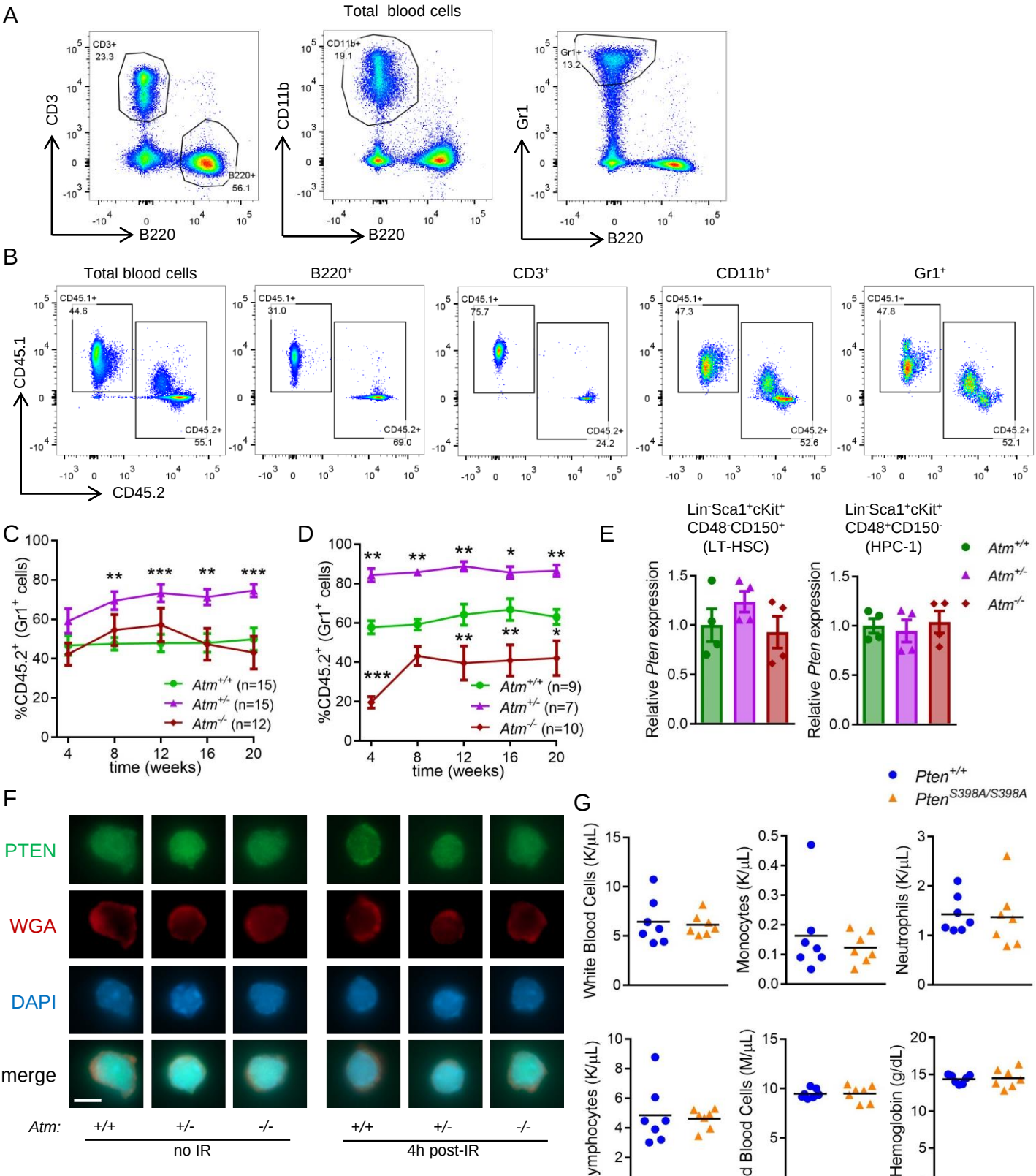
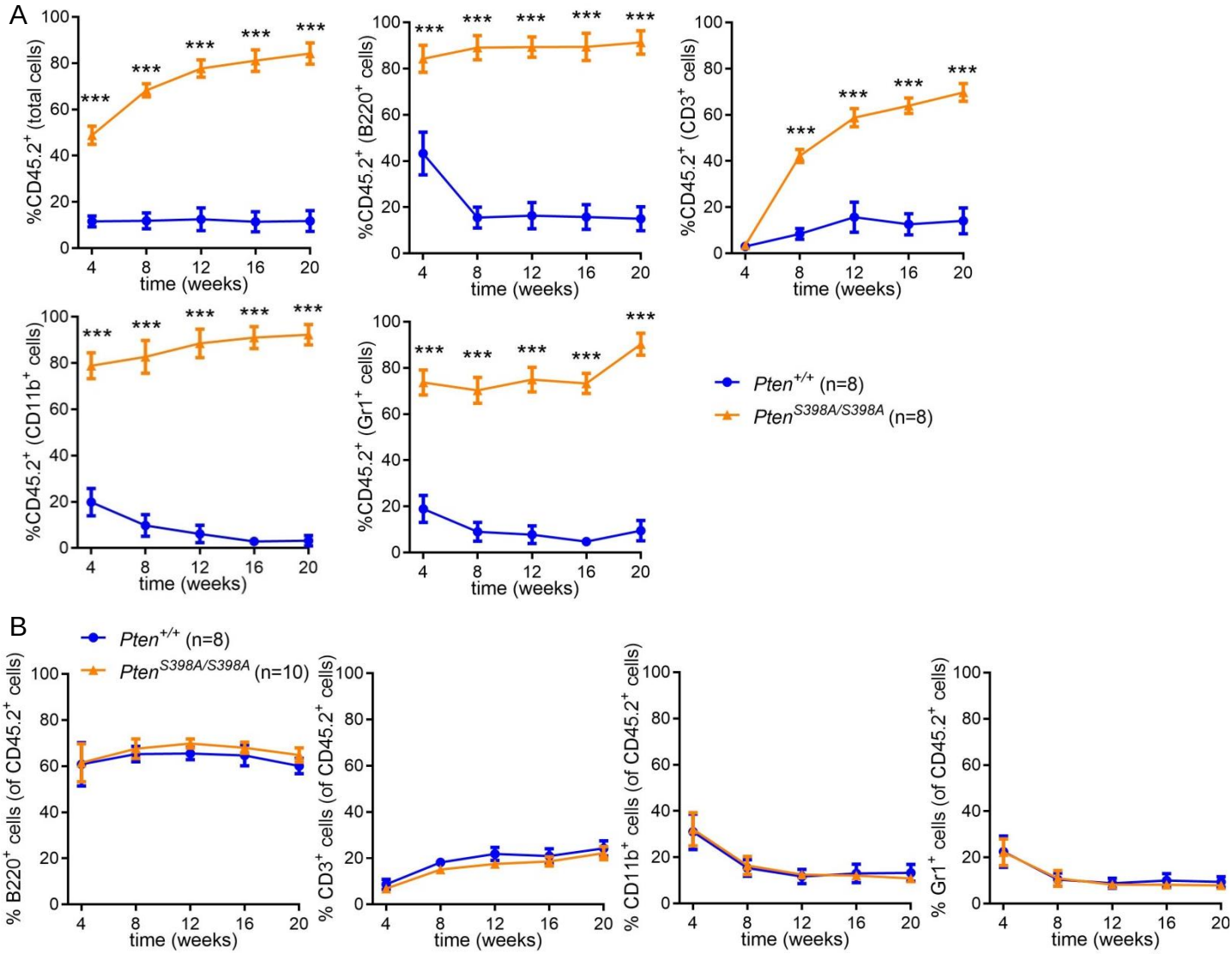



Supplemental Figure 1

**Supplemental Figure 1. Enhanced competitive fitness of *Atm*$^{+/-}$ hematopoietic cells.**

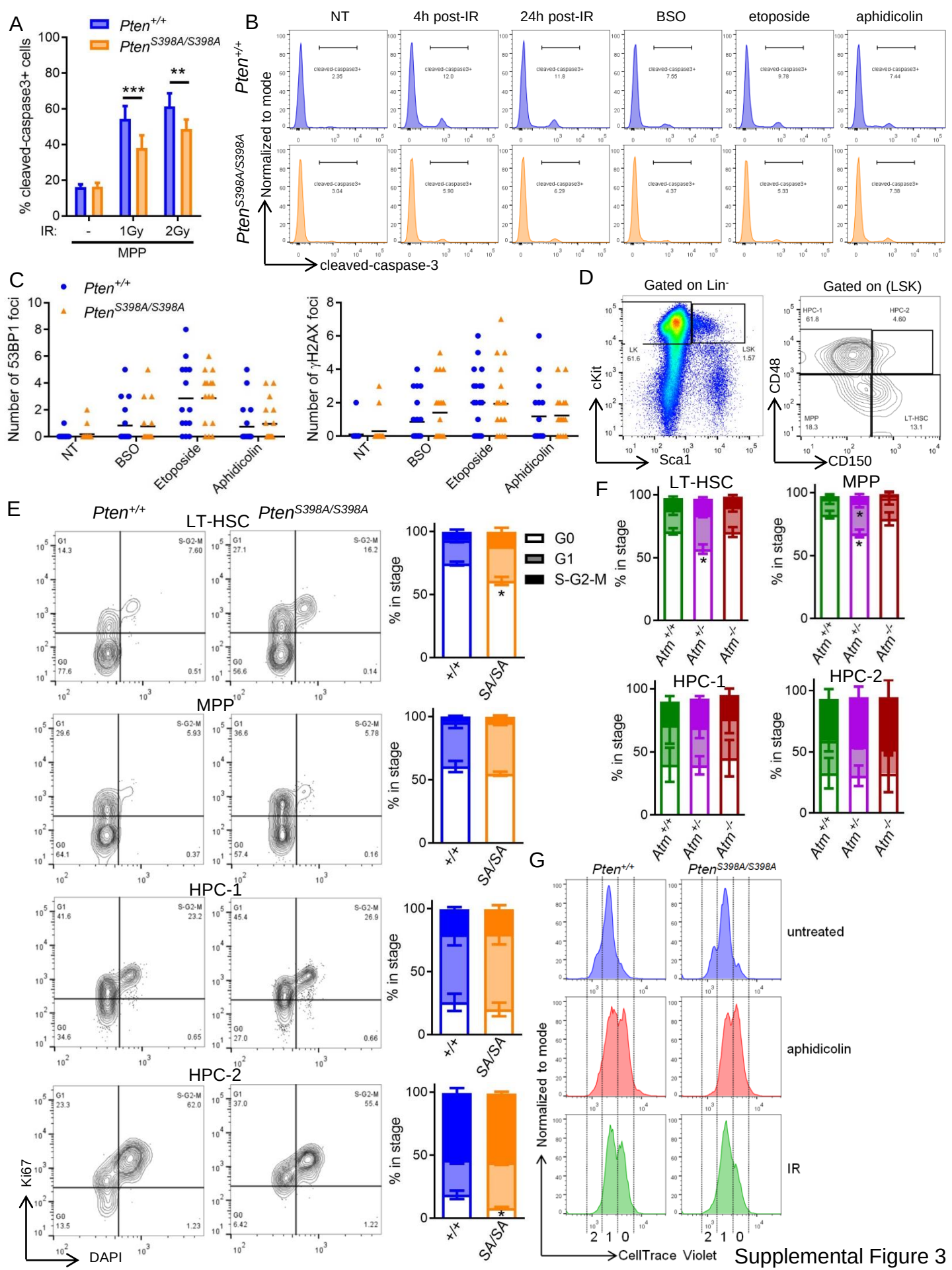
(**A**) Flow cytometry plots depicting the gating strategy to identify B220$^{+}$ cells, CD3$^{+}$ cells , CD11b$^{+}$ cells, and Gr1$^{+}$ cells in the peripheral blood of lethally irradiated mice competitively transplanted with CD45.1$^{+}$ and CD45.2$^{+}$ bone marrow cells. The antibodies used are indicated on the axes. (**B**) Flow cytometry plots depicting the gating strategy to identify CD45.1$^{+}$ and CD45.2$^{+}$ cells within total blood cells, B220$^{+}$ cells, CD3$^{+}$ cells, CD11b$^{+}$ cells, and Gr1$^{+}$ cells, in the peripheral blood of lethally irradiated mice competitively transplanted with CD45.1$^{+}$ and CD45.2$^{+}$ bone marrow cells. The antibodies used are indicated on the axes. (**C**) Proportion of Gr1$^{+}$ cells expressing the CD45.2 marker in the peripheral blood of lethally irradiated mice transplanted with *Atm*$^{+/+}$, *Atm*$^{+/-}$, or *Atm*$^{-/-}$ CD45.2$^{+}$ bone marrow cells mixed 1:1 with wild-type CD45.1$^{+}$ competitors. Data are combined from three independent experiments in which transplanted cells were pooled from 2-3 donor animals per genotype. “n” indicates the number of transplanted mice. (**D**) Proportion of Gr1$^{+}$ cells expressing the CD45.2 marker in the peripheral blood of lethally irradiated mice secondarily transplanted with bone marrow cells isolated from primary transplanted mice that received *Atm*$^{+/+}$, *Atm*$^{+/-}$, or *Atm*$^{-/-}$ CD45.2$^{+}$ bone marrow cells mixed 1:1 with wild-type CD45.1$^{+}$ competitors. Data are from two independent experiments. “n” indicates the number of recipient mice. (**E**) Expression of *Pten*, normalized to the housekeeping gene *Actb*, measured by qPCR in the indicated cell subsets isolated from *Atm*$^{+/+}$, *Atm*$^{+/-}$, and *Atm*$^{-/-}$ mice. Each dot represents data from an individual mouse. (**F**) Immunofluorescence microscopy images of cultured HSCs sorted from *Atm*$^{+/+}$, *Atm*$^{+/-}$, and *Atm*$^{-/-}$ mice, and exposed or not to 1 Gy irradiation 4 hours prior to cell fixation. Cells were stained for PTEN, Wheat Germ Agglutinin (WGA), and DAPI. Scale bar: 5 µm. (**G**) Complete blood counts showing the circulating number (in thousands per µL) of white blood cells, monocytes, neutrophils, lymphocytes, and red blood cells (in millions per µL) as well as hemoglobin levels in *Pten*$^{+/+}$ and *Pten*$^{S398A/S398A}$ mice. Each dot represents an individual animal. In all panels, mean and SEM are shown. In panels (**C**) and (**D**), *: $p<0.05$; **: $p<0.01$; ***:$p<0.001$, analyzed using two-way ANOVA with Dunnett (*Atm*$^{+/-}$ and *Atm*$^{-/}$ compared to *Atm*$^{+/+}$ mice) multiple comparisons tests.




