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1 **Disruption of the 5S RNP-Mdm2 interaction significantly improves the**
2 **erythroid defect in a mouse model for Diamond-Blackfan anemia**

3
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31

32 **ABSTRACT**

33 Diamond-Blackfan anemia (DBA) is a congenital erythroid hypoplasia caused by
34 haploinsufficiency of genes encoding ribosomal proteins (RPs). Perturbed
35 ribosome biogenesis in DBA has been shown to induce a p53-mediated
36 ribosomal stress response. However, the mechanisms of p53 activation and its
37 relevance for the erythroid defect remain elusive. Previous studies have indicated
38 that activation of p53 is caused by the inhibition of Mdm2, the main negative
39 regulator of p53, by the 5S ribonucleoprotein particle (RNP). Meanwhile, it is not
40 clear whether this mechanism solely mediates the p53-dependent component
41 found in DBA. To approach this question, we crossed our mouse model for
42 RPS19-deficient DBA with Mdm2^{C305F} knock-in mice that have a disrupted 5S
43 RNP-Mdm2 interaction. Upon induction of the Rps19 deficiency, Mdm2^{C305F}
44 reversed the p53 response and improved expansion of hematopoietic progenitors
45 *in vitro*, and ameliorated the anemia *in vivo*. Unexpectedly, disruption of the 5S
46 RNP-Mdm2 interaction also led to selective defect in erythropoiesis. Our findings
47 highlight the sensitivity of erythroid progenitor cells to aberrations in p53
48 homeostasis mediated by the 5S RNP-Mdm2 interaction. Finally, we provide
49 evidence indicating that physiological activation of the 5S RNP-Mdm2-p53
50 pathway may contribute to functional decline of the hematopoietic system in a
51 cell-autonomous manner over time.

52

53

54 **INTRODUCTION**

55

56 Diamond-Blackfan anemia (DBA) is a congenital erythroid hypoplasia
57 characterized by macrocytic anemia with selective absence of erythroid
58 precursors, physical abnormalities, and cancer predisposition¹⁻³. DBA is most
59 often caused by mutations in *ribosomal protein S19 (RPS19; eS19)* and other
60 genes encoding ribosomal proteins (RPs)⁴⁻¹¹. Studies in zebrafish and mouse
61 DBA model systems *in vivo* and human hematopoietic cells *in vitro* have
62 suggested that the anemia is caused by the activation of p53 generated by the

63 RP deficiency¹²⁻¹⁵. Mice that have a missense mutation in *Rps19* exhibit dark
64 skin, retarded growth and a reduction in the number of erythrocytes, and all of
65 these features are rescued in a p53-null background¹². Similarly, the lethal
66 hematopoietic phenotype of the transgenic *Rps19* knockdown mice is reversed
67 upon loss of p53¹⁵. Additionally, Dutt *et al.* demonstrated that inhibition of p53
68 with the small molecule pifithrin alpha rescues the erythroid defect in RPS19-
69 deficient human bone marrow (BM) cell cultures¹⁴.

70 The identification of p53 as one of the mediators of the DBA phenotype could
71 have therapeutic implications for the treatment of DBA and related disorders.
72 However, strong concerns have to be raised towards therapeutic strategies
73 based on direct interference with p53, given its prominent role as an orchestrator
74 of genetic programs leading to cell cycle arrest, senescence and apoptosis¹⁶.
75 Therefore, it is important to delineate the components upstream of p53 activation
76 upon RP deficiency as these might provide novel disease-specific targets for
77 therapeutic intervention. The level of p53 is negatively regulated by mouse
78 double minute 2 (Mdm2; HDM2 in humans), which functions as an ubiquitin
79 ligase that targets p53 to the proteasome in the absence of stress¹⁷. Cellular
80 stress signals, such as DNA damage and oncogenic insults, disrupt the
81 interaction between Mdm2 and p53 resulting in the stabilization and activation of
82 p53¹⁷. A body of *in vitro* studies indicates that perturbed ribosome biogenesis
83 activates the p53 response through the nuclear accumulation of RPL5 and
84 RPL11 that in turn bind to Mdm2 and inhibit its ubiquitin ligase function toward
85 p53^{14,18-20}. More recently, RPL5 and RPL11 were shown to regulate p53 as part
86 of the 5S ribonucleoprotein particle (RNP), in which the 5S ribosomal RNA
87 (rRNA) is also critical for p53 regulation^{21,22}. Finally, the physiological relevance
88 of the 5S RNP-Mdm2 interaction was further confirmed by the generation of
89 Mdm2^{C305F} knock-in mice that harbor a missense mutation in the zinc finger
90 region of Mdm2 preventing its binding to 5S RNP^{23,24}. Disruption of the 5S RNP-
91 Mdm2 interaction in these mice attenuated the p53 activation upon chemically
92 induced ribosome biogenesis stress, and shortened the latency of c-Myc driven
93 tumorigenesis²³. However, the physiological relevance of the 5S RNP-Mdm2-p53

94 pathway for generation of the erythroid defect of DBA is not known. By
95 intercrossing Rps19-deficient mice¹⁵ with the Mdm2^{C305F} knock-in mice²³, we
96 show in the current study that the 5S RNP-Mdm2 interaction has a dominant role
97 in mediating the p53 activation upon Rps19 deficiency and its disruption partially
98 improves the erythropoiesis of Rps19-deficient mice *in vivo*. We also report the
99 unexpected finding that the loss of 5S RNP-Mdm2 interaction *per se* causes mild
100 anemia by triggering a p53 signature in erythroid progenitor cells. Finally, our
101 results indicate that disruption of the 5S RNP-Mdm2 interaction has a positive
102 impact on the reconstitution capacity of serially transplanted hematopoietic stem
103 cells (HSCs), suggesting that the 5S RNP-Mdm2-p53 pathway may contribute to
104 the functional decline of the hematopoietic system upon replicative stress.

105

106

107 **MATERIAL AND METHODS**

108

109 **Mice**

110 Generation of transgenic Rps19 knockdown mice and the Mdm2^{C305F} knock-in
111 mice has been described earlier^{15,23}. Mice were maintained in C57BL/6
112 background and litter mate controls were used. Rps19 deficiency was induced by
113 administering doxycycline in the food (Bio-Serv; 200 mg/kg). Mice were
114 maintained at Lund University animal facility and experiments were performed
115 with consent from the Lund University animal ethics committee.

116

117 **Blood and bone marrow cellularity**

118 Peripheral blood was collected from the tail vein into Microvette tubes (Sarstedt)
119 and analyzed using Sysmex XE-5000 and Sysmex KX-21 hematology analyzers.
120 BM cells were isolated by crushing hip bones, femurs and tibiae in PBS
121 containing 2 % FCS (GIBCO), stained with Türk's solution (Merck) and counted
122 in Bürker chambers, or alternatively counted using the Sysmex KX-21
123 hematology analyzer.

124

125 **Flow cytometry**

126 FACS analysis of the myeloerythroid compartment in BM was performed as
127 previously described^{15,25}. Antibodies used are listed in the supplementary table 1.
128 To evaluate lineage distribution between myeloid, B and T cells in the peripheral
129 blood, erythrocytes were removed by sedimentation with Dextran (2%, Sigma-
130 Aldrich) and ACK lysis. Following the lysis of erythrocytes, cells were stained for
131 surface markers. For cell cycle analyses, c-Kit-enriched BM cells were cultured
132 for 72 hours and 0.5×10^6 cells were stained for c-Kit. BD Cytofix/Cytoperm™
133 Fixation/Permeabilization Kit (BD Biosciences; 554714) was utilized for
134 intracellular staining according to manufacture's instructions and DNA was
135 stained with DAPI (Sigma-Aldrich). Experiments were performed using FACS
136 Aria cell sorter (Becton Dickinson) and LSR II flow cytometer (Becton Dickinson),
137 and analyzed using FlowJo software (Tree Star, v9.7.6).

138

139 **Cell culture**

140 c-Kit⁺ cells were enriched using CD117 MicroBeads and MACS separation
141 columns (Miltenyi), and cultured in StemSpan®SFEM medium (StemCell
142 Technologies) supplemented with penicillin/streptomycin (GIBCO), mSCF (100
143 ng/mL, PeproTech), mL-3 (10 ng/mL, PeproTech) and hEPO (2 U/mL, Janssen-
144 Cilag) ±doxycycline (1 µg/mL; Sigma-Aldrich).

145

146 **Expression analyses**

147 Total RNA was isolated from cultured cells after 48 hours using the RNAeasy
148 micro kit (Qiagen). cDNA was transcribed using SuperScript III reverse
149 transcriptase (Life Technologies). Real-time PCR reactions were performed using
150 pre-designed assays (Life Technologies) with the exception of *Rps19* that was
151 quantified using the SYBR GreenER™ system (5'-
152 GCAGAGGCTCTAAGAGTGTGG-3'; 5'-CCAGGTCTCTGTCCCTGA-3')
153 according to manufacturer's instructions (Life Technologies, 11761-500).

154

155 **Immunoblotting**

156 Whole cell extracts were prepared by harvesting of cells directly into Laemmli
157 sample buffer (Bio-Rad) followed by sheering of the chromatin using a needle
158 and boiling of the extracts. Proteins were separated on ready-made SDS-PAGE
159 gels (Life Technologies). Proteins on gels were transferred to nitrocellulose
160 membranes using a Trans-Blot Turbo machine (Bio-Rad). Equal loading of
161 protein was verified by Ponceau S staining and confirmed by immunoblotting for
162 β -actin. Blots were blocked in 5% milk in PBS for at least 20 min and
163 subsequently incubated with the appropriate primary antibodies overnight at 4°C
164 on a shaker. After three 5-minute washes with PBS, the blots were incubated
165 with the appropriate secondary horse radish peroxidase conjugated antibody at
166 room temperature for two hours followed by detection using the SuperSignal
167 West Pico reagent (Thermo Scientific). Antibodies used are listed in the
168 supplementary table 2. Immunoblots were quantified using the Image J software.

169

170 **Statistical analyses**

171 Student's *t* test was used to determine statistical significance, and two-tailed *P*
172 values are shown. Cell culture and expression analysis-related *n* represents
173 individual biological repeat experiments. A minimum of three biological replicate
174 experiments was performed to justify the use of chosen statistical test.

175

176

177 **RESULTS**

178

179 **Disruption of the 5S RNP-Mdm2 interaction improves the expansion of** 180 **Rps19-deficient hematopoietic progenitor cells**

181 To study the relevance of the 5S RNP-Mdm2-p53 pathway in DBA we took
182 advantage of our mouse model for RPS19-deficient DBA¹⁵. This model contains
183 an *Rps19*-targeting shRNA (shRNA-B) that is expressed by a doxycycline-
184 responsive promoter located downstream of the *Collagen A1* gene, allowing for
185 an inducible and dose-dependent *Rps19* downregulation (Figure 1A). All
186 experimental animals were bred homozygous for the *M2-rtTA* at the *Rosa26*

187 locus. Administration of doxycycline to the shRNA-B mice results in a severe
188 erythroid phenotype that is reversed in the p53-deficient background¹⁵.
189 Importantly, the hematopoietic phenotype in these mice is specific to *Rps19*
190 downregulation as it can be cured by enforced expression of *RPS19*^{15,26}. We
191 intercrossed the shRNA-B mice with the *Mdm2*^{C305F} knock-in mice that have a
192 point mutation in the zinc finger domain of *Mdm2*, which prevents its binding to
193 5S RNP (Figure 1B)^{23,24}. As a consequence these mice present with an
194 attenuated p53 response upon ribosome biogenesis stress, but retain a normal
195 response to DNA damage²³. As a working model, we hypothesized that if the p53
196 response in *Rps19*-deficient mice is mediated through the 5S RNP-*Mdm2*
197 interaction, the *Mdm2*^{C305F} background should reverse the p53 activity and
198 subsequently improve the erythroid phenotype of these mice (Supplementary
199 figure 1). To confirm that the *Mdm2*^{C305F} background had no effect on the *Rps19*
200 knockdown efficiency in our model system, we quantified the expression of
201 *Rps19* in cultures initiated with c-Kit-enriched hematopoietic progenitor cells from
202 heterozygous (B/+; 1 shRNA-B allele) and homozygous (B/B; 2 shRNA-B alleles)
203 shRNA-B mice. After 48 hours of doxycycline treatment, the B/+ and B/B cells
204 showed on average 45% and 75% reduction in *Rps19* expression, respectively,
205 and *Mdm2*^{C305F} had no significant impact on the *Rps19* knockdown efficiency
206 (Figure 1C).

207 We have previously shown that induction of *Rps19* deficiency impairs expansion
208 of hematopoietic progenitor cells¹⁵. Therefore, we first assessed the effect of
209 *Mdm2*^{C305F} on the expansion of *Rps19*-deficient hematopoietic progenitor cells *in*
210 *vitro*. After 5 days, the uninduced control cultures with wild-type *Mdm2* or
211 homozygous *Mdm2*^{C305F} showed no difference in cell number ($5.04 \pm 0.91 \times 10^6$
212 and $5.04 \pm 0.83 \times 10^6$, respectively) (Figure 1D). In contrast, the doxycycline-
213 treated B/+ cultures with wild-type *Mdm2* showed a significant reduction in cell
214 number ($2.99 \pm 0.82 \times 10^6$), and this was improved in a dose-dependent manner in
215 the heterozygous and homozygous *Mdm2*^{C305F} background ($3.87 \pm 0.56 \times 10^6$ and
216 $4.68 \pm 0.93 \times 10^6$, respectively) (Figure 1D). The B/B cultures with wild-type *Mdm2*
217 failed to expand and had $0.32 \pm 0.12 \times 10^6$ cells after 4 days of culture, while the

218 heterozygous and homozygous Mdm2^{C305F} background resulted in significant,
219 partial restoration of cell numbers ($0.63 \pm 0.21 \times 10^6$ and $0.99 \pm 0.14 \times 10^6$,
220 respectively) (Figure 1D).

221 In order to assess whether the reduced expansion of Rps19-deficient
222 hematopoietic progenitors was mainly due to altered proliferation or apoptosis,
223 we performed DNA content analysis of the B/B cultures using flow cytometry.
224 Induction of Rps19 deficiency resulted in a dramatic increase in the frequency of
225 apoptotic cells as indicated by accumulation of these cells in the sub-G1 cell
226 cycle phase that corresponds to cells with hypodiploid DNA content (Figure 1E).
227 Furthermore, and similar to our previous study demonstrating a delay in G1/S
228 transition in Rps19-deficient erythroblasts *in vivo*¹⁵, following the exclusion of
229 apoptotic cells in the sub-G1 fraction from our analysis we observed a significant
230 accumulation of Rps19-deficient cells in the G1 cell cycle phase (Figure 1E).
231 While the heterozygous Mdm2^{C305F} background resulted in a partial rescue of
232 both viability and cell cycle progression, the extent of rescue in the homozygous
233 Mdm2^{C305F} background was close to complete (Figure 1E).

234

235 **5S RNP-Mdm2 interaction underlies the p53 activation in Rps19-deficient** 236 **hematopoietic progenitor cells regardless of the level of Rps19** 237 **downregulation**

238 Next, we assessed the impact of Rps19 deficiency on p53 response by
239 quantifying the expression of p53 transcriptional target genes (*Cdkn1a*, *Ptp4a3*,
240 *Zmat3*, *Bax*, *Ccng1* and *Phlda3*)¹⁵ in c-Kit-enriched hematopoietic progenitor
241 cells. Comparison of uninduced cultures with wild-type or homozygous
242 Mdm2^{C305F} background revealed no differences in the expression of the p53
243 target genes with the exception of *Zmat3* that was found to be significantly
244 upregulated by Mdm2^{C305F} (Figure 2A). However, the induction of Rps19
245 deficiency triggered a profound p53 response that was dependent on the level of
246 Rps19 deficiency. For instance, the fold increase in expression of *Cdkn1a* (*p21*)
247 in B/+ and B/B cultures was 1.8 and 4, respectively. Mdm2^{C305F} significantly

248 reduced the expression of p53 target genes in a dose-dependent manner
249 regardless of the level of Rps19 downregulation (Figure 2A).

250 Analysis of the doxycycline-treated B/B cells revealed a robust downregulation of
251 Rps19 protein (Figure 2B and 2C). Furthermore, correlating with the activation of
252 p53 transcriptional response, doxycycline-treated B/B cells with wild-type Mdm2
253 showed accumulation of the p53 protein as well as its transcriptional target Mdm2
254 (Figure 2B and 2C). Remarkably, a homozygous Mdm2^{C305F} background
255 reversed the accumulation of p53. Finally, in agreement with previous
256 studies^{27,28}, Rps19-deficient cells showed a decrease in the total level of Rps6,
257 but the pool of phosphorylated Rps6 was increased (Figure 2C). Interestingly,
258 Mdm2^{C305F} had no effect on the phosphorylation of Rps6.

