Summer 2020

The Role of Chromatin Remodeling Protein and Transcriptional Repressor, Ing4, in Hematopoiesis Regulation

Melanie Rodriguez

The Role of Chromatin Remodeling Protein and Transcriptional Repressor, Ing4, in Hematopoiesis Regulation

by

Melanie Rodriguez

Bachelor of Science
University of Richmond, 2017

Submitted in Partial Fulfillment of the Requirements

For the Degree of Master of Science in

Biological Sciences

College of Arts and Sciences

University of South Carolina

2020

Accepted by:

Katie Kathrein, Director of Thesis

Alissa Richmond Armstrong, Reader

Maria Marjorette Peña, Reader

Cheryl L. Addy, Vice Provost and Dean of the Graduate School

ACKNOWLEDGEMENTS

There have been so many people who have lifted me up and supported my efforts through graduate school. I would like to first give a huge thank you to Dr. Katie Kathrein. I am so incredibly grateful to have been under your mentorship and scientific guidance the past two years. You have taught me more than you know, including skills that go beyond the lab, and for that I am thankful. You have built a lab culture that is inviting and encourages critical thinking and open discussion, a lab culture that not only allows us to learn from our mistakes but laugh at them. Thank you for always asking me, "why do you think that is" instead of just telling me. You always pushed me to come to my own conclusions, and this simple sentence has forced me to grow as a scientist. Thank you Dr. Kathrein.

You can't talk about the Kathrein lab and its atmosphere, without mentioning Zanshe´ Thompson and Seth Gabriel. Thank you for the afternoon coffee pick me ups and for always making the long lab days aka transplant days more bearable. I will always carry our laughs, science discussions, and life talks with me. It is difficult to imagine my two years in South Carolina without you.

I would also like to thank my committee members, Dr. Marj Peña, and Dr. Alissa Armstrong, for taking interest in my project and my success. I greatly appreciate your time and the questions that made me think more deeply about my research.

I am especially grateful for the PREP program and the directors, Dr. Bert Ely and Dr. Richard Hunt, who gave me the initial opportunity to learn at the University of South

Carolina. Thank you for making it your goal to prepare us for graduate school and for giving me advice along the way.

Finally, I would like to thank my mother, my grandmother, my sister, and my best friends, Arlene, Heidi, and Justine, who have continuously supported me during my academic journey; I dedicate this piece of work to them. Thank you for encouraging me and for believing in me, even in the moments that I doubted myself. Thank you for always telling me that I make you proud. Thank you for being there for me! I would be nowhere without my support system.

ABSTRACT

The hematopoietic compartment is tasked with the establishment and maintenance of the entire blood program. Key to this process are hematopoietic stem cells (HSCs), which possess the unique ability to self-renew and differentiate to replenish blood cells throughout an organism's lifetime. In a screen for epigenetic regulators of hematopoiesis in zebrafish, we identified chromatin remodeling protein and transcription repressor, Ing4, as a requirement for hematopoietic stem and progenitor cell (HSPC) specification. Ing4, a member of the inhibitor of growth (ING) family, is a subunit of the HB01-JADE-hEAF6 histone acetyltransferase complex responsible for most nucleosomal histone H4 acetylation in eukaryotes. Ing4 is known to form an inhibitory complex with RelA, the large subunit of the NF-kB protein complex, limiting expression of NF-kB target genes. NF-kB is a dimeric complex of Rel proteins that can activate, and sometimes repress transcription of cytokine genes. Thus, loss of Ing4 in mice causes hyper inflammatory responses because there is no inhibition of NF-kB. Ing4 has also been suggested to regulate a wide variety of cellular processes, including DNA repair, apoptosis, cell-cycle regulation, metastasis, angiogenesis, and tumor suppression. The role of Ing4 in hematopoiesis has not been established.

Here, we present data that shows Ing4 is critical for HSC gene regulation to direct both stem cell maintenance and differentiation in mice. Utilizing competitive transplantation, flow cytometry analysis of Ing4-deficient mouse bone marrow and peripheral blood, as well as colony-forming unit (CFU) assays, we have revealed that loss

of Ing4 in mice resulted in a shift in the endogenous HSC sub-populations towards short term-HSCs (ST-HSCs)/long term-HSCs (LT-HSCs) and skewed HSC differentiation towards granulocyte-macrophage progenitors (GMPs). Furthermore, when sorted LT-HSC, ST-HSC, and MPP cells were transplanted into irradiated mice, Ing4-/- LT HSCs failed to reconstitute recipient BM, while Ing4-/- MPPs were greater contributors to multilineage engraftment compared to WT MPPs. Interestingly, MPPs are a population that have lost the self-renewal capacity of a stem cell, but our data suggests that under stress hematopoiesis, the loss of Ing4 likely results in reprogramming of MPPs to gain stem-like features. Further characterization of this HSC sub-population is underway. Moreover, the inability of Ing4-/- LT HSCs to engraft suggests an evolutionary requirement for Ing4 in normal hematopoiesis and gives us insight into how Ing4 modulates inflammatory pathways to regulate HSC function.

Through a number of *in situ* hybridization studies, we have previously shown that Ing4-deficient zebrafish embryos have decreased expression of HSC specific genes, *runx1* and *c-myb*, in the aorta—gonad—mesonephros (AGM) region, while mRNA based overexpression of human ING4 results in increased expression of these same genes, suggesting that normal expression levels of Ing4 are required for HSC specification. As in mice, Ing4 deficiency in zebrafish results in increased expression of NF-kB target genes. Interestingly, simultaneous inhibition of NF-kB and Ing4 restores HSC formation in embryonic zebrafish. Based on these observed effects and the literature published on our mouse model, we conducted a mini screen of NF-kB inhibitors with known modes of action, to identify those that could compensate for the consequences of Ing4 loss of function. These experiments revealed that NF-kB inhibitors, MRT673007, Senexin, and

Parthenolide significantly reduced cytokine expression in Ing4-deficient zebrafish and as expected, rescued HSC specification in this system.

Our findings support a novel role for Ing4 in blood development and highlight a potential molecular mechanism by which Ing4 regulates hematopoiesis through the NF-κB pathway. This provides further insight into the characterization of factors and pathways involved in hematopoiesis and the specific genetic defects in these regulators that can be targeted for therapeutic treatment of blood disorders.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

HSC..... Hematopoietic Stem Cell

Ing4 .....Inhibitor of growth 4

AGM ..... Aorta–Gonad–Mesonephros

HSPC..... Hematopoietic Stem Progenitor Cell

(W)BM.....(Whole) Bone Marrow

PB..... Peripheral Blood

WT .....Wildtype

CFU.....Colony Forming Unit

MPP.....Multipotent Progenitor

LT-HSC.....Long-Term Hematopoietic Stem Cell

ST-HSC..... Short-Term Hematopoietic Stem Cell

CFU-GEMM .....Granulocyte, Erythrocyte, Monocyte, Megakaryocyte

CFU-GM .....Granulocyte, Macrophage

CFU-G.....Granulocyte

BFU-E ..... Burst Forming Unit-Erythroid

RBC..... Red Blood Cells

MEP ..... Megakaryocyte Erythroid Progenitor

GMP.....Granulocyte Macrophage Progenitor

CMP ..... Common Myeloid Progenitor

MK .....Megakaryocyte

CHAPTER 1

INTRODUCTION

Hematopoiesis is a tightly regulated process that relies on a complex network of regulatory mechanisms to establish the blood system. Essential to this program are hematopoietic stem cells (HSCs), which differentiate to produce a series of progenitor and precursor populations, ultimately giving rise to all mature hematopoietic lineages throughout the lifetime of an organism. Various cells produced through hematopoiesis perform a multitude of roles for the body. Red blood cells oxygenate the entire body system, myeloid-derived and lymphoid-derived white blood cells provide host defense mechanisms through the innate and adaptive immune responses, and platelets promote blood clotting and tissue repair following vessel injury. The hematopoietic compartment must maintain homeostasis and promote these processes when needed, thus preserving a careful balance between HSC maintenance and differentiation under steady-state conditions. A hallmark of HSCs is their ability to differentiate and self-renew due in part to the interworking of transcription factors and chromatin remodeling enzymes that activate or inhibit target gene expression. These factors provide an entry point for clarifying how HSCs develop during embryogenesis and how specification is programmed (Hsia et al., 2005).

To uncover chromatin factors that are necessary for the establishment of HSCs, we conducted the first large-scale *in vivo* reverse genetic morpholino-based screen targeting zebrafish orthologues of 425 human chromatin factors (Huang et al., 2013). In this study,

over a dozen chromatin remodeling factors, with no previously known association to HSCs, were found to be involved in blood formation, including the histone acetylation complex, Hbo1. (Fig. 1.1.) This complex functions to promote gene expression through the binding of H3K4me3 by inhibitor of growth 4 (Ing4), which results in localized Hbo1 histone acetylation (Coles et al. 2009)(Orkin, 2008). Ing4, a member of the inhibitor of growth (ING) family, has been suggested to regulate a wide variety of cellular processes, including DNA repair, apoptosis, cell-cycle regulation, metastasis, angiogenesis, and tumor suppression (Li et al., 2015) (Coles et al., 2010). While no role for Ing4 has been established in HSCs, we have previously shown, through a number of *in situ* hybridization studies, that Ing4-deficient zebrafish embryos have decreased expression of HSC specific genes, *runx1* and *c-myb*, while mRNA based overexpression of human ING4 results in increase expression of these same genes (Fig. 1.2.). This suggests that normal expression levels of Ing4 are required for HSC specification.

The extent of altered hematopoiesis upon the loss of Ing4 expression in HSCs, however, has not yet been tested. Our lab is particularly interested in understanding how Ing4 affects the development of stem and progenitor cells and cell lineage fate. The body of literature presented here will highlight what is known about the interrelation of transcriptional regulators and inflammation, thus providing the framework for our studies on elucidating the role of Ing4 in hematopoiesis and the onset of human disease.

1.1. Transcriptional Regulation of Hematopoiesis

Blood production is a dynamic process that happens in two waves during development: the primitive and the definitive wave.

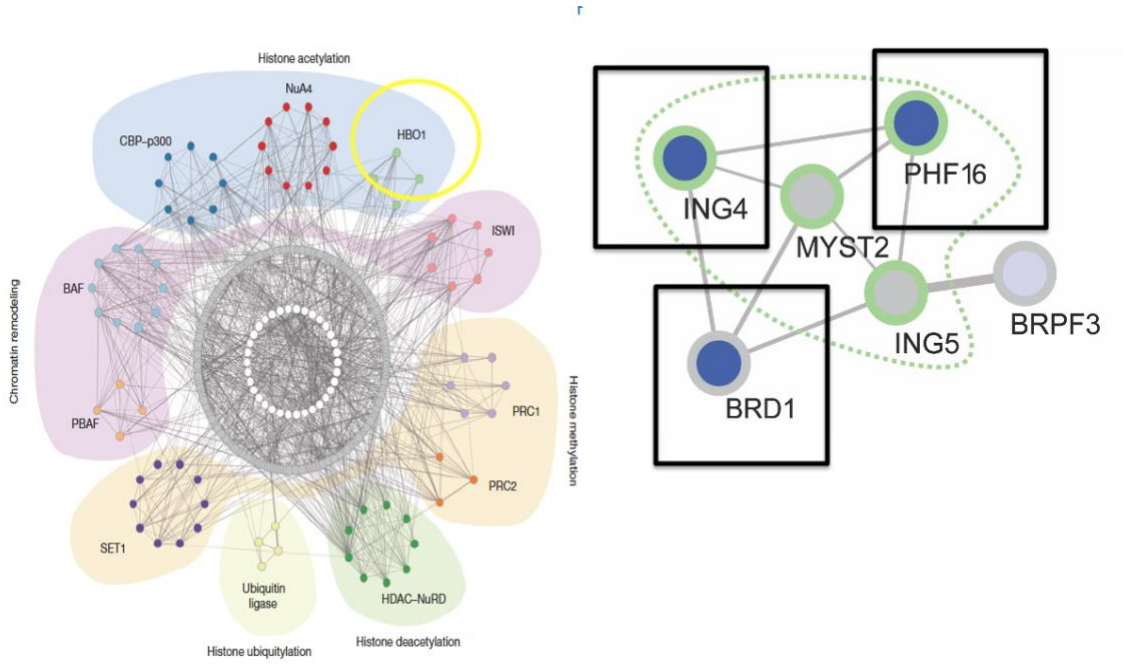

Figure 1.1. **Protein interaction network of genes involved in hematopoietic cell development.** Screen identified several genes and chromatin factors involved in hematopoiesis. Each module is assigned a unique color, and those that share the same chromatin function are highlighted in the same background color. Grey circles represent chromatin factors that not associated with isolated complexes.

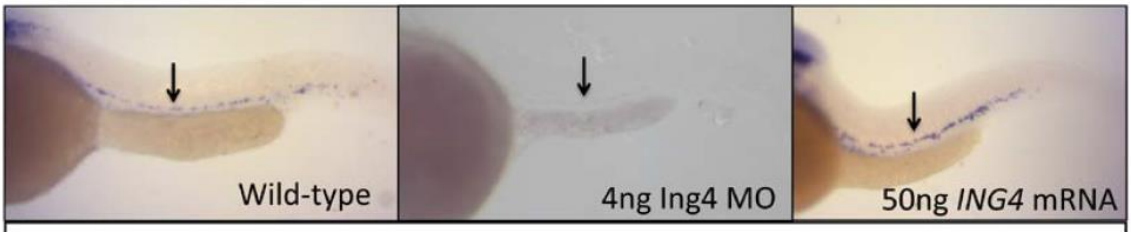

Figure 1.2. **Normal expression levels of Ing4 are required for HSC specification.** From left to right, wild-type zebrafish with observable expression of HSC markers, *runx1* and *c-myb* in the AGM, Ing4-deficient zebrafish embryos with significantly reduced expression of HSC markers in the same region and mRNA-based overexpression of human ING4 in zebrafish, which restored HSC expression.

