The Transcription Factor Gfi1b Regulates Cell Fate in Hematopoietic Stem Cells and Associated Malignancies

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1. Abstract

The efficacy of bone marrow transplantation is critically dependent on the transfer of sufficient hematopoietic stem cells (HSCs) which possess the capacity for self-renewal and can fully reconstitute the hematopoietic system. By transiently manipulating the factors that govern HSC homeostasis it has been proposed that HSCs can be expanded without the loss of essential stem cell characteristics. Previously it was observed that ablation of Gfi1b *in-vivo* using the interferon based Mx-Cre system results in a dramatic expansion and mobilization of hematopoietic stem cells in the bone marrow and periphery. I was able to replicate this finding using a non-inflammatory, tamoxifen inducible, deletion system indicating this expansion is a property of Gfi1b ablation and so it was hypothesized that Gfi1b regulates the fate of HSCs and is a potential target for their *ex-vivo* expansion. Indeed, when deletion of *Gfi1b* was induced in whole bone marrow *ex-vivo* HSCs expanded both in absolute number and in terms of proportion of bone marrow by approximately 5-fold.

Furthermore, in *ex-vivo* expansion cultures of primary HSCs tracking of surface levels of cd48, which indicates an HSC has transitioned to a differentiation committed multi-potent progenitor, revealed that Gfi1b null HSCs underwent symmetric self-renewal type cell divisions at a significantly increased frequency. Importantly it has also been shown that HSCs lacking Gfi1b cycle at a faster rate than control HSCs. This combination of increased cell division and preferential self-renewal of *Gfi1b*$^{-/-}$ HSCs indicates that inhibition of Gfi1b is an ideal strategy for *ex-vivo* HSC expansion. As well, in accordance with this preference for self-renewal, Gfi1b null HSCs that were cultured

under myeloid differentiation conditions remained primarily in an undifferentiated state as defined by a lack of the myeloid surface markers Gr1 and Mac1. These cultures also demonstrated increased long term colony forming capacity versus controls, further supporting an undifferentiated phenotype in *Gfi1b*$^{-/-}$ cells.

Because the stem cell niche is a highly complex and heterogeneous environment I also investigated whether bone marrow in which *Gfi1b* has been deleted exerts paracrine effects that contributed to HSC expansion. Co-Culture assays demonstrated that *Gfi1b*$^{-/-}$ bone marrow was able to induce an expansion of progenitors in wild-type bone marrow of more than 10 fold compared to *Gfi1b*$^{-/+}$ bone marrow. Interestingly cells co-cultured with Gfi1b null bone marrow also exhibited an overall proliferation advantage of approximately 3 fold after short-term cultures. This indicates that not only does loss of Gfi1b induce HSC expansion via cell intrinsic mechanisms, but also through paracrine factors that alter the bone marrow homeostasis.

I also investigated the role of Gfi1b in HSC associated malignancies. Previously it had been described that Gfi1b is overexpressed in BCR-ABL driven CML and B-ALL. As well work from our lab showed that transgenic overexpression of Gfi1b in model B-ALL accelerate disease progression and morbidity. I was able to show that deletion of *Gfi1b* in an established B-ALL leads to remission of disease. As well pharmacological inhibition of the core Gfi1b co-factor LSD1 was shown to kill CML cells *in-vitro* proportionally to concentration. This suggests that Gfi1b plays a central functional role in both normal and malignant hematopoietic cells.

Résumé

L'efficacité d'une greffe de moelle osseuse dépend de façon critique du transfert d'un nombre suffisant de cellules souches hématopoïétiques (CSHs) qui possèdent 1- la capacité de s'auto-renouveler et 2- la capacité d'entièrement reconstituer le système hématopoïétique suite à leur différentiation. En manipulant transitoirement les facteurs qui régissent l'homéostasie des CSHs, il a été proposé qu'une expansion de ces dernières peut être réalisée sans entraîner la perte de leurs caractéristiques essentielles. Il avait préalablement été observé que l'ablation de l'expression de Gfi1b *in vivo,* grâce au système Mx-Cre basé sur l'interféron, entraînait une expansion et une mobilisation spectaculaires du nombre de CSHs dans la moelle osseuse et la périphérie. J'ai pu reproduire ce résultat en utilisant un système de suppression de l'expression de Gfi1 inductible au tamoxifen et non-inflammatoire, suggérant que l'expansion du nombre de CSHs observée est bien une propriété de l'ablation de l'expression de Gfi1b. Nous avons par conséquent émis l'hypothèse que Gfi1b régie le sort des CSHs et est une cible potentielle pour leur expansion *ex-vivo*. En accord avec cette hypothèse, nous avons observé une expansion d'environ 5 fois du nombre de CSHs en nombre absolu et en termes de proportion de la moelle osseuse lorsque la délétion de l'expression de Gfi1b est induite dans l'ensemble des cellules de moelle osseuse *ex-vivo*.

De plus, le suivi des niveaux d'expression dans les cultures d'expansion de CSHs du marqueur de surface CD48, qui indique qu'une CSH s'est différentiée en progéniteur multipotent, a permis de révéler que les CSHs Gfi1b-nulles se sont

divisées de façon symétrique en conservant leur capacité d'auto-renouvellement avec une fréquence significativement plus élevée que les cellules contrôles. Il a également été démontré que les CSHs Gfi1b-nulle se divisent à un rythme plus élevé. Cette combinaison de l'augmentation de la division cellulaire et de l'auto-renouvellement des CSHs *Gfi1b*$^{-/-}$ indique que l'inhibition de Gfi1b est une stratégie idéale pour l'expansion des CSHs ex-vivo. Conformément à cette préférence pour l'auto-renouvellement, les CSHs Gfib-nulles qui ont été cultivées dans des conditions de différenciation myéloïde sont restées principalement dans un état indifférencié tel que défini par l'absence de marqueurs de surface myéloïdes Gr1 et MAC1. Ces cultures ont également démontré une augmentation de leur capacité à former des colonies à long terme en comparaison des contrôles. Ces résultats soutiennent le phénotype non différencié des cellules *Gfi1b*$^{-/-}$.

Parce que la niche de cellules souches est un environnement très complexe et hétérogène, j'ai aussi cherché à savoir si la moelle osseuse où l'expression de Gfi1b a été supprimée exerce des effets paracrines qui ont contribué à l'expansion des CSHs. Des essais de co-culture ont démontré que la moelle osseuse *Gfi1b*$^{-/-}$ était capable d'induire une expansion des progéniteurs de la moelle osseuse de type sauvage de plus de 10 fois en comparaison à la moelle osseuse *Gfi1b*$^{-/+}$. Il est intéressant de noter que les cellules co-cultivées avec la moelle osseuse Gfi1b-nulle ont également montré un avantage global de prolifération d'environ 3 fois suite à leur culture à court terme. Cela signifie que l'élimination de l'expression de Gfi1b induit l'expansion des CSHs grâce à des mécanismes cellulaires intrinsèques, mais aussi

grâce à la sécrétion de facteurs paracrines qui altèrent l'homéostasie de la moelle osseuse.

J'ai également étudié le rôle de Gfi1b dans les tumeurs malignes associées aux CSHs. Auparavant, il avait été décrit que l'expression de Gfi1b est augmentée dans les CML et les B-ALL induites par BCR-ABL. De plus, des études provenant de notre laboratoire ont montré que la surexpression de Gfi1b dans un modèle de B-ALL accélère la progression de la maladie et de la morbidité. En contraste, j'ai pu démontrer que la suppression de l'expression de Gfi1b dans un modèle établi de B-ALL entraîne une rémission de la maladie. De plus, l'inhibition pharmacologique de LSD1, un cofacteur de Gfi1b, entraîne la mort des cellules leucémiques *in vitro* proportionnellement à sa concentration. Ces études suggèrent que Gfi1b joue un rôle fonctionnel central dans les cellules hématopoïétiques normales et malignes.

“All can be measured by the standard of the capybara.
Everyone is lesser than or greater than the capybara.
Everything is taller or shorter than the capybara.
Everything is mistaken for a Brazilian dance craze
more or less frequently than the capybara”

- from “Units of Measure” by Sandra Beasley

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A thesis, much like the rest of the things in existence it turns out, does not just come to be. Rather it is made, it is shaped and it evolves. Kind of like a horrific tropical parasite really, a thesis feeds off the people around it; it consumes their hard work, their ideas and inspiration, both personal and professional. Then one day something wonderful happens: the thesis finds itself metamorphosed into a mature work of scholarship. Or at least one hopes so. Although it is my name on the cover of this thesis, indicating I was its primary host, it could not have come to be without the generous support of a great many people. For this I am extremely grateful.

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3. Contribution of Authors

Tarik Moroy supervised research. Cyrus Khandanpour and Tarik Moroy helped design research. Lothar Vassen performed all experiments on Gfi1b transgenic mice including survival experiments for BCR-ABL B-ALL and B-ALL micro-arrays. Charles Vadnais helped with array analysis.

4. List of Figures

5. List of Abbreviations

- AK – Adenylate Kinase
- ATCC – American type culture collection
- ATP – Adenosine triphosphate
- B-ALL – B cell acute lymphoblastic leukemia
- BCL-XL – B-cell lymphoma-extra large
- BLK – B lymphocyte kinase
- BM – Bone marrow
- BMC – Bone marrow cell
- BMI-1 – polycomb ring finger oncogene
- BSA – Bovine serum Albumin
- ChIP – chromatin immunoprecipitation
- CLP – Common lymphoid progenitor
- CML – Chronic Myeloid Leukemia
- CMP – common myeloid progenitor
- DMEM – Dulbecco's modified eagle medium
- EBF – Early B-cell factor
- EKLF – Erythroid Kruppel-like factor
- ERG – ETS related gene
- FACS – Fluorescence-activated cell sorting
- FAIRE – formaldehyde-assisted isolation of regulatory elements
- FCS – Fetal Calf Serum
- Flt3-L – Flt3 ligand

- Foxo3a – Forkhead box O3
- G-CSF – Granulocyte colony stimulating factor
- Gfi1(b) – Growth Factor Independence 1(b)
- GFP – green fluorescent protein
- GMP – Granulocyte-monocyte progenitor
- HDAC – Histone deacetylase
- HIF-1-alpha – Hypoxia inducible factor-1-alpha
- HSC – Hematopoietic Stem Cell
- IMDM – Isocove's Modified Dulbecco's Medium
- IL – Interleukin
- KO - Knockout
- LIF – Leukemia inhibitory factor
- LMMP – Lymphoid-primed multi-potent progenitor
- LNA – Locked nucleic acid
- LSC – leukemia stem cell
- LSD1 – Lysine Specific Demethylase 1
- MEP – megakaryocyte erythrocyte progenitor
- MYB – Myeloblastosis viral oncogene
- MYC – Myelocytomatosis viral oncogene
- MPP – Multi-potent progenitor
- NAC – N-acetyl cysteine
- NF-Y – Nuclear transcription factor Y

- PAX5 – Paired box protein 5
- PBS – Phosphate Buffered Saline
- PCR – Polymerase Chain Reaction
- Ph – Philadelphia chromosome
- pIpC – polyInosinic polycytidylic acid
- PNS – peripheral nervous system
- RAG – Recombination activating Gene
- RPM – Rotations per minute
- RUNX1 – Runt-related transcription factor 1
- SCF – Stem cell factor
- SCL/TAL1 – Stem cell leukemia protein/T-cell acute leukemia protein
- SOCS1 – Suppressor of cytokine signaling 1
- TGF-beta – Transforming growth factor-beta
- TK – Thymidine Kinase
- TPO – Thrombopoietin
- WT – Wild-type

6. Introduction and Overview

All cells of the blood and immune system begin their development from an initial progenitor, the hematopoietic stem cell (HSC). These cells must be maintained throughout the life of an organism and generally speaking a break in hematopoietic homeostasis can lead to a wide variety of pathologies. Given this it is not surprising that a complex regulatory network governs the fate of a HSC, controlling the balance between self-renewal, death and production of mature blood and immune cells in response to various hematopoietic needs.

Previously it was observed that the transcription factor Growth Factor Independence 1b (Gfi1b) is a core component of the HSC regulatory machinery. When *Gfi1b* is deleted conditionally in adult mice HSCs expand in the bone marrow by approximately 30-fold, however the total bone marrow cellularity is unchanged [1]. The first objective of this project was to confirm this result in an alternative conditional knockout system in order show that the expansion phenotype was indeed due to loss of Gfi1b and not an artifact of the deletion system used in the original study.

Based on the selective expansion of HSCs after Gfi1b ablation without compromising the production of mature myeloid and lymphoid cells it was hypothesized that Gfi1b restricted the symmetric self renewal division of HSCs. As such this study also aimed to assess the level of symmetric division in HSCs lacking Gfi1b by tracking their division in culture under various conditions. This assay

identified a shift in the distribution of cell fates in *Gfi1b* null HSCs and confirmed an increase in symmetric self-renewal as one component of the mechanism underlying the *Gfi1b* knockout phenotype. In this work I also investigated the extent to which this finding is cell intrinsic and whether Gfi1b controls paracrine regulatory elements as well.

HSCs are also the critical cell type in a bone marrow graft where they are required to reconstitute all hematopoiesis in the recipient. Because material for such grafts is often limited, particularly in the case of cells harvested from umbilical cord blood, a method for the *ex-vivo* expansion of HSCs is of significant clinical interest. Given that HSCs in which *Gfi1b* is conditionally deleted expand *in-vivo* without a concordant loss of function one of the primary objectives of this project was to apply the inhibition of Gfi1b in culture towards accomplishing the *ex-vivo* expansion of HSCs.

