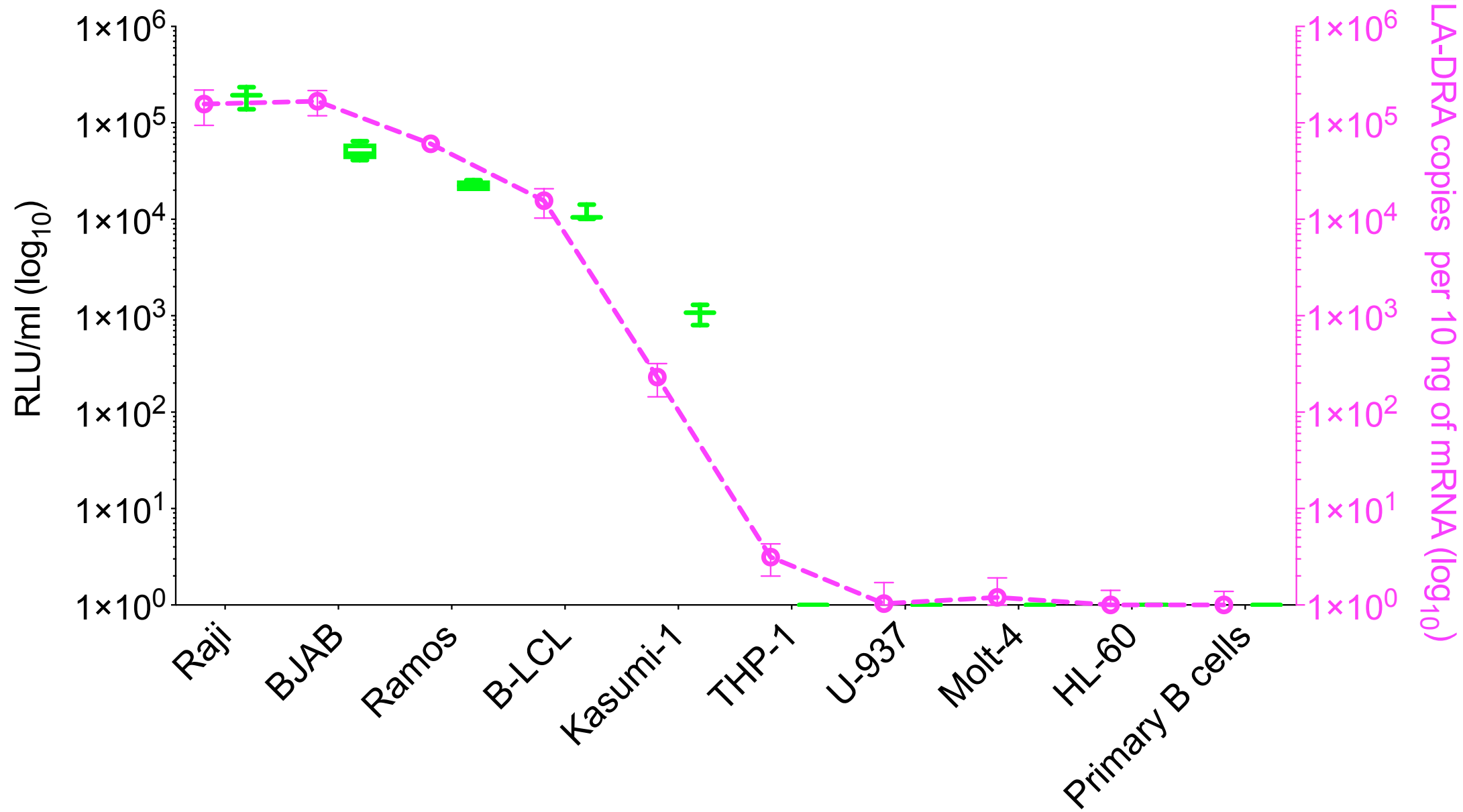
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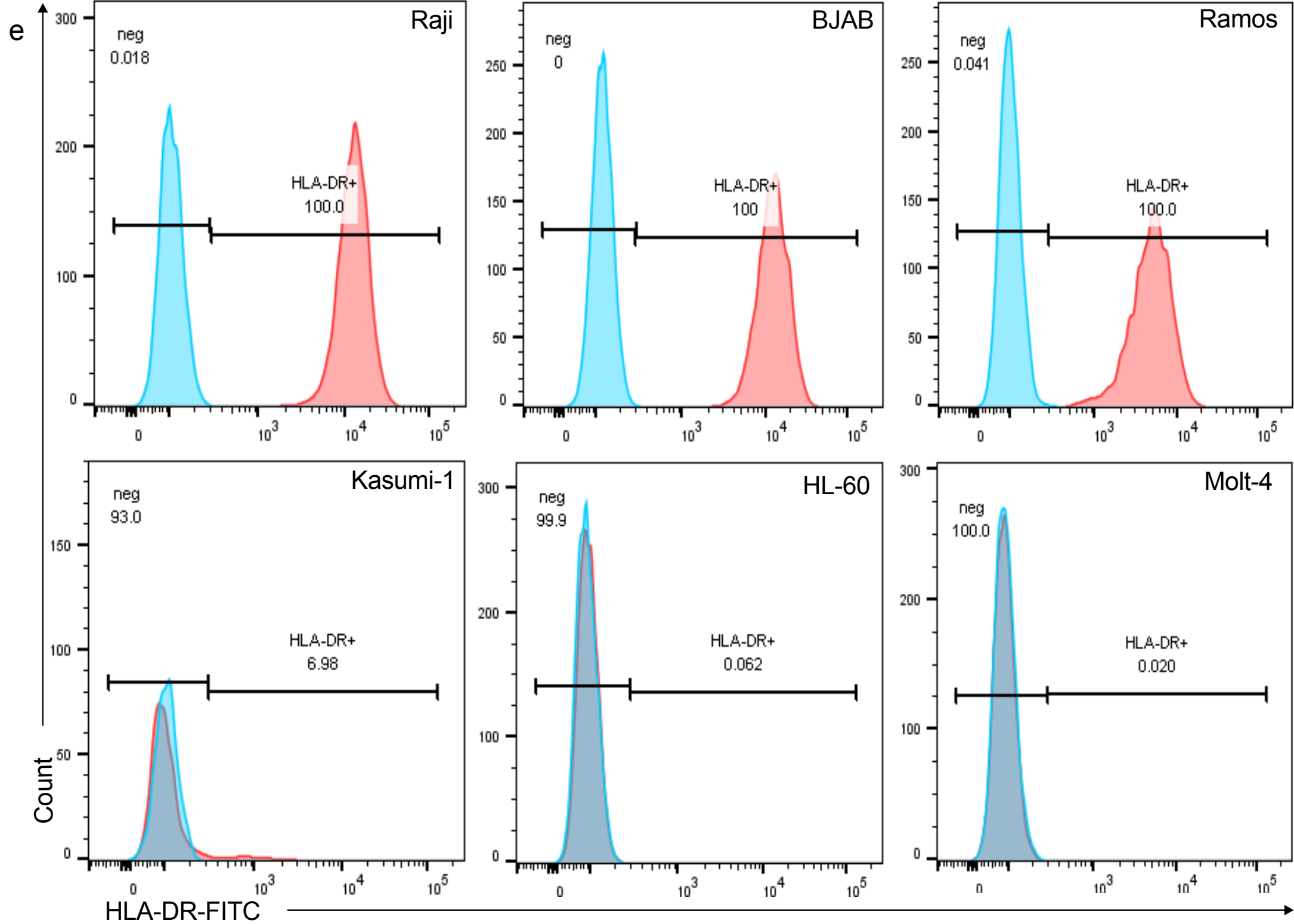


