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Deciphering the Role of Gfi1b Gene Target Rgs18 in Erythro-megakaryocytic Lineage Divergence

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Deciphering the role of Gfi1b gene target Rgs18 in erythro-megakaryocytic lineage divergence.

By

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A dissertation submitted to the Graduate Faculty in Biology (Molecular Cellular and Developmental biology subdivision) in partial fulfillment of the requirements for the degree of doctor of philosophy

The City University of New York

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This manuscript has been read and accepted for the Graduate Faculty in Biology in satisfaction of the dissertation requirement for the degree of Doctor of Philosophy.

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Abstract

Deciphering the role of Gfi1b gene target Rgs18 in erythro-megakaryocytic lineage divergence.

By

Ananya Sengupta

Adviser: Dr. Shireen Saleque

The molecular programs that govern the specification of erythroid and megakaryocytic lineages remain incompletely defined. Gene targeting experiments have shown the transcriptional repressor Gfi (Growth factor independence) 1b to be essential for the generation of both erythroid and megakaryocyte cells. Transcriptional repression of Gfi1b target genes is mediated mainly by the cofactors LSD (lysine demethylase) 1 and CoREST/Rcor1 (REST corepressor 1) or other Rcor factors. To understand the mechanism of Gfi1b action, its target genes were identified by chromatin immunoprecipitation (ChIP on Chip) screens. In this study we present the role of Rgs18 (Regulator of G protein signaling 18), a GTPase activating protein (GAP) and a transcriptional target of Gfi1b, in mediating erythro-megakaryocytic lineage specification in murine and human contexts.

Following identification of Rgs18 as a potential Gfi1b and LSD1 target, its regulation by these factors was ascertained in erythro-megakaryocytic cells. Subsequently, the role of Rgs18 in
erythro-megakaryocyte differentiation was interrogated by cDNA and shRNA mediated manipulation of expression in primary hematopoietic progenitors and cell lines. To determine the role of Rgs18 in vivo rgs18/-/ mice have been generated and their phenotypes will be analyzed shortly. In parallel, to trace the underlying mechanistic alterations responsible for the phenotypes obtained by the above manipulations, the status of two branches of the MAPK (mitogen activated protein kinase) pathway and gene expression patterns of the mutually antagonistic transcription factors Fli1 (Friend leukemia integration [site] 1/ Klf1 (Kruppel like factor 1) were determined in Rgs18 manipulated cells. Also Rgs18 interacting proteins were identified in megakaryocytic cells. Rgs18 expression was found to be low in immature megakaryoblasts in keeping with strong Gfi1b and LSD1 expression, but was reciprocally upregulated in mature megakaryocytes following declining Gfi1b and LSD1 levels in cells and on the rgs18 promoter. In contrast, expression of Gfi1b was strong in immature erythroid cells and increased further in mature cells. Manipulation of Rgs18 expression in murine hematopoietic progenitors and a multi-potential human hematopoietic cell line produced opposite outcomes in the two lineages, with expression augmenting megakaryocytic, and potently suppressing erythroid differentiation and vice versa. These phenotypes resulted from differential impact of Rgs18 expression on the p38 and ERK branches of MAPK signaling in the erythroid and megakaryocytic lineages. Repercussions of these signaling changes impacted relative expression of the mutually antagonistic transcription factors Fli1 and Klf1 and were compensated by ectopic Fli1 expression demonstrating activity of this transcription factor downstream of Rgs18. These results identify Rgs18 as a critical downstream effector of Gfi1b and an upstream regulator of MAPK signaling and Klf1/Fli1 gene expression. Sustained Gfi1b expression during erythroid differentiation represses Rgs18 and limits megakaryocytic gene expression in these cells. However
during the progress of megakaryocytic differentiation, declining Gfi1b levels result in robust expression of Rgs18 and lineage progression. Preliminary analysis of Rgs18 interactors in megakaryocytes indicates that Rgs18 likely modulates the MAPK pathway by impacting Gai, cAMP, Ras signaling and certain cytoskeletal proteins. These results will be further extended and confirmed by phenotypic analysis of rgs18-/- mice, and by characterization of novel Rgs18 interacting proteins.
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Abbreviations

AGM- Aorta gonad mesonephros.

BFU- Burst forming unit.

Cas9- CRISPR associated protein 9.

CFU- Colony forming unit.

ChIP- Chromatin immunoprecipitation.

CoREST- Corepressor of repressor element-1 silencing transcription factor.

CRISPR- Clustered regularly interspersed short palindromic repeats.

DMSO- Dimethyl sulfoxide.

EB3- Erythrocyte band 3.

EPO- Erythropoietin.

ERK- Extracellular signal regulated kinase.

EKLF- Erythroid Kruppel like factor.

FACS- Fluorescence-activated cell sorting.

FBS- Fetal bovine serum.

FLC- Fetal liver cell.

Fli1- Friend leukemia virus integration 1.

GAP- GTPase activating protein.

GDP- Guanosine diphosphate.
Gfi1b- Growth factor independence 1b.

GPCR- G protein coupled receptor.

GPIIb- Glycoprotein IIb (also known as integrin αIIb).

GTP- Guanosine triphosphate.

GTPase- Guanosine triphosphate hydrolases.

GYPA- Glycophorin A.

HDAC- Histone deacetylase.

HPRT- Hypoxanthine-guanine phosphoribosyl transferase.

HSC- Hematopoietic stem cell.

IL3- Interleukin 3.

Klf1- Kruppel like factor 1.

LSD1- Lysine specific demethylase 1.

MAPK- Mitogen activated protein kinase.

MEL- Murine erythroid leukemia.

MEP- Megakaryocyte erythroid progenitor.

PBS- Phosphate buffered saline.

PF4- Platelet factor 4.

qPCR- Quantitative real time PCR.
RBC- Red blood cell.

Rgs- Regulator of G protein signaling.

SCF- Stem cell factor.

shRNA- short hairpin RNA.

T7E1- T7 endonuclease.

TPO- Thrombopoietin.

vWF- Von Willebrand factor
Chapter 1: Introduction

1.1. Hematopoiesis.

Hematopoiesis is a process by which mature blood cells capable of performing specific and specialized functions are produced from multipotent stem and progenitor cells as a result of coordinated cell specific gene expression [1-3]. Hematopoiesis starts during embryogenesis in the “blood islands” (clusters of erythroblasts surrounded by endothelial cells) of the extra-embryonic yolk sac shortly after the onset of gastrulation. The first wave of hematopoiesis known as primitive hematopoiesis occurs at embryonic day (E) 7-8.5 in mice. The primitive wave of hematopoiesis was initially defined as being uni-lineage comprising of primitive erythroid cells originating from hemangioblast precursors (multipotent precursor cells that can differentiate into both hematopoietic or endothelial cells) and distinguished from subsequent fetal and adult erythroid cells by their large size and embryonic globin gene expression [4]. These primitive erythroid cells function as oxygen delivery vehicles for the rapidly growing embryo. These cells also play a crucial role in vascular remodeling during development and hence are considered critical for the transition of an embryo to a fetus in the developing mammal [4]. Recent reports of the presence of megakaryocyte progenitors in the yolk sac around E7.5 along with the primitive erythroid cells indicates that the first wave of hematopoiesis is bilineage in nature [5-7]. As primitive hematopoiesis is not sufficient for embryonic survival, the second wave of hematopoiesis starts around E10.5-12.5 in the aorta gonad mesonephros (AGM) region of the embryo with the emergence of hematopoietic stem cells (HSCs) [3, 5]. The transition from yolk sac to HSC derived hematopoiesis establishes long term hematopoiesis where HSCs with their self-renewal capacity
and their potential to give rise to lineage specific progenitors then migrate to and populate the fetal liver around E11 [8] [6].

The murine fetal liver functions as the major hematopoietic organ for the remainder of fetal development with its hematopoietic function reaching a peak on E13-14 and ceasing during postnatal day 2-4 [9]. The function of the fetal liver as a major hematopoietic organ is dependent on its specific microenvironment. This microenvironment is created by various epithelial and mesenchymal cell types which produce cytokines, chemo-attractants and extracellular matrix components that interact directly with hematopoietic cells to maintain the hematopoietic potential of the developing fetal liver [9]. After birth expanding HSCs in the fetal liver eventually move to the bone marrow establishing the last key event of hematopoietic migration [2, 10] (Figure 1). Long Term-HSCs possess self-renewal capability while short Term-HSCs produce multi-lineage progenitor cells which retain limited proliferative potential and a greater capacity to differentiate into various hematopoietic lineages (Figure 2).

1.2. Emergence and differentiation of Megakaryocyte Erythroid Progenitors (MEPs).

Bipotential megakaryocyte-erythroid progenitors (primitive MEPs) emerge during early gastrulation around E7.5-8 along with unipotential primitive erythroid and megakaryocyte progenitors (Meg-Colony Forming Cells [CFCs]) from the hemangioblast precursors present within the yolk sac of the murine embryo [5]. Around E10.5-11 during the initial stage of development of the fetal liver as the primary hematopoietic organ, these megakaryocyte progenitors and primitive MEPs migrate and seed the nascent the fetal liver where definitive hematopoiesis begins. However, these primitive cells are soon replaced by definitive MEPs arising from AGM derived HSCs migrating to the fetal liver [5, 6]. Tracking the fate of single HSCs has
shown that Megakaryocyte erythroid (MegE) cells or MEPs in turn originate from common myeloid progenitors during adult hematopoiesis [11, 12] [13] (Figure 2).

MEPs were initially identified from hematopoietic colonies composed of both erythroid and megakaryocyte cells in vitro that were obtained from single cells and subsequently from isolation of these progenitors from adult bone marrow, spleen, fetal liver and yolk sacs of mice [5, 14]. Following commitment of MEPs towards the erythroid lineage, the cell progresses to form a mature red blood cell (RBC) after proceeding through three major stages: The erythroid progenitor stages (BFU-E, CFU-E, EryP-CFC), Erythroid precursor stages (pro-erythroblast, basophilic erythroblast, polychromatic, orthochromatic, reticulocyte), and the mature RBC stage [15, 16]. Similarly once committed to the megakaryocyte lineage, the cell proceeds through progenitor stages (BFU-Mk, CFU-MK) then differentiates into immature megakaryocytes (promegakaryoblast, megakaryoblast) and to mature megakaryocytes which are large polyploid cells capable of producing platelets [17].

Apart from arising from a common progenitor (MEP), both erythroid cells and megakaryocytes initially share common multifactorial transcriptional complexes and their protein partners, but these associations become significantly different as differentiation proceeds along each lineage [18]. These transcriptional complexes regulate the lineage commitment and terminal maturation of the two lineages by coordinating the chromatin organization of lineage specific genes and priming their expression [19]. Although the origin and differentiation of MEPs into erythroid and megakaryocytic cells is well known, the mechanisms that lead to their divergence from a common progenitor are still incompletely defined [19, 20].
Thus the process of adult hematopoiesis (Figure 2) is sustained throughout an organism’s life time by a critical balance of two processes (stochastic and instructive): 1. A tight control of lineage specific transcriptional programs (stochastic) in each cell type that produce and maintain stem cells or allow lineage commitment and 2. By growth factor (instructive) stimulated signaling pathways which also promote survival, proliferation and/or maturation of progenitors [10, 21] [22, 23].

1.3. Transcription factors in erythro-megakaryocytic development.

To understand the regulation of hematopoiesis by transcription factors in mammals, researchers have mostly relied on gene deletion (knockout) and retroviral infection/over-expression experiments [24-27]. Diverse results from various such experiments have demonstrated the importance of transcription factors as essential regulators of the production, maintenance, lineage commitment and specification of HSCs, progenitors like MEPs and of their differentiated progeny [3, 28-30]. During the primitive hematopoiesis the transcription factor SCL/tal1 along with LMO2 specifies the initiation of a hematopoietic program in hemangioblast. Similarly, Runx1 is necessary for the generation of HSCs during definitive hematopoiesis in the AGM. Apart from the production of HSCs, transcription factors like Runx1, Scl/tal-1, and GATA2 are also responsible for cell fate determination and maintenance of HSCs [3, 30].

As hematopoiesis progresses some transcription factors act as key lineage determinants [3, 30] thus restricting the fate of multipotential cells by promoting one and/or inhibiting alternative programs [3]. Dual expression of GATA1 and PU.1 differentiates HSCs to Common myeloid precursors (CMP). The subsequent commitment of CMPs to the MEP fate is regulated by the predominance of GATA1 over PU.1 expression (Figure 3). Therefore, the MEP versus (Granulocyte macrophage progenitor [GMP]) fate decision from CMPs is controlled by the cross-antagonism between them. Overexpression experiments have shown that these two transcription
factors physically interact and inhibits each other’s functions. Ectopic expression of GATA1 can reprogram myeloid cells into MEPs while that of PU.1 directs CMPs towards the GMP fate [31-33].

Similar to the GATA1/PU.1 antagonism, functional antagonism between the transcription factors Klf1 (Kruppel like factor1) and Fli1 (Friend leukemia integration (site) 1) is known to be responsible for the divergence of erythroid versus megakaryocytic cell fates from a bipotent MEP along with an array of other transcription factors (GATA1, FOG1, GATA2, SCL) and micro RNAs (like miR-150 and miR-451) [34]. Fli1 with its ETS (E-twenty six) repression domain is an essential pro-megakaryopoietic transcription factor which represses Klf1, a pro-erythropoietic zinc finger transcription factor in an MEP cell inclined to a megakaryocytic fate [35]. Similarly, Klf1 inhibits Fli1 expression by binding and repressing the fli1 promoter in a MEP cell leaning toward an erythroid fate. The mutual antagonism between these two transcription factors controls the differentiation of erythro-megakaryocytic cells from MEPs [3, 34, 35] (Figure 4). A recent study has further shown that erythroid or megakaryocyte lineage progression from a MEP cell depends not only on the anti-correlation between Klf1 and Fli1 levels as mentioned earlier but also the cross-inhibition of the receptors for erythropoietin and thrombopoietin that specify the erythroid and megakaryocytic lineages respectively, by these factors [23] (Figure 5).

Apart from transcription factors, experimental studies have revealed chromatin modifiers, cell cycle regulators, and multiple signaling molecules/cascades to be essential during the process of hematopoietic lineage commitment [10, 13, 36]. Vincenta et al. 2012 in their model of erythro-megakaryocyte development have shown the stage specific expression and requirement of important transcriptional and signaling regulators during erythro-megakaryopoiesis [37] (Figure 6).
1.4. Growth factor independence (Gfi) 1b.