Supplemental Figure 2

**Supplemental Figure 2. Enhanced competitive fitness of *Pten*$^{S398A/S398A}$ hematopoietic cells.**

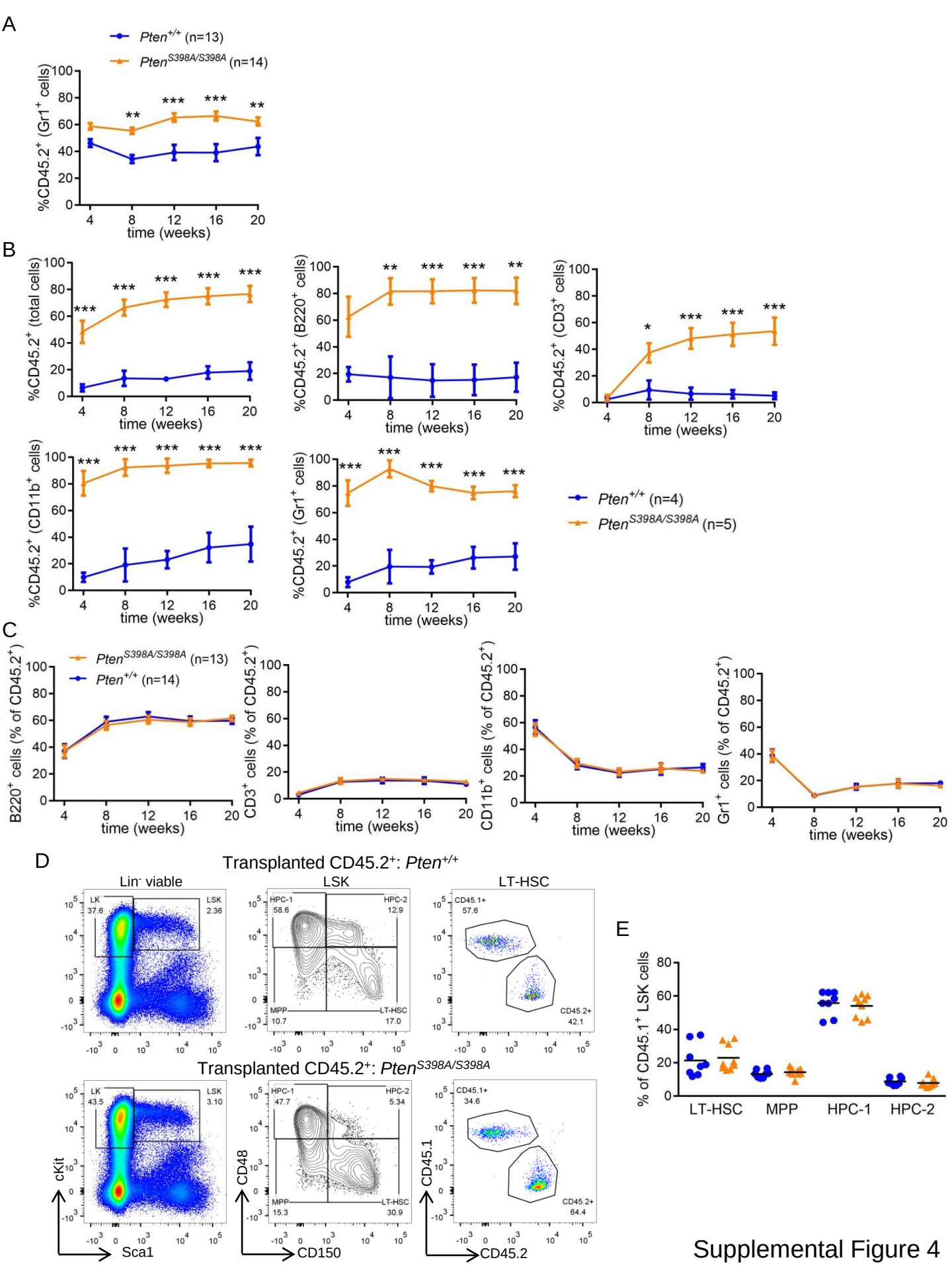
**(A**) Proportion of total circulating blood cells, B220$^{+}$ cells, CD3$^{+}$ cells, CD11b$^{+}$ cells, and Gr1$^{+}$ cells expressing the CD45.2 marker, in lethally irradiated mice secondarily transplanted with bone marrow cells isolated from primary transplanted mice that received *Pten*$^{+/+}$ or *Pten*$^{S398A/S398A}$ CD45.2$^{+}$ bone marrow mixed 1:1 with wild-type CD45.1$^{+}$ competitors. Data are combined from two independent experiments. "n" indicates the number of transplanted mice. (**B**) Proportion of circulating CD45.2$^{+}$ cells expressing B220, CD3, CD11b, or Gr1 in the peripheral blood of lethally irradiated mice transplanted with *Pten*$^{+/}$ or *Pten*$^{S398A/S398A}$ CD45.2$^{+}$ bone marrow mixed 1:1 with wild-type CD45.1$^{+}$ competitors. Data are combined from two independent experiments in which transplanted cells were pooled from 3 donor animals per genotype. "n" indicates the number of transplanted mice. In all panels, mean and SEM are shown. ***:$p$<0.001, assessed by two-way ANOVA with Sidak multiple comparisons tests.




Supplemental Figure 3

**Supplemental Figure 3. Altered response to genotoxic stress in *Pten*$^{S398A/S398A}$ stem and progenitor cells**

(**A**) Quantification of the proportion of cleaved caspase 3-positive multipotent progenitors (MPP), isolated from sorted *Pten*$^{+/+}$ and *Pten*$^{S398A/S398A}$ mice, fixed for immunofluorescence 4 hours after 1 Gy or 2 Gy irradiation, or no irradiation. N=3 independent experiments. (**B**) Flow cytometry histograms depicting the gating strategy to identify cleaved caspase 3-positive cells in cultured *Pten*$^{+/+}$ and *Pten*$^{S398A/S398A}$ LT-HSCs that were not treated (NT), analyzed 4 hours or 24 hours after 1 Gy irradiation (IR), or exposed to 100 µM buthionine sulfoximine (BSO), 0.25 µM etoposide, or 50 ng/mL aphidicolin for 24 hours. (**C**) Number of 53BP1 (left) and γH2AX foci (right), assessed by immunofluorescence, in cultured *Pten*$^{+/+}$ and *Pten*$^{S398A/S398A}$ LT-HSCs that were not treated (NT), or exposed to 100 µM buthionine sulfoximine (BSO), 0.25 µM etoposide, or 50 ng/mL aphidicolin for 24 hours. Each dot represents an individual cell. These results are from the same experiments as those presented in Figure 3D, and data from the "NT" condition are included in both figures. (**D**) Flow cytometry plots depicting the gating strategy to identify: Lin$^-$cKit$^+$Sca1$^-$ cells (LK), Lin$^-$cKit$^+$Sca1$^+$ cells (LSK), long-term hematopoietic stem cells (LT-HSC), multipotent progenitors (MPP), and two subsets of hematopoietic progenitors (HPC-1 and HPC-2), for cell cycle assessment. The parent populations are indicated on top of the plots, and the antibodies used are indicated on the axes. (**E**) Flow cytometry plots (left) and quantification (right) of the cell cycle stage distribution in LT-HSC, MPP, HPC-1 and HPC-2 cells in *Pten*$^{+/+}$ and *Pten*$^{S398A/S398A}$ mice. N= 4 mice per genotype. Significance symbols reflect differences in the proportion of cells in a given stage between genotypes. (**F**) Quantification of the cell cycle stage distribution in LT-HSC, MPP, HPC-1 and HPC-2 cells in *Atm*$^{+/+}$, *Atm*$^{+/-}$, and *Atm*$^{-/-}$ mice. N = 4 *Atm*$^{+/+}$, 7 *Atm*$^{+/-}$ and 4 *Atm*$^{-/-}$ mice. Significance symbols reflect differences in the proportion of cells in a given stage in *Atm*$^{+/-}$ mice compared with both *Atm*$^{+/+}$ and *Atm*$^{-/-}$ mice. In all panels, mean and SEM are shown. *: $p<0.05$; **: $p<0.01$; ***:$p<0.001$, assessed by two-way ANOVA with Sidak (**A** and **E**) or Tukey (**F**) multiple comparisons tests.

