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14. ABSTRACT Myelodysplastic syndromes (MDS) are acquired bone marrow failure syndromes that affects between 20,000 and 45,000 people each year in the US. As <i>TP53</i> mutations are associated with short survival and drug resistance in patients with MDS, the goal of this study is to understand the role of these <i>TP53</i> mutations in MDS in order to develop novel treatments for MDS patients. We found that the inflammasome is activated in MDS stem cells expressing mutant p53, and these cells release more inflammatory cytokines to impair normal stem cell function. In addition, we found that the expression of spliceosome genes were downregulated in MDS stem cells expressing mutant p53. Further, we observed that mRNAs are not properly spliced in MDS stem cells with mutant p53. Importantly, we found that human MDS cells with <i>TP53</i> mutations are sensitive to inflammasome and spliceosome inhibitor treatment. Thus, mutant p53 proteins activate inflammasome and alter RNA splicing, leading to the formation of MDS stem cells. Upon completion of this research, we expect to establish both inflammasome and spliceosome as new druggable targets for clinical trials for MDS patients					
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1. Introduction

Myelodysplastic syndromes (MDS) are acquired bone marrow failure syndromes characterized by dysplastic hematopoiesis, bone marrow failure, peripheral cytopenias, and frequent progress to acute myelogenous leukemia (AML). The incidence of MDS in the US is at least 20,000 cases per year, making it the most common bone marrow failure syndrome in adults. As the clinical outcome of MDS patients is still very poor, despite progress in treatment approaches, there is a critical need to identify novel treatments for MDS. Somatic mutations in the tumor suppressor gene *TP53* are present in 10% of MDS cases and in 20–30% of secondary MDS, arising after exposure to radiation or alkylating agents. *TP53* mutations are associated with advanced disease and poor prognosis in patients with MDS. Recently, acquired somatic mutations in *TP53* were identified in 0.13% of aged healthy individuals. Therefore, the frequency of *TP53* mutations ranks top five among mutations identified in the elderly. Evolution of mutant clonal hematopoiesis with age predisposes the elderly to MDS and other aging-associated diseases, suggesting that mutations identified in hematopoietic stem and progenitor cells contribute to disease development. Our objective here is to characterize the role of mutant p53 in the pathogenesis of MDS and identify novel therapeutic targets for MDS treatment. Identification of the key regulators of MDS stem cells will significantly advance our understanding of the initiation and progression of MDS. Delineating the effects of GOF mutant p53, inflammasome, and spliceosome on MDS stem cells will likely identify novel druggable targets.

2. Keywords

MDS, TP53, inflammasome, inflammatory cytokines, spliceosome, and MDS stem cells

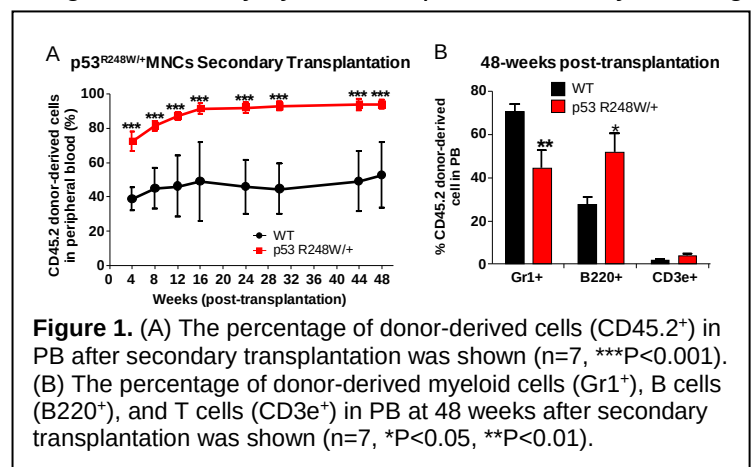
3. Accomplishments

Major goals and accomplishments: From August 1, 2018 to March 31, 2021, we have carried out experiments proposed in both Aim 1 and Aim 2. We have completed all proposed research as indicated in the Statement of Work (SOW). As our research progress, we made new discoveries and further advanced the mission of Bone Marrow Failure Research Program.

Specific Aim 1: Determine the impact of NLRP1 inflammasome activation on HSCs.

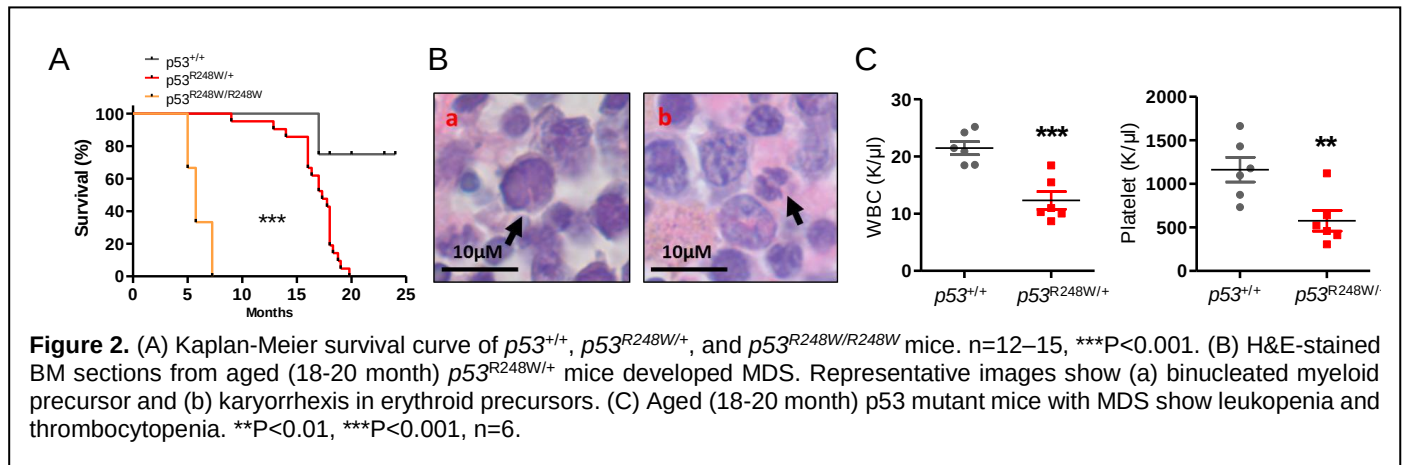
Rationale: Inflammasomes are cytosolic protein complexes that serve as platforms for the recruitment and activation of the pro-inflammatory Caspase-1 protease. Caspase-1 activation leads to processing and maturation of pro-inflammatory cytokines such as IL-1 β and a lytic form of cell death termed pyroptosis. Inflammasome assembly is initiated by cytosolic sensors in response to microbial infections. The NLRP1 inflammasome is highly expressed in hematopoietic progenitor cells. Its activation increases secretion of pro-inflammatory cytokine IL-1 β and inhibits wild-type HSPC function. MDS stem cells have been shown to impair normal hematopoietic cell function via activating inflammatory cytokine responses, thereby allowing propagation of MDS clones. We hypothesize that mutant p53 activates the NLRP1 inflammasome and increases the release of inflammatory cytokines to inhibit wild-type HSC function, thereby promoting the clonal expansion of mutant HSCs. We will determine the effects of genetic and pharmacological inhibition of the NLRP1 inflammasome on clonal expansion of mutant p53 HSCs.

Major Task 1: Determine the impact of genetic inhibition of NLRP1 on HSCs expressing mutant p53



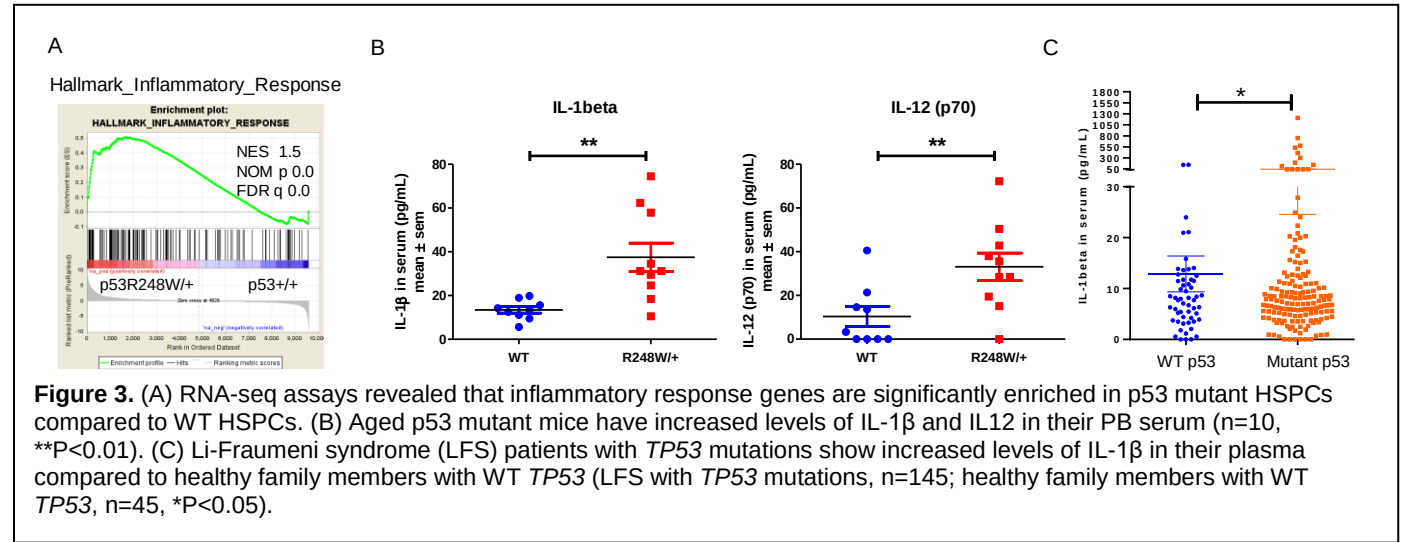
Mutant p53 promotes HSC expansion and impairs hematopoietic differentiation during aging. To determine the role of mutant p53 in HSC aging, we utilized the humanized knock-in mice of $p53^{R248W}$, expressing human mutant p53 protein from the murine *Trp53* promoter. As $p53^{+/+}$ and $p53^{R248W/+}$ mice have similar number of HSPCs in the BM, we transplanted 5×10^5 young (3-4 month) donor BM cells ($p53^{+/+}$ or $p53^{R248W/+}$, CD45.2⁺) into lethally irradiated (9.5 Gy) young recipient mice (CD45.1⁺CD45.2⁺) along with 5×10^5 young competitor BM cells (CD45.1⁺). Donor BM cells were pooled from age- and sex-matched littermates. At 16 weeks post transplantation, the repopulating ability of $p53^{R248W/+}$ cells was significantly higher than WT cells (data not shown). We then transplanted 3×10^6 BM cells isolated from the primary recipients into lethally irradiated secondary recipients. Forty-eight weeks after secondary transplantation, $p53^{R248W/+}$ cells continued to show increased repopulating ability (**Fig. 1A**). In addition, we found decreased frequency of donor-derived myeloid cells and increased frequency of lymphoid cells in PB of the secondary recipients repopulated with $p53^{R248W/+}$ BM cells (**Fig.1B**). These findings suggest that $p53^{R248W/+}$ mice may develop MDS during aging.

Aged heterozygous p53 mutant mice develop MDS. Most homozygous p53 knockout and mutant mice develop lymphoma and sarcoma and die within 3 to 6 months after birth. As the majority of *TP53* mutations found in CHIP and MDS are mono-allele missense mutations, we tested whether heterozygous p53 mutant mice would develop MDS during aging. We maintained age- and sex-matched young $p53^{+/+}$ and $p53^{R248W/+}$ mice for more than a year and monitored their survival and tumor development. $p53^{R248W/+}$ mice had an extended life span compared to $p53^{R248W/R248W}$ mice in that most heterozygous mutant mice died within 18-20 months after birth (**Fig. 2A**). Approximately 60% of aged (18-20 month) $p53^{R248W/+}$ mice of both sexes developed MDS based upon pathological analysis of BM and PB. Other aged $p53^{R248W/+}$ mice developed lymphoma and sarcoma. Aged $p53^{R248W/+}$ mice with MDS showed myeloid and erythroid precursor dysplasia in the BM (**Fig. 2B**). Aged $p53^{R248W/+}$ PB smears revealed hyper-segmented neutrophils, Pseudo-Pelger-Huet, and nucleated red cells (data not shown). Significant leukopenia, thrombocytopenia, and anemia were seen in aged p53 mutant mice with MDS compared to age- and sex-matched $p53^{+/+}$ mice (**Fig. 2C** and data not shown). Further, we found that mutant p53 driven-MDS is transplantable (data not shown). Thus, we demonstrated that mutant p53 drives MDS development during aging, thereby providing validity for the rodent model of MDS.

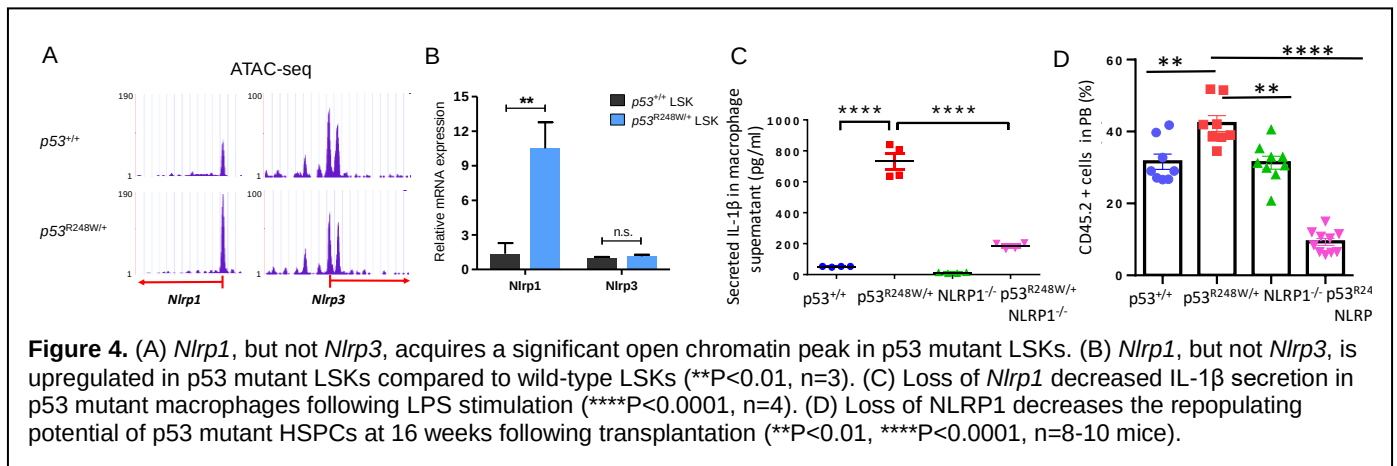


Mutant p53 generates a chronic inflammatory microenvironment in mice during aging. To understand how mutant p53 confers a competitive advantage to HSPCs and drives MDS development, we performed RNA-seq studies to compare gene expression in $p53^{+/+}$ and $p53^{R248W/+}$ HSPCs (LSKs). To ensure reproducibility, three biological replicates were performed, and LSK cells were pooled from age and sex matched littermates. We then employed Gene Set Enrichment Assays (GSEA) to group potential $p53^{R248W}$ target genes into specific pathways important for HSC behavior. GSEA analysis revealed that inflammatory response genes are significantly enriched in $p53^{R248W/+}$ HSPCs compared to $p53^{+/+}$ HSPCs (**Fig. 3A**). To determine the impact of mutant p53 on inflammatory response, we measured the levels of cytokines in PB serum and BM fluid utilizing cytokine arrays (Eve Technologies: Mouse Cytokine / Chemokine Array 31-Plex). While the levels of cytokines in PB serum from young mice are comparable (data not shown), we observed increased levels of pro-inflammatory cytokines, including IL-1 β and IL-12, in the serum of aged p53 mutant mice compared to that of the age- and sex-matched WT mice (**Fig. 3B**). We also observed increased levels of IL-1 β and IL-12 in the BM fluid of aged p53 mutant mice (data not

shown). Thus, we demonstrated that mutant p53 generates a chronic inflammatory microenvironment in mice during aging. Chronic inflammation is a well-characterized driver of cancer initiation, progression, and metastasis. During aging, chronic and low-grade inflammation (i.e. **inflammaging**) develops, thereby contributing to the pathogenesis of age-related diseases such as CHIP and MDS. Thus, we have uncovered a novel mechanism by which mutant p53 promotes CHIP progression and drives MDS pathogenesis during aging.



Plasma from individuals with Li-Fraumeni syndrome (LFS) have increased levels of IL-1 β : Li-Fraumeni syndrome (LFS), a rare, autosomal dominant disease, predisposes individuals to hereditary cancer linked to *TP53* germline mutations, and some LFS patients develop MDS and AML. As the *p53*^{R248W/+} mice is a mouse model of human LFS, we then examined the levels of IL-1 β in the plasma of LFS patients with *TP53* mutations and their healthy family members who have WT *TP53*. Consistent with our observation in aged p53 mutant mice, we found increased levels of IL-1 β in the plasma of LFS patients with *TP53* mutations (Fig. 3C). As IL-1 β has been implicated in human cancer, our findings suggest that mutant p53-driven chronic inflammation may promote cancer development in individuals with LFS.



Mutant p53 increases the chromatin accessibility to the NLRP1 inflammasome gene in HSPCs. To investigate how mutant p53 activates inflammatory response, we performed ATAC-seq assays in HSPCs (LSKs) to identify differential regions of chromatin accessibility. To ensure reproducibility, three biological replicates were performed, and LSK cells were pooled from age- and sex-matched littermates. Notably, we found that mutant p53 increases the chromatin accessibility to inflammasome gene *Nlrp1*, and we confirmed the upregulation of *Nlrp1* in *p53*^{R248W/+} LSKs as compared to *p53*^{+/+} LSKs (Figs. 4A and 4B). While NLRP3 inflammasome is activated in HSPCs from low to high risk MDS, we found that mutant p53 affects neither the chromatin accessibility nor the expression of *Nlrp3* in LSK cells (Figs. 4A and 4B). These findings suggest that the NLRP1 inflammasome may be specifically activated in p53 mutant mice.

Increased secretion of IL-1 β seen in p53 mutant macrophages is dependent on NLRP1. NLRP1 inflammasome activation increases IL-1 β secretion and IL-1 β in serum could come from stromal cells, immune cells, and/or endothelial cells. Given that macrophages are major sources of cytokine production, we examined the impact of mutant p53 on IL-1 β secretion from BM derived macrophages following lipopolysaccharide (LPS) stimulation. We cultured low-density BM mononuclear cells from $p53^{+/+}$, $p53^{R248W/+}$, $Nlrp1^{-/-}$, and $p53^{R248W/+}Nlrp1^{-/-}$ mice in the presence of GM-CSF (100ng/ml) for 7 days. We treated these cells with 100 ng/ml LPS for 16 hours to induce pro-inflammatory activation. Supernatants were then collected and subjected to cytokine array analysis. We observed increased secretion of IL-1 β from mutant macrophages compared to WT macrophages following LPS stimulation (**Fig. 4C**). Importantly, loss of *Nlrp1* significantly decreased IL-1 β secretion from p53 mutant macrophages following LPS stimulation (**Fig. 4C**).

NLRP1 is essential for p53 mutant HSPC repopulating potential: To determine the role of NLRP1 in p53 mutant HSPCs, we performed both colony formation assays and competitive BM transplantation assays. We found that loss of NLRP1 significantly decreases the colony formation of p53 mutant HSPCs (data not shown). Further, we found that loss of NLRP1 significantly decreases the repopulating potential of p53 mutant HSPCs (**Fig. 4D**), demonstrating that NLRP1 is important for p53 mutant HSPC function.

Major Task 2: Determine the impact of pharmacological inhibition of the NLRP1 inflammasome on HSCs expressing mutant p53

Given that IL-1 β is a key effector molecule of the NLRP1 inflammasome and there are no NLRP1 specific inhibitors available, we decided to focus on determine the impact of inhibiting IL-1 β /IL-1R signaling on HSCs.

IL-1 β /IL-1R signaling is important for enhanced fitness of p53 mutant HSPCs.

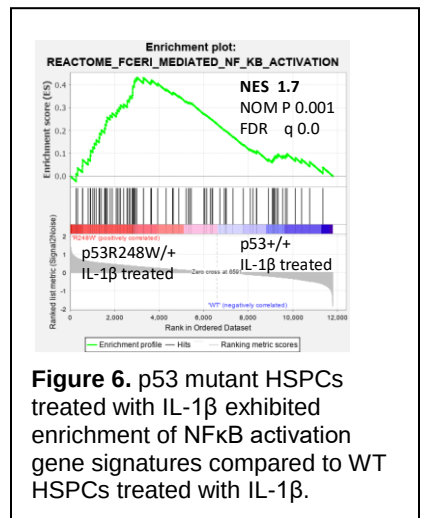
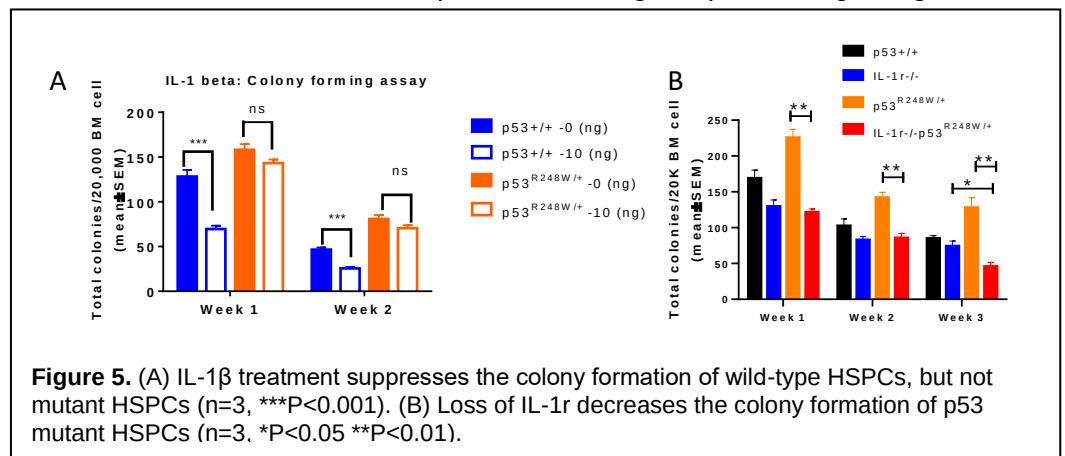
IL-1 β is known to exert suppressive effects on HSPC activity. To assess the effects of IL-1 β on mutant HSPCs, we performed serial replating assays and found that IL-1 β

treatment decreases the

serial replating potential of WT HSPCs, but not mutant HSPCs (**Fig. 5A**). We then examined the impact of IL-1 β neutralizing antibody treatment on colony formation of HSPCs. While IL-1 β neutralizing antibody treatment has no effect on WT HSPC colony formation, it decreases the replating potential of p53 mutant HSPCs (data not shown). The extracellular forms of the IL-1 cytokines are active through binding to the IL-1 receptor (IL-1R) on the surface of target cells. To determine the role of IL-1R in p53 mutant HSPCs, we have generated $p53^{R248W/+}IL-1r^{-/-}$ mice. We found that loss of *IL-1r* decreases the colony formation of p53 mutant HSPCs (**Fig. 5B**). These findings demonstrate that IL-1 β /IL-1R signaling is important for enhanced fitness of p53 mutant HSPCs.

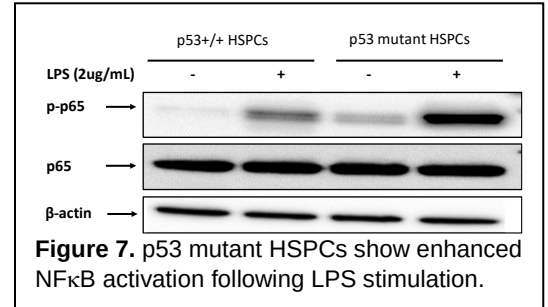
p53 mutant HSPCs are enriched with NF κ B activation gene signatures following IL-1 β treatment.

To determine the mechanism by which p53 mutant HSPCs are resistant to IL-1 β treatment, we performed RNA sequencing in WT ($p53^{+/+}$) and $p53^{R248W/+}$ LSKs treated with IL-1 β (10 ng/ml) or PBS *in vitro* for 90 minutes. To ensure reproducibility, three biological replicates were performed, and LSK cells were pooled from age- and sex-matched littermates. Gene Set Enrichment Analysis (GSEA) revealed that p53 mutant LSKs treated with IL-1 β are enriched with NF κ B

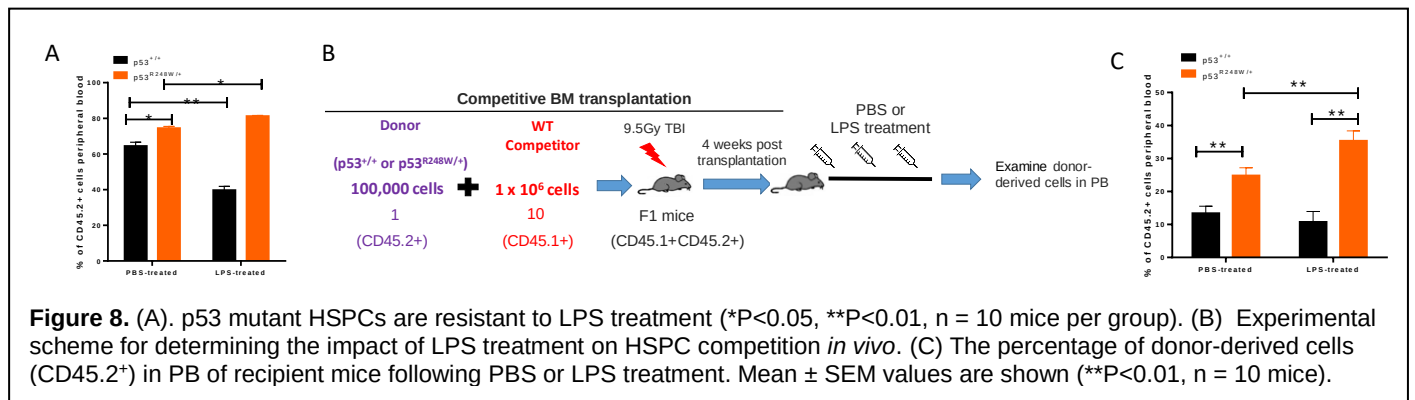


activation gene signatures compared to IL-1 β treated WT LSKs (**Fig. 6**). In addition, p53 mutant LSKs treated with IL-1 β were enriched with MYC target genes compared to IL-1 β treated WT LSKs (data not shown). Thus, we demonstrate that p53 mutant HSPCs have altered responses to IL-1 β treatment compared to WT HSPCs, and these responses, which involved NF κ B activation and MYC-dependent survival pathways, may confer a competitive advantage to mutant HSPCs.

p53 mutant HSPCs show enhanced NF κ B activation: NF κ B is a key mediator of LPS/TLR-4 and IL-1 β /IL-1R signaling in immune cells. The NF- κ B family consists of several proteins, including RelA (commonly known as p65), RelB, c-Rel, p50, and p52, operating as heterodimers or homodimers. Upon LPS or IL-1 β stimulation, p65 is phosphorylated and then translocates into nucleus to activate NF- κ B target genes. To determine the response of HSPCs to LPS, we treated p53^{+/+} and p53^{R248W/+} HSPCs with or without LPS (2 μ g/ml) for 5 minutes and then examined NF κ B activation (p65 phosphorylation). While p53 mutant HSPCs displayed high levels of p65 phosphorylation in steady state, LPS treatment further enhanced p65 phosphorylation in p53 mutant HSPCs compared to WT HSPCs (**Fig. 7**), demonstrating that p53 mutant HSPCs exhibit enhanced NF- κ B activation following LPS treatment.



p53 mutant HSPCs are resistant to LPS treatment: Chronic LPS treatment impairs the repopulating potential of WT HSPCs. We next evaluated the effects of chronic inflammation on p53 mutant HSPC function *in vivo*. p53^{+/+} and p53^{R248W/+} mice were treated with PBS or LPS (1mg/kg, I.P.) every other day for 4 weeks. We then utilized live BM cells from PBS or LPS-treated p53^{+/+} and p53^{R248W/+} mice as donor cells to perform competitive BM transplantation assays. The ratio of donor cells (CD45.2⁺) to competitor cells (CD45.1⁺) is 1 to 1. While BM cells from LPS-treated p53^{+/+} mice show decreased engraftment compared to that of PBS-treated p53^{+/+} mice, LPS treatment increased the engraftment of BM cells from p53^{R248W/+} mice at 16 weeks following transplantation (**Fig. 8A**). To determine whether functional TP53 mutations promote clonal expansion of HSPCs following LPS treatment, we generated mixed BM chimaeras containing donor BM cells (p53^{+/+} or p53^{R248W/+}, CD45.2⁺) and WT competitor BM cells (CD45.1⁺). To mimic the human scenario of clonal hematopoiesis, we used a 1:10 ratio of donor cells to competitor cells in the experiment. Four weeks following transplantation, recipient mice were treated with PBS or LPS (1mg/kg, I.P.) every other day for 4 weeks (**Fig. 8B**). While LPS treatment did not affect the repopulating ability of donor WT HSPCs, LPS treatment significantly expanded p53 mutant HSPCs at 16 weeks following LPS treatment (**Fig. 8C** and data not shown).



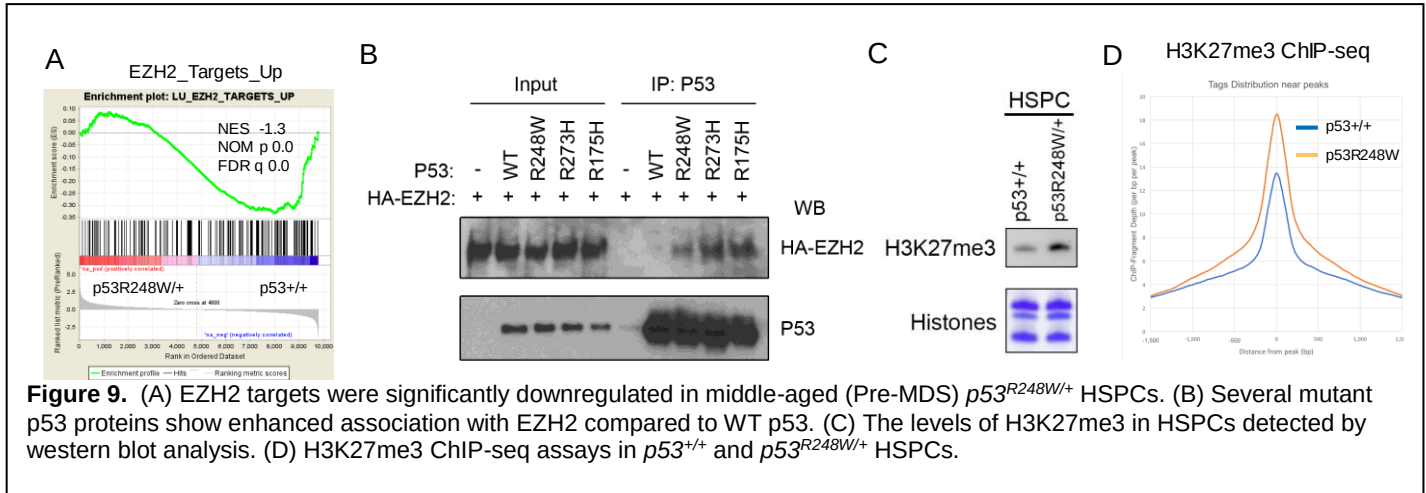
Specific Aim 2: Elucidate the mechanism by which mutant p53 alters pre-mRNA splicing in HSCs.

Rationale: Splicing of precursor messenger RNA by spliceosome is a critical step in regulating gene expression and alterations in splicing may contribute to MDS pathogenesis. We hypothesize that GOF mutant p53 drives MDS development through altering the splicing of key regulators of hematopoiesis and spliceosome. We will determine the role of mutant p53 in regulating spliceosome gene expression and pre-mRNA splicing in aged HSCs. Given that MDS cells with spliceosome gene mutations are susceptible to

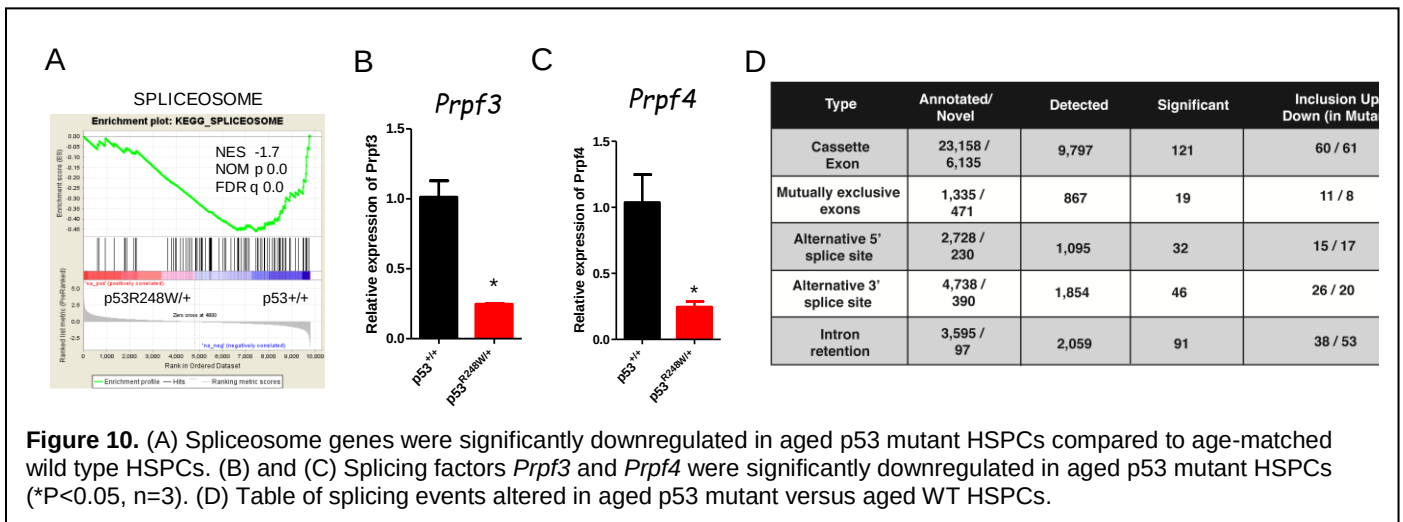
spliceosome inhibitor treatment, we will treat MDS cells with *TP53* mutations with an EZH2 or a spliceosome inhibitor (E7107). These studies will delineate the mechanism by which mutant p53 alters pre-mRNA splicing and identify spliceosome as a novel therapeutic target for the treatment of MDS patients with *TP53* mutations.

Major Task 3: Determine the role of mutant p53 in spliceosome gene expression and splicing in aged HSCs.

Aged p53 mutant HSPCs show enhanced EZH2 activity. RNA-seq assays revealed that EZH2 targets



were significantly downregulated in middle-aged (Pre-MDS) p53 mutant HSPCs (**Fig. 9A**). EZH2 is the catalytic component of the Polycomb repressive complex 2 (PRC2) that trimethylates histone H3 at lysine 27 (H3K27me3) to repress the transcription of target genes. Downregulation of EZH2 targets in p53 mutant HSPCs suggests that EZH2 activity may be enhanced. Indeed, we found that several mutant p53 proteins, but not wild-type p53, interact with EZH2 (**Fig. 9B**). p53 mutant HSPCs showed increased levels of H3K27me3 compared to wild-type HSPCs (**Fig. 9C**). Further, H3K27me3 ChIP-seq assays revealed that mutant HSPCs exhibit significantly high levels of H3K27me3 compared to that of the WT HSPCs (**Fig. 9D**). Thus, these studies identify enhanced EZH2 activity as a potential mechanism for mutant p53 in MDS.

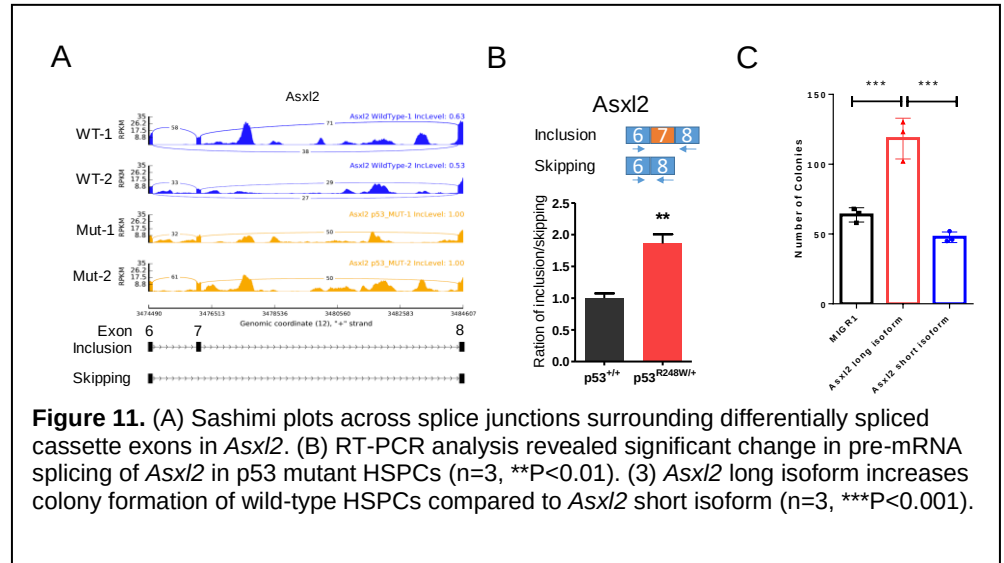


Mutant p53 alters pre-mRNA splicing in aged HSPCs. RNA-seq assays also revealed that spliceosome genes were significantly downregulated in aged p53^{R248W/+} HSPCs compared to p53^{+/+} HSPCs (**Fig. 10A**). We confirmed that the expression of splicing factors, including *Prpf3* and *Prpf4*, was significantly downregulated in aged p53^{R248W/+} HSPCs (**Fig. 10B and 10C**). Given *Prpf3* and *Prpf4* deficiencies comprise spliceosome assembly, we speculated that downregulation of *Prpf3* and *Prpf4* in p53 mutant HSPCs may alter pre-mRNA splicing. We then conducted comprehensive pre-mRNA splicing analysis

utilizing the MISO (Mixture of Isoforms) algorithm to identify differences in exon inclusion ratio between p53 mutant versus WT HSPCs. We further utilized ExonImpact, a novel tool developed in Dr. Yunlong Liu's laboratory, to prioritize identified exons. We observed differential splicing of all classes of alternative splicing events, including Cassette Exons, Competing 5' and 3' splice sites, Mutually Exclusive Exons, and Retained Introns in *p53*^{R248W/+} cells (**Fig. 10D**), the magnitude of which is similar to what seen in MDS patients with spliceosome gene mutations. Importantly, we found that human CD34⁺ cells expressing mutant p53 displayed significant alterations in pre-mRNA splicing compared to CD34⁺ cells expressing WT p53 (data not shown), demonstrating that the impact of mutant p53 on pre-mRNA splicing in HSPCs is conserved across species.

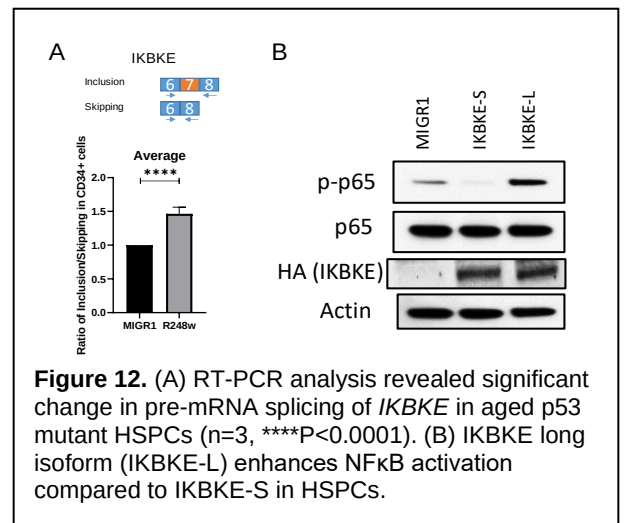
Aged p53 mutant HSPCs display aberrant pre-mRNA splicing in key regulators of hematopoiesis

We found that aged p53 mutant HSPCs show dysregulated pre-mRNA splicing in key regulators of hematopoiesis such as *Asx12* and *Setd3* (**Fig. 11A** and data not shown). Polycomb group protein ASXL2 is frequently mutated in AML patients with t(8;21). We recently reported that *Asx12* is required for normal HSC self-renewal and deletion of *Asx12* leads to MDS development in mice. We confirmed that p53 mutant HSPCs display significant change in pre-mRNA splicing of *Asx12*, mainly expressing the long isoform of *Asx12* (**Fig. 11B**). Further, we found that ectopic expression of *Asx12* long isoform, but not the *Asx12* short isoform, increases the colony formation of WT HSPCs (**Fig. 11C**).



Aged p53 mutant HSPCs display aberrant pre-mRNA splicing in key regulators of inflammatory response

We found that aged p53 mutant HSPCs show dysregulated pre-mRNA splicing in key regulators of NFκB such as *IKBKE* and *USP15* (data not shown). *IKBKE* (inhibitor of nuclear factor kappa B kinase subunit epsilon), a member of the nonclassical IKK (IκB kinase) family, can indirectly activate the NFκB signaling pathway by forming a kinase complex with TBK1 and TANK. We confirmed that aged p53 mutant HSPCs display significant change in splicing of *IKBKE*, mainly expressing the long isoform of *IKBKE* (*IKBKE*-L) (**Fig. 12A**). We introduced *MIGR1* (GFP), HA-tagged *IKBKE*-L or *IKBKE*-S into LSKs using retroviruses and examined p65 phosphorylation in transduced cells (GFP⁺). We found that ectopic expression of *IKBKE*-L enhances NFκB activation, whereas *IKBKE*-S expression decreases NFκB activation in LSKs (**Fig. 12B**).

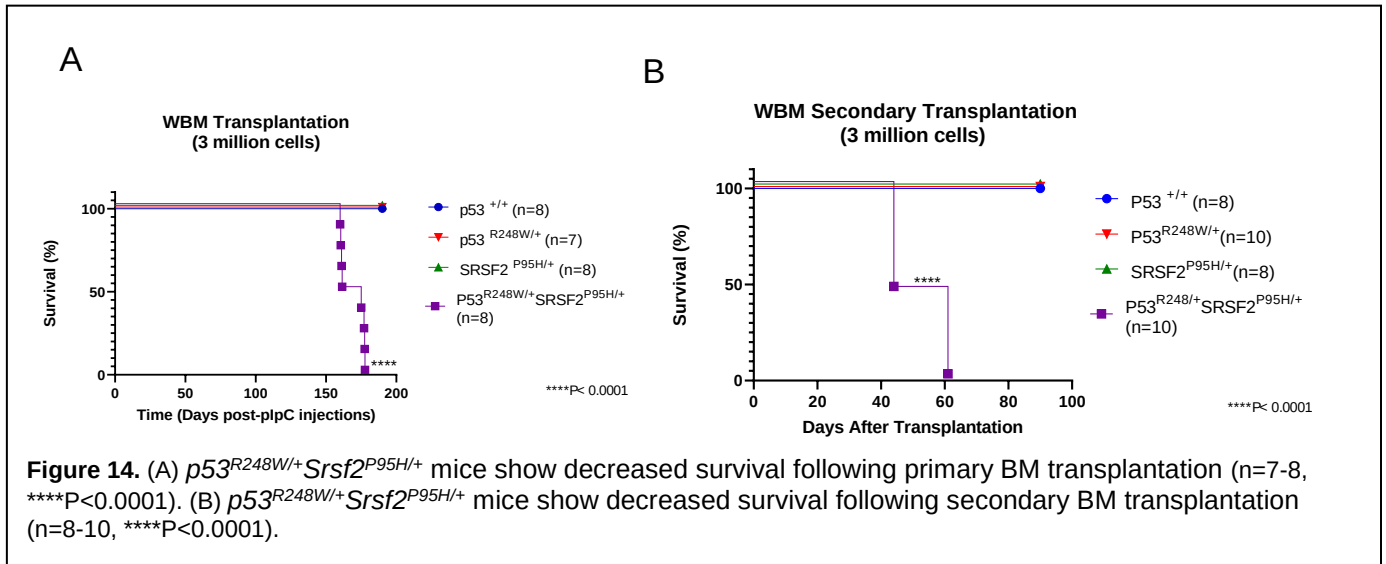
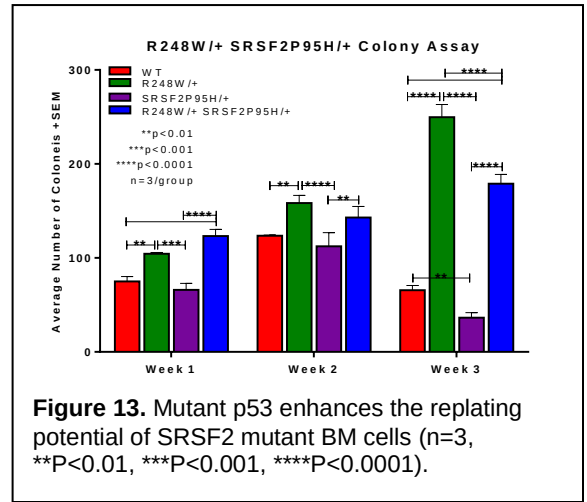


Major Task 4: Determine the impact of spliceosome inhibitor on hematopoietic progenitor cells and MDS cells with *TP53* mutations

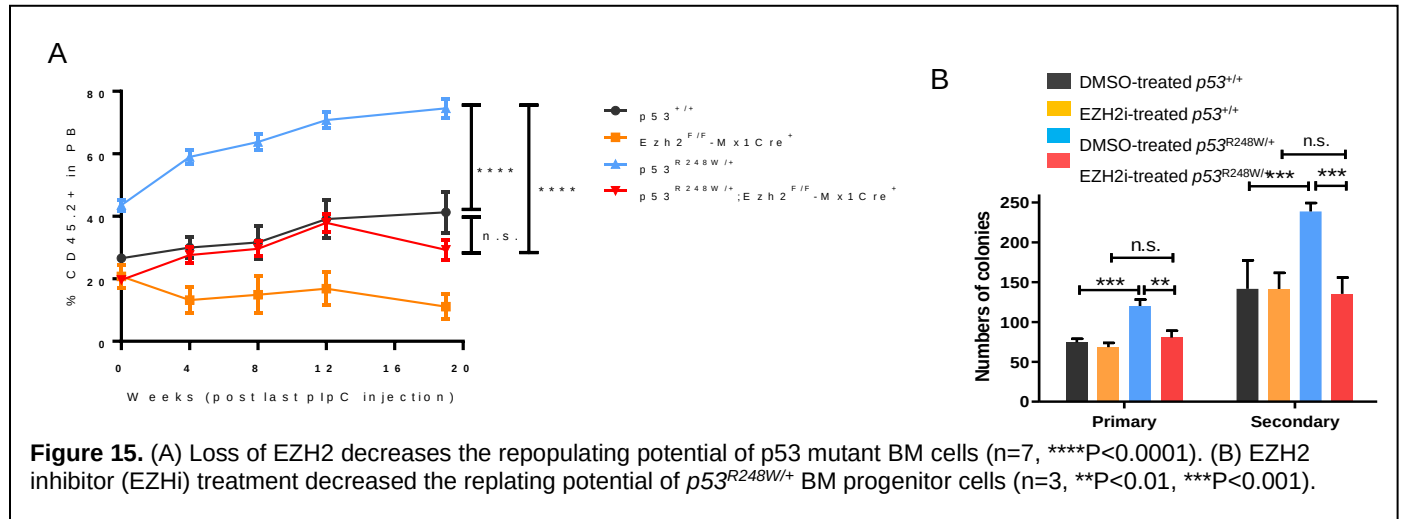
***TP53* and *SRSF2* mutations identified in CHIP and MDS cooperate in the pathogenesis of hematological malignancies**. While coexisting mutations with *TP53* mutations in MDS are limited, previous studies indicate that *TP53* mutations co-occur with splicing factor mutations such as *SRSF2* mutations in human MDS. Splicing factor *SRSF2*^{P95H} mutant is frequently found in CHIP and MDS.

We received *Srsf2*^{P95H/+}-*Mx1-Cre*⁺ mice from our collaborator Dr. Omar Abdel-Wahab at the Memorial Sloan-Kettering Cancer Center and have generated *p53*^{R248W/+}*Srsf2*^{P95H/+}-*Mx1-Cre*⁺ mice. We first utilized BM cells from *p53*^{+/+}, *p53*^{R248W/+}, *Srsf2*^{P95H/+}-*Mx1-Cre*⁺, and *p53*^{R248W/+}*Srsf2*^{P95H/+}-*Mx1-Cre*⁺ mice and performed serial replating assays. We found that mutant p53 enhances the replating potential of *Srsf2* mutant BM cells (Fig. 13).

To determine whether *TP53* and *SRSF2* mutations cooperate in pathogenesis of hematological malignancies, we performed BM transplantation assays. We transplanted 3 x 10⁶ middle-aged (12-14 month) *p53*^{+/+}, *p53*^{R248W/+}, *Srsf2*^{P95H/+}-*Mx1-Cre*⁺, and *p53*^{R248W/+}*Srsf2*^{P95H/+}-*Mx1-Cre*⁺ BM cells (CD45.2⁺) into lethally irradiated young recipient mice (CD45.1⁺). Donor BM cells were pooled from age- and sex-matched littermates. Four weeks after

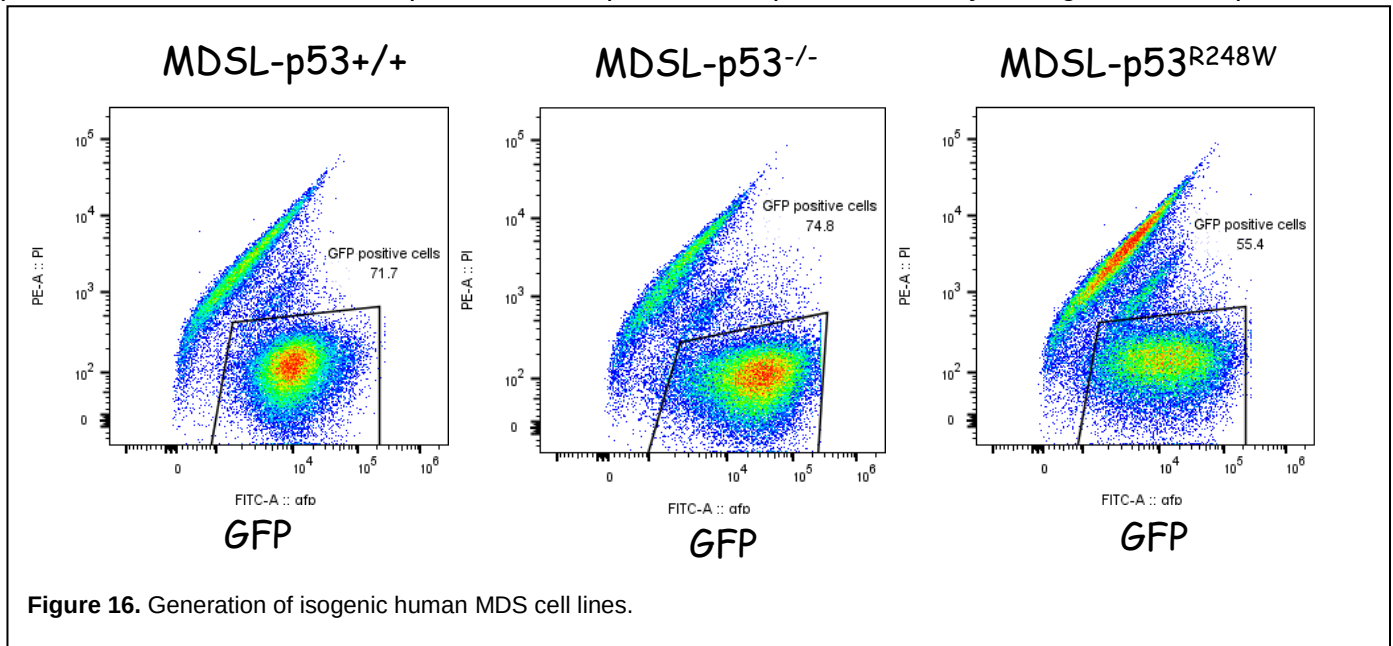


transplantation, we treated recipient mice with pl:pC to induce the expression of SRSF2^{P95H} mutant in hematopoietic cells. We then monitored the survival and disease development in recipient mice. We found that the survival of recipient mice repopulated with *p53*^{R248W/+}*Srsf2*^{P95H/+} BM cells was significantly decreased compared to recipients repopulated with WT, *p53*^{R248W/+}, or *Srsf2*^{P95H/+} BM cells in both primary and secondary transplantation experiments (Fig. 14 A and 14B). All recipient mice repopulated with *p53*^{R248W/+}*Srsf2*^{P95H/+} BM cells died within 6 month following pl:pC treatment and developed hematological



malignancies, including AML and acute lymphoblastic leukemia (data not shown). These findings demonstrate that mutant p53 synergizes with *Srsf2* mutation in pathogenesis of hematological malignancies, manifested by decreased survival and accelerate disease onset.

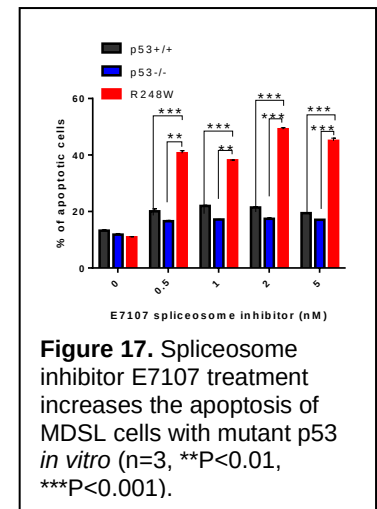
Inhibiting Ezh2 decreases p53 mutant HSPC function. To determine the impact of Ezh2 deficiency on p53 mutant HSPC function, we performed competitive transplantation assays using HSCs from *p53*^{+/+},



p53^{R248W/+}, *Ezh2*^{-/-}, and *p53*^{R248W/+} *Ezh2*^{-/-} mice. We found that Ezh2-deficiency significantly decreases the repopulating potential of p53 mutant HSCs (**Fig. 15A**). In addition, we found that pharmacological inhibition of Ezh2 activity with an EZH2-specific inhibitor (EPZ011989, 3μM) decreases the replating potential of p53 mutant HSPCs, but not WT HSPCs (**Fig. 15B**).

Human MDS cells with TP53 mutations are sensitive to spliceosome inhibitor treatment. Human MDS cells with splicing factor mutations are dependent on the remaining WT spliceosome gene allele for survival. Thus, these MDS cells are sensitive to spliceosome inhibitor treatment. Due to COVID-19 pandemic, IRB protocol application was dramatically delayed and we could not obtain MDS patient samples for proposed studies. To overcome this limitation, we decided to use human MDS cell lines to perform the experiments. MDSL is a human MDS cell line (*p53*^{-/-}) derived from a MDS patient with refractory anemia-ringed sideroblasts. We transduced parental MDSL cells with retroviruses expressing WT p53, GFP, or p53R248W, and generated three isogenic MDSL lines, including MDSL-*p53*^{+/+}, MDSL-*p53*^{-/-}, and MDSL-*p53*^{R248W} (**Fig. 16**).

To determine the response of human MDS cells to spliceosome inhibitor treatment, we cultured MDSL-*p53*^{+/+}, MDSL-*p53*^{-/-}, and MDSL-*p53*^{R248W} cells with DMSO or increased concentrations of spliceosome inhibitor E7107 for 24 hours and then examined their survival by flow cytometry analysis. We found that MDSL-*p53*^{R248W} cells show increased apoptosis and decreased proliferation compared to MDSL-*p53*^{+/+} and MDSL-*p53*^{-/-} cells following spliceosome inhibitor treatment (**Fig. 17** and data not shown).



Opportunities for training and professional development:

Dr. Yan Liu has trained four graduate students, including Sergio Barajas, Wenjie Cai, Shiyu Xiao, and Christopher Bochers, and two postdoctoral fellows, including Sasidhar Vemula and Hongxia Chen, from 2018 to 2021. Sasidhar, Sergio and Wenjie attended American Society of Hematology annual meeting in Orlando in 2019. Hongxia Chen attended leukemia and epigenetics symposium at Miami in 2020. The

award has enriched their knowledge in hematology and leukemia and improved their skills in leukemia research.

Dissemination of results to communities of interest:

Invited speaker (Dr. Yan Liu):

- | | |
|------|---|
| 2018 | Genetic and Epigenetic Control of Hematopoietic Stem Cell Expansion and pathogenesis of MDS. Wells Center for Pediatric Research, Indiana University School of Medicine. |
| 2018 | Understanding and Targeting Mutant p53 in Hematological Malignancies. Simon Cancer Center, Indiana University School of Medicine. |
| 2018 | PRL2 phosphatase in normal and malignant hematopoiesis. Cooperative Center of Excellence in Hematology, Indiana University School of Medicine. |
| 2018 | Developing Novel Therapeutic Approaches to improve childhood leukemia treatment, the St. Baldrick's Foundation, CA |
| 2018 | Mutant p53 promotes hematopoietic stem and progenitor cell expansion through interacting with epigenetic modulator EZH2. The 60 th American Society of Hematology (ASH) Conference, San Diego, CA. |
| 2018 | Developing Novel Therapeutic Approaches to improve leukemia treatment, Indiana Science and Technology Club, IN |
| 2019 | Genetic and Epigenetic Regulation of Leukemia Stem Cells, University of Notre Dame, South Bend, Indiana. |
| 2019 | Mutant p53 drives clonal hematopoiesis through modulating epigenetic pathways, the 8 th Mutant p53 Workshop, Lyon, France. |
| 2019 | Genetic and Epigenetic Control of Pre-leukemia Stem Cells and Leukemia Stem Cells, University of Minnesota, Minnesota. |
| 2019 | Understanding and Targeting of Pre-leukemia Stem Cells and Leukemia Stem Cells, Northwestern University. |
| 2019 | Understanding and Targeting of Pre-leukemia Stem Cells and Leukemia Stem Cells, University of Miami. |
| 2020 | Understanding and Targeting of Pre-leukemia Stem Cells and Leukemia Stem Cells, University of Florida. |
| 2021 | Understanding and Targeting of Pre-leukemia Stem Cells and Leukemia Stem Cells, Chicago Blood Club. |

4. Impact

What was the impact on the development of the principal discipline(s) of the project?

The proposed research is **innovative** because our strategy represents a substantial departure from the status quo; our work provided first-time experimental evidence that mutant p53 drives MDS development in mice during aging. We discovered that NLRP1 inflammasome is specifically activated in mutant p53 HSCs utilizing innovative techniques, such as ATAC-seq analysis. Further, we found that mutant p53 alters the splicing of key regulators of hematopoiesis and spliceosome in HSCs to promote MDS development. Collectively, these **innovative** studies are expected to establish both **NLRP1 inflammasome** and **spliceosome** as novel druggable targets in MDS with **mutant p53**.

Identification of the key regulators of MDS stem cells will significantly advance our understanding of the initiation and progression of MDS. Delineating the effects of GOF mutant p53, inflammasome, and spliceosome on MDS stem cells will likely identify novel druggable targets. Indeed, targeting **NLRP1 inflammasome** and **spliceosome** in human MDS may have therapeutic potential for eliminating drug-resistant MDS stem cells. Thus, successful completion of these studies would be expected to have an important *positive impact* on **military personnel, veterans, and their family members**.

What was the impact on other disciplines?

Nothing to report

What was the impact on technology transfer?

Nothing to report

What was the impact on society beyond science and technology?

Nothing to report

5. Changes/Problems

Due to COVID-19 pandemic, IRB protocol approval was dramatically delayed and we could not obtain MDS patient samples for proposed studies. To overcome this limitation, we decided to use human MDS cells lines to perform the experiments. We have included IRB approval in the Appendices.

6. Products

Publications, conference papers, and presentations

a. Journal publications.

1. Chen S, Gao R, Yao C, Kobayashi M, Liu SZ, Yoder MC, Broxmeyer HE, Kapur R, Boswell HS, Mayo LD, and **Liu Y***. Genotoxic stresses promotes the clonal expansion of hematopoietic stem cells expressing mutant p53. *Leukemia*. 2018 Mar;32(3):850-854. PMCID:PMC5842141
2. Chen S and **Liu Y***. Battle in stem cell niches: canonical versus non-canonical Wnt signaling. *Journal of Leukocyte Biology*. 2018 Mar;103(3):377-379. PubMed PMID: 29393968.
3. Cai Z, Kotzin JJ, Ramdas R, Chen S, Nelanuthala S, Palam LR, Pandey R, Mali RS, **Liu Y**, Kelley MR, Sandusky G, Mohseni M, Williams A, Henao-Mejia J, and Kapur R. Downregulation of *Morrbid* in Tet2-deficient preleukemic cells overcomes resistance to inflammatory stress and mitigates clonal hematopoiesis. *Cell Stem Cell*. 2018 Dec 6;23(6):833-849.e5. PMID:30526882
4. Nabinger S, Chen S, Yao C, Gao R, Kobayashi M, Vemula S, Fahey AC, Wang C, Daniels C, Boswell HS, Sandusky GE, Mayo LD, Kapur R, and **Liu Y***. Mutant p53 enhances leukemia-initiating cell self-renewal to promote leukemia development. *Leukemia*. 2019 Jun;33(6):1535-1539. PMID: 30675010
5. Chen S, **Liu Y***. p53 involvement in clonal hematopoiesis of indeterminate potential. *Curr Opin Hematol*. 2019 Jul;26(4):235-240. doi: 10.1097/MOH.0000000000000509.

6. Chen S, Wang Q, Yu H, Capitano ML, Vemula S, Nabinger SC, Gao R, Yao C, Kobayashi M, Geng Z, Fahey AC, Henley D, Liu SZ, Barajas S, Cai W, Wolf ER, Ramdas B, Cai Z, Gao H, Luo N, Sun Y, Wong TN, Link DC, Liu Y, Boswell HS, Mayo LD, Huang G, Kapur R, Yoder MC, Broxmeyer HE, Gao Z, and **Liu Y***. Mutant p53 Drives Clonal Hematopoiesis through Modulating Epigenetic Pathway. *Nature Communications*. 2019 Dec 11;10(1):5649. doi: 10.1038/s41467-019-13542-2. PMCID: PMC6906427.

7. Kobayashi M, Lin Y, Mishra A, Shelly C, Gao R, Wang P, Xi R, Wenzel P, Liu Y, **Liu Y***, and Yoshimoto M. Bmi1 maintains the self-renewal property of innate -like B lymphocytes. *Journal of Immunology*. 2020 Jun 15;204(12):3262-3272. **(Co-corresponding author)**.

8. Broxmeyer HE, **Liu Y**, Kapur R, Orschell CM, Aljoufi A, Ropa JP, Trinh T, Burns S, Capitano ML. Fate of Hematopoiesis During Aging. What Do We Really Know, and What are its Implications?. *Stem Cell Rev Rep*. 2020 Dec;16(6):1020-1048.

9. Chen S, Yu H, Gao R, Liu X, Vemula S, Fahey AC, Henley D, Kobayashi M, Liu SZ, Wang Q, Qian Z, Kapur R, Broxmeyer H, Gao Z, Xi R and **Liu Y***. Bmi1 regulates Wnt signaling in hematopoietic stem and progenitor cells. *Stem Cell Reviews and Report* (Submitted, 2021).

a. Books or other non-periodical, one-time publications.

Nothing to Report

b. Other publications, conference papers, and presentations.

- | | |
|------|---|
| 2018 | Mutant p53 promotes hematopoietic stem and progenitor cell expansion through interacting with epigenetic modulator EZH2. The 60 th American Society of Hematology (ASH) Conference, San Diego, CA. |
| 2019 | Mutant p53 drives clonal hematopoiesis through modulating epigenetic pathways, Dr. Yan Liu, the 8 th Mutant p53 Workshop, Lyon, France. |

Website(s) or other Internet site(s)

Nothing to Report

Technologies or techniques

Nothing to Report

Inventions, patent applications, and/or licenses

Nothing to Report

Other Products

Identify any other reportable outcomes that were developed under this project. Reportable outcomes are defined as a research result that is or relates to a product, scientific advance, or research tool that makes a meaningful contribution toward the understanding, prevention, diagnosis, prognosis, treatment, and/or rehabilitation of a disease, injury or condition, or to improve the quality of life. Examples include:

a. data or databases;

Nothing to Report

b. biospecimen collections;

Nothing to Report

c. *audio or video products;*

Nothing to Report

d. *software;*

Nothing to Report

e. *models;*

Nothing to Report

f. *educational aids or curricula;*

Nothing to Report

g. *instruments or equipment;*

Nothing to Report

h. *research material (e.g., Germplasm; cell lines, DNA probes, animal models);*

Human MDS cells lines: MDSL-p53, MDSL-p53-/- and MDSL-p53R248W

*Animal models: P53R248W/+EZH2-/-, P53R248W/+NLRP1-/-, and P53R248W/+IL-1R-/- mice;
Mouse models of human MDS and patient-derived xenograft (PDX) models of MDS.*

i. *clinical interventions;*

Nothing to Report

j. *new business creation;*

Nothing to Report

k. *other.*

Nothing to Report

7. Participants & Other Collaborating Organizations

What individuals have worked on the project?

Name:	<i>Yan Liu</i>
Project Role:	<i>PI</i>

Researcher Identifier (e.g. ORCID ID):	0000-0003-4878-9111
Nearest person month worked:	2
Contribution to Project:	<i>Dr. Liu supervised the conduct of the experiments, analyzed data, developed hypotheses, planned future experiments and writing, revising, and submitting manuscripts describing the findings from this project.</i>
Funding Support:	<i>NIH and Leukemia and Lymphoma Society</i>

- a. *Provide the following information for: (1) PDs/PIs; and (2) each person who has worked at least one person month per year on the project during the reporting period, regardless of the source of compensation (a person month equals approximately 160 hours of effort). If information is unchanged from a previous submission, provide the name only and indicate "no change."*

Name:	<i>Yunlong Liu</i>
Project Role:	<i>Co-I</i>
Researcher Identifier (e.g. ORCID ID):	N/A
Nearest person month worked:	<i>1</i>
Contribution to Project:	<i>Dr. Liu provided statistical support for the project, including study design, sample size calculation, statistical inference, and any related requests.</i>
Funding Support:	<i>NIH</i>

Name:	Omar Abdel-Wahab
Project Role:	<i>Co-I</i>
Researcher Identifier (e.g. ORCID ID):	N/A
Nearest person month worked:	<i>1</i>
Contribution to Project:	Dr. Abdel-Wahab was responsible for supervising the conduct of the experiments, analyzing data, and obtaining human MDS patient samples.
Funding Support:	<i>NIH and Leukemia and Lymphoma Society</i>

Name:	<i>Sasidhar Vemula</i>
Project Role:	<i>Postdoc</i>

Researcher Identifier (e.g. ORCID ID):	N/A
Nearest person month worked:	12
Contribution to Project:	Dr. Vemula designed and conducted the experiments listed in this proposal.
Funding Support:	N/A

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

Nothing to Report

What other organizations were involved as partners?

i. **Organization Name:** Memorial Sloan Kettering Cancer Center

ii. **Location of Organization:** *New York*

iii. **Partner's contribution to the project** (*identify one or more*)

1. **Financial support;**

Nothing to Report

2. **In-kind support** (*e.g., partner makes software, computers, equipment, etc., available to project staff*);

Nothing to Report

3. **Facilities** (*e.g., project staff use the partner's facilities for project activities*);

Nothing to Report

4. **Collaboration** (*e.g., partner's staff work with project staff on the project*);

Dr. Abdel-Wahab's lab performed experiments and provided reagents to the study.

5. **Personnel exchanges** (*e.g., project staff and/or partner's staff use each other's facilities, work at each other's site*); and

Nothing to Report

6. **Other.**

Nothing to Report

8. Special Reporting Requirements

COLLABORATIVE AWARDS: Not applicable

QUAD CHARTS: Not applicable

9. Appendices

- A. IRB approval
- B. 8 published papers and 1 manuscript
- C. Yan Liu CV



Memorial Sloan Kettering
Cancer Center™

Institutional Review Board/Privacy Board

TO: Omar Abdel-Wahab, MD
Department of Medicine/ Leukemia

FROM: Eileen M O'Reilly, MD
Chair, Institutional Review Board/Privacy Board-B

DATE: 02/24/2021

RE: IRB Approval: Protocol # 21-092

Your protocol entitled "Development of therapeutic strategy for the treatment of Myelodysplastic syndrome", was reviewed on 02/23/2021 by the Institutional Review Board/Privacy Board-B and was approved by expedited review process as per 45 CFR 46.110 and 21 CFR 56.110 for 12 months.

The IRB/PB has determined that the investigation, presented for approval, is at a low protocol risk level.

The IRB/PB determined that Progress Reports are sufficient for future reviews.

The request to waive the requirement to obtain written informed consent and a research authorization has been granted as per 45 CFR 46.116(c)(d) and 45 CFR 164.512(i)(1)(ii).

The IRB Reviewed the Following:

- Abdel-Wahab_EmailCorr_IoR
- MED20-379_DOD Research Summary Sheet
- MED20-379_Protocol

Genetic sequencing on collected samples:	Yes
Research investigating inherited genetic factors:	No
Tumor and normal samples used for genetic analysis to learn about the causes of cancer:	N/A
Normal sample (germline) used as part of the project:	N/A
Germline subtracted for participants with limited consent:	N/A

Type of Biomaterial	Disease Name	Disease Site	Sample required	Amount required for each case	Number of cases required	Source
Bone marrow/blood	Myelodysplastic Syndrome	BLOOD/BONE MARROW	Frozen	1-5x10 ⁶ cells	20	06-107, 09-141, 12-245, pre-HIPAA

If you have any questions, please contact the IRB Office.

IRB-B Determination of Risk

Protocol: Low

Children: N/A

Device: N/A

LETTER TO THE EDITOR

Genotoxic stresses promote clonal expansion of hematopoietic stem cells expressing mutant p53

Leukemia advance online publication, 16 January 2018;
 doi:10.1038/leu.2017.325

Clonal hematopoiesis increases with age, where a single mutant hematopoietic stem or progenitor cell (HSPC) contributes to a significant, measurable clonal proportion of mature blood lineages.^{1–5} Evolution of mutant clonal hematopoiesis with age predisposes the elderly to myelodysplastic syndromes (MDS), acute myeloid leukemia (AML) and other aging-associated diseases, suggesting that mutations identified in hematopoietic stem cells (HSCs) contribute to disease development.^{1–5} However, the mechanisms by which age-induced stem cell mutations cause clonal expansion of HSCs are largely unknown.

Recently, acquired somatic mutations in *TP53* gene were identified in the blood of aged healthy individuals and the frequency of *TP53* mutations increases with age.^{3–5} *TP53* mutations rank top five among mutations identified in aged healthy individuals.^{3–5} The *TP53* gene, which encodes the tumor-suppressor p53, is the most frequent target for mutation in human cancer, including hematological malignancies.⁶ *TP53* mutations are present in 10% of MDS cases and 20–30% in secondary MDS arising after exposure to radiation or alkylating agents.^{1,7} The frequency of *TP53* mutations in AML is approximately 10%. However, in AML with complex karyotype, the frequency of *TP53* mutations and/or deletions is almost 70%.⁸ Further, *TP53* mutations are associated with poor prognosis and decreased survival in MDS and AML.^{7–9} However, the role of *TP53* mutations in clonal hematopoiesis and the pathogenesis of hematological malignancies is largely unknown.

