

DISCOVERY AND FUNCTIONAL CHARACTERIZATIONS OF PLAG1
IN
HUMAN HEMATOPOEITIC STEM AND PROGENITOR CELLS

**DISCOVERY AND FUNCTIONAL CHARACTERIZATIONS OF PLAG1 IN
HUMAN HEMATOPOEITIC STEM AND PROGENITOR CELLS**

BY

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ABSTRACT

Discovery and functional characterization of self-renewal regulatory factors constitute one of the central themes in the field of human hematopoietic stem and progenitor cell (HSPC) biology both for advancing the fundamental science as well as harnessing the knowledge to expand hematopoietic stem cells (HSCs) for regenerative therapeutics purposes.

Musashi-2 (MSI2) is a marker and essential positive regulator of HSCs but knowledge of the transcription factors (TFs) that ensure its appropriate HSC-specific expression is lacking. I believed that uncovering the transcriptional circuitry of MSI2 could also offer opportunities for the discovery of candidate TFs with similar or superior functions in human HSPCs. To that end, initial investigations were set out to identify a conserved minimal promoter region bound by TFs upstream of the translation start site of human MSI2. *In silico* analysis of the minimal promoter region and series of TF binding-site mutagenesis biochemical assays identified USF2 and PLAG1 as key co-regulators of MSI2 expression and function in the model K562 cell line and cord blood HSPCs. The approach identified PLAG1 as a candidate TF whose function in HSPCs was not previously reported.

I have characterized PLAG1 as an important regulator of HSPCs self-renewal using *in vitro* and xenotransplantation functional genomics coupled with RNA sequencing and downstream pathway analysis. *In vitro*, overexpression of PLAG1 (PLAG1^{OE}) imparts sustained pro-survival advantage to progenitors and produces significantly more CD34⁺ cells compared to control culture. PLAG1^{OE} enhances BFU-E and total colony forming

unit (CFU) output. Complementary *in vitro* assays using shRNA-mediated knockdown (PLAG1^{KD}) elucidated impaired CFU output, increased apoptosis and significantly reduced CD34⁺ cell counts. *In vivo* transplantation of PLAG1^{OE} CD34⁺ cells into NSG mice at limiting doses robustly enhances the frequency and absolute number of HSCs by a magnitude of 24.1-fold compared to control. Serial transplantation of bone marrow cells into secondary recipients demonstrated enhanced self-renewal of HSCs compared to control. Mechanistically, transcriptome-wide gene-set enrichment and network analysis revealed that ribosome biogenesis and proteostasis pathways are significantly attenuated. PLAG1^{OE} up-regulates expression of H19 and MEG3 imprinted long non-coding RNA (lncRNA)-derived miR-675 and miR-127 species respectively. *In silico* overlap analysis with experimentally validated targets of both miR-127 and miR-675 revealed ribosome biogenesis and proteins synthesis pathways components as prime targets. PLAG1 also represses the pan-ribosome biogenesis transcriptional regulator MYC. Down regulation of MYC demonstrates another layer of PLAG1-mediated transcriptional attenuation of protein synthesis. Additionally, we observed dampening of IGF1R/PI3K/AKT/mTOR signaling pathway. Conversely, PLAG1^{KD} down regulated H19 and MEG3 expression and reverses global gene-set enrichment observed by PLAG1^{OE} in reciprocal fashion. Taken together, our study presents PLAG1 as master regulator of ribosome biogenesis and protein synthesis from multiple checkpoints to enhance clonogenicity, survival and long-term self-renewal capacity of HSCs.

ACKNOWLEDGMENTS

It has been quite a journey! A former full-time, well-paid (Huh, at least I thought so) research lab technician/manager transitioned to be a graduate student willing to tradeoff with student stipend! Well, curse/praise (however you interpret it) that childhood passion for life science instilled in me by otherwise “uneducated” curious mother who still to date is so inquisitive about the nature of things.

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Jon and Andre, you have been a great source of guidance, support and critical evaluation of my experimental results and bring my PhD projects to completions. I am indebted for your supports whenever I needed especially during that summer of 2016. Thank you indeed!

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LIST OF ABBREVIATIONS

3'-untranslated region	3'-UTR
7-aminoactinomycin-D	7-AAD
Acute myeloid leukemia	AML
Adeno-associated viruses	AAV
Alexa fluour	AF
Angiogenin	ANG
Aryl hydrocarbon receptor	AHR
Bacterial artificial chromosome	BAC
Blast forming unit erythroid	BFU-E
Blue fluorescent protein	BFP
Bone marrow	BM
Bone marrow transplant	BMT
CCCTC-binding factor	CTCF
Chelator tetraethylenepentamine	TEPA
Chromatin Immuno-Precipitation-sequencing	ChIP-seq
Chromatin immunoprecipitation	ChIP
Chromatin immunoprecipitation-quantitative PCR	ChIP-qPCR
Chronic myeloid leukemia	CML
Cobblestone area-forming cells	CAFC
Colony-forming units	CFU

Colony-forming units in culture	CFU-C
Colony-forming units in spleen	CFU-S
Common lymphoid progenitors	CLP
Common myeloid progenitors	CMP
Cord blood	CB
Cross-Linking Immune-Precipitation-sequencing	CLIP-seq
Cytomegalo virus	CMV
Dendritic cells	DCs
Developmental pluripotency-associated 5	Dppa5
Differential expression	DE
Differentially methylated region	DMR
Dimethyl sulfoxide	DMSO
Dimethyl-prostaglandin E2	dmPGE2
Dipeptidylpeptidase 4	DPP4
DNA methyl transferase	DNMT
Dulbecco's Modified Eagle's Media	DMEM
Encyclopedia of DNA elements	ENCODE
Endoplasmic reticulum UPR	UPR ^{ER}
Erythroid progenitors	EP
Eukaryotic initiation factor	eIF
Extra physiological oxygen shock/stress	EPHOSS
Extreme limiting dilution analysis	ELDA

False discovery rate	FDR
Fetal bovine serum	FBS
FLT3 Ligand	FLT3
Fluorescence activated cell sorting	FACS
Forward-scatter	FSC
Gene ontology	GO
Gene set enrichment analysis	GSEA
Graft-versus-host disease	GVHD
Granulocyte-colony stimulating factor	G-CSF
Granulocyte-erythroid-megakaryocyte-monocyte	GEMM
Granulocyte-monocyte progenitors	GMP
Green fluorescent protein	GFP
Hematopoietic stem and progenitor cell	HSPC
Hematopoietic stem cell	HSC
Histone deacetylase	HDAC
Human embryonic kidney	HEK
Human leukocyte antigen	HLA
Immuno-histochemical	IHC
Immunofluorescence	IF
Immunoglobulin G	IgG
Imprinted genes	IGs
Imprinting control region	ICR

Induced-pluripotent cell	iPSC
Insulin-like growth factor	IGF
Insulin-like growth factor receptor	IGFR
Integrated genome viewer	IGF
Intergenic germline-derived DMR	IG-DMR
Iscoe's Modified Dulbecco Media	IMDM
Knockdown	KD
Leukemic inhibitory factor receptor	LIFR
Limiting dilution analysis	LDA
Long non-coding RNA	lncRNA
Long-term culture initiating cells	LTC-IC
Long-term-HSCs	LT-HSCs
Luciferase	Luc
Mammalian target of rapamycin	mTOR
<i>Maternally expressed gene-3</i>	<i>MEG3</i>
Median fluorescent intensity	MFI
Megakaryocyte-erythroid progenitor	MEP
Mesenchymal stem cell	MSC
Messenger RNA	mRNA
MicroRNA	miRNA
Mitogen-activated protein kinase	MAPK
Mobilized peripheral blood	MPB

Mobilized peripheral blood	MPB
Mouse stromal	MS
Multi-lymphoid progenitors	MLPs
MUltiple sequence Local AligNment	MULAN
Multipotent progenitors	MPP
Murine stem cell virus	MSCV
Musashi-2	MSI2
Myelodysplastic syndrome	MDS
Myeloid progenitors	MP
N-acetyl-cysteine	NAC
Natural killer	NK
Next-generation sequencing	NGS
NOD-Scid-Gamma chain null	NSG
Non-obese diabetic	<i>NOD</i>
Nuclease accessible site	NAS
O-propargyl-puromycin	O-p-puro
Osteoblast secreted extracellular vesicles	Osteoblast-EVs
Over expression	OE
Oxidative phosphorylation	OxPhos
Pleomorphic adenoma gene -1	PLAG1
Pleomorphic adenoma gene-like -1	PLAGL-1
Pleomorphic adenoma gene-like -2	PLAGL-1

Polyacrylamide gel electrophoresis	PAGE
Polycomb repressor complex	PRC
Polymerase chain reaction	PCR
Quantitative real time –PCR	qRT-PCR
Reactive oxygen species	ROS
Red fluorescent protein	RFP
Revolutions per minute	RPM
Ribosomal DNA	rDNA
Ribosomal proteins	RP
Ribosomal RNAs	rRNAs
Ribosome biogenesis	Ribi
RNA binding proteins	RBP
RNA interference	RNAi
RNA polymerase	RNA Pol
RNA sequencing	RNA-seq
SCID repopulating cells	SR
Severe Combined Immune-Deficient	SCID
Short-hairpin RNA	shRNA
Short-term-HSCs	ST-HSCs
Side-scatter	SSC
Signaling lymphocyte activation molecule	SLAM
Small nucleolar RNA	snoRNA

Spleen focus forming virus	SFFV
Stem cell factor	SCF
StemRegenin-1	SR1
Thrombopoietin	TPO
Total nucleated cells	TNCs
Transcription factor	TF
Ubiquitin-proteasome system	UPS
Unfolded protein response	UPR
Upstream stimulating factor-2	USF2
Valproic acid	VPA
Vesicular stomatitis virus –G	VSV-G

DECLARATION OF ACADEMIC ACHIEVEMENT

I designed the experiments, interpreted and analyzed the results and wrote the respective manuscripts. Dr. Kristin Hope supervised the projects, helped with interpreting results and writing of the manuscripts. Dr. Stefan Rentas performed experiments. Drs. Veronique Voisin and Gary Bader supervised and performed all the bioinformatics pathway analysis. Specific contributions and additional acknowledgments are included in the respective Chapters.

CHAPTER 1

INTRODUCTION

1.0 Preamble

Human hematopoietic stem cell (HSC) self-renewal is a process critical for normal functioning of the blood system however its regulation is still not fully understood. The new knowledge presented in this thesis was in direct follow up to the seminal works identifying and characterizing the novel HSC self-renewal regulating roles of Musashi-2 (MSI2) (Hope et al., 2010a; Rentas et al., 2016). With the goal of identifying novel HSC self-renewal transcriptional circuitry, I explored the promoter region and associated trans acting factors of human MSI2, the results of which are presented in **Chapter 2**. This approach led to the discovery of the transcription factor (TF) PLAG1 in particular as a previously uncharacterized and yet important regulator HSC self-renewal, the hematopoietic stem and progenitor (HSPC)-specific characterization of which I thoroughly carried out and discuss in **Chapter 3**. The significances, limitations and further implications of the findings are thoroughly discussed in **Chapter 4**. In **Chapter 1**, I aim to outline foundational observations and contemporary approaches for the study of human HSC function and regulation. Strategies towards *ex vivo* expansion of HSCs for the purpose of regenerative medicine are also discussed. The chapter ends with summary of intent presenting the thesis research questions, formulated hypotheses and specific experimental objectives formulated to address these.

1.1 Historical perspectives: Foundational observations and experiments in the study of HSCs

The diversity and sustained production of all mature blood cells is made possible by self-renewing hematopoietic stem cells (HSCs) primarily residing in the bone marrow (BM) (Doulatov et al., 2012; Orkin and Zon, 2008). HSCs progressively differentiate into lineage specific precursors, which in turn produce different types of terminally and functionally differentiated effector cells of the erythroid, myeloid and lymphoid lineages that together fulfill the daily needs of an individual. The blood system and its organizational structure through development and in adult homeostasis has been the subject of decades of detailed study and as such constitutes a now relatively well-deciphered stem-cell directed system functional in higher organisms.

Historically, the study of HSCs had humble but profound beginnings that date back to the atomic weaponry era in the early to mid 20th century. Patients exposed to lethal doses of radiation were found to suffer early mortality largely as a result of acute BM failure. Inadvertent transplantations (without the knowhow and understanding of regenerative cell therapy) of marrow ‘humour’ from individuals that were not exposed to the radiation dramatically rescued the health of radiation-exposed recipient patients (Lorenz et al., 1952; Lorenz et al., 1951). Around the same time, Jacobson et al observed that syngeneic and congenic BM intravenous injections were able to provide radioprotection to recipient mice and to regenerate within them the hematopoietic system (Jacobson et al., 1950; Jacobson et al., 1951). Seminal works of Thomas et al pioneered the therapeutic power of BM transplantation in humans(Thomas et al., 1957). However, none of those studies

attempted to operationally demonstrate the presence of multipotent cells capable of regenerating the entire hematopoietic lineages. Clearly, those observations set the stage for subsequent seminal experimental demonstrations by Till and McCulloch (McCulloch and Till, 1960; Till and Mc, 1961). They provided the first evidence for the presence of multipotent cells responsible for the clonal regeneration of cells from each of the erythrocyte, megakaryocyte and granulocyte lineages. These cells appear as macroscopic nodules in the spleen of irradiated recipient mice 2 weeks post-transplant. Till and McCulloch termed the cells that made the nodules colony-forming units in spleen (CFU-S) (Becker et al., 1963; McCulloch and Till, 1960; Till and Mc, 1961). These observations brought further impetus to characterize and quantify the multipotent cells based on the extent of lymphoid and myeloid blood cells produced in the repopulated recipient mice. Consequently, they injected limiting doses of donor BM cells into myeloablated recipient mice and quantified the number of CFU-Ss 14 days later (Till and Mc, 1961). Interestingly, they observed a direct correlation between the number of cells injected and colonies counted. Next, in order to define whether each CFU-S is generated from a single donor-derived multipotent cell, they treated the donor marrow with ionizing gamma-irradiation and induced chromosomal lesions that can subsequently be detected by standard cytogenetic analysis (Becker et al., 1963). Many of the CFU-S were a mixture of lineages of myeloid cells (erythroid, megakaryopoietic and granulocyte) and yet, strikingly, each single lineage colony was derived from a single donor-derived multipotent cell as defined by the common presence of a unique cytogenetic mark and hence were concluded to be clonal (Becker et al., 1963). Further dose-dependent serial

transplantation of multilineage CFU-S into secondary recipient mice generated distinct and multi-lineage colonies - a demonstration of the primitiveness of the precursor cells in the primary colonies (Siminovitch et al., 1963; Siminovitch et al., 1964; Till et al., 1964b). Moreover, size, number and types of colonies in both primary and secondary transplantations were heterogeneous, a phenomenon thought to be a result of stochastic effects imparted by the splenic microenvironment (Till et al., 1964b). Subsequently, Becker et al elegantly demonstrated using a 20 minute *in vitro* nucleoside analogue incorporation assay that under steady state hematopoiesis colony forming cells are non-cycling (Becker et al., 1965). However, when regenerative demands arise, 40 - 65 % of these cells are found undergoing DNA synthesis and thus in a proliferative phase. Later, Wu et al showed that the precursor cells that made each CFU-S have also the predisposition to make functional lymphoid cells in the thymus of the recipient mice - a demonstration of common ancestry of lymphoid and myeloid cells (Wu et al., 1968).

Despite their elegance at the time, with advances in the field the seminal insights these experiments are now recognized to have provided were restricted to progenitors as the assays used lacked the capacity to measure true “stemness”. In fact, lineage committed progenitors with limited proliferative potential we now know are capable of producing the respective CFU. In addition, the 14 day post-transplant endpoint readouts used by Till and McCulloch do not permit a ‘long-term’ assessment of primitiveness and measure progenitor activities at best. Nonetheless, these pioneering experiments were the foundation for the explosion of discoveries in the field of HSC biology and inspired other adult stem cell biologists to follow suit. Indeed the fundamentals of the assays used to

determine CFU-S activity are still used today to characterize aspects of HSPC biology. These include, the short-term *in vitro* progenitor assays used to measure clonogenicity and which were inspired by the *in vivo* CFU-S assay; serial transplantation assays to assess extensive self-renewal and clonal propagation ability of HSCs; the theoretical framework for clonal tracking of genetically labeled single cells during *in vivo* transplantation; heterogeneity within a seemingly homogenous HSC compartment as determined by limiting dilution analysis (LDA). In addition these early findings set the stage for the proposition of a rudimentary hematopoietic hierarchy at the apex of which are BM precursor cells producing all the lymphoid and myeloid cells. This forms the basis for the classic model of hematopoiesis which has lasted for 60 years. Most importantly, the CFU-S assays laid the foundation for the birth of a new field of clinical therapy using donor-derived cells for regenerative medicine.

