Fall 12-2019

# Identification of Chromatin Regulators Perturbed in Hematopoietic Stem and Progenitor Cell Aging

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# IDENTIFICATION OF CHROMATIN REGULATORS PERTURBED IN HEMATOPOIETIC STEM AND PROGENITOR CELL AGING

By

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B.S. LUMS 2013

A DISSERTATION

Submitted in Partial Fulfillment of the

Requirements for the Degree of

Doctor of Philosophy

(in Biomedical Sciences)

The Graduate School

The University of Maine

December 2019

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Thesis Advisor: Dr. Jennifer Trowbridge

An Abstract of the Dissertation Presented
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As lifespan is increasing globally, there is a critical need to identify strategies to extend healthspan and prevent chronic diseases into older age. The long-term goal of my research is to identify novel strategies to ameliorate aging-induced decline in hematopoietic stem cell (HSC) function. HSCs give rise to all mature blood and immune cells. With age, HSCs undergo defects in their differentiation ability which correlates with a decline in immune function. Lack of comprehensive knowledge of gene regulatory and epigenetic mechanisms underlying this defect is a barrier to developing therapies to ameliorate aging-associated decline in HSC function. Therefore, my project focuses on understanding the gene regulatory mechanisms underlying this decline in HSC function. Before delving into the gene regulatory mechanisms that go awry with age, it is important to identify which mechanisms are important for the differentiation of HSCs to mature cells. The majority of screening approaches for the identification of novel genes and gene

regulatory elements rely on robust *in vitro* assays. I have demonstrated that one such assay widely used in the field to differentiate hematopoietic stem and progenitor cells (HSPCs) to B-lymphoid cells performs in a qualitative rather than a quantitative manner which calls into question interpretations of results of this assay. Also, by mining publicly available gene expression data sets and data from an unpublished shRNA knockdown screen, I have identified that the epigenetic regulator lysine acetyltransferase 6b (*Kat6b*) is important for HSC function as well as demonstrated that KAT6B levels are significantly decreased in expression in aged long term-hematopoietic stem cells (LT-HSCs) at the transcript and protein levels using qPCR and immunofluorescence. In addition, I have observed that knockdown of *Kat6b* leads to enhanced myeloid differentiation from LT-HSCs by using *in vitro* and *in vivo* assays which partially replicates aging-associated hematopoietic phenotypes. Transcriptome analysis suggests that *Kat6b* knockdown in LT-HSCs leads to dysregulation of differentiation signatures and an increase in inflammation. These data support increasing the levels of *Kat6b* as a novel therapeutic strategy for ameliorating aging-associated hematopoietic decline.

## DEDICATION

To my family, and in memory of the students who succumbed to challenges of mental health.

## ACKNOWLEDGEMENTS

I acknowledge my Ph.D advisor, Dr. Jennifer Trowbridge, for giving me the opportunity to perform this work and for supporting me in every way during the course of my graduate studies. I also express my gratitude to my current committee members: Dr. Christopher Baker, Dr. Luanne Peters, Dr. Derry Roopenian, Dr. Dustin Updike and past committee member, Dr. Mary Ann Handel, for their valuable advice during my Ph.D. I further acknowledge past and present members of the Trowbridge Lab for their help and support in conducting this research and in my graduate school career in general. I also appreciate the help of computational services, genetic engineering technologies, genome technologies, microscopy core and flow cytometry core at the Jackson Laboratory. I thank past and present Directors of GSBSE, Dr David Neivandt and Dr. Clarissa Henry respectively, for giving me the opportunity to be a part of GSBSE program. I appreciate Tammy Crosby's and Zhen Zhang's continuous support in administrative and logistic issues of the program. Lastly, I acknowledge members of the Braun Lab and the Lu Lab for their help.

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# CHAPTER 1

# INTRODUCTION

## 1.1. The hematopoietic system generates blood and immune cells

Blood development (hematopoiesis) is the process of generating and replenishing blood and immune cells throughout the lifespan of an organism (Orkin, 2000). The cellular products of the hematopoietic system can be broadly classified into three main lineage types: erythroid, myeloid and lymphoid lineages (Figure 1.1).

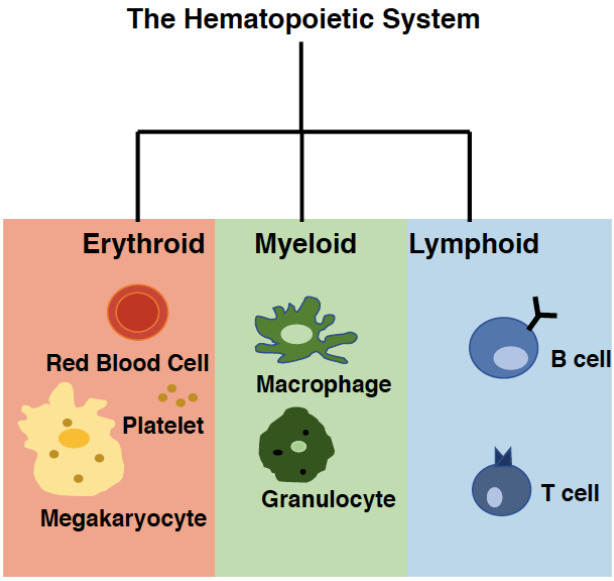



**Figure 1.1. The hematopoietic system generates immune and blood cells.**

The lymphoid lineage consists of B, T and natural killer (NK) cells. B and T cells constitute the adaptive part of the immune system whereas NK cells contribute to the innate immune system (Olsen Saraiva Camara, Lepique, & Basso, 2012). These lymphocytes execute their functions in several ways including direct interrogation of foreign pathogens and activation of components of the innate immune system like

macrophages (Olsen Saraiva Camara et al., 2012). The myeloid compartment consists of dendritic cells, monocytes, macrophages and, granulocytes (Bassler, Schulte-Schrepping, Warnat-Herresthal, Aschenbrenner, & Schultze, 2019). These cells contribute to the immune system by phagocytosis of foreign particles and by secreting inflammatory cytokines (Kawamoto & Minato, 2004). The erythroid lineage broadly incorporates megakaryocytic and erythroid lineages which are responsible for the production of platelets and red blood cells respectively. A rare population of cells in the bone marrow, HSCs, is responsible for generating all these cells through by tightly regulated processes of differentiation and self-renewal (Orkin, 2000) where self-renewal consists of stem cells dividing to produce more stem cells (He, Nakada, & Morrison, 2009).

### 1.1.1. Historical identification of HSCs

Roots of stem cells can be traced to Ernst Haeckel, a German Biologist, who first employed the term "Stammzelle" (stem cell) to represent a unicellular organism from which all other organisms evolved (Ramalho-Santos & Willenbring, 2007). He later proposed that a fertilized egg can be also described as a stem cell (Ramalho-Santos & Willenbring, 2007). Later, Boveri proposed that cells that lie on the developmental trajectory between a fertilized egg and committed germ cells should be called stem cells. These ideas represented the first characteristic of a stem cell, that is, the ability to give rise to multiple cell lineages. Later, Häcker observed that a particular cell in *Cyclops*, which he also called a stem cell, went through asymmetric differentiation and gave rise to mesoderm and a germ cell (Ramalho-Santos & Willenbring, 2007). This provided the second key characteristic of stem cells, that is, the ability to self-renew. Although, we now know that "stem cells" mentioned in these early studies represented germline stem

cells (Ramalho-Santos & Willenbring, 2007), these studies were critical in establishing the key characteristics of stem cells. Roughly at the same time the aforementioned ideas were being investigated, the identification of different types of white blood cells by Paul Ehlrich had sparked a debate about the existence of a single precursor of the hematopoietic system (Ramalho-Santos & Willenbring, 2007). One school of thought held the theory that myeloid and lymphoid cells originated from distinct progenitors and took place in distinct anatomical locations, whereas the other school of thought held the opinion that myeloid, erythroid and lymphoid cells originate from a single precursor (Ramalho-Santos & Willenbring, 2007).

This issue was resolved when key studies done in the 1960s by Till and colleagues and others showed the presence of a single hematopoietic progenitor cell which could give rise to myeloid, erythroid and lymphoid cells and had the ability to self-renew. Transplantation of nucleated bone marrow cells in irradiated recipients produced colony forming units (CFUs) in recipients' spleens (Till & McCulloch, 2012). These CFUs consisted of erythroblast, myelocytes, metamyelocytes as well as megakaryocytes (Till & McCulloch, 2012). Similar experiments also provided evidence that these CFUs in the spleen (CFU-S) could also give rise to the lymphoid cells in the thymus (Wu, Till, Siminovitch, & McCulloch, 1968). These were the first reports which provided evidence for the existence of cells in the bone marrow having the ability to differentiate into the three hematopoietic lineages, which is one of the key characteristics of HSCs. When CFU-S were harvested from spleens of recipient mice and transplanted into secondary recipients, spleens of these recipients also harbored CFU-S (Siminovitch, McCulloch, & Till, 1963), which further showed that progenitor cells exist in the bone marrow that have

multilineage differentiation as well as self-renewal abilities. However, these studies still did not provide proof that a single progenitor cell in the bone marrow had the ability to give rise to a complete CFU-S. This proof was provided when donor bone marrow cells were irradiated before transplantation into recipients (Becker, McCulloch, & Till, 1963). Irradiation of these donor bone marrow cells resulted in each cell having a distinct karyotype. The cells within a single CFU-S colony in recipients were observed to contain similar karyotypes (Becker et al., 1963). This study showed that CFU-S arises from a single progenitor cell in the bone marrow of donor mice which had multilineage differentiation and self-renewal ability. Therefore, these studies provided the evidence, in the context of spleen colony units, for the existence of a stem cell that satisfied the key characteristics of HSC. However, the identity of HSCs was not revealed until cells were prospectively isolated by distinct cell surface markers (Weissman & Shizuru, 2008), starting from the enrichment of HSCs based on the $Sca1^{+}$ cell surface maker (Spangrude, Heimfeld, & Weissman, 1988). This ability to isolate HSCs allowed elucidation of the journey of HSCs during development and adult stages.

### 1.1.2. Developmental hematopoiesis

Hematopoiesis in vertebrates occurs in two waves; the primitive wave and the definitive wave (Galloway & Zon, 2003). The primitive wave generates unipotent progenitors like nucleated erythrocytes whereas the definitive wave involves the production of multipotent progenitors like HSCs (Dzierzak & Bigas, 2018; Galloway & Zon, 2003). The definitive wave can sustain hematopoiesis for the lifespan of an organism. These two waves of hematopoiesis take place in distinct anatomical locations (Galloway & Zon, 2003) which will be described in the following sections.

#### 1.1.2.1. Primitive hematopoiesis

The hematopoietic system in mice arises in the mesodermal germ layer (Dzierzak & Medvinsky, 1995). This primitive hematopoiesis primarily produces cells of erythroid, megakaryocytic and myeloid lineages (Palis, 2016) with limited lymphoid potential, and originates from the yolk sac blood islands which are considered to be the first site for development of blood and vascular systems in vertebrate embryos (Ferkowicz & Yoder, 2005). In mice, blood islands, also known as mesodermal cells masses or angioblastic cords, originate from mesodermal cells in the yolk sac between E7-E7.5 (Palis & Yoder, 2001). Visceral endoderm in the yolk sac provides the signals which lead to formation of blood cells (Palis & Yoder, 2001). Extra-embryonic mesodermal sheet gives rise to mesodermal masses which serve as precursors for blood islands which contain common endothelial and hematopoietic progenitors called hemangioblasts.(Ferkowicz & Yoder, 2005). Hemangioblasts give rise to a common erythroid and megakaryocyte progenitor (Tober et al., 2007).

The cells on the outer layer of the blood island differentiate into endothelial cells whereas inner cells differentiate into primitive erythroblasts (Palis & Yoder, 2001). These erythroblasts start circulation in the embryo proper when cardiac contractions start at E8.25 (Palis, 2016). These primitive erythroblasts go through several changes involving reduction in cell size, production of hemoglobin, mitochondria loss, nuclear condensation and, express embryonic globin genes (Palis, 2016). Primitive erythroblasts become enucleated around E12.5-E16.5 and are found to be in circulation a few days after birth (Palis, 2016).

Megakaryocyte progenitors also start to appear around E7.5-E10.5 with limited potential for endoreduplication (Tober et al., 2007), which is the process of replication of the genome without cell division (Ullah, Lee, Lilly, & DePamphilis, 2009). A limited number of platelets are also found to be circulating starting from E9.5 (Palis, 2016).

Similar to primitive megakaryocyte cells, primitive myeloid cells originate at E7.25 having predominantly macrophage differentiation potential (Palis, 2016). However, myeloid progenitors with neutrophil, mast cell, and granulocyte-macrophage differentiation potential start to appear from E8.25 (Palis, 2016). In addition, E8 - E9 yolk sac was found to have B and T-cell reconstitution potential in recipient mice which were deficient for B and T-cells (Palacios & Imhof, 1993). Tracking of labeled yolk sac cells in recipient mice also revealed that these cells gave rise to adult HSCs (Samokhvalov, Samokhvalova, & Nishikawa, 2007). Therefore, these studies showed that after E8-9 hematopoietic progenitor cells in the yolk can be defined as stem cells because of their multilineage differential and self-renewal potential.

**1.1.2.2. Definitive hematopoiesis**

The aorta, gonad, mesonephros (AGM) region is the primary site for definitive hematopoiesis (Zon, 1995). Cells from the AGM were observed to have 200 times more CFU-S as compared to yolk sac on day 10 days post coitum (DPC) in mice (Medvinsky, Samoyllna, Miillert, & Dzlerzakt, 1993; Sánchez, Holmes, Miles, & Dzierzak, 1996) which shows that cells from the AGM contain cells with characteristics similar to the HSCs. CFU-S content starts to decline in the AGM from DPC 11 which coincides with an increase in CFU-S in the fetal liver (Medvinsky et al., 1993). In addition, cells from the aorta were found to migrate into the lumen of the aorta which showed the exit of

potential HSCs towards fetal liver (Galloway & Zon, 2003). This results in the fetal liver becoming the next site for the hematopoietic development.

The fetal liver provides the next niche for definitive hematopoiesis where hematopoietic progenitors undergo expansion and maturation (Ciriza, Thompson, Petrosian, Manilay, & García-Ojeda, 2013). At around 12.5 DPC HSCs go through expansion and differentiation mainly in the fetal liver (Mikkola & Orkin, 2006). Initially, fetal liver hematopoiesis focuses on the production of erythroid cells, which later incorporates the production of myeloid and lymphoid cells as well (Mikkola & Orkin, 2006).

As mentioned before, nucleated erythroblasts decline and nonnucleated erythroid cells increase in circulation by 12 DPC (Galloway & Zon, 2003). HSCs start to populate long bones from E17.5 (Mikkola & Orkin, 2006).

Fetal liver HSCs contain several differences as compared to adult HSCs (Beaudin et al., 2016; Bowie et al., 2007; Matsuoka et al., 2001). Transplantation of $CD34^-$ and $CD34^+$ HSCs from fetal liver and adult bone marrow in irradiated mice showed that fetal HSCs are marked with $CD34^+$ whereas adult HSCs (after 8 weeks of age) are marked by $CD34^-$ (Matsuoka et al., 2001). Besides, fetal HSCs have a higher capacity for regeneration of HSCs as compared to their 4-week old counterparts (Bowie et al., 2007). This is accompanied by higher expression of genes associated with HSC cycling/self-renewal (e.g. IKAROS family zinc finger 1 (*Ikaros*), Polyhomeotic 1 (*Rae-28*)) in fetal HSCs as compared to adult HSCs (Bowie et al., 2007). Fetal HSCs also produced a higher percentage of myeloid cells when transplanted into irradiated recipients (Bowie et al., 2007). These observations show that fetal HSCs perform the function of generating a

higher number of HSCs which can sustain hematopoiesis for the lifespan for an organism. However, in adulthood, the focus of the hematopoietic system switches to the maintenance of numbers of HSCs.

### 1.1.3. Bone marrow hematopoiesis

Adult HSCs are responsible for production of all mature blood and immune cells. However, the differentiation pathways that lead to mature cells from HSCs have been revised several times (Y. Zhang, Gao, Xia, & Liu, 2018). In the 2000s, HSCs were considered to undergo sequential differentiation steps producing progenitors with successive restriction in self-renewal and differentiation potential ultimately leading to the generation of mature cells (Adolfsson et al., 2005; Akashi K, Traver D, Miyamoto T, & IL, 2000; Kondo, Weissman, & Akashi, 1997; Morrison, Wandycz, Hemmati, Wright, & Weissman, 1997; L. Yang et al., 2005).

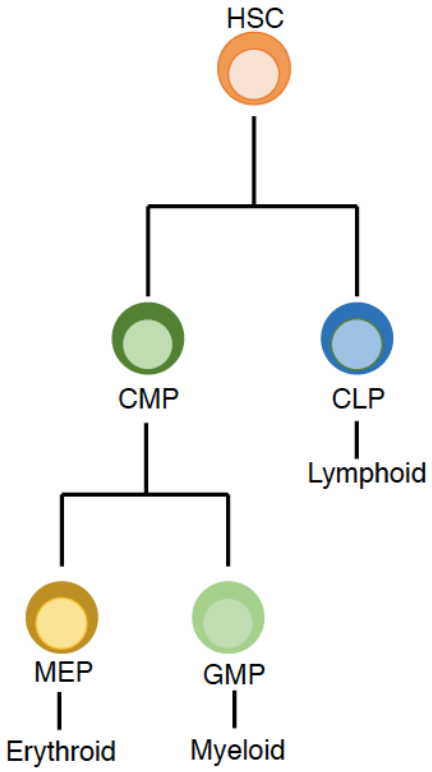



**Figure 1.2. The classical hierarchy of the hematopoietic system.**

According to this classical view of the hematopoietic hierarchy (Figure 1.2), LT-HSCs have long term reconstitution ability which was assessed by multilineage chimerism of donor LT-HSCs for 15 weeks after being transplanted into the primary recipient mice (Christensen & Weissman, 2001; Mastake, Hanada, Hamada, & Nakauchi, 1996) as well as self-renewal ability which was assessed by chimerism in the secondary recipients (Smith, Weissman, & Heimfeldtt, 1991). These LT-HSCs then differentiate into short-term HSCs (ST-HSCs) which still retain multilineage differentiation ability but do not have long term (greater than 15-16 weeks) reconstitution ability like LT-HSCs (L. Yang et al., 2005). ST-HSCs have faster kinetics of reconstituting recipients as compared to LT-HSCs (L. Yang et al., 2005), suggesting that ST-HSCs might contribute more towards steady-state hematopoiesis as compared to LT-HSCs. ST-HSCs give rise to

multipotent progenitors (MPPs) which have multi-lineage reconstitution ability with no self-renewal potential (Y. Zhang et al., 2018). After MPPs, myeloid and lymphoid lineages separate into lineage-committed progenitors; common lymphoid progenitors (CLPs) and common myeloid progenitors (CMPs) respectively (Akashi K et al., 2000; Kondo et al., 1997). CLPs, identified by the expression of the interleukin 7 receptor (IL7RA) marker, were found to harbor B and T-cell reconstitution ability with no myeloid reconstitution ability *in vivo* (Kondo et al., 1997) and expressed low levels of genes like GATA binding protein 3 (*Gata-3*) and paired box 5 (*Pax5*) which are important for the lymphoid lineage (Akashi K et al., 2000). Whereas, CMPs, identified by the absence of IL7RA marker, were found to have *in vitro* and *in vivo* myeloid and erythroid differentiation ability (Akashi K et al., 2000) but had no B and T-cell reconstitution ability (Boyer et al., 2019). CMPs gave rise to granulocyte-macrophage progenitors (GMPs) and megakaryocyte erythrocyte progenitors (MEPs). Transplantation of MEPs in irradiated recipients only produced $TER119^+$ erythroid cells, whereas GMPs only produced $MAC1^+/GR1^+$ myeloid cells in similar experiments (Akashi K et al., 2000). GMPs and MEPs were found to express genes important for myeloid and erythroid lineages respectively (Akashi K et al., 2000). Therefore, according to these earlier studies HSCs went through stepwise restriction of lineage fates starting from separation of the lymphoid lineage from the myeloid/erythroid lineages, followed by the separation of the erythroid lineage from the myeloid lineage.

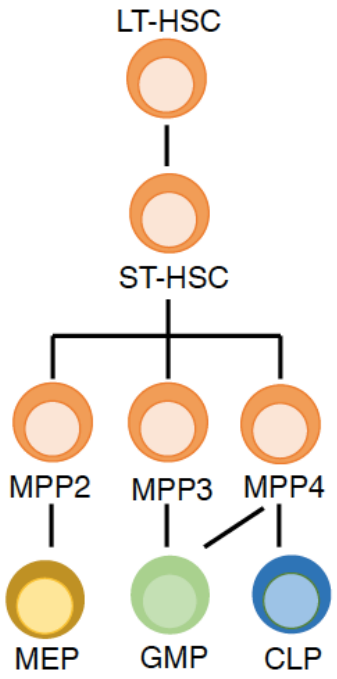



**Figure 1.3. The revised hierarchy of the hematopoietic system.**

The aforementioned classical view of the hematopoietic hierarchy began to be revised (Figure 1.3) with the identification and characterization of different types of MPPs (Cabezas-Wallscheid et al., 2014; Pietras et al., 2015b; Wilson et al., 2008). New MPPs (MPP1, MPP2, MPP3, and MPP4) were identified by varied expression patterns of CD150, CD48, and CD135 cell surface markers (Wilson et al., 2008). HSPCs were defined by following cell surface markers: LT-HSCs (lineage$^-$ Sca1$^+$ cKit$^+$ Flt3$^-$ CD150$^+$ CD48$^-$), ST-HSCs (lineage$^-$ Sca1$^+$ cKit$^+$ Flt3$^-$ CD150$^-$ CD48$^-$), MPP2 (lineage$^-$ Sca1$^+$ cKit$^+$ Flt3$^-$ CD150$^+$ CD48$^+$), MPP3 (lineage$^-$ Sca1$^+$ cKit$^{+ve}$ Flt3$^-$ CD150$^-$ CD48$^+$) and MPP4s (lineage$^-$ Sca1$^+$ cKit$^+$ Flt3$^+$ CD150$^-$) (Pietras et al., 2015a).

These MPPs also showed different quiescence and cell cycling properties, with MPP1s being the most quiescent and MPP2, MPP3, and MPP4s showing higher cycling (Wilson et al., 2008). Using a combination of *in vitro* CFU and *in vivo* reconstitution

experiments, MPP2s were found to be biased towards the megakaryocyte/erythroid lineage, MPP3s were biased towards myeloid lineages whereas MPP4s had a lymphoid bias (Pietras et al., 2015a). However, all of these MPPs were found to have minor contributions towards the rest of the two lineages which they were not biased towards: MPP2s had myeloid and lymphoid contributions, MPP3s had lymphoid and erythroid contributions, and MPP4s had myeloid and erythroid differentiation potential (Pietras et al., 2015a). These studies provided evidence against strict restriction of lineage potential during differentiation of HSCs to mature cells which had been presented by earlier studies.

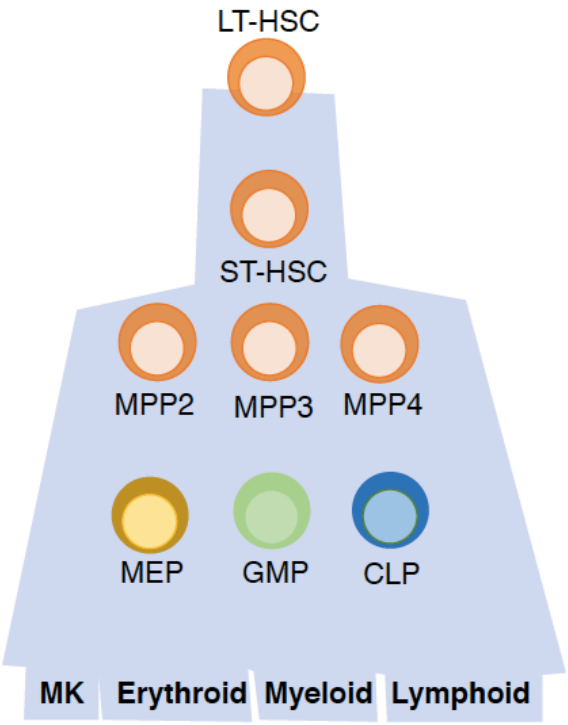



**Figure 1.4. Hematopoiesis is a process of continuous differentiation.**

The advent of single-cell genome technologies enabled a more thorough interrogation of the differentiation potential of hematopoietic cells (Y. Zhang et al.,

2018). Single-cell RNA-seq (scRNA-seq) of HSPCs has now shown that the differentiation potential of these cells changes along a continuum rather than discrete states in mice (Dahlin et al., 2018). These findings were also observed in human hematopoiesis, where investigation of single-cell chromatin accessibility (scATAC-seq) revealed a continuous relationship among hematopoietic cells and also showed heterogeneity among cell populations which were considered to be homogenous by characteristic cell surface maker patterns (Buenrostro et al., 2018). These studies support a model of hematopoiesis in which HSPCs lie on a continuous spectrum of differentiation potential rather than following discrete steps which then ultimately produces mature cells (Figure 1.4).

### 1.1.4. Assays for assessing differentiation of HSPCs

Studies involving hematopoiesis have relied on a variety of *in vitro* and *in vivo* assays for assessing the functional potential of HSPCs.

