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**Graphical abstract**

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Concerted roles of PTEN and ATM in controlling hematopoietic stem cell fitness and dormancy

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In order to sustain proficient life-long hematopoiesis, hematopoietic stem cells (HSCs) must possess robust mechanisms to preserve their quiescence and genome integrity. DNA-damaging stress can perturb HSC homeostasis by affecting their survival, self-renewal, and differentiation. Ablation of the kinase ataxia telangiectasia mutated (ATM), a master regulator of the DNA damage response, impairs HSC fitness. Paradoxically, we show here that loss of a single allele of Atm enhances HSC functionality in mice. To explain this observation, we explored a possible link between ATM and the tumor suppressor phosphatase and tensin homolog (PTEN), which also regulates HSC function. We generated and analyzed a knockin mouse line (PtenS398A/S398A), in which PTEN cannot be phosphorylated by ATM. Similar to Atm+/−, PtenS398A/S398A HSCs have enhanced hematopoietic reconstitution ability, accompanied by resistance to apoptosis induced by genotoxic stress. Single-cell transcriptomic analyses and functional assays revealed that dormant PtenS398A/S398A HSCs aberrantly tolerate elevated mitochondrial activity and the accumulation of reactive oxygen species, which are normally associated with HSC priming for self-renewal or differentiation. Our results unveil a molecular connection between ATM and PTEN, which couples the response to genotoxic stress and dormancy in HSCs.

Introduction

Life-long hematopoiesis is required to produce all the blood cell types that maintain vital functions such as immunity, tissue repair, and oxygen transport. Hematopoietic dysregulation can have severe consequences for the organism, including impaired immune response, anemia, myelodysplastic syndromes, and leukemias (1–6). Hematopoietic stem cells (HSCs) sit at the apex of a cellular self-renewal and differentiation hierarchy that maintains hematopoiesis throughout life (7). Robust mechanisms must exist to ensure HSC fitness and integrity, thus preserving their unique capacity to self-renew and give rise to differentiating progenies (7). Accordingly, the most primitive HSCs remain largely dormant, dividing infrequently (8). Despite decades of intensive studies since their initial functional identification (9, 10), the mechanisms controlling whether HSCs remain dormant, self-renew, differentiate, or become senescent remain incompletely understood (7). Upon exit from dormancy, HSCs undergo profound transcriptional changes to reach an active state, primed to enter the cell cycle or differentiate (11). HSC activation is associated with enhanced metabolic activity and the production of reactive oxygen species (ROS), which can be a source of endogenous DNA-damaging stress (11, 12).

A proper DNA damage response is critical for maintaining HSC function (12, 13). The kinase ataxia telangiectasia mutated (ATM) is a master regulator of the cellular response to DNA damage (14). ATM exerts its functions by phosphorylating a large number of targets, which in turn control specific aspects of the DNA damage response, including DNA repair, apoptosis, and cell cycle arrest (14). Genetic deletion of Atm impairs HSC function, at least in part due to the uncontrolled accumulation of ROS (15). Inactivating various ATM targets can disrupt a subset of its functions, while preserving others, sometimes resulting in opposite phenotypes to those caused by complete Atm ablation (16–20). Phosphatase and tensin homolog (PTEN) has recently been identified as being directly regulated by ATM through phosphorylation on serine 398 (S398) (21). PTEN is a tumor suppressor that controls a plethora of nuclear function, or other, remains unknown. Here, by combining the analysis of genetically engineered mouse models, single-cell transcriptomic studies, and in vivo and in vitro functional assays, we show that phosphorylation of PTEN by ATM controls HSC dormancy, response to DNA damage, and fitness.

