Characterization of Genome-Wide Binding of NUCLEAR-FACTOR I-X in Hematopoietic Progenitor Cells

Megan J. Walker

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Characterization of Genome-Wide Binding of NUCLEAR-FACTOR I-X in Hematopoietic Progenitor Cells

Abstract
We report that ectopic expression of Nfix in primary mouse HSPC extended their ex vivo culture from 20 to 40 days. HSPC overexpressing Nfix displayed hypersensitivity to supportive cytokines and reduced apoptosis when subjected to cytokine deprivation compared to controls. Ectopic Nfix resulted in elevated levels of c-Mpl transcripts and cell surface protein on primary murine HSPC as well as increased phosphorylation of STAT5, which is known to be activated down-stream of c-MPL signaling. Blocking c-MPL signaling by removal of its ligand, thrombopoietin (TPO), or addition of a c-MPL neutralizing antibody negated the anti-apoptotic effect of Nfix overexpression on cultured HSPC. Furthermore, NFIX-FLAG was capable of binding to and transcriptionally activating a proximal c-Mpl promoter fragment. In sum, these data suggest that NFIX-mediated up-regulation of c-Mpl transcription can protect primitive hematopoietic cells from stress ex vivo. Understanding the direct transcriptional targets or co-binding partners of NFIX would provide further insight into the mechanisms HSPC employ to maintain steady-state hematopoiesis or overcome stress hematopoiesis.

To this end, we combined global transcriptional profiling and genome-wide binding to identify direct transcriptional targets of NFIX in Nfix+/+ and Nfix-/- HPC5 cells, a primitive multi-potent hematopoietic cell line. We find that NFIX preferentially binds enhancer and promoter genomic regions. Integrative analysis revealed >500 differentially expressed genes of which 58% were direct NFIX targets. Many of these genes were downregulated in the absence of NFIX, indicating that NFIX functions primarily as a transcriptional activator in this context. PANTHER pathway analysis implicated NFIX in the regulation of apoptosis, myeloid cell differentiation and cell-cell adhesion. Using archived ChIP-seq data, we revealed significant co-localization of NFIX with other well-known hematopoietic transcription factors, including pSTAT1, RUNX1, RAD21, STAT3, ETO2, FLI1, GATA2, LYL1, LDB1 and PU.1. We showed NFIX and PU.1 together target genes regulating hematopoietic cell adhesion, cell death and differentiation in hematopoietic cells. Our data support a model in which NFIX collaborates with PU.1 to regulate differentiation and apoptosis in hematopoietic cells. In summary, we identified direct transcription targets and putative co-regulatory partners of NFIX.

In sum, the work here further characterizes the complex role of the NFI family member, NFIX. We show minor perturbations in PB lineages when transplanting NfixΔ/Δ HSPC. During secondary transplants, NfixΔ/Δ donor chimerism was similar to controls. Also, during steady-state hematopoiesis, we did not observe any overt phenotypes in the NfixΔ/Δ mice. We discovered that overexpressing Nfix ex vivo imparts cells with hypersensitivity to cytokines and resistance to apoptosis. These characteristics were due to an increase in c-MPL signaling and could be abolished if this signaling was blocked. We identified c-Mpl as the first transcriptional target of NFIX in a hematopoietic context. Finally, we have rigorously characterized the genome-wide binding of NFIX in a hematopoietic cell line as well as identified 291 putative transcriptional targets. This work provides more data towards illuminating the role of an NFI family member during hematopoiesis.

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Characterization of Genome-Wide Binding of NUCLEAR FACTOR I-X in Hematopoietic Progenitor Cells

Author: Megan J. Walker
Advisor: Shannon McKinney-Freeman, PhD

A Dissertation Presented for The Graduate Studies Council of The University of Tennessee Health Science Center in Partial Fulfillment of the Requirements for the Doctor of Philosophy degree from The University of Tennessee in Biomedical Sciences: Cancer and Developmental Biology College of Graduate Health Sciences

October 2020
DEDICATION

★★★

For all the personal triumphs and mistakes that became lessons.

This is for Stuert, my most beloved friend. For me, it is the highest privilege and honor to call him my husband. When I think of the billions of people occupying this planet and our serendipitous meeting, I am grateful every day we chose each other. Through all this, Stuert imparted a strength and courage to me that I had not realized I needed. I could not have done this without his unwavering support, unconditional love and indescribable sacrifice. Stuert celebrated small victories with me and helped me piece myself back together when I wanted to quit. I thank him for sharing this dream with me. This is as much his accomplishment as it is mine.
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I would first like to acknowledge my mentor, Shannon McKinney-Freeman. I am forever grateful for her training me to be a better scientist, her patience and for her nurturing disposition. I appreciate Shannon giving me a place in her lab in 2014 alongside some truly brilliant people. Her mentorship has helped me evolve into a more confident, thoughtful and robust scientist. I appreciate her relentless editing of abstracts and manuscripts. I am thankful for the opportunity to travel and for introductions made in our field. I am excited to leave her lab and continue to build on the tremendous foundations she has helped me develop.

I first began meeting regularly with Joe Opferman in 2018. I want to declare my appreciation for his mentorship and always advocating for my progress and success. I would also like to thank the rest of my committee members: Suzanne Baker, John Cox, Erin Schuetz, Tiffany Seagroves and Brian Sorrentino.

I would also like to thank my past and present lab members: Chaïma Benaksas, Marie Bordas, Lina-Marie Bruu, Ashley Chabot, Maheen Ferdous, Miguel Ganuza, Per Holmfeldt, Charly Le Maout, Himangi Marathe, Alice Martinat, Shobhit Saxena and Albert Vacheron. I have had several interactions with each of these people that I believe have contributed to me becoming a more experienced scientist and mature individual. I especially would like to thank Miguel. We have been in Shannon’s lab together the longest and I have learned an incredible amount about our field and science in general. He is the most hard-working and rigorous scientist I know. I am happy we are friends and colleagues. Trent Hall, Antonio Morales, Chris Nevitt, Aditya Barve, James Johnson, Claire Caprio, Emilia Kooienga and Pramika Sriram are all people I am currently interacting with daily. I am so glad to have Emilia, Claire and Pramika as helping hands and for keeping other aspects of the lab running smoothly. They have been a tremendous help and are invaluable assets to our lab. I thank Trent for completing the Stem Cells paper with me and training me when I first entered the lab. He is also one of the most selfless and kind individuals that has challenged me to become a more caring person inside and outside the lab. Antonio and Chris are both scientist I am striving to be like in the future. Antonio is amazingly thoughtful, well-read and organized in the execution of his projects. Chris has challenging and thought-provoking questions that I believe only come from a seasoned and meticulous scientist. I wish I had more time to learn from Aditya, but from our first interaction, I have envied his enthusiasm, curiosity and charm. My conversations with James have helped me personally reflect on what will ultimately make me happy going forward in life. I think James is quirky in the best way possible and takes time for consideration before acting. It is my intention to continuously implement each of these qualities in myself as I go forward. These have been some of the most valuable lessons and I am forever indebted to these individuals for helping me become the scientist and person I am today.

Dick Ashmun, Liusheng He, Scott Perry, Sandy Schwemberger and Stacie Woolard and other staff in the Flow Cytometry Core have been integral to the success of
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This project would never have gotten off the ground as well as it did without Scott Brown. I thank Scott for working with Clay Christian early in the project to develop and validate the antibody used in this work. To my knowledge this reagent is the first that has been rigorously tested and validated for specificity during chromatin immunoprecipitation.

I do not think there is enough thanks for the work done by the Cheng lab. I would first like to acknowledge the mentorship provided by Yong Cheng himself. I am appreciative for our conversations and his input that made this work more impactful. I secondly would like to thank Yong’s wife, Qian Qi. She has been tremendously helpful sharing with me her optimized protocols and reagents. I appreciate her letting me peer over her shoulder while I was learning their protocols and for providing direction while I performed these critical experiments. Finally, I would like to express my gratitude for a post-doctoral fellow in Yong’s lab, Yichao Li. Yichao and I have conversed extensively, and I am glad to consider him more than a colleague, but a friend. He has been one of the best teachers I have met during my graduate studies. I appreciate him taking two to three hours of his time to sit with me each week to go over my data and explain the gritty details of bioinformatics in way I could understand. And in March, when we were all sent to work from home, he always made himself available to talk and discuss different ways of analyzing our data.

Lastly, I would like to thank my parents again. I dedicated my Master’s thesis to them and I want to thank them again for their support during the last seven years. They have always advocated my personal endeavors and made my happiness their priority. Again, I attribute their parenting and personal and emotional investment as the reason for my success. Whenever I felt things were becoming too difficult, my parents reminded me that my feelings were an indication I was in the best possible place for me. Because I would not become smarter or better trained if I was not surrounded by people who were already smarter and better trained. If I never “failed”, I would never learn.

I must also include my sister, Jamie. If it were not for her moving to Memphis suddenly and her husband who is friends with everyone he meets, I probably would never had met Stuart.
Hematopoietic stem cells (HSC) are responsible for maintaining all cells in the hematopoietic hierarchy. During periods of stress, such as infection or exposure to radiation, HSC can replenish the hematopoietic system. This inherent characteristic is regularly exploited in the clinic to treat diseases such as, leukemia, lymphomas and other non-malignant disorders. However, there are still several morbidities and mortality associated with HSC transplant (HSCT). Several efforts have been made since the first successful bone marrow transplant (BMT) to improve transplant outcomes. Our lab has contributed to better understanding the molecular regulators of HSCT by identifying 18 genes required for successful HSCT. We reported the transcription factor, nuclear factor I-X (Nfix), functions as a positive regulator of HSC biology during transplantation.

To confirm that Nfix is required for HSCT, we utilized a genetic model to temporally delete Nfix in vivo specifically in hematopoietic cells. We found that the Nfix-shRNA originally used to suggest the importance of Nfix in HSCT, targets other genes in addition to Nfix. Interrogating the need for Nfix during HSCT using our genetic model, we found that Nfix-deficient hematopoietic stem and progenitor cells (HSPC) exhibit a modest, but significant, increase in peripheral blood (PB) donor chimerism. Specifically, there was a small but significant increase in donor chimerism in the lymphoid lineage but not in the myeloid lineage. We learned these HSPC are capable of secondary transplant and do not display a decrease in colony-forming ability, suggesting the HSC are functionally similar to controls. During steady-state hematopoiesis, Nfix-deleted HSPC could contribute to normal hematopoiesis and mice had normal complete blood counts (CBC). The cells did not exhibit any perturbations in lineage distribution. Together, these data suggest Nfix is an unessential factor during HSCT and steady-state hematopoiesis. However, we cannot exclude the possibility of redundancy or compensation within the NFI family. More work with double or triple NFI-knockout models is necessary to truly interrogate these hypotheses. Nonetheless, in contrast we observed that ectopic expression of Nfix in HSPC ex vivo results in significantly reduced apoptosis.

We report that ectopic expression of Nfix in primary mouse HSPC extended their ex vivo culture from 20 to 40 days. HSPC overexpressing Nfix displayed hypersensitivity to supportive cytokines and reduced apoptosis when subjected to cytokine deprivation compared to controls. Ectopic Nfix resulted in elevated levels of c-Mpl transcripts and cell surface protein on primary murine HSPC as well as increased phosphorylation of STAT5, which is known to be activated down-stream of c-MPL signaling. Blocking c-MPL signaling by removal of its ligand, thrombopoietin (TPO), or addition of a c-MPL neutralizing antibody negated the anti-apoptotic effect of Nfix overexpression on cultured HSPC. Furthermore, NFIX-FLAG was capable of binding to and transcriptionally activating a proximal c-Mpl promoter fragment. In sum, these data suggest that NFIX-mediated up-regulation of c-Mpl transcription can protect primitive hematopoietic cells from stress ex vivo. Understanding the direct transcriptional targets or co-binding partners of NFIX would provide further insight into the mechanisms HSPC employ to maintain steady-state hematopoiesis or overcome stress hematopoiesis.
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<thead>
<tr>
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<th>Description</th>
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<tbody>
<tr>
<td>AA</td>
<td>Aplastic Anemia</td>
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<td>aGVHD</td>
<td>Acute Graft-Versus-Host Disease</td>
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<td>Ad</td>
<td>Adenovirus</td>
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<td>ALL</td>
<td>Acute Lymphoblastic Leukemia</td>
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<td>bFGF</td>
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<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
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</tr>
<tr>
<td>HD</td>
<td>Hodgkin's Disease</td>
</tr>
<tr>
<td>hFGF-a</td>
<td>Human Fibroblast Growth Factor Acidic</td>
</tr>
<tr>
<td>hIL-6</td>
<td>Human Interleukin-6</td>
</tr>
<tr>
<td>HLA</td>
<td>Human Leukocyte Antigen</td>
</tr>
<tr>
<td>HSC</td>
<td>Hematopoietic Stem Cells</td>
</tr>
<tr>
<td>HSCT</td>
<td>Hematopoietic Stem Cell Transplant</td>
</tr>
<tr>
<td>HSPC</td>
<td>Hematopoietic Stem and Progenitor Cells, Lineage-cKit+Sca-1+</td>
</tr>
<tr>
<td>IMDM</td>
<td>Iscove’s Modified Dulbecco’s Medium</td>
</tr>
<tr>
<td>JAM</td>
<td>Junctional Adhesion Molecule</td>
</tr>
<tr>
<td>KI</td>
<td>Knockin</td>
</tr>
<tr>
<td>KO</td>
<td>Knockout</td>
</tr>
<tr>
<td>LepR</td>
<td>Leptin Receptor+ Cells</td>
</tr>
<tr>
<td>LSK</td>
<td>Lineage-cKit+Sca-1+, Hematopoietic Stem and Progenitor Cells</td>
</tr>
<tr>
<td>mAb</td>
<td>Monoclonal Antibody</td>
</tr>
<tr>
<td>MADCAM1</td>
<td>Mucosal Addressing Cell Adhesion Molecule-1, α4β7</td>
</tr>
<tr>
<td>MSC</td>
<td>Mesenchymal Stem Cells</td>
</tr>
<tr>
<td>MDS</td>
<td>Myelodysplastic Syndrome</td>
</tr>
<tr>
<td>mIGF-2</td>
<td>Murine Insulin-Like Growth Factor 2</td>
</tr>
<tr>
<td>mm9</td>
<td>Mouse Genome Assembly 9</td>
</tr>
<tr>
<td>MMTV</td>
<td>Mouse Mammary Tumor Virus</td>
</tr>
<tr>
<td>MPN</td>
<td>Myeloproliferative Neoplasms</td>
</tr>
<tr>
<td>MSC</td>
<td>Mesenchymal Stem Cell</td>
</tr>
<tr>
<td>mSCF</td>
<td>Murine Stem Cell Factor</td>
</tr>
<tr>
<td>MTG</td>
<td>1-Thioglycerol, Monothioglycerol</td>
</tr>
<tr>
<td>mTPO</td>
<td>Murine Thrombopoietin</td>
</tr>
<tr>
<td>NFI</td>
<td>Nuclear Factor I</td>
</tr>
<tr>
<td>NFIA</td>
<td>Nuclear Factor I-A</td>
</tr>
<tr>
<td>NFIB</td>
<td>Nuclear Factor I-B</td>
</tr>
<tr>
<td>NFIC</td>
<td>Nuclear Factor I-C</td>
</tr>
<tr>
<td>NFIX</td>
<td>Nuclear Factor I-X</td>
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<tr>
<td>NGS</td>
<td>Next Generation Sequencing</td>
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<td>NHL</td>
<td>Non-Hodgkin's Lymphomas</td>
</tr>
<tr>
<td>NPY</td>
<td>Neuropeptide Y</td>
</tr>
<tr>
<td>NS</td>
<td>Not significant</td>
</tr>
<tr>
<td>NSC</td>
<td>Neural Stem Cells</td>
</tr>
<tr>
<td>NTC</td>
<td>Non-Tissue Culture</td>
</tr>
<tr>
<td>PB</td>
<td>Peripheral Blood</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate Buffered Saline</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase Chain Reaction</td>
</tr>
<tr>
<td>PDGFβ</td>
<td>Platelet Derived Growth Factor β</td>
</tr>
<tr>
<td>PDGFBB</td>
<td>Platelet Derived Growth Factor Subunit BB</td>
</tr>
<tr>
<td>pIpc</td>
<td>Polyinosinic:Polycytidylic Acid</td>
</tr>
<tr>
<td>PTN</td>
<td>Pleiotropin</td>
</tr>
<tr>
<td>pTP</td>
<td>Precursor Terminal Protein</td>
</tr>
<tr>
<td>qRT-PCR</td>
<td>Quantitative Real-Time Polymerase Chain Reaction</td>
</tr>
<tr>
<td>R-Niche</td>
<td>Reconstituting Niche</td>
</tr>
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</table>
rhG-CSF  Recombinant Human Granulocyte-Colony Stimulating Factor
rhGM-CSF Recombinant Human Granulocyte-Macrophage Colony Stimulating Factor
RNA       Ribonucleic Acid
ROS       Reactive Oxygen Species
SA        Streptavidin
SCF       Stem Cell Factor
SE        Standard Error
SFEM      Serum-Free Expansion Media
sgRNA     Short Guide Ribonucleic Acid
shRNAs    Short-Hairpin Ribonucleic Acids
SNS       Sympathetic Nervous System
ssDNA     Single Stranded Deoxyribonucleic Acid
TF        Transcription Factor
TGF-β     Transforming Growth Factor-β
tn-c      Tenascin-C
TPO       Thrombopoietin
VCAM1     Vascular Cell Adhesion Molecule-1
VE        VCAM1+EMBIGIN+ Cells
VEGFa     Vascular Endothelial Growth Factor A
VLA4      Very Late Antigen-4, α4β1
VSV-G     Vesicular Stomatitis Virus Glycoprotein G
WBM       Whole Bone Marrow
WT        Wild Type
4-OHT     4-Hydroxytamoxifen
5-FU      5-Fluorouracil
CHAPTER 1. INTRODUCTION

The Race to Define the Source of Hematopoietic Regeneration: What Are Hematopoietic Stem Cells?

Early Studies of Hematopoiesis

The possible existence of hematopoietic stem cells (HSC) had been entertained as early as 1896 by Artur Pappenheim who proposed a precursor cell capable of giving rise to red and white blood cells (Figure 1-1) [1]. Pappenheim had been inspired by previous work done in the field of embryology, leading him to the concept of stem cells. In 1906, Alexander Maximow proposed the monophyletic theory of hematopoiesis, suggesting all blood cells from each lineage were derived from a single precursor. He also suggested stem cells were influenced by the marrow stroma [2]. Through the rest of the early 20th century, research was devoted to better understanding stem cells during development, anemia and leukemia. In 1945, Ray Owen published a paper explaining how twin cattle could share identical blood types [3]. He postulated that the factual explanation was the existence of an “embryonal cell ancestral to erythrocytes” that could lodge in the bone marrow of their twin and go on to supply blood distinct from the host. Owen’s paper was published about six weeks after the end of World War II.

Conservative estimates report over 200,000 people were killed in the nuclear blast created by the United States in 1945 during World War II. At this time, there was increased interest in determining how to protect humans from unintended nuclear radiation. Jacobson et al. described their observations when the spleens of lethally irradiated mice were shielded [4]. Here they show increased erythropoiesis and myelopoiesis at the expense of lymphopoiesis. They concluded there may be a factor or factors that contribute to the rapid acceleration of erythropoiesis from the spleen. Jacobson follows this article with a thorough review making a case for a humoral factor. He highlighted work describing enhanced recovery of hematopoiesis when: normal spleens were transplanted to irradiated recipient mice, mashed mouse embryos were injected intraperitoneally into irradiated mice, irradiated mice were joined with non-irradiated littermates via parabiosis or when intravenous injection of homologous bone marrow into irradiated mice was performed [5]. Interestingly, Jacobson only speculates that the injected cells may produce a factor (or factors) that significantly enhance hematopoiesis after radiation injury.

Figure 1-1.  Early depiction of the hematopoietic hierarchy.
Artur Pappenheim’s 1905 drawing of the hematopoietic tree detailing the origination of all blood cells at “1”.
http://dx.doi.org/10.1016/j.stem.2007.05.013
Discoveries of the 1950s and 1960s Characterizing HSC

Four years later in 1956, two groups, Ford et al. and Nowell et al., presented evidence of bone marrow chimeras after transplanting donor bone marrow into irradiated recipients [6] [7]. Ford et al. used a distinct chromosome found in donor mice to show chimerism in lethally irradiated recipient mice [6]. His group showed that the donor cells persisted for more than 30 days, suggesting these cells become residents of the host bone marrow and replicate to give rise to new blood cells. Nowell et al. used the dichotomy of alkaline phosphatase staining found in rat and mouse bone marrow cells [7]. Mouse bone marrow cells stained negative for alkaline phosphatase while rat bone marrow cells were strongly positive. With elegant controls, they show that bone marrow from irradiated mice injected with mouse bone marrow stained negative for alkaline phosphatase. On the other hand, bone marrow from irradiated mice injected with rat bone marrow reacted strongly to alkaline phosphatase staining. These works showed that cells are, in part, necessary for hematopoietic recovery post-irradiation. Finally, in 1959, Miya et al. reported that cell-free extracts prepared from spleens do not protect irradiated murine recipients from bone marrow failure, solidifying that recovery is not singularly dependent on a humoral factor [8].