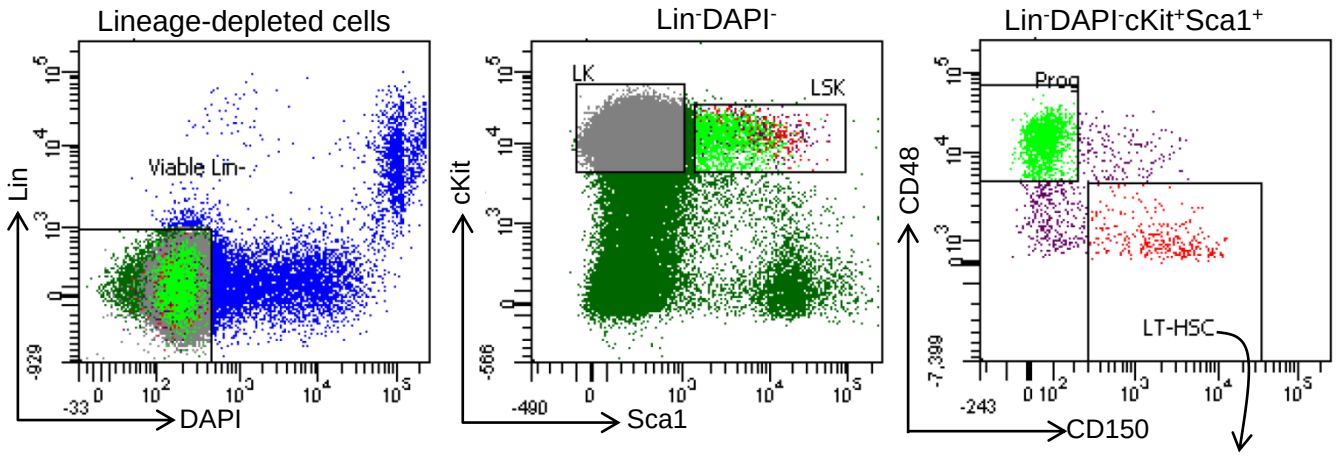



Supplemental Figure 4

**Supplemental Figure 4. Enhanced competitive fitness of *Pten*$^{S398A/S398A}$ hematopoietic cells after irradiation.**

(**A**) Proportion of circulating Gr1$^+$ cells expressing the CD45.2 marker in the peripheral blood of lethally irradiated mice transplanted with *Pten*$^{+/+}$ or *Pten*$^{S398A/S398A}$ CD45.2$^+$ bone marrow subjected to 2 Gy irradiation and mixed 10:1 with non-irradiated wild-type CD45.1$^+$ competitors. Data are combined from two independent transplantation experiments. In each, experiment, transplanted cells were pooled from 3 donor animals per genotype. "n" indicates the number of transplanted mice. (**B**) Proportion of circulating total blood cells, B220$^+$ cells, CD3$^+$ cells, CD11b$^+$ cells, and Gr1$^+$ cells expressing the CD45.2 marker, in lethally irradiated mice secondarily transplanted with bone marrow cells isolated from the primary transplanted mice described in panel (**A**). Transplanted cells were pooled from all the primary transplanted mice. "n" indicates the number of transplanted mice. (**C**) Proportion of CD45.2$^+$ cells expressing B220, CD3, CD11b, or Gr1 in the peripheral blood of the transplanted mice described in panel (**A**). "n" indicates the number of transplanted mice. (**D**) Flow cytometry plots depicting the gating strategy to identify stem and progenitor cell populations as well as CD45.2 and CD45.1-expressing cells in the bone marrow of mice transplanted as described in panel (**A**), at 20 weeks after transplantation. The parent populations are indicated on top of the plots, and the antibodies used are indicated on the axes. (**E**) Proportion of the indicated stem and progenitor cell subsets within CD45.1-expressing LSK cells in the bone marrow from mice transplanted as described in panel (**A**), analyzed 20 weeks after transplantation. Each dot represents an individual mouse. In all panels, mean and SEM are shown. *: $p<0.05$; **: $p<0.01$; ***:$p<0.001$, assessed by two-way ANOVA with Sidak multiple comparisons tests.

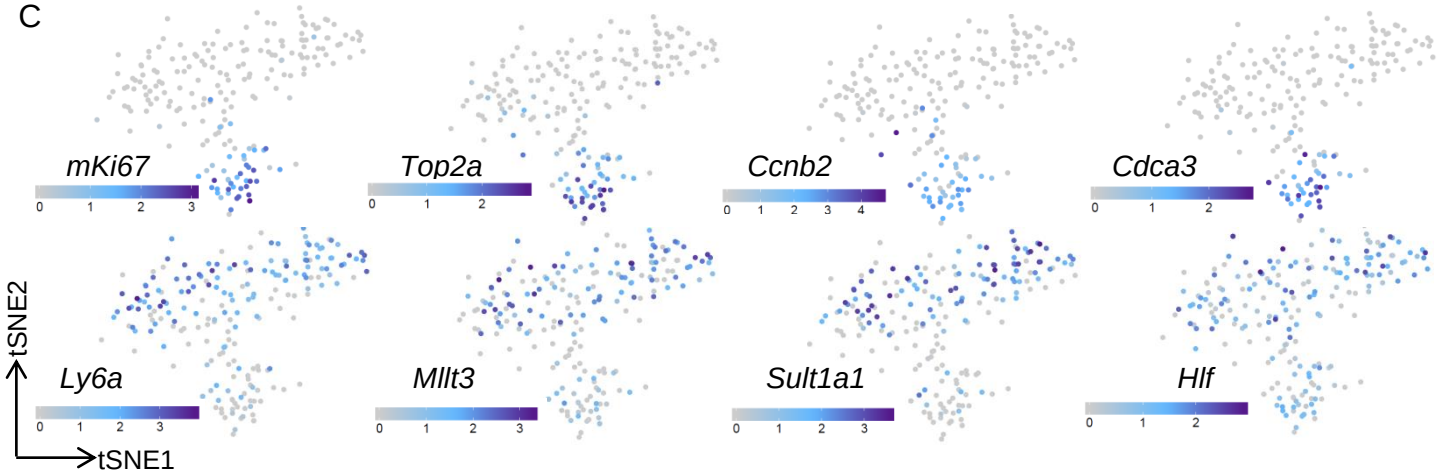
A




B

| Genotype | Sex | Number of mice | Number of viable single cells captured |
|---|---|---|---|
| *Pten*$^{+/+}$ | ♀ | 3 | 80 |
| | ♂ | 3 | 50 |
| *Pten*$^{S398A/S398A}$ | ♀ | 3 | 48 |
| | ♂ | 3 | 54 |

C




D

unsupervised clustering

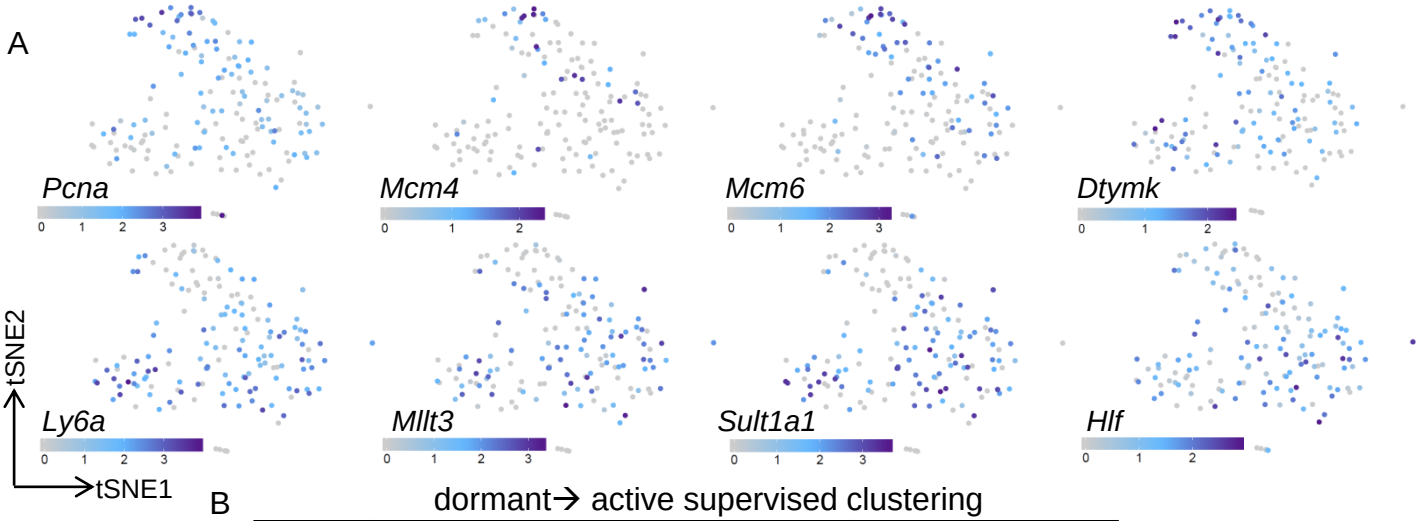
| | Number of cells and proportion | | |
|---|---|---|---|
| Cluster | *Pten*$^{+/+}$ | *Pten*$^{S398A/S398A}$ | |
| 1 | 32 (28%) | 55 (61%) | $p<0.0001$ |
| 2 | 55 (47%) | 21 (23%) | |
| 3 | 29 (25%) | 14 (16%) | |

Supplemental Figure 5

**Supplemental Figure 5. Isolation and transcriptomic analysis of single *Pten*$^{+/+}$ and *Pten*$^{S398A/S398A}$ hematopoietic stem cells.**

(**A**) Flow cytometry plots depicting the gating strategy to isolate long-term hematopoietic stem cells (LT-HSC), by fluorescence-activated cells sorting, for single-cell transcriptomic analyses. LK: Lin$^-$cKit$^+$Sca1$^-$ cells. LSK: Lin$^-$cKit$^+$Sca1$^+$ cells. Prog.: progenitors. Only the LT-HSC subset was sorted in these experiments. The parent populations are indicated on top of the plots, and the antibodies or fluorescent dyes used are indicated on the axes. (**B**) Table indicating the genotype, number, and sex of mice used, and the number of viable cells captured on the C1 chip, in each sorting experiment. (**C**) t-SNE plots depicting the relative expression of selected genes in single HSCs sorted from *Pten*$^{+/+}$ and *Pten*$^{S398A/S398A}$ mice, and visualized as in Figure 5A. (**D**) Tables showing the number and proportion of *Pten*$^{+/+}$ and *Pten*$^{S398A/S398A}$ LT-HSCs found in each of the clusters identified in Figure 5A. Data were analyzed using Chi-Square tests.

A



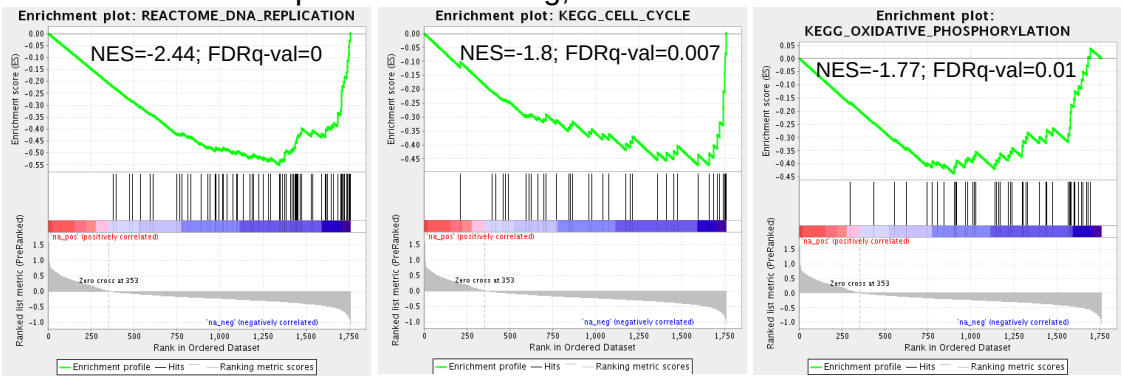

B

dormant→ active supervised clustering

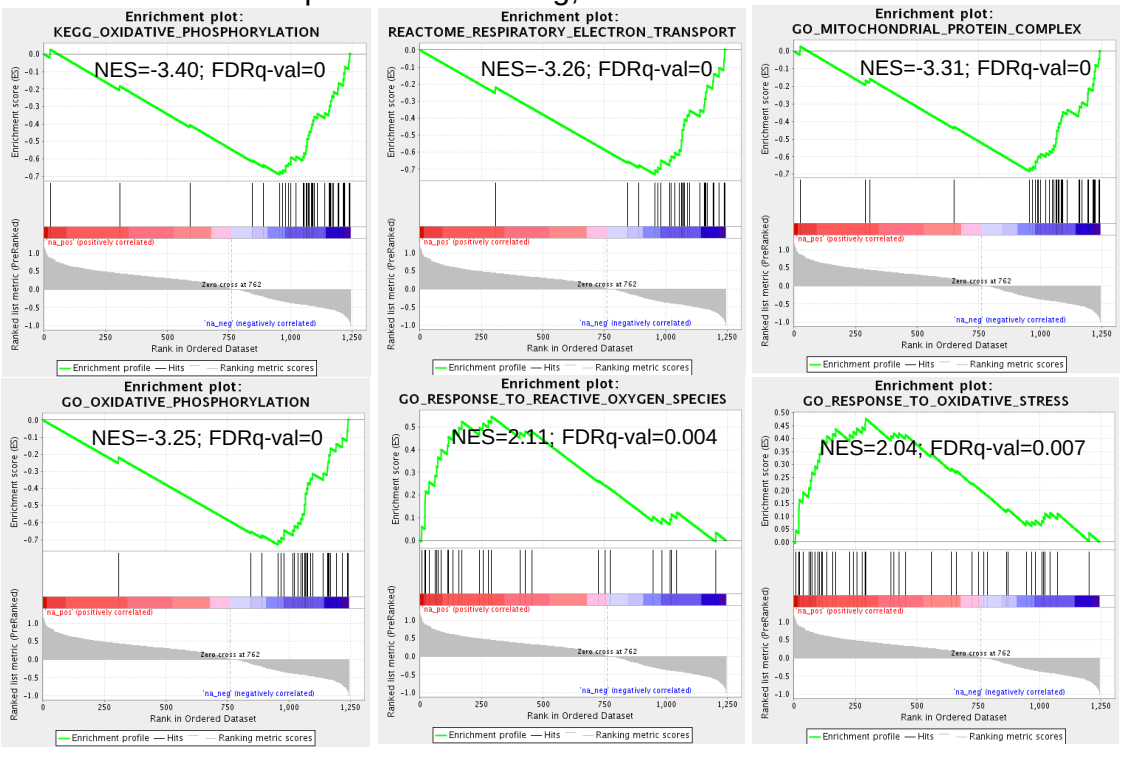
| | Number of cells and proportion | | |
|---|---|---|---|
| Cluster | *Pten*$^{+/+}$ | *Pten*$^{S398A/S398A}$ | |
| A | 50 (59%) | 20 (26%) | *p*<0.0001 |
| B | 31 (36%) | 25 (33%) | |
| C | 4 (5%) | 29 (38%) | |