#### 1.1.4.1. *In vitro* assays

*In vitro* assays for interrogating the differentiation potential of HSPCs can be broadly classified into liquid culture assays and semi-solid methylcellulose-based assays. Liquid culture-based assays involve culturing of HSPCs in presence of growth factors which promote differentiation to towards each lineage or combination of erythroid/myeloid and lymphoid lineages which is followed by an assessment of the differentiation potential by flow cytometry (Adolfsson et al., 2005; Young et al., 2016a). For assessment of B-lymphoid differentiation potential, co-culture with OP-9 stromal cells has been employed (Nakano, Kodama, & Honjo, 1994; Pietras et al., 2015a; Viera & Cumano, 2004). Colony forming unit (CFU) assays provide a measure of number and

type of progenitor cells by using defined methylcellulose media (Purton & Scadden, 2007). Specific methylcellulose media are commercially available which promote differentiation to myeloid, erythroid or lymphoid lineages. These CFU assays produce colonies whose number and morphology give a measure of the respective type of progenitor present in the population of cells that was plated in the assay.

#### 1.1.4.2. *In vivo* assays

*In vivo* assays for assessment of hematopoietic stem/progenitor cells involve transplantation of the respective population into myeloablated recipients (Adolfsson et al., 2005; Pietras et al., 2015a). The ability of transplanted cells to reconstitute the recipient hematopoietic system is measured by the peripheral blood composition of the recipient mice by flow cytometry. For assessment of long-term reconstitution potential transplanted mice are observed for 4-6 months (Purton & Scadden, 2007). For determination of true hematopoietic stem cell function involving self-renewal, bone marrow from primary recipients is transplanted into secondary myeloablated recipients to assess multilineage reconstitution potential. The ability to reconstitute secondary recipients shows self-renewal potential of the transplanted population.

## 1.2. Changes in the hematopoietic system with age

### 1.2.1 Functional changes in the immune system with age

Aging involves a progressive decline in a variety of cellular systems which results in a decline in the healthspan, the period of time from birth until the organism remains free from chronic diseases and aging-associated diseases (Kaeberlein, 2018). Among these changes, reduction in immune system function has a significant contribution to the shortening of healthspan. As the immune system is responsible for detection and

neutralizing pathogens and foreign particles (Parkin & Cohen, 2001), the elderly become more susceptible to infections leading to more frequent and severe infections (Dorshkind, & Swain, 2009). For example, 80-90% of the mortalities due to infection by influenza occur in individuals 65 years or older (Dorshkind, Montecio-Rodriguez, & Signer, 2009). Aging also involves deterioration in the efficiency of vaccination and the diminished ability of immune cells to detect malignant cells (Ponnappan & Ponnappan, 2011). In the context of human bone marrow transplantation, marrow from older individuals is less successful at reconstituting the immune system in recipients (Dorshkind et al., 2009). Another functional consequence of aging is an increase in the incidence of anemia, which is linked to dementia and cardiovascular disease (Ferrucci & Balducci, 2003).

### 1.2.2. Changes in the adaptive immune system with age

Functional degeneration of the immune system is associated with changes in both the adaptive and innate systems. The adaptive immune system consists of antigen-specific responses to pathogens mounted by T and B lymphocytes, whereas the innate immune system involves chemical, physical and microbiological barriers and cellular responses by monocytes, neutrophils, macrophages, complement, cytokines and acute phase proteins (Parkin & Cohen, 2001). Age-associated regression in adaptive immunity involves changes in both T cell and B cells. With age, T cell proliferation decreases *in vitro* and *in vivo* and number of naive T cells decrease with a corresponding increase in memory T cells (Miller, 1996). Contrary to T cells, overall numbers of B cells in peripheral lymphoid organs do not change with age (Kogut, Scholz, Cancro, & Cambier, 2012). However, there is an increase in the proportion of B cells derived from a relatively small number of clones (Miller, 1996). Germinal centers are a collection of B-cells that

are responsible for the generation of diverse and specific antibodies which also involves somatic hypermutation (Victora & Nussenzweig, 2012). Antibody diversity (generated by hypermutation in germinal centers) also diminishes with age and overall antibody production declines as well (Miller, 1996).

### 1.2.3. Changes in the innate immune system with age

Activation of the innate immune system with age results in misregulation of inflammatory responses (Shaw, Goldstein, & Montgomery, 2013). This involves upregulation of pro-inflammatory cytokines like interleukin-6 (IL-6), interleukin-18 (IL-18), interleukin-1β (IL-1β) and tumor necrosis factor (TNFα) (Mcmichael, Simon, & Hollander, 2015). Neutrophils and macrophages are effectors of the innate immune system (Solana, Pawelec, & Tarazona, 2006). Neutrophils are the first to arrive at sites infected by pathogens, and their ability to migrate in and out of infectious sites decreases with age (Shaw et al., 2013). Aging is also associated with a decline in the phagocytotic ability of both neutrophils and macrophages as well as decreased cytotoxic activity towards phagocytosed pathogens (Shaw et al., 2013).

### 1.2.4. Changes in HSCs with age:

Intuitively, the frequency of HSCs should decrease with age, which would correspond with a decline in the immune function. However, quantification of HSCs with different types of HSC specific staining schemes shows that the frequency of HSCs actually increases with age in mice (Dykstra, Olthof, Schreuder, Ritsema, & De Haan, 2011; Morrison, Andycz1, & Weissman, 1996; Rodrigues, Maciel-Filho, Asenjo, Zaror, & Maugeri, 1997; Sudo, Ema, Morita, & Nakauchi, 2000) and humans (Taraldsrud et al., 2009). Increased variability in the frequency of HSCs among old mice was also observed

(Dykstra et al., 2011). However, increased HSCs' frequency is not followed by a corresponding increase in the function of HSCs. Approximately ten HSCs from 2-14-month old mice were able to achieve 63% limiting dilution frequency (the frequency of transplanted cells at which 63% of transplanted recipients showed reconstitution of hematopoietic system) of irradiated recipients when co-injected with 2 x $10^5$ support cells, whereas 40 HSCs from 24-month-old mice were required to achieve the same results (Morrison et al., 1996). Reduction in the ability of individual HSC to form colonies was also shown by decreased cobblestone area forming capacity, which measured the in vitro differentiation ability of stem cells (Theilgaard-Mönch, 2008), of old HSCs (Dykstra et al., 2011). This shows that the overall reconstitution ability of HSCs decreases with age. In addition to increases in numbers of HSCs, the lineage capacity of HSCs also changes with age. HSCs from old mice are biased towards the myeloid lineage at the expense of the lymphoid lineage (Chung & Park, 2017). Transplantation of old HSCs compared to young HSCs leads to higher myeloid reconstitution and lower lymphoid reconstitution capacity which included increased percentages of GMPs and decreased percentages of CLPs (Rodrigues et al., 1997). Therefore, aged HSCs have higher myeloid and lower lymphoid differentiation potential (Figure 1.5).

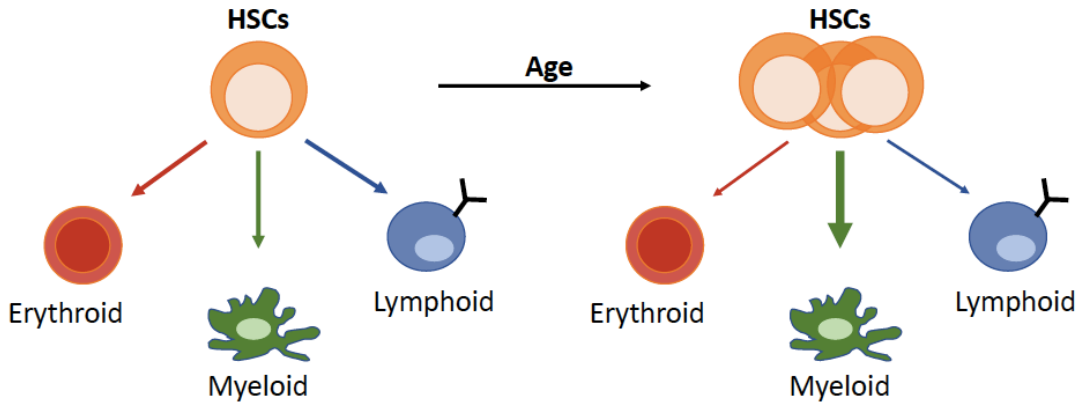



**Figure 1.5. Cellular changes in HSC lineage potential with age.**

However, these studies involved transplantations that are conducted under non-physiological conditions involving isolation of cells from their normal environment inside the bone marrow and exposure various kind of stresses in the process. The results of those experiments might not provide an accurate picture of HSC function in endogenous conditions (Säwen et al., 2018). Native labeling, which involves labelling of the cells in endogenous niches like bone marrow, of old and young HSCs has shown that multilineage differentiation ability decreases with age (Säwen et al., 2018). The frequency of platelet-biased HSCs increases with age as well, along with enrichment for gene signatures corresponding to pre-MegE (megakryocyte/erythroid) and megakaryocyte genes (Grover et al., 2016). Apart from impaired regenerative and differentiation abilities, HSCs from old mice also have reduced homing capability (Dykstra et al., 2011).

Therefore, HSCs undergo several changes during aging, consisting of an increase in frequency, a bias towards differentiation to the myeloid cell types, and a decrease towards differentiation to the lymphoid cell types. That is why it is important to identify gene regulatory and epigenetic mechanisms driving these changes in HSCs with age.

## 1.3. Epigenetics and hematopoiesis

### 1.3.1. Introduction to epigenetics

The development of complex multicellular organisms from single cells involves a series of differentiation and division cycles while maintaining the same genome sequence. Changes in gene expression profiles are critical processes for the regulation of cell fate which aids in the development of a complex organism. Additionally, homeostasis and preservation of cellular identity through cellular division requires specific gene expression patterns to be maintained. Epigenetic mechanisms orchestrate these changes in gene expression profiles via changes in chromatin accessibility. The term 'epigenetics' was coined by Conrad Waddington and was defined as "the branch of biology which studies the causal interactions between genes and their products, which bring the phenotype into being" (Goldberg, Allis, & Bernstein, 2007). This involves heritable variation that occurs without changes in the DNA sequence (Butler & Dent, 2013a). DNA is wrapped around histones that are made up of a tetramer of histone proteins H2A, H2B, H3 and H4 which constitute a nucleosome (T. Chen & R Dent, 2013). N- and C- terminal tails of these histone proteins are exposed and have the ability to interact with surrounding nucleosomes and linker DNA that connects adjacent nucleosomes (T. Zhang, Cooper, & Brockdorff, 2015). Covalent modifications of DNA

and histone tails can lead to direct reversible changes in chromatin structure and also serve as binding sites for recruitment of other non-histone proteins like adenosine triphosphate (ATP) dependent chromatin remodeling complexes, which exchange different types of histone and change position of histones by using energy from ATP hydrolysis (T. Chen & R Dent, 2013). Packaging and accessibility status of chromatin on target loci dictate their pattern of expression. Tightly packaged chromatin is considered heterochromatin and is associated with transcriptional repression, whereas accessible chromatin is considered euchromatin and is associated with transcriptional activation (T. Chen & R Dent, 2013). Epigenetic processes that lead to changes in chromatin architecture involve DNA methylation and post-translational modifications of the histone tails (Butler & Dent, 2013a). Replacement of canonical histones by non-canonical histones like H3.3 and H2A.Z can also serve to modify the chromatin accessibility and modify the expression of target loci (T. Zhang et al., 2015). In addition, non-coding RNAs, functional RNAs that are not translated, have the ability to regulate transcription, splicing, and translation to regulate expression of genes (T. Chen & R Dent, 2013).

#### 1.3.1.1. DNA methylation

DNA methylation involves the covalent attachment of a methyl group to the cytosine base in a CpG dinucleotide context (Conerly & Grady, 2010) and follows a broad spectrum of variation in levels and patterns in animals (Bird, 2002). Cytosine methylation is generally associated with gene repression and heterochromatin formation and is considered a stable epigenetic mark (Butler & Dent, 2013a). DNA methylation is catalyzed by two classes of enzymes: the maintenance methyltransferase, DNA methyltransferase 1 (DNMT1), and the de-novo methyltransferases which include DNA

methyltransferase 3a (DNMT3A) and DNA methyltransferase 3b (DNMT3B) (Conerly & Grady, 2010). In addition, DNMT3L aids in the stimulation of de-novo methyltransferase activity of Dnmt3a (Dé Ric Ché, Lieber, & Hsieh, 2002). Patterns of cytosine methylation established by these enzymes play important roles in chromatin organization and gene regulation during organogenesis and gametogenesis (Goldberg et al., 2007). DNA methylation is found throughout vertebrate genomes (Schübeler, 2015). Most of the CpGs found in mammalian genomes are methylated (Field et al., 2018). When present in gene bodies, DNA methylation aids in gene expression, whereas when found on distal regulatory sequences, like enhancers, it promotes repression (Field et al., 2018). These changes in gene expression are brought about by methylation-dependent interactions with transcriptional activators and repressors (Conerly & Grady, 2010). In addition, DNA methylation is critical for numerous cellular processes like the silencing of the repetitive sequences, X-chromosome inactivation and genomic imprinting.

#### 1.3.1.2. Histone modifications

Residues on histone tails can be post-translationally modified, which include methylation, acetylation, sumoylation, phosphorylation, ubiquitination, de-amination, adenosine diphosphate (ADP)-ribosylation and buturylation (Lawrence, Daujat, & Schneider, 2016a). According to the "histone code" hypothesis histone modifications can serve as recruitment sites for effector proteins (Jenuwein & David Allis, 2001). These histone fold modifications can disrupt the interaction between nucleosomes, for example acetylation of lysine 16 of histone H4 (H4K16ac) has been associated with a decrease in chromatin compaction and increase in transcription (Lawrence, Daujat, & Schneider, 2016b). Whereas, histone modifications like trimethylation at lysine 20 of histone H4

(H4K20me3) have been shown to enhance chromatin compaction (Lawrence et al., 2016b). Some common histone modifications and their functions are listed in Table1.1.

| **Histone Modification** | **Function** | **Enzyme** |
|---|---|---|
| H3K4me1/3 | Enhancer Function/Gene activation | MLL3/MLL4 |
| H3K9me3 | Gene Repression | SUV39H1 |
| H3K27ac | Gene Activation | CBP, P300, GCN5 |
| H3K27me3 | Gene Repression | EZH2 |
| H3K36me1-3 | Gene Activation | SETD2 |
| H3K36ac | Marks Active Promoters | GCN5 |
| H4K16ac | Gene Activation | ATF2 |
| H4K20me1 | Gene Repression | SETD8 |

**Table 1.1. Common histone modifications and their associated function in gene regulation (adapted from** (Zhao & Garcia, 2015)**).**

### 1.3.2. Chromatin modifiers

#### 1.3.2.1. Histone methylation

Histone methylation has been observed on the lysine and arginine residues of H3 and H4. There are seven residues which can be mono, di or tri-methylated: lysine 4 of histone H3 (H3K4), lysine 9 of histone H3 (H3K9), arginine 17 of histone H3 (H3R17), lysine 27 of histone H3 (H3K27), lysine 36 of histone H3 (H3K36), arginine 3 of histone H4 (H4R3) and lysine 20 of histone H4 (H4K20) (Lawrence et al., 2016a). Three main protein families are responsible for catalyzing the methylation of these residues: the PRMT1 family, the SET domain family and the DOT1/DOT1L containing non-SET domain proteins (Martin & Zhang, 2009). The functional effects of lysine methylation in terms of gene expression are context-dependent and can result in either activation or inhibition of gene expression. For example, trimethylation at lysine 4 of histone H3 (H3K4me3) is associated with transcriptional elongation whereas trimethylation at lysine

9 of histone H3 (H3K9me3) is linked with gene repression (Table 1.1) (Lawrence et al., 2016a).

Methylated lysines can recruit other protein complexes collectively known as "reader" proteins. Methylated lysine binding domains include the ankyrin repeat, the plant homeodomain (PHD) finger, chromo, tudor, and malignant brain tumor (PWWP) domains (Butler & Dent, 2013a).

Histone demethylases are responsible for catalyzing the demethylation of methylated lysine and arginine residues on histone tails. Demethylation of lysine residues is performed by two main families: jumonji-C domain-containing enzymes and amine oxidases (Butler & Dent, 2013b). Jumonji domain-containing protein 6 (JMJD6), peptidylarginine deiminase 4 (PADI4) and lysine-specific demethylases (KDMs) like KDM3A, KDM4E KDM5C, and KDM6B are candidates for arginine demethylation (Wesche et al., 2017). Because of conflicting reports in the literature regarding their arginine demethylases activities, arginine demethylases have not been well characterized (Wesche et al., 2017).

#### 1.3.2.2. Histone acetylation

Histone acetyltransferases are enzymes that catalyze acetylation of histone tails which results in effects on chromatin architecture and gene regulation. There are two main families that contain histone acetyltransferase (HAT) domains: Gcn5 N-acetyltransferases and the MYST family of acetyltransferases (Lee & Workman, 2007). There are other proteins which have the capacity to acetylate histones like E1A binding protein p300 (EP300)/CREB binding protein (CBP), TATA-box binding protein associated factor 1 (TAF1) and a number of nuclear coreceptors, however these are

considered to be an orphan class of HAT enzymes because they do not harbor consensus HAT domains (Lee & Workman, 2007). HATs can be recruited to their target loci by specific proteins that contain tudor domain, PHD fingers, chromodomain, bromodomain, and WD40 repeats. HATs play important roles in DNA repair and genome stability (Lee & Workman, 2007). HATs also have non-histone acetylation targets for example transformation-related protein 53 (P53) and general transcription factor IIB (TFIIB) (Yang, 2004). Histone deacetylases (HDACs) catalyze the removal of acetyl marks from histone residues (Butler & Dent, 2013a). Eleven HDACs are encoded by the mammalian genome which share a conserved histone deacetylase domain and are divided into four families: class I, IIa, IIb and IV (Haberland. Michael, Montgomery, & Olson., 2009).

### 1.3.3. MYST Family of acetyltransferases

The MYST family of acetyltransferases consists of five members: MOZ (KAT6a), MORF(KAT6b), HBO1 (KAT7), TIP60 (KAT5) and MOF (KAT8) (Avvakumov & Coˆte´, 2007). Proteins of the MYST family are characterized by the presence of a MYST region, an acetyl co-enzyme A binding site and C2HC type zinc finger motif (Valerio, Xu, Chen, et al., 2017). The presence of acetyl co-enzyme binding site allows acetyl co-enzyme A to bind, which is required for the acetyltransferase reaction (Sebastián & Mostoslavsky, 2017). The C2HC zinc finger motif aids in substrate recognition and acetyltransferase activity (Akhtar & Becker, 2001).

#### 1.3.3.1. KAT8

Lysine acetyltransferase 8 (KAT8) was initially identified as a regulator of dosage compensation in flies (Su, Wang, Cai, Jin, & Chi-shing Cho, 2016.). KAT8 is responsible for acetylation at H4K16 and *Kat8* depletion can lead to abnormal gene transcription.

Irregular expression of *Kat8* and H4K16ac has been observed in various types of tumors and cancer cells. HAT activity of KAT8 is necessary for MLL-AF9 tumorigenesis (Valerio, Xu, Chen, et al., 2017). In addition, *Kat8* is involved in lung cancer by promoting S phase entry via regulation of H4K16ac. *Kat8* is important for hematopoiesis in newborn and adult mice, conditional deletion of *Kat8* leads to hematopoietic failure resulting in death of pups after 8-11 days of birth (Valerio, Xu, Eisold, et al., 2017). Acetyltransferase activity of KAT8 was found to be important for its role in hematopoiesis (Valerio, Xu, Eisold, et al., 2017). Transduction of *Kat8* in *Kat8*-conditionally deleted Lineage$^-$ cKit$^+$ Sca1$^+$ (LSK) cells, which consist of HSPCs, was able to rescue colony forming potential in an *in vitro* methylcellulose based assay whereas *Kat8* with an inactive acetyltransferase domain was not able to rescue it (Valerio, Xu, Eisold, et al., 2017).

**1.3.3.2. KAT6A**

Lysine acetyltransferase 6a (*Kat6a*) was originally identified as a fusion partner with *Cbp* in translocation t(8;16)(p11;p13) which is found in 4-7/1000 cases of acute myeloid leukemia (AML) (Borrow et al., 1996). In addition, fusions of *Kat6a* with Ep300, Nuclear receptor coactivator 2 (*Tif2*) and developing brain homeobox 1 (*Leutx*) have been found in other hematological malignancies (X.-J. Yang, 2015). KAT6A is responsible for acetylation at lysine 9 of histone H3 (H3K9ac) at its target loci (Sheikh et al., 2016). Loss of *Kat6a* in embryogenesis results in a reduction in the number of HSCs (Katsumoto et al., 2006). Conditional deletion of *Kat6a* in bone marrow leads to a decline in the number of HSCs and fewer of them were found to be in a quiescent stage as compared to wild type controls (Sheikh et al., 2016). Fetal liver from mice with a

catalytically inactive KAT6A histone acetyltransferase domain was also found to have a diminished number of HSPCs (Perez-Campo, Borrow, Kouskoff, & Lacaud, 2009). Hematopoietic progenitors containing catalytically inactive KAT6A have increased expression of cyclin-dependent kinase inhibitor 2a (*p16*) and enhanced nuclear localization of chromobox 3 (HP-1γ), markers for senescence (Perez-Campo et al., 2014). This suggests that *Kat6a* is required for the maintenance of fetal and adult HSCs and that histone acetyltransferase activity of KAT6A is important for this function by inhibiting *p16* expression.

#### 1.3.3.3. KAT6B

Lysine acetyltransferase 6b (*Kat6b*) is a paralogue of *Kat6a* and shares more than 90% sequence similarity in functional domains. Homozygous *Kat6b* mutants survive until three weeks of age and show craniofacial abnormalities and cerebral defects (Thomas, Voss, Chowdhury, & Gruss, 2000). In a fluorescent reporter tagged overexpression mouse model of *Kat6b*, high expression of *Kat6b* corresponded with cells showing features of neural stem cells (Sheikh, Dixon, Thomas, & Voss, 2011) and overexpression of *Kat6b* also resulted in increased neuronal production (Merson et al., 2006). A mutation in *Kat6b* has been observed in a patient with Noonan syndrome which involves hyperactivation of the mitogen-activated protein kinase (MAPK) pathway (Kraft et al., 2011). KAT6B has been found to acetylate H3K23 (H3K23ac) (Sim O-Riudalbas et al., 2015). Therefore, *Kat6b* is important for the function and generation of neural stem cells. Although, the role of KAT6B in HSCs has not been studied yet, its importance in neural stem cell function and its similarity to KAT6A suggests that KAT6B might also be important for HSC function.

## 1.4. Epigenetic regulation of HSC function

### 1.4.1 Role of histone writers and erasers in HSC differentiation

The dynamic nature of hematopoiesis relies on the tightly regulated process of self-renewal and differentiation of HSCs (D. Hu & Shilatifard, 2016). Chromatin modifying enzymes play important roles during hematopoiesis by regulating histone modifications (Butler & Dent, 2013b). The importance of epigenetic regulators in hematopoietic function is also demonstrated by the fact that mutations in these epigenetic regulators are common in myeloid malignancies and also play a role in hematopoietic transformation (Shih, Abdel-Wahab, Patel, & Levine, 2012). Epigenetic regulators involve histone writers who perform the task of catalyzing the addition of post-translational modifications (PTMs) on histones, whereas histone erasers remove those histone modifications (Gillette & Hill, 2015). A number of histone writers and erasers responsible for marks associated with both gene activation and repression have been identified with respect to their role in HSC function.

*Cbp*, and its paralogue, *p300*, are histone lysine acetyltransferases responsible for depositing the activating acetylation at lysine 27 of histone H3 (H3K27ac) mark (Chan et al., 2011)(Tan, Nimer, Sun, Man, & Wang, 2015). Conditional deletion of *Cbp* in adult bone marrow led to a decrease in HSPCs, an increase in myeloid and a decrease in the lymphoid compartment, showing that *Cbp* is critical for differentiation of HSCs(Chan et al., 2011). In addition, serial transplantation of conditionally deleted *Cbp* bone marrow and HSCs had reduced reconstitution ability thus showing that *Cbp* is also important for HSC self-renewal (Chan et al., 2011; Rebel et al., 2002).

Histone deacetylases (HDACs) remove the acetylation mark deposited by acetyltransferases like CBP. Studies involving the treatment of human $CD34^+$ cells with valproic acid, an HDAC inhibitor, have shown that inhibition of HDACs results in increased proliferation of $CD34^+$ cells as well as increased self-renewal of HSPCs as observed by competitive repopulation assays (Zheng et al., 2005).

