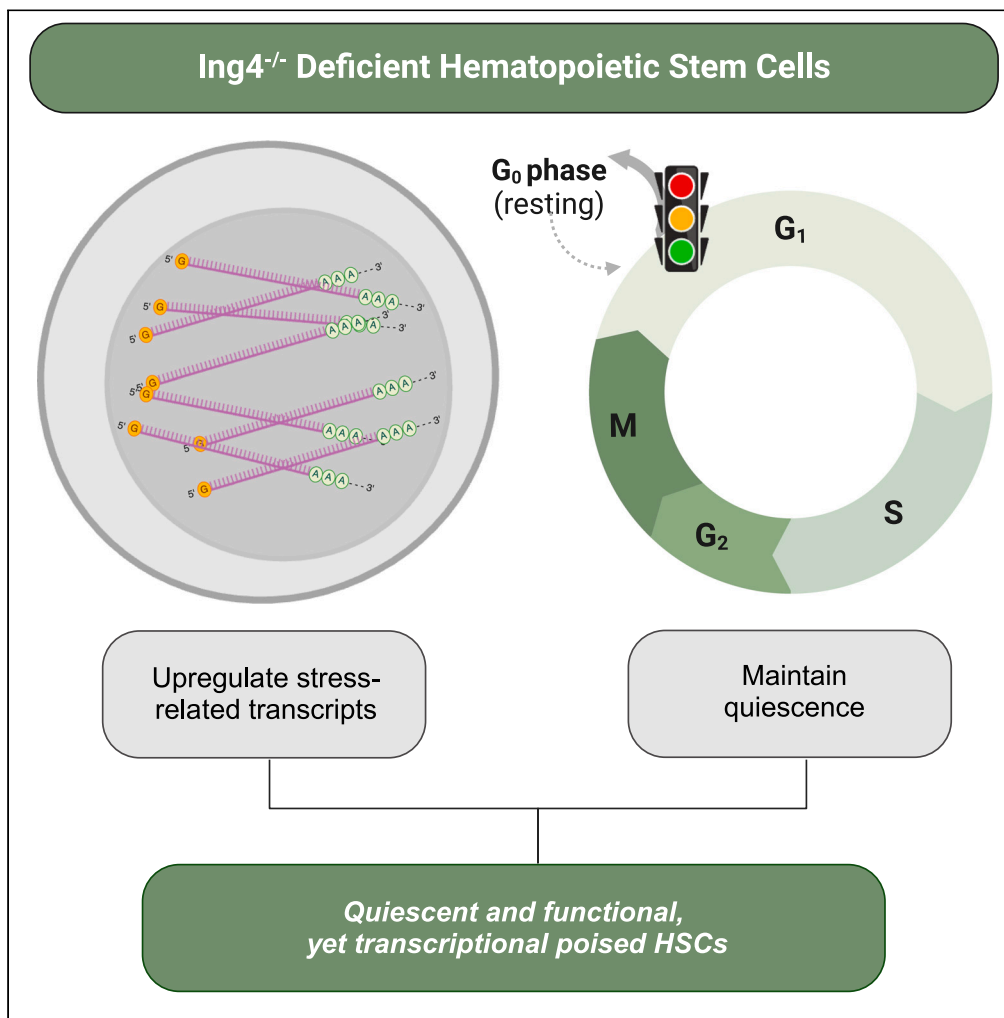


Article

Ing4-deficiency promotes a quiescent yet transcriptionally poised state in hematopoietic stem cells



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Highlights

Loss of Ing4 promotes a poised state in hematopoietic stem cells (HSCs)

Ing4^{-/-} HSCs have a transcriptional profile that reflects an activated, stressed HSC

Despite this, Ing4^{-/-} HSCs are quiescent and functional in transplantation

Reducing c-Myc activity stimulates Ing4^{-/-} HSCs into cycling

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Article

Ing4-deficiency promotes a quiescent yet transcriptionally poised state in hematopoietic stem cells

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SUMMARY

Defining the mechanisms that regulate stem cell maintenance, proliferation, and differentiation is critical for identifying therapies for improving stem cell function under stress. Here, we have identified the tumor suppressor, inhibitor of growth 4 (Ing4), as a critical regulator of hematopoietic stem cell (HSC) homeostasis. Cancer cell line models with Ing4 deficiency have shown that Ing4 functions as a tumor suppressor, in part, due to Ing4-mediated regulation of several major signaling pathways, including c-Myc. In HSCs, we show Ing4 deficiency promotes gene expression signatures associated with activation, yet HSCs are arrested in G₀, expressing several markers of quiescence. Functionally, Ing4-deficient HSCs demonstrate robust regenerative capacity following transplantation. Our findings suggest Ing4 deficiency promotes a poised state in HSCs, where they appear transcriptionally primed for activation but remain in a resting state. Our model provides key tools for further identification and characterization of pathways that control quiescence and self-renewal in HSCs.

INTRODUCTION

Hematopoiesis is the process by which hematopoietic stem cells (HSCs) give rise to all mature blood cells.¹ HSCs simultaneously maintain quiescence and promote activation through tightly regulated cell-extrinsic and cell-intrinsic frameworks of proteins that maintain, promote, and repress specific gene expression patterns to enable production of blood for the lifespan of an organism.^{2–7} This delicate balance allows for maintenance of a constant source for immune cell production while retaining a robust stem cell pool.^{8–16} Disruption of the signaling pathways that maintain the stem cell pool can have significant consequences on the responsiveness, longevity, and strength of the immune system, often resulting in HSC exhaustion through chronic activation.^{17–20} The importance of understanding hematopoiesis is clinically relevant, as hematopoietic stem exhaustion can result in bone marrow failure diseases, such as anemia, β -thalassemia, and inherited metabolic disorders.²¹

To uncover critical regulators of HSCs, we recently identified inhibitor of growth 4 (Ing4) in a zebrafish reverse genetic screen.¹³ Ing4 is a tumor suppressor protein, generally localized to the nucleus, which is associated with a high frequency of acquired, inactivating mutations and poor prognoses in diverse human cancers.^{22–26} Ing4 has many regulatory roles, both as a chromatin remodeling protein within the Hbo1 complex and as a direct regulator of several major signaling pathways: nuclear factor κ B (NF- κ B), c-Myc, p53, and HIF-1 α .^{27–32}

Previous work using a mouse model of Ing4 deficiency showed that Ing4^{-/-} macrophages and neutrophils have increased NF- κ B target gene expression and enhanced cytokine production upon exposure to lipopolysaccharides, suggesting a role for Ing4 in regulating differentiated immune cell responses.³² Ing4 binds to the p65/RelA subunit of NF- κ B, preventing p65 from binding to DNA, resulting in suppression of NF- κ B target genes and inflammatory pathways.^{29–31,33} In cancer cell line models, Ing4 has also been shown to interact directly with the c-Myc inhibitor, AUF1, to disrupt c-Myc function,³⁴ and the HIF-1 α inhibitor, HPH-2, to disrupt HIF-1 α function.³⁵ In addition, Ing4 enhances p53 function through acetylation.³⁶ These studies suggest that Ing4 is a critical regulatory protein, yet its role in hematopoiesis remains uncharacterized.

In this study, we identified an unexpected role for Ing4 as a negative regulator of HSC quiescence and self-renewal. Using an Ing4-deficient mouse model,³² we show that Ing4 deficiency disrupts normal HSC populations. Ing4^{-/-} HSCs display increased quiescence and they maintain low reactive oxygen species (ROS) levels and mitochondrial potential. Strikingly, loss of Ing4 expression has dueling outcomes for

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Table 1. Cell types and corresponding surface markers used for identification

Cell Type	Surface Markers
LSK	Lin ⁻ Sca-1 ⁺ c-Kit ⁺
LT-HSC	Lin ⁻ Sca-1 ⁺ c-Kit ⁺ CD150 ⁺ CD34 ⁻
ST-HSC	Lin ⁻ Sca-1 ⁺ c-Kit ⁺ CD150 ⁺ CD34 ⁺
CLP	Lin ⁻ Sca-1 ^{low} c-Kit ^{low} CD127 ⁺
MEP	Lin ⁻ Sca-1 ⁺ c-Kit ⁺ CD16/32 ⁻ CD34 ⁻
CMP	Lin ⁻ Sca-1 ⁺ c-Kit ⁺ CD16/32 ⁻ CD34 ⁺
GMP	Lin ⁻ Sca-1 ⁺ c-Kit ⁺ CD16/32 ⁺ CD34 ⁺
T cell	CD3e ⁺
B cell	B220 ⁺
Macrophage	Mac11b ⁺
Granulocyte	Gr-1 ⁺
RBC	Ter119 ⁺

HSCs; they become more quiescent while simultaneously upregulating pathways associated with activation, creating a poised state. The quiescent state can be overcome through inhibition of c-Myc activity, which induces *Ing4*^{-/-} HSCs into cycling. Our findings display an essential role for *Ing4* in stem cell maintenance and activation.

RESULTS

The hematopoietic program is disrupted in the absence of *Ing4*

To investigate the role of *Ing4* in hematopoiesis, we used a previously described *Ing4*-deficient mouse model to profile hematopoietic stem cells in the absence of *Ing4* (Table 1).³² *Ing4* is highly expressed in murine HSCs, suggesting it may have a specific role in HSC function (see Figure S1A).^{37,38} When compared with wild-type (WT) mice, there was no significant difference observed in the percentage and absolute number of CD48⁻ Lin⁻ Sca1⁺ c-Kit⁺ cells (LSKs) observed in the whole bone marrow (WBM) of *Ing4*^{-/-} mice (see Figures S1B and S1C). Total nucleated cell numbers were similar for WT and *Ing4*^{-/-} bone marrow (see Figure S1D). Recent publications have identified two distinct populations of HSCs: quiescent, repopulating long-term HSCs (LT-HSCs), and more actively dividing short-term HSCs (also termed HSCST, MPP1, and ST-HSCs; here designated ST-HSC)^{4,39-45} (Gating strategy in Figure 1A). Analysis of these specific HSC populations showed that, in *Ing4*^{-/-} mice, percentages and total numbers of LT-HSCs (LSK CD48⁻ CD34⁻ CD150⁺) (Figures 1B and 1C) and ST-HSCs (LSK CD48⁻ CD34⁺ CD150⁺) (Figures 1D and 1E) were significantly increased. These results suggest *Ing4* deficiency may result in a differentiation block or expansion in HSCs.

Analysis of lineage committed cells in the WBM revealed no significant differences in progenitor populations or lineage positive (Lin⁺) cell populations in the WBM of *Ing4*^{-/-} mice (Gating Strategy in Figure 1F, population percentages shown in Figures 1G–1J, cell counts shown in Figures S1G–S1J). Thus, *Ing4* is largely dispensable for maintenance of the committed progenitor pool.

