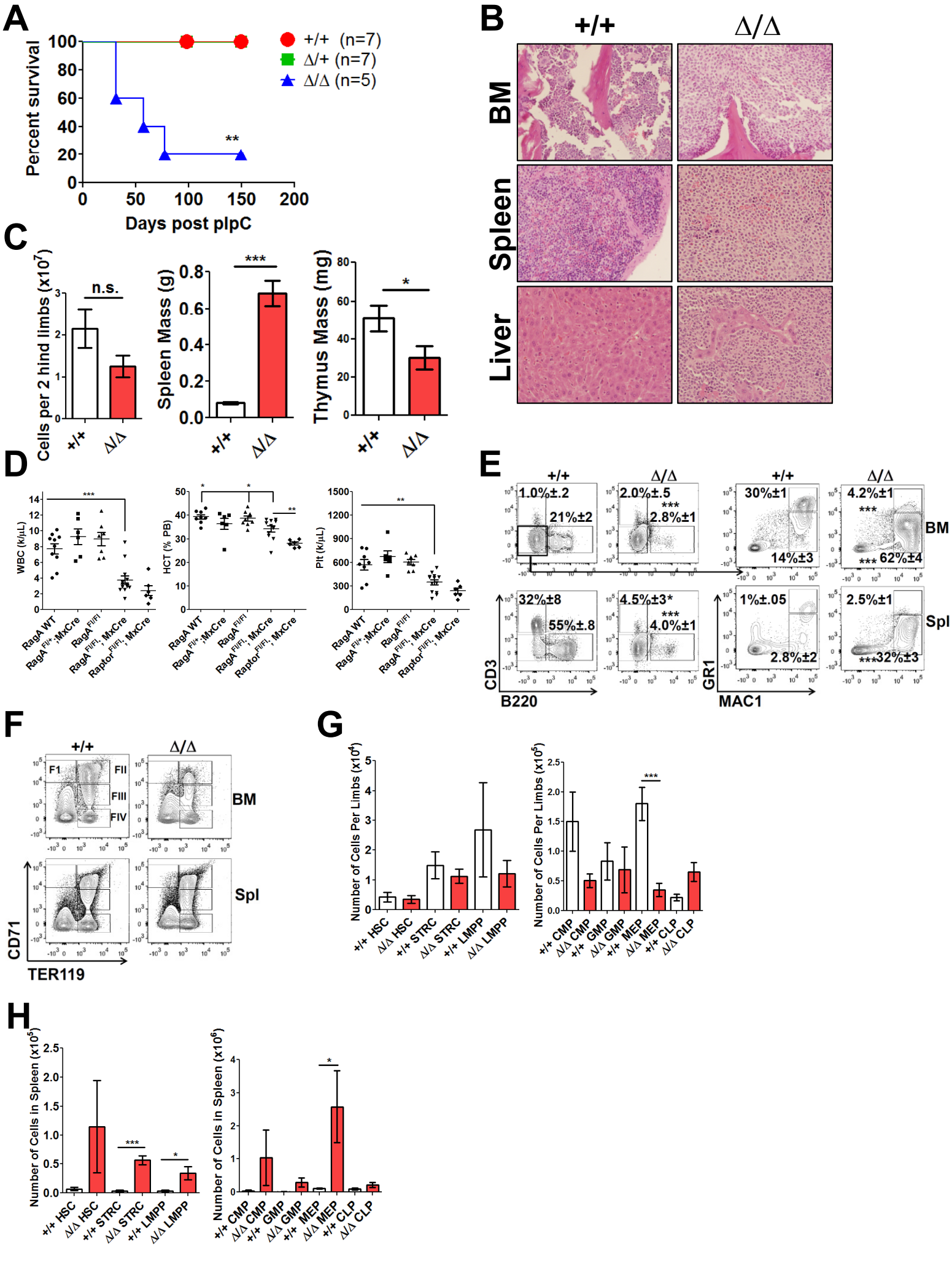

A
Percent survival
Days post plpC
+/+ (n=7)
Δ/+ (n=7)
Δ/Δ (n=5)
**
B
+/+
Δ/Δ
BM
Spleen
Liver
C
Cells per 2 hind limbs (x10^7)
n.s.
Spleen Mass (g)
***
Thymus Mass (mg)
*
+/+
Δ/Δ
D
WBC (K/μL)
HCT (% PB)
Plt (K/μL)
RagA WT
RagA Fl/+; MxCre
RagA Fl/Fl; MxCre
RagA Fl/Fl; MxCre
Raptor Fl/Fl; MxCre
E
+/+
Δ/Δ
1.0%±.2
21%±2
2.0%±.5
2.8%±1
30%±1
14%±3
4.2%±1
62%±4
BM
32%±8
55%±.8
4.5%±3*
4.0%±1
1%±.05
2.8%±2
2.5%±1
32%±3
Spl
CD3
B220
GR1
MAC1
F
+/+
Δ/Δ
F1
FII
FIII
FIV
BM
Spl
CD71
TER119
G
Number of Cells Per Limbs (x10^4)
Number of Cells Per Limbs (x10^5)
+/+ HSC
Δ/Δ HSC
+/+ STRC
Δ/Δ STRC
+/+ LMPP
Δ/Δ LMPP
+/+ CMP
Δ/Δ CMP
+/+ GMP
Δ/Δ GMP
+/+ MEP
Δ/Δ MEP
+/+ CLP
Δ/Δ CLP
H
Number of Cells in Spleen (x10^5)
Number of Cells in Spleen (x10^6)

Supplemental Figure 1. (A) Kaplan-Meier survival curve from mice of the indicated *RagA* genotypes using *MxCre* induced excision of floxed alleles after pIpC treatment (p value derived by log-rank test).