The primitive wave, which occurs in the mammalian yolk sac (Moore & Metcalf, 1970), is the initial wave of hematopoiesis, that gives rise to erythrocytes and macrophages during early embryonic development (Palis and Yoder, 2001). The primary purpose of the

primitive wave is to produce red blood cells that can facilitate tissue oxygenation as the embryo undergoes rapid growth (Orkin and Zon, 2008). This stage is transient and is later replaced by the definitive wave. In contrast, the definitive wave begins autonomously in the aorta–gonad–mesonephros (AGM) region surrounding the dorsal aorta (Medvinsky, 1996, de Bruijn, 2000) to produce smaller erythroblasts that express adult hemoglobin's and various other mature blood cells (Chen, 2014). These definitive HSCs then migrate from the AGM to colonize the fetal liver and ultimately the bone marrow (Takeuchi, 2002). Multipotent long-term HSCs (LT-HSCs), which are responsible for sustaining and replenishing the blood system, are generated during this second wave (Müller, 1994).

Targeted disruption of several genes, including the hematopoietic transcription factors Scl/Tal-1, Lmo2, Gata-1, and Gata-2, diminishes or completely abrogates primitive hematopoiesis (Silver and Palis, 1997). Scl/Tal-1 (T-cell acute lymphocytic leukemia 1) is a basic helix-loop-helix transcription factor that forms a DNA-binding complex with Lmo2 (LIM domain only 2) (Nam and Rabbitts, 2006). Loss of function murine models for Scl/Tal-1 and Lmo2 result in embryonic death due to the absence of primitive erythropoiesis and myelopoiesis (Shivdasani and Mayer, 1995)(Yamada et al., 1998). Similarly, Gata-1 and Gata-2 mutant mice die mid-gestation due to arrested development of embryonic erythrocyte precursors that results in severe anemia (Fujiwara, 1996) (Silver and Palis, 1997). Both Gata-1 and Gata-2 govern the development of erythrocytes (Moriguchi, 2014) and megakaryocytes during primitive and definitive hematopoiesis (Lugus, 2007)(Belele, 2009) and thus are important for HSC regulation.

Gata-1 was the first erythroid master regulator identified and remains the best-studied hematopoietic transcription factor (Cantor & Orkin, 2002)(Welch et al., 2004). By

contrast, SPI1/PU.1, is the master regulator of myelopoiesis (Scott et al., 1994), which results in the development of neutrophils, dendritic cells, and monocytes, from a myeloid progenitor cell. As master regulators, Gata-1 and PU.1 find themselves at odds during hematopoietic development, mutually inhibiting each other to control cell lineage fate. To promote erythroid differentiation, Gata-1 binds PU.1's upstream regulatory element, consequently repressing PU.1 (Chou et al., 2009) and many of the myelolymphoid downstream target genes of PU.1 (Wontakal et al., 2012). Previous studies have shown that forced expression of Gata-1 is sufficient to reprogram myeloid into erythroid cells in both avian and murine cell lines (Kulessa et al., 1995)(Yamaguci et al., 1998). Conversely, although PU.1 is highly expressed in myeloid and B-cells, it is also normally present in immature erythroid progenitors (Back et al., 2004), where it antagonizes erythroid differentiation by binding to and inhibiting the genes encoding Gata-1 and Scl, and important Gata-1 cofactors (Wontakal et al., 2012).

In definitive hematopoiesis, runx1 and c-myb have been established as the earliest markers due to their expression in the AGM during HSC specification (Thompson et al., 1998)(Kataoka et al., 2000). Runx1, a member of the runt family of transcription factors, plays an important role in hematopoiesis. Wang et al. demonstrated that loss of runx1 in mice resulted in embryonic lethality and a block on definitive erythropoiesis and myelopoiesis (Wang et al., 1996). More recently, runx1 has been shown to be required for the switch of endothelial cells to hematopoietic progenitor stem cells (HSPCs) (Chen et al., 2009)(Yzaguirre, 2017). Similarly, c-myb, which belongs to the myb family of proto-oncogenes (Greco et al., 2001), is a critical transcription factor for definitive hematopoiesis. c-myb is highly expressed in immature HSCs and its expression is down

regulated as they become more differentiated (Gonda and Metfcalf, 1984). Soza-Reid et al. showed that c-myb is dispensable for primitive erythropoiesis in zebrafish even though it is expressed in these cells (Soza-Ried, 2010). By contrast, c-myb-/- mice died in utero at E15.5 with severe anemia and displayed defects in erythroid and myeloid development (Mucenski et al., 1991). Comparatively, c-myb mutant zebrafish survived into early adulthood, but as seen in the knockout mice, definitive hematopoiesis was permanently extinguished upon the loss of c-myb (Soza-Ried, 2010). Taken together, due to their roles in HSC regulation, these transcripts can be widely used in animal knockout models and cell lines to study developmental hematopoiesis.

1.2. Inflammation and Hematopoiesis

Inflammatory signaling pathways regulate many aspects of hematopoiesis, including embryonic development, emergency granulopoiesis, and age-associated hematopoietic defects. Several recent publications have identified new mechanisms for regulation of inflammatory signaling in HSPCs. Using zebrafish and mouse models, the Molero laboratory recently reported an evolutionary-conserved signaling pathway linking inflammasomes with HSPC fate decision, where inflammasomes can refine protein quantities of the lineage-specific transcription factor, Gata-1 (Trysalka, 2019). Their work suggests that inflammasomes activate caspases capable of cleaving Gata-1 and inactivating it, promoting myeloid cell fate at the expense of erythropoiesis.

Skewed differentiation towards the myeloid lineage from HSPCs is a frequent stress-induced hematopoietic response (Chavakis, Zhao 2015). Cytokines are essential in regulating hematopoiesis and promoting myeloid-biased differentiation. Previous work on proinflammatory cytokine TNF-α suggest its complicated role in hematopoiesis (Baldridge

et al., 2011)(Ishida et al., 2017)(Pearl-Yafe et al., 2010)(Pronk et al., 2011) and essential function in HSC specification (Espin-Palazon et al., 2018). Recently, the Passegué laboratory clarified the role of TNF-α in HSCs as a pro-survival and pro-regeneration factor. TNF-α-induced p65-NF-κB activity protected HSCs from necroptosis-mediated elimination and triggered a strong regenerative program, resulting in emergency myelopoiesis via upregulation of lineage-specific transcription factor PU.1 (Yamashita et al., 2019) Similarly, Etzrodt et al. reported TNF-α as the principal PU.1 inducing signal in HSCs (Etzrodt et al., 2019) while the chromatin remodeling protein, Phf6, was recently identified as a modulator of TNF-α signaling (Miyagi et al., 2019).

Inflammatory cues from toll-like receptor (TLR) ligands, interferons (IFNs) and interleukins (ILs) have also been shown to activate proliferation, differentiation, and HSC emergence. Using an in *vitro* model of B cell maturation, Hayashi et al. demonstrated that TLR4 and TLR2 have distinct effects on B lymphocyte differentiation. While TLR4 signaling favored B lymphocyte maturation, TLR2 arrested the differentiation process (Hayashi et al., 2005). In a separate study, lymphoid-biased progenitors were directed to a dendritic cell fate in response to TLR signaling, that suppressed B lymphopoiesis (Nagai et al., 2006).

In an emerging infectious disease model for human monocytic ehrlichiosis (HME), IFN-γ signaling resulted in altered myeloid differentiation and changes in hematopoietic progenitor cell function. By contrast, IFN-γ was dispensable for lymphoid differentiation during infection (MacNamara et al., 2012). In an in *vivo* mouse model of *Mycobacterium avium* infection, LT-HSCs, which remain mainly quiescent in the BM niche, were found to be actively cycling in response to IFNγ stimulation during chronic infection. This

resulted in rapid expansion of downstream progenitors to restore and maintain homeostasis during inflammatory stress. The effects of this response on LT-HSCs was exhibited by a transplantation experiment, where LT-HSCs from infected mice failed to regenerate. Similarly, in response to IFNα treatment, HSCs showed decreased quiescence, as they exited G0 and entered active cell cycling (Essers et al., 2009).

Historically, interleukins have also been associated with hematopoiesis. In an earlier study, purified, bacterially synthesized IL-3 was injected intraperitoneally into mice over the course of six days. This inflammatory stimulus promoted progenitor differentiation at the expense of self-renewal (Metcalf et al., 1986). In a colitis disease murine model, the loss of quiescence in LT-HSCs, skewed progenitor activity, and other changes in the HSPC compartments, were a direct response to IL-23-driven-inflammation (Griseri et al., 2012). Using zebrafish models of IL-6 pathway deficiencies, Tie et al. showed the necessity of IL-6 signaling for embryonic HSC specification. Myeloid cells from both the early primitive wave of hematopoiesis and the later definitive erythro-myeloid progenitors (EMPs) were revealed as major sources of IL-6 (Tie et al., 2019). These studies demonstrate the impact of cytokines in regulating hematopoiesis and suggest the potential unintended consequences to the hematopoietic system associated with the use of cytokine inhibitors in treating inflammatory diseases.

HSCs must maintain a balancing act between two inflammatory signaling extremes. On one hand, fully activated HSCs expressing little NF-κB have low regeneration potential (Yamashita et al., 2019); conversely, increased NF-κB activity is associated with decreased quiescence, rapid expansion of progenitor cells, and impaired long-term engraftment upon transplantation (Weisser et al., 2016)(Nakagawa et al., 2018)(Ramalingam et al., 2020).

Ramalingam et al. showed that sustained inflammation in murine BM adversely impacted hematopoiesis via chronic activation of endothelial MAPK and NF-κB signaling. Donor BM cells from a competitive transplant displayed proliferative stress and decreased functionality. The resulting proinflammatory cytokines expressed in the BM niche drove myeloid-biased differentiation in HSCs at the expense of self-renewal (Ramalingam et al., 2020). These studies underscore the versatile role of cytokine signaling and inflammatory stress in regulating cell fate and hematopoiesis lineage bias.

1.3. Ing4

The founding member of the inhibitor of growth (ING) family, Ing1, was the first of the ING genes to be characterized using overexpression and antisense knockdown of Ing1 normal human diploid fibroblasts (Garkavtsev et al., 1996). It was found that ectopic overexpression of Ing1 blocked cell cycle progression, while inactivation in cell lines, resulted in malignant cell transformation (Garkavstev et al., 1996). Garkavstev et al. also highlighted a novel role of Ing1 as a transcription regulator of tumor suppressor, p53, as functional loss of either of these genes, prevented their ability to inhibit cellular growth (Garkavtsev et al., 1998). This study indicated Ing1 as an important component of the p53 signaling pathway. Though Ing1 remains the most widely studied of the Ing proteins, research has also linked Ing2-5 as regulators of several cellular processes, such as cell cycle and apoptosis, chromatin remodeling, and inflammation (Nagashima, 2003)(Doyon, 2006) (Shen et al., 2007) (Kumamoto et al., 2010).

Ing proteins contain highly conserved plant homeodomain (PHD) zinc fingers, a common characteristic for chromatin-modifying nuclear proteins (Kalkhoven, 2002). In particular, the PHD fingers of the Ing proteins specifically bind to H3K4me2 and

H3K4me3 (Pena, 2006), which supports the role of Ing family members as chromatin remodeling proteins and transcription factors. Our protein of interest, Ing4, is a key subunit of the histone acetylation complex, HBO1-JADE-hEAF6, responsible for the majority of histone H4 acetylation in eukaryotes (Doyon, 2006). Ing4 is required for efficient chromatin modification by Hbo1, as depletion of Ing4, greatly reduces the ability of this complex to acetylate nucleosomal histones (Doyon, 2006), and thus affecting gene expression. Moreover, Ing4 is often mutated in various human cancer cell lines (Kim, 2004) and in brain (Garkavstev, 2004), breast (Keenan, 2016), and colon (Chen et al., 2016) cancers, where Ing4-deficiency is associated with poor prognosis due to aberrant cell proliferation. On the other hand, overexpression of Ing4 results in decreased S-phase cellular activity (Shiseki, 2003) (Li et al., 2015). Therefore, in cancers, Ing4 expression is inversely correlated with cell cycle progression.

Ing4 is among the few known regulatory proteins that can directly interact with chromatin as well as with transcription factors, including p53, NF-κB and HIF-1α. Overexpression of Ing4 results in p53-dependent apoptosis in a human colorectal cell line (Shiseki, 2003) and reduction of p53 expression in osteosarcoma cells (Li et al., 2015). Ozer et al. demonstrated that presence of Ing4, suppressed expression of HIF-1α target genes under hypoxic conditions. Ing4 does not bind directly to HIF-1α but instead to HPH-2, a regulator of HIF-1α stability (Ozer et al., 2005). As part of the Hbo1 complex, Ing4 sequesters RelA/p65, the large subunit of the NF-kB protein complex, in the cytoplasm thus resulting in suppression of NF-κB target genes (Coles, 2010). The extent of altered NF-kB signaling upon the loss of Ing4 was furthered demonstrated by Nozell et al. In human gliomas, absence of Ing4 resulted in higher levels of NF-κB and its target genes,

MMP-9 and COX-2 (Nozell et al., 2008). Similarly, in an Ing4-deficient mouse model, loss of Ing4 caused hyper inflammatory responses to LPS, a known activator of NF-κB signaling. The aberrant activation of NF-κB, and subsequent overexpression of some NF-κB target genes, is attributed to the loss of signaling regulation by Ing4 (Coles, 2010)(Fig. 1.3.). These mice are developmentally normal. They display elevated cytokine responses but fail to develop spontaneous tumor, suggesting Ing4 deficiency itself may not be sufficient so initiate tumorigenesis (Coles et al., 2008)(Du et al., 2019).