James E. Till and Ernest A. McCulloch are credited for rigorously characterizing HSC through the 1960s. The two described an experiment that is now the gold standard for quantifying viable, self-renewing HSC by essentially transplanting dilutions of bone marrow into lethally irradiated mice [9]. They found the number of macroscopic nodules or colonies that appeared on the spleen was proportional to the number of injected bone marrow cells. Using a distinct chromosome marker in 1963, Becker et al. described the clonal nature of spleen nodules that formed when irradiated mice were injected with bone marrow [10]. These data together allowed them to propose that a single cell from the bone marrow, a “colony-forming cell”, could give rise to many differentiated cells within a single spleen nodule. These nodules became known as spleen colony-forming units (CFU-S) (Figure 1-2). Later the following year, Till and McCulloch tried to quantify the number of stem cells from the bone marrow by performing serial transplants from these CFU-S [11]. Here, their work described the self-renewal ability and multi-lineage differentiation capacity of these colony-forming cells, which we now know are hematopoietic stem and progenitor cells (HSPC). They also described the stochastic nature of self-renewal and differentiation [12]. Till et al. used these data to show that the colony-forming cells, or HSPC, do not have an unlimited capacity for self-renewal. They showed that with repeated transplantations, eventually these cells become exhausted and self-renewal ability is lost. They also reasoned that the progeny of these colony-forming cells, which also have some self-renewing abilities, must not be identical to their parent colony-forming cell as the progeny have reduced self-renewing abilities. Till and McCulloch also worked with a group who characterized mutant mouse models [13 14]. Russell et al. showed that mice with the genetic mutation W/Wv were anemic at steady state, significantly more sensitive to irradiation and the W/Wv HSPC had significantly reduced colony-forming ability when transplanted but that this condition could be cured by transplantation of isologous bone marrow from wild-type (w/w) littermates. Another
Figure 1-2. Experimental schematic for spleen colony-forming assay. Bone marrow would be collected from a donor mouse and injected intravenously into a heavily irradiated recipient mouse [12]. Ten days after injection, the spleen from the recipient mouse would be isolated and observed for nodules. These nodules consisted of clones of differentiated cells from a single colony-forming cell.
model, the S1/S1\textsuperscript{d} mouse model had a similar phenotype as the W/W\textsuperscript{v} model, except w/w bone marrow transplantation could not rescue the anemia. These two groups coordinated together and reasoned that in the W/W\textsuperscript{v} model there was a cell-intrinsic defect, resulting in anemia and reduced colony forming activity after transplantation. Conversely, in the S1/S1\textsuperscript{d} model, a cell-extrinsic signal must be compromised during transplantation. In a paper published in 1965, McCulloch et al. showed that when bone marrow from S1/S1\textsuperscript{d} mice was transplanted into W/W\textsuperscript{v} mice, erythropoiesis was restored [15]. For much of their careers, Till and McCulloch remained focused on understanding HSC kinetics and dissecting how the stem cells balanced self-renewal, an explosive proliferative capacity and differentiation. Concurrently, the field of bone marrow transplant in humans was garnering success. The first successful bone marrow transplant was conducted in 1956, and by the 1960s, donor selection was improved by identifying the necessity for human leukocyte antigen (HLA) matching between donors and recipients.

Hematopoietic Stem Cell Transplant as Treatment for Hematopoietic Malignancy and Disease

The Nobel Prize in Physiology and Medicine was awarded to E. Donnall Thomas in 1990 for his discoveries concerning organ and cell transplantation to treat human disease, and thus is recognized as the “Father of bone marrow transplantation”. Thomas’ first attempts of allogeneic bone marrow transplant with humans began in 1957 [16]. These six transplants did not yield life-long maintenance of the hematopoietic system but were the starting point for Thomas’ career in improving BM transplants. Studies in 1958 by Uphoff and Law revealed that donor histocompatibility genes (H-1, H-2, H-3), coding for antigens that would be present on the surface of cells, deserved important consideration for successful allogeneic transplants in mice [17]. In this work, differences especially in the H-2 phenotype between donors and recipients resulted in significant loss of transplanted grafts and overall reduced survival, supporting the concept of an immune response elicited by donor marrow. Thomas conducted an experiment where he showed lethally irradiated canines could be reconstituted with bone marrow from littermates but failed to survive due to infection and other issues, otherwise known as secondary syndromes [18]. Thomas successfully transplanted bone marrow from two sets of identical twins in 1959 to treat leukemia (Figure 1-3) [19]. Through the 1960s Thomas continued BM transplant experimentation in canines. Thomas recognized the importance of radiation dosage resulting in the destruction of the host’s immunologic defense allowing a graft to be transplanted successfully [18] as well as administration of the immunosuppressive drug, Methotrexate [20 21]. He also worked on improving the procurement and storage of marrow cells [22 23]. In 1968, Storb et al. dramatically improved allogeneic transplantation in canines by including histocompatibility assays in donor selection [24 25], something that had been shown previously in mice [17 26]. Later these assays would be known as HLA matching. By December 1968, two groups had reported case studies where patients with immunological disorders (i.e. Wiskott-Aldrich syndrome and X-linked severe combined immunodeficiency) could be transplanted with bone marrow from histocompatibility-gene matched siblings, effectively curing the
Figure 1-3. A timeline of the history of bone marrow transplantation.
this time, allogeneic transplantation rapidly expanded as a treatment option for many malignancies and disorders.

**Indications for HSCT and Complications**

The previous two decades have witnessed an increased trend in survival of people diagnosed with hematopoietic diseases and treated with HSCT [27]. Figure 1-4 indicates, leukemia and myelodysplastic syndromes (MDS) are the most common indications for allogeneic HSCT, in addition to aplastic anemia (AA) and other non-malignant diseases. Autologous HSCT is more commonly used to treat lymphomas and myelomas, as well as other cancers (i.e. Breast cancer, germ cell tumors, etc.) (Figure 1-4). HSCT patients are normally treated with a preparative or conditioning regimen consisting of high doses of chemotherapy or radiotherapy and immunosuppressive drugs. There is ongoing work trying to improve these regimens by reducing toxicities and dampening acute adverse effects. However, there are still many complications associated with HSCT, such as engraftment failure, infection and mortality (Figure 1-5). For example, graft-versus-host disease (GVHD) is a common obstacle encountered by recipients of allogeneic HSCT. GVHD can be classified as either acute (aGVHD) or chronic (cGVHD), based on the amount of time between HSCT and disease presentation, organ involvement, and other histological analyses [28]. GVHD develops when donor derived T-cells identify recipient cells as foreign and an immune response is mounted against the host. This disease is associated with impaired hematopoiesis and poor prognosis. The precise mechanism for how GVHD adversely affects BM recovery is still being explored. Knowledge of the regulatory mechanisms of HSC is highly complex and made more complicated by the surrounding microenvironment.

**Extrinsic and Intrinsic Factors Necessary for Successful HSCT**

**Bone Marrow Reconstituting Niche**

As mentioned previously, the first successful human BM transplant was reported in 1959, and by the 1960s, donor selection was improved by identifying the necessity for HLA matching between donors and recipients. In 1984, it was established that human HSC expressed the surface antigen CD34 [29 30]. Shortly after, CD34+ cells were shown to repopulate irradiated baboons [31]. Finally, transplantation protocols were drastically transformed by the discovery that recombinant human granulocyte-macrophage colony stimulating factor (rhGM-CSF) and recombinant human granulocyte-colony stimulating factor (rhG-CSF) could mobilize and enhance the number of CD34+ HSC collected from a patient’s blood [32]. Mobilized peripheral blood stem cells are now the most commonly used source of HSC for transplantation [27]. In 2018, over 14,000 autologous HSCT and over 9,000 allogeneic HSCT were performed in the United States to treat hematologic disease; the vast majority of stem cells being isolated by means of mobilization. Several distinct strategies have been implemented to induce PB mobilization of HSC for
Figure 1-4. Indications for HCT in the United States as of 2018.
Most transplants conducted involve the patient’s own marrow (autologous) and are used to treat myeloma, non-Hodgkins lymphomas (NHL) and Hodgkins disease (HD). Acute leukemias (AML, ALL) and MDS (combined with MPNs) are the most common indications for allogeneic transplants accounting for 75% of allogeneic HCTs.
Figure 1-5. Causes of death after HCT.

(A) Causes of death after autologous HCT. (B) Causes of death after HLA-matched sibling HCT. (C) Causes of death after unrelated donor HCT. These data represent 3-year mortality and conducted in 2016-2017.

transplantation. The most commonly used HSC mobilizer is G-CSF (filgrastim). During homeostasis, serum G-CSF protein levels in healthy individuals are relatively low and produced primarily by macrophages and monocytes [33]. However, during challenge, such as bacterial infections or cytotoxic therapies, the BM endothelium and fibroblasts are induced to produce G-CSF [34]. In the niche, administered rhG-CSF can significantly increase numbers of neutrophils, which release proteolytic enzymes that cleave adhesion molecules bonding HSC to its BM niche and thereby mobilize HSC to the periphery. Fundamentally, all HSC mobilization strategies depend on disrupting HSC engagement with its homeostatic niche (i.e., h-niche) in the BM.

The BM HSC niche, as a hypothesis, was first proposed by Raymond Schofield about 20 years after the first successful BM transplant [35]. He suggested that HSC are juxtaposed with other cells that guide HSC self-renewal, asymmetric division and quiescence. This hypothesis is now well supported as many distinct cell types are now known to support the HSC niche (both directly and indirectly) including macrophages, endothelial cells, sympathetic nerve cells, adipocytes, megakaryocytes, osteolineage cells, mesenchymal stem cells (MSC), and neutrophils (reviewed in [36]). Secreted factors and the extracellular matrix (ECM) also contribute to the regulation of the niche and HSC. Although significant advances have been made in the mobilization and collection of HSC for transplant, the challenge still remains that very few transplanted HSC actually participate in the stable engraftment and reconstitution of patients [37 38]. Thus, strategies focused on improving the efficiency of HSC engraftment could significantly reduce both the number of HSC required for transplant and transplant morbidity by allowing for milder conditioning regimens. Developing these strategies requires a deep understanding of the cell autonomous and non-cell autonomous mechanisms influencing the successful engagement of HSC with the BM niche. Transplanted HSC must overcome multiple bottlenecks during transplantation. Paramount among these is engaging with a niche damaged by disease, myeloablative therapy and conditioning regimens. Megakaryocytes, osteoblasts, adipocytes and endothelial cells represent multiple examples of cellular components of the BM niche perturbed by conditioning regimens, both directly and indirectly. Outlined in Table 1-1 is our current understanding of the post-ablated niche, designated here as the “BM reconstituting niche (r-niche),” and the key players involved in fostering transplanted HSC.

BM stromal cells and secreted factors contribute to recovery of the r-niche and hematopoiesis

Vascular regeneration is critical for successful HSC engraftment. Transplant conditioning dramatically alters the BM microenvironment. Irradiation or myelosuppression induces the BM vasculature to regress, become dilated, leaky, and highly unorganized [39]. Three to seven days after lethal irradiation of mice, 20% of endothelial cells die. For efficient BM regeneration, new construction or repair of damaged blood vessels must occur. Extrinsic factors that promote neovascularization after myelosuppression include vascular endothelial growth factor A (VEGFA) and angiopoietin-1 (ANGPT-1), which are secreted by cells expressing the Leptin
Table 1-1. Comparison of bone marrow cell types and their function in the h-niche and r-niche.

<table>
<thead>
<tr>
<th>Cell type</th>
<th>h-Niche</th>
<th>r-Niche</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adipocytes</td>
<td>• Source of Adiponectin</td>
<td>• Source of SCF</td>
<td>[40 41]</td>
</tr>
<tr>
<td></td>
<td>• More common with age and obesity</td>
<td>• Promotes BM recovery</td>
<td></td>
</tr>
<tr>
<td></td>
<td>• Mouse thoracic vertebra bone marrow is adipocyte rich with fewer HSC</td>
<td>• Adipose-deficient mice have increased BM repopulating ability</td>
<td></td>
</tr>
<tr>
<td>Endothelial Cells</td>
<td>• Conduit for HSC movement in and out of marrow</td>
<td>• Damaged, leaky</td>
<td>[39 42-45]</td>
</tr>
<tr>
<td></td>
<td>• Component of vascular niche</td>
<td>• Upregulates EGF, TIE2, G-CSF, PTN, TN-C, VCAM1, E-SELECTIN</td>
<td></td>
</tr>
<tr>
<td></td>
<td>• Supportive scaffold for BM stromal cells</td>
<td>• Promotes BM recovery</td>
<td></td>
</tr>
<tr>
<td>Leptin Receptor+MSC</td>
<td>• Source of SCF, CXCL12, ANGPT1</td>
<td>• Critical source of SCF, CXCL12, ANGPT1, VEGFA</td>
<td>[40 42]</td>
</tr>
<tr>
<td></td>
<td>• Regulates HSC maintenance</td>
<td>• Increased proliferation post-irradiation</td>
<td></td>
</tr>
<tr>
<td></td>
<td>• Located near sinusoids</td>
<td>• Promotes BM recovery</td>
<td></td>
</tr>
<tr>
<td>Macrophages</td>
<td>• Source of CXCL12</td>
<td>• Proposed signaling axis with NPY</td>
<td>[46]</td>
</tr>
<tr>
<td></td>
<td>• Depletion leads to HSC mobilization</td>
<td>• NPY-induced elevated Tgfb expression</td>
<td></td>
</tr>
<tr>
<td></td>
<td>• Regulates Nestin⁶ MSC gene expression</td>
<td>• Promotes BM recovery</td>
<td></td>
</tr>
<tr>
<td>Megakaryocytes</td>
<td>• Source of platelets</td>
<td>• Relocate to endosteum</td>
<td>[47]</td>
</tr>
<tr>
<td></td>
<td>• Source of TPO</td>
<td>• Upregulate PDGFb, PDGFBb, bFGF</td>
<td></td>
</tr>
<tr>
<td></td>
<td>• Perivascular</td>
<td>• Promotes BM recovery</td>
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Table 1-1. Continued.

<table>
<thead>
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<th>r-Niche</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nestin+ MSC</td>
<td>• Source of ANGPT1, CXCL12</td>
<td>• CXCL12 supports BM recovery</td>
<td>[36 42 48]</td>
</tr>
<tr>
<td></td>
<td>• Regulates HSC maintenance and retention</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Osteolineage Cells</td>
<td>• Maintenance of bone</td>
<td>• Multi-layered</td>
<td>[49-51]</td>
</tr>
<tr>
<td></td>
<td>• Source of CXCL12</td>
<td>• Upregulates CXCL12, IL-18, EMBIGIN, ANG</td>
<td></td>
</tr>
<tr>
<td></td>
<td>• Component of endosteal niche</td>
<td>• Primary site of HSC homing</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>• Upregulates CXCL12, IL-18, EMBIGIN, ANG</td>
<td></td>
</tr>
<tr>
<td>Sympathetic Nerve Fibers</td>
<td>• Source of TGF-β</td>
<td>• Upregulates NPY, β-adrenergic signaling</td>
<td>[46 52 53]</td>
</tr>
<tr>
<td></td>
<td>• Regulates HSC quiescence and maintenance</td>
<td>• Promotes regeneration of endothelium and Nestin+ cells</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>• Promotes BM recovery</td>
<td></td>
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</table>

receptor (LepR) and HSPC, respectively [40]. LepR+ cells are a sub-population of MSC with multipotent potential and are critical for HSC maintenance in the h- and r-niche [40 42 48]. Regenerating BM endothelial cells upregulate expression of the ANGPT-1 receptor, epidermal growth factor homology domains-2 (also known as TIE2). When ANGPT-1/TIE2 signaling is disrupted, regeneration of BM endothelial cells is delayed, resulting in a concomitant delay in HSC reconstitution. Other factors secreted by endothelial cells are also increased shortly after myelosuppression, including epidermal growth factor (EGF) and EGF receptor agonist, AMPHIREGULIN [54]. In mice, systemic addition of EGF represses PUMA-mediated apoptosis in HSC resulting in increased HSC survival and BM regeneration [43 45]. Further, conditional deletion of Jagged-1, a NOTCH ligand, specifically in endothelial cells results in impaired HSC regeneration after lethal irradiation [55]. Although the NOTCH-pathway appears dispensable for normal hematopoiesis, JAG1-signaling via NOTCH in BM endothelial cells is necessary for efficient hematopoietic regeneration. Another BM endothelial secreted factor of interest is pleiotropin (PTN). PTN administration increases mouse survival after lethal irradiation and HSC transplantation via induction of RAS/MEK/ERK signaling, which promotes HSC quiescence [56]. PTN also regulates HSC homing and retention post-irradiation. In summary, VEGFA, ANGPT-1, EGF, and JAG1 are all upregulated by endothelial cells, promoting BM recovery (Figure 1-6). The vasculature is the conduit for transplanted HSPC to travel to the BM or from the BM to the periphery during mobilization or inflammation. Recovery of the vasculature is one of the first critical steps in successful HSC engraftment. Our further understanding of how endothelial cells mechanistically regulate HSPC function and how we can lessen the collateral damage inflicted on vasculature will facilitate efforts to improve mobilization and engraftment.

**Mesenchymal stem and progenitor cells maintain HSC during homeostasis and BM regeneration.** Interestingly, some BM niche cells tolerate radiation better than others. MSC are more resistant to radiation-induced ablation than HSC [57]. MSC are a subset of multipotent progenitors that give rise to non-hematopoietic cells in the BM, especially during regeneration. The LepR marks MSC capable of generating bone, adipocytes, and cartilage in culture and after transplantation. Although LepR+ cells are essential for maintaining HSC in the h-niche, less is known about their role during stress or after myeloablation [48]. Recent evidence suggests that they also support HSCs in the r-niche. For example, after irradiation, LepR+ MSC are the main source of new BM osteoprogenitors and adipocytes, which are required to coordinately support blood regeneration in the r-niche after HSCT [40]. Both during homeostasis and after HSCT, LepR+ cells are a critical source of stem cell factor (SCF) and C-X-C motif chemokine ligand 12 (CXCL12). SCF is key supportive cytokine of HSC in the niche and CXCL12, with its receptor C-X-C chemokine receptor type 4 (CXCR4), is a master regulator of HSC migration and survival. Although many cell types in the BM produce SCF (e.g., endothelial cells, osteolineage cells, adipocytes, LepR+ cells, and HSPC), only LepR+ cell-derived SCF is critical for HSC reconstitution post-transplant . Overall, MSC are essential for HSC reconstitution in the r-niche, where they function as a key source of multiple critical supportive secreted factors (e.g., SCF and CXCL12) and differentiate into osteoprogenitors and adipocytes (Figure 1-6). There is currently great interest in
Figure 1-6. Proposed bone marrow reconstituting niche recovering from irradiation.
Endothelial cells are damaged and vessels become leaky (no distinction between arterioles and sinusoids have been made). Some endothelial cells succumb to irradiation-induced apoptosis. Surviving endothelial upregulate synthesis of TIE2, G-CSF, PTN, and EGF. The endosteal niche has become multi-layered and radio-resistant megakaryocytes have relocated from the vasculature to near the endosteum, where they secrete ECM proteins and upregulate osteoblast growth-promoting factors (PDGFβ, PDGFBB, bFGF). Sympathetic nerve fibers are damaged post-irradiation. LepR+ MSC cells secrete survival factors (depicted by ↑) and differentiate (shown as a bold arrow) into adipocytes and osteoprogenitors. DKK1 secreted by osteoprogenitor cells acts on (shown as an arrow) HSPC, reducing ROS, apoptosis, and senescence (depicted by ↓).
exploiting MSC clinically in regenerative therapies. They have been shown capable of repairing damaged cardiac tissue and bone defects [58]. In the future, it will be interesting to unravel the full multipotent potential of MSC in repairing the r-niche.

**Osteolineage cells and many secreted factors aid in BM recovery post-transplant.** Irradiation results in a dramatic expansion of endosteal osteoblasts, transforming them from a single cell layer to many cell layers [59]. This expansion is accompanied by increased secretion of CXCL12 by osteoblasts, which perturbs the localization of BM megakaryocytes from perivascular BM to the endosteal bone surface. Megakaryocytes may then encourage further osteoblast proliferation by secreting growth factors like platelet-derived growth factor β (PDGF-β), PDGF-BB and basic-fibroblast growth factor (bFGF). CXCL12 promotes megakaryocyte homing and survival [47]. During homeostasis, megakaryocytes respond to endothelial-derived CXCL12 and protrude into the vessels to release platelets directly into the bloodstream. Vascular damage caused by irradiation contributes to thrombocytopenia in patients by disrupting these endothelial-associated megakaryocytes. Administration of TPO in combination with CXCL12 expands megakaryocytes and increases platelets in irradiated mice [60]. TPO regulates the DNA-damage response in HSC post-irradiation and pre-treatment improves engraftment. These data implicate secreted factors, TPO and CXCL12, as tantalizing targets for improving outcomes in patients undergoing HSCT. In 2008, a nonpeptide TPO receptor agonist, eltrombopag, was approved by the FDA for treatment of chronic immune (idiopathic) thrombocytopenic purpura. In a small case study, eltrombopag was used to treat prolonged thrombocytopenia in patients who had received HSCT [61 62]. This treatment resulted in platelet recovery and negated the need for platelet transfusion. Osteoprogenitor cells also secrete the WNT inhibitor, Dipkkof-1 (D KK1) [63]. DKK1 promotes increased recovery of HSC numbers after irradiation, while its deletion results in reduced HSPC numbers after irradiation. Thus, BM osteoprogenitors are a critical source of DKK1. Mechanistically, DKK1 reduces mitochondrial reactive oxygen species (ROS), decreasing apoptosis and senescence in HSPC. DKK1 can also induce secretion of EGF from BM endothelial cells, which further aids in HSC recovery post-transplant [64]. Several novel secreted factors were recently discovered that are expressed by osteolineage cells residing close to transplanted HSPC [50]. Three of these, including the proinflammatory cytokine IL-18, the cell adhesion molecule EMBIGIN, and the secreted RNase ANGIOGENIN (ANG) have been found to play a key role in regulating HSC in both the h-niche and r-niche. ANG expression in the r-niche is required for efficient HSC transplantation [65]. Further, HSC transplanted into mice that lack ANG display compromised serial transplantation activity. Therefore, ANG likely functions as a non-cell autonomous factor that regulates HSC quiescence and self-renew in the h-niche. IL-18 appears to act specifically on short-term reconstituting HSC, thus enhancing acute hematopoietic repopulation post-transplant [50]. EMBIGIN regulates HSC quiescence and homing post-transplant. HSC transplanted into EMBIGIN-deficient mice display increased proliferation. Interestingly, osteolineage cells expressing vascular cell adhesion molecule-1 (VCAM1) and EMBIGIN (termed VE cells) respond to myeloablation by upregulating the expression of CXCL12, VCAM1 and cell–cell adhesion genes. These findings implicate VE cells as a novel population whose response to conditioning may actually be critical in supporting the stable engraftment of
transplanted HSPC. Indeed, osteolineage cells appear to form prime real estate for transplanted HSC via their production of multiple factors that improve HSC survival and proliferation, such as CXCL12, DKK1, IL-18, EMBIGIN, and ANG (Figure 1-6). More work is needed to determine if these observations can be exploited to improve engraftment in the clinic.

