



LUND UNIVERSITY

Loss of HIF-1 α accelerates murine FLT-3ITD-induced myeloproliferative neoplasia.

Velasco, Talia; Tornero Prieto, Daniel; Cammenga, Jörg

Published in:
Leukemia

DOI:
[10.1038/leu.2015.156](https://doi.org/10.1038/leu.2015.156)

2015

[Link to publication](#)

Citation for published version (APA):

Velasco, T., Tornero Prieto, D., & Cammenga, J. (2015). Loss of HIF-1 α accelerates murine FLT-3ITD-induced myeloproliferative neoplasia. *Leukemia*, 29(12), 2366-2374. <https://doi.org/10.1038/leu.2015.156>

Total number of authors:
3

General rights

Unless other specific re-use rights are stated the following general rights apply:

Copyright and moral rights for the publications made accessible in the public portal are retained by the authors and/or other copyright owners and it is a condition of accessing publications that users recognise and abide by the legal requirements associated with these rights.

- Users may download and print one copy of any publication from the public portal for the purpose of private study or research.
- You may not further distribute the material or use it for any profit-making activity or commercial gain
- You may freely distribute the URL identifying the publication in the public portal

Read more about Creative commons licenses: <https://creativecommons.org/licenses/>

Take down policy

If you believe that this document breaches copyright please contact us providing details, and we will remove access to the work immediately and investigate your claim.

LUND UNIVERSITY

PO Box 117
221 00 Lund
+46 46-222 00 00

1 **Loss of HIF-1 α accelerates murine FLT-3^{ITD}-induced**
2 **myeloproliferative neoplasia**

3

4 Talia Velasco-Hernandez¹, Daniel Tornero² and Jörg Cammenga^{1, 3, 4, 5}

5 ¹Department of Molecular Medicine and Gene Therapy, Lund Stem Cell Center, Lund
6 University, Lund, Sweden; ²Laboratory of Stem Cells and Restorative Neurology, Lund
7 Stem Cell Center, Skanes University Hospital, Lund, Sweden; ³Department of
8 Hematology, Skanes University Hospital, Lund, Sweden, ⁴Department of Hematology,
9 Linköping University Hospital and ⁵Institution for Clinical and Experimental Medicine,
10 Linköping University, Linköping, Sweden.

11

12 **Running title:** HIF-1 α in FLT-3^{ITD} myeloid neoplasia

13 **Key words:** Hypoxia, HIF-1 α , myeloproliferative neoplasm (MPN), *Flt-3*, tumor
14 suppressor gene.

15

16 Correspondence should be addressed to:

17 Jörg Cammenga

18 Department of Molecular Medicine and Gene Therapy

19 Lund Stem Cell Center BMC A12, Sölvegatan 17, 22184 Lund, Sweden

20 e-mail: jorg.cammenga@med.lu.se

21

22 The authors declare no conflict of interests.

23

24

25

26 **Abstract:**

27

28 Hypoxia-induced signaling is important for normal and malignant hematopoiesis. The
29 transcription factor hypoxia-inducible factor-1 α (HIF-1 α) plays a crucial role in
30 quiescence and self-renewal of hematopoietic stem cells (HSCs) as well as leukemia-
31 initiating cells (LICs) of acute myeloid leukemia (AML) and chronic myeloid leukemia
32 (CML). We have investigated the effect of HIF-1 α loss on the phenotype and biology of
33 FLT-3^{ITD}-induced myeloproliferative neoplasm (MPN). Using transgenic mouse models,
34 we show that deletion of HIF-1 α leads to an enhanced MPN phenotype reflected by
35 higher numbers of white blood cells, more severe splenomegaly and decreased
36 survival. The proliferative effect of HIF-1 α loss is cell-intrinsic as shown by
37 transplantation into recipient mice. HSCs loss and organ specific changes in number
38 and percentage of long-term hematopoietic stem cells (LT-HSCs) were the most
39 pronounced effects on a cellular level after HIF-1 α deletion. Furthermore, we found a
40 metabolic hyperactivation of malignant cells in the spleen upon loss of HIF-1 α . Some of
41 our findings are in contrary to what has been previously described for the role of HIF-
42 1 α in other myeloid hematologic malignancies and question the potential of HIF-1 α as
43 a therapeutic target.

44

45

46

47

48

49

50 Introduction:

51 Hypoxia has been proposed to be a physiologic condition in the adult hematopoietic
52 stem cell (HSC) niche and previous publications have provided experimental evidence
53 for this hypothesis¹. Additionally, it has been shown that hypoxia signaling, through
54 hypoxia-inducible factors (HIFs), is required for proper HSCs function, and that this
55 might be mediated by HIF-induced pyruvate dehydrogenase kinase (PDK) and vascular
56 endothelial growth factor (VEGF) expression²⁻⁴. Further dissection of the HIF signaling
57 pathway has indicated that HIF-1 α , but not HIF-2 α , seems to be the major player in
58 HSCs self-renewal⁵. Interestingly, deletion of both members of the HIF family had
59 surprisingly little effect on hematopoiesis in steady state and functional HSC defects
60 were only apparent after serial transplantations⁵.

61 Normally, HIF-1 α activity is regulated on a post-transcriptional, post-translational
62 level by oxygen dependent hydroxylation of two proline residues in the oxygen-
63 dependent domain (ODD) of the HIF-1 α subunit followed by binding of the von Hippel
64 Lindau (VHL) protein and degradation by the ubiquitination pathway⁶. Other
65 mechanisms than oxygen tension might regulate HIF-1 α expression in the HSC niche.
66 Cytokines, like stem cell factor (SCF) and thrombopoetin (TPO) that are highly
67 expressed in the adult bone marrow (BM) niche have been shown to lead to HIF-1 α
68 stabilization^{7,8}. Moreover, the homeobox gene Meis1, highly expressed in HSCs, also
69 stabilizes HIF-1 α contributing to the quiescence of HSCs⁹.

70 Members of the HIF family have been proposed to be crucial for self-renewal of human
71 acute myeloid leukemia-initiating cells (AML-ICs). shRNA expression against HIF-1/2 α
72 in human AMLs showed impaired engraftment in NOD/SCID mice^{10,11}. Whether HIF
73 was a direct target of the genetic alterations in the used AML samples has not been

74 addressed in these studies. On the other hand, Meis1, commonly overexpressed in
75 AML, induces HIF-1 α stabilization, and accordingly, its deletion in transgenic mice
76 leads to HSCs exhaustion due to their inability to up-regulate HIF-1 α ^{9, 12}.
77 Additionally, it has been shown that the t(9;21) fusion protein BCR-ABL signals
78 directly to HIF-1 α leading to its activation¹³. Deletion of HIF-1 α in a murine model of
79 chronic myeloid leukemia (CML) showed that CML-initiating cells (CML-ICs) lacking
80 HIF-1 α failed to generate leukemia in secondary transplanted mice arguing for an
81 important role of HIF-1 α in CML-ICs self-renewal¹⁴.
82 Taking all these data into consideration, HIF-1 α might be a good therapeutic target for
83 different types of leukemia if HSCs and leukemic initiating cells (LICs) had a different
84 requirement for HIF signaling. Since previous studies have used methods (shRNA
85 expression, unspecific inhibitors of HIF in human AML cells or retroviral
86 transduction/transplantation assays)^{10, 11} that could bear potential technical problems
87 in the evaluation of LICs self-renewal, we have tested the requirement of HIF-1 α in
88 myeloproliferative neoplasms (MPN) using a FLT-3^{ITD} transgenic mouse model.
89 Internal tandem duplications (ITD) in the *FLT3* gene are found in approximately 25%
90 of AML cases, constitutively activating this receptor and predicting increased relapse
91 rates and reduced overall survival¹⁵.
92 Here, we show that loss of HIF-1 α leads to an enhanced FLT-3^{ITD}-induced MPN
93 phenotype, indicated by higher numbers of white blood cells (WBC) and myeloid cells,
94 more severe splenomegaly and a shorter survival. The increased proliferation is cell-
95 intrinsic and this phenotype transplantable to primary recipient mice. Our data
96 question the role of HIF-1 α as a target to eliminate LICs in MPN and show that loss of
97 HIF-1 α can aggravate the disease.

98 **Materials and methods:**

99

100 **Transgenic mice**

101 *Hif-1 α* ^{flox/flox} mice¹⁶ were crossed with the interferon-inducible *Mx1-Cre* mice¹⁷ and
102 with the knock-in *Flt-3*^{ITD} mice¹⁸ to generate conditional knock-out *Hif-1 α* ^{flox/flox}; *Mx1-*
103 *Cre*; *Flt-3*^{ITD/+} mice. All animals were bred and maintained in accordance with Lund
104 University's ethical regulations (Ethical permit M86-12).

105

106 **Monitoring of mice and bone marrow transplantation assays**

107 Leukemia development was analyzed by measuring myeloid cells and total WBC in
108 peripheral blood (PB) every 4 weeks. Myeloid cells were analyzed by flow cytometry
109 and WBC counts were determined by a cell counter (KX-21N, Sysmex, Norderstedt
110 Germany).

111 For HSC transplantation, 8 to 12-week-old B6SJL (CD45.1) recipient mice were lethally
112 irradiated with 900 cGy 4-15 hours prior to transplantation. BM *c-kit*⁺ cells from
113 donors (CD45.2) were isolated using the MACS[®] magnetic separation system and anti-
114 *c-kit* magnetic beads (Miltenyi Biotec, Bergisch Gladbach, Germany). 5 x 10⁵ *c-kit*⁺ cells
115 were injected into the tail vein of recipient mice accompanied by 2 x 10⁵ freshly
116 isolated total BM supporting cells from B6SJL x C57BL/6J (CD45.1-CD45.2) mice.
117 Donor chimerism and myeloproliferative development were determined after
118 transplantation by PB analysis every 4 weeks.

119 For the transplantation of the cells kept *in vitro*, *c-kit*⁺ cells were cultured in serum free
120 expansion media (StemCell Technologies, Vancouver, BC, Canada) supplemented with
121 20 ng/mL murine interleukin 3 (PeproTech, Stockholm, Sweden), 50 ng/mL human

122 interleukin 6 (PeproTech), 50 ng/mL human TPO (PeproTech) and 50 ng/mL murine
123 SCF (PeproTech) for 48 hours before transplant.

124 Animals that had to be euthanized due to non-MPN-associated symptoms were
125 excluded from the survival analysis.

126 Deletion of *Hif-1 α* was verified by polymerase chain reaction (PCR) analysis of DNA
127 from BM cells of primary animals using the following primers: *HIF Δ -forward*: 5' -
128 GCAGTTAAGAGCACTAGTTG-3' and *HIF Δ -reverse*: 5' -TTGGGGATGAAAACATCTGC-3'.

129

130 **Flow cytometry analysis**

131 Expansion of the MPN and engraftment of transplanted cells were monitored by flow
132 cytometry analysis of PB, BM and spleen cells. PB samples were lysed with ammonium
133 chloride (StemCell Technologies) prior to staining. 4,6 diamidino-2-phenylindole
134 (DAPI) (Sigma-Aldrich, St. Louis, MO, USA) or 7-amino-actinomycin D (7-AAD, BD
135 Pharmingen, San Diego, CA, USA) was used to exclude dead cells. For chimerism and
136 lineage analysis the following antibodies were used: Gr1-PE, -PECy5 (RB6-8C5), Mac1-
137 PE, -PECy5 (M1/70), B220-PE, -APC, -PECy5 (RA3-6B2), CD3-PE, -PECy5 (145-2C11),
138 Ter119-PECy5 (TER-119), CD45.1-PECy7 (A20), Sca1-BV, -APC (D7), CD48-FITC
139 (HM48-1) and CD150-APC, -PECy7 (TC15-12F12.2) from BioLegend (San Diego, CA,
140 USA) and CD45.2-APCe780 (104) and c-kit-APCe780 (2B8) from eBiosciences (San
141 Diego, CA, USA).

142

143 For cell cycle analysis, cells were fixed in 0.4% formaldehyde (Merck, Darmstadt,
144 Germany) and permeabilized with 0.1% Triton-X (Sigma-Aldrich). Thereafter, cells
145 were stained with Ki-67-PE (B56) antibody (BD Pharmingen) and DAPI (Sigma-
146 Aldrich). Cellular reactive oxygen species (ROS) production was analyzed using

147 CellROX® Deep Red Reagent (Life Technologies, Stockholm, Sweden); mitochondrial
148 ROS production was analyzed using MitoSOX™ Red mitochondrial superoxide
149 indicator (Life Technologies) at 2 μM concentration; mitochondrial activity was
150 evaluated using MitoTracker® Deep Red FM probe (Life Technologies) at 10 nM
151 concentration; apoptosis analysis was performed using the BD Pharmingen™ PE
152 Annexin Apoptosis Detection Kit (BD Pharmingen); all according to manufacturer's
153 instructions. Samples were analyzed using a FACSCantoII (BD Biosciences, Stockholm,
154 Sweden) and data was analyzed with FlowJo software (TreeStar, Ashland, OR, USA).