Gfi1b is a transcriptional repressor reported to bind to the DNA sequence (TAAATCAC(A/T)GCA) on its gene target via three of its six carboxy-terminal C2-H2 type zinc fingers [38, 39]. It belongs to the Gfi1 family of transcription factors which comprises of two small nuclear proteins: Gfi1 (55-kDa) and Gfi1b (37-kDa). Apart from harboring the six highly conserved C-terminal zinc fingers, vertebrate Gfi1 family members also possess well conserved 20 amino acid SNAG (Snail/Gfi) repressor domains at their N-termini and non-conserved intermediate domains between the SNAG domain and the zinc fingers [40-42] (Figure 7).

Gfi1 and Gfi1b auto-regulate and cross-regulate each other’s expression in a context dependent manner [42]. Both genes are expressed in hematopoietic cells of which Gfi1b is highly expressed in erythro-megakaryocyte lineages [43] [44] [45]. Gfi1b is expressed in megakaryocyte erythroid progenitors (MEPs) and throughout erythroid lineage development with highest expression reported at the erythroblast stage [42] and differential regulation of Gfi1b expression was reported during megakaryocyte maturation in Gfi1b:GFP knock-in mutant mice with high expression of Gfi1b in the progenitors and less in mature megakaryocytes [42]. Due to reports of differential expression pattern of Gfi1b during megakaryopoiesis, the function and expression pattern of Gfi1b during megakaryocyte differentiation remains to be further elucidated.

Accordingly disruption of the gfi1b gene in mice is embryonic lethal at ~E15 as gfi1b-/- embryos display a total failure of definitive (adult type) erythro-megakaryopoiesis and death during the transition from primitive to definitive hematopoiesis. The lethality results from delayed maturation of primitive erythrocytes and the complete absence of definitive enucleated erythrocytes [40] (Figure 8). gfi1b-/- embryos also exhibit abnormal and arrested primitive (yolk sac) hematopoiesis. The presumptive megakaryocytic precursors present in gfi1b-/- embryos proliferate
in the presence of thrombopoetin, but fails to mature further (Figure 9). A recent report with conditional gfi1b knockout mice has shown lineage restricted requirement of Gfi1b expression in erythro-megakaryocyte development in adult bone marrow cells. The conditional mouse strain was generated by flanking exons 2-5 of gfi1b which encode the N terminal portion of the protein (from the initiator ATG to zinc finger 2) with loxP sites. For conditional disruption, a floxed gfi1b allele was combined with germline excised gfi1b knockout allele, so that the single excision event results in bi-allelic absence of intact gfi1b. Excision was mediated by the Mx-Cre transgene which excised floxed alleles in the bone marrow upon injection of polyinosinic: polycytidylic acid or by TetO-Cre which is the doxycycline-inducible Cre transgene. By either of these systems, conditional disruption of gfi1b resulted in hematopoietic abnormalities and lethality within less than 3 weeks. Disruption of gfi1b lead to the arrest of the erythroid program as early as the bipotential MEP stage [46]. In case of megakaryocytes, MEPs progress to the megakaryocyte lineage in conditional gfi1b mutant mice but then are arrested at the pro-megakaryocyte stage following nuclear polyploidization, but prior to cytoplasmic maturation. Thus precluding the production of platelets altogether [46].

Conversely, Gfi1b overexpression has been linked with erythro-megakaryocytic and other malignancies. A three-fold increase in Gfi1b expression has been reported in patients with erythroleukemia and megakaryocytic leukemia [47]. A novel single nucleotide insertion in gfi1b gene has been reported in patients with a unique inherited bleeding disorder causing a frame shift mutation that disrupts the integrity of the fifth zinc finger and eliminates the coding sequence of the sixth zinc finger domain. Thus the mutated Gfi1b protein is unable to bind DNA and losses its transcriptional repression capacity. The patients affected by this disorder exhibit red blood cells of unequal size, large platelets and moderate thrombocytopenia [48]. A recent study of the hereditary
condition, Gray platelet syndrome has revealed an autosomal dominant nonsense mutation that introduces a premature stop codon at amino acid 287 of gfi1b in a subset of the patients. This mutation results in the deletion of forty four carboxy-terminal amino acids corresponding to zinc finger 5 leading to a truncated Gfi1b protein. Since zinc finger 5 is known to be required for DNA binding, the inability of the mutant protein to bind to DNA is likely the primary cause of the inherent bleeding disorder. The overexpression of mutant Gfi1b in the mouse bone marrow resulted in severe dysplastic megakaryocytes and platelets showing that Gray platelet syndrome is caused by dominant negative inhibition of non-mutant protein by the mutant Gfi1b [49]. The disease is characterized by the defective production of alpha granules in platelets leading to their grey color and larger than normal size and low count. This is due to failure of megakaryocytes to efficiently channelize endogenous proteins to the granules. The patients with gray platelets thus suffer from bleeding tendencies with variable severity [49].

Gfi1b is an epigenetic regulator that modifies chromatin structure on the basis of its biochemical function as a transcriptional repressor and recruiter of histone modifiers. Gfi1b recruits the chromatin modifying proteins LSD1 (Lysine specific demethylase1) and CoREST/Rcor1 (REST corepressor 1), histone methyl transferases such as G9A and histone deacetylase (HDACs) to its target gene promoters. Together Gfi1b and its cofactors reversibly inhibit target gene expression including its own promoter by catalyzing serial histone modifications which lead to gradual gene silencing [50] (Figure 10). Recruitment of HDACs and LSD1 to its target DNA results in deacetylation of H3-K9 residues and demethylation of histone (H) 3-lysine (K) 4 residues respectively. Further recruitment of G9A by Gfi1b facilitates methylation of H3-K9 residues [50-52].
1.5. Gfi1b gene targets.

In order to fully understand the mechanism of action of Gfi1b, knowledge of its target genes is necessary. Previous studies have demonstrated various potential targets (p21, SOCS1&3, CD3γ, lymphotoxin-A and Gfi1b itself) [39, 53, 54] in hematopoietic cells. However these targets cannot completely account for its function and mechanism of action in specifying erythro-megakaryocyte development. Hence, to understand the transcriptional repression mechanism of Gfi1b and its associated proteins (LSD1 and CoREST), additional gene targets were identified by chromatin immunoprecipitation screens (CHIP on chip) for all three proteins (Figure 11).

These screens identified 653 common target genes of the Gfi1b/LSD1/CoREST complex in erythroid cells [50]. Gene ontology analysis of these Gfi1b/LSD1/CoREST transcriptional targets revealed them to belong to groups with diverse functions indicating that Gfi1b regulates multiple biological pathways and processes [50]. Subsequent expression profiling was carried out in control and LSD1 inhibited (knocked down) erythroid cells to determine the regulation of these genes by the Gfi1b transcriptional complex [50]. LSD1 knockdown cells were chosen instead of Gfi1b inhibited cells because of auto-regulation of the gfi1b promoter which prevents effective repression of this factor by small hairpin (sh) or short interfering (si) RNAs. Depletion of LSD1 from cells leads to the up-regulation of Gfi1b target gene expression including itself.

Among the prominent targets of Gfi1b identified by the ChIP screen were three family members of the Rgs (Regulators of G protein signaling) family- Rgs2, Rgs14 and Rgs18 (Figure 12). Up-regulation of these three Rgs transcripts in gfi1b/- fetal liver and LSD1 knockdown cells further confirmed them to be bona fide and likely direct targets of Gfi1b.
1.6. Regulators of G protein signaling (Rgs).

Rgs proteins are a diverse group of signaling proteins, with a conserved 120 amino acid long signature RGS domain that binds directly to Gα subunits of G proteins. Hetero-trimeric G proteins consist of a GDP-bound Gα subunit associated with a Gβγ heterodimer. The Gβγ subunit assist the coupling of Gα to the G protein coupled receptors (GPCRs) and also prevent the dissociation of GDP from Gα. GPCRs represent the largest family of receptors that receive extracellular cues from the environment and relay them into the cell to elicit appropriate intracellular responses. Upon binding of an activating ligand the GPCR undergoes conformational changes which result in the release of GDP and subsequent binding of GTP to the Gα subunit (Figure 13). This Gα-GTP association also results in the dissociation of Gβγ subunit from Gα-GTP and the activation downstream effectors like adenylate cyclases, phospholipase-C isoforms, Rho GEFs and several ion channels that in turn activate downstream signaling pathways. These downstream signaling pathways remain activated until the hydrolysis of GTP by facilitation from the Rgs proteins. After binding to the Gα subunit via their RGS domains, the Rgs proteins accelerate the hydrolysis of GTP thus returning G proteins to their Gα-GDP bound state and resulting in termination of downstream signaling [55-57].

Rgs proteins interact either directly or via intermediate scaffolding proteins like Spinophilin, and 14-3-3 with G-protein-coupled-receptors (GPCRs) resulting in desensitization of the latter by the above mentioned mechanism. This leads to termination of downstream signaling that in turn regulate cell growth, differentiation, motility and intercellular trafficking [55, 57, 58]. Recent studies have also shown the regulation of Rgs proteins by various mechanisms thus providing evidence of cross-talk between multiple GPCRs and other signaling pathways [58].
So far 37 mammalian Rgs proteins have been identified that can be categorized into 7 subfamilies based on their molecular mass, primary sequence homology and presence of additional domains. All members of the Rgs family accelerate the GTPase activity of Gα subunits with varying degrees of selectivity for Gαi, Gαq/11 and Gα12/13 proteins. To perform their canonical GAP activity all Rgs proteins are localized to the plasma membrane along with GPCRs and G proteins [59-61]. Apart from the 120 amino acid long RGS box, many Rgs proteins possess extensions of various lengths containing one or more domains with which they mediate G protein independent functions as well as interactions with non-canonical partners [58]. Thus a single Rgs protein is often regulated by various mechanisms while several Rgs proteins can be regulated within a cell by a single mechanism. Hence the role and regulation of one Rgs protein in one cell type may vary from other cell types. Some Rgs proteins (Rgs19, Rgs14, Rgs10, Rgs2 and Rgs3) are broadly expressed and found in most tissues while others have limited patterns of expression like Rgs20 which is specifically expressed in the brain and Rgs5 which is found in vascular smooth muscles [62]. Among the three Rgs members (Figure 12) obtained as Gfi1b targets from CHIP on Chip screen, Rgs18 was reported to be highly expressed in megakaryocytes and platelets [63-68]. Since Gfi1b is a major transcriptional regulator responsible for erythro-megakaryopoiesis and Rgs18 was identified as one of its target in erythroid cells, we decided to investigate the role and mechanism of action of this protein during erythroid and megakaryocyte lineage differentiation and its regulation by Gfi1b and its cofactors during the divergence and determination of these two lineages.

1.7. Regulator of G protein signaling (Rgs) 18.

Rgs18 was independently identified by two groups upon isolation of novel thrombopoietin (TPO)-inducible transcripts in primary megakaryocytes [69] and by sequencing unknown transcripts from
a LT-HSC cDNA library [65], respectively. Rgs18 is a 235 amino acid long protein that belongs to the R4 subfamily of Rgs proteins and acts as a GAP for Gα1 and Gαq subunits [65, 66, 69]. Consistent with high level expression megakaryocytes and platelets, Rgs18 exhibits is also highly expressed in predominantly hematopoietic tissues such as the fetal liver, bone marrow and spleen [65, 66, 69]. It is also expressed in stem cells (LT-HSCs and ST-HSCs) and in more differentiated progenitors such as GMPs, MEPs [65] [66, 69]. Rgs18 depletion was shown to cause thrombocytopenia in zebrafish while over-expression increased megakaryocyte differentiation of mouse HSCs [63]. Unlike its stimulatory role in megakaryocytes, Rgs18 appeared to limit activation of platelets by restricting the duration of G-protein dependent signaling.

The complex of scaffolding proteins Spinophilin (SPL) and the tyrosine phosphatase (SHP-1) modulates platelet activation by sequestering two members of the Rgs family Rgs18 and Rgs10. Before injury The SPL/Rgs/SHP1 complex helps to maintain the quiescence of circulating platelets while after injury the complex releases the Rgs proteins to limit platelet activation by inhibiting GPCR signaling. Thus Rgs proteins help to maintain optimal platelet response to vascular injury by regulating excessive platelet aggregation which can result in arterial occlusion [64].

1.8. Rgs proteins and mitogen activated protein kinase (MAPKs) signaling.

MAPKs (ERKs, JNKs and p38MAPKs) are a family of kinases connecting cell-surface receptors to changes in transcription programs. These kinases are expressed and involved in regulation of cellular functions like proliferation, differentiation, migration and apoptosis. The binding of MAPK signaling complexes to scaffold proteins determine the location and duration of MAPK activation, thus regulating their different signaling outcomes [70].
In hematopoietic cells, ERK1 and ERK2 also known as p44\textsuperscript{MAPK} and p42\textsuperscript{MAPK} respectively are activated by a variety of stimuli including growth factors, ligands for GPCRs, cytokines and other cell stresses. Upon activation, these proteins phosphorylate and regulate the activity of cytoplasmic and nuclear proteins that further control myeloid, erythroid and megakaryocytic progenitor proliferation and differentiation. Thus proper activation and duration of the ERK pathway is required for ultimate regulation of the balance between proliferation, survival and differentiation of hematopoietic cells [71] (Figure 14).

The p38 MAPK family consists of four splice variants – p38\textalpha{}, p38\textbeta{}, p38\textgamma{}, and p38\textdelta{}. Of these, deficiency of p38\textalpha{} is embryonic lethal and p38\textalpha{} deficient mice die around E16.5 due to anemia as a result of stress induced abnormal erythropoiesis. Thus p38 MAPK has been reported to be necessary for initiation of erythroid differentiation and has been reported to be activated by the erythropoietin (EPO) receptor. p38 MAPK favors erythroid differentiation by stimulating heme biosynthesis, iron uptake [72]. In contrast, in the bipotent erythro-megakaryoblastic cell line (K562) p38MAPK down-regulation accompanies megakaryocyte maturation [73].

ERK1/2 MAPK signaling inhibits erythroid differentiation as the inhibitors of ERK pathway in MEL cell line resulted in promotion of erythroid differentiation and increase in hemoglobin protein as well as \textbeta{}-globin transcript level. In case of megakaryocytes, ERK1/2 activation was reported may be essential for megakaryocyte differentiation. But Conde et al. 2010 have shown that inhibition of ERK failed to block expression of megakaryocyte markers in K562 cells when added 24 hours after stimulation with megakaryocyte differentiation inducer PMA (phorbol 12-myristate-13-acetate). Thus ERK activity is essential to initiate megakaryocyte differentiation but once cells enter the differentiation program, signaling through the pathway is not critical [73]. ERK1-2 (p44/42 MAPK) can be activated by ligands for GPCRs. The G protein and cytokine
signaling converge to activate the MAPK pathway. Since Rgs proteins limit GPCR signaling by accelerating GTP hydrolysis of G proteins, they also inhibit their downstream signal transduction processes including MAPK signaling [74].

