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***Ptk7* deficient mice have decreased Hematopoietic Stem Cell pools as a result of  
deregulated proliferation and migration**

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**Keywords:** PTK7, planar cell polarity, hematopoietic stem cells, homing

**Running title:** Role of the polarity protein PTK7 in murine haematopoiesis.

25 **Abstract**

26 Hematopoietic Stem Cells (HSCs) located in adult bone marrow or fetal liver in mammals  
27 produce all cells from the blood system. At the top of the hierarchy are long-term HSCs  
28 endowed with lifelong self-renewal and differentiation properties. These features are  
29 controlled through key microenvironmental cues and regulatory pathways such as Wnt  
30 signaling. We have previously shown that PTK7, a tyrosine kinase receptor involved in  
31 planar cell polarity (PCP), plays a role in epithelial Wnt signaling. However, its function in  
32 hematopoiesis has remained unexplored. Here we show that PTK7 is expressed by  
33 hematopoietic stem and progenitor cells (HSPCs) with the highest protein expression level  
34 found on HSCs. Taking advantage of a *Ptk7* deficient mouse strain, we demonstrate that  
35 loss of *Ptk7* leads to a diminished pool of HSCs, but does not affect *in vitro* or *in vivo*  
36 hematopoietic cell differentiation. This is correlated with increased quiescence and reduced  
37 homing capacities of *Ptk7* deficient HSPCs, unravelling novel and unexpected functions for  
38 planar cell polarity pathways in HSC fate.

39

40

## 41 **Introduction**

42 Hematopoiesis is a biological process consisting on the production of all blood cell  
43 types from hematopoietic stem cells (HSCs) located in the bone marrow (BM) of adult  
44 mammals. Fetal hematopoiesis occurs in different sites, particularly in the liver, during its  
45 earliest phases. Long term hematopoietic stem cells (LT-HSCs) are characterized by their  
46 capacity to provide lifelong reconstitution of all blood cell lineages after transplantation  
47 into lethally irradiated recipients, whereas short term HSCs (ST-HSCs) do so for only 8-10  
48 weeks.

49 In the mouse, LT-HSCs belong to the LSK cell compartment defined by the  
50 Lineage<sup>-low</sup> Scd1<sup>+</sup>c-Kit<sup>+</sup> phenotype irrespectively of their fetal liver (FL) or adult bone  
51 marrow (BM) origin. Additional markers known as SLAM (Signal Lymphocytes Activating  
52 Molecules) can further subdivide this compartment (1)(2). Indeed, CD150<sup>+</sup>CD48<sup>-</sup> LSK have  
53 been shown to contain around 40% of HSCs with long-term reconstitution potential in BM  
54 as well as in FL (3).

55 Hematopoiesis is a highly controlled multi-step process that relies on complex  
56 interaction networks involving cell surface receptors, growth factors, and adhesion  
57 molecules expressed by HSCs and their environment (4)(5)(6)(7)(8). Because HSCs  
58 generate mature hematopoietic cells, including immune cells, their replacement must be  
59 adjusted to homeostatic or stress conditions such as infections, inflammation or blood loss,  
60 and their expansion must be controlled to avoid exhaustion. This control is made possible  
61 by the coordinated regulation of quiescence, self-renewal, and differentiation through  
62 appropriate signals delivered by functional BM niches (9). HSCs are retained in these BM  
63 niches by cell surface molecules endowed with adhesive and/or signalling functions  
64 expressed by HSCs. These receptors belong to different protein families: integrins (VLA-4)  
65 (10), Immunoglobulin superfamily (Ig Sf) adhesion molecules (6) (11), G Protein Coupled

66 Receptors (CXCR4)(12), or tyrosine kinase receptors (TIE2, c-Kit)(13)(14)(15) interacting  
67 with ligands present within the BM microenvironment (16). Discovery of novel molecules  
68 implicated in this multi-step program is of particular importance to embrace its complexity,  
69 develop new strategies aiming at the regeneration of damaged hematopoietic tissues and to  
70 understand hematopoietic diseases.

71 PTK7 is a planar cell polarity (PCP) receptor belonging to the Immunoglobulin  
72 Superfamily (Ig Sf) and playing important roles during development (17)(18). PCP is  
73 controlled by a non-canonical Wnt pathway, organizes polarization of many epithelial  
74 tissues and organs within the plane and drives cell migration and cell intercalation of non-  
75 epithelial cells such as mesenchymal cells (19)(20). *Ptk7* deficient mouse embryos show all  
76 signs of typical PCP defects such as severe tube neural and abdomen closure defect with  
77 secondary development abnormalities (21). PTK7 is a type I protein composed of seven Ig  
78 loops in its extracellular region, a transmembrane domain, an intracellular region with a  
79 tyrosine kinase domain (17)(18)(22)(23). While PTK7 is catalytically inactive, the  
80 cytoplasmic domain containing the tyrosine kinase domain is mandatory for receptor  
81 function *in vitro* and *in vivo* (24)(25)(26)(27). Both extracellular and intracellular domains  
82 of PTK7 can be released from the plasma membrane following the sequential cleavage of  
83 the receptor by membrane type 1-matrix metalloproteinase (MT1-MMP)/ADAM-17, and  $\gamma$ -  
84 secretase, interfering with its PCP and promigratory activities (24)(28)(29).

85 We have recently found that PTK7 expression is not restricted to epithelial cells.  
86 Indeed, PTK7 is expressed at the cell surface of human hematopoietic progenitors and acute  
87 myeloid leukemia (AML) patient blast cells. Overexpression of the PCP protein leads to  
88 increased cell survival and cell migration of leukemic cells and confers a poor prognosis to  
89 patients independently of other risk factors (27). Similar conclusions were recently drawn

90 from studies done in chronic lymphocytic leukemia in which a set of PCP proteins is  
91 upregulated (30).

92 Although functions of canonical and non-canonical Wnt pathways have been  
93 extensively studied in hematopoiesis (for review see (31)(32)(33)), the role of PTK7 has  
94 never been explored in this process. In the present study, we have thus addressed the  
95 function of PTK7 in physiological hematopoiesis using a mouse model deficient for *ptk7*  
96 expression. Using flow cytometry, we first mapped expression of PTK7 on HSCs and  
97 progenitors. As PTK7 deficient mice die perinatally, we then studied function of PTK7 in  
98 fetal hematopoiesis and revealed a decreased number of HSPCs in FL of *Ptk7* deficient  
99 mice. Although HSC differentiation was not affected, we found a dramatic decrease in  
100 proliferation and migration properties of *Ptk7* deficient HSPCs that reveal novel unexpected  
101 functions for this planar cell polarity molecule.

102

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103 **Methods**

104 *Mice*

105 C57BL/6 and C57BL/6-CD45.1 were purchased from Janvier Laboratory and Charles River  
106 Laboratories, respectively. All mice used for transplantation experiments are 6 to 12 weeks  
107 old. Lethal irradiation was done with an X-ray irradiator (X-RAD® 160 X-Ray, PXi) using  
108 a single dose of 8Gy.

109 PTK7 gene-trap mice were obtained by injection of gene-trap ES XST087 (Bays Genomics,  
110 USA) in E3.5 blastocyst from C57BL/6 mouse. Chimeric mice were back-crossed with B6  
111 mice for seven generations to obtain homogenous genetic background for experiments. All  
112 mice were housed and maintained under specific pathogen-free condition. E14.5 and 15.5  
113 were used in all FL experiments. All animal experiments were performed in agreement with  
114 the French Guidelines for Animal Handling.

115

116 *Genotyping*

117 Genotyping of adult mice and embryos was performed by genomic PCR using the  
118 following pairs of primers: 5'- ACGTCACTGGAGAGGAAGTCCGCAG-3' and 5'-  
119 GATCGAAGCAGGTCCTGTGGTCCT-3' for wild type allele and 5'-  
120 TTGTGAAGTGGAGGCTCCGGACC-3' and 5'-  
121 CGATCTTCCTGAGGCCGATACTGTC-3' for gene trap insertion allele.

122

123 *Flow cytometry and cell sorting*

124 For the analysis of hematopoietic stem/progenitor cells in FLs, FLs were dissected from  
125 E14.5 embryos and single cell suspension was made in phosphate-buffered saline (PBS)-  
126 2% Fetal Calf Serum (FCS).

127 For analysis of hematopoietic stem/progenitor cells in BM, thymus or blood cells obtained  
128 from adult mice were suspended in PBS-2% FCS. For BM and thymus, cells were filtered  
129 using 70 µm cell strainer (BDFalcon) and lysis of red blood cells was done using ACK lysis  
130 buffer (0.15 M NH<sub>4</sub>Cl, 10mM KHCO<sub>3</sub>, 0.1mM Na<sub>2</sub>EDTA, Life Technology) for 2 min at  
131 room temperature. Cells were spun and resuspended in PBS-2%FCS +/- 0.125mM EDTA  
132 for staining. For blood samples, red blood cell lysis was done after staining using BD FACS  
133 Lysing buffer solution (BD Biosciences) following manufacturer instructions.

134 For flow cytometry analysis and cell sorting, cells were stained with monoclonal antibodies  
135 purchased from Ebiosciences or Biolegend against the following molecules: B220 (clone  
136 6B2), CD3 (145-2C11), CD4 (RM4-5), CD8 (53-6.7), CD11b/Mac-1 (M1/70), CD11c  
137 (N418), CD16/32 (93), CD19 (6D5), CD24 (M1/69), CD34 (RAM34), CD45 (30F11),  
138 CD45.1/Ly5.1 (A20), CD45.2/Ly5.2 (104), CD48 (HM48-1), CD49b (DX5), CD49d (R1-  
139 2), CD117/c-Kit (2B8 and/or ACK2), CD135 (A2F10), CD150 (TC15-12F12.2), F4/80  
140 (BM8), Gr1 (RB6-8C5), Sca-1 (D7), Ter-119. Antibody human PTK7 and glycoprotein A  
141 (11E4B-7-6) were purchased from Miltenyi biotech and Beckman Coulter, respectively.

142 All experiments were done by multicolour parametric analysis using FITC, PE, APC, PE-  
143 Cy7, APC-Cy7, PerCPCy5.5, AlexaFluor700, AlexaFluor594, Pacific blue, PE-Cy5, Q-dot  
144 605 fluorochroms or spectral equivalents. Commercial antibodies were used either directly  
145 conjugated or biotinylated. For mouse PTK7 expression, polyclonal rabbit antibody raised  
146 against recombinant PTK7-Fc was used in the experiments. For detection of interesting  
147 antigen, PE or APC-conjugated goat-anti-rabbit (Jackson ImmunoResearch Laboratories)  
148 were used as secondary antibodies. To exclude lineage positive cells, streptavidin  
149 conjugated with Alexa A594 (Invitrogen) or Pacific blue (Ebiosciences) were used.  
150 Irrelevant isotype-matched antibodies were used as controls. Cells were stained 20 minutes  
151 at 4°C and rinsed in PBS-2% FCS solution. Viability marker (vivid dye, Invitrogen) was



152 added in last staining in PBS solution. Stained cells were analysed by LSR II SORP (laser  
153 405, 488, 561, and 633; BD Bioscience) or sorted by ARIA III (BD Bioscience). LSK cell  
154 populations were purified by cell sorting using a FACS-ARIA with > 95% purity. Results  
155 were analyzed using BD-DIVA Version 6.1.2 software (BD Biosciences) or FlowJo  
156 Version 7.6.2 (TreeStar) software.

157

### 158 *Homing experiments*

159 E14.5 embryos were sacrificed and were immediately genotyped. FL cells from wild type  
160 or gene trap mice were suspended, counted and stained with cell tracker calcein AM and  
161 calcein red-orange (Invitrogen), respectively, according manufacturer instructions. Cells  
162 were mixed at 1/1 ratio and  $4 \cdot 10^6$  total cells were injected through retro-orbital vein in  
163 previously irradiated (8 Gy) mice. 12h after injection, mice were sacrificed and blood, bone  
164 marrow, spleen, liver and thymus samples were analysed for fluorescent cells.

