

1 **Enkurin: A novel marker for myeloproliferative neoplasms from platelet,**  
2 **megakaryocyte, and whole blood specimens.**

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17 **Key Points**

- 18 1. Enkurin, a calcium adaptor protein, is identified as a novel marker of pathogenesis in MPNs.
- 19 2. MPN megakaryocyte and platelet expression of enkurin at RNA and protein levels is inversely  
20 associated with a cell differentiation cycle gene, CDC20.
- 21 3. Likely role for dysregulated calcium homeostasis, and ER and protein folding stress in MPN  
22 transformation.

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33 **Abstract**

34 Impaired protein homeostasis, though well established in age-related disorders, has been linked in  
35 recent research with the pathogenesis of myeloproliferative neoplasms (MPNs). As yet, however,  
36 little is known about MPN-specific modulators of proteostasis, thus impeding our ability for increased  
37 mechanistic understanding and discovery of additional therapeutic targets. Loss of proteostasis, in  
38 itself, is traced to dysregulated mechanisms in protein folding and intracellular calcium signaling at  
39 the endoplasmic reticulum (ER). Here, using *ex vivo* and *in vitro* systems (including *CD34<sup>+</sup>* cultures  
40 from patient bone marrow, and healthy cord/peripheral blood specimens), we extend our prior data  
41 from MPN patient platelet RNA sequencing, and discover select proteostasis-associated markers at  
42 RNA and/or protein levels in each of platelets, parent megakaryocytes, and whole blood specimens.  
43 Importantly, we identify a novel role in MPNs for enkurin (*ENKUR*), a calcium mediator protein,  
44 implicated originally only in spermatogenesis. Our data reveal consistent *ENKUR* downregulation at  
45 both RNA and protein levels across MPN patient specimens and experimental models, with a  
46 concomitant upregulation of a cell cycle marker, *CDC20*. Silencing of *ENKUR* by shRNA in *CD34<sup>+</sup>*  
47 derived megakaryocytes further confirm this association with *CDC20* at both RNA and protein levels;  
48 and indicate a likely role for the *PI3K/Akt* pathway. The inverse association of *ENKUR* and *CDC20*  
49 expression was further confirmed upon treatment with thapsigargin (an agent that causes protein  
50 misfolding in the ER by selective loss of calcium) in both megakaryocyte and platelet fractions at  
51 RNA and protein levels. Together, our work sheds light on enkurin as a novel marker of MPN  
52 pathogenesis beyond the genetic alterations; and indicates further mechanistic investigation into a  
53 role for dysregulated calcium homeostasis, and ER and protein folding stress in MPN transformation.

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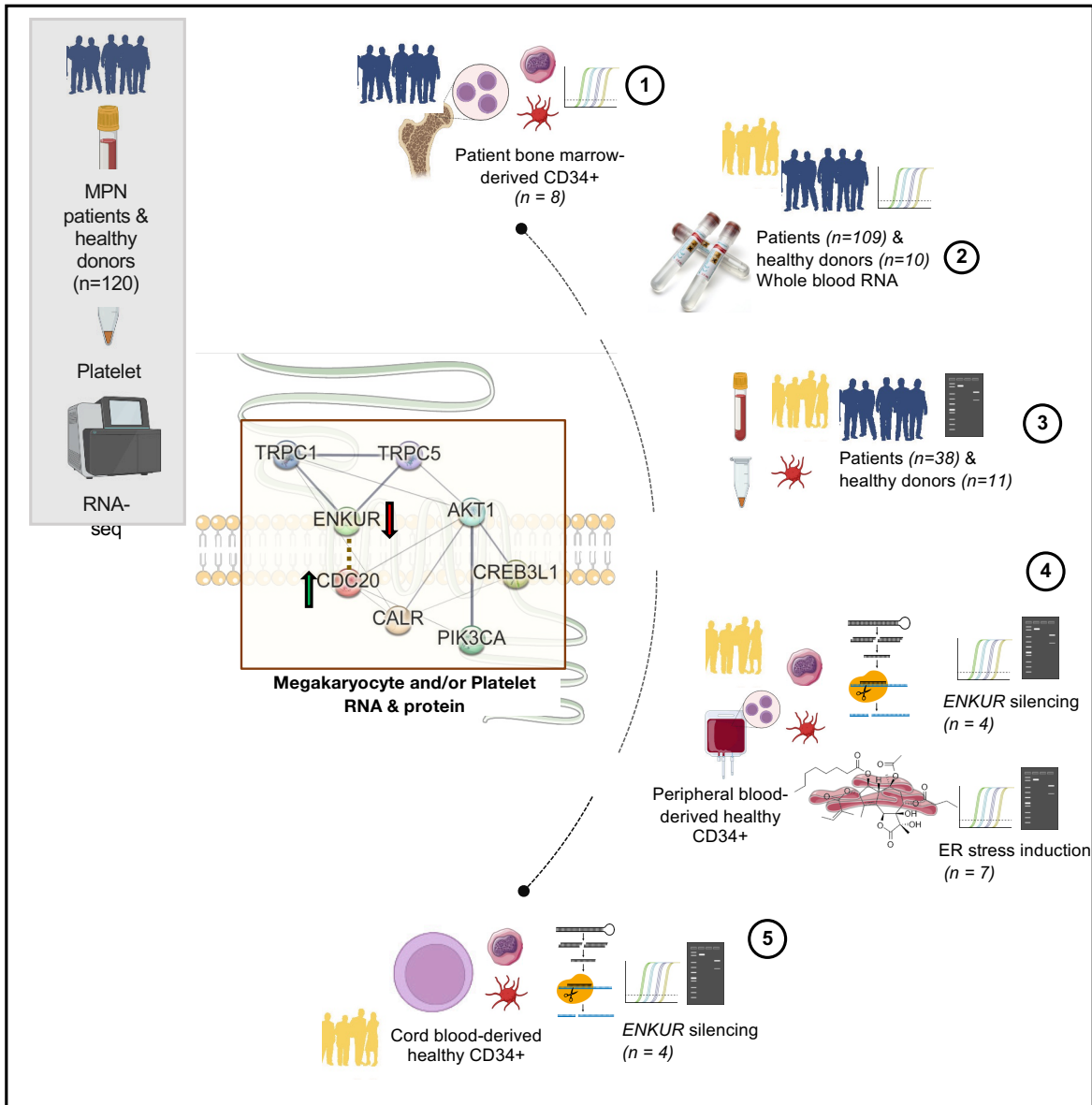
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## VISUAL ABSTRACT



## 63 **Introduction**

64 Myeloproliferative neoplasms (MPNs) are a group of malignant disorders of the bone marrow where a  
65 dysregulated balance between proliferation and differentiation gives rise to abnormal numbers of  
66 blood cells in circulation. Classical MPNs<sup>1,2</sup> are defined by a combination of clinical, laboratory,  
67 morphological and molecular genetic features, and constitute three phenotypic subtypes: essential  
68 thrombocythemia (ET), polycythemia vera (PV), and myelofibrosis (MF, primary or secondary if  
69 transformed from prior ET/PV). Somatic mutations in one of three driver genes (*JAK2*, *CALR*, *MPL*)  
70 constitute their shared molecular genetic pathogenesis; causing constitutive JAK/STAT signaling in  
71 hematopoietic stem cells (HSC)<sup>3-7</sup>. The mutated *CALR* also induces oncogenic effects by binding and  
72 constitutively activating the thrombopoietin receptor and the downstream signaling cascade<sup>7,8</sup>  
73 causing cellular transformation and abnormal megakaryopoiesis. To date, therapeutic strategies have  
74 largely focused on *JAK2* inhibition (e.g. ruxolitinib<sup>9</sup>) and are effective in alleviating MPN symptoms,  
75 but only partially, given their primary role in suppression of inflammation and reduction of  
76 circulating pro-inflammatory cytokines<sup>10,11</sup>. Additional therapeutic strategies that target MPN  
77 pathogenetic mechanisms<sup>12-17</sup> would be critical in resolving patient disease burden.

78  
79 A recent study<sup>18,19</sup> assessing mechanisms of MPN pathogenesis identifies dysregulation of proteostasis  
80 and protein quality control at the endoplasmic reticulum (ER) as crucial transformative events, and  
81 therefore, a strong therapeutic target. However, this study was primarily in mice and remains to be  
82 confirmed in independent additional MPN models and patient-derived specimens. Our recent  
83 investigation, profiling the blood platelet transcriptome in all three subtypes of MPN patients<sup>20</sup>  
84 identified high expression of genes and pathways associated with impaired proteostasis, ER stress and  
85 unfolded protein response (UPR) well-recognized in other age-related disorders<sup>21-25</sup>. Here, we extend  
86 our prior MPN patient platelet transcriptomic data with a focus on genes associated with proteostasis,  
87 cell proliferation, ER stress or calcium signaling at high statistical significance (FDR <0.01), and  
88 representing four types of trends in platelet RNA expression across MPN subtypes: progressively  
89 downregulated, progressively upregulated, consistently upregulated, and uniquely upregulated in MF  
90 alone. Genes selected include i) enkurin, *ENKUR*<sup>26</sup>, a little-studied TRPC (transient receptor potential  
91 cation channel<sup>27,28</sup>) adaptor protein with known function primarily in the context of sperm motility  
92 alone<sup>26,29</sup>, ii) *CALR*, a well-known ER chaperone and calcium binding protein<sup>30-34</sup>, also relevant in the

93 context of megakaryocyte to platelet differentiation<sup>35-38</sup>, iii) cyclic AMP-responsive element-binding  
94 protein *CREB3L1*, an ER-Golgi stress transducer and transcription protein involved in the UPR  
95 response<sup>39-42</sup> and lastly iv) *CDC20*, involved in cell division and proliferation<sup>43-45</sup>, and known to be  
96 upregulated in other cancers<sup>45</sup>. Using MPN patient bone marrow- and healthy cord- and peripheral  
97 blood-derived CD34+ cells differentiated into megakaryocyte and platelet cultures *ex vivo*, we  
98 consistently identify *ENKUR* as a novel peripheral and marrow biomarker in MPNs at both RNA and  
99 protein levels. Our data also demonstrate a negative correlation of *ENKUR* with *CDC20* expression in  
100 the megakaryocyte/platelet cultures, and in independent banked MPN whole blood RNA specimens.  
101 Beyond the utility of enkurin as a potential marker of MPN chronic vs advanced subtypes, more  
102 research is warranted to elucidate the mechanisms of its downregulation in MPNs, and how that may  
103 target cell proliferation pathways.

104

## 105 **Methods**

106 Please see **Table 1** for a tabulated overview of all RNA and protein measurements in this study.

## 107 **Ethics Statement**

108 All MPN patient and healthy donor samples were obtained under written informed patient consent  
109 and were fully anonymized. Study approval was provided by the Stanford University Institutional  
110 Review Board. All relevant ethical regulations were followed.

111

## 112 **Materials**

113 We collected MPN patient bone marrow specimens from the Stanford Cancer Center, and whole  
114 blood from healthy donors at the Stanford Blood Center. Cell culture supplies including plates, fetal  
115 bovine serum, penicillin, streptomycin, phosphate-buffered saline (PBS), and culture medium were  
116 procured from GIBCO BRL (Frederick, MD). Antibodies for CALR, CDC20, p-PI3K, p-Akt, b-Actin  
117 and anti-rabbit goat IgG were procured from Cell Signaling Technologies (Danvers, MA), for enkurin  
118 from Sigma Aldrich (St. Louis, MO) and CREB3L1 from Thermo Scientific (Rockford, IL). Antibodies  
119 for Alexa647-tagged CD41, PE-CD42b, PE-CD61, and PE-CD45 were from BioLegend (San Diego,  
120 CA). shRNA for ENKUR silencing were synthesized from Protein and Nucleic acid facility at  
121 Stanford University (Stanford, CA). The human HEK 293 FT cells and THP1 human macrophage cells  
122 were purchased from the American Type Culture Collection (ATCC, Manassas, VA) and cultured

123 following supplier instructions. The CD34, CD45 microbeads, LS and MS columns and magnetic  
124 separators were purchased from Miltenyi Biotec (Cambridge, MA). Cytokines IL6, thrombopoietin  
125 TPO, and Flt3 ligand were from PeproTech Inc. (Cranbury, NJ); SCF and SFEM II media was from  
126 Stem Cell Technologies (Kent, WA). Lenti-X™ GoStix™ Plus was purchased from TaKaRa Bio Inc.  
127 (San Jose, CA).

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### 129 **Peripheral blood platelet isolation**

130 Peripheral blood was collected in acid citrate-dextrose (ACD, 3.2%) sterile yellow-top tubes (Becton,  
131 Dickinson and Co.) and was processed within 4 h of collection for all samples. Platelets were isolated  
132 by established<sup>46-49</sup> purification protocols. Briefly, the ACD-tube whole blood was first centrifuged at  
133 200xg for 20min at room temperature (RT). The platelet-rich plasma (PRP) was removed and  
134 Prostaglandin E1 was added to the PRP to prevent exogenous platelet activation. The PRP was then  
135 centrifuged at 1000xg for 20min at RT. The platelet pellet was re-suspended in warmed (37 deg C)  
136 PIPES saline glucose (PSG). Leukocytes were depleted using CD45<sup>+</sup> magnetic beads (Miltenyi Biotec).  
137 Isolated platelets were further resuspended in Trizol or LDS buffer for RNA (PCR) and protein  
138 (Western Blot) analyses.

139

### 140 **RNA extraction and quantification from whole blood (PAXgene tubes)**

141 Whole blood from MPN patients and healthy donors was collected into PAXgene tubes (BD  
142 Biosciences) containing RBC lysis buffer . RNA was isolated using PAXgene Blood RNA kit following  
143 manufacturer instructions (762164, PreAnalytix, Switzerland).

144

### 145 **CD34<sup>+</sup> cell isolation from MPN bone marrow and healthy peripheral and cord blood cultures**

146 CD34<sup>+</sup> cells were isolated<sup>50,51</sup> using microbeads positive selection (Miltenyi Biotec) from source  
147 specimens of peripheral blood or cord blood of healthy donors or bone marrow of MPN patients  
148 (collected as part of their clinical care with research consent). Briefly, the RBCs were lysed using  
149 RBC lysis buffer and the cells pelleted. Cells were suspended in MACS running buffer and incubated  
150 with the CD34<sup>+</sup> microbeads for 1 hour on ice and then passed through MACS LS columns pre-  
151 equilibrated with MACS running buffer. CD34<sup>+</sup> cells thus collected were washed using MACS  
152 running buffer and resuspended in the SFEM II media (4-5x10<sup>5</sup> cells/mL) containing TPO (20ng/ml),

153 SCF (25ng/ml) and Gentamicin (1:1000) and plated/transferred to 12 well plates at 37 °C in 5% CO<sub>2</sub>.  
154 On day 3, cells were resuspended in fresh media (SFEM II) containing TPO (20ng/ml), SCF (25ng/ml)  
155 and Gentamicin (1:1000), and on day 6, 9, and 12, in TPO (40ng/ml) and Gentamicin (1:1000). The  
156 cells were harvested on day 15. Cells were collected and centrifuged at 300xg for 10 min to pellet the  
157 larger megakaryocyte fraction and at 3000xg for 30 min for the smaller platelet fraction. With both  
158 peripheral- and cord-blood-derived CD34<sup>+</sup> cells, the isolated cells were expanded using hematopoietic  
159 stem cell expansion media (Cell Genix) containing 100ng/ml of IL6, Flt3 ligand, SCF, and TPO for 5  
160 days before starting the culture. The formation of MK cells and platelets were confirmed by surface  
161 marker analysis with CD41, CD42b and CD61.

162

### 163 **Cell surface marker analysis using flow cytometry**

164 Following isolation of the megakaryocyte and platelet fractions from the 14-day (+ TPO) CD34<sup>+</sup>  
165 culture, cells were collected and washed with PBS. For the CD41, CD42b and CD61 stains, cells were  
166 resuspended in MACS running buffer and stained with Alexa 647 anti-human CD41, PE anti-human  
167 CD42b, and PE anti-human CD61, and incubated at RT protected from light for 45 min. Cells were  
168 washed with PBS and resuspended at a final concentration of  $2 \times 10^7$  cells/ml in PBS, prior to running  
169 the sample on the flow cytometer. All experiments were performed using GUAVA Flow Cytometer  
170 and all flow cytometry analysis was performed in the FlowJo Software. Data from flow cytometry  
171 experiments was acquired by gating for events that were in focus.