Results

Reducing Atm genetic dosage or ablating ATM-dependent PTEN phosphorylation enhances long-term hematopoietic reconstitution potential. While investigating mechanisms linking the control of genomic stability and HSC fitness, we explored whether HSC
ability, we competitively transplanted equal numbers of wild-type CD45.1+ and Atm+/+, Atm+/−, or Atm−/− CD45.2+ bone marrow cells into lethally irradiated CD45.1+ mice (Figure 1C). Multilineage reconstitution potential was measured over 20 weeks after transplant in primary and in secondary recipients, a well-established assay to measure HSC fitness (26, 27). Consistent with previous results (15), Atm−/− bone marrow cells were impaired in their relative ability to reconstitute long-term hematopoiesis, indicated by functionality may be sensitive to the genetic dosage of ATM, given its role in orchestrating multiple aspects of the DNA damage response. As expected, Atm expression was lower in Atm+/− hematopoietic stem and progenitor cells compared with their Atm+/+ counterparts, and was undetectable in Atm−/− cells (Figure 1A). Similar gene dosage–dependent differences in ATM protein levels were observed in lineage-negative (Lin−) bone marrow cells (Figure 1B). To functionally evaluate their hematopoietic reconstitution
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RESEARCH ARTICLE

A

B

C

D

E

CD45.2\(^+\) Pten\(^{-/-}\) or Pten\(^{S398A/S398A}\)

CD45.1\(^+\) (wild-type)

1:1

1

3'UTR

S398A

PGK-Neo

Cre or FLPe

loxP

7

8

9

3'UTR

S398A

PGK-Neo

Cre or FLPe

loxP

S398A

TUBULIN

Merge

WGA

DAPI

PTEN

pAKT

AKT

Control

+ SCF

4 h after IR

No IR

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a decreased proportion of Atm−/−-derived circulating blood cells, compared with their Atm+/− counterparts (Figure 1D and Supplemental Figure 1, A–C; supplemental material available online with this article; https://doi.org/10.1172/JCI131698DSI). Interestingly, this was largely restricted to the lymphoid lineages (B220+ B cells and CD3+ T cells) in primary transplantation recipients (Figure 1D and Supplemental Figure 1, A–O). The defective reconstitution potential of Atm−/− cells was accentuated in secondary recipients, where it also extended to the myeloid lineages (CD11b+ and Gr1+; Figure 1E and Supplemental Figure 1D). In stark contrast, Atm+/− cells displayed enhanced multilineage reconstitution potential, which was maintained over the 20-week primary posttransplant period, and extended to secondary recipients, suggesting that it reflects improved HSC functionality (Figure 1, D and E, and Supplemental Figure 1, C and D).

To explain these paradoxical results, we reasoned that, while complete Atm ablation causes the loss of all ATM-regulated processes, partial Atm deficiency may preferentially impair the function of specific ATM targets. A potential role for ATM-dependent regulation of PTEN in controlling HSC fitness has not yet been examined. To selectively disrupt PTEN phosphorylation by ATM in vivo, without affecting its lipid phosphatase activity, we generated a knockin allele in mice, in which serine 398 is mutated to alanine (PtenS398A) (Figure 2A). PtenS398A mice were obtained at Mendelian ratio, were viable, and developed normally. PtenS398A mice and Pten−/− Lin− bone marrow cells had comparable PTEN protein levels, as well as similar AKT phosphorylation levels with and without stem cell factor (SCF, also known as KIT ligand) stimulation (Figure 2B). Accordingly, PTEN expression was normal in Atm−/− and Atm+/− hematopoietic stem and progenitor cells (Figure 1B and Supplemental Figure 1E). Previous work has shown that in tumor cells, PTEN is excluded from the nucleus in response to irradiation (IR), through a mechanism that requires its phosphorylation by ATM (21). In cultured Pten−/− HSCs, PTEN was partially redistributed toward the plasma membrane 4 hours after 1 Gy IR, but this did not occur in PtenS398A Atm+/−, and Atm−/− HSCs (Figure 2C and Supplemental Figure 1F).

We next investigated steady-state hematopoiesis in PtenS398A mice. Peripheral blood analyses indicated comparable circulating numbers of lymphocytes, monocytes, and red blood cells in PtenS398A and Pten+/+ littermates (Supplemental Figure 1G). PtenS398A mice had normal overall numbers of stem and progenitor cells in the bone marrow, defined as Lin−cKit+Sca1+ (LSK) cells, but a slight expansion of Lin−cKit+Sca1−CD150− (LK) myeloid progenitor cells (Figure 2D). This increase appeared to be distributed between common myeloid progenitors (CMPs; Lin−cKit+Sca1 CD34+CD16/32−), granulocyte/macrophage progenitors (GMPs; Lin−cKit+Sca1 CD34+CD16/32+), and megakaryocytes/erythroid progenitors (MEPs; Lin−cKit+Sca1 CD34−CD16/32−) (Figure 2D). Within LSK cells, PtenS398A mice had normal numbers of long-term HSCs (LT-HSCs; Lin−cKit+Sca1+CD48+CD150−), multipotent progenitors (MPPs; Lin−cKit+Sca1+CD48+CD150+), and hematopoietic progenitor cells (HPC-1 and HPC-2; Lin−cKit+Sca1−CD48−CD150−) (Figure 2D). Overall, these results suggest that steady-state hematopoiesis is largely normal in PtenS398A mice.

