



HAL
open science

Erythropoietin is a major regulator of thrombopoiesis in thrombopoietin-dependent and -independent contexts

Salima Hacein-Bey-Abina, Machadiya Estienne, Stephanie Bessoles, Hamid Echchakir, Magali Pederzoli-Ribeil, Andrada Chiron, Lydia Aldaz-Carroll, Valentin Leducq, Yanyan Zhang, Michele Souyri, et al.

► To cite this version:

Salima Hacein-Bey-Abina, Machadiya Estienne, Stephanie Bessoles, Hamid Echchakir, Magali Pederzoli-Ribeil, et al. Erythropoietin is a major regulator of thrombopoiesis in thrombopoietin-dependent and -independent contexts. *Experimental Hematology*, 2020, 88, pp.15-27. 10.1016/j.exphem.2020.07.006 . hal-03295372

HAL Id: hal-03295372

<https://cnrs.hal.science/hal-03295372>

Submitted on 22 Aug 2022

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.



Distributed under a Creative Commons Attribution - NonCommercial 4.0 International License

Erythropoietin is a major regulator of thrombopoiesis in thrombopoietin- dependent and – independent context

Salima Hacein-Bey-Abina^{1,2*}, Machadiya Estienne³, Stéphanie Bessoles¹, Hamid Echchakir³, Magali Pederzoli-Ribeil¹, Andrada Chiron^{1,2}, Lydia Aldaz-Carroll¹, Valentin Leducq³, Yanyan Zhang⁴, Michèle Souyri⁵, Fawzia Louache^{4,6}, Amine M. Abina^{1,3*}

¹UTCBS CNRS UMR 8258, INSERM U1267, Faculté de Pharmacie de Paris, Université de Paris, 4 av. de l'Observatoire, 75006 Paris, France

²Clinical Immunology Laboratory, Groupe Hospitalier Universitaire Paris-Sud, Hôpital Kremlin-Bicêtre, Assistance Publique-Hôpitaux de Paris, 78 r. du Général-Leclerc, 94270 Le-Kremlin-Bicêtre, France.

³NOKAD, 4 r. Pierre Fontaine, 91000, Evry, France

⁴INSERM U1170, CNRS GDR 3697 MicroNiT, Institut Gustave Roussy, 94805 Villejuif, France

⁵INSERM UMRS 1131, Institut Universitaire d'Hématologie, Université Paris Diderot, Hôpital Saint Louis, 1 av. Claude Vellefaux 75010, Paris France

⁶Université Paris-Sud, 91405 Orsay, France

***Corresponding authors:**

Salima Hacein-Bey-Abina, UTCBS CNRS UMR 8258, INSERM U1267, Faculté de Pharmacie de Paris, Université de Paris, 4 av. de l'Observatoire, 75006 Paris, France; Tel: 00 33 1 53 73 97 46; Fax: 00 33 1 70 64 94 52 ; e-mail : salima.hacein-bey@aphp.fr

Amine.M Abina, UTCBS CNRS UMR 8258, INSERM U1267, Faculté de Pharmacie de Paris, Université de Paris, 4 av. de l'Observatoire, 75006 Paris, France; Tel: 00 33 1 53 73 97 46; Fax: 00 33 1 70 64 94 52 ; e-mail : a.abina@wanadoo.fr

Non-corresponding authors' e-mail addresses:

macha.muslmani@gmail.com (ME); stephanie.bessoles@parisdescartes.fr (SB); hamid.echchakir@inserm.fr (HE); magali.pederzoli-ribeil@parisdescartes.fr (MPR); andrada.chiron@aphp.fr (AC); lydia.aldaz-carroll@parisdescartes.fr (LAC) ; valentin.leducq@upmc.fr (VL); yanyan_gh@yahoo.com (YZ); michele.souyri@inserm.fr (MS); Fawzia.LOUACHE@gustaveroussy.fr (FL).

Category for the Table of Contents: Normal Hematopoiesis (myelopoiesis, erythropoiesis, lymphopoiesis, megakaryocytopoiesis)

Key words: thrombopoiesis, large-platelets, EPO, Mpl, TPO

Word count: 3695 words

1 **Abstract**

2 Thrombopoietin (TPO), through activation of its cognate receptor Mpl, is the major regulator of
3 platelet production. However, residual platelets observed in TPO- and Mpl-loss-of-function (LOF)
4 mice suggest the existence of an additional factor to TPO in platelet production. Since
5 erythropoietin (EPO) showed both *in vitro* megakaryocytic potential, in association with other
6 early acting cytokines, and *in vivo* platelet activation activity, we sought to investigate its role in
7 this setting. Here, we used multiple LOF models to decipher the reciprocal role of EPO and TPO
8 in the regulation of platelet production in TPO- and Mpl-LOF mice and of platelet size
9 heterogeneity in wild type mice. We first identified EPO as the major thrombopoietic factor in the
10 absence of TPO-Mpl pathway. Based on the study of several mouse models we found that the
11 EPO-EPO receptor pathway acts on late-stage megakaryopoiesis and is responsible of large-size
12 platelet production, while the TPO-Mpl pathway promotes small-size platelet production. ~~Our data~~
13 ~~support the use of EPO for thrombocytopenia supportive therapy in congenital~~
14 ~~amegakaryocytopoiesis.~~ **Based on our data, EPO might be used for thrombocytopenia supportive**
15 **therapy in congenital amegakaryocytopoiesis.** Furthermore, since a distribution skewed towards
16 large platelets is an independent risk factor and a poor prognosis indicator in atherothrombosis, the
17 characterization of EPO's role in the production of large-size platelets, **if confirmed in human,**
18 may open new perspectives in the understanding of the role of EPO-induced platelets in
19 atherothrombosis.

20

21

22

23

24

25

26 **Introduction**

27 The process of differentiation of hematopoietic stem cells into functional blood cells involves a
28 continuum of organized, stable and hierarchical intermediate stages ranging from multipotent to
29 unipotent progenitor cells. In the canonical hematopoiesis, megakaryocyte and erythroid lineages
30 are closely related and share a common bipotent megakaryocytic and erythroid progenitor (MEP)
31 (1, 2). Recently, several studies have demonstrated the existence of hematopoietic stem cells
32 (HSC) with megakaryocyte-biased differentiation potential at the apex of the hematopoietic
33 hierarchy in normal and stress hematopoiesis suggesting that commitment to the megakaryocyte
34 lineage could also occur at an earlier stage, bypassing erythroid versus megakaryocyte lineage
35 bifurcation (3-8). In addition, a unipotent megakaryocyte progenitor population (MegP) upstream
36 of the MEP, which resides in the common myeloid progenitor (CMP) population, was identified
37 recently (9).

38 ~~Although~~—The megakaryocytic and erythroid lineages have distinct physiologic roles and
39 regulatory mechanisms **but share many cytokine receptors** (10-14). After activation by their
40 specific ligands (thrombopoietin- TPO and erythropoietin- EPO), Mpl and EPO receptor (EpoR)
41 promote the differentiation, survival and proliferation of the megakaryocytic and erythroid
42 lineages, respectively. In addition to a major role on their respective erythroid and megakaryocytic
43 target cells, EPO and TPO have the ability to cross-stimulate the other lineage. TPO is able to
44 restore erythroid differentiation blockade of EpoR deficient erythroid progenitors (15) and
45 increases erythroid recovery in myelo-suppressed mice (16, 17). Conversely, EPO was already
46 identified as a potent megakaryocyte (MK) differentiation factor *in vitro* (18-23). Strikingly, EpoR
47 is expressed in up to 59% of total MKs (24) and EPO administration activates platelets (25-30),
48 while it induces only modest platelet production (27, 29-35).

49 Moreover, recombinant human EPO (rHuEPO) administration is associated with increased

50 atherothrombosis and venous thromboembolism in cancer (36, 37) and chronic kidney disease
51 patients (38, 39). However, EPO's specific physiologic role in *in vivo* basal platelet production
52 and function has not been established yet.

53 Marked size heterogeneity is a unique feature of platelets. Platelet size curve follows a log
54 Gaussian distribution, rather than the Gaussian distribution observed for mammalian cells (40, 41).
55 Relative to small platelets, large platelets show greater metabolic activity and have a higher
56 activation potential (41, 42). The mean platelet volume (MPV) (the platelet index most frequently
57 used in clinic), is a marker of activity and an independent cardiovascular risk factor in prospective
58 studies of patients or healthy subjects (43-47). Yet, despite extensive investigations, the regulation
59 of platelet size heterogeneity is elusive (48-51).

60 Here we identified that EPO is the long-sought-after residual thrombopoietic factor in *Mpl*^{-/-} and
61 *TPO*^{-/-} mice. We also reveal a dual and sequential regulation of platelet production with opposing
62 impact of EPO and TPO on platelet size heterogeneity in wild type mice. Our study links EPO,
63 whose production is inversely correlated with oxygen availability (52), to the physiologic
64 regulation of large platelets.

65 **Materials and Methods**

66 **Mice**

67 Wild-type female C57BL/6J01aHsd (WT), used at 8-12 weeks-old, were purchased from Harlan
68 Envigo (Horst, The Netherlands). Male and female *Mpl*^{-/-} and *TPO*^{-/-} mice (53, 54), kindly
69 provided by F.J. de Sauvage (Roche Genentech, CA, USA), were used at 8-12 weeks-old for
70 experiments

71 Anti-EPO polyclonal antibodies used for the generation of functional knock-out (KO) of EPO
72 (EPO-fKO) mice were produced by subcutaneous vaccination of 8-12 weeks female Sprague Dawley
73 rats (Harlan Envigo) with recombinant human Adenovirus-5 vectors encoding human EPO (Ad.huEPO).

74 Rat anti-human EPO polyclonal antibodies were validated by their ability to induce profound anemia
75 both in rat and in mice. Polyclonal non-relevant antibodies, used as negative controls, were produced by
76 injecting rats with an adenovirus vector devoid of any recombinant sequence (Ad.null). Each control
77 was also validated for absence of anemia in mice injected with 100 μ l of control serum 5 times per week
78 during 2 weeks.

79 Functional-KO (fKO) of EPO (EPO-fKO) mice were generated by intraperitoneal injections of 100 μ l of
80 rat serum containing anti-EPO polyclonal antibodies (poAb), 5 times per week during 2 weeks. Control
81 mice were injected with non-relevant poAb.