In addition to its role in HSCs, Gfi1b is overexpressed in the HSC-associated malignancies CML and B-ALL [2]. These leukemias can both be driven by a chromosomal translocation resulting in a BCR-ABL fusion kinase. Interestingly *Gfi1b* is carried along in this translocation. As well, *Gfi1b* is further upregulated in these tumors in response to treatment with the BCR-ABL inhibitor imatinib. Taken together these results suggest that Gfi1b plays a functional role in the pathogenesis of CML and B-ALL and so the final aim of this project was to determine whether Gfi1b was a viable therapeutic target for the treatment of these malignancies.

7. Literature Review

7.1 Hematopoietic progenitors and differentiation

7.1.1 Hierarchy of hematopoiesis

Hematopoiesis refers to the development of all the varying lineages of blood and immune cells including lymphocytes, granulocytes, macrophages, dendritic cells, platelets and red blood cells. Despite these diverse outcomes though, all hematopoiesis begins from an initial progenitor, the hematopoietic stem cell (HSC), which differentiates into a series of intermediary progenitors before committing to a specific lineage and terminally differentiating. Thus the so-called hierarchy of hematopoiesis branches out from the early progenitors with increasing functional specificity at the expense of potentiality [3].

The first step in any hematopoietic development is the commitment of an HSC to differentiation and the subsequent transition into a multi-potent progenitor (MPP). MPPs still retain the capacity to form cells of any hematopoietic lineage, however they no longer have long-term self-renewal and reconstitution potential [4-6]. The current model holds that the first branch point in hematopoiesis is the separation of MPPs into Megakaryocyte-Erythrocyte progenitors (MEPs) and lymphoid-primed MPPs (LMPPs), which retain both myeloid and lymphoid development capacity [7]. Within lymphocyte development LMPPs will then further differentiate into common lymphoid progenitors (CLPs) and early lymphoid progenitors (ELPs) [8, 9]. As well LMPPs may instead continue towards the myeloid

lineage, becoming granulocyte-macrophage progenitors (GMPs) [7]. Each of these lineage specific progenitors is then able to undergo terminal differentiation into a variety of mature cell types: CLPs and ELPs will eventually form all the various subsets of B and T lymphocytes, respectively, MEPs will become red blood cells or platelet producing megakaryocytes, and GMPs will make either granulocytes, macrophages or dendritic cells. Progression through and maintenance of this hematopoietic hierarchy is governed by a number of factors including intrinsic regulation by transcription factors and extrinsic signaling through cytokines, growth factors or cell-cell contact; all of this with the goal of maintaining blood cell homeostasis within the organism.

7.1.2. Regulation of hematopoiesis by transcription factors

Transcription factors act in a coordinated fashion, executing specific programs of gene expression over time to define cellular states throughout hematopoiesis. This may take the form of so-called master regulators instructing a lineage decision during development or more complex networks made up of many factors that can integrate various signals in order to decide the fate of multi-potent cells. As well, regardless of cell type, it is now well documented that integrity of transcriptional regulation is essential to maintaining hematopoietic homeostasis.

There are a number of examples of transcription factors associated with specific hematopoietic lineages. For instance the transcription factors PAX5 and EBF

are expressed exclusively in the B-cell lineage and activate each other's expression to form a feed-forward loop promoting B-cell development [10, 11]. Another excellent case of lineage specific transcriptional regulation is the control of globin gene expression in developing erythrocytes, which requires the erythroid transcription factor EKLF for up-regulation of beta globin genes and cellular maintenance [12, 13]. These regulatory events do not occur in isolation though, but are rather downstream of earlier transcription factors and signaling cascades. In the case of B cell development, PAX5 and EBF are both induced together with other B-Cell factors by the more widely expressed E2A proteins [13]. Similarly EKLF expression is dependent on binding by GATA-1 centered complexes [14, 15]. Moreover, aberrant expression patterns of EKLF are associated with hematopoietic disorders including leukemia [16]. As such it is clear that although the timing and integrity of terminal lineage specific transcription is crucial in proper hematopoiesis, it is only a part of a much larger developmental program.

More complex transcription factor interactions are found at the branch points of the hematopoietic hierarchy. In the case of the divide between myeloid and erythrocyte-megakaryocyte lineages the ratio of expression between the transcription factors PU.1 and GATA-1 controls cell fate. These two proteins cross regulate each other's expression, activating their own promoters and inhibiting the other's. Thus based on the stochastic state of the expression of these factors within a given cell one will eventually become dominant with PU.1 directing myeloid development and GATA-1 promoting megakaryocyte-erythrocyte development [17-

19]. PU.1 is also involved in a similar type of regulatory switch with the transcription factor Gfi1 in the separation of B-cells from myeloid progenitors in early hematopoiesis. The mechanism in this case involves repression of *PU.1* by Gfi1, with high *PU.1* expression generating myeloid cells and its repression leading to B-Cell development [20]. These types of molecular switches are in fact responsible for the majority of decisions during hematopoietic development and allow progenitors to integrate various signals, which are reflected in the concentrations of core lineage switch factors.

Although these two-protein switches provided an elegant mechanism for determining cell fate during development, it is important to consider that they typically lie downstream of much larger transcriptional networks. For instance by analyzing the expression patterns of 11 hematopoietic transcription factors a group recently placed the GATA-1/PU.1 switch into a much larger network consisting of 28 regulatory interactions throughout hematopoiesis [21]. As well, recent studies capitalizing on advances in deep sequencing and chromatin Immunoprecipitation – so-called ChIP-seq experiments – have revealed far more complex interactions governing gene expression involving as many as seven core factors in one cellular context [22]. In addition to this, these studies have shown that transcriptional networks are far from linear, but rather that many factors may regulate a single node as in the case of transcriptional control of Gfi1 by various combinations of SCL/TAL1, GATA2, ERG, MEIS1, PU.1 and RUNX1 [23]. As such, although single gene

studies are still informative in investigating hematopoiesis the larger regulatory context must be considered as well.

The study of hematopoietic transcription factors is also further complicated by the now well-documented fact that many factors play different roles and institute different patterns of gene expression depending on the cell type or environment in which they are expressed. SCL/TAL1 for instance is a core hematopoietic transcription factor that has been demonstrated to have an almost entirely unique set of target genes in transformed T cells compared to normal erythrocytes [24]. Such changes may be due to the differential availability of binding sites and co-factors between cell types [25]. As well, external factors can impact transcription factor function. In the case of Foxo3a activity in erythrocytes its effect on target genes varies dramatically depending on whether the cell has encountered erythropoietin, which initiates a cascade of events converting Foxo3a into a transcriptional activator as opposed to a repressor ultimately promoting development instead of inhibiting it [26]. Similarly, a recent study making use of high-throughput single-cell expression analysis to study various hematopoietic subsets showed that cross regulation of the transcription factors Gfi1 and Gfi1b is dramatically altered by the expression of Gata2 in the cell, ultimately affecting lineage decisions [7]. Taken together these results highlight the importance of context in studying a transcription factor, particularly given that most hematopoietic transcription factors have now been shown to participate in regulatory networks across multiple cell lineages [27].

From these discoveries it also followed that, although the conventional model of hematopoietic regulation used to hold that differentiating cells would acquire the expression of lineage specific factors that would lock in a particular cell fate, in fact lineage and cell fate is at least to some extent primed in very early progenitors that express factors belonging to multiple lineages [27]. Early studies identified some "promiscuity" in the expression of lineage specific factors in early progenitors, however it was unclear what the functional importance of this observation was [28]. Later the concept of priming in MPPs was introduced, explaining the gene expression patterns observed in LMPPs as the result of downregulation of megakaryocyte-erythrocyte factors rather than the gain of myeloid and lymphoid gene expression [29]. It was not until recently though, that so-called "anticipatory binding" by lineage associated groups of transcription factors was observed upstream in hematopoietic development [27]. This adds an additional layer of complexity to hematopoietic transcriptional regulation in which the pattern of genes associated with a lineage or cellular state are dynamically controlled throughout all lineages.

7.1.3. Properties of Hematopoietic Stem Cells

Hematopoietic stem cells (HSCs) are the initial progenitors of all hematopoiesis and are required for its maintenance throughout the life of an organism. They are defined functionally by this capacity to give rise to all the

lineages of blood and immune cells as well as the ability to self-renew themselves and to repopulate the hematopoietic system after bone marrow transplantation. In addition to this HSCs are typically very long lived compared to other hematopoietic cells and undergo cell division very infrequently, instead residing mainly in a quiescent state. These so-called dormant HSCs primarily reside in a protected niche within the bone marrow adjacent to osteoblast cells [30]. When HSCs become active however they typically relocalize to an alternate perivascular niche where they interact with endothelial cells and are exposed to a different set of growth factors [30]. It has recently been described that HSCs can actually cycle back and forth between active and dormant states depending the homeostatic needs within the bone marrow [6]. Generally speaking though, HSCs are limited to finite set of fates during any hematopoietic event: they can remain quiescent within the osteoblastic niche; they can undergo apoptosis; or they can become active, leave the niche and divide producing either two daughter HSCs, termed symmetric self-renewal division, one HSC and one differentiation committed MPP, termed asymmetric division, or two daughter MPPs termed symmetric commitment division [31]. Maintaining the correct balance between these cell fates both under normal and stress conditions is central to hematopoietic homeostasis.

Hematopoietic stem cells represent a unique regulatory challenge compared to more mature cells because they must maintain their stem cell properties as well as the potential for all lineage decisions. On a transcriptional level a large number of factors are known to be required for the self-renewal/maintenance of HSCs

including Gfi1, GATA-2, BMI1 (a member of the poly-comb repressive complex), PU.1, MYB and many others [32-36]. In a properly functioning HSC all of these factors work in concert, and as a part of a larger network, to maintain self-renewal [27]. However, HSCs must also be able to respond to hematopoietic need in the event of injury or inflammation and accordingly are regulated by external factors on top of the underlying transcriptional program [37]. Cytokines such as SCF and TPO are essential external stimuli for the maintenance of HSCs and accordingly loss of expression of their receptors also abolishes HSC self-renewal [38-40]. Similarly WNT ligands also play an essential role in HSC survival, with the strength of WNT signaling being associated with the balance of self-renewal and differentiation [41, 42]. Other core development pathways are also active in HSC regulation including Sonic Hedgehog and NOTCH and similarly exposure to these ligands can have dramatic effects on HSC survival and proliferation [43] [44]. These findings indicate that HSCs are highly dependent on external regulation in order to maintain the complex internal network responsible for self-renewal and differentiation decisions.

The protected environment of the bone marrow niche is itself a key regulator of HSCs in that it controls exposure to these various growth factors. Located far from the blood vessel, the HSC niche is removed from the majority of diffusible factors in the blood stream. As well niche cells themselves are a source of specific growth factors. For example osteoblasts in the niche may produce Notch ligands and drive HSC expansion [45]. Niche cells also express a number of regulatory factors on their surface in membrane bound forms including SCF, osteopontin and angiopoietin-1

[46-48]. An equally important regulatory component of the niche though is its control of oxygen homeostasis. HSCs within the osteoblastic niche have low levels of reactive oxygen species (ROS) under normal circumstances and are protected against oxidative stress [49]. This is crucial for maintaining them in a quiescent state, preventing DNA damage and prolonging their lifespan [50, 51]. Interestingly, at lower, non-pathological levels, ROS also acts as a signaling molecule in HSCs in response to various growth factors further underscoring the importance of tightly regulating oxidative stress [52]. Given these varied and essential roles the bone marrow niche plays in HSC biology it is no surprise that mice with osteoblastic defects also display profoundly disturbed hematopoiesis [53].

For experimental purposes HSCs have historically been defined phenotypically by expression of surface markers using flow cytometry. One of the earlier markers of bone marrow progenitors was the combined surface expression of C-Kit (CD117) and SCA-1 and the absence of any mature lineage markers. These cells, termed LSKs, are a heterogeneous population and contain approximately 3-4% HSCs as determined by functional assay[54]. As well no cells from outside this population display any HSC functionality. In addition to the LSK signature, HSCs were also defined in the past based on the exclusion of the DNA dye Hoechst, which is rapidly removed by transport molecules primarily expressed on HSCs [55]. Currently though, the highest purity of HSCs isolated from mouse bone marrow has been obtained by further subdividing the LSK population based on the expression CD150 and the absence of both CD48 and CD34 which provides approximately 50%

functional HSCs [4]. Therefore it is important to consider in any experimentation that a single FACS defined HSC does not necessarily correspond to a functional one.

7.1.4. Ex-Vivo expansion and Bone marrow transplantation

Bone marrow transplantation is an intensive therapy for the treatment of a number of hematopoietic malignancies involving the ablation and replacement of a patient's hematopoietic system, however these procedures are often contingent on obtaining sufficient material, specifically hematopoietic stem cells from a matched donor, for reconstituting the patients blood and immune system. As such techniques for the rapid expansion of HSCs for clinical use are of great interest, particularly when working with limited cell populations like those obtained from umbilical cord blood. In any such expansion procedure though, it is critical to maintain the integrity of the hematopoietic stem cells within the prospective graft including both the ability to engraft permanently in the bone marrow and the ability to produce all required hematopoietic progeny.

At the moment there are two main methods for obtaining HSCs for bone marrow transplantation: harvesting from the blood of a donor and purification from frozen umbilical cord blood. Although HSCs typically reside within the bone marrow and enter circulation at a very low frequency injection with mobilizing agents, such as G-CSF, can be used to cause the egress of HSCs into the blood of the donor for collection [56]. This type of procedure is, however, contingent on finding a suitable

donor-patient match which may limit the usage of transplant. For this reason the banking of umbilical cord blood has become an area of increasing interest; if a patient's cord blood has been viably stored, then there is no need to find a matching donor and far fewer complications occur with transplantation [57]. Furthermore once a bank has been established cord blood is readily available for use immediately and can be pre-screened for HLA-type compatibility [58]. Cord blood transplants are also subject to technical limitations though, mainly in the availability and viability of material [57]. It is for this reason that the *ex-vivo* expansion of HSCs from cord blood for transplantation holds so much promise.

