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TITLE: Targeting Leukemia-initiating Cells to Improve Leukemia Treatment

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CONTRACTING ORGANIZATION: Indiana University, Indianapolis, IN

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14. ABSTRACT Pediatric leukemias account for almost 35% of all childhood cancers, leaving leukemia as the leading cause of cancer death for children. In addition to children, leukemia also affects adults. Adult leukemia usually occurs around age 60 and carries a very poor prognosis, with most patients live less than 18 months. Leukemia is initiated and maintained by a rare population of leukemia-initiating cells (LICs). LICs, and in particular those that are in a dormant state, are resistant to chemotherapy or targeted therapies. As we found that protein tyrosine phosphatase PRL2 is highly expressed in MLL leukemias, the objective of this proposal is to determine the effects of genetic and pharmacological inhibition of PRL2 on human leukemia-initiating cells in order to further assess its clinical potential. We found that PRL2 is essential for the self-renewal and survival of LICs expressing MLL-AF9. We developed a novel PRL2-specific inhibitor (PRLi) and found that PRLi treatment decreases the proliferation and survival of human MLL leukemia cells <i>in vitro</i> . Importantly, we found that <i>in vivo</i> PRLi treatment significantly increases the survival of MLL leukemia mice. The proposed work will facilitate the clinical application of PRL2 inhibitors in treating military personnel, veterans and their dependents with leukemia, thus improving their quality of life.					
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1. Introduction

This proposed research is designed to address the critical knowledge gap as identified by the **FY18 PRCRP Program: "Blood Cancers"**. Pediatric leukemias account for almost 35% of all childhood cancers, leaving leukemia as the leading cause of cancer death for children. In addition to children, leukemia also affects adults. Adult leukemia usually occurs around age 60 and carries a very poor prognosis, with most patients live less than 18 months. **Active duty military members** are frequently exposed to ionizing irradiation, chemicals, infectious agents and/or environmental carcinogens. This exposure can cause mutations in blood cells that lead to blood cancer (leukemia). For instance, there are increasing numbers of **Gulf War veterans** returning from theater with irradiation or toxin exposure-related leukemia. Despite significant progress in treating leukemia, some patient populations response poorly to conventional chemotherapy. Unfortunately, little progress has been made in treating leukemia over the past 4 decades. Clearly, new treatment strategies are urgently needed. Leukemia is initiated and maintained by a rare population of leukemia-initiating cells (LICs). LICs, and in particular those that are in a dormant state, are resistant to chemotherapy or targeted therapies. This proposal seeks to validate and pharmacologically modulate new leukemia targets with an eye toward clinical translation. As we found that protein tyrosine phosphatase PRL2 is highly expressed in MLL leukemias, the objective of this proposal is to determine the effects of genetic and pharmacological inhibition of PRL2 on human leukemia-initiating cells in order to further assess its clinical potential. We found that PRL2 is essential for the self-renewal and survival of LICs expressing MLL-AF9. We developed a novel PRL2-specific inhibitor (PRLi) and found that PRLi treatment decreases the proliferation and survival of human MLL leukemia cells *in vitro*. Importantly, we found that *in vivo* PRLi treatment significantly increases the survival of MLL leukemia mice. The proposed work will facilitate the clinical application of PRL2 inhibitors in treating military personnel, veterans and their dependents with leukemia, thus improving their quality of life.

2. Keywords

Leukemia, leukemia-initiating cells, PRL2, PRLi, targeted therapy, and MLL-AF9

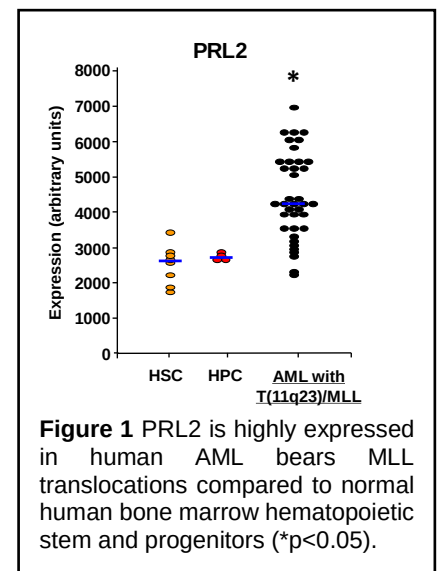
3. Accomplishments

Major goals and accomplishments: From September 1, 2020 to August 31, 2021, 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).

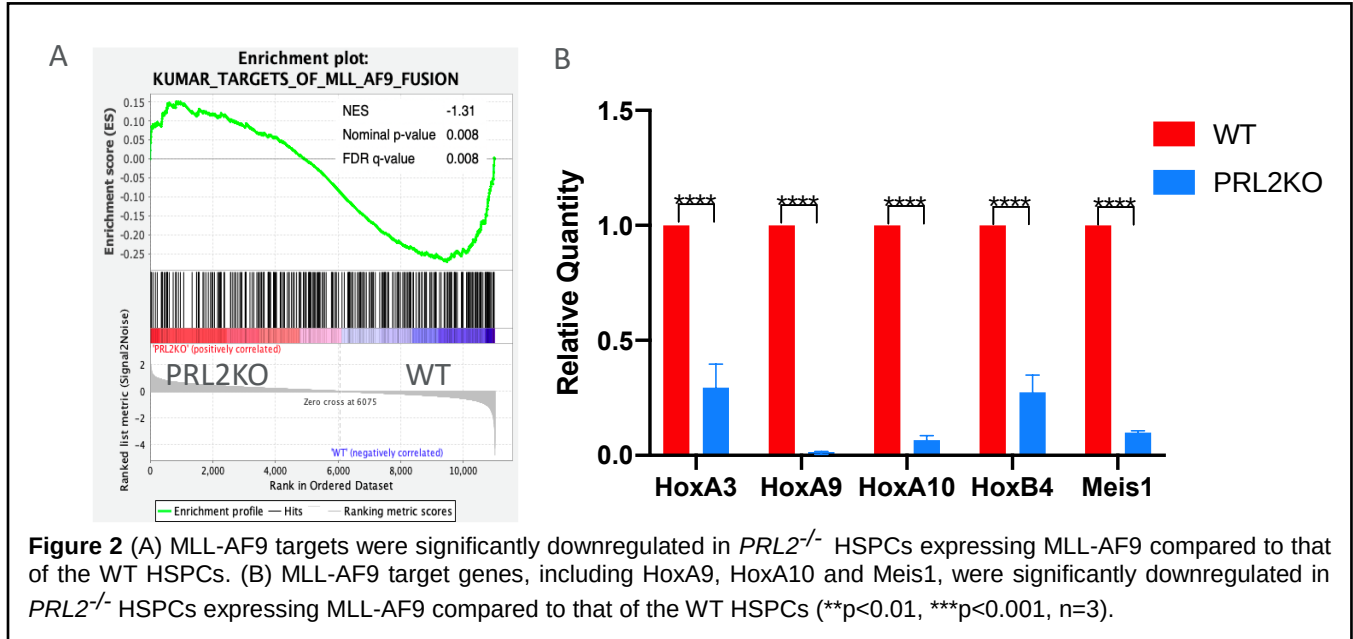
Specific Aim 1: Determine the impact of genetic and pharmacological inhibition of PRL2 on leukemia-initiating cells.

Rationale: MLL-rearranged leukemias represent about 10% of all leukemia cases, including acute lymphoblastic leukemia (ALL) and acute myeloid leukemia (AML). We found that PRL2 is highly expressed in human AML with MLL translocations (**Fig. 1**). Given that elevated PRLs have been shown to promote cancer cell proliferation and survival, PRL2 may be a potential therapeutic target in MLL leukemias. Increased self-renewal potential and enhanced survival are two key behaviors of leukemia-initiating cells. The objective of this aim is to determine the impact of inhibiting PRL2 on LIC self-renewal and survival in MLL leukemias. Our working hypothesis is that inhibition of PRL2 activity will decrease LIC self-renewal and survival. To test this hypothesis, we will determine the impact of genetic and pharmacological inhibition of PRL2 on human leukemia cells with MLL translocations by employing a mouse model of human AML and Patient-Derived Xenograft (PDX) models.

Major Task 1: Determine the impact of genetic inhibition of PRL2 on human leukemia cells with MLL translocations.

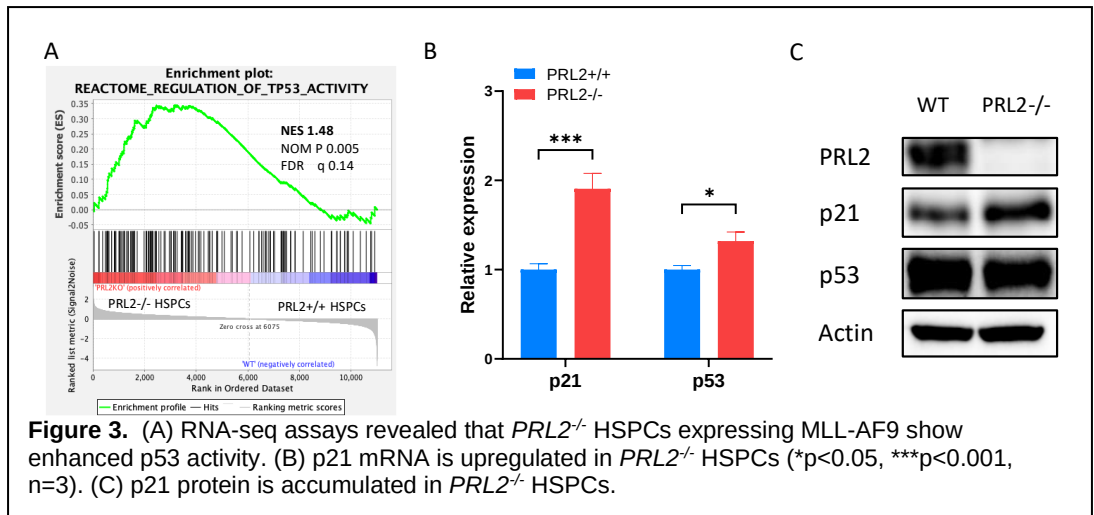


MLL-AF9 targets were significantly downregulated in *Prl2*^{-/-} HSPCs: To determine the mechanisms by which PRL2 enhances LIC self-renewal, we performed RNA-seq analysis to compare gene expression in *Prl2*^{+/+} and *Prl2*^{-/-} fetal liver HSPCs (LSKs) expressing MLL-AF9. To ensure reproducibility, three biological replicates were performed. We then employed Gene Set Enrichment Analysis (GSEA) to group potential PRL2 target genes into specific pathways important for LIC behavior. MLL-AF9 targets were significantly downregulated in *Prl2*^{-/-} HSPCs compared to *Prl2*^{+/+} HSPCs (**Fig. 2A**). We confirmed that MLL-AF9 target



genes that are important for LIC self-renewal, including *HoxA9*, *HoxA10*, and *Meis1*, are downregulated in *MLL-AF9*⁺*Prl2*^{-/-} HSPCs (**Fig. 2B**). Thus, we demonstrate that PRL2 is important for MLL-AF9-induced gene expression in HSPCs.

PRL2 null HSPCs expressing MLL-AF9 show enhanced p53 activity: The tumor suppressor protein p53, encoded by the *TP53* gene, plays an important role in preventing cancer development. However, p53 is frequently found mutated in human cancer. While the reported frequency of *TP53* mutations in AML is low (5-10%), p53 dysfunction is highly prevalent in AML. Previous studies have shown that p53 is functionally inactivated, but is rarely mutated in AML with *MLL* rearrangements.

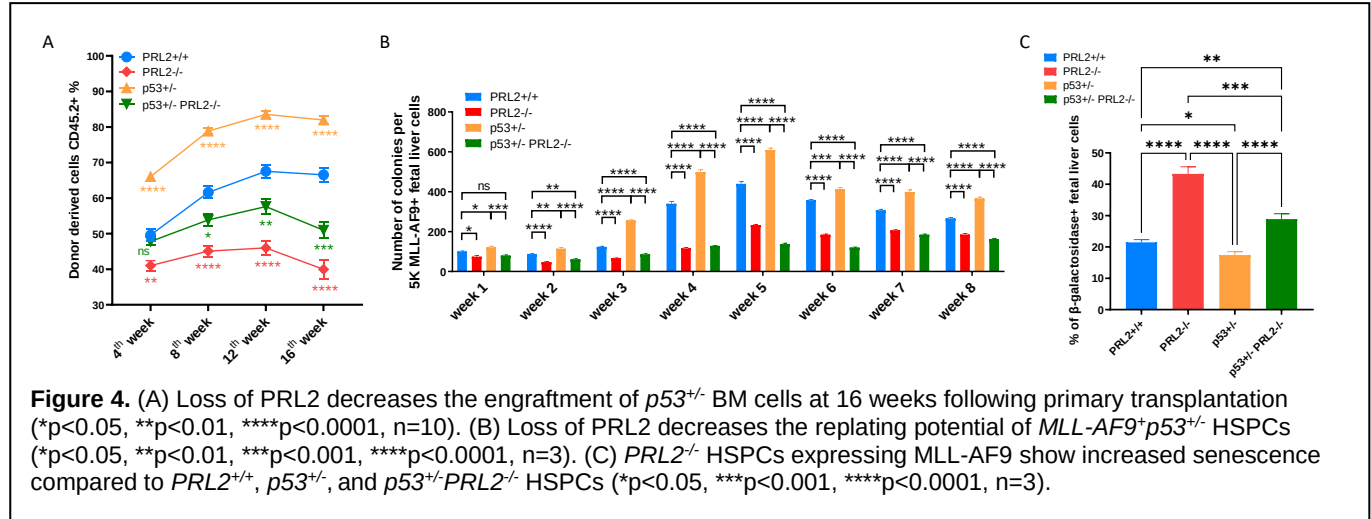


MLL fusion proteins such as MLL-AF9 are associated with suppression of WT p53 activity in leukemia cells. Our RNA-seq data revealed that p53 activity is increased in HSPCs expressing MLL-AF9 (**Fig. 3A**). While p53 expression is modestly increased in *Prl2*^{-/-} HSPCs, p53 target gene *p21* is significantly upregulated in *Prl2*^{-/-} HSPCs (**Fig. 3B**). We observed increased levels of p21 protein in *Prl2*^{-/-} HSPCs, whereas p53 levels are comparable in *Prl2*^{+/+} and *Prl2*^{-/-} HSPCs (**Fig. 3C**). These findings suggest that the p53 activity is increased in *Prl2*^{-/-} HSPCs.

Loss of PRL2 decreases the repopulating potential of *p53*^{+/+} HSPCs: To determine the interaction between p53 and PRL2 in leukemogenesis, we have generated *p53*^{+/+}*Prl2*^{-/-} mice. We performed competitive BM

transplantation assays and found that loss of PRL2 significantly decreases the engraftment of $p53^{+/-}$ BM cells at 16 weeks following transplantation (**Fig. 4A**), demonstrating that PRL2 is important for $p53^{+/-}$ BM cells to repopulate lethally irradiated host *in vivo*.

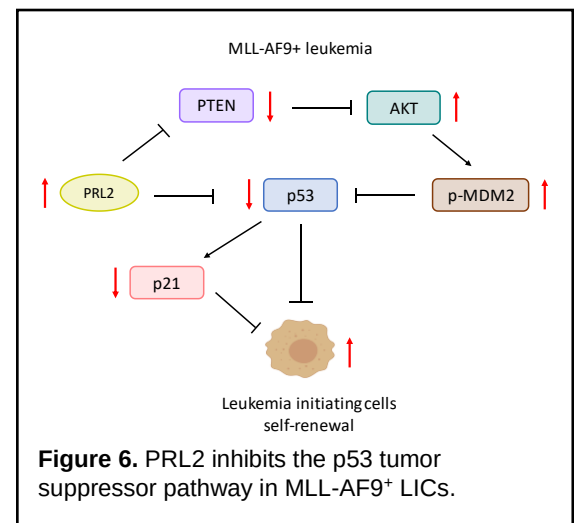
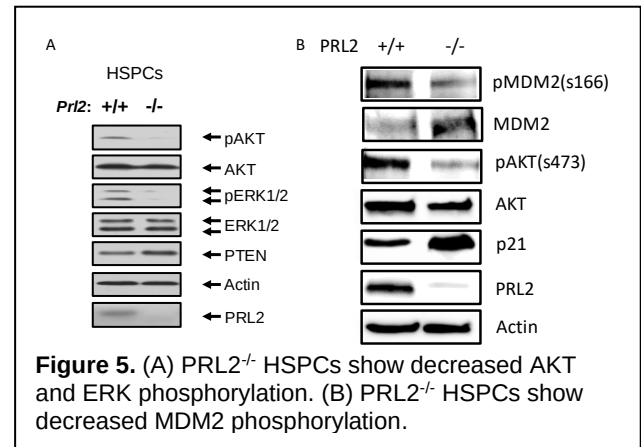
Loss of PRL2 decreases the replating potential of $p53^{+/-}$ HSPCs expressing MLL-AF9: To determine the role of PRL2 and p53 in MLL-AF9-driven leukemia, we introduced MLL-AF9 into $Prl2^{+/+}$, $p53^{+/-}$, $Prl2^{-/-}$, and $p53^{+/-}Prl2^{-/-}$ HSPCs using retroviruses and performed serial replating assays. We found that loss of PRL2 significantly decreases the replating potential of $p53^{+/-}$ MLL-AF9⁺ HSPCs (**Fig. 4B**). Further, we found that $Prl2^{-/-}$ HSPCs expressing MLL-AF9 show enhanced p53-dependent senescence compared to $Prl2^{+/+}$ HSPCs (**Fig. 4C**). These findings suggest that PRL2 may enhance MLL-AF9-driven leukemia through inhibiting p53 activity.