172

### 173 **Total RNA isolation and PCR with CD34<sup>+</sup> derived megakaryocyte and platelet fractions**

174 Total RNA was isolated from the megakaryocyte (MK) and platelet fractions using a mirVana RNA  
175 extraction kit (Life Technologies, Grand Island, NY) in accordance with the manufacturer's  
176 instructions. Briefly, MKs/platelets were homogenized into 300µl lysis buffer, then incubated with  
177 30µl homogenate additive for 10 min. RNA was extracted with acid-phenol and column purified,  
178 then washed three times with washing buffer, and eluted in 40µl of sterile elution buffer. The total  
179 RNA was quantified first using a Nanodrop spectrophotometer, then 100ng of total RNAs were  
180 reverse-transcribed using a Reverse-Transcription Kit (Life Technologies) and RT primers to  
181 synthesize cDNA. Then, qRT-PCR was performed using the TaqMan-PCR primers and probe mix  
182 combined with the cDNA derivatives. qRT-PCR was performed through 2 min incubation at 50 °C,

183 then followed by DNA polymerase activation at 95 °C for 10 min; plus 60-cycles at 95 °C for 15 s, and  
184 60 °C for 60 s in the BioRad CFX96 thermocycler system (BioRad). The qRT-PCR reaction procedure  
185 was executed in a 20µl final reaction volume. The expression of target genes was analyzed by the  
186  $2^{-\Delta\Delta C_t}$  method.

187

### 188 **Thapsigargin-induced ER stress**

189 Isolated human CD34<sup>+</sup> cells from the peripheral blood of healthy volunteers were cultured for 15  
190 days with the supplementation of both SCF and TPO for the first 6 days and then with TPO alone till  
191 day 15. Fresh media containing either SCF and/ or TPO was added every 3 days. To induce ER stress  
192 in these cells, known ER stressor drug Thapsigargin (125nM, as optimum dosage identified via  
193 preliminary experiments) was added to the culture on day 7. The culture was maintained till day 15  
194 with media change every 3 days. The cells were harvested and analyzed for gene expression and  
195 immunoblotting for the target proteins.

196

### 197 **Western Blotting for protein expression in megakaryocytes and platelets**

198 Isolated MKs were aliquoted for flow cytometry, RNA expression and western blotting. For western  
199 blotting, an aliquot of the MK cells was pelleted and lysed using cell lysis buffer containing protease  
200 inhibitor cocktail, EDTA. The samples were then resolved on a reducing SDS-PAGE (10%  
201 acrylamide). Blots were stained using appropriate primary (anti-human rabbit antibody; 1:1000 v/v)  
202 and secondary antibodies (anti-rabbit IgG HRP conjugate; 1:2000 v/v).

203 Protein expression was determined based on the detection of a band. The intensity of the protein  
204 bands observed was semi-quantified using IVIS or ImageJ software with normalization of each  
205 protein against beta-actin. Blots were visualized using ECL reagent by Amersham Imager 680 or by  
206 IVIS imaging systems.

207

### 208 **ENKUR gene silencing**

#### 209 **a. Construction of the vector**

210 PLKO1 vector was used to construct the ENKUR-silenced cell lines in HEK293T cells (known for  
211 their ease of transfection and fast growth rate). The PLKO1 vector was cut using EcoR1 and Age1 to  
212 ligate the shRNA using T4 ligase.



213 The shRNA sequences used were:

214 shRNA1: PCCGGTCCGGCCAACCTCGATACTCTTATTTCTCGAGAAATAAGAGTATCG

215 AGGTTGGTTTTTTTTTGG

216 shRNA 2:

217 PCCGGTCCGGCATGGGAGTGGCTAAAAAGCCCTCGAGGGCTTTTTAGCC

218 ACTCCCATGTTTTTTTTTGG

219 Scrambled: PCCGGTCCGGGTGCGTTGCTAGTACCAACTCTCGAGAGTTGGTACTA

220 GCAACGCACTTTTTTTTTGG

221

## 222 **b. Validation of the construct**

223 The silencing effect of the shRNA construct was assessed using HEK293 FT cells by transfection of  
224 the shRNA containing plasmids (pLKO1-shRNA) using lipofectamine method. Briefly, 100K HEK293  
225 FT cells were seeded in a 12-well plate 24h prior to the transfection. Transfection was performed by  
226 adding a master mix containing lipofectamine and the shRNA in reduced serum media for 4 hours,  
227 before adding the complete media. The cells were harvested 48h after transfection and the silencing  
228 effect assessed by western immunoblotting against enkurin.

229

## 230 **Cell culture for ENKUR silencing**

231 HEK293 FT cells were cultured in Dulbecco's Modified Eagle's Medium/high glucose with 10% FBS,  
232 0.1% streptomycin, and 100 U mL<sup>-1</sup> penicillin at 37 °C, 5% CO<sub>2</sub>, and 95% air environment, while  
233 THP-1 cells were cultured in RPMI media containing 10% FBS, 0.1% streptomycin, and 100 U mL<sup>-1</sup>  
234 penicillin at 37 °C, 5% CO<sub>2</sub>, and 95% air environment. The cells were tested for any mycoplasma  
235 contamination using a MycoAlert kit (Lonza, Allendale, NJ), and were maintained at optimum  
236 cultural conditions.

237

## 238 **Production of lentiviral particles**

239 After the confirmation of the silencing effect of the shRNA construct, they were packaged along with  
240 the viral plasmids to produce the lentivirus. Briefly, the lentiviral vectors were transfected to HEK293  
241 FT cells using calcium phosphate method. The cells were supplemented with chloroquine and  
242 HEPES. After 48h of incubation, the media was carefully aspirated, and the viral particles were

243 concentrated by ultracentrifugation. The IFU value was determined by Lenti-X GoStix following  
244 manufacturer instructions (Cat#631280).

245

#### 246 **Validation of the lentivirus silencing in HEK cells**

247 As above, 100K HEK293 FT cells were plated in a 12-well plate 24h prior to lentiviral infection  
248 (transduction). The media was aspirated and lentivirus master mix containing polybrene in reduced  
249 serum media was added to the cells. After 4h of incubation, the cells were supplemented with  
250 complete media and was incubated for 48h before harvesting. The cells were lysed and  
251 immunoblotted against enkurin to assess the gene silencing effect.

252

#### 253 **ENKUR gene silencing in CD34<sup>+</sup> derived megakaryocytes**

254 The shRNA expressing lentiviral particles were infected into the CD34<sup>+</sup> cells in culture on day 9 (MKs  
255 derived from CD34<sup>+</sup> cells) at a ratio of 1:20 (cell: viral particle). The culture was maintained for  
256 another 6 days with supplementation of TPO every 3 days. Cells were harvested on day 15; and  
257 centrifuged at two speeds; low (300xg) and high (3000xg) to separate the MK and platelet fractions  
258 respectively. Each fraction was divided into 3 aliquots for assessing each of cell surface markers (50 –  
259 100K cells), RNA (~50K cells) and protein (100 – 200K cells) expression.

260

#### 261 **Statistical Analyses**

262 Continuous variables from all experiments were assessed for normality. Data that were normally  
263 distributed were expressed as a mean plus or minus the standard error of the mean. For analyses  
264 involving two groups, a parametric two-tailed student t-test was used. When three or more groups  
265 were analyzed, an ANOVA with a Tukey's post-hoc test was performed. When data were not  
266 normally distributed a Mann-Whitney was used when two groups were analyzed while a Kruskal-  
267 Wallis with a Dunn's multiple comparison post-hoc test was used for analyses of three or more  
268 groups. When appropriate a two-way ANOVA with post-hoc test was used as described. Summary  
269 statistics were used to describe the study cohort and clinical variables were expressed as the mean ±  
270 error of the mean or as a number and percentage (%). Statistical analyses were performed by using  
271 GraphPad Prism (version 9, San Diego, CA), and a p-value < 0.05 was considered statistically  
272 significant.

## 273 **Results**

### 274 **Validation of RNA and protein expression of 4 MPN candidate markers**

275 Four candidate markers from our prior platelet RNA-seq analyses<sup>20</sup> were identified based on specific  
276 trends in progressive expression across the 3 MPN subtypes vis-à-vis healthy donors (FDR<0.01,  
277 n=120) independent of patient driver mutational status. *ENKUR* and *CALR*, in particular, were  
278 identified based on opposing trends in progressive expression (**Figure 1A and Figure S1A**), *CDC20* on  
279 its specific high expression in MF patients alone in contrast with healthy donors and ET or PV  
280 patients (**Figure 1A**, top right), and *CREB3L1* based on its substantially increased (> 50-fold)  
281 expression in all MPNs vs healthy (**Figure S1A**, top right). Given that the platelet RNA profile is a  
282 composite of influences from the peripheral circulation as well as of the genetic (and MPN  
283 mutational) profile of megakaryocytes in the bone marrow, we hypothesized *a priori* that our MPN  
284 candidate markers are likely to be variably validated in one versus the other.

285

286 First, in whole blood RNA of MPN patients versus healthy donors, expression of *ENKUR* and *CDC20*  
287 was confirmed by qPCR (**Figure 1B**, n=109, p < 0.005 ) as nearly 6-fold downregulated and 3-fold  
288 upregulated respectively in the advanced MPN subtype, MF. Expression of *CREB3L1* was also  
289 confirmed as over 15-fold upregulated across ET, PV, and MF (**Figure S1B**, right panel), whereas  
290 expression of *CALR* (**Figure S1B**, left panel) was variable and not uniformly consistent with the  
291 platelet transcriptome (likely owing to lower resolution for detection of transcripts in whole blood).  
292 Next, in assessing platelet protein levels, trends in expression of ENKUR was significantly different in  
293 PV and MF versus healthy donors, and CDC20 in ET and MF (**Figure 1C**), whereas CALR and  
294 CREB3L1 were not significantly different between patient and healthy donor specimens (**Figure S1C**).

295

### 296 **Megakaryocyte and platelet fractions from CD34<sup>+</sup> cultures of MPN patient bone marrow**

297 To further delineate RNA expression of the candidate markers as specific to platelets in circulation  
298 alone or also in marrow-derived megakaryocytes, we established an *ex vivo* culture of CD34<sup>+</sup> stem  
299 cells from MPN patient bone marrow (n=8, 4 MF) and generated megakaryocyte and platelet  
300 fractions (**Figure 2A-B**, and Methods and **Figure S2A-B** for flow cytometric and histological  
301 confirmation of cultured megakaryocyte and platelet fractions). Given the variable differentiation  
302 states of the nascent platelet fraction and its lower cell quantity than the megakaryocytic fraction, we

303 focused entirely on the megakaryocytes harvested. Downregulation of *ENKUR* was recapitulated in  
304 these patient-derived megakaryocytic RNA (mean approx. 2-fold reduction in MF alone and 5-fold all  
305 MPN) with associated upregulation of *CDC20*, *CREB3L1* (mean of approx. 1-fold) and *CALR* (~4-  
306 fold) in all MPN (n=8, **Figure 2C**).

307

### 308 ***ENKUR* gene silencing**

309 To better evaluate the negative correlation between *ENKUR* and *CDC20/CALR* expression, we  
310 sought lentiviral knockdown in primary CD34<sup>+</sup> cells (derived from healthy peripheral blood and cord  
311 blood specimens) using two distinct *ENKUR* shRNA constructs. **Figure 3A-B** describes our  
312 experimental framework. The shRNAs were first expressed in pLKO.1 plasmid that was amplified and  
313 isolated from *E.coli*, and the lentiviral plasmids were then transfected into HEK293 FT cells to  
314 generate the lentivirus. *ENKUR* shRNA silencing and downregulation of enkurin protein was  
315 confirmed at a concentration of 1:20 HEK cell:viral particle. Primary CD34<sup>+</sup> cells from healthy  
316 peripheral (n=4) and cord blood (n=4) specimens were cultured at four conditions: i) control with no  
317 *ENKUR* silencing, ii) and iii) lentiviral knockdown with the two shRNA constructs, and finally iv)  
318 scramble control shRNA. Where relevant, shRNA infection was introduced at day 7 of culture and  
319 continued for another 7 days (totaling a 15-day culture, **Figure 3B**). Megakaryocyte and platelet  
320 fractions thus derived were confirmed by flow cytometric analysis (**Figure 4A**). Downregulated RNA  
321 expression of *ENKUR* was significantly associated with concomitant high expression of *CDC20* in  
322 both of the megakaryocyte and platelet fractions (**Figure 4B**). Cell quantity in the platelet fraction  
323 was just sufficient for RNA qPCR measurements alone, and therefore, protein levels could be assessed  
324 only in the megakaryocytes. Inverse correlation between *ENKUR* and *CDC20* was validated even in  
325 megakaryocytic protein expression (**Figure 4C**). However, *ENKUR* silencing alone (without the  
326 associated patient factors in our *in vitro* experiments, e.g. mutational status) was insufficient to  
327 generate a statistically significant differential in *CALR* or *CREB3L1* expression at both RNA and  
328 protein levels (**Figure S3A-B**).

329

330 One classical downstream pathway linked previously to ER stress response<sup>18,19</sup> in MPNs<sup>52</sup> and other  
331 cancers<sup>53-55</sup> is the PI3K/Akt signaling cascade. Here with sh*ENKUR*, we briefly evaluated possible

332 association with the PI3K/Akt pathway and found that silencing of *enkurin* correlated with increased  
333 expression of phosphorylated PI3K in megakaryocytes (**Figure 4C**).

334

### 335 **Effect of ER stress on CD34<sup>+</sup>-derived megakaryocytes**

336 Considering that the differential and inversely correlated expression of *ENKUR* and *CDC20* extends  
337 to the CD34<sup>+</sup> cell-derived megakaryocytes, and our hypothesis on the role of ER stress in this  
338 response, we assessed RNA and protein levels of these markers following treatment of the cells with  
339 the sarco/endoplasmic reticulum Ca<sup>2+</sup>-ATPase inhibitor, thapsigargin (125nM). CD34<sup>+</sup> cells from  
340 peripheral blood of healthy donors were differentiated into megakaryocyte and platelet fractions,  
341 with thapsigargin introduced at day 7 of a 15-day culture (see Methods and **Figure 5A**; limited cell  
342 quantity of the platelet fraction enabled protein analyses in the megakaryocytic fraction alone).  
343 Thapsigargin evoked a similar expression profile in megakaryocytes (**Figure 5B-C**) as that of MPN  
344 patient bone marrow-derived CD34<sup>+</sup> cells with downregulated *ENKUR* and upregulated *CDC20*  
345 expression at both RNA and protein levels.

346

### 347 **Discussion**

348 Here, we present the first report, to our knowledge, of *enkurin* (*ENKUR*) as a potential new  
349 peripheral biomarker and therapeutic strategy in myeloproliferative neoplasms. Our prior study<sup>20</sup>  
350 profiling the platelet transcriptome in patients with chronic MPNs (n=120) identified progressive  
351 association in expression of several proteostasis-associated genes with advancing disease subtype. Our  
352 findings were consistent with other studies demonstrating dysregulated proteostasis as a primary  
353 effector of myeloid transformation<sup>18,56,19,57</sup>. The MPN platelet RNA-seq data also confirmed the limited  
354 impact<sup>58</sup> of treatment by JAK2-inhibitor ruxolitinib (RUX) relative to the substantial disease burden  
355 in myelofibrosis; and urged the need for development of novel candidate drugs to be used alone or in  
356 combination with RUX for the treatment of MPNs.

357

358 Four candidate markers from the prior study (*ENKUR*, *CDC20*, *CALR*, and *CREB3LI*) were evaluated  
359 in this work based not only on their significant association with pathways related to proteostasis, cell  
360 proliferation, ER stress or calcium signaling, but also progressive increase or decrease in differential  
361 expression across the MPN chronic vs advanced subtypes (ET/PV to MF versus healthy donors)

362 regardless of patient *JAK2/CALR* mutational status. Using CD34<sup>+</sup> cells isolated from MPN patient  
363 bone marrow as well as healthy donor peripheral and cord blood, we generated *ex vivo*  
364 megakaryocyte and platelet fractions and discovered at both RNA and protein levels, an inverse  
365 correlation between *ENKUR*, a calmodulin and TRPC channel modulator<sup>26,29</sup> (not previously  
366 associated with MPNs) and *CDC20* (also termed Fizzy<sup>44</sup>), a cell cycle gene and an anaphase promoting  
367 complex activator (with known associations with malignancies broadly<sup>43,59</sup> and select studies in  
368 MPNs<sup>60</sup>). Lentiviral-mediated silencing of *ENKUR* was utilized to further confirm the negative  
369 association between *ENKUR* and *CDC20*; and indicating a potential interaction via the PI3K-Akt  
370 pathway (**Figure 6**). Our data on the inverse association between enkurin, a membrane calcium influx  
371 adaptor protein, and *CDC20*, a crucial cell cycle and proliferation regulator point to a role for  
372 dysregulated calcium signaling in MPNs; and the possibility for the ratio of expression between the  
373 two to be developed as a potential biomarker of MPN disease.