165

### 166 *Transplantation assays*

167 For LSK sorting, FL cells (CD45.2+) were labelled for lineage positive (lin+) cells with  
168 following biotinylated primary rat antibodies (lineage mix): CD3, CD4, CD8, CD19, B220,  
169 DX5, TER-119, GR-1, CD11c. Lin+ cells were depleted by magnetic separation using anti-  
170 rat Ig coated magnetic beads (DynaBeads, Lifetechnology). Lin<sup>Neg</sup> cells were also stained  
171 and sorted by FACS according to the phenotype Lin<sup>Neg</sup>, CD45.2<sup>+</sup>, Kit<sup>+</sup> and Sca-1<sup>+</sup>. CD45.1  
172 mice were lethally irradiated and grafted with 1000 or 400 CD45.2<sup>+</sup> LSK cells through the  
173 retro-orbital vein along with  $1 \cdot 10^5$  total BM recipient cells to allow short term reconstitution  
174 and mice survival. Recipient mice survival was followed and BM engraftment was  
175 examined by monitoring donor (CD45.2<sup>+</sup> cells) and host cell (CD45.1<sup>+</sup> cells) frequency in

176 the blood. After lethal irradiation, mice were maintained on water containing neomycin  
177 sulfate 1g/500mL for 2 weeks.

178

#### 179 *Serial transplantation assay*

180 Lethally irradiated (8 Gy) mice (C57Bl/6, CD45.1) received  $3 \cdot 10^6$  total fetal liver cells (first  
181 transplantation) or BM total cells (next transplantations). BM engraftment was monitored as  
182 described above.

183

#### 184 *Competition assay*

185 Lethally irradiated (8 Gy) mice (C57Bl/6, CD45.1) received  $2 \cdot 10^6$  total FL cells from  
186 CD45.2 PTK7<sup>-/-</sup> mutant embryos and  $2 \cdot 10^6$  total FL cells from CD45.1<sup>+</sup>CD45.2<sup>+</sup> PTK7<sup>+/+</sup>  
187 embryos, issues from crossing of CD57Bl/6, CD45.1 and CD57Bl/6, CD45.2. Plugs are  
188 obtained the same day. BM engraftment was examined by monitoring frequency of  
189 CD45.2<sup>+</sup> cells from PTK7 mutant donors and CD45.1<sup>+</sup>CD45.2<sup>+</sup> cells from wild type donor,  
190 excluding CD45.1<sup>+</sup> cells from host cells, in the blood. After lethal irradiation, mice were  
191 maintained on water containing neomycin sulfate 1g/500mL for 2 weeks.

192

#### 193 *Cell cycle analysis and apoptosis analysis*

194 FL progenitors were enriched as previously described and stained with cKit-APC-  
195 eFluor750, CD150-APC, Sca-1-PerCP-Cy5.5, and CD48-PECy7. The biotin-conjugated  
196 lineage combination was revealed with AlexaFluor 594-streptavidin (Invitrogen). For cell  
197 cycle analysis, after extracellular staining, cells were permeabilized, fixed, and stained with  
198 anti-Ki67-FITC MoAb (BD Biosciences) and DAPI as previously described (34). For  
199 apoptosis analysis, cells were stained with PE-annexine V to evaluate annexine V  
200 externalization (BD biosciences) and DAPI.

201

202 *In vitro methylcellulose assays*

203 For methylcellulose colony assays, FL or BM cells suspensions were prepared in Iscove  
204 modified Dulbecco medium (IMDM) supplemented with 2% FBS.  $3 \cdot 10^4$  cells of FL or  
205  $1 \cdot 10^5$  cells of BM were seeded in Methocult M3434 (containing rmSCF 50 ng/mL, rmIL-3  
206 10ng/mL, rhIL-6 10ng/mL, and EPO 3U/mL) (from Stem Cell Technologies, Grenoble,  
207 France) in 35 mm dishes accordingly to manufacturer's instructions. All assays were done  
208 in triplicated. Colonies (>30 cells) were counted after 13 days of incubation at 37°C, 5%  
209 CO<sub>2</sub> and humidified atmosphere.

210

211 *Adhesion assays*

212 MS5 stromal cells were plated on 96-well flat bottom plates, at a concentration of  $5 \cdot 10^3$   
213 cells/well 1 day before the assay. MS5 is a stromal cell line that has been generated by  
214 irradiating adherent cells in long-term BM culture. Lin<sup>Neg</sup> FL cells ( $5 \cdot 10^5$ ) obtained as  
215 previously described were cocultured for 2 hours with MS5 stromal cells. Plates were  
216 washed 3 times with PBS and adherent cells were removed by strong pipetting in PBS  
217 1.25mM EDTA 2% FCS. The phenotype of adherent cells was determined using anti-  
218 CD117, Sca-1, CD150, CD48, and FITC-lineage mix to exclude any residual mature cells  
219 contaminating the Lin<sup>Neg</sup> preparation. Absolute numbers were measured using the HTS  
220 coupled to the BD-LSR2 SORP (BD Bioscience).

221

222 *Migration assays*

223 Lin<sup>Neg</sup> FL cells were enriched as previously described and suspended in Iscove modified  
224 Dulbecco medium (IMDM) containing 2% of bovine serum albumine. About  $2 \cdot 10^5$  cells in  
225 75 µl were loaded in 5-µm pore Transwell system (Corning Life Sciences). The lower

226 chamber was filled with Serum free Media supplemented with the chemoattracting murine  
227 stromal-derived factor-1 (mSDF-1 $\alpha$ , 100 ng/mL, Peprotech). The same number of cells was  
228 plated in distinct wells to be used as control for migration. Plates were then incubated 4  
229 hours at 37°C, 5% CO<sub>2</sub>. All experiments were performed in duplicates. Cells were also  
230 stained using anti-CD117, Sca-1 and streptavidin coupled to Pacific Blue as described  
231 above. Absolute numbers were measured using the HTS coupled to the BD-LSR2 SORP  
232 (BD Bioscience) and the migration ratio was defined as the number of migrating cells  
233 between migration wells and control wells.

234

#### 235 *TF1 cell differentiation*

236 Growth of the human erythroid/macrophage progenitor cell line TF1 is dependent on GM-  
237 CSF or IL3 and can be induced to differentiate in the presence of growth factors. TF1 cells  
238 were grown in IMDM medium supplemented with 5 ng/ml GM-CSF and supplemented  
239 with EPO (2 UI/ml) for erythroid differentiation. TF1 cells were infected by viral particles  
240 containing shRNA against PTK7 as previously described (27). A scramble non-targeting  
241 shRNA was used as a control. shRNA efficiency for PTK7 downregulation was controlled  
242 by flow cytometry before and at the end of experiments. TF1 cells were then differentiated  
243 in IMDM with 2 IU/ml EPO and without GM-CSF for a total of seven days as previously  
244 described (35). Erythroblastic differentiation was evaluated by glycophorin A expression.

245

#### 246 *Statistical analysis*

247 Statistical significance was determined with nonparametric Mann-Whitney *U* test using  
248 Prism Version 5 software (Graphpad Software Inc).

249

250 **Results**

251

252 *PTK7 is expressed on HSCs, myeloid progenitors, lymphoid and thymic progenitors*

253 In a first approach, we studied PTK7 pattern of expression in adult murine hematopoietic  
254 cells. To this end, a rabbit polyclonal antibody generated against the extracellular region of  
255 PTK7 was used. This antibody recognizes mouse PTK7 endogenously expressed on  
256 endothelial and hematopoietic cells by FACS analysis in wild type but not *ptk7* deficient  
257 mice (Supplementary Fig. S1). Analysis of PTK7 expression in peripheral blood and in  
258 splenocytes revealed that none of the mature mouse leukocytes subsets (granulocytes,  
259 monocytes, B cells and T cells) expressed PTK7 to detectable level (Fig. 1A and data not  
260 shown). We next analysed PTK7 expression in murine BM hematopoietic progenitors using  
261 multiparametric flow cytometry and gating strategies described in Figure 1B. When LSK  
262 subsets were examined, PTK7 expression was found to be inversely correlated to  
263 differentiation stages with the highest expression on HSCs and multipotent progenitors  
264 (MPP) type 1 HSC-MPP1 (RFI=40), and intermediate expression on MPP2 (RFI=20) and  
265 MPP3-MPP4 (RFI=15) (Fig. 1C). More committed progenitors including common myeloid  
266 progenitors (CMP) and common lymphoid progenitors (CLP) expressed PTK7 at lower  
267 levels while the protein was almost undetectable on the surface of granulocyte-macrophage  
268 progenitor (GMP), and megakaryocyte–erythroid progenitor (MEP) (Fig. 1D). A similar  
269 phenotypic analysis was conducted on differentiating T cells isolated from the thymus. T  
270 cell progenitors do not express CD4 and CD8 markers and are called double negative (DN)  
271 cells before differentiation into double positive (DP) and single positive (SP) cells. Within  
272 the DN compartment, four thymic progenitor populations can be identified according to  
273 CD44 and CD25 expression from the most immature DN1 to the most mature DN4 subsets.  
274 PTK7 is expressed by the CD44+CD25- (DN1) population and expression is conserved

275 during thymic differentiation within the DN2, DN3 and DN4 subpopulations  
276 (Supplementary Fig. S2). PTK7 expression is also detected on DP thymocytes before  
277 becoming progressively lost at the SP stage. Altogether, these phenotypic analyses reveal  
278 that PTK7 expression is inversely correlated to hematopoietic differentiation with the  
279 highest expression found in HSCs. This suggests that PTK7 may play a role at this early  
280 stage of hematopoietic differentiation.

281

282 *Ptk7<sup>-/-</sup> embryos have a quantitative defect of hematopoietic stem cells*

283 We next questioned the function of PTK7 in hematopoiesis. To this end, we generated a  
284 *Ptk7* deficient mouse strain using a gene-trapped ES cell available through the International  
285 Gene Trap Consortium. As expected from the previous description of *Ptk7* deficient strains,  
286 we observed a perinatal lethality due to profound developmental defects (21). *Ptk7<sup>-/-</sup>*  
287 embryos harbour a severe phenotype with cranial neural tube closure defect, gastroschisis,  
288 kidney and forelimb abnormalities (Fig. 2A, 2B). Considering this dramatic phenotype, it  
289 was impossible to study adult hematopoiesis in these mice and we thus decided to study FL  
290 hematopoiesis. In FL, as well as in the BM, HSCs are comprised within the LSK cell  
291 compartment. Using the same gating strategy as in Figure 1, we analysed the consequences  
292 of PTK7 deficiency in the different LSK cell populations (Fig. 2C). We observed a 25%  
293 decrease of total cellularity at E14.5 in PTK7 deficient FL compared to wild type FL (Fig.  
294 2D). However proportion of leucocytes (e.g. CD45<sup>+</sup> cells) is equivalent between deficient  
295 and wild type FL (Fig. 2E) suggesting that the decrease in FL cellularity in *Ptk7<sup>-/-</sup>* embryos  
296 is likely due to a global effect of *ptk7* deficiency on FL size. We next examined the  
297 hematopoietic progenitor compartment. A decrease of about 50% of LSK cell frequency  
298 was observed in *Ptk7* deficient FL compared to control FL (1.33% *Ptk7<sup>+/+</sup>* and *+/+* LSK  
299 cells versus 0.67% *Ptk7<sup>-/-</sup>* LSK cells) (Fig. 2F). In the different LSK cell populations, the

300 most affected one was the HSC-MPP1 subset (Fig. 2C and 2G). No significant difference in  
301 the frequency of MMP2 and MPP3-MPP4 subsets was observed in *Ptk7* deficient FL  
302 compared to wild type or heterozygous FL. When we examined the absolute number of the  
303 different subsets of hematopoietic progenitors, we noticed that all LSK subsets were  
304 affected by PTK7 deficiency. Indeed, there was a 68% decrease in the LSK cell absolute  
305 number in *Ptk7* deficient embryos compared to control embryos (Fig. 2H). The most  
306 dramatic decrease was observed in the HSC compartment. Indeed, there was up to 75%  
307 decrease in HSC number in *Ptk7*<sup>-/-</sup> embryos compared to control one whereas there was  
308 around a 50% decrease in MPP2 and MPP3/4 cell numbers (Fig. 2I)

309 To assess the frequency of functional HSC in FL, we transplanted 1000 and 400 sorted LSK  
310 cells from *Ptk7*<sup>+/+</sup> or *Ptk7*<sup>-/-</sup> FL bearing the CD45.2 allele into two groups of lethally  
311 irradiated mice bearing the CD45.1 allele. As expected, all mice included in the group  
312 injected with 1000 LSK cells survived. However, with 400 injected cells, *Ptk7*<sup>+/+</sup> FL LSK  
313 cells were able to reconstitute recipient mice between 2 and 12 weeks after injection  
314 whereas all mice except one injected with *Ptk7*<sup>-/-</sup> LSK cells died within 6 weeks (Fig. 2J).