Ing4-deficient HSCs are quiescent and have low intracellular levels of reactive oxygen species and mitochondrial potential

We next investigated the cell-cycle status of *Ing4*^{-/-} HSCs. The balance of quiescence and proliferation is essential for maintaining HSC populations.⁴⁶ Cell-cycle status of HSCs was analyzed using DAPI for DNA content and Ki-67 for proliferative status. Surprisingly, we found an increased proportion of cells in G₀ in both LT- and ST-HSC populations from *Ing4*^{-/-} mice (Figures 2A–2D). To further interrogate if the increased proportions of cells in G₀ in LT- and ST-HSCs result from senescence in these populations or a phenotypic difference between the WT and *Ing4*^{-/-} populations, we analyzed senescence-associated β-galactosidase (SA-β-gal) staining in WT and *Ing4*-deficient HSCs. We found slight increases in SA-β-gal activity in *Ing4*^{-/-} LT- and ST-HSCs populations, but these differences were not statistically significant (Figures 2E–2I), suggesting that *Ing4* deficiency likely does not trigger senescence in the majority of quiescent cells at this time point. As cell size correlates with quiescence, we also analyzed the forward scatter profiles of HSCs. *Ing4*^{-/-} LT-HSCs are slightly smaller than WT LT-HSCs, though no significant difference in cell size was observed between WT and *Ing4*^{-/-} ST-HSCs (see Figures S1E and S1F). These results further characterize the quiescent nature of *Ing4*^{-/-} HSCs.

Ing4 has been shown to promote apoptosis through a direct interaction with p53.⁴⁷ Absence of *Ing4* gives a competitive advantage to tumor cells by limiting p53 activity and, therefore, apoptosis. We therefore conducted assays with WT and *Ing4*^{-/-} HSCs to determine if loss of *Ing4* decreases apoptosis in these populations. When compared with WT HSCs, there was no significant difference in the frequency of cells undergoing apoptosis (annexin V⁺) in the *Ing4*^{-/-} HSC populations (see Figures S2D and S2E).

Previous studies show a direct link between HSC quiescence and low levels of intracellular ROS content.^{48,49} To determine whether ROS levels are altered in *Ing4*^{-/-} HSCs, we treated freshly isolated lineage depleted cells with 2',7'-dichlorofluorescein diacetate (H₂DCFDA).

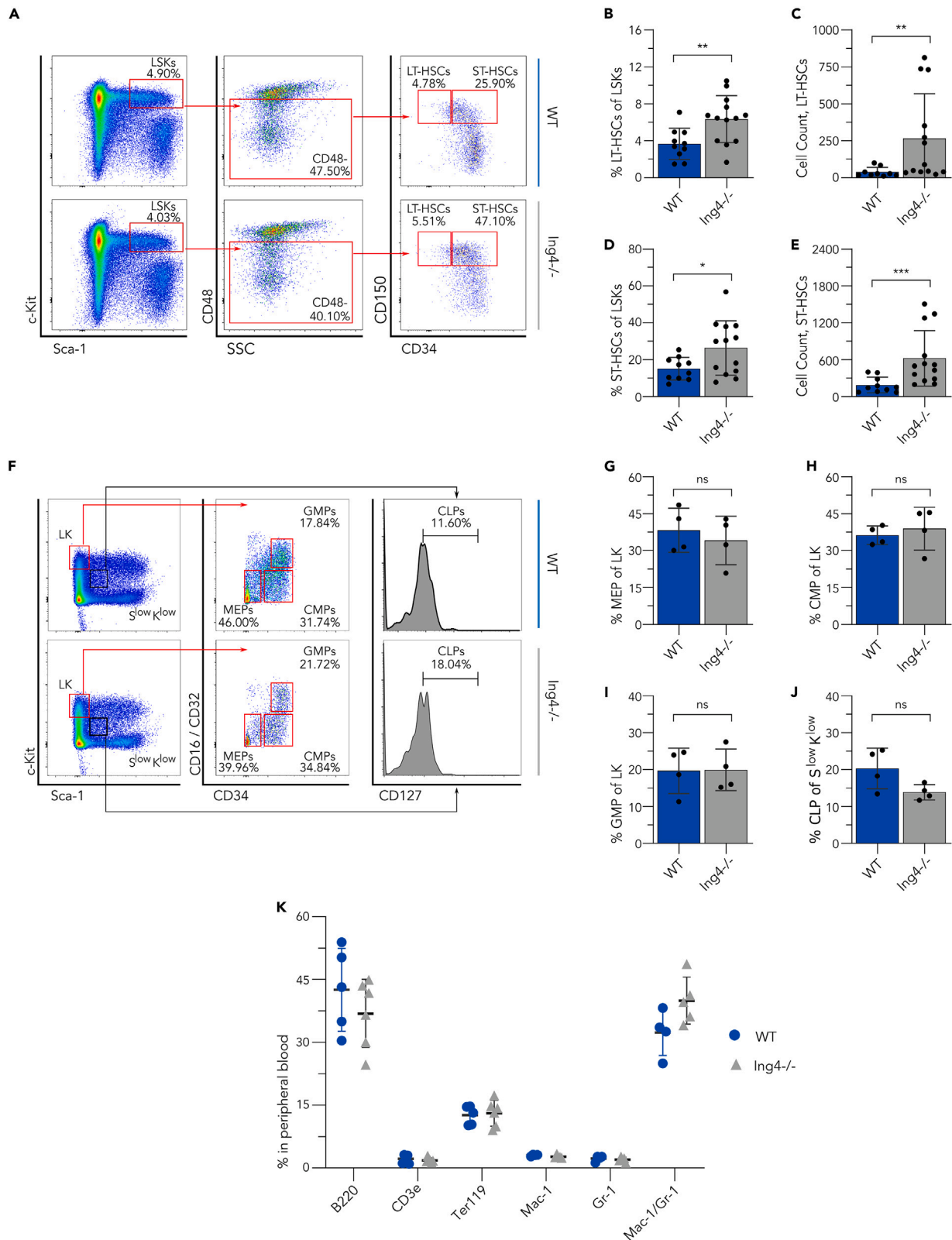


Figure 1. Deletion of *Ing4* leads to skewed hematopoiesis in the bone marrow

- (A) Representative gating strategy of flow cytometric analysis for LT-HSCs and ST-HSCs.
 (B) LT-HSCs from WBM isolated from individual WT and *Ing4*^{-/-} steady state mice as a percentage of LSK cells. (n = 10–13; **p < 0.01).
 (C) Cell counts of LT-HSCs from WBM isolated from individual WT and *Ing4*^{-/-} steady-state mice. (n = 10–13; **p < 0.01).
 (D) ST-HSCs from WBM isolated from individual WT and *Ing4*^{-/-} steady state mice as a percentage of LSK cells. (n = 10–13; *p < 0.05).
 (E) Cell counts of ST-HSCs from WBM isolated from individual WT and *Ing4*^{-/-} steady-state mice. (n = 10–13; ***p < 0.005).
 (F) Representative flow cytometric analysis of megakaryocyte-erythroid progenitors (MEP), common myeloid progenitor (CMP), granulocyte-macrophage progenitor (GMP), and common lymphoid progenitor (CLP) cell populations in WBM of WT and *Ing4*^{-/-} steady-state mice.
 (G) Percentages of lineage committed cell populations in the peripheral blood of WT and *Ing4*^{-/-} steady-state mice as analyzed via flow cytometry. (n = 5–6; ns = p > 0.05).
 (H) MEP from WBM isolated from individual WT and *Ing4*^{-/-} steady-state mice as a percentage of LK cells. (n = 4; ns = p > 0.05).
 (I) CMP from WBM isolated from individual WT and *Ing4*^{-/-} steady-state mice as a percentage of LK cells. (n = 4; ns = p > 0.05).
 (J) GMP from WBM isolated from individual WT and *Ing4*^{-/-} steady-state mice as a percentage of LK cells. (n = 4; ns = p > 0.05).
 (K) CLP from WBM isolated from individual WT and *Ing4*^{-/-} steady-state mice as a percentage of S^{low}K^{low} cells. (n = 4; ns = p > 0.05).
 Data in (B), (D), (H), (I), (J), and (K) reflect mean values ± SD. Statistical significance was assessed using Student's t test with Welch's correction. Data in (C) and (E) reflect mean values ± SD. Statistical significance was assessed using Mann-Whitney analysis.

Ing4^{-/-} LT- and ST-HSCs trend toward lower, but not significantly different, levels of ROS content compared to WT controls (Figures 3A–3E). These results correlate with *Ing4*^{-/-} HSC quiescence levels.

Finally, to investigate metabolic activation, we looked at tetramethylrhodamine methyl ester (TMRM) levels for the detection of mitochondrial membrane potential state as a marker for cell activation. Upon activation, HSCs undergo a switch from glycolysis to oxidative phosphorylation (OXPHOS) to generate ATP, creating electrical potential difference across the mitochondrial membrane that results in TMRM staining.^{50,51} Comparing WT and *Ing4*^{-/-} HSCs, we observe similar TMRM levels, suggesting that *Ing4*^{-/-} HSCs have similar membrane potential to WT, denoting similar OXPHOS usage as an energy source to WT HSCs (Figures 3F–3J). Taken together with our findings of cell cycle and ROS profiling of *Ing4*^{-/-} HSCs, these data provide evidence that *Ing4*^{-/-} HSCs persist in a sustained resting state.

***Ing4*^{-/-} HSCs simultaneously express genes associated with activation and quiescence**

To elucidate the molecular consequences of *Ing4* loss in HSC regulation, we conducted a genome-wide expression analysis using bulk RNA-sequencing (RNA-seq) of purified *Ing4*^{-/-} LT-HSCs and ST-HSCs and compared the profiles of these populations, pooled, against pooled wild-type LT-HSC and ST-HSC gene expression. This analysis revealed 2,420 differentially expressed genes in the combined populations (1,139 upregulated and 1,281 downregulated, p < 0.05) (Figures 4A and 4B). Surprisingly, gene set enrichment analysis (GSEA) of this dataset using the gene ontology biological process (GOBP) gene set showed upregulated genes in *Ing4*^{-/-} HSCs that were associated with oxidative phosphorylation, ribosomal biogenesis, and c-Myc target gene expression. Downregulated genes were associated with mitotic spindle formation and cytoskeletal modification (Figure 4A). All dysregulated genes are represented in a volcano plot shown in Figure 4B. The 100 genes with greatest magnitude of up- or downregulation are shown in Figures S3A and S3B. Several genes upregulated in RNA-seq were validated with qPCR (Figures 4C–4F). Results from qPCR analysis show a statistically significant positive correlation with RNA-seq data (Figure 4C). These data are also shown as individual genes, with relative fold changes analyzed by qPCR and compared to housekeeping gene expression (Figures 4D and 4F). A set of genes that are associated with HSC quiescence and cell cycle regulation were also upregulated, including the cell-cycle regulator p57 in both the RNA-seq data (Figure 4B) and by qPCR (Figure 4F). Together, these signatures suggest that *Ing4* deficiency may promote a transcriptionally poised state in HSCs, whereby *Ing4* loss upregulates genes associated with activation while also inducing quiescence-associated genes to maintain HSCs in a resting state.