Histone lysine methyltransferases are responsible for catalyzing methylation on lysine residues at histone tails. Methylation at lysine 4 and lysine 3 of histone 3 are the most extensively studied among histone methylation marks (Greer & Shi, 2012). The MLL family of histone methyltansferases is responsible for methylation of H3K4 residues which is associated with open chromatin (W. Yang & Ernst, 2017). Lysine specific methyltransferase 2a (*MLL1*)-/- HSCs show a defect in self-renewal potential and lack of success in engrafting recipients (W. Yang & Ernst, 2017). *MLL3* is related to *MLL1* and both have been found to be mutated in acute lymphoid leukemia and acute myeloid leukemia (C. Chen et al., 2014). Knockdown of lysine-specific methyltransferase 2c (*MLL3*) leads to an increase in LT-HSCs and a block in differentiation in a *p53* background (C. Chen et al., 2014). Another member of the MLL family, lysine specific methyltransferase 2d (*MLL4*), has also been found to be important for HSC function (Santos et al., 2014). Conditional deletion of *MLL4* in adult bone marrow leads to an increase in the number of stem and progenitor cells (Santos et al., 2014). However, *MLL4* deletion leads to decreased self-renewal capacity of HSCs which was shown by a reduction in asymmetric division of HSCs and decreased competitive repopulation ability of HSPCs (Santos et al., 2014). Apart from the MLL family, enhancer of zeste 2 polycomb repressive complex 2 subunit (EZH2) is responsible for trimethylation at lysine

27 of histone H3 (H3k27me3) which is associated with gene inactivation (Mochizuki-Kashio et al., 2011). Expression of *Ezh2* decreases after the differentiation of HSPCs (Abboud & Berman, 2013). Conditional deletion of *Ezh2* in fetal liver leads to a decrease in efficiency of repopulation ability in fetal liver, however, competitive repopulation of adult bone marrow with *Ezh2* deletion did not result in decrease in repopulation ability in both primary and secondary transplants, showing that *Ezh2* is more important for fetal liver HSCs as compared to adults (Mochizuki-Kashio et al., 2011). Histone demethylases play the role of removing methyl marks from methylated histone tails. Lysine specific demethylase 1a (LSD1) is a histone demethylase that is responsible for removing methylation from H3K4 residues (Kerenyi et al., 2013). Conditional deletion of *Lsd1* in adult mouse bone marrow leads to a reduction in stem cell self-renewal as well as a reduction in myeloid progenitors (Kerenyi et al., 2013). Therefore, these studies show that histone-modifying enzymes are important for various aspects in the function and the differentiation of HSCs.

### 1.4.2. Role of enhancers in HSC differentiation

Enhancers are cis-regulatory DNA elements that regulate the transcription of target genes (Halfon, 2019). Usually consisting of a few hundred base pairs in length, enhancers function as a platform for recruitment of transcription and chromatin-modifying machinery which aids in the transcription of its target genes (Halfon, 2019). Enhancers can perform these functions while being situated at long distances (> 1 Mb) (Cico, Andrieu-Soler, & Soler, 2016a) and can be present both upstream or downstream of their target genes as well as in introns of their target genes (Pennacchio, Bickmore, Dean, Nobrega, & Bejerano, 2013).

The first enhancer, SV40, was discovered around 40 years ago and was responsible for increasing transcription of a *β-globin* gene in the rabbit by more than 200 fold (Banerji, Rusconi, & Schaffner, 1981). Since then, the important role of enhancers in development and disease has been well established (Halfon, 2019). Enhancer-associated chromatin goes through a number of changes that correlates with the activity of the enhancer (Figure 1.6). Lineage determining transcription factors bind closed enhancer associated chromatin to make it more accessible to histone methyltransferases which then deposit monomethyl at lysine 4 of histone H3 (H3K4me1) on enhancer associated chromatin (Heinz, Romanoski, Benner, & Glass, 2015). This marks the enhancer in a primed state (Heinz et al., 2015). After this, histone acetyltransferases deposit H3K27ac which marks the enhancer in an active state (Heinz et al., 2015).

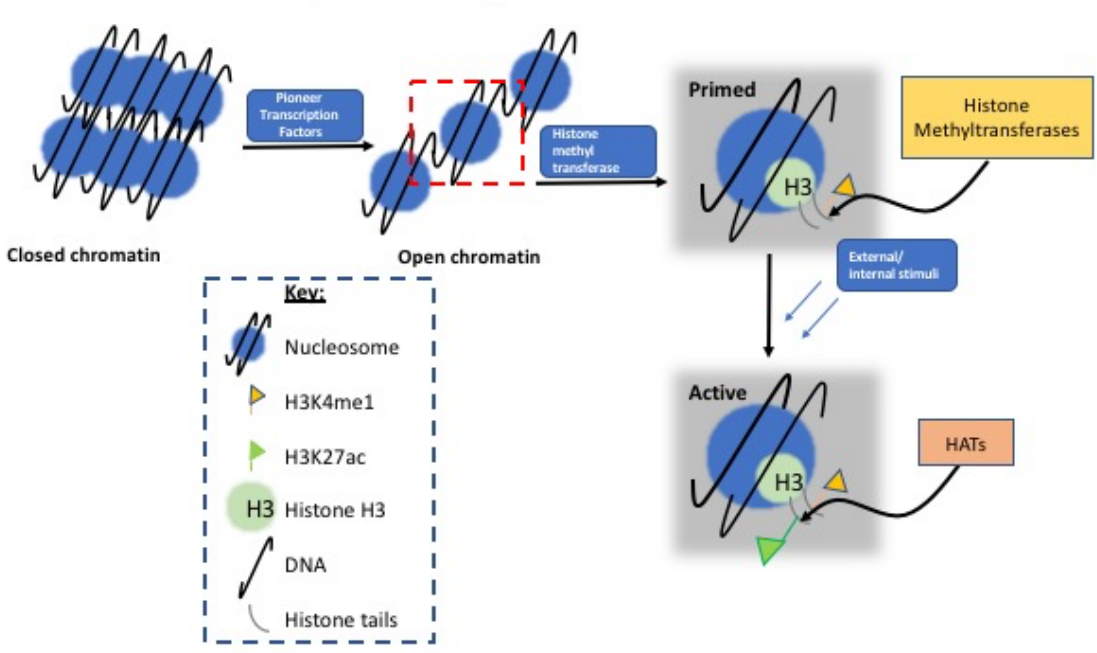



**Figure 1.6. Schematic for priming and activation of enhancers.**

In the context of hematopoiesis, studies involving focused investigation of gene regulatory elements have demonstrated that enhancers play a role in hematopoietic function (Bauer et al., 2013; Johnson et al., 2015). Whole-genome technologies have accelerated the discovery of putative enhancers and have also allowed the study of a larger number of enhancers. Using these approaches it was found that enhancers undergo considerable changes in priming and activation status during differentiation of HSCs to mature cells, the nature of these changes being dependent on the lineage type (Choukrallah, Song, Rolink, Burger, & Matthias, 2015; Lara-Astiaso et al., 2014; Luyten, Zang, Liu, & Shivdasani, 2014). These studies suggest that the enhancers which are active in mature erythroid and myeloid cells become established in HSPCs (Lara-Astiaso et al., 2014) whereas, the enhancers that are activated in mature lymphoid cells become established in those mature cell types (Choukrallah et al., 2015; Lara-Astiaso et al., 2014). This shows that there is a difference in the enhancer establishment and setting up of the lineage-specific programs between erythroid, myeloid and lymphoid lineages.

### 1.4.3. Epigenetic changes in HSCs with age

HSCs undergo a variety of epigenetic changes with age involving DNA methylation, histone modifications and expression of epigenetic regulators (Buisman & Haan, 2019) which raises the possibility of the contribution of epigenetic regulators towards HSC aging. There are overall changes in gene expression profiles with age (Beerman et al., 2013; Sun et al., 2014; Wahlestedt et al., 2013). Specifically, the expression of important epigenetic regulators declines with age (Sun et al., 2014). Expression of genes associated with chromatin modification like *Ezh2* and *Cbx2* as well

genes liked to DNA methylation like Tet methylcytosine dioxygenase 1 (*Tet1*) and Tet methylcytosine dioxygenase 2 (*Tet2*) decreases with age (Sun et al., 2014).

In addition to variations in gene expression profiles, HSC aging involves changes in levels and localization of histone modifications (Florian et al., 2012; Grigoryan et al., 2018; Sun et al., 2014). Levels of gene activating marks H3K4me3 and H4K16ac increase in old HSCs compared to young HSCs (Florian et al., 2012; Sun et al., 2014). On the other hand, the number peaks of the repressive mark H3K27me3 do not change, however the coverage of peaks becomes broader with age in HSCs (Sun et al., 2014). Histone modifications like H4K16ac have found to distributed asymmetrically in the nucleus of HSCs (Florian et al., 2012). Analysis of nuclear localization of histone modifications showed that this polarity of H4K16ac and H3K27ac decreases significantly whereas polarity of acetylation at lysine 8 of histone H4 (H4K8ac) and acetylation at lysine 5 of histone H4 (H4K5ac) undergo modest decreases in polarity with age in HSCs (Grigoryan et al., 2018). Restoration of polarity of H3K16ac by inhibition of CDC42 was associated with functional rejuvenation of HSCs (Florian et al., 2012).

These studies suggest that gene-regulatory elements and epigenetic regulators are important for function of HSPCs and that changes in epigenetic regulators and their activities correlate with HSC aging. Which epigenetic regulators and gene regulatory elements drive aging in HSCs and which related molecular pathways are perturbed in aging by these epigenetic factors needs to be identified.

# CHAPTER 2

## RESULTS

Hematopoiesis is a well-coordinated system of differentiation from HSCs to mature cells which relies on precise changes in gene expression profiles at each point in the differentiation continuum. Regulation of these gene expression profiles is dependent on a dynamic collaboration between lineage-determining transcription factors, chromatin-modifying enzymes and cis-regulatory elements (Cico, Andrieu-Soler, & Soler, 2016b). Misregulation of these chromatin-modifying enzymes and cis-regulatory elements can disrupt the balance in the orchestration of precise gene expression profiles leading to disease and aging-associated phenotypes. My thesis involves the identification and characterization of gene regulatory elements and epigenetic regulators that are important for HSPC differentiation to mature cells so that this knowledge can be utilized in ameliorating the defects in the differentiation process due to aging.

Although genome-wide technologies have facilitated the identification of putative enhancers, the number of functionally characterized enhancers is negligible compared to the total number of putative enhancers. Activity of enhancers in the hematopoietic progenitors has been observed to change with age (Poplineau et al., 2019). In the context of lymphoid lineage, identification of functionally characterized enhancers is important for developing novel therapeutic strategies to overcome the decline in lymphoid function which is observed with aging. It has been reported that the majority of lymphoid specific enhancers are established in mature cells and only a few are established in HSPCs (Choukrallah et al., 2015; Lara-Astiaso et al., 2014). Therefore, I hypothesize that a few

key enhancers which are established in HSPCs are important for commitment to the lymphoid lineage. In the first part of results I outline the investigation of this hypothesis.

It is known that there is a decrease in the expression of key epigenetic factors and changes in levels of histone modifications in LT-HSCs during aging in mice (Sun et al., 2014) and humans (Adelman et al., 2019). However, precise molecular mechanisms underlying changes in epigenetic regulators and hematopoietic aging have not been elucidated yet. This serves as a barrier for developing novel therapeutic strategies for ameliorating aging-associated hematopoietic decline. I hypothesized that changes in expression/activity of key epigenetic regulators drive age-associated functional decline in HSCs. The second part of the results section consists of an investigation of this hypothesis.

### 2.1. Optimization of assay for *in vitro* differentiation of HSPCs to the lymphoid lineage and identification and characterization of lymphoid specific enhancers

Enhancers are cis-regulatory elements that are capable of activating lineage-specific gene expression patterns (Heinz et al., 2015). This is supported by the observation that distal cis-regulatory elements in mature lymphoid cells were found to be enriched for motifs of transcription factors which are important for the lymphoid lineage (Heinz et al., 2010). Although putative lymphoid specific enhancers have been identified by genome-wide technologies, functional characterization of these enhancers is a barrier for ameliorating the decline in production and function of lymphoid cell types which is observed in aging (Dykstra et al., 2011; Miller, 1996; Rodrigues et al., 1997). Studies have shown that the majority of lymphoid specific enhancers are established in mature cell stages. I hypothesized that a few key enhancers that are established in HSPCs are

important for commitment to lymphoid lineage. In this section I test this hypothesis by optimizing culture conditions for *in vitro* OP9 co-culture of HSPCs to assess B-lymphoid differentiation potential, followed by development of a knockout strategy for putative lymphoid enhancers, ultimately employing these two techniques to characterize the importance of putative lymphoid enhancers for differentiation of HSPCs to B-lymphoid cells.

### 2.1.1 Optimization of co-culture conditions

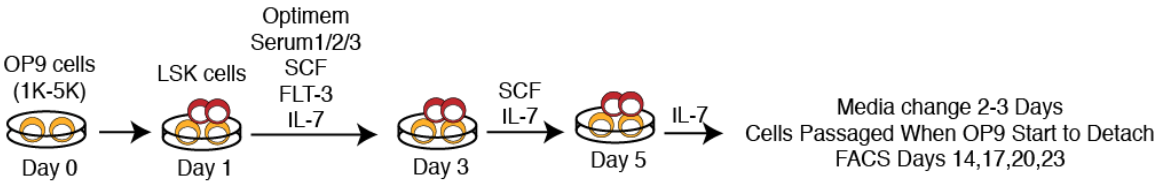



**Figure 2.1. Schematic for *in vitro* differentiation of HSPCs to B-lymphoid cells.**

The *in vitro* assay for differentiation of HSPCs to B-lymphoid cells by co-culture with OP9 stromal cells was adapted from Pietras et al. (Pietras et al., 2015a). For optimization of starting cell number of OP9 cells appropriate for co-culture, I seeded 1000, 2000 or 5000 OP9 cells in flat-bottom 96-well plate on Day 0 of the assay (Figure 2.1). On day 1, 500 Lineage$^-$ cKit$^+$ Sca1$^+$ (LSK) cells, which consist of HSPCs, were seeded on top of OP9 layer in OptiMEM supplemented with serum1(Hyclone defined; AYM175301), serum 2 (Hyclone Defined; AB1016350) or serum 3(VWR) and stem cell factor (SCF;), FMS tryosine like-3 ligand (FLT-3) and interleukin-7 (IL-7). On day 3 of the assay half of the media was replaced with fresh media supplemented with SCF and IL-7 (Figure 2.1). On day 5 of the assay half of the media was replaced with fresh media

supplemented with IL-7 (Figure 2.1). Subsequently, the media was changed every 2-3 days with IL-7 supplementation. When the OP9 cell layer started to detach, the cells were harvested by trypsinization and transferred to a new well in a 24-well plate with pre-seeded OP9 cells. Cells were analyzed by fluorescence-activated cell sorting (FACS) on days 14, 17, 20 and 23 of the assay (Figure 2.1) and number of $CD45^+$ cells and percentage of $CD19^+$ cells were used for determining the optimal conditions for the assay.

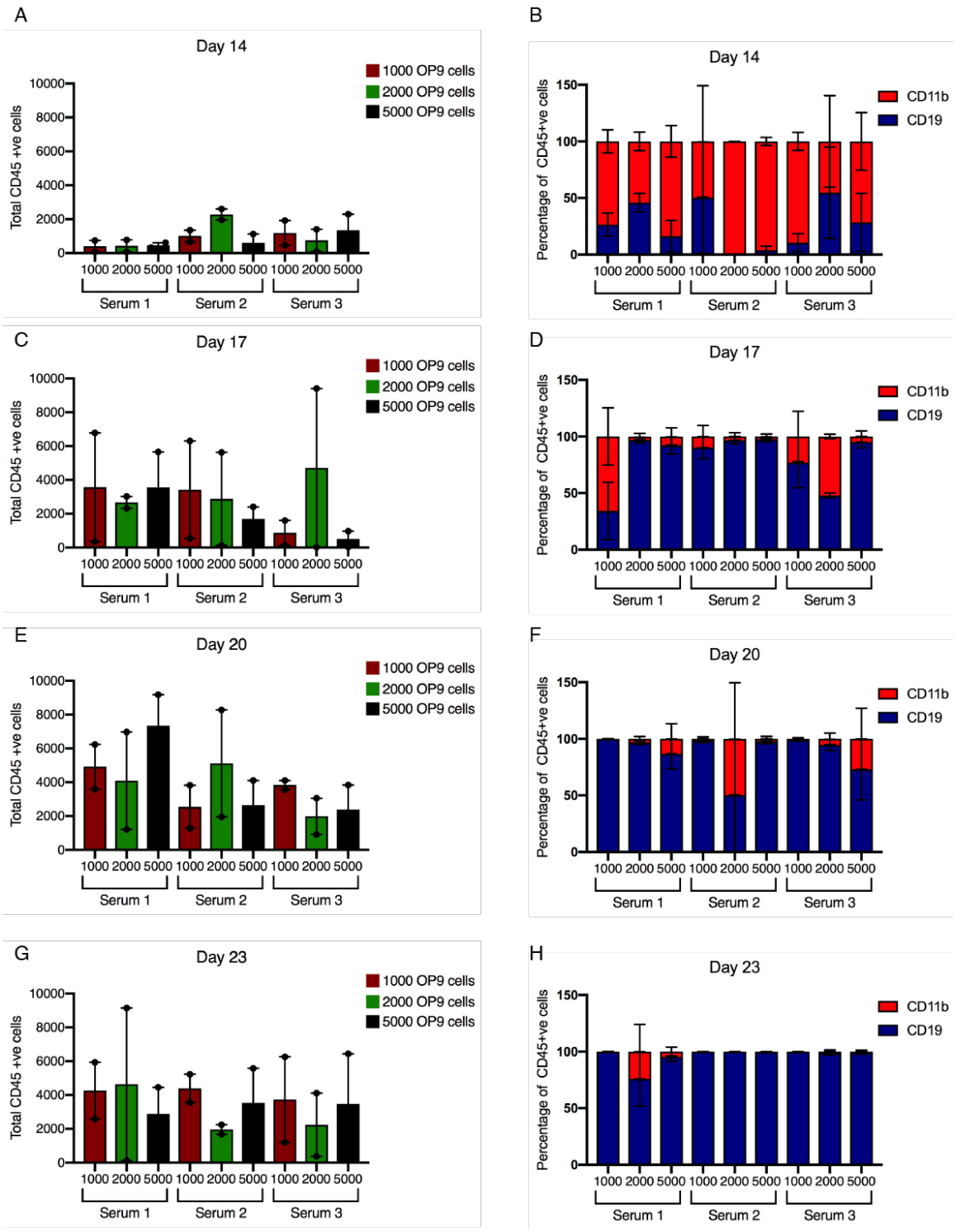



**Figure 2.2. Optimization of OP9 seeding density, serum type and read-out time point.** 1000, 2000 or 5000 OP9 cells were seeded in 96-well plate, supplemented with serum 1, serum 2 or serum 3 and analyzed by FACS on Day 14,17,20 and 21 of assay. Total $CD45^{+}$ cells obtained on day 14 (A), day 17 (C), day 20 (E), day 23 (G), Percentage

of $CD19^+$ and $CD11b^+$ cells obtained on day 14 (B), day 17 (D), day 20 (F), day 23 (H). Dots represent $n$ =2 biological replicates.

As the purpose of this assay was to differentiate HSPCs to $CD19^+$ B-lymphoid cells, I determined which readout time point produced the highest percentage of $CD19^+$ cells. Day 23 seemed to be most efficient for the production of $CD45^+$ $CD19^+$ cells compared to the rest of the time points across all conditions (Figure 2.2). day 23 was used as the reference time point to assess the effect of serum type and OP9 seeding density on differentiation to B-lymphoid cells. Assays started with 1000 OP9 cells produced the highest mean number of $CD45^+$ cells across all serum types (Figure 2.2G) and also had the highest percentage of $CD19^+$ cells (Figure 2.2H) compared to assays started with 2000 and 5000 OP9 cells.

To ascertain the type of serum with the highest differentiation potential to $CD45^+$ cells I compared total $CD45^+$ cells obtained on day 23 with assays seeded at 1000 OP9 cells. I observed that cells supplemented with Serum 2 had the highest mean number of $CD45^+$ cells compared to Serum 1 and Serum 3 (Figure 2.2G).

To conclude, I observed that OP9 co-culture B-lymphoid differentiation assay performs optimally when started with 1000 OP9 cells, supplemented with Serum 2 and readout on day 23 of the assay.

### 2.1.2. Minimizing variability in OP9 co-culture assay

Although the conditions described the in last section were able to differentiate HSPCs to $CD19^+$ cells efficiently, I observed significant variation in the overall differentiation ability between replicates for each condition (Figure 2.2G). In order to test if this variation is dependent on the number of HSPCs that was used to start the assay, I

seeded 10, 50, 100, 250 and 500 Lineage$^-$ cKit$^+$ (LK) cells, which consist of HSPC and lineage-committed progenitors, and compared their ability to differentiate to CD45$^+$ CD19$^+$ cells (Figure 2.3A). Assays started with all of the aforementioned LK cell numbers produced CD19$^+$ cells efficiently (Figure 2.3C); however, no correlation was found between the total number of CD45$^+$ cells obtained and the number of LK cells used to start the assay (Figure 2.3B). This led me to hypothesize that the passaging conditions involving trypsinization and transfer to a new well is the source of variability for this assay.

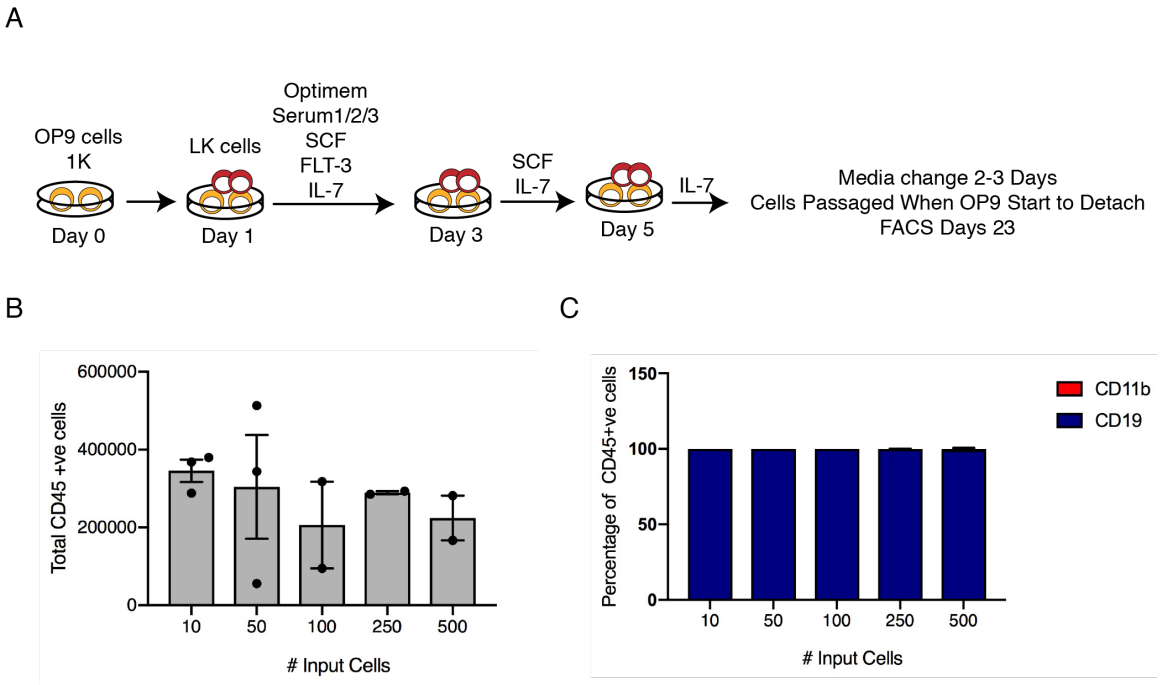



**Figure 2.3. Correlation between HSPC number and lymphoid differentiation ability with OP9 stromal cells.** (A) Schematic of lymphoid differentiation assay (B) Total CD45$^+$ cells obtained on day 23 of assay. (C) Percentage of CD19$^+$ and CD11b$^+$ cells obtained on day 23 of assay.

To test this hypothesis, I performed the OP9 differentiation assay by seeding 1000 OP9 cells in a 96-well plate (Figure 2.4A) and 5000 OP9 cells in a 24-well plate (Figure 2.4D), followed by FACS analysis on day 12 without passaging during the assay. My

results showed that the 96-well assay without passaging produced less $CD45^+$ cells as compared to an equivalent number of 500 LK cells readout on day 23 with passaging (Figure 2.3B) and also less than 50% of $CD45^+$ cells were $CD19^+$ cells at the end of the assay (Figure 2.4C). Although the 24-well assay without passaging produced a higher number of $CD45^+$ cells (Figure 2.4E) as compared to 96-well without passaging (Figure 2.4B) the percentage of $CD19^+$ (Figure 2.4F) was still less than what had been obtained with cells without passaging (Figure 2.3C), and the total number of $CD45^+$ cells still seemed variable (Figure 2.4D). Therefore, elimination of passaging did not mitigate the variation in $CD45^+$ numbers produced at end of assay and was also not efficient in differentiation to $CD19^+$ cells.

