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Characterization of RUNX1 function in hematopoiesis

A dissertation submitted in partial satisfaction of the requirements for the degree Doctor of Philosophy

in

Biomedical Sciences

by

Kenton Lam

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2014
The Dissertation of Kentson Lam is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

Chair

University of California, San Diego
2014
# TABLE OF CONTENTS

Signature page .......................................................................................................................... iii

Table of Contents ....................................................................................................................... iv

List of Figures ............................................................................................................................ vii

List of Tables ............................................................................................................................... xii

Acknowledgements ................................................................................................................... xiii

Vita ............................................................................................................................................... xvii

Abstract of the Dissertation ...................................................................................................... xix

1. Introduction ............................................................................................................................. 1
   1.1. RUNX1 structure and regulation .................................................................................. 3
       1.1.1. RUNX1 promoters (proximal and distal) and RUNX1 isoforms .......... 3
       1.1.2. RUNX1 protein structure, domains and functions .................................. 7
   1.2. RUNX1 as a master regulator of hematopoiesis ......................................................... 10
       1.2.1. The role of RUNX1 in the specification and development of the
              definitive hematopoietic stem cell using mouse models ....................... 10
       1.2.2. RUNX1 target genes as they relate to hematopoiesis ............................... 13
   1.3. RUNX1 in human disease .............................................................................................. 15
       1.3.1. Mutations in RUNX1 lead to aberrant hematopoiesis ......................... 15
       1.3.2. RUNX1 is a commonly found constituent in chromosomal
              translocations associated with cancer .................................................. 17
   1.4 Summary ......................................................................................................................... 19

2. Hmga2 is a direct target gene of RUNX1 and regulates expansion of
myeloid progenitors .................................................................................................................. 20
   2.1. Introduction ................................................................................................................... 22
   2.2. Results ........................................................................................................................... 24
       2.2.1. Hmga2 is a direct RUNX1 target gene in HSPCs ................................. 24
       2.2.2. The Hmga2 promoter is regulated by RUNX1 in a cell type-
              dependent manner and is independent of canonical binding sites ......... 34
       2.2.3. RUNX1 intronic and intergenic binding regions mediate Hmga2
              transcription .............................................................................................. 39
       2.2.4. HMGA2 does not modulate the effect of RUNX1 deficiency on
              HSPC expansion ....................................................................................... 42
       2.2.5. Hmga2 contributes to myeloid progenitor expansion caused by
              the loss of RUNX1 .................................................................................... 46
   2.3. Summary and discussion ............................................................................................... 49
   2.4. Materials and Methods ................................................................................................. 55
       2.4.1. Mice .............................................................................................................. 55
2.4.2. Cells and cell culture ................................................................. 55
2.4.3. Chromatin immunoprecipitation (ChIP) and sequencing .......... 56
2.4.4. RT-qPCR .................................................................................. 56
2.4.5. Luciferase assays ....................................................................... 58
2.4.6. Flow cytometry .......................................................................... 58
3. Loss of RUNX1 function results in hypersensitivity to G-CSF in mice ...... 61
3.1. Introduction .................................................................................... 63
3.2. Results ............................................................................................ 66
  3.2.1. Runx1Δ/Δ mice are hypersensitive to G-CSF-induced mobilization of 
        HSPCs. ....................................................................................... 66
  3.2.2. Dominant-negative RUNX1SF results in higher degree of 
        hypersensitivity to G-CSF-induced mobilization of HSPCs. .......... 71
  3.2.3. Enhanced HSPC mobilization in Runx1Δ/Δ mice is not solely due to 
        decreased expression of Cxcr4. ................................................ 73
  3.2.4. Utilization of a mini-library approach to discover RUNX1 target 
        genes involved in mediating HSPC mobilization. ....................... 76
  3.2.5. Hypersensitivity to G-CSF mobilization is not mediated by a single 
        gene but via a combination of RUNX1 target genes....................... 81
3.3. Summary and discussion ................................................................. 85
3.4. Materials and Methods ................................................................. 88
  3.4.1. Mice ....................................................................................... 88
  3.4.2. Retroviral transduction and bone marrow transplantation .......... 88
  3.4.3. Colony forming unit and replating assays ................................ 89
4. GIMAP4 mediates expansion of HSPCs due to the loss of RUNX1 function 
   in mice ............................................................................................. 90
  4.1. Introduction ................................................................................... 92
  4.2. Results .......................................................................................... 95
    4.2.1. Gimap4 is up-regulated in the absence of RUNX1 function ....... 95
    4.2.2. Loss of Gimap4 attenuates enhanced in vitro proliferation and self- 
           renewal caused by loss of RUNX1 function. ......................... 97
    4.2.3. Loss of Gimap4 results in fewer GMPs in RUNX1SF mice. ...... 99
    4.2.4. Gimap4 contributes to the expansion of HSPCs due to the loss of 
           RUNX1. .................................................................................. 102
    4.2.5. Loss of Gimap4 leads to expansion of the CLP population...... 104
    4.2.6. Loss of Gimap4 affects myeloid progenitor expansion due to the 
           loss of RUNX1. .................................................................... 106
    4.2.7. Modulation of GIMAP4 induces apoptosis in a tissue/cell type 
           specific manner. ..................................................................... 108
4.3. Summary and discussion ............................................................... 111
4.4. Materials and Methods ............................................................... 114
  4.4.1. Mice ..................................................................................... 114
  4.4.2. Retroviral transduction and bone marrow transplantation .......... 114
  4.4.3. RT-qPCR .............................................................................. 115
  4.4.4. Colony forming unit and replating assays ............................... 115
  4.4.5. Flow cytometry ..................................................................... 116
5. Conclusions and future studies ................................................................. 117

References ...................................................................................................... 122
LIST OF FIGURES

Figure 1.1 ......................................................................................................................... 4
RUNX1 isoforms and genomic locus. (A) The RUNX1 genomic locus on chromosome 21 is shown with the location of the proximal and distal promoters and exons based on National Center for Biotechnology Information Nucleotide database. (B) The three main transcriptional isoform of RUNX1 are shown. RUNX1a is consists of exons 4a...

Figure 1.2 ......................................................................................................................... 8
RUNX1 protein domains and interaction partners. The RUNX1b isoform is shown with the major domains listed above and interaction partners listed below.

Figure 2.1 ......................................................................................................................... 25
RUNX1 genome-wide occupancy in HSPCs as determined by ChIP-seq. (A) The 6370 RUNX1 ChIP-seq peaks with a FDR less than 1% are shown in relation to the absolute distance of the TSS of known genes in kb. (B) Distribution of RUNX1 ChIP-seq peaks are grouped based on promoter (>3 kb upstream of TSS), intron, exon,…

Figure 2.2 ......................................................................................................................... 32
Overlap between microarray and ChIP-seq results. Venn diagram showing the overlap between the common differentially expressed genes in Runx1Δ/Δ and RUNX1SF HSPCs (n=59)[1] and the genes associated with RUNX1 ChIP-seq peaks.

Figure 2.3 ......................................................................................................................... 32
Expression of Hmga2 upon loss of RUNX1 function. (A) Relative expression of Hmga2 in wildtype, Runx1Δ/Δ and RUNX1SF HSPCs based on microarray data published in Matsuura et al10. (B) Validation of up-regulation of Hmga2 in microarray results by RT-qPCR using cDNA from wildtype, Runx1Δ/Δ, and RUNX1SF HSPCs…

Figure 2.4 ......................................................................................................................... 33
RUNX1 ChIP for the Hmga2 locus. (A) Location of RUNX1 ChIP-seq peaks relative to the Hmga2 gene locus on chromosome 10. IgG control and one RUNX1 ChIP-seq replicate are shown. (B) Confirmation of RUNX1 occupancy regions in relation to the Hmga2 gene locus by ChIP-qPCR. RUNX1 ChIP was compared relative to IgG…

Figure 2.5 ......................................................................................................................... 35
Schematic of Hmga2 promoter-luciferase construct and results. (A) Diagram of promoter-luciferase construct showing RUNX consensus Sites 1 and 2 (*) at bp -363 and -213, respectively. (B) The adherent non-hematopoietic NIH3T3 and 293T cell lines were transfected with the full length Hmga2 promoter-luciferase construct and…

Figure 2.6 ......................................................................................................................... 36
Mutated Hmga2 promoter-luciferase construct results in NIH3T3 cells. (D) NIH3T3 cells were transfected with promoter-luciferase constructs with Site1, Site2, or both sites mutated. Luciferase assays were performed 24 hours after transfection. Co-expression was conducted with an empty expression vector (Control) or RUNX1…
The effect of RUNX1 on the Hmga2 promoter is independent of canonical RUNX binding sites. (A) Diagram of promoter-luciferase construct showing locations of RUNX consensus sites and other constructs used in assays, which are denoted (i) through (v). (B) NIH3T3 cells were transfected with the indicated...

Mutated Hmga2 promoter-luciferase construct results in K562 cells. (E) K562 cells were transfected with the constructs indicated in (C) and luciferase assays were performed 24 hours after transfection. Co-expression was conducted with an empty expression vector (Control) or RUNX1 and CBFβ expression vectors. Error bars...

RUNX1 distal binding regions exert differential effects on Hmga2 expression. (A) Schematic of distal element-promoter luciferase constructs. The distal elements being tested are the RUNX1 binding regions that are downstream, upstream, and in the intron of the Hmga2 locus. Distal element sequences were cloned downstream of...

HMGA2 does not modulate the effect of RUNX1 deficiency on HSPC expansion. Percentage of hematopoietic stem cell populations analyzed by flow cytometry, including (A) LSK cells, (B) LT-HSCs, and (C) SH-HSCs. For LSK: Wildtype (Hmga2+/+ or +/-, Runx1(fl/fl), Mx1Cre-, n=13), Hmga2/-/- (Hmga2/-/-, Runx1(fl/fl))...

Percentage of CLP populations were analyzed by flow cytometry. For CLPs: Wildtype (n=5), Hmga2/-/- (n=5), Runx1Δ/Δ (n=10), Double KO (n=4).e (n=3).

Loss of HMGA2 results in higher frequencies of apoptosis cells. Averages of frequencies of Annexin V-positive/7AAD-negative cells from the LSK cells of each genotype (n=4 each) are shown. (*) indicates p<0.05.

Lack of HMGA2 rescues myeloid progenitor expansion due to loss of RUNX1. Percentage of hematopoietic stem cell populations analyzed by flow cytometry, including (A) GMPs, (B) MEPs, and (C) CMPs. For myeloid progenitor staining, mice used were: Wildtype (Hmga2+/+ or +/-, Runx1(fl/fl), Mx1Cre-, n=5), Hmga2/-/-...

Decrease in apoptosis in Runx1Δ/Δ mice is mediated by Hmga2. Averages of frequencies of Annexin V-positive/7AAD-negative cells from the (E) GMP, (F) MEP, and (G) CMP gates of each genotype (n=4 each) are shown. (*) indicates p<0.05.

Common genes between RUNX1 gene expression profiling and RUNX1 ChIP-seq analyses. Venn diagram showing the Runx1 differentially expressed genes that have Runx1 ChIP-seq peaks associated with their loci. Runx1 ChIP-seq studies from Wu et al. and Wilson et al. are also included.
Figure 3.1................................................................. 69
Splenomegaly is induced in Runx1Δ/Δ mice after G-CSF treatment. Picture of spleens and spleen weights are shown. (n=5 for each genotype)

Figure 3.2........................................................................ 69
Peripheral blood colony assay after G-CSF treatment. Runx1Δ/Δ mice have significantly higher numbers of colonies compared to wildtype mice after treatment. (n=5 for each genotype).

Figure 3.3........................................................................ 70
HSPC frequencies after G-CSF treatment in wildtype and Runx1Δ/Δ mice. (A) Percent of LSK cells in bone marrow of G-CSF injected mice. (B) Percent of LSK cells in spleens of G-CSF injected mice. (C) Percent of LSK cells in the peripheral blood of G-CSF injected mice.

Figure 3.4........................................................................ 70
HSPC frequencies without any treatment in wildtype and Runx1Δ/Δ mice. (A) Percent of LSK cells in bone marrow. (B) Percent of LSK cells in spleens. The numbers of mice were n = 2 for each genotype.

Figure 3.5........................................................................ 72
HSPC frequencies after G-CSF treatment in MigR1 and RUNX1SF-transplanted mice. (A) Percent of GFP-positive LSK cells in bone marrow of G-CSF injected MigR1 (n=4) and RUNX1SF (n=5) mice. (B) Percent of GFP-positive LSK cells in spleens of G-CSF injected mice. (C) Percent of GFP-positive LSK cells in the peripheral blood...

Figure 3.6........................................................................ 72
Peripheral blood colony assay after G-CSF treatment. RUNX1SF mice have significantly higher numbers of colonies compared to wildtype mice after treatment. (n=4 for MigR1 and n=5 for RUNX1SF).

Figure 3.7........................................................................ 74
HSPC frequencies after combination G-CSF and AMD3100 treatment in wildtype and Runx1Δ/Δ mice. (A) Percent of LSK cells in bone marrow of G-CSF injected mice. (B) Percent of LSK cells in spleens of G-CSF injected mice. (C) Percent of LSK cells in the peripheral blood of G-CSF injected mice.

Figure 3.8........................................................................ 75
Peripheral blood colony assay after combination G-CSF and AMD3100 treatment. Runx1Δ/Δ mice have significantly higher numbers of colonies compared to wildtype mice after treatment. (n=4 for each).

Figure 3.9........................................................................ 77
Schematic of retroviral barcode vector used in cDNA screen. Each cDNA was cloned downstream of the 5' long terminal repeat (LTR). A universal forward primer sequence and a barcode specific to each cDNA was added 3' to the cDNA. Common primers specific for the universal sequence and part of the IRES sequence will be...
Barcode mice exhibit myeloid differentiation bias to G-CSF treatment. (A) Cell surface marker staining of peripheral blood from mice transplanted with barcode-transduced cells for myeloid markers CD11b and Gr-1. (B) Cell surface marker staining of peripheral blood from mice transplanted with barcode-transduced cells...

Barcode mice exhibit hypersensitivity to G-CSF treatment. (A) Percent of LSK cells in the peripheral blood after regimen of G-CSF injections into MigR1 and Barcode transplanted mice. (B) Colony assay using cells from peripheral blood of G-CSF injected mice. (n=9 for MigR1, n=17 for Barcode)

Frequency of barcode counts. (A) Counts from bone marrow LSK cells after two months of engraftment but before any treatment. (B) Counts from peripheral blood LSK cells after G-CSF treatment.

Ratio of barcode counts. The counts for each barcode, and hence cDNA, in peripheral blood LSK cells after treatment was compared to bone marrow LSK cells before G-CSF treatment.

Gimap4 expression is up-regulated in the absence of RUNX1 function. (A) The relative expression of Gimap4 between various HSPC and blood spleen cells are shown. (B) The relative expression of Gimap4 in wildtype, Runx1 Δ/Δ and RUNX1SF HSPCs based on microarray data published in Matsuura et al10. (C) Validation of...

Loss of RUNX1 function results in enhanced in vitro proliferation and self-renewal which is attenuated by loss of GIMAP4. Representative results of colony formation and replating assay from 4 independent replicates. Total bone marrow cells from WT or Gimap4−/− mice were transduced with MIP or RUNX1SF and plated in...

Loss of GIMAP4 possibly attenuates the ability of RUNX1SF to expand HSPCs. Percentage of hematopoietic stem cell populations analyzed by flow cytometry, including (A) LSK cells, (B) LT-HSCs, and (C) SH-HSCs are shown. (n=5 for wildtype (WT) cells transduced with MigR1, n=2 for Gimap4−/− (KO) cells transduced with...

Loss of GIMAP4 results in fewer GMPs in RUNX1SF mice. Percentage of myeloid progenitor cell populations analyzed by flow cytometry, including (A) GMPs, (B) CMPs, and (C) MEPs. (n=5 for wildtype (WT) cells transduced with MigR1, n=2 for Gimap4−/− (KO) cells transduced with MigR1, n=5 for WT cells transduced with...

GIMAP4 contributes to the expansion of HSPCs due to loss of RUNX1. Percentage of hematopoietic stem cell populations analyzed by flow cytometry, including (A) LSK
cells, (B) LT-HSCs, and (C) SH-HSCs are shown. Numbers for each genotype include: Wildtype (n=4), Runx1$^{Δ/Δ}$ (n=5), Gimap4$^{-/-}$ (n=12), and Double KO (n=11)...

**Figure 4.6**
Loss of GIMAP4 leads to expansion of the CLP population. Percentage of CLPs analyzed by flow cytometry are shown. Numbers for each genotype include: Wildtype (n=4), Runx1$^{Δ/Δ}$ (n=5), Gimap4$^{-/-}$ (n=14), and Double KO (n=13). (*) indicates p<0.05.

**Figure 4.7**
Loss of Gimap4 affects myeloid progenitor expansion due to the loss of RUNX1. Percentage of myeloid progenitor populations analyzed by flow cytometry are shown, including (A) GMPs, (B) MEPs, and (C) CMPs. For myeloid progenitor staining, numbers for each genotype include: Wildtype (n=2), Runx1$^{Δ/Δ}$ (n=2)...

**Figure 4.8**
Apoptosis induction by GIMAP4 is cell type-specific. (A) Percent of Annexin V-positive/7-AAD-negative cells in MIP control (black bars) or MIP-GIMAP4 (gray bars) transfected Jurkat cells. Flow cytometry charts for each condition are given below. (B) Percent of Annexin V-positive/7-AAD-negative cells...

**Figure 4.9**
Loss of GIMAP4 does not affect rates of apoptosis in LSK and myeloid progenitor cells. Percent of Annexin V-positive/7-AAD-negative cells are shown for (A) LSK, (B) GMP, (C) MEP, and (D) CMP populations. n=2 for each genotype.
LIST OF TABLES

Table 2.1............................................................................................................................................. 28
Common genes differentially expressed in Runx1Δ/Δ and RUNX1SF HSPCs that overlap with RUNX1 genome occupancy.

Table 2.2............................................................................................................................................. 57
Primer sequences for RT-qPCR and ChIP-qPCR.

Table 3.1............................................................................................................................................. 68
Top networks and genes from Ingenuity IPA analysis on differentially expressed genes in Runx1Δ/Δ HSPCs.