A number of factors have now been shown to expand HSCs *ex-vivo*, however progression to clinical trials has been slow due to potential developmental biases or other permanent effects on the expanded HSCs. Initial studies made use of stromal layers to support *ex-vivo* growth, which although effective is undesirable for clinical application due to the potential for contaminating cells in grafts [59]. More recently a number of molecular strategies have been developed using liquid cultures instead of stromal co-culture setups. One of the earliest and best characterized HSC expansion techniques is the over-expression of the transcription factor HOXB4 – either by retroviral transduction or using cell permeable HOXB4 peptides – which expands HSCs by approximately 40-fold in two week cultures [60-62]. Unfortunately though, expansion with HOXB4 appears to significantly limit the myeloid and lymphoid developmental capacity of HSCs, more or less precluding its use in the clinic [63]. Other strategies using small molecules or growth factors, such as aryl-

hydrocarbon receptor antagonists and pleiotrophin, in combination with cytokine treatments have shown promise in recent studies, producing large expansions of HSCs without apparent lineage bias in xenograft models however they have yet to be translated to the clinic [64, 65]. Similarly Prostaglandin E2, fibroblast growth factor 1, Angiopoietin-like 5 and insulin-like growth factor binding protein 2 can all contribute to HSC expansion, but only in combination with a number of other growth factors, which has posed financial and technical barriers to translation [66, 67]. At this point early clinical trials are in progress for only a few techniques including fixed Notch ligands and copper chelating molecules [68, 69]. It remains to be seen, however, if these protocols will be amenable to large-scale clinical translation.

7.2. Growth Factor Independence 1b (Gfi1b)

7.2.1. The Gfi1 Protein Family

Growth factor independence 1 (Gfi1) was discovered in 1993 in a retroviral insertion-activation screen for IL-2 independent growth and shortly thereafter the highly related Gfi1b protein was identified by homology-based search [70-73]. Both Gfi1 and Gfi1b are transcription factors – each having 6 C-terminal C_2H_2 type zinc fingers which mediate both DNA binding and interactions with other proteins – and have primarily been implicated in transcriptional repression. As well the two proteins share a conserved SNAG domain at the N terminus which is also found in Snail, Slug, Scratch and the Insulinoma-associated transcription factors 1 and 2 [74].

Although highly similar in these respects, the two proteins are differentiated by unique intermediate domains that are implicated in the formation of specific protein complexes. This three domain "SNAG-mid-zinc finger" structure is generally conserved between homologs and defines the Gfi1 family.

Proper functionality of Gfi1 family proteins is dependent on the integrity of all three domains. A single proline to alanine mutation at position 2, which lies within the SNAG domain, abrogates the majority of Gfi1 functions [71, 75]. Interestingly, knock-in mice carrying this mutation present a similar, but not identical phenotype to *Gfi1* Knockout mice suggesting that there is a subset of Gfi1 functions that are SNAG domain independent [75]. As well, a variant human GFI1 allele that expresses a protein with an amino acid exchange at position 36 (GFI136N) has been show to abrogate normal repression of many genes and is associated with acute myeloid leukemia [76, 77]. In addition to this, deletion of any of the six zinc fingers in a Gfi1 protein will lead to a loss of function although only zinc fingers 3,4 and 5 are required for DNA binding [78]. Taken together these results indicate that integrity of the entire protein is crucial for proper Gfi1 activity.

Gfi1s are not universally expressed, but rather are differentially expressed in a specific set of tissues including intestinal epithelium, certain nervous tissues and the immune system. Moreover, experiments using mice in which one allele of either *Gfi1* or *Gfi1b* has been replaced with GFP have shown expression of the two proteins to be highly cell-type specific within a given tissue. In the case of the immune system

Gfi1 is expressed primarily in differentiated granulocytes, activated macrophages, GMPs and HSCs as well as various lymphoid cells. Gfi1b on the other hand is expressed in megakaryocytes, erythrocytes, MEPs, B cell progenitors and HSCs [79]. In the intestinal epithelium, much like the immune system, Gfi1 and Gfi1b are expressed in distinct cellular subsets with Gfi1 being present in the secretory lineage while Gfi1b expression is restricted to brush cells [80, 81]. Gfi1 is also expressed in the inner ear hair cells of the adult mouse and associated neurons as well as in various PNS sensory cells during development[82]. These diverse, but not ubiquitous, patterns of protein expression suggest that Gfi1 and Gfi1b are involved in regulating complex programs of genes which define cellular state and that their function is mediated by cell type specific co-factors.

Complete knockouts have been generated for both *Gfi1* and *Gfi1b* and these experiments also revealed specific and independent roles for the two proteins. Germ-line deletion of *Gfi1* in mice leads to severe neutropenia in offspring and accordingly an increased risk of infection [83, 84]. These mice also display hyperactive inflammatory responses from their macrophages and accordingly are more susceptible to LPS induced septic shock than wild-type animals [84, 85]. Loss of Gfi1 also leads to defects in the lymphoid compartment including developmental defects in both the T and B cell lineages [86, 87]. Mice lacking Gfi1 also display neurological defects. In particular they fail to develop inner ear hair cells, which leads to ataxia, deafness, and balance issues [82]. *Gfi1b* deletion on the other hand is embryonic lethal at day 14.5 probably due to a developmental failure in red blood

cell development [88], although this has not ben established unequivocally yet. Interestingly, knock in of *Gfi1b* into the *Gfi1* locus restores the majority of hematopoietic defects in *Gfi1* knockout mice but fails to rescue the neurological phenotype, presumably due to an interaction specific to the intermediate domain which differs between the two paralogues [75]. Thus there appear to be three crucial components to the function Gfi1 family proteins: specificity of protein-protein interactions, timing or level of expression and structural integrity of the protein.

7.2.2. Mechanisms of Gfi1b Action and Regulation

Gfi1b regulates transcription via a set of mechanisms that are generally conserved between it and Gfi1 and mainly consist of the recruitment of specific regulatory protein complexes to target genes resulting in histone modification [89, 90]. This is accomplished, at least in part, by the recognition of a specific DNA target sequence (with the core being "AATC") that is common between Gfi1 and Gfi1b [71, 78]. The most well characterized interaction partner of Gfi1b is Lysine Specific Demethylase 1 (LSD1) which docks onto the SNAG domain of Gfi1b and recruits the REST corepressor (CoREST) [90]. Recruitment of both LSD1 and CoREST by Gfi1b leads to the repression of target genes via demethylation of histones at the H3K4 [91, 92]. ChIP-on-chip experiments demonstrated that LSD1 is recruited to approximately 80% of Gfi1b binding sites and that the ternary Gfi1b-LSD1-CoREST complex is present at 50% of Gfi1b sites indicating that this is a major functional

interaction of Gfi1b [90]. In accordance with this, knock down of LSD1 leads to the upregulation of Gfi1b target genes and an associated increase in H3K4 methylation [90]. It follows from this that although LSD1 plays a large cellular role independent of its interaction with Gfi1b [93, 94] it is a critical mediator of Gfi1b repressive function.

In addition to H3K4 demethylation, Gfi1b induced repression is also associated with histone deacetylation and H3K9 methylation via interaction with HDAC1/2 and the histone methyl transferase G9A respectively [89, 90]. As would be expected then, the loss of Gfi1b leads to increased acetylation and decreased H3K9 methylation at target genes and a general active chromatin state [89, 95]. In addition to this the tethering of both Gfi1b – as well as Gfi1 – to the nuclear matrix via association with ETO has been shown to be essential for repression [96]. Thus it is likely that all of these mechanisms – histone acetylation, methylation and demethylation – act in concert and that Gfi1b is responsible for targeting general repressive machinery to a specific gene.

Given that when present in the cell Gfi1b will bind specific target genes and induce transcriptional repression, the regulation of Gfi1b expression itself is also crucial to the maintenance of normal cellular function. Control of the timing and dose of Gfi1b in a cell is achieved dynamically through a number of mechanisms including auto-regulation, cross-regulation with Gfi1, induction by external signals and feedback from other core transcription factors. For example erythrocyte specific

expression of Gfi1b, which is crucial to proper red blood cell development, is induced by the combination of GATA1 and NF-Y [97, 98]. As would be expected the forced suppression of Gfi1b during erythrocyte development also delays terminal differentiation [99]. Although the Gfi1b promoter remains active throughout erythropoiesis this upregulation is also attenuated by external feedback in the form of Erythropoietin [95, 100]. Thus by combining multiple regulatory mechanisms precise expression of Gfi1b is achieved ensuring proper development.

The converse of this is also true: if Gfi1b regulation is disrupted then various hematological disorders may result. For instance Gfi1b is overexpressed in CML, erythrocytic and megakaryocytic malignancies although whether this disregulation is causal for malignancy remains open for debate [2, 101]. As well forced expression of Gfi1b blocks both normal and malignant T-cell differentiation and activation [102, 103]. This type of perturbation also occurs naturally through promoter mutations affecting Gfi1b expression. In particular mutations in GATA-1 and Oct-1 binding sites in the *Gfi1b* promoter have been identified in human patients with various types of acute leukemia [104]. These mutations lead to a loss of DNA binding by GATA-1 and Oct-1 and subsequently a respective decrease or increase of Gfi1b expression. These transcription factors though, don't act independently but are rather linked together in larger regulatory networks [105, 106].

A crucial and rather unique component of the Gfi1b regulatory network is the role that it and Gfi1 play through auto and cross-regulation. Gfi1b has been

described to directly repress expression of both itself and Gfi1 in-vitro and in-vivo, however this function is cell type specific implying that repression is not an isolated event but rather part of the overall cellular transcriptional state [107]. Indeed, at the minimum this regulatory loop is mediated by interaction with GATA-1 although presumably there are more factors involved [97]. Furthermore, formaldehyde-assisted isolation of regulatory elements and DNase1 hypersensitivity assays revealed a greater complexity of autoregulation to due to Gfi1b binding at multiple sites in it's own promoter in order to dynamically repress itself during development through the recruitment of various complexes [108].

Gfi1b function is also regulated through alternative splicing of mRNA and specific variants have been associated with normal and malignant hematopoietic development [2, 109]. The shorter splice variant of *Gfi1b* was reported in patients with CML more than 10 years after the protein itself was first described, but was not detected in healthy controls. This variant is missing exon 9, resulting in the reorganization of the c-terminal zinc fingers to form four rather than the usual six [2]. Importantly, *in vitro* this shorter variant of Gfi1b is also able to bind DNA and repress the *Gfi1b* promoter in a reporter assay [2]. Although its specific function in CML is unknown this shorter isoform, termed Gfi1b-p32, was later shown to have an essential role in erythrocyte differentiation and to associate with co-factors with greater efficiency than the more common p37 isoform [109].

As described above, there are numerous facets to the regulation of Gfi1b and in turn its own regulatory function on target genes. Although these may include interaction with other transcription factors, auto-regulation, alternative splicing or external signaling depending on the cellular context it is evident that regardless of the dominant mechanism it is the maintenance of precise timing and levels of Gfi1b expression that is crucial for cellular development and function.

6.2.3 Roles of Gfi1b throughout Hematopoiesis

The differential expression of Gfi1b in various hematopoietic subsets corresponds to a set of cell-type specific roles for the protein, which have been identified using various deletion models. Peak Gfi1b expression is found in the megakaryocyte and erythrocyte lineages as well as in hematopoietic stem cells and it is also expressed at lower levels in early B cell lineage cells [1, 79]. As discussed germline deletion of *Gfi1b* is embryonic lethal but in accordance with the adult expression patterns these embryos show a profound defect in both the erythrocyte and megakaryocyte compartments [88]. In addition to this, by conditionally deleting *Gfi1b* in adult mice it is now also known that Gfi1b plays a crucial role in hematopoietic stem cell homeostasis and in the maintenance of specific B-cell functions [110-112].

Both Gfi1 and Gfi1b are expressed in hematopoietic stem cells, but interestingly mice display quite different phenotypes in the HSC compartment when

the two proteins are knocked out. Conditional deletion of *Gfi1b*, which is expressed at higher levels HSCs than Gfi1, leads to an expansion of functional HSCs that maintain their long-term hematopoietic reconstituting capacity [1]. *Gfi1* knockout HSCs on the other hand undergo increased apoptosis and accordingly fail at bone marrow reconstitution [32, 113, 114]. This indicates that despite some compensatory behavior, Gfi1 and Gfi1b are not fully redundant in HSC homeostasis. Interestingly it has also been shown that Gfi1b is down-regulated in HSCs upon commitment to differentiation while Gfi1 expression is maintained which has lead to the hypothesis that the two transcription factors function as a switch between HSC differentiation and self-renewal [1].

The first cell type specific role described for Gfi1b was in the development of megakaryocytes and erythrocytes, the two cell types in which it is most highly expressed [79, 88]. Although complete deletion of *Gfi1b* is embryonic lethal, analysis of these embryos revealed that both erythrocyte and megakaryocyte development fail in the absence of Gfi1b presumably leading to the death of the embryo [88]. In the case of erythrocytes the converse is also true: forced expression of Gfi1b in hematopoietic progenitors results in a significant expansion of immature erythroblasts [115]. The specific functions of Gfi1b in erythropoiesis are not fully elucidated, however it is known that it mediates the erythropoietin response in progenitor cells and that it regulates the core erythrocyte transcription factor BCL-xl [99, 116]. In addition to this Gfi1b is highly expressed in the joint megakaryocyte-erythrocyte progenitors, or MEPs. At this phase in development Gfi1b is thought to

help instruct the cell fate decision of these progenitors, at least in part through the regulation of TGF-beta signaling [117].