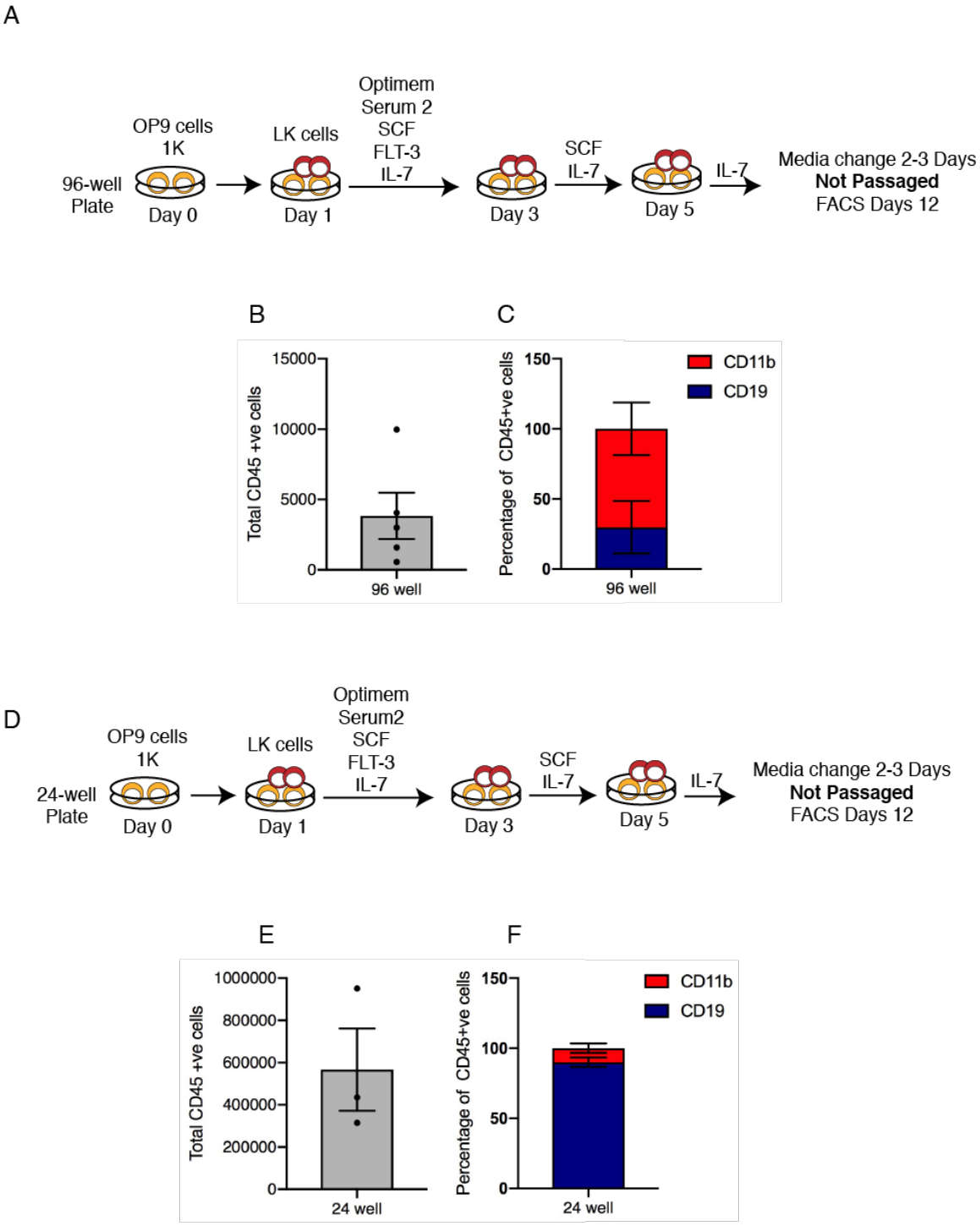



**Figure 2.4. Effect of passaging on differentiation ability in OP9 co-culture assay.** Schematic of assay without passaging in (A) 96-well and (D) 24-well plate. Total $CD45^+$ cells obtained on day 12 of assay from (B) 96-well and (E) 24-well plate. Percentage of $CD19^+$ and $CD11b^+$ cells obtained on day 12 of assay (C) 96-well and (F) 24-well plate.

To further attempt to overcome the issue of heterogeneity arising due to the trypsinization and harvest of cells during replating in this assay, I cultured 500 and 1000 LK cells with Transwells with readout on day 12 of the assay (Figure 2.5A). This method of culture involved no contact between LKs and OP9 cells and transwells containing LK cells were transferred to new wells on day 8 of the assay. There was still variability between technical replicates of in total number of $CD45^{+}$ cells produced (Figure 2.5B) and was also inefficient at producing $CD19^{+}$ cells (Figure 2.5C).

A

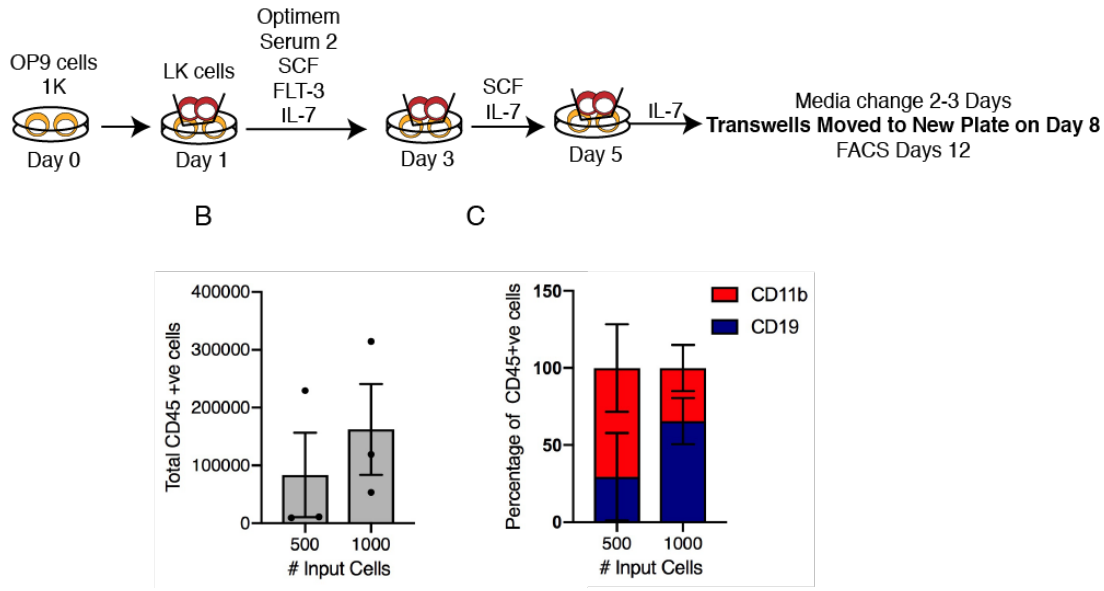



**Figure 2.5. Effect of OP9-HSPC contact on differentiation ability in OP9 co-culture assay.** (A) Schematic of assay with transwells. (B) Total $CD45^{+}$ cells obtained on day 12 of assay with 500 or 1000 starting HSPCs. (C) Percentage of $CD19^{+}$ and $CD11b^{+}$ cells obtained on day 12 of assay with 500 or 1000 starting HSPCs.

Therefore, out of all the culture conditions that I have investigated, culture for 23 days with serum 2 starting with 1000 OP9 cells with passaging seem to be the most optimal conditions for efficient differentiation of HSPCs to B-lymphoid $CD19^{+ve}$ cells

and my experiments also suggest that this assay is more suitable for qualitative rather than quantitative analysis.

### 2.1.3. Identification and characterization of lymphoid specific enhancers

#### 2.1.3.1. *In silico* identification of putative lymphoid specific enhancers

The goal of this part of the study was to test the hypothesis that lymphoid specific enhancers established in HSPCs are important for commitment to lymphoid lineage. I tested this hypothesis by identifying and characterizing cis-regulatory elements that were functionally important for the commitment of HSPCs to B-lymphoid lineage. To test this hypothesis, I utilized a published catalog of putative enhancers in the hematopoietic system (Lara-Astiaso et al., 2014). Lara-astiaso *et al* identified these putative enhancers by using levels of H3K4me1, di-methylation at lysine 4 of histone H3 (H3K4me2), H3K27ac and expression of nearest genes from hematopoietic stem, progenitor and mature cells (Lara-Astiaso et al., 2014). To identify B-lymphoid specific enhancers from this catalogue I used H3K4me1 levels (<25 was considered not primed; >25 was considered primed) to assess status of priming and H3K27ac levels (<25 was considered inactive; >50 was considered active) to assess status of activation at a particular enhancer as defined by Lara-Astiaso *et al*, 2014.

| Enhancer Type A | LT-HSC | ST-HSC | MPP | CLP | B |
|---|---|---|---|---|---|
| H3K4me1 | >50 | >50 | >50 | >50 | >50 |
| H3K27ac | <25 | <25 | <25 | >25 | >25 |

| Enhancer Type B | LT-HSC | ST-HSC | MPP | CLP | B |
|---|---|---|---|---|---|
| H3K4me1 | >50 | >50 | >50 | >50 | >50 |
| H3K27ac | <25 | <25 | <25 | <25 | >25 |

| Enhancer Type C | LT-HSC | ST-HSC | MPP | CLP | B |
|---|---|---|---|---|---|
| H3K4me1 | <25 | <25 | >50 | >50 | >50 |
| H3K27ac | <25 | <25 | <25 | >25 | >25 |

| Enhancer Type D | LT-HSC | ST-HSC | MPP | CLP | B |
|---|---|---|---|---|---|
| H3K4me1 | <25 | <25 | >50 | >50 | >50 |
| H3K27ac | <25 | <25 | >25 | >25 | >25 |

| Enhancer Type E | LT-HSC | ST-HSC | MPP | CLP | B |
|---|---|---|---|---|---|
| H3K4me1 | <25 | <25 | <25 | <25 | >50 |
| H3K27ac | <25 | <25 | <25 | <25 | >25 |

**Table 2.1. Criteria for identification of putative lymphoid specific enhancers.** Numbers represent processed read counts for H3K4me1 and H3K27ac ChIP-seq data sets.

Therefore, by using levels of H3K4me1 and H3K27ac ChIP-seq data in HSCs, MPPs, CLPs, and B-cells (Table 2.1), I mined for enhancers that would be predicted to be primed and activated at various stages during differentiation from HSCs. My results revealed a total of 665 putative enhancers which were active in B-cells and which can be divided into 5 broad categories: Type A, Type B, Type C, Type D and Type E (Figure 2.7).

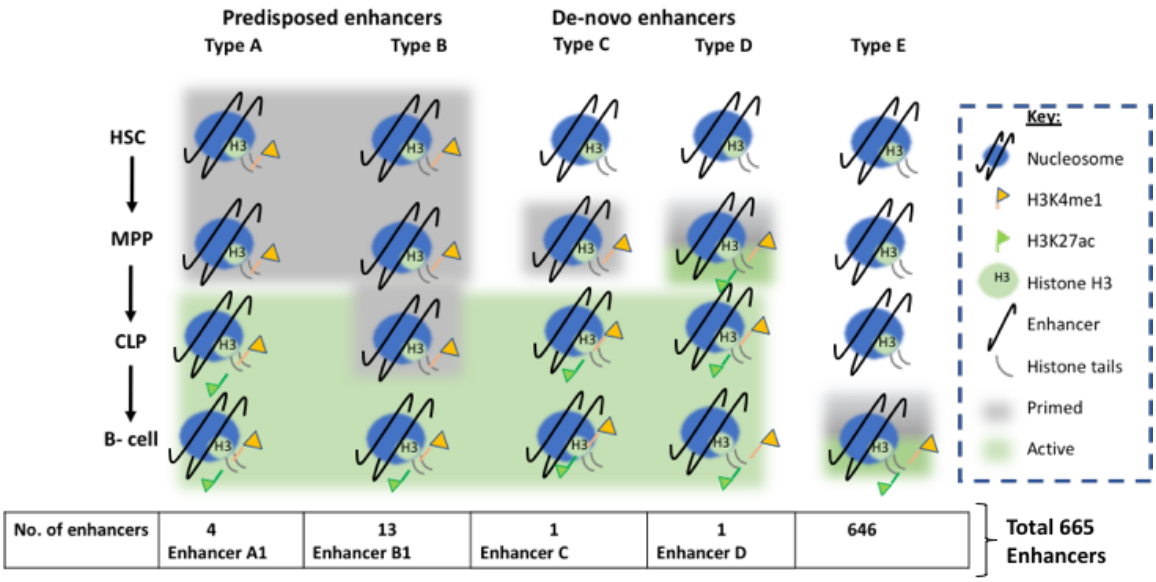



**Figure 2.6. Graphical representation of priming and activation status of putative enhancers active in B-cells.**

Enhancers in Type A and Type B both got primed in HSCs however, Type A enhancers got activated in CLPs, whereas Type B enhancers got activated in B-cells (Figure 2.6). Because Type A and Type B enhancers got primed at HSCs these enhancers were denoted as "Pre-disposed enhancers" and contained 4 and 13 enhancers respectively. Type C and Type D enhancers both got primed in MPPs (Figure 2.6). Type C enhancers became activated in CLPs, whereas Type D enhancers became activated in MPPs (Figure 2.6). The final category, Type E, consisted of enhancers that got primed and activated in B-cells. Type C, Type D and Type E enhancers were denoted as "De-novo Enhancers" because these got established or primed In MPP or later stages and contained 1, 1 and 646 enhancers respectively (Figure 2.6). Since I was interested in enhancers which are important for commitment to lymphoid lineage I decided to pursue enhancers in categories Type A, Type B, Type C and Type D for validation. One

enhancer was randomly selected from each category (Enhancer A1, Enhancer B1, Enhancer C, Enhancer D) for initial functional characterization (Table 2.2).

| Enhancer Type | Chromosome # | Coordinates |
|---|---|---|
| Enhancer A1 | 5 | 37059418-37061418 |
| Enhancer B1 | 10 | 98591289-98593289 |
| Enhancer C | 8 | 55069106-55071106 |
| Enhancer D | 1 | 16748743-16750743 |

**Table 2.2. Genome loci of enhancers short-listed for functional characterization.**

### 2.1.3.2. Strategy for *ex vivo* CRISPR/Cas9 mediated enhancer knockout in primary cells

To characterize putative B-lymphoid enhancers identified in Section 2.1.3.1, I employed CRISPR/Cas9 mediated knockout strategy. It consisted of performing knockout of each putative enhancer by using targeting 2 sgRNAs flanking each enhancer (Table 3.3). Initially, I employed a strategy consisting of delivery of Cas9 expressing plasmid in primary cells by lentiviral transduction.

#### 2.1.3.2.1. Determining the efficiency of lentiviral transduction of Cas9

For optimization of gene delivery of Cas9 by lentiviral transduction, I used 3$^{rd}$ and 4$^{th}$ generation systems of lentiviral packaging. I prepared lentivirus containing pLentiCRISPR-EGFP, which expresses Cas9 along with GFP, using 3$^{rd}$ and 4$^{th}$ generation lentiviral systems (Figure 2.7) and transduced whole bone marrow (BM) mononuclear cells from wildtype C57BL/6J mice. 3$^{rd}$ generation lentiviral systems consist of two viral packaging plasmids: one encodes Gag/Pol and second encodes Rev ("Lentiviral guide," 2019). Whereas the 4$^{th}$ generation consists of 4 packaging plasmids with each containing a component for viral assembly ("Fourth-generation lentiviral packagin overview," 2019).

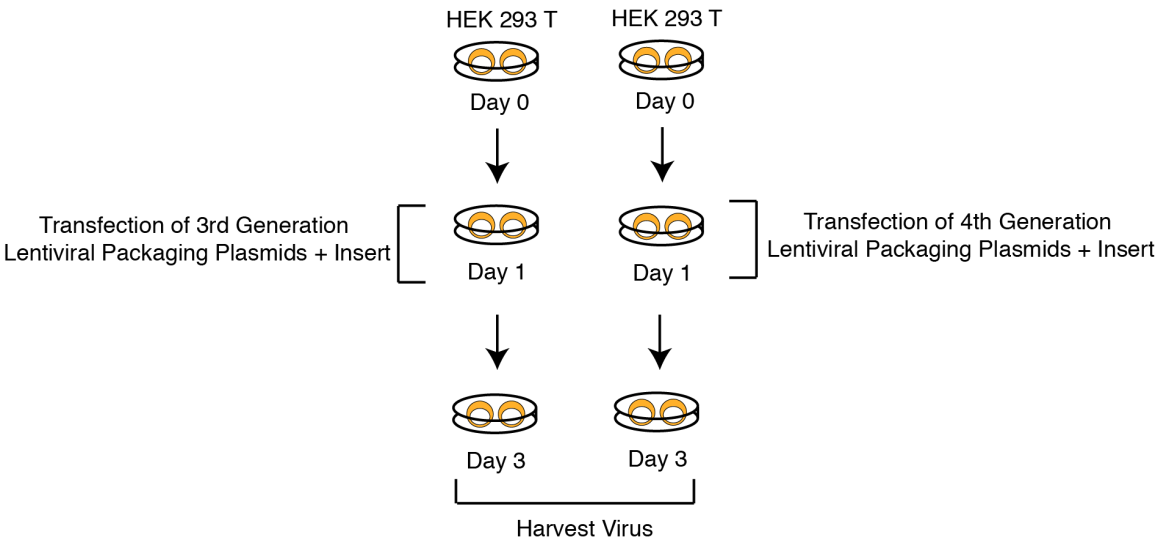
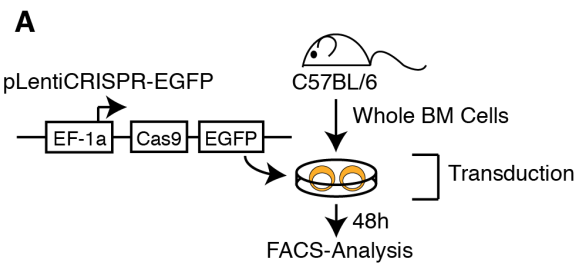



**Figure 2.7. Schematic of generation of viral supernatant containing pLentiCRIPSR-EGFP with 3rd and 4th generation lentiviral packaging systems.**

Cells were analyzed with FACS analysis after 48 hours post-transduction (Figure 2.8A). My results showed that the transduction efficiency from both of the packaging system was almost similar and was considerably low (< 0.5% GFP cells) to obtain a sufficient number of transduced cells for downstream analysis (Figure 2.8B). Since transduction of the 4th generation system was not lower than the 3rd generation system and 4th generation system is safer than 3rd generation, I selected the 4th generation packaging system for use in further experiments.

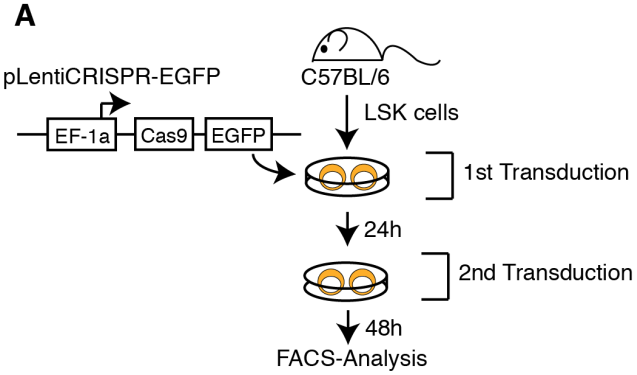



B

| Sample | % GFP+ |
|---|---|
| 3rd Generation | 0.30 |
| 4th Generation | 0.34 |

**Figure 2.8. No difference in transduction efficiency of whole BM cell transduced with lentivirus packaged with 3rd and 4th generation packaging systems.** (A) Schematic of transduction of whole BM cells (B) Transduction efficiency (%GFP$^+$) cells obtained after transduction with 3rd generation and 4th generation virus.

To improve the transduction efficiency, I switched to a more homogenous population, LSKs, as compared to whole bone marrow mononuclear cells and transduced twice (once at day 0 and a second time 24 hours after the 1st transduction) (Figure 2.9A). However, I still obtained a negligible transduction efficiency (Figure 2.9B).




B

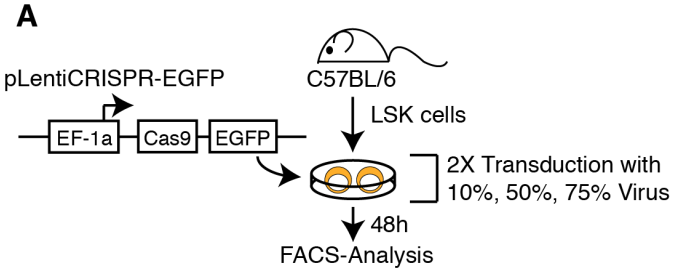
| Sample | % GFP+ |
|---|---|
| pLentiCRISPR-EGFP | 0.20 |

**Figure 2.9. Effect of number of transductions on transduction efficiency** (A) Schematic for transduction of whole BM cells with viral supernatant containing pLentiCRISPR-EGFP. (B) Transduction efficiency (%GFP$^+$ cells) obtained after two consecutive transductions of LSK cells.

Next, I investigated the effect of the cell type and the amount of virus used for transduction on transduction efficiency. I transduced LSK cells with 10%, 50% and 75% virus by volume. Transduction was repeated after 24 hours. I analyzed the cells by FACS

48 hours post-transduction (Figure 2.10A). My results showed that the cells transduced with 10%, 50% and 75% virus produced approximately 0.17%, 0.56% and 0% $GFP^+$ cells (Figure 2.10B).

**A**



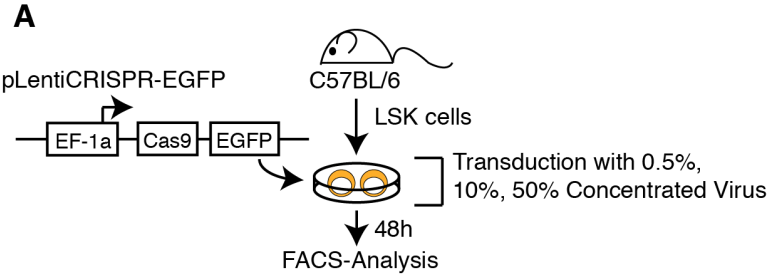

**B**

| Sample | % $GFP^+$ |
|---|---|
| 10% Virus | 0.17 |
| 50% Virus | 0.56 |
| 75% Virus | 0.00 |

**Figure 2.10. Effect of viral concentration by volume of culture media on transduction efficiency** (A) Schematic for transduction of LSKs with viral supernatant containing pLentiCRISPR-EGFP using different viral concentrations. (B) Transduction efficiency (%$GFP^+$) cells obtained after transduction with 10%, 50% and 75% by volume virus concentrations.

After this, I prepared concentrated virus containing pLentiCRISPR-EGFP by ultracentrifugation of viral supernatant, and transduced LSK cells with 0.5%, 10% and 50% virus by volume, followed by FACS analysis 48 hours post-transduction (Figure 2.11A). However, my results showed no $GFP^+$ cells with these conditions (Figure 2.11B).

A




B

| Sample | % GFP+ |
|---|---|
| 0.5 % Virus | 0.00 |
| 10% Virus | 0.00 |
| 50% Virus | 0.00 |

**Figure 2.11. Effect of concentrated virus on transduction efficiency.** (A) Schematic for transduction of LSKs with concentrated viral supernatant containing pLentiCRISPR-EGFP using different viral concentrations. (B) Transduction efficiency (%GFP) cells obtained after transduction with 0.5%, 10% and 50% by volume virus concentrations.

Toxicity due to concentrated virus could be responsible for these results.

Overall, Cas9 delivery by lentiviral transduction seemed an inefficient method for gene transfer in primary hematopoietic cells. Therefore, I concluded that this method was not appropriate for validating B-lymphoid specific enhancers.

### 2.1.3.2.2. Knockout by electroporation of *in vitro* transcribed sgRNA and Cas9 protein

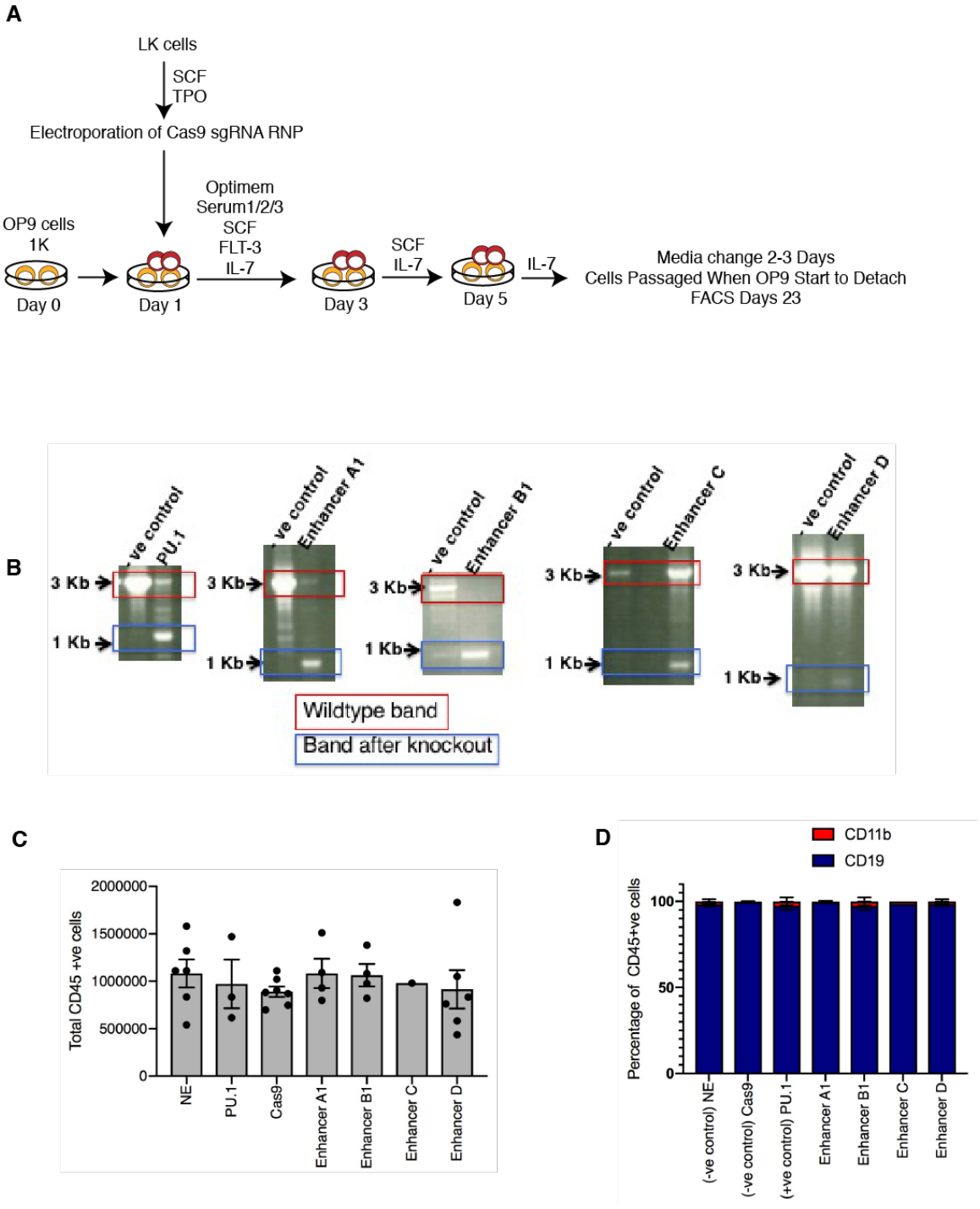



**Figure 2.12. Knockout of enhancer B1 results in a reduction in the percentage of CD19$^{+}$ cells.** (A) Schematic for electroporation of Cas9-sgRNA RNPs in LK cells and

assessment of differentiation potential by OP9 co-culture. (B) Verification of knockout. (C) Total $CD45^+$ cells and (D) Percentage of $CD19^+$ and $CD11b^+$ cells obtained on day 23 of assay.