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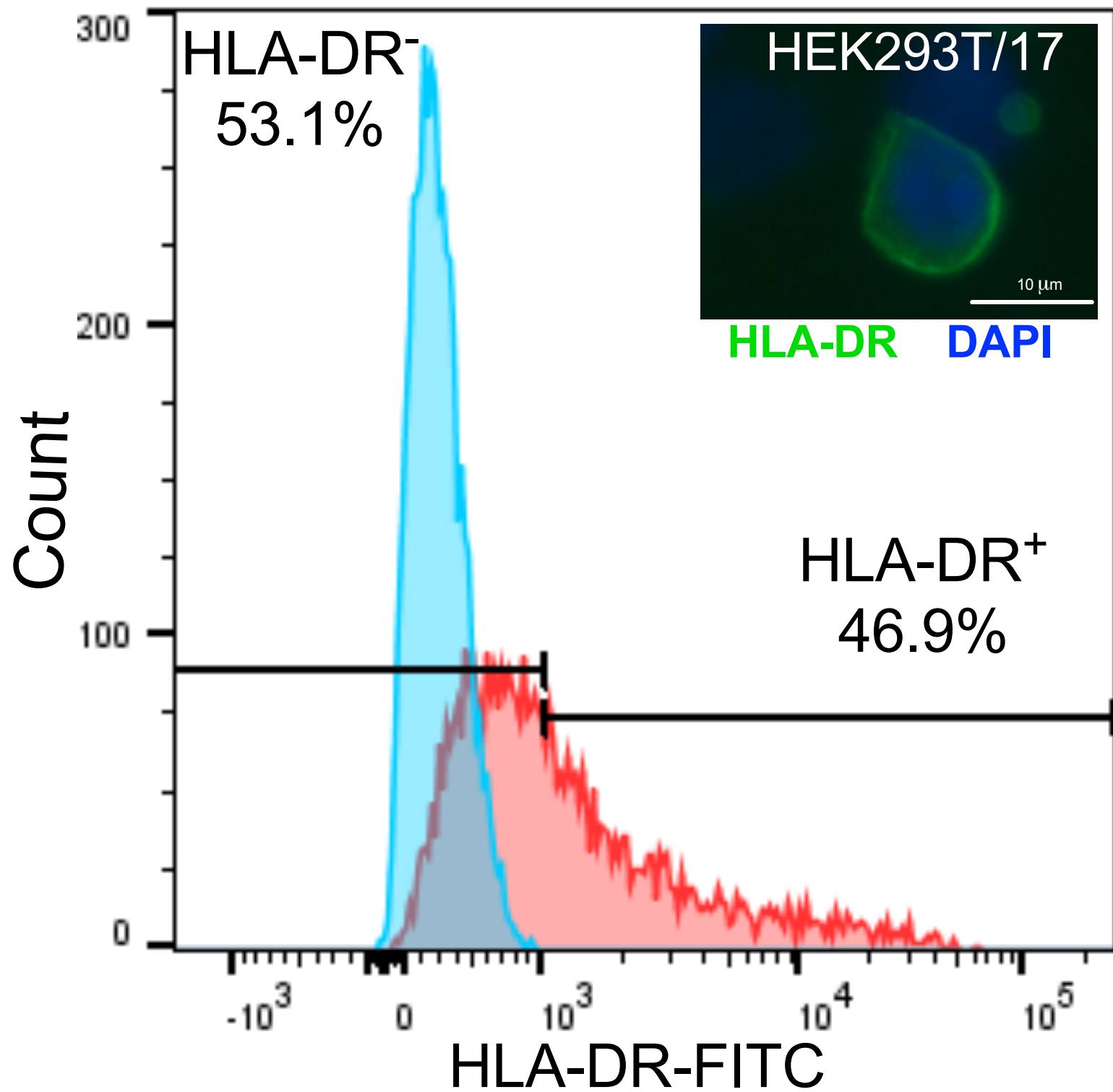