***Ing4*-deficient HSCs demonstrate normal responses in stress hematopoiesis**

To analyze the functional role of *Ing4* deficiency in HSC reconstitution, we competitively transplanted WBM cells from *Ing4*^{-/-} and WT mice into lethally (9.5Gy) irradiated WT recipients (1:4 ratio) (Figures 5A and 5B). Peripheral blood analysis at 4- and 12-week post-transplantation showed no significant difference in donor contribution of *Ing4*^{-/-} cells in comparison to WT cells (Figures 5C and 5D) nor was there significantly altered lineage distribution observed (Figures 5E–5G). This suggests that *Ing4*-deficient bone marrow is capable of engraftment at wild-type levels.

To compare the reconstitution and maintenance capacity of *Ing4*-deficient HSC subsets, we transplanted sorted LT-HSCs and ST-HSCs from CD45.2 *Ing4*^{-/-} and WT mice in a competitive setting. To this end, we transplanted either 10 LT-HSCs or 50 ST-HSCs combined with 200,000 CD45.1 unfractionated WBM cells. PB analysis at 4-, and 12-week post-transplantation revealed a significant absence of donor contribution in the *Ing4*^{-/-} LT-HSCs in comparison to WT LT-HSC, though chimerism is very low for both groups (see Figure S4). There were no significant differences in the chimerism observed in transplanted ST-HSC populations (Figure 4G). These results suggest *Ing4*^{-/-} LT-HSCs may be incapable of repopulation, whereas ST-HSCs have robust repopulating activity.

To examine if proliferation may be impaired in the absence of *Ing4* and contributing to loss of LT-HSC function in transplantation, we performed an *in vitro* cell proliferation assay. WBM was harvested, stained with cell trace violet (CTV), and cultured for 7 days. *Ing4*^{-/-} ST-HSCs proliferate at similar rates to WT ST-HSCs, but *Ing4*^{-/-} LT-HSCs are less proliferative than their WT counterparts, with more cells that have not undergone cell division by 7 days (Figures 5H–5J).

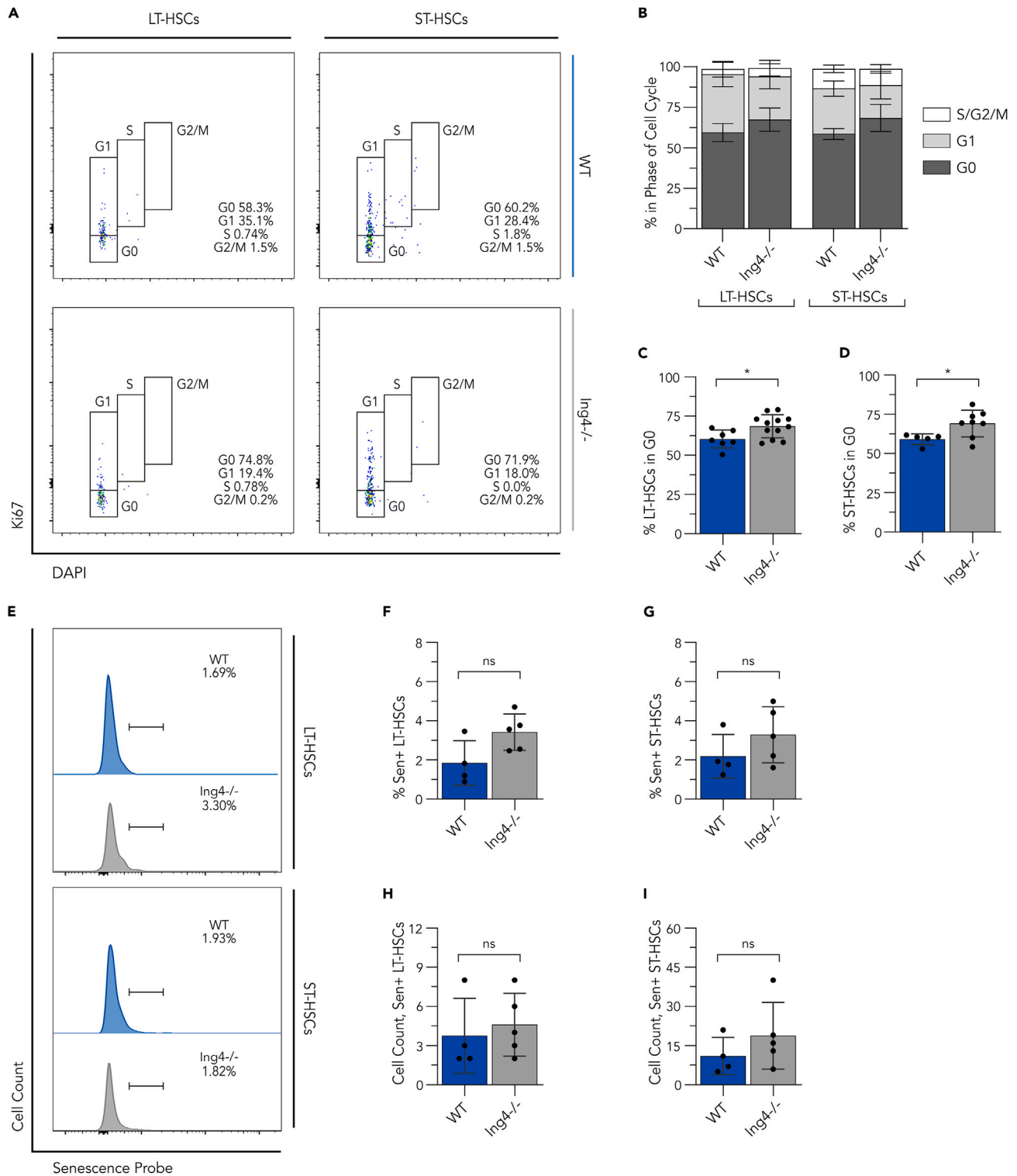


Figure 2. Ing4-deficient HSCs have increased quiescence

(A) Representative flow cytometric analysis of LT-HSCs and ST-HSCs using Ki-67 and DAPI for cell cycle profile.

(B) Mean values and standard deviations of all cell-cycle phases for LT-HSC and ST-HSC populations in WT and Ing4^{-/-} bone marrow at steady state.

(C) LT-HSCs in G₀ isolated from bone marrow of individual WT and Ing4^{-/-} steady-state mice as a percentage of the LT-HSC population. (n = 5–9; * = p < 0.05).

(D) ST-HSCs in G₀ isolated from bone marrow of individual WT and Ing4^{-/-} steady-state mice as a percentage of the ST-HSC population. (n = 5–9; * = p < 0.05).

Figure 2. Continued

(E) Representative flow cytometric analysis of β -galactosidase staining for senescence in LT-HSC and ST-HSC populations in WT and $\text{Ing4}^{-/-}$ bone marrow at steady state. [Figure S2A](#) shows negative and positive histogram profiles for senescence.
 (F) Senescence-positive LT-HSCs isolated individual WT and $\text{Ing4}^{-/-}$ steady-state mice as a percentage of all LT-HSCs. ($n = 4-5$; $ns = p > 0.05$).
 (G) Senescence-positive ST-HSCs isolated from individual WT and $\text{Ing4}^{-/-}$ steady-state mice as a percentage of all ST-HSCs. ($n = 4-5$; $ns = p > 0.05$).
 (H) Cell counts of senescence-positive LT-HSCs from WBM isolated from individual WT and $\text{Ing4}^{-/-}$ steady-state mice. ($n = 4-5$; $ns = p > 0.05$).
 (I) Cell counts of senescence-positive ST-HSCs from WBM isolated from individual WT and $\text{Ing4}^{-/-}$ steady-state mice. ($n = 4-5$; $ns = p > 0.05$).
 Data in (C), (D), (F), (G), and (I) reflect mean values \pm SD. Statistical significance was assessed using Student's t test with Welch's correction. Data in (H) reflect mean values \pm SD. Statistical significance was assessed using Mann-Whitney analysis.

To further dissect hematopoietic function in the absence of Ing4 , we studied the response of $\text{Ing4}^{-/-}$ HSCs to hematopoietic stress induced by the myeloablative agent 5-fluorouracil (5-FU) as a mechanism to trigger HSC activation ([Figure 6A](#)). Following single dose 5-FU- or mock-treatment, there was no significant difference in $\text{Ing4}^{-/-}$ LT-HSCs populations in the bone marrow at 15 days ([Figures 6B](#) and [6C](#)). In contrast, bone marrow from WT mice treated with 5-FU exhibited a significant increase in LT-HSCs as a proportion of LSKs at 15 days post-treatment compared to mice receiving mock treatment ([Figure 6B](#)). This suggests that $\text{Ing4}^{-/-}$ LT-HSCs are not responding to 5-FU treatment.

We found $\text{Ing4}^{-/-}$ ST-HSCs to be significantly decreased in mice treated with 5-FU as compared to those receiving mock treatment at 15 days post-injection, while at the same time point, there was no significant difference between ST-HSCs of WT mice who received 5-FU or mock ([Figures 6D](#) and [6E](#)). These data indicate that 5-FU ablation may remove a differentiation block or trigger apoptosis in $\text{Ing4}^{-/-}$ ST-HSCs at 15 days post-5-FU or -mock treatment.

By 30 days post-treatment, no significant differences between $\text{Ing4}^{-/-}$ HSCs were observed in mice who received 5-FU or mock treatment ([Figures 6F-6I](#)) by percent of LSK. We noted lower trending LT- and ST-HSCs in the bone marrow of $\text{Ing4}^{-/-}$ mice treated with 5-FU than WT mice receiving 5-FU injections at 30 days post-treatment ([Figures 6F-6I](#)), but this is only significant by cell count in ST-HSCs. These results suggest $\text{Ing4}^{-/-}$ HSCs ultimately respond to ablation and return to near pretreatment levels, albeit the response is delayed compared to WT HSCs.

c-Myc inhibitor enhances HSC recovery

We also used sublethal (3.5Gy), low-dose irradiation (LDI) as an alternative insult to target dividing cells ([Figure 7A](#)). 14 days post-irradiation, $\text{Ing4}^{-/-}$ LT- and ST-HSCs showed significantly more cells in G_0 compared to WT LT- and ST-HSCs, suggesting a diminished or delayed response to LDI in $\text{Ing4}^{-/-}$ HSCs ([Figures 7B](#) and [7E](#)).

Next, we sought to determine if targeted chemical perturbation of $\text{Ing4}^{-/-}$ HSCs could impact the quiescent state of $\text{Ing4}^{-/-}$ HSCs. We focused on the c-Myc pathway for several reasons: (1) c-Myc target genes are over-represented in our RNA-seq data, (2) c-Myc lies upstream of several of the other mis-regulated pathways observed in $\text{Ing4}^{-/-}$ HSCs, and (3) Ing4 was previously reported to negatively regulate c-Myc target gene expression.^{34,52} Recognizing the myriad impact of the c-Myc pathway, we hypothesized that by targeting c-Myc activity, we could trigger a dampening of many of the upregulated genes in $\text{Ing4}^{-/-}$ HSCs through diminishing c-Myc target gene expression, thus achieving a level of c-Myc target gene expression closer to WT levels. Considering our observation of increased quiescence in $\text{Ing4}^{-/-}$ HSCs, we conducted an *in vivo* assay whereby WT and $\text{Ing4}^{-/-}$ mice were treated with a c-Myc dimerization inhibitor, 10058-F4, following hematopoietic insult by sublethal irradiation. This allowed us to ascertain whether $\text{Ing4}^{-/-}$ HSC quiescence could be relieved through inhibition of c-Myc activity.^{53,54} We found that, in $\text{Ing4}^{-/-}$ mice, the inhibitor stimulated both LT-HSCs and ST-HSCs to begin cycling ([Figures 7B-7G](#)). Cell-cycle analysis of c-Myc inhibitor-treated HSCs revealed decreased percentages of $\text{Ing4}^{-/-}$ LT- and ST-HSCs in the G_0 phase of the cell cycle compared to untreated $\text{Ing4}^{-/-}$ HSCs ([Figures 7C](#) and [7F](#)). These data demonstrate inhibition of c-Myc activity releases $\text{Ing4}^{-/-}$ HSCs from G_0 arrest, allowing them to proceed through the cell cycle.