To evaluate PtenS398A HSC functionality in vivo, we measured their relative ability to reconstitute hematopoiesis in competitive transplantation experiments. Lethally irradiated CD45.1+ mice were transplanted with equal numbers of wild-type CD45.1+ and Pten−/− or PtenS398A CD45.2+ bone marrow cells. Compared with Pten−/−, PtenS398A cells displayed enhanced multilineage reconstitution potential, as indicated by an increased proportion of PtenS398A CD45.2−-derived circulating blood cells (Figure 2E). This difference was maintained over the 20-week posttransplant period (Figure 2E), and further enhanced upon serial transplantation into secondary recipients (Supplemental Figure 2A). Similar to Atm−/−, the enhanced reconstitution potential of PtenS398A mice was seen across B220+ B cells, CD3+ T cells, CD11b+ and Gr1+ myeloid cells (Figure 2F and Supplemental Figure 2A), and was not associated with lineage skewing (Supplemental Figure 2B). Together, these results suggest that PtenS398A HSCs have enhanced fitness.

PtenS398A HSCs are resistant to genotoxic stress. We next investigated whether the increased in vivo functionality of PtenS398A HSCs might be associated with an abnormal response to genotoxic stress. We first evaluated the response of PtenS398A mice to a 10 Gy IR challenge, which normally causes lethality as a result of hematopoietic failure (10). Whereas all of the Pten−/− mice succumbed following IR with a median survival of 13.5 days, lethality was delayed in their PtenS398A littermates, with half of the animals surviving until at least 30 days after IR (Figure 3A). This was reminiscent of the phenotype observed in mice lacking the ATM target CHK2, which regulates p53-dependent cell cycle arrest and apoptosis in response to DNA damage (20, 28). Therefore, we sorted LT-HSCs and progenitors from Pten−/− and PtenS398A littermates, and measured their propensity to undergo...
go apoptosis upon an IR challenge in vitro, as judged by the accumulation of cleaved caspase 3. Four hours after 1 Gy or 2 Gy IR, PtenS398A/S398A LT-HSCs and progenitors had a lower proportion of cells expressing cleaved caspase 3 compared with Pten+/+ cells, suggesting a defective apoptotic response to DNA damage (Figure 3B and Supplemental Figure 3A). Accordingly, the colony-forming ability of PtenS398A/S398A bone marrow cells in methylcellulose was enhanced, both under baseline conditions and in response to 2 Gy IR (Figure 3C). This was also observed with Atm−/− cells, whereas Atm+/+ cells were profoundly impaired in their ability to generate colonies, particularly after IR (Figure 3C).

To assess whether the apparent resistance of PtenS398A/S398A cells to IR was associated with an abnormal response to DNA damage or its repair, we measured the emergence and resolution of γH2AX and 53BP1 foci following ex vivo IR (1 Gy) in sorted LT-HSCs. PtenS398A/S398A and Pten+/+ cells accumulated similar numbers of foci 4 hours after IR, and these were largely resolved by 24 hours (Figure 3, D and E). In contrast, Atm−/− cells showed more γH2AX and 53BP1 foci at 4 hours, and were impaired in damage resolution at 24 hours after IR (Figure 3F). PtenS398A/S398A and Atm−/− cells tended to retain more foci at the 24-hour time point, such that Atm−/− cells were not significantly different than either the Atm+/+ or Atm−/− LT-HSCs (Figure 3F). Similarly, assessment of γH2AX immunoreactivity in freshly isolated bone marrow cells pointed to an accumulation of endogenous DNA damage by Atm−/− LT-HSCs in vivo, whereas PtenS398A/S398A and Atm−/− cells showed nonsignificant trends compared with their respective wild-type controls (Figure 3G). To assess whether PtenS398A/S398A LT-HSCs had similar responses to different types of DNA damage, we measured γH2AX and 53BP1 foci, as well as cleaved caspase 3 immunoreactivity, 4 hours and 24 hours following IR, or upon topoisomerase II poisoning (etoposide), oxidative stress (buthionine sulfoximine; BSO), or replication stress (aphidicolin). Interestingly, PtenS398A/S398A cells were resistant to IR-, etoposide-, and BSO-induced apoptosis, but had normal sensitivity to aphidicolin (Figure 3H and Supplemental Figure 3I). Induction of γH2AX and 53BP1 foci in response to all treatments was similar in PtenS398A/S398A and Pten+/+ LT-HSCs (Supplemental Figure 3C). Overall, these results suggest that PtenS398A/S398A cells have a higher tolerance to the toxic effects of certain types of DNA damage.