315 We concluded that loss of PTK7 leads to a defect in functional HSC frequency. *In vitro*  
316 clonogenicity assays confirmed the defect in HSCs and early precursor frequency as we  
317 observed a twofold reduction in colony-forming units starting from 3.10<sup>4</sup> total FL cells.  
318 (Fig. 2K).

319

320 *PTK7 deficient fetal liver cells are more quiescent than wild type fetal liver cells*

321 To determine if the absence of PTK7 could alter HSC quiescence, we performed cell cycle  
322 phenotypic analysis of LSK FL cells using an antibody directed against Ki-67, a nuclear  
323 marker to measure cell cycle, and DAPI to evaluate the DNA content (Fig. 3A). We found  
324 that a greater proportion of HSCs and early progenitors were in G0 phase in *Ptk7* deficient  
325 embryos as compared to control. Indeed, 56% of HSC-MPP1 cells were quiescent in *Ptk7*  
326 deficient embryos with respect to 46% in control embryos (Fig. 3B). This significant  
327 increase in quiescent cells was maintained throughout hematopoietic differentiation with  
328 respectively 46% versus 39% and 19% versus 14% of the cells in G0 phase at the MMP2  
329 and MPP3-MPP4 stages (Fig. 3B). To exclude that these unbalanced proportion of  
330 quiescent cells was not due to selective apoptosis of cycling *Ptk7* deficient cells, we  
331 analyzed the percentage of apoptotic cells in *Ptk7* deficient and control FL cells. We did not  
332 observed a difference of apoptosis as measured by Annexin V staining between wild type  
333 and mutant mice in total FL cells and LSK cells (Fig. 3C). These results demonstrate that  
334 PTK7 delivers proliferative signals to HSCs but does not affect HSC survival.

335

336 *PTK7 deficient cells are able to reconstitute definitive hematopoiesis in recipient mice*

337 To investigate the functional capacity of *Ptk7* deficient HSCs, we generated chimeric mice  
338 using FL cell transplantation. CD45.1 C57Bl/6 mice were lethally irradiated (8 Gy) and  
339 then reconstituted with  $3 \cdot 10^6$  total FL cells from *Ptk7* deficient or wild type FL embryos  
340 (CD45.2). Reconstitution and chimerism were followed over time by monitoring the  
341 frequency of donor cells (CD45.2) versus recipient cells (CD45.1) in the peripheral blood  
342 of recipient mice (Fig. 4A). As expected, all recipient mice injected with control FL cells  
343 were reconstituted between 2 and 12 weeks. The same results were obtained with PTK7  
344 deficient FL cells demonstrating that in absence of any competition, *Ptk7* deficient HSCs



345 are able to reconstitute all mature hematopoietic lineages of lethally irradiated recipients  
346 (Fig. 4B-C). To investigate whether *Ptk7* deficiency could lead to more subtle  
347 hematopoietic defects, BM of chimeric mice was analyzed after twelve weeks. No  
348 significant difference was found in frequencies of mature and progenitor cells in the BM  
349 (Fig. 4E-F). In agreement with reconstitution experiments, *in vitro* differentiation assays  
350 showed that *Ptk7* deficient FL cells are able to give rise to all subtypes of colony forming  
351 units in the same proportion than control FL cells (Fig. 4G, left panel and supplementary  
352 Fig.S4). However, we observed fewer colonies generated by *Ptk7* deficient HSPC than by  
353 control cells (Fig. 4G, right panel). This is likely due to decreased cell cycle activity of *Ptk7*  
354 deficient HSPCs while the potential of differentiation is not affected. Since quiescence of  
355 HSCs could be correlated to loss of self-renewal (36), we then tested self-renewal capacities  
356 of *Ptk7* deficient HSCs by serial transplantation assays. Recipient CD45.1 mice were  
357 lethally irradiated and reconstituted with CD45.2 donor cells from wild type and mutant  
358 mice. The first injection was performed with  $3.10^6$  total FL cells. After 12 weeks, BM cells  
359 were harvested and  $3.10^6$  cells were then injected to a second lethally irradiated recipient.  
360 The same protocol was applied for the following transplantations. We monitored chimerism  
361 by flow cytometry analysis and obtained a very good reconstitution with about 95 to 100%  
362 of donor cells in BM for all transplantations (Fig. 4H). We also investigated erythroblastic  
363 reconstitution in mice and did not find any differences in term of hemoglobin level and red  
364 blood cell count (Supplementary Fig. S3A and S3B). *In vitro* differentiation using TF1 cells  
365 that are able to differentiate in response to EPO did not reveal any difference in the  
366 presence or absence of PTK7 (Supplementary Fig. S3C and S3D). We thus concluded that  
367 PTK7 is not required for hematopoietic differentiation.

368 To get further insights into PTK7 function in early hematopoiesis, we performed mixed  
369 chimera competition experiments. CD45.1 C57Bl/6 mice were lethally irradiated (8 Gy)

370 and then reconstituted with  $2 \cdot 10^6$  total FL cells from *Ptk7* deficient (CD45.2) and  $2 \cdot 10^6$  total  
371 FL cells from wild type FL embryos (CD45.2xCD45.1). Reconstitution and chimerism  
372 were followed over time by monitoring the frequency of *PTK7*<sup>-/-</sup> cells (CD45.2) and  
373 *PTK7*<sup>+/+</sup> (CD45.2<sup>+</sup>CD45.1<sup>+</sup>) versus recipient cells (CD45.1) in the peripheral blood of  
374 recipient mice. We did not observe significant differences at D30 and D60, but observed an  
375 advantage of *PTK7*<sup>-/-</sup> cells at long term (>120 jours) (Fig. 5A). Since maintenance of  
376 quiescence is a stem cell property, we tested cell cycle activity of adult HSC in chimeric  
377 mice. Recipient CD45.1 mice were lethally irradiated and reconstituted with CD45.2 donor  
378 cells from wild type and mutant FL mice as previously described. We performed cell cycle  
379 analysis on hematopoietic progenitors more than 6 months post transplantation, when stable  
380 hematopoietic reconstitution is obtained. We confirmed that hematopoietic progenitors  
381 isolated from mice reconstituted with *PTK7* deficient cells are more quiescent than wild  
382 type counterparts (Fig. 5B and 5C). Clonogenic experiments confirmed that fewer colonies  
383 are generated by *Ptk7* deficient HSPC than by control from chimeric mice (Fig. 5D). These  
384 results suggest that *PTK7* is essential for quiescence independently of the  
385 microenvironment.

386

### 387 *Homing and migration defects of Ptk7 deficient cells*

388 Since we have previously described a pro-migratory function of *PTK7* in leukemic cell  
389 lines and because increased HSC quiescence may not be the unique cause of the functional  
390 defect of *Ptk7*<sup>-/-</sup> HSCs (Fig. 2J), we decided to investigate the role of *PTK7* in  
391 hematopoietic homing. FL cells from several *Ptk7*<sup>-/-</sup> or *Ptk7*<sup>+/+</sup> embryos were pooled and  
392 stained separately with two different fluorescent trackers. Cells were injected according to a  
393 1/1 ratio and mice were sacrificed 12h after injection. Analysis of homing was performed  
394 by flow cytometry (Fig. 6A-C). Although we observed comparable levels of residual

395 circulating cells regardless the PTK7 status in peripheral blood, *Ptk7* deficient cells had a  
396 very poor capacity to home to the BM, spleen, liver, and thymus as compared to control  
397 (Fig. 6D). Since homing defect can be explained by loss of cell adhesion to BM stromal  
398 cells and/or by a defect of cell migration toward a chemoattractant, we tested these two  
399 hypotheses. Adhesion of *Ptk7* deficient LSK cells on the murine MS5 stromal cell line was  
400 not significantly different from control LSK cells (Fig. 7A). In contrast, we found a  
401 decrease in spontaneous transmigration of *Ptk7* deficient FL cells as compared to control  
402 cells in Boyden chamber assay (8% versus 5%, Fig. 7B). No effect was observed with  
403 heterozygous FL cells. We also observed significant differences when the chemokine  
404 SDF1 $\alpha$  was used as a chemoattractive molecule. Indeed, whereas 12% of control FL cells  
405 migrated toward SDF1 $\alpha$ , only 8% of PTK7 deficient FL cells were able to do so (Fig. 7C).  
406 Without chemoattractant, less than 5% of LSK cells migrated across the transwell  
407 regardless the PTK7 status (Fig. 7D). When SDF1 $\alpha$  was used as a chemoattractive molecule,  
408 a twofold decrease in LSK transmigration was observed for *Ptk7* deficient cells as  
409 compared to control cells (20% versus 40%, Fig. 7E).

410

411

412 **Discussion**

413 Discovery of novel molecular pathways implicated in HSC maintenance is of  
414 particular interest to develop new strategies aiming at the regeneration of damaged  
415 hematopoietic tissues and to understand hematopoietic diseases such as leukemia. We have  
416 previously identified the cell polarity protein, PTK7, as a novel tyrosine kinase receptor  
417 overexpressed in acute myeloid leukemia (AML). High expression of PTK7 is correlated to  
418 poor prognosis and resistance to chemotherapeutic regimens (27). Recently, another study  
419 revealed that PTK7 is also upregulated in T acute lymphocytic leukemia (T-ALL)(37). In  
420 humans, we and others have evidenced that PTK7 expression is mostly restricted to normal  
421 early progenitors committed to myeloid and T lymphoid lineages.

422 In the present study, we show that PTK7 expression is similar between mouse and  
423 human systems. PTK7 is absent in mature blood cells and its expression in BM  
424 hematopoietic progenitors is inversely correlated with differentiation. The highest  
425 expression of PTK7 is found in HSC-MPP1 compartment. PTK7 expression is also  
426 conserved between mice and human when the thymic compartment is considered. PTK7 is  
427 easily detectable at the cell surface of CLP and immature DN and DP populations whereas  
428 its expression is lost in single positive CD4 or CD8 lymphocytes. These data correlate with  
429 results from other groups that demonstrated expression of PTK7 by recent thymic emigrants  
430 cells and thymocytes (38)(37).