The nervous system promotes regeneration of other important niche cells: endothelium and nestin+ cells. The sympathetic nervous system (SNS) also plays a significant role in promoting hematopoietic regeneration after myeloablation. Indeed, chemotherapies that damage nerves also compromise hematopoietic regeneration while protection of sympathetic nerve fibers from apoptosis via Trp53 deletion or induced neuroregeneration via 4-methylcatechol or glial-derived neurotrophic factor treatment promotes hematopoietic regeneration [66]. The SNS appears to promote hematopoietic regeneration indirectly by activating β-adrenergic signaling and secreting neuropeptides in the niche. Denervation of the BM or blocking adrenergic signaling results in an increased loss of BM endothelial and Nestin+ mesenchymal cells following myeloablation, which stifles hematopoietic regeneration. Nestin+ cells are key sources of CXCL12 during homeostasis to which HSC home after lethal irradiation and transplant. Nestin+ cells crosstalk with macrophages in the h-niche and maintain HSC retention. In vitro, macrophages respond to Neuropeptide Y (NPY), a secreted neurotransmitter from sympathetic nerves, via the NPY receptor, Y1 [67]. Evidence suggests NPY, acting via the macrophage Y1 receptor, induces release of transforming growth factor β (Tgf-β) to suppress HSC cell cycling; preventing premature exhaustion. Whether these findings translate in vivo is unclear. Indeed, NPY treatment reduces irradiation-induced nerve damage and improves engraftment. HSC cannot efficiently engraft the BM of mice lacking NPY due to reduced homing and increased apoptosis in both stromal populations and HSC themselves [68]. Thus, NPY is a critical regulator of HSC during homeostasis and regeneration, making it ripe for clinical exploitation to improve outcomes in patients. A better understanding of how the nervous system regulates HSC is likely to illuminate additional factors that could be exploited to improve HSC engraftment and BM recovery. In sum, β-adrenergic signaling and NPY are key SNS-derived players that regulate the r-niche.

Bone morphogenic proteins negatively regulate HSC repopulating activity. During homeostasis, sympathetic nerves ensheathed by nonmyelinating Schwann cells secrete factors, such as TGF-β, that regulate HSPC cell cycle dynamics and quiescence [52]. Multiple bone morphogenic proteins (BMP), which belong to the TGF-β family, modulate bone mass and maintain bone homeostasis in the h-niche. Systemic infusion of BMP7 decreases HSC expansion and engraftment [69]. In contrast, infusion of NOGGIN, a BMP antagonist, has the opposite effect. BMP4 is critical for HSC maintenance during homeostasis and after transplantation [70]. Interestingly, wild type (WT) HSC are capable of multilineage reconstitution in lethally irradiated BMP4-deficient recipients. However, HSC previously transplanted to a BMP4-deficient recipient displayed poor repopulating activity when transplanted into secondary recipients. The critical source of BM BMP4 post-transplant is unclear, as osteolineage cells, vascular endothelial cells, perivascular cells, radio-resistant T-cells and megakaryocytes all produce BMP4 in
response to radiation. BMP4 also induces BM adipogenesis in irradiated mice by inducing up-regulation of peroxisome proliferator activated receptor-g, a key transcriptional regulator of adipogenesis [71]. In sum, BMP4 and BMP7 negatively regulate HSC repopulating activity; in contrast, treatment with a BMP4 antagonist improves engraftment. The TGF-β gene family is large and has been implicated in homeostatic regulation of HSC and other somatic stem cells. It will be interesting to see how other family members are involved in HSC repopulating activity.

**Adipocytes: a source for SCF.** Adipocytes have been implicated as both negative and positive regulators of hematopoietic regeneration [40 41]. They appear to be a key source of BM SCF during HSC regeneration post-irradiation and deletion of SCF from adipogenic-restricted LepR+ progenitors results in reduced HSC regeneration in mice post-transplant. Indeed, co-transplantation of BM CD45-CD31-Sca1+CD24+ multipotent progenitors that give rise to adipogenic progenitors and express LepR and CXCL12, enhances HSC repopulation. However, high adipocyte content also correlates with low HSC numbers in different BM compartments and mice with severely diminished adipogenesis possess HSC with enhanced repopulating ability. It appears that very early adipogenic progenitors promote HSC regeneration and function while downstream adipogenic progenitors suppress HSC function. Relatively little is currently known about the factors secreted by BM adipocytes (i.e., adipokines) during homeostasis and regeneration. Adiponectin, a secreted adipokine, regulates HSC growth and HSC express the cognate receptor. However, adiponectin is elevated in HSCT patients diagnosed with GVHD and has antiangiogenic properties [72]; not effective in an environment requiring vascular repair. There is controversy in the field as to whether adiponectin has pro- or anti-inflammatory mechanisms. Chronic inflammation is associated with BM dysfunction. Although more work is needed to resolve these conflicting results and to clarify the role of adipocytes in the r-niche, most current evidence suggests a positive role for BM adipocytes regulating BM repopulation. Further, given that BM adipocytes increase with both age and obesity, as obesity rates increase and a large flux of people enter old age, identifying the critical roles of adipocytes during homeostasis and regeneration is paramount.

**Direct cell-to-cell communication promotes HSC engraftment and BM regeneration**

Cells communicate directly with other cells via integrins, gap junctions and adhesion molecules. Cells also interact with elements in their surrounding milieu. Multiple components of the ECM are important for hematopoietic recovery posttransplant (**Figure 1-6** and **Table 1-2**). Significant cellular components of the r-niche, such as megakaryocytes, upregulate fibrous ECM proteins like laminin, Type IV collagen and fibronectin in response to myeloablation; facilitating the physical interaction of HSC with the BM niche. Many of these proteins also promote viability and expansion of HSC in *ex vivo* cultures. HSC survival post-transplant can be mediated by direct cell–cell communication. Gap junctions are channels between cells that allow for the direct exchange of small molecules. These channels are composed of connexins, which are dodecamers that link together on juxtaposed cells. Connexin 43 (Cx43) is required for
Table 1-2. Comparison of forms of cellular communication in the bone marrow and their function in the h-Niche and r-Niche.

<table>
<thead>
<tr>
<th>Cellular communication</th>
<th>h-Niche</th>
<th>r-Niche</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Connexin-43</td>
<td>• Regulates HSC traffic to and from BM</td>
<td>• Facilitate ROS transfer between cells</td>
<td>[73 74]</td>
</tr>
<tr>
<td></td>
<td>• Expressed by osteolineage cells</td>
<td></td>
<td></td>
</tr>
<tr>
<td>E-Selectin</td>
<td>• Leukocyte rolling</td>
<td>• Aids in homing, engraftment and HSC proliferation</td>
<td>[44 75-77]</td>
</tr>
<tr>
<td>Junctional Adhesion</td>
<td>• Involved in immune response, inflammation, and leukocyte migration</td>
<td>• Required for survival and engraftment</td>
<td>[78 79]</td>
</tr>
<tr>
<td>Molecules</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MADCAM1</td>
<td>• Undetermined</td>
<td>• Promotes engraftment</td>
<td>[80]</td>
</tr>
<tr>
<td>Tenascin-C</td>
<td>• KO has normal hematopoiesis</td>
<td>• Upregulated after myeloablation</td>
<td>[81]</td>
</tr>
<tr>
<td></td>
<td>• Part of adhesion molecule family</td>
<td>• Induces HSC proliferation</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>• Regulates cell cycle genes</td>
<td></td>
</tr>
<tr>
<td>VCAM1</td>
<td>• Regulates HSC egress from BM</td>
<td>• HSC homing post-transplant</td>
<td>[75]</td>
</tr>
</tbody>
</table>

HSC regeneration after myeloablation in mice [73]. It is expressed by both HSPC and osteolineage cells and facilitates the transfer of ROS from HSPC to non-hematopoietic cells in the r-niche [74]. This likely helps to diminish ROS levels, which are elevated post-irradiation and toxic to cells. VCAM1 is expressed by endothelial cells and its receptor, very-late-antigen 4 (VLA4, also known as α4β1), is expressed by HSPC. Blocking VCAM1 inhibits HSPC homing to the BM [75]. Mucosal addressing cell adhesion molecule-1 (MADCAM1, also known as α4β7) is also expressed by endothelial cells [80]. Endothelial expression of MADCAM1 is induced by irradiation and blocking MADCAM1 is detrimental to HSCT. Junctional adhesion molecules (JAM) are critical regulators of the physical interaction of HSC with their niche. JAM-A, JAM-C, JAM4, and ESAM are all expressed by HSC [79]. In contrast, JAM-B is expressed by BM stromal cells and is required for survival and engraftment of lethally irradiated recipients [78]. Tenascin-C (TN-C) is a non-fibrous component of the ECM. After myeloablation, TN-C is secreted by endothelial cells and CXCL12-abundant reticular cells in the r-niche [81]. HSPC express integrin α9, which is the ligand for TN-C. TN-C promotes HSPC proliferation in an integrin α9-dependent manner and also increases expression of cell-cycle-promoting genes while suppressing inhibitory cell cycle genes. α9 integrin regulates HSPC–niche interactions, as do α4, α6, and β1 integrins [82-84]. While integrins are one means of maintaining cellular interactions, cadherins and selectins also facilitate cell–cell interactions. P-selectin and E-selectin are canonical regulators of leukocyte rolling [76]. E-selectin, expressed on endothelial cells, aids in the homing, engraftment, and proliferation of HSPC. E-selectin is upregulated after irradiation in mice and E-selectin+ endothelial cells are found mostly near the endosteum, where HSPC localize, proliferate and differentiate [77]. An E-selectin antagonist reduces HSC cycling, increases HSC survival post-transplant and enhances neutrophil recovery in the peripheral blood [44]. Thus, this antagonist may be an attractive clinical target for transplant patients.

More studies are needed to further understand the roles of fibrous ECM proteins such as, laminin, Type IV collagen and fibronectin. For example, HSC cultured on a fibronectin scaffold display significant expansion [85]. Furthering knowledge of these components could improve the ex vivo expansion of HSC, which will be useful during HSCT. Additionally, Cx43 is required for ROS transfer between HSC and stromal cells. Vascular cell adhesion molecule 1 and MADCAM1 are expressed by endothelial cells and are essential for HSC homing and BM regeneration, respectively. Junctional adhesion molecules, TN-C, integrins, and E-selectin are necessary for BM regeneration in the r-niche after irradiation.

**Functional Screen Identifies Regulators of Murine Hematopoietic Stem Cell Repopulation**

Successful HSCT requires that transplanted hematopoietic cells migrate to the appropriate marrow space, engage with the r-niche, receive survival cues and proliferate sufficiently to repopulate a recipient whose own hematopoietic system has been ablated or compromised. Considerable effort has been directed toward understanding the bone...
marrow niche, as described previously. Our lab conducted a screen of genes as potential molecular regulators of HSPC during transplant [86]. Genes of interest were knocked down via shRNAs in HSPC and transplanted into lethally irradiated mice. This study revealed 15 genes that were required for hematopoietic repopulation ability, 13 of which not previously implicated in HSC biology. These genes include Arhgef5, Cadps2, Crispd1, Emen, Foxa3, Fstl1, Glis2, Gpr56, Myct1, Nbea, P2ry14, Smarca2, Sox4, Stat4, Zfp521 and Nfix (Figure 1-7A). Genes found not be required for hematopoietic repopulation in HPSC is included in Figure 1-7B. In general, there was a significant decrease in the donor chimerism in the PB but overall the PB lineage distribution remained similar to controls, except in recipients of HSPC treated with Cadps2 and Foxa3 shRNAs (Figure 1-7C). In order to interrogate functional defects in HPSC where each of these genes were knocked down, the colony forming ability, cell cycle status, apoptosis and immunophenotype for each was examined. It was shown that the total colony forming ability was only significant in HSPC treated with Arhgef5-shRNAs, Emen-shRNAs and Fstl1-shRNAs (Figure 1-8A, top). The distribution of the different colonies (CFU-E, CFU-G/M/GM, CFU-GEMM) formed was significantly different from controls in HSPC treated with Cadps2-shRNAs, Emen-shRNAs, Fstl1-shRNAs, Nbea-shRNAs and p2ry14-shRNAs (Figure 1-8A, lower). HSPC treated with Arhgef5-shRNAs resulted in a significant increase of cells occupying G1 and a concomitant loss of cells in G2/S/M and G0 of the cell cycle (Figure 1-8B, top). HSPC treated with Glis2-shRNAs had begun losing their LSK phenotype five days post-transduction compared to controls (Figure 1-8B, middle). The only shRNAs to results in less HSPC cell death included those targeting Glis2 (Figure 1-8C, lower). This study also identified two genes that enhanced HSPC repopulation, Gprasp2 and Armcx1. Figure 1-8C summarizes the mean percentage of donor chimerism in each of the cells making up the heterogeneous HSPC population. Each of these genes are canonically associated with regulating vesicular trafficking, cell surface receptor turnover and secretion of ECM components suggesting active cross talk between HSPC and the niche. The work discussed here specifically focuses on the gene Nfix.

The Nuclear Factor I Family of Site-Specific DNA Binding Proteins

Initial Discoveries of the NFI Family

The nuclear factor I (NFI) protein family was first identified in HeLa cells where it was shown to be essential for initiating the formation of a complex between the adenovirus (Ad) precursor terminal protein (pTP) and 5′-dCMP, the 5′-terminal nucleotide of all human Ad DNA sequences [87]. Thus, making NFI required for Ad DNA replication in vitro. Later, it was discovered that NFI, as well as another factor, Oct-1, recruit Ad DNA polymerase to the origin of replication as well as the pTP via specific recognition sites. These recognition sites were identified as a palindromic sequence consisting of 15-16 nucleotides, TGG(N6-7)GCCA [88 89]. It was also noted that the proteins can bind half of the consensus sequence but with lower affinity [90]. Shortly after its identification, the NFI binding sequence was observed in other virus
Figure 1-7.  shRNA-mediated knockdown of genes in HPSC identifies 15 genes required for robust HSPC repopulating activity.

(A) Verified loss-of-function hits. A one sample Student’s t test was performed testing the null hypothesis that the normalized measurements = 1. P-values are two-sided. §, P < 0.1; *, P < 0.05; **, P < 0.005; ***, P < 0.0001. Only p-values calculated >16 wk after transplant are shown. (B) Functional screen non-hits. In E and F, each gene was interrogated with at least two independent shRNAs (labeled as a, b, or c) and the percentage of CD45.2 PB at 4 and >16 wk after transplant of recipients of gene-specific shRNA–treated Test cells normalized to that of recipients of control shRNA–treated Test cells is shown. (C) Distribution of T, B, and myeloid PB lineages in mCherry+CD45.2+ compartment of genes that scored as hits after retesting >16 wk after transplant. In E–G, each bar is the average of at least four recipient mice, and error bars represent SD. In G, asterisks denote a statistically significant difference in distribution of at least one lineage relative to control for both shRNAs tested (P < 0.05). P-values were calculated using the exact Wilcoxon Mann-Whitney test. ND, not determined.

Figure 1-8. Functional analysis of HSPC treated with gene-specific shRNAs. (A) 500 mCherry+ LSK cells transduced with control or gene-specific shRNAs were assayed for CFU potential 5 days after transduction. Values are the mean of two to three independent experiments normalized to control ± SE. (B) Cell cycle status of the mCherry+ LSK cell compartment, the frequency of mCherry+ LSK cells, and apoptosis of mCherry+ LSK cells was analyzed 5 days after transduction with control or gene-specific shRNAs. Values are the mean of two to three independent experiments normalized to control ± SE. For A and B, a one-sample Student’s t test was performed testing the null hypothesis that the normalized measurements = 1. P-values are two-sided. §, P < 0.1; *, P < 0.05; **, P < 0.005. (C) Heat map summarizing mean percentage of CD45.2+ (Test cell–derived) HSC, MPP, CMP, CLP, GMP, and MEP in recipients >16 wk after transplant. Values are normalized to control recipients (i.e., 1 = yellow). Higher chimerism relative to control = darker green; lower chimerism relative to control = darker red. ND, not determined.

promoters or long terminal repeats, including the mouse mammary tumor virus (MMTV) [91] human cytomegalovirus (HCMV) [92] and the hepatitis B virus (HBV). The Ad, MMTV, HCMV and HBV are grouped together, as each consist of double-stranded DNA (dsDNA). There is data showing NFI proteins preferentially bind dsDNA and have very little affinity for single stranded DNA (ssDNA) [89]. In higher eukaryotes NFI binding sequences were identified in the promoters or regulatory regions of genes such as chicken lysozyme [93], human IgM [94], human c-myc [95] vertebrate globin genes [96] and mouse alpha 2(I) collagen [97]. Activation of the mouse alpha 2(I) collagen by NFI was shown to be induced by the cytokine, TGFβ, suggesting gene regulation functions. Eventually, there were four separate genes identified as belonging to the NFI protein family: NFIA [98], NFIB, NFIC and NFIX [99].

**NFI Family Role as Transcriptional Regulators**

There is tremendous homology among the NFI family members, especially in the amino-terminal region (Figure 1-9B, C) [100]. The amino-terminal region contains the DNA binding and dimerization domain while the carboxy-terminal region is responsible for controlling transcription activation or repression (Figure 1-9A). These proteins bind as homo- or hetero-dimers [101] to regulate cellular and viral gene transcription and viral replication. The NFI proteins bind with high affinity to the three guanine residues that are part of the consensus sequence (TGG(N6-7)GCCA) and occur mainly at the major groove [102]. Substitution of one of these guanine abolishes the ability for NFI to bind DNA. The spacer region also influences the binding of NFI. Depending on the composition of the bases, 6-7bp results in a range of binding affinity [103 104]. The NFI proteins initiate Ad5 replication by inducing a 60° bend in the replication origin of Ad5 [105]. This bend in the DNA was shown to depend on the presence of NFI and an A/T rich region upstream of the NFI binding sequence. The NFI binding site has been identified in promoters, enhancers and silencer regions of the human genome.

**Expression of NFI Family Members During Development and in Adult Tissue**

The NFI family is well studied in the nervous system. During mouse embryogenesis, Nfia, Nfib and Nfix are all expressed in the developing and postnatal brain [106]. Knockout (KO) models of Nfia and Nfib display forebrain defects, enlarged lateral ventricles, agenesis of the corpus callosum and perinatal lethality [107-109]. The Nfix KO model also has neurological defects and need to be kept on a soft chow diet in order for most to avoid death at P21-P28 [110]. The NFI family members are expressed in a variety of cell types, including multiple adult stem cell compartments [111-115]. NFIC regulates the differentiation, proliferation and apoptosis of dental follicle stem cells [112]. Nfib is expressed by epithelial hair follicle stem cells, promoting proliferation and differentiation [113]. Nfia functions as a transcriptional switch in multiple stem and progenitor cell compartments. It promotes gliogenesis in the developing chick neural tube while inhibiting further neurogenesis of ventricular zone progenitor cells [114] and regulates the granulocytic/erythroid fate choice of human hematopoietic progenitors.
Figure 1-9. NFI domain structure, protein sequence alignment and protein sequence homology in vertebrates.

(A) Exons are numbered 1–12. Full-length Nfib transcripts contain 12 exons, while full length Nfia, Nfix, and Nfic transcripts contain 11 exons. The N-terminal region contains a DNA binding and dimerization domain (bracketed and labeled DNA binding and dimerization) and is largely encoded by exon 2. This domain contains four conserved cysteine residues (labeled C) which are required for DNA binding and redox control. There is also a basic alpha helical domain (bracketed and labeled alpha helix) at the start of exon 2. The C-terminal transactivation and repression domain (labeled transactivation and repression) is encoded from exon 3 onwards. The deduced nuclear localization signals are labeled NLS and are at the border of exon 5 and exon 6 (colored gray). The proline-rich region of the activation/repression domain is bracketed.

(B) Homology of the NFI family members in mouse. The predicted amino acid sequences of full length transcripts of the NFIA, NFIB, NFIX, and NFIC family members were aligned using Clustal W (MegAlign). The PubMed accession numbers of the messenger RNA transcripts for NFIA, NFIB, NFIX, and NFIC used in this alignment are NM_010905, NM_001113209, NM_001081982, and NM_008688, respectively. Conserved amino acids between all four family members are shaded blue. NFIB has an extended C-terminus of ∼60 amino acids.

(C) Percent homologies of all family members in mouse. Analysis was performed with the aid of Clustal W, with the N-terminus arbitrarily taken as the first 210 amino acids and the C-terminus taken as the remainder of the predicted polypeptide. NFIA and NFIX are the most homologous, with an overall homology of ∼73%.

during *in vitro* differentiation [115]. The *Nfi* genes have been identified in almost every organ and tissue-type (reviewed in [116]).

### Nuclear Factor I-X

*Nfix* is a novel regulator of murine hematopoietic stem and progenitor cell survival

Nuclear factor I-X (*Nfix*) was a gene included in the functional screen described above. In this study, shRNA-mediated knock-down of *Nfix* in HSPC resulted in severe loss of repopulating ability observed as early as four weeks post-transplantation, increased apoptosis in the HSPC compartment and reduced colony-forming ability [117]. Genes also known to promote HSPC maintenance and survival, such as *c-Mpl, Mecom* and *Erg*, were significantly down-regulated in *Nfix*-deficient HSPC. Holmfeldt *et al.* report data supporting a model where *Nfix* serves as a novel regulator of HSCT in HSPC.