Genotoxic stress can induce cell cycle arrest, a process that is regulated by ATM (14). We therefore assessed whether LT-HSCs, which are predominantly quiescent, had an altered cell cycle distribution in PtenS398A/S398A mice. The proportion of freshly isolated LT-HSCs in G0 phase was slightly lower in PtenS398A/S398A mice compared with their Pten+/+ littermates, with a concomitant increase in the proportion of S-phase–primed G1 cells (Supplemental Figure 3, D and E). The cell cycle distribution in more differentiated progenitor subsets was similar between genotypes (Supplemental Figure 3E). Interestingly, this mild loss of quiescence of PtenS398A/S398A LT-HSCs under homeostatic conditions was also observed in Atm−/−, but not in Atm+/+ animals (Supplemental Figure 3F). To evaluate whether PtenS398A/S398A LT-HSCs are deficient in DNA damage–induced cell cycle arrest, we sorted LT-HSCs from PtenS398A/S398A and Pten+/+ littermates, pulsed them with a fluorescent cell division tracer ex vivo, exposed them to IR or aphidicolin, and subjected them to short-term culture to allow 1 or 2 cell divisions (Figure 3I and Supplemental Figure 3G). The proportion of cells having divided was decreased by either IR or aphidicolin (Figure 3I). Compared with Pten+/+, PtenS398A/S398A LT-HSCs were less sensitive to the effect of IR (Figure 3I). In contrast, cells with either genotype responded similarly to aphidicolin (Figure 3I).

To evaluate the functional consequences of the enhanced tolerance of PtenS398A/S398A HSCs to DNA damage, we performed competitive bone marrow transplantation following an ex vivo IR challenge (29). Pten+/+ or PtenS398A/S398A CD45.2− bone marrow cells were subjected to 2 Gy IR, and mixed at a 10:1 ratio with nonirradiated wild-type CD45.1− cells prior to transplantation into lethally irradiated CD45.1− recipients (Figure 4A and Supplemental Figure 4A), and sustained in secondary recipient mice (Supplemental Figure 4B). This enhanced reconstitution potential was seen across multiple mature cell types (Figure 4A and Supplemental Figure 4A) and was not associated with preferential expansion of a particular lineage (Supplemental Figure 4C). To examine the HSC and progenitor reconstitution potential of irradiated Pten+/+ and PtenS398A/S398A cells, we analyzed the bone marrow of recipient mice 20 weeks after transplantation. Compared with recipients of Pten+/+ cells, animals transplanted with PtenS398A/S398A cells had a higher proportion of CD45.2− LT-HSCs (Figure 4B and Supplemental Figure 4D). Interestingly, within the CD45.2− LSK compartment, PtenS398A/S398A bone marrow recipients had a higher proportion of LT-HSCs, and a lower proportion of more differentiated HPC-2 cells (Figure 4C). By contrast, the proportion of these subsets within CD45.1+ LSK cells was similar between conditions, as expected given that CD45.1+ competitor cells were wild type,
and were derived from the same pool of donors for all the transplanted mice (Supplemental Figure 4E). Overall, these results suggest that PTEN S398A/S398A HSCs preserve enhanced survival and fitness in response to DNA damage in vitro and in vivo.