1.9. Specific Aims.

1) Determining the regulation of Rgs18 by Gfi1b and its cofactors.
Since the Rgs18 promoter was found to be a prominent target of the Gfi1b/LSD1/Rcor1 repressor complex in erythroid cells we determined its regulation in both erythroid and megakaryocyte lineages by these factors. Expression profile of Rgs18 was monitored along with that of Gfi1b and LSD1 in wild type erythroid and megakaryocytic cells as well as in gfi1b-/- mutant fetal liver cells. These expression patterns were correlated with Gfi1b and LSD1 enrichment on rgs18 promoter sequences in different stages of erythroid and megakaryocytic differentiation.

2) Establishing the role of Rgs18 in erythropoiesis and megakaryopoiesis.
Since differential expression of Rgs18 was observed in the erythroid and megakaryocytic lineages, I next interrogated its physiological role in these cells and determined how it affected the differentiation of erythro-megakaryocytic cells as follows:

a) Examining the role of Rgs18 in murine and human cell lines and progenitors by manipulating expression.

The effect of Rgs18 on erythro-megakaryopoiesis was established by manipulating its expression in appropriate cell lines and in primary cells from mice and determining the resulting phenotypes. Rgs18 was knocked down or over-expressed by shRNAs and cDNAs respectively.

b) in vivo manipulation of Rgs18 to understand its physiological function.
Accordingly, a CRISPR-Cas9 mediated complete knockout of \textit{rgs18} was performed in mice to determine its physiological functions \textit{in vivo}.

3) Determining the mechanism of action of Rgs18.

To delineate the underlying mechanistic alterations responsible for the phenotypes ensuing from Rgs18 manipulation, I undertook the following approaches.

a) Determining downstream effectors and processes regulated by Rgs18.

To understand how Rgs18 alters MAPK signal transduction processes I investigated the activation (phosphorylation) status of p38 MAPK, ERK-MAPK proteins and expression pattern of lineage determining genes like EKLF/Klf1 and Fli1 in Rgs18 manipulated erythroid and megakaryocyte lineages.

b) Identification of proteins associated with Rgs18 in megakaryocytic cells.

To identify novel Rgs18 associated proteins in megakaryocytes, we performed affinity purification of Rgs18 protein complexes from megakaryocytes using a recombinant epitope tagged bait followed by analysis of the complex by mass spectrometry.
Chapter 2: Materials and methods.

2.1. Plasmid construction.

2.1.1. cDNA preparation and PCR amplification.

Pooled full length cDNAs were obtained from total RNA of L8057 cells using Transcriptor High Fidelity cDNA synthesis kit (Roche # 05081955001). Murine Rgs18 cDNA (Accession No. NM_022881) and Fli1 cDNA (Accession No. NM_008026) were PCR amplified using the Expand long template PCR system kit (Roche # 116818340001) and the primers listed below.

Rgs18: atgcaggtttctcttcatt and taaccaaatggcaacatctgacttacat

Rgs18-Biotag- attcccggggccaccatgatatgctcatttttttc and attccgggtaaccaatggcaacatctgacctt

Fli1- atggacgactattaaggagct and gtatgggtagctgccctagttgaag

2.1.2. Insertion into TOPO vector.

The PCR amplified products were inserted into TOPO sequencing vector (pcr4 –TOPO Cat # 45-0030; Thermo fisher Scientific) and the identity of the insert confirmed by sequence verification.

2.1.3. Sub-cloning into expression vectors.

After sequence verification the inserts were excised out of TOPO vector by digestion with specific restriction endonucleases and ligated with suitably digested pEF4/myc-His vector (Invitrogen), pCDH-MSCV™ vector (System Biosciences) and pEF1α Biotag vector. Inserted cDNAs were confirmed by sequence analysis and protein expression ascertained following transient transfection into 293T cells.
2.1.4. shRNAs.

Commercially available Rgs18 shRNAs were purchased from the Mission™ collection (Sigma Aldrich) and their sequences are listed below:

RGS18 shRNA1 (coding): ccggctcctgsgaagcagtgaaatctcgagatttcactgcttcttcaggagtttttg

RGS18shRNA2 (3’UTR): ceggagtaatgtcacatcttagtttgctcgagcaaactagatgtgtgacattaacttttttg

RGS18shRNA3 (3’UTR): cgggatcatcatcttcagaaactcggagcaggagtttttg

The cDNA and shRNAs expressing plasmids were transduced into L8057, MEL and K562 cell lines to create Rgs18 inhibited or over-expression stable lines.

2.2. Production and maintenance of stable cell lines.

2.2.1. Cell lines.

The murine hematopoietic cell lines MEL (erythroid), L8057 (megakaryoblastic) and K562 (myeloerythroid) were used for the study as indicated. Provided below is a brief description of each one.

MEL: Murine erythroleukemia (MEL) cells represent murine erythroid progenitor cells transformed by Friend’s leukemia virus and arrested at the proerythroblast stage of erythroid differentiation. MEL cells can be induced to differentiate to more mature orthochromatic erythroblast like cells with compounds like dimethyl sulfoxide (DMSO) and sodium butyrate [75].

L8057: L8057 cells are a murine megakaryoblastic cell line obtained from irradiated mice. These cells can be induced to differentiate into mature megakaryocytes with phorbol ester (12-O-tetradecanoyl phorbol-13-acetate) [76].
K562: The human multipotent hematopoietic cell line (K562 ATCC® CCL243™) [77] was derived from a chronic myeloid leukemia patient [78]. It has the potential to differentiate into erythroid, megakaryocyte and other hematopoietic lineages [79, 80]. In this study they were induced to differentiate into erythroid cells with sodium butyrate and into megakaryocytes with phorbol ester.

2.2.2. Stable Cell line production.

Stable Rgs18 overexpressing cell lines were generated by nucleofection (Amaxa cell line nucleofector kit V # VCA-1003) of Rgs18-pEF4 vector into L8057, MEL and K562 cells. Stable Rgs18 shRNA expressing (knock down) cell lines were generated by transduction with Rgs18 shRNA expressing lentiviruses (also see below).

2.2.3. Cell culture.

MEL, L8057 and K562 cells were cultured in their respective media: MEL (DMEM medium with 10% FBS), L8057 (50% IMDM, 50% DMEM and 10% FBS), K562 (IMDM with 10% FBS). Rgs18 inhibited or over-expressing stable cell lines were maintained by suitable antibiotic selections as listed below:

Rgs18-pCDH, Rgs18shRNA inhibited cell lines - 2µg/ml puromycin

Rgs18-pEF4 cell lines - 0.5 mg/ml zeocin

BirA and Rgs18 bio-tag containing cells - 0.4mg/ml G418 (BirA) and 2µg/ml puromycin (Rgs18-biotag).
2.2.4. Differentiation of MEL, L8057 and K562 cells.

To initiate erythroid cell differentiation, immature MEL (~10^5) cells were induced to differentiate with 1.5% dimethyl sulfoxide (DMSO) for 4 days. L8057 cells were induced to differentiate with 50nM 12-O-tetradecanoyl phorbol-13-acetate (TPA) for 4-5 days. The multipotent K562 cells were induced to differentiate into erythroid lineage by treatment with 2mM sodium butyrate and megakaryocyte lineage with 50nM of TPA for 4-5 days. The uninduced and induced cells were harvested as needed for protein and RNA collection or histological staining.

2.3. Primary cell collection, manipulation and culture.

2.3.1. Lenti-viral production in HEK293T cells and transduction of murine cells.

50-60% confluent HEK293T cells in 10 cm tissue culture plates were transfected with a mixture containing 500µl of BioWhittaker™UltraMEM (reduced serum media # 12-743F Lonza), 72ul of Trans IT® 293 reagent Mirus (transfection reagent # MIR2705), 12µg of shRNA or cDNA plasmid DNA, 9 µg of pPAX2 (lentiviral packaging plasmid) and 3 µg of PMD.2G plasmid (lentiviral envelope plasmid) DNA to produce shRNA or cDNA packaged lentiviruses as a delivery vehicles for transduction of primary cells or cell lines.

The following day, 293T medium was replaced with the appropriate target cell medium. Viral supernatants were collected ~48 hours post transfection and either used directly for transduction or stored at -80°C for future use.

~10^5 (total fetal liver or cell line) cells were infected with lentiviruses by treatment with 3 ml of the filtered viral supernatant, 20ng/ml IL3 (only for FLCs) and 8µg/ml polybrene in a 6 well plate and centrifuged for 2hrs at 2000rpm at 22°C and then incubated overnight at 37°C. After ~ 24 hrs
the virus and polybrene were washed off the cells and transduced cells were plated in their corresponding medium supplemented with antibiotics. The shRNAs were maintained with puromycin (2µg/ml for cell lines and 1µg/ml for FLCs) and the FLCs and cell lines transduced with cDNA constructs were maintained with the antibiotics respective to their vector backbone specific antibiotic resistant gene (pCDH: puromycin 2µg/ml for cell lines and 0.75-1µg/ml for FLCs).

2.3.2. Culture and manipulation of fetal liver cells.

Total or sorted fetal liver cells (~10⁵) from embryonic day 12.5 (e12.5) staged embryos were harvested following timed mouse matings and either cultured directly or transduced with Rgs18 cDNA, Rgs18 shRNA and /or Flil cDNA carrying lentiviruses as detailed above. Cells were differentiated into erythroid and megakaryocyte lineages using cytokines (10ng/ml IL3 [#403-ML R&D system], 20ng/ml thrombopoietin [# 488-TO R&D system] and 10ng/ml stem cell factor [SCF] [#403-ML] for megakaryocytes, 2Units/ml erythropoietin [#287-TC], 25ng/ml SCF for erythroid differentiation) either in liquid culture or in semi-solid methylcellulose medium (# M3234; Stem cell Technologies™). The cells were selected for lentivirus retention with 0.5-1µg/ml of puromycin and harvested after 4-5 days for erythroid and 5-6 days for megakaryocytic cultures for various assays and/or megakaryocytic colonies were counted in methylcellulose cultures.

2.4. Histological staining.

For histochemical analysis, 100 µl of cell suspension (~10⁵ cells) were cyto-centrifuged at 400 rpm for 4 minutes onto glass slides, air dried and then subjected to various histological staining procedures as discussed below:
2.4.1. May-Grunwald Giemsa staining.

The slides were immersed in May-Grunwald stain (Sigma Aldrich #MG1L) (undiluted) for 3 minutes with periodic dipping; then transferred immediately to freshly prepared 1:20 diluted Giemsa stain ( #620G-75 Giemsa stain EMD™) for 15 minutes with periodic dipping and rinsed with water to remove the extra stain [40]. The air dried slides were mounted with Permount (Fisher Scientific # SP15-100) and coverslipped.

2.4.2. Acetylcholine esterase assay.

This assay was used to detect the production of acetylcholine esterase enzyme which is a marker of mature mouse megakaryocytes. The acetylthiocholine substrate used in this assay reacts with the enzyme and colors mature megakaryocyte cells bright brown [81]. The sample containing slides were incubated with a substrate solution comprised of 2.3mM of acetylthiocholine iodide (Sigma life science # A5751-G), 0.1M dibasic sodium phosphate (VWR # BDH8022-5200G), 0.1M sodium citrate ( VWR# BDH8017-500), 30mM copper sulphate (VWR# 7758-99-8), 5mM potassium ferricyanide (EMD # PX-14552) for 3-5 hours. The stain was fixed with 95% ethanol for 10 minutes and further counter stained with Harris hematoxylin and saturated lithium carbonate [40]. The slides were washed, air dried mounted and coverslipped.

2.4.3. Benzidine staining.

Benzidine staining was used to detect mature (heme containing) erythroid cells [82]. O-dianisidine stock solution (4.9 mM of O-diansidine [# Sigma D-Q143] and glacial acetic acid [0.2% final solution]) was mixed with one tenth volume of 30% H₂O₂. 50 µl of the H₂O₂/dianisidine solution was added to 500 µl of cell suspension containing ~10^5 cells. The cells were stained for 10 minutes at room temperature and centrifuged at 5000 rpm for 5 minutes. The 450 µl of supernatant was
removed and 100 µl of cells were cytocentrifuged onto glass slides. The air dried slides containing
the cells were then counterstained with May-Grunwald solution [40]. The air dried slides were
mounted and coverslipped.

Stained cells were photographed at 100X and 200X magnifications. The number of positively
stained cells relative to the total number of cells in a defined area was determined using the Image
J™ cell imaging and counting software [83].

2.5. Preparation of total RNA and cDNA.

Total RNA was isolated from 10⁶-10⁷ cells, using the EZNA Total RNA kit (#R6834-02).

cDNA for subsequent qPCR analysis was prepared from the isolated total RNA using the qScript™
cDNA synthesis kit from Quanta Bioscience (#95047-100).

2.6. Quantitative real time PCR.

Quantified expression profiling of different transcripts was performed by qPCR on an ABI 7500
machine (Applied Biosciences) using Absolute Blue QPCR SYBR Green low ROX Mix
(#AB4323 Thermo Fisher Scientific). Expression was normalized to that for HPRT (hypoxanthine
phosphoribosyl transferase) a house keeping gene. The primers used for qRT PCR amplification
are listed below:

muRgs18: caaagaaccaagactctctctgaa, taagaaatctggtaaaagcatccac
mu/hu Rgs18: cttggccaaagaaacaagagtctc, cactgaattcagttttaagaaatctgg
muKlf1: tcttaccctccatcagtacactca, atttcagactcacgtgatgggac;
muFlI1: ggaattgtgtaaatgaacaagag, ttctgtatgggaggtgttgtttagg.
muRgs2: ctcagaagaggtgtacagtttgat, gtggtttttacataagtcttggtta
muRgs6: cagtatatgttgtgacagacagac, gttttcggatgtcatcttttgtagt
muRgs10: atctacatgaacctctgttcaata, acttcatgagattgagatctggc
muRgs14: gaagtattgctgcgtgtatctacc, gtagacctttatgtcaggeagagag
muRgs16: agatgtactgggatggagagagtc, gtctcgtgatctatgttcaccttt
muHPRT: gttggatatgccttttgacta, ctaattttactggaacact
muEB3: actctcatgacagaaagag, aggtaagcagatcttttt
muBeta-globin major: ctatcatgggtaatgccaaa,agcctgaagttctcaggat
muGYPA: gggattatcggaac, aggagtctgctcaa
muPF4: gcaagcacccctatataag, ataacatgggaagattgg
muvWF: ctacagcctgcactattcag, agtctgacctctctctcg
muGPIIb: gtttatgttctgagcc,acagaatatectattagcc
muRgs18bio: tgtatagaggaagacctcagagace, atacacatacatatcaaaaagaagaca
hu HPRT: aaattccagacaaggttagttgtagg, tcaacttgaacttcatettagge
huGlobin: catttcggaaagaattcaccce, tatcctgaagctctgaatcatgg
huCD36: ttgagagaactgttatggggctata, actccatgctgcatagttgtgtaag
huGlycophorinA: tgtgcttttctcagcaacgtaac, tgtctctctctcttctctctt
2.7. Chromatin immunoprecipitation (ChIP).