82 TPO-fKO mice were generated by vaccination with an adenovirus encoding heterologous human
83 thrombopoietin as previously described (55). All adenovirus vectors were produced by Atlantic Gene
84 Therapies (Nantes, France).

85 For stimulation studies, mice were subcutaneously injected with 20 U of recombinant
86 erythropoietin (rHuEPO) (Neorecormon®, Roche) diluted in PBS, 3 times per week during 2-3
87 weeks. Control mice were injected with PBS.

88 **Blood counts**

89 Mice were bled retro-orbitally and blood samples collected on EDTA-containing tubes were
90 analyzed within 30 min on a MS9-5 blood cell analyzer (Melet-Schloesing, Osny, France).

91 **Flow cytometry**

92 WT or *TPO*^{-/-} mice were injected with anti-EPO poAbs or non-relevant poAb. Mice were
93 sacrificed when reaching a hematocrit between 30-15% (EPO-fKO) in peripheral blood counts for
94 BM or spleen cells collection. Cells prepared in 10% FCS (fetal calf serum) DMEM, were stained
95 first with a biotin mouse lineage panel (#559971, Becton Dickinson Biosciences,
96 Le-Pont-De-Claix, France) and visualized with streptavidin-pacific orange (#S32365, Thermo
97 Fischer Scientific, Rockford, IL) and then with fluorochrome-conjugated antibodies against CD41

98 (#11-0411, eBioscience/Thermo Fischer Scientific), c-Kit (#135136, BioLegend, Uithoorn,
99 Netherlands), Sca-1 (#108114, BioLegend), and CD150 (#115910, BioLegend) before data
100 acquisition. ~~For MKP characterization CD41⁺ gates included both high and low expressing cells,~~
101 ~~while high and low CD41⁺ expressing cells were gated separately for cell size and granularity~~
102 ~~determination.~~ MKPs were characterized as Lin⁻ cKit⁺ Sca-1⁻ CD150⁺ CD41⁺. CD41⁺ gates
103 included both high and low expressing cells, while high and low CD41⁺ expressing cells were
104 gated separately for cell size and granularity determination.

105 Megakaryocyte ploidization studies were performed on BM and spleen cell suspensions prepared
106 in PBS with 0.5% BSA (bovine serum albumin). Cells ($1-5 \times 10^6$) were incubated for 30 min on
107 ice after addition of 1.25 μg of anti-CD41 antibody (#11-0411, eBioscience/Thermo Fischer
108 Scientific) and washed with PBS-citrate-BSA buffer. The pellet was then suspended in 200 μL
109 PBS, and 4 mL of 70% cold ethanol in PBS was added. After 1 hour incubation at 4°C, the
110 suspension was centrifuged and suspended in a PBS solution containing 50 $\mu\text{g}/\text{mL}$ propidium
111 iodide and 100 $\mu\text{g}/\text{mL}$ RNase A. After 30 minutes at 37°C, cells were analyzed by flow cytometry.
112 All acquisitions were performed using either a FC-500, FACS-CantoII or FACSCalibur cell
113 analyzers (Becton Dickinson Biosciences). Flow cytometry data analyzes were evaluated using
114 FlowJo version 10.0.8 software (Tree Star, Ashland, OR, USA).

115 **Quantitative real-time PCR**

116 Whole blood, pooled from 10-20 mice, was collected in citrated tubes then centrifuged at 185 g
117 for 30 min at room temperature. The upper plasma supernatant, Platelet-Rich-Plasma (PRP), was
118 collected and then centrifuged at 2000 g for 15 min at room temperature. The platelet-containing
119 pellet was immediately dissolved in 1ml TRIzol reagent (#15596018, Thermo Fischer Scientific,
120 Massachusetts, USA), maintained at room temperature for 3 min, then stored at -80 °C. The
121 remaining blood obtained after PRP collection was used to purify the leukocyte fraction using
122 Histopaque-1077 (#10771, Sigma-Aldrich) gradient density. The collected leukocytes were

123 immediately dissolved in 1ml TRIzol reagent and stored at -80°C.

124 Total RNA was obtained and then reverse-transcribed into cDNA using a High Capacity RNA to
125 cDNA Kit (#4387406, Thermo Fischer Scientific, Massachusetts, USA) according to manufacturer
126 instructions. SYBR Green I (#04887352001, Roche, Meylan, France) was used to quantify the
127 PCR-amplification products. All primers were purchased from Eurofins-MWG/Operon
128 (Ebersberg, Germany) (Table 1). The mRNA expression levels of the target genes were
129 normalized to a selected endogenous reference gene, depending on the cell population, according
130 to $\Delta\Delta$ -CT method. The reference genes were selected based on their stability in the studied
131 population. β -2-microglobulin (B2M) was used for mouse BM cells, while PPIA was selected for
132 mice platelets and human primary MK cells.

133 RNAs from each human primary MK ploidization stage were kindly provided by Hana Raslova
134 (INSERM U1170, Villejuif, France) and were previously described (56). Briefly, cytopheresis
135 samples from healthy individuals, obtained with their informed consent, were used to purify
136 CD34+ cells. Cells were then cultured with 10 ng/mL human TPO (Kirin, Tokyo, Japan). After 8
137 days of culture, cells were recovered and incubated for 30 min at 4°C with FITC-conjugated
138 anti-CD41a monoclonal antibody (mAb) (eBioscience/Thermo Fisher). After washing with
139 PBS-EDTA, cells were incubated with 0.01 mM Hoechst 33342 (Sigma-Aldrich) at 37 °C for 2 h.
140 2N and 4N or 8N and 16N cell fractions were then purified by flow cytometry.

141 **Table 1.:**

Primer for qPCR	SOURCE	Sequence
mB2M Fw	Eurofins-MWG/Operon	GACTGATACATACGCCTGCAGAGTT
mB2M Rv	Eurofins-MWG/Operon	TCACATGTCTCGATCCCAGTAGA
mEpoRFw	Eurofins-MWG/Operon	CCCAAGTTTGAGAGCAAAGC
mEpoRRv	Eurofins-MWG/Operon	TGCAGGCTACATGACTTTTCG
mPPIAFw	Eurofins-MWG/Operon	TGGAGAGAAAGGATTTGGCTA
mPPIARv	Eurofins-MWG/Operon	AAAACCTGGGAACCGTTTGTG
mGYPAFw	Eurofins-MWG/Operon	CTGTGGTGGCTTCAACTGTA
mGYPARv	Eurofins-MWG/Operon	CGATAATCCCTGCCATCACG
mGPIIbFw	Eurofins-MWG/Operon	GCAGGTCAGGAGCAAGAACA
mGPIIbRv	Eurofins-MWG/Operon	TCACACTCCACCACCGTACA
hB2M Fw	Eurofins-MWG/Operon	CTCACGTCATCCAGCAGAGA
hB2M Rv	Eurofins-MWG/Operon	CGGCAGGCATACTCATCTTT
hEpoRFw	Eurofins-MWG/Operon	GACTTGGTGTGTTTCTGGGAG
hEpoRRv	Eurofins-MWG/Operon	GGCGTCTAGGAGCACTACTTC
hMplFw	Eurofins-MWG/Operon	TCCTGCACACTACAGGAGACTGA
hMplRv	Eurofins-MWG/Operon	TCCAATTCTTCACAGGTATCTGAGACT
hPPIAFw	Eurofins-MWG/Operon	GTCAACCCACCGTGTCTT
hPPIARv	Eurofins-MWG/Operon	CTGCTGTCTTTGGGACCTTGT

142

143 **Table 1. List of the primer pairs used in this study.** Abbreviations: Fw=forward, h=human,

144 m=murine, Rv=reverse

145 **Statistical analysis**

146 Data were analyzed using the adapted test for each experiment and are indicated in the figure

147 legends. Statistical analyses were performed using Prism software (GraphPad 7.0, La Jolla, CA).

148 For all tests: *= $P < 0.05$; **= $P < 0.01$; ***= $P < 0.001$; ****= $P < 0.0001$; ns=not significant.149 **Study approval**

150 All animal experiments were conducted in compliance with European guidelines (European

151 Directive 2010/63/EU) and were approved by the ethical and scientific board of the CERFE

152 animal facility (Evry, France) under the reference 2011-41.

153 **Results**

154 **EPO is the major regulator of platelet production in *TPO*^{-/-} and *Mpl*^{-/-} mice**

155 Despite the fact that TPO is the major regulator for platelet production, *TPO*^{-/-} and *Mpl*^{-/-} mice, still
156 result in platelet production which may reach up to 20% of normal levels (53, 54). The search for
157 the factors responsible for such residual thrombopoiesis using double KO-mutants involving *Mpl*
158 and other putative genes with MK stimulation potential, including IL-3, IL-6, IL-11, LIF, G-CSF,
159 IL-5, GM-CSF, and SCF, was unsuccessful so far (57-62). We selected EPO as a candidate
160 because *in vitro* data show that it influences megakaryopoiesis both on WT and *Mpl*^{-/-} mice (18-23,
161 63), although the prior action of other early acting megakaryocytic growth factors such as IL-3,
162 IL-6, IL-11, TPO and SCF is required (23, 64-66). In addition, previous reports showed that
163 rHuEPO treatment increases platelet activation both in patients and healthy individuals (25-30,
164 67).

165 Since both *EPO*^{-/-} and *EpoR*^{-/-} mice die during embryonic development (68), we designed a
166 functional knock-out (fKO) strategy in which an antibody neutralizes EPO's activity *in vivo* (55,
167 69). The erythroid cell count starts to fall one week after anti-EPO injection, and the hematocrit
168 drops down to around 10% at three weeks (Figure 1A). The EPO-fKO phenotype was confirmed
169 by the marked reduction in mRNA expression of two specific markers of the erythroid lineage
170 (glycophorin A -GYPA and *EpoR*) (Figure 1B) as early as 1 week following anti-EPO injection.
171 We then evaluated the effects of EPO-fKO on the residual platelets produced by *TPO*^{-/-} and *Mpl*^{-/-}
172 mice.