155

156 **Histology**

157 For morphological analysis, cells from BM and spleen were subjected to cytopspin
158 preparation onto glass slides and PB smears were stained with May-Grünwald (Merck)
159 and Giemsa (Merck). For microscopic examination, an Olympus IX70 microscope and
160 an Olympus DP72 camera were used (Olympus, Tokyo, Japan).

161

162 **Statistical analysis**

163 All data are expressed as the mean ± SEM. Differences between groups were assessed
164 by unpaired two-tailed Student's t-test. Statistical analysis of survival curves was
165 performed using Mantel-Cox log-rank test. All analyses were performed with Prism
166 software version 6.0 (GraphPad Software, San Diego, CA, USA). Animal cohort size was
167 chosen according to the published literature and our previous studies. Since all
168 experiments were performed with mice homogeneous regarding strain and age, no
169 randomization method was used. Since this study did not include objective
170 measurements, no blinding was performed.

171

172 **Results:**

173

174 **Spontaneous deletion of *Hif-1 α* by *Mx1*-Cre in the FLT-3^{ITD} mice**

175 To investigate the role of HIF-1 α in FLT-3^{ITD}-induced MPN and self-renewal of LICs, *Flt-*
176 *3*^{ITD} knock-in mice were crossed with *Hif-1 α* conditional knock-out mice and *Mx1*-Cre
177 mice to obtain *Flt-3*^{ITD/+}; *Hif-1 α* ^{flox/flox}; *Mx1*-Cre mice and *Flt-3*^{ITD/+}; *Hif-1 α* ^{flox/flox} control
178 mice (refer hereafter as *Hif-1 α* ^{Δ/Δ} and *Hif-1 α* ^{+/+}, respectively). The phenotype of the
179 FLT-3^{ITD}-induced MPN mouse model resembles human chronic myelomonocytic
180 leukemia (CMML), with an expansion of the myeloid/monocytic compartment¹⁸. We
181 intended to delete the *Hif-1 α* gene using poly(deoxyinosinic/deoxycytidylic) acid
182 (pIpC) for induction of the Cre recombinase under the control of the *Mx1* promoter.
183 However, investigation of untreated mice showed that recombination had occurred
184 spontaneously, probably due to activation of signaling pathways downstream of the
185 *Flt3* receptor, triggering an interferon response and leading to the expression of Cre
186 recombinase (Figure 1a and Supplementary Figure S1). Due to the very high
187 spontaneous deletion frequency of the floxed HIF-1 α gene none of the mice were
188 treated with pIpC.

189

190 **FLT-3^{ITD}-induced MPN is aggravated by the loss of HIF-1 α**

191 Primary *Hif-1 α* ^{Δ/Δ} mice surprisingly showed a more severe FLT-3^{ITD} MPN phenotype
192 than *Hif-1 α* ^{+/+} animals. While control mice suffered from a chronic MPN that mice
193 normally did not succumb to, *Hif-1 α* ^{Δ/Δ} mice started to die at week 26 of age, reaching
194 50% of survival at week 68 (Figure 1b). This enhanced MPN phenotype was also
195 reflected in higher WBC counts (Figure 1c) and a higher percentage of Gr1⁺/Mac1⁺

196 cells (myeloid cells) in PB at 8, 12, 16 and 20 weeks of age (Figure 1d-e and
197 Supplementary Figure S2). Blood smears also showed more mature granulocytes in
198 *Hif-1 α ^{$\Delta\Delta$}* mice (Figure 1f).

199 20-week-old mice were sacrificed to analyze other MPN symptoms. Bones from mice
200 lacking HIF-1 α presented pale aspect (femurs, tibiae and hips) (Figure 2a), probably
201 due to a combination of anemia (Table 1) and infiltration of myeloid cells in the BM.
202 FLT-3^{ITD}-induced MPN in the absence of HIF-1 α was characterized by a more severe
203 splenomegaly and hepatomegaly indicated by higher spleen and liver weights (Figure
204 2a-b) in *Hif-1 α ^{$\Delta\Delta$}* mice. Percentages of Gr1⁺/Mac1⁺ cells in BM and spleen were also
205 increased upon loss of HIF-1 α (Figure 2c-d). Taken together, our data indicate that loss
206 of HIF-1 α in an FLT-3^{ITD}-induced MPN model accelerates disease progression and
207 aggravates its severity.

208

209 **Loss of HIF-1 α affects cell cycle status of FLT-3^{ITD}-induced MPN cells**

210 To investigate whether higher number of myeloid cells in PB, BM and spleen was due
211 to increased proliferation or decreased apoptosis, we first investigated cell cycle status
212 in malignant myeloid cells from these tissues. Cell cycle analysis revealed that there
213 was a higher percentage of cycling cells in spleen (G2/S/M phase) of *Hif-1 α ^{$\Delta\Delta$}* mice
214 compared to controls (Figure 2e and Supplementary Figure S3). Interestingly we
215 observed no differences in BM cells from mice with different HIF-1 α status. Overall
216 these data indicate that loss of HIF-1 α in FLT-3^{ITD}-induced MPN results in an increased
217 number of Gr1⁺/Mac1⁺ myeloid cells by enhance proliferation in spleen as shown by a
218 higher percentage of cycling cells, even when expressing maturation surface markers.

219 When studying cell death of these neoplastic myeloid cells, we observed that loss of
220 HIF-1 α leads to a decrease in apoptosis both in BM and spleen (Figure 2f-g).

221 Our data point to a dual role of HIF-1 α loss in the observed phenotype by increasing
222 the percentage of cells in active cycling and at the same time decreasing apoptosis
223 resulting in an overall more severe MPN phenotype.

224

225 **Loss of HIF-1 α leads to an organ-specific change in stem and progenitor cell**
226 **numbers**

227 It has been previously shown that *Flt-3^{ITD}/+* mice present an expansion of multi-potent
228 progenitor cells (MPPs) and a severe decrease in long-term hematopoietic stem cells
229 (LT-HSCs) using either CD48/CD150 (SLAM) or FLT-3/CD34 staining of Lin⁻, Sca-1⁺, c-
230 Kit⁺ (LSK) cells^{15, 19, 20}. Having shown that deletion of *Hif-1 α* leads to an enhanced FLT-
231 3^{ITD}-induced MPN we wanted to further characterize at which level of the
232 hematopoietic hierarchy the effects occur. For this reason we analyzed and
233 enumerated different hematopoietic stem and progenitor populations using staining
234 for LSK and SLAM markers. Since expression of FLT-3^{ITD} leads to an expansion of
235 mature granulocytes in BM resulting in a change of cellular composition, both,
236 percentages and total numbers of cells were analyzed (Figure 3a-c and Supplementary
237 Figure S4). We observed the previously described reduction of LT-HSCs (LSK CD48-
238 CD150⁺) in FLT-3^{ITD} expressing mice compared to wild type (wt) mice, but even to a
239 bigger extent when *Hif-1 α* was deleted. When comparing BM cells from *Hif-1 α ^{Δ/Δ}* mice
240 to controls, we found an expansion of the more mature compartment (LK cells: Lin⁻ c-
241 Kit⁺ Sca1⁻) and a progressive decrease towards LT-HSCs, through the different levels
242 of differentiation (LSK and MPPs (defined as LSK CD48- CD150⁻)). In spleen, the

243 scenario was different, showing an increment (although non-significant in some cases)
244 of cells of all these different undifferentiated populations in the *Hif-1 α ^{Δ/Δ}* mice.
245 When analyzing cell cycle status of these populations, we only observed higher
246 percentage of cycling cells (G1-G2 phase) in *Hif-1 α ^{Δ/Δ}* mice in the LSK population in
247 spleen (Figure 3d and Supplementary Figure S5). These results, together with data
248 presented in Figure 2e, indicate that the production of malignant cells in the *Hif-1 α ^{Δ/Δ}*
249 mice occurs mainly in the spleen through proliferation of committed progenitors.
250 Thus, the lost of HIF-1 α changes the phenotype of FLT-3^{ITD} malignancies in terms of
251 proportions and location of primitive cells.

252

253 **The effect of HIF-1 α loss on FLT-3^{ITD}-induced MPN is cell-intrinsic**

254 To investigate whether the aggravation of the FLT-3^{ITD}-induced MPN by HIF-1 α loss is
255 a cell intrinsic effect or a result mediated by cells in the microenvironment, and if this
256 loss results in a defect in LICs homeostasis and self-renewal, transplantation assays
257 were performed. To this end, c-kit⁺ BM cells from the different genotypes were
258 transplanted into lethally irradiated recipient wt mice.

259 The disease was transplantable and additionally, the acceleration of the MPN
260 phenotype by loss of HIF-1 α was also observed in transplanted mice with *Hif-1 α ^{Δ/Δ}*
261 cells, arguing for a cell-intrinsic effect of HIF-1 α on FLT-3^{ITD}-induced MPN (Figure 4a
262 and Supplementary Figure S6). However, the reduction in LT-HSCs observed in
263 transgenic animals (Supplementary Figure S6f) is more severe in transplanted mice
264 and accordingly, secondary recipients showed a loss of *Hif-1 α ^{Δ/Δ}* donor contribution to
265 PB (Supplementary Figure S7). The fact that *Hif-1 α ^{Δ/Δ}* FLT-3^{ITD} MPN was transplantable
266 into primary recipients indicates that loss of HIF-1 α did not result in a defect in LICs

267 engraftment, although the numbers of LT-HSCs are severely affected in primary
268 recipients. Whether the effect in the secondary recipients is due to a loss of LIC self-
269 renewal or a displacement of LT-HSC by expanding MPPs in the bone marrow remains
270 elusive.

271 Previous studies, investigating the role of HIF-1 α in CML, have used a retroviral
272 transduction/transplantation model that requires cycling of the hematopoietic stem
273 and progenitor cells (HSPCs), which is normally induced by *in vitro* culturing in the
274 presence of cytokines. To test whether these experimental differences could explain
275 the conflicting results between our experiments and previously published data, we
276 cultured c-kit⁺ BM cells in presence of cytokines, as normally performed when using
277 the transduction/transplantation method, and transplanted afterwards. Mice
278 transplanted with *in vitro* cultured *Hif-1 $\alpha^{\Delta\Delta}$* BM cells showed, not only, a similar
279 phenotype regarding the expansion of myeloid cells over time, but also a shorter
280 survival ($P=0.0046$)(Figure 4b and Supplementary Figure S8a-b). Mice transplanted
281 with *Hif-1 $\alpha^{\Delta\Delta}$* cultured BM cells started to die of progressive MPN around week 20
282 with most of the animals dead by week 40, while no animal died in the control group.

283 To evaluate the level of competition in our transplantation experiments, we calculated
284 the number of LT-HSCs injected in each group. According to the obtained values in
285 Figure 3 of c-kit⁺ cells and LT-HSCs in wt, *Hif-1 $\alpha^{+/+}$* and *Hif-1 $\alpha^{\Delta\Delta}$* animals, we estimated
286 that the ratio of competitor:donor cells was 1:3 for *Hif-1 $\alpha^{\Delta\Delta}$* and 1:65 for *Hif-1 $\alpha^{+/+}$* cells
287 respectively. Beside the higher competition in the *Hif-1 $\alpha^{\Delta\Delta}$* group, we observed similar
288 levels of donor contribution to the myeloid compartment 20 weeks post-
289 transplantation (Supplementary Figure S8c). To analyze donor contribution to the
290 other cell lineages discarding the effect of the percentage variation when one

291 population is highly increased, we calculated donor contribution to each lineage in
292 total number of cells. We observed equal contribution to the T cells from *Hif-1 α ^{+/+}* and
293 *Hif-1 α ^{$\Delta\Delta$}* animals. However, we observed more contribution from *Hif-1 α ^{$\Delta\Delta$}* donor cells
294 to myeloid and less to B cell lineage most likely due to the MPN phenotype resulting in
295 an expansion of myeloid cells at the expense of B-cells (Supplementary Figure S8d).

296 In summary, our data indicate that FLT-3^{ITD}-induced MPN is cell-intrinsic and
297 transplantable, independently of HIF-1 α status. Self-renewal of LICs was not lost even
298 when cells were cultured *in vitro* for 48 hours prior to primary transplantations,
299 although LT-HSCs numbers are highly reduced in recipient mice transplanted with *Hif-*
300 *1 α ^{$\Delta\Delta$}* .