ChIP experiments were performed with MEL and L8057 cells as previously described [50, 84-86] with anti-Gfi1b (Santa-cruz; # Sc8559) and anti-LSD1 (Abcam; #ab17721) antibodies. \(10^6\) cells were used per ChIP reaction, crosslinked with 1% formaldehyde, sonicated, precleared and incubated with 5-10\(\mu\)g of antibody. Complexes were washed with high and low salt buffers, and the DNA was extracted and precipitated.

Primers used for qPCR amplification of ChIP DNA were:
- Rgs18 promoter (upstream): tcatttccttcaacaattcagtaca, cgaatctttcctcagatttttctta.
- Rgs18 promoter (downstream): atgtgtgaatcaaaagagaaaacttt, cacagatattcatcaatcatgctactt
- Sμ: cttgagccaaaatgaagtagactgt, acagtccagtgtagcagtagt

2.8. Flow cytometric analysis.

For flow cytometric analyses of surface markers, 2x10^5 cells fresh or cultured primary cells were resuspended in 100 \(\mu\)l of FACS buffer (3% FBS in PBS) along with 1:500 dilution of Fc blocking reagent anti- mouse CD16/32 (e bioscience #2014-11) and incubated on ice for 5 minutes then washed and incubated with FITC-conjugated anti-mouse CD9 or CD71 antibodies and APC-conjugated anti-mouse CD41 or Ter119 antibodies (eBioscience) [1:200 dilution] at 4°C for 30 minutes. The cells were then washed, strained and analyzed on a BD LSRII Analyzer (Becton
Dickinson). For FACs sorting the cells were labeled with lin-FITC and c-kit-PE antibodies and the lin^{-}kit^{+} cells sorted on a BD FACS Aria (Becton Dickinson).

2.9. Preparation and analysis of protein samples.

2.9.1. Whole cell lysate preparation.

~5X10^5 cells were collected by centrifugation and lysed with whole cell lysis buffer (50mM Tris HCl, 150 mM NaCl, 1mM EDTA, 10% Triton) or NP40 lysis buffer (0.5% NP-40, 50mM Tris pH 7.5, 100mM NaCl, 0.1mM EDTA, 10% glycerol) supplemented with protease inhibitors (#P8340-1M; Sigma Aldrich) by incubating for 30 minutes at 4°C with agitation. The cell lysates were centrifuged at 13000 rpm for 10 minutes at 4°C and the supernatant (protein) was collected in a chilled tube. The protein concentration of the cell lysate was determined by Bradford assay (Bio-Rad reagent, # 5000006) and 30-60 µg of total protein was loaded in 10% or 15% SDS-PAGE gels for analysis.

2.9.2. Analysis of protein samples.

SDS gels were either stained with Coomassie blue (Invitrogen# LC6060) for visualization of total protein or transferred onto PVDF membranes and blotted with different antibodies. Antibodies used were anti-Gfi1b (Santa-cruz; #Sc8559), anti-LSD1 (Abcam; #ab17721), anti-Rgs18 (Abcam; #ab25917), and anti-P42/44-MAPK (#4695), anti-phosphoP42/44-MAPK (#4370S), anti-P38 (#2371), anti-phosphoP38 (#4511S) from Cell Signaling Technologies.

2.10. Streptavidin-agarose affinity chromatography.

To obtain protein complexes with recombinant biotagged Rgs18, 1-2 liters L8057 (~1X 10^9) cells were lysed with NP40 lysis buffer (0.5% NP40, 10% glycerol, 50mM Tris pH 7.5, 150mM NaCl,
0.1 mM EDTA) as described above. The supernatants from the lysed cells were pre-cleared by mixing with a 1:10 ratio of a 50% prewashed agarose bead slurry and the mixture was incubated at 4°C for 2 hours with agitation. The mixture was centrifuged at 3000 rpm for 10 minutes at 4°C and the supernatant was added to the 1:10 ratio of a 50% prewashed streptavidin-agarose bead slurry. The mixture was incubated overnight at 4°C with rocking and then centrifuged at 3000 rpm for 10 minutes followed by 3 washes with NP40 wash buffer (0.5% NP40, 50 mM Tris pH 7.5, 150 mM NaCl, 0.1 mM EDTA). After washing away non-specific material, the proteins bound to the streptavidin-agarose beads were eluted with of 2X SDS loading buffer (1:1 ratio of beads plus buffer) by heating at 95-100°C for 5 minutes followed by centrifugation at room temperature at 13000 rpm for 1 minute. Affinity precipitation of Rgs18 was confirmed by Western blotting of 10% of the precipitated material. The remainder of the precipitate was resolved on a 4-15% SDS-PAGE gel for tryptic digestion and analysis.

2.10.1. Simply Blue staining.

The SDS-PAGE gel was incubated with ~20 ml of SimplyBlue™ SafeStain (Invitrogen# LC6060) for 30 minutes and destained by multiple washes with deionized water and then submitted for mass spectrometric analysis.


2.11.1 Rgs18 guide RNA design strategy.

Rgs18 mutant mice were produced using CRISPR (clustered regularly interspersed short palindromic repeats) Cas9 (CRISPR associated protein 9) mediated gene editing [87]. This strategy consists of two components - a guide RNA (gRNA) and a CRISPR associated endonuclease (Cas9). The gRNA is a short synthetic RNA composed of a “scaffold” sequence
necessary for Cas9-binding and a user-defined ∼20 nucleotide “targeting” sequence with which the genomic target is modified. This system can be used to generate knock-out cells or animals by co-expressing both gRNAs specific to the gene to be targeted along with the Cas9 endonuclease in appropriate cells. Upon co-expression the Cas9 and gRNAs form a riboprotein complex at the target loci by annealing of the 3’ end of the guide RNA to the target sequence. This complex formation also activates Cas9 from an inactive conformation to an active DNA binding state. The Cas9 endonuclease then cleaves the target DNA to produce a double stranded break which is subsequently repaired by the cells non-homologous end joining (NHEJ) machinery with the introduction of errors at a certain frequency [87].

We utilized the transgenic facility at MSKCC (Memorial Sloan Kettering Cancer Center) to perform gRNAs targeting of the \textit{rgs18} locus in the vicinity of the initiator ATG codon (on exon1). Plasmids expressing hybrid gRNAs composed of a segment complementary to the targeting sequence and a tracrRNA (trans-activating CRISPR RNA), the cas9 endonuclease and a selectable marker (puro\textsuperscript{R}) were introduced into zygotes. These injected embryos were then transferred to foster mothers who then gave birth to \textit{rgs18} genome edited mosaic founder mice.

Following is the list of four gRNA sequences used for targeting the first \textit{rgs18} exon in the vicinity of the initiator ATG codon.  #1: caagcatcgaggccaaaatc, #2: ttcaaactaatgcatgggtc, #3: caaactaatgcatgggtca, #4: aaagaagaaacaagcatc. The \textit{rgs18} genomic sequence in the vicinity of exon1 and the sequences of the gRNAs are shown in Figure 15.

2.11.2 Determination of \textit{rgs18} mutations in founder mice.

a) T7 endonuclease 1 assay: This assay was also performed at MSKCC to identify \textit{rgs18} gene targeted founders. The T7 endonuclease1 assay identifies heteroduplex DNA that results from the
annealing of modified DNA strands produced upon guide RNA-Cas9 mediated editing of DNA strands with wild type strands. This assay relies on the property of the T7 endonuclease to recognize and cleave the mis-matched DNA duplexes. In this assay, Rgs18 exon 1 sequence was amplified from the genomic tail DNA of rgs18 gene edited founder mice with the following primers- atctgaggaaagattcggga, cactccaatcaatatattttcaac. The PCR samples were loaded on the gel and the correct bands were cut to extract the DNA. The purified PCR amplified DNA were denatured, reannealed and then subjected to T7 endonuclease digestion. Amplicons from WT-DNA form only homoduplexes and are not digested while gene edited mice generally possess both the wild type and one or more mutant loci which produce heteroduplexes with kinks resulting from mismatches that are recognized and cleaved by the endonuclease. The products are then resolved on a 1.5% agarose gel. The gel image obtained from T7 endonuclease assays on rgs18 gene edited mice at MSKCC is shown in the result section. The presence of two or more bands being indicative of T7 endonuclease digestion of indels in the rgs18 locus.

b) Insertion into TOPO cloning vector for sequence verification: Tail genomic DNA obtained from rgs18 founder mosaic mutant mice were PCR amplified with the primers atctgaggaaagattcggga, cactccaatcaatatattttcaac and were inserted into the TOPO (pcrR4–TOPO # 45-0030 Thermo fisher Scientific) vector for sequencing. The sequences obtained were further compared with the wild type rgs18 sequence to determine changes in the DNA and especially in the open reading frame due to generation of the CRISPR induced indels.

2.11.3. Design of alternative PCR strategies to genotype rgs18 mutants.

Based on the above mentioned initial sequence analyses, founders #12, #34 and #38 were selected for breeding to produce germline transmission.
Following the determination of the sequences corresponding to the different gene edited lesions in the \textit{rgs18} locus, specific primers were designed as indicated in Figure 16 to specifically amplify the DNA corresponding to a particular lesion versus that for wild type DNA.

The Rgs18 mutant mice were genotyped by PCR using the following primers:

Rgs18 (WT): atggatatgtcactgttttcttc, cctgattttggcctcgatgcttgt

Rgs18 (#12 and #34): atggatatgtcactgttttcttc, aattttacctgattttggcctctg

Rgs18 (#38): atggatatgtcactgttttcttc, tacctgattttggcctcgatttttgct

\textbf{2.12. Software and computer analysis.}

ImageJ: Quantitative analysis of histochemical samples was performed by counting numbers of positively stained cells relative to the total cell population for a defined image area using the Image J\textsuperscript{TM} cell imaging and counting software [83].

FlowJo: The data from the flow cytometric experiments were analyzed and represented using FlowJo V10 software (FlowJo Enterprise).

ANOVA: Data from all qPCR reactions were calculated for standard deviation from three independent experiments and \( p \) values were calculated by “One way analysis of variance” (ANOVA) or by multiple t test followed by Halm-Sidak post-hoc test as applicable.
Chapter 3: Results

3.1. Specific Aim1: Determining the regulation of Rgs18 by Gfi1b and its cofactors.

3.1.1. Differential repression of Rgs18 by Gfi1b and LSD1 in erythro-megakaryocytic lineages.

3.1.1.1 Analysis of \textit{rgs18} promoter sequence and identification of putative Gfi1b binding sites.

The Rgs18 promoter was identified as a prominent target of the Gfi1b/LSD1/Rcor1 transcriptional complex following chromatin immunoprecipitation (ChIP on chip) screens with antibodies against all three proteins [50] in erythroid (MEL) cells. Examination of \textit{rgs18} promoter sequences obtained from the mouse promoter arrays of these ChIP-on-chip screens [84, 85] revealed the presence of quasi-consensus (AAATCT) and consensus (AAATCA) Gfi1/1b binding sites [88] in the 5’ UTR and proximal protein coding regions of murine \textit{rgs18} respectively (Figure 17). This identified the \textit{rgs18} promoter as a putative target of the transcriptional of the Gfi1b transcriptional complex and indicated possible regulation of Rgs18 by Gfi1b and its cofactors in erythroid cells.

3.1.1.2. Stage specific expression patterns of Rgs18, Gfi1b and LSD1 in erythro-megakaryocytic cells.

As discussed above, Gfi1b and its cofactor LSD1 generally repress their gene targets [84, 89]. Therefore we first monitored expression of Rgs18, Gfi1b, LSD1 and β-actin (as a loading control) in immature and mature erythroid (MEL) and megakaryocytic (L8057) cells to determine the reciprocal relationship, if any, between them.

The MEL (murine erythroleukemia) cell line is a murine erythroid progenitor line arrested at the
proerythroblast stage that can be induced to differentiate into relatively mature cells (orthochromic erythroblasts) with dimethyl sulfoxide (DMSO) or sodium butyrate [90]. L8057 is a murine megakaryoblastic cell line that can be induced to differentiate into mature megakaryocytes with phorbol ester [76].

As shown in Figure 18, Rgs18 was found to be low in uninduced L8057 in keeping with strong Gfi1b and LSD1 expression in these cells, but was strongly upregulated upon differentiation of these cells to megakaryocytes following a sharp decline in Gfi1b and LSD1 levels in the induced cells. In contrast, expression of Gfi1b was strong in uninduced proerythroblast-like MEL cells and increased further in DMSO induced MEL cells (Figure 18). Conversely, Rgs18 expression which was moderate in immature erythroid cells, exhibited a further a reciprocal decline during maturation. As reported previously [89], LSD1 levels remained approximately uniform during maturation of MEL cells. These experiments confirmed inverse expression patterns of Rgs18 and Gfi1b/LSD1 in erythro-megakaryocytic cells.

3.1.1.3 Dose dependent enrichment of Gfi1b and LSD1 on the rgs18 promoter elements.

To confirm association of Gfi1b and LSD1 with the Rgs18 promoter, ChIP-qPCR was performed in uninduced (immature) and induced (mature) MEL and L8057 cell lines. (These ChIP experiments were performed by Ghanshyam Upadhyay; Figure 19). In accordance with their expression profiles, Gfi1b and LSD1 showed substantial enrichment on both putative Gfi1b binding elements in uninduced L8057 cells but greatly reduced enrichment in induced L8057 cells. In contrast, marginally higher enrichment of these proteins was observed in mature (induced) versus immature (uninduced) MEL cells. Surprisingly, the upstream “semi-consensus” Gfi1b binding element consistently showed greater enrichment for Gfi1b and LSD1 relative to the downstream “consensus” element as defined by previous reports [17], in both lineages, demonstrating the relatively greater affinity of the upstream
site for these proteins.

3.1.2. Expression of Rgs18 in LSD1 inhibited and gfi1b deficient erythro-megakaryocytic cells.

To demonstrate repression of the rgs18 promoter by LSD1, Rgs18 expression was measured in immature erythroid and megakaryocytic cells upon LSD1 inhibition. Up-regulation of Rgs18 expression was seen in both cell types although to different extents (Figure 20 A-B). These results demonstrated repression of Rgs18 by LSD1 in the two cell lines.