259

260 **Disruption of the 5S RNP-Mdm2 interaction significantly improves the** 261 **erythroid defect in Rps19-deficient mice**

262 To study the physiological relevance of our *in vitro* findings we transplanted BM
263 from the B/B mice into lethally irradiated wild-type recipients (Figure 3A). We
264 chose this experimental strategy since we have previously shown that
265 doxycycline treatment of the B/B mice results in *Rps19* haploinsufficiency and the
266 resulting hematopoietic phenotype is autonomous to the blood system¹⁵.
267 Furthermore, this strategy allowed us to monitor the impact of Mdm2^{C305F} on the
268 hematopoietic recovery from transplantation before the onset of Rps19
269 deficiency. To assess whether Mdm2^{C305F} had an effect on hematopoiesis *per se*,
270 we analyzed the peripheral blood one month after transplantation. No obvious
271 differences in hematological parameters were observed between the recipients
272 transplanted with control or heterozygous Mdm2^{C305F} BM (Figure 3B,
273 Supplementary table 3). By contrast, when compared to mice with wild-type
274 Mdm2, the recipients with homozygous Mdm2^{C305F} BM showed a significant
275 decrease in the number of erythrocytes (93 % of the recipients with wild-type
276 Mdm2), hemoglobin concentration (95 % of the recipients with wild-type Mdm2),
277 number of white blood cells (74 % of the recipients with wild-type Mdm2), and

278 macrocytosis (Figure 3B). No differences in the number of reticulocytes were
279 observed (Supplementary table 3).

280 Next the recipient mice were administered doxycycline for two weeks in order to
281 induce Rps19 deficiency. The onset of Rps19 deficiency leads to a rapid
282 reduction in blood cellularity that is rescued in the p53-null background¹⁵. As
283 Rps19-deficient mice are partly able to compensate for the erythroid defect
284 caused by the induction of Rps19 deficiency, monitoring the blood and BM
285 cellularity during the first weeks of doxycycline administration provides a valid
286 model to assess the effect of Mdm2^{C305F} on the erythroid recovery of Rps19-
287 deficient mice²⁵. Doxycycline administration to the recipients transplanted with
288 B/B BM cells resulted in a robust reduction in the number of erythrocytes (58 %
289 of control), reticulocytes (67 % of control), and hemoglobin concentration (59 %
290 of control) (Figure 3C, Supplementary table 4). Heterozygous and homozygous
291 Mdm2^{C305F} background led to a significant increase in all erythroid parameters,
292 demonstrating the physiological relevance of the 5S RNP-Mdm2-p53 pathway for
293 the erythroid defect in Rps19-deficient mice (Figure 3C, Supplementary table 4).
294 In addition to improved erythropoiesis, the recipients with Mdm2^{C305F} showed an
295 increase in the number of platelets and total BM cellularity, further demonstrating
296 the more pronounced hematological recovery after induction of Rps19 deficiency
297 in these mice (Figure 3C-D). Finally, despite the reduction in white blood cells
298 prior to doxycycline administration by Mdm2^{C305F} (Figure 3B), Mdm2^{C305F} had no
299 negative effect on white blood cell number in the recipients with Rps19-deficient
300 BM (Figure 3C).

301

302 **Mdm2^{C305F} knock-in mice show selective defect in erythropoiesis**

303 Given the negative impact of Mdm2^{C305F} *per se* on the hematopoietic recovery
304 after transplantation (Figure 3B), we decided to characterize the hematopoietic
305 phenotype of the Mdm2^{C305F} knock-in mice in more detail. Indeed, careful
306 elucidation of the hematopoietic phenotype caused by Mdm2^{C305F} is essential in
307 order to estimate the significance of the 5S RNP-Mdm2-p53 pathway on Rps19-
308 deficient erythropoiesis (Figure 3C). Peripheral blood analysis revealed no

309 significant differences between control and heterozygous Mdm2^{C305F} mice
310 (Figure 4A). However, similar to recipients transplanted with the homozygous
311 Mdm2^{C305F} BM (Figure 3B), the homozygous Mdm2^{C305F} knock-in mice showed
312 significant decrease in the number of erythrocytes that was associated with
313 macrocytosis, and decrease in hemoglobin concentration (Figure 4A). In addition
314 to the erythroid phenotype we observed an increase in the number of platelets
315 (Figure 4A).

316 To gain further insights on the nature of the hematopoietic defect in the
317 homozygous Mdm2^{C305F} knock-in mice we applied FACS strategies that allow for
318 fractionation of HSCs, myeloerythroid progenitors and erythroid precursors in the
319 BM^{15,25} (Supplementary figure 2A and 2B). Correlating with the reduced number
320 of erythrocytes in the peripheral blood, BM of the homozygous Mdm2^{C305F} mice
321 showed a distinct reduction in the frequency of erythroid-committed preCFU-E
322 (64 % of control) and CFU-E (57 % of control) progenitor cells, and more mature
323 erythroblasts in otherwise normocellular BM (Figure 4B and 4C, and
324 Supplementary figure 2C).

325 Although we observed no major differences in p53 response between uninduced
326 (Rps19-proficient) wild-type and homozygous Mdm2^{C305F} cultures of c-kit-
327 enriched hematopoietic stem and progenitor cells (Figure 2A), we wanted to
328 confirm the impact of Mdm2^{C305F} on the p53 pathway in highly purified progenitor
329 subpopulations. Given the strict correlation between the expression of p53
330 transcriptional target genes and the level of p53 protein (Figure 2A-C), we used
331 the expression of p53 transcriptional targets as an indicator of p53 activity.
332 Strikingly, quantification of the p53 transcriptional targets in freshly isolated
333 immature (Lineage⁻Sca-1⁺c-Kit⁺; LSK), myeloid-committed (preGM/GMP) and
334 erythroid-committed (CFU-E) hematopoietic progenitor cells revealed a relatively
335 selective and pronounced activation of the p53 signature in the erythroid-
336 committed CFU-E progenitors of the homozygous Mdm2^{C305F} knock-in mice
337 (Figure 4D). Corroborating these data, expression of two additional p53
338 transcriptional target genes *Phlda3* and *Bax* was increased on average 3.9 fold
339 and 1.7 fold, respectively, in CFU-Es by Mdm2^{C305F} (Supplementary figure 3).

340

341 **Mdm2^{C305F} ameliorates the functional decline of the hematopoietic system**
342 **upon replicative stress**

343 To study the impact of Mdm2^{C305F} on hematopoiesis upon stress, we
344 transplanted lethally irradiated wild-type recipients with unfractionated control or
345 Mdm2^{C305F} BM cells together with unfractionated congenic wild-type support BM
346 (Figure 5A). In contrast to the primary Mdm2^{C305F} knock-in mice, the recipients of
347 Mdm2^{C305F} BM cells developed a profound decrease in white blood cell
348 reconstitution that appeared especially pronounced for the lymphoid lineages
349 (Figure 5B). These results are in line with the significant decrease in the total
350 number of white blood cells upon transplantation of homozygous Mdm2^{C305F} BM
351 (Figure 3B), and demonstrate that the negative impact of Mdm2^{C305F} on
352 hematopoiesis is not restricted to the erythroid lineage upon transplantation-
353 mediated stress. As would be expected due to the competitive nature of the
354 transplantation experiments, BM analysis of the recipients four to five months
355 after transplantation revealed no differences in the total BM cellularity or in the
356 frequency of HSCs and multipotent progenitor cells (MPPs) (Figure 5C). While
357 the frequency of donor-derived MPPs was modestly decreased in the recipients
358 with Mdm2^{C305F} BM, no difference was observed in the frequencies of donor-
359 derived HSCs (Figure 5D).

360 To directly assess the effect of Mdm2^{C305F} on regenerative properties of HSCs,
361 we FACS-sorted highly purified donor HSCs (CD45.2 LSK CD150+CD48-) from
362 the control or homozygous Mdm2^{C305F} primary recipients, and transplanted them
363 into lethally irradiated secondary recipients together with fresh unfractionated
364 wild-type BM (Figure 5E). After five months, the recipients transplanted with
365 control HSCs showed a robust myeloid reconstitution, while the reconstitution of
366 the lymphoid lineages was low (Figure 5F). Surprisingly, the homozygous
367 Mdm2^{C305F} HSCs gave rise to at least equivalent myeloid and lymphoid
368 reconstitution compared to the control HSCs (Figure 5F). Therefore, when taking
369 into account the significant negative impact mediated by Mdm^{C305F} in primary
370 transplantations (Figure 5B), these data strongly indicate that Mdm2^{C305F} may

371 ameliorate the functional decline that normally associates with HSCs upon
372 replicative stress.

373

374

375 **DISCUSSION**

376

377 Studies using animal models and primary human hematopoietic cells have
378 suggested that the increased activation of p53 may be the underlying cause of
379 the anemia in DBA¹²⁻¹⁵. Our current study confirms the prevailing hypothesis that
380 the p53 activation upon Rps19 deficiency is dominantly mediated through the 5S
381 RNP-Mdm2 interaction and demonstrates for the first time the physiological
382 relevance of the 5S RNP-Mdm2-p53 pathway for the erythroid defect in DBA.
383 Finally, our results suggest that the 5S RNP-Mdm2-p53 pathway may contribute
384 to the replicative stress-associated functional decline of the hematopoietic
385 system.

386 Our cell culture studies revealed an activation of the p53 pathway upon induction
387 of Rps19 deficiency in c-Kit-enriched hematopoietic stem and progenitor cells
388 and that the degree of the p53 response was strictly dependent on the level of
389 Rps19 downregulation. Mdm2^{C305F} caused a significant reduction in the level and
390 activity of p53 regardless of the extent of Rps19 downregulation, demonstrating a
391 dominant role of the 5S RNP-Mdm2 interaction for p53 activation upon Rps19
392 deficiency. Reduction of the p53 activity in Rps19-deficient hematopoietic stem
393 and progenitor cells by Mdm2^{C305F} normalized their viability and improved their
394 proliferative capacity, resulting in significantly increased expansion of these cells
395 *in vitro*. Importantly, reduction in p53 activity in cells with haploinsufficient
396 expression of *Rps19*, a condition similar to DBA, led to a close to complete
397 reversal of the expansion defect.

398 We and others have previously demonstrated that the hematopoietic phenotype
399 upon Rps19 deficiency is reversed in the absence of p53^{12,13,15}. However, the
400 use of p53-deficient mice to study the relevance of p53 for the hematopoietic
401 defect in DBA is problematic due to its additional role in restraining hematopoietic

402 stem and progenitor cell proliferation^{29,30}. Indeed, increased influx of
403 hematopoietic progenitor cells into the erythroid lineage in p53-deficient mice
404 may mask the effect of p53-independent components that contribute to the
405 erythroid defect in Rps19-deficient mice. Given that the Mdm2^{C305F} mice have
406 wild-type p53, this model addresses more accurately the impact of p53 on
407 Rps19-deficient hematopoiesis in absence of the physiological compensatory
408 mechanisms present in the p53-deficient background. The significant
409 improvement of the Rps19-deficient erythropoiesis by Mdm2^{C305F} clearly
410 demonstrates the physiological and cell-autonomous relevance of the 5S RNP-
411 Mdm2-p53 pathway for the disease phenotype *in vivo*. Interestingly, extent of the
412 erythroid rescue by Mdm2^{C305F} was only partial, which was initially surprising
413 given the robust improvement of the expansion defect of Rps19-deficient
414 hematopoietic stem and progenitors *in vitro*. This finding can be explained in part
415 by the subsequent identification of the selective erythroid defect caused by
416 Mdm2^{C305F}. Indeed, the homozygous Mdm2^{C305F} knock-in mice showed a
417 reduction in the frequency of erythroid-committed preCFU-E and CFU-E
418 progenitor cells that was associated with a marked increase in the expression of
419 p53 transcriptional target genes at these cellular stages. This erythroid-
420 pronounced activation of the p53 signature was not detected in our cell culture
421 experiments due to the low frequency of preCFU-E and CFU-E progenitors within
422 the c-Kit-enriched hematopoietic stem and progenitor cells in the homozygous
423 Mdm2^{C305F} mice. Finally, in contrast to the dose-dependent reversal of the p53
424 response by Mdm2^{C305F} *in vitro*, the heterozygous and homozygous Mdm2^{C305F}
425 background resulted in comparable erythroid rescue in the Rps19-deficient
426 recipients, further highlighting the negative impact of the Mdm2^{C305F} *per se* on
427 erythropoiesis. Together with our earlier study locating the most severe erythroid
428 defect in Rps19-deficient mice at the CFU-E stage¹⁵, these data collectively
429 indicate that both activation as well as disruption of the 5S RNP-Mdm2
430 interaction result in defective erythropoiesis at the level of CFU-E progenitor cell.
431 Erythroid progenitor cells therefore appear especially sensitive to aberrations in
432 p53 homeostasis mediated by the 5S RNP-Mdm2 interaction, with the 5S RNP-

433 Mdm2-p53 pathway representing a likely contributor to the complex pathogenesis
434 of DBA, although the impact of additional and to date unidentified effects caused
435 by inactivation of the Mdm2 zinc finger cannot be entirely ruled out.
436 In contrast to steady-state hematopoiesis, transplantation of Mdm2^{C305F} BM
437 revealed a significant reconstitution disadvantage compared to the wild-type
438 cells, and this appeared especially pronounced for the lymphoid lineages. These
439 data demonstrate that the defect caused by Mdm2^{C305F} extends beyond the
440 erythroid lineage upon hematopoietic stress. Despite an initial negative impact of
441 Mdm2^{C305F} on hematopoietic regeneration, serial transplantation of purified wild-
442 type and homozygous Mdm2^{C305F} HSCs resulted in comparable myeloid and
443 lymphoid peripheral blood chimerism, suggesting that Mdm2^{C305F} may provide
444 HSCs with a reconstitution advantage over time. Since serial transplantation
445 mimics many aspects of normal aging of the hematopoietic system³¹, our data
446 therefore implicate that the 5S RNP-Mdm2-p53 pathway may contribute to this
447 process. Interestingly, aging-associated decrease in lymphopoiesis has been
448 attributed to increased levels of the tumor suppressors p16 and p19Arf over
449 time³². Given that the activation of p53 by p19Arf is at least partially dependent
450 on the 5S RNP-Mdm2 interaction²², we speculate that disruption of this pathway
451 by Mdm2^{C305F} in serially transplanted HSCs and their progeny could underlie the
452 observed relative increase in their lymphoid reconstitution capacity.
453 Taken together, our study demonstrates the dominant role of the 5S RNP-Mdm2
454 interaction as a mediator of the p53 response upon Rps19 deficiency and
455 provides the first physiological evidence that the 5S RNP-Mdm2-p53 pathway
456 contributes to the anemia in DBA, and may also contribute to normal aging of the
457 hematopoietic system.

458
459

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468

469

470 **CONFLICT OF INTEREST DISCLOSURE**

471 The authors declare no conflict of interests.

472

473

474 **CORRESPONDENCE**

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478 Supplementary information is available at Leukemia's website.

479

480

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583
584

585 **FIGURE LEGENDS**

586

587 **Figure 1. Mdm2^{C305F} improves the expansion of Rps19-deficient**
588 **hematopoietic progenitors *in vitro*.** (A) Schematic overview of the transgenic
589 Rps19 knockdown mouse model. This model contains an *Rps19*-targeting
590 shRNA (shRNA-B) under the control of doxycycline-regulatable control element
591 downstream of the *Collagen A1* gene, and constitutively active M2 reverse
592 tetracycline transactivator (M2-rtTA) under the endogenous *Rosa26*¹⁵. (B)
593 Schematic overview of the Mdm2^{C305F} knock-in mouse model. This model has a
594 missense mutation in the central zinc finger domain of Mdm2 that disrupts its
595 binding to the 5S RNP²³. (C) Quantitative real-time PCR analysis of *Rps19*
596 mRNA levels in cultures initiated with c-Kit-enriched BM cells isolated from
597 heterozygous [B/+] and homozygous [B/B] shRNA-B mice. Samples for RNA
598 extraction were collected after 48 hours (n= 6 for the groups without doxycycline;
599 n= 3 for the groups with doxycycline). (D) Expansion of 0.5 x10⁶ c-Kit-enriched
600 BM cells in cultures (n= 6 for the groups without doxycycline; n= 4 for the groups
601 with doxycycline). (E) Cell cycle analysis of c-Kit-enriched BM cells from in
602 cultures. DNA content analysis was performed after 72 hours on c-Kit+ cells (n=
603 3 per group). Dean-Jett-Fox model was used to calculate percentages of cells in
604 G0/G1, S, and G2/M phases (shown in brown). Data are presented as mean±
605 standard deviation.

606

607 **Figure 2. 5S RNP-Mdm2 interaction underlies the p53 activation in Rps19-**
608 **deficient hematopoietic progenitor cells.** (A) Quantitative real-time PCR
609 analysis of previously identified p53 transcriptional target genes¹⁵ in cultures
610 initiated with c-Kit-enriched BM cells. Samples for RNA extraction were collected
611 after 48 hours (n= 5 for the groups without doxycycline; n= 3 for the groups with
612 doxycycline). (B) Representative immunoblots of Mdm2, p53 and Rps19 of
613 cultures initiated with c-Kit-enriched B/B BM cells. Samples were collected after
614 48 hours. (C) Densitometry analysis of p53 and Rps19 protein shown in (B) (n=
615 3). (D) Representative immunoblots of Rps6 and p-Rps6 of cultures initiated with

616 c-Kit-enriched B/B BM cells. Samples were collected after 48 hours. Data are
617 presented as mean± standard deviation.

