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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, 2019 to August 31, 2020, we carried out some experiments proposed in both Aim 1 and Aim 2. We have completed majority of proposed research as indicated in the Statement of Work (SOW).

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

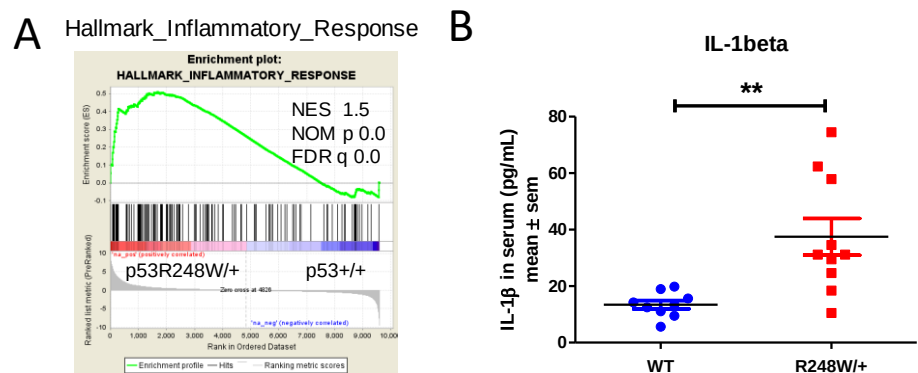


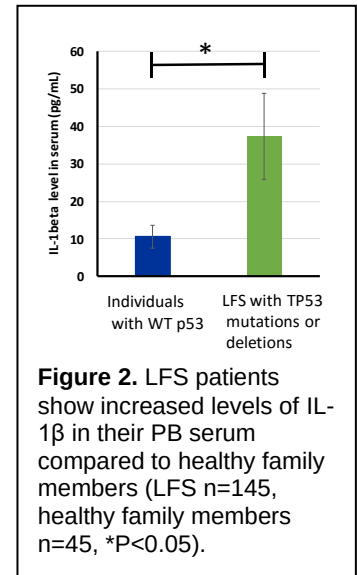
Figure 1. (A) RNA-seq assays revealed that inflammatory response genes are significantly enriched in p53 mutant HSPCs compared to WT HSPCs. (B) p53 mutant mice show increased levels of IL-1 β in their peripheral blood serum (n=10, **P<0.01).

the effects of genetic and pharmacological inhibition of the NLRP1 inflammasome on clonal expansion of mutant p53 HSCs.

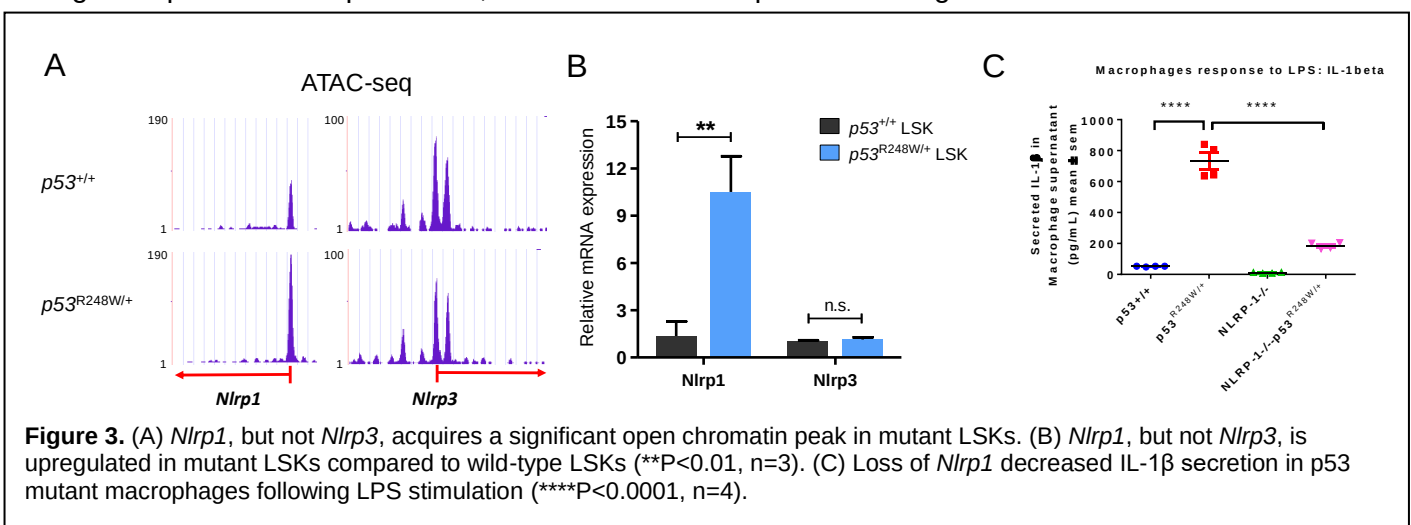
Major Task 1: Determine the impact of genetic inhibition of NLRP1 on HSCs expressing mutant p53

Mutant p53 generates a chronic pro-inflammatory microenvironment in mice. To understand how mutant p53 confers a competitive advantage to HSPCs, 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. 1A**). 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). We observed increased levels of pro-inflammatory cytokines, including IL-1 β and IL-6, in the serum of p53 mutant mice compared to that of the age-matched WT mice (**Fig. 1B** and data not shown). BM microenvironment is the niche of HSCs and we observed increased levels of IL-1 β and IL-6 in BM fluid of p53 mutant mice (data not shown). Thus, these findings demonstrate that mutant p53 generates a systematic chronic inflammatory microenvironment, which may play an important role in conferring a competitive advantage to p53 mutant HSPCs during leukemia initiation.

Peripheral blood serum from individuals with LFS show increased levels of pro-inflammatory cytokines: As the $p53^{R248W/+}$ is a mouse model of human Li-Fraumeni syndrome (LFS), we then examined the levels of IL-1 β and IL-6 in PB serum of LFS patients with *TP53* mutations and their family members who have WT *TP53*. Consistent with our observation in p53 mutant mice, we found increased levels of IL-1 β and IL-6 in LFS compared to their healthy family members (**Fig. 2** and data not shown). As both IL-1 β and IL-6 have been implicated in human cancer, our findings suggest that mutant p53-driven chronic inflammation may promote cancer development in individuals with LFS.



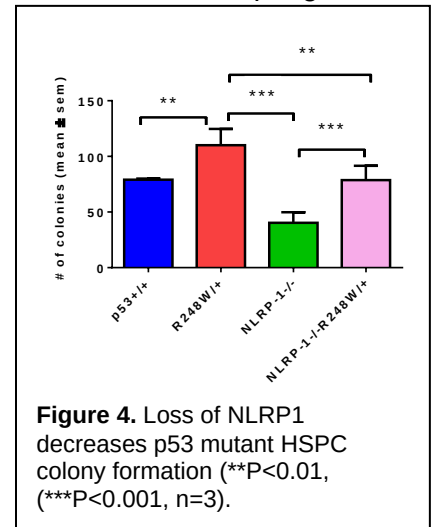
Mutant p53 increases the chromatin accessibility to the NLRP1 inflammasome gene in HSPCs. To investigate how mutant p53 activates inflammatory response genes, we performed ATAC-seq assays in HSPCs (LSKs) to identify differential regions of chromatin accessibility. To ensure reproducibility, three biological 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. 3A and 3B**). While NLRP3 inflammasome is activated in MDS HSPCs, we found that mutant p53 affects neither the chromatin accessibility nor the expression of *Nlrp3* in LSK cells (**Figs. 3A and 3B**). These findings suggest

that the NLRP1 inflammasome is specifically activated in p53 mutant mice.

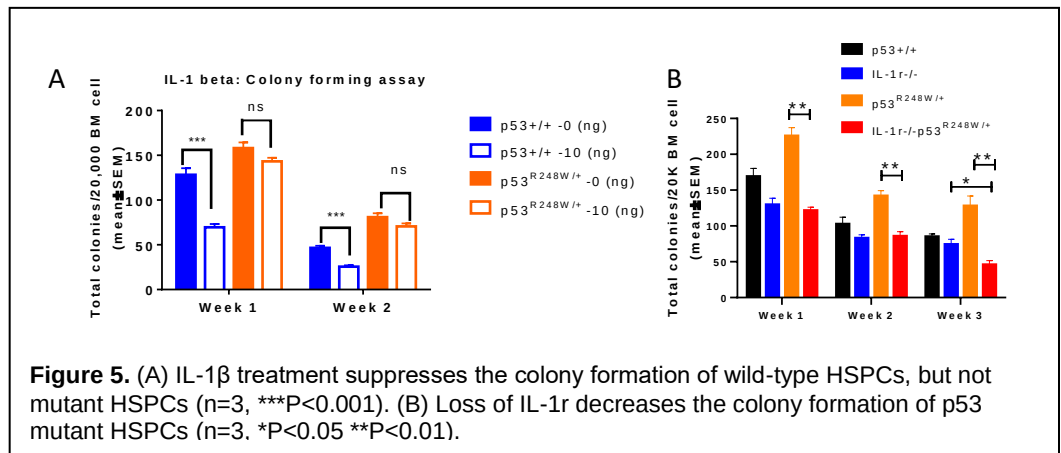
Increased secretion of IL-1 β seen in p53 mutant macrophages is dependent on NLRP1. NLRP1 activation increases IL-1 β secretion that inhibits wild-type HSPC function. IL-1 β in serum could come from stromal cells, immune cells and/or endothelial cells. Given that macrophages are major sources of cytokine production, we examined the impact of mutant p53 on IL-1 β secretion from BM derived macrophages following 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 then 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. While we observed increased secretion of IL-1 β from mutant macrophages compared to WT macrophages following LPS stimulation, loss of Nlrp1 significantly decreased IL-1 β secretion from p53 mutant macrophages following LPS stimulation (**Fig. 3C**). These findings implicate NLRP1 as a critical factor in increased secretion of IL-1 β from p53 mutant hematopoietic cells.



NLRP1 is essential for p53 mutant HSPC colony formation: To determine the role of NLRP1 in p53 mutant HSPCs, we performed colony formation assays and found that loss of NLRP1 significantly decreases the colony formation of p53 mutant HSPCs (**Fig. 4**), demonstrating that NLRP1 is important for the enhanced fitness of p53 mutant HSPCs.

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

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.



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 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. 6A**). 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. 6B**). 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. 6C** and data not shown).

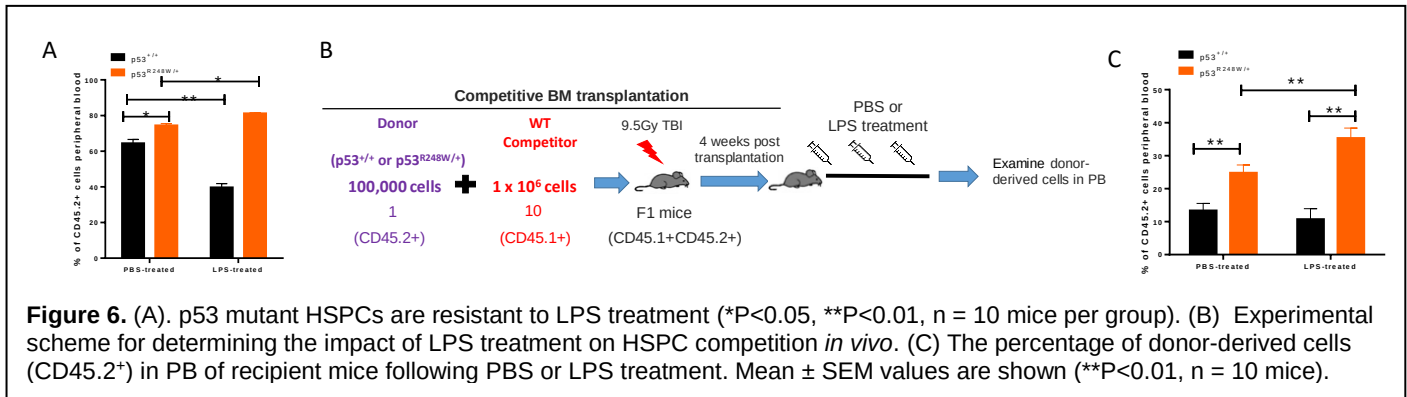


Figure 6. (A). $p53$ mutant HSPCs are resistant to LPS treatment (* $P < 0.05$, ** $P < 0.01$, $n = 10$ mice per group). (B) Experimental scheme for determining the impact of LPS treatment on HSPC competition *in vivo*. (C) The percentage of donor-derived cells (CD45.2⁺) in PB of recipient mice following PBS or LPS treatment. Mean $\pm$ SEM values are shown (** $P < 0.01$, $n = 10$ mice).

$p53$ 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.

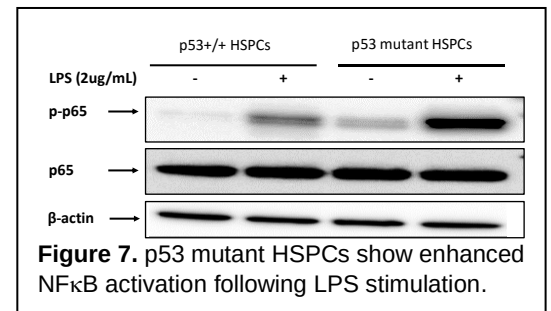


Figure 7. $p53$ mutant HSPCs show enhanced NF κ B activation following LPS stimulation.

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.

Mutant $p53$ alters pre-mRNA splicing in aged HSPCs. RNA-seq assays also revealed that spliceosome genes were significantly downregulated in middle-aged (Pre-MDS) $p53^{R248W/+}$ HSPCs compared to $p53^{+/+}$ HSPCs (**Fig. 8A**). We confirmed that the expression of splicing factors, including *Prpf3* and *Prpf4*, was significantly downregulated in aged $p53^{R248W/+}$ HSPCs (**Fig. 8B and 8C**). 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. 8D**), **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),

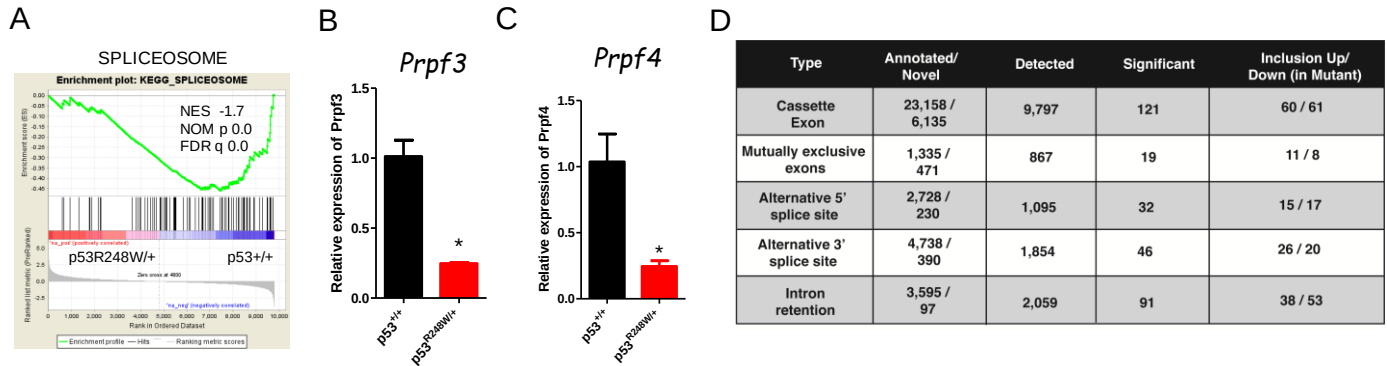


Figure 8. (A) Spliceosome genes were significantly downregulated in middle-aged (Pre-MDS) p53 mutant HSPCs compared to age-matched wild type HSPCs. (B) and (C) Splicing factors *Prpf3* and *Prpf4* were significantly downregulated in middle-aged p53 (Pre-MDS) mutant HSPCs (* $P<0.05$, $n=3$). (D) Table of splicing events altered in aged p53 mutant versus aged WT HSPCs.

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 (Pre-MDS) p53 mutant HSPCs show dysregulated pre-mRNA splicing in key regulators of hematopoiesis such as *Asx2* and *Setd3* (**Fig. 9A** and data not shown).

Polycomb group protein ASXL2 is frequently mutated in AML patients with t(8;21). We recently reported that *Asx2* is required for normal HSC self-renewal and deletion of *Asx2* leads to MDS development in mice [80-81]. We confirmed that p53 mutant HSPCs display significant change in pre-mRNA splicing of *Asx2*, mainly expressing the long isoform of *Asx2* (**Fig. 9B**). Further, we found that ectopic expression of *Asx2* long isoform, but not the *Asx2* short isoform, increases the colony formation of WT HSPCs (**Fig. 9C**).

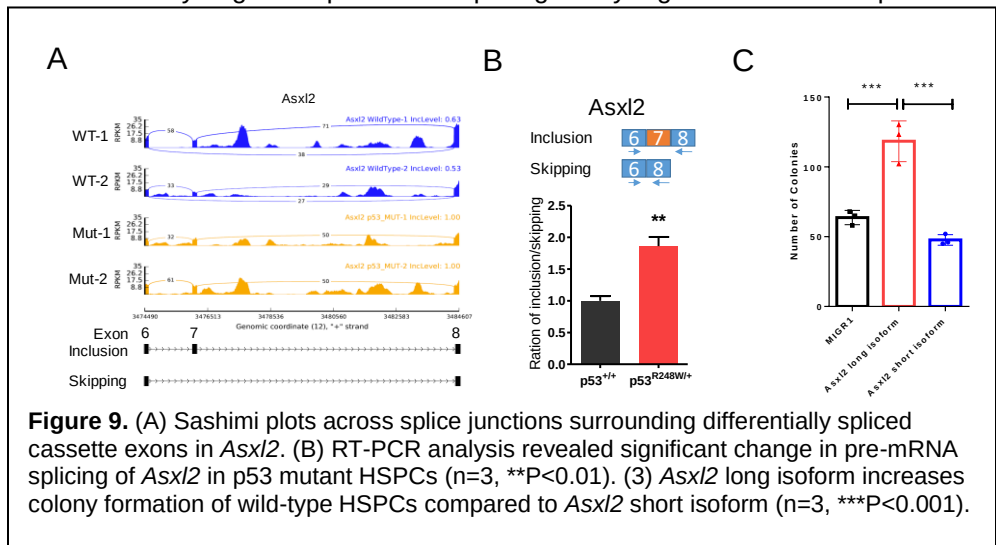


Figure 9. (A) Sashimi plots across splice junctions surrounding differentially spliced cassette exons in *Asx2*. (B) RT-PCR analysis revealed significant change in pre-mRNA splicing of *Asx2* in p53 mutant HSPCs ($n=3$, ** $P<0.01$). (3) *Asx2* long isoform increases colony formation of wild-type HSPCs compared to *Asx2* short isoform ($n=3$, *** $P<0.001$).

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

TP53 and *SRSF2* mutations cooperate on MDS pathogenesis:

While coexisting mutations with *TP53* mutations in MDS are limited, previous studies indicate that *TP53* mutations co-occur with splicing factor mutations, including *SRSF2*, *SF3B1*, and *U2AF1* mutations, in human MDS. To determine the impact of *TP53* and *SRSF2* mutations in MDS pathogenesis, we have generated $p53^{R248W/+}$ *Srsf2*^{P95H/+} *-Mx1-Cre*⁺. 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. 10**).

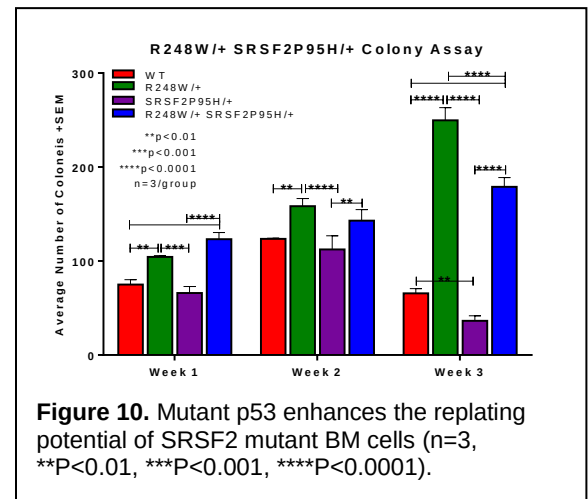


Figure 10. Mutant p53 enhances the replating potential of *Srsf2* mutant BM cells ($n=3$, ** $P<0.01$, *** $P<0.001$, **** $P<0.0001$).

We then transplanted $p53^{+/+}$, $p53^{R248W/+}$, $Srsf2^{P95H/+}$ - $Mx1$ - Cre^{+} , and $p53^{R248W/+}$ $Srsf2^{P95H/+}$ - $Mx1$ - Cre^{+} BM cells into lethally irradiated recipient mice. Four weeks after transplantation, we treated recipient mice with pl:pC. We then monitor survival and MDS development in recipient mice. We found that the survival of $p53^{R248W/+}$ $Srsf2^{P95H/+}$ mice was significantly decreased compared to WT, $p53^{R248W/+}$, and $Srsf2^{P95H/+}$ mice (Fig. 11A and 11B), demonstrating that mutant p53 synergizes with $Srsf2$ mutation in MDS pathogenesis, manifested by decreased survival and accelerate disease onset.

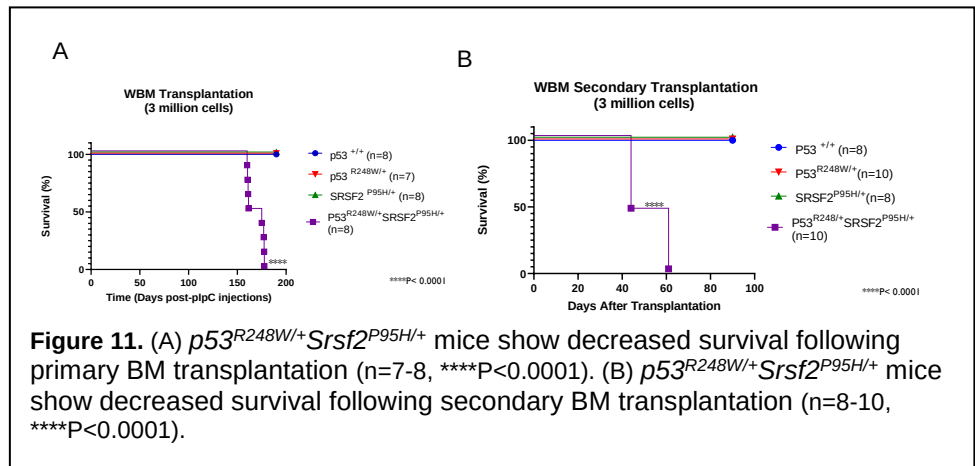


Figure 11. (A) $p53^{R248W/+}$ $Srsf2^{P95H/+}$ mice show decreased survival following primary BM transplantation (n=7-8, ****P<0.0001). (B) $p53^{R248W/+}$ $Srsf2^{P95H/+}$ mice show decreased survival following secondary BM transplantation (n=8-10, ****P<0.0001).

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 (data not shown). 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. 12A).

Human MDS cells with TP53 mutations are sensitive to spliceosome inhibitor treatment.

We cultured MDL-p53^{+/+}, MDL-p53^{-/-}, and MDL-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 MDL-p53^{R248W} cells show increased apoptosis and decreased proliferation compared to MDL-p53^{+/+} and MDL-p53^{-/-} cells following spliceosome inhibitor treatment (Fig. 12B and data not shown).

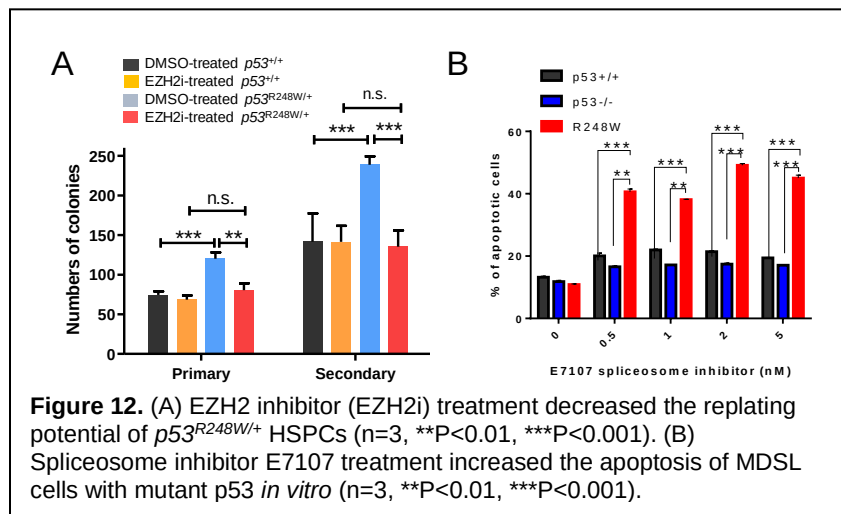


Figure 12. (A) EZH2 inhibitor (EZH2i) treatment decreased the replating potential of $p53^{R248W/+}$ HSPCs (n=3, **P<0.01, ***P<0.001). (B) Spliceosome inhibitor E7107 treatment increased the apoptosis of MDL cells with mutant p53 *in vitro* (n=3, **P<0.01, ***P<0.001).

Opportunities for training and professional development:

Dr. Yan Liu has trained two graduate students, including Sergio Barajas and Wenjie Cai, and two postdoctoral fellows, including Sasidhar Vemula and Hongxia Chen, from 2019 to 2020. 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):

- 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 8th 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.

Future plans:

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

We will determine the effect of *Nlrp1* deficiency on the self-renewal and differentiation of HSCs expressing mutant p53. We will also determine the impact of NLRP1 inhibitor on HSCs expressing mutant p53 both in vitro and in vivo

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

We will determine the impact of spliceosome inhibitor E7107 on human MDS cells with *TP53* mutations in vivo.

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

Nothing to Report

6. Products

Publications, conference papers, and presentations

a. Journal publications.

1. 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.
2. 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.
3. 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**).
4. 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, 2020).
5. 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, 2020).

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

Nothing to Report

b. Other publications, conference papers, and presentations.

- 2019 Mutant p53 drives clonal hematopoiesis through modulating epigenetic pathways, Dr. Yan Liu, the 8th 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;*

Human MDS patient samples

- 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);*

Animal models: 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?