1.2 Development of functional assays to measure human HSPCs activity

The eternal challenge in the field of HSC biology is the designing of assays that unambiguously decipher the functional heterogeneity inherent within the stem and progenitor cell compartments. Assays measuring progenitor activity could not be recapitulated to measure primitive stem cells and vice versa, because of differences in underlying factors controlling these respective populations. The complexity has also been exacerbated by the poor predictive power of cell surface markers traditionally used for characterizations of HSPCs. It is established now that *in vivo* transplantations using immune-compromised mice is the gold standard assay for measuring the extensive human HSC self-renewal as well as their ability to undergo the full extent of lymphomyeloid

differentiation decisions. However *in vitro* assays are restricted to measuring progenitor activity only.

1.2.1 Development of *in vitro* functional assays for progenitor cells

Pluznik and Sachs pioneered the recapitulation of the CFU-S assay by dissociating and plating single cell suspensions of spleen in agar for ~10 days. The *in vitro* assay marked the identification of macroscopic colony forming units in culture (CFU-C) (Pluznik and Sachs, 1965). Using density gradient centrifugation and bone marrow fractionation, Worton et al. later indicated the developmental primitiveness of CFU-S as compared to CFU-C (Worton et al., 1969). The establishment of a murine CFU-C assay coupled with the discovery of growth promoting cytokines inspired others in pursuit of complementary *in vitro* assays to identify and functionally characterize human BM progenitors (Entringer et al., 1977; Moore et al., 1973; Pike and Robinson, 1970). Coincidentally, the advent of monoclonal antibodies coupled with multi-parametric fluorescence activated cell sorting (FACS) facilitated prospective isolation of human progenitor cells (discussed in section 1.3). FACS is a rapid tool for prospective isolation of cells and their phenotypic characterization however it does not measure the functional properties of progenitor cells until it is coupled with CFU assays by plating cells in semi-solid methylcellulose media supplemented with growth promoting cytokines. Myeloid progenitors measurable through this assay can create blast forming erythroid (BFU-E), granulocyte (CFU-G), monocyte/macrophage (CFU-M), granulocyte-monocyte (GM) or more primitive mixed lineage colonies (GEMM) within a culture period of 10 - 14 days. Serial re-plating of GEMM colonies can also provide information on the self-renewal ability of the

progenitors that derive them and hence replating is a surrogate functional assay for measuring primitive progenitor activity. As compared to CFU assays, measurements of cobblestone area-forming cells (CAFC) and long-term culture initiating cells (LTC-IC) are lengthier (Conneally et al., 1997; Gartner and Kaplan, 1980; Lansdorp and Dragowska, 1992; Petzer et al., 1996; Sutherland et al., 1989; Sutherland et al., 1991; Sutherland et al., 1990). In both CAFC and LTC-IC assay systems, primitive cells are co-cultured with irradiated stromal “feeder” layers and are able to generate myeloid clonogenic progenitors and mature granulocytes and macrophages for more than 5 weeks under appropriate cytokine supplemented culture conditions. Primarily this culture system is used to quantify primitive progenitors capable of initiating and sustaining myeloid cell production for many weeks (Sutherland et al., 1990) though the culture system has also been developed and demonstrated for natural killer (NK) cells as well (Miller et al., 1992). As a long-term culture surrogate system, albeit shorter in duration compared to long-term xenotransplantation, the cells assayed with this approach share some functional and phenotypic properties with SCID repopulating cells (SRCs) (discussed in section 1.2.2) (Conneally et al., 1997; Hogge et al., 1996). Operationally, quantitation of LTC-IC requires culturing the primitive cells on the irradiated “feeder” cell layer followed by seeding of precursor cells for CFU assays at limiting cell doses. Hence, operationally, LTC-ICs are developmentally more primitive than CFU-Cs. The assay assumes that each CFU is a product of terminal differentiation of each of the plated cells. LDA is then applied to determine the frequency of LTC-IC as well as the number of CFU produced per each dose of precursor LTC-IC using Poisson statistics (Sutherland et al., 1990). As is

the case during the evolution of xenograft mouse models (discussed in section 1.2.2), improvements to the LTC-IC assay have been established. In particular, these resulted in increased reproducibility, longevity and sensitivity of readout through the use of genetically engineered stromal layers expressing human cytokines (Sutherland et al., 1991). Nonetheless, the appreciation of LTC-IC declined with the advent of xenograft models capable of measuring both stem and progenitor activity in near physiological conditions. Importantly, conventional CFU assays are deficient in assessing lymphopoiesis *in vitro*. This is partly due to the lack of an appropriate ‘cocktail’ of cytokines to support *in vitro* differentiation of lymphoid cells (B, T and NK cells). Nonetheless, 2 weeks co-culture of human progenitors with mouse stromal (MS) MS5 and MS17 cells (with SCF, TPO, IL-7, and IL-2 cytokines) are able to generate B and NK colonies (Collins and Dorshkind, 1987). While co-culture with the OP9-DL1 stromal line (expressing the Notch ligand Delta-like 1) support only T-lymphopoiesis (La Motte-Mohs et al., 2005; Van Coppenolle et al., 2009). Overall, the spectrum of *in vitro* assays developed to date provided important measures to quantify and characterize progenitor cells but the limitations of these *in vitro* assays ‘primed’ the field towards the discovery and optimization of xenograft models to functionally characterize primitive HSPCs.

1.2.2 Development of xenograft models of human hematopoiesis

The road to development of the SCID model

Unlike the very elegant, extensive studies of murine HSCs using syngeneic and/or congenic models (Purton and Scadden, 2007), progress in the study of human HSCs was severely limited by the absence of a surrogate model system recapitulating homeostasis.

To circumvent these limitations initial efforts were attempted by *in vivo* transplantations into non-human primates and large mammals (because of closeness in size and life span). At first this was done through *in utero* injections of human BM CD34⁺ cells into immune-competent fetal sheep which demonstrated the persistence of all erythroid, granulocytic/monocytic and megakaryocytic human hematopoietic cells for more than 7 months (Srour et al., 1992; Srour et al., 1993). Alternative autologous *ex vivo* expanded mobilized peripheral blood (MPB) CD34⁺ HSC transplantation was performed in monkeys with the idea of extrapolating the findings to the human context (Norol et al., 2002). Issues of cost and space confound the sustainability of using large animals for xenotransplantation however and this prompted the development of immuno-compromised mouse models. Skeptics argued against the relevance of the latter due to species-specific differences in body size, lifespan, and kinetics of hematopoietic development (Dick, 2008). Epistemologically, the atomic weaponry era ignited the profound observations that laid the foundations for the study of HSCs (discussed above). The AIDS epidemic in the 1980's set the stage for a profound breakthrough in the discovery and experimental exploitation of an immune-compromised mouse model - the severe combined immune-deficient (*Scid*) mouse strain, which lacks functional B and T lymphocytes (Bosma et al., 1983; Fulop and Phillips, 1990). Fulop and Phillips (1990) mapped the mutations in the *Scid* strain to the gene *Prkdc*, responsible for V(D)J recombination and immunoglobuline expression (Fulop and Phillips, 1990).

The *Scid* model was superior in modelling elements of human hematopoiesis that included: 1) the reconstitution of human B and T cells after peripheral blood infusion

(*Scid-PBL*) in order to study human immune response to infections (such as HIV)(Boyle et al., 1995); 2) *de novo* lympho/myeloid differentiation of HSCs after surgical transplantation of fragments of human fetal BM, liver and thymus tissues (*Scid-hu*) (McCune et al., 1988; Mosier et al., 1991); 3) demonstration of hematopoietic reconstitution after intravenous injections of adult human BM HSC (*Scid-bnx*), as primarily spearheaded by John Dick and colleagues (Kamel-Reid and Dick, 1988; Lapidot et al., 1992). Technical impediments and heavy reliance on human fetal tissues in the *Scid-hu* model defaulted the study of HSC to *Scid-bnx* (or *Scid*). Contemporaneously, the *Scid-bnx* model was demonstrated to support long-term propagation of LTC-IC and CFU-C and serial transplantation of cells isolated from primary transplant(Cashman et al., 1997). This approach demonstrated sustained lymphomyeloid engraftment for over 4 months and hence achieved the criteria for the functional definition and quantification of HSCs as *Scid*-repopulating cells (SRCs). To overcome the very limitation of the *Scid* model (a lack of sustained myeloid engraftment), improvements to the model involved preconditioning mice with sub-lethal irradiation and concomitantly infusing pro-myeloid growth factors (IL-3, GM-CSF and SCF) (Clutterbuck et al., 1985). The *Scid* model laid the foundation upon which decades of human HSC work have relied in order to understand in detail the primitive hematopoietic hierarchy.

Development of humanized Scid xenograft models

‘Leaky’ B and T cell adaptive immune function that re-emerges with age hampered long term engraftment of human HSCs. Schultz et al perfected the *Scid* mouse by crossing it with non-obese diabetic (*NOD*) strain which in itself has defective innate immunity. The

resulting *NOD-Scid* mouse model supports superior levels of human HSC-derived engraftment (Shultz et al., 1995). Interestingly neither non-obese resistant (NOR) or BALB/c crossing was able to support human engraftment due to a polymorphic defect in the expression of the transmembrane protein *Sirpa*. The *Sirp* proteins (*Sirpa* and *Sirpb*) bind to their ligand CD47 and induce inhibitory signal preventing phagocytosis by resident macrophages (Jaiswal et al., 2009; Takenaka et al., 2007). The conserved expression of CD47 in human HSPCs made the *NOD-Scid* strain an ideal alternative.

Despite advances, the *NOD-Scid* strain has also other limitations. In particular they retain active NK cells and develop lethal thymic lymphomas (Shultz et al., 1995) ultimately hampering sought-after long-term human HSC engraftment. Therefore, further fine-tuning of the *NOD-Scid* strain was necessary by totally disabling B, T and NK cell function which can be enabled by deletion of the IL-2R common γ chain expressed on these cells and required as a cell surface receptor for cytokines IL-2, IL-4, IL-7, IL-9, IL-15, and IL-21. Crossing of NOD-Scid mice with IL-2R Gamma null mice resulted in the NSG (NOD-Scid-Gamma chain null) mouse strain which significantly increases human HSC-based long-term engraftment and lymphomyeloid reconstitution capacity by a magnitude of 6-fold compared to NOD-Scid (Ito et al., 2002; Shultz et al., 2005). In pursuit of further strain ‘humanization’, the NSG mouse has been genetically engineered to constitutively express human specific IL-3, TPO and GM-CSF and other cytokines. This conditions the BM environment for enhanced human HSC engraftment (Rongvaux et al., 2011; Willinger et al., 2011). Additionally, technical considerations regarding the route of injections (e.g. intra-femoral versus peripheral vein to overcome homing

inefficiencies) and choice of sex of the mouse (e.g. female NSG mice are superior over male mice) have been demonstrated to influence the engraftment and lymphomyeloid reconstitution outcome (Futrega et al., 2016; Gao et al., 2009; Mazurier et al., 2003; Notta et al., 2010; Wang et al., 2003). Advances in the development of more sensitive xenograft mouse models will derive better characterizations of human HSCs and facilitate their therapeutic applications. Development of the NSG strain has been one of the significant strides in the field of HSC biology facilitating important landmark studies (Doulatov et al., 2010; Notta et al., 2016).

Contemporary molecular biology tools to study hematopoiesis

The AIDS epidemic in the 1980s' also paved way for the repurposing of retro- and lentiviral genetic elements and the generation of replication-incompetent vectors for gene transfer (Naldini et al., 1996). Contemporaneously, RNAi post-transcriptional gene silencing technology (Fire et al., 1998) and green fluorescent protein (GFP) as a reporter of gene transfer (Chalfie et al., 1994) were discovered. By combining lentivirus-based RNAi (and overexpression), high throughput genetic screens and FACS, important HSC regulators have been identified. Such regulators include epigenetic modifiers and TFs (Deneault et al., 2009), miRNAs (O'Connell et al., 2010), lncRNAs (Luo et al., 2015) and RBPs (Hope et al., 2010a). Recent advances in next-generation sequencing (NGS) technologies such as chromatin immunoprecipitation sequencing (ChIP-seq) and RNA-seq coupled with global methylome and proteome profiling of purified primitive hematopoietic cell subpopulations further revolutionized the discovery of novel and complex epigenetic and transcriptional regulatory networks in HSCs (Cabezas-

Wallscheid et al., 2014; Novershtern et al., 2011; Sun et al., 2014). The advent of NGS also offered high-resolution single cell viral genetic barcoding and *in vivo* tracking to understand clonal heterogeneity within HSPCs (Cheung et al., 2013; Lu et al., 2011). Finally, the revolution in genome editing using CRISPR/Cas9 has already begun to fuel targeted genetic manipulations in HSPCS (e.g. deletion, replacement, tagging and correction) at unprecedented resolution (Gundry et al., 2016).

1.3 The human hematopoietic hierarchy

The capacity to self-renew and differentiate are two characteristics inherent to HSCs. Capitalizing on their observations of the intrinsic clonogenic heterogeneity within the CFU-S to produce macroscopic colonies of varying lineages upon serial transplantation (Siminovitch et al., 1963; Till et al., 1964a), Till and McCulloch, also pioneered the prospective isolation and enrichment of primitive hematopoietic cells capable of engraftment upon serial transplantations using density gradient centrifugation on fetal calf serum (Worton et al., 1969). Of note, their strategy marked the dawn of an era to operationally define ‘stemness’ in the context of hematopoiesis. The advent of multi-parametric flow cytometry techniques (Hulett et al., 1969) coupled with the availability of fluorescent molecule-conjugated antibodies against cell surface antigens (Kohler and Milstein, 1975) revolutionized the field of hematopoietic stem cell biology.

The discussion of the mouse hematopoietic hierarchy here is beyond the major themes of my PhD research. However, to provide historical perspective and fundamental parallels, I will highlight landmark studies in the prospective isolation and phenotypic characterization of mouse HSCs where the relative ease in prospectively purifying and

functionally characterizing mouse HSPCs pushed the field forward at unprecedented pace. Mouse HSCs were purified by the phenotypic marker combination of lineage depleted (Lin^-) $\text{Sca1}^+\text{c-Kit}^+$ (LSK) by Irving Weissmann's lab (Spangrude et al., 1988). Further incorporation of the Flk-2 and Thy1.1 markers into the LSK combination delineated HSCs into three phenotypically and functionally different sub-populations: $\text{Thy}^{(lo)} \text{Flk-2}^-$ (long-term (LT)-HSCs), $\text{Thy}^{(lo)} \text{Flk-2}^+$ (short-term (ST)-HSCs), and $\text{Thy}^- \text{Flk-2}^+$ multipotent progenitors (MPP) (Christensen and Weissman, 2001). The loss of Thy-1.1 and gain of Flk-2 expression marks the loss of self-renewal in HSC maturation. Further discovery of phenotypic markers better defined and enriched LT-HSC isolation from the mouse BM. These include low or negative expression of CD34 in combination ($\text{LSK CD34}^{-/lo}$) (Osawa et al., 1996), and the signaling lymphocyte activation molecule (SLAM) family of receptor $\text{Lin}^- \text{CD150}^+\text{CD48}^-$ (Kiel et al., 2005; Kim et al., 2006; Yilmaz et al., 2006a).