618

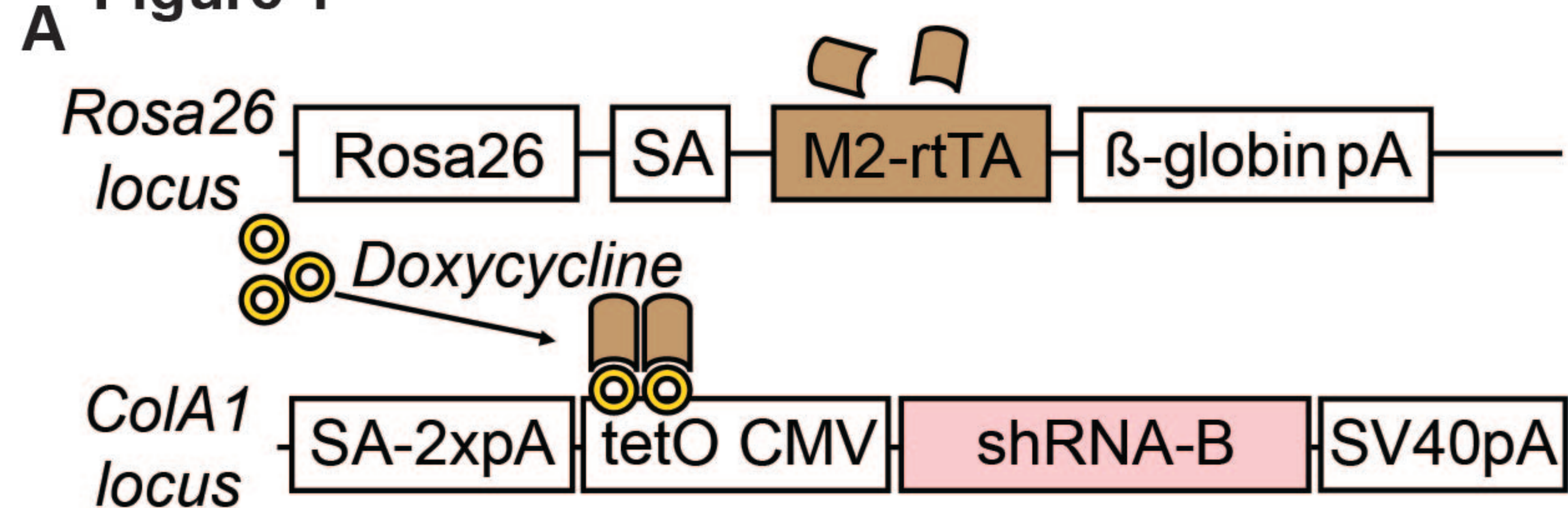
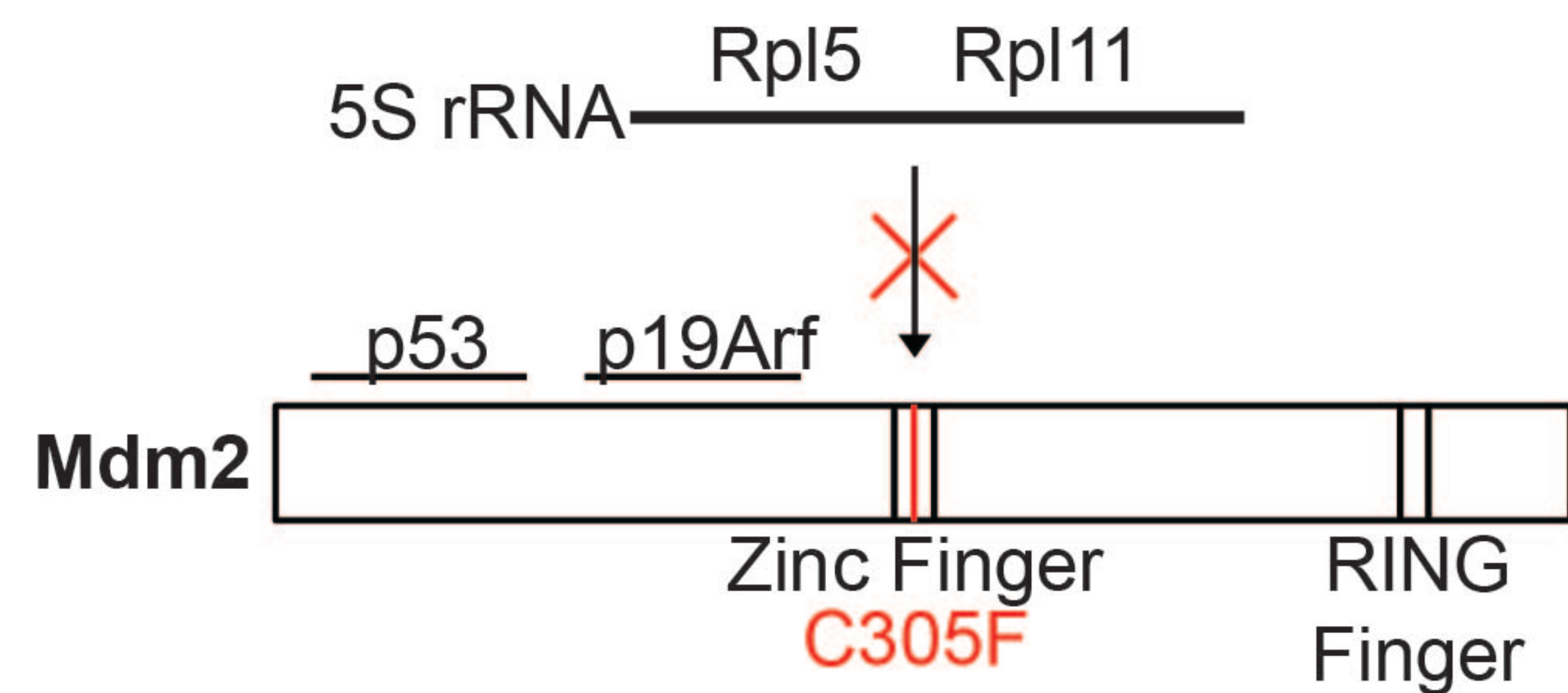
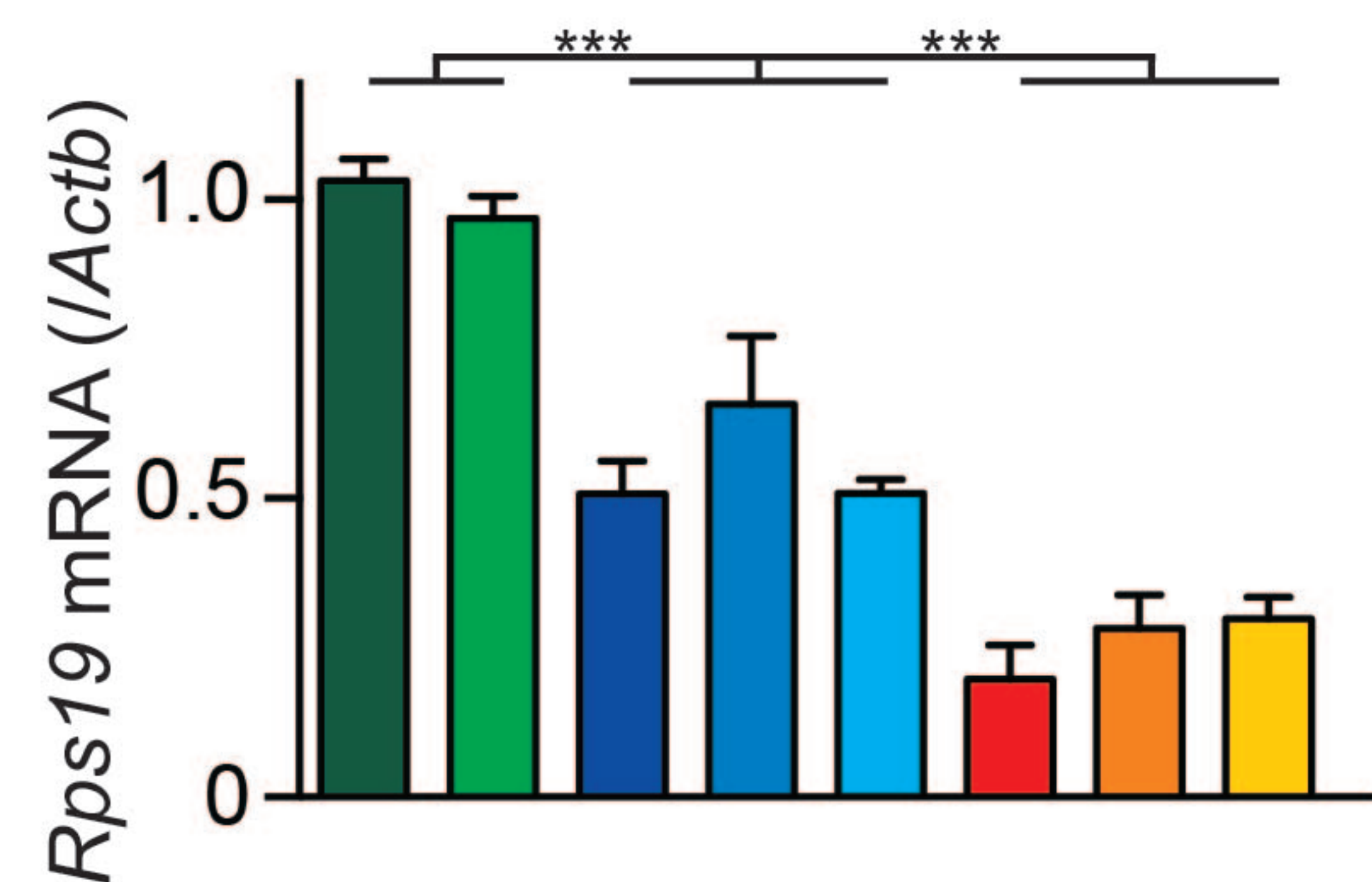
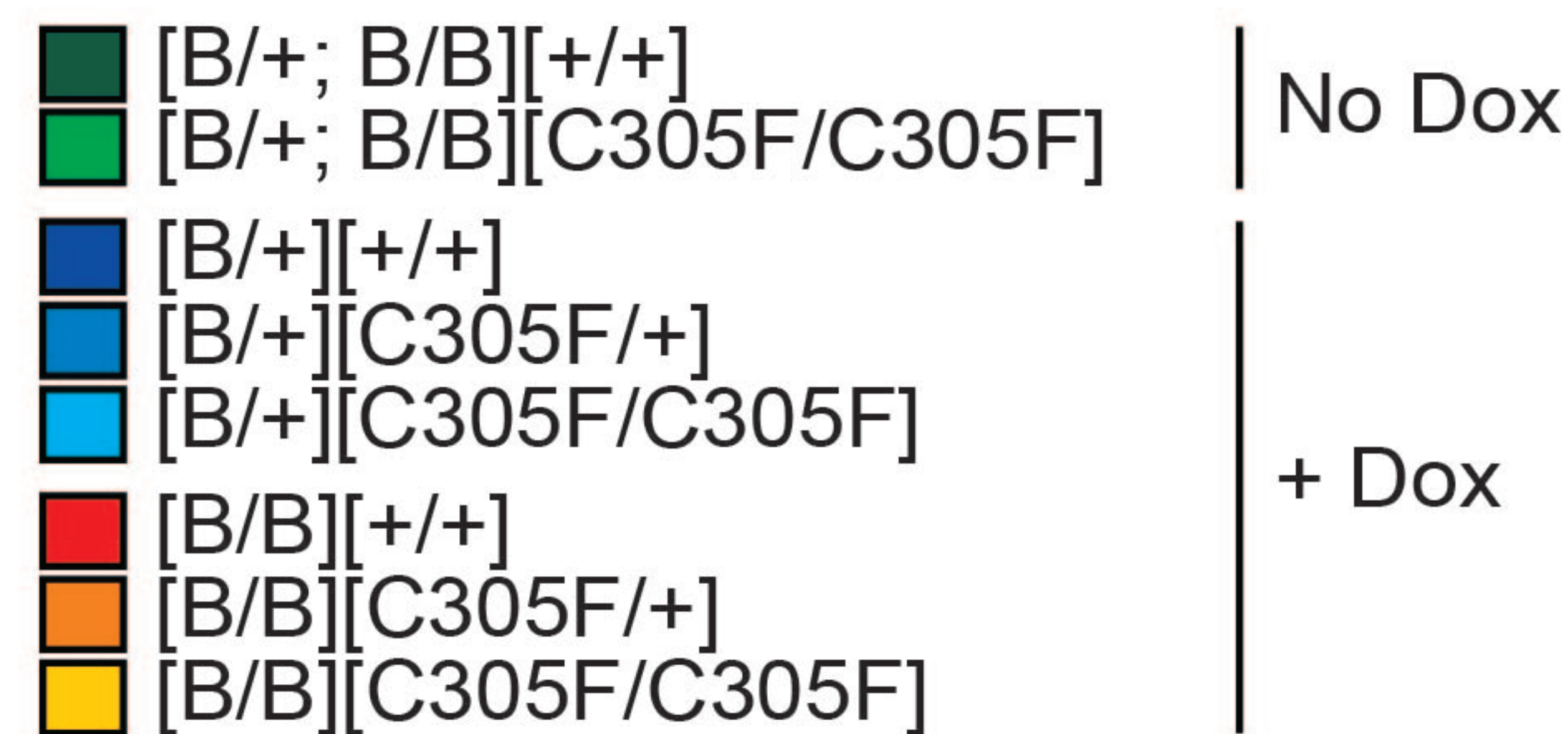
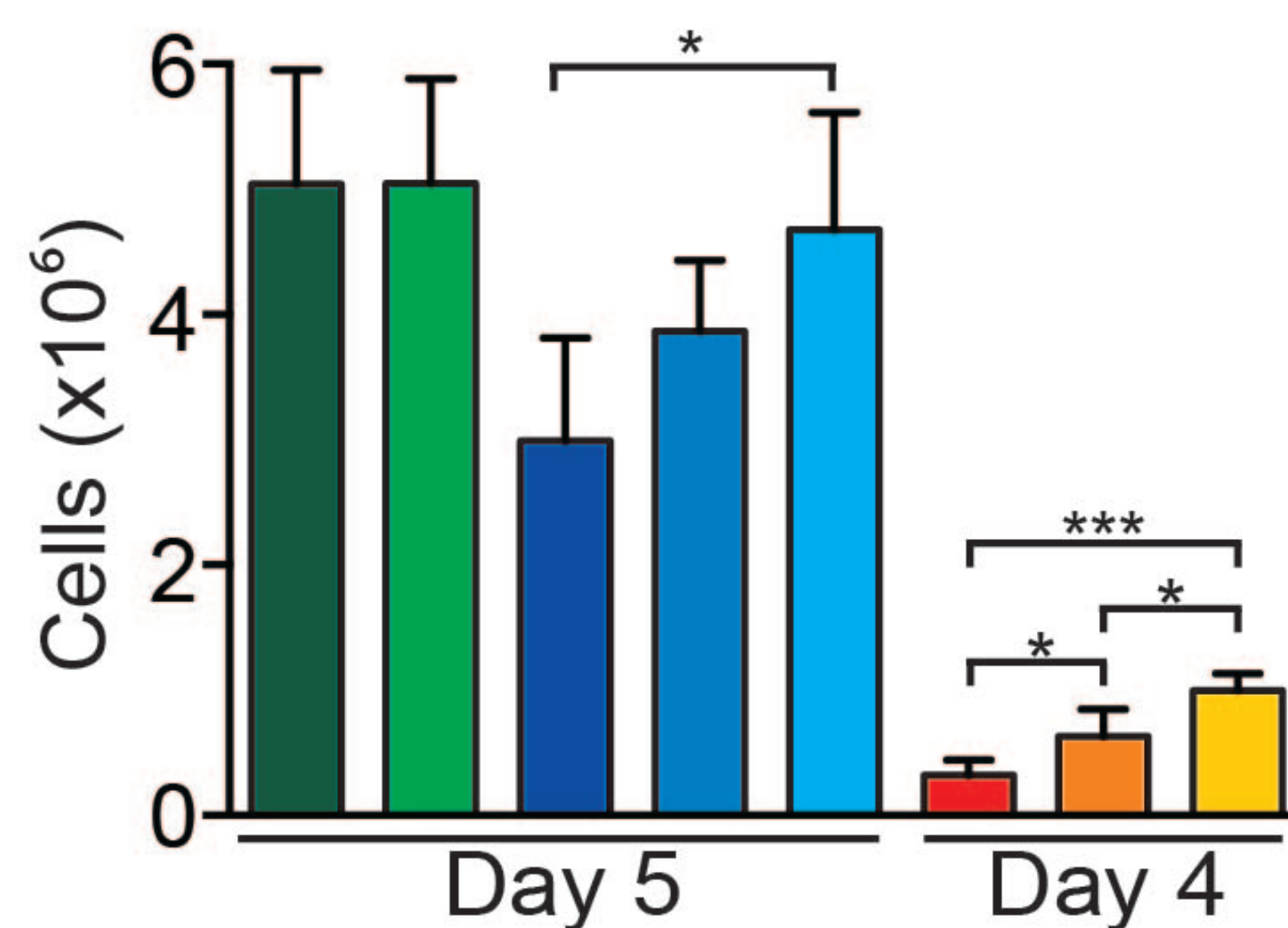
619 **Figure 3. Mdm2^{C305F} ameliorates the anemia in Rps19-deficient mice.** (A)
620 Experimental strategy to assess the effect of Mdm2^{C305F} on Rps19-proficient
621 hematopoietic recovery following transplantation and on Rps19-deficient
622 hematopoiesis. One million uninduced unfractionated BM cells were transplanted
623 in 300 µL PBS into the tail vein of lethally irradiated (900 cGy) wild-type
624 recipients. (B) Erythrocyte number, hemoglobin concentration, mean corpuscular
625 volume (MCV), white blood cell and platelet numbers before the administration of
626 doxycycline (n= 25, 24 and 33 for the [B/B; +/+], [B/B; C305F/+], and [B/B;
627 C305F/C305F] groups, respectively). (C) Erythrocyte number, hemoglobin
628 concentration, MCV, reticulocyte, white blood cell and platelet numbers two
629 weeks after doxycycline administration (n= 28, 20, 19 and 19 for the [+/+; +/+],
630 [B/B; +/+], [B/B; C305F/+], and [B/B; C305F/C305F] groups, respectively). (D) BM
631 cellularity two weeks after doxycycline administration (n= 27, 18, 18 and 17 for
632 the [+/+; +/+], [B/B; +/+], [B/B; C305F/+], and [B/B; C305F/C305F] groups,
633 respectively). Data are presented as mean± standard deviation.