- 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>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>

Name:	<i>Yunlong Liu</i>
Project Role:	<i>Co-I</i>
Researcher Identifier (e.g. ORCID ID):	N/A
Nearest person month worked:	1
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:	1
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>Hongxia Chen</i>
Project Role:	<i>Postdoc</i>
Researcher Identifier (e.g. ORCID ID):	N/A
Nearest person month worked:	<i>12</i>
Contribution to Project:	Dr. Chen designed and conducted the experiments listed in this proposal.
Funding Support:	

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. 3 published papers and 2 manuscripts
- B. Yan Liu CV

ARTICLE

There are amendments to this paper

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OPEN

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

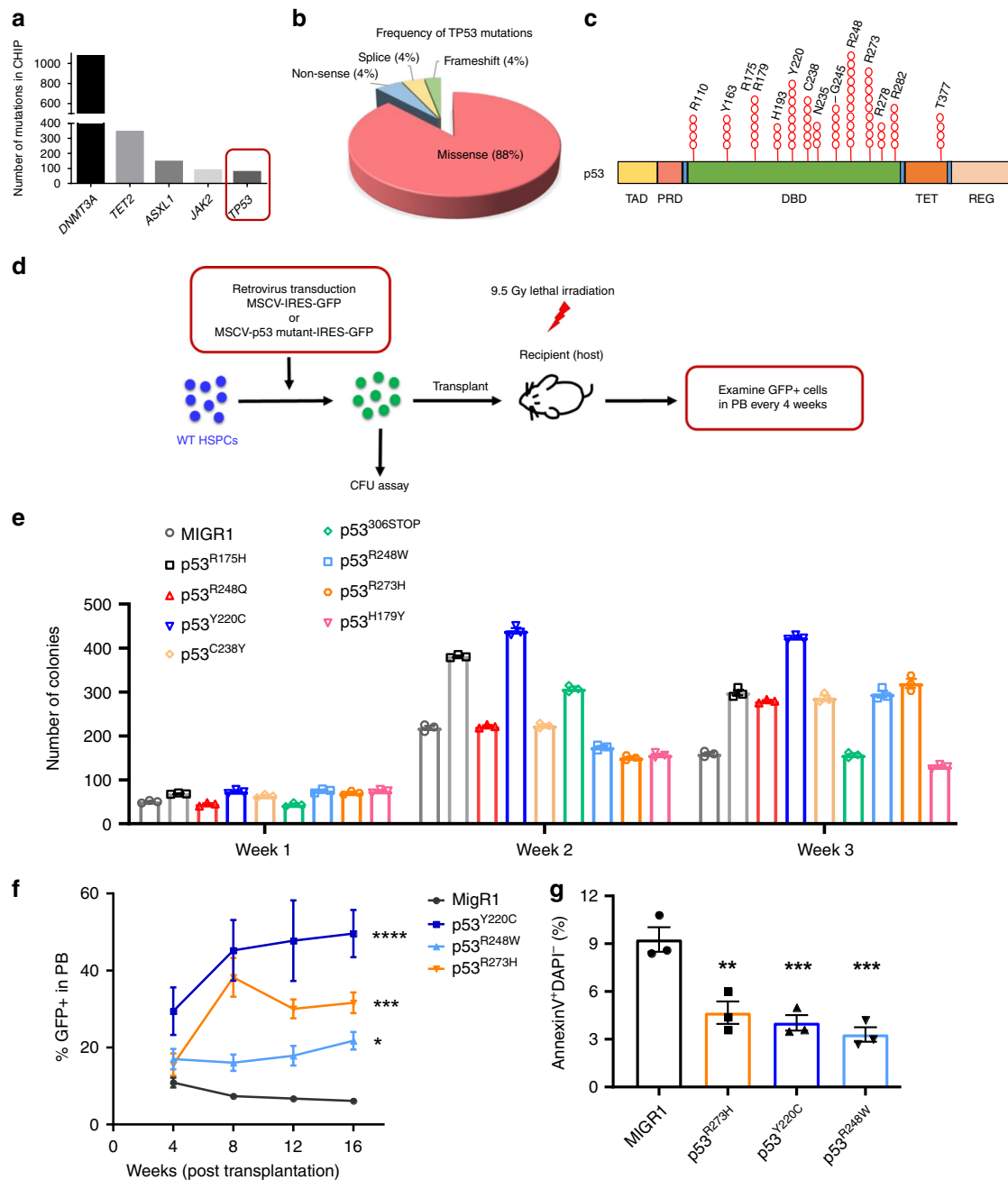
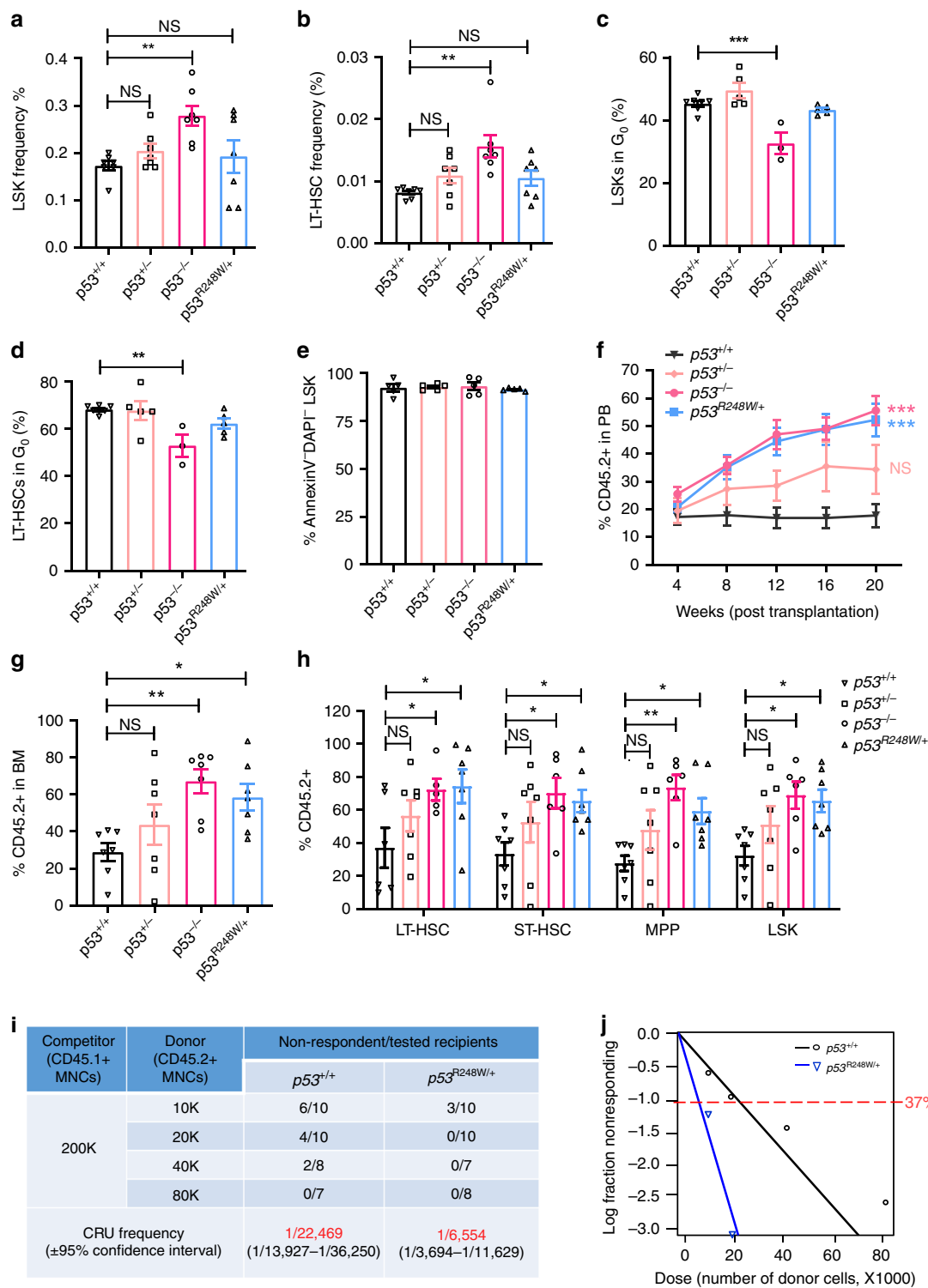


Fig. 1 *TP53* mutations identified in CHIP enhance HSPC repopulating potential. **a** Tumor suppressor gene *TP53* ranks in the top five among genes that were mutated in clonal hematopoiesis with indeterminate potential (CHIP). **b** Pie chart representing different types of *TP53* mutations identified 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*.

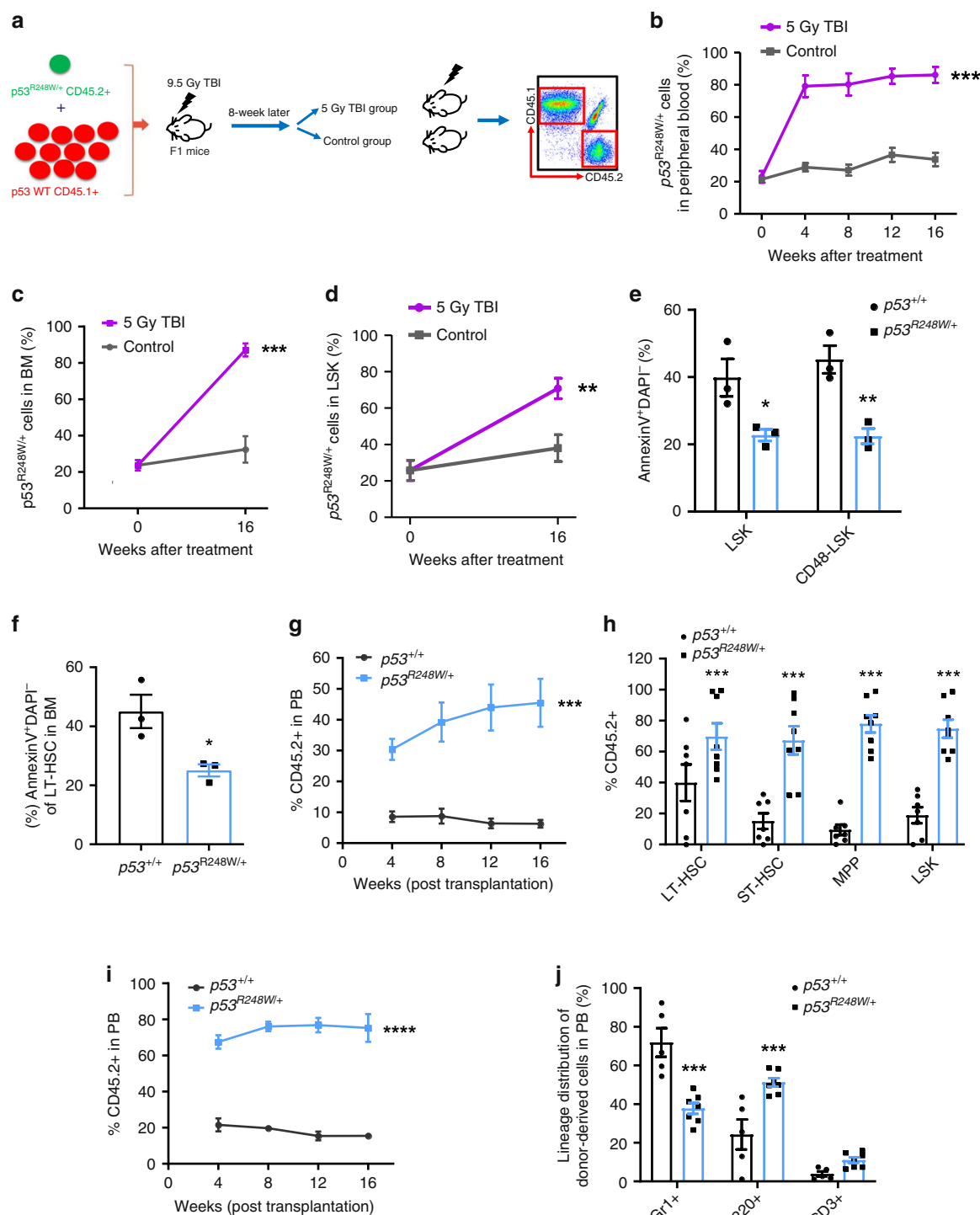
To determine the impact of radiation on $p53^{+/+}$ and $p53^{R248W/+}$ mice, we irradiated these mice and monitored their survival. While most $p53^{+/+}$ mice died 5 weeks following 9 Gy TBI, most $p53^{R248W/+}$ mice were still alive (Supplementary Fig. 2a). Further, $p53^{R248W/+}$ HSCs show decreased 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 alpha 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).

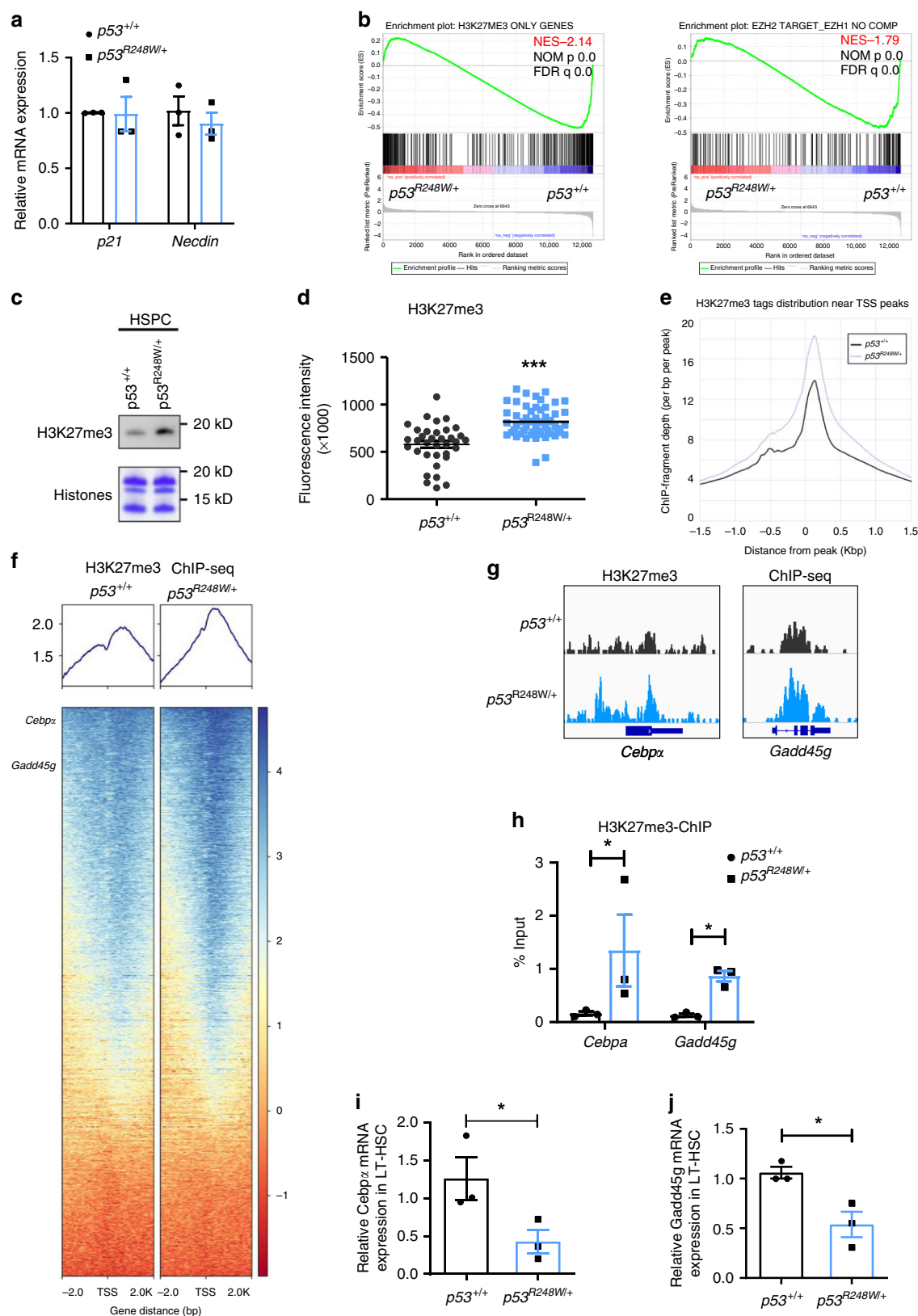
Stimulation of WT HSPCs with thrombopoietin (TPO) dramatically increased *Gadd45g* expression; however, TPO treatment only modestly increased *Gadd45g* expression in p53 mutant HSPCs (Supplementary Fig. 5a), suggesting that mutant p53 may repress *Gadd45g* expression upon cytokine stimulation. To determine 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.

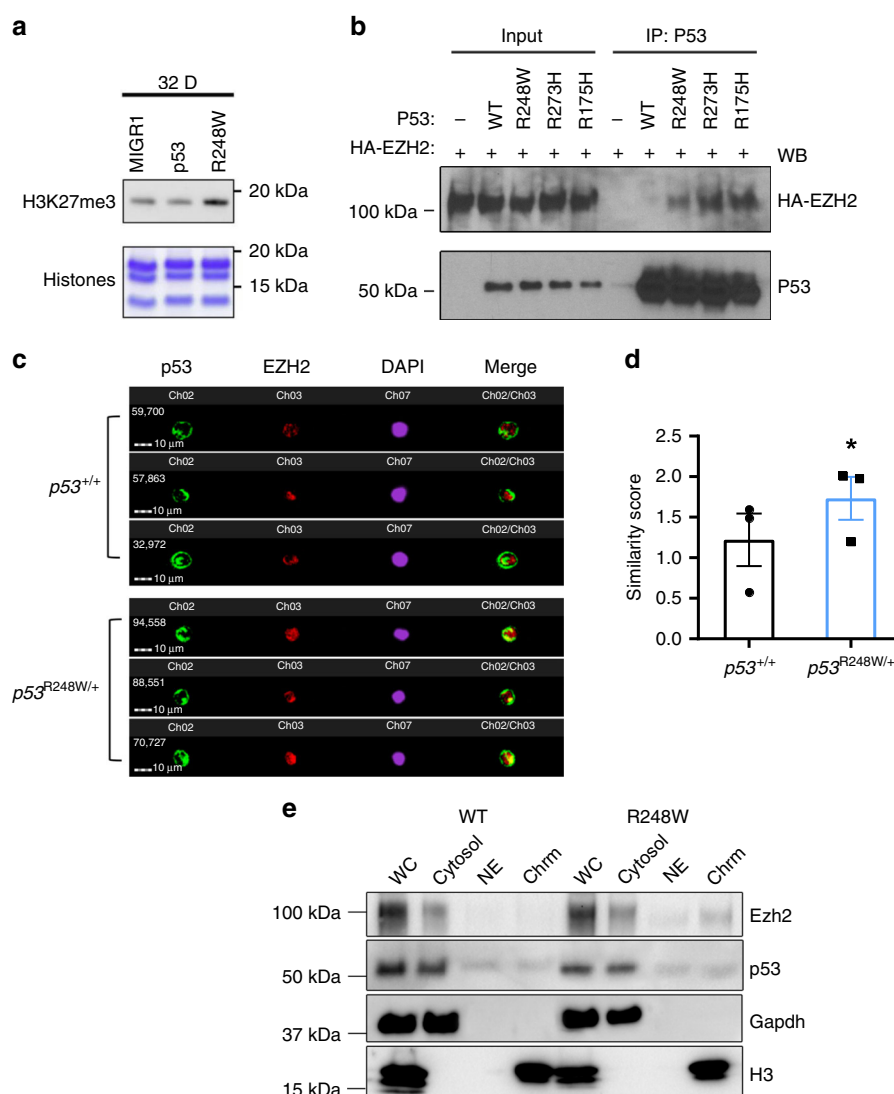


Fig. 5 Mutant p53 enhances the association of EZH2 with the chromatin in HSPCs. **a** 32D cells expressing mutant p53, but not wild-type (WT) p53, displayed increased levels of H3K27me3 as determined by immuno-blot analysis. **b** Several mutant p53 proteins, but not wild-type p53, show enhanced association 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^{f/+}-Mx1Cre⁺*, *p53^{R248W/+}*, and *p53^{R248W/+}Ezh2^{f/+}-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

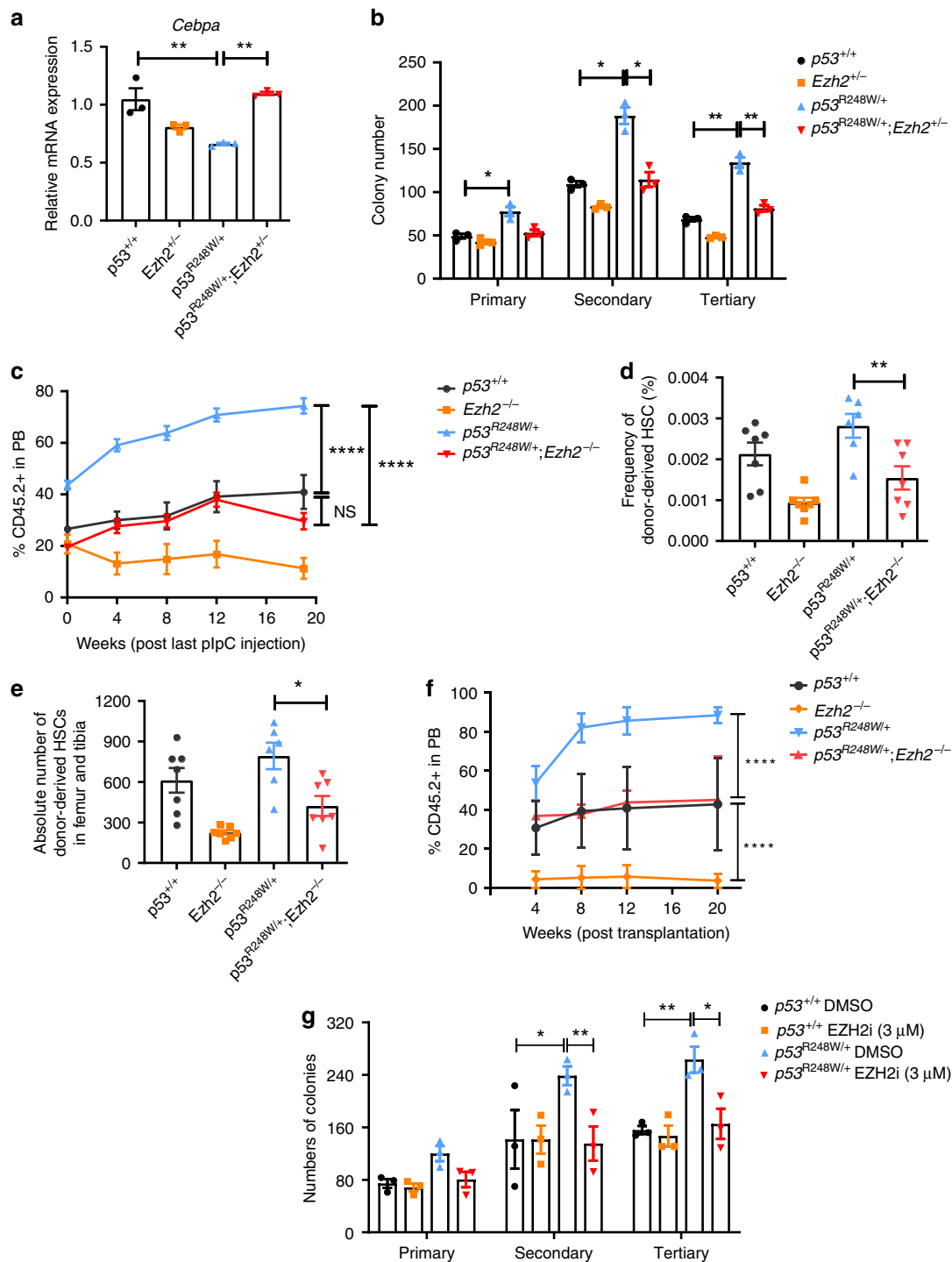


Fig. 6 Loss of EZH2 decreases the repopulating potential of p53 mutant HSPCs. **a** *Cebpa* expression in $p53^{+/+}$, $Ezh2^{-/-}$, $p53^{R248W/+}$, and $p53^{R248W/+};Ezh2^{-/-}$ HSPCs; $n = 3$ biological replicates. **b** Serial replating assays of BM cells from $p53^{+/+}$, $Ezh2^{-/-}$, $p53^{R248W/+}$ 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 mouse) 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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References

1. Steensma, D. P. et al. Clonal hematopoiesis of indeterminate potential and its distinction from myelodysplastic syndromes. *Blood* **126**, 9–16 (2015).
2. Sperling, A. S., Gibson, C. J. & Ebert, B. L. The genetics of myelodysplastic syndrome: from clonal haematopoiesis to secondary leukaemia. *Nat. Rev. Cancer* **17**, 5–19 (2017).
3. Jan, M., Ebert, B. L. & Jaiswal, S. Clonal hematopoiesis. *Semin. Hematol.* **54**, 43–50 (2017).
4. Genovese, G. et al. Clonal hematopoiesis and blood-cancer risk inferred from blood DNA sequence. *N. Engl. J. Med.* **371**, 2477–2487 (2014).
5. Jaiswal, S. et al. Age-related clonal hematopoiesis associated with adverse outcomes. *N. Engl. J. Med.* **371**, 2488–2498 (2014).
6. Xie, M. et al. Age-related mutations associated with clonal hematopoietic expansion and malignancies. *Nat. Med.* **20**, 1472–1478 (2014).
7. Young, A. L., Challen, G. A., Birmann, B. M. & Druley, T. E. Clonal haematopoiesis harbouring AML-associated mutations is ubiquitous in healthy adults. *Nat. Commun.* **7**, 12484 (2016).
8. Zink, F. et al. Clonal hematopoiesis, with and without candidate driver mutations, is common in the elderly. *Blood* **130**, 742–752 (2017).
9. Jaiswal, S. et al. Clonal hematopoiesis and risk of atherosclerotic cardiovascular disease. *N. Engl. J. Med.* **13**, 111–121 (2017).
10. Coombs, C. C. et al. Therapy-related clonal hematopoiesis in patients with non-hematologic cancers is common and associated with adverse clinical outcomes. *Cell Stem Cell* **21**, 374–382.e4 (2017).
11. Xia, J. et al. Somatic mutations and clonal hematopoiesis in congenital neutropenia. *Blood* **131**, 408–416 (2018).
12. Wong, T. N. et al. Cellular stressors contribute to the expansion of hematopoietic clones of varying leukemic potential. *Nat. Commun.* **9**, 455 (2018).
13. Levine, A. J. & Oren, M. The first 30 years of p53: growing ever more complex. *Nat. Rev. Cancer* **9**, 749–758 (2009).
14. Brosh, R. & Rotter, V. When mutants gain new powers: news from the mutant p53 field. *Nat. Rev. Cancer* **9**, 701–713 (2009).
15. Talwalkar, S. S. et al. Myelodysplastic syndromes arising in patients with germline TP53 mutation and Li-Fraumeni syndrome. *Arch. Pathol. Lab Med.* **134**, 1010–1015 (2010).
16. Bejar, R. et al. Clinical effect of point mutations in myelodysplastic syndromes. *N. Engl. J. Med.* **364**, 2496–2506 (2011).
17. Peller, S. & Rotter, V. TP53 in hematological cancer: low incidence of mutations with significant clinical relevance. *Hum. Mutat.* **21**, 277–284 (2003).
18. Rücker, F. G. et al. TP53 alterations in acute myeloid leukemia with complex karyotype correlate with specific copy number alterations, monosomal karyotype, and dismal outcome. *Blood* **119**, 2114–2121 (2012).
19. Welch, J. S. et al. TP53 and decitabine in acute myeloid leukemia and myelodysplastic syndromes. *N. Engl. J. Med.* **375**, 2023–2036 (2016).
20. Liu, Y. et al. p53 Regulates hematopoietic stem cell quiescence. *Cell Stem Cell* **4**, 37–48 (2009).
21. Asai, T. et al. Ncdin, a p53 target gene, regulates the quiescence and response to genotoxic stress of hematopoietic stem/progenitor cells. *Blood* **120**, 1601–1612 (2012).
22. Chen, S. et al. Genotoxic stresses promotes the clonal expansion of hematopoietic stem cells expressing mutant p53. *Leukemia* **32**, 850–854 (2018).
23. Bondar, T. & Medzhitov, R. p53-Mediated Hematopoietic Stem and Progenitor Cell Competition. *Cell Stem Cell* **6**, 309–322 (2010).