C

dormant→ active supervised clustering; Cluster A vs Cluster B



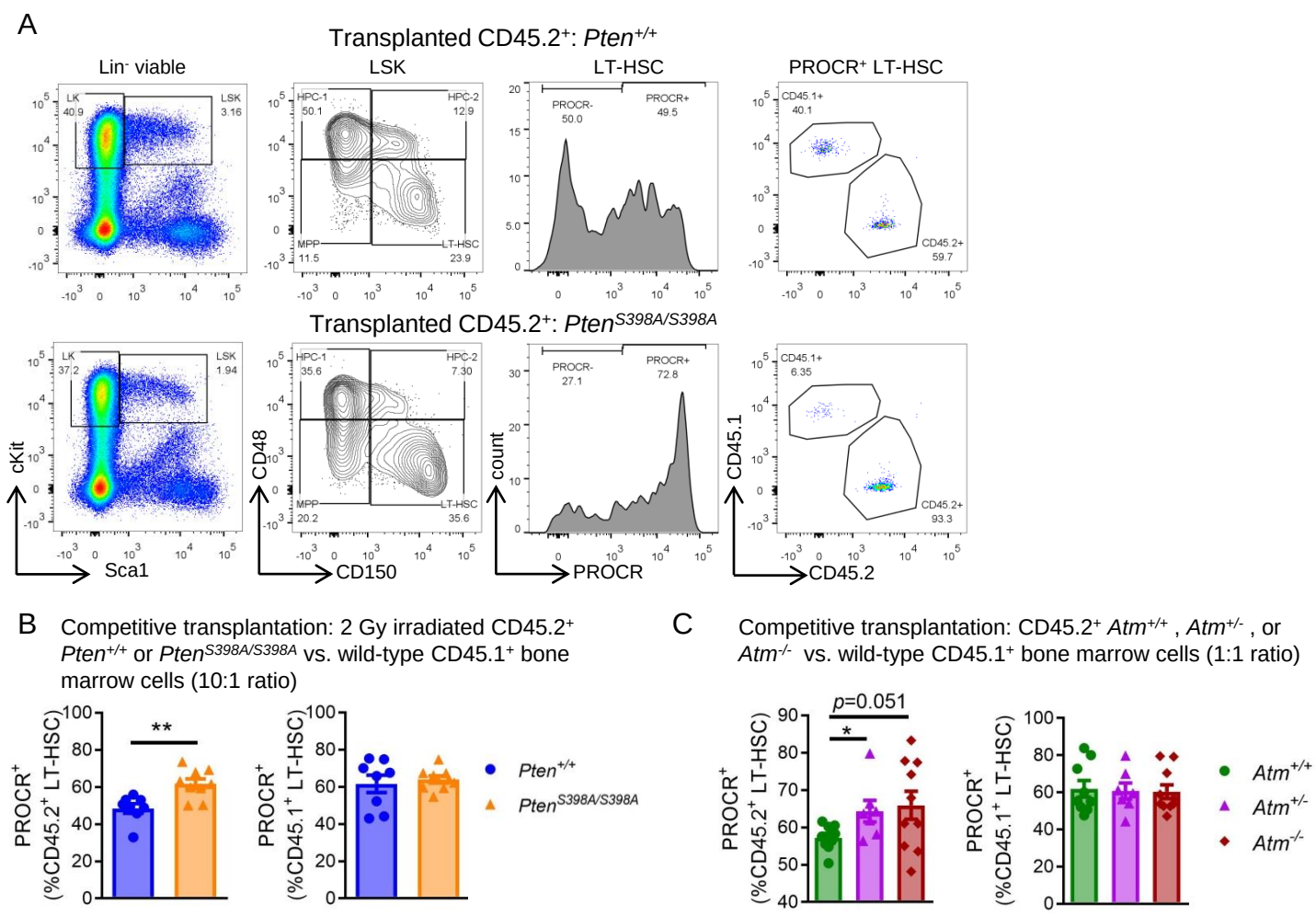

dormant→ active supervised clustering; Cluster A vs Cluster C




Supplemental Figure 6

**Supplemental Figure 6. Altered transcriptome of quiescent *Pten*$^{S398A/S398A}$ hematopoietic stem cells.**

(**A**) Table showing the number and proportion of *Pten*$^{+/+}$ and *Pten*$^{S398A/S398A}$ HSCs found in each of the clusters identified in Figure 5B. Data were analyzed using Chi-Square tests. (**B**) t-SNE plots depicting the relative expression of selected genes in single quiescent HSCs sorted from *Pten*$^{+/+}$ and *Pten*$^{S398A/S398A}$ mice, and visualized as in Figure 5B. (**C**) Gene Set Enrichment Analysis performed on genes differentially expressed between Cluster A vs Cluster B (top panels), and Cluster A vs Cluster C (bottom panels) cells identified by supervised clustering of *Pten*$^{+/+}$ and *Pten*$^{S398A/S398A}$ HSCs with a gene signature defining the dormant → active state transition (see Figure 5). Normalized Enrichment Scores (NES) and False Discovery Rate q-value (FDR q-val) are indicated in each plot.





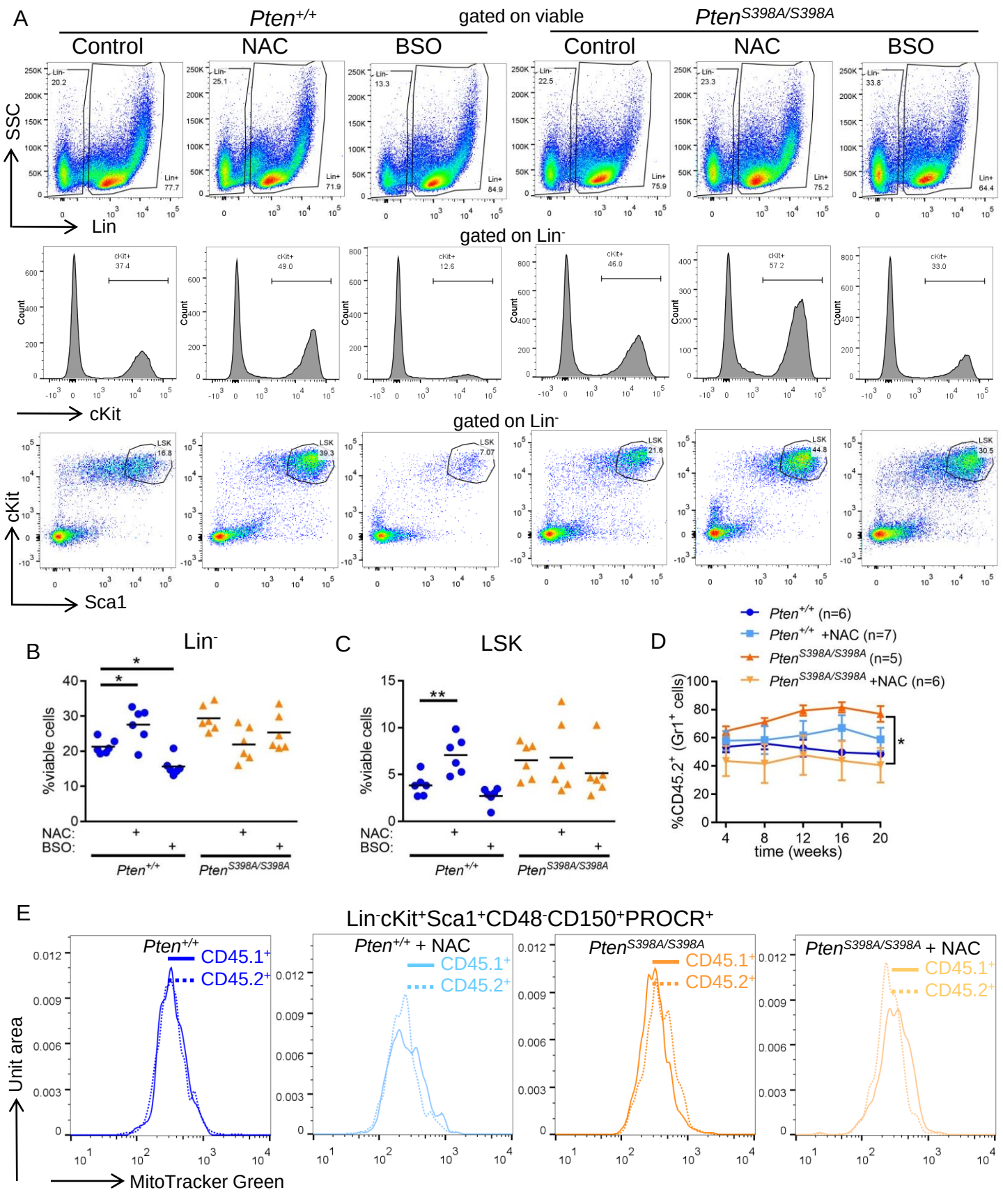





Supplemental Figure 7

**Supplemental Figure 7. Expansion of *Pten*$^{S398A/S398A}$ PROCR$^{+}$ LT-HSCs in the bone marrow of competitively transplanted mice.**

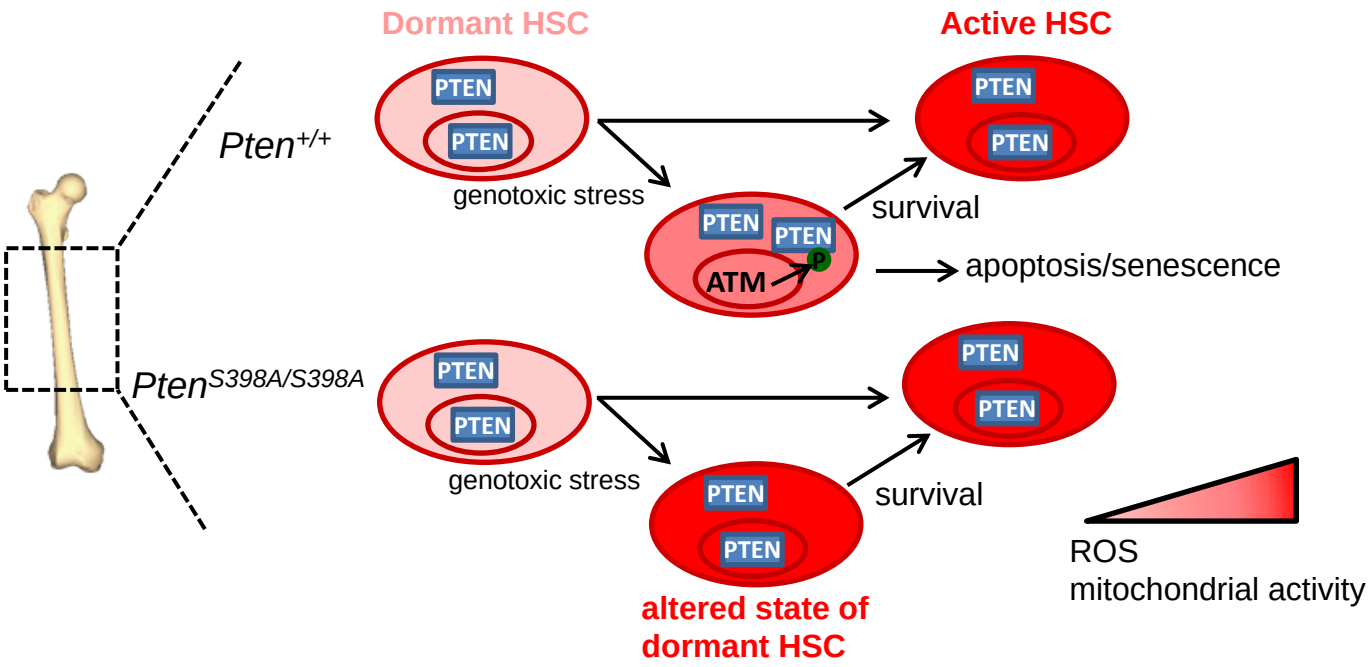
(**A**) Flow cytometry plots depicting the gating strategy to identify: Lin$^{-}$cKit$^{+}$Sca1$^{-}$ cells (LK), Lin$^{-}$cKit$^{+}$Sca1$^{+}$ cells (LSK), long-term hematopoietic stem cells (LT-HSC), multipotent progenitors (MPP), two subsets of hematopoietic progenitors (HPC-1 and HPC-2), PROCR-expressing LT-HSCs, as well as CD45.1 and CD45.2-positive cells, in the bone marrow of lethally irradiated mice transplanted with 300 *Pten*$^{+/+}$ or *Pten*$^{S398A/S398A}$ CD45.2$^{+}$ PROCR$^{+}$ LT-HSCs mixed with 2 x $10^{5}$ wild-type CD45.1$^{+}$ competitors, 20 weeks after transplantation (see Figure 7). **B-C**) Proportion of CD45.2$^{+}$ (left) and CD45.1$^{+}$ (right) LT-HSCs expressing PROCR in the bone marrow from mice transplanted as described above the plots, analyzed at 20 weeks after transplantation. In all panels, mean and SEM are shown. *: $p$<0.05; **: $p$<0.01, assessed by *t*-test.