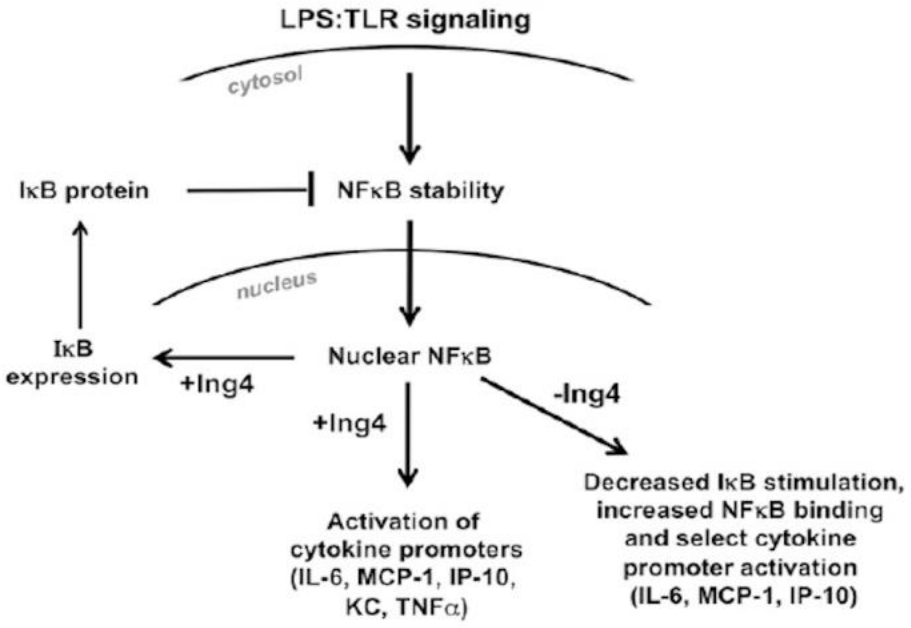

Figure 1.3. **Schematic diagram for the role of Ing4 in regulation of IkB expression and NF-κB activity.** Up-regulation of nuclear NF-κB by LPS treatment leads to increased RelA-responsive promoter activation and cytokine production in Ing4 transgenic mice. *Figure adapted from Coles, A. H., Gannon, H., Cerny, A., Kurt-Jones, E., & Jones, S. N. (2010). Inhibitor of growth-4 promotes IkB promoter activation to suppress NF-kB signaling and innate immunity.*

As in mice, Ing4 deficiency in zebrafish results in increased expression of NF-κB target genes. We have previously shown that simultaneous inhibition of NF-κB and Ing4 restores HSC formation in embryonic zebrafish (Fig. 1.4.). Based on these observed effects

and the literature published on our mouse model, we conducted a mini screen of NF-κB inhibitors with known modes of action, to identify those that could compensate for the consequences of Ing4 loss of function. The zebrafish is a desirable model system due to their high fecundity, rapid development, evolutionary conservation, and ease in generating genetic knockdowns (Huang et al., 2013). We generated an Ing4 knockdown zebrafish model by designing an anti-sense morpholino oligo against Ing4 and injecting it into zebrafish embryos at the single cell stage.

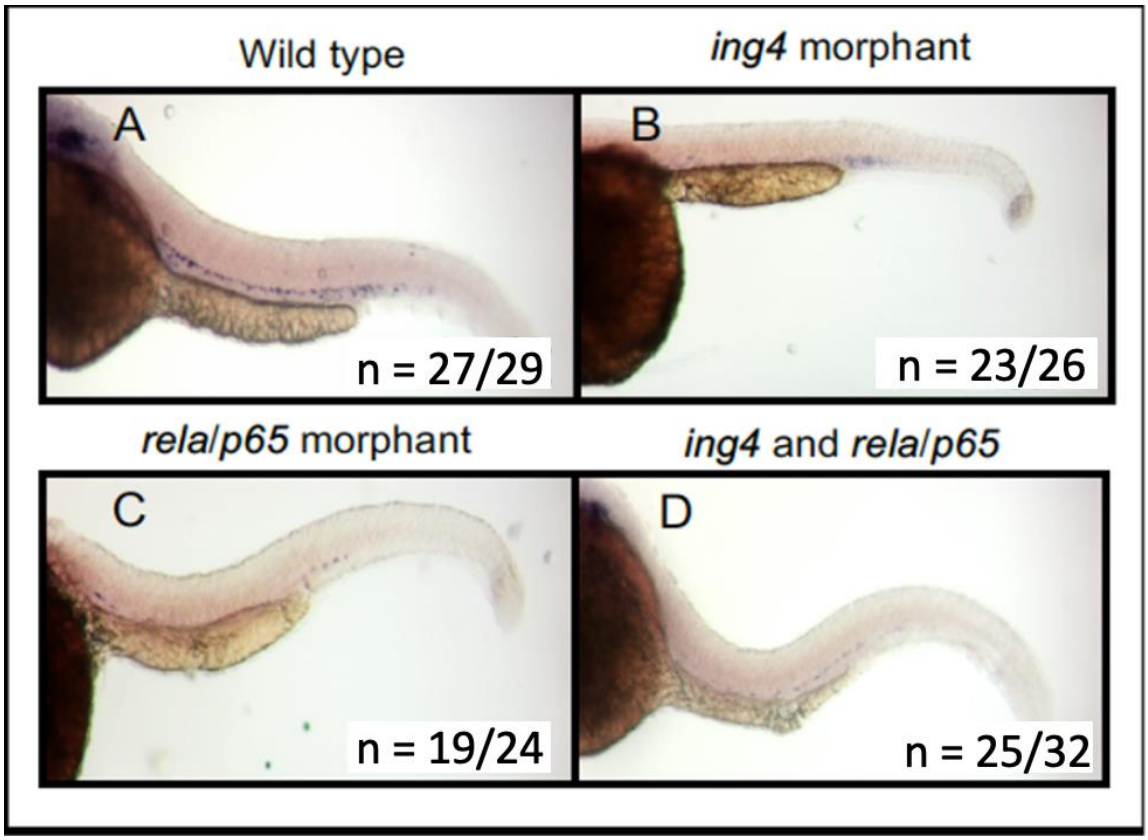

Figure 1.4. **Rescue of HSC specification due to Ing4 inactivation through inhibition of NF-kB.** HSC marker expression, *runx1* and *c-myb*, in the AGM region is shown. **A** uninjected wildtype, **B** *ing4* morphant, **C** *RelA/p65* morphant, **D** *ing4* and *RelA/p65* double morphant.

We propose that Ing4 is required for HSC gene regulation to direct both stem cell maintenance and differentiation. Utilizing Ing4-deficient mouse and zebrafish model systems, our work aims to establish Ing4 as a critical regulator of HSC specification through *in* vivo and *in* vitro experiments and highlight a potential molecular mechanism by which Ing4 regulates hematopoiesis through the NF-κB pathway.

This data will broaden our understanding on the basic biology of HSCs and the relationship between inflammatory pathways and hematopoietic development, which could lead to improved therapeutic treatments for blood disorders and Ing4-deficient cancers and become helpful for marrow and cord blood transplantation.

CHAPTER 2

MATERIALS AND METHODS

Mice

C57BL/6J (CD45.2) and B6.SJL-*Ptpcr$_a$Pepc$_b$/BoyJ* (CD45.1) were purchased from the Jackson Laboratory (Bar Harbor, ME). Ing4 knockout mice (C57BL/6 background) were obtained from Stephen Jones at the University of Massachusetts Medical School (Coles, 2010). Ing4 knockout mice were bred and maintained on a C57BL/6J (CD45.2) genetic background. Six- to 12-week old mice were used as donors for transplantation assays and eight- to 12-week old mice were used as recipients. Similarly, six- to 12-week old mice were used for in *vitro* experiments. Respective littermates or aged-matched WT animals were used as controls and as competitive donors. All mice were housed in a barrier unit maintained in The Department of Laboratory Animal Resources (DLAR) at the University of South Carolina. Specifically, immunocompromised recipient mice (CD45.1) were housed in sterile conditions for two weeks following transplantation. These mice were maintained on water supplemented with Baytril (0.01 mg/mL) and sterilized food. All experiments were done in accordance with the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) and National Institute of Health (NIH) Office of Laboratory Animal Welfare (OLAW) guidelines and under the protocols approved by Institutional Animal Care and Use Committee (IACUC) at the University of South Carolina.

Zebrafish

Zebrafish (*Danio rerio*) Tübingen strain were bred and maintained in accordance with the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) and National Institute of Health (NIH) Office of Laboratory Animal Welfare (OLAW) guidelines and under the protocols approved by Institutional Animal Care and Use Committee (IACUC) at the University of South Carolina. Zebrafish were bred and embryos were collected for microinjection within 45minutes post fertilization. Ing4 antisense oligo morpholino microinjections were performed at the 1-2 cell stage. Injection volumes were measured using a stage micrometer and injected once into the yolk of the embryo. Post microinjection, embryos were raised to 24 hpf in 100mm Petri dishes at a density of 100 embryos or less per dish. Ing4-morphant and control embryos were dechorionated and treated with DMSO, 5μM MRT67307, 10μM Senexin, or 2μM Parthenolide. in 96-well plate format with 5-7 embryos per well.

Ing4 Morpholino Design and Synthesis

Ing4 sequence was analyzed to identify most suitable target sites for morpholino knockdown. Translation blocking, or ATG, morpholinos were designed if the transcriptional start site was well annotated. Target regions fall within a 25 base pair range surrounding the ATG site. Once the target site was selected, DNA sequences were submitted for morpholino oligonucleotide design and synthesis (Gene-Tools). To prepare morpholinos for injection, lyophilized morpholinos were dissolved in nuclease-free water to a stock concentration of 2mM and stored at room temperature. Before microinjection, morpholinos were diluted to the appropriate concentration and heated for 5 min at 65 C to minimize secondary structures.

In-situ hybridization

Zebrafish embryos were dechorionated by hand or using pronase and fixed in 4% paraformaldehyde at 4$_{o}$ Celsius. Embryos 24hpf and older were bleached to remove pigmentation, then dehydrated in methanol and stored at -20$_{o}$ Celsius. Complementary DNA probes were synthesized from restriction enzyme linearized plasmid and transcribed with T7, SP6, or T3 polymerase in the presence of RNA DIG labelling mix to generate probes according to manufacturer's protocol (Qiagen). When the embryos reached 36hpf, they were harvested and subjected to *in situ* hybridization using probes against c-myb and runx1 expression, two HSC specific markers.

RT-PCR

Treated and control embryos were collected in Trizol (Invitrogen) and homogenized using a homogenizer. RNA was extracted according to the Cold Harbor Spring Protocol (Lan et al., 2009). For reverse transcription reactions, 1 μg of total RNA was used. cDNA was synthesized using Superscript III First Strand Synthesis SuperMix for qRT-PCR kit (Invitrogen) according to the manufacturer's instructions. All gene-specific primers used are listed below. *gapdh* was used as the housekeeping gene.

Colony Forming Unit Assays

Colony-forming units (CFUs) in methylcellulose were quantified to assess hematopoietic progenitor activity. All cultures were performed at 37$_{o}$C in a 5% CO_2 water jacket incubator (Thermo Scientific). Whole bone marrow (WBM) was isolated from femurs by gently crushing with a mortar and pestle, washed with cold mouse buffer (2.5% FBS, 1% Pen-strep, 1x PBS) and filtered over a 40 μm filter (Fisher) to obtain single-cell suspension.

Table 2.1. **Primers for small molecule screen.** Following pharmacological inhibition of NF-kB, we used the following primers to detect cytokine expression in WT and Ing4 morphant zebrafish.

zIL6F– TCAACTTCTCCAGCGTGATG zIL6R– TCTTTCCCTCTTTTCCTCCTG	IL-1b
zIL2RgaF– TCAGGGCTCGGATAACCTA zIL2RgaR– CGTCACAGACATGGAACCCA	IL-2R
zIL62F– AGACCGCTGCCTGTCTAAAA zIL62R– CAACTTCTCCAGCGTGATGA	IL-6
zIL42KKF– GCAGCATATACCGGGACTGG zIL42KKR– ATGGCAGCATGCTTTGGTTT	IL-4
zGapdhF- GTGGAGTCTACTGGTGTCTTC zGapdhR- GTGCAGGAGGCATTGCTTACA	Gapdh

Viable cell counts were determined with a hemocytometer using Trypan Blue (Life Technologies). WT and Ing4-/- WBM cells ($1x10_4$ cells/well) were plated in triplicate into a six-well plate containing Methocult GF M3434 methylcellulose (StemCell Technologies) according to the manufacturer's suggestions. Colonies were scored after 10 days for phenotypic CFU-GEMM, CFU-GM, CFU-M, CFU-G, and BFU-E colonies using a Motic Swift Line AE31R inverted microscope (Fisher). This experiment was repeated five times under the same conditions.

Competitive Transplantation

Adult CD45.1 recipient mice (n= 20) were pre-conditioned with lethal irradiation (9.5 Gy) in a split dose prior to transplantation. WBM was isolated from femurs by gently crushing with a mortar and pestle, washed with cold mouse buffer (2.5% FBS, 1% Pen-strep, 1x PBS) and filtered through a 40 μm filter (Fisher). Viable cell counts were determined with a hemocytometer using Trypan Blue (Life Technologies). After processing the sample, $1x10_5$ WT or Ing4-/- WBM cells (CD45.2) were transplanted with $4x10_5$ competitor WBM cells (CD45.1) via retro-orbital injections into irradiated recipient (CD45.1) mice. For sorted transplant, adult CD45.1 recipient mice (n= 60) were pre-conditioned with lethal irradiation (9.5 Gy) in a split dose prior to transplantation. After processing the sample as aforementioned, 10 LT-HSCs, 50 ST-HSCs, and 100 MPPs WT or Ing4-/- WBM cells (CD45.2) were transplanted with $2x10_5$ competitor WBM cells (CD45.1) via retro-orbital injections into irradiated recipient (CD45.1) mice. Retro-orbital bleeds using 75mm heparinized glass capillary tubes (Fisher) were used to assess multilineage hematopoietic engraftment at four weeks, 12 weeks 16 weeks, and a year post-transplantation. Prior to staining for flow cytometry, peripheral blood (PB) was spun for seven minutes at 1200rpm, resuspended in 1X Red Blood Cell (RBC) lysis buffer (Miltenyi) then washed with cold mouse buffer (2.5% FBS, 1% Pen-strep, 1x PBS). Cell populations were stained and analyzed using flow cytometry.