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375 Next, in evaluating calreticulin (*CALR*), a well-established driver mutation in MPNs<sup>31,61</sup>, as well as  
376 *CREB3L1*, an ER/UPR stress response transcription factor, high expression noted previously across  
377 MPN patient platelet RNA-seq<sup>20</sup> was confirmed in patient-derived specimens (platelet, whole-blood,  
378 and bone-marrow-CD34<sup>+</sup>-cultured megakaryocytes) but only at the RNA (and not protein) levels.  
379 Future investigations applying genome editing in MPN models will be needed to ascertain  
380 overlapping mechanisms of somatic alterations in *CALR* and its effect on function in altered calcium  
381 signaling and dysregulated ER protein folding<sup>62,63</sup> in MPNs.

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383 Taken together, we offer two inversely associated MPN markers, *ENKUR* and *CDC20*, whose whole  
384 blood, platelet and megakaryocyte RNA and protein expression reflect MPN pathobiology  
385 irrespective of patient driver mutational status. These candidate markers also seek to expand our  
386 understanding of MPN pathology beyond the classical inflammatory signatures<sup>64-67</sup>.

387

### 388 **Limitations of the study**

389 There are several limitations to our study. First, higher statistical power and longitudinal prospective  
390 data will be necessary to further confirm *ENKUR/CDC20* as markers for MPN disease progression.  
391 Second, future investigations evaluating these signatures in patient-derived CD34<sup>+</sup> cells may identify

392 additional functional aspects of bone marrow and MPN pathology. Third, we recognize that our data  
393 are not assessing mechanistic signaling of how loss of *ENKUR* expression or impaired calcium  
394 signaling contributes to myeloproliferation; and will need to be specifically interrogated in MPN  
395 murine and other models. Follow-on studies evaluating a potential role of the *PI3K/Akt* pathway, in  
396 particular the effect of pharmaceutical inhibitors already in use in MF will add significant value to  
397 the current study. Studies evaluating platelet function under impaired enkurin expression and  
398 calcium modulation are promising immediate future directions. And above all, it will be important to  
399 assess how enkurin downregulation might be more broadly relevant to other hematological cancers,  
400 such as myelodysplastic syndromes and acute myeloid leukemia, or potentially unique to MPNs  
401 alone.

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422 **Acknowledgements**

423 This work was funded by the MPN Research Foundation and the US National Institutes of Health  
424 grants 1K08HG010061-01A1 and 3UL1TR001085-04S1 (research re-entry award) to A.K. Other  
425 support included 1S10OD018220 and 1S10OD021763 shared instrumentation grants to the Stanford  
426 Functional Genomics Facility and the Stanford Research Computing Center, and the Charles and Ann  
427 Johnson Foundation to J.G. All authors thank the patients at the Stanford Cancer Center and the  
428 healthy donors at the Stanford Blood Center for their generous participation in this research, and the  
429 Canary Center at Stanford for Cancer Early Detection for access to flow cytometry and microscopy.  
430 Authors also thank Dr. Diwash Jangam for his assistance with the initial Paxgene PCR setup.

431

432 **Author Contributions** AK conceived of the study and the team, secured funding, and wrote the  
433 manuscript. LF and JG provided samples and clinical annotation and reviewed the clinical data. AK  
434 and RP coordinated and oversaw sample acquisition and processing. SS, YL, JW, and KM performed  
435 the experiments and interpreted the analyses with AK, RP, HM, JG, and JZ. All authors critically  
436 reviewed and edited the manuscript. #SS and YL contributed equally. #JG and JZ also contributed  
437 equally. All authors approved the final manuscript.

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440 **Conflict of Interest Disclosures:** Authors declare no conflict of interest.



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47 PMC6913459 Celgene, Apexx Oncology, CTI Biopharma, and Inctye and research funding from Roche,  
48 Genentech, Incyte, Celgene, Imago Biosciences, Samus Therapeutics, and Gilead.

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2 **Tables**

3 **Table 1:** Summary of all cell types used, their sources and the respective analyses performed.

4

Table 1: Experimental Framework and Components							
#	Cell type	Source	Megakaryocyte (MK) culture	ENKUR silencing	ER stress induction	RNA expression (MK and/or Platelet)	Protein expression (MK and/or Platelet)
1	Platelets	Peripheral blood (n = 49)				X <i>CALR, CREB3L1, CDC20, ENKUR</i>	X CALR, CREB3L1, CDC20, ENKUR
2	CD34 <sup>+</sup>	Bone marrow (n = 8)	X			X <i>CALR, CREB3L1, CDC20, ENKUR</i>	
3	CD34 <sup>+</sup>	Cord blood (n = 4)	X	X		X <i>CALR, CREB3L1, CDC20, ENKUR</i>	X CALR, CREB3L1, CDC20, ENKUR, PI3K and Akt
4	CD34 <sup>+</sup>	Peripheral blood (n = 7)	X	X	X	X <i>CALR, CREB3L1, CDC20, ENKUR</i>	X CALR, CREB3L1, CDC20, ENKUR, PI3K and Akt

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1 **Figure Legends**

2 **Figure 1: RNA expression in platelets and whole blood of MPN patients and protein expression in**  
3 **platelets**

4 (A) MPN Platelet RNA-seq normalized expression data: MPN patients (total n=99: ET n=24; PV n=33;  
5 MF n=42) vs healthy donors (n=21) (B) whole blood RNA: MPN patients (total n=109: ET n=39; PV  
6 n=38; MF n=32) vs healthy donors (n=10) (C) platelet protein: peripheral blood platelets were isolated  
7 from MPN patients (total n=38: MF n=12; PV n=19; ET n=7) and healthy donors (n=11). Bonferroni  
8 multiple comparisons adjusted p values \*\*\*\*p<0.0001, \*\*\*p<0.001 and \*\*p<0.01 when compared with  
9 controls (healthy donors). All RNA expression is normalized to GAPDH and expressed as log2 fold  
10 change.

11

12 **Figure 2: Culturing of CD34<sup>+</sup> cells from the bone marrow of MPN patients and RNA expression**

13 (A) Microscopic images of the CD34<sup>+</sup> cells isolated from the bone marrow of MPN patients over the  
14 15-day culture period at 20X magnification. Notice the formation of megakaryocytes indicated by the  
15 red arrow. (B) Flow cytometric analysis of cell surface markers to confirm the production of  
16 megakaryocyte and platelet fractions after 15 days of culture. (C) megakaryocytic RNA: cell culture  
17 from fresh MPN patient bone marrow (n=8) derived CD34<sup>+</sup> cells then differentiated over 15 days into  
18 megakaryocytes. All RNA expression is normalized to GAPDH and expressed as log2 fold change.  
19 Bonferroni adjusted p values \*\*p<0.01, \*p<0.05.

20

21 **Figure 3: Experimental design and workflow of Lentiviral transduction-induced silencing of ENKUR**  
22 **gene in CD34<sup>+</sup> stem cells**

23 (A) Workflow of the shRNA construction and generation of the lentivirus (B) experimental plan for  
24 the silencing of the ENKUR gene in CD34<sup>+</sup> stem cells. The CD34<sup>+</sup> cells were isolated from both  
25 peripheral and cord blood.

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1 **Figure 4: Lentiviral transduction induced silencing of ENKUR gene in CD34<sup>+</sup> stem cells**

2 (A) Flow cytometric analysis of cell surface markers to confirm the megakaryocytic and platelet  
3 fractions after 15 days of culture. (B) RNA expression levels in the CD34<sup>+</sup> cell-derived megakaryocytes  
4 and platelets. (C) (i) Western blot of the CD34<sup>+</sup> cells derived megakaryocytes (n=4 cord blood and n=4  
5 peripheral blood) and (ii) densitometry for the blots. Densitometry was performed using the IVIS  
6 imaging software (\*\*\*\*p<0.0001, \*\*\*p<0.001, \*\*p<0.01 and \*p<0.05 when compared with non-silenced  
7 controls).

8

9 **Figure 5: Effect of ER stress on megakaryocytes derived from CD34<sup>+</sup> cells from healthy donors**

10 (A) Representative microscopic images of the CD34<sup>+</sup> cells isolated from the peripheral blood of healthy  
11 donors over the 15-day culture period at 20X magnification. The ER stressor, thapsigargin (125nM) was  
12 added to the culture at day 7 and cultured for another 7 days. (B) RNA expression: cell culture from  
13 thapsigargin-treated CD34<sup>+</sup> derived megakaryocytes (n=7). (C) (i) Western blot of the thapsigargin-  
14 treated CD34<sup>+</sup> cells derived megakaryocytes (n=3 in triplicate) and (ii) densitometry for the blots.  
15 Densitometry was performed using the ImageJ software (\*\*\*\*p<0.0001, \*\*p<0.01 when compared with  
16 untreated controls).

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18 **Figure 6: Probable mechanism for the role of ENKUR downregulation in cell proliferation**

19 We hypothesize that, under normal conditions, enkurin at sufficient physiological levels is bound to  
20 PI3K via the SH3 binding domain, thus regulating its activation<sup>26</sup>. However, in the setting of MPNs,  
21 downregulation of enkurin likely contributes to free PI3K and its prolonged activation, further  
22 activating Akt and potentially other signaling mechanisms prompting overexpression of cell  
23 differentiation cycle genes including CDC20<sup>68</sup> and cell proliferation.

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1 **Supplementary Figure legends**

2 **Figure S1: RNA expression in platelets and whole blood of MPN patients and protein expression in**  
3 **platelets**

4 (A) MPN Platelet RNA-seq Normalized Expression Data: MPN patients (n=99: ET n=24; PV n=33; MF  
5 n=42) vs healthy donors (n=21) (B) whole blood RNA: MPN patients (n=109: ET n=39; PV n=38; MF  
6 n=32) vs healthy donors (n=10) (C) platelet protein: peripheral blood platelets were isolated from  
7 MPN patients (n=38: MF n=12; PV n=19; ET n=7) and healthy donors (n=11).

8

9 **Figure S2: Culturing of CD34<sup>+</sup> cells from the bone marrow of MPN patients and RNA expression**

10 (A) Confocal microscopy: Microscopic images of the CD34<sup>+</sup> cells isolated from the bone marrow of  
11 MPN patients over the 15-day culture period at 20X magnification. The cells were treated with cell  
12 surface markers for CD34<sup>+</sup> cells (CD34), Megakaryocytes (CD41, CD42b, and CD61) (B)  
13 Quantification of CD41<sup>+</sup> megakaryocytes from CD34<sup>+</sup> cells isolated from the bone marrow of MPN  
14 patients over the 15-day culture period (C) Megakaryocytic RNA: cell culture from fresh MPN  
15 patient bone marrow (n=8) derived CD34<sup>+</sup> cells then differentiated over 15 days into  
16 megakaryocytes. All RNA expression is normalized to GAPDH and expressed as log<sub>2</sub> fold change.

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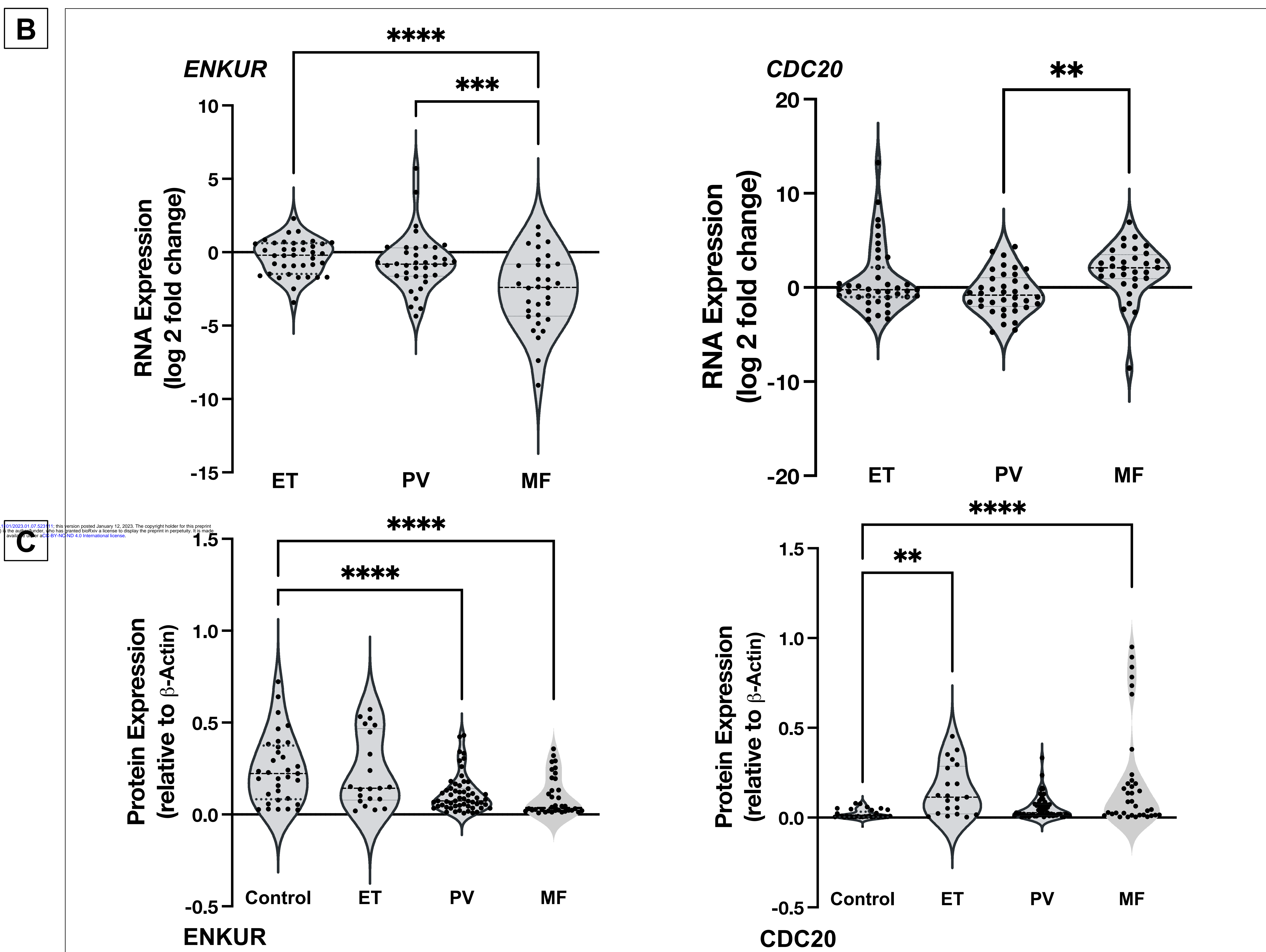
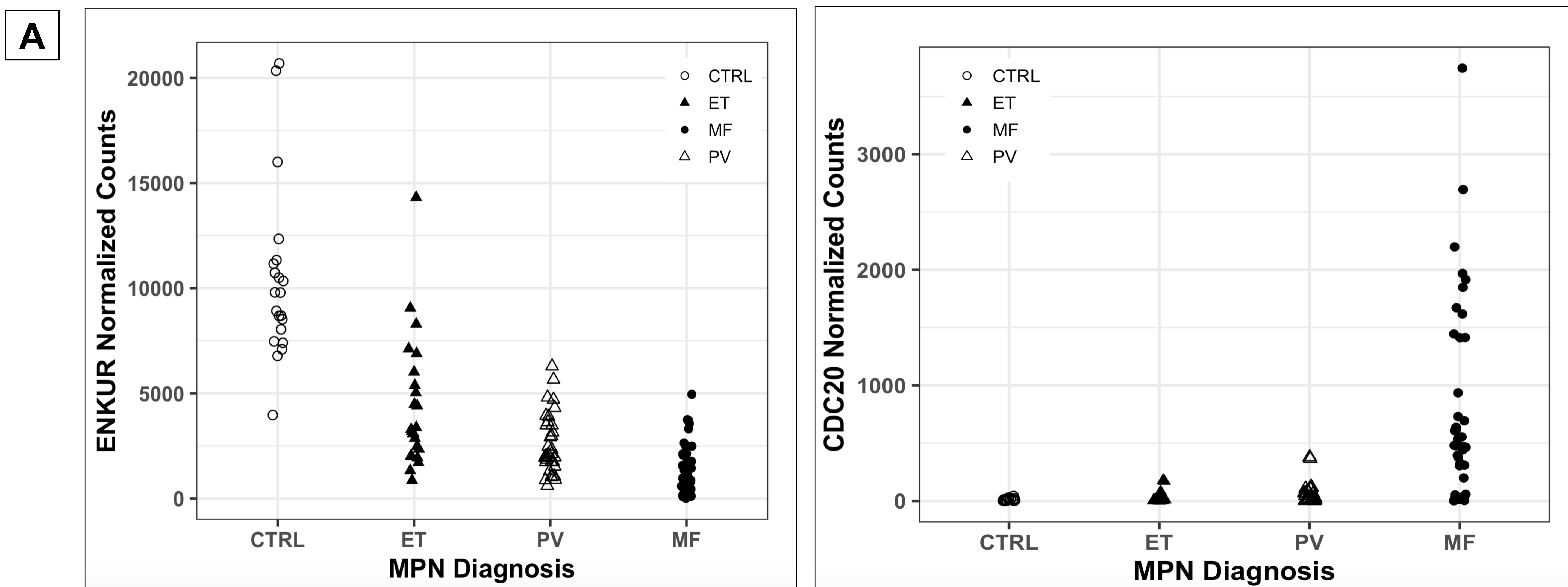
18 **Figure S3: Effect of Lentiviral transduction induced silencing of ENKUR gene on CALR and CREB3L1**  
19 **gene expression in CD34<sup>+</sup> stem cells**

20 (A) RNA expression levels in the CD34<sup>+</sup> cell-derived megakaryocytes and platelets. Log<sub>2</sub> fold change  
21 was plotted by normalizing with the control (B) (i) Western blot of the CD34<sup>+</sup> cell-derived  
22 megakaryocytes (n=4 cord blood and n=4 peripheral blood) and (ii) densitometry for the blots.  
23 Densitometry was performed using IVIS imaging software.