(B) BM, spleen and liver H&E staining from mice of the indicated genotypes showing monocytoid expansion and infiltration. Images are representative of induced *MxCre;RagA*$^{Fl/Fl}$ mice that required euthanasia typically due to abdominal distension.

(C) The number of BM cells (n=8), spleen mass (n=9) and thymus mass (n=8) were measured from mice of the indicated genotype 1-2 months post deletion.

(D) White blood cell counts (WBC) (left panel), hematocrit (Hct) (middle panel) and platelet (Plt) (right panel) measurements from mice of the indicated genotype treated with pIpC 1-1.5 months prior to bleeding (n=6-13).

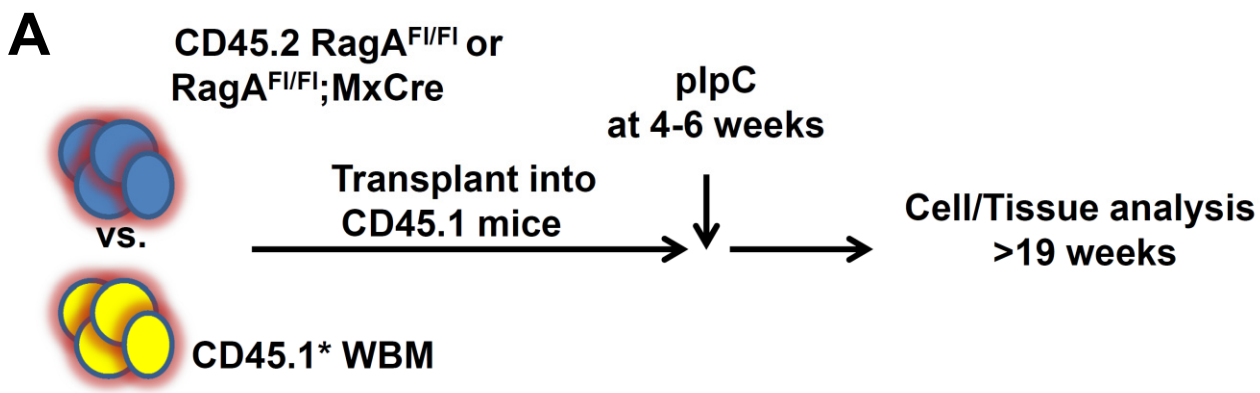
(E) The frequencies of B cells (B220), T cells (CD3), granulocytes ($Mac1^+Gr1^+$) and $Mac1^+Gr1^{lo}$ cells are shown in the BM and spleen (Spl) from mice of the indicated *RagA* genotype 1-1.5 months post deletion (n=3-4).

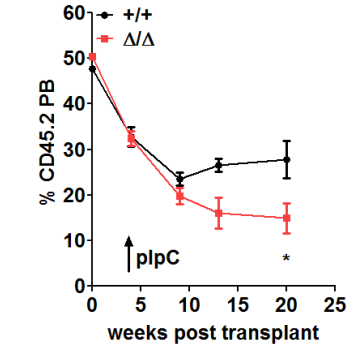
(F) Ter119/CD71 staining was performed from BM and Spl to assess erythroid fractions (F1-FIV) in control and *RagA* deleted mice 1-1.5 months post deletion (n=3).