Progenitor Activity

To further characterize the hematopoietic progenitor population in the murine bone marrow, femurs were gently crushed with a mortar and pestle, washed with cold mouse buffer (2.5% FBS, 1% Pen-strep, 1x PBS) and filtered through a 40 μm filter (Fisher) to

obtain a single cell suspension. For optimal flow cytometry immunophenotyping, RBC were removed from the samples using 1x RBC lysis buffer (Miltenyi). Additionally, viable cell counts were determined with a hemocytometer using Trypan Blue (Life Technologies) for subsequent enrichment of lineage negative (Lin-) cells using the Direct Lineage Cell Depletion Kit (Miltenyi). For depletion, cells were magnetically labeled with a cocktail of microbeads conjugated to antibodies against lineage markers (CD5, CD45R (B220), CD11b, Gr-1 (Ly-6G/C), 7-4, and Ter-119) (32). Bead-labeled cells were separated with manual LS columns according to the manufacturer's instruction (Miltenyi). The resulting cell suspension of isolated unlabeled Lin- cells was stained and used for flow cytometry analysis. This experiment was repeated four times with (n = 3) for each experiment.

Flow cytometry

As described above, HSCs were purified from murine bone marrow and characterized using phenotypic cell surface markers. Sample data were collected and analyzed using an LSR II Sorp (BD Biosciences) with FACS Diva (BD Biosciences) and FlowJo software. Gates were established using unstained controls. The antibody panels used for immunofluorescence staining for HSC and progenitor isolation were as followed:

Table 2.2. **Flow cytometry stain for chimerism analysis of WBM transplantation.** For transplantation experiments, PB chimerism and multilineage hematopoietic engraftment of donor-derived cells were assessed. Recipient (CD45.1), donor (CD45.2), competitor (CD45.1), CD3e (T cells), B220 (B cells), Mac1/CD11b (Macrophages), Ter119 (Erythrocytes), Gr-1 (Granulocytes).

CD45.1	PE
CD45.2	FITC
CD3e	APC
B220	e-Fluor450
Mac1/CD11b	BV510
Ter119	APC-Cy7
Gr-1	PE-Cy7

Quantitation and Statistical Analysis

All experiments were repeated as indicated; n indicates the number of biological replicates. Data are presented as the mean ± standard error of the mean (SEM). Student's *t*-test was used to evaluate statistical comparison between two groups. Statistical significance was indicated by *($P < 0.05$), **($P < 0.01$), ***($P < 0.001$), and n.s (not significant.

Table 2.3. **Flow cytometry stain for HSC sorted transplantation assay.** Isolation of donor-derived HSC-subpopulations for sorted transplantation experiments. Lin- fraction: CD34, CD48, CD150, Sca-1, c-Kit (LSK). Lin+ fraction: Mac1/CD11b (Macrophages), B220 (B cells), Ter119 (Erythrocytes), Gr-1 (Granulocytes), CD3e (T cells).

CD34	FITC
CD150	APC-Cy7
Sca-1	APC
CD48	PE
c-Kit	PE-Cy7
Lin CD11b	PE
Lin B220	PE
Lin Ter119	PE
Lin Gr-1	PE
Lin CD3e	PE

Table 2.4. **Progenitor stain for flow cytometry analysis.** BM cells were stained to characterize the progenitor compartment in WT and Ing4-deficient mice. Lin- fraction: CD34, CD48, Sca-1, c-Kit (LSK), CD16/32 (myeloid progenitors), CD127 (lymphoid progenitors). Lin+ fraction: Mac1/CD11b (Macrophages), B220 (B cells), Ter119 (Erythrocytes), Gr-1 (Granulocytes), CD48 (B cells), CD3e (T cells).

CD34	FITC
CD48	BV510
Sca-1	APC
c-Kit	APC-Cy7
CD16/32	PB
CD127	PE-Cy7
Lin CD11b	BV510
Lin B220	BV510
Lin Ter-119	BV510
Lin Gr-1	BV510
Lin CD48	BV510
Lin CD3e	BV510

CHAPTER 3

RESULTS

3.1. Loss of Ing4 results in skewed HSC differentiation

The hallmark of HSCs is their ability to differentiate and self-renew. The process of differentiating from a multipotent stem cell to a lineage-committed cell is tightly regulated by transcription factors and chromatin remodeling proteins that change gene expression. To assess the ability of HSCs to proliferate and differentiate in the absence of Ing4, we cultured WT and Ing4-/- BM cells in methylcellulose and scored colony phenotypes at 10 days. Marrow collected from mice in steady-state conditions showed that Ing4 inactivation hindered colony-forming abilities of BM progenitor cells (Fig. 3.1.). This was reflected in the significant decrease of CFU-M (macrophages) (WT vs. Ing4-/-) (24.64 vs. 32.16), CFU-GEMM (granulocytes, erythrocytes, megakaryocytes, monocytes) (2.32 vs. 6.71), and CFU-GM (granulocytes, macrophages) (5.11 vs. 8.72) colonies in the Ing4 null mice. We found no significant change between the WT and the Ing4-/- CFU-G and BFU-E colonies.

To determine if loss of Ing4 affected HSC lineage specification, we analyzed the PB of Ing 4-/- and WT mice in steady-state conditions. As expected, (B220+/CD3+) progenitor cells had a negligible presence in the PB of recipient mice (Fig. 3.1.). Generally, these cell populations are found in small numbers circulating in the blood. Similar to the CFU assays, there was no difference between the WT and Ing4 null populations of mature erythrocyte (Ter119+) and granulocyte (Gr-1+) cells.

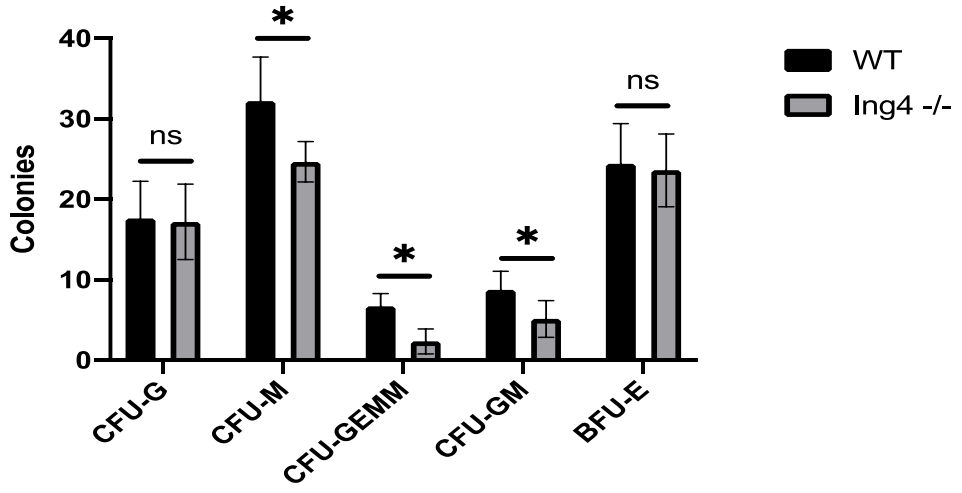

Figure 3.1. **CFU analysis of progenitor activity in mouse BM.** Methylcellulose based progenitor assay from WT and Ing4-deficient BM cells. Bar graphs indicate number of CFUs per 10_4 WBM; CFU-G (granulocytes), CFU-M (macrophages), CFU-GEMM (Granulocyte, erythroid, monocyte, megakaryocyte), CFU-GM (granulocyte, macrophage), BFU-E (erythroblasts). **P < 0.05; **P <0 .01; ***P < 0.001, $_{ns}$P> 0.05.*

Notably, Ing4-/- PB showed an increase in the frequency of T cells (CD3+) and a significant decrease in B cells (B220+). This suggests that Ing4 affects HSC specification. Interestingly, PB showed a significant increase in circulating macrophages (Mac-1+), compared to the WT, indicating that the absence of Ing4 promotes a myeloid-bias in the PB. However, the opposite was observed in the methylcellulose-based colony assays of BM cells (Fig. 3.2.).

To characterize the progenitor compartment upon Ing4 inactivation, we analyzed the murine BM niche using flow cytometry. We saw a significant increase in the GMP (52.2% vs. 42.1%) population in the Ing4-/- marrow. A dramatic decrease in the MEPs (2.17% vs. 16.8%) and a subtle increase in the CMP (34.2% vs. 33.7%) population. This indicates that loss of Ing4 drives skewed myelopoiesis (Fig 3.3.). Taken together, these data demonstrate that there is variability in how Ing4 regulates the ability of progenitor cells to proliferate and differentiate into lineage committed cells.

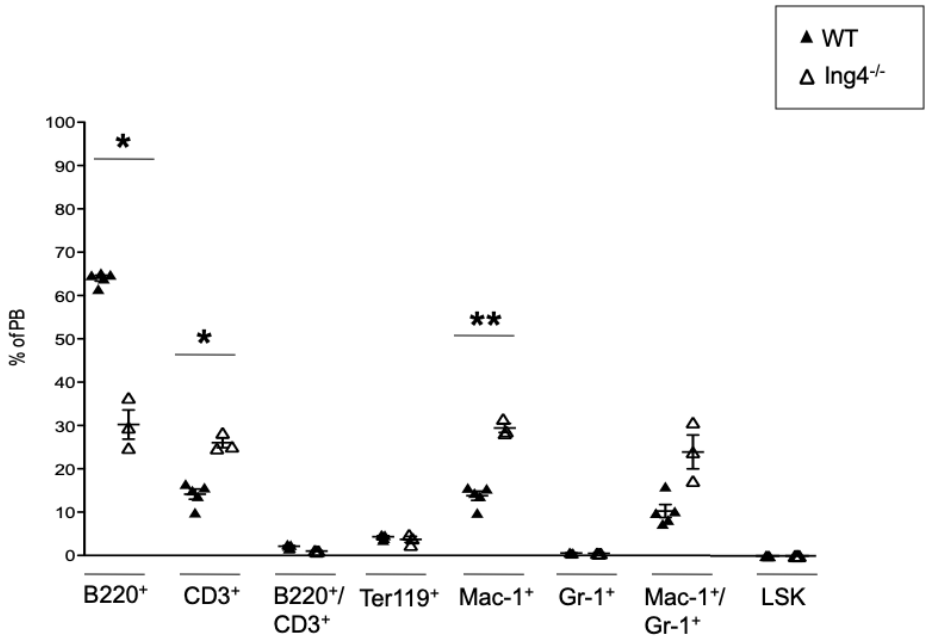

Figure 3.2. **Lineage specific cell populations in mouse PB.** Flow cytometry analysis of lineage specific cells in the PB of Ing4-/- and WT mice in steady-state conditions. CD3 (T cells), B220 (B cells), Mac1/CD11b (Macrophages), Ter119 (Erythrocytes), Gr-1 (Granulocytes), LSK (Lin–Sca-1+c-Kit+). **P < 0.05; **P <0 .01; ***P < 0.001, $_{ns}P>0.05$.*

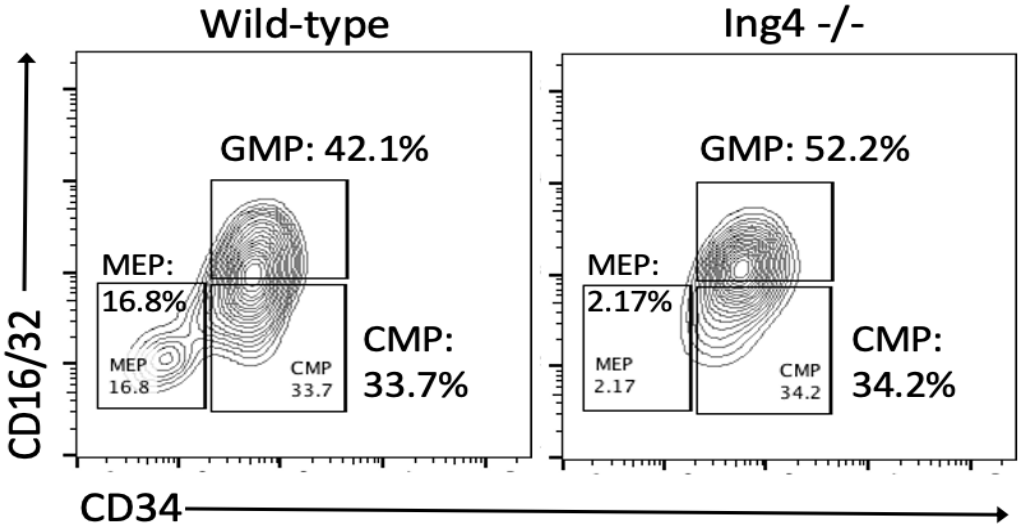

Figure 3.3. **Analysis of progenitor populations in the BM niche.** Representative contour plots demonstrating differentiation of WT and Ing4-deficient cells in steady-state conditions. The myeloid progenitor population can be identified in the Sca- portion of the LSK stain. This fraction can be further subdivided into common-myeloid progenitor (CMP) (CD34+CD16/32-), megakaryocyte-erythrocyte progenitor (MEP) (CD34-CD16/32-), and granulocyte-macrophage progenitor (GMP) (CD34+CD16/32).

3.2. The absence of Ing4 disrupts mouse hematopoiesis

A comparison of the HSC-subpopulations present in the BM niche in steady state, revealed that absence of Ing4 disrupted mouse hematopoiesis by forcing a shift towards ST-HSC/LT-HSC in the HSPC compartment (Fig. 3.4.). Compared to WT marrow, Ing4-deficiency resulted in a dramatic increase of ST-HSCs (31.7% vs. 11.4%) and decrease in MPPs (19.3% vs. 47.9%). This data demonstrates that Ing4 is required for normal mouse hematopoiesis.