### NFIX roles in other tissues

*Nfix* regulates the molecular switch from embryonic skeletal muscle to fetal skeletal muscle

*Nfix* has been shown to activate fetal specific genes while repressing embryonic specific genes, functioning as a molecular switch [118]. Messina *et al.* shows early *Nfix* expression turns on transcription of fetal genes and reduces expression of embryonic genes in embryonic muscle. Alternatively, preventing *Nfix* expression results in sustained expression of embryonic genes in the fetus. *Nfix* has also been implicated in skeletal muscle regeneration and progression of muscular dystrophies [119 120]. Interestingly, deletion of *Nfix* in muscular dystrophy mouse models results in improvement of the pathology while overexpression of *Nfix* exacerbates the pathology and increases muscle regeneration.

*Nfix* regulates neural stem cell quiescence, proliferation and migration

Mentioned previously, the *Nfi* members have been well studied in the nervous system. *Nfix* has been implicated in many biological functions in the brain, specifically in neural stem cells (NSC). In quiescent NSC cultures, the NFI motif was enriched for in enhancer regions and that NFIX is up-regulated when NSC become quiescent [121]. *In vivo*, Martynoga *et al.* found significantly more NSC cycling in *Nfix*-KO brain. Another group showed *Nfix* facilitates correct migration and proliferation of NSC in the mouse brain [122]. GSEA analysis also suggests that *Nfix* expression in quiescent NSC may be involved with the ECM and cell adhesion, which pairs well with *Nfix* regulated NSC migration [121].
Hypothesis and Specific Aims

The accumulated data shown by others implicates $N\text{fix}$ in regulating hematopoiesis. However, the exact molecular mechanism is not understood. Our hypothesis was that NFIX regulates the transcription of genes during steady-state and stress hematopoiesis. In addition, we predicted genes targeted by NFIX would be involved in cellular differentiation, apoptosis and cell adhesion. It was also expected NFIX might cooperate with other transcription factors known to be important for hematopoiesis. In this study, we aimed to:

- Examine the role of $N\text{fix}$ during steady-state and stress hematopoiesis.
- Interrogate the direct transcriptional targets of NFIX.
- Investigate other key hematopoietic transcription factors that may co-occupy regions of the genome.
CHAPTER 2. MATERIALS AND METHODOLOGY

Mice and Genotyping

C57BL/6J and C57BL/6.SJL-Ptprca Pep3b BoyJ mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and housed in a pathogen-free facility. All animal experiments were carried out according to procedures approved by the St. Jude Children’s Research Hospital Institutional Animal Care and Use Committee. Nfix<sup>fl</sup>/fl mice [110] were a gift from the laboratory of Dr. Richard Gronostajski (University of Buffalo, Buffalo). C57BL/6 HSC-Scl-Cre-ERT mice [123] were a gift from the laboratory of Dr. Joachim Götbert (University of Duisburg-Essen, Germany). The following genotyping primers were used to identify wild-type and mutant Nfix progeny: (NfixF5 -5’ atggacatgtcatgggtgcgacag -3’), (NfixR1-5’ aaccagagcacagagcttgtc -3’), (NfixR2 -5’ aagcccctagctctagcacagag -3’). Polymerase chain reaction (PCR) was completed using 1X Colorless GoTaq Flexi Buffer (Promega; Madison, WI), 2mM MgCl<sub>2</sub>, 0.2mM dNTPs, 0.1µM NfixF5, 0.1µM NfixR1, 0.1µM NfixR2, 0.05U GoTaq DNA Polymerase (Promega; Madison, WI) (Figure 2-1A).

The following genotyping primers were used to identify wild-type and mutant Rosa26-Cre-ERT2 progeny: (olMR883 -5’aaagtcgctctgagttatat -3’), (olMR4982 -5’ aaagtcgctctgagttatat -3’), (olMR316-5’ ggcgagagcagagttatat -3’). Polymerase chain reaction (PCR) was completed using 1X Colorless GoTaq Flexi Buffer (Promega; Madison, WI), 1.5mM MgCl2, 0.2mM deoxynucleoside triphosphates (dNTPs), 2µM olMR883, 2µM olMR4982, 2µM olMR316, 0.025U GoTaq DNA Polymerase (Promega; Madison, WI) (Figure 2-1B).

The following genotyping primers were used to identify progeny with an Slc-Cre-ERT allele: 6E5-SCL locus (6E51-5’ aacaacagggctgagag -3’), Cre-ER[T] (CREr1-5’ atgtttagctgccaatact -3’). PCR was completed using 1X Colorless GoTaq Flexi Buffer (Promega; Madison, WI), 1.6mM MgCl2, 0.2mM dNTPs, 0.2µM 6E51, 0.2µM CREr1, 0.02U GoTaq DNA Polymerase (Promega; Madison, WI) (Figure 2-1C).

Vector Construction

Mouse Nfix cDNA was purchased from GE Healthcare Dharmacon Inc. (Lafayette, Colorado) (Accession: BC003766; Clone ID: 3491917). Nfix was cloned into the Gateway entry vector pDONR221 (Thermo Fisher Scientific, Waltham, MA) by BP clonase reaction, followed by transfer into pCCL-MND-U3-Gateway-PGK-GFP

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Figure 2-1. Representative genotyping results.
(A) Genotyping results for the presence of the Nfix floxed alleles. Expected size for: wild-type amplicon is 214 bp and floxed allele(s) is 420 bp. (B) Genotyping results for the presence of the Rosa26-Cre-ERT2 knock-in allele. Expected size for: wild-type amplicon is 320 bp and knock-in is 650 bp. (C) Genotyping results for the presence or absence of the Scl-Cre-ERT allele. Expected size for: transgenic mutant allele is 328 bp. L lane: 1kb plus DNA ladder, W lane: wild-type control, M lane: mutant control, N lane: no template control.
by LR clonase reaction to produce pCCL-MND-U3-\textit{Nfix}-PGK-GFP (MND-\textit{Nfix}). pCCL-MND-U3-Gateway-PGK-GFP was prepared by transferring the Gateway cassette from pRFA (Thermo Fisher Scientific) to pCCL-MND-U3-PGK-GFP downstream of the MND-U3 promoter. pCCL-MND-U3-PGK-GFP was used as a control vector (MND-Control).

**Lentivirus Production**

A four plasmid system (transfer vector (i.e. \textit{Nfix}), Gag/Pol, Rev/Tat, and vesicular stomatitis virus glycoprotein G (VSV-G) envelope plasmid) was used to produce VSV-G-pseudotyped lentivirus. Briefly, plasmids were co-transfected into 293T cells using TransIT 293 (Mirus, Madison, WI) and viral supernatant was collected 48 hours post-transfection. 293T cells were maintained in Dulbecco’s Modified Eagle Medium (DMEM) (GE Healthcare Life Sciences, Logan, UT) supplemented with 10% fetal calf serum (FCS) (Omega Scientific, Tarzana, CA).

**Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)**

Total RNA was isolated from 70-200,000 cells after 4-7 days in \textit{ex vivo} culture using the Qiagen RNeasy Micro Kit (Qiagen, Valencia, CA), followed by reverse transcription using the High Capacity cDNA Reverse Transcription Kit with RNase Inhibitor (Thermo Fisher Scientific). qRT-PCR was performed on an ABI StepOnePlus thermal cycler using SYBR Green (Thermo Fisher Scientific). \textit{Tbp} was used as a housekeeping gene, and changes in gene expression between test and control samples were calculated using the \(\Delta\Delta C_t\) method. Primer sequences can be found in (Table 2-1)

**Fluorescence-Activated Cell Sorting (FACS)**

Bone marrow was harvested from the femurs, tibias, pelvic bones, and spines of mice by crushing. c-KIT+ cells were enriched by staining the bone marrow with anti-cKIT microbeads (Miltenyi Biotec, San Diego, CA), followed by magnetic separation on an autoMACS Pro Separator (Miltenyi Biotec). Following separation, cells were stained with the following antibodies: c-KIT-APC (2B8) (eBioscience, Inc., San Diego, CA) and SCA-1-FITC (E13-161.7) (BD Biosciences, San Jose, CA). The Lin-c-KIT+SCA-1+ (LSK) fraction was sorted on a FACSAriaIII (BD Biosciences). 4’,6-diamidino-2-phenylindole (DAPI) (Thermo Fisher Scientific) was used to exclude dead cells. Gating strategy is included in Figure 2-2.
Table 2-1  List of primers used for qRT-PCR.

<table>
<thead>
<tr>
<th>Target</th>
<th>Forward primer</th>
<th>Reverse primer</th>
<th>Amplicon size (bp)</th>
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<td>CACTGGGGCGAGCTTGTAGAG</td>
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<td>Nfia</td>
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<tr>
<td>c-Mpl</td>
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<tr>
<td>Bcl-xL</td>
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<tr>
<td>Erg</td>
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<td>Gata3</td>
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<tr>
<td>Tbp</td>
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<td>CCTTATAGGGAACCTACTCATACAG</td>
<td>129</td>
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</table>
Figure 2-2. Gating strategy for isolating HSPC.
BM cells enriched for the cell surface marker, cKit, are sorted for live, singlets and Sca-1+ cKit+ cells, representing LSK cells (i.e. HSPC).
**Lentiviral Transduction**

96-well non-tissue culture (NTC) treated plates (Thermo Fisher Scientific) were coated with Retronectin (Takara Bio USA, Inc., Mountain View, CA) according to the manufacturer’s instructions. After coating, lentivirus was spin loaded onto the plates for one hour at 1000g at room temperature at 2.5×10^6 virus/cm^2. Wells were then washed once with phosphate buffered saline (PBS) (Thermo Fisher Scientific) and 15,000 sorted LSK cells resuspended in 200 µL of serum-free expansion media (SFEM) (STEMCELL Technologies Canada, Inc., Vancouver, BC) were added to each well. SFEM was supplemented with 10 ng/mL murine stem cell factor (mSCF), 20 ng/mL murine thrombopoietin (mTPO), human fibroblast growth factor acidic (hFGF-a) (Peprotech, Rocky Hill, NJ), 20 ng/mL murine insulin-like growth factor 2 (mIGF-2) (R&D Systems, Inc., Minneapolis, MN), and 5 µg/mL protamine sulfate (Sigma-Aldrich Corp., St. Louis, MO). This cytokine combination will hereafter be referred to as “STIF.”

**HSPC Ex Vivo Culture**

Following 24-48 hours of lentiviral transduction, cells were washed of any residual viral particles with PBS/2% FCS. After washing, 15,000 cells resuspended in 200 µL SFEM supplemented with STIF and 10 µg/mL heparin (Sigma-Aldrich) were added to each well of a 96-well NTC plate. Cells were collected and passaged 1:4 into new media every 48-72 hours. 50 µL of cells not used for passaging were simultaneously assessed for relative growth and GFP% via flow cytometry analysis using BD LSRFortessa (BD Biosciences) and data analysis using FlowJo (FlowJo, LLC, Ashland, OR). To determine relative cell number, the 50 µL fraction was collected for 30 seconds at medium speed on the same instrument for every time point assessed, and the number of live cells collected was recorded. DAPI was used to exclude dead cells. This value was then entered into the following equation (Equation 2-1):

\[
\text{relative growth} = \left( \frac{\text{live cell number} \times 4}{\text{previous live cell number}} \right) \times \text{previous relative growth value}
\]

(Eq. 2-1)

In this equation, “4” corresponds to the dilution factor of the previous passage. For cytokine deprivation experiments, the same procedure was followed with the exception that 25% of the normal concentrations of STIF cytokines were used.

**Cytospin Preparation**

Cytospins were prepared and stained using cytopads with caps (Fisher) in a 7120 Aerospray Hematology Slide Stainer/Centrifuge (Wescor, Logan, UT). Briefly, 75,000 GFP+ control or GFP+ NF1X+ cells in 100µL were centrifuged for three minutes at 1000 rpm. After letting the slides air dry, slides were stained using the Romanowsky staining
method: eosin (Wescor), Thiazin (azure B, methylene blue) (Wescor) and light eosin rinse (Wescor). Anhydrous methanol (Wescor) was used for fixation.

**Bone Marrow Transplantation**

CD45.2 “test” HSPC cells were collected and transduced with lentivirus as described above. Twenty-four hours post-transduction, 5000 test cells were washed with PBS and transplanted with 5000 mock-transduced CD45.1 HSPC cells into lethally irradiated CD45.1/CD45.2 recipients. For lethal irradiation, CD45.1/CD45.2 mice received two doses of 5.5 Gy administered three hours apart. Experimental schematic is included in Figure 2-3.

**TPO Removal and AMM2 Treatment**

Ex vivo HSPC were transduced with lentivirus and plated as described above. After 72 hours in culture, 15,000 cells were collected, washed with PBS, and replated in 200µL SFEM supplemented with 25% STIF ± TPO or ± 2 µg/mL of the c-MPL neutralizing antibody AMM2 (Takara Bio USA). After another 72 hours in culture, cells were collected and counted via hemacytometer, as well as analyzed for GFP%, c-KIT%, and apoptosis via flow cytometry on a BD LSRFortessa.

**Colony Forming Unit (CFU) Assays**

Control and NFIX+ cells were sorted for GFP+ cells as described above and plated in methylcellulose M3434 (STEMCELL Technologies). Colonies were scored and counted 10-12 days after plating. For identification of CFU-Megs, sorted cells were plated in MegaCult-C medium with collagen (STEMCELL Technologies), along with 50ng/mL TPO, 20ng/mL IL-6, and 10ng/mL IL-3. Colonies were stained and counted 6-8 days after plating, according to manufacturer’s instructions (STEMCELL Technologies).

**Phosphoflow**

Cells were transduced with lentivirus and plated as described above. After four to seven days in culture, 15,000-40,000 cells were collected, washed with PBS, and replated in 200µL SFEM without cytokines for two hours. After the incubation period, cells were collected and treated with 20 ng/mL mTPO for 5, 10, 25, 60, or 120 minutes at 37 ºC, followed by fixation in 1.6% formaldehyde (Avantor Performance Materials, Center Valley, PA) for 10 minutes at room temperature. Fixed cells were then pelleted and resuspended in ice cold methanol (Thermo Fisher Scientific), followed by 30 minutes incubation on ice or storage at -20 ºC for later analysis. After permeabilization, cells were washed with PBS/2% FCS and stained with fluorescent conjugated antibodies for phosphorylation of STAT5, ERK1/2, or AKT for analysis by flow cytometry.
Figure 2-3. Schematic of competitive transplantation with transduced CD45.2+ HSPC and mock transduced CD45.1+ HSPC.
Luciferase Reporter Activity Assay

Constructs for luciferase reporter assays were designed by using primers listed in Table 2.2. HindIII and XhoI restriction sites were included during primer design (bold, lowercase in Table 2.2). Regions of the c-Mpl promoter were amplified via PCR using these primers and fragments were purified with Wizard SV Gel and PCR Clean-up system (Promega, Madison, WI). Purified fragments and promoterless luciferase vector pGL4.14 (Promega) were incubated with restriction enzymes HindIII-High Fidelity and XhoI (New England Biolabs, Ipswich, MA) and purified. Each fragment of the c-Mpl promoter and digested pGL4.14 backbone were ligated together with T4 DNA ligase overnight at 16°C. Ligation reactions were transformed into Escherichia coli TOP10 One Shot competent cells (Invitrogen) and plated onto LB agar plates supplemented with 100ug/mL Ampicillin. Resulting colonies were sequenced using the RVprimer3 sequencing primer. The MND-control or MND-NFIX constructs (2.5 ug) were co-transfected with pGL4.70 (hRluc) (0.125 ug) and one of the luciferase constructs described earlier (0.875 ug) into 10^6 K562 cells using nucleofector kit V (Amaxa; Lonza Group, Basel, Switzerland) according to the manufacturer’s protocol. K562 cells were maintained in DMEM (GE Healthcare Life Sciences) supplemented with 10% FCS (Omega Scientific). 24 hours post-transfection, cells were lysed with passive lysis buffer and tested for reporter activity using the Dual-Luciferase Reporter Activity Assay Stop-and-Glo Kit (Promega) and a BioTek Synergy H1 Multi-Mode Microplate Reader (BioTek Instruments, Winooski, VT) according to manufacturer’s instructions.

Flow Cytometry

All flow cytometry analysis was performed on a BD LSRFortessa and all data was analyzed by FlowJo. For determination of Lineage ‘SCA-1’c-KIT’ immuno-phenotype of ex vivo cells, the following antibodies were used: [CD3 (145-2C11), CD4 (GK1.5), CD19 (6D5), GR-1 (Rb6-8C5), TER-119 (TER-119) (BioLegend, San Diego, CA), CD8 (53-6.7), B220 (RA3-6B2) (BD Biosciences)]-PerCP; SCA-1-PerCP-Cy5.5 (E13-161.7) (BioLegend); c-KIT-APC-eFluor780 (2B8) (eBioscience). A complete list of antibodies used for flow cytometry is included in Table 2-3. For determination of c-KIT% of ex vivo cells in the TPO and AMM2 experiments, c-KIT-PE-Cy7 (2B8) (BioLegend) was used. For flow cytometry analysis of cell cycle, cells were collected, washed with PBS/2% FCS, then fixed and permeabilized followed by DAPI staining. For flow cytometry analysis of apoptosis, cells were collected, washed with PBS/2% FCS and resuspended in Annexin Binding Buffer (BD Biosciences). Cells were then stained with DAPI and Annexin V-APC (BD Biosciences). For phosphorflow, the following antibodies were used: STAT5(pY694) (47); ERK1/2(pT202/pY204) (20A); AKT(pS473) (M89-61) (BD Biosciences). For peripheral blood analysis of recipients, blood was collected from the retro orbital plexus in heparinized capillary tubes and lysed in red blood cell lysis buffer (Sigma-Aldrich Corp.). Cells were then stained with CD45.1-APC (A20), CD45.2-v500 (104) (eBioscience), [B220 (RA3-6B2), Gr-1 (RB6-8C5), CD11b (M1/70)]-PerCP-Cy5.5, [B220 (RA3-6B2), CD4 (GK1.5), CD8 (53-6.7)]-PE-Cy7 (BioLegend). For staining of megakaryocyte progenitors, cells were stained with c-KIT-APC-eFluor780.
Table 2-2. Primer sequences used for luciferase constructs.

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<tr>
<th>Sites included</th>
<th>Forward (5’-3’)</th>
<th>Reverse (5’-3’)</th>
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<tr>
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<td>GGGGctcgagAATATATACCTCTGTGTCCCTGCCC</td>
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<tr>
<td>-127,-101,-18</td>
<td>GGGGctcgagATATATACCTCTGTGTCCCTGCCC</td>
<td>GGGaagcttCACTGTGTGCCTGCCTTA</td>
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<tr>
<td>-18</td>
<td>GGGGctcgagGGACGTGGGGCTGTATCTGA</td>
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</tr>
</tbody>
</table>

Note: HindIII and XhoI sites are bolded and lowercase in primer sequences.

Table 2-3. List of antibodies and respective clones used in study.

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<tr>
<th>Target</th>
<th>Clone</th>
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<td>CD4</td>
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<tr>
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<td>Ter119</td>
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<td>53-6.7</td>
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<tr>
<td>B220</td>
<td>RA3-6B2</td>
</tr>
<tr>
<td>Sca-1</td>
<td>E13-161.7</td>
</tr>
<tr>
<td>cKit</td>
<td>2B8</td>
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<tr>
<td>Annexin V</td>
<td></td>
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<tr>
<td>STAT5 (pY694)</td>
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</tr>
<tr>
<td>ERK1/2 (pT202/pY204)</td>
<td>20A</td>
</tr>
<tr>
<td>AKT (pS473)</td>
<td>M89-61</td>
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<td>A20</td>
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<td>A7R34</td>
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<td>CD9</td>
<td>KMC8</td>
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<tr>
<td>CD16/CD32</td>
<td>93</td>
</tr>
<tr>
<td>CD41</td>
<td>eBIOMWReg30</td>
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</table>
(2B8) (eBioscience), SCA-1-PE (E13-161.7) (BioLegend), CD127-PE-Cy7 (A7R34) (Tonbo Biosciences, San Diego, CA), CD9-A647 (KMC8) (BD Biosciences), CD32/CD16-A700 (93) (eBioscience), and CD41-PerCP-e710 (eBioMWReg30) (eBioscience).

**Chromatin Immunoprecipitation qRT-PCR**

HPC5 cells were infected with lentivirus carrying MND-Nfix-FLAG or MND-control constructs. 48 hours after infection, cells were sorted for GFP+ cells. GFP+ HPC5 cells were expanded to $10^7$ in culture for no more than one week. $10^7$ GFP+ HPC5 cells were crosslinked with 1% formaldehyde and stored at -80°C. After crosslinking, cells were sheared and diluted. At this time, 10% of the total lysate volume was reserved as the total input sample. Each sample received 1μg of an anti-FLAG antibody (CST, clone: D6W5B) or rabbit IgG antibody (CST, 2729). Samples were incubated overnight at 4°C with gentle rocking. Next, Protein G Dynabeads (Fisher) were added to samples and incubated for two hours at 4°C with gentle rocking. Samples were washed with a series of buffers and then immunoprecipitated chromatin and total input chromatin were eluted. Chromatin were de-crosslinked and RNA and protein were digested by overnight incubations with RNase and Proteinase K in a sodium chloride rich buffer. DNA from ChIP samples and total input samples was extracted using phenol/chloroform. Total input samples were diluted ten times before qPCR. The following primers were designed to encompass NFI consensus binding sites in the Mus musculus c-Mpl proximal promoter [Forward: cccattccccctctcttg] and [Reverse: cctgtcagatacagccccac]. Primers used for ChIP-qPCR were validated with serial dilutions of HPC5 genomic DNA. Total input samples were first adjusted to represent 100% of the total chromatin present in samples. Finally, percent input was calculated as follows (Equation 2-2):

$$\text{% Input} = 100 \times 2(\text{adjusted input Ct} - \text{ChIP Ct})$$  \hspace{1cm} (Eq. 2-2)

**Statistical Analysis**

Statistical significance was determined using two-sample/one sample Student’s t-tests or exact Wilcoxon rank sum tests, depending on the normality of the data as determined by the Shapiro-Wilk test. In Chapter 4, a linear regression model was used to examine the reduction in cell number in controls compared with NFIX+ cells. P-values < 0.05 or < 0.1 (where indicated) were considered statistically significant in all analyses.