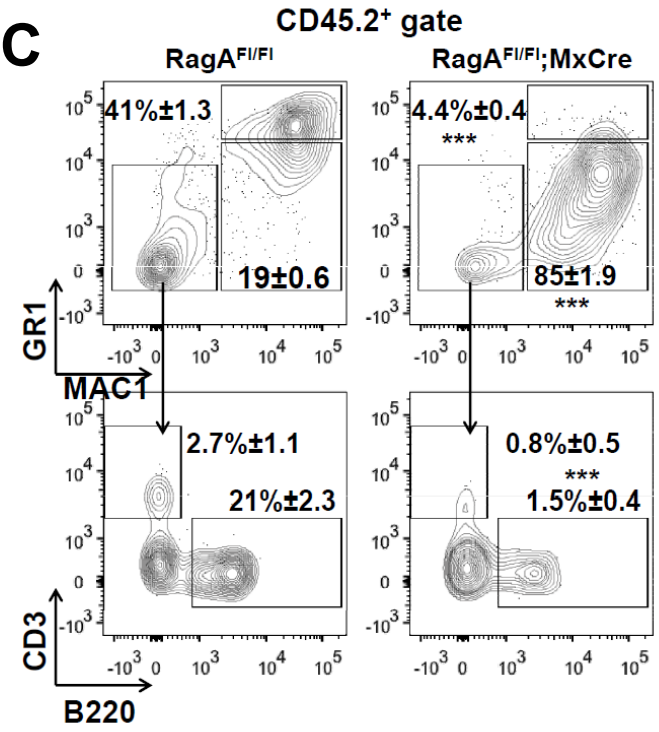
(G) The absolute number of HSPC and committed progenitors are shown in BM from the mice of the indicated genotypes 1-1.5 months post pIpC. (n=7 for BM and n=6-7 for Spl). (HSC = $Lin^-7AAD^-CD127^-Sca1^+cKit^+$ $CD34^-Flt3^-$, STRC = $Lin^-7AAD^-CD127^-Sca1^+cKit^+$ $CD34^+Flt3^-$, LMPP = $Lin^-7AAD^-CD127^-Sca1^+cKit^+$ $CD34^+Flt3^+$).

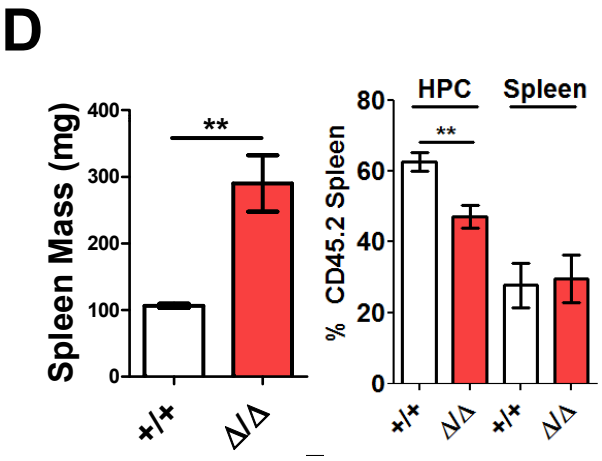
(H) The absolute number of HSPC and committed progenitors are shown in Spleen from the mice of the indicated genotypes 1-1.5 months post pIpC. (n=7 for BM and n=6-7 for Spl). (HSC = $Lin^-Sca1^+cKit^+CD34^-Flt3^-$, STRC = $Lin^{--}Sca1^+cKit^+CD34^+Flt3^-$, LMPP = $Lin^-Sca1^+cKit^+CD34^+Flt3^+$).

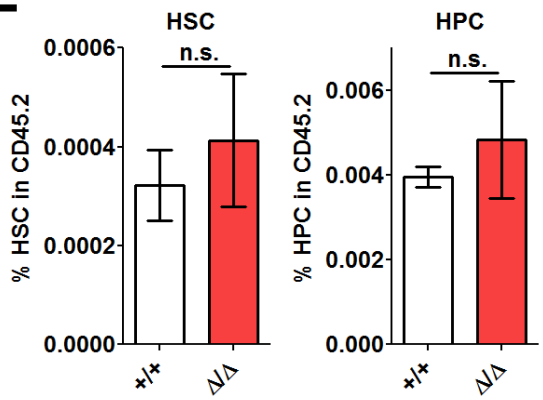
Error bars indicated the S.E.M. (* $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, n.s. = not statistically significant).