We next determined if Ing4-/- HSCs maintain the ability to repopulate recipient mice. Donor chimerism can be determined by flow cytometry analysis of the peripheral blood and the bone marrow, using cell surface markers that distinguish donor cells from recipient. Though we observed changes in the progenitor compartment and the distribution of HSC sub-populations, the competitive WBM transplantation revealed that total CD45.2+ cell engraftment was similar for Ing4-/- and WT BM cells (Fig. 3.5.) over a period of three months. Indicating that HSCs lacking Ing4 can still regenerate in the BM niche.

3.3. Ing4 is required for LT-HSC engraftment but not MPP cells

We determined overall short-term and long-term engraftment was similar in Ing4 null and WT recipient. Next, we wanted to examine if the loss of Ing4 would impact the reconstitution abilities of each HSC-subpopulation, independently. This data would tell us if Ing4-dependent altered hematopoiesis drives HSCs to rapidly exhaust. In a sorted competitive transplant, PB analysis indicated functional differences between WT and Ing4 null HSCs (Fig. 3.6.). Ing4-/-LT-HSCs failed to engraft at six months.

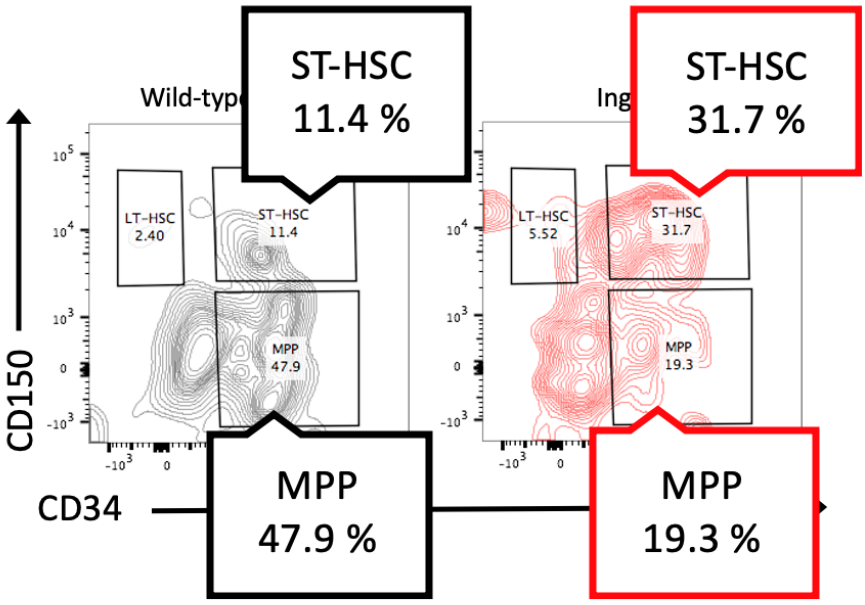

Figure 3.4. **Representative contour plots demonstrating HSC sub-populations present in WT and Ing4-deficient mice.** LT-HSC, ST-HSCs, and MPPs were identified using LSK (Lin-c-Kit+Sca-1+) and SLAM markers. LT-HSCs (CD34-CD150+), ST-HSCs (CD34+CD150+), and MPPs (CD34+CD150-).

Surprisingly, Ing4 null MPPs were greater contributors to the BM niche (19% vs. 60%), compared to WT MPPs. Moreover, there was no significant difference in the repopulation of WT vs. Ing4-deficient ST-HSCs. Collectively, these findings support a role of Ing4 as an evolutionary requirement for engraftment of bona fide LT-HSCs and a potential transcriptional repressor of MPPs, as Ing4-/-MPPs gained stem-like features (i.e. ability to self-renew) in order to reconstitute the BM niche in irradiated mice.

3.4. NF-κB inhibitors can rescue loss of HSC specification in zebrafish

As in our mouse model (Coles, 2010) loss of function of Ing4 in zebrafish results in increased NF-kB genes. We have previously shown that genetic inhibition of NF-kB in Ing4-deficient zebrafish restores HSC formation (Fig. 3.7.), thus we wanted to confirm if this was also true for chemical inhibition of NF-kB.

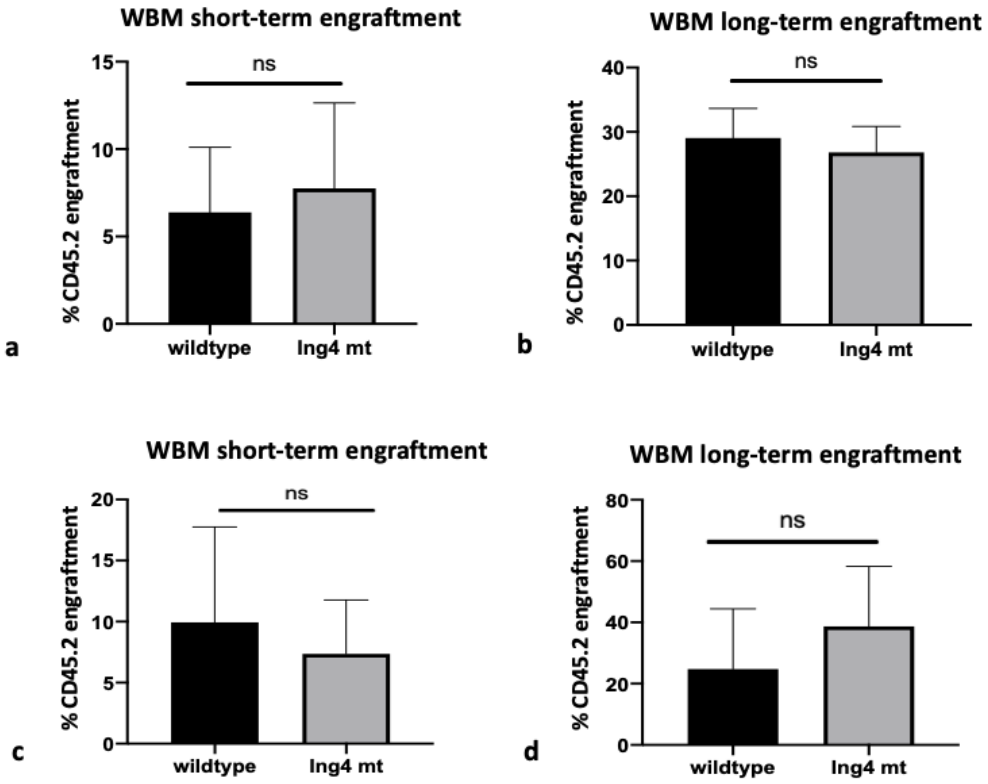

Figure 3.5. **PB %chimerism following WBM competitive transplantation assay.** 1x10$_5$ WT or Ing4-/- WBM cells (CD45.2) were transplanted with 4x10$_5$ competitor WBM cells (CD45.1) via retro-orbital injections into *(n = 20)* irradiated recipient (CD45.1) mice. **A, B, C, D** Assessment of total CD45.2 short-term engraftment (four weeks) and long-term engraftment (12 weeks) (n = 20 recipients; n = 5 donors) for two separate experiments. Top **(A,B):** 9/24/20, Bottom **(C,D):** 7/9/19. **P < 0.05; **P <0 .01; ***P < 0.001, $_{ns}$P> 0.05.*

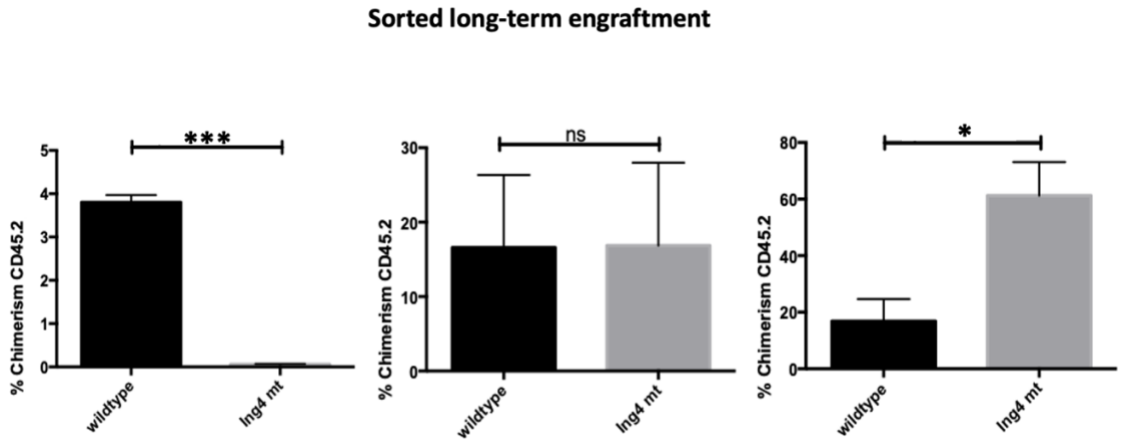

Figure 3.6. **Sorted competitive repopulation assay assessing total CD45.2 engraftment of LT-HSCs, ST-HSCs and MPP cells.** For sorted transplant, 10 LT-HSCs, 50 ST-HSCs, and 100 MPPs WT or Ing4-/- WBM cells (CD45.2) were transplanted with $2x10_5$ competitor WBM cells (CD45.1) via retro-orbital injections into (*n = 60)* irradiated recipient (CD45.1) mice. Long-term engraftment (6 months). **P < 0.05; **P <0 .01; ***P < 0.001*, *nsP> 0.05.*

We conducted a mini screen of NF-kB inhibitors with known modes of action, to identify those that could compensate for the consequences of Ing4 loss of function. These experiments revealed that NF-kB inhibitor, MRT673007 significantly reduced expression of cytokines IL-1b, IL-4, IL-6, and IL-2R. While Senexin only reduced the expression of IL-1b and IL-4 (Fig. 3.8.), and Parthenolide reduced expression of II-1b, IL-4, and IL-6. All of these drugs have different modes of action. For example, MRT67307 is an IKKε inhibitor (Hassan, 2016), Senexin inhibits CDK8/19 (Chen, 2017), and Parthenolide is an IκB inhibitor (Saadane, 2007). All of which target components of the NF-κB pathway. As hypothesized, *in-situ* hybridization results demonstrated that NF-kB inhibitors instigated varying levels of dose-dependent rescue of the HSC marker, *runx1*, in the AGM of treated Ing4-/- zebrafish (Fig. 3.9.). Taken together, these data confirm that both genetic and chemical inhibition of NF-kB can partially rescue the defects caused by Ing4 deficiency in zebrafish.

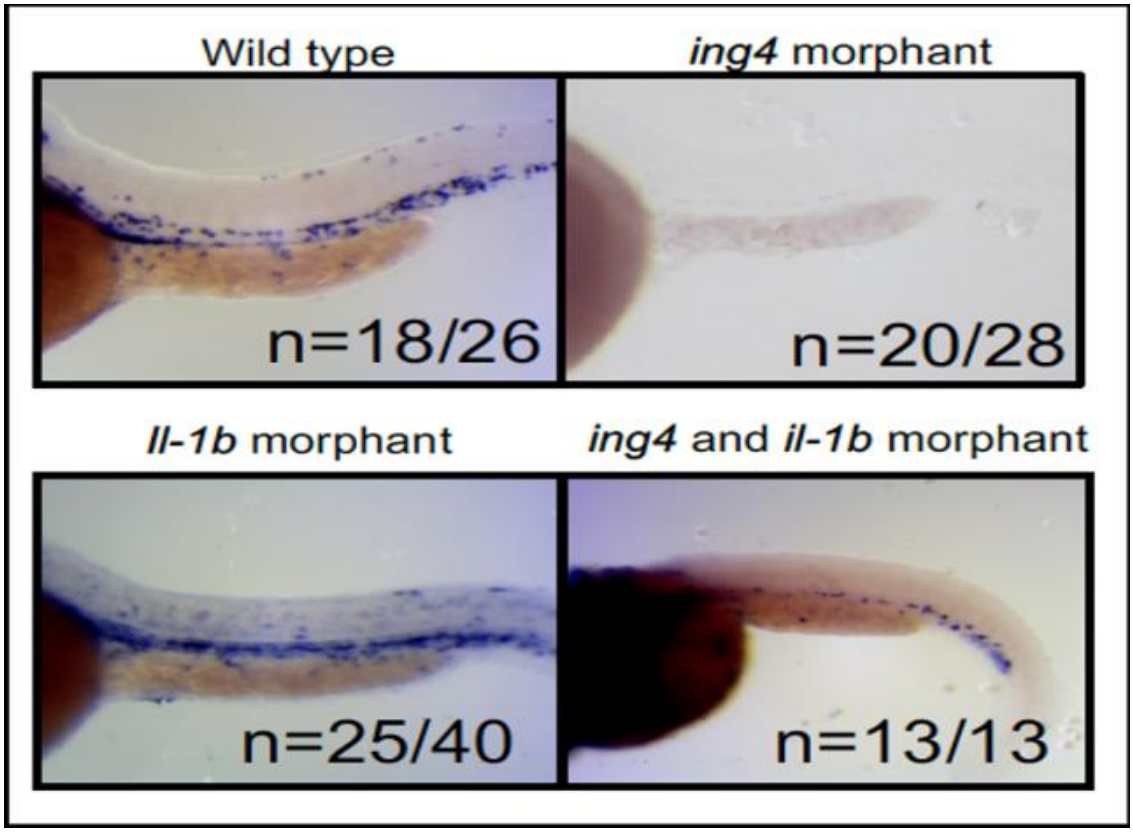

Figure 3.7. **Genetic inhibition of Ing4 and NF-kB target gene restores HSCs in the AGM.** HSC marker expression, *runx1* and *c-myb*, in the AGM region of embryonic zebrafish 36hpf is shown. **A** uninjected wildtype, **B** *ing4* morphant, **C** *Il-1b* morphant, **D** *ing4* and *Il-1b* double morphant.