301

302 **HIF-1 α status influences mitochondrial activity and ROS levels in FLT-3^{ITD}-** 303 **induced MPN**

304 Mitochondrial respiratory chain constitutes the main intracellular source of ROS in
305 most of the tissues. Because HIF-1 α status influences the metabolism of cells³, which
306 could affect the malignant properties of the FLT-3^{ITD} cells, we examined mitochondrial
307 membrane function and levels of ROS of these neoplastic myeloid cells. Two different
308 tests showed an increment of cellular and mitochondrial ROS levels in *Hif-1 α ^{$\Delta\Delta$}* mice in
309 BM and an opposite effect in spleen (Figure 5a-b).

310 According to mitochondrial membrane function, we found three well-defined different
311 populations, named as M1, M2 and M3 (Figure 5c). Whereas in BM there was an
312 increase in the population with less mitochondrial activity (M1) when *Hif-1 α* is
313 deleted, we observed a reduction of this population in the splenic myeloid cells. This

314 could be indicating that high ROS production is a result of higher mitochondrial
315 function caused by higher metabolic activity of *Hif-1 α ^{$\Delta\Delta$} cells.*

316 Together this data could indicate a metabolic adaptation of these malignant cells to
317 their new niches and an improvement of their tumorigenic capacities (less quiescence
318 and more proliferation) when HIF-1 α is lost in these cells.

319

320 **Discussion:**

321 Hypoxia signaling, mainly mediated by transcription factors HIF-1 α and HIF-2 α and
322 their target genes, has been shown to play an important role in stem cell biology,
323 particularly in normal and malignant HSPCs. Previous work has provided evidence that
324 HIF-1 α is required for HSCs quiescence and self-renewal² as well as HIF-2 α has a role
325 in protecting HSCs from endoplasmic reticulum stress-induced apoptosis¹¹.
326 Surprisingly, loss of HIF-1 α function (and combined loss of HIF-1 α and HIF-2 α) seems
327 rather weak since the phenotype comes only apparent after challenging HSCs by serial
328 transplantation^{2, 5}.

329 Additionally, it has been demonstrated that HIF might play a role in murine and human
330 leukemia. Inhibitors and shRNA against HIF-1 α and HIF-2 α have been used to show
331 the requirement of these two transcription factors in human AML-ICs self-renewal and
332 ability to induce AML in immune-compromised mice^{10, 11}.

333 The concept to target HIF in AML is intriguing but whether there is a therapeutic
334 window for the treatment of leukemia by targeting HIF-1/2 α without inducing major
335 hematologic toxicity has not been extensively studied. It also remains elusive whether
336 HIF can function as a therapeutic target for all genetic subtypes of AML. It has been
337 shown that some genetic alterations in AML stabilize HIF in a hypoxia-independent

338 manner, making these specific subtypes maybe prime targets for therapy against HIF.
339 Requirement of HIF-1 α for CML-ICs self-renewal has been confirmed using retroviral
340 overexpression of BCR-ABL oncogene in BM cells from HIF-1 α conditional knock-out
341 mice¹⁴. All these approaches have some technical caveats that could influence the
342 viability of LICs. First, inhibitors of HIF-1 α are rather unselective making it difficult to
343 evaluate whether their effect is primarily caused by HIF-1 α inhibition. Expression of
344 shRNA against HIF family members requires retroviral transduction of AML-LICs and
345 might have off-target effects even though scrambled shRNA was used as control.
346 Retroviral transduction of BM cells requires cytokine stimulation of HSPCs *in vitro*,
347 which can change the properties of these cells. Therefore, we have investigated the role
348 of HIF-1 α in FLT-3^{ITD}-induced MPN using just transgenic mouse models for both
349 genetic alterations.

350 The first unexpected result was a spontaneous deletion of the floxed *Hif-1 α* gene in *Flt-*
351 *3^{ITD}*; *Mx1*-Cre background. We assume that FLT-3^{ITD} signaling triggers an interferon
352 response that leads to activation of Cre recombinase via activation of *Mx1* promoter.
353 Our finding is in accordance with the published literature^{20, 21} in which mice with
354 either *Runx1^{flox/flox}* or *Npm1^{flox-cA/+}* in combination with a *Flt-3^{ITD}* and *Mx1*-Cre genotype
355 develop AML spontaneously.

356 In contrary to the previously described role of HIFs in LICs of AML and CML, we found
357 that HIF-1 α loss exaggerates FLT-3^{ITD}-induced MPN phenotype, as indicated by a
358 shorter survival, higher number of myeloid cells in PB, BM and spleen, leading to a
359 more severe splenomegaly.

360 It has been previously shown that *Flt-3^{ITD}* knock-in mice have an expansion of the
361 myeloid progenitor compartment while the LT-HSC population was severely

362 decreased^{15, 19, 20}. We observed that this effect is even more aggravated when *Hif-1 α* is
363 deleted. Surprisingly, reduction of LT-HSCs was not associated with a dramatic loss of
364 LICs homeostasis, since the MPN could be transplanted into primary recipient animals
365 even after *in vitro* incubation. Interestingly, transplanted MPNs lacking HIF-1 α showed
366 an even more aggressive phenotype as indicated by a shorter survival of transplanted
367 mice. Whether the reduction in LT-HSCs in the bone marrow of primary recipients and
368 the associated low contribution of *Hif-1 α ^{$\Delta\Delta$}* cells in the secondary recipients is due to a
369 defect in self-renewal or a displacement by progenitors and mature myeloid cells
370 needs further investigation.

371 Hypoxia-induced HIF expression has been linked to metabolic switch due to a shift
372 from oxidative phosphorylation (OXPHOS) to glycolysis, an effect that has been first
373 described by Warburg and carries his name²². Tumors that become hypoxic heavily
374 depend on this mechanism but even tumors that are not hypoxic switch to
375 energetically very inefficient glycolysis for reasons that still remain elusive. Therefore,
376 we investigated the metabolic profile (mitochondrial function and ROS levels) of FLT-
377 3^{ITD}-MPN cells lacking HIF-1 α . It has been described that changes in these parameters
378 can affect self-renewal and differentiation of HSCs. For instance, when mitochondrial
379 potential is blocked, HSCs are unable to initiate differentiation²³ and high levels of ROS
380 force cells to go out of quiescence^{24, 25}. We found a correlation between cycling profile,
381 ROS levels and mitochondrial function in FLT-3^{ITD}-MPN cells (Figure 5d), indicating a
382 more active status of *Hif-1 α ^{$\Delta\Delta$}* cells in spleen, the main (malignant) hematopoietic
383 organ in animals with MPN and leading to a more aggressive disease.

384 The fact that, in animals with the MPN, hematopoiesis is taking place in an
385 extramedullary niche (spleen), with very different microenvironmental properties

386 from the BM, including oxygen tension, lead us to postulate that these observed
387 differences are established by cell-intrinsic mechanisms that stabilize HIF-1 α ,
388 imprinted already in the primary niche (BM) or by originating genetic alterations of
389 the malignancy.

390 These results are in concordance with our recently findings regarding the role of HIF-
391 1 α in AML pathogenesis using oncogenes that either do or do not signal directly
392 towards HIF-1 α . Expression of *MLL-AF9* and *MEIS1/HOXA9*, that are supposedly
393 activating HIF-1 α , and *AML/ETO9a*, a truncated version of the AML1/ETO fusion
394 protein with no known connection to HIF-1 α , induced AML independent of HIF-1 α
395 status²⁶. HIF-1 α was not needed for LIC self-renewal, but loss of HIF-1 α rather lead to
396 an accelerated and more severe phenotype, similar to the observations made for the
397 FLT-3^{ITD}-induced MPN model described in this paper.

398 Based on previous studies, it has been proposed that HIF might be used as molecular
399 therapeutic target to interfere with self-renewal of LICs. Our data indicate that
400 targeting HIF-1 α in FLT-3^{ITD}-induced MPN rather leads to disease acceleration and a
401 more severe phenotype. Whether the inhibition of HIF in combination with
402 chemotherapy or targeted small molecules can be a useful therapeutic strategy needs
403 further investigation.

404

405 **Conflict of Interest**

406 The authors declare no conflict of interest.

407

408

409

410 Acknowledgements

411 The authors thank Dr. D. Gary Gilliland (University of Pennsylvania, Philadelphia, USA)
412 for kindly providing *Flt-3^{ITD}* knock-in mice and Dr. David Bryder (Lund University,
413 Lund, Sweden) for helpful discussions and advice on flow cytometry.

414 This work was supported by project grants from Barncancerfonden, Cancerfonden and
415 SSF through the Hemato-Linné and StemTherapy consortium (Sweden) to J.C. and a
416 post-doctoral grant from Barncancerfonden (Sweden) to T.V.-H.

417

418 Author's contributions

419 T.V.-H. designed the research, performed experiments, analyzed data, created figures,
420 and wrote the manuscript; D.T. performed experiments; J.C. conceived and supervised
421 the project, designed the research, and wrote the manuscript. All authors read and
422 approved the final manuscript.

423

424

425

426

427

428

429

430

431

432

433

434

435 **References**

- 436 1. Nilsson SK, Johnston HM, Coverdale JA. Spatial localization of transplanted
437 hemopoietic stem cells: inferences for the localization of stem cell niches. *Blood*
438 2001 Apr 15; **97**(8): 2293-2299.
- 439
- 440 2. Takubo K, Goda N, Yamada W, Iriuchishima H, Ikeda E, Kubota Y, *et al*.
441 Regulation of the HIF-1alpha level is essential for hematopoietic stem cells. *Cell*
442 *stem cell* 2010 Sep 3; **7**(3): 391-402.
- 443
- 444 3. Takubo K, Nagamatsu G, Kobayashi CI, Nakamura-Ishizu A, Kobayashi H,
445 Ikeda E, *et al*. Regulation of glycolysis by Pdk functions as a metabolic
446 checkpoint for cell cycle quiescence in hematopoietic stem cells. *Cell stem cell*
447 2013 Jan 3; **12**(1): 49-61.
- 448
- 449 4. Rehn M, Olsson A, Reckzeh K, Diffner E, Carmeliet P, Landberg G, *et al*.
450 Hypoxic induction of vascular endothelial growth factor regulates murine
451 hematopoietic stem cell function in the low-oxygenic niche. *Blood* 2011 Aug 11;
452 **118**(6): 1534-1543.
- 453
- 454 5. Guitart AV, Subramani C, Armesilla-Diaz A, Smith G, Sepulveda C, Gezer D,
455 *et al*. Hif-2alpha is not essential for cell-autonomous hematopoietic stem cell
456 maintenance. *Blood* 2013 Sep 5; **122**(10): 1741-1745.
- 457
- 458 6. Semenza GL. Hypoxia-inducible factors in physiology and medicine. *Cell*
459 2012 Feb 3; **148**(3): 399-408.

460

461 7. Pedersen M, Lofstedt T, Sun J, Holmquist-Mengelbier L, Pahlman S,
462 Ronnstrand L. Stem cell factor induces HIF-1alpha at normoxia in hematopoietic
463 cells. *Biochemical and biophysical research communications* 2008 Dec 5; **377**(1):
464 98-103.

465

466 8. Kirito K, Fox N, Komatsu N, Kaushansky K. Thrombopoietin enhances
467 expression of vascular endothelial growth factor (VEGF) in primitive
468 hematopoietic cells through induction of HIF-1alpha. *Blood* 2005 Jun 1;
469 **105**(11): 4258-4263.

470

471 9. Simsek T, Kocabas F, Zheng J, Deberardinis RJ, Mahmoud AI, Olson EN, *et*
472 *al.* The distinct metabolic profile of hematopoietic stem cells reflects their
473 location in a hypoxic niche. *Cell stem cell* 2010 Sep 3; **7**(3): 380-390.

474

475 10. Wang Y, Liu Y, Malek SN, Zheng P, Yang L. Targeting HIF1alpha eliminates
476 cancer stem cells in hematological malignancies. *Cell stem cell* 2011 Apr 8; **8**(4):
477 399-411.

478

479 11. Rouault-Pierre K, Lopez-Onieva L, Foster K, Anjos-Afonso F, Lamrissi-
480 Garcia I, Serrano-Sanchez M, *et al.* HIF-2alpha protects human hematopoietic
481 stem/progenitors and acute myeloid leukemic cells from apoptosis induced by
482 endoplasmic reticulum stress. *Cell stem cell* 2013 Nov 7; **13**(5): 549-563.