Table 3.2............................................................................................................................................. 78
List of genes used in mini library barcode screen.

Table 3.3............................................................................................................................................. 83
Ratio of barcode counts in peripheral blood LSK cells after G-CSF treatment compared to counts in bone marrow LSK cells before any treatment.
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ABSTRACT OF THE DISSERTATION

Characterization of RUNX1 function in hematopoiesis

by

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Doctor of Philosophy in Biomedical Sciences

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Professor Dong-Er Zhang, Chair

RUNX1 or runt-related transcription factor 1 is a transcription factor in the RUNX family of proteins. This family is composed of RUNX1, RUNX2, and RUNX3—all of which contain the runt homology domain, which mediates interaction with DNA and other transcriptional co-factors. RUNX1 has garnered much attention over the past decades primarily because its disruption has been associated with a variety of human diseases and malignancies. Several chromosomal translocations in leukemias involve RUNX1. The most famous one is between chromosomes 8 and 21 resulting in
the RUNX1-ETO fusion and leads to acute myeloid leukemia. Since then, RUNX1 mutations have been found in acute lymphoid leukemia, myelodysplastic syndrome, myeloproliferative neoplasm, familial platelet disease, and others. Although RUNX1 has been a major focus in the field of hematology, many of the molecular and cellular mechanisms of how RUNX1 disruption leads to disease remain unknown. Uncovering these mechanisms will result in a better understanding of these diseases and may lead to meaningful therapies.

As a transcription factor, RUNX1 primarily exerts its functions by regulating target genes. This dissertation explores how these target genes ultimately facilitate RUNX1 functions in hematopoiesis and in hematopoietic stem and progenitor cells (HSPCs). We first focus on Hmga2 or high mobility group AT-hook 2, which was found to be up-regulated in RUNX1 loss-of-function HSPCs. Hmga2 is an established oncogene and has roles in inducing cellular proliferation. Notably, Hmga2 was found to contribute to myeloid progenitor cell expansion, which is a main characteristic of RUNX1 loss-of-function. Next, we analyzed differential gene expression data from wildtype and RUNX1 loss-of-function HSPCs and determined that these genes are involved in pathways associated with cell-to-cell interaction and signaling. Upon further examination, we discovered that RUNX1 loss-of-function mice exhibit hypersensitivity to HSPC mobilization regimens. Finally, we focus on another RUNX1 target gene known as Gimap4 or GTPase of the immunity-associated protein 4 and confirm that up-regulation of Gimap4 contributes to
HSPC expansion, a finding also found in RUNX1 loss-of-function mice. Together, these studies provide critical insight in the role of RUNX1 in hematopoiesis and blood-related diseases, and offer additional avenues for therapies for these diseases.
1. Introduction

RUNX1, also known as AML1, CBFalpha2, and PEBP2alphaB, belongs to the family of runt-related transcription factors (RUNXs)[2]. The runt protein is encoded by the Drosophila runt gene, which is required for normal segmentation, sex determination, and neurogenesis during Drosophila embryogenesis[3]–[5]. Other RUNX family members include RUNX2 and RUNX3. This family of proteins was first described as a component of Moloney murine leukemia virus enhancer core binding factor (CBF) and Polyomavirus enhancer binding protein 2 (PEBP2)[6], [7]. RUNX1 is also known as acute myeloid leukemia 1 due to the discovery of its gene sequence from human acute myeloid leukemia (AML) patient samples[8]. These patients harbored the well studied translocation between chromosomes 8 and 21, which results in the RUNX1-ETO fusion protein. RUNX1 has subsequently been implicated in a variety of other chromosomal translocations associated with hematological malignancies. Point mutations in RUNX1 have also been associated with other blood disorders like myelodysplastic syndrome (MDS), myeloprolifetive disease/neoplasm (MPN), and familial platelet disease with propensity to develop AML (FPD/AML).

The wide prevalence of RUNX1 mutations in human disease has made RUNX1 a highly interesting topic of study for past few decades. Although these studies have elucidated many important functions of RUNX1 in transcriptional regulation, hematopoietic development, and hematopoietic
stem cell homeostasis, much still remains unknown. As a transcription factor, RUNX1 exerts its primary functions by controlling the expression of its target genes, and these target genes presumably directly affect biological functions. In this dissertation, I will focus on the various biological roles and functions RUNX1 mediates throughout hematopoiesis and the target genes that RUNX1 regulates to fulfill these roles.
1.1. RUNX1 structure and regulation

1.1.1. RUNX1 promoters (proximal and distal) and RUNX1 isoforms

RUNX1 was first cloned from DNA obtained from an AML patient with t(8;21)-positive leukemia[8]. Although there may be at least 12 different RUNX1 mRNA isoforms, three main protein isoforms of RUNX1 are primarily discussed[9]. These are known as RUNX1a, RUNX1b, and RUNX1c (Figure 1.1A-C). These three major isoforms all contain the Runt domain located in the N-terminal region. RUNX1a, consisting of 250 amino acids, and RUNX1b, consisting of 453 amino acids, share the same N-terminal region and are the result of alternative splicing[10]. RUNX1a lacks the transcriptional regulatory domains present in the C-terminal region common in the other two RUNX1 isoforms[11]. RUNX1c, consisting of 480 amino acids, is the longest of the RUNX1 isoforms and its transcripts is transcribed from a distal promoter in the RUNX1 locus, while RUNX1a and RUNX1b are transcribed from the proximal promoter[10]. RUNX1b and RUNX1c have the same C-terminal region.

Interestingly, the various RUNX1 isoforms play specific roles in specifying the hematopoietic stem cell (HSC) and regulating embryonic hematopoiesis. A study done by Tsuzuki et al. demonstrated that the RUNX1a isoform is found relatively more abundantly in the CD34+ progenitor population in human cord blood and that over-expression of RUNX1a compared with RUNX1b in mouse bone marrow progenitor cells can potentiate
Figure 1.1. RUNX1 isoforms and genomic locus. (A) The RUNX1 genomic locus on chromosome 21 is shown with the location of the proximal and distal promoters and exons based on National Center for Biotechnology Information Nucleotide database. (B) The three main transcriptional isoform of RUNX1 are shown. RUNX1a is consists of exons 4a through 8. RUNX1b consists of exons 4a through 10, but excludes exon 8. RUNX1c includes exons 1 through 3 and exons 4b through 10, but also excludes exon 8. (C) The three main RUNX1 isoforms are shown with the Runt homology domain shaded.
engraftment ability upon competitive transplantation[12]. Studies from our own laboratory have further demonstrated that expression of the RUNX1a isoform in embryonic stem cells can enhance hemaopoietic commitment[13]. Hence, manipulating the levels of RUNX1a may be used to drive proliferation of human bone marrow cells for use in transplantation therapy. Another study, however, showed that over-expression of RUNX1a may also lead to the development of leukemia in a mouse transplantation model[14]. These studies suggest that RUNX1a, because it includes the Runt domain but lacks the C-terminal regulatory domains, may act as a dominant-negative regulator of the other RUNX1 isoforms[11], [14]. As discussed in subsequent sections, the C-terminal domains are necessary for normal RUNX1 function and over-expression of a RUNX1 isoform lacking these domains may lead to abnormal hematopoiesis.

The ability of the RUNX1a isoform to direct a program of self-renewal reflects its importance in embryonic development. Early work using oligonucleotide PCR primers specific for either the proximal or distal transcriptional forms of RUNX1 in T cells showed that the distal form is more prevalent in developing T cells[15]. However, when the proximal form, which in this case is RUNX1b, was retrovirally over-expressed in the 32Dcl.3 myeloid progenitor line, significantly more proliferation and neutrophil differentiation was observed when compared to over-expression of the distal RUNX1c isoform[15]. In zebrafish, where the transcriptional regulation of RUNX1 by use of two promoters is conserved, transgenic lines that express fluorescently
labeled RUNX1 isoforms specific for each of the two promoters showed that the distal isoform is expressed in areas where erythromyeloid progenitors arise while the proximal isoform originates where definitive HSCs develop[16]. More recent studies using mouse knock-in models to label the expression patterns of the RUNX1 distal and proximal promoters have also shown that the proximal promoter may be important for the initial development of definitive hematopoietic cells from hemogenic endothelium while the distal promoter is active in more mature progenitors[17]. Furthermore, mice hypomorphic for the proximal promoter make it term but die perinatally, while mice null for the distal promoter show no overt phenotype[18], [19]. The aforementioned studies indicate that the two RUNX1 promoters may have varying yet overlapping functions and interestingly, the proximal RUNX1 isoform has a more involved role in defining the HSC.

The two promoters of RUNX1 rely on a cis-regulatory element located approximately 23.5 kilobases downstream of the transcriptional start site of the distal promoter[20], [21]. This promoter contains sites for various essential hematopoietic transcription factors like Gata2, Ets family members, and Lmo2[20]. Furthermore, this element can drive specific expression of genetic markers like lacZ or green fluorescent protein (GFP) in HSCs and the hemogenic endothelium in transgenic mice[18], [21]. More studies regarding the expression patterns, transcriptional control, and dosage levels of the various RUNX1 isoforms in HSCs and during embryonic hematopoiesis will be
needed to further elucidate their ability to regulate HSCs and to potentiate leukemia development.

1.1.2. RUNX1 protein structure, domains and functions

RUNX1 is defined by its 128 amino acid Runt domain found in the N-terminal region (Figure 1.2). The Runt domain mediates binding to DNA and interaction with its heterodimerization partner, core-binding factor beta (CBFbeta), which itself does not bind to DNA. Heterodimerization of RUNX1 and CBFbeta increases the DNA-binding affinity of RUNX1[22], [23]. The complex consisting of RUNX1, CBFbeta, and DNA was one of the first gene regulatory complexes where detailed structural investigations have been conducted. NMR spectroscopy studies of the RUNX1/CBFbeta/DNA complex have revealed that it resembles an immunoglobulin fold similar to the DNA-binding domains of NF-kappaB, NFAT1, p53, and the STAT proteins[24], [25]. Crystal structure studies of this complex have demonstrated that CBFbeta interacts with RUNX1 allosterically to stabilize its ability to bind DNA and that diseases associated with mutations in RUNX1 correspond to sites in its DNA-binding domain[26]–[28].

The other protein domains of RUNX1 also help to regulate its ability to control transcription of its target genes. Various deletion studies of full-length RUNX1 have shown that the N-terminal and C-terminal regions directly adjacent to the Runt domain inhibit DNA binding[29], [30]. Binding to CBFbeta
Figure 1.2. RUNX1 protein domains and interaction partners. The RUNX1b isoform is shown with the major domains listed above and interaction partners listed below.
relieves this inhibition and allows RUNX1 to bind to DNA at its full potential[29]. Furthermore, RUNX1 contains a nuclear matrix targeting signal (NMTS), a 31 amino acid region in the C-terminal region, which aids in transcriptional activation[31]. At the very C-terminal end of RUNX1 is a VWRPY motif that is conserved among all Runt family members[32]. This motif mediates the Groucho/TLE-dependent transcriptional repressor activities of RUNX1[32], [33]. The NMTS and the VWRPY motif have roles in mediating T cell development. By itself, the C-terminal VWPRY motif is not required for developing thymocytes to properly repress CD4 expression[34]. When the region containing both the nuclear matrix targeting signal and the VWRPY motif are deleted, however, the thymocytes can no longer repress CD4[34].

In addition to interacting with CBFbeta, RUNX1 is very versatile in interacting with various other transcription factors and transcriptional co-regulators. For example, RUNX1 and ETS1 interact to coordinate transcriptional activity of the T cell receptor via the Runt domain of RUNX1, including regions just adjacent to the domain[35], [36], [30]. RUNX1 has also been shown to interact with PU.1, C/EBPalpha, p300, mSin3a, GATA1, and Fli1 among many other factors[37]–[42]. These studies indicate that RUNX1 function relies heavily on its interaction partners and that these interaction partners may help to regulate its target genes in a cell type- or tissue-specific manner.
1.2. RUNX1 as a master regulator of hematopoiesis

1.2.1. The role of RUNX1 in the specification and development of the definitive hematopoietic stem cell using mouse models

RUNX1 plays an essential role in specifying the definitive hematopoietic stem cell (HSC). Two waves of hematopoiesis occur during embryonic development. The first wave is known as primitive hematopoiesis, which describes the differentiation of primitive macrophages and early erythrocytes from progenitors in the yolk sac to aid in the rapid development of the embryo[43]. After this initial wave, definitive hematopoiesis, which describes the process of generating the various lineages of mature blood cell types from a common definitive HSC, takes place. One of the first sites that HSCs are detected in mammals is the aorta-gonad-mesonephros region at 10.5 days post conception (dpc) in the mouse embryo, where they bud off from the ventral aspect of the dorsal aorta and eventually colonize the fetal liver[44], [45]. Runx1 was detected in both locations during embryogenesis, indicating that expression of RUNX1 marks the earliest hematopoietic precursor cells[46]. One of the most important pieces of evidence implicating the role of RUNX1 in specifying the HSC was the generation of Runx1-null mice[47], [48]. While heterozygous mice are healthy and fertile, the homozygous knockout mice die between 12.5 to 13.5 dpc with severe hemorrhaging along the central nervous system. Such extensive hemorrhage is most likely due to defects in angiogenesis caused by a lack of angiopoietin-1 expression in these knockout
mice[49]. Furthermore, although these mice have nucleated primitive erythrocytes, indicating that there was no major defect in primitive hematopoiesis, they lacked definitive hematopoiesis. Cells from embryonic hematopoietic tissues, such as the yolk sac and liver, do not show colony forming units when cultured in vitro, and chimeric mice made from Runx1-deficient embryonic stem (ES) cells and wild type mouse blastocysts do not show any Runx1-deficient ES cell contribution to adult hematopoietic cells. More recent studies have further established the essential role that RUNX1 plays in derivation of HSCs from the hemogenic endothelium[50], [51]. Interestingly, however, once the HSC is defined, RUNX1 is no longer essential for hematopoiesis[50]. These results indicate that RUNX1 plays a fundamental role in the establishment of definitive HSCs.

Although the role of RUNX1 in programming the HSC from embryonic development is well established from the investigations discussed above, one remaining question is whether RUNX1 is important in adult HSC function. Sun et al. described how mice that were haploinsufficient for Runx1 displayed a higher number of HSCs as defined by the cell surface marker phenotype, lineage-negative, Sca-1+, c-Kit+ (LSK), but contrastingly has lower number of functional long-term HSCs as assayed by limiting dilution analyses[52]. To delve into this issue further, several groups have utilized a conditional Runx1 knockout model with the Mx1-Cre transgenic mouse line[53]–[55]. The Mx1 gene is an interferon-inducible gene and its promoter allows expression of Cre to occur in HSCs when exposed to interferon (IFN) or other IFN-inducing
agents like the synthetic double-stranded RNA polyinosinic/polycytidylic acid (polyIC)[56]. The most striking phenotypes of conditional Runx1 knockout mice are a significant expansion of the putative HSC population (LSK cells) and myeloid progenitors, thrombocytopenia, and lymphopenia[53], [54]. Analysis of the spleen and thymus revealed a myeloproliferative phenotype[54], [55]. Although Runx1-deficient cells showed enhanced proliferative ability, they are functionally impaired in their ability to engraft upon competitive transplantation with wild type hematopoietic cells into an irradiated host[54], [57]. Jacob et al. go further and state that lack of RUNX1 causes stem cell exhaustion and use a retroviral insertional mutagenesis screen to identify Evi5 as a gene that can ameliorate this exhaustion phenotype when over-expressed. Moreover, they attribute the phenotype to a defect in the interaction between HSCs and the niche. 

Cxcr4, a gene important in stem cell homing and niche interactions, is a direct target of RUNX1. This transcript is down-regulated in the absence of RUNX1 but levels are rescued when Evi5 is over-expressed[57]. Hence, absence of RUNX1 may disrupt the HSC-niche interaction leading to aberrant hematopoiesis characterized as a stem cell exhaustion phenotype observed in Runx1 conditional knockout mice. The HSC proliferation displayed in Runx1 conditional knockout mice may be partially explained by a niche interaction defect, or by decreased apoptosis or increased proliferation[58].

In addition to genetic models of Runx1 deficiency, our lab has used bone marrow transplantation of transduced blood progenitor cells as a model study how aberrant forms of RUNX1 affect hematopoiesis. Transplanting cells
expressing solely the Runt homology domain, composed of amino acids 41 to 214 of RUNX1b, into mice resulted in MDS-like disease[1]. In addition, these mice had increased numbers of HSCs in the bone marrow and features found in Runx1 conditional knockout mice described earlier but to a more exaggerated extent[1]. A similar study using expression of a mutated form of RUNX1 revealed similar results[59]. These findings support the notion that mutated forms of RUNX1 act as dominant-negative regulators of endogenous RUNX1 and other RUNX proteins.

1.2.2. RUNX1 target genes as they relate to hematopoiesis

In addition to the mouse models described earlier, numerous studies have focused on RUNX1 as a DNA-binding transcription factor and on the genes that it regulates. The Runt domain of RUNX1 mediates binding to the TG(T/C)GGT consensus sequence[60]. In various adult blood types, target genes of RUNX1 have been fairly well characterized. In the myeloid lineage, RUNX1 directly binds and regulates the promoter activities of genes related to myeloid growth factor signaling such as IL-3, GM-CSF, the M-CSF receptor, and c-Mp[61]–[64], and to the function of myeloid cells such as myeloperoxidase, neutrophil elastase, and mast cell protease 6[65], [66]. In the T cell lineage, RUNX1 targets promoters and enhancers of T cell receptors and the CD11a promoter[67]–[69]. In the B cell lineage, RUNX1 targets a B
cell specific src family tyrosine kinase known as blk, Ig promotors, and the immunoglobulin antigen receptor enhancers[70]–[72].