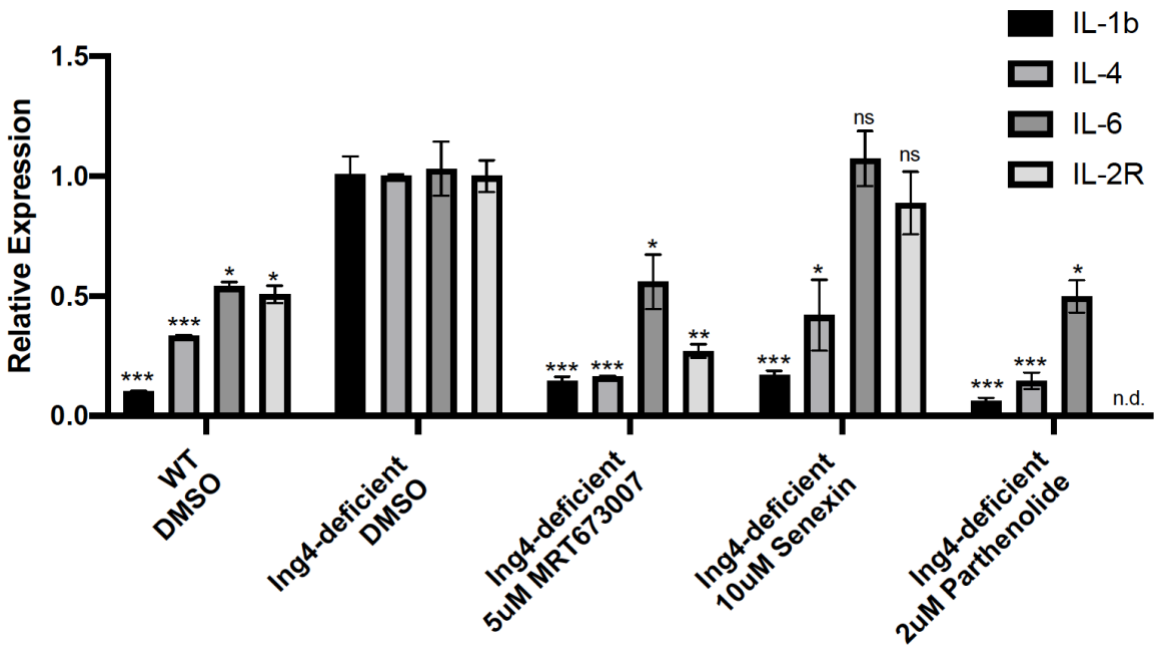

Figure 3.8. **Pilot chemical screen of NF-kB inhibitors, MRT673007, Senexin, and Parthenolide.** Graphical representation of qRT-PCR results from chemical inhibition of Ing4 is shown. WT and Ing4-deficient zebrafish were chemically treated with NF-kB inhibitors or DMSO. *Gapdh* (not pictured) was used as a housekeeping gene. IL-1b, IL-4, IL-6, and IL-2r are NF-kB target genes upregulated upon the loss of Ing4 in zebrafish. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$, $_{ns}P > 0.05$.

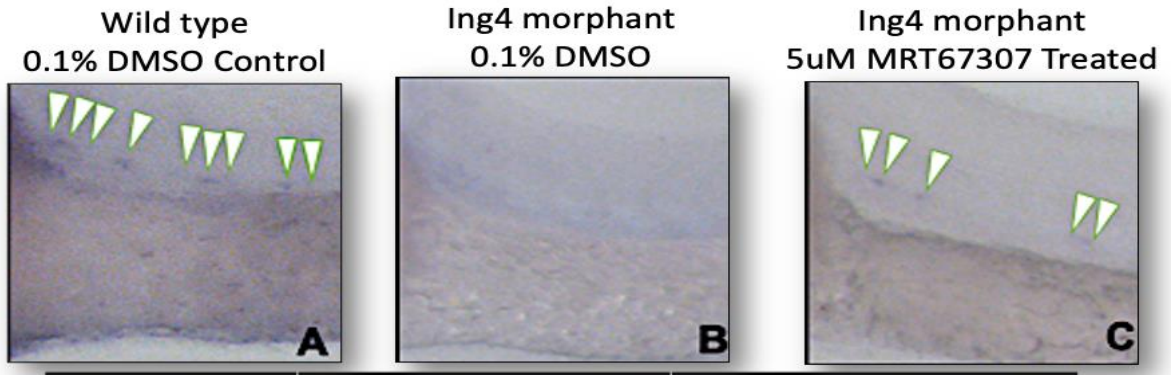

Figure 3.9. **Whole mount *in-situ* hybridization results of screen for small molecules that restore HSC specification.** Expression of HSC marker, *runx1,* in the AGM region of zebrafish embryos is shown. **A)** WT zebrafish treated with 0.1% DMSO control. **B)** Ing4 MO zebrafish treated with 0.1% DMSO. **C)** Ing4 MO zebrafish treated with MRT67307. Arrows indicate HSC marker staining.

CHAPTER 4

DISCUSSION

Hematopoietic stem cells are central to the blood program. Their unique ability to self-renew and differentiate into multipotent progenitor cells, gives rise to all the distinct blood lineages in a hierarchical manner, while maintaining homeostasis throughout an organism's life. This process is under the control of cell-specific transcription factors and chromatin remodeling proteins that regulate gene expression and consequently, stem cell fate. We previously have found that normal expression levels of Ing4 are required for HSC specification in zebrafish (Fig. 1.2.). However, the role of Ing4 in hematopoiesis has not yet been established.

In this study, we demonstrate Ing4 is critical in HSC self-renewal and differentiation. First, we analyzed the bone marrow and peripheral blood of Ing4-deficient and WT mice using CFU assays and flow cytometry in steady-state conditions. Using methylcellulose-based experiments gives us information about the ability of individual hematopoietic progenitor cells to proliferate and differentiate into a colony of lineage committed cells. Similarly, immunophenotyping via flow cytometry, identifies what lineages are present in mouse PB. In the CFU assays, it was observed that CFU-M (macrophage), CFU-GEMM (granulocyte, erythrocyte, monocyte, megakaryocyte), and CFU-GM (granulocyte, macrophage) colonies were significantly decreased in Ing4-/- BM cells (Fig. 3.1.). Contrastingly, in the PB of Ing4-/- mice, there was an increase in

macrophages (Fig. 3.2.). The differences we examined in the PB and the marrow can be explained by the nature of our model system. According to literature published on our Ing4-/- mouse model, these mice are hyper responsive to LPS treatment, a known activator of NF-κB signaling. It is likely that we see more macrophages circulating in the PB in these mice because of the activated inflammatory signaling upon the loss of Ing4. To explore this further, flow cytometry analysis of HSC lineage specification in the mouse BM will have to be done.

In addition, we sought to examine if there were changes in the progenitor compartment when Ing4 is inactivated. Compared to the WT, Ing4-deficient mice showed enhanced frequency of GMPs (granulocyte/macrophage progenitors) (Fig. 3.3.). This was interesting since we previously observed a decrease in macrophages in CFU assays. It is possible that the absence of Ing4 causes progenitor cells to become more quiescent or "stuck," thus losing the ability to efficiently differentiate into their lineage specific cells. Miyai et al. demonstrated that inactivation of early genes *Nr4a2, Klf4,* and *Egr1* in B-cell progenitors, led to the disruption of their genetic program and loss of B-cell identity (Miyai et al., 2018). Mature blood cells arise from pluripotent stem cells through a series of lineage restriction steps; therefore, it is likely that the absence of Ing4, a chromatin remodeling protein and transcription regulator, leads to inhibition of genes involved in transcriptional priming of GMPs.

Notably, both MEPs (megakaryocyte/erythroid progenitors) and CFU-GEMMs were decreased in Ing4-/- mice (Fig. 3.3.) (Fig. 3.1.). Since there was no significant difference between the erythrocytes in WT and Ing4-/-, we hypothesize that the myeloid-lineage cells contributing to this phenotype are megakaryocytes (Mk). Currently, the

antibody panels we use for immunofluorescence staining of HSPCs does not stain for Mks. In addition, a shortcoming of CFU assays using Methocult medium is that CFU-Mks can be difficult to distinguish morphologically from other cell lineages, such as macrophages (Nikougoftar, 2011). For further analysis, we will have to stain for Mk surface markers and determine what the distribution of this population is within the Ing4-deficient BM niche, in conjunction with a Megacult-C assay, which is a collagen-based system that optimally detects CFU-Mk progenitor cells in human and mouse samples. Taken together, these experiments elucidate that Ing4 regulates HSC specification and loss of function of Ing4 results in skewed differentiation.

In trying to uncover if Ing4 is a requirement for hematopoiesis, we evaluated HSC activity through WBM competitive transplantation assays in mice. We hypothesized that Ing4-deficient HSCs would rapidly lose quiescence and transiently repopulate recipient mice in response to enhanced stress. The efficacy of bone marrow reconstitution is determined in the PB output. Conversely, analysis of the PB of recipient mice, revealed similar short-term and long-term engraftment of WT and Ing4-/- donor-derived cells. This was interesting, as Ramalingam et al. demonstrated that chronic inflammation, via activation of NF-κB, resulted in poor long-term engraftment (Ramalingam, 2020). These results not only support a functional role for Ing4 in normal hematopoiesis, but they highlight the complex relationship between Ing4 and NF-κB and the possibility of other signaling pathways regulating hematopoiesis through their interaction with Ing4.

In addition, examination of the HSC-subpopulations in murine BM, revealed a shift towards LT-HSC/ST-HSCs and a drastic decrease in the MPP compartment (Fig. 3.4.) in the Ing4 null mice. To determine if the HSC-subpopulations maintained function, such as

reconstitution potential, we sorted mouse bone marrow into LT-HSCs, ST-HSCs and MPPs then transplanted the sorted cells into irradiated mice. Interestingly, compared to the WT, Ing4-/-LT HSCs failed to engraft, while Ing4-/-MPPs showed dramatic increase in peripheral blood multilineage chimerism up to 9 months post-transplantation (Fig. 3.6.). Though HSCs remain mainly dormant in the BM niche, they become activated in response to BM injury or inflammatory stimulation (Wilson et al., 2008) in order to self-renew and regenerate the blood system. Therefore, the inability of donor-derived Ing4-/-LT-HSCs to engraft long-term, indicates an evolutionary conserved requirement of Ing4 in self-renewal and differentiation of bona fide LT-HSCs and subsequently, their ability to reconstitute the marrow. In this context, Ing4-deficient LT-HSCs are either actively cycling thus rapidly exhausting due to increased inflammation or they are more quiescent, resulting in slower HSC expansion. Future cell cycle analysis can further elucidate this HSC activity.

Based on the engraftment ability of Ing4-/-MPPs in the BM, we hypothesize that Ing4 normally functions as a critical suppressor for genes required for self-renewal and developmental potency in MPPs, thus the absence of Ing4 results in reprogramming of MPPs to act like stem-cells. This is an exciting finding as MPPs are a cell population that have lost the self-renewal capacity of HSCs (Morrison et al., 1994). Moreover, it has been shown that there is heterogeneity within the MPP compartment. Pietras et al. demonstrated that this population is composed of distinct lineage biased MPPs that are produced in parallel by HSCs, but with different kinetics and at variable levels depending on the demands of hematopoiesis (Pietras et al., 2016). Given this, further characterization of Ing4-/- MPP cells is underway to determine which subset is contributing to the enhanced regeneration abilities observed in transplantations.

As previously mentioned, HSCs must find a careful balance between two inflammatory signaling extremes. Enhanced NF-κB activity is associated with decreased quiescence, rapid expansion of progenitor cells, and impaired long-term engraftment upon transplantation (Weisser et al., 2016) (Nakagawa et al., 2018) (Ramalingam et al., 2020). While fully activated HSCs expressing little NF-κB have low regeneration potential (Yamashita et al., 2019). Similar to our mouse model, we have generated an Ing4 morpholino zebrafish model that exhibits an inflammatory phenotype. Evident by the increase in relative expression of cytokines, IL-1b, IL-4, IL-6, and IL-2R. Specifically, our data shows that chemical inhibition of NF-κB in Ing4-deficient zebrafish, reduces cytokine expression and restores HSC specification in the AGM (Fig. 3.8.) (Fig. 3.9.). This correlates with what we have seen when genetically inhibiting NF-κB in this same model system (Fig. 3.7.). Overall, these results demonstrate that Ing4 regulates hematopoiesis through suppression of NF-κB, elucidating a molecular mechanism by which it functions during HSC specification. A major challenge for cancer researchers and clinicians is how to identify those patients that are most likely to benefit from NF-κB inhibitors in cancer therapy, thus the expansion of this pilot assay to examine various other targets and their ability to restore HSC specification, is promising for the treatment of Ing4-deficient cancers and hematological disorders.

To gain a better understanding of the molecular changes upon losing Ing4, RNA seq is a useful tool. By harvesting bone marrow and sorting the HSC-sub populations, this method would reveal genes that are upregulated/downregulated when Ing4 is inactivated. We expect to see an increase in some NF-κB target genes since loss of Ing4 causes hyper response of the innate immune system, but also a decrease in others because a subset is

regulated by H4 acetylation. We also expect an observed difference in expression of tumor suppressors such as p53, as Ing4 has been shown to complex with p53 and induce apoptosis (Shiseki, 2003), along with HIF-1α related genes. Ing4 is upstream of several known signaling pathways but we anticipate that RNA seq results will elucidate new pathways de-regulated by Ing4-deficiency.

In summary, our was aim was to define the role of Ing4 in developmental hematopoiesis in HSCs by examining the effects of Ing4 inactivation in mice in-*vitro* and in-*vivo*. The clinical importance of HSCs in marrow transplantations for the treatment of bone marrow failure, cancers, and autoimmune disorders, highlights the significance of understanding the mechanisms that regulate HSC function and their ability to engraft. Our research delineates the scope of altered hematopoiesis upon the loss of Ing4, lending us a blueprint for how this complex system is regulated and how it can be better targeted to improve stem cell function for therapeutic treatments.