483

- 484 12. Hisa T, Spence SE, Rachel RA, Fujita M, Nakamura T, Ward JM, *et al.*
485 Hematopoietic, angiogenic and eye defects in Meis1 mutant animals. *The EMBO*
486 *journal* 2004 Jan 28; **23**(2): 450-459.
- 487
- 488 13. Mayerhofer M, Valent P, Sperr WR, Griffin JD, Sillaber C. BCR/ABL induces
489 expression of vascular endothelial growth factor and its transcriptional
490 activator, hypoxia inducible factor-1alpha, through a pathway involving
491 phosphoinositide 3-kinase and the mammalian target of rapamycin. *Blood* 2002
492 Nov 15; **100**(10): 3767-3775.
- 493
- 494 14. Zhang H, Li H, Xi HS, Li S. HIF1alpha is required for survival maintenance
495 of chronic myeloid leukemia stem cells. *Blood* 2012 Mar 15; **119**(11): 2595-
496 2607.
- 497
- 498 15. Chu SH, Heiser D, Li L, Kaplan I, Collector M, Huso D, *et al.* FLT3-ITD
499 knockin impairs hematopoietic stem cell quiescence/homeostasis, leading to
500 myeloproliferative neoplasm. *Cell stem cell* 2012 Sep 7; **11**(3): 346-358.
- 501
- 502 16. Ryan HE, Poloni M, McNulty W, Elson D, Gassmann M, Arbeit JM, *et al.*
503 Hypoxia-inducible factor-1alpha is a positive factor in solid tumor growth.
504 *Cancer research* 2000 Aug 1; **60**(15): 4010-4015.
- 505
- 506 17. Kuhn R, Schwenk F, Aguet M, Rajewsky K. Inducible gene targeting in
507 mice. *Science* 1995 Sep 8; **269**(5229): 1427-1429.
- 508

- 509 18. Lee BH, Tothova Z, Levine RL, Anderson K, Buza-Vidas N, Cullen DE, *et al.*
510 FLT3 mutations confer enhanced proliferation and survival properties to
511 multipotent progenitors in a murine model of chronic myelomonocytic
512 leukemia. *Cancer cell* 2007 Oct; **12**(4): 367-380.
513
- 514 19. Li L, Piloto O, Nguyen HB, Greenberg K, Takamiya K, Racke F, *et al.* Knock-
515 in of an internal tandem duplication mutation into murine FLT3 confers
516 myeloproliferative disease in a mouse model. *Blood* 2008 Apr 1; **111**(7): 3849-
517 3858.
518
- 519 20. Mead AJ, Kharazi S, Atkinson D, Macaulay I, Pecquet C, Loughran S, *et al.*
520 FLT3-ITDs instruct a myeloid differentiation and transformation bias in
521 lymphomyeloid multipotent progenitors. *Cell reports* 2013 Jun 27; **3**(6): 1766-
522 1776.
523
- 524 21. Mupo A, Celani L, Dovey O, Cooper JL, Grove C, Rad R, *et al.* A powerful
525 molecular synergy between mutant Nucleophosmin and Flt3-ITD drives acute
526 myeloid leukemia in mice. *Leukemia* 2013 Sep; **27**(9): 1917-1920.
527
- 528 22. Denko NC. Hypoxia, HIF1 and glucose metabolism in the solid tumour.
529 *Nature reviews Cancer* 2008 Sep; **8**(9): 705-713.
530
- 531 23. Yu WM, Liu X, Shen J, Jovanovic O, Pohl EE, Gerson SL, *et al.* Metabolic
532 regulation by the mitochondrial phosphatase PTPMT1 is required for
533 hematopoietic stem cell differentiation. *Cell stem cell* 2013 Jan 3; **12**(1): 62-74.

534

535 24. Tothova Z, Gilliland DG. FoxO transcription factors and stem cell
536 homeostasis: insights from the hematopoietic system. *Cell stem cell* 2007 Aug
537 16; **1**(2): 140-152.

538

539 25. Ito K, Suda T. Metabolic requirements for the maintenance of self-
540 renewing stem cells. *Nature reviews Molecular cell biology* 2014 Apr; **15**(4):
541 243-256.

542

543 26. Velasco-Hernandez T, Hyrenius-Wittsten A, Rehn M, Bryder D, Cammenga
544 J. HIF-1alpha can act as a tumor suppressor gene in murine acute myeloid
545 leukemia. *Blood* 2014 Dec 4; **124**(24): 3597-3607.

546

547

548

549

550

551

552

553

554

555

556

557

558

559 **Figure legends**

560

561 **Figure 1. Loss of HIF-1 α accelerates FLT-3^{ITD}-induced MPN phenotype.**

562

563 (a) Deletion of *Hif-1 α* was checked by PCR amplification of DNA extracted from BM
 564 cells of the analyzed mice. Shown is a representative gel indicating deletion of *Hif-1 α* in
 565 non-treated *Hif-1 α ^{fllox/fllox}; Mx1-Cre; Flt-3^{ITD/+}* mice. (b) Kaplan-Meier survival curve of
 566 FLT-3^{ITD} mice (*Hif-1 α ^{Δ/Δ}* , n= 10; *Hif-1 α ^{+/+}*, n= 14). Log-rank (Mantel-Cox) test was used
 567 to assess statistical significance. (c, d) Blood analysis of mice at different ages, showing
 568 increased WBC (c) and myeloid cells (Gr1⁺/Mac1⁺ cells) (d) in *Hif-1 α ^{Δ/Δ}* mice (*Hif-*
 569 *1 α ^{+/+}*, n=23; *Hif-1 α ^{Δ/Δ}* , n=18; wt, n=6). (e) Representative FACS plots of PB cells of 12-
 570 week-old mice, showing an increased myeloid population in PB of *Hif-1 α ^{Δ/Δ}* mice.
 571 Differentiated populations are stained with the following antibodies: CD3 for T cells
 572 (T), B220 for B cells (B) and Gr1/Mac1 for myeloid cells (M). (f) Representative blood
 573 smears of 12-week-old mice of both genotypes. Scale bar= 10 μ m.
 574 Plots represent mean \pm SEM. Unless otherwise stated, 2-tailed Student *t* test was used
 575 to assess statistical significance. **P*<0.05, ***P*<0.01, ****P*<0.001. wt = wild type.

576

577 **Figure 2. Accumulation of *Hif-1 α ^{Δ/Δ}* -mature myeloid cells in spleen is due to**
 578 **increased cycling and reduced cell death.**

579

580 (a) Representative phenotype of bones and spleens from 20-week-old mice with the
 581 indicated genotypes. (b) Increment in spleen and liver weight in *Hif-1 α ^{Δ/Δ}* 20-week-
 582 old-mice as a consequence of accelerated MPN in these mice (n=12; wt, n=6). (c)

583 Percentage of mature myeloid cells (Gr1⁺/Mac1⁺) in BM and spleen of 20-week-old
584 mice (n=12; wt, n=6). **(d)** Cytospins of BM cells with different genotypes showing the
585 mature myeloid aspect of predominant cells in this compartment. Scale bar= 10µm. **(e)**
586 Cell-cycle analysis of myeloid cells (Gr1⁺/Mac1⁺) from 3 independent experiments (16
587 to 20-week-old mice) (n= 12; wt, n=6). **(f)** Apoptosis analysis of myeloid cells
588 (Gr1⁺/Mac1⁺) from 2 independent experiments (20-week-old mice) (wt, n=6; *Hif-1α*^{+/+},
589 n=7; *Hif-1α*^{Δ/Δ}, n=8). Plots represent mean ± SEM. Two-tailed Student *t* test was used to
590 assess statistical significance. **P*<0.05, ***P*<0.01, ****P*<0.001

591

592 **Figure 3. LT-HSCs are highly reduced in BM and expanded in spleen of *Hif-1α*^{Δ/Δ}**
593 **mice with FLT-3^{ITD}-induced MPN.**

594

595 **(a)** Percentage of the indicated populations of undifferentiated cells from total BM or
596 spleen cells (wt, n=5; *Hif-1α*^{+/+}, n=12; *Hif-1α*^{Δ/Δ}, n=13). **(b)** Absolute number of cells
597 from the indicated population of undifferentiated cells in the BM (6 bones: 2 femurs, 2
598 tibiae and 2 hips) or spleen (wt, n=5; *Hif-1α*^{+/+}, n=8; *Hif-1α*^{Δ/Δ}, n=7). **(c)** Representative
599 FACS plots of BM samples from both genotypes showing the gating strategy used for
600 the analysis of LT-HSCs, MPPs, LSK and LK cells. First shown plots derived from a
601 previous gating of singlets, alive, lineage negative cells. **(d)** Cell-cycle analysis of the
602 indicated populations of undifferentiated cells from BM or spleen (20-week-old mice)
603 (n= 3; wt, n=6). Plots represent mean ± SEM. Two-tailed Student *t* test was used to
604 assess statistical significance. **P*<0.05, ***P*<0.01, ****P*<0.001

605

606

607

608 **Figure 4. *Hif-1 α* -deleted-FLT-3^{ITD} MPN-initiating cells are able to engraft and**
609 **recapitulate the disease in recipient mice.**

610

611 (a) BM cells from a single donor were transplanted, without previous culturing *in vitro*,
612 into 2-3 lethally irradiated wt mice. We show the phenotype of transplanted disease by
613 several parameters: Kaplan-Meier survival curve, WBCs and myeloid (Gr1⁺/Mac1⁺)
614 cells in PB at the indicated time points after transplantation (*Hif-1 α* ^{+/+}, n=9; *Hif-1 α* ^{Δ/Δ} ,
615 n=7). (b) BM cells from 3 different donors of each genotype were pooled together, kept
616 in culture for 2 days and transplanted into 7 lethally irradiated wt mice. We show the
617 phenotype of transplanted disease by several parameters: Kaplan-Meier survival
618 curve, WBCs and myeloid (Gr1⁺/Mac1⁺) cells in PB at the indicated time points after
619 transplantation (n=7). Log-rank (Mantel-Cox) test was used to assess statistical
620 significance of the survival curve. Plots represent mean \pm SEM. Unless otherwise
621 stated, 2-tailed Student *t* test was used to assess statistical significance. **P*<0.05,
622 ***P*<0.01, ****P*<0.001

623

624 **Figure 5. Malignant infiltrating cells in spleen present higher metabolic profile**
625 **and oxidative stress.**