173
174 Notably, EPO-fKO induced a marked reduction in the platelet count of EPO-fKO-*TPO*^{-/-} and
175 EPO-fKO-*Mpl*^{-/-} mice (Figure 2A-B). In parallel to decreased platelet counts, EPO-fKO resulted in
176 a significant decrease in the numbers of CD41+CD150+ megakaryocytic progenitor (MKP) cells

177 in the BM and spleen suggesting a direct effect of EPO on megakaryopoiesis (Figure 2C-F).
178 To confirm the EPO's thrombopoietic role, we administered rHuEPO to *TPO*^{-/-} and *Mpl*^{-/-} mice.
179 We found that rHuEPO injection increased platelet counts in *Mpl*^{-/-} and *TPO*^{-/-} mice up to the
180 normal values observed in WT mice (Figure 2G-H) and confirmed the full thrombopoietic potency
181 of EPO.

182

183 **Platelets characteristics in *TPO*^{-/-} and *Mpl*^{-/-} mice**

184 Initial studies in the *TPO*^{-/-} and *Mpl*^{-/-} mice showed that despite profound thrombocytopenia
185 (Figure 3A), these mice have no spontaneous bleeding and display normal platelet function (70).
186 We sought to evaluate platelet characteristics in these models. We found that both models had
187 very high MPV and P-LCR (Platelet-large cell ratio) values compared to WT mice (Figure 3B-C).
188 This result suggests that EPO contributes to the platelet mass that maintains normal hemostasis in
189 the absence of the TPO/Mpl pathway.

190

191 **MK ploidy in *TPO*^{-/-} and *Mpl*^{-/-} mice**

192 Platelets are derived from megakaryocytes during a maturation process that involves ploidy and
193 cell size increase, cytoplasm fragmentation and, ultimately, platelet shedding (71, 72).
194 Interestingly, *in vitro*, EPO was able to stimulate megakaryocyte production from bone marrow
195 harvested from *Mpl*^{-/-} mice (63).

196 We thus elected to study MK characteristics in *TPO*^{-/-} and *Mpl*^{-/-} mice. Notably, we found a higher
197 proportion of polyploid MKs in *TPO*^{-/-} and *Mpl*^{-/-} mice than in WT mice (Figure 4A). We also
198 analyzed the size through the use of the forward size scatter (FSC) and density or granularity using
199 the side scatter parameter (SSC) of both CD41+highCD150+ and CD41+lowCD150+ MKP cells
200 of *TPO*^{-/-} mice compared to WT mice. The results of these experiments confirmed a higher
201 ploidy of MKP cells in *TPO*^{-/-} than in WT mice (Figure 4B). These observations suggest that
202 ~~EPO regulates positively the MK ploidy~~ **EPO might be associated with MK ploidy but**

203 further *in vivo* studies are needed to definitively confirm the direct role of EPO as a physiologic
204 regulator of late MK stages.

205

206 **EPO and TPO have opposing effects on platelet size heterogeneity**

207 To determine whether EPO has a specific physiologic activity in the MK lineage, we then
208 evaluated the impact of modulating EPO levels (Figure 5A) on platelet characteristics in WT mice.
209 Interestingly, the platelet size indices were significantly reduced after EPO-fKO (Figure 5B-C).
210 As expected, the megakaryocyte-erythroid progenitors' skewed differentiation towards the MK
211 lineage, as a result of the blockade of the erythroid lineage, induced an increase in platelet counts
212 (Fig 5D) (2, 73). Hence, EPO has a major role in the regulation of platelet size distribution and
213 more specifically on large-size platelet production, independently of the increased platelet renewal
214 observed in this context. We next evaluated the impact of EPO supplementation in WT mice
215 (Figure 5A). Elevated MPV and P-LCR values were observed when compared to control mice
216 (Figure 5B-C), whereas the platelet counts fell, as expected (Figure 5D) (2, 34).

217 To gain insight into the respective influences of TPO and EPO on platelet size heterogeneity, we
218 compared the variation of platelet indices in a progressive TPO-neutralized model (TPO-fKO),
219 generated through a vaccination strategy using an adenovirus encoding the heterologous human
220 TPO (55), to that of EPO-fKO mice. TPO-dependent platelet (TPL) counts were inversely
221 correlated with MPV and P-LCR (Figure 5E-F), whereas EPO-dependent platelet (EPL) counts
222 were directly correlated with MPV and P-LCR (Figure 5B-C). **Indeed, when TPO is not yet**
223 **neutralized (to the right of the graphs depicted on Figure 5E and 5F), platelets counts are high**
224 **(1400-1200 $\times 10^3/\text{mm}^3$), the action of EPO (physiologically produced) is not visible and the**
225 **platelets produced are small TPL with low MPV (~6 fL) and low P-LCR (10%). Conversely, as**
226 **TPO is neutralized (to the left on Figure 5E and 5F), platelets count decrease and platelet indices**
227 **increase, because the effect of EPO manifests and larger platelets are produced (for example,**
228 **when the TPO is neutralized to the point of reaching a platelet count of 600 $\times 10^3/\text{mm}^3$, these**

229 platelets have a high MPV of about 10 fL and high proportion of large platelets (P-LCR of 30%).
230 The progress towards loss-of-TPL production revealed the major role of EPO in the regulation of
231 the platelet functional mass. The impact of EPO neutralization or EPO supplementation on
232 platelets indices when TPO/Mpl axis is abrogated has also been studied. As shown on Figure 5H
233 and 5I, EPO neutralization (EPO-fKO) in *Mpl*^{-/-} mice induces a significant decrease in platelet
234 indices. Conversely, the administration of EPO to this mouse model increases the MPV (Figure 5L)
235 and the P-LCR (Figure 5M). Overall, these data reveal opposing effects of TPO and EPO on
236 platelet size heterogeneity.

237

238 **EPO's impact on the MK lineage**

239 We next sought to characterize the mechanisms underlying the effects of EPO on platelet size
240 heterogeneity when the TPO/MPL pathway is still functional. Three types of studies were
241 performed for this purpose.

242 First, given that Glycoprotein IIb (GpIIb) levels are known to increase with ploidy (56,
243 74-76) we wondered whether EPO injection in WT mice would impact the expression of this
244 marker on platelets. Indeed, platelets do not transcribe the nuclear genome since they are
245 anucleated, thus, variation in their RNA levels reflects changes in megakaryocyte transcriptional
246 activity (77). Interestingly, we found that rHuEPO injection for 2-3 weeks induced a
247 simultaneous increased expression of GpIIb and EpoR in platelets, at significantly higher relative
248 levels as compared to control mice (Figure 6A-B).

249 We next evaluated if EPO neutralization, when TPO/Mpl pathway is functional, impacts the
250 megakaryocyte profile. Surprisingly, we found that EPO-fKO has no impact on total bone marrow
251 MKP counts in this context (Figure 6 C-D). Accordingly, the study of the size (FSC) and
252 granularity or density (SSC) of CD41+CD150+ cells further confirmed the absence of variation of
253 the MK profile in EPO-fKO on a WT background (Figure 6E-F).

254 Finally, in view of human translation of our mouse results, we evaluated the balance between

255 EPO-EpoR and TPO-Mpl pathways in primary human MK, through an RNA expression approach.
256 We showed that progress through MK ploidy was associated with a marked rise in EpoR
257 expression, whereas Mpl expression levels remained relatively stable (Figure 6G). Taken together,
258 these data unraveled **EPO's potential impact on late MK differentiation stages to achieve its**
259 **physiologic role in platelet mass homeostasis.**, ~~through its prominent action on late MK~~
260 ~~differentiation stages. This action is in permanent coordination with TPO in a physiologic context.~~

261 **Discussion**

262 Platelets' primary physiological function is to enable hemostasis in response to vascular
263 endothelial injury, through the differential action and localization of highly activated and less
264 activated platelets within the thrombi (78-81). Platelets are also key actors in the pathogenesis of
265 atherothrombosis, through the formation and immobilization of stable platelets and
266 leukocyte-platelet aggregates at the site of atherosclerotic plaque rupture (82).

267 Despite compelling evidence for the role of large-size platelets in the pathogenesis of
268 atherothrombosis, their origin and regulation remain elusive. Previous studies showed that platelet
269 size at basal state is influenced by a number of genetic factors, regulating protein structure,
270 cellular trafficking, signaling and transcription (48-51), as well as various conditions such as
271 IL-1 α release (83), diabetes (84, 85), hypoxia(86-89), acute blood loss(90), and treatment with
272 rHuEPO (28). In addition, several studies have shown that EPO increases platelet activation in
273 both patients and healthy individuals (25-30, 67).

274 By studying various mouse models, we have demonstrated herein that TPO and EPO have major,
275 sequential and differential effects on the regulation of platelet-size heterogeneity. Specifically, we
276 first identified EPO as the major residual thrombopoietic factor in *TPO*^{-/-} and *Mpl*^{-/-} mice. We next
277 showed that EPO supplementation in *TPO*^{-/-} and *Mpl*^{-/-} mice induced a rebound of platelet counts
278 up to normal values, supporting its use as a potential therapy of human thrombocytopenia

279 secondary to *Mpl* mutations such as congenital amegakaryocytic thrombopocytopenia. We also
280 characterized the major regulatory role of EPO on the basal and induced production of large-size
281 platelets. EPO neutralization was associated with increased platelet production and reduced MPV
282 and P-LCR, while TPO abrogation reduced platelet production but with increased MPV and
283 P-LCR. These results suggest that platelet count and size are modulated (at least in mice) by the
284 coordinated action of TPO and EPO, to maintain a stable platelet mass.