REFERENCES

1. Back, J., Dierich, A., Bronn, C., Kastner, P., & Chan, S. (2004). PU.1 determines the self-renewal capacity of erythroid progenitor cells. *Blood*. https://doi.org/10.1182/blood-2003-11-4089
2. Baldridge, M. T., King, K. Y., Boles, N. C., Weksberg, D. C., & Goodell, M. A. (2010). Quiescent haematopoietic stem cells are activated by IFN-γ in response to chronic infection. *Nature*. https://doi.org/10.1038/nature09135
3. Baldridge, M. T., King, K. Y., & Goodell, M. A. (2011). Inflammatory signals regulate hematopoietic stem cells. In *Trends in Immunology*. https://doi.org/10.1016/j.it.2010.12.003
4. Barrio, L., Saez de Guinoa, J., & Carrasco, Y. R. (2013). TLR4 Signaling Shapes B Cell Dynamics via MyD88-Dependent Pathways and Rac GTPases. *The Journal of Immunology*. https://doi.org/10.4049/jimmunol.1301623
5. Belele, C. L., English, M. A., Chahal, J., Burnetti, A., Finckbeiner, S. M., Gibney, G., Kirby, M., Sood, R., & Liu, P. P. (2009). Differential requirement for Gata1 DNA binding and transactivation between primitive and definitive stages of hematopoiesis in zebrafish. *Blood*. https://doi.org/10.1182/blood-2009-05-224709

6. Cantor, A. B., & Orkin, S. H. (2002). Transcriptional regulation of erythropoiesis: An affair involving multiple partners. *Oncogene*. https://doi.org/10.1038/sj.onc.1205326
7. Chavakis, T., Mitroulis, I., & Hajishengallis, G. (2019). Hematopoietic progenitor cells as integrative hubs for adaptation to and fine-tuning of inflammation. In *Nature Immunology*. https://doi.org/10.1038/s41590-019-0402-5
8. Chen, B., Mao, B., Huang, S., Zhou, Y., Tsuji, K., & Ma, F. (2014). Human Embryonic Stem Cell-Derived Primitive and Definitive Hematopoiesis. In *Pluripotent Stem Cell Biology - Advances in Mechanisms, Methods and Models*. https://doi.org/10.5772/58628
9. Chen, M., Liang, J., Ji, H., Yang, Z., Altilia, S., Hu, B., Schronce, A., McDermott, M. S. J., Schools, G. P., Lim, C. U., Oliver, D., Shtutman, M. S., Lu, T., Stark, G. R., Porter, D. C., Broude, E. V., & Roninson, I. B. (2017). CDK8/19 Mediator kinases potentiate induction of transcription by NFκB. *Proceedings of the National Academy of Sciences of the United States of America*. https://doi.org/10.1073/pnas.1710467114
10. Chen, M. J., Yokomizo, T., Zeigler, B. M., Dzierzak, E., & Speck, N. A. (2009). Runx1 is required for the endothelial to haematopoietic cell transition but not thereafter. *Nature*. https://doi.org/10.1038/nature07619
11. Chen, Y., Huang, Y., Hou, P., Zhang, Z., Zhang, Y., Wang, W., Sun, G., Xu, L., Zhou, J., Bai, J., & Zheng, J. (2016). ING4 suppresses tumor angiogenesis and functions as a prognostic marker in human colorectal cancer. *Oncotarget*. https://doi.org/10.18632/oncotarget.12984

12. Chou, S. T., Khandros, E., Bailey, L. C., Nichols, K. E., Vakoc, C. R., Yao, Y., Huang, Z., Crispino, J. D., Hardison, R. C., Blobel, G. A., & Weiss, M. J. (2009). Graded repression of PU.1/Sfpi1 gene transcription by GATA factors regulates hematopoietic cell fate. *Blood*. https://doi.org/10.1182/blood-2009-03-207944
13. Clapes, T., Lefkopoulos, S., & Trompouki, E. (2016). Stress and non-stress roles of inflammatory signals during HSC emergence and maintenance. *Frontiers in Immunology*. https://doi.org/10.3389/fimmu.2016.00487
14. Coles, A. H., Gannon, H., Cerny, A., Kurt-Jones, E., & Jones, S. N. (2010). Inhibitor of growth-4 promotes IκB promoter activation to suppress NF-κB signaling and innate immunity. *Proceedings of the National Academy of Sciences of the United States of America*. https://doi.org/10.1073/pnas.0912116107
15. Coles, A. H., & Jones, S. N. (2009). The ING gene family in the regulation of cell growth and tumorigenesis. *Journal of Cellular Physiology*. https://doi.org/10.1002/jcp.21583
16. de Bruijn, M. F. T. R. (2000). Definitive hematopoietic stem cells first develop within the major arterial regions of the mouse embryo. *The EMBO Journal*. https://doi.org/10.1093/emboj/19.11.2465
17. Doyon, Y., Cayrou, C., Ullah, M., Landry, A. J., Côté, V., Selleck, W., Lane, W. S., Tan, S., Yang, X. J., & Côté, J. (2006). ING tumor suppressor proteins are critical regulators of chromatin acetylation required for genome expression and perpetuation. *Molecular Cell*. https://doi.org/10.1016/j.molcel.2005.12.007

18. Du, Y., Cheng, Y., & Su, G. (2019). The essential role of tumor suppressor gene ING4 in various human cancers and non-neoplastic disorders. *Bioscience Reports*. https://doi.org/10.1042/BSR20180773

19. Espin-Palazon, R., Weijts, B., Mulero, V., & Traver, D. (2018). Proinflammatory Signals as Fuel for the Fire of Hematopoietic Stem Cell Emergence. *Trends in Cell Biology*. https://doi.org/10.1016/j.tcb.2017.08.003

20. Essers, M. A. G., Offner, S., Blanco-Bose, W. E., Waibler, Z., Kalinke, U., Duchosal, M. A., & Trumpp, A. (2009). IFNα activates dormant haematopoietic stem cells in vivo. *Nature*. https://doi.org/10.1038/nature07815

21. Etzrodt, M., Ahmed, N., Hoppe, P. S., Loeffler, D., Skylaki, S., Hilsenbeck, O., Kokkaliaris, K. D., Kaltenbach, H. M., Stelling, J., Nerlov, C., & Schroeder, T. (2019). Inflammatory signals directly instruct PU.1 in HSCs via TNF. *Blood*. https://doi.org/10.1182/blood-2018-02-832998

22. Garkavtsev, I., Grigorian, I. A., Ossovskaya, V. S., Chernov, M. V., Chumakov, P. M., & Gudkov, A. V. (1998). The candidate tumour suppressor p33(ING1) cooperates with p53 in cell growth control. *Nature*. https://doi.org/10.1038/34675

23. Garkavtsev, I., Kazarov, A., Gudkov, A., & Riabowol, K. (1996). Suppression of the novel growth inhibitor p33(ING1) promotes neoplastic transformation. *Nature Genetics*. https://doi.org/10.1038/ng1296-415

24. Garkavtsev, I., Kozin, S. V., Chernova, O., Xu, L., Winkler, F., Brown, E., Barnett, G. H., & Jain, R. K. (2004). The candidate tumour suppressor protein ING4 regulates brain tumour growth and angiogenesis. *Nature*. https://doi.org/10.1038/nature02329

25. Gonda, T. J., & Metcalf, D. (1984). Expression of myb, myc and fos proto-oncogenes during the differentiation of a murine myeloid leukaemia. *Nature*. https://doi.org/10.1038/310249a0

26. Greco, C., Alvino, S., Buglioni, S., Assisi, D., Lapenta, R., Grassi, A., Stigliano, V., Mottolese, M., & Casale, V. (2001). Activation of c-myc and c-myb proto-oncogenes is associated with decreased apoptosis in tumor colon progression. *Anticancer Research*.

27. Griseri, T., McKenzie, B. S., Schiering, C., & Powrie, F. (2012). Dysregulated Hematopoietic Stem and Progenitor Cell Activity Promotes Interleukin-23-Driven Chronic Intestinal Inflammation. *Immunity*. https://doi.org/10.1016/j.immuni.2012.08.025

28. Hayashi, E. A., Akira, S., & Nobrega, A. (2005). Role of TLR in B Cell Development: Signaling through TLR4 Promotes B Cell Maturation and Is Inhibited by TLR2. *The Journal of Immunology*. https://doi.org/10.4049/jimmunol.174.11.6639

29. Hayashi, E. A., Granato, A., Paiva, L. S., Bertho, Á. L., Bellio, M., & Nobrega, A. (2010). TLR4 Promotes B Cell Maturation: Independence and Cooperation with B Lymphocyte-Activating Factor. *The Journal of Immunology*. https://doi.org/10.4049/jimmunol.0903253

30. Hsia, N., & Zon, L. I. (2005). Transcriptional regulation of hematopoietic stem cell development in zebrafish. *Experimental Hematology*. https://doi.org/10.1016/j.exphem.2005.06.013

31. Huang, H. T., Kathrein, K. L., Barton, A., Gitlin, Z., Huang, Y. H., Ward, T. P., Hofmann, O., Dibiase, A., Song, A., Tyekucheva, S., Hide, W., Zhou, Y., & Zon, L. I. (2013). A Network of Epigenetic Regulators Guides Developmental Haematopoiesis in Vivo. *Nature Cell Biology*. https://doi.org/10.1038/ncb2870

32. Hung, T., Binda, O., Champagne, K. S., Kuo, A. J., Johnson, K., Chang, H. Y., Simon, M. D., Kutateladze, T. G., & Gozani, O. (2009). ING4 Mediates Crosstalk between Histone H3 K4 Trimethylation and H3 Acetylation to Attenuate Cellular Transformation. *Molecular Cell*. https://doi.org/10.1016/j.molcel.2008.12.016

33. Iizuka, M., Sarmento, O. F., Sekiya, T., Scrable, H., Allis, C. D., & Smith, M. M. (2008). Hbo1 Links p53-Dependent Stress Signaling to DNA Replication Licensing. *Molecular and Cellular Biology*. https://doi.org/10.1128/mcb.00662-07

34. Ishida, T., Suzuki, S., Lai, C. Y., Yamazaki, S., Kakuta, S., Iwakura, Y., Nojima, M., Takeuchi, Y., Higashihara, M., Nakauchi, H., & Otsu, M. (2017). Pre-Transplantation Blockade of TNF-α-Mediated Oxygen Species Accumulation Protects Hematopoietic Stem Cells. *Stem Cells*. https://doi.org/10.1002/stem.2524

35. Kalkhoven, E., Teunissen, H., Houweling, A., Verrijzer, C. P., & Zantema, A. (2002). The PHD Type Zinc Finger Is an Integral Part of the CBP Acetyltransferase Domain. *Molecular and Cellular Biology*. https://doi.org/10.1128/mcb.22.7.1961-1970.2002

36. Kulessa, H., Frampton, J., & Graf, T. (1995). GATA-1 reprograms avian myelomonocytic cell lines into eosinophils, thromboblasts, and erythroblasts. *Genes and Development*. https://doi.org/10.1101/gad.9.10.1250

37. Kumamoto, K., Fujita, K., Kurotani, R., Saito, M., Unoki, M., Hagiwara, N., Shiga, H., Bowman, E. D., Yanaihara, N., Okamura, S., Nagashima, M., Miyamoto, K., Takenoshita, S., Yokota, J., & Harris, C. C. (2009). ING2 is upregulated in colon cancer and increases invasion by enhanced MMP13 expression. *International Journal of Cancer*. https://doi.org/10.1002/ijc.24437

38. Lan, C. C., Tang, R., Leong, I. U. S., & Love, D. R. (2009). Quantitative real-time RT-PCR (qRT-PCR) of zebrafish transcripts: Optimization of RNA extraction, quality control considerations, and data analysis. *Cold Spring Harbor Protocols*, *4*(10). https://doi.org/10.1101/pdb.prot5314

39. Li, M., Zhu, Y., Zhang, H., Li, L., He, P., Xia, H., Zhang, Y., & Mao, C. (2014). Delivery of inhibitor of growth 4 (ING4) gene significantly inhibits proliferation and invasion and promotes apoptosis of human osteosarcoma cells. *Scientific Reports*. https://doi.org/10.1038/srep07380

40. Lugus, J. J., Chung, Y. S., Mills, J. C., Kim, S. Il, Grass, J., Kyba, M., Doherty, J. M., Bresnick, E. H., & Choi, K. (2007). GATA2 functions at multiple steps in hemangioblast development and differentiation. *Development*. https://doi.org/10.1242/dev.02731

41. MacNamara, K. C., Oduro, K., Martin, O., Jones, D. D., McLaughlin, M., Choi, K., Borjesson, D. L., & Winslow, G. M. (2011). Infection-Induced Myelopoiesis during Intracellular Bacterial Infection Is Critically Dependent upon IFN-γ Signaling. *The Journal of Immunology*. https://doi.org/10.4049/jimmunol.1001893

42. Mann, M., Mehta, A., De Boer, C. G., Kowalczyk, M. S., Lee, K., Haldeman, P., Rogel, N., Knecht, A. R., Farouq, D., Regev, A., & Baltimore, D. (2018). Heterogeneous Responses of Hematopoietic Stem Cells to Inflammatory Stimuli are Altered with Age One sentence summary. *Cell Rep*, *25*(11), 2992–3005. https://doi.org/10.1016/j.celrep.2018.11.056

43. Medvinsky, A., & Dzierzak, E. (1996). Definitive hematopoiesis is autonomously initiated by the AGM region. *Cell*. https://doi.org/10.1016/S0092-8674(00)80165-8

44. Metcalf, D., Begley, C. G., Johnson, G. R., Nicola, N. A., Lopez, A. F., & Williamson, D. J. (1986). Effects of purified bacterially synthesized murine Multi-CSF (IL-3) on hematopoiesis in normal adult mice. *Blood*. https://doi.org/10.1182/blood.v68.1.46.bloodjournal68146