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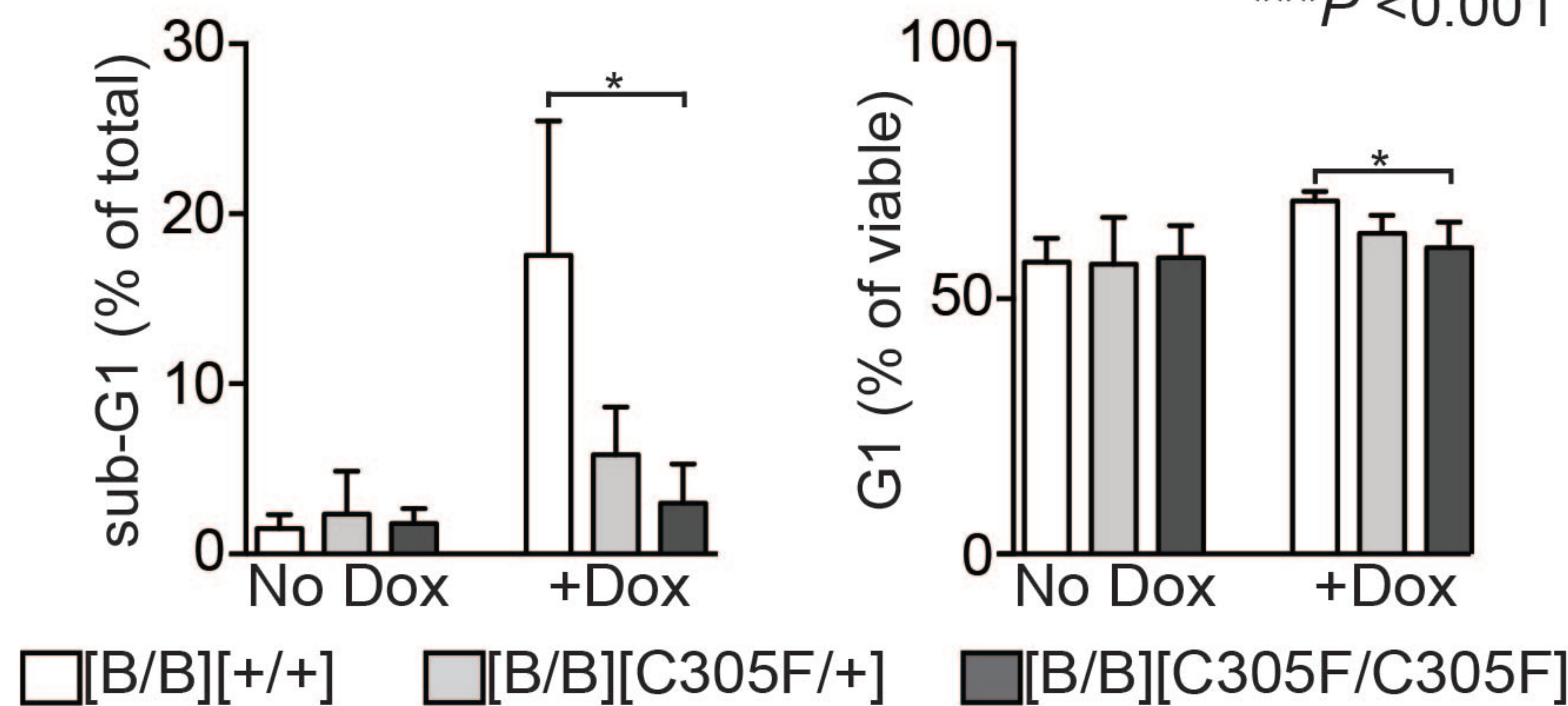
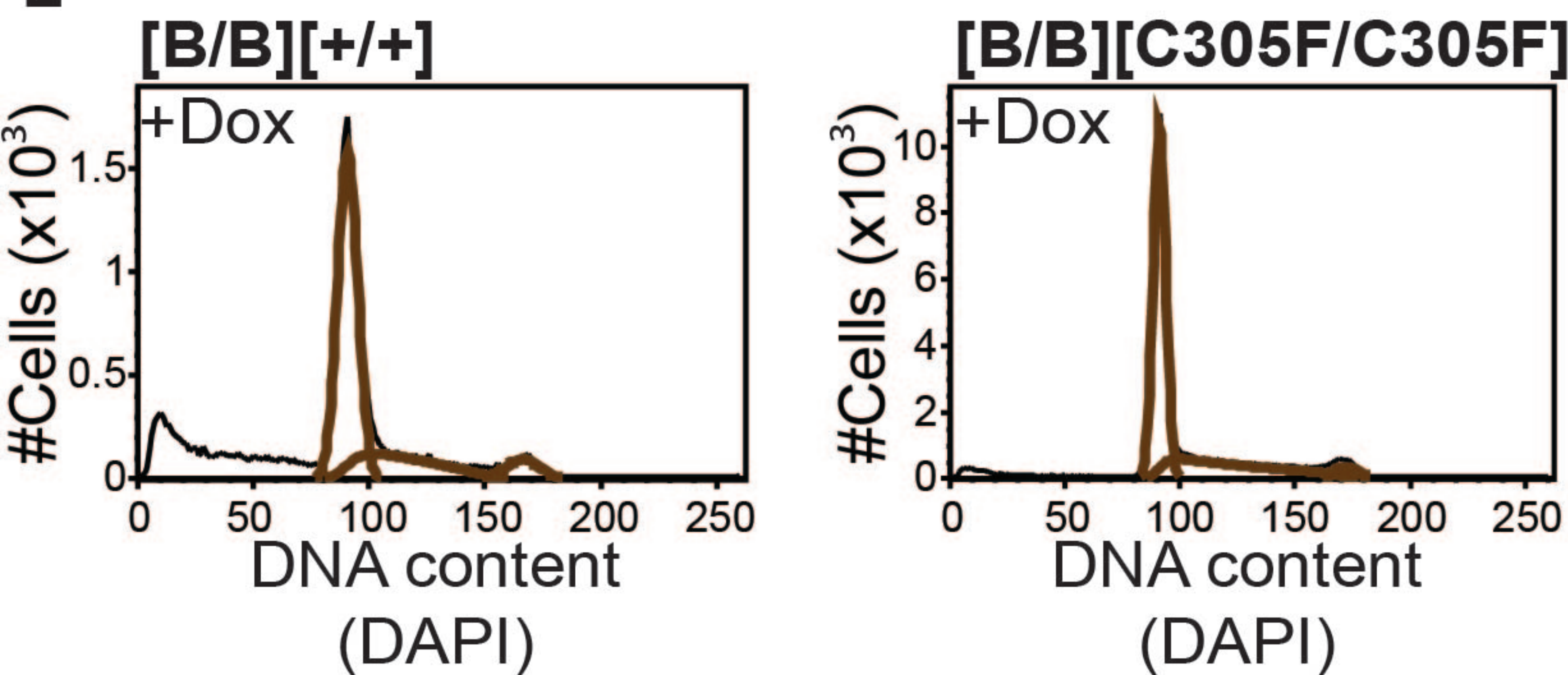
635 **Figure 4. Mdm2^{C305F} knock-in mice show selective defect in erythropoiesis.**
636 (A) Erythrocyte number, hemoglobin concentration, mean corpuscular volume
637 (MCV), platelet number and white blood cell number in primary Mdm2^{C305F} knock-
638 in mice (n= 10, 16 and 9 for the [+/+], [C305F/+], and [C305F/C305F] groups,
639 respectively). (B) Bone marrow cellularity (n= 8 per group). (C) Frequencies of
640 erythroid committed preCFU-E and CFU-E progenitor cells, and erythroblasts in
641 the BM of primary Mdm2^{C305F} knock-in mice (n= 8 per group). (D) Quantitative
642 real-time PCR analysis of previously identified p53 transcriptional target genes¹⁵
643 in freshly isolated LSK, preGM/GMP and CFU-E progenitor populations (n= 4 per
644 group). Data are presented as mean± standard deviation. EB=erythroblast.

645

646 **Figure 5. Mdm2^{C305F} ameliorates the functional decline of the hematopoietic**
647 **system upon replicative stress.** (A) Experimental strategy to assess the impact
648 of Mdm2^{C305F} on transplantation-mediated hematopoietic stress. 0.66 million
649 unfractionated donor BM cells were transplanted together with 0.33 million
650 unfractionated support BM cells in 300 μ L PBS into the tail vein of lethally
651 irradiated (900 cGy) wild-type recipients. (B) Myeloid (CD11b+), B cell (CD19+)
652 and T cell (CD3+) donor reconstitution in primary recipients one and four months
653 after transplantation (n= 26, 14 and 26 for the [+/+], [C305F/+] and
654 [C305F/C305F] groups, respectively). (C) Bone marrow cellularity and (D)
655 frequencies of total and donor-derived HSCs (CD45.2 LSK CD150+ CD48-) and
656 multipotent progenitor (MPP; CD45.2 LSK CD150- CD48-) cells in primary
657 recipients four to five months after transplantation (n= 27, 13 and 26 for the [+/+],
658 [C305F/+] and [C305F/C305F] groups, respectively). (E) Experimental strategy to
659 assess the impact of Mdm2^{C305F} on regenerative properties of HSCs. BM from
660 primary recipients were pooled per genotype and 375 FACS-sorted donor HSCs
661 (CD45.2 LSK CD150+ CD48-) were transplanted together with 0.4 million fresh
662 unfractionated support BM cells in 300 μ L PBS into the tail vein of lethally
663 irradiated (900 cGy) wild-type recipients. (F) Myeloid (CD11b+), B cell (CD19+)
664 and T cell (CD3+) donor reconstitution in secondary recipients five months after
665 transplantation (n= 11 per group and represents the average of two biological
666 experiments). Data are presented as mean \pm standard deviation.