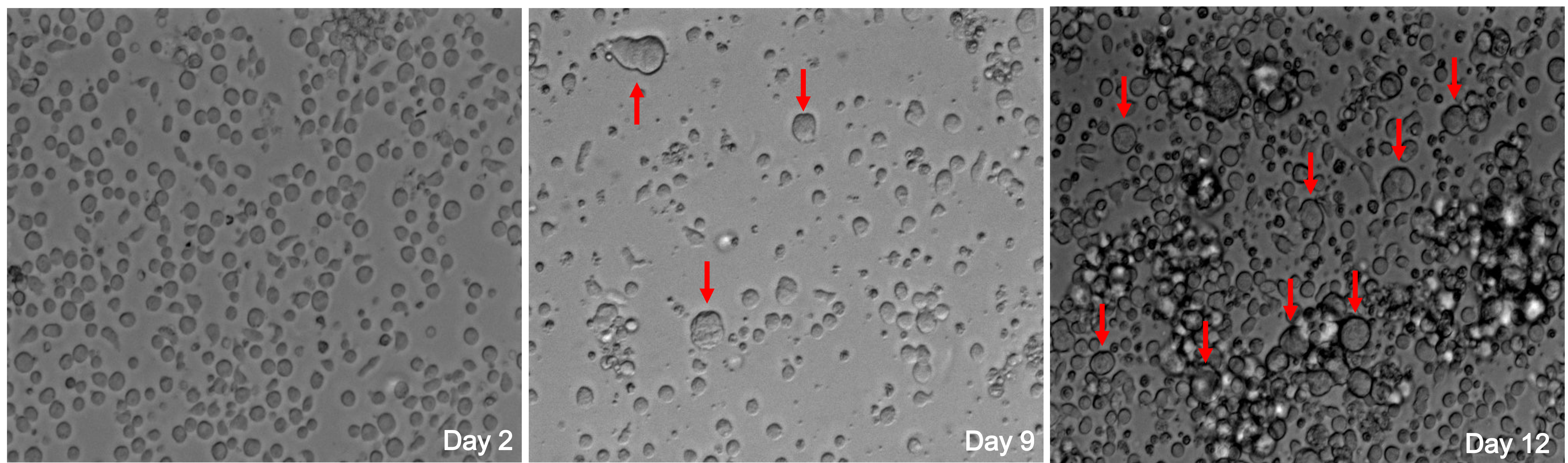
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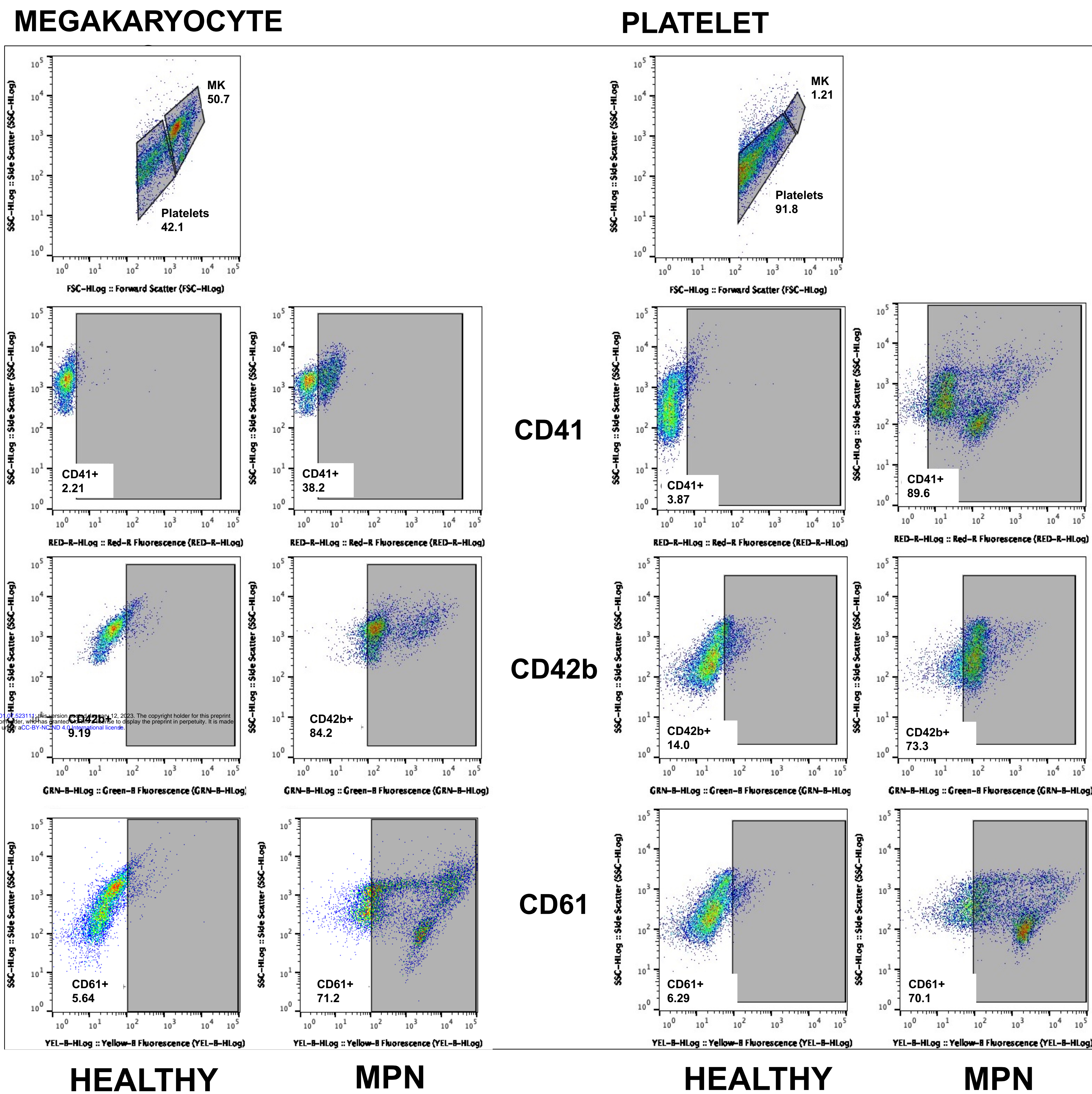
**FIGURE 1**



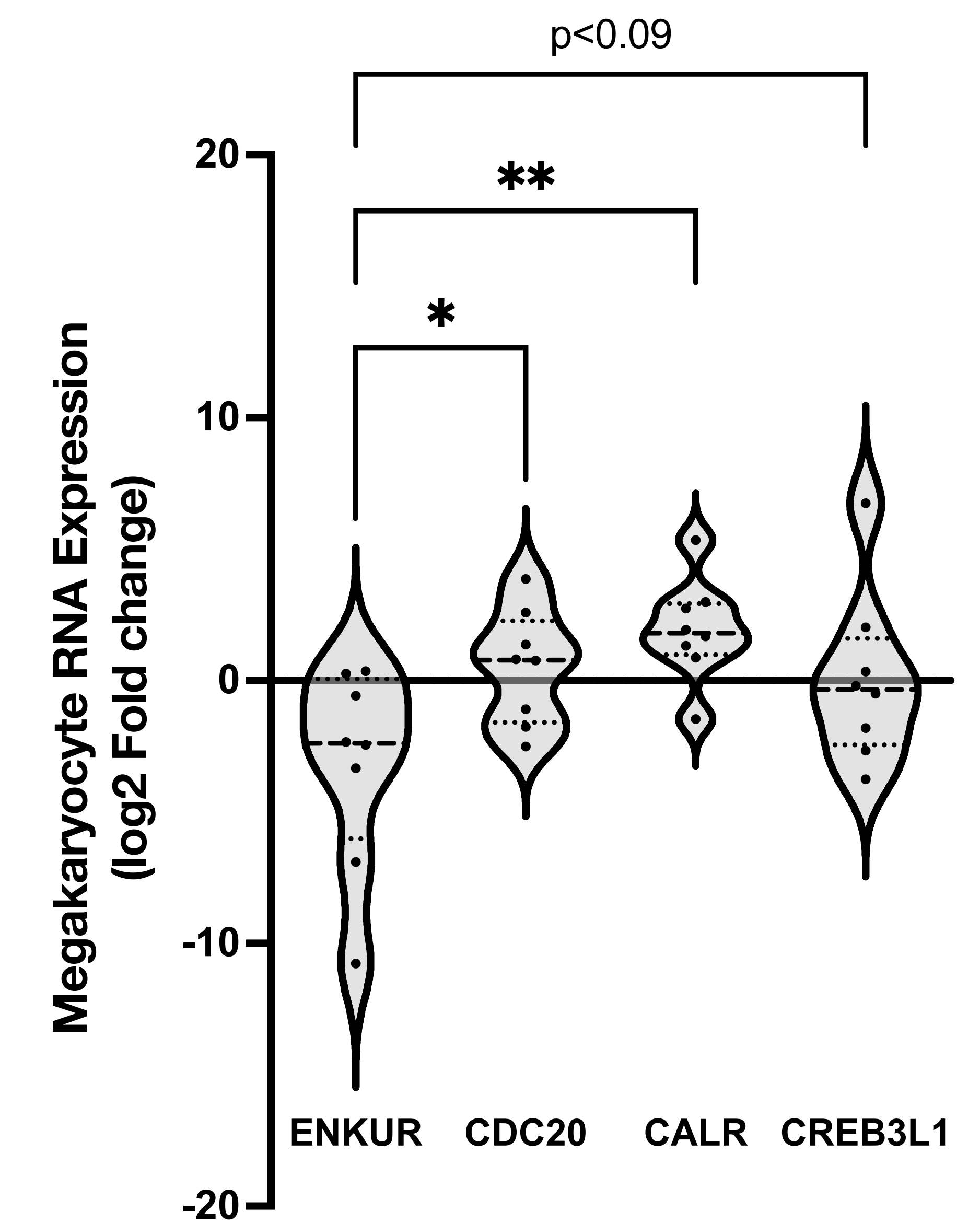
A



B

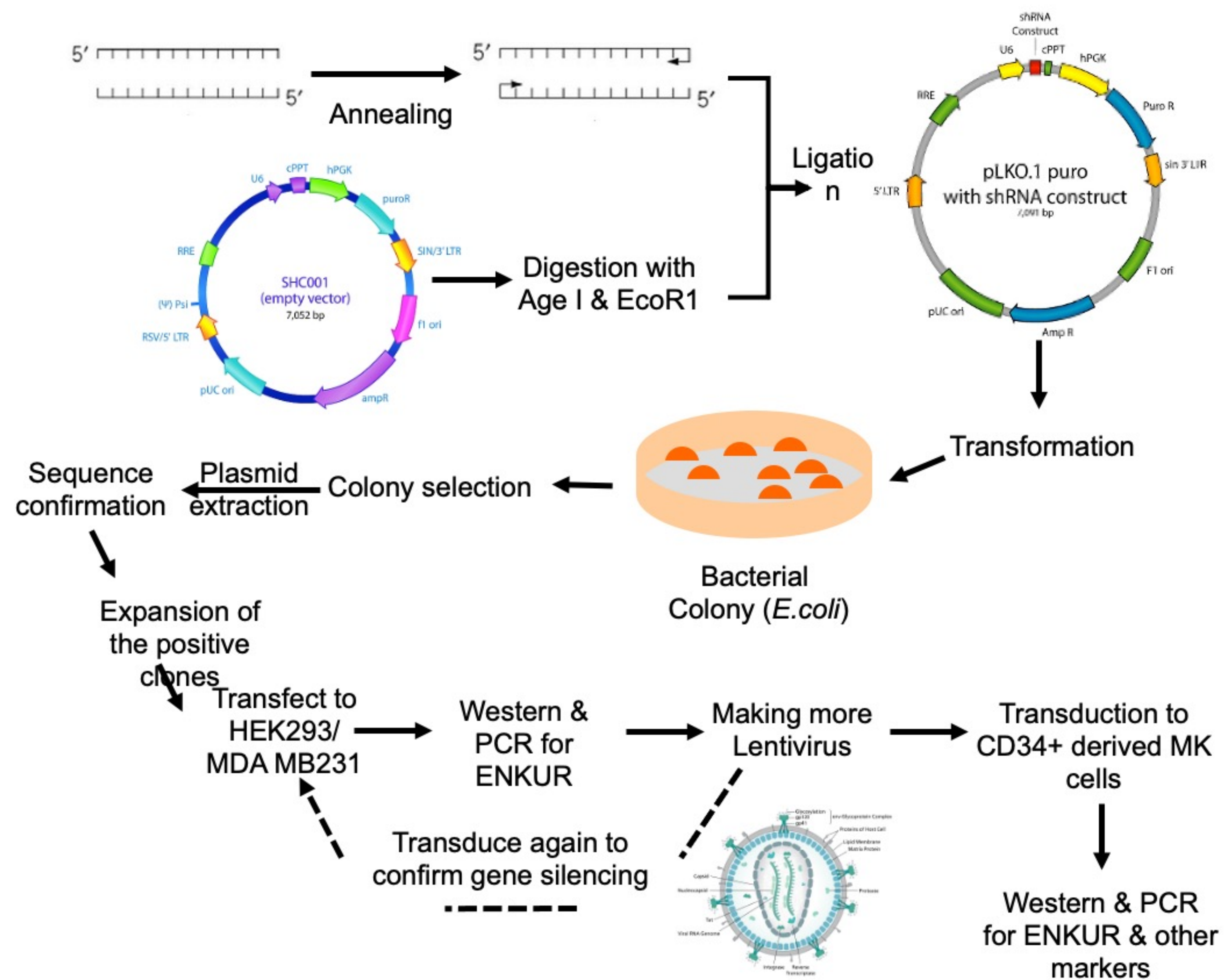


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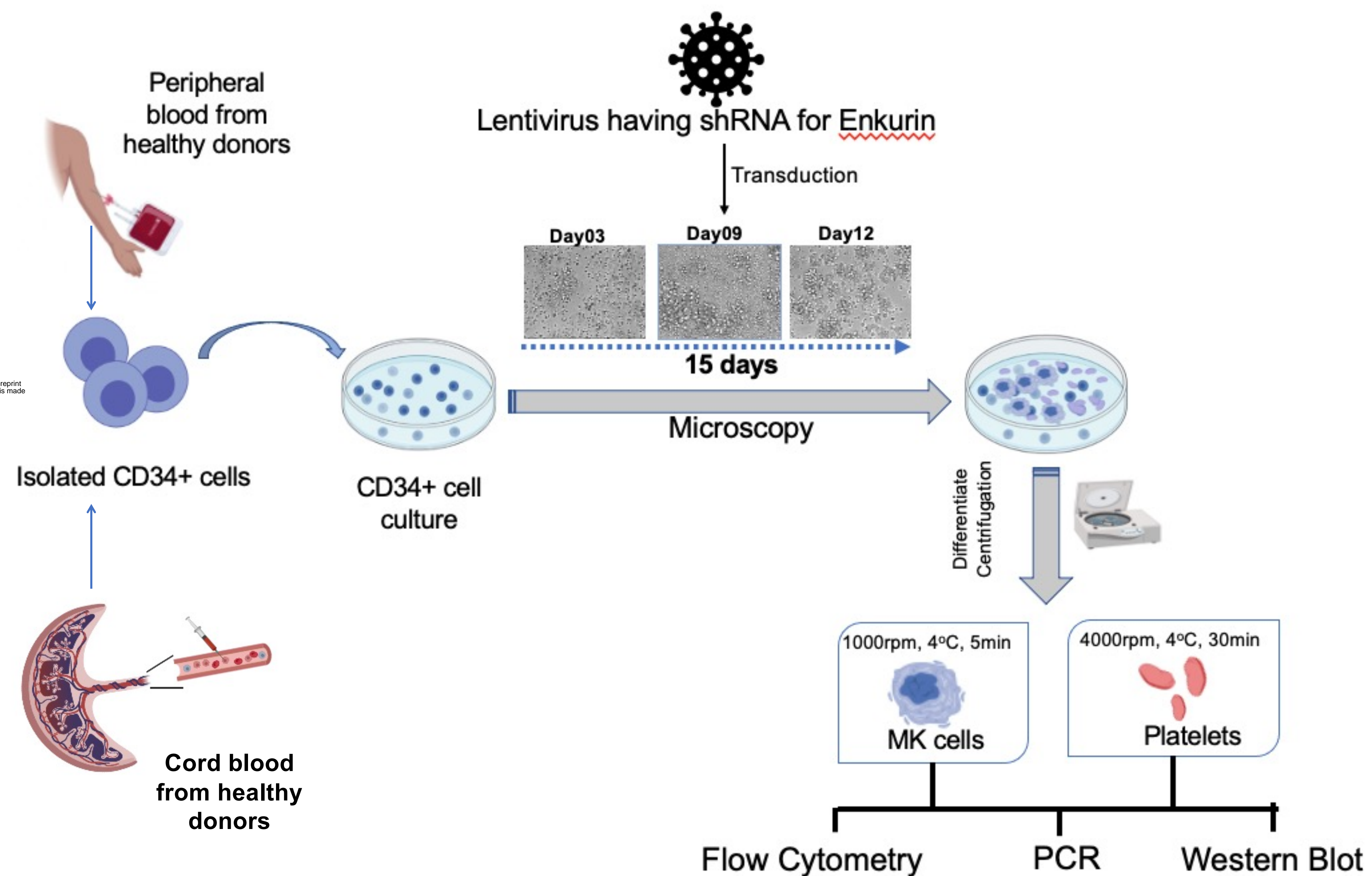


