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Supplementary Materials for

Conditionally pathogenic genetic variants of a hematopoietic disease–suppressing enhancer

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This PDF file includes:

Supplementary Text

Developmental requirements for the *Gata2* +9.5 E-box

While the majority of E15.5 embryos revealed no phenotypic abnormalities (Fig. S3), 17% of live +9.5(E-box)$^{-/-}$ embryos (11/63) had edema and 3% (2/63) hemorrhaging. Regardless of vascular defects, no significant differences in fetal liver cellularity or *Gata2* expression in the fetal liver were seen in +9.5(E-box)$^{-/-}$ embryo.

HSC genesis in the +9.5$^{-/-}$ or +9.5(E-box;Ets)$^{-/-}$ aorta-gonad-mesonephros (AGM) is defective, based on lack of HSC-containing clusters and depletion of long-term repopulating HSCs (Gao et al., (*33*); Soukup et al., (*32*)). Point mutation of the +9.5 Ets motif led to an ~50% reduction in emerging hematopoietic cells (Soukup et al., (*32*)). To determine if the +9.5(E-box)$^{-/-}$ mutation impacts the endothelial to hematopoietic transition, 3D confocal analysis of embryos was conducted to quantify emergence of hematopoietic cells. Endothelial and hematopoietic cells express CD31, and hematopoietic, but not endothelial, cells, express c-Kit (Yokomizo et al., (*63*)). While CD31^{+}c-Kit^{+} clusters were abundant in E10.5 wild type AGM, +9.5(E-box)$^{-/-}$ embryos had 2.8-fold fewer clusters (Fig. S3).

To assess whether defects in hematopoietic cell emergence persisted throughout embryogenesis, we quantified immunophenotypic HSPCs in E14.5 fetal liver. +9.5(E-box)$^{-/-}$ E14.5 fetal liver LSKs and MPPs were increased 2.2- and 2.5-fold, respectively, vs. wild type, with no significant alterations in HSC levels. By contrast, GMP levels were decreased 28% in +9.5(E-box)$^{+/-}$ and 2.3-fold in +9.5(E-box)$^{-/-}$ embryos (Fig S3).

Deregulation of *Gata2* through deletion of the -77 enhancer leads to increased LSKs in the embryo as well as skewed levels of myeloid progenitor populations (Johnson et al., (*14*)). Competitive transplantation of these aberrant HSPCs revealed increased repopulating activity as well as increased monocytic differentiation. We therefore asked whether the defects seen in +9.5(E-box)$^{-/-}$ embryos translated to altered regenerative capacity after transplantation. In contrast to what was seen from -77 embryos, competitive transplantation of E15.5 fetal liver from WT and +9.5(E-box)$^{-/-}$ revealed no significant differences in repopulation capacity and only minor changes in lymphoid output (Fig. S4). Terminal analysis of bone marrow from transplanted animals revealed no significant differences in any of the HSPC populations assessed. Thus, the E-box is dispensable for developmental hematopoiesis and embryogenesis yet contributes to select +9.5 functions.

Figure S1

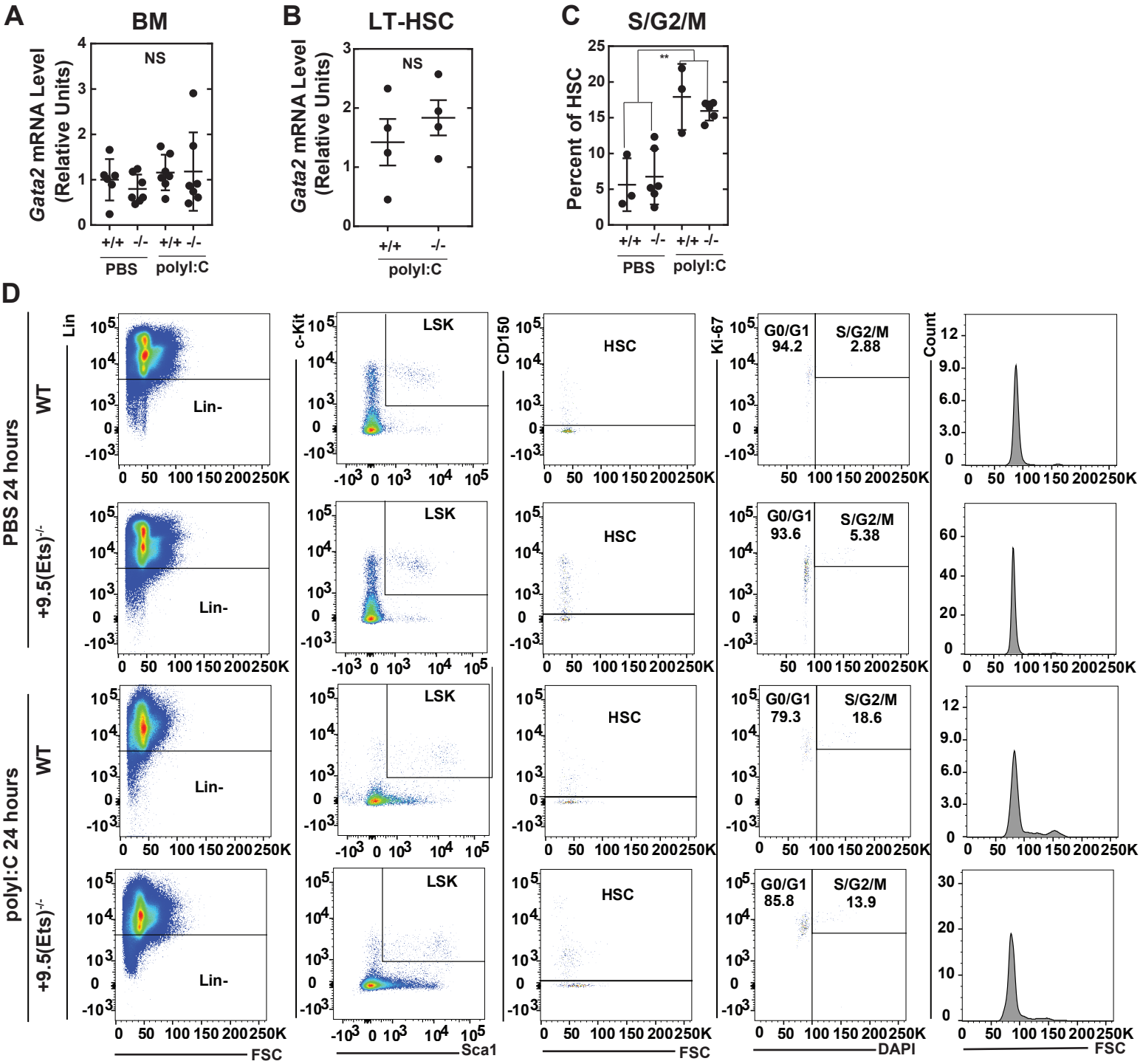
A
BM
Gata2 mRNA Level (Relative Units)
NS
+/+ -/- +/+ -/-
PBS polyI:C
B
LT-HSC
Gata2 mRNA Level (Relative Units)
NS
+/+ -/-
polyI:C
C
S/G2/M
Percent of HSC
**
+/+ -/- +/+ -/-
PBS polyI:C
D
PBS 24 hours
WT
+9.5(Ets)-/-
polyI:C 24 hours
WT
+9.5(Ets)-/-
Lin
c-Kit
CD150
Ki-67
Count
Lin-
LSK
HSC
G0/G1 94.2
S/G2/M 2.88
G0/G1 93.6
S/G2/M 5.38
G0/G1 79.3
S/G2/M 18.6
G0/G1 85.8
S/G2/M 13.9
FSC
Sca1
FSC
DAPI
FSC