Figure 1**B****C****D**

* $P < 0.05$
 ** $P < 0.01$
 *** $P < 0.001$

E

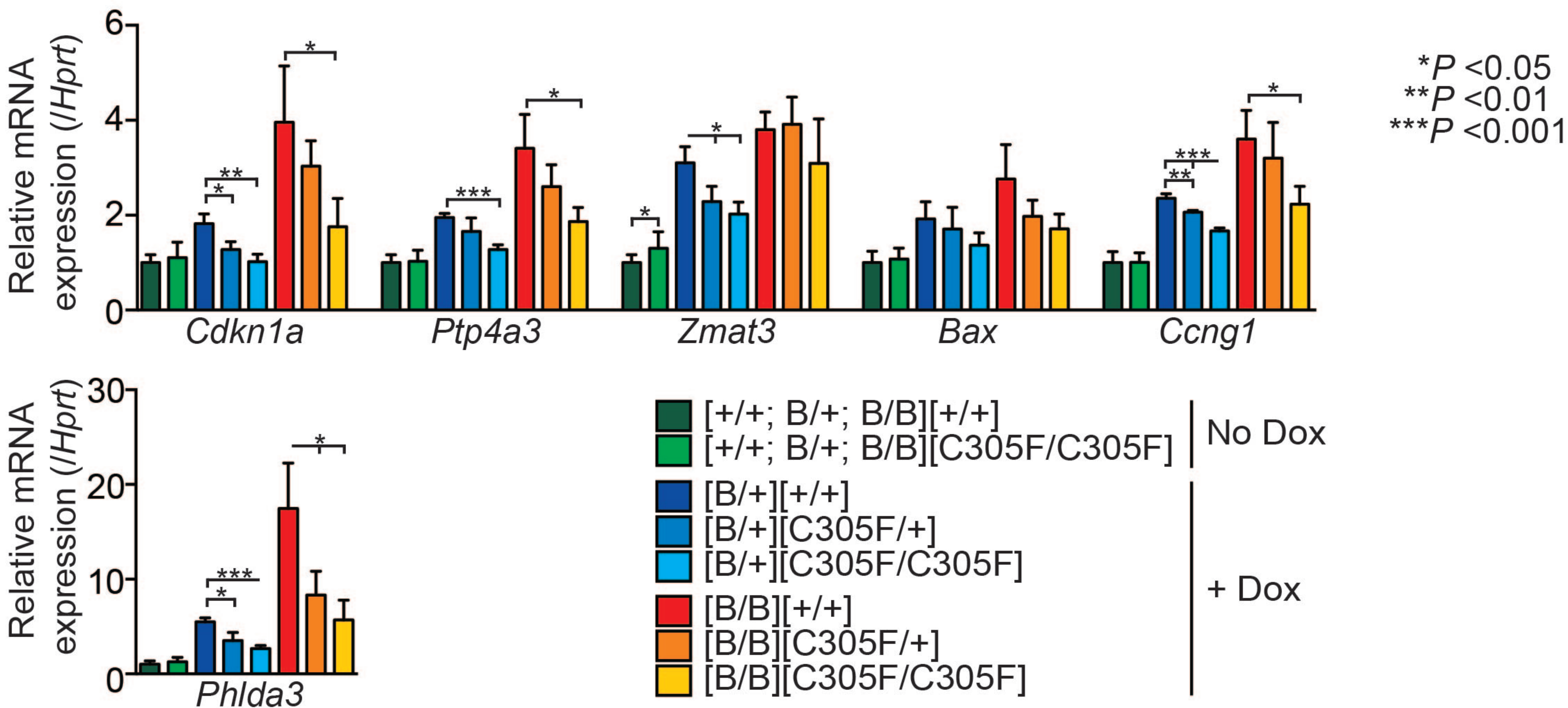
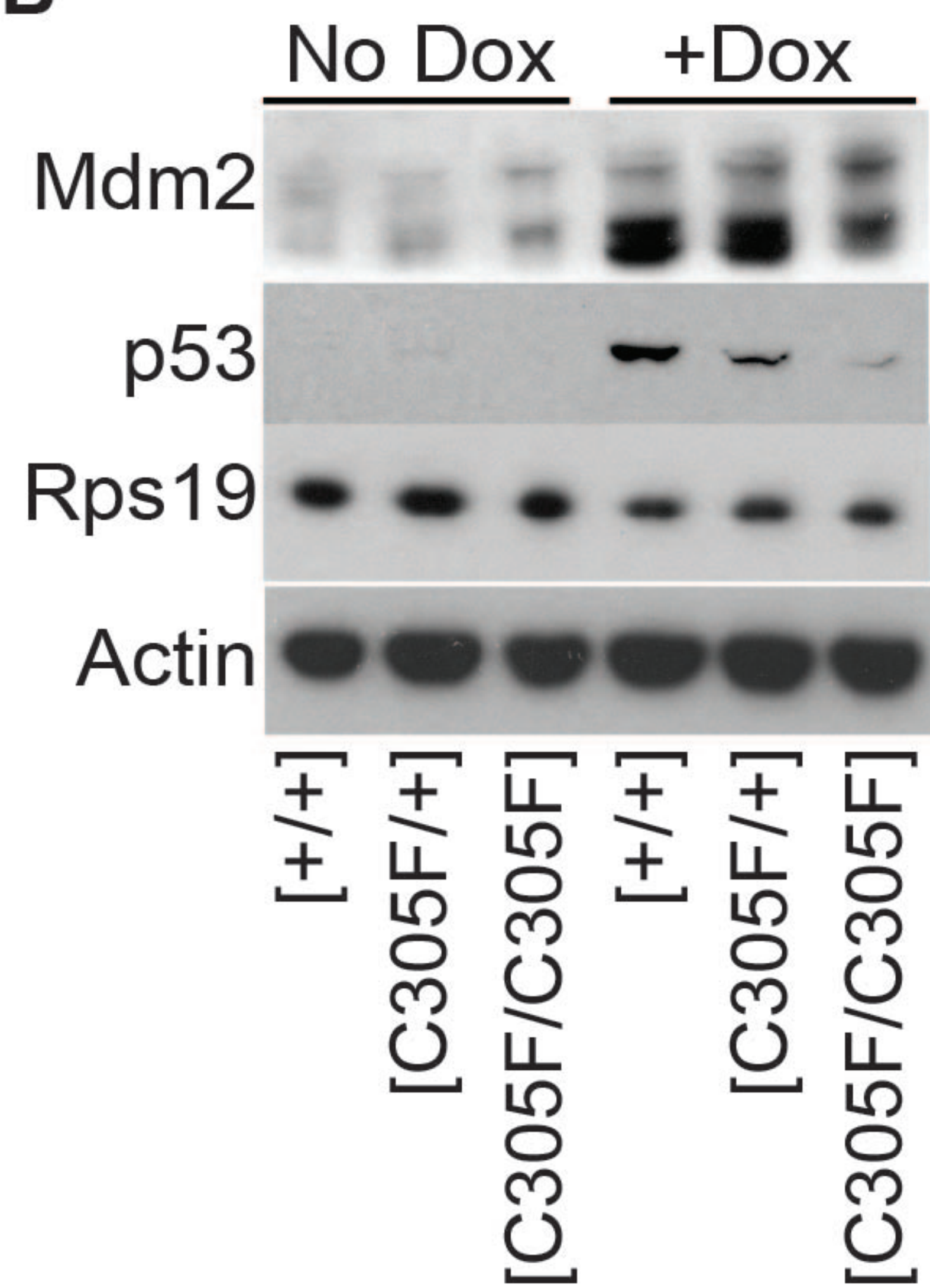
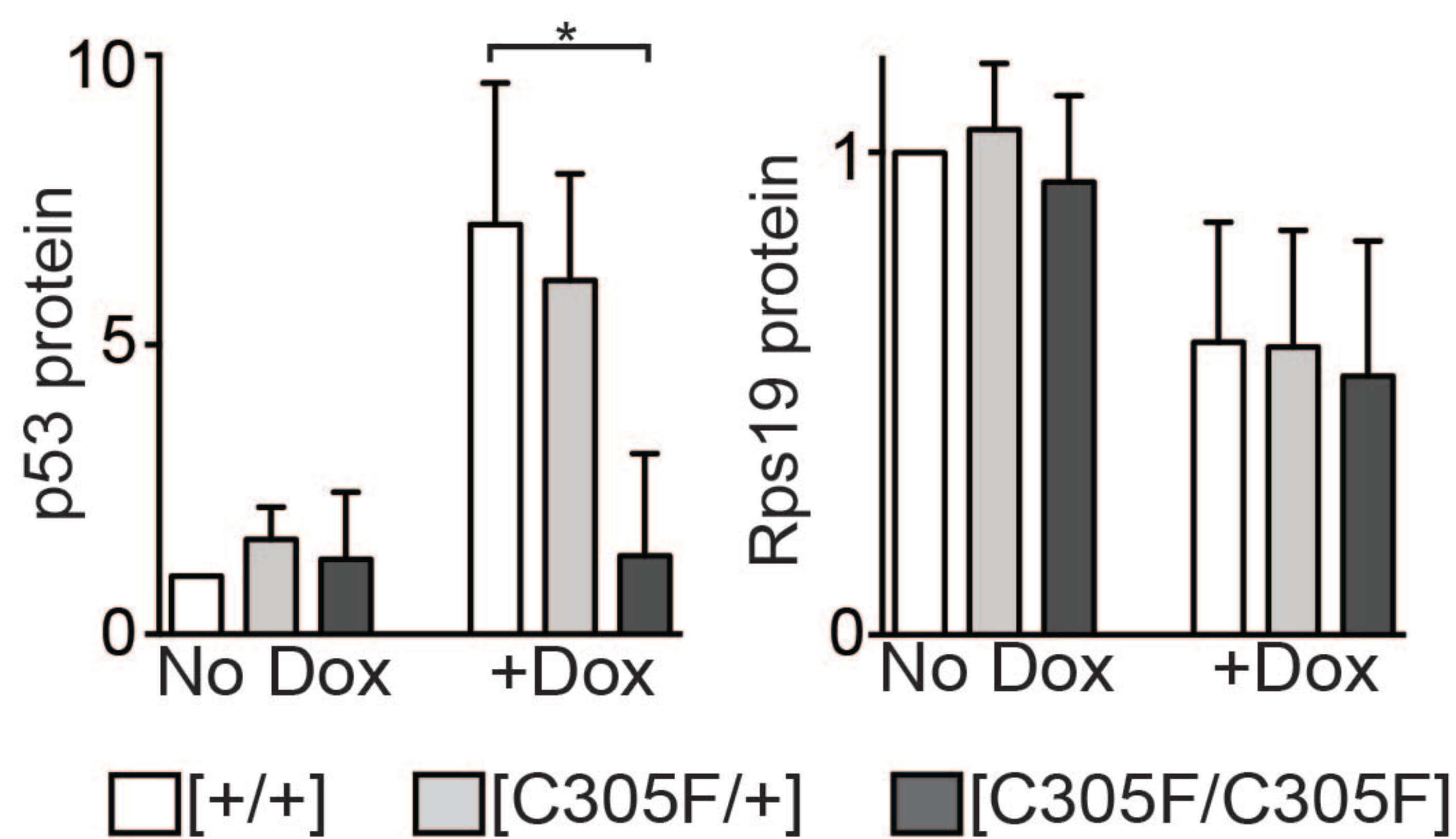
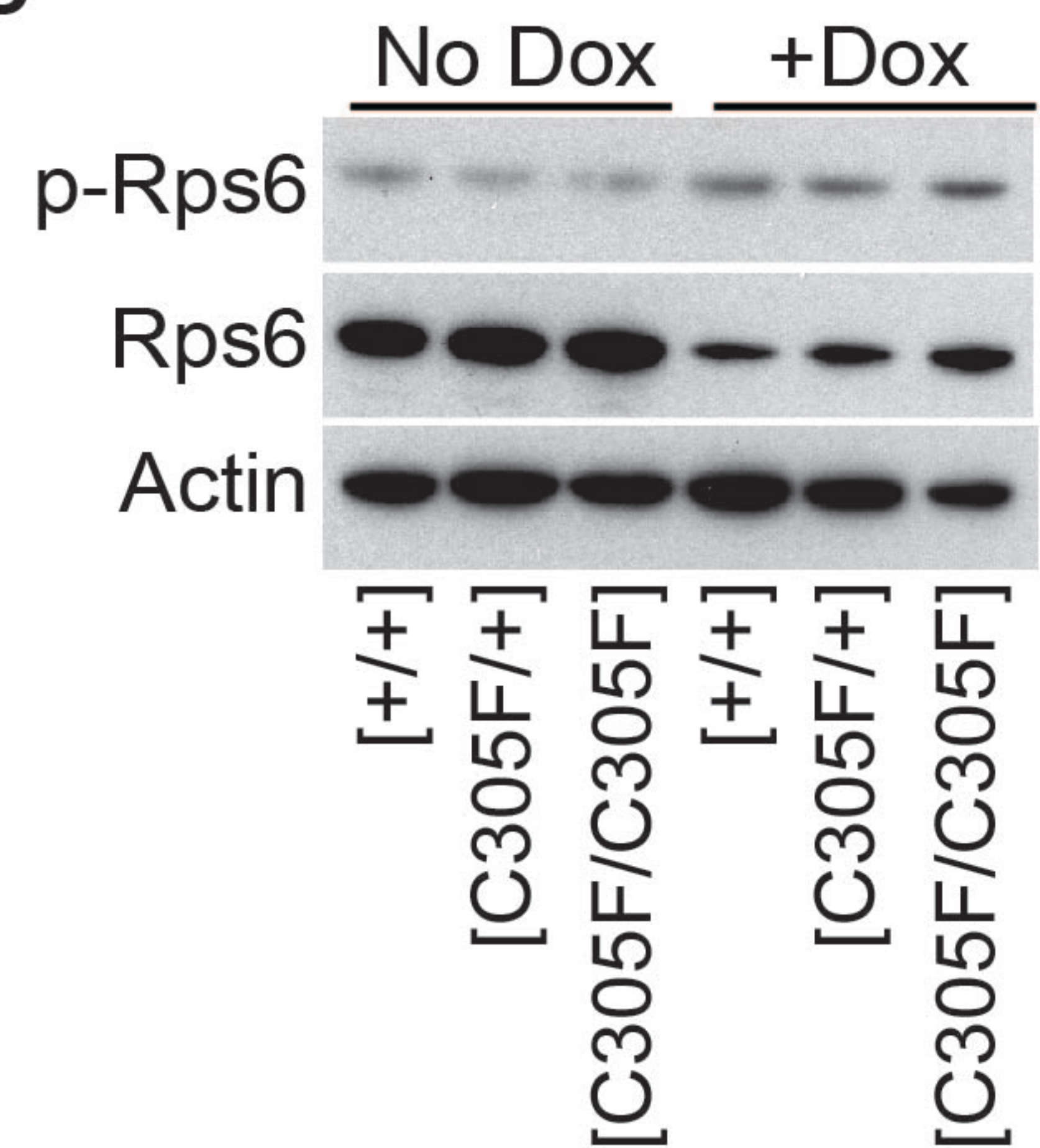
A Figure 2**B****C****D**