$Prl2^{-/-}$ HSPCs show decreased MDM2

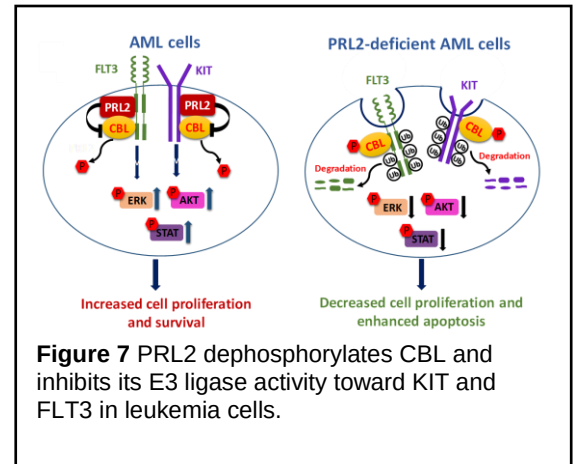
phosphorylation and increased levels of p21: To understand how PRL2 regulates p53 activity, we investigated the effect of PRL2 deficiency on the activation of signaling pathways in HSPCs. We found that the levels of pAKT and pERK were significantly lower in $Prl2$ null HSPCs compared with wild-type cells (**Fig. 5A**). We found that PRL2 null HSPCs show decreased MDM2 phosphorylation at Serine 166 (**Fig. 5B**). In addition, we observed increased levels of p21 in PRL2 null HSPCs (**Fig. 5B**), demonstrating that p53 activity is increased in PRL2 null HSPCs.

MLL fusion proteins such as MLL-AF9 are associated with suppression of WT p53 activity in leukemia cells. Notably, our RNA-seq data revealed that p53 activity is increased in $PRL2^{-/-}$ HSPCs expressing MLL-AF9 (**Fig. 3A**). Indeed, we discovered that cell cycle regulator p21, a key p53 target in hematopoietic cells, is accumulated in $PRL2^{-/-}$ HSPCs (**Fig. 3C**). One regulator of p53 activity is MDM2. In order for MDM2 to block p53 activity, MDM2 is phosphorylated by AKT, thereby gaining nuclear entry and facilitating nuclear exit of p53 to functionally inactivate it. We found that PRL2 null HSPCs show decreased MDM2 phosphorylation at Serine 166 (one of the AKT phosphorylation sites) (**Fig. 5B**), which may contribute to increased p53 activity in PRL2 null HSPCs. Based upon our preliminary studies and the literature, we hypothesize that PRL2 promotes LIC self-renewal and survival through inhibiting the p53 tumor suppressor pathway (**Fig. 6**).



Specific Aim 2: Determine the mechanisms by which PRL2 contributes to the pathogenesis of leukemia.

Rationale: While PRL2 is overexpressed in AML with *MLL* translocations (**Fig. 1**), its role in *MLL* leukemia is largely unknown. We found that loss of PRL2 significantly delayed the development of *MLL*-AF9-driven leukemia in mice and that PRL2 is essential for LIC self-renewal. Human leukemia harboring chromosomal translocations involving the *MLL* gene possess high-level of expression and frequent activating mutations of *FLT3*. Recent studies suggest *FLT3* mutations are present in human LICs, underscoring the importance of *FLT3* signaling in AML. While *MLL*-AF9 has been shown to cooperate with *FLT3*-ITD in leukemogenesis, the underlying mechanisms are not known. Most human leukemia cells depend on aberrant receptor tyrosine kinase signaling and the subsequent downstream effectors for proliferation and survival. KIT and *FLT3* are two of the major oncogenic receptor tyrosine kinases that are aberrantly activated in leukemia. We found that PRL2 enhances KIT activation in hematopoietic stem and progenitor cells following cytokine stimulation. KIT inactivation can be mediated by removal from the cell surface and intracellular degradation. Indeed, we found that PRL2 modulates KIT internalization in hematopoietic progenitor cells and leukemia cells. Further, KIT half-life is reduced in PRL2 null hematopoietic progenitor cells due to enhanced ubiquitination. 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. 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. We found that PRL2 showed enhanced association with CBL, KIT, and *FLT3* in human leukemia cells, suggesting that CBL may be a substrate of PRL2 in leukemia cells. We hypothesize that PRL2 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 (**Fig. 7**, 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* (**Fig. 7**, right panel).



Major Task 3: Cbl in *PRL2*^{-/-} LIC self-renewal.

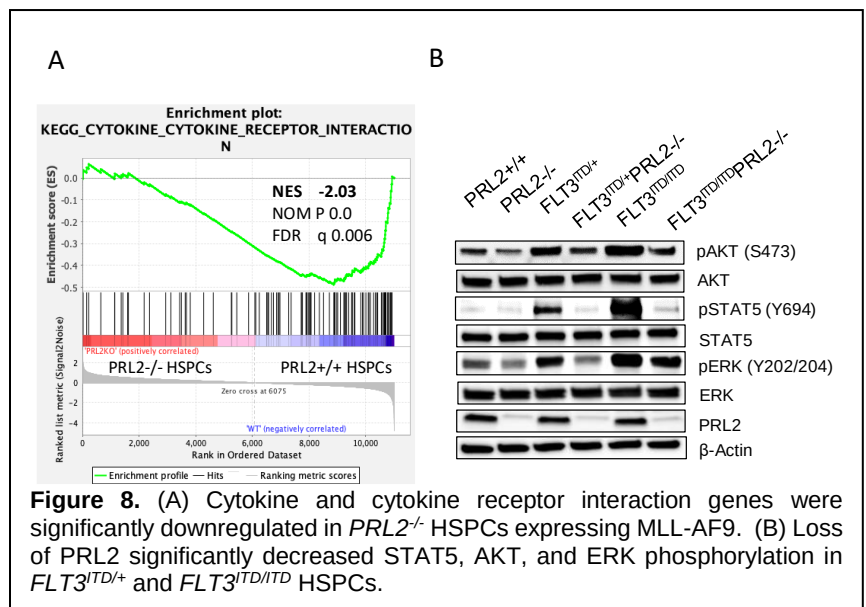
Generating *Cbl*^{-/-}*Prl2*^{-/-} mice: We obtained *Cbl* conditional knockout mice (*Cbl*^{fl/fl}-*Mx1Cre*⁺) from Dr. Hamid Band in 2020. We have crossed *Prl2*^{-/-} mice with *Cbl*^{fl/fl}-*Mx1Cre*⁺ mice. We expect to obtain *Prl2*^{-/-}*Cbl*^{fl/fl}-*Mx1Cre*⁺ mice by the end of 2021 and then start characterizing hematopoiesis in these mice.

Cytokine and cytokine receptor interaction genes are downregulated in *Prl2*^{-/-} HSPCs expressing *MLL*-AF9:

Our RNA-seq data also revealed that cytokine and cytokine receptor interaction genes are significantly downregulated in *Prl2*^{-/-} HSPCs compared to that of the WT HSPCs (**Fig. 8A**).

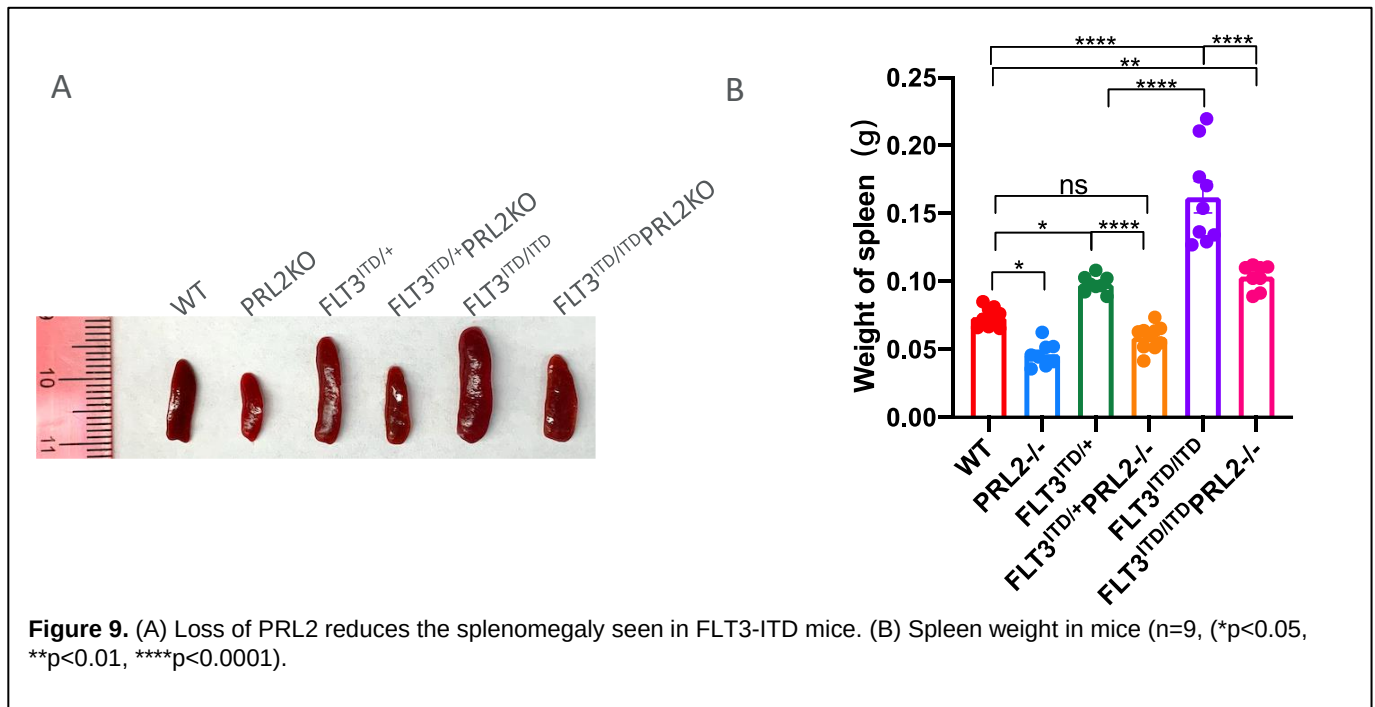
Notably, *Flt3* is one of the downregulated genes in *Prl2*^{-/-} HSPCs, suggesting that *FLT3* signaling is diminished in PRL2-deficient LICs.

Loss of PRL2 decreases STAT5, AKT, and ERK phosphorylation in *FLT3*-ITD⁺ HSPCs: To determine the impact of PRL2 on oncogenic *FLT3* signaling, we have generated *Prl2*^{-/-}*FLT3*^{ITD/+} and *Prl2*^{-/-}*FLT3*^{ITD/ITD} mice.



We examined STAT5, AKT, and ERK phosphorylation and found that loss of PRL2 decreases STAT5, AKT, and ERK phosphorylation in both $FLT3^{ITD/+}$ and $FLT3^{ITD/ITD}$ HSPCs (**Fig. 8B**), demonstrating that PRL2 is a key mediator of FLT3 signaling in HSPCs.

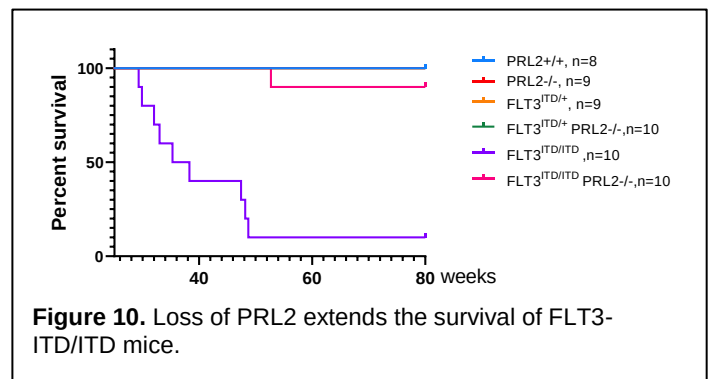
Loss of PRL2 reduces splenomegaly seen in FLT3-ITD mice: FLT3-ITD KI mice develop MPN overtime and show splenomegaly. We found that loss of PRL2 significantly reduced splenomegaly seen in FLT3-ITD mice (**Fig. 9 A and 9B**).



Loss of PRL2 extends the survival of $FLT3^{ITD/ITD}$ mice: To determine the impact of PRL2 in FLT3-ITD-driven MPN, we performed bone marrow transplantation assays. We transplanted 3×10^6 BM cells from PRL2^{+/+}, PRL2^{-/-}, $FLT3^{ITD/+}$, $FLT3^{ITD/ITD}$, PRL2^{-/-} $FLT3^{ITD/+}$, and PRL2^{-/-} $FLT3^{ITD/ITD}$ mice into lethally irradiated recipient mice and then monitored survival and disease development in these mice. We found that loss of PRL2 significantly extends the survival of $FLT3^{ITD/ITD}$ mice (**Fig. 10**).

Loss of PRL2 decreases the repopulating potential of $FLT3^{ITD/+}$ HSPCs: We performed serial competitive BM transplantation assays and found that loss of PRL2 significantly decreases the engraftment of $FLT3^{ITD/+}$ BM cells in both primary and secondary transplantation assays (**Fig. 11A** and data not shown), demonstrating that PRL2 is essential for the self-renewal of $FLT3^{ITD/+}$ HSPCs *in vivo*.

Loss of PRL2 decreases replating potential of MLL -AF9⁺ $FLT3$ -ITD⁺ HSPCs: To determine the role of PRL2 in MLL-AF9-driven leukemia, we introduced MLL-AF9 into PRL2^{-/-} $FLT3^{ITD/+}$ HSPCs. We performed serial replating assays and found that loss of PRL2 significantly decreases the replating potential of MLL -AF9⁺ $FLT3$ -ITD⁺ HSPCs (**Fig. 11B**), suggesting that PRL2 may be important for MLL-AF9 and FLT3-ITD-driven leukemia *in vivo*.



Opportunities for training and professional development:

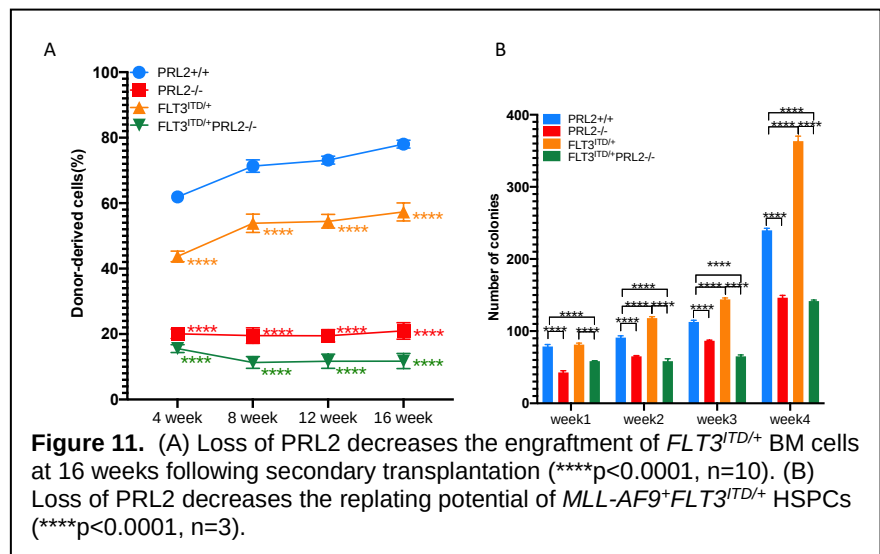
Dr. Yan Liu has trained four graduate students, including Sergio Barajas, Wenjie Cai, Shiyu Xiao and Christopher Borchers and three postdoctoral fellows, including Sasidhar Vemula and Hongxia Chen, from 2020 to 2021.

Dissemination of results to communities of interest:

Invited speaker (Dr. Yan Liu):

2020 Understanding and Targeting of Pre-leukemia Stem Cells and Leukemia Stem Cells, University of Florida.

2021 Understanding and Targeting of Pre-leukemia Stem Cells and Leukemia Stem Cells, Chicago Blood Club.



Future plans:

Specific Aim 1: Determine the impact of genetic and pharmacological inhibition of PRL2 on leukemia-initiating cells.

Major Task 2: Determine the impact of pharmacological inhibition of PRL2 on human leukemia cells with *MLL* translocations.

We will determine the effect of PRL2 inhibitor on human *MLL* leukemia cells *in vivo*.

Specific Aim 2: Determine the mechanisms by which PRL2 contributes to the pathogenesis of leukemia.

Major Task 4: PRL2 in CBL phosphorylation.

We will (1) determine the impact of PRL2 on CBL phosphorylation, and (2) map CBL phosphorylation sites in leukemia cells.

Major Task 5: CBL E3-dead mutant on KIT and FLT3 ubiquitination and degradation

We will determine the impact of CBL E3-dead mutant on KIT and FLT3 ubiquitination and degradation following SCF stimulation.

Major Task 6: Determine the impact of KIT and FLT3 inhibitors on human leukemia cells.

We will determine the effect of KIT and FLT3 inhibitors on human leukemia cells *in vivo*.

4. Impact

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

Pediatric leukemias account for almost 35% of all childhood cancers, leaving leukemia as the leading cause of cancer death for children. In addition to children, leukemia also affects adults. Adult leukemia usually occurs around age 60 and carries a very poor prognosis, with most patients live less than 18 months. **Active duty military members** are frequently exposed to ionizing irradiation, chemicals, infectious agents and/or environmental carcinogens. This exposure can cause mutations in blood cells that lead to blood cancer (leukemia). For instance, there are increasing numbers of **Gulf War veterans**

returning from theater with irradiation or toxin exposure-related leukemia. Despite significant progress in treating leukemia, some patient populations response poorly to conventional chemotherapy. Unfortunately, little progress has been made in treating leukemia over the past 4 decades. Clearly, new treatment strategies are urgently needed.

The **long-term goal** of this research is to develop new treatments for leukemia patients that decrease relapse and improve cure rates. Given that leukemia-initiating cells (LICs), and in particular those that are in a quiescent state and capable of self-renewal, are resistant to chemotherapy or targeted therapies, the **objective** of this proposal is to develop therapeutic strategies in order to eradicate LICs and improve leukemia treatment. At the completion of this proposed work, the anticipated **short-term outcome** is to establish PRL2 as a new druggable target in leukemia treatment and provide new translational information from preclinical animal models for the development of more effective therapeutic approaches that can eliminate drug-resistant leukemia-initiating cells (LICs) and improve leukemia treatment.

The **next translation goal** would be to move PRL2 inhibitors into clinical trials for both pediatric and adult leukemia patients. This advancement will also likely to change the standard of care for patients with leukemia. To facilitate the fast transition from bench to bedside, Dr. Liu has assembled a strong translational team consisting of basic scientists and clinicians. Dr. Boswell is the Co-Investigator on this grant. He is an attending physician of the Hematology/Oncology service at the Indiana University Simon Cancer Center and the Richard Roudebush VA Medical Center. He is involved in both patient care and patient-oriented research. In addition, we will collaborate with Dr. James Croop, an attending physician of the Pediatric Hematology/Oncology section at the Riley Hospital for Children. He is involved in both patient care and patient-oriented research, as well.