To date, no studies have investigated the *in vivo* effect of *TP53* mutations expressed from the endogenous locus in hematopoietic cells, which might allow delineation of how these mutations contribute to the pathogenesis of hematological malignancies.^{10–13} The vast majority of missense *TP53* mutations are mapped to the DNA-binding domain of p53 protein and usually abrogate its sequence-specific DNA-binding activity.⁶ Previous studies show that codon 248 of the p53 protein is most frequently mutated in

MDS and AML.^{7,8} p53^{R248W} mutant has also been identified in aged healthy individuals.^{3–5} To better understand the role of mutant p53 in hematopoiesis when expressed physiologically from its endogenous promoter, we utilized the humanized p53^{R248W} knock-in mice, expressing human p53 mutant protein from the endogenous murine *Trp53* promoter.¹⁴ Given that most *TP53* mutations in clonal hematopoiesis and hematological malignancies are monoallelic missense mutations,^{3–9} we examined HSC function in the heterozygous p53^{R248W/+} mice to recapitulate clinical conditions. We first analyzed the peripheral blood (PB) and bone marrow (BM) of p53^{+/+} and p53^{R248W/+} mice (8–12 week old). PB cell counts and BM cellularity were comparable among these mice (Supplementary Figures S1a–f). In p53 knockout mice, there is a dramatic increase of long-term HSCs¹¹; however, we found that p53^{+/+} and p53^{R248W/+} mice have a similar number of long-term HSCs (Figure 1a and Supplementary Figure S1g). In addition, the expression of p53^{R248W} did not affect the frequency of myeloid progenitors (Figures 1a and b, and Supplementary Figure S1g). To determine the role of p53^{R248W} in hematopoiesis *in vivo*, we performed competitive BM transplantation assays as shown in Supplementary Figure S1h. We transplanted 5 × 10⁵ donor BM cells (p53^{+/+} or p53^{R248W/+}, CD45.2⁺) into lethally irradiated (9.5 Gy) recipient mice (CD45.1⁺CD45.2⁺) along with 5 × 10⁵ competitor marrow cells (CD45.1⁺). At 16 weeks after transplantation, the repopulating ability of p53^{R248W/+} cells was significantly higher than that of the wild-type cells (Figure 1c). We also observed increased frequency of donor-derived HSPCs in the BM of recipient mice repopulated with p53^{R248W/+} cells (Figures 1d and e and Supplementary Figure S1i). p53^{R248W} did not affect myeloid and lymphoid differentiation in the PB and the BM of the primary recipient mice (Figures 1f and g). We then transplanted 3 × 10⁶ BM cells isolated from the primary recipient mice into lethally irradiated secondary recipients. Sixteen weeks after transplantation, p53^{R248W/+} cells continued to show increased repopulating ability (Figure 1h). p53^{R248W} did not affect myeloid and lymphoid differentiation in the PB of secondary recipients (Figure 1i). These findings suggest that the expression of mutant p53 in normal

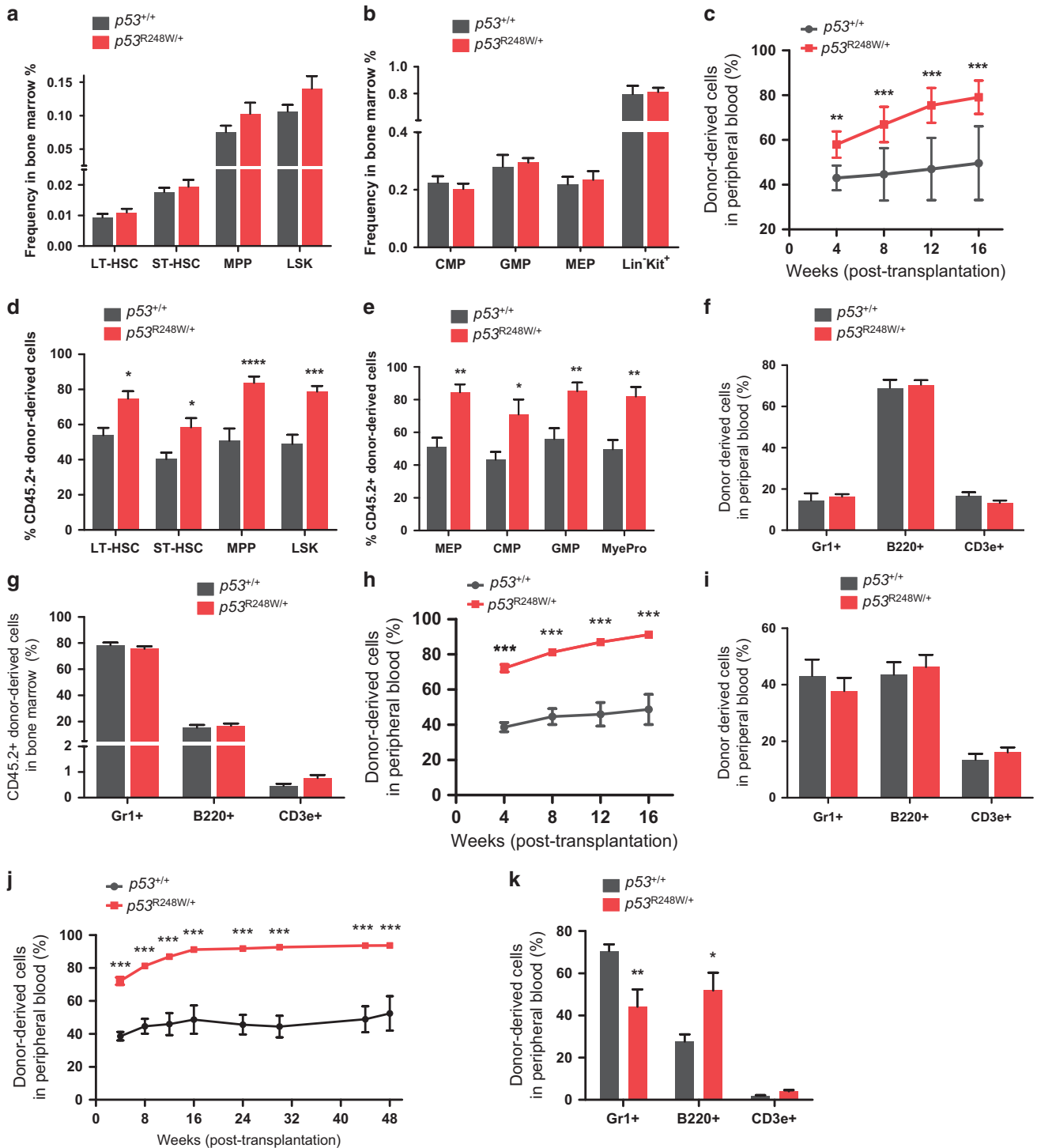
Figure 1. Mutant p53 enhances the repopulating potential of BM cells. **(a)** The frequency of long-term (LT)-HSCs, short-term (ST)-HSCs, multipotent progenitors (MPPs) and lineage[−]Sca-1⁺c-kit⁺ (LSKs) in the BM of young p53^{+/+} and p53^{R248W/+} mice. *n* = 10 mice per genotype. **(b)** The frequency of common myeloid progenitors (CMPs), megakaryocyte/erythroid progenitors (MEPs), granulocyte/macrophage progenitors (GMPs) and Lin[−]Kit⁺ cells in the BM of young p53^{+/+} and p53^{R248W/+} mice. *n* = 10 mice per genotype. **(c)** Percentage of donor-derived (CD45.2⁺) cells in the PB of primary recipient mice posttransplantation, measured at 4-week intervals. ***P* < 0.01, ****P* < 0.001, *n* = 7 mice per group. **(d)** The frequency of donor-derived LT-HSCs, ST-HSCs, MPPs and LSKs in the BM of primary recipient mice 16 weeks following transplantation. **P* < 0.05, ****P* < 0.001, *****P* < 0.0001, *n* = 7 mice per group. **(e)** The frequency of donor-derived MEPs, CMPs, GMPs and myeloid progenitors (Lin[−]Kit⁺) in the BM of primary recipient mice 16 weeks following transplantation. **P* < 0.05, ***P* < 0.01, *n* = 7 mice per group. **(f)** The percentage of donor-derived myeloid cells (Gr1⁺), B cells (B220⁺) and T cells (CD3e⁺) in the PB of primary recipient mice 16 weeks following transplantation. *n* = 7 mice per group. **(g)** The percentage of donor-derived myeloid cells, B cells and T cells in the BM of primary recipient mice 16 weeks following transplantation. *n* = 7 mice per group. **(h)** The percentage of donor-derived cells in the PB of secondary recipient mice. ****P* < 0.001, *n* = 7 mice per group. **(i)** The percentage of donor-derived myeloid cells, B cells and T cells in the PB of secondary recipient mice 16 weeks following transplantation. *n* = 7 mice per group. **(j)** The percentage of donor-derived cells in the PB of secondary recipients 48 weeks after transplantation. ****P* < 0.001, *n* = 7 mice per group. **(k)** The percentage of donor-derived myeloid cells, B cells and T cells in the PB of secondary recipient mice 48 weeks after transplantation. **P* < 0.05, ***P* < 0.01, *n* = 7 mice per group.

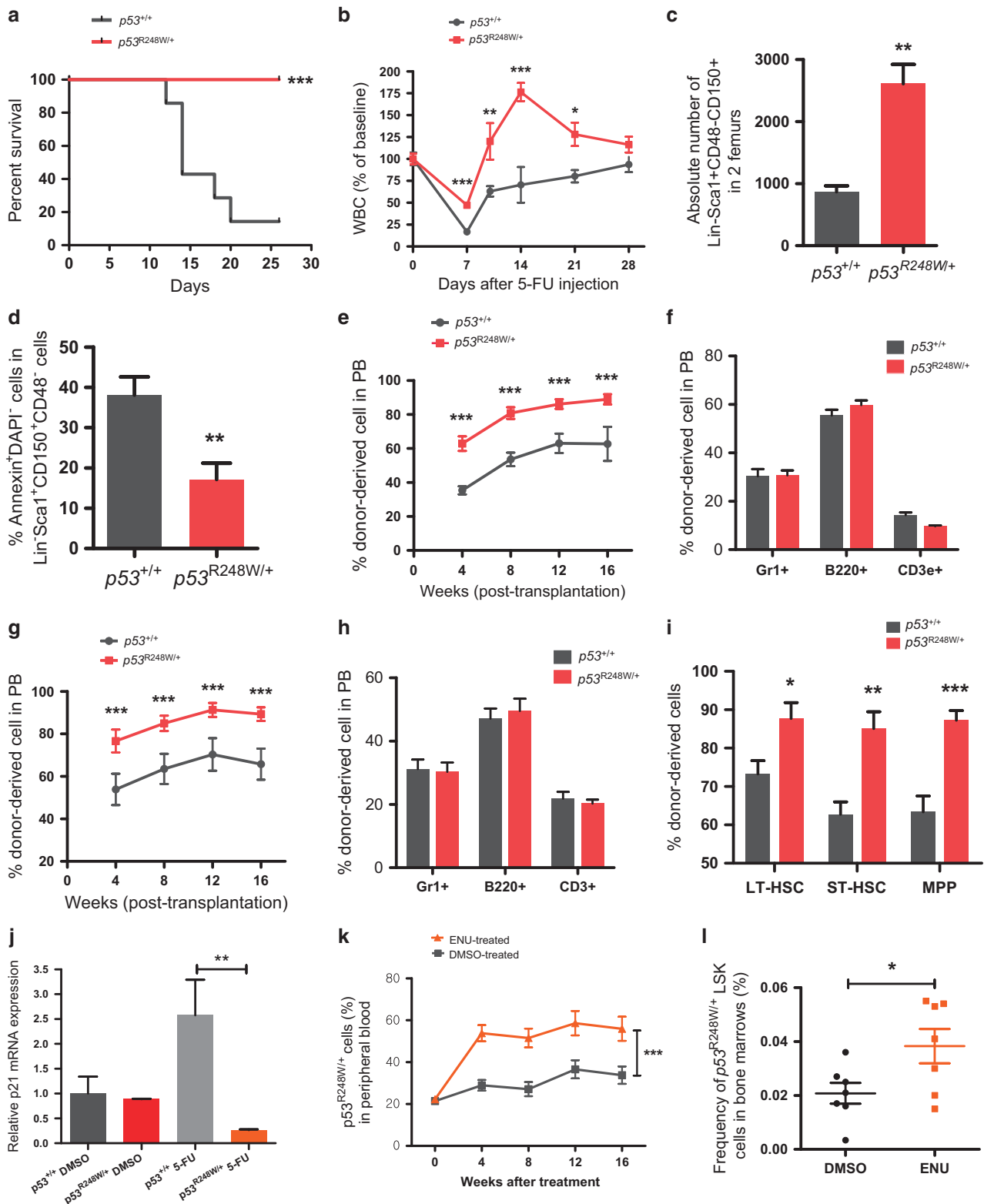
HSCs does not cause leukemic transformation but rather generates a premalignant state.¹⁵

HSCs in aged mice have decreased per cell repopulating activity, self-renewal and homing abilities, myeloid skewing of differentiation and increased apoptosis with stress.^{1,16} To determine the role of mutant p53 in hematopoiesis during aging, we maintained the secondary transplantation recipient mice shown in Figure 1h for >12 months. We observed enhanced repopulating potential of $p53^{R248W/+}$ BM cells compared with $p53^{+/+}$ cells 48 weeks after transplantation (Figure 1j). We found decreased frequency of donor-derived myeloid cells and increased frequency

of lymphoid cells in the PB of the recipients repopulated with $p53^{R248W/+}$ cells (Figure 1k).

Tumor suppressor p53 regulates HSC quiescence and response to genotoxic stress.¹¹ Given that $p53^{-/-}$ mice are resistant to 5-fluorouracil (5-FU) treatment,¹⁰ we hypothesized that $p53^{R248W/+}$ mice may be resistant to chemotherapy treatment. We treated $p53^{+/+}$ and $p53^{R248W/+}$ mice with 5-FU weekly and then monitored their survival. Although most wild-type mice died 4 weeks following 5-FU treatment, all $p53^{R248W/+}$ mice were still alive (Figure 2a). To measure the kinetics of hematopoietic recovery, we administered a single dose of 5-FU (200 mg/kg)





and serially followed PB cell counts. 5-FU-treated $p53^{R248W/+}$ mice had less severe leukopenia than $p53^{+/+}$ mice, with more rapid recovery (Figure 2b, Supplementary Figures S2a–d). We treated $p53^{+/+}$ and $p53^{R248W/+}$ mice with one dose of 5-FU (200 mg/kg) and then examined BM cellularity, HSC frequency and apoptosis 1 week later. We found that $p53^{R248W/+}$ mice exhibited higher BM cellularity compared with $p53^{+/+}$ mice following 5-FU treatment (Supplementary Figure S2e). There were more HSCs in the BM of

5-FU-treated $p53^{R248W/+}$ mice compared with that of the $p53^{+/+}$ mice (Figure 2c, Supplementary Figures S2f and g). Moreover, $p53^{R248W/+}$ HSCs were less apoptotic compared to $p53^{+/+}$ HSCs following 5-FU treatment (Figure 2d and Supplementary Figure S2h).

To determine the impact of 5-FU treatment on $p53^{R248W/+}$ hematopoietic cells *in vivo*, we performed serial competitive transplantation assays using live BM cells isolated from $p53^{+/+}$ and

Figure 2. Chemotherapy treatment promotes clonal expansion of HSCs expressing mutant p53. **(a)** Kaplan–Meier survival curve of $p53^{+/+}$ and $p53^{R248W/+}$ mice following weekly 5-FU treatment. 5-FU was administered intraperitoneally weekly (the initial dose was 125 mg/kg, with subsequent doses of 90 mg/kg) for 3 weeks and the survival rates of 5-FU-treated mice were measured. Results were analyzed with a log-rank nonparametric test and expressed as Kaplan–Meier survival curves ($***P < 0.001$, $n = 10$ mice per group). **(b)** Hematopoietic recovery of $p53^{+/+}$ and $p53^{R248W/+}$ mice following a single dose of 5-FU treatment (200 mg/kg intraperitoneally). White blood cell (WBC) counts are shown at each point after 5-FU administration as a percentage of the initial values for each group of mice. Mean $\pm$ s.e.m. values are shown ($*P < 0.05$, $**P < 0.01$, $***P < 0.001$, $n = 5$ mice per group for each time point). **(c)** Absolute number of HSCs ($\text{Lin}^{-}\text{Sca1}^{+}\text{CD48}^{-}\text{CD150}^{+}$ cells) in the BM of $p53^{+/+}$ and $p53^{R248W/+}$ mice 7 days after a single dose of 5-FU treatment (200 mg/kg intraperitoneally). Mean $\pm$ s.e.m. values are shown ($**P < 0.01$, $n = 5$ mice per group). **(d)** The percentage of early apoptotic (AnnexinV⁺DAPI[−]) HSCs ($\text{Lin}^{-}\text{Sca1}^{+}\text{CD48}^{-}\text{CD150}^{+}$ cells) in the BM of $p53^{+/+}$ and $p53^{R248W/+}$ mice 7 days after a single dose of 5-FU treatment (200 mg/kg intraperitoneally). Mean $\pm$ s.e.m. values are shown ($**P < 0.01$, $n = 5$ mice per group). **(e)** The percentage of donor-derived cells in the PB of primary recipient mice reconstituted with BM cells from 5-FU-treated $p53^{+/+}$ and $p53^{R248W/+}$ mice. Mean $\pm$ s.e.m. values are shown ($***P < 0.001$, $n = 7$ –8 mice per group). **(f)** The percentage of donor-derived myeloid cells (Gr1⁺), B cells (B220⁺) and T cells (CD3e⁺) in the PB of secondary recipient mice 16 weeks after transplantation. Mean $\pm$ s.e.m. values are shown (NS, $P > 0.05$, $n = 7$ –8 mice per group). **(g)** The percentage of donor-derived cells in the PB of secondary recipient mice. Mean $\pm$ s.e.m. values are shown ($***P < 0.001$, $n = 7$ –8 mice per group). **(h)** The percentage of donor-derived myeloid cells, B cells and T cells in the PB of secondary recipient mice 16 weeks after transplantation. Mean $\pm$ s.e.m. values are shown (NS, $P > 0.05$, $n = 7$ –8 mice per group). **(i)** The chimerism of donor-derived long-term HSCs (LT-HSCs), short-term HSCs (ST-HSCs) and multipotent progenitors (MPPs) in the BM of secondary recipient mice at 16 weeks following transplantation. Mean $\pm$ s.e.m. values are shown ($*P < 0.05$, $**P < 0.01$, $***P < 0.001$, $n = 7$ –8 mice per group). **(j)** The mRNA levels of p53 target gene *p21* in hematopoietic stem and progenitor cells isolated from $p53^{+/+}$ and $p53^{R248W/+}$ mice treated with DMSO or 5-FU were determined by quantitative real-time PCR analysis. $**P < 0.01$, $n = 3$. **(k)** Mutant p53 promotes clonal expansion of HSCs following chemotherapy treatment. The percentage of $p53^{R248W/+}$ cells (CD45.2⁺) in the PB of recipient mice following ENU or DMSO treatment was determined by flow cytometric analysis at monthly intervals. Mean $\pm$ s.e.m. values are shown ($***P < 0.001$, $n = 7$ mice per group). **(l)** The frequency of $p53^{R248W/+}$ LSK cells (CD45.2⁺) in the BM of recipient mice at 16 weeks following ENU or DMSO treatment. Mean $\pm$ s.e.m. values are shown ($*P < 0.05$, $n = 7$ mice per group).

$p53^{R248W/+}$ mice treated with one dose of 5-FU (Supplementary Figure S2i). We found that 5-FU-treated $p53^{R248W/+}$ BM cells showed enhanced repopulation potential compared with 5-FU-treated $p53^{+/+}$ cells at 16 weeks following primary transplantation (Figure 2e). Multi-lineage differentiation of HSCs in the PB was comparable in $p53^{+/+}$ and $p53^{R248W/+}$ mice (Figure 2f). We then transplanted 3×10^6 BM cells isolated from primary recipient mice into lethally irradiated secondary recipient mice. $p53^{R248W/+}$ BM cells continued to show enhanced engraftment compared with $p53^{+/+}$ cells at 16 weeks following secondary transplantation (Figure 2g). Mutant p53 did not affect differentiation in the PB and the BM of secondary recipient mice (Figure 2h and Supplementary Figure S2j). The frequency of donor-derived HSPCs was significantly increased in the BM of secondary recipient mice repopulated with mutant BM cells (Figure 2i). To determine the impact of mutant p53 on DNA damage response, we treated wild-type and mutant p53 mice with dimethyl sulfoxide (DMSO) or 5-FU and then examined the expression of p53 target gene *p21* in HSPCs. Although the mRNA levels of *p21* were significantly increased in wild-type cells following 5-FU treatment, the expression of *p21* in mutant cells was not induced by 5-FU treatment (Figure 2j).

To determine whether functional *TP53* mutations promote clonal expansion of HSCs following chemotherapy treatment, we generated mixed BM chimaeras containing both $p53^{+/+}$ and $p53^{R248W/+}$ cells (the ratio of $p53^{+/+}$ to $p53^{R248W/+}$ cells is 10:1). Eight weeks following transplantation, recipient mice were treated with DMSO or chemotherapy drug *N*-ethyl-*N*-nitrosourea (ENU), respectively (Supplementary Figure S3a). Although DMSO treatment did not affect the repopulating ability of $p53^{R248W/+}$ HSCs, $p53^{R248W/+}$ HSCs outcompeted $p53^{+/+}$ cells and become clonal dominance upon ENU treatment (Figure 2k). ENU treatment did not affect the differentiation of $p53^{+/+}$ and $p53^{R248W/+}$ HSCs in the PB of recipient mice (Supplementary Figure S3b). The frequency of mutant LSKs was significantly increased in the BM of recipient mice at 16 weeks following ENU treatment (Figure 2i and Supplementary Figure S3c). We sacrificed the recipient mice at 16 weeks following DMSO or ENU treatment and then performed pathological analysis to detect tumor formation. Although there were no tumors in recipient mice treated with DMSO, four out of the seven recipient mice treated with ENU developed lymphoma or thymoma (data not shown).

Given p53-null HSCs are less sensitive to irradiation,^{11–13} we examined the impact of mutant p53 on HSC function following irradiation. We found that HSCs expressing mutant p53 show decreased apoptosis and increased repopulating potential compared with wild-type HSCs following irradiation. Furthermore, $p53^{R248W/+}$ HSCs show competitive advantage over $p53^{+/+}$ cells and underwent clonal expansion following total body irradiation (SC and YL, unpublished data).

Thus we have identified a critical role for mutant p53 in regulating the response of HSCs to genotoxic stresses. Further, we discovered that chemotherapy and radiotherapy cause the expansion of HSCs expressing mutant p53. Although mutant p53 proteins accumulate in cancer cells, the levels of mutant p53 proteins in normal cells are very low.^{14,17} Genotoxic stresses stabilize mutant p53 in hematopoietic cells.^{14,17–18} Given that the $p53^{R248W}$ mutant has both dominant-negative and gain-of-function roles in human cancer,^{6,14} stabilized mutant p53 may inhibit the wild-type p53 function or gain new oncogenic functions through protein–protein interactions to promote leukemic transformation. In addition, the expansion of mutant HSCs following genotoxic stresses may increase the possibility of acquiring additional genetic and/or epigenetic changes that facilitate leukemic transformation.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGEMENTS

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AUTHOR CONTRIBUTIONS

SC and YL designed the research. SC, RG, CY, MK and SZL performed the research. SC and YL analyzed the data and performed the statistical analysis. MCY, HB, RK, HSB and LDM provided reagents and/or input to the study. SC and YL wrote the manuscript. All authors read, commented on and approved the manuscript.

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Supplementary Information accompanies this paper on the Leukemia website (<http://www.nature.com/leu>)

Genotoxic stresses promote clonal expansion of hematopoietic stem cells expressing mutant p53

Supplemental Methods

Mice

The humanized *p53* knock-in mice (*HUPKI*, $p53^{+/+}$) and $p53^{R248W/+}$ mice have been backcrossed to the *C57BL6* background for at least 8 generations (Song et al., 2007). All $p53^{+/+}$ and $p53^{R248W/+}$ mice used in these studies are 8 to 12 weeks old and are tumor free. Wild type *C57BL/6* ($CD45.2^+$), *B6.SJL* ($CD45.1^+$) and F1 mice ($CD45.2^+ CD45.1^+$) mice were obtained from an on-site core breeding colony. All mice were maintained in the Indiana University Animal Facility according to IACUC-approved protocols.

Flow cytometry

Flow cytometry analysis of hematopoietic stem and progenitor cells was performed as described previously (Liu et al., 2009). Murine hematopoietic stem and progenitor cells were identified and evaluated by flow cytometry using a single cell suspension of bone marrow mononuclear cells (BMMCs). Hematopoietic stem and progenitors are purified based upon the expression of surface markers. Bone marrow (BM) cells were obtained from femurs by flushing cells out of the bone using a syringe and phosphate-buffered saline (PBS) with 2mM EDTA. Red blood cells (RBCs) were lysed by RBC lysis buffer (eBioscience) prior to staining. Experiments were performed on FACS LSR IV cytometers (BD Biosciences) and analyzed by using the FlowJo Version 9.3.3 software (TreeStar).

Transplantation

For the competitive repopulation assays, we injected 5×10^5 BM cells from $p53^{+/+}$ and $p53^{R248W/+}$ mice ($CD45.2^+$) plus 5×10^5 competitor BM cells ($CD45.1^+$) into 9.5Gy lethally irradiated F1 mice ($CD45.1^+CD45.2^+$). Peripheral blood was obtained by tail vein bleeding every 4-week after transplantation, RBC lysed, and the PB mononuclear cells stained with anti- $CD45.2$ FITC and anti-

CD45.1 PE, and analyzed by flow cytometry. 16 weeks following transplantation, bone marrow cells from recipient mice were analyzed to evaluate donor chimerism in bone marrows. For secondary transplantation, 3×10^6 BM cells from mice reconstituted with $p53^{+/+}$ or $p53^{R248W/+}$ BM cells were injected into 9.5Gy lethally irradiated F1 mice (CD45.1⁺CD45.2⁺).

We treated $p53^{+/+}$ and $p53^{R248W/+}$ mice (CD45.2⁺) with a single dose of 5-FU (200 mg/kg intraperitoneally). 7 days later, we injected 5×10^5 live BM cells from 5-FU treated $p53^{+/+}$ and $p53^{R248W/+}$ mice (CD45.2⁺) plus 5×10^5 competitor BM cells (CD45.1⁺) into lethally irradiated (9.5Gy) F1 mice (CD45.1⁺CD45.2⁺). Peripheral blood was obtained by tail vein bleeding every 4-week after transplantation, RBC lysed, and the PB mononuclear cells stained with anti-CD45.2 FITC and anti-CD45.1 PE, and analyzed by flow cytometry. For secondary transplantation, 3×10^6 BM cells from primary recipient mice reconstituted with $p53^{+/+}$ or $p53^{R248W/+}$ BM cells were injected into lethally irradiated (9.5Gy) F1 mice (CD45.1⁺CD45.2⁺).

Apoptosis assays

Bone marrows were stained with cell surface markers as described above. After staining, cells were washed with 0.2% BSA in PBS, and suspended in 1 x Annexin V binding buffer (eBiosciences) and then incubated with FITC-conjugated-AnnexinV (eBiosciences) and 4',6-diamidino-2-phenylindole (DAPI) (Sigma) at room temperature for 30 minutes. Cells were acquired using LSR IV flow cytometer. Data analysis was performed using FlowJo software. Early apoptotic cells were defined as Annexin V⁺ DAPI⁻ cells.

Statistical Analysis

Statistical analysis was performed with GraphPad Prism 6 software (GraphPad software, Inc). All data are presented as mean $\pm$ standard error of the mean (SEM). The sample size for each experiment are included in the figure legends. Statistical analyses were performed using unpaired, two-tailed Student's t

test where applicable for comparison between two groups, and a One-way ANOVA test or Two-way ANOVA was used for experiments involving more than two groups. Statistical significance was defined as * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; ns, not significant.

Supplementary Figure Legends

Figure S1. (a) White blood cell, (b) Neutrophil, (c) lymphocyte, (d) Red blood cell, and (e) Platelet counts in the peripheral blood of $p53^{+/+}$ and $p53^{R248W/+}$ mice (8 to 12 weeks old). $n=5$ mice per group. (f) Bone marrow cell numbers from $p53^{+/+}$ and $p53^{R248W/+}$ mice. $n=10$ mice per group. (g) The frequency of LT-HSCs ($CD48^-CD150^+LSKs$), ST-HSCs ($CD48^-CD150^-LSKs$), MPPs ($CD48^+CD150^-LSKs$), CMPs ($Lin^-Sca1^-Kit^+Fc\gamma RII/III^{low}CD34^{high}$), GMPs ($Lin^-Sca1^-Kit^+Fc\gamma RII/III^{high}CD34^{high}$) and MEPs ($Lin^-Sca1^-Kit^+Fc\gamma RII/III^{low}CD34^{low}$) in the bone marrow of $p53^{+/+}$ and $p53^{R248W/+}$ mice was determined by flow cytometry analysis of cell surface markers. Representative flow cytometry plots of hematopoietic stem and progenitor cells are shown. (h) Experimental schema of competitive bone marrow transplantation assays. (i) The percentage of donor-derived cells ($CD45.2^+$) in the bone marrow of primary recipient mice 16 weeks following transplantation was determined by flow cytometry analysis. $n = 7$ mice per group, * $p<0.05$.

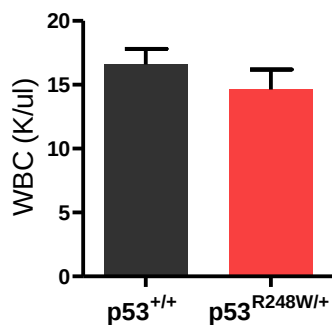
Figure S2. (a) Lymphocyte, (b) Neutrophil, (c) Red blood cell, and (d) Platelet counts in the peripheral blood of $p53^{+/+}$ and $p53^{R248W/+}$ mice following a single dose of 5-FU treatment (200 mg/kg intraperitoneally). Cell counts are shown at each point after 5-FU administration as a percentage of the initial values for each group of mice. Mean $\pm$ SEM values are shown (* $p<0.05$, ** $p<0.01$, *** $p<0.001$, $n = 5$ mice per group for each time point). (e) Bone marrow cell counts of $p53^{+/+}$ and $p53^{R248W/+}$ mice seven days after a single dose of 5-FU treatment (200 mg/kg intraperitoneally). Mean $\pm$ SEM values are shown (*** $p<0.001$, $n = 5$ mice per group). (f) The frequency of HSCs ($Lin^-Sca1^+CD48^-CD150^+$ cells) in the

bone marrow of $p53^{+/+}$ and $p53^{R248W/+}$ mice seven days after a single dose of 5-FU treatment (200 mg/kg intraperitoneally). Mean $\pm$ SEM values are shown (* $p < 0.05$, $n = 5$ mice per group). (g) The frequency of MPPs (Lin⁻Sca1⁺CD48⁺CD150⁻ cells) in the bone marrow of $p53^{+/+}$ and $p53^{R248W/+}$ mice seven days after a single dose of 5-FU treatment (200 mg/kg intraperitoneally). Mean $\pm$ SEM values are shown (* $p < 0.05$, $n = 5$ mice per group). (h) The percentage of early apoptotic (AnnexinV⁺DAPI⁻) MPPs (Lin⁻Sca1⁺CD48⁺CD150⁻ cells) in the bone marrow of $p53^{+/+}$ and $p53^{R248W/+}$ mice seven days after a single dose of 5-FU treatment (200 mg/kg intraperitoneally). Mean $\pm$ SEM values are shown (** $p < 0.01$, $n = 5$ mice per group). (i) Experimental schema of competitive bone marrow transplantation assays using live bone marrow cells isolated from $p53^{+/+}$ and $p53^{R248W/+}$ mice treated with 5-FU. (j) The percentage of donor-derived myeloid cells, B cells, and T cells in the BM of secondary recipient mice 16-weeks after transplantation. Mean $\pm$ SEM values are shown (n.s, $p > 0.05$, $n = 7-8$ mice per group).

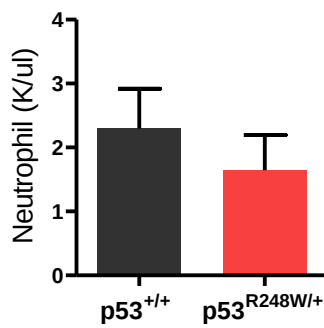
Figure S3. (a) Bone marrow chimaeras were generated by transplanting a 10:1 ratio of $p53^{+/+}$ cells (CD45.1⁺) to $p53^{R248W/+}$ cells (CD45.2⁺) into irradiated recipient mice (CD45.1⁺CD45.2⁺). After hematopoietic reconstitution (8-weeks after transplantation), mice were treated with DMSO or ENU (100mg/kg two doses, 9 days apart). (b) The percentage of $p53^{R248W/+}$ (CD45.2⁺) myeloid cells (Gr1⁺), B cells (B220⁺) and T cells (CD3e⁺) in the peripheral blood of recipient mice 16-weeks following ENU or DMSO treatment. Mean $\pm$ SEM values are shown (n.s, $p > 0.05$, $n = 7$ mice per group). (c) The frequency of $p53^{R248W/+}$ cells (CD45.2⁺) in the bone marrow of recipient mice 16-weeks following ENU or DMSO treatment. Mean $\pm$ SEM values are shown (* $p < 0.05$, $n = 7$ mice per group).

Figure S1

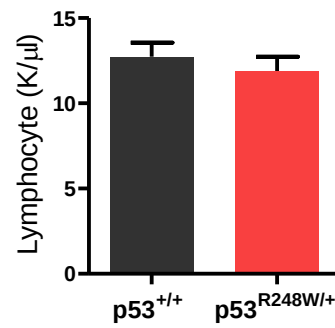
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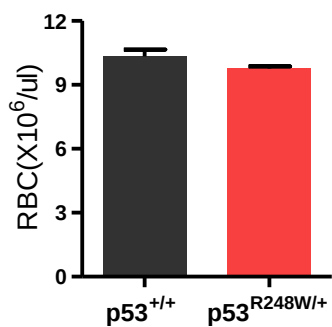
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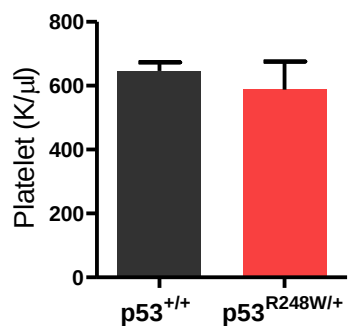
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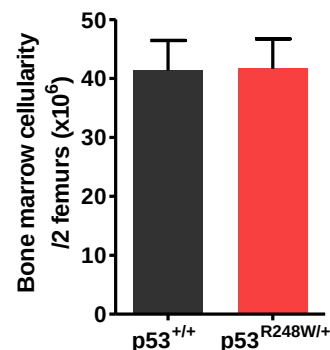
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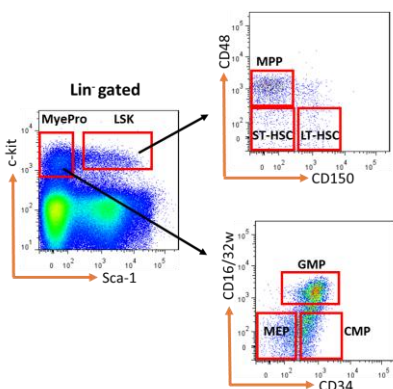
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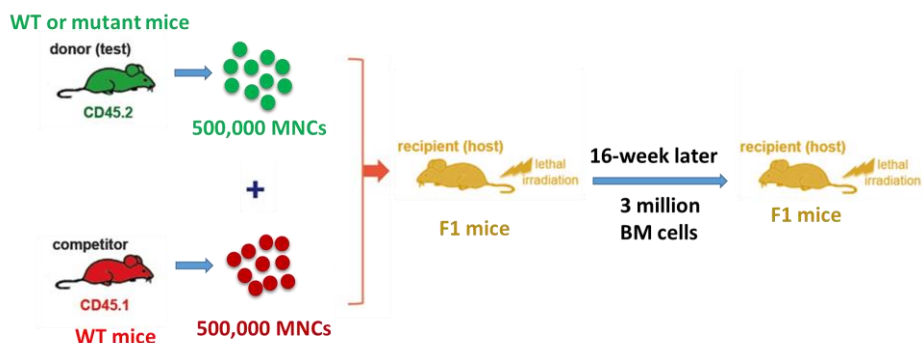
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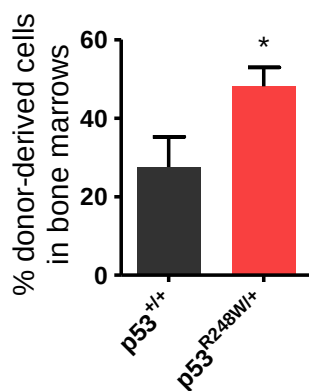
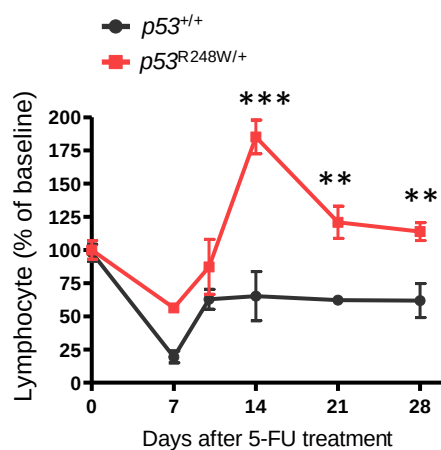
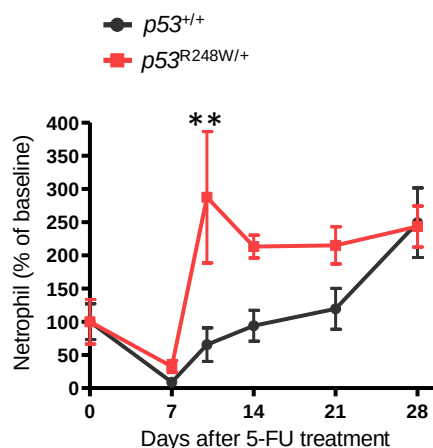


Figure S2

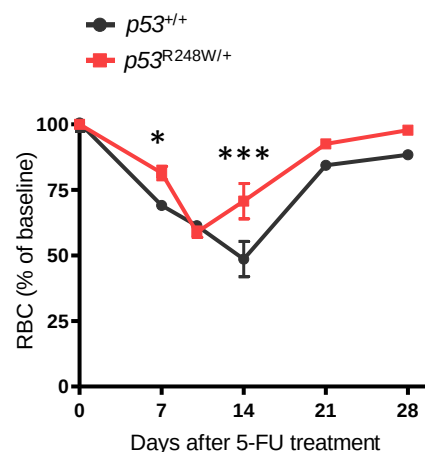
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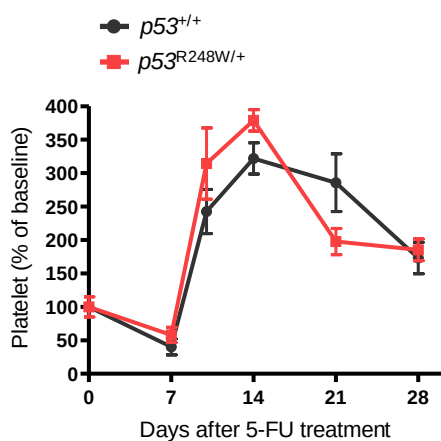
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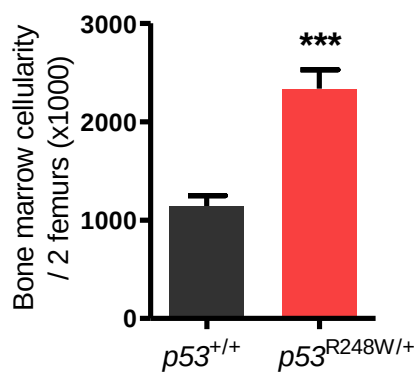
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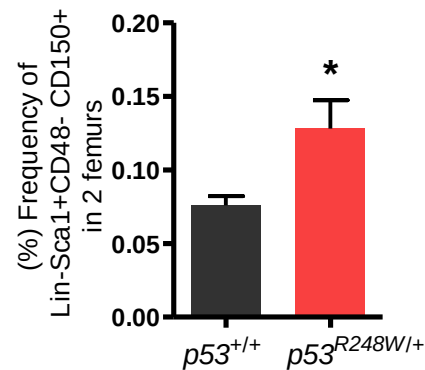
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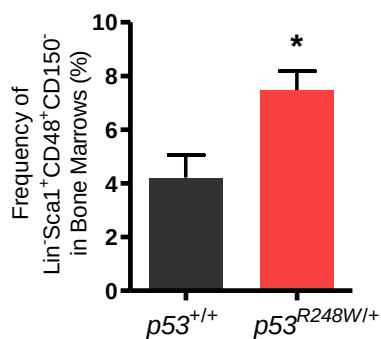
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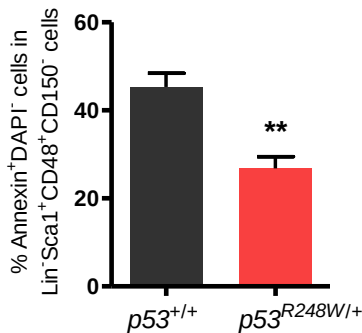
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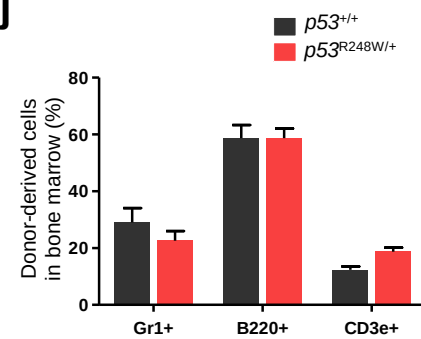
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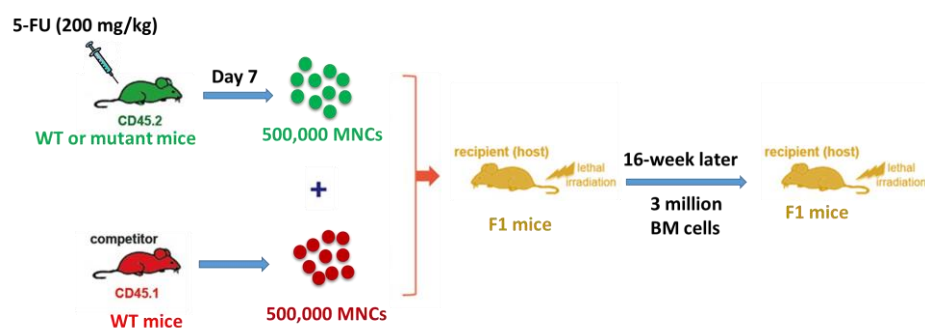
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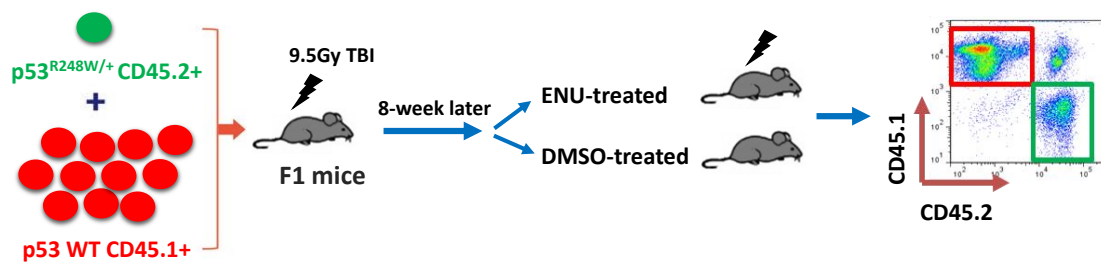
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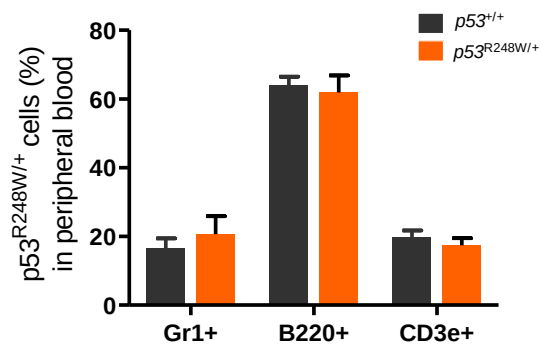
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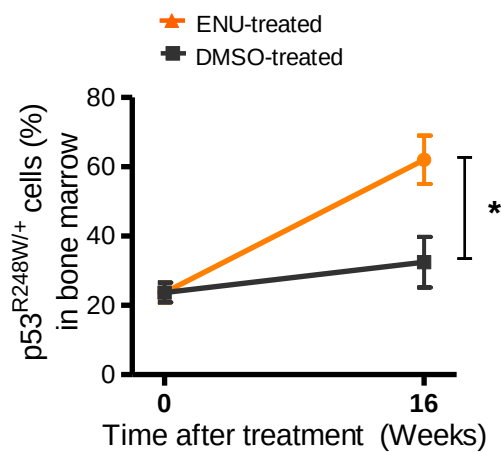
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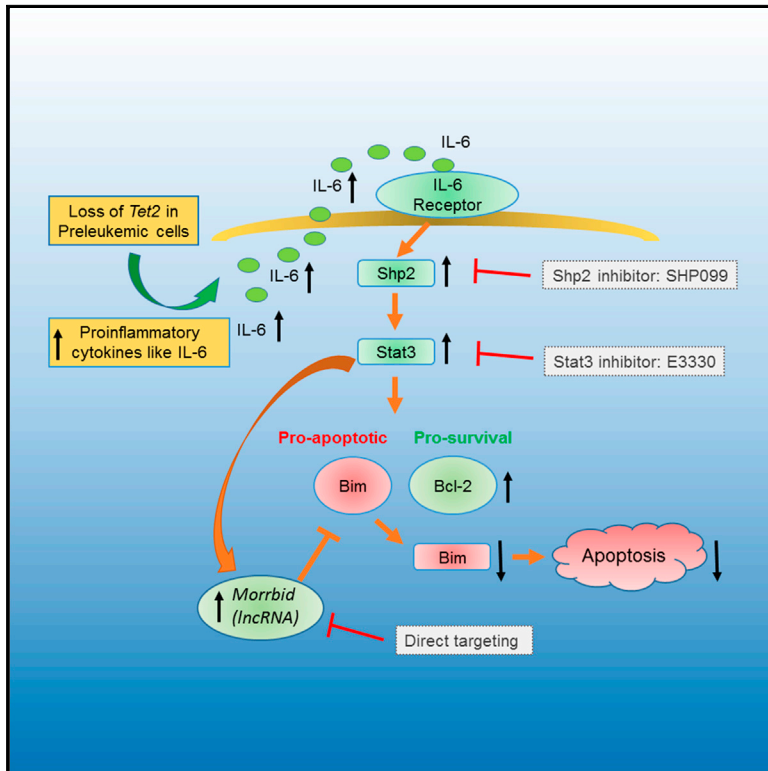


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Inhibition of Inflammatory Signaling in *Tet2* Mutant Preleukemic Cells Mitigates Stress-Induced Abnormalities and Clonal Hematopoiesis

Graphical Abstract



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In Brief

Cai et al. report that *Tet2*-deficient hematopoietic stem and progenitor cells manifest a hyperactive IL-6/Shp2/Stat3/*Morbid* pathway, which promotes cell survival under basal conditions and in response to inflammatory stress. Blocking this pathway using anti-inflammatory drugs (E3330 and SHP099) or by genetic loss of *Morbid* mitigates this response.

Highlights

- *Tet2*-KO HSPCs maintain repopulation advantage after acute inflammatory insult
- Hyperactivation of IL-6/Shp2/Stat3/*Morbid* and reduced apoptosis occurs in *Tet2*-KO HPSCs
- Anti-inflammation drugs E3330 or SHP099 inhibit survival advantage of *Tet2*-KO HSPCs
- *Morbid*^{+/-} counters emergency granulopoiesis and clonal hematopoiesis in *Tet2*^{+/-}



Inhibition of Inflammatory Signaling in *Tet2* Mutant Preleukemic Cells Mitigates Stress-Induced Abnormalities and Clonal Hematopoiesis

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SUMMARY

Inflammation is a risk factor for cancer development. Individuals with preleukemic *TET2* mutations manifest clonal hematopoiesis and are at a higher risk of developing leukemia. How inflammatory signals influence the survival of preleukemic hematopoietic stem and progenitor cells (HSPCs) is unclear. We show a rapid increase in the frequency and absolute number of *Tet2*-KO mature myeloid cells and HSPCs in response to inflammatory stress, which results in enhanced production of inflammatory cytokines, including interleukin-6 (IL-6), and resistance to apoptosis. IL-6 induces hyperactivation of the Shp2-Stat3 signaling axis, resulting in increased expression of a novel anti-apoptotic long non-coding RNA (lncRNAs), *Morrbid*, in *Tet2*-KO myeloid cells and HSPCs. Expression of activated Shp2 in HSPCs phenocopies *Tet2* loss with regard to hyperactivation of Stat3 and *Morrbid*. *In vivo*, pharmacologic inhibition of Shp2 or Stat3 or genetic loss of *Morrbid* in *Tet2* mutant mice rescues inflammatory-stress-induced abnormalities in HSPCs and mature myeloid cells, including clonal hematopoiesis.

INTRODUCTION

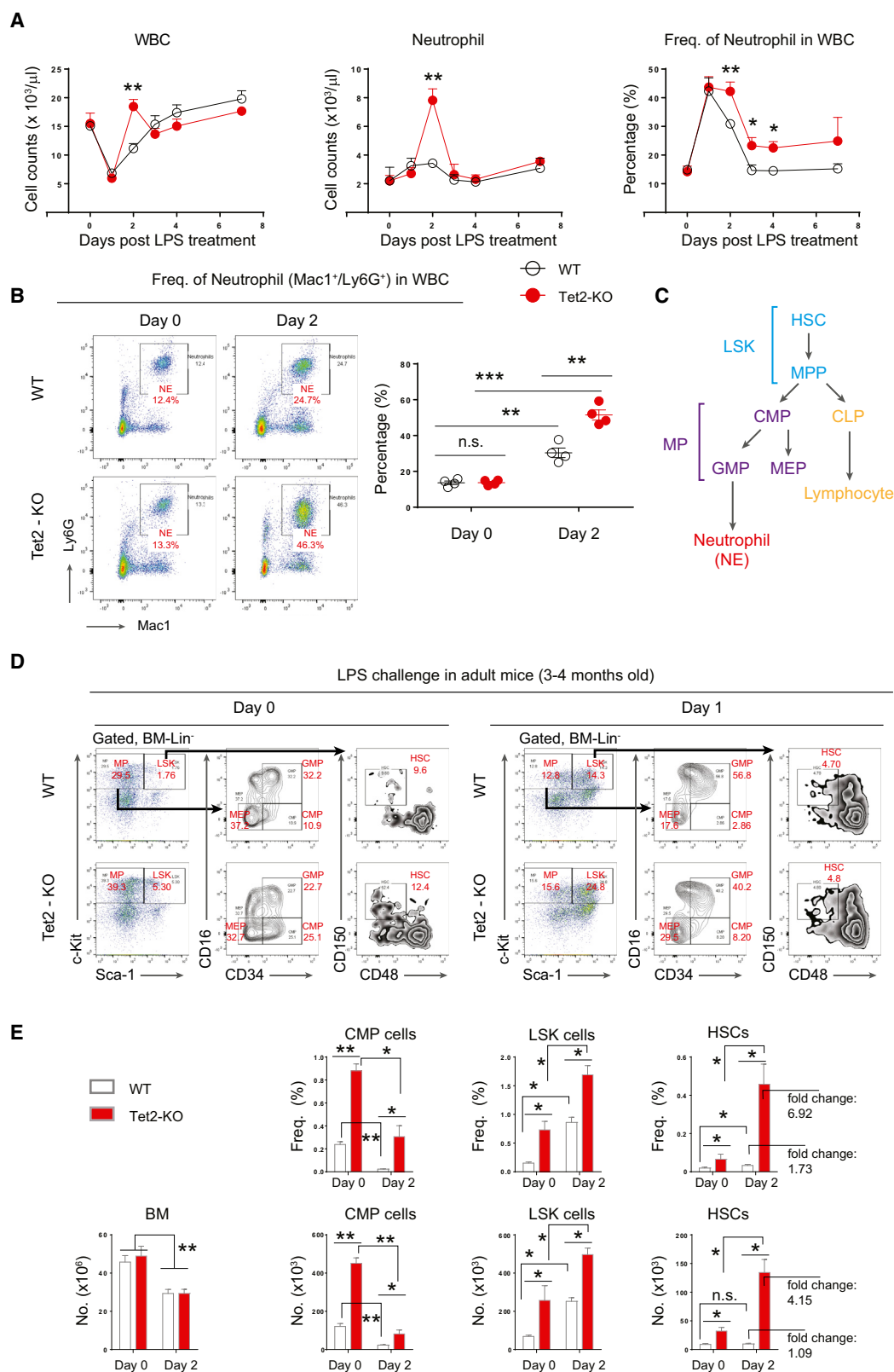
Myeloid malignancies such as acute myeloid leukemia (AML), chronic myelomonocytic leukemia (CMML), myeloproliferative neoplasia (MPN), and myelodysplastic syndromes (MDS) are considered clonal blood disorders. Hematopoietic stem and progenitor cells (HSPCs) with mutations in AML-related genes such as *TET2* or *DNMT3A* represent what are commonly defined

as preleukemic HSPCs (Jan et al., 2012; Shlush and Minden, 2015; Shlush et al., 2014; Sperling et al., 2017). The selection and expansion of preleukemic-HSPC clones precede the development of AML (Abkowitz 2014; Grove and Vassiliou, 2014; Jan et al., 2012). Additionally, preleukemic HSPCs can transform through serial acquisition of additional somatic mutations over time and contribute to the development of full-blown AML. What is unclear is the nature of environmental signals that might contribute to the “switch” from a preleukemic state to a leukemic state in cells bearing these mutations. In this context, inflammation has been hypothesized to play an essential role, but precisely how inflammatory signals influence the growth, survival, differentiation, and overall engraftment potential of preleukemic HSPCs is poorly understood.

Older mice carrying loss-of-function alleles in *Tet2* or *Dnmt3a* manifest an expanded HSPC pool, including a hematopoietic stem cell (HSC)-enriched fraction defined by the cell-surface markers Lineage[−]/Sca-1⁺/c-Kit⁺ (LSK). Some of these genetically modified mice go on to develop CMML or MPN with modest penetration when aged (Challen et al., 2011; Ko et al., 2011; Li et al., 2011; Moran-Crusio et al., 2011; Quivoron et al., 2011). The majority of preleukemic mutations on their own are insufficient to cause AML in mice, suggesting that a single mutation among the above-described mutations defines a preleukemic state and perhaps additional cooperating mutations and/or environmental factors are necessary to provide a more effective selection advantage for preleukemic HSPCs leading to the development of full-blown leukemia.

Inflammation has been linked to tumor induction and transformation in solid tissues and has recently been speculated as an enabling characteristic of cancer and its malignancies (Grivnick et al., 2010; Hanahan and Weinberg, 2011; Mantovani et al., 2008). Inflammation caused by environmental exposure, infection, autoimmunity, or aging may result in mutations and genomic instability in somatic cells as well as in reprogramming of the tumor microenvironment through regulating angiogenesis





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and expression of cytokines and chemokines. Considering that both innate and adaptive immune cells are generated from HSPCs and are involved in regulating local as well as whole-body inflammatory processes, the relationship between inflammation and hematopoietic malignancies is likely to be complex. While the influence of inflammatory stress on normal HSPCs has recently gained significant attention, recent studies have begun to address how preleukemic HSPCs respond or contribute to inflammation (Abegunde et al., 2018; Hasselbalch, 2012; Kobayashi et al., 2016; Meisel et al., 2018; Mirantes et al., 2014; Sano et al., 2018; Takizawa et al., 2012; Zhao and Baltimore, 2015). Because HSPCs in adults reside in the bone marrow and are surrounded by mature immune cells, the inflammatory microenvironment is likely to influence the growth and self-renewal of these cells in part by producing pro-inflammatory cytokines and chemokines. In support of this hypothesis are epidemiologic findings demonstrating that infection may act as a trigger for AML development in humans (Kristinsson et al., 2011).

In the present study, we asked whether and how *Tet2*-KO HSPCs maintain survival advantage during pathological stress by examining how *Tet2*-KO preleukemic HSPCs respond to inflammatory stress.

RESULTS

A Transient but Enhanced Neutrophil Response to Acute Inflammatory Stress in *Tet2*-KO Mice

Lipopolysaccharide (LPS), a ligand that functions by stimulating the Toll-like receptor 4 (TLR4) and nuclear factor κ B (NF- κ B) signaling pathway, is widely used to induce inflammation in mice (Manz and Boettcher, 2014). To assess how *Tet2*-KO preleukemic stem, progenitor, and mature cells respond to acute inflammation, we injected LPS in *Tet2*-KO mice and their wild-type counterparts and followed these mice for 7 days to assess changes in peripheral blood (PB) hematologic parameters (Rodriguez et al., 2009). On day 2 (48 hr) after LPS treatment, a significant increase in white blood cells (WBC) was observed in *Tet2*-KO mice relative to controls (Figure 1A). The increase in WBCs in these mice was associated with an increase in the absolute number and the frequency of neutrophils (NEs), consistent with enhanced “emergency granulopoietic” response (Figures 1A and 1B) (Manz and Boettcher, 2014). The number of monocytes (MO) was comparable between wild-type and *Tet2*-KO mice after LPS treatment, while eosinophil (EO) and basophil (BA) counts were also elevated like neutrophils in *Tet2*-KO mice (Figure S1A; data not shown). In addition, the numbers of lymphocytes (LYs), platelets (PLTs), and red blood cells (RBCs) were comparable between wild-type and *Tet2*-KO mice (Figure S1A). Although no gross changes in

the histology of bone marrow and spleen was seen upon LPS challenge (Figure S2B), flow cytometry analysis revealed a significant increase in the frequency of Mac1⁺ myeloid cells in *Tet2*-KO mice on day 2 after LPS treatment compared to wild-type controls (Figure S1C). Taken together, these data suggest that *Tet2*-KO mice manifest a transient but significant increase in both number and frequency of neutrophils in response to acute inflammatory stress.

Acute Inflammatory Stress Results in Increased Numbers of Myeloid Progenitors and HSCs in the Bone Marrow of *Tet2*-KO Mice

Infection induces acute inflammation and modulates hematopoiesis at the level of both HSCs and hematopoietic progenitor cells (HPCs) to adapt to pathological insult (King and Goodell, 2011; Takizawa et al., 2012). In contrast to steady-state hematopoiesis (naïve; hematopoietic hierarchy illustrated in Figure 1C), infection or stress-induced hematopoiesis in the bone marrow (BM) is recognized as “emergency hematopoiesis” (Trumpp et al., 2010; Wilson et al., 2008). After LPS challenge, we analyzed the LSK and HSC compartments (defined by LSK/CD48⁺/CD150⁺) in wild-type and *Tet2*-KO mice (Kiel et al., 2005). We also analyzed various BM progenitor cell subsets composed of common myeloid progenitors (CMPs; defined by Lin[−]/Sca-1[−]/c-Kit⁺/CD16[−]/CD34⁺), granulocyte-macrophage progenitors (GMPs; defined by Lin[−]/Sca-1[−]/c-Kit⁺/CD16⁺/CD34⁺), and megakaryocyte-erythroid progenitors (MEPs; defined by Lin[−]/Sca-1[−]/c-Kit⁺/CD16[−]/CD34[−]) in these mice (Figure 1D). We observed a similar drop in the overall BM cellularity in both wild-type and *Tet2*-KO mice after LPS challenge (Figure 1E). In contrast, LPS treatment resulted in significant differences in the frequencies and absolute number of BM progenitors in *Tet2*-KO mice relative to controls after LPS challenge. Quantitatively, LPS challenge of *Tet2*-KO mice resulted in increased number and frequencies of CMPs and GMPs, but not MEPs (Figure 1E; data not shown). A significant enrichment in LSK and HSC frequency and numbers was also observed in *Tet2*-KO mice compared to controls after LPS treatment (Figure 1E). Similarly, *Tet2*-KO mice exhibit an increase in multipotent progenitors (MPPs; defined by LSK/CD48⁺/CD150[−]) and short-term HSCs (ST-HSCs; defined by LSK/CD48⁺/CD150⁺) after LPS challenge (Figure S1D). Given that adult (12–16 weeks old) *Tet2*-KO mice also manifest increased basal (day 0, before LPS challenge) levels of CMPs, LSKs, and HSCs compared to controls, we assessed if juvenile (younger) *Tet2*-KO mice, which show a similar frequency and number of CMPs, LSKs, and HSCs as wild-type controls under basal conditions, respond to LPS challenge in a manner similar to older *Tet2*-KO mice. As shown in Figure S1E, juvenile *Tet2*-KO mice also exhibit enhanced response to LPS challenge in all the BM

Figure 1. *Tet2*-KO Mice Exhibit Transient but Amplified Neutrophils and HSPCs in Response to Acute Inflammatory Challenge

(A) Hematologic changes in the peripheral blood (PB) of LPS-treated WT and *Tet2*-KO mice over a 7-day period. A single dose of LPS (intraperitoneally [i.p.], 0.8 mg/kg) was administered to adult WT or *Tet2*-KO mice (~3–4 months old). n = 4–10 mice per group; results are pooled from multiple experiments.

(B) LPS induces aberrant emergency granulopoiesis in *Tet2*-KO mice, revealed by flow cytometry analysis using the neutrophil markers Mac1 and Ly6G. n = 4 mice per group.

(C) A schematic demonstrating hematopoietic hierarchy.

(D) Representative flow cytometry profiles showing gating strategy and changes in various BM HSPC subsets.

(E) Quantification of the frequency (Freq.) and absolute cell number (No.) of CMPs, LSK, and HSCs on days 0 and 2 after LPS treatment in adult WT and *Tet2*-KO mice. n = 4 mice per group.

Results are representative of two independent experiments. *p < 0.05, **p < 0.01, ***p < 0.001; n.s., not significant. Statistical analysis was performed using an unpaired, two-tailed Student's t test. See also Figure S1 for additional supporting data.

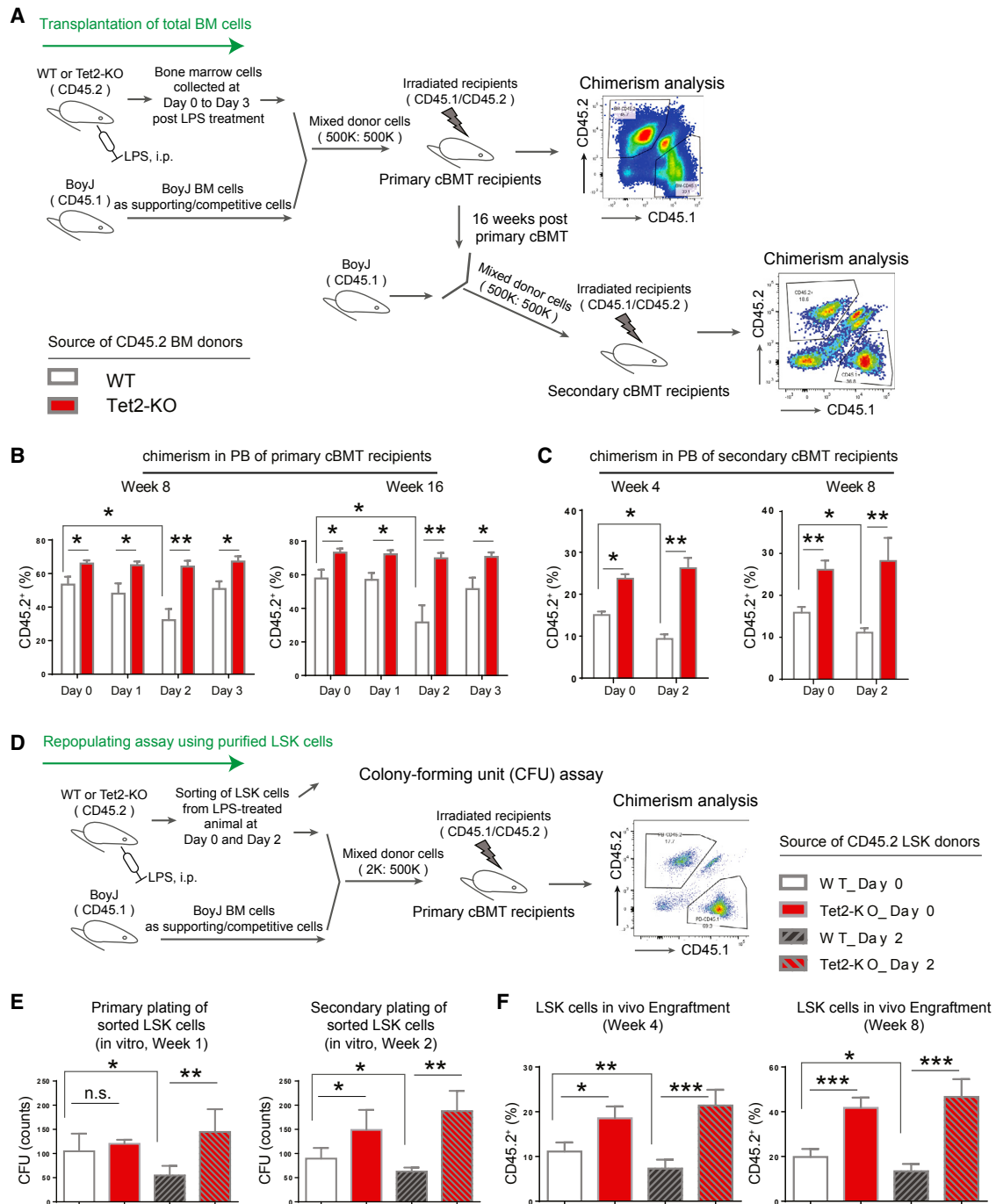
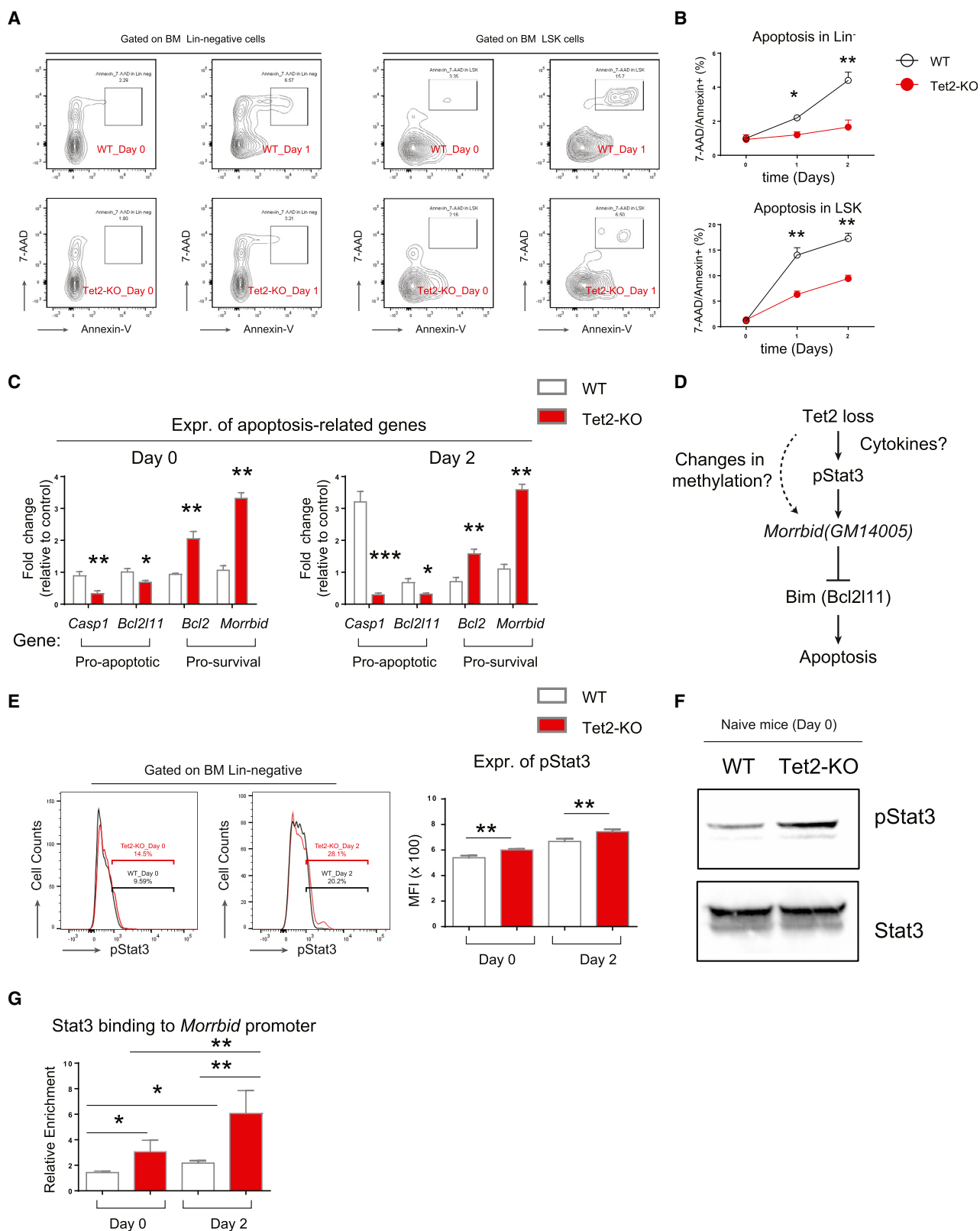


Figure 2. LPS-Stressed *Tet2*-KO BM Cells Maintain a Repopulating Advantage

(A) A schematic describing the primary and secondary competitive BM transplantation (cBMT) assays. The age of the mice for donor cells was ~3–4 months old. (B and C) *Tet2*-KO BM cells with or without LPS treatment manifest significantly higher engraftment in primary (B) and secondary (C) recipients compared to WT controls.

(D–F) An identical number of LSK cells were purified from WT and *Tet2*-KO mice before and after LPS treatment and subjected to an *in vitro* CFU assay (E) and an *in vivo* cBMT assay (F), respectively. Transplant experiments were conducted as described in (D).

Data in (B) are from a representative experiment ($n = 5$ recipients for cBMT analysis; mean $\pm$ SEM). Results are representative of two independent experiments. Data in (C) are from a single experiment ($n = 5$ recipients for cBMT analysis; mean $\pm$ SEM). Data in (E) are from pooled analysis of two mice per group and performed in replicates of 4 (mean $\pm$ SEM; $n = 4$ replicates). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; n.s., not significant. Statistical analysis was performed using an unpaired, two-tailed Student's *t* test. See also Figure S2 for additional supporting data.



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progenitor subsets examined, including CMPs, LSKs, and HSCs, compared to controls. Collectively, these data suggest that deficiency of Tet2 in HSPCs primes them to respond to acute inflammatory stress much more efficiently than WT controls.

Differential Impact of Acute Inflammatory Stress on the Function of Normal versus Tet2-KO HSCs

Recent studies have shown that LPS challenge or bacterial infection in mice not only expands the HSC and/or LSK population in the BM but also potentially depletes HSCs and impairs their self-renewing capability (Esplin et al., 2011; Matatall et al., 2016; Rodriguez et al., 2009). We asked whether LPS-challenged Tet2-KO HSPCs also demonstrate impaired stem cell activity after being exposed to an inflammatory stress. To assess this, we first performed a competitive repopulation assay using whole BM cells before and after LPS challenge. The scheme for conducting competitive BM transplantation (cBMT) is shown in Figure 2A. PB chimerism revealed that the repopulating activity of CD45.2 donor cells from wild-type (WT) mice on day 2 after LPS treatment (measured by percentage of CD45.2_WT_day 2 in primary recipients) was significantly reduced compared to non-LPS-treated WT donor cells (measured by percentage of CD45.2_WT_day 0 in primary recipients) (percentage of CD45.2_WT_day 2 versus percentage of CD45.2_WT_day 0, $^*p < 0.05$; Figure 2B). In contrast, LPS-stressed Tet2-KO CD45.2 donor cells did not show any decline in repopulating ability on any of the post-LPS-treatment time points examined (Figure 2B). Further, at every time point after LPS treatment, Tet2-KO CD45.2 BM donor cells demonstrated a higher repopulating ability than WT CD45.2 donor controls, and the greatest difference was observed on day 2 after LPS treatment (i.e., percentage of CD45.2_WT_Day 2 versus percentage of CD45.2_Tet2-KO_Day 2, $^{**}p < 0.01$; Figures 2B and S2A). Secondary cBMT experiments further confirmed that WT CD45.2 donor cells on day 2 after LPS treatment showed a significant decrease in the repopulating activity while Tet2-KO CD45.2 donor cells were resistant to LPS stress and maintained robust engraftment advantage (Figure 2C). Chimerism analysis of various fractions of BM cells, including Lin-negative (Lin⁻) cells, LSK cells, myeloid cells (labeled by Mac1), B cells (labeled by CD19), and T cells (labeled by CD3), in the BM of primary cBMT recipients also demonstrated that the repopulation of Tet2-KO donor cells was higher than controls (Figure S2A–S2D).