1.3.1 The human hematopoietic stem and progenitor compartment

For a long time the prospective isolation and enrichment of human HSCs has been made challenging by the extremely rare nature of these cells in the human BM (Wang et al., 1997), lack of reliable phenotypic markers to study these cells at a single cell or clonal level (Notta et al., 2011) and absence of standard post-xenograft endpoint durations to capture all the hematopoietic lineages outputs at any given time point unlike the case for syngeneic mouse HSC transplantations (Benveniste et al., 2010; Glimm et al., 2001). In the early 1980's, CD34^+ was identified as the first cell surface marker to, in the cell compartment devoid of lineage positive cells, enrich for human hematopoietic stem and

progenitor cells (Civin et al., 1984). Later CD34⁻ fractions also called CD34neg-SCID repopulating cells (CD34neg-SRC), came into the picture purportedly marking HSCs with distinctive and extensive lymphomyeloid repopulating ability (Bhatia et al., 1998; Ishii et al., 2011). Despite the historic utilization of the CD34⁺ marker for isolating HSPCs in experimental and clinical hematology (Kang et al., 2008; Vogel et al., 2000), additional markers were necessary to purify LT-HSCs. CD90 (Thy-1)⁺ was the next surface marker identified that when combined with CD34⁺ enriches for HSC from human fetal BM cells (Baum et al., 1992; Murray et al., 1995). Towards the end of the 20th century, further discoveries of monoclonal antibodies against differentiation markers CD45RA and CD38 enabled stringent isolation of HSCs using multi-parametric FACS (ie. Lin⁻CD34⁺CD90⁺CD38⁻CD45RA⁻) (Bhatia et al., 1997b; Conneally et al., 1997; Lansdorp et al., 1990). These phenotypic markers were functionally validated to demarcate a still heterogeneous HSC compartment capable of long term engraftment and multilineage reconstitution capacity and the consensus has been that gradual loss of CD90 and acquisition of CD38 expression marks the onset of MPP or rapid SRC identity. The latter population is characterized by its capacity to achieve only transient engraftment capacity in the *NOD-Scid* model (Majeti et al., 2007; Mazurier et al., 2003). One of the ultimate goals of utilizing xenograft models, as pioneered by the works of John Dick and colleagues in the 1980's, was to be able to use this system to uncover ideally one marker that could be used purify the 'mother of all LT- HSCs' under the most stringent sets of criteria. Work that has gotten the field closer to this goal showed that CD49f expression within the Lin⁻CD34⁺CD90⁺CD38⁻CD45RA⁻ fraction unequivocally marks LT-HSCs

capable of engrafting NSGs for more than 8 months, though it should be noted that LT-HSCs are still not isolated to purity in this scheme but are present at a frequency of 1 in 10.5. Whereas the $\text{Lin}^- \text{CD34}^+ \text{CD90}^- \text{CD38}^- \text{CD45RA}^- \text{CD49f}^-$ fraction constitutes MPPs with transient reconstitution capacity that peak for about 4 weeks post transplant (Notta et al., 2011) (Figure 1.1 and 1.2).

1.3.2 Lineage commitment in human hematopoiesis

The classic model of hematopoiesis postulates a pyramidal model at the apex of which are HSCs followed by the MPPs and immediate divergence of lymphoid and myeloid lineages through common lymphoid progenitors (CLPs) and common myeloid progenitors (CMPs) respectively, ultimately producing the 10 different mature blood cell types (Dick, 2008; Eaves, 2015) (Figure 1.2A).

Gradual differentiation produces bivalent oligopotential intermediates granulocyte-monocyte progenitors (GMPs) that proceed to make granulocytes and monocytes and megakaryocyte-erythroid progenitors (MEPs) that go on to produce megakaryocytes and platelets (Dick, 2008; Eaves, 2015; Spangrude et al., 1991; Till and McCulloch, 1980). According to this model CMPs ($\text{CD34}^+ \text{CD38}^+ \text{CD45A}^+ \text{IL-3R } \alpha^+$) hold a critical position in the lineage commitment to GMP and MEP as shown by the CFU assay (Manz et al., 2002). GMPs in turn differentiate into unipotent granulocyte and monocyte producing progenitors. MEPs, on the other hand, differentiate into megakaryocytes, platelet and erythroid producing unipotent progenitors. The CMP, GMP and MEP progenitors differ in the expression of CD123 (IL-3 receptor- α chain), CD135 (FLT3 receptor) and CD45RA (Manz et al., 2002). Gradual loss and/or gain of expression of

CD135 and CD45RA determine commitment of CMP to GMP or MEP. Single CMPs ($\text{CD135}^+\text{CD45RA}^-$) produce all the GMPs ($\text{CD135}^+\text{CD45R}^+$) and MEPs ($\text{CD135}^-\text{CD45RA}^-$) in stromal *in vitro* and *in vivo* transplantation assays. However, there has never been clear consensus on the early stages of multi-lymphoid commitment. At first, The BM CLP ($\text{CD34}^+\text{CD38}^+\text{CD10}^+\text{CD45RA}^+$) origin of B, T and NK cells was first discovered in a stromal co-culture system (Galy et al., 1995). Subsequently, incorporation of CD24 ($\text{CD34}^+\text{CD38}^+\text{CD10}^+\text{CD45RA}^+\text{CD24}^-$) was shown to identify thymus-seeding CLPs from both BM and cord blood (CB) (Six et al., 2007). Nonetheless, others have argued that lymphoid lineage origin can be ascribed to a more primitive BM population marked as $\text{Lin}^- \text{CD34}^+\text{CD38}^-\text{CD7}^+$ (Hao et al., 2001; Hoebeke et al., 2007).

Doulatov et al introduced rigorous functional clonal analysis combined with purification of cells possessing a complex cell surface immunophenotype ($\text{CD34}^+\text{CD38}^-\text{CD90}^{\text{lo}}\text{CD45RA}^+\text{CD7}^+$) to elucidate the HSC-like origin of multi-lymphoid progenitors (MLPs) with concomitant T, B, NK (lymphoid) and macrophage and dendritic cell (myelomonocytic) potential from BM and CB sources (Doulatov et al., 2010). Furthermore, MLPs harbor a hybrid transcriptional state of stem, lymphoid and myeloid programs suggestive of their primitive origin in the hierarchy (Laurenti et al., 2013). Around the same time, MLPs were also identified in BM acute myeloid leukemia (AML) samples indicating the multi-lympho/myeloid nature of this population (Goardon et al., 2011). Monocytes, macrophages and dendritic cells belong to the mononuclear phagocyte network of immune cells sharing a common origin progenitor (Fogg et al., 2006; Geissmann et al., 2010). While DCs are known to have lymphomyeloid origin, the

discovery of an MLP-origin of monocytes and macrophages by Doulatov et al (2010) was a novel insight and made a strong case for revision of the older model of lymphopoiesis as arising from a discrete lymphoid-restricted progenitor.

In a seminal clonal functional analysis in mice, Yamamoto et al elucidated the HSC origin of megakaryocyte/erythroid progenitors bypassing the MPP stepwise progression - a challenge to the classic model of myelopoiesis (Yamamoto et al., 2013). Interestingly also, overlap in the differentiation programs between megakaryocyte/erythroid cells and LT-HSCs (Novershtern et al., 2011; Pronk et al., 2007) and the discovery of platelet-biased LT-HSCs (Sanjuan-Pla et al., 2013) point to the HSC origin of the megakaryocyte/erythroid lineage. However, there was not certainty as to whether this postulated developmental process exists in fetal and adult human hematopoiesis. Because the megakaryocyte-platelet-erythroid component constitutes 99% of the blood cells in the human body, accounting for the bulk of the billions of daily cell turnover, a clearer map of fate commitment of this lineage is critical. In yet another breakthrough from the Dick laboratory, Notta et al identified that oligopotent myeloid progenitors constitute a very negligible component of the adult hematopoietic hierarchy (Notta et al., 2016). Rather each of the myeloid, erythroid and megakaryocyte lineages are derived from their respective multipotent progenitors (MPPs) in the bone marrow presenting a ‘two-tier’ concept of the hematopoietic hierarchy comprised in the top-tier of HSCs and MPPs and in the bottom tier committed lineage specific progenitors) (Notta et al., 2016). In summary, together the above studies, utilizing the most advanced high resolution cell sorting and *in vitro* and *in vivo* clonal assays, provide concrete evidence for substantial

revision of the traditional stepwise lineage commitment organizational structure of the human hematopoietic hierarchy (Figure 1.2B).

1.4 Ex vivo expansion of HSCs and regenerative medicine

Discovery and functional characterization of intrinsic and extrinsic factors regulating the fine balance between HSC self-renewal and differentiation constitutes one of the central questions in the field of HSC biology (Doulatov et al., 2012; Eaves, 2015; Orkin and Zon, 2008). Such discoveries not only advance our understanding of HSCs but also pave the way to harnessing their clinical use for cell replacement and gene therapies (e.g. treating malignant and non-malignant hematological disorders). HSC-based BM transplantation is the sole clinically approved regimen of cell therapy for treating deadly hematological disorders such as leukemia (Daley, 2012; Walasek et al., 2012) and induced BM failure in breast cancer patients undergoing high dose chemo and radio therapy (McNiece et al., 2000; Vavrova et al., 1999).

Currently there are three sources to obtain HSCs for the allogeneic transplantation setting. These are BM aspirates, MPB aphereses and umbilical CB harvests from newborns (Ballen et al., 2013). Each of these sources comes with their own benefits and risks. First, BM aspirates have the advantage of being a source of copious adult HSCs capable of engrafting a recipient's bone marrow with shorter median time to platelet and immune cell recovery. For that reason, patients receiving BM derived HSCs have very minimal risk of acquiring transplantation-related opportunistic infections. However, allogeneic BM HSCs come with their own set of risks. Achieving perfect human leukocyte antigen (HLA) matching is extremely difficult, especially for patients from diverse ethnic and

racial backgrounds (Appelbaum, 2012). The long-term quality of life after partial matching and transplantation is complicated by chronic graft-versus-host disease (GVHD), a condition which can be severe and potentially life threatening. In addition, operationally, drawing BM HSCs from donor BM is relatively invasive procedure which adds another layer of procedural risk.

As a transplant product, MPB apheresis shares all the benefits associated with BM aspirates. Mobilization predominantly uses G-CSF albeit sub-optimally (Petit et al., 2002) to induce elastase activation from neutrophils which then interferes with the SDF-1/CXCR4 interaction maintaining HSCs in their niche and thus leads to the release of HSCs into the circulation. Alternatively, other novel and efficient mobilizing agents have been approved recently (e.g. AMD3100) (Devine et al., 2004). Procedurally, the process of apheresis is less invasive compared to BM aspiration but this aside, the issues and risks associated with HLA mismatching still prevail when transplanting MPB derived HSCs (Eapen et al., 2004).

Given the long-term quality of life post-transplant depends on the presence/absence of GVHD, a viable alternative of using CB HSCs has been in the practice for the past 30 years (Broxmeyer, 2016). The first CB HSC transplant was done in 1988 in France for a 6 year old suffering from Fanconi anaemia (Broxmeyer et al., 1989; Gluckman et al., 1989). Quickly, CB-based transplant was extended to treat other malignant haematological disorders (Wagner et al., 1995). Since then over 30,000 CB transplants have been performed and over 600,000 umbilical CB units donated to more than 100 private and public blood banks (Ballen et al., 2013). One of the benefits of using CB HSCs is the lack

of immune reactions following transplantations even when the donor cells possess half-HLA disparity. This benefits those patients with very diverse genetic backgrounds (Barker et al., 2010). Importantly, HSCs are harvested from the umbilical cord, which was once considered as medical waste. Hence the process of harvesting the CB cells themselves is entirely non invasive. Important also, CB HSCs can be cryopreserved indefinitely and transplanted into the recipient after thawing without significantly losing their repopulating potency (Broxmeyer et al., 2011). The major drawback of using CB as a sole source is the very limited number of HSCs per unit (Broxmeyer et al., 1992). For a successful graft, clinical data from paediatric centres indicate that recipient patient requires at least $2 - 4 \times 10^7$ total nucleated cells (TNCs) (Rubinstein and Stevens, 2000) and/or more than 1.7×10^5 CD34⁺ cells per kilogram of body weight (Wagner et al., 2002). These specifications represent a major hurdle and for that reason use of CB HSCs has been restricted to paediatric cases only (Ballen et al., 2013). The other problem that compounds CB derived HSCs, unlike those from MPB or BM aspirates, is the relatively long median time that is required for these cells to achieve the required neutrophil, platelet and immune cell recovery post transplant. As a result patients receiving CB HSCs are at heightened risk of acquiring transplantation-related opportunistic infections (Kurtzberg, 1996).

Given the benefits outweigh the risks associated with CB HSCs, pre-clinical models have been demonstrating *ex vivo* expansion as viable means to circumvent the limited number of HSCs from this source (Broxmeyer, 2016). The advantage is two-fold as this increases the use of a large ready source of CB HSCs and also economizes the harvesting, isolation

and processing of future samples. Ultimately, any successful approach of *ex vivo* expansion aims to: 1) increase the number of durably self-renewing HSCs; 2) offer increased numbers of progenitors capable of quickly recovering immune functions with shorter median time to immune cell recovery post-transplant.

1.4.1 Approaches for *ex vivo* HSC expansion

Pro-proliferative cytokines, growth media supplements and co-culture systems

Suspension culture with ‘cocktails’ of growth promoting cytokines Flt-3 ligand (Yonemura et al., 1997), TPO (Yagi et al., 1999), SCF, IL-3, IL- 6, and G-CSF constituted the earliest attempts to expand HSCs *ex vivo* (Bhatia et al., 1997a; Conneally et al., 1997; Gammaitoni et al., 2003; Shpall et al., 2002; Ueda et al., 2000). Those approaches however, promoted differentiation at the expense of long-term repopulating cells with lymphomyeloid regeneration capacity and yielded only a 2 to 5-fold net increase compared to input. Yet, the strategy seemed a viable option to recover neutrophils and other immune cells. Further clinical trials on autologous and allogeneic *ex vivo* expanded HSCs using these strategies did not show any difference compared to unmanipulated CB counterparts (Bachier et al., 1999; Williams et al., 1996). The alternative to the suspension culture-based approach was recapitulating the BM microenvironment through co-culture with a mesenchymal stem cell (MSC) derived stromal layer (Kelly et al., 2009; Li et al., 2007; McNiece et al., 2004). Li et al (2007). The co-culture approach delayed differentiation while promoting self-renewal and proliferation of durably repopulating HSCs. Clinical trials using CB HSC expanded with the co-culture approach has yielded a shorter time to immune cell recovery (15 days vs

the historical 24 days) and better engraftment in the majority of the recipient patients (de Lima et al., 2012).

Culture supplements such as the copper ion chelator tetraethylenepentamine (TEPA) and Nicotinamide were also reported to delay differentiation of HSCs. TEPA enhanced preferential expansion of HSPCs in CB-derived CD34⁺ cell culture (Peled et al., 2004a; Peled et al., 2002; Peled et al., 2004b). Using this strategy, Horwitz et al demonstrated a substantial *ex vivo* expansion of long-term HSCs in phase I/II clinical trial setting (Horwitz et al., 2014). Similarly, the Bernstein group achieved another extrinsic factor-mediated *ex vivo* expansion using DLK1 to activate the Notch signaling pathway in CB CD34⁺CD38⁻ cells which specifically resulting in rapid myeloid reconstitution and improved long-term engraftment of HSCs (Delaney et al., 2010; Delaney et al., 2005; Ohishi et al., 2002). Additionally, endothelial derived growth factors such as Angiogenin, Angiopoietin-like 5 (Angptl5), IGFBP2, and pleiotrophin resulted in sizable expansion in the numbers of human stem and progenitor cells *ex vivo* when combined with a standard cytokine cocktail (Goncalves et al., 2016; Himburg et al., 2010; Zhang et al., 2008).