Although the role of RUNX1 in regulating these genes are well described in adult blood cells, more recent studies have focused on RUNX1 target genes that are important in the regulation of hematopoiesis at the stages of stem cells and early progenitors. For example, RUNX1 has been demonstrated to play an important role in the differentiation and function of regulatory T cells by targeting and interacting with FoxP3[73], [74] RUNX1 also has an essential role in regulating Pu.1, considered to be a critical transcription factor in myeloid progenitors and other mature myeloid cells[75]. In this study, an upstream regulatory element located 14 kilobases upstream to the Pu.1 locus was found to have binding sites for RUNX1, and mice harboring mutations in these binding sites exhibited a phenotype similar to the Runx1 conditional knockout phenotype described earlier. In addition, RUNX1 regulates miR-27a, which is involved in a feedback loop by binding to sites on the 3’ UTR of RUNX1, thereby mediating megakaryopoiesis[76]. Since RUNX1 encompasses such a large role in hematopoiesis, more work is needed on the identification of its target genes, especially as they relate to HSCs or early progenitors.
1.3. RUNX1 in human disease

1.3.1. Mutations in RUNX1 lead to aberrant hematopoiesis

The importance of RUNX1 in hematopoiesis is further exemplified by the mutations found in RUNX1 in patients with various hematological diseases. In one study, eight out of 160 patients with AML were found to have various mutations in the RUNX1 gene[77]. Interestingly, these mutations were located in the Runt domain and further molecular analyses indicated that some of these mutations resulted in abnormal DNA binding, which altered transactivation of the M-CSF promoter, a gene known to be regulated by RUNX1[77].

In another study, six pedigrees of patients with familial platelet disorder with predisposition to acute myeloid leukemia (FPD/AML) were analyzed and found to be linked to a region of chromosome 21 encoding RUNX1 and concluded that haploinsufficiency of RUNX1 may be one cause of FPD/AML[78]. While haploinsufficiency of RUNX1 may predispose patients to FPD/AML, biallelic mutations in RUNX1 resulting from a second hit may lead to full on leukemia[79].

Cases of myelodysplastic syndrome (MDS) have also been found to contain mutations in RUNX1[80]–[82]. Many mutations were found to be associated with the Runt domain of RUNX1, which leads to defective DNA binding or altered interaction with its heterodimer partner CBFbeta. Other mutations can lead to C-terminal truncations, thereby eliminating the
transactivation domain of RUNX1 resulting in dominant-negative regulators of RUNX1. In any case, RUNX1 mutations are typically with worse prognosis than patients without RUNX1 mutations[81].

Patients with myeloproliferative neoplasms (MPN) were also found to have point mutations in RUNX1[83]. When these mutated forms of RUNX1 were used to transduce human CD34+ MPN cells, proliferation was promoted. The heightened proliferation may allow for leukemic transformation in patients with MPN[83]. RUNX1 has also been to be more highly expressed in a variety of MPNs and up-regulates the target gene NF-E2, whose over-expression in mice serves as an MPN disease model[84], [85].

More recently patients of chronic myelomonocytic leukemia (CMML) have been found to harbor mutations in RUNX1[86], [87]. Although most mutations were in the Runt homology domain, some patients had mutations in the C-terminal region. Interestingly these patients progressed to full blown AML much faster when compared to patients without any mutations[87]. The prevalence of RUNX1 mutations in the various hematological diseases described above highlights the important role that RUNX1 plays in normal hematopoiesis and what may happen when normal RUNX1 function goes awry.
1.3.2. RUNX1 is a commonly found constituent in chromosomal translocations associated with cancer

As discussed in the previous section, RUNX1 is commonly found to be mutated in diseases associated with disrupted hematopoiesis. Many of the patients suffering from these diseases are one step away from developing full blown leukemia. Hence it is not surprising that RUNX1 is the most common target of chromosomal translocations found in acute leukemia. The three most common chromosomal translocations involving RUNX1 are: t(8;21), t(12;21), and t(3;21)[88]. RUNX1-ETO (also known as AML1-ETO, RUNX1-MTG8, and RUNX1-RUNX1T1) is a result from t(8;21) and is found in about 12% of AML and 40% of the M2 subtype of AML[89]. TEL-RUNX1 is a result of t(12;21) and was originally cloned from two patients with pediatric precursor B-cell acute lymphoblastic leukemia (ALL)[90]. The translocation is present in about 25% of patients with childhood pre-B cell ALL and produces a fusion with the N-terminal HLH domain of the TEL protein and almost the entire RUNX1 protein, including its Runt and transactivation domains[90], [91]. The third most common translocation involving RUNX1 is t(3;21), which was first discovered in patients in the blast crisis phase of chronic myelogenous leukemia and later in approximately 3% of therapy-related MDS and AML[92], [93]. Cloning of fusion transcripts from patient tissue samples revealed that the N-terminal portion of RUNX1, including its Runt domain, was fused with either one of three genes on chromosome three including EVI, MDS1, or EAP[94]. Less common chromosomal translocations involving RUNX1 have also been
described in patients with a variety of other leukemias and hematological neoplasms[90], [95], [96]. The variety of leukemias found in these patients, which have shown to include those of both myeloid and lymphoid origin, suggests that RUNX1 acts relatively upstream in the hematopoietic lineage tree to regulate the activity of HSCs. The prevalence of RUNX1 mutations in these diseases obviously substantiates its role in normal blood development, and further establishes it as a master regulator of hematopoiesis.
1.4 Summary

RUNX1 is undoubtedly a master regulator of hematopoiesis. RUNX1 is critical in defining the HSC and in mediating its differentiation into various lineages. These attributes have encouraged numerous investigations into uncovering its molecular and cellular mechanisms for regulating hematopoiesis and HSCs. Further study and understanding of these mechanisms will undoubtedly yield potential therapies for a variety of disorders. The vital nature of RUNX1 has been demonstrated experimentally by mouse models and has been further confirmed by its prevalence in a variety of hematological diseases and neoplasms. Again, further insight into its roles and functions also offers insight into understanding the pathology of these diseases. As a transcription factor, RUNX1 must exert its actions through its target genes. In this thesis, we explore and characterize how these target genes play a role in mediating hematopoiesis and HSCs.

Chapter 1, in part, has been published in Frontiers in Bioscience. Kentson Lam and Dong-Er Zhang. "RUNX1 and RUNX1-ETO: roles in hematopoiesis and leukemogenesis." The dissertation author is the primary investigator and author of the manuscript.
2. Hmga2 is a direct target gene of RUNX1 and regulates expansion of myeloid progenitors

RUNX1 is a master transcription factor in hematopoiesis, and mediates the specification and homeostasis of hematopoietic stem and progenitor cells (HSPCs). Disruptions in RUNX1 either by mutation or involvement in chromosomal translocations are well known to lead to hematological disease. In this study, we sought to identify and characterize RUNX1 target genes in HSPCs by performing RUNX1 chromatin immunoprecipitation coupled with high throughput sequencing (ChIP-seq) using a murine HSPC line and complementing this data with our previously described gene expression profiling of primary wildtype and RUNX1-deficient HSPCs (Lineage-/c-Kit+/Sca-1+). From this analysis, we identified and confirmed that Hmga2, a known oncogene, as a direct target of RUNX1. Hmga2 was strongly up-regulated in RUNX1-deficient HSPCs, and the promoter of Hmga2 was responsive in a cell-type dependent manner upon co-expression of RUNX1. Conditional Runx1 knockout mice exhibit expansion of its HSPCs and granulocyte-macrophage progenitors (GMPs) as hallmark phenotypes. To further validate and establish that Hmga2 plays a role in inducing HSPC expansion, we generated mouse models of HMGA2 and RUNX1 deficiency. Although mice lacking both factors continued to display higher frequencies of HSPCs, the expansion of GMPs was effectively rescued. The data presented
here establish *Hmgα2* as a transcriptional target of RUNX1 and a critical regulator of myeloid progenitor expansion.
2.1. Introduction

RUNX1, also known as AML1 and CBFα2, is a member of the runt family of proteins. The major function of RUNX1 is operating as a DNA binding transcription factor. Studies over the past 20 years have established RUNX1 as a critical player in hematopoiesis and specification of hematopoietic stem and progenitor cells (HSPCs), for neither process can occur without RUNX1[47], [48], [50]. The importance of RUNX1 is further validated by its prevalence in a variety of hematological diseases and malignancies including myelodysplastic syndrome (MDS), myeloproliferative neoplasms (MPN), and multiple forms for acute myeloid leukemia (AML)[97]–[99]. As a transcription factor, RUNX1 binds to DNA regulatory regions in order to guide the expression of its target genes. Most confirmed RUNX1 target genes are mainly expressed in differentiated blood cells[63], [62], [70], [98]. Direct targets of RUNX1 in HSPCs, however, have largely remained unexplored. The identification of these genes offers an insightful view into how this master regulator influences HSPC biology. To elucidate RUNX1 target genes in HSPCs, we performed genome-wide occupancy analysis with chromatin immunoprecipitation coupled with high-throughput sequencing (ChIP-seq) using RUNX1 antibodies and a murine HSPC cell line. This data was combined with gene expression signatures from wildtype and RUNX1-deficient HSPCs (Lineage-/cKit+/Sca-1+)[1]. One of the genes identified is Hmga2.
HMGA2 is a non-histone chromatin binding protein typically associated with enhancers but lacks its own transcriptional activity[100]. Its expression is generally highest in stem cells and during embryogenesis[101]. Aberrant HMGA2 expression has been associated with a variety of mesenchymal tumors including several examples in the hematopoietic system[102], [103]. Hence, HMGA2 is considered to be an oncogene. More recently, ectopic expression of Hmga2 in transgenic mouse models or bone marrow transplantation with cells expressing Hmga2 have been demonstrated to result in myeloproliferative disease[104], [105]. In these models, the HSPC populations are expanded as well as the myeloid progenitors. All of these studies suggest that HMGA2 serves as a critical regulator of proliferation and survival. Conditional deletion of Runx1 in mice has allowed for the study of its role in HSPCs since mice that are null for Runx1 display embryonic lethality[47], [48], [53], [54]. Interestingly, one of the most striking phenotypes in Runx1 conditional knockout mice is a marked expansion of its HSPCs and myeloid progenitors[54], [106]. In this study, we identified Hmga2 as a target gene using RUNX1 ChIP-seq and analyzed the role of RUNX1 in regulating Hmga2 expression. Furthermore, using genetic models of RUNX1 and HMGA2 deficiency, we establish that Hmga2 is a crucial regulator of myeloid progenitor expansion.
2.2. Results

2.2.1. Hmga2 is a direct RUNX1 target gene in HSPCs

To gain further insight into which genes are directly regulated by RUNX1 in HSPCs, we performed RUNX1 ChIP-seq in the murine HSPC-like EML cell line. EML cells rely on stem cell factor for survival and has the capability of differentiating into the erythroid, myeloid, and lymphoid lineages[107]. Their multipotency and capacity to be grown in large cultures make the EML cell line an ideal cell system for ChIP-seq studies. Peak calling revealed 6370 peaks with a FDR of less than 1%. Interestingly, the location of the majority of peaks (73.3%) lay in regions more than 5 kilobases (kb) away from a transcriptional start site (TSS) (Figure 2.1A). Only a small percentage (10.8%) of peaks was in promoter regions, defined as 3 kb upstream of a TSS (Figure 2.1B). De novo motif analyses using the MEME suite revealed the most enriched motif to be TGTGGT, which is the known RUNX consensus motif (Figure 2.1C)[60]. Other top motifs correspond to the ETS and GATA family of transcription factors, both of which are also important players in hematopoiesis and further confirm that RUNX1 often works with these factors to exert its functions[108]–[111].

We previously reported the gene expression signatures of HSPCs (Lineage-/c-Kit+/Sca-1+ or LSK cells) from wildtype, Runx1 conditional knockout (Δ/Δ) and RUNX1(41-214)-transplanted mice[1]. RUNX1(41-214)
Figure 2.1. RUNX1 genome-wide occupancy in HSPCs as determined by ChIP-seq. (A) The 6370 RUNX1 ChIP-seq peaks with a FDR less than 1% are shown in relation to the absolute distance of the TSS of known genes in kb. (B) Distribution of RUNX1 ChIP-seq peaks are grouped based on promoter (>3 kb upstream of TSS), intron, exon, and intergenic regions. (C) The 500 peaks with the lowest FDR were submitted to the MEME suite. The top motifs are shown and given with E-values, the associated motif family, and the frequency of the motif found in the 500 peaks.
(from here on, RUNX1 short form (SF)) is primarily a dominant-negative competitor of endogenous RUNX proteins. Of the 59 genes that were commonly differentially expressed in Runx1Δ/Δ and RUNX1SF HSPCs, 27 have RUNX1 ChIP-seq peaks associated with their loci (Table 2.1, Figure 2.2).

To further explore the biological effect of RUNX1 on hematopoiesis, we focused on its ability to regulate the expression of its target genes. We decided to concentrate our studies on high mobility group AT-hook 2 (HMGA2). HMGA2 has been reported to play important roles in regulating cellular proliferation and cancer development[112], [102]. Our gene expression profiling data obtained from three biologically independent sets of RNA prepared from LSK cells indicate that Hmga2 expression is significantly increased (Figure 2.3A). Using RT-qPCR studies, we demonstrated that Hmga2 transcript levels were 6.06- and 15.12-fold higher in Runx1Δ/Δ and RUNX1SF HSPCs, respectively, relative to wildtype controls (Figure 2.3B). In addition, Hmga2 is up-regulated in Runx1Δ/Δ myeloid progenitor populations relative to wildtype controls (Figure 2.3C).

Upon examination of the Hmga2 gene locus, ChIP-seq revealed three RUNX1 binding regions in the promoter, upstream, downstream, and third intron regions (Figure 2.4A). All except the promoter region were determined to be significant by MACS[113]. These three significant RUNX1 binding regions are in agreement with recently published RUNX1 ChIP-seq data using murine HSPC lines[110]. The binding of RUNX1 to these regions was confirmed using ChIP-qPCR (Figure 2.4B). The locations of these regions to
the Hmga2 TSS suggest that they may serve as enhancers and/or silencers for the regulation of Hmga2 expression. Together, our combined gene expression and ChIP analyses show that RUNX1 directly binds to the Hmga2 locus and influences its expression.
Table 2.1. Common genes differentially expressed in Runx1ΔΔ and RUNX1SF HSPCs that overlap with RUNX1 genome occupancy. Fold chances from the microarray are given for Runx1ΔΔ and RUNX1SF relative to the wildtype values.

<table>
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<tr>
<th>Symbol</th>
<th>Description</th>
<th>Runx1ΔΔ array</th>
<th>RUNX1SF array</th>
<th>Peak location</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adamtsl4</td>
<td>ADAMTS-like 4</td>
<td>2.42</td>
<td>3.37</td>
<td>chr3:95511225-95512034</td>
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<tr>
<td>Alcam</td>
<td>activated leukocyte cell adhesion molecule</td>
<td>-2.05</td>
<td>-4.28</td>
<td>chr16:54016417-54016980</td>
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<tr>
<td>Atp8a2</td>
<td>ATPase, aminophospholipid transporter, class I, type 8A, member 2</td>
<td>2.05</td>
<td>2.84</td>
<td>chr14:60691216-60748880</td>
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Table 2.1 continued Common genes differentially expressed in Runx1Δ/Δ and RUNX1SF HSPCs that overlap with RUNX1 genome occupancy. Fold changes from the microarray are given for Runx1Δ/Δ and RUNX1SF relative to the wildtype values.

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Description</th>
<th>Runx1Δ/Δ array</th>
<th>RUNX1SF array</th>
<th>Peak location</th>
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Table 2.1.continued  Common genes differentially expressed in Runx1Δ/Δ and RUNX1SF HSPCs that overlap with RUNX1 genome occupancy. Fold changes from the microarray are given for Runx1Δ/Δ and RUNX1SF relative to the wildtype values.

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Table 2.1.continued Common genes differentially expressed in Runx1\(^{Δ/Δ}\) and RUNX1SF HSPCs that overlap with RUNX1 genome occupancy. Fold changes from the microarray are given for Runx1\(^{Δ/Δ}\) and RUNX1SF relative to the wildtype values.

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Figure 2.2. Overlap between microarray and ChIP-seq results. Venn diagram showing the overlap between the common differentially expressed genes in Runx1Δ/Δ and RUNX1SF HSPCs (n=59)[1] and the genes associated with RUNX1 ChIP-seq peaks.

Figure 2.3. Expression of Hmga2 upon loss of RUNX1 function. (A) Relative expression of Hmga2 in wildtype, Runx1Δ/Δ and RUNX1SF HSPCs based on microarray data published in Matsuura et al10. (B) Validation of up-regulation of Hmga2 in microarray results by RT-qPCR using cDNA from wildtype, Runx1Δ/Δ, and RUNX1SF HSPCs. (C) Up-regulation of Hmga2 as demonstrated by RT-qPCR using cDNA from wildtype and Runx1Δ/Δ GMP, CMP, and MEP populations. RNA extraction and RT-qPCR were performed from at least 2-3 independent batches of mice.
Figure 2.4. RUNX1 ChIP for the Hmga2 locus. (A) Location of RUNX1 ChIP-seq peaks relative to the Hmga2 gene locus on chromosome 10. IgG control and one RUNX1 ChIP-seq replicate are shown. (B) Confirmation of RUNX1 occupancy regions in relation to the Hmga2 gene locus by ChIP-qPCR. RUNX1 ChIP was compared relative to IgG ChIP and normalized to a negative control region. Data represents 3 replicates of ChIP followed by qPCR.
2.2.2. The *Hmga2* promoter is regulated by RUNX1 in a cell type-dependent manner and is independent of canonical binding sites

To study how RUNX1 regulates *Hmga2* expression, a fragment consisting of base pairs (bp) -800 to +197 relative to the published TSS of *Hmga2* was inserted into the multiple cloning site of the pGLX promoterless luciferase reporter vector[114]. The pGLX vector is a modified version of pGL2, which does not contain any RUNX consensus binding sites[115]. The cloned *Hmga2* promoter DNA fragment has two RUNX consensus sites at bp -363 and -213 and a polypyrimidine/polypurine tract spanning bp -84 to -25, which gives the promoter much of its activity (Figure 2.5A)[114]. To examine whether RUNX1 affects *Hmga2* promoter activity, we conducted transient transfection assays in two non-hematopoietic cell lines (NIH3T3 and 293T) and two hematopoietic cell lines (K562 and Jurkat). In NIH3T3 and 293T cells, co-expression of the full length promoter with RUNX1 reduces luciferase activity to 72% and 47%, respectively (Figure 2.5B). These results suggest that RUNX1 acts as a transcriptional repressor on the *Hmga2* promoter in these two adherent cell lines. Interestingly, in the K562 and Jurkat leukemia cell lines, RUNX1 increases luciferase activity by 3- and 1.6-fold, respectively (Figure 2.5C). Thus, RUNX1 regulates *Hmga2* promoter activity in all of the cell lines tested, but acts on the promoter in a positive or negative manner depending on the cell and/or tissue type.
Figure 2.5. Schematic of Hmga2 promoter-luciferase construct and results. (A) Diagram of promoter-luciferase construct showing RUNX consensus Sites 1 and 2 (*) at bp -363 and -213, respectively. (B) The adherent non-hematopoietic NIH3T3 and 293T cell lines were transfected with the full length Hmga2 promoter-luciferase construct and luciferase assays were performed 24 hours after transfection. Co-expression was conducted with an empty expression vector (Control) or RUNX1 and CBFβ expression vectors. Error bars indicate standard error and are from at least 3 replicates. (C) The hematopoietic K562 and Jurkat cell lines were transfected with the full length Hmga2 promoter-luciferase construct and luciferase assay was performed 24 hours after transfection. Co-expression was conducted with an empty expression vector (Control) or RUNX1 and CBFβ expression vectors. Error bars indicate standard error and are from at least 3 replicates.
Since the *Hmga2* promoter contains two RUNX consensus sites, we sought to ascertain whether these sites mediate RUNX1 interaction with the promoter by utilizing promoter constructs with these regions truncated or mutated. In NIH3T3 cells, mutation of either or both of the presumptive RUNX1 binding sites in the promoter does not abrogate the repression of luciferase upon co-expression of RUNX1 and CBFβ (Figure 2.6). Constructs harboring truncations of the promoter at the 5’ or 3’ end also failed to affect RUNX1-mediated repression (Figure 2.7A-B). The same constructs were used in K562 cells. The co-expression of RUNX1 and CBFβ was still able to activate luciferase activity with the three mutation constructs (Figure 2.8). The same was observed with the truncation constructs (Figure 2.7C). These results suggest that RUNX1 does not regulate the *Hmga2* promoter through these two sites and likely exerts its effect through other intermediary factors.