To further compare the repopulating activity of stem cells after LPS-induced inflammatory damage in WT and Tet2-KO mice, identical numbers of LSK cells from pre- and post-LPS-treated

WT and Tet2-KO mice were sorted and subjected to a colony-forming unit (CFU) assay *in vitro* and BM transplantation assay *in vivo* (Figure 2D). Because the frequencies of HSCs in the LSK pool were comparable between WT and Tet2-KO mice (Figure S2E), the analysis using LSK cells allowed us to assess the functional difference between the two groups with regards to HSCs upon LPS challenge. Consistent with our data utilizing whole BM cells (Figure 2B), purified WT LSK cells derived from mice 2 days after LPS treatment showed impaired colony-forming ability *in vitro* and significantly reduced repopulating activity *in vivo* compared to Tet2-KO LSK cells, which were significantly more resistant to inflammatory insult (Figures 2E and 2F). Taken together, our functional data demonstrate that while the repopulating activity of WT HSPCs is significantly impaired in response to LPS-induced inflammatory stress, LPS-treated Tet2-KO HSPCs maintain greater engraftment and are resistant to inflammatory stress.

HSPCs Lacking Tet2 Resist Apoptosis in Response to Acute Inflammatory Stress

Given our results demonstrating increased frequency, increased number, and enhanced engraftment of Tet2-KO HSPCs upon LPS challenge, we sought to determine whether Tet2-KO and WT HSPCs manifest differences in apoptosis and/or proliferation upon encountering inflammatory stress. We analyzed apoptosis in HSPCs by Annexin-V and 7-AAD staining followed by flow cytometry analysis after LPS treatment (Figure 3A). We also determined proliferation by assessing the percentage of Ki67⁺ HSPCs (Figure S3A). Although we observed only subtle changes in proliferating LSK pool (Figure S3A), remarkable and consistent differences in apoptosis were detected in both Lin⁻ and LSK pool between WT and Tet2-KO cells upon LPS challenge (Figures 3A and 3B). Furthermore, the phenotypic observation with regard to reduced apoptosis in Tet2-KO cells after LPS treatment was consistent with changes in the expression of pro-apoptotic genes, including *Casp1* (encoding Caspase-1) and *Bcl2l11* (encoding Bim), which were significantly reduced in Tet2-KO cells (Figure 3C). Furthermore, pro-survival genes, including *Bcl2* and *Morbid*, showed increased expression in Tet2-KO cells in both naive condition and upon LPS challenge (Figure 3C). *Morbid* is a recently identified long non-coding RNA (lncRNA) involved both in regulating the expression of Bim and in the survival of myeloid cells, including neutrophils; however, the role of *Morbid* in the survival of preleukemic HSPCs is not known (Kotzin et al., 2016). We hypothesized that the hyperactivation of *Morbid* in Tet2-KO HSPCs could be mediated via direct

Figure 3. Tet2-KO Hematopoietic Progenitor Cells Show Sustained Cell Survival and Heightened Activation of Morbid in Response to Acute Inflammatory Challenge

(A and B) Level of apoptosis in Lin⁻ cells and LSK cells before and after LPS treatment as assessed by Annexin-V and 7-AAD staining. (A) Representative flow profile of Annexin and 7-AAD staining. (B) Quantification of apoptosis level.
(C) Differential expression of pro-apoptotic and pro-survival genes in Lin⁻ cells before and after LPS treatment.
(D) A scheme for two possible mechanisms by which Tet2 loss induces hyperactivation of *Morbid*. One is through direct regulation of the *Morbid* promoter, and another is through cytokine-modulated phosphorylation of Stat3 (pStat3), which may bind to the *Morbid* promoter and activate its expression.
(E and F) Tet2-KO HSPCs maintain elevated expression of pStat3, as revealed by flow cytometry (E) and western blot (F).
(G) Stat3 binds to the *Morbid* locus, as revealed by a chromatin immunoprecipitation (ChIP) qPCR enrichment assay. Shown is relative enrichment in binding (1% of input as unit 1).

Data in (A) show a representative profile of flow cytometry. Data in (B)–(G) are from a representative experiment ($n = 4$ mice per group, mean $\pm$ SEM). Results are representative of two independent experiments. $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$; n.s., not significant. Statistical analysis was performed using an unpaired, two-tailed Student's *t* test. See also Figure S3 for additional supporting data.

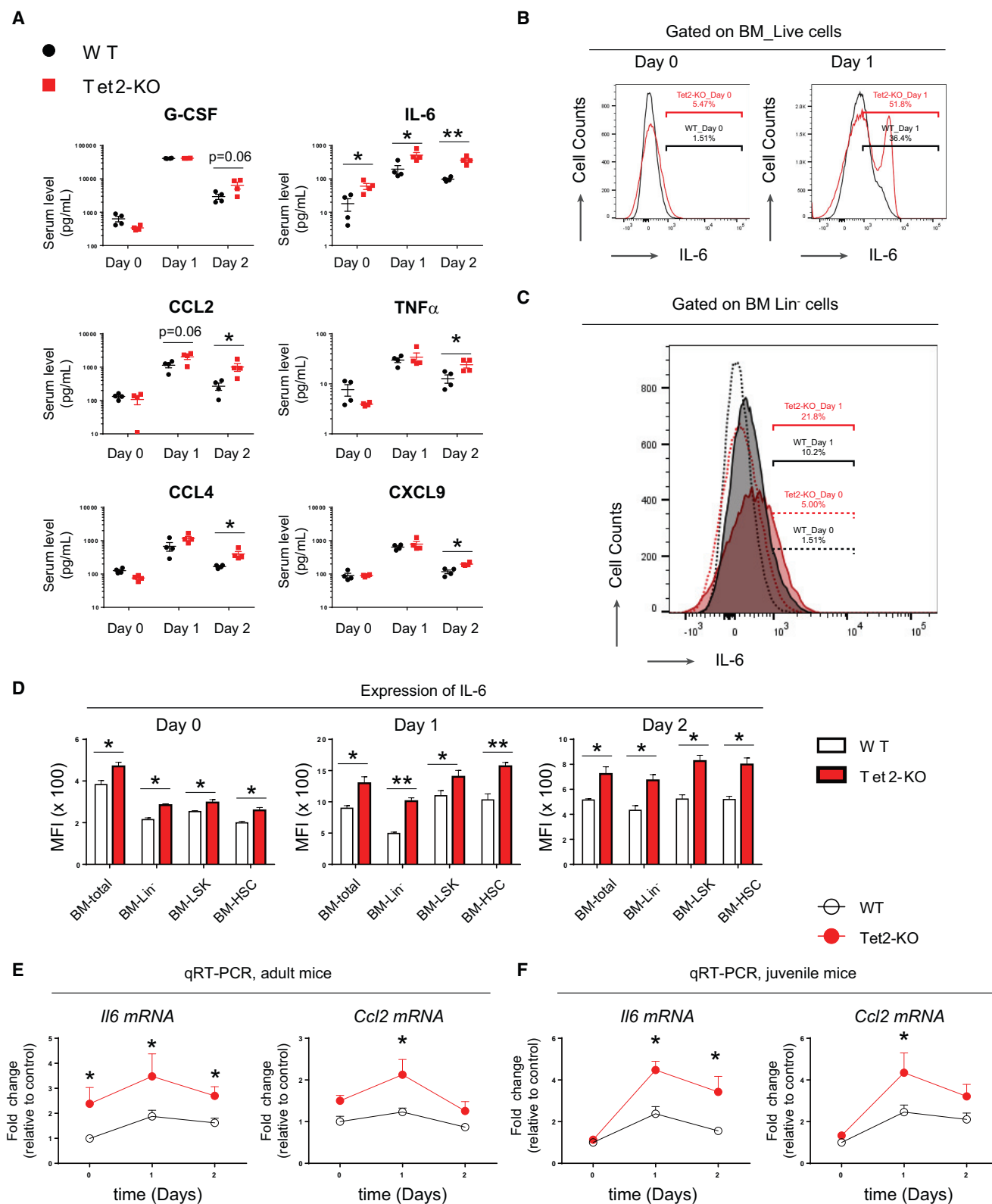


Figure 4. Tet2-KO Mice Show Increased Expression of IL-6 in Serum and in Various BM Subsets

(A) Multiple cytokines and chemokines were increased on day 1 and/or day 2 after LPS treatment in Tet2-KO mice compared to WT controls.
(B and C) Intracellular flow cytometry analysis (ICFC) of IL-6 expression in total BM cells (B) and Lin⁻ BM cells (C) before and after LPS treatment.
(D) Expression of IL-6 in indicated BM subsets as assessed by flow cytometry and mean fluorescence intensity (MFI) quantification.

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regulation of *Morbid* promoter by Tet2 or perhaps via an indirect mechanism involving transcription factors that regulate inflammation (See Figure 3D). It has been reported that knockout of a specific promoter region (~800 bp) of *Morbid* impairs its activity (Kotzin et al., 2016). Detailed methylation analysis on the ~800-bp promoter region of *Morbid* showed no profound difference in either 5-mC or 5-hmC modifications (Figures S3B–S3E). In contrast, we readily detected hyperactive phospho-Stat3 (pStat3) in *Tet2*-KO cells by flow cytometry and western blot analysis (Figures 3E and 3F). Furthermore, we detected increased binding of Stat3 to the *Morbid* promoter (Figure 3G). Taken together, these results suggest that *Tet2* loss results in survival advantage in preleukemic cells, which is associated with increased expression of *Morbid* and activation of Stat3.

Tet2-KO Mice Show Enhanced Expression of Pro-inflammatory Cytokines

Acute inflammatory stress can induce an immediate and transient “cytokine storm” to regulate emergency hematopoiesis and granulopoiesis (Manz and Boettcher, 2014). To determine the upstream regulators of Stat3-*Morbid* survival pathway and see if the observed resistance to inflammatory stress in *Tet2*-KO HSPCs is in part due to dysregulated expression of inflammatory cytokines in *Tet2*-KO mice, we quantified 31 cytokines and chemokines to assess their levels in serum. 15 cytokines or chemokines (G-CSF, interleukin-6 [IL-6], CCL2, CCL4, CXCL1, CCL5, tumor necrosis factor α (TNF- α), CXCL9, CXCL10, IL-10, GM-CSF, IL-1 α , IL-1 β , M-CSF, IL-2) were found to be stimulated in serum by LPS on day 1 and/or day 2 compared to day 0 in WT and *Tet2*-KO mice (Figures 4A and S4A). However, we consistently observed that loss of Tet2 resulted in a profound increase in serum IL-6 levels on day 0, while a further increase was observed on days 1 and 2 after LPS treatment (Figure 4A). CCL2, CCL4, TNF- α , and CXCL9 were also increased in *Tet2*-KO mice on day 2 (Figure 4A). G-CSF, one of the most essential cytokines for granulopoiesis, was also slightly, but not significantly, increased in *Tet2*-KO mice on day 2 after LPS treatment compared to WT ($p = 0.06$, Figure 4A). Recent studies suggest that LPS can directly induce the production of multiple cytokines, including IL-6, IL-1 β , GM-CSF, and TNF- α , in WT HSPCs (Zhao et al., 2014). We therefore examined whether *Tet2*-KO HSPCs also produce these cytokines. IL-6 was expressed more highly by mature BM cells as well as immature BM cells (HSPCs) lacking Tet2 upon LPS treatment compared to WT controls (Figures 4B–4D and S4B). TNF- α was expressed at a higher level on day 1 in Lin[−] BM cells derived from *Tet2*-KO mice (Figure S4C). Expression of IL-1 β and GM-CSF was also enhanced by LPS treatment but was comparable between WT and *Tet2*-KO HSPCs (Figures S4D and S4E). qRT-PCR analysis on Lin[−] BM cells confirmed that *Il6* mRNA was significantly elevated on days 0, 1, and 2 after LPS treatment in adult *Tet2*-KO mice relative to controls (Figure 4E). Likewise, in juvenile mice, where serum IL-6 and expression of *Il6* were com-

parable between WT and *Tet2*-KO cells on day 0, LPS challenge resulted in significantly higher expression of *Il6* in Lin[−] cells derived from *Tet2*-KO mice relative to controls (Figure 4F). Expression of *Ccl2* was also increased in the serum and in Lin[−] cells on day 1 after LPS treatment in *Tet2*-KO mice relative to controls (Figures 4A, 4E, and 4F). Taken together, these data suggest that *Tet2*-KO preleukemic hematopoietic cells produce increased levels of pro-inflammatory cytokines, including IL-6, upon LPS challenge.

A Selective Shp2 Inhibitor (SHP099) or Stat3 Inhibitor (E3330) Blocks IL-6-Induced Hyperactivation of *Morbid* In Vitro

LPS activates canonical TLR4/NF- κ B signaling, which induces the expression of inflammatory cytokines such as IL-6 to induce emergency hematopoiesis (a schematic of the TLR4/NF- κ B/IL-6 and IL-6/Shp2/Stat3 signaling pathways is illustrated in Figure 5A) (Lu et al., 2008). Interestingly, multiple components of the TLR4/NF- κ B/IL-6/Stat3 signaling pathway, including *Tlr4*, *Nfkbiz*, *Il6*, and *Stat3*, were elevated in *Tet2*-KO Lin[−] cells with or without LPS challenge, consistent with a heightened inflammatory response in *Tet2*-KO HSPCs (Figures S5A–S5C). Given the increased expression of IL-6 in *Tet2*-KO HSPCs, we asked how IL-6 might contribute to the enhanced survival of *Tet2*-KO HSPCs. *In vitro* stimulation of Lin[−] *Tet2*-KO cells with IL-6 resulted in reduced apoptosis of these cells compared to controls (Figure 5B). Importantly, the reduced apoptosis of *Tet2*-KO cells in the presence of IL-6 was associated with increased activation of Stat3 and expression of lncRNA, *Morbid* (Figures S5D and 5C). Given the importance of IL-6 in activating Stat3 via Shp2 (Heinrich et al., 2003), we examined the consequence of constitutively active Shp2 expression in Lin[−] cells on *Morbid* activation. Consistent with IL-6 stimulation of *Morbid* *in vitro*, HSPCs with expression of a constitutive Shp2 isoform, Shp2E76K, also demonstrated reduced apoptosis, enhanced activation of Stat3, increased binding of Stat3 to the *Morbid* promoter, increased expression of *Morbid*, and reduced expression of Bim, essentially phenocopying the heightened survival advantage of *Tet2*-KO preleukemic cells (Figures 4D and S5E). Taken together, these gain-of-function studies further indicate the importance of the IL-6/Shp2/Stat3/*Morbid* axis in driving the enhanced survival observed in *Tet2*-KO Lin[−] cells.

To further verify the role of Stat3 and Shp2 in regulating the IL-6/*Morbid* axis in *Tet2*-KO Lin[−] cells, we treated the cells with E3330, a pan inhibitor of both NF- κ B and Stat3 (Cardoso et al., 2012; Jedinak et al., 2011), and SHP099, a novel selective allosteric inhibitor of Shp2 (Chen et al., 2016). E3330 is being advanced in cancer clinical trials as a novel, oral, first-in-class drug in humans to target APE-1 (investigative new drug application #125360). SHP099 (TN0155) is also being tested in phase 1 clinical trial (NCT03114319) for solid tumors with mutations in the receptor tyrosine kinases. We observed a significant inhibition in replating efficiency of *Tet2*-KO Lin[−] cells relative to controls

(E and F) qRT-PCR analysis of *Il6* and *Ccl2* expression in BM Lin[−] cells derived from adult (E) and juvenile (F) pre- and post-LPS-treated WT and *Tet2*-KO mice. Data in (A) are from a single experiment ($n = 4$ mice per group, mean $\pm$ SEM). Data in (B) and (C) are from a representative experiment. Results are representative of two independent experiments. Data in (D)–(F) are from a representative experiment ($n = 4$ mice per group, mean $\pm$ SEM). Results are representative of two independent experiments. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; n.s., not significant. Statistical analysis was performed using an unpaired, two-tailed Student's t test. See also Figure S4 for additional supporting data.

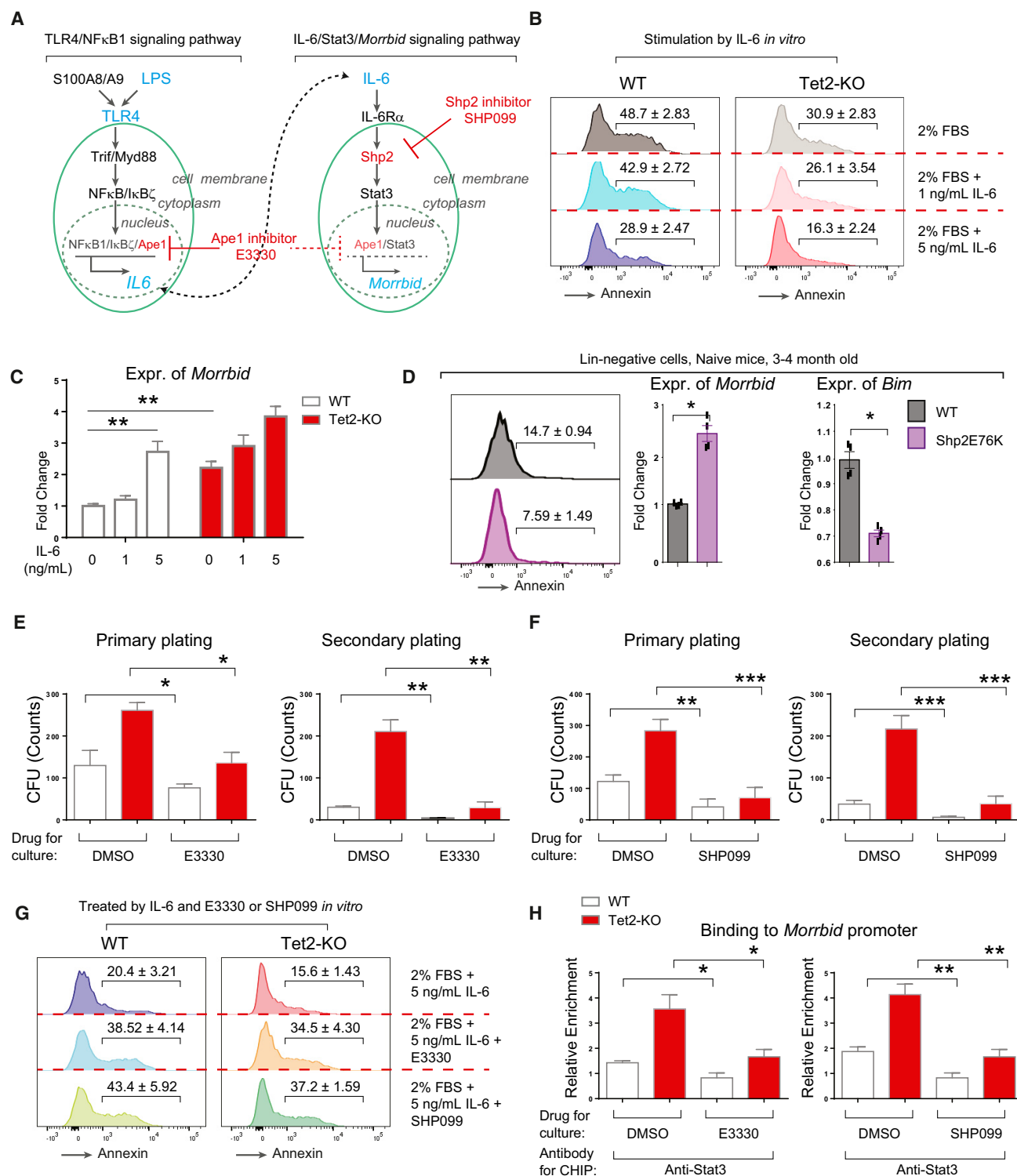


Figure 5. IL-6 Stimulates a Shp2/Stat3/Morbid-Mediated Pro-survival Pathway *In Vitro*, which Is Inhibited by the Pharmacologic Inhibitors SHP099 and E3330

(A) A schematic describing an abbreviated form of the canonical TLR4/NF- κ B/IL-6 and putative IL-6/Stat3/Morbid signaling pathways.

(B) IL-6 promotes the survival of Lin[−] cells in a liquid culture assay.

(C) IL-6 induces the expression of activation of *Morbid* in WT and *Tet2*-KO Lin[−] cells.

(D) Lin[−] cells expressing Shp2E76K, a gain-of-function isoform of Shp2, manifest decreased apoptosis, decreased expression of Bim, and elevated expression of *Morbid* compared to WT controls.

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in the presence of E3330 or SHP099 (Figures 5E and 5F), which was associated with reduced expression of IL-6, reduced activation of Stat3, and enhanced apoptosis (Figures S5F and 5G). Of note, the repressive activity of E3330 and SHP099 exhibited a similar trend in CFU assays in control and *Tet2*-KO cells, though the repression was more profound in *Tet2*-KO cells. Consistent with these observations, we also observed a significant reduction in the binding of Stat3 to the *Morrbid* promoter in the presence of these drugs (Figure 5H). Taken together, these data suggest that IL-6, Shp2, and Stat3 are upstream regulators of *Morrbid* and that E3330 and SHP099 impair the growth of *Tet2*-KO Lin[−] cells in part by modulating the expression of *Morrbid*.

***Tet2*-Loss-Induced Aberrant Hematopoietic Expansion and Emergency Granulopoiesis Is Repressed by E3330 or SHP099**

We next asked if SHP099 or E3330 are also effective in mice that mimic *Tet2*-loss-induced clonal hematopoiesis or early signs of myeloid neoplasia. We first examined the *in vivo* impact of E3330 and SHP099 treatment on LPS-induced emergency hematopoiesis. A significant decrease in LPS-induced neutrophilia in PB, as well as in the frequency of LSK cells, in the BM of *Tet2*-KO mice was observed as early as 2 days after LPS treatment in the presence of E3330 or SHP099 treatment, demonstrating that these two drugs possess potent anti-inflammatory activity not only *in vitro* but also *in vivo* (Figure S6A). Following a similar approach as described before (Fuster et al., 2017), we next developed an experimental model of clonal hematopoiesis (or clonal expansion) by mixing *Tet2*-KO BM cells (from *Tet2* mutant mice [CD45.2]) with WT cells (from BoyJ mice [CD45.1]) at a low ratio (1:5) prior to transplantation into recipient animals (F1 mice [CD45.2/CD45.1]) (Figure 6A). The proportion of *Tet2*-KO cells consistently expanded with time, and the ratio of *Tet2*-KO to WT cells almost doubled every month (ratio value was calculated by assessing the percentage of CD45.2⁺ cells to percentage of CD45.1⁺ cells) (Figure 6B; data not shown). Next, we tested if continuous administration of LPS (mimicking chronic infection), E3330, or SHP099 alters *Tet2*-loss-induced clonal expansion (procedure scheme illustrated in Figure 6A). As shown in Figures 6B and 6C, stimulation of chimeric mice with LPS further increased the ratio of *Tet2*-KO to WT cells in this model; daily treatment with E3330 or SHP099 significantly repressed the hematopoietic cell expansion of *Tet2*-KO cells compared to controls. These results demonstrate that *Tet2*-loss-induced clonal expansion of preleukemic HSPCs is inhibited by the treatment of these mice with E3330 or SHP099.

In addition to examining the role of these two drugs in chimeric mice demonstrating inhibition of clonal expansion, we also assessed the efficacy of E3330 and SHP099 in a cohort of *Tet2*-KO mice. Since *Tet2*-KO mice develop age-dependent CMML-like disease symptoms (Figure S6B), we chose both juvenile

(~1-month-old) and old *Tet2*-KO (~6-month-old) mice for the drug treatment. Although WT control mice were tolerant of the drug treatment and showed no changes in any of the hematologic parameters tested (Figure S6C), a two-month continuous treatment of *Tet2*-KO mice with E3330 or SHP099 reversed multiple early signs of aberrant HSPC dysregulation associated with *Tet2*-deficiency, including enhanced frequency of CMPs, LSKs, HSCs and splenomegaly (Figure 6D). Importantly, and consistent with our *in vitro* observations, expression of IL-6, pStat3, *Morrbid*, and Bim were significantly corrected in *Tet2*-KO cells treated with E3330 or SHP099 (Figure 6D). In a short-term treatment regimen (14 days) in aged *Tet2*-KO mice, E3330 and SHP099 failed to reverse the frequency of LSKs and CMPs. However, both drugs were able to promptly and significantly reduce the neutrophil and WBC burden and also restored the anemia (including RBC and platelet counts) in *Tet2*-KO mice relative to controls (Figures 6E and S6D). In addition, a reduction in spleen weight was observed in SHP099-treated *Tet2*-KO mice, although it did not reach statistical significance ($p = 0.09$; Figure S6D). Importantly, serum IL-6 levels were also reduced upon E3330 or SHP099 treatment in *Tet2*-KO mice (Figure 6E). Taken together, these findings suggest that through targeted inhibition of IL-6/Shp2/Stat3/*Morrbid* signaling axis, treatment with E3330 or SHP099 offers an anti-inflammatory benefit and rescues clonal hematopoiesis as well as some aspects of *Tet2*-deficiency-associated HSPC aberrations, including CMML-like disease.

Genetic Loss of *Morrbid* in *Tet2*^{+/-} HSPCs Rescues Inflammatory-Stress-Induced Defects Associated with Loss of *Tet2*

Given the observed upregulation of *Morrbid* via the enhanced binding of Stat3 to the *Morrbid* gene in *Tet2*-KO Lin[−] cells, we next directly tested the requirement for *Morrbid* in *Tet2*-loss-induced aberrant hematopoietic phenotypes, including clonal hematopoiesis and CMML-like disease. We inhibited the expression of *Morrbid* by knocking down its expression using a lentivirus in *Tet2*-KO cells (Figure S7A) or breeding *Morrbid*^{+/-} mice to *Tet2*^{+/-} mice, given that both heterozygous mutants closely mimic the phenotype observed in complete knockout for these genes (Figure 7A) (Kotzin et al., 2016; Quivoron et al., 2011).

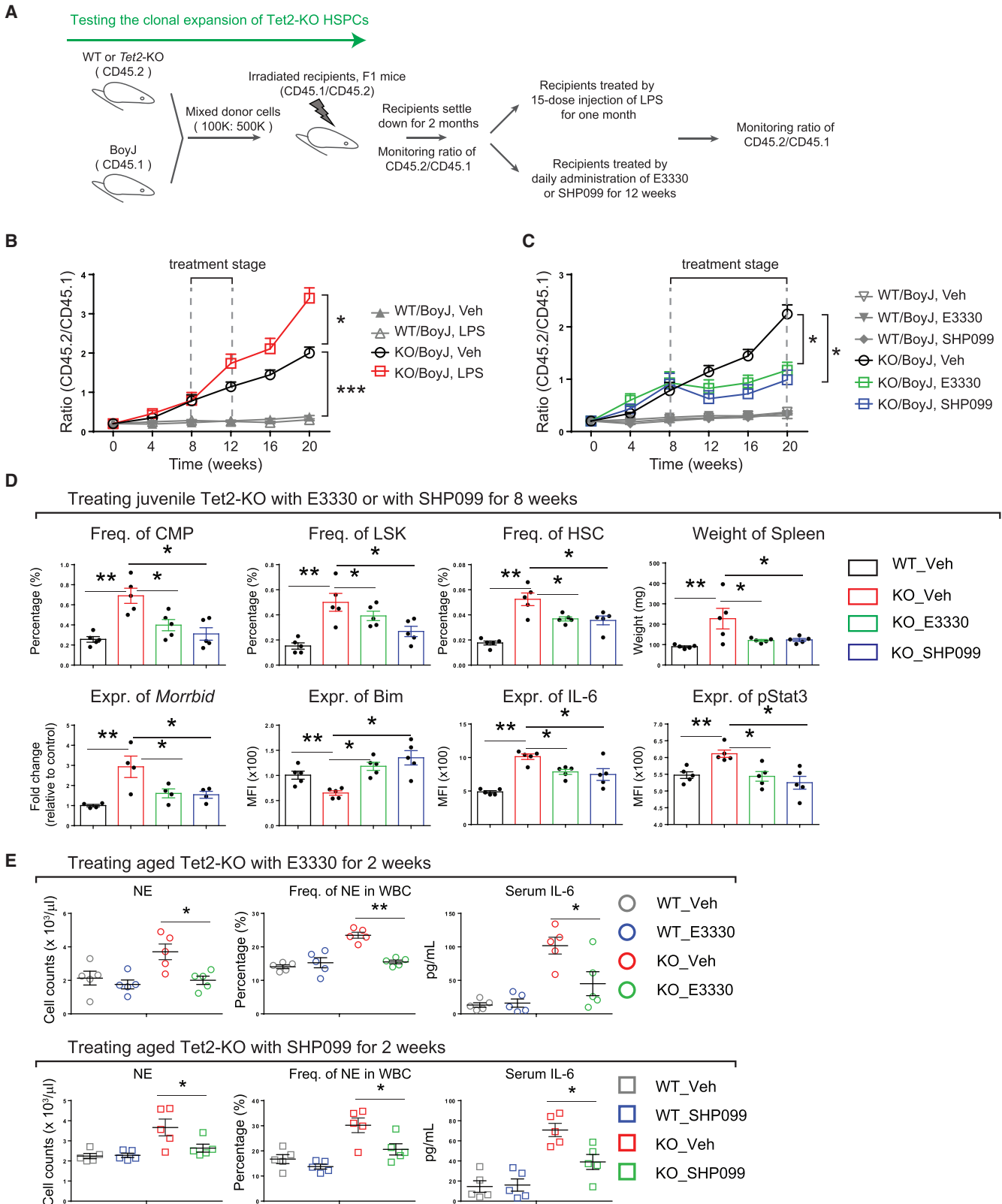
When analyzing the compound mutant mice, loss of *Morrbid* (*Morrbid*^{+/-}) mitigated the enhanced emergency granulopoiesis in *Tet2*^{+/-} mice upon LPS challenge and reversed the splenomegaly normally observed in these mice (Figures 7B and 7C). Similarly, genetic loss of IL-6 or TLR4 (*IL-6*^{-/-} or *TLR4*^{-/-}) also reversed LPS-induced heightened emergency granulopoiesis in *Tet2*-KO mice (Figures S7E and S7F). As shown in Figures S7B and S7C, targeting *Morrbid* by two distinct small hairpin RNAs (shRNAs) decreased the replating activity of *Tet2*-KO cells in a CFU assay *in vitro* and engraftment in a short-term

(E and F) E3330 (E) or SHP099 (F) treatment of *Tet2*-KO Lin[−] BM cells corrects the aberrant colony-forming ability of *Tet2*-KO cells in primary and secondary replating assays. E3330, 0.5 μ M; SHP099, 0.1 μ M.

(G) E3330 or SHP099 treatment of *Tet2*-KO Lin[−] BM cells inhibits IL-6-induced cell survival.

(H) E3330 or SHP099 inhibits Stat3 binding to the *Morrbid* promoter, as revealed by a chromatin immunoprecipitation (ChIP)-qPCR assay. E3330, 0.5 μ M; SHP099, 0.1 μ M.

Data in (B)–(H) are from a representative experiment ($n = 3$ –4 per group, mean $\pm$ SEM). Experiments were repeated twice. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; n.s., not significant. Statistical analysis was performed using an unpaired, two-tailed Student's *t* test. See also Figure S5 for additional supporting data.



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transplantation assay *in vivo*. Consistent with these findings, *Tet2*^{+/-}; *Morrbid*^{+/-} Lin⁻ cells failed to manifest replating advantage seen in *Tet2*^{+/-} Lin⁻ cells in a CFU assay *in vitro* and enhanced the repopulating advantage in a cBMT assay *in vivo* (Figures 7D and 7E). The phenotypic corrections observed in *Tet2*^{+/-}; *Morrbid*^{+/-} mice were due in part to increased expression of Bim, which was associated with enhanced apoptosis in both mature myeloid cells and progenitor cells compared to controls (Figure S7D). Taken together, these data demonstrate that *Morrbid* is an essential mediator of aberrant emergency hematopoiesis and clonal hematopoiesis due to loss of *Tet2* in HSPCs.

DISCUSSION

In this study, we examined the impact of inflammation on the function of preleukemic stem and progenitor cells lacking the enzyme Tet2. *TET2* is frequently mutated in patients with myeloid malignancies, and these mutations are also present in normal individuals who lack signs of AML but demonstrate clonal hematopoiesis (Busque et al., 2012; Genovese et al., 2014; Jaiswal et al., 2014). Some of these individuals develop full-blown hematologic malignancies later on in life. It is, however, unclear what risk factors in these individuals push the progression from clonal hematopoiesis to full-blown leukemia. In solid tumors, infection or inflammation contribute to colon cancer (Bernstein et al., 2001; Newman et al., 2001). Here, we have examined how acute inflammation changes the behavior of HSPCs in a mouse model of preleukemic stem and progenitor cells that shows signs of clonal hematopoiesis. Specifically, we studied if *Tet2*-KO preleukemic stem and progenitor cells themselves produce inflammatory cytokines and how they respond to an inflammatory challenge. Our findings demonstrate that *Tet2*-KO preleukemic stem and progenitor cells show an enhanced response to inflammatory stimuli within the more primitive and mature stem and progenitor cell compartment of the BM. *Tet2*-KO HSPCs show sustained survival in response to inflammation. Serum IL-6 levels and the expression of IL-6 are significantly upregulated in *Tet2*-KO HSPCs with or without LPS stimulation. Functionally, *Tet2*-KO HSCs demonstrate resistance to inflammation-induced damage. Pharmacologically, an anti-inflammatory drug (E3330) or an SHP2 inhibitor (SHP099) normalizes inflammation-induced emergency hematopoiesis, represses aberrant hematopoietic

phenotypes in the BM of young *Tet2*-KO mice, and reverses the imbalance of WBCs and RBCs in aged *Tet2*-KO mice. Mechanistically, hyperactive *Morrbid* in part contributes to the aberrant growth advantage caused by Tet2 loss. Taken together, these findings suggest that *Tet2*-KO preleukemic HSPCs are powered with a selection advantage under conditions of inflammation-induced stress. The growth advantage seen in *Tet2*-KO preleukemic stem cells is likely due to elevated NF- κ B/IL-6/Stat3/*Morrbid* signaling in both mature cells (supplying IL-6) and immature cells (supplying and responding to IL-6), which likely forms a feed-forward loop to promote myeloid malignancies with age (Figure S7G).

TET2 catalyzes the 5-hydroxylation of 5-methylcytosine (5-mc) to 5-hydroxymethylcytosine (5-hmc) and is an essential epigenetic regulator of the genome (Shih et al., 2012). TET2 is a tumor suppressor in myeloid and solid cancer (Ko et al., 2010; Lian et al., 2012). Although studies have shown that *Tet2*-deficient (*Tet2*^{-/-} or *Tet2*^{+/-}) LSK/HSC cells have increased self-renewal activity, the underlying molecular mechanisms that control this process are largely unknown. During the course of our studies, Zhang et al. (2015) utilized *Tet2*-KO dendritic cells and macrophages and showed that upon LPS treatment, *Tet2*-KO mature immune cells produce higher levels of IL-6 and fail to resolve inflammation as efficiently as WT cells, implicating an important role of Tet2 in regulating inflammation. Jaiswal et al. (2017) and Fuster et al. (2017) also reported that Tet2 loss results in aberrant inflammation and clonal hematopoietic expansion, which further promotes myeloid cell-driven diseases such as cardiovascular disease. In the current study, we observed higher levels of IL-6 in the serum and in HSPCs lacking Tet2 upon LPS challenge. Surprisingly, we also found that adult and older naive *Tet2*-KO mice also possess increased expression of IL-6 in serum and in HSPCs. This observation has not been appreciated before and strongly suggests that *Tet2*-KO HSPCs perpetuate a baseline high level of inflammation, which may be one of the mechanisms involved in driving clonal hematopoiesis (Figure S7G).

IL-6 is one of the major pro-inflammatory cytokines circulating in the blood and also functions locally (Hunter and Jones, 2015). IL-6 can be synthesized in large amounts in response to LPS challenge. In addition to playing an essential role in regulating immunity, IL-6 can also regulate hematopoietic cell development and leukemia transformation. Recent studies utilizing a mouse

Figure 6. E3330 and SHP099 Treatment Represses Clonal Expansion of *Tet2*-KO HSPCs and Prevents Aberrant Early Signs of MPN in Both Young and Aged *Tet2* Mutant Mice

(A) A schematic showing the experimental procedure used for generating chimeric mice mimicking clonal expansion of *Tet2*-KO cells. *Tet2*-KO BM cells (CD45.2) were mixed with BoyJ BM cells (CD45.1) at a ratio 1 to 5 (100,000:500,000). Two months post-transplant, the chimeric mice were subjected to continuous treatment with LPS (0.8 mg/kg injected i.p. every other day for 1 month) or a daily injection of E3330 or SHP099 (oral gavage, 20 mg/kg for E3330 and 50 mg/kg for SHP099 for 3 months). Chimerism in PB was analyzed monthly at the indicated time points.

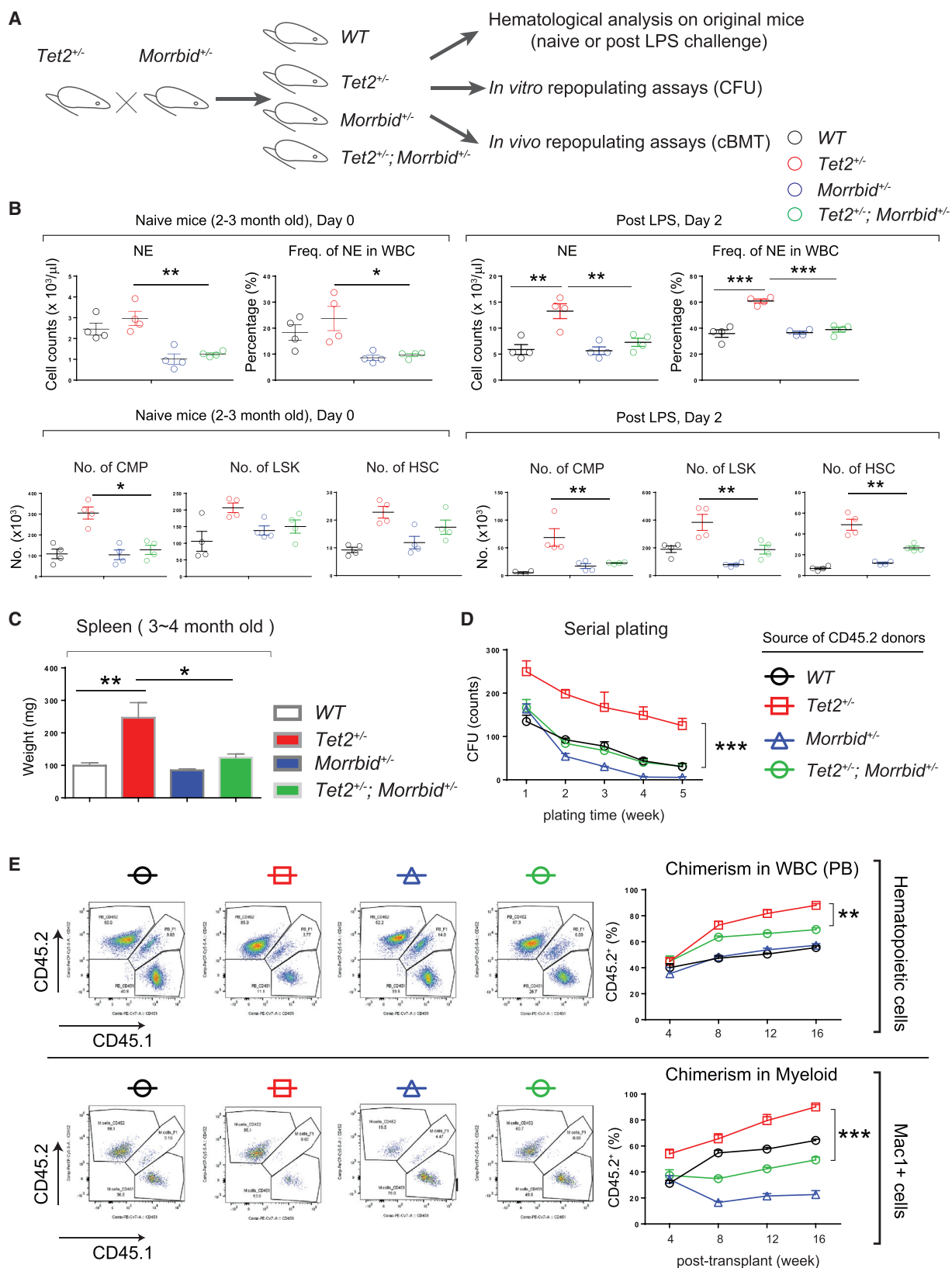
(B) Clonal expansion of *Tet2*-KO HSPCs is heightened by continuous administration of LPS.

(C) Clonal expansion of *Tet2*-KO HSPCs is repressed by daily injection of E3330 or SHP099.

(D) Long-term treatment of juvenile *Tet2*-KO mice with E3330 or SHP099 results in the rescue of multiple phenotypic defects associated with Tet2 deficiency, including frequency of CMPs, LSKs, and HSCs; expression of *Morrbid*, Bim, IL-6, and pStat3; and spleen weight.

(E) Short-term treatment of aged *Tet2*-KO mice with E3330 or SHP099 prevents early hematological signs of CMML in the PB. E3330, 20 mg/kg; SHP099, 50 mg/kg (oral gavage).

Transplant data in (B) and (C) were derived from 5 recipient mice per group (mean $\pm$ SEM). 15 doses of LPS were given at 0.8 mg/kg over a period of 1 month (injected i.p. every other day). E3330 (20 mg/kg) and SHP099 (50 mg/kg) were administered by oral gavage daily for 8 weeks (D) or 2 weeks (E). Drug treatment studies in (D) and (E) are pooled data from two independent experiments ($n = 4$ –5 mice per group; mean $\pm$ SEM). * $p < 0.05$, ** $p < 0.01$; n.s., not significant. Statistical analysis was performed using an unpaired, two-tailed Student's *t* test. See also Figure S6 for additional supporting data.



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model of chronic myeloid leukemia (CML) showed that leukemia in this model is dependent on increased levels of the inflammatory cytokine IL-6 and that blocking IL-6 signaling prevents CML development (Reynaud et al., 2011; Welner et al., 2015). These observations and our present findings support a persuasive hypothesis that increased levels of the pro-inflammatory cytokine IL-6 is an essential trigger of CMML-like disease observed in *Tet2*-KO mice with age (Li et al., 2011; Moran-Crusio et al., 2011; Quivoron et al., 2011). In addition to IL-6, other cytokines such as IFN α , IFN γ , IL-1 α , and IL-1 β can also directly activate HSCs (Baldridge et al., 2010; Essers et al., 2009; Pietras et al., 2014, 2016). As we only tested the levels of intracellular IL-6, IL-1 β , TNF- α , and GM-CSF at defined time points, we cannot rule out the possibility that the presence of additional cytokines may also contribute to the observed phenotype seen in *Tet2*-KO mice. Interestingly, we detected increased expression of TLR4 in *Tet2*-KO HSPCs (Figure S5C). Meisel et al. (2018) have recently reported increased expression of IL6R α in *Tet2*-KO GMPs. These observations may provide further explanation as to why *Tet2*-deficient (*Tet2*^{-/-} or *Tet2*^{+/-}) preleukemic cells are primed and more sensitive to LPS and IL-6 signaling than WT cells.

While *Bim*^{-/-} mice manifest increased myeloid cells in the PB (Bouillet et al., 1999), *Morbid* negatively regulates *Bcl2l12* (encoding Bim) expression, and *Morbid*^{+/-} or *Morbid*^{-/-} mice manifest decreased myeloid cells in the PB (Kotzin et al., 2016). We show that HSPCs with shRNA-mediated knockdown of *Morbid* or genetic loss of *Morbid* in the setting of *Tet2* deficiency failed to undergo aberrant hematopoietic clonal expansion and that *Tet2*^{+/-}; *Morbid*^{+/-} HSPCs failed to manifest amplified emergency granulopoiesis upon LPS challenge. Likewise, when a cohort of young *Tet2*-KO mice were treated with E3330 or SHP099, recovery of Bim and repression of IL-6 were observed, suggesting that these two drugs largely function via the NF- κ B/IL-6/Stat3/*Morbid* pathway for inducing the disease phenotype associated with *Tet2* loss.

It is generally accepted that cancer, including solid tumors and leukemia, undergoes an evolutionary process relying on adaptive advantages of acquired somatic mutations (intrinsic factors) with fitness for niche selection (extrinsic factors), with bioecological principles similar to those indicated in Darwinian natural selection (Greaves and Maley, 2012). A recent study showed that once lymphocyte progenitors are primed with constitutive expression of oncogenes such as *BCR-ABL*, *NRAS*^{V12}, or *Myc*, progenitor cells have a selection advantage in age-induced inflammatory niche and transform into leukemia (Henry et al., 2015). By conducting primary and secondary cBMT assays, we show that *Tet2*-KO HSPCs always outperform

WT control cells. Essentially, when WT donor cells are isolated from their endogenous microenvironment on day 2 after LPS treatment, they lose their normal repopulating activity in recipient mice. In contrast, and in agreement with the finding by Ko et al. (2011), even though the primary cBMT only received half the number of *Tet2*-KO BM donor cells, the recipient animals still developed early signs of MPN or CMML, strongly indicating that *Tet2* deficiency induces a growth and repopulating advantage in HSCs.

Based on the results of our study and those of others, we have created a schematic that summarizes our findings (Figure S7G). Our data suggest that loss of *Tet2* results in multiple changes in the levels of key molecules, including IL-6 and *Morbid*, which may render the self-renewal, differentiation, survival, and clonal evolution of mutant HSCs to include myeloid skewing and development of MPN or CMML-like disease with age. Given the hyperactivation of the IL-6/Shp2/Stat3/*Morbid* pathway in *Tet2*-KO cells, we employed small-molecule inhibitors (E3330 and SHP099) to determine their impact on emergency hematopoiesis. Our results show that both E3330 and SHP099 can effectively repress LPS-induced emergency granulopoiesis and LSK expansion. More importantly, our results show that E3330 and SHP099 treatment prevents aberrant hematopoietic phenotypes in the BM of young *Tet2*-KO mice and reverses neutrophil counts and serum IL-6 levels in aged naive *Tet2*-KO mice, suggesting that these drugs, through their specific inhibition of Ape1-NF- κ B activation or Shp2-Stat3 activation, might provide an anti-inflammatory benefit to AML patients bearing *TET2* mutations and normal healthy individuals bearing *TET2* mutations and clonal hematopoiesis. Given that emerging evidence suggests that inflammation very likely plays a causative role in the pathology of MPN (Fleischman, 2015; Koschmieder et al., 2016), testing these drugs in other preleukemic models may be of clinical benefit.

STAR★METHODS

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

- KEY RESOURCES TABLE
- CONTACT FOR REAGENT AND RESOURCE SHARING
- EXPERIMENTAL MODEL
 - Mice
- METHOD DETAILS
 - *In vivo* treatments
 - Hematological cell counts
 - Flow cytometry

Figure 7. Genetic Loss of *Morbid* Represses the Growth Advantage in *Tet2*-Deficient Preleukemic Cells

(A) A schematic showing the experimental procedures for testing genetic requirement of *Morbid* in *Tet2*-deficiency-mediated aberrant hematopoietic phenotypes in naive mice or LPS-challenged mice and in *in vitro* repopulation assays and *in vivo* cBMT assays.

(B) *Tet2*^{+/-}; *Morbid*^{+/-} mice fail to manifest amplified emergency hematopoiesis seen in *Tet2*^{+/-} mice upon LPS treatment.

(C) Rescue of splenomegaly in *Tet2*^{+/-}; *Morbid*^{+/-} mice relative to *Tet2*^{+/-} mice.

(D) *Tet2*^{+/-} HSPCs with *Morbid* haploinsufficiency correct the enhanced self-renewing ability of *Tet2*^{+/-} HSPCs in an *in vitro* serial replating assay.

(E) *Tet2*^{+/-} HSPCs with *Morbid* haploinsufficiency rescue the enhanced clonal expansion seen with *Tet2*^{+/-} HSPCs, as assessed in a chimeric cBMT assay.

Data in (B), (C), and (E) are from a representative experiment (n = 4–5 mice per group; mean $\pm$ SEM). Data in (D) are pooled from two mice performed in replicates of 4. Data in (E) are from 4 to 5 recipient mice for every group at every time point (4–16 weeks) (n = 4 to 5, mean $\pm$ SEM). Similar results were seen in another independent experiment (n = 4–5 mice per group; analyzed at 8 weeks). *p < 0.05, **p < 0.01, ***p < 0.001; n.s., not significant. Statistical analysis was performed using an unpaired, two-tailed Student's t test. See also Figure S7 for additional supporting data.

- Multiplex cytokine assays
- Isolation of Lin-negative BM cells, LSK cells and qRT-PCR assays
- CFU assay
- *In vitro* suspension culture with IL-6 and drugs
- CHIP-qPCR assay
- Analysis of 5mC and 5hmC level on the *Morbid* promoter
- Competitive bone marrow transplantation (cBMT)
- shRNA generation and approach for knocking down the expression of *Morbid*

● QUANTIFICATION AND STATISTICAL ANALYSIS

SUPPLEMENTAL INFORMATION

Supplemental Information includes seven figures and one table and can be found with this article online at <https://doi.org/10.1016/j.stem.2018.10.013>.

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AUTHOR CONTRIBUTIONS

R.K. conceived the project. Z.C. and R.K. designed the experiments, analyzed data, and wrote the manuscript. Z.C. performed most of the experiments and acquired the data. G.S. analyzed the histology and pathology of bone marrow and spleen. A.W., J.H.-M., J.J.K., M.M., M.R.K., and Y.L. provided reagents, shared unpublished data, and contributed to the drafting and revision of the manuscript. B.R., S.C., S.N., R.P., L.R.P., and R.S.M. assisted with certain experiments and acquired part of the data.

DECLARATION OF INTERESTS

M.R.K. has licensed E3330 (APX3330) through Indiana University Research and Technology Corporation to Apexian Pharmaceuticals. Apexian Pharmaceuticals had neither control nor oversight of the studies or the interpretation or presentation of the data in this manuscript. M.M. is an employee of Novartis Institutes of Biomedical Research. The remaining authors declare no competing interests.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Experimental Models: Organisms/Strains		
Mouse: <i>Tet2</i> ^{-/-} (<i>Tet2</i> -KO), <i>Tet2</i> ^{+/-}	Li et al., 2011	N/A
Mouse: <i>Morbid</i> ^{+/-}	Kotzin et al., 2016	N/A
Mouse: Shp2E76K	Xu et al., 2011	N/A
Mouse: Tlr4-KO	Jackson Laboratory	Cat #007727
Mouse: IL6-KO	Jackson Laboratory	Cat #002650
Mouse: C57/B6	Jackson Laboratory	Cat #000664
Mouse: BoyJ	Jackson Laboratory	Cat #002014
Critical Commercial Assays		
Multiplex cytokine assays	Eve Technologies	#MD31
CFU assays	StemCell	#M3434
EasySep Mouse Hematopoietic Progenitor Cell Isolation Kit	StemCell	#19856
Rneasy Mini Kit	QIAGEN	#74104
SuperScript II Reverse Transcriptase	Fisher Scientific	#18064014
SYBR Green master mix	Life Technologies	#4385612
MAGnify Chromatin Immunoprecipitation System	Fisher Scientific	#492024
Genomic DNA purification Kit	Fisher Scientific	#K0512
EpiTect Bisulfite Kit	QIAGEN	#59104
Quest 5-hmC Detection Kit	Zymo	#5415
MspI	NEB	R0106S
HpaI	NEB	R0105S
Chemicals, Peptides, and Recombinant Proteins		
LPS	Sigma	#L8643
Cremophor	Sigma	#C5135
Methylcellulose	Sigma	#M0262
Tween-80	Fisher Scientific	#BP338-500
E3330	Kelley Laboratory	N/A
SHP099	Mohseni Laboratory (Novartis)	N/A
Lysis Buffer	BD	#555899
Cytofix/Cytoperm Kit	BD	#554714
mIL-3 peptide	PeproTech	#213-13
mIL-6 peptide	PeproTech	#216-16
mSCF peptide	PeproTech	#250-03
Antibodies for Western Blot or CHIP assays		
phospho-Stat3	Cell Signaling Tech	#9145
Stat3	Cell Signaling Tech	#9139
Antibodies for Flow Cytometry		
TER-119, PE	BioLegend	#116208
Gr1, PE	BioLegend	#108408
Mac1, PE	BioLegend	#101208
B220, PE	BioLegend	#103208
CD3, PE	BioLegend	#100206
CD4, PE	BioLegend	#116006

(Continued on next page)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
CD8a, PE	BioLegend	#100708
c-Kit, APC	BioLegend	#105812
Sca-1, APC/Cy7	BioLegend	#108126
CD150, PE/Cy5	BioLegend	#115912
CD48, PE/Cy7	BioLegend	#103424
CD127, PE/Cy5	BioLegend	#135016
CD16/32, PE/Cy7	BioLegend	#101318
CD34, FITC	eBioscience	#11-0341-85
Annexin V, FITC	BioLegend	#640906
7-AAD	BioLegend	#79993
TLR4, PE/Cy7	BioLegend	#145408
IL-6, AF488	BD	#561363
TNF α , AF488	BioLegend	#506315
IL-1b, FITC	eBioscience	#11-7114-80
GM-CSF, FITC	BioLegend	#505403
Ki67, FITC	BioLegend	#652410
NFkB1 (p50), PE	Cell Signaling Tech	#24961
phospho-Stat3, AF488	Cell Signaling Tech	#4323
Bim mAb (C34C5), AF488	Cell Signaling Tech	#94805S
CD45.2, PerCP/Cy5.5	BioLegend	#109928
CD45.1, PE/Cy7	BioLegend	#110730
Ly-6G, FITC	BioLegend	#127606
Oligonucleotides		
Part-I: Key oligonucleotides for qRT-PCR, See also Table S1 for information of additional oligonucleotides for qRT-PCR		N/A
Morrbid, Forward TCTGAGAATGAGGGGACTGG	Kotzin et al., 2016	N/A
Morrbid, Reverse TGTGCTGTGAAGATCCCAAG	Kotzin et al., 2016	N/A
Il6, Forward AGTTGCCTTCTTGGGACTGA	https://mouseprimerdepot.nci.nih.gov/	N/A
Il6, Reverse TCCACGATTTCCAGAGAAC	https://mouseprimerdepot.nci.nih.gov/	N/A
Part-II: Oligonucleotides for CHIP-qPCR		
Promoter of Morrbid (~130 bp), Forward AGCACGAGTCATCTGGTTCC	Kotzin et al., 2016	N/A
Promoter of Morrbid (~130 bp), Reverse ACCCAGTCCCCTCATTCTCA	Kotzin et al., 2016	N/A
Part-III: Oligonucleotides for 5mC/5hmC analysis		
Promoter of Morrbid (~800 bp), Forward ACC CCC AAG TCT CCTA ACCA	Kotzin et al., 2016	N/A
Promoter of Morrbid (~800 bp), Reverse GTT CAA CCT CAG TGC CCACT	Kotzin et al., 2016	N/A
Promoter Region with 4 CpG island sites, Forward ATTTAAGGTTTGGGAAGTTGTTTTT	This paper	N/A
Promoter Region with 4 CpG island sites, Reverse CAAACACCTCAATCTTCATTATCACTA	This paper	N/A
Software and Algorithms		
FlowJo	FlowJo	V10.2
Prism	GraphPad Software	V6.0
Adobe Illustrator	Adobe	CC-2015

CONTACT FOR REAGENT AND RESOURCE SHARING

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Reuben Kapur (rkapur@iupui.edu).

EXPERIMENTAL MODEL

Mice

All mice were bred and maintained under specified pathogen-free (SPF) conditions in the animal facility at Indiana University School of Medicine with 12-hr light/dark cycle and were provided food and water *ad libitum*. The Institutional Animal Care and Use Committee (IACUC) at Indiana University School of Medicine approved experiments with mice. *Tet2*-deficient mice (*Tet2*^{-/-} or *Tet2*^{+/-}, CD45.2) and *Morbid*-deficient mice (*Morbid*^{+/-}, CD45.2) are on C57BL/6 genetic background and have been described previously (Kotzin et al., 2016; Li et al., 2011). *Tet2*^{+/-} mice were bred with *Morbid*^{+/-} mice to generate *Tet2*^{+/-}; *Morbid*^{+/-} mice and all the controls. Wild-type C57BL/6 (CD45.2) mice were purchased from The Jackson Laboratory and used as controls. BoyJ mice (CD45.1) were purchased from The Jackson Laboratory for transplantation and chimerism analysis. The Shp2E76K mice have been described (Xu et al., 2011). *TLR4*^{-/-} and *IL6*^{-/-} were purchased from Jackson Laboratory (Cat #007727; Cat #002650). Whenever possible littermates were used as controls for all experiments. The age of mice has been indicated specifically for certain analysis in Figures 1, 2, 3, 4, 5, 6, and 7. In general, juvenile mice were between 1~2 months of age, adult mice between 3~4 months of age and older mice between 6~12 months of age. Mice of both sexes were used for experiments. Mice were not mated or used in previous procedure before experimentation.

METHOD DETAILS

In vivo treatments

Lipopolysaccharide (LPS) was purchased from Sigma (Cat # L8643) and dissolved in sterile phosphate-buffered saline (PBS) prior to being administered to mice (single dose; 0.8 mg/kg, i.p.). E3330, also called APX3330 was synthesized as described (Jiang et al., 2010; Nyland et al., 2010). E3330 was dissolved in Cremophor: EtOH (1:1) (Cremophor was purchased from Sigma, Cat # C5135) for stock solution generation and diluted in PBS prior to use for pre-LPS treatment or post-LPS treatment (20 mg/kg, twice a day, i.p. for Figure S6A; 20 mg/kg, one dose per day, daily by gavage for experiments in Figures 6C–6E, S6C, and S6D). SHP099 (provided by Novartis), was dissolved in 0.5% Methylcellulose (Sigma, Cat# M0262) and 0.1% Tween-80 (Fisher Scientific, Cat #BP338-500) and given to animals by gavage (daily, 50 mg/kg) for all the *in vivo* treatments.

Hematological cell counts

Hematologic parameters in peripheral blood (PB, from tail bleeding) were analyzed by an automated cell counter machine (Drew Hemavet 950). Total bone marrow (BM) cells were harvested from two femurs and two tibias and kept on ice or in refrigerator and stored in sterile blocking buffer containing 2% rat-serum prior to analysis. BM cellularity (viable cell counts) was analyzed by an automated cell counter (Beckman the Vi-CELL Cell Counter for Cell Viability Analyzer).

Flow cytometry

Non-lysed BM cells were used for analysis of erythroid lineage progenitor cells (Ter119 and CD71 staining). Remaining flow cytometry analysis was performed on lysed bone marrow cells (Lysis Buffer, BD, Cat # 555899). Antibodies against Ter119, Mac1, Gr1, B220, CD3, CD4 and CD8 were used for detecting mature cells (Lineage labeling). Progenitor cells were labeled and analyzed using the indicated antibodies. Antibody labeled BM cells were analyzed using a BD FACS-CANTO II machine with a two-laser and six-filter configuration. The properly compensated flow data were analyzed by Flow Jo software (V10.2). Events plotting, calculation of frequency, mean fluorescence intensity (MFI), and histogram overlays were analyzed by Flow Jo software. A panel of PE-conjugated antibodies against TER-119, Gr1, Mac1, B220, CD3, CD4 and CD8a surface antigens were mixed as a cocktail for lineage labeling (Lin-PE cocktail). A panel of antibodies containing Lin-PE cocktail, c-Kit APC, Sca-1 APC/Cy7, CD150 PE/Cy5 and CD48 PE/Cy7 were used for LSK/HSC labeling. A panel of antibodies containing Lin-PE cocktail, c-Kit APC, Sca-1 APC/Cy7, CD127 PE/Cy5, CD16/32 PE/Cy7 and CD34 FITC were used for CMP/GMP/MEP/CLP labeling. A panel of antibodies containing Lin-PE cocktail, c-Kit APC, Sca-1 APC/Cy7, Annexin FITC and 7-AAD were used for apoptosis labeling. For detecting the expression of TLR4 in mature cells, TLR4 PE/Cy7 was mixed with Mac1 PE, B220 APC and CD3 PE. For detecting the expression of TLR4 in HSPCs, TLR4 PE/Cy7 was mixed with Lin-PE cocktail, c-Kit APC, Sca-1 APC/Cy7 and CD150 PE/Cy5. For chimerism analysis in mature cells, CD45.2 PerCP/Cy5.5 and CD45.1 PE/Cy7 were mixed with Mac1 PE, B220 APC and CD3 PE. For chimerism analysis in HSPCs, CD45.2 PerCP/Cy5.5 and CD45.1 PE/Cy7 were mixed with Lin-PE cocktail, c-Kit APC, Sca-1 APC/Cy7 and CD150 PE/Cy5. Mac1 PE and Ly6G FITC were mixed for neutrophil labeling. Intracellular flow cytometry (ICFC) was performed to detect the expression of Bim, GM-CSF, IL-6, IL-1 β , Ki-67, NF κ B, phospho-Stat3, and TNF α . Briefly, freshly prepared bone marrow cells were pre-stained by using cell surface antibodies for mature cells or for HSPCs and then fixed with BD Cytofix and washed using BD Cytoperm three times. The pre-stained cells were then re-stained with the appropriate indicated antibodies. Staining with an Annexin-V and 7-AAD kit (BioLegend, Cat # 640922) was performed according to the manufacturer's instruction for apoptosis analysis, along with labeling of LSK cells.

A full list of antibodies is provided in the [Key Resources Table](#).

Multiplex cytokine assays

Serum samples were prepared from PB (tail-bleeding) and diluted in sterile PBS (1 to 2 dilution). Thirty-one cytokines or chemokines were quantified by multiplex immunoassay with a BioPlex 200 instrument (Eve Technologies, Mouse Cytokine Array/Chemokine Array 31-Plex, Cat # MD31).

Isolation of Lin-negative BM cells, LSK cells and qRT-PCR assays

Lin⁻ BM cells ($\sim 1 \times 10^6$) were purified by an EasySep Mouse Hematopoietic Progenitor Cell Isolation Kit (StemCell, Cat # 19856) according to manufacturer's instruction. LSK cells were purified from Lin⁻ negative BM cells by staining the cells with antibodies against c-Kit and Sca-1 followed by sorting them (Fluorescence-activated cell sorting, FACS) (BD FACSARIA). Total RNA was extracted from Lin⁻ cells using RNeasy Mini Kit (QIAGEN, Cat # 74104) according to the manufacturer's instructions. Isolated RNA was quantified by spectrophotometer and RNA concentrations were normalized. cDNA was synthesized by SuperScript II Reverse Transcriptase (ThermoFisher Scientific, Cat # 18064014). Resulting cDNA was analyzed by SYBR Green master mix (Life Technologies, Cat # 4385612) with indicated primers on a ViiA7 Real-Time PCR instrument. Expression of *Actin-b* was used as an internal control (Forward, 5'-GACGGCCAGGTCATCACTATTG-3' and Reverse 5'-AGGAAGGCTGGAAAAGAGCC-3') for calculating fold changes of indicated genes. The information of qRT-PCR primers for *Morbid* and *IL-6* is provided in the [Key Resources Table](#). See also [Table S1](#) for a list of additional qRT-PCR primers.

CFU assay

Bone marrow Lin⁻ negative cells or LSK cells were isolated as described above and plated in 24-well plate (10K cells per 0.5 mL medium per well) using MethoCult GF M3434 (Stem Cell). E3330 and SHP099 were dissolved in DMSO as inhibitors. Colonies were counted after 7-days of culture.

In vitro suspension culture with IL-6 and drugs

Bone marrow Lin⁻ negative cells were isolated as described above and cultured in basal medium (GIBCO IMDM containing 2% FBS) or with IL-6 as indicated in [Figures 6](#) and [S6](#). Drugs E3330 or SHP099 were added as indicated. After cultured for 36~48 hours, cells were washed by PBS and analyzed by flow cytometry or by qRT-PCR.

CHIP-qPCR assay

BM Lin-negative cells were used to extract chromatin DNA using MAGnify Chromatin Immunoprecipitation System (ThermoFisher, Cat # 492024) according to the manufacturer's instruction. CHIP purified chromatin DNA and input DNA were normalized to identical concentration for qPCR validation and enrichment analysis (1% enrichment of input level was defined as unit 1). Anti-Stat3 (Cell Signaling Technologies, #9319) was used for chromatin precipitation. Primers for CHIP-qPCR analysis are listed in the [Key Resources Table](#).

Analysis of 5mC and 5hmC level on the *Morbid* promoter

Genomic DNA from Lin-negative cells derived from wild-type and *Tet2*-KO mice was purified by Thermo Scientific Genomic DNA Purification Kit (Cat #K0512). For analyzing 5mC level of the indicated CCGG site by MspI and HpaI enzymes, the DNA samples were pretreated by QIAGEN EpiTect Bisulfite Kits (Cat #59104) for bisulfite conversion, followed by MspI or HpaI digestion. The digested products were used as templates for quantitative PCR (qPCR) analysis and/or DNA gel running by the indicated primers targeting the promoter region (oligos used for the ~800-bp promoter are included in the [Key Resources Table](#)). For analyzing 5hmC level of the indicated CCGG site, the DNA was pretreated by Zymo Quest 5-hmC Detection Kit for 5hmC conversion, followed by MspI digestion (Cat #D5415). Similarly the digested products were used as templates for quantitative PCR (qPCR) analysis and/or DNA gel running by the indicated primers targeting the promoter region. For analyzing the indicated CpG sites 1 to 4 by bisulfite sequencing PCR (BSP), the DNA samples were pretreated by QIAGEN EpiTect Bisulfite Kit (Cat #59104) for bisulfite conversion and then amplified by indicated primers (oligos used for the 4 CpG sites are included in the [Key Resources Table](#)). The amplified products were cloned into T-vector (Promega, Cat #A3610). Twenty-four proper clones were randomly selected for sequencing analysis to determine the methylation level on the CpG sites. 5mC level (percentage) is measured by the percentage of "C-T" conversion.

Competitive bone marrow transplantation (cBMT)

B6.SJL-Ptprc^a Pepc^b/Boy (BoyJ, CD45.1) mice were purchased from The Jackson Laboratory. Recipient animals (F1, CD45.2/CD45.1) were lethally irradiated (700 cGy plus 400 cGy) one day prior to transplantation (intravenous tail injection) of donor cells. For primary cBMT, CD45.2 donor BM cells from naive or LPS-treated mice were mixed with BoyJ CD45.1 competitor BM donor cells (with an equal number of viable total cells, 500K: 500K). For secondary cBMT, donor BM cells from primary cBMT recipients were mixed with BoyJ CD45.1 competitor BM cells (with an equal number of viable total cells). For LSK cell engraftment, 2000 LSK cells from LPS treated or control mice were mixed with 500,000 BoyJ CD45.1 supporting cells (2K: 500K). For generating chimeric mice mimicking hematopoietic clonal expansion, *Tet2*-KO donor cells and BoyJ donor cells were mixed at a ratio of 1: 5 (100K: 500K). For assessing the repopulating advantage of *Tet2*^{+/-}; *Morbid*^{+/-} cells, bone marrow donor cells from *Tet2*^{+/-}; *Morbid*^{+/-}

mice and all other controls were equally mixed with BoyJ donor cells respectively (500K: 500K). Chimerism analysis for progressive engraftment was analyzed on PB samples monthly (every 4-week interval) post BM transplantation.

shRNA generation and approach for knocking down the expression of *Morrbid*

Empty vector for pGreen shRNA cloning and shRNA-containing vectors for knocking-down *Morrbid*, shRNA1 and shRNA2 have been described before (Kotzin et al., 2016). Lentivirus supernatants were generated by transfecting 293T cells with an envelope plasmid (Addgene: #12259), packaging plasmid (Addgene: #12260), and the shRNA-containing vector or empty vector (1 μ g: 3 μ g: 6 μ g) per 10-cm dish. Virus soup was collected on day 1, day 2 and day 3 after transfection, combined, filtered and enriched by high-speed centrifugation (10×10^3 g). Lin⁻ cells from wild-type or *Tet2*-KO mice were freshly isolated and pre-cultured over-night at 37°C in 6-well plate (1×10^6 per mL) with medium containing mL-3 (10 ng/mL), mL-6 (5 ng/mL) and mSCF (50 ng/mL) (all are from PeproTech). Cell number was readjusted to make them equal at equal in 2 mL at 2.0×10^6 per well in a 6-well plate and spininfected for 2 hours with 5 μ g/mL polybrene. After overnight culture, the transfection efficiency was determined by flow cytometry for GFP expression. Approximately 60%–70% infection efficiency was achieved in most cases. For CFU assays, infected cells (GFP+) were purified by FACS. For cBMT assays, infected cells were washed three times in PBS, diluted and mixed with BoyJ cells (100K: 500K) prior to transplantation. Cells targeted by *Morrbid*-shRNA or empty vector were monitored by examining the GFP+% in the CD45.2 pool.

QUANTIFICATION AND STATISTICAL ANALYSIS

All experimental procedures on *Tet2*-KO samples were always run in parallel with wild-type controls (sex and age matched littermate controls when possible) for observing experiment variabilities. Analysis of grouped data was not blinded and no samples were excluded. Aged *Tet2*-KO mice were randomized into two groups for treatment with E3330 or vehicle, SHP099 or vehicle (Figure 6). *P* value was calculated using an unpaired t test for comparing means of two groups (GraphPad Prism 6.0). Error bars indicate the standard error of mean (s.e.m.).



p53 involvement in clonal hematopoiesis of indeterminate potential

Sisi Chen^a and Yan Liu^{a,b}

Purpose of review

Clonal hematopoiesis of indeterminate potential (CHIP) increases with age and occurs when a single mutant hematopoietic stem cell (HSC) contributes to a significant clonal proportion of mature blood lineages. Somatic mutations in the *TP53* gene, which encodes the tumor suppressor protein p53, rank in the top five among genes that were mutated in CHIP. This review focuses on mechanisms by which mutant p53 promotes CHIP progression and drives the pathogenesis of hematological malignancies, including myelodysplastic syndromes, and acute myeloid leukemia.

Recent findings

TP53 was frequently mutated in individuals with CHIP. Although clinical studies suggest that expansion of HSCs with *TP53* mutations predisposes the elderly to hematological neoplasms, there is a significant gap in knowledge regarding the mechanisms by which *TP53* mutations promote HSC expansion. Recent findings suggest that several cellular stressors, including hematopoietic transplantation, genotoxic stress, and inflammation, promote the expansion of HSCs with *TP53* mutations. Further, *TP53* mutations identified in CHIP cooperate with genetic and/or epigenetic changes in leukemogenesis.

Summary

TP53 mutations identified in CHIP are associated with increased risks of de novo and therapy-related hematological neoplasms. Thus, targeting mutant p53 and related pathways holds great potential in preventing CHIP progression and treating hematological malignancies.