Supplemental Figure 8

**Supplemental Figure 8. Normalization of the competitive fitness of *Pten*$^{S398A/S398A}$ HSCs by antioxidant treatment.**

(**A**) Flow cytometry plots depicting the proportion of cells negative or positive for lineage markers (Lin$^-$ and Lin$^+$, respectively), negative for lineage markers and positive for cKit (cKit$^+$), or negative for lineage markers and positive for cKit and Sca1 (LSK), assessed by flow cytometry, in colonies formed by bone marrow cells isolated from *Pten*$^{+/+}$ and *Pten*$^{S398A/S398A}$ mice in normal M3434 medium, or M3434 medium supplemented with 1 mM N-acetylcysteine (NAC) or 1 µM buthionine sulfoximine (BSO). The parent populations are indicated on top of the plots, and the antibodies used are indicated on the axes. **B-C**) Quantification of the Lin$^-$ (**B**) and LSK (**C**) cell populations depicted in panel (**A**), expressed as a proportion of viable cells. Each dot represents a culture from an individual animal. (**D**) Proportion of circulating Gr1$^+$ cells expressing the CD45.2 marker in the peripheral blood of lethally irradiated mice transplanted with *Pten*$^{+/+}$ or *Pten*$^{S398A/S398A}$ CD45.2$^+$ bone marrow mixed 1:1 with wild-type CD45.1$^+$ competitors. Mice had access to normal drinking water or water supplemented with 1 g/L N-acetylcysteine (NAC), as indicated. Transplanted cells were pooled from 3 donor animals per genotype. “n” indicates the number of transplanted mice. For clarity, statistical analyses are indicated only for the 20 week time-point. (**E**) Flow cytometry plots depicting the fluorescence intensity for MitoTracker Green, an indicator of mitochondrial content, in Lin$^-$cKit$^+$Sca1$^+$CD48$^-$CD150$^+$PROCR$^+$ cells that are either CD45.1$^+$ (wild-type; solid lines), or CD45.2$^+$ (*Pten*$^{+/+}$ or *Pten*$^{S398A/S398A}$; dotted lines) isolated from the bone marrow-transplanted mice described in panel (**D**), and represented with the same color and symbols scheme. In all panels, mean and SEM are shown. *: $p<0.05$; **: $p<0.01$, assessed by one-way ANOVA (**B**; each genotype analyzed separately) or two-way ANOVA (**D**) with Tukey multiple comparisons tests.




Supplemental Figure 9

**Supplemental Figure 9. Control of HSC fitness and dormancy by ATM phosphorylation of PTEN.**

Model summarizing the findings presented in this manuscript, and integrated with other published observations. Transition from the dormant to active state in HSC is associated with increased mitochondrial activity and ROS accumulation. *Pten*$^{S398A/S398A}$ HSCs can tolerate elevated ROS levels while retaining dormant characteristics, likely due in part to impaired PTEN nuclear export directed by ATM phosphorylation.

## Supplemental Methods

### Mice

*Pten*$^{S398A/S398A}$ mice were generated by gene-targeting in embryonic stem cells. A targeting construct was engineered, containing: an upstream homology arm comprising a portion of the 8$^{th}$ intron, a PGK-Neo selection cassette flanked by *LoxP* and *Frt* sites, a downstream homology arm covering part of exon 9 (including some of the 3' untranslated region), and a diphtheria toxin fragment A negative selection cassette. The PGK-Neo cassette was excised in vivo by breeding with "FLP deleter" mice (B6.129S4-*Gt(ROSA)26Sor*$^{tm1(FLP1)Dym}$/RainJ, obtained from The Jackson Laboratory). All the experiments in this paper were performed with mice backcrossed on a C57Bl/6 background for at least 8 generations. CD45.1 mice (B6.SJL-*Ptprc*$^{a}$ *Pepc*$^{b}$/BoyJ; stock #002014) were obtained from The Jackson Laboratory. *Atm*$^{-/-}$ mice were previously described (1), and maintained on a pure C57Bl/6 background (backcrossed >10 generations).

### Flow cytometry

Bone marrow cells were flushed from tibia, femur and humerus bones in FACS buffer (phosphate-buffered saline without $MgCl_2$ and $CaCl_2$, supplemented with 1% heat-inactivated fetal bovine serum, 2mM EDTA pH8.0 and 0.05% sodium azide) or MACS buffer (phosphate-buffered saline without $MgCl_2$ and $CaCl_2$, supplemented with 0.5% bovine serum albumin and 2mM EDTA pH8.0) using a syringe fitted with a 26 gauge needle. Dissociated cells were passed through a 70 µm nylon mesh, and centrifuged at 300 x g for 5 minutes at 4°C. The liquid was aspirated, and red blood cell lysis was performed by resuspending the cells in 1 mL red blood cell lysis buffer (Sigma) and incubating 5 minutes at ambient temperature. The red blood cell lysis reaction was neutralized with the addition of 10 mL cold FACS buffer, and the cells were centrifuged at 300 x g for 5 minutes a 4°C. The liquid was aspirated, the cell pellet resuspended in 1 mL cold FACS buffer, and the cells counted on a ViCell Counter. Cells were then washed by addition of 5 mL cold FACS buffer, and centrifuged 300 x g for 5 minutes a 4°C. The liquid was aspirated, at the cell pellet resuspended at the desired cell density for downstream processing.

For enumeration of the stem and progenitor subtypes within whole bone marrow, a 1 x $10^8$ cells/mL suspension was prepared, and 100 µl (1 x $10^7$ cells) transferred to 5 mL polystyrene tubes. 1 µl Fc blocking antibody (Tonbo Biosciences # 70-0161-M001) was added,

and the cells incubated on ice for 20 minutes, except in experiments where progenitor subsets were identified with fluorophore-conjugated anti-CD16/32 antibodies. A 2X antibody staining cocktail was then prepared by diluting the appropriate antibodies in FACS buffer at twice the desired final concentration. To identify differentiated hematopoietic cells, this antibody cocktail included a panel of biotin-conjugated antibodies against different lineages (NK1.1, CD3, C5, CD8, B220, CD11b, Gr1 and Ter119 – see antibody table below). 100 µl of this 2X antibody mixture was added to the cells, and staining was performed on ice for 30 minutes, protected from light, except in experiments using CD34 antibodies, where the incubation time was lengthened to 90 minutes. After staining, cells were washed twice by adding 3 mL cold FACS buffer and spinning at 300 x g for 5 minutes at 4°C. Cells were resuspended in 100 µl FACS buffer containing streptavidin-APC/Cy7 (1:50), incubated for 30 minutes on ice, and washed as above. Cells were resuspended in 500 µl cold FACS buffer, and filtered through at 25 µm nylon mesh into new 5 mL polystyrene tubes immediately prior to data acquisition. Data were acquired using a Fortessa flow cytometer (BD Biosciences)

In experiments where lineage-negative cell enrichment was required, bone marrow cells were prepared as above, but resuspended in MACS buffer (phosphate-buffered saline without $MgCl_2$ and $CaCl_2$, supplemented with 0.5% bovine serum albumin and 2mM EDTA pH8.0) after red blood cell lysis. Cells were processed using the Mouse Lineage Cell Depletion Kit (Miltenyi Biotec # 130-090-858), following the manufacturer's instructions: cells were resuspended at 2.5 x $10^8$ cells/mL in MACS buffer. 10 µl of biotinylated mouse lineage antibody cocktail was added per 10 million cells, followed by incubation on ice for 15 minutes. An additional 30 µl of MACS buffer and 20 µl of streptavidin-conjugated magnetic microbeads were added per 10 million cells, followed by incubation ice for 15 minutes. The cells and beads mixture was then diluted in 10 mL cold MACS buffer, filtered through a 30 µm nylon mesh, and centrifuged at 300 x g for 5 minutes at 4°C. The pellet was resuspended in 1 mL cold MACS buffer, and processed for lineage separation on and an AutoMACS Pro instrument (Miltenyi Biotec) using the "Deplete" settings. Lineage-negative and –positive fractions were collected, and processed for downstream applications.

For cell cycle analysis, bone marrow cells were isolated and subjected to lineage-negative enrichment as described above. Cell-surface antibody staining was performed in MACS buffer containing ScaI-alexa700 (1:100), CD150-PE (1:50), CD48-APC (1:100), cKit-PE/Cy7 (1:100), and Streptavidin-APC/Cy7( 1:50). Following staining, cells were washed twice in MACS buffer and centrifuged at 300 x g for 5 minutes at 4°C. Cells were resuspended in 1 mL

BD Cytofix/Cytoperm solution (BD Biosciences), and incubated on ice for 30 minutes. Cells were washed once in BD Perm/Wash buffer (BD Biosciences), centrifugation at 300 x g for 5 minutes at 4°C, and resuspended in 500 µL BD Perm/Wash buffer containing 1:100 Ki67-FITC, 2ug/mL DAPI, and 100ug/mL RNAse A (Thermo Fisher). Cells were incubated for 1 hour on ice, washed twice with MACS buffer and centrifugation at 300 x g for 5 minutes at 4°C, and resuspended in 500 µL prior to analysis by flow cytometry. For data analysis, gating was restricted to cells with a DNA content between 2n and 4n, based on DAPI signal intensity, excluding <2n ("sub-G1; presumably dead cells) and >4n (possibly doublets) events.

For DNA damage and apoptosis analyses, cells were isolated, stained for cell surface antigens, fixed and permeabilized as above. The cell pellet was then resuspended in 500 µl 1X Perm/Wash buffer containing 1:50 anti-cleaved caspase 3-AF647, and incubated on ice for 30 minutes. Cells were then washed a processed for flow cytometry as described above.

For ROS measurements, ROS-MACS buffer (phosphate-buffered saline with $MgCl_2$ and $CaCl_2$, supplemented with 0.5% bovine serum albumin and 2mM EDTA pH8.0) was used for all the cell isolation and lineage-negative enrichment steps. To detect ROS, ROS-MACS buffer containing 2 µM dihydrorhodamine 123 (DHR) was warmed at 37°C prior to addition to the cells. Cells were incubated in 500 µL buffer for 30 minutes at 37°C in a tissue culture incubator. After the incubation, 3 mL cold MACS buffer was added, and cells were centrifuged at 300 x g for 5 minutes at 4°C. The liquid was removed, and the cell pellet processed for antibody staining as described above.

To measure mitochondrial content, cells were processed for antibody staining as described above, in buffer containing 200 nM MitoTracker Green (Thermo Fisher Scientific).

All flow cytometry data were analyzed using FlowJo Version 10.