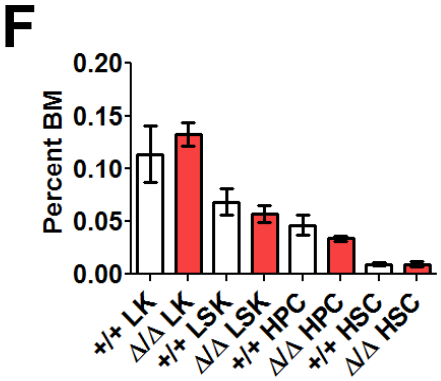

A
CD45.2 RagA^Fl/Fl or
RagA^Fl/Fl;MxCre
vs.
CD45.1* WBM
Transplant into
CD45.1 mice
pIpC
at 4-6 weeks
Cell/Tissue analysis
>19 weeks


B
+/+
Δ/Δ
% CD45.2 PB
pIpC
*
weeks post transplant


C
CD45.2+ gate
RagA^Fl/Fl
RagA^Fl/Fl;MxCre
41%±1.3
19±0.6
4.4%±0.4
***
85±1.9
***
GR1
MAC1
2.7%±1.1
21%±2.3
0.8%±0.5
***
1.5%±0.4
CD3
B220


D
Spleen Mass (mg)
**
+/+
Δ/Δ
HPC
Spleen
% CD45.2 Spleen
**


E
HSC
n.s.
% HSC in CD45.2
HPC
n.s.
% HPC in CD45.2


F
Percent BM
+/+ LK
Δ/Δ LK
+/+ LSK
Δ/Δ LSK
+/+ HPC
Δ/Δ HPC
+/+ HSC
Δ/Δ HSC

Supplemental Figure 2. (A) Experimental schematic to assess HSC function and cell autonomous hematopoietic functions of RagA using either CD45.1 or CD45.1^{STEM} (CD45.1*) competitor cells.

(B) A 1:1 mixture of CD45.2 test cells (indicated) were transplanted with CD45.1^{STEM} (CD45.1*) competitor cells. At the indicated time point mice were treated with pIpC and peripheral blood (PB) was collected and analyzed for CD45.2 chimerism.

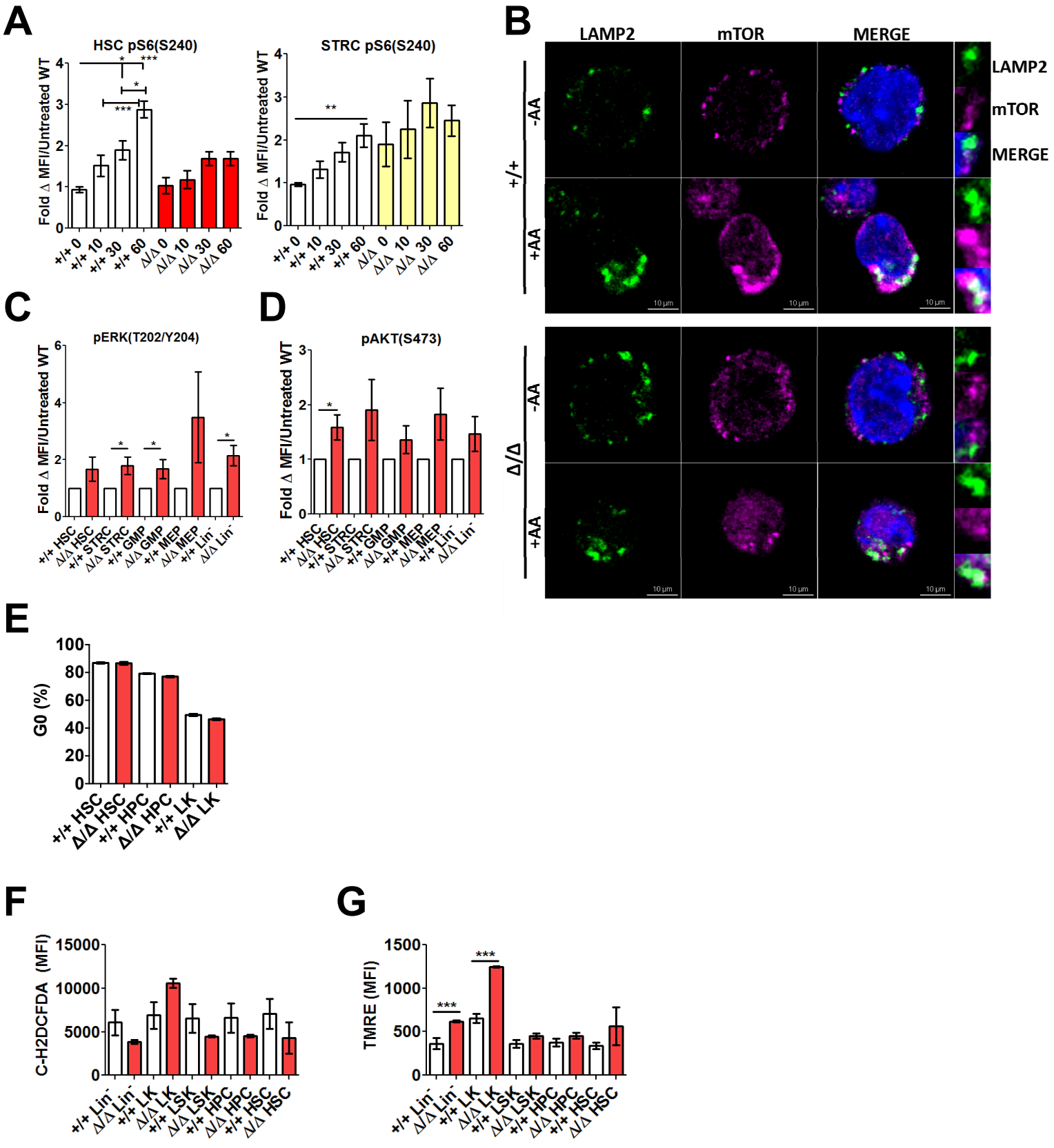
(C) The type and percentage of mature hematopoietic cells produced in transplant recipients from the bone marrow of transplant recipients at >19 weeks post pIpC is shown (n=4).