To confirm repression of rgs18 by Gfi1b in primary cells, their message (mRNA) levels were monitored in control (wild type and heterozygous) versus gfi1b mutant hematopoietic cells. Since auto-repression of the gfi1b promoter by itself [84, 91] prevents effective inhibition of this factor by shRNA mediated mechanisms, we were unable to monitor Rgs18 mRNA levels following inhibition of Gfi1b.

We determined Gfi1b and Rgs18 expression in total embryonic day 12.5 (e12.5) fetal liver cells from all three genotypes (gfi1b/+, gfi1b+/-, gfi1b-/-). Wild type e12.5 fetal livers are predominantly erythroid in composition with ~80% of cells exhibiting robust ter119 (mature erythroid cell marker) expression) in addition to having limited numbers of HSCs, MEPs and megakaryocyte progenitors (Figure 21; courtesy G. Upadhyay). Consistent with the indistinguishable phenotypes of wild type versus gfi1b+/- fetal livers as reported previously [92], we observed a marginal decrease in Gfi1b, and a similarly modest (~20%) increase in Rgs18, message levels respectively, in the heterozygous cells relative to wild type controls (Figure 22). In contrast complete absence of Gfi1b in the homozygotes produced a substantial elevation of Rgs18 message relative to wild type cells in this primarily erythroid tissue (Figure 22).

Next, to investigate the expression of Gfi1b and Rgs18 in primary megakaryocytes we cultured wild
type e12.5 fetal liver cells with megakaryocytic cytokines (TPO, IL3) to preferentially expand this lineage, which is under represented in the fetal liver relative to erythroid cells [93]. These cytokines induce megakaryocyte progenitors to differentiate into mature megakaryocytes as well as promote uncommitted HSCs and MEPs present in the fetal liver populations to choose this fate. Differentiation of fetal liver progenitors to megakaryocytes was monitored by visual inspection and expression of the megakaryocytic differentiation marker glycoprotein IIb (GPIIb, also known as CD41) (Figure 23A). Differentiation of wild type progenitors into megakaryocytes was accompanied by a gradual decline in Gfi1b, and a concomitant and reciprocal increase in Rgs18, expression (Figure 23A), demonstrating progressively attenuated repression of the rgs18 promoter upon decreasing Gfi1b expression. In contrast, although Rgs18 levels were higher in gfi1b-/- fresh (d0) fetal liver cells relative to controls, it remained unchanged upon differentiation of mutant cells along the megakaryocyte lineage relative to that observed on day 0 (Figure 23B). This lack of a net increase in Rgs18 mRNA levels in gfi1b-/- cells relative to the day 0 control confirms minimal or no repression of Rgs18 by Gfi1b during megakaryocytic differentiation and therefore no noticeable rise in Rgs18 expression in the absence of gfi1b unlike the increase observed in wild type cells.

Overall, the results depicted in Specific Aim1 collectively demonstrate dose dependent and differential repression of Rgs18 by Gfi1b and its cofactor LSD1 in erythroid versus megakaryocytic cells resulting in their reciprocal expression in the two lineages.

3.2. Specific Aim2: Determining the role of Rgs18 in erythropoiesis and megakaryopoiesis.

3.2.1. Examining the role of Rgs18 in murine and human cell lines and progenitors by manipulating expression.
3.2.1.1. Manipulation of Rgs18 expression in murine (MEL and L8057) cell lines, and in murine fetal liver (hematopoietic) cells.

Given that Rgs18 is expressed both in erythroid and megakaryocytic cells, we next interrogated its role in their differentiation. Rgs18 expression was manipulated in L8057 (Figure 24A) and MEL cell lines as well as in fetal liver progenitor cells (Figure 25C) by shRNA mediated inhibition and cDNA i.e. protein over-expression. Consistent with previous reports in other systems [67, 94], inhibition of Rgs18 retarded megakaryocytic differentiation of induced L8057 cells (Figure 24) as well as fetal liver progenitors cultured in vitro (Figure 25). This effect was demonstrated by a decrease in the expression of differentiation markers relative to a housekeeping gene (HPRT) (Figure 24B, Figure 25A), a reduction in the number of acetylcholine esterase (a mouse megakaryocyte marker) positive cells (Figure 24D, Figure 25D) and a diminution in the number of cells displaying the mature megakaryocytic cell surface markers CD9 and CD41 (Figure 25E). The reverse result, namely augmentation of differentiation was obtained upon over-expression of Rgs18 in this lineage (Figure 24C and D, Figure 25B, D, E). These results confirmed previous reports documenting the stimulatory role of Rgs18 in megakaryocytic differentiation [67, 94].

To confirm the validity of the Rgs18 knockdown phenotype and rule out shRNA mediated off-target effects, we over-expressed the protein coding segment of the Rgs18 cDNA in L8057 cells depleted for endogenous Rgs18 by shRNAs targeting the 3’UTR (3’ untranslated region) of the native transcript. Following induction with TPA, cells expressing Rgs18 shRNAs and cDNAs reverted back to the control state (Figure 24E) confirming the fidelity of the Rgs18 knock down phenotypes.

In sharp contrast to the above phenotype, manipulation (inhibition and over-expression) of Rgs18 produced exactly the opposite outcome in both the murine MEL (erythroid) cell line (Figure 26) and
in primary erythroid cells (Figure 27). Rgs18 inhibition produced a marked increase in differentiation (Figure 26B, C and Figure 27A, C), as judged from differentiation marker analysis, benzidine staining (for heme positivity), and relative expression of surface markers Ter119 (a marker of mature erythroid cells) versus c-Kit (a common marker of uncommitted progenitors) (Figure 27D). Conversely, Rgs18 over-expression resulted in decreased differentiation of erythroid cells (Figure 27 B, C, D).

Once again, to confirm the validity of the Rgs18 knockdown phenotypes we performed similar rescue experiments in erythroid cells as discussed above for megakaryocytes (Figure 26 D). Collectively, these results demonstrated a positive role for Rgs18 in megakaryocytic differentiation and a hitherto unknown function of Rgs18 in effectively inhibiting erythroid differentiation.

3.2.1.2. Manipulation of Rgs18 expression in lineage negative (Lin-) c-Kit+ FLCs.

To further substantiate the analogous effects of Rgs18 on hematopoietic progenitors and to understand whether Rgs18 regulates cell fate choice of MEPs, Lin+c-Kit+ cells representing lineage marker (CD2-8, NK1.1, Ter119, Gr-1) negative and progenitor (c-kit) marker positive hematopoietic progenitors comprising HSCs, MEPs as well as other undifferentiated progenitors were sorted out from e12.5 fetal livers (courtesy G. Upadhyay; Figure 28 A) and transduced with Rgs18 shRNAs or cDNAs.

The transduced progenitors were then co-cultured with a mixture of cytokines (erythropoietin, SCF-erythroid, TPO, IL3- megakaryocytic) to promote simultaneously differentiation along both lineages and then analyzed for relative CD41 versus Ter119 expression. The FACS analysis data showed that inhibition of Rgs18 increased the percentage of Ter119 (mature erythroid marker) positive cells (quadrant 3 of histogram) as compared to the control shRNA treated cells. Whereas over-expression of Rgs18 resulted in increased percentage of mature megakaryocyte marker CD41 (quadrant 2 of FACS histogram) positive cells and vice versa. These results further confirmed the stimulatory effect
of Rgs18 expression on megakaryocytic, and suppressive effect on erythroid, differentiation in a progenitor population subjected to mixed culture conditions (Figure 28 B).

3.2.1.3 **Manipulation of Rgs18 expression in the human hematopoietic K562 cell line.**

Finally, to delineate the effects of Rgs18 expression in human cells, Rgs18 cDNA was over-expressed in the human multipotent hematopoietic cell line K562 [77] followed by differentiation along either lineage [79, 80]. The K562 (erythromyeloblastoid) cell line has the potential to differentiate into erythroid and megakaryocytic cells following induction with sodium butyrate and TPA, respectively. Expression of murine Rgs18 cDNA which is ~90% identical to its human counterpart (NP_570138) also resulted in suppression of erythroid, and stimulation of megakaryocytic, differentiation relative to a vector control as evidenced by differentiation marker mRNA analysis and histochemical staining, in these human cells (Figure 29 A-E).

The cumulative results of these multiple approaches described in Specific aim 2.1 unequivocally demonstrate the dual role of Rgs18 in actively promoting megakaryopoiesis and in restricting erythropoiesis in both mouse and human contexts. This, in conjunction with the reciprocal expression of Rgs18 and its repressor Gfi1b as demonstrated in Specific aim 1, suggest that the high level of expression of Gfi1b in MEPs and erythroid cells promotes this fate by repressing Rgs18 and keeping megakaryocytic differentiation in check. However, in cells adopting a megakaryocyte fate, declining Gfi1b levels upregulate Rgs18 expression and drive differentiation of these cells.

3.2.2. **In vivo disruption of the rgs18 gene to determine its physiological functions.**

3.2.2.1. **CRISPR/Cas9 mediated rgs18 knockout mouse design and generation.**

Expression of Rgs18 is most robust in hematopoietic cells such as megakaryocytes, platelets [69], and to a lesser extent in erythroid cells, MEPs, HSCs and lymphocytes [65] and in tissues like fetal
liver, bone marrow and spleen [65]. Therefore we decided to delete rgs18 in the germline with the expectation that the most prominent phenotype would be obtained in these cells. To do so we utilized, a CRISPR-Cas9 targeting (clustered regularly interspaced short palindromic repeat-CRISPR associated nuclease 9) strategy [87]. rgs18 genome edited mosaic founder mice were generated in the transgenic facility of MSKCC (Memorial Sloan Kettering Cancer Center) by Dr. Peter Romanienko and the transgenic team lead by Dr. Willy Marks. The procedure followed to generate these mosaic founder mice is explained in detail in the Methods section of the thesis. The founder generation was screened for editing of the rgs18 locus by the T7E1 (T7 endonuclease) assay also at MSKCC to identify the insertion or deletion of sequences (indels) created at target locus. T7 endonuclease is a mismatch-specific DNA endonuclease that is used for detecting insertion/deletion mutations generated by genome engineering. This enzyme targets and digests mismatched hetero-duplex double stranded DNAs obtained by PCR to produce multiple smaller DNA fragments that can be visualized by agarose gel electrophoresis [95]. Figure 30 shows the indels (multiple bands) generated by the T7 assay from three such founder mice (#12, #34 and #38).

Subsequently the sequences corresponding to the indels was determined by PCR amplification, sub-cloning and sequencing of the DNA in the region of the indels from the founder mice. A sampling of indel sequences and the corresponding translational frame shifts resulting from them is presented in Figure 31. The figure shows the sequences of the four founder mice that exhibited frameshift indels leading to premature stop codons. These rgs18 edited loci encode mutant RNAs that should produce either severely truncated proteins or none at all if they are rapidly degraded by nonsense mediated decay.
These mosaic founder mice (some with multiple lesions) were then backcrossed with wild type mice to produce heterozygotes. *rgs18* heterozygous mice harboring lesions depicted in Figure 31 were genotyped by PCR amplification with lesion specific primers described in “Materials and Methods”.

Currently we are breeding the heterozygous Rgs18 mutant population in our own CCNY mouse facility to expand the colony and back cross them into the C57Bl/6 strain. Future experiments with the mutant mice are elaborated in the “Discussion” section of the thesis.


3.3.1. Determining downstream effectors and processes regulated by Rgs18.

3.3.1.1. Differential regulation of MAPK signaling by Rgs18 in erythroid and megakaryocytic cells.

To delineate the underlying mechanistic alterations responsible for the phenotypes ensuing from Rgs18 manipulation, we interrogated the status of two branches of MAPK (mitogen activated protein kinase) signaling in these cells. The p38-MAPK and the P44/42-ERK (extra-cellular signal regulated kinase) pathways are known to be impacted by G protein signaling [96], while also being implicated in erythro-megakaryocytic differentiation [71]. Specifically, ERK and P38MAPK proteins oppositely regulate these lineages by promoting proliferation of hematopoietic (megakaryocytic), and differentiation of erythroid, cells respectively [71]. Since Rgs proteins attenuate G protein activation, they are known to negatively impact MAPK signaling [96, 97]. Accordingly, Rgs18 levels and activity were expected to correlate inversely with MAPK signaling.

This was indeed found to be the case for ERK signaling in megakaryocytes (L8057 cells) where Rgs18 inhibition resulted in enhanced, and over-expression in diminished, phospho-ERK (pERK) levels.
(Figure 32A and B) indicating an inverse correlation between Rgs18 expression and the activity of this MAPK pathway. Likewise, p38MAPK phosphorylation was also sharply attenuated upon Rgs18 over-expression and enhanced upon inhibition (Figure 32D and E). These observations indicated that both p38 and ERK pathways are negatively impacted by Rgs18 activity in megakaryocytes. These results were confirmed by time course induction experiments that clearly demonstrated attenuated decay of ERK and p38 phosphorylation during differentiation in Rgs18 inhibited cells relative to controls (Figure 33).

Although the effect of ERK signaling on proliferation versus differentiation of megakaryoblasts is controversial with different reports supporting its stimulation of one or the other process [71, 98], our observation of heightened differentiation of L8057 cells following downregulation of this pathway (by Rgs18) clearly demonstrates an inverse relationship between ERK signaling and the differentiation of these cells. On the other hand reduced p38 signaling upon Rgs18 over-expression, likely contributes further to the attenuation of erythroid characteristics and gene expression, in these cells (Figure 32 G).

In contrast Rgs18 depletion in erythroid (MEL) cells, diminished pERK levels (Figure 32 C) while augmenting both P38 protein and phosphorylation levels (Figure 32 F) to co-operatively increase differentiation. Thus, the phospho-p38 (p-p38) response was similar in both lineages and correlated inversely with Rgs18 activity, while the pERK response was diametrically opposite in erythroid and megakaryocytic cells. Overall, the alterations in MAPK signaling upon Rgs18 manipulation illustrate the likely molecular mechanisms responsible for the corresponding phenotypic alterations in differentiation, in each lineage (Figure 32 G).

3.3.1.2. Rgs18 determines the equilibrium between Klf1 and Fli1.

To investigate gene expression changes downstream of Rgs18 and to connect MAPK signaling with
expression of Eklf/Klf1 and Fli1, we interrogated expression of the mutually antagonistic transcription factors Eklf/Klf1 and Fli1 in Rgs18 manipulated cells. Klf1 and Fli1 are essential for the generation of the erythroid and megakaryocytic lineages respectively [99-101], while also actively suppressing alternative lineage fates downstream of MEPs [102]. Likewise, the relative abundance of these factors determines the lineage choices adopted by the progeny of these bipotent progenitors. Yet how one factor gains dominance over the other and vice versa during lineage specification remains unclear.