Transcription factor-mediated ex vivo expansion

The retroviral expression of the TF HOXB4 in murine HSCs represented one of the first successful TF-mediated *ex vivo* HSC expansion protocols (Antonchuk et al., 2002). The reported extraordinary 1000-fold expansion over 10 - 14 days culture period quickly drove the search for translation of these findings to human HSCs. Contemporaneously, by using recombinant TAT-HOXB4 chimeric protein in 4 day culture, Krosl et al expanded murine HSCs 4- to 6-fold compared to control (Krosl et al., 2003). Despite the

significantly less expansion, this approach offered proof of principle for non-viral-based utilization of HOXB4. Consequently, Amesellem et al managed to expand human CB CD34⁺ cells in MS-5 stromal ‘feeder’ layer expressing a TAT-HOXB4 chimeric fusion albeit only 2.5-fold compared to control (Amsellem et al., 2003). Such discrepancies between human and murine HSC expansion outcomes demonstrate the fundamental underlying differences and the unreliability of the later as preclinical model. Notably, the TAT-chimeric fusion strategy was the inspiration for Codispoti et al, Bird et al, Aguila et al and Domshenko et al to employ BMI-1, MYC/BCL2, SALL4 and NFYA respectively for expanding human CB, MPB and BM CD34⁺ cells *ex vivo* (Aguila et al., 2011; Bird et al., 2014; Codispoti et al., 2017; Domashenko et al., 2010). For instance, CD34⁺ cells cultured on a stromal layer secreting TAT-BMI-1 for 3 days displayed a 2-fold higher engraftment and long-term multi-lineage repopulating activity in NSG mice (Codispoti et al., 2017). Similarly, human BM CD34⁺ cells treated with recombinant TAT-NFYA demonstrated a 4-fold higher human chimerism in transplanted NSG mice. Codispoti et al and Domashenko et al however failed to rigorously determine the resultant HSC *ex vivo* and *in vivo* expansions by using LDA. The studies discussed above highlight the possibility that human HSCs can be expanded *ex vivo* by modulating intrinsic regulatory pathways without the need to modify them genetically.

With decades of historical findings, TF-mediated *ex vivo* expansion still holds center stage as a subject of HSC research as TF’s are amongst the penultimate orchestrators of genes known to dictate HSC self-renewal. Identification of HSC self-renewal-regulating TFs through functional genomics approach thus continues to be an essential step in their

optimized use for *in vivo* and *ex vivo* expansion. For example, lentiviral mediated over expression of ID2 in CB CD34⁺ cells results a 10-fold *in vivo* expansion and long-term self-renewal ability albeit its insignificant effect *ex vivo* (van Galen et al., 2014b). The *in vivo* expansion by van Galen et al (2014b) is robust compared to previous similar approaches using TFs such as HOXB4 (see above). Through chemical ligand-mediated activation of the pluripotent TF OCT4 (OCT4 activating chemical 1, OAC1) in CB CD34⁺ cells, Hal Broxmeyer's group demonstrated a 3.5- and 6.3-fold increase in the number of SRCs compared to uncultured cells and the vehicle control respectively (Huang et al., 2016). Mechanistically, using RNAi, Huang et al elucidated OAC1 functions through up-regulation of HOXB4, demonstrating the modular nature of HSC self-renewal regulatory networks. Recently, Humphries' group reported *ex vivo* expansion effects of the transcriptional co-activator Nucleoporin98 fused to HOX proteins (Nup98-HOXB4, NUP98-HOXA10, NUP98-HOX10HD) (Abraham et al., 2016) in murine HSCs as achieving superior effects (1000- to 10,000-fold over input) to the same lab's historical control HOXB4 (discussed above) (Ohta et al., 2007). Next, Abraham et al lentivirally expressed NUP98-HOX10HD in MPB CD34⁺ cells and observed only modest 2–3 fold higher short-term engraftment as compared to controls (Abraham et al., 2016). The lack of reproducibility between murine and human HSC expansion is not necessarily surprising and it should be taken as additional convincing evidence for the underlying differences in the self-renewal machinery between human and murine HSCs.

Post-transcriptional regulation as alternative avenue for ex vivo expansion

Post-transcriptional regulators of gene expression such as RNA binding proteins (RBPs), miRNAs and LncRNAs have been demonstrated to be important modulators of human HSC self-renewal and differentiation. For example, our group reported that the RBP Musashi-2 (MSI2) imparts a net 17-fold *in vivo* expansion for short-term repopulating HSCs and 23-fold expansion of long-term repopulating cells (Rentas et al., 2016). Interestingly, RNA-seq and cross-linking immune-precipitation-sequencing (CLIP-seq) profiling with ectopic expression of MSI2 demonstrated direct post-transcriptional attenuation of the canonical components of the AHR signalling pathway, the same pathway identified by Boitano et al (2010) (discussed in section 1.4.1). The Developmental pluripotency-associated 5 (Dppa5) is another RBP whose ectopic expression imparts enhanced survival and long-term reconstitution potential to murine HSCs by reducing ER-stress (see also section 1.7.1) (Miharada et al., 2014). At the present time, the functional role of DPPA5 in possibly achieving *ex vivo* expansion of CB HSCs remains to be validated.

Also, miRNAs have been shown to play a role in the regulation of human HSC self-renewal and differentiation and harnessed for the purpose of CB expansion in numerous pre-clinical models. For example, Lechman et al showed miR-126 plays a pivotal role in critically regulating cell-cycle progression of HSC *in vitro* and *in vivo* through a gain- and loss- of function approach (Lechman et al., 2012). While miR-126 knockdown increased HSC proliferation, resulting in expansion of human long-term repopulating HSC, its ectopic overexpression impaired cell-cycle entry, negatively modulating HSC function.

Mechanistically, antagonizing expression of miR-126 increases HSC self-renewal through activation of the mTOR signalling pathway (Lechman et al., 2012). Of note, the absence of HSC exhaustion with activated mTOR signalling is intriguing despite the overwhelming evidence thereof (Kharas et al., 2010b; Lee et al., 2010; Yilmaz et al., 2006b; Zhang et al., 2006). It remains to be elucidated whether there are unidentified counteracting repressive pathways that prevent HSC exhaustion in these contexts. A related study Wojtowicz et al also reported that miR-125a when ectopically expressed, offers MPPs very extensive self-renewal potential with robust long-term reconstitution capability (Wojtowicz et al., 2016). Through a quantitative proteomics approach this study identified a significant repression of genes involved in the p38/mitogen-activated protein kinase (MAPK) signalling pathway likely responsible for the observed effect of miR-125a (Wojtowicz et al., 2016). David Baltimore's group indicated optimized expression of miRNAs such as miR-125b promote long-term murine HSC self-renewal (O'Connell et al., 2010). However, pre-clinical demonstrations have yet to be completed to see if the same holds true for human HSCs overexpressing miR-125b. miRNAs are not restricted in their function intrinsically. They are also secreted to the extracellular environment within the niche and can influence HSC fate in a paracrine fashion. For example, osteoblast secreted extracellular vesicles (osteoblast-EVs) maintain in as cargo small miRNAs which have been found to promote the *ex vivo* expansion of CB CD34⁺ HSPCs with *in vivo* repopulating and multi-lineage potential as well as *in vitro* proliferation of immature cells (CD34⁺CD90⁺) (Morhayim et al., 2016).

Unbiased screens for bioactive molecules and regulatory networks expanding HSCs

Rational selection of growth factors or intrinsic modulators of HSC self-renewal and proliferation have yielded significant strides in their application for *ex vivo* expansion (discussed above). The advent of modern high throughput chemical screening technologies have enabled unbiased approaches to discover novel small-molecules regulating stem cell chemistry and opened a new field called ‘Stemistry’ (Davies et al., 2015). Noteworthy is also the rapid proliferation of next generation sequencing, proteome and methylome profiling offering high throughput mechanistic characterization of candidate targets from small-molecule screens. For example, North et al identified dimethyl-prostaglandin E2 (dmPGE2) as a potent expander of HSCs in a zebrafish model with just a very brief *ex vivo* pulse (~ 2hrs) (North et al., 2007). In human HSCs a dmPGE2 pulse has demonstrated its efficacy in increasing total CFU and enhanced engraftment of CB derived CD34⁺ cells and sustained multi-lineage reconstitution potential (Cutler et al., 2013; Goessling et al., 2011). The precise signaling pathway(s) through which dmPGE2 operates are not however clearly deciphered yet though it has been implicated to interact with Wnt pathway and enhances cell survival and homing through upregulation of Survivin and CXCR4 respectively (Goessling et al., 2009; Hoggatt et al., 2009). In another chemical screen, demonstrating the importance of an unbiased approach in identifying human HSC modulators, Boitano et al at Novartis identified the aryl hydrocarbon receptor (AHR) antagonist, purine derivative small molecule they termed StemRegenin-1 (SR1) among as capable of maintaining CD34⁺ cells *in vitro* (Boitano et al., 2010). Most importantly, using a very rigorous limiting

dilution quantitative analysis, they showed that a 3-week culture with SR1 increased the number of functional, durably repopulating (in immunodeficient mice) CB HSCs by 17-fold compared to uncultured or cytokine-only treated CD34⁺ cells. Global gene expression profiling of the SR1 treated CD34⁺ cells revealed a massive suppression of genes networks of the AHR pathway such as CYP1B1 and AHRR (Boitano et al., 2010). Recent data from the subsequent and still ongoing phase I/II clinical trial of SR1 has been very encouraging. Here patients receiving SR1 treated CB have responded with no graft failure and significantly faster time to neutrophil and platelet recovery compared to those receiving unmanipulated CB or to historical control (Wagner et al., 2016). Historically, time to neutrophil and platelet recovery is relatively longer for CB HSC-based transplantations and thus a peripheral goal of *ex vivo* expansions has been to produce committed myeloid and erythroid/megakaryocyte progenitors, cells that can regenerate immune cell and platelets to protect the recipient patient from opportunistic infections. Smith et al and Strassel et al achieved that goal using SR1 in induced-pluripotent cell (iPSC)-derived hematopoietic progenitors and peripheral blood CD34⁺ respectively (Smith et al., 2013; Strassel et al., 2016). In less than 5 years after the discovery of SR1, Guy Sauvageau's group performed an independent and methodologically novel bioactive screen for HSC self-renewal agonists using a fed-batch culture system identifying the Pyrimidoindole derivative molecule UM171 (Fares et al., 2014). Interestingly, while both SR1 and UM171 share the commonality of suppressing differentiation of CD34⁺ cells compared to untreated cultured cells, UM171 seems to favourably expand more the primitive population (CD34⁺CD45RA⁻) in comparison to SR1 (CD34⁺CD45RA⁺) *in vitro*.

The *in vivo* observations from both studies are difficult to directly compare for two reasons. First while the Boitano et al study used 3 week standard ‘open incubator’ culture conditions with SCF, FLT3, IL-6 and TPO growth factor supplements, the UM171 study used a 12-day closed and automated fed-batch culture system with all of the above cytokines except IL-6. Second, the post-transplant end points measured were 3 months for the SR1 versus 6 months for UM171. Nonetheless, relying on the rigorous 6 month post-transplant time point which is clearly measuring the output of LT-HSCs, it is clear that UM171 is potent regulator of these cells as it achieves a 14-fold expansion of LT-HSC compared to uncultured CB control. Importantly, further gene expression analysis also demonstrated AHR independent and rather global down regulation of differentiation genes for UM171 (Fares et al., 2014). Such disparity in the signaling pathways targeted by SR1 and UM171 suggest that additive effects are possible in enhancing the activity of primitive cells. Indeed, Fares et al observed that when combined, the addition of UM171 and SR1 increase the frequency and number of mixed GEMM colonies (Fares et al., 2014). Also, in an exploratory study, adult BM HSC cultured for 5 days using a combination of SR1, UM171 and the p38-MAPK14 inhibitor LY2228820 (Ly) produces significantly more long-term engrafting HSCs (CD34⁺CD38⁻CD90⁺) compared to untreated or UM171 alone control (Psatha et al., 2017). This important preliminary work thus presents a proof of principle for integrating and repurposing other novel *ex vivo* CB HSC expansions strategies for achieving similar expansions of adult BM and mobilized peripheral blood HSCs.

In a semi-unbiased small-scale chemical screen using inhibitors of histone deacetylases (HDACIs) in CB CD34⁺ cells, Chaurasia et al identified valproic acid (VPA) as a potent expander of HSCs (Chaurasia et al., 2014). Further phenotypic characterization and gene expression analysis demonstrated also up regulation of pluripotency genes (e.g. OCT4 and NANOG), increased aldehyde dehydrogenase activity, and enhanced expression of CD90, c-Kit (CD117), integrin α 6 (CD49f), and CXCR4 (CD184). Subsequent primary and secondary *in vivo* transplantations experiments with VPA treated CB CD34⁺ cells indicated robust SRCs with lymphomyeloid capacity compared to untreated control (Chaurasia et al., 2014). As a pre-clinical model this study elucidated for the first time that epigenetic reprogramming using reversible inhibitors could be one avenue to expand functional and engrafting CB HSCs.

Unbiased genetic screens for regulatory networks/signalling pathways also provide ‘druggable’ targets used for *ex vivo* expansion of CB HSCs (Baudet et al., 2012; Galeev et al., 2016). Baudet et al used an RNAi screen against kinases/phosphatases in CD34⁺ cells and identified MAPK14 coding for p38-alpha, a member of the p38 family of MAPK, as critical regulator of HSCs (Baudet et al., 2012; see also Psatha et al., 2017). Pharmacological inhibition of MAPK14 leads to significant expansion of HSCs through reduction of ROS levels (Baudet et al., 2012). Noteworthy is an independent study carried out by the Dick lab wherein they identified that the MAPK pathway as targeted by miRNA-125a, is an important target for HSC expansion (Wojtowicz et al., 2016). In another similar RNAi screen, the Larsson group identified the phosphatase PLCZ1 and EXT1 (implicated in Wnt and TGF-beta signalling) as important for *ex vivo* HSC

expansion (Ali et al., 2009). More recently, a genome wide RNAi screen identified members of the Cohesin genes family (*STAG1*, *STAG2*, *RAD21*, and *SMC3*) as critical for CB HSC self-renewal and differentiation. RNAi against *STAG2* in particular was found to significantly expand HSCs *ex vivo* and to increased the level engraftment mediated by HSCs in NSG mice (Galeev et al., 2016). Mechanistically, knockdown of Cohesin family members induced an HSC self-renewal signature gene expression suggesting that these proteins represent another rational target for *ex vivo* HSC expansion (Galeev et al., 2016).

Incorporation of technological innovations and HSC conditioning regimens into ex vivo expansion approaches

The introduction of technological innovations at any of the stages in the processing of CB HSCs has been implicated as key factor to maximizing their transplantability. For instance, the use of hypoxic chambers during harvesting and processing of CB HSCs was shown to significantly improve their engraftment and lineage reconstitution capacity (Mantel et al., 2015). Mantel et al has demonstrated that the exposure to ambient air significantly underestimates the number of HSC maintained in a culture due to ‘extra physiological oxygen shock/stress’ (EPHOSS) mediated by the production of reactive oxygen species (ROS). To minimize the risk of EPHOSS these researchers introduced a hypoxia mimicking pharmacological agent called cyclosporine A into the CB samples, ultimately mitigating the damaging effect of oxygen shock. Yet another powerful technological advance is the use of the aforementioned closed fed-batch culture system to automatically replenish fresh growth factors and media to the culture of HSCs so as to

increase the efficacy of their *ex vivo* expansion (Csaszar et al., 2012). By using the fed-batch system Csaszar et al minimized (if not completely eliminated) the secreted inhibitory feedback signals from the rapidly differentiating cells resulting in an 11-fold increase of HSCs with self-renewing, multilineage repopulating ability at the end of 12 days of *ex vivo* culture.

In addition to enhancing the *ex vivo* expansion of HSCs, delayed engraftment (and BM homing) of human HSC remains another major obstacle impeding the promise of CB HSC-based cell therapy. For example, Robinson et al incubated CB CD34⁺ cells for 30-minute *ex vivo* with fucosyltransferase-VI and its substrate (GDP-fucose) (resulting in fucosylation of surface ligands) and found significantly enhanced homing and interaction with the BM microvasculature post-transplant (Robinson et al., 2012). Another strategy is the use of Diprotin A, pharmacological inhibitor of CD26, Dipeptidylpeptidase 4 (DPP4) (Christopherson et al., 2007). DPP4 cleaves a wide variety of substrates, including the chemokine stromal cell-derived factor-1 (SDF-1) and CXCL12, reducing HSPC engraftment, homing and mobilization. HSPCs treated with a DPP4 inhibitor have enhanced *ex vivo* expansion, survival, colony replating potential and *in vivo* engraftment frequency (Broxmeyer et al., 2012; Campbell et al., 2007; Christopherson et al., 2007). In addition, a high throughput small-molecule screen by Broxmeyer's group identified glucocorticoid (GC) hormone signaling as enhancing HSC self-renewal, homing and engraftment (Guo et al., 2017). Mechanistically, GC receptor activation facilitates CXCR4 transcription promoting SDF-1-mediated chemotaxis to the BM niche. Also, as discussed above, dmPGE2's effect on CXCR4 also enhances the homing and engraftment

efficiency of CB HSCs (Goessling et al., 2009; Hoggatt et al., 2009). Interestingly, Capitano et al demonstrated that mild heat pre-treatment of CB HSCs (39.5°C) for 4 hrs, called hyperthermia, primes these cells for enhanced *in vivo* engraftment through increased CXCR4 expression (Capitano et al., 2015). Altogether, the preceding studies exhibit important evidence for in support of the incorporation and integration of identifying improved cell conditioning regimens to enhance the potency of *ex vivo* expanded CB HSCs.