Although Gfi1b is expressed at lower levels within the B lymphocyte lineage compared to the megakaryocyte and erythrocyte lineages it is still required for proper functionality in at least two ways. In the developing B-cell there is a critical molecular switch in the responsiveness to Il-7 during the pro-B to pre-B cell transition. The presence of Gfi1b has been shown to enhance the responsiveness of pro-B cells to Il-7 and is thought to counterbalance SOCS-1 mediated inhibition of Il-7 signaling [112]. Thus the balance between Gfi1b and SOCS-1 may regulate the pro to pre-B cell transition and in accordance with this hypothesis Gfi1b is downregulated in late B-cell development [79]. In addition to this developmental role, Gfi1b is also required in mature B-cells to negatively regulate expression of the core B-cell receptor rearrangement factor RAG. It was shown that Gfi1b binds to the promoters of both RAG itself and the transcriptional activator FoxO1, repressing them [111]. This repression of RAG is crucial to proper cell function, for *Gfi1b* knockout B-cells have increased RAG expression and subsequently increased DNA damage and cell cycle defects as well [111].

7.3. BCR-ABL fusion protein driven malignancies

7.3.1. Chronic Myeloid Leukemia

Chronic Myeloid Leukemia (CML) is a malignancy thought to arise from oncogenic transformation of hematopoietic stem cells. The disease generally presents as an excess of granulocytes as well as erythrocytes and platelets in the blood and granulocyte infiltration of the bone marrow. Without treatment a patient will progress in 3 to 5 years from chronic myeloid cell hyperplasia to a blast crisis similar in appearance to many acute leukemias [118]. Although CML was once a fatal disease the therapies available have improved greatly in the last 20 years, particularly with the advent of the specific BCR-ABL kinase inhibitor imatinib, and the majority of patients now achieve cytological remission. Despite these advances though, a complete cure for CML remains elusive as many patients relapse into disease upon withdrawal of treatment or development of drug resistance.

The initial molecular insult leading to CML is typically a reciprocal translocation between chromosomes 9 and 22 producing a BCR-ABL fusion kinase. The fusion protein formed may be of varying sizes, but typically the p210 translocation product is associated with CML, while the more potent p190 form is found primarily in B-ALL [119]. BCR-ABL is constitutively active and drives the activation of a wide variety of oncogenes including Phosphatidylinositol-3 kinase, MYC and RAS [120-122]. Accordingly, cells transformed with BCR-ABL are able to grow independently of cytokines and are protected against apoptosis [123, 124]. As

well mice that transgenically express BCR-ABL succumb to acute leukemia shortly after birth, further highlighting the potent oncogenicity of the fusion kinase [125]. Thus it is not surprising that the major breakthrough in the treatment of CML was the discovery of the drug imatinib, which specifically targets the ATP binding of BCR-ABL and inhibits kinase activity [126, 127].

Although CML was initially described as a clonal disease, in recent years a leukemia stem cell (LSC) population has been identified that is thought to underlie relapse and drug resistance in CML patients. LSCs have a similar surface phenotype to hematopoietic precursors – $Lin^{-}Sca1^{+}C\text{-}kit^{+}$ in mice and $CD34^{+}CD38^{-}$ in humans – and similarly are absolutely required to transfer disease between congenic animals [128-130]. One of the key features of these LSCs in CML is that they function independently of BCR-ABL catalytic activity and thus are not eliminated by kinase inhibitor therapies [128, 131]. As well LSCs proliferate at a very low rate, which may offer further protection against chemotherapy [132]. Because of this resistance to conventional therapies a great deal of research has gone into dissecting the molecular pathways that maintain LSCs in the hopes of developing targeted therapies. Similar to HSCs, LSCs have a complex network of factors involved in their regulation. To date factors know to be required for LSC maintenance in mice include beta-Catenin, Sonic Hedgehog, FOXO and HIF-1-alpha amongst others [129, 133-135]. Unfortunately though, despite the fact that ablation of these factors in mice selectively kills LSCs none of these findings has yet translated into a viable therapy to combat CML relapse in the clinic.

7.3.2. B-cell Acute lymphoblastic leukemia

B-cell Acute lymphoblastic leukemia (B-ALL) refers to a heterogeneous group of malignancies that arise clonally from varied B-cell precursors. Accordingly, B-ALLs have different prognoses and progressions depending on the molecular subtype driving the malignancy. The BCR-ABL fusion kinase is responsible for approximately 5% of pediatric B-ALL and almost 25% of adult cases [136]. Despite the success of BCR-ABL kinase inhibitors in improving outcome though, this particular subtype of B-ALL is still associated with poor prognosis overall [137, 138].

Although driven by similar hyperactive BCR-ABL kinases these Philadelphia chromosome$^+$ (Ph^+) B-ALLs differ from CML on a molecular level, which has impacted the development of therapeutics. Ph^+ B-ALL for instance is dependent on the SRC family kinases Lyn, HCK and FGR while CML is not, perhaps providing a unique drug target in these tumors [139]. Interestingly, Vav3 was also recently demonstrated to be a viable therapeutic target specifically in Ph^+ B-ALL [140]. Typically though, BCR-ABL expressing B-ALL is associated with a worse prognosis than CML primarily because the fusion kinase tends to be associated with a loss of the transcription factors Ikaros and PAX5 which renders the tumors resistant to kinase inhibitors, promotes the acquisition of B-Cell lineage markers and enforces

the more aggressive phenotype [141]. As such there is still a definitive need for novel therapies for the treatment of these tumors.

7.3.3. Therapeutic Strategies and Challenges

The progression of treatments available for CML patients from crude chemotherapy and bone marrow transplantation to highly targeted molecular therapies is commonly viewed as a triumph of modern drug design. Initially a patient presenting with CML was treated with Bisulphan or hydroxeaurea to limited effect and would eventually require a complete bone marrow transplant, carrying significant risk [142]. In the 1980s though interferon-alpha was approved for trial in the treatment of CML and eventually showed significantly improved response over conventional chemotherapy [143, 144]. Unfortunately interferon alpha treatment has severe side effects throughout the body and thus a failure of patient compliance with treatment regimens – one study showed as many as 87% of patients withdrawing from therapy within two years – limited the practical use of interferon for CML patients [145, 146]. The more significant breakthrough in CML treatment came with the discovery of targeted BCR-ABL inhibitors. The first of these drugs, imatinib, was developed in the late 1990's and quickly progressed through clinical trials to become the standard front line treatment for CML, with up to 90% of patients achieving complete cytological remission after initial treatment [127, 147]. Imatinib works by blocking the binding domain of BCR-ABL in a highly specific fashion and inhibiting kinase function. Unfortunately though frequent mutations in

this domain lead to the development of resistance in a large proportion of patients [148]. As such a second generation of specific kinase inhibitors targeting other domains of BCR-ABL were developed including Dasatinib and Nilotinib. Both of these drugs show improved efficacy over imatinib as a first line treatment and achieved some responsiveness in imatinib resistant cases [149]. Despite the breakthrough of BCR-ABL kinase inhibitors though, both resistance and relapse are still serious problems in the treatment of CML and Ph^+ B-ALL. This is most likely due to the resistant leukemia stem cell population, which is not eradicated by conventional therapy. As such, if a complete cure for CML is to be developed more research is required into pathways that can selectively target these resistant cells and work in conjunction with kinase inhibitors to eliminate disease.

7.4 Gfi1b in Hematopoietic malignancies

7.4.1. Over-expression of Gfi1b in CML and B-ALL

Gfi1b is located at chromosomal position 9q34 and is therefore carried along with *Abl1* in the reciprocal 9:22 translocation that results in the Philadelphia chromosome causative for most CMLs and a subset of B-ALLs [2]. This translocation then leads to an approximately 20-fold increase in the expression of Gfi1b in Ph+ patients compared to healthy controls [2]. Given that Gfi1b is known to play anti-apoptotic role in many contexts [82, 115] it is likely that this over-expression is not passive, but rather helps to drive leukemia progression. As well a hyperactive form of Gfi1b, which is also found in normal erythrocyte development, is preferentially

expressed on both CML and B-ALL also suggesting a functional role for Gfi1b in leukemogenesis [2, 109]. Interestingly Gfi1b is further upregulated in CML patients who have been treated with the BCR-ABL kinase inhibitor imatinib, which was suggested to be part of a compensatory mechanism leading to BCR-ABL independent tumors [2]. All of this implies that Gfi1b is functionally active CML and may contribute to drug resistance or disease relapse.

In accordance with this hypothesis a recent paper demonstrated that siRNA mediated inhibition of Gfi1b *in-vitro* reduced the viability of CML cells [150]. This study also demonstrated an additive effect when inhibiting Gfi1b together with BCR-ABL suggesting the possibility of targeting Gfi1b in conjunction with conventional kinase inhibitor therapies in the clinic [150]. It remains to be seen however, if this finding will translate into *in-vivo* models of disease.

7.4.2. Over-expression of Gfi1b in Megakaryocytic and Erythrocytic malignancies

In additional to its described role in CML and B-ALL, Gfi1b has also been implicated *in vitro* in tumors deriving from the megakaryocyte and erythrocyte lineages. In both primary patient samples from erythrocytic and megakaryocytic leukemias as well as corresponding immortalized cell lines Gfi1b was found to be significantly over-expressed compared to healthy patient samples and other types of leukemia [101]. In accordance with a functional role in these tumors, *in-vitro* knock-down of Gfi1b in HEL, K563 and NB4 cells induces apoptosis and reduces the rate of

proliferation [101]. As well, gene profiling of K562 cells in which Gfi1b has been knocked-down revealed at least indirect regulation of a number of genes involved in oncogenesis including MYC, p21 and several pathways required for normal immune function and chemokine signaling [151]. Given this, it is highly likely that Gfi1b contributes to the pathogenesis of these tumors.

7.5 Gfi1b in Hematopoietic Stem Cells

7.5.1 Conditional deletion of Gfi1b expands HSCs in adult mice

Flow cytometric studies on *Gfi1b:GFP* knock-in mice revealed that Gfi1b is highly expressed in HSCs and is downregulated more than 10 fold upon commitment to differentiation and progression to the MPP1 phase of development [1, 79]. In addition to this Gfi1b had previously been implicated in HSC regulatory networks, although only at the level of DNA binding [152]. These observations, together with the known role of its cross-regulatory homologue Gfi1 in HSC homeostasis, implied an important function of Gfi1b in HSC biology.

As discussed above, germline deletion of *Gfi1b* is embryonic lethal and so conditional deletion is a valuable tool for studying its function. By crossing *Gfi1b*$^{flox/flox}$ mice onto an inducible Mx-Cre background hematopoietic cell specific deletion of G*fi1b* can be obtained in adult mice by serial injection of pIpC to activate the Cre-recombinase [1]. These conditionally *Gfi1b* deleted mice show a dramatic expansion of HSCs and LSKs in the bone marrow of approximately 30-fold and 3-

fold respectively. As well Gfi1b ablation leads to an even greater expansion of HSCs in the periphery, with almost 100 times as many HSCs present in the blood and spleen, indicating activation and mobilization of these cells in addition to expansion [1]. This expansion was also confirmed by limiting dilution assay, which showed approximately a 6-fold increase in HSC functionality. Importantly, HSCs lacking Gfi1b are still able to give rise to all lineages upon transplantation with the exception of platelets, which fail due to a downstream defect in the megakaryocyte lineage [1, 88]. This implies that HSCs lacking Gfi1b expand without compromising their "stemness" or reconstituting capacity, making Gfi1b an ideal target for inducing the *ex-vivo* expansion of HSCs for clinical use in transplantation.

7.5.2. Characteristics of Gfi1b KO HSCs

HSCs that have lost Gfi1b present a unique phenotype that combines increased cycling and activation with maintenance of self-renewal and overall "stemness". Typically HSCs that are hyperactive due to some genetic perturbation will eventually exhaust themselves resulting in failure of the hematopoietic system. *Gfi1b*$^{-/-}$ HSCs on the other hand retain their long-term reconstitution ability while expanding numerically [1]. As might be expected given the expansion observed, HSCs lacking Gfi1b cycle faster than control HSCs, notably though they also undergo apoptosis at an increased rate indicating some loss of cellular integrity. Given that fidelity of DNA repair is required for HSC maintenance and that the highly related Gfi1 protein was recently described to mediate the DNA damage response in certain

cell types [153, 154] the partial loss of integrity in Gfi1b null HSCs is not surprising, although it is clearly compensated for by factors driving expansion. In addition to this *Gfi1b* knockout HSCs exhibit significantly increased levels of ROS further suggesting that some homeostatic mechanisms are perturbed in these cells. Of significant interest though is the finding that treatment with the anti-oxidant NAC blocks the expansion of HSCs in response to conditional *Gfi1b* deletion suggesting that increased ROS is in fact causative for the phenotype observed [1]. In an additional attempt to reveal the mechanism underlying the HSC expansion observed in *Gfi1b*$^{-/-}$ mice expression arrays were performed comparing deleted and control HSCs. This analysis revealed disregulation of a large set of genes involved in HSC function including various surface markers that regulate niche interactions such as integrins, Vcam1, P-Selectin and Cxcr4 which were proposed to contribute to the break in oxygen homeostasis in these cells [1]. Although some normal processes are disturbed in *Gfi1b* deleted HSCs, they are of particular interest because they retain their self-renewal and differentiation potential despite this perturbation.

8. Materials and Methods

HSC purification:

Primary murine hematopoietic stem cells were purified from the marrow of the femur, tibia, hip and humerus by FACS. To do so, bones were isolated and flushed with PBS containing 2.5% FCS and 1% Penicillin/Streptomycin. Subsequently red blood cells were lysed for 7 minutes in ammonium chloride buffer and bone marrow was resuspended in PBS with FCS and Pen/Strep. Cells were then stained with antibodies for Sca1, C-Kit, CD150, CD48 and mature lineage markers (Gr1, Mac1, Ter119, B220, CD19, CD3, CD4, CD8, Nk1.1 and Il7R-alpha, Flt3) and HSCs – defined as Lin^-, $Sca1^+$, $C\text{-}kit^+$, $cd150^+$, $cd48^-$ – were sorted using a MoFLO cytometer from Beckman Coulter Inc. Primary antibodies were incubated for 15 minutes before washing and secondary fluorescent Streptavidin was incubated for 10 minutes before washing. All centrifugation of primary bone marrow for washing steps or otherwise was done at 1100 RPM and 4^oC for 5 minutes.