Keywords

acute myeloid leukemia, age-related clonal hematopoiesis, clonal hematopoiesis of indeterminate potential, myelodysplastic syndromes, p53, *TP53*

INTRODUCTION

Human aging is associated with an exponential increase in the occurrence of clonal hematopoiesis of indeterminate potential (CHIP) in aged individuals. CHIP occurs when a single mutant hematopoietic stem cell (HSC) contributes to a significant, measurable clonal proportion of mature blood lineages [1,2[■],3]. CHIP is also known as age-related clonal hematopoiesis (ARCH) [4[■]]. CHIP is associated with an increased risk of hematological malignancies, such as myelodysplastic syndromes (MDS) and acute myeloid leukemia (AML), as well as a higher incidence of other age-related pathological conditions such as cardiovascular disease (CVD) [5–10,11[■]]. Given that CHIP is an age-dependent risk factor for MDS, AML, and CVD, preventing CHIP progression may prove to be beneficial for human health. However, mechanisms by which somatic mutations in blood cells contribute to the pathogenesis of age-related diseases are largely unknown. The vast majority of the mutations identified in

CHIP are dispersed across the genome. However, five genes, including *DNMT3A*, *TET2*, *ASXL1*, *JAK2*, and *TP53*, have disproportionately high numbers of somatic mutations [5–7]. *TP53* ranks in the top five among genes that were mutated in CHIP and the frequency of *TP53* mutations increases with age [5–9,12[■],13[■]]. *TP53* gene encodes the tumor suppressor p53 protein [14]. This review focuses on the involvement of p53 in CHIP progression and pathogenesis of hematological malignancies, including MDS and AML.

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KEY POINTS

- Tumor suppressor gene *TP53* was frequently mutated in individuals with CHIP.
- Several cellular stressors, including transplantation, genotoxic stress, and inflammation, promote the expansion of HSCs with *TP53* mutations.
- *TP53* mutations identified in CHIP cooperate with genetic and/or epigenetic changes in leukemogenesis.
- Targeting mutant p53 and related pathways holds great potential in preventing CHIP progression and treating hematological malignancies.

p53 IN CLONAL HEMATOPOIESIS OF INDETERMINATE POTENTIAL

p53 is a transcription factor that regulates a large number of genes in response to a variety of cellular insults, including oncogene activation, DNA damage, and inflammation. These stressors activate p53 through posttranslational modifications that result in augmented levels of p53 protein and transactivation activity [14,15]. Activated p53 induces growth arrest, apoptosis, DNA repair, and differentiation in damaged cells to suppress cellular transformation [14,15]. p53 is a critical regulator of hematopoietic stem cell behavior and we discovered that p53 maintains HSC quiescence and regulates DNA damage response [16,17]. p53 activates the transcription of target genes to mediate DNA damage repair, growth arrest or apoptosis [14,18]. We have identified both *Necdin* and *Gfi1* as p53 target genes in HSCs [16,17].

Like solid tumors, p53 function is always compromised in hematological malignancies, usually as a result of somatic mutations and deletions [14,15,19–23]. *TP53* ranks in the top five among genes that were mutated in CHIP (Fig. 1). The majority of *TP53* mutations in CHIP are missense mutations. The remainder of the *TP53* mutations are nonsense, frameshift, and splice site mutations [5–7,12*,13*]. *TP53* mutation spectrums in CHIP are similar to hematological malignancies, including MDS and AML [19–23]. Although clinical studies suggest that expansion of HSCs with *TP53* mutations predisposes the elderly to hematological neoplasms [5–7,12*,13*], the mechanisms by which *TP53* mutations promote HSC expansion during aging are largely unknown. We discovered that *TP53* mutations identified in CHIP enhances the repopulating potential of HSCs, thereby conferring a competitive advantage to HSCs following transplantation stress [24*]. Hematopoietic transplantation also expanded p53 mutant clones in patients with hematological malignancies [13*].

Modulation of gene transcription is an important mechanism for mutant p53 in cancer development [15]; however, how mutant p53 regulates gene expression in HSCs is largely unknown. Loss of epigenetic regulation of gene expression in aged HSCs contributes to aging phenotypes and dysregulated epigenetic control has been implicated in the pathogenesis of hematological malignancies [20,21,25,26]. Indeed, recent studies revealed that some mutant p53 proteins increase the expression of epigenetic factors, including MLL1, MLL2, and MOZ, in human cancer cells [27]. However, we found that the expression of *Mll1*, *Mll2*, and *Moz* is comparable in p53 wild-type and mutant HSCs (S.C. and Y.L., unpublished data). As mutant p53 proteins have been shown to play context dependent roles in human cancer [15], it is possible that mutant p53 proteins may utilize different mechanisms to modulate gene expression in HSCs. We found that mutant p53 interacts with epigenetic regulator EZH2. *EZH2*, a key component of Polycomb repressive complex 2, catalyzes the trimethylation of lysine 27 of histone H3 (H3K27me3) in cells [28]. Hematopoietic-specific deletion of *Ezh2* impairs HSC self-renewal and terminal differentiation [29]. We discovered that mutant p53 enhanced the association of EZH2 with the chromatin and increased the levels of H3K27me3 in genes regulating HSC self-renewal and differentiation (S.C. and Y.L., unpublished data). Thus, we have uncovered a novel mechanism by which mutant p53 drives clonal hematopoiesis. As common mutations identified in CHIP affect epigenetic modulators, including DNMT3A, TET2, and ASXL1 [5–7], our findings that mutant p53 modulates EZH2 activity and increases the levels of H3K27me3 in HSCs underscore the importance of dysregulated epigenetic control in CHIP development.

Most of *TP53* mutations in human cancer lost their tumor suppressor function [15]. However, mounting evidence suggests that some mutant p53 proteins not only lose their tumor suppressor function, but also acquire new oncogenic properties that are independent of wild-type p53, known as gain-of-function (GOF) properties [15]. To ascertain the oncogenic effects of GOF *TP53* mutations, *p53*^{R248W} and *p53*^{R273H} mutant were engineered into the endogenous *Trp53* locus in mice [30]. Homozygous *p53*^{R248W/R248W} and *p53*^{R273H/R273H} mice developed novel tumors compared with *p53*^{-/-} mice [30]. Thus, GOF mutant p53 proteins have enhanced oncogenic potential beyond the simple loss of p53 function. As EZH2 interacts with mutant p53 but not wild-type p53, our findings suggest that some *TP53* mutations identified in CHIP may have GOF properties.

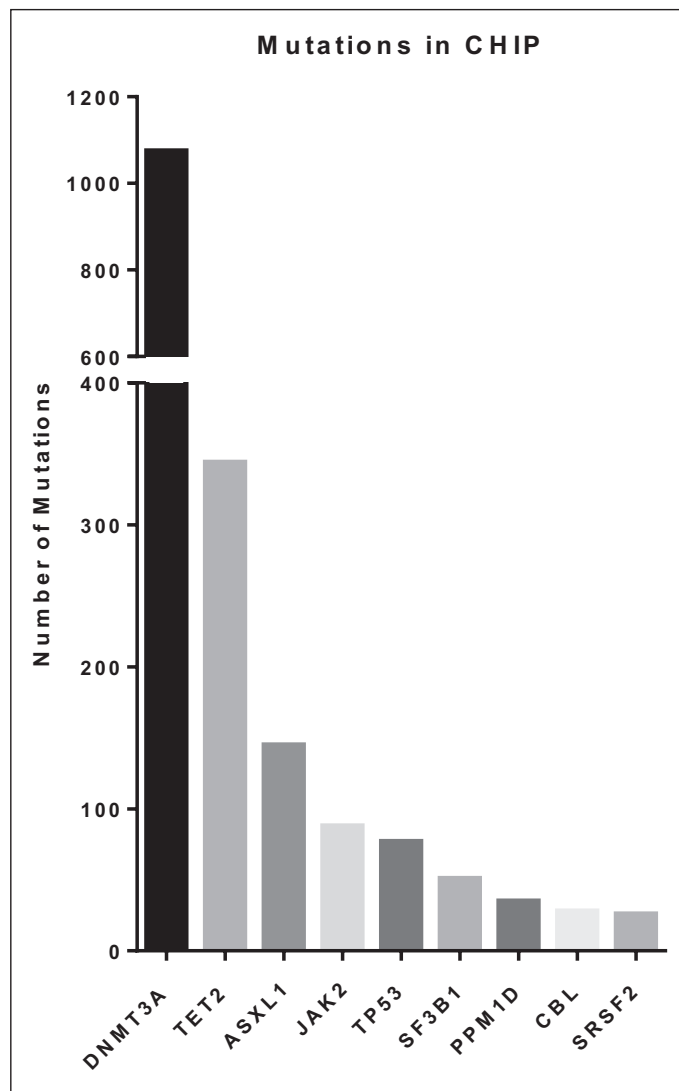


FIGURE 1. Tumor suppressor gene *TP53* ranks in the top five among genes that were mutated in clonal hematopoiesis of indeterminate potential.

In transplantation assays, p53 mutant hematopoietic cells always outcompeted wild-type competitor cells and became clonal dominance [13[■],24[■]]. Although we have identified a cell autonomous mechanism by which mutant p53 drives clonal hematopoiesis, recent studies indicate that mutations identified in CHIP may utilize cell nonautonomous mechanisms to promote clonal hematopoiesis [11[■],31[■]]. For example, mutant hematopoietic cells displayed increased secretion of proinflammatory cytokines, including IL-1 β and IL-6 [11[■],31[■]]. We found that inhibition of inflammatory signaling in Tet2 mutant preleukemic cells mitigates clonal hematopoiesis [31[■]]. RNA-seq analysis revealed that inflammatory response genes are enriched in p53 mutant HSCs (S.C. and Y.L., unpublished data), suggesting that mutant p53 may drive clonal hematopoiesis through activating proinflammatory pathways. The

potential cell nonautonomous mechanisms by which mutant p53 promotes HSC expansion during aging await future investigation.

p53 IN THERAPY-RELATED CLONAL HEMATOPOIESIS OF INDETERMINATE POTENTIAL

TP53 mutations are commonly found in therapy related CHIP as well as in secondary MDS and AML [12[■],13[■],24[■],32]. Indeed, somatic *TP53* mutations are present in 5–10% of MDS cases and in 30% of secondary MDS patients arising after exposure to radiation or chemotherapy [20,21]. *TP53* mutations in CHIP are associated with prior exposure to chemotherapy or radiotherapy [12[■],13[■]]. Chemotherapy treatment resulted in expansion of both *p53*^{+/-} and *p53*^{R28W/+} HSCs [13[■],24[■],32]. *p53*^{-/-} HSCs are

resistant to radiation [16] and we observed that *TP53* mutations identified in CHIP confer radiation resistance, leading to selective expansion of *TP53*-mutant HSCs (S.C. and Y.L., unpublished data). *TP53* mutations in therapy-related CHIP are also associated with smoking [12[■]], suggesting that smoking-induced DNA damage may promote the expansion of hematopoietic clones with *TP53* mutations.

Mutations in protein phosphatase Mg^{2+}/Mn^{2+} 1D (PPM1D) have been identified in CHIP and myeloid neoplasms, with an enrichment in patients previously exposed to chemotherapy [5–7,12[■],13[■],33[■],34[■]]. *PPM1D* negatively regulates p53 and several proteins involved in the DNA damage response [35,36]. *PPM1D* mutations confer chemotherapy resistance, resulting in the selective expansion of *PPM1D*-mutant hematopoietic cells [33[■],34[■]]. However, *PPM1D* mutants lack an advantage under bone marrow transplantation stress [13[■],33[■],34[■]]. Thus, mutant p53 appears to play distinct roles in driving clonal hematopoiesis compared with *PPM1D* mutants.

p53 IN THE PATHOGENESIS OF HEMATOLOGICAL MALIGNANCIES

The frequency of *TP53* mutations in AML is approximately 10% [22]. However, in AML with complex karyotype, the frequency of *TP53* mutations and/or deletions is almost 70% [23]. *TP53* mutations and/or deletions were thought to be secondary events that occur during leukemic transformation [37–39]. However, an aged individual with *TP53* mutation in hematopoietic cells developed AML after acquiring additional chromosomal changes [5]. Significantly, Li–Fraumeni syndrome (LFS), a rare, autosomal dominant disease, predisposes individuals to hereditary cancer linked to *TP53* germline mutations, and some LFS patients develop MDS and AML as they age [15,40], suggesting that *TP53* mutations may be early events in leukemia development.

Most homozygous p53 knockout and mutant mice develop spontaneous tumors, including lymphoma and sarcoma, and die within 3–6 months after birth [30]. As MDS is an age-related hematological disorder [20,21], we reasoned that heterozygous p53 mutant mice may develop myeloid malignancies with age. We maintained *p53*^{+/+} and *p53*^{R248W/+} mice for more than a year and monitored tumor development. We found that some *p53*^{R248W/+} mice developed MDS with age based upon pathological analysis of bone marrow and peripheral blood. Other *p53*^{R248W/+} mice developed lymphoma and sarcoma (S.C. and Y.L., unpublished data).

Thus, we demonstrated that mutant p53 drives MDS in mice with age.

Although coexisting mutations with *TP53* mutations in AML are limited, previous studies indicate that *TP53* mutations do co-occur with AML driver mutations in oncogenic signaling molecules such as NRAS (neuroblastoma RAS) and FMS-like tyrosine kinase receptor-3 (FLT3) [41[■],42,43[■]]. Loss of p53 has been shown to cooperate with *Nras* mutations in AML development in mice. Mechanistically, *p53*^{-/-} synergizes with enhanced oncogenic *Nras*^{G12D} signaling to transform megakaryocyte-erythroid progenitors into leukemia-initiating cells (LICs) thereby driving AML development [43[■]]. We found that mutant p53 cooperates with FLT3-ITD (internal tandem duplication) in chronic myeloid leukemia (CML) development in mice. Further, we found that mutant p53 enhanced the self-renewal potential of FLT3-ITD⁺ LICs [44[■]]. Given that *TP53* mutations are present in both chronic and blast crisis phases of CML [45], our studies underscore the importance of mutant p53 in CML pathogenesis.

TARGETING MUTANT p53 TO PREVENT CLONAL HEMATOPOIESIS OF INDETERMINATE POTENTIAL PROGRESSION AND TREAT HEMATOLOGICAL MALIGNANCIES

TP53 is the most frequently mutated gene across all cancer types [14,15,46[■]]. The presence of mutant p53 predisposes to tumor development and is associated with ineffective therapeutic responses and unfavorable prognosis [15,46[■]]. Indeed, somatic *TP53* mutations are associated with advanced disease and poor prognosis in both MDS and AML [19–23]. Despite these effects, no drug that abrogates the oncogenic functions of mutant p53 has yet been approved for the treatment of cancer [46[■]]. To date, there are no effective treatment for MDS and AML patients with *TP53* mutations, and most patients die within 2 years of diagnosis [19–23]. Thus, there is an urgent need to develop therapeutic strategies that can target mutant p53 and related pathways, enhancing our abilities to prevent CHIP progression and treat age-related diseases. Although hematopoietic cell transplantation has curative potential, *TP53* mutations are unfavorable prognostic markers for transplantation in MDS patients and negatively affect posttransplant survival [47[■],48]. While MDS and AML patients with *TP53* mutations have been observed to have favorable clinical response and robust mutation clearance after receiving a 10-day courses of decitabine, the response is not durable [49]. Epigenetic factor EZH2 is rarely mutated in CHIP [5–7] and we found that genetic and pharmacological inhibition of EZH2 decrease the

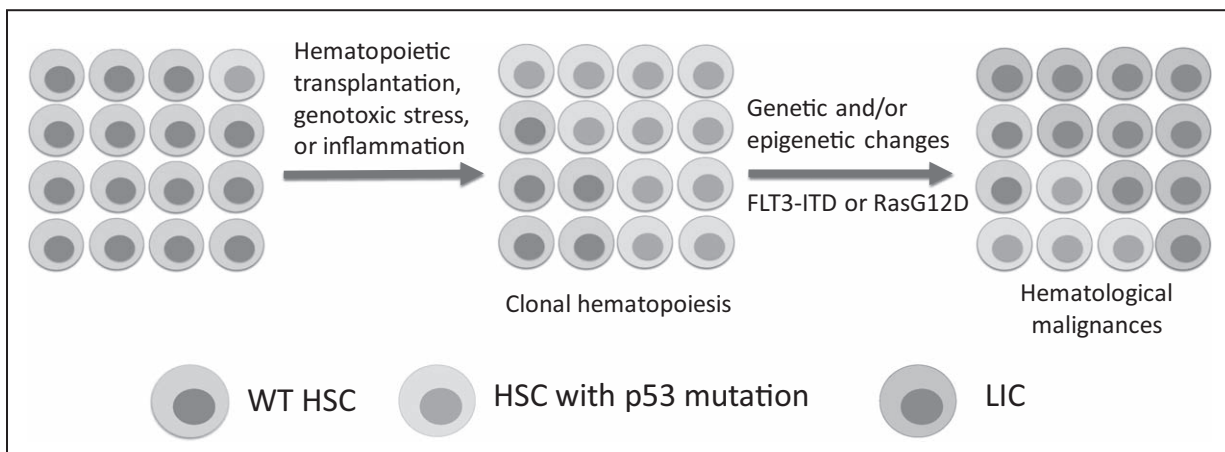


FIGURE 2. *TP53* mutations identified in clonal hematopoiesis of indeterminate potential utilize distinct mechanisms to promote hematopoietic stem cell expansion during aging. Expanded mutant hematopoietic stem cells become fully transformed leukemia-initiating cells after acquiring additional genetic and/or epigenetic changes.

repopulating potential of p53 mutant HSCs (S.C. and Y.L., unpublished data), suggesting that EZH2 may be a novel target for preventing CHIP progression in aged individuals with *TP53* mutations.

CONCLUSION

Tumor suppressor gene *TP53* was frequently mutated in individuals with CHIP and the frequency of *TP53* mutations increases with age [5–9]. Recent findings suggest that several cellular stressors, including hematopoietic transplantation, genotoxic stress, and inflammation, promote the expansion of HSCs with *TP53* mutations [11[–]13[–], 24[–], 31[–], 32]. Further, *TP53* mutations identified in CHIP cooperate with genetic and/or epigenetic changes in leukemogenesis [43[–], 44[–]] (Fig. 2). As *TP53* mutations identified in CHIP are associated with increased risks of de novo and therapy-related hematological neoplasms [5–9, 12[–], 13[–]], targeting mutant p53 and related pathways holds great potential in preventing CHIP progression and treating hematological malignancies.

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Conflicts of interest

There are no conflicts of interest.

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Mutant p53 enhances leukemia-initiating cell self-renewal to promote leukemia development

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To the Editor

Acute myeloid leukemia (AML) is an aggressive blood cancer with poor prognosis [1]. AML is thought to be initiated and maintained by a rare population of leukemia stem cells (LSCs) or leukemia-initiating cells (LICs) that have acquired the capacity for self-renewal and is blocked in their ability to differentiate by the accumulation of a series of mutations and/or epigenetic changes [2, 3]. Clinical studies show that LICs are resistant to conventional chemotherapy and/or targeted therapies [3]. Thus, there is an unmet need to elucidate the molecular mechanisms governing LIC self-renewal and develop novel therapeutic approaches that can target LICs and improve leukemia treatment [3].

The tumor suppressor p53 is a stress response protein that regulates a large number of genes in response to a variety of cellular insults, including oncogene activation,

DNA damage, and inflammation [4]. These signals activate p53 primarily through post-translational modifications that result in augmented levels of p53 protein and transactivation activity [4]. Activated p53 suppresses cellular transformation mainly by inducing growth arrest, apoptosis, DNA repair, and differentiation in damaged cells [4]. Accordingly, p53 function is always compromised in tumor cells, usually as a result of somatic mutations and deletions, which occur in approximately half of all human cancers [5]. The *TP53* gene encodes the tumor suppressor p53. The frequency of *TP53* mutations in AML is ~10%. However, in AML with complex karyotype, the frequency of *TP53* mutations and/or deletions is almost 70% [6]. While *TP53* mutations confer drug resistance and poor prognosis in AML, the role of mutant p53 in the initiation and progression of AML is largely unknown [6, 7].

We have been investigating the role of tumor suppressor p53 in normal and malignant hematopoiesis. We found that wild-type p53 maintains HSC quiescence and inhibits HSC self-renewal [8]. Codon 248 of p53 is frequently mutated in AML and p53^{R248W} has been shown to be a gain-of-function (GOF) mutant in human cancer cells as well as in animal models [6, 7, 9]. We recently reported that p53^{R248W} enhances HSC self-renewal in steady state and promotes HSC expansion following genotoxic stresses [10]. Of note, homozygous *p53*^{-/-} and *p53*^{R248W/R248W} mice develop lymphoid tumors, including lymphoma and thymoma, but not myeloid malignancies [9], suggesting that expression of mutant p53 is not sufficient for inducing myeloid leukemia in mice. This has led to a search for potential second hits that cooperate with mutant p53 in the pathogenesis of myeloid malignancies, primarily focused on using mouse models.

While coexisting mutations with *TP53* mutations in AML are limited [6, 7], previous studies indicate that *TP53* mutations co-occur with AML driver mutations in oncogenic

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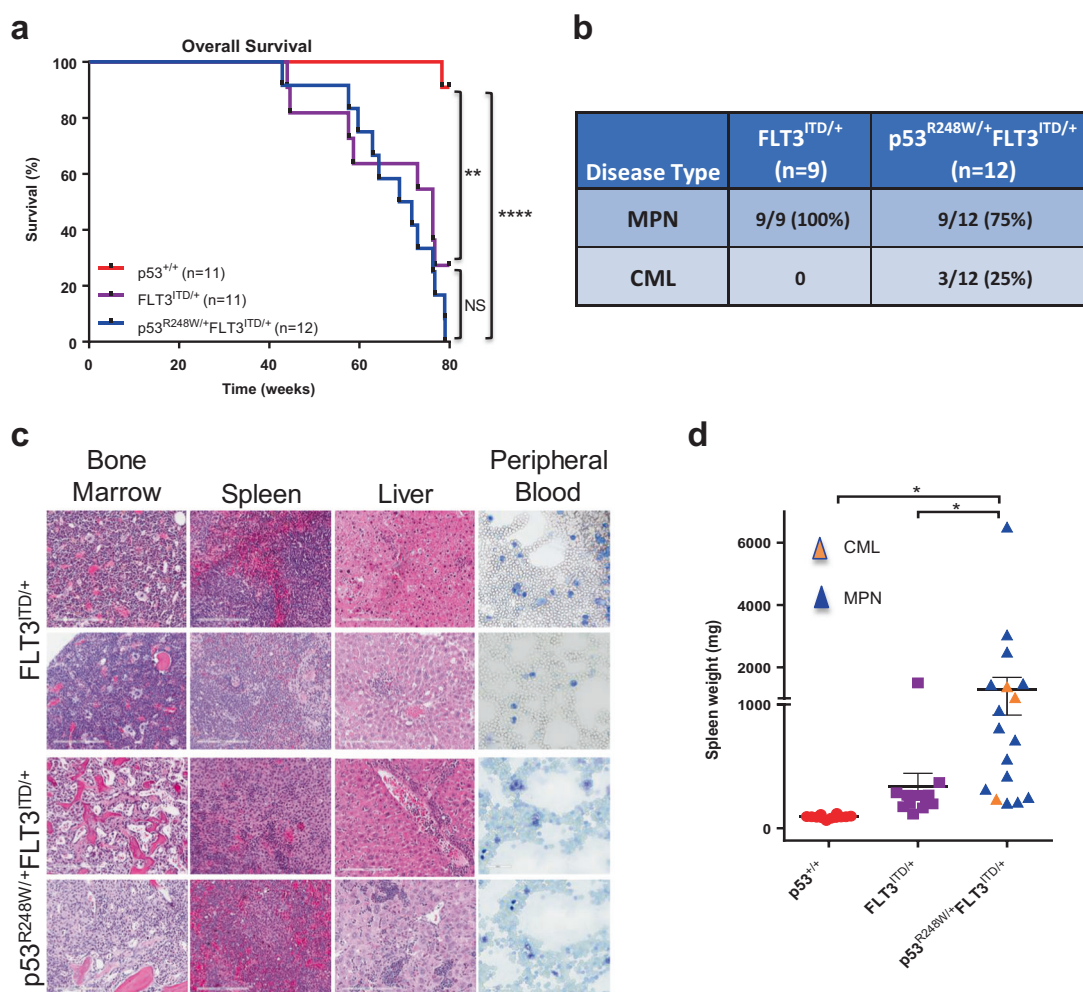


Fig. 1 Mutant p53 cooperates with FLT3-ITD in the pathogenesis of myeloid leukemia. **a** $FLT3^{ITD/+}$ and $p53^{R248W/+}FLT3^{ITD/+}$ mice show decreased survival compared to $p53^{+/+}$ mice ($n = 11$, $p53^{+/+}$; $n = 11$, $FLT3^{ITD/+}$; $n = 12$, $p53^{R248W/+}FLT3^{ITD/+}$, $**p < 0.01$, $****p < 0.0001$). **b** Disease spectrums in $FLT3^{ITD/+}$ and $p53^{R248W/+}FLT3^{ITD/+}$ mice were determined by pathological analysis of the bone marrow, spleen, liver, and peripheral blood ($n = 9$, $FLT3^{ITD/+}$; $n = 12$, $p53^{R248W/+}FLT3^{ITD/+}$).

c Representative H&E (20 $\times$) images of the bone marrow, spleen, liver, and peripheral blood smears from $FLT3^{ITD/+}$ mice with MPN and $p53^{R248W/+}FLT3^{ITD/+}$ mice with CML. **d** Spleen weight of $p53^{+/+}$, $FLT3^{ITD/+}$, and $p53^{R248W/+}FLT3^{ITD/+}$ mice. Mean values ($\pm$ SEM) are shown ($n = 12$, $p53^{+/+}$; $n = 12$, $FLT3^{ITD/+}$; $n = 17$, $p53^{R248W/+}FLT3^{ITD/+}$, $*p < 0.05$).

signaling molecules, such as FMS-like tyrosine kinase receptor-3 (FLT3) [11]. Mutations in FLT3 have been identified in myeloid malignancies, including myeloproliferative neoplasms (MPN) and AML [12]. Internal tandem duplications in the juxtamembrane domain (FLT3-ITD) and mutations in the activating loop of FLT3 (FLT3-TKD) are seen in 30–35% of AML patients [12]. Both ITD and TKD mutations of FLT3 lead to constitutive activation of the tyrosine kinase, promoting proliferation, and survival of leukemic blasts [12]. Given that expression of FLT3-ITD in the hematopoietic compartment results in MPN in mice and that FLT3-ITD impairs HSC self-renewal in vivo [13], we reasoned that mutant p53 might synergize with FLT3-ITD in driving the development of myeloid leukemia through enhancing LIC self-renewal.

To test this hypothesis, we generated $p53^{R248W/+}FLT3^{ITD/+}$ mice and monitored overall survival and tumor development of these mice. We observed that both $FLT3^{ITD/+}$ and $p53^{R248W/+}FLT3^{ITD/+}$ mice have decreased life span compared with $p53^{+/+}$ mice (Fig. 1a). Some $p53^{R248W/+}$ mice develop myelodysplastic syndromes (MDS) with age and other $p53^{R248W/+}$ mice developed lymphoma and sarcoma based upon pathological analysis of the bone marrow (BM), spleen, liver, and peripheral blood (PB) (SC and YL, unpublished data). However, the majority of $p53^{R248W/+}FLT3^{ITD/+}$ mice developed MPN as seen in $FLT3^{ITD/+}$ mice (Fig. 1b, c and data not shown), suggesting that FLT3-ITD-induced MPN development does not depend on mutant p53. Histological observation of spleen sections from MPN mice showed disarray of normal splenic architecture with a

reduction and almost total absence of the white pulp in some cases and increased red pulp area with increased extramedullary hematopoiesis (Fig. 1c). These features appeared in conjunction with hepatosplenomegaly, variable leukocytosis and overproduction of myeloid cells in the bone marrow, spleen, and peripheral blood (Fig. 1c). We noted that the bone marrow cellularity decreased as splenomegaly increased, consistent with increased extramedullary hematopoiesis. Notably, ~25% of $p53^{R248W/+}FLT3^{ITD/+}$ mice developed chronic myeloid leukemia (CML) [14]. Upon necropsy, mice with CML displayed severe splenomegaly, and some also displayed hepatomegaly. Morphological analysis of peripheral blood smears revealed increased myeloid cells with dysplastic features (Fig. 1c). The bone marrow cellularity varied from hypocellular to hypercellular among animals. Increased number of myeloid cells (blast to immature myeloid cells) was observed in the bone marrow with extensive spread of myeloid elements in the spleen and in a few livers (Fig. 1c). While $p53^{R248W/+}FLT3^{ITD/+}$ mice showed marked splenomegaly compared with $p53^{+/+}$ and $FLT3^{ITD/+}$ mice (Fig. 1d), this is not likely due to CML development as majority of double-mutant mice developed MPN (Fig. 1b, c).

Given that patients with homozygous FLT3-ITD mutations have a more severe disease compared with those with heterozygous FLT3-ITD mutations [11, 12], we examined whether mutant p53 cooperates with homozygous FLT3-ITD mutant in leukemia development. We transplanted 3×10^6 whole bone marrow cells from $p53^{+/+}$, $FLT3^{ITD/ITD}$, or $p53^{R248W/+}FLT3^{ITD/ITD}$ mice into lethally irradiated recipient mice and measured their overall survival. Both $FLT3^{ITD/ITD}$ and $p53^{R248W/+}FLT3^{ITD/ITD}$ recipient mice had decreased life spans compared with $p53^{+/+}$ recipient mice (Figure S1a). Interestingly, ~30% of the $p53^{R248W/+}FLT3^{ITD/ITD}$ transplanted animals developed CML (Figure S1b), similar to that seen in $p53^{R248W/+}FLT3^{ITD/+}$ animals (Fig. 1b). Rest of the $p53^{R248W/+}FLT3^{ITD/ITD}$ mice developed MPN (Figure S1b).

Given that some $p53^{R248W/+}FLT3^{ITD/+}$ mice develop CML, we next examined the impact of mutant p53 on FLT3-ITD⁺ hematopoietic stem and progenitor cells (HSPCs) in order to understand the underlying mechanisms. We first analyzed peripheral blood (PB), the bone marrow (BM), and the spleen of $p53^{+/+}$, $p53^{R248W/+}$, $FLT3^{ITD/+}$, and $p53^{R248W/+}FLT3^{ITD/+}$ mice (8–12 week old). PB white blood cell (WBC) counts, BM cellularity, and spleen weight were comparable among the four groups of mice (Figures S1c, S1d, and S1e). We then examined the frequency of hematopoietic stem and progenitor cells in the BM of $p53^{R248W/+}FLT3^{ITD/+}$ mice. While the number of LT-HSCs and ST-HSCs was comparable among these mice, LSKs and MPPs were expanded in the $p53^{R248W/+}FLT3^{ITD/+}$ mice compared with that of the $p53^{+/+}$, $p53^{R248W/+}$, and $FLT3^{ITD/+}$ mice

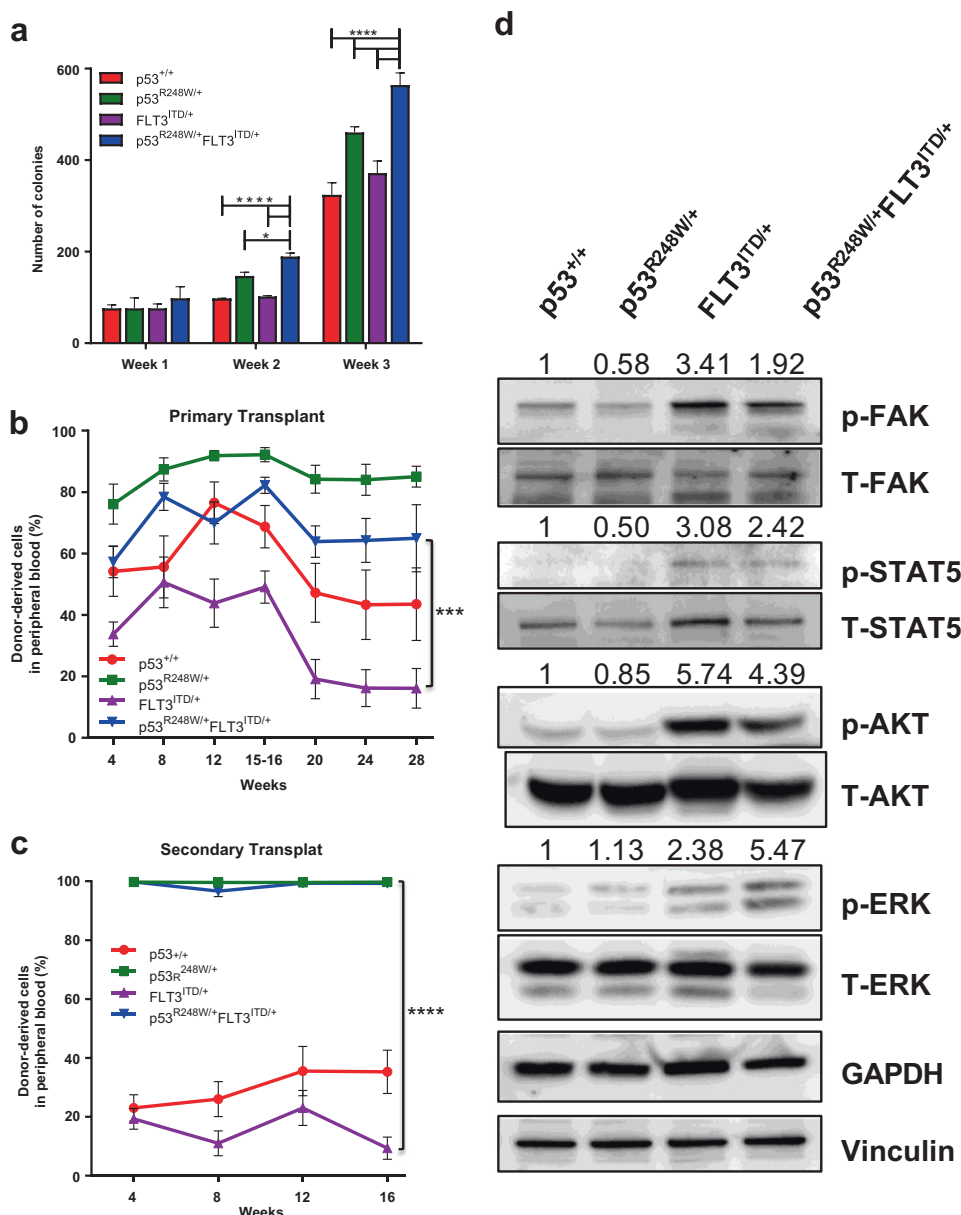
(Figure S2a). We also observed increased frequency of myeloid progenitors (Lin[−]Kit⁺ cells) in the bone marrow of $p53^{R248W/+}FLT3^{ITD/+}$ mice (Figure S2b). These findings suggest that the effects of mutant p53 and FLT3-ITD on myeloid progenitor cell expansion appears additive. However, the number of common lymphoid progenitors (CLPs) was comparable among four group of mice (Figure S2c).

FLT3 mutations have been shown to enhance the proliferation of hematopoietic stem and progenitor cells [13]. We then examined the cell cycle status of LSKs isolated from $p53^{+/+}$, $p53^{R248W/+}$, $FLT3^{ITD/+}$, and $p53^{R248W/+}FLT3^{ITD/+}$ mice. We confirmed that $FLT3^{ITD/+}$ LSKs shown enhanced proliferation compared with $p53^{+/+}$ LSKs (Figure S2d). However, mutant p53 did not alter the proliferation of $FLT3^{ITD/+}$ LSKs (Figure S2d). To determine the impact of mutant p53 on myeloid progenitors, we performed serial replating assays using BM cells from $p53^{+/+}$, $p53^{R248W/+}$, $FLT3^{ITD/+}$, and $p53^{R248W/+}FLT3^{ITD/+}$ mice. While the colony formation potential of $p53^{+/+}$ and $FLT3^{ITD/+}$ BM cells was comparable in serial replating assays, $p53^{R248W/+}FLT3^{ITD/+}$ BM cells show enhanced replating potential compared with $p53^{R248W/+}$ and $FLT3^{ITD/+}$ BM cells (Fig. 2a), suggesting that expanded myeloid progenitors in $p53^{R248W/+}FLT3^{ITD/+}$ mice are functional in vitro.

To examine the impact of mutant p53 on HSCs in vivo, we performed serial competitive bone marrow transplantation assays. We transplanted 5×10^5 donor BM cells ($p53^{+/+}$, $p53^{R248W/+}$, $FLT3^{ITD/+}$ or $p53^{R248W/+}FLT3^{ITD/+}$, and CD45.2⁺) into lethally irradiated (11 Gy) F1 recipient mice (CD45.1⁺ CD45.2[−]) along with 5×10^5 competitor BM cells (CD45.1⁺). Peripheral blood white blood cell counts were comparable among the four groups of mice following transplantation (Figure S2e). While $FLT3^{ITD/+}$ BM cells showed decreased repopulating ability compared with $p53^{+/+}$ cells 16 weeks post transplantation, $p53^{R248W/+}FLT3^{ITD/+}$ BM cells displayed enhanced engraftment compared to $FLT3^{ITD/+}$ BM cells (Fig. 2b). We then killed the recipient mice and examined the frequency of donor-derived hematopoietic stem and progenitor cells in their bone marrow. We found increased number of donor-derived LSKs in the BM of recipient mice repopulated with $p53^{R248W/+}$ BM cells compared with that of the $p53^{+/+}$ and $FLT3^{ITD/+}$ BM cells, whereas the frequency of donor-derived LSKs in the BM of recipient mice repopulated with $p53^{R248W/+}$ and $p53^{R248W/+}FLT3^{ITD/+}$ cells was comparable (Figure S2f). We found increased number of donor-derived GMPs in the BM of recipient mice repopulated with $p53^{R248W/+}FLT3^{ITD/+}$ bone marrow cells compared with that of the $FLT3^{ITD/+}$ BM cells (Figure S3a). The spleen size was comparable in recipient mice repopulated with four group of BM cells (Figure S3b).

To determine the impact of mutant p53 on the self-renewal potential of FLT3-ITD⁺ HSCs, we transplanted 3×10^6 BM cells isolated from the primary recipient mice

Fig. 2 Mutant p53 enhances the self-renewal potential of FLT3-ITD⁺ LICs. **(a)** Serial replating assays of bone marrow cells from young $p53^{+/+}$, $p53^{R248W/+}$, $FLT3^{ITD/+}$ and $p53^{R248W/+}$ + $FLT3^{ITD/+}$ mice. Mean values ($\pm$ SD) are shown ($n = 3$, * $p < 0.05$, *** $p < 0.0001$). **(b)** $p53^{R248W}$ enhances the repopulating potential of $FLT3^{ITD/+}$ hematopoietic cells. Percentage of donor-derived (CD45.2⁺) cells in the peripheral blood of primary recipient mice post-transplantation, measured at 4-week intervals. Mean values ($\pm$ SEM) are shown ($n = 7$, *** $p < 0.001$). **(c)** The percentage of donor-derived cells in the peripheral blood of secondary recipient mice. Mean values ($\pm$ SEM) shown, ($n = 7$, $p53^{R248W/+}$ vs $FLT3^{ITD/+}$ and $FLT3^{ITD/+}$ vs $p53^{R248W/+}$ + $FLT3^{ITD/+}$, *** $p < 0.0001$). **(d)** Western blot analysis of activated and total FAK, STAT5, AKT, and ERK protein levels in $p53^{+/+}$, $p53^{R248W/+}$, $FLT3^{ITD/+}$ and $p53^{R248W/+}$ + $FLT3^{ITD/+}$ mononuclear cells differentiated into macrophage progenitors. Loading controls GAPDH and Vinculin are also shown. Quantification of phosphorylated proteins was calculated relative to total protein level and is displayed above each respective phospho-protein



repopulated with $p53^{+/+}$, $p53^{R248W/+}$, $FLT3^{ITD/+}$, or $p53^{R248W/+}$ + $FLT3^{ITD/+}$ cells into lethally irradiated secondary F1 recipients. Sixteen weeks after transplantation, $p53^{R248W/+}$ + $FLT3^{ITD/+}$ cells continued to show increased repopulating ability compared with $FLT3^{ITD/+}$ BM cells (Fig. 2c). These findings suggest that mutant p53 may promote leukemic transformation through enhancing LIC self-renewal.

To examine the impact of mutant p53 on oncogenic signaling pathways, we performed western blot analysis on macrophage progenitor cells derived from $p53^{+/+}$, $p53^{R248W/+}$, $FLT3^{ITD/+}$, or $p53^{R248W/+}$ + $FLT3^{ITD/+}$ bone marrow cells. Consistent with previous studies, cells from $FLT3^{ITD/+}$ mice had activated FAK, STAT5, and AKT (Fig. 2d). Further, expressing FLT3-ITD in a mutant p53 background

enhances activated ERK levels, but slightly decreases activated FAK and STAT5 levels (Fig. 2d). We found increased levels of FLT3 in $p53^{R248W/+}$ + $FLT3^{ITD/+}$ macrophage progenitor cells (Figure S3c). Thus, expressing FLT3-ITD in a mutant p53 background has no effect on FLT3-ITD-induced activation of signaling pathways. However, ERK inhibitor treatment decreased the replating potential of $p53^{R248W/+}$, $FLT3^{ITD/+}$, and $p53^{R248W/+}$ + $FLT3^{ITD/+}$ bone marrow cells (Figure S3d). These findings suggest that mutant p53 and FLT3-ITD may function through different signaling pathways in the pathogenesis of hematological malignancies. In future, we will elucidate the mechanisms by which mutant p53 upregulates FLT3 in HSPCs.

While *TP53* and *FLT3* mutations are rarely co-occur in MPN and AML [11], the underlying mechanisms are not known. We found that the majority of *p53*^{R248W/+} *FLT3*^{ITD/+} mice developed MPN, as seen in *FLT3*^{ITD/+} mice [13]. Further, we discovered that mutant p53 and FLT3-ITD cooperate in CML development in mice. Functionally, mutant p53 synergizes with FLT3-ITD to expand the myeloid progenitor cell pool and enhance the self-renewal potential of LICs. *TP53* mutations are present in both chronic and blast crisis phase of CML [15], underscoring the importance of mutant p53 in CML pathogenesis. Delineating the role of mutant p53 and FLT3-ITD in LIC self-renewal and pathogenesis of hematological malignancies may facilitate the development of novel therapeutic approaches that can improve leukemia treatment.

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Author contributions SCN and YL designed the research. SCN, SC, RG, CY, MK, SV, ACF, CW, and CD performed the research. SCN, SC, and YL analyzed the data and performed the statistical analysis. GES performed pathological analysis. HSB, LDM, and RK provided reagents and constructive advice to the study. SCN, SC, and YL wrote the paper. All authors read, commented on, and approved the paper.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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Mutant p53 drives clonal hematopoiesis through modulating epigenetic pathway

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Clonal hematopoiesis of indeterminate potential (CHIP) increases with age and is associated with increased risks of hematological malignancies. While *TP53* mutations have been identified in CHIP, the molecular mechanisms by which mutant p53 promotes hematopoietic stem and progenitor cell (HSPC) expansion are largely unknown. Here we discover that mutant p53 confers a competitive advantage to HSPCs following transplantation and promotes HSPC expansion after radiation-induced stress. Mechanistically, mutant p53 interacts with EZH2 and enhances its association with the chromatin, thereby increasing the levels of H3K27me3 in genes regulating HSPC self-renewal and differentiation. Furthermore, genetic and pharmacological inhibition of EZH2 decreases the repopulating potential of p53 mutant HSPCs. Thus, we uncover an epigenetic mechanism by which mutant p53 drives clonal hematopoiesis. Our work will likely establish epigenetic regulator EZH2 as a novel therapeutic target for preventing CHIP progression and treating hematological malignancies with *TP53* mutations.

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Clonal hematopoiesis of indeterminate potential (CHIP), also known as age-related clonal hematopoiesis (ARCH), occurs when a single mutant hematopoietic stem and progenitor cell (HSPC) contributes to a significant clonal proportion of mature blood lineages during aging^{1–3}. CHIP is common in aged healthy individuals and associated with increased risks of hematological neoplasms, including myelodysplastic syndromes (MDS) and acute myeloid leukemia (AML)^{4–8}. CHIP is also associated with increased all-cause mortality and risk of cardio-metabolic disease^{4–6,9}. While these findings suggest that mutations identified in CHIP likely drive disease development, mechanisms by which these mutations promote HSPC expansion are largely unknown^{4–9}.

Most individuals with CHIP carry hematological malignancy-associated mutations, including *DNMT3A*, *TET2*, *ASXL1*, *JAK2*, and *TP53*^{4–6}. The *TP53* gene, which encodes the tumor suppressor protein p53, ranks in the top five among genes that were mutated in CHIP^{4–6,10–12}. p53 bears the usual hallmarks of a transcription factor and regulates a large number of genes in response to a variety of cellular insults, including oncogene activation, DNA damage, and inflammation, to suppress tumorigenesis^{13,14}. *TP53* mutations and deletions were found in approximately half of all human cancers, including hematological malignancies^{13,14}. Recently, somatic *TP53* mutations were identified in CHIP^{4–6}. *TP53* mutations were also commonly found in therapy-related CHIP^{10,12}. Interestingly, some individuals with Li-Fraumeni syndrome (LFS), who carry germline *TP53* mutations, develop MDS and AML as they age^{14,15}. Indeed, somatic *TP53* mutations are present in 10% of MDS and AML cases and in 30% of secondary MDS and AML patients arising after exposure to radiation or chemotherapy^{2,16–19}. While *TP53* mutations are associated with adverse clinical outcomes in MDS and AML^{2,16–19}, how mutant p53 drives the pathogenesis of hematological malignancies are not fully understood.

We have been investigating the role of p53 in normal and malignant hematopoiesis. We discovered that wild-type (WT) p53 maintains hematopoietic stem cell (HSC) quiescence and identified Necdin as a p53 target gene that regulates DNA damage response (DDR) in HSCs^{20,21}. We extended our research to mutant p53 to generate additional knowledge in order to develop therapeutic strategies that can enhance our abilities to prevent CHIP progression and treat hematological diseases. We discovered that mutant p53 enhances the repopulating potential of HSPCs²², similar to what has been reported previously^{23,24}. While clinical studies suggest that expansion of HSPCs with *TP53* mutations predisposes the elderly to hematological neoplasms^{4–6,10–12}, the role of *TP53* mutations in CHIP progression remains elusive.

Polycomb group (PcG) proteins are epigenetic regulators that have been implicated in stem cell maintenance and cancer development^{25–28}. Genetic and biochemical studies indicate that PcG proteins exist in at least two protein complexes, Polycomb repressive complex 2 (PRC2) and Polycomb repressive complex 1 (PRC1), that act in concert to initiate and maintain stable gene repression^{25–28}. EZH2, a key component of PRC2 complex, catalyzes the trimethylation of lysine 27 of histone H3 (H3K27me3) in cells²⁷. While EZH2 plays important roles in HSCs and MDS development^{16,29,30}, its regulation in HSPCs is not fully understood.

In the present study, we discovered that mutant p53 confers a competitive advantage to HSPCs following transplantation and promotes HSPC expansion after radiation-induced stress. Mechanistically, mutant p53 interacts with EZH2 and enhances its association with the chromatin, thereby increasing the levels of H3K27me3 in genes regulating HSPC self-renewal and differentiation. Thus, we have uncovered an epigenetic mechanism by which mutant p53 drives clonal hematopoiesis.

Results

TP53 mutations identified in CHIP enhance HSPC functions.

TP53 ranks in the top five among genes that were mutated in CHIP (Fig. 1a)^{4–6,10–12}. Approximately 90% of somatic *TP53* mutations in CHIP are missense mutations in the DNA-binding domain (DBD) of the p53 protein (Fig. 1b)^{4–6,10–12}. The most frequently mutated codon in p53 was 248, followed by codons 273, 220, and 175 (Fig. 1c). *TP53* mutation spectrums in CHIP are similar to hematological malignancies. Different mutant p53 proteins have been shown to exhibit distinct functions in promoting cancer initiation, progression, or metastasis¹⁴. To determine the impact of *TP53* mutations on HSPC functions, we introduced eight hot-spot *TP53* mutations identified in CHIP^{4–6,10–12} (Fig. 1c), into WT mouse HSPCs using retrovirus-mediated transduction and performed in vitro and in vivo assays (Fig. 1d). Ectopic expression of some mutant p53, including p53^{R248W}, p53^{R248Q}, p53^{R175H}, p53^{R273H}, p53^{C238Y}, and p53^{Y220C}, enhanced the replating potential of WT HSPCs compared to control viruses (MIGR1) transduced cells (Fig. 1e).

p53^{R248W}, p53^{R273H}, and p53^{Y220C} are hot-spot *TP53* mutations in CHIP, MDS, and AML and predict leukemia development^{4–6,10–12,17–19}. These mutations have also been shown to gain oncogenic properties in mouse models of human cancer^{14,31–33}. We introduced p53^{R248W}, p53^{R273H}, or p53^{Y220C} into WT HSPCs (CD45.2⁺) using retrovirus-mediated transduction, and then transplanted transduced cells (GFP⁺) together with competitor bone marrow (BM) cells (CD45.1⁺) into lethally irradiated recipient mice. We observed increased number of GFP⁺ cells in peripheral blood (PB) of recipient mice repopulated with HSPCs expressing mutant p53 compared to that of control viruses transduced cells at 16 weeks following transplantation (Fig. 1f). Increases in total GFP⁺ cells in PB at 16 weeks from mice with mutant p53 proteins were highly suggestive of enhanced HSPC repopulating potential.

p53 is important for HSC survival following genotoxic stress and p53 null HSCs are less sensitive to irradiation as manifested by decreased apoptosis²⁰. We found that ectopic expression of p53^{R248W}, p53^{R273H}, or p53^{Y220C} in WT HSPCs resulted in decreased apoptosis following 2 Gy irradiation (Fig. 1g), suggesting that HSPCs expressing these mutant p53 proteins are not sensitive to radiation-induced stress.

TP53 mutations in CHIP confer competitive advantage to HSPCs.

Given that codon 248 of the p53 protein (p53^{R248}) is most frequently mutated in CHIP, MDS, and AML^{4,6,10–12,18,19}, we focused our investigation on p53^{R248W} in hematopoiesis. Since overexpression of mutant p53 from an MSCV promoter may not reflect accurate function when expressed at physiological levels, we utilized the p53^{R248W} knock-in mice, where p53^{R248W} was introduced into the humanized p53 knock-in (*HUPKI*) allele in mice, expressing human p53 mutant protein from the endogenous murine *Trp53* promoter³³. The *HUPKI* allele encodes a human/mouse chimeric protein consisting primarily of human p53 sequence (amino acids 33–332) flanked by the conserved extreme amino and carboxyl-termini of mouse p53³⁴. *HUPKI* mice were described as p53^{+/+} mice in the text.

Since the majority of *TP53* mutations in CHIP are mono-allele missense mutations (Fig. 1b)^{4–6,10–12}, we utilized heterozygous mutant mice (p53^{R248W/+}) to investigate the biological impact of mutant p53 on primitive HSPC populations. As nonsense, frameshift, and splice site mutations result in *TP53* deletions¹⁴, we also included p53^{+/-} and p53^{-/-} mice in the experiments. We first analyzed the BM of p53^{+/+}, p53^{+/-}, p53^{-/-}, and p53^{R248W/+} mice and found that BM cellularity is comparable among these mice (Supplementary Fig. 1a). We observed increased number of

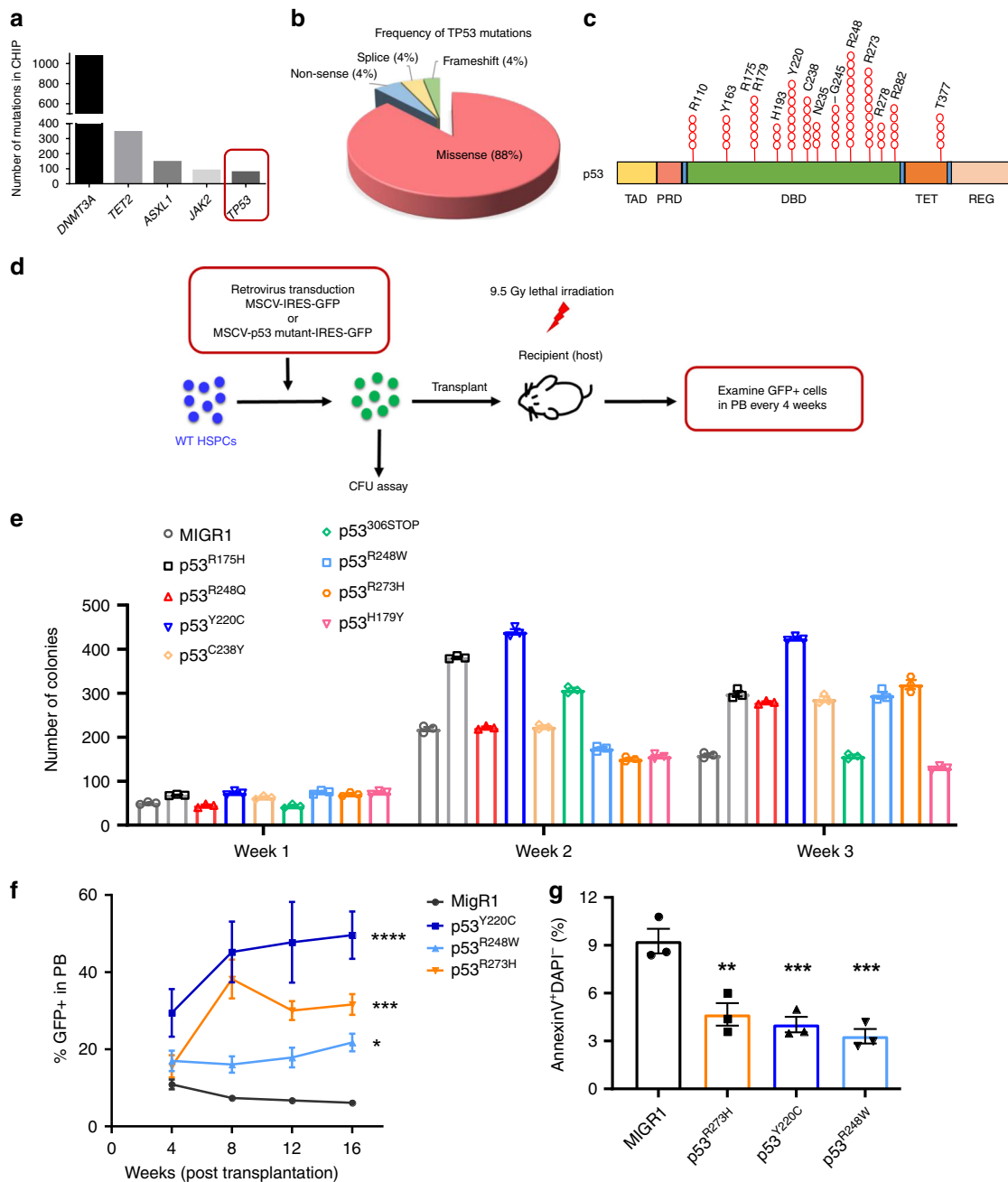
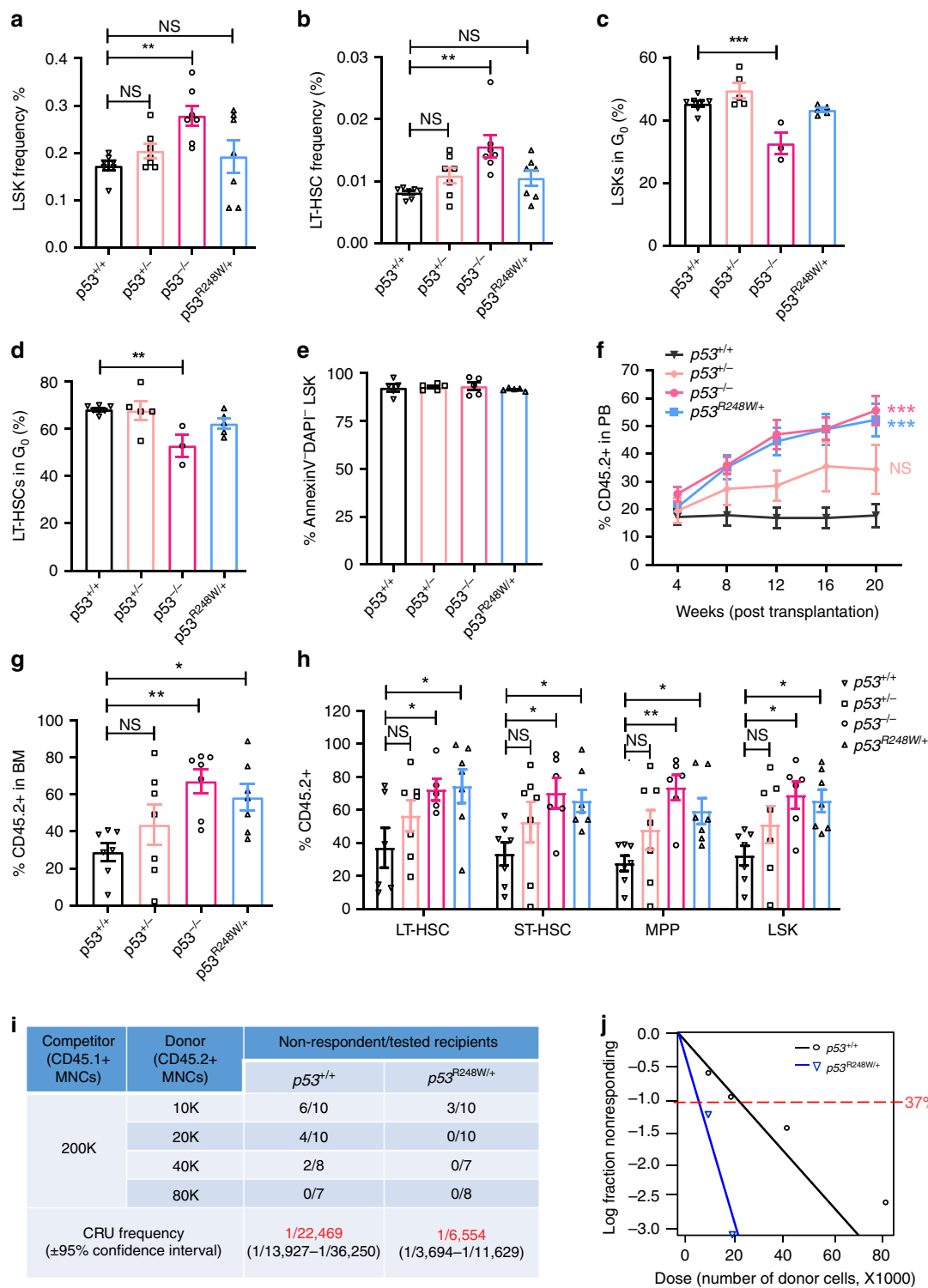


Fig. 1 *TP53* mutations identified in CHIP enhance HSPC repopulating potential. **a** Tumor suppressor gene *TP53* ranks in the top five among genes that were mutated in clonal hematopoiesis with indeterminate potential (CHIP). **b** Pie chart representing different types of *TP53* mutations identified in CHIP. **c** *TP53* mutations in CHIP are enriched in the DNA-binding domain (DBD) of the p53 protein. TAD, transactivation domain; PRD, proline-rich domain; DBD, DNA-binding domain; TET, tetramerization domain; and REG, carboxy-terminal regulatory domain. **d** Several hot-spot *TP53* mutations identified in CHIP were introduced into wild-type hematopoietic stem and progenitor cells (HSPCs) using retrovirus-mediated transduction. In vitro and in vivo stem and progenitor cell assays were then performed using sorted GFP (green fluorescent protein)-positive cells. **e** Serial replating assays of HSPCs expressing different mutant p53 proteins. The methylcellulose cultures were serially replated, weekly, for 3 weeks; $n = 3$ independent experiments performed in duplicate. **f** Percentage of GFP+ cells in the peripheral blood (PB) of recipient mice following competitive transplantation; $n = 3$ –5 mice per group. **g** HSPCs expressing mutant p53 proteins were assessed for apoptosis at 24 h after radiation (2 Gy); $n = 3$ independent experiments. Data are represented as mean $\pm$ SEM. P -values were calculated using two-way ANOVA (analysis of variance) with Dunnett's multiple comparisons test in **e** and **f**, one-way ANOVA with Dunnett's multiple comparisons test in **g**; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$. Source data are provided as a Source Data file.

LSKs and LT-HSCs in the BM of $p53^{-/-}$ mice as reported²⁰; however, $p53^{R248W}$ affects neither the frequency nor the number of HSPCs in the BM (Fig. 2a, b, and Supplementary Fig. 1b, c). While loss of p53 decreases the quiescence of LSKs and LT-HSCs²⁰, mutant p53 does not affect HSPC quiescence (Fig. 2c, d). In addition, we observed similar number of apoptotic HSPCs in

$p53^{+/+}$, $p53^{+/-}$, $p53^{-/-}$, and $p53^{R248W/+}$ mice in steady state (Fig. 2e). Thus, mutant p53 does not affect the frequency, quiescence, or the survival of HSPCs when expressed at physiological levels.

Hematopoietic transplantation is a cellular stressor that has been shown to promote the expansion of mutant HSPCs^{12,35,36}.



We recently reported that *p53*^{R248W} enhances the repopulating potential of BM cells²². To further establish that the enhanced repopulating potential is HSC intrinsic, we purified HSCs (CD48⁻CD150⁺LSKs, CD45.2⁺) from *p53*^{+/+}, *p53*^{+/-}, *p53*^{-/-}, and *p53*^{R248W/+} mice and performed HSC transplantation assays. Both *p53*^{-/-} and *p53*^{R248W/+} HSCs exhibited a substantially higher contribution to PB production compared to *p53*^{+/+} and *p53*^{+/-} HSCs at 20 weeks following primary transplantation (Fig. 2f). In addition, the percentage of donor-derived hematopoietic cells and HSPCs in the BM of recipient mice repopulated

with *p53*^{-/-} and *p53*^{R248W/+} HSCs was significantly higher than that of the *p53*^{+/+} HSCs (Fig. 2g, h). Mutant *p53* did not affect myeloid and lymphoid differentiation in PB and the BM of recipient mice following HSC transplantation (Supplementary Fig. 1d, e). To determine the impact of mutant *p53* on HSC self-renewal, we performed secondary BM transplantation assays. We found that both *p53*^{-/-} and *p53*^{R248W/+} HSCs continue to show enhanced engraftment compared to *p53*^{+/+} and *p53*^{+/-} HSCs at 20 weeks following secondary transplantation. Interestingly,

Fig. 2 $p53^{R248W/+}$ confers a competitive advantage to HSPCs. **a** The frequency of Lin[−]Sca1⁺Kit⁺ cells (LSKs) in the bone marrow (BM) of $p53^{+/+}$, $p53^{+/-}$, $p53^{-/-}$, and $p53^{R248W/+}$ mice; $n = 7$ mice per genotype. **b** The frequency of long-term hematopoietic stem cells (LT-HSCs) in the BM of $p53^{+/+}$, $p53^{+/-}$, $p53^{-/-}$, and $p53^{R248W/+}$ mice; $n = 7$ mice per genotype. **c** The quiescence of LSKs was determined by Ki67 and DAPI (4',6-diamidino-2-phenylindole) staining followed by flow cytometry analysis; $n = 3-7$ mice per genotype. **d** The quiescence of LT-HSCs was determined by Ki67 and DAPI staining and flow cytometry analysis; $n = 3-7$ mice per genotype. **e** The apoptosis of LSKs was determined by Annexin V and DAPI staining and flow cytometry analysis; $n = 5$ mice per genotype. **f** Percentage of donor-derived cells in PB of recipient mice at 20 weeks following HSC transplantation; $n = 7$ mice per group. **g** Percentage of donor-derived cells in the BM of recipient mice at 20 weeks following HSC transplantation; $n = 7$ mice per group. **h** Percentage of donor-derived LT-HSCs, short-term hematopoietic stem cells (ST-HSCs), multi-potent progenitors (MPPs), and LSKs in the BM of recipient mice repopulated with $p53^{+/+}$, $p53^{+/-}$, $p53^{-/-}$, or $p53^{R248W/+}$ HSCs; $n = 7$ mice per genotype. **i** Measuring the number of functional HSCs in the BM of $p53^{+/+}$ and $p53^{R248W/+}$ mice utilizing limiting dilution transplantation assays. Recipients with <2% donor-derived cells in the peripheral blood were defined as non-respondent; $n = 7-10$ mice per group. $P = 0.00114$. **j** Poisson statistical analysis of data from Fig. 2i using L-Cal software. Shapes represent the percentage of negative mice for each dose of cells. Solid lines indicate the best-fit linear model for each dataset. Data are represented as mean $\pm$ SEM. P -values were calculated using one-way ANOVA with Dunnett's multiple comparisons test in **a**, **b**, **c**, **d**, **e**, **g**, and **h**, two-way ANOVA with Dunnett's multiple comparisons test in **f**, and χ^2 test in **i** and **j**; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. Source data are provided as a Source Data file.

$p53^{+/-}$ HSCs show increased repopulating potential compared to $p53^{+/+}$ HSCs in secondary transplantation assays (Supplementary Fig. 1f). We observed increased number of donor-derived hematopoietic cells in the BM of secondary recipients repopulated with $p53^{+/-}$, $p53^{-/-}$, and $p53^{R248W/+}$ cells compared to $p53^{+/+}$ cells (Supplementary Fig. 1g). However, neither *TP53* mutation nor *p53*-deficiency alters terminal differentiation of HSCs (Supplementary Fig. 1h).

To enumerate the numbers of functional HSCs in the BM of $p53^{R248W/+}$ mice, we performed competitive BM transplantation experiments with limiting-dilution of donor cells. The frequency of competitive repopulation units (CRU) in the BM of $p53^{R248W/+}$ mice is three- to four-fold higher than that of the $p53^{+/+}$ mice (Fig. 2i, j). Enhanced repopulating potential of $p53^{R248W/+}$ BM cells could be due to changes in homing capacities of donor cells. We performed homing assays but did not detect difference in the frequency of donor-derived cells in the BM of recipient mice repopulated with $p53^{R248W/+}$ BM cells compared to $p53^{+/+}$ BM cells (Supplementary Fig. 1i, j). Taken together, we demonstrate that mutant *p53* identified in CHIP confers a competitive advantage to HSPCs following transplantation.

***TP53* mutations promote HSPC survival following radiation.**

Therapy-related CHIP in patients with non-hematologic cancers is common and associated with adverse clinical outcomes^{10,12}. Cytotoxic therapy results in the expansion of clones carrying *TP53* mutations^{10,12,37}. Indeed, we found that chemotherapy treatment expands HSPCs expressing mutant *p53*²². Given that HSPCs expressing mutant *p53* are not sensitive to radiation (Fig. 1g), we then examined the impact of radiation on mutant HSPC expansion. We generated mixed BM chimeras containing both $p53^{R248W/+}$ (CD45.2⁺) and $p53^{+/+}$ (CD45.1⁺) cells with a 1:10 ratio of mutant to WT cells. Eight weeks following transplantation, recipient mice were treated with or without 5 Gy total body irradiation (TBI) (Fig. 3a). We found that mutant BM cells outcompeted $p53^{+/+}$ cells, and became clonally dominant following TBI (Fig. 3b). Further, TBI significantly increased frequency of mutant HSPCs in BM of recipient mice (Fig. 3c, d). Thus, we demonstrate that radiation promotes the expansion of HSPCs with mutant *p53*.

To determine the impact of radiation on $p53^{+/+}$ and $p53^{R248W/+}$ mice, we irradiated these mice and monitored their survival. While most $p53^{+/+}$ mice died 5 weeks following 9 Gy TBI, most $p53^{R248W/+}$ mice were still alive (Supplementary Fig. 2a). Further, $p53^{R248W/+}$ HSCs show decreased apoptosis both in vitro and in vivo following 2 Gy irradiation (Fig. 3e, f). Using phosphorylation of histone H2AX (γ H2AX) as an indicator of DNA damage, we found that $p53^{+/+}$ HSCs stained positive for γ H2AX, whereas

$p53^{R248W/+}$ HSCs were largely devoid of γ H2AX foci (Supplementary Fig. 2b, c).

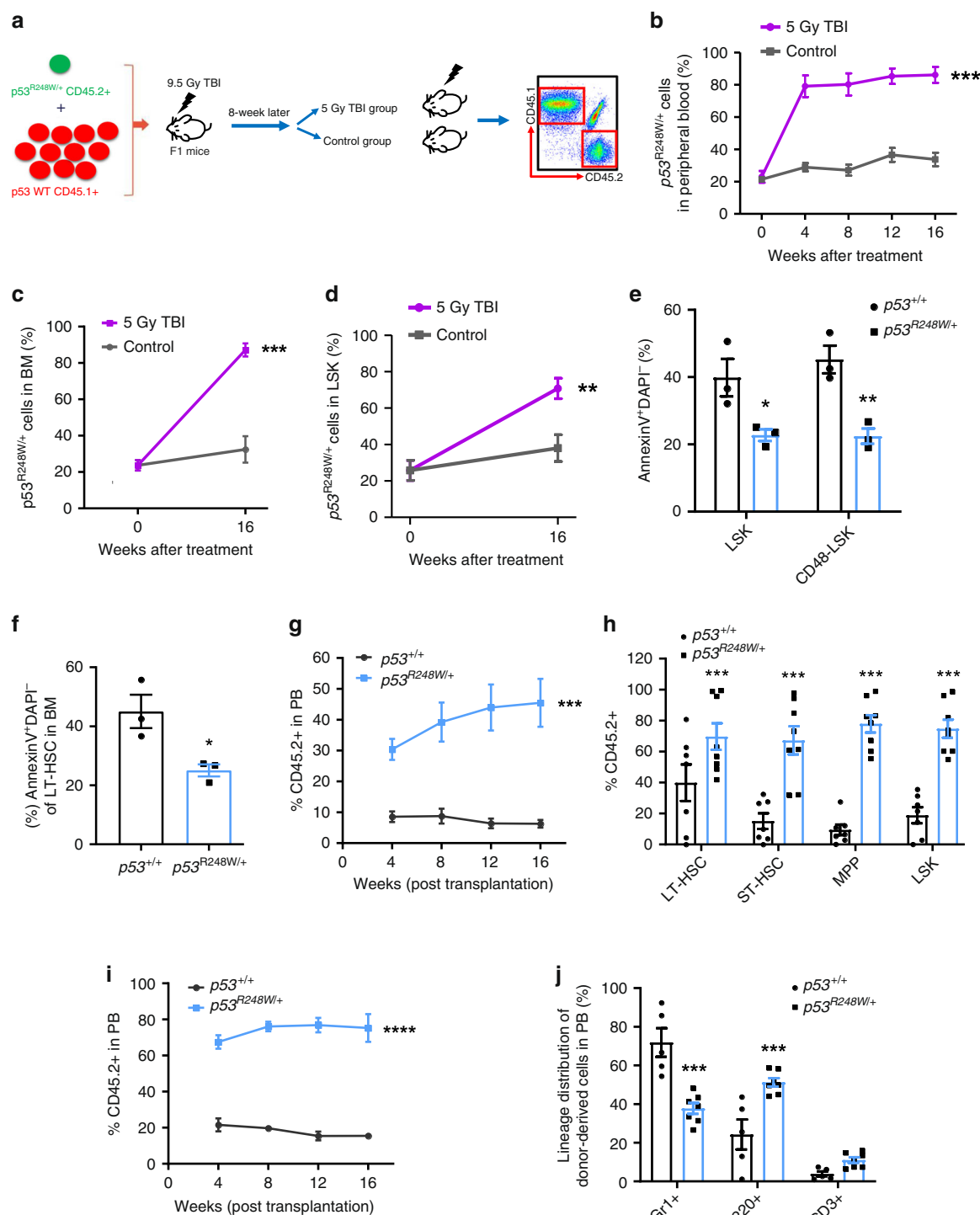
To determine the impact of radiation on $p53^{R248W/+}$ HSPC function in vivo, we treated $p53^{+/+}$ and $p53^{R248W/+}$ mice with 2 Gy TBI. Two hours following TBI, we isolated live BM cells from irradiated mice and performed competitive transplantation assays. Irradiated mutant BM cells displayed enhanced repopulating potential in primary transplantation assays compared to irradiated WT cells (Fig. 3g). We observed increased number of donor-derived HSPCs in the BM of primary recipient mice repopulated with live $p53^{R248W/+}$ BM cells (Fig. 3h and Supplementary Fig. 2d). Sixteen weeks after secondary transplantation, $p53^{R248W/+}$ cells continued to show increased repopulating ability (Fig. 3i). While mutant *p53* had no effect on multi-lineage differentiation in PB of primary recipient mice (Supplementary Fig. 2e), we found decreased myeloid differentiation and increased B cell differentiation in the PB of secondary recipient mice repopulated with mutant BM cells (Fig. 3j). Thus, we demonstrate that *TP53* mutations identified in CHIP confer resistance to radiation, leading to the selective expansion of *TP53*-mutant HSPCs.