Drs. Boswell and Croop have been involved in multiple clinical trials involving adult and pediatric leukemia patients at the Simon Cancer Center and Riley Hospital for Children. We will discuss a project design for moving PRL2 inhibitor into clinical trials with the Simon Cancer Center and the Riley Hospital for Children Clinical Research Program. Upon completion of this study, we believe the approach for pharmacological targeting PRL2 in LICs will allow for the commencement of efficacy and safety phase I/II clinical trials in the near term, providing novel therapeutic options for leukemia patients. Thus, the proposed research is highly translational; it will facilitate the development of novel strategies for the treatment of leukemia and other human cancers. Indeed, the translation of the discovery from the proposed studies will likely expand the horizon for both leukemia research and for patients suffering from such a life-threatening disease.

Approximately **2,000 military personnel, veterans and their dependents** are expected to develop leukemia in the United States in 2018. Within the next decade, it is expected that patients with leukemia will receive new and effective medications that can eradicate the drug resistant leukemia-initiating cells, which will significantly benefit the **US military community**. Thus, the successful completion of these studies would be expected to have a potentially important **positive impact** on **US military personnel, veterans and their family members**. In addition, the novel information gained while conducting this work will be of great value to the **military**, as these approaches will facilitate the clinical application of PRL2 inhibitors and lead to life-saving therapies, improving the quality of life of **active duty military members, military families and, the US veterans**.

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

Dr. Yan Liu at the Indiana University has been the PI of this award since 2019. Dr. Liu moved to Northwestern University on April 1, 2021. Dr. James Croop at the Indiana University became the PI of this award on April 1, 2021. Dr. Yan Liu at the Northwestern University became the co-investigator (Co-I) of this award on April 1, 2021. Due to COVID pandemic, our research was significantly delayed. DOD granted one year no cost extension (NCE) to the award. We have been working closely with IRB office at Indiana and Northwestern University. We have submitted IRB and hope that it will be approved soon and we can start experiments related to leukemia patient samples. The Liu lab at the Northwestern University will work closely with Dr. James Croop at the Indiana University and complete experiments proposed in SOW by August 2022.

6. Products

Publications, conference papers, and presentations

a. Journal publications.

1. Broxmeyer HE, **Liu Y**, Kapur R. *et al.* Fate of Hematopoiesis During Aging. What Do We Really Know, and What are its Implications? ***Stem Cell Rev and Rep.*** 2020; (16): 1020–1048.
2. 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; 204(12):3262-3272. (**Co-corresponding author**).
3. 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. (manuscript in preparation, 2021).
4. Chen S, Yu H, Gao R, Liu X, Vemula S, Fahey AC, Henley D, Kobayashi M, Liu SZ, Wang Q, Qian Z, Kapur R, Broxmeyer H, Gao Z, Xi R and **Liu Y**. Bmi1 regulates Wnt signaling in hematopoietic stem and progenitor cells. ***Stem Cell Rev and Rep.*** (Submitted, 2021).

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

Nothing to Report

b. Other publications, conference papers, and presentations.

Nothing to Report

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

- a. *data or databases;*

Nothing to Report

- b. *biospecimen collections;*

Human leukemia 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);*

Cell lines: MV4-11 (control shRNA) and MV4-11 (PRL2 shRNA); Molm-13 (control shRNA) and Molm-13 (PRL2 shRNA)

Animal models: Cbl^{-/-}Prl2^{-/-}, FLT3-ITD-Prl2^{-/-}, and p53^{+/-}Prl2^{-/-} mice; Mouse models of human leukemia (MLI-AF9); and patient-derived xenograft (PDX) models of leukemia.

- 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>James Croop</i>
Project Role:	<i>PI</i>
Researcher Identifier (e.g. ORCID ID):	N/A
Nearest person month worked:	<i>1</i>
Contribution to Project:	Dr. Croop was responsible for supervising the conduct of the experiments, analyzing data, and obtaining human AML patient samples.
Funding Support:	

Name:	<i>Yan Liu</i>
Project Role:	<i>Co-I</i>
Researcher Identifier (e.g. ORCID ID):	0000-0003-4878-9111
Nearest person month worked:	<i>3</i>
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:	<i>1</i>

Contribution to Project:	<i>Dr. Liu provided statistical support for the project, including study design, sample size calculation, statistical inference, and any related requests.</i>
Funding Support:	<i>NIH</i>

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

Name:	<i>Wei Tong</i>
Project Role:	<i>Co-I</i>
Researcher Identifier (e.g. ORCID ID):	<i>N/A</i>
Nearest person month worked:	<i>2</i>
Contribution to Project:	<i>Dr. Tong supervised the conduct of the experiments, analyzed data, developed hypotheses, planned future experiments.</i>
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):	<i>N/A</i>
Nearest person month worked:	<i>12</i>
Contribution to Project:	<i>Dr. Chen generated mouse models of human AML and determined the impact of genetic and pharmacological inhibition of PRL2 on leukemia treatment. He designed and conducted the experiments listed in this proposal.</i>
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:** University of Pennsylvania
- ii. **Location of Organization:** Philadelphia
- 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. Wei Tong'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

What other organizations were involved as partners?

- i. **Organization Name:** Northwestern University
- ii. **Location of Organization:** Chicago
- iii. **Partner's contribution to the project** (*identify one or more*)

- 7. **Financial support;**

Nothing to Report

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

Nothing to Report

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

Nothing to Report

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

Dr. Yan Liu's lab performed experiments and provided reagents to the study.

11. **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

12. **Other.**

Nothing to Report

8. Special Reporting Requirements

COLLABORATIVE AWARDS: Not applicable

QUAD CHARTS: Not applicable

9. Appendices

- A. James Croop CV
- B. Yan Liu CV
- C. Publications

Name: James Merrill Croop

Address: Section of Pediatric Hematology/Oncology
James Whitcomb Riley Hospital for Children
Room 4340
705 Riley Hospital Drive
Indianapolis, Indiana 46202

Telephone:

Fax: (317) 948-0616

Email: jcroop@iupui.edu

Place of Birth: Pittsburgh, Pennsylvania

Education:

9/71-8/74 B.A. Department of Biology, University of Pennsylvania

9/74-6/80 Ph.D. Department of Anatomy, University of Pennsylvania

9/74-6/80 M.D. School of Medicine, University of Pennsylvania

Postdoctoral Training:

7/80-6/83 Pediatric Residency
Children's Hospital of Philadelphia
7/83-6/86 Pediatric Hematology/Oncology Fellowship
Dana-Farber Cancer Institute and
Children's Hospital Medical Center, Boston

Licensure and Certification:

1982 Pennsylvania Medical License
1983 Massachusetts Medical License
1985 Diplomat, American Board of Pediatrics
1990 Diplomat, Pediatric Hematology and Oncology
1996 Recertification, Pediatric Hematology and Oncology
1997 Indiana Medical License
2014 Recertification, Pediatric Hematology and Oncology

Academic Appointments:

7/80-6/83	Assistant Instructor, Department of Pediatrics University of Pennsylvania, School of Medicine
7/83-6/86	Research Fellow in Pediatrics, Harvard Medical School
7/86-6/88	Instructor in Pediatrics, Harvard Medical School
7/88-6/97	Assistant Professor, Department of Pediatrics, Harvard Medical School
7/97-6/03	Associate Professor, Department of Pediatrics, Indiana University School of Medicine
7/03-	Professor, Department of Pediatrics, Indiana University School of Medicine

Hospital Appointments:

7/86-6/88	Instructor in Medicine, The Children's Hospital, Boston
7/86-6/97	Clinical Associate in Pediatrics Dana-Farber Cancer Institute
7/88-6/97	Associate in Medicine, Hematology/Oncology The Children's Hospital, Boston
7/97-	James Whitcomb Riley Hospital for Children, Indianapolis

Principal Clinical and Hospital Service Responsibilities:

7/86-6/97	Staff Physician, Jimmy Fund Clinic Dana-Farber Cancer Institute
7/86-6/97	Clinic Leader, Jimmy Fund Clinic Dana-Farber Cancer Institute
7/87-6/97	Attending Physician, Oncology Ward The Children's Hospital, Boston
7/97-	Attending Physician, Section of Hematology/Oncology James Whitcomb Riley Hospital for Children

Visiting Appointments:

7/84-6/88	Visiting Scientist, Massachusetts Institute of Technology, Center for Cancer Research
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Clinical Investigation

- 1997-2004 Gene Therapy Working Group
Indiana University Medical Center
- 1999- 2004 Gene Therapy Working Group
Chair, Quality Assurance Committee
- 1998- Institutional Principal Investigator, COG Phase 1 Trials,
Riley Hospital for Children
- 1998-2013 Principal Investigator, A Pilot Study Of Dose-intensified
Procarbazine, CCNU, Vincristine (PCV) For Poor
Prognosis Pediatric And Adult Brain Tumors Utilizing
Fibronectin-assisted, Retroviral- Mediated Modification Of
CD34+ Peripheral Blood Cells With O6-Methylguanine
DNA Methyltransferase.
- 1998-1999 Principal Investigator, A Pilot Study of Recombinant
Human Interleukin 11 for Patients With Fanconi Anemia
and Inherited Bone Marrow Failure Syndromes.
- 1999-2004 Principal Investigator, Gene Therapy For Patients With
Fanconi Anemia: A Pilot Study. (Development Phase)
- 1999-2012 Principal Investigator, Fibronectin-Assisted, retroviral-Mediated
Transduction of CD34+ Peripheral blood Cells with gp91phox in
Patients with X-Linked Chronic Granulomatous Disease: A Phase 1
Study
- 2002-2003 Institutional PI, Phase II Irinotecan Study, Bristol Meyers.
- 2001-2004 Institutional PI, Zofran Study, GlaxoSmithKline
- 2006-2007 Institutional PI, COG Phase I Protocol, Phase I Study of PT-523
(Talotrexin) in Children and Adolescents with Recurrent Solid
Tumors (Development Phase).
- 2008-2009 Institutional PI, COG Phase I Protocol, Phase I Study of
GSK923295A, a centromere associated protein E (CENP-E)
inhibitor in pediatric patients with recurrent/refractory solid tumors.
- 2010--2011 Institutional PI, COG Phase I Protocol, A Phase 1 Study of OSI-906
in Pediatric Patients with Recurrent/Refractory Solid, including
CNS, Tumors
- 2014-- Co-Principal Investigator, COG Phase I Protocol, A Phase 1
Ramucirumab, a Humanized, Monoclonal Antibody Against the
Vascular Endothelial Growth Factor-2 (VEGFR-2) Receptor in
Children with Recurrent or Refractory Solid Tumors, including
CNS Tumors.

- 2016- Institutional PI, A Phase 1, Multi-center, Open-label, Dose De-escalation Study to Evaluate the Safety and Efficacy of Talimogene Laherparepvec in Pediatric Subjects with Advanced Non Central Nervous System Tumors That are Amenable to Direct Injection (Amgen).
- 2017- Institutional PI, A Phase 1/2 Study OF Lenvatinib in combination with Everolimus in Recurrent and Refractory Pediatric Solid Tumors, including CNS tumors” (COG Protocol ADVL1711)

Awards and Honors:

- 1974 Phi Beta Kappa, University of Pennsylvania
 1975-1980 Trainee, Medical Scientist Training Program
 1987-1992 NIH K08 Clinical Investigator Award
 1989-1990 DFCI BRSG Analysis of MDR in Drosophila
 1988-1993 NIH R01 Immunological Analysis of MDR
 1992-1993 DFCI BRSG Mechanisms of MDR Interactions
 1992-1993 Sandoz Pharmaceutical Development Award
 1992-1995 American Cancer Society Junior Faculty Award
 1993-1994 DFCI BRSG Solid Tumor Models of MDR
 1994-1995 DFCI BRSG Detection of MDR in vivo
 1994-1997 Sandoz Pharmaceutical Research Grant
 1995-1996 DFCI BRSG Characterization of Drosophila MDR Genes
 1997-1998 IUPUI Cancer Center Pediatric Development Grant
 1997-1998 NCCF Cancer Center Pediatric Development Grant
 1997-1998 Genetics Institute Clinical Trial Grant

Invited Presentations

- 1987 Platform Session, American Society of Hematology
 1988 Invited Lecture, Multidrug Resistance, Gordon Conference
 1990 Invited Lecture, Multidrug Resistance, Gordon Conference
 1991 Invited Lecture, AACR Conference on Drug Resistance
 1992 Invited Lecture, Annual Drosophila Research Conference
 1994 Pediatric Oncology Grand Rounds, Multidrug Resistance
 1995 Pediatric Oncology Grand Rounds, Multidrug Resistance
 1995 Presentation, AACR Conference on Drug Resistance
 1997 Presentation, National Pediatric Blood Club, Gene Therapy

1998 Invited Lecture, AML Workshop, Hospital St. Louis, Paris
 1998 Indiana University Cancer Center Grand Rounds, Gene Therapy
 1998 Pediatric Grand Rounds, Riley Hospital for Children, Gene Therapy
 2000 Clarian Hospital Nurses Pediatrics Seminar, Gene Therapy
 2001 Indiana University Cancer Center Grand Rounds, Pediatric Oncology
 2002 Pediatric Grand Rounds, Riley Hospital for Children, Phase I Studies
 2006 Child Care Conference, Hematology for the Pediatrician
 2011 Psi Iota Xi, Key Note Speaker, Leukemia

Teaching:

1977 Teaching Assistant in Histology
 University of Pennsylvania School of Medicine
 1987-1997 Resident and Medical Student Lectures
 Children's Hospital, Boston
 1988-1997 Laboratory Advisor, Undergraduate/Medical Students
 Post-doctoral students/Thesis Advisor
 1991/1995 Introduction to Clinical Medicine, Instructor
 1992-1996 Tutor, The Human Body, Harvard Medical School
 1992-1996 Histology Instructor, Human Body, Harvard Medical School
 1999 Medical Student Clinical Trial Elective Preceptor
 1999-2002 OSCE Development Committee, IUPUI School of Medicine
 2005-2014 Fellow Mentoring Committees (4)
 2018-2019 Fellow Mentoring Committee (1)
 2020- Medical Student Mentor (4)

Committee Assignments:

1977 Admissions Committee, U of Pennsylvania School of Medicine
 1992-1997 Pediatric Scientific Review Committee
 Dana-Farber Cancer Institute and Boston Children's Hospital
 1995, 1998 Experimental Therapeutics Study Section, NCI, Special Reader
 1995-1996 Quality Improvement Committee, Dana-Farber Cancer Institute
 1997-1999 Riley Children's Cancer Committee
 1997-1999 Scientific Review Committee, Member
 Indiana University Simon Cancer Center
 1999 -2017 Chair, Scientific Review Committee
 Indiana University Simon Cancer Center
 1999 - Clinical Director, Pediatric Hem/Onc Clinical Research Office
 1999 - Institutional Biohazard Committee, Alternate

1999-2015	Clarian/IU Health Cancer Committee
1999-2006	National Gene Vector Laboratory Advisory Committee, Alternate
1999-2017	Clinical Research Committee, IU Simon Cancer Center
2000	Inter-institutional Investigator Responsibilities, IRB Committee
2001-2004	Conflict of Interest Committee, IU School of Medicine
2001-2006	Research Subject Advocate, GCRC
2001-2003	Executive Committee, Society of Research Subject Advocates
2002-2004	Biomedical Human Subjects Research Education Committee
2001-2019	Member, COG Developmental Therapeutics Study Committee
2005-2011	Chair, COG Phase I Performance Monitoring Committee
2011-	Member, COG Membership Committee
2011-	Chair, COG Institutional Performance Monitoring Committee
2014-2015	Interim Director, Clinical Research Office, Simon Cancer Center
2015-	Fellowship Clinical Competency Committee
2015-2019	Member, COG Developmental Therapeutics Executive Committee
2015-	Member, Quality Council, Pediatric Hem/Onc Riley Hospital
2017-2018	Member, Primary Pediatric Dossier Committee, Riley Hospital
2016-2019	Member, COG Group Chair Advisory Council.
2018 -	Chair, Data and Safety Monitoring Committee, Simon Cancer Center
2020-	Member, Powerchart Oncology Committee, Riley Hospital
2020-	Chair, Growth Factor Communication QI Committee, Riley Hospital
2020-	Chair, Chemotherapy Consent Committee, Riley Hospital

Professional Societies:

1990-2008	American Association for Cancer Research
1992-2009	American Society of Hematology
1997-1999	Children's Cancer Group
1998-2008	American Society of Gene Therapy
1998-	American Society of Pediatric Hematology/Oncology
1999-	Children's Oncology Group

Major Research Interests:

1. Pediatric Oncology
2. Drug Development
3. Gene Therapy
4. Drug Resistance Genes
5. Hematopoietic Stem Cells

Publications

Journal Articles

1. Croop, J. and Holtzer, H. Response of myogenic and fibrogenic cells to Cytochalasin-B and Colcemid. *J. Cell Biol.* 1974; 65:271-285.
2. Holtzer, H., Croop, J., Gershon, M. and Somlyo, A. Effects of Cytochalasin B and Colcemid on cells in muscle cultures. *Am J. Anat.* 1975; 141:291-296.
3. Holtzer, H., Croop, J., Dienstman, S., Ishakawa, H. and Somlyo, A. Effects of Cytochalasin B and Colcemid on myogenic cultures. *Proc. Natl. Acad. Sci.* 1975; 72:513-517.
4. Bennett, G.S., Fellini, S., Croop, J., Otto, J., Bryan, J. and Holtzer, H. Differences among 100 Å Filament subunits from different cell types. *Proc. Natl. Acad. Sci.* 1978; 75:4364-4368.
5. Schmid, E., Tapscott, S., Bennett, G.S., Croop, J., Fellini, S., Holtzer, H., and Franke, W. Differential Location of different types of intermediate sized filaments in various tissues of the chick embryo. *Differentiation* 1979; 15:27-40.
6. Croop, J., Toyama, Y., Dlugosz, A. and Holtzer, H. Selective effects of phorbol-12-myristate-13-acetate on myofibrils and 10nm filaments. *Proc. Natl. Acad. Sci.* 1980; 77:5273-5277.
7. Holtzer, H., Pacifici, M., Croop, J., Boettiger, D., Toyama, Y., Payette, R., Biehl, J., Dlugosz, A. and Holtzer, S. Properties of cell lineages as indicated by the effects of ts-RSV and TPA on the generation of cell diversity. *Fortschritte der Zell.* 1982; 26:207-225.
8. Croop, J., Dubyak, G., Toyama, Y., Dlugosz, A., Scarpa, A., and Holtzer, H. Effects of TPA on myofibril integrity and calcium content in developing myotubes. *Dev. Biol.* 1982; 89:460-474.