![Figure 2.6](image)

**Figure 2.6.** Mutated *Hmga2* promoter-luciferase construct results in NIH3T3 cells. (D) NIH3T3 cells were transfected with promoter-luciferase constructs with Site1, Site2, or both sites mutated. Luciferase assays were performed 24 hours after transfection. Co-expression was conducted with an empty expression vector (Control) or RUNX1 and CBFβ expression vectors. Error bars indicate standard error and are from at least 2 replicates. (*) indicates p<0.05)
Figure 2.7. The effect of RUNX1 on the Hmga2 promoter is independent of canonical RUNX binding sites. (A) Diagram of promoter-luciferase construct showing locations of RUNX consensus sites and other constructs used in assays, which are denoted (i) through (v). (B) NIH3T3 cells were transfected with the indicated promoter-luciferase construct and luciferase assays were performed 24 hours after transfection. Co-expression was conducted with an empty expression vector (Control) or RUNX1 and CBFβ expression vectors. Error bars indicate standard error from at least 2 replicates. (C) K562 cells were transfected with the indicated promoter-luciferase construct and luciferase assays were performed 24 hours after transfection. Co-expression was conducted with an empty expression vector (Control) or RUNX1 and CBFβ expression vectors. Error bars indicate standard error from at least 3 replicates. (* indicates p<0.05).
Figure 2.8. Mutated *Hmgα2* promoter-luciferase construct results in K562 cells. (E) K562 cells were transfected with the constructs indicated in (C) and luciferase assays were performed 24 hours after transfection. Co-expression was conducted with an empty expression vector (Control) or RUNX1 and CBFβ expression vectors. Error bars indicate standard error from at least 3 replicates. (* indicates p<0.05)
2.2.3. RUNX1 intronic and intergenic binding regions mediate *Hmga2* transcription

Significant RUNX1 binding regions were discovered in intronic and intergenic regions of the *Hmga2* locus (Figure 2.4A). We sought to determine how these regions affect the transcription of *Hmga2* and whether they contribute to the effect of RUNX1 on the *Hmga2* promoter. The three regions were cloned downstream of the luciferase gene in the *Hmga2* promoter-luciferase construct (Figure 2.9A). The upstream binding element adds significant basal luciferase activity relative to the full length promoter alone in all cell lines tested (Figure 2.9B-C). Interestingly in NIH3T3 cells, addition of the upstream element induces enough transcriptional activation to counteract the repressive effect due to co-expression of RUNX1 and CBFβ (Figure 2.9B). In the promoter and upstream case, repression is still observed but is no longer significant. Accordingly, the upstream element continues to result in heightened transcriptional activity in K562 cells (Figure 2.9C).

The downstream element was also tested in NIH3T3 and K562 cells. In both cell lines, the effect of co-expression of RUNX1 and CBFβ remained intact and did not differ significantly from the promoter element by itself. Finally, the constructs containing the intron element with the *Hmga2* promoter were used for promoter-luciferase assays. In contrast to the upstream element, the intron element significantly represses basal luciferase activity relative to the full length promoter in all of the cell lines tested (Figure 2.9B-C).
In NIH3T3 cells, the promoter and intron construct continues to result in transcriptional repression when co-expressed with RUNX1 and CBFβ (Figure 2.9B). In K562 cells, addition of the intron element induces enough transcriptional repression to counteract the activation due to co-expression of RUNX1 and CBFβ (Figure 2.9C). Similar to the case of the promoter and upstream element in NIH3T3 cells where the trend of repression is still observed but no longer significant, in K562 cells the activation due to co-expression of RUNX1 and CBFβ is still observed but no longer significant. These results suggest that the upstream and intron regions are enhancer and silencer elements, respectively.
Figure 2.9. RUNX1 distal binding regions exert differential effects on Hmga2 expression. (A) Schematic of distal element-promoter luciferase constructs. The distal elements being tested are the RUNX1 binding regions that are downstream, upstream, and in the intron of the Hmga2 locus. Distal element sequences were cloned downstream of the Hmga2 promoter in the promoter-luciferase constructs. (B) These constructs were co-transfected with empty vector (Control) or with RUNX1 and CBFβ expression constructs into NIH3T3 cells. Luciferase activity was performed 24 hours after transfection. Error bars indicated standard error from 4 biological replicates. (C) Same experiment as described in (B) performed using K562 cells. Luciferase activity was performed 24 hours after transfection. Error bars indicated standard error from 3 biological replicates. (*)
2.2.4. HMGA2 does not modulate the effect of RUNX1 deficiency on HSPC expansion

Our current studies indicate that *Hmga2* expression is directly regulated by RUNX1 and that *Hmga2* expression is significantly increased in HSPCs in the absence of RUNX1 or in the presence of RUNX1SF (Figure 2.3). One of the most dramatic phenotypes of *Runx1Δ/Δ* mice and RUNX1SF mice is expansion of their HSPC populations[1], [53], [54]. HMGA2 is generally thought to be a proliferation-inducing factor. Transgenic mice expressing *Hmga2* and over-expression of *Hmga2* via retroviral transduction and bone marrow transplantation have both been recently shown to induce myeloproliferative disease in mice[104], [105]. Since *Hmga2* is a target gene of RUNX1, up-regulation of *Hmga2* in RUNX1-deficient HSPCs may contribute to their expansion. To examine the role of Hmga2 in HSPC expansion due to loss of RUNX1 function, we generated littermates of wild type, *Hmga2*−/−, *Runx1Δ/Δ*, and *Hmga2*−/− and *Runx1Δ/Δ* double knockout (Double KO) mice for further studies. The bone marrow compartments of these four genotypes of mice were analyzed by staining with cell surface markers followed by flow cytometry (Figure 2.10). As expected, RUNX1 deficiency led to a significant expansion of LSK, LT-HSC, and ST-HSC populations (Figure 2.10A-D). However, lack of HMGA2 did not have any effects on HSPC frequency. The Double KO also displayed HSPC expansion, suggesting that loss of HMGA2 does not affect HSPC expansion due to the deficiency of RUNX1.
Figure 2.10. HMGA2 does not modulate the effect of RUNX1 deficiency on HSPC expansion. Percentage of hematopoietic stem cell populations analyzed by flow cytometry, including (A) LSK cells, (B) LT-HSCs, and (C) ST-HSCs. For LSK: Wildtype (Hmga2+/+ or +/-, Runx1(fl/fl), Mx1Cre-, n = 13), Hmga2-/- (Hmga2-/-, Runx1(fl/fl), Mx1Cre-, n = 8), Runx1Δ/Δ (Hmga2+/+ or +/-, Runx1(fl/fl), Mx1Cre+, n = 19), Double KO (Hmga2-/-, Runx1(fl/fl), Mx1Cre+, n=7). For LT-HSCs and ST-HSCs: Wildtype (n = 8), Hmga2-/- (n = 3), Runx1Δ/Δ (n = 9), Double KO (n = 3). (D) Representative flow cytometry gating of lineage-negative, LSK, and SLAM populations for each of the four genotypes of mice and their averages are shown.
In addition, the common lymphoid progenitor (CLP) populations were assessed. Although we observed a trend toward higher frequencies of CLPs in the absence of Runx1, this difference was not significant (Figure 2.11). In addition, neither Hmga2$^{−/}$ nor Double KO mice exhibited any significant differences in CLP frequency.

The HSPCs in Runx1$^{Δ/Δ}$ mice have been described to have lower levels of apoptosis overall[58]. In addition, ectopic expression of Hmga2 has also been demonstrated to lead to lower levels of apoptosis[116]. To ascertain whether Hmga2 has any role in mediating apoptosis in the context of RUNX1 loss-of-function, we examined the frequency of apoptosis in LSK cells by using Annexin V staining (Figure 2.12). Hmga2$^{−/}$ and Double KO mice exhibited higher levels while Runx1$^{Δ/Δ}$ mice generally exhibited lower levels of Annexin V-positive cells, demonstrating that Hmga2 plays an important role in regulating apoptosis in this population and that it is necessary for the decreased apoptosis associated with loss of RUNX1.
Figure 2.11. Percentages of CLP populations were analyzed by flow cytometry. For CLPs: Wildtype (n = 5), Hmga2-/- (n = 5), Runx1Δ/Δ (n = 10), Double KO (n = 4).e (n = 3).

Figure 2.12. Loss of HMGA2 results in higher frequencies of apoptosis cells. Averages of frequencies of Annexin V-positive/7AAD-negative cells from the LSK cells of each genotype (n = 4 each) are shown. (*) indicates p<0.05.
2.2.5. **Hmga2 contributes to myeloid progenitor expansion caused by the loss of RUNX1**

Another major phenotype of Runx1Δ/Δ mice is their expansion of the myeloid progenitors, specifically the granulocyte-macrophage progenitor (GMP) population[54], [106]. Intriguingly, the role of HMGA2 in inducing proliferation in the hematopoietic system has primarily been biased towards the myeloid lineage[104], [105]. To test the involvement of HMGA2 in regulating myeloid progenitors in RUNX1-deficient mice, we analyzed the bone marrow compartments of wildtype, Hmga2−/−, Runx1Δ/Δ and Double KO mice and focused on their myeloid progenitor cells (Figure 2.13A). As expected, Runx1Δ/Δ mice displayed an expansion of the GMP population compared to wildtype (0.64% of total bone marrow versus 1.33%, p = 0.004). Other myeloid progenitors such as the megakaryocyte-erythrocyte (MEP) and common myeloid progenitors (CMP) were not affected (Figure 2.13B-C). Hmga2−/− mice, on the other hand, had significantly fewer GMPs compared to wildtype (0.23%, p = 0.01). In Double KO mice, the expansion of the GMPs is significantly rescued compared to Runx1Δ/Δ mice (0.64%, p = 0.0137). Representative flow cytometry charts and averages for each genotype are shown (Figure 2.13D). Thus, the loss of Hmga2 in a RUNX1-deficient genetic background brings the GMP frequency back down to wildtype levels. Together these results suggest that Hmga2 contributes to the GMP expansion due to the loss of RUNX1 function.
Figure 2.13. Lack of HMGA2 rescues myeloid progenitor expansion due to loss of RUNX1. Percentage of hematopoietic stem cell populations analyzed by flow cytometry, including (A) GMPs, (B) MEPs, and (C) CMPs. For myeloid progenitor staining, mice used were: Wildtype (Hmga2+/+ or +/−, Runx1(fl/fl), Mx1Cre−, n = 5), Hmga2−/− (Hmga2−/−, Runx1(fl/fl), Mx1Cre−, n = 5), Runx1Δ/Δ (Hmga2+/+ or +/−, Runx1(fl/fl), Mx1Cre+, n = 10), and Double KO (Hmga2−/−, Runx1(fl/fl), Mx1Cre+, n = 4). (D) Representative flow cytometry gating of GMP, MEP, and CMP populations on each of the four genotypes of mice and the averages are shown. (*) indicates p<0.05.
In addition, \textit{Hmga2} regulates the decreased apoptosis observed in the myeloid progenitors of \textit{Runx1}^{Δ/Δ} mice since Double KO myeloid progenitors continue to show increased frequencies of Annexin V-positive cells (Figure 2.14A-C).

\textbf{Figure 2.14.} Decrease in apoptosis in Runx1Δ/Δ mice is mediated by \textit{Hmga2}. Averages of frequencies of Annexin V-positive/7AAD-negative cells from the (E) GMP, (F) MEP, and (G) CMP gates of each genotype (n = 4 each) are shown. (*) indicates p<0.05.
2.3. Summary and discussion

RUNX1 has been implicated in a variety of blood-related diseases and neoplasms[98]. Many of these diseases originate from mutations in RUNX1 that occur at the stage of the HSPC and result in the loss of normal RUNX1 function. As a transcription factor, the primary biological activity of RUNX1 is to control the expression of its target genes. The goal of this study was to determine which genes RUNX1 directly targets at the level of the HSPC and whether those genes have any functions in regulating HSPCs. To this end, we used a combination of differential gene expression analyses and genome-wide transcription factor occupancy to identify prospective RUNX1 direct targets. For gene expression analyses, two models of RUNX1 deficiency were employed—an inducible conditional knockout model for RUNX1, and a bone marrow transplantation model employing RUNX1SF as a dominant-negative regulator of endogenous RUNX proteins. To elucidate DNA occupancy of RUNX1 on a genome-wide scale, ChIP-seq was conducted on a HSPC-like cell line using RUNX1 antibody. The result was a condensed list of high potential direct target genes that are regulated by RUNX1 at the level of the HSPC (Table 1). Furthermore, we performed similar comparisons using recently published ChIP-seq data (Figure 2.15)[110], [111]. The prospect of modulating the activity of these genes provides potential candidates for creating therapies against diseases caused by the loss of RUNX1 function,
Figure 2.15. Common genes between RUNX1 gene expression profiling and RUNX1 ChIP-seq analyses. Venn diagram showing the Runx1 differentially expressed genes that have Runx1 ChIP-seq peaks associated with their loci. Runx1 ChIP-seq studies from Wu et al. and Wilson et al. are also included.
All of these genes have diverse roles in mediating various functions both inside and outside of the hematological system. Briefly, Csf2rb codes for the common beta chain subunit found in the GM-CSF, IL-3, and IL-5 receptors and is important in regulating specific cytokine responses[117]. Gzmb and Igf2r have both been implicated in regulating cytotoxic T-cell-mediated apoptosis[118]. Lcp2 is necessary for T-cell development[119]. Another subset of genes are involved in cell-to-cell interactions in blood cells like Alcam[120] and Jam3[121], and may help to mediate interactions between HSPCs and the stem cell niche. These are just a few examples of genes that have blood-specific functions. Others like Krt80[122] and Tjp1[123] have described functions primarily in non-hematopoietic cells. Some genes like Fhdc1 and Zcchc18 have biological roles that are relatively unstudied or unknown. The diversity of functions in this list further confirms that RUNX1 is a master transcription factor and that disrupting RUNX1 function can have a variety of consequences resulting from disruption of its target genes. Ultimately, we decided that Hmga2 was an attractive candidate for further study primarily because of its known roles in mediating cellular proliferation[102]. A recent study described the role of HMGA2 in both fetal and adult HSPCs but interactions between RUNX1 and HMGA2 have remained largely unexplored[116]. The connection of these two genes in regulating various hematopoietic processes is a highly interesting avenue for further investigation.
Promoter-luciferase assays demonstrated that Hmga2 is directly regulated by RUNX1, but the regulation is cell type-specific as shown by contrasting results in a variety of cell lines. Blood cell lines like K562 and Jurkat cells express different sets of transcriptional regulators and co-factors that may not be present in adherent cell lines like NIH3T3 and 293T cells. Various members of the ETS and GATA families offer just a few examples of transcription factors that primarily function in the hematopoietic system. These hematopoietic-specific transcription factors may collaborate with RUNX1 to result in hematopoietic-specific regulation of Hmga2. Furthermore, the factors cooperating with RUNX1 in regulating Hmga2 expression can differ in HSPCs, differentiated hematopoietic cells, or leukemia cells. Identification of which partner factor(s) RUNX1 interacts with in these various contexts is a topic of ongoing study.

For the first time, distal regulatory regions in and around the Hmga2 locus were demonstrated to have effects on Hmga2 expression. Interestingly, the effects exerted by the upstream and intron regions were constant and did not depend on the cell type in which they were tested. When these regions were added to the promoter-luciferase assays, the upstream and intron regions were associated with transcriptional activation and repression, respectively, but did not affect RUNX1-mediated transcription specific to each cell line tested. Hence, the upstream and intron regions most likely serve as enhancer and silencers of Hmga2, respectively. Another possibility is that RUNX1 binding to these regulatory elements contributes to the regulation of
endogenous *Hmga2* gene expression in HSPCs. Under conditions of transient transfection, however, the relatively higher levels of exogenous RUNX1 expression is sufficient for binding with its collaborating factors at the *Hmga2* proximal promoter region which may overwhelm its effects from the binding to these other regulatory regions.