Fig. S1. Related to Figure 1. 10 mg/kg polyI:C or vehicle control was administered to WT and +9.5(Ets)$^{-/-}$ animals. Bone marrow was harvested after 24 hours. (A) mRNA quantification from bone marrow (B) mRNA quantification from FACS-isolated LT-HSCs ($Lin^{-}CD48^{-}Sca1^{+}Kit^{+}CD150^{+}$). (C) Cell cycle analysis. (D) Representative flow cytometry plots for analysis of HSC cell cycle ($Lin^{-}CD48^{-}Sca1^{+}Kit^{+}CD150^{+}$).

Figure S2

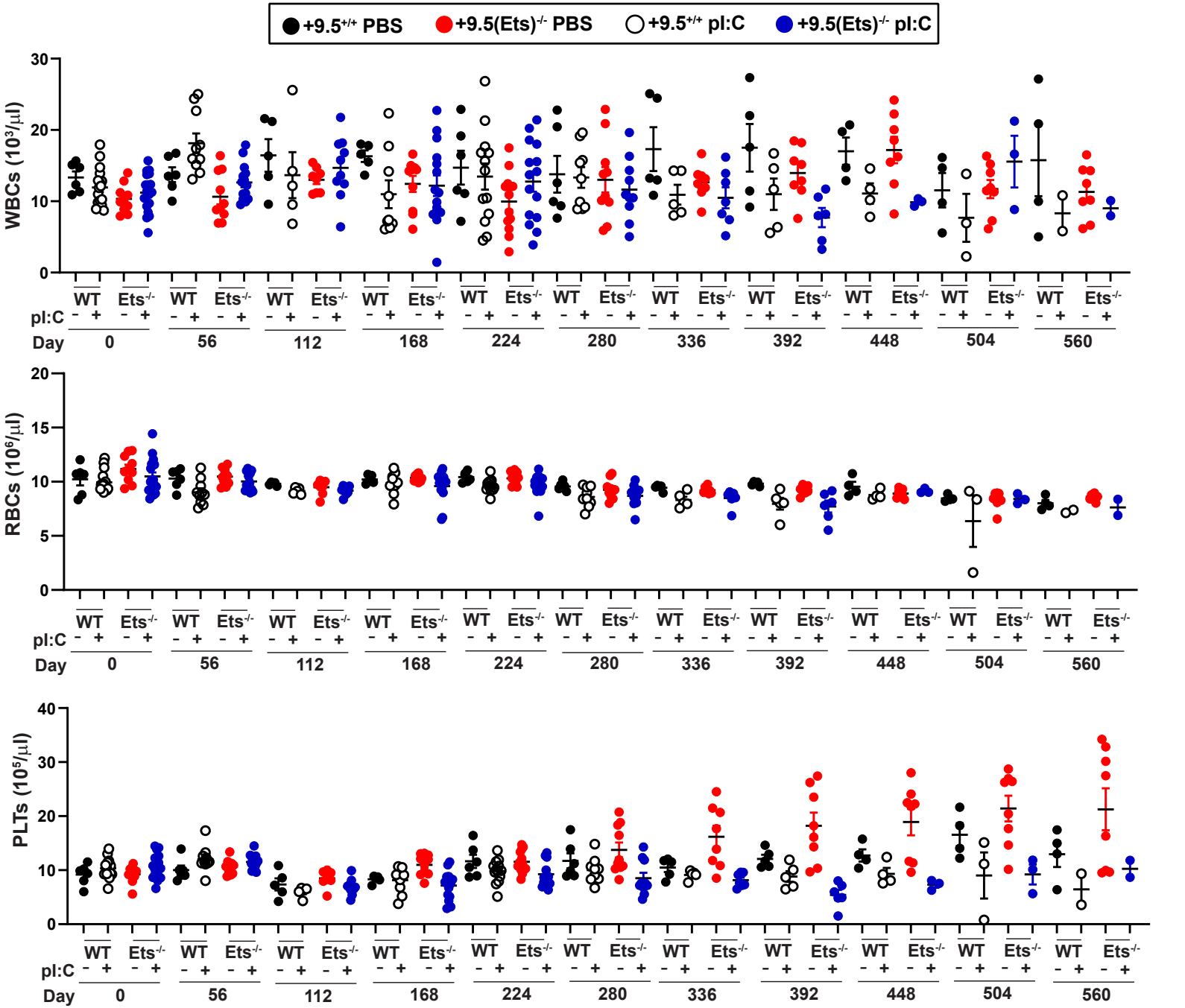
+9.5+/+ PBS
+9.5(Ets)-/- PBS
+9.5+/+ pI:C
+9.5(Ets)-/- pI:C
WBCs (10³/μl)
RBCs (10⁶/μl)
PLTs (10⁵/μl)
WT
Ets-/-
pI:C
Day
0
56
112
168
224
280
336
392
448
504
560

Fig. S2. Related to Figure 1. Quantification of peripheral blood parameters. ~0.1 ml of peripheral blood was drawn from the retroorbital sinus and parameters quantified on a HemaVet analyzer.