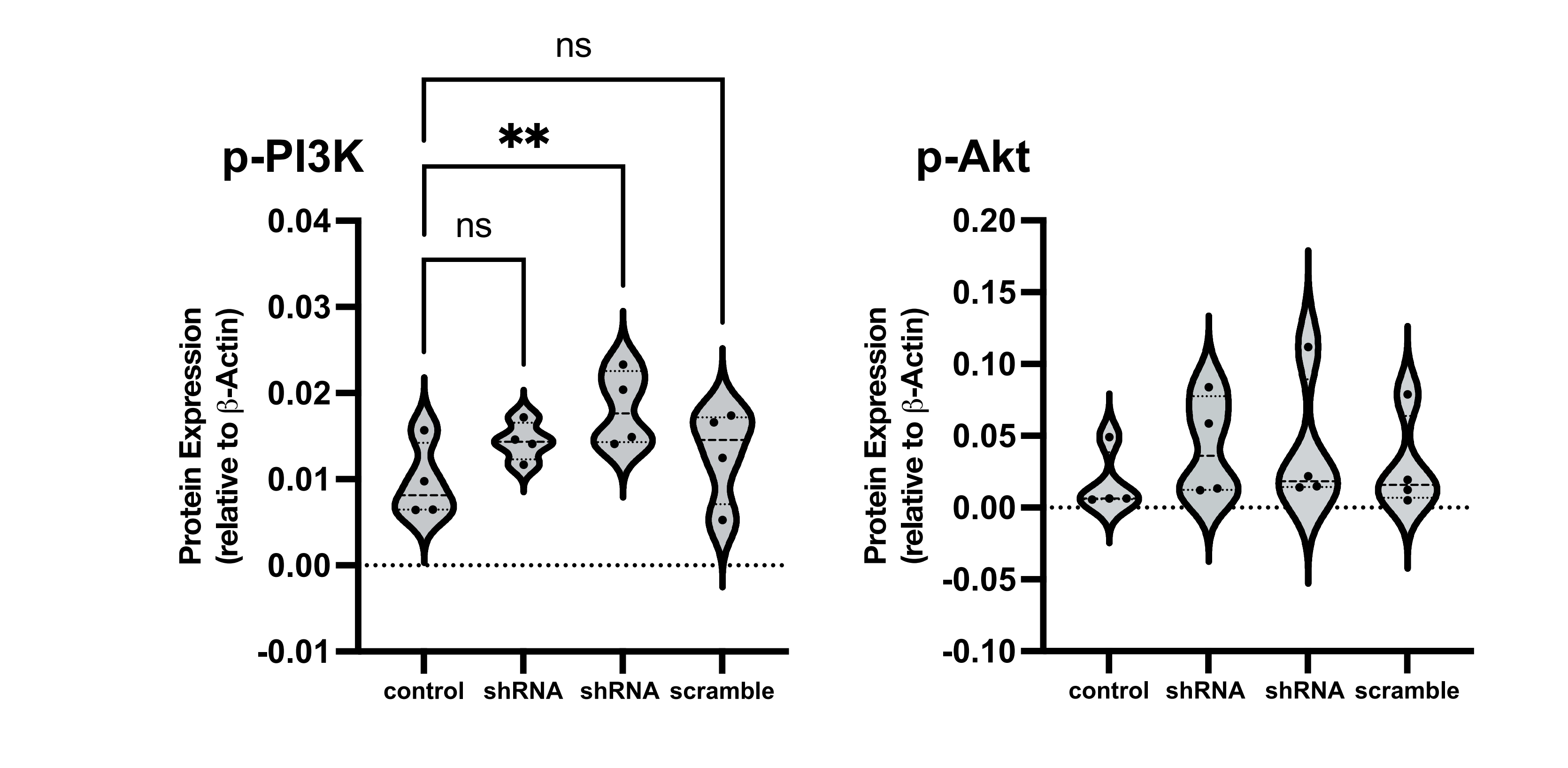
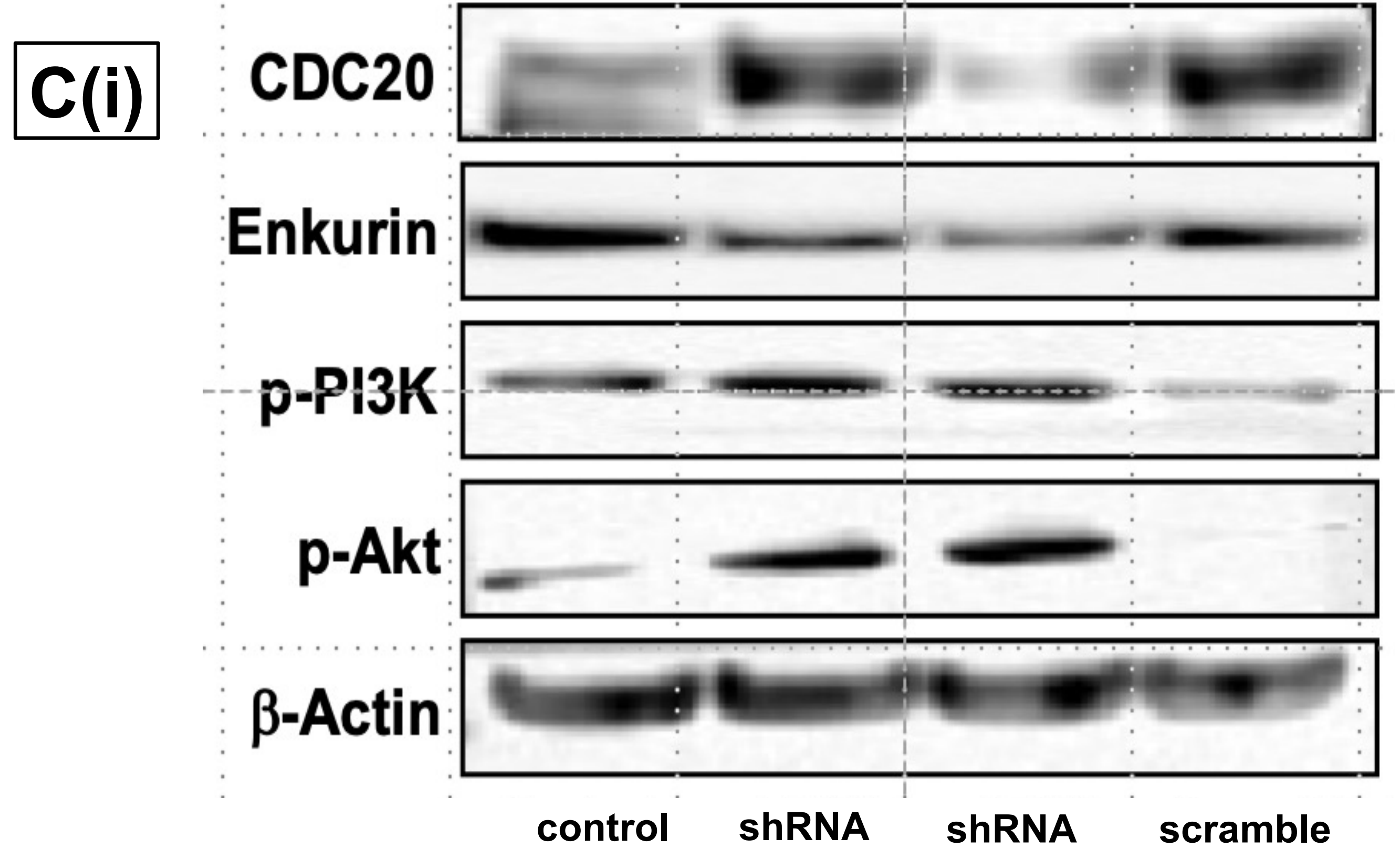
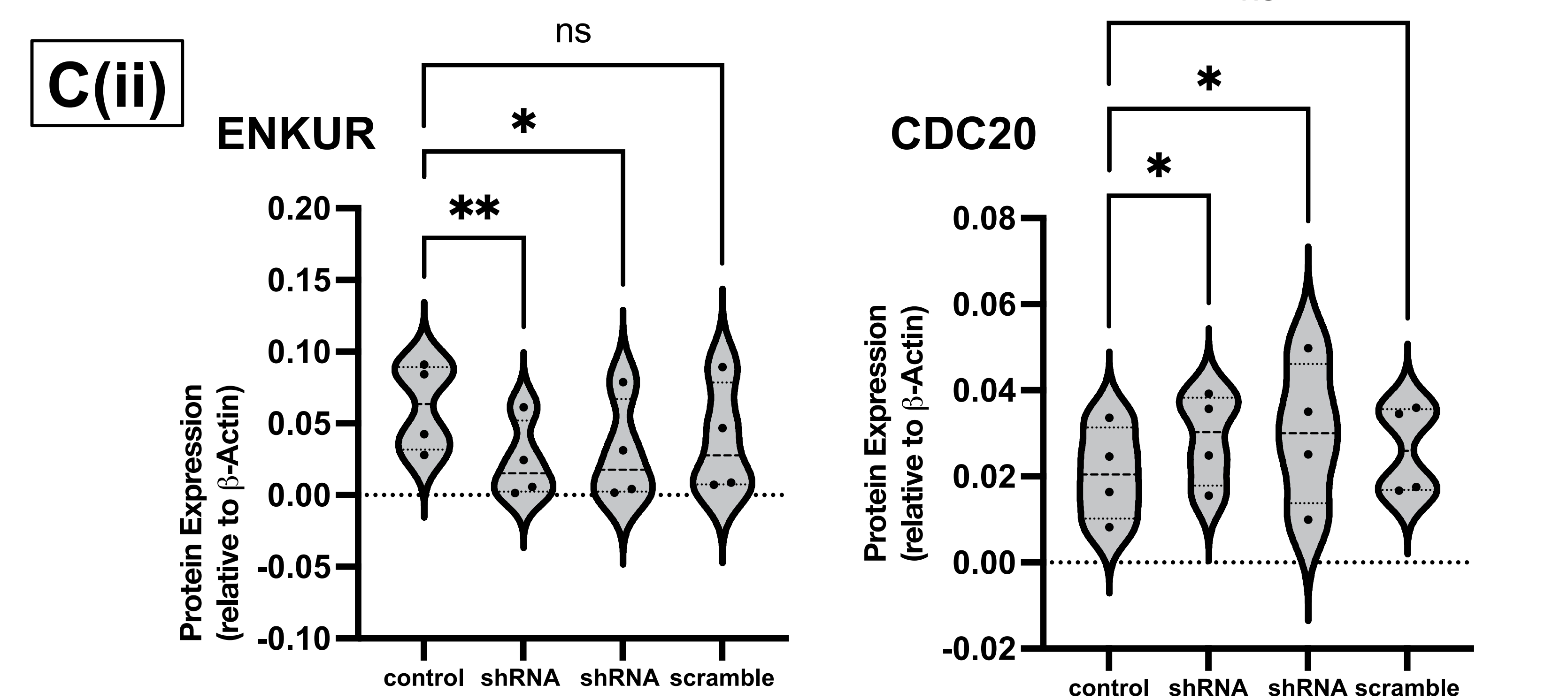
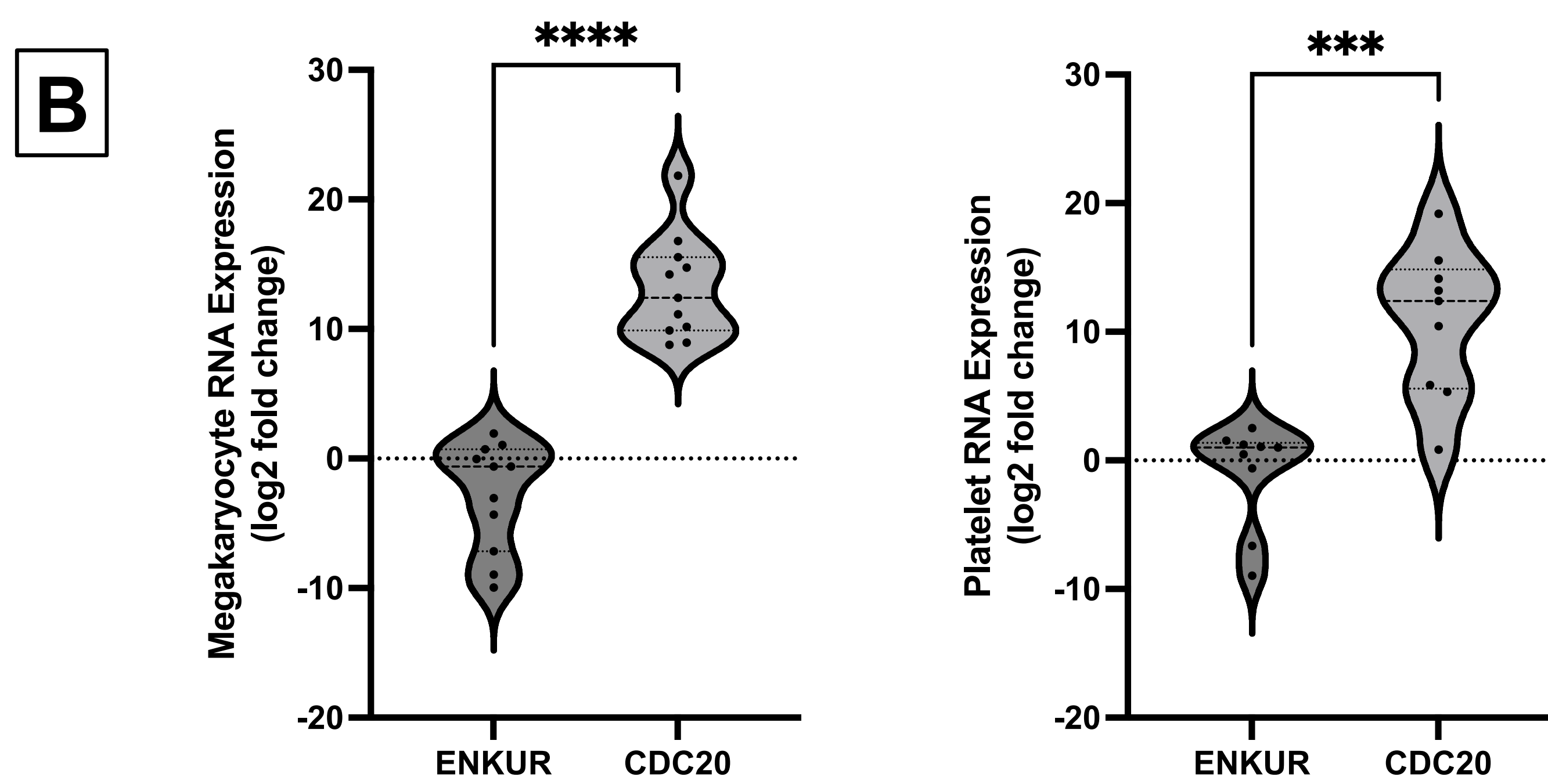
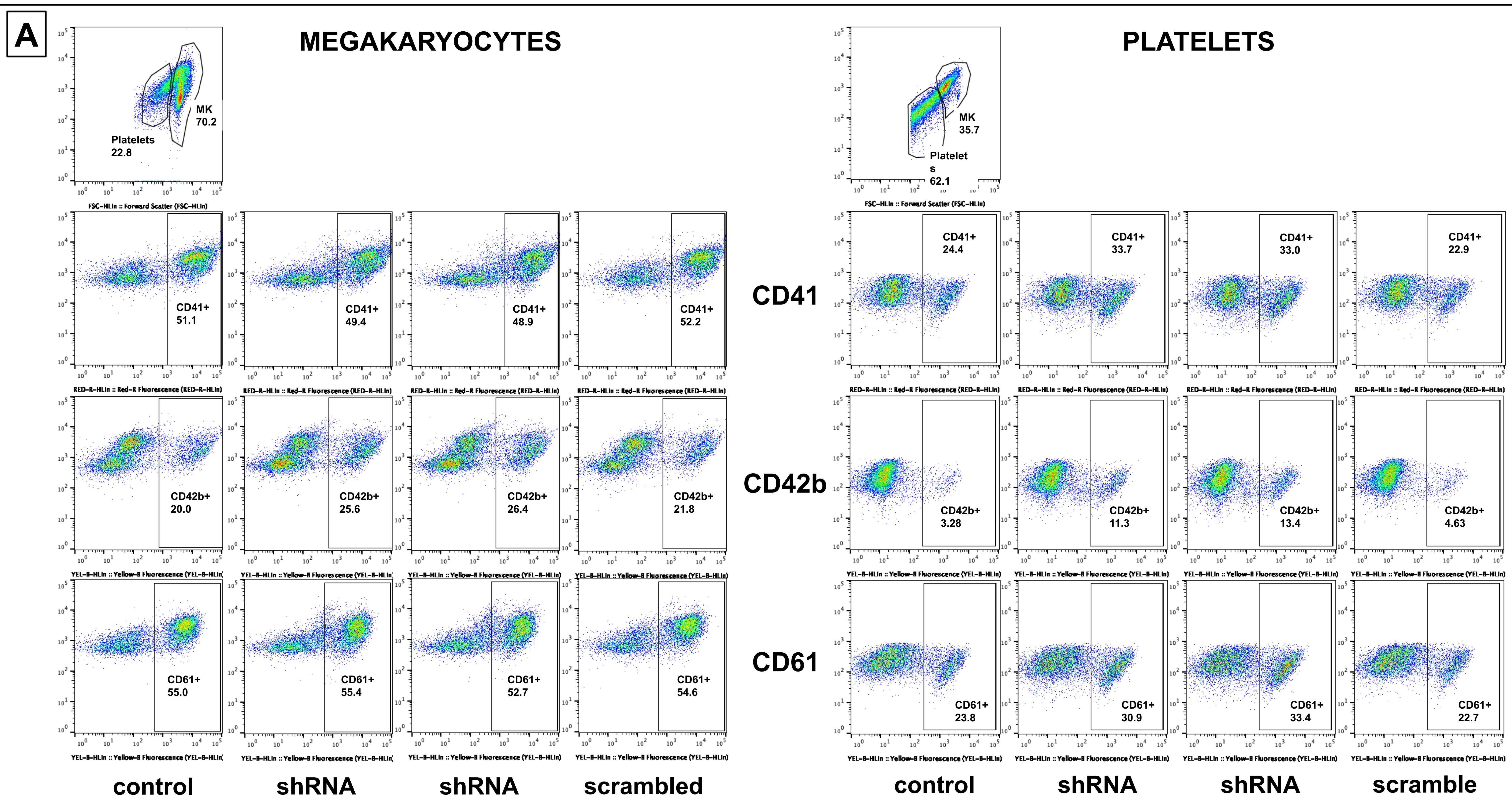
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A