Therefore, to determine if these factors act downstream of Rgs18 we assessed their levels in cells manipulated for the latter. In both lineages, Rgs18 levels correlated directly with Fli1, and inversely with Klf1, message levels indicating that Rgs18 arbitrates alternative fates by ultimately regulating relative Klf1 and Fli1 stoichiometries (Figure 34 A-D).

Additionally to confirm Rgs18 action upstream of Fli1, Fli1 was ectopically expressed in fetal liver cells depleted of Rgs18 and then induced to differentiate along the megakaryocyte lineage. Ectopic expression of Fli1 “rescued” the megakaryocytic potential of these cells as evidenced by the recovery of expression of megakaryocytic differentiation markers (Figure 35 A) and histochemical (Ache positive cells) staining of the doubly manipulated cells (Figure 35 B) to control levels. This was further confirmed by surface expression of the megakaryocytic markers CD9 and CD41 by FACS analysis of Rgs18k/d+Fli1o/e doubly manipulated cells relative to controls (Figure 35 C).

In addition to validate the above mentioned results further, Rgs18-Fli1 manipulated cells were grown in methylcellulose medium supplemented with megakaryocytic cytokines and the number of megakaryocyte colonies per $10^5$ cells determined (Figure 36 A-B). Presence of similar megakaryocyte colony counts in Rgs18k/d+Fli1o/e group relative to control confirmed the role of Fli1 downstream of Rgs18 in promoting megakaryocyte differentiation.

Overall, these results established new and important functional connections between Gfi1b, Rgs18,
3.3.2. Identifying proteins associated with Rgs18 in megakaryocytic cells.

Consistent with its identity as a GAP factor, Rgs18 is known to interact with Goi and Gaq subunits of G proteins and with scaffolding proteins like 14-3-3 and Spinophilin in platelets to mediate its GAP activity [64]. However, these may represent only a small subset of Rgs18 interacting proteins. Additionally Rgs18 partners in erythroid cells if distinct from those in megakaryocytes are entirely unknown. Therefore, to understand the mechanism of Rgs18 action during erythro-megakaryocyte development particularly its differential regulation of MAPK signaling in these lineages, it seems essential to determine its full repertoire of interacting proteins in both lineages. Moreover, since Rgs18 produces opposite effects in the erythroid and megakaryocytic lineages, it may well do so by engaging with lineage specific factors in addition to common ones in these cells. Therefore, we endeavored to identify additional Rgs18 interacting proteins by affinity purification followed by mass spectrometric analysis initially in megakaryocytic cells while similar experiments have been planned for erythroid cells. For this purpose the Rgs18 coding sequence was fused to a short epitope tag known to undergo in vivo biotinylation by the bacterial biotin ligase (BirA). The Rgs18-biotag expression vector was co-transfected with the BirA expression vector into L8057 (megakaryoblastic) and MEL (erythroid) cells to generate stable cell lines (Figure 37). L8057 cells stably expressing Rgs18-biotag and BirA was used for the subsequent affinity purification of the Rgs18 interactome.

Biotinylated Rgs18 along with its associated proteins were captured from whole cells extracts on streptavidin agarose beads. The affinity purified protein complexes from L8057 expressing either BirA alone (control) or BirA and Rgs18-biotag were resolved by SDS-PAGE and subjected to
whole lane mass spectrometry. **Figure 38** shows a selective list of peptides mostly unique to the Rgs18-biotagged lane in megakaryocytic cells obtained from three mass spectrometry analysis. Although preliminary, this screen has begun to reveal potentially interesting interaction partners of Rgs18 that on further analysis should produce major insights into the mechanism of action of this crucial signaling molecule.

Preliminary analysis of Rgs18 interacting proteins in megakaryocytes has identified G proteins (Gαi2, Gβ like1, cluster of G(i)G(s)G(t), Gαs), proteins of the cAMP pathway (PKA, CAP1, Adenylase cyclase type 10), proteins of the Ras-MAPK signaling cascade (Rap1, Ras GTPase activating like protein, Ras GAP binding protein, MAPK1-ERK2), scaffolding protein like 14-3-3, integrins and cytoskeletal proteins like Talin1 and Kindlin3 of which the latter are also Gfi1b targets (D. Singh *et.al*, manuscript in preparation). A cumulative list of selective peptides obtained from the three mass spectrometric analyses is shown in **Figure 38**. The Rgs18 interacting proteins are categorized based on their ontology and affiliation. Among these proteins 86 peptides of Gαi2 was obtained in the experimental Rgs18-biotagged lane in the third experiment compared to the control lane which showed 2 peptides. This confirms previous reports that noted interaction of Rgs18 with Gαi in megakaryocytes and strongly indicates that Gαi2 is one of the most potent Rgs2 interactors [66, 69, 103]. If Rgs18 accelerates GTP hydrolysis by Gαi2 then it should activate the cAMP pathway which operates downstream of and is inhibited by Gαi proteins (Figure 41). Interestingly, apart from interacting with Gαi2, Rgs18 also interacts directly and/or intimately with other proteins of the cAMP pathway (PKA, Adenylase cyclase associated protein 1 and Adenylase cyclase type 10). Rgs18 was also found to associate with multiple Ras family proteins and RAS-MAPK pathway proteins in this analysis. The cAMP pathway and PKA inhibits ERK-MAPK phosphorylation [96]. Although the consequence of the interaction of Rgs18 with the other
components of the cAMP pathway is not clear from our present results, Rgs18 likely inhibits ERK signaling by activating the cAMP pathway either via G\textsubscript{ai} inhibition or by direct engagement. Although there are conflicting reports in the literature regarding the role of ERK signaling in the proliferation versus differentiation of megakaryocyte progenitors[71, 98], our results would suggest that suppression of ERK signaling by Rgs18 is responsible at least partly for increased differentiation.

On the other hand as members of the Ras pathway also came up as Rgs18 interacting proteins in this analysis, it can be interpreted that Rgs18 by interacting with RasGAPs may also directly accelerate GTP hydrolysis by Ras proteins and thus inhibits the Ras-Rac-MEK-ERKMAPK pathway in ways not previously appreciated and which could further enhance megakaryocyte differentiation (Figure 41).

Integrins (integrin \(\beta_2\), integrin \(\beta_1\), integrin \(\alpha_4\), integrin L), Rap1 and the cytoskeletal proteins Kindlin3 and Talin1 obtained in these screens are also known to activate platelet aggregation [104]. Previous reports have shown the direct interaction of Kindlin3 and Talin1 with \(\alpha\text{IIb}/\beta_3\) integrin [104, 105] facilitates terminal differentiation of mature megakaryocytes into nascent proplatelets [106]. Rap1 also a member of the Ras subfamily is stimulated by GPCRs, cytokine receptors and cell adhesion molecules. Rap1 activation induces its association with Talin1 which in turn leads to integrin-Talin1 interactions [104], while Kindlin3 and talin1 cooperatively bind with and activate integrins [107]. It is not clear how interaction of Rgs18 with these cytoskeletal proteins leads to megakaryocytic differentiation, given the complexity of the interactions involving Rgs18 with these and other proteins. Disrupting specific associations may help illustrate their individual or collective contributions.
Chapter 4: Discussion and Future Directions.

During the process of lineage specification from multipotent progenitor cells in hematopoiesis, a bipotent progenitor cell usually undergoes a discrete cell fate decision by committing to one of two fates of predefined cell lineages [13]. This lineage commitment is achieved by expression of cell type specific transcriptomes that are either stochastic or determined by extracellular signals in the form of hematopoietic cytokines. Previous studies have shown differential expression of various transcription factors (GATA1, GATA2, Klf1 and Fli1) during specification of the erythroid and megakaryocytic cell fates from a bipotent MEP cell in determining outcomes [34]. In this report we have identified a signaling molecule Rgs18, a prominent transcriptional target of Gfi1b in instigating lineage specification downstream of MEPs along with its repressor.

4.1. Regulation of Rgs factors during differentiation of hematopoietic cells.

We identified Rgs18 as a prominent target of Gfi1b and its co-repressor LSD1 in erythro-megakaryocytic cells. Rgs18 was found to be stringently repressed by Gfi1b and LSD1 in erythroid cells while being robustly expressed in megakaryocytes due to reduced expression of these repressors. These expression patterns then support stimulation of megakaryocytic, and suppression of erythroid, differentiation respectively, by Rgs18. These results thus introduce Rgs18 as a new arbitrator of erythro-megakaryocytic differentiation and provide a novel perspective on the regulation of these lineages by Gfi1b and its target, Rgs18. Given that Gfi1b performs similar functions in fetal liver and bone marrow hematopoiesis [40, 46], this paradigm may hold true for the divergence of bone marrow MEPs as well. Whether Rgs18 arbitrates similar lineage divergence at other cellular branch points and/or is prototypic of the function of other Rgs proteins widely expressed in multiple tissues, hematopoietic and otherwise, are fascinating and germane questions that need to be investigated in light of the above observations.
Germline and conditional *gfi1b* deletions have demonstrated the essential requirement of this gene for the normal production and development of MEPs and their erythroid and megakaryocytic progeny [40, 46]. Hence absence of *gfi1b* leads to an ablation of both lineages and produces either embryonic lethality upon germline deletion [40] or adult mortality upon conditional deletion of this gene in the bone marrow [46]. What our current results highlight is the differential expression of, and requirement for, this factor subsequent to the initial specification of these lineages. Robust and sustained expression of Gfi1b in the progeny of MEPs is required for erythroid identity and differentiation, in part by suppressing megakaryocytic gene expression including that of Rgs18 until the erythroblast stage. Any decline in Gfi1b levels subsequent to this stage may then be required for terminal erythroid maturation [42]. Conversely, downregulation of Gfi1b in maturing megakaryoblasts and subsequent stages is necessary for de-repression of genes like Rgs18 that promote differentiation of this lineage. Other reports have shown Rgs18 to be repressed in erythroid cells by GATA1 [108], although it is not clear if this repression pattern is maintained in megakaryocytes as GATA1 expression is also downregulated during differentiation [109]. Therefore the *rgs18* promoter could potentially be co-operatively repressed by Gfi1b and GATA1 in erythroid cells while being derepressed by the downregulation of both factors in megakaryocytes.

In addition to Rgs18, several other Rgs factors are derepressed to varying degrees upon loss of *gfi1b* (Figure 39). Of these Rgs14 and Rgs2 are also chromatin targets of Gfi1b and its cofactors (LSD1 and Rcor1) in erythroid cells (S. Saleque *et al.* unpublished). Therefore other Rgs factors may co-operate with Rgs18 in promoting one (megakaryocytic) versus the other (erythroid) lineage to mediate multi-factorial regulation of this lineage divergence.

Rgs18 is also prominently expressed in platelets [110] where it potently inhibits processes like platelet activation and aggregation by inhibiting G protein signaling [68, 111]. Therefore, this GAP factor
performs distinct and even opposite functions in megakaryopoiesis versus thrombopoiesis.

4.2. Antagonistic regulation of erythro-megakaryocytic differentiation by Gfi1b and Rgs18.

The molecular events initiating the divergence of erythroid (red blood cell) and megakaryocytic (platelet precursor) lineages from a bipotent progenitor are not entirely clear. As mentioned earlier several transcription factors along with Gfi1b, signaling molecules and lineage specific genes have been identified as essential for erythro-megakaryocytic lineage development where their expression drives progenitor cells towards either of the two lineages [28]. Despite the recognition of these molecules as key regulators of these lineage specifications, their mechanism of action and the signaling cascades regulated by them are still not entirely clear. Here we have identified a reciprocal relationship between the transcriptional repressor Gfi1b and its gene target Rgs18 during specification of the erythroid and megakaryocyte lineages. In our current model (Figure 40) we propose that a gradual decrease in Gfi1b levels produces an inverse increase in Rgs18 levels to promote the differentiation of MEPs along the megakaryocytic lineage. Rgs18 then stimulates differentiation by negatively regulating both the p38 and ERK branches of the MAPK pathway and expression of Klf1 while positively regulating the expression of Fli1. In contrast, robust and increasing Gfi1b expression during erythroid differentiation results in suppression of Rgs18 expression during the differentiation of these cells. Reduced Rgs18 expression in turn promotes activation of the p38-MAPK pathway and down regulation of ERK-MAPK signaling to boost Klf1 over Fli1 expression and stimulate erythroid differentiation.

4.3. Role of signaling pathways and Rgs18 interactors in facilitating erythromegakaryocytic lineage specification.

Several studies have demonstrated the role of p38MAPK and ERK signaling in the regulation of
hematopoietic stem and progenitor cell expansion and differentiation [71, 98]. Yet their relationship with upstream or downstream transcription factors and the processes regulated by them continue to remain nebulous. The observations reported here now establish coherent connections between the erythro-megakaryocytic transcription factor Gfi1b and MAPK pathways via G protein signaling mechanisms. They further extend the signaling chain to reveal regulation of Klf1 and Fli1 by these pathways.

Previously, manipulation of Rgs16 but not Rgs18, had been found to impact MAPK signaling in megakaryocytes. According to that study, both Rgs16 and Rgs18 mRNAs were upregulated during megakaryocyte differentiation in cord blood cells. But over-expression of Rgs16 mRNA in M07e (human megakaryoblastic myeloid leukemia) cell line inhibited MAPK (mitogen activated protein kinase) and AKT (protein kinase B) expression as shown by reduced phosphorylation of these proteins in Western blots but Rgs18 over-expression had so such effect on these proteins [97]. Since our results now clearly illustrate the effect of Rgs18 manipulation on p38 and ERK signaling in both erythroid and megakaryocytic cells, this apparent discrepancy between the earlier results and ours may be a consequence of the different cell types (human MO7e versus murine L8057 and MEL) or assays employed in each study. Moreover our subsequent mass spectrometric data reveal that inhibition of MAPK pathways which have been implicated in proliferation [71, 98] by Rgs18 are likely due to a combination of inhibition of Ga_i and Ras signaling by this factor (Figure 41).

Based on the Rgs18 interaction proteins obtained from the mass-spectrometry results a possible link between Rgs18-MAPK-Klf1 has been outlined. p38-MAPK and Klf1 are both known to be required for and to stimulate erythropoietic differentiation [72, 100]. So even though we do not known if Klf1 is directly regulated by p38-MAPK pathway we found its expression to correlate directly with p38 phosphorylation levels. Even though a direct connection cannot be determined but a causal link
between p38 phosphorylation and Klf1 may be tested by inhibiting one or the other.