285 The identification of the reciprocal role of EPO and TPO in the regulation of the MPV is **might be**
286 important in the context of cardiovascular and respiratory diseases, since long-term evaluation of
287 large cohort studies of healthy subjects has shown that a high MPV is associated with greater
288 incidence of venous thromboembolism (91), myocardial infarction and a higher all-cause mortality
289 rate (45). Furthermore, the MPV is elevated in patients with acute myocardial infarction (43, 92),
290 ischemic stroke (93) and peripheral arterial disease (46, 47). Strikingly, treatment with rHuEPO is
291 associated with thrombotic events in patients suffering from cancer or chronic kidney disease
292 (36-39). **The demonstration in clinical setting of the direct role of EPO in human thrombosis**
293 **associated with high MPV is still needed.** Our results, confirming previous observations, indicate
294 that EPO has a limited basal action at the early MK differentiation steps since TPO, thanks to high
295 *Mpl* expression relative to EpoR levels, controls the early MKPs proliferative events in
296 coordination with the other early acting cytokines (23, 64-66). In contrast, given the relatively
297 stable expression of *Mpl* observed all along the MK ploidy stages (our results), increased EpoR in
298 parallel with higher DNA contents might allow EPO to become more active at late MK
299 ploidy stages. It is noteworthy that EPO binding reportedly increases at late MK ploidy
300 stages ~~in line with our data on human MK ploidy stages~~ (12). In contradiction with the initial
301 conclusions based only on data from bone marrow cells from *TPO*^{-/-} mice (53), we observed that
302 both bone marrow and spleen cells from *TPO*^{-/-} and *Mpl*^{-/-} exhibited a higher MK ploidy in these
303 two mouse models producing platelets mainly through an EPO-EpoR pathway. Furthermore,

304 increased GpIIb relative expression levels (RLs) in platelets issued from rHuEPO-supplemented
305 mice is indicative of higher MK ploidy than the one induced by TPO suggests an increased
306 MK ploidy in this context. These results confirm previous studies showing that *in vitro*
307 stimulation of MKs with EPO increased their ploidy (19, 94, 95). Similarly, EPO supplementation
308 was also associated with elevated MK ploidy in rodents (96, 97).

309 The very high proportion of large-size platelets observed in *Mpl*^{-/-} and *TPO*^{-/-} mice, produced
310 mainly through an EPO-driven pathway, and the parallel increase in GpIIb and EpoR in platelets
311 purified from EPO-treated WT mice, which display elevated MPV in this context, warranted a
312 strong control of EPL production associated with high ploidy. Regarding the association of
313 increased platelet renewal with reduced MPV and P-LCR observed in the absence of the basal
314 action of EPO (EPO-fKO-WT mice), we hypothesize that TPO compensates for the reduction of
315 large-size platelets by maintaining a similar ploidy pattern, thereby stabilizing the platelet
316 mass through increased platelet production-renewal. Alternatively, the megakaryocytic-biased
317 HSC (3-8), or the MegP (which resides in the CMP progenitors) (9) might be involved in this
318 thrombopoiesis-stress response in order to rapidly increase the platelet count and then to regulate
319 the platelet mass when EPO is inactive.

320 Thus, our identification in mice models of a bipolarized process involving EPO and TPO and their
321 respective cognate receptors in the modulation of platelet size heterogeneity is an important step in
322 the understanding of late stage MK physiology.

323 Interplay between the two pathways in steady-state and under stress conditions may might
324 contribute to the regulation of platelet homeostasis. Since platelets can be produced throughout the
325 MK differentiation process (98), the prominent expression of EpoR in high ploidy MKs
326 suggests that the overproduction of EPO during a hemostatic stress (e.g. acute or chronic blood
327 loss) is an emergency mechanism to rapidly increase the production of large (and possibly more
328 active) platelets to stop a bleeding. Since mouse models may differ in some hematopoiesis aspects

329 from human, these results should be confirmed in human settings.

330

331

332 **Acknowledgments**

333 We thank F.J. de Sauvage for providing *TPO*^{-/-} and *Mpl*^{-/-} mice, Hana Raslova for providing MK
334 mRNA, Olivier Vidalin and Estienne Clément for help with FACS and qPCR experiments,
335 Jean-Philippe Rosa and Lotfi Hacein-Bey for critical reading of the manuscript. This work was
336 supported in part by a grant from the Agence Nationale de la Recherche (grant N°
337 ANR-08-BIOT-023-01) (F.L and A.M.A).

338

339 **Figure Legends**

340 **Figure 1** (No colour should be used, *small column size (90mm)*)

341 **Figure 1. Reduced red blood cells and markers of the erythroid lineage in the BM of**
342 **EPO-neutralized mice.** (A) Hematocrit (Ht) and red blood cells (RBC) in peripheral blood were
343 measured in C57BL/6 mice treated with anti-EPO polyclonal antibodies (poAb) (EPO-fKO-WT) (*n* = 12)
344 or with a non-relevant poAb (CTRL-WT) (*n* = 8), 5 times a week during 3 weeks. The results show a
345 blockade of the RBC production in EPO-neutralized mice. (B) RT-qPCR analyses were conducted on total
346 bone marrow cells obtained from EPO-fKO-WT mice (*n* = 8) and control WT mice (*n* = 5) to measure
347 mRNA relative expression levels (RL) of Glycophorin A (GYPA) and EpoR selected as markers of the
348 erythroid lineage. The results show a major reduction of EpoR and GYPA mRNA relative expression in
349 total BM cells of EPO-neutralized mice. Data are displayed as mean ± SEM. Significance was determined
350 by Mann-Whitney test (A,B). **P*<0.05, ***P*<0.01, ****P*<0.001, *****P*<0.0001, ns: not significant

351

352 **Figure 2** (Colour should be used, *full page width (190 mm)*)

353 **Figure 2. EPO regulates the residual platelet production in *TPO*^{-/-} and *Mpl*^{-/-} mice.** (A) Lower
354 platelet (PLT) count in EPO-fKO-*TPO*^{-/-} (*n* = 16) and (B) EPO-fKO-*Mpl*^{-/-} mice (*n* = 15) than in *TPO*^{-/-} (*n* =
355 15) and *Mpl*^{-/-} (*n* = 8) control mice (C-F) FACS analysis of **Lin-c-Kit⁺ Sea-1⁺ Sca-1⁻ CD150⁺ CD41⁺**
356 megakaryocyte progenitors (MKPs) in the BM (C) and spleen (D) of EPO-fKO-*TPO*^{-/-} mice and *TPO*^{-/-} mice
357 indicates a low residual MKP counts in the BM (E) (*n* = 6) and spleen (*n* = 3) (F) cells of EPO-fKO-*TPO*^{-/-}
358 mice quoted as absolute count when compared to *TPO*^{-/-} control mice (*n* = 4 and 7 respectively) (G and H)
359 Time course of PLT recovery in *Mpl*^{-/-} and *TPO*^{-/-} mice (*n* = 17-19/group) after stimulation with rHuEPO.
360 PLT: platelet; Ht: hematocrit; MKP: megakaryocytic progenitors; EPO-fKO: EPO-functional-KO. Data are
361 quoted as the mean ± SEM. **P*<0.05, ***P*<0.01, ****P*<0.001, *****P*<0.0001, ns: not significant.
362 Statistical significance was determined in a Mann-Whitney test (A,B, D-H).

363

364 **Figure 3** (No colour should be used, *small column size (90mm)*)

365 **Figure 3. Platelet characteristics of *Mpl*^{-/-} and *TPO*^{-/-} compared to WT mice.** (A-C) Platelet

366 indices (MPV, P-LCR) and platelet counts (PLT) of $Mpl^{-/-}$ ($n = 159$) and $TPO^{-/-}$ ($n = 97$) were compared to
367 C57BL/6 WT mice ($n = 55$). Results show high MPV and P-LCR values in $Mpl^{-/-}$ and $TPO^{-/-}$ mice, relative to
368 WT mice. Data are quoted as the mean \pm SEM. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$, ns: not
369 significant. Statistical significance was determined in a Mann-Whitney test.

370
371 **Figure 4** (No colour should be used, *one and a half page width (140mm)*)
372 **Figure 4. EPO acts predominantly in the late stages of MK differentiation.** (A) Elevated MK
373 ploidy in $TPO^{-/-}$ ($n = 3$) and $Mpl^{-/-}$ ($n = 7$) mice vs. WT mice ($n = 7$). The percentage of spleen and BM
374 MKs (evaluated separately) were pooled and normalized against values for WT mice at the same ploidy
375 stage. (B, C) Increased of CD41+low CD150+ and CD41+high CD150+ cell size (FSC) and cell density
376 (SSC) in $TPO^{-/-}$ ($n = 4$) compared to WT mice ($n = 8$). Statistical significance was determined in a multiple t
377 test (A-C). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$, ns: not significant.

378
379 **Figure 5** (Colour should be used, *full page width (190 mm)*)
380 **Figure 5. EPO is the major regulator of large platelets production.** (A-D) Platelets (PLT) count
381 and indices (MPV, P-LCR) and hematocrit (Ht) of C57BL/6 WT mice were measured after stimulation with
382 rHuEPO for 2 weeks ($n = 15$, $n = 13$ for controls (control-e) or after EPO neutralization (anti-EPO) ($n = 15$,
383 $n = 8$ for controls (control-a)). Results showed a direct correlation between EPO action and large-size platelet
384 production. (E, F) Inverse correlation between MPV or P-LCR and the PLT count during generation of the
385 TPO-fKO phenotype ($n = 73$). (G-N) Platelets (PLT) count and indices (MPV, P-LCR) and hematocrit (Ht)
386 of $Mpl^{-/-}$ mice were measured after EPO neutralization (G-J) (EPO-fKO- $Mpl^{-/-}$, $n = 10$ and $n = 16$ for
387 controls (CTRL- $Mpl^{-/-}$ for which the values were pooled from data collected from individual mice during all
388 the follow-up), and after stimulation with rHuEPO for 2 weeks (K-N) (post-EPO stimulation: $n = 17$, $n = 7$
389 for controls ($Mpl^{-/-}$)). Control-e: PBS; control-a: non-relevant antibody. Data are quoted as the mean \pm SEM.
390 * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, ns: not significant. Statistical significance was determined in a
391 Mann-Whitney test (A-D) and (G-N) or a Pearson's correlation test (E, F). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$,
392 **** $P < 0.0001$, ns: not significant.

393
394 **Figure 6** (Colour should be used, *full page width (190 mm)*)
395 **Figure 6. EPO acts predominantly in the late stages of MK differentiation.** (A, B) Increased
396 mRNA relative expression levels (RL) of GpIIb and EpoR in PLTs purified from C57BL/6 after rHuEPO
397 stimulation relative to controls. (C, D) FACS analysis showed that MKP counts (Lin- c-Kit+ Sca-1- Sea-1+
398 CD150+ CD41+ megakaryocyte progenitors) counts were similar in the BM of EPO-fKO-WT ($n = 16$) and
399 C57BL/6-WT ($n = 12$) mice. (E, F) Cell size (FSC) and cell density (SSC) of CD41+low CD150+ or
400 CD41+high CD150+ cells of C57BL/6-WT mice ($n = 7$) were similar to those observed in EPO-fKO-WT mice

401 (n = 16). (G) Changes in EpoR and Mpl mRNA relative expression levels (RL) normalized against the
402 values observed at the 2N stage in pooled purified human MKs at different ploidy stages, indicate an
403 increased EpoR expression along MK maturation whereas Mpl levels remained relatively stable. Data are
404 quoted as the mean \pm SEM. Significance was determined using Mann-Whitney test (A, B, D), and multiple *t*
405 test (E-G). **P*<0.05; ***P*<0.01; ****P*<0.001*****P*<0.0001, ns: not significant.