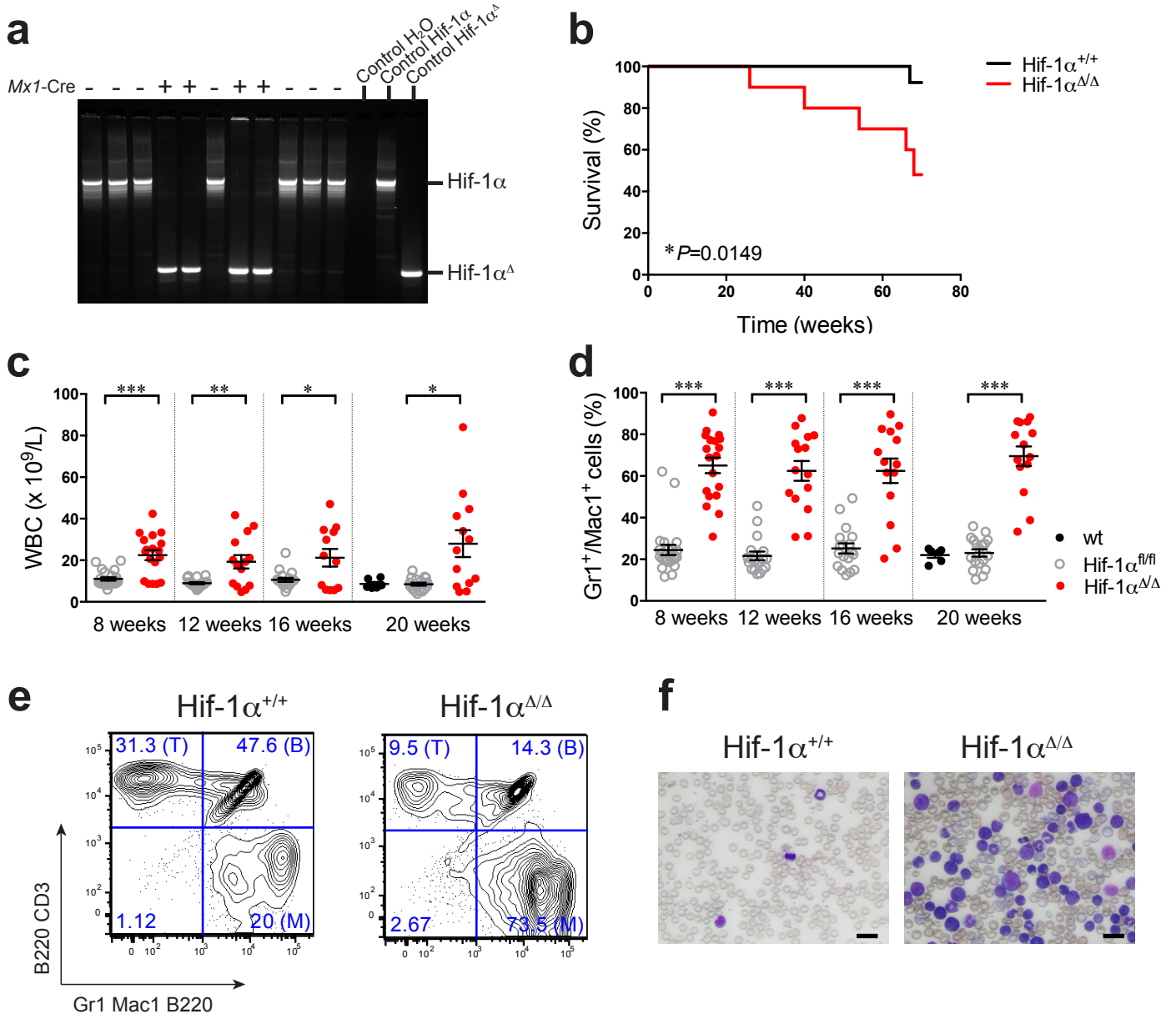
626

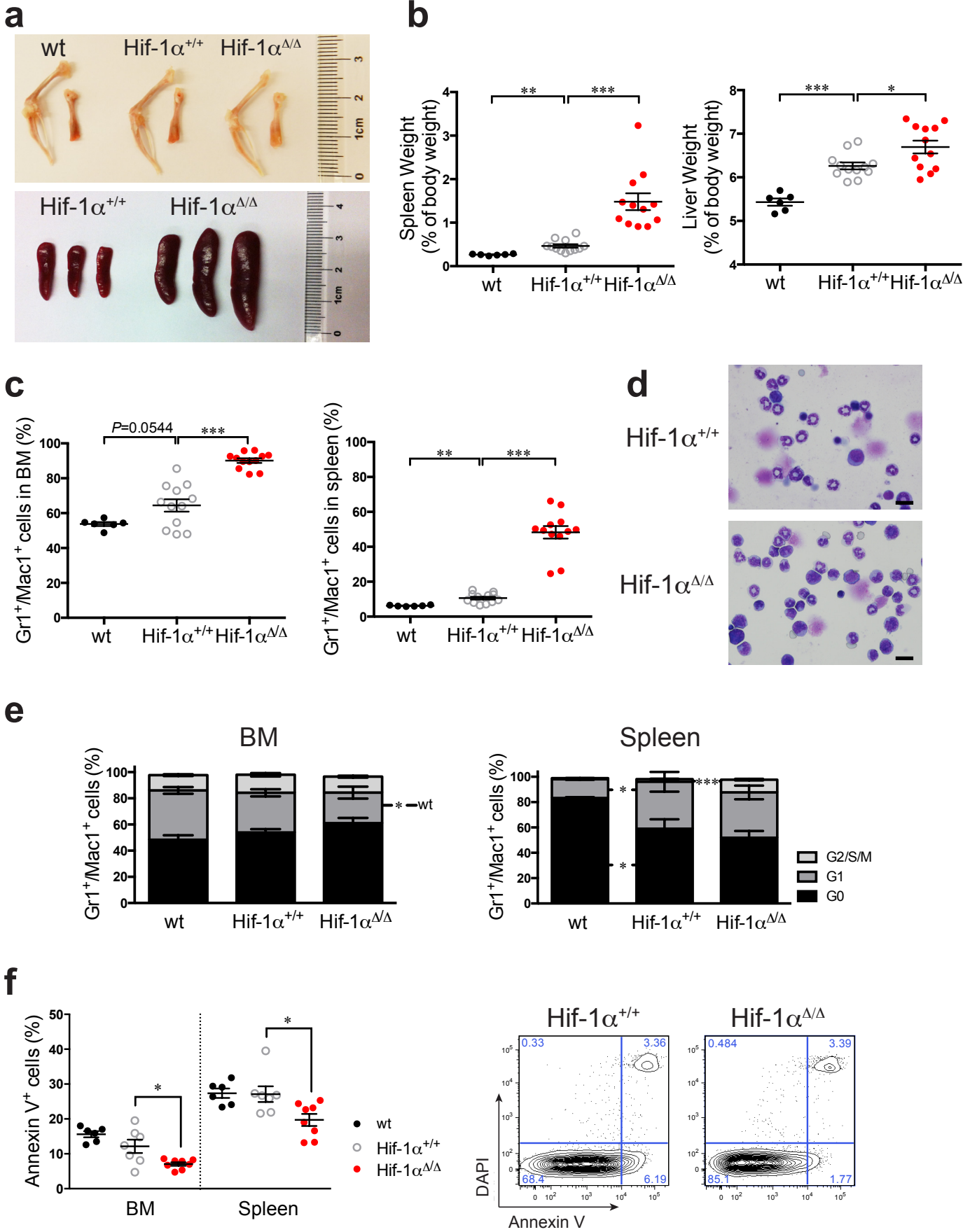
627 We analyzed cellular levels of ROS (a), measured by CellROX Deep Red staining (n=16),
628 and mitochondrial levels of ROS (b) measured by MitoSOX staining (*Hif-1 α* ^{+/+}, n=7; *Hif-*
629 *1 α* ^{Δ/Δ} , n=8) in myeloid cells (Gr1⁺/Mac1⁺) of BM and spleen. Plots represent
630 normalized MFI respect to controls (mean MFI set at 100%) of each experiment (2-4

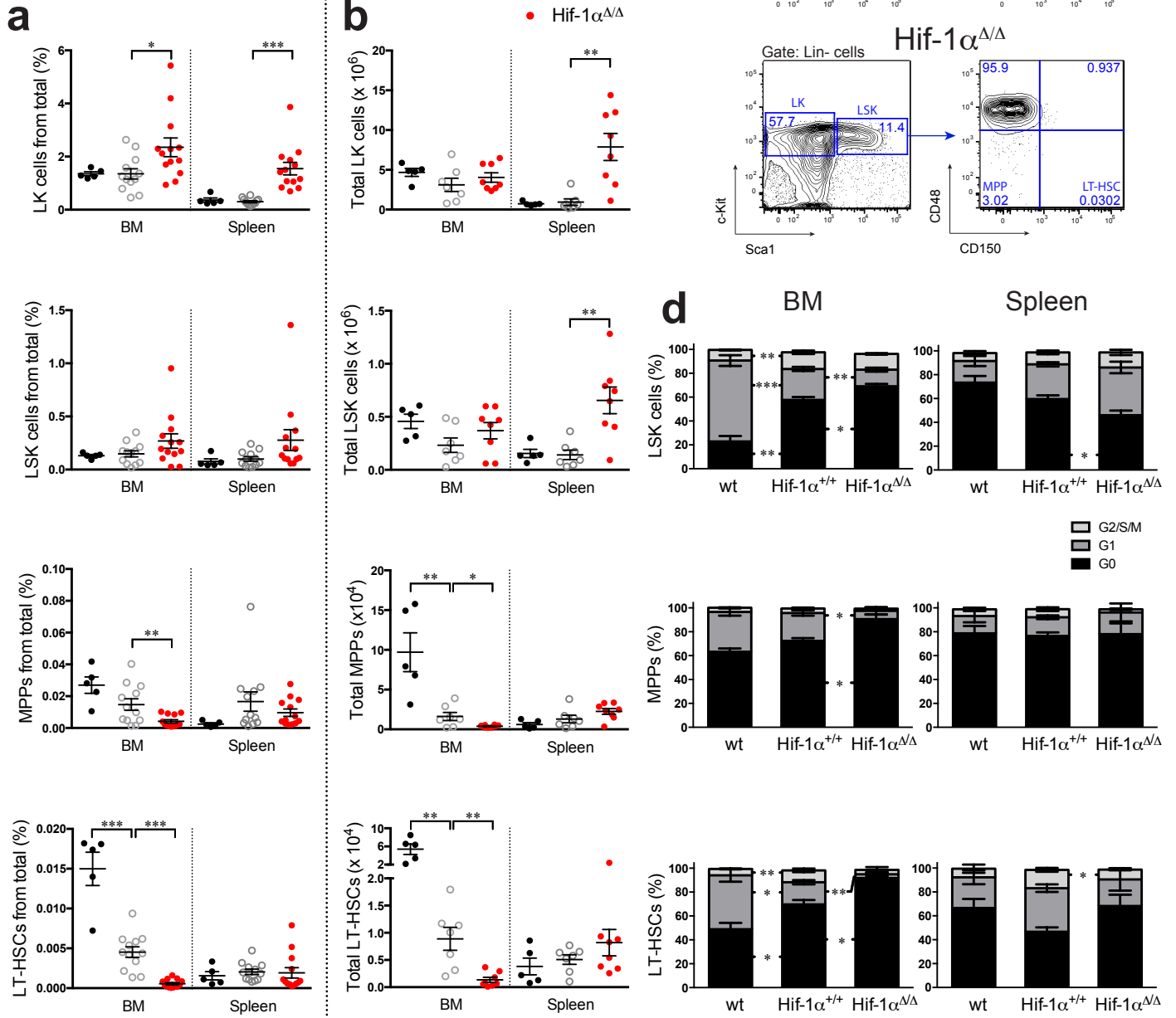
631 independent experiments; 20-week-old mice). (c) Mitochondrial membrane function,
632 measured by MitoTracker Deep Red staining (*Hif-1 α ^{+/+}*, n=8; *Hif-1 α ^{Δ/Δ}* , n=9), in
633 myeloid cells (Gr1⁺/Mac1⁺) of BM and spleen. We observed 3 different populations
634 according to MitoTracker staining that we named M1, M2 and M3 from lower to higher
635 intensity. We determined the percentage of myeloid cells in each of these populations
636 (3 independent experiments; 20-week-old mice). (d) Summary of the metabolic profile
637 of *Hif-1 α ^{Δ/Δ}* and *Hif-1 α ^{+/+}* myeloid cells in the BM and spleen. Dashed lines indicate
638 statistically non significant data. Plots represent mean \pm SEM. Two-tailed Student *t* test
639 was used to assess statistical significance. **P*<0.05, ***P*<0.01, ****P*<0.001