24. Marusyk, A., Porter, C. C., Zaberezhnyy, V. & DeGregori, J. Irradiation Selects for p53-Deficient Hematopoietic Progenitors. *PLoS Biology* **8**, e1000324 (2010).
25. Valk-Lingbeek, M. E., Bruggeman, S. W. & van Lohuizen, M. Stem cells and cancer; the polycomb connection. *Cell* **118**, 409–418 (2004).
26. Sparmann, A. & van Lohuizen, M. Polycomb silencers control cell fate, development and cancer. *Nat. Rev. Cancer* **6**, 846–856 (2006).
27. Bracken, A. P. & Helin, K. Polycomb group proteins: navigators of lineage pathways led astray in cancer. *Nat. Rev. Cancer* **9**, 773–784 (2009).
28. Simon, J. A. & Kingston, R. E. Mechanisms of polycomb gene silencing: knowns and unknowns. *Nat. Rev. Mol. Cell. Biol.* **10**, 697–708 (2009).
29. Xie, H. et al. Polycomb repressive complex 2 regulates normal hematopoietic stem cell function in a developmental-stage-specific manner. *Cell Stem Cell* **14**, 68–80 (2014).
30. Mochizuki-Kashio, M. et al. Dependency on the polycomb gene *Ezh2* distinguishes fetal from adult hematopoietic stem cells. *Blood* **118**, 6553–6561 (2012).
31. Olive, K. P. et al. Mutant p53 gain of function in two mouse models of Li-Fraumeni syndrome. *Cell* **119**, 847–860 (2004).
32. Loizou, E. et al. A gain-of-function p53-mutant oncogene promotes cell fate plasticity and myeloid leukemia through the pluripotency factor FOXH1. *Cancer Discov.* **9**, 962–979 (2019).
33. Song, H., Hollstein, M. & Xu, Y. p53 gain-of-function cancer mutants induce genetic instability by inactivating ATM. *Nat. Cell. Biol.* **15**, 376–388 (2007).
34. Luo, J. L. et al. Knock-in mice with a chimeric human/murine p53 gene develop normally and show wild-type p53 responses to DNA damaging agents: a new biomedical research tool. *Oncogene* **20**, 320–328 (2001).
35. Hsu, J. I. et al. PPM1D mutations drive clonal hematopoiesis in response to cytotoxic chemotherapy. *Cell Stem Cell* **23**, 700–713.e6 (2018).
36. Kahn, J. D. et al. PPM1D-truncating mutations confer resistance to chemotherapy and sensitivity to PPM1D inhibition in hematopoietic cells. *Blood* **132**, 1095–1105 (2018).
37. Wong, T. N. et al. Role of TP53 mutations in the origin and evolution of therapy-related acute myeloid leukaemia. *Nature* **518**, 552–555 (2015).
38. Zhu, J. et al. Gain-of-function p53 mutants co-opt chromatin pathways to drive cancer growth. *Nature* **525**, 206–211 (2015).
39. Krivtsov, A. V. & Armstrong, S. A. MLL translocations, histone modifications and leukaemia stem-cell development. *Nat. Rev. Cancer* **7**, 823–833 (2007).
40. Rao, R. C. & Dou, Y. Hijacked in cancer: the KMT2 (MLL) family of methyltransferases. *Nat. Rev. Cancer* **15**, 334–346 (2015).
41. Voss, A. K., Collin, C., Dixon, M. P. & Thomas, T. Moz and retinoic acid coordinately regulate H3K9 acetylation, Hox gene expression, and segment identity. *Dev. Cell* **17**, 674–686 (2009).
42. Sun, X. J., Man, N., Tan, Y., Nimer, S. D. & Wang, L. The role of histone acetyltransferases in normal and malignant hematopoiesis. *Front. Oncol.* **5**, 108 (2015).
43. Zhang, P. et al. Enhancement of hematopoietic stem cell repopulating capacity and self-renewal in the absence of the transcription factor C/EBP alpha. *Immunity* **21**, 853–863 (2004).
44. Pulikkan, J. A., Tenen, D. G. & Behre, G. C/EBPα deregulation as a paradigm for leukemogenesis. *Leukemia* **31**, 2279–2285 (2017).
45. Thalheimer, F. B. et al. Cytokine-regulated GADD45G induces differentiation and lineage selection in hematopoietic stem cells. *Stem Cell Rep.* **3**, 34–43 (2014).
46. Campbell, J. E. et al. EPZ011989, a potent, orally-available EZH2 inhibitor with robust in vivo activity. *ACS Med. Chem. Lett.* **6**, 491–495 (2015).
47. Kenzelmann Broz, D. et al. Global genomic profiling reveals an extensive p53-regulated autophagy program contributing to key p53 responses. *Genes Dev.* **27**, 1016–1031 (2013).
48. Lindsley, R. C. & Ebert, B. L. Molecular pathophysiology of myelodysplastic syndromes. *Annu. Rev. Pathol.* **8**, 21–47 (2013).
49. Sun, D. et al. Epigenomic profiling of young and aged HSCs reveals concerted changes during aging that reinforce self-renewal. *Cell Stem Cell* **14**, 673–688 (2014).
50. Beerman, I. & Rossi, D. J. Epigenetic control of stem cell potential during homeostasis, aging, and disease. *Cell Stem Cell* **16**, 613–625 (2015).
51. Van der Meulen, J., Speleman, F. & Van Vlierberghe, P. The H3K27me3 demethylase UTX in normal development and disease. *Epigenetics* **9**, 658–668 (2014).
52. Burchfield, J. S., Li, Q., Wang, H. Y. & Wang, R. F. JMJD3 as an epigenetic regulator in development and disease. *Int. J. Biochem. Cell Biol.* **67**, 148–157 (2015).
53. Boettcher, S. et al. A dominant-negative effect drives selection of TP53 missense mutations in myeloid malignancies. *Science* **365**, 599–604 (2019).
54. Fiscella, M. et al. Wip1, a novel human protein phosphatase that is induced in response to ionizing radiation in a p53-dependent manner. *Proc. Natl Acad. Sci. USA* **94**, 6048–6053 (1997).
55. Lu, X. et al. The type 2C phosphatase Wip1: an oncogenic regulator of tumor suppressor and DNA damage response pathways. *Cancer Metastasis Rev.* **27**, 123–135 (2008).
56. Fuster, J. J. et al. Clonal hematopoiesis associated with TET2 deficiency accelerates atherosclerosis development in mice. *Science* **355**, 842–847 (2017).
57. Cai, Z. et al. Inhibition of inflammatory signaling in Tet2 mutant preleukemic cells mitigates stress-induced abnormalities and clonal hematopoiesis. *Cell Stem Cell* **23**, 833–849 (2018).
58. Prokocimer, M., Molchadsky, A. & Rotter, V. Dysfunctional diversity of p53 proteins in adult acute myeloid leukemia: projections on diagnostic workup and therapy. *Blood* **130**, 699–712 (2017).
59. Nabinger, S. C. et al. Mutant p53 enhances leukemia-initiating cell self-renewal to promote leukemia development. *Leukemia* **33**, 1535–1539 (2019).
60. Gao, Z. et al. PCGF homologs, CBX proteins, and RYBP define functionally distinct PRC1 family complexes. *Mol. Cell* **45**, 344–356 (2012).
61. Wang, Q. et al. WDR68 is essential for the transcriptional activation of the PRC1-AUTS2 complex and neuronal differentiation of mouse embryonic stem cells. *Stem Cell Res.* **33**, 206–214 (2018).
62. Egan, B. et al. An alternative approach to ChIP-seq normalization enables detection of genome-wide changes in histone H3 lysine 27 trimethylation upon EZH2 inhibition. *PLoS ONE* **11**, e0166438 (2016).

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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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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.

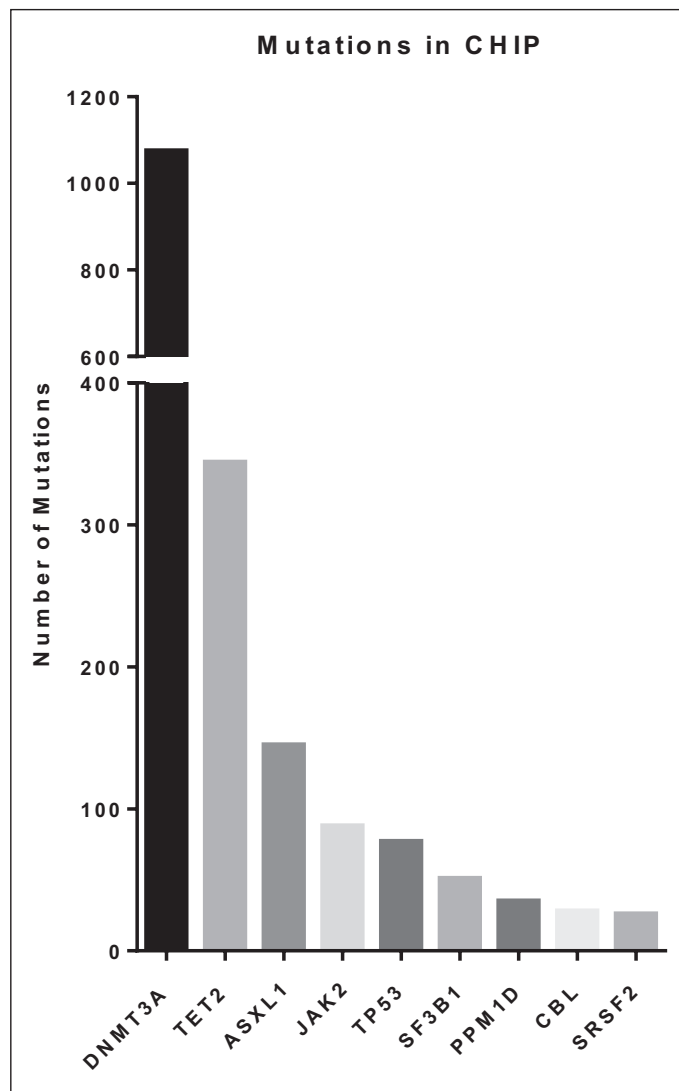


FIGURE 1. Tumor suppressor gene *TP53* ranks in the top five among genes that were mutated in clonal hematopoiesis of indeterminate potential.

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

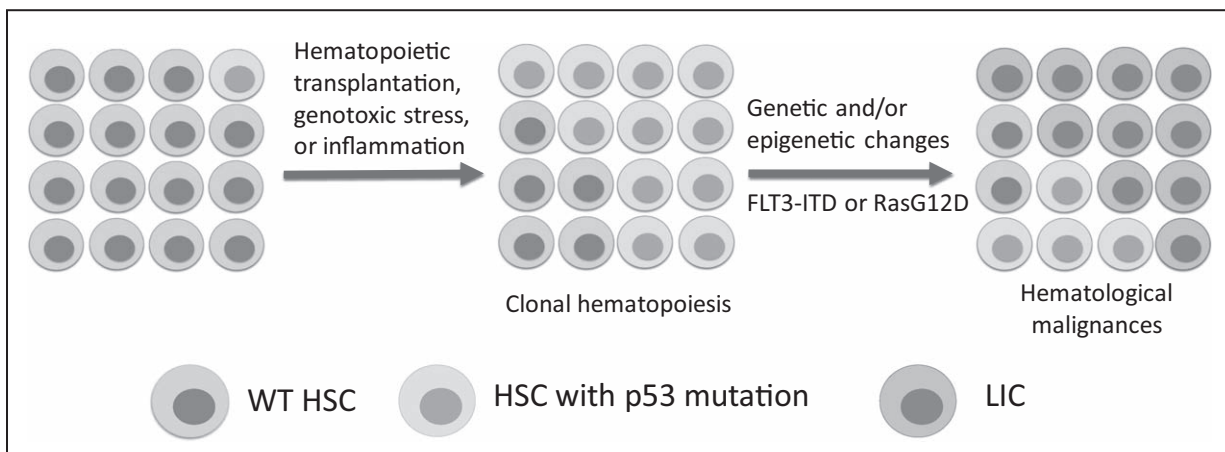


FIGURE 2. *TP53* mutations identified in clonal hematopoiesis of indeterminate potential utilize distinct mechanisms to promote hematopoietic stem cell expansion during aging. Expanded mutant hematopoietic stem cells become fully transformed leukemia-initiating cells after acquiring additional genetic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

REFERENCES AND RECOMMENDED READING

Papers of particular interest, published within the annual period of review, have been highlighted as:

- of special interest
- of outstanding interest

1. Jan M, Ebert BL, Jaiswal S. Clonal hematopoiesis. *Semin Hematol* 2017; 54:43–50.
2. Sperling AS, Gibson CJ, Ebert BL. The genetics of myelodysplastic syndrome: from clonal haematopoiesis to secondary leukaemia. *Nat Rev Cancer* 2017; 17:5–19.
- Comprehensive review on clonal hematopoiesis of indeterminate potential (CHIP) development and myelodysplastic syndromes (MDS) pathogenesis.
3. Steensma DP, Bejar R, Jaiswal S, et al. Clonal hematopoiesis of indeterminate potential and its distinction from myelodysplastic syndromes. *Blood* 2015; 126:9–16.
4. Busque L, Buscarlet M, Mollica L, Levine RL. Concise review: age-related clonal hematopoiesis: stem cells tempting the devil. *Stem Cells* 2018; 36:1287–1294.
- Comprehensive review on ARCH.
5. Genovese G, Kähler AK, Handsaker RE, et al. Clonal hematopoiesis and blood-cancer risk inferred from blood DNA sequence. *N Engl J Med* 2014; 371:2477–2487.
6. Jaiswal S, Fontanillas P, Flannick J, et al. Age-related clonal hematopoiesis associated with adverse outcomes. *N Engl J Med* 2014; 371:2488–2498.
7. Xie M, Lu C, Wang J, et al. Age-related mutations associated with clonal hematopoietic expansion and malignancies. *Nat Med* 2014; 20:1472–1478.
8. Young AL, Challen GA, Birmann BM, Druley TE. Clonal haematopoiesis harbouring AML-associated mutations is ubiquitous in healthy adults. *Nat Commun* 2016; 7:12484.
9. Zink F, Stacey SN, Norddahl GL, et al. Clonal hematopoiesis, with and without candidate driver mutations, is common in the elderly. *Blood* 2017; 130:742–752.
10. Jaiswal S, Natarajan P, Silver AJ, et al. Clonal hematopoiesis and risk of atherosclerotic cardiovascular disease. *N Engl J Med* 2017; 13:111–121.
11. Fuster JJ, MacLauchlan S, Zuriaga MA, et al. Clonal hematopoiesis associated with *TET2* deficiency accelerates atherosclerosis development in mice. *Science* 2017; 355:842–847.
- Identifying a novel mechanism by which *TET2* mutations drive cardiovascular diseases during aging.

12. Coombs CC, Zehir A, Devlin SM, *et al.* Therapy-related clonal hematopoiesis in patients with nonhematologic cancers is common and associated with adverse clinical outcomes. *Cell Stem Cell* 2017; 21:374–382.e4. Comprehensive clinical reports on therapy-related CHIP.
13. Wong TN, Miller CA, Jotte MRM, *et al.* Cellular stressors contribute to the expansion of hematopoietic clones of varying leukemic potential. *Nat Commun* 2018; 9:455. Showing distinct effects of hematopoietic transplantation and chemotherapy on the expansion of hematopoietic cells with different mutations.
14. Levine AJ, Oren M. The first 30 years of p53: growing ever more complex. *Nat Rev Cancer* 2009; 9:749–758.
15. Brosh R, Rotter V. When mutants gain new powers: news from the mutant p53 field. *Nat Rev Cancer* 2009; 9:701–713.
16. Liu Y, Elf SE, Miyata Y, *et al.* p53 regulates hematopoietic stem cell quiescence. *Cell Stem Cell* 2009; 4:37–48.
17. Asai T, Liu Y, Di Giandomenico S, *et al.* Necdin, a p53 target gene, regulates the quiescence and response to genotoxic stress of hematopoietic stem/progenitor cells. *Blood* 2012; 120:1601–1612.
18. Kenzelmann Broz D, Spano Mello S, Bieging KT, *et al.* Global genomic profiling reveals an extensive p53-regulated autophagy program contributing to key p53 responses. *Genes Dev* 2013; 27:1016–1031.
19. Peller S, Rotter V. TP53 in hematological cancer: low incidence of mutations with significant clinical relevance. *Hum Mutat* 2003; 21:277–284.
20. Lindsley RC, Ebert BL. Molecular pathophysiology of myelodysplastic syndromes. *Annu Rev Pathol* 2013; 8:21–47.
21. Bejar R, Stevenson K, Abdel-Wahab O, *et al.* Clinical effect of point mutations in myelodysplastic syndromes. *N Engl J Med* 2011; 364:2496–2506.
22. Grossmann V, Schnittger S, Kohlmann A, *et al.* A novel hierarchical prognostic model of AML solely based on molecular mutations. *Blood* 2012; 120:2963–2972.
23. Rücker FG, Schlenk RF, Bullinger L, *et al.* TP53 alterations in acute myeloid leukemia with complex karyotype correlate with specific copy number alterations, monosomal karyotype, and dismal outcome. *Blood* 2012; 119:2114–2121.
24. Chen S, Gao R, Yao C, *et al.* Genotoxic stresses promotes the clonal expansion of hematopoietic stem cells expressing mutant p53. *Leukemia* 2018; 32:850–854. Showing the effects of genotoxic stresses, including chemotherapy and radiation, on clonal expansion of hematopoietic progenitor cells with TP53 mutations identified in CHIP.
25. Beerman I, Rossi DJ. Epigenetic control of stem cell potential during homeostasis, aging, and disease. *Cell Stem Cell* 2015; 16:613–625.
26. Sun D, Luo M, Jeong M, *et al.* Epigenomic profiling of young and aged HSCs reveals concerted changes during aging that reinforce self-renewal. *Cell Stem Cell* 2014; 14:673–688.
27. Zhu J, Sammons MA, Donahue G, *et al.* Gain-of-function p53 mutants co-opt chromatin pathways to drive cancer growth. *Nature* 2015; 525:206–211.
28. Bracken AP, Helin K. Polycomb group proteins: navigators of lineage pathways led astray in cancer. *Nat Rev Cancer* 2009; 9:773–784.
29. Mochizuki-Kashio M, Mishima Y, Miyagi S, *et al.* Dependency on the polycomb gene Ezh2 distinguishes fetal from adult hematopoietic stem cells. *Blood* 2012; 118:6553–6561.
30. Song H, Hollstein M, Xu Y. p53 gain-of-function cancer mutants induce genetic instability by inactivating ATM. *Nat Cell Biol* 2007; 15:376–388.
31. Cai Z, Kotzin JJ, Ramdas B, *et al.* Inhibition of inflammatory signaling in Tet2 mutant preleukemic cells mitigates stress-induced abnormalities and clonal hematopoiesis. *Cell Stem Cell* 2018; 23:833–849.e5. Identifying a novel mechanism by which TET2 mutations drive clonal hematopoiesis.
32. Wong TN, Ramsingh G, Young AL, *et al.* Role of TP53 mutations in the origin and evolution of therapy-related acute myeloid leukaemia. *Nature* 2015; 518:552–555.
33. Hsu JI, Dayaram T, Tovy A, *et al.* PPM1D mutations drive clonal hematopoiesis in response to cytotoxic chemotherapy. *Cell Stem Cell* 2018; 23:700–713.e6. Determining the role of PPM1D mutations in clonal hematopoiesis.
34. Kahn JD, Miller PG, Silver AJ, *et al.* PPM1D-truncating mutations confer resistance to chemotherapy and sensitivity to PPM1D inhibition in hematopoietic cells. *Blood* 2018; 132:1095–1105. Determining the role of PPM1D mutations in clonal hematopoiesis.
35. Fiscella M, Zhang H, Fan S, *et al.* Wip1, a novel human protein phosphatase that is induced in response to ionizing radiation in a p53-dependent manner. *Proc Natl Acad Sci U S A* 1997; 94:6048–6053.
36. Lu X, Nguyen TA, Moon SH, *et al.* The type 2C phosphatase Wip1: an oncogenic regulator of tumor suppressor and DNA damage response pathways. *Cancer Metastasis Rev* 2008; 27:123–135.
37. Nakai H, Misawa S, Toguchida J, *et al.* Frequent p53 gene mutations in blast crisis of chronic myelogenous leukemia, especially in myeloid crisis harboring loss of a chromosome 17p. *Cancer Res* 1992; 52:6588–6593.
38. Harutyunyan A, Klampfl T, Cazzola M, *et al.* p53 lesions in leukemic transformation. *N Engl J Med* 2011; 364:488–490.
39. Rampal R, Ahn J, Abdel-Wahab O, *et al.* Genomic and functional analysis of leukemic transformation of myeloproliferative neoplasms. *Proc Natl Acad Sci U S A* 2014; 111:E5401–E5410.
40. Talwalkar SS, Yin CC, Naeem RC, *et al.* Myelodysplastic syndromes arising in patients with germline TP53 mutation and Li–Fraumeni syndrome. *Arch Pathol Lab Med* 2010; 134:1010–1015.
41. Prokocimer M, Molchadsky A, Rotter V. Dysfunctional diversity of p53 proteins in adult acute myeloid leukemia: projections on diagnostic workup and therapy. *Blood* 2017; 130:699–712. Comprehensive review of the role of mutant p53 in pathogenesis and treatment of acute myeloid leukemia (AML).
42. Garg M, Nagata Y, Kanojia D, *et al.* Profiling of somatic mutations in acute myeloid leukemia with FLT3-ITD at diagnosis and relapse. *Blood* 2015; 126:2491–2501.
43. Zhang J, Kong G, Rajagopalan A, *et al.* p53^{-/-} synergizes with enhanced NrasG12D signaling to transform megakaryocyte-erythroid progenitors in acute myeloid leukemia. *Blood* 2017; 129:358–370. Showing the synergy between p53 loss and NRAS mutation in AML pathogenesis in mice.
44. Nabinger S, Chen S, Yao C, *et al.* Mutant p53 enhances leukemia-initiating cell self-renewal to promote leukemia development. *Leukemia* 2019. [Epub ahead of print] Showing the synergy between mutant p53 and FLT3 mutation in chronic myeloid leukemia pathogenesis in mice.
45. Menezes J, Salgado RN, Acquadro F, *et al.* ASXL1, TP53 and IKZF3 mutations are present in the chronic phase and blast crisis of chronic myeloid leukemia. *Blood Cancer J* 2013; 3:e157.
46. Sabapathy K, Lane DP. Therapeutic targeting of p53: all mutants are equal, but some mutants are more equal than others. *Nat Rev Clin Oncol* 2018; 15:13–30. Comprehensive review on therapeutic targeting of mutant p53 in human cancer.
47. Lindsley RC, Saber W, Mar BG, *et al.* Prognostic mutations in myelodysplastic syndrome after stem-cell transplantation. *N Engl J Med* 2017; 376:536–547. Showing that TP53 mutations are poor prognostic markers in MDS after stem-cell transplantation.
48. Yoshizato T, Nannya Y, Atsuta Y, *et al.* Genetic abnormalities in myelodysplasia and secondary acute myeloid leukemia: impact on outcome of stem cell transplantation. *Blood* 2017; 129:2347–2358.
49. Welch JS, Petti AA, Miller CA, *et al.* TP53 and decitabine in acute myeloid leukemia and myelodysplastic syndromes. *N Engl J Med* 2016; 375:2023–2036.