DISCUSSION

The exact molecular mechanisms that allow for the maintenance of HSC homeostasis in the bone marrow, including quiescence, self-renewal, and activation, remain elusive. In this study, we describe a role for Ing4 in regulation of HSC homeostasis. Prior to our work, no role for Ing4 had been identified in murine hematopoiesis. Here, we show that Ing4 plays a critical role as a vital regulator of HSC maintenance and self-renewal in several key ways: (1) Ing4 deficiency induces a poised state in HSCs, where HSCs are quiescent, yet transcriptionally primed for activation, (2) Ing4 -deficient HSCs are functional, with the ability to repopulate the bone marrow in transplantation assays and recover from 5-FU treatment or LDI, and (3) modulation of c-Myc activity can mitigate the cell-cycle arrest in Ing4 -deficient HSCs.

Ing4 deficiency results in skewed hematopoiesis, with a subtle increase in LT-HSCs, and a more marked increase in ST-HSCs. Both LT-HSCs and ST-HSCs are more quiescent than their wild-type counterparts. Transcriptional profiling of $\text{Ing4}^{-/-}$ HSCs show that they more closely resemble activated cells or more differentiated hematopoietic progenitors rather than quiescent HSCs. Specifically, markers of oxidative phosphorylation, ribosomal biogenesis, and other c-Myc target genes are significantly upregulated in $\text{Ing4}^{-/-}$ HSCs, more closely matching profiles of differentiating HSCs or progenitors.⁵⁵⁻⁵⁹ Surprisingly, examination of the levels of ROS, mitochondrial potential, and cell size in $\text{Ing4}^{-/-}$ HSCs showed no differences from wild type. These data, coupled with the quiescent status of the $\text{Ing4}^{-/-}$ HSCs, suggest that although we observe transcriptional upregulation of activation-associated genes, the resting, quiescent state of the HSCs may prevent the conversion from this poised state to an active state.

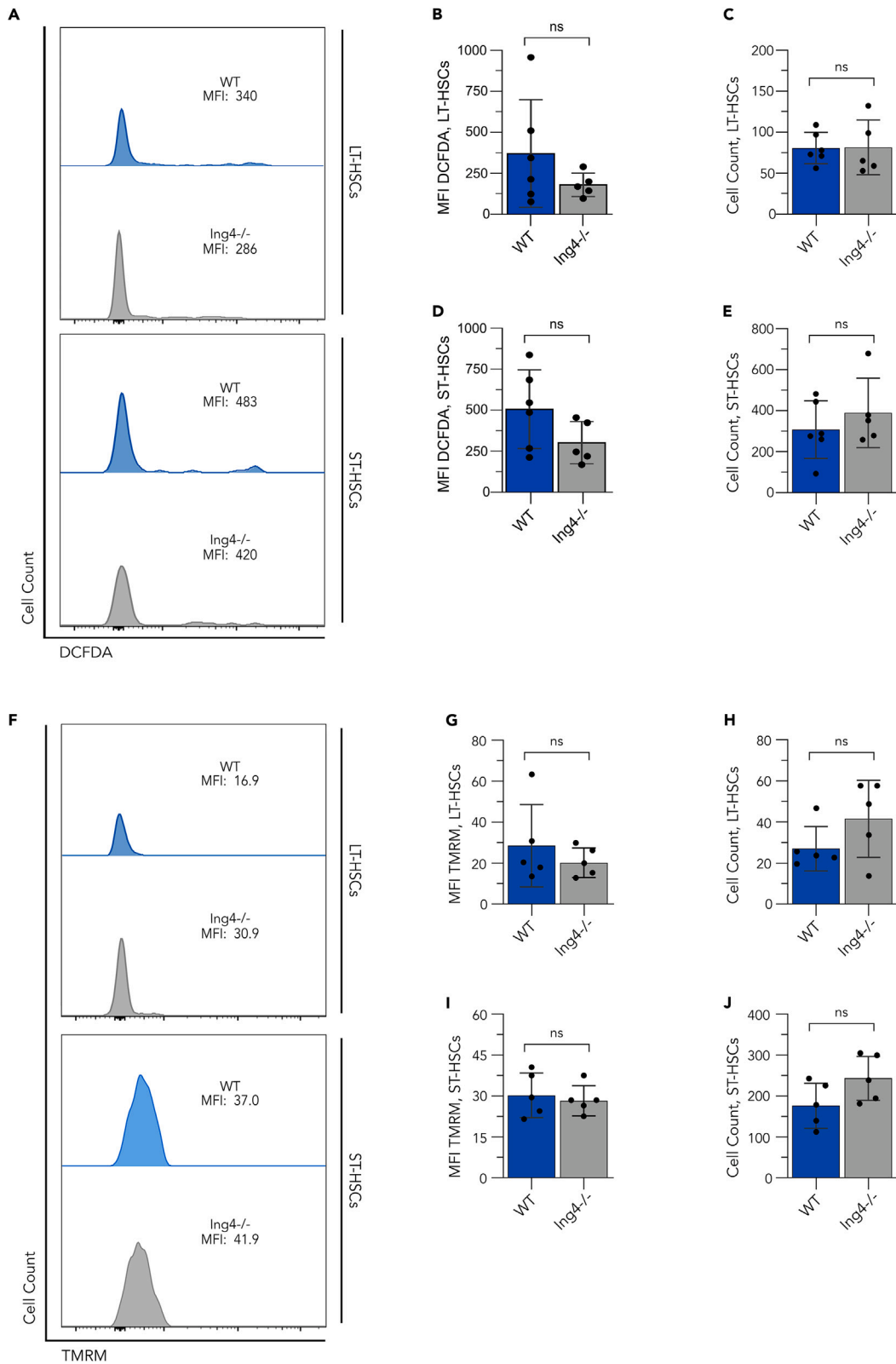


Figure 3. *Ing4*-deficient HSCs have low levels of reactive oxygen species and mitochondrial potential

(A) Representative flow cytometric analysis of staining with DCFDA for ROS in LT-HSC and ST-HSC populations in WT and *Ing4*^{-/-} bone marrow at steady state. Figure S2B shows negative and positive histogram profiles for ROS staining via flow cytometry.
 (B) MFI of DCFDA in LT-HSCs isolated from individual WT and *Ing4*^{-/-} steady-state mice. (n = 5–6; ns = p > 0.05).
 (C) Cell counts of LT-HSCs from WBM isolated from individual WT and *Ing4*^{-/-} steady-state mice. (n = 5–6; ns = p > 0.05).
 (D) MFI of DCFDA in ST-HSCs isolated from individual WT and *Ing4*^{-/-} steady-state mice. (n = 5–6; ns = p > 0.05).
 (E) Cell counts of ST-HSCs from WBM isolated from individual WT and *Ing4*^{-/-} steady-state mice. (n = 5–6; ns = p > 0.05).
 (F) Representative flow cytometric analysis of staining for TMRM in LT-HSC and ST-HSC populations in WT and *Ing4*^{-/-} bone marrow at steady state. Figure S2C shows negative, carbonyl cyanide m-chlorophenylhydrazone (CCCP) treated, and positive histogram profiles for TMRM staining via flow cytometry.
 (G) MFI of TMRM-treated LT-HSCs isolated from individual WT and *Ing4*^{-/-} steady-state mice. (n = 5; ns = p > 0.05).
 (H) Cell counts of LT-HSCs from WBM isolated from individual WT and *Ing4*^{-/-} steady-state mice. (n = 5; ns = p > 0.05).
 (I) MFI of TMRM-treated ST-HSCs isolated from individual WT and *Ing4*^{-/-} steady-state mice. (n = 5; ns = p > 0.05).
 (J) Cell counts of ST-HSCs from WBM isolated from individual WT and *Ing4*^{-/-} steady-state mice. (n = 5; ns = p > 0.05).
 Data in (B), (C), (E), (G), (I), and (J) reflect mean values ± SD. Statistical significance was assessed using Student's t test with Welch's correction. Data in (D) and (H) reflect mean values ± SD. Statistical significance was assessed using Mann-Whitney analysis.

Enhanced or enforced HSC quiescence is a hallmark of a subset of dormant or latent hematopoietic stem cells.^{60–62} Although the transcriptional profiles of latent HSCs vary from *Ing4*^{-/-} HSCs, these studies suggest that *Ing4*^{-/-} HSCs may retain functional latency despite their transcriptional profiles. Quiescence markers such as p57 are transcriptionally upregulated in *Ing4*^{-/-} HSCs, providing a potential mechanism for latency maintenance.^{7,63–70} Future studies will focus on whether *Ing4* directly regulates these quiescence markers.

To overcome the enforced quiescence in *Ing4*^{-/-} HSCs, we used LDI followed by treatment with a c-Myc inhibitor to potentially target several of the pathways transcriptionally overexpressed in our RNA-seq analysis, including ribosomal biogenesis, oxidative phosphorylation, and c-Myc target gene expression. This inhibitor triggered proliferation in both LT-HSCs and ST-HSCs. These results suggest that quiescence in *Ing4*-deficient HSCs can be partially overcome through targeting of c-Myc activity.⁷¹ Our data suggest that a relationship between *Ing4* and c-Myc activity, either direct or indirect, may be the driving force behind the poised state we observe in *Ing4*-deficient HSCs, but the exact nature of this interaction remains to be determined. c-Myc has long been considered to play a vital role in cell cycle progression, and recent studies have added insight into the complexity of c-Myc function in hematopoiesis, more specifically as a mediator of stem cell quiescence and self-renewal.^{72,73}

Mechanistically, c-Myc is believed to selectively activate transcription of genes encoding for oxidative phosphorylation (IDH3A, IDH3B, IDH3G, NDUFB7, SDHB, UQCRC1, and COX5B) in p27^{high}Ki67^{low} quiescent, rather than cycling, cells.⁷⁴ Notably, this is similar to results observed in our *Ing4*^{-/-} LT- and ST-HSCs. Studies using melanoma and lung cancer cells have reported upregulated expression of both c-Myc and p27, which drive cancer cells into quiescence.⁷⁴ Loss of a single c-Myc allele resulted in decreased HSC quiescence and impaired repopulation capacity, further highlighting its necessity for HSC quiescence.⁷⁵ Yet, c-Myc inactivation can result in an accumulation of HSCs and diminished HSC function,^{72,76} although an *in vitro* study has suggested that c-Myc loss enhances HSC expansion in culture.⁷⁷ Enforced expression of c-Myc results in HSC loss of function, at least in part through N-cadherin and integrin downregulation. These findings suggest that specific levels of c-Myc activity may dictate HSC function and could account for the disparate data in the literature, and may suggest a mechanism for *Ing4*^{-/-} HSC deregulation. Future work will be focused on determining the exact mechanism for this phenotype, specifically if c-Myc activity is directly regulated by *Ing4* and gives rise to the resulting c-Myc target gene expression, or if *Ing4* functions downstream of c-Myc to regulate c-Myc target gene expression.