406

407

408 **References**

- 409 1. Han ZC, Bellucci S, Pidadr D, Caen JP. Coexpression in the same cell in marrow culture of antigens
410 from erythroid and megakaryocytic lineage. C. K-G, N. S, Ch. S, J. M, editors. London: John Libbey
411 Eurotext; 1991. 17-8 p.
- 412 2. McDonald TP, Sullivan PS. Megakaryocytic and erythrocytic cell lines share a common precursor
413 cell. *Exp Hematol.* 1993;21(10):1316-20.
- 414 3. Yamamoto R, Morita Y, Ooehara J, Hamanaka S, Onodera M, Rudolph KL, et al. Clonal analysis
415 unveils self-renewing lineage-restricted progenitors generated directly from hematopoietic stem cells. *Cell.*
416 2013;154(5):1112-26.
- 417 4. Sanjuan-Pla A, Macaulay IC, Jensen CT, Woll PS, Luis TC, Mead A, et al. Platelet-biased stem cells
418 reside at the apex of the haematopoietic stem-cell hierarchy. *Nature.* 2013;502(7470):232-6.
- 419 5. Shin JY, Hu W, Naramura M, Park CY. High c-Kit expression identifies hematopoietic stem cells
420 with impaired self-renewal and megakaryocytic bias. *J Exp Med.* 2014;211(2):217-31.
- 421 6. Haas S, Hansson J, Klimmeck D, Loeffler D, Velten L, Uckelmann H, et al. Inflammation-Induced
422 Emergency Megakaryopoiesis Driven by Hematopoietic Stem Cell-like Megakaryocyte Progenitors. *Cell*
423 *Stem Cell.* 2015;17(4):422-34.
- 424 7. Psaila B, Barkas N, Iskander D, Roy A, Anderson S, Ashley N, et al. Single-cell profiling of human
425 megakaryocyte-erythroid progenitors identifies distinct megakaryocyte and erythroid differentiation
426 pathways. *Genome Biol.* 2016;17:83.
- 427 8. Psaila B, Mead AJ. Single-cell approaches reveal novel cellular pathways for megakaryocyte and
428 erythroid differentiation. *Blood.* 2019;133(13):1427-35.
- 429 9. Miyawaki K, Iwasaki H, Jiromaru T, Kusumoto H, Yurino A, Sugio T, et al. Identification of
430 unipotent megakaryocyte progenitors in human hematopoiesis. *Blood.* 2017;129(25):3332-43.
- 431 10. Emerson SG, Yang YC, Clark SC, Long MW. Human recombinant granulocyte-macrophage colony
432 stimulating factor and interleukin 3 have overlapping but distinct hematopoietic activities. *J Clin Invest.*
433 1988;82(4):1282-7.
- 434 11. Fraser JK, Lin FK, Berridge MV. Expression of high affinity receptors for erythropoietin on human
435 bone marrow cells and on the human erythroleukemic cell line, HEL. *Exp Hematol.* 1988;16(10):836-42.
- 436 12. Fraser JK, Tan AS, Lin FK, Berridge MV. Expression of specific high-affinity binding sites for
437 erythropoietin on rat and mouse megakaryocytes. *Exp Hematol.* 1989;17(1):10-6.
- 438 13. Messner HA, Yamasaki K, Jamal N, Minden MM, Yang YC, Wong GG, et al. Growth of human
439 hemopoietic colonies in response to recombinant gibbon interleukin 3: comparison with human recombinant
440 granulocyte and granulocyte-macrophage colony-stimulating factor. *Proc Natl Acad Sci U S A.*
441 1987;84(19):6765-9.
- 442 14. Huang E, Nocka K, Beier DR, Chu TY, Buck J, Lahm HW, et al. The hematopoietic growth factor
443 KL is encoded by the Sl locus and is the ligand of the c-kit receptor, the gene product of the W locus. *Cell.*
444 1990;63(1):225-33.
- 445 15. Kieran MW, Perkins AC, Orkin SH, Zon LI. Thrombopoietin rescues in vitro erythroid colony
446 formation from mouse embryos lacking the erythropoietin receptor. *Proc Natl Acad Sci U S A.*
447 1996;93(17):9126-31.
- 448 16. Kaushansky K, Lin N, Grossmann A, Humes J, Sprugel KH, Broudy VC. Thrombopoietin expands
449 erythroid, granulocyte-macrophage, and megakaryocytic progenitor cells in normal and myelosuppressed
450 mice. *Exp Hematol.* 1996;24(2):265-9.
- 451 17. Abina MA, Tulliez M, Lacout C, Debili N, Villeval JL, Pflumio F, et al. Major effects of TPO
452 delivered by a single injection of a recombinant adenovirus on prevention of septicemia and anemia
453 associated with myelosuppression in mice: risk of sustained expression inducing myelofibrosis due to
454 immunosuppression. *Gene Ther.* 1998;5(4):497-506.
- 455 18. McLeod DL, Shreve MM, Axelrad AA. Induction of megakaryocyte colonies with platelet formation
456 in vitro. *Nature.* 1976;261(5560):492-4.
- 457 19. Ishibashi T, Koziol JA, Burstein SA. Human recombinant erythropoietin promotes differentiation of
458 murine megakaryocytes in vitro. *J Clin Invest.* 1987;79(1):286-9.
- 459 20. Dessypris EN, Gleaton JH, Armstrong OL. Effect of human recombinant erythropoietin on human
460 marrow megakaryocyte colony formation in vitro. *Br J Haematol.* 1987;65(3):265-9.

- 461 21. Ishida Y, Yano S, Yoshida T, Tanaka H, Yamada Y, Kawano M, et al. Biological effects of
462 recombinant erythropoietin, granulocyte-macrophage colony-stimulating factor, interleukin 3, and
463 interleukin 6 on purified rat megakaryocytes. *Exp Hematol.* 1991;19(7):608-12.
- 464 22. Tsukada J, Misago M, Kikuchi M, Sato T, Ogawa R, Ota T, et al. Interactions between recombinant
465 human erythropoietin and serum factor(s) on murine megakaryocyte colony formation. *Blood.*
466 1992;80(1):37-45.
- 467 23. An E, Ogata K, Kuriya S, Nomura T. Interleukin-6 and erythropoietin act as direct potentiators and
468 inducers of in-vitro cytoplasmic process formation on purified mouse megakaryocytes *Exp Hematol.*
469 1994;22(2):149-56.
- 470 24. Pronk CJ, Rossi DJ, Mansson R, Attema JL, Norddahl GL, Chan CK, et al. Elucidation of the
471 phenotypic, functional, and molecular topography of a myeloerythroid progenitor cell hierarchy. *Cell Stem*
472 *Cell.* 2007;1(4):428-42.
- 473 25. Moia M, Mannucci PM, Vizzotto L, Casati S, Cattaneo M, Ponticelli C. Improvement in the
474 haemostatic defect of uraemia after treatment with recombinant human erythropoietin. *Lancet.*
475 1987;2(8570):1227-9.
- 476 26. Wolf RF, Gilmore LS, Friese P, Downs T, Burstein SA, Dale GL. Erythropoietin potentiates
477 thrombus development in a canine arterio-venous shunt model. *Thromb Haemost.* 1997;77(5):1020-4.
- 478 27. Wolf RF, Peng J, Friese P, Gilmore LS, Burstein SA, Dale GL. Erythropoietin administration
479 increases production and reactivity of platelets in dogs. *Thromb Haemost.* 1997;78(6):1505-9.
- 480 28. Sharpe PC, Desai ZR, Morris TC. Increase in mean platelet volume in patients with chronic renal
481 failure treated with erythropoietin. *J Clin Pathol.* 1994;47(2):159-61.
- 482 29. Stohlawetz PJ, Dzirlo L, Hergovich N, Lackner E, Mensik C, Eichler HG, et al. Effects of
483 erythropoietin on platelet reactivity and thrombopoiesis in humans. *Blood.* 2000;95(9):2983-9.
- 484 30. Homoncik M, Jilma-Stohlawetz P, Schmid M, Ferlitsch A, Peck-Radosavljevic M. Erythropoietin
485 increases platelet reactivity and platelet counts in patients with alcoholic liver cirrhosis: a randomized,
486 double-blind, placebo-controlled study. *Aliment Pharmacol Ther.* 2004;20(4):437-43.
- 487 31. Pirisi M, Fabris C, Soardo G, Cecchin E, Toniutto P, Bartoli E. Thrombocytopenia of chronic liver
488 disease corrected by erythropoietin treatment. *J Hepatol.* 1995;23(2):233.
- 489 32. McDonald TP, Cottrell MB, Clift RE, Cullen WC, Lin FK. High doses of recombinant erythropoietin
490 stimulate platelet production in mice. *Exp Hematol.* 1987;15(6):719-21.
- 491 33. McDonald TP, Cottrell MB, Steward SA, Clift RE, Swearingen CJ, Jackson CW. Comparison of
492 platelet production in two strains of mice with different modal megakaryocyte DNA ploidies after exposure
493 to hypoxia. *Exp Hematol.* 1992;20(1):51-6.
- 494 34. McDonald TP, Clift RE, Cottrell MB. Large, chronic doses of erythropoietin cause
495 thrombocytopenia in mice. *Blood.* 1992;80(2):352-8.
- 496 35. Beguin Y. Erythropoietin and platelet production. *Haematologica.* 1999;84(6):541-7.
- 497 36. Bohlius J, Schmidlin K, Brillant C, Schwarzer G, Trelle S, Seidenfeld J, et al. Recombinant human
498 erythropoiesis-stimulating agents and mortality in patients with cancer: a meta-analysis of randomised trials.
499 *Lancet.* 2009;373(9674):1532-42.
- 500 37. Bohlius J, Wilson J, Seidenfeld J, Piper M, Schwarzer G, Sandercock J, et al. Recombinant human
501 erythropoietins and cancer patients: updated meta-analysis of 57 studies including 9353 patients. *J Natl*
502 *Cancer Inst.* 2006;98(10):708-14.
- 503 38. Pfeffer MA, Burdmann EA, Chen CY, Cooper ME, de Zeeuw D, Eckardt KU, et al. A trial of
504 darbepoetin alfa in type 2 diabetes and chronic kidney disease. *N Engl J Med.* 2009;361(21):2019-32.
- 505 39. Skali H, Parving HH, Parfrey PS, Burdmann EA, Lewis EF, Ivanovich P, et al. Stroke in patients
506 with type 2 diabetes mellitus, chronic kidney disease, and anemia treated with Darbepoetin Alfa: the trial to
507 reduce cardiovascular events with Aranesp therapy (TREAT) experience. *Circulation.* 2011;124(25):2903-8.
- 508 40. Martin JF, Kristensen SD, Mathur A, Grove EL, Choudry FA. The causal role of
509 megakaryocyte-platelet hyperactivity in acute coronary syndromes. *Nat Rev Cardiol.* 2012;9(11):658-70.
- 510 41. Karpatkin S, Khan Q, Freedman M. Heterogeneity of platelet function. Correlation with platelet
511 volume. *Am J Med.* 1978;64(4):542-6.
- 512 42. Karpatkin S. Heterogeneity of human platelets. II. Functional evidence suggestive of young and old
513 platelets. *J Clin Invest.* 1969;48(6):1083-7.
- 514 43. Martin JF, Plumb J, Kilbey RS, Kishk YT. Changes in volume and density of platelets in myocardial
515 infarction. *Br Med J (Clin Res Ed).* 1983;287(6390):456-9.