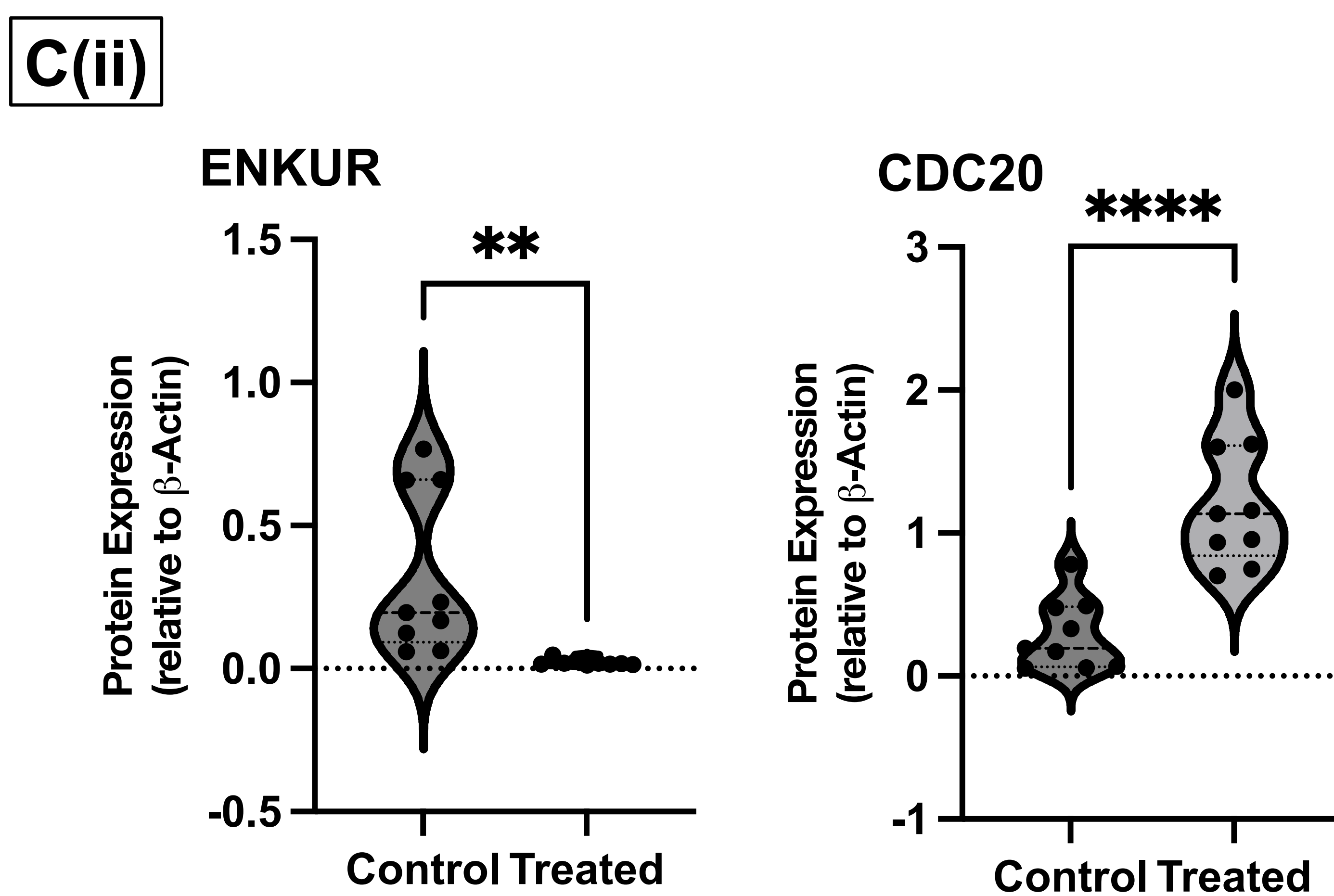
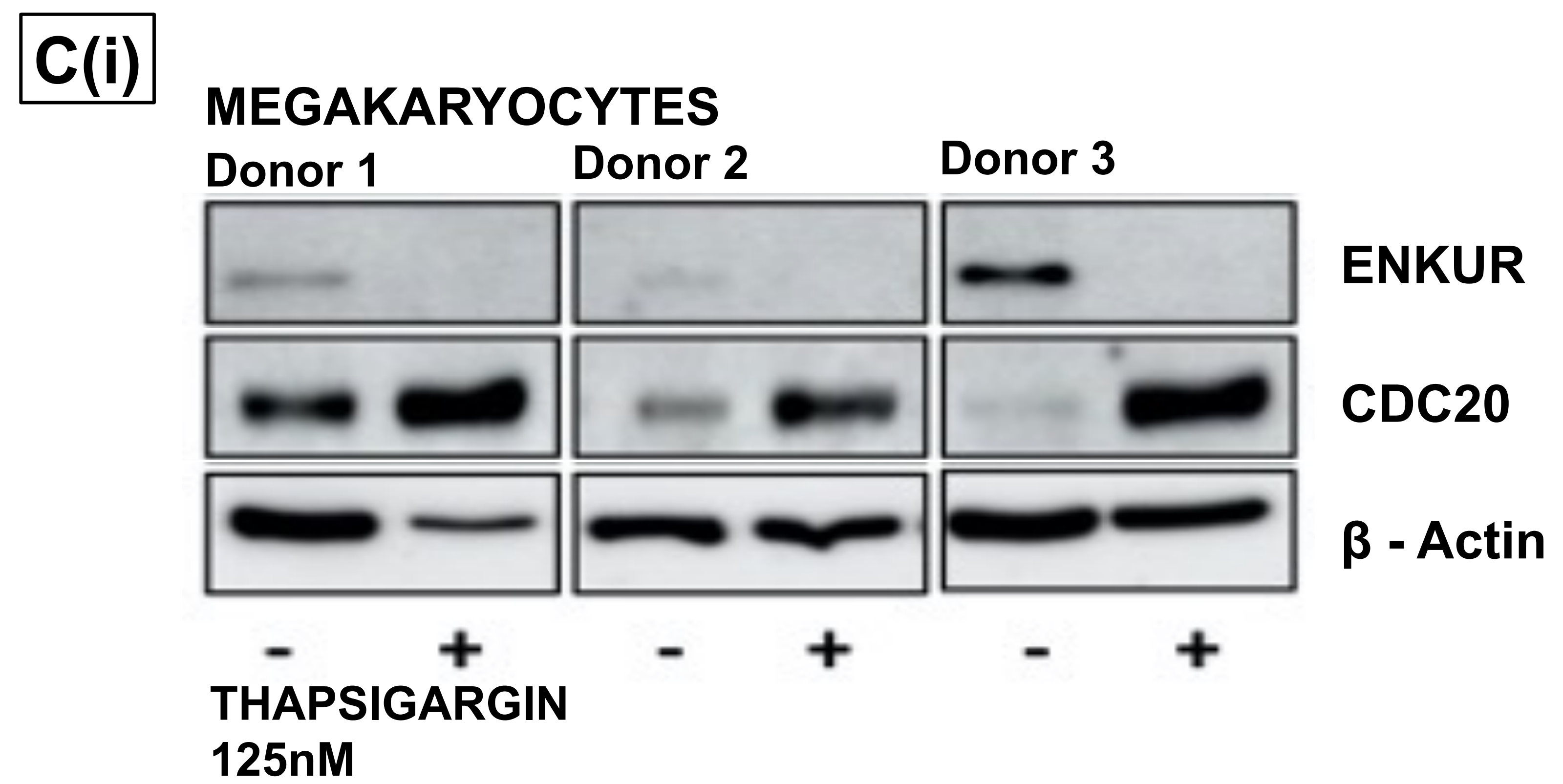
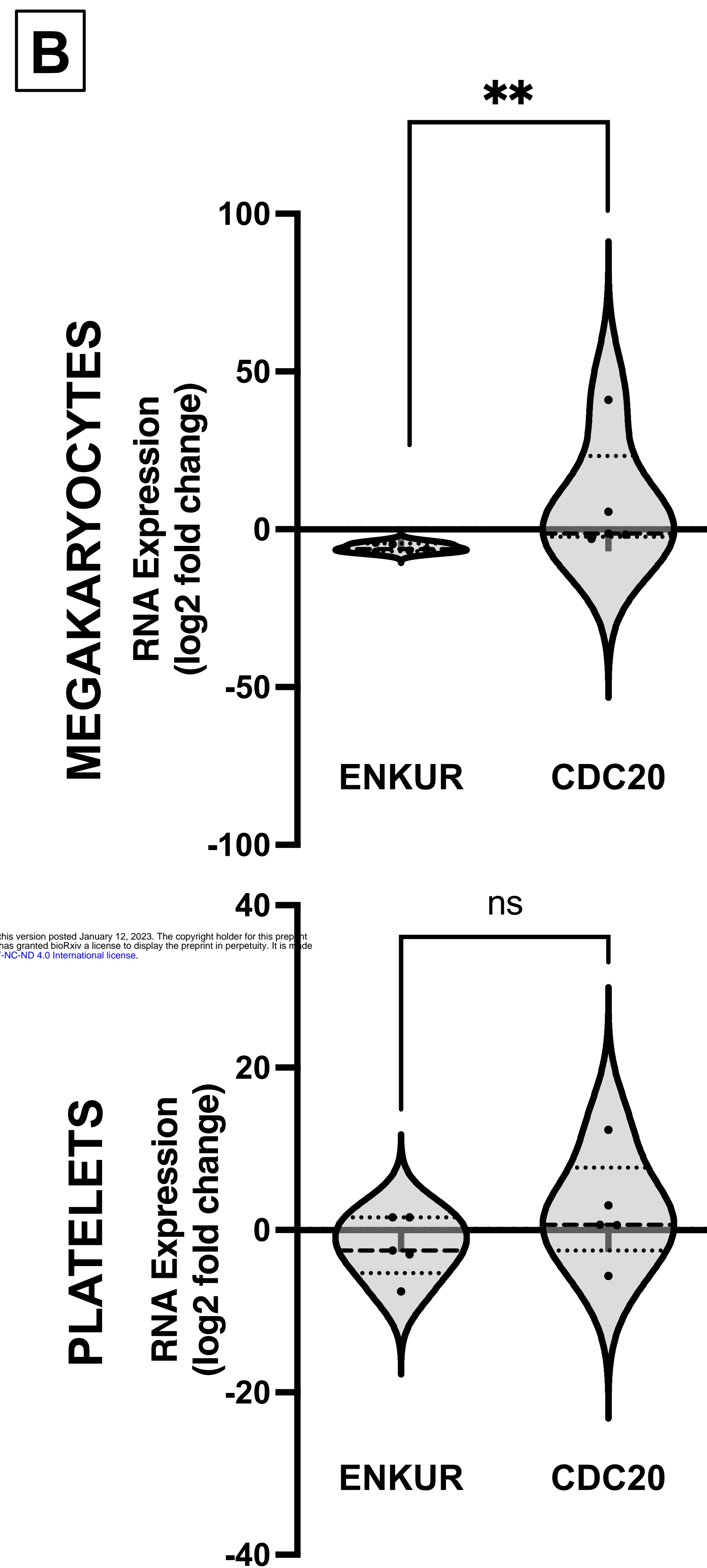
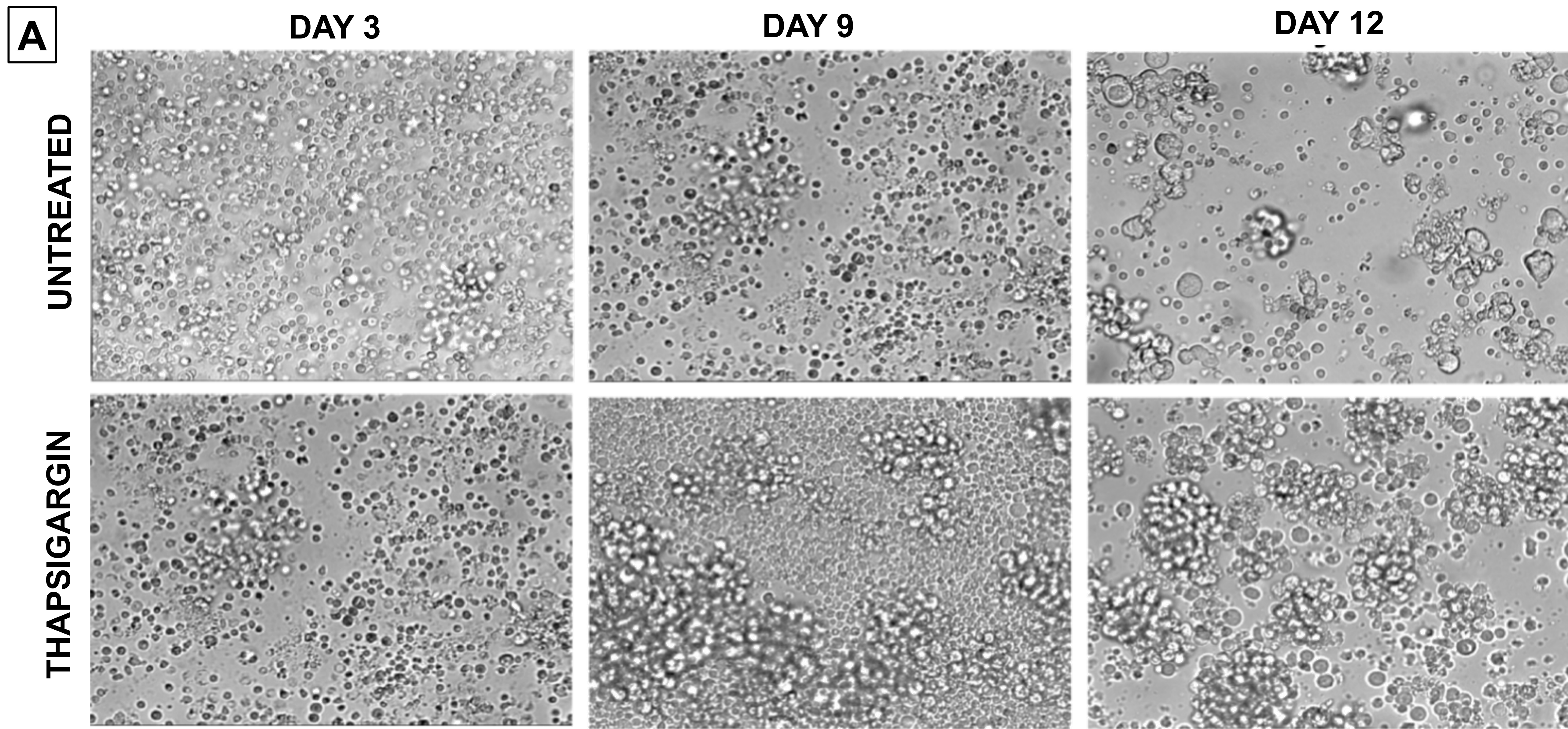