431 In order to study the role of PTK7 in hematopoiesis, we generated *Ptk7* deficient mice using  
432 available gene-trapped ES cells. Our *Ptk7* deficient strain exhibited expected planar cell  
433 polarity defects as compared to another strain of mice issued from the same ES clone (21).  
434 Since these mutant mice cannot reach adulthood, functions of PTK7 were investigated in  
435 FL hematopoietic cells. The major hematopoietic phenotype of *Ptk7* deficient mice consists  
436 in increased quiescence of HSPCs and shrinkage of the FL HSPC pool. Although these

437 phenotypes may sound logical if one considers that less proliferation produces less cells,  
438 they are difficult to reconcile with loss of functional reconstitution observed when  
439 recipient mice are engrafted with limited numbers of LSK cells (Fig. 2J). Indeed, PTK7 is  
440 implicated in canonical and non-canonical Wnt pathway (17)(39), which are thought to be  
441 the guardians of the balance between HSC self-renewal and HSC differentiation (32). We  
442 have recently shown that PTK7 plays a role in canonical pathway downstream of Wnt3a  
443 suggesting that deficiency in PTK7 may mirror deficiency in Wnt3a (25). This would be  
444 consistent with results showing that Wnt3a deficiency leads to a reduction in the numbers  
445 of HSPCs in the FL but would not fit with the reduced reconstitution capacity observed in  
446 secondary transplantation assays of Wnt3a deficient cells which is not observed with *Ptk7*  
447 deficient cells (40). Alternatively, it may well be that PTK7 controls non-canonical pathway  
448 through interaction with Wnt ligands (41) (42). In this way, Wnt5a antagonizes Wnt3a-  
449 mediated canonical signaling and increases quiescence of HSCs (43). This occurs through  
450 activation of the small Rho GTPase Cdc42 that increases HSC polarity (44)(45). Such a  
451 function of PTK7 in Wnt5a-mediated non-canonical pathway would fit with the planar cell  
452 polarity function of PTK7 and with altered homing and migrating properties of *Ptk7*  
453 deficient cells that likely depend on small Rho GTPase activation. Some studies show that  
454 PTK7 regulates the balance between Wnt canonical and non-canonical pathways by  
455 interacting with Dishevelled (26)(46), ROR2 (42)(47),  $\beta$ -catenin (25) and LRP6 (39). We  
456 unsuccessfully tried to identify downstream pathways that could be regulated by Ptk7 in  
457 HSCPs using a candidate gene/protein approach. We also looked at gene expression  
458 differences between Ptk7 knock-out and control hematopoietic cells using unbiased gene  
459 expression profiling. Although we identified differences (data not shown), we could not  
460 find obvious Gene Ontology enrichments or pathways that could be experimentally tested.  
461 Further studies will be needed to unravel how Ptk7 functions in HSPCs.

462 In our model of FL hematopoietic cells, we confirmed the role of PTK7 in cell  
463 migration and found a new role of this protein in cell homing to hematopoietic organs and  
464 in the control of cell proliferation. This observation confirms our previous data obtained  
465 from human leukemia samples (27) can be linked with already described functions of PTK7  
466 in cell migration and in metastatic dissemination. Indeed, we and others have found that  
467 PTK7 expression on various cancer cells is linked with higher metastasis potential  
468 (27)(29)(48)(49). Recently, we also described a poor prognosis of PTK7 expression in  
469 colon cancer cells linked with higher metastatic events (50). In addition, our data show for  
470 the first time that PTK7 is involved in the regulation of hematopoietic and stem and  
471 progenitors cell (HSPC) cycle. Lack of PTK7 expression increases quiescence and  
472 enhances the long term repopulating potential after six months, while a trend toward  
473 decreased chimerism is observed after one or two months. This could reflect the dual  
474 function of PTK7 loss of expression in decreasing short term homing to the bone marrow  
475 and increasing long term establishment of hematopoiesis. Alternatively, it may well be that  
476 homing assay performed with total liver cells reveals a constitutive promigratory function  
477 of PTK7, in contrast to long term engraftment reflecting bone marrow micro-environmental  
478 signaling through PTK7 in HSCs. Similar complex mechanisms of cell cycle regulation and  
479 differential homing of mature cells versus HSPCs homing to the bone marrow have also  
480 been attributed to CXCR4 (51) (52) (53) (54). Whether PTK7 and CXCR4 signaling share  
481 more in common remains to be addressed. In conclusion, PTK7 overexpression in  
482 immature blasts cells of acute myeloid leukemia has been associated to bad prognosis, early  
483 relapse and chemotherapeutic resistance and a recent study shows that PTK7 can be used as  
484 a stem cell marker of colon cancer stem cells (27)(55). Taken together, all these data form a  
485 set of arguments to involve PTK7 expression in stem cells including cancer stem cells but  
486 also to prove a functional role of PTK7 in stem cell biology. We think that these new data

487 on PTK7 involvement on cell cycle are very promising to understand molecular  
488 mechanisms of leukemia/cancer development.

489 Altogether, our results report a novel unexpected function for the planar cell polarity  
490 molecule PTK7 in the crosstalk between expansion of HSPC compartment and HSPC  
491 migration. This opens new avenue for manipulation of HSC fate using agonists or  
492 antagonists of PTK7.

493

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498

499 **Author contribution**

500 A.-C.L designed experiments, wrote the paper, performed all experiments and generated

501 deficient mice; M.D.G, M.G., F.L., F.B., S.M. helped to perform experiments; J.-C.O.

502 generated deficient mice; M-L.A., M.A-L, J-P.B. designed experiments and wrote the

503 paper.

504

505 **Disclosure of potential conflicts of interest**

506 All authors declare no conflict of interest.

507

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508 **References**

- 509 1. Kiel, M. J., O. H. Yilmaz, T. Iwashita, O. H. Yilmaz, C. Terhorst, and S. J. Morrison.  
510 2005. SLAM family receptors distinguish hematopoietic stem and progenitor cells and  
511 reveal endothelial niches for stem cells. *Cell* 121: 1109–1121.
- 512 2. Oguro, H., L. Ding, and S. J. Morrison. 2013. SLAM family markers resolve functionally  
513 distinct subpopulations of hematopoietic stem cells and multipotent progenitors. *Cell Stem*  
514 *Cell* 13: 102–116.
- 515 3. Kim, I., S. He, O. H. Yilmaz, M. J. Kiel, and S. J. Morrison. 2006. Enhanced purification  
516 of fetal liver hematopoietic stem cells using SLAM family receptors. *Blood* 108: 737–744.
- 517 4. Golan, K., Y. Vagima, A. Ludin, T. Itkin, S. Cohen-Gur, A. Kalinkovich, O. Kollet, C.  
518 Kim, A. Schajnovitz, Y. Ovadya, K. Lapid, S. Shvitiel, A. J. Morris, M. Z. Ratajczak, and  
519 T. Lapidot. 2012. S1P promotes murine progenitor cell egress and mobilization via S1P1-  
520 mediated ROS signaling and SDF-1 release. *Blood* 119: 2478–2488.
- 521 5. Lapidot, T., P. Goichberg, K. Lapid, A. Avigdor, and O. Kollet. 2007. The endosteum  
522 region keeps human leukemic stem cells alive. *Cell Stem Cell* 1: 483–484.
- 523 6. Arcangeli, M.-L., V. Frontera, F. Bardin, E. Obrados, S. Adams, C. Chabannon, C.  
524 Schiff, S. J. C. Mancini, R. H. Adams, and M. Aurrand-Lions. 2011. JAM-B regulates  
525 maintenance of hematopoietic stem cells in the bone marrow. *Blood* 118: 4609–4619.
- 526 7. Ooi, A. G. L., H. Karsunky, R. Majeti, S. Butz, D. Vestweber, T. Ishida, T. Quertermous,  
527 I. L. Weissman, and E. C. Forsberg. 2009. The adhesion molecule esam1 is a novel  
528 hematopoietic stem cell marker. *Stem Cells Dayt. Ohio* 27: 653–661.
- 529 8. Winkler, I. G., V. Barbier, B. Nowlan, R. N. Jacobsen, C. E. Forristal, J. T. Patton, J. L.  
530 Magnani, and J.-P. Lévesque. 2012. Vascular niche E-selectin regulates hematopoietic stem  
531 cell dormancy, self renewal and chemoresistance. *Nat. Med.* 18: 1651–1657.
- 532 9. Morrison, S. J., and D. T. Scadden. 2014. The bone marrow niche for haematopoietic  
533 stem cells. *Nature* 505: 327–334.
- 534 10. Papayannopoulou, T., C. Craddock, B. Nakamoto, G. V. Priestley, and N. S. Wolf.  
535 1995. The VLA4/VCAM-1 adhesion pathway defines contrasting mechanisms of  
536 lodgement of transplanted murine hemopoietic progenitors between bone marrow and  
537 spleen. *Proc. Natl. Acad. Sci. U. S. A.* 92: 9647–9651.
- 538 11. Hidalgo, A., F. Sanz-Rodríguez, J. L. Rodríguez-Fernández, B. Albella, C. Blaya, N.  
539 Wright, C. Cabañas, F. Prósper, J. C. Gutierrez-Ramos, and J. Teixidó. 2001. Chemokine  
540 stromal cell-derived factor-1alpha modulates VLA-4 integrin-dependent adhesion to  
541 fibronectin and VCAM-1 on bone marrow hematopoietic progenitor cells. *Exp. Hematol.*  
542 29: 345–355.
- 543 12. Ara, T., K. Tokoyoda, T. Sugiyama, T. Egawa, K. Kawabata, and T. Nagasawa. 2003.  
544 Long-term hematopoietic stem cells require stromal cell-derived factor-1 for colonizing  
545 bone marrow during ontogeny. *Immunity* 19: 257–267.
- 546 13. Arai, F., A. Hirao, M. Ohmura, H. Sato, S. Matsuoka, K. Takubo, K. Ito, G. Y. Koh,  
547 and T. Suda. 2004. Tie2/angiopoietin-1 signaling regulates hematopoietic stem cell  
548 quiescence in the bone marrow niche. *Cell* 118: 149–161.
- 549 14. McCulloch, E. A., L. Siminovitch, J. E. Till, E. S. Russell, and S. E. Bernstein. 1965.  
550 The cellular basis of the genetically determined hemopoietic defect in anemic mice of  
551 genotype Sl-Sld. *Blood* 26: 399–410.
- 552 15. Barker, J. E. 1994. Sl/Sld hematopoietic progenitors are deficient in situ. *Exp. Hematol.*  
553 22: 174–177.
- 554 16. Wilson, A., and A. Trumpp. 2006. Bone-marrow haematopoietic-stem-cell niches. *Nat.*  
555 *Rev. Immunol.* 6: 93–106.