Efficient CRIPSR/Cas9 mediated gene editing involving gene delivery by electroporation of ribonucleoprotein (RNP) complexes containing Cas9 protein and sgRNAs has been reported in the literature (Gundry et al., 2016). Electroporation, in this case, employed the neon transfection system (Invitrogen), which allows electroporation of low input cells, which was suitable for my experiments. For preliminary experiments, one enhancer from each of the shortlisted enhancer categories was selected for validation. In addition, spleen focus forming virus (SFFV) proviral integration oncogene (*Pu.1*) was selected as a positive control because of its importance in the differentiation of HSCs to lymphoid progenitors (Iwasaki et al., 2005). sgRNAs were designed flanking each enhancer and flanking exons 4 and 5 of *Pu.1* gene using Benchling. Oligos containing sgRNAs linked to the T7 promoter were designed and gRNA scaffold was amplified from pX458 plasmid using these oligos, followed by *in vitro* transcription to produce sgRNAs as described (Gundry et al., 2016).

LK cells were isolated from wildtype C57BL/6J young mice and cultured in the presence of SCF and TPO for three hours. RNP complexes for each enhancer and *Pu.1* were made with 1 ug of Cas9 and 0.5ug of each of two flanking sgRNAs for each condition. These RNPs were electroporated in LK cells after three hours of culture, and cells were plated into the OP9 lymphoid differentiation assay. Cells were analyzed by FACS analysis on Day 23 and $CD45^+$ cells were sorted for genomic DNA extraction to determine knockout efficiency (Figure 2.12A). I observed efficient knockout of target

regions in almost all conditions (Figure 2.12B). Total $CD45^+$ cells were not significantly different between positive and negative controls and enhancer knockout cells (Figure 2.12C). There was a slight decrease in the percentage of $CD19^+$ cells from Enhancer B1 knockout cells (Figure 2.12D) which was similar to change observed from the positive control (*Pu.1*) although the result was not statistically significant

Therefore, electroporation of RNP complexes is an efficient method for gene targeting in primary HSPCs. In addition, this preliminary data shows that knockout of enhancer B1 in HSPCs shows a subtle reduction in $CD19^+$ cells as compared to negative controls. However, this will need to be reproduced in a more robust assay to draw definite conclusions.

## 2.2. Aging-Associated decrease in the histone acetyltransferase KAT6B is linked to myeloid-biased hematopoietic stem cell differentiation with age

HSCs give rise to all mature blood cells. However, with age, cell-intrinsic changes within HSCs contribute to aging-associated hematopoietic decline such as increased HSC frequency, enhanced differentiation toward myeloid cells, and decreased ability to return to quiescence after activation (Verovskaya, Dellorusso, & Passegué, 2019). Molecular features that contribute to these phenotypes include loss of cell polarity, impaired DNA damage repair, increased production of reactive oxygen species (ROS), and declines in mitochondrial function (Verovskaya et al., 2019). In addition, previous studies support the occurrence of epigenetic drift in aged HSCs. This involves decreased expression of key epigenetic regulators, a global increase in DNA methylation (Beerman et al., 2013; Sun et al., 2014), and altered levels of histone H3 lysine 4 trimethylation (H3K4me3) and lysine 27 trimethylation (H3K27me3) in both aged murine and human

HSCs (Adelman et al., 2019; Sun et al., 2014). Moreover, diminished levels and polarity of histone H4 lysine 16 acetylation (H4K16ac) is associated with loss of regenerative capacity and gain of myeloid lineage skewing in aged LT-HSCs (Florian et al., 2012). While these studies support involvement of epigenetic regulatory processes in HSC aging, there remains a lack of comprehensive knowledge of the extent to which epigenetic alterations are linked to aging-associated changes in HSC function. Therefore, the goal of this study was to identify novel role of epigenetic regulators in the context of myeloid skewed differentiation of HSCs.

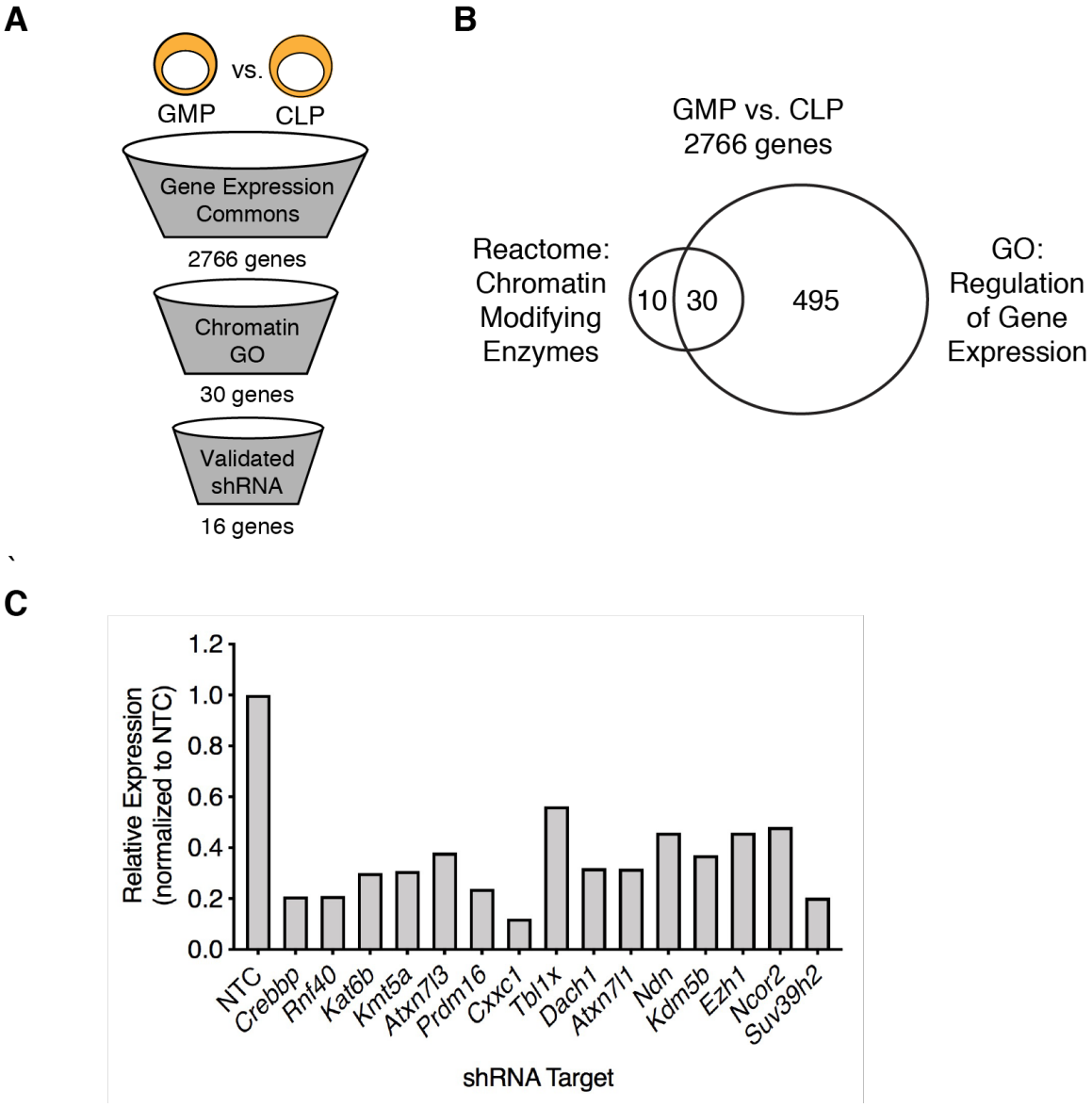



**Figure 2.13. Candidate screen for epigenetic regulators of myeloid versus B-lymphoid differentiation.** (A) Schematic of candidate selection criteria to identify chromatin regulatory genes involved in myeloid versus B-lymphoid differentiation of HSPCs. GMP; granulocyte-macrophage progenitors, CLP; common lymphoid progenitors. (B) GO enrichment analysis of 2,766 genes identified as differentially expressed between GMP versus CLP. (C) Relative expression of shRNA target genes following knockdown in NIH/3T3 cells. Bars represent mean of $n = 3$ technical replicates.

To identify epigenetic regulators that have a functional role in aging-associated myeloid lineage-biased differentiation of HSCs a graduate student in our lab, Sneha Borikar, conducted an *in vitro* shRNA screen. Using gene expression commons (GEXC) (Seita et al., 2012), she identified 2,766 differentially expressed genes between granulocyte macrophage progenitors (GMPs) and common lymphoid progenitors (CLPs) (Figure 2.13A), which are committed progenitors for the myeloid and lymphoid lineages, respectively (Motonari, 2013). Among these 2,766 differentially expressed genes, gene ontology (GO) enrichment analysis of Reactome pathways (Ashburner et al., 2000; Mi et al., 2017; The Gene Ontology Consortium 2019) revealed significant enrichment of chromatin modifying enzymes ($P = 0.000158$, FDR = 0.00111). The 40 most enriched genes encoding chromatin modifying enzymes were further filtered to 30 genes based on overlap with the GO annotation "regulation of gene expression" (GO:0010468) (Figure 2.13B). Lastly, this gene list was filtered to include those with commercially available shRNA constructs with verified knockdown in murine cell lines, resulting in 16 genes (Table 2.3).

| Gene Symbol | Gene Name |
|---|---|
| *Kat6b* | K(lysine) acetyltransferase 6B |
| *Kmt5a* | lysine methyltransferase 5A |
| *Tbl1x* | transducin (beta)-like 1 X-linked |
| *Kdm5b* | lysine (K)-specific demethylase 5B |
| *Ncor2* | nuclear receptor co-repressor 2 |
| *Suv39h2* | suppressor of variegation 3-9 2 |
| *Mta3* | metastasis associated 3 |
| *Carm1* | coactivator-associated arginine methyltransferase 1 |
| *Dot1l* | DOT1-like, histone H3 methyltransferase (S. cerevisiae) |
| *Kmt2e* | lysine (K)-specific methyltransferase 2E |
| *Arid1a* | AT rich interactive domain 1A (SWI-like) |
| *Kdm5c* | lysine(K)-specific demethylase 5C |
| *Smarcc2* | SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily c, member 2 |
| *Sap30l* | SAP30-like |
| *Jmjd6* | jumonji domain containing 6 |
| *Padi2* | peptidyl arginine deiminase, type II |

**Table 2.3. Sixteen chromatin regulatory genes for shRNA screening identified using an unbiased differential expression approach.**

To begin functional screening, shRNA expression plasmids for six of these 16 genes were obtained. For negative and positive controls, she used a scrambled shRNA-

expressing non-targeting control (NTC) vector and a shRNA vector targeting CREB-binding protein (*Crebbp*), respectively. Conditional knockout of *Crebbp* is known to cause loss of HSPCs and result in myeloid-biased hematopoiesis (Chan et al., 2011). In addition, shRNA constructs were obtained for eight genes hypothesized to regulate lineage differentiation using a candidate gene approach (Table 2.4). After cloning, she validated reduced target gene expression from each of these shRNA constructs in murine 3T3 cell lines (Figure 2.13C).

| Gene Symbol | Gene Name |
|---|---|
| ***Rnf40*** | **ring finger protein 40** |
| ***Atxn7l3*** | **ataxin 7-like 3** |
| ***Prdm16*** | **PR domain containing 16** |
| ***Cxxc1*** | **CXXC finger 1 (PHD domain)** |
| ***Dach1*** | **dachshund family transcription factor 1** |
| ***Atxn7l1*** | **ataxin 7-like 1** |
| ***Ndn*** | **necdin** |
| ***Ezh1*** | **enhancer of zeste 1 polycomb repressive complex 2 subunit** |

**Table 2.4. Eight chromatin regulatory genes for shRNA screening identified using a candidate gene approach.**

To perform the *in vitro* shRNA screen, HSCs would seem to be the most appropriate cell type however, we have observed in our lab (data not shown) that HSCs do not perform well in B-lymphoid CFU assays. MPP4 cells have both lymphoid and myeloid differentiation potential (Pietras et al., 2015a) and, in contrast to LT-HSCs, have

efficient clonal *in vitro* differentiation capacity giving rise to both lymphoid and myeloid cells (Young et al., 2016). Thus, we chose to utilize lymphoid-primed multipotent progenitor cells (MPP4) as our starting cell population to conduct this screen. MPP4 ($Lin^-$ $Sca^+$ c-$Kit^+$ $Flt3^+$) cells were isolated by FACS from young adult (8-10 weeks old) mice, transduced with lentiviral particles containing individual shRNA expression plasmids, and cultured for two days with growth factors that we previously identified as supporting both lymphoid and myeloid differentiation from this population (Young et al., 2016b) (Figure 2.14A).

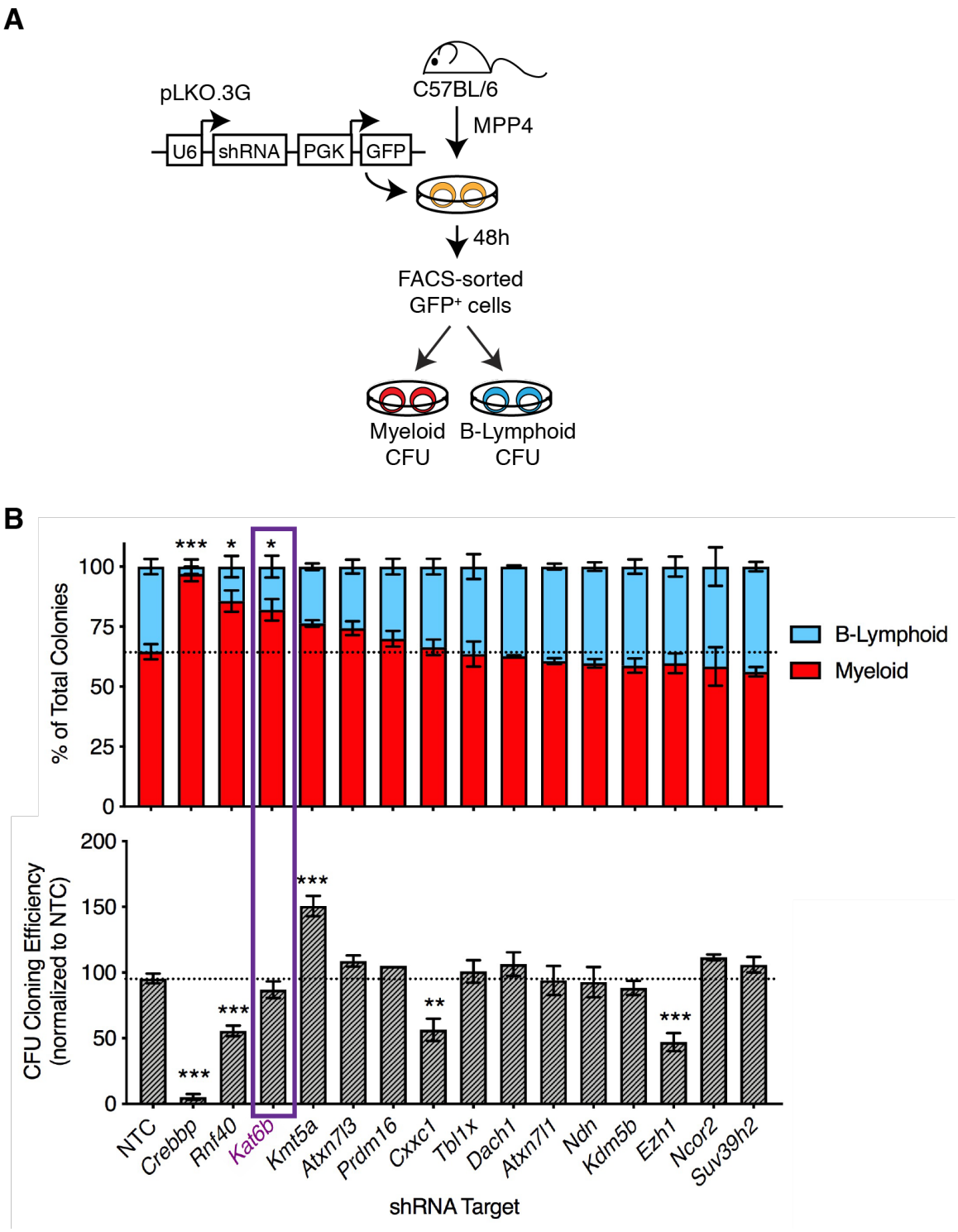



**Figure 2.14. Functional shRNA screen for epigenetic regulators of myeloid versus B-lymphoid differentiation identifies *Kat6b*.** (A) Schematic of experimental design to test epigenetic regulatory gene candidates using shRNA-mediated knockdown in lymphoid-primed multipotent progenitor cells (MPP4) and colony-forming unit (CFU) assays. (B)

(Top panel) Frequency of myeloid and B-lymphoid colonies out of total colonies and (Bottom panel) CFU cloning efficiency calculated as the total number of myeloid and B-lymphoid colonies following shRNA knockdown of the indicated target genes divided by the total number of myeloid and B-lymphoid colonies in NTC. NTC; non-targeting control. Bars represent mean ± SEM of $n \geq 2$ biological replicates. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$ by two-way ANOVA and Dunnett's multiple comparisons test or one-way ANOVA and Holm-Sidak's multiple comparisons test.

After two days, GFP-expressing cells were isolated by FACS and plated into parallel myeloid and B-lymphoid colony-forming unit (CFU) differentiation assays. To identify genes responsible for myeloid versus B-lymphoid differentiation, I sought genes whose knockdown produced a significant change in the proportion of myeloid relative to B-lymphoid colonies while maintaining overall cloning efficiency. Relative to NTC, I found that knockdown of our positive control *Crebbp* resulted in a near-complete loss of CFU capacity and the residual colonies that formed were predominantly myeloid (Figure 2.14B), consistent with the expected phenotype of *Crebbp* loss (Chan et al., 2011). In two out of the 14 shRNA constructs evaluated, targeting *Kat6b* and *Rnf40* (ring finger protein 40), I observed a significant increase in the proportion of myeloid relative to B-lymphoid colonies (Figure 2.14B, Top panel). Of these, only knockdown of *Kat6b* was found not to alter overall cloning efficiency (Figure 2.14B, Bottom panel) and thus was pursued as a candidate epigenetic regulator of aging-associated myeloid lineage bias.

### 2.2.1. KAT6B decreases at the transcript and protein level in aged LT-HSCs

As the goal of this study was to gain insight into the functional role of *Kat6b* in aging-associated myeloid lineage-biased differentiation of HSCs, I sought to determine if *Kat6b* is abundantly expressed within the HSCs and how this expression may be altered in aging. I isolated LT-HSCs ($Lin^- Sca^+$ c-$Kit^+$ $CD150^+$ $CD48^-$) by FACS from young (2-4

month) and aged (20-23 month) mice. By real-time PCR, I observed that the *Kat6b* transcript is expressed in LT-HSCs and that its expression decreases 2.8-fold with age in LT-HSCs (Figure 2.15A).

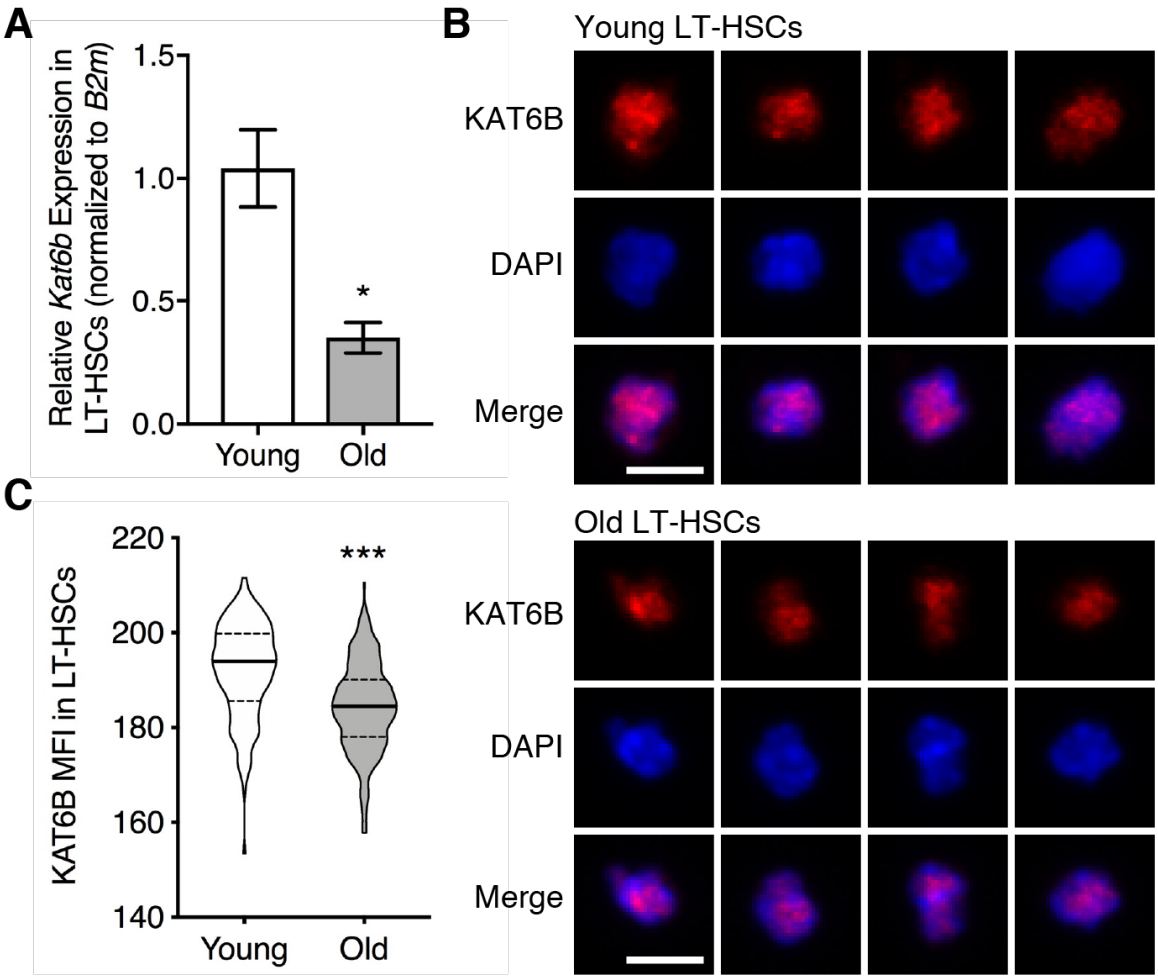



**Figure 2.15. KAT6B is decreased in aged LT-HSCs.** (A) Relative expression of *Kat6b* in LT-HSCs isolated from young (2-4 month) and aged (20-23 month) mice. Bars represent mean ± SEM of $n \geq 3$ biological replicates. *$P < 0.05$ by unpaired *t* test. (B) Representative immunofluorescence images of KAT6B and DAPI in LT-HSCs isolated from young and aged mice. Scale bar equals 5 µm. (C) Violin plots of mean fluorescence intensity (MFI) of KAT6B in LT-HSCs isolated from young and aged mice. Solid lines indicate median and dotted lines indicate quartiles. Data points include $n = 17$-64 individual cells sampled from $n = 4$ biological replicate animals. ***$P < 0.001$ by unpaired *t* test.

Previous work comparing transcriptional changes between young and aged mouse LT-HSCs found a 1.2-fold decrease in *Kat6b* expression in aged LT-HSCs (FDR = 0.0413) (Sun et al., 2014) and a recent study comparing human HSPCs ($Lin^-$ $CD34^+$ $CD38^-$) isolated from young (18-30 year-old) and aged (65-75 year-old) individuals (Adelman et al., 2019) identified that *KAT6B* transcript decreases 1.2-fold in aging (*P*adj = 0.0397), supporting my finding that *Kat6b* levels decrease with age in HSCs. To analyze KAT6B at the protein level, I immuno-stained LT-HSCs isolated by FACS from young and aged mice with an antibody against KAT6B and the nuclear stain DAPI (Figure 2.15B). I observed that the mean fluorescence intensity (MFI) of KAT6B in LT-HSCs isolated from aged mice is significantly lower than in young mice (Figure 2.15C). Together, my results show that KAT6B is significantly decreased at both the transcript and protein levels in aged LT-HSCs.