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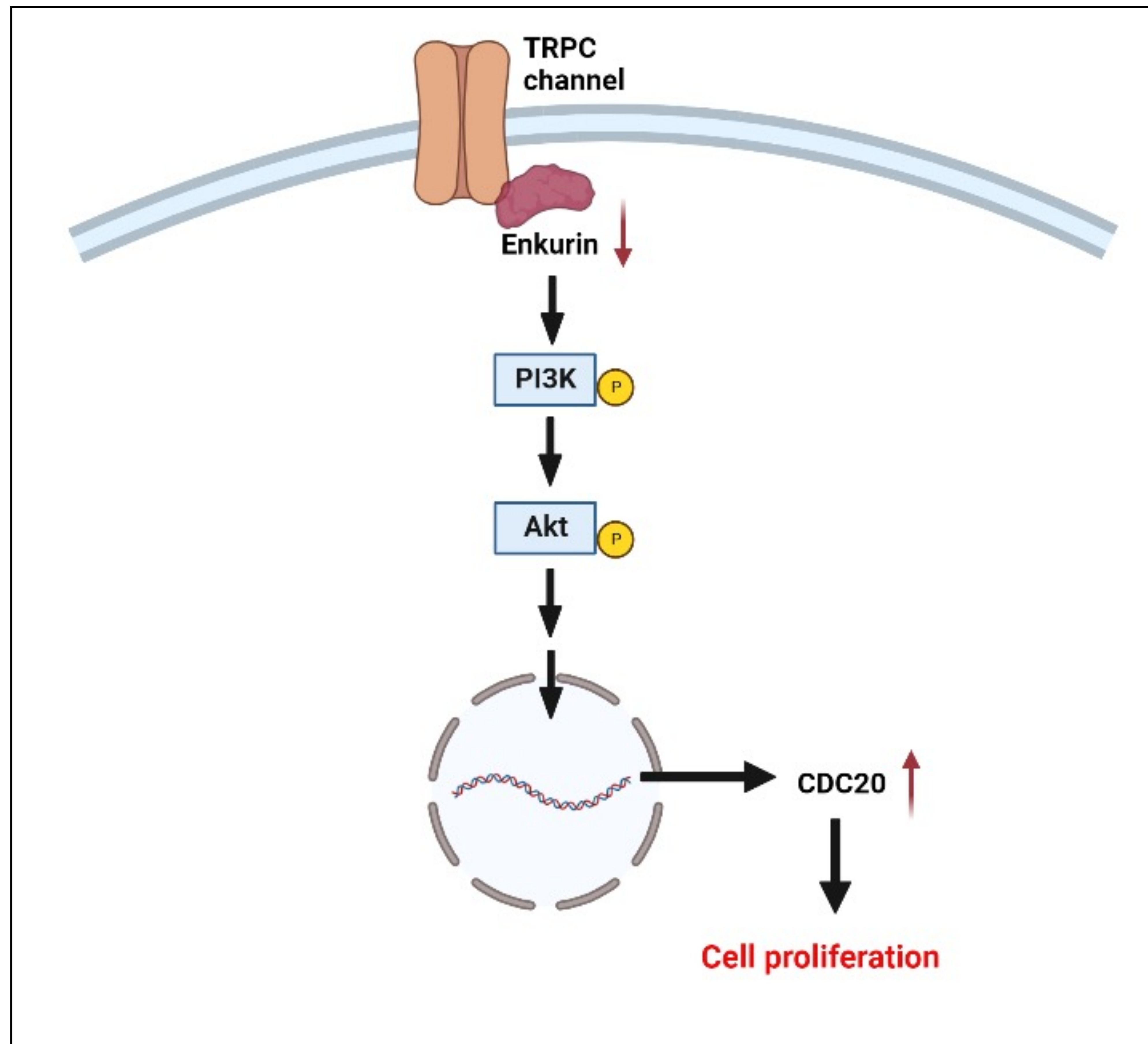




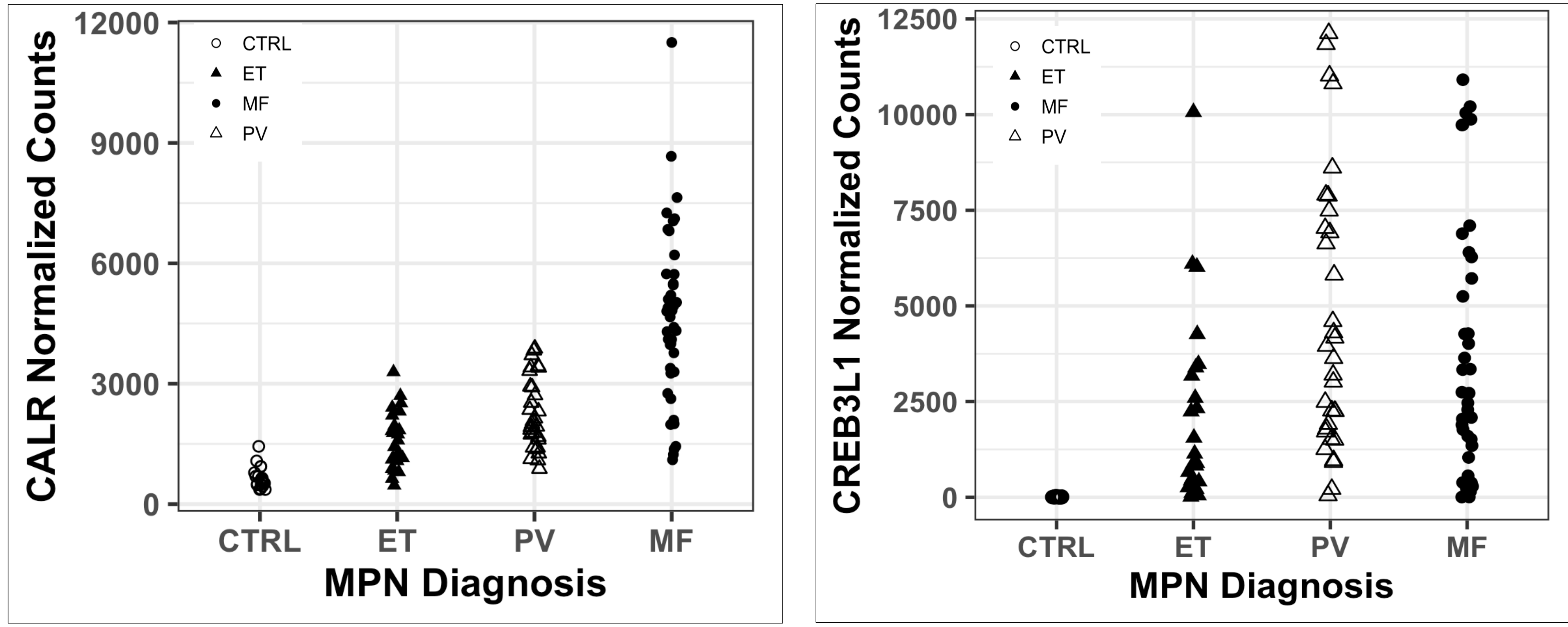
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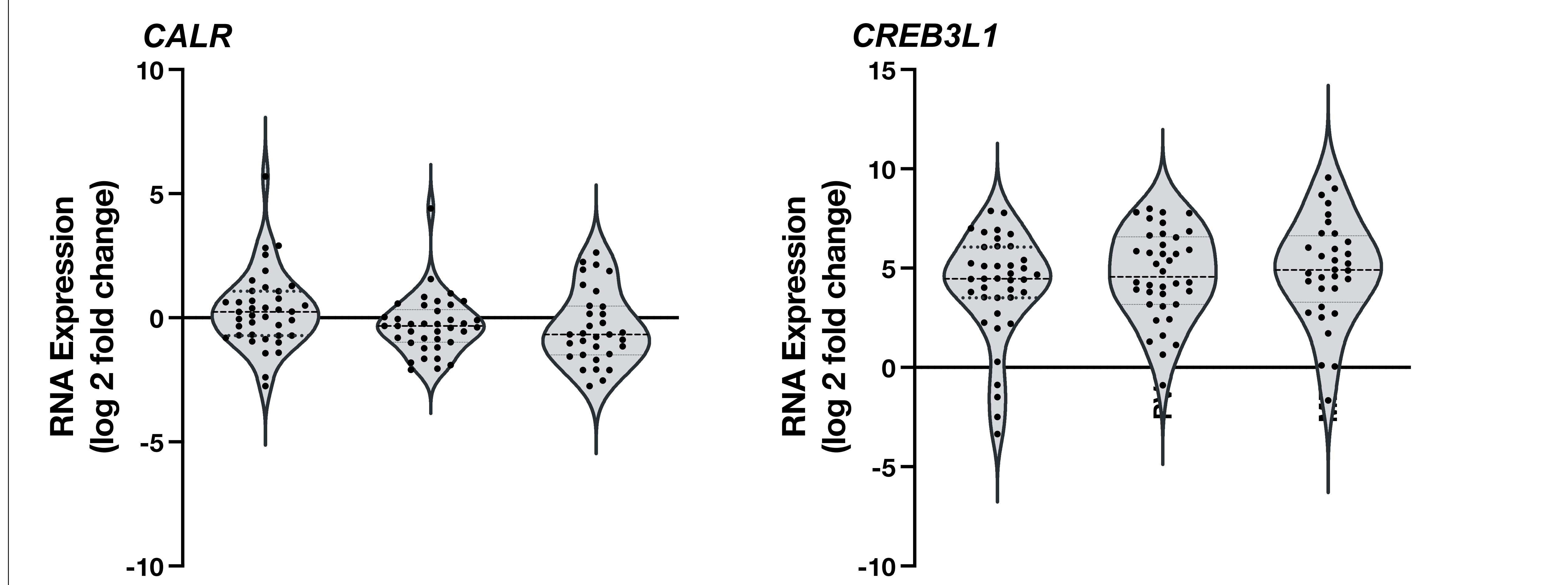
**FIGURE 6**