Our results demonstrate for the first time the importance of *Ing4* regulation of several pathways required for hematopoiesis. *Ing4* deficiency places hematopoietic stem cells in a peculiar state, with a transcriptional profile of activation, but without the normally observed functional HSC phenotypes typically associated with this profile, including the inability to contribute to chimerism during transplantation, skewed hematopoiesis, and exhaustion. Our results suggest a potential disconnect between transcription and production of functioning proteins in the absence of *Ing4*. However, normal HSCs have been shown to maintain high levels of ribosomal biogenesis during quiescence without translational changes, so *Ing4*^{-/-} HSCs may utilize normal pathways to maintain further elevated levels of ribosomal components and other transcripts while maintaining normal levels of translation.^{78,79} Alternatively, *Ing4* has been linked to regulation of mRNA processing as a mechanism for regulation of c-Myc target gene expression.^{34,80} Perhaps there is an expanded role for this pathway in the absence of *Ing4* in hematopoietic stem cells. As well, expression of the mRNA m⁶a reader, YTHDF2, is transcriptionally upregulated in *Ing4*^{-/-} HSCs, providing a potential mechanism for mRNA decay, which might contribute to the observed disconnect between *Ing4*^{-/-} HSCs transcriptional profile and functional outcomes.⁵⁵ Future work will focus on determining the exact mechanism for this phenotype.

Limitations of the study

Many similarities are observed between the *Ing4*^{-/-} LT- and ST-HSCs, but we do observe some differences that remain to be fully elucidated and represent a limitation of the scope of this study. Specifically, at 15 days post 5-FU treatment we observed differing effects on *Ing4*^{-/-} ST-HSCs, with an observed a loss of ST-HSCs following 5-FU treatment that is not observed for LT-HSCs, which do not respond to 5-FU treatment. Both cell types return to mock treatment levels by 30 days by percent of LSK. We did observe a trend of lower LT- and ST-HSCs in the bone marrow of *Ing4*^{-/-} mice treated with 5-FU than WT mice receiving 5-FU injections at 30 days post-treatment (Figures 6F–6I), but this is only significant by cell count in ST-HSCs and becomes less significant from 15 days to 30 days post-treatment. Further work will focus on if ST-HSCs are more sensitive to hematopoietic insults than their wild-type counterparts.

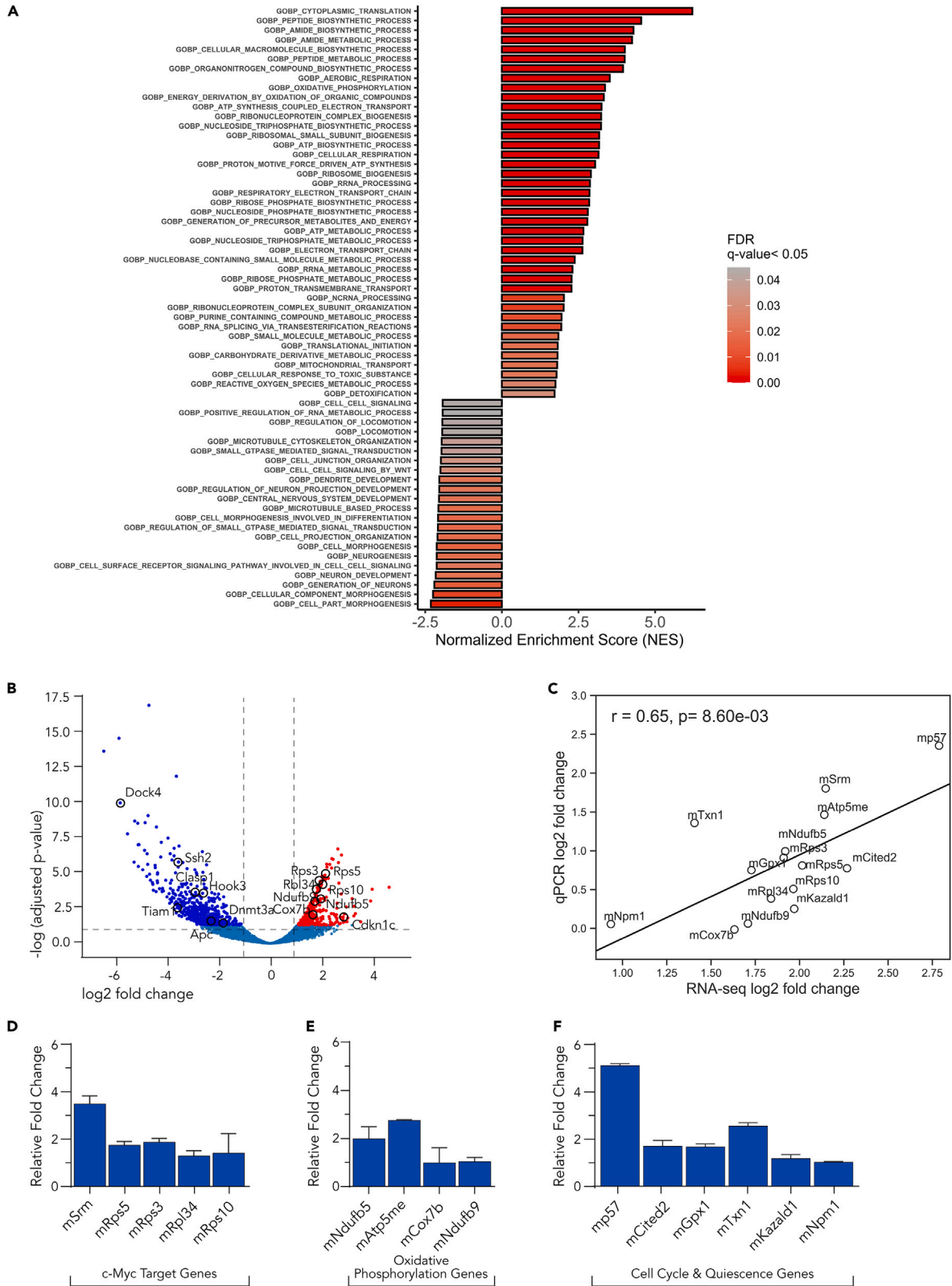


Figure 4. Gene expression analysis of *Ing4*^{-/-} HSC populations

(A) Normalized enrichment scores (NES) from a gene ontology biological process (GOBP) gene set analysis comparing two populations (WT LT- and ST-HSCs pooled), and (null LT- and ST-HSCs pooled). A total of 63 enrichment pathways (FDR-adjusted *p* value < 0.05) were identified. Bar colors indicate statistical significance.

(B) Differentially expressed genes in pooled WT LT-HSCs and ST-HSCs compared to pooled *Ing4*^{-/-} LT-HSCs and ST-HSCs, with a statistically significant threshold of *p* < 0.05, and a Log₂Fold change = 1. Upregulated genes are represented in red, downregulated in blue. Highlighted genes in red are validated from qPCR data. Highlighted genes in blue are associated with the mitotic spindle formation.

(C) Positive correlation of RNA-sequencing expression data and qPCR expression data. Log₂-fold change values were analyzed and plotted for 14 differentially expressed genes. The solid reference line represents the linear relationship.

(D) qPCR analysis of *c-Myc* target gene expression in cDNA generated from RNA collected from pooled LT- and ST-HSCs of WT or *Ing4*^{-/-} WBM.

(E) qPCR analysis quantifying expression of genes associated with oxidative phosphorylation in cDNA generated from RNA collected from pooled LT- and ST-HSCs of WT or *Ing4*^{-/-} WBM.

(F) qPCR analysis quantifying expression of genes associated with cell cycle regulation and quiescence in cDNA generated from RNA collected from pooled LT- and ST-HSCs of WT or *Ing4*^{-/-} WBM.

We also observe a difference in contribution to chimerism in the sorted LT-HSC and ST-HSC transplantations. The chimerism in our LT-HSC transplantation assay is low and needs further exploration to make definitive conclusions, though the data suggest that loss of *Ing4* impairs function of LT-HSCs but not ST-HSCs. Additionally, thus far we have only looked at gene expression changes for *Ing4*^{-/-} HSCs as a combined group compared to wild type. Identifying pathways that are differentially expressed between *Ing4*^{-/-} LT- and ST-HSCs may guide in understanding some of the observed differences. Nevertheless, we have identified several compelling phenotypes that warrant further exploration.

In summary, our work demonstrates a unique model where genetic deletion of *Ing4* triggers HSCs to maintain quiescence despite transcriptional upregulation of activation markers. This model may provide target pathways for limiting translational activation of HSCs to prevent exhaustion. Manipulation of these pathways may provide a powerful mechanism to improve HSC function during hematopoietic stress.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

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SUPPLEMENTAL INFORMATION

Supplemental information can be found online at <https://doi.org/10.1016/j.isci.2024.110521>.