In addition to demonstrating that *Hmga2* is a transcriptional target of RUNX1, we showed that it contributes to GMP expansion resulting from loss of RUNX1 function. Importantly, the GMP population has often been associated with harboring leukemia stem cells (LSCs) in a variety of different leukemias[124]–[126]. A recent study focused specifically on the RUNX1-ETO (RE) fusion protein described the ability of RE to induce expansion of the GMP population and that the GMP RUNX1-ETO-expressing population can induce a leukemia-like state in an in vivo mouse model[126]. Loss of RUNX1 in various conditional knockout models has also been shown to expand the GMP population and induce altered hematopoietic states[54], [106]. Hence, loss of RUNX1 function either through mutation or by involvement in chromosomal translocation leads to an increase of cells capable of eventually transitioning to leukemia. Without RUNX1 acting as a tumor suppressor, *Hmga2* is allowed to become up-regulated to induce a pre-malignant state. Limiting the expansion of GMPs by decreasing or regulating the amounts of HMGA2 may provide another method of controlling leukemia progression through keeping the number of LSCs in check.
Various cases of *HMGA2* disruption associated with hematological disease have been described and are typically associated with perturbing the myeloid lineages[127]–[129]. Notably, some mutations result in truncation of the 3’ untranslated region of *HMGA2*. This region of *HMGA2* contains binding sites for the let-7 family of miRNAs, which target a variety of cellular mediators and is a major regulator of *HMGA2* levels[130], [131]. Let-7 family members have been reported to be lower in RE-positive leukemia in patients and mouse models of RE[132], [133], but various large gene expression studies in RE-positive leukemia patients have reported contrasting levels of *HMGA2* when compared to non-RE-positive leukemia patients (The Cancer Genome Atlas). No comprehensive study has been conducted comparing the expression of let-7 family members or *HMGA2* in MDS or MPN patients based on *RUNX1* mutation status, but such an investigation offers an interesting future direction to further establish the role HMGA2 in the context of RUNX1 loss-of-function.

In summary, our study establishes *Hmga2* as a target gene of RUNX1 and that *Hmga2* mediates myeloid progenitor expansion due to loss of RUNX1 function.
2.4. Materials and Methods

2.4.1. Mice

All experimental protocols were approved by the UCSD Institutional Animal Care and Use Committee and all animals were housed at UCSD. *Runx1*\textsuperscript{floxed/floxed} and *Mx1-Cre* mice were kindly provided by Dr. Nancy Speck[54] and *Hmga2*\textsuperscript{-/-} mice were kindly provided by Dr. Kiran Chada[134]. For induction of Cre excision, 6-10 week old mice were injected every other day for a total of 3 to 6 injections with polyinosinic:polycytidylic acid (Sigma, St. Louis, USA) based on the following formula: mouse weight (g) × 10 + 50 = µl of 1 mg/ml polyIC[58]. Mice were analyzed 4-12 weeks after the injection regimen.

2.4.2. Cells and cell culture

EML cells were cultured in Iscove’s DMEM with 20% horse serum supplemented with stem cell factor-conditioned medium, 10 mM glutamine and penicillin (100 IU)/streptomycin (100 µg/ml)[107]. K562, Jurkat, U937 cells were cultured in RPMI 1640 supplemented with 10% fetal bovine serum (FBS), glutamine and penicillin/streptomycin. 293T cells were cultured in DMEM supplemented with 10% bovine calf serum, glutamine and penicillin/streptomycin. NIH3T3 cells were cultured in DMEM supplemented with 10% FBS, glutamine and penicillin/streptomycin.
2.4.3. Chromatin immunoprecipitation (ChIP) and sequencing

ChIP procedure and antibody was previously described[135]. Approximately $5 \times 10^7$ EML cells were used to perform two replicates. High-throughput sequencing was conducted on a Genome Analyzer II (Illumina, San Diego, USA) resulting in $2.7 \times 10^7$ and $2.6 \times 10^7$ reads. Sequences were aligned to the reference mouse genome (version mm9). Peak calling was performed using the MACS algorithm[113]. Peaks with less a false discovery rate of less than 1% were associated with the closest transcriptional start site using PeakAnalyzer[136]. De novo motif finding was performed using the MEME software suite (http://meme.nbcr.net)[137]. Primers are provided in Table 2.2.

2.4.4. RT-qPCR

RNA was extracted using the TRIzol method (Life Technologies, Carlsbad, USA) or RNeasy Micro Plus kit (Qiagen, Venlo, Netherlands). cDNA was synthesized using Superscript III (Life Technologies). Primers are provided in Table 2.2.
Table 2.2. Primer sequences for RT-qPCR and ChIP-qPCR.

<table>
<thead>
<tr>
<th>Description</th>
<th>Method</th>
<th>Primer sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hmga2 forward</td>
<td>RT-qPCR</td>
<td>ACAAGTTGTTTCAAGAGAGCCTG</td>
</tr>
<tr>
<td>Hmga2 reverse</td>
<td>RT-qPCR</td>
<td>AGTGGAAAGACCATTGGAATAC</td>
</tr>
<tr>
<td>Gapdh forward</td>
<td>RT-qPCR</td>
<td>GGCCTGAGTACGTCGGAGTCCA</td>
</tr>
<tr>
<td>Gapdh reverse</td>
<td>RT-qPCR</td>
<td>AAAGTTGTCATGGATGACCTTGG</td>
</tr>
<tr>
<td>Hmga2 promoter forward</td>
<td>ChIP-qPCR</td>
<td>GAGTCACCCTGGAGAGCTTG</td>
</tr>
<tr>
<td>Hmga2 promoter reverse</td>
<td>ChIP-qPCR</td>
<td>CTTTGTCACGCTTTTGT</td>
</tr>
<tr>
<td>Hmga2 upstream forward</td>
<td>ChIP-qPCR</td>
<td>GATGAAGCTCAGCGATCGTCA</td>
</tr>
<tr>
<td>Hmga2 upstream reverse</td>
<td>ChIP-qPCR</td>
<td>GGGGAGACTGTACGTGGAGA</td>
</tr>
<tr>
<td>Hmga2 downstream forward</td>
<td>ChIP-qPCR</td>
<td>GCCCTGCTATTCTGAGC</td>
</tr>
<tr>
<td>Hmga2 downstream reverse</td>
<td>ChIP-qPCR</td>
<td>TGCAACTTCCTCCAGACCAT</td>
</tr>
<tr>
<td>Hmga2 intron forward</td>
<td>ChIP-qPCR</td>
<td>GCACGGAGACAACATTCTGA</td>
</tr>
<tr>
<td>Hmga2 intron reverse</td>
<td>ChIP-qPCR</td>
<td>ACAGCCTAAAAACTCAGCATTTC</td>
</tr>
<tr>
<td>Negative region forward</td>
<td>ChIP-qPCR</td>
<td>GTCATCCAAACCAGCCTCTA</td>
</tr>
<tr>
<td>Negative region reverse</td>
<td>ChIP-qPCR</td>
<td>ACCCTGAGGAGAACTTTGA</td>
</tr>
</tbody>
</table>
2.4.5. Luciferase assays

Cells were transfected with PEI or by Nucleofection (Amaxa, Basal, Switzerland) with 1-2 μg luciferase construct, 0.5 μg of pCMV5-RUNX1b[38], 0.5 μg of pCMV5-CBFβ[115], and 50-250 ng of Renilla luciferase construct (pRL-null from Promega, Madison, USA) as an internal transfection control for 293T, NIH3T3, and K562 cells. Jurkat cells were transfected by electroporation with these constructs in the same ratios with a total DNA amount of 20-25 μg. Cells were collected 24 hours after transfection and lysates were prepared. Manufacturer’s protocol for the Dual-Luciferase Reporter Assay System (Promega) and Monolight 3010 Luminometer (BD Biosciences, San Jose, USA) were followed for ascertaining luciferase activity. Firefly luciferase activity was normalized to Renilla luciferase activity.

2.4.6. Flow cytometry

Cells were collected from mice, treated in ammonium-chloride-potassium buffer, stained. The following antibodies were used: PerCP-Cy5.5 or PE-Cy5-conjugated lineage antibodies (CD3, CD4, CD8a, B220, CD19, Gr-1, CD11b, and Ter119), APC or PE-Cy5-conjugated Sca-1, PE-Cy7 or APC-conjugated c-Kit, PE-conjugated CD48, biotin-conjugated CD150 with streptavidin-conjugated APC-Cy7, PE-conjugated FcyR, FITC-conjugated CD34, and PerCP-Cy5.5 or PE-conjugated IL7Ra (all antibodies from eBioscience, San Diego, USA). For apoptosis studies, APC-conjugated
Annexin V and 7AAD (BD Pharmingen) were used. After staining, cells were analyzed on a BD FACS Canto flow cytometer.
Chapter 2, in part, is now in press at Blood for publication. Kentson Lam, Alexander D. Muselman, Randal Du, Yuka Harada, Ming Yan, Shinobu Matsuura, Stephanie Weng, Hironori Harada, Dong-Er Zhang. "Hmga2 is a direct target gene of RUNX1 and regulates expansion of myeloid progenitors in mice." The dissertation author is the primary investigator and author of the manuscript.
3. Loss of RUNX1 function results in hypersensitivity to G-CSF in mice

Transcription factors are versatile proteins capable of controlling critical cellular and molecular processes. For example, expression of just a few transcription factors are capable of inducing terminally differentiated cells to transition back into a stem cell-like state, which can then differentiate into completely different lineages[138]. They are able accomplish such amazing functions because they can modulate the expression of hundreds if not thousands of target genes. RUNX1 is a transcription factor that is considered critical in hematopoiesis. By regulating its target genes, RUNX1 specifies the first definitive hematopoietic stem cells and subsequently regulates their differentiation into a variety of blood lineages. By analyzing differentially expressed genes from hematopoietic stem and progenitor cells (HSPCs) from wildtype and Runx1 conditional knockout mice, we have identified cellular and molecular networks that RUNX1 may potentially regulate and control. Interestingly, the top networks include terms like cellular movement, and cell-to-cell signaling and interaction, suggesting that RUNX1 may play a role in mediating HSPC interaction with the bone marrow stromal niche. To pursue this idea further, we used granulocyte-colony stimulating factor as agent to induce mobilization of HSPCs from the bone marrow into peripheral blood
tissues, and identified that loss of RUNX1 function presents with a novel phenotype of hypersensitivity to mobilization.
3.1. Introduction

RUNX1 is a transcription factor that is critical in hematopoiesis. Mice knocked out for RUNX1 die during embryogenesis and lack definitive hematopoiesis[47], [48]. A variety of studies have thoroughly established that RUNX1 is necessary for specification of definitive hematopoietic stem cells (HSCs) and their derivation from the endogenic hemothelium[139], [140]. Furthermore, knocking out RUNX1 conditionally in adult mice results in abnormal homeostasis of HSCs[53]–[55]. The importance of RUNX1 in hematopoiesis displayed through these various mouse models is further corroborated by the preponderance of RUNX1 mutations found in human disease, particularly those involving the hematological system[98]. Although cell autonomous functions of RUNX1 like HSC specification, cell cycle status, differentiation have been described using these models, the effect of RUNX1 on non-cell autonomous functions remains poorly understood. One recent publication recently suggested some features related to non-cell autonomous effects due to the loss of RUNX1 including an increase of HSCs in peripheral blood tissues like the spleen and peripheral blood[57]. In this chapter of the thesis, we focus on the role of RUNX1 in mediating the interactions between hematopoietic stem and progenitor cells (HSPCs) and the bone marrow niche.

We recently performed differential gene expression analysis on HSPCs, defined as negative for lineage markers and positive for c-Kit and Sca-1 markers (LSK), from wildtype mice, Runx1 conditional knockout mice
(Runx1Δ/Δ), and mice transplanted with a dominant-negative form of RUNX1[1]. Pathway analysis was performed, which revealed pathways and biological functions enriched for cell-to-cell interaction and signaling and cellular movement. This gene expression analysis further supports the notion that RUNX1 and its target genes mediate HSPC and bone marrow niche interactions. To test the functions of RUNX1 regarding niche interaction, we employed stem cell mobilization induction by treating wildtype and RUNX1 loss-of-function mice with granulocyte colony stimulating factor (G-CSF). Interestingly, we observed that mice with loss of RUNX1 function, either by conditional knockout or expression of the dominant-negative form, demonstrated a hypersensitivity to G-CSF-mediated mobilization. Cxcr4, which codes for the receptor to stem cell niche chemokine SDF-1, has been described as a RUNX1 target gene and may contribute to these phenotypes[57]. Nonetheless, a combination of regimen of G-CSF and AMD3100, a CXCR4 antagonist, elicited a heightened mobilization response in RUNX1 loss-of-function mice compared to G-CSF alone. Using a mini-library approach of over-expression a variety of RUNX1 target genes simultaneously, we sought to identify which target genes are most responsible for this enhanced mobilization. Interestingly, over-expression of no single gene was sufficient to result in enhanced mobilization, suggesting that a combination of RUNX1 target genes is critical for this phenotype.

G-CSF-mediated mobilization is considered a “gold standard” for the induction of HSPC mobilization from the bone marrow into the peripheral blood
tissues. Currently, G-CSF is commonly utilized for bone marrow donations and for patient suffering from neutropenia, or low neutrophil counts, deriving from a variety of medical conditions. Unfortunately, a subset of patients fail to respond to G-CSF therapy. Modulating the function levels of RUNX1 may serve as a novel method of increasing the mobilization response from G-CSF.
3.2. Results

3.2.1. Runx1\(^{Δ/Δ}\) mice are hypersensitive to G-CSF-induced mobilization of HSPCs.

We previously reported the gene expression signatures of HSPCs (Lineage-/-c-Kit+/Sca-1+ or LSK cells) from wildtype and Runx1\(^{Δ/Δ}\) mice[1]. To gain further insight into the differentially expressed genes caused by the loss of RUNX1 activity, pathway analyses on these genes were performed using Ingenuity's IPA software. The top three networks all involved some description of cellular movement and/or cell-to-cell signaling and interaction (Table 3.1). The potential for RUNX1 to be involved in some way in regulating the interactions between the bone marrow niche and HSPCs has been suggested previously, but has not been thoroughly explored.

Granulocyte-colony stimulating factor (G-CSF) is commonly used as a mobilization reagent to drive HSPCs from the bone marrow into peripheral blood tissues, and is one method to assess proper interaction between stem cells and the niche[141]. Runx1\(^{Δ/Δ}\) mice were injected with G-CSF daily for five days and were analyzed one day after the final injection. Spleens from Runx1\(^{Δ/Δ}\) mice were noticeably enlarged by gross observation and by weight (Figure 3.1A-B). Peripheral blood from Runx1\(^{Δ/Δ}\) mice after G-CSF treatment produced significantly more colonies than blood from wildtype mice (Figure 3.2). The increase in HSPCs in the peripheral blood tissues was further confirmed by measuring the frequencies of LSK cells in the bone marrow,
spleen, and blood (Figure 3.3A-C). Comparing wildtype with Runx1Δ/Δ mice, more LSK cells were found in the spleen (n = 5 each, 0.18% vs 0.44%, p = 0.075) and in peripheral blood (n = 5 each, 0.07% vs 0.34%, p < 0.0001), and significantly fewer were found in the bone marrow (n = 5 each, 0.23% vs 0.09%, p < 0.01). Without any treatment, LSK frequencies in the bone marrow in wildtype and Runx1Δ/Δ mice were 0.38% and 0.94% (n = 2 each), respectively (Figure 3.4A). In the spleen, LSK frequencies were 0.04% and 0.29% in wildtype and Runx1Δ/Δ mice (n = 2 each), respectively (Figure 3.4B). These results suggest that loss of RUNX1 leads to hypersensitivity to G-CSF treatment and more mobilization of HSPCs.
Table 3.1. Top networks and genes from Ingenuity IPA analysis on differentially expressed genes in Runx1Δ/Δ HSPCs. Bold denotes genes in differential gene expression analysis.

<table>
<thead>
<tr>
<th>ID Associated Network Functions</th>
<th>Genes</th>
<th>Score</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Cellular Movement, Cell-To-Cell Signaling and Interaction, Hematological System Development and Function</strong></td>
<td>14-3-3, Akt, Alpha catenin, BHLHE40, CD3, CD274, CLDN5, COL4A1, CPA3, CSF2RB, DEPTOR, EPS8, F Actin, GJA1, GZMB, HCK, HNRNPAB, IL12 (complex), LCP2, MYO7A, NCF1, NOV, PTN, RHOJ, RUNX1, SOCS2, SPP1, SRC (family), STAT5a/b, SYNJ2, TCR, TEK, TJP1, TMPRSS4, VAV</td>
<td>55</td>
</tr>
<tr>
<td><strong>Cell-To-Cell Signaling and Interaction, Nervous System Development and Function</strong></td>
<td>Acot1, ACP2, ADAMTSL4, ANKH, BDH1, BDNF, CYB561, DECR1, DNAJ3, EDN1, ELF5, ERBB2, GCSAM, IFNG, IL4, KCNA3, mir-155, MLLT3, NOV, PLAT, PTN, PRPM, SERPINB9, SLC39A4, SPINT1, STK17B, TEAD2, TGFB1, TJP1, TJP2, TP53, VASN, Wdfdc18, WNT1, ZCCHC18</td>
<td>28</td>
</tr>
<tr>
<td><strong>Cell-To-Cell Signaling and Interaction, Tissue Development, Reproductive System Development and Function</strong></td>
<td>ADAM2, Adam3, ADAM7, ADAM9, ADAM15, ADAM28, ADAM33, Adam1a, ALCAM, beta-carotene, CAV1, CHD7, CLDN7, CTNN, DHR53, DSC2, DSG2, EGFR, FADS3, HMGA2, Integrinα, ITGA9, ITG81, ITG88, JAM2, JAM3, MIA, PLXDC2, Sod, SOX2, Tenascin, TJP1, TNF, TREML1, TULP3</td>
<td>21</td>
</tr>
<tr>
<td><strong>Metabolic Disease, Cancer, Cellular Function and Maintenance</strong></td>
<td>26s Proteasome, Actin, ADCY, ATP8A2, BCL2, caspase, CAV2, CD72, CD200R1, Collagen(s), ECM1, ERK, ERK1/2, GP5, IgE, IgG1, IL1, IL12 (family), Immunoglobulin, Interferon alpha, ITGA2B, Jnk, LHCGCR, MAP3K6, Mapk, NFκB (complex), P38 MAPK, PDE2A, Pdgf (complex), PI3K (complex), Pka, Pkc(s), PODXL, Tgf beta, Vegf</td>
<td>20</td>
</tr>
<tr>
<td><strong>Cellular Development, Cellular Growth and Proliferation, Reproductive System Development and Function</strong></td>
<td>ACTRT3, ALK, beta-estradiol, CFTR, Ck2, DDX4, DUSP6, FAM118B, FGFR4, FSCN1, HOXA7, HSD17B3, IGF2R, KIF21A, LAMP2, LMO1, LYL1, MGP, NDRG2, NfkB-RelA, NFKBIA, NRK, OCLN, PCDH7, PFN3, Prl2c2 (includes others), SCAPER, SLC24A3, SSBBP2, STX3, TAL1, TAL2, tretinoin, VCP, VCPIP1</td>
<td>14</td>
</tr>
</tbody>
</table>
Figure 3.1. Splenomegaly is induced in Runx1Δ/Δ mice after G-CSF treatment. Picture of spleens and spleen weights are shown (n = 5 for each genotype).