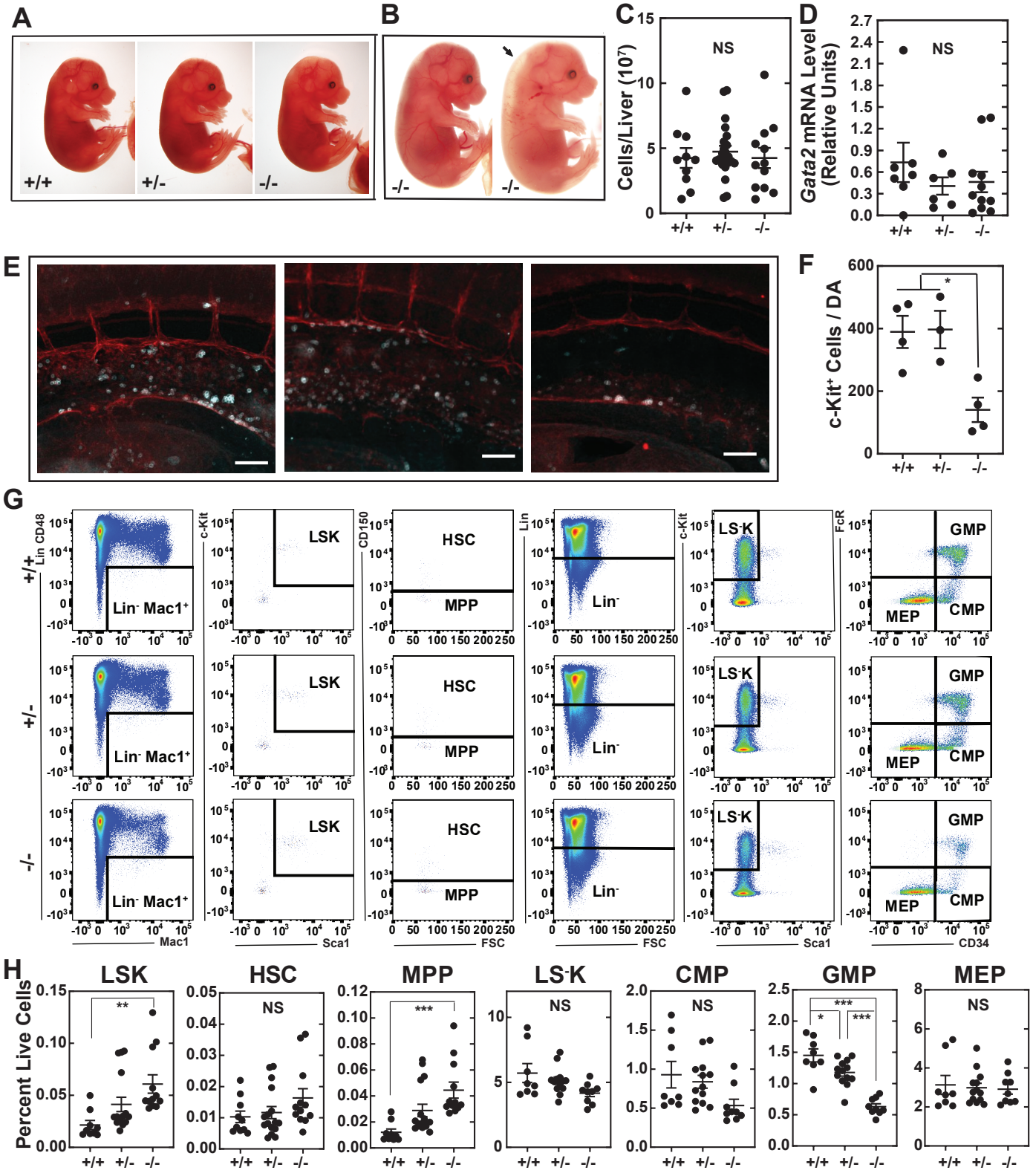
A
+/+
+/-
-/-
B
-/-
-/-
C
Cells/Liver (10^7)
NS
+/+ +/- -/-
D
Gata2 mRNA Level (Relative Units)
NS
+/+ +/- -/-
E
F
c-Kit+ Cells / DA
*
+/+ +/- -/-
G
+/+
+/-
-/-
Lin CD48
Lin- Mac1+
c-Kit
LSK
CD150
HSC
MPP
Lin
Lin-
LS-K
FcR
GMP
MEP
CMP
Mac1
Sca1
FSC
CD34
H
Percent Live Cells
LSK
HSC
MPP
LS-K
CMP
GMP
MEP
NS
**
***
*
+/+ +/- -/-