1.5 Transcription factor and epigenetic networks and HSC homeostasis

HSC homeostasis is controlled by a number of transcriptional regulatory networks. In this regard, TFs orchestrate gene expression to ultimately regulate important cellular functions such as differentiation, proliferation and survival, and thus are often designated as ‘master regulators’(Gottgens, 2015).

The microarray era heralded the first transcriptome-wide profiling of HSPCs prospectively isolated from BM, CB and MPB (Georgantas et al., 2004; Ivanova et al., 2002). Bhatia’s group also identified the TFs Hairy Enhancer of Split-1 (HES-1) and Hepatocyte Leukemia Factor (HLF) through global gene expression profiling in CD34⁺CD38⁻ primitive HSCs isolated from fetal liver, CB and adult BM (Shojaei et al., 2005). Shojaei et al further validated the increased short-term *in vivo* reconstitution capacity conferred by both HES-1 and HLF. HES-1 is the downstream effector of the canonical Notch signaling pathway initiated by binding of Jagged, Delta-like or CCN3 (NOV) as extracellular modulators (Gupta et al., 2007; Pajcini et al., 2011). Constitutive activation of the Notch signaling pathway was demonstrated to enhance HSC expansion -

a concept that led to its utilization in CB expansion protocols (Delaney et al., 2010; Varnum-Finney et al., 2000). In unbiased high throughput comparative transcriptome analysis Gazit et al also picked HLF (among other transcriptional regulators) as a positive regulator of stem and progenitor cells (Gazit et al., 2013). Retrovirally-mediated expression of HLF resulted in robust induction of a primitive immunophenotype, sustained colony-formation activity, and enhanced self-renewal *in vitro* mirroring the previous observations (Gazit et al., 2013; Shojaei et al., 2005).

1.5.1 Computational prediction of global TF interaction networks in HSPCs

There is limited comprehensive data predicting network interaction between key TFs individually identified to regulate HSPCs. Rationally identified ‘2- factor’ or ‘3-factor’ interaction models have however been highly informative and allowed for the development of global network interactions that regulate HSPCs (Gottgens, 2015; Narula et al., 2010). For example, using systematic and integrated pairwise analysis, Wilson et al mapped a genome-wide *cis*-element binding and interactions profile for 10 key, previously identified TFs with relevance to primitive hematopoietic cell biology (SCL/TAL1, LYL1, LMO2, GATA2, RUNX1, MEIS1, PU.1, ERG, FLI-1 and GFI1B) (Wilson et al., 2010). This approach brought to light novel genetic and protein interactions between the key HSPC TFs based on target site occupancy patterns. It also provided a new computational analysis pipeline for mapping TF interaction networks. Indeed, Gottgens’s group extended their pipeline to 17 additional core TFs (17 TFs) (CEBP α , CEBP β , cFOS, cMYC, E2F4, EGR1, ELF1, ETO2, c-JUN, LDB1, MAX, MYB, NFE2, p53, RAD21, pSTAT1, and STAT3) based on ChIP-seq data generated

using primitive murine hematopoietic HPC-7 cell line (Wilson et al., 2016), a study which to date represents the only comprehensive genome-scale ChIP-seq data with relevance to HSPC biology to have been carried out.

The technical requirements of ChIP-seq limit our understanding of TF networks in rare primary HSCs. To circumvent this, Novershtern et al integrated global transcriptome profiling with pre-identified TF network interactions using computational predictions to find novel regulatory circuitry in rare primary HSPCs (CD34⁺CD38⁻ or CD34⁺CD133⁺) (Novershtern et al., 2011). They used probabilistic and sophisticated computational prediction models of gene expression and analysis of *cis*-elements in promoters to decipher the general organization of the modular regulatory circuitry of TFs. One of their breakthroughs is the validation of previous observations that TFs are “reused” between multiple lineages and between HSCs and committed progenitors (Carotta et al., 2010; Novershtern et al., 2011; van der Meer et al., 2010). The cases in point of such transcription factor “re-usage” are the versatile ETS family transcription factor PU.1 and growth factor independence 1 (Gfi1) (Carotta et al., 2010; van der Meer et al., 2010). While PU.1 is required for many aspects of lymphoid and myeloid cell development, Gfi1 is required for multilineage blood cell development, from stem and progenitor cells to differentiated lymphoid and myeloid cells.

Recent advances in high-resolution immunophenotyping efforts by Notta et al and others have enabled identification of critical transcriptome profiles in HSCs (Cabezas-Wallscheid et al., 2014; Laurenti et al., 2013; Notta et al., 2011; Notta et al., 2016). In this

regard, the computational pipelines developed by Novershtern et al could be revised to integrate and unveil previously unidentified TF networks in HSCs.

1.5.2 Epigenetic Regulation and HSC homeostasis

Recruitment of histone and DNA epigenetic modifiers is thought to be a key mechanism through which transcription factors regulate important functions in HSC proliferation, survival and differentiation. Some of the well-studied epigenetic modifiers in the context of HSC biology include the histone acetyltransferases MOZ, CBP, P300 (Chan et al., 2011b; Katsumoto et al., 2006; Rebel et al., 2002), H3K79 methyltransferase Dot11 (Jo et al., 2011), H3K4 methyltransferase MLL1, MLL5 (Jude et al., 2007; Zhang et al., 2009), multiple polycomb group (PcG) proteins such as BMI1 (Iwama et al., 2004; Majewski et al., 2010; Rizo et al., 2008; Rizo et al., 2009) and DNA methyltransferases such as DNMT1-3 and TET2 (Challen et al., 2011; Ko et al., 2011; Tadokoro et al., 2007; Trowbridge et al., 2009). The epigenetic landscape of young and old HSCs was recently reported to differ and accordingly affect their self-renewal kinetics (Sun et al., 2014).

To elucidate functional crosstalk between epigenetic modification and transcription factor activity in the context of HSC homeostasis, I highlight here the polycomb-group proteins (histone modifiers) and DNMT and TET2 (DNA modifiers) as representative factors. The evolutionarily conserved chromatin modifier polycomb-group forms two structurally and functionally distinct complexes, polycomb repressive complex 1 and 2 (PRC1 and PRC2). While PRC1 activity involves H2AK119 monoubiquitination, PRC2 trimethylates H3K27 the results of which are often antagonistic in HSC function

(Majewski et al., 2010). An important PRC1 core component BMI1 for example is key regulator of the proliferative activity of hematopoietic stem and progenitor cells (Iwama et al., 2004; Lessard and Sauvageau, 2003; Park et al., 2003). Using a functional genomics approach Rizo et al elucidated in human CD34⁺ cells that its loss leads to impairment of clonal repopulation capacity while overexpression significantly boosts multi-lineage and serial re-plating potential (Rizo et al., 2008; Rizo et al., 2009). In addition BMI1 has been identified to have functional cross talk with MLL/HOXA9 in establishing HSC homeostasis (Smith et al., 2011). Taken together, the available evidence presents BMI1 as an essential regulator of HSC self-renewal.

DNA methylation as 5-cytosine methylation (5-mC) in a CpG dinucleotide context is essential for HSC homeostasis. Through conditional deletion of DNMT1 which is involved in maintaining DNA methylation patterns, Trowbridge et al showed that deficient HSCs have impaired self-renewal and an inability to give rise to multilineage reconstitution by upregulating expression of genes such as GATA1 and THY1 (Trowbridge et al., 2009). On the other hand loss of DNMT3a/b which is involved in *de novo* DNA methylation in long-term HSCs, leads to impaired differentiation and expansion of the primitive cell pool in the bone marrow through CpG hypomethylation of multipotency genes such as GATA3 and PBX1 (Challen et al., 2011; Tadokoro et al., 2007). The TET2 gene encodes a member of TET family enzymes that alters the epigenetic status of DNA by oxidizing 5-methylcytosine to 5-hydroxymethylcytosine (5hmC). TET2 is the most expressed member of the TET family of genes in HSCs. Targeted disruption of the TET2 catalytic domain delayed HSC differentiation enhancing

their competitive and multilineage reconstitution capacity likely by controlling the level of 5hmC in self-renewal genes (Ko et al., 2011).

1.6 Genomic imprinting, long-non coding RNAs and HSC homeostasis

Genomic imprinting is a highly regulated epigenetic-marking that dictates the expression of a set of ~150 genes in a maternal- or paternal-origin-specific pattern (Peters, 2014). Accordingly these genes are classified as imprinted genes (IGs). The discovery of the first group of IGs, namely IGF2, IGF1R and H19 (Barlow et al., 1991; Bartolomei et al., 1991; DeChiara et al., 1991) ignited genuine efforts toward elucidating mechanisms of genomic imprinting. Most IGs are organized into allelic clusters of 3–12 genes spread over 20 kb to 3.7 Mb of DNA that contain a differentially methylated region (DMR) as a *cis*-acting imprinting control region (ICR). Each cluster contains protein-coding RNAs, long non-coding RNAs (lncRNAs) (> 200 nucleotides), miRNAs, and/or snoRNAs expressed only from either the paternal or maternal allele but not both. The *de novo* DNA methyltransferases 3A/B(DNMT3A/B) mediate imprint establishment during gametogenesis. Subsequently, DNMT1 primarily mediates imprinting maintenance to protect from genome-wide epigenetic modifications occurring in the pre-implantation embryo (Li and Sasaki, 2011). Finally, genome-wide imprinting erasure is mediated by the TET1/2– induced demethylation of the DMR during early germ cell development completing the life cycle of genomic imprinting (Yamaguchi et al., 2013).

The canonical model of IG regulation dictates that epigenetic access to the DMR is orchestrated by lncRNAs or blocked through the insulating action of the CCCTC-binding factor (CTCF) (Kurukuti et al., 2006; Lee and Bartolomei, 2013; Zhang et al., 2014).

Evidence has also started to emerge for a non-canonical miRNA-mediated post-transcriptional repressive circuitry within the IG clusters (Venkatraman et al., 2013).

Imprinted genes are known to regulate fetal and post-natal development in a gene dosage dependent pattern. As such, altered gene dosage can compromise cell proliferation and differentiation leading to prenatal size and developmental aberrations such as overgrowth, intrauterine restriction, embryonic lethality and malignant transformations (Charalambous et al., 2012; Varrault et al., 2006). Interestingly, several IGs have differential expression across the hematopoietic hierarchy. Some of them have elevated expression and are associated with limited proliferation and quiescence in LT-HSCs (Qian et al., 2016; Venkatraman et al., 2013). Others are highly expressed in ST-HSCs and MPPs and in these contexts promote proliferation and increased metabolism (Cabezas-Wallscheid et al., 2014; Qian et al., 2016; Venkatraman et al., 2013). Finally, some IGs show peak expression in fetal HSCs and a decrease after birth while others have ontogenic conservation between embryonic stem cells (ESCs) and adult HSCs (Cabezas-Wallscheid et al., 2014; Frost et al., 2010; Varrault et al., 2006).

In the hematopoietic system exploration of lncRNA expression and function has lagged behind that in other systems. Novel HSC-specific lncRNAs (LncHSCs) have just recently begun to be identified, annotated and validated in the murine model (Cabezas-Wallscheid et al., 2014; Luo et al., 2015; Qian et al., 2016; Venkatraman et al., 2013). For instance, recent functional genomics approaches revealed key aspects of murine HSC self-renewal machineries including reactive oxygen species (ROS) production (Qian et al., 2016; Venkatraman et al., 2013) and TF-binding site co-occupancy as being critically regulated

by lncRNAs (Luo et al., 2015). It remains to be explored if lncRNAs govern similar molecular functions in human HSCs.

1.6 1 IGF2-H19 imprinted cluster

The protein coding IGF2 and the lncRNA H19 are transcribed from the paternal and maternal alleles respectively. Binding of CTCF in the DMR silences IGF2 expression from the maternal allele by insulating/blocking transactivation by distal enhancers (Kurukuti et al., 2006). In turn, methylation of the paternal DMR prevents CTCF binding allowing IGF2 expression while preventing H19 transcription. IGF2 expressed from fetal liver CD3⁺Ter119⁻ stromal cells was thought to support extensive proliferation of hematopoietic repopulating cells *ex vivo* (Zhang and Lodish, 2004). However, under homeostatic condition using conditional knockout (KO) mouse models, Venkatraman et al. elucidated the dispensability of IGF2 for maintaining adult LT-HSCs (Venkatraman et al., 2013). They subsequently disrupted the DMR upstream of H19 (H19-DMR) from the maternal allele which was found to result in constitutive upregulation of IGF2, whose elevated binding to its receptor IGF1R induced FOXO3-mediated inhibition of HSC cell cycle arrest ultimately leading to their exhaustion (Venkatraman et al., 2013). The H19 lncRNA is also a developmental reservoir of the miRNA-675 microRNA that is known to post-transcriptionally repress IGF1R (Keniry et al., 2012). Hence, disruption of the H19-DMR interferes with miR-675 biogenesis and IGF1R repression. Of note, IGF1R is known to activate the PI3K-AKT-mTOR signalling pathway and as such the functional consequence of H19-DMR disruption on the mTOR pathway needs further investigation. It is important to point out that this study, which clearly demonstrated the role of H19 in

maintaining murine LT-HSC quiescence, awaits follow up in the human setting in order to define the possible functional relevance of H19 and miR-675 in the context of human HSCs self-renewal and thus their potential as targets for clinically relevant therapeutic applications.

1.6 2. DLK1-MEG3 imprinted cluster

The DLK1-MEG3 (Gtl2) genomic cluster contains the paternally expressed protein coding imprinted genes DLK1, RTL1, and DIO3 and the maternally expressed lncRNAs MEG3 (Gtl2), MEG8 (RIAN), and antisense RTL1 (asRTL1) embedding miRNAs and snoRNAs. Both the intergenic germline-derived DMR (IG-DMR) and the Gtl2-DMR determine reciprocal expression of ncRNAs and protein-coding genes from the respective maternal and paternal alleles (Lin et al., 2003). Interestingly, this locus embeds one of the largest (~10%) miRNA clusters in the entire genome and many of these miRNAs are differentially expressed across the hematopoietic hierarchy (Qian et al., 2016). Qian et al elegantly demonstrated that constitutive ablation of the IG-DMR from the maternal allele (mat *ΔIG*) repressed biogenesis of ncRNAs from the Gtl2 locus and functionally impaired adult and fetal HSCs without affecting the frequency or the number of progenitors and mature hematopoietic cells. Mechanistically, the miRNAs transcribed from Gtl2 locus invariably repress multiple components of the PI3K-AKT-mTOR pathway. The mTOR pathway is a known essential regulator of HSC self-renewal (Hirao and Hoshii, 2013; Lee et al., 2010; Zhang et al., 2006). Qian et al showed activated mTOR leads to enhanced mitochondrial biogenesis, metabolic activity and ROS levels in HSCs which was partly rescued though pharmacological treatment using N-acetyl-

cysteine (NAC) (Laplane and Sabatini, 2012; Morita et al., 2013; Qian et al., 2016) (also discussed in section 1.7). In summary, Li's group (Qian et al., 2016) deciphered a functional link between the epigenetic regulation of IGs expression and HSC self-renewal using systematic ablation of the DMR in mouse models. However, it remains to be validated whether MEG3 plays similar roles in regulating human HSC self-renewal, knowledge that if garnered could have implications for therapeutic applications i.e. *ex vivo* expansion.