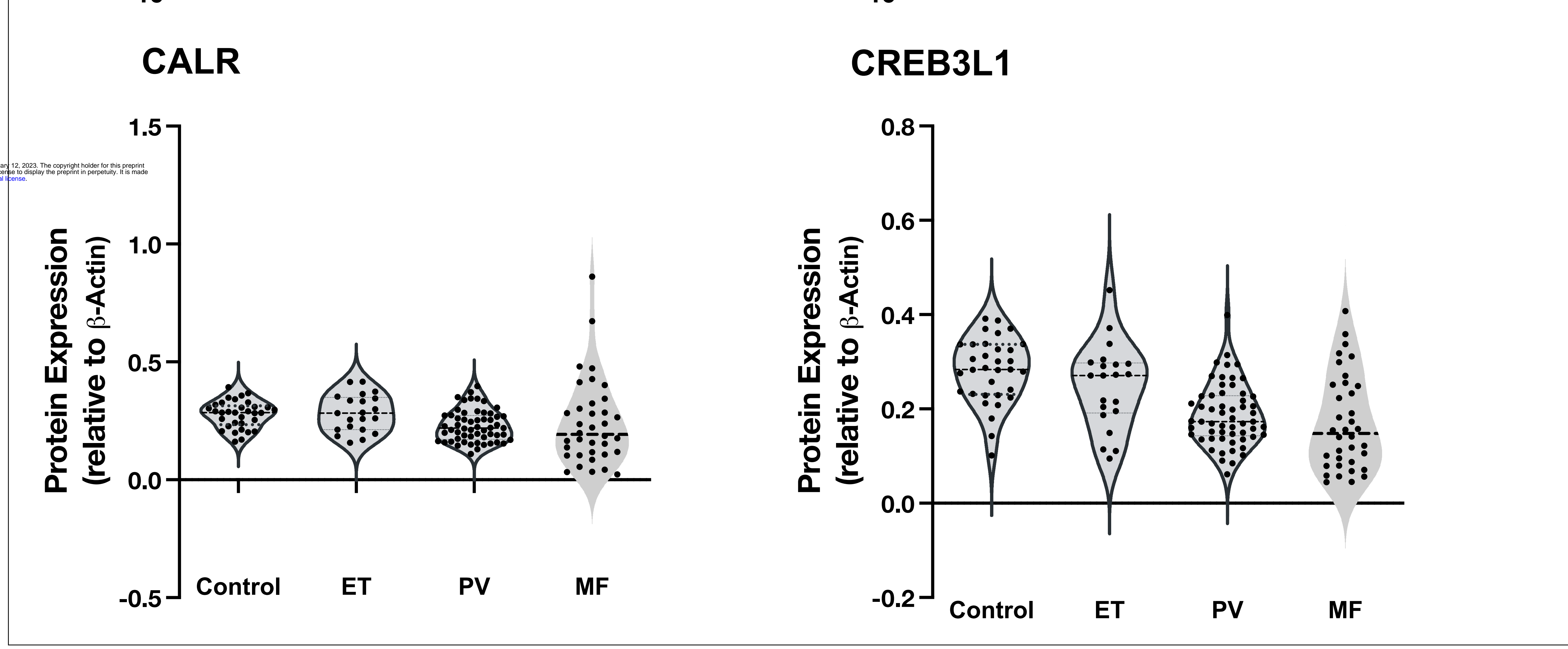
**A**



**B**



**C**

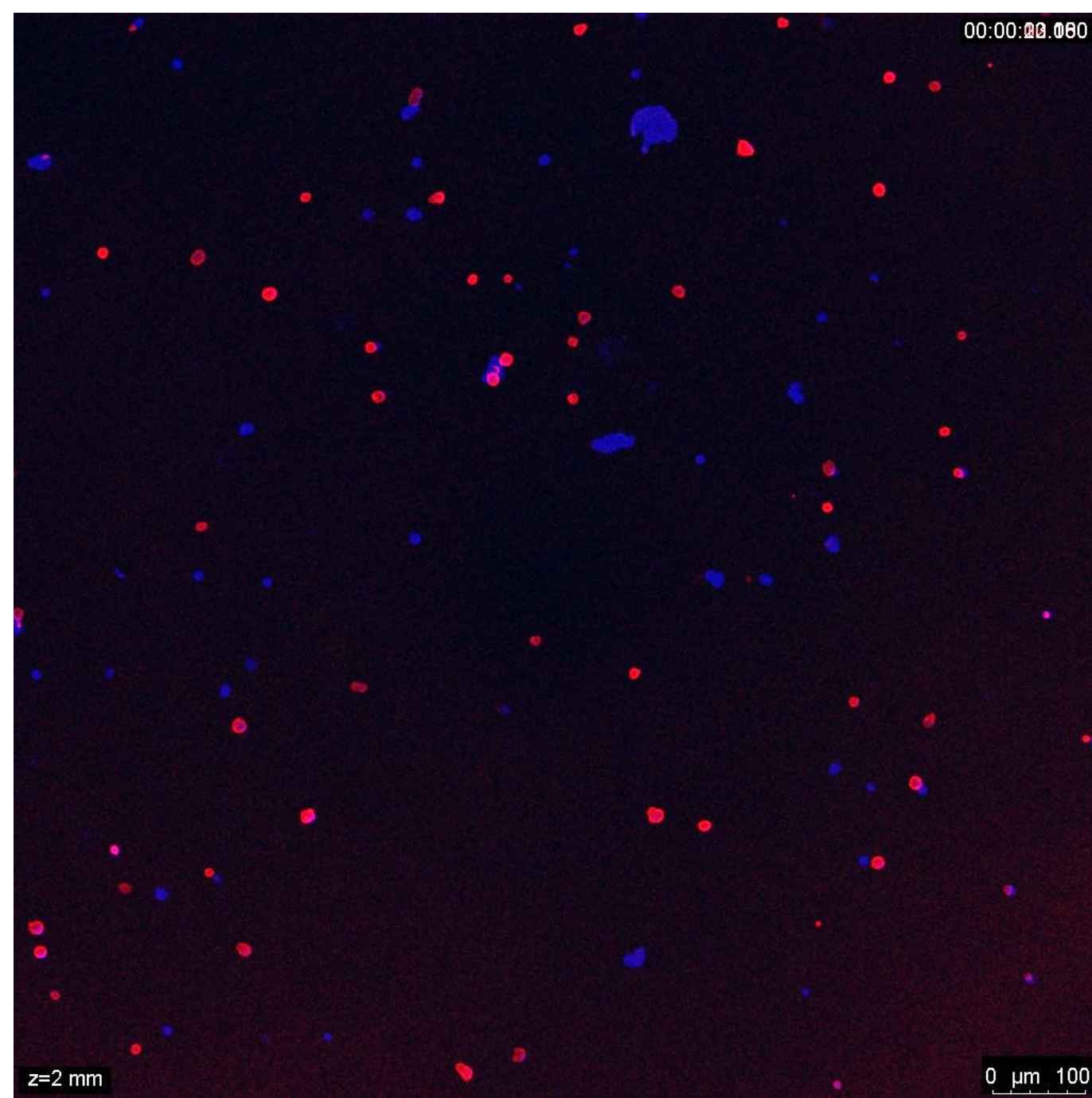


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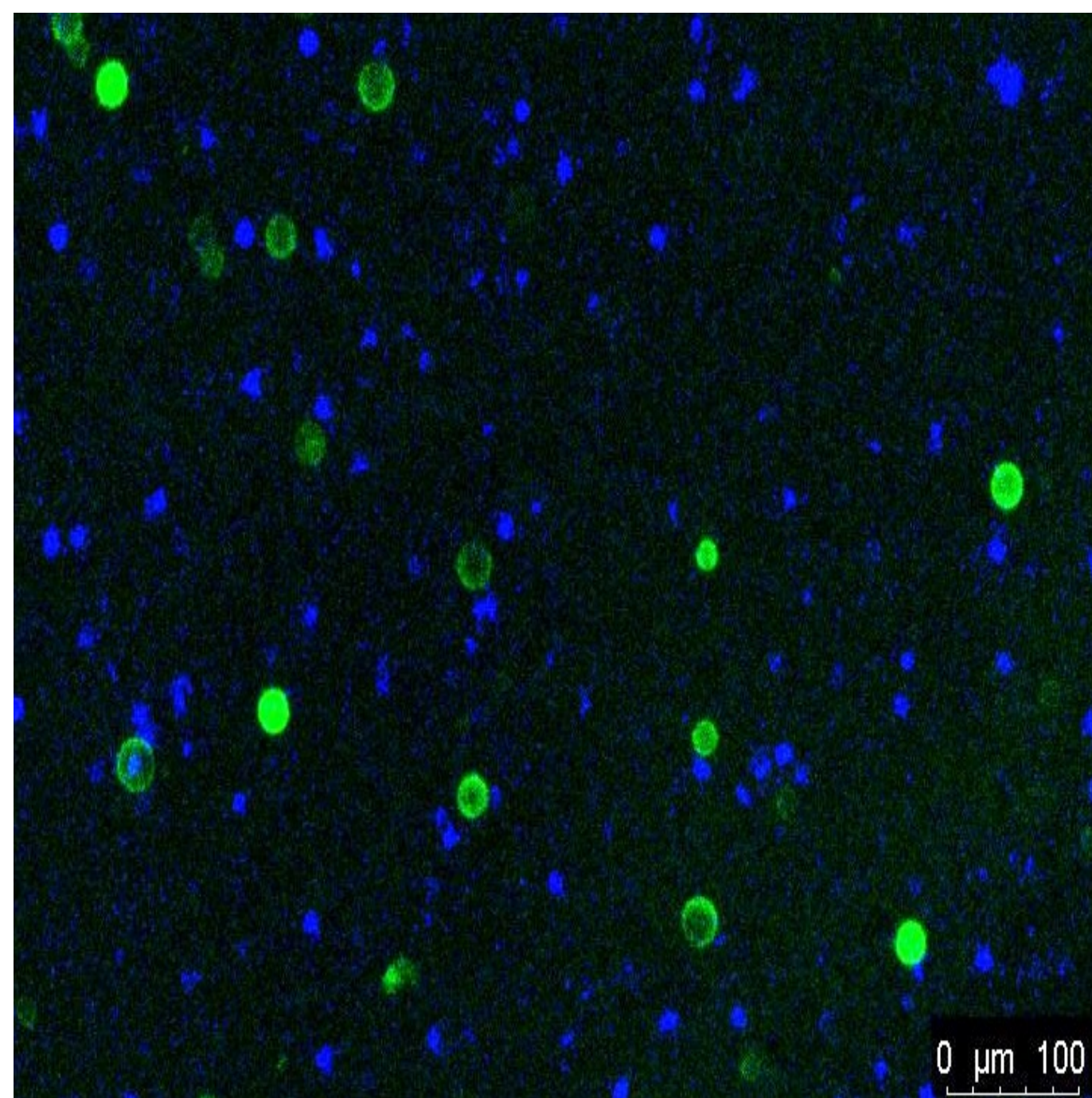
**A**

Day 1



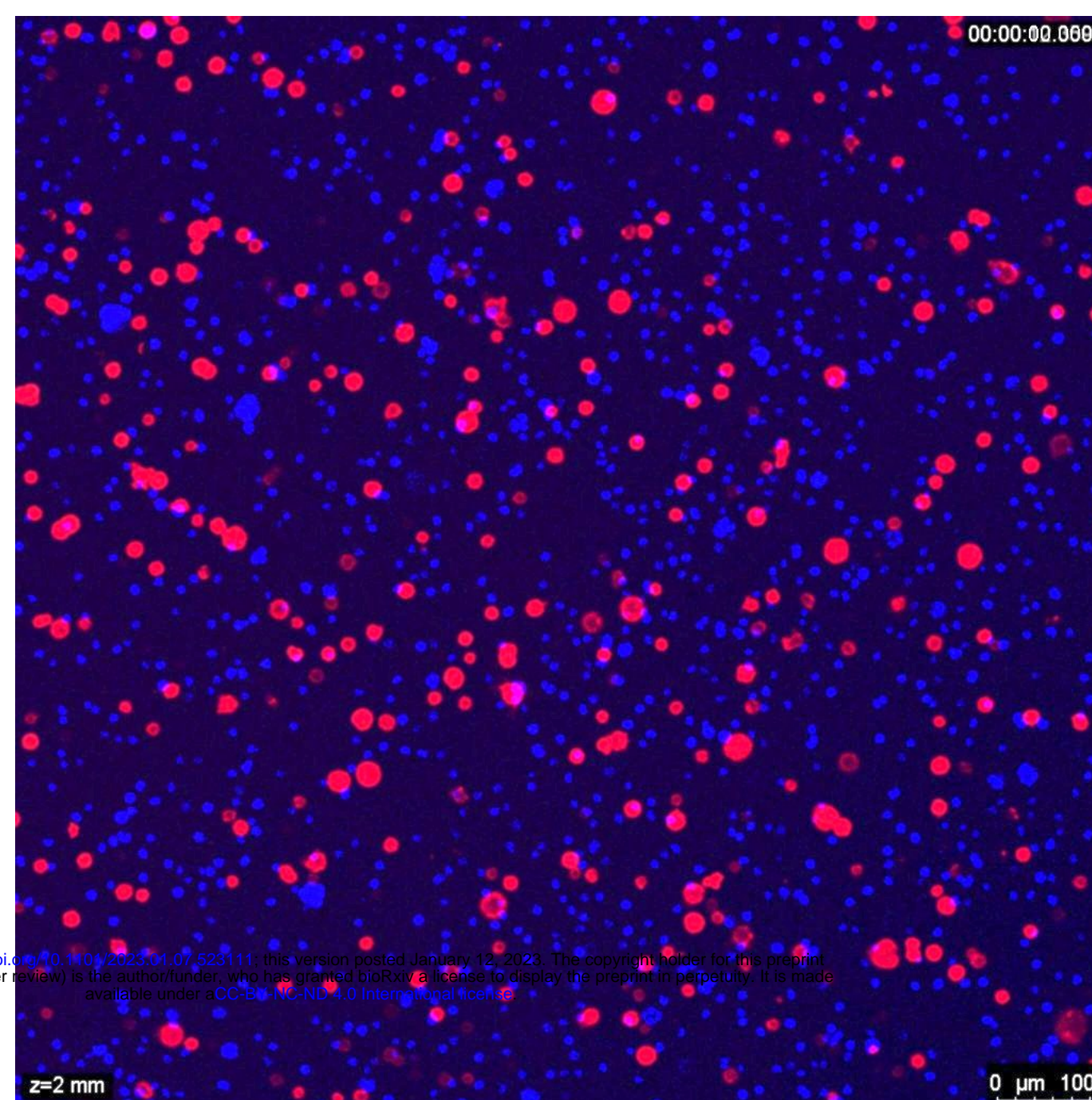
CD34  
CD42b  
Hoechst

Day 9



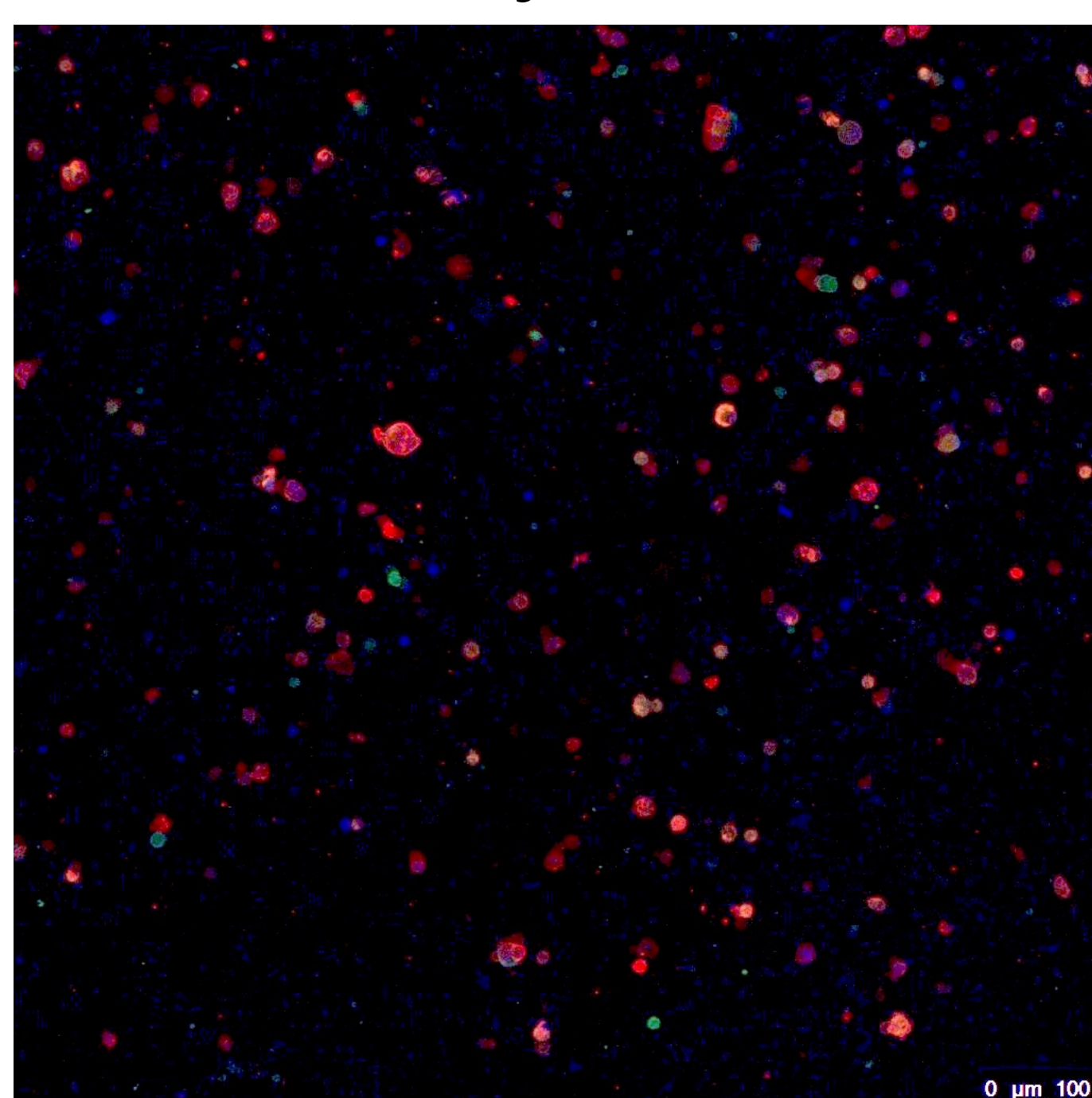
CD34  
CD42b  
Hoechst

Day 9



CD41  
Hoechst

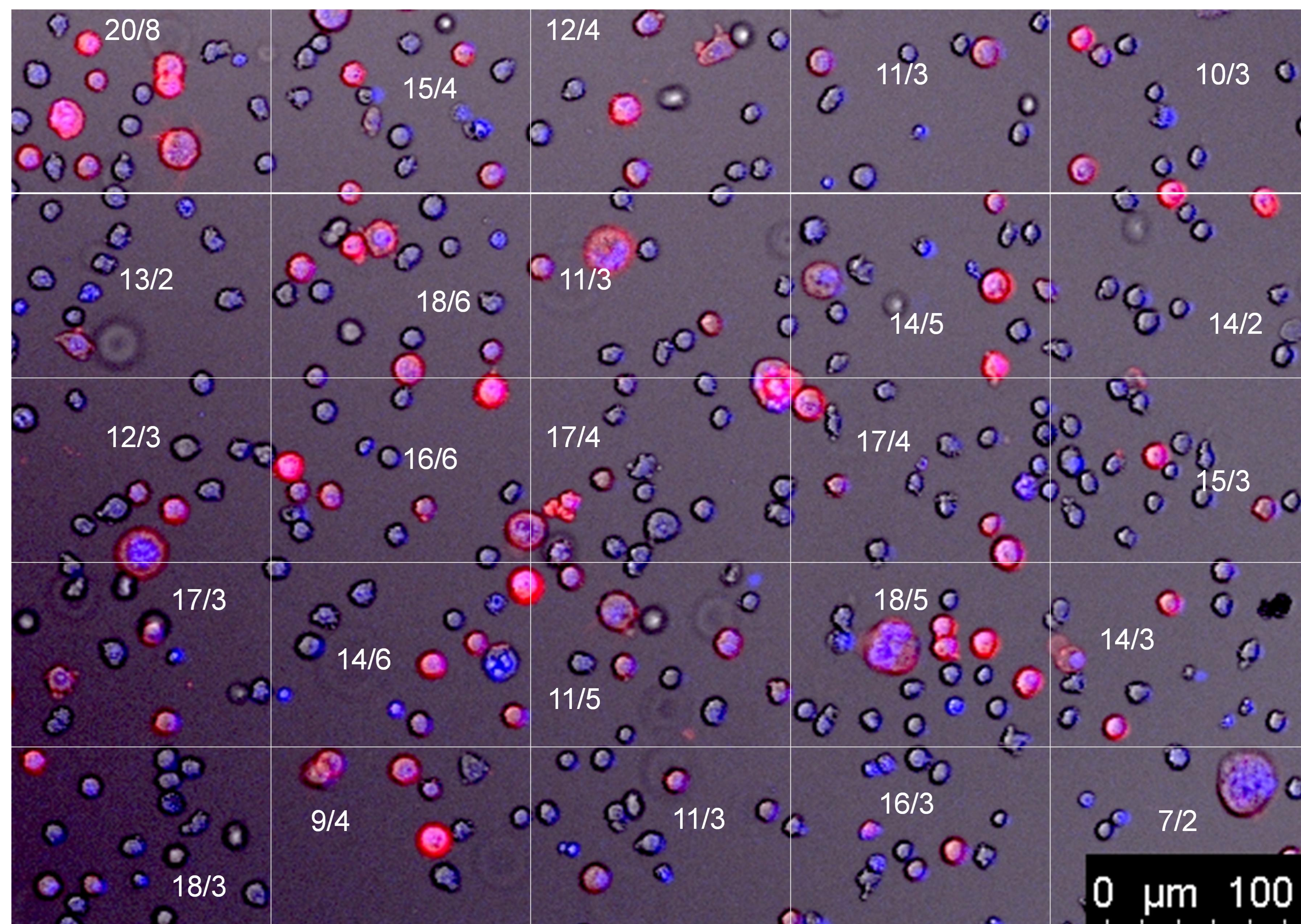
Day 13



CD41  
CD42b  
Hoechst

**B**

Day 9



CD41  
Hoechst  
Bright field

Total: 350 / CD41+ : 97 (27.7%)