- 556 17. Lhoumeau, A.-C., F. Puppo, T. Prébet, L. Kodjabachian, and J.-P. Borg. 2011. PTK7: a  
557 cell polarity receptor with multiple facets. *Cell Cycle Georget. Tex* 10: 1233–1236.
- 558 18. Peradziryi, H., N. S. Tolwinski, and A. Borchers. 2012. The many roles of PTK7: a  
559 versatile regulator of cell-cell communication. *Arch. Biochem. Biophys.* 524: 71–76.
- 560 19. Angers, S., and R. T. Moon. 2009. Proximal events in Wnt signal transduction. *Nat.*  
561 *Rev. Mol. Cell Biol.* 10: 468–477.
- 562 20. Sebbagh, M., and J.-P. Borg. 2014. Insight into planar cell polarity. *Exp. Cell Res.* .
- 563 21. Lu, X., A. G. M. Borchers, C. Jolicœur, H. Rayburn, J. C. Baker, and M. Tessier-  
564 Lavigne. 2004. PTK7/CCK-4 is a novel regulator of planar cell polarity in vertebrates.  
565 *Nature* 430: 93–98.
- 566 22. Jung, J. W., A. R. Ji, J. Lee, U. J. Kim, and S. T. Lee. 2002. Organization of the human  
567 PTK7 gene encoding a receptor protein tyrosine kinase-like molecule and alternative  
568 splicing of its mRNA. *Biochim. Biophys. Acta* 1579: 153–163.
- 569 23. Mossie, K., B. Jallal, F. Alves, I. Sures, G. D. Plowman, and A. Ullrich. 1995. Colon  
570 carcinoma kinase-4 defines a new subclass of the receptor tyrosine kinase family.  
571 *Oncogene* 11: 2179–2184.
- 572 24. Na, H.-W., W.-S. Shin, A. Ludwig, and S.-T. Lee. 2012. The cytosolic domain of  
573 protein-tyrosine kinase 7 (PTK7), generated from sequential cleavage by a disintegrin and  
574 metalloprotease 17 (ADAM17) and  $\gamma$ -secretase, enhances cell proliferation and migration in  
575 colon cancer cells. *J. Biol. Chem.* 287: 25001–25009.
- 576 25. Puppo, F., V. Thomé, A.-C. Lhoumeau, M. Cibois, A. Gangar, F. Lembo, E. Belotti, S.  
577 Marchetto, P. Lécine, T. Prébet, M. Sebbagh, W.-S. Shin, S.-T. Lee, L. Kodjabachian, and  
578 J.-P. Borg. 2011. Protein tyrosine kinase 7 has a conserved role in Wnt/ $\beta$ -catenin canonical  
579 signalling. *EMBO Rep.* 12: 43–49.
- 580 26. Shnitsar, I., and A. Borchers. 2008. PTK7 recruits dsh to regulate neural crest  
581 migration. *Dev. Camb. Engl.* 135: 4015–4024.
- 582 27. Prebet, T., A.-C. Lhoumeau, C. Arnoulet, A. Aulas, S. Marchetto, S. Audebert, F.  
583 Puppo, C. Chabannon, D. Sainty, M.-J. Santoni, M. Sebbagh, V. Summerour, Y. Huon, W.-  
584 S. Shin, S.-T. Lee, B. Esterni, N. Vey, and J.-P. Borg. 2010. The cell polarity PTK7  
585 receptor acts as a modulator of the chemotherapeutic response in acute myeloid leukemia  
586 and impairs clinical outcome. *Blood* 116: 2315–2323.
- 587 28. Golubkov, V. S., A. V. Chekanov, P. Cieplak, A. E. Aleshin, A. V. Chernov, W. Zhu, I.  
588 A. Radichev, D. Zhang, P. D. Dong, and A. Y. Strongin. 2010. The Wnt/planar cell polarity  
589 protein-tyrosine kinase-7 (PTK7) is a highly efficient proteolytic target of membrane type-1  
590 matrix metalloproteinase: implications in cancer and embryogenesis. *J. Biol. Chem.* 285:  
591 35740–35749.
- 592 29. Golubkov, V. S., N. L. Prigozhina, Y. Zhang, K. Stoletov, J. D. Lewis, P. E. Schwartz,  
593 R. M. Hoffman, and A. Y. Strongin. 2014. Protein-tyrosine pseudokinase 7 (PTK7) directs  
594 cancer cell motility and metastasis. *J. Biol. Chem.* 289: 24238–24249.
- 595 30. Kaucká, M., K. Plevová, S. Pavlová, P. Janovská, A. Mishra, J. Verner, J. Procházková,  
596 P. Krejčí, J. Kotasková, P. Ovesná, B. Tichy, Y. Brychtová, M. Doubek, A. Kozubík, J.  
597 Mayer, S. Pospíšilová, and V. Bryja. 2013. The planar cell polarity pathway drives  
598 pathogenesis of chronic lymphocytic leukemia by the regulation of B-lymphocyte  
599 migration. *Cancer Res.* 73: 1491–1501.
- 600 31. Kokolus, K., and M. J. Nemeth. 2010. Non-canonical Wnt signaling pathways in  
601 hematopoiesis. *Immunol. Res.* 46: 155–164.
- 602 32. Malhotra, S., and P. W. Kincade. 2009. Wnt-related molecules and signaling pathway  
603 equilibrium in hematopoiesis. *Cell Stem Cell* 4: 27–36.

604 33. Florian, M. C., K. J. Nattamai, K. Dörr, G. Marka, B. Uberle, V. Vas, C. Eckl, I. Andrä,  
605 M. Schieman, R. A. J. Oostendorp, K. Scharffetter-Kochanek, H. A. Kestler, Y. Zheng,  
606 and H. Geiger. 2013. A canonical to non-canonical Wnt signalling switch in haematopoietic  
607 stem-cell ageing. *Nature* 503: 392–396.

608 34. Wilson, A., G. M. Oser, M. Jaworski, W. E. Blanco-Bose, E. Laurenti, C. Adolphe, M.  
609 A. Essers, H. R. Macdonald, and A. Trumpp. 2007. Dormant and self-renewing  
610 hematopoietic stem cells and their niches. *Ann. N. Y. Acad. Sci.* 1106: 64–75.

611 35. Wang, F., J. Travins, B. DeLaBarre, V. Penard-Lacronique, S. Schalm, E. Hansen, K.  
612 Straley, A. Kernysky, W. Liu, C. Gliser, H. Yang, S. Gross, E. Artin, V. Saada, E.  
613 Mylonas, C. Quivoron, J. Popovici-Muller, J. O. Saunders, F. G. Salituro, S. Yan, S.  
614 Murray, W. Wei, Y. Gao, L. Dang, M. Dorsch, S. Agresta, D. P. Schenkein, S. A. Biller, S.  
615 M. Su, S. de Botton, and K. E. Yen. 2013. Targeted inhibition of mutant IDH2 in leukemia  
616 cells induces cellular differentiation. *Science* 340: 622–626.

617 36. Fleming, H. E., V. Janzen, C. Lo Celso, J. Guo, K. M. Leahy, H. M. Kronenberg, and  
618 D. T. Scadden. 2008. Wnt signaling in the niche enforces hematopoietic stem cell  
619 quiescence and is necessary to preserve self-renewal in vivo. *Cell Stem Cell* 2: 274–283.

620 37. Jiang, G., M. Zhang, B. Yue, M. Yang, C. Carter, S. Z. Al-Quran, B. Li, and Y. Li.  
621 2012. PTK7: a new biomarker for immunophenotypic characterization of maturing T cells  
622 and T cell acute lymphoblastic leukemia. *Leuk. Res.* 36: 1347–1353.

623 38. Haines, C. J., T. D. Giffon, L.-S. Lu, X. Lu, M. Tessier-Lavigne, D. T. Ross, and D. B.  
624 Lewis. 2009. Human CD4+ T cell recent thymic emigrants are identified by protein  
625 tyrosine kinase 7 and have reduced immune function. *J. Exp. Med.* 206: 275–285.

626 39. Bin-Nun, N., H. Lichtig, A. Malyarova, M. Levy, S. Elias, and D. Frank. 2014. PTK7  
627 modulates Wnt signaling activity via LRP6. *Dev. Camb. Engl.* 141: 410–421.

628 40. Luis, T. C., B. A. E. Naber, W. E. Fibbe, J. J. M. van Dongen, and F. J. T. Staal. 2010.  
629 Wnt3a nonredundantly controls hematopoietic stem cell function and its deficiency results  
630 in complete absence of canonical Wnt signaling. *Blood* 116: 496–497.

631 41. Peradziryi, H., N. A. Kaplan, M. Podleschny, X. Liu, P. Wehner, A. Borchers, and N. S.  
632 Tolwinski. 2011. PTK7/Otk interacts with Wnts and inhibits canonical Wnt signalling.  
633 *EMBO J.* 30: 3729–3740.

634 42. Martinez, S., P. Scerbo, M. Giordano, A. M. Daulat, A.-C. Lhoumeau, V. Thomé, L.  
635 Kodjabachian, and J.-P. Borg. 2015. The PTK7 and ROR2 Protein Receptors Interact in the  
636 Vertebrate WNT/Planar Cell Polarity (PCP) Pathway. *J. Biol. Chem.* 290: 30562–30572.

637 43. Nemeth, M. J., L. Topol, S. M. Anderson, Y. Yang, and D. M. Bodine. 2007. Wnt5a  
638 inhibits canonical Wnt signaling in hematopoietic stem cells and enhances repopulation.  
639 *Proc. Natl. Acad. Sci. U. S. A.* 104: 15436–15441.

640 44. Florian, M. C., K. Dörr, A. Niebel, D. Daria, H. Schrezenmeier, M. Rojewski, M.-D.  
641 Filippi, A. Hasenberg, M. Gunzer, K. Scharffetter-Kochanek, Y. Zheng, and H. Geiger.  
642 2012. Cdc42 activity regulates hematopoietic stem cell aging and rejuvenation. *Cell Stem*  
643 *Cell* 10: 520–530.

644 45. Schlessinger, K., E. J. McManus, and A. Hall. 2007. Cdc42 and noncanonical Wnt  
645 signal transduction pathways cooperate to promote cell polarity. *J. Cell Biol.* 178: 355–361.

646 46. Wehner, P., I. Shnitsar, H. Urlaub, and A. Borchers. 2011. RACK1 is a novel  
647 interaction partner of PTK7 that is required for neural tube closure. *Dev. Camb. Engl.* 138:  
648 1321–1327.

649 47. Podleschny, M., A. Grund, H. Berger, E. Rollwitz, and A. Borchers. 2015. A  
650 PTK7/Ror2 Co-Receptor Complex Affects Xenopus Neural Crest Migration. *PLoS One* 10:  
651 e0145169.

- 652 48. Shin, W.-S., J. Kwon, H. W. Lee, M. C. Kang, H.-W. Na, S.-T. Lee, and J. H. Park.  
653 2013. Oncogenic role of protein tyrosine kinase 7 in esophageal squamous cell carcinoma.  
654 *Cancer Sci.* 104: 1120–1126.
- 655 49. Jin, J., H. S. Ryu, K. B. Lee, and J.-J. Jang. 2014. High expression of protein tyrosine  
656 kinase 7 significantly associates with invasiveness and poor prognosis in intrahepatic  
657 cholangiocarcinoma. *PLoS One* 9: e90247.
- 658 50. Lhoumeau, A.-C., S. Martinez, J.-M. Boher, G. Monges, R. Castellano, A. Goubard, M.  
659 Doremus, F. Poizat, B. Lelong, C. de Chaisemartin, F. Bardin, P. Viens, J.-L. Raoul, T.  
660 Prebet, M. Aurrand-Lions, J.-P. Borg, and A. Gonçalves. 2015. Overexpression of the  
661 Promigratory and Prometastatic PTK7 Receptor Is Associated with an Adverse Clinical  
662 Outcome in Colorectal Cancer. *PLoS One* 10: e0123768.
- 663 51. Cashman, J., I. Clark-Lewis, A. Eaves, and C. Eaves. 2002. Stromal-derived factor 1  
664 inhibits the cycling of very primitive human hematopoietic cells in vitro and in NOD/SCID  
665 mice. *Blood* 99: 792–799.
- 666 52. Broxmeyer, H. E., L. Kohli, C. H. Kim, Y. Lee, C. Mantel, S. Cooper, G. Hangoc, M.  
667 Shaheen, X. Li, and D. W. Clapp. 2003. Stromal cell-derived factor-1/CXCL12 directly  
668 enhances survival/antiapoptosis of myeloid progenitor cells through CXCR4 and G(alpha)i  
669 proteins and enhances engraftment of competitive, repopulating stem cells. *J. Leukoc. Biol.*  
670 73: 630–638.
- 671 53. Foudi, A., P. Jarrier, Y. Zhang, M. Wittner, J.-F. Geay, Y. Lecluse, T. Nagasawa, W.  
672 Vainchenker, and F. Louache. 2006. Reduced retention of radioprotective hematopoietic  
673 cells within the bone marrow microenvironment in CXCR4<sup>-/-</sup> chimeric mice. *Blood* 107:  
674 2243–2251.
- 675 54. Nie, Y., Y.-C. Han, and Y.-R. Zou. 2008. CXCR4 is required for the quiescence of  
676 primitive hematopoietic cells. *J. Exp. Med.* 205: 777–783.
- 677 55. Jung, P., C. Sommer, F. M. Barriga, S. J. Buczacki, X. Hernando-Momblona, M.  
678 Sevillano, M. Duran-Frigola, P. Aloy, M. Selbach, D. J. Winton, and E. Batlle. 2015.  
679 Isolation of Human Colon Stem Cells Using Surface Expression of PTK7. *Stem Cell Rep.* 5:  
680 979–987.