1.7 Ribosome biogenesis and proteostasis in HSCs

There is now an accumulating body of evidence demonstrating the key roles played by protein homeostasis (hereafter termed as proteostasis) and metabolic cellular bioenergetics on HSC quiescence, proliferation and survival (Garcia-Prat et al., 2017). Under steady state hematopoiesis, HSCs are quiescent but when regenerative demands arise (such as in BM transplantation and under genotoxic stress), they exit quiescence, enter cell cycle and proliferate to repopulate the hematopoietic system (Chandel et al., 2016; Doulatov et al., 2012; Kohli and Passegue, 2014). HSC proliferation requires a steady supply of metabolic energy through oxidative phosphorylation that inevitably generates ROS and free radicals that could damage organelles and macromolecules such as nucleic acids, proteins and lipids (Ito et al., 2006; Simsek et al., 2010; Suda et al., 2011). To circumvent the damage and maintain the integrity and homeostasis of the proteome, HSCs have also developed multi-layered proteostatic checkpoint mechanisms (Garcia-Prat et al., 2017). These involve ribosome biogenesis (Ribi) and protein synthesis pathways (Cai et al., 2015; Signer et al., 2014), the unfolded protein response (UPR)

(Rouault-Pierre et al., 2013; Sigurdsson et al., 2016; van Galen et al., 2014a), the ubiquitin proteasome system (UPS) and autophagy (Ho et al., 2017; Ito et al., 2006; Moran-Crusio et al., 2012; Warr et al., 2013). The UPR system is triggered when proper protein folding processes are disrupted producing misfolded and unfolded proteins accumulating in the endoplasmic reticulum, cytoplasm and mitochondria. As the penultimate protein quality control mechanism, the ubiquitin-proteasome system (UPS) ubiquitinates and proteolytically degrades misfolded/unfolded proteins. Ultimately, autophagy targets damaged, misfolded and unfolded proteins for lysosome-mediated degradation and recycling.

1.7.1 Protein synthesis in HSCs

Eukaryotic ribosome biogenesis is a complex and intricate biological process involving step-wise maturation and assembly of ribosomal proteins (RPs) and ribosomal RNAs (rRNAs) into the smaller 40S and larger 60S subunits. The ribosomal DNA (rDNA) is first pre-transcribed as a single 47S rRNA precursor (pre-rRNA) by RNA Pol I in the nucleolus (Rodnina and Wintermeyer, 2009). The pre-rRNA is subsequently processed into 18S, 5.8S and 28S matured rRNAs (Roeder and Rutter, 1970). RNA Pol III transcribes the 5S rRNA from a chromosomal region outside the nucleolus (Weinmann and Roeder, 1974) while ribosomal proteins of the smaller (RPS) and larger (RPL) subunits are transcribed by RNA Pol II (Schlosser et al., 2003; van Riggelen et al., 2010). RPs are then synthesized in the cytoplasm and imported into the nucleus for assembly with rRNAs. The 40S subunit is assembled from the 18S rRNA and approximately 32 RPSs. However, the 60S subunit is assembled from 5S, 5.8S and 28S rRNA and

approximately 47 RPLs (Rodnina and Wintermeyer, 2009). Effective ribosome biogenesis culminates with export of the 40S and 60S subunits into the cytoplasm and their eventual assembly into the 80S mature ribosome (Gebauer and Hentze, 2004) (Figure 1.3A).

Protein synthesis is co-ordinately executed by concerted multi-step and multifactor-based organization of the 80S ribosome, tRNAs, mRNA and translation initiation and elongation factors. First, the 43S pre-initiation complex is formed by the incorporation of eukaryotic initiation factors (eIFs) eIF2 and eIF3, tRNA and GTP into a 40S ribosomal subunit. Next, eIF4E is recruited into the pre-initiation complex to bind eIF4F and 5'-methylated mRNA to form a 48S complex (Asano et al., 2000; Gebauer and Hentze, 2004). eIF4E acts as scaffold for eIF3 and poly-A binding proteins such as eIF4G and PABP. Translation elongation is achieved by the formation of the 80S complex which is comprised of the interaction of the 48S complex with 60S subunit (Hernandez and Vazquez-Pianzola, 2005) and can be inhibited by physical interaction of eIF4E and its binding partners the 4E-BPs (Richter and Sonenberg, 2005).

One critical role of proteostasis in HSCs is to exert tight regulation on the complex process of ribosome biogenesis and protein synthesis. Indeed, recent landmark studies using murine models elucidated that under homeostatic conditions HSCs have limited a protein synthesis rate compared to the highly proliferating committed progenitor and other hematopoietic cells (Cai et al., 2015; Signer et al., 2014). Dysregulated increases or decreases of the protein synthesis rate was also found in these studies to significantly impair HSC self-renewal/fitness, regenerative potential and resistance to stress (Cai et al.,

2015; Signer et al., 2014). For example increasing protein synthesis through deletion of the tumor suppressor PTEN leads to attenuated HSC function which Signer et al (and others) found also predisposed to leukemogenesis (Signer et al., 2014; Signer et al., 2016; Yilmaz et al., 2006b). Conversely, significant decreases of protein synthesis by interfering with ribosome biogenesis also impairs the reconstitution ability of HSCs. Importantly, Signer et al demonstrated that the dampened basal protein synthesis rate in murine HSCs is partly dependent on 4E-binding protein 1 (4E-BP1) and 4E-BP2 but independent of other proteostatic pathways (such as UPS or autophagy), RNA content or cell division rate (Signer et al., 2014; Signer et al., 2016).

In related studies, through a comprehensive transcriptome/methylome analysis comparing young and aged murine HSCs, Sun et al identified ribosome biogenesis as one of the highly deregulated biological process that accompanied aging (Signer and Morrison, 2013; Sun et al., 2014). Interestingly, older HSCs have increased transcription of RPs, rRNAs and hypomethylation of rRNA genes that result in a skewing towards myeloid-biased proliferation and a likely predisposition to myeloid leukemic transformation. In a conserved mechanism, Goncalves et al identified the niche-secreted RNase, angiogenin (ANG), also known as RNase5, as reciprocally regulating proliferation of myeloid progenitors and HSPCs (Goncalves et al., 2016). ANG simultaneously promotes cell survival and suppresses global protein translation through the production of tRNA-derived stress-induced small RNAs (tiRNA). Mouse HSCs and human CD34+ HSPCs treated with ANG have reduced proliferation and enhanced self-renewal capacity upon transplantations compared to untreated control.

The above studies suggest that discovery and functional validation of upstream regulators of any aspect of the ribosome biogenesis machinery is of great importance for therapeutic interventions and *ex vivo* expansion. Indeed, Cai et al demonstrated that in the murine system, the TF Runx1 regulates transcription of rRNAs (Cai et al., 2015). Functionally, Runx1 deletion attenuated ribosome biogenesis and yet also enhanced HSC competitive repopulation capacity while imparting a simultaneous resistance to irradiation and chemically induced insults. Of interest, MYC is also an important known regulator of the transcription of the trilogy of RNA polymerases and hence multiple stages of ribosome biogenesis (Oskarsson and Trumpp, 2005). Specifically, MYC enhances transcription of rRNAs by directly binding to cognate E-box sequences in the respective rDNA, interacting with transcriptional co-factors and facilitating recruitment RNA Polymerase I (Grandori et al., 2005). Numerous functional studies and gene expression analysis have established direct correlation between MYC and transcription of RPs (Boon et al., 2001; Coller et al., 2000; Kim et al., 2000). Importantly, Wu et al combined ChIP and microarray analysis to demonstrate direct regulation of the transcription of ribosomal proteins by MYC (Wu et al., 2008). MYC also regulates the transcription of auxiliary proteins involved in the processing, nuclear-cytoplasm transport and assembly of rRNAs and ribosome subunits (Boon et al., 2001; Schlosser et al., 2003) and eIFs (Schmidt, 2004). The functional consequence of MYC regulated aspects of ribosome biogenesis and protein synthesis on HSC homeostasis remains to be elucidated.

The endoplasmic reticulum UPR (UPR^{ER}) system is another proteostatic step HSCs use to avoid stress-induced apoptosis. Mitigation mechanisms include the cell's utilization of

chemical and heat shock protein chaperones to promote proper protein folding (Tai-Nagara et al., 2014), limiting mitochondrial activity and reducing ROS production through HIF2-alpha (Rouault-Pierre et al., 2013). The RNA binding protein Dppa5 (Miharada et al., 2014) and Taurocholic bile acid (Sigurdsson et al., 2016) are other known factors that can also increase efficient protein folding in HSCs and failure of these mitigation mechanisms induces UPR leading to loss of HSC function and ultimately apoptosis. Indeed, van Galen et al (2014a) demonstrated the relative sensitivity of HSCs to tunicamycin-induced UPR with concomitant loss of long-term self-renewal capacity (van Galen et al., 2014a). Together, these studies have therefore led to the appreciation of the requirement for efficient protein folding, and the maintenance of an inactive UPR^{ER} for maintenance of HSC quiescence and overall function.

As the ultimate proteome quality control mechanism, HSCs resort to UPS and autophagy to degrade and remove or recycle damaged or improperly folded proteins. The UPS is particularly well known to play a key role in assuring the integrity of the HSC transcriptional ‘master’ regulators such as NOTCH, MYC, GATA2 and STAT5 (Goh et al., 2002; Moran-Crusio et al., 2012; Naujokat and Saric, 2007). Likewise, HSCs activate the autophagy system in response to damage to organelles or the proteome to maintain sustained self-renewal and enhance ‘stemness’ (Warr et al., 2013). Defects in autophagy pathways leads instead to extensive ROS induced proteome damage resulting in apoptosis and predisposition to leukemia - a phenomenon that, not surprisingly, is therefore usually observed with aged HSCs (Ho et al., 2017; Mortensen et al., 2011; Warr et al., 2013).

1.7.2 mTOR signalling in proteostasis, metabolism and HSC homeostasis

The mechanistic/mammalian target of rapamycin (mTOR) is a signalling hub integrating multiple intracellular and external signals such as growth factors, energy status and oxygen consumption (Laplane and Sabatini, 2013). Accordingly, mTOR activation induces/represses multiple downstream biological functions such as ribosome biogenesis and protein synthesis, autophagy and mitochondrial functions (Laplane and Sabatini, 2013). In response to growth factor binding, membrane receptor tyrosine kinases such as IGF1R activate the PI3K signalling complex. PI3K phosphorylates AKT and suppresses TSC1/2- negative regulators of mTORC1 phosphorylation. Activated mTOR increases protein synthesis via two arms: 1) it phosphorylates 4E-BP1 and thus suppresses its inhibitory function on CAP-dependent translation initiation; 2) it phosphorylates and activates the p70 ribosomal kinase 1 RPS6K1 and facilitates the translation of ribosomal proteins, their maturation and assembly (Ma and Blenis, 2009; Richter and Sonenberg, 2005) (Figure 1.3B). Importantly, hyperactivation of mTOR, among other things, increases ribosome biogenesis and protein synthesis and also alters HSC homeostasis (Cai et al., 2015; Lee et al., 2010; Qian et al., 2016; Yilmaz et al., 2006b). For instance, rapamycin (an allosteric inhibitor of mTORC1) treatment of CB and murine HSCs lead to enhanced *ex vivo* expansion and long-term self-renewal in serial transplantation assays (Huang et al., 2012; Rohrabough et al., 2011). Also, compelling evidences in murine contexts demonstrate that intrinsically, HSCs achieve low mTOR activity to maintain long-term self-renewal and survival using negative regulators such as PTEN and TSC1/2 (Gan and DePinho, 2009; Lee et al., 2010; Signer et al., 2014; Yilmaz et al., 2006b). Loss

of PTEN brings short-term extensive HSC proliferation leading to exhaustion or predisposition to leukemia (Yilmaz et al., 2006b; Zhang et al., 2006). It remains to be demonstrated whether similar mechanism is employed by human HSCs to achieve low mTOR activity during homeostasis. Noteworthy, Chen et al observed that pharmacological inhibition of AKT (or AKT RNAi) enhances CB CD34⁺ cells quiescence, colony formation potential and short-term repopulating potential *in vivo* (Chen et al., 2017). Interestingly however, constitutive loss of components of mTOR activation pathway such as AKT1/2 in HSCs leads to defects in their bone marrow regeneration capacity suggesting a requirement for a minimum threshold of activity (Juntilla et al., 2010; Kalaitzidis et al., 2012). Transcriptionally, mTOR signalling activates key transcription effectors such as MYC (Gan et al., 2010; Gurumurthy et al., 2010) and RUNX (Cai et al., 2015) to regulate ribosome biogenesis and protein synthesis. In response to low oxygen and low energy demand, the master sensor AMP-activated protein kinase (AMPK) is activated and phosphorylates TSC1/2, which in turn attenuates mTORC1 activation (Gwinn et al., 2008; Inoki et al., 2003; Ito et al., 2006) (Figure 1.3B). Hence, the energy status of HSCs is orchestrated by mTOR mainly through AMPK (Ito et al., 2006). HSCs mainly use anaerobic respiration through the glycolytic pathway as a sole source of ATP for metabolic function, likely an evolutionary adaptation to the low oxygen (hypoxic) bone marrow niche (Simsek et al., 2010; Suda et al., 2011; Takubo et al., 2013). Also, emerging evidence supports that the balance between HSC quiescence/cycling and self-renewal/commitment is regulated by mitochondrial dynamics and functions such as mitochondrial membrane potential (Luchsinger et al., 2016; Piccoli

et al., 2013; Romero-Moya et al., 2013; Vannini et al., 2016). Dependence on glycolysis as opposed to oxidative phosphorylation (OxPhos) protects HSCs from the long-term damaging effects of ROS generated by the latter (Ito et al., 2006; Takubo et al., 2013). Yet, when proliferative demands arise such as in BM repopulation, HSCs transiently switch to OxPhos (Lewandowski et al., 2010; Takubo et al., 2013). Hence, as a mitigation mechanism from the unrelenting insults by ROS accumulation, HSCs switch to AMPK activation, which in turn induces FoxO3-dependent activation of autophagy to maintain ‘stemness’ (Ito et al., 2006; Rimmele et al., 2015; Warr et al., 2013). Also, Ho et al and Vannini et al demonstrated that HSCs utilize autophagy to keep the mitochondrial number in check to reduce metabolic activity and enhance long-term regeneration potential in return. Together, these represent important links between proteostasis and metabolism in controlling HSC function (Ho et al., 2017; Vannini et al., 2016).

1.8 Summary of Intent

HSCs are the most widely and successfully clinically applied stem cell type, having the therapeutic potential to treat hematologic malignancies when introduced through bone marrow transplantation (BMT) (Daley, 2012; Walasek et al., 2012). The success of BMT is critically hindered by the availability of HLA-matched donor HSCs. Additionally, to achieve clinically successful transplantation, sufficient numbers of HSCs must be infused in order to provide sustained recovery of the blood system. Expanding HSCs *ex vivo* prior to transplant remains an attractive solution to overcome both of these barriers. Attempts have been made to expand HSCs *ex vivo* using extrinsic factors including pro-proliferative cytokines and conditions emulating the bone marrow niche such as co-

culture with supporting stromal cell layers or three dimensional scaffold extra-cellular matrices (Delaney et al., 2010; Feng et al., 2006; Huang et al., 2007; Magin et al., 2009). The unyielding challenge with the preceding protocols is that culture conditions tend to favor differentiation and commitment to mature cells. To circumvent this problem, different groups have tried and succeeded in overexpressing HSC-specific transcription factors to expand functional mouse HSCs *ex vivo* and *in vivo* (Aguila et al., 2011; Antonchuk et al., 2002; Ohta et al., 2007; Watts et al., 2012). Nonetheless, these approaches have not produced similar outstanding results for human HSCs – a bottleneck in the clinical application of *ex vivo* expanded HSCs. Interestingly, a bioactive high throughput chemical screen found an antagonist of the aryl-hydrocarbon receptor called stemregenin1 (SR1) produced dramatic expansions of human HSCs thereby opening up a new paradigm in the search for other promising candidate molecules using drug discovery platforms (Boitano et al., 2010). Subsequently similar other studies have shown dimethyl-prostaglandin E2 (dmPGE2), VPA and inhibitor of p38 MAPK as capable of enhancing human HSCs *ex vivo* (Baudet et al., 2012; Chaurasia et al., 2014; Goessling et al., 2011). A recent high profile study identified UM171 as a superior agent of *ex vivo* expansion thereby keeping the promise alive for robust clinical applications of HSC transplant (Fares et al., 2014). Despite all these advances, our integrated understanding of the detailed molecular mechanisms of human HSC self-renewal is still inadequate. As a result, identifying novel critical molecular players enhancing HSC fitness could advance their full-fledged use for regenerative therapeutic purposes.