Conditional deletion of Gfi1b using the mx-cre system:

Gfi1b flox, Mx-Cre transgenic mice were obtained as previously described [1]. Briefly, Cre activation and subsequent excision of Gfi1b were achieved by intra-peritoneal injection of 200 µL of 2mg/ml pIpC (sigma P1530) every other day for 10 days. 30 days were allowed after initial injection for interferon to clear the system before analysis of animals.

Conditional deletion of Gfi1b using the ROSA-cre-ERT2 system:

Gfi1b flox, *Rosa-Cre* transgenic mice were injected intra-peritoneally with 10 mg/kg body weight tamoxifen on two successive days to induce cre activation and recombination.

Cell culture:

K562 cells (ATCC) were cultured in IMDM supplemented with 10% FCS and 1% Penicillin/Streptomycin. Cells were maintained between 10^5 and 10^6 cells/ml.

For culture of primary bone marrow and hematopoietic stem cells in progenitor expansion or maintenance experiments cells were cultured in StemSpan SFEM (Stem Cell Technologies) supplemented with 100 ng/ml SCF and 20 ng/ml TPO.

For myeloid differentiation of primary cells, cultures were done in IMDM supplemented with 10% FCS, 1% Penicillin/Streptomycin, 100 ng/ml SCF, 50 ng/ml Flt3-L, 10 ng/ml Il-3, and 10 ng/ml Il-6.

For all primary cell cultures the outer-most wells of the tissue culture plate used were filled with PBS to prevent differential evaporation between wells from impacting experiments.

Flow cytometry assay for symmetric division:

200 sorted HSCs were plated in triplicate in 96 well u-bottom plates in 200uL of StemSpan SFEM medium supplemented with 100 ng/ml SCF and 20 ng/ml TPO. At 3 day timepoints half of each culture was taken for FACS and

cultures were refreshed with 100 uL of complete medium. Cells were stained directly with antibodies for cd48, c-kit and mature lineage markers (CD4, CD8, Gr1, Mac1, B220) and analyzed using a LSR cytometer from BD.

Colony forming assays:

Cells were plated in 1.5 ml of semi-solid methylcellulose (M3434, Stem Cell technologies) in a $4cm^2$ dish and cultured at 37 degrees for 7 days. After culture acquisition was performed on an axiovert S100TV microscope using Matlab. Colonies were scored according to the guidelines provided by Stem Cell Technologies. Briefly, a colony was defined as at least 50 cells proximally centered on a single point or clear network of points. It is also crucial that any scored colony contains a significantly higher percentage of cells than the background. Erythrocytic colonies were identified based on their red colour. B cell colonies were identified by the regular circular shape of the colony as well as the nucleus to cytoplasm ratio of the cells. Granulocyte and Macrophage colonies were identified by tinted and large cytoplasmic areas respectively as well as the irregular shape of the colony and apparent motility of the cells.

Bone marrow transplantation:

For bone marrow transplantation experiments antibiotic treatment of recipient mice was commenced four days prior to transplantation. Six to 24 hours before transplantation recipients were lethally irradiated (9.5 Gy). Transplantations were performed via tail vein injection. Antibiotic treatment

was continued for 10 days after transplantation. A minimum of 500 000 viable bone marrow cells were transplanted into recipients.

Retroviral production:

All retroviral stocks were prepared by transfecting the PlatE cell line and collecting the supernatant [155]. PlatE cells were cultured in DMEM supplemented with 10% FCS, 1 % Penicillin/Streptomycin, 1% Sodium Pyruvate and 0.1% β-Mercapto ethanol. One day prior to viral production PlatE cells were plated so as to be approximately 80% confluent the following day. 2 hours prior to transfection media was replaced with Glutamax (GIbco) supplemented with 10% FCS and 1% Penicillin/Streptomycin. Transfections were performed with Purefection reagent (System Biosciences) according to the manufacturers specifications and at a ration of 40 ug of DNA to 80 ul of perfection reagent per 25 cm^2 plate of cells. The morning following transfection media was replaced with PlatE media as described above, supplemented with 10 mM Sodium Butyrate and incubated for 6-8 hours. After incubation media was replaced with PlatE media supplemented with 10mM HEPES. Supernatant was collected at 24-36 hours and concentrated using a vivaspin-12 column. Retroviral stocks were stored at -80 ^oC if not used fresh.

Generation of model B-ALL:

For BCR-ABL driven malignancy bone marrow was harvested in sterile PBS and red blood cells were lysed. Remaining cells were then transduced with a retrovirus carrying the BCR-ABL p210 fusion protein and

a separate GFP marker using retronectin to increase efficiency (Takara). To do this, retronectin was coated onto an untreated 24-well tissue culture plate at room temperature for 2 hours according to the manufacturers specifications. After coating, plates were blocked for 30 minutes with 2% BSA in sterile PBS and then washed once with sterile PBS. PBS was then aspirated and approximately 1 ml of concentrated virus was added to coated wells and centrifuged for at least 90 minutes at 4000 RPM and 4 ^{o}C. Virus was then aspirated, leaving approximately 150 uL behind in the well and 1 million bone marrow cells were plated into the wells in 1 ml of StemSpan SFEM (Stem Cells technology) supplemented with 50 ng/ml Il-7 and 50 ng/ml Flt3-L. Plates were then centrifuged for 10 minutes at 1500 RPM and 20 ^{o}C. After spin-down cells were cultured in a 37^{o}C incubator overnight. The following day transduced cells were transplanted into lethally irradiated recipient mice as described above together with 500 000 fresh carrier bone marrow cells.

Disease progression was monitored by checking for the presence of GFP positive cells in the blood. All animals were euthanized when moribund in accordance with institutional animal facility protocols. Disease was confirmed post-mortem by flow cytometry with antibodies target B220 and CD19 as well as by blood analysis performed on an advia analyzer (Seimans).

Transwell co-culture assays:

Co-culture experiments were performed using a 24-well plate transwell nsert (Falcon) with a 3 um pore to allow the passage of diffusible

factors but not cells. 200 000 fresh wild type bone marrow cells were plated in a 24 well plate in 500 µl of IMDM supplemented with 10% FCS, 1% Penicillin/streptomycin, 100ng/ml SCF and 20 ng/ml TPO. The transwell insert was then placed in the wells and 500 000 Gfi1b$^{-/-}$ bone marrow cells or control cells were plated into the insert in 500 ul of the same media. Media was refreshed as needed and cultures were split at 3 day timepoints for analysis or continued culture.

In-vitro knockout of Gfi1b in primary murine bone marrow:

Bone marrow from *Gfi1b flox, mx-Cre* mice or control mice was purified as described above and plated in 1 ml of StemSpan SFEM (Stem Cells Technologies) supplemented with 100ng/ml SCF and 20 ng/ml TPO as well as 1 µl interferon alpha to activate Cre recombination. Deletion was assessed using genotyping PCR specific for Gfi1b flox and knockout alleles.

9. Results:

9.1. Gfi1b regulates symmetric division of HSCs

It has previously been reported that conditional deletion of Growth Factor Independence 1b (*Gfi1b*) in adult mice using the Mx-Cre system leads to a dramatic expansion of hematopoietic stem cells in the bone marrow and periphery of 30 and 100-fold respectively [1]. Using the same protocol of serial injection of pIpC to induce Cre activation and subsequent excision of floxed *Gfi1b* alleles I was able to replicate this result convincingly (data not shown). This model, however, is dependent on the systemic production of interferon-alpha in response to pIpC injection [156] and notably it has been demonstrated that interferon-alpha treatment leads to an activation and loss of quiescence in murine HSCs [157]. Although in all experiments involving Mx-Cre mice 30 days were allowed for animals to recover from interferon treatment before analysis and control mice also received pIpC injection it was still of interest to replicate this finding in a non-inflammatory model.

To perform this experiment *Gfi1b*$^{flox/flox}$ mice were crossed onto the tamoxifen inducible *ROSA-Cre-ERT2* background [158]. Deletion was achieved by injecting tamoxifen intra-peritoneally into 8-12 week old mice as described above. Although incomplete excision of the *Gfi1b* alleles was obtained in the bone marrow, a proportional expansion was still observed one month after treatment in both the LSK and HSC compartments of 4.5 and 22-fold respectively **Fig. 1A**. Additionally this

system allowed for analysis at earlier time points because, unlike the Mx-Cre model, there is no required clearance time after Cre activation. As expected, time course experiments revealed a gradual expansion of HSCs after Gfi1b ablation, which increased over time to a plateau of approximately a 40-fold over controls **Fig 1b**.

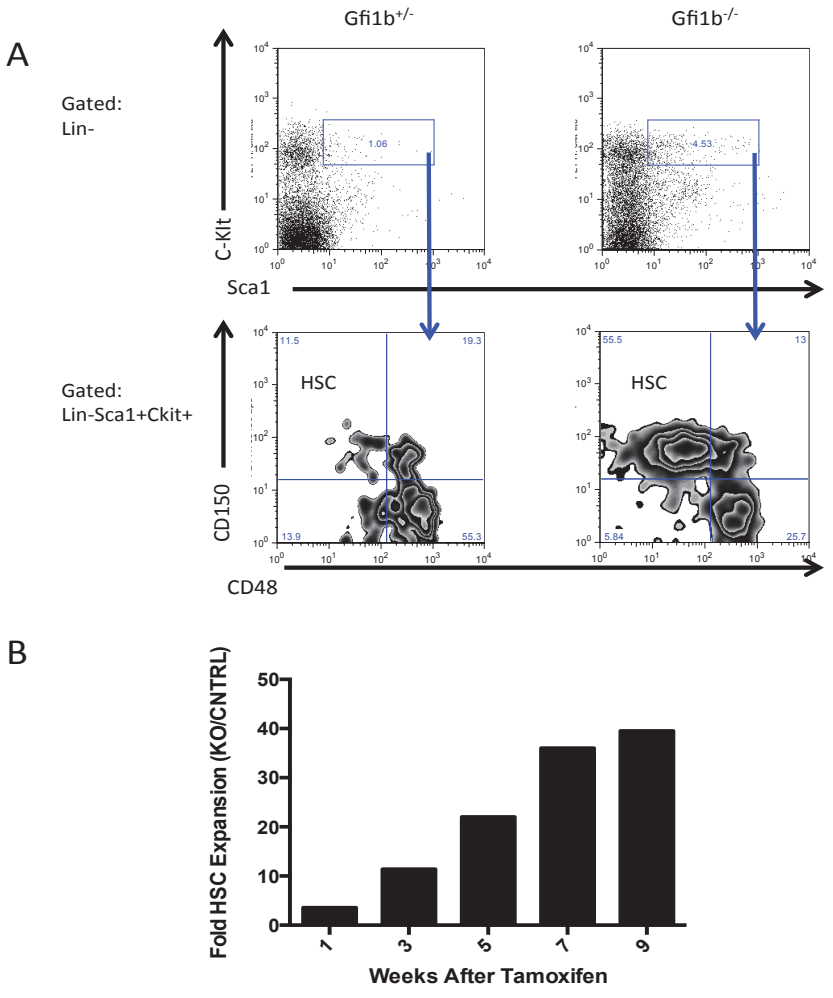


Figure 1: Conditional deletion of Gfi1b in adult mice using the tamoxifen inducible ROSA-Cre system expands hematopoietic Progenitors. A) Representative FACS plots showing bone marrow from Gfi1b$^{-/-}$ or control mice stained for HSCs 1 month after tamoxifen treatment. Both LSKs and HSCs (LSK, CD150^{+}, CD48^{-}) have expanded. B) Fold expansion of HSCs in ROSA-Cre, *Gfi1b*$^{flox./flox}$ mice versus control ROSA-Cre, *Gfi1b*$^{flox./wt}$ mice over time after tamoxifen injection.

We next hypothesized that one of the mechanisms contributing to the expansion of HSCs in *Gfi1b* conditional knockout mice was a shift in the ratio of self-renewal and differentiation during HSC division towards symmetric self-renewal type divisions. To assess this, HSCs ($Lin^{-}Sca1^{+}C\text{-}kit^{+}CD48^{-}CD150^{+}$) were FACS sorted from either *Gfi1b* knockout mice or control mice in which only a single allele had been deleted and cultured *ex-vivo* in conditions known to support the maintenance of stemness in hematopoietic progenitors. These cells were allowed to divide and then checked for the upregulation of CD48 indicating the transition of an HSC to a multi-potent progenitor (MPP) that has committed to terminal differentiation and lost its long-term self-renewal capacity **Fig 2A**. As expected cultures of $Gfi1b^{-/-}$ HSCs displayed significantly higher proportions of CD48 negative cells as early as 3 days *ex-vivo*, or after approximately two cell divisions per HSC, with 90% remaining uncommitted compared to 75% in controls **Fig 2B and 2C**. This difference became even more pronounced after longer culture periods. After 6 days only 42% of cells in control cultures were CD48 negative whereas knockout cultures averaged 83% consistent with an intrinsic effect in the $Gfi1b^{-/-}$ HSCs **Fig 2B and 2C**. As well, *Gfi1b* knockout HSCs appeared to proliferate in culture at a slightly increased rate compared to controls, just as they do in-vivo (data not shown). Taken together, these results implied that loss of Gfi1b shifts the balance of cell divisions in HSCs towards symmetric self-renewal without compromising their ability to proliferate.