**Transcriptomic anomalies in single quiescent PTEN S398A/S398A HSCs.**

To begin deciphering the molecular mechanisms underlying enhanced HSC functionality in PTEN S398A/S398A mice, we profiled the transcriptomes of sorted LT-HSCs by single-cell RNA sequencing (RNA-seq), using the microfluidic chip-based Fluidigm C1 platform (see Supplemental Figure 5A for sorting strategy). After quality control filtering (see Supplemental Methods), we obtained transcriptomic data for 116 PTEN+/+ and 90 PTEN S398A/S398A cells, derived from a total of 6 mice from 2 independent sorting procedures for each genotype (Supplemental Figure 5B). Clustering of the single cells using the Seurat pipeline (30) and visualization by t-distributed stochastic neighbor embedding (t-SNE) identified 3 cell clusters (Clusters 1, 2, and 3; Figure 5A). Marker gene analysis and machine learning–based cell-cycle phase assignment (31) indicated that Cluster 3 contained actively proliferating (S, G2, or M phase) cells, as indicated by high expression of genes such as *mKi67*, *Top2a*, *Ccnb2*, and *Cdca3* (Supplemental Figure 5C).

Clusters 1 and 2 represented quiescent HSCs, highlighted by low expression of cell-cycle regulators, and high expression of quiescent HSC markers such as *Ly6a*, *Mllt3*, *Sult1a1*, and *Hlf* (Supplemental Figure 5C and refs. 11, 32, 33). Interestingly, the PTEN+/+ and PTEN S398A/S398A cells markedly differed in their distribution among the 2 quiescent cell clusters, being enriched in Cluster 2 and Cluster 1, respectively (Figure 5A and Supplemental Figure 5D). Therefore, we sought to identify transcriptional differences between PTEN+/+ and PTEN S398A/S398A quiescent HSCs.

Recently, quiescent HSCs have been shown to undergo a transition from a dormant to an active state under homeostatic conditions (8, 11). We reclustered quiescent HSCs (Clusters 1 and 2 from the above analysis), based on a gene signature that defines...
the transition from the dormant to the active state in single-cell RNA-seq analyses (11). This procedure allocated quiescent Pten+/− and PtenS398A/S398A HSCs to 3 new clusters (Clusters A, B, and C; Figure 5B). Pten+/− cells were largely restricted to 2 clusters (A and B) (Figure 5B and Supplemental Figure 6A). To functionally annotate these clusters, we computed scores measuring the expression of genes that characterize dormant and active HSCs (Figure 5C). Cluster B cells could be assigned to the active state, as reflected by elevated expression of genes involved in DNA replication and nucleotide synthesis such as PcnA, Mcm4, Mcm6, and Ditynk (Supplemental Figure 6B and ref. 11). Gene set enrichment analysis (GSEA) confirmed this classification (Supplemental Figure 6C). Similar proportions of Pten+/− and PtenS398A/S398A cells were assigned to this active cell cluster, suggesting that the ability of PtenS398A/S398A cells to transition through that state is largely normal (Figure 5B and Supplemental Figure 6A). Cells in Cluster A displayed high dormant and low active scores, and therefore could readily be assigned to the dormant state (Figure 5C). These cells showed particularly high expression of quiescent HSC markers such as Ly6a, Sult1a1, and Hlf (Supplemental Figure 6B) (11, 32, 33). Importantly, PtenS398A/S398A cells were underrepresented in Cluster A compared with Pten+/− cells, and made up almost the entirety of Cluster C (Figure 5B and Supplemental Figure 6A). Cells in Cluster C showed a low active score similar to Cluster A, but an intermediate dormant score that fell between that of Cluster A and Cluster B cells (Figure 5C). Together with an underrepresentation of PtenS398A/S398A cells in the dormant Cluster A, these data suggest that Cluster C represents an altered dormant state (hereafter, “dormantPTEN-S398A”).

PtenS398A/S398A dormant HSCs tolerate elevated levels of oxidative stress and have improved fitness. To understand how dormantPTEN-S398A HSCs differ from their dormant counterparts, we
expression of Procr (Figure 5D), a cell-surface marker of exception-
ally multipotent HSCs (34). PROCR + LT-HSCs were slightly, but
significantly, expanded in $Pten^{S398A/S398A}$ mice compared with their
$Pten^{+/+}$ littermates (Figure 6A). Atm+/– mice also had a higher pro-
portion of PROCR + LT-HSCs, whereas healthy Atm –/– mice were
more variable and not significantly different from
Atm +/+ animals (Figure 6B). Furthermore, $Pten^{S398A/S398A}$
PROCR + LT-HSCs had higher intracellular ROS levels and mitochondrial content than
their $Pten^{+/+}$ counterparts (Figure 6C). The elevation in mitochon-
drial content was further confirmed using a qPCR assay to measure
the ratio of mitochondrial to nuclear DNA (ref. 35 and Figure 6D).