1.8.1. Musashi-2 and hematopoiesis

The study of gene regulation at the posttranscriptional level is a rapidly evolving field of research in developmental biology and involves among other mechanisms, the control of RNA splicing, mRNA stability, and mRNA translation (Moore, 2005). In particular, RNA binding proteins (RBPs) have been demonstrated to regulate the spatio-temporal biogenesis, stability, expression, function, transport and cellular localization of fate determinants during development (Glisovic et al., 2008). The eukaryotic Musashi (MSI) family is an evolutionarily conserved group of RBPs including MSI1 and MSI2. MSI was first discovered in *Drosophila* as a cell-intrinsic regulator of the asymmetric cell division of neural precursors during sensory organ development (Nakamura et al., 1994). Mechanistically, the MSI family of RBPs has been shown to inhibit translation by binding to the 3'-untranslated region (3-UTR) of target genes such as the fate determinant NUMB (Kawahara et al., 2008) as well as to affect processing of primary miRNA to their mature form (Choudhury et al., 2013). Subsequent studies have identified Msi1 and Msi2 both as master regulators of multiple stem cell populations in mammals (Kayahara et al., 2003; Lagadec et al., 2014; Sakakibara et al., 2001) however in the hematopoietic system only Msi2 is expressed in HSCs and its expression gradually decreases with differentiation (de Andrés-Aguayo et al., 2011; Hope et al., 2010b; Ito et al., 2010a; Kharas et al., 2010a; Park et al., 2014). Additionally, Park et al., de Andrés-Aguayo et al. and Hope et al. showed that loss of Msi2 impairs the self-renewal potential of mouse HSCs. Conversely, it was shown that overexpression of Msi2 enhances the bone marrow repopulating capacity of mouse HSCs. The discovery of novel functional roles for Msi2 in the

hematopoietic system has opened a window of hope for harnessing its capacity to regulate stem cell self-renewal for *ex vivo* expansion of HSCs for regenerative medicine. Indeed, through integrative functional genomics using *in vivo* transplantations, RNA-seq and CLIP-seq, we reported the critical role MSI2 plays in regulating human HSC self-renewal (Rentas et al., 2016). Ectopic expression of MSI2 imparts a net 23-fold *ex vivo* expansion of long-term repopulating CB HSCs. Mechanistically, MSI2 significantly attenuates components of AHR signalling, findings that provide potentially the first-ever insights into how this pathway is repressed at steady-state to maintain HSC self-renewal. Taken together, these studies suggest that the precise molecular regulation of MSI2 gene expression may be among the critical mechanisms underlying balanced HSC self-renewal/differentiation. Despite the clear importance of understanding how MSI2 maintains an appropriate HSC-specific expression level, very little is understood of the TFs that mediate this.

Therefore, the goal of my PhD research was to dissect the MSI2 promoter, use this as a discovery tool to explore, identify and functionally characterize the associating TFs for their role in regulating MSI2 expression and HSC function. Deciphering the upstream regulators of MSI2 could identify: 1) Transcription factors that could be amenable targets to boost MSI2 expression in HSCs and thus enhance its power for *ex vivo* expansion (Chapter 2). 2) Transcription factors that could be novel master regulators of HSC self-renewal and be used as potent *ex vivo* HSC expanders in their own right (independent of MSI2) (Chapter 3).

In **Chapter 2**, I employed an exploratory approach to identify and validate transcription factors regulating the expression of MSI2 in the model K562 cell line and primary HSPCs.

Rationale and Hypothesis

The hematopoietic expression and function of MSI2 is conserved between human and murine HSPCs as outlined in section 1.8.1. Therefore, its transcriptional regulation is suggestively mediated by promoter sequences bound by conserved TFs. *Given its convincingly demonstrated novel functions, my hypothesis is that MSI2's expression and function is (co)- regulated by novel hematopoietic stem cell-enriched TFs.*

To achieve this, I set the following specific objectives:

1. Systematically identify a putative minimal promoter region for MSI2 conserved between mouse and human.
2. Screen for and identify putative TFs binding to the minimal promoter region using *in silico* TF binding prediction tools, literature review, site-directed mutagenesis biochemical reporter assays and chromatin immunoprecipitation (ChIP) assays.
3. Functionally validate candidate TFs using reporter assays in K562 cells
4. Functionally validate candidate TFs for their downstream functions in HSPCs

I undertook a systematic approach to map and define relevant regulatory elements of the MSI2 minimal promoter. I focused on dissecting the approximate 3.5 kb region 5' upstream of MSI2's translational start site (TSS) shared between mouse and human and thus having the greatest potential of containing regulatory elements key to a conserved MSI2 HSC-specific expression program. Progressive 5'-terminal deletions of this region

coupled with *in silico* prediction of TFs that bind this region, identified Upstream Stimulating Factor-2 (USF2) and Pleomorphic Adenoma Gene -1 (PLAG1) (**Chapter 2**). This approach identified PLAG1 as co-regulator of MSI2 expression in HPSCs. However, its individual contribution towards MSI2 expression and function was negligible. Despite this, I found novelty in PLAG1 as a transcription factor whose function has never been explored, the basis for the studies carried out in Chapter 3.

In **Chapter 3**, I aimed to comprehensively decipher the functional role of PLAG1 in CB HSPCs and the molecular circuitry it uses to achieve this.

Rationale and Hypothesis

PLAG1 is a developmentally regulated C₂H₂ zinc finger TF recognizing a bi-partite DNA consensus sequence consisting of core sequence (GRGGC) and G-cluster (RGGK) separated by 6-8 random nucleotides (Voz et al., 2004). It was discovered in human pleomorphic adenomas of the salivary glands as proto-oncogene frequently rearranged and activated by chromosomal translocations with beta-Catenin (CTNNB1) (Debiec-Rychter et al., 2001; Kas et al., 1997) and leukemic inhibitory factor receptor (LIFR) (Voz et al., 1998) producing t(3,8)(p21;q12) and t(5,8) (p13;q12) respectively. Therefore, 20 years have passed since its discovery without any reported comprehensive functional studies in the hematopoietic context. Recent, transcriptome-wide gene expression analysis for transcriptional regulators of human HSCs identified PLAG1 as one of top six highly expressed transcription factors (Gazit et al., 2013). Further re-analysis of the same database and qRT-PCR from CB samples shows PLAG1 expression is enriched within the HSC compartment and its expression decreases with differentiation into more

committed progenitors and matured myeloid cells. However, no functional role linking PLAG1 expression with HSC phenotypes such as self-renewal, proliferation, differentiation, cell cycle, etc has been reported as yet. Given that PLAG1 is a novel TF and highly expressed in the HSC compartment, my hypothesis is that ectopic PLAG1 will induce and enhance the self-renewal machinery of HSCs.

To address the hypothesis, I set the following specific objectives:

1. Characterize *in vitro* the effect of PLAG1 overexpression and knockdown in CB HSPCs using CFU, cell proliferation and counts, immunophenotyping, apoptosis and cell cycle analysis.
2. Characterize *in vivo* the effect of PLAG1 modulation in CB HSPCs. I used LDA to determine effect of PLAG1 overexpression on the number and frequency of HSCs and serial transplantations to determine effect on long-term HSC self-renewal. I used RNAi to determine the consequence of loss of PLAG1 on HSC engraftment and lineage reconstitution.
3. Perform RNA-sequencing for transcriptome-wide global analysis of HSPC circuitry upon PLAG1 modulation to provide mechanistic insights that explain observations gleaned from objectives 1 and 2.

Complementary over expression and knockdown experiments were undertaken to decipher the functional relevance of PLAG1 in CB HPSCs (**Chapter 3**). Ectopic PLAG1 over expression in CB CD34⁺ cells offers significant pro-survival and CFU proliferative advantages to progenitors *in vitro*. PLAG1 over expression also cumulatively increases total nucleated and CD34⁺ cells count in *in vitro* culture. Importantly, ectopic expression

of PLAG1 in CD34⁺ cells *ex vivo* followed by transplantation into NSG mice at limiting doses robustly enhanced the frequency of engrafting HSCs with lymphomyeloid capacity. Complementary RNA sequencing in CB CD34⁺ cells with PLAG1 over expression and knockdown identified significant modulation of ribosome biogenesis (**Chapter 3**). Mechanistically, PLAG1 induces expression of H19 and MEG3 imprinted lncRNA-derived miRNAs to globally dampen proteostasis pathways including ribosome biogenesis. Furthermore, PLAG1 represses the pan-ribosome biogenesis TF MYC to exert another layer of control on protein synthesis pathways. In addition, PLAG1 enforces global stem cell gene signature to maintain long-term HSC self-renewal. Taken together, the discovery and follow up functional characterizations of PLAG1 coupled with RNA sequencing and subsequent pathway analysis paves the way for novel cellular and molecular mechanisms governing self renewal and commitment decision in CB HSCs — an immense body of knowledge that could help us in the therapeutic manipulations of these cells such as in *ex vivo* expansion.

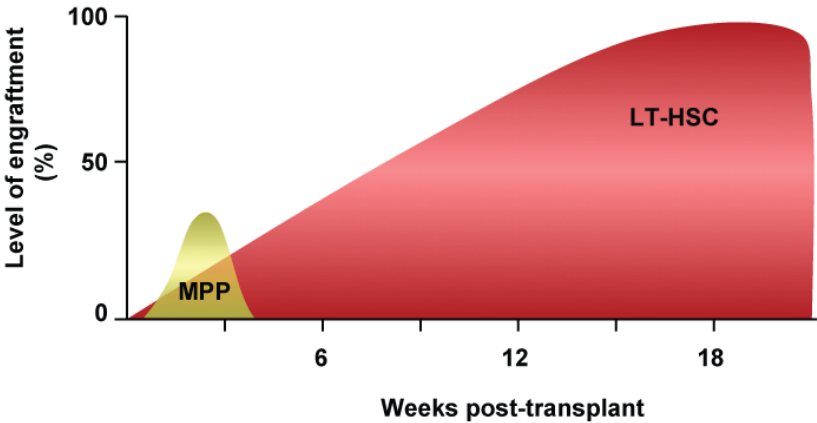


Figure 1.1. Cartoon schematics of the kinetics of human hematopoietic stem and progenitor cells xenotransplantation. LT-HSCs are operationally characterized by their ability to engraft immune-compromised mice for more than 16 weeks where as MPPs engraft transiently.

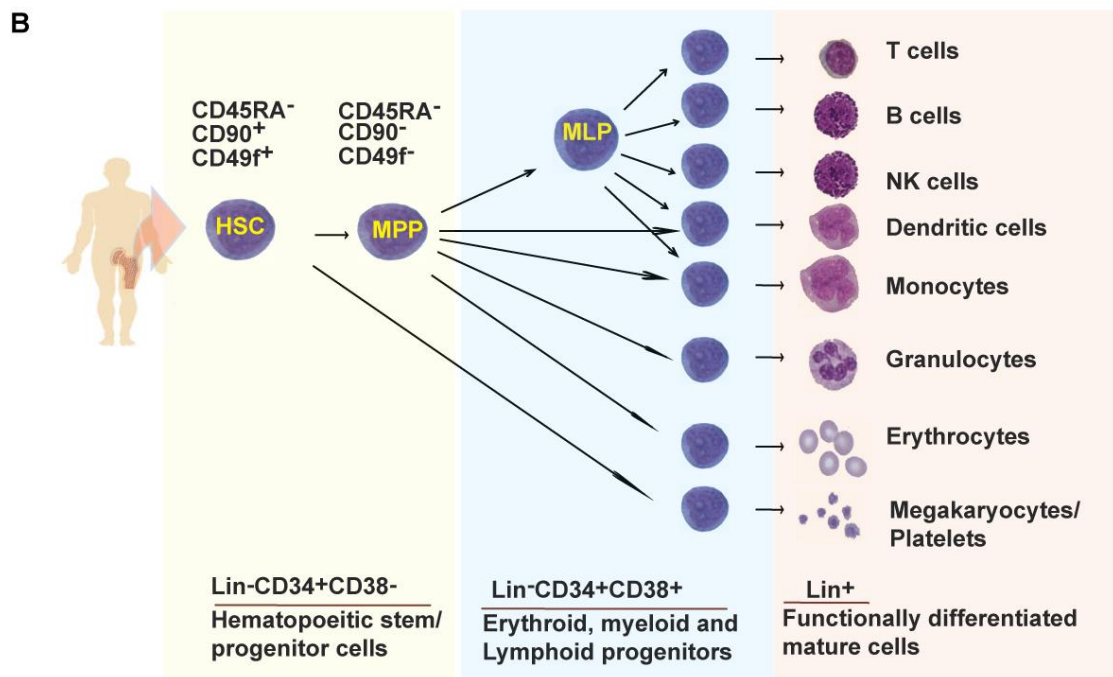
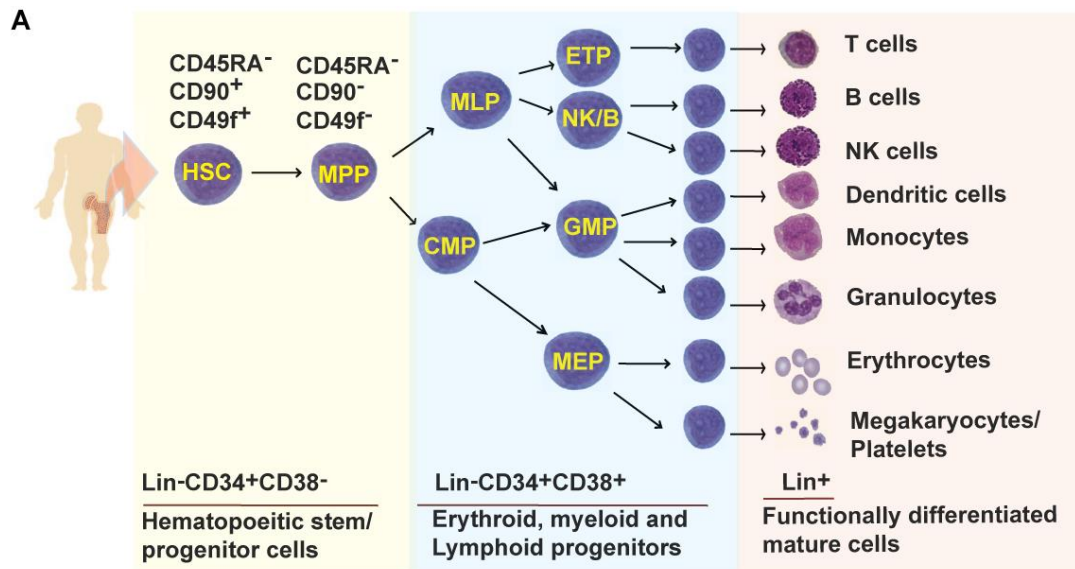
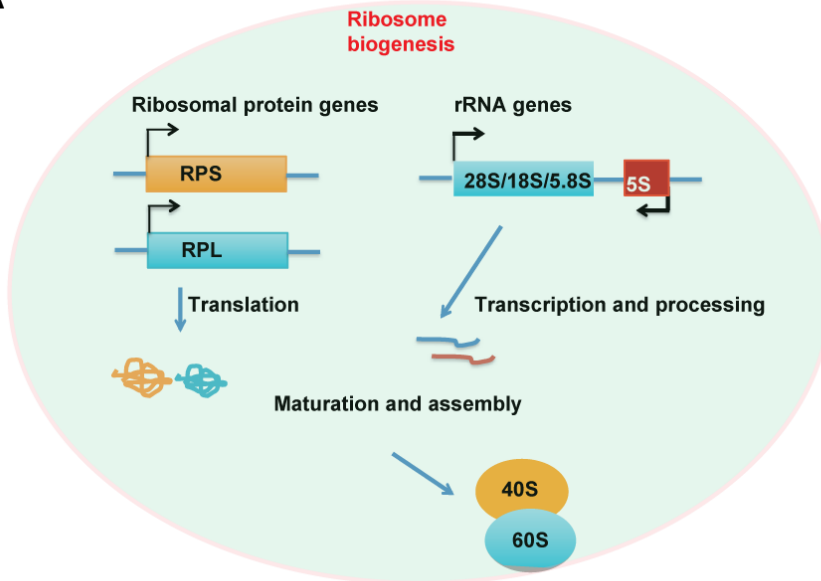


Figure 1.2. Models of adult human hematopoiesis. (A) Classic model of adult hematopoiesis. (B). Revised model of adult hematopoiesis (adapted from Notta et al 2016).