9. Menko, A.S., Croop, J., Toyama, Y., Holtzer, H., and Boettiger, D. The response of chicken embryo fibroblasts to Cytochalasin B is altered by RSV induced cell transformation. *Mol. and Cell. Biol.* 1982; 2:320-330.
10. Rosenberg, H., Coleman, B., Croop, J., Granowetter, L., and Evans, A. Pseudocyesis in an adolescent patient. *Clin Ped* 1983; 22:708-712.
11. Croop, J., Shapiro, B., Gershon, A., and Campo, J. Arizona hinshawii osteomyelitis from a pet snake. *Ped Inf Disease* 1984; 3:188.
12. Gros, P., Croop, J., Roninson, I., Varshavsky, and Housman, D. Isolation and characterization of DNA Sequences Amplified in Multidrug Resistant Hamster cells. *Proc. Natl. Acad. Sci.* 1986; 83:337-341.
13. Gros, P., Ben Neriah, Y., Croop, J. and Housman, D.E. Isolation and Expression of a cDNA (mdr) that Confers Multidrug Resistance. *Nature* 1986; 323:728-731.
14. Gros, P., Fallows, D., Croop, J. and Housman, D.E. Chromosome Mediated Gene Transfer of Multidrug Resistance. *Mol. and Cell. Biol.* 1986; 6:3785-3790.
15. Gros, P., Croop, J., and Housman, D.E. Mammalian Multidrug Resistance Gene: Complete cDNA Sequence Indicates Strong Homology to Bacterial Transport Proteins. *Cell* 1986; 47:371-380.
16. Croop, J., Guild, B., Gros, P., and Housman, D. Genetics of Multidrug Resistance: Relationship of a Cloned Gene to the Complete Multidrug Resistance Phenotype. *Cancer Research* 1987; 47:5982-5988.
17. Arceci, R., Croop, J., Horwitz, S., and Housman, D. The mdr Gene is Induced and Expressed at High Levels During Pregnancy in the Secretory Epithelium of the Uterus. *Proc. Natl. Acad. Sci.* 1988; 85:4350-4354.
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19. Greenberger, L. Croop, J. Horwitz, S. and Arceci, R. P-glycoproteins encoded by *mdr1b* in murine gravid uterus and multidrug resistant tumor cell lines are differentially glycosylated. *FEBS Letters* 1989; 257:419-421.
20. Mazzanti, R., Gatmaitan, Z., Croop, J. Shu, H., and Arias, I. Quantitative image analysis of Rhodamine 123 transport by adriamycin sensitive and resistant NIH 3T3 and human hepatocellular carcinoma (Alexander) cells. *J. Cell. Pharmacol.* 1990; 1:50-56.
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22. Gros, P., Dhir., R., Talbot, F., Croop, J., Groulx, N. A single amino acid substitution strongly modulates the activity and substrate specificity of the mouse *mdr1* and *mdr3* drug efflux pumps. *Proc. Natl. Acad. Sci.* 1991; 88:7289-7293.
23. Wu, C-t., Budding, M., Griffin, M., and Croop, J. Isolation and characterization of *Drosophila* multidrug resistance gene homologues. *Mol. Cell. Biol.* 1991; 11:3940-3948.
24. Buschman, E., Arceci, R.J., Croop, J.M. Mingxin, C., Arias, I.M., Housman, D.E., Gros, P. Isoform specific antibodies identify the bile canalicular membrane as the primary site of expression of the P-glycoprotein encoded by mouse *mdr2*. *J. Biol. Chem.* 1992; 267:18093-18099.
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27. Piwnica-Worms, D., Chiu, M.L., Budding, M., Kronauge, J.F., Kramer, R.A., and Croop, J. Functional Imaging of Multidrug Resistance P-glycoprotein with and Organotechnetium Complex. *Cancer Research* 1993; 53:977-984.

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30. Dunussi-Joannopoulos, K., Nickerson, P., Strom, T., Weinstein, H., Burakoff, S., Croop, J.M., and Arceci, R.J. B-7 costimulatory immunotherapy in a mouse model of acute myelogenous leukemia. *Blood.* 1996; 87:2938-2946.
31. Bosch, I., Jackson, G.R., Croop, J.M., and Cantiello, H.F. Expression of *Drosophila melaogaster* P-glycoproteins is associated with ATP-channel activity. *Amer J Physiol.* 1996; 40:C1527-C1538.
32. Croop, J.M., Tiller, G.E., Fletcher, J.A., Lux, M.L., Raab, E., Goldenson, D., Son, D., Arciniegas, S., and Wu, R.L. Isolation and Characterization of a mammalian homolog of the *Drosophila* white gene. *Gene.* 1997; 185:77-85.
33. Dunussi-Joannopoulos, K., Krenger, K., Weinstein, H., Ferrara, J., Croop, J.M. CD8+ T-cells activated during the course of murine AML elicit therapeutic responses to late B7 vaccines after cytoreductive treatment. *Blood.* 1997; 89:2915-2924.
34. Bosch, I., Dunussi-Joannopoulos, K., Wu, R.L., Furlong, S., and Croop, J.M. Phosphatidylcholine and phosphatidylethanolamine are substrates of human MDR1 P-glycoprotein. *Biochemistry.* 1997; 36:5685-5694.
35. Bosch, I., Crankshaw, C.L., Piwnica-Worms, D, Croop, J.M. Characterization of Functional Assays of P-glycoprotein Transport Activity. *Leukemia.* 1997; 11:1131-1137.
36. Dunussi-Joannopoulos, K., Dranoff, G., Weinstein, H., Ferrara, J., Bierer, B. E., Croop, J.M. Gene Immunotherapy in murine Acute Myeloid Leukemia: Granulocyte-Macrophage colony-stimulating factor tumor cell vaccines elicit more potent antitumor immunity compare with B7 family and other cytokine vaccines. *Blood.* 1998; 91:222-230.

37. Barbarics, E., Kronauge, J.F., Cohen, D., Davison, A., Jones, A.G. Croop, J.M. Characterization of P-glycoprotein transport and inhibition in vivo. 1998; Cancer Res 58:276-282.
38. Lee, G.Y., Croop, J.M. Anderson, E. Multidrug resistance gene expression correlates with progesterone production in dehydroepiandrosterone-induced polycystic and equine chorionic gonadotropin-stimulated ovaries of prepubertal rats. Biology of Reproduction 1998; 58:330-337.
39. Croop J.M., Cooper, R., Seshadri R., Fernandez, C., Graves, V., Kreissman, S., Smith, F.O., Cornetta, K., Williams, D.A., Abonour R. Large Scale Mobilization and Isolation of CD34+ Cells from Normal Donors. Bone Marrow Transplantation. 2000; 26:1271-1279.
40. Chen, J. Reeves, L. Sanburn, N., Croop, J. Willaims, D.A., Cornetta, K. Packaging cell line DNA contamination of vector supernatants: Implication for laboratory and clinical research. Virology. 2001; 282:186-197
41. Croop, J.M., Cooper, R., Fernandez, C., Graves, V., Kreissman S., Hanenberg, H., Smith, F.O., Cornetta K., Williams D.A. Mobilization and Collection of Peripheral Blood CD34+ Cells from Patients with Fanconi Anemia. Blood 2001; 98: 2917-2921
42. Kumar, M., Vik, T., Johnson, C. Southwood, M.E., Croop, J.M. Treatment, outcome and cost of care in children with idiopathic thrombocytopenic purpura. Am J Hem 2005; 78:181-187.
43. Cavalier M.E., Davis M.M., Croop J.M. Germline p53 mutation presenting as synchronous tumors. Journal of Pediatric Hematology/Oncology. 2005 27:441-443.
44. Daw N.C., Furman W.L., Stewart C.F., Iacono L.C., Krailo M., Bernstein M.L., Dancey J.E., Speights R.A., Blaney S.M., Croop J.M., Reaman G.H., Adamson P.C. Phase I and pharmacokinetic study of gefitinib in children with refractory solid tumors: a Children's Oncology Group Study. Journal of Clinical Oncology. 2005 23:6172-6180.
45. Cornetta K. Croop J. Dropcho E. Abonour R. Kieran MW. Kreissman S. Reeves L. Erickson LC. Williams DA. A pilot study of dose-intensified procarbazine, CCNU, vincristine for poor prognosis brain tumors utilizing

fibronectin-assisted, retroviral-mediated modification of CD34+ peripheral blood cells with O6-methylguanine DNA methyltransferase. *Cancer Gene Therapy*. 2006 13:886-895.

46. Mondick J.T., Johnson BM, Haberer L.J., Sale M.E., Adamson P.C., Coté C.J., Croop J.M., Russo M.W., Barrett J.S., Hoke J.F. Population pharmacokinetics of intravenous ondansetron in oncology and surgical patients aged 1-48 months. *European Journal of Clinical Pharmacology*. 2010 66:77-86.

47. Creary S, Heiny M, Croop J, Fallon R, Vik T, Hulbert M, Knoderer H, Kumar M, Sharathkumar A. Clinical course of postthrombotic syndrome in children with history of venous thromboembolism. *Blood Coagul Fibrinolysis*. 2012 23:39-44.

48. Robertson KA, Nalepa G, Yang FC, Bowers DC, Ho CY, Hutchins GD, Croop JM, Vik TA, Denne SC, Parada LF, Hingtgen CM, Walsh LE, Yu M, Pradhan KR, Edwards-Brown MK, Cohen MD, Fletcher JW, Travers JB, Staser KW, Lee MW, Sherman MR, Davis CJ, Miller LC, Ingram DA, Clapp DW. Imatinib mesylate for plexiform neurofibromas in patients with neurofibromatosis type 1: a phase 2 trial. *Lancet Oncol*. 2012 13:1218-1224.

49. Mueller, E.L., Croop, J., Carroll, AE. Fever and neutropenia hospital discharges in children with cancer: A 2012 update. *Pediatr Hematol Oncol*. 2016 33:39-48.

50. Kobayashi M, Bai Y, Chen S, Gao R, Yao C, Cai W, Cardoso AA, Croop J, Zhang ZY, Liu Y. Phosphatase PRL2 promotes oncogenic NOTCH1-Induced T-cell leukemia. *Leukemia*. 2017 31:751-754.

Reviews

1. Holtzer, H., Croop, J., Toyama, Y., Bennett, G.S., Fellini, S. and West, C. Differences in differentiation programs between presumptive myoblasts and their daughters the definitive myoblast and myotubes. In: *Plasticity of Muscle*. Pette, D. (Ed) Gruter & Co., Berlin, 1980:133-146.

2. Holtzer, H., Bennett, G.S., Tapscott, S. J., Croop, J., Dlugosz, A. and Toyama, Y. Changes in intermediate-sized filaments during myogenesis and neurogenesis. In: *International Cell Biology*. Schweiger (Ed) Springer-Verlang, Berlin, 1981:294-304.

3. Holtzer, H., Pacifici, M., Payette, R., Croop, J., Dlugosz, A. and Toyama, Y. TPA reversibly blocks the differentiation of chick myogenic, chondrogenic and melanogenic cells. In : Carcinogenesis, Vol. 7, Hecker, E., et. al. (Ed) Raven Press, N.Y., 1982:347-357.
4. Holtzer, H., Bennett, G.S., Tapscott, S.J., Croop, J., and Toyama, Y. Intermediate-size filaments: Changes in synthesis and distribution in cells of the myogenic and neurogenic lineages. Cold Spring Harb Symp 1982; 46:317-329.
5. Housman, D., Croop, J., Mukaiyama, T., Abelson, H., Roninson, I., and Gros, P. A molecular genetics approach to the problem of drug resistance in chemotherapy. In: New Avenues in Developmental Cancer Chemotherapy. Academic Press, 1987:503-517.
6. Croop, J., Gros, P., and Housman, D. Genetics of multidrug resistance. J. Clin. Invest. 1988; 81:1303-1309.
7. Croop, J. P-glycoprotein structure and evolutionary homologies. In: Multiple Drug Resistance in Cancer, Clynes, M. (Ed), Kluwer Academic Publishers, 1994:1-32.
8. Bosch, I., Croop J. P-glycoprotein and cancer. BBA Reviews on Cancer Biochim Biophys Acta, 1996; 1288:F37-F54.
9. Dunussi-Joannopoulos, K., Weinstein, H., Arceci, R.J., and Croop, J.M. B-7 Gene therapy with B7.1 and GM-CSF vaccines in a murine AML model. J Ped Hem/Onc. 1997 19:536-540.
10. Bosch, I., and Croop, J.M. P-glycoprotein structure and evolutionary homologies. Cytotechnology. 27:1-30, 1998.
11. Croop, J.M. Evolutionary relationships among ABC Transporters. Meth. Enz. 1998 292:101-116.
12. Abonour R, Croop, J., Cornetta, K. Multidrug-resistance gene therapy in hematopoietic cell transplantation. In, Gene Therapy of Cancer, Ed. Lattime, E.C., and Zgerson, S.L., Academic Press, San Diego, 2001 pp. 355-364.

13. Croop, J. Gene Therapy for Fanconi Anemia. *Current Hematology Reports*. 2003; 2:335-340

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Abstracts

1. Croop, J., Gros, P., and Housman, D. Mutational analysis of a site involved in colchicine resistance in the mouse *mdr1* gene. *Proc. AACR* 32:(1991).

2. Abonour, R., L. Einhorn, R. Hromas, M.J. Robertson, E. Srour, C.M. Traycoff, A. Bank, I. Kato, K. Asada, J. Croop, F.O. Smith, D.A. Williams, K. Cornetta Highly Efficient Mdr-1 Gene Transfer Into Humans Using Mobilized CD34+ Cells Transduced Over Recombinant Fibronectin CH-296 Fragment. 1998; *Blood* 92(Suppl):690a.

3. J.M. Croop, R. Cooper, H. Hanenberg, S. Kreissman, C. Thompson, V. Graves, F.O. Smith, D.A. Williams. Feasibility Of Large Scale Peripheral Blood CD34+ Cell Collections In Normal Donors And In Patients With Fanconi Anemia. *Blood* 1998; 92(Suppl):441a.

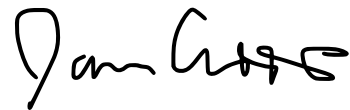
4. Pradhan, K., Berg, S.L., Liu, X., Minard, C.J., Croop, J., Reid, J.M., Fox, E., and Weigel, B.J. A dose escalation and toxicity study using pre-determined target concentration of Ramucirumab, a novel anti-angiogenic agent in children with recurrent / refractory solid tumors: A report from the children's oncology group phase I/ pilot consortium (ADVL1416). 2018: *Cancer Res* (13 Supplement): 78

5. Filemon S. Dela Cruz, Elizabeth Fox, Jodi Ann Muscal, Ivan Kirov, James I. Geller, Steven G. DuBois, AeRang Kim, James Croop, Theodore Willis Laetsch, Charles G. Minard, Xiaowei Liu, Brenda Weigel, Chinyere E. Okpara, Jie Huang, Lea Dutta, Jagadeesh Aluri, Julia Lynne Glade Bender. A Phase 1 Study of Talimogene Laherparepvec (T-Vec) in Pediatric Patients with Advanced Solid Tumors. 2020: *Journal of Clinical Oncology* 38:15(Suppl)

Patents

1. Croop, J.M., Gros, P., and Housman, D. DNA Sequence That Encodes the Multidrug Resistance Gene. Patent No. 5,198,344; Issued March 30, 1993.
2. Arceci, R. J., and Croop, J.M. Antibodies for P-glycoprotein encoded by the MDR1 Gene and Uses Thereof. Patent No. 5,369,009; Issue November 29, 1994.

3/16/21

A handwritten signature in black ink, appearing to read "Jan Giese". The signature is fluid and cursive, with a large initial "J" and a stylized "G".

CURRICULUM VITAE

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

PROFESSIONAL ORGANIZATIONS

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

PROFESSIONAL SERVICE

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

HONORS AND AWARDS

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

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

RESEARCH SUPPORT

CURRENT ACTIVE SUPPORT:

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

PENDING SUPPORT:

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

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

Role: Principal Investigator

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

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

Role: Principal Investigator

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

Understanding and Targeting PRL2 Phosphatase in Acute Myeloid Leukemia

Role: Principal Investigator

COMPLETED SUPPORT:

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

Indiana University School of Medicine (IUSM)

p53 in normal and malignant hematopoiesis

Role: Principal Investigator

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

Mutant p53 rejuvenates aged stem cells through modulating epigenetic regulators

Role: Principal Investigator

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

Molecular mechanisms underlying clonal expansion of hematopoietic stem cells

Role: Principal Investigator

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

Modulating Leukemia-initiating Cell Quiescence to Improve Leukemia Treatment

Role: Principal Investigator

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

Understanding and Targeting Mutant p53 in Myelodysplastic Syndromes

Role: Principal Investigator

NCE

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

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

Role: Co-investigator

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

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

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

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

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

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

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

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

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

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

Role: Principal Investigator

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

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

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

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

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

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

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

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

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

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

Role: Principal Investigator

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

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

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

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

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

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

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

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

PUBLICATIONS (*Corresponding author)

PUBLICATIONS SUBMITTED/IN PREPARATION

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

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

2. Kobayashi M, Bai Y, Chen S, Nabinger SC, Vemula S, Henley D, Fahey AC, Yao C, Sun J, Boswell HS, Croop JM, Rönnstrand L, Kapur R, Zhang ZY, and **Liu Y***. PRL2 phosphatase enhances oncogenic cytokine signaling in leukemia cells. (Manuscript in preparation)
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. ***Stem Cell Rev and Rep*** (Submitted, 2021).
4. Olivos D, Hauck P, Jacobsen M, Sandusky G, **Liu Y**, and Mayo L. Emergence of Tumor-Initiating Cells with the Loss of Mdm2. *Molecular Cancer research* (Submitted, 2021).