Fig. S3. Related to Figure 3. The +9.5 E-box is not required for developmental hematopoiesis. HSPC genesis in +9.5(E-box)$^{-/-}$ embryos (A) Representative E15.5 littermates (B) Representative E15.5 littermates. Arrow indicates area of edema. (C) Quantification of E15.5 liver cellularity [+9.5(E-box;Ets$^{+/+}$) (n = 10), +9.5(E-box)$^{+/-}$ (n = 22), and +9.5(E-box)$^{-/-}$ (n = 12) from 4 experiments]. (D) *Gata2* mRNA quantification from E15.5 fetal liver [+9.5(E-box)$^{+/+}$ (n = 7), +9.5(E-box)$^{+/-}$ (n = 7), and +9.5(E-box)$^{-/-}$ (n = 11) from 4 experiments]. (E) Whole-mount immunostaining of E10.5 dorsal aorta (DA). CD31^{+} cells, magenta; c-Kit^{+} cells, green. Scale bars, 80 μm. (F) c-Kit^{+} cell quantification within the DA [+9.5$^{+/+}$ (n = 4), +9.5(E-box)$^{+/-}$ (n = 3), and +9.5(E-box)$^{-/-}$ (n = 4)]. (two-tailed unpaired Student's t test). (G) Flow cytometric analysis of E15.5 fetal liver for LSK (Lin^{-}CD41^{-}CD48^{-}Mac1^{+}Sca1^{+}Kit^{+}), HSC (Lin^{-}CD41^{-}CD48^{-}Mac1^{+}Sca1^{+}Kit^{+}CD150^{+}), MPP (Lin^{-}CD41^{-}CD48^{-}Mac1^{+}Sca1^{+}Kit^{+}CD150^{-}), LS^{-}K (Lin^{-}Sca1^{-}Kit^{+}), MEP (Lin^{-}Sca1^{-}Kit^{+}FcgR^{-}CD34^{-}), CMP (Lin^{-}Sca1^{-}Kit^{+}FcgR^{-}CD34^{+}) and GMP (Lin^{-}Sca1^{-}Kit^{+}FcgR^{+}CD34^{+}). (H) HSPC quantification (percentage of live fetal liver cells). [+9.5$^{+/+}$ (n = 13), +9.5(E-box)$^{+/-}$ (n = 16), and +9.5(E-box)$^{-/-}$ (n = 12) from 5 experiments]. Quantitative data from n > 4 are represented as a box-and-whisker plot, with bounds from 25th to 75th percentile, median line, and whiskers ranging from minimum to maximum values. **P* < 0.05, ***P* < 0.01, ****P* < 0.001 (two-tailed unpaired Student's t test with Benjamini-Hochberg correction).

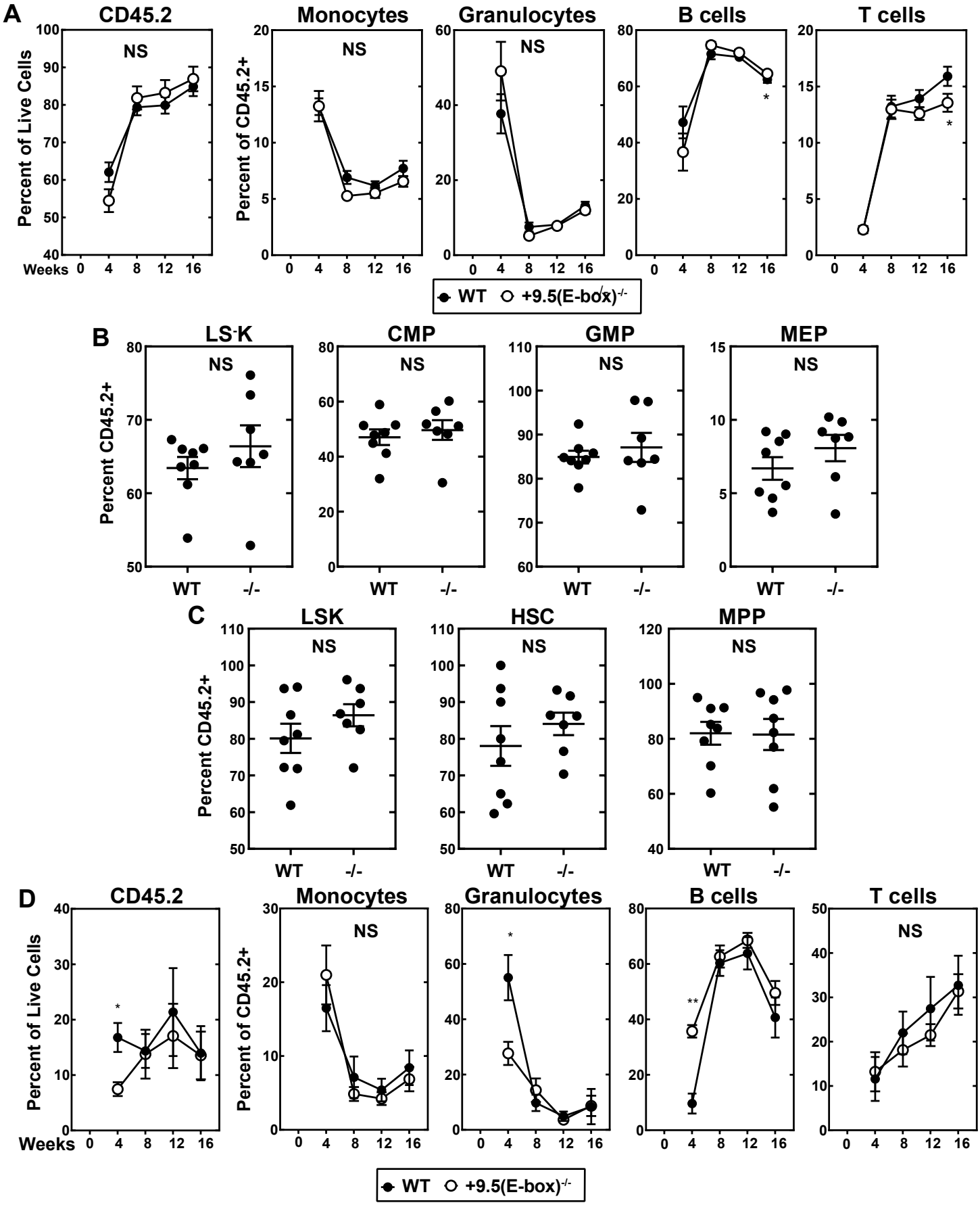
A
CD45.2
Monocytes
Granulocytes
B cells
T cells
Percent of Live Cells
Percent of CD45.2+
NS
Weeks
WT
+9.5(E-box)-/-
B
LS-K
CMP
GMP
MEP
Percent CD45.2+
WT
-/-
C
LSK
HSC
MPP
D
CD45.2
Monocytes
Granulocytes
B cells
T cells
Percent of Live Cells
Percent of CD45.2+
Weeks
WT
+9.5(E-box)-/-