HSC and AML signatures were enriched in *p53* mutant HSPCs.

WT *p53* is a transcription factor that activates the transcription of several target genes in HSCs, including *p21* and *Necdin*^{20,21}. However, we found that mutant *p53* does not alter the expression of *p21* and *Necdin* in HSCs (Fig. 4a). To understand how mutant *p53* enhances HSPC self-renewal, we performed transcript profiling (using RNA-seq studies and quantitative real-time PCR (qRT-PCR) analysis) to compare gene expression in HSPCs isolated from $p53^{+/+}$ and $p53^{R248W/+}$ mice. We employed Gene Set Enrichment Assays (GSEA) to group potential mutant *p53* target genes into specific pathways important in HSPC behavior. HSC and AML signatures were significantly enriched in *p53* mutant HSPCs compared to $p53^{+/+}$ HSPCs (Supplementary Fig. 3a, b). Several pathways important for HSC maintenance, including Regulation of hematopoiesis, Hematopoietic organ development, Immune response, and Positive regulation of cytokine response, were significantly enriched in $p53^{R248W/+}$ HSPCs compared to $p53^{+/+}$ HSPCs (Supplementary Fig. 3c). Collectively, the gene expression profiling data suggest that mutant *p53* modulates specific pathways associated with HSC maintenance and leukemogenesis.

EZH2 targets were downregulated in *p53* mutant HSPCs.

While we found that several hundred genes are either upregulated or downregulated in *p53* mutant HSPCs compared to WT HSPCs, how mutant *p53* regulates gene expression in HSPCs is



unknown. Recent studies revealed that some mutant p53 proteins increase the expression of epigenetic factors, including *MLL1*, *MLL2*, and *MOZ* (*KAT6A*), in human cancer cells³⁸. *MLL1* and *MLL2* are key components of the MLL complexes that confer histone H3K4 trimethylation (H3K4me3), which is an active histone mark important for gene expression^{39,40}. *MOZ* is a histone acetyltransferase and mediates histone H3K9 acetylation (H3K9ac)^{41,42}. However, we found that the expression of *MLL1*, *MLL2*, and *MOZ* is comparable in p53 WT and mutant HSPCs (Supplementary Fig. 3d), suggesting mutant p53 may utilize other mechanisms to modulate gene expression in hematopoietic cells.

Interestingly, RNA-seq assays revealed that genes that only marked by H3K27me3 were negatively enriched with significance in mutant HSPCs compared to that of the WT HSPCs (Fig. 4b, left panel). EZH2 target gene signature (without EZH1 compensation) was also negatively enriched with significance in mutant HSPCs (Fig. 4b, right panel). However, loss of p53 in HSPCs did not significantly change EZH2 target gene signature (Supplemental Fig. 3e). Western blot analysis showed increased levels of H3K27me3 in p53 mutant HSPCs compared to WT HSPCs (Fig. 4c). Further, $p53^{R248W/+}$ HSCs displayed higher levels of H3K27me3 compared to $p53^{+/+}$ HSCs quantified by flow cytometry analysis (Fig. 4d).

Fig. 3 $p53^{R248W/+}$ confers a survival advantage to HSPCs after radiation. **a** BM chimeras were generated by transplanting a 1:10 ratio of $p53^{R248W/+}$ cells (CD45.2⁺) to $p53^{+/+}$ cells (CD45.1⁺) into irradiated recipient mice (CD45.1⁺CD45.2⁺). After hematopoietic reconstitution (8 weeks), mice were treated with or without 5 gray (Gy) total body irradiation (TBI). **b** Percentage of $p53^{R248W/+}$ (CD45.2⁺) cells in PB of recipient mice following TBI treatment; $n = 7$ mice per group. **c** Percentage of $p53^{R248W/+}$ cells (CD45.2⁺) in the BM of recipient mice at 16 weeks following TBI treatment; $n = 7$ mice per group. **d** Percentage of $p53^{R248W/+}$ LSK cells (CD45.2⁺) in the BM of recipient mice at 16 weeks following TBI treatment; $n = 7$ mice per group. **e** Hematopoietic stem and progenitor cells from $p53^{+/+}$ and $p53^{R248W/+}$ mice were assessed for apoptosis 2 h after 2 Gy TBI; $n = 3$ mice per group. **f** HSCs purified from the BM of $p53^{+/+}$ and $p53^{R248W/+}$ mice were treated with 2 Gy TBI and then assessed for apoptosis; $n = 3$ mice per group. **g** Competitive transplantation assays using BM cells isolated from $p53^{+/+}$ and $p53^{R248W/+}$ mice treated with 2 Gy TBI. Two hours following TBI, we isolated BM cells from irradiated mice and transplanted 500,000 live BM cells together with equal number of competitor BM cells into lethally irradiated recipient mice. The percentage of donor-derived cells in the PB of recipient mice; $n = 7$ –8 mice per group. **h** Percentage of donor-derived LT-HSCs, ST-HSCs, MPPs, and LSK cells in the PB of the primary recipient mice 16 weeks following transplantation; $n = 7$ –8 mice per group. **i** Contribution of $p53^{+/+}$ and $p53^{R248W/+}$ BM cells to recipient mouse PB in secondary transplantation assays; $n = 5$ –7 mice per group. **j** Lineage contribution of donor-derived cells in the PB of secondary recipient mice 16 weeks following transplantation; $n = 5$ –7 mice per group. Data are represented as mean $\pm$ SEM. P -values were calculated using two-way ANOVA with Bonferroni's multiple comparisons test in **b**, **e**, **g**, **i**, and **j**, unpaired t -test with Welch's correction in **c**, **d**, **f**, and **h**; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$. Source data are provided as a Source Data file.

To further understand how mutant $p53$ modulates gene expression in hematopoietic cells, we performed H3K27me3 ChIP-seq assays in HSPCs from $p53^{+/+}$ and $p53^{R248W/+}$ mice. As expected, H3K27me3 is enriched at the transcription start site (TSS). Large regions of H3K27me3 enrichments are also found covering entire gene regions as well as intragenic regions. We found that $p53$ mutant HSPCs exhibited significantly higher levels of H3K27me3 at TSS compared to that of the WT HSPCs (Fig. 4e). The heat map of H3K27me3 ChIP-seq also revealed that many genes show increased H3K27me3 enrichment in mutant HSPCs compared to that of the WT HSPCs (Fig. 4f). The majority of H3K27me3 peaks (2582 out of 2669) in WT HSPCs are overlapped with H3K27me3 peaks in $p53$ mutant HSPCs (Supplementary Fig. 4a). By using the same enrichment threshold, we obtained 1232 additional peaks in mutant HSPCs. These peaks are likely targeted by H3K27me3 in WT cells but failed to reach the threshold. Indeed, these peaks show a similar pattern of fold enrichment between mutant and WT HSPCs (Supplementary Fig. 4b). We also observed increased H3K27me3 enrichment in other genes in $p53$ mutant HSPCs (Supplementary Fig. 4c, d).

Increased levels of H3K27me3 were found in genes regulating HSC self-renewal and differentiation, including *Cebpa* and *Gadd45g*^{43–45}, in $p53^{R248W/+}$ HSPCs compared to $p53^{+/+}$ HSPCs (Fig. 4f, g). The transcription factor C/EBP α is required for granulopoiesis and frequently disrupted in human AML. Loss of *Cebpa* enhances HSC repopulating capability and self-renewal^{43,44}. Tumor suppressor GADD45G induces HSC differentiation following cytokine stimulation, whereas loss of GADD45G enhances the self-renewal potential of HSCs⁴⁵. We confirmed that there were increased levels of H3K27me3 at both *Cebpa* and *Gadd45g* genes by ChIP experiments (Fig. 4h). Consistently, both *Cebpa* and *Gadd45g* were significantly down-regulated in $p53^{R248W/+}$ HSCs compared to $p53^{+/+}$ HSPCs (Fig. 4i, j).

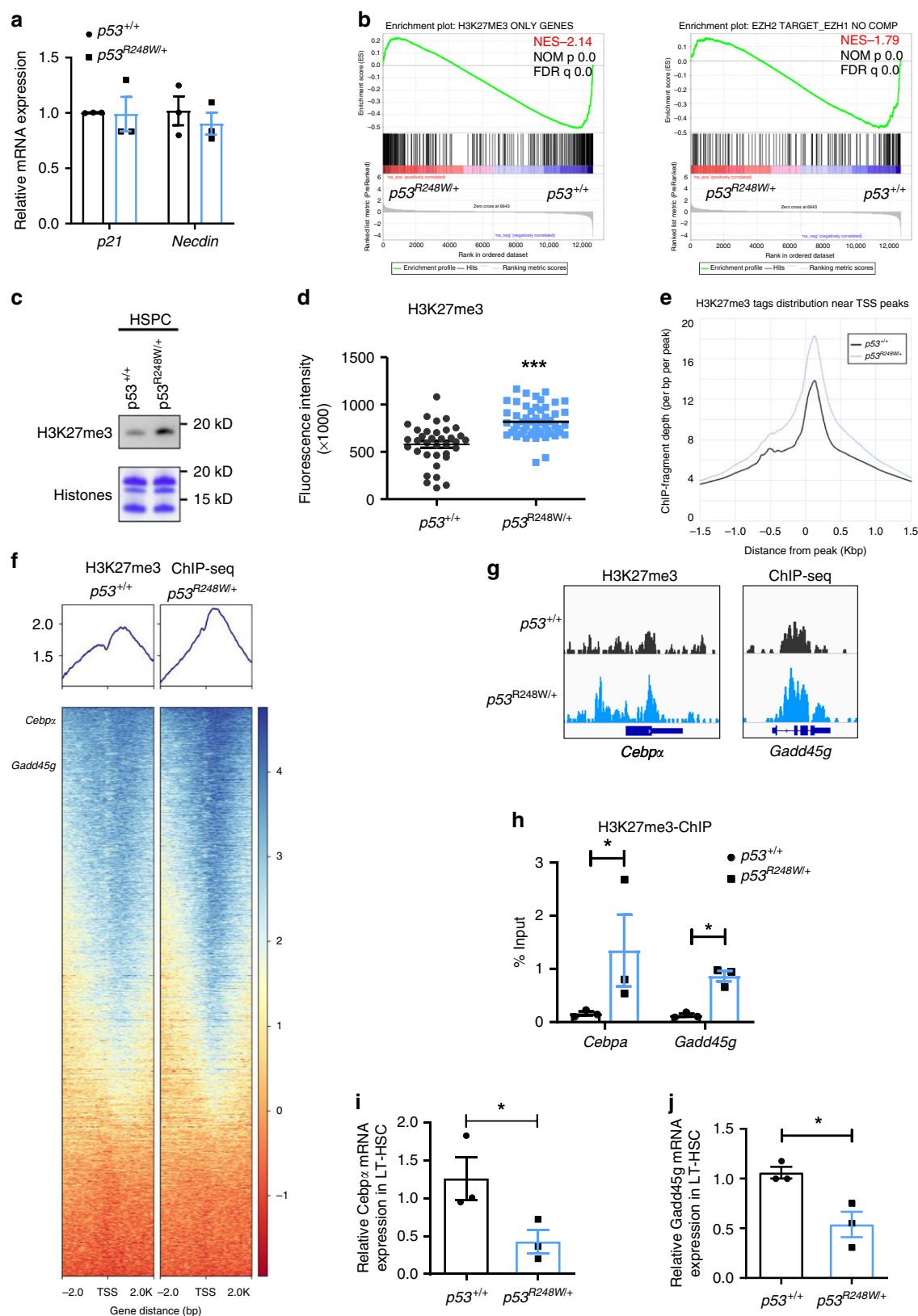
Stimulation of WT HSPCs with thrombopoietin (TPO) dramatically increased *Gadd45g* expression; however, TPO treatment only modestly increased *Gadd45g* expression in $p53$ mutant HSPCs (Supplementary Fig. 5a), suggesting that mutant $p53$ may repress *Gadd45g* expression upon cytokine stimulation. To determine the impact of *Gadd45g* on HSPC function in vitro, we introduced *Gadd45g* into BM cells from $p53^{+/+}$ and $p53^{R248W/+}$ mice using retroviruses and performed colony formation as well as transplantation assays. We found that ectopic *Gadd45g* expression decreases the colony formation of $p53$ mutant BM cells (Supplementary Fig. 5b). Further, ectopic *Gadd45g* expression decreased the engraftment of $p53$ mutant BM cells in vivo (Supplementary Fig. 5c). Given that loss of *Gadd45g* increases HSC self-renewal⁴⁵, it is possible that inactivation of *Gadd45g* is responsible for increased self-

renewal and colony formation seen in $p53$ mutant HSPCs. We also found that ectopic *Cebpa* expression decreases the colony formation of $p53$ mutant BM cells (Supplementary Fig. 5d). These data suggest that mutant $p53$ may repress gene expression in HSPCs through increasing the levels of H3K27me3.

Mutant $p53$ enhances the association of EZH2 with the chromatin. The PRC2 complex consists of EZH2/EZH1, EED, and SUZ12²⁷. While the levels of EZH2 was modestly increased in mutant HSPCs, the expression of other PRC2 core components was comparable between $p53$ WT and mutant HSPCs (Supplementary Fig. 6a). As the protein levels of PRC2 complex in mouse HSPCs were very low, we determined the impact of mutant $p53$ on the expression of PRC2 complex in murine hematopoietic progenitor 32D cells. We found that ectopic expression of mutant $p53$, but not WT $p53$, increased levels of H3K27me3 in 32D cells (Fig. 5a). However, ectopic expression of neither WT nor mutant $p53$ affected the protein levels of PRC2 core components in 32D cells (Supplementary Fig. 6b). Thus, the increased H3K27me3 in $p53$ mutant HSPCs may not be due to increased expression of catalytic components of PRC2, including EZH2 and EZH1, or other components of the PRC2 complex.

We then tested whether mutant $p53$ interacts with EZH2. We performed co-immunoprecipitation assays and found that several mutant $p53$ proteins, including $p53^{R248W}$, $p53^{R273H}$, and $p53^{R175H}$, displayed enhanced association with EZH2 compared to WT $p53$ (Fig. 5b). The recruitment and displacement of the PRC2 complex on chromatin are a dynamic process and tightly regulated to activate or repress transcription^{25–28}. Genome-wide H3K27me3 ChIP-seq assays revealed that the majority of H3K27me3 peaks in $p53$ mutant HSPCs are overlapped with that of the WT HSPCs (Fig. 4e, f, Supplementary Fig. 4a), suggesting that mutant $p53$ may enhance the association of EZH2 with the chromatin, thereby increasing the levels of H3K27me3. To test this, we examine the co-localization of $p53$ and *Ezh2* in $p53$ WT and mutant HSPCs utilizing the ImageStream flow cytometry analysis. The median fluorescent intensity (MFI) of $p53$ and *Ezh2* in the nucleus was comparable between $p53^{+/+}$ and $p53^{R248W/+}$ HSPCs (Supplementary Fig. 6c, d). However, we found that mutant $p53$, but not WT $p53$, show increased co-localization with *Ezh2* in the nucleus (Fig. 5c, d).

To determine whether mutant $p53$ enhances the association of *Ezh2* with the chromatin, we separated proteins in $p53$ WT and mutant HSPCs into cytosol, nuclear cytosol, and chromatin bound fractions. While EZH2 was present in the cytosol of both $p53$ WT and mutant HSPCs, we observed increased levels of *Ezh2* in the chromatin bound fraction of $p53$ mutant HSPCs compared to that of the $p53$ WT HSPCs (Fig. 5e). We then performed $p53$ and EZH2 ChIP assays in $p53^{+/+}$ and $p53^{R248W/+}$ HSPCs



(Lin⁻Kit⁺ cells) and found that both mutant p53 and EZH2 show increased association with *Cebpa* (Supplementary Fig. 6e, f). Thus, we demonstrate that mutant p53 interacts with EZH2 and enhances its association with the chromatin, thereby increasing the levels of H3K27me3 in HSPCs.

Inhibiting EZH2 decreases p53 mutant HSPC expansion. Hematopoietic-specific deletion of *Ezh2* impairs HSC self-renewal and terminal differentiation³⁰. To determine the functional impacts of mutant p53 and *Ezh2* interaction on hematopoiesis, we generated *p53*^{R248W/+} *Ezh2*^{fl/f} *Mx1Cre*⁺ mice³⁰. While *Ezh2*-

Fig. 4 EZH2 targets were significantly downregulated in p53 mutant HSPCs. **a** Quantitative reverse transcription polymerase chain reaction (RT-PCR) analysis of mRNA levels of p53 target genes, including *p21* and *Necdin*, in HSCs; $n = 3$ biological replicates. **b** Gene Set Enrichment Assays (GSEA) analysis shows that EZH2 targets were significantly downregulated in p53 mutant HSPC compared to $p53^{+/+}$ HSPCs. **c** p53 mutant HSPCs display increased levels of H3K27me3 (trimethylation at lysine 27 of histone H3) determined by immuno-blot analysis. **d** Lineage depleted HSPCs were stained with SLAM (signaling lymphocyte activation molecule) surface markers (CD48 and CD150) before fixation. Median fluorescence intensity of H3K27me3 in $p53^{+/+}$ and $p53^{R248W/+}$ HSCs (Lin⁻Sca1⁺Kit⁺CD48⁻CD150⁺ cells) was detected by ImageStream flow cytometry analysis. $p53^{+/+}$ $n = 35$ cells, $p53^{R248W/+}$ $n = 52$ cells. **e** H3K27me3 ChIP-seq (chromatin immunoprecipitation sequencing) tag density in $p53^{+/+}$ and $p53^{R248W/+}$ HSPCs, centered on TSS (transcription start site). **f** Heat map shows genes in HSPCs marked by H3K27me3. **g** Genome browser views of H3K27me3 ChIP-seq profiles of *Cebpa* (CCAAT/enhancer-binding protein alpha) and *Gadd45g* (growth arrest and DNA-damage-inducible 45 gamma). **h** H3K27me3 enrichment on *Cebpa* and *Gadd45g* genes in $p53^{+/+}$ and $p53^{R248W/+}$ HSPCs were examined by H3K27me3-ChIP assays; $n = 3$ independent experiments. **i** Quantitative RT-PCR analysis of mRNA levels of *Cebpa* in $p53^{+/+}$ and $p53^{R248W/+}$ LT-HSCs; $n = 3$ biological replicates. **j** Quantitative RT-PCR analysis of mRNA levels of *Gadd45g* in $p53^{+/+}$ and $p53^{R248W/+}$ LT-HSCs; $n = 3$ biological replicates. Data are represented as mean $\pm$ SEM. P -values were calculated using unpaired t test with Welch's correction in **a** and **d**, paired t -test in **h**, **i**, and **j**, and GSEA software in **b**; * $P < 0.05$, *** $P < 0.001$. Source data are provided as a Source Data file.

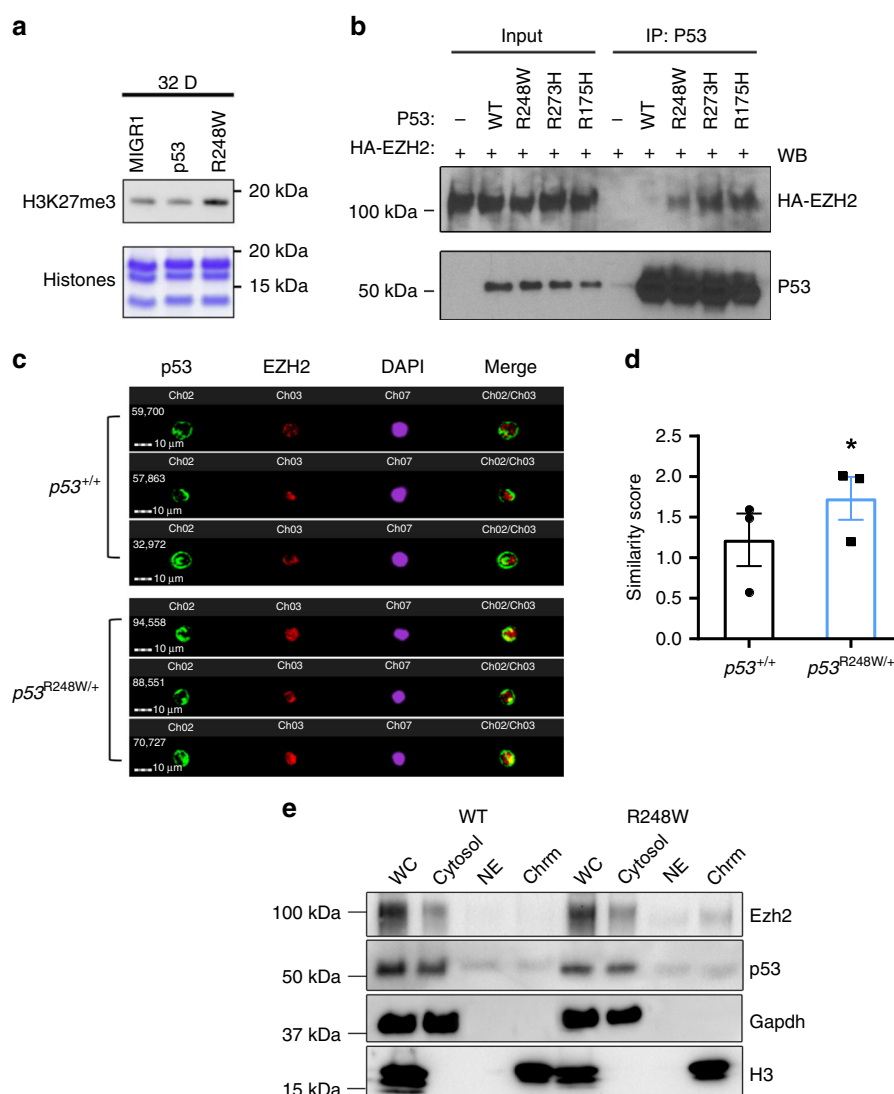


Fig. 5 Mutant p53 enhances the association of EZH2 with the chromatin in HSPCs. **a** 32D cells expressing mutant p53, but not wild-type (WT) p53, displayed increased levels of H3K27me3 as determined by immuno-blot analysis. **b** Several mutant p53 proteins, but not wild-type p53, show enhanced association with EZH2 as assayed by co-IP (co-immunoprecipitation) experiments. **c** Mutant p53 and EZH2 localization in HSPCs (Lin⁻Sca1⁺Kit⁺CD150⁺) as determined by ImageStream flow cytometry analysis. **d** Quantification of p53 and Ezh2 co-localization in the nucleus of HSPCs (Lin⁻Sca1⁺Kit⁺CD150⁺). A similarity feature determined the amount of overlay between p53 and Ezh2 within the DAPI mask. The higher the similarity score is, the more co-localized staining is within the nucleus; $n = 3$ biological replicates. **e** Cellular fractionation shows increased EZH2 association with the chromatin fraction in p53 mutant HSPCs. The absence of Gapdh (glyceraldehyde 3-phosphate dehydrogenase) and exclusive distribution of histone H3 in the chromatin fraction indicates no cross contamination between different cellular compartments. WC whole cell extract, Cyto cytosol, NE nuclear cytosol, Chrm chromatin. Data are represented as mean $\pm$ SEM. P -values were calculated using paired t -test in **d**; * $P < 0.05$. Source data are provided as a Source Data file.

deficiency did not affect *Cebpa* and *Gadd45g* expression, *Ezh2* deficiency brought the expression of *Cebpa* and *Gadd45g* back to normal in the mutant p53 background (Fig. 6a and Supplementary Fig. 7a).

To determine the impact of genetic inhibition of *Ezh2* on p53 mutant HSPC function in vitro, we performed serial replating assays using BM cells from $p53^{+/+}$, $Ezh2^{fl/+}$ -*Mx1Cre*⁺, $p53^{R248W/+}$, and $p53^{R248W/+}$ *Ezh2*^{fl/+}-*Mx1Cre*⁺ mice following polyinosinic:polycytidylic acid (pI:pC) treatment. We found that *Ezh2*-deficiency brings the replating potential of p53 mutant BM cells back to the WT cell level (Fig. 6b). To determine the impact of *Ezh2* deficiency on HSPC function in vivo, we performed competitive BM transplantations. We treated recipient mice with pI:pC at 8 weeks after transplantation to delete *Ezh2* and then examined donor cell engraftment every 4 weeks for 20 weeks. $p53^{R248W/+}$ BM cells exhibited a substantially higher contribution to PB production compared to $p53^{+/+}$ cells at 20 weeks following pI:pC treatment, whereas loss of *Ezh2* decreased the engraftment of mutant BM cells to the WT cell level (Fig. 6c). While both the frequency and the absolute number of donor-derived HSPCs in the BM of recipient mice repopulated with $p53^{R248W/+}$ BM cells was significantly higher than that of the WT cells, the frequency and the number of donor-derived HSPCs in the BM of recipient mice repopulated with $p53^{+/+}$ and $p53^{R248W/+}$ *Ezh2*^{-/-} BM cells were comparable (Fig. 6d, e). In addition, we observed decreased number of donor-derived CMPs and MEPs in the BM of recipient mice repopulated with $p53^{R248W/+}$ *Ezh2*^{-/-} BM cells compared to that of the $p53^{R248W/+}$ BM cells (Supplementary Fig. 7b, c), whereas the number of donor-derived GMPs was comparable in the BM of recipient mice repopulated with $p53^{R248W/+}$ cells and $p53^{R248W/+}$ *Ezh2*^{-/-} cells (Supplementary Fig. 7d). To determine the impact of *Ezh2*-deficiency on mutant HSC self-renewal, we performed secondary BM transplantation assays and found that *EZH2*-deficiency decreases the repopulating potential of $p53^{R248W/+}$ HSCs following secondary transplantation assays (Fig. 6f).

To determine the effect of pharmacological inhibition of *Ezh2* activity on mutant p53 HSPCs, we treated $p53^{+/+}$ and $p53^{R248W/+}$ BM cells with DMSO or *EZH2* specific inhibitor EPZ011989⁴⁶ and performed serial replating assays. While *EZH2* inhibitor had no effect on the colony formation of WT BM cells, *EZH2* inhibitor treatment decreased the replating potential of $p53^{R248W/+}$ BM cells to the WT level (Fig. 6g). Thus, we demonstrate that *EZH2* is important for mutant p53 HSPC functions both in vitro and in vivo.

Discussion

WT p53 is a transcription factor that activates the transcription of target genes to mediate DNA damage repair, growth arrest, or apoptosis^{13,47}. Most TP53 mutations observed in human cancers abrogate or attenuate the binding of p53 to its consensus DNA sequence (p53 responsive element) and impede transcriptional activation of p53 target genes¹⁴. However, we found that mutant p53 does not alter the expression of p53 target genes, including p21 and *Necdin*, in HSCs (Fig. 4a). Genome-wide transcriptome assays revealed that HSC and AML signatures are enriched in p53 mutant HSPCs, which is different from gene expression signatures regulated by the WT p53 protein^{20,47}. Thus, our findings provide experimental evidence that TP53 mutations identified in CHIP regulate gene expression in a distinct manner compared to WT p53.

Some mutant p53 proteins have been shown to promote cancer development through modulating gene transcription¹⁴. Dysregulated epigenetic control has been implicated in HSC aging and the pathogenesis of hematological malignancies^{16,48–50}. RNA-seq

assays revealed that *Ezh2* target genes are significantly down-regulated in $p53^{R248W/+}$ HSPCs compared to $p53^{+/+}$ HSPCs. We observed increased levels of H3K27me3 in p53 mutant HSPCs. Further, H3K27me3 ChIP-seq assays revealed that p53 mutant HSPCs exhibit significantly high levels of H3K27me3. Genes important for HSC self-renewal and differentiation, including *Cebpa* and *Gadd45g*^{43–45}, were occupied with increased levels of H3K27me3 in p53 mutant HSPCs. Thus, mutant p53 may enhance HSPC self-renewal through increasing the levels of H3K27me3 in genes involved in HSC self-renewal and differentiation.

Then, how does mutant p53 enhance H3K27me3 in HSPCs? One possible mechanism is that mutant p53 upregulates the expression of the core PRC2 components, thereby increasing PRC2 activity. However, the expression of the core PRC2 components was comparable between p53 WT and mutant HSPCs. Mutant p53 is incapable of binding to its normal binding sites and has been shown to be targeted by interactions with other transcription factors, including ETS family and SREBP^{14,38}. We discovered that several mutant p53 proteins show enhanced association with *EZH2* compared to WT p53. H3K27me3 ChIP-seq assays revealed that the increase in *EZH2*-dependent H3K27me3 is broad across genes, pointing toward an alternative *EZH2*/PRC2 targeting strategy or an increase in enzymatic activity but with normal targeting mechanisms. As shown in Supplementary Fig. 4a, 96.7% H3K27me3 peaks in WT cells also show H3K27me3 occupation in p53 mutant cells. Although additional peaks are enriched in p53 mutant cells, they are indeed associated with H3K27me3 in WT cells as well albeit lower enrichment. Further, we found that mutant p53 interacts with *EZH2* and enhances its association with the chromatin in HSPCs. Thus, mutant p53 appears to enhance H3K27me3 occupation rather than change its genome-wide distribution in HSPCs.

While the PRC2 complex controls dimethylation and trimethylation of H3K27, the Jumonji domain containing-3 (*Jmjd3*, *KDM6B*) and ubiquitously transcribed X-chromosome tetratricopeptide repeat protein (*UTX*, *KDM6A*) have been identified as H3K27 demethylases that catalyze the demethylation of H3K27me2/3^{51,52}. Decreased activity of *KDM6A*/*UTX* and *JMJD3* may be an alternative mechanism leading to increased *EZH2* activity. Future studies will be needed to investigate the potential impact of *UTX* and *JMJD3* on regulating H3K27me3 in p53 mutant HSPCs.

Most of *TP53* mutations in human cancer result in either partial or complete loss of tumor suppressor function¹⁴. Some mutant p53 proteins acquire new oncogenic properties that are independent of WT p53, known as the gain-of-function (GOF) properties¹⁴. Most GOF properties are believed to stem from binding of mutant p53 to cellular proteins such as transcription factors and altering their activity¹⁴. These (neomorphic) GOF properties can be experimentally demonstrated in the absence of a functional WT p53. Homozygous $p53^{R248W/R428W}$ and $p53^{R273H/R273H}$ mice developed novel tumors compared to $p53^{-/-}$ mice^{31,33}, demonstrating that some mutant p53 proteins have enhanced oncogenic potential beyond the simple loss of p53 function. WT p53 has not been shown to be associated with *EZH2* activity or H3K27me3. RNA-seq assays revealed that PRC2-related gene signature was not significantly different between $p53^{+/+}$ and $p53^{-/-}$ HSPCs, which is different from what we observed in p53 mutant HSPCs, suggesting that loss of WT p53 may affect neither *EZH2* activity nor H3K27me3 in HSPCs. Ectopic expression of mutant p53, but not WT p53, enhances H3K27me3 in 32D cells. Further, we found that several mutant p53 proteins show enhanced association with *EZH2* compared to WT p53. While both loss of p53 ($p53^{-/-}$) and mutant p53 ($p53^{R248W/+}$) enhances HSC repopulating potential, our data

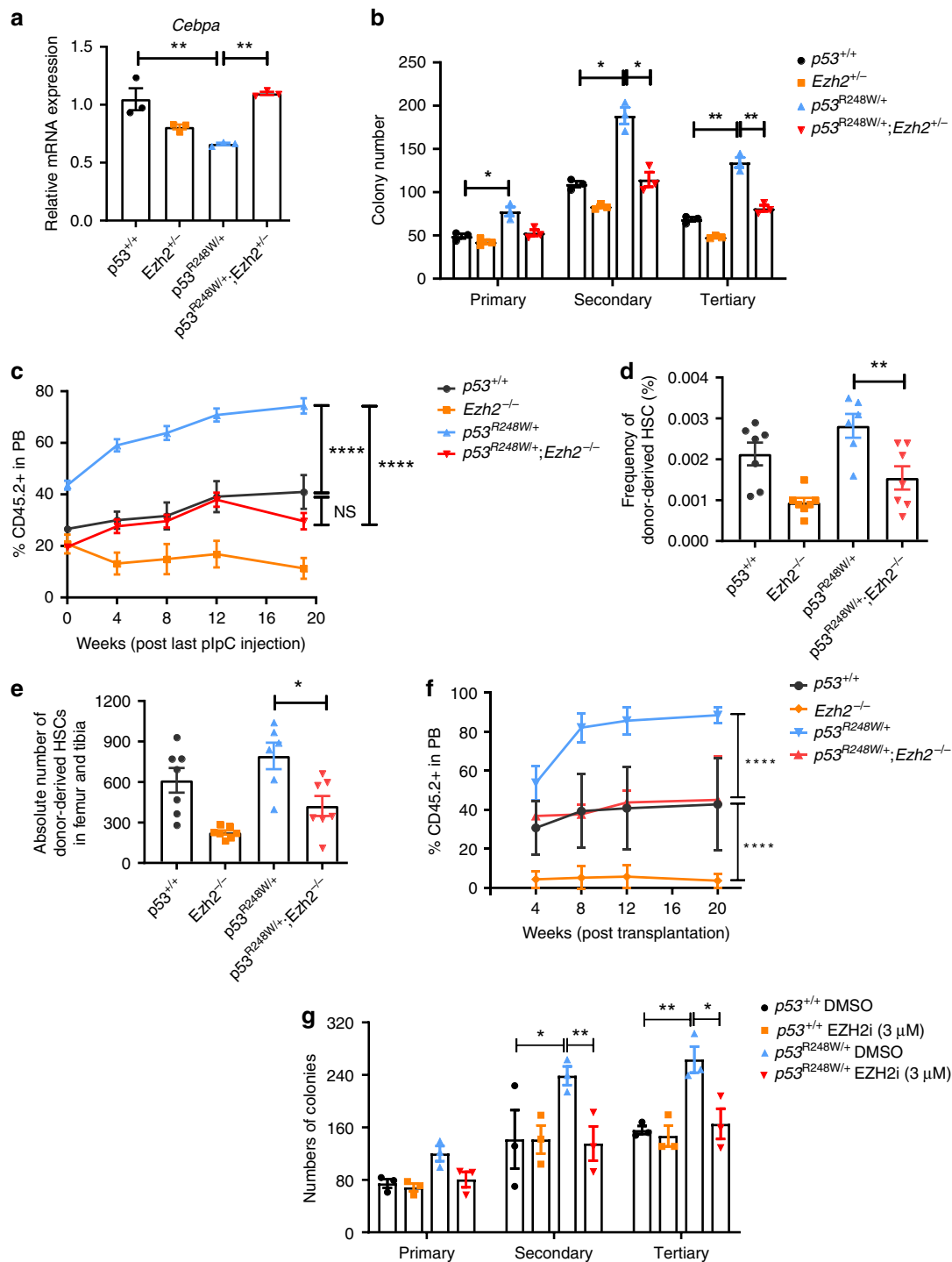


Fig. 6 Loss of EZH2 decreases the repopulating potential of p53 mutant HSPCs. **a** *Cebpa* expression in $p53^{+/+}$, $Ezh2^{-/-}$, $p53^{R248W/+}$, and $p53^{R248W/+}; Ezh2^{-/-}$ HSPCs; $n = 3$ biological replicates. **b** Serial replating assays of BM cells from $p53^{+/+}$, $Ezh2^{-/-}$, $p53^{R248W/+}$ and $p53^{R248W/+}; Ezh2^{-/-}$ mice; $n = 3$ independent experiments. **c** Percentage of donor-derived cells in the PB of recipient mice at 20 weeks following pl:pC (polyinosinic:polycytidylic acid) treatment; $n = 7$ mice per group. **d** Percentage of donor-derived HSCs in the BM of recipient mice at 20 weeks following pl:pC treatment; $n = 6-7$ mice per group. **e** The absolute number of donor-derived HSCs in the BM of recipient mice at 20 weeks following pl:pC treatment; $n = 6-7$ mice per group. **f** Percentage of donor-derived cells in the PB of recipient mice at 20 weeks following secondary transplantation; $n = 7$ mice per group. **g** Serial replating assays using $p53^{+/+}$ and $p53^{R248W/+}$ BM cells treated with DMSO (dimethyl sulfoxide) or EZH2 inhibitor (3 μ M); $n = 3$ independent experiments. Data are represented as mean $\pm$ SEM. P -values were calculated using one-way ANOVA with Tukey's multiple comparisons test in **a**, **d**, and **e**, two-way ANOVA with Tukey's multiple comparison test in **b**, **c**, **f**, and **g**; $*P < 0.05$, $**P < 0.01$, $****P < 0.0001$. Source data are provided as a Source Data file.

suggest that increased levels of H3K27me3 in p53 mutant HSPCs is likely due to the presence of the mutant allele, but not the result of losing WT p53 activity. While a dominant-negative (DN) effect has been shown to drive selection of *TP53* missense mutations in

myeloid malignancies⁵³, GOF mutant p53 appears to play an important role in myeloid leukemia³². Our work suggests that both DN and GOF properties may contribute to enhanced HSC self-renewal seen in $p53^{R248W/+}$ mice.

Clinical studies revealed that hematopoietic clones harboring specific mutations in individuals with CHIP may expand over time^{4–6}. However, how different cellular stressors affect clonal expansion is largely unknown. Recently, two different stressors, including hematopoietic transplantation and cytotoxic therapy, have been shown to expand hematopoietic clones^{10,12,22,35–37}. We discovered that *TP53* mutations identified in CHIP confer a competitive advantage to HSPCs following transplantation. *TP53* mutations are associated with prior exposure to chemotherapy^{10,12} and we observed that *TP53* mutations confer radiation resistance, leading to selective expansion of *TP53*-mutant HSPCs. Recently, *PPM1D* mutations were found in CHIP, especially in patients previously exposed to chemotherapy^{10,12,35,36}. *PPM1D* is a phosphatase that negatively regulates p53 and several proteins involved in the DDR pathway^{54,55}. While *PPM1D* mutations result in the expansion of *PPM1D*-mutant hematopoietic cells following chemotherapy treatment^{35,36}, they do not confer competitive advantage to HSPCs following BM transplantation^{12,35,36}. Thus, p53 and *PPM1D* appear to play distinct roles in driving clonal hematopoiesis.

While we have identified a stem cell intrinsic mechanism by which mutant p53 drives clonal hematopoiesis, recent studies indicate that mutations identified in CHIP may utilize cell extrinsic mechanisms to promote clonal hematopoiesis^{56,57}. We will investigate the cell extrinsic mechanisms by which mutant p53 drives CHIP in the future. Some individuals with CHIP developed AML with age^{2,3}. However, the role of mutant p53 in the initiation and progression of AML is largely unknown^{18,58}. We recently reported that mutant p53 synergizes with FLT3-ITD in leukemia development⁵⁹. We will elucidate the mechanisms by which mutant p53 drives leukemia development.

In summary, we discovered that *TP53* mutations drive clonal hematopoiesis in response to distinct cellular stressors. Mechanistically, mutant p53 interacts with EZH2 and enhances its association with the chromatin, increasing the levels of H3K27me3 in genes regulating HSPC self-renewal and differentiation. EZH2 is rarely mutated in CHIP^{4–6} and we found that genetic and pharmacological inhibition of EZH2 decrease the repopulating potential of p53 mutant HSPCs. Thus, our work will likely establish epigenetic regulator EZH2 as a novel therapeutic target for preventing CHIP progression and treating hematological malignancies with *TP53* mutations.

Methods

Mice. The HUPKI (*p53*^{+/+}) and *p53*^{R248W/+} mice used in our studies have been backcrossed to the C57BL/6 background for 12 generations^{22,33}. All young *p53*^{+/+}, *p53*^{+/-}, *p53*^{-/-}, and *p53*^{R248W/+} and *Ezh2*^{ΔF/Mx1-Cre} mice used in these studies are 8–12 weeks old and are tumor free. WT C57BL/6 (CD45.2⁺), B6.SJL (CD45.1⁺), and F1 mice (CD45.2⁺ CD45.1⁺) mice were obtained from an on-site core breeding colony. We have complied with all relevant ethical regulations for animal testing and research. All animal-related experiments have received ethical approval from the Indiana University Institutional Animal Care and Use Committee (IACUC). All mice were maintained in the Indiana University Animal Facility according to IACUC-approved protocols.

Generation of retroviruses and infection of HSPCs. Retroviral vectors were produced by transfection of Phoenix E cells with the MIGR1 control or MIGR1 full-length mutant p53 cDNA plasmids, according to standard protocols. Mouse HSPCs were infected with high-titer retroviral suspensions in the presence of Retronectin. Forty-eight hours after infection, the GFP-positive cells were sorted by FACS^{20,21}.

Stem and progenitor cell assays. Clonogenic progenitors were determined in methylcellulose medium (MethoCult GF M3234, StemCell Technologies) using 2 × 10⁴ BM cells per well (6-well plate). Colonies were scored after 7 days of the initial culture, and all cells were collected and washed twice in PBS. Subsequently cells were cultured in the same medium. Colony scoring and replating were repeated every 7 days for at least two times^{20,21}.

Flow cytometry. Murine HSPCs were identified and evaluated by flow cytometry using a single cell suspension of bone marrow mononuclear cells (BMMCs). Hematopoietic stem and progenitors are purified based upon the expression of surface markers: LT-HSC (Lin⁻ Sca1⁺ Kit⁺ CD48⁻ CD150⁺), ST-HSC (Lin⁻ Sca1⁺ Kit⁺ CD48⁻ CD150⁻), MPP (Lin⁻ Sca1⁺ Kit⁺ CD48⁺ CD150⁻), CMP (Lin⁻ Sca1⁻ Kit⁺ CD16/32^{low} CD34^{high}), GMP (Lin⁻ Sca1⁻ Kit⁺ CD16/32^{high} CD34^{high}), and MEP (Lin⁻ Sca1⁻ Kit⁺ CD16/32^{low} CD34^{low}). BM cells were obtained from tibia, femur, and iliac crest (6 from each mice) by flushing cells out of the bone using a syringe and phosphate-buffered saline (PBS) with 2 mM EDTA. Red blood cells (RBCs) were lysed by RBC lysis buffer (eBioscience) prior to staining. Experiments were performed on FACS LSR IV cytometers (BD Biosciences) and analyzed by using the FlowJo software (TreeStar).

Ki-67 staining. BM cells were stained for cell surface markers as described above. After staining, cells were washed with 0.2% BSA in PBS, fixed and permeabilized using Cytofix/Cytoperm buffer (BD Biosciences) and then incubated with PE-conjugated-antibody against Ki-67 (BD Biosciences) for more than 30 min on ice. Cells were washed, incubated with 4',6-diamidino-2-phenylindole (DAPI) (Sigma) and acquired using LSR IV flow cytometer machine^{20,21}. Data analysis was performed using FlowJo software.

Hematopoietic cell transplantation. For HSC transplantation, we injected 200 CD48⁻ CD150⁺ LSK cells from *p53*^{+/+}, *p53*^{+/-}, *p53*^{-/-}, and *p53*^{R248W/+} mice (CD45.2⁺) plus 3 × 10⁵ competitor BM cells (CD45.1⁺) into lethally irradiated F1 mice (CD45.1⁺ CD45.2⁺). The percentage of donor-derived (CD45.2⁺) cells in PB was analyzed every 4 weeks after transplantation as described above. Twenty weeks following transplantation, we harvested BM cells from recipient mice and performed flow cytometry analysis to evaluate HSC repopulating capability. For secondary transplantation assays, 3 × 10⁶ BM cells from mice repopulated with *p53*^{+/+}, *p53*^{+/-}, *p53*^{-/-}, and *p53*^{R248W/+} HSCs were transplanted into lethally irradiated F1 mice.

For the competitive BM repopulation assays, we injected 5 × 10⁵ BM cells from *p53*^{+/+} and *p53*^{R248W/+} mice (CD45.2⁺) plus 5 × 10⁵ competitor BM cells (CD45.1⁺) into 9.5 Gy lethally irradiated F1 mice (CD45.1⁺ CD45.2⁺). PB was obtained by tail vein bleeding every 4 weeks after transplantation, RBC lysed, and the PB mononuclear cells stained with anti-CD45.2 FITC and anti-CD45.1 PE, and analyzed by flow cytometry. Sixteen weeks following transplantation, BM cells from recipient mice were analyzed to evaluate donor chimerisms in BMs. For secondary transplantation, 3 × 10⁶ BM cells from mice reconstituted with *p53*^{+/+} or *p53*^{R248W/+} BM cells were injected into 9.5 Gy lethally irradiated F1 mice (CD45.1⁺ CD45.2⁺).

Limiting dilution assays. Different doses (10,000, 20,000, 40,000, 80,000) of BM cells from *p53*^{+/+} and *p53*^{R248W/+} mice (CD45.2⁺) together with 200,000 competitor cells (CD45.1⁺) were transplanted into lethally irradiated (9.5 Gy) F1 recipient mice (CD45.2⁺ CD45.1⁺). The percentage of donor-derived (CD45.2⁺) cells were analyzed 16-weeks following transplantation as described above. HSC frequency was calculated using L-Cal software (StemCell Technologies Inc.) and ELDA software (bioinf.wehi.edu.au/software/elda/). Poisson statistics was used to calculate the *P* value.

Homing assays. A total of 1 × 10⁷ *p53*^{+/+} and *p53*^{R248W/+} BM cells (CD45.2⁺) were injected into lethally irradiated recipient mice (CD45.1⁺). BM cells were harvested 18 h following injection and the frequency of donor-derived cells (CD45.2⁺) was evaluated by flow cytometry.

Quantitative real-time PCR. Total RNA was extracted from cells using RNeasy Plus Micro Kit (Qiagen) and cDNA was prepared from total RNA using SuperScript IV First-Strand cDNA Synthesis Kit (Invitrogen Life Technologies) and oligo (dT) primers, following manufacturer's instructions. qRT-PCR assay was performed by using the 7500 Real Time PCR machine (Applied Biosystems) with FastStart Universal SYBR Green Master (ROX) (Roche).

ImageStream flow cytometry analysis. To quantify γ-H2Ax foci in HSPCs, lineage-depleted BM cells were first stained with antibodies against appropriate HSPC surface markers, then fixed and permeabilized using the Cytofix/Cytoperm Kit (BD Biosciences), as described by the manufacturer, and finally stained with an Alexa-488-conjugated anti-γ-H2Ax antibody (Cell Signaling Technology).

To quantify the intensity of H3K27me3 in HSPCs, lineage-depleted BM cells were first stained with antibodies against HSPC surface markers, then fixed and permeabilized using the Cytofix/Cytoperm Kit (BD Biosciences), as described by the manufacturer, and finally stained with an Alexa-488-conjugated anti-H3K27me3.

For quantitative image analysis of p53 and Ezh2 co-localization within the nucleus, fluorescent cell images (×40) were acquired using an ImageStream flow cytometry system (Amnis; Seattle, WA, <http://www.amnis.com>). Between 171 and 554 Lin⁻ Sca1⁺ cKit⁺ CD150⁺ cell images were analyzed per sample using IDEAS software (Amnis; Seattle, WA, <http://www.amnis.com>). In focus cells were evaluated after gating on live, single, Lin⁻ Sca1⁺ cKit⁺ CD150⁺ cells. Utilizing DAPI staining, we were able to create a nucleus mask and instruct the program to only look at the staining of p53 and Ezh2 within the DAPI/nucleus mask. Bright detail intensity of

FITC-p53, PE-Ezh2, and DAPI staining was used to quantify mean and geo mean intensity and co-localization within the nucleus. A similarity feature determined the amount of overlay between p53 and Ezh2 within the DAPI mask. The higher the similarity score is, the more co-localized the staining within the nucleus.

Co-IP. H1299 cells (p53 null) were co-transfected with FLAG-HA-EZH2 and WT or mutant p53, respectively, or transfected with FLAG-HA-EZH2 alone. Nuclear extract (NE) was prepared from these cells and incubated with a polyclonal p53 antibody (FL393, Santa Cruz) prior to addition of protein G beads. After overnight incubation, beads were then washed five times and eluted with glycine (0.1 M, pH 2.0), and then neutralized by adding Tris solution (1.5 M, pH 8.8). The eluates were mixed with SDS sample buffer and analyzed by SDS-PAGE, followed by immunoblotting⁶⁰.

Cellular fractionation. Briefly, p53^{+/+} and p53^{R248W/+} HSPCs were harvested and lysed in Buffer A (10 mM Tris-HCl, pH 7.9, 1.5 mM MgCl₂, 10 mM KCl, 0.5 mM DTT, 0.2 mM PMSF, 1 µg/ml pepstatin A, 1 µg/ml leupeptin, 1 µg/ml aprotinin). The cell lysate was then homogenized by a dounce homogenizer for 10 strokes and centrifuged at 4 °C and 15,000×g for 10 min. The supernatant was saved as the cytosolic fraction. The pellet was washed with Buffer A and then re-suspended with Buffer C (20 mM Tris-HCl, pH 7.9, 25% glycerol, 420 mM NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA, 0.5 mM DTT, 0.2 mM PMSF, 1 µg/ml pepstatin A, 1 µg/ml leupeptin, 1 µg/ml aprotinin) and dounced for 10 strokes. Suspension was rotated at 4 °C for 30 min and centrifuged at 4 °C and 7500×g for 5 min. The supernatant was saved as the nucleoplasmic fraction. The pellet was saved as the chromatin fraction and re-suspended with Buffer C. Cell fractions were mixed with SDS sample buffer and heated at 95 °C for 5 min⁶¹. Whole-cell extraction, nucleoplasmic, and chromatin extractions were sonicated for 15 s using a probe sonicator before loading to SDS-PAGE.

RNA sequencing. Total RNA is extracted from LSKs using RNeasy MicroPlus Kit (Qiagen). Then the mRNA is enriched with the oligo(dT) magnetic beads (for eukaryotes), and is fragmented into short fragments (about 100 bp). With random hexamer-primer, the first strand of cDNA is synthesized, and then the second strand is synthesized. The double-strand cDNA is purified with magnetic beads. The ends of the double strand cDNA are repaired, and a single nucleotide A (adenine) is added to the 3'-ends. Finally, sequencing adaptors are ligated to the fragments. The ligation products are amplified with PCR. For quality control, RNA and library preparation integrity are verified using Agilent 2100 BioAnalyzer system and ABI StepOnePlus Real-Time PCR System. RNA sequencing library was then constructed and then sequenced with HiSeq 4000.

Gene Set Enrichment Analysis (GSEA) was performed on gene sets from the Molecular Signatures Database (MSigDB, <https://www.broadinstitute.org/msigdb>) and additional gene sets curated from publications. Gene sets with FDR q-value <0.05 were considered significantly enriched.

ChIP sequencing. Lin⁻Kit⁺ cells were fixed with 1% formaldehyde for 15 min and quenched with 0.125 M glycine. Chromatin was isolated by the addition of lysis buffer, followed by disruption with a Dounce homogenizer. Lysates were sonicated and the DNA sheared to an average length of 300–500 bp. Genomic DNA (Input) was prepared by treating aliquots of chromatin with RNase, proteinase K and heat for de-crosslinking, followed by ethanol precipitation. Pellets were resuspended and the resulting DNA was quantified on a NanoDrop spectrophotometer. An aliquot of chromatin (10 µg, spiked-in with 200 ng of Drosophila chromatin) was precleared with protein A agarose beads (Invitrogen). Genomic DNA regions of interest were isolated using 4 µg of antibody against Histone H3K27me3 (clone: 39155, Active Motif). Antibody against H2Av (0.4 µg) was also present in the reaction to ensure efficient pull-down of the spike-in chromatin⁶². Complexes were washed, eluted from the beads with SDS buffer, and subjected to RNase and proteinase K treatment. Crosslinks were reversed by incubation overnight at 65 °C, and ChIP DNA was purified by phenol-chloroform extraction and ethanol precipitation.

Illumina sequencing libraries were prepared from the ChIP and Input DNAs by the standard consecutive enzymatic steps of end-polishing, dA-addition, and adaptor ligation. After a final PCR amplification step, the resulting DNA libraries were quantified and sequenced on Illumina's NextSeq 500 (75 nt reads, single end). Reads were aligned consecutively to the mouse genome (mm10) and to the Drosophila genome (dm3) using the BWA algorithm (default settings). Duplicate reads were removed and only uniquely mapped reads (mapping quality >= 25) were used for further analysis. The number of mouse alignments used in the analysis was adjusted according to the number of Drosophila alignments that were counted in the samples that were compared. Mouse alignments were extended in silico at their 3'-ends to a length of 200 bp, which is the average genomic fragment length in the size-selected library, and assigned to 32-nt bins along the genome. The resulting histograms (genomic "signal maps") were stored in bigWig files. H3K27me3 enriched regions were identified using the SICER algorithm with a MaxGap parameter setting of 600 bp. Signal maps and peak locations were used as input data to Active Motifs proprietary analysis program, which creates Excel tables containing detailed information on sample comparison, peak metrics, peak locations, and gene annotations.

Statistical information. Statistical analysis was performed with GraphPad Prism 8 software (GraphPad software, Inc.). All data are presented as mean ± standard error of the mean (SEM). The sample size for each experiment and the replicate number of experiments are included in the figure legends. Statistical analyses were performed using Student's *t* test where applicable for comparison between two groups, and a one-way ANOVA test or two-way ANOVA was used for experiments involving more than two groups. Statistical significance was defined as **P* < 0.05, ***P* < 0.01, ****P* < 0.001, *****P* < 0.0001; ns, not significant.

Reporting summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability

All RNA-seq and ChIP-seq data from this study were deposited in the Gene Expression Omnibus (GEO) with the accession number of [GSE137126](https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE137126). The source data underlying all figures are provided as Source Data files. All other remaining data are available within the article and Supplemental Files, or available from the authors upon request.

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Author contributions

S.C., Z.G., and Y.L. conceived the concept, designed the experiments, analyzed and interpreted the data, and wrote the manuscript. S.C., Q.W., H.Y., M.L.C., S.V., S.C.N., R.G., C.Y., M.K., Z.G., A.F., D.H., S.Z.L., S.B., W.C., E.R.W., B.R., Z.C., and N.L. performed the experiments. Q.W. performed the ChIP-seq data analysis. H.G. and Yunlong L performed the RNA-seq data analysis. Y.S., T.N.W., D.C.L., H.S.B., L.D.M., G.H., R.K., M.C.Y., and H.E.B. provided reagents and constructive advice to the study.

Competing interests

The authors declare no competing interests.

Additional information

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Bmi1 Maintains the Self-Renewal Property of Innate-like B Lymphocytes

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The self-renewal ability is a unique property of fetal-derived innate-like B-1a lymphocytes, which survive and function without being replenished by bone marrow (BM) progenitors. However, the mechanism by which IgM-secreting mature B-1a lymphocytes self-renew is poorly understood. In this study, we showed that *Bmi1* was critically involved in this process. Although *Bmi1* is considered essential for lymphopoiesis, the number of mature conventional B cells was not altered when *Bmi1* was deleted in the B cell lineage. In contrast, the number of peritoneal B-1a cells was significantly reduced. Peritoneal cell transfer assays revealed diminished self-renewal ability of *Bmi1*-deleted B-1a cells, which was restored by additional deletion of *Ink4-Arf*, the well-known target of *Bmi1*. Fetal liver cells with B cell-specific *Bmi1* deletion failed to repopulate peritoneal B-1a cells, but not other B-2 lymphocytes after transplantation assays, suggesting that *Bmi1* may be involved in the developmental process of B-1 progenitors to mature B-1a cells. Although *Bmi1* deletion has also been shown to alter the microenvironment for hematopoietic stem cells, fat-associated lymphoid clusters, the reported niche for B-1a cells, were not impaired in *Bmi1*^{-/-} mice. RNA expression profiling suggested lysine demethylase 5B (*Kdm5b*) as another possible target of *Bmi1*, which was elevated in *Bmi1*^{-/-} B-1a cells in a stress setting and might repress B-1a cell proliferation. Our work has indicated that *Bmi1* plays pivotal roles in self-renewal and maintenance of fetal-derived B-1a cells. *The Journal of Immunology*, 2020, 204: 3262–3272.

Murine B-1 cells are innate-like mature B lymphocytes distinct from conventional adoptive immune B lymphocytes (B-2 cells). B-1 cells harbor unique characteristics, including specific surface markers (IgM^{high}IgD^{low}CD19^{hi}B220^{lo}), production of natural IgM Abs, and their primal localization in the

pleural and peritoneal cavities (1). One of the most striking characteristics of CD5⁺ B-1 cells (referred to as B-1a cells) is their unique origin and self-replenishing ability. Cell transfer studies using fetal liver (FL) and bone marrow (BM) progenitors demonstrated that only FL progenitors, not BM cells, efficiently reconstitute peritoneal B-1a cells upon transplantation (2). Lin⁻AA4.1⁺CD19⁺B220^{lo-neg} B-1-specific progenitors have been found in the FL, and neonatal BM and were shown to decline in number during aging, suggesting that B-1 cells are mostly derived from fetal and neonatal progenitors (3, 4) and represent a distinct lineage from conventional B-2 cells. Additionally, long-term hematopoietic stem cells (LT-HSCs) in the FL and adult BM reportedly failed to reconstitute peritoneal B-1a cells in transplantation assays (5, 6), indicating that some (if not most) B-1a cells develop independently of hematopoietic stem cells (HSCs) (7, 8) and are maintained throughout life without being replenished by adult HSC-derived progenitors (9). Finally, adoptive transfer of mature B-1a cells into congenic recipient mice was demonstrated to be sufficient to repopulate and maintain the B-1a cell compartment in the long term, supporting the notion that B-1a cells are maintained in vivo by self-renewal mechanisms.

The self-renewal ability is one of the most important features of stem cells. Both HSCs and neural stem cells rely on the *Bmi1* polycomb ring finger proto-oncogene (*Bmi1*) for their long-term self-renewal (10–12). BMI1 consists of the polycomb repressor complex 1 (PRC1), which represses gene expression. In *Bmi1*^{-/-} mice, the number of HSCs and all lymphoid cell subsets were shown to be significantly reduced, and the self-renewal ability of HSCs upon transplantation was lost (11). The *Ink4-Arf* locus is a known target of *Bmi1* in both HSCs and neural stem cells, and deletion of *Ink4-Arf* in *Bmi1*^{-/-} mice was demonstrated to dramatically correct the number and self-renewal ability of HSCs (13). Moreover, *Bmi1* has also been shown to regulate the regeneration of

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Abbreviations used in this article: B/J, Boy/J; BM, bone marrow; *Bmi1*^{F/F}, *Bmi1*-flox/flox; ChIP, chromatin immunoprecipitation; DE, differential expression; DKO, double knockout; F, forward; FALC, fat-associated lymphoid cluster; FL, fetal liver; HSC, hematopoietic stem cell; *Kdm5b*, lysine demethylase 5B; LT-HSC, long-term hematopoietic stem cell; MNC, mononuclear cell; NSG, NOD/SCID/IL-2R γ c^{-/-}; PRC1, polycomb repressor complex 1; qPCR, quantitative PCR; R, reverse; WT, wild-type.

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skeletal muscles (14) and the proliferation and self-renewal of intestinal stem cells (15). Additionally, it has been reported that overexpression of *Bmi1* induced an extensive capacity for self-renewal in adult BM erythroblasts to similar levels as those seen in embryo-derived self-renewing erythroblasts (16). We recently reported that overexpressing *Bmi1* in mouse embryonic stem cell-derived B-1 cells enhanced long-term engraftment in recipient mice upon transplantation (17). Therefore, we hypothesized that *Bmi1* might play an important role in the homeostatic ability of self-renewal in B-1a cells.

In this study, we report the critical role of *Bmi1* in the maintenance of B-1a cells in vivo. We found that, compared with levels in other lymphoid cell subsets, *Bmi1* is highly expressed in B-1a cells. We confirmed that the total number of T and B lymphocytes were reduced in the spleen and BM of *Bmi1*^{-/-} mice, as previously reported (18). However, the percentage and number of total peritoneal B-1a cells were specifically decreased at a higher rate than those of B-2 and B-1b cells. Importantly, in B cell-specific *Bmi1* knockout mice, only B-1a cells, not other mature B cell subsets (follicular, marginal zone, and B-1b B cells), showed a reduction in their number and frequency. Accordingly, *Bmi1*-deficient peritoneal B-1a cells lost their self-renewal ability upon transplantation, which was restored by overexpression of *Bmi1* or deletion of a well-known target of *Bmi1*, *Ink4-Arf*. Although this restoration by *Ink4-Arf* deletion is consistent with previous reports on HSCs (13), microarray analysis suggested lysine demethylase 5B (*Kdm5b*) as another novel candidate target gene for *Bmi1*. Our results indicated that mature B-1a lymphocytes use *Bmi1* for their maintenance of self-renewal ability.

Materials and Methods

Mice

All mice were bred and maintained under specific pathogen-free conditions in the Indiana University School of Medicine Laboratory Animal Resource Center and the Center for Laboratory Animal Medicine and Care at University of Texas Health Science Center at Houston. C57BL/6J, Boy/J (B/J), NOD/SCID/IL-2R γ ^{-/-} (NSG), *Bmi1*-flox/flox (*Bmi1*^{f/f}), and CD19-Cre knock-in mice (CD19^{Cre/Cre}) were purchased from The Jackson Laboratory. *Bmi1*^{f/f} mice were also provided by Dr. X. Liu (National Institute of Biological Science, Beijing, China). *Bmi1*^{-/-} mice were provided by Dr. M. van Lohuizen (The Netherlands Cancer Institute). *Ink4a-Arf*^{-/-} mice were provided by Dr. R. A. DePinho (MD Anderson Cancer Center). *Bmi1*^{-/-} and *Bmi1*^{-/-}*Ink4-Arf*^{-/-} mice had CD45.1⁺CD45.2⁺ or CD45.2⁺ (depending on the experiment). The experimental procedures were approved by the Institutional Animal Care and Use Committee at Indiana University and the Animal Welfare Committee in University of Texas Health Science Center at Houston.

Flow cytometry

FL, spleen, BM, and peritoneal cells were prepared as single-cell suspension. For analysis and sorting of hematopoietic subsets (Supplemental Table I), the following Abs were used at different fluorescent color combinations: anti-mouse AA4.1 (AA4.1), CD19 (1D3), B220 (RA3-6B2), IgM (II/41), CD21 (8D9), CD23 (B3B4), CD11b (M1/70), Gr1 (RB6-8C5), CD5 (53-7.3), CD3e (145-2C11), Ter119 (TER-119), c-kit (2B8), Sca-1 (D7), CD150 (TC15-12F12.1), CD48 (HM48-1), IL-7Ra (A7R34), and annexin V (all purchased from eBioscience or BioLegend). Cells were analyzed on LSR II or sorted on FACS Aria (Becton Dickinson).

Real-time PCR

Briefly, total RNA was extracted with RNeasy Micro (QIAGEN), followed by reverse-transcription with SuperScript III (Invitrogen). The input cDNA was standardized and then amplified with an ABI Prism 7500 HT (Applied Biosystems) with SYBR Green Master Mix (Applied Biosystems); the following primer sets were used: β -actin forward (F), 5'-CCTAAGGCCAACCCTGAAAAG-3' and reverse (R), 5'-CAGAGGCATACAGGGACAGCA-3'; *HPRT* F, 5'-TCCTCCTCAGACCGCTTTT-3' and R, 5'-CCTGGTTCATCATCGCTAATC-3'; *Bmi1* F, 5'-CAGGTTCAACAACACAGACCAC-3' and R, 5'-TGACGGGTGAGCTGCATAAA-3'; *Arf*

F, 5'-CATGTTGTGAGGCTAGAGAGGA-3' and R, 5'-CTGCACCGTAGTTGAGCAGA-3'; *Kdm5b* F, 5'-TCAGGGGACATTATGAGCGAA-3' and R, 5'-TCGGAGGTCAGGTTAGGCTC-3'; p21 F, 5'-AACATCTCAGGGCCGAAAAC-3' and R, 5'-TCCTACCCACAGCAGAGGA-3'; and *Bcl-2* F, 5'-TGAGTACCTGAACCGGCATCT-3' and R, 5'-TCAAACAGAGGTCGCATGCTG-3'.

Retrovirus production and infection

The knockdown plasmid pMKO was purchased from Addgene (no. 10676) and pMKO-shLuc or -sh-Kdm5b (sh1-3) were prepared with standard cloning strategy. For retrovirus production, pMigR1-Mock or -*Bmi1* or knockdown plasmids were transfected into Phoenix-ECO producer cells respectively, followed by collection and concentration of the virus supernatant with the standard method (19). The established spin-infection was used for infection into FL cells. Target sequences are the following: control (luciferase) 5'-TAAGGCTATGAAGAGATAC-3', Ms_Kdm5b-sh1 5'-GCAGAGGC-TATGAATATTA-3', Ms_Kdm5b-sh3 5'-CGCCAGTGTGTGGAGCA-TTA-3', and Ms_Kdm5b-shB 5'-GAGATGCACTCCGATACAT-3'.

Transplantation

For peritoneal cell transplantation, donor peritoneal cells (CD45.2 or CD45.1⁺CD45.2⁺) were injected into the peritoneal cavity of sublethally irradiated (250 rad) NSG mice (CD45.1). FL lineage⁻ cells with or without *Bmi1*-expressing vector were injected into the tail vein of lethally irradiated (950 rad) B/J mice. FL B-1 progenitor cells were injected into the peritoneal cavity of sublethally irradiated (150 rad) NSG neonates.

Microarray analysis

Peritoneal B-1a cells were collected from *Bmi1*^{-/-} and wild-type (WT) mice, and RNA was extracted using RNeasy Micro Kit (QIAGEN) and submitted to Miltenyi Biotec for gene expression analysis using Agilent Whole Mouse Genome Oligo Microarrays. Each seven samples were examined. Complete raw dataset and normalized data were produced by Miltenyi Biotec. The microarray data were submitted to Gene Expression Omnibus (accession code: GSE97202; <https://www.ncbi.nlm.nih.gov/geo/>).

Bioinformatics analysis of microarray

The normalized expression data from Miltenyi Biotec were first filtered by the expression level with a cutoff value of 5. If a gene is expressed higher than this cutoff level in four or more WT/knockout samples, it is kept for the downstream differential expression (DE) analysis. The DE analysis was performed on Partek Genomic Suite (Partek, St. Charles, MO). We then calculated Benjamini-Hochberg multitest adjusted false discovery rate based on the *p* values from the DE analysis. Genes with a false discovery rate <0.2 were used for the Ingenuity Pathway Analysis.

For hierarchical clustering, filtered data were imported into GenePattern (v. 3.9.9; Broad Institute) for hierarchical clustering. Clustering and distance measurements were calculated using pairwise complete linkage and Pearson correlation. Output was used to render heat map data.

Chromatin immunoprecipitation

Chromatin immunoprecipitation (ChIP) was done with standard protocols. DNA from Baf-3 cell (1×10^7) was cross-linked with 1% formaldehyde and was sheared into fragments ~200–500 bp in length with a sonicator (505 Sonic Dismembrator; Thermo Fisher Scientific). All immunoprecipitation used 5 μ g control IgG, anti-Bmi1 (AF27; Active Motif) or ring finger protein 1b (Ring-1b) (D139-3; MBL International) Ab with Protein A Dynabeads (Invitrogen). Quantification of precipitated genomic DNA relative to input was done in triplicate after real-time PCR with SYBR Green Master Mix (Applied Biosystems). The following primer sets were used: Ms_Kdm5b/ChIP+700 F, 5'-GTCTGGAGCGGCTGGTTGAG-3' and R, 5'-CCCACATCTCTCAAAGTGTGCG-3'; Ms_Kdm5b/ChIP-200 F, 5'-GTCTGTCC-TTGCTGCTCCTTG-3' and R, 5'-AAACCCGAGAAGCAGAGTACT-3'; Ms_Kdm5b/ChIP-2000 F, 5'-TCAGGCTCCAAATCCCTGTAGA-3' and R, 5'-GCTCTATCGAAGTACCTGGCC-3'; Ms_Kdm5b/ChIP-3300 F, 5'-ACTGAGATGGTGCATTGCTGA-3' and R, 5'-CCTGGCCTTACTGT-TAGTGCG-3'; Ms_Arf/ChIP F, 5'-AAAACCCCTCTTGTGAGTGGG-3' and R, 5'-GCAGGTTCTTGGTCACTGTGAG-3'; Ms_Ink4a/ChIP F, 5'-GCCCGAGAAATCCTAGAGATCC-3' and R, 5'-GGATTCTCTAGGA-TTTCTCGGGC-3'; and Ms_GAPDH/ChIP F, 5'-CCCACTTGCCTCTGTAT-TGG-3' and R, 5'-CTGTGGGGAGTCCTTTTCAG-3'.

Fat-associated lymphoid cluster staining

Fat tissues were removed from the peritoneal cavity and fixed with 4% paraformaldehyde for 1 h at 4°C. The samples were stained with 1:200

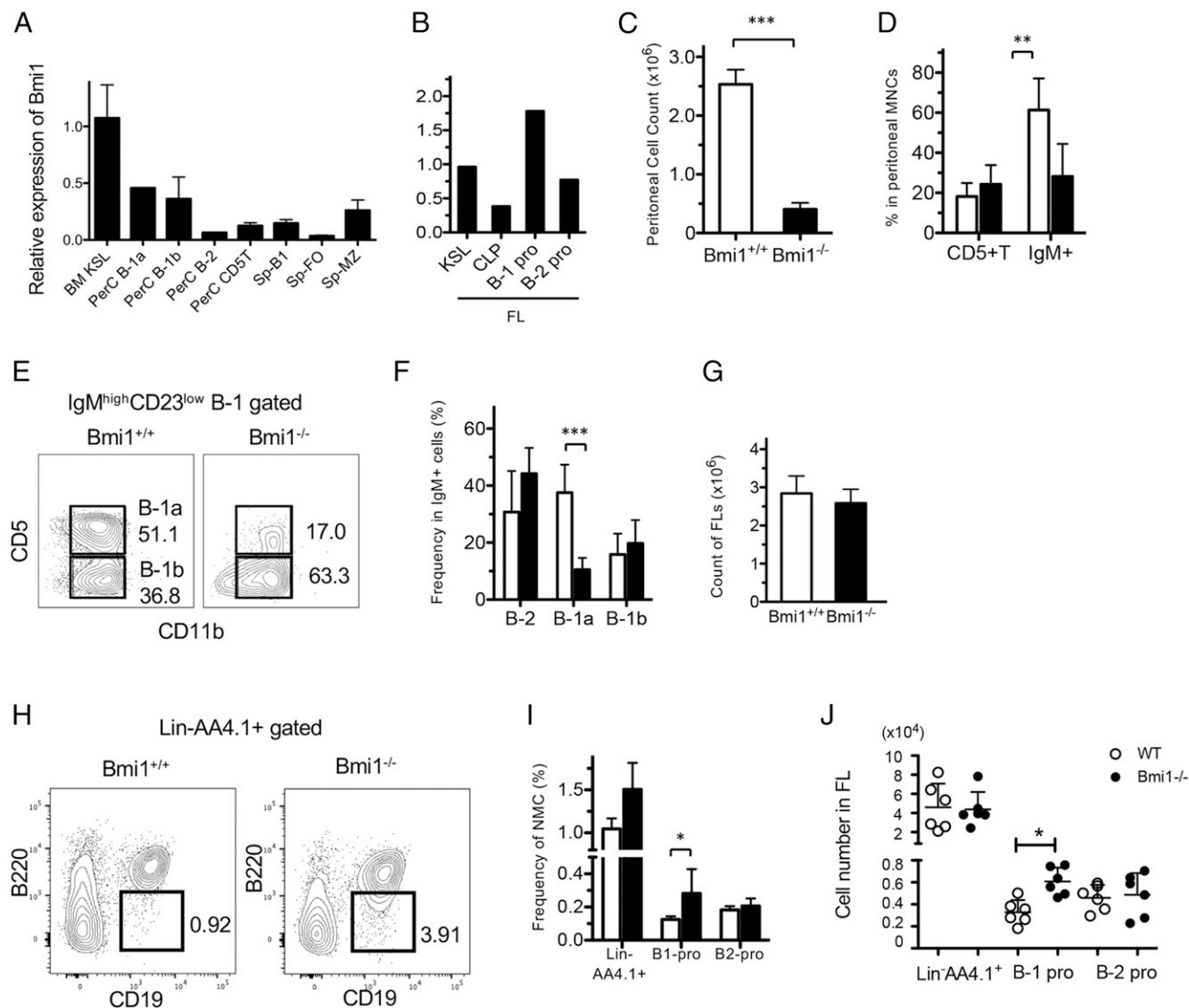


FIGURE 1. Peritoneal B-1a cells have higher *Bmi1* dependency than other lymphoid subsets. (A) *Bmi1* mRNA expression measured by qPCR in various sorted populations from adult BM, peritoneal cells (PerC), or spleen (Sp). (B) *Bmi1* expressions in various FL progenitor populations. (C) Marked reduction of PerC count in *Bmi1*^{-/-} mice ($n = 6$). (D) The frequency of IgM⁺ B cell and CD5⁺ T cells in the WT and *Bmi1*^{-/-} peritoneal cavity is depicted ($n = 5$). (E) Representative FACS plots for peritoneal B-1a cells in WT and *Bmi1*^{-/-} mice are depicted. (F) The frequency of B cell subsets among the peritoneal IgM⁺ cells is depicted. The percentage of B-1a cells is reduced ($n = 5$). (G–J) Total cell number of FLs (G), representative FACS plots for Lin⁻AA4.1⁺CD19⁺B220⁺ B-1 progenitor population in the FL (H), and the frequency (I) and absolute number (J) of FL B-1 and B-2 progenitor cells in WT and *Bmi1*^{-/-} embryos are depicted. B-1 progenitor cell number is increased in the *Bmi1*^{-/-} FL ($n = 6$). * $p < 0.05$. White bar and circle, WT; black bar and circle, *Bmi1*^{-/-}. All data were obtained from experiments more than three times. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. CLP, common lymphoid progenitor; FO, follicular B cell; KSL, kit⁺Sca-1⁺lin⁻ HSC population; MZ, marginal zone B cell.