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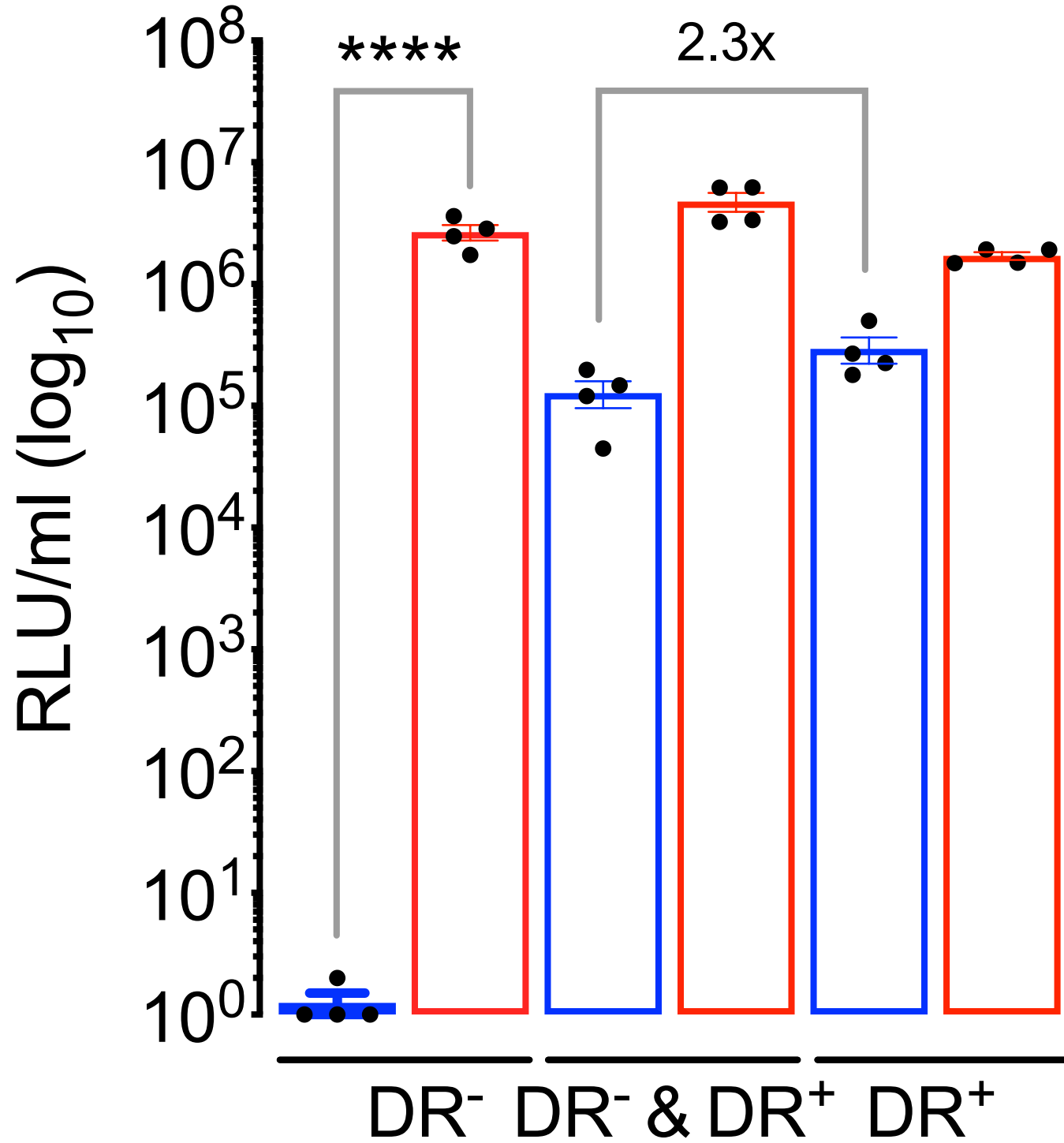


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