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686

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693

694 **Figure Legends:**

695

696 **Figure 1: PTK7 expression in wild type bone marrow.** (A) Expression of PTK7 in blood

697 mature cells. Granulocytes are defined as Gr1<sup>+</sup>CD11b<sup>+</sup> cells. Monocytes are defined as

698 F4/80<sup>+</sup> CD11b<sup>+</sup>CD115<sup>+</sup> cells. Lymphocytes are gated using B220, CD19, CD3, CD4 and

699 CD8 combination. MFI ratios are indicated in dots plots. (B) Strategy of bone marrow

700 progenitors gating. First panel: Dot plot showing the LSK<sup>+</sup> gate within Lin<sup>-</sup> bone marrow

701 cells. Second panel: dot plot showing early progenitors gating using SLAM markers.

702 Hematopoietic stem cells (HSC) are defined as CD150<sup>+</sup>CD48<sup>-</sup> cells. More mature

703 multipotents progenitors (MPP) are defined as CD150<sup>+</sup>CD48<sup>+</sup> (MPP2) and then CD150<sup>-</sup>

704 CD48<sup>+</sup> (MPP3/4). Third panel: dot plot showing engaged progenitors within c-Kit<sup>+</sup>Sca-1<sup>-</sup>

705 Lin<sup>-</sup> cells. CMP (Common Myeloid Progenitor) is defined as CD16/32<sup>lo</sup>CD34<sup>+</sup>. GMP

706 (Granulocyte-Macrophage Progenitor) is defined as CD34<sup>+</sup>CD16/32<sup>hi</sup>. MEP

707 (Megakaryocyte-Erythroid Progenitor) is defined as CD16/32<sup>-</sup>CD34<sup>-</sup>. Fourth panel: Gating

708 of CLP (Common Lymphoid Progenitor) defined as Lin<sup>-</sup>IL7Ra<sup>+</sup>B220<sup>-</sup>CD24<sup>lo</sup>CD117<sup>lo</sup>. (C)

709 Overlays showing expression of PTK7 during differentiation. Black line: PTK7, gray

710 histogram: isotypic control (pre-immune rabbit antiserum). (D) Quantitative comparison of

711 expression levels between different progenitor populations: normalization of PTK7 mean of

712 fluorescence (MFO) with CD117 MFO on LSK subpopulations and progenitors

713 (CD117<sup>+</sup>Sca-1<sup>-</sup> cells). Statistical significance was calculated by nonparametric Mann-

714 Whitney test. All data are shown as the mean ± s.d. \*p<0.05. \*\*p<0.01.\*\*\*p<0.001.

715

716 **Figure 2: Phenotype of PTK7 deficient mouse and analysis of fetal liver at E14.5.** (A)

717 PTK7 mutants embryos showing severe planar cell polarity and developmental

718 abnormalities. (B) Western blot of murine embryonic fibroblasts (MEFs) obtained from a



719 litter of our PTK7 gene trapped established mouse line. (C) Dot plots showing LSK gate  
720 within  $lin^-$  FL cells and SLAM gating within LSK cells. (D) Quantification of total number  
721 fetal liver cells. Data are pooled from 6 litters:  $Ptk7^{-/-}$  N=12;  $Ptk7^{+/-}$  N=19;  $Ptk7^{+/+}$  N=12  
722 (E) Percentage of  $CD45^+$  cells within fetal liver. Data are pooled from 8 litters:  $Ptk7^{-/-}$   
723 N=15;  $Ptk7^{+/-}$  N=26;  $Ptk7^{+/+}$  N=9. (F) Percentage of LSK cells within  $CD45^+$  FL cells. (G)  
724 Percentage of LSK subpopulation using SLAM markers. (H) Absolute number of LSK cells  
725 in E14.5 FL. (I) Absolute number of LSK subpopulations using SLAM markers. From (F)  
726 to (I) data are pooled from 10 litters:  $Ptk7^{-/-}$  N=19;  $Ptk7^{+/-}$  N=29;  $Ptk7^{+/+}$  N=10. (J) Survival  
727 curves of  $CD45.1$  C57Bl/6 mice engrafted with 400 LSK cells sorted from  $CD45.2$  FL  
728 together with  $1.10^5$  congenic  $CD45.1$  total bone marrow cells. Black line: engraftment with  
729  $Ptk7^{+/+}$  FL cells; Dashed line: engraftment with  $Ptk7^{-/-}$  FL cells. Shown experiment is  
730 representative from 3 separate experiments. N=6 mice per group. (K) Clonogenic capacity  
731 of FL total cells.  $3.10^4$  FL cells are seeded in M3434 methylcellulose. Shown experiment is  
732 representative from 3 separate experiments.  $Ptk7^{-/-}$  N=6;  $Ptk7^{+/+}$  N=4. All statistical  
733 significance was calculated by nonparametric Mann-Whitney test. All data are shown as the  
734 mean  $\pm$  s.d. \* $p < 0.05$ . \*\* $p < 0.01$ . \*\*\* $p < 0.001$ .

735

736 **Figure 3: Cell cycle and apoptosis analysis of LSK FL cells.** (A) Representative dot plots  
737 of DNA content (DAPI) plotted versus Ki-67 nuclear antigen staining on LSK cells. The  
738 cell-cycle phases were defined as G0 (Ki-67<sup>-</sup> and  $2n$  DNA), G1 (Ki-67<sup>+</sup> and  $2n$  DNA) and S-  
739 G2-M (Ki-67<sup>+</sup> and DNA  $>2n$ ). (B) Percentage of LSK FL cells in cell-cycle phases using  
740 SLAM markers. Data are pooled from 3 separate experiments and embryos ( $Ptk7^{-/-}$  N=10;  
741  $Ptk7^{+/+}$  N=11) from 7 different litters. \* $p < 0.05$ . \*\* $p < 0.01$ . (C) Determination of percentage  
742 of apoptotic cells by staining with annexin V and DAPI. Apoptotic cells were defined as  
743 annexin V<sup>+</sup> DAPI<sup>-</sup>. Results are representative of 8  $Ptk7^{-/-}$  and 6 WT embryos from 5

744 different litters. All statistical significance was calculated by nonparametric Mann-Whitney  
745 test. All data are shown as the mean  $\pm$  s.d. \* $p < 0.05$ . \*\* $p < 0.01$ . \*\*\* $p < 0.001$ .

746

747 **Figure 4: Hematological capacity of reconstitution and differentiation.** Lethally  
748 irradiated CD45.1 mice (8 Gy) were reconstituted with  $3 \cdot 10^6$  CD45.2 total FL cells isolated  
749 from  $Ptk^{+/+}$  and  $Ptk^{-/-}$  embryos. Percentage of each hematological populations from donor  
750 (CD45.2) was monitored by flow cytometry (A). Percentage of matures cells in recipient  
751 blood (B) and in spleen (C). Percentage of BM mature cells (D) and BM progenitors (E) in  
752 recipient mice. (F) Percentage of LSK cells and LSK subpopulation in recipient BM. White  
753 histograms depict mice reconstituted with  $Ptk^{-/-}$  FL. Black histograms depict mice  
754 reconstituted with  $Ptk^{+/+}$  FL. N=6 mice per group. (G) *In vitro* differentiation capacity of FL  
755 cells.  $3 \cdot 10^4$  FL cells were seeded in methylcellulose. Left panel: Quantification and  
756 characterization of CFU-C. Right panel: Proportion of CFU. (H) Serial transplantation. First  
757 transplantation: Lethally irradiated mice were injected with  $3 \cdot 10^6$  of total E14.5 FL cells  
758 (T1: transplantation 1). Next transplantations were done by injection of  $3 \cdot 10^6$  total bone  
759 marrow cells from previously reconstituted mice in irradiated C57Bl/6 CD45.1 mice (T2 to  
760 T5). Percentage of donor cells (CD45.2) *versus* recipient cells (CD45.1) was monitored.  
761 Each data represents chimerism of definitive hematopoiesis 3 months after irradiation. Data  
762 are the mean  $\pm$  s.d. N=6 mice per group.

763

764 **Figure 5: Competition experiments and cell cycle in adult mice.** (A)  $2 \cdot 10^6$  cells from  
765  $Ptk^{+/+}$  (CD45.1xCD45.2) and  $2 \cdot 10^6$  cells from  $Ptk^{-/-}$  (CD45.2) are injected in recipient  
766 (CD45.1) mice. Chimerism is followed in blood samples. Data are representative of 4  
767 independent experiments (N=6 mice per group). (B-D) Recipient CD45.1 mice  
768 reconstituted with CD45.2 donor cells from wild type and mutant FL mice (B)

769 Representative dot plots of DNA contents (DAPI) plotted versus Ki-67 nuclear antigen  
770 staining on LSK cells. (C) Percentage of LSK cells in cell cycle phases using SLAM  
771 markers. (D) Clonogenic capacity of BM total cells.  $1.10^5$  BM cells are seeded in M3434  
772 methylcellulose. Data are representative of 2 independent experiments (N=6 mice per  
773 group). All statistical significance was calculated by nonparametric Mann-Whitney test.  
774 \* $p < 0.05$ . \*\* $p < 0.01$ . \*\*\* $p < 0.001$ .

775

776 **Figure 6: Homing capacities of deficient PTK7 cells.** (A)  $2.10^6$  of total fetal liver cells  
777 from *Ptk7<sup>-/-</sup>* or *Ptk7<sup>+/+</sup>* embryos were stained with cell tracker calcein AM and calcein red-  
778 orange respectively, pooled in 1/1 ratio and injected in recipient mice. (B-D) 12h after  
779 injection, mice were sacrificed and biological samples were removed and analyzed to  
780 evaluate homing capacities of each cells. (B) Bone marrow of uninjected mice was used as  
781 control for staining specificity. (C) Dot plots of stained cells removed from blood, BM,  
782 spleen, liver and thymus. (D) Quantification and statistical analysis. Data are pooled from 3  
783 independent experiments or representative (for spleen panel). N=6 mice per group. All  
784 statistical significance was calculated by nonparametric Mann-Wihtney test. \* $p < 0.05$ .  
785 \*\* $p < 0.01$ . \*\*\* $p < 0.001$ .