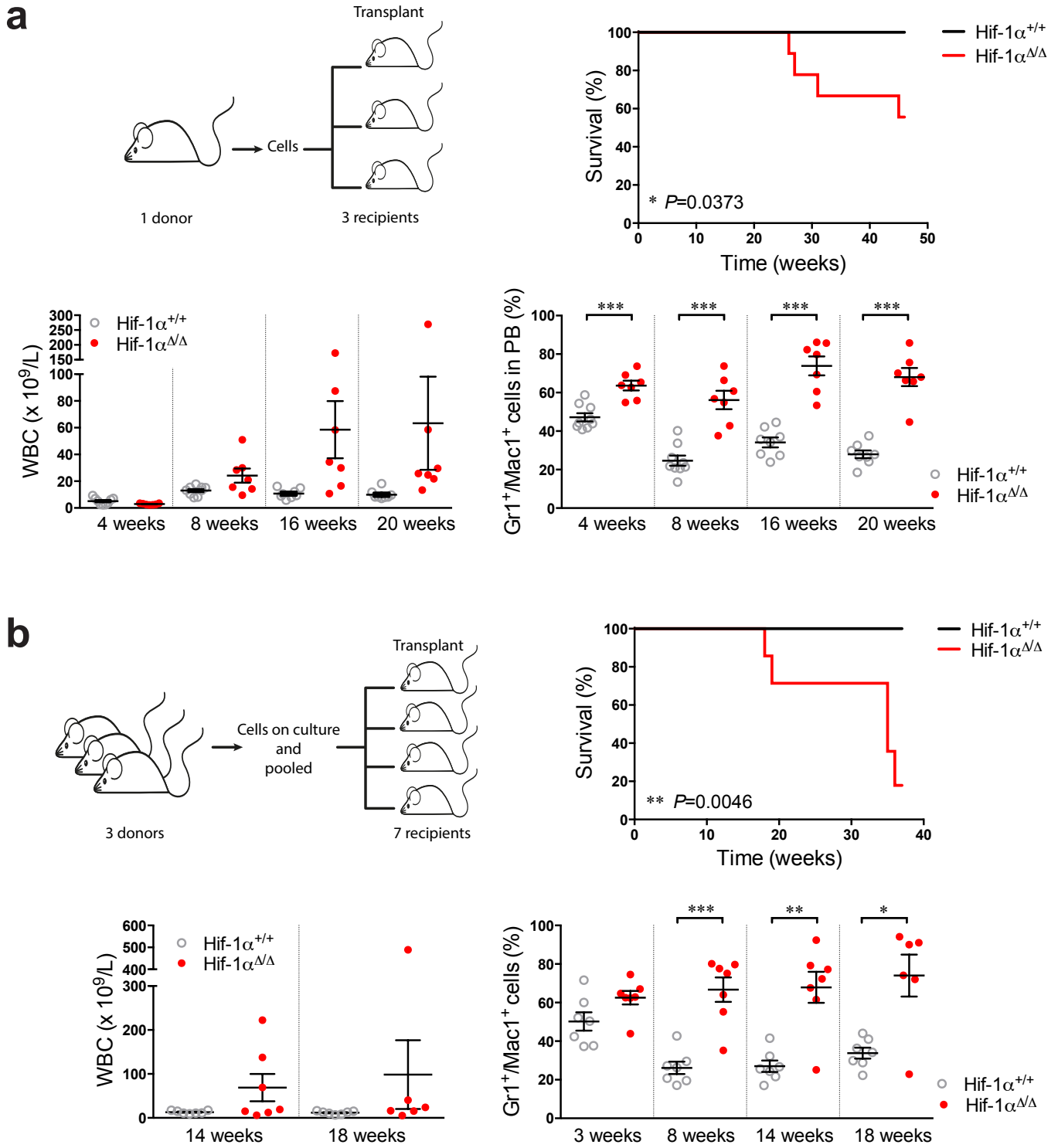
640

641









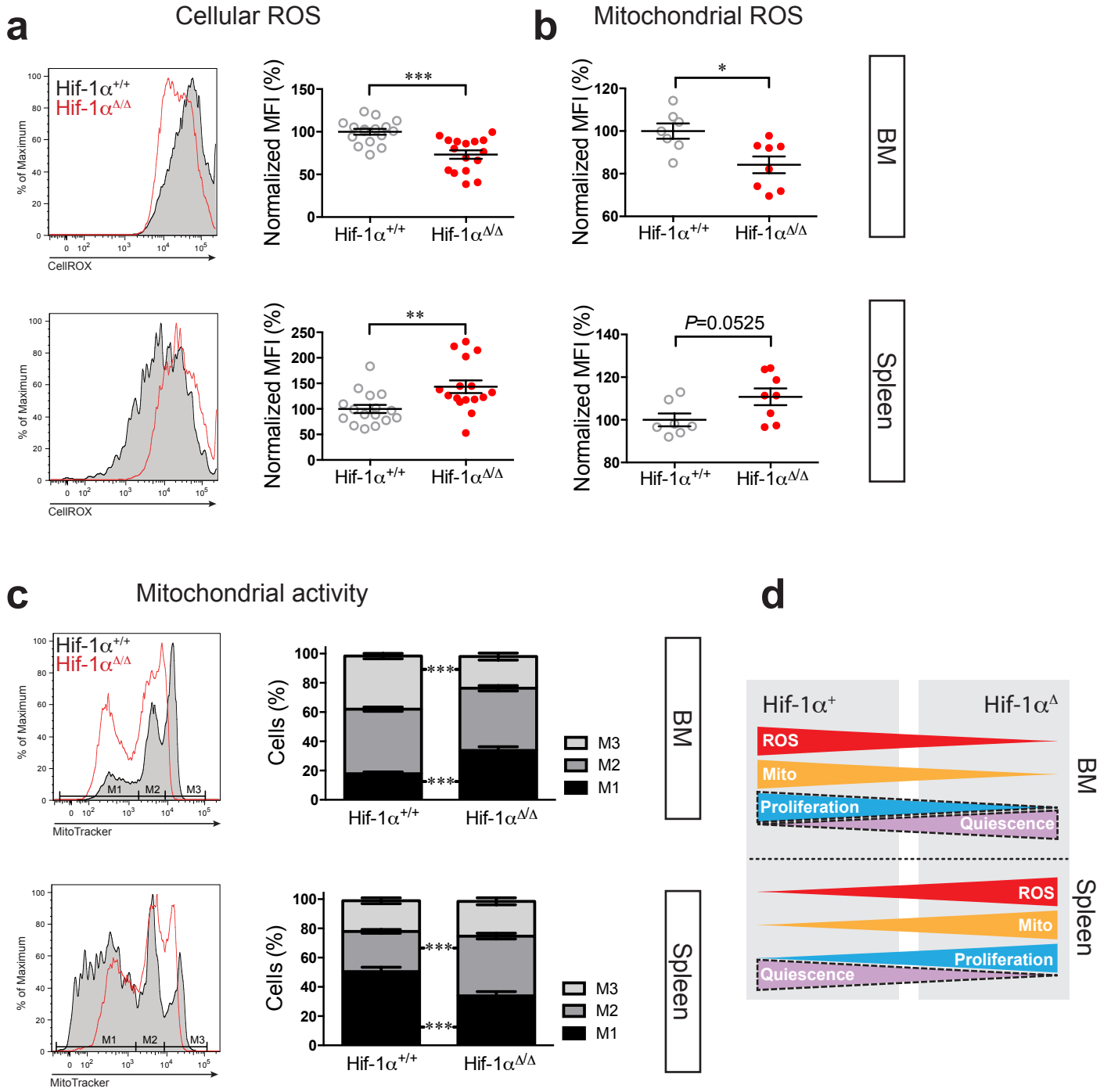


Table 1. Peripheral blood counts

Parameter	wt mice	<i>Hif-1α^{+/+}</i> mice	<i>Hif-1α^{$\Delta\Delta$}</i> mice	<i>P</i> -value (+/ Δ)
RBC (x10¹²/L)	10.04 \pm 0.11	9.275 \pm 0.19	7.707 \pm 0.39	0.0007 ***
HGB (g/L)	148.7 \pm 1.59	142.8 \pm 2.44	137.3 \pm 3.68	0.2069
HCT	0.5158 \pm 0.006	0.4795 \pm 0.009	0.4507 \pm 0.014	0.0835
Platelets (x10⁹/L)	1140 \pm 61.11	1093 \pm 93.76	880.0 \pm 127.2	0.1829

RBC: red blood cells; HGB: hemoglobin; HCT: hematocrit. Wt: n=6, *Hif-1 α ^{+/+}*: n=13, *Hif-1 α ^{$\Delta\Delta$}* : n=9. Values represent mean \pm SEM. *P*-values are calculated between *Hif-1 α ^{+/+}* and *Hif-1 α ^{$\Delta\Delta$}* groups.

Supplementary Information

Loss of HIF-1 α accelerates murine FLT-3^{ITD}-induced myeloproliferative neoplasia

Talia Velasco-Hernandez¹, Daniel Tornero² and Jörg Cammenga^{1, 3, 4, 5}

¹Department of Molecular Medicine and Gene Therapy, Lund Stem Cell Center, Lund University, Lund, Sweden; ²Laboratory of Stem Cells and Restorative Neurology, Lund Stem Cell Center, Skanes University Hospital, Lund, Sweden; ³Department of Hematology, Skanes University Hospital, Lund, Sweden, ⁴Department of Hematology, Linköping University Hospital and ⁵Institution for Clinical and Experimental Medicine, Linköping University, Linköping, Sweden.

Correspondence should be addressed to:

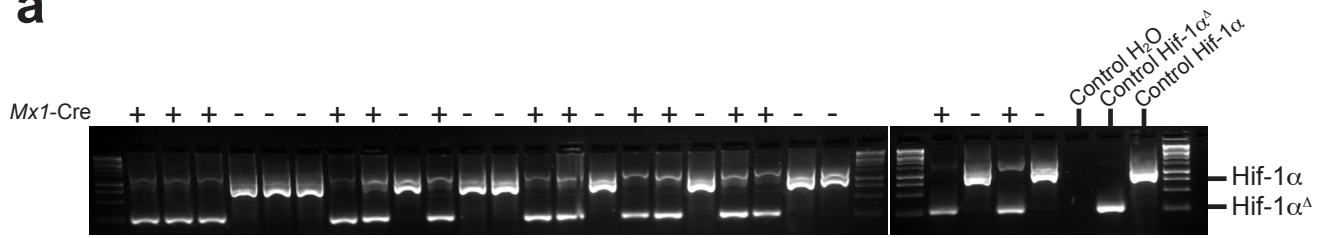
Jörg Cammenga

Department of Molecular Medicine and Gene Therapy

Lund Stem Cell Center BMC A12, Sölvegatan 17, 22184 Lund, Sweden

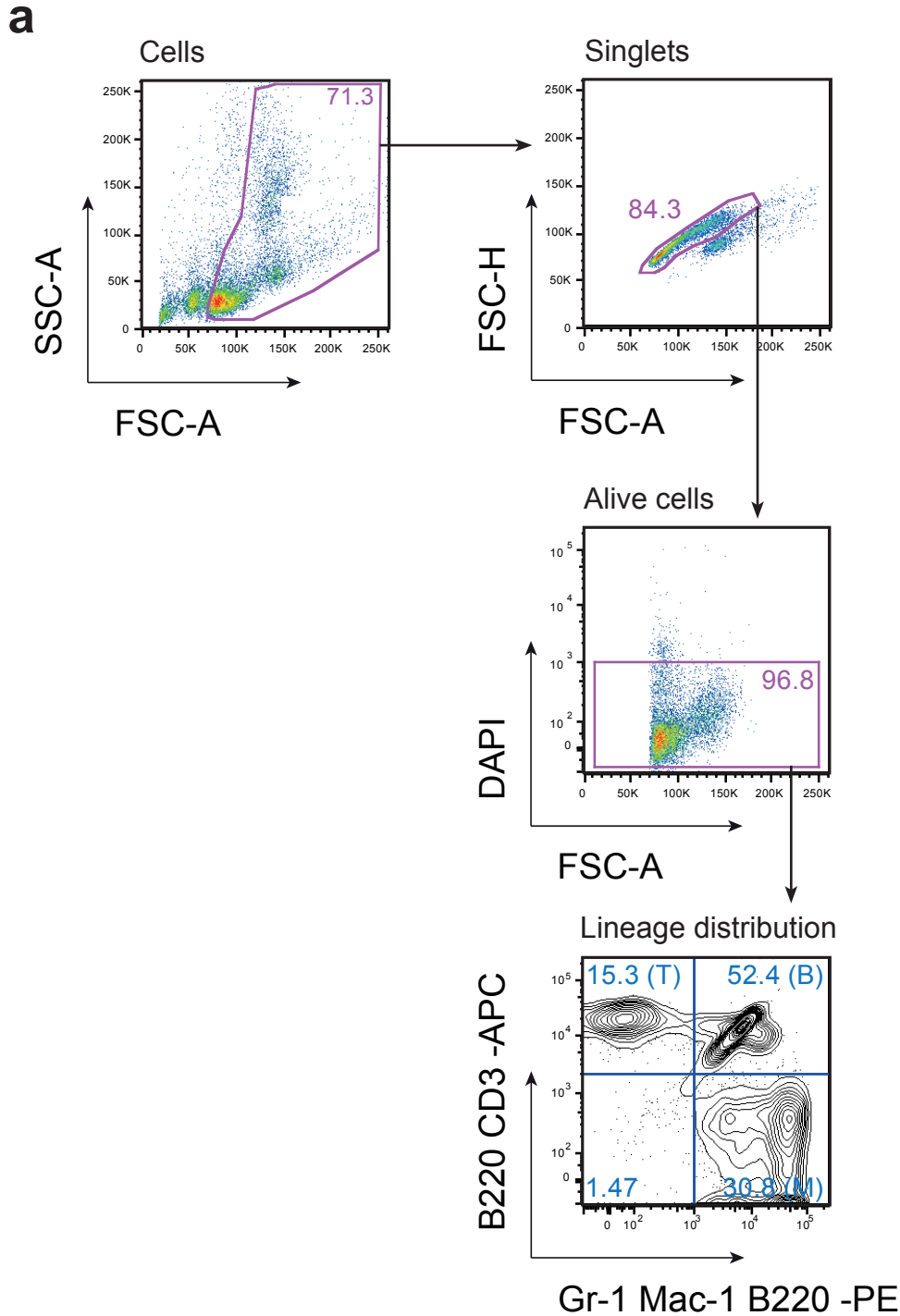
e-mail: jorg.cammenga@med.lu.se

a



Supplementary Figure S1. Spontaneous deletion of *Hif-1α*.