Figure S4. Related to Figure 3. +9.5 E-box corruption does not impair donor-derived hematopoiesis (**A**) Repopulating activity in peripheral blood 16 weeks post-competitive transplant of E15.5 Fetal Liver (n = 9-10 per genotype) (two-tailed unpaired Student's t test). (**B**) Donor-derived hematopoiesis of $LS^{-}K$, CMP, GMP, and MEP within BM 16 weeks post-competitive transplant (n = 7-8 per genotype) (two-tailed unpaired Student's t test) (**C**) Donor-derived hematopoiesis of LSK, HSC, and MPP within BM 16 weeks post-competitive transplant (n = 7-8 per genotype) (two-tailed unpaired Student's t test). (**D**) Repopulating activity in peripheral blood 16 weeks post-secondary transplant (n = 9-10 per genotype) (two-tailed unpaired Student's t test).

Figure S5

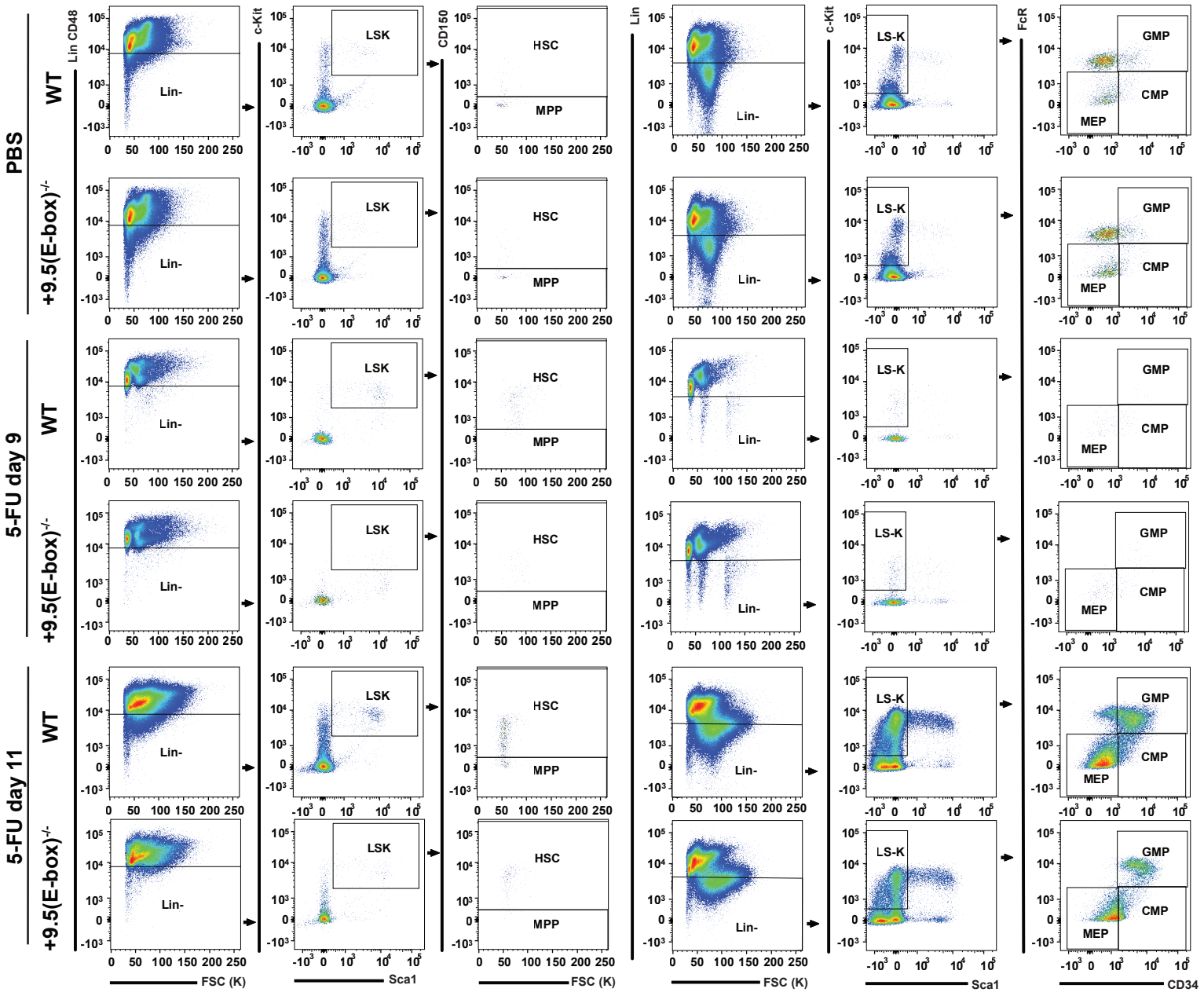
PBS
5-FU day 9
5-FU day 11
WT
+9.5(E-box)-/-
Lin CD48
c-Kit
CD150
Lin
c-Kit
FcR
Lin-
LSK
HSC
MPP
LS-K
GMP
CMP
MEP
FSC (K)
Sca1
CD34

Figure S5. Related to Figure 3. Supporting flow cytometry graphs (A) Representative flow cytometry graphs for quantification of LSK ($Lin^{-}CD48^{-}Sca1^{+}Kit^{+}$), HSC ($Lin^{-}CD48^{-}Sca1^{+}Kit^{+}CD150^{+}$), MPP ($Lin^{-}CD48^{-}Sca1^{+}Kit^{+}CD150^{-}$), $LS^{-}K$ ($Lin^{-}Sca1^{-}Kit^{+}$), MEP ($Lin^{-}Sca1^{-}Kit^{+}FcgR^{-}CD34^{-}$), CMP ($Lin^{-}Sca1^{-}Kit^{+}FcgR^{-}CD34^{+}$) and GMP ($Lin^{-}Sca1^{-}Kit^{+}FcgR^{+}CD34^{+}$) from total bone marrow after vehicle (PBS), 9 days 5-FU, or 11 days 5-FU (250 mg/kg) treatment.