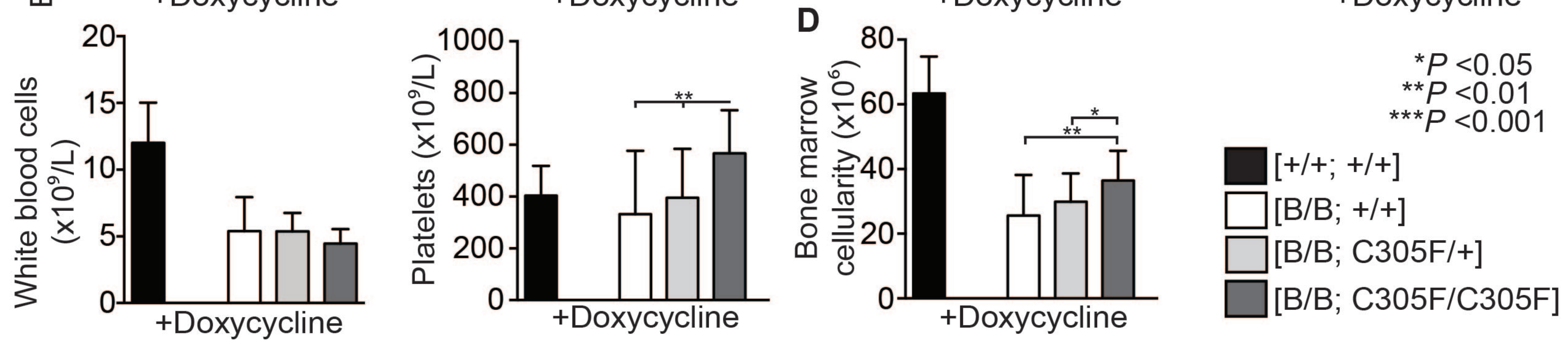
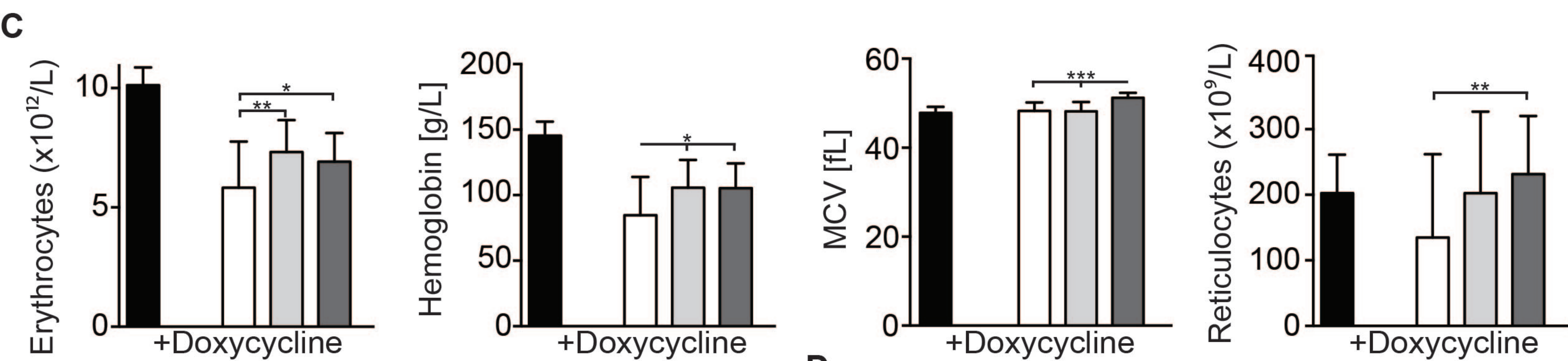
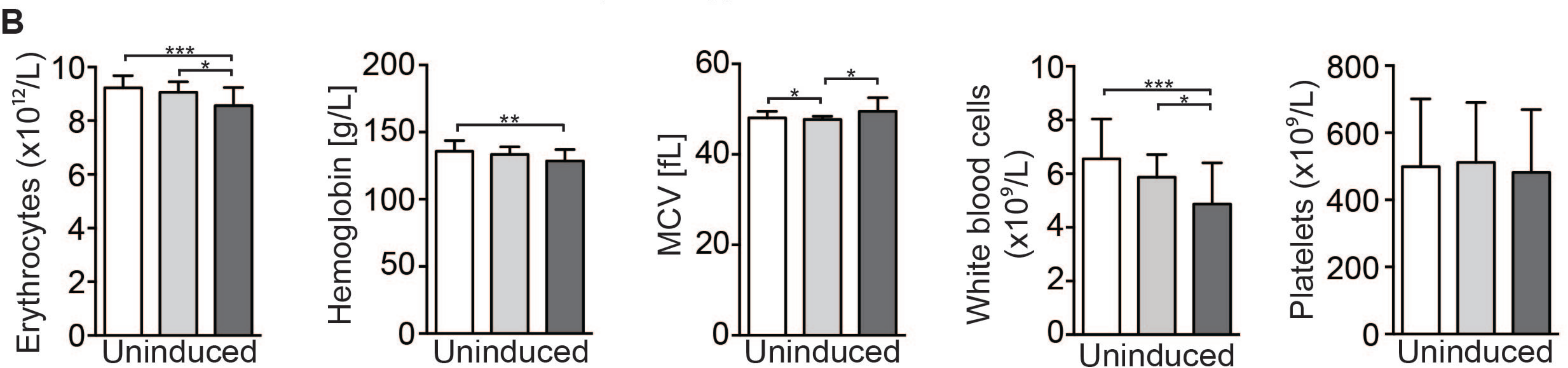
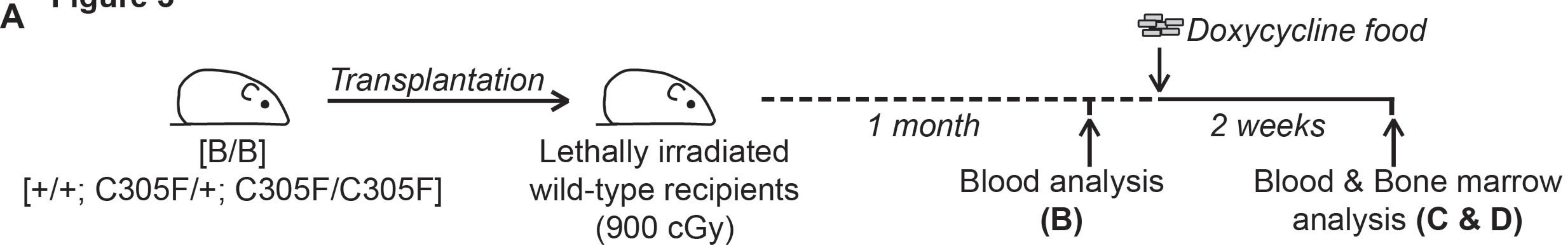
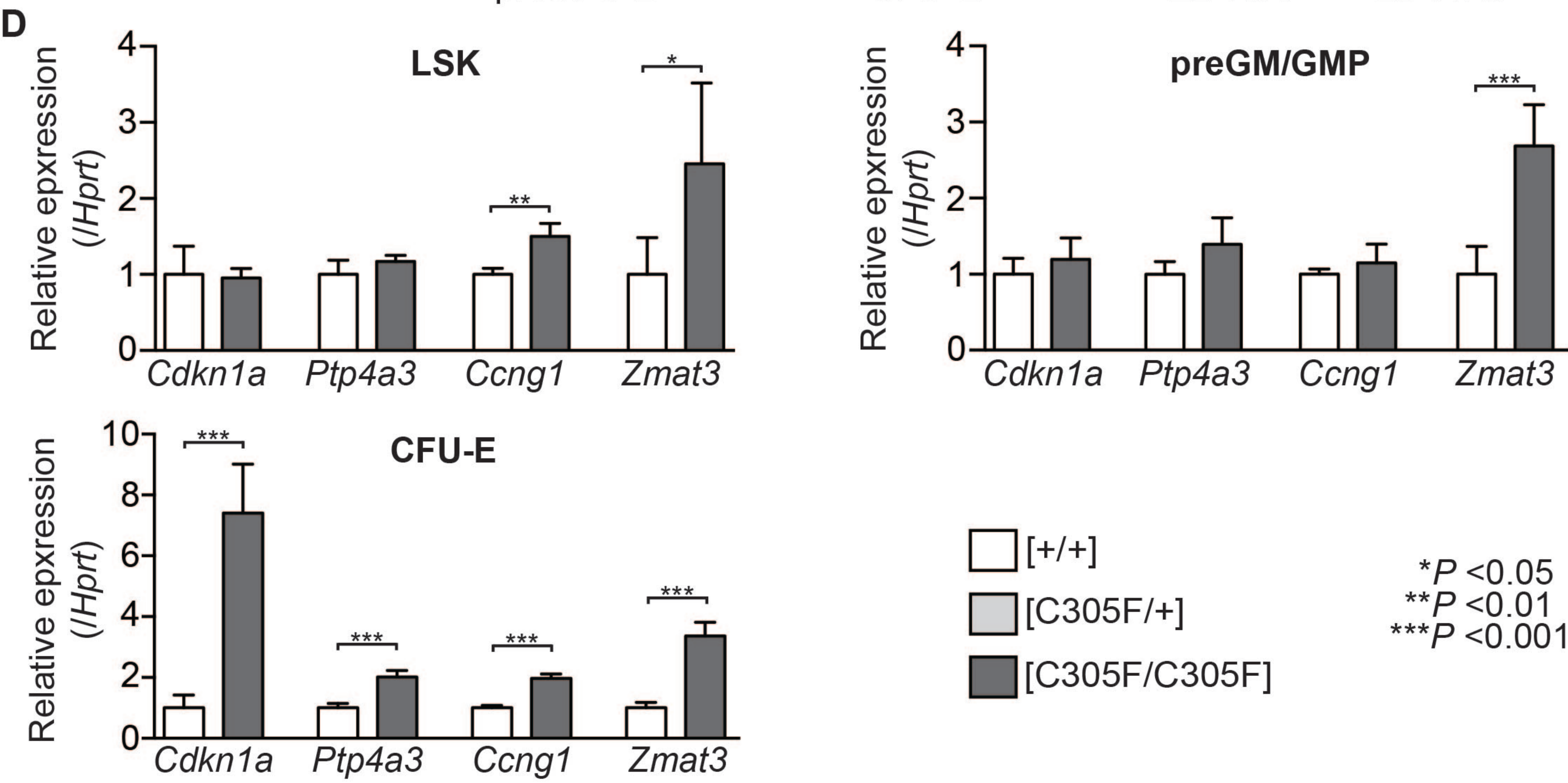
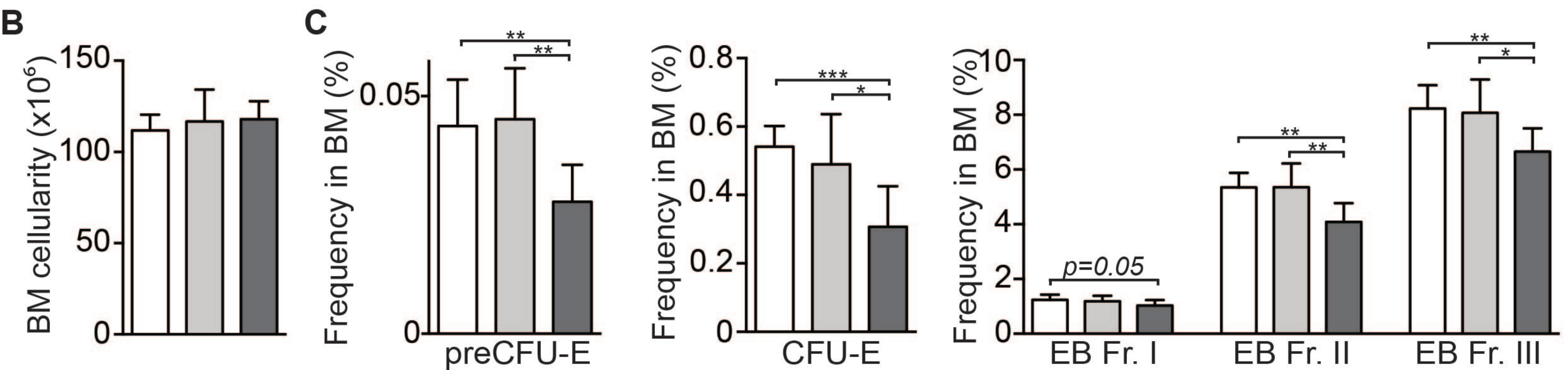
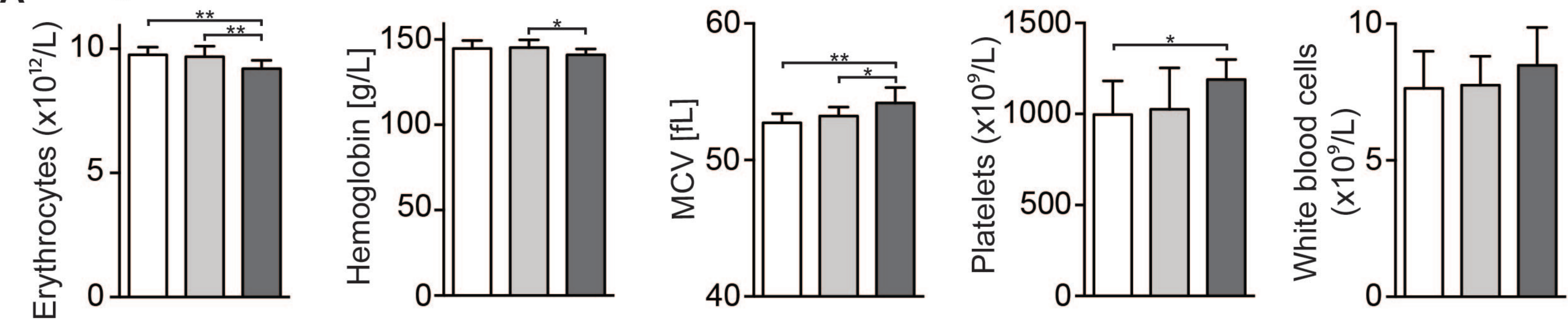
Figure 3