**Cell Culture**

Nfix<sup>+/+</sup> and Nfix<sup>-/-</sup> HPC5 cells were maintained in IMDM with GlutaMax (Gibco; Carlsbad, CA) supplemented with 5% FCS (Atlanta Biologicals; Flowery Branch, GA), 1.5×10<sup>-4</sup> M monothiolglycerol (MTG) (Sigma-Aldrich; St. Louis, MO), 100 ng/mL murine stem cell factor (SCF) (PeproTech; Rocky Hill, NJ) and 10 ng/mL human
interleukin-6 (hIL-6) (PeproTech; Rocky Hill, NJ). Cells were maintained at 0.5×10^6 cells/mL and 2×10^6 cells/mL and kept at 37°C and 5% CO₂ in a humidified cabinet. 293T cells were cultured in DMEM (Gibco; Carlsbad, CA) with 10% FBS.

**Drug Treatment**

10mg/mL Tamoxifen (Sigma-Aldrich; St. Louis, MO) was resuspended in 10% ethanol and 90% sunflower seed oil (Sigma-Aldrich; St. Louis, MO). 1mg was administered to mice via oral gavage each day for five consecutive days.

**HSPC Isolation**

Bone marrow was harvested from femurs, tibias, pelvic bones and spines of 10- to 14-week-old mice by crushing. c-Kit+ cells were enriched magnetically using anti-c-Kit microbeads (Miltenyi Biotec; San Diego, CA) and an AutoMACS (Miltenyi Biotec; San Diego, CA). Cells were then stained with fluorescently conjugated antibodies for Sca-1 (E13-161.7, BD Biosciences; San Jose, CA) and c-Kit (2B8, eBioscience, Inc.; San Diego, CA) and HSPC (Lineage-Sca-1+cKit+) were isolated via cell sorting using a FACS Aria III (BD Biosciences; San Jose, CA). DAPI (Sigma-Aldrich; St. Louis, MO) was used to exclude dead cells.

**Antibody Production**

Three peptides (peptide 1 (Q26-R46): QARKRKYFKKHEKRMSKDEER, peptide 2 (V96-D110): VLSNPDKGKIRRID, peptide 3 (T177-K195): TPESGQSDSSNQQGDADIK) were designed based on the murine NFIX protein sequence (P70257.2) and injected into three cohorts of mice. Sera were then collected from injected mice and screened in both enzyme-linked immunosorbent assay (ELISA) using the respective peptide bound to the plate and a flow-based screening technique. The flow-based assay used paramagnetic streptavidin (SA) beads (Promega; Madison, WI), biotinylated rat anti-FLAG monoclonal antibody (mAb) (LSBio; Seattle, WA), goat anti-mouse IgG (H+L) rat adsorbed (SouthernBiotech; Birmingham, AL), cellular lysate from 293T cells transfected with either a FLAG-NFIX overexpression vector or a GFP expression control vector, and a FACSCalibur cell analyzer with CellQuest Pro software for analyses. This was performed by incubating the SA beads with the biotinylated anti-FLAG mAb at 4°C in one tube and the lysate with sera (diluted 1:100 in the lysate) in a second tube at 4°C overnight. The beads were washed three times to removed unbound mAb. The lysate/sera mixture was then added to the beads and allowed to incubate at 4°C for a minimum of four hours or overnight. Following the incubation, beads were washed and stained with the goat anti-mouse IgG secondary at a 1:1000 dilution for 30 minutes on ice. Beads were then analyzed by flow cytometry. This assay identified peptide three as the most promising and the mouse with the highest titer was selected for fusion.
Briefly, the spleen was harvested and splenocytes fused to an equal number of Sp2/mIL-6 (ATCC® CRL-2016™) cells [124]. The fusion was transferred to 96 well plates and incubated in HAT (Sigma-Aldrich; St. Louis, MO) supplemented media (ClonaCell-HY Medium E, Stemcell Technologies; Vancouver, BC) for 10 days. The supernatants from wells containing clones were then screened by an ELISA for reactivity to the peptide. Positive wells were then transferred to 24 well plates and supernatant was used in the SA bead/lysate assay as described above. The cells from positive wells in this assay were then single cell sorted into 96 well plates to confirm they were monoclonal.

**Generation of Nfix<sup>−/−</sup> HPC5 Cells**

Nfix<sup>−/−</sup> HPC5 cells were generated using clustered, regularly interspaced, palindromic repeats (CRISPR) and CRISPR-associated protein 9 (Cas9). Briefly, short guide RNAs (sgRNAs) were designed with at least three base pairs of mismatch to any other site in the mouse genome to mitigate the risk of off-target editing. 500,000 HPC5 cells were transiently co-transfected with 0.5 µg pmaxGFP plasmid (Lonza; Basel, Switzerland), 33 pmol spCas9 protein and 100 pmol chemically modified sgRNAs (5’-ucagauauguaccacca-3’) (Synthego; Redwood City, CA) via nucleofection (4D-Nucleofector X-unit, Lonza; Basel, Switzerland), using solution P3 and program CA-137 in a small (20 µL) cuvette according to the manufacturer’s recommended protocol. Five days post nucleofection, cells were single-cell sorted by FACS to enrich for GFP+ (transfected) cells, clonally expanded, and verified for the desired modification via targeted deep sequencing using gene specific primers with partial Illumina adapter overhangs (mNfix.F – 5’ cctgagcagtggtaacagtc-3’ and mNfix.R – 5’ ccgacctctagcctcccacata-3’, overhangs not shown). Next generation sequencing (NGS) analysis of clones was performed using CRIS.py [125].

**Chromatin Immunoprecipitation and Library Preparation**

20 million Nfix<sup>+/+</sup> and Nfix<sup>−/−</sup> HPC5 cells were fixed with 1% formaldehyde (Sigma-Aldrich; St. Louis, MO) for 10 minutes at room temperature with gentle stirring. Fixing was quenched with 0.125 M glycine (Sigma-Aldrich; St. Louis, MO) and further stirring for five minutes at room temperature. Cells were collected by centrifugation and snap frozen for storage at -80°C. Cells were then resuspended in buffer L1 (50mM HEPES pH 7.5, 140mM NaCl, 1mM EDTA pH 8.0, 10% glycerol, 0.5% NP40, 0.25% Triton X-100) supplemented with one Protease Inhibitor tablet (Roche; Basel, Switzerland). During lysis, samples were kept on ice for 10 minutes with gentle agitation every two minutes. Samples were centrifuged at 1693 rcf for 10 minutes at 4°C in a swing bucket rotor. Supernatant was removed and cells were resuspended in buffer L2 (200mM NaCl, 1mM EDTA pH 8.0, 0.5mM EGTA, 10mM Tris pH8.0) supplemented with one Protease Inhibitor tablet (Roche; Basel Switzerland). Samples were rocked at room temperature for 10 minutes. Nuclei were isolated by centrifugation at 1693 rcf for 10 minutes at 4°C. Supernatant was carefully removed and 1X RIPA buffer was added to samples. Nuclei were sonicated using a cell disruptor (SFX250) (Branson Ultrasonics
Corporation; Brookefield, CT). Lysate was clarified with centrifugation at 18879 rcf for 15 minutes at 4°C. The supernatant was carefully transferred to a 15mL conical vial. Samples were incubated with protein A/G sepharose beads (Pierce; Waltham, MA) overnight to preclear supernatant. 20ug of each antibody listed in anti-PU.1 (sc-390405, Santa Cruz; Dallas, TX) and anti-NFIX (clone: 7B5.3) antibodies were also pre-bound to protein A/G agarose beads (Pierce; Waltham, MA) for overnight incubation. Pre-cleared chromatin was centrifuged at 1000 rcf for three minutes at 4°C. Pre-cleared chromatin was added to respective antibody-bead complex in Eppendorf tubes while some pre-cleared chromatin was reserved for input. Samples were rotated at 4°C for four hours. After incubation, samples were centrifuged at 5418 rcf for five minutes at 4°C. Supernatant was aspirated and beads were washed with various buffers: once with IP Wash Buffer I (20mM Tris pH8.0, 50mM NaCl, 2mM EDTA, 1% Triton X-100, 0.1% SDS), twice with IP High Salt Wash Buffer (20mM Tris pH8.0, 500mM NaCl, 2mM EDTA, 1% Triton X-100, 0.1% SDS), once with IP Wash Buffer II (10mM Tris pH8.0, 250mM LiCl, 1mM EDTA, 1% NP-40, 1% deoxycholic acid), and twice with TE buffer. DNA:protein complexes from beads were eluted twice with freshly made elution buffer (1% SDS, 100mM sodium bicarbonate). To reverse crosslinking, 5M NaCl (final concentration 370mM), 10mg/mL RNase (final concentration 0.45mg/mL), and 20mg/mL proteinase-K (final concentration 0.26mg/mL) were added to eluates and input samples. Incubate samples at 37°C for 30 minutes, 45°C for 30 minutes, and 65°C overnight. DNA was eluted using the Qiagen MinElute (Hilden, Germany) kit following manufacturer’s protocol except DNA was eluted in 28uL with provided EB buffer. DNA was quantified using the QuantiT PicoGreen assay (Life Technologies; Carlsbad, CA) Qubit dsDNA HS Assay Kit (Thermo Fisher Scientific; Waltham, MA) or SpectraMax Quant AccuBlue Pico dsDNA assay kit (Molecular Devices; San Jose, CA). Libraries were prepared with KAPA HyperPrep Library Preparation Kits (KK8504) (Roche; Basel, Switzerland). Libraries were analyzed for insert size distribution on a 2100 BioAnalyzer High Sensitivity kit (Agilent Technologies; Santa Clara, CA), 5300 Fragment Analyzer System HS Large Fragment Kit (Agilent Technologies; Santa Clara, CA), 4200 TapeStation D1000 ScreenTape assay (Agilent Technologies; Santa Clara, CA) or Caliper LabChip GX DNA High Sensitivity Reagent Kit (PerkinElmer; Waltham, MA). Libraries were quantified using the QuantiT PicoGreen ds DNA assay (Life Technologies; Carlsbad, CA) or low pass sequencing with a MiSeq nano kit (Illumina; San Diego, CA). Single read 50 cycle sequencing was performed on a NovaSeq 6000 (Illumina; San Diego, CA) or NextSeq 550 (Illumina; San Diego, CA).

**Peak Calling**

The sequencing reads were mapped to the mouse genome (mm9) using BWA (version 0.7.16a). ChIP-seq peaks were called using MACS2. De novo motif discovery on ChIP-seq peaks was performed using HOMER (v4.9.1) [126] with parameters “-size 200 -mask”.

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RNA Extraction, Sequencing and Transcript-Level Abundance Calculation

RNA was extracted from 0.6x10^6-0.8x10^6 cells using the Qiagen RNase Mini kit (Hilden, Germany) according to the manufacturer, including the on-column DNase digestion. RNA was quantified using the Quant-iT RiboGreen assay (Life Technologies; Carlsbad, CA) and quality checked by 2100 Bioanalyzer RNA 6000 Nano assay (Agilent Technologies; Santa Clara, CA), 4200 TapeStation High Sensitivity RNA ScreenTape assay (Agilent Technologies; Santa Clara, CA), or LabChip RNA Pico Sensitivity assay (PerkinElmer; Waltham, MA) prior to library generation. Libraries were prepared from total RNA with the TruSeq Stranded mRNA Library Prep Kit according to the manufacturer’s instructions (20020595, Illumina; San Diego, CA). Libraries were analyzed for insert size distribution on a 2100 BioAnalyzer High Sensitivity kit (Agilent Technologies; Santa Clara, CA), 4200 TapeStation D1000 ScreenTape assay or Caliper LabChip GX DNA High Sensitivity Reagent Kit (PerkinElmer; Waltham, MA). Libraries were quantified using the Quant-iT PicoGreen ds DNA assay (Life Technologies; Carlsbad, CA) or low pass sequencing with a MiSeq nano kit (Illumina; San Diego, CA). Paired end 100 cycle sequencing was performed on a NovaSeq 6000 (Illumina; San Diego, CA). Transcript-level abundance was quantified using Kallisto [20] with pre-built genome index for mm9 and differential analysis was done using Sleuth [21]. Source code for Kallisto is available at: https://github.com/pachterlab/kallisto. Source code for Sleuth is available at: https://github.com/pachterlab/sleuth.

Bioinformatics Pipeline Description

NFIX target genes were identified based on observed differential expression in Nfix+/+ versus Nfix-/- HPC-5 cells and binding to either gene promoters (+- 10kb around TSS, ensembl v67) or enhancers (based on CaptureC experiments from GSE119339). NFIX peaks were then extracted and used to search for co-binding factors, including SCL/TAL1, LYL1, LMO2, GATA2, RUNX1, MEIS1, PU.1, ERG, FLI-1 and GFI1B (GSE22178). Co-binding analysis based on Chi-square test was performed at both in vivo transcription factor binding sites (i.e., ChIP-seq peaks) and in-silico for known motifs). Source code is available at: https://github.com/YichaoOU/TF_target_finder.
CHAPTER 3. NFIX IS DISPENSABLE DURING HSPC TRANSPLANT AND STEADY STATE HEMATOPOIESIS

Introduction

Transplant-induced stress exerted on HSPC has been well documented, resulting in reduced stem cell pools and decreased self-renewal ability [127-129]. Regulation of their ability to overcome this stress and successfully replenish hematopoiesis is not well understood. Cell-extrinsic and cell-intrinsic regulators of HSCT have been implicated in HSPC self-renewal, mobilization and homing (see Figure 1-6 and Tables 1-1, 2) (reviewed in [130]) [131-133]. Better understanding the mechanisms that allow HSPC engraftment post-transplant will facilitate efforts to improve transplantation protocols and clinical outcomes.

Recently, our lab completed a functional screen that identified 18 novel regulators of murine HSCT, including Nfix [86]. NFI family members function as transcriptional activators and repressors [116 134]. NFIX and NFIA, a related family member, have also been implicated in regulating hematopoietic lineage fate decisions, with ectopic expression of NFIA or Nfix promoting HSPC differentiation to erythropoiesis or myelopoiesis and depletion promoting granulopoiesis or lymphopoiesis, respectively [115 135]. Although NfixΔ/Δ mice display no overt hematopoietic phenotypes during native hematopoiesis, shRNA-mediated knockdown or genetic deletion of Nfix in HSPC results in a profound loss of competitive in vivo repopulating potential and increased apoptosis [117]. However, it is prudent to validate shRNAs specificity.

Thus, we sought to better characterize the function of Nfix during hematopoiesis utilizing a mouse model where Nfix could be spatially and temporally deleted in vivo in hematopoietic cells, avoiding the use of viral integration and shRNAs. Also, to our knowledge, no work has been done characterizing the hematopoietic compartment of NfixΔ/Δ mice.

Results and Discussion

Nfix-shRNA Do Not Specifically Target Nfix

The second exon of Nfi family members consists of the DNA binding domain [101]. This exon has been targeted for deletion by insertion of loxP sites in the introns flanking the entire second exon [110]. We crossed this Nfixflox/flox mouse with a Rosa26-Cre-ERT2 mouse model. We show consistently that in Nfixflox/flox Rosa26-Cre-ERT2 mice treated with Tamoxifen there is near 100% deletion in HSPC (Figure 3-1A, B).
Figure 3-1. Genotyping isolated colonies grown in methylcellulose.

(A) Top, colonies were collected from methylcellulose that had previously been plated with 300 HSPC from Tamoxifen-treated $Nfix^{flox/flox}$ $Rosa26$-Cre-ER$^{T2}$, except in lane 1 and lane 3. In lane 1 and lane 3, these represent colonies from $Nfix^{+/+}$ $Rosa26$-Cre-ER$^{T2}$ mice treated with Tamoxifen. In lane 5 and lane 8, the floxed allele is still detected, suggesting partial deletion. Lower, genotyping confirming the colonies have a Cre allele.

(B) Genotyping of colonies were collected from methylcellulose that had previously been plated with 300 HSPC from Tamoxifen-treated $Nfix^{+/+}$ $Rosa26$-Cre-ER$^{T2}$. Expected amplicon sizes: $Nfix^{+/+} = 214$bp, $Nfix^{flox/flox} = 400$bp and $Nfix^{Δ/Δ} = 309$bp.
HSPC were isolated from Tamoxifen-treated \( Nfix^{\text{floxfloxflox}} \) \textit{Rosa26-Cre-ER\textsuperscript{T2}} (denoted as \( Nfix^{\Delta/\Delta} \)) and transduced these HSPC with the \( Nfix\)-shRNA used previously in Holmfeldt \textit{et al.} \[117\]. The \( Nfix\)-shRNA transduced \( Nfix^{\Delta/\Delta} \) HSPC were transplanted into lethally irradiated recipient mice with mock transduced competitor cells (CD45.1\+). Four weeks post-transplant the PB was taken from recipient mice and analyzed for donor cell chimerism. Previously, when HSPC were treated with \( Nfix\)-shRNAs, there was a significant decrease in donor cell chimerism seen as early as four weeks post-transplant [117]. We observed no significant difference in the frequency of donor cell chimerism measured as %mCherry\+ CD45.2\+ between recipients of \( Nfix^{+/+} \) HSPC transduced with \( Nfix\)-shRNA and \( Nfix^{\Delta/\Delta} \) HSPC transduced with \( Nfix\)-shRNA (Figure 3-2). These data strongly imply the shRNA used to target \( Nfix \) is not specific. Transplantation with two other \( Nfix\)-shRNAs were attempted, however, the transduction efficiencies (%mCherry\+) were not consistent across cohorts. More work may be necessary to identify an shRNA that specifically targets \( Nfix \). Of note, there is no difference in donor cell chimerism between recipients transplanted with \( Nfix^{+/+} \) HSPC or \( Nfix^{\Delta/\Delta} \) HSPC. If \( Nfix \) was necessary during HSCT, we would have expected a decrease in donor cell chimerism in PB of recipient mice. The experiment in Figure 3-2 was terminated four weeks post-transplant. Perhaps, in our genetic deletion model, we would need to allow for more time to observe a phenotype. The pool of cells we are transplanting (\textit{i.e.} HSPC) is a heterogeneous mix of stem and progenitor cells. Acutely post-transplant, transplanted progenitor cells give rise to the PB lineages [136]. Thus, perturbations in donor cell chimerism early post-transplant may implicate defects with progenitor cells. Perturbations observed later post-transplant may indicate functionally compromised HSC. In order to further assess the necessity of \( Nfix \), we performed competitive transplants with \( Nfix^{+/+} \) and \( Nfix^{\Delta/\Delta} \) HSPC.

\textbf{\( Nfix \) Is Dispensable for Murine HSCT}

To mirror the acute deletion of \( Nfix \), similar to that when shRNAs were used, we isolated HSPC from \( Nfix^{+/+} \) \textit{Rosa26-Cre-ER\textsuperscript{T2}} and \( Nfix^{\text{floxfloxflox}} \) \textit{Rosa26-Cre-ER\textsuperscript{T2}} mice. These cells were treated with 4-Hydroxytamoxifen (4-OHT), the active metabolite of Tamoxifen, \textit{in vitro} for 24 hours. After 24 hours, the HSPC were mixed 1:1 with CD45.1\+ HSPC and transplanted into lethally irradiated recipient mice. Every four weeks post-transplant, PB was sampled from the recipient mice and donor chimerism as well as frequency of PB lineages was examined. We observed only a modest increase in PB chimerism 12 and 16 weeks post-transplant (Figure 3-3, top left). We also see a modest increase in the lymphoid lineage of the PB in recipients (Figure 3-3, lower left and right). There is no difference in the donor chimerism in the myeloid lineage (Figure 3-3, top right). These results suggest that perhaps the HSC are more proliferative and may exhaust if stressed further. To interrogate this hypothesis, we performed secondary transplants. Whole BM (WBM) from primary transplant recipients was collected and \( 1x10^6 \) WBM cells were transplanted in lethally irradiated secondary recipients. At this time, \( 0.04x10^6 \) WBM cells were plated in methylcellulose in order to interrogate the deletion efficiency of cells. As previously mentioned, every four weeks post-transplant, PB was sampled from the recipient mice and donor chimerism as well as frequency of PB lineages was examined. At no point during these secondary transplants...
Figure 3-2. %mCherry+ CD45.2+ frequency in PB of recipient mice transplanted with NfixΔ/Δ HSPC transduced with Nfix-shRNA. Four weeks post-transplant the %mCherry+ CD45.2+ frequency of donor chimerism in recipients of NfixΔ/Δ HSPC transduced with Nfix-shRNA and Nfix+/+ HSPC transduced with Nfix-shRNA is not significantly (NS) different. N=4-3 mice/cohort. One-way ANOVA followed by Tukey post-hoc test. *P < 0.05, **P < 0.01.
Figure 3-3. Frequency of PB chimerism and lineages in primary Nfix<sup>Δ/Δ</sup> transplants.
Top left, Frequency of PB chimerism in recipient mice. At 12 and 16 weeks post-transplant we observed a modest but significant increase in PB chimerism. Lower left, frequency of donor-derived B-cells in PB of recipients. At 12 and 16 weeks post-transplant we observed a modest but significant increase in B-cell chimerism. Lower right, frequency of donor-derived T-cells in PB of recipients. At 12 and 16 weeks post-transplant we observed a modest but significant increase in T-cell chimerism. Top right, there was no significant difference in the frequency of donor-derived myeloid cells comparing recipient mice transplanted with Nfix<sup>Δ/Δ</sup> HSPC (■) and Nfix<sup>+/+</sup> HSPC (●). N=4-5 independent transplants, 4-5 mice/cohort. A two-way ANOVA with Sidak’s multiple comparisons test was performed to determine significance. *P = <0.05.
**P = <0.01
were there any significant differences revealed \( \text{Figure 3-4} \). These data suggest there are no functional defects in \( \text{Nfix}^{\Delta/\Delta} \) HSC.