To verify whether the enhanced reconstitution potential of transplanted $Pten^{S398A/S398A}$
bone marrow cells was specifically due to improved fitness of quiescent LT-HSCs (34), PROCOR: LT-HSCs were slightly, but significantly, expanded in $Pten^{S398A/S398A}$ mice compared with their $Pten^{+/+}$ litters (Figure 6A). Atm+/– mice also had a higher proportion of PROCOR: LT-HSCs, whereas healthy Atm –/– mice were more variable and not significantly different from Atm +/+ animals (Figure 6B). Furthermore, $Pten^{S398A/S398A}$ PROCOR: LT-HSCs had higher intracellular ROS levels and mitochondrial content than their $Pten^{+/+}$ counterparts (Figure 6C). The elevation in mitochondrial content was further confirmed using a qPCR assay to measure the ratio of mitochondrial to nuclear DNA (ref. 35 and Figure 6D).

To verify whether the enhanced reconstitution potential of transplanted $Pten^{S398A/S398A}$ bone marrow cells was specifically due to improved fitness of quiescent LT-HSCs, we competitively transplanted 300 sorted $Pten^{+/+}$ or $Pten^{S398A/S398A}$ CD45.2+ PROCR+ LT-HSCs alongside $2 \times 10^5$ wild-type CD45.1+ competitors into lethally irradiated CD45.1+ recipients (Figure 7A). Compared with $Pten^{+/+}$, $Pten^{S398A/S398A}$ cells showed improved relative multilineage hematopoietic reconstitution (Figure 7A). Twenty weeks after transplantation, we analyzed the bone marrow of recipient

Figure 7. Improved multilineage hematopoietic reconstitution ability of $Pten^{S398A/S398A}$ PROCR– hematopoietic stem cells in vivo. (A) Diagram representing the experimental setup, and plots showing the proportion of circulating total blood cells, B220+ cells, CD3+ cells, CD11b+ cells, and Gr1+ cells expressing the CD45.2 marker in the peripheral blood of lethally irradiated mice transplanted with 300 $Pten^{+/+}$ or $Pten^{S398A/S398A}$ CD45.2+ PROCR+ LT-HSCs mixed with $2 \times 10^5$ wild-type CD45.1+ competitors. Transplanted cells were isolated from a pool of 3 donor animals per genotype: n indicates the number of transplanted mice. (B) Proportion of LT-HSCs expressing CD45.2+ in the bone marrow from mice transplanted as depicted in panel A, analyzed at 20 weeks after transplantation. The gating strategy is depicted in Supplemental Figure 7A. (C) Proportion of CD45.2+ (left) and CD45.1+ (right) LT-HSCs expressing PROCR in the bone marrow from mice transplanted as depicted in panel A, analyzed at 20 weeks after transplantation. In all panels, mean and SEM are shown. **P < 0.05; ***P < 0.01; ****P < 0.001; assessed by 2-way ANOVA with Sidak’s multiple-comparison test (A) or t test (B and C).
mice. Compared with Pten+/+ recipients, animals transplanted with PtenS398A/S398A cells had a higher proportion of CD45.2+ PROCR+ LT-HSCs (Figure 7B and Supplementary Figure 7A). In addition, the proportion of CD45.2+ LT-HSCs expressing PROCR was higher in the bone marrow of PtenS398A/S398A recipients (Figure 7C). In contrast, the proportion of CD45.1+ LT-HSCs expressing PROCR was similar between the conditions, as expected given that the transplanted CD45.1+ cells were derived from the same group of wild-type donor mice (Figure 7C). Notably, we observed a similar preferential expansion of PROCR-expressing CD45.2+ LT-HSCs in the bone marrow of animals competitively transplanted with wild-type CD45.1+ and Atm+/–, Atm+/–, or IR-exposed PtenS398A/S398A CD45.2+ cells (Supplemental Figure 7, B and C). Collectively, these results suggest that PtenS398A/S398A quiescent LT-HSCs have improved functional fitness in vivo.