### 2.2.2. Knockdown of *Kat6b* in LT-HSCs causes myeloid-biased *in vitro* differentiation in CFU assays

To evaluate the functional consequence of reduced expression of *Kat6b* as observed in aged LT-HSCs, I utilized a shRNA knockdown approach. LT-HSCs isolated from young mice were transduced with lentiviral particles containing NTC or one of two *Kat6b* shRNA expression plasmids and cultured for two days with growth factors supporting LT-HSC maintenance (Figure 2.16A) (Holmfeldt et al., 2016). After two days, $GFP^+$ cells were isolated by FACS and evaluated for *in vitro* myelo-erythroid differentiation using CFU assays. From the resultant colonies, I determined that *Kat6b* transcript was reduced by 4.8-fold and 1.5-fold using *Kat6b* shRNA1 (sh1) and *Kat6b*

shRNA2 (sh2), respectively (Figure 2.16B). The total number of colonies was not significantly altered in sh1 or sh2 compared to NTC (Figure 2.16C).

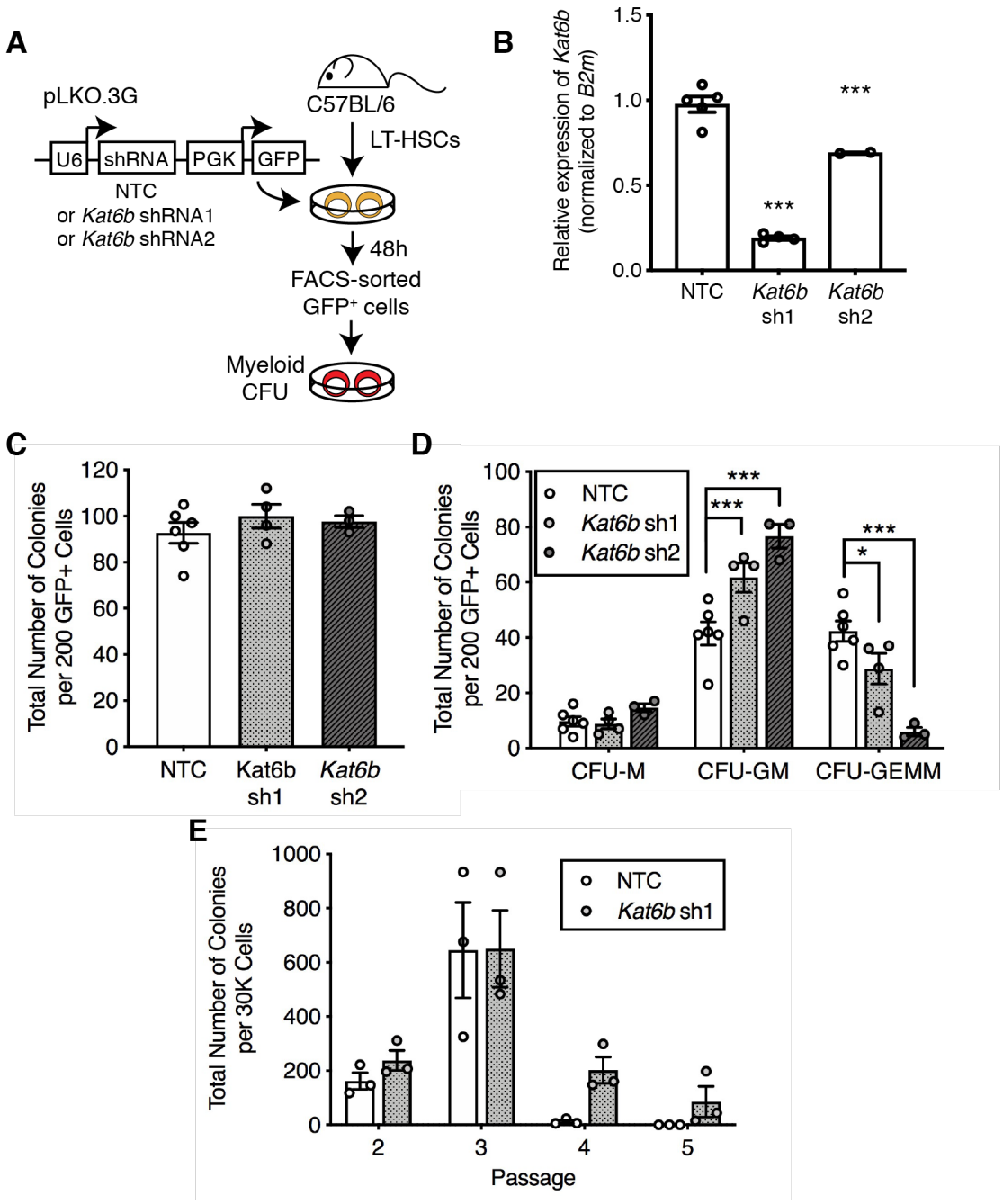



**Figure 2.16. *Kat6b* knockdown alters myeloid differentiation of LT-HSCs *in vitro*.** (A) Schematic of experimental design to knockdown *Kat6b* in LT-HSCs and assess differentiation in the myeloid CFU assay. (B) Relative expression of *Kat6b* in colonies following shRNA-mediated knockdown of *Kat6b* using two independent hairpins (sh1 or

sh2) or NTC. Bars represent mean ± SEM of $n \geq 3$ biological replicates. ***$P < 0.001$ by one-way ANOVA and Holm-Sidak's multiple comparisons test. (C) Total number of colonies produced and (D) colony subtype distribution from 200 $GFP^+$ cells post-transduction of LT-HSCs. CFU-M; macrophage, CFU-GM; granulocyte-macrophage, CFU-GEMM; granulocyte-erythrocyte-macrophage-megakaryocyte. Dots denote biological replicates and bars represent mean ± SEM of $n \geq 3$ biological replicates. *$P < 0.05$; ***$P < 0.001$ by two-way ANOVA and Dunnett's multiple comparisons test. (E) Total number of colonies produced upon passage of 30K cells harvested from the primary CFU assay. Dots denote biological replicates and bars represent mean ± SEM of $n = 3$ biological replicates.

However, differences were observed with respect to colony composition, determined based upon cellular morphology within each colony to distinguish macrophage-only (CFU-M), granulocyte-macrophage (CFU-GM) and granulocyte-erythrocyte-macrophage-megakaryocyte (CFU-GEMM) colonies. Upon knockdown of *Kat6b*, I observed a significant increase in the number of CFU-GM colonies and a significant and consistent decrease in the number of CFU-GEMM colonies (Figure 2.16D), consistently with both shRNA constructs. In addition, I investigated the effect of *Kat6b* knockdown on colony replating capacity for further assessment of myeloid differentiation potential. While NTC colonies did not replate past passage three, I observed that *Kat6b* knockdown colonies replated to passage five, representing an increase in CFU replating capacity (Figure 2.16E). Together, my results demonstrate that knockdown of *Kat6b* results in myeloid-biased *in vitro* differentiation from LT-HSCs and increased serial replating capacity of the resultant myeloid progenitor cells.

### 2.2.3. Knockdown of *Kat6b* in LT-HSCs causes myeloid-biased differentiation *in vivo*

To evaluate the functional consequence of reduced levels of *Kat6b* in LT-HSCs *in vivo*, I transduced LT-HSCs with *Kat6b* sh1 or NTC and transplanted $GFP^+$ cells into lethally irradiated B6.CD45.1 recipient mice (Figure 2.17A).

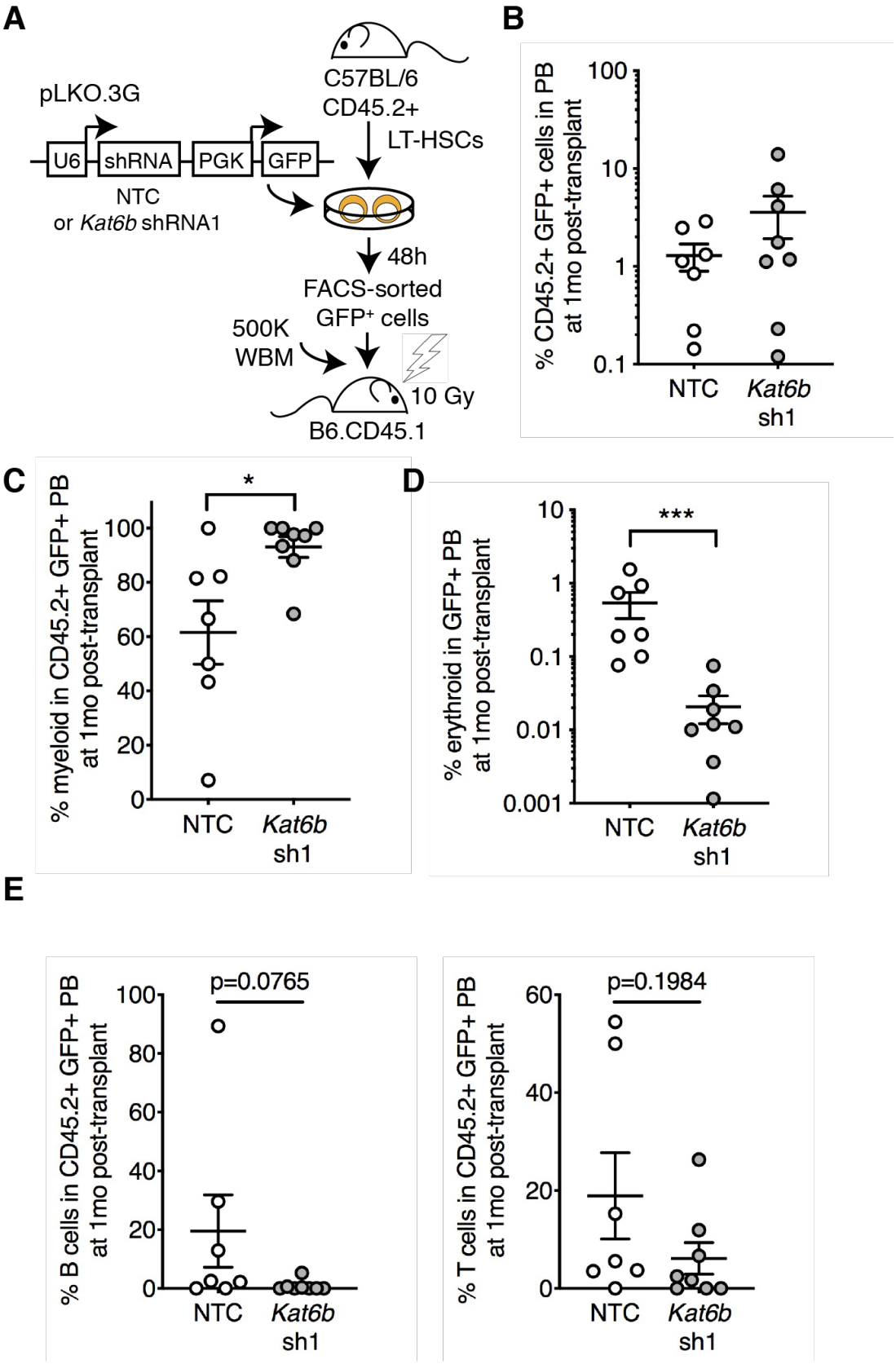

A
pLKO.3G
U6
shRNA
PGK
GFP
NTC
or Kat6b shRNA1
C57BL/6
CD45.2+
LT-HSCs
48h
FACS-sorted
GFP+ cells
500K
WBM
10 Gy
B6.CD45.1
B
% CD45.2+ GFP+ cells in PB
at 1mo post-transplant
NTC
Kat6b
sh1
C
% myeloid in CD45.2+ GFP+ PB
at 1mo post-transplant
*
D
% erythroid in GFP+ PB
at 1mo post-transplant
***
E
% B cells in CD45.2+ GFP+ PB
at 1mo post-transplant
p=0.0765
% T cells in CD45.2+ GFP+ PB
at 1mo post-transplant
p=0.1984

**Figure 2.17. *Kat6b* knockdown alters myeloid and erythroid differentiation of LT-HSCs *in vivo*.** (A) Schematic of experimental design to knockdown *Kat6b* in LT-HSCs and assess hematopoietic reconstitution in lethally irradiated recipient mice compared to NTC-transduced LT-HSCs. (B) Frequency of donor-derived cells ($CD45.2^+$ $GFP^+$) in the peripheral blood (PB) of recipient mice, (C) myeloid cells ($CD11b^{+ve}$) within donor-derived PB cells ($CD45.2^+$ $GFP^+$), and (D) erythroid cells ($Ter119^+$) within donor-derived PB cells ($GFP^+$) at 1 month (1mo) post-transplant. Each dot represents one recipient mouse. Lines represent mean ± SEM of $n \geq 7$ biological replicates. $*P < 0.05$; $***P < 0.001$ by Mann-Whitney test. (E) Frequency of B ($B220^+$) and T ($CD3e^+$) cells within donor-derived PB cells ($CD45.2^+$ $GFP^+$) at one month post-transplant. Each dot represents one recipient mouse. Lines represent mean ± SEM of $n \geq 7$ biological replicates. *P* values calculated by unpaired *t* test.

In total, 15 recipient mice were transplanted with NTC-transduced cells and 16 recipient mice were transplanted with *Kat6b* sh1-transduced cells. From these, 7/15 (46%) and 8/16 (50%) were found to have multilineage engraftment above a threshold of 0.1% donor-derived peripheral blood cells at one-month post-transplant. At this time point, donor-derived engraftment (% $CD45.2^+$ $GFP^+$) was not significantly different between NTC and *Kat6b* sh1 (Figure 2.17B). However, mice transplanted with *Kat6b* knockdown LT-HSCs had a significant increase in the proportion of donor-derived myeloid cells in the peripheral blood as compared to NTC (Figure 2.17C). In addition, there was a significant decrease in donor-derived erythroid cells in the peripheral blood of mice transplanted with *Kat6b* knockdown LT-HSCs compared to NTC (Figure 2.17D). A trend toward decreased frequency of donor-derived B and T lymphocytes in *Kat6b* knockdown compared to NTC did not reach statistical significance ($P = 0.0765$ and $P = 0.1984$, respectively) (Figure 2.17E).

At four months post-transplant, 3/15 (20%) and 3/16 (18.8%) of recipients were found to have sustained multilineage engraftment above a threshold of 0.1% donor-derived peripheral blood cells (Figure 2.18A).

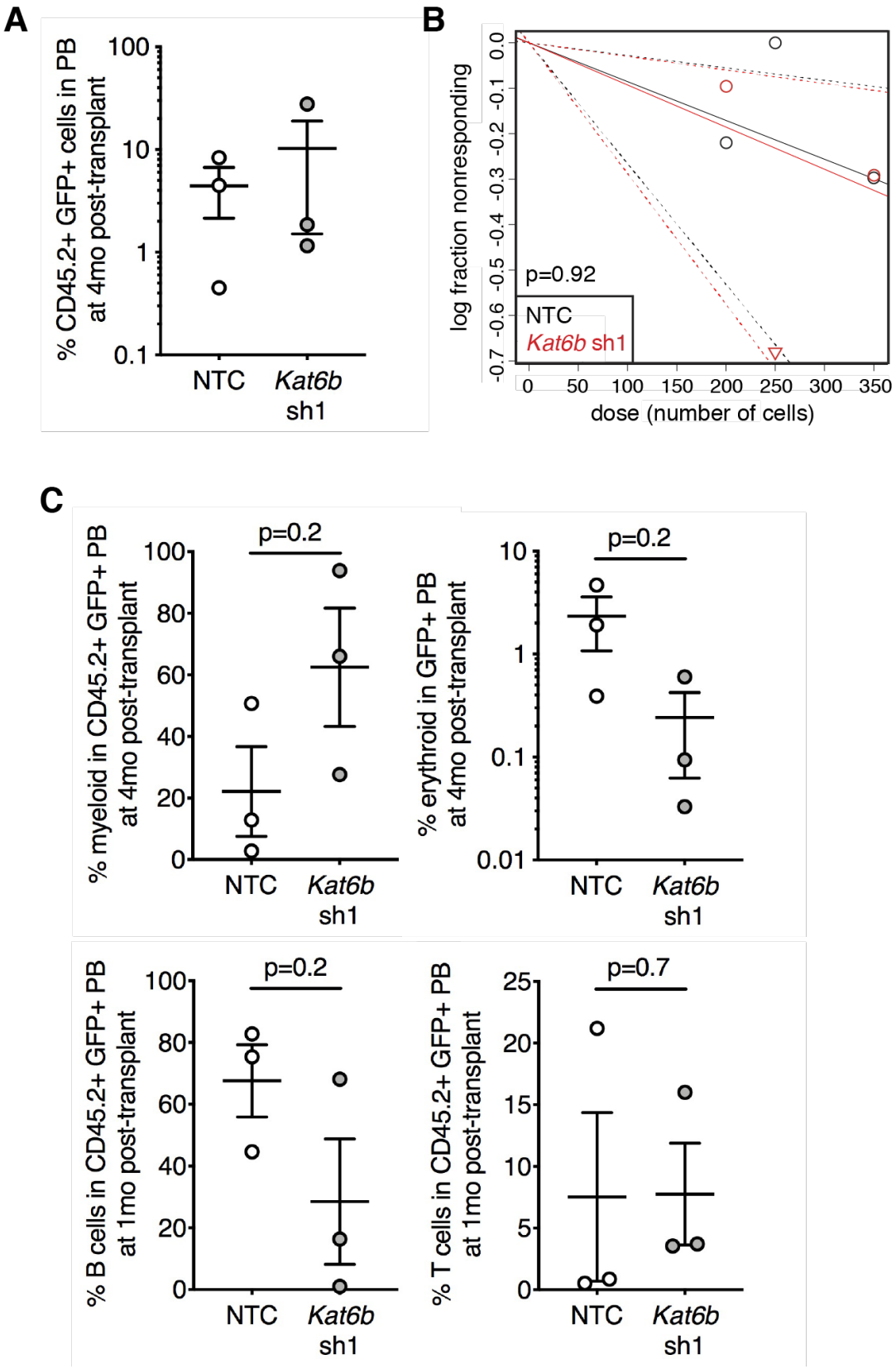

A
% CD45.2+ GFP+ cells in PB at 4mo post-transplant
100
10
1
0.1
NTC
Kat6b sh1
B
log fraction nonresponding
0.0
-0.1
-0.2
-0.3
-0.4
-0.5
-0.6
-0.7
p=0.92
NTC
Kat6b sh1
0
50
100
150
200
250
300
350
dose (number of cells)
C
% myeloid in CD45.2+ GFP+ PB at 4mo post-transplant
p=0.2
100
80
60
40
20
0
NTC
Kat6b sh1
% erythroid in GFP+ PB at 4mo post-transplant
p=0.2
10
1
0.1
0.01
NTC
Kat6b sh1
% B cells in CD45.2+ GFP+ PB at 1mo post-transplant
p=0.2
100
80
60
40
20
0
NTC
Kat6b sh1
% T cells in CD45.2+ GFP+ PB at 1mo post-transplant
p=0.7
25
20
15
10
5
0
NTC
Kat6b sh1

**Figure 2.18. Multilineage engraftment of LT-HSCs following *Kat6b* knockdown.** (A) Frequency of donor-derived cells in the peripheral blood (PB) of recipient mice at 4mo post-transplant. Each dot represents one recipient mouse. Lines represent mean ± SEM of $n = 3$ biological replicates. (B) Limiting dilution analysis of repopulating cell frequency from NTC or *Kat6b*-transduced LT-HSCs at 4mo post-transplant**.** (C) Frequency of myeloid ($CD11b^+$), erythroid ($Ter119^+$), B ($B220^+$) and T ($CD3e^+$) cells within donor-derived PB cells at four months post-transplant. Each dot represents one recipient mouse. Lines represent mean ± SEM of $n = 3$ biological replicates. *P* values calculated by Mann-Whitney test.

These data were utilized to calculate repopulating cell frequency by limiting dilution analysis (Y. Hu & Smyth, 2009). In the NTC group, the repopulating cell frequency was calculated to be 1/1170 (1/3644 to 1/376; 95% Confidence Interval (CI)), similar to the repopulating cell frequency in *Kat6b* knockdown (1/6720, 1/1683 to 1/422; 95% CI) (Figure 2.18B). In addition, frequency of donor-derived lymphocytes, erythroid and myeloid cells showed similar trends at 4-month transplant as were observed at 1-month post-transplant in *Kat6b* knockdown compared to NTC, but did not reach statistical significance (Figure 2.18C). Together, these results show that knockdown of *Kat6b* causes myeloid-biased differentiation from LT-HSCs in vivo without altering repopulation capacity.

### 2.2.4. Knockdown of *Kat6b* in LT-HSCs decreases multilineage priming and promotes expression of inflammation-associated gene signatures

To investigate the molecular mechanisms underlying myeloid-biased differentiation after *Kat6b* knockdown, I transduced LT-HSCs with NTC or *Kat6b* sh1 and performed RNA-seq on sorted $GFP^+$ cells. Unsupervised clustering separated NTC and *Kat6b* knockdown samples (Figure 2.19A). 252 significantly differentially expressed genes ($FDR < 0.05$) were identified, out of which 127 genes were upregulated and 125

genes were downregulated in *Kat6b* knockdown compared to NTC (Figure 2.19B). *Kat6b* itself was found to be downregulated by 1.2-fold in *Kat6b* knockdown compared to NTC samples. In addition, I observed downregulation of *Aldh3a1* (aldehyde dehydrogenase family 3), loss of which impairs B cell development and HSC function (Gasparetto et al., 2012) and *Apoe* (apolipoprotein E), loss of which has been demonstrated to cause monocytosis and neutrophilia in mice (Murphy et al., 2011).

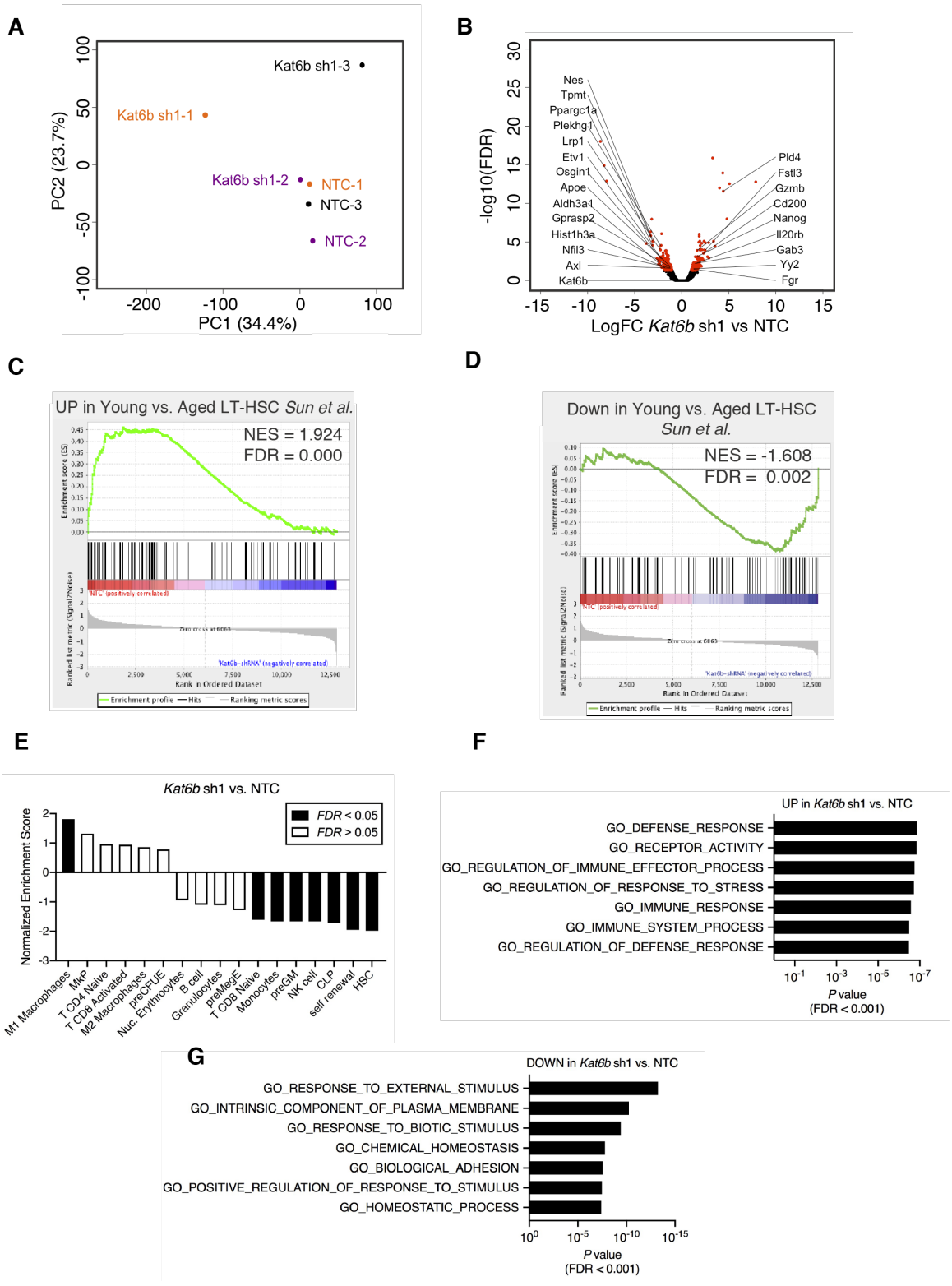



**Figure 2.19. *Kat6b* knockdown alters gene expression programs critical for multilineage differentiation.** (A) PCA plot showing unsupervised clustering of gene

expression profiles from *Kat6b* sh1 ($n = 3$) and NTC ($n = 3$). Each color represents a set of biological replicate samples. (B) Volcano plot showing log fold changes of genes against -log10 of *FDR*. Points in red highlight genes with $FDR < 0.05$. (C) Gene set enrichment analysis (GSEA) of genes upregulated in young versus aged LT-HSCs (Sun et al., 2014). Red denotes NTC and blue denotes *Kat6b* sh1. (D) Gene set enrichment analysis (GSEA) of genes upregulated in aged versus young LT-HSCs (Sun et al., 2014). Red denotes NTC and blue denotes *Kat6b* sh1. (E) Normalized enrichment score from GSEA analysis of the indicated datasets in *Kat6b* sh1 versus NTC. Black bars indicate $FDR < 0.05$, white bars indicate $FDR > 0.05$. (F) Top gene ontology (GO) terms enriched in genes found to be significantly upregulated in *Kat6b* sh1 versus NTC (fold change > 2 and $P < 0.05$). (G) Top gene ontology (GO) terms enriched in genes found to be significantly downregulated in *Kat6b* sh1 versus NTC (fold change > 2 and $P < 0.05$).