In sum, we find the \( \text{Nfix}-\text{shRNA} \) used initially does not specifically target \( \text{Nfix} \). The primary and secondary transplants supported a model where \( \text{Nfix} \) may be unnecessary during stress hematopoiesis. These data may be confounded by compensation from other \( \text{Nfi} \) family members. We find both \( \text{Nfic} \) and \( \text{Nfia} \) are expressed throughout the hematopoietic hierarchy. However, neither \( \text{Nfic} \) nor \( \text{Nfia} \) KO models display hematopoietic defects. This suggests there may be compensation or redundancy within the family and alludes to the importance of \( \text{Nfi} \) expression during hematopoiesis.

### Deletion of \( \text{Nfix} \) Does Not Perturb Steady-State Hematopoiesis

To determine the importance of \( \text{Nfix} \) at steady-state hematopoiesis we utilized a mouse model where we could spatially and temporally delete \( \text{Nfix} \) in hematopoietic cells, including HSPC. We crossed \( \text{Nfix}^{\text{floxed/floxed}} \) mice with \( \text{HSC-Scl-Cre-ER}^{\text{T}} \) mice to generate \( \text{Nfix}^{\text{floxed/floxed}} \) \( \text{HSC-Scl-Cre-ER}^{\text{T}} \) mice. Controls were \( \text{Nfix}^{\text{floxed/floxed}} \) without \( \text{HSC-Scl-Cre-ER}^{\text{T}} \) knockin (KI) allele. \( \text{Nfix}^{\text{floxed/floxed}} \) mice and \( \text{Nfix}^{\text{floxed/floxed}} \) \( \text{HSC-Scl-Cre-ER}^{\text{T}} \) (denoted \( \text{Nfix}^{\Delta/\Delta} \) after treatment with Tamoxifen) mice were treated with Tamoxifen for five consecutive days via oral gavage. This mouse model also exhibits efficient deletion of \( \text{Nfix} \) in HSPC. Peripheral blood from these mice were collected for 47 weeks \( \text{Figure 3-5} \). At no point were there any significant differences in PB lineages comparing \( \text{Nfix}^{\text{floxed/floxed}} \) mice and \( \text{Nfix}^{\Delta/\Delta} \) mice. These mice also retained the complete deletion of \( \text{Nfix} \) in HSPC after 47 weeks and had normal complete blood counts (CBC).

In conclusion, we have discovered that our \( \text{Nfix}-\text{shRNA} \) does not specifically target \( \text{Nfix} \). We also show that during primary transplants with \( \text{Nfix}^{\Delta/\Delta} \) HSPC there is a slight but significant increase in donor cell chimerism as well as an increase in donor-derived lymphoid cells at 12- and 16-weeks post-transplant. This phenotype later in the transplant suggests that \( \text{Nfix}^{\Delta/\Delta} \) HSC may be cycling faster and eventually exhaust resulting in defective performance during another round of stress hematopoiesis. To test this hypothesis, we performed secondary transplants with WBM from primary transplant recipients. In these transplants, we did not observe any significant differences in donor-cell chimerism or PB lineages. It may be interesting to repeat the primary transplants but include a different stress, such as, polyl:polyC (plpC) or 5-fluorouracil (5-FU) treatment. Repeated treatments may be necessary to stress the \( \text{Nfix}^{\Delta/\Delta} \) HSC. The modest increase in B-cell and T-cell lineages may suggest \( \text{Nfix} \) regulates lineage-specific transcriptional programs. Our data are in agreement with previous observations where it enforced \( \text{Nfix} \) resulted in increased myelopoiesis at the expense of lymphopoiesis [137]. In another cellular context, during skeletal muscle development \( \text{Nfix} \) acts as a switch to activate a fetal transcriptional program while repressing embryonic gene expression [118]. ChIP-seq and RNA-seq in the T- and B-cell lineages using \( \text{Nfix}^{\Delta/\Delta} \) samples may reveal \( \text{Nfix} \) functions similarly during hematopoietic lineage determination.
Figure 3-4. Frequency of PB chimerism and lineages in secondary Nfix^{−/−} transplants.
The secondary transplants utilizing WBM from primary transplants reveals no significant differences in donor-cell chimerism or donor-derived lineages. N=2-4 independent transplants, 4-5 mice/cohort. A two-way ANOVA with Sidak’s multiple comparisons test was performed to determine significance.
Figure 3-5.  Frequency of PB lineages comparing $Nfix^{\Delta/\Delta}$ mice and $Nfix^{+/+}$ mice. N=2 independent cohorts, 6-7 mice/genotype. Student’s T-test was used to determine significance. Errors bars represent standard error (SE).
CHAPTER 4. ENFORCED NFIX PROMOTES SURVIVAL OF IMMATURE HEMATOPOIETIC CELLS EX VIVO

Introduction

In the previous chapter, our data suggested that Nfix was not necessary for HSCT or to maintain steady-state hematopoiesis. This finding could result from redundancy or compensatory mechanisms from other Nfi family members, especially Nfia or Nfic, suggesting the importance of the gene family for hematopoiesis. Previously, both NFIA and Nfix were shown to promote HSPC differentiation [115 135], however, little is known about how NFIX regulates HSPC at the molecular and cellular level. Here we report that enforced Nfix can promote ex vivo growth, cytokine hypersensitivity, and survival of primitive hematopoietic populations ex vivo. We further demonstrate that these effects are in part mediated via up-regulation of the TPO receptor, c-Mpl, thus revealing NFIX as a novel transcriptional regulator of c-Mpl and illuminating one molecular pathway targeted by NFIX in HSPC.

Results and Discussion

Nfix Extends the Ex Vivo Growth of Immature Hematopoietic Cells

To further interrogate the role of Nfix in HSPC biology, we ectopically expressed Nfix in Lineage- Sca-1- c-Kit+ (LSK; HSPC) cells cultured under serum-free conditions (Figure A-1A). During culture, cells were assessed for growth rate, retention of vector+ (NFIX+) cells, and persistence of an LSK phenotype (Figure 4-1A). Nfix was over-expressed 20-fold in NFIX+ cells, while other NFI genes remained unperturbed (Figure 4-1B). Remarkably, ectopic Nfix expression prolonged hematopoietic cell cultures two-fold, allowing cells to persist up to 40 days ex vivo (Figure 4-1C). However, the relative growth of control and NFIX+ cultures did not significantly diverge until control cells began to display culture exhaustion (p = 0.036) (Figure 4-1C). During this extended time, a steady selection for NFIX+ cells was apparent (Figure 4-1D). These data suggest that Nfix can promote the extended cell culture of hematopoietic progenitors. By seven days of culture, the majority of cells in both control and NFIX+ cultures had lost the LSK cell surface phenotype (Figure 4-1E), with immunophenotypic LSK cells being almost completely lost from culture by day 14 (Figure A-1B, C).

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Figure 4-1.  Nfix induces longevity in hematopoietic stem and progenitor cell ex vivo culture.

(A) Experimental schematic. 48 hours post-transduction, LSK cells transduced with MND-Control or MND-Nfix were re-plated in 96-well non-tissue culture treated plates in serum-free expansion medium (SFEM) supplemented with mSCF, mTPO, mIGF-2, and hFGF-a (STIF). Every 48-72 hours of culture, cells were counted, assessed for GFP+ cells, and passaged 1:4. Cells were also periodically assessed for LSK immuno-phenotype. (B) Relative expression of NFI-family genes in NFIX+ cells compared to control cells, quantified by qRT-PCR (n = 3). Tbp was used as a housekeeping gene. (C) Relative growth of control and NFIX+ cells during ex vivo culture (n = 4). Dotted line indicates the divergence in relative growth between control and NFIX+ cells. (D) GFP percentage of control and NFIX+ cells during ex vivo culture, assessed by flow cytometry (n = 4). (E) Percentage of control and NFIX+ cells with an LSK immuno-phenotype at day seven of ex vivo culture, depicted as a (top) representative dot plot and (lower) bar plot (n = 6). All values represent mean ± standard deviation. NS denotes not significant.
However, Nfix overexpression appeared to accelerate the loss of this phenotype, evident by the appearance of a Sca-1<sup>−</sup> c-Kit<sup>−</sup> population in NFIX+ cells and reduced overall levels of cell surface c-Kit, relative to control (Figure 4-1E, top and lower and Figure A-1D). These data suggest that Nfix might promote LSK cell differentiation during ex vivo culture. At day seven of culture, control and NFIX+ cells displayed a similar blast-like morphology, with NFIX+ cells retaining this morphology through day 30 of culture (Figure A-2). However, LSK cells overexpressing Nfix displayed a loss of in vivo competitive hematopoietic repopulating potential, a myeloid bias in peripheral blood production and a loss of CFU potential compared to control cells by seven days of culture, with a significant loss in CFU potential by 21 days in culture (p = 0.023) (Figure A-3A through D and Figure A-4). A majority of expanded control and NFIX+ cells were negative for all major lineage markers (except CD8) and expressed c-Kit and CD71, which is a marker of proliferating progenitors (Figure A-5A, B). High CD71 expression can also be indicative of erythroid progenitors, and while NFIX+ cells show a significantly higher percentage of a CD71hi population compared to controls (p = 0.017), this population represents only a small portion (15-25%) of cells throughout the entirety of the culture (Figure A-5C). Together, these data suggest that Nfix promotes differentiation of LSK cells towards a heterogeneous immature progenitor population that ultimately lacks CFU potential, suggesting arrested differentiation potential.

**Enforced Expression of Nfix in HSPC Results in Hypersensitivity to Growth Cytokines and Protection From Apoptosis**

This unimpeded growth led us to question if the cells were resistant to apoptosis or accelerating through the cell cycle. Towards this, NFIX+ HSPC were cultured under normal or reduced cytokine conditions and monitored for growth rate, NFIX+ cell selection, cell cycle, and apoptosis (Figure 4-2A). Control cells cultured in reduced cytokines displayed a significantly lower growth rate by day 13 (p = 0.048) and an attenuated culture lifespan relative to cells maintained at normal cytokine levels (Figure 4-2B). Remarkably, reduced cytokine levels had no effect on the extended culture of NFIX+ cells (Figure 4-2B). NFIX+ cells cultured under reduced cytokines were selected for at a significantly accelerated rate compared to NFIX+ cells cultured under normal cytokine levels (p = 0.041) (Figure 4-2C). There were no significant differences in cell cycle status between control and NFIX+ cells regardless of cytokine levels (Figure 4-2D), suggesting that the reduced growth rate of cytokine-deprived control cells was not due to a reduction in cycling. However, control cells displayed a significant increase in apoptosis (p = 0.032) when cultured in reduced cytokines (Figure 4-2E). In contrast, the apoptotic status of NFIX+ cells was unaffected by reduced cytokines (Figure 4-2E), even in immunophenotypic HSPC (Figure A-6). These data reveal that Nfix promotes primitive hematopoietic cell survival ex vivo.
Figure 4-2. Hematopoietic stem and progenitor cells overexpressing Nfix can withstand cytokine deprivation and display reduced apoptosis during ex vivo culture.

(A) Experimental schematic. 48 hours post-transduction, LSK cells transduced with MND-Control or MND-Nfix were re-plated in 96-well non-tissue culture treated plates in serum-free expansion medium (SFEM) supplemented with either normal (100%) or reduced (25%) levels of STIF cytokines. Every 48-72 hours of culture, cells were counted, assessed for GFP+ cells, and passaged 1:4. Cells were also assessed by flow cytometry for apoptosis via Annexin V and cell cycle via DAPI at day seven of culture.

(B) Relative growth of control and NFIX+ cells during ex vivo culture (n = 4). Dotted line indicates the divergence in relative growth between control 100% and control 25% cells.

(C) GFP percentage of control and NFIX+ cells during ex vivo culture, assessed by flow cytometry (n = 4). Dotted line indicates significant selection of NFIX+ cells under 25% cytokines.

(D) Cell cycle analysis of GFP+ control or NFIX+ cells at day seven of ex vivo culture (n = 3).

(E) Percentage of GFP+ apoptotic cells within control or NFIX+ cell cultures at day seven of ex vivo culture (n = 6). All values represent mean ± standard deviation. NS denotes not significant.
c-Mpl Expression Is Increased in Nfix Overexpressing Cells

We previously observed by global gene expression analyses [117] that Nfix knockdown in HSPC reduced expression of multiple genes implicated in HSPC survival and maintenance including c-Mpl, a known regulator of HSC maintenance in the bone marrow niche that has been shown to affect apoptosis via multiple downstream signaling cascades [138-141]. c-MPL is the receptor for TPO, which is added as a supplement to our *ex vivo* serum-free cultures of HSPC. To further explore possible regulation of c-Mpl levels by NFIX, we assessed the expression of c-Mpl in NFIX+ cells after seven days of *ex vivo* culture by qRT-PCR and flow cytometry (Figure 4-3A, B). We found that c-Mpl transcripts increased two-fold in NFIX+ cells (p = 0.028) (Figure 4-3A). We also observed a two-fold increase in c-MPL cell surface antigen on NFIX+ cells relative to control via flow cytometry (p = 0.042) (Figure 4-3B top and lower). Also, from the number of additional HSPC genes previously observed to be perturbed by loss of Nfix [117], Erg was significantly upregulated (p = 0.022) (Figure A-7). TPO/c-MPL signaling is classically involved in megakaryopoiesis and platelet production [142-144]. Thus, as expected, NFIX+ cells also displayed a substantial increase in the cell-surface antigen CD41 (Figure 4-3C left and right), a known marker of megakaryocytes [145]. Since our data suggested that Nfix was driving HSPC towards an immature progenitor population (Figure A-2 through 5), we further interrogated our cultures for CFU-Megakaryocytes (CFU-Megs). NFIX+ cells appeared to generate more CFU-Megs than control cells after seven days of culture (p = 0.052), but the absolute frequency of CFU-Megs in NFIX+ cultures was minute (0.016), revealing that megakaryocytic progenitors with colony forming potential are rare in NFIX+ cultures (Figure A-8B). This is consistent with the observed low percentage of immunophenotypic megakaryocyte progenitors (c-Kit+Sca-1−CD127−CD9+CD32/CD16loCD41+) (Figure A-8C) [146]. Indeed, by day 30 almost no CFU-Megs were present in NFIX+ cultures (Figure A-8A).

TPO/c-MPL can activate JAK/STAT, PI3K/AKT, and MAPK/ERK downstream signaling pathways [147]. To determine if NFIX-mediated up-regulation of c-Mpl also increased TPO/c-MPL signaling, we examined the phosphorylation status of STAT5, AKT, and ERK1/2 via flow cytometry. NFIX+ cells displayed significant enhancement of STAT5 phosphorylation compared to control cells (p = 0.018), while AKT trended towards enhanced phosphorylation after prolonged TPO treatment (Figure 4-3D, left, middle, right and Figure A-9A). Also, NFIX+ cells showed no difference in phosphorylation of ERK1/2 compared to control cells (Figure 4-3D, right and Figure A-9A). This suggests that the anti-apoptotic effects displayed by NFIX+ HSPC may be mediated through the STAT5 signaling pathway. Indeed, expression of *Bcl-XL*, an anti-apoptotic factor induced by STAT5 [148], was also significantly upregulated in NFIX+ cells by two weeks of culture compared to controls (p = 0.0038) (Figure A-9B). In sum, these data reveal that up-regulation of Nfix induces both c-Mpl expression and signaling downstream of c-MPL in primitive hematopoietic cells.
Figure 4-3. NFIX upregulates c-Mpl expression and downstream signaling in HSPC during ex vivo culture.

(A) Expression of c-Mpl in LSK cells transduced with MND-Nfix relative to controls at day seven of culture, quantified by qRT-PCR (n = 3). Tbp was used as a housekeeping gene. (B) Percentage of c-MPL+ cells at four days of ex vivo culture for control and NFIX+ HSPCs, depicted as (top) representative dot plots and (lower) bar plots (n = 3).

(C) Relative level of CD41 cell surface expression in NFIX+ cells compared to controls after four days of ex vivo culture, measured by flow cytometry as gMFI, depicted as (left) representative fluorescence histogram and (right) bar plot (n = 3). (D) Relative levels of STAT5, AKT, and ERK1/2 phosphorylation in NFIX+ cells compared to controls after four days of ex vivo culture, measured by flow cytometry as geometric mean fluorescence intensity (gMFI). Depicted as (top) representative fluorescence histograms and (lower) bar plots (n = 3). (E) (top) Quantitative ChIP analysis of c-Mpl proximal promoter in HPC5 cells. Data are presented as a percentage of total input chromatin (n = 3). (lower) Left, Schematic representation of the c-Mpl promoter with half and full NFI consensus sites cloned into luciferase reporter backbone pGL4.14. Right, Results showing luciferase activity normalized to Renilla luminescence and relative to MND-control samples (n = 3-5). Values represent mean ± SE. NS denotes not significant.
NFIX Regulates the Expression of c-Mpl

Examination of the c-Mpl locus revealed palindromic NFI binding sites within the c-Mpl promoter (Figure 4-3E, lower). Promoter analysis by TRANSFAC revealed full NFI consensus sites 101 and 127 nucleotides upstream of the c-Mpl transcription start site (TTS, +1) (Figure 4-3E, lower). NFI members are known to bind both full and half NFI consensus sites [149]. Two half sites were identified 18 and 189 nucleotides upstream of the c-Mpl TSS (Figure 4-3E, lower). To assess NFIX transcriptional activity against these putative NFI binding sites in the c-Mpl proximal promoter, a c-Mpl 243 bp genomic fragment 5′ of the c-Mpl promoter containing the four identified putative NF consensus sites was sub-cloned into the pGL4.14 promoterless luciferase vector. Transient transfection of this vector into K562 cells yielded nearly three-fold higher levels of promoter activity when co-transfected with MND-NFIX relative to co-transfection with MND-Control (Figure 4-3E, lower). This enriched activity was diminished when the half NFI site (−189) furthest from the TSS was removed and was significantly reduced by the additional removal of the two full NFI sites (−127 and −101) (p = 0.0056) (Figure 4-3E, lower). Further, chromatin immunoprecipitation (ChIP) was used to show direct NFIX binding to the c-Mpl proximal promoter in the HPC5 bone marrow derived cell line. Primers were designed and validated to amplify the promoter region containing two full NFI consensus sites. In Figure 4-3E (top), a near 9-fold enrichment is observed in samples where a FLAG-tagged NFIX is present compared to controls. These data suggest that NFIX may directly activate c-Mpl promoter activity in a hematopoietic cell line.

Nfix-Induced Protection From Apoptosis Can Be Abrogated by Blocking c-MPL/TPO Signaling

To determine if the anti-apoptotic effects of ectopic Nfix in primitive hematopoietic cells depends on enhanced TPO/c-MPL signaling, we cultured NFIX+ HSPC in reduced cytokines while also either removing TPO or blocking ligand binding to c-MPL via a neutralizing antibody (AMM2) [138] for 72 hours. Although removal of TPO and neutralization of c-MPL led to reduced cell expansion in both control and NFIX+ cultures, NFIX+ cultures were significantly more sensitive to the loss of c-MPL stimulation after TPO removal or the addition of AMM2 (p = 0.0021 and 0.033, respectively) (Figure 4-4A, left and right). The selection for NFIX+ cells under reduced cytokines was also lost when TPO/c-MPL signaling was blocked by TPO removal or the addition of AMM2 (p = 0.0054 and 0.0019, respectively) (Figure 4-4B, left and right), suggesting an enhanced reliance on TPO/c-MPL signaling for expansion of NFIX+ cells. NFIX+ cells display an accelerated loss of the LSK immuno-phenotype (Figure 4-1E), possibly due to enhanced differentiation towards a downstream progenitor (Figure A.3 through 5). This loss of immuno-phenotype was mostly due to down-regulation of c-Kit cell surface expression (Figure 4-1E, top). When NFIX+ cells were culture in the absence of TPO or the presence of AMM2, c-Kit was no longer rapidly down-regulated relative to control (Figure 4-4C). Finally, while apoptosis was relatively unaffected by a loss of c-MPL signaling in control cells, NFIX+ cells displayed
Figure 4-4. The antiapoptotic effect of Nfix in hematopoietic stem and progenitor cell depends on c-MPL signaling.

(A) Relative number of cells in control and NFIX+ cultures (left) with or without TPO and (right) with or without AMM2 72 hours after replating (TPO, n = 3; AMM2, n = 6). Bar indicates significant difference in the extent of cell loss after 72 hours of culture between control and NFIX+ cells. (B) Percentage of GFP+ cells in control and NFIX+ cultures (left) with or without TPO and (right) with or without AMM2 72 hours after replating (TPO, n = 3; AMM2, n = 6). (C) Representative fluorescence histograms of GFP+ control and NFIX+ cultures to illustrate shift in c-KIT intensity 72 hours after removal of mTPO or addition of AMM2 (TPO, n = 3; AMM2, n = 6). (D) Relative levels of apoptosis in GFP+ compartment of control and NFIX+ cultures (left) with or without TPO and (right) with or without AMM2 72 hours after replating (TPO, n = 3; AMM2, n = 6). All values represent mean ± standard deviation. NS denotes not significant.
a significant increase in apoptosis after TPO removal (p = 0.045) or addition of AMM2 (p = 0.0098) (Figure 4-4D, left and right). These data reveal that NFIX-mediated up-regulation of c-MPL, and subsequent downstream signaling, functionally contributes to Nfix-induced protection from apoptosis and accelerated differentiation in primitive hematopoietic cells ex vivo.