Figure S6

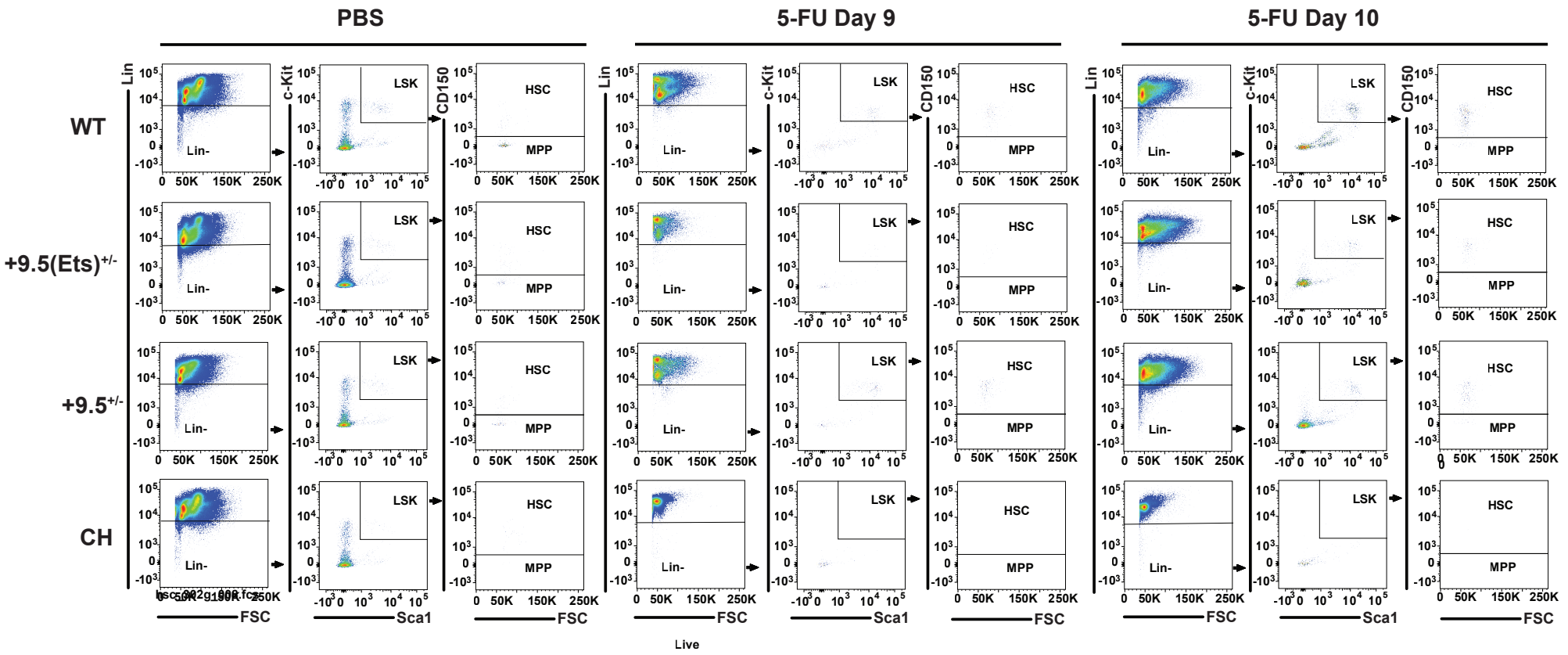
PBS
5-FU Day 9
5-FU Day 10
WT
+9.5(Ets)+/-
+9.5+/-
CH
Lin
Lin-
c-Kit
LSK
CD150
HSC
MPP
FSC
Sca1
Live

Figure S6. Related to Figure 5. Supporting flow cytometry graphs. Representative flow cytometry graphs for quantification of LSK ($Lin^{-}CD48^{-}Sca1^{+}Kit^{+}$), HSC ($Lin^{-}CD48^{-}Sca1^{+}Kit^{+}CD150^{+}$), and MPP ($Lin^{-}CD48^{-}Sca1^{+}Kit^{+}CD150^{-}$) from total bone marrow after vehicle (PBS), 9 days 5-FU, or 11 days 5-FU (250 mg/kg) treatment.