786

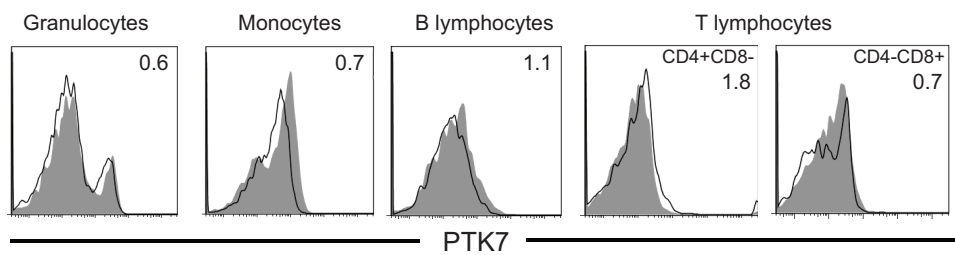
787 **Figure 7: Adhesion and migration assays** (A). Adhesion assay on MS5 stromal cells.  
788 Percentage of adherent FL cells on MS5 stromal bone marrow cell line after 2 hours of  
789 incubation. Left panel: total FL cells. Right panel: LSK cells. Data are representative of 3  
790 independent experiments. *Ptk<sup>-/-</sup>* N=4; *Ptk<sup>+/-</sup>* N=6; *Ptk<sup>+/+</sup>* N=3. (B to E) Migration in Boyden  
791 chamber assay. Number of migrating cells are normalized to the number of input cells for  
792 each sample (duplicate wells). (B) Migration of  $\text{lin}^{\text{Neg}}$  enriched FL cells without  
793 chemoattractant. (C) Migration of  $\text{lin}^{\text{Neg}}$  enriched FL cells with SDF1a as chemoattracting



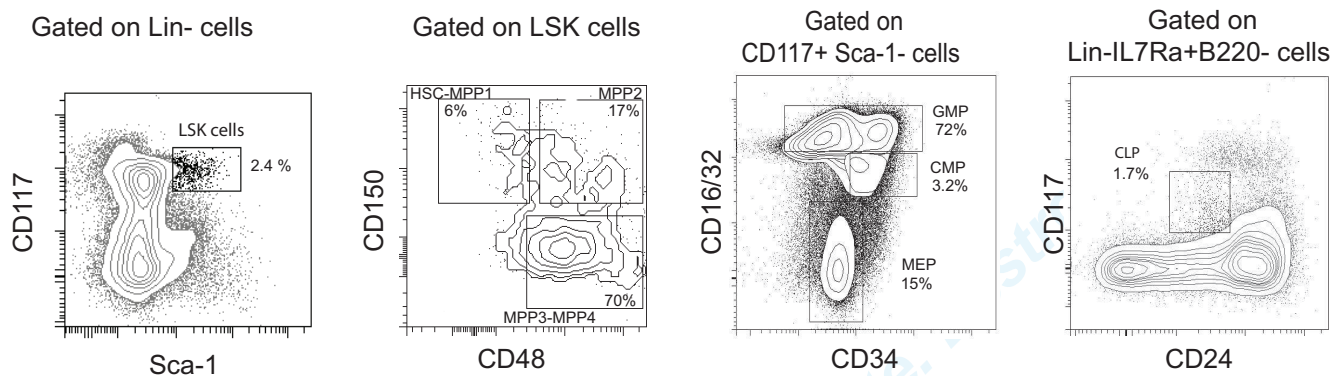
794 reagent. (D). Migration of LSK FL cells without chemoattractant. (E). Migration of LSK  
795 FL cells with SDF1 $\alpha$  as chemoattracting reagent. All statistical significance was calculated  
796 by nonparametric Mann-Wihtney test. \*p<0.05. \*\*p<0.01. \*\*\*p<0.001.

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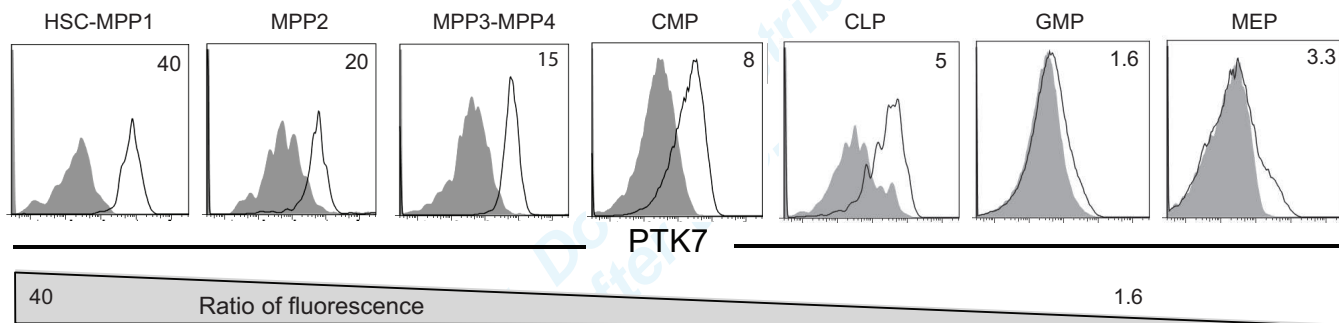
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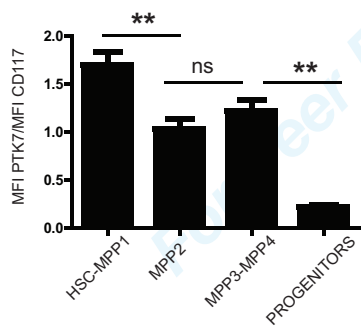


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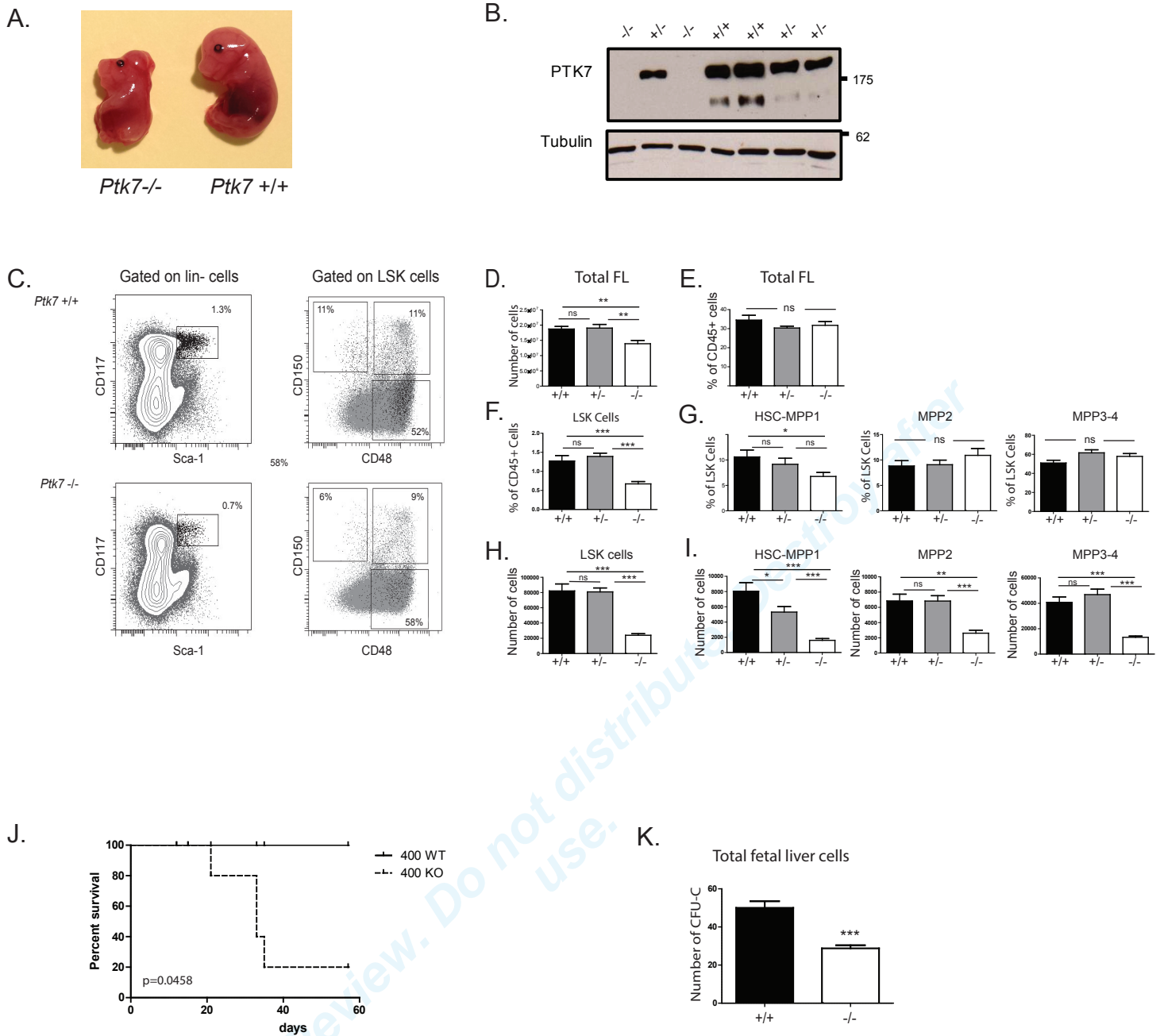


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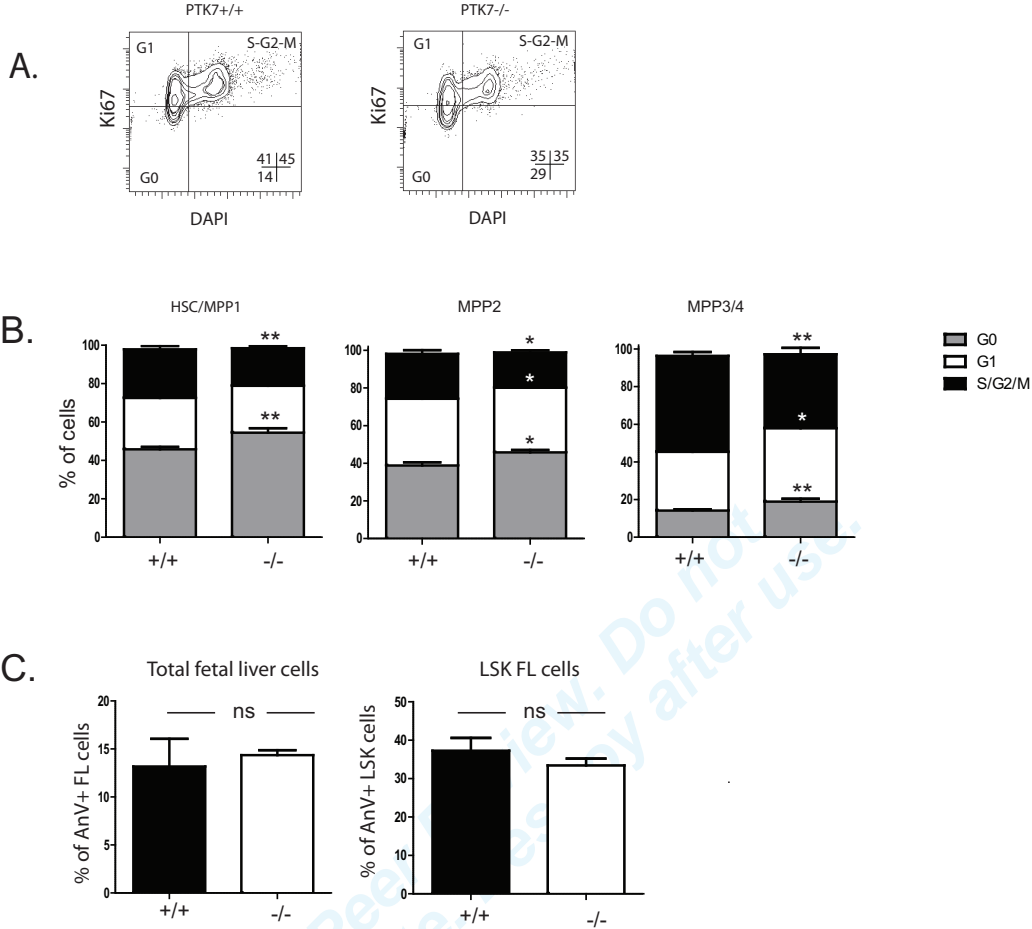