(D) Spleen mass (n=4) and percent chimerism (n=7) in each cell type of the indicated genotypes is shown at >19 weeks post transplantation (HPC = Lin$^-$Sca1$^+$cKit$^+$CD48$^+$).

(E) 600 RagA-deleted HSCs (CD48$^-$CD150$^+$LSK) were transplanted into lethally-irradiated CD45.1 mice. HSC (Lin$^-$Sca1$^+$cKit$^+$CD150$^+$CD48$^-$) and HPC (Lin$^-$ Sca1$^+$cKit$^+$CD48$^+$) CD45.2 donor BM chimerism was assessed 2 weeks post transplantation in mice receiving cells of the indicated genotype (n=5).

(F) The frequencies of HSPCs in RagA-deficient mice on day 7 post-exposure to 150 mg/kg 5-FU (n=5).

Error bars indicated the S.E.M. (* $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, n.s. = not statistically significant).


A
HSC pS6(S240)
STRC pS6(S240)
Fold Δ MFI/Untreated WT
+/+ 0
+/+ 10
+/+ 30
+/+ 60
Δ/Δ 0
Δ/Δ 10
Δ/Δ 30
Δ/Δ 60
B
LAMP2
mTOR
MERGE
+/+
Δ/Δ
-AA
+AA
10 μm
C
pERK(T202/Y204)
D
pAKT(S473)
+/+ HSC
Δ/Δ HSC
+/+ STRC
Δ/Δ STRC
+/+ GMP
Δ/Δ GMP
+/+ MEP
Δ/Δ MEP
+/+ Lin-
Δ/Δ Lin-
E
G0 (%)
+/+ HSC
Δ/Δ HSC
+/+ HPC
Δ/Δ HPC
+/+ LK
Δ/Δ LK
F
C-H2DCFDA (MFI)
+/+ Lin-
Δ/Δ Lin-
+/+ LK
Δ/Δ LK
+/+ LSK
Δ/Δ LSK
+/+ HPC
Δ/Δ HPC
+/+ HSC
Δ/Δ HSC
G
TMRE (MFI)

Supplemental Figure 3. (A) Lineage depleted (Lin^{Dep}) BM from mice of the indicated *RagA* genotypes was placed in amino acid (AA) free media containing 10% dialyzed FBS for ~1 hour (AA deprivation) and then stimulated with AA for the indicated time points (minutes). Intracellular flow cytometry was performed to assess levels of pS6 (S240) in the indicated gated populations after amino acid stimulation for the indicated time points in minutes. The fold change in levels of pS6 (S240) relative to unstimulated wt cells is indicated for Lin^{Dep} HSC ($CD34^{-}Sca1^{+}cKit^{+}$) (n=5-7) (left panel) and STRC (short term reconstituting cells, $CD34^{+}Sca1^{+}cKit^{+}$) (n=6-7) (right panel). One-way ANOVA with Tukey's Multiple Comparison Test was applied (left panel) or One-way ANOVA (right panel) within AA treated groups of the indicated genotype and relevant statistically significant differences are noted.