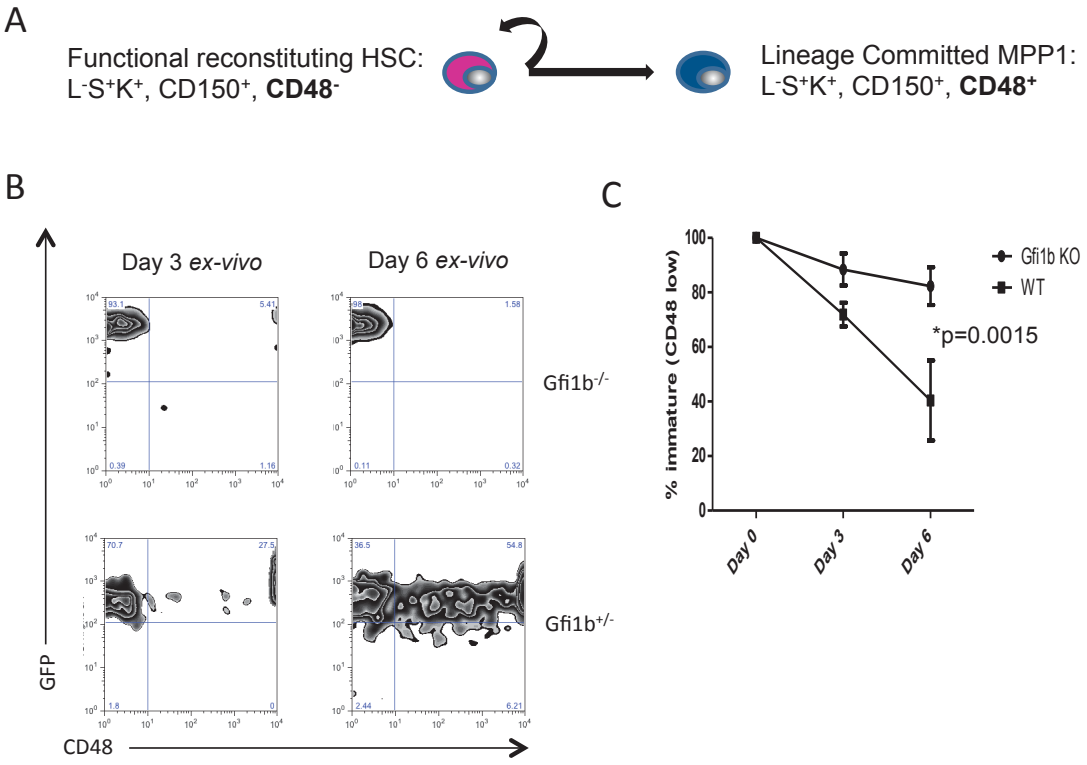


Figure 2: Loss of Gfi1b increases the proportion of symmetric self-renewal in HSCs cultured *ex-vivo* as determined by tracking the surface marker CD48 which indicates commitment to differentiation and transition of an HSC to an MPP.
A) Schematic of markers defining HSCs and MPPs. During a cell division event an HSC may either produce two HSCs, two MPPs or one of each cell type. The production of the $CD48^-$ HSCs is termed symmetric self-renewal division. B) FACS plots for both*Gfi1b*$^{-/-}$ and *Gfi1b*$^{-/+}$ cultures indicating the scheme for identifying $CD48^-$ HSCs after culture. GFP Indicates a viable hematopoietic cell that has not terminally differentiated. C) *Gfi1b*$^{-/-}$ cultures retain a significantly larger proportion of cells as uncommitted, self renewing HSCs as defined by CD48 expression.

9.2. Gfi1b is required for normal hematopoietic differentiation in vitro

Given that HSCs lacking Gfi1b appeared to produce committed progenitors at a lower frequency during cell division, I then investigated whether there would be a concordant decrease in their capacity to produce terminally differentiated cells as well. To test this, sorted HSCs were cultured *ex-vivo* under conditions that promote the development of myeloid cells. After 9 days of differentiation upwards of 80% of control HSCs had upregulated the myeloid lineage markers Gr1 and Mac1, however the majority of cells in *Gfi1b* deleted cultures failed to differentiate, with fewer than 40% expressing Gr1 or Mac1 **Fig 3A**. Since this block in differentiation is not complete and the overall cellularity of cultures was the same in Gfi1b null cultures and controls it supports the idea that HSCs in which *Gfi1b* has been deleted are still able to stochastically differentiate into committed progenitors, but preferentially self-renew.

Additionally *Gfi1b*$^{-/-}$ myeloid differentiation cultures exhibited replating capacity in methylcellulose colony forming assays, which is indicative of progenitors remaining in the cultures, whereas control cultures were exhausted after the first plating **Fig 3B**. As well upon initial plating *Gfi1b* knockout cultures showed a two-fold increase in the number of colonies produced further supporting a tendency towards undifferentiated cells in Gfi1b ablated cultures **Fig 3B.**

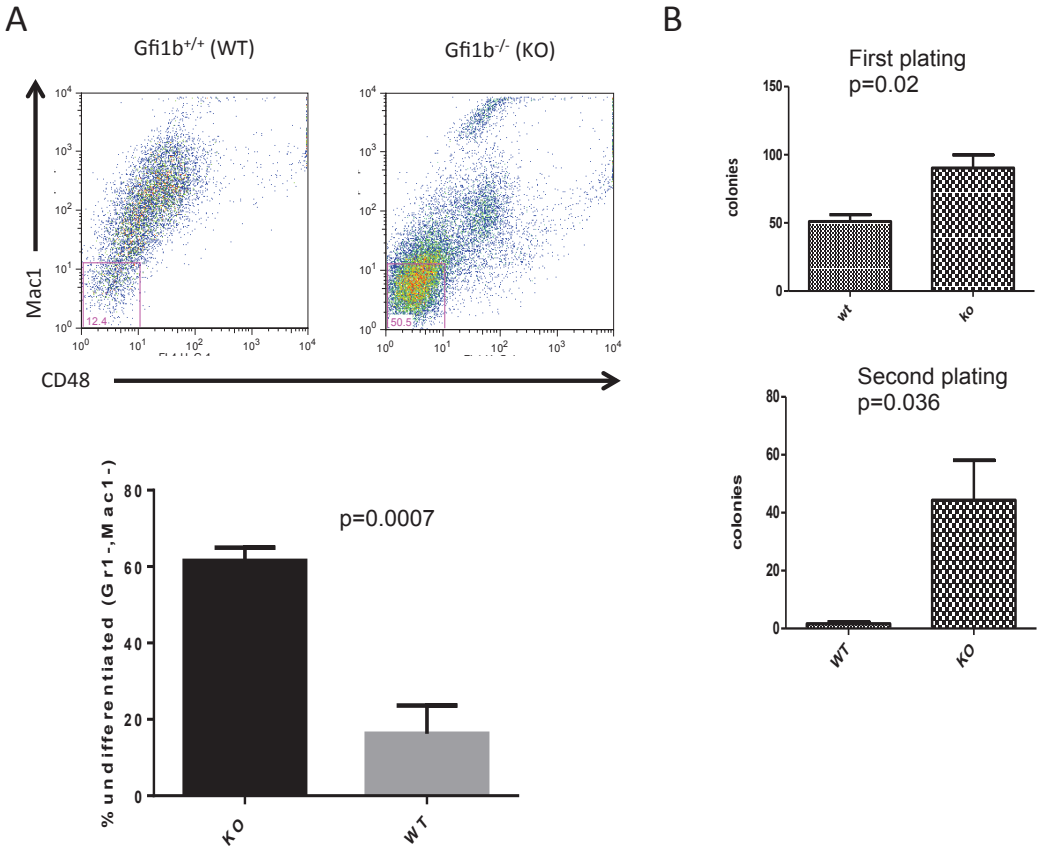


Figure 3: After 9 days in culture sorted HSCs from *Gfi1b*$^{-/-}$ mice retain a large proportion of cells in an undifferentiated state compared to wild-type HSCs when undergoing *in-vitro* myeloid differentiation. A) Representative FACS plots and quantifications of undifferentiated cells from KO and WT cultures. The majority of WT cells upregulate the myeloid markers Gr1 and Mac1 while KO cells remain primarily undifferentiated. B) Colony counts from semi-solid methylcellulose cultures of KO and WT myeloid differentiation cultures. *Gfi1b*$^{-/-}$ cells have increased colony forming ability after myeloid differentiation upon initial plating in methylcellulose and retain colony forming capacity upon replating whereas WT cells are exhausted. This indicates that only *Gfi1b*$^{-/-}$ cultures retain functional progenitors.

9.3 Gfi1b regulates paracrine bone marrow interactions

The bone marrow stem cell niche is a highly complex environment and one way in which it is governed is through diffusible regulatory factors that signal between cells. In order to assess whether deletion of *Gfi1b* affected this component of hematopoietic regulation bone marrow from G*fi1b* knockout or control mice was co-cultured with wild-type bone marrow using a transwell system that allowed diffusible factors to pass between the two cell populations but prevented physical interaction **Fig 4A**. Strikingly, bone marrow co-cultured with *Gfi1b*$^{-/-}$ bone marrow displayed a 3-fold growth advantage over 6 day cultures **Fig 4B**. As well an expansion of phenotypic hematopoietic progenitors by a factor of 44 was observed in bone marrow co-cultured with Gfi1b null HSCs compared to controls **Fig 4C and 4D**. Cells co-cultured with *Gfi1b*$^{-/-}$ bone marrow also exhibited methylcellulose replating capacity after control co-culture cells had been exhausted, supporting a concordant functional expansion of progenitors **Fig 4E**. Additionally, in order to ensure all effects were due to a diffusible factor, wild-type bone marrow was genotyped for genetic markers unique to *Gfi1b*$^{-/-}$ and control bone marrow in order to demonstrate that no contamination from cells across the transwell insert occurred (data not shown).

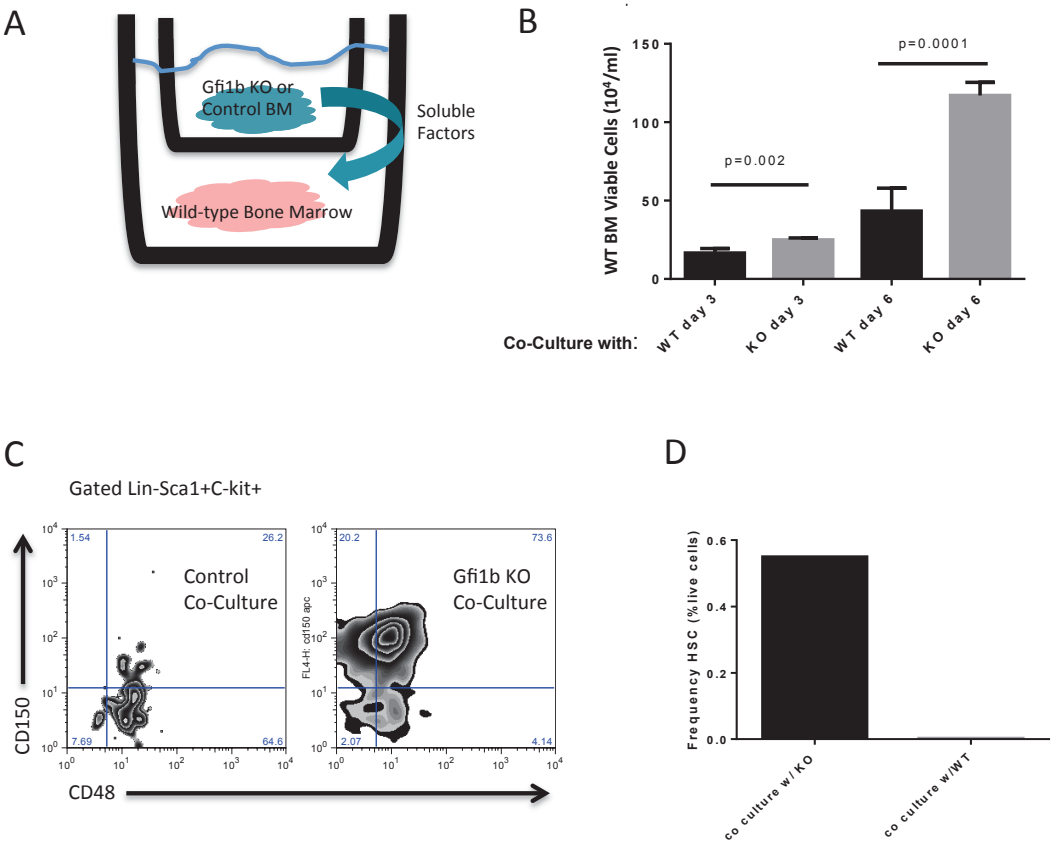


Figure 4: Gfi1b regulates paracrine bone marrow interactions. A) Schematic of transwell cultures setup. Soluble factors pass between bone marrow populations, but direct contact is blocked. B) Trypan blue viable cell counts of bone marrow co-cultured with Gf1ib KO or control bone marrow. Co-culture with KO bone marrow provides a significant growth advantage. C) Representative FACS plots of HSC stainings in wild-type bone marrow from KO and control co-cultures after 6 days D) Quantification of HSC frequency as determined by FACS in in wild-type bone marrow from KO and control co-cultures after 6 days E) Colony forming assay. Counts shown are from 3rd platings of wild-type bone marrow from KO and control co-cultures. Retention of colony forming capacity indicates functional expansion of progenitors.

9.4. Gfi1b is a target for ex-vivo expansion of HSCs

We next investigated whether deletion of *Gfi1b ex-vivo* produced the same HSC expansion phenotype observed with the *in-vivo* conditional deletion models. Bone marrow from *Mx-Cre tg, Gfi1b*$^{flox/flox}$ or *Mx-Cre tg, Gfi1b*$^{wt/flox}$ mice was purified and put into culture under conditions that support the survival of hematopoietic progenitors. Interferon-alpha was added to these cultures to induce Cre activation and excision of the floxed *Gfi1b* alleles. Although an incomplete deletion was obtained with this methodology **Fig 5A** there was still a 18-fold expansion of HSCs in the *Gfi1b*$^{flox/flox}$ bone marrow compared to the control culture in just six days as determined by surface marker expression **Fig 5B and C**. Thus ablation of Gfi1b is sufficient to expand HSCs independently of an intact bone marrow environment.