Alexa Fluor 555-conjugated anti-mouse IgM Ab (SouthernBiotech) and FITC-conjugated anti-CD31 Ab or FITC-conjugated anti-CD11b Ab in 0.1% Triton X-100-PBS for 1 h at room temperature. Then Alexa Fluor 555⁺ (red) areas were dissected out under a Leica mz9.5 fluorescent stereomicroscope and then mounted on Superfrost Plus Gold Slides (Thermo Fisher Scientific) with ProLong Gold Antifade solution with DAPI (Thermo Fisher Scientific). Confocal images were taken using an Olympus II microscope with UAp0N340 20×/0.7W objectives (Olympus). ImageJ software was used to adjust and output the images.

Ag stimulation assays

The sorted B-1a cells (20–30 × 10³) from each genogroup were plated in the 96-well plate in IMDM with 10% FBS, 10 ng/ml IL-5, 5 μg/ml R848 (TLR7/8 ligand), and 5 μg/ml LPS. Forty-eight hours later, cells were collected, and cell numbers were counted.

Statistical analysis

Student *t* test was used for all statistical analysis.

Results

B-1a cells show higher dependency on *Bmi1* than do other lymphoid subsets

We examined the expression of the *Bmi1* gene by quantitative PCR (qPCR) in various FACS-sorted lymphocyte subsets as shown in Supplemental Table I. Notably, *Bmi1* was highly expressed in peritoneal B-1a and B-1b cells compared with other lymphocyte subsets (Fig. 1A). In the FL, *Bmi1* was highly expressed in B-1-specific progenitors (Lin⁻AA4.1⁺CD19⁺B220⁺) compared with other progenitor subsets (Fig. 1B). Although previous reports have indicated that the lymphoid compartment is globally reduced in the lymphoid organs of *Bmi1*^{-/-} mice, the effect of *Bmi1* deletion on peritoneal B-1 cells has not been reported. Therefore, we examined the peritoneal cavity and found that the total number of peritoneal cells was markedly reduced in *Bmi1*^{-/-} mice (Fig. 1C).

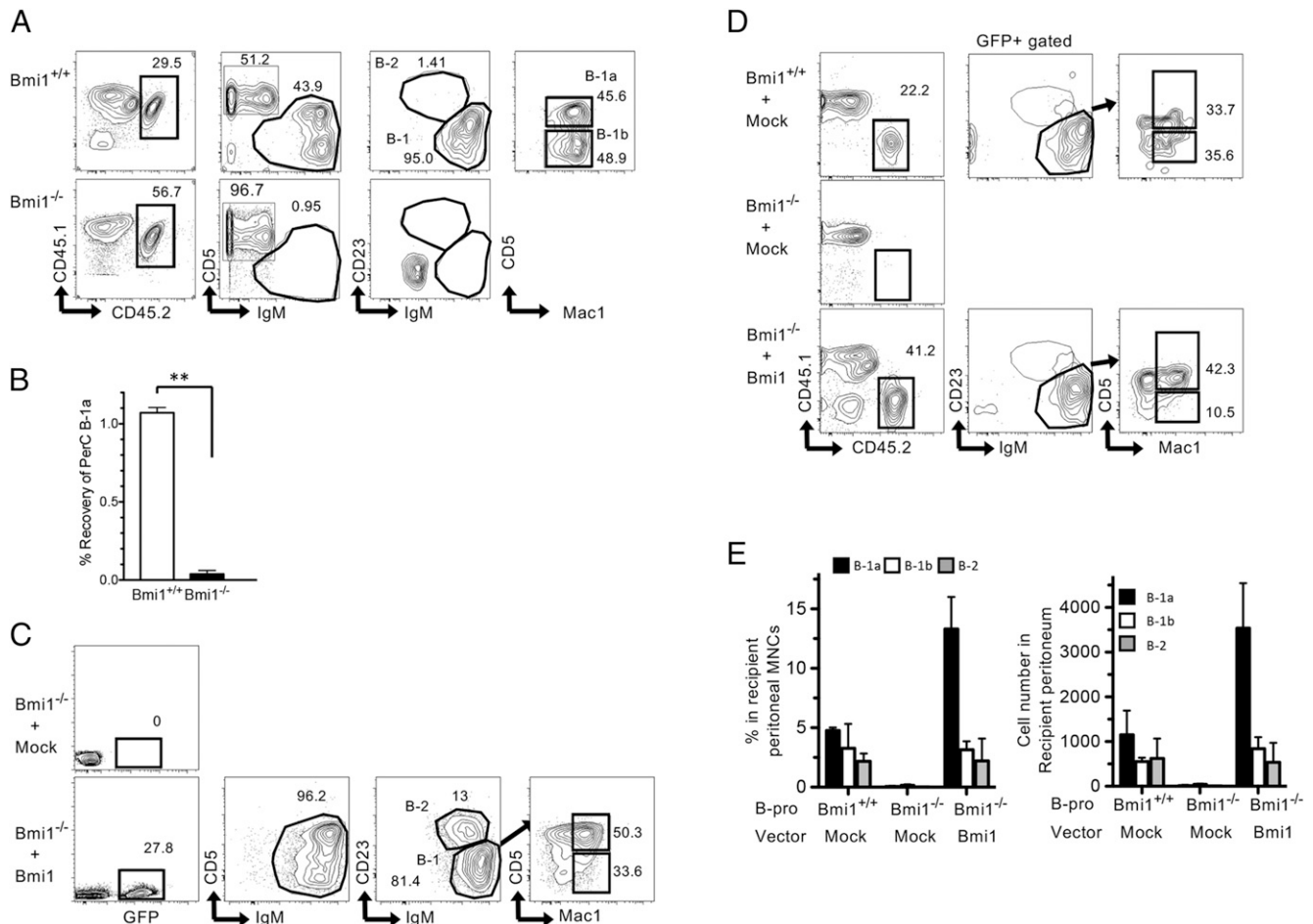


FIGURE 2. Loss of B-1a self-renewal by *Bmi1* disruption. **(A)** Representative FACS plots of recipient peritoneal cells at 12 wk posttransplant. The peritoneal cells of the recipient NSG mice transplanted with *Bmi1*^{+/+} (upper panel) or *Bmi1*^{-/-} (lower panel) peritoneal cells are depicted ($n = 3$). **(B)** Recovery ratio of WT and *Bmi1*^{-/-} B-1a cells after transplantation, calculated by (number of recovered B-1a cells)/(number of injected B-1a cells) 12 wk after transplantation ($n = 3$ for each group). Around 4,000–80,000 B-1a cells were injected. $**p < 0.01$. **(C)** Retrovirus with mock or *Bmi1* vector was infected into *Bmi1*^{-/-} FL Lin⁻ cells. Subsequently, infected cells were transplanted into sublethally irradiated (250 rad) NSG mice. Representative FACS plots at 4 mo posttransplant are depicted ($n = 3$). **(D)** WT and *Bmi1*^{-/-} FL B-1 progenitor cells with a mock or *Bmi1*-overexpressing retrovirus were injected into sublethally irradiated NSG mice. The recipient peritoneal cell analysis 4 mo after transplantation is depicted ($n = 3$). **(E)** The percentage (left) and cell number (right) of donor-derived B cell population in the peritoneal cavity of NSG mice transplanted with *Bmi1*^{-/-} FL B-1 progenitor cells with or without *Bmi1*-overexpressing vector are depicted ($n = 3$). All data were obtained from experiments more than three times.

Although the frequency of CD5⁺ T cells was not altered, the frequency of IgM⁺ B cells was significantly reduced (Fig. 1D). Interestingly, among the peritoneal IgM⁺ cells, the percentage of B-1a cells was specifically reduced, whereas B-2 and B-1b subsets showed similar percentages to those in WT mice (Fig. 1E, 1F). The absolute number of B-1a cells was also dramatically reduced. Both the frequency and absolute number of B-1 progenitors in the *Bmi1*^{-/-} FL were modestly increased compared with those of WT FL (Fig. 1G–J). Thus, loss of *Bmi1* resulted in a profound reduction in both the frequency and absolute number of peritoneal B-1a cells.

Loss of *Bmi1* impairs self-renewal ability of peritoneal B-1a cells

B-1a cells have a self-renewal ability; their numbers are maintained without being replenished by BM progenitors. Because *Bmi1* is crucial for the self-renewal capacity of various stem cells (10–15), we hypothesized that *Bmi1* might also play a critical role in self-renewal ability of B-1a cells. Accordingly, we measured the self-renewal ability of peritoneal B-1 cells using transplantation assays in which peritoneal cells containing the same number of B-1a cells (4,000–80,000 cells) from either WT or *Bmi1*^{-/-} mice

were transplanted into sublethally irradiated (250 rad) NSG mice. Our results showed that 3 wk after transplantation, WT donor peritoneal B-1 cells repopulated both B-1a and B-1b subsets, whereas *Bmi1*^{-/-} donor B-1a cells showed poor engraftment (Supplemental Fig. 1A). Furthermore, 12 wk after transplantation, WT B-1a cells were fully reconstituted in the recipient peritoneal cavity, whereas *Bmi1*^{-/-} B-1a cells were diminished (Fig. 2A, 2B). Taken together, these results indicate that B-1a cells lost the self-renewal ability in the absence of *Bmi1*.

Overexpression of *Bmi1* rescues the self-renewal ability of B-1a cells

To confirm that *Bmi1* deficiency is the primary cause of the defect in B-1a cell self-renewal in *Bmi1*^{-/-} mice, we retrovirally overexpressed *Bmi1* in *Bmi1*^{-/-} FL lineage-negative cells (Supplemental Fig. 1B) and transplanted them into sublethally irradiated NSG mice. Although *Bmi1*^{-/-} FL cells transduced with mock vector failed to reconstitute in recipient mice, *Bmi1*^{-/-} FL cells overexpressing *Bmi1* efficiently repopulated all lineages in the BM, including HSCs (Supplemental Fig. 1C). Donor-derived B-1a, B-1b, and B-2 cells were all reconstituted in the peritoneal cavity of the recipient (Fig. 2C).

Next, *Bmi1*^{-/-} FL B-1-specific progenitors retrovirally transduced with *Bmi1* were injected into sublethally irradiated NSG mice to determine the requirement for *Bmi1* in B-1 progenitors. We found that 4 mo after transplantation, *Bmi1*^{-/-} B-1 progenitors transduced with mock vector failed to repopulate B-1a cells, as expected (Fig. 2D). However, *Bmi1*-overexpressing *Bmi1*^{-/-} B-1 progenitors exhibited efficient B-1a cell repopulation in recipient NSG mice (Fig. 2E). Interestingly, donor-derived B-1a cells were more frequent in mice transplanted with *Bmi1*-expressing *Bmi1*^{-/-} B-1 progenitors relative to those transplanted with WT B-1 progenitors (Fig. 2D). Thus, *Bmi1* overexpression not only restored, but also enhanced the self-renewal ability of *Bmi1*^{-/-} B-1a cells.

B cell-restricted deletion of Bmi1 leads to B-1a-specific reduction and loss of self-renewal ability

To exclude the potential influence of defects in either HSCs or the microenvironment on the reduction of B-1a cells in *Bmi1*^{-/-} mice, we used a mouse model in which *Bmi1* was conditionally deleted in the B cell lineage by crossing *CD19*^{Cre/+} and *Bmi1*^{F/F} mice. Notably, because homozygous *CD19*^{Cre/Cre} knock-in mice show defects in B-1a cells (20–22), we used *CD19*^{Cre/+} heterozygous mice to examine B cell-specific depletion of *Bmi1* to avoid any confusing results. Efficient deletion of *Bmi1* alleles was confirmed in sorted peritoneal B-1 cells from *CD19*^{Cre/+}*Bmi1*^{F/F} mice by genomic PCR, as well as by Western blotting of sorted spleen B220⁺ cells, including B-1, marginal zone, and follicular B cells (Fig. 3A, 3B).

Because CD19 is known to play a key role in BCR signaling, we investigated any adverse effects of CD19 heterozygosity on B-1a cells by comparing the frequency and absolute numbers of B-1a cells and other lymphoid subsets in the peritoneal cavity and spleen of WT, *CD19*^{Cre/+}, *CD19*^{Cre/+}*Bmi1*^{F/+}, and *CD19*^{Cre/+}*Bmi1*^{F/F} mice at different developmental ages (Fig. 3C). Although the numbers of peritoneal B-1a cells were significantly lower in any of the *CD19*^{Cre/+} groups at <3 mo old compared with those in WT mice, after 3 mo, only *CD19*^{Cre/+}*Bmi1*^{F/F} knockout mice showed a significant reduction in the numbers of peritoneal B-1a cells among the four groups (Fig. 3C, 3D). When we compared the numbers of B-1a cells in *CD19*^{Cre/+}*Bmi1*^{F/+} control and *CD19*^{Cre/+}*Bmi1*^{F/F} mice in the same litter, the reduction of B-1a cells was clear (Fig. 3E). Therefore, we evaluated the effect of B cell-specific deletion of *Bmi1* in mice older than 3 mo. The frequency and absolute number of peritoneal and splenic B-1a cells were significantly reduced in *CD19*^{Cre/+}*Bmi1*^{F/F} mice among the four groups, whereas no significant change was seen in the other B cell subsets (B-1b and B-2 cells) (Fig. 3F–H, Supplemental Fig. 2A). In addition, no significant change was observed in FL B-1 progenitors (Supplemental Fig. 2B).

To compare the self-renewal ability of B-1a cells from *CD19*^{Cre/+}*Bmi1*^{F/F} and WT mice in the same microenvironment, we performed competitive transplantation assays. To this end, 1×10^5 peritoneal B-1a cells from *Bmi1*^{F/F} or *CD19*^{Cre/+}*Bmi1*^{F/F} mice (CD45.2) were injected into the peritoneal cavities of NSG mice along with an equal number of congenic B/J WT B-1a cells (CD45.1, competitor). We noted that 12 wk after the injection, *CD19*^{Cre/+}*Bmi1*^{F/F} B-1a cells were able to reconstitute only ~8% of total B-1a cells in recipient mice, whereas both the control *Bmi1*^{F/F} and WT B/J B-1a cells achieved comparable levels of reconstitution (Fig. 3I, 3J). Thus, the self-renewal ability of B-1a cells was severely impaired in the absence of *Bmi1*.

The in vivo environment in Bmi1^{-/-} mice permits development and maintenance of B-1a cells

The difference between the severities of the reductions in B-1a cells of *Bmi1*^{-/-} and *CD19*^{Cre/+}*Bmi1*^{F/F} mice led us to hypothesize

that the microenvironment supporting maintenance of B-1a cells might be altered in *Bmi1*^{-/-} mice, similar to the impaired *Bmi1*^{-/-} BM environment that reportedly failed to support HSC self-renewal (13, 23, 24). Fat-associated lymphoid clusters (FALCs) have been reported as a niche for B-1 cells (25, 26), with IgM⁺ cell colonies surrounded by CD31⁺ endothelial cells (Fig. 4A). In *Bmi1*^{-/-} mice, the IgM⁺ lymphoid clusters/colonies were smaller in size and number than were those in WT mice ($p < 0.01$; Fig. 4B, 4C). Interestingly, FALCs in the omentum of *Bmi1*^{-/-}*Ink4a-Arf*^{-/-} mice were partially rescued in size compared with those in *Bmi1*^{-/-} mice (Fig. 4D, 4E). However, it was not clear whether the reduced number and size of FALCs in *Bmi1*^{-/-} mice was the result of environmental defects or of the reduction of B-1a cells itself. To investigate this, we examined the peritoneal environment of *Bmi1*^{-/-} mice using reciprocal transplantation assays. Sorted WT B-1a cells were injected into the peritoneal cavity of sublethally irradiated *Bmi1*^{-/-} and *Bmi1*^{F/F} mice. Against our expectations, the percentage of donor-derived B-1a cells was greater in *Bmi1*^{-/-} than in *Bmi1*^{F/F} recipient mice, although the actual cell numbers were similar (Fig. 4F, 4G). These results indicated that there were no environmental defects related to the engraftment/maintenance of B-1a cells in the peritoneal cavities of *Bmi1*^{-/-} mice.

Development of B-1a cells from FL progenitors is impaired in the absence of Bmi1

Next, to understand the effect of *Bmi1* deletion during development of B-1a cells, we transplanted E14.5 FL mononuclear cells (MNCs) containing B-1 progenitors from *Bmi1*^{F/F}, *CD19*^{Cre/+}, or *CD19*^{Cre/+}*Bmi1*^{F/F} embryos into sublethally irradiated adult NSG mice. Accordingly, 9 mo after transplantation, *CD19*^{Cre/+}*Bmi1*^{F/F} FL MNCs failed to repopulate the peritoneal B-1a cells but successfully repopulated the B-2 and B-1b cells to the same levels as did *Bmi1*^{F/F} FL MNCs (Fig. 3L). Strikingly, defects in the reconstitution of B-1a cells in both the peritoneum and spleen of recipient mice could be observed as early as 6 wk after transplantation (Fig. 3K, Supplemental Fig. 2C), suggesting that *Bmi1* might also be important for the development of B-1a cells from B-1 progenitors in the FL.

TLR-dependent proliferation of B-1a is impaired in the absence of Bmi1

Based on our findings that *Bmi1* was essential to B-1a cell self-renewal, we hypothesized that *Bmi1*-deficient B-1a cells might lose their proliferation ability upon Ag stimulation. To this end, we examined the ability of B-1 cells to proliferate upon stimulation with TLRs. FACS-sorted B-1a cells from each genotype were stimulated in vitro using 5 μg/ml R848 (TRL7/8 agonist) (27). Our results showed that 48 h after stimulation, *Bmi1*-deficient B-1a cells failed to proliferate and, instead, decreased in number, whereas B-1a cells from all control groups showed robust proliferation (Fig. 3M). Taken together, these data suggested that, in addition to its role in self-renewal, *Bmi1* was essential for the proliferation of B-1a cells upon stimulation.

Ink4-Arf locus is a target of Bmi1 in B-1a cells

Because the *Ink4a-Arf* locus is a well-known target of *Bmi1* in HSCs and loss of *Ink4a-Arf* in *Bmi1*^{-/-} mice has been shown to rescue the numbers of HSCs and their ability to self-renew (13), we hypothesized that *Ink4a-Arf* might also be a target of *Bmi1* in B-1a cells. Correspondingly, qPCR analysis showed a marked elevation of the expression of *Arf* in *Bmi1*^{-/-} peritoneal B-1a cells (Fig. 5A). In *Bmi1*^{-/-}*Ink4a-Arf*^{-/-} (double knockout [DKO]) mice, the absolute number of peritoneal cells was significantly higher than that in *Bmi1*^{-/-} mice but still lower than that in WT

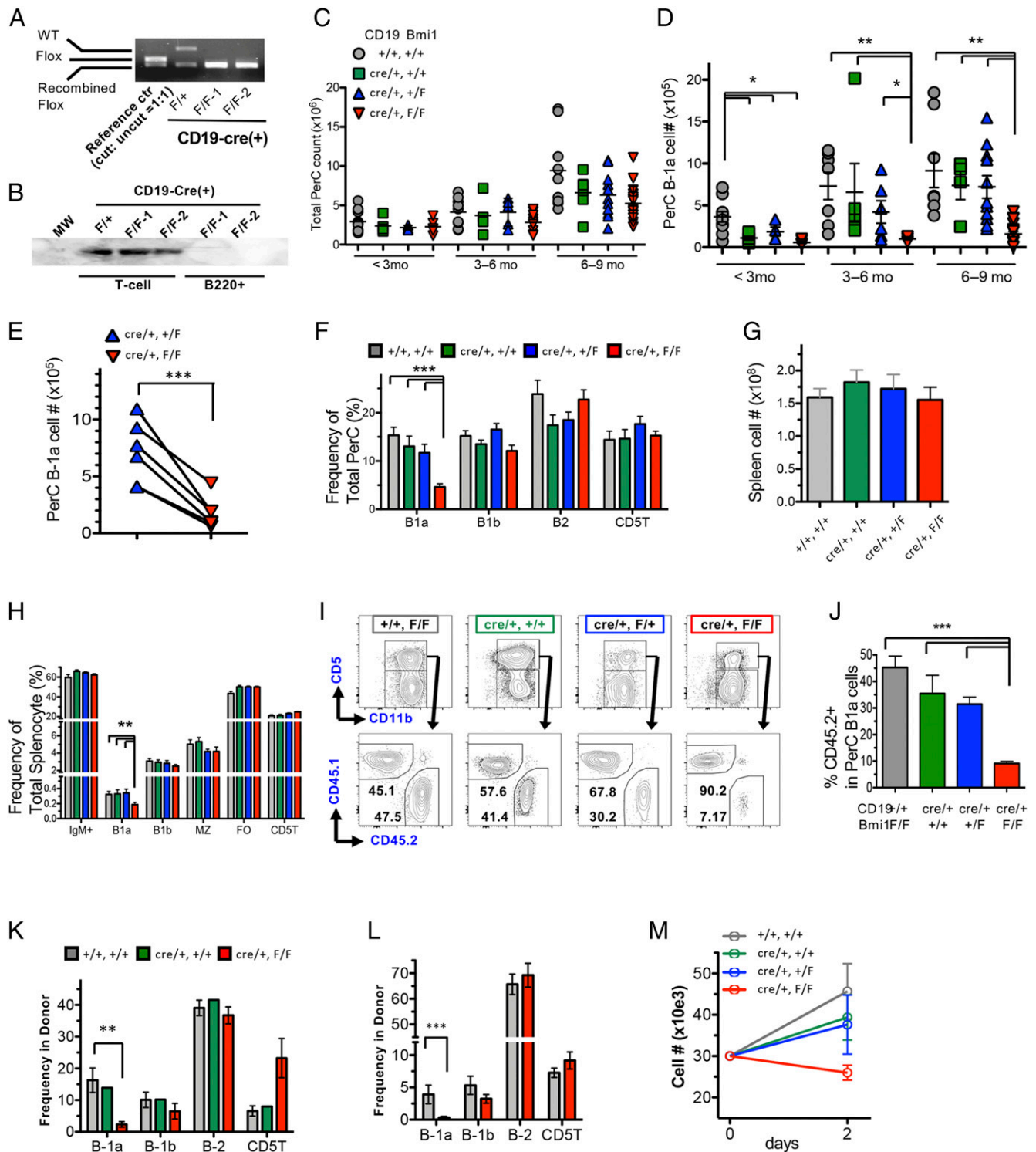


FIGURE 3. B cell-specific deletion of *Bmi1* leads to reduction in the B-1a cell pool and loss of self-renewal ability. The efficiency of CD19-Cre recombinase was confirmed by genomic PCR (**A**) in FACS-sorted B-1 cells or bead-selected spleen B cells (**B**). (**C**) Total peritoneal cell numbers in WT, *CD19^{Cre/+}*, *CD19^{Cre/+} Bmi1^{F/+}*, and *CD19^{Cre/+} Bmi1^{F/F}* mice are shown. No obvious difference was found among the four groups. (**D**) The numbers of peritoneal B-1a cells from four groups at different ages are shown. (*n* = 7–12 for each genotype for each age point). (**E**) The number of B-1a cells in *CD19^{Cre/+} Bmi1^{F/+}* and *CD19^{Cre/+} Bmi1^{F/F}* mice at 3–9 mo of age in the same litter is depicted. The line connects the mice in the same litter. The frequency of lymphoid subsets in the peritoneal cavities (**F**) and the spleens (**G** and **H**) of WT, *CD19^{Cre/+}*, *CD19^{Cre/+} Bmi1^{F/+}*, and *CD19^{Cre/+} Bmi1^{F/F}* mice are shown (*n* = 6). (**I** and **J**) Competitive peritoneal cell transplantation assays. The FACS plots of peritoneal B-1 cells of competitive peritoneal cell-transplanted mice (**I**) and the donor percentage (**J**) are shown. (**K** and **L**) FL MNC transplantation assays. The percentage of donor-derived lymphoid subsets in the peritoneal cavities of transplanted mice with WT or *CD19^{Cre/+} Bmi1^{F/F}* FL MNCs at 6 wk (**K**) and 9 mo (**L**) posttransplant (*n* = 3 for each genotyping, at each time point). (**M**) B-1a cell proliferation upon stimulation with TRL7 agonist. Cell number was counted 48 h after stimulation (*n* = 3). All data were obtained from experiments more than three times. **p* < 0.05, ***p* < 0.01, ****p* < 0.001. CD5T, CD5⁺ T cell; FO, follicular B cell; MZ, marginal zone B cell.

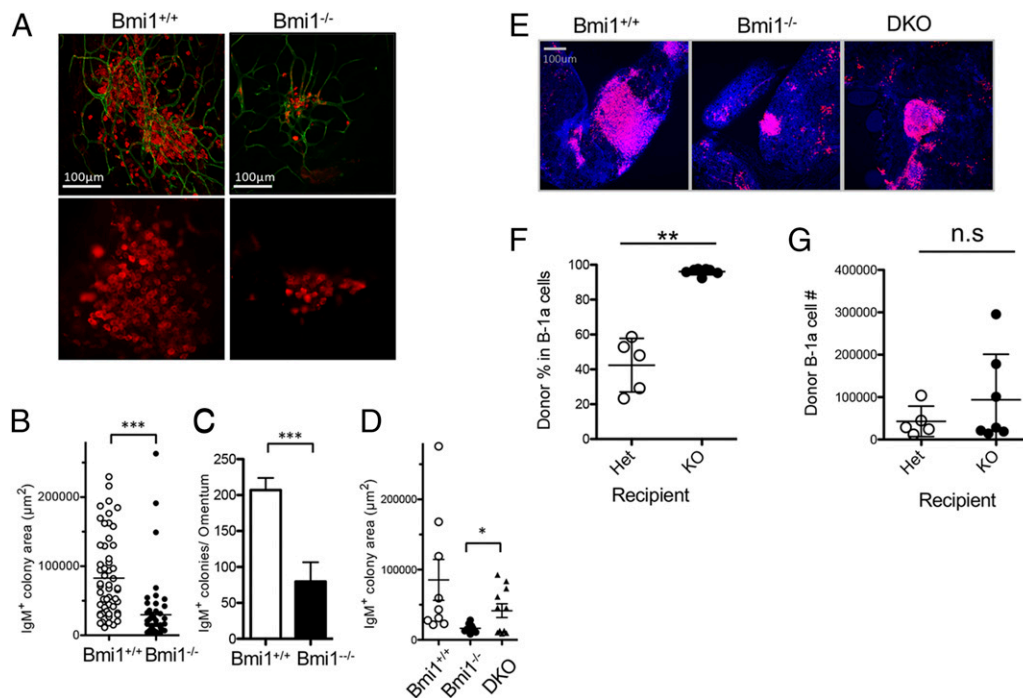


FIGURE 4. FALCs in *Bmi1*^{-/-} peritoneum and reciprocal transplantation assays. **(A)** Representative pictures of FALCs marked by IgM staining (red) in WT or *Bmi1*^{-/-} peritoneum. CD31⁺ endothelial cells are also stained (green). The size **(B)** and number **(C)** of FALCs in the omentum are depicted. **(D)** IgM⁺ colony areas in the omentum among WT, *Bmi1*^{-/-}, and DKO mice. **(E)** Representative pictures of FALCs in WT, *Bmi1*^{-/-}, or DKO mice are shown. The percentage **(F)** and number **(G)** of donor-derived B-1a cells in recipient peritoneal cavity 6 wk after reciprocal transplantation assays. Scale bar, 100 μ m. The data were obtained from three to four animals for each genotype from three experiments. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

mice probably because of their smaller body sizes (WT: $2.7 \pm 0.1 \times 10^6$, DKO: $1.2 \pm 0.2 \times 10^6$, and *Bmi1*^{-/-}: $0.5 \pm 0.2 \times 10^6$; $p < 0.01$ between DKO and *Bmi1*^{-/-}). The percentage of B-1a cells in DKO mice was similar to that in WT mice (Fig. 5B, 5C), suggesting that deletion of *Ink4-Arf* in *Bmi1*^{-/-} mice was able to rescue the maintenance of B-1a cells, although the recovery of absolute numbers of B-1a cells in DKO was only partial (Fig. 5D). When we performed a competitive assay by transplanting DKO along with B/J WT peritoneal B-1a cells into sublethally irradiated NSG mice, DKO cells achieved similar chimerism to that in WT cells, whereas *Bmi1*^{-/-} B-1a cells failed to repopulate, indicating that deletion of *Ink4-Arf* in *Bmi1*^{-/-} B-1a cells completely restored the self-renewal ability of B-1a cells (Fig. 5E). These results demonstrated that the *Ink4-Arf* locus appears to be a critical target of *Bmi1* in B-1a cells and might play a key role in the self-renewal ability of peritoneal B-1a cells.

Dysregulation of epigenetic gene modifiers in *Bmi1*^{-/-} B-1a cells suggested *Kdm5b* as a target of *Bmi1*

To investigate the *Bmi1*-related genes involved in the maintenance of B-1a cells, we performed microarray analysis of sorted peritoneal B-1a cells from WT and *Bmi1*^{-/-} mice. We identified 364 genes that were shown to be significantly changed (>2-fold) between the two groups. Significantly upregulated and downregulated genes are shown in Fig. 6. Multiple genes mediating genome integrity and translational control, including AT-rich interaction domain 1B (*Arid1b*) and PHD finger protein 12 (*Phf12*), were downregulated (Fig. 6C). Interestingly, various epigenetic modifiers, including DNA methyltransferase 3 α (*Dnmt3a*), 3b, enhancer of zeste 1 polycomb repressive complex 2 subunit (*Ezh1*), and chromobox 1 (*Cbx1*), were also downregulated (Fig. 6D).

Because *Bmi1* represents a *PRC1* gene repressor, many transcription factors were elevated in *Bmi1*^{-/-} B-1a cells. Among

them, we were interested in *Kdm5b* because it has been reported to play a role in the self-renewal and proliferation of cells (28). The expression of *Kdm5b* was significantly increased in the peritoneal B-1a cells, adult LT-HSCs, FL B-1 progenitors, and FL LT-HSCs in the *Bmi1*^{-/-} mice (Fig. 7A, 7B). However, the expression of *Kdm5b* was not elevated in *CD19*^{Cre/+} *Bmi1*^{F/F} B-1a cells in steady states (pre in Fig. 7C). Forty-eight hours after transplantation into sublethally irradiated NSG mice, *Kdm5b* expression significantly increased, and *Arf* expression in *CD19*^{Cre/+} *Bmi1*^{F/F} peritoneal B-1a cells markedly increased (Fig. 7C). In addition, the expression of p21 (cell cycle repressor) at 48 h was not repressed in *CD19*^{Cre/+} *Bmi1*^{F/F} peritoneal B-1a cells as it was in the control cells (Fig. 7C). Moreover, a higher percentage of dead cells was observed among *CD19*^{Cre/+} *Bmi1*^{F/F} B-1a cells (Fig. 7D). These results suggested that *Bmi1*-deficient B-1a cells could not proliferate after transplantation, presumably because of overexpression of *Arf* and *p21*.

To understand the molecular role of *Kdm5b* in cell proliferation, we knocked down the expression of *Kdm5b* in the Baf3 pro-B cell line using a sh-*Kdm5b* retroviral construct (Fig. 7E). sh-*Kdm5b*-transduced cells showed more proliferation than did control cells (sh-Luc) (Fig. 7F). Next, we introduced the viral sh construct into *Bmi1*^{-/-} FL cells and performed colony-forming assays. The number of colonies that formed from *Bmi1*^{-/-} FL cells was significantly lower than that formed from WT cells, as previously reported (12) (Fig. 7G). However, the knockdown of *Kdm5b* in *Bmi1*^{-/-} FLs induced an increase in the number of colonies formed (Fig. 7G). Thus, knockdown of *Kdm5b* led to enhanced cell proliferation in Baf3 and FL cells. In addition, a ChIP assay showed that *Bmi1* bound to the upstream region of *Kdm5b* (Fig. 7H), as well as *Arf*, a known target of *Bmi1* (Fig. 7I). We also confirmed that *Ring1b*, a main component of the *PRC1* complex protein, bound to the same regions. Collectively, our data suggested that *Kdm5b* appears to be a downstream target of *Bmi1*, and

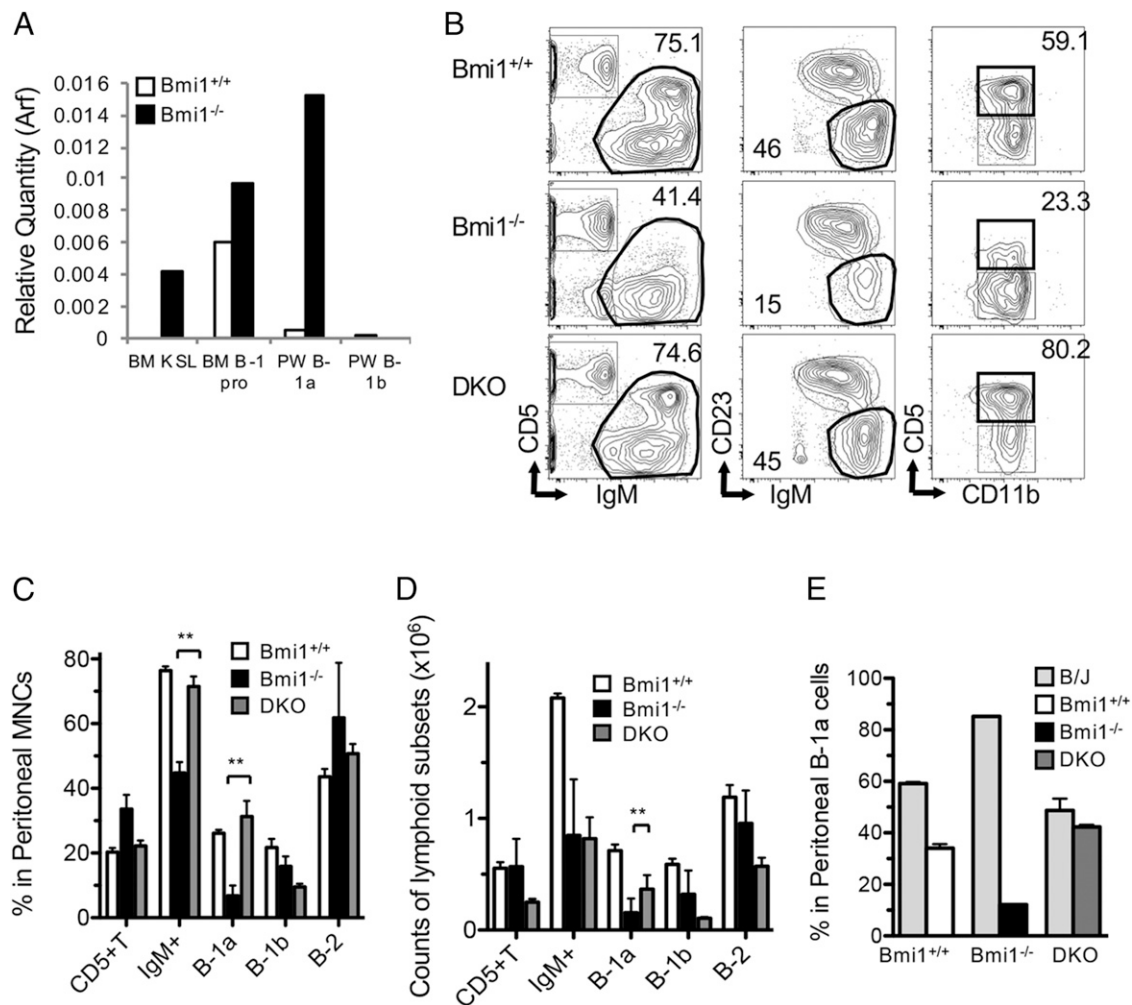


FIGURE 5. Arf-Ink4a is a major target for self-renewal of *Bmi1*^{-/-} B-1a cells. (A) Arf expression in WT and *Bmi1*^{-/-} B cells was measured by qPCR. (B) Representative FACS plots of peritoneal cells from WT, *Bmi1*^{-/-}, and *Bmi1*^{-/-} Arf-Ink4^{-/-} (DKO) mice are depicted. (C) Frequencies of CD5⁺ T cell and B cell subsets in the peritoneal cavity of WT, *Bmi1*^{-/-}, and DKO mice are shown (*n* = 3). (D) Absolute numbers of the peritoneal subpopulation from the same three groups (C) were measured. (E) Equal numbers of sorted B-1a cells from WT, *Bmi1*^{-/-}, and DKO mice were injected into sublethally irradiated NSG mice in a competitive manner against B/J B-1a cells, followed by analysis at 6 wk. The results of donor contribution are shown. The *Bmi1*^{-/-} B-1a cells failed to repopulate, whereas the DKO cells achieved repopulation similar to that of WT (*n* = 3 for each genotyping). The data were obtained from three experiments. ***p* < 0.01. PW, peritoneal wash.

an optimal low expression level of *Kdm5b* seems to be important for cell proliferation.

Discussion

B-1a lymphocytes are derived from embryonic precursors and maintained in the long-term through their self-renewal ability independently of HSCs (5–9). However, despite extensive studies on the development and function of B-1 cells, the molecular mechanism of B-1a cell self-renewal remains largely unknown. Traditionally, the spleen is known as the organ important for the maintenance of B-1a cells, in addition to their specific BCR and CD19 signaling, all of which continuously stimulate B-1a cells (22, 29–31). A recent study showed that deletion of the basic helix-loop-helix family member E41 (*Bhlhe41*) transcription factor significantly reduced the number of peritoneal B-1a cells, likely by altering BCR signaling and preventing the maturation of transitional B-1 progenitors in the spleen (32). Another interesting report has demonstrated that the autophagy-related 7 (*Atg7*) gene was required for the self-renewal of B-1a cells because of their specific metabolic status that is different from that of B-2 cells (33). These findings have gradually started to elucidate the mechanisms

that support B-1a cell self-renewal and suggest its complexity. In this study, we demonstrated that expression of *Bmi1* was indispensable for the self-renewal ability of peritoneal B-1a cells. The importance of this finding is that mature IgM-secreting B-1a lymphocytes use BMI1 for their self-renewal ability in a similar way to HSCs.

Bmi1, a component of PRC1, is a widely known critical factor in the self-renewal of various stem cells. Importantly, *Bmi1* is also considered to be involved in lymphopoiesis because a large reduction has been observed in lymphocyte counts in the spleen and thymus of *Bmi1*^{-/-} mice (18). However, the frequency and number of B-2 lymphocytes were not altered when *Bmi1* was deleted in the B cell lineage (using CD19-Cre mice). Therefore, it seems that a defect in stem cells (differentiation to B progenitors) mainly contributed to the reduction of B-2 cells in *Bmi1*^{-/-} mice and that, among the mature B cell subsets, only B-1a cells depend on *Bmi1* expression. In the field of HSC biology, self-renewal ability is confirmed only by transplantation assays. Therefore, we carried out peritoneal cell transfer assays in which WT peritoneal B-1a cells repopulate the recipient peritoneal cavity in the long-term, and we further demonstrated a defect in the self-renewal

A Top Canonical Pathways

name	p-value	Ratio
CXCR4 Signaling	3.28E-04	9/152 (0.059)
Signaling by Rho Family GTPases	6.17E-04	11/244 (0.045)
RhoGDI Signaling	9.01E-04	9/184 (0.049)
Role of NFAT in Regulation of the Immune Response	2.56E-03	8/173 (0.046)
Cholecystokinin/Gastrin-mediated Signaling	3.08E-03	6/97 (0.062)

B Molecular and Cellular Functions

name	p-value	# Molecules
Cellular Development	0.0006-0.04	29
Cell-to-Cell Signaling and Integration	6.17E-04	17
Cellular Assembly and organization	9.01E-04	23
Gene Expression	2.56E-03	55
Cellular Function and Maintenance	3.08E-03	35

C Fold change downregulated

Molecules	Exp. Value
Agbl1	-159.10
Ms4a4a	-102.24
Wipf1	-70.39
Dido1	-59.09
Rlim	-58.93
Zcchc2	-56.11
Plekhb2	-46.63
Slbp	-43.02
Gtf2h2	-40.81
Fzd6	-39.05
Nup188	-36.04
Phf12	-31.28
Rab27a	-29.63
Phf8	-19.34
Arid1b	-17.36
phf13	-12.70
phf3	-7.84
Eif5b	-6.54

Fold change upregulated

Molecules	Exp. Value
Hoxc9	84.49
Hcn3	14.38
Zfp873	12.44
Anxa9	8.96
Msrb1	8.83
Cript	7.28
Zeb1	7.09
Eef1g	4.35
Jmjd1c	4.01
Kdm5b	3.70
Il9r	3.47

D

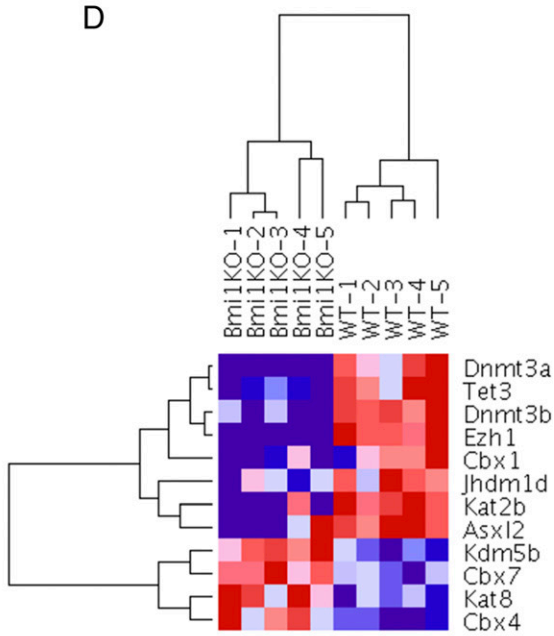


FIGURE 6. Gene expression profiling of *Bmi1*^{-/-} reveals dysregulation of epigenetic genes. B-1a cells were sorted from WT and *Bmi1*^{-/-} peritoneal cavity and subjected to microarray analysis. Affected canonical signaling pathways (**A**) and affected biofunctions (**B**) analyzed by Ingenuity Pathway Analysis are listed. (**C**) Differentially upregulated and downregulated transcripts are listed. (**D**) Hierarchical clustering of epigenetic genes is shown (*n* = 5 for each group).

ability of *Bmi1*-deficient B-1a cells, which was restored by additional deletion of *Ink4-Arf*. Proliferation of *Bmi1*-deficient B-1a cells was also impaired after exposure to a TLR7/8 agonist, as well as after transplantation. *Bmi1*-deficient B-1a cells showed upregulation of *Arf* and failure to downregulate p21 after stimulation, which suggested that *Bmi1* maintains the self-renewal ability of B-1a cells through the *Arf-p21* axis. Although *Bmi1* seems to play a role in the self-renewal of B-1a cells similar to its role in HSCs, we have further identified *Kdm5b* as a possible target of *Bmi1* in B-1a cells. The KDM5B histone modifier specifically demethylates H3 at lysine 4 and is known to be required for self-renewal of embryonic stem cells, HSC function, and leukemia/cancer progression and plays different roles depending on cell type and physiological context (28, 34–36). Our results showed that *Kdm5b* was upregulated in *Bmi1*-deficient B-1a cells in a stress setting, but not in steady state, suggesting that *Kdm5b* suppresses proliferation of B-1a cells in the absence of *Bmi1*. Indeed, knockdown of *Kdm5b*-enhanced proliferation of Baf3 cells and rescued the colony-forming ability of *Bmi1*^{-/-} FL cells (Fig. 7F, 7G). Therefore, it can be speculated that B-1a cells fail to proliferate and undergo apoptosis upon Ag exposure in the absence of *Bmi1* followed by upregulated *Kdm5b*. As such, further

investigation on the precise roles of *Kdm5b* in B-1a cells will be required.

Furthermore, FL MNCs that contain *Bmi1*-deleted B progenitors failed to repopulate only B-1a cells, and not other mature B cell subsets, even at an early time point after transplantation. This result suggests a possibility that *Bmi1* may also be required for the developmental process of fetal B-1 progenitor cells into mature B-1a cells. In addition, it is notable that overexpressing *Bmi1* in *Bmi1*^{-/-} FL MNCs not only rescued, but further enhanced B-1a cell repopulation ability (Fig. 2E). This result is consistent with previous reports showing enhanced self-renewal after overexpressing *Bmi1* in BM HSCs and erythroblasts (12, 16). Thus, *Bmi1* seems to enhance both B-1a cell differentiation from FL progenitors and self-renewal of mature B-1a cells. Because the developmental pathway from FL B-1 progenitors to mature peritoneal B-1a cells has not been fully characterized, further study to determine the stage at which *Bmi1* might be involved in the differentiation into B-1a cells is required.

Recent reports have shown the important role of *Bmi1* in controlling the fat volume in the HSC niche (23, 24). We also sought to investigate the effect of the lack of *Bmi1* on FALCs, a niche for B-1a cells, but found that the microenvironment of B-1a cells was

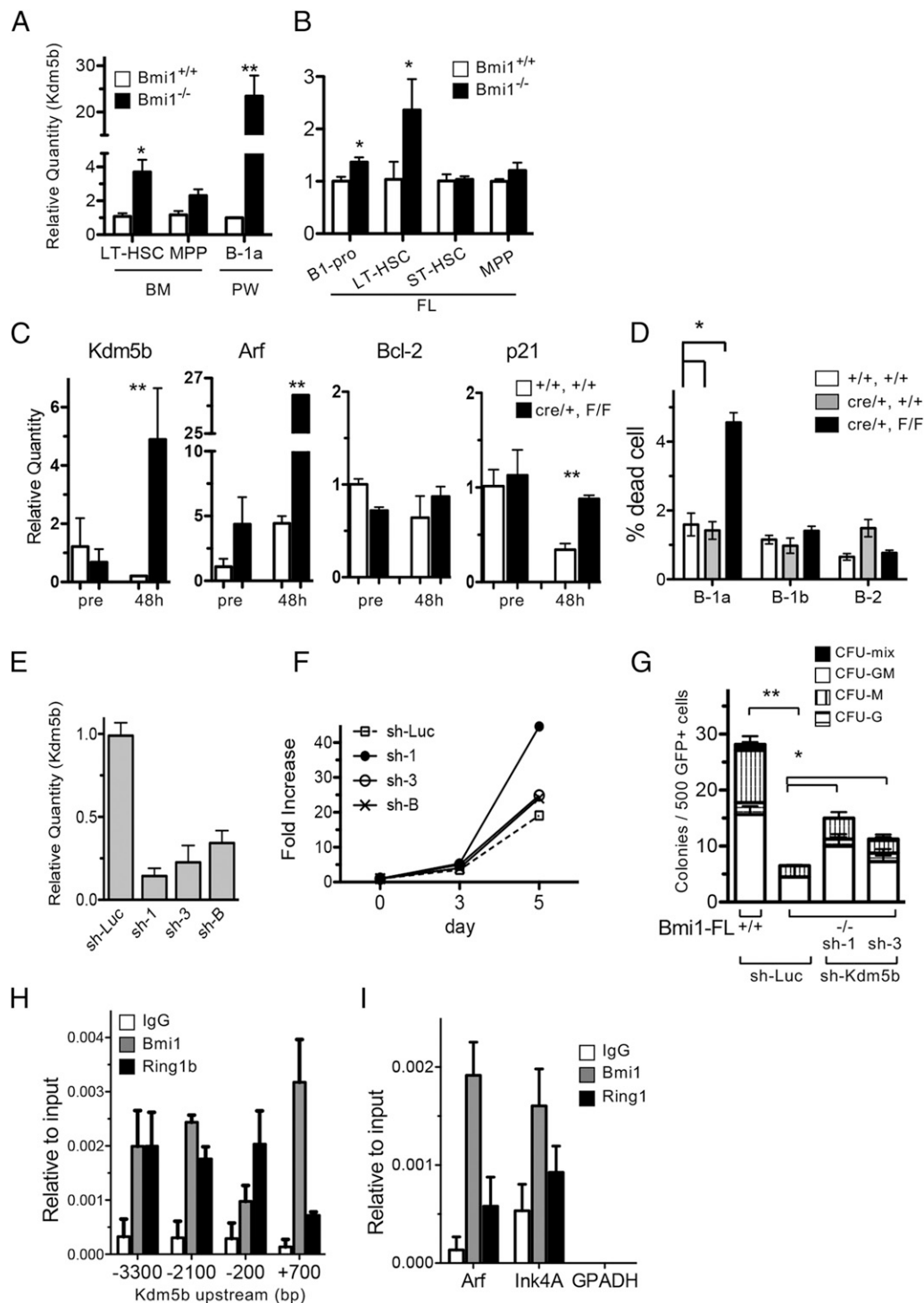


FIGURE 7. *Kdm5b* is a possible *Bmi1* target and crucial for posttransplant B-1a cell expansion. Expression of *Kdm5b* in various cell fractions from WT and *Bmi1*^{-/-} BM and peritoneum (**A**) or FL (**B**) ($n = 3$). * $p < 0.03$, ** $p < 0.01$. (**C**) Sorted B-1a cells from *CD19*^{Cre/+}*Bmi1*^{F/F} or control mice were injected into sublethally irradiated NSG mice, followed by harvesting of B-1a cells 48 h after transplantation by FACS sorting. Changes in the expression of various mRNAs before and after transplantation (day 2) were measured ($n = 3$). ** $p < 0.01$. (**D**) Cell death in experiment (C) was measured by annexin V and DAPI staining ($n = 3$). * $p < 0.05$. (**E**) Confirmation of knockdown of *Kdm5b*. Short hairpin RNAs (sh-RNAs) targeting *kdm5b* or control (sh-Luc) were introduced retrovirally into Baf/3 pro-B cells, and GFP⁺ cells were sorted. The quantity of *Kdm5b* mRNA in each clone is shown. (**F**) Cell proliferation of each Baf/3 subline targeting *Kdm5* is shown. (**G**) Colony-forming assays of *Kdm5* knockdown *Bmi1*^{-/-} FL cells. *Bmi1*^{-/-} FLs with *sh-Kdm5b* formed more colonies than did the sh-control ($n = 3$). ** $p < 0.01$, * $p < 0.05$. (**H** and **I**) ChIP assays were performed in Baf3 cells to confirm *Bmi1* binding to the *Kdm5b* or *Arf*-*Ink4* regions. Binding to upstream regions of *Kdm5b* (**H**) and *Arf*-*Ink4* (**I**) is shown. The data were obtained from three experiments.

not altered. Therefore, *Bmi1* appears to maintain the self-renewal ability of B-1a cells in a cell-intrinsic manner.

In summary, our studies reviewed in this report showed that the self-renewal ability of B-1a lymphocytes depends heavily on

Bmi1 expression, and this dependency is not observed in other mature lymphocyte subsets. Because B-1a cells are not replenished by adult BM progenitors, the loss of B-1a cells after BM transplantation might thus result in acquired immunodeficiency

(37). The knowledge presented in this study could pave the way to fully elucidate the mechanisms of the *in vivo* self-renewal of B-1a cells, an important step toward producing pluripotent stem cell-derived B-1 cells for immune cell therapy (17).

Disclosures

The authors have no financial conflicts of interest.

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Fate of Hematopoiesis During Aging. What Do We Really Know, and What are its Implications?

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Abstract

There is an ongoing shift in demographics such that older persons will outnumber young persons in the coming years, and with it age-associated tissue attrition and increased diseases and disorders. There has been increased information on the association of the aging process with dysregulation of hematopoietic stem (HSC) and progenitor (HPC) cells, and hematopoiesis. This review provides an extensive up-to date summary on the literature of aged hematopoiesis and HSCs placed in context of potential artifacts of the collection and processing procedure, that may not be totally representative of the status of HSCs in their *in vivo* bone marrow microenvironment, and what the implications of this are for understanding aged hematopoiesis. This review covers a number of interactive areas, many of which have not been adequately explored. There are still many unknowns and mechanistic insights to be elucidated to better understand effects of aging on the hematopoietic system, efforts that will take multidisciplinary approaches, and that could lead to means to ameliorate at least some of the dysregulation of HSCs and HPCs associated with the aging process.

Keywords Hematopoiesis · Hematopoietic stem and progenitor cells · Aging · Cytokines/Chemokines · Microenvironment · Oxygen · Inflammation · Microbiome · CHIP

Aging is an inevitable process if one lives long enough. There is an ongoing shift in demographics such that older persons will outnumber young persons in the coming years, and with it age-associated tissue attrition and increased diseases and disorders. There has been an increased influx in literature on the association of the aging process with dysregulation of hematopoietic stem (HSC) and progenitor (HPC) cells, and hematopoiesis. Most such hematopoietic aging studies have been carried out in mice, where it has been reported that the aging process (in this case mice in the range of 2 years old)

compared to that of younger mice is associated with increased absolute numbers of phenotypically defined HSCs identified by cell surface antigens in the bone marrow (BM). Yet, the functional capacities of these increased numbers of HSCs are grossly deficient in their engrafting capability in competitive and non-competitive HSC transplants in lethally irradiated mice. Moreover, the differentiation capacity of the engrafted donor BM cells from the old mice is different from that of young donor BM cells; there is a shift in the lymphoid/myeloid ratio of engrafting cells such that the older donor BM cells manifest greater numbers of myeloid to lymphoid cell output. This is the opposite of that of young engrafting mouse BM HSCs. How informative this and other information on aged hematopoiesis is remains to be determined by further investigation. Recent work from our laboratory [1] has observed that at least some of the abnormalities of HSCs from old mice may be more of an artifact of the collection and processing of mouse BM cells, rather than how they manifest their numbers and functional capacities *in vivo*.

This review provides an extensive, although not necessarily complete, summary on the literature of aged hematopoiesis, HSCs and HPCs. When placed in context of potential artifacts of the collection and processing procedure, that may

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not be totally representative of the status of HSCs in the *in vivo* microenvironment of BM, the site in which HSCs, HPCs, and hematopoiesis are nurtured for self-renewal, proliferation, survival, and differentiation some of what we know may have to be re-evaluated. This review encompasses the following sections: A) Aging and Stem Cells in General, B) Age-Related Changes in HSCs/HPCs and Hematopoiesis, C) Age-Related Clonal Hematopoiesis of Indeterminant Potential (CHIP) and Inflammation, D) DNA Damage, Transcriptional and Epigenetic Changes During Aging, E) Metabolic Processes, Mitochondria and Reactive Oxygen Species (ROS) During Aging, F) Apoptosis, Autophagy, Radiation and a Role for the Sirtuin Family of Proteins During Aging, G) The Microbiome, Hematopoiesis, and Aging, H) Additional Age-Related Information, The Microenvironment, Exosomes, Leptin (Lep) and Leptin Receptors (R), and Means to Better Evaluate and Understand Hematopoiesis During Aging in part in context of our recent studies [1], I) COVID-19, SARS-CoV-2, Aging and Hematopoiesis, and J) Conclusions in Context of Potential Future Interventions for Better Health of the Hematopoietic System During Aging. One of the authors (HEB) of this review had an interest in Gerontology, the study of aging, over 50 years ago, but it is only most recently, that he, his lab members, and collaborators have been involved in actual experiments in this area, having previously focused on the regulation of hematopoiesis in the young.¹

A) Aging and Stem Cells in General

It has been suggested that aging is not caused by active gene programming, but that it rather evolved through limitations in maintenance of somatic cells in which there was a build up of damage [2], which in fact is associated with gene mutations that affect endocrine signaling, stress responses, metabolism and telomere length [3]. Thus, aging is believed to entail

¹ While HEB was pursuing a Masters degree in microbiology, prior to his PhD studies in blood cell development and regulation, he was especially interested in reading the literature on Gerontology. In 1969, he attended as an on-looker an International Conference on Gerontology, the first conference he ever attended. The conference was held in Washington, DC, where he heard talks by all the leading experts in this area of research, including Leonard Hayflick, the original proponent on identifying the limited life-span of normal cells. On a whim, HEB registered to attend a closed discussion group of 20 individuals in the conference to discuss the ramifications of what was then currently known about the biology of aging. While he was the only one in the discussion group without an advanced degree, the others in the group were understanding when the Discussion participants were asked to describe their academic background and interest in the field of Gerontology. When it came time for him to talk he apologized, as he had not worked in the area, but noted his interest in the field, and told them his very limited academic training to that time. To his surprise, he was warmly welcomed into the discussion group, which included amongst those present, Dr. Hayflick. The other participants made it clear that the field of Gerontology was still in its infancy, and their words of advice to him, in not so many words, was to find a field to work in that was more advanced and then when he became an expert in that field to integrate the knowledge from that field into the studies of aging. This he did, a half century later.

“damage” due to multiple mechanisms, some information of which may possibly be used to slow some of the “damage” during aging for healthier outcome. Covered in these papers [2, 3] are the areas of: why aging occurs, is it programmed, how does evolutionary genetics and physiology fit into these processes, how aging is “caused” in terms of molecular mechanisms, mitochondria, and network themes. Whether we yet know enough about the aging process of cells, their organelles, and organisms is still open for debate, although more insight into problems and causes associated with aging could provide the means to potentially intervene at least partially in the future.

Human aging is associated with a number of diseases and defects including the heart, muscle wasting, osteoporosis, and in some cases mental deterioration [4]. Senescent cells and their accumulating damage can contribute to aging, through a number of intracellular signaling pathways including the p53 and RB tumor suppressors, and the influences of neighboring cells in the environment [5]. These, and a number of other genetic pathways have been implicated in aging [6], including nutrient sensing pathways. Over thirty genetic mutations have been reported to extend the lifespan of mice, and a number of genes have been associated in genome wide association studies with longevity of humans [6]. Ames Dwarf mice which harbor a spontaneous mutation in the *Prop1^{df}* gene resulting in the lack of growth hormone, prolactin, and thyroid-stimulating hormone are known to live close to two times the lifespan of other mouse strains [7–10], a situation mimicking certain conditions in humans. Why we age has been commented on from an evolutionary point of view [11]. While somatic cells have a limited lifespan, the lifespan of stem cells, which have the property of making more of themselves (self-renewal) and being able under the appropriate stimuli for differentiation to more mature cell types has not yet been conclusively defined, although transcriptional fingerprinting and other pathway analyses suggest that stem cells do themselves age as one gets older [12, 13].

B) Age-Related Changes in HSCs/HPCs and Hematopoiesis

A number of articles and reviews appeared in this area of research since 2005 [14–37] which will be mainly described in chronological order so that the reader can see what research was reported first and then subsequently, as not all research findings may agree. It was reported that the aging of long-term (LT) HSCs was associated with autonomous changes that increased the self-renewal of these cells, but that these HSCs manifested decreased potential for lymphoid cell differentiation and production [14]. This was associated with down-modulation of genes that mediate lymphoid specification, and up-modulation of myeloid fate decisions and functions

[14]. Competitive transplants were done using the congenic CD45.1/CD45.2 mouse system with relatively purified populations of donor HSCs to assess engraftment, and self-renewal was estimated by secondary transplants. This paper [14] did not note the decreased engrafting capacity of HSCs from BM of older mice that most of the other numerous publications in this area have reported, and lacked detailed month by month chimerism data comparing engraftment of old vs. young mice; nor did it quantitate numbers of functional HSCs using limiting dilution analysis to calculate competitive repopulating units (CRUs, a measure of the numbers of functional HSCs [38]). Age-related defects in lymphoid-bias [15] and B-lymphopoiesis [16] have been reported by others, and have been suggested to underlie the dominance of myeloid cells in adult leukemia [17]. In order for HSCs to engraft, they must first home to the BM after IV injection. This process of homing for BM cells of old mouse CRUs was about 3-fold lower than the homing efficiency of CRUs from young mice [18], hence one potential reason for decreased engrafting capability noted by others. Of some interest, although not completely understood, the ability to mobilize HSCs from old mice with G-CSF to the blood was increased compared to that of G-CSF mobilization of HSCs from younger mice [19]. This correlated with a reduced adhesion capacity of an immature cell population (not a purified HSC population) to stromal cells, and with increased activation of Cdc42, a small RhoGTPase. This work has not yet been reproduced to the knowledge of the authors of this review, and more rigorous analysis is needed to fully understand this interesting phenomenon. What has not been defined yet is the mobilization of lymphoid vs. myeloid-biased HSCs in old vs. young mice. It will also be of interest to assess the mobilizing capacity of bonified HSCs to the combination of G-CSF plus AMD3100 (Plerixafor), as G-CSF and AMD3100 synergize to mobilize HSCs and HPCs from young mice [39].

Other reviews have noted age-related changes in hematopoiesis of old vs. young mice [20, 21], with one short report [22] not seeing differences in engraftment of sorted populations of HPCs from elderly (>70 years old vs. young) human BM in immune deficient NSG mice. This clearly needs more rigorous investigation in terms of numbers and engrafting capability of rigorously purified populations of functional human BM HSCs (not HPCs) from old and young donors.

While an earlier report [14] suggested increased self-renewal of HSCs from BM of old mice, a later report by others with more in-depth analysis demonstrated that HSCs from the BM of old mice manifested significantly reduced self-renewal in secondary transplants using highly purified populations of LT-HSCs [23] for both primary and secondary engraftment. They [23] as did others [18] showed decreased homing efficiency of HSCs from the BM of old mice. Moreover, they [23] showed significantly delayed proliferative responses of old vs. young BM HSCs.

What is clear is that all studies thus far that have assessed old vs. young BM engrafting HSCs have shown a bias of the myeloid vs. lymphoid production capability of HSCs from old mice [14, 16–18, 20–24]. Whether this apparent bias of donor HSCs from old mice might be due to potential artifacts in how donor cells were collected, processed, and injected into recipient mice [1] will be discussed in Section H.

The impact of hematopoiesis in aging primates was investigated by clonal tracking in which clonal output of thousands of genetically barcoded HSCs and HPCs was determined in old vs. young macaques after autologous transplantation [25, 26]. Delayed output from multipotent clones was observed in old macaques with persistence of lineage biased clones noted; in contrast to aging studies in mice which showed persistence of myeloid-biased clones with old age, there was persistent output from both B-lymphoid- and myeloid-biased clones. Whether or not macaque vs. mouse differences were due to aging differences between species requires further investigation as these studies [25] were based on only two old macaques 18 and 25 years of age, which were considered “aged” on their lifespans of captivity of 20–30 years.

The multipotential progenitor (MPP) cell compartment is a composite of 4 different cell types, with the MPP4 compartment being considered to be lymphoid-primed [27]. A yet to be understood observation in context of lymphoid-biased aging studies is the progressive loss and increased cycling of the MPP4 population with aging; other cells and factors may be involved in lymphoid-biased output from engrafted aged HSCs.

Two intriguing reviews on HSC aging are entitled: “The slippery slope of hematopoietic stem cell aging” [30], and “Age-related clonal hematopoiesis: Stem cells tempting the devil” [29]. The latter review touches on clonal hematopoiesis of indeterminate potential (CHIP), an area that will be covered in detail in Section C, and is associated with increased risk of hematological cancers, as well as that of the mortality associated with cardiovascular problems. A number of other more recent reviews are worth noting including: “Aging of hematopoietic stem cells” [31], “Anemia at older age: etiologies, clinical implications and management” [32], “Aged murine stem cells drive aging-associated immune remodeling” [33], “The global complexity of the murine blood system declines throughout life and after serial transplantation” [34], “Hematopoietic stem cells aging, life span and transplantation” [35], “The ageing hematopoietic stem cell compartment” [36], and “Relationships between aging and hematopoietic cell transplantation” [37]. All these reviews suggest that intervention in age-related dysfunction of HSCs may be possible, in part by targeting selected intracellular regulatory pathways. We suggest in Section H, the potential use based on studies in mice of HSCs and HPCs from older individuals for efficient hematopoietic cell transplantation (HCT), if the cells are more appropriately collected and processed under conditions that

maintain their *in vivo* numbers and functional characteristics [40–42]. How these cells are collected may be crucial, as cells are currently collected in almost all mouse studies, except those noted by us [40–42], and in all human studies, in ambient air (~21% O₂ tension). Collection of cells in ambient air subjects them to a phenomenon which we termed Extra Physiological Oxygen Shock Stress (EPHOSS) [40, 41]. Ambient air causes the very rapid loss of HSCs and a concomitant increase in numbers of HPCs due to EPHOSS-induced differentiation of HSCs [40]. This differentiation process during collection of cells in air occurs within minutes, and may likely be needed to be considered in interpretation of at least some of the published information presented. This may require some re-evaluation of past studies to ensure that studies accurately describe the situation of numbers and functions of these cells as when they are present in their BM microenvironment, before removal for collection and analysis, as we reported in [1] and discuss in Section H.

C) Age-Related Clonal Hematopoiesis of Indeterminant Potential (CHIP) and Inflammation

Age-Related Clonal Hematopoiesis

CHIP, also known as age-related clonal hematopoiesis (ARCH), is characterized by expansion of somatic mutations in various hematopoietic lineages of older persons and is associated with risks of developing leukemia [43], as well as other age-associated disorders including cardiovascular disease [44]. Human aging is associated with an exponential increase in the occurrence of CHIP in aged individuals. It is an emerging public health issue that affects at least 15–20% of individuals aged 70 or above [43–59]. A number of reviews and reports on clonal hematopoiesis have been published [45–59]. This is currently a heavily researched area of investigation, with the causes still relatively unknown. Clarity is needed on why some cells with mutations, likely involving and caused by several factors [57] noted in the below Sections, persist and/or expand with resultant disorders such as leukemias, myelodysplasias, and cancers associated with aging individuals.

The vast majority of the mutations identified in CHIP are dispersed across the genome. However, five genes, including *DNMT3A*, *TET2*, *ASXL1*, *JAK2*, and *TP53*, have high numbers of somatic mutations [54–59]. The most common base-pair change in the somatic variants identified in CHIP was a cytosine to thymine (C to T) transition, a somatic mutational signature of aging [54–56]. CHIP is an age-dependent risk factor for both hematological malignancies and cardiovascular disease [53–59]. Thus, preventing CHIP progression may prove to be beneficial for human health. However,

mechanisms by which somatic mutations in HSCs and other blood cells contribute to the pathogenesis of age-related diseases are largely unknown.

Clinical studies revealed that hematopoietic clones harboring specific mutations in individuals with CHIP may expand over time [54–58]. However, how different cellular stressors affect clonal expansion is largely unknown. Recently, three different stressors, including hematopoietic transplantation, cytotoxic therapy and inflammation, have been shown to expand hematopoietic clones. *TP53* mutations identified in CHIP confer a competitive advantage to HSCs and HPCs following transplantation through modulating epigenetic pathways [52]. Considering that common mutations identified in CHIP affect epigenetic modulators, including *DNMT3A*, *ASXL1*, and *TET2*, these findings underscore the importance of dysregulated epigenetic control in CHIP development.

PPM1D is a phosphatase that negatively regulates p53 and several proteins involved in the DNA damage response (DDR) pathway [60]. Recently, *PPM1D* mutations were found in CHIP [54–58]. *PPM1D* mutations result in the expansion of *PPM1D*-mutant hematopoietic cells following chemotherapy treatment. However, *they do not confer* competitive advantage to HSCs and HPCs following bone marrow transplantation [61, 62]. *TP53* mutations are associated with prior exposure to chemotherapy [63]. Genotoxic stresses selectively expand *TP53*-mutant HSPCs [50, 64]. While both p53 and *PPM1D* are involved in the DDR pathway, they appear to play distinct roles in promoting of HSCs and HPCs expansion.

The Effects of Chronic, Low-Grade Inflammation Associated with Aging

During aging, chronic and low-grade inflammation - inflammaging - develops, which contributes to the pathogenesis of age-related diseases [65, 66]. Aberrant innate immune activation and pro-inflammatory signaling within the malignant clone and the BM microenvironment have been identified as key pathogenic drivers of myelodysplastic syndrome (MDS), an age-related disease [67]. Mutations identified in CHIP may utilize cell extrinsic mechanisms to promote clonal hematopoiesis. For example, *TET2*-deficient macrophages exhibit an increased in NLRP3 inflammasome-mediated interleukin-1 β secretion [68]. Inflammasomes are multiprotein complexes that activate Caspase-1 and increase the release of pro-inflammatory cytokines such as IL-1 β , leading to caspase-1-dependent death, known as pyroptosis [69]. HSCs and HPCs from low to high-risk human patients with MDS manifest activated NLRP3 inflammasome [70]. NLRP1 inflammasome activation increases IL-1 β secretion that inhibits wild-type HSPC function through inducing pyroptosis [71]. The NLRP1 inflammasome, but not the NLRP3 inflammasome, is specifically activated in p53 mutant

HSPCs, leading to increased secretion of IL-1 β , which induces pyroptosis of wild-type HSPCs in a paracrine fashion (YL and HEB, unpublished data). Tet2-deficient hematopoietic stem and progenitor cells manifest a hyperactive IL-6 pathway, which promotes cell survival under basal conditions and in response to inflammatory stress. Inhibiting inflammatory signaling in Tet2 mutant preleukemic cells mitigates stress-induced abnormalities and clonal hematopoiesis [72].

Splicing of pre-mRNAs by the spliceosome plays a key role in tissue development [73, 74]. Genome wide splicing analysis revealed an increased number of spliced genes during aging [75, 76]. Changes in spliceosome gene expression and alterations in pre-mRNA splicing are associated with lifespan in mice and humans [77]. Notably, both human and mouse HSPCs display dysregulated pre-mRNA splicing with age [78, 79]. Further, spliceosome gene mutations, including *SF3B1*, *SRSF2* and *U2AF1*, were frequently found in CHIP and MDS [54–57, 80–82], implicating that aberrant splicing in hematopoietic cells may contribute to CHIP and pathogenesis of MDS. Although both *SRSF2* and *SF3B1* mutations alter mRNA splicing, these mutations functionally converge with hyperactivation of NF- κ B, a key mediator of the inflammatory response [83]. These findings underscore the importance of chronic inflammation in promoting CHIP development during aging.

Inflammation

Inflammation is a double-edged sword in hematopoiesis and disease. The hematopoietic system gives rise to the immune cells of the body and is, therefore, closely linked to inflammation. Even at early stages of hematopoietic development, inflammatory cytokines, such as interleukin-1 (IL-1), interferon- γ (IFN- γ), tumor necrosis factor (TNF), and granulocyte colony stimulating factor (G-CSF), play critical roles in the specification of hematopoietic stem cells (HSCs) [84]. Remarkably, different levels and combinations of inflammatory signaling molecules can elicit opposing responses, suggesting that context may be significant. For example, while IFN- γ and TNF are linked to bone marrow failure and decreased self-renewal capacity in adults, they enable hematopoiesis during development [84]. Under normal conditions in the mature hematopoietic system, cytokines influence HSC proliferation, differentiation, and self-renewal [84]. IFN- γ , IL-3, and IL-1 can influence the differentiation of HSCs toward myeloid lineages by activating myeloid transcription factors [84]. These same inflammatory factors stimulate hematopoiesis to support the immune system during infection and injury in a process called emergency granulopoiesis [85]. This process leads to the expansion of myeloid cells, which serve as the first line of defense against foreign pathogens [85]. These mechanisms rely on inflammatory mediators and are essential for maintaining homeostasis.