(B) Confocal microscopy was performed on sorted LSK cells from the indicated genotypes from Lin^{Dep} BM that was placed in AA free media for ~1 hr (AA deprivation) then stimulated for 10 minutes with AA prior to fixation. Cells were stained with anti mTOR (violet) and LAMP2 (green) antibodies along with DAPI (blue). Colocalization of mTOR and LAMP2 appears white.

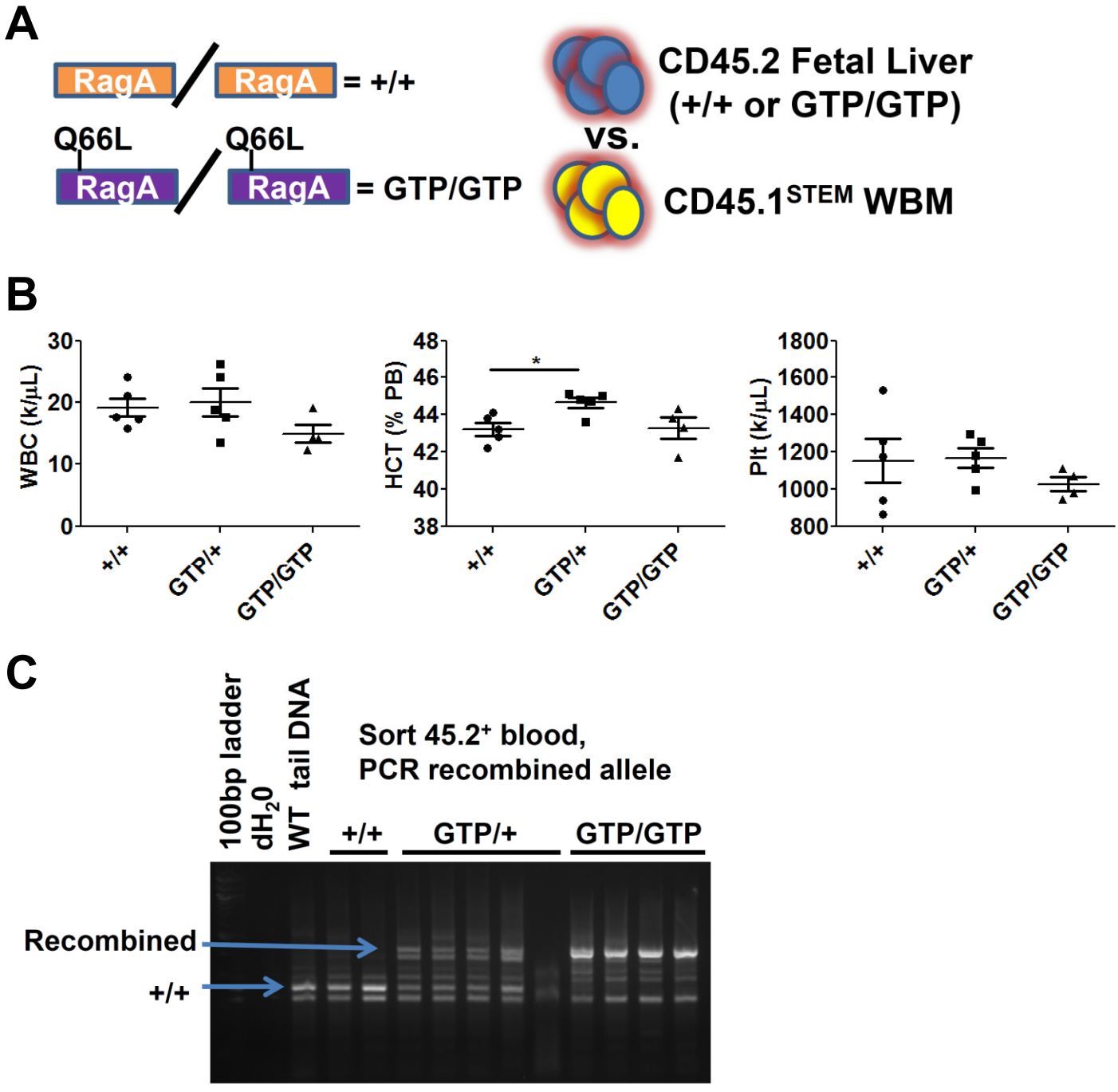
(C and D) Basal levels (cells grown in AA free media + 10% dialyzed FBS for ~1 hr (AA deprivation)) of **(D)** pERK (T202/Y204) and **(E)** pAKT (S473) from gated events of the indicated BM populations and genotype is shown. Fold change is normalized with each individual +/+ unstimulated population set to 1 (n=4-5).