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

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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-2Rγc^{-/-}; 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'-CCTGGTTTCATCATCGCTAATC-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'-GCAGAGGCTATGAATATTA-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'-AAAACCCCTCTTGGAGTGGG-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

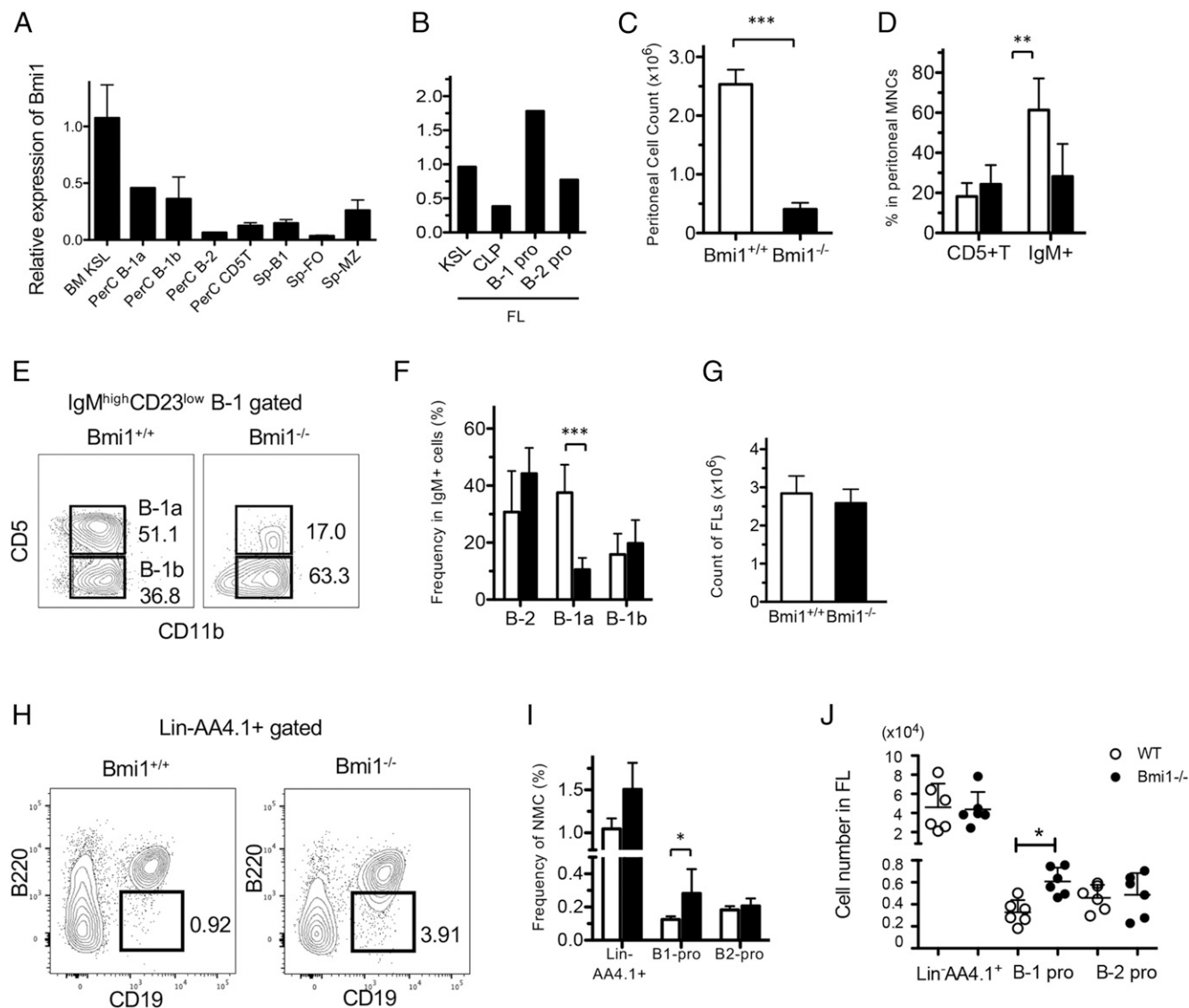


FIGURE 1. Peritoneal B-1a cells have higher *Bmi1* dependency than other lymphoid subsets. **(A)** *Bmi1* mRNA expression measured by qPCR in various sorted populations from adult BM, peritoneal cells (PerC), or spleen (Sp). **(B)** *Bmi1* expressions in various FL progenitor populations. **(C)** Marked 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. The percentage of B-1a cells is reduced ($n = 5$). **(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^{low}) 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).

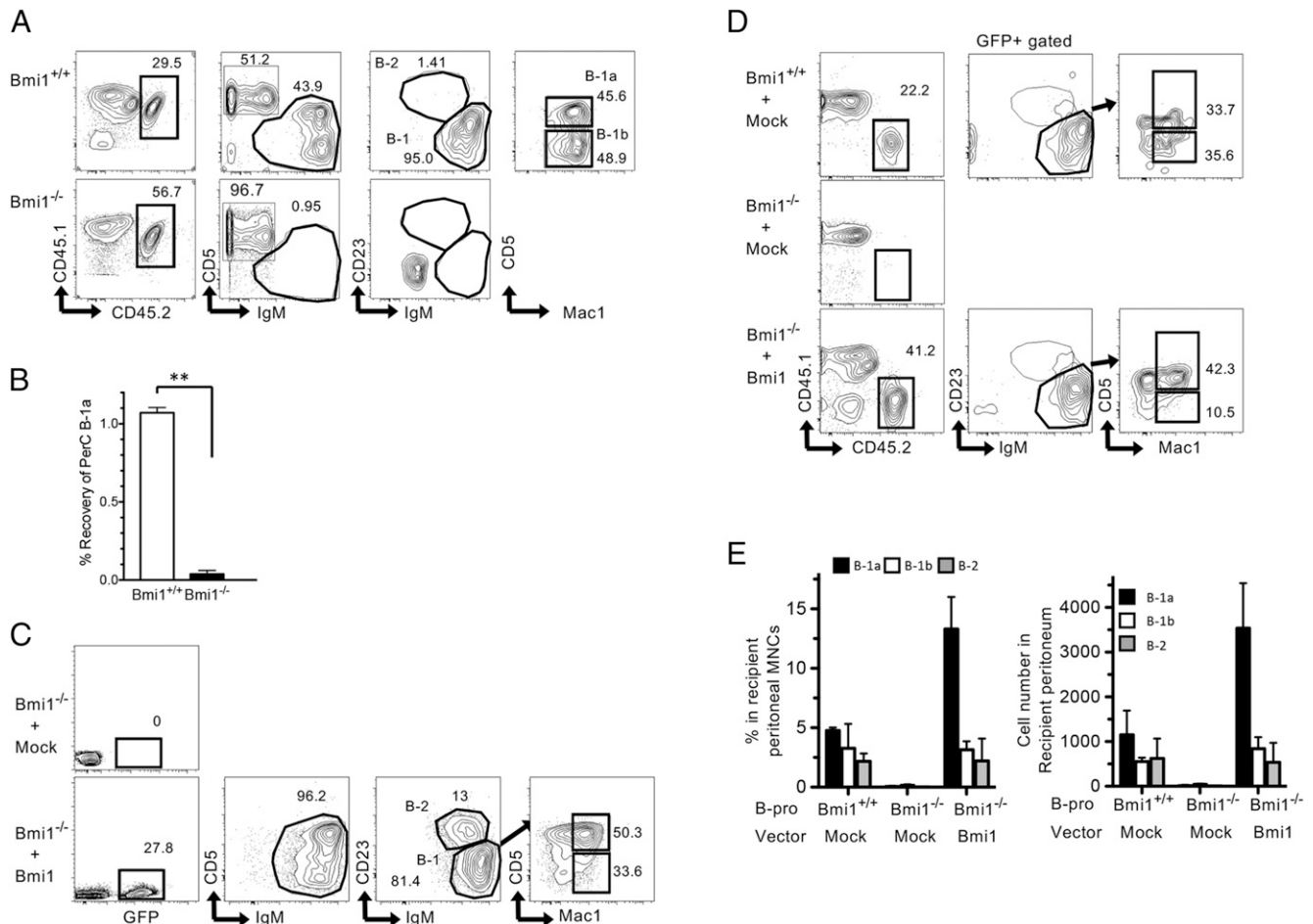


FIGURE 2. Loss of B-1a self-renewal by *Bmi1* disruption. **(A)** Representative FACS plots of recipient peritoneal cells at 12 wk posttransplant. The peritoneal cells of the recipient NSG mice transplanted with *Bmi1*^{+/+} (upper panel) or *Bmi1*^{-/-} (lower panel) peritoneal cells are depicted ($n = 3$). **(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

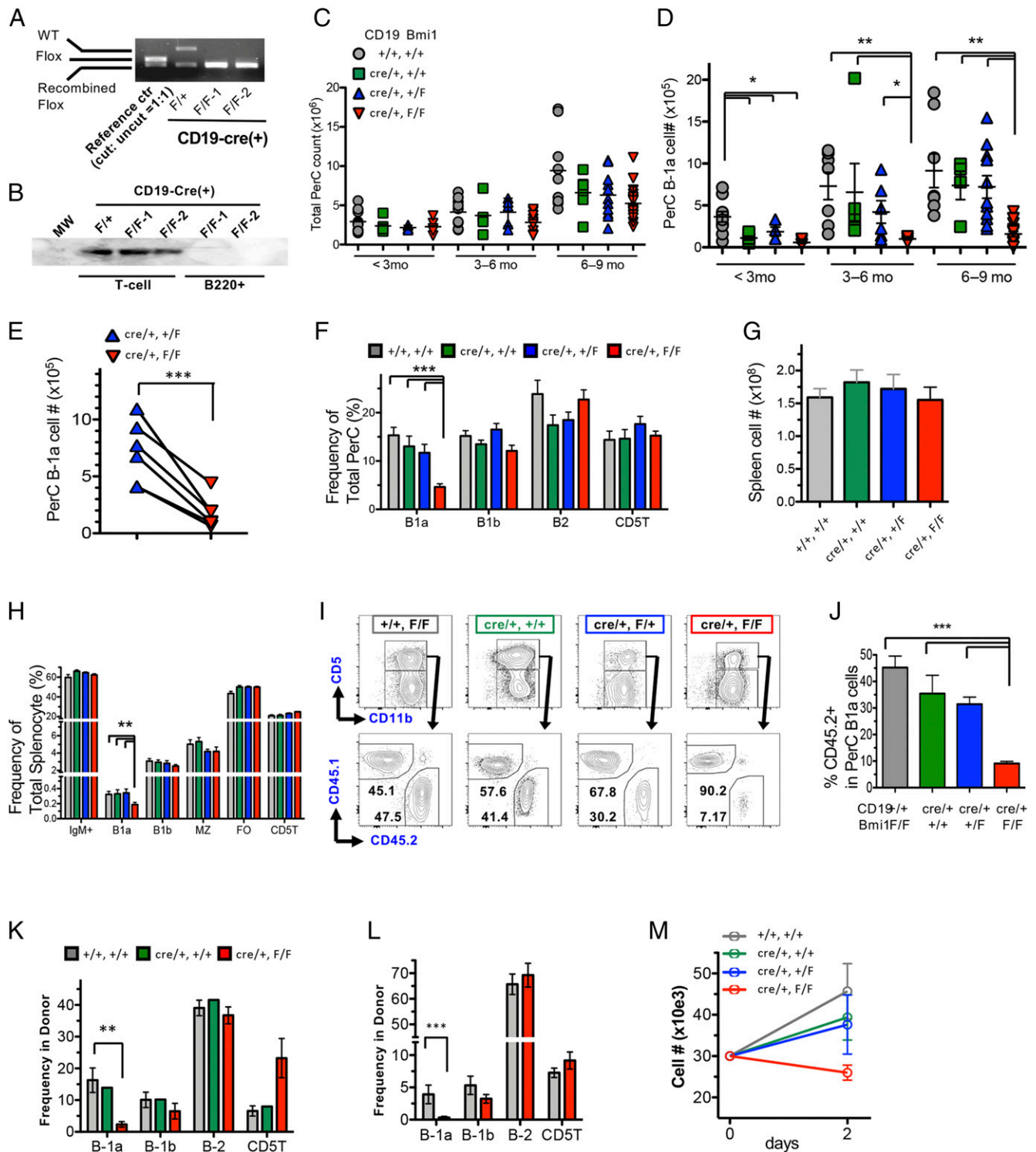


FIGURE 3. B cell-specific deletion of *Bmi1* leads to reduction in the B-1a cell pool and loss of self-renewal ability. The efficiency of CD19-Cre recombinase was confirmed by genomic PCR (**A**) in FACS-sorted B-1 cells or bead-selected spleen B cells (**B**). (**C**) Total peritoneal cell numbers in 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 (**J**) 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.

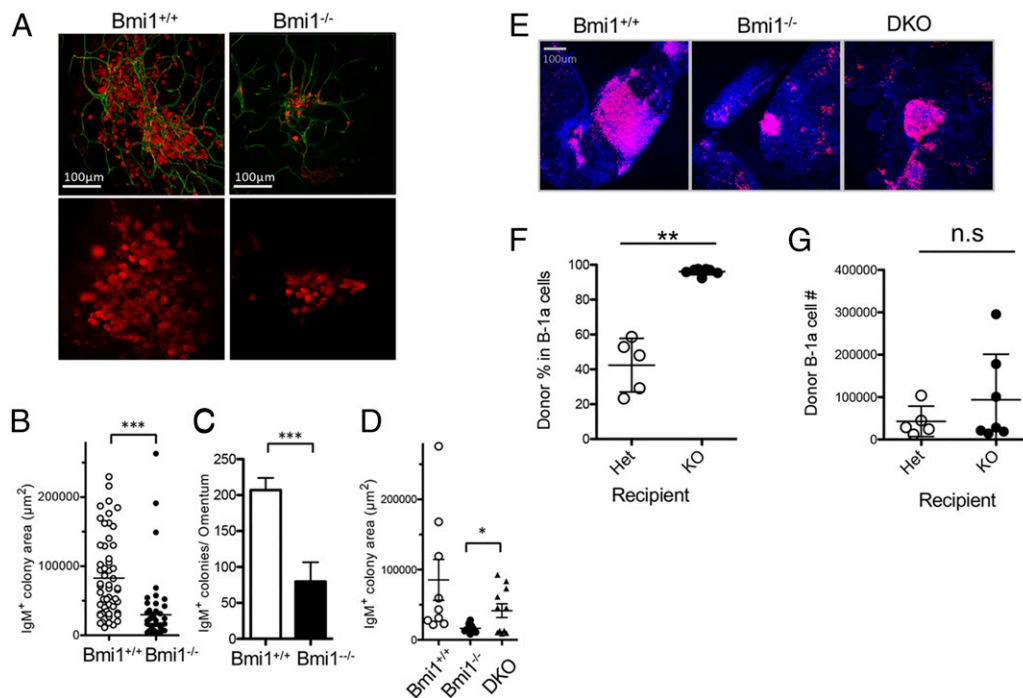


FIGURE 4. FALCs in *Bmi1*^{-/-} peritoneum and reciprocal transplantation assays. **(A)** Representative pictures of FALCs marked by IgM staining (red) in WT or *Bmi1*^{-/-} peritoneum. CD31⁺ endothelial cells are also stained (green). The size **(B)** and number **(C)** of FALCs in the omentum are depicted. **(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

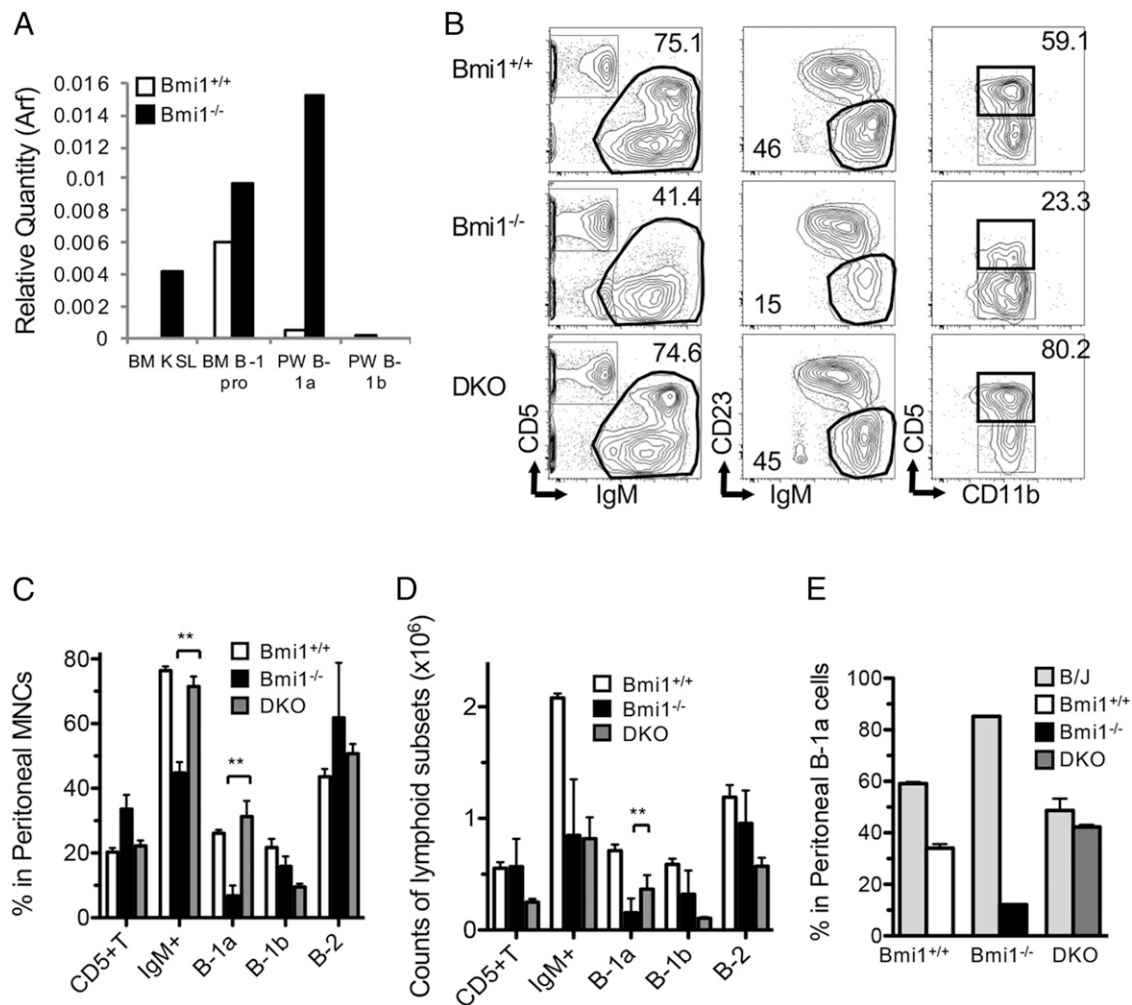


FIGURE 5. Arf-Ink4a is a major target for self-renewal of *Bmi1*^{-/-} B-1a cells. (A) Arf expression in WT and *Bmi1*^{-/-} B cells was measured by qPCR. (B) Representative FACS plots of peritoneal cells from WT, *Bmi1*^{-/-}, and *Bmi1*^{-/-} Arf-Ink4^{-/-} (DKO) mice are depicted. (C) Frequencies 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

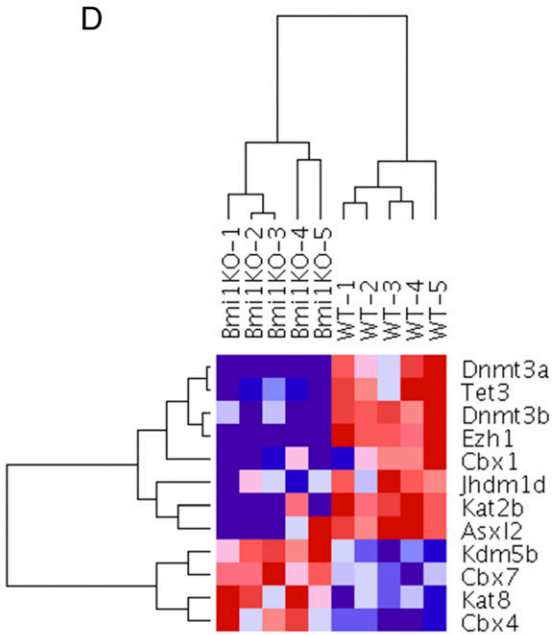


FIGURE 6. Gene expression profiling of *Bmi1*^{-/-} reveals dysregulation of epigenetic genes. B-1a cells were sorted from WT and *Bmi1*^{-/-} peritoneal cavity and subjected to microarray analysis. Affected canonical signaling pathways (**A**) and affected biofunctions (**B**) analyzed by Ingenuity 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

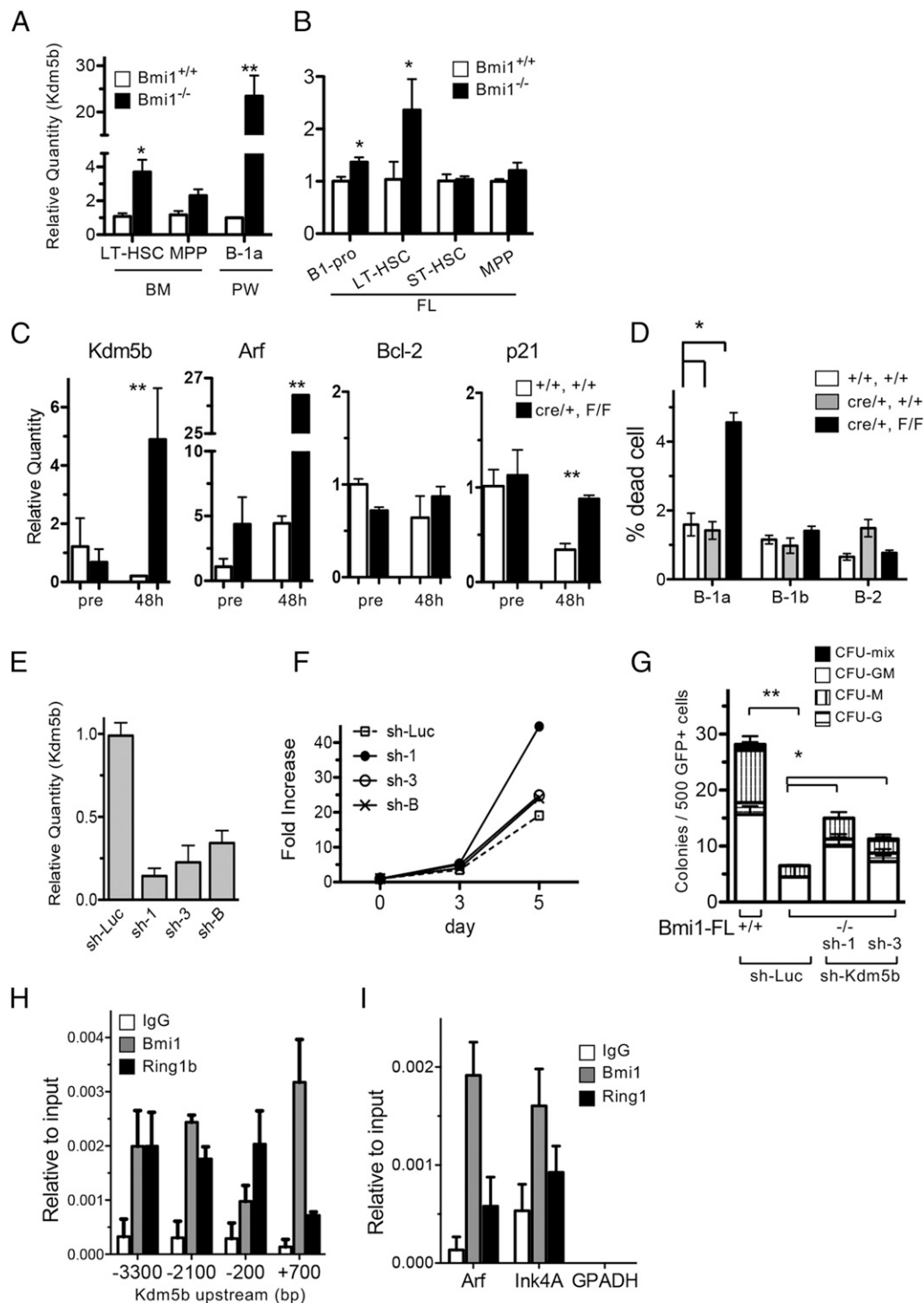


FIGURE 7. *Kdm5b* is a possible *Bmi1* target and crucial for posttransplant B-1a cell expansion. Expression of *Kdm5b* in various cell fractions from WT and *Bmi1*^{-/-} BM and peritoneum (A) or FL (B) (*n* = 3). **p* < 0.03, ***p* < 0.01. (C) Sorted B-1a cells from *CD19*^{Cre/+}*Bmi1*^{F/F} or control 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.