Figure 4

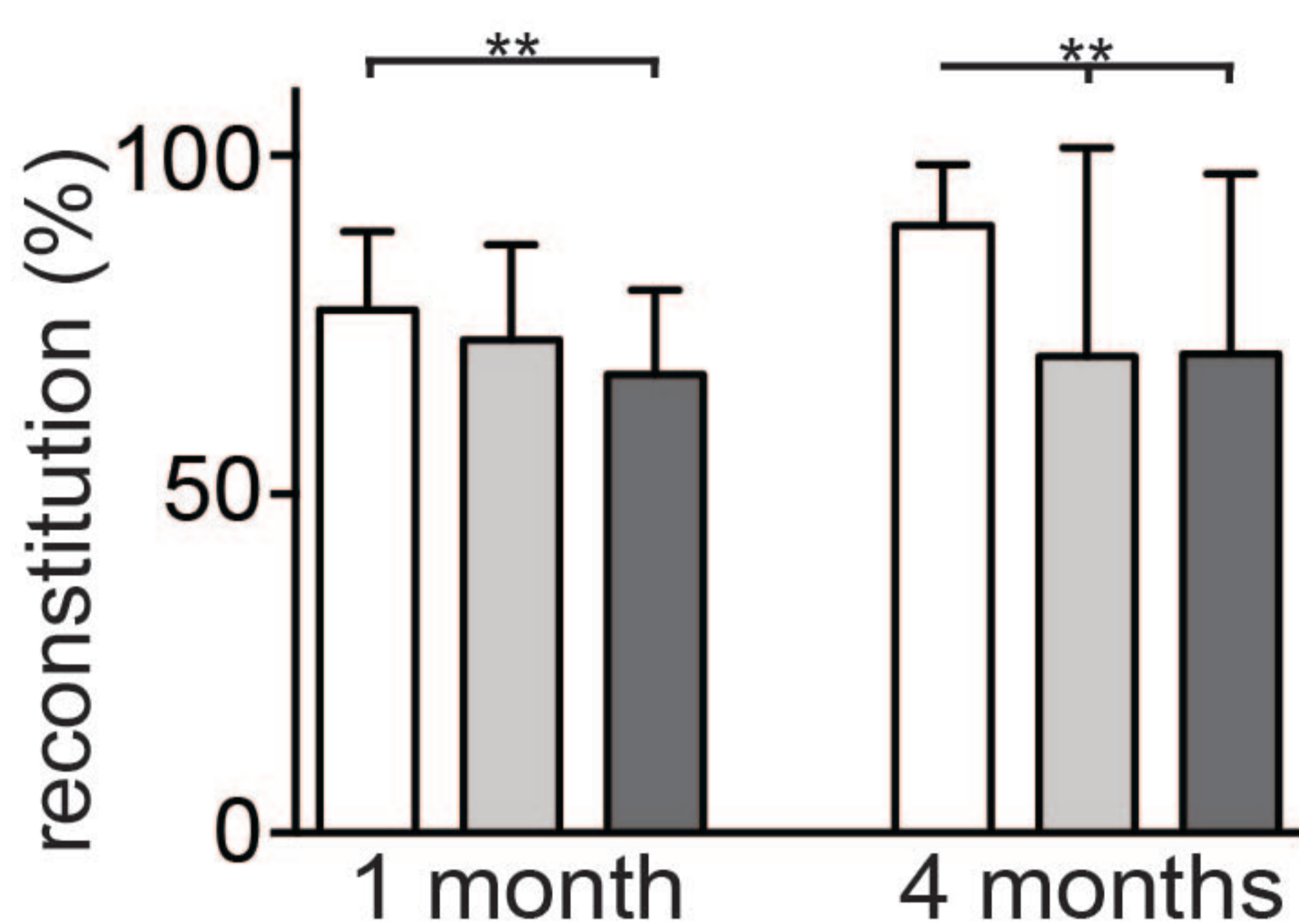
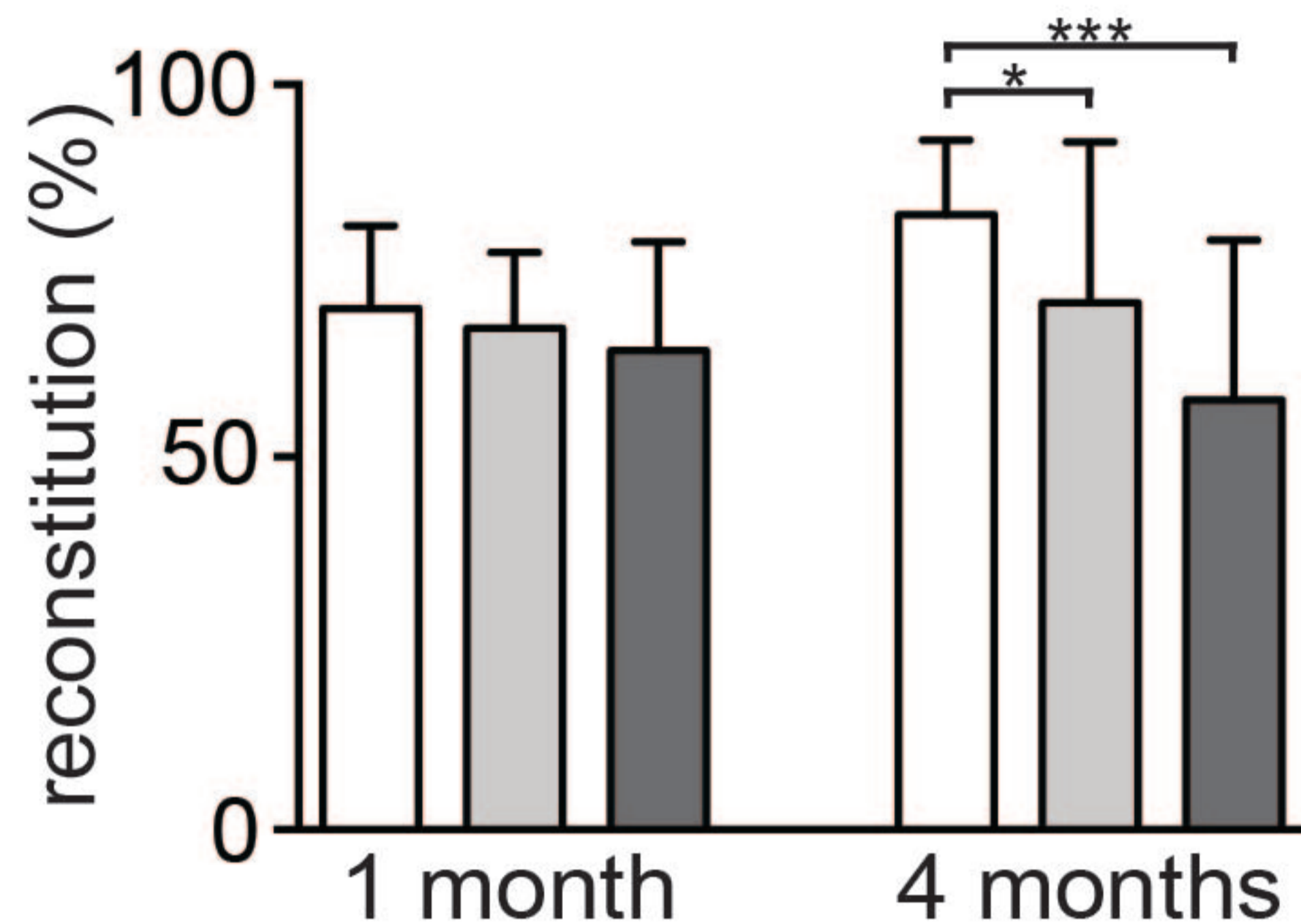
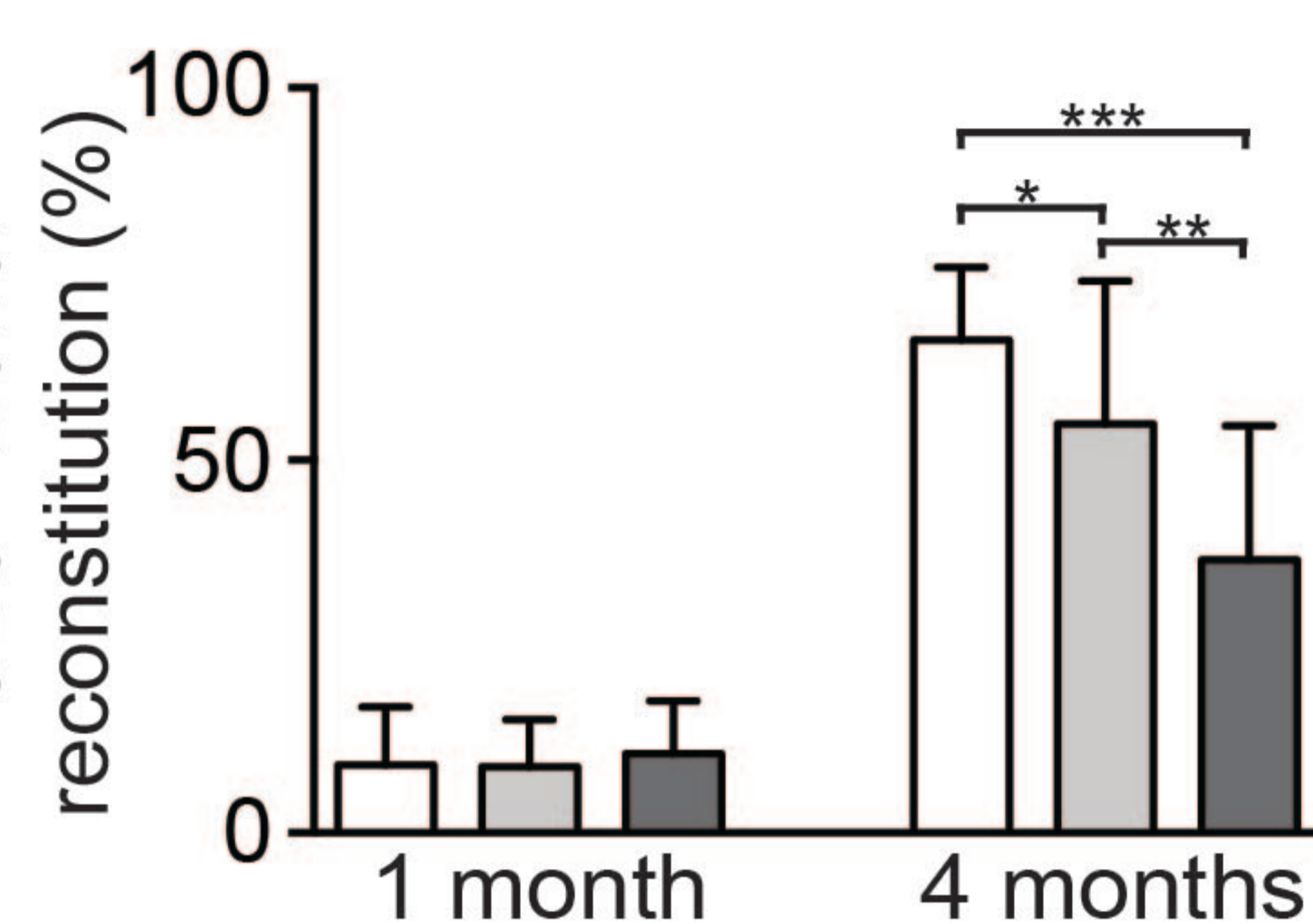
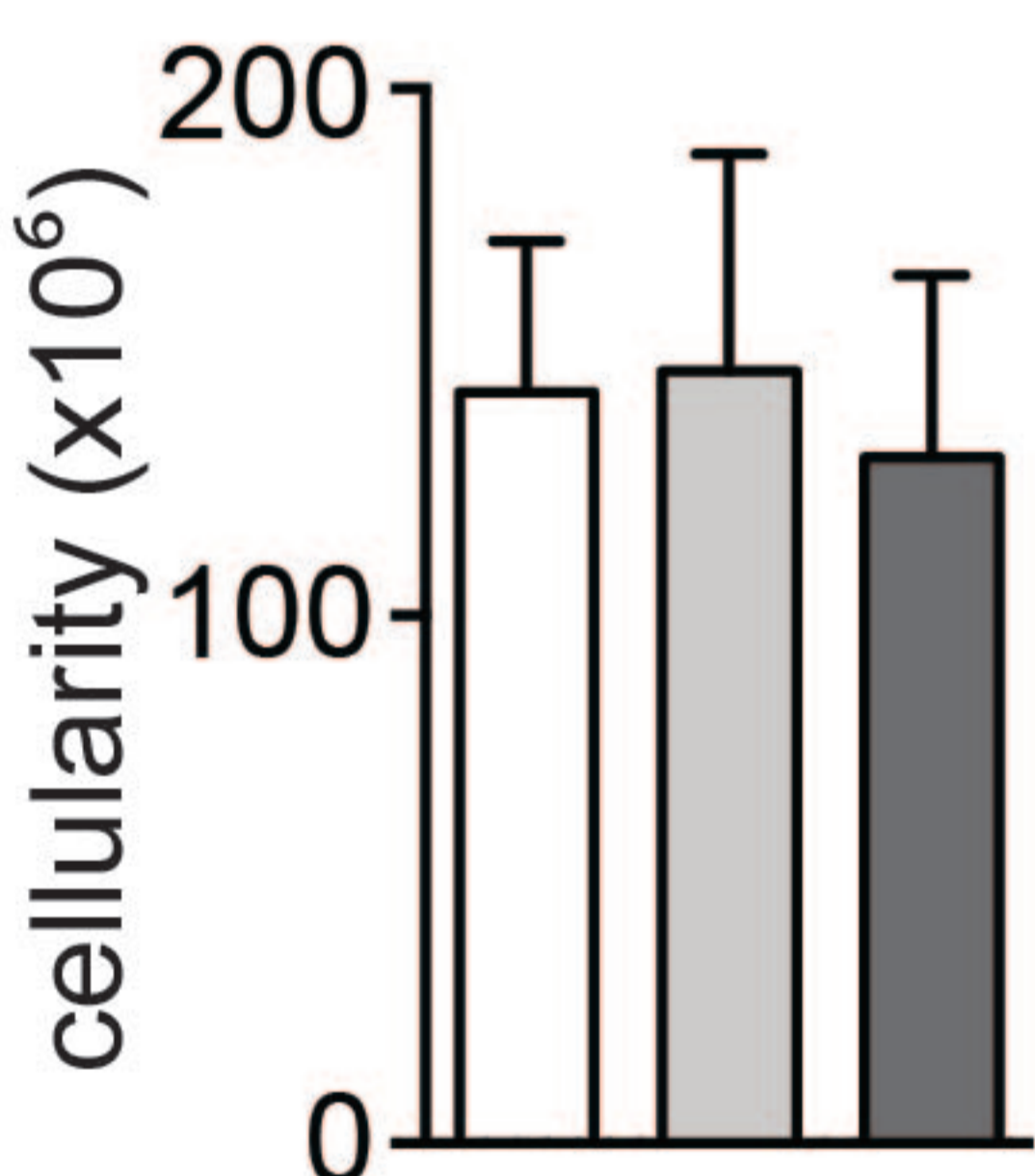
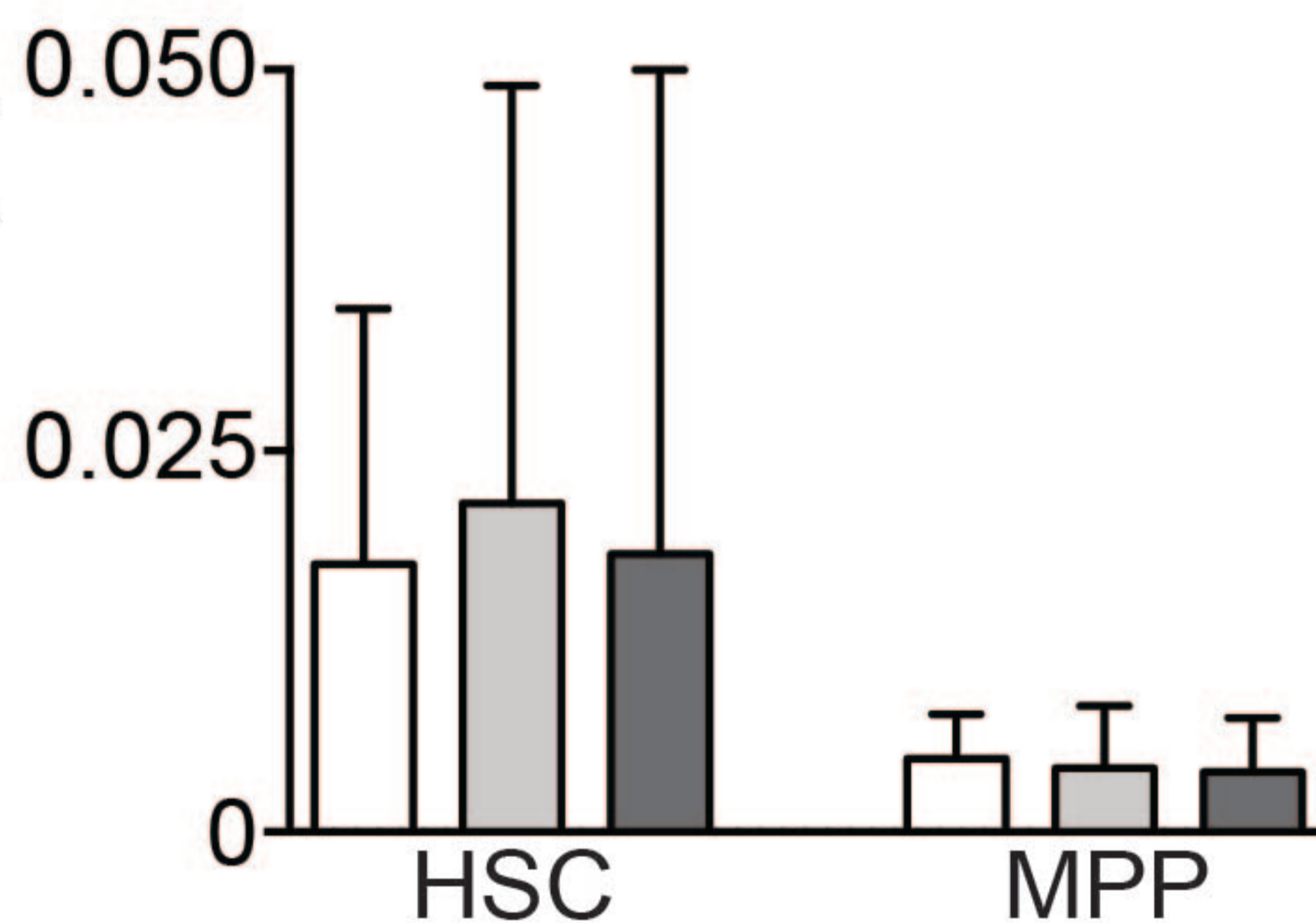
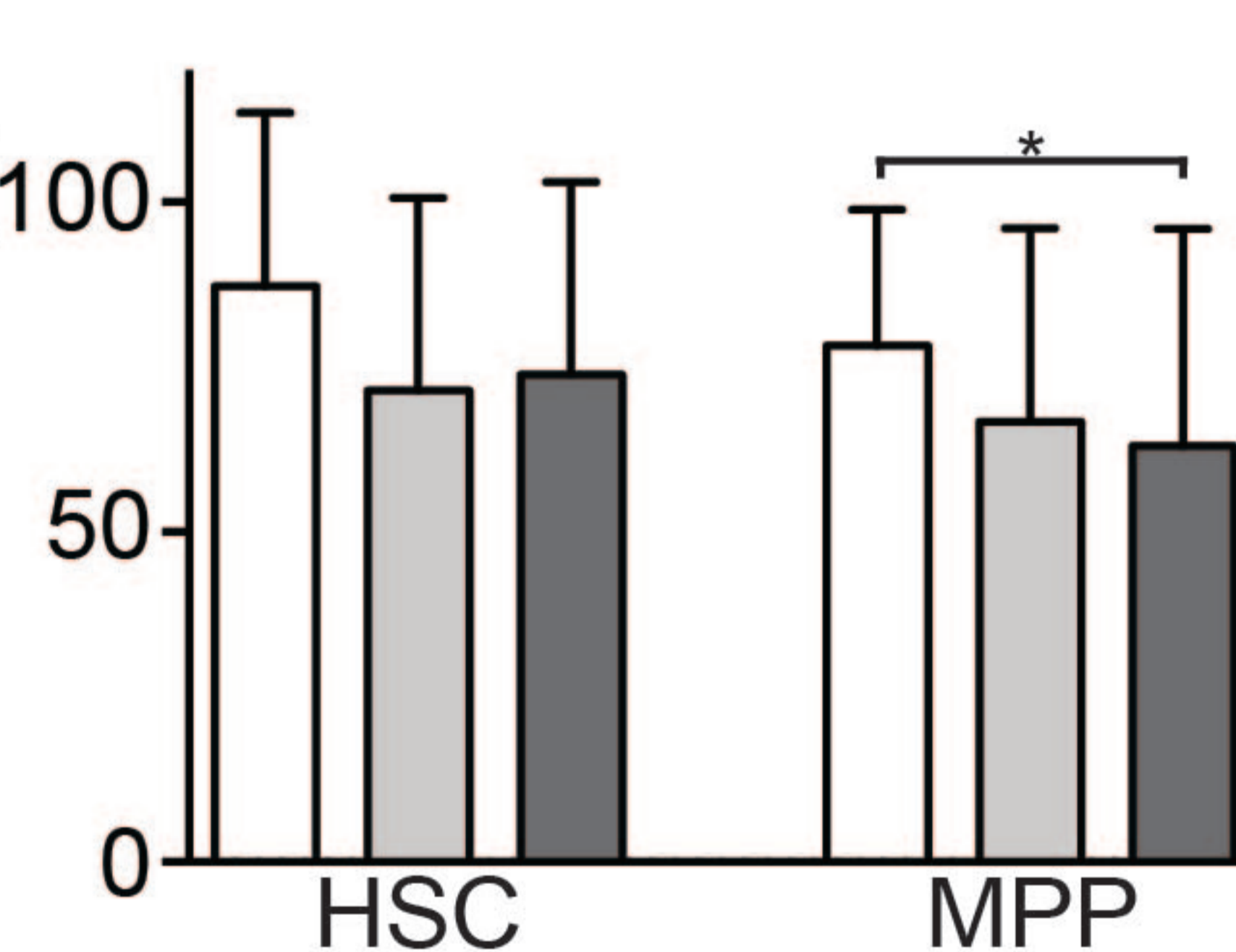
A Figure 5**Transplantation** 0.66×10^6 BM (donor; CD45.2) 0.33×10^6 BM (support; CD45.1)

[+/+; C305F/+; C305F/C305F]

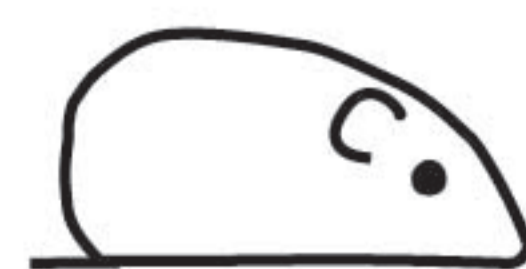
Lethally irradiated
wild-type recipients
(CD45.1; 900 cGy)