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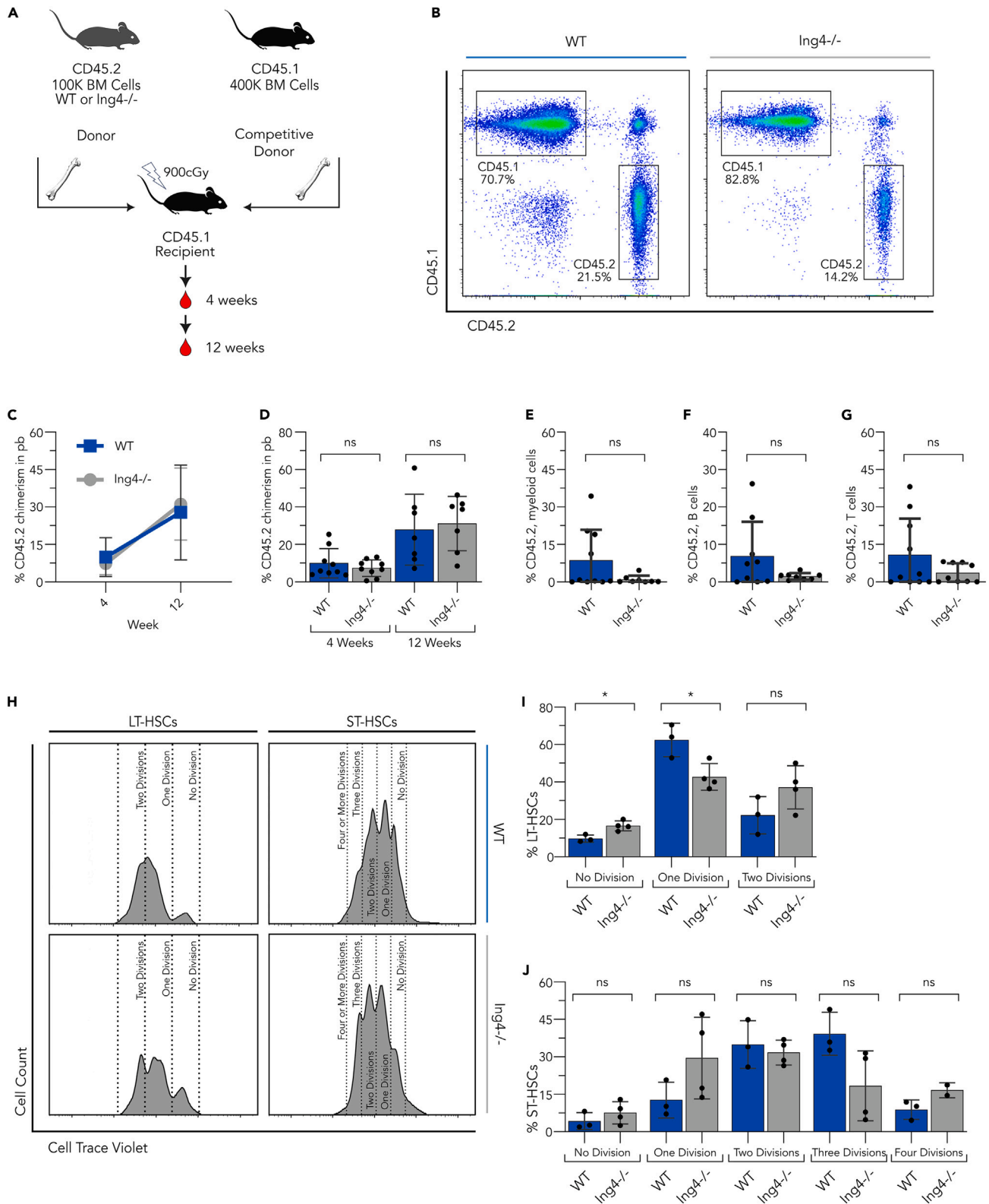


Figure 5. In competitive whole bone marrow transplantation assays, *Ing4*^{-/-} cells contribute to chimerism at levels comparable to WT

- (A) Schematic overview of the competitive transplantation assay using WBM from WT and *Ing4*^{-/-} mice.
 (B) Representative flow cytometric analysis of CD45.1 and CD45.2 chimerism in peripheral blood collected from recipient mice 12 weeks following transplantation with WBM from WT and *Ing4*^{-/-} mice.
 (C) Mean and standard deviation depicting percentages of CD45.2 chimerism in the peripheral blood of recipient mice collected 4 and 12 weeks following competitive WBM transplantation. (n = 7–9).
 (D) CD45.2 chimerism in individual mice from the peripheral blood of recipient mice collected 4 and 12 weeks following competitive WBM transplantation. (n = 7–9; ns = p > 0.05).
 (E) CD45.2 chimerism of myeloid cells in the peripheral blood of recipient mice collected 12 weeks following competitive WBM transplantation. (n = 8–10; ns = p > 0.05).
 (F) CD45.2 chimerism of B-cells in the peripheral blood of recipient mice collected 12 weeks following competitive WBM transplantation. (n = 8–10; ns = p > 0.05).
 (G) CD45.2 chimerism of T cells in the peripheral blood of recipient mice collected 12 weeks following competitive WBM transplantation. (n = 8–10; ns = p > 0.05).
 (H) Representative flow cytometric analysis using CellTrace Violet to show cell division events for 7 days culture.
 (I) Proliferation assay quantifying cell division events for LT-HSCs for 7 days in culture. (n = 3–4; ns = p > 0.05; *p < 0.05).
 (J) Proliferation assay quantifying cell division events for ST-HSCs undergoing no, one, two, three or four cell divisions in 7 days (n = 3–4; ns = p > 0.05).
 Data in (E), (F), (G), (I), and (J) reflect mean values ± SD. Statistical significance was assessed using Student's t test with Welch's correction.

AUTHOR CONTRIBUTIONS

Conceptualization, K.L.K. and Z.T.; methodology, K.L.K., Z.T., G.A., M.R., M.H., C.A.Q., S.G., A.M., and V.B.; data analysis, K.L.K., Z.T., G.A., M.H., C.A.Q., A.M., V.B., and A.M.T.; writing, K.L.K., Z.T., and G.A.; funding acquisition, K.L.K.; supervision, K.L.K.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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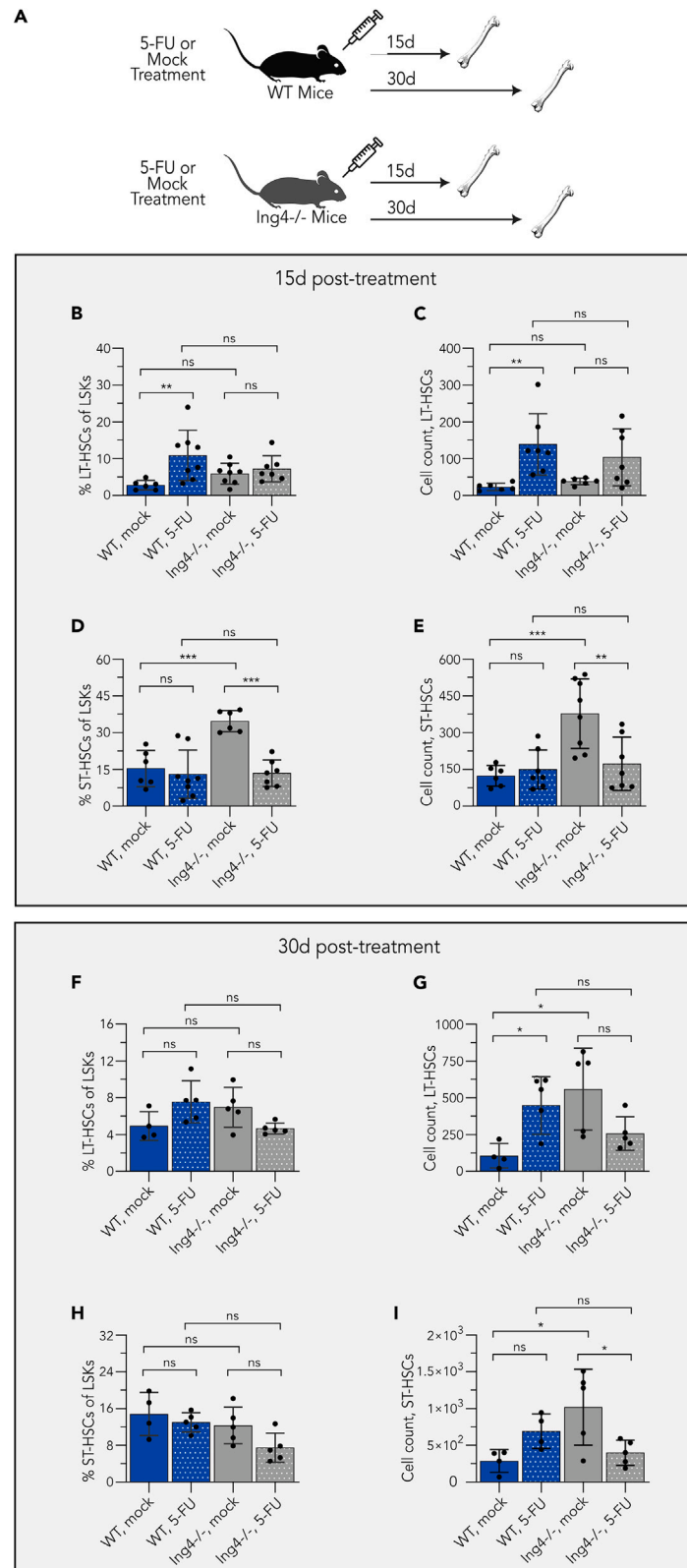


Figure 6. Ing4-deficient HSCs show altered stress hematopoiesis following chemotoxic insult

- (A) Schematic overview of the 5-FU assay in WT and *Ing4*^{-/-} mice.
 (B) LT-HSCs isolated from individual 5-FU or mock treated WT and *Ing4*^{-/-} mice, as a percentage of the LSK population at 15 days post-treatment. (*n* = 6–8; *ns* = *p* > 0.05; ** = *p* < 0.01).
 (C) Cell count of LT-HSCs isolated from individual 5-FU or mock treated WT and *Ing4*^{-/-} mice, at 15 days post-treatment. (*n* = 5–6; *ns* = *p* > 0.05; ** = *p* < 0.01).
 (D) ST-HSCs isolated from individual 5-FU or mock treated WT and *Ing4*^{-/-} mice, as a percentage of the LSK population at 15 days post-treatment. (*n* = 6–8; *ns* = *p* > 0.05; *** = *p* < 0.005).
 (E) Cell count of ST-HSCs isolated from individual 5-FU or mock treated WT and *Ing4*^{-/-} mice, at 15 days post-treatment. (*n* = 4–5; *ns* = *p* > 0.05; ** = *p* < 0.01; *** = *p* < 0.005).
 (F) LT-HSCs isolated from individual 5-FU or mock treated WT and *Ing4*^{-/-} mice, as a percentage of the LSK population at 30 days post-treatment. (*n* = 6–8; *ns* = *p* > 0.05).
 (G) Cell count of LT-HSCs isolated from individual 5-FU or mock treated WT and *Ing4*^{-/-} mice, at 30 days post-treatment. (*n* = 6–8; *ns* = *p* > 0.05; * = *p* < 0.05).
 (H) ST-HSCs isolated from individual 5-FU or mock treated WT and *Ing4*^{-/-} mice treated, as a percentage of the LSK population at 30 days post-treatment. (*n* = 6–8; *ns* = *p* > 0.05).
 (I) Cell count of ST-HSCs isolated from individual 5-FU or mock treated WT and *Ing4*^{-/-} mice, at 30 days post-treatment. (*n* = 4–5; *ns* = *p* > 0.05; * = *p* < 0.05).
 Data in (B), (C), (D), (E), (F), (G), (H), and (I) reflect mean values ± SD. Statistical significance was assessed using a two-way ANOVA analysis followed by Tukey's post hoc test.