PEER-REVIEWED PUBLICATIONS

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

12. Chen S, Gao R, Yao C, Kobayashi M, Liu SZ, Yoder MC, Broxmeyer HE, Kapur R, Boswell HS, Mayo LD, and **Liu Y***. Genotoxic stresses promotes the clonal expansion of hematopoietic stem cells expressing mutant p53. *Leukemia*. 2018 Mar;32(3):850-854. PMID:PMC5842141
13. 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**
14. Yao C, Kobayashi M, Chen S, Nabinger S, Gao R, Liu SZ, Asai T, and **Liu Y***. Necdin modulates leukemia-initiating cell quiescence and chemotherapy response. *Oncotarget*. 2017; 8:87607-87622. PMID: PMC5675657.
15. Kobayashi M, Chen S, Bai Y, Yao C, Gao R, Sun XJ, Mu C, Twiggs TA, Yu ZH, Boswell HS, Yoder M, Kapur R, Mulloy J, Zhang ZY, and **Liu Y***. Phosphatase PRL2 promotes AML1-ETO-induced acute myeloid leukemia. *Leukemia*. 2017 June; 31(6):1453-1457. PubMed PMID: 28220038. PMID:PMC5695226
16. Kobayashi M, Bai Y, Yoshimoto M, Gao R, Chen S, Yao C, Dong Y, Zhang L, Rodriguez S, Carlesso N, Yoder MC, Kapur R, Kaplan MK, Lacorazza HD, Zhang ZY, and **Liu Y***. Protein tyrosine phosphatase PRL2 mediates Notch and Kit signals in early T cell progenitors. *Stem Cells*. 2017 Apr;35(4):1053-1064. PMID: PMC5367971.
17. Kobayashi M, Bai Y, Chen S, Gao R, Yao C, Cai W, Croop J, Zhang ZY and **Liu Y***. Phosphatase PRL2 promotes oncogenic Notch-induced T cell leukemia. *Leukemia*. 2017 Mar;31(3):751-754. PMID:PMC5695227
18. Chen S, Gao R, Kobayashi M, Yu H, Yao C, Kapur R, Yoder M, and **Liu Y***. Pharmacological inhibition of AKT activity in human CD34+ cells enhances their engraftment in immunodeficient mice. *Experimental Hematology*. 2017 Jan;45:74-84. PubMed Central PMID: PMC5859321.
19. Ghosh J, Kobayashi M, Ramdas B, Chatterjee A, Ma P, Mali RS, Carlesso N, **Liu Y**, Plas DR, Chan RJ, and Kapur R. S6K1 regulates Stem Cell Self-Renewal and Leukemia Maintenance. *Journal of Clinical Investigation*. 2016 Jul 1;126(7):2621-5. PMID:PMC4922705.
20. Gao R, Chen S, Kobayashi M, Yu H, Young SK, Soltis A, Zhang Y, Wan Y, Vemula S, Fraenkel E, Cantor A, Xu Y, Yoder MC, Wek R, Kapur R, Ellis S, Zhu X and **Liu Y***. Bmi1 promotes erythropoiesis through regulating ribosome biogenesis. *Stem Cells*. 2015 Mar;33(3):925-38. PMID:PMC4380436.
21. Chatterjee A, Ghosh J, Ramdas B, Mali RS, Martin H, Kobayashi M, Vemula S, Canela VH, Waskow ER, Visconte V, Tiu RV, Smith CC, Shah N, Bunting KD, Boswell HS, **Liu Y**, Chan RJ, Kapur R. Regulation of Stat5 by FAK and PAK1 in Oncogenic FLT3- and KIT-Driven Leukemogenesis. *Cell Reports*. 2014 Nov 20;9(4):1333-1348. PMID:PMC4380442.

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

33. Hu P, Carlesso N, Xu M, **Liu Y**, Nebreda AR, Takemoto C, and Kapur R. Genetic evidence for critical roles of P38 α in regulating mast cell differentiation and chemotaxis through distinct mechanisms. *J. Biol. Chem*, June 8;287(24):20258-69, 2012. PMCID: PMC3370208
34. Huang G, Zhao X, Wang L, Elf S, Xu H, Zhao X, Sashida G, **Liu Y**, Lee J, Menendez S, Yang Y, Yan Y, Zhang P, Tenen DG, Osato M, Hsieh J, and Nimer SD. The ability of MLL to bind RUNX1 and methylate H3K4 at PU.1 regulatory regions is impaired by MDS/AML-associated RUNX1/AML1 mutations. *Blood*, Dec 15;118(25):6544-52, 2011. PMCID: PMC3242717
35. Asai T, **Liu Y**, Bae N, and Nimer SD. The p53 tumor suppressor protein regulates hematopoietic stem cell fate. *J Cell Physiol*, Sep; 226(9):2215-21, 2011. PMCID: PMC3575444.
36. Sashida G, Bae N, Di Giandomenico S, Asai T, Gurvich N, Bazzoli E, **Liu Y**, Huang G, Zhao X, Menendez S, Nimer SD. (2011). The Mef/Elf4 transcription factor fine tunes the DNA damage response. *Cancer Research*, July 15; 71(14): 4857-65, 2011. PMCID: PMC4073677
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INVITED PRESENTATIONS

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

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

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

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

PROFESSIONAL SERVICE

EDITORIAL BOARD:

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

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

UNIVERSITY SERVICE

INSTITUTIONAL COMMITTEE:

2012 to 2017	Member, Institutional Animal Care and Use Committee (IACUC), Indiana University School of Medicine
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2017 to 2020	Member, Graduate Faculty Council, Indiana University
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REVIEWER FOR GRANTS:

2011	IUSCC ITRAC pilot project
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2012	Simon Clinical Research pilot project
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2013	IUSCC Pancreatic Center pilot project
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2013	IUSCC Bioinformatics Core Pilot projects
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2013-2016	IUSM Wright Scholarship
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2015	Indiana CTSI Core Pilot projects
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2017	IUSCC ITRAC pilot project
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2017	IUSCC Associate Member pilot project
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2018	IUSCC Associate Member pilot project
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2018	Wells Center Translational Research Grant
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2019	Wells Center Translational Research Grant
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OTHER SERVICE:

2012 to 2016	Associate Editor, the Wells Center Newsletter
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2012	Poster Judge, Research Day, Department of Biochemistry and Molecular Biology, March 2012
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2013 - 2017	Poster Judge, Cancer Research Day, Indiana University Cancer Research Day
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2013 - 2015	Panelist and Judge, Wells Center Summer Intern Program, Oral Presentation
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2013 -2015	Poster Judge, Student Research Program in Academic Medicine Poster Presentations
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2013	Interview prospective international graduate students for the IBMG program, IUSM, March 2013
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2014 -2018	Career Panelist, Molecular Medicine in Action (MMIA) program, IUSM
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TEACHING ASSIGNMENTS

UNDERGRADUATE SUMMER STUDENTS AND MEDICAL STUDENTS:

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

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

GRADUATE SCHOOL THESIS COMMITTEES:

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

Ph.D. THESIS DIRECTOR:

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

POSTDOCTORAL FELLOWS:

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

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

AWARDS TO STUDENTS AND POSTDOCTORAL FELLOWS:

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

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

GRADUATE TEACHING ASSIGNMENTS:

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

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

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

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

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

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

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

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

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

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

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



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

Hal E. Broxmeyer¹ · Yan Liu² · Reuben Kapur² · Christie M. Orschell³ · Arafat Aljoufi¹ · James P. Ropa¹ · Thao Trinh¹ · Sarah Burns² · Maegan L. Capitano¹

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Abstract

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

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

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

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

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

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

A) Aging and Stem Cells in General

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

Age-Related Clonal Hematopoiesis

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

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

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

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

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

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

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

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

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

Inflammation

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

Aging-Related Apoptosis and Autophagy

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

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

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

Radiation Effects and Aging

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

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

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

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

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

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

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

The Role of Sirtuins in Regulating Aged HSC Function

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

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

G) The Microbiome, Hematopoiesis, and Aging

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

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

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

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

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

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

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

Role of Collection/Processing of Cells

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

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

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

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

The Microenvironment

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

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

Leptin (Lep) and Lep Receptors (R)

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

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

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

More on Inflammation

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

Exosomes

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

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

Biological Time Keeping and Circadian Rhythms

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

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

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

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

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

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

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

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

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

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

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

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

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

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

Cytokines, Chemokines and Intracellular Signaling

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

Dipeptidylpeptidase (DPP)4

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

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

Concluding Thoughts

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

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

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

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

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

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

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Bmi1 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'-CCTGGTTCATCATCGCTAATC-3'; *Bmi1* F, 5'-CAGGTTCAACAACACAGACCAC-3' and R, 5'-TGACGGGTGAGCTGCATAAA-3'; *Arf*

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

Retrovirus production and infection

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

Transplantation

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

Microarray analysis

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

Bioinformatics analysis of microarray

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

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

Chromatin immunoprecipitation

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

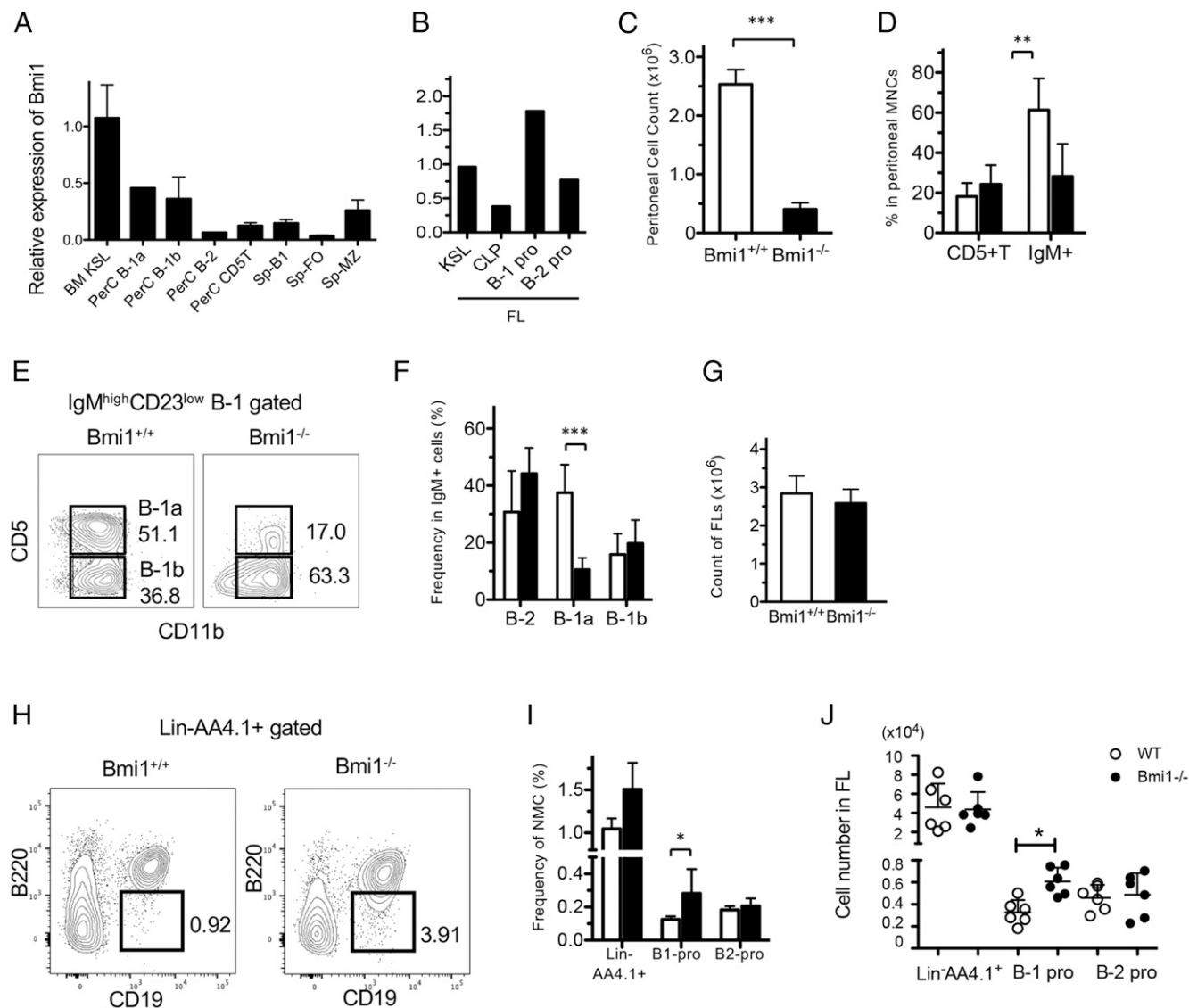


FIGURE 1. Peritoneal B-1a cells have higher *Bmi1* dependency than other lymphoid subsets. **(A)** *Bmi1* mRNA expression measured by qPCR in various sorted populations from adult BM, peritoneal cells (PerC), or spleen (Sp). **(B)** *Bmi1* expressions in various FL progenitor populations. **(C)** Marked 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 ($n = 6$). * $p < 0.03$. 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 ($20\text{--}30 \times 10^3$) from each genogroup were plated in the 96-well plate in IMDM with 10% FBS, 10 ng/ml IL-5, 5 $\mu\text{g/ml}$ R848 (TLR7/8 ligand), and 5 $\mu\text{g/ml}$ LPS. Forty-eight hours later, cells were collected, and cell numbers were counted.

Statistical analysis

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

Results

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

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

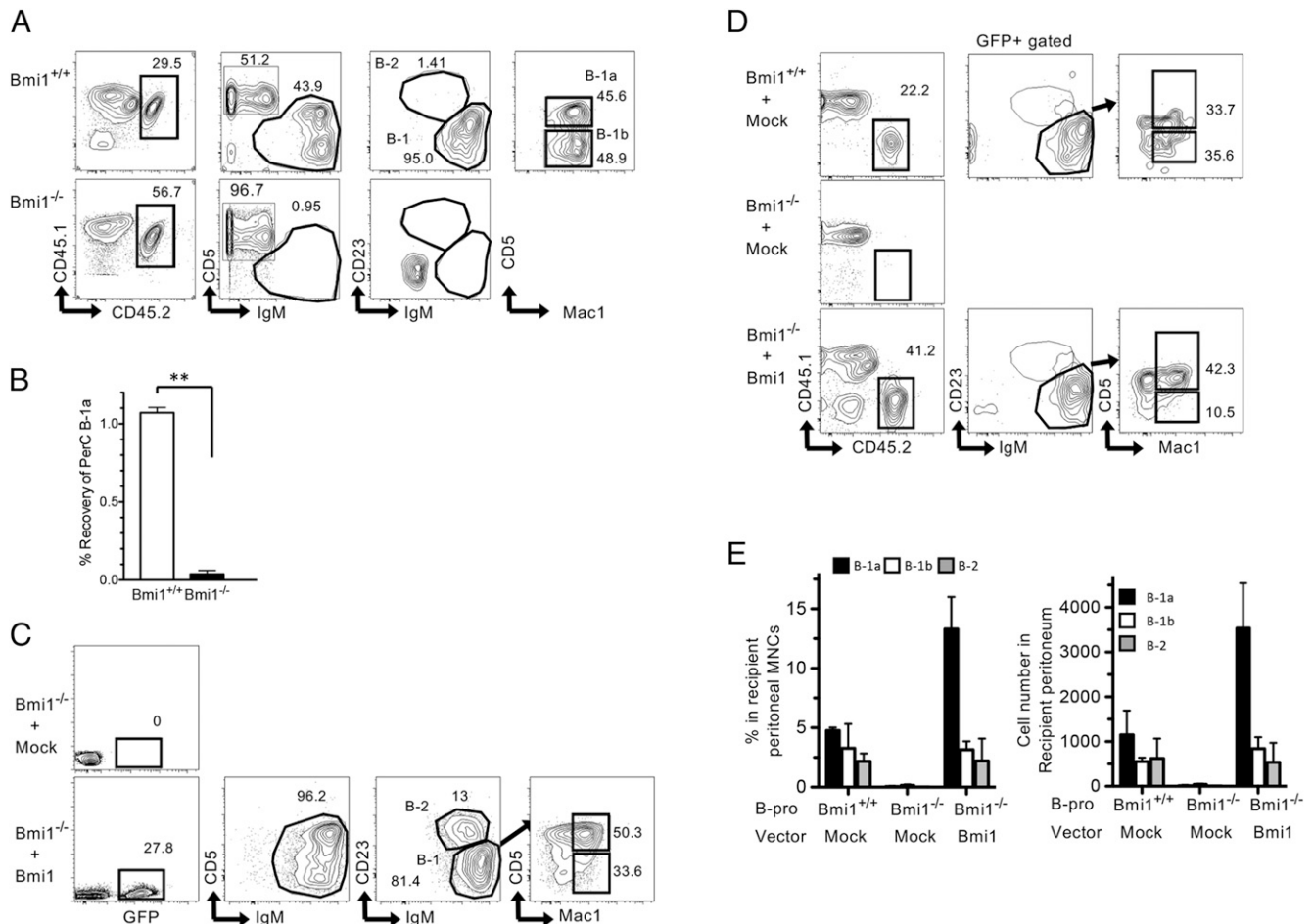


FIGURE 2. Loss of B-1a self-renewal by *Bmi1* disruption. **(A)** Representative FACS plots of recipient peritoneal cells at 12 wk posttransplant. The peritoneal cells of the recipient NSG mice transplanted with *Bmi1*^{+/+} (upper panel) or *Bmi1*^{-/-} (lower panel) peritoneal cells are depicted ($n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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