While inflammation enables hematopoietic development and stimulation of HSCs during illness and injury, it also contributes to pathogenesis and disease progression. During infection, inflammatory signals, such as IFN- γ and IL-27, trigger the proliferation and differentiation of HSCs to bolster the immune response either by acting directly on HSCs or indirectly via mature hematopoietic cells, endothelial cells, or the bone marrow microenvironment [86–90]. However, prolonged bacterial or viral infection can hinder self-renewal and competitive repopulation capacity, leading to HSC depletion [91, 92]. This exhaustion of HSCs during chronic inflammation may be attributed to increased myeloid differentiation [93]. Notably, dysregulation of the myeloid cell compartment also occurs in patients with severe COVID-19 [94]. This inflammation-mediated HSC dysfunction may occur via the TLR4-TRIF-ROS-p38 signaling pathway rather than Myd88 signaling, suggesting that the mechanism underlying chronic inflammation may be distinct from that of emergency granulopoiesis [91]. In the context of sepsis, activation of Myd88 caused myelosuppression without significant effects on HSCs, whereas activation of TRIF strongly inhibited HSC self-renewal without direct effects on myeloid cells, inferring cell type-specific effects of these inflammatory mechanisms [95]. Targeting these two pathways may have therapeutic value. While foreign infections can alter hematopoiesis by triggering inflammation, the normal microbiome can also influence the hematopoietic system. See more on this in Section G. Antibiotic-treated mice exhibit depletion of HSCs and progenitor cells as well as anemia, thrombocytosis, pan-lymphopenia, and leukopenia [96]. The complexity of the intestinal microbiome regulates the size of the myeloid cell population in the bone marrow via Myd88 signaling [97]. The disruption of these interactions may have implications for the potential contribution of infection to the progression of preleukemic conditions to hematological disease. For example, disruption of the intestinal barrier promotes myeloproliferation in mice lacking the preleukemic gene Tet2, whereas germ-free Tet2-deficient mice do not exhibit myeloproliferation [98]. Germline Tet2 loss of function is associated with immunodeficiency and lymphoma in children [99, 100]. Importantly, myeloproliferation was alleviated in Tet2-deficient mice with loss of intestinal integrity by treatment with antibiotics [98, 101]. Similarly, bacterial signals cause the expansion of HSCs lacking Tet2 and induce the production of IL-6 from HSCs, bolstering the role of infection and inflammation in the pathogenesis of hematological malignancy [102]. A link to these effects in aged animals remains to be better elucidated, with effects of bacteria on tumor progression and metastasis covered in Section I.

Remarkably, many of the same inflammatory pathways that guide hematopoietic development and strengthen the immune system under normal conditions can also drive leukemia in the context of infection or other inflammatory conditions.

For example, IL-6 facilitates the development of chronic myelogenous leukemia in mice, and IL-33 contributes to myeloproliferative neoplasms by altering myelopoiesis [103, 104]. In addition, inflammation can cause genotoxic stress, the accumulation of mutations, and the progression of preleukemic conditions to leukemia [105, 106]. An unexplored area is the elucidation of the factors and events responsible for the transition from the normal inflammatory response to the deleterious inflammation that can promote hematological disease. As during hematopoietic development, the context of the inflammatory response may be important in shaping disease outcomes. For example, chronic IL-1 signaling can reduce HSC self-renewal, limit hematopoietic lineages, and impair the response of HSCs to replicative challenges [107]. A recent study of MDS indicates that inflammation acts as a selective pressure that specifically fosters expansion of preleukemic or malignant HSCs compared to normal HSCs [108]. Distinct hematopoietic cell subtypes may exhibit differential responses to specific inflammatory signaling molecules, further supporting heterogeneous HSC populations that can drive leukomogenesis [84, 109].

The effect of inflammation on HSCs has valuable implications for age-associated diseases, as older individuals exhibit elevated levels of inflammation [110]. Inflammation plays a significant role in expansion of HSC clones carrying preleukemic mutations in CHIP. Characterized by acquisition of somatic mutations in hematopoietic lineages with age, CHIP is associated with both hematological malignancy and cardiovascular disease (CVD), broadening the role of preleukemic mutations in disease states [111, 112]. The pro-inflammatory response observed in HSCs carrying preleukemic mutations indicates the existence of intrinsic mechanisms of inflammation for HSCs [102, 113, 114]. The addition of a pro-inflammatory preleukemic mutation can alter the presentation of hematological malignancy [115]. It has been proposed that HSCs and mature hematopoietic lineages propagate the inflammatory response via a feedforward mechanism, in which inflammatory signals from one cell type amplifies the other [116]. Pro-inflammatory macrophages secreting IL-1 α enable CHIP-associated CVD, underscoring that some of the same pathways involved in hematopoietic homeostasis and leukemogenesis also contribute to the non-hematopoietic manifestations of CHIP [68]. In addition, as in emergency granulopoiesis, aging promotes myeloid expansion, further emphasizing potential mechanistic overlap [109]. While intrinsic factors appear to be important to the inflammatory response involved in the pathogenesis of CHIP-associated diseases, extrinsic mechanisms may also influence this process as the conditioned media from aged mesenchymal stromal cells impairs the function of young HSCs [117]. In addition to acting directly on HSCs, inflammation can also remodel the bone marrow microenvironment, which regulates

hematopoiesis [118]. Additional studies are needed to understand the ways in which these pathways are altered during aging and pathogenesis, and how they may be modified for health benefit.

Elderly populations may be uniquely susceptible to the effects of inflammation and CHIP as comorbidities and CHIP are more common in aging individuals. Several comorbidities with inflammatory components, such as ulcerative colitis, rheumatoid arthritis, and systemic sclerosis, have been linked to increased clonal hematopoiesis [119–121]. Pro-inflammatory features of CHIP-associated HSCs/HPCs may influence outcomes of hematopoietic stem cell transplants, leading to cytopenias, chronic graft vs. host disease, and/or donor-derived leukemia (DDL) [122]. These patient populations may especially be vulnerable to development of hematological malignancies and CHIP-associated diseases following infection, as infection may exacerbate the existing inflammatory response in these patients. Inflammation may be a key factor contributing to the heterogeneity observed in hematological malignancies and CHIP-associated diseases and should be considered in the clinical management of patients with these conditions. In particular, differences in the clinical presentation between donors and recipients that develop DDL highlight the potential role of inflammation and comorbidities in promoting leukemogenesis. It is yet not known if these different sources of inflammation influence hematological malignancies and CHIP-associated diseases via common mechanisms.

Intrinsic and extrinsic sources of inflammation may represent potential therapeutic targets for hematological malignancies and CHIP-associated diseases. Inhibition of inflammation can impede clonal expansion in response to inflammatory stimuli [102]. Blocking inflammation may also be a valuable therapeutic approach in CVD, as inhibiting IL-1 receptor signaling from pro-inflammatory macrophages can prevent CHIP-associated CVD in mice [68]. It is currently unclear whether suppressing inflammatory HSCs or mature hematopoietic lineages is more effective. Inflammatory signals from the bone marrow microenvironment may also be viable therapeutic targets but have not yet been investigated. A more thorough understanding of the timing of the inflammatory response and the cell types involved will facilitate the effective inhibition of inflammation in hematological malignancies and CHIP-associated diseases. Strategies to both prevent pathogenesis and to treat existing disease will be valuable. A critical aspect of targeting inflammation in these disease contexts will be to maintain the normal inflammatory response necessary for responses to infection and injury while targeting aberrant inflammatory pathways that promote disease; however, additional studies are needed to elucidate factors governing these processes. Thus, Inflammation acts as a dysregulator of tissue maintenance and regeneration during aging as evidenced by

the fact that HSC do not regenerate well after inflammatory challenge [123].

D) DNA Damage, Transcriptional, and Epigenetic Changes During Aging

DNA damage accumulates with age, and defects in DNA repair can cause cellular changes that resemble a premature aging phenomenon [124–127]. Tables on selected models of premature aging in mice and their common features have been summarized in a review [126] and p53 implicated in DNA damage [87]. While DNA damage to HSCs and HPCs during aging is clearly impacted, such damage to the microenvironmental niche cannot be overlooked [124]. Transcriptional changes in stem cell populations have been profiled for HSCs and other stem cell types, but it is not clear yet if a common age-related signature has been identified [12]. A role for epigenetics in the aging process is also considered [128]. Epigenetic hallmarks of aging and senescence have been diagrammed, as have been the pros and cons of using model systems to study aging and senescence in a variety of species, along with a short listing of repositories and tools for evaluating a role for research in the aging process [128].

Repair of damage has been shown to offset deficient HSC function during aging [129]. This was especially apparent under stress conditions, in which DNA damage led to loss of the potential of HSC reconstitution, proliferative capacity, self-renewal activity, enhanced apoptosis, and then exhaustion of function [129]. It was suggested that the accrual of DNA damage may be a means contributing to HSC functional defects of these cells to respond to acute stress or injury [129].

A shift from canonical to non-canonical signaling by Wnt, in response to elevated expression of Wnt5a was associated with the process of HSC aging [130]. Treatment of cells from young mice with Wnt5a induced aging associated HSC apolarity, reduced their capacity for regeneration, and resulted in a age-related shift in myeloid/lymphoid differentiation, that was associated with activation of the GTPase Cdc42 [130]. Moreover, haploinsufficiency of Wnt5a resulted in the attenuation of the aging phenotype of HSCs [130]. Other studies defined replication stress as a driver of functional declines in HSCs during aging [131]. This was associated with decreased expression of mini-chromosomal maintenance helicase components and altered DNA replication fork dynamics [132].

There are reports on a role for epigenetics in abnormalities associated with HSCs in old mice [132–135]. While the decline of HSC function seemed to be dependent on their proliferative history, it was noted to be independent of the length of their telomeres [133]. HSCs from old mice manifested reduced signaling of transforming growth factor-beta with changes in genes involved in proliferation and differentiation of HSCs [135]. HSCs from old mice had broader peaks of

H3K4me3 with increased methylation of DNA at the transcription factor binding sites that were associated with genes involved in promotion of differentiation, and a reduction of genes associated with maintenance of HSCs [135]. Ribosomal biogenesis was found to be a particular target of this age-related HSC phenotype; there was increased transcription of ribosomal protein and RNA genes, and the hypomethylation of genes for ribosomal RNA [135].

Proteasome analysis [136] and single-cell RNA sequencing [137] have been performed on HSCs from old mice. How much these analyses really inform us about the prime drivers in HSC dysfunction remains to be determined, especially since the cells were collected in ambient air prior to analysis, which may not be optimal for assessing physioxia associated effects [1]. Of some interest, deletion of inhibition of DNA binding1 (Id1), a helix-loop-helix transcription factor protected HSCs from both the effects of stress-induced exhaustion and that of aging [138].

E) Metabolic Processes, Mitochondria and Reactive Oxygen Species (ROS) During Aging

Metabolism, mitochondria and ROS are important aspects of HSC function [40, 41], as well as for other stem and progenitor cell types [139–141]. Aging is associated with extensive changes in metabolism [75–77]. A short report questioned whether or not metabolic mechanisms of stem cell maintenance might explain aging and its associated impact on stem cells [142]. Another review concentrated on mitochondrial contributions to dysfunction of somatic stem cells in general and in context of aging [143] and a review on mitochondrial metabolic checkpoints and aging of HSCs implicated mitochondrial maintenance mechanisms including mitophagy and asymmetric segregation of “aged” mitochondria [144]. This is an area that clearly requires more detailed investigation, although it has been suggested that mutations in mitochondrial DNA are not a primary driver of stem cell aging [145].

ROS has been implicated in various stem cell functions [40, 41, 146–148], and STAT3, mitochondrial dysfunction, and overproduction of ROS has been associated with a rapid aging-like phenotype [149]. Symmetric divisions of stem cells, including HSCs, results in increased stem cell numbers with maintenance of stem cell characteristics of the original “mother” cell. However, asymmetric division of stem cells can result in one daughter cell maintaining the original stem cell characteristics of the “mother” stem cell, while the other cell can be a more differentiated progenitor cell. To assess selective apportioning of subcellular contents between “daughter” cells using mammary stem like cells, it was found that “daughter” cells that received fewer “old” mitochondria were associated with maintenance of stem cell traits; inhibition of mitochondrial

fission disrupted age-dependent subcellular localization and segregation of mitochondria with resultant loss of stem cell properties in the progeny [150]. It is not clear if such studies with mitochondria apportioning between HSCs undergoing symmetric or asymmetric divisions have yet been done, but it is certainly an area of interest if done in context of HSC from young and old bone marrow HSCs, and their collection and processing under physioxia conditions as noted [1].

There is still much to be learned regarding how stem cells maintain metabolic homeostasis. The unfolded protein response has been implicated as modulating the HSC pool during stress [151], but has apparently not yet been evaluated in HSCs from aged mice. However, a regulatory branch of the mitochondrial unfolded protein response, mediated by the interplay of the sirtuin, SIRT7 (more on sirtuins in Section F), and nuclear respiratory factor 1 (NRF1) which is a master regulator of mitochondria, was interrogated in HSCs [152]. It was noted that inactivation of SIRT7 resulted in reduced quiescence, increased mitochondrial protein folding stress, and decreased regenerative capacity of HSCs. Moreover, expression of SIRT7 was decreased in HSCs from old mice, and up-regulation of SIRT7 in the aged HSCs improved their regenerative capacity. This implicated the mitochondrial unfolded protein response-mediated metabolic checkpoint as a contributor to HSCs in old mice [152]. In addition, mitochondrial DNA polymerase, when defective, has been associated with premature aging in mice [153], but how, if at all, this relates to HSC function from old mice remains to be determined.

Thioredoxin-interacting protein (TXNIP) is a 397 amino acid residue, belonging to the arrestin family of proteins. It has been reported to regulate HSC quiescence and mobilization after stress [154–156], and is likely to be involved in HSC function, but has not to our knowledge been extensively investigated. Reasons to evaluate this during aging is that *Txnip*^{-/-} mice have decreased HSC reconstitution resulting in HSC exhaustion, effects associated with hyperactive signaling of Wnt, an active cell cycle, and reduced expression of p21^{cip1}. These stresses also affect the BM microenvironment resulting in decreased expression of CXCL12 (a chemotactic and homing chemokine)- and osteopontin-mediated interactions between HSCs and the BM [154]. TXNIP helps to maintain the pool of HSCs by functional switching of p53 after oxidative stress, effects that have been reviewed [155].

There is much to be learned regarding metabolic influences in aging, and molecular mechanisms underlying aging effects on HSCs still remain unclear. Elevated activity of the small RhoGTPase cdc42, previously noted by the investigators in another paper was linked casually to effects on HSCs in old mice [152], with a correlation of the loss of polarity in these cells. Moreover, by inhibiting cdc42 activity by pharmacological means, it “rejuvenated” the aged populations of HSCs by increasing the percent of polarized cells and restoring the level and spatial distribution of histone H4 lysine16 acetylation

such that it was similar to that in HSCs isolated from young mice [152, 157]. This information further identified epigenetic regulatory changes in functional effects of HSCs from old mice, and may relate to metabolic changes.

Other studies linked the interaction of ROS dependent DNA damage, mitochondria, and p38 MAPK with senescence of adult mesenchymal stem/stroma cells (MSCs) from humans, with pharmacological inhibition of p38 MAPK partially recovered the senescence phenotype by partial prevention of hydrogen peroxide-induced senescence [158]. How linked senescence phenotypes are to the function of HSCs in aged persons remains to be determined. Somatic cell mitochondrial DNA (mtDNA) mutations contribute to such age-related disorders as those associated with myelodysplasia (MDS), and it was noted that the mito-protective effect of autophagy was impaired in erythroid cells of old mice [159]. mtDNA-mutated mice had somatic mtDNA mutations that were a targeted defect in the function of proofreading mtDNA polymerase, PolgA, and developed macrocytic anemia similar that seen in MDS patients. Mechanistic insight into these processes was reported [159], but whether or not these processes reflected changes in HSCs from old mice was not explored.

F) Apoptosis, Autophagy, Radiation, and a Role for the Sirtuin Family of Proteins During Aging

Aging-Related Apoptosis and Autophagy

Apoptosis, the phenomenon of programmed cell death, and autophagy, a self-degradative process responsible for eliminating cytosolic constituents such as long-lived proteins, aggregated proteins, and damaged organelles (mitochondria, ribosomes, peroxysomes) [160] have been linked to functional changes noted during aging [161, 162] and HCT [163]. Autophagy is associated with repair pathways that can protect hematopoiesis from injury due to nuclear radiation [164, 165]. Inhibition of autophagy by genetic manipulation was associated with normal and pathological aging, with its inhibition compromising the “longevity-promoting” effects of restriction of calories, the activation of SIRT1, inhibition of insulin and insulin growth factor signaling, and the administration of rapamycin, resveratrol, or spermine [161]. Autophagy was shown to maintain the metabolism of HSCs from both young and old mice [164]. These influences were not noted in all HSCs from old mice, with about a third of HSCs from aged mice demonstrating high autophagy levels being associated with a low metabolic state and high potential for regeneration [165]. This suggests that not all HSCs in aged mice are functionally compromised, an important point in aging HSC research that can be overlooked when studying HSCs from old mice at a total HSC population level. It is known that there are

subsets of rigorously purified HSC populations that differ in mitotic history [166], and intracellular characteristics [1]. FOXO4 was suggested as a pivotal agent in the area of cellular senescence [167]. Using a FOXO4 peptide that disrupted the FOXO4 interactions with p53 *in vivo* where it was tolerated, restored certain functions in naturally aged and in fast aging Xpd^{TTD/TTD} mice. How this relates to HSCs in old mice remains to be evaluated.

Mitophagy is a process that is evolutionary conserved involving autophagic targeting and clearance of mitochondria that are destined for removal [168]. It is induced by short ubiquitin chains on the mitochondria [169]. Reviews on this process have been reported [168, 169] and discuss how metformin, an oral diabetes medication, both enhances and normalizes mitochondrial function that leads to alleviation of inflammation associated with aging [170]. What remains to be determined is if there is a role for mitophagy in HSC and HPC during aging, and if this can be modulated for health benefit.

Radiation Effects and Aging

Like aging, exposure to radiation is an additional stressor to the hematopoietic (H) system, the most sensitive tissue in the body to radiation damage. Therapeutic radiation, nuclear accidents, and malicious exposure from radiologic-warfare put mankind at risk for life-threatening acute radiation syndromes (ARS) and the delayed effects of acute radiation exposure (DEARE) in those fortunate to survive ARS. H-ARS, due to direct and indirect effects of radiation exposure on all classes of hematopoietic cells, leads to death within weeks if untreated [171, 172].

Hematopoietic DEARE, also known as residual bone marrow damage (RBMD), is characterized by diminished immunity and decreased production of blood cells persisting for years after radiation exposure [173–179]. Survivors of H-ARS exhibit severe lifelong damage to HSC, characterized by significantly decreased complete blood count, loss of HSC repopulating potential, loss of HSC quiescence, decreased numbers of HPC, and dramatic myeloid skewing, all most evident under stress [173–183]. An increased incidence of lymphoid malignancies, shortened life span, decreased mesenchymal stem/progenitor cell (MSC) number, and aberrant levels of endothelial cell-derived HSC niche proteins in aged H-ARS survivors have also been documented (Orschell, unpublished data). Long-term damage to the HSC-supportive niche also likely contributes to HSC dysfunction and RBMD. As enhanced cycling of HSC is believed to lead to loss of self-renewal potential and is detrimental to engraftment potential [184], it seems likely that the enhanced cycling of HSC from H-ARS survivors is a major contributor to RBMD. These data illustrate an unrecoverable loss of HSC self-renewal and differentiation potential, the two hallmarks of HSC [185–191], in survivors of H-ARS and suggest that compensatory

mechanisms of hematopoietic support cannot overcome the “second hit” imparted by aging [14, 124, 129, 192, 193].

The DEARE are generally thought to result from persistent inflammation and chronic oxidative stress [194–201], leading to fibrosis [202] and loss of stem cell self-renewal functions. Indeed, elevated levels of pro-inflammatory cytokines associated with oxidative stress [203] have been reported in Japanese atomic bomb survivors [198, 202]. Other studies in atomic bomb survivors have shown possible reductions in self-renewal capability of HSC secondary to dose-dependent DNA damage [204], as well as detriments in immune function [205], corroborating mouse H-ARS data. NAD(P)H oxidase, xanthine oxidase, and mitochondria have all been implicated as primary oxidant sources in various models and conditions [203, 206–208], and ROS has been documented in HSC post-irradiation as well [181]. NF-κB, one of several transcription factors activated by ionizing radiation [209], plays a central role in inflammation [210], is activated by oxidative stress and induces oxidative stress through interactions with cytokines [211], creating a potential feed-forward mechanisms to maintain chronic inflammation and oxidative stress.

Cellular senescence, and its associated oxidative/pro-inflammatory phenotype, has recently emerged as a causative mechanism of DEARE [211–214], making senescent cells a new therapeutic target for RBMD and other DEARE. Importantly, senolytic drugs have the potential to be used as an effective treatment for DEARE even after DEARE becomes a progressive disease [212], but it is not yet clear how this might be used in and for elderly exposed individuals.

As mice age, like humans, health issues and phenotypic changes begin to manifest and variability in experimental endpoints increases, necessitating the need for larger group sizes for sufficient statistical power. For example, mice of similar strains have been shown to exhibit significantly different life spans [215, 216] and radiation sensitivities when aged (Orschell, unpublished data), as well as differing susceptibilities to radiation-induced swollen muzzle syndrome [217], all depending on the vendor from which they were sourced. Mice from different vendors have also been shown to possess different fecal microbiota [218], which may contribute to vendor-specific phenotypic differences. For these reasons, investigators should consider stringent control of the vendor and barrier of their mice for aging studies to ensure optimal stability of their experimental models.

It is noted that more profound effects of aging may be produced not by life-threatening ARS (where the majority of those are exposed to high dose radiation), but rather by moderate or even low dose exposure.

The Role of Sirtuins in Regulating Aged HSC Function

Sirtuins are part of a large family of molecules, some of which have been linked in longevity/aging studies. The role of the

sirtuin SIRT1 in stem cell biology, the aging process and in HSC function in old mice had been reviewed [219]. In this review it was noted that although the role of SIRT1 in telomere maintenance was not resolved, its role in mitochondria and generation of ROS was highly implicated. It was observed that the genetic, hormonal, or drug manipulation of stem cell mitochondria may be useful as an intervening tool for manipulating HSCs from old mice. It was later reported that deficiency of SIRT1 compromised mouse embryonic stem cell hematopoietic cell differentiation in addition to embryonic and adult mouse hematopoiesis [220]. SIRT1 was reported to be required for maintenance of HSCs and lineage specification, in part by the transcription factor FOXO3 [221]. These investigators also suggested that SIRT1 may be involved in HSC function during aging by “delaying” HSC functional abnormalities [221], but this has not been rigorously studied. Although the role of SIRT1 and other sirtuins in the caloric restriction of modifying the aging process have been extensively reviewed [222–224], such studies do not always take into account the sex and mouse strains utilized [225] which could influence the reported results. SIRT3, while found to be dispensable for maintenance of HSCs and homeostasis of tissues during young age, was reported to be essential following stress and with the aging process [226]. SIRT3 expression was decreased with aging and upregulation of SIRT3 expression in HSCs of old mice improved their regenerative capacity, effects involving a role for mitochondrial homeostasis [226]. As noted above in Section E, SIRT7 in the mitochondrial unfolded protein response and aging-associated changes in HSCs in the old mice were linked [152]. This was discussed more thoroughly in a short commentary [227].

G) The Microbiome, Hematopoiesis, and Aging

The microbiome describes microorganisms such as bacteria, viruses, and fungi that colonize the human and animal body and influence various biological processes. Most studies that explored microbiome–hematopoiesis interactions are based on characterizing HSC and HPC populations in germ-free (GF) or broad-spectrum antibiotic-treated mice and in human subjects under prolonged antibiotic regimens or diagnosed with gut dysbiosis such as inflammatory bowel syndrome [228–230]. GF mice demonstrated myelosuppression, smaller HSC, MPP, and common lymphoid progenitor (CLP) populations, and impaired neutrophils, monocytes, and T-cell functions. Recolonization of GF mice restored immune response to infection [96, 228, 231]. However, a closer evaluation of HSCs and HPCs in oral antibiotic-treated mice revealed normal HSC and HPC populations but reduced mature T cell, B cell, and granulocyte populations, suggesting impaired differentiation of mature immune cells in microbiota-depleted mice

after oral antibiotics treatment and introduced some discrepancies between animal models used to study the microbiome [232]. Following HCT, microbiota-depleted recipient mice immune reconstitution was significantly lower than their control counterparts [232] supporting the conclusion that the microbiome plays a role in regulating mature immune cell development. Several studies have linked the human gut microbiome imbalance or dysbiosis in conditions such as inflammatory bowel syndrome, malnutrition, and obesity to altered hematopoiesis [229].

The microbiome and hematopoiesis have been intimately linked [233–238]. Gut microbiota are known to sustain hematopoiesis [233], microbiota can regulate HSC differentiation by altering the BM niche [234], and CX3CR1⁺ mononuclear cells influence HPCs [235]. Reduced mPB is noted in mice receiving antibiotics [236] and microbiota modification has been discussed in context of hematology [237]. Moreover, gut microbiota are known to control bacterial infection by promoting hematopoiesis [238], but definitive and rigorous comparative studies on a role of the microbiome on hematopoiesis in the young and old are yet to be done.

Bacteria and the microbiome present a not uncomplicated scenario that has not been adequately addressed in context of aged hematopoiesis and this needs adequate attention. Certain bacteria, using that of *Fusobacterium nucleatum* as an example, have been implicated in enhancing metastasis of cancer cells [239–248]. If such bacteria have this capacity for cancer cell metastasis, then why not for HSC and HPC, migration and/or homing an area worthy of investigation.

Up to 15% of patients with a history of prolonged antibiotic use have suffered hematological adverse effects in the form of neutropenia, anemia, and pancytopenia [249, 250]. Although associations between the hematopoietic system and microbiota imbalance is apparent in mice and humans, mechanistic understanding of this interaction is limited. In the signal transducer and activator of transcription protein 1 (STAT1) knockout mouse, the antibiotic effects on HSC and HPC numbers were abrogated [96]. In another report, administering a ligand of the pathogen recognition receptor 1 (NOD1) restored HSC and HPC numbers in GF mice [251]. To the authors' knowledge, aged-HSC and HPC function and phenotyping in relation to the microbiome have not yet been reported in mice or humans. Aging is associated with perturbation of intestinal epithelial integrity and upregulation of permeability, allowing microbiota entrance to the circulation and induces a chronic inflammatory state in the aged subject. Aging results in microbiota-associated increases in pro-inflammatory cytokine levels (e.g., TNF- α , TGF- β , IL-6, etc), changes in T-cell numbers (e.g., Treg, Th1, and Th2 T-cell subsets), and activation of TLR2, NF- κ B and mTOR [252]. Considering how the well-characterized low-grade, chronic inflammation associated with aging affects the hematopoietic system, a role for microbiota promoting such inflammation is a strong

possibility. How this might regulate hematopoiesis in the young and old remains an unexplored area to be better studied in steady- and stressed-states.

H) Additional Age-Related Information and Means to Better Evaluate/Understand Hematopoiesis During Aging

Role of Collection/Processing of Cells

Many studies have acknowledged the probable effects of oxidative stress on functional changes in stem cells during aging [253]. This oxidative stress is associated with damage to macromolecules including that of nucleic acids, proteins, lipids, and carbohydrates that could contribute to changes in HSC function. This however, did not consider how even the mere removing of HSC-containing populations of cells from mouse BM [40], mouse mobilized peripheral blood (mPB) [42], or human cord blood [40] could so quickly change HSC numbers and impinge on the function of the removed HSCs. As previously mentioned, collecting and processing cells under ambient air conditions for as little as 15 minutes exposes the cells to extra physiological oxygen stress/shock or EPHOSS. EPHOSS is associated with increased differentiation of HSCs to HPCs through a sequence of events involving p53, the opening of the mitochondrial permeability transition pore, cyclophilin D, hypoxia inducing factor (HIF)1- α and the hypoxamir, miR210 [40, 41]. By collecting and processing BM and mPB from mice and cord blood from humans in a hypoxic chamber set at 3% O₂ and taking care to make sure that the cells are never exposed to ambient air conditions, it is possible to obtain many more phenotyped and functional HSCs [40–42]. Increased numbers of collected HSCs under hypoxia has also been reported for BM cells from *Fancc*^{-/-} and *Fancc*^{-/-} mice [254]. It is possible that some of the EPHOSS-related effects on HSCs exposed to ambient air can be compensated for by collection and processing of these cells at ~4°C [255]. Such cold collections and processing of human cord blood and mouse BM cells mimic at least some of the effects seen during hypoxia collection/processing of cells including increased numbers of collected HSCs. However, the mechanisms involved with preserving HSC numbers/function following cold collection/processing of cord blood cells have not yet been worked out and may differ somewhat from that of physioxia/hypoxia collected/processed cell populations.

Collecting/processing BM cells from old vs. young mice under different oxygen tensions [1], allowed us to demonstrate that functional engrafting HSCs from old mouse BM collected/processed at 3% O₂ were equal in number to that of ambient air (~21% O₂) collected/processed young mouse BM HSCs. Perhaps more importantly, the abnormal myeloid to lymphoid ratio seen when aged BM cells were engrafted into lethally

irradiated recipient mice in a competitive transplant setting was not noticeable and completely resembled that seen after engraftment of young mouse BM. This was consistent with increased CLP numbers and decreased CMP and GMP numbers in the donor BM from old mice following their collection in hypoxia. These phenomena seen with old BM HSCs collected/processed in hypoxia, were associated with decreased total and mitochondria ROS, and decreased expression of stress-induced proteins [1]. Hence, aged mouse BM HSC function may not be as dysregulated as many others have reported, with differences perhaps due to their increased response to EPHOSS following *ex vivo* analysis in ambient air. A corollary of this may suggest that with the more physiological collection and processing of HSCs (a.k.a. in hypoxia conditions, or by means of a physiological strategy (e.g. collecting cells in the cold [255]) from BM of older individuals may be more functional in context of clinical HCT than if they were collected/processed as usual in ambient air O₂. It is known that there are distinct populations of even rigorously purified populations of HSCs [1, 256]. Which of these HSC populations survive EPHOSS effects when cells are collected/processed in ambient air from young and old mouse BM remains to be better elucidated. While much more obviously needs to be done to more mechanistically and physiologically understand the true state of HSCs isolated from older human individuals or mice, it may be that much of the literature on aged HSCs needs to be re-evaluated for their functional status as they are within their *in vivo* physioxia microenvironment. Studies to be done with hypoxia vs. ambient collected HSCs include more in-depth intracellular events and signaling pathways that include gene and protein expression profiles, as well as epigenetic changes, work currently ongoing.

Molecular chaperones and heat shock responses have been postulated to play a role in longevity and aging [257], but rigorous studies in the area of aged HSCs are lacking. Age and organ specific differences in bioenergetics in Brown Norway rats have been noted [258], and p53 deficiency induces diverse dysregulated processes under physiological oxygen/physioxia [259], but we know that such events are influenced by modes of cell collection and processing [40, 41].

The Microenvironment

Not to be ignored in studies of aged HSCs is the role that their microenvironment *in vivo* plays in the functional cellular and intracellular abnormalities/defects associated with their engrafting deficiencies and biased differentiation patterns. The microenvironment niche for HSCs has been studied for young mice [260], but more in-depth studies of the aged BM microenvironment is warranted, especially in context of oxygen content [260], as we noted [1]. There is a report of the rejuvenation of progenitors from old mice when placed into and exposed to a young BM environment [261], and a more

recent report demonstrated that degeneration of adrenergic nerves in BM affects aging of the HSC niche [262], and that aging in humans alters the special organization between populations of CD34⁺ cells (contains mainly HPCs, but also a small percentage of HSCs) and adipocytes in the BM [263]; this is of potential relevance as increased adiposity is associated with the aged BM microenvironment and can alter the functionality of surrounding cells. MSCs showed aging related expression of *cxcr4* [264], a “homing” HSC/HPC receptor, but this has not yet been evaluated under conditions of hypoxia collection and processing such as in [40, 41].

Leptin (Lep) and Lep Receptors (R)

Metabolic activities of cells are adaptively regulated by systemic signals that reflect the nutritional status of an organism throughout its life [265, 266]. This is particularly crucial in the case of HSCs as they are rare, and they maintain the integrity of the entire hematopoietic organ system. One way that the body communicates nutritional cues to HSCs is via systemic hormonal regulations. Various metabolic hormones have been documented to influence hematopoiesis, and aging can significantly alter these metabolic messengers, hence indirectly affecting HSC functional behavior [267–269].

Among them, leptin controls the body energy expenditure and storage through both central and peripheral mechanisms [270]. As an adipokine, it is recognized to have broad spectrum effects on numbers and functions of different immune cells under homeostasis [271]. Aging is known to be associated with multiple dysregulations of the immune system, including a declined adaptive immune response [272]. Lep induces gene expression of p16, a marker of cellular immunosenescence in human B cells from young lean adults. These cells also exhibited lowered class switching activity and ability to produce influenza-specific IgG [273, 274]. Unfortunately, the study was limited to *in vitro* treatment only and did not provide full mechanistic insights. In line with this finding, it was demonstrated that lep induced significantly higher levels of IL-10, TNF- α , and IL-6 from aged human B cells as compared to young controls, and this effect was mediated through the STAT3 signaling pathway downstream of lepR activation [275]. Another group reported that sustainably higher lep levels were found in LPS-treated older (24 month) compared to young (2 month) rats, and the old rats showed delayed but longer febrile response. Elevated lep concentrations were accompanied by increased levels of pro-inflammatory cytokines including IL-6 and IL-1R α ; however, it was not determined whether the increase in lep level was causative and how [276]. Although lep signaling was consistently reported to be altered in aged animals, more rigorously designed studies are needed to help us understand how this well-known proinflammatory neuroendocrine adipokine may play any roles in aging-associated immunological changes [277, 278].

Beyond its role in immunity, lepR-expressing MSCs in murine hematopoiesis have been well-characterized as an indispensable source of stem cell factor for maintenance of both HSCs and more differentiated progenitor cells [279–282]. In addition, it has been demonstrated that BM adipose tissue possessed brown fat properties that declined in old or diabetic mice [283]. It was demonstrated that during the process of aging or in obesity, MSCs preferentially differentiated into adipocytes, which impaired hematopoietic recovery [284]. Since BM adipocytes could be a potential source of lep, it is important to know how BM adipocyte-derived lep can directly or indirectly alter hematopoiesis as the animal ages. In the context of HSC biology, we recently discovered that LepR marks a small subset of robustly repopulating and self-renewing long-term HSCs in adult murine BM; LepR⁺ HSCs (defined as LSKCD150⁺CD48⁺) were found to generate equal myeloid-lymphoid outputs as compared to LepR⁺ HSCs [285]. Given that lepR (OB-R) was also reported to be expressed by different types of both myeloid and lymphoid leukemic cells [286–289], it will be intriguing and important to determine whether aging has a differential selection pressure on LepR⁺ HSCs as compared to the rest of total BM HSCs, particularly in the context of clonal hematopoiesis. Although RNA-seq data suggested that LepR⁺ HSCs and LepR⁺ MPPs (defined as LSKCD150⁺CD48⁺) predominantly expressed the truncated short isoforms *OB-Ra* and *OB-Rc*, it remains to be determined whether aged HSCs or leukemic cells can express the long functional isoform *OB-Rb* (*LepR*). Future studies with more mechanistic insight should be able to address these questions, perhaps providing potential meaningful clinical implications.

More on Inflammation

Aging-related inflammation promoted aging characteristics of HSCs through a tumor necrosis (TNF)- α , ERK, ETS1, IL-27 receptor (R) pathway [290]. TNF- α increases during aging and induced expression of HSC IL-27R- α by ERK-ETS1 intracellular signaling, with deletion of IL-27R- α rescuing functional decline and myeloid bias of HSCs. Old IL-27R- α knockout mice had reduced proportions of myeloid-biased HSCs. Thus, this is another report implicating factors external to HSCs that effect the functional capacity of HSCs from old mice. Somatotrophic/Insulin-Like Growth Factor has also been implicated in aging effects on stem cells as well, in these cases on a population of very small embryonic like stem cells [291, 292].

Exosomes

Exosomes are a subset of small extracellular vesicles that range in size from 30–150nm, that are produced by normal and malignant cells [293, 294], originate from the endocytic compartment of producer cells [295], and have emerged as a

universal intercellular communication system [296, 297]. Exosomes are in a protective protein/lipid bilayer, are delivered to recipient cells without degradation, and freely cross biological barriers [296, 297]. Exosomes [298] influence proliferation of HPCs [299], but there is no information on effects of exosomes on HSCs and HPCs during aging.

Biological Time Keeping and Circadian Rhythms

Biological time keeping [300] and circadian variations are known to influence numbers of HSCs and HPCs in BM and blood, but how this might occur in old mice has not yet been explored. Such studies may be of interest, based on when it is best to collect HSCs and HPCs from older individuals from BM and mPB for use in HCT. Studies of circadian deep sequencing revealed stress-response genes that adapted to high rhythmic expression during aging [301], and daily onset of light and darkness have been reported to manifest control over HSC maintenance and differentiation [302]. Moreover, circadian host and microbiome interactions have been suggested to play relevant roles [303]. There is a noted sexual dimorphism in body clock regulation [304] which has not been adequately addressed, especially in context of aging, and aged HSCs and HPCs.

HSC homing to the BM plays an important role in the engrafting capability of the HSCs [305], and more consideration needs to be given to this capacity of aged HSCs. There are a number of means to enhance the homing of HSCs for enhanced engraftment [306–309], but these have not yet been evaluated in context of HSCs from old mice, or when cells are collected/processed in hypoxia vs. ambient air. Notably, there are a number of animal models to study aging, with an example being the Ames hypopituitary dwarf mice [7–10], that live approximately two times longer than that of most other mice. Imbalanced myelopoiesis was noted in myelopoiesis between BM and spleen in Ames dwarf mice [310].

I) COVID-19/SARS-CoV-2, Aging, and Hematopoiesis

Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) is the pathogen responsible for causing Coronavirus Disease 2019 (COVID-19), a disease which has spread worldwide, infected over 32 million people and claimed the lives of nearly 1 million people as of September 2020 [311]. SARS-CoV-2 is a virus which infects cells by binding to cell surface proteins via its Spike protein that extends out from the viral envelope, facilitating entry to the host cell and allowing for viral replication within the cell [312–314]. The most well-studied presentation of COVID-19 is an infection of the lungs, with symptoms ranging from a mild cough and fever to a severe pneumonia [315, 316].

The prognosis and severity of COVID-19 in patients appears strongly tied to the age of the patient [317]. The risk for SARS-CoV-2 infection leading to symptomatic disease rises dramatically with age. According to the Center for Disease Control (CDC), as age increases the likelihood of being hospitalized for COVID-19 increases, with adults over 65 having 5–13x higher hospitalization rate [318]. Older people are, as presently known, also more likely to die from the disease, with a 90–630x higher death rate in patients over 65 [318], although it is likely that the full story on this is not yet known. This is not likely due to older people being more susceptible to infection, as RT-PCR tests for the presence of SARS-CoV-2 in mild to moderate cases of COVID-19 demonstrate that the host cells of younger patients contain more viral RNA than older patients [319]. A potential interpretation of this is that a higher viral load is necessary for younger people to display symptoms compared to older people. This suggests that children are equally likely to be infected, but may be less likely to display symptoms of the disease, likely due to a general lack of additional contributing factors, possibly including a predisposition to hematologic disorders.

Understandably, many studies of SARS-CoV-2 have focused on its infection of and impact on the lungs. However, it has become increasingly evident that COVID-19 is systemic in nature [320], affecting many different systems including primitive and mature hematopoietic cells [317, 321–324]. The impact that the disease has on the hematopoietic system is evident in the hematological manifestations of COVID-19. A review looking at hematological factors in COVID-19 patients found that both lymphocytopenia and thrombocytopenia are common symptoms with hospitalized patients [322]. It is also evident that more severe cases of COVID-19 were more frequently associated with these hematological factors [322]. Further, one of the more devastating effects of SARS-CoV-2 infection is the induction of a "cytokine storm" [325, 326]. Cytokine storm refers to a toxic excessive release of immune cytokines leading to an autoimmune response. Thus, there is a strong need to address the mechanisms and effects of SARS-CoV-2 exposure on primitive and mature hematopoietic cells and the impact it may have for COVID-19 patients, especially in older individuals.

Recently it was demonstrated that ACE2 is expressed on the cell surface of small numbers of HPCs and mature immune cells [324] and moderate to large numbers of HSCs [323, 324]. Importantly, exposure of human HSCs to SARS-CoV-2 Spike protein induces increases in expression of inflammatory molecules such as *NLRP3* and *IL-1beta* [323], indicating that the cytokine storm may be mediated in part through primitive hematopoietic cells. Further, human HSCs and HPCs exposed to Spike protein *ex vivo* have decreased capacity for functional HPC colony formation, exhibit decreased cell growth, and decreased expansion of HSCs, HPCs, and functional HPC colony forming units compared to cells that were unexposed to the

viral protein [324]. These effects can be neutralized by co-treatment with Angiotensin1-7 [323, 324], a peptide linked to ACE2 regulation of hypertension [327]. The effects of the SARS-CoV-2 Spike protein on colony forming capacity and expansion can also be neutralized by treatment with an antibody targeting SARS-CoV-2 Spike protein or by treatment with soluble human ACE2 [324]. Human peripheral blood mature immune cells also exhibit a response to exposure to Spike protein *ex vivo*, with monocytes upregulating CD14 and undergoing aberrant changes in morphology [324]. It is clear that SARS-CoV-2 does not have to infect HSCs and HPCs to cause some of the above noted effects of the SARS-CoV-2 Spike protein [323, 324]. These, and that yet to be reported, data are important because they may help to explain the origin of hematological manifestations of COVID-19 such as lymphocytopenia and thrombocytopenia and provide insight into neutralizing these effects on the hematopoietic system.

It is very possible that one of the contributing factors to COVID-19 disease severity in the aged population is due to the impact of the disease on a hematopoietic system that has already been accumulating alterations and damage for many years. Additionally, effects of cytokine storm associated inflammation may further damage aged hematopoietic cells, possibly making them even more vulnerable to the development of hematological disorders even after recovery from COVID-19. The relationships between hematologic manifestations of COVID-19 and age should be further studied, as should the effects of SARS-CoV-2 exposure on HSC/HPC and mature immune cells from the aged versus young. It will also be important to determine whether the hematological manifestations of COVID-19 may be neutralized by specifically targeting the effects on the hematopoietic system, thus potentially relieving some of the disease burden on more severely affected patients, including older patients.

J) Conclusions in Context of Potential Future Intervention for Better Health of the Hematopoietic System During Aging

A number of studies have reviewed the aging process in general including protein sequestration at the nuclear periphery, and pathways of cellular proteostasis, the effects of aging on stem cell populations, and potential therapeutic interventions including that for aged HSCs [328–334]. Organoids have been suggested as experimental means to study the process of cellular aging [335], but much more rigorous work is needed in this area, especially with analysis *ex vivo* of HSCs in a relevant physioxia microenvironment BM niche model.

It has been noted that there are molecules in aged blood that promote the spread of cancer [336] and the accumulation of methylmalonic acid promotes tumor progression in the aged

[337]. How these phenomenon relate to HSC, HPC, and hematopoiesis and pre-leukemia and leukemia, and to effects on cells collected in hypoxia/physioxia remains to be determined. A recent book [338] has described the aging process from the perspective of a long-time investigator in this field and noted the mTOR, AMPK and sirtuin pathways as main longevity signaling pathways.

Other considerations in context of aging and hematopoiesis to be elucidated are: how mitochondrial ROS acts as a double-edged conundrum for that of host defense in contrast to infection associated pathological inflammation [339] and its effects on HSC and HPC [340]. More in-depth insights can be gained from approaches incorporating single cell multiomics [341] and what role the mechanoregulation [342] of hematopoiesis might play during aging and disorders associated with aging. There is also the question of a role for mitochondrial transfer from Cx43-expressing HPC to stroma [343] during aging of the hematopoietic system and its interaction with the bone marrow microenvironment during regeneration. As noted in more recent reviews on CHIP [344–346], there is still much we do not understand about this phenomenon and its relationship to aging and aged hematopoiesis.

Cytokines, Chemokines and Intracellular Signaling

HSCs, HPCs, and hematopoiesis are regulated by numerous interacting cytokines and chemokines [347], effects mediated by receptor-induced intracellular signaling [348; Broxmeyer; submitted solicited review on Cytokines/Chemokines/Other Growth Regulators and their Receptors, 8th Edition, Hematology: Basic Principles and Practice, Eds. R. Hoffman, et. al., 2020). Some recent intracellular players involved in HSC and HPC function have been reported [307–309, 349, 350]. However, all such intracellular signaling events have been carried out with HSCs, HPCs, and immune cells of young mice, or human CB or human BM or mPB from younger individuals. Whether or not such regulatory intracellular signaling is similar in cells from old vs. the young remains to be determined, and such studies need to be assessed with purified populations of HSCs and HPCs, and in context of such cells isolated and processed under physioxia conditions, so that they are not exposed to ambient air oxygen which will likely modify the signaling events.

Dipeptidylpeptidase (DPP)4

We had noted that it may be feasible to use HSCs from older individuals for hematopoietic cell transplantation (HCT) if the cells are collected so that they are not induced by EPHOSS to decrease HSC numbers [1] when stressed by ambient air collection [40–42], but are there means to decrease the acute GVHD associated with allogeneic HCT, which perhaps has the potential to be more aggressive when donor cells are from

more aged individuals? Inhibition of the enzyme dipeptidylpeptidase (DPP)4/CD26 has been shown to enhance mouse BM HCT and to accelerate recovery after radiation and selected chemotherapeutic drugs [351, 352] and to enhance time to engraftment of cord blood (CB) HCT [353–355]. This enhancement in time to neutrophil engraftment was also associated with a decrease in the already low acute GVHD noted for CB HCT [356]. It is now clear that the orally active DPP4 inhibitor, sitagliptin used in the CB Trials also greatly dampens acute GVHD in the setting of clinical mPB HCT [357]. Hence, there may be a role for DPP4 inhibitors such as sitagliptin in context of aged hematopoiesis and HCT. DPP4 has also been implicated in exosomes from patients with acute myeloid leukemia [299]. More in depth information on DPP4 during the aging process is clearly warranted. There are many proteins that have purported DPP4-truncation sites [358, 359]. This is of relevance as DPP4-truncated proteins can have decreased activity and block the effects of the full length molecules [352, 360]. Hence, a better undertaking of DPP4 on hematopoiesis in the old, as well as young, could uncover additional means to enhance hematopoiesis during health, aging and disease.

Concluding Thoughts

There are still many unknowns, and still to be elucidated mechanistic insights to understand changes in hematopoiesis during aging. Enhanced genomics may provide additional clues to age-related hematopoiesis [361]. Some of these areas for further investigation are noted in Table 1. It will likely take

Table 1 Suggested areas of investigation requiring more thorough and rigorous analysis to better understand HSC and HPC function and hematopoiesis during the aging process

Listing [In context of physioxia (*in vivo* microenvironmental hypoxia) Collection and Processing Analysis [1, 40–42, 254] or perhaps non-physiological cold collection and processing [255] using purified subset populations of HSCs, HPCs, stromal and accessory cells]:

- Cytokine, chemokine and other growth regulatory factors, and their receptor-mediated intracellular signaling events
- Role for the enzyme dipeptidylpeptidase (DPP)4/CD26 and perhaps other enzymes for modifying protein actions
- Gene Expression
- Epigenetics
- Age-Related Clinal Hematopoiesis of Indeterminant Potential (CHIP), Preleukemia (MDS, MPN), Leukemia
- Metabolism, mitochondria, ROS, apoptosis, autophagy, mitophagy
- Microenvironmental and accessory cells
- Inflammation
- Microbiome

Legend: This is not meant to be a complete listing but a start on areas needing further investigation.

a multidisciplinary approach to fully understand the causes of abnormalities associated with the aging process, not only of the hematopoietic system. This information might at least partially control what might not be the inevitable consequences of the time- and disease-induced aging process. Future studies of hematopoiesis and HSCs during the aging process that investigate these processes in context of their status in an *in vivo*/physioxia environment, and *ex-vivo* under conditions that more closely mimic their *in vivo* condition of O₂ tension and with microenvironmental niche cell interactions [362] will bring us closer to better understanding aged HSCs and what their true functional capacities and abnormalities are. Only with this enhanced understanding can we truly know how this information can best be used and better modified, if necessary and possible, for health benefit.

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Bmi1 regulates Wnt signaling in hematopoietic stem and progenitor cells

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Summary

Polycomb group protein Bmi1 is essential for hematopoietic stem cell (HSC) self-renewal and terminal differentiation. However, its target genes in hematopoietic stem and progenitor cells are largely unknown. We performed gene expression profiling assays and found that genes of the Wnt signaling pathway are significantly elevated in Bmi1 null hematopoietic stem and progenitor cells (HSPCs). Bmi1 is associated with several genes of the Wnt signaling pathway in hematopoietic cells. Further, we found that Bmi1 represses Wnt gene expression in HSPCs. Importantly, loss of β -catenin, which reduces Wnt activation, partially rescues the HSC self-renewal and differentiation defects seen in the Bmi1 null mice. Thus, we have identified Bmi1 as a novel regulator of Wnt signaling pathway in HSPCs. Given that Wnt signaling pathway plays an important role in hematopoiesis, our studies suggest that modulating Wnt signaling may hold potential for enhancing HSC self-renewal, thereby improving the outcomes of HSC transplantation.

Keywords Bmi1, HSC, self-renewal, differentiation, Wnt, and β -catenin

Introduction

Hematopoietic stem cells (HSCs) are multipotent, self-renewing progenitors that generate all mature blood cells [1-2]. In order to maintain hematopoietic homeostasis throughout the lifetime of an organism, the HSC pool must be maintained, which is achieved by the process of self-renewal [3-4]. Although practiced clinically for more than 40 years, the use of HSC transplants remains limited by the ability to expand functional HSCs *ex vivo* [5]. Deciphering the molecular mechanisms controlling HSC self-renewal is essential for developing clinical strategies that can enhance *ex vivo* HSC expansion [3-5].

Polycomb group (PcG) proteins are epigenetic gene silencers that have been implicated in stem cell maintenance and cancer development [6-11]. Genetic and biochemical studies indicate that Polycomb group proteins exist in at least two protein complexes, Polycomb repressive complex 2 (PRC2) and Polycomb repressive complex 1 (PRC1), that act in concert to initiate and maintain stable gene repression [10-11]. Bmi1, a key component of the Polycomb repressive complex 1 (PRC1), is essential for both HSC and leukemia stem cell (LSC) self-renewal [12-15]. We demonstrate that Bmi1 is a substrate of AKT and that AKT-mediated phosphorylation of Bmi1 inhibits HSC self-renewal [16]. In addition to HSC self-renewal, Bmi1 also plays key roles in multi-lineage differentiation [17]. We found that Bmi1 enhances erythroid differentiation through upregulating ribosomal genes [18]. We also found that Bmi1 maintains the self-renewal property of innate-like B lymphocytes [19]. While Bmi1 plays critical roles in hematopoiesis [12-19], its target genes in hematopoietic stem and progenitor cells (HSPCs) are largely unknown. Bmi1 is a potent negative regulator of the *Ink4a/Arf* locus, which encodes the cell cycle regulator and tumor suppressor p16^{Ink4a} and p19^{Arf} proteins [20-21]. In *Bmi1*^{-/-} bone marrow (BM) cells, there is an upregulation of both p16^{Ink4a} and p19^{Arf} [22]. However, loss of both p16 and p19 only partially rescues the self-renewal defects of *Bmi1*^{-/-} HSCs [22], suggesting that Bmi1 may regulate the expression of other genes in HSPCs.

The Wnt signaling pathway has pivotal roles during the development of many organ systems, and dysregulated Wnt signaling is a key factor in the initiation of various tumors [23]. In the canonical Wnt pathway, Wnt ligand binds to its receptor Frizzled at the cell surface and inhibits glycogen synthase kinase-

3 β (GSK-3 β)-mediated phosphorylation and degradation of β -catenin. Stabilized β -catenin then translocates to the nucleus where it binds to T cell factor (TCF)/lymphoid enhancer factor (LEF) transcription factors and induces the expression of Wnt target genes, such as *Tcf* and *Axin2*. In the absence of Wnt ligand, GSK-3 β phosphorylates β -catenin and targets it for ubiquitination and degradation [23]. Canonical Wnt signaling is differentially activated during hematopoiesis [24-26], suggesting an important regulatory role for specific Wnt signaling levels [27-29]. The Adenomatous polyposis coli gene (*Apc*) is a negative modulator of the canonical Wnt pathway and loss of *Apc* in hematopoietic compartment leads to stabilization of β -catenin and activation of the Wnt signaling pathway [29-30]. By combining different targeted hypomorphic alleles and a conditional deletion allele of *Apc*, a gradient of five different Wnt signaling levels were obtained *in vivo* [26]. By analyzing the effect of different mutations of *Apc* on hematopoiesis, Luis and colleagues demonstrated that the canonical Wnt signaling regulates hematopoiesis in a dosage-dependent fashion: Low levels of Wnt signaling activation (2-fold increase above normal) result in the maintenance of a multipotent state, therefore resulting in increased reconstitution in stem cell transplantation assays, and that high level of Wnt signaling (more than 10-fold increase above normal) results in impaired HSC self-renewal and a block in differentiation [26]. While Wnt signaling plays an important role in hematopoiesis [24-26], how Wnt signaling is regulated in HSCs remains elusive. Identify key regulators of Wnt signaling in HSCs may lead to novel approaches to expand human HSCs *ex vivo* and improve transplantation efficiency.

In this study, we discovered that several genes of the Wnt signaling pathway are upregulated in *Bmi1* null hematopoietic stem and progenitor cells (HSPCs). *Bmi1* binds to several Wnt genes on the chromatin and represses their transcription in HSPCs. Importantly, we found that loss of β -catenin partially rescues the HSC self-renewal and differentiation defects seen in the *Bmi1*^{-/-} mice. Thus, we have identified *Bmi1* as a novel regulator of Wnt signaling pathway in hematopoietic stem and progenitor cells.

Materials and Methods

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Mice

Global *Bmi1* knockout mice were provided by Dr. Martin van Lohuizen at the Netherlands Cancer Institute, the Netherlands [20]. Conditional *Bmi1* knockout mice (*Bmi1^{F/F}*) in the *C57BL6* background were generated at the National Institute of Biological Science, Beijing, China. The *Ctnnb1^{F/F}* mice were obtained from the Jackson Labs and have been backcrossed to the *C57BL6* background for at least 8 generations. Wild type *C57BL/6* (*CD45.2⁺*), *B6.SJL* (*CD45.1⁺*) and *F1* mice (*CD45.2⁺ CD45.1⁺*) mice were obtained from an on-site core breeding colony. All mice were maintained in the Indiana University Animal Facility according to IACUC-approved protocols.

Flow cytometry

Flow cytometry analysis of hematopoietic stem and progenitor cells was performed as described previously [31-32]. Murine hematopoietic stem and progenitor cells were identified and evaluated by flow cytometry using a single cell suspension of bone marrow mononuclear cells (BMMCs). Hematopoietic stem and progenitors are purified based upon the expression of surface markers. Bone marrow (BM) cells were obtained from femurs by flushing cells out of the bone using a syringe and phosphate-buffered saline (PBS) with 2mM EDTA. Red blood cells (RBCs) were lysed by RBC lysis buffer (eBioscience) prior to staining. Experiments were performed on FACS LSR IV cytometers (BD Biosciences) and analyzed by using the FlowJo Version 9.3.3 software (TreeStar).

Gene expression and Pathways Analyses

Transcript profiling of HSCs and MPPs from *Bmi1^{+/+}* and *Bmi1^{-/-}* mice were analyzed by Agilent Whole Mouse Genome Oligo Microarrays. Raw data will be available for download from Gene Expression Omnibus (<http://ncbi.nlm.nih.gov/geo/>, accession number in progress). Genes whose expressions are increased or decreased more than 2-fold in *Bmi1^{-/-}* cells compared to wild-type cells are shown. The Microarray data were analyzed using the Ingenuity Pathways Analysis program (Ingenuity Systems,

www.ingenuity.com); to identify the pathways that met the < or > 2-fold change cutoff and were associated with a canonical pathway in the Ingenuity Pathways Knowledge base were considered for the analysis. The significance of the association between the data set and the identified canonical pathway was measured in 2 ways: (1) A ratio of the number of genes from the data set that map to the pathway divided by the total number of genes from the data set that map to the canonical pathway and (2) Fischer's exact test, to calculate a p value determining the probability that the association between the genes in the data set and the canonical pathway is explained by chance alone.

ChIP assays

For ChIP assays, Kit⁺ BM cells and Baf3 cells were fixed with 1% (vol/vol) formaldehyde for 10 min at room temperature. ChIP assays were performed using the EZ-Magna ChIP A/G Kit (Millipore). Anti-Bmi1 antibody (Active Motif, AF27) and normal mouse IgG were used for immunoprecipitation. ChIP DNA was then subjected to real-time PCR analysis using primers targeting different region of genes of the canonical Wnt pathway and *Ink4a/Arf* locus.

Reporter assays

The Top-Flash luciferase construct encodes the firefly luciferase reporter gene under the control of a minimal CMV promoter and 8 tandem repeats of the TCF/LEF transcriptional response element (TRE). A Top-Flash mutant plasmid, containing defective TCF/LEF transcriptional response element, was used as a negative control in this assay. Each reporter is premixed with constitutively expressing Renilla luciferase, which serves as an internal control for normalizing transfection efficiencies and monitoring cell viability. 293T cells were transfected using Lipofectamine 2000 transfection reagent (ThermoFisher), according to the manufacturer's instructions. Transfected cells were harvested 24 hours later, and processed using Dual-Luciferase Reporter Assay (Promega).

Generation of retroviruses and infection of murine HSCs and MPPs

Retroviral vectors were produced by transfection of Phoenix E cells with the MIGR1 control (MSCV-IRES-GFP) or MIGR1 full-length Bmi1 c-DNA plasmid (MSCV-Bmi1-IRES-GFP), according to

standard protocols [16]. Murine HSCs and MPPs were infected with high-titer retroviral suspensions in the presence of 8 µg/mL polybrene (Sigma-Aldrich). Forty-eight hours after infection, the GFP-positive cells were sorted by FACS.

Transplantation

We transplanted 500,000 BM mononuclear cells isolated from *Bmi1^{F/F}-Ctnnb1^{F/F}-Mx1Cre⁻*, *Bmi1^{+/+}-Ctnnb1^{F/F}-Mx1Cre⁺*, *Bmi1^{F/F}-Ctnnb1^{+/+}-Mx1Cre⁺*, and *Bmi1^{F/F}-Ctnnb1^{F/F}-Mx1Cre⁺* mice (CD45.2⁺) together with 250,000 competitor BM cells (CD45.1⁺) into lethally irradiated recipient mice (CD45.1⁺CD45.2⁺). Eight weeks following transplantation, we injected pI:pC to delete *Bmi1* and/or *Ctnnb1* from hematopoietic cells. Peripheral blood was obtained by tail vein bleeding every 4-week after pI:pC treatment, RBC lysed, and the PB mononuclear cells stained with anti-CD45.2 FITC and anti-CD45.1 PE, and analyzed by flow cytometry. 20 weeks following transplantation, bone marrow cells from recipient mice were analyzed to evaluate donor chimerism in bone marrows. For secondary transplantation, 3 x 10⁶ BM cells from primary recipient mice reconstituted with *Bmi1^{F/F}-Ctnnb1^{F/F}-Mx1Cre⁻*, *Bmi1^{+/+}-Ctnnb1^{F/F}-Mx1Cre⁺*, *Bmi1^{F/F}-Ctnnb1^{+/+}-Mx1Cre⁺*, and *Bmi1^{F/F}-Ctnnb1^{F/F}-Mx1Cre⁺* BM cells were injected into lethally irradiated F1 mice (CD45.1⁺CD45.2⁺).

Statistical Analysis

Statistical analysis was performed with GraphPad Prism 8 software (GraphPad software, Inc). All data are presented as mean ± standard error of the mean (SEM). The sample size for each experiment are included in the figure legends. Statistical analyses were performed using unpaired, two-tailed Student's t test where applicable for comparison between two groups, and a One-way ANOVA test or Two-way ANOVA was used for experiments involving more than two groups. Statistical significance was defined as *p < 0.05, **p < 0.01, ***p < 0.001; ns, not significant.

Results

Genes of the Wnt signaling pathway are upregulated in *Bmi1* null hematopoietic stem and progenitor cells

To understand how Bmi1 regulates HSC self-renewal and differentiation, we performed gene expression profiling assays (using microarray analysis and quantitative RT-PCR analysis) to identify Bmi1 target genes in HSCs (CD48⁻CD150⁺Lin⁻Sca1⁺Kit⁺ cells) and MPPs (CD48⁺CD150⁻Lin⁻Sca1⁺Kit⁺ cells). Microarray analysis revealed that several genes in the canonical Wnt signaling pathway are significantly elevated in both HSCs and MPPs from *Bmi1*^{-/-} mice (Fig. 1A-B), suggesting that Wnt signaling may be activated in the absence of Bmi1. We first confirmed upregulation of several genes of the canonical Wnt pathway, including *Fzd4*, *Fzd7*, *Fzd9*, *Tcf3*, and *Axin2*, in *Bmi1*^{-/-} HSCs compared to *Bmi1*^{+/+} HSCs using quantitative RT-PCR analysis (Fig. 1C). *Fzd4*, *Lgr4*, *Lef1*, *Tcf3*, and *Axin2* are significantly upregulated in *Bmi1*^{-/-} MPPs compared to *Bmi1*^{+/+} MPPs. (Fig. 1D). These findings suggest that Bmi1 may be associated with genes in the Wnt pathway, thereby repressing their expression in HSPCs.

Bmi1 is associated with Wnt genes in hematopoietic cells

To determine if Bmi1 is directly associated with genes in the Wnt pathway, we performed chromatin immunoprecipitation (ChIP) assays using an antibody against Bmi1 or normal mouse IgG. As expected, Bmi1 was associated with the *Ink4a/Arf* locus in both Kit⁺ BM and Baf3 cells (Fig. 2A-B). We detected the association of Bmi1 with the *Lef1*, *Fzd4*, and *Axin2* promoters in these cells (Fig. 2A-B). Ring1b is the catalytic component of the PRC1 complex and genome-wide ChIP-seq analysis showed Ring1b binding to several Wnt target genes, including *Ccnd2*, *Fzd7*, *Ckn2a* and *Tcf3*, in murine L8057 megakaryoblastic cells (Fig. 2C) [33]. Thus, we demonstrate that Bmi1/PRC1 is associated with Wnt genes in hematopoietic cells.

Bmi1 represses Wnt gene expression in hematopoietic stem and progenitor cells

To determine the impact of Bmi1 expression on Wnt activation in cells, we used the Top-Flash Wnt reporter system. The Top-Flash reporter system is designed to monitor the activity of Wnt signal

transduction pathway in cultured cells [34]. While Wnt3a readily activates the Wnt reporter in 293T cells, Wnt activation is efficiently repressed by ectopic Bmi1 expression, demonstrating that Bmi1 indeed can inhibit Wnt signaling activation in cultured cells (Fig. 3A).

To determine whether Bmi1 represses the expression of Wnt signaling genes in HSPCs, we introduced Bmi1 (MSCV-Bmi1-IRES-GFP) or GFP (MSCV-IRES-GFP) into HSCs and MPPs purified from *Bmi1*^{+/+} and *Bmi1*^{-/-} mice using retroviruses mediated transduction. 48 hours after transduction, we isolated mRNA from transduced cells (GFP⁺) and performed qRT-PCR assays for genes involved in the canonical Wnt signaling pathway. We found that that ectopic *Bmi1* expression results in downregulation of *Fzd4*, *Fzd7*, and *Lef1* expression in *Bmi1*^{+/+} HSCs compared to that of the control viruses (MSCV-IRES-GFP) transduced cells (Fig. 3B). We also found that that ectopic *Bmi1* expression leads to downregulation of *Fzd4*, *Fzd7*, *Fzd9*, *Lef1*, and *Axin2* expression in *Bmi1*^{+/+} MPPs compared to that of the control viruses transduced cells (Fig. 3C). In addition, ectopic *Bmi1* expression represses *Fzd4*, *Fzd7*, and *Fzd9* expression in *Bmi1*^{-/-} HSCs compared to that of the control viruses transduced cells (Fig. 3D). Further, ectopic *Bmi1* expression leads to downregulation of *Fzd4*, *Fzd9*, and *Lef1* in *Bmi1*^{-/-} MPPs (Fig. 3E). Thus, we demonstrate that Bmi1 represses Wnt gene expression in HSPCs.

Loss of β -catenin partially rescued HSC self-renewal and differentiation defects seen in Bmi1 null mice

The *Ctnnb1* gene encodes β -catenin and loss of β -catenin reduces Wnt activation [35]. To determine the impact of β -catenin deficiency on *Bmi1*^{-/-} HSPCs, we generated *Bmi1*^{F/F}-*Ctnnb1*^{F/F}-*Mx1Cre*⁺ mice. We transplanted 500,000 BM cells isolated from *Bmi1*^{F/F}-*Ctnnb1*^{F/F}-*Mx1Cre*⁻, *Bmi1*^{+/+}-*Ctnnb1*^{F/F}-*Mx1Cre*⁺, *Bmi1*^{F/F}-*Ctnnb1*^{+/+}-*Mx1Cre*⁺, and *Bmi1*^{F/F}-*Ctnnb1*^{F/F}-*Mx1Cre*⁺ mice (CD45.2⁺) together with 250,000 competitor BM cells (CD45.1⁺) into lethally irradiated recipient mice (CD45.1⁺CD45.2⁺). Eight weeks following transplantation, we injected pI:pC to delete *Bmi1* and/or *Ctnnb1* from hematopoietic cells and examined the frequency of donor-derived cells (CD45.2⁺) in peripheral blood every 4 weeks for twenty

weeks. Conditional deletion of *Ctnnb1* does not affect the repopulating ability of BM cells (Fig. 4A). While the percentage of donor-derived cells in PB of recipient mice repopulated with *Bmi1*^{-/-} BM cells decreases, loss of *Ctnnb1* significantly increased the engraftment of *Bmi1*^{-/-} BM cells at twenty weeks following pI:pC treatment (Fig. 4A). We observed increased number of donor-derived HSCs in the BM of recipient mice repopulated with *Bmi1*^{-/-}*Ctnnb1*^{-/-} BM cells compared to that of the *Bmi1*^{-/-} cells (Fig. 4B-C). Loss of *Bmi1* resulted in decreased myeloid differentiation and increased lymphoid differentiation in the BM, whereas *Ctnnb1* deficiency rescued differentiation defects seen in *Bmi1*^{-/-} mice (Fig. 4D).

To determine the impact of genetic deletion of *Ctnnb1* on *Bmi1*^{-/-} HSC self-renewal, we performed secondary BM transplantation assays. We found that *Bmi1*^{-/-}*Ctnnb1*^{-/-} BM cells show increased engraftment compared to *Bmi1*^{-/-} cells following secondary transplantation (Fig. 4E). Thus, we demonstrate that loss of β -catenin, which reduces Wnt activation, partially rescues HSC self-renewal and differentiation defects seen in *Bmi1*^{-/-} mice.

Discussion

Hematopoietic stem cell (HSC) self-renewal requires a complex crosstalk between extrinsic signals from the microenvironment and the HSC-intrinsic regulators to maintain an undifferentiated state [1-4]. However, the crosstalk between signaling pathways and HSC-intrinsic regulators has not been well defined at the molecular level [1-4]. Thus, there remains a critical need to improve our understanding of the interactions between signaling pathways and HSC-intrinsic regulators and develop novel strategies that can enhance *ex vivo* HSC expansion and improve the efficiency and outcome of HSC transplantation [5].

The role of Wnt signaling in adult hematopoiesis has been controversial [24-25]. While loss of Wnt3a impairs HSCs self-renewal and differentiation [37-38], blocking the secretions of Wnt proteins in the hematopoietic system does not affect hematopoiesis [39]. Constitutive beta-catenin activation impairs HSC self-renewal and blocks terminal differentiation [36]; however, mice lacking β - and γ -catenin have normal stem cell self-renewal and differentiation [35]. These findings indicate canonical Wnt signaling is far more complicated than expected. Indeed, the canonical Wnt signaling appears to regulate adult hematopoiesis in a dosage-dependent manner: low level of canonical Wnt activation enhances HSC self-renewal, whereas high level of canonical Wnt activation impairs HSC self-renewal and blocks terminal differentiation [26].

Polycomb group protein Bmi1 plays an important role in cellular homeostasis by maintaining a balance between proliferation and senescence [12-15, 20]. It is often overexpressed in cancer cells and is required for stem cell self-renewal [14-15]. However, the downstream targets that mediate Bmi1 function remain elusive. To explore the mechanism by which Bmi1 enhances hematopoiesis, we performed transcript profiling assays to compare gene expression in HSCs and MPPs isolated from wild type and Bmi1^{-/-} mice. We found that the expression of several genes of the Wnt signaling pathway was upregulated in hematopoietic stem and progenitor cells (HSPCs). Further, Bmi1 directly associates with the promoter of these genes in hematopoietic cells. Importantly, we found that loss of β -catenin partially rescued self-renewal defects seen in Bmi1 null mice. Given that activation of the canonical Wnt pathway leads to loss of hematopoietic stem cell repopulation and multilineage differentiation block [26, 29-30, 36], our studies uncover a novel mechanism by which Bmi1 enhances HSC self-renewal and promotes terminal differentiation,

Previous studies have implicated Bmi1 in regulating WNT signaling in some cancer cells [40-41]. BMI1 has been shown to autoactivate its own promoter via an E-box present in its promoter. Further, BMI1 activates the WNT pathway by repressing DKK family of WNT inhibitors. BMI1 mediated repression of DKK1 results in up-regulation of WNT target c-Myc, leading to transcriptional

autoactivation of BMI1 [36]. This positive feedback loop regulating BMI1 expression may be relevant to the role of BMI1 in promoting cancer and maintaining stem cell phenotype [40]. For example, BMI1 is upregulated in colon cancer tissues and cell lines. Overexpression of BMI1 in primary epithelial colon cells promotes cellular growth and activates WNT pathway, whereas knocking down of BMI1 expression in colon cancer cells represses these effects [41]. Mechanistically, BMI1 activates WNT signaling in colon cancer by negatively regulating the WNT antagonist IDAX [41]. These findings suggest that Bmi1 may play a context-dependent role in regulating Wnt signaling during development and tumorigenesis.

Although BMI1 is upregulated in many cell types, very little is known about the signaling pathways that regulate its expression. Wnt signaling plays a key role in intestinal stem cells and Bmi1 has been shown to be a potential marker for intestinal stem cells [42]. The expression of Bmi1 in human colon cancers is associated with nuclear β -catenin, a hallmark for the activated Wnt signaling. Thus, these studies suggest that Wnt signaling may regulate the expression of Bmi1 in colon cancer cells [42]. Whether Wnt signaling regulates Bmi1 expression in hematopoietic stem and progenitor cells is not known, thereby awaiting future investigation.

In summary, we have identified Bmi1 as a negative regulator of canonical Wnt signaling pathway in hematopoietic stem and progenitor cells. Our studies suggest that modulating canonical Wnt signaling may hold potential for enhancing HSC self-renewal, thereby improving the outcomes of HSC transplantation,.

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293 **Author Contributions**

294 H.Y., R.G., S.C., R.X., and Y.L. Designed the research. H.Y., R.G., S.C., X.L., Q.W., W.C., S.V.,
295 A.C.F., D.H., M.K., and S.Z.L. Performed the research; H.Y., R.G., S.C., Q.W., and Y.L. Analyzed the
296 data and performed the statistical analysis. Z.Q., R.K., H.E.B., and Z.G. Provided reagents and
297 constructive advice to the study. S.C., R.X., and Y.L. Wrote the manuscript. All authors read,
298 commented on, and approved the manuscript.