References

- Herzenberg, L. A., A. M. Stall, P. A. Lalor, C. Sidman, W. A. Moore, D. R. Parks, and L. A. Herzenberg. 1986. The Ly-1 B cell lineage. *Immunol. Rev.* 93: 81–102.
- Hardy, R. R., and K. Hayakawa. 1991. A developmental switch in B lymphopoiesis. *Proc. Natl. Acad. Sci. USA* 88: 11550–11554.
- Montecino-Rodriguez, E., H. Leathers, and K. Dorshkind. 2006. Identification of a B-1 B cell-specified progenitor. *Nat. Immunol.* 7: 293–301.
- Barber, C. L., E. Montecino-Rodriguez, and K. Dorshkind. 2011. Reduced production of B-1-specified common lymphoid progenitors results in diminished potential of adult marrow to generate B-1 cells. *Proc. Natl. Acad. Sci. USA* 108: 13700–13704.
- Ghosh, E. E., R. Yamamoto, S. Hamanaka, Y. Yang, L. A. Herzenberg, H. Nakauchi, and L. A. Herzenberg. 2012. Distinct B-cell lineage commitment distinguishes adult bone marrow hematopoietic stem cells. *Proc. Natl. Acad. Sci. USA* 109: 5394–5398.
- Ghosh, E. E., J. Waters, M. Phillips, R. Yamamoto, B. R. Long, Y. Yang, R. Gerstein, C. A. Stoddart, H. Nakauchi, and L. A. Herzenberg. 2016. Fetal hematopoietic stem cell transplantation fails to fully regenerate the B-lymphocyte compartment. *Stem Cell Reports* 6: 137–149.
- Kobayashi, M., W. C. Shelley, W. Seo, S. Vemula, Y. Lin, Y. Liu, R. Kapur, I. Taniuchi, and M. Yoshimoto. 2014. 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. USA* 111: 12151–12156.
- Yoshimoto, M., E. Montecino-Rodriguez, M. J. Ferkowicz, P. Porayette, W. C. Shelley, S. J. Conway, K. Dorshkind, and M. C. Yoder. 2011. Embryonic day 9 yolk sac and intra-embryonic hemogenic endothelium independently generate a B-1 and marginal zone progenitor lacking B-2 potential. *Proc. Natl. Acad. Sci. USA* 108: 1468–1473.
- Sawai, C. M., S. Babovic, S. Upadhyaya, D. J. H. F. Knapp, Y. Lavin, C. M. Lau, A. Goloborodko, J. Feng, J. Fujisaki, L. Ding, et al. 2016. Hematopoietic stem cells are the major source of multilineage hematopoiesis in adult animals. *Immunity* 45: 597–609.
- Molofsky, A. V., R. Pardo, T. Iwashita, I. K. Park, M. F. Clarke, and S. J. Morrison. 2003. Bmi-1 dependence distinguishes neural stem cell self-renewal from progenitor proliferation. *Nature* 425: 962–967.
- Park, I. K., D. Qian, M. Kiel, M. W. Becker, M. Pihlaja, I. L. Weissman, S. J. Morrison, and M. F. Clarke. 2003. Bmi-1 is required for maintenance of adult self-renewing hematopoietic stem cells. *Nature* 423: 302–305.
- Iwama, A., H. Oguro, M. Negishi, Y. Kato, Y. Morita, H. Tsukui, H. Ema, T. Kamijo, Y. Katoh-Fukui, H. Koseki, et al. 2004. Enhanced self-renewal of hematopoietic stem cells mediated by the polycomb gene product Bmi-1. *Immunity* 21: 843–851.
- Oguro, H., A. Iwama, Y. Morita, T. Kamijo, M. van Lohuizen, and H. Nakauchi. 2006. Differential impact of Ink4a and Arf on hematopoietic stem cells and their bone marrow microenvironment in Bmi1-deficient mice. *J. Exp. Med.* 203: 2247–2253.
- Di Foggia, V., X. Zhang, D. Licastro, M. F. Gerli, R. Phadke, F. Muntoni, P. Mourikis, S. Tajbakhsh, M. Ellis, L. C. Greaves, et al. 2014. Bmi1 enhances skeletal muscle regeneration through MT1-mediated oxidative stress protection in a mouse model of dystrophinopathy. *J. Exp. Med.* 211: 2617–2633.
- Yan, K. S., L. A. Chia, X. Li, A. Ootani, J. Su, J. Y. Lee, N. Su, Y. Luo, S. C. Heilshorn, M. R. Amieva, et al. 2012. The intestinal stem cell markers Bmi1 and Lgr5 identify two functionally distinct populations. *Proc. Natl. Acad. Sci. USA* 109: 466–471.
- Kim, A. R., J. L. Olsen, S. J. England, Y. S. Huang, K. H. Fegan, L. F. Delgadillo, K. E. McGrath, P. D. Kingsley, R. E. Waugh, and J. Palis. 2015. Bmi-1 regulates extensive erythroid self-renewal. *Stem Cell Reports* 4: 995–1003.
- Lin, Y., M. Kobayashi, N. Azevedo Portilho, A. Mishra, H. Gao, Y. Liu, P. Wenzel, B. Davis, M. C. Yoder, and M. Yoshimoto. 2019. Long-term engraftment of ESC-derived B-1 progenitor cells supports HSC-independent lymphopoiesis. *Stem Cell Reports* 12: 572–583.
- Bruggeman, S. W., M. E. Valk-Lingbeek, P. P. van der Stoop, J. J. Jacobs, K. Kieboom, E. Tanger, D. Hulsman, C. Leung, Y. Arsenijevic, S. Marino, and M. van Lohuizen. 2005. Ink4a and Arf differentially affect cell proliferation and neural stem cell self-renewal in Bmi1-deficient mice. *Genes Dev.* 19: 1438–1443.
- Gao, R., S. Chen, M. Kobayashi, H. Yu, Y. Zhang, Y. Wan, S. K. Young, A. Soltis, M. Yu, S. Vemula, et al. 2015. Bmi1 promotes erythroid development through regulating ribosome biogenesis. *Stem Cells* 33: 925–938.
- Rickert, R. C., J. Roes, and K. Rajewsky. 1997. B lymphocyte-specific, Cre-mediated mutagenesis in mice. *Nucleic Acids Res.* 25: 1317–1318.
- Rickert, R. C., K. Rajewsky, and J. Roes. 1995. Impairment of T-cell-dependent B-cell responses and B-1 cell development in CD19-deficient mice. *Nature* 376: 352–355.
- Sato, S., N. Ono, D. A. Steeber, D. S. Pisetsky, and T. F. Tedder. 1996. CD19 regulates B lymphocyte signaling thresholds critical for the development of B-1 lineage cells and autoimmunity. *J. Immunol.* 157: 4371–4378.
- Hu, T., A. Kitano, V. Luu, B. Dawson, K. A. Hoegenauer, B. H. Lee, and D. Nakada. 2019. Bmi1 suppresses adipogenesis in the hematopoietic stem cell niche. *Stem Cell Reports* 13: 545–558.
- Kato, Y., L. B. Hou, S. Miyagi, E. Nitta, K. Aoyama, D. Shinoda, S. Yamazaki, W. Kuribayashi, Y. Isshiki, S. Koide, et al. 2019. Bmi1 restricts the adipogenic differentiation of bone marrow stromal cells to maintain the integrity of the hematopoietic stem cell niche. *Exp. Hematol.* 76: 24–37.
- Bénézech, C., N. T. Luu, J. A. Walker, A. A. Kruglov, Y. Loo, K. Nakamura, Y. Zhang, S. Nayar, L. H. Jones, A. Flores-Langarica, et al. 2015. Inflammation-induced formation of fat-associated lymphoid clusters. *Nat. Immunol.* 16: 819–828.
- Moro, K., T. Yamada, M. Tanabe, T. Takeuchi, T. Ikawa, H. Kawamoto, J. Furusawa, M. Ohtani, H. Fujii, and S. Koyasu. 2010. Innate production of T(H)2 cytokines by adipose tissue-associated c-Kit(+)Sca-1(+) lymphoid cells. *Nature* 463: 540–544.
- Genestier, L., M. Taillardet, P. Mondiere, H. Gheit, C. Bella, and T. Defrance. 2007. TLR agonists selectively promote terminal plasma cell differentiation of B cell subsets specialized in thymus-independent responses. *J. Immunol.* 178: 7779–7786.
- Cellot, S., K. J. Hope, J. Chagraoui, M. Sauvageau, É. Deneault, T. MacRae, N. Mayotte, B. T. Wilhelm, J. R. Landry, S. B. Ting, et al. 2013. RNAi screen identifies Jarid1b as a major regulator of mouse HSC activity. *Blood* 122: 1545–1555.
- Kretschmer, K., J. Stopkiewicz, S. Scheffer, T. F. Greten, and S. Weiss. 2004. Maintenance of peritoneal B-1a lymphocytes in the absence of the spleen. *J. Immunol.* 173: 197–204.
- Pedersen, G. K., X. Li, S. Khoenkhon, M. Ádori, B. Beutler, and G. B. Karlsson Hedestam. 2018. B-1a cell development in splenectomized neonatal mice. *Front. Immunol.* 9: 1738.
- Wardemann, H., T. Boehm, N. Dear, and R. Carsetti. 2002. B-1a B cells that link the innate and adaptive immune responses are lacking in the absence of the spleen. *J. Exp. Med.* 195: 771–780.
- Kreslavsky, T., B. Vilagos, H. Tagoh, D. K. Poliakova, T. A. Schwickert, M. Wöhner, M. Jaritz, S. Weiss, R. Taneja, M. J. Rossner, and M. Busslinger. 2017. Essential role for the transcription factor Bhlhe41 in regulating the development, self-renewal and BCR repertoire of B-1a cells. *Nat. Immunol.* 18: 442–455.
- Clarke, A. J., T. Riffelmacher, D. Braas, R. J. Cornall, and A. K. Simon. 2018. B1a B cells require autophagy for metabolic homeostasis and self-renewal. *J. Exp. Med.* 215: 399–413.
- Kidder, B. L., G. Hu, Z. X. Yu, C. Liu, and K. Zhao. 2013. Extended self-renewal and accelerated reprogramming in the absence of Kdm5b. *Mol. Cell. Biol.* 33: 4793–4810.
- Wang, H., C. Song, Y. Ding, X. Pan, Z. Ge, B. H. Tan, C. Gowda, M. Sachdev, S. Muthusami, H. Ouyang, et al. 2016. Transcriptional regulation of JARID1B/KDM5B histone demethylase by Ikaros, histone deacetylase 1 (HDAC1), and casein kinase 2 (CK2) in B-cell acute lymphoblastic leukemia. *J. Biol. Chem.* 291: 4004–4018.
- Stewart, M. H., M. Albert, P. Sroczynska, V. A. Cruickshank, Y. Guo, D. J. Rossi, K. Helin, and T. Enver. 2015. The histone demethylase Jarid1b is required for hematopoietic stem cell self-renewal in mice. *Blood* 125: 2075–2078.
- Moins-Teisserenc, H., M. Busson, A. Herda, S. Apete, R. Peffault de Latour, M. Robin, A. Xhaard, A. Toubert, and G. Socié. 2013. CD19+CD5+ B cells and B1-like cells following allogeneic hematopoietic stem cell transplantation. *Biol. Blood Marrow Transplant.* 19: 988–991.

Bmi1 regulates Wnt signaling in hematopoietic stem and progenitor cells

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28 Polycomb group (PcG) proteins are epigenetic gene silencers that have been implicated in stem cell
29 maintenance and cancer development.¹ Bmi1, a key component of the Polycomb repressive complex 1
30 (PRC1), is essential for both hematopoietic stem cell (HSC) and leukemia stem cell (LSC) self-
31 renewal.²⁻⁴ We demonstrate that Bmi1 is a substrate of AKT and that AKT-mediated phosphorylation of
32 Bmi1 inhibits HSC self-renewal.⁵ In addition to HSC self-renewal, Bmi1 also plays key roles in multi-
33 lineage differentiation.⁶ We found that Bmi1 enhances erythroid differentiation through upregulating
34 ribosomal genes.⁷ While Bmi1 plays critical roles in hematopoiesis, its target genes in hematopoietic
35 stem and progenitor cells (HSPCs) are largely unknown.

36 Bmi1 is a potent negative regulator of the *Ink4a/Arf* locus, which encodes the cell cycle regulator
37 and tumor suppressor p16^{Ink4a} and p19^{Arf} proteins.⁸ In *Bmi1*^{-/-} bone marrow (BM) cells, there is an
38 upregulation of both p16^{Ink4a} and p19^{Arf}.⁹ However, we found that loss of both p16 and p19 only
39 partially rescues the self-renewal defects of *Bmi1*^{-/-} HSCs,⁹ suggesting that Bmi1 may regulate the
40 expression of other genes in HSPCs. To understand how Bmi1 regulates HSC self-renewal and
41 differentiation, we performed gene expression profiling assays (using microarray analysis and
42 quantitative RT-PCR analysis) to identify Bmi1 target genes in HSCs (CD48⁺CD150⁺Lin⁻Sca1⁺Kit⁺
43 cells) and MPPs (CD48⁺CD150⁻Lin⁻Sca1⁺Kit⁺ cells). Microarray analysis revealed that several genes in
44 the canonical Wnt signaling pathway are significantly elevated in both HSCs and MPPs from *Bmi1*^{-/-}
45 mice (Figure 1A-B), suggesting that Wnt signaling may be activated in the absence of Bmi1.

46 Wnt signaling pathways regulate cell-fate decisions at all stages of development in multiple
47 tissues.¹⁰ In the canonical Wnt pathway, Wnt ligand binds to its receptor Frizzled at the cell surface and
48 inhibits glycogen synthase kinase-3 β (GSK-3 β)-mediated phosphorylation and degradation of β -catenin.
49 Stabilized β -catenin then translocates to the nucleus where it binds to T cell factor (TCF)/lymphoid
50 enhancer factor (LEF) transcription factors and induces the expression of Wnt target genes, such as *Tcf*
51 and *Axin2*. In the absence of Wnt ligand, GSK-3 β phosphorylates β -catenin and targets it for
52 ubiquitination and degradation.¹⁰ Wnt signaling appears to regulate hematopoiesis in a dosage-

53 dependent fashion: Low levels of Wnt activation (2-fold) maintains a multipotent state and increases
54 reconstitution, whereas high levels of Wnt activation (more than 10-fold) results in impaired HSC self-
55 renewal and block of differentiation.¹¹ However, the regulators of Wnt signaling in HSPCs remain
56 elusive.

57 We first confirmed upregulation of several genes of the canonical Wnt pathway, including *Fzd4*,
58 *Fzd7*, *Fzd9*, *Tcf3*, and *Axin2*, in *Bmi1*^{-/-} HSCs compared to *Bmi1*^{+/+} HSCs using quantitative RT-PCR
59 analysis (Figure 1C). *Fzd4*, *Lgr4*, *Lef1*, *Tcf3*, and *Axin2* are significantly upregulated in *Bmi1*^{-/-} MPPs
60 compared to *Bmi1*^{+/+} MPPs (Figure 1D). These findings suggest that Bmi1 may be associated with
61 genes in the Wnt pathway, thereby repressing their expression in HSPCs. To determine if Bmi1 is
62 directly associated with genes in the Wnt pathway, we performed chromatin immunoprecipitation
63 (ChIP) assays using an antibody against Bmi1 or normal mouse IgG. As expected, Bmi1 was associated
64 with the *Ink4a/Arf* locus in both Kit⁺ BM cells and Baf3 cells (Figure 1E, *Online Supplementary Figure*
65 *S1A*). We detected the association of Bmi1 with the *Lef1*, *Fzd4*, and *Axin2* promoters in these cells
66 (Figure 1E, *Online Supplementary Figure S1A*). Ring1b is the catalytic component of the PRC1
67 complex and genome-wide ChIP-seq analysis showed Ring1b binding to several Wnt target genes,
68 including *Ccnd2*, *Fzd7*, *Ckn2a* and *Tcf3*, in murine L8057 megakaryoblastic cells (*Online*
69 *Supplementary Figure S1B*).¹² Thus, we demonstrate that Bmi1/PRC1 is associated with Wnt genes in
70 hematopoietic cells.

71 To determine the impact of Bmi1 expression on Wnt activation in cells, we used the Top-Flash
72 Wnt reporter system. The Top-Flash reporter system is designed to monitor the activity of Wnt signal
73 transduction pathway in cultured cells. While Wnt3a readily activates the Wnt reporter in 293T cells,
74 Wnt activation is efficiently repressed by ectopic Bmi1 expression, demonstrating that Bmi1 indeed can
75 inhibit Wnt signaling activation in cultured cells (Figure 1F). To determine whether Bmi1 represses the
76 expression of Wnt signaling genes in HSPCs, we introduced Bmi1 (MSCV-Bmi1-IRES-GFP) or GFP
77 (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 (Online Supplementary Figure S2A). 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 (Online Supplementary Figure S2B). In addition, ectopic *Bmi1* expression represses *Fzd4*, *Fzd7*, and *Fzd9* expression in *Bmi1*^{-/-} HSCs compared to that of the control viruses transduced cells (Online Supplementary Figure S2C). Further, ectopic *Bmi1* expression leads to downregulation of *Fzd4*, *Fzd9*, and *Lef1* in *Bmi1*^{-/-} MPPs (Online Supplementary Figure S2D). Thus, we demonstrate that *Bmi1* represses Wnt gene expression in HSPCs.

Next, we determined the impact of Wnt activation on *Bmi1*^{-/-} HSPCs *in vitro* and *in vivo*. The *Ctnnb1* gene encodes β -catenin and loss of β -catenin reduces Wnt activation.¹³ 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 (Figure 2A). 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 (Figure 2A). 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 (Figures 2B, Online Supplementary Figure S3A). Loss of *Bmi1* resulted in decreased myeloid

103 differentiation and increased lymphoid differentiation in the BM, whereas *Ctnnb1* deficiency rescued
104 differentiation defects seen in *Bmi1*^{-/-} mice (Figure 2C). To determine the impact of genetic deletion of
105 *Ctnnb1* on *Bmi1*^{-/-} HSC self-renewal, we performed secondary BM transplantation assays. We found that
106 *Bmi1*^{-/-}*Ctnnb1*^{-/-} BM cells show increased engraftment compared to *Bmi1*^{-/-} cells following secondary
107 transplantation (Figure 2D). Thus, we demonstrate that loss of β -catenin, which reduces Wnt activation,
108 partially rescues HSC self-renewal and differentiation defects seen in *Bmi1*^{-/-} mice.

109 APC is involved in the regulation of the Wnt signaling pathway via its ability to control the
110 degradation of β -catenin.¹⁰ The *APC* gene is located at chromosome band 5q23, and loss of a single
111 allele of *APC* occurs in more than 95% of patients with myeloid neoplasms and -5/del(5q).¹⁴ We found
112 that loss of one allele of *Apc*, which enhances Wnt activation, impairs HSC self-renewal and survival in
113 mice.¹⁴ As we found that Bmi1 represses Wnt genes in HSPCs, we predicted that Bmi1 overexpression
114 might counteract the negative impact of *Apc* loss on hematopoiesis. We have generated a mouse line
115 (*R26Stop^FBmi1*), where Bmi1 can be conditionally overexpressed under the control of the endogenous
116 Rosa26 promoter.¹⁵ We named the mice *Bmi1-Tg*. We crossed *Bmi1-Tg* mice with *Apc*^{F/+}-*Mx1Cre*⁺
117 mice and generated *Bmi1-Tg-Apc*^{F/+}-*Mx1Cre*⁺ mice. Three weeks after pI:pC treatment, we found that
118 the peripheral blood counts, including WBC, RBC, Hb, MCV and HCT, are comparable in *Apc*^{F/+}-
119 *Mx1Cre*⁻, *Bmi1-Tg-Mx1Cre*⁺, *Apc*^{F/+}-*Mx1Cre*⁺ and *Bmi1-Tg-Apc*^{F/+}-*Mx1Cre*⁺ mice (*Online*
120 *Supplementary Figure S3B*). We observed similar number of LT-HSCs, ST-HSCs, MPPs, CMPs,
121 GMPs, MEPs and LSKs in the BM of four group of mice (*Supplementary Figures S4a and S4b*).
122 Further, overexpression of Bmi1 and/or loss of one allele of *Apc* did not affect terminal differentiation in
123 BM at three weeks following pI:pC treatment (*Online Supplementary Figure S4C*). We then performed
124 colony formation assays using BM cells from these mice. While loss of *Apc* decreased colony
125 formation, overexpression of Bmi1 increased the colony formation of *Apc*^{+/-} BM cells (Figure 2E). To
126 determine the impact of Bmi1 on *Apc*-deficient HSPCs *in vivo*, we performed competitive BM
127 transplantation assays. While *Apc*^{+/-} BM cells show decreased engraftment as reported,¹⁴ overexpression

128 of Bmi1 significantly increased the engraftment of *Apc*^{+/-} BM cells (Figure 2F). Thus, we demonstrate
129 that activation of Wnt signaling contributes to impaired HSC self-renewal and terminal differentiation
130 seen in *Bmi1*^{-/-} mice.

131 In summary, we discovered that several genes of the Wnt signaling pathway are upregulated in
132 *Bmi1*^{-/-} HSCs and MPPs. Bmi1 is associated with several genes in the Wnt pathway and represses their
133 transcription in HSPCs. We found that loss of β -catenin partially rescues the HSC self-renewal and
134 differentiation defects seen in the *Bmi1*^{-/-} mice. Further, we discovered that overexpression Bmi1
135 enhances both the colony formation and repopulating potential of *Apc*^{+/-} BM cells. Thus, we have
136 identified Bmi1 as a novel regulator of canonical Wnt signaling pathway in hematopoietic stem and
137 progenitor cells. Our studies provide additional evidence that modulating canonical Wnt signaling holds
138 potential for enhancing HSC self-renewal, thereby improving the outcomes of HSC transplantation.

139

140 **CONFLICT OF INTEREST**

141 The authors declared that no conflict interest exists.

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151

152 **AUTHOR CONTRIBUTIONS**

153 SC, HY, RG, RX, and YL. Designed the research. SC, HY, RG, XL, QW, SV, ACF, DH, WC, CB, MK,
154 and SZL. Performed the research; SC, HY, RG, QW, and YL. Analyzed the data and performed the
155 statistical analysis. AI, HK, ZQ, RK, HEB, and ZG. Provided reagents and constructive advice to the
156 study. SC, RX and YL. Wrote the manuscript. All authors read, commented on, and approved the
157 manuscript.

158

159 **REFERENCES**

- 160 1. Bracken AP, and Helin K. Polycomb Group Proteins: Navigators of Lineage Pathways Led Astray in
161 Cancer. Nat Rev Cancer. 2009; 9: 773-84.
- 162 2. Konuma T, Oguro H, Iwama A. Role of the polycomb group proteins in hematopoietic stem cells.
163 Dev Growth Differ. 2010; 52:505-16.
- 164 3. Park IK, Qian D, Kiel M, Becker MW, Pihalja M, Weissman IL, Morrison SJ, Clarke MF. Bmi-1 is
165 required for maintenance of adult self-renewing haematopoietic stem cells. Nature. 2003; 423:302-5.
- 166 4. Lessard J, Sauvageau G. Bmi-1 determines the proliferative capacity of normal and leukaemic stem
167 cells. Nature. 2003;423:255-60.
- 168 5. Liu Y, Liu F, Yu H, Zhao X, Sahsida G, Deblasio A, Chen Z, Lin HK, Di Giandomenico S, Elf SE,
169 Yang YY, Miyata Y, Huang G, Menendez S, Mellinshoff I, Pandolfi PP, Hedvat CV and Nimer SD.
170 Akt Phosphorylates the Transcriptional Repressor Bmi1 to Block Its Association with Tumor
171 Suppressing *Ink4a-Arf* locus. Science Signaling. 2012; 5: ra77.
- 172 6. Oguro H, Yuan J, Ichikawa H, Ikawa T, Yamazaki S, Kawamoto H, Nakauchi H, Iwama A. Poised
173 lineage specification in multipotential hematopoietic stem and progenitor cells by the polycomb
174 protein Bmi1. Cell Stem Cell. 2010;6:279-86.
- 175 7. Gao R, Chen S, Kobayashi M, Yu H, Young SK, Soltis A, Zhang Y, Wan Y, Vemula S, Fraenkel E,
176 Cantor A, Xu Y, Yoder MC, Wek R, Ellis S, Kapur R, Zhu X and Liu Y. Bmi1 promotes erythroid
177 development through regulating ribosome biogenesis. Stem Cells. 2015; 33:925-38.
- 178 8. Bracken AP, Kleins-Kohlbrecher D, Dietrich N, Pasini D, Gargiulo G, Beekman C, Theilgaard-
179 Mönch K, Minucci S, Porse BT, Marine JC, Hansen KH, Helin K. The Polycomb group proteins
180 bind throughout the INK4A-ARF locus and are disassociated in senescent cells. Genes Dev.
181 2007;21:525-30.

- 182 9. Oguro H, Iwama A, Morita Y, Kamijo T, van Lohuizen M, Nakauchi H. Differential impact of Ink4a
183 and Arf on hematopoietic stem cells and their bone marrow microenvironment in Bmi1-deficient
184 mice. *J Exp Med*. 2006; 203:2247-53.
- 185 10. Staal FJ, Chhatta A, Mikkers H. Caught in a Wnt storm: Complexities of Wnt signaling in
186 hematopoiesis. *Exp Hematol*. 2016; 44: 451-7.
- 187 11. Luis TC, Naber BA, Roozen PP, Brugman MH, de Haas EF, Ghazvini M, Fibbe WE, van Dongen
188 JJ, Fodde R, Staal FJ. Canonical wnt signaling regulates hematopoiesis in a dosage-dependent
189 fashion. *Cell Stem Cell*. 2011; 9:345-56.
- 190 12. Yu M, Mazor T, Huang H, Huang HT, Kathrein KL, Woo AJ, Chouinard CR, Labadorf A, Akie TE,
191 Moran TB, Xie H, Zacharek S, Taniuchi I, Roeder RG, Kim CF, Zon LI, Fraenkel E, Cantor AB.
192 Direct recruitment of polycomb repressive complex 1 to chromatin by corebinding transcription fact
193 ors. *Mol Cell*. 2012;45:330-43.
- 194 13. Jeannet G, Scheller M, Scarpellino L, Duboux S, Gardiol N, Back J, Kuttler F, Malanchi I,
195 Birchmeier W, Leutz A, Huelsken J, Held W. Long-term, multilineage hematopoiesis occurs in the
196 combined absence of beta-catenin and gamma-catenin. *Blood*. 2008; 111:142-9.
- 197 14. Wang J, Fernald AA, Anastasi J, Le Beau MM, Qian Z. Haploinsufficiency of Apc leads to
198 ineffective hematopoiesis. *Blood*. 2010;115:3481-8.
- 199 15. Nakamura S, Oshima M, Yuan J, Saraya A, Miyagi S, Konuma T, Yamazaki S, Osawa M, Nakauchi
200 H, Koseki H, Iwama A. Bmi1 confers resistance to oxidative stress on hematopoietic stem cells.
201 *PLoS One*. 2012;7:e36209.