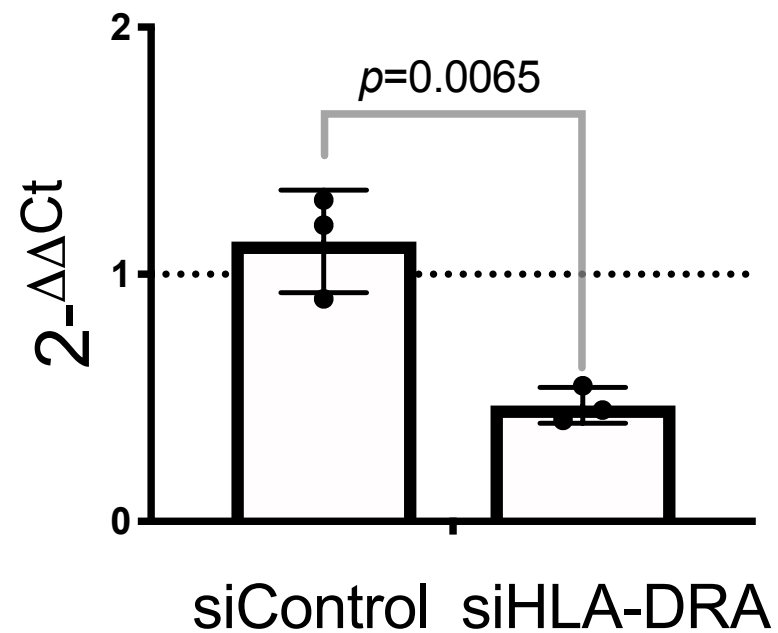
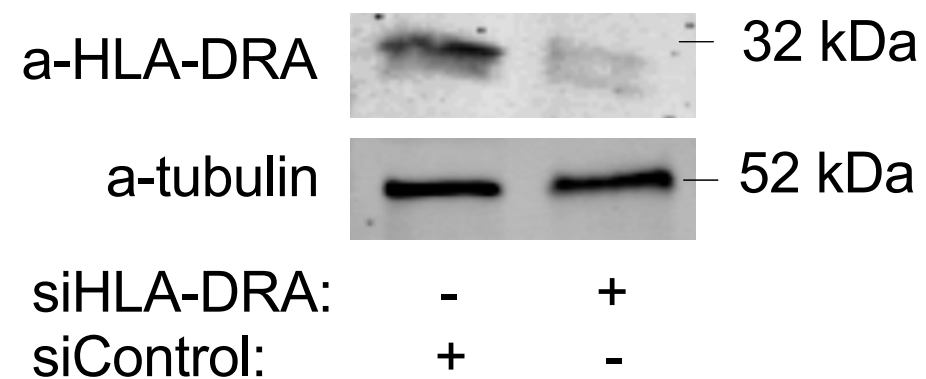
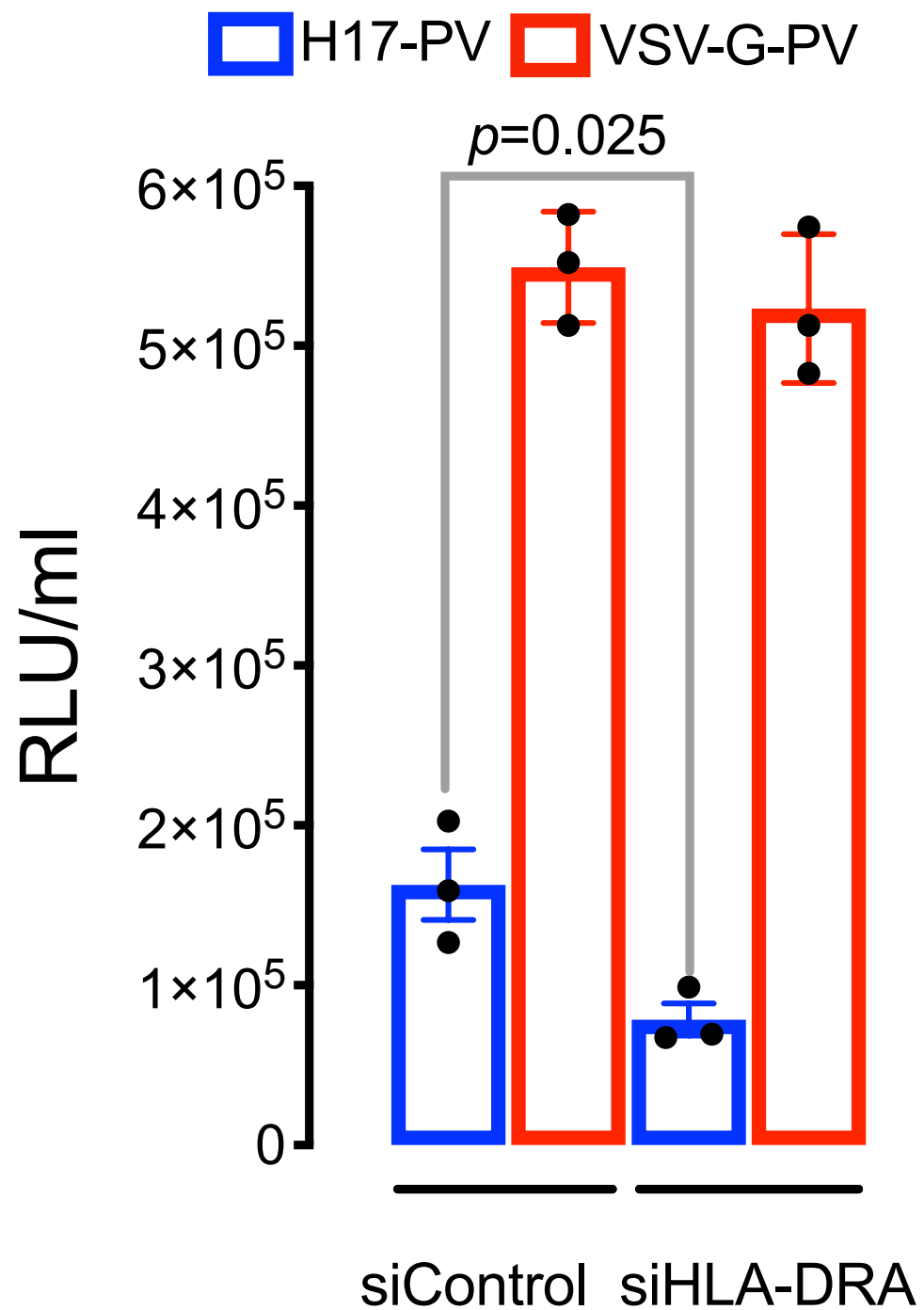


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H17-PV VSV-G-PV



h



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