516 44. Martin JF, Bath PM, Burr ML. Influence of platelet size on outcome after myocardial infarction.
517 Lancet. 1991;338(8780):1409-11.

518 45. Klovaite J, Benn M, Yazdanyar S, Nordestgaard BG. High platelet volume and increased risk of
519 myocardial infarction: 39,531 participants from the general population. *J Thromb Haemost.* 2011;9(1):49-56.

520 46. Berger JS, Eraso LH, Xie D, Sha D, Mohler ER, 3rd. Mean platelet volume and prevalence of
521 peripheral artery disease, the National Health and Nutrition Examination Survey, 1999-2004. *Atherosclerosis.*
522 2010;213(2):586-91.

523 47. Chu SG, Becker RC, Berger PB, Bhatt DL, Eikelboom JW, Konkle B, et al. Mean platelet volume as
524 a predictor of cardiovascular risk: a systematic review and meta-analysis. *J Thromb Haemost.*
525 2010;8(1):148-56.

526 48. Gieger C, Radhakrishnan A, Cvejic A, Tang W, Porcu E, Pistis G, et al. New gene functions in
527 megakaryopoiesis and platelet formation. *Nature.* 2011;480(7376):201-8.

528 49. Meisinger C, Prokisch H, Gieger C, Soranzo N, Mehta D, Roskopf D, et al. A genome-wide
529 association study identifies three loci associated with mean platelet volume. *Am J Hum Genet.*
530 2009;84(1):66-71.

531 50. Soranzo N, Spector TD, Mangino M, Kuhnel B, Rendon A, Teumer A, et al. A genome-wide
532 meta-analysis identifies 22 loci associated with eight hematological parameters in the HaemGen consortium.
533 *Nat Genet.* 2009;41(11):1182-90.

534 51. Panova-Noeva M, Schulz A, Hermanns MI, Grossmann V, Pefani E, Spronk HM, et al. Sex-specific
535 differences in genetic and nongenetic determinants of mean platelet volume: results from the Gutenberg
536 Health Study. *Blood.* 2016;127(2):251-9.

537 52. Caro J, Erslev AJ, Silver R, Miller O, Birgegard G. Erythropoietin production in response to anemia
538 or hypoxia in the newborn rat. *Blood.* 1982;60(4):984-8.

539 53. de Sauvage FJ, Carver-Moore K, Luoh SM, Ryan A, Dowd M, Eaton DL, et al. Physiological
540 regulation of early and late stages of megakaryocytopoiesis by thrombopoietin. *J Exp Med.*
541 1996;183(2):651-6.

542 54. Gurney AL, Carver-Moore K, de Sauvage FJ, Moore MW. Thrombocytopenia in c-mpl-deficient
543 mice. *Science.* 1994;265(5177):1445-7.

544 55. Abina MA, Tulliez M, Duffour MT, Debili N, Lacout C, Villeval JL, et al. Thrombopoietin (TPO)
545 knockout phenotype induced by cross-reactive antibodies against TPO following injection of mice with
546 recombinant adenovirus encoding human TPO. *J Immunol.* 1998;160(9):4481-9.

547 56. Raslova H, Kauffmann A, Sekkai D, Ripoche H, Larbret F, Robert T, et al. Interrelation between
548 polyploidization and megakaryocyte differentiation: a gene profiling approach. *Blood.* 2007;109(8):3225-34.

549 57. Gainsford T, Nandurkar H, Metcalf D, Robb L, Begley CG, Alexander WS. The residual
550 megakaryocyte and platelet production in c-Mpl-deficient mice is not dependent on the actions of
551 interleukin-6, interleukin-11, or leukemia inhibitory factor. *Blood.* 2000;95(2):528-34.

552 58. Gainsford T, Roberts AW, Kimura S, Metcalf D, Dranoff G, Mulligan RC, et al. Cytokine
553 production and function in c-mpl-deficient mice: No physiologic role for interleukin-3 in residual
554 megakaryocyte and platelet production. *Blood.* 1998;91(8):2745-52.

555 59. Antonchuk J, Hyland CD, Hilton DJ, Alexander WS. Synergistic effects on erythropoiesis,
556 thrombopoiesis, and stem cell competitiveness in mice deficient in thrombopoietin and steel factor receptors.
557 *Blood.* 2004;104(5):1306-13.

558 60. Kaushansky K, Fox N, Lin NL, Liles WC. Lineage-specific growth factors can compensate for stem
559 and progenitor cell deficiencies at the postprogenitor cell level: an analysis of doubly TPO- and G-CSF
560 receptor-deficient mice. *Blood.* 2002;99(10):3573-8.

561 61. Scott CL, Robb L, Mansfield R, Alexander WS, Begley CG. Granulocyte-macrophage
562 colony-stimulating factor is not responsible for residual thrombopoiesis in mpl null mice. *Exp Hematol.*
563 2000;28(9):1001-7.

564 62. Chen Q, Solar G, Eaton DL, de Sauvage FJ. IL-3 does not contribute to platelet production in
565 c-Mpl-deficient mice. *Stem Cells.* 1998;16 Suppl 2:31-6.

566 63. Carver-Moore K, Broxmeyer HE, Luoh SM, Cooper S, Peng J, Burstein SA, et al. Low levels of
567 erythroid and myeloid progenitors in thrombopoietin-and c-mpl-deficient mice. *Blood.* 1996;88(3):803-8.

568 64. Cardier JE, Erickson-Miller CL, Murphy MJ, Jr. Differential effect of erythropoietin and GM-CSF
569 on megakaryocytopoiesis from primitive bone marrow cells in serum-free conditions. *Stem Cells.*
570 1997;15(4):286-90.

- 571 65. Broudy VC, Lin NL, Kaushansky K. Thrombopoietin (c-mpl ligand) acts synergistically with
572 erythropoietin, stem cell factor, and interleukin-11 to enhance murine megakaryocyte colony growth and
573 increases megakaryocyte ploidy in vitro. *Blood*. 1995;85(7):1719-26.
- 574 66. Metcalf D, Di Rago L, Mifsud S. Synergistic and inhibitory interactions in the in vitro control of
575 murine megakaryocyte colony formation. *Stem Cells*. 2002;20(6):552-60.
- 576 67. Huraib S, al-Momen AK, Gader AM, Mitwalli A, Sulimani F, Abu-Aisha H. Effect of recombinant
577 human erythropoietin (rHuEpo) on the hemostatic system in chronic hemodialysis patients. *Clin Nephrol*.
578 1991;36(5):252-7.
- 579 68. Wu H, Liu X, Jaenisch R, Lodish HF. Generation of committed erythroid BFU-E and CFU-E
580 progenitors does not require erythropoietin or the erythropoietin receptor. *Cell*. 1995;83(1):59-67.
- 581 69. Vidalin O, Muslmani M, Estienne C, Echchakir H, Abina AM. In vivo target validation using gene
582 invalidation, RNA interference and protein functional knockout models: it is the time to combine. *Curr Opin*
583 *Pharmacol*. 2009;9(5):669-74.
- 584 70. Bunting S, Widmer R, Lipari T, Rangell L, Steinmetz H, Carver-Moore K, et al. Normal platelets
585 and megakaryocytes are produced in vivo in the absence of thrombopoietin. *Blood*. 1997;90(9):3423-9.
- 586 71. Trowbridge EA, Martin JF, Slater DN, Kishk YT, Warren CW. Platelet production: a computer
587 based biological interpretation. *Thromb Res*. 1983;31(2):329-50.
- 588 72. Breton-Gorius J, Reyes F. Ultrastructure of human bone marrow cell maturation. *Int Rev Cytol*.
589 1976;46:251-321.
- 590 73. McDonald TP. Platelet production in hypoxic and RBC-transfused mice. *Scand J Haematol*.
591 1978;20(3):213-20.
- 592 74. Hancock V, Martin JF, Lelchuk R. The relationship between human megakaryocyte nuclear DNA
593 content and gene expression. *Br J Haematol*. 1993;85(4):692-7.
- 594 75. Lepage A, Leboeuf M, Cazenave JP, de la Salle C, Lanza F, Uzan G. The alpha(IIb)beta(3) integrin
595 and GPIb-V-IX complex identify distinct stages in the maturation of CD34(+) cord blood cells to
596 megakaryocytes. *Blood*. 2000;96(13):4169-77.
- 597 76. Raslova H, Roy L, Vourc'h C, Le Couedic JP, Brison O, Metivier D, et al. Megakaryocyte
598 polyploidization is associated with a functional gene amplification. *Blood*. 2003;101(2):541-4.
- 599 77. Rowley JW, Schwertz H, Weyrich AS. Platelet mRNA: the meaning behind the message. *Curr Opin*
600 *Hematol*. 2012;19(5):385-91.
- 601 78. Bellido-Martin L, Chen V, Jasuja R, Furie B, Furie BC. Imaging fibrin formation and platelet and
602 endothelial cell activation in vivo. *Thromb Haemost*. 2011;105(5):776-82.
- 603 79. van Gestel MA, Heemskerk JW, Slaaf DW, Heijnen VV, Sage SO, Reneman RS, et al. Real-time
604 detection of activation patterns in individual platelets during thromboembolism in vivo: differences between
605 thrombus growth and embolus formation. *J Vasc Res*. 2002;39(6):534-43.
- 606 80. Hechler B, Nonne C, Eckly A, Magnenat S, Rinckel JY, Denis CV, et al. Arterial thrombosis:
607 relevance of a model with two levels of severity assessed by histologic, ultrastructural and functional
608 characterization. *J Thromb Haemost*. 2010;8(1):173-84.
- 609 81. Hayashi T, Mogami H, Murakami Y, Nakamura T, Kanayama N, Konno H, et al. Real-time analysis
610 of platelet aggregation and procoagulant activity during thrombus formation in vivo. *Pflugers Arch*.
611 2008;456(6):1239-51.
- 612 82. Davi G, Patrono C. Platelet activation and atherothrombosis. *N Engl J Med*. 2007;357(24):2482-94.
- 613 83. Nishimura S, Nagasaki M, Kunishima S, Sawaguchi A, Sakata A, Sakaguchi H, et al. IL-1alpha
614 induces thrombopoiesis through megakaryocyte rupture in response to acute platelet needs. *J Cell Biol*.
615 2015;209(3):453-66.
- 616 84. Papanas N, Symeonidis G, Maltezos E, Mavridis G, Karavageli E, Vosnakidis T, et al. Mean platelet
617 volume in patients with type 2 diabetes mellitus. *Platelets*. 2004;15(8):475-8.
- 618 85. Muscari A, De Pascalis S, Cenni A, Ludovico C, Castaldini N, Antonelli S, et al. Determinants of
619 mean platelet volume (MPV) in an elderly population: relevance of body fat, blood glucose and ischaemic
620 electrocardiographic changes. *Thromb Haemost*. 2008;99(6):1079-84.
- 621 86. Wedzicha JA, Cotter FE, Empey DW. Platelet size in patients with chronic airflow obstruction with
622 and without hypoxaemia. *Thorax*. 1988;43(1):61-4.
- 623 87. McDonald TP, Cottrell M, Clift R. Effects of short-term hypoxia on platelet counts of mice. *Blood*.
624 1978;51(1):165-75.