202

203

204 **FIGURE LEGENDS**

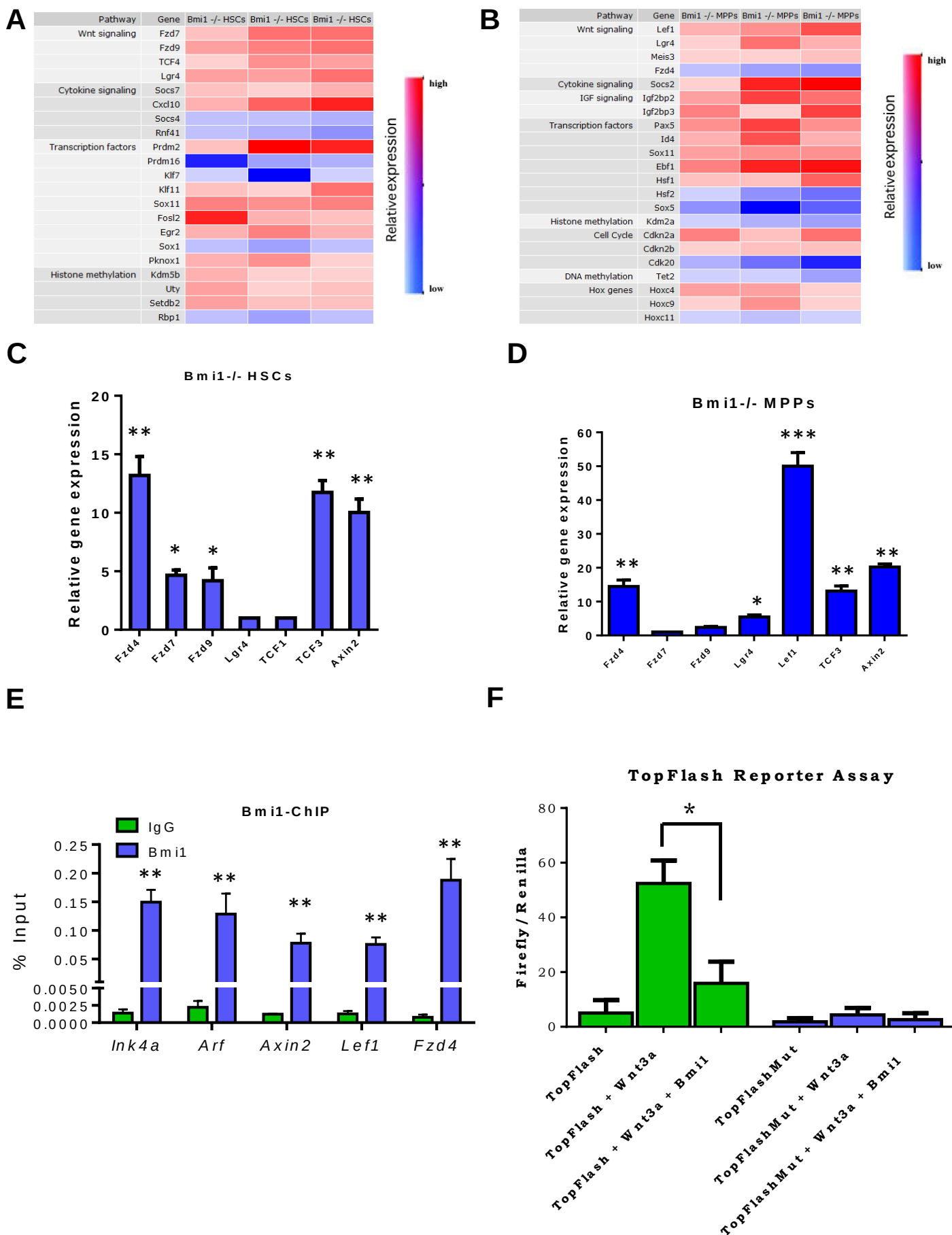
205
206 **Figure 1. *Bmi1* regulates Wnt gene expression in hematopoietic stem and progenitor cells.** (A)
207 Transcript profiling of HSCs (CD48⁻CD150⁺LSKs) from *Bmi1*^{+/+} and *Bmi1*^{-/-} mice were analyzed by
208 Agilent Whole Mouse Genome Oligo Microarrays. Genes that are differentially expressed in *Bmi1*^{-/-}
209 HSCs compared to wild-type cells are shown. We utilized Ingenuity pathways Analysis (Ingenuity
210 Systems) to group genes into specific canonical pathways. Values are shown for three biological
211 replicates. Color red indicates genes that are upregulated in *Bmi1*^{-/-} HSCs and color blue indicates genes
212 that are downregulated in *Bmi1*^{-/-} HSCs. (B) Transcript profiling of MPPs (CD48⁺CD150⁻LSKs) from
213 *Bmi1*^{+/+} and *Bmi1*^{-/-} mice were analyzed by Agilent Whole Mouse Genome Oligo Microarrays. Genes
214 that are differentially expressed in *Bmi1*^{-/-} MPPs compared to wild-type cells are shown. We utilized
215 Ingenuity pathways Analysis (Ingenuity Systems) to group genes into specific canonical pathways.
216 Values are shown for three biological replicates. Color red indicates genes that are upregulated in *Bmi1*^{-/-}
217 MPPs and color blue indicates genes that are downregulated in *Bmi1*^{-/-} MPPs. (C) Real-time RT-PCR
218 analysis of gene expression in *Bmi1*^{+/+} and *Bmi1*^{-/-} HSCs. Data shown are relative expression as
219 compared to *Bmi1*^{+/+} HSCs (set as 1), n = three biological replicates, *p<0.05, **p<0.01. (D) Real-time
220 RT-PCR analysis of gene expression in *Bmi1*^{+/+} and *Bmi1*^{-/-} MPPs. Data shown are relative expression as
221 compared to *Bmi1*^{+/+} MPPs (set as 1), n = three biological replicates, *p<0.05, **p<0.01, ***p<0.001.
222 (E) *Bmi1* binds to promoters of genes of the Wnt pathway *in vivo*. Chromatin bound DNA from Kit⁺
223 BM cells was immunoprecipitated with a *Bmi1*-specific antibody or with normal mouse IgG. qRT-PCR
224 amplification was performed on corresponding templates using primers for indicated genes, n = three
225 biological replicates, **p<0.01. (F) *Bmi1* represses Wnt3a-induced Wnt reporter activation. Luciferase
226 activity was assayed 24 hours after transfection of 293T cells. Values are means (±SEM), n = three
227 biological replicates, *p<0.05.

228

229 **Figure 2. Activation of Wnt signaling impairs HSC self-renewal and differentiation in *Bmi1*^{-/-}**
230 **mice.** (A) Percentage of donor-derived cells (CD45.2⁺) in the peripheral blood of recipient mice

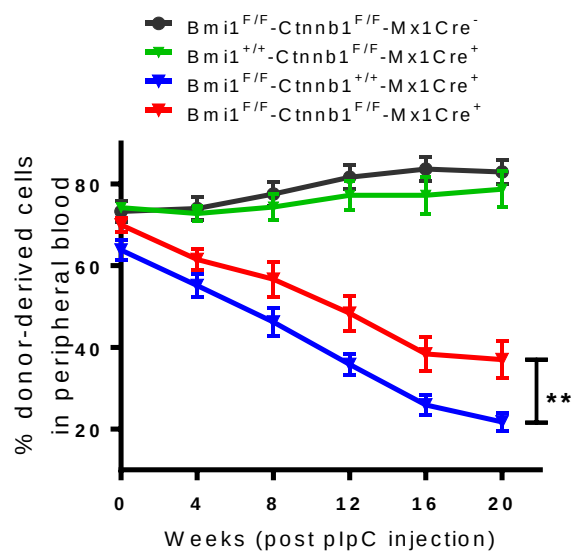
231 following pI:pC treatment. n=7 mice per group, **p<0.01. (B) The frequency of donor-derived HSCs in
232 the BM of recipient mice at 20 weeks following pI:pC treatment. n=4 mice per group, *p<0.05. (C)
233 Lineage distribution of donor-derived cells in the bone marrow of primary recipient mice at 20 weeks
234 following pI:pC treatment. n=4 mice per group, **p<0.01, ***p<0.001. (D) Percentage of donor-
235 derived cells (CD45.2⁺) in the peripheral blood of recipient mice at 8 weeks following secondary
236 transplantation. n=7-8 mice per group, ***p<0.001. (E) Colony formation assays using BM cells from
237 *Apc^{F/+}-Mx1Cre⁻*, *Bmi1-Tg-Mx1Cre⁺*, *Apc^{F/+}-Mx1Cre⁺* and *Bmi1-Tg-Apc^{F/+}-Mx1Cre⁺* mice treated with
238 pI:pC. n=3 independent experiment performed in duplicate, *p<0.05, **p<0.01. (F) Percentage of
239 donor-derived cells (CD45.2⁺) in the peripheral blood of recipient mice at 8 weeks following
240 transplantation. n=5-6 mice per group, *p<0.05, **p<0.01.

Figure 1



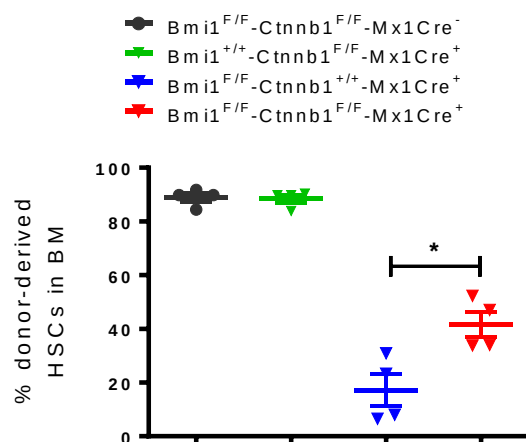
A

Primary BM transplantation



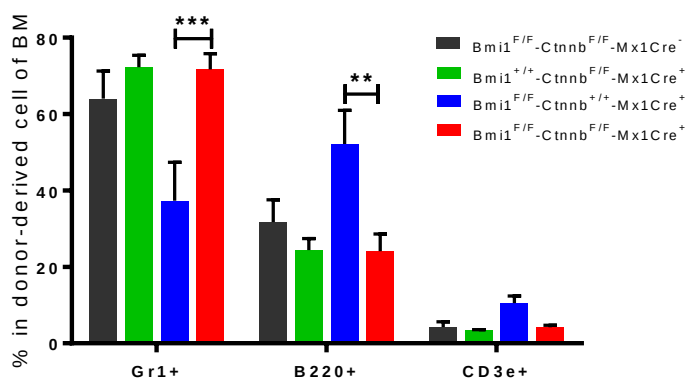
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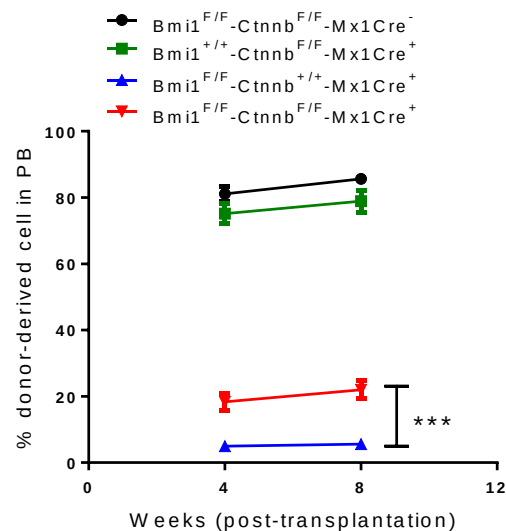


C

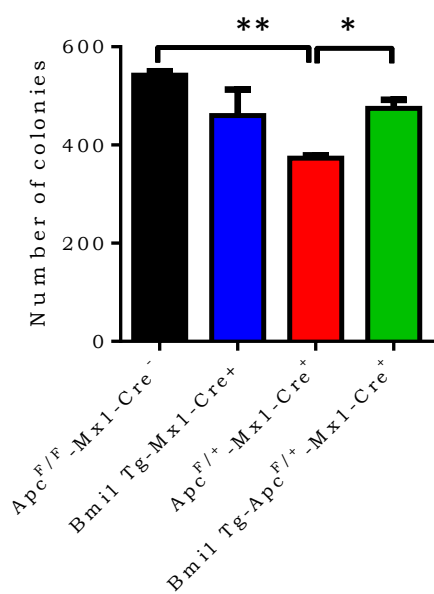
Multilineage differentiation in BM at 20 week after plpC



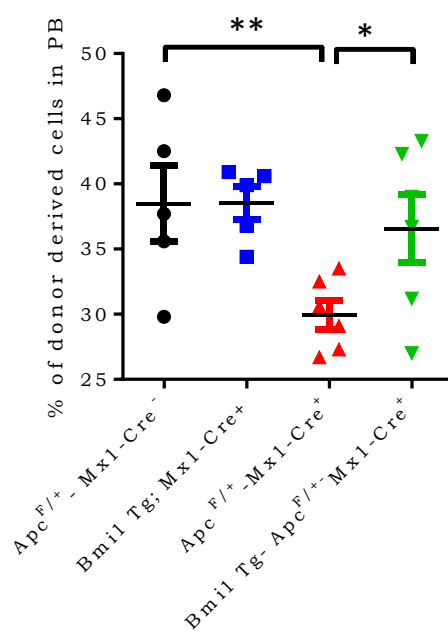
D



E



F



Bmi1 regulates Wnt signaling in hematopoietic stem and progenitor cells

Supplemental Methods

Mice

Global Bmi1 knockout mice were provided by Dr. Martin van Lohuizen at the Netherlands Cancer Institute, the Netherlands. Conditional Bmi1 knockout mice (*Bmi1^{F/F}*) in the *C57BL6* background were generated at the National Institute of Biological Science, Beijing, China. *R26Stop^FBmi1* (*Bmi1-Tg*) mice were generated at the RIKEN Research Center for Allergy and Immunology, Yokohama, Japan. The *Ctnnb1^{F/F}* and *Apc^{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 (Liu et al., *Cell Stem Cell*, 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

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⁺).

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. 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.

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.

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).

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.

Statistical Analysis

Statistical analysis was performed with GraphPad Prism 8 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) 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, * $p < 0.05$, ** $p < 0.01$. (B) 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.

Figure S2. (A) 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, * $p < 0.05$, ** $p < 0.01$. (B) 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, * $p < 0.05$, ** $p < 0.01$. (C) 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, * $p < 0.05$, ** $p < 0.01$. (D) 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, * $p < 0.05$, *** $p < 0.001$.

Figure S3. (A) 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, ** $p < 0.01$. (B) Peripheral blood of *Apc*^{F/+}-*Mx1Cre*⁻, *Bmi1-Tg-Mx1Cre*⁺, *Apc*^{F/+}-*Mx1Cre*⁺ and *Bmi1-Tg-Apc*^{F/+}-*Mx1Cre*⁺ mice at three weeks after pI:pC treatment were collected and analyzed. $n = 5-6$ mice per group.

Figure S4. (A) The frequency of long-term (LT)-HSCs and short-term (ST) HSCs in the bone marrow of *Apc^{F/+}-Mx1-Cre⁻*, *Bmi1-Tg-Mx1-Cre⁺*, *Apc^{F/+}-Mx1-Cre⁺* and *Bmi1-Tg-Apc^{F/+}-Mx1-Cre⁺* mice three weeks after pI:pC treatment. n=5 mice per group. (B) The frequency of multipotent progenitors (MPPs), common myeloid progenitors (CMPs), megakaryocyte and erythroid progenitors (MEPs), granulocyte and macrophage progenitors (GMPs), Lin⁻Sca1⁺Kit⁺ (LSKs) and Lin⁻Kit⁺ (LKs) in the bone marrow of *Apc^{F/+}-Mx1-Cre⁻*, *Bmi1-Tg-Mx1-Cre⁺*, *Apc^{F/+}-Mx1-Cre⁺* and *Bmi1-Tg-Apc^{F/+}-Mx1-Cre⁺* mice three weeks after pI:pC treatment. n=5 mice per group. (C) The frequency of B220⁺, CD3⁺, Mac1⁺Gr1⁺ and Ter119⁺ cells in the bone marrow of *Apc^{F/+}-Mx1-Cre⁻*, *Bmi1-Tg-Mx1-Cre⁺*, *Apc^{F/+}-Mx1-Cre⁺* and *Bmi1-Tg-Apc^{F/+}-Mx1-Cre⁺* mice three weeks after pI:pC treatment. n=5 mice per group.

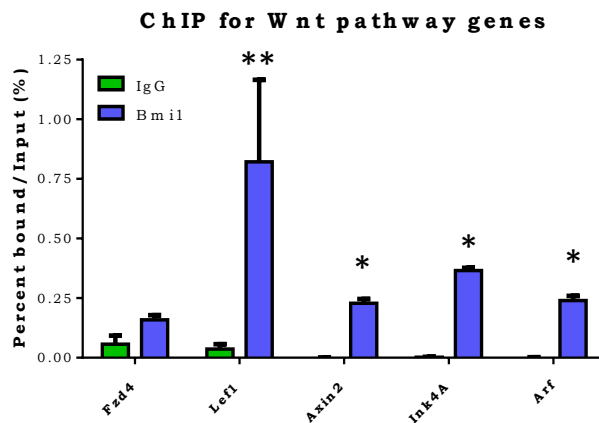
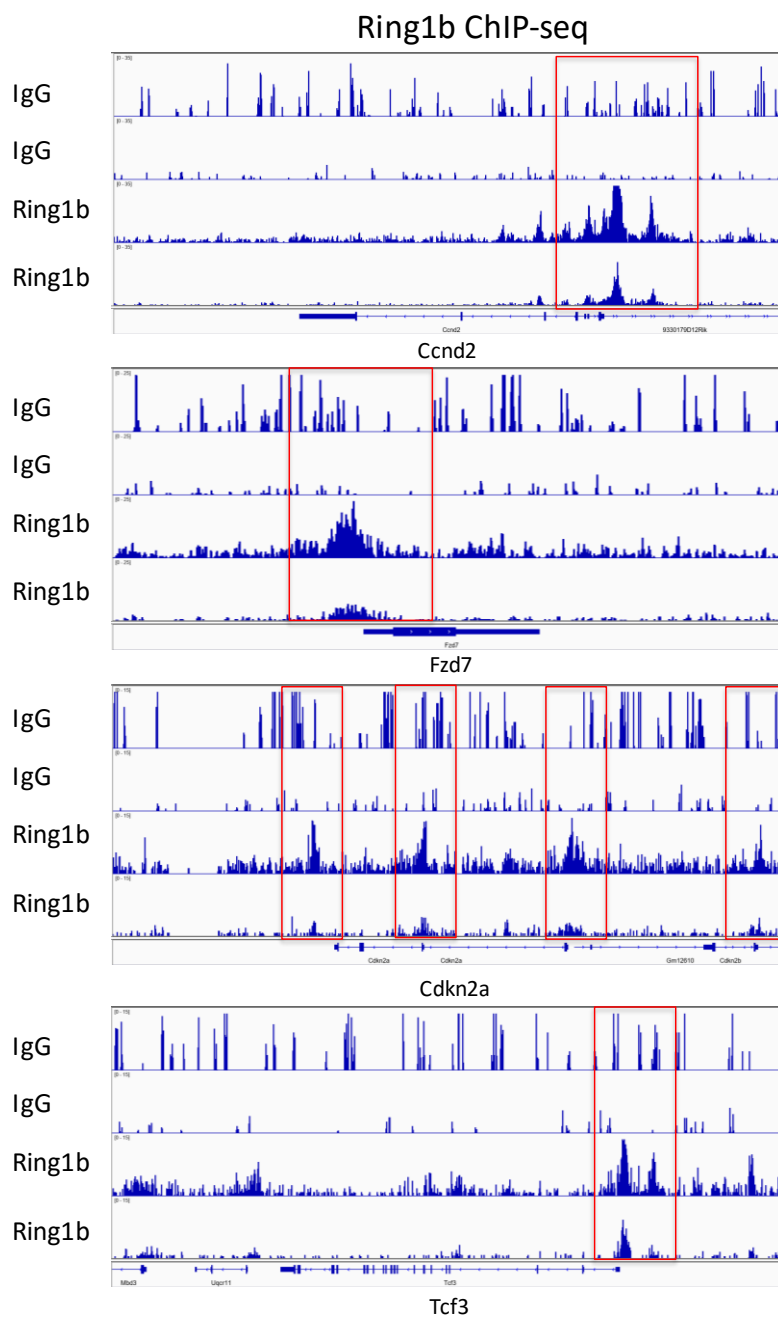
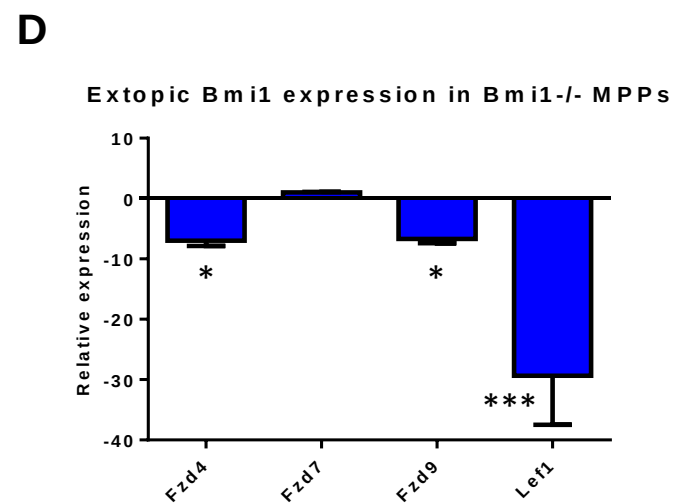
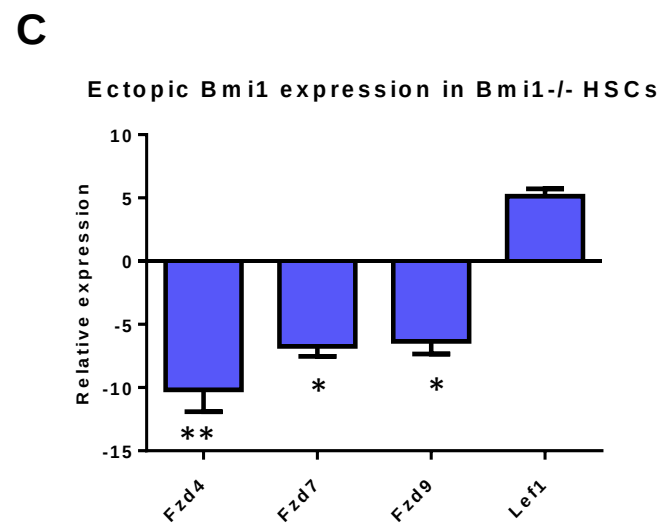
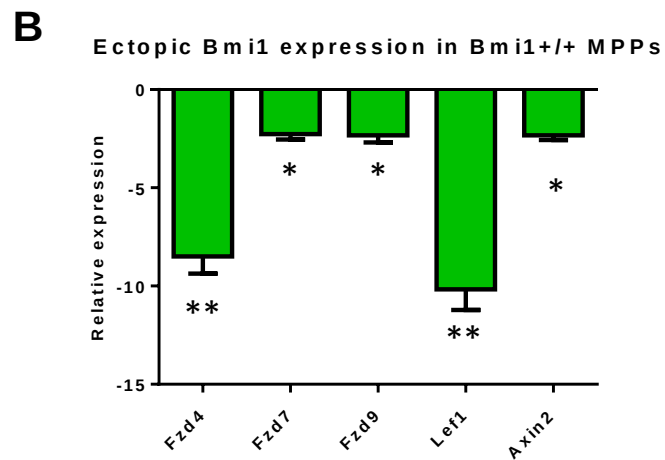
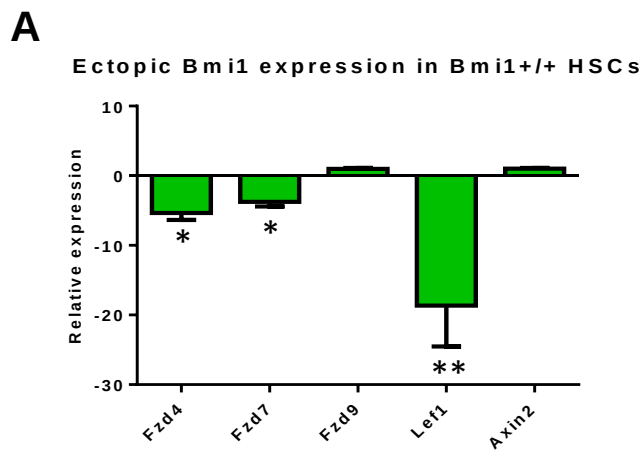
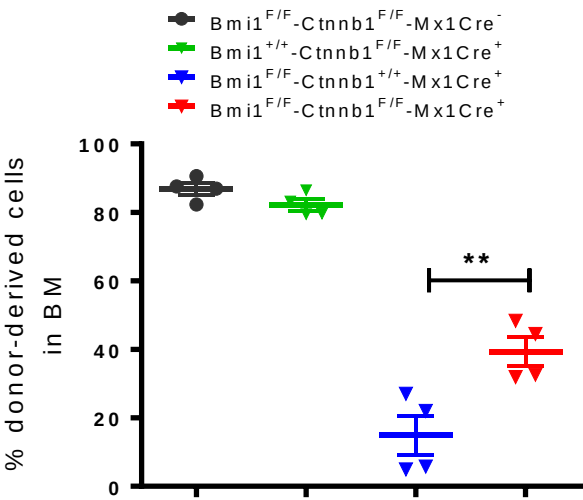
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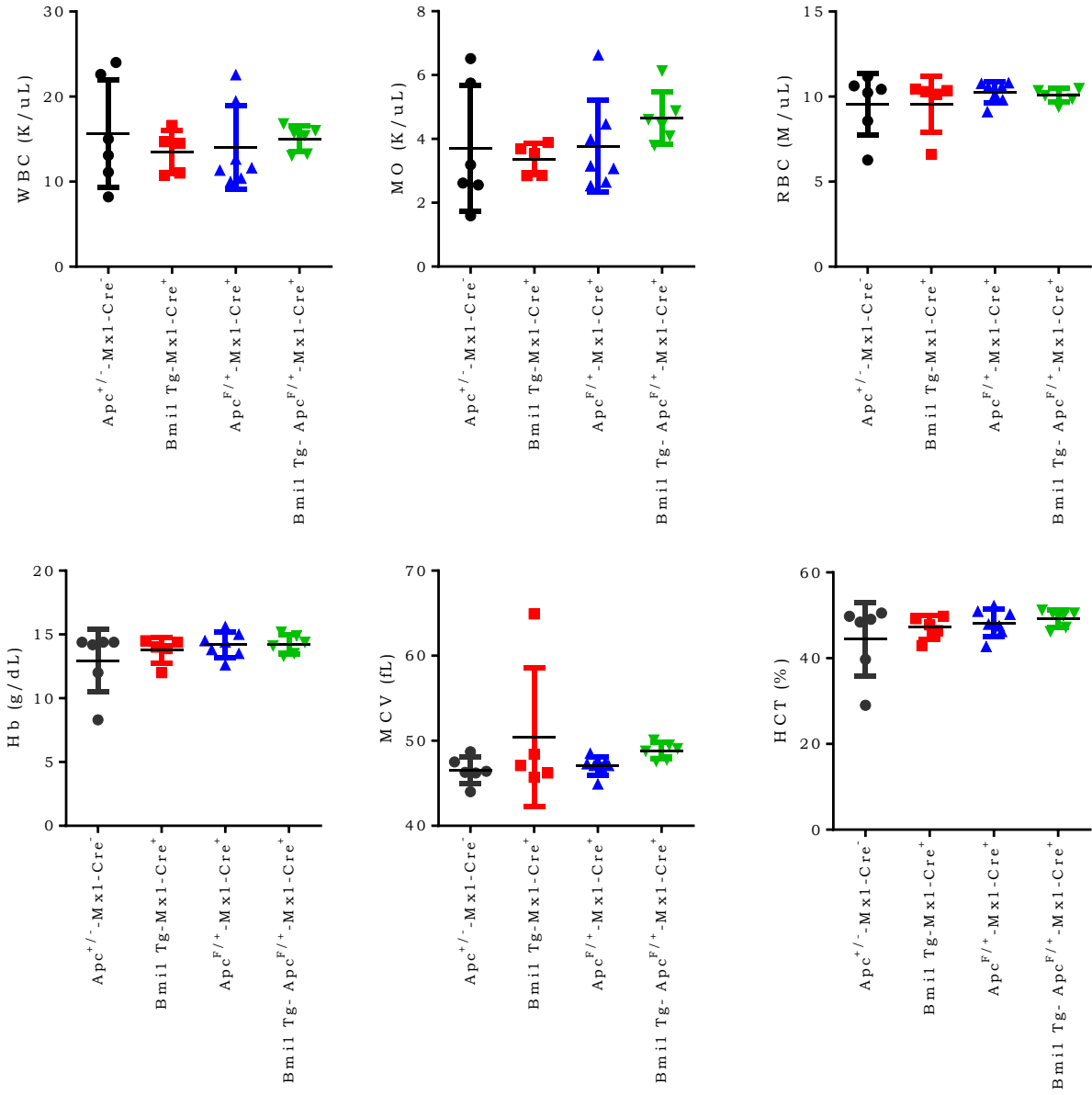
Figure S2