1 month

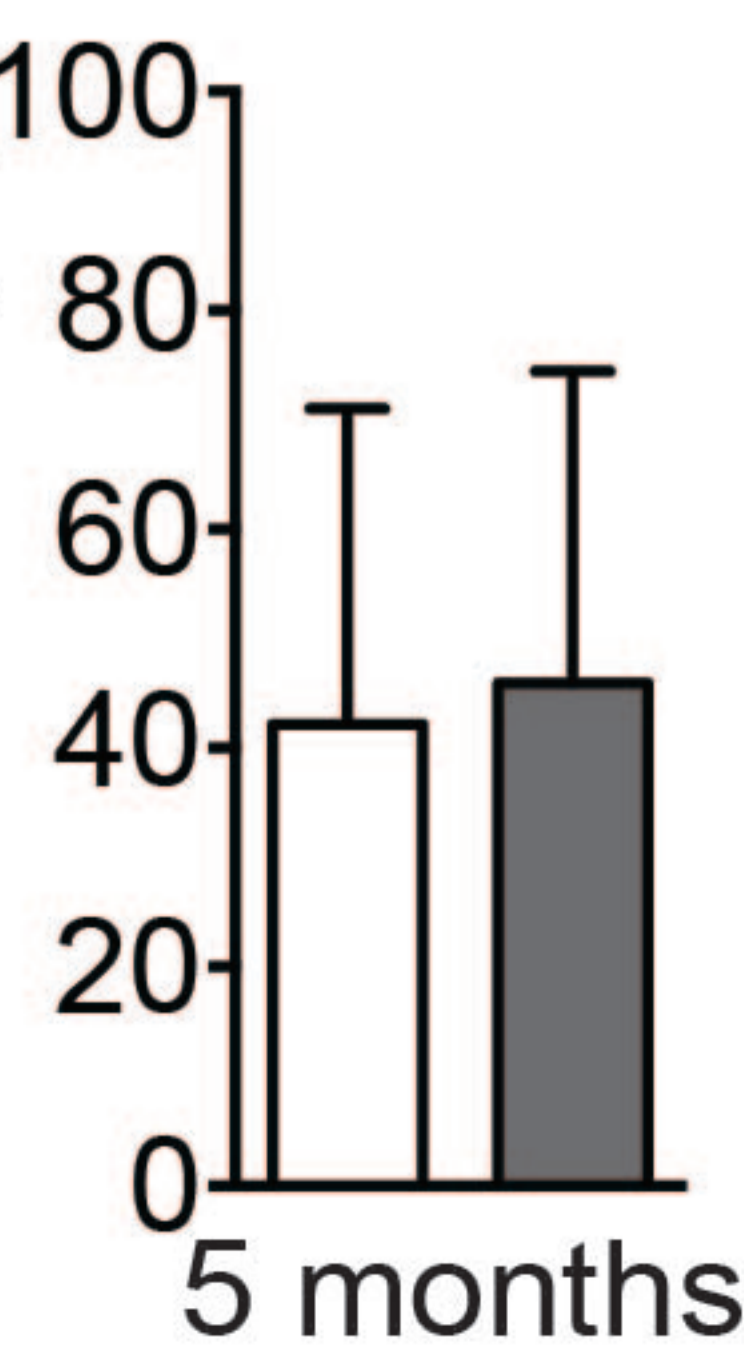
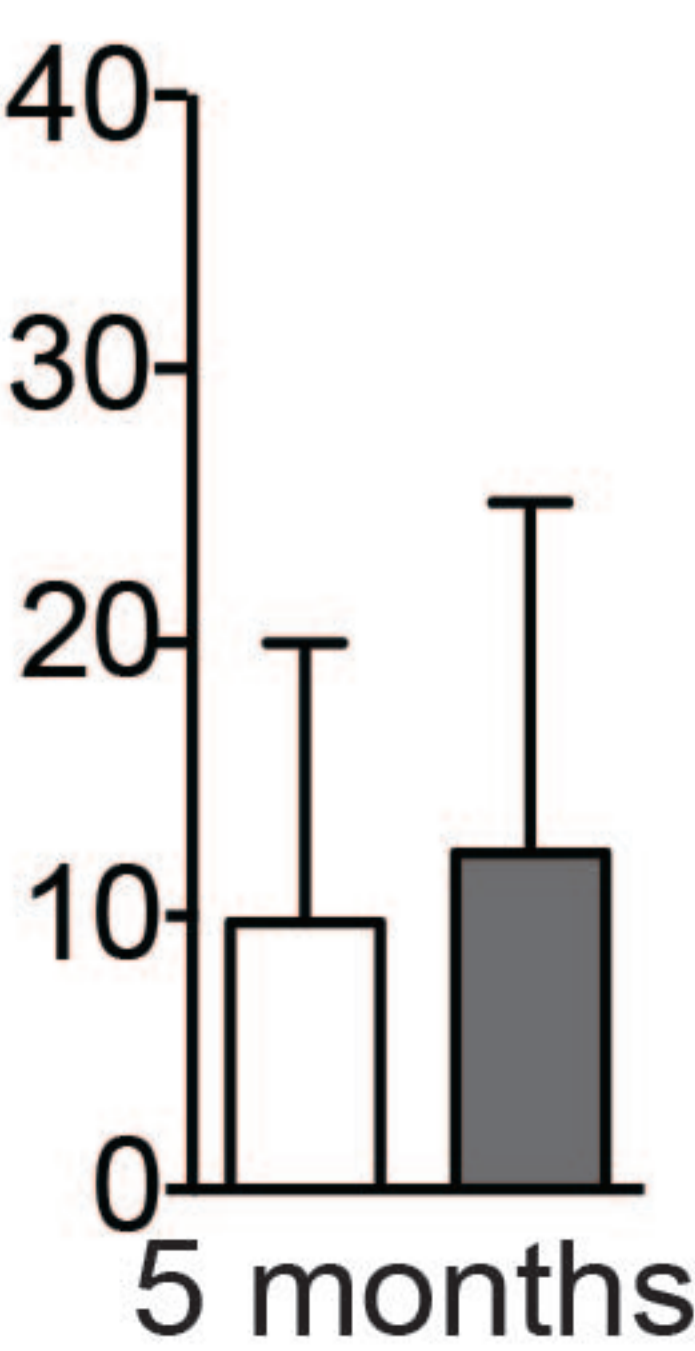
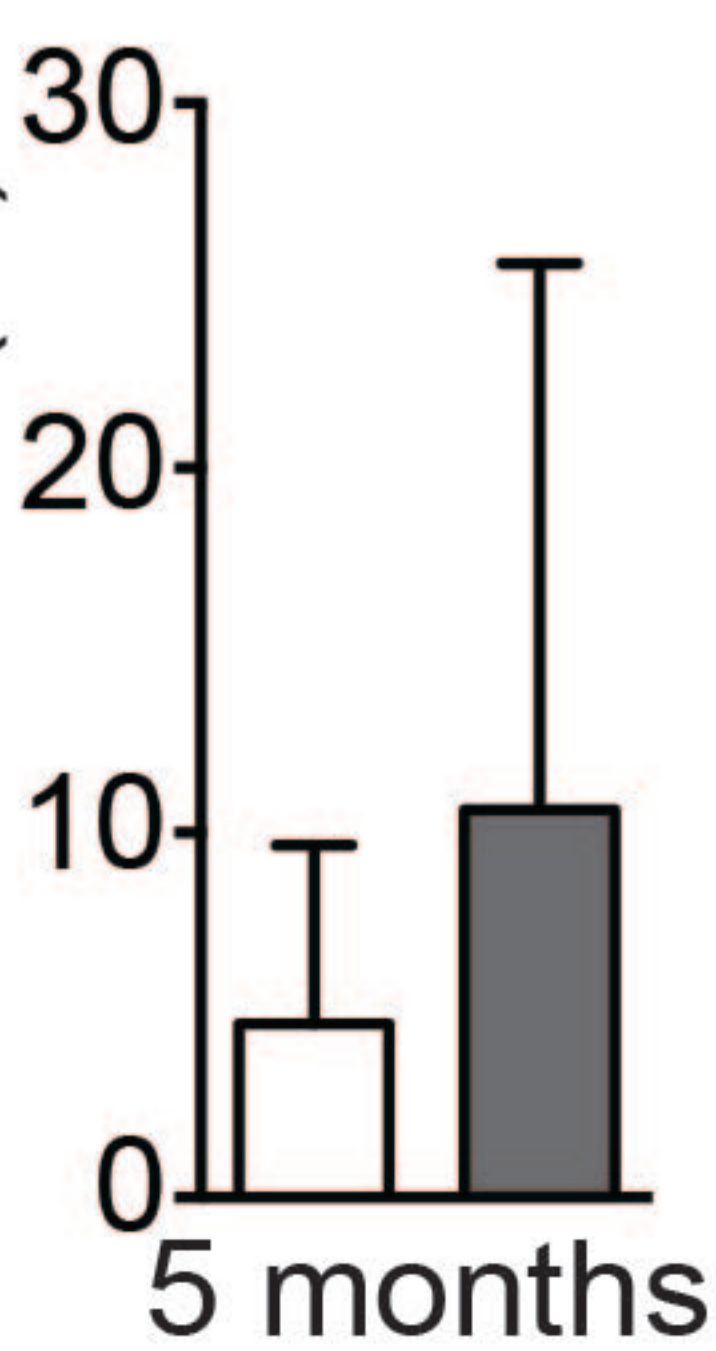
4 months

Blood analysis
(B)Blood analysis
(B)BM analysis
(C-D)**B**CD11b+ Donor
reconstitution (%)CD19+ Donor
reconstitution (%)CD3+ Donor
reconstitution (%)**C**Bone marrow
cellularity ($\times 10^6$)**D**Frequency in
bone marrow (%)Donor
reconstitution (%)* $P < 0.05$
** $P < 0.01$
*** $P < 0.001$ **E****2ary transplantation**

375 HSCs (donor; CD45.2)

 0.4×10^6 BM (support; CD45.1)

[+/+; C305F/C305F]

Lethally irradiated
wild-type recipients
(CD45.1; 900 cGy)**F**CD11b+ Donor
reconstitution (%)CD19+ Donor
reconstitution (%)CD3+ Donor
reconstitution (%)

Supplementary table 1. List of antibodies and reagents used for flow cytometry.

	Fluorochrome	Cat#	Manufacturer
BM analysis:			
CD45.2	FITC	109806	Biolegend
CD71	FITC	113806	Biolegend
CD41	PE	12-0411-83	eBioscience
CD45.1	PE	110708	Biolegend
GR1	PE-Cy5 (Lineage)	108410	Biolegend
CD11b	PE-Cy5 (Lineage)	101210	Biolegend
B220	PE-Cy5 (Lineage)	103210	Biolegend
CD3	PE-Cy5 (Lineage)	100310	Biolegend
Ter119	PE-Cy7	25-5921-82	eBioscience
CD150	APC	115910	Biolegend
c-Kit	APC-eFluor780	47-1171-82	eBioscience
Endoglin	Biotin	120404	Biolegend
Sca-1	Pacific blue	122520	Biolegend
Streptavidin	QD605	Q10101MP	Life Technologies
Peripheral blood analysis:			
CD45.2	FITC	109806	Biolegend
CD45.1	PE	110708	Biolegend
CD19	PE-Cy7	25-0193-82	eBioscience
CD11b	APC	101212	Biolegend
CD3	Alexa Fluor® 700	100216	Biolegend
Cell cycle analysis:			
c-Kit	APC	105812	Biolegend

Supplementary table 2. List of antibodies used for immunoblotting experiments.

	Clone/Cat#	Manufacturer
β -actin	AC-15	Sigma-Aldrich
p53	sc-6243	Santa Cruz
MDM2	SMP14	Sigma-Aldrich
RPS19	ab57643	Abcam
RPS6	#2217	Cell Signaling Technologies
p-RPS6	#2215	Cell Signaling Technologies

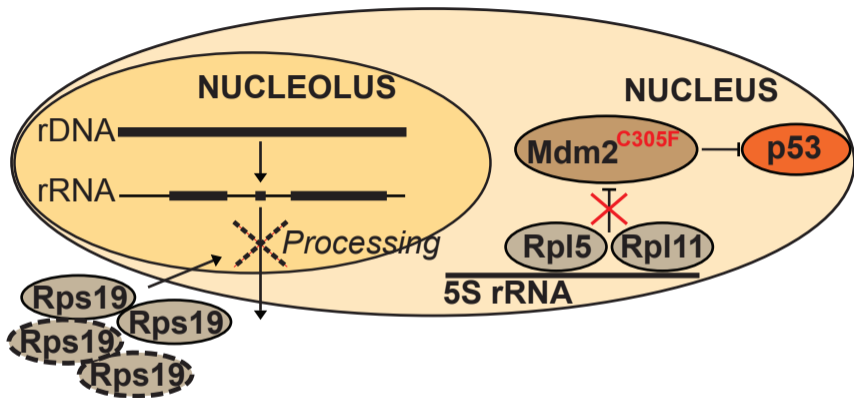
Supplementary table 3. Erythrocyte number, hemoglobin concentration, mean corpuscular volume (MCV), reticulocyte, platelet number and white blood cell number before the administration of doxycycline.

	[B/B][+/+] (n=25)	[B/B][C305F/+] (n=24)	[B/B][C305F/C305F] (n=33)
Erythrocytes (x10 ¹² /L)	9.23±0.44 (100 %)	9.06±0.39 (98 %)	8.56±0.67 (93 %)
Hemoglobin (g/L)	136±8 (100 %)	133±6 (98 %)	129±8 (95 %)
MCV (fL)	48.1±0.3	47.7±0.7	49.5±3.0
Reticulocytes (x10 ⁹ /L)	257±71 (100 %)	255±39 (99 %)	267±76 (104 %)
Platelets (x10 ⁹ /L)	499±202 (100 %)	512±179 (103 %)	482±187 (97 %)
White blood cells (x10 ⁹ /L)	6.55±1.48 (100 %)	5.87±0.84 (90 %)	4.87±1.53 (74 %)

Supplementary table 4. Erythrocyte number, hemoglobin concentration, mean corpuscular volume, reticulocyte number, platelet number and white blood cell number 2 weeks after doxycycline administration.

	[+/+][+/+] (n=28)	[B/B][+/+] (n=20)	[B/B][C305F/+] (n=19)	[B/B][C305F/C305F] (n=19)
Erythrocytes (x10 ¹² /L)	10.13±0.74 (100 %)	5.83±1.93 (58 %)	7.32±1.34 (72 %)	6.91±1.2 (68 %)
Hemoglobin (g/L)	145±11 (100 %)	85±29 (59 %)	106±21 (73 %)	105±19 (72 %)
MCV (fL)	47.8±1.3	48.3±1.9	48.2±2.1	51.2±1.1
Reticulocytes (x10 ⁹ /L)	202±59 (100 %)	135±127 (67 %)	203±124 (100 %)	231±88 (114 %)
Platelets (x10 ⁹ /L)	404±115 (100 %)	332±245 (82 %)	396±189 (98 %)	567±166 (140 %)
White blood cells (x10 ⁹ /L)	12±3 (100 %)	5.39±2.55 (45 %)	5.38±1.39 (45 %)	4.45±1.09 (37%)

Supplementary figure 1

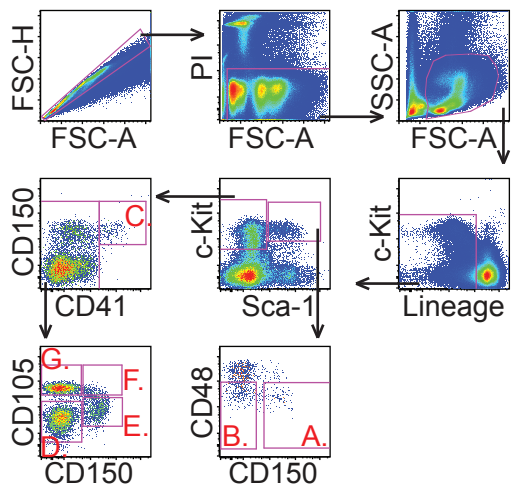


5S RNP =

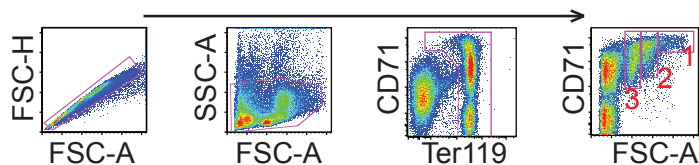


Supplementary figure 1. A working model of the effect of Mdm2^{C305F} on p53 activation upon Rps19 deficiency. Rps19 deficiency impairs the processing of rRNA (Jaako et al., Blood 2011), which leads to nuclear accumulation of the 5S ribonucleoprotein particle (5S RNP) that can bind to and inhibit Mdm2, resulting in the stabilization of p53. As Mdm2^{C305F} fails to bind the 5S RNP, it is expected to prevent the activation of p53 upon Rps19 deficiency.

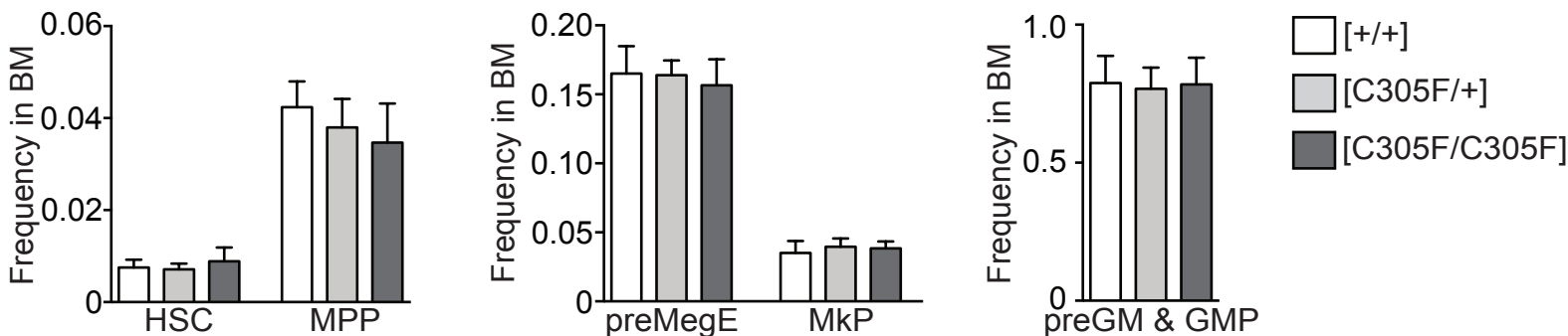
A Supplementary figure 2.



B

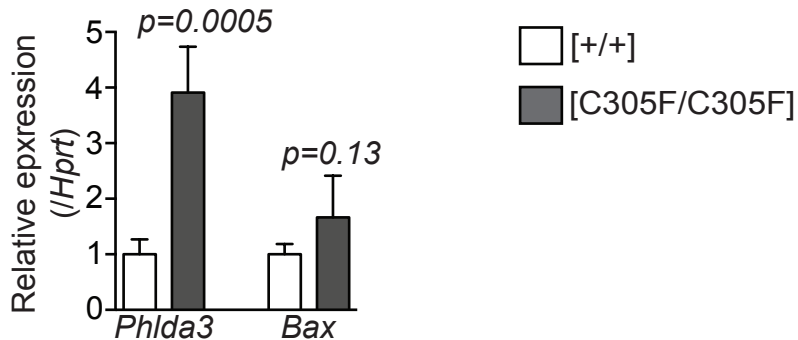


C



Supplementary figure 2. (A) FACS strategy allowing for the fractionation of HSCs and myeloerythroid progenitors. (B) FACS strategy allowing for the fractionation of erythroid precursors. (C) Frequency of HSCs, MPPs, preMegEs, MkPs and preGM/GMPs in the bone marrow ($n=8$ per genotype).

Supplementary figure 3



Supplementary figure 3. CFU-E erythroid progenitor cells from $Mdm2^{C305F}$ knock-in mice show elevated expression of p53 transcriptional target genes *Phlda3* and *Bax*. $n=4$ per genotype. Student's t test was used to determine statistical significance and two-tailed P -values are shown. Data are presented as mean \pm standard deviation.