Figure S7

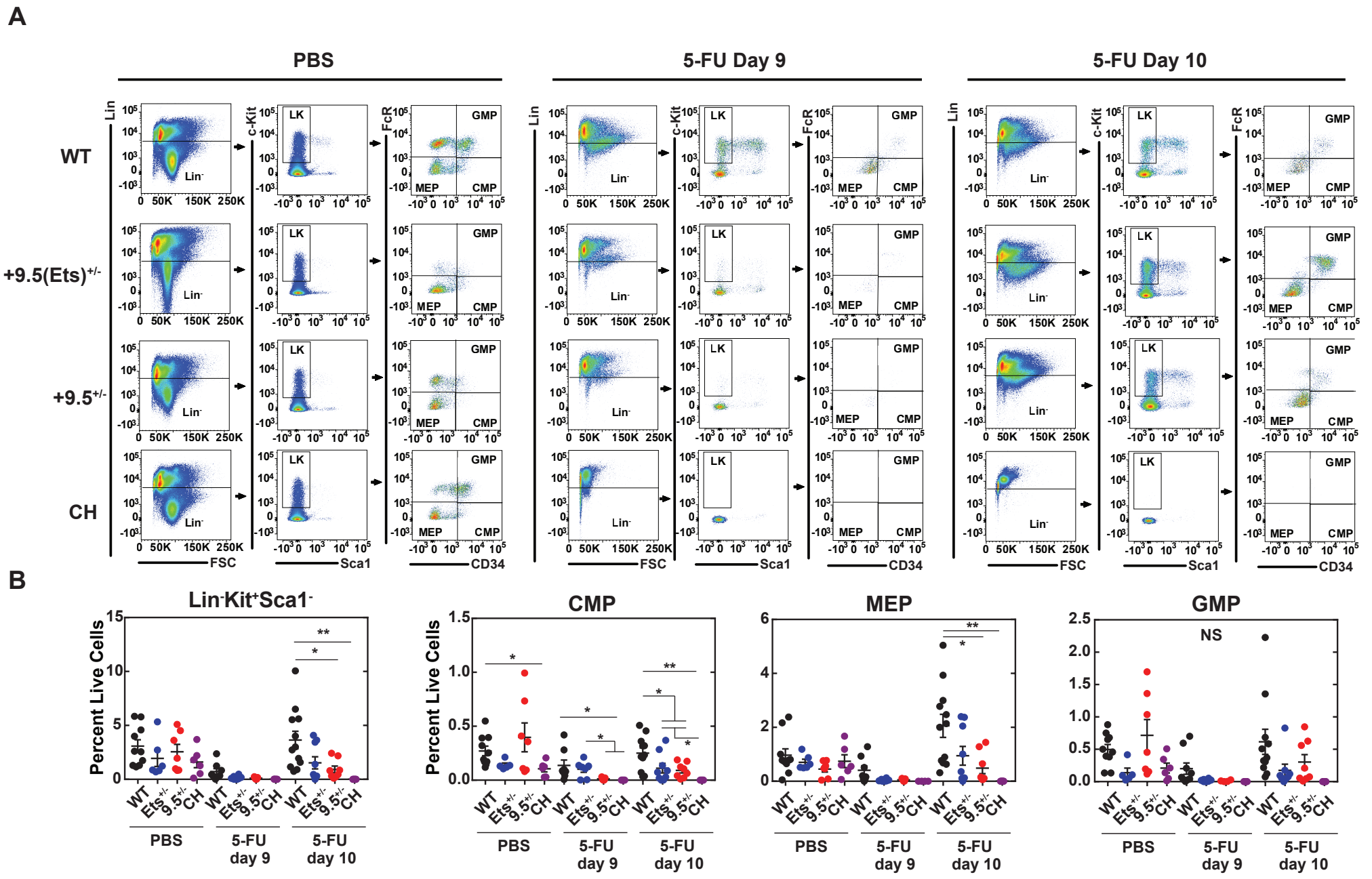
A
PBS
5-FU Day 9
5-FU Day 10
WT
+9.5(Ets)+/-
+9.5+/-
CH
Lin
c-Kit
FcR
LK
GMP
MEP
CMP
Lin-
FSC
Sca1
CD34
B
Lin-Kit+Sca1-
CMP
MEP
GMP
Percent Live Cells
NS
WT
Ets+/-
9.5+/-
CH
PBS
5-FU day 9
5-FU day 10

Figure S7. Related to Figure 5. CH mutation blocks regeneration of blood progenitors following myeloablation. Representative flow cytometry graphs for quantification of $LS^{-}K$ ($Lin^{-}Sca1^{-}Kit^{+}$), MEP ($Lin^{-}Sca1^{-}Kit^{+}FcgR^{-}CD34^{-}$), CMP ($Lin^{-}Sca1^{-}Kit^{+}FcgR^{-}CD34^{+}$) and GMP ($Lin^{-}Sca1^{-}Kit^{+}FcgR^{+}CD34^{+}$) from total bone marrow after vehicle (PBS), 9 days 5-FU, or 11 days 5-FU (250 mg/kg) treatment.

Figure S8

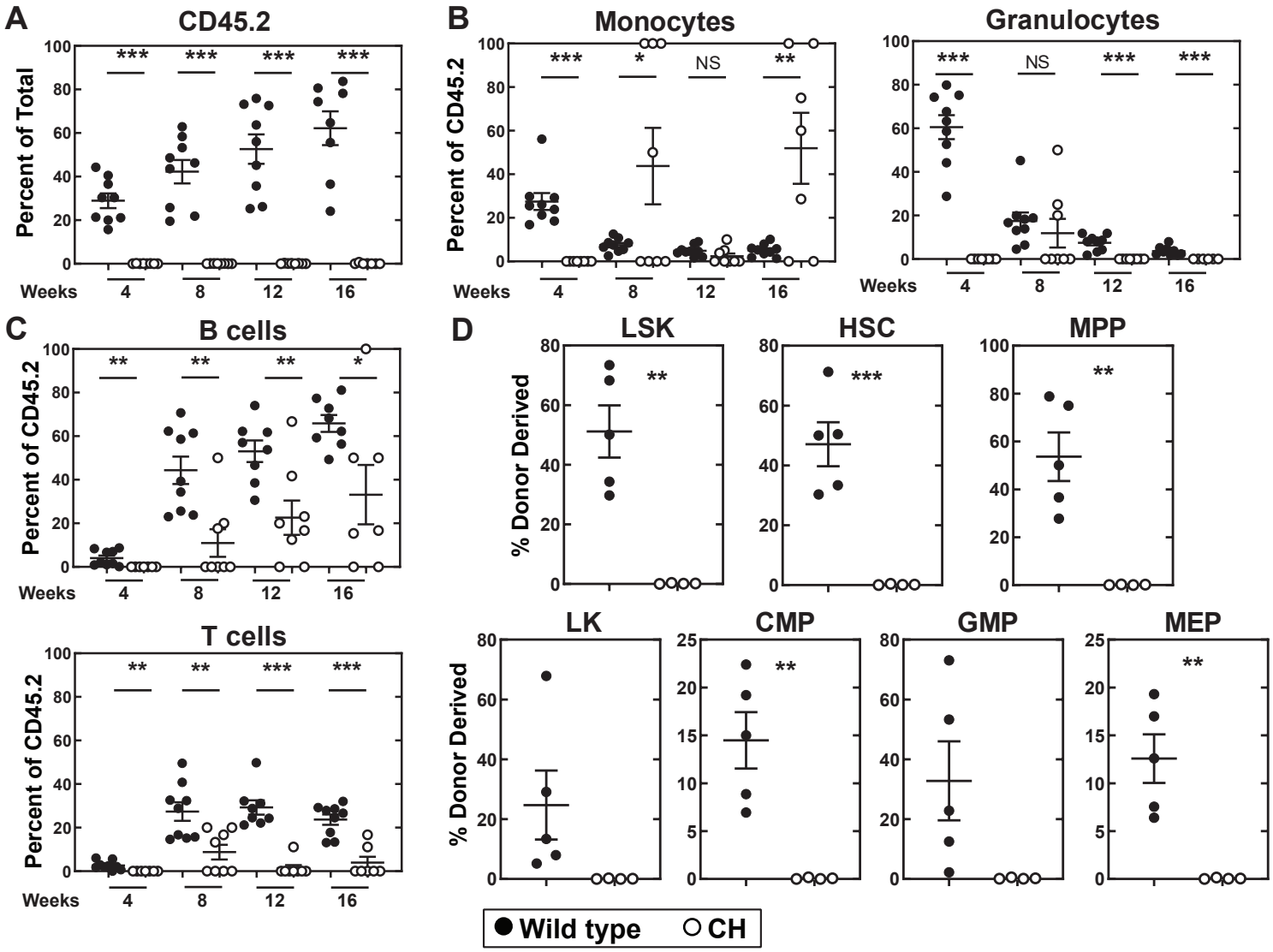
A
CD45.2
Percent of Total
Weeks
4
8
12
16
B
Monocytes
Granulocytes
Percent of CD45.2
NS
C
B cells
T cells
D
LSK
HSC
MPP
LK
CMP
GMP
MEP
% Donor Derived
Wild type
CH