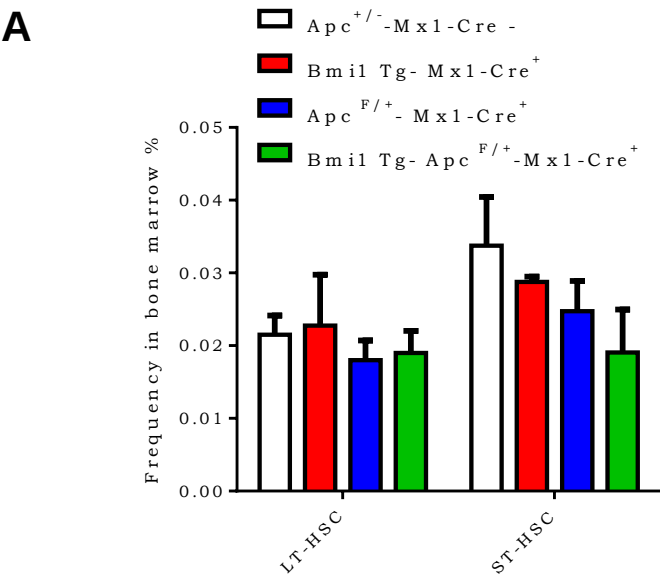


A Primary BM transplantation

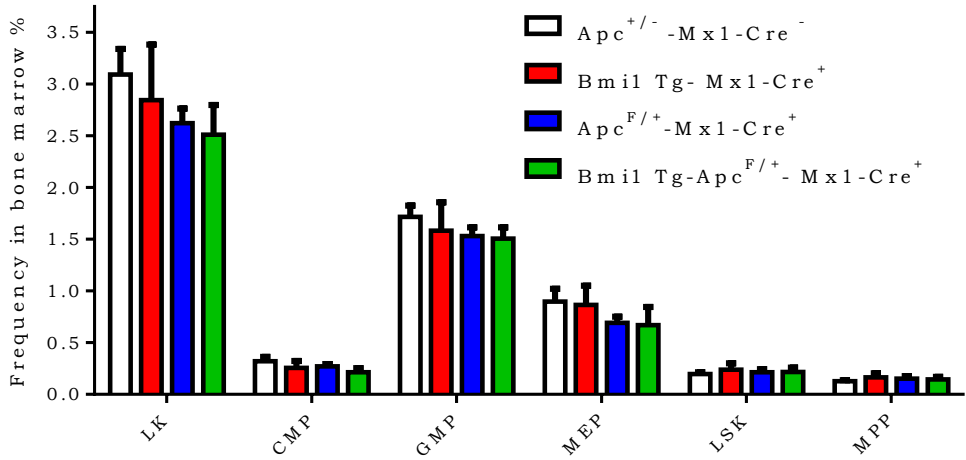


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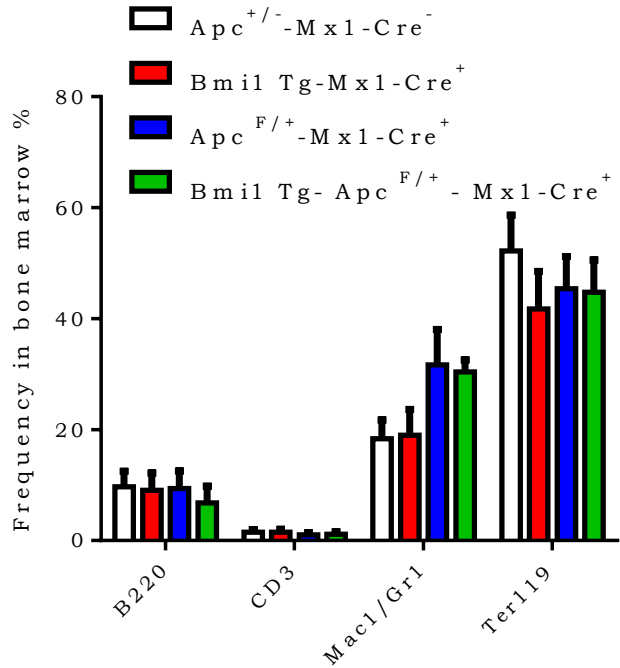




B



C



PRL2 phosphatase enhances oncogenic cytokine signaling in leukemia cells

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The **p**hosphatase of **r**egenerating **l**iver (PRL) family of phosphatases, consisting of PRL1, PRL2, and PRL3, represents an intriguing group of proteins that are being pursued as biomarkers and therapeutic targets in human cancers.¹⁻³ While PRL2 is highly expressed in AML and acute lymphoblastic leukemia (ALL), its roles in normal and malignant hematopoiesis are not known.⁴ We found PRL2 is important for the proliferation and self-renewal of hematopoietic stem cells (HSCs).⁵ PRL2 is highly expressed in some subtypes of AML, including AML1-ETO⁺ AML and AML with mixed lineage leukemia (MLL) translocations.⁶ We found that PRL2 promotes AML1-ETO-induced leukemia *in vivo*.⁷ Importantly, genetic and pharmacological inhibition of PRL2 decreased the proliferation and survival of human AML cells.⁷⁻⁸ Furthermore, we found that PRL2 regulates T cell development and that PRL2 promotes oncogenic NOTCH1-Induced T-cell leukemia.⁹⁻¹⁰ While PRL2 is important for normal and malignant hematopoiesis, the mechanisms by which PRL2 promotes leukemia development are not fully understood.

Most human leukemia cells depend on aberrant receptor tyrosine kinase signaling and the subsequent downstream effectors for proliferation and survival.¹¹⁻¹² Gain-of-function mutations in receptor tyrosine kinase KIT, including mutation at codon 816 in human (KITD816V; KitD814V in mouse), are associated with poor prognosis and reduced survival in AML.¹³⁻¹⁵ Given that PRL2 is important for SCF-mediated HSPC proliferation,⁵ we reasoned that PRL2 may promote leukemia development through enhancing oncogenic KIT signaling. Indeed, we found that loss of PRL2 decreases the ability of oncogenic KitD814V mutant in promoting HSPC proliferation.⁵ Enforced KitD814V expression in HSPCs induces myeloproliferative neoplasms (MPN) in mice¹⁶ and we found that PRL2 deficiency significantly delays the onset of MPN induced by the KitD814V mutant (Figure 1A). Kasumi-1 is a human AML cell line with KIT mutation and we found that knockdown of PRL2 decreases the proliferation of Kasumi-1 cells.⁷ Thus, we demonstrate that PRL2 is important for the proliferation of leukemia cells bearing KIT mutations.

FLT3 is a key driver of AML and internal tandem duplications in FLT3 (FLT3-ITD) are seen in ~30% of AML patients, and these duplications are associated with poor prognosis.¹⁷⁻¹⁸ We introduced wild-type FLT3 or FLT3-ITD mutant into Lin⁻ cells purified from wild-type and *Prl2* null mice and found that *Prl2* null Lin⁻ cells that carry the FLT3-ITD mutant exhibit decreased proliferation compared to wild-type cells both in the absence of cytokines and in the presence of FLT3 ligand (Figure 1B). As expected, ectopic expression of FLT3-ITD increased the colony formation of wild-type HSPCs (Figure 1C). While *Prl2* deficiency did not affect the colony formation of HSPCs expressing wild-type FLT3, loss of *Prl2* decreased the colony formation of HSPCs expressing FLT3-ITD (Figure 1C). Enforced FLT3-ITD expression in HSPCs induces myeloproliferative neoplasms (MPN) in mice.¹⁹ We found that ectopic expression of PRL2 dominant-negative mutant (PRL2-CSDA) decreased the proliferation of murine hematopoietic progenitor cell line 32D expressing FLT3-ITD (Figure 1D).⁷ While ectopic expression of PRL2 had no effect on the survival of C3H/HeJ mice transplanted with 32D cells expressing FLT3-ITD, expression of PRL2-CSDA significantly extended the survival of C3H/HeJ mice (Figure 1E). Molm-13 and MV4-11 are human AML cell line with FLT3 mutations and we found that knockdown of PRL2 decreases the proliferation of both Molm-13 and MV4-11 cells (Figure 1F).⁷ Thus, we demonstrate that PRL2 is important for the proliferation of leukemia cells bearing FLT3 mutations.

Attenuation of c-Kit signaling is important to obtain a suitable intensity and duration of signal transduction to meet the biological needs.²⁰ There are at least three levels of c-Kit downregulation that function in concert: (1) tyrosine dephosphorylation, (2) inactivation of the kinase domain by serine phosphorylation, and (3) removal from the cell surface and intracellular degradation.²⁰ We first examined c-Kit activation in *Prl2*^{-/-} HSPCs following SCF stimulation. PRL2 null HSPCs showed decreased c-Kit phosphorylation at tyrosine 703 as well as Erk and Akt phosphorylation following SCF stimulation (Figure 2A), suggesting that c-Kit^{Y703} is not a substrate of PRL2. To further characterize c-Kit phosphorylation in the absence of PRL2, we utilized purified c-Kit^{bright} mast cells (MCs) from BM cells 4 weeks following induction.²¹ Given that PKC-dependent phosphorylation is a known negative

feedback mechanism of c-Kit,²² we examined the phosphorylation status of c-Kit^{S744} (human S746) in MCs following SCF stimulation using a phospho-specific antibody.²² The level of c-Kit^{S744} phosphorylation exhibited a similar pattern compared to that of the p-Kit^{Y703} between the *Prl2*^{+/+} and *Prl2*^{-/-} cells upon SCF stimulation (Figure S1A), suggesting that the negative feedback mechanism does not function in MCs.

We found that c-Kit internalization was enhanced in *Prl2*^{-/-} MCs compared to *Prl2*^{+/+} cells following SCF stimulation (Figure 2B). Importantly, PRL2 null MCs had progressively reduced c-Kit expression on cell surface during SCF-induced differentiation (Figure S1B). In addition, we observed reduced c-Kit expression in human AML cells expressing a shRNA targeting PRL2 or overexpressing a PRL2 dominant-negative mutant (PRL2-CSDA) (Figures 2C and S1C). We then treated serum starved MCs with cycloheximide and measured the half-life of c-Kit protein. The half-life of c-Kit in *Prl2*^{-/-} MCs was significantly shorter than that of the *Prl2*^{+/+} MCs (Figure 2D). Furthermore, *Prl2*^{-/-} MCs showed enhanced c-Kit ubiquitination compared to WT cells following SCF stimulation (Figure 2E). Thus, we demonstrate that PRL2 deficiency results in less c-Kit on the cell surface and a lower global c-Kit level in hematopoietic cells due to accelerated c-Kit turnover.

While PRL2 is a dual specificity protein phosphatase, its substrates in cells are largely unknown.¹⁻⁴ To identify potential PRL2 substrates in leukemia cells, we performed protein phosphatase substrate trapping experiments. We utilized a GST-tagged PRL2-CSDA mutant, in which the catalytic cysteine and aspartic acid were mutated to serine and alanine respectively, so that the mutant PRL2 would bind to its substrates stronger but be unable to catalyze substrate turnover.²³ We found that the mutant PRL2-CSDA displays enhanced association with E3 ubiquitin ligase CBL compared to WT PRL2 in Kasumi-1 cells (Figure 2F). In addition to CBL, we also detected the association of PRL2 with c-Kit (Figures 2F). We further confirmed the PRL2 and CBL interaction by using the co-immunoprecipitation (co-IP) assays in 293 cells expressing KitD814V (Figure S2A). The CBL family E3 ubiquitin ligases, including CBL and CBL-B, are responsible for the ubiquitination and degradation of KIT and FLT3 in hematopoietic

cells.²⁴⁻²⁵ Indeed, we found that PRL2-CSDA shows enhanced association with CBL and FLT3 compared to wild-type PRL2 in MV4-11 cells (Figure S2B). Upon SCF stimulation, KIT binds to and induces the phosphorylation of CBL proteins, which in turn act as E3 ligases, mediating the ubiquitination and degradation of KIT and themselves.²⁵ We found decreased levels of CBL in PRL2 null MCs following SCF stimulation (Figure 2A). These findings suggest that CBL may be a substrate of PRL2 in leukemia cells.

In summary, we demonstrated that PRL2 enhances KIT and FLT3 activation through regulating their internalization and turnover in leukemia cells. CBL becomes phosphorylated on tyrosine residues within the proline-rich domain, including Tyr 700, Tyr 731, and Tyr 774, following cytokine stimulation.²⁴⁻²⁵ Given that we found that PRL2 associates with KIT and FLT3 in leukemia cells (Figures 2F and S2B), we predict that PRL2 dephosphorylates CBL on tyrosine residues and inhibits the E3 ligase activity of CBL toward KIT and FLT3, leading to decreased ubiquitination and degradation of KIT and FLT3, thereby activating downstream signaling pathways in leukemia cells (Figure S2C, left panel). We further speculate that CBL is hyper-phosphorylated in PRL2-deficient leukemia cells, leading to enhanced ubiquitination and degradation of both KIT and FLT3 (Figure S2C, right panel). Despite substantial efforts devoted to the development of FLT3 and KIT inhibitors, the effectiveness of these agents in AML has been limited.^{11-14,17-18} Establishing PRL2 as a key mediator of oncogenic cytokine signaling in leukemia cells will facilitate the development of PRL2 inhibitors that are useful for treating human leukemia.

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Authorship

Contribution: MK, YB, ZYZ, and YL. Designed the research. MK, YB, SC, SCN, SV, DH, ACF, CY, and JS. Performed the research; MK and YL. Analyzed the data and performed the statistical analysis. HSB, JMC, LR, LDM, and RK. Provided reagents to the study. MK, YB, ZYZ, and YL. Wrote the manuscript. All authors read, comment on, and approved the manuscript.

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REFERENCES

1. Rios P, Li X, Köhn M. Molecular mechanisms of the PRL phosphatases. *FEBS J.* 2013; 280(2):505-24.
2. Bessette DC, Qiu D, Pallen CJ. PRL PTPs: mediators and markers of cancer progression. *Cancer Metastasis Rev.* 2008; 27: 231–252.
3. Campbell AM, Zhang ZY. Phosphatase of regenerating liver: a novel target for cancer therapy. *Expert Opin Ther Targets.* 2014;18(5):555-69.
4. Kobayashi M, Chen S, Gao R, *et al.* Phosphatase of regenerating liver in hematopoietic stem cells and hematological malignancies. *Cell Cycle.* 2014;13:2827-35.
5. Kobayashi M, Bai Y, Dong Y, *et al.* PRL2/PTP4A2 phosphatase is important for hematopoietic stem cell self-renewal. *Stem Cells.* 2014; 32:1956-67.
6. Bagger FO, Rapin N, Theilgaard-Mönch K, *et al.* HemaExplorer: a Web server for easy and fast visualization of gene expression in normal and malignant hematopoiesis. *Blood.* 2012; 119:6394-5.
7. Kobayashi M, Chen S, Bai Y, *et al.* Phosphatase PRL2 promotes AML1-ETO-induced acute myeloid leukemia. *Leukemia.* 2017;31:1453-1457.
8. Bai Y, Yu ZH, Liu S, *et al.* Novel Anticancer Agents Based on Targeting the Trimer Interface of the PRL Phosphatase. *Cancer Res.* 2016;76:4805-15.
9. Kobayashi M, Nabinger SC, Bai Y, *et al.* Protein Tyrosine Phosphatase PRL2 Mediates Notch and Kit Signals in Early T Cell Progenitors. *Stem Cells.* 2017; 35:1053-1064.
10. Kobayashi M, Bai Y, Chen S, *et al.* Phosphatase PRL2 promotes oncogenic NOTCH1-induced T cell leukemia. *Leukemia.* 2016;31:751-754.
11. Toffalini F, Demoulin JB. New insights into the mechanisms of hematopoietic cell transformation by activated receptor tyrosine kinases. *Blood.* 2010;116(14):2429-37.
12. Stirewalt DL, Meshinchi S. Receptor tyrosine kinase alterations in AML - biology and therapy. *Cancer Treat Res.* 2010;145:85-108.

13. Longley BJ, Reguera MJ, Ma Y. Classes of c-KIT activating mutations: proposed mechanisms of action and implications for disease classification and therapy. *Leuk Res.* 2001;25(7):571-576.
14. Stankov K, Popovic S, Mikov M. C-KIT signaling in cancer treatment. *Curr Pharm Des.* 2014;20(17):2849-80.
15. Schnittger S, Kohl TM, Haferlach T, *et al.* KIT-D816 mutations in AML1-ETO-positive AML are associated with impaired event-free and overall survival. *Blood.* 2006;107(5):1791-9.
16. Ma P, Mali RS, Martin H, *et al.* Role of intracellular tyrosines in activating KIT-induced myeloproliferative disease. *Leukemia.* 2012;26(7):1499-506.
17. Kindler T, Lipka DB, Fischer T. FLT3 as a therapeutic target in AML: still challenging after all these years. *Blood.* 2010;116(24):5089-102.
18. Swords R, Freeman C, Giles F. Targeting the FMS-like tyrosine kinase 3 in acute myeloid leukemia. *Leukemia.* 2012;26(10):2176-85.
19. Lee BH, Tothova Z, Levine RL, *et al.* FLT3 mutations confer enhanced proliferation and survival properties to multipotent progenitors in a murine model of chronic myelomonocytic leukemia. *Cancer Cell.* 2007;12:367-80.
20. Lennartsson J, Rönnstrand L. Stem cell factor receptor/c-Kit: from basic science to clinical implications. *Physiol Rev.* 2012;92(4):1619-49.
21. Hu P, Carlesso N, Xu M, *et al.* Genetic evidence for critical roles of P38 α protein in regulating mast cell differentiation and chemotaxis through distinct mechanisms. *J Biol Chem.* 2012; 287(24):20258-69.
22. Edling CE, Pedersen M, Carlsson L, *et al.* Haematopoietic progenitor cells utilise conventional PKC to suppress PKB/Akt activity in response to c-Kit stimulation. *Br J Haematol.* 2007;136(2):260-8.
23. Hardy S, Uetani N, Wong N, *et al.* The protein tyrosine phosphatase PRL-2 interacts with the magnesium transporter CNNM3 to promote oncogenesis. *Oncogene.* 2015;34(8):986-95.
24. Thien CB, Langdon WY. Cbl: many adaptations to regulate protein tyrosine kinases. *Nat Rev Mol Cell Biol.* 2001;2(4):294-307.

25. Zeng S, Xu Z, Lipkowitz S, Longley JB. Regulation of stem cell factor receptor signaling by Cbl family proteins (Cbl-b/c-Cbl). *Blood*. 2005;105(1):226-32.

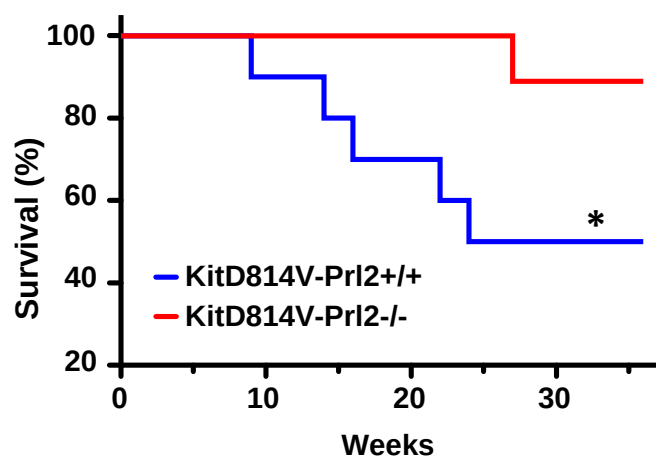
FIGURE LEGENDS

Figure 1. PRL2 is important for oncogenic cytokine signaling-mediated hematopoietic progenitor cell proliferation. (A) PRL2 deficiency extended the survival of recipient mice transplanted with hematopoietic progenitor cells expressing KitD814V (* $p < 0.05$, $n = 9$). (B) PRL2 deficiency decreased the proliferation of hematopoietic progenitor cells expressing FLT3-ITD both in the absence of cytokines and in the presence of FLT3 ligand (* $p < 0.05$, $n = 3$). (C) Wild-type FLT3 or FLT3-ITD were introduced into Lin⁻ cells purified from wild-type and *Prl2* null mice. Loss of PRL2 decreased the colony formation of HSPCs expressing FLT3-ITD (** $p < 0.01$, $n = 3$). (D) Ectopic PRL2-CSDA expression decreased the proliferation of 32D cells expressing FLT3-ITD (** $p < 0.01$, $n = 3$). (E) Expressing PRL2-CSDA, but not the WT PRL2, extended the survival of C3H/HeJ mice transplanted with 32D cells expressing FLT3-ITD (* $p < 0.05$, $n = 7$). (F) Knockdown of PRL2 decreased the proliferation of Molm-13 cells (** $p < 0.01$, $n = 3$).

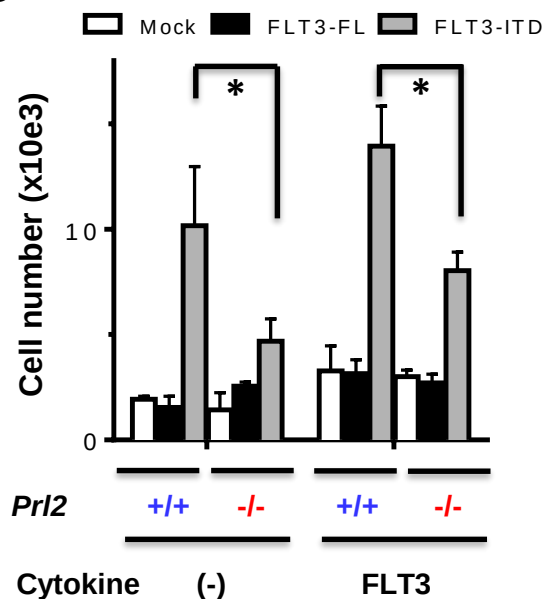
Figure 2. PRL2 regulates the internalization and turnover of c-Kit in hematopoietic cells. (A) PRL2 is important for c-Kit activation following SCF stimulation. Immunoblot analysis of Kit, Erk, and Akt phosphorylation in WT and *Prl2* null hematopoietic progenitor cells following SCF stimulation. (B) Mast cells (MCs) were induced from WT and PRL2 null KSL cells followed by SCF stimulation. Levels of c-Kit on the surface of MCs were determined by flow cytometry analysis (** $p < 0.01$, $n = 3$). (C) Knockdown of PRL2 in human AML cell line MO7e decreased the level of KIT on the cell surface. (D) PRL2 null MCs showed decreased half-life of c-Kit following SCF stimulation. (E) Serum-starved MCs were stimulated with SCF, collected at each time point, and followed by immunoprecipitation with a Kit antibody. Ubiquitinated proteins were detected by immunoblotting with an anti-ubiquitin antibody. (F) GST pull-down assays. Total cellular proteins from Kasumi-1 cells were isolated, incubated with GST, GST-PRL2, or GST-PRL2-CSDA and immunoblotted with antibody against KIT or CBL.

Figure 1

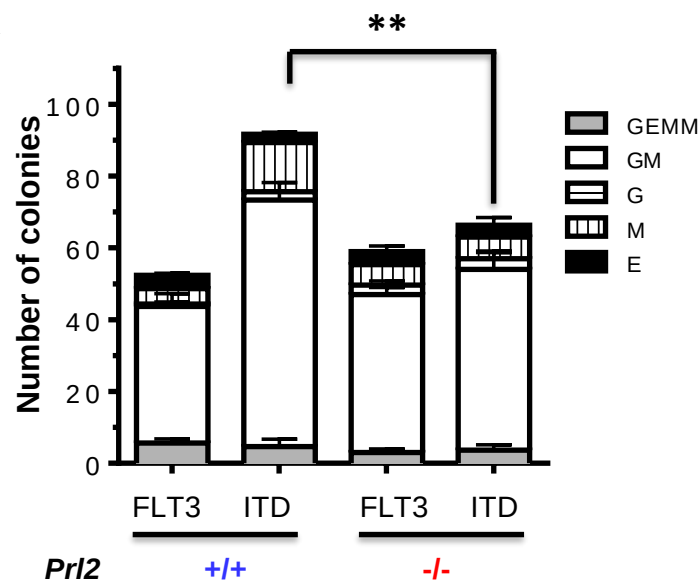
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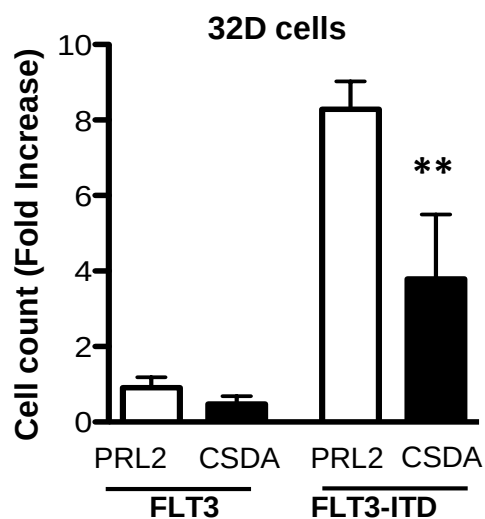
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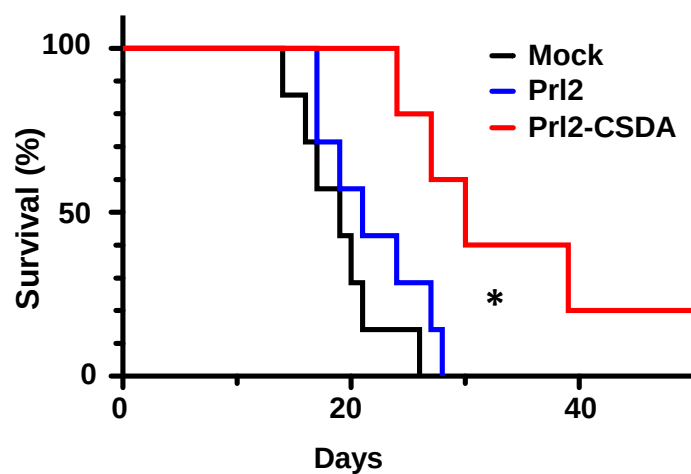
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D