To test the hypothesis that *Kat6b* knockdown alters expression of gene programs associated with aging and differentiation of LT-HSCs, we performed gene set enrichment analysis (GSEA) (Mootha et al., 2003; Subramanian et al., 2005). Gene set enrichment analysis compares the expression of query gene list between our control or experiment samples and then ranks the genes based on enrichment in either the control or experimental samples. Comparing our RNA-seq data to LT-HSC aging gene signatures (Sun et al., 2014) revealed that genes more highly expressed in young versus aged LT-HSCs were significantly enriched in NTC versus *Kat6b* knockdown (Figure 2.19C). On the other hand gene more highly expressed in old vs young LT-HSCs were significantly enriched in *Kat6b* knockdown vs NTC from (Sun et al., 2014) (Figure 2.19D. This might also explain the heterogeneity among Kat6b sh1 biological replicates. as heterogeneity due to epigenetic dysregulation in HSCs is a feature of aging (Buisman & Haan, 2019).

I then compared our dataset to previously defined gene signatures representing HSCs (Chambers et al., 2007), the self-renewal program (Krivtsov et al., 2006), hematopoietic progenitor cell populations (lymphoid (CLP), granulocyte-macrophage (preGM) and erythroid-megakaryocyte (preMegE, preCFU-E, MkP)) (Sanjuan-Pla et al.,

2013), and mature hematopoietic cell populations (M1 and M2 macrophages, monocytes, granulocytes, erythrocytes, $CD4^+$ naïve T cells, $CD8^+$ naïve and activated T cells, B cells and NK cells) (Chambers et al., 2007; Engler, Robinson, Smirnov, Hodgson, & Berger, 2012; Mantovani, Sozzani, Locati, Allavena, & Sica, 2002; Martinez, Gordon, Locati, & Mantovani, 2006). This analysis revealed that *Kat6b* knockdown LT-HSCs had a significant enrichment of an M1 macrophage signature while NTC LT-HSCs were enriched in HSC/self-renewal, preGM, monocyte, CLP, NK and $CD8^+$ naïve T cell signatures (Figure 2.19E). This result is consistent with literature demonstrating downregulation of *Kat6b* during macrophage polarization and M1 activation (Shukla et al., 2018). To further interrogate mechanisms underlying the observed myeloid differentiation bias of *Kat6b* knockdown LT-HSCs, unbiased GO enrichment analysis was utilized. This analysis revealed significant upregulation of signatures associated with defense response, immune processes and immune response in *Kat6b* knockdown LT-HSCs (Figure 2.19F) and downregulation of signatures associated with response to external stimulus and homeostasis (Figure 2.19G). Together, these data suggest that decreased expression of *Kat6b* in LT-HSCs impairs multilineage differentiation, and permits a transcriptional program promoting differentiation toward pro-inflammatory-type macrophages and that is associated with aging.

### 2.2.5. H3K23ac levels trend towards decline in aged LT-HSCs

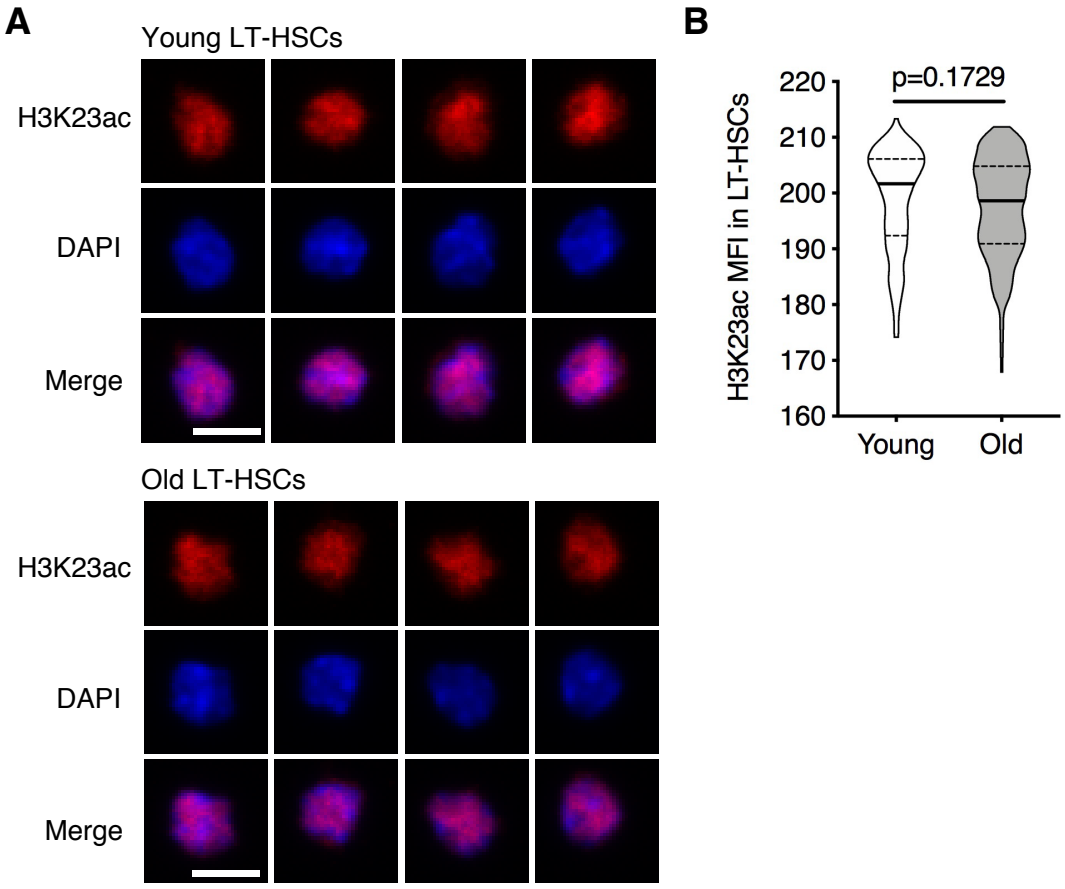



**Figure 2.20: Alterations in H3K23ac with aging.** (A) Representative immunofluorescence images of H3K23ac and DAPI in LT-HSCs isolated from young and aged mice. Scale bar equals 5 μm. (B) Violin plots of mean fluorescence intensity (MFI) of H3K23ac in LT-HSCs isolated from young and aged mice. Solid lines indicate median and dotted lines indicate quartiles. Data points include $n$ = 25-69 individual cells sampled from $n$ = 4 biological replicate animals. $P$ values calculated by unpaired $t$ test.

As KAT6B is known to catalyze H3K23 acetylation, I hypothesized that this modification would be decreased in LT-HSCs with aging. To test this hypothesis, I isolated LT-HSCs from young and aged mice by FACS and immunostained with an

antibody against H3K23ac and DAPI (Figure 2.20A). I observed a trend toward decrease in mean fluorescence intensity of H3K23ac in LT-HSCs isolated from aged mice ($P$ = 0.1729) (Figure 2.20B). These results suggest that H3K23ac may be modestly reduced with age in LT-HSCs, consistent with reduction in KAT6B at the transcript and protein levels.

# CHAPTER 3

# METHODS

## 3.1. Experimental animals

Young C57BL/6J (2-4 months old), aged C57BL/6J (20-24 months old) and B6.SJL-*Ptprc*$^{a}$*Pepc*$^{b}$/BoyJ (B6.CD45.1) (2-4 months old) were obtained from, and aged within, The Jackson Laboratory. All mice used in this study were females. All experiments were approved by The Jackson's Laboratory Institutional Animal Care and Use Committee (IACUC).

## 3.2. Plasmids

shRNA expression plasmids (in pLKO.1 or pLKO1.5) were obtained from Sigma (St. Louis, MO) (Table 3.1).

**Table 3.1. shRNA plasmids.**

| Target Gene Symbol | Target Gene Name | Clone Number (Sigma) |
|---|---|---|
| NTC | MISSION® pLKO.1-puro Non-Target shRNA Control Plasmid DNA or TRC2 pLKO.5-puro Non-Mammalian shRNA Control Plasmid DNA | SHC202 or SHC016 |
| *Kat6b* sh1 | K(lysine) acetyltransferase 6B | TRCN0000287544 |
| *Kat6b* sh2 | K(lysine) acetyltransferase 6B | TRCN0000039341 |
| *Crebbp* | CREB binding protein | TRCN0000012725 |
| *Rnf40* | ring finger protein 40 | TRCN0000041048 |

| | | |
|---|---|---|
| *Kmt5a* | lysine methyltransferase 5A | TRCN0000241070 |
| *Atxn7l3* | ataxin 7-like 3 | TRCN0000251735 |
| *Tbl1x* | transducin (beta)-like 1 X-linked | TRCN0000109355 |
| *Atxn7l1* | ataxin 7-like 1 | TRCN0000348933 |

Table 3.1. Continued

| | | |
|---|---|---|
| *Ndn* | necdin | TRCN0000312951 |
| *Ezh1* | enhancer of zeste 1 polycomb repressive complex 2 subunit | TRCN0000317140 |
| *Kdm5b* | lysine (K)-specific demethylase 5B | TRCN0000113491 |
| *Ncor2* | nuclear receptor co-repressor 2 | TRCN0000095281 |
| *Suv39h2* | suppressor of variegation 3-9 2 | TRCN0000353741 |
| *Prdm16* | PR domain containing 16 | TRCN0000075459 |
| *Cxxc1* | CXXC finger 1 (PHD domain) | TRCN0000257088 |
| *Dach1* | dachshund family transcription factor 1 | TRCN0000433533 |

pLKO.3G was a gift from Christophe Benoist & Diane Mathis (Addgene, Watertown, MA; plasmid #14748). The GFP cassette from pLKO.3G was sub-cloned into each shRNA plasmid. Colony PCR with eGFP primers (Table 3.2) was performed to identify clones containing the GFP insert

**Table 3.2. Primer sequences.**

| **Target** | **Purpose** | **Forward Primer (5')** | **Reverse Primer (5')** |
|---|---|---|---|
| eGFP | Cloning | GCAGTCGGCTCCCTCGTTGACCGA | CTGCACGCTCCCGTCCTCGATGTT |
| *B2m* | Real-time PCR | CAGTATGTTCGGCTTCCCATTC | TTCTGGTGCTTGTCTCACTGA |
| *Kat6b* set 1 | Real-time PCR | AGAAGAAAAGGGGTCGTAAACG | GTGGGAATGCTTTCCTCAGAA |

| | | | |
|---|---|---|---|
| *Kat6b* set 2 | Real-time PCR | AGCTTCTGTTTGGGGACTAAAG | GTGTCCACTACTGCCACAATC |
| *Crebbp* | Real-time PCR | CCAAACGAGCCAAACTCAGC | TTTGGACGCAGCATCTGGAA |
| *Rnf40* | Real-time PCR | GACCCTACGGTGACGGAAGT | CCAGTAGCGGTTGACGATGT |
| *Kmt5a* | Real-time PCR | CAGACCAAACTGCACGACATC | CTTGCTTCGGTCCCCATAGT |

Table 3.2. Continued

| | | | |
|---|---|---|---|
| *Atxn7l3* | Real-time PCR | AAGGAGTGTGTTTGCCCCAA | AGACTTGGATCTTCGAGGGGA |
| *Tbl1x* | Real-time PCR | CACAAGTTGCACGGCTCG | ACTGTGGCTTTACTCGGTGG |
| *Atxn7l1* | Real-time PCR | CAAGCCCTAGAACAGCGTCA | AGCAAGTTTCTGCCCTCACA |
| *Ndn* | Real-time PCR | CCAGAGGAGCTAGACAGGGT | ACGCCTGGGGATCTTTCTTG |
| *Ezh1* | Real-time PCR | CAACACTTCCCGCTGCATTC | GGCGCTTCCGTTTTCTTGTT |
| *Kdm5b* | Real-time PCR | CGAGCTGGGAAGAGTTCGC | ATCACAAGCGAATGGTGGCT |
| *Ncor2* | Real-time PCR | CCTGGTGGAAGTTCGTGGAC | GCTCCTGAGACCGTTCACTC |
| *Suv39h2* | Real-time PCR | GACCGCGCCAGTTTGAATG | CTAAAGGTGGGCCCTCCAAG |
| *Prdm16* | Real-time PCR | ATGGATCCCATCTACAGGGTA | CATTGCATATGCCTCCGGGT |
| *Cxxc1* | Real-time PCR | GATGATCACGGCCTACCCTG | GCCGTTTGTACCTCTCCTCC |
| *Dach1* | Real-time PCR | GGCTTTCGACCTGTTCCTGA | AGGAAGTTCCAGTCCAACACT |
| *PU1* | Knockout Screen | AACAGATGCACGTCCTCGAT | GAAGGCTGTGCTTGTGGAGT |
| *Enhancer A1* | Knockout Screen | ATGGCCAGTCATACAAAGGATTT | AATGGCACAAAGAAGTCTTGAGAA |
| *Enhancer B1* | Knockout Screen | ACCGTCTAAGAGGCTTTGGC | GCTACTAAGGCCACCGCTAG |

| | | | |
|---|---|---|---|
| *Enhancer C* | Knockout Screen | TTGGTGCCATTTTTCACATGATGC | AGATGTTCTTTGGCCATCACTCT |
| *Enhancer D* | Knockout Screen | GTCCTCCAAGAATGGGGGTG | CACCCCTCCGGTTAGGTTTC |
| Px458 Reverse | sgRNA synthesis | AGCACCGACTCGGTGCCACT | |

pLENTI-CRISPR-EGFP (EGFP) was a gift from Dr. Rick Maser. PX458 plasmid was obtained from addgene.

**3.3. Lentiviral supernatant for *Kat6b* knockdown experiments**

shRNA expression plasmids, RC-CMV-Rev1b, HDM-Hgpm2 (*gag-pol)*, HDM-tat1b, HDM-VSV-G (at a mass ratio of 10:2:2:2:1) were transfected into 5 x $10^6$ HEK-293T cells (ATCC, Manassas, VA), seeded 24 hours before transfection. CalPhos™ Mammalian Transfection Kit (Takara Bio, Mountain View, CA) was used for transfection. Growth media was replaced 24 hours after transfection. For viral supernatant collection, media was collected 48 hours after transfection, centrifuged at 1250 rpm for 5 min at 4$^o$C and aliquots of supernatant were stored at -80$^o$C.

**3.4. Lentiviral supernatant for pLenti-CRISPR-EGFP transduction optimization experiments**

For 4$^{th}$ generation system, pLenti-CRISPR-EGFP, RC-CMV-Rev1b, HDM-Hgpm2 (*gag-pol)*, HDM-tat1b, HDM-VSV-G, or for 3$^{rd}$ generation system PMD2.G and pxPAX2 were transfected into 5 x $10^6$ HEK-293T cells (ATCC, Manassas, VA), seeded 24 hours before transfection. CalPhos™ Mammalian Transfection Kit (Takara Bio, Mountain View, CA) was used for transfection. Growth media was replaced 24 hours

after transfection. For viral supernatant collection, media was collected 48 hours after transfection, centrifuged at 1250 rpm for 5 min at 4ºCand used either fresh or aliquots of supernatant were stored at -80ºC.

**3.5. Titering of viral supernatant**

To titer lentiviral supernatant, 2 x $10^5$ NIH/3T3 cells (ATCC) were seeded in a 6-well plate 24 hours before supernatant was thawed and added at 1:2, 1:10, 1:50, 1: 250 and 1:1250 diluted in DMEM+10%FBS with 5 ug/ml polybrene. Plates were spun at 2500 rpm for 60 min at room temperature followed by incubation at 37ºC and 5% $CO_2$ for 48 hours with media change after 24 hours. After this 48h culture period, cells were harvested and run on a LSRII (BD Biosciences, San Jose, CA) to assess frequency of $GFP^+$ cells. These frequencies were used to calculate the titer of each preparation of lentiviral supernatant. In addition, RNA was isolated from transduced NIH/3T3 cells to assess shRNA knockdown efficiency by real-time PCR.

**3.6. Primary cell isolation**

LT-HSCs and MPP4 cells were isolated as described previously (Young et al., 2016b). Femurs, tibiae, iliac crests from each mouse were pooled, crushed and filtered to prepare single-cell suspensions of bone marrow (BM). Ficoll-Paque (GE Healthcare, Chicago, IL) density centrifugation was used to isolate BM mononuclear cells (MNCs). These cells were stained with fluorochrome-conjugated antibodies from BioLegend (San Diego, CA), eBiosciences (ThermoFisher Scientific, Waltham, MA), BD Biosciences (San Jose, CA): c-Kit (clone 2B8), CD48 (clone HM48-1), CD150 (clone TC15-12F12.2), Sca-1 (clone D7), FLT3 (clone A2F10), mature lineage (Lin) marker mix (B220 (clone RA3-6B2), CD11b (clone M1/70), CD4 (clone RM4-5), CD8a (clone 53-

67), Ter-119 (clone TER-119), Gr-1 (clone RB6-8C5), CD5 (clone 53-7.3)) and viability stain propidium iodide (PI). Cells were sorted on a FACSAria (BD Biosciences) with these surface marker profiles: LT-HSC ($Lin^-$ $Sca^+$ c-$Kit^+$ $CD150^+$ $CD48^-$), MPP4 cells ($Lin^-$ $Sca^+$ c-$Kit^+$ $Flt3^+$), LSK cells ($Lin^-$ $Sca^+$ c-$Kit^+$) and LK cells ($Lin^-$ c-$Kit^+$).

**3.7. Transduction of LT-HSCs, MPP4 Cells**

LT-HSCs were resuspended in SFEMII (StemCell Technologies, Vancouver, Canada) supplemented with growth factors described previously (Holmfeldt et al., 2016); Stem cell factor (SCF; 10 ng/ml), thrombopoietin (TPO; 20 ng/ml), insulin-like growth factor 2 (IGF2; 20 ng/ml) and fibroblast growth factor (FGF; 10 ng/ml) (BioLegend or StemCell Technologies) along with 5ug/ml polybrene (Sigma) and viral supernatant. Lentiviral supernatant was added at concentration of 1000 MOI in a total volume of 200 ul in a 96-well U-bottom plate. The plate was spun at 2500 rpm for 60 min at room temperature. After this, cells were cultured at 37$^o$C and 5% $CO_2$ for 36 hours. Transduced cells (viable $GFP^+$) were sorted on a FACSAria (BD Biosciences). MPP4 cells were handled as above in IMDM (ThermoFisher Scientific) supplemented with 10% FBS (VWR, Radnor, PA), interleukin-3 (IL-3, 10 ng/ml ), interleukin-6 (IL-6, 10 ug/ml), interleukin-7 (IL-7, 20 ng/ul), SCF (100 ng/ml), leukemia inhibitory factor (LIF, 20 ng/ml) (Peprotech, Rocky Hill, NJ) and polybrene (5 ug/ml) (Sigma).

**3.8. Generation of concentrated virus by ultracentrifugation**

shRNA expression plasmids, RC-CMV-Rev1b, HDM-Hgpm2 (*gag-pol)*, HDM-tat1b, HDM-VSV-G (at a mass ratio of 10:2:2:2:1) were transfected into 5 x $10^6$ HEK-293T cells (ATCC, Manassas, VA), seeded 24 hours before transfection. CalPhos™ Mammalian Transfection Kit (Takara Bio, Mountain View, CA) was used for

transfection. Growth media was replaced 24 hours after transfection. For viral supernatant collection, media was collected 48 hours after transfection, centrifuged at 1250 rpm for 5 min at 4$^{o}$C and filtered through a 0.45uM filter. Filtrate was centrifuged at 25000 rpm for 90 mins. Viral pellet was incubated at 4C overnight, followed by resuspension. Aliquots of supernatant were stored at -80$^{o}$C.

**3.9. Electroporation of Cas9-sgRNA RNPs in HSPCs:**

Electroporation protocol was adapted from (Gundry et al., 2016).LK cells were cultured for three in presence of 10 ng/ml SCF 100 ng/ml TPO in SFEMII media for three hours. Cells were resuspended in 10 ul of Buffer T (Neon Transfection Kit). 1 ug of Cas9 protein (PNABio) was incubated with 1 ug of *in vitro* transcribed sgRNA (0.5 ug of each sgRNA was used which flanked the target knockout region) in a total volume of 2 ul for 15 mins at RT. RNP complexes were combined with resuspended cells and electroporated at 1700 V, 20 ms (Neon Transfection System) followed by cell count and plating into OP9 co-culture assay.

Screen for knockout was performed by isolation of genomic DNA of cells with DNeasy Blood and Tissue Kit (Qiagen) and performing PCR by using primers listed in Table 3.2.

**3.10. *In vitro* synthesis of sgRNAs**

Oligos containing T7 promoter and variable region of sgRNAs against candidate loci were ordered from Integrated DNA Technologies (Table 3.3).

**Table 3.3. Oligos for CRISPR/Cas9 knockout.**

| Target | Purpose | sgRNA 1 | sgRNA2 |
|---|---|---|---|
| *PU1* | Cas9 Knockout | GAAATTAATACGACTCACTATAAGTCCATAAGGGATAGCCCAGTTTTAGAGCTAGAAATAGC | GAAATTAATACGACTCACTATAAGACTCCCAAGGAAGCACCGGTTTTAGAGCTAGAAATAGC |
| *Enhancer A1* | Cas9 Knockout | GAAATTAATACGACTCACTATAGGGCAAGGGTGTAAAGAAGTGTTTTAGAGCTAGAAATAGC | GAAATTAATACGACTCACTATAGGGCAAGGGTGTAAAGAAGTGTTTTAGAGCTAGAAATAGC |
| *Enhancer B1* | Cas9 Knockout | GAAATTAATACGACTCACTATAAGAAGAGAGTGCACTCACAGGTTTTAGAGCTAGAAATAGC | GAAATTAATACGACTCACTATAAGATTGGCAAGGGCTCACAGGTTTTAGAGCTAGAAATAGC |
| *Enhancer C* | Cas9 Knockout | GAAATTAATACGACTCACTATAAGTAGCTATTTGGAACCTGAGTTTTAGAGCTAGAAATAGC | GAAATTAATACGACTCACTATAGGTTGATGATCAGTATGTGAGTTTTAGAGCTAGAAATAGC |
| *Enhancer D* | Cas9 Knockout | GAAATTAATACGACTCACTATAGAACCTTGGGAATACAACACGTTTTAGAGCTAGAAATAGC | GAAATTAATACGACTCACTATAGGGAGTACCTGGTGTTCCAAGTTTTAGAGCTAGAAATAGC |

These oligos were used as primers to PCR amplify scaffold region of sgRNA from PX458 plasmid followed by purification of PCR products by QIAquick PCR Purification Kit (Qiagen). *In vitro* RNA transcription of these PCR products was performed by HiScribe T7 High Yield RNA Synthesis Kit (NEB) followed by purification and concentration by RNA Clean & Concentrator-25 (Zymo Research).

### 3.11. OP9 co-culture for differentiation to B-lymphoid cells

OP9 co-culture protocol was adapted from (Pietras et al., 2015a). OP9 cells were cultured with MEM-α media 89%, FBS 10%, Pen/Strep 1%. On Day -1 OP-9 cells were seeded in 96-well or 24-well plate. LSKs or LKs were added to pre-seeded OP9 cells in

200 ul of B-cell media (optiMEM 93.9% , FBS 5%, Pen/strep 1%, 1% of 55mM B-mercaptoethanol 0.1%, SCF 10 ng/ml, Flt3L 10 ng/ml, IL-7 5 ng/ml). On Day 2, half media was replaced with fresh media **(**optiMEM 93.9% , FBS 5%, Pen/strep 1%, 55mM B-mercaptoethanol 0.1%, SCF 10 ng/ml, IL-7 5 ng/ml). On Day 5, half media was replaced with fresh media (optiMEM 93.9% , FBS 5%, Pen/strep 1%, 55mM B-mercaptoethanol 0.1%, IL-7 5 ng/ml). After this media was changed every 2-3 days same as done on Day 5. When the OP9 layer started to detach from the periphery, 5000 OP9 cells were seeded in 1 well of a 24 well plate. Cells were harvested by trypsinization. Resuspended in 200 ul of B-cell media (same composition as Day 5 of assay) along with old media from 96-well plate.

**3.12. Transduction optimization experiments for pLenti-CRISPR-EGFP**

For Figure 2.8 1 x $10^6$ whole bone marrow cells were resuspended in 1.8 ml of respective viral supernatant supplemented with 5% FBS, SCF (100 ng/ml) and Polybrene (13.5 ug/ml). Cells were spun at 2500 rpm for 1 hour. After overnight culture, cells were resuspended in SFEMII with SCF (100 ng/ml).