4.4. Relevance of Rgs18 functions to hematopoietic diseases and their control.

Various lines of evidence demonstrate that Rgs18 stimulates megakaryocytic differentiation [63, 67] while limiting platelet aggregation and activation by turning off G_q signaling [64, 68, 111]. We now demonstrate the key role played by this factor in ensuring erythro-megakaryocytic homeostasis by actively suppressing the erythroid and promoting megakaryocyte differentiation. These insights could potentially be utilized to rectify imbalances between erythroid and megakaryocytic cell generation that lead to hematopoietic abnormalities. Accordingly, a deficit in erythropoiesis could conceivably be compensated by inhibiting Rgs18 (or other Rgs proteins that work similarly and co-operatively in these cells) and/or by indirectly stimulating G protein signaling. Conversely, thrombocytopenia resulting from G protein signaling problems or other deficits could be offset by stimulating Rgs18 expression or activity, and/or by inhibiting specific G proteins.

In conclusion, our study presents Rgs18 as a key regulator of erythro-megakaryocyte lineages specification, downstream of the transcription factor Gfi1b. These effects are mediated by differential MAPK signaling and alterations in Klf1:Fli1 levels. As mentioned above, these molecular insights into the divergence of the erythroid and megakaryocytic lineages from MEPs could also provide rational platforms for developing strategies for stimulating one or the other lineage when depleted by diseases or environmental assaults.

4.5. Summary.

In this report, the dual role of a signaling molecule Rgs18 was shown in specification of erythroid and megakaryocyte lineages in hematopoiesis. Rgs18, a GTPase activating protein was identified as one of the gene targets of the transcriptional repressor Gfi1b through ChIP on Chip screen in erythroid
cells. Previous studies have already established the essential role of the site specific transcriptional repressor Gfi1b in erythro-megakaryopoiesis but its mechanism of action and functional role in these processes remains elusive.

Now, we have demonstrated that Rgs18 along with its transcriptional repressor Gfi1b arbitrate specification of erythroid and megakaryocyte lineages in human and mouse cells. Gfi1b stringently represses Rgs18 expression in erythroid cells, while during megakaryocytic differentiation, declining Gfi1b levels facilitate robust induction of the latter. Concordantly, alterations in Rgs18 expression produce disparate outcomes by augmenting megakaryocytic and potently suppressing erythroid differentiation and vice versa. These phenotypes reflect differential impact of Rgs18 on p38 and ERK signaling in the two lineages, which in turn alter the balance between the mutually antagonistic transcription factors, Fli1 and Klf1. Overall these results identify Rgs18 as a new and critical effector of Gfi1b that regulates downstream signaling and gene expression programs to orchestrate erythro-megakaryocytic lineage choices. Since Rgs18 was identified along with two other members (Rgs2, Rgs14) of the Rgs family in ChIP on Chip screen, the dual role of Rgs18 in reciprocally regulating divergent lineages presented here could exemplify generic mechanisms characteristic of these and other family members in specifying alternative lineages in different contexts.

4.6. Future directions.

4.6.1. Analysis and prediction of rgs18 mutant physiology.

To understand the role of Rgs18 in mammalian physiology, we have generated rgs18 mutant mice. Two recent reports of functional Rgs domain deleted rgs18 mutant mice have revealed either a gain of function phenotype in platelets with enhanced occlusion time at sites of vascular injury and enhanced bleeding time or defective megakaryopoiesis followed by mild thrombocytopenia in
mice [67, 68]. However, these recent studies with Rgs18 mutants did not report any differences in erythropoiesis between the controls and mutants. Since our *ex-vivo* experiments have demonstrated enhanced differentiation of erythroid cells upon Rgs18 inhibition in FLCs it is likely that these mice may have mild to moderate erythrocytosis (increased number of RBC) that may have been undetected or overlooked by these researchers given that the regulation of erythropoiesis by Rgs18 was previously unknown and/or may not noticeably impair the health of these mice over and above that due to the defects in megakaryopoiesis and clotting. Moreover these studies have reported phenotypes resulting from mutants with partially deleted RGS domain of Rgs18. Therefore it is not clear if these knock outs represent null or hypomorphic mutations of *rgs18*. Since the 235 amino acid codons of *rgs18* are distributed over 5 exons with the RGS domain (comprised of 127 amino acids) spanning exons 3-5, deletion of the RGS domain from exon 4 onward does not completely delete the gene and may not completely abrogate its function. Therefore, we have generated *rgs18* mutants with frame-shift mutations in the first exon of the *rgs18* gene that should result in premature termination of the open reading frame with severely truncated proteins or none at all and so result in a true null mutations. Following their initial characterization by genotyping, three independent *rgs18* mutations were identified and gene edited heterozygous mice were derived from them. These mice are currently being mated with wild type mice to expand the colony and the resulting heterozygotes will be mated to produce homozygotes for phenotypic analysis as outlined below:

a) Firstly E12.5 day embryos obtained from the timed mating of *rgs18* +/- mice will be genotyped. The expected Mendelian ratio (1:2:1) of wild type, heterozygous and mutant embryos would be verified by genotyping and morphology of the mutant embryos would also be examined. Peripheral blood of *rgs18*+/+, *rgs18*+/- and *rgs18*/-/- embryos will be compared at E12.5-14.5
gestation stages by staining the yolk sac blood cells with May-Grunwald-Giemsa stain (MGG). FACS analysis of the WT versus mutant FLCs doubly stained with antibodies against Ter119 (mature erythroid marker) and c-kit (marker for immature hematopoietic cells) will be performed. Since we know from the *ex vivo* studies that Rgs18 is a negative regulator of erythropoiesis, we are expect that absence of Rgs18 to result in a higher ratio of mature erythroid cells compared to the wild type. Thus, the MGG stain should show more mature adult enucleated red blood cells in the *rgs18*-/- blood samples and higher percentage of c-kit^-Ter119^+ cells in FACs analysis compared to the wild type cells.

b) Since Rgs18 positively regulates megakaryopoiesis and negatively erythropoiesis, we expect absence of Rgs18 to result in defects in production, maturation and total counts of these cells. Thus to analyze the effect of Rgs18 deletion in megakaryopoiesis, the FLCs from E12.5 *rgs18*-/- and wild-type embryos will be cultured *ex vivo* with the megakaryocytic cytokines. The deficit in megakaryopoiesis, if any, will be examined by acetylcholine esterase (Ache) assays, qPCR analysis of megakaryocyte markers and flow cytometric analysis of CD9 and CD41 expression of *rgs18*-/- versus *rgs18*+/+ cells. Since we predict at least a reduction in megakaryocytic differentiation in *rgs18*-/- cells, therefore we expect to obtain fewer of Ache (+) ve cells, lower expression of megakaryocyte markers and lower numbers of CD9+CD41+ cells in the mutants relative to wild type or heterozygous controls.

c) In parallel, the expression pattern of different Rgs proteins especially the ones known to be highly expressed in megakaryocytes along with Rgs18 (Rgs16, Rgs10) as well as the other Gfi1b targets from the Rgs family (Rgs2 and Rgs14) will be quantified in *rgs18*-/- fresh FLCs and those induced towards erythroid and megakaryocytic differentiation. This will be performed to determine if their expression levels were affected due to *rgs18* deletion and may
indicate compensation at the level of expression or function among Rgs family members, if any. Although, prior rgs18 knock outs [68] have reported no detectable change in Rgs16 and Rgs10 expression, we may see an increase in the expression of these proteins as well as Rgs2 and Rgs14 in our null animals.

d) Whether or not we observe over-expression of other Rgs proteins rgs18-/—FLCs they will be knocked them systematically and combinatorially in the rgs18-/—cells to determine functional synergy between them.

4.6.2. Determine Rgs18 interacting proteins in erythroid cells.

As mentioned in the result section, preliminary data from the mass spectrometric analysis have identified different interactors of Rgs18 in megakaryocytes. Since manipulation of Rgs18 shows distinct and opposite phenotypes (positive regulation of megakaryopoiesis while negative effect on erythropoiesis) in the two lineages, there may be a different interacting partners of Rgs18 in the two lineages. Thus in the future Rgs18 interactors in the erythroid lineage will be similarly identified by affinity chromatography and mass spectrometry. The data obtained from these experiment will very likely divulge the mechanism(s) responsible for the differential even opposite effects exerted by Rgs18 on ERK signaling in erythroid versus megakaryocytic cells which may be a major determinant in specifying divergent outcomes in the two lineages. Thus the knowledge of Rgs18 interactors will provide unprecedented insights into the regulation of signal transduction, particularly MAPK pathways, by Gfi1b via its transcriptional target Rgs18.
Figure 1: Ontogeny of mouse hematopoiesis.

Mesoderm forms at gastrulation (E6.5), then blood islands develop within the yolk sac (E7.5), followed by emergence of HSCs in the AGM region (E10.5) and in placenta (E10.5-11). Finally fetal liver hematopoiesis occurs between E11.5-E14.5 and shifts to the bone marrow at E18.5 and then continues throughout adult life.

**Figure 2: Classical model of adult hematopoiesis.**

ST-HSCs produce multipotent progenitors such as the common myeloid progenitor, which further diverge to cells with megakaryocyte erythroid potential (MEPs) and granulocyte macrophage (GMP) potentials. The MEPs subsequently diverge to produce to erythroid and megakaryocyte cells. The yellow highlight shows the divergence of erythroid and megakaryocyte lineage from MEPs.

(Ref: Wang et al Nature reviews 2011)
Figure 3: Transcription factors regulating commitment of HSCs to MEPS.

Dual expression of GATA1 and PU.1 causes HSCs to differentiate into common myeloid progenitors. Subsequently dominance of GATA1 expression over PU.1 directs CMPs to the EMP/ MEP fate.

**Figure 4: Mutual antagonism between EKLF/Klf1 and Fli1 during erythro-megakaryopoiesis.**

Erythro-megakaryocyte cell fate determining factors Klf1 and Fli1 establish cell fate by cross inhibiting each other’s promoters.


**Figure 5: Regulation of erythro-megakaryocytic cytokine receptors by EKLF/ Klf1 and Fli1.**

Mutually antagonistic transcription factors Klf1 and Fli1 not only cross-inhibit each but also regulate erythropoietin and thrombopoietin receptor expression.

Figure 6: Regulators of erythro-megakaryocyte development.

The development of mature erythroid and megakaryocyte cells from their progenitors is controlled by transcription factors and signaling proteins. Those aberrantly expressed in erythroleukemias are highlighted in red. Grey boxes indicate factors necessary for initial specification of erythro-megakaryopoiesis. The yellow box highlights the transcription factor Gfi1b and shows its role at different stages of erythro-megakaryocyte development.

Figure 7: Schematic representation of Gfi1 and Gfi1b.

Their respective SNAG repression domain and DNA binding Zinc finger domain are highlighted in red and blue respectively.


Figure 8: Gfi1b is required for definitive erythropoiesis.

Control and gfi1b -/- embryos and blood at embryonic day 10.5, 12.5 and 14.5, respectively. gfi1b -/- embryos show aberrant primitive erythroid cell morphology and delayed cell maturation and a complete lack of enucleated definitive erythroid cells.

Figure 9: *gfi1b* -/- fetal livers shows arrested megakaryopoiesis.

*gfi1b* -/- fetal liver cells when induced to differentiate in culture exhibit a complete differentiation arrest as seen from visualization of cell morphology (e) and absence of acetylcholine esterase staining (f).

Figure 10: Mechanism of transcriptional repression by Gfi1b.

Gfi1b recruits its chromatin modifiers to chromatin targets to alter histone marks. Recruitment of LSD1 and HDAC to the DNA results in demethylation of K4 and deacetylation of K9 residues respectively. Recruitment of G9A by Gfi1b facilitates methylation of H3-K9, resulting in stable, long-term silencing of the locus.

Figure 11: Diagrammatic representation of Gfi1b, LSD1, and CoREST targets.

CHIP on Chip screen data of Gfi1b and its cofactors (LSD1 and CoREST). Each factor has its independent target genes indicated in brackets. The targets that are either unique to each other or common to one or more of them are indicated in the corresponding region of the diagram. All three shares 653 common target genes between each other.

Figure 12: Domain architecture of three Rgs family members that are gene targets of Gfi1b.

Rgs2 and Rgs18 belongs to the R4 subfamily of Rgs proteins which contain only one RGS domain while Rgs14 belongs to the R12 subfamily that has 1RGS domain, 2 Raf like Ras-binding domains, 1 GoLoco domain.

Figure 13: Regulation of G protein signaling by Rgs proteins.

Rgs proteins are GTPase activating proteins (GAPs) that accelerate GTP hydrolysis by Gα subunits of heteromeric G proteins and downregulate GPCR and the G protein mediated signaling.

(Ref: http://koslofflab.haifa.ac.il/index.htm)
Figure 14: Role of the MAPK pathway in hematopoiesis.
Initial activation of p38 and ERK pathways are essential for development of megakaryocyte erythroid progenitors from CMP. p38 is essential for the final maturation of erythroid lineage. Positive effect on lineage development is indicated in green whereas the inhibitory effects are depicted in red.
**Figure 15:** Schematic representation of Rgs18 guide RNA design strategy.

Section of Rgs18 genomic DNA with exon1 highlighted in yellow. Numbers and underlines represent the sequences corresponding to the four gRNAs. Sequences of primers used to amplify Rgs18 exon1 are highlighted in blue. The initiator ATG is highlighted in orange.
Figure 16: Rgs18 mutant primers sequence positions.

Sequences of primers specific for amplifying lesions present in founders, #34 and #38 are underlined and the alterations in the genomic sequence highlighted in pink (deletion) and yellow (insertion).
Figure 17: Annotated Rgs18 promoter sequence.

Rgs18 promoter sequence obtained from ChIP-on-chip sequence experiments with Gfi1b/LSD1/Rcor1 in MEL cells. The transcriptional start site is indicated by the underlined bold maroon letter G. The initiator ATG codon is indicated in bold green letters. The consensus (AAATCA) and quasi-consensus (AAATCT) Gfi1/1b binding elements are indicated in bold blue letters and the sequences of the primers used for ChIP qPCR amplification used in Figure 19 are underlined.

Figure 18: Expression pattern of Rgs18, Gfi1b, and LSD1 proteins in erythro-megakaryocytic cells.

Western blot documenting Gfi1b, LSD1, Rgs18 and b-actin (as loading control) protein levels in uninduced (-TPA and -DMSO) and induced (+TPA and +DMSO) L8057 (Mk line) and MEL (Ery line) cells. 60µg protein was loaded in each lane.

Figure 19: ChIP experiment showing enrichment of Gfi1b/LSD1 on \textit{rgs18} promoter segments.

Enrichment of Gfi1b and its cofactor LSD1 on upstream (u) and downstream (d) \textit{rgs18} promoter segments (rgs18p) in uninduced and induced (A) megakaryocytic (meg) and (B) erythroid (ery) cell lines relative to a mock (IgH switch Sm) locus. Mean and sd (±) from three independent ChIP experiments are shown. P values were <0.0001 (****), <0.001 (***) , <0.01(***), <0.01(**) and <0.05 (*) respectively were calculated using Anova and student t test (Courtesy: G. Upadhyay).