E



F

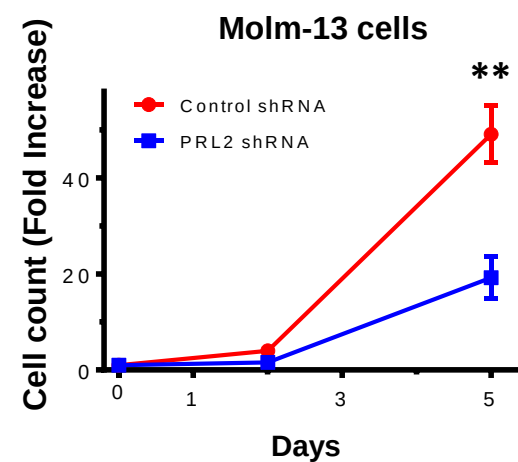
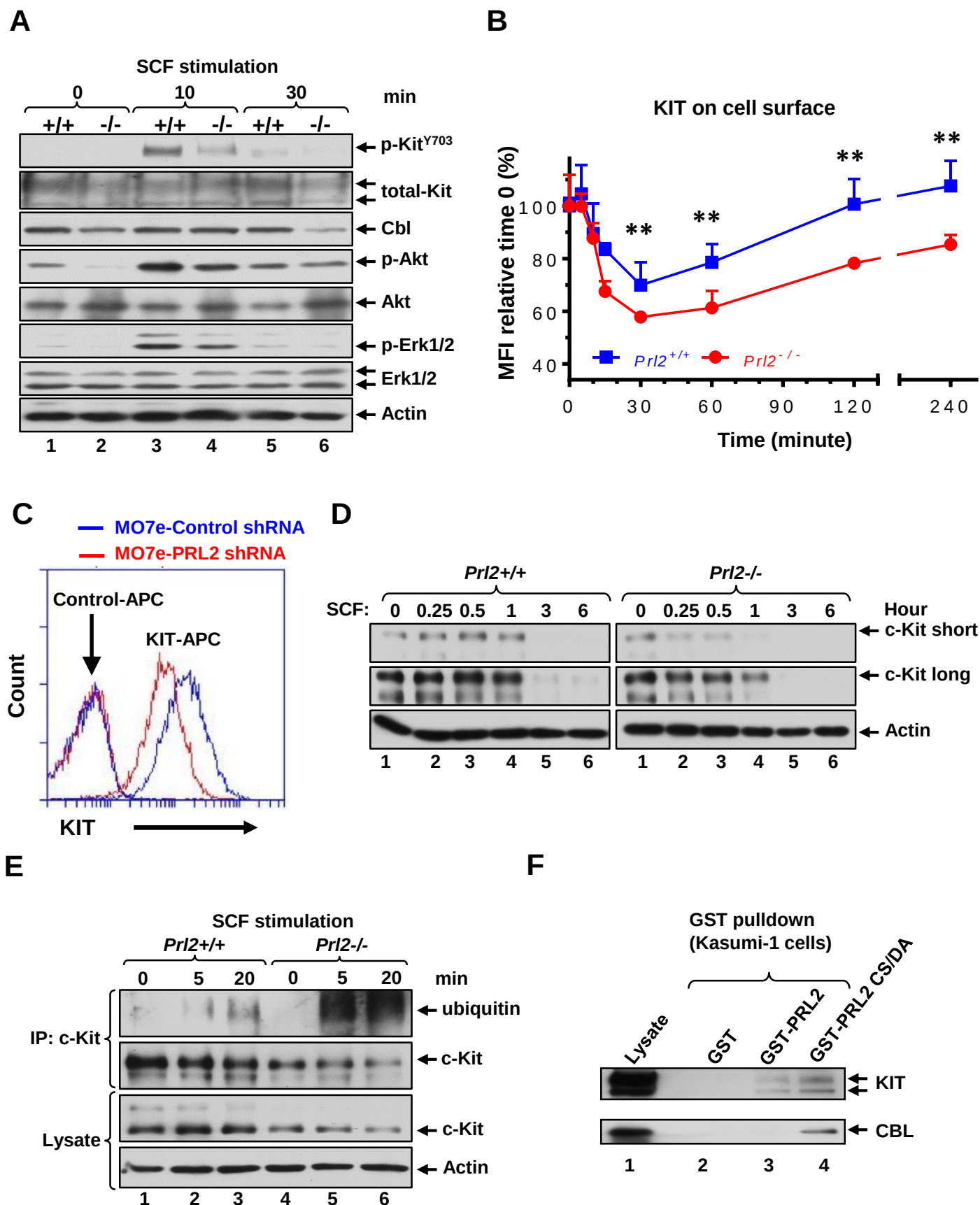


Figure 2



PRL2 phosphatase enhances oncogenic cytokine signaling in leukemia cells

Supplemental Methods

Mice

Wild type C57BL/6 (CD45.2⁺), B6.SJL (CD45.1⁺), and C3H/HeJ mice were purchased from the Jackson Laboratories. *Prl2*^{+/+} and *Prl2*^{-/-} mice were maintained in the Indiana University Animal Facility and kept in Thorensten units with filtered germ-free air. All mice were 10-12 weeks of age at the time of analysis. Both male and female mice were utilized in the experiments. The Institutional Animal Care and Use Committee (IACUC) of the Indiana University School of Medicine approved all experimental procedures.

Production of Retrovirus

Retroviral particles were produced by transfection of Phoenix E cells with the MSCV-IRES-GFP or MSCV-PRL2-IRES-GFP, MSCV-PRL2-CSDA-IRES-GFP, MSCV-KITD814V-IRES-GFP, MSCV-FLT3-IRES-GFP, or MSCV-FLT3-ITD-IRES-GFP plasmids, according to standard protocols. Mouse hematopoietic progenitor cells were transduced on retronectin (Takara)-coated non-tissue culture plates with high-titer retroviral suspensions. Twenty-four hours after infection, GFP-positive cells were sorted by FACS. Transduced cells were then transplanted into lethally irradiated recipient mice. The presence of GFP⁺ cells in the peripheral blood was measured by flow cytometry analysis.

Human AML cell lines

Human AML cell lines, including Kasumi-1, MV4-11, and MO7e were obtained from ATCC. All cell lines were authenticated by SRT profiling and tested for mycoplasma contamination.

Production of Lentivirus

Lentiviral shRNA plasmid (pLB) was purchased from Addgene (11619). Oligonucleotides targeting control (Luciferase) and human PRL2 cDNAs were cloned into the pLB plasmid. Oligonucleotide sequences are available upon request. Lentiviral particles were generated by standard method using the third generation packaging system (pMDL, pMD2.G, and pRSV-Rev). Human AML cell lines were infected with high-titer lentiviral suspensions. Twenty-four hours after infection, GFP-positive cells were sorted by FACS. The reduction of PRL2 proteins was determined by immunoblot analysis.

Immunoblotting analysis

Cells were washed with ice-cold PBS, and lysed on ice for 30 min in lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% Triton X-100, and 10% glycerol) supplemented with a Complete Protease Inhibitor tablet (Roche Applied Science). Cell lysates were cleared by centrifugation at 15,000 rpm for 10 min. The lysate protein concentration was estimated using a BCA protein assay kit (Pierce). The protein samples were boiled with sample buffer, separated by SDS-PAGE, transferred electrophoretically to a nitrocellulose membrane, and immunoblotted with appropriate antibodies, followed by incubation with horseradish peroxidase-conjugated secondary antibodies. The blots were developed by the enhanced chemiluminescence technique (ECL kit, GE Healthcare). Representative results from at least two independent experiments are shown. Antibodies were purchased from Cell Signaling (c-Kit, pKit^{Y703}, ERK1/2, pERK1/2, AKT, pAKT, ubiquitin), Santa Cruz (Actin and FLT3), Transduction (CBL) and Sigma (Flag). The generation of pKit^{S746} antibody was described previously (Edling et al., 2007).

GST pull down assays

1 x 10⁹ Kasumi-1 or MV4-11 cells were treated with 1 mM pervanadate for 30 minutes and collected by centrifugation. The cell pellet was lysed with 3 ml lysis buffer (20 mM Tris, pH 7.5, 100 mM NaCl, 1% Triton X-100, 10% glycerol, supplemented with 5 mM iodoacetic acid, 1 mM orthovanadate, and proteases inhibitors). 10 mM DTT was added in the lysate and incubated for 15 min on ice to inactivate

any unreacted iodoacetic acid and pervanadate. Supernatant was collected by centrifugation at 14,000 g for 15 min. 25 µg GST, GST-PRL2 or GST-PRL2-CSDA were coupled to GST beads in lysis buffer, incubated at 4°C for 1h. Cell lysates were incubated with GST proteins conjugated to beads at 4 °C for 2h. The beads were pelleted and washed 3 times for 5 min with lysis buffer. Bound proteins were re-suspended in 50 µL Laemmli sample buffer, boiled for 5 min, and the samples are resolved by SDS-PAGE gels.

Statistical Analysis

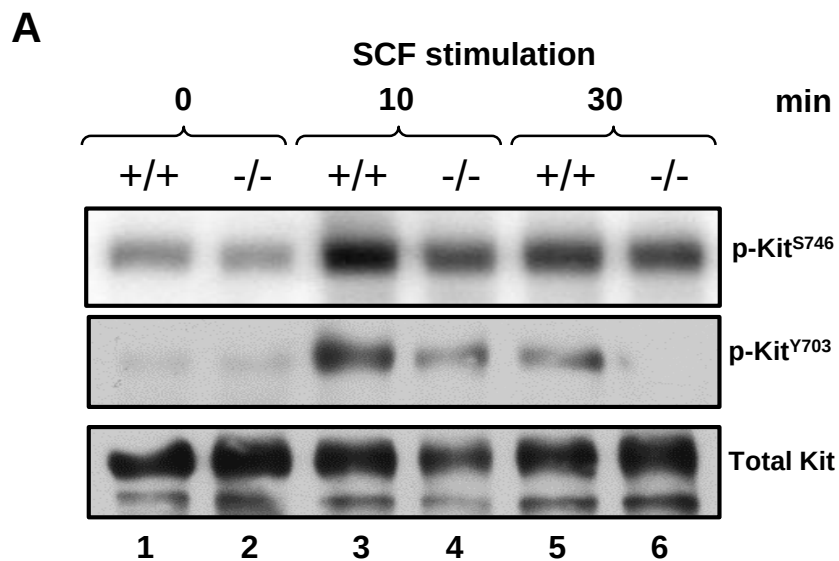
The animal sample size was based on previous studies evaluating the roles of AML1-ETO9a in leukemia and POWER analysis. Using Chi-Square analysis, 7 mice per group will provide 80% POWER in detecting difference with 95% difference. Gehan-Breslow-Wilcoxon test was used for Kaplan-Meier survival curves. The other data were analyzed by paired or unpaired t test using GraphPad Prism 6. *, $p < 0.05$; **, $p < 0.01$. All experiments were repeated at least once.

Supplementary Figure Legends

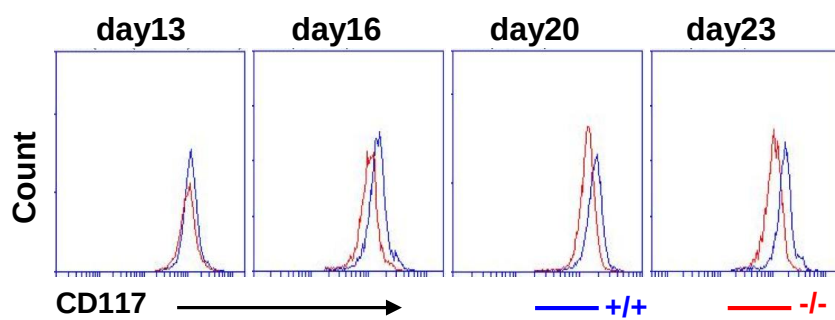
Figure S1. (A) Evaluation of PKC-mediated negative regulation of c-Kit activation in PRL2 null mast cells (MCs). MCs were induced from WT and PRL2 null KSL cells, serum starved, followed by stimulation of SCF. Total cell lysates harvested at each time point were subjected to western blot analysis using antibodies against Kit, Kit^{Y703} or Kit^{S746} (mouse Kit^{S744}). (B) Expression of c-Kit is progressively declined in PRL2 null MCs. MCs were induced from KSLs and c-Kit median fluorescent intensity (MFI) was measured by flow cytometry at each time point. (C) MO7e cells were transduced with retroviruses expressing GFP, PRL2 or PRL2-CSDA mutant and the levels of KIT on cell surface were assessed by flow cytometry analysis.

Figure S2. (A) PRL2 interacts with KITD814V and CBL in 293 cells. (B) GST pull-down assays. Total cellular proteins from MV4-11 cells were isolated, incubated with GST, GST-PRL2, or GST-PRL2-

CSDA and immunoblotted with antibody against KIT and CBL. PRL2 associates with FLT3 and CBL in MV4-11cells in GST pull-down assays. (C) Schematic model of potential mechanisms by which PRL2 mediates oncogenic cytokine signaling in leukemia cells. PRL2 dephosphorylates CBL and inhibits its E3 ligase activity toward KIT and FLT3 in leukemia cells, leading to increased activation KIT and FLT3 signaling. .



B



C

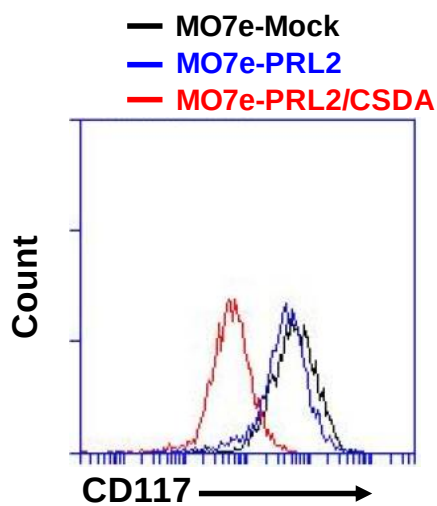
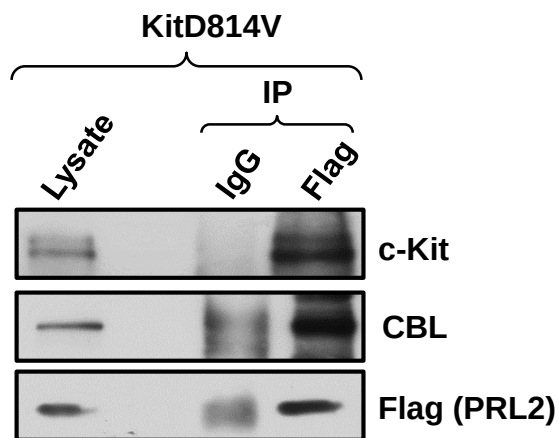
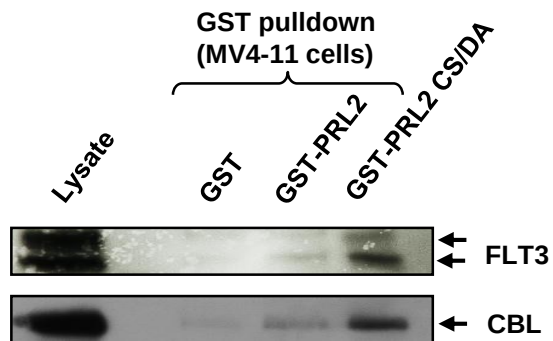


Figure S2

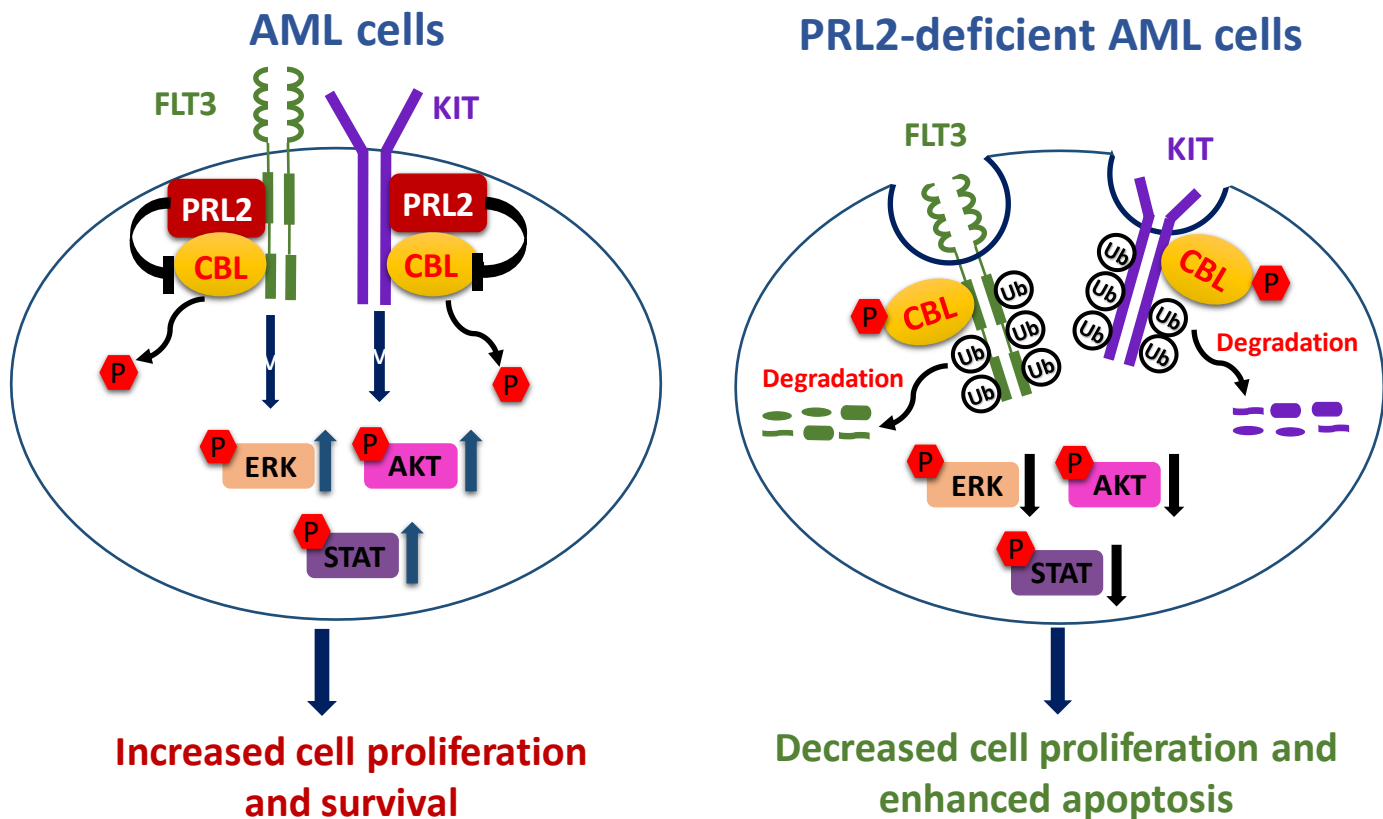
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C



CURRICULUM VITAE

Yan Liu, Ph.D.

Associate Professor of Pediatrics, Biochemistry and Molecular Biology (Tenured)

St. Baldrick's Foundation Scholar

Showalter Scholar

Department of Pediatrics

Wells Center for Pediatric Research

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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, New York

2008-2010 Senior Research Scientist, Memorial Sloan-Kettering Cancer Center, New York, New York

2010-2016 Assistant Professor of Pediatrics, Biochemistry and Molecular Biology (tenure-track), Indiana University School of Medicine, Indianapolis, IN

2016-present Associate Professor of Pediatrics, Biochemistry and Molecular Biology (tenured), Indiana University School of Medicine, Indianapolis, IN

2011-present Member, Indiana University Simon Cancer Center

2011-present Member, Indiana University/NIDDK Cooperative Center of Excellence in Hematology (CCEH)

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	(PI: Liu)	07/15/19-06/30/23	3.6 calendar
NIH/NHLBI		\$1,550,000	
Molecular mechanisms underlying clonal expansion of hematopoietic stem cells			
Role: Principal Investigator			
W81XWH-19-1-0575	(PI: Liu)	08/01/19-06/30/21	2.4 calendar
DOD/PRCRP Idea Development Award		\$596,000	
Targeting Leukemia-initiating Cells to Improve Leukemia Treatment			
Role: Principal Investigator			

W81XWH-18-1-0265 (PI, Liu) 07/01/18-06/30/21 1.2 calendar
DOD/BMFRP Idea Development Award \$484,967
Understanding and Targeting Mutant p53 in Myelodysplastic Syndromes
Role: Principal Investigator
NCE

6581-20 (PI: Liu) 07/01/19-06/30/22 0.6 calendar
Leukemia & Lymphoma Society (LLS) TRP Grant \$600,000
Development of therapeutic strategy for the treatment of MDS
Role: Principal Investigator

Scholar Award (PI: Liu) 07/01/19-06/30/22
Ralph and Grace Showalter Research Trust Fund \$75,000
The role of mutant p53 in clonal hematopoiesis
Role: Principal Investigator

PENDING SUPPORT:

1 R01 CA258175 (PIs: Liu and Mayo) 04/01/21-03/31/26 2.4 calendar
NIH/NCI \$2,287,885
Chronic inflammation drives leukemogenesis through enhancing mutant hematopoietic stem and progenitor cell fitness
Role: Principal Investigator
(Submitted in June 2020. It will be reviewed in November 2020)

COMPLETED SUPPORT:

Startup Fund (PI, Liu) 11/01/10-10/31/15
Indiana University School of Medicine (IUSM) \$1,050,000
p53 in normal and malignant hematopoiesis
Role: Principal Investigator

R56AG052501 (PI, Liu) 09/15/17-08/31/18 2.4 calendar
NIH/NIA \$255,000
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 \$150,000
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 \$374,400
Modulating Leukemia-initiating Cell Quiescence to Improve Leukemia Treatment
Role: Principal Investigator

R01 AI121197-01A1 (PI: Yoshimoto) 06/13/16-05/31/17 1.2 calendar
NIH/NIAID \$1,968,000

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 \$250,000
Embryonic origin and self-renewal of B-1a cells
Role: Co-investigator

1F32CA203049-01 (PI: Nabinger) 07/01/16 – 6/30/18
NIH/NCI \$125,382
Targeting PRL2 Phosphatase in acute myeloid leukemia
Role: Mentor

Scholar Award (PI: Liu) 07/11/14-12/31/19 7.2 calendar
St. Baldrick's Foundation \$560,000
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 \$125,000
Targeting PRL2 in Acute Myeloid Leukemia
Role: Principal Investigator

New Investigator Award (PI: Liu) 07/01/16-12/31/17
Leukemia Research Foundation \$100,000
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 \$60,000
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 \$60,000
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 \$40,000
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 \$30,000
Targeting PRL2 in acute myeloid leukemia
Role: Principal Investigator

Research Grant (PI: Liu) 09/02/14-08/31/15
Alex's Lemonade Stand Foundation \$20,000

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) \$20,000
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) \$20,000
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) \$55,000
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 \$50,000
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 \$50,000
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) \$40,000
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) \$40,000
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 \$35,000
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 \$35,000
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 \$25,000

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) \$20,000
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) \$20,000
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 \$60,000
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) \$50,000
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 \$40,000
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) \$20,000
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, Rönstrand L, Kapur R, Zhang ZY, and **Liu Y***. PRL2 phosphatase

enhances oncogenic cytokine signaling in leukemia cells. *Blood* (In revision, 2020).

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, 2020).
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, 2020).

PEER-REVIEWED PUBLICATIONS

5. 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**).
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. 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.
8. 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
9. Cai Z, Kotzin JJ, Ramdas R, Chen S, Nelanthala 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 *Morbid* 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
10. 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.
11. 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
12. Sayar H, **Liu Y***, Cripe LD, Wilson-Weekes AM, Weisenbach J, Cangany M, Sargent KJ, Nassiri M, Li L, Al Baghdadi Y, Gupta S, Suvannasankha A, Gao R, Pan F, Shanmugam R, Goswami C, Xu M, Boswell HS. Consecutive Epigenetically-active Agent Combinations act in ID1-RUNX3-TET2 and HOXA pathways for Flt3ITD+ve AML. *Oncotarget*. 2017 Dec 25;9(5):5703-5715. PubMed PMID: 29464028; PubMed Central PMCID: PMC5814168. **Co-corresponding author**

13. 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. PMCID: PMC5675657.
14. 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. PMCID:PMC5695226
15. 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. PMCID: PMC5367971.
16. 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. PMCID:PMC5695227
17. 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 PMCID: PMC5859321.
18. 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. PMCID:PMC4922705.
19. 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. PMCID:PMC4380436.
20. 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. PMCID:PMC4380442.
21. 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
22. 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
23. 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.

24. 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
25. 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
26. **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.
27. 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
28. **Liu Y***, Liu F, Yu H, Zhao X, Sahsidia 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**
29. 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
30. Hu P, Nebreda AR, **Liu Y**, Carlesso N, Kaplan M, Kapur R. P38 α 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
31. 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**.
32. 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
33. 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
34. 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.

35. 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
36. 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
37. 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
38. **Liu Y**, Asai T, and Nimer SD. Myelodysplasia: battle in the bone marrow. *Nat Med*, Jan; 16(1):30-2, 2010.
39. **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.
40. **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
41. Sashida G, **Liu Y**, Elf S, Miyata Y, Ohyashiki K, Izumi M, Menendez S, and Nimer SD. ELF4/MEF activates MDM2 expression and blocks oncogene-induced p16 activation to promote transformation. *Mol Cell Biol*, Jul; 29(13):3687-99, 2009. PMCID: PMC2698769
42. Lin HK, Wang G, Chen Z, Teruya-Feldstein J, **Liu Y**, Chan CH, Yang WL, Erdjument-Bromage H, Nakayama KI, Nimer S, Tempst P, and Pandolfi PP. Phosphorylation-dependent regulation of cytosolic localization and oncogenic function of Skp2 by Akt/PKB. *Nat Cell Biol*, Apr; 11(4):420-32, 2009. PMCID: PMC2830812
43. **Liu Y**, Elf SE, Miyata Y, Sashida G, Liu Y, Huang G, Di Giandomenico S, Lee JM, DeBlasio A, Menendez S, Antipin J, Reva B, Koff A, and Nimer SD. p53 regulates hematopoietic stem cell quiescence. *Cell Stem Cell*, Jan 9; 4(1):37-48, 2009. PMCID: PMC2839936
44. Morales M, **Liu Y**, Laiakis EC, Morgan WF, Miner SD, and Petrini JH. DNA damage signaling in hematopoietic cells: a role for Mre11 complex repair of topoisomerase lesions. *Cancer Res*, Apr 1; 68(7):2186-93, 2008. PMCID: PMC2996041
45. Yao JJ, **Liu Y**, Lacorazza HD, Soslow RA, Scandura JM, Nimer SD, and Hedvat CV. Tumor promoting properties of the ETS protein MEF in ovarian cancer. *Oncogene*, Jun 7; 26(27):4032-7, 2007.
46. **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
47. Lacorazza HD, Yamada T, **Liu Y**, Miyata Y, Sivina M, Nunes J, and Nimer SD. The transcription factor MEF/ELF4 regulates the quiescence of primitive hematopoietic cells. *Cancer Cell*, Mar; 9(3):175-87, 2006.

48. 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.
- 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 8th 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.

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

Ph.D. THESIS DIRECTOR:

2012-2018	Sisi Chen, PhD Candidate, Department of Biochemistry and Molecular Biology, IUSM
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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-present 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-present Sasidhar Vemula, PhD, Postdoctoral Fellow, Indiana University School of Medicine

2019-present 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