- 625 88. McDonald TP, Cottrell MB, Clift RE, Jackson CW. Effects of hypoxia on megakaryocyte size and
626 number of C3H and BALB/c mice. *Proc Soc Exp Biol Med.* 1992;199(3):287-90.
- 627 89. McDonald TP, Cullen WC, Cottrell M, Clift R. Effects of hypoxia on the small
628 acetylcholinesterase-positive megakaryocyte precursor in bone marrow of mice. *Proc Soc Exp Biol Med.*
629 1986;183(1):114-7.
- 630 90. Minter FM, Ingram M. Platelet volume: density relationships in normal and acutely bled dogs. *Br J*
631 *Haematol.* 1971;20(1):55-68.
- 632 91. Braekkan SK, Mathiesen EB, Njolstad I, Wilsgaard T, Stormer J, Hansen JB. Mean platelet volume
633 is a risk factor for venous thromboembolism: the Tromso Study, Tromso, Norway. *J Thromb Haemost.*
634 2010;8(1):157-62.
- 635 92. Enticknap JB, Gooding PG, Lansley TS, Avis PR. Platelet size and function in ischaemic heart
636 disease. *J Atheroscler Res.* 1969;10(1):41-9.
- 637 93. Butterworth RJ, Bath PM. The relationship between mean platelet volume, stroke subtype and
638 clinical outcome. *Platelets.* 1998;9(6):359-64.
- 639 94. Kuter DJ, Greenberg SM, Rosenberg RD. Analysis of megakaryocyte ploidy in rat bone marrow
640 cultures. *Blood.* 1989;74(6):1952-62.
- 641 95. Teramura M, Mizoguchi H. The effect of cytokines on the ploidy of megakaryocytes. *Int J Cell*
642 *Cloning.* 1990;8(4):245-52.
- 643 96. Shikama Y, Ishibashi T, Kimura H, Kawaguchi M, Uchida T, Maruyama Y. Transient effect of
644 erythropoietin on thrombocytopoiesis in vivo in mice. *Exp Hematol.* 1992;20(2):216-22.
- 645 97. Yonemura Y, Kawakita M, Fujimoto K, Sakaguchi M, Kusuyama T, Hirose J, et al. Effects of
646 short-term administration of recombinant human erythropoietin on rat megakaryopoiesis. *Int J Cell Cloning.*
647 1992;10(1):18-27.
- 648 98. Mattia G, Vulcano F, Milazzo L, Barca A, Macioce G, Giampaolo A, et al. Different ploidy levels of
649 megakaryocytes generated from peripheral or cord blood CD34+ cells are correlated with different levels of
650 platelet release. *Blood.* 2002;99(3):888-97.