In this study we have utilized ex vivo culture of HSPC to interrogate the molecular regulation of HSPC by Nfix. HSPC overexpressing Nfix persist in culture significantly longer than control cells, even when severely deprived of cytokines. We show that this persistence is due to enhanced survival that is mediated, in part, by up-regulation of the TPO receptor, c-Mpl. Nfix appears to promote differentiation of cultured HSPC towards a heterogeneous mixture of immature progenitors that lack transplantation and CFU potential (Figure A-2 through 5 and 8). We further demonstrate that NFIX may function as a transcriptional regulator of c-Mpl. Indeed, NFIX was capable of activating a promoter containing multiple NFI consensus binding sites located upstream of the c-Mpl promoter. We also show NFIX-FLAG directly associated with the proximal promoter. NFIX may also regulate downstream effectors of the TPO/c-MPL signaling pathway, as Stat5a is significantly upregulated in NFIX+ cells compared to controls (p = 0.0012) (Figure A-9C). We proposed the increased Bcl-xL may be driving protection from apoptosis in NFIX+ cells. To test this hypothesis, we could collect HSPC from Bcl-xL<sup>lox/lox</sup> Rosa26-Cre-ER<sup>T2</sup> mice, transduce these cells with MND-Nfix and grow these vector+ cells under reduced cytokines. A less specific method could be the use of a BH3-mimetic drug or Roscovitine in NFIX+ cells. Roscovitine was initially used to target STAT5, however, it has been shown to also affect cyclin dependent kinases (CDK).
CHAPTER 5. CHARACTERIZATION OF GENOME-WIDE NFIX BINDING IN HEMATOPOIETIC CELLS

Introduction

Nfix is well-characterized as a vital regulator of transcription in immature cells, however, little is known about other direct transcriptional targets of NFIX in hematopoietic progenitors. Furthermore, it is also currently unknown if NFIX collaborates with other well-characterized transcriptional regulators of hematopoiesis. In the previous chapter, we revealed data identifying a putative direct gene target of NFIX, c-Mpl. Here, we sought to more broadly identify the direct transcriptional targets and transcriptional partners of NFIX in HSPC.

Towards this goal, we created and validated an anti-NFIX mAb (clone: 7B5.3) and an Nfix−/− hematopoietic cell line. We exploited these tools to perform anti-NFIX ChIP-seq and RNA-seq in a hematopoietic context. These data allowed for comparison with public ChIP-seq datasets, which implicated putative NFIX transcriptional partners. Our results support a model in which NFIX and PU.1 collaborate to regulate genes implicated in hematopoietic cell proliferation, apoptosis and differentiation.

Results and Discussion

To identify direct transcriptional targets of NFIX, we engineered an NFIX-specific mAb (clone: 7B5.3). Towards this, we identified a sequence downstream of the DNA-binding domain that is conserved amongst NFIX splice variants (Figure B-1A). As the NFI family members share homology in their N-terminal region [100], we assessed the cross-reactivity of our anti-NFIX mAb with another NFI family member, NFIA. Indeed, the anti-NFIX mAb exclusively immune-precipitated FLAG-NFIX in 293T cells overexpressing FLAG-NFIX but not in samples overexpressing FLAG-NFIA (Figure B-1B). In sum, we have engineered a mAb that specifically recognizes and immune-precipitates FLAG-NFIX.

NFIX Binds Accessible and Inaccessible Regions of Chromatin

Due to the paucity of primary HSPC, we utilized HPC5 cells, an immortalized hematopoietic progenitor cell line, as an experimental surrogate [150]. HPC5 cells derive from adult murine bone marrow progenitors and express NFIX. To rigorously control for antibody specificity, we exploited CRISPR-Cas9 technology to perform targeted editing of Nfix. sgRNAs combined with Cas9 was employed to target a region in the third exon of Nfix (Figure B-2A). Successfully transfected clones were isolated, expanded and sequenced to assess insertion and deletions (InDels) in the targeted region of Nfix. A clone was identified with InDels in both Nfix alleles: a 10 bp InDel and a seven bp InDel, each of which resulted in frameshifts and multiple pre-termination codons.
(Figure B-2A). Nfix expression was decreased by 70% in this targeted clone relative to parental HPC5 cells (Figure B-2B). In contrast, Nfia and Nfic were upregulated by 40% and 48%, respectively, in Nfix-/- HPC5 cells compared to parental HPC5 cells (Figure B-2B). Consistent with our previous findings [117], c-Mpl expression was decreased by about 25% in Nfix-/- HPC5 cells relative to parental HPC5 cells (Figure B-2B).

Exploiting our NFIX-specific mAB, we performed ChIP-seq using chromatin from Nfix+/+ and Nfix-/- HPC5 cells. We recorded 6,831 total peaks from Nfix+/+ samples. NFIX peaks observed in Nfix+/+ cells were significantly enriched for the NFI-binding motif sequence (p = 1x10^{-1844}) (Figure 5-1A). Conversely, Nfix-/- samples show no enrichment for the NFI-binding motif (Figure 5-1B) and 98% of peaks appearing in Nfix+/+ samples were absent from Nfix-/- samples. The only enriched motifs identified in Nfix-/- cells belonged to MAFB (p = 1x10^{-14}) and SRF (p = 1x10^{-13}) (Figure 5-1B). NFIX peaks were also enriched for ETS-binding motifs and the MYOGENIN-binding (MYOG) motif (p = 1x10^{-31} and 1x10^{-17}, respectively) (Figure 5-1A). An NFI family member was previously shown to complex with and enhance the transcriptional activity of MYOG [151]. Further, the NFI-binding motif is present in the MyoG promoter. However, we do not find NFIX binding to promoter or enhancer regions of MyoG in HPC5 cells. Together, these results indicate the high specificity of our novel antibody. We also found NFIX binding enriched in promoter regions and 5’-untranslated regions (UTR) of the HPC5 cells genome (Figure 5-1C).

NFI proteins have been shown to bind genomic regions associated with methylated histone marks, such as histone 3 lysine 4 mono-methylation (H3K4me1) and histone 3 lysine 4 tri-methylation (H3K4me3) [152]. These marks tend to display enrichment in active enhancer and transcription start genomic regions, respectively [26]. NFIX binding has also been seen to correlate with the enhancer-associated histone mark, histone 3 lysine 27 acetylation (H3K27ac) [121]. Thus, we performed ChIP-seq against H3K4me1, H3K4me3 and H3K27ac using Nfix+/+ HPC5 cell-derived chromatin. We observed an enrichment of H3K4me1 histone marks centered around NFIX peaks (Figure 5-2A), confirming NFIX binding in enhancer regions. We found additional histone marks, such as H3K4me3 and H3K27ac, also enriched around NFIX binding (Figure 5-2B, C, respectively). Thus, NFIX binds active promoters and enhancers.

Promoters and/or CCCTC-binding factor (CTCF) often demarcate chromatin domain boundaries [153]. NFIX is known to also associate with chromatin boundaries [152]. We thus assessed the co-localization of CTCF and NFIX. We find NFIX and CTCF peaks overlap in HPC5 cells (Figure 5-3A), indicating that NFIX might also have a function at chromatin boundaries. We performed ATAC-seq using our Nfix+/+ and Nfix-/- HPC5 cells. These data suggest 55% of NFIX peaks bind to accessible regions of chromatin in HPC5 cells (Figure 5-3B). Furthermore, the NFI motif is only present in 20% of these accessible regions (Figure 5-3B, light orange, p = 1x10^{-609}). Conversely, NFIX-occupied inaccessible chromatin were highly enriched for the NFI motif (Figure 5-3B, light blue, p = 1x10^{-2877}). Recently, NFIB and NFIX were shown to regulate chromatin accessibility at super-enhancers to maintain stem cell identity [154].
Figure 5-1. NFIX is enriched in promoter regions of the HPC5 cell genome.

(A) HOMER de novo motif scanning reveals the NFI binding motif as the most significant motif detected in Nfix+/+ samples. P = 1x10^{-1844}; n=2. (B) For Nfix−/− samples, HOMER de novo motif scanning detected only the MAFB (P = 1x10^{-14}) and SRF (P = 1x10^{-13}) binding motifs (n=2). (C) The log2 fold enrichment of NFIX peaks. NFIX is enriched in promoter regions and 5’UTR regions (n=2). (D) Comparison of Nfix+/+ and Nfix−/− ChIP-seq tracks.
Figure 5-2.  NFIX significantly associates with active promoter and enhancers histone marks.
(A) Enrichment of H3K4me1, H3K4me3 and H3k27ac signal centered around NFIX. Left panel is signal plot showing H3K4me1 signal centered around NFIX. (B) Top, NFIX binding with H3K4me1. Middle, NFIX and H3K4me3 binding near the Irs2 promoter. Bottom, NFIX overlapping H3K27ac and H3K4me1 suggesting the presence of an active enhancer. Promoter captureC from publicly available data is included to show NFIX interacting with promoters from distant enhancers.
Figure 5-3. **CTCF and NFIX co-localize in HPC5 cells.**

(A) CTCF read density centered around NFIX. (B) Top, 45% of NFIX peaks are located in inaccessible regions of chromatin (blue) while the remaining are located in regions with an ATAC-seq signal (orange). Lower, within accessible regions of chromatin where NFIX peaks are found the NFI motif only makes up 20% of these targets however, still highly enriched ($p = 1 \times 10^{-609}$). Inaccessible regions where NFIX is binding the NFI motif accounts for 67% of these target regions and is highly enriched (left, light blue, $p = 1 \times 10^{-2877}$). (C) More than half of the identified super-enhancers also contain at least one NFIX peak in HPC5 cells.
Super-enhancers can be identified by binding of H3K27ac marks and large open regions of chromatin. We were curious if NFIX binds to super-enhancers in our cellular context. We found that NFIX overlaps with 52% of identified super-enhancers in HPC5 cells (Figure 5-3C).

NFIX Functions as a Transcriptional Activator in Hematopoietic Cells

We next sought to identify direct transcriptional targets of NFIX in hematopoietic cells. To better understand the transcriptional landscape regulated by NFIX, we performed RNA-sequencing on Nfix\textsuperscript{+/+} and Nfix\textsuperscript{−/−} HPC5 cells. We observed 531 differentially expressed genes (Figure 5-4A) (logFC=\(|1.0|\), FDR<0.05). 80% of these genes were down-regulated (Figure 5-4B). These data, combined with our data showing NFIX binding is enriched for histone marks associated with active transcription, suggest that NFIX acts as a transcriptional activator in hematopoietic cells. Globally, gene set enrichment analysis (GSEA) also revealed that perturbed genes were enriched for gene sets associated with p53 signaling, gene targets of HOXA9 and MEIS1, tumor necrosis factor-alpha (TNF-α) signaling activation via nuclear factor κB, toll-like receptor signaling and genes down-regulated in hematopoietic stem cells. Gene ontology (GO) analysis using PANTHER revealed that the downregulated differentially expressed genes were mostly involved in regulation of cell adhesion, proliferation and regulation of immune system process (Figure 5-4C and Table B-1). Conversely, genes upregulated in Nfix\textsuperscript{−/−} HPC5 cells were not significantly associated with any GO terms.

To determine the direct gene targets of NFIX in HPC5 cells, we combined promoter capture-C data (E-MTAB-3954), to identify enhancer regions, and known transcription start site locations, to identify promoters, with our ChIP-seq dataset (Figure 5-5A). This generated a list of putative NFIX target genes. We then filtered this list with differentially expressed genes from our RNA-seq dataset (logFC=\(|1.0|\), FDR<0.05). This produced a list of 301 differentially expressed genes with NFIX binding in enhancer or promoter regions. GO terms for these putative direct targets were related to negative regulation of apoptosis in hematopoietic cells, positive regulation of hematopoietic cell differentiation and proliferation, positive regulation of adaptive immune response and cell-substrate adhesion (Table 5-1). Interrogating only the downregulated NFIX-target genes showed GO terms associated with regulation of localization, cell differentiation and regulation of cell adhesion. As expected, the NFI binding motif was the most enriched known motif in NFIX peaks present in these putative direct targets (p = 1x10\(^{-69}\)) followed by NFIX and the NFI-half-site (Figure 5-5B). NFI family members are capable of binding NFI-half-sites [90]. Consensus binding motifs for the known hematopoietic transcriptional regulators, RUNX and PU.1, were also significantly enriched (p = 1x10\(^{-4}\) and 1x10\(^{-2}\), respectively) (Figure 5-5B). The PU.1 motif is specifically enriched in NFIX peaks putatively targeting downregulated genes (p = 1x10\(^{-52}\)). RUNX1 is required for HSC emergence during development, is expressed throughout the hematopoietic hierarchy and vital for HSC homeostasis [155]. PU.1 is also required for fetal hematopoietic development and necessary for hematopoietic progenitor cell commitment and directs lymphopoiesis/granulopoiesis.
Figure 5-4. NFIX functions as a transcriptional activator in HPC5 cells.
(A) Volcano plot showing 80% of differentially expressed genes (red dots) in Nfix−/− versus Nfix+/+ HPC5 cells are downregulated (n=3). (B) GSEA plots reveal terms associated with the p53 signaling pathway, Hoxa9 and Meis1 targets, TNFα signaling and genes associated with hematopoietic stem cells. Below each GSEA plot is a representative heatmap showing expression of genes that significantly contribute to the leading edge of the analysis.
Figure 5-5. **NFIX peaks regulating putative NFIX-target genes are enriched for NFI and PU.1 binding motifs.**

(A) Target genes were identified based on observed differential expression in Nfix^{-/-} versus Nfix^{+/+} HPC5 cells and binding to either promoters or enhancers regions. NFIX peaks were then extracted and used to search for co-binding factors. (B) Homer *de novo* motif scanning reveals the NFI consensus motif as significantly enriched in enhancer and promoter regions of direct gene targets \((p = 1 \times 10^{-69})\). (C) Known PU.1 target genes are differentially regulated in Nfix^{-/-} HPC5 cells. (D) Signal plots show ChIP peak enrichment of key hematopoietic factors including, STAT3, PU.1, ETO2, RAD21, pSTAT1 and RUNX1 centered around NFIX ChIP peaks.
Table 5-1. GO terms, determined by PANTHER analysis of genes directly regulated by NFIX, suggests roles in regulating hematopoiesis.

<table>
<thead>
<tr>
<th>Term</th>
<th>FDR</th>
<th>Genes</th>
</tr>
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<tbody>
<tr>
<td>Regulation of response to external stimulus</td>
<td>1.32x10^{-4}</td>
<td>Casp12, Tbxa2r, Lgmn, Nenf, Sema69, Nupr1, Ly86, Dbn1, Dscam, Cd74, Cd28, Nos2, Cdh5, Xrcc5, Tgm2, Ephb2, Ccl5, Fgr, Soc5, Apol2, Gpr183, Clcf1, Serpinf1, Phldb2</td>
</tr>
<tr>
<td>Myeloid cell differentiation</td>
<td>7.55x10^{-3}</td>
<td>Cdkn1c, Hlf, TSPAN2, Meis1, Tgfbr3, Dab2, Tnfsf9, Sox6, Fam20c, Gpr183</td>
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<tr>
<td>Cell-cell adhesion via plasma-membrane adhesion molecules</td>
<td>1.93x10^{-3}</td>
<td>Mpz, Celsr2, Alcam, Dscam, Celsr1, Plxnb2, Cd74, Epcam, Mag, Pvr12</td>
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<td>Regulation of lymphocyte apoptotic process</td>
<td>4.23x10^{-2}</td>
<td>Lgals3, Irs2, Cd74, Pdcd1, Ccl5</td>
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<tr>
<td>Positive regulation of angiogenesis</td>
<td>3.86x10^{-2}</td>
<td>Prkcb, Tbxa2r, Pde3b, Lgals3, Hmga2, Ephal, Cd74, Emp2, Ccl5, Serpinf1</td>
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<tr>
<td>Positive regulation of lymphocyte proliferation</td>
<td>2.86x10^{-2}</td>
<td>Tnfsf9, Cd74, Cd28, Ccl5, Gpr183, Clcf1, Irs2</td>
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</tbody>
</table>

Notes: Genes listed are differentially expressed NFIX target genes that belong to the term listed. FDR, false discovery rate.
Indeed, multiple putative direct targets of NFIX are genes previously shown to be transcriptionally regulated by PU.1 or their expression depends on PU.1 (Figure 5-5C) [156-165].

To further assess if NFIX collaborates with key hematopoietic transcription factors in gene regulation, we examined publicly available ChIP-seq data (GSE22178 and E-MTAB-3594) of the genomic binding of multiple hematopoietic transcription factors (i.e. SCL/TAL1, LYL1, LMO2, GATA2, RUNX1, MEIS1, PU.1, ERG, FLI-1, GFI1B, CEBPB, CFOS, CMYC, E2F4, EGR1, ELF1, ETO2, JUN, LDB1, MAX, MYB, NF32, TP53, RAD21, pSTAT1, STAT3) in HPC7 cells [166]. HPC7 cells are a hematopoietic progenitor cell line that was immortalized similarly to HPC5 cells [167].

Here, we compared the co-localization between the hematopoietic transcription factor peaks in HPC7 cells with NFIX peaks found in promoters and enhancers in our dataset. We observed significant co-localization with STAT3 (p = 3.429x10^{-4}), RAD21 (p = 7.675x10^{-5}), pSTAT1 (p = 9.840x10^{-6}), ETO2 (p = 0.002), FLI1 (p = 0.004), GATA2 (p = 0.008), LYL1 (p = 0.017), LDB1 (p = 0.041), RUNX1 (p = 9.640x10^{-6}) and PU.1 (p = 0.003) (Table 5-2) (Figure 5-5D). Indeed, 33% of PU.1 peaks in HPC7 cells overlapped with NFIX peaks in HPC5 cells (Table 5-2). This analysis suggests that PU.1 and NFIX may collaborate to co-regulate a subset of target genes. In sum, NFIX may cooperate other key hematopoietic transcription factors, including RUNX1 and/or PU.1, to regulate target genes in HPC5 cells.

**NFIX and PU.1 Co-Localize to Regulate Genes in Hematopoietic Cells**

To validate these results, we performed ChIP-seq using a PU.1 antibody in Nfix^{+/+} and Nfix^{-/-} HPC5 cells. Indeed, we found significant co-occupancy of PU.1 peaks and NFIX peaks in Nfix^{+/+} and Nfix^{-/-} HPC5 cells at promoter and enhancer regions (p = 0.016 and 0.008, respectively) (Figure 5-6A). We generated a list of >40 target genes based on NFIX and PU.1 co-localization (Figure 5-6C). GO terms associated with genes targeted by both NFIX and PU.1 include many linked to cell adhesion, cell death and differentiation (Figure 5-6B). Indeed, NFIX and PU.1 gene targets included Pdcd1, Socs3 and Meis1 (Figure 5-6D). Additionally, we observed the PU.1 signal is significantly less in the Nfix^{-/-} HPC5 cells (p < 0.0001) (Figure 5-6D). These data suggest that NFIX and PU.1 work together as transcriptional co-regulators to target genes in hematopoietic cells.

In sum, we sought to identify the genome-wide binding, direct transcriptional targets and transcriptional binding partners of NFIX. We have now shown that NFIX preferentially binds at promoter and 5'UTR regions and associates with histone marks, H3K4me1, H3K4me3 and H3K27ac. Our work reveals that NFIX mainly function as a transcriptional activator in primitive hematopoietic cells. >300 putative direct targets of NFIX (i.e. differentially expressed genes with NFIX occupying promoter or enhancer regions of these genes) were identified using a novel mAb. NFIX and multiple key hematopoietic transcription factors, including STAT3, RAD21, pSTAT1, ETO2, FLI1,
<table>
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<th>% overlap of TF ChIP-seq peaks with NFIX peaks</th>
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Notes: TF, transcription factor. TF ChIP-seq peaks are from GSE22178 and E-MTAB-3594.
Figure 5-6. NFIX and PU.1 co-localize and regulate genes associated with cell adhesion, cell differentiation and cell death.

(A) Signal plot showing the enrichment of PU.1 ChIP peaks center around NFIX. The antibody used for each ChIP is shown in parentheses. (B) PANTHER GO terms associated with genes that are putatively regulated by NFIX and PU.1. (C) Heatmap showing expression of all genes directly targeted by NFIX and PU.1. (D) Representative tracks from Nfix+/+ and Nfix−/− samples used for ChIP. The antibody used for ChIP is indicated as either NFIX or PU.1. Asterisks (*) are placed between PU.1 ChIP tracks where there was significantly different signal obtained (p = <0.0001).
GATA2, LYL1, LDB1, RUNX1 and PU.1, were significantly overlapping at promoter and enhancer regions, suggesting possible co-regulatory functions. We validated co-localization of NFIX with PU.1 in HPC5 cells. Additionally, our work illuminates the molecular pathways regulated by NFIX and PU.1 in these cells, such as regulating cell adhesion, cell differentiation and cell death.
CHAPTER 6. SUMMARY

Hypothesis and Specific Aims

Our original hypothesis endeavored to better understand the role of Nfix during steady-state and stress hematopoiesis. We also sought to identify the direct transcriptional targets of NFIX and what other transcription factors were involved in co-regulating gene expression.

Nfix Is Dispensable During HSPC Transplant and Steady State Hematopoiesis

It is possible that Nfix is not necessary during hematopoiesis, however, it may also be that the other Nfi members are compensating for the absence of Nfix. Previously, androgen receptor (AR) target genes were shown to be influenced by pan-NFI knockdown [168]. In a prostate cancer cell line, individual NFI gene knockdown was shown to influence AR target gene expression in a gene-dependent manner [169]. In general, when NFIB or NFIX were knocked-down AR target genes were repressed. Conversely, when NFIA or NFIC were knocked down AR target genes were activated. Also, in these experiments, knockdown of NFIX resulted in increased expression of both NFIA and NFIB. It will be interesting to evaluate hematopoiesis from double or triple knockout models to interrogate the necessity of Nfi family members. Indeed, more studies are needed to understand how this gene family with often overlapping expression regulates transcription.