(E) Cell cycle G_0 status in *RagA*-deleted HSPCs from *RagA* deleted mice 1 month post deletion (n=4).

(F) ROS levels were measured upon *RagA* deletion in HSPCs by C-H2CDCFDA staining. The level of signal from each indicated population is shown from *RagA* deleted mice 1 month post deletion (n=4).

(G) Mitochondrial membrane potential measurement with TMRE staining followed by flow cytometry analysis from *RagA* deleted mice 1 month post deletion (n=4).

Error bars indicated the S.E.M. (* $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, n.s. = not statistically significant).


A
RagA
RagA
= +/+
Q66L
RagA
Q66L
RagA
= GTP/GTP
CD45.2 Fetal Liver
(+/+ or GTP/GTP)
vs.
CD45.1STEM WBM
B
WBC (k/µL)
30
20
10
0
+/+
GTP/+
GTP/GTP
HCT (% PB)
48
46
44
42
40
38
*
+/+
GTP/+
GTP/GTP
Plt (k/µL)
1800
1600
1400
1200
1000
800
+/+
GTP/+
GTP/GTP
C
100bp ladder
dH2O
WT tail DNA
Sort 45.2+ blood,
PCR recombined allele
+/+
GTP/+
GTP/GTP
Recombined
+/+

Supplemental Figure 4. (A) Schematic of "gain-of-function" *RagA* alleles and transplantation experiment utilizing $0.5x10^6$ test fetal liver cells vs. $0.5x10^6$ adult $CD45.1^{STEM}$ competitor cells.
(B) Peripheral blood (PB) parameters from mice receiving fetal liver cells of the indicated genotypes 5 months post transplantation. One-way ANOVA with Tukey's Multiple Comparison Test was applied and relevant statistically significant differences are noted.
(C) PCR was performed on DNA isolated from sorted CD45.2 PB cells to assess allele recombination from cells of the indicated genotype 5 months post transplantation.
Error bars indicated the S.E.M. (* $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, n.s. = not statistically significant).

Supplemental Methods and Materials

Flow Cytometry Antibodies

The following antibody cocktails/antibodies were used:

For HSPC analysis:

Antibody	**Company**	**Dilution**
PE-Cy5-CD3 (145-2C11)	BD	1:100
PE-Cy5-CD4 (RM4-5)	BD	1:100
PE-Cy5-CD8 (53-6.7)	Biolegend	1:100
PE-Cy5-CD19 (6D5)	Biolegend	1:100
PE-Cy5-B220R (RA3-6B2)	eBioscience	1:100
PE-Cy5-Gr1 (RB6-8C5)	eBioscience	1:100
PE-Cy5-Ter119 (TER119)	eBioscience	1:400
BV605-Sca1 (D7)	BD	1:100
BUV395-cKit (2B8)	BD	1:100
PE-CD150 (TC15-12F12.2)	Biolegend	1:50
FITC-CD48 (HM48-1)	Biolegend	1:100 or BV510 BD 1:100
eFluor450-CD34 (RAM34)	eBioscience	1:33
APC-CD135 (A2F10)	Biolegend	1:25
PE-Cy7-CD16/32 (93)	eBioscience	1:200
APC-eFluor780-CD127 (A7R34)	eBioscience	1:33

7AAD was added prior to FACS analysis.

For C-H2DCFDA, TMRE and 5-FU analysis:

Antibody	**Company**	**Dilution**
Biotin-CD3 (145-2C11)	BD	1:100
Biotin-CD4 (GK1.5)	BD	1:100
Biotin-CD8 (53-6.7)	BD	1:100
Biotin-CD11b (M1/70)	BD	1:100
Biotin-B220R (RA3-6B2)	BD	1:100
Biotin-Gr1 (RB6-8C5)	BD	1:100
Biotin-Ter119 (TER119)	BD	1:100
Streptavidin-Pacific Orange	Invitrogen	1:500
BV421-Sca1 (D7)	Biolegend	1:200
APC-cKit (2B8)	BD	1:100

PE-Cy7-CD150 (TC15-12F12.2)	Biolegend	1:100
APC-Cy7-CD48 (HM48-1)	BD	1:500

7AAD was added prior to FACS analysis.