Figure 3.2. Peripheral blood colony assay after G-CSF treatment. Runx1Δ/Δ mice have significantly higher numbers of colonies compared to wildtype mice after treatment (n = 5 for each genotype).
**Figure 3.3.** HSPC frequencies after G-CSF treatment in wildtype and Runx1Δ/Δ mice. (A) Percent of LSK cells in bone marrow of G-CSF injected mice. (B) Percent of LSK cells in spleens of G-CSF injected mice. (C) Percent of LSK cells in the peripheral blood of G-CSF injected mice. The numbers of mice were n = 5 for each genotype pooled from three independent experiments.

**Figure 3.4.** HSPC frequencies without any treatment in wildtype and Runx1Δ/Δ mice. (A) Percent of LSK cells in bone marrow. (B) Percent of LSK cells in spleens. The numbers of mice were n = 2 for each genotype.
3.2.2. Dominant-negative RUNX1SF results in higher degree of hypersensitivity to G-CSF-induced mobilization of HSPCs.

To further confirm that loss of RUNX1 function results in hypersensitivity to G-CSF, we employed a previously described mouse model which utilizes expression of a dominant-negative regulator of RUNX proteins[1]. This regulator consists of amino acids 41 to 214 of RUNX1, which resembles a C-terminal truncation and includes primarily the runt homology domain. Hereafter, this model will be referred to as RUNX1 short form (SF). Mice transplanted with cells expressing RUNX1SF display many of the same phenotypes as Runx1Δ/Δ mice but to a more dramatic degree. When RUNX1SF mice were treated with the G-CSF regimen, HSPC mobilization was even more pronounced as evidenced by a significant increase in LSK cells found in the spleen and peripheral blood (Figure 3.5A-B). In the spleen, RUNX1SF mice (n = 5) had an average frequency of 2.38% LSK cells compared to control MigR1 mice (n = 4) which had an average frequency of 0.21% (p = 0.025). In the peripheral blood, RUNX1SF mice displayed 1.03% LSK cells compared to 0.02% for MigR1 mice (p = 0.005). Interestingly, in contrast to what was observed in Runx1Δ/Δ mice, RUNX1SF did not exhibit a decrease in LSK cells in the bone marrow (Figure 3.5C, MigR1 vs. RUNX1SF, 0.50% vs. 0.46%). This result may be due to a heightened proliferative response to G-CSF in addition to enhanced proliferation by bone marrow LSK cells in RUNX1SF mice. Methylcellulose colony assays using the peripheral
blood from mobilized animals confirmed that there were significantly more mobilized progenitors in RUNX1 SF-transplanted mice (Figure 3.6, 23-fold, p < 0.001).

**Figure 3.5.** HSPC frequencies after G-CSF treatment in MigR1 and RUNX1SF-transplanted mice. (A) Percent of GFP-positive LSK cells in bone marrow of G-CSF injected MigR1 (n = 4) and RUNX1SF (n = 5) mice. (B) Percent of GFP-positive LSK cells in spleens of G-CSF injected mice. (C) Percent of GFP-positive LSK cells in the peripheral blood of G-CSF injected mice. Data was pooled from three independent experiments.

**Figure 3.6.** Peripheral blood colony assay after G-CSF treatment. RUNX1SF mice have significantly higher numbers of colonies compared to wildtype mice after treatment. (n = 4 for MigR1 and n = 5 for RUNX1SF).
3.2.3. Enhanced HSPC mobilization in \textit{Runx1}^{Δ/Δ} mice is not solely due to decreased expression of \textit{Cxcr4}.

CXCR4 is the chemokine receptor for SDF1 or stromal-derived factor 1. This receptor and ligand pair mediates the homing of HSPCs to their bone marrow niche[142]. Down-regulating this signaling axis has been implicated as one of the mechanisms of how G-CSF induces mobilization of HSPCs[143], [144]. RUNX1 has also been suggested to act as a transcriptional activator of \textit{Cxcr4}[57]. Hence at steady state, \textit{Runx1}^{Δ/Δ} HSPCs express lower levels of \textit{Cxcr4} and are more frequently found in the peripheral circulation. Lower expression of \textit{Cxcr4} may also offer one explanation of why \textit{Runx1}^{Δ/Δ} mice may be more sensitive to G-CSF mobilization. To test this hypothesis, wildtype and \textit{Runx1}^{Δ/Δ} mice were treated with a regimen of G-CSF injections followed by one injection of AMD3100, a CXCR antagonist also known as plerixafor. This treatment protocol typically results in a synergistic effect on mobilization when compared to using either reagent alone[145]. \textit{Runx1}^{Δ/Δ} mice displayed even more mobilization of LSK cells into the spleen (p = 0.014) and peripheral blood (p = 0.029) using the combination of G-CSF and AMD3100 treatment (Figure 3.7A-B). The frequency of LSK cells in the bone marrow was also significantly lower after treatment (Figure 3.7C, p = 0.010). The increase in mobilized HSPCs was further confirmed by peripheral blood colony assay, which demonstrated more colonies from \textit{Runx1}^{Δ/Δ} mice compared to wildtype (Figure 3.8, p < 0.001). When a combined G-CSF and AMD3100 regimen was used,
hypersensitivity to mobilization was still observed in Runx1Δ/Δ mice. The combination regimen also mobilized better than using G-CSF alone, suggesting that although Cxcr4 has been shown to be down-regulated by RUNX1, Runx1Δ/Δ mice are still capable of mobilization when treated with a CXCR4 antagonist. Hence, other targets are involved in regulating the hypersensitivity to G-CSF due to the loss of RUNX1.

**Figure 3.7.** HSPC frequencies after combination G-CSF and AMD3100 treatment in wildtype and Runx1Δ/Δ mice. (A) Percent of LSK cells in bone marrow of G-CSF injected mice. (B) Percent of LSK cells in spleens of G-CSF injected mice. (C) Percent of LSK cells in the peripheral blood of G-CSF injected mice. The numbers of mice were n = 5 for wildtype and n = 4 for Runx1Δ/Δ pooled from two independent experiments.
Figure 3.8. Peripheral blood colony assay after combination G-CSF and AMD3100 treatment. Runx1ΔΔ mice have significantly higher numbers of colonies compared to wildtype mice after treatment. (n = 4 for each).
3.2.4. Utilization of a mini-library approach to discover RUNX1 target genes involved in mediating HSPC mobilization.

Differentially expressed genes between wildtype and Runx1Δ/Δ HSPCs have been previously described[1]. From this list, up-regulated genes that have involvement in cell-to-cell interaction or mobilization, and that have cDNAs readily available for purchase were selected for further study (Table 3.2). At this time, we chose not to focus on down-regulated genes by knockdown studies because expression levels for these genes may be variable and knockdown efficiency would be technically difficult to ascertain. These cDNAs were cloned into the MigR1 retroviral vector and were pooled together to create a mini-library (mLib) of cDNAs. In addition to cloning of the various cDNAs into MigR1, a common forward primer sequence and a 6 base pair (bp) DNA barcode specific to each unique cDNA were added 3' to the cDNA coding sequence (Figure 3.9). The barcode allows for recognition of the cDNA upon insertion into the host cell genome. By deep sequencing, the abundance of individual cDNAs can be correlated to the counts of each barcode read.
Figure 3.9. Schematic of retroviral barcode vector used in cDNA screen. Each cDNA was cloned downstream of the 5' long terminal repeat (LTR). A universal forward primer sequence and a barcode specific to each cDNA was added 3' to the cDNA. Common primers specific for the universal sequence and part of the IRES sequence will be used to sequence the barcode region.
Table 3.2. List of genes used in mini library barcode screen.

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Gene Name</th>
<th>Location</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD274</td>
<td>CD274 molecule</td>
<td>Plasma Membrane</td>
</tr>
<tr>
<td>CPA3</td>
<td>carboxypeptidase A3 (mast cell)</td>
<td>Extracellular Space</td>
</tr>
<tr>
<td>DEPTOR</td>
<td>DEP domain containing MTOR-interacting protein</td>
<td>Unknown</td>
</tr>
<tr>
<td>ECM1</td>
<td>extracellular matrix protein 1</td>
<td>Extracellular Space</td>
</tr>
<tr>
<td>F2R</td>
<td>coagulation factor II (thrombin) receptor</td>
<td>Plasma Membrane</td>
</tr>
<tr>
<td>FAM189B</td>
<td>family with sequence similarity 189, member B</td>
<td>Unknown</td>
</tr>
<tr>
<td>GNA14</td>
<td>guanine nucleotide binding protein (G protein), alpha 14</td>
<td>Plasma Membrane</td>
</tr>
<tr>
<td>GP5</td>
<td>glycoprotein V (platelet)</td>
<td>Plasma Membrane</td>
</tr>
<tr>
<td>HMGA2</td>
<td>high mobility group AT-hook 2</td>
<td>Nucleus</td>
</tr>
<tr>
<td>ITGA2B</td>
<td>integrin, alpha 2b (platelet glycoprotein IIb of IIb/IIIa complex, antigen CD41)</td>
<td>Plasma Membrane</td>
</tr>
<tr>
<td>JAM3</td>
<td>junctional adhesion molecule 3</td>
<td>Plasma Membrane</td>
</tr>
<tr>
<td>KRT80</td>
<td>keratin 80</td>
<td>Unknown</td>
</tr>
<tr>
<td>MLLT3</td>
<td>myeloid/lymphoid or mixed-lineage leukemia (trithorax homolog, Drosophila); translocated to, 3</td>
<td>Nucleus</td>
</tr>
<tr>
<td>NOV</td>
<td>nephroblastoma overexpressed</td>
<td>Extracellular Space</td>
</tr>
<tr>
<td>PDE2A</td>
<td>phosphodiesterase 2A, cGMP-stimulated</td>
<td>Cytoplasm</td>
</tr>
<tr>
<td>PODXL</td>
<td>podocalyxin-like</td>
<td>Plasma Membrane</td>
</tr>
<tr>
<td>RHOJ</td>
<td>ras homolog family member J</td>
<td>Cytoplasm</td>
</tr>
<tr>
<td>SLC24A3</td>
<td>solute carrier family 24 (sodium/potassium/calcium exchanger), member 3</td>
<td>Plasma Membrane</td>
</tr>
<tr>
<td>SOCS2</td>
<td>suppressor of cytokine signaling 2</td>
<td>Cytoplasm</td>
</tr>
<tr>
<td>TULP3</td>
<td>tubby like protein 3</td>
<td>Extracellular Space</td>
</tr>
</tbody>
</table>
The mLib was used to produce retrovirus particles used to transduce fetal liver hematopoietic cells for injection into irradiated recipients. Three months after transplantation, mice with the Barcode cDNA pool displayed myeloid bias in their peripheral blood as indicated by higher frequencies of CD11b and Gr-1 positive cells (Figure 3.10A). The mice also exhibited lower frequencies of T cells but B cells remained unchanged (Figure 3.10B-C). The transplanted mice were treated with a G-CSF regimen and assessed for mobilization. Significantly higher percentages of LSK cells were found in the peripheral blood of Barcode mice compared to MigR1 control (Figure 3.11A, p < 0.0001). The increase in mobilization of HSPCs was further supported by a trend of higher colony numbers using mobilized peripheral blood (Figure 3.11B, p = 0.14).

Figure 3.10. Barcode mice exhibit myeloid differentiation bias to G-CSF treatment. (A) Cell surface marker staining of peripheral blood from mice transplanted with barcode-transduced cells for myeloid markers CD11b and Gr-1. (B) Cell surface marker staining of peripheral blood from mice transplanted with barcode-transduced cells for T cells markers CD4 and CD8. (C) Cell surface marker staining of peripheral blood from mice transplanted with barcode-transduced cells for B cell marker B220. Three months after transplantation. (n = 6 for MigR1, n = 20 for Barcode)
Figure 3.11. Barcode mice exhibit hypersensitivity to G-CSF treatment. (A) Percent of LSK cells in the peripheral blood after regimen of G-CSF injections into MigR1 and Barcode transplanted mice. (B) Colony assay using cells from peripheral blood of G-CSF injected mice. (n = 9 for MigR1, n = 17 for Barcode)
3.2.5. Hypersensitivity to G-CSF mobilization is not mediated by a single gene but via a combination of RUNX1 target genes.

To gain further insight into which of the cDNAs contribute to G-CSF mobilization, the fetal liver cells used for transplantation, the bone marrow LSK cells after a two month engraftment period but before any treatment, and the peripheral blood LSK cells after G-CSF treatment were collected and submitted for high-throughput sequencing. The frequency of each cDNA was attributed to the barcode count (Figure 3.12A-C). The ratio of the frequency of barcodes in LSK cells after and before treatment was used as an indicator of which cDNAs were enriched post-treatment (Figure 3.13). Four biological replicates followed by sequencing were performed. Using the criterion where a cDNA is considered to be enriched if its ratio of reads after treatment compared to before treatment is greater than 1.5 in all replicates, no single cDNA stands out (Table 3.3). These results suggest that a combination of RUNX1 target genes expressed simultaneously is capable of eliciting a heightened mobilization response to G-CSF, but single genes alone may not be sufficient. Our study only assessed the roles of 20 genes, so the possibility exists that other RUNX1 target genes may contribute to this phenotype.
Figure 3.12. Frequency of barcode counts. (A) Counts from fetal liver cells after transduction to be used for transplantation. (B) Counts from bone marrow LSK cells after two months of engraftment but before any treatment. (C) Counts from peripheral blood LSK cells after G-CSF treatment.
Table 3.3. Ratio of barcode counts in peripheral blood LSK cells after G-CSF treatment compared to counts in bone marrow LSK cells before any treatment. Ratios from four biological replicates are shown.

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Figure 3.13. Ratio of barcode counts. The counts for each barcode, and hence cDNA, in peripheral blood LSK cells after treatment was compared to bone marrow LSK cells before G-CSF treatment.
3.3. Summary and discussion

To gain further insights into RUNX1 as a transcription factor, we performed pathway analyses on its differentially expressed genes obtained from comparing wildtype to Runx1Δ/Δ HSPCs. The top networks and biological functions generated from Ingenuity’s IPA were enriched for cell-to-cell interaction and signaling, movement, and immune cell trafficking. These associations support the notion that one of RUNX1’s functions in HSPCs is to regulate genes critical in mediating their interactions with the bone marrow niche. To study this issue further, we used HSPC mobilization as an assay to study how loss of RUNX1 function may affect these interactions. Using G-CSF or the combination of G-CSF with a CXCR4 antagonist, we demonstrate a novel finding in that mice deficient in RUNX1 displayed enhanced mobilization and confirm that RUNX1 and its target genes indeed play a role in mediating these interactions.

Looking further into the list of differentially expressed genes between wildtype and Runx1Δ/Δ HSPCs, many of these genes operate at the plasma membrane or in the extracellular space/matrix. Some of these have documented roles in hematopoiesis or in HSPCs. For example, one target gene Itga2b, which codes for an integrin protein, cooperates with ITGB3 to form the fibrinogen receptor, an integral member of the clotting cascade[146]. ITGA2B is also used as a marker for fetal HSCs and was recently found to be expressed in adult HSPCs, where it modulates their survival and
quiescence[147]. *Podxl*, part of the CD34 family, codes for a cell surface protein involved in mediating cell-to-cell interactions like adhesion, and has been utilized as a marker for HSPCs and hemangioblasts[148], [149]. *Nov* codes for an extracellular growth factor that regulates the functions of HSPCs, and addition of recombinant NOV has been shown to expand HSPCs ex vivo[150]. To further study the effects of these and other RUNX1 target genes, we selected a subset that were the most up-regulated to make a mini-library of cDNAs. Many of the target genes used in the mLib screen code for proteins that are at the cell surface (*Cd274, Gp5, Itga2b, Jam3, Podxl, Slc24a3, F2r*) or extracellular (*Cpa3, Ecm1, Nov, Tulp3*). By this method, we were able to assess the effects of multiple genes simultaneously. Interestingly, the combination of genes in the mLib resulted in higher percentages of LSK cells in the peripheral blood and spleen tissues. These results confirm the fact that RUNX1 target genes, even a small subset of them, can modulate interactions between HSPCs and the niche.

HSPCs reside in the bone marrow niche microenvironment. Disruptions in the interaction between HSPCs and the niche are now widely accepted as a mechanism that can lead to disease[151], [152]. The confirmation that RUNX1 may play a role in this interaction opens up a new avenue of research for this master transcription factor. Other genetic models, like mouse knockouts for Rb or RARγ, that disrupt the bone marrow microenvironment has been shown to result in myeloproliferative disorder and failure of the HSPCs to stay in the niche[153], [154]. These phenotypes are similar to those observed in *Runx1Δ/Δ*
mice. Furthermore, RUNX1 mutations have been associated with patients with myeloproliferative diseases[83], [155]. The findings suggest that further examination into the role of RUNX1 in mediating niche interactions is highly warranted.