Figure S8. Related to Figure 6. Impaired donor derived hematopoiesis of CH marrow after secondary transplant. (**A**) Repopulating activity in peripheral blood 16 weeks post-secondary transplant of total bone marrow (n = 9 per genotype) (two-tailed unpaired Student's t test). (**B**) Percentage of donor derived myeloid cells. (**C**) Percentage of donor derived lymphoid cells. (n = 8-9 per genotype) (two-tailed unpaired Student's t test). (**D**) Donor-derived hematopoiesis of LSK, HSC, and MPP within BM 16 weeks post-competitive transplant. Donor-derived hematopoiesis of $LS^{-}K$, CMP, GMP, and MEP after BM 16 weeks post-competitive transplant (n = 4-5 per genotype) (two-tailed unpaired Student's t test).

Figure S9

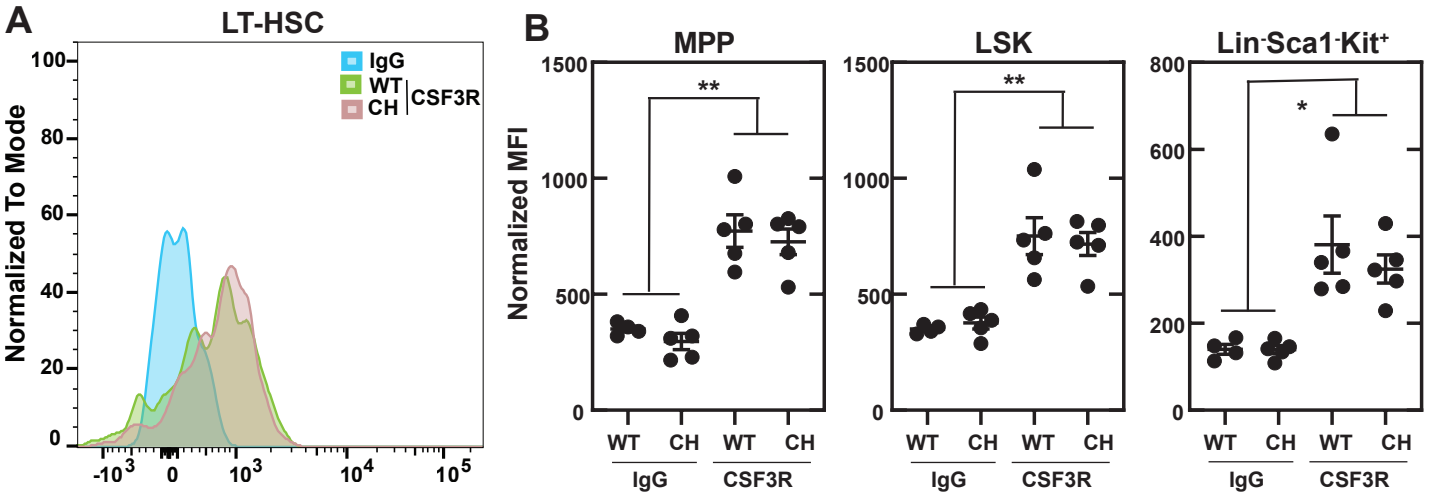
A
LT-HSC
IgG
WT
CH
CSF3R
Normalized To Mode
100
80
60
40
20
0
-10^3
0
10^3
10^4
10^5
B
MPP
LSK
Lin-Sca1-Kit+
Normalized MFI
1500
1000
500
0
800
600
400
200
**
**
*
WT
CH
WT
CH
IgG
CSF3R

Figure S9. Related to Figure 8. CSF3R levels are unaltered in CH HSPCs. (A) Representative phospho-flow plots of CSF3R levels on LT-HSCs ($Lin^{-}CD48^{-}Sca1^{+}Kit^{+}CD150^{+}$) **(B)** Quantitation of CSF3R on MPPs ($Lin^{-}CD48^{-}Sca1^{+}Kit^{+}CD150^{-}$), LSKs, and $Lin^{-}Sca1^{-}Kit^{+}$. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$; Tukey's multiple comparisons test.