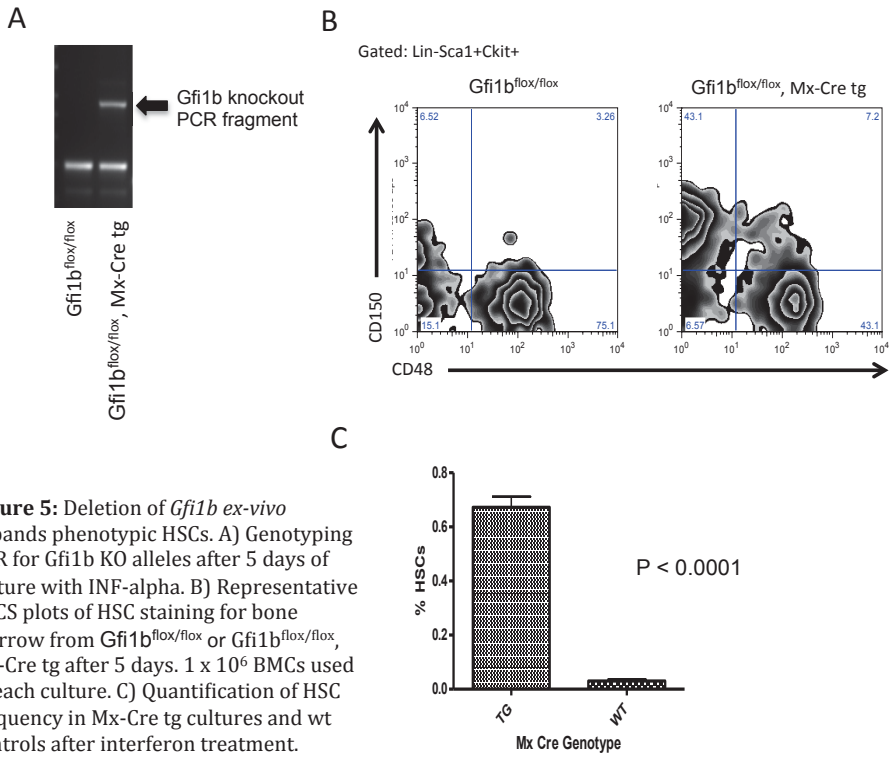


Figure 5: Deletion of *Gfi1b ex-vivo* expands phenotypic HSCs. A) Genotyping PCR for Gfi1b KO alleles after 5 days of culture with INF-alpha. B) Representative FACS plots of HSC staining for bone marrow from Gfi1b$^{flox/flox}$ or Gfi1b$^{flox/flox}$, Mx-Cre tg after 5 days. 1 x 10^6 BMCs used in each culture. C) Quantification of HSC frequency in Mx-Cre tg cultures and wt controls after interferon treatment. Deletion of Gfi1b significantly expands phenotypic HSCs.

9.5. Gfi1b is a potential therapeutic target in BCR-ABL malignancies

BCR-ABL$^+$ B-cell acute lymphoblastic leukemia was generated in mice as described above, consistently producing tumors positive for the B-cell markers B220 and CD19 **Fig 6A**. Previous work from our group had indicated that Gfi1b might play a role in the pathogenesis of BCR-ABL driven malignancies [2]. Indeed mice were observed to succumbed to model B-ALL that transgenically overexpressed Gfi1b significantly faster than to wild-type disease with a 50% survival of 35 days compared to 70 in two experiments **Fig 6B**. As well expression arrays comparing Gfi1b over-expressing tumors to wild type ones revealed the differential regulation of a number of factors involved in leukemogenesis **(Table 1).** Of particular interest, the CML associated tumor suppressor BLK was significantly downregulated in the more aggressive Gfi1b-transgenic tumors. Other tumor suppressors were also downregulated in these cells including Magi1 and Leukemia Inhibitory Factor. Not surprisingly, this was also accompanied by an upregulation of negative prognosis markers such as thymidine kinase and adenylate kinase.

Gene	Fold Change	Description
BLK	-6.16	Tumor Suppressor in CML stem cells
Magi1	-3.21	Tumor suppressor in many cancer including ALL
LIF	-2.54	Leukemia tumor suppressor
TK	+3.75	Negative prognosis marker
AK	+9.28	Negative prognosis marker

Table 1: Selected genes differentially expressed in model B-ALL tumors overexpressing Gfi1b. From NCBI accession GSE33709.

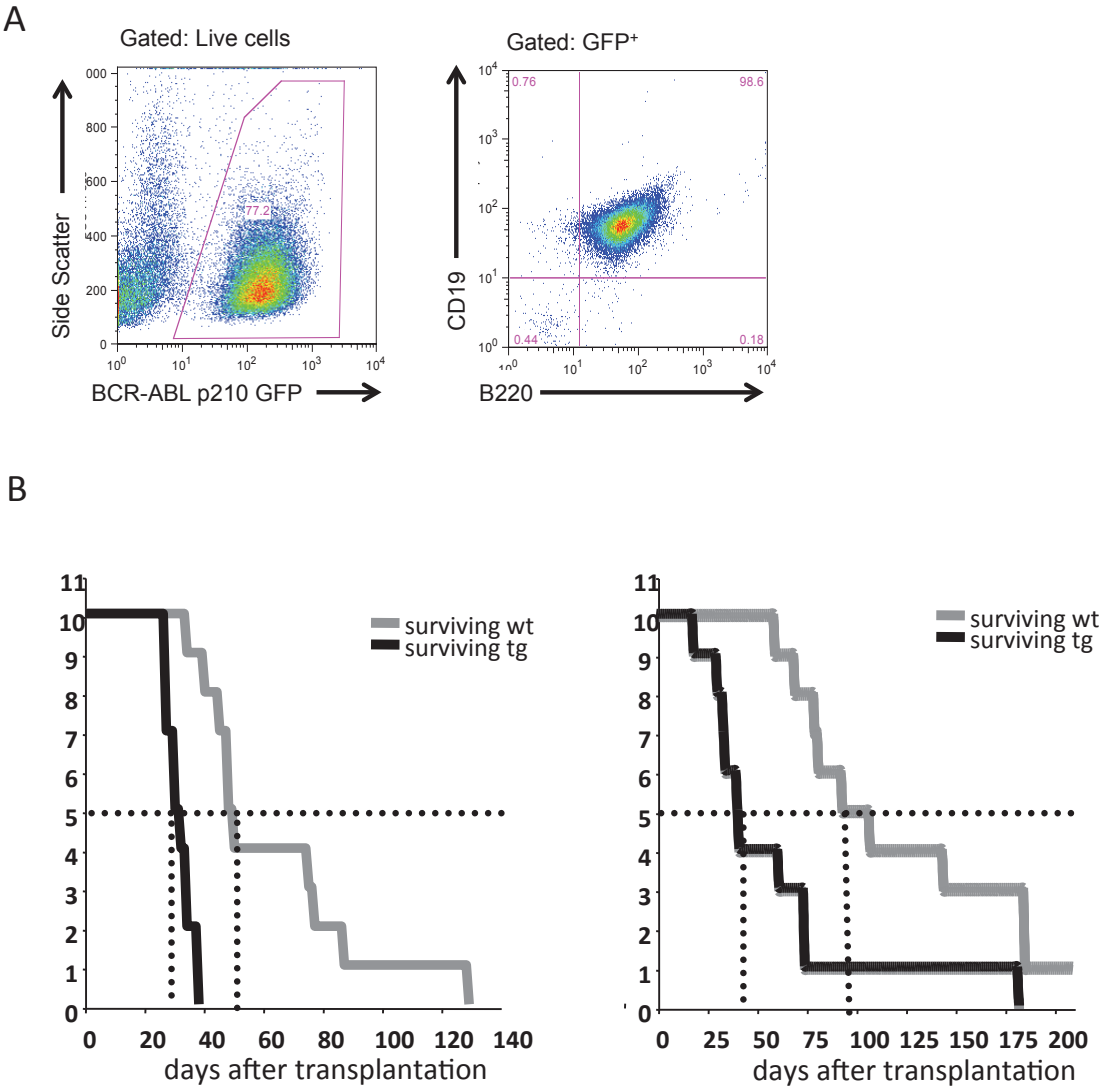
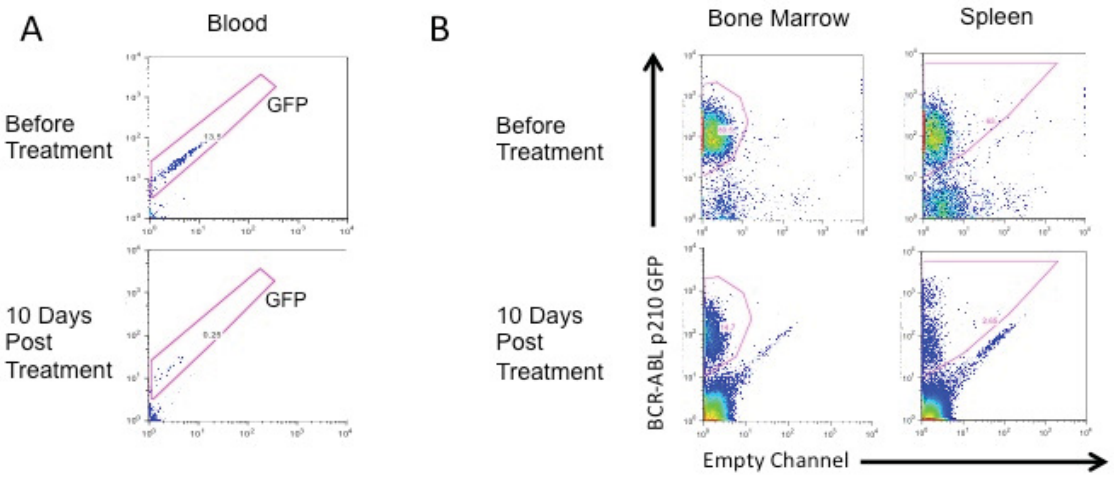


Figure 6: Transduction with BCR-ABL p210 oncogene induces more aggressive tumors when transgenically overexpressing Gfi1b. A) Representative FACS plots of tumor phenotypes. Tumors are GFP+ and express B220 and CD19. B) Survival curves of two independent experiments indicating that transgenic overexpression of Gfi1b accelerates morbidity in model B-ALL. These survival curves are the work of Lothar Vassen.

Experiments comparing the survival of mice with wild-type or Gfi1b ablated tumors are ongoing, however I have also observed that deletion of Gfi1b in an already established tumor appears to lead to remission of the disease. Mice with tumors bearing the *Mx-Cre* transgene and with floxed *Gfi1b* alleles were injected with pIpC once the tumor burden in the blood surpassed 10% BCR-ABL positive cells as determined by GFP expression. Within 10 days of injection there was a decrease in the proportion of BCR-ABL expressing cells in the blood by as much as 50-fold in some animals **Fig 7A**. Similarly the bone marrow and spleen of mice analyzed either the day of pIpC treatment or 10 days after showed a dramatic decrease in the tumor burden in both organs **Fig 7B**.

At this point no viable technique for inhibiting Gfi1b *in-vivo* has been developed, however given that the majority of Gfi1b functionality is dependent in interaction with LSD1 I posited that inhibiting it might have a similar effect in leukemic cells as Gfi1b knock down. Parnate (or transcyclopromine) is a generic LSD1 inhibitor approved for clinical use in a number of contexts. It was observed that treating the CML K52 cell line with parnate induced apoptosis proportionally to concentration *in-vitro* **Fig 7C**. Thus LSD1 inhibition offers a potential strategy for indirectly blocking the function of Gfi1b.



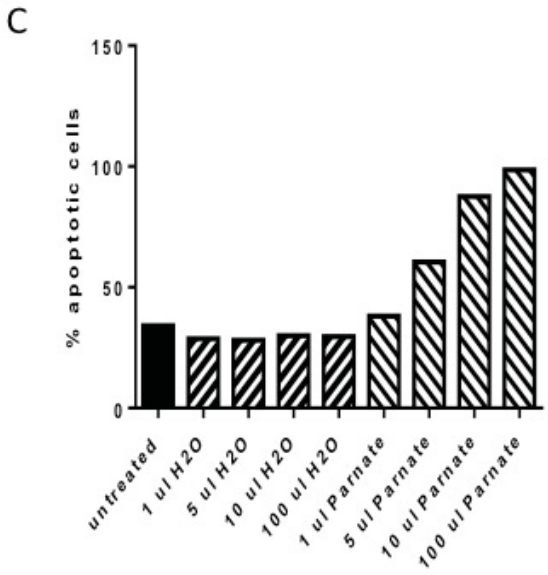


Figure 7: In preliminary studies Gfi1b and the Gfi1b co-factor LSD1 are required for tumor maintenance. A) Deletion of Gfi1b in an established tumor using the Mx-Cre system abolishes the majority of tumor cells in the blood after 10 days. B) Gfi1b deletion also reduces the tumor burden in the bone marrow and spleen. C) Treatment of the CML K562 cell line with the LSD1 inhibitor Parnate induces apoptosis proportionally to concentration as measured by Annexin V/ propidium iodide staining.

10. Discussion and Perspectives

Evidence that Gfi1b may restrict symmetric self-renewal division in HSCs

Throughout the life of a hematopoietic stem cell both intrinsic and extrinsic factors regulate the balance of quiescence and activation as well as the fate of the cell during division. The previous finding that conditional deletion of *Gfi1b* in adult mice leads to a dramatic expansion of HSCs in the bone marrow without compromising their functionality lead us to hypothesize that Gfi1b regulates the balance of self-renewal and differentiation in these cells. As well the fact that I was able to replicate this finding using a non-inflammatory, tamoxifen inducible, deletion system indicated that this in-vivo expansion of HSCs was specifically a result of the loss of Gfi1b providing further impetus to investigate the mechanism behind the expansion phenotype.