G-CSF is currently the gold standard for inducing mobilization in human patients[156]. Other mobilization agents affecting different aspects of HSPC homing, niche interaction, or mobility have been explored over the past decade, but G-CSF is still the most clinically effective mobilizer of HSPCs. As reviewed in Pelus et al., cells mobilized with G-CSF engraft better than HSPCs coming from other sources like bone marrow or cord blood, are better tolerated by patients, and induce fewer side effects[156]. However, a small percentage of patients fail to respond to G-CSF. These include cancer patients that have undergone chemotherapy[157], [158], patients with genetic blood disorders like Fanconi’s anemia[159], and even healthy donors[160]. For this reason, other agents like CXCR4 antagonists, CXCR2 ligands[161], and IL-8[162] have been investigated for use in non-responders to G-CSF. By uncovering a novel role for RUNX1 in mediating mobilization, we can now add RUNX1 and/or its target genes for further exploration as therapies for inducing HSPC mobilization.
3.4. Materials and Methods

3.4.1. Mice

All experimental protocols were approved by the UCSD Institutional Animal Care and Use Committee and all animals were housed at UCSD. \textit{Runx1}\textsuperscript{floxed/floxed} and \textit{Mx1-Cre} mice were kindly provided by Dr. Nancy Speck[54]. For induction of Cre excision, 6-10 week old mice were injected every other day for a total of 3 or 4 injections with polyinosinic:polycytidylic acid (Sigma, St. Louis, USA) based on the following formula: mouse weight (g) × 10 + 50 = µl of 1 mg/ml polyIC[58]. Mice were analyzed 4-12 weeks after the injection regimen. For G-CSF (Neupogen from Amgen, Thousand Oaks, USA) treatment, mice were weighed and injected once daily for five days subcutaneously at a dose of 250 µg per kg body weight. For AMD3100 (Sigma, St. Louis, USA) treatment, mice were weighed and injected once subcutaneously at a dose of 5 mg per kg body weight. For the combination treatment, mice were treated with G-CSF for four days followed by AMD3100 treatment on the fifth day using the doses described above.

3.4.2. Retroviral transduction and bone marrow transplantation

Procedure was performed as previously described[1]. Briefly, 293T cells were transfected with 5 µg of Ecopac and 5 µg of MSCV-IRES-EGFP (MigR1) or MigR1-RUNX1(41-214) constructs to produce retroviral particles. Retroviral
supernatant was collected two days after transfection. Fetal liver cells were collected from embryonic day 14.5 to 16.5 embryos and resuspended in retroviral supernatant with polybrene added to a concentration of 4 μg/ml. Cells were then placed in a centrifuge for spin infection at 3000 rpm at 32 degrees for 3 hours. The procedure was repeated the following day. The next day, transduced cells were washed with PBS and resuspended at a concentration of 5x10⁶ cells/mL and 1x10⁶ cells were injected per mouse. Transplanted mice were monitored regularly and peripheral blood was collected monthly to assess engraftment efficiency and blood parameters.

3.4.3. Colony forming unit and replating assays

Base methylcellulose was supplemented with fetal bovine serum and BIT (all from Stemcell Technologies, Vancouver, Canada). In addition, rmSCF, rmIL-1, and rhIL-6 (all from PeproTech, Rocky Hill, USA) were added. Peripheral blood was directly added to the supplemented methylcellulose and colonies were scored 7 days later. For replating assay, transduced bone marrow cells were added to the supplemented methylcellulose with puromycin. Colonies were scored every 7 days after inoculation, and 5x10⁴ cells were used for the next plating.
4. GIMAP4 mediates expansion of HSPCs due to the loss of RUNX1 function in mice

RUNX1 is a master transcription factor in hematopoiesis and in hematopoietic stem cells (HSCs). Mice null for Runx1 die during embryogenesis and never develop definitive HSCs[47], [48]. Mice conditionally knocked out for Runx1 (Runx1Δ/Δ) exhibit a variety of hematopoietic defects including abnormal megakaryopoiesis, B and T cell defects, splenomegaly, and others[53]–[55]. Perhaps the most striking phenotype is that of hematopoietic stem and progenitor cell (HSPC) expansion. The defects observed in mice are translatable to those seen in patients with diseases arising from RUNX1 mutations. Hence a more thorough understanding of the molecular mechanisms leading to these defects may uncover novel therapies for patients suffering from these disorders.

We have previously conducted differential gene expression analyses using HSPCs from wildtype and Runx1Δ/Δ mice. Gimap4 was identified as one of the most up-regulated genes in Runx1Δ/Δ HSPCs. Using a dominant-negative RUNX1 model, we demonstrate that loss of Gimap4 attenuates the ability of RUNX1 loss-of-function progenitor cells to proliferate and self-renew in vitro. Furthermore, mice depleted of both Gimap4 and Runx1 exhibit significantly less expansion of HSPCs compared to single Runx1Δ/Δ mice, confirming that up-regulation of Gimap4 plays an important role in mediating
this phenotype. The insights provided by this study establish *Gimap4* as an important regulator of RUNX1 in HSPC homeostasis.
4.1. Introduction

RUNX1 is considered a master transcription factor in hematopoiesis. Its expression is pervasive throughout most blood cells, lack of RUNX1 results in embryonic lethality, and RUNX1 mutations are found in a wide array of blood diseases and neoplasms (reviewed in Lam and Zhang[98]). We wanted to focus on RUNX1’s role as a transcription factor in order to discover novel target genes that it regulates in hematopoietic stem or progenitor cells (HSPCs). Toward this goal, we conducted differential gene expression analyses on HSPCs, defined as lineage-negative, Sca-1-positive, and c-Kit-positive (LSK), obtained from wildtype mice and mice with loss of RUNX1 function[1]. One of the highest up-regulated genes in HSPCs with loss of RUNX1 function is Gimap4 (GTPase, immunity-associated protein family member 4).

GIMAP4 is part of the GIMAP family of proteins, which are primarily expressed during T cell development and maturation. Hence, most studies on this family have focused on its roles during lymphopoiesis[163], [164]. Seven family members are present in humans while eight are present in mice. GIMAP3, GIMAP4, and GIMAP5 are the most well studied members of the family and all have attributed roles in T cell apoptosis and survival. One prevailing hypothesis is that GIMAP family members may serve as the link during T cell development when T cells with incompatible T cell receptors (TCRs) must undergo programmed cell death. B cell lymphoma 2 (BCL2)
family members contribute to this step in T cell maturation[165]. Recently, multiple GIMAP family members have been shown to interact with a variety of BCL2 proteins, suggesting that GIMAP proteins may serve as the physical connection between TCR signaling and BCL2-mediated apoptosis or survival[166]. The functions of GIMAP family members outside of T cell biology are not well studied, but one recent report suggests that they may also serve vital roles in other blood cells like HSPCs. Chen et al. characterized GIMAP5 and its role in maintaining HSPC survival by mediating and preserving BCL2 family member stability[167]. From previous differential gene expression analysis, we found that Gimap4 was one of the up-regulated genes upon loss of RUNX1 function in HSPCs. This led us to pursue its roles in HSPCs further.

GIMAP4 is capable of binding to both GDP and GTP, and has intrinsic GTPase activity[168]. Although GIMAP4 protein was found to be expressed in B, T, and NK cells, macrophages, and splenocytes, its expression patterns in HSPCs are not well understood[169]. Gimap4<sup>−/−</sup> mice have been generated, but have a generally normal phenotype in regards to T cell distribution, maturation, and homeostasis[169]. The major difference between wildtype and Gimap4<sup>−/−</sup> mice was a delay in the transition from early apoptosis, designated by Annexin V-positive and 7-aminoactinomycin (7-AAD)-negative staining, to late apoptosis or cell death, designated by Annexin V-positive and 7-AAD-positive staining, and suggests that one of GIMAP4’s normal functions is to enhance the kinetics of apoptosis[169]. Using in vitro fetal thymus organ
culture (THOC) assays, over-expression of GIMAP4 was also shown to lead frequencies to higher Annexin V-positive/7-AAD-negative cells[166]. Thus far, the functions of GIMAP4 in HSPCs have not been investigated and examining its functions is one of the main goals of this study.

Two models of RUNX1 loss-of-function include the conditional Runx1 knockout mouse model (Runx1Δ/Δ)[53], [54] and bone marrow transplantation of cells over-expressing a dominant-negative form of RUNX1[1]. The combination of Runx1floxed/floxed alleles and the Mx-1Cre+ transgene allows for the generation of mice with excision of Runx1 upon injection of interferon-inducing agents like polyinosinic/polycytidylic acid. Over-expression of a shortened form of RUNX1, consisting of amino acids 41 to 214, into HSCs for transplantation produces a model where endogenous RUNX proteins are negatively affected. Both models interestingly lead to expansion of HSPCs and myeloid progenitors[1], [53], [54]. In this study, we sought to define the role of Gimap4 in mediating this myeloproliferative phenotype. Lack of Gimap4 indeed results in reduced expansion of HSPCs upon loss of RUNX1 as demonstrated by both in vitro and in vivo models. In addition, GIMAP4 regulates apoptosis in a cell-specific manner. Finally, we uncovered a novel finding that Gimap4−/− mice have an increased frequency of common lymphoid progenitors (CLPs).
4.2. Results

4.2.1. *Gimap4* is up-regulated in the absence of RUNX1 function.

*GIMAP4* is known to be expressed in the hematopoietic system, but its expression pattern in HSPCs is not well studied. Cells were isolated from various stem cell populations including LSK, CLP, common myeloid progenitor (CMP), megakaryocyte-erythrocyte progenitor (MEP) and granulocyte-macrophage progenitor (GMP) cells (Figure 4.1A). Cells from the spleen were used as a reference for differentiated blood cells. The CLP population displayed the highest levels of *Gimap4* among the HSPC populations while the CMP population displayed the lowest levels. The LSK population exhibited relatively intermediate but still substantial levels of *Gimap4* compared to the rest of the populations assessed, suggesting that *Gimap4* may have important functions in these cells.

Upon further analysis of previously reported gene expression experiments comparing LSK cells from wildtype, *Runx1Δ/Δ*, and RUNX1SF mice[1], *Gimap4* was found to be up-regulated in both cells with loss of RUNX1 function (Figure 4.1B). This up-regulation was validated by RT-qPCR experiments using cDNA generated from LSK cells from the indicated mice (Figure 4.1C). Short-term transduction of lineage-depleted cells with control Murine Stem Cell Virus (MSCV)-Internal Ribosomal Entry Site (IRES)-Puromycin Resistance (MIP) or MIP-RUNX1SF retrovirus also resulted in up-regulation of *Gimap4* after two days of puromycin treatment (Figure 4.1D).
Hence upon loss of RUNX1 function, either by conditional knockout or by expression of a dominant-negative form of RUNX1, Gimap4 expression levels are up-regulated.

**Figure 4.1.** Gimap4 expression is up-regulated in the absence of RUNX1 function. (A) The relative expression of Gimap4 between various HSPC and blood spleen cells is shown. (B) The relative expression of Gimap4 in wildtype, Runx1Δ/Δ and RUNX1SF HSPCs based on microarray data published in Matsuura et al10. (C) Validation of up-regulation of Gimap4 by RT-qPCR using cDNA from wildtype, Runx1Δ/Δ, and RUNX1SF HSPCs. (D) Expression of Gimap4 is up-regulated upon short-term expression of RUNX1SF in lineage-negative cells. RNA extraction and RT-qPCR were performed from at least 2-3 independent batches of mice.
4.2.2. Loss of *Gimap4* attenuates enhanced in vitro proliferation and self-renewal caused by loss of RUNX1 function.

To examine the effect of GIMAP4 on HSPC proliferation and self-renewal, we utilized the in vitro colony forming unit and serial replating assay. The numbers of colonies present after plating a fixed number of cells and the ability of those cells to generate more colonies week after week are surrogate markers for cell proliferation and self-renewal ability, respectively. Wildtype and *Gimap4*<sup>−/−</sup> cells were used for the assays and were transduced with either MIP control virus or MIP-RUNX1SF virus. In both wildtype and *Gimap4*<sup>−/−</sup> cells, MIP colonies were only able to serially replate until week 4 and had substantially lower numbers of colonies compared to cells transduced with RUNX1SF (Figure 4.2). Cells transduced with RUNX1SF, on the other hand, consistently resulted in 5 to 10 times higher numbers of colonies and indefinite replating ability (Figure 4.2). Interestingly, while *Gimap4*<sup>−/−</sup> cells transduced with RUNX1SF were also capable of forming higher numbers of colonies compared to MIP, they consistently formed fewer colonies than wildtype cells transduced with RUNX1SF. This trend was observed in all of the replicates performed and suggests that *Gimap4* contributes to the ability of RUNX1SF to induce proliferation and self-renewal in vitro.
Figure 4.2. Loss of RUNX1 function results in enhanced in vitro proliferation and self-renewal which is attenuated by loss of GIMAP4. Representative results of colony formation and replating assay from 4 independent replicates. Total bone marrow cells from WT or Gimap4−/− mice were transduced with MIP or RUNX1SF and plated in methylcellulose for 7 days to assess colony formation units. Assays were replated weekly using 5x10^4 cells from the previous plating.
4.2.3. Loss of Gimap4 results in fewer GMPs in RUNX1SF mice.

One of the most prominent phenotypes of mice transplanted with cells over-expressing RUNX1SF is an expansion of their HSPCs. Since Gimap4 was found to be up-regulated in RUNX1SF HSPCs, we sought to determine whether this change in expression contributes to the expansion phenotype. Fetal liver cells from wildtype or Gimap4<sup>−/−</sup> mice were transduced with MigR1 (MSCV-IRES-green fluorescent protein) or RUNX1SF viruses, and these cells were used for bone marrow transplantation into lethally irradiated wildtype mice. Approximately three months post-transplantation, the HSPC compartments were collected and analyzed. In agreement with previously published results[1], over-expression of RUNX1SF results in significant expansion of the LSK population compared to MigR1 mice when using wildtype donor cells (Figure 4.3A-C). Interestingly although the difference was not significant, Gimap4<sup>−/−</sup> donor cells expressing RUNX1SF displayed less expansion than wildtype cells expressing RUNX1SF.

In addition to the expansion of the LSK compartment observed in RUNX1SF mice, a myeloid differentiation bias exists which is accompanied by an increase in myeloid progenitor frequency. In particular, the GMP population is significantly expanded in RUNX1SF mice[1]. To ascertain whether up-regulation of Gimap4 contributes to this expansion, we compared the myeloid progenitor populations of mice transplanted with wildtype or Gimap4<sup>−/−</sup> cells as described above. Mice transplanted with RUNX1SF-expressing Gimap4<sup>−/−</sup>
donor cells had significantly fewer GMPs compared to mice transplanted with RUNX1SF-expressing wildtype donor cells (Figure 4.4A). This result suggests that *Gimap4* contributes to the GMP expansion observed in RUNX1SF mice. RUNX1SF expression also led to an increase of CMPs and MEPs, but the presence or absence of *Gimap4* did not affect the ability of RUNX1 to expand these populations (Figure 4.4B-C).

**Figure 4.3.** Loss of GIMAP4 possibly attenuates the ability of RUNX1SF to expand HSPCs. Percentage of hematopoietic stem cell populations analyzed by flow cytometry, including (A) LSK cells, (B) LT-HSCs, and (C) ST-HSCs are shown. (n = 5 for wildtype (WT) cells transduced with MigR1, n = 2 for Gimap4-/- (KO) cells transduced with MigR1, n = 5 for WT cells transduced with RUNX1SF, and n = 4 for KO cells transduced with RUNX1SF).
Figure 4.4. Loss of GIMAP4 results in fewer GMPs in RUNX1SF mice. Percentage of myeloid progenitor cell populations analyzed by flow cytometry, including (A) GMPs, (B) CMPs, and (C) MEPs. (n=5 for wildtype (WT) cells transduced with MigR1, n=2 for Gimap4/- (KO) cells transduced with MigR1, n=5 for WT cells transduced with RUNX1SF, and n=4 for KO cells transduced; (*) indicates p<0.05).
4.2.4. *Gimap4* contributes to the expansion of HSPCs due to the loss of RUNX1.

The *Runx1*Δ/Δ mouse model is an additional model for studying the effects of loss of RUNX1 function and demonstrates characteristics of abnormal hematopoiesis as evidenced by its thrombocytopenia, increase in myeloid progenitors, and defects in lymphocyte maturation among other features [53], [54]. These mice also prominently display a myeloproliferative syndrome as evidenced by an expansion of HSPCs upon deletion of *Runx1*. As with the RUNX1SF model, we sought to determine whether up-regulation of *Gimap4* contributes to stem cell expansion. To address this question, *Mx-1*Cre*+Runx1*Δ/Δ murine line was crossed with the *Gimap4*−/− line to eventually yield *Mx-1*Cre*+Runx1*Δ/Δ*Gimap4*−/− mice (Double KO), which lack both *Runx1* and *Gimap4*. The HSPC compartments of *Mx-1*Cre*−Runx1*f/f*Gimap4*+/+ mice (wildtype), *Mx-1*Cre*+Runx1*Δ/Δ*Gimap4*−/+ mice (*Runx1* conditional knockout or *Runx1*Δ/Δ), *Mx-1*Cre*−Runx1*f/f*Gimap4*+/+ mice (*Gimap4*−/−), and Double KO mice were collected and analyzed. In agreement with previously published reports [53], [54], *Runx1*Δ/Δ mice exhibited significant expansion of the LSK population compared to wildtype mice (Figure 4.5A, D). They also exhibit higher frequencies of long-term and short-term HSCs (Figure 4.5B-D). In all three populations, the Double KO showed significantly less expansion compared to *Runx1*Δ/Δ mice. However, Double KO mice still exhibited expansion compared to *Gimap4*−/− mice, suggesting that although up-regulation
or activation of \textit{Gimap4} expression contributes to HSPC expansion, other factors are also likely involved.

\textbf{Figure 4.5.} GIMAP4 contributes to the expansion of HSPCs due to loss of RUNX1. Percentage of hematopoietic stem cell populations analyzed by flow cytometry, including (A) LSK cells, (B) LT-HSCs, and (C) ST-HSCs are shown. Numbers for each genotype include: Wildtype (n=4), Runx1\textsuperscript{Δ/Δ} (n=5), Gimap4\textsuperscript{-/-} (n=12), and Double KO (n=11). (D) Representative flow cytometry gating of LSK and SLAM populations for each of the four genotypes of mice and their averages are shown. (*) indicates p<0.05.)
4.2.5. Loss of *Gimap4* leads to expansion of the CLP population.