The increase in chimerism in the lymphoid compartment could be due to the absence of NFIX tipping the scales towards that lineage. Indeed, our data agrees with previous findings that enforced Nfix favors myelopoiesis at the expense of lymphopoiesis [135]. Our data fit well with O’Connor et al. findings where we also see an increase in the myeloid lineage when HSPC overexpressing Nfix are transplanted into lethally irradiated recipients (Figure A-3). Additionally, this same trend has been observed when expression of NFIA is altered: increased NFIA favors erythroid lineage differentiation while suppression of NFIA leads to granulopoiesis [115].

Enforced Nfix Promotes Survival of Immature Hematopoietic Cells Ex Vivo

We present work in agreement with other literature in skeletal muscle, where overexpression of Nfix resulted in exacerbated proliferation of skeletal muscle [119]. Here, we show Nfix promotes differentiation of cultured HSPC towards a mix of immature progenitors that lack transplantation and CFU potential (Figure A-2 through 5 and 8). This possibly indicates a block in differentiation. It is also possible that Nfix expression selects for a cell in these cultures that depends on c-MPL signaling for survival. However, the enhanced survival of NFIX+ cells can also be observed in immunophenotypic HSPC (Figure A-6), demonstrating that this phenomenon is not
confined to a particular population. We further demonstrate that NFIX may function as a transcriptional regulator of c-Mpl. Indeed, NFIX was capable of activating a promoter containing multiple NFI consensus binding sites located upstream of the c-Mpl promoter. We also show NFIX-FLAG directly associated with the proximal promoter. NFIX may also regulate downstream effectors of the TPO/c-MPL signaling pathway, as Stat5a is significantly upregulated in NFIX+ cells compared to controls (p = 0.0012) (Figure A-9C). However, this effect may be indirect as there are no NFI consensus binding sites proximal to the Stat5a promoter (data not shown).

c-Mpl is a well-known regulator of HSPC function, as it is required for the maintenance of adult quiescent HSCs and protection from DNA-damage induced apoptosis in vivo [110 170 171]. Our data further implicate Nfix as a novel regulator of this important HSPC regulatory axis. Further work will be required to determine if Nfix-mediated regulation of HSPC responsiveness to TPO contributes to HSPC survival and niche retention.

Characterization of Genome-Wide Binding of NUCLEAR FACTOR I-X in Hematopoietic Cells

NFIX is the first NFI member identified as functionally relevant to HSPC biology in vivo, thus necessitating a more thorough understanding of its function. NFI proteins have been previously implicated in the regulation of differentiation, proliferation and survival in many cellular contexts [111-115]. Here, we show that NFIX direct transcriptional gene targets are enriched for GO terms such as negative regulation of apoptosis in hematopoietic cells, positive regulation of hematopoietic cell differentiation and proliferation, positive regulation of adaptive immune response and cell-substrate adhesion. These data are congruent with earlier findings that Nfix-KO NSC exhibit increased apoptosis, delayed differentiation and stochastic migration while ectopic Nfix protects HSPC from apoptosis induced by cytokine-deprivation in vitro. While the exact mechanism for NFIX regulation of apoptosis is not well understood in hematopoiesis or neurogenesis, here we identify several target genes (i.e. Il10ra, Irs2, Pdcd1) that are responsive to external stimuli and anti-apoptotic [172-174]. NFIX also appears to positively regulate galectin-3 (Lgals3), a lectin family member that associates with anti-apoptotic BCL2 [175] and supports mitochondrial integrity during apoptotic stimuli and stress [176]. The chemokine, CC-chemokine ligand 5 (CCL5), was also positively regulated by NFIX. CCL5 can have both anti-apoptotic and pro-apoptotic functions [177 178]. Further investigation of the exact mechanisms affected by NFIX will clarify its precise role in promoting cell survival.

In primary astrocytes, NFIX and STAT3 form a complex to positively regulate the expression of YKL-40, a migratory factor [179]. In the same study, ectopic expression of NFIX in glioma cells negatively affected expression of SERPINE1, a serpin family member. Here, we observed significant co-localization of NFIX and STAT3 at gene targets in hematopoietic cells, including another serpin family member, Serpinf1. Serpinf1 encodes pigment epithelium-derived factor (PEDF). PEDF has been
shown to inhibit the migration of endothelial cells [180] and cancer cell lines [181]. While PEDF is reported as dispensable for HSPC steady-state and regeneration function [182], there are 60 functional Serpin genes identified in the mouse [183] and 37 in humans [184]. Thus, PEDF may be readily compensated for by a family member or other proteinase inhibitors. Whether NFIX and STAT3 together coordinate migration in hematopoietic cells is an intriguing question. STAT3 has also been implicated a positive regulator of HSPC regeneration [185]. We had previously implicated NFIX as an essential factor required for optimal HSPC transplantation [117]. Here, we find co-localization with STAT3 at genes also implicated in hematopoietic reconstitution, such as Hmga2 [185], Cdkn1c [186] and Clcf1 [187]. Thus, NFIX and STAT3 may cooperate to promote HSPC reconstitution post-transplant.

We found that NFIX targets genes related to hematopoietic cell differentiation, such as Meis1. PU.1 induces myeloid lineage commitment in HSPC [188] and directs progenitors towards the lymphoid lineage [189]. PU.1 is another key hematopoietic regulator that we found co-localized at gene targets with NFIX. Interestingly, genes putatively targeted by both NFIX and PU.1 are enriched for GO terms involving cell proliferation, regulation cell adhesion, cell death and differentiation. Target genes include Pdcd1, Socs3 and Meis1. NFIX has been shown to modulate HSPC lineage commitment: enforced NFIX expression skews towards the myeloid lineage at the expense of B cells [135]. Given the importance of PU.1 across the hematopoietic hierarchy and its co-localization with NFIX, a relatively novel HSPC transcriptional regulator, future studies should focus on unraveling the relationship between NFIX and PU.1.

Another important consideration is the function of PU.1 as a pioneering factor. Pioneering factors are proteins that can bind to regions of condensed chromatin and whose occupancy precedes that of other factors, imparting competency for transcriptional activity. During hematopoiesis, PU.1 acts as a prototypical pioneer factor as it does not bind to nucleosomal DNA in vitro. Here we show reduced binding of PU.1 in the absence of NFIX (Figure 5-6E). Work focused on understanding the binding kinetics between these two factors will be important in understanding changes in the chromatin landscape during hematopoiesis. In a prostate cancer cell line, NFIX interacts with FOXA1, another pioneer factor. However, NFIX represses expression of AR-regulated genes regardless of FOXA1 [169]. Currently, without ATAC-seq datasets, it is difficult to establish if NFIX is acting as a pioneer factor in these other cellular contexts. ATAC-seq and ChIP-seq experiments will be vital in showing how NFIX may function as a pioneer factor.

In summary, ours is the first study to uncover the direct transcriptional targets of NFIX in hematopoietic cells. These direct targets include genes that regulate apoptosis, differentiation and cell adhesion. We show NFIX prefers genomic promoters and enhancers. NFIX also co-localizes with other key hematopoietic transcription factors, including STAT3, RAD21, pSTAT1, ETO2, FLI1, GATA2, LYL1, LDB1, RUNX1 and PU.1. We confirmed PU.1 and NFIX genomic co-localization. NFIX appears to be multifaceted; having roles in binding chromatin boundaries and co-localizing with CTCF, regulating an anti-apoptotic gene signature, interacting with STAT3 in hematopoietic cells to promote migration and modulating cell proliferation/differentiation putatively
with PU.1. Future work towards understanding the role of NFIX with each of these biological functions will be illuminating, especially its function in the molecular processes of HSPC transplantation and cooperation with other key hematopoietic factors.
CHAPTER 7. FUTURE DIRECTIONS

Importantly, the data presented in this work suggests \textit{Nfix} has a complex role in hematopoiesis. Despite the advances reported here, there are still questions that would further reveal the involvement of \textit{Nfix} during hematopoiesis.

Do Other NFI Family Members Compensate for the Absence of \textit{Nfix} During HSCT?

There is evidence of overlapping expression of \textit{Nfi} family members suggesting other members may compensate for the loss of another or redundancy. In order to test the hypothesis that NFI members compensate for the absence of \textit{Nfix}, performing RNA-seq on transplanted HSPC from \textit{Nfix}\textsuperscript{-/-} mice would be illuminating. RNA-seq has the advantage over qRT-PCR by being more sensitive and providing substantially more data. In addition to being able to determine the quantity of transcripts of other NFI family members, one could perform differential gene expression analysis and use this list of genes to assign GO terms. This differs from our current data as our data was collected without transplantation or any other form of stress.

Is the NFI Family Dispensable for Hematopoiesis?

Our lab and others have implicated the NFI family in hematopoiesis. Most of these studies have involved cell lines, \textit{in vitro} analyses and single KO mouse models. I propose that working with a triple KO or double KO mouse models would be the most rigorous way to test this hypothesis. \textit{Nfic}\textsuperscript{-/-} mice are viable and require soft chow. We currently have the \textit{Nfix}\textsuperscript{flox/flox} mouse model and an \textit{Nfia}\textsuperscript{flox/flox} mouse model exists, although would need to be backcrossed onto a C57Bl6/J background. Breeding strategies to generate a \textit{Nfic}\textsuperscript{-/-} \textit{Nfix}\textsuperscript{flox/flox} \textit{Nfia}\textsuperscript{flox/flox} HSC-Scl-Cre-ER\textsuperscript{T} mouse model would allow for fairly normal development (\textit{i.e.} the mice would need to be fed soft chow as the \textit{Nfic}\textsuperscript{-/-} mice have defective molar root formation). Thus, the two floxed genes could be spatially and temporally deleted in HSPC and transplanted into lethally irradiated recipient mice. Controls would lack the HSC-Scl-Cre-ER\textsuperscript{T} knockin allele but still be treated with Tamoxifen.

In the same way, one could imagine having double KO models to interrogate if a specific NFI family member is required for hematopoiesis (\textit{i.e.} \textit{Nfic}\textsuperscript{-/-} \textit{Nfix}\textsuperscript{flox/flox} HSC-Scl-Cre-ER\textsuperscript{T} or \textit{Nfic}\textsuperscript{-/-} \textit{Nfia}\textsuperscript{flox/flox} HSC-Scl-Cre-ER\textsuperscript{T}). We have not found evidence for the expression of \textit{Nfib} in the hematopoietic hierarchy (data not shown).

How Does NFIX Regulate the Globin Genes?

Recently, NFIX was implicated as a repressor of fetal hemoglobin (HbF) [190]. Briefly, during development there is a developmentally regulated switch from the
expression of HbF to adult hemoglobin. To better understand which transcription factors may be involved in this switching, Chaand et al. used ATAC-seq and transcriptionally profiled differentiated CD34+ cells from adult BM, CD34+ cells from cord blood (CB) and HUDEP-2 cells, an immortalized human erythroid progenitor cell line. Their results show the NFI motif was enriched in regions of chromatin accessibility in cells from adult BM compared to cells from CB. More differentiated HUDEP-2 cells compared to a less differentiated stage. They also show an increase in NFIX mRNA in adult BM compared to CB. These data suggest NFIX plays a role in repressing HbF during the developmental switch to adult hemoglobin. To test this hypothesis, Chaand et al. knocked-down NFIX in CD34+ cells from adult BM and HUDEP-2 cells. This caused an increase in HbF comparable to when BCL11A, a known HbF repressor, is knocked-down. Together, their data strongly implies that NFIX represses HbF during development. This has implications in treating diseases where an increase in HbF would have therapeutic impact, such as with sickle cell anemia. Regulation of the globin gene locus is very complex and has limited the ability of researchers to fully understand how it is regulated. Performing ChIP-seq using HUDEP-2 cells or CD34+ cells from adult BM and CB may offer more insight into how this important cluster of genes is regulated, especially in combination with Chaand et al. ATAC-seq data and using their shRNAs as a control.

**Does NFIX Require PU.1 Binding First or Does NFIX Function as a Pioneering Factor?**

We have data suggesting NFIX and PU.1, a master transcription factor, co-localize regions of the HPC5 cell genome. There is an obvious question about which factor binds first? To better understand the answer to this question, one could imagine knocking down PU.1 and then perform ChIP with our anti-NFIX antibody to test if NFIX binding is still present or reduced. Also, there is a competitive PU.1 inhibitor (PU.1-ets) that encodes only the DNA binding domain of PU.1. PU.1 requires its protein-protein interaction domains in order to bind DNA and affect transcription.

Pioneering factors are transcription factors that can bind to regions of condensed chromatin and whose occupancy precedes that of other factors, imparting competency for transcriptional activity. Pioneering factors are especially important during development and lineage determination. It will be advantageous to use a cellular model where differentiation in vitro is possible to track the presence of NFIX at different regulatory enhancers and promoters. For example, previous work has used a skeletal muscle cell line to study the role of NFIX when differentiating embryonic myoblasts to fetal myoblasts [118]. In these studies, there is a clear role for NFIX regulating differentially expressed genes between the two developmental stages; however, use of our anti-NFIX antibody would determine if NFIX is affecting condensed regions of chromatin and “priming” these areas for other transcription factor occupancy. Additionally, the NFI members have established roles in NSC development. It will be informative to test the occupancy of NFIX throughout hierarchies of development (e.g. hematopoiesis, NSC development).
What Other Proteins Interact With NFIX?

Immunoprecipitation followed by mass spectrophotometry (IP-MS) relies on protein abundance as well as a robust, specific antibody for the protein of interest. With our novel antibody, we can globally interrogate what other proteins are physically interacting with NFIX. Furthermore, editing of NFI domains followed by IP-MS may be informative towards understanding what domains of NFIX are important for protein-protein interactions. The caveat here is to make certain the anti-NFIX antibody is still useable/specific when NFIX has been modified/truncated.

We show there is overlapping signal between NFIX and CTCF in HPC5 cells. The NFI proteins have been known to re-shape DNA, remodel chromatin and bind chromatin boundaries. To determine how NFIX and CTCF interact, it would be worth performing HiChIP. HiChIP combines ChIP with chromosome conformation capture coupled with high-throughput sequencing. Using our anti-NFIX antibody, we could perform HiChIP to interrogate long range interaction by NFIX. Including the Nifix−/− HPC5 cells would further inform if NFIX is necessary for CTCF binding or if NFIX functions as a transcriptional insulator.
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Figure A-1. NFIX overexpression promotes accelerated differentiation of LSK cells during ex vivo culture.

(A) FACS plots depicting the sorting schematic for freshly isolated LSK cells. (B) LSK immunophenotype of control and NFIX+ cells at day 0, 7, and 14 of culture depicted a representative dot plot from three independent experiments. (C) LSK immunophenotype of control and NFIX+ cells at day 14 of culture depicted as bar plot (n = 3). (D) One-way FACS histogram depicting a reduction in c-KIT+ cells among the lineage negative population of control and NFIX+ cells after seven days of ex vivo culture. All values represent mean ± standard deviation. NS denotes not significant.

Figure A-2. HSPC overexpressing Nfix display an immature blast-like morphology similar to control cells.

Figure A-3. HSPCs overexpressing Nfix fail to repopulate the bone marrow of irradiated recipients and display a myeloid bias in lineage distribution.

(A) Schematic displaying competitive transplantation assay to assess hematopoietic repopulation potential of HSPC. CD45.2 “test” LSK cells were harvested from bone marrow and transduced with either MND-control or MND-Nfix lentiviral vectors. CD45.1 “competitor” LSK cells were mock transduced. 24 hours post-transduction, 5000 test and 5000 competitor cells were harvested and transplanted into irradiated recipients. (B) Percentage of CD45.2 “test” cells in the peripheral blood of transplanted recipients over a 16 week period. (C) Percentage of GFP+ cells within CD45.2 “test” cells in the peripheral blood of transplanted recipients over a 16 week period. (D) Percentage of T-, B-, and myeloid cells within CD45.2 “test” cells in the peripheral blood of transplanted recipients over a 16 week period.

Figure A-4. HSPC overexpressing Nfix display reduced CFU potential.  
(A) Frequency of colony-forming units among GFP+ control and NFIX+ cells cultured for seven days ex vivo (n = 3).  
(B) Frequency of colony-forming units among GFP+ control and NFIX+ cells cultured for 21 days ex vivo (n = 3). The frequency of colony forming units refers to the number of colonies scored divided by the total number of cells plated in methylcellulose. All values represent mean ± standard deviation. NS denotes not significant.

Figure A-5. Nfix-overexpressing cells display no major lineage markers and an immature progenitor immuno-phenotype.

(A) Percentage of lineage+ cells among GFP+ control and NFIX+ cells as one (n = 3), three (n = 2), and four (n = 3) weeks in ex vivo culture. (B) Percentage of c-Kit+ CD71+ cells among GFP+ control and NFIX+ cells at various time-points during ex vivo culture. (C) Representative FACS plot depicting the percentage of CD71hi cells in GFP+ control and NFIX+ cultures at day seven and day 30 of ex vivo culture. All values represent mean ± standard deviation. Note: all comparisons in (A) are not significant.

Figure A-6.  LSK cells overexpressing Nfix display reduced apoptosis under cytokine deprivation during ex vivo culture.

Percentage of GFP+ apoptotic cells within control or NFIX+ LSK cells at day seven of ex vivo culture (n = 3). All values represent mean ± standard deviation. NS denotes not significant.

Figure A-7. Nfix overexpression affects the expression of other known regulators of HSPC biology.
Relative expression of several regulators of HSPC biology in NFIX+ cells compared to control cells known to be down-regulated upon shRNA-induced Nfix knockdown [10] at day seven of ex vivo culture (n = 3). All values represent mean ± standard deviation. NS denotes not significant.
Figure A-8. HSPCs overexpressing Nfix are not enriched for megakaryocyte progenitors or CFU-Megs.
(A) Frequency of GFP+ CFU-Megs from control day seven, NFIX+ day seven, and NFIX+ day 30 ex vivo cells (n = 2). (B) Representative images of CFU-Megs from control day seven, NFIX+ day seven, and NFIX+ day 30 ex vivo cells (n = 2). (C) Percentage of megakaryocyte progenitors (c-Kit+Sca-1-CD127-CD9+CD32/CD16loCD41+) among GFP+ control and GFP+ NFIX+ cells (n = 4). All values represent mean ± standard deviation. Reprinted with permission from John Wiley and Sons. Hall, T.*, Walker, M.*, Ganuza, M., Holmfeldt, P., Bordas, M., Kang, G., Bi, W., Palmer, L.E., Finkelstein, D. and McKinney–Freeman, S. (2018), Nfix Promotes Survival of Immature Hematopoietic Cells via Regulation of c-Mpl . Stem Cells, 36: 943-950. (*Co-first author). doi: http://dx.doi.org/10.1002/stem.2800.
Figure A-9. NFIX+ cells display enhanced TPO/c-MPL signaling sensitivity to mTPO exposure.

(A) Relative phosphorylation status of STAT5, AKT, and ERK1/2 in NFIX+ cells during a time-course of mTPO exposure following cytokine starvation, as measured by phosphoflow (STAT5, ERK1/2: n = 4; AKT: n = 3). gMFI: Geometric mean fluorescence intensity. (B) Relative expression of Bcl-xL in NFIX+ cells compared to control cells at different time points during ex vivo culture, quantified by qRT-PCR (n = 3). Tbp was used as a housekeeping gene. (C) Relative expression of Stat5a and Stat5b in NFIX+ cells compared to control cells at day seven of ex vivo culture, quantified by qRT-PCR (n = 6). Tbp was used as a housekeeping gene. All values represent mean ± standard deviation. NS denotes not significant.

Figure B-1. mAb 7B5.3 specifically detects murine NFIX.
(A) Alignment of the four characterized NFIX protein isoforms. The underlined portion is the 3’ end of the DNA binding domain. The red font, downstream of the DNA binding domain, is the sequence used to generate a peptide for antibody production and is conserved between the four NFIX isoforms. (B) Western blot showing immunoprecipitation from 293T cells lysates overexpressing FLAG-NFIX or FLAG-NFIA. Expected size of FLAG-NFIX: 50kDa. Expected size of FLAG-NFIA: 55kDa.
Figure B-2. NFIX expression in Nfix^{+/+} and Nfix^{−/−} HPC5 cells. (A) Red font indicates guide RNA (gRNA) designed for CRISPR-Cas9 editing. Top row shows wild-type Nfix sequence and bottom rows show deep sequencing results of edited Nfix^{−/−} alleles from clonal cell line. (C) Real-time polymerase chain reaction results show expression of Nfix, Nfic, Nfia and c-Mpl. Tata binding protein (Tbp) was used for normalization. n=3.
Table B-1. Gene set enrichment analysis of differentially expressed genes in Nfix\textsuperscript{−/−} HPC5 cells compared to Nfix\textsuperscript{+/+} HPC5 cells.

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Figure B-3.  *Sfpi1* expression in *Nfix*<sup>−/−</sup> HPC5 cells.
(A) Log2 transcript per million (TPM) for *Sfpi1* in *Nfix*<sup>+/+</sup> HPC5 cells and *Nfix*<sup>−/−</sup> HPC5 cells. These values are from the RNA-seq dataset described in Chapter 5 (n=3).
VITA

Megan Walker was born in 1987. She was always curious and believed she had been made for learning. She graduated from high school and attended a community college, graduating with an Associate of Science degree concentrating in Chemistry. She continued her education at Austin Peay State University and graduated with two Bachelor in Science degrees concentrating in Biochemistry and Biology. During her undergraduate, she performed research interrogating the expression of a gap junction in a neuronal cell line. She remained at Austin Peay State University to continue pursuing a Master of Science degree. Her thesis was based on work evaluating surface proteins allowing for host immune evasion of the causative agent of Lyme disease, *Borrelia burgdorferi*. Afterwards, she moved to Memphis in 2013 and expects to graduate from the University of Tennessee Health Science Center with a Doctor of Philosophy Degree in October 2020. In the future, Megan plans learning more about bioinformatics, next generation sequencing and gardening.