Tracking of CD48 upregulation on sorted HSCs cultured ex-vivo under conditions that promote progenitor maintenance showed significantly fewer differentiation-committed CD48+ cells in cultures of *Gfi1b*$^{-/-}$ HSCs compared to Gfi1b$^{+/-}$ controls. Importantly these cultures also showed a trend towards increased proliferation indicating that the difference in the ratio of CD48+ to CD48- cells between *Gfi1b* knockout and control cultures was not due to a failure to divide or an increase of quiescence in the HSCs lacking Gfi1b. Therefore, this finding suggests that the balance of cell fates is shifted towards symmetric self-renewal type division in *Gfi1b* knockout HSCs. As well, the fact that *Gfi1b*$^{-/-}$ HSCs display this property

when sorted out from the rest of the bone marrow and cultured without stromal support or other cell-cell contacts implies that the phenotype observed in *Gfi1b* conditional deletion mice is at least to some extent intrinsic to HSCs themselves and partially independent of bone marrow microenvironment interactions.

The idea that HSCs lacking Gfi1b preferentially undergo self-renewal during cell division events is also supported by the *ex-vivo* differentiation results. In these experiments both knockout and control cultures produced mature myeloid cells as defined by surface expression of Gr1 and Mac1, however the HSCs in which Gfi1b had been deleted also retained a large population of phenotypically undifferentiated cells. This is consistent with a decrease in the proportion of committed progenitors amongst the daughter cells of dividing *Gfi1b*$^{-/-}$ HSCs and accordingly colony-forming experiments indicate retention of progenitor cells the *Gfi1b* knockout myeloid differentiation cultures. It remains to be seen whether similar patterns of limited differentiation and increased progenitor retention occur during the *ex-vivo* development of other lineages, but significantly Gfi1b knockout mice are known to produce all cells of the myeloid and lymphoid lineages in competitive transplantation assays [1]. Given this it seems probable that the tendency towards undifferentiated cells during *ex-vivo* differentiation of Gfi1b null HSCs is indeed due to the production of fewer differentiation-committed daughter cells during division rather than an inherent inability to form mature myeloid cells. One can then hypothesize based on this that in the *in-vivo* deletion models the increased absolute

number of HSCs compensates for the decreased frequency of commitment explaining why those mice display normal levels of myeloid and lymphoid cells.

In the future it would be interesting to validate this finding of increased symmetric self-renewal in *Gfi1b*$^{-/-}$ HSCs using another experimental setup, for instance live tracking of Numb distribution or Notch expression, both validated methods of determining HSC daughter cell symmetry [31]. Another interesting experiment that should be performed is the single cell transplantation of HSCs cultured *ex-vivo*. This would provide a functional description of the ratio of self-renewal to commitment in *Gfi1b*$^{-/-}$ HSCs to accompany the phenotypic data described here. Lastly the CD48 tracking experiment developed in this project also provides an elegant system with which to rapidly test potential candidates for a rescue of the *Gfi1b* knock out phenotype.

Re-analysis of the HSC expression arrays generated by Khandanpour et al. when describing the initial conditional deletion phenotype for Gfi1b [1] has identified some candidate Gfi1b target genes that may contribute to the increased proportion of symmetric self-renewal in the knockout HSCs. Of particular interest two microRNA clusters, mir-302 and mir-24, are significantly upregulated in HSCs lacking Gfi1b. Mir-302 is a well-defined pluripotency factor and may contribute to HSC expansion in this capacity, while mir-24 is known to target p16 which in turn can regulate symmetry of HSCs amongst other processes [159-161]. Expression of p16 is also involved in the ROS response in HSCs and interestingly inhibition of ROS

partially rescues the *Gfi1b* knockout phenotype perhaps by shutting down ROS signaling pathways that are dysfunctional in the absence of p16 [162]. Another intriguing finding from the array data is that both the retinoic acid receptor and retinoic acid response genes are upregulated in *Gfi1b*$^{-/-}$ HSCs. Retinoic acid signaling has been directly implicated in regulating symmetry in other cell types and more generally it has been shown to expand hematopoietic progenitors and promote their self-renewal similarly to what is described in the Gfi1b conditional knockout mouse [163-165]. Although these are promising targets, it remains to be seen whether miRNA-blocking treatment or retinoic acid inhibitors can impact the phenotype of *Gfi1b* knockout animals and cells.

The model described here, in which Gfi1b represses a set of genes that upon activation following Gfi1b ablation will lead to an increase in symmetric self-renewal of HSCs, is dependent on the tacit assumption that the result of an HSC division is to some extent stochastic. There is a great deal of evidence that certain factors such as cell-cell contacts or availability of growth factors can instruct symmetric or asymmetric division in HSCs, but in all such cases – and in the case of Gfi1b ablation specifically – HSCs appear to retain some plasticity in terms of cell fate [31, 166]. Thus it seems that rather than enforcing a particular function, Gfi1b – and presumably other hematopoietic transcription factors – regulates the probability of an outcome given a specific input into the cell's regulatory network.

Inhibition of Gfi1b is a potential technique for ex-vivo expansion of HSCs

The development of a reliable method for the *ex-vivo* expansion of HSCs without compromising their functionality has been an active area of research for more than a decade. Despite this though, even the most successful strategies are still in early clinical trials and have posed problems scaling up to useful size [167]. Given the expansion of HSCs observed in *Gfi1b* conditional knockout mice and the fact that *Gfi1b* knockout HSCs maintain their long-term functionality, Gfi1b can be considered an ideal target for ex-vivo expansion.

Critically when Gfi1b deletion was induced *ex-vivo* in whole bone marrow the same expansion of HSCs as seen with the *in-vivo* conditional deletion models was observed. This suggests that the expansion phenotype will viably translate to the simplified culture systems used for clinical *ex-vivo* expansion. As well it is promising that deletion of *Gfi1b* selectively expands HSCs out of a heterogeneous population indicating that a Gfi1b-inhibition based expansion protocol would not require the enrichment of progenitors from the similarly heterogeneous cord blood population but could be applied directly reducing processing and cellular stresses involved.

In order to translate this finding to cells that have not been genetically manipulated *a priori*, in particular human HSCs, I have begun developing a number of techniques to inhibit Gfi1b function *ex-vivo*. Morpholino technology (from Genetools) proved too inefficient in my hands to mediate knock down of Gfi1b in

primary human cord blood (data not shown). As such I have moved towards an LNA based knock-down approach. Any such inhibitor would ideally selectively target Gfi1b and not the highly related Gfi1 protein, which is also expressed in HSCs. An alternative method to this RNAi based approach is to directly block the binding of Gfi1b to its co-factor LSD1. The Gfi1b-LSD1 interaction is dependent on LSD1 docking onto the SNAG domain and homologous interactions between LSD1 and SNAIL have been shown to be susceptible to competitive inhibition by peptides that mimic the SNAG domain [168]. Given this I designed peptides homologous to the Gfi1b SNAG domain in an attempt to specifically block Gfi1b function. This methodology though is dependent on cell permeability of the peptides and so TAT-fusion peptides will also be used as a positive control for uptake and as a potential means to deliver the SNAG peptide itself. It remains to be seen though, whether these strategies will provide sufficient inhibition of Gfi1b to induce the type of genetic reprograming observed in Cre-based conditional deletion models.

Gfi1b regulates paracrine bone marrow interactions

The significant growth advantage afforded by co-culture with *Gfi1b*$^{-/-}$ bone marrow suggests a previously unknown role of the protein in bone marrow homeostasis. Based on these experiments it is likely that Gfi1b normally represses, either directly or indirectly, the production of a growth factor (or set of factors) in hematopoietic cells in order to maintain normal growth and differentiation patterns. When Gfi1b is perturbed, a shift in paracrine regulatory signals leads to an

expansion of progenitor cells as well as an overall increase in bone marrow growth. Given that both of those characteristics are associated with oncogenicity it is not surprising that tight regulatory mechanisms would exist to control the expression of such a growth factor, although at this point no such tumor suppressor function for Gfi1b has been explicitly described. It is also interesting to consider whether this type of action might contribute to the application of Gfi1b inhibition for *ex-vivo* expansion of HSCs. If this is the case, then the specific growth factors controlled by Gfi1b could be used to promote HSC expansion without the broader genetic consequences of inhibiting a transcription factor like Gfi1b.

In order to identify specifically which secreted factor(s) are differentially expressed by *Gfi1b*$^{-/-}$ bone marrow chromatographic separation of conditioned media from primary bone marrow cultures should be performed, followed by mass spectrometry to identify individual active components of fractions. An early hypothesis, based on expression arrays comparing *Gfi1b*$^{-/-}$ HSCs with controls, is that the growth factor pleiotrophin is upregulated after Gfi1b ablation. Interestingly pleiotrophin was recently shown to expand HSCs *ex-vivo* and is the active component behind the success of co-culturing hematopoietic cells with human brain endothelial cells to support their growth [65, 169].

The bone marrow cells (BMCs) used in these experiments comprise a heterogeneous population and it should also be investigated whether one particular subset of BMCs is responsible for this finding. As such, transwell experiments should

be repeated with bone marrow that has been fractioned into purer components, such as lineage negative/positive or LSK cells. These experiments would reveal whether this particular regulatory mechanism is specific to one cell type or rather a general phenomenon throughout hematopoiesis. As well *Gfi1b*$^{-/-}$ bone marrow has a significantly increased proportion of progenitors within it and it is possible that this is causative for the differential net expression of growth factors in conditioned media rather than a result of it.

Gfi1b plays a role in BCR-ABL driven oncogenesis

The retroviral transduction of murine bone marrow with BCR-ABL p210 to model oncogenesis was first described by David Baltimore's group in 1990 [170]. Consistent with published protocols I generated BCR-ABL+ B-ALLs that expressed the surface markers CD19 and B220. The finding that transgenic overexpression of Gfi1b in these model tumors accelerates disease progression and limits survival suggests that the overexpression of Gfi1b previously described in patient samples [2] is a contributing component of malignancy rather than a passive variation. As such survival experiments comparing model B-ALL with and without Gfi1b should be completed in order to determine whether Gfi1b is required for the initiation of BCR-ABL driven tumorigenesis in addition to promoting it.

The experiments in which *Gfi1b* is deleted in an already established tumor suggest that Gfi1b will be a valid therapeutic target showed a promising reduction in

tumor burden. It is important to consider though, that excision of *Gfi1b* alleles was achieved using the interferon based Mx-Cre system and interferon itself is a well-established treatment for CML. Thus these experiments must be repeated in a non-inflammatory system such as the ROSA-Cre model described above and with proper controls. As well, given the success in my hands of LSD1 inhibitors for inducing apoptosis in CML cells *in-vitro* this strategy should also be translated to the *in-vivo* disease models.

In the case of CML it is also interesting to consider whether Gfi1b plays a specific role in the leukemia stem cell population. This seems likely based on both its HSC specific functions in normal hematopoiesis and the association between high Gfi1b expression and resistance to imatinib treatment, which in turn is thought to depend on LSCs [2]. It should be investigated whether Gfi1b is differentially expressed in these cells, as in normal HSCs, and what LSC specific effects deletion of Gfi1b has both in terms of survival and ability to reconstitute a tumor in a secondary recipient.

The mechanisms by which Gfi1b contributes to oncogenesis are not yet clear, however based on what is know about Gfi1b's functions in hematopoiesis a few interesting hypotheses have emerged. Array data comparing tumors transgenically overexpressing Gfi1b to wild type ones revealed the downregulation of a number of tumor suppressors by Gfi1b, which likely contributes to disease. As well, Gfi1b deletion leads to increased ROS levels in HSCs. If this effect translates to BCR-ABL

transformed cells it would be of clinical significance because ROS suppression is known to be crucial to LSC survival in CML [171-173]. Regardless of the mechanism though, Gfi1b appears to be a promising therapeutic target for BCR-ABL driven malignancies.

11. Conclusions and Summary:

In the study presented above I investigated a number of functionalities of the transcription factor Growth Factor Independence 1b (Gfi1b) in both normal and malignant cells. Although a great deal of work remains to be done before this protein is fully understood, I was able to show that Gfi1b appears to restrict the symmetric self renewal of dividing hematopoietic stem cells in order to maintain normal blood cell homeostasis. Concordantly, loss of Gfi1b in *ex-vivo* cultures lead to a reduced frequency of differentiated cells when HSCs are instructed to undergo myeloid lineage commitment. As well it was shown using co-culture experiments that Gfi1b controls paracrine regulatory factors in the bone marrow and that *Gfi1b*$^{-/-}$ bone marrow was able to expand co-cultured hematopoietic progenitors across a transwell insert and provided a general proliferative advantage. It is clear from this data then, that there are a number of mechanisms that contribute to the expansion of HSCs in conditional *Gfi1b* knockout mice including both intrinsic and extrinsic factors.

As well it was shown in this project that Gfi1b is a promising target for inhibition in order to expand HSCs *ex-vivo* for clinical use. I demonstrated that genetic ablation of *Gfi1b* was sufficient to selectively expand murine phenotypic HSCs from whole bone marrow cultured *ex-vivo*. As well the development of translational strategies including small peptide inhibitors and LNA based knock-down was initiated. Theoretically if these methodologies prove efficient enough at inhibiting Gfi1b they should be amenable for clinical application.

This work also indicated that Gfi1b has the potential for clinical translation in the treatment of CML and B-ALL. The finding that Gfi1b overexpression accelerated disease progression on model B-ALL combined with the preliminary data indicating it is required for tumor maintenance suggests that inhibition of Gfi1b is a driving component of these malignancies and a potential drug target.

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