The antibodies used for flow cytometry were:

| **Antibody and fluorophore** | **Manufacturer and catalog #** | **Dilution** |
|---|---|---|
| B220-APC/Cy7 | BD Biosciences # 552094 | 1:100 |
| B220-biotin | BioLegend # 103204 | 1:100 |
| CD11b-biotin | BioLegend # 101204 | 1:100 |
| CD11b-PE | BioLegend # 101208 | 1:200 |
| CD150-BV421 | BioLegend # 115926 | 1:50 |
| CD150-BV605 | BioLegend # 115927 | 1:50 |
| CD150-PE | BioLegend # 115904 | 1:50 |
| CD150-PerCP/Cy5.5 | BioLegend # 115922 | 1:50 |
| CD16/32-PerCP/Cy5.5 | BD Biosciences # 560540 | 1:100 |

| | | |
|---|---|---|
| CD3-biotin | BioLegend # 100304 | 1:100 |
| CD3-eFluor450 | ThermoFisher # 48-0033-82 | 1:100 |
| CD34-eFluor450 | ThermoFisher # 48-0341-82 | 1:50 |
| CD34e-FITC | BD Biosciences # 560238 | 1:50 |
| CD45.1-APC | BioLegend # 110714 | 1:100 |
| CD45.1-PerCP/Cy5.5 | BioLegend # 109828 | 1:50 |
| CD45.2-AF700 | BioLegend # 109822 | 1:50 |
| CD45.2-FITC | BioLegend # 109806 | 1:50 |
| CD48-APC | BioLegend # 103412 | 1:100 |
| CD5-biotin | BioLegend # 100604 | 1:100 |
| CD8-biotin | BioLegend # 100704 | 1:100 |
| cKit-PE/Cy7 | BioLegend # 105814 | 1:100 |
| cleaved caspase 3-AF647 | BD Biosciences # 560626 | 1:50 |
| Gr1-biotin | BioLegend # 108404 | 1:100 |
| Gr1-PE/Cy5 | BioLegend # 108410 | 1:200 |
| γH2AX-FITC | Millipore # 16-202A | 1:100 |
| Ki-67-FITC | ThermoFisher # 11-5698-82 | 1:100 |
| NK1.1-biotin | BioLegend # 108704 | 1:100 |
| PROCR-PE | Stemcell Technologies # 60038PE | 1:100 |
| ScaI-alexa700 | BioLegend # 108141 | 1:50 |
| Sca1-APC | BioLegend # 108112 | 1:50 |
| ScaI-BV421 | BioLegend # 108128 | 1:50 |
| ScaI-FITC | BioLegend #108106 | 1:100 |
| ScaI-PE | BioLegend # 108108 | 1:50 |
| Streptavidin-APC/Cy7 | BioLegend # 405208 | 1:50 |
| Ter119-biotin | BioLegend # 116204 | 1:100 |

**Complete blood count**

For complete blood count, a small volume of blood was collected from the tail vein in Microvette 100 K3E tubes (Sarstedt), and analyzed on a Hemavet HV950 instrument (Drew Scientific).

**Bone marrow transplantation and chimerism assessment**

For bone marrow transplantation, recipient mice received 10 Gy full-body irradiation, using an X-RAD 320 (Precision X-Ray) instrument, 15-18h prior to bone marrow transplantation. Bone marrow cells were isolated as described above, under sterile conditions. Equal numbers of cells from each CD45.2$^+$ donor animal of the same genotype were pooled, and mixed 1:1 with bone marrow cells from CD45.1$^+$ mice. Importantly, the same pool of wild-type CD45.1$^+$ cells was used for all the recipient animals within each experiment. There may be differences in the fitness of hematopoietic stem cells between the CD45.1$^+$ and the CD45.2$^+$ strains. Furthermore,

by necessity, mice with either CD45 allele were derived from distinct breeding stocks. Therefore, in all of our analyses, we compared the relative reconstitution ability of $CD45.2^+$ cells between mutant and control *Pten* and *Atm* genotypes, rather than between $CD45.2^+$ and $CD45.1^+$ cells. The cells were diluted in sterile PBS at a final concentration of $2.5 \times 10^6$ cells/mL. 200 µL of this cell suspension, equivalent to $5 \times 10^5$ cells (equivalent to $2.5 \times 10^5$ $CD45.1^+$ and $2.5 \times 10^5$ $CD45.2^+$ cells) were injected the tail vein of recipient mice.

For competitive transplantation of irradiated cells, bone marrow cells were prepared as above, except that $CD45.2^+$ cells from $Pten^{+/+}$ and $Pten^{S398A/S398A}$ mice were subjected to 2 Gy irradiation prior to mixing at a 10:1 ratio with non-irradiated $CD45.1^+$ cells from $Pten^{+/+}$ mice, and injection into lethally irradiated recipients as described above.

For competitive transplantation of $PROCR^+$ LT-HSCs, bone marrow cells were isolated, processed, and stained as described above, and sorted on a FACSAria Fusion fluorescence-activated cell sorter (BD Biosciences) into 1.5 mL tubes containing MACS buffer. Cells were spun at 300 x g for 5 minutes and resuspended in PBS. Cells were diluted and mixed with $CD45.1^+$ whole bone marrow cells, such that each 200 µL contained 300 $PROCR^+$ LT-HSCs ($CD45.2^+$) and $2 \times 10^5$ $CD45.1^+$ cells. 200 µL of this cell suspension was then injected in the tail vein of recipient mice.

For mice treated with N-Acetyl-L-Cysteine, transplantation procedures were as described above. Starting from the day of transplantation, mice has access to normal drinking water, or water supplemented with 1 g/L N-Acetyl-L-Cysteine (NAC). The drinking water was replaced every other day for the full duration of the experiment.

To assess blood cell chimerism, approximately 50 µL blood was collected from the tail vein in Micro-Hematorit capillary tubes with Heparin (VWR), and diluted in 50 µL PBS containing 20 mM EDTA. 1 µL Fc blocking antibody was added, and the cells incubated on ice for 20 minutes. 100 µL of antibody cocktail in FACS buffer, containing anti-CD45.1-APC (1:50), anti-CD45.2-FITC (1:25), anti-CD11b-PE (1:100), anti-Gr1-PE/Cy5 (1:100), anti-cKit-PE/Cy7 (1:50), anti-B220-APC/Cy7 (1:50) and anti-CD3-PacificBlue (1:50), was added (final concentration are 1:2 of those indicated above). Blood samples were incubated with the antibodies for 30 minutes at ambient temperature, protected from light. 2 mL FACS buffer was added, samples were centrifuged at 300 x g for 5 minutes at 4°C, and resuspended in 1 mL BD Cytofix/Cytoperm solution (BD Biosciences) to fix the cells and lyse the red blood cells. Samples were incubated for 5 minutes at ambient temperature, centrifuged at 300 x g for 5 minutes at 4°C, resuspended

in 1 mL Cytofix/Cytoperm solution, filtered through a 35 µm nylon mesh into polystyrene 5 mL FACS tubes, centrifuged at 300 x g for 5 minutes at 4°C, and resuspended in 500 µL FACS buffer prior to analysis by flow cytometry. We consistently observed, as reported by others (e.g. (2, 3)), that the reconstitution kinetics of $CD3^+$ cells was delayed compared with myeloid lineages in these experiments.

**Survival in response to whole-body irradiation**

Mice were subjected to total-body irradiations using a self-contained X-ray system for the delivery of precise radiation dosage (X-RAD 320, Precision X-Ray). Irradiation of conscious mice was performed in ventilated Plexiglas® boxes. Irradiation was given a dose rate of 3 Gy/min, for a total dose of 10 Gy, as a single exposure. After TBI, the mice were returned to their home cages, and monitored over 30 days until reaching humane endpoints.

**HSC sorting, culture, irradiation and immunofluorescence**

For immunofluorescence experiments, bone marrow cell isolation and lineage-negative enrichment was performed as described above. Cells were stained in an antibody cocktail containing ScaI-FITC (1:100), CD150-PE (1:50), CD48-APC (1:100), cKit-PE/Cy7 (1:100), Streptavidin-APC/Cy7 (1:50) and DAPI (5 ng/µL). Cells were sorted on a FACSAria II or FACSAria Fusion fluorescence-activated cell sorter (BD Biosciences) into culture medium (see below for specific culture media for each experiment). Cells were centrifuged at 300 x g for 5 minutes at 4°C, and resuspended in a small volume of culture medium. A 30 µL drop was deposited on BioCoat™ Poly-D-Lysine/Laminin 12 mm coverslips (Corning) in 24-well tissue culture plates. Cells were allowed to settle down and attach to the coverslips for 30 minutes at 37°C in a tissue culture incubator. An additional 500 µL of culture medium was then added, and the cells were treated or subjected to irradiation using an X-RAD 320 (Precision X-Ray) instrument as indicated.

For cleaved caspase 3 immunofluorescence experiments, cells were sorted, subjected or not to irradiation, and cultured for 4 hours in Iscove's Modified Dulbecco's Medium (IMDM) supplemented with 10% FBS and 1:1000 beta-mercaptoethanol. For 53BP1, γH2AX, and PTEN immunofluorescence experiments, cells were cultured for 24 hours in StemPro-34 medium (Thermo Fisher) containing 1X StemPro Nutrient Supplement, and the following cytokines (all from Peprotech): SCF (25 ng/mL), FLT3L (25 ng/mL), IL-11 (25 ng/mL), IL-3 (10 ng/mL), GM-CSF (10 ng/mL), EPO (4 U/mL), and TPO (25 ng/mL). Where indicated, cells were subjected to

irradiation 24 hours or 4 hours prior to cell fixation, or the following drugs or compounds were added 24 hours prior to cell fixation: buthionine sulfoximine (BSO; Millipore Sigma # B2515,100 µM), Etoposide (Millipore Sigma #E1383, 0.25 µM), or aphidicolin (Millipore Sigma #A4487, 25 ng/mL).

At the end of the treatment period, the culture medium was removed, cells were washed in 500 µL PBS, and then fixed in 500 µL 4% PFA for 10 minutes at ambient temperature with gentle motion. For experiments involving WGA staining, cells were washed twice in HBSS, and incubated for 10 minutes with 500 µL of a solution of 5 µg/mL Alexa Fluor 647-WGA in HBSS. Cells were then washed three times with HBSS before proceeding to permeabilization. For other staining conditions, cells were washed with 1 mL PBS after fixation, and permeabilized by incubation with 500 µL PBS containing 0.25% Triton X-100 for 5 minutes at ambient temperature with gentle motion. The permeabilization buffer was removed, and blocking was performed by incubating for 1 hour in 1 mL PBS containing 1% BSA with gentle motion. The blocking buffer was removed, and 300uL of blocking buffer containing primary antibodies was added. Antibody incubation was performed overnight a 4$^{o}$C with gentle motion. The antibody solution was then removed, and cells were washed three times with 500 µL PBS (each wash for 5 minutes with gentle motion). Cells were incubated with 400uL of blocking buffer containing secondary antibodies for 1 hour at ambient temperature, protected from light. The antibody solution was then removed, and cells were washed three times with 500 µL PBS (each wash for 5 minutes at ambient temperature, with gentle motion, protected from light). Cells were then stained with DAPI (500 µL of 2.5 µg/mL DAPI in PBS) for 5 minutes at ambient temperature. The DAPI solution was then removed, and cells were washed three times with 500 µL PBS (each wash for 5 minutes at ambient temperature, with gentle motion, protected from light). Coverslips were mounted by inverting on a drop of VECTASHIELD® Antifade Mounting Medium (Vector Laboratories), or Fluoromount-G mounting medium (Thermo Fisher) on +ASSURE®+ frosted slides (Epic Scientific). Cells were visualized and imaged using a Zeiss AxioImager microscope fitted with a sCMOS camera, and ZEN software. Antibodies and lectins used for immunofluorescence were:

| **Antibody** | **Manufacturer and catalog #** | **Dilution** |
|---|---|---|
| 53BP1 | Bethyl # A300-081A | 1:1000 |
| cleaved caspase 3 | Cell Signaling #9661 | 1:1000 |
| γH2AX | Millipore # 05-636 | 1:250 |
| PTEN | Cell Signaling #9559 | 1:250 |
| WGA-Alexa Fluor 647 | Thermo Fisher # W32466 | 5 µg/mL |
| Goat anti-mouse-Alexa Fluor 488 | Thermo Fisher # A-11001 | 1:500 |

| | | |
|---|---|---|
| Goat anti-rabbit-Alexa Fluor 568 | Thermo Fisher # A-11011 | 1:500 |
| Goat anti-rabbit-Alexa Fluor 647 | Thermo Fisher # A-21244 | 1:500 |

**HSC sorting, culture, and flow cytometry for cell division analysis**

LT-HSCs were sorted from freshly isolated bone marrow, as described above, and collected in MACS buffer in 1.5 mL tubes. Sorted cells were spun at 300 x g for 5 minutes. The liquid was removed, and the cells were resuspended in PBS containing 5 µM CellTrace Violet (Thermo Fisher #C34557). The cells were incubated for 20 minutes are ambient temperature with gentle motion. The labeling reaction was quenched by transferring the sample to a 5 mL tube containing 3 mL MACS buffer with 1.25% BSA, and incubated for 5 minutes. Cells were spun at 300 x g for 5 minutes, and resuspended in StemPro-34 medium containing supplements and cytokines, as described above. Cells were then seeded in round-bottom 96-well plates. Where indicated, cells where exposed to 2 Gy irradiation, or cultured in the presence of 50 ng/mL aphidicolin. 72 hours after culture initiation, cells were collected in MACS buffer, incubated with 7-AAD Viability Staining Solution (BioLegend) at a 1:500 dilution for 30 minutes on ice, washed twice in MACS buffer, and analyzed by flow cytometry.