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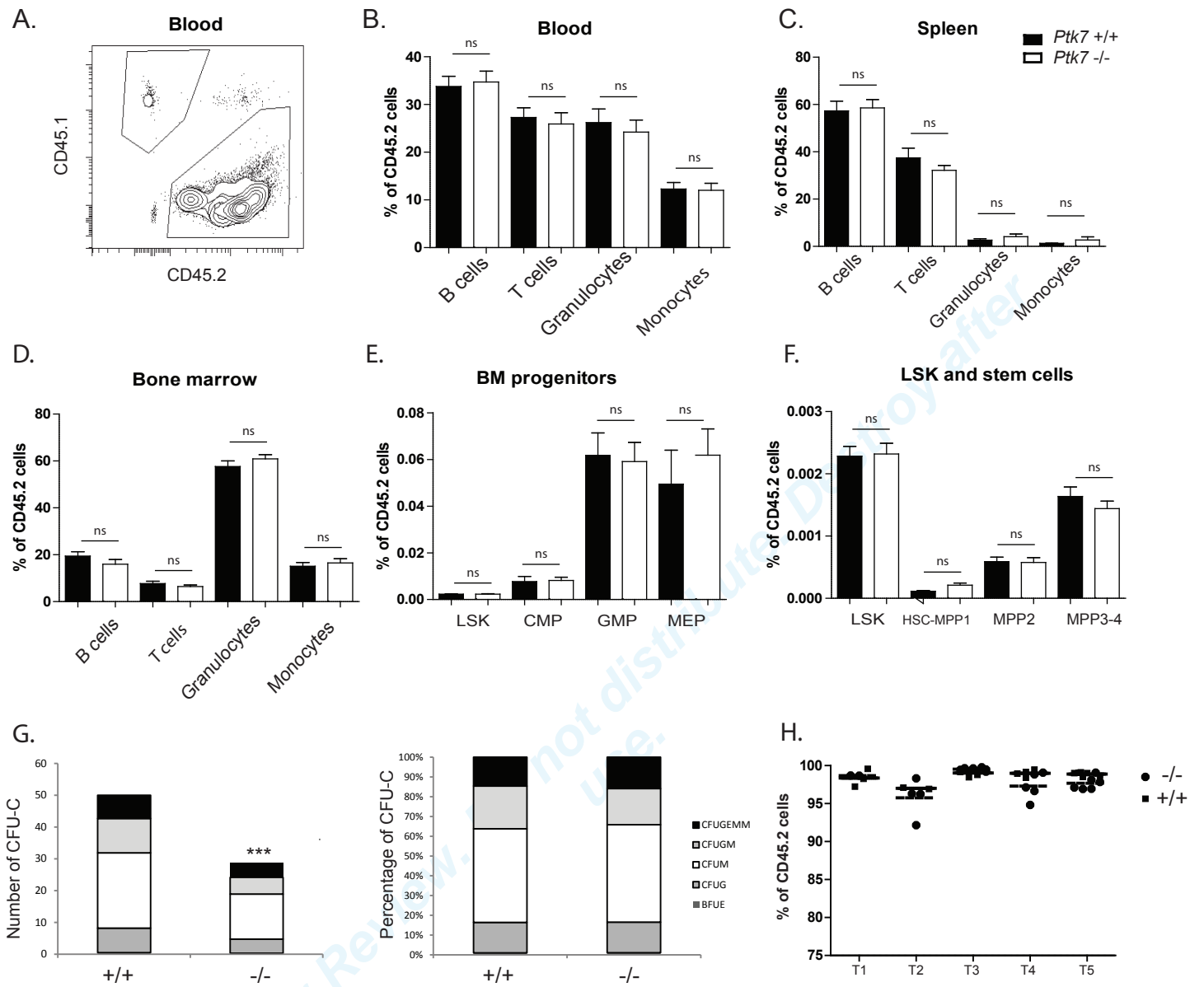
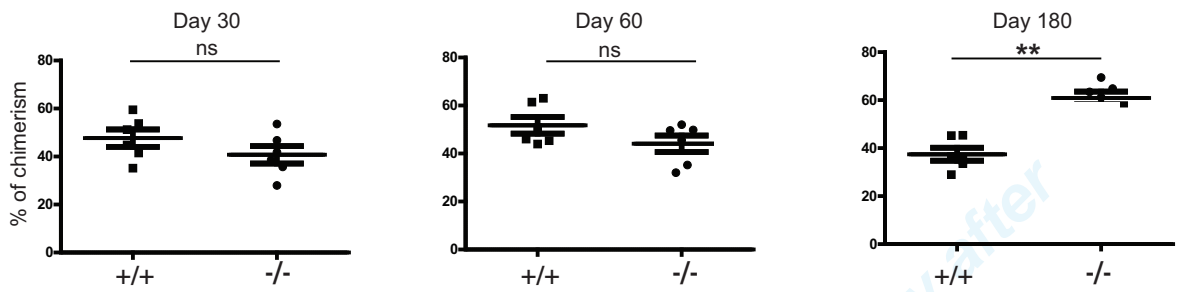
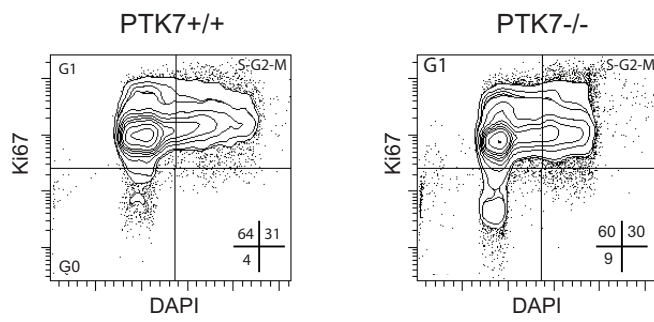


Figure 4

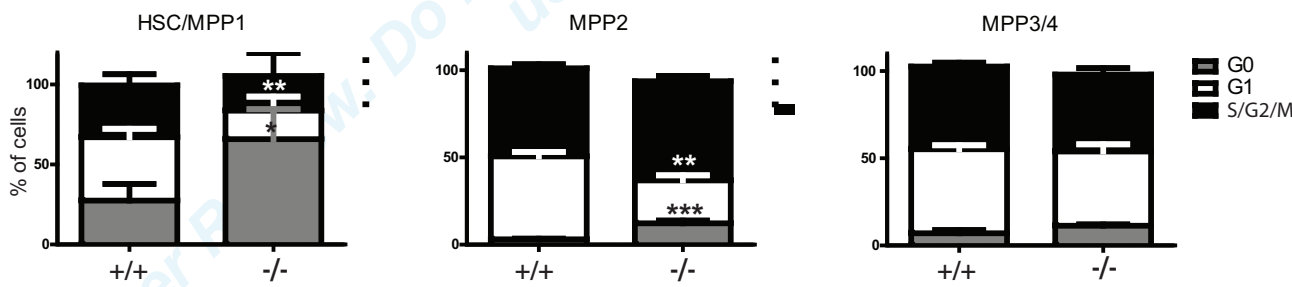
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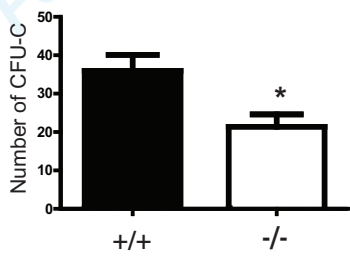


Figure 5

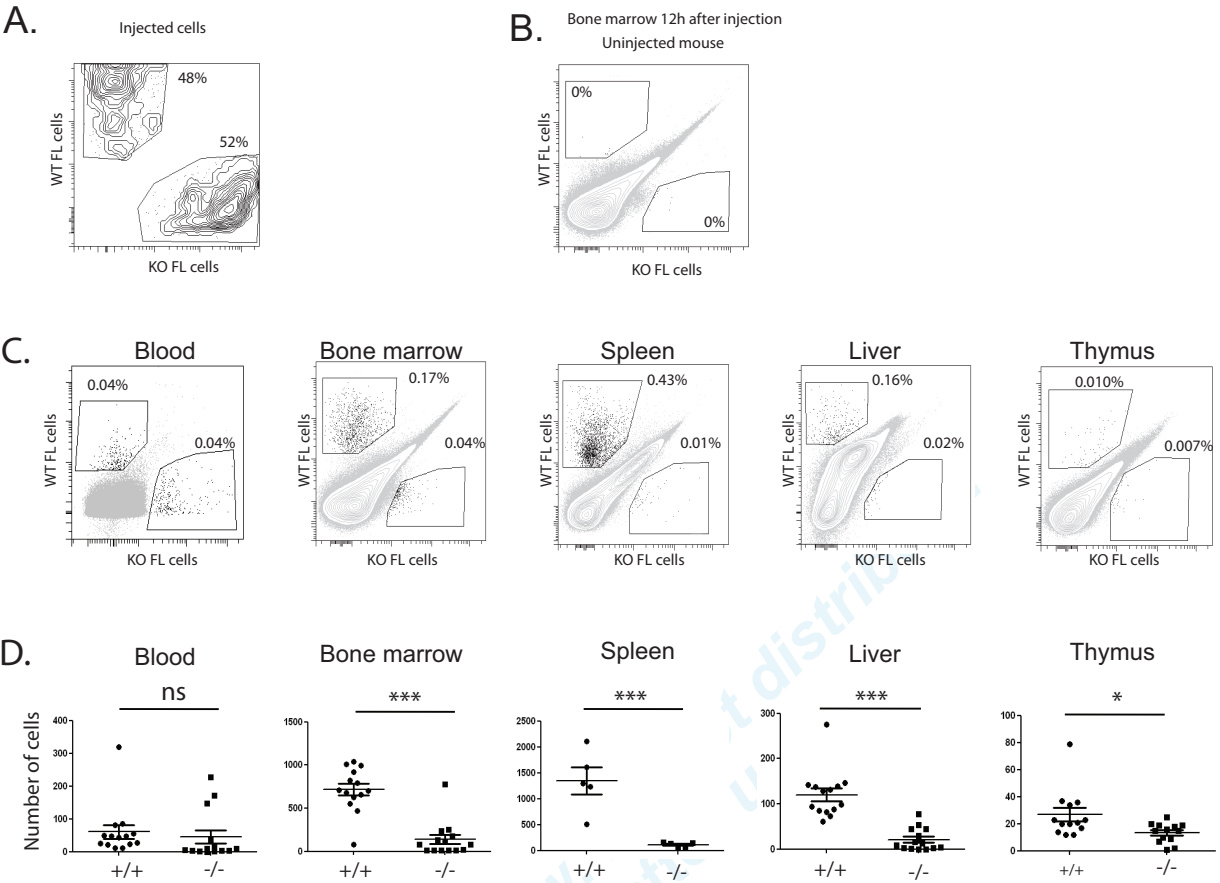


Figure 6

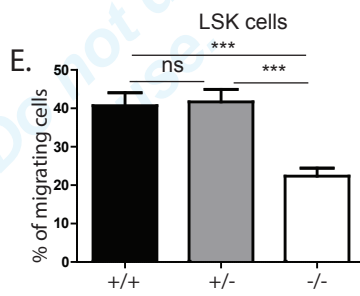
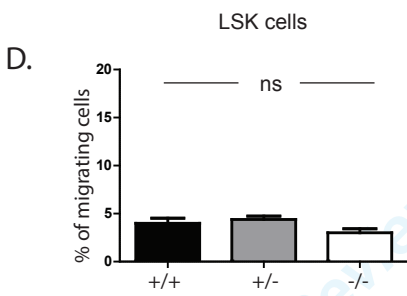
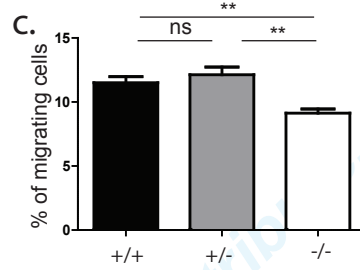
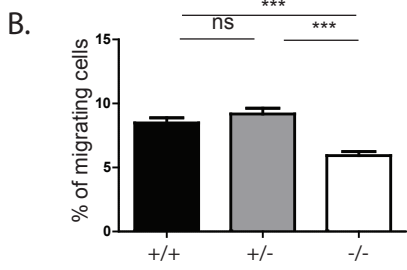
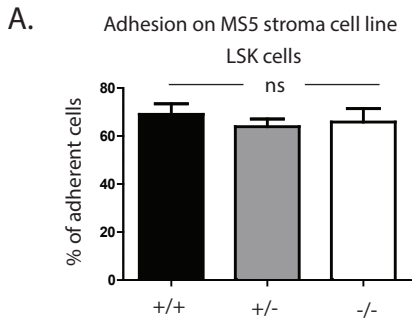


Figure 7