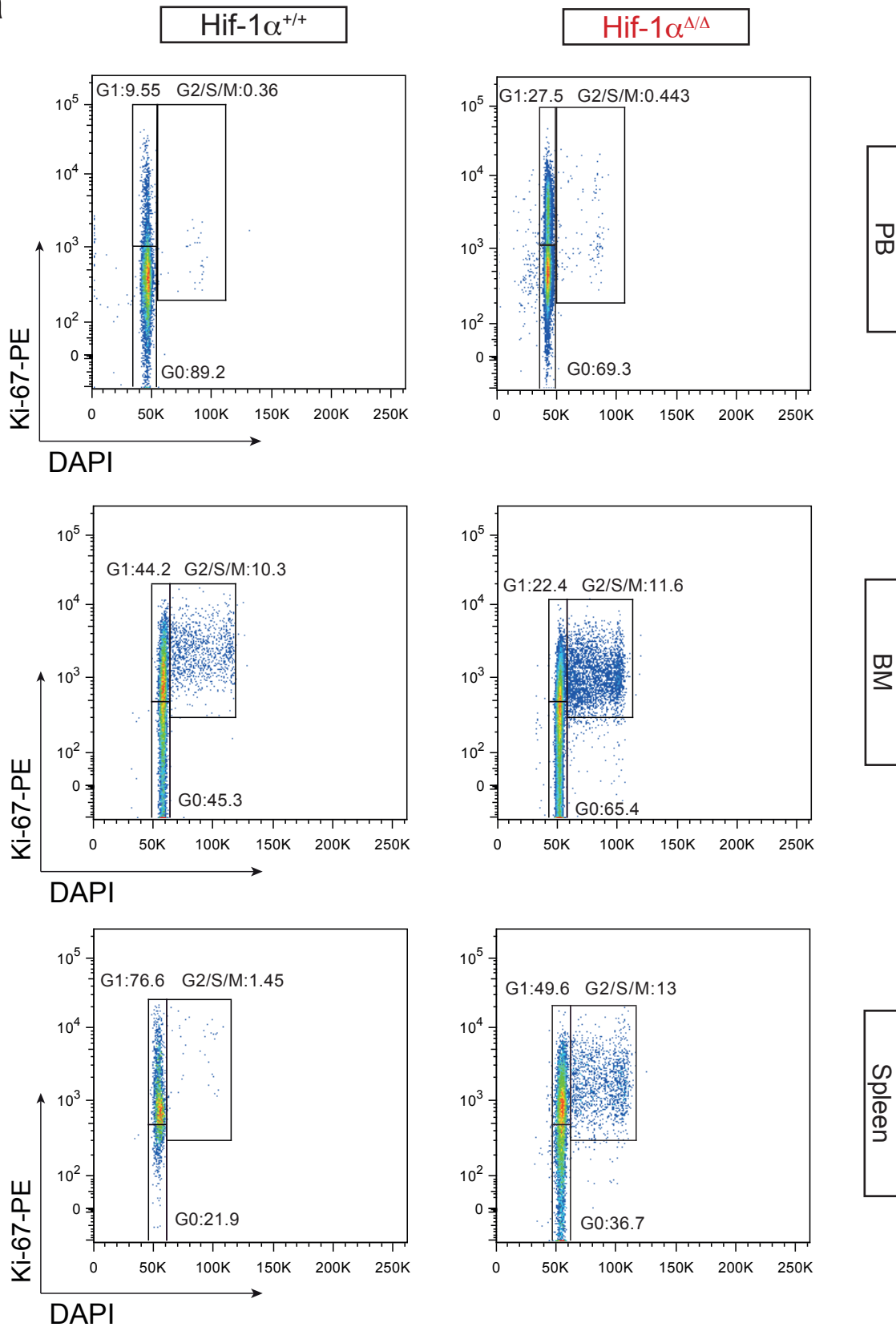
(a) Deletion of Hif-1^α was checked by PCR amplification of the DNA extracted from BM cells of the mice used in this manuscript. All 14 additionally genotyped mice showed deletion of the HIF-1^α gene, providing evidence that spontaneous recombination is a general phenomenon in these mice.



Supplementary Figure S2. Gating strategy used for the identification of the different cell lineages.

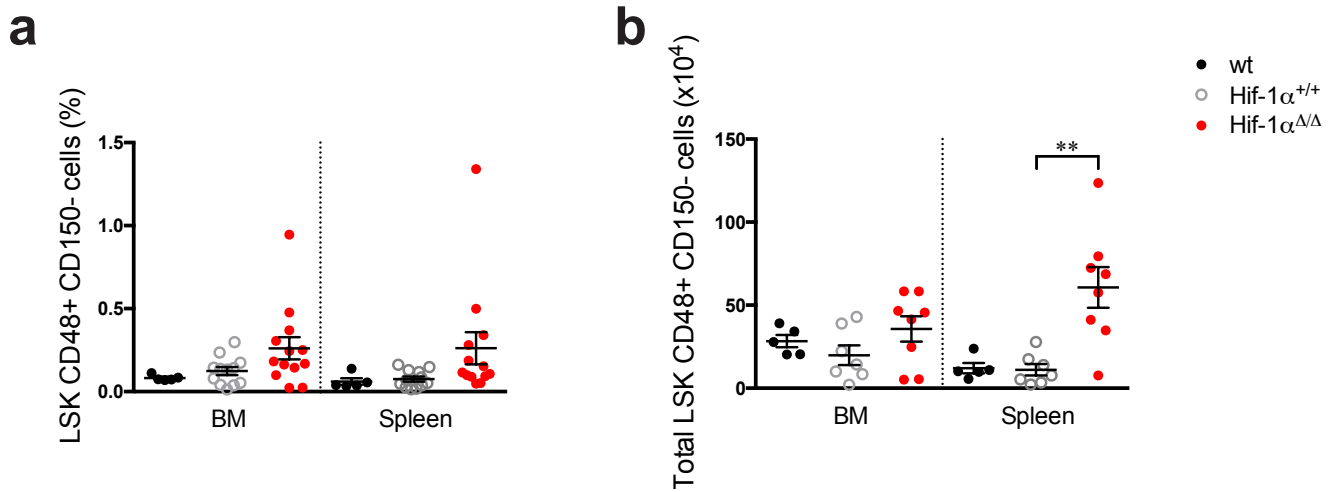
(a) For the identification of the specific lineage populations, we gated entire cells using FSC-A/SSC-A, singlets according to FSC-A/FSC-H, alive cells discarding the DAPI positive cells and finally a combination of Gr1-PE, Mac1-PE, B220-PE, B220-APC and CD3-APC. PE⁺ cells are myeloid cells (M) (Gr1⁺/Mac1⁺), PE⁺APC⁺ are B cells (B) (B220⁺) and APC⁺ are T cells (T) (CD3⁺).

a



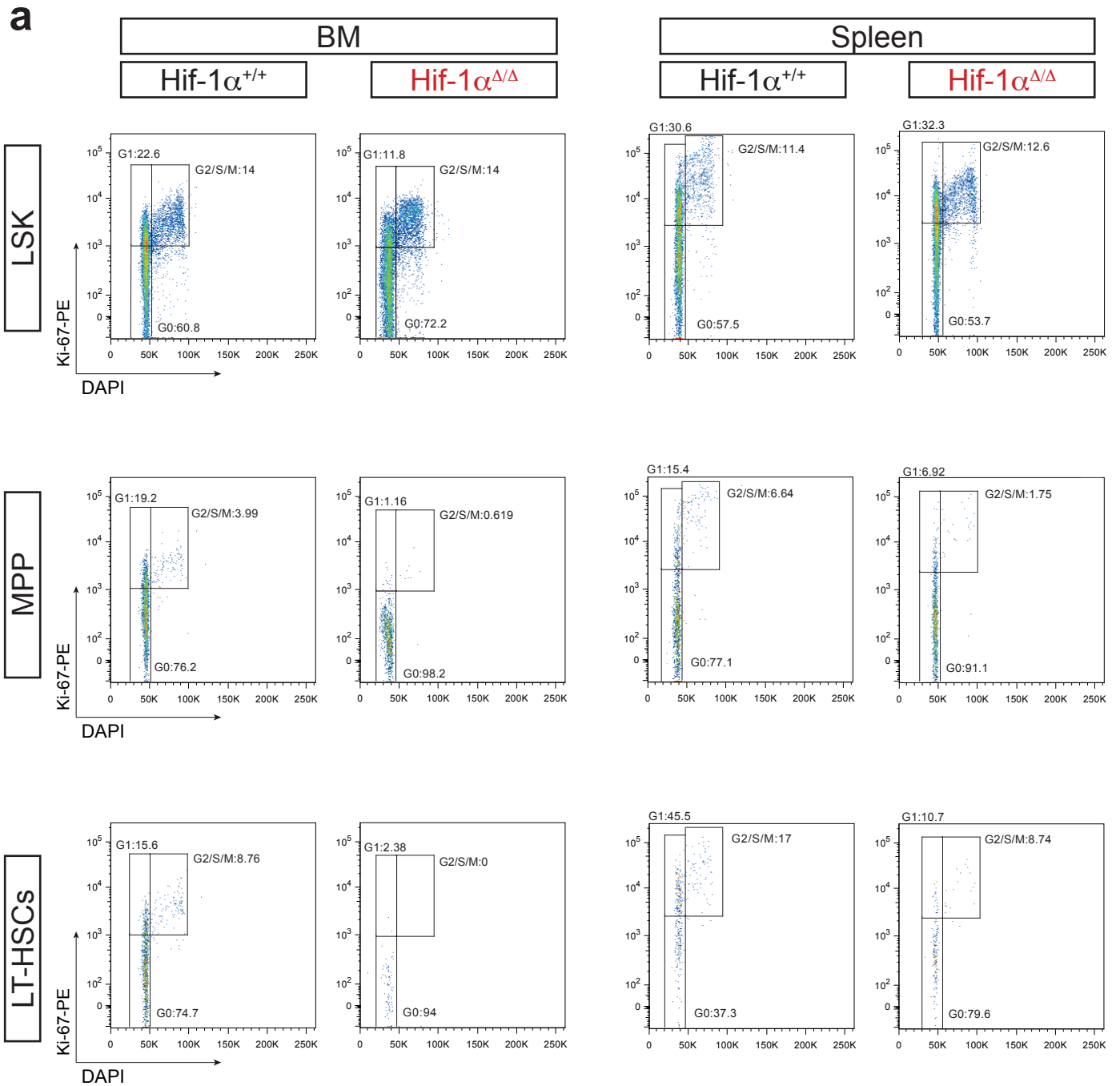
Supplementary Figure S3. Cell-cycle analysis of myeloid cells.

(a) Representative plots of the cell-cycle analysis of myeloid cells ($Gr1^+/Mac1^+$) located in BM and spleen of $HIF-1\alpha^{\Delta/\Delta}$ and $HIF-1\alpha^{+/+}$ mice .



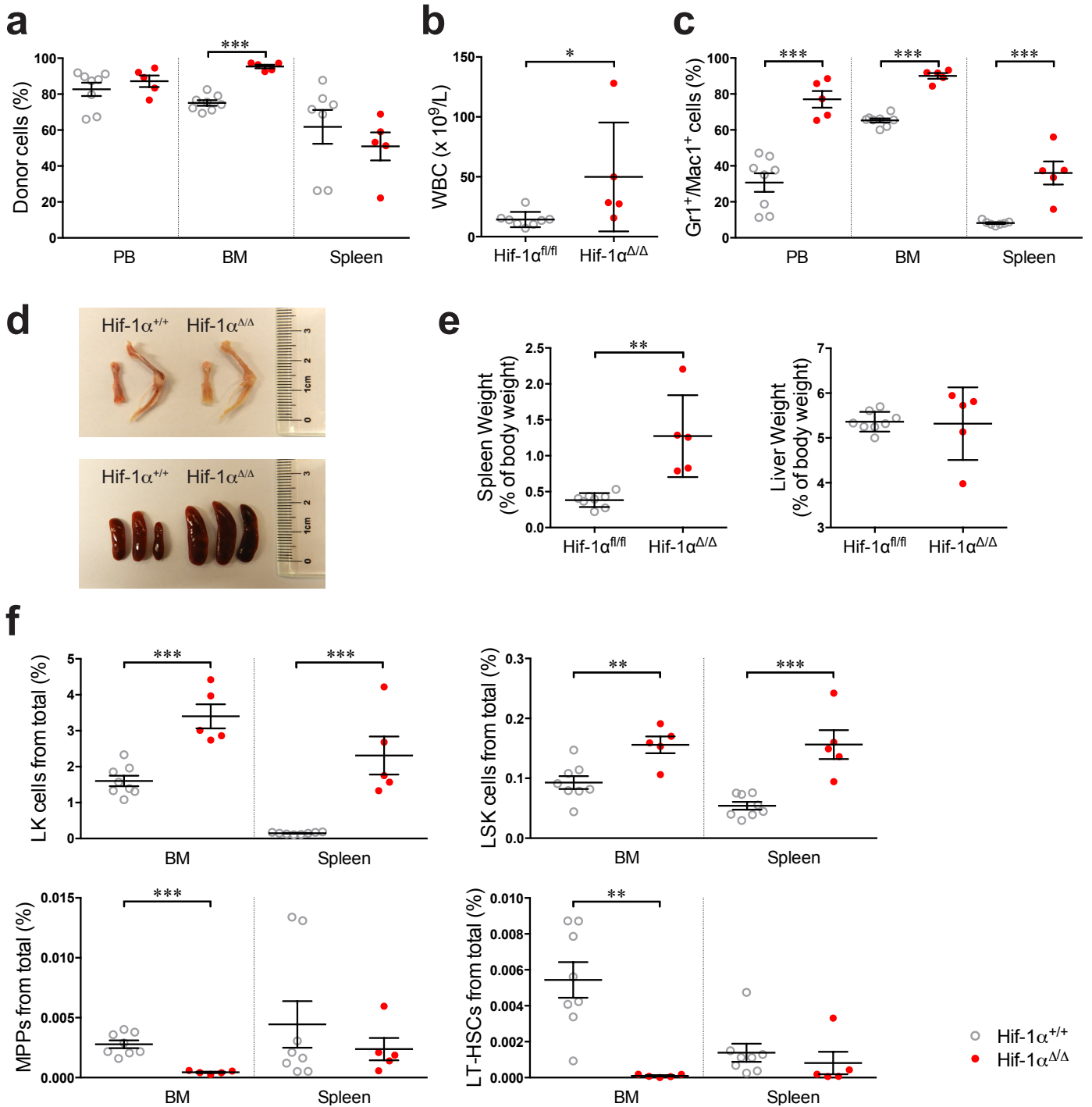
Supplementary Figure S4. Percentages and total cell numbers of LSK CD48+ CD150- cells in FLT-3^{ITD} mice.