For Figure 2.9 approximately 9000 LSKs were resuspended in SFEMII media supplemented with SCF (100 ng/ml), Polybrene (5 ug/ml) with 8% by volume pLENTI-CRISPR-EGFP viral supernatant. Cells were spun at 1800 rpm for 30 min. After overnight culture, cells were transduced with similar conditions as Day 1.

For Figure 2.10 approximately 4000 LSK cells were resuspended in SFEMII media supplemented with SCF (100 ng/ul), Polybrene (8 ug/ml) and 10%, 50% or 70%

pLENTI-CRISPR-EGFP viral supernatant. Cells were spun at 2500 rpm for 60 mins. After overnight culture, transduction was repeated as performed on Day 1.

For Figure 2.11 approximately 2000 LSKs were resuspended in IMDM medium with 0.5%, 10% or 50% of concentrated viral supernatant, SCF (100 ng/ul), Polybrene (5ug/ml) made up to 200 ul total volume with IMDM 10% FBS. Cells were spun at 2500 rpm for 60 mins. After overnight culture cells were resuspended in IMDM 10% FBS.

### 3.13. Colony forming unit (CFU) Assays

For B-lymphoid CFU assays, 100 $GFP^+$ cells from transduced MPP4 cells were plated in Methocult M3630 (StemCell Technologies) supplemented with FMS-like tyrosine kinase like 3 ligand (FLT3L, 25 ng/ml) and SCF (50 ng/ml) (Peprotech). For myeloid CFU assays, 100 $GFP^+$ cells from transduced MPP4 cells or 200 $GFP^+$ cells from transduced LT-HSCs were plated in Methocult GF M3434 (StemCell Technologies) and cultured at 37$^o$C and 5% $CO_2$. Scoring of colonies was done between days 7 and 10 using a Nikon Eclipse TS100 inverted microscope. CFU cloning efficiency was calculated as the sum of the myeloid and B-lymphoid colonies divided by the sum of the myeloid and B-lymphoid colonies in the NTC group. For analysis of replating potential, colonies were harvested and total viable counts were obtained, followed by plating of 1-3 x $10^4$ cells in Methocult GF M3434. Remaining cells were resuspended in RLT buffer (Qiagen, Hilden, Germany). RNA was isolated by RNeasy Micro or RNeasy Mini kits (Qiagen) followed by cDNA synthesis using the SuperScript™ III First-Strand Synthesis SuperMix (ThermoFisher Scientific).

### 3.14. Real-time PCR

Real-time PCR was performed using $RT^2$ SYBR Green ROX qPCR Mastermix (Qiagen) using the Viaa7 or QuantStudio 7 Flex (Applied Biosystems, Foster City, CA). Refer to Supplemental Table 5 for primer sequences used.

### 3.15. Immunofluorescence staining of LT-HSCs

Staining was performed as previously described (Florian et al., 2012). Sorted LT-HSCs were seeded on retronectin-coated glass coverslips in SFEMII supplemented with SCF (10 ng/ml), TPO (20 ng/ul), IGF2 (20 ng/ul), FGF (10 ng/ul) (BioLegend or StemCell Technologies) for at least 2 hours. Cells were fixed in 4% PFA (ThermoFisher Scientific). Cells were washed with PBS, permeabilized with 0.2% Triton X-100 (ThermoFisher Scientific) in PBS for 20 mins and blocked with 10% goat serum (ThermoFisher Scientific) for 20 mins. Cells were stained by either α-KAT6B (NBP1-92036; Novus Biologicals, Centennial, CO) or α-H3K23ac (ab61234; Abcam, Cambridge, UK) for one hour at room temperature. For secondary antibody, cells were stained with α-Rabbit conjugated with Alexa-568 (A-11036; ThermoFisher Scientific) for one hour. Coverslips were mounted on slides with Gold Antifade with DAPI (ThermoFisher Scientific). Imaging was performed with Leica SP8 confocal microscope. Z-stack images were summed and quantification of individual fluorescence intensities was performed by Fiji software (Schindelin et al., 2012). Cells were detected by Fiji in range of 150-250 pixel units. Scale bars in images represent 5 um.

### 3.16. *In Vivo* transplantation

200-350 transduced $GFP^+$ cells were combined with 5 x $10^5$ bone marrow mononuclear cells (MNCs) from B6.CD45.1 mice and retro-orbitally injected into recipient B6.CD45.1 mice after 1000 rads gamma irradiation (split dose). Peripheral blood from recipient mice was analyzed by flow cytometry every 4 weeks after transplantation. Blood was collected by retro-orbital bleeding and stained with a combination of fluorochrome-conjugated antibodies from BioLegend or BD Biosciences with or without red blood lysis: CD45.1 (clone A201.7 or clone A20), CD45.2 (clone 104), B220 (clone RA3-6B2), CD3e (clone 145-2C11), CD11b (clone M1/70), Ly6g (clone 1A8), Ly6c (clone HK1.4), Ter-119 (clone TER-119), GR1 (clone RB6-8C5), CD4 (clone GK1.5), CD8a (clone 53-6.72) and CD41 (clone MWReg30). Stained cells were run on a FACSymphony A5 (BD Biosciences) and data was analyzed using FlowJo software (FlowJo, LLC, Ashland, OR).

### 3.17. RNA-Seq

Transduced $GFP^+$ LT-HSCs from 3 independent biological replicates were sorted directly into 350 ul of RLT buffer (Qiagen). Total RNA was isolated from cells using the RNeasy Micro kit (Qiagen), according to manufacturers' protocols, including the optional DNase digest step. Sample concentration and quality were assessed using the Nanodrop 2000 spectrophotometer (ThermoFisher Scientific) and the RNA 6000 Pico LabChip assay (Agilent Technologies, Santa Clara, CA). Libraries were prepared by the Genome Technologies core facility at The Jackson Laboratory using the Ovation RNA-seq System V2 (NuGEN Technologies, Redwood City, CA) and Hyper Prep Kit (Kapa Biosystems, Wilmington, MA). Briefly, the protocol entails first and second strand cDNA synthesis

and cDNA amplification utilizing NuGEN's Ribo-SPIA technology, cDNA fragmentation, ligation of Illumina-specific adapters containing a unique barcode sequence for each library, and PCR amplification. Libraries were checked for quality and concentration using the D5000 ScreenTape assay (Agilent Technologies) and quantitative PCR (Kapa Biosystems), according to the manufacturers' instructions. Libraries were pooled and sequenced 75 bp single-end on the NextSeq 500 (Illumina, San Diego, CA) using NextSeq High Output Kit v2.5 reagents. Raw and processed data was deposited in the Gene Expression Omnibus (GEO accession GSE133304).

**3.18. RNA-seq analysis**

Trimmed alignment files (with trimmed base quality value < 30, and 70% of read bases surpassing that threshold) were processed using the RSEM (v1.2.12; RNA-Seq by Expectation-Maximization) software (B. Li & Dewey, 2011) and the Mus Musculus reference GRCm38. Alignment was completed using Bowtie 2 (v2.2.0) (Langmead & Salzberg, 2012) and processed using SAMtools (v0.1.18) (H. Li et al., 2009). Fragment length mean was set to 280 and standard deviation to 50. Expected read counts per gene produced by RSEM were rounded to integer values, filtered to include only genes that have at least two samples within a sample group having a cpm > 1.0, and were passed to edgeR (v3.5.3) (Robinson, Mccarthy, & Smyth, 2010) for differential expression analysis. The negative binomial conditional common likelihood was maximized to estimate a common dispersion value across all genes. Exact tests were used to elucidate statistical differences between the two sample groups of negative-binomially distributed counts producing *p*-values per test. The Benjamini and Hochberg's algorithm (*p*-value adjustment) was used to control the false discovery rate (FDR). Features with an *FDR*-

adjusted $p$-value < 0.05 were declared significantly differentially expressed. Gene set enrichment analysis (GSEA) (Daly et al., 2003; Subramanian et al., 2005) was performed using previously published aged LT-HSC RNA-seq data (Sun et al., 2014) and previously defined gene signatures representing HSCs (Chambers et al., 2007), the self-renewal program (Krivtsov et al., 2006), hematopoietic progenitor cell populations (lymphoid (CLP), granulocyte-macrophage (preGM) and erythroid-megakaryocyte (preMegE, preCFU-E, MkP)) (Sanjuan-Pla et al., 2013), and mature hematopoietic cell populations (M1 and M2 macrophages, monocytes, granulocytes, erythrocytes, CD4+ naïve T cells, CD8+ naïve and activated T cells, B cells and NK cells) (Chambers et al., 2007; Engler et al., 2012; Mantovani et al., 2002; Martinez et al., 2006) (Supplemental Table 3).

**3.19. Statistical analysis**

Sample groups were compared using an unpaired *t* test, Mann-Whitney test, one-way ANOVA and Holm-Sidak's multiple comparisons test, or two-way ANOVA and Dunnett's multiple comparisons test as indicated in figure legends.Prism software (GraphPad Software, San Diego, CA) was utilized for statistical calculations and graphing.

# CHAPTER 4

# DISCUSSION

## 4.1. OP9 co-culture of HSPCs is a qualitative assay

I have reproduced the finding that the OP9 co-culture of HSPCs is successful at the production of $CD19^+$ B-lymphoid cells as has been earlier reported (Pietras et al., 2015b). However, I observed that this assay shows considerable heterogeneity with respect to efficiency of differentiation of HSPCs to mature cells. I did not find any correlation between the number of HSPCs which were input in the start of assay and the number of $CD45^+$ cells produced at the end. This was the first indication that this assay was suitable for qualitative measure of B-lymphoid differentiation potential rather than quantitative assessment. However, I attributed this heterogeneity in the performance of this assay to the culture conditions. After 7-10 days of culture the OP9 layer started to detach from the surface of a 96-well plate, at which time OP9 and HSPCs were harvested by trypsinization and centrifugation and were transferred to a well 24 well plate containing pre-seeded OP9 cells for rest of the assay. I hypothesized that this step was the source of variability of this assay and so I tested this hypothesis by performing the assay starting from 96-well and 24-well and continuing the culture for 12 days without re-plating. I observed that these conditions produced $CD19^+$ cells with lower efficiency as compared to the 23-day long culture with re-plating and there was still heterogeneity among technical replicates. I also attempted to culture HSPCs in transwells which allowed transfer the of HSPCs to a new well without trypsinization steps, however, the percentage of $CD19^+$ cells was considerably lower as compared to cells cultured with initial conditions and variability in output persisted. Although this experiment did not solve the

problem of variability in output, it did provide evidence that HSPCs differentiation into $CD19^+$ cells is dependent on contact with OP9 cells. It can also be inferred from these experiments that the variability in OP9 co-culture of HSPCs could stem from the heterogeneity in OP9 cells themselves which would result in heterogeneity in the performance of the assay. Although another cell line, S17, has been used for *in vitro* differentiation to lymphoid cells, OP9 cells were found to be 30% more efficient (Viera & Cumano, 2004). Therefore, among current methods, OP9 coculture of HSPCs cells is the most appropriate method for assessment of *in vitro* B-lymphoid differentiation potential. That is why this assay is widely used in the field (Adolfsson et al., 2005; Akashi K et al., 2000; Arinobu et al., 2007; Pietras et al., 2015a). My findings provide evidence that OP9 co-culture assay is more suitable for qualitative assessment of B-lymphoid differentiation potential, rather than quantitative analyses. Therefore, these findings provide important considerations for the application of the OP9 co-culture assay. It is also possible that the heterogeneity that I observed in my results could stem from the use of a heterogeneous population of HSPCs. The use of HPSCs for these experiments was important due to the biological question that I was asking regarding their differentiation. Therefore, I propose that future studies should involve studying the effect of using more purified hematopoietic progenitor populations like CLPs in the OP9 co-culture. I also propose that further studies should involve the generation of a more homogenous OP9 cell line and determining the exact cell receptors or molecules secreted by these cells which promote B-lymphoid differentiation, which will allow more controlled in vitro differentiation culture systems.

### 4.2. The OP9 assay is not suitable for enhancer loss of function studies

Enhancers often act in a combinatorial manner in which activities of multiple enhancers contribute to the expression of a single target gene (Maekawa, Imamoto, Merlino, Pastan, & Ishii, 1989; Osterwalder et al., 2018). *In vivo* knockout of experimentally validated limb specific enhancers did not produce any limb morphology phenotypes, whereas deletion of two enhancers did produce in a discernable phenotype showing the presence of redundancy in enhancer function as well (Osterwalder et al., 2018). In context of the hematopoietic system, synergy between two enhancers, HS1 and HS2, of *Gata1*, a gene important for erythroid lineage, has also been established (Testa et al., 2004). Therefore, in the context of the loss of function studies involving the deletion of a single enhancer that I attempted, the expected phenotype is likely to be subtle. And, these subtle phenotypes were probably masked by the heterogeneity and qualitative nature the of OP9 assay. This is a possible explanation that why I did not observe a significant reduction in B-lymphoid differentiation of HSPCs after knockout of *Pu.1*. These technical challenges could be overcome by performing an *in vivo* screen for these putative lymphoid specific enhancers. Such a screen would involve targeting enhancers by a library of genome-wide sgRNAs in HSPCs isolated from Cas9 expressing mice, followed by transplantation in conditioned recipients. Prevalence of sgRNA tags in donor-derived B-cells would a measure of relative importance of each enhancer for differentiation to B-cells.

### 4.3. *Kat6b* is a novel therapeutic target for ameliorating aging-associated decline in HSCs

By employing a shRNA-mediated screen of epigenetic regulators, we have discovered a novel role for *Kat6b* in the context of LT-HSC differentiation with relevance to aging. I have found that KAT6B decreases in aged HSCs at the transcript and protein levels. The knockdown of *Kat6b* in young LT-HSCs resulted in an increase in the proportion of myeloid cells and a decrease in the proportion of erythroid cells *in vitro* and *in vivo*, demonstrating myeloid lineage-biased differentiation. I did not observe any BFU-E/CFU-E colonies *in vitro*, perhaps due to the effect of 2-day culture conditions on the differentiation potential of LT-HSCs before setting up CFU assays. Transcriptome data revealed that knockdown of *Kat6b* resulted in loss of HSC-associated expression signatures as well as multilineage priming, while gaining an M1 pro-inflammatory macrophage expression signature. HSPCs have been known to express myeloid genes (Iwasaki and Akashi, 2007) and my *in vitro* and *in vivo* data show an increase in differentiation towards myeloid cells. Thus, knockdown of *Kat6b* might be causing expression of myeloid associated genes which might have led to an enrichment in gene signatures associated with M1 macrophages. In addition, it has been reported that *Kat6b* expression is reduced in macrophages under LPS stimulation which results in M1 activation (Shukla et al., 2018). LPS results in immune activation and we have shown that knockdown of *Kat6b* also results in GO terms associated with immune response (Figure 5E). So, it is possible that immune response mediated by reduction in *Kat6b* is resulting in the activation of M1 gene expression profiles. Together, my results support that *Kat6b* functions as a regulator of HSC self-renewal and multilineage differentiation

and decrease in *Kat6b* promotes myeloid lineage-biased HSC differentiation, similar to results observed in aging.

This work builds upon literature demonstrating the importance of the MYST family of acetyltransferases for LT-HSC function. KAT6A, a paralogue of KAT6B with structural similarity (Simpson et al., 2012), also has important functions in the regulation of HSPCs (Sheikh et al., 2016). My results support overlapping but non-redundant roles for KAT6A and KAT6B in hematopoiesis. *In vitro, Kat6a*-deficient bone marrow has reduced total number of colonies in the CFU assay including reduction in all colony subtypes (Sheikh et al., 2016), whereas I observed that *Kat6b* knockdown results in no change in total colony numbers and a proportional increase in myeloid-only colonies. *In vivo*, conditional knockout of *Kat6a* resulted in impaired competitive repopulation capacity and increased ratio of myeloid to lymphoid differentiation (Sheikh et al., 2016), whereas *Kat6b* knockdown resulted in no significant change in repopulation capacity or B cell frequency, increased frequency of myeloid cells and decreased frequency of RBCs. Thus, I propose that KAT6A and KAT6B have overlapping but distinct roles in proper multilineage differentiation from LT-HSCs.

In the context of my experiments, LT-HSCs were cultured under *ex vivo* conditions which have been reported to promote HSC self-renewal (Holmfeldt et al., 2016). However, this requirement for *ex vivo* culture for lentiviral transduction is also a caveat in the interpretation of our results. It is possible that some or all of the LT-HSCs seeded into *ex vivo* culture differentiate to progenitors during the 36h transduction culture period. Thus, the *Kat6b* knockdown phenotype I observe may be manifest in either HSCs or their myeloid progenitor progeny. In addition, *ex vivo* culture conditions of LT-HSCs

before CFU assay might also explain lack of observation of erythroid colonies like BFU-E and CFU-E from my experiments. Therefore, to overcome these caveats and further test the hypothesis that *Kat6b* is important for regulation of HSC differentiation to myeloid and erythroid lineages, it would be important to perform loss of function studies of *Kat6b* in adult bone marrow by using an inducible system. These experiments would involve engineering a *Kat6b* inducible knockout mouse consisting of floxed *Kat6b*. The knockout would be induced specifically in bone marrow at adult (2-4 months) stage. Uninduced floxed mice will be used as negative controls. After induction of knockout of *Kat6b*, HSCs will be isolated from experimental and control mice and transplanted in conditioned recipients. Peripheral blood and bone marrow composition of these mice will be measured at 6 months post transplantation to test the hypothesis that knockout of *Kat6b* results in increase in differentiation to myeloid and reduction in differentiation to erythroid cell types as I observed in *in vivo* experiments involving transplantation of HSCs after Kat6b knockdown by lentiviral transduction. To assess that if *Kat6b* knockout affects stem cell self-renewal, HSCs from recipient mice will be isolated and transplanted into secondary recipients.

Both NTC and *Kat6b* sh1 transplanted mice had low donor-derived reconstitution ability and also declined across 4-month time period post-transplant. This observation can be attributed to culture conditions during transduction not being sufficient to keep the HSCs in stem cell state completely. Another possible reason is that effect of lentiviral transduction on reconstitution ability of stem cells. It has been reported that transduction followed by transplantation usually results in low reconstitution (Barrette et al., 2000; Hosokawa et al., 2010; Kinkel et al., 2015; Kumar et al., 2019; Mazumdar et al., 2015)

and also declines with time (Passegué et al., 2004). Higher engraftment by transduction and transplantation methods are observed in proliferation inducing phenotypes (Hope et al., 2004). Since I did not observe any evidence that *Kat6b* knockdown is inducing any malignant transformation of cells, that's why low reconstitution of transplanted cells can be explained by their transduction.

As a proof of principle that aged HSCs can be rejuvenated by therapeutic interventions that target epigenetic regulatory processes, it has been shown that inhibition of CDC42 by CASIN restored the polarity of H4K16ac, Cdc42 and Tubulin in aged HSCs (Florian et al., 2012). These CASIN-treated aged HSCs had re-balanced B-lymphoid and myeloid differentiation potential *in vivo* and HSC frequency resembling young animals (Florian et al., 2012), although a subsequent study has shown that simply increasing the levels of H4K16ac using a pan-HDAC inhibitor is not sufficient to restore HSC function (Grigoryan et al., 2018). In addition, reprogramming of aged HSCs to induced pluripotent stem cells (iPSCs) has been shown to rejuvenate *in vivo* engraftment and T cell differentiation potential (Wahlestedt et al., 2013). My work suggests that therapeutically increasing levels of KAT6B in aged HSCs may also rejuvenate aspects of altered functionality, particularly with respect to lineage-balanced differentiation. A recent report by Adelman et al. demonstrated a reduction in active enhancer-associated chromatin modifications at a *KAT6B*-proximal enhancer region in aged versus young human HSCs (Adelman et al., 2019), suggesting that therapeutic approaches to increase enhancer activity may be a viable strategy to boost *Kat6b* expression in aged HSCs. Further studies will be required to test whether restoring expression of *Kat6b* in aged HSCs to levels observed in young HSCs is sufficient to restore balanced lineage differentiation.

# CHAPTER 5

# CONCLUSIONS

Hematopoiesis is a well-coordinated continuous process of differentiation from HSCs to mature blood and immune cells. Investigations of molecular mechanisms of this process have not only provided us with knowledge regarding the origin of blood disorders and malignancies but have also been instrumental in elucidating basic molecular and cellular mechanisms underlying stem cell function and differentiation. These studies have relied on a variety of *in vitro* and *in vivo* assays, that is why robustness and correct applications of a particular assay are key to interpreting the results obtained from that assay. In my thesis work, I have investigated the robustness of OP9 co-culture assay for the differentiation of HSPCs to B-lymphoid cells. My experiments have reproduced findings from literature which support that this assay is successful in differentiation of HSPCs to B-lymphoid cells. However, I have contributed to the body of literature in this field by showing that this assay provides a qualitative measure of differentiation potential and therefore is not suitable for studies involving quantitative assessment of differentiation potential. This provides novel insights into the application of this assay for assessment of B-lymphoid differentiation capacity. I have also demonstrated that one (enhancer B1) of out of 4 putative lymphoid enhancers showed a subtle defect in differentiation towards B-lymphoid cells after knockout as compared to the negative control, whereas rest of investigated enhancers did not show any difference. There can be a couple of interpretations from these results. Since enhancer B1 belonged to the category of pre-disposed enhancers, therefore, it can be inferred that pre-disposed enhancers are more important for commitment and differentiation to lymphoid lineage as compared to *de*

*novo* established enhancers. Secondly, it is possible that these enhancers are acting in a redundant manner which might explain subtle or no phenotypes after knockout of single enhancers.

In addition, I have also studied the cellular and molecular mechanism underlying differentiation of HSCs to myeloid cells with links to aging-associated phenotypes. Some of the key cellular phenotypes hematopoietic aging are a reduction in differentiation to erythroid and increase in the production of myeloid cell types. I have demonstrated that loss of function of *Kat6b* by knockdown approaches results in impaired differentiation to erythroid cell types and enhanced differentiation to myeloid cell types *in vitro* and *in vivo* from HSCs (Figure 5.1).

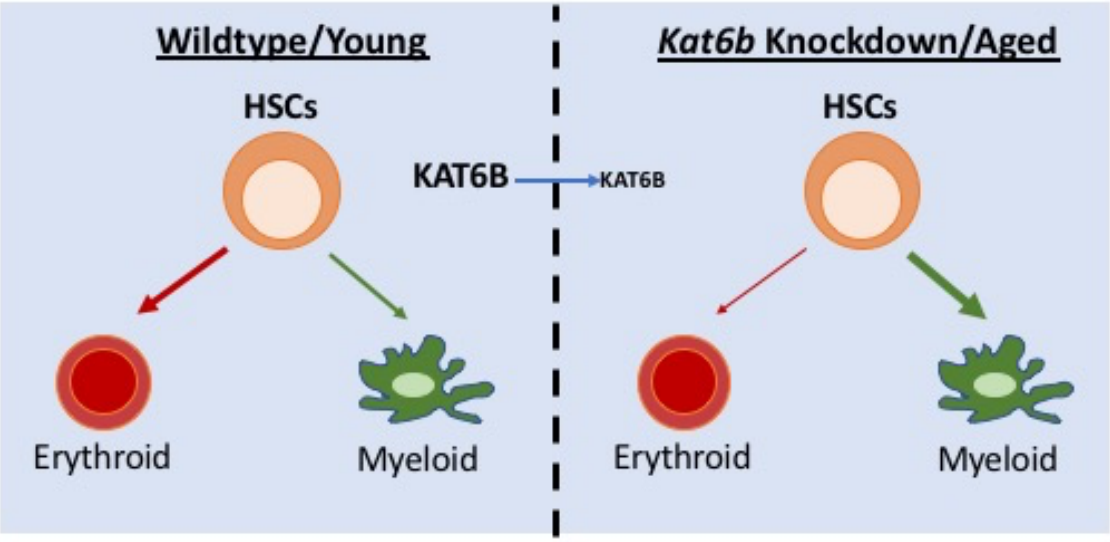



**Figure 5.1. Model of link between *Kat6b* and aging-associated hematopoietic decline.**

By performing transcriptomic analysis after *Kat6b* knockdown I have demonstrated that reduction in *Kat6b* expression results in gene expression profiles that partially

recapitulate aging-associated gene signatures. Furthermore, I have shown that transcript and protein levels of KAT6B decline with age in murine HSCs. This provides *Kat6b* as a novel therapeutic target whose over-expression in aged HSCs could potentially rejuvenate aging-associated decline in the hematopoietic system.

Therefore, I have demonstrated in my thesis that gene regulatory and epigenetic elements are important for the differentiation of HSPCs to mature cells. I have also shown that epigenetic regulators like *Kat6b* are linked with aging-associated decline in the hematopoietic systems and alterations in levels of such epigenetic regulators can be one of the contributing factors which drive aging-associated hematopoietic decline. Future studies in these areas should involve investigation of combinatorial approaches involving targeting gene regulatory elements and associated epigenetic regulators to boost hematopoietic function with age. These studies will consist of *in vivo* genome-wide screens to identify cis-regulatory elements which are important for differentiation to mature lineages, and tracking changes in the activation status of these cis-regulatory elements. These will be followed by the recruitment of dCas9 fused epigenetic effectors to these cis regulatory elements in old HSCs to correct the changes that have accrued due to aging. The long-term goal of such studies would be to obtain comprehensive knowledge about molecular and cellular mechanisms that go awry with aging, so that therapeutic strategies to treat aging-associated diseases can be developed.

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