Figure 1

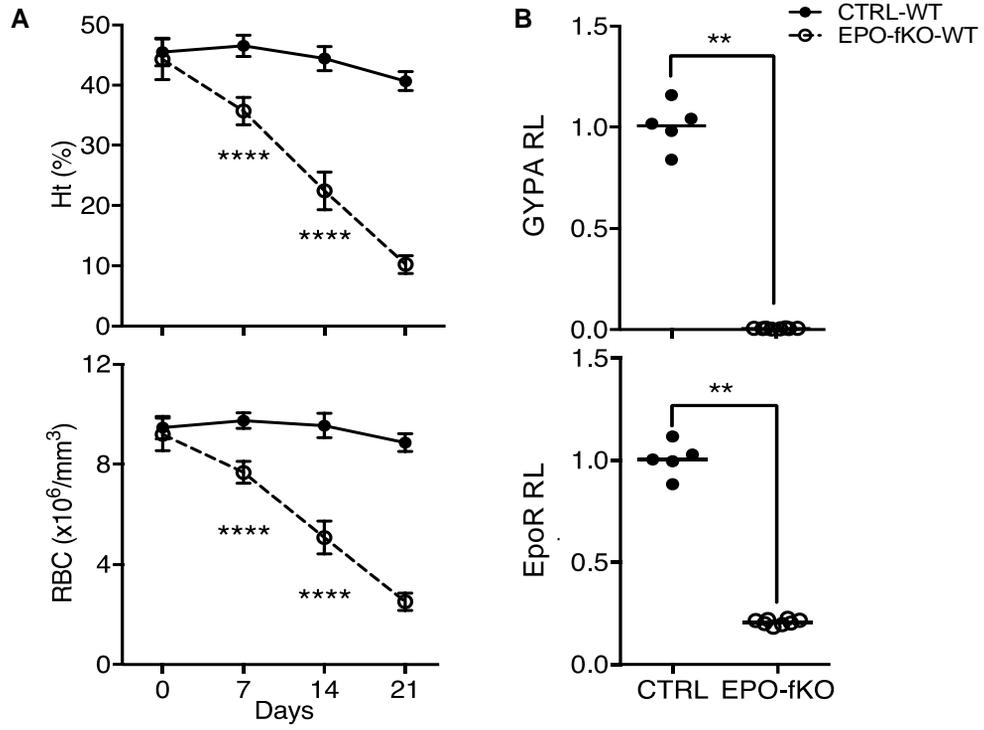


Figure 2

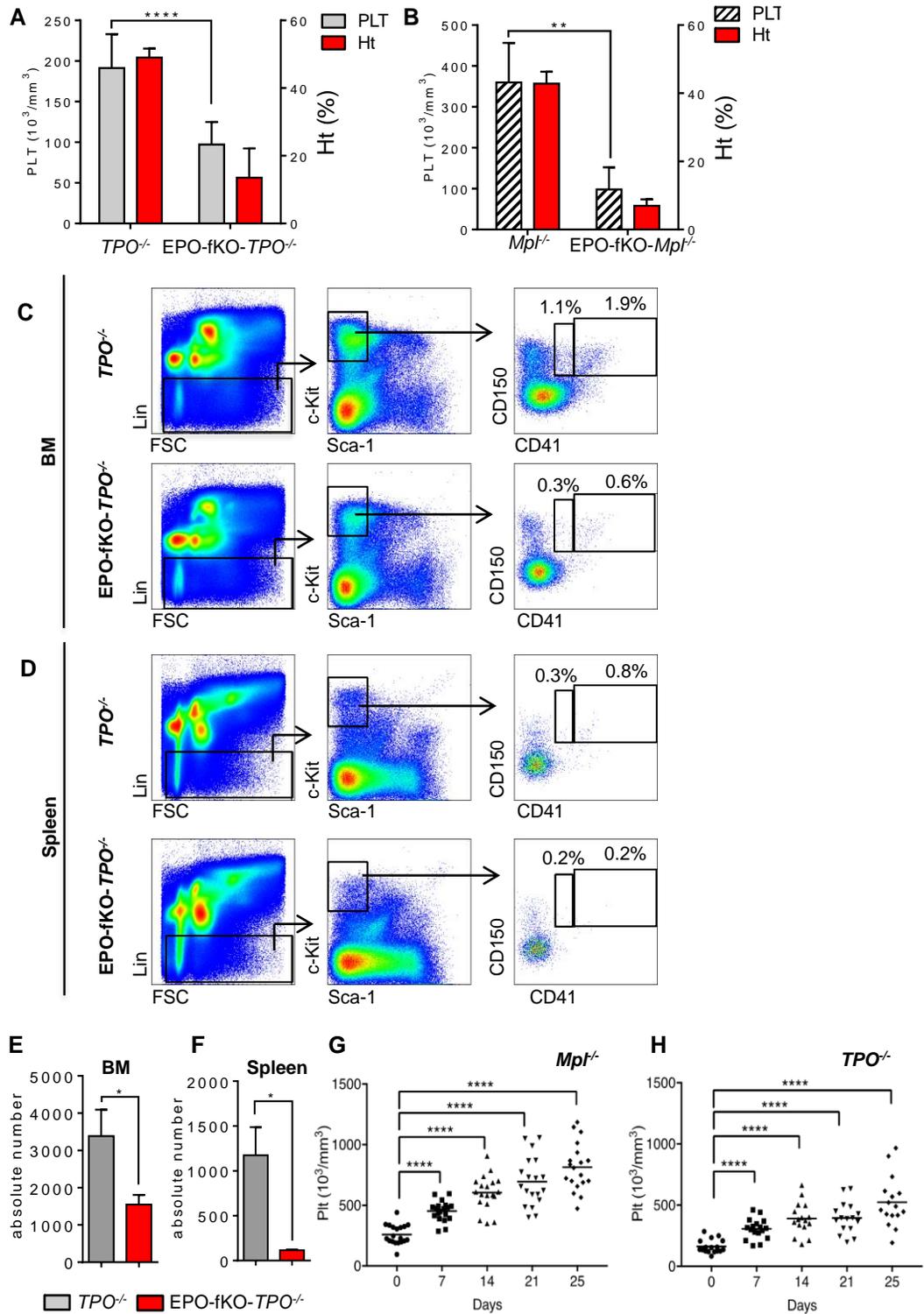


Figure 3

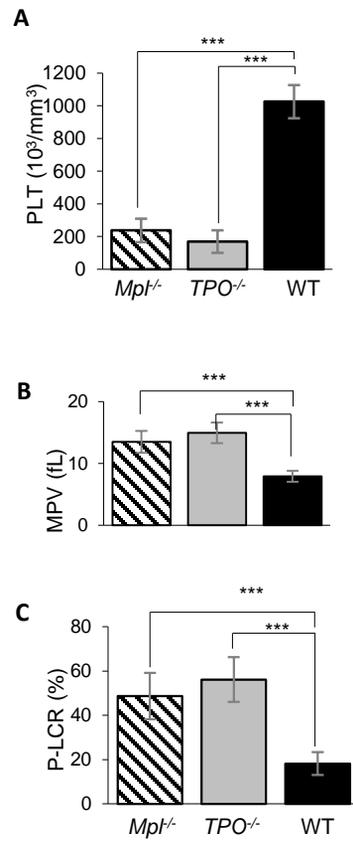


Figure 4

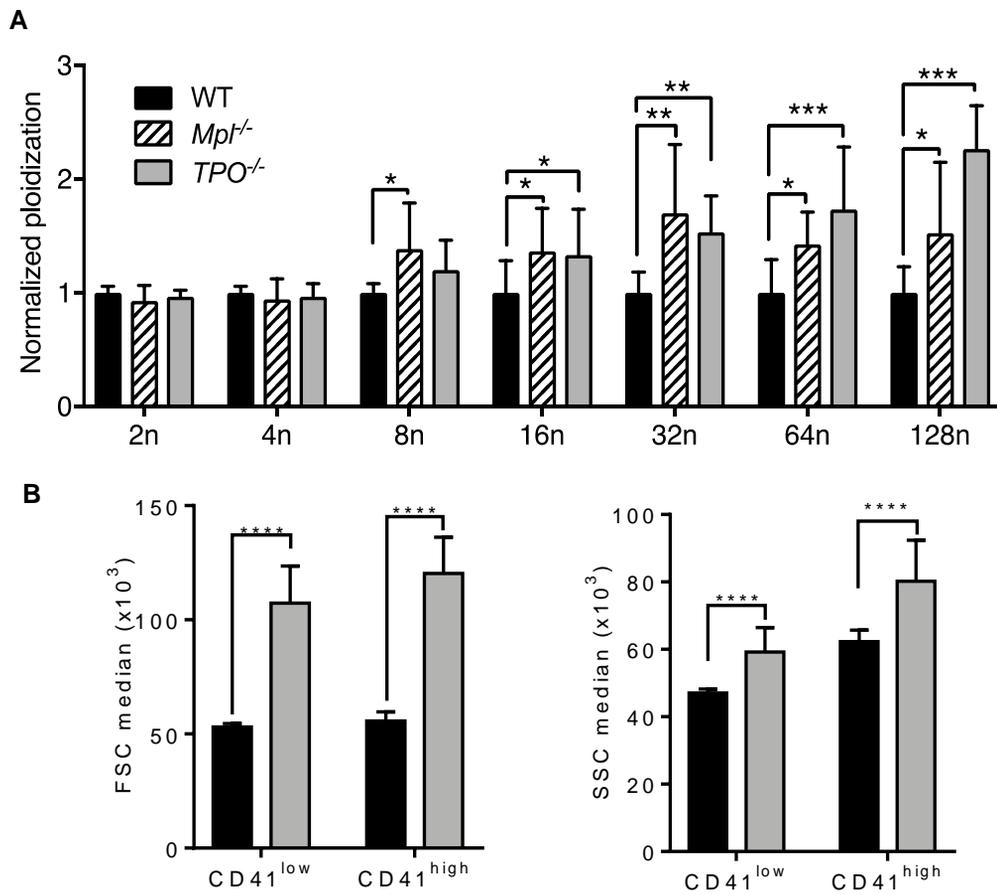


Figure 5

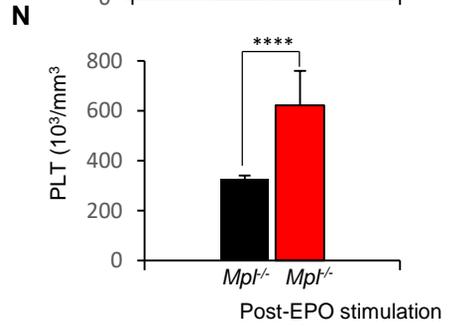
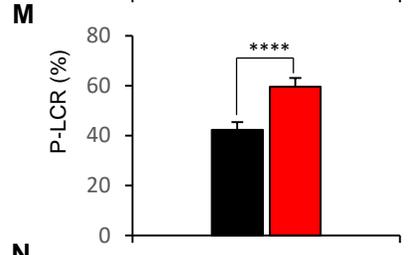
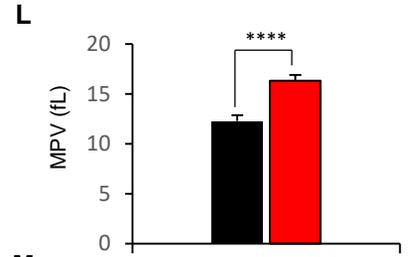
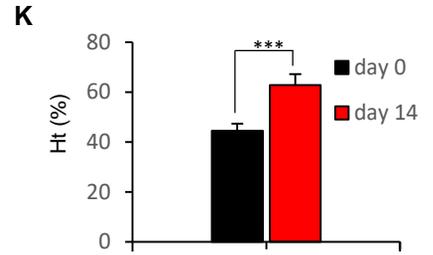
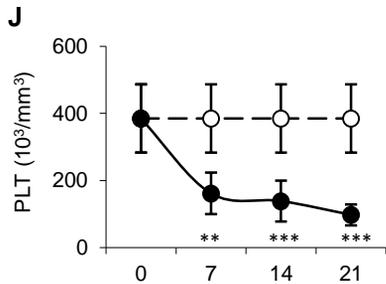
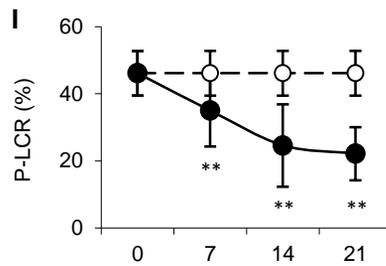
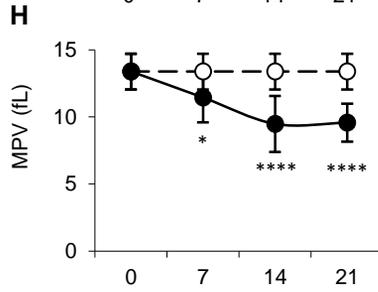
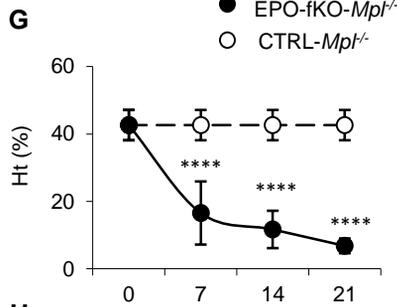
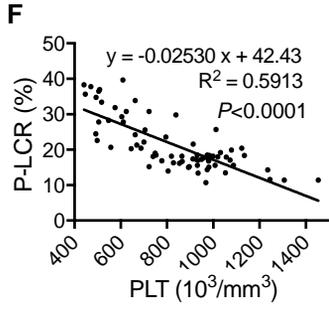
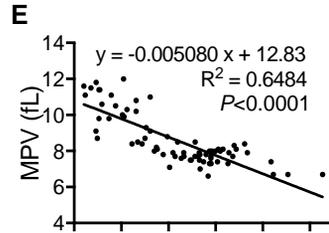
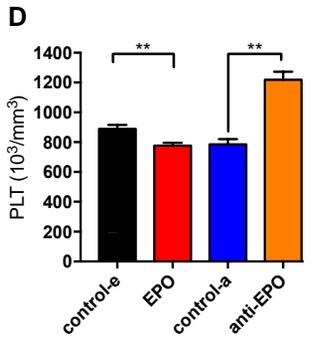
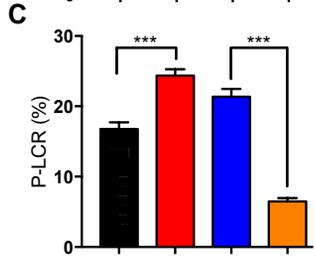
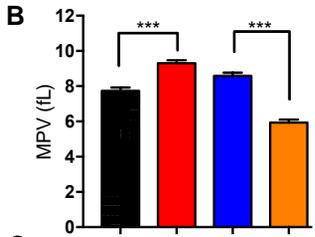
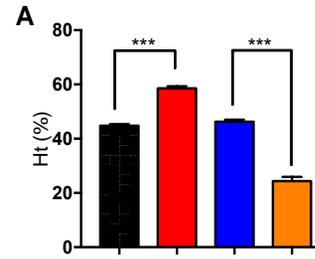


Figure 6