(a) Percentage of LSK CD48+ CD150- cells of total BM or spleen cells (wt, n=5; HIF-1 $\alpha^{+/+}$, n=12; HIF-1 $\alpha^{\Delta/\Delta}$, n=13). (b) Absolute number of LSK CD48+ CD150- cells in BM (6 bones: 2 femurs, 2 tibiae and 2 hips) or spleen. Plots represent mean \pm SEM. Two-tailed Student *t* test was used to assess statistical significance. ***P*<0.01.



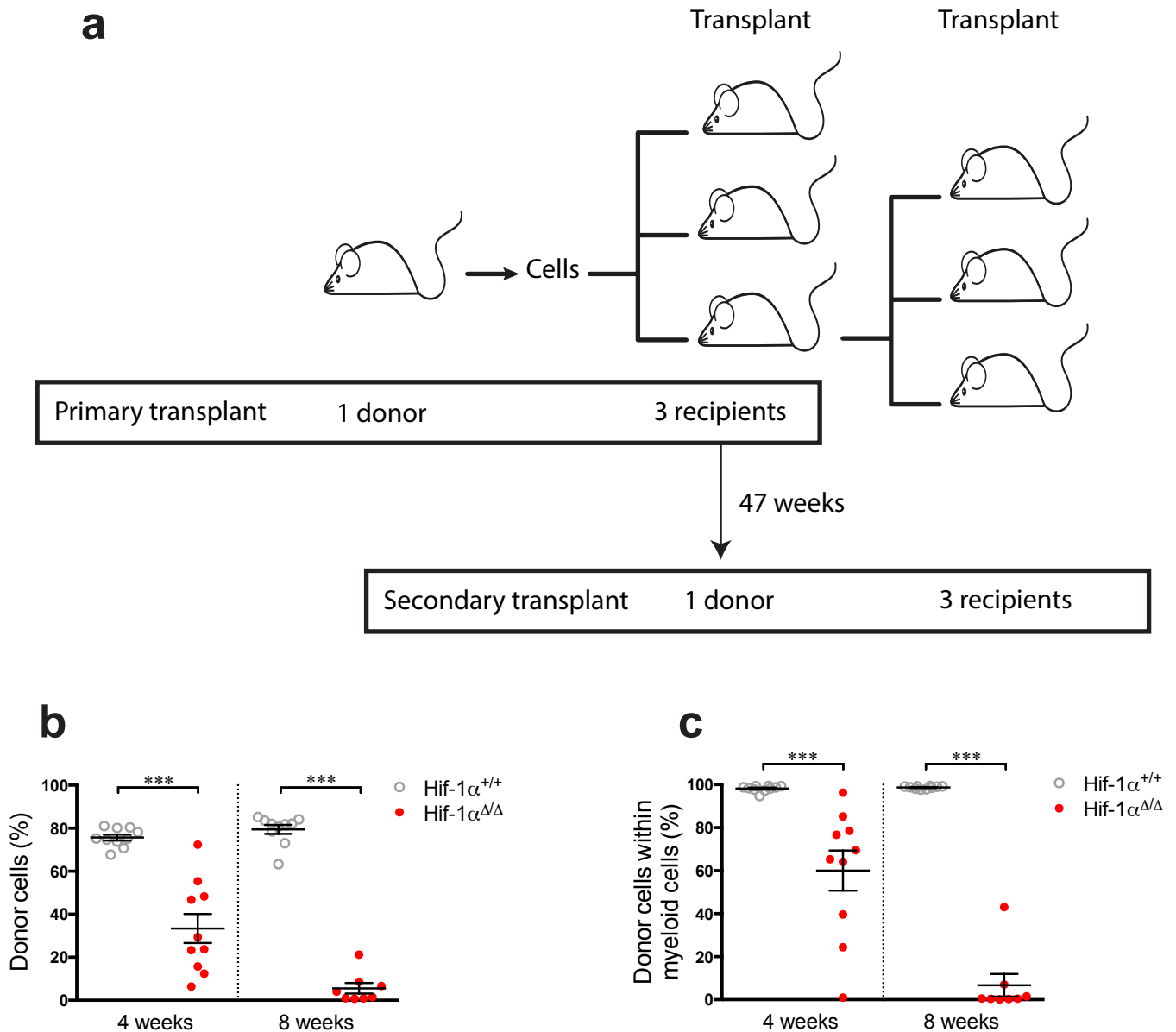
Supplementary Figure S5. Cell-cycle analysis of primitive populations.

(a) Representative plots of the cell-cycle analysis of the indicated primitive populations located in BM and spleen of HIF-1 $\alpha^{\Delta/\Delta}$ and HIF-1 $\alpha^{+/+}$ mice .



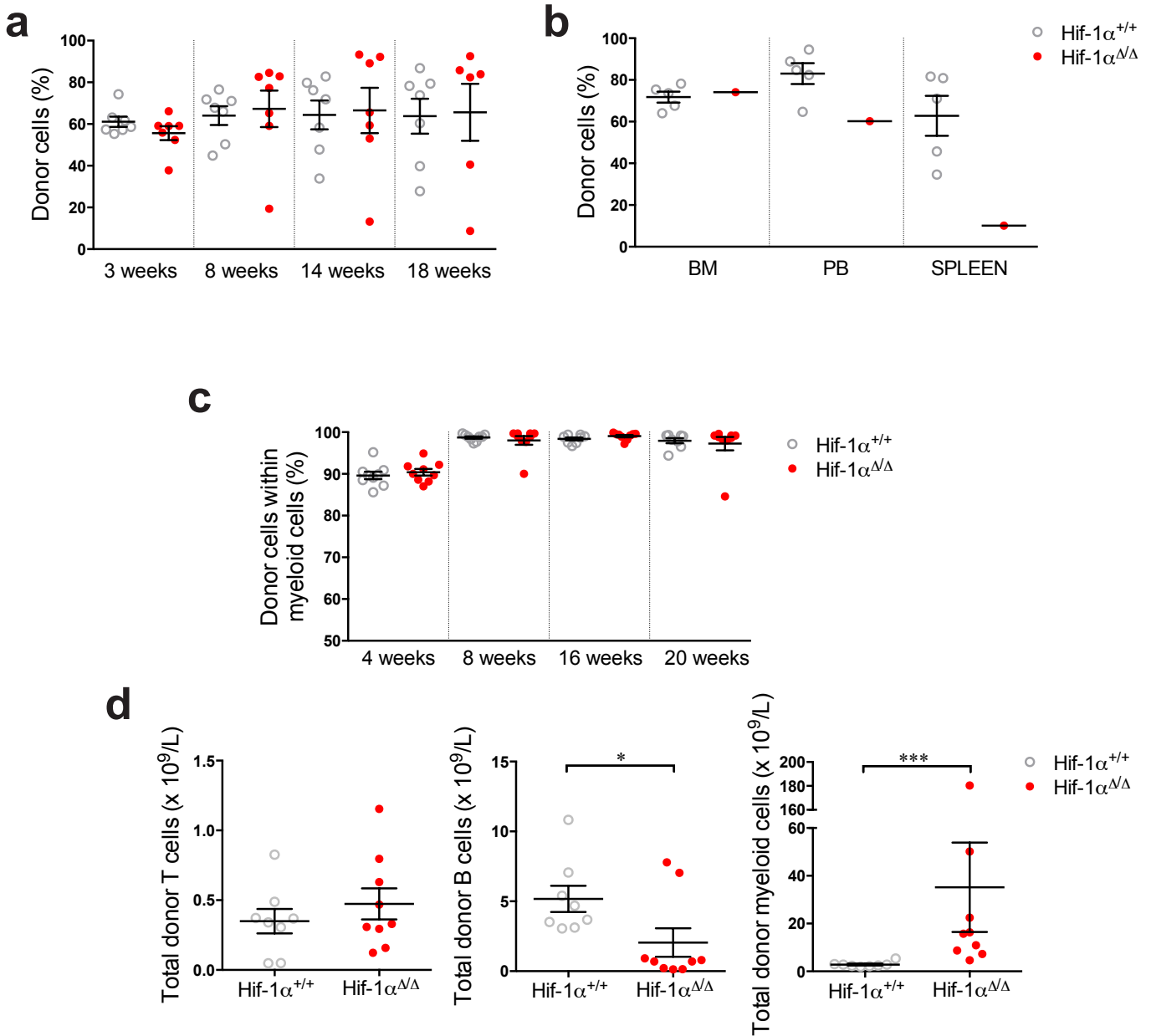
Supplementary Figure S6. Enhanced FLT-3^{ITD}-induced MPN phenotype in mice transplanted with HIF-1 $\alpha^{\Delta/\Delta}$ BM cells.

(a) Donor reconstitution of transplanted mice 47 weeks after transplantation (end point) (n=5-8). (b) Blood analysis of mice at the end point, showing increased WBC (n=5-8). (c) Percentage of mature myeloid cells (Gr1 $^+$ /Mac1 $^+$) in PB, BM and spleen at the end point (n=5-8). (d) Representative phenotype of bones and spleens at the end point from mice with the indicated genotypes. (e) Spleen and liver weights in relation to body weight for the different genotypes (n=5-8). (f) Percentage of the indicated populations of undifferentiated cells from total BM or spleen cells (n=5-8). Plots represent mean \pm SEM. Two-tailed Student *t* test was used to assess statistical significance. **P*<0.05, ***P*<0.01, ****P*<0.001



Supplementary Figure S7. Donor contribution of FLT-3^{ITD} cells in secondary recipients.

(a) Experimental design of the secondary transplantation assay. Donor cells from one primary recipient were transplanted into 3 secondary recipients. Donor cells were harvested 47 weeks after transplantation (end point of the experiment). (b) Donor reconstitution of transplanted mice in PB at different time points after transplantation. (n=10). (c) Donor contribution to the myeloid compartment in PB at different time points after transplantation (n=10). Plots represent mean \pm SEM. Two-tailed Student *t* test was used to assess statistical significance. ***P<0.001.



Supplementary Figure S8. Donor contribution of transplanted FLT-3^{ITD} cells.

(a) Donor contribution of mice transplanted with *in vitro* cultured cells (see also Figure 4b) in PB at different time points after transplantation (n=7) and their donor reconstitution (b) in BM, PB and spleen at 62 weeks after transplantation (end point). Notice that at this time point, there is only one HIF-1 $\alpha^{\Delta/\Delta}$ survivor. (c) Donor contribution to the myeloid compartment in secondary recipients (see also Figure 4a) at different time points after transplantation cells and (d) donor contribution to the different lineages in total cell numbers from the same transplantation at 20 weeks after transplantation. Plots represent mean \pm SEM. Two-tailed Student *t* test, and Mann-Whitney test for (d) due to the different variances, were used to assess statistical significance. *P<0.05, ***P<0.001.