Although the function of GIMAP4 is not well understood, much of the studies regarding this protein have focused on its roles in T cell biology. Interestingly, *Gimap4*<sup>−/−</sup> mice do not have any overt phenotypes in T cell development, maturation, or peripheral distributions[169]. Considering that *Gimap4* contributes at least in part toward regulating HSPC expansion, we sought to investigate whether it played similar roles in lymphoid progenitor populations like the CLPs. Interestingly, *Gimap4*<sup>−/−</sup> mice had a significant increase in CLPs compared to wildtype mice (Figure 4.6). *Runx1<sup>Δ/Δ</sup>* mice did not show any differences in CLP frequencies compared to wildtype mice, but Double KO mice also demonstrated an increase similar to that of *Gimap4*<sup>−/−</sup> mice, confirming that *Runx1* likely plays no role in regulating the CLP population (Figure 4.6).
Figure 4.6. Loss of GIMAP4 leads to expansion of the CLP population. Percentage of CLPs analyzed by flow cytometry are shown. Numbers for each genotype include: Wildtype (n=4), Runx1ΔΔ (n=5), Gimap4Δ (n=14), and Double KO (n=13). (*) indicates p<0.05.)
4.2.6. Loss of *Gimap4* affects myeloid progenitor expansion due to the loss of RUNX1.

In addition to exhibiting expansion of the HSPCs, *Runx1*Δ/Δ mice also have an increase in myeloid progenitors, especially the GMP population. To examine whether up-regulation of *Gimap4* contributes to expansion of this population, we analyzed the myeloid progenitors from the same four genotypes of mice introduced earlier. While *Runx1*Δ/Δ mice demonstrate increases in GMPs and CMPs compared to wildtype mice, in Double KO mice these increases are no longer significant (Figure 4.7A-C). These results suggest that *Gimap4* may also contribute to the expansion of these populations due to the loss of RUNX1. Moreover, *Gimap4*Δ/Δ mice show significantly lower frequencies of CMPs and a trend toward lower GMPs and MEPs compared to wildtype mice, suggesting that lack of *Gimap4* lead to some loss of these progenitor populations (Figure 4.7A-C).
Figure 4.7. Loss of Gimap4 affects myeloid progenitor expansion due to the loss of RUNX1. Percentage of myeloid progenitor populations analyzed by flow cytometry are shown, including (A) GMPs, (B) CMPs, and (C) MEPs. For myeloid progenitor staining, numbers for each genotype include: Wildtype (n=2), Runx1Δ/Δ (n=2), Gimap4-/- (n=14), and Double KO (n=13). (*) indicates p<0.05.
4.2.7. Modulation of GIMAP4 induces apoptosis in a tissue/cell type specific manner.

Previous studies on the functions of GIMAP4 have so far suggested that it is an inducer of apoptosis or cellular arrest. For example, over-expression of GIMAP4 induces apoptosis in developing thymocytes in THOC assays[166] and leads to cell cycle arrest in Baf3 cells upon cytokine withdrawal[168]. Reports on Gimap4\textsuperscript{-/-} mice have also suggested that GIMAP4 accelerates T cell death[169]. Our own studies utilizing GIMAP4 over-expression have produced contrasting results depending on the cell or tissue type used. In Jurkat cells, a T cell leukemia line, over-expression of GIMAP4 results in heightened numbers of apoptotic cells, designated by Annexin V-positive and 7-AAD-negative staining (Figure 4.8A). However, in K562 cells, a BCR-ABL-positive myeloid leukemia cell line, over-expression of GIMAP4 has no significant effects on apoptosis (Figure 4.8B).

To analyze the roles of GIMAP4 in apoptosis in vivo, we also compared frequencies of Annexin V-positive and 7-AAD-negative between wildtype and Gimap4\textsuperscript{-/-} cells including the LSK, GMP, CMP, and MEP populations. In all groups analyzed, no significant differences in apoptosis was observed between the two lines of mice (Figure 4.9A-D). These results suggest that at least under steady-state conditions, loss of GIMAP4 does not induce apoptosis in these cells.
Figure 4.8. Apoptosis induction by GIMAP4 is cell type-specific. (A) Percent of Annexin V-positive/7-AAD-negative cells in MIP control (black bars) or MIP-GIMAP4 (gray bars) transduced Jurkat cells. Flow cytometry charts for each condition are given below. (B) Percent of Annexin V-positive/7-AAD-negative cells in MIP control (black bars) or MIP-GIMAP4 (gray bars) transfected K562 cells. Flow cytometry charts for each condition are given below. Chart is representative of two independent experiments.
Figure 4.9. Loss of GIMAP4 does not affect rates of apoptosis in LSK and myeloid progenitor cells. Percent of Annexin V-positive/7-AAD-negative cells are shown for (A) LSK, (B) GMP, (C) MEP, and (D) CMP populations. n=2 for each genotype.
4.3. Summary and discussion

In this study, we identify *Gimap4* as a novel RUNX1 target gene that contributes to HSPC homeostasis. *Gimap4* is up-regulated in HSPCs that are deficient in RUNX1 either by conditional knockout or by expression of a dominant-negative regulator of RUNX1. The up-regulation of *Gimap4* has functional consequences in HSPCs by contributing to their expansion, which is observed in RUNX1 loss-of-function mice. Accordingly, the loss of both RUNX1 and GIMAP4 results in significantly less HSPC expansion compared to loss of RUNX1 alone.

Exactly how GIMAP4 mediates expansion is still not entirely understood. Previous reports have so far indicated that over-expression of GIMAP4 leads to a decrease in cell survival, increase in apoptosis, or cell cycle arrest[166], [168]. Our own studies expressing GIMAP4 in Jurkat cells have confirmed that GIMAP4 acts as an inducer of apoptosis. However, these negative effects on cell growth and survival are primarily described in T lymphoid cells. Over-expression of GIMAP4 in K562 cells, a myeloid leukemia cell line, does not induce apoptosis. The induction of apoptosis caused by GIMAP4 over-expression may be explained by cell- or tissue-specific regulators that are present in T cells but that may not necessarily behave in the same manner in other cell types. In addition, factors may be present in other cell types like HSPCs that would allow GIMAP4 to be switched from an inducer of apoptosis into a negative regular of apoptosis.
The BCL2 family comes to mind when searching for factors that can affect apoptosis in opposite directions. BCL2 is the founding member of this family and has been established as a pro-survival regulator[170], [171]. Its pro-survival actions were first observed by over-expression of BCL2 in cytokine-dependent cell lines, which endows these cells with the ability to become cytokine-independent[172]. Since then, numerous other BCL2 family members have been discovered and their primary effects on apoptosis elucidated. BCL-X\textsubscript{L} is another family member that contributes to pro-survival effects, while BAX and BAK are representative members that are pro-apoptotic[170], [171]. Nitta et al. were able to show that certain GIMAP family members like GIMAP3 and GIMAP5 are capable are interacting with numerous BCL2 family members, including the four just mentioned, by using over-expression and co-immunoprecipitation (co-IP) studies in 293T cells[166]. GIMAP4, however, was only found to co-IP with BAX, which suggests that it may have some specificity for pro-apoptotic effects. Perhaps GIMAP4 primarily interacts with BAX in T cell lineages to increase rates of apoptosis, but it may inhibit BAX in other cell types like HSPCs or myeloid lineages and thereby enhance survival. GIMAP4 may also interact with other pro-survival BCL2 family members in different cellular contexts. More studies will need to be conducted in order to address these important questions.

One very striking phenotype in Gimap4\textsuperscript{-/-} mice is an expansion of the CLPs, which is present independent of Runx1 excision status. Although Gimap4\textsuperscript{-/-} mice have previously been reported to exhibit normal T cell
maturation and distribution, an increase in CLPs suggest that other lymphoid lineages may be affected. Given its functions in T cell survival and apoptosis, GIMAP4 may play similar roles in regulating CLPs. For example, lack of GIMAP4 in CLPs may decrease the amount of apoptosis and hence lead to an increase in this population in Gimap4−/− mice. Possible roles of GIMAP4 in lymphoid lineage commitment or differentiation have not been adequately explored and offer exciting new avenues for future study. In summary, we establish Gimap4 as another RUNX1 target gene that mediates HSPC homeostasis.
4.4. Materials and Methods

4.4.1. Mice

All experimental protocols were approved by the UCSD Institutional Animal Care and Use Committee and all animals were housed at UCSD. \( \text{Runx1}^{\text{floxed/floxed}} \) and \( \text{Mx1-Cre} \) mice were kindly provided by Dr. Nancy Speck[54]. \( \text{Gimap4}^{-/-} \) mice were kindly provided by Dr. Heinz Jacobs[169]. For induction of Cre excision, 6-10 week old mice were injected every other day for a total of 3 or 4 injections with polyinosinic:polycytidylic acid (Sigma, St. Louis, USA) based on the following formula: mouse weight (g) \( \times \) 10 + 50 = \( \mu \)l of 1 mg/ml polyIC[58]. Mice were analyzed 4-12 weeks after the injection regimen.

4.4.2. Retroviral transduction and bone marrow transplantation

Procedure was performed as previously described[1]. Briefly, 293T cells were transfected with 5 \( \mu \)g of Ecopac and 5 \( \mu \)g of MSCV-IRES-EGFP (MigR1) or MigR1-RUNX1SF constructs to produce retroviral particles. Retroviral supernatant was collected two days after transfection. Fetal liver cells were collected from embryonic day 14.5 to 16.5 embryos and resuspended in retroviral supernatant with polybrene added to a concentration of 4 \( \mu \)g/ml. Cells were then placed in a centrifuge for spin infection at 3000 rpm at 32 degrees for 3 hours. The procedure was repeated the following day. The next
day, transduced cells were washed with PBS and resuspended at a concentration of $5 \times 10^6$ cells/mL and $1 \times 10^6$ cells were injected per mouse. Transplanted mice were monitored regularly and peripheral blood was collected monthly to assess engraftment efficiency and blood parameters.

### 4.4.3. RT-qPCR

RNA was extracted using the TRIzol method (Life Technologies, Carlsbad, USA) or RNeasy Micro Plus kit (Qiagen, Venlo, Netherlands). cDNA was synthesized using Superscript III (Life Technologies). Primers used:

- **Gimap4 forward**: TGGTGCGGTTGTCATCCAGAA
- **Gimap4 reverse**: TCTTTCCATCCAGGTGCTCACCA
- **Gapdh forward**: GGTGCTGAGTATGTCGTGGAGTCTA
- **Gapdh reverse**: AAAGTTGTCCATGGATGACCTTGG

### 4.4.4. Colony forming unit and replating assays

Base methylcellulose was supplemented with fetal bovine serum and BIT (all from Stemcell Technologies, Vancouver, Canada). In addition, rmSCF, rmIL-1, and rhIL-6 (all from PeproTech, Rocky Hill, USA) were added. Peripheral blood was directly added to the supplemented methylcellulose and colonies were scored 7 days later. For replating assay, transduced bone marrow cells were added to the supplemented methylcellulose with puromycin. Colonies were scored every 7 days after inoculation, and $5 \times 10^4$ cells were
used for the next plating.

### 4.4.5. Flow cytometry

Cells were collected from mice, treated in ammonium-chloride-potassium buffer, and stained. The following antibodies were used: PerCP-Cy5.5 or PE-Cy5-conjugated lineage antibodies (CD3, CD4, CD8a, B220, CD19, Gr-1, CD11b, and Ter119), APC or PE-Cy5-conjugated Sca-1, PE-Cy7 or APC-conjugated c-Kit, PE-conjugated CD48, biotin-conjugated CD150 with streptavidin-conjugated APC-Cy7, PE-conjugated FcγR, FITC-conjugated CD34, and PerCP-Cy5.5 or PE-conjugated IL7Rα (all antibodies from eBioscience, San Diego, USA). For apoptosis studies, APC-conjugated Annexin V and 7-AAD (BD Pharmingen) were used. After staining, cells were analyzed on a BD FACS Canto flow cytometer.
5. Conclusions and future studies

The first member of the RUNX family of transcription factors was discovered in 1980 in Drosophila as a critical regulator of segmentation[3]. Roughly ten years later, RUNX1 was discovered to be a constituent of one of the most prevalent chromosomal translocation-related fusion proteins in human leukemia[8]. Since then, various studies have elucidated other roles of RUNX1 in embryonic development, transcriptional regulation, and human disease. More recently, RUNX1 has even been associated in diseases outside of the hematopoietic system like in colon[173] and breast cancer[174]. These findings have inspired additional forays into ascertaining the roles of RUNX1 in the context of epithelial biology and the stem cells in these tissues[175], [176]. Overall, these studies implicate the importance of RUNX1 in a large variety of tissues and biological mechanisms. Hence these studies will not only reveal insights into hematopoiesis and HSPCs, but the whole field of biomedical research.

Transcription factors are powerful proteins, and in certain combinations can even induce pluripotency in terminally differentiated cells[138]. In 2012, a Nobel Prize was awarded to Shinya Yamanaka for the discovery that expressing the combination of Oct4, Sox2, c-Myc, and Klf4 transcription factors were capable of reprogramming mouse fibroblasts into induced pluripotent stem cells. All cellular mechanisms depend on an optimal level tissue-specific gene expression in order to maintain proper cellular
homeostasis. Transcription factors are the primary workhorses for controlling
the expression of the genes vital to these mechanisms. Hence, cellular
dysfunction and ultimately disease may arise as a result from disruptions to
transcription factors.

As a transcription factor, RUNX1 exerts its action by regulating
hundreds, if not thousands, of target genes. In addition, these targets may
differ depending on the cellular context and where the cell currently resides on
the hematopoietic lineage tree. We decided to focus on RUNX1 transcriptional
targets in hematopoietic stem and progenitor cells (HSPCs) since this
population serves such important roles in sustaining and supporting the blood
system. These cells are also of highly beneficial therapeutic usage as
exemplified by hematopoietic stem cell transplantation (HSCT), which is still
considered one of the gold standards in curative therapies for a variety of
blood diseases and malignancies[177], [178]. Obtaining these cells is often a
huge hurdle toward utilizing HSCT. Discovering new methods to obtain or
expand HSPCs has been one of the main reasons for the enormous interest in
studying stem cell and HSPC biology. In addition, HSPCs often serve as the
cellular origin of leukemia stem cells (LSCs)[179], [180]. A better
understanding of what stimuli may convert HSPCs into LSCs may lead to
novel therapies for malignancies associated with LSCs.

RUNX1 is a master regulator in hematopoiesis, so its own disruption
can lead to a variety of blood-related disorders and neoplasms. Thus
investigating the functions of RUNX1 and its target genes will offer insight into
fundamental mechanisms of HSPCs as well as disorders caused by RUNX1 mutations. Although Runx1 null mice exhibit embryonic lethality[47], [48], Runx1 conditional knockout (Runx1$^{Δ/Δ}$) mice serve as a representative model for RUNX1-related diseases like familial platelet disease, myelodysplastic syndrome (MDS), and myeloproliferative disease (MPD)[53], [54]. These mice have notable defects in HSPCs and in various blood lineages. We sought to tackle the question of what target genes are regulated by RUNX1 in HSPCs and what processes these genes may modulate. Toward this goal, we conducted a combination of differential gene expression analyses using wildtype and RUNX1 loss-of-function HSCPs, and RUNX1 chromatin-immunoprecipitation followed by high-throughput sequencing. The result was a high potential list of RUNX1 target genes.

High mobility group AT-hook 2 (Hmga2) was the first gene that we focused on given its well established roles as an oncogene and inducer of proliferation[102]. Hmga2 is up-regulated in RUNX1 loss-of-function and was validated to be a RUNX1 target gene. Furthermore, Hmga2 expression contributes to myeloid progenitor expansion observed in the context of RUNX1 loss-of-function. In addition to RUNX1, various members of the let-7 family of micro RNAs (miRNAs) regulate Hmga2 expression by binding to its 3’ untranslated region[130], [131]. An interesting study would be to compare the expression of let-7 family members or HMGA2 in MDS or MPD patients with or without mutations in RUNX1. Besides the known mechanism of let-7 family members regulating HMGA2, a recent study confirmed the ability of full length
HMGA2 transcript to act as a competing endogenous RNA of the let-7 family in lung cancer[181]. This mechanism has not yet been tested in the hematopoietic system or in HSPCs and serves as yet another interesting study to investigate how RUNX1 may regulate miRNAs.

The whole list of RUNX1 target genes contains a very diverse group of genes, which have been implicated in a variety of cellular processes. After performing pathway analyses on these genes, we ascertained that RUNX1 has a critical role in mediating the interaction between HSPCs and the bone marrow stromal niche. Runx1Δ/Δ mice exhibit slight mobilization of HSPCs under steady-state conditions[57]. For example, more HSPCs are found in the spleens and peripheral blood of Runx1Δ/Δ mice. In order to examine this niche defect further, we utilized granulocyte-colony stimulating factor (G-CSF)-induced mobilization as an assay to further exacerbate the defect. By doing so, we observed and established a novel phenotype caused by the loss of RUNX1 function. Loss of RUNX1 leads to a heightened response to G-CSF and other mobilization agents. The role of RUNX1 as a transcription factor suggests that its target genes play an important part in regulating this mechanism. Elucidating exactly which target genes, or which combination of them, are important and which cellular mechanisms they regulate would be a highly interesting future study.

Finally, we focused on Gimap4 which is also known GTPase, immunity-associated protein family member 4. In contrast to Hmga2, Gimap4 has not been well characterized. In fact, most studies to date have focused on its roles
in T cell development and survival. However, *Gimap4* was one of the most highly up-regulated genes in our differential gene expression analyses, and *Gimap5*, which is another member of the Gimap family of proteins, was recently described to have critical roles in HSPC survival. Upon further analyses of *Gimap4*, we discovered that it indeed has vital roles in HSPC proliferation and self-renewal. Further studies will need to be conducted to ascertain how the cellular context affects GIMAP4 function. For example, GIMAP4 appears to have contrasting roles in mediating apoptosis depending on whether it is expressed in the T cell lineage or stem/myeloid cell lineages. Another possibility may be that the levels of GIMAP4 expression determine whether apoptosis is induced or inhibited. Nonetheless, GIMAP4 presents a novel entry into the role of how GTPases can regulate HSPC biology.

Characterization of RUNX1 and its target genes in hematopoiesis and HSPCs will undoubtedly unveil novel insights that may be employed to develop for a variety of therapeutic usages. These insights will benefit patients suffering from RUNX1-related diseases and malignancies. Because RUNX1 serves as such an important transcription factor, basic transcriptional mechanisms that apply to all transcription factors may also be elucidated by the continued study of RUNX1. Our body of work has contributed to the RUNX1 field by characterizing new target genes and biological mechanisms that RUNX1 regulates. In time, we hope that these findings and other investigations focusing on RUNX1 will ultimately lead to real cures for these diseases.
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