Enhanced competitive fitness of PtenS398A/S398A LT-HSCs is normalized by antioxidant treatment. To functionally test whether the enhanced fitness of PtenS398A/S398A HSCs may be due to their ability to tolerate endogenous oxidative stress, we first measured the colony-forming ability of Pten+/+ and PtenS398A/S398A bone marrow cells in methylcellulose cultures in the presence of N-acetyl-L-cysteine (NAC), or the pro-oxidant BSO. Similar to previous results (Figure 3C), PtenS398A/S398A cells had enhanced colony-forming potential compared with their Pten+/+ counterparts (Figure 8A). Addition of NAC lead to an increase in the number of colonies generated by PtenS398A/S398A cells, but it was still substantially higher than in Pten+/+ cultures under the same conditions (Figure 8A). We also assessed the differentiation status of colony-forming cells after 7 days of culture. Similar to their effects on the number of colonies, NAC and BSO increased and decreased, respectively, the proportion of Lin–, Lin cKit+, and Lin cKitScal+ cells in the Pten+/+ cultures (Figure 8B and Supplemental Figure 8, A–C). By contrast, PtenS398A/S398A cultures, which contained a higher proportion of Lin+ cells under basal conditions, were largely insensitive to the effect of NAC or BSO across the Lin–, Lin cKit+, and Lin cKitScal+ subsets (Figure 8B and Supplemental Figure 8, A–C).

The above results raised the possibility that treatment with antioxidants may equalize the reconstitution potential of Pten+/+ and PtenS398A/S398A cells in bone marrow chimeras. To test this, we competitively transplanted equal numbers of wild-type CD45.1+ and Pten+/+ or PtenS398A/S398A CD45.2+ bone marrow cells in lethally irradiated CD45.1+ recipients. The transplanted animals were then assigned to either have access to normal drinking water, or to water supplemented with NAC (Figure 8C). Similar to previous experiments, over the course of 20 weeks after transplantation, PtenS398A/S398A CD45.2+–derived cells displayed better multilineage reconstitution potential than their Pten+/+ CD45.2+ counterparts in mice with normal water (Figure 8D and Supplemental Figure 8D). Importantly, differences between Pten+/+ and PtenS398A/S398A cells were largely abolished in mice receiving NAC (Figure 8D). The apparent decrease in relative fitness of PtenS398A/S398A cells in the presence of NAC may reflect a balancing positive effect of antioxidant treatment on the competing CD45.1+ cells. To relate the effect of NAC to oxidative stress levels within HSCs, we measured intracellular ROS levels in PROCR+ LT-HSCs within the bone marrow of transplanted mice. Consistent with the above observations, ROS levels were higher in PtenS398A/S398A CD45.2+ cells compared with their wild-type CD45.1+ competitors, unlike those in mice transplanted with Pten+/+ CD45.2+ cells (Figure 8, E and F). The differences in the relative ROS levels between the competing PROCR+ LT-HSC populations were normalized by NAC treatment (Figure 8F), while similar trends were observed for the mitochondrial content of PROCR+ LT-HSCs (Figure 8G and Supplemental Figure 8E). Thus, the enhanced function of PtenS398A/S398A cells in bone marrow chimeras may be explained, at least in part, by their enhanced ability to tolerate endogenous oxidative stress.