**Gene expression analysis and measurement of nuclear and mitochondrial DNA**

Cells were sorted from freshly isolated bone marrow, as described above, into 1.5 mL tubes containing MACS buffer, maintained at 4°C throughout the sorting procedure. The sorted cells were then spun at 300 x g for 5 minutes. The liquid was removed, and the cells were immediately processed for RNA or DNA extraction. DNA was isolated using the QIAamp DNA Micro Kit (Qiagen) following the manufacturer's protocol, and eluted in a final volume of 20 µL $H_2O$. RNA was isolated using the RNeasy Plus Micro Kit (Qiagen) according to the manufacturer's protocol, and eluted in 18 µL $H_2O$. 15 µL of the eluate was used for cDNA synthesis using the iScript cDNA Synthesis Kit (Bio-Rad) in a final volume of 20 µL (containing 4 µL iScript Reaction Mix and 1 µL iScript Reverse Transcriptase) according to the manufacturer's protocol. The cDNA samples were then diluted 2-fold by adding 20 µL $H_2O$. qPCR reactions were assembled in 384-well plates, each reaction containing 5 µL 2X Power SYBR Green PCR Sample Mix (Thermo Fisher), 3 µL $H_2O$, 0.5 µL each of 10 µM forward and reverse primers, and 1 µL DNA or cDNA. Samples were run on an ABI 7900HT Fast Real-Time PCR instrument (Applied Biosystems) with the following parameters: 95°C for 10 minutes, followed by 45 cycles

of 95°C for 15 seconds and 60°C for 1 minute. Data were analyzed using the comparative Ct method. The primers used in this study were:

| **Primer** | **Sequence** |
|---|---|
| *Actb* (for genomic DNA) (forward) | 5'-CGGCTTGCGGGTGTTAAAA-3' |
| *Actb* (for genomic DNA) (reverse) | 5'-CGTGATCGTAGCGTCTGGT-3' |
| *Atm* (forward) | 5'-TGTCATGCAGCAGGTCTTCC-3' |
| *Atm* (reverse) | 5'-GTGCACCACTCGAGAACAC-3' |
| *Cytb* (for mitochondrial DNA) (forward) | 5'-CTTCATGTCGGACGAGGCT-3' |
| *Cytb* (for mitochondrial DNA) (reverse) | 5'-TGTGGCTATGACTGCGAAC-3' |
| *Pten* (forward) | 5'-ACACCGCCAAATTTAACTGC-3' |
| *Pten* (reverse) | 5'-TACACCAGTCCGTCCCTTTC-3' |
| *Rpl19* (forward) | 5'-CGGGAATCCAAGAAGATTGA-3' |
| *Rpl19* (reverse) | 5'-TTCAGCTTGTGGATGTGCTC-3' |

**Western blotting**

Lineage-negative cells were isolated from the bone marrow as described above. For SCF stimulation experiments, cells were starved in 1.5 mL tubes in serum-free IMDM for 30 minutes at 37°C in a cell culture incubator. Where indicated, SCF was added (50 ng/mL), and the cells were incubated at 37°C with gentle motion for 15 minutes. The cells were then collected by centrifugation at 300 x g. The liquid was removed, and the cell pellet was snap-frozen on dry ice and stored at -80°C until further processing. Cells were lysed in 100 µL Laemmli buffer (2% SDS; 62.5 mM Tris-HCl pH 6.8) by passing repeatedly through a 27 gauge needle. Samples were prepared by mixing lysates (equivalent amounts of protein) and Laemmli buffer, adding up to a volume of 26 µL, with 10 µL of 4X 1X Bolt LDS Sample Buffer (Thermo Fisher #B0007), and 4 µL 1M DTT. The samples were heated at 95°C for 5 minutes, and loaded on Bolt 4-12% Bis-Tris Plus (AKT blots) or NuPAGE 3-8% Tris-Acetate (ATM blots) gels (Thermo Fisher). The gels were run at 165 V for 45 minutes (4-12% gels) or 100 V for 90 minutes (3-8% gels), and proteins were transferred on methanol-activated Immobilon-P PVDF membranes in Bolt Transfer Buffer (Thermo Fisher) for 1 hour at 30 V (4-12% gels) or 18 hours at 150 mA at 4°C (3-8% gels). Protein detection was then performed using the LI-COR system (LI-COR Biotechnology): The membranes were blocked for 1 hour at ambient temperature in Intercept (TBS) Blocking Buffer, and then incubated overnight at 4°C with primary antibodies diluted in blocking buffer containing 0.1% Tween-20. Membranes were washed 3 times in TBST buffer (50 mMTris-HCl pH7.5, 150 mM NaCl, 0.1% Tween-20), incubated for 1 hour at ambient temperature with fluorophore-conjugated secondary antibodies diluted in blocking buffer

containing 0.1% Tween-20 and 0.01% SDS, and washed 3 times in TBST. Fluorescence detection was performed with an Odyssey CLx instrument (LI-COR).

Antibodies used for western blotting were:

| **Antibody** | **Manufacturer and catalog #** | **Dilution** |
|---|---|---|
| AKT | Cell Signaling # 4691 | 1:1000 |
| Phospho-AKT-S473 | Cell Signaling # 4051 | 1:1000 |
| ATM | Santa Cruz # sc-23921 | 1:300 |
| PTEN | Cell Signaling # 9559 | 1:1000 |
| α-Tubulin | Sigma # T6199 | 1:5000 |
| Anti-rabbit-Alexa Fluor 680 | Thermo Fisher # A21109 | 1:5000 |
| Anti-mouse-IRDye 800CW | LI-COR # 926-32210 | 1:10 000 |

**Methylcellulose colony-forming assays**

To measure hematopoietic colony-forming ability, bone marrow cells were isolated as described above, and diluted at 2 x $10^5$ cells/mL in MACS buffer. 400 µL of this cell suspension (equivalent to 8 x $10^4$ cells) was added in 4 mL MethoCult GF M3434 methylcellulose medium (STEMCELL Technologies) in 15 mL polypropylene tubes, vortexed vigorously, and left at rest for 5 minutes. In some experiments, N-Acetyl-L-Cysteine (NAC; 1 mM) or L-Buthionine-sulfoximine (BSO; 100 µM) were added along with the cells prior to vortexing. 1.1 mL of the cell preparation was dispensed using 3 mL syringes fitted with 18 gauge needles in each of three wells of 6 well cell culture dishes. PBS was added between the wells to maintain humidity, and the cells were placed in a humidified cell culture incubator at 37°C under 5% $CO_2$. In some experiments, cells were subjected to 2 Gy irradiation immediately after plating. Colonies were scored 7 days after plating, and identified as colony-forming unit; granulocyte-erythroid-macrophage- megakaryocyte (CFU-GEMM), blast-forming unit; erythroid (BFU-E), or colony-forming unit; granulocyte-macrophage (CFU-GM), following the guidelines of the technical manual accompanying the MethoCult GF M3434 methylcellulose medium.

For flow cytometric assessment of methylcellulose colonies, 1 mL TrypLE (Thermo Fisher) was added to each well. The methylcellulose medium and TrypLE were mixed by pipetting, and transferred to 5 mL polystyrene tubes. An additional 1 mL TrypLE was added to each well, and transferred to the same tubes. Cells were centrifuged at 300 x g for 5 minutes at 4°C, and washed once with 3 mL FACS buffer. Cells were resuspended in 100 µL FACS buffer containing 1:100 Fc blocking antibody, and incubated at ambient temperature for 20 minutes. 100 µL of antibody cocktail containing cKit-PE/Cy7 (1:75), Sca1-PE (1:50), Mouse Lineage

Detection cocktail (1:20; Miltenyi Biotec # 130-092-613), and 2 µg/mL DAPI was added to the cells (final concentrations are 1:2 of those indicated above), and the cells incubated on ice for 15 minutes. 2 µL Streptavidin-PE/Cy5 (final concentration 1:100) was added, and the cells incubated for an additional 20 minutes on ice. Cells were washed twice with 3 mL FACS buffer with centrifugation at 300 x g for 5 minutes at 4$^{o}$C, resuspended in 500 µL FACS buffer, and analyzed by flow cytometry.

**Single-cell sorting, capture, and cDNA synthesis**

To sort long-term hematopoietic stem cells (LT-HSCs) for single-cell transcriptomic analyses, bone marrow cells were isolated and subjected to lineage-negative enrichment as described above. For each genotype and sex, lineage-negative cells from 3 animals were pooled and resuspended in 300 µL MACS buffer containing 1:100 Fc blocking antibody, and incubated on ice for 15 minutes. The following antibodies were added at the indicated concentrations: Sca1-FITC (1:100), CD150-PE (1:50), CD48-APC (1:100), cKit-PE/Cy7 (1:100), Streptavidin-APC/Cy7 (1:50), and DAPI (5 µg/mL). Cells were incubated with antibodies on ice for 30 minutes. 3.5 mL MACS buffer was added, and the cells were filtered through a 30 µm nylon mesh. An additional 3.5 mL MACS buffer was added through the nylon mesh. Cells were centrifuged at 300 x g for 5 minutes at 4$^{o}$C, and resuspended in 400 µL MACS buffer. Cells were filtered through a 35 µm nylon mesh into a 1.5 mL microtube immediately prior to sorting.

Before sorting, a small-sized (5-10 µm) C1 Single-Cell AutoPrep IFC microfluidic chip (Fluidigm) was primed using the script “SMART-Seq v4: Prime Rev B (1771x) for 5-10 µm diameter cells” according to the manufacturer’s instruction. 2.5 µL of Cell Suspension-Viability reagent, prepared by mixing Cell Suspension buffer (Fluidigm) and LIVE/DEAD Viability/Cytotoxicity Kit for mammalian cells (Thermo Fisher) components at a ratio of 1:1:50 (Ethidium homodimer-1 : Calcein AM : Cell Suspension buffer) was added in the cell loading inlet of the C1 chip. Cells were sorted directly onto the cell loading inlet of the C1 chip, using optimized single-cell sorting parameters on a BD FACSARIA instrument (BD Biosciences). Typically, cells were sorted at an approximate speed of 3500 events/second with low-pressure settings, yielding approximately 1000 LT-HSCs (~0.2% of the total number of events), in a volume of 4.5-5 µL, in around 10 minutes.

After sorting, the cell loading inlet of the C1 chip contained approximately 7.5 µL of sorted LT-HSCs at a final concentration of ~130-140 cells/µL and cell buoyancy ratio of ~60-65% (murine primary HSCs : Cell Suspension-Viability reagent). The C1 chip was then

processed on a Fluidigm C1 instrument (Fluidigm) using the script “SMART-Seq v4: Cell Load Rev B (1771x) for 5-10 µm diameter cells”. This protocol was designed to capture individual LT-HSC in up to 96 capture chambers in the C1 chip in approximately 30 minutes. The chip was then transferred to an EVOS FL Cell Imaging System (Thermo Fisher), and each cell was imaged under bright-field and fluorescence illumination to visualize the viability stain. C1 reagents (Fluidigm) and SMART-Seq v4 Ultra Low Input RNA Kit for the Fluidigm C1 System (Clontech) were added to the designated wells according to the manufacturers’ instructions. The chip was then returned to the Fluidigm C1 system and the script “SMART-Seq v4: Sample Prep Rev B (1771x), for 5-10 um diameter cells” was executed, which took ~8 h and included single cell lysis, reverse transcription, and full-length cDNA synthesis. After completion of the protocol, the amplified single cell cDNAs were harvested in a total of 8 µL C1 DNA Dilution Reagent (Fluidigm). 1 µL of the diluted cDNA was analyzed using Agilent High Sensitivity DNA Kit on an Agilent BioAnalyzer 2100 (Agilent Technologies, Inc.) and quantified using Quant-iT PicoGreen dsDNA Assay kit (Thermo Fisher) on a SpectraMax M3 Microplate Reader (Molecular Devices). The average size of the single cell cDNA synthesized was between 1300 to 1,700 bp in length and the typical yield was ~400-1200 pg/µL.