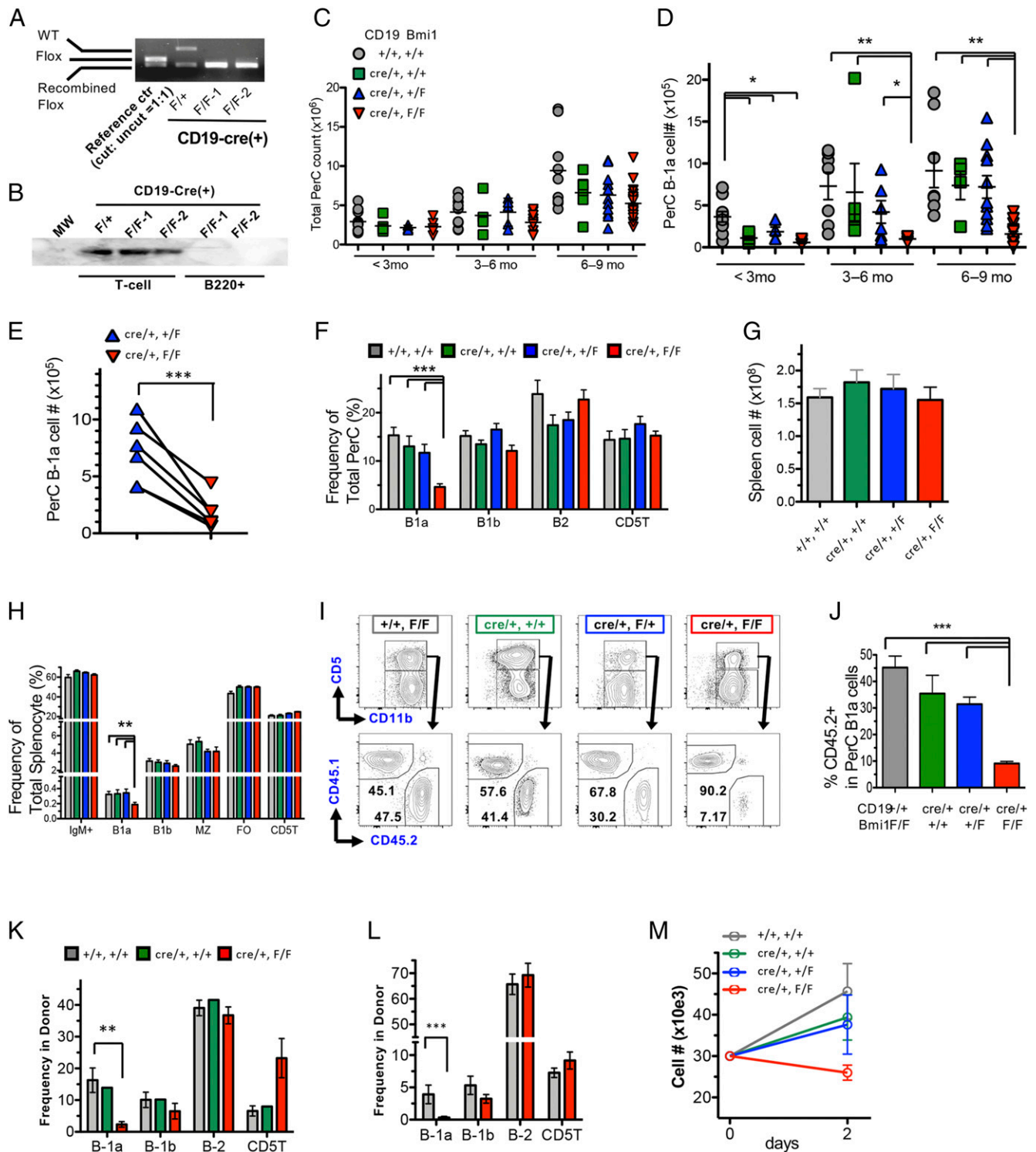


FIGURE 3. B cell-specific deletion of *Bmi1* leads to reduction in the B-1a cell pool and loss of self-renewal ability. The efficiency of CD19-Cre recombinase was confirmed by genomic PCR (**A**) in FACS-sorted B-1 cells or bead-selected spleen B cells (**B**). (**C**) Total peritoneal cell numbers in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

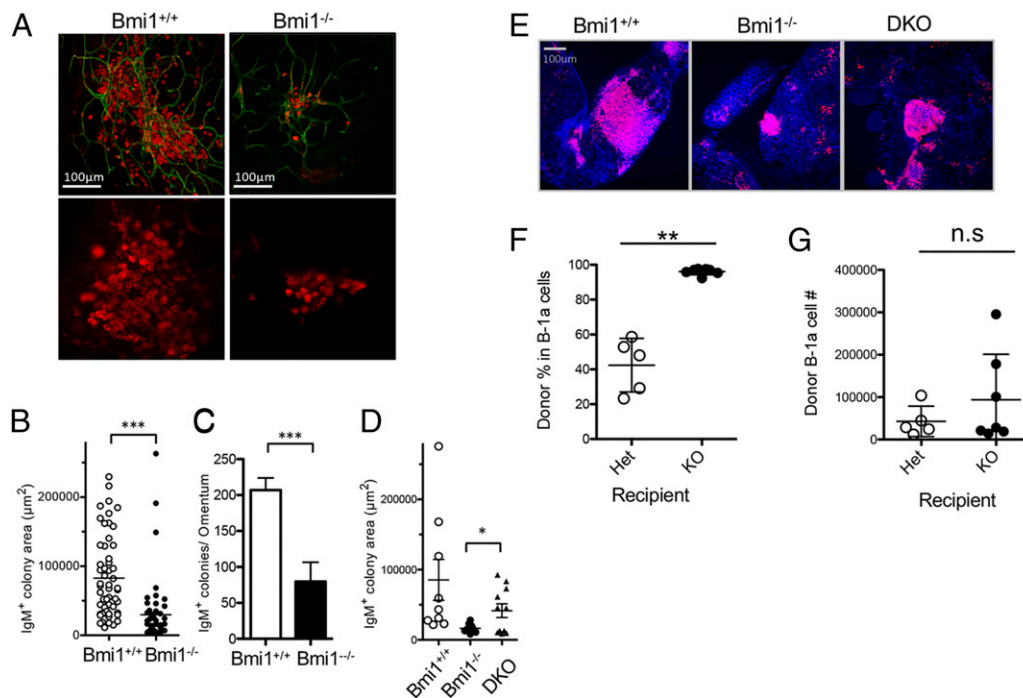


FIGURE 4. FALCs in *Bmi1*^{-/-} peritoneum and reciprocal transplantation assays. **(A)** Representative pictures of FALCs marked by IgM staining (red) in WT or *Bmi1*^{-/-} peritoneum. CD31⁺ endothelial cells are also stained (green). The size **(B)** and number **(C)** of FALCs in the omentum are depicted.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

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

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

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

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

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

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

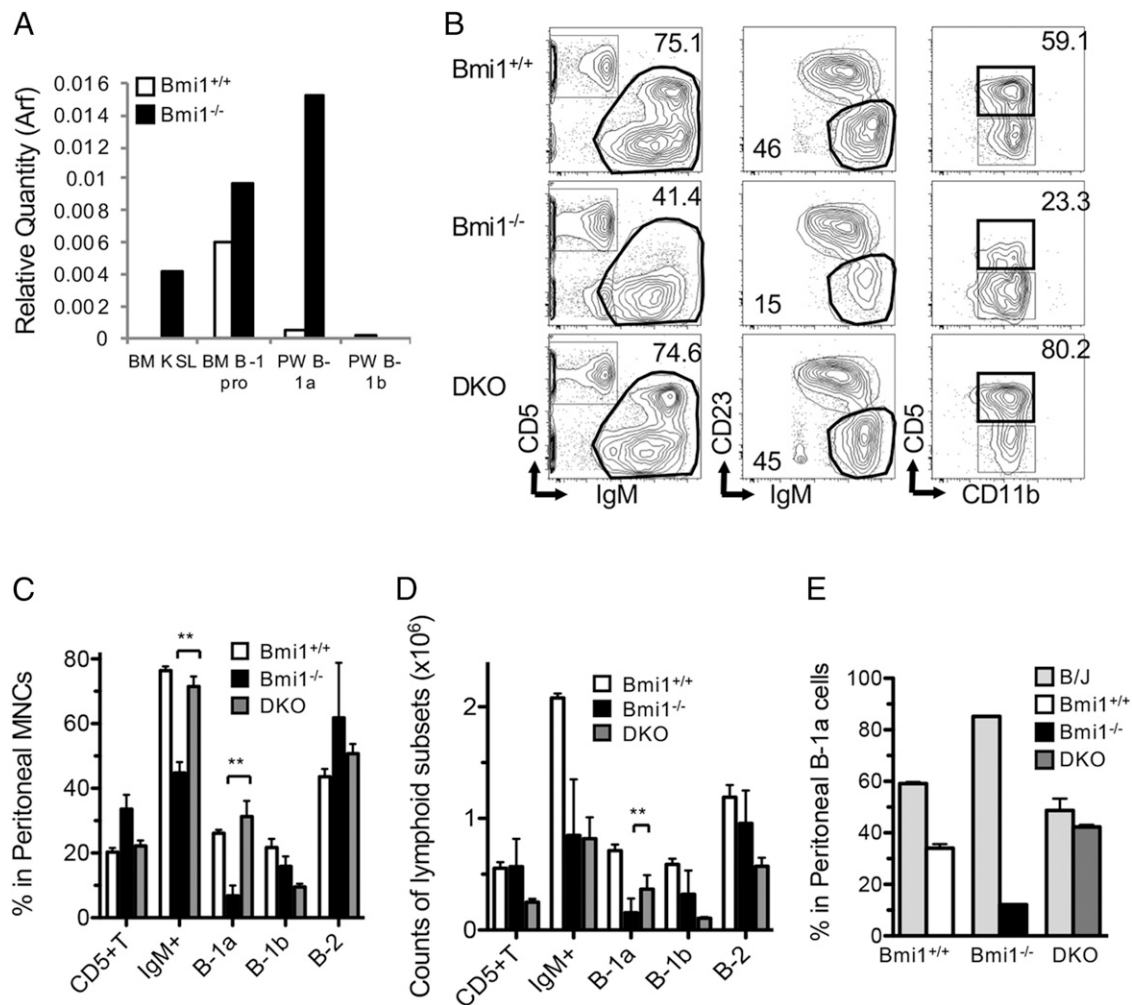


FIGURE 5. Arf-Ink4a is a major target for self-renewal of *Bmi1*^{-/-} B-1a cells. (A) Arf expression in WT and *Bmi1*^{-/-} B cells was measured by qPCR. (B) Representative FACS plots of peritoneal cells from WT, *Bmi1*^{-/-}, and *Bmi1*^{-/-} Arf-Ink4^{-/-} (DKO) mice are depicted. (C) Frequencies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

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

Discussion

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

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

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

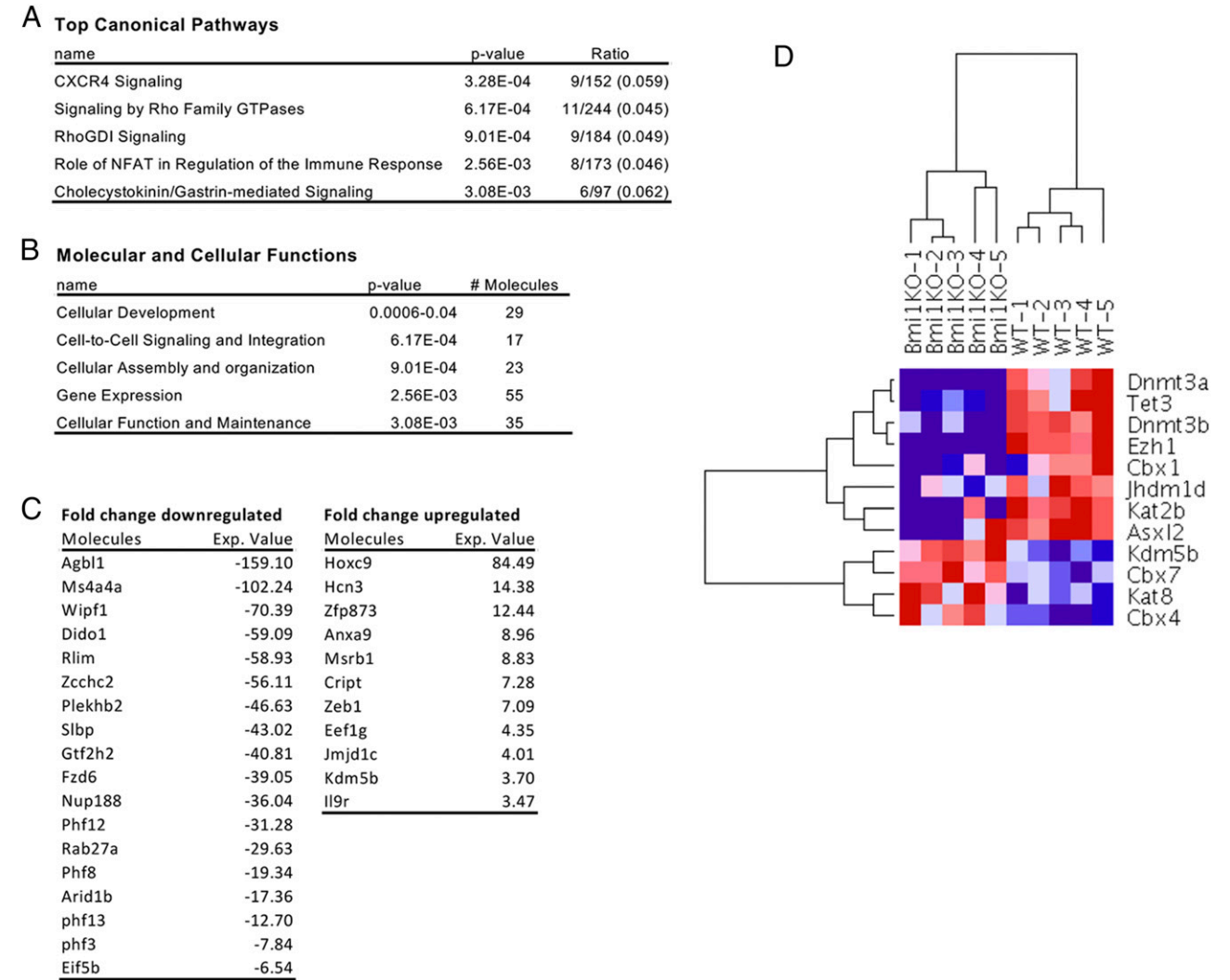


FIGURE 6. Gene expression profiling of *Bmi1*^{-/-} reveals dysregulation of epigenetic genes. B-1a cells were sorted from WT and *Bmi1*^{-/-} peritoneal cavity and subjected to microarray analysis. Affected canonical signaling pathways (**A**) and affected biofunctions (**B**) analyzed by Ingenuity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

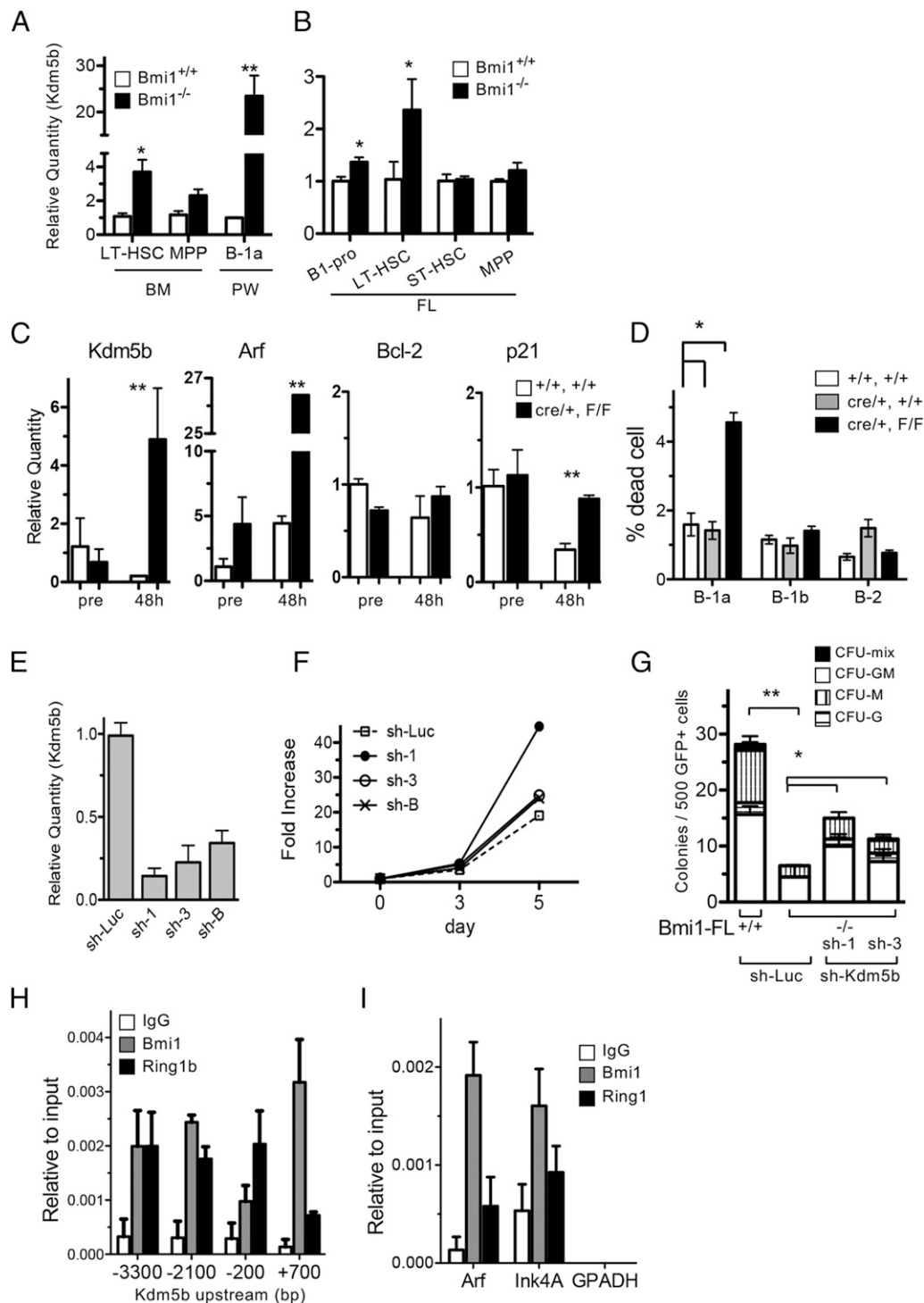


FIGURE 7. *Kdm5b* is a possible *Bmi1* target and crucial for posttransplant B-1a cell expansion. Expression of *Kdm5b* in various cell fractions from WT and *Bmi1*^{-/-} BM and peritoneum (**A**) or FL (**B**) ($n = 3$). $*p < 0.03$, $**p < 0.01$. (**C**) Sorted B-1a cells from *CD19*^{Cre/+}*Bmi1*^{F/F} 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 Baf/3 cells to confirm *Bmi1* binding to the *Kdm5b* or *Arf-Ink4* regions. Binding to upstream regions of *Kdm5b* (**H**) and *Arf-Ink4* (**I**) is shown. The data were obtained from three experiments.