Figure 20: Rgs18 expression pattern in LSD1 inhibited uninduced MEL and L8057 cells.

A. LSD1 mRNA levels in uninduced L8057 (meg) and MEL (ery) cell lines knocked down for LSD1 (sh-LSD1) versus controls (Scr-ctrl). B. Relative Rgs18 mRNA levels in control (Scr-Ctrl; scrambled) and LSD1 inhibited (sh-LSD1) erythroid (ery; MEL) and megakaryoblastic (meg; L8057) cells. Mean and sd (±) from three independent experiments are shown. P values were <0.0001 (****), <0.001 (**), <0.01(***), and <0.05 (*) respectively were calculated using Anova and student t test.

Figure 21: Composition of E12.5 fetal liver cells.

Expression of CD71 and Ter119 surface markers in FACs sorted E12.5 fetal liver cells. 84.5% cells exhibited robust ter119 (mature erythroid marker) expression.


Figure 22: Expression pattern of Gfi1b and Rgs18 in Gfi1b mutant and wild type FL cells.

Gfi1b and Rgs18 mRNA levels in gfi1b+/+, gfi1b+-, and gfi1b-/- e12.5 total fetal liver (FL cells).

Figure 23: Time course of Rgs18 expression pattern in $gfi1b^{+/+}$ and $gfi1b^{-/-}$ fetal liver cells.

A. Time course (from day 0-5) of GPIIb (glycoprotein IIb/CD41), Gfi1b and Rgs18 message levels in wild type e12.5 fetal liver cells differentiated along the megakaryocyte lineage relative to day 0 (d0), following normalization for HPRT. B. Rgs18 mRNA levels in WT ($Gfi1b^{+/+}$) and mutant ($Gfi1b^{-/-}$) fetal liver cells cultured along the megakaryocytic lineage for the indicated periods. Mean and sd (±) from three independent experiments are shown. P values were $<0.0001$ (****), $<0.001$ (**), $<0.01$ (***), and $<0.05$ (*) respectively were calculated using Anova and student T test.

**Figure 24: Rgs18 promotes differentiation of the megakaryocytic cell line, L8057.**

A. Western blot of Rgs18 protein levels upon inhibition and over-expression in the megakaryocytic line L8057. 50µg protein was loaded in each lane. B. qPCR analysis of Rgs18 mRNA level and differentiation markers upon Rgs18 inhibition. C. qPCR analysis of Rgs18 mRNA level and differentiation markers upon Rgs18 over-expression. D. Acetylcholine esterase staining of Rgs18 manipulated cells. Mean ± sd of acetylcholine positive cells as a % of the total population from three independent experiments is indicated in parentheses. E. Megakaryocytic marker expression in cells expressing both Rgs18 shRNAs and cDNA (±Rgs18) versus controls. Mean and sd (±) from three independent experiments are shown. P values were <0.0001 (****), <0.001 (***), <0.01(**) and <0.05 (*) respectively were calculated using Anova and student T test.

Figure 25a: Augmentation of megakaryocytic differentiation by Rgs18.

A. qPCR analysis of Rgs18 mRNA levels and differentiation markers GPIIb, PF4 (platelet factor 4) and vWF (vonWillebrand factor) upon shRNA mediated Rgs18 inhibition (sh-Rgs18) relative to scrambled shRNA controls (Scr-Ctrl) in e12.5 fetal liver cells differentiated into megakaryocytes. B. qPCR analysis of analogous markers upon Rgs18 cDNA/protein over-expression (Rgs18 o/e) relative to vector control (pCDH-Ctrl) in the same cells. Mean and sd (±) from three independent experiments are shown. P values were <0.0001 (****), <0.001 (**), <0.01 (**) and <0.05 (*) respectively were calculated using Anova and student T test. C. Western blot of total Rgs18 protein levels following inhibition and over-expression as indicated, in primary (fetal liver derived) megakaryocytes relative to b-actin. 35µg of protein was loaded in each lane.
Figure 25b: Augmentation of megakaryocytic differentiation by Rgs18.

D. Acetylcholine esterase staining of Rgs18 manipulated cells as indicated. Mean ± standard deviation of acetylcholine positive cells as a percent of the total population from three independent experiments is indicated in parentheses. E. FACs analysis of CD9 and CD41 expression in fetal liver cells cultured into megakaryocytes following the indicated manipulations into as indicated. One of three representative experiments is shown.

Figure 26: Rgs18 suppresses differentiation of the erythroid cell line, MEL.

A. Western blot of Rgs18 protein levels upon inhibition in MEL (erythroid) cells. 50 μg protein was loaded in each lane. B. qPCR analysis of Rgs18 message and differentiation markers upon Rgs18 inhibition. C. Benzidine staining of Rgs18 manipulated cells. Mean ± standard deviation of benzidine positive cells as a % of the total population from three independent experiments is indicated in parentheses. D. Erythroid marker expression in cells expressing both Rgs18 shRNAs and cDNA (±Rgs18) versus controls. Mean and sd (±) from three independent experiments are shown. P values were <0.0001 (****), <0.001 (**), <0.01(**), and <0.05 (*) respectively were calculated using Anova and student T test.

Figure 27: Suppression of erythroid differentiation by Rgs18.

A. qPCR analysis of Rgs18 message levels and differentiation markers EB3 (erythrocyte band 3), GypA (glycophorinA/ter119) and Globin (b major globin) upon shRNA mediated Rgs18 inhibition (sh-Rgs18) relative to controls (Scr-Ctrl) in e12.5 fetal liver cells differentiated along the erythroid lineage. B. qPCR analysis of the same erythroid markers and cells upon Rgs18 cDNA/protein over-expression (Rgs18 o/e) relative to vector control (pCDH-Ctrl). P values were <0.0001 (****), <0.001 (**), <0.01 (**) and <0.05 (*) respectively. C. Benzidine staining of Rgs18 manipulated erythroid cells as indicated. Mean ± standard deviation of acetylcholine positive cells as a percent of the total population from three independent experiments is indicated in parentheses. D. FACS analysis of the surface markers ter119 and c-kit in control and Rgs18 manipulated fetal liver cells cultured into erythroid cells as indicated. Averages and standard deviations from 3 independent experiments are shown in A-C. Results from one of three representative experiments is shown in D.

Figure 28. Reciprocal regulation of erythro-megakaryocytic lineage specification by Rgs18.

A. Isolation of hematopoietic (lin-c-kit+) progenitors from e12.5 fetal liver cells for transduction with Rgs18 cDNAs (Rgs18o/e) and shRNAs (sh-Rgs18) in B. B. Expression of erythroid and megakaryocytic surface markers ter119 and CD41 respectively, in lin-c-kit+ progenitors co-cultured with a mixture of erythroid and megakaryocytic cytokines following the indicated manipulations. Results from one of three representative experiments is shown in B (courtesy G. Upadhyay).

Figure 29. Expression and manipulation of Rgs18 in K562 cell line.

Expression of Rgs18 (A) and other messages as indicated in the human hematopoietic cell line K562 transduced with Rgs18 cDNA and differentiated along the erythroid (+Na but) (B) or megakaryocytic (+TPA) (C) lineages. D. Total Rgs18 and b-actin protein levels in uninduced K562 cells (Un; top two panels) and upon differentiation into erythroid (+Na-but; middle two panels) and megakaryocytic cells (+TPA; bottom two panels), respectively. E. Histochemical staining of K562 cells differentiated along the erythroid (top panels) or megakaryocytic (bottom panels) lineages following transduction with empty vector (pEF4-Ctrl) or Rgs18 cDNA (Rgs18 o/e). Erythroid cells were stained for benzidine (top panels), megakaryocytic cells were visualized by generic May-Grunwald Giemsa staining (bottom panels). Mean and sd (±) from three independent experiments are shown. P values were <0.0001 (****), <0.001 (**), <0.01(***), <0.05 (*) respectively were calculated using Anova and student T test. Averages and standard deviations from 3 independent experiments are shown in A-C. (Ref: Sengupta, A. et al. Journal of Cell Science 2015 pii: jcs.177519.)
**Figure 30. Identification of indels in rgs18 founders.**

A. Identification of indels in select rgs18 founders by the T7 endonuclease assay.

B. Nucleotide and amino acid sequence of exon1 of rgs18. The sequence altered in the founders is indicated in bold maroon letters.

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<th>Peptide sequence (predicted)</th>
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**Figure 31. Characterization of mutations in rgs18 founders.**

Nucleotide sequence analysis of rgs18 founder DNA flanking the lesion along with the predicted amino acid sequence. Deletions in DNA sequence are indicated in red and the insertions in blue. Altered amino acid sequence is indicated in red.
Figure 32: Differential regulation of MAPK pathways by Rgs18.

A-B. Effect of Rgs18 inhibition (A) and over-expression (o/e) (B) relative to controls, on pERK1/2 levels in induced L8057 [megakaryocytic (Mk) line +TPA]. Top panel: pERK1/2; middle panel: total ERK1/2; bottom panel: β-actin. C. Effect of Rgs18 inhibition on the MAPK-ERK1/2 pathway in induced MEL cells [Erythroid (Ery) line +DMSO). D-E. Effect of Rgs18 inhibition (D) and over-expression (E) on p38-MAPK protein and phosphorylation levels in megakaryocytes. Top panel: phospho p38; middle panel: p38α; bottom panel: β-actin. F. Effect of Rgs18 inhibition on the p38-MAPK pathway in erythroid cells. 50µg of protein was loaded in panels A,D,E,F and 80µg loaded in B&C. One of 3 representative experiments is shown. G. Table summarizing the effects of Rgs18 manipulation on ERK1/2 and p38 pathways versus differentiation of erythro-megakaryocyte cells. Arrows indicate increase (↑) or decrease (↓) in activity and differentiation (Diff), respectively.

Figure 33: MAPK phosphorylation kinetics in differentiating megakaryocytes.

A. Time course of ERK phosphorylation (top panel) versus total ERK protein (middle panel) and b-actin (loading control; bottom panel) in L8057 cells induced to differentiated for the indicated periods (hours). B. Time course of p38 phosphorylation (top panel) versus p38α protein (middle panel) and b-actin (loading control; bottom panel) in L8057 cells induced to differentiated for the indicated periods (hours).

Figure 34: Changes in expression pattern of transcriptional effectors of Rgs18.

A-D. Klf1 and Fli1 mRNA levels (normalized for HPRT) in control versus Rgs18 inhibited (sh-Rgs18; A, B) or over-expressing (C and D), fetal liver derived erythroid (A and C) and megakaryocytic (B and D) cells. Mean and sd (±) from three independent experiments are shown. P values were <0.0001 (****), <0.001 (**), <0.01(*) and <0.05 (*) respectively were calculated using Anova and student T test.

Figure 35: Rescue of megakaryocytic differentiation in Rgs18-inhibited primary fetal liver cells.

A. qPCR assay showing rescue of megakaryocytic differentiation in Rgs18-inhibited (sh-Rgs18) primary megakaryocytic cells upon Fli-1 overexpression (sh-Rgs18+Fli1o/e) as assessed by marker analysis and acetylcholine esterase staining. B. The mean and sd± of acetylcholine positive cells as a percentage of total population from three independent experiments as indicated in parentheses. P values were <0.0001 (****), <0.001 (**), <0.01(*) and <0.05 (*) respectively were calculated using Anova and student T test. C. Megakaryocyte markers CD9 and CD41 expression in fetal liver cells cultured for 5 days along the megakaryocytic lineage following indicated manipulation.

Figure 36: Samples of megakaryocytic colonies upon differentiation of fetal liver cells.

A. Megakaryocyte colony numbers obtained from fetal liver cells cultured for 5 days in methyl cellulose supplemented with IL-3 and thrombopoietin following Rgs18 inhibition (sh-Rgs18) and Rgs18 inhibition and Fli1 over-expression (shRgs18; Fli1 o/e) relative to scrambled controls (control). B. Samples of 5 day old megakaryocytic colonies from control (scrambled), Rgs18 inhibited (sh-Rgs18) and Rgs18 inhibited and Fli1 over expressing (sh-Rgs18+Fli1o/e) fetal liver cells following in vitro culture in methyl cellulose supplemented with IL-3 and thrombopoietin. Mean and sd (±) from three independent experiments are shown. P values were <0.0001 (****), <0.001 (***) and <0.01 (**) respectively were calculated using Anova and student T test in A.

Figure 37: Diagrammatic representation of the process of purification of biotinylated protein and its interactors.

Rgs18 was inserted into a vector with biotin tag. Both Rgs18-biotag and BirA plasmid were expressed in L8057 (megakaryoblastic) and MEL (erythroid) cells. The interacting proteins were pulled along with the biotinylated protein using streptavidin-agarose beads.
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Figure 38: Potential Rgs18 interacting proteins.

A selective list of the number of independent peptides obtained from three independent mass spectrometric analysis of affinity purified Rgs18 and protein complexes relative to a BirA (control) from the uninduced L8057 (megakaryoblastic) cells. The peptides are categorized based on their ontology, affiliation and highlighted with different colors. The yellow color represents G protein group, blue Ras-MAPK pathway proteins, cAMP pathway proteins are represented in orange, peptides from integrins and cytoskeletal proteins are represented in green, scaffolding protein is represented by light blue. The peptides from proteins that cannot be categorized in any section are written in black. Rgs18 is highlighted in red.
Figure 39: Expression profiling of Rgs proteins in gfi1b+/+ and gfi1b-/- cells.

Relative message levels of multiple Rgs proteins known to be expressed in hematopoietic cells, in gfi1b+/+ versus gfi1b-/- cells from e12.5 total fetal livers representing predominantly erythroid populations. Mean and sd (±) were calculated from three independent experiments. P values were <0.0001 (****), <0.001 (**), <0.01 (***), <0.05 (*) respectively calculated by using student t test and Anova.

**Figure 40: Proposed model of regulation of erythro-megakaryocytic differentiation.**

Proposed trajectory of erythro-megakaryocytic lineage specification from progenitors (MEPs) as mediated by Gfi1b and Rgs18. Reciprocal expression of Gfi1b and Rgs18 in erythroid and megakaryocytic cells is indicated by colored trapezoids; green being stimulatory and red inhibitory. Alterations in signaling pathways and downstream gene expression following differential Gfi1b and Rgs18 expression, are indicated by vertical arrows.

Figure 41: Possible link between G protein signaling, cAMP and MAPK pathways and their regulation by Rgs18 during megakaryocyte differentiation.

Rgs18 regulates the cAMP and Ras-MAPK pathways by possibly interacting with Ga_i, CAP1, PKA, RasGAP binding proteins and Ras-GTPase activating proteins that are essential for modulation of AC-cAMP-PKA and Ras-MAPK pathways.
References