**Single-cell library preparation and sequencing**

Single cell cDNA libraries were constructed using Nextera XT DNA Library Kit (Illumina) according to the modified protocols described in the SMART-Seq V4 Ultra Low Input RNA Kit for the Fluidigm C1 System, IFCs User Manual (Clontech). 100-300 pg of each amplified single cell cDNA was used as the input for Nextera XT library construct, and Illumina N7 and S5 indexes were added (Illumina) for tagmentation followed by PCR amplification using an Applied Biosystems Veriti 96-Well Thermal Cycler (Thermo Fisher). cDNA libraries were pooled and purified with Agencourt AMPure XP beads (Beckman Coulter, Inc.) twice. Final cDNA libraries sizes were validated using the Agilent Bioanalyzer 2100 and quantified by quantitative PCR using KAPA SYBR FAST qPCR kit (Kapa Biosystems, Inc.) on a CFX96 Real-Time system (BioRad). The libraries were then sequenced on the NextSeq 500 system (Illumina, Inc.) using a pair-end 75 cycle sequencing run to achieve one million reads per cell.

**Single-cell RNA sequencing data analysis**

*Raw data quality control, read-alignment, and count-matrix assembly*

The raw 76-basepair paired-end reads from the sequencer, obtained as compressed FASTQ files with one file per cell, were first quality-checked using FASTQC v0.11.5 (https://www.bioinformatics.babraham.ac.uk/projects/fastqc) and MultiQC v0.8(4) software packages, and then aligned to the *Mus musculus* genome assembly version GRCm38 (mm10) from the Genome Reference Consortium using the STAR aligner v2.5.2b(5). The aligned transcripts were quantified using RSEM v1.3.0(6). The RSEM quantification output files were then further processed using in-house R scripts to assemble a consolidated gene-by-cell read-count matrix denoting single-cell gene expression, with rows corresponding to genes and columns corresponding to cells.

*Filtering, normalization, and variable gene selection*

The assembled matrix of raw count (in which each row is a gene, and each column is a cell), was then fed into the standard workflow of the single-cell data processing R package, Seurat v2.3.4 (run in R v3.5.1). Only genes that were expressed (read count > 0) in at least 3 cells, and only cells that expressed at least 200 genes, were retained for downstream processing. Furthermore, cells expressing more than 7000 genes (potential multiplets), and cells with more than 30% of the reads mapping to mitochondrial genes, were removed from the analysis. The final filtered matrix contained 11908 genes and 206 cells, with 116 $Pten^{+/+}$ cells and 90 $Pten^{S398A/S398A}$ cells. The filtered matrix was log-normalized using global scaling in Seurat with the scaling factor value set to 10000. To identify highly varying genes, the *FindVariableGenes* module of Seurat was employed to establish the mean-variance relationship of the normalized counts of each gene across cells. Genes whose log-mean was between 0.1 and 8 and whose dispersion was above 1, were chosen, resulting in a total of 858 highly varying genes.

*Dimensionality reduction, clustering, t-SNE visualization, and canonical markers*

The log-normalized matrix was subjected to dimensionality reduction by carrying out principal component analysis (PCA) on the highly varying genes. Upon a visual inspection of the PCA elbow plot, which plots the standard deviations of the principal components (PCs), the top 10 PCs were chosen for further analysis. Clustering was performed on the chosen PCs using the shared nearest neighbor (SNN) modularity optimization algorithm in Seurat, with default parameters (resolution = 0.6). To visualize the clusters in two dimensions, a t-stochastic neighbor embedding (t-SNE) map was computed and plotted using the appropriate modules in Seurat. Cluster-defining canonical marker genes were identified by comparing the gene-by-gene

average expression levels within a cluster with the average levels across the rest of the cell population using the Wilcoxon rank sum test.

*Dormant and active scores*

To compute dormant and active HSC scores, we used a previously published gene signature defining the dormant-to-active state transition derived from single-cell HSC transcriptomic data (7). This signature was restricted to the top 100 genes enriched in dormant cells and to the top 300 genes enriched in active cells. Upon filtering out the genes that were not expressed in our dataset, these lists were further trimmed to 92 genes enriched in dormant cells and 295 genes enriched in active cells. Each gene was weighted by its relative average expression in dormant vs. active cells, using data from (7). To compute the scores for each cell, a weighted average of the log-normalized expressions of the corresponding gene subsets were computed and these averages were then z-scored.

*Re-clustering the quiescent HSCs*

To identify transcriptional differences between *Pten*$^{+/+}$ and *Pten*$^{S398A/S398A}$ quiescent HSCs, we re-clustered only the quiescent HSCs (Clusters 1 and 2 from the original analysis), with the clustering driven by the dorman-to-active state transition gene signature described above (7). The same general pipeline as for the original clustering analysis was applied until the step before dimensionality reduction (PCA). The initial filtering steps yielded a final matrix of 11908 genes and 161 cells, with 85 *Pten*$^{+/+}$ cells and 76 *Pten*$^{S398A/S398A}$ cells. A sub-matrix containing only the state-transition gene signature (the set of 387 genes from above) was then given as input to the PCA step instead of the default sub-matrix corresponding to the highly varying genes. Upon a visual inspection of the PCA elbow plot, the top 6 PCs were chosen for clustering (resolution = 0.8). The clusters were then visualized using t-SNE plots in the usual manner, and the marker genes were computed using the Wilcoxon rank sum test, as before.

**Gene Set Enrichment Analysis**

Gene Set Enrichment Analysis (GSEA) was performed using the GenePattern platform (Broad Institute, MIT). The “GSEAPreranked” module was used on ranked lists of differentially expressed genes (filtered to include only genes with an uncorrected $p$ value <0.05). Gene sets queried were: the “Canonical Pathways” subset of C2 (Curated Gene Sets), and C5 (Gene Ontology Gene Sets).

**Publicly depostited data and accession numbers**

The single-cell RNA sequencing data have been deposited in Gene Expression Omnibus, under accession number: GSE164388

**Statistics**

Data were analyzed using one- or two-way analysis of variance (ANOVA), *t*-tests, or Mantel-Cox analysis, followed by multiple-comparisons tests (Tukey, Dunnett or Sidak) where appropriate, using GraphPad Prism version 7.03. Throughout the manuscript, the following notation was used to indicate statistical significance: *:$p<0.05$; **:$p<0.01$; ***:$p<0.001$. All the error bars represent the standard error of the mean (SEM).

**Study approval**

All animal experiments were performed in accordance with institutional and federal guidelines, and approved by the institutional Animal Care Committee (protocols #985 and #5975).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Uncropped images of the western blot figures shown in this paper

Figure 1B

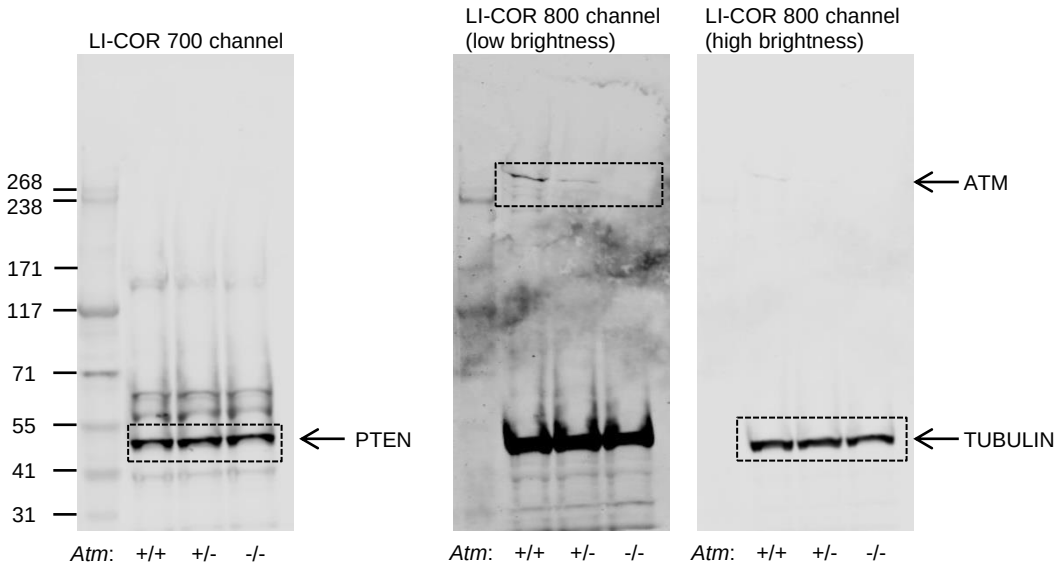
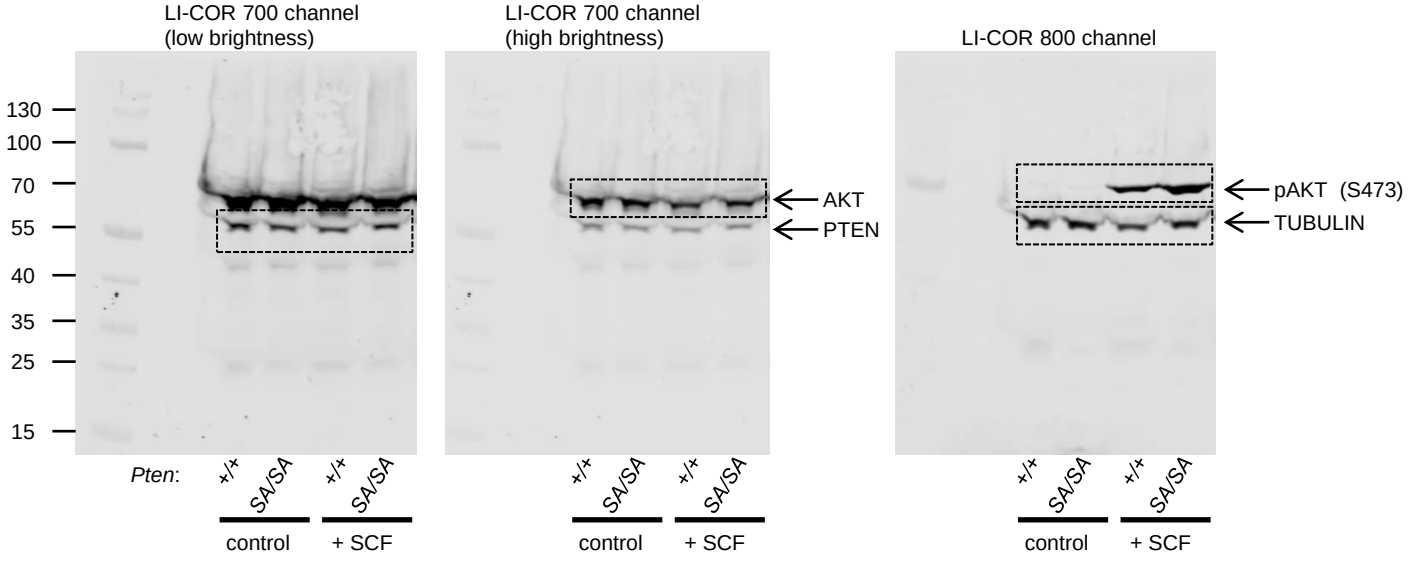



Figure 2B




Uncropped images of the western blots shown in this paper. The membranes were probed simultaneously will all of the indicated antibodies, and imaged in the Odyssey CLX 700 channel (rabbit antibodies) and 800 channel (mouse antibodies). Note that for Figure 2B, sample preparation and gel running conditions caused all the proteins to run slightly above their expected molecular weight in comparison to the ladder. (Ladder: 2 µL directly loaded into the gel without additional buffer, following standard procedures for LI-COR experiments. Samples: 40 µL containing 50 µg protein in Laemmli buffer).