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

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

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

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

Disclosures

The authors have no financial conflicts of interest.

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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.

Acknowledgements

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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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FIGURE LEGENDS

Figure 1. PRL2 is important for oncogenic cytokine signaling-mediated hematopoietic progenitor cell proliferation.

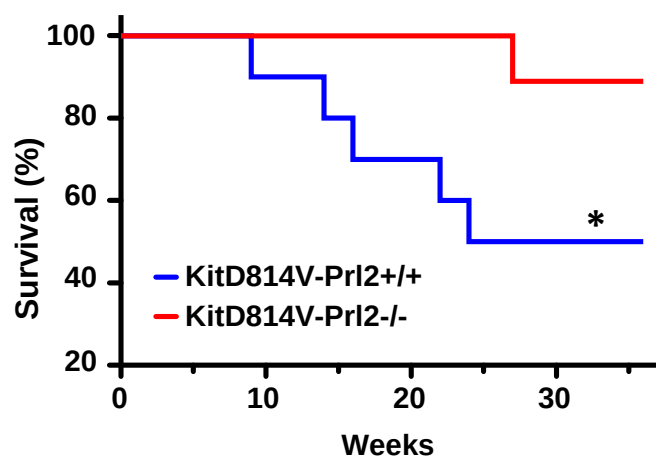
(A) PRL2 deficiency extended the survival of recipient mice transplanted with hematopoietic progenitor cells expressing KitD814V (* $p < 0.05$, $n = 9$). (B) PRL2 deficiency decreased the proliferation of hematopoietic progenitor cells expressing FLT3-ITD both in the absence of cytokines and in the presence 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.

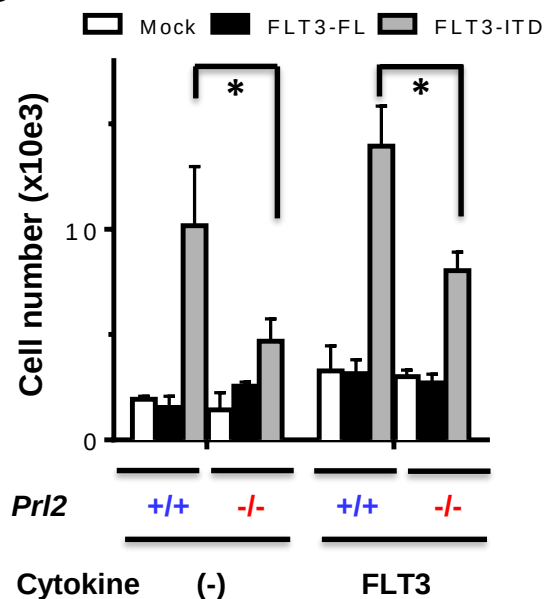
(A) PRL2 is important for c-Kit activation following SCF stimulation. Immunoblot analysis of Kit, Erk, and Akt phosphorylation in WT and *Prl2* null hematopoietic progenitor cells following SCF stimulation. (B) Mast cells (MCs) were induced from WT and PRL2 null KSL cells followed by SCF stimulation. 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

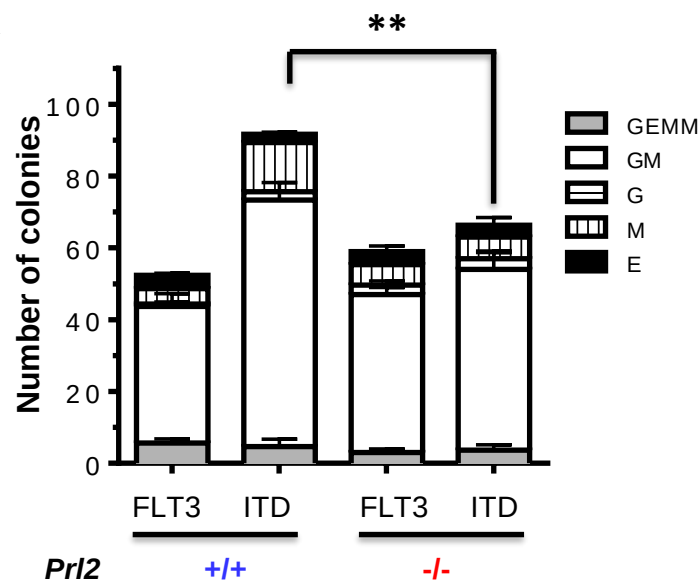
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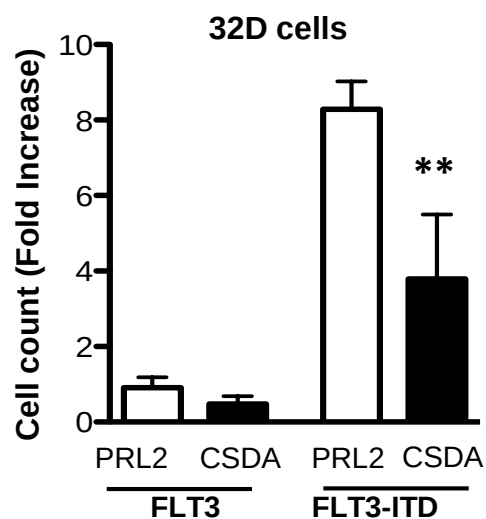
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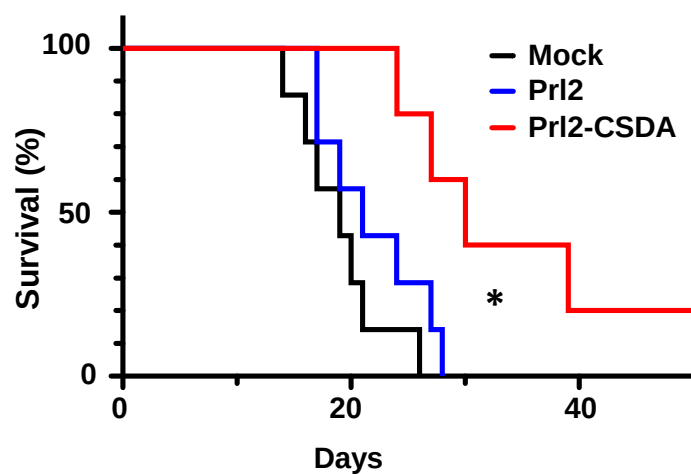
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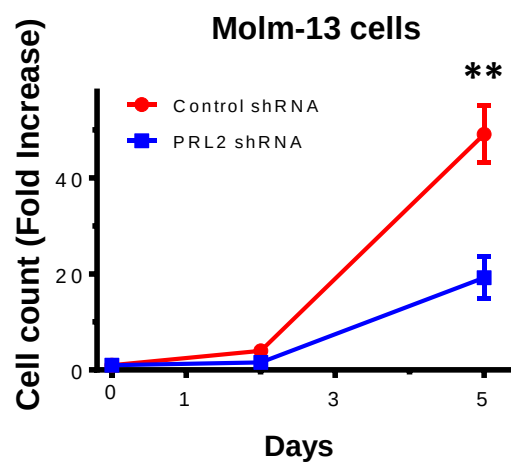
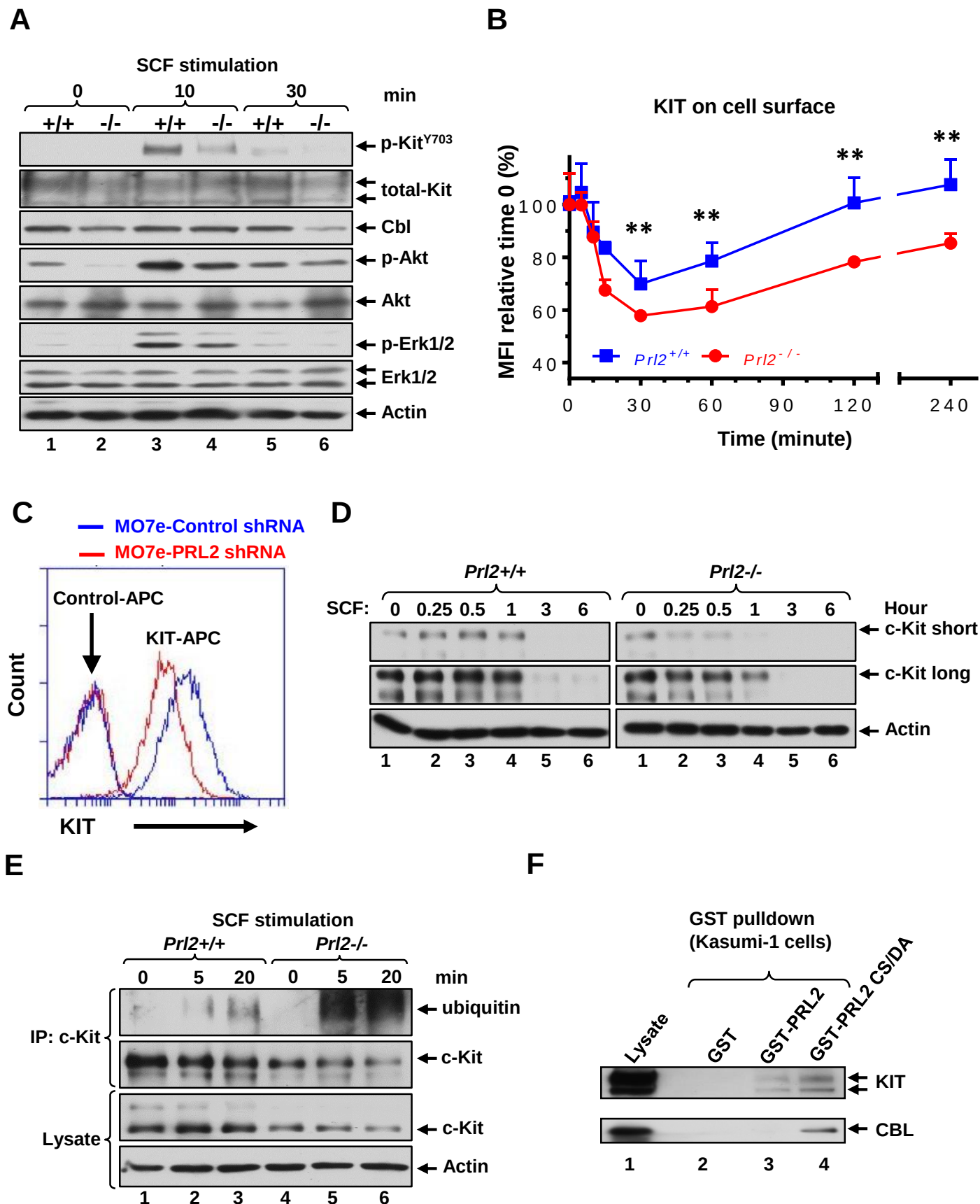


Figure 2



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

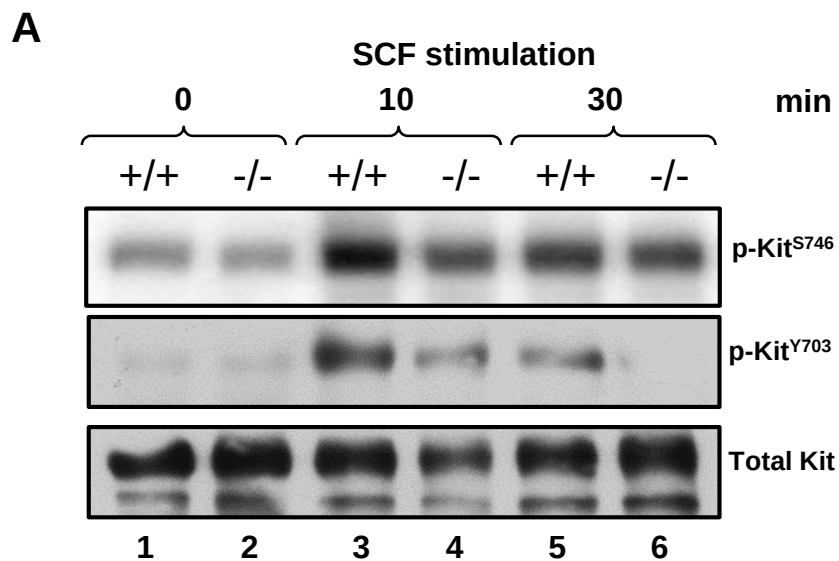
The animal sample size was based on previous studies evaluating the roles of AML1-ETO9a in leukemia and POWER analysis. Using Chi-Square analysis, 7 mice per group will provide 80% POWER in detecting difference with 95% difference. Gehan-Breslow-Wilcoxon test was used for Kaplan-Meier survival curves. 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

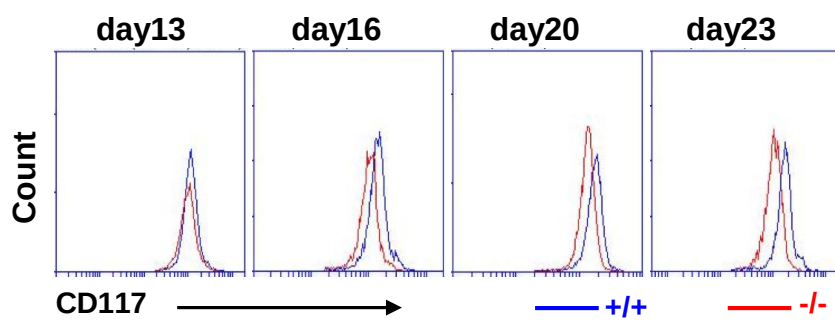
Figure S1. (A) Evaluation of PKC-mediated negative regulation of c-Kit activation in PRL2 null mast cells (MCs). MCs were induced from WT and PRL2 null KSL cells, serum starved, followed by stimulation of SCF. Total cell lysates harvested at each time point were subjected to western blot analysis using 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

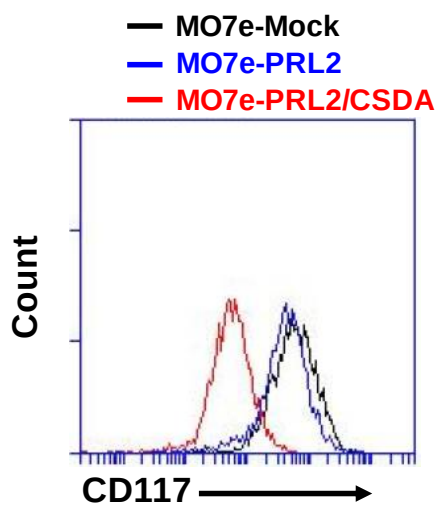
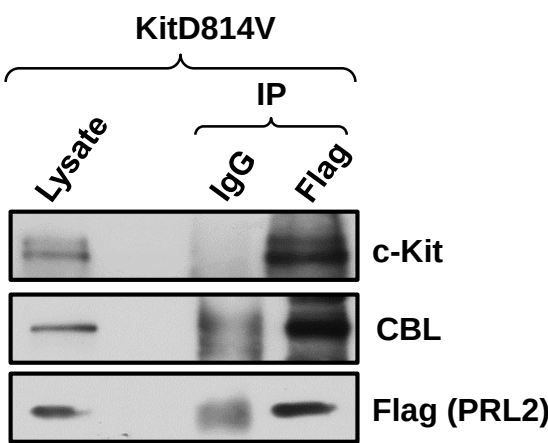
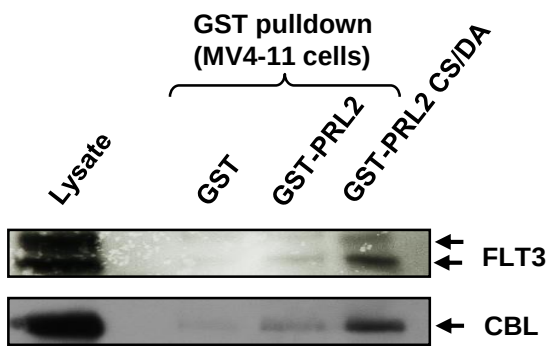


Figure S2

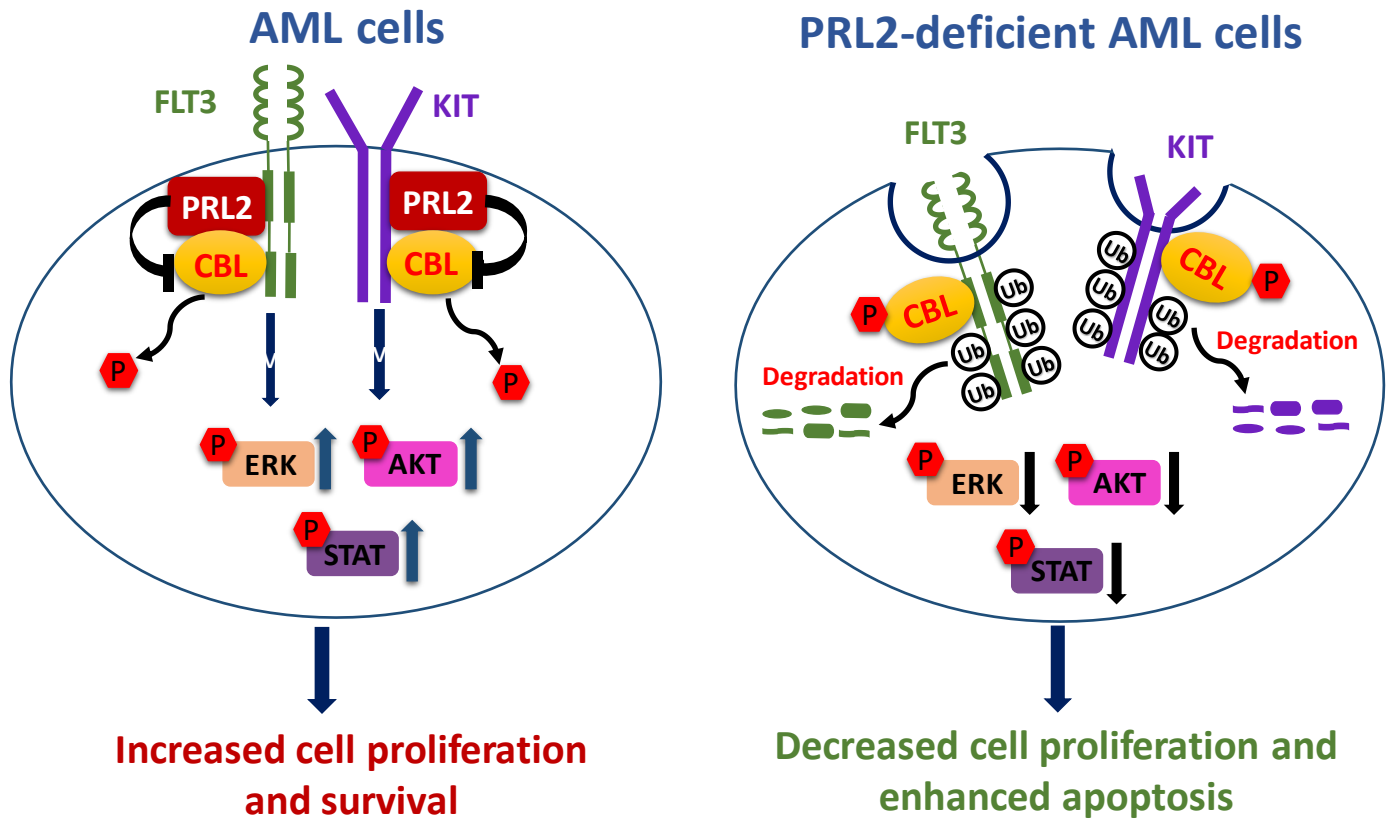
A



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Bmi1 regulates Wnt signaling in hematopoietic stem and progenitor cells

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Summary

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

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

Introduction

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

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

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

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

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

Materials and Methods

Mice

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

Flow cytometry

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

Gene expression and Pathways Analyses

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

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

ChIP assays

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

Reporter assays

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

Generation of retroviruses and infection of murine HSCs and MPPs

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

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

Transplantation

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

Statistical Analysis

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

Results

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

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

Bmi1 is associated with Wnt genes in hematopoietic cells

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

Bmi1 represses Wnt gene expression in hematopoietic stem and progenitor cells

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

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

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

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

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

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

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

Discussion

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

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

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

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

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

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

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

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Declarations

Ethical Approval All mouse experiments were approved by the Indiana University Institutional Animal Use and Care Committee.