45. Miyagi, S., Sroczynska, P., Kato, Y., Nakajima-Takagi, Y., Oshima, M., Rizq, O., Takayama, N., Saraya, A., Mizuno, S., Sugiyama, F., Takahashi, S., Matsuzaki, Y., Christensen, J., Helin, K., & Iwama, A. (2019). The chromatin-binding protein Phf6 restricts the self-renewal of hematopoietic stem cells. *Blood*. https://doi.org/10.1182/blood.2019000468

46. Miyai, T., Takano, J., Endo, T. A., Kawakami, E., Agata, Y., Motomura, Y., Kubo, M., Kashima, Y., Suzuki, Y., Kawamoto, H., & Ikawa, T. (2018). Three-step transcriptional priming that drives the commitment of multipotent progenitors toward B cells. *Genes and Development*. https://doi.org/10.1101/gad.309575.117

47. Moore, M. A. S., & Metcalf, D. (1970). Ontogeny of the Haemopoietic System: Yolk Sac Origin of In Vivo and In Vitro Colony Forming Cells in the Developing Mouse Embryo. *British Journal of Haematology*. https://doi.org/10.1111/j.1365-2141.1970.tb01443.x

48. Moriguchi, T., & Yamamoto, M. (2014). A regulatory network governing Gata1 and Gata2 gene transcription orchestrates erythroid lineage differentiation. In *International Journal of Hematology*. https://doi.org/10.1007/s12185-014-1568-0

49. Morrison, S. J., & Weissman, I. L. (1994). The long-term repopulating subset of hematopoietic stem cells is deterministic and isolatable by phenotype. *Immunity*. https://doi.org/10.1016/1074-7613(94)90037-X

50. Mucenski, M. L., McLain, K., Kier, A. B., Swerdlow, S. H., Schreiner, C. M., Miller, T. A., Pietryga, D. W., Scott, W. J., & Potter, S. S. (1991). A functional c-myb gene is required for normal murine fetal hepatic hematopoiesis. *Cell*. https://doi.org/10.1016/0092-8674(91)90099-K

51. Müller, A. M., Medvinsky, A., Strouboulis, J., Grosveld, F., & Dzierzakt, E. (1994). Development of hematopoietic stem cell activity in the mouse embryo. *Immunity*. https://doi.org/10.1016/1074-7613(94)90081-7

52. Nagai, Y., Garrett, K. P., Ohta, S., Bahrun, U., Kouro, T., Akira, S., Takatsu, K., & Kincade, P. W. (2006). Toll-like Receptors on Hematopoietic Progenitor Cells Stimulate Innate Immune System Replenishment. *Immunity*. https://doi.org/10.1016/j.immuni.2006.04.008

53. Nagashima, M., Shiseki, M., Pedeux, R. M., Okamura, S., Kitahama-Shiseki, M., Miura, K., Yokota, J., & Harris, C. C. (2003). A novel PHD-finger motif protein, p47ING3, modulates p53-mediated transcription, cell cycle control, and apoptosis. *Oncogene*. https://doi.org/10.1038/sj.onc.1206115

54. Nakagawa, M. M., & Rathinam, C. V. (2018). Constitutive Activation of the Canonical NF-κB Pathway Leads to Bone Marrow Failure and Induction of Erythroid Signature in Hematopoietic Stem Cells. *Cell Reports*. https://doi.org/10.1016/j.celrep.2018.10.071

55. Nam, C. H., & Rabbitts, T. H. (2006). The role of LMO2 in development and in T Cell leukemia after chromosomal translocation or retroviral insertion. In *Molecular Therapy*. https://doi.org/10.1016/j.ymthe.2005.09.010

56. Nozell, S., Laver, T., Moseley, D., Nowoslawski, L., DeVos, M., Atkinson, G. P., Harrison, K., Nabors, L. B., & Benveniste, E. N. (2008). The ING4 Tumor Suppressor Attenuates NF-κB Activity at the Promoters of Target Genes. *Molecular and Cellular Biology*. https://doi.org/10.1128/mcb.00697-08

57. Orkin, S. H., & Zon, L. I. (2008). Hematopoiesis: An Evolving Paradigm for Stem Cell Biology. *Cell*. https://doi.org/10.1016/j.cell.2008.01.025

58. Ozer, A., Wu, L. C., & Bruick, R. K. (2005). The candidate tumor suppressor ING4 represses activation of the hypoxia inducible factor (HIF). *Proceedings of the National Academy of Sciences of the United States of America*. https://doi.org/10.1073/pnas.0502716102

59. Palis, J., & Yoder, M. C. (2001). Yolk-sac hematopoiesis: The first blood cells of mouse and man. *Experimental Hematology*. https://doi.org/10.1016/S0301-472X(01)00669-5

60. Passegué, E., Wagers, A. J., Giuriato, S., Anderson, W. C., & Weissman, I. L. (2005). Global analysis of proliferation and cell cycle gene expression in the regulation of hematopoietic stem and progenitor cell fates. *Journal of Experimental Medicine*. https://doi.org/10.1084/jem.20050967

61. Pearl-Yafe, M., Mizrahi, K., Stein, J., Yolcu, E. S., Kaplan, O., Shirwan, H., Yaniv, I., & Askenasy, N. (2010). Tumor necrosis factor receptors support murine hematopoietic progenitor function in the early stages of engraftment. *Stem Cells*. https://doi.org/10.1002/stem.448

62. Peña, P. V., Davrazou, F., Shi, X., Walter, K. L., Verkhusha, V. V., Gozani, O., Zhao, R., & Kutateladze, T. G. (2006). Molecular mechanism of histone H3K4me3 recognition by plant homeodomain of ING2. *Nature*. https://doi.org/10.1038/nature04814

63. Pietras, E. M. (2017). Inflammation: A key regulator of hematopoietic stem cell fate in health and disease. *Blood*, *130*(15), 1693–1698. https://doi.org/10.1182/blood-2017-06-780882

64. Pietras, E. M., Reynaud, D., Kang, Y. A., Carlin, D., Calero-Nieto, F. J., Leavitt, A. D., Stuart, J. A., Göttgens, B., & Passegué, E. (2015). Functionally Distinct Subsets of Lineage-Biased Multipotent Progenitors Control Blood Production in Normal and Regenerative Conditions. *Cell Stem Cell*. https://doi.org/10.1016/j.stem.2015.05.003

65. Pronk, C. J. H., Veiby, O. P., Bryder, D., & Jacobsen, S. E. W. (2011). Tumor necrosis factor restricts hematopoietic stem cell activity in mice: Involvement of two distinct receptors. *Journal of Experimental Medicine*. https://doi.org/10.1084/jem.20110752

66. Ramalingam P, Poulos MG, Lazzari E, et al. (2020). Chronic activation of endothelial MAPK disrupts hematopoiesis via NFKB dependent inflammatory stress reversible by SCGF. *Nature Communication.* https://doi.org/10.1038/s41467-020-14478-8

67. Saadane, A., Masters, S., DiDonato, J., Li, J., & Berger, M. (2007). Parthenolide inhibits IκB kinase, NF-κB activation, and inflammatory response in cystic fibrosis cells and mice. *American Journal of Respiratory Cell and Molecular Biology*. https://doi.org/10.1165/rcmb.2006-0323OC

68. Scott, E. W., Simon, M. C., Anastasi, J., & Singh, H. (1994). Requirement of transcription factor PU.1 in the development of multiple hematopoietic lineages. *Science*. https://doi.org/10.1126/science.8079170

69. Shen, J. C., Unoki, M., Ythier, D., Duperray, A., Varticovski, L., Kumamoto, K., Pedeux, R., & Harris, C. C. (2007). Inhibitor of growth 4 suppresses cell spreading and cell migration by interacting with a novel binding partner, liprin α. *Cancer Research*. https://doi.org/10.1158/0008-5472.CAN-06-3870

70. Shiseki, M., Nagashima, M., Pedeux, R. M., Kitahama-Shiseki, M., Miura, K., Okamura, S., Onogi, H., Higashimoto, Y., Appella, E., Yokota, J., & Harris, C. C. (2003). p29ING4 and p28ING5 bind to p53 and p300, and enhance p53 activity. *Cancer Research*.

71. Shivdasanl, R. A., Mayer, E. L., & Orkin, S. H. (1995). Absence of blood formation in mice lacking the T-cell leukaemia oncoprotein tal-1/SCL. *Nature*. https://doi.org/10.1038/373432a0

72. Silver, L., & Palis, J. (1997). Initiation of murine embryonic erythropoiesis: A spatial analysis. *Blood*. https://doi.org/10.1182/blood.v89.4.1154

73. Soza-Ried, C., Hess, I., Netuschil, N., Schorpp, M., & Boehm, T. (2010). Essential role of c-myb in definitive hematopoiesis is evolutionarily conserved. *Proceedings of the National Academy of Sciences of the United States of America*. https://doi.org/10.1073/pnas.1004640107

74. Takeuchi, M., Sekiguchi, T., Hara, T., Kinoshita, T., & Miyajima, A. (2002). Cultivation of aorta-gonad-mesonephros-derived hematopoietic stem cells in the fetal liver microenvironment amplifies long-term repopulating activity and enhances engraftment to the bone marrow. *Blood*. https://doi.org/10.1182/blood.V99.4.1190

75. Tie, R., Li, H., Cai, S., Liang, Z., Shan, W., Wang, B., Tan, Y., Zheng, W., & Huang, H. (2019). Interleukin-6 signaling regulates hematopoietic stem cell emergence. *Experimental and Molecular Medicine*, *51*(10), 1–12. https://doi.org/10.1038/s12276-019-0320-5

76. Tyrkalska, S. D., Pérez-Oliva, A. B., Rodríguez-Ruiz, L., Martínez-Morcillo, F. J., Alcaraz-Pérez, F., Martínez-Navarro, F. J., Lachaud, C., Ahmed, N., Schroeder, T., Pardo-Sánchez, I., Candel, S., López-Muñoz, A., Choudhuri, A., Rossmann, M. P., Zon, L. I., Cayuela, M. L., García-Moreno, D., & Mulero, V. (2019). Inflammasome Regulates Hematopoiesis through Cleavage of the Master Erythroid Transcription Factor GATA1. *Immunity*, *51*(1), 50-63.e5. https://doi.org/10.1016/j.immuni.2019.05.005

77. Wang, Q., Stacy, T., Binder, M., Marín-Padilla, M., Sharpe, A. H., & Speck, N. A. (1996). Disruption of the Cbfa2 gene causes necrosis and hemorrhaging in the central nervous system and blocks definitive hematopoiesis. *Proceedings of the National Academy of Sciences of the United States of America*. https://doi.org/10.1073/pnas.93.8.3444

78. Weisser, M., Demel, U. M., Stein, S., Chen-Wichmann, L., Touzot, F., Santilli, G., Sujer, S., Brendel, C., Siler, U., Cavazzana, M., Thrasher, A. J., Reichenbach, J., G Essers, M. A., Schw, J., & Grez, M. (2016). Hyperinflammation in patients with chronic granulomatous disease leads to impairment of hematopoietic stem cell functions. *The Journal of Allergy and Clinical Immunology*. https://doi.org/10.1016/j.jaci.2015.11.028https://doi.org/10.1016/j.jaci.2015.11.028

79. Welch, J. J., Watts, J. A., Vakoc, C. R., Yao, Y., Wang, H., Hardison, R. C., Blobel, G. A., Chodosh, L. A., & Weiss, M. J. (2004). Global regulation of erythroid gene expression by transcription factor GATA-1. *Blood*. https://doi.org/10.1182/blood-2004-04-1603

80. Wilson, A., Laurenti, E., Oser, G., van der Wath, R. C., Blanco-Bose, W., Jaworski, M., Offner, S., Dunant, C. F., Eshkind, L., Bockamp, E., Lió, P., MacDonald, H. R., & Trumpp, A. (2008). Hematopoietic Stem Cells Reversibly Switch from Dormancy to Self-Renewal during Homeostasis and Repair. *Cell*. https://doi.org/10.1016/j.cell.2008.10.048

81. Wontakal, S. N., Guo, X., Smith, C., MacCarthy, T., Bresnick, E. H., Bergman, A., Snyder, M. P., Weissman, S. M., Zheng, D., & Skoultchi, A. I. (2012). A core erythroid transcriptional network is repressed by a master regulator of myelo-lymphoid differentiation. *Proceedings of the National Academy of Sciences of the United States of America*. https://doi.org/10.1073/pnas.1121019109

82. Woo, H., Ha, S. D., Lee, S. B., Buratowski, S., & Kim, T. S. (2017). Modulation of gene expression dynamics by co-Transcriptional histone methylations. In *Experimental and Molecular Medicine*. https://doi.org/10.1038/emm.2017.19

83. Yamada, Y., Warren, A. J., Dobson, C., Forster, A., Pannell, R., & Rabbitts, T. H. (1998). The T cell leukemia LIM protein Lmo2 is necessary for adult mouse hematopoiesis. *Proceedings of the National Academy of Sciences of the United States of America*. https://doi.org/10.1073/pnas.95.7.3890

84. Yamaguehi, Y., Zon, L. I., Ackerman, S. J., Yamamoto, M., & Suda, T. (1998). Forced GATA-1 expression in the murine myeloid cell line M1: Induction of c-Mpl expression and megakaryocytic/erythroid differentiation. *Blood*. https://doi.org/10.1182/blood.v91.2.450.450_450_457

85. Yamashita, M., & Passegué, E. (2019). TNF-α Coordinates Hematopoietic Stem Cell Survival and Myeloid Regeneration. Cell stem cell, 25(3), 357–372.e7. https://doi.org/10.1016/j.stem.2019.05.019

86. Zarif, M. N., Soleimani, M., Abolghasemi, H., Amirizade, N., Abroun, S., & Kaviani, S. (2011). The high yield expansion and megakaryocytic differentiation of human umbilical cord blood CD133 + cells. *Cell Journal*.

87. Zhao, J. L., & Baltimore, D. (2015). Regulation of stress-induced hematopoiesis. In *Current Opinion in Hematology*. https://doi.org/10.1097/MOH.0000000000000149