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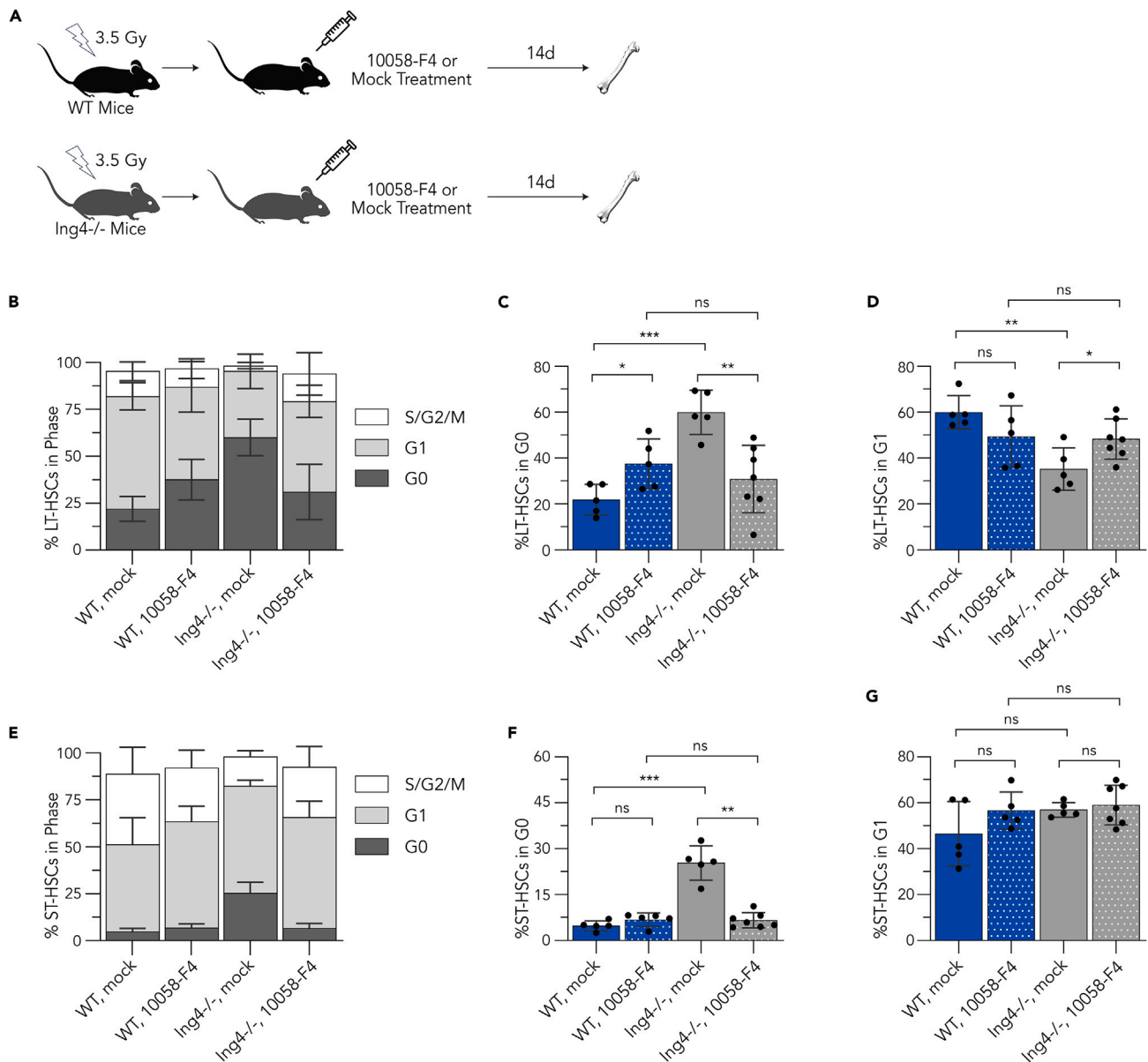


Figure 7. Inhibition of c-Myc activity induces cell cycling in $lng4^{-/-}$ HSCs after low-dose irradiation

(A) Schematic overview of the c-Myc inhibitor assay in WT and $lng4^{-/-}$ mice.

(B) Mean values and standard deviations of all cell cycle phases for mock or 10058-F4 treated LT-HSCs in WT and $lng4^{-/-}$ mice, as a percentage of LT-HSC population. ($n = 5$).

(C) LT-HSCs in G_0 from individual mock or 10058-F4 treated WT and $lng4^{-/-}$ mice, as a percentage of the LT-HSC population. ($n = 5$; $ns = p > 0.05$; $*p < 0.05$; $**p < 0.01$; $*** = p < 0.005$).

(D) LT-HSCs in G_1 from individual mock or 10058-F4 treated WT and $lng4^{-/-}$ mice, as a percentage of the LT-HSC population. ($n = 5$; $ns = p > 0.05$; $*p < 0.05$; $**p < 0.01$).

(E) Mean values and standard deviations of all cell cycle phases for individual mock or 10058-F4 treated ST-HSCs in WT and $lng4^{-/-}$ mice, as a percentage of ST-HSC population. ($n = 5$).

(F) ST-HSCs in G_0 from individual mock or 10058-F4 treated WT and $lng4^{-/-}$ mice, as a percentage of the ST-HSC population. ($n = 5$; $ns = p > 0.05$; $**p < 0.01$; $*** = p < 0.005$).

(G) ST-HSCs in G_1 from individual mock or 10058-F4 treated WT and $lng4^{-/-}$ mice, as a percentage of the ST-HSC population. ($n = 5$; $ns = p > 0.05$).

Data in (C), (D), (F), and (G) reflect mean values \pm SD. Statistical significance was assessed using a two-way ANOVA followed by Tukey's post hoc test. Total cell counts for LT- and ST-HSCs are shown in Figures S4A and S4B.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
TER-119 Monoclonal Antibody (TER119), PE	Invitrogen	Cat#MA5-17824; RRID: AB_2539208
CD3e Monoclonal Antibody (145-2C11), PE	Invitrogen	Cat#12-0031-82; RRID: AB_465496
Ly-6G/Ly-6C Monoclonal Antibody (RB6-8C5), PE	Invitrogen	Cat#12-5931-82; RRID: AB_466045
CD11b/c Monoclonal Antibody (OX42), PE	Invitrogen	Cat#12-0110-82; RRID: AB_11150971
CD45R (B220) Monoclonal Antibody (HIS24), PE	Invitrogen	Cat#12-0460-82; RRID: AB_465692
CD48 Monoclonal Antibody (HM48-1), PE	Invitrogen	Cat#12-0481-82; RRID: AB_465694
CD34 Monoclonal Antibody (RAM34), FITC	Invitrogen	Cat#11-0341-82; RRID: AB_465021
Ki-67 Monoclonal Antibody (SolA15), PE-Cyanine7	Invitrogen	Cat#14-5698-82; RRID: AB_10854564
PE/Cyanine7 anti-mouse CD150 (SLAM) Antibody	BioLegend	Cat#115913; RRID: AB_2900547
APC/Fire™ 750 anti-mouse CD150 (SLAM) Antibody	BioLegend	Cat#115939; RRID: AB_2629586
Brilliant Violet 510™ anti-mouse Ly-6A/E (Sca-1) Antibody	BioLegend	Cat#108129; RRID: AB_2561593
APC anti-mouse Ly-6A/E (Sca-1) Antibody	BioLegend	Cat#108111; RRID: AB_313348
APC/Cyanine7 anti-mouse CD117 (c-kit) Antibody	BioLegend	Cat#135135; RRID: AB_2632808; RRID: AB_2632808
CD3e Monoclonal Antibody (145-2C11), APC, eBioscience™	Invitrogen	Cat#17-0031-82; RRID: AB_469315
CD45R (B220) Monoclonal Antibody (RA3-6B2), eFluor™ 450, eBioscience™	Invitrogen	Cat#48-0452-82; RRID: AB_1548761
Brilliant Violet 510™ anti-mouse/human CD11b Antibody	Invitrogen	Cat#101245; RRID: AB_2561390
APC/Cyanine7 anti-mouse TER-119/Erythroid Cells Antibody	BioLegend	Cat#116223; RRID: AB_2137788
Ly-6G/Ly-6C Monoclonal Antibody (RB6-8C5), PE-Cyanine7, eBioscience™	Invitrogen	Cat#25-5931-82; RRID: AB_469663
CD45.1 Monoclonal Antibody (A20), PE, eBioscience™	Invitrogen	Cat#12-0453-82; RRID: AB_465675
CD45.2 Monoclonal Antibody (104), Alexa Fluor™ 700, eBioscience™	Invitrogen	Cat#56-0454-82; RRID: AB_657752
CD34 Monoclonal Antibody (RAM34), Alexa Fluor 700	Invitrogen	Cat#56-0341-82; RRID: AB_493998
Chemicals, peptides, and recombinant proteins		
H2DCFDA (H2-DCF, DCF)	Invitrogen	Cat#D399
Bovine Serum Albumin (BSA)	Invitrogen	Cat#B14
Fetal Bovine Serum, qualified, United States	Invitrogen	Cat#26140079
PBS, pH 7.4	Invitrogen	Cat#100100002
Red Blood Cell Lysis Solution (10x)	Miltenyi Biotec	Cat#130-094-183
Hematopoietic Lineage Labeling Cocktail, anti-mouse, Biotin	Miltenyi Biotec	Cat#130-092-613
LS Columns	Miltenyi Biotec	Cat#130-042-401
eBioscience™ Intracellular Fixation & Permeabilization Buffer Set	Invitrogen	Cat#88-8824-00
Buffer RLT Plus	Qiagen	Cat#1053393
RNeasy Plus Kits for RNA Isolation	Qiagen	Cat#74034
SYBR™ Green PCR Master Mix	ThermoFisher	Cat#4309155

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
CellTrace™ Violet Cell Proliferation Kit, for flow cytometry	Thermofisher	Cat#C34571
StemPro™-34 SFM (1X)	Thermofisher	Cat#10639011
Mouse SCF Recombinant Protein, PeproTech®	Thermofisher	Cat#250-03-1MG
Mouse TPO (Thrombopoietin) Recombinant Protein, PeproTech®	Thermofisher	Cat#315-14-1MG
5-Fluorouracil, 99%	Thermofisher	Cat#228440250
10058-F4, Thermo Scientific Chemicals	Thermofisher	Cat#J65650.MA
Annexin V Recombinant Protein, APC, eBioscience™	Thermofisher	Cat#BMS306APC-100
DAPI Solution	Thermofisher	Cat#62248
CellEvent™ Senescence Green Detection Kit	Thermofisher	Cat#C10851
LIVE/DEAD™ Fixable Red Dead Cell Stain Kit, for 488 nm excitation	Thermofisher	Cat#L34971
NEBNext® Single Cell/Low Input cDNA Synthesis & Amplification Kit	New England Biolabs	Cat#E6421S
MitoProbe™ TMRM Assay Kit for Flow Cytometry	Thermofisher	M20036

Deposited data

RNA-sequencing	NCBI	https://www.ncbi.nlm.nih.gov/bioproject/1130539
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Experimental models: Organisms/strains

Mouse: C57BL/6 (CD45.2)	The Jackson Laboratory	Cat#000664
Mouse: SJL-Ptpr ^a Pepc ^b /BoyJ (CD45.1)	The Jackson Laboratory	Cat#002014
Mouse: lng4 ^{-/-} (CD45.2)	Stephen N. Jones	N/A