For cell cycle analysis:

Antibody	**Company**	**Dilution**
Biotin-CD3 (145-2C11)	BD	1:100
Biotin-CD4 (RM4-5)	BD	1:100
Biotin-CD8 (53-6.7)	BD	1:100
Biotin-CD11b (M1/70)	BD	1:100
Biotin-B220R (RA3-6B2)	BD	1:100
Biotin-Gr1 (RB6-8C5)	BD	1:100
Biotin-Ter119 (TER119)	BD	1:100
Streptavidin-PE-Cy7	Biolegend	1:500
PE-Cy5.5-Sca1 (D7)	Biolegend	1:200
APC-cKit (2B8)	BD	1:100
PE-CD150 (TC15-12F12.2)	Biolegend	1:200
APC-Cy7-CD48 (HM48-1)	BD	1:500
FITC-KI67 (35/Ki67)	BD	1:10
DAPI	Invitrogen	2 µg/ml

Mature Cell Lineages:

Antibody	**Company**	**Dilution**
APC-CD11b (M1/70)	eBioscience	1:200 or PE 1:200
PE-Cy5-Gr1 (RB6-8C5)	eBioscience	1:200
Pacific Blue-CD3 (17A2)	Biolegend	1:100
FITC-B220R (RA3-6B2)	eBioscience	1:100 or PE-Cy7 1:100

If RBC lineage analyzed:

Antibody	**Company**	**Dilution**
PE-Cy7-Ter119 (TER-119)	BD	1:400
PE-CD71 (R17217)	Biolegend	1:400

For CD45.2/45.1 Analysis:

Antibody	Company	Dilution
PE-CD45.1 (A20)	BD	1:100
APC-45.1 (A20)	eBioscience	1:100
FITC-CD45.2 (104)	BD	1:100
APC-Cy7 CD45.2 (104)	Biolegend	1:100

Confocal Microscopy

Lineage depleted BM was placed at 37C in AA-free RPMI containing 10% dialyzed FBS for 1 hr. Cells were then treated -/+ AA for 10 minutes then fixed with PFA (1.5% final concentration). Cells were surface stained and LSK cells were FACS sorted. Sorted cells were then permeabilized with 0.05% TritonX on ice for 10 min. Cells were then stained with mTOR Ax647 (7C10) (CST), and Lamp2/CD107b (M3/84) Ax488 (Biolegend) antibodies and DAPI in 0.5%BSA/PBS/0.05%Triton, washed and imaged after being placed in chamber slides (Lab-Tek) in 0.5%BSA/PBS. Images were acquired by confocal microscopy using Nikon Ti-8000 inverted microscope equipped with 60X Plan Apo NA 1.4 objective and Qimaging 5.0 RTV micropublisher color CCD camera. Images were processed with NIS-Elements software. Images were prepared with Adobe Photoshop.

Measurement of ROS and Mitochondrial Membrane Potential

Cellular ROS were measured by staining cells with 5-(and -6)-carboxy-20, 70-dichlorodihydrofluorescein diacetate (carboxy-H2DCFDA, 10 µM) (Life Technologies), followed by flow cytometry analysis (1). And mitochondrial membrane potential was analyzed with flow cytometry for TMRE staining (0.1 µM) (Life Technologies) (1). To analyze the ROS and Mitochondrial Membrane Potential by flow cytometry, cells were first stained with antibodies for surface markers and then incubated C-H2DCFDA (10 µM) and TMRE (0.1 µM) for 30min at 37C. 7AAD was added prior to FACS analysis. FACS analysis was performed with an LSRII flow cytometer (BD).

Cell Cycle Assay

To analyze the cell cycle by flow cytometry, cells were first stained with antibodies for surface markers and then fixed and permeabilized according to the manufacturer's instruction (BD) (1, 2). Ki67 and DNA were detected using a FITC anti-Ki67 antibody (35/Ki67) and DAPI (2 µg/ml), respectively.

Supplemental References

1. Wang, Y.H., Israelsen, W.J., Lee, D., Yu, V.W., Jeanson, N.T., Clish, C.B., Cantley, L.C., Vander Heiden, M.G., and Scadden, D.T. 2014. Cell-state-specific metabolic dependency in hematopoiesis and leukemogenesis. *Cell* 158:1309-1323.

2. Goncalves, K.A., Silberstein, L., Li, S., Severe, N., Hu, M.G., Yang, H., Scadden, D.T., and Hu, G.F. 2016. Angiogenin Promotes Hematopoietic Regeneration by Dichotomously Regulating Quiescence of Stem and Progenitor Cells. *Cell* 166:894-906.