300 **Conflict Interest**

301 The authors declared that no conflict interest exists.

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FIGURE LEGENDS

Fig. 1 Genes of the Wnt signaling pathway are upregulated in hematopoietic stem and progenitor cells.

(A) Transcript profiling of HSCs (CD48⁻CD150⁺LSKs) from *Bmi1*^{+/+} and *Bmi1*^{-/-} mice were analyzed by Agilent Whole Mouse Genome Oligo Microarrays. Genes that are differentially expressed in *Bmi1*^{-/-} HSCs compared to wild-type cells are shown. We utilized Ingenuity pathways Analysis (Ingenuity Systems) to group genes into specific canonical pathways. Values are shown for three biological replicates. Color red indicates genes that are upregulated in *Bmi1*^{-/-} HSCs and color blue indicates genes that are downregulated in *Bmi1*^{-/-} HSCs. (B) Transcript profiling of MPPs (CD48⁺CD150⁻LSKs) from *Bmi1*^{+/+} and *Bmi1*^{-/-} mice were analyzed by Agilent Whole Mouse Genome Oligo Microarrays. Genes that are differentially expressed in *Bmi1*^{-/-} MPPs compared to wild-type cells are shown. We utilized Ingenuity pathways Analysis (Ingenuity Systems) to group genes into specific canonical pathways. Values are shown for three biological replicates. Color red indicates genes that are upregulated in *Bmi1*^{-/-} MPPs and color blue indicates genes that are downregulated in *Bmi1*^{-/-} MPPs. (C) Real-time RT-PCR analysis of gene expression in *Bmi1*^{+/+} and *Bmi1*^{-/-} HSCs. Data shown are relative expression as compared to *Bmi1*^{+/+} HSCs (set as 1), n = three biological replicates, *p<0.05, **p<0.01. (D) Real-time RT-PCR analysis of gene expression in *Bmi1*^{+/+} and *Bmi1*^{-/-} MPPs. Data shown are relative expression as compared to *Bmi1*^{+/+} MPPs (set as 1), n = three biological replicates, *p<0.05, **p<0.01, ***p<0.001.

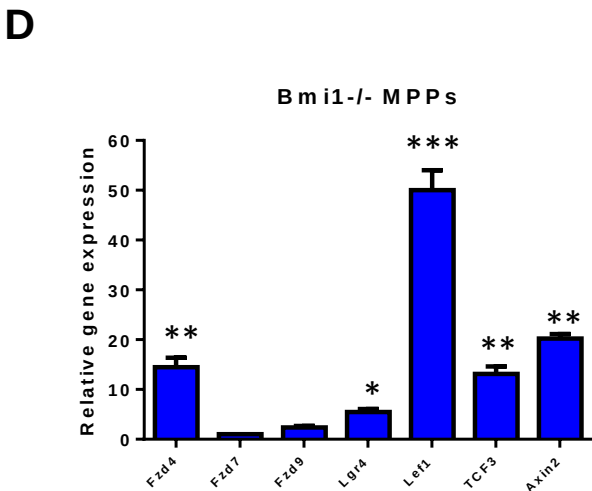
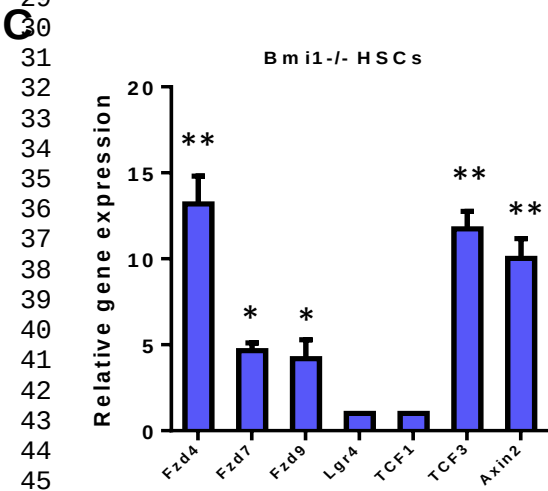
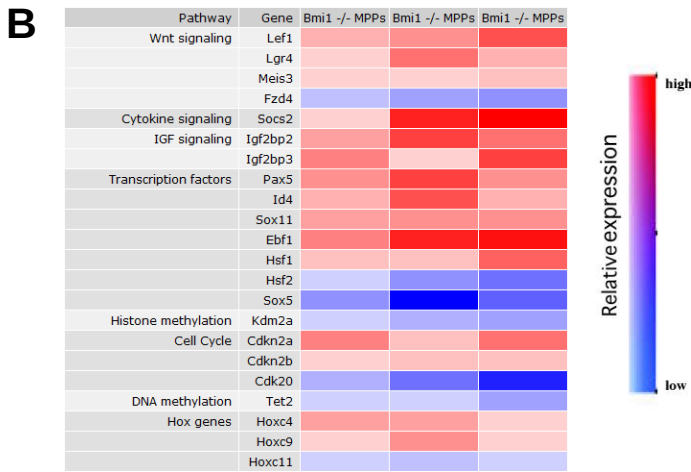
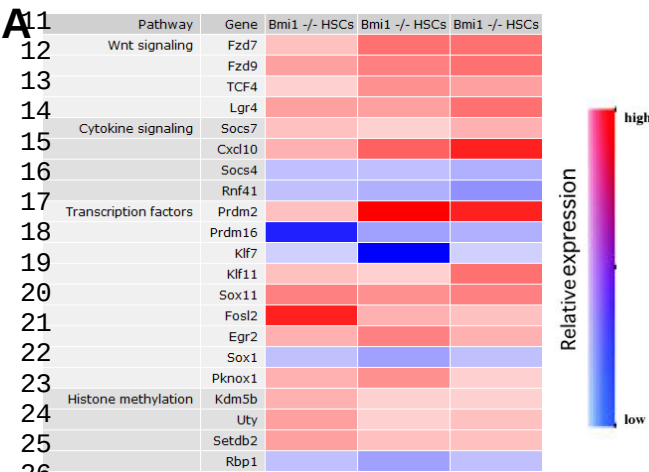
Fig. 2 Bmi1 is associated with Wnt genes in hematopoietic cells. (A) Bmi1 binds to promoters of genes of the Wnt pathway *in vivo*. Chromatin bound DNA from Kit⁺ BM cells was immunoprecipitated with a Bmi1-specific antibody or with normal mouse IgG. qRT-PCR amplification was performed on corresponding templates using primers for indicated genes, n = three biological replicates, **p<0.01. (B) Bmi1 binds to promoters of genes of the Wnt pathway *in vivo*. Chromatin bound DNA from Baf3 cells was immunoprecipitated with a Bmi1-specific antibody or with normal mouse IgG. qRT-PCR amplification was performed on corresponding templates using primers for indicated genes, n = three biological replicates, *p<0.05, **p<0.01. (C) Representative Ring1b and IgG ChIP-seq profiles of loci

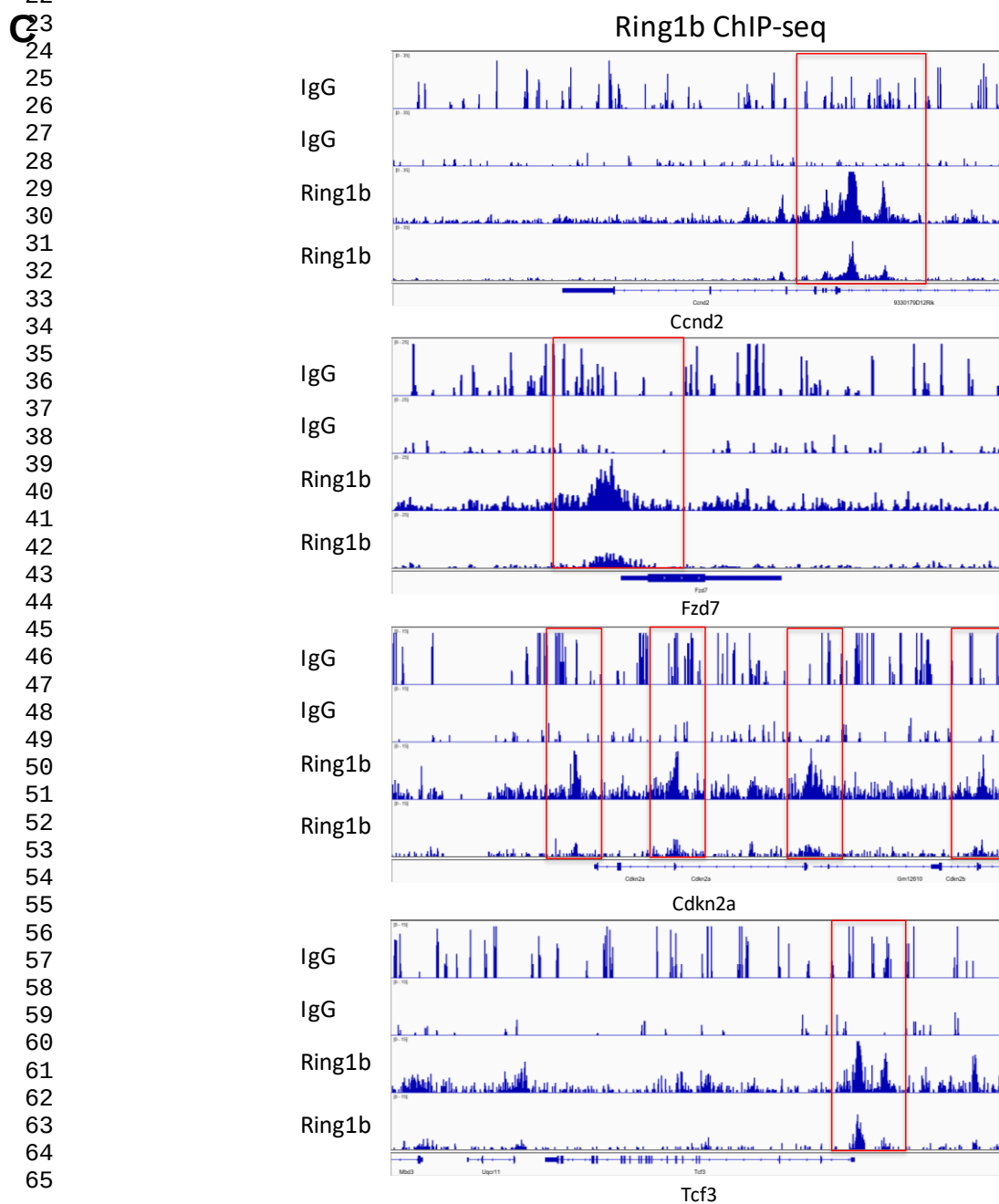
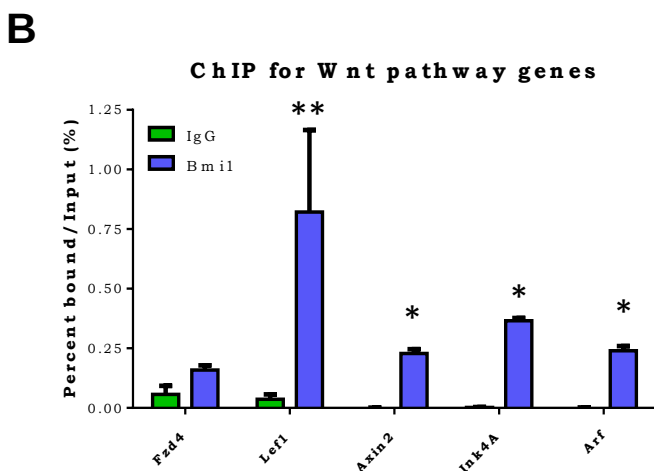
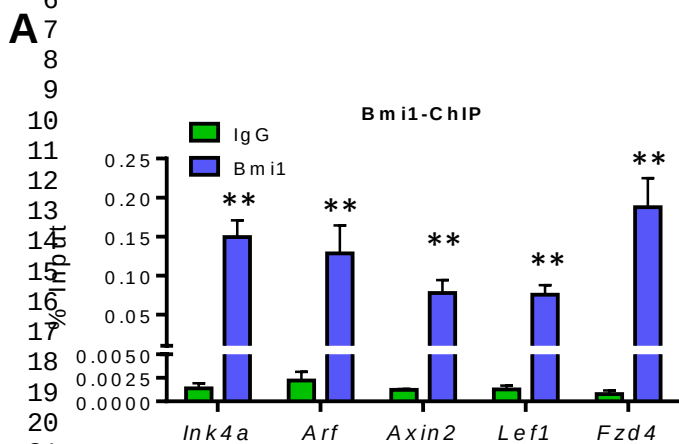
occupied by Ring1b in murine L8057 megakaryoblastic cells. It appears that Ring1b associates with several Wnt target genes, including *Ccnd2*, *Fzd7*, *Cdkn2a* and *Tcf3*, in L8057 cells.

Fig. 3 Bmi1 regulates Wnt gene expression in hematopoietic stem and progenitor cells. **(A)** Bmi1 represses Wnt3a-induced Wnt reporter activation. Luciferase activity was assayed 24 hours after transfection of 293T cells. Values are means ($\pm$ SEM), n = three biological replicates, *p<0.05. **(B)** Ectopic Bmi1 expression represses Wnt gene expression in *Bmi1*^{+/+} HSCs. Data shown are relative expression compared to control viruses (MSCV-IRES-GFP) transduced HSCs, n = three biological replicates, *p<0.05, **p<0.01. **(C)** Ectopic Bmi1 expression represses Wnt gene expression in *Bmi1*^{+/+} MPPs. Data shown are relative expression compared to control viruses transduced MPPs, n = three biological replicates, *p<0.05, **p<0.01. **(D)** Ectopic Bmi1 expression represses Wnt gene expression in *Bmi1*^{-/-} HSCs. Data shown are relative expression compared to control viruses transduced *Bmi1*^{-/-} HSCs, n = three biological replicates, *p<0.05, **p<0.01. **(E)** Ectopic Bmi1 expression represses Wnt gene expression in *Bmi1*^{-/-} MPPs. Data shown are relative expression compared to control viruses transduced *Bmi1*^{-/-} MPPs, n = three biological replicates, *p<0.05, ***p<0.001.

Fig. 4 Loss of β -catenin partially rescued HSC self-renewal and differentiation defects seen in Bmi1 null mice. **(A)** Percentage of donor-derived cells (CD45.2⁺) in the peripheral blood of recipient mice following pI:pC treatment. n=7 mice per group, **p<0.01. **(B)** Percentage of donor-derived cells (CD45.2⁺) in the BM of recipient mice at 20 weeks following pI:pC treatment. n=4 mice per group, **p<0.01. **(C)** The frequency of donor-derived HSCs in the BM of recipient mice at 20 weeks following pI:pC treatment. n=4 mice per group, *p<0.05. **(D)** Lineage distribution of donor-derived cells in the bone marrow of primary recipient mice at 20 weeks following pI:pC treatment. n=4 mice per group, **p<0.01, ***p<0.001. **(E)** Percentage of donor-derived cells (CD45.2⁺) in the peripheral blood of recipient mice at 8 weeks following secondary transplantation. n=7-8 mice per group, ***p<0.001.

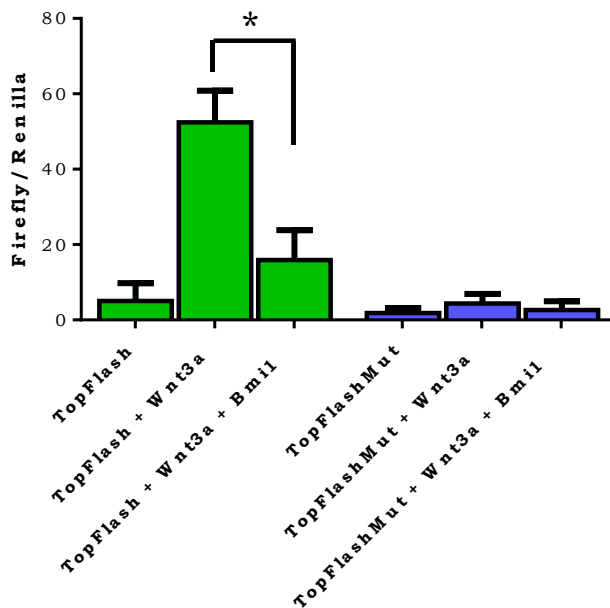
Figure 1



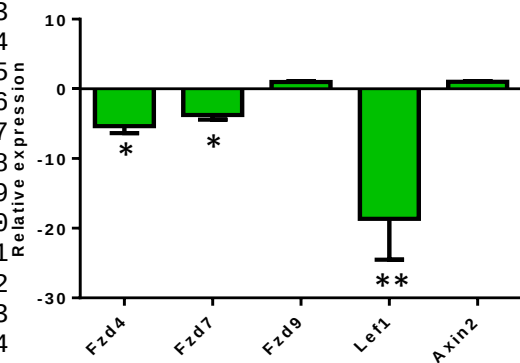


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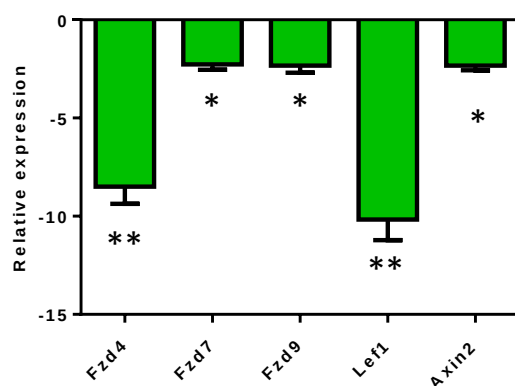
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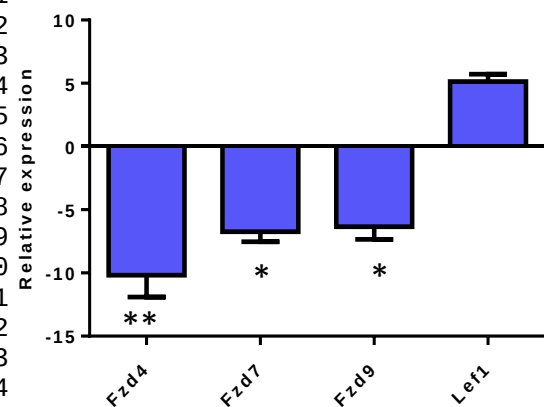
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Ectopic Bmi1 expression in Bmi1^{+/+} HSCs

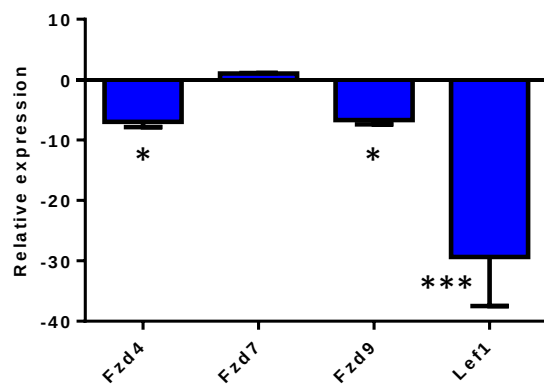
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Ectopic Bmi1 expression in Bmi1^{+/+} MPPs

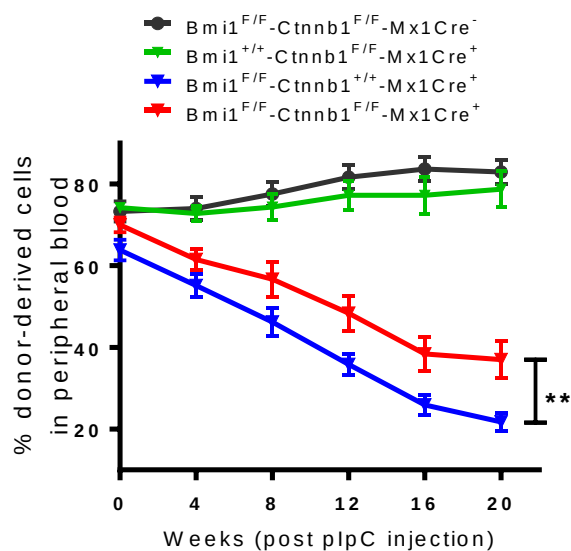
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Ectopic Bmi1 expression in Bmi1^{-/-} HSCs

E

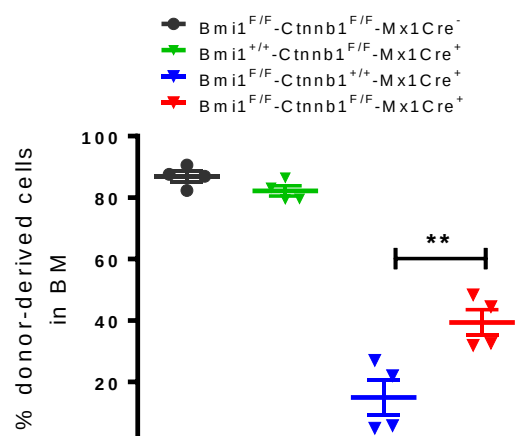
Ectopic Bmi1 expression in Bmi1^{-/-} MPPs

Primary BM transplantation

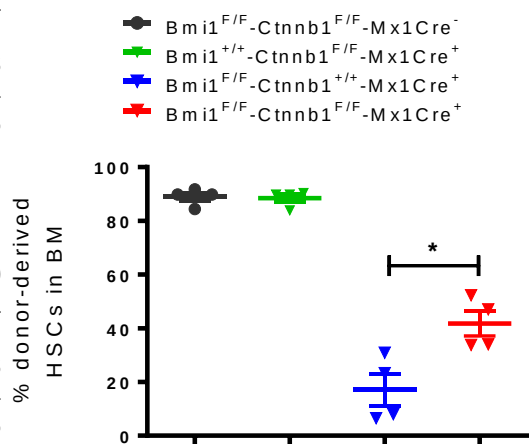


B

Primary BM transplantation

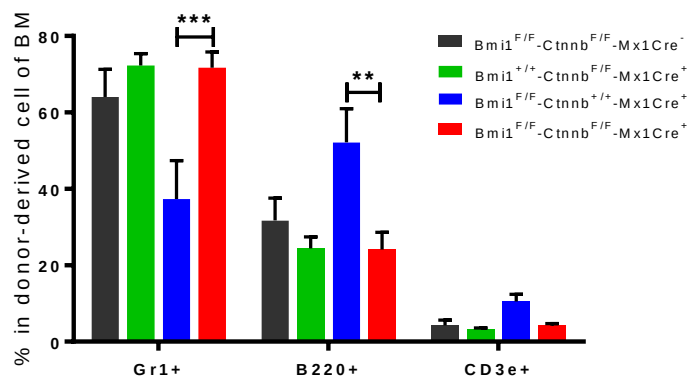


Primary BM transplantation

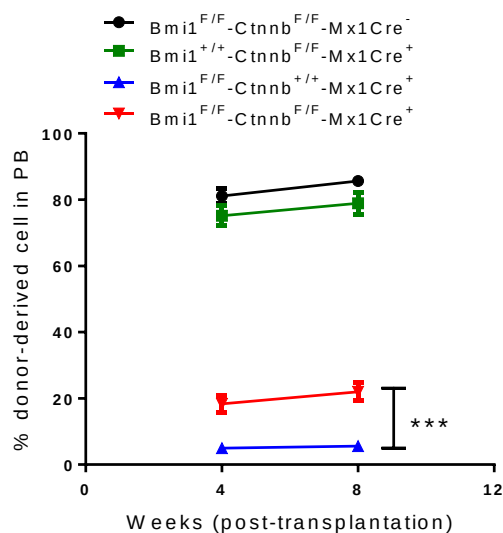


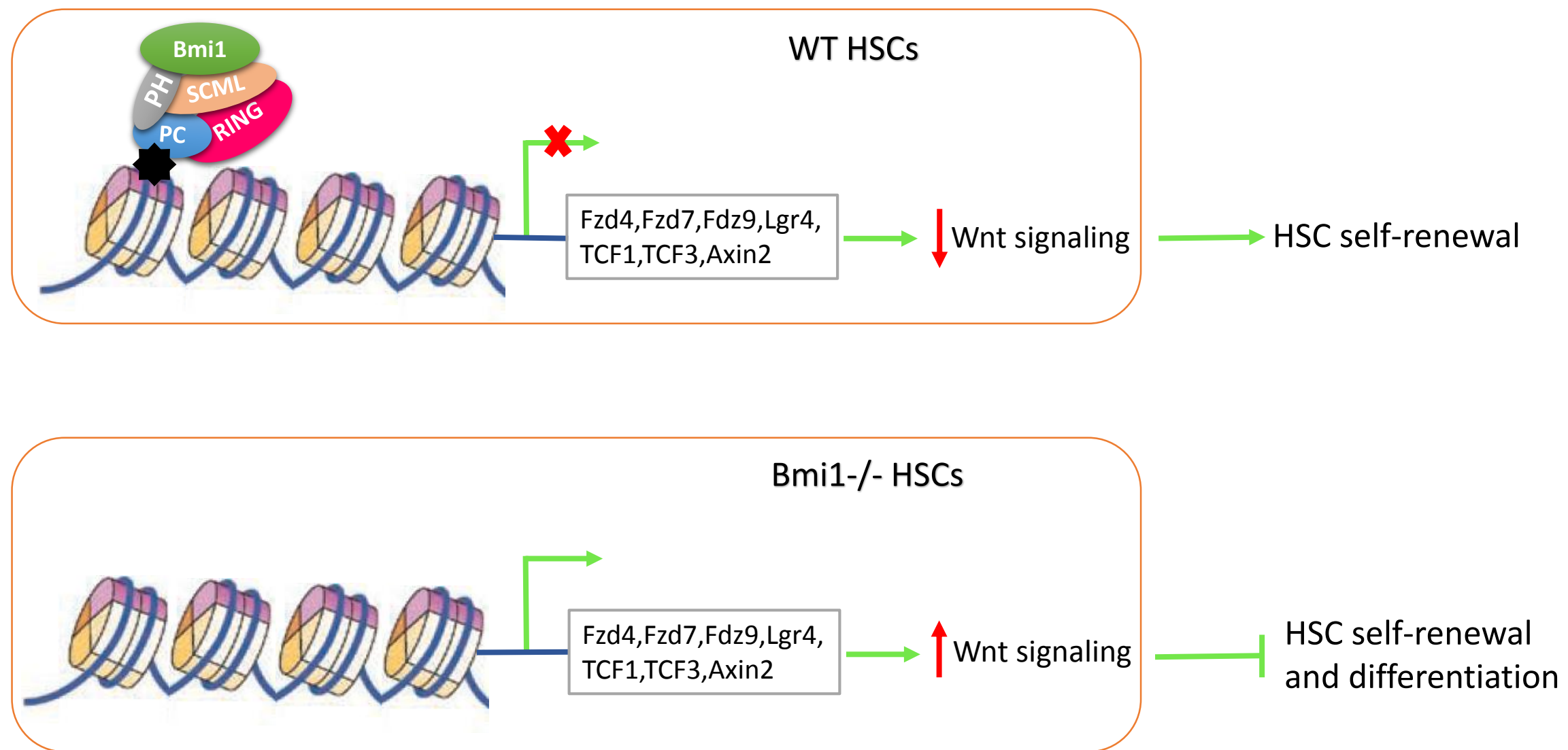
D

Multilineage differentiation in BM at 20 week after plpC



E





CURRICULUM VITAE

Yan Liu, Ph.D.

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Feinberg School of Medicine
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Robert H. Lurie Comprehensive Cancer Center
Northwestern University
Email: yan.liu@northwestern.edu

EDUCATION

- 1988-1992 **B.S.**, Jilin University, China
- 1992 - 1997 **Ph.D.**, Chinese Academy of Sciences, Beijing, China, Xuexian Peng and Jingrui Dai - Mentors
- 1998 - 2001 Postdoctoral Fellow, University of Arizona, Tucson, Arizona, Brian Larkins - Mentor
- 2001 - 2006 Research Fellow, Memorial Sloan-Kettering Cancer Center, New York, New York, Stephen Nimer - Mentor

ACADEMIC APPOINTMENTS

- 2006-2008 Research Associate, Memorial Sloan-Kettering Cancer Center, New York, NY
- 2008-2010 Senior Research Scientist, Memorial Sloan-Kettering Cancer Center, New York, NY
- 2010-2016 Assistant Professor of Pediatrics, Biochemistry and Molecular Biology (tenure-track), Indiana University School of Medicine, Indianapolis, IN
- 2016-2021 Associate Professor of Pediatrics, Biochemistry and Molecular Biology (tenured), Indiana University School of Medicine, Indianapolis, IN
- 2011-2021 Member, Indiana University Simon Cancer Center
- 2011-2021 Member, Indiana University/NIDDK Cooperative Center of Excellence in Hematology (CCEH)
- 2021-present Associate Professor of Medicine (tenured), Feinberg School of Medicine, Northwestern University, Chicago, IL
- 2021-present Co-Leader, Hematologic Malignancies Program, Robert H. Lurie Comprehensive Cancer Center

PROFESSIONAL ORGANIZATIONS

2002-present Member, American Society of Hematology (ASH)
2007-present Member, International Society for Stem Cell Research (ISSCR)
2011-present Member, International Society for Experimental Hematology (ISEH)
2011-present Member, Chinese Biological Investigators Society (CBI)
2015-present Member, Society of Chinese Bioscientists in America (SCBA)
2016-present Faculty Member, Faculty of 1000 Medicine

PROFESSIONAL SERVICE

2013-present Member, NYSTEM Study Section, New York State Department of Health and the Empire State Stem Cell Board
2013-2017 Member, the Early Career Reviewer (ECR) program at the Center for Scientific Review (CSR), National Institutes of Health
2013-present Foreign Expert (Member), Hematology Study Section, National Natural Science Foundation of China (NSFC), China.
2014-present Member, Career Development Award (CDA) Program, International Human Frontier Science Program Organization (HFSP), France
2015-present Reviewer, St. Baldrick's Foundation, USA
2017 Ad hoc Reviewer, Cancer Genetics (CG) Study Section, NIH
2019- Reviewer, Leukemia Research Foundation (LRF), USA

HONORS AND AWARDS

2009 International Society for Stem Cell Research (ISSCR) Travel Award
2010 NYSTEM Idea Award
2012 Showalter Research Trust Fund New Investigator Award
2013 DOD/PRCRP Career Development Award
2013 American Cancer Society (ACS) New Investigator Award

2014	Elsa U. Pardee Foundation New Investigator Award
2014	St. Baldrick's Foundation Scholar Award
2014	Alex's Lemonade Stand Foundation Research Award
2016	Leukemia Research Foundation (LRF) New Investigator Award
2016	Children's Leukemia Research Association Research Award
2016	Showalter Research Trust Fund Young Scholar Award
2017	St. Baldrick's Foundation Extended Scholar Award
2017	NIH/NIA R56 Award
2018	DOD/BMFRP Idea Development Award
2018	NIH/NIDDK R56 Award
2018	St. Baldrick's Foundation Extended Scholar Award
2019	NIH/NHLBI R01 Award
2019	DOD/PRCRP Idea Development Award
2019	Leukemia & Lymphoma Society (LLS) TRP Award
2019	Showalter Research Trust Fund Scholar Award

RESEARCH SUPPORT

CURRENT ACTIVE SUPPORT:

R01HL150624 NIH/NHLBI	(PI: Liu)	07/15/19-06/30/23	3.6 calendar
Molecular mechanisms underlying clonal expansion of hematopoietic stem cells Role: Principal Investigator			
W81XWH-19-1-0575 DOD/PRCRP Idea Development Award	(PI: Liu)	08/01/19-06/30/21	2.4 calendar
Targeting Leukemia-initiating Cells to Improve Leukemia Treatment Role: Principal Investigator			
6581-20 Leukemia & Lymphoma Society (LLS) TRP Grant	(PI: Liu)	07/01/19-06/30/22	0.6 calendar
Development of therapeutic strategy for the treatment of MDS Role: Principal Investigator			

PENDING SUPPORT:

R01 CA258175 (PIs: Liu and Mayo) 04/01/21-03/31/26 2.4 calendar
NIH/NCI

Chronic inflammation drives leukemogenesis through enhancing mutant hematopoietic stem and progenitor cell fitness

Role: Principal Investigator

U01 CA263686-01 (PI: Liu) 06/01/21-05/31/26 3.6 calendar
NIH/NCI

Molecular mechanisms underlying the development and outgrowth of leukemia-initiating cells during aging

Role: Principal Investigator

R01 CA266331 (PI: Liu) 09/01/21-08/31/26 3.6 calendar
NIH/NCI

Understanding and Targeting PRL2 Phosphatase in Acute Myeloid Leukemia

Role: Principal Investigator

COMPLETED SUPPORT:

Startup Fund (PI, Liu) 11/01/10-10/31/15

Indiana University School of Medicine (IUSM)

p53 in normal and malignant hematopoiesis

Role: Principal Investigator

R56AG052501 (PI, Liu) 09/15/17-08/31/18 2.4 calendar
NIH/NIA

Mutant p53 rejuvenates aged stem cells through modulating epigenetic regulators

Role: Principal Investigator

R56DK119524 (PI, Liu) 09/15/18-08/31/19 1.2 calendar
NIH/NIDDK

Molecular mechanisms underlying clonal expansion of hematopoietic stem cells

Role: Principal Investigator

W81XWH-13-1-0187 (PI: Liu) 07/01/13-06/30/15 2.4 calendar
DOD/PRCRP Career Development Award

Modulating Leukemia-initiating Cell Quiescence to Improve Leukemia Treatment

Role: Principal Investigator

W81XWH-18-1-0265 (PI, Liu) 07/01/18-03/31/21 1.2 calendar
DOD/BMFRP Idea Development Award

Understanding and Targeting Mutant p53 in Myelodysplastic Syndromes

Role: Principal Investigator

NCE

R01 AI121197-01A1 (PI: Yoshimoto) 06/13/16-05/31/17 1.2 calendar
NIH/NIAID

Embryonic origin and self-renewal of B-1a cells

Role: Co-investigator

R56AI110831 (PI: Yoshimoto) 03/10/15 – 02/29/17 1.2 calendar
NIH/NIAID
Embryonic origin and self-renewal of B-1a cells
Role: Co-investigator

1F32CA203049-01 (PI: Nabinger) 07/01/16 – 6/30/18
NIH/NCI
Targeting PRL2 Phosphatase in acute myeloid leukemia
Role: Mentor

Scholar Award (PI: Liu) 07/01/19-03/31/21
Ralph and Grace Showalter Research Trust Fund The role
of mutant p53 in clonal hematopoiesis
Role: Principal Investigator

Scholar Award (PI: Liu) 07/11/14-12/31/19 7.2 calendar
St. Baldrick's Foundation
Targeting PRL2 phosphatase in T cell acute lymphoblastic leukemia
Role: Principal Investigator
NCE

New Investigator Award (PI: Liu) 01/01/14-12/31/14 1.2 calendar
Elsa U. Pardee Foundation
Targeting PRL2 in Acute Myeloid Leukemia
Role: Principal Investigator

New Investigator Award (PI: Liu) 07/01/16-12/31/17
Leukemia Research Foundation
Targeting PRL2 phosphatase in Acute Myeloid Leukemia
Role: Principal Investigator

Research Grant (PI: Liu) 07/01/12-06/30/13 1.2 calendar
Showalter Research Trust Fund
The role of gain-of-function mutant p53 in hematopoietic stem cell self-renewal
Role: Principal Investigator

Young Scholar Award (PI: Liu) 7/01/16-6/30/18 1.2 calendar
Showalter Research Trust Fund
The role of Polycomb repressive complex 1 in hematopoietic stem cells
Role: Principal Investigator

Research Grant (PI: Liu) 06/01/13-05/31/14
American Cancer Society and Indiana University Simon Cancer Center The
role of PRL2 Phosphatase in Acute Myeloid Leukemia
Role: Principal Investigator

Research Grant (PI: Liu) 01/01/16-12/31/16 0.6 calendar
Children's Leukemia Research Association
Targeting PRL2 in acute myeloid leukemia

Role: Principal Investigator

Research Grant (PI: Liu) 09/02/14-08/31/15
Alex's Lemonade Stand Foundation
Targeting PRL2 phosphatase in pediatric acute myeloid leukemia
Role: Principal Investigator

Research Grant (PI: Liu) 10/01/11-09/30/12
NIDDK Cooperative Center of Excellence in Hematology (CCEH) The
role of mutant p53 in hematopoietic stem cell self-renewal
Role: Principal Investigator

Research Grant (PI: Liu) 7/01/16-6/30/17
NIDDK Cooperative Center of Excellence in Hematology (CCEH)
Polycomb repressive complex 1 in hematopoietic stem cells
Role: Principal Investigator

Research Grant (PI: Liu) 07/01/11-12/31/12
Indiana University Simon Cancer Center (IUSCC)
Targeting mutant p53 to improve leukemia treatment
Role: Principal Investigator

Research Grant (PI: Liu) 10/01/18 - 02/28/19
Wells Center for Pediatric Research/IUSM
Molecular mechanisms underlying clonal expansion of hematopoietic stem cells
Role: Principal Investigator

Research Grant (PI: Liu) 01/01/19 -12/31/19
Wells Center for Pediatric Research/IUSM
Molecular mechanisms of stem cell aging and pathogenesis of age-related diseases
Role: Principal Investigator

Biomedical Research Grant (PI: Liu) 07/1/13 - 06/30/14
Indiana University School of Medicine (IUSM)
Bmi1 is a key regulator of Wnt signaling in hematopoietic stem cells
Role: Principal Investigator

Biomedical Research Grant (PI: Liu) 03/1/11 - 02/28/12
Indiana University School of Medicine (IUSM)
The role of Bmi1 in hematopoietic stem cell self-renewal
Role: Principal Investigator

RSFG Grant (PI: Liu) 7/01/16-6/30/17
Indiana University-Purdue University Indianapolis
Mutant p53 rejuvenates aged stem cells through modulating epigenetic regulators
Role: Principal Investigator

Research Support Funds Grants Award (PI: Liu) 07/01/13-06/30/14
Indiana University-Purdue University Indianapolis
The role of mutant p53 in acute myeloid leukemia

Role: Principal Investigator

Seed Fund (PIs: Liu and Mayo) 01/1/18 - 02/28/19
Wells Center for Pediatric Research
Genotoxic stresses in mutant p53-driven MDS pathogenesis
Role: Principal Investigator

Seed Fund (PI: Liu) 06/1/15 - 12/31/15
Wells Center for Pediatric Research \$25,000
PRL2 phosphatase as a novel therapeutic target in Acute Myeloid Leukemia
Role: Principal Investigator

Pilot Grant (PI: Liu) 5/01/15-4/30/16
Indiana University Simon Cancer Center (IUSCC)
Targeting mutant p53 to improve leukemia treatment
Role: Principal Investigator

Pilot Project Grant (PI: Liu) 07/01/12-12/31/13
Indiana University Simon Cancer Center (IUSCC)
The role of Bmi1 in the pathogenesis of myelodysplastic syndromes
Role: Principal Investigator

Research Grant (PI: Kobayashi) 07/01/14 - 06/30/15
Ralph W. and Grace M. Showalter Research Trust Fund
Targeting PRL2 in Acute Myeloid Leukemia
Role: Mentor

Biomedical Research Grant (PI: Kobayashi) 11/1/15 - 10/31/16
Indiana University School of Medicine (IUSM)
Targeting PRL2 phosphatase in Acute Myeloid Leukemia
Role: Mentor

Research Grant (PI: Kobayashi) 06/01/14 - 05/31/15
American Cancer Society and Indiana University Simon Cancer Center
The role of PRL2 Phosphatase in the pathogenesis of T-ALL
Role: Mentor

Pilot Project Grant (PI: Kobayashi) 11/01/14 - 10/30/15 NIDDK
Cooperative Center of Excellence in Hematology (CCEH)
The role of PRL2 Phosphatase in T cell development
Role: Mentor

PUBLICATIONS (*Corresponding author)

PUBLICATIONS SUBMITTED/IN PREPARATION

1. Vemula S, Chen S, Nabinger SC, Capitano ML, Fahey AC, Henley D, Barajas S, Cai W, Boswell HS, Broxmeyer HE, Savage S, Mayo LD, and **Liu Y***. Chronic inflammation

promotes p53 mutant hematopoietic stem and progenitor cell expansion.
(Manuscript in preparation)

2. Kobayashi M, Bai Y, Chen S, Nabinger SC, Vemula S, Henley D, Fahey AC, Yao C, Sun J, Boswell HS, Croop JM, Rönnstrand L, Kapur R, Zhang ZY, and **Liu Y***. PRL2 phosphatase enhances oncogenic cytokine signaling in leukemia cells. *Blood* (In revision, 2021).
3. Chen S, Yu H, Gao R, Liu X, Vemula S, Fahey AC, Henley D, Kobayashi M, Liu SZ, Wang Q, Qian Z, Kapur R, Broxmeyer H, Gao Z, Xi R and **Liu Y***. Bmi1 regulates Wnt signaling in hematopoietic stem and progenitor cells. *Leukemia* (In revision, 2021).
4. Olivos D, Hauck P, Jacobsen M, Sandusky G, **Liu Y**, and Mayo L. Emergence of Tumor-Initiating Cells with the Loss of Mdm2. *Molecular Cancer research* (In revision, 2021).

PEER-REVIEWED PUBLICATIONS

5. Broxmeyer HE, **Liu Y**, Kapur R. *et al.* Fate of Hematopoiesis During Aging. What Do We Really Know, and What are its Implications? ***Stem Cell Rev and Rep.*** 2020; (16): 1020–1048.
6. Kobayashi M, Lin Y, Mishra A, Shelly C, Gao R, Wang P, Xi R, Wenzel P, Liu Y, **Liu Y***, and Yoshimoto M. Bmi1 maintains the self-renewal property of innate -like B lymphocytes. ***Journal of Immunology.*** 2020 Jun 15;204(12):3262-3272. (Co-corresponding author).
7. Chen S, Wang Q, Yu H, Capitano ML, Vemula S, Nabinger SC, Gao R, Yao C, Kobayashi M, Geng Z, Fahey AC, Henley D, Liu SZ, Barajas S, Cai W, Wolf ER, Ramdas B, Cai Z, Gao H, Luo N, Sun Y, Wong TN, Link DC, Liu Y, Boswell HS, Mayo LD, Huang G, Kapur R, Yoder MC, Broxmeyer HE, Gao Z, and **Liu Y***. Mutant p53 Drives Clonal Hematopoiesis through Modulating Epigenetic Pathway. ***Nature Communications.*** 2019 Dec 11;10(1):5649. doi: 10.1038/s41467-019-13542-2. PMCID: PMC6906427.
8. Chen S, **Liu Y***. p53 involvement in clonal hematopoiesis of indeterminate potential. ***Curr Opin Hematol.*** 2019 Jul;26(4):235-240. doi: 10.1097/MOH.0000000000000509.
9. Nabinger S, Chen S, Yao C, Gao R, Kobayashi M, Vemula S, Fahey AC, Wang C, Daniels C, Boswell HS, Sandusky GE, Mayo LD, Kapur R, and **Liu Y***. Mutant p53 enhances leukemia-initiating cell self-renewal to promote leukemia development. *Leukemia.* 2019 Jun;33(6):1535-1539. PMID: 30675010
10. Cai Z, Kotzin JJ, Ramdas R, Chen S, Nelanuthala S, Palam LR, Pandey R, Mali RS, **Liu Y**, Kelley MR, Sandusky G, Mohseni M, Williams A, Henao-Mejia J, and Kapur R. Downregulation of *Morrbid* in Tet2-deficient preleukemic cells overcomes resistance to inflammatory stress and mitigates clonal hematopoiesis. *Cell Stem Cell.* 2018 Dec 6;23(6):833-849.e5. PMID:30526882
11. Chen S and **Liu Y***. Battle in stem cell niches: canonical verse non-canonical Wnt signaling. *Journal of Leukocyte Biology.* 2018 Mar;103(3):377-379. PubMed PMID: 29393968.

12. Chen S, Gao R, Yao C, Kobayashi M, Liu SZ, Yoder MC, Broxmeyer HE, Kapur R, Boswell HS, Mayo LD, and **Liu Y***. Genotoxic stresses promotes the clonal expansion of hematopoietic stem cells expressing mutant p53. *Leukemia*. 2018 Mar;32(3):850-854. PMID:PMC5842141
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14. Yao C, Kobayashi M, Chen S, Nabinger S, Gao R, Liu SZ, Asai T, and **Liu Y***. Necdin modulates leukemia-initiating cell quiescence and chemotherapy response. *Oncotarget*. 2017; 8:87607-87622. PMID: PMC5675657.
15. Kobayashi M, Chen S, Bai Y, Yao C, Gao R, Sun XJ, Mu C, Twiggs TA, Yu ZH, Boswell HS, Yoder M, Kapur R, Mulloy J, Zhang ZY, and **Liu Y***. Phosphatase PRL2 promotes AML1-ETO-induced acute myeloid leukemia. *Leukemia*. 2017 June; 31(6):1453-1457. PubMed PMID: 28220038. PMID:PMC5695226
16. Kobayashi M, Bai Y, Yoshimoto M, Gao R, Chen S, Yao C, Dong Y, Zhang L, Rodriguez S, Carlesso N, Yoder MC, Kapur R, Kaplan MK, Lacorazza HD, Zhang ZY, and **Liu Y***. Protein tyrosine phosphatase PRL2 mediates Notch and Kit signals in early T cell progenitors. *Stem Cells*. 2017 Apr;35(4):1053-1064. PMID: PMC5367971.
17. Kobayashi M, Bai Y, Chen S, Gao R, Yao C, Cai W, Croop J, Zhang ZY and **Liu Y***. Phosphatase PRL2 promotes oncogenic Notch-induced T cell leukemia. *Leukemia*. 2017 Mar;31(3):751-754. PMID:PMC5695227
18. Chen S, Gao R, Kobayashi M, Yu H, Yao C, Kapur R, Yoder M, and **Liu Y***. Pharmacological inhibition of AKT activity in human CD34+ cells enhances their engraftment in immunodeficient mice. *Experimental Hematology*. 2017 Jan;45:74-84. PubMed Central PMID: PMC5859321.
19. Ghosh J, Kobayashi M, Ramdas B, Chatterjee A, Ma P, Mali RS, Carlesso N, **Liu Y**, Plas DR, Chan RJ, and Kapur R. S6K1 regulates Stem Cell Self-Renewal and Leukemia Maintenance. *Journal of Clinical Investigation*. 2016 Jul 1;126(7):2621-5. PMID:PMC4922705.
20. Gao R, Chen S, Kobayashi M, Yu H, Young SK, Soltis A, Zhang Y, Wan Y, Vemula S, Fraenkel E, Cantor A, Xu Y, Yoder MC, Wek R, Kapur R, Ellis S, Zhu X and **Liu Y***. Bmi1 promotes erythropoiesis through regulating ribosome biogenesis. *Stem Cells*. 2015 Mar;33(3):925-38. PMID:PMC4380436.
21. Chatterjee A, Ghosh J, Ramdas B, Mali RS, Martin H, Kobayashi M, Vemula S, Canela VH, Waskow ER, Visconte V, Tiu RV, Smith CC, Shah N, Bunting KD, Boswell HS, **Liu Y**, Chan RJ, Kapur R. Regulation of Stat5 by FAK and PAK1 in Oncogenic FLT3- and KIT-Driven Leukemogenesis. *Cell Reports*. 2014 Nov 20;9(4):1333-1348. PMID:PMC4380442.

22. Kobayashi M, Shelly WC, Seo W, Vemula S, **Liu Y**, Kapur R, Taniuchi I, Yoshimoto M. Functional B-1 progenitor cells are present in the hematopoietic stem cell deficient embryo and depend on Cbfb for their development. *Proc Natl Acad Sci U S A*. 111(33):12151-6, 2014. PMCID: PMC4143017
23. Kobayashi M, Chen S, Gao R, Bai Y, Zhang ZY, and **Liu Y***. Phosphatase of regenerating liver in hematopoietic stem cell self-renewal and hematological malignancies. *Cell Cycle*, 2014;13(18): 2827-2835. PMCID: PMC4614371
24. Kobayashi M, Bai Y, Dong Y, Yu H, Chen S, Gao R, Zhang L, Yoder MC, Kapur R, Zhang ZY and **Liu Y***. PRL2/PTP4A2 phosphatase is important for hematopoietic stem cell self-renewal. *Stem Cells*. 32(7):1956-67, 2014. PMCID: PMC4063874.
25. Vu LP, Perna F, Wang L, Voza F, Figueroa ME, Tempst P, Erdjument-Bromage H, Gao R, Chen S, Paietta E, Deblasio T, Melnick A, **Liu Y**, Zhao X and Nimer SD. PRMT4 blocks myeloid differentiation by assembling a methyl-RUNX1-dependent repressor complex. *Cell Reports*. 5(6):1625-38, 2013. PMCID: PMC4073674
26. Sabelli PA, **Liu Y**, Dante RA, Lizarraga LE, Nguyen HN, Brown SW, Klingler JP, Yu J, LaBrant E, Layton TM, Feldman and Larkins BA. Control of Cell Proliferation, Endoreduplication, Cell Size and Cell Death by the Retinoblastoma-Related Pathway in Maize Endosperm. *Proc Natl Acad Sci U S A*. 110(19):E1827-36, 2013. PMCID: PMC3651506
27. **Liu Y***, Yu H, Nimer SD. PI3K-Akt pathway regulates polycomb group protein and stem cell maintenance. *Cell Cycle*. 12(2):199-200, 2013. PMCID: PMC3757235.
28. Asai T, **Liu Y** and Nimer SD. Necdin, a p53 target, in normal and cancer stem cells. *Oncotarget*. 4(6):806-7, 2013. PMCID: PMC3757235
29. **Liu Y***, Liu F, Yu H, Zhao X, Sahsida G, Deblasio A, Chen Z, Lin HK, Di Giandomenico S, Elf SE, Yang YY, Miyata Y, Huang G, Menendez S, Mellinghoff I, Pandolfi PP, Hedvat CV and Nimer SD. Akt Phosphorylates the Transcriptional Repressor Bmi1 to Block Its Association with Tumor Suppressing *Ink4a-Arf* locus. *Science Signaling*, October 23; 5, ra77, 2012. PMCID: PMC3784651. **Co-corresponding author**
30. Vemula S, Shi J, Mali RS, Ma P, **Liu Y**, Hanneman P, Koehler KR, Hashino E, Wei L, and Kapur R. ROCK1 functions as a critical regulator of stress erythropoiesis and survival by regulating p53. *Blood*, Oct 4; 120(14):2868-78, 2012. PMCID: PMC3466968
31. Hu P, Nebreda AR, **Liu Y**, Carlesso N, Kaplan M, Kapur R. P38α negatively regulates T helper type 2 responses by orchestrating multiple TCR-associated signals. *Journal of Biological Chemistry*, Sep 28; 287(40): 33215-26, 2012. PMCID: PMC3460427
32. Asai T, **Liu Y**, Di Giandomenico S, Bae N, Xu H, Nadiaye-Lobry D, Deblasio A, Menendez S, Antipin J, Reva B, Wevrick R, and Nimer SD. Necdin, a p53 target gene, regulates hematopoietic stem/progenitor cell quiescence and response to genotoxic stress. *Blood*, Aug 23;120(8):1601-12, 2012. PMCID: PMC3429304. **Co-first author**.

33. Hu P, Carlesso N, Xu M, **Liu Y**, Nebreda AR, Takemoto C, and Kapur R. Genetic evidence for critical roles of P38 α in regulating mast cell differentiation and chemotaxis through distinct mechanisms. *J. Biol. Chem*, June 8;287(24):20258-69, 2012. PMCID: PMC3370208
34. Huang G, Zhao X, Wang L, Elf S, Xu H, Zhao X, Sashida G, **Liu Y**, Lee J, Menendez S, Yang Y, Yan Y, Zhang P, Tenen DG, Osato M, Hsieh J, and Nimer SD. The ability of MLL to bind RUNX1 and methylate H3K4 at PU.1 regulatory regions is impaired by MDS/AML-associated RUNX1/AML1 mutations. *Blood*, Dec 15;118(25):6544-52, 2011. PMCID: PMC3242717
35. Asai T, **Liu Y**, Bae N, and Nimer SD. The p53 tumor suppressor protein regulates hematopoietic stem cell fate. *J Cell Physiol*, Sep; 226(9):2215-21, 2011. PMCID: PMC3575444.
36. Sashida G, Bae N, Di Giandomenico S, Asai T, Gurvich N, Bazzoli E, **Liu Y**, Huang G, Zhao X, Menendez S, Nimer SD. (2011). The Mef/Elf4 transcription factor fine tunes the DNA damage response. *Cancer Research*, July 15; 71(14): 4857-65, 2011. PMCID: PMC4073677
37. Sashida G, Bazzoli E, Menendez S, **Liu Y**, and Nimer SD. The oncogenic role of the ETS transcription factors MEF and ERG. *Cell Cycle*, Sep 1; 9(17):3457-9, 2010. PMCID: PMC3230474
38. Miyata Y, **Liu Y**, jankovic V, Sashida G, Lee JM, Shieh JH, Naoe T, Moore M, and Nimer SD. Cyclin C regulates human hematopoietic stem/progenitor cell quiescence. *Stem Cells*, Feb; 28(2):308-17, 2010. PMCID: PMC3144254
39. **Liu Y**, Asai T, and Nimer SD. Myelodysplasia: battle in the bone marrow. *Nat Med*, Jan; 16(1):30-2, 2010.
40. **Liu Y**, Hoya-Arias R, and Nimer SD. The role of p53 in limiting somatic cell reprogramming. *Cell Res*, Nov; 19(11):1227-8, 2009.
41. **Liu Y**, Elf SE, Asai T, Miyata Y, Liu Y, Sashida G, Huang G, Di Giandomenico S, Koff A, and Nimer SD. The p53 tumor suppressor protein is a critical regulator of hematopoietic stem cell behavior. *Cell Cycle*, Oct1; 8(19):3120-4, 2009. PMCID: PMC4637974
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46. Yao JJ, **Liu Y**, Lacorazza HD, Soslow RA, Scandura JM, Mimer SD, and Hedvat CV. Tumor promoting properties of the ETS protein MEF in ovarian cancer. *Oncogene*, Jun 7; 26(27):4032-7, 2007.
47. **Liu Y**, Hedvat CV, Mao S, Zhu XH, Yao J, Nguyen H, Koff A, and Nimer SD. The ETS protein MEF is regulated by phosphorylation-dependent proteolysis via the protein-ubiquitin ligase SCFSkp2. *Mol Cell Biol*, Apr; 26(8):3114-23, 2006. PMCID: PMC1446966
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49. Larkins BA, Dilkes BP, Dante RA, Coelho CM, Woo YM, **Liu Y**. Investigating the hows and whys of DNA endoreduplication. *J Exp Bot*, Feb;52(355):183-92, 2001. PMID: 11283162.

INVITED PRESENTATIONS

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|------|---|
| 2007 | Transcriptional regulation of the Normal and Leukemic Hematopoiesis. Sloan Kettering Institute Scientific Colloquium, New York, NY. |
| 2007 | Regulation of Hematopoietic Stem Cell Quiescence - a Novel Role for p53. The 49 th American Society of Hematology (ASH) Conference, Atlanta, GA. |
| 2009 | Using cord blood to identify cell cycle regulatory targets for treating leukemia. The 7 th Annual International Umbilical Cord Blood Transplantation Symposium, Los Angeles. |
| 2009 | Necdin regulates hematopoietic stem cell quiescence. The 7 th Annual International Society for Stem Cell Research (ISSCR), Barcelona, Spain. |
| 2009 | Molecular Basis of Hematopoietic Stem Cell Quiescence and Self Renewal. Children's Hospital Oakland Research Institute, Oakland, CA. |
| 2010 | Molecular Basis of Hematopoietic Stem Cell Quiescence and Self Renewal. Rochester University School of Medicine, Rochester, NY. |
| 2010 | Molecular Basis of Hematopoietic Stem Cell Quiescence and Self Renewal. New York University School of Medicine. New York, NY. |
| 2010 | Molecular Basis of Hematopoietic Stem Cell Quiescence and Self Renewal. St. Jude Children's Research Hospital |
| 2010 | Molecular Basis of Hematopoietic Stem Cell Quiescence and Self Renewal. Department of Pediatrics, Indiana University School of Medicine, Indianapolis, IN. |

- 2011 Molecular basis of hematopoietic stem cell quiescence and self-renewal. Institute of Hematology & Blood Diseases Hospital, Chinese Academy of Medical Sciences, Tianjin, China
- 2011 Akt-mediated phosphorylation of Bmi1 regulates hematopoietic stem cell self-renewal. Midwest Blood Club, Cincinnati, Ohio
- 2012 Molecular basis of hematopoietic stem cell self-renewal and leukemogenesis. National Institute of Biological Sciences, Beijing, China
- 2012 Molecular basis of hematopoietic stem cell self-renewal and leukemogenesis. Tsinghua University, Beijing, China
- 2012 Molecular basis of hematopoietic stem cell quiescence and self-renewal. Department of Biochemistry and Molecular Biology seminar, Indiana University, Indianapolis, IN
- 2012 Molecular basis of hematopoietic stem cell self-renewal and leukemogenesis. Neonatology morning conference, Department of Pediatrics, Indiana University.
- 2012 Akt-mediated phosphorylation of Bmi1 inhibits hematopoietic stem cell self-renewal. Midwest Blood Club, Indianapolis, Indiana
- 2013 PRL2 maintains hematopoietic stem and progenitor cells through regulating SCF/KIT signaling. Midwest Blood Club, Cincinnati, Ohio
- 2013 Bmi1 regulates hematopoietic stem cell self-renewal and lineage commitment. Center of Excellence of Molecular Hematology, Indiana University
- 2013 Bmi1 regulates ribosome biogenesis during erythroid differentiation. The 55th American Society of Hematology (ASH) Conference, New Orleans, LA.
- 2014 PRL2 phosphatase in normal and malignant hematopoiesis. Institute of Hematology & Blood Diseases Hospital, Chinese Academy of Medical Sciences, Tianjin, China
- 2014 Polycomb group protein Bmi1 regulates hematopoietic stem cell self-renewal and differentiation. National Institute of Biological Sciences, Beijing, China
- 2015 Polycomb Repressive Complex 1 enhances HSC self-renewal through repressing the canonical Wnt signaling pathway. Midwest Blood Club, French Lick, Indiana
- 2015 Gain-of-function Mutant p53 enhances HSC self-renewal. Keystone Symposia, Hematopoiesis (B6), Keystone, CO.
- 2015 Polycomb Repressive Complex 1 enhances HSC self-renewal through repressing the canonical Wnt signaling pathway. The 57th American Society of Hematology (ASH) Conference, Orlando, FL.

- 2016 Epigenetic Control of Hematopoietic Stem Cells. Institute of Health, Chinese Academy of Sciences, Shanghai, China
- 2016 Epigenetic Control of Hematopoietic Stem Cells. Fudan University, Shanghai, China
- 2016 Mutant p53 drives the development of pre-leukemic HSCs through modulating epigenetic regulators. The 11th Bi-annual Chinese Biological Investigator Society (CBIS), Chengdu, China
- 2016 Epigenetic Control of Hematopoietic Stem Cells. Penn State College of Medicine, Hershey, PA
- 2016 Genetic and Epigenetic Control of Hematopoietic Stem Cells. Wells Center for Pediatric Research, Indiana University.
- 2016 PRL2 Phosphatase Is a Key Mediator of Oncogenic Cytokine Signaling in Leukemia Stem Cells. The 58th American Society of Hematology (ASH) Conference, San Diego, CA.
- 2016 Polycomb Repressive Complex 1 in Hematopoietic Stem Cell Self-renewal and Differentiation. NIDDK Cooperative Center of Excellence in Hematology (CCEH) Meeting. Seattle, WA.
- 2017 Gain of function mutant p53 drives the development of pre-leukemic HSCs through epigenetic modulations, Indiana University Cancer Research Day Symposium.
- 2018 Genetic and Epigenetic Control of Hematopoietic Stem Cell Expansion and pathogenesis of MDS. Wells Center for Pediatric Research, Indiana University School of Medicine.
- 2018 Understanding and Targeting Mutant p53 in Hematological Malignancies. Simon Cancer Center, Indiana University School of Medicine.
- 2018 PRL2 phosphatase in normal and malignant hematopoiesis. Cooperative Center of Excellence in Hematology, Indiana University School of Medicine.
- 2018 Developing Novel Therapeutic Approaches to improve childhood leukemia treatment, the St. Baldrick's Foundation, CA
- 2018 Mutant p53 promotes hematopoietic stem and progenitor cell expansion through interacting with epigenetic modulator EZH2. The 60th American Society of Hematology (ASH) Conference, San Diego, CA.
- 2018 Developing Novel Therapeutic Approaches to improve leukemia treatment, Indiana Science and Technology Club, IN
- 2019 Chronic inflammation increases cancer development in LFS patients. Wells Center for Pediatric Research, Indiana University School of Medicine.

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| 2019 | Genetic and Epigenetic Regulation of Leukemia Stem Cells, University of Notre Dame, South Bend, Indiana. |
| 2019 | Mutant p53 drives clonal hematopoiesis through modulating epigenetic pathways, the 8 th Mutant p53 Workshop, Lyon, France. |
| 2019 | Genetic and Epigenetic Control of Pre-leukemia Stem Cells and Leukemia Stem Cells, University of Minnesota, Minnesota. |
| 2019 | Understanding and Targeting of Pre-leukemia Stem Cells and Leukemia Stem Cells, Northwestern University. |
| 2019 | Understanding and Targeting of Pre-leukemia Stem Cells and Leukemia Stem Cells, University of Miami. |
| 2020 | Understanding and Targeting of Pre-leukemia Stem Cells and Leukemia Stem Cells, University of Florida. |
| 2021 | Understanding and Targeting of Pre-leukemia Stem Cells and Leukemia Stem Cells, Chicago Blood Club. |

PROFESSIONAL SERVICE

EDITORIAL BOARD:

- | | |
|-----------------|--|
| 2014 to present | International Journal of Neonatal Science and Therapeutics |
| 2014 to present | Journal of Stem Cell Research and Transplantation |
| 2014 to present | Stem Cells Research, Development and Therapy |
| 2014 to present | Journal of Stem Cells and Translational Investigation |
| 2014 to present | Pediatrics and Neonatal Biology |
| 2016 to present | Cancer Translational Medicine |
| 2020 to present | Leukemia & Lymphoma |

REVIEWER FOR JOURNALS: Cell Stem Cell, JCI, PNAS, Blood, Leukemia, Stem Cells, Journal of Leukocyte Biology, BMC Cell Biology, PLoS ONE, Frontiers in Oncology, Cancer Biotherapy & Radiopharmaceutics, Molecular and Cellular Biochemistry, Tumor Biology, Journal of Hematology and Oncology Research, In Vitro Cellular & Developmental Biology, International Journal of Biochemistry and Molecular Biology, Oncotarget, Blood Advances, and Cell Communication and Signaling.

UNIVERSITY SERVICE

INSTITUTIONAL COMMITTEE:

2012 to 2017 Member, Institutional Animal Care and Use Committee (IACUC), Indiana University School of Medicine

2017 to 2020 Member, Graduate Faculty Council, Indiana University

REVIEWER FOR GRANTS:

2011 IUSCC ITRAC pilot project

2012 Simon Clinical Research pilot project

2013 IUSCC Pancreatic Center pilot project

2013 IUSCC Bioinformatics Core Pilot projects

2013-2016 IUSM Wright Scholarship

2015 Indiana CTSI Core Pilot projects

2017 IUSCC ITRAC pilot project

2017 IUSCC Associate Member pilot project

2018 IUSCC Associate Member pilot project

2018 Wells Center Translational Research Grant

2019 Wells Center Translational Research Grant

OTHER SERVICE:

2012 to 2016 Associate Editor, the Wells Center Newsletter

2012 Poster Judge, Research Day, Department of Biochemistry and Molecular Biology, March 2012

2013 - 2017 Poster Judge, Cancer Research Day, Indiana University Cancer Research Day

2013 - 2015 Panelist and Judge, Wells Center Summer Intern Program, Oral Presentation

2013 -2015 Poster Judge, Student Research Program in Academic Medicine Poster Presentations

2013 Interview prospective international graduate students for the IBMG program, IUSM, March 2013

2014 -2018 Career Panelist, Molecular Medicine in Action (MMIA) program, IUSM

TEACHING ASSIGNMENTS

UNDERGRADUATE SUMMER STUDENTS AND MEDICAL STUDENTS:

2007	Youyang Yang, Summer Student, Harvard University
2008-2009	Narae Bae, Graduate Student, Cornell University
2011	Joel Pollack, Undergraduate Student, Wells Center Summer Intern program, Boston University
2012	Sisi Chen, Graduate Student, Indiana University School of Medicine
2012	Zhixiang Zhang, Undergraduate Student, Wells Center Summer Intern program, Indiana University
2012	Awahngie Akwo, Medical Student, SRPAM program, Indiana University
2013	Levi Hadley, Undergraduate Student, Wells Center Summer Intern program, Indiana University
2013	Hyewon Hwang, Undergraduate Student, CEMH Summer Intern Program, Inje University, Korea
2014	Wenjing Cai, Undergraduate Student, Indiana University
2014	Chen Mu, Medical Student, SRPAM program, Indiana University
2015	Taylor Twiggs, Medical Student, SRPAM program, Indiana University
2015	Christine Wang, Medical Student, Wells Center Summer Intern program, Indiana University
2016	Cecil Daniels, Undergraduate Student, Undergraduate Research for Prospective Physician-Scientists, Indiana University
2017	David Schmitz, Medical Student, SRPAM program, Indiana University
2017	Aidan Fahey, Graduate student, Ulster University at Coleraine
2018	Odelia Satchivi, Undergraduate Student, Indiana University
2018	Danielle Henley, Undergraduate Student, University of South Alabama
2018	Sergio Barajas, Graduate Student, Indiana University School of Medicine
2019	Wenjie Cai, Graduate Student, Indiana University School of Medicine

2019 Katherine Strube, Undergraduate Student, Butler University
2019 Javona Freeman, Undergraduate Student, Indiana University

GRADUATE SCHOOL THESIS COMMITTEES:

2011-2012 Wenjuan Liao, PhD Candidate, Department of Biochemistry and Molecular Biology, IUSM
2017-2018 Yang Lin, PhD Candidate, Department of Biochemistry and Molecular Biology, IUSM
2017-2018 Aidan Fahey, Graduate student, Ulster University at Coleraine, UK
2020-2021 Aditi Khatpe, PhD Candidate, Department of Biochemistry and Molecular Biology, IUSM

Ph.D. THESIS DIRECTOR:

2012-2018 Sisi Chen, PhD Candidate, Department of Biochemistry and Molecular Biology, IUSM
2019-present Wenjie Cai, PhD Candidate, Department of Biochemistry and Molecular Biology, IUSM
2019-present Sergio Barajas, PhD Candidate, Department of Biochemistry and Molecular Biology, IUSM
2020-present Shiyu Xiao, PhD Candidate, Department of Biochemistry and Molecular Biology, IUSM
2020-2021 Christopher Borchers, PhD Candidate, Department of Biochemistry and Molecular Biology, IUSM

POSTDOCTORAL FELLOWS:

2003-2006 Jinjuan Yao, MD & PhD, Postdoctoral Fellow, MSKCC
2004-2005 Yasuhiko Miyata, MD & PhD, Postdoctoral Fellow, MSKCC
2005-2006 Goro Sashida, MD & PhD, Postdoctoral Fellow, MSKCC
2007-2008 Fabiana Perna, MD, Postdoctoral Fellow, MSKCC
2008-2010 Takashi Asai, MD & PhD, Postdoctoral Fellow, MSKCC
2011-2013 Hao Yu, PhD, Postdoctoral Fellow, Indiana University School of Medicine
2011-2015 Rui Gao, PhD, Postdoctoral Fellow, Indiana University School of Medicine

2011-2016	Michihiro Kobayashi, MD & PhD, Postdoctoral Fellow, Indiana University School of Medicine
2015-2017	Chonghua Yao, MD & PhD, Postdoctoral Fellow, Indiana University School of Medicine
2014-2018	Sarah Nabinger, PhD, Postdoctoral Fellow, Indiana University School of Medicine
2017-2021	Sasidhar Vemula, PhD, Postdoctoral Fellow, Indiana University School of Medicine
2019-2021	Yuxia Yang, PhD, Postdoctoral Fellow, Indiana University School of Medicine
2019-present	Hongxia Chen, MD & PhD, Postdoctoral Fellow, Indiana University School of Medicine

AWARDS TO STUDENTS AND POSTDOCTORAL FELLOWS:

F32 Award	Sarah Nabinger, PhD, Postdoctoral Fellow Mentor, Yan Liu, PhD., NIH/NCI 2016-2018
New Investigator Award	Sisi Chen, Graduate Student; Mentor, Yan Liu, PhD, 45 th International Society for Experimental Hematology (ISEH) Annual Meeting, 2016
Abstract Achievement Award	Sisi Chen, Graduate Student; Mentor, Yan Liu, PhD, the 59 th American Society of Hematology (ASH) Conference, Atlanta, GA, 2017.
Abstract Achievement Award	Sarah Nabinger, PhD, Postdoctoral Fellow; Mentor, Yan Liu, PhD, the 57 th American Society of Hematology (ASH) Conference, Orlando, FL, 2015.
Abstract Achievement Award	Sisi Chen, Graduate Student; Mentor, Yan Liu, PhD, the 57 th American Society of Hematology (ASH) Conference, Orlando, FL, 2015.
Abstract Achievement Award	Sisi Chen, Graduate Student; Mentor, Yan Liu, PhD, the 56 th American Society of Hematology (ASH) Conference, San Francisco, CA, 2014.
Abstract Achievement Award	Michihiro Kobayashi, MD, PhD, Postdoctoral Fellow; Mentor, Yan Liu, PhD, the 55 th American Society of Hematology (ASH) Conference, New Orleans, LA, 2013.
Graduate Student Travel Award	Sisi Chen, Graduate Student; Mentor, Yan Liu, PhD, IUPUI, 2016.

Graduate Student Travel Award	Sisi Chen, Graduate Student; Mentor, Yan Liu, PhD, IUPUI, 2014.
Honorary Mention	Sisi Chen, Graduate Student; Mentor, Yan Liu, PhD, Biochemistry Research Day, IUSM, 2014.
Honorary Mention	Sisi Chen, Graduate Student; Mentor, Yan Liu, PhD, Biochemistry Research Day, IUSM, 2017.
Honorary Mention	Sisi Chen, Graduate Student; Mentor, Yan Liu, PhD, Indiana University Cancer Research Day, 2015.
Oral presentation First Prize	Chen Mu, Medical Student; Mentor, Yan Liu, PhD, the Summer Research Program in Academic Medicine (SRPAM), Indiana University, 2014.
Oral presentation First Prize	Taylor Twiggs, Medical Student; Mentor, Yan Liu, PhD, the Summer Research Program in Academic Medicine (SRPAM), Indiana University, 2015.
Oral presentation Second Prize	Christine Wang, Medical Student; Mentor, Yan Liu, PhD, Wells Center Summer Intern program, Indiana University, 2015.

GRADUATE TEACHING ASSIGNMENTS:

Developmental Genetics-G726 Spring 2012, Lecture 8: Hematopoiesis
Role: Instructor

Developmental Genetics-G726 Spring 2013, Lecture 8: Hematopoiesis
Role: Instructor

Clinical Problem Solving-X604 Spring 2013, Cases: 5-8
Role: Instructor

Clinical Problem Solving-X604 Spring 2014, Cases: 5-8
Role: Instructor

Clinical Problem Solving-X604 Spring 2015, Cases: 5-8
Role: Instructor

Stem Cell Biology-G720 Spring 2015, Lecture 8: Cancer Stem Cells
Role: Instructor

Stem Cell Biology-G720 Spring 2016, Lecture 8: Cancer Stem Cells
Role: Instructor

Stem Cell Biology-G720 Spring 2017, Lecture 8: Cancer Stem Cells
Role: Instructor

Stem Cell Biology-G720 Spring 2019, Lecture 8: Cancer Stem Cells
Role: Instructor

Stem Cell Biology-G720 Spring 2020, Lecture 8: Cancer Stem Cells
Role: Instructor

Stem Cell Biology-G720 Spring 2021, Lecture 8: Cancer Stem Cells
Role: Instructor