Oligonucleotides

Actin-Forward	CATCCGTAAAGACCTCTATGCCAAC	N/A
Actin-Reverse	ATGGAGCCACCGATCCACA	N/A
Gapdh-Forward	ATGTGTCCGTCGTGGATCTGAC	N/A
Gapdh-Reverse	AGACAACCTGGTCCTCAGTGTAG	N/A
mSrm-Forward	ACATCCTCGTCTCCGCAGTA	N/A
mSrm-Reverse	GGCAGGTGGCGATCATCT	N/A
mRps5-Forward	TGGCAGAGACCCTGACAT	N/A
mRps5-Reverse	GGGCAGGTAAGTGGCATACT	N/A
mRps3-Forward	ATGGCGGTGCAGATTCCAA	N/A
mRps3-Reverse	GTAAGTCCGACTTCAACTCCAG	N/A
mRpl34-Forward	TCCAGCGTTTGACATACCGC	N/A
mRpl34-Reverse	TAGGTGCTTTCCCAACTTCT	N/A
mNdufb5-Forward	CAGGCTGGACTCAGCTACATC	N/A
mNdufb5-Reverse	AGTCTTCATGGCGTTTGCTT	N/A
mNdufb4-Forward	CGCTTGGCACTGTTTAATCCA	N/A
mNdufb4-Reverse	TCCATGGCTCTGGGTGTTC	N/A
mAtp5me-Forward	GTTCAAGTCTCTCCACTCATCA	N/A
mAtp5me-Reverse	CGGGGTTTTAGGTAAGTGTAGC	N/A
mCited2-Forward	TGCCGCCCAATGTCATAGACAC	N/A
mCited2-Reverse	AGAGTTCGGGCAGCTCCTTGAT	N/A
mCavin3-Forward	AAGCTGCACGTCCTGCTTCA	N/A
mCavin3-Reverse	CCAACCTCATCCTCTGGCTGATC	N/A

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
mHAT1-Forward	GATGGAGCTACGCTCTTTGCGA	N/A
mHAT1-Reverse	GCCCTGACCTTGAAATGGAGTC	N/A
mActr3-Forward	CAGGCTGAAGTTAAGCGAGGAG	N/A
mActr3-Reverse	CCTCCAAACCCAGACTGCATACC	N/A
mRgs1-Forward	AATGCAGTGGTCTCAGTCTCTGG	N/A
mRgs1-Reverse	ATAGTCCTCACAAGCCAACCAGA	N/A
mGpx1-Forward	CGCTCTTTACCTTCCTGCGGAA	N/A
mGpx1-Reverse	CGCTCTTTACCTTCCTGCGGAA	N/A
mTxn1-Forward	CAAATGCATGCCGACCTTCCAG	N/A
mTxn1-Reverse	GCTGGTTACACTTTTCAGAGCATG	N/A

Software and algorithms

FlowJo v10.9	FlowJo™	N/A
GraphPad Prism 10	Dotmatics	N/A
Biorender	Biorender	N/A

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Dr. Katie Kathrein (klk@sc.edu).

Materials availability

This study did not generate any plasmids.

This study did not generate any new mouse lines.

This study did not generate new, unique reagents.

Data and code availability

RNA-seq data are publicly accessible through NCBI using accession number [PRJNA1130539](https://www.ncbi.nlm.nih.gov/submit/query.fcgi?tbl=SequenceReads&tbl_info=sra&tbl_id=PRJNA1130539).

This paper does not report original code.

Any additional information required to reanalyze the data reported in this paper is available from the [lead contact](#) upon request.

EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS

Mice

All mice were bred and maintained at an AALAC-accredited animal facility at the University of South Carolina. Animal experiments were performed according to Institutional Animal Care and Use Committee animal guidelines, and all animal experiments were performed with consent from the local ethical committee. Both female and male mice were used in experiments. Mice of 8–12 weeks of age were used for analysis unless otherwise specified in the text.

Ing4^{-/-} mice (CD45.2) were provided by Stephen N. Jones (University of Massachusetts Medical School) and colony was maintained on site. Wildtype (WT) C57BL/6 (CD45.2) and SJL-Ptprc^a Pepc^P/BoyJ (CD45.1) served as controls. CD45.1 mice were purchased from The Jackson Laboratory.

METHOD DETAILS

Bone marrow and peripheral blood preparation

For HSC isolation (Lin⁻Sca⁺c-Kit⁺[LSK]CD48⁻CD150⁺ cell fraction), WBM cells isolated by crushing long bones from each mouse then filtered through a 40µm nylon cell strainer. Cells were incubated with 1x RBC Lysis Solution (Miltenyi Biotec) to lyse red blood cells. Cells were lineage depleted using a Lineage Cell Depletion Kit (Miltenyi Biotec) using LS columns and an LS magnetic separator. Under anesthesia, peripheral blood was collected from retroorbital venous sinus into heparinized (0.05 IU/mL; Alfa Aesar) micro-hematocrit capillary tubes (Kimble). Cells were incubated with 1x RBC Lysis Solution (Miltenyi Biotec) to lyse red blood cells. All experiments have been repeated at least twice unless otherwise noted.

Flow cytometry

After lineage depletion, WBM cells were incubated for in the dark for 30 min at 4°C with antibodies for HSC profiling (Lineage: B220, CD3e, CD11b, Gr-1, Ter119; Sca-1, c-Kit, CD48, CD34, CD150). Markers used for progenitor cells also included CD16/32 and CD127. For mature blood cells, markers were CD3e, CD45R, Ter119, Ly-6G/C, and CD11b. Following antibody staining, cells were rinsed twice with phosphate-buffered saline (PBS)-bovine serum albumin (BSA). Antibodies against CD45.2 and CD45.1 were used to assess engraftment following transplantation assays. See [STAR methods](#) for complete list of antibody sources.

Cells were analyzed on BD LSR II, FACSAria II, or FACSymphony (BD). For sorted cell assays, FACSAria II was used. FACS Diva was used in conjunction with flow cytometers for acquisition and FlowJo (version 10) was used for analysis of HSC populations.

Cell cycle analysis

Lineage-depleted, stained WBM cells were fixed and permeabilized with Cytofix/Cytoperm kit (Invitrogen) according to manufacturer's instructions. Cells were incubated in the dark overnight at 4°C with Ki67 antibody (Invitrogen), then rinsed with PBS. Prior to analysis via flow cytometry, cells were incubated for 15 min at room temperature with DAPI (0.2 µL/mL, Invitrogen) and rinsed with PBS.

Reactive oxygen species analysis

Lineage-depleted WBM cells were stained for HSC profiling WBM, then incubated with 7.5µM H₂DCFDA (Invitrogen) in PBS for 30 min at 37°C. Cells were rinsed with PBS.

Senescence assay

Lineage-depleted, stained WBM cells were fixed and permeabilized with Cytofix/Cytoperm kit (Invitrogen) according to manufacturer's instructions. Fixed and permeabilized cells were incubated with CellEvent Senescence Probe (Thermo) for 2 h at 37°C. Cells were rinsed with PBS.

RNA-seq analysis

Bone marrow from 8 to 10 mice was harvested and pooled, and lineage depleted. LT- and ST-HSCs were sorted separately into Buffer RLT Plus (Qiagen) for both WT or *Ing4*^{-/-} mice. Total RNA was isolated with the RNeasy Micro column (QIAGEN). Library construction, sequencing by Illumina HiSeq, and initial bioinformatics analyses were conducted by GENEWIZ. Counts obtained were used to perform Gene Set Enrichment Analysis. *Ing4*^{-/-} LT- and ST-HSCs were compared together against WT LT- and ST-HSCs.

Quantitative real-time PCR

Bone marrow from 8 to 10 mice was harvested, pooled, and lineage depleted. LT- and ST-HSCs were sorted together into Buffer RLT Plus (Qiagen) for both WT or *Ing4*^{-/-} mice. Total RNA was isolated with the RNeasy Micro column (QIAGEN). Lineage depleted LT- and ST-HSCs from pools of 8–10 WT or *Ing4*^{-/-} mice were sorted into Buffer RLT Plus. Total RNA from WT and *Ing4*^{-/-} mice was extracted with RNA Isolation kit system (QIAGEN) and purified with RNeasy system (QIAGEN). cDNA was prepared with Single Cell/Low Input cDNA Synthesis & Amplification System for RT-PCR (NEB) using oligo-dT primers associated RNA-seq data. (Primer sequences shown in [STAR methods](#)) qRT-PCR was performed using the SYBR Green PCR Master Mix (Applied Biosystems). Gene expression changes were quantified as linear ratio to Gapdh.

Transplantation assays

For competitive WBM transplantation assays, 1×10^5 total lineage-depleted WBM cells were isolated from *Ing4*^{-/-} donor mice (on CD45.2 background) or WT donor mice (on CD45.2 background). An additional 4×10^5 lineage-depleted WBM marrow cells were isolated from WT mice (on CD45.1 background) as competitor cells. Donor (CD45.2) and competitor (CD45.1) cells were transplanted via retroorbital injection into 8- to 12-week-old recipient mice (B6. SJL-CD45.1) that had been lethally irradiated (9.5 Gy, administered as two doses at least 3 h apart). Peripheral blood of recipient mice was analyzed at 4 and 12 weeks post-transplant.

For competitive transplantation assays, 10 LT-HSCs or 50 ST-HSCs isolated from *Ing4*^{-/-} or WT donors (CD45.2) were combined with 200,000 CD45.1 total, unfractionated WBM cells and transplanted into lethally irradiated recipient mice (CD45.1). Flow cytometry analysis was performed using a BD LSR II and sorting was performed using a BD FACSAria II cytometer. Data was analyzed using FlowJo version 10.6.2 software. Antibodies used are provided in the [STAR methods](#).

Cell proliferation assay

Lineage-depleted WBM cells were stained with 5µM CellTrace Violet (ThermoFisher Scientific) for 20 min at 37°C. Cells were then incubated in complete culture medium for 5 min at 37°C to absorb unbound dye. Washed cells were incubated in STEMPRO medium (Gibco) supplemented with SCF (10 ng/mL, Peprotech) and TPO (100 ng/mL, Peprotech) for 7 days and analyzed by flow cytometry for HSC panel.

5-Fluorouracil treatment

Under anesthesia, 8- to 12-week-old $\text{Ing4}^{-/-}$ and WT mice received one retroorbital venous sinus injection of 5-fluorouracil (5-FU, 150 mg/kg, Sigma) or PBS. WBM was harvested 15 or 30 days following 5-FU or mock treatment, lineage depleted, stained using HSC panel, and analyzed via flow cytometry.

c-Myc inhibition

Mice were subjected to 3.5 Gy total body irradiation (Rad Source). c-Myc inhibitor 10058-F4 was dissolved in a mixture of ethanol, Kolliphor, and saline (1:1:8 v/v/v) to a final concentration of 2 mg/mL. Sublethally irradiated (3.5 Gy) 8- to 12-week-old $\text{Ing4}^{-/-}$ and WT mice were treated (retroorbital injection) with 20 mg/kg of 10058-F4 or vehicle (ethanol, Kolliphor, saline only) once per day for 14 days. On day 15, bone marrow was analyzed via flow cytometry.

QUANTIFICATION AND STATISTICAL ANALYSIS

Mean values \pm SD are shown. Student's t test with Welch's correction was used for single comparisons (GraphPad Prism v.10.1.1). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.005$, **** $p < 0.001$. Two-way ANOVA was used for multiple comparisons for *in vivo* inhibitor assays (GraphPad Prism v.10.1.1). Mann-Whitney test was used to determine chimerism post-WBM transplant.