Consent to Participate None.

Consent to Publish All authors concur with the publication of the manuscript.

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

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Competing Interests The authors declared that no conflict interest exists.

Availability of data and materials Raw data will be available for download from Gene Expression Omnibus (<http://ncbi.nlm.nih.gov/geo/>, accession number in progress).

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FIGURE LEGENDS

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

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

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

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

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

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

Figure 1

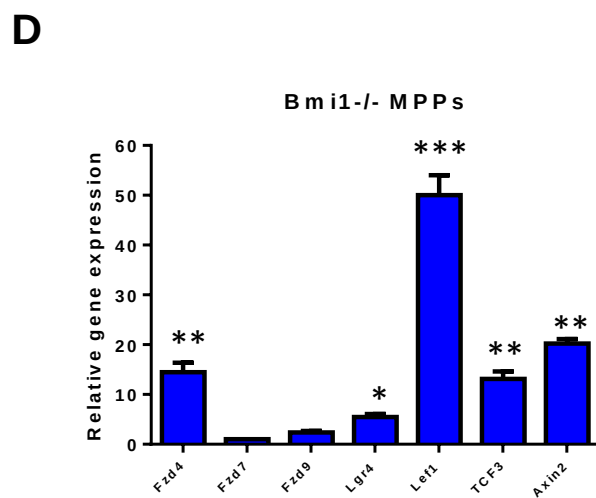
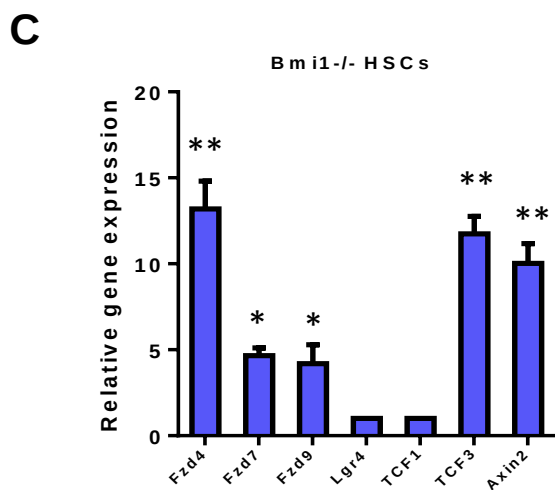
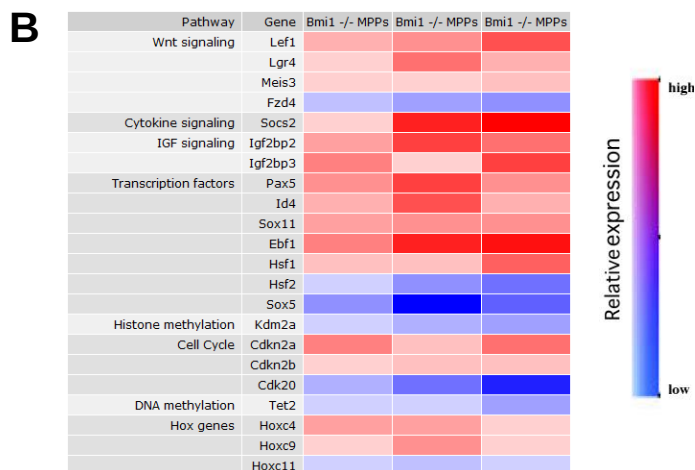
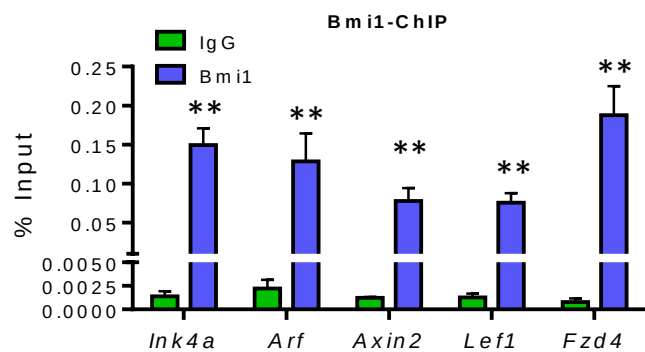
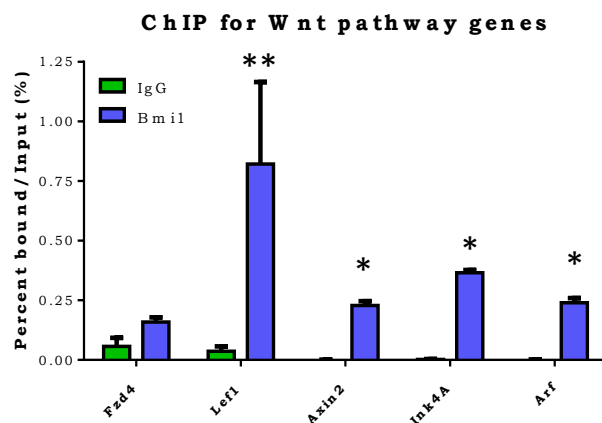


Figure 2

A



B



C

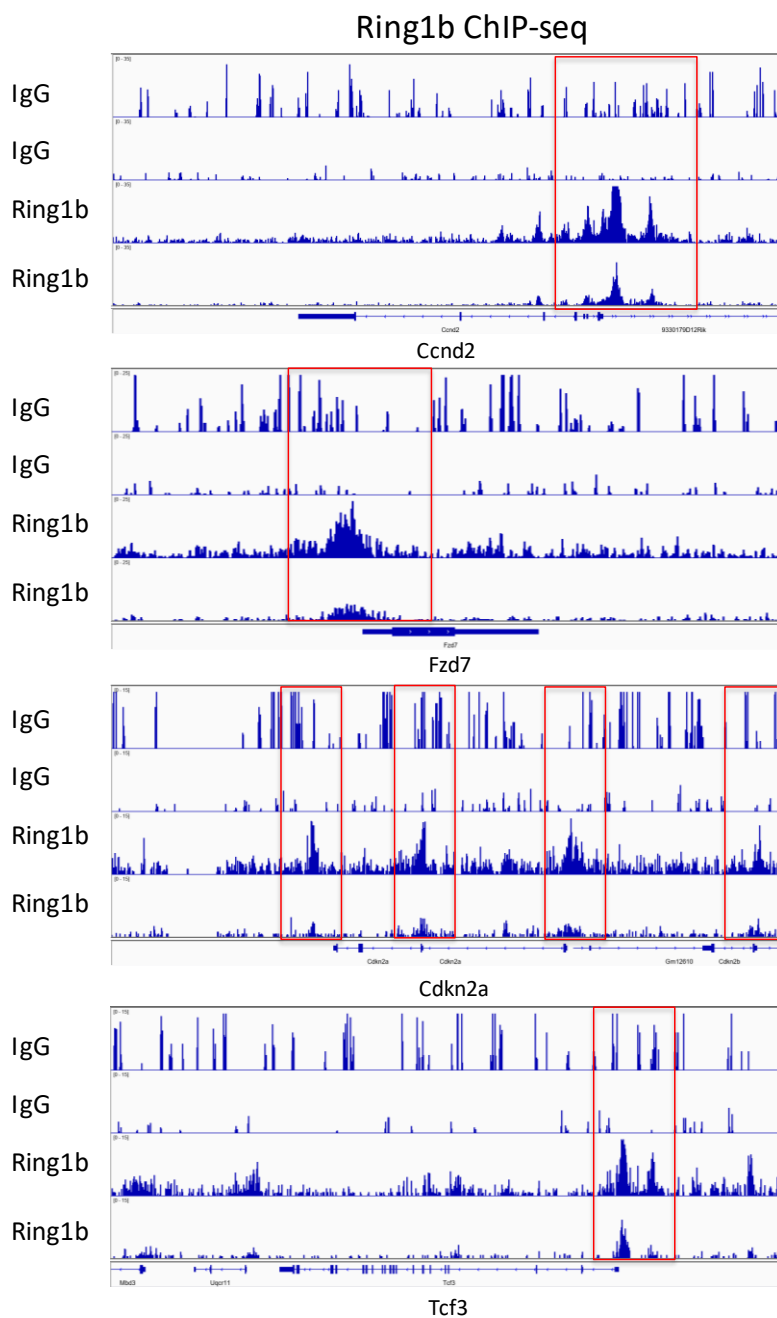
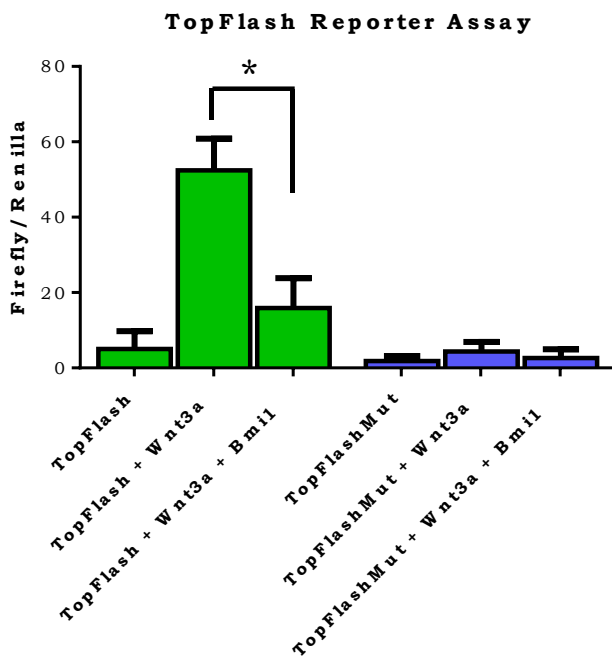


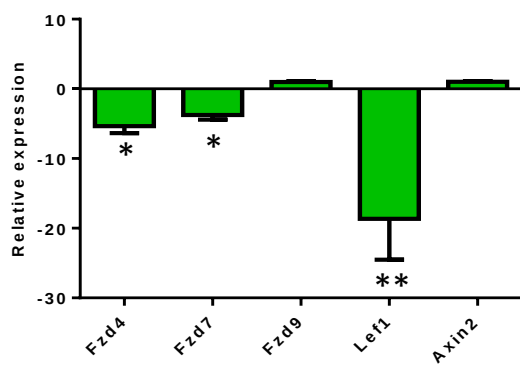
Figure 3

A



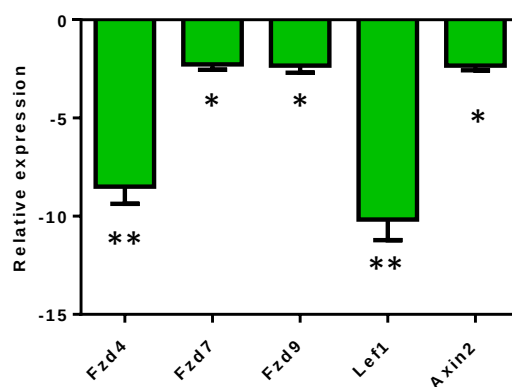
B

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



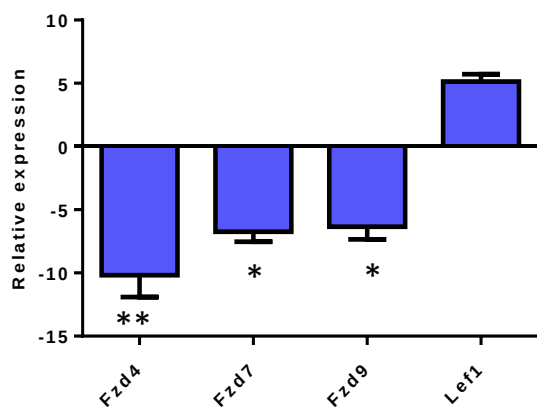
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Ectopic Bmi1 expression in Bmi1^{+/+} MPPs



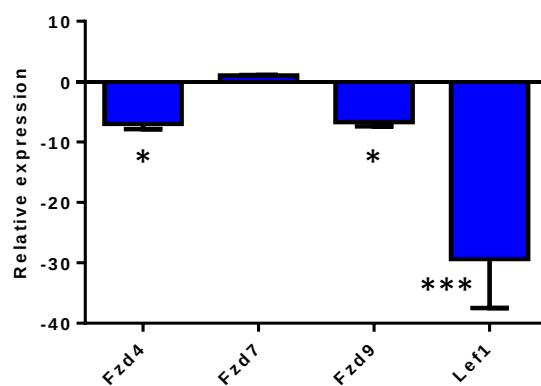
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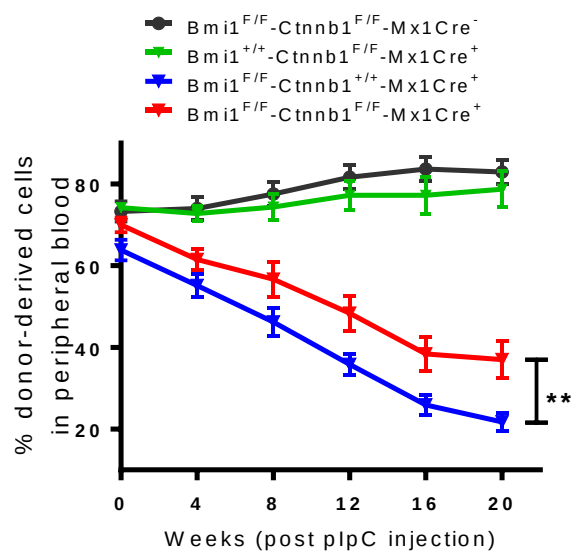


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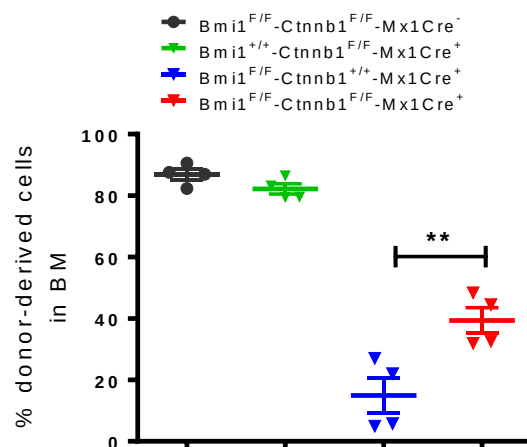
Ectopic Bmi1 expression in Bmi1^{-/-} MPPs



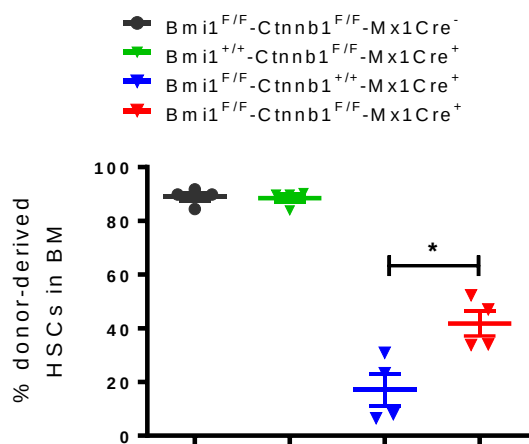
A Primary BM transplantation



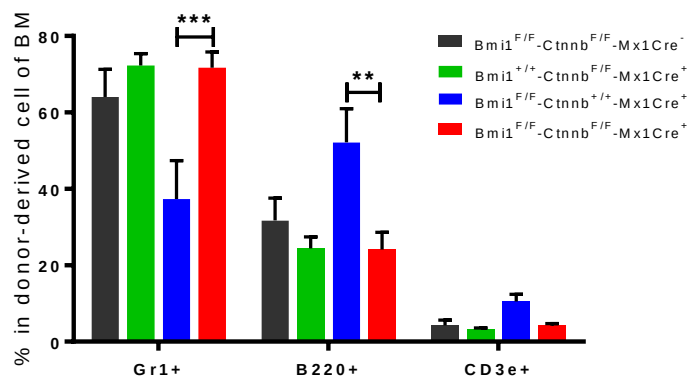
B Primary BM transplantation



C Primary BM transplantation



D Multilineage differentiation in BM at 20 week after plpC



E