Discussion

HSC functionality is tightly dependent on the control of genomic stability, which is extensively governed by ATM. Here, we show that HSCs in which PTEN cannot be phosphorylated by ATM have improved fitness and resistance to genotoxic stress, associated with an altered dormant state. These results suggest that the ATM/PTEN axis forms an essential part of the mechanisms that control HSC dormancy. Our single-cell transcriptomic and functional analyses indicated that a subset of PtenS398A/S398A dormant HSCs can aberrantly tolerate increased ROS levels. A concomitant elevation in mitochondrial activity suggests that ROS accumulation is possibly due to enhanced oxidative phosphorylation. Metabolic activity in HSCs is tightly
controlled, and increased oxidative phosphorylation is associated with a transition from the dormant to active state (11, 36, 37). This could form part of the mechanisms that safeguard HSC integrity, by coupling the potential genotoxic effect of ROS to differentiation priming. Indeed, studies in multiple mouse models have identified ROS elevation as a main cause of HSC dysfunction following disruption of regulators of the DNA damage response (15, 19, 38–40). We speculate that an abnormal response to ROS in Pten+/−, S398A HSCs may enable some Pten+/−, S398A dormant HSCs to acquire the biosynthetic capabilities of active cells, while retaining the quiescence and cellular characteristics necessary to maintain their multipotency (Supplemental Figure 9). Such a mechanism, which remains to be formally tested, could conceivably endow Pten+/−, S398A HSCs with superior fitness, as we observe in competitive transplantation assays. In support of this possibility, a recent study has demonstrated that elevated mitochondrial activity enhances the functionality of aged HSCs (41). It would be interesting to investigate whether this phenomenon is associated with an altered response to oxidative stress, coupled with the reported resistance of aged HSCs to genotoxins (42). Furthermore, PTEN itself is sensitive to the cellular redox state (43–45), and it is possible that the Pten−/− mutation could disrupt this regulation. Additional alterations in Pten+/−, S398A HSCs, such as defective apoptotic priming in response to DNA damage, may also contribute to improved HSC functionality by permitting the survival of HSCs that would normally be eliminated.

PTEN can localize to multiple subcellular compartments, where it exerts diverse functions (23). Previous work has shown that PTEN is excluded from the nucleus in response to genotoxic stress and phosphorylation on S398 by ATM (21). In the present study, we observed a similar redistribution of PTEN in HSCs following IR, which was impaired by the PtenS398A mutation. Such exclusion of PTEN from the nucleus could enhance its activity in other compartments, including at the plasma membrane where it functions as a lipid phosphatase to negatively regulate AKT signaling (46, 47). This may affect multiple AKT-dependent signaling pathways that control HSC function and response to genotoxic stress, including mTOR complexes and FOXO transcription factors (24, 48–51). The cellular response to signals from the microenvironment that are transduced via AKT signaling, such as SCF, or ablation) can have a paradoxical tumor-protective effect (39, 59). These observations highlight the complex role of the DNA damage response in controlling homeostasis and tumorigenesis in the hematopoietic system.

Overall, our studies identify ATM phosphorylation of PTEN as essential to safeguard HSC dormancy and response to genotoxic stress. These results could inform strategies for hematopoietic regeneration, and for anticancer treatments in hematologic malignancies.

Methods
Detailed descriptions of animals and experimental procedures are provided in Supplemental Methods.

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).

Publicly deposited data and accession numbers. The single-cell RNA-seq data have been deposited in NCBI’s Gene Expression Omnibus database (GEO GSE164388).

Author contributions
JF and CB conceived the study, designed and performed experiments, analyzed the results, and wrote the manuscript with input from all the authors. PR performed single-cell bioinformatics analyses. WYL designed and performed single-cell transcriptomic experiments. RT, IZ, and GH performed experiments and analyzed the results. BES, JH, CT, KH, and AW generated mice and performed analyses. VS and TWM conceived and supervised the study. All authors revised and approved the manuscript.

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The phenotype of Pten+/−, S398A HSCs bears some resemblance to aging HSCs, which preserve enhanced functionality compared with young HSCs in response to DNA-damaging insults (42). Interestingly, this property of aging HSCs is at least partially dependent on reduced ATM function (42), which could be recapitulated by loss of ATM-dependent PTEN regulation in our Pten+/−, S398A model, as well as in Atm−/− mice. A drawback of higher tolerance to genotoxic stress might be an increased susceptibility to acquire oncogenic lesions (58). We did not observe higher incidence of spontaneous hematologic malignancies throughout the lifespan of Pten+/−, S398A mice, or in bone marrow chimeras transplanted with Pten+/−, S398A cells, suggesting that additional oncogenic hits may be required to uncover a possible tumor-promoting effect of the Pten+/− mutation in the hematopoietic system. Furthermore, previous work has shown that, by promoting differentiation, disruption of the DNA damage response in hematopoietic cells (including by ATM inhibition or ablation) can have a paradoxical tumor-protective effect (39, 59). These observations highlight the complex role of the DNA damage response in controlling homeostasis and tumorigenesis in the hematopoietic system.

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