A



B

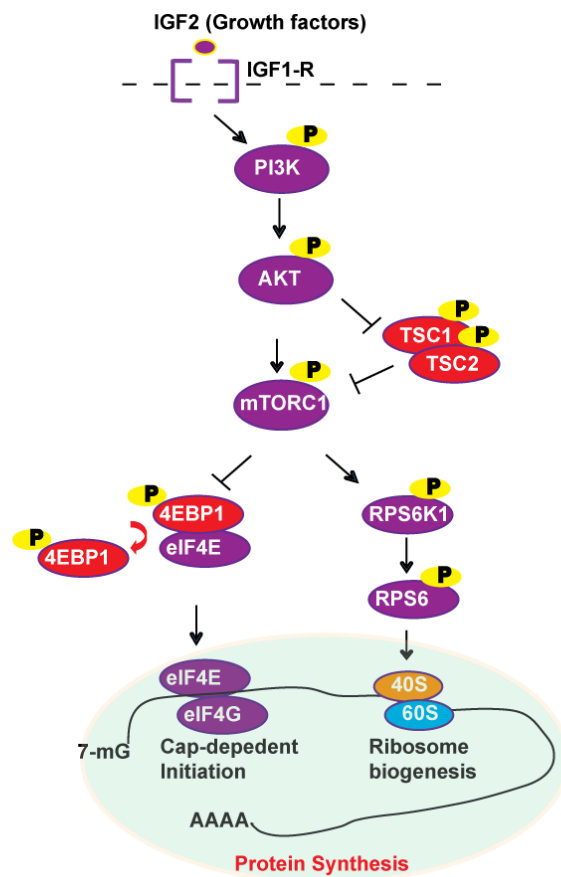


Figure 1.3. Cartoon schematics of ribosome biogenesis and mTOR signaling pathways. (A). Simplified schematics of the steps in the assembly of RPs and rRNAs. Homeostatic ribosome biogenesis involves translation of ribosomal proteins (RPs) and transcription and processing of ribosomal RNAs (rRNAs). (B). Simplified schematics of IGF1R-PI3K-AKT-mTOR signaling pathway as related to ribosome biogenesis/protein synthesis. mTOR signaling is activated by growth factors such as IGF2 binding to IGF1-R (adapted from Laplante and Sabatini, 2013).

CHAPTER 2

PLAG1 and USF2 Co-regulate Expression of MSI2 in Human Hematopoietic Stem and Progenitor Cells

Preamble

1. This chapter is an original article submitted and currently in revision for publication at the journal *Stem Cell Reports*. It is presented in its submitted form with the following authorships and title:

“Belew MS, Rentas, S and Hope KJ. PLAG1 and USF2 Co-regulate Expression of MSI2 in Human Hematopoietic Stem and Progenitor Cells”

2. The findings presented in this Chapter are also incorporated into the provisional patent filing:

“Belew MS and Hope KJ: Methods and compositions for expansion of hematopoietic stem and/or progenitor cells” Filed July 7, 2017; US62/529,694

MSB designed and performed experiments, interpreted results and wrote the manuscript. SR assisted with cord blood experiments and manuscript preparation. KJH supervised the project, interpreted results and wrote the manuscript.

3. I have significantly contributed towards the *Nature* (2016) publication from our laboratory reporting on the functional characterizations of MSI2 in HSPCs:

“Rentas S., Holzapfel NT, **Belew MS***, Pratt G., Voisin V., Wilhelm BT, Bader GT., Yeo GW, Hope KJ. Musashi-2 Attenuates AHR Signaling to Expand Human Hematopoietic Stem Cells. **Nature**. April 28, 2016, 532, 508–511, doi:10.1038/nature17665. Copyright© Nature Publishing Group. *Authors contributed equally “*

MSI2 is a marker and essential positive regulator of human hematopoietic stem cells (HSC) but knowledge of the mechanisms that ensure its appropriate expression in this context are lacking. Here I provide the first mapping of the MSI2 promoter functional in hematopoietic cells and identify the USF2 and PLAG1 transcription factors as co-operative regulators of endogenous MSI2 expression in human HSC. It is important to underline that future detailed characterization of the promoter and enhancer regions will very likely uncover additional transcription factors that also contribute to regulating physiological MSI2 expression. Here, I demonstrate that co-expression of PLAG1 and USF2 upregulates MSI2 expression. Functionally, the co-expression of PLAG1 and USF2 leads to post-transcriptional repression of CYP1B1 similar to what we have reported occurs with direct MSI2 overexpression (Rentas et al., 2016). Most importantly, co-expression of PLAG1 and USF2 increased total nucleated cells (TNCs) and CD34⁺ frequency, revealing novel transcriptional circuitry amenable for *ex vivo* HSC expansion.

CHAPTER 2

PLAG1 and USF2 Co-regulate Expression of MSI2 in Human Hematopoietic Stem and Progenitor Cells

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SUMMARY

MSI2 is expressed predominantly in human hematopoietic stem and progenitor cells (HSPCs), enforces HSPC expansion *ex vivo* when overexpressed and is upregulated in poor prognosis myeloid leukemias indicating its carefully regulated transcription is critical to balanced HSC self-renewal and restraint of leukemia progression. Despite this, little is understood of the transcription factors that enforce appropriate physiological levels of MSI2 in the blood system. We undertook a systematic approach to define a minimal conserved promoter region that reports on endogenous expression of MSI2. Coupled with *in silico* prediction of transcription factors binding this region and a mutagenesis/reporter assay screen we identified USF2 and PLAG1 as transcription factors whose binding to the MSI2 promoter drive reporter activity. Loss and gain of function studies showed that these factors co-regulate, and are required for, efficient transactivation of endogenous MSI2. Moreover, PLAG1 is most highly expressed within primitive cells of the hematopoietic hierarchy compared to the ubiquitous USF2 and we demonstrate that these factors preferentially bind the MSI2 minimal promoter in primary human CD34⁺ hematopoietic cells. Finally, we reveal that coincident overexpression of both USF2 and PLAG1 in primitive cord blood cells enhanced MSI2 transcription and yielded cellular phenotypes, including expansion of CD34⁺ cells during *in vitro* culture, consistent with that achieved by direct MSI2 overexpression. By identifying PLAG1 and USF2 cooperation as an important contributor to stem cell-specific expression of MSI2, we have pinpointed novel transcriptional circuitry that governs stemness in the blood system.

Introduction

The unique ability of HSCs to self-renew while giving rise to mature cells by differentiation is what enables their regenerative capacity. As such, identification and characterization of key regulators of this property is of both fundamental and clinical interest. The MSI2 RBP is now recognized as one such key HSC regulator. Its expression is highest in the primitive HSC compartment and progressively decreases upon commitment (de Andres-Aguayo et al., 2011; Hope et al., 2010a; Park et al., 2014; Rentas et al., 2016). MSI2 loss of function results in a significant depletion in the reconstitution capacity of primitive murine hematopoietic cells (de Andres-Aguayo et al., 2011; Hope et al., 2010a; Ito et al., 2010b; Park et al., 2014). By contrast, when moderately overexpressed, it imparts enhanced HSC self-renewal activity as measured by increased competitiveness in *in vivo* reconstitution assays (Hope et al., 2010a). In the human system we have shown an analogous detrimental effect on cord blood HSC-mediated reconstitution when MSI2 is repressed. These same stem cells undergo significant ex vivo expansion when MSI2 is overexpressed (Rentas et al., 2016). MSI2 has also been implicated in aspects of leukemia pathogenesis (Ito et al., 2010b; Kharas et al., 2010a; Park et al., 2015). For instance, in mouse models of chronic myeloid leukemia (CML) and myelodysplastic syndrome (MDS), ectopic expression of MSI2 encourages promotion of the disease to acute phases (Kharas et al., 2010a; Taggart et al., 2016). In the human context aberrantly high expression of MSI2 correlates with more aggressive CML disease states and is associated with poor prognosis in acute myeloid leukemia (AML) and MDS (Ito et al., 2010b; Kharas et al., 2010a; Taggart et al., 2016). Taken together, these studies

suggest that the precise molecular regulation of MSI2 gene expression may be among the critical mechanisms underlying balanced HSC self-renewal/differentiation and the restraint of leukemia progression. Despite the importance of understanding how MSI2 maintains an appropriate stem cell-specific expression level, very little is known of the promoter elements or transcription factors that mediate this. Here we report a systematic strategy that provides the first mapping of the MSI2 promoter functional in hematopoietic cells and identify the USF2 and PLAG1 transcription factors as novel co-operative regulators of endogenous MSI2 expression and function in human HSPCs.

Results and Discussion

Dissection of the MSI2 minimal promoter

MSI2 expression is evolutionarily conserved in both mouse and human HSPCs. Therefore, as an initial step in mapping its promoter we concentrated on the region directly upstream of the translational start site sharing extensive sequence similarity between the two species. This corresponded to a region extending to ~3.2 kb upstream wherein homology peaks were detected throughout as identified by the Multiple sequence Local Alignment and visualization tool (MULAN) (Ovcharenko et al., 2004) (Figure 2.1A, middle panel). Multiple sequence features including a nuclease accessible site (NAS), CpG island and TF binding sites as identified by ChIP-seq within a conserved region ~1 kb upstream of the translational start site further suggested the potential for this region to function in a promoter capacity (Figure 2.1A). Introduction of this 3.2 kb region upstream of firefly luciferase in pGL3-basic yielded significantly greater reporter activity as compared to the promoterless construct in MSI2-expressing K562 and HEK293 cell

lines (3-fold and 7.5-fold respectively) (Figure 2.1A, data not shown). Using variations in the extent of homology peaks as end points, we generated a set of luciferase reporter constructs with serial 5'-truncations of the ~3.2 kb sequence. A significant drop in reporter activity resulted only when the upstream sequence driving reporter expression was reduced from -588 to -203 bp (Figure 2.1A). In confirmation that a minimal promoter region containing essential *cis* elements governing MSI2 expression is contained within this 385 bp region we found its deletion from the full-length 3.2 kb fragment was sufficient to repress luciferase activity to the level of the promoterless reporter (Figure 2.1A).

USF2 and PLAG1 binding sites are required for MSI2 promoter activity

We next implemented a mutagenesis screen to systematically test the functionality of TF consensus sites within the minimal promoter region in order to pinpoint key MSI2-regulators. First we used *in silico* TF binding site prediction (Matinspector) to identify a total of 107 TF candidates representing 65 different TF families. From within this set we removed those TFs not appreciably expressed in both murine and human HSPCs where MSI2 expression is known to be concentrated. A final refinement based on an extensive literature search to identify novel TFs, or those implicated in the regulation of stem cell function and/or leukemia yielded a list of 10 candidate regulators of primitive hematopoietic cell expression of MSI2 (Figure 2.1B, C). Using the -588-Luc reporter construct as a template we generated a series of constructs possessing mutations of the consensus binding sites of each of these TFs and screened for loss of luciferase reporter activity (Figure 2.1D, E). The only TFs whose binding site mutagenesis resulted in a

significant reduction in reporter activity were the E-box motif-binding USF2 and the C₂H₂ zinc finger PLAG1 (Figure 2.1E). Moreover, combinatorial binding site mutagenesis of USF2 and PLAG1 binding sites simultaneously yielded a more significant reduction in minimal promoter activity than when PLAG1 or USF2 binding sites were mutated in isolation (Figure 2.1F). The results of our rationalized screen thus indicate that intact PLAG1 and USF2 binding sites are required for full MSI2 promoter activity and highlight a potential co-operativity between PLAG1 and USF2 in MSI2 transactivation.

USF2 and PLAG1 bind the promoter of MSI2 and promote its transcription

The minimal MSI2 promoter sequence contains a total of three consensus E-box motifs for USF2 (Bendall and Molloy, 1994) and three G-rich bipartite consensus sequences for PLAG1 (Hensen et al., 2002) (Figure 2.2A). In support of the functionality of these binding sites, ChIP-qPCR demonstrated that PLAG1 and USF2 yield significant fold-enrichment at the minimal promoter region of MSI2 in K562 cells (Figure 2.2B). Importantly, USF1 and MYC TFs known to bind to the same E-box motif as USF2 were not enriched in the *MSI2* minimal promoter region (Supplementary Figure 2.1). Combined with the mutagenesis data, these results are consistent with the direct binding of USF2 and PLAG1 to their consensus recognition elements as requirements for full activity of the MSI2 minimal promoter. To quantify the transactivation potential of USF2 and/or PLAG1 on the promoter region we independently overexpressed each gene. USF2-overexpressing (USF2) cells exhibited a significant 40% (1.4-fold) enhanced reporter activity as compared to vector controls (Figure 2.2C, D). In addition, USF2 overexpressing cells did not exhibit any elevation of luciferase reporter levels when

transfected with a promoter construct that had mutations in all three USF2 binding sites, thus further confirming the specificity of USF2 binding to the predicted sites (Figure 2.2E). Lastly, USF2 overexpression resulted in a 1.4-fold increased MSI2 transcript level compared to negative controls, validating the reporter results and confirming the direct connection of USF2 levels with endogenous MSI2 expression (Figure 2.2F).

There are three human PLAG1 transcript variants encoding two protein isoforms. The longer isoform (PLAG1-A) contains all annotated functional domains whereas the shorter form (PLAG1-B) is missing Zn fingers F1 and F2. A third reported slightly smaller PLAG1 translational isoform exists through translation from methionine 100 (PLAG1-S) is missing the first 17 N-terminal residues of PLAG1-B and thus co-migrates with it on SDS-PAGE (Debiec-Rychter et al., 2001) (Figure 2.2G, Supplemental Figure 2.2). In both K562 and primary primitive human CB cells PLAG1 appears to be contributed largely by the short-form(s) (Figure 2.2G, H). Thus to characterize their respective contributions to MSI2 transactivation we overexpressed PLAG1-B and PLAG1-S in K562 cells (Figure 2.2I). Both forms independently enhanced reporter activity ~1.4 fold in a PLAG1 binding site dependent manner (Figure 2.2J, K) and most importantly, resulted in a significant 1.6-fold increase in endogenous MSI2 transcription suggesting they behave interchangeably with respect to MSI2 regulation (Figure 2.2L). Together, these findings indicate that both USF2 and PLAG1 directly interact with the MSI2 minimal promoter and are capable of ectopically transactivating its expression.

USF2 and PLAG1 function co-operatively to promote MSI2 transactivation

As binding site co-mutagenesis suggested that USF2 and PLAG1 potentially co-operate to regulate MSI2 promoter activity we sought to further address this possibility using PLAG1-S and USF2 co-overexpression in K562 cells. Luciferase activity following co-overexpression was approximately 2-fold greater than in negative controls and over 1.5-fold greater than in cells overexpressing either USF2 or PLAG1-S alone (Figure 2.3A). In addition, the same assay performed in singly and doubly USF2- and PLAG1-S-overexpressing cells transfected with USF2/PLAG1 binding site co-mutated promoter reporter constructs demonstrated background level reporter activity confirming the specificity of the predicted binding sites and mandatory requirement of coincident USF2 and PLAG1-S binding for full transactivation (data not shown and Figure 2.3B). Strikingly, co-overexpression increased endogenous MSI2 transcription more than 4.5-fold in comparison to vector transfected control cells, an increase that was also manifested at the protein level (Figure 2.3C, D). This robust increase in MSI2 transcription is more than 2.5-fold higher than that observed in cells overexpressing USF2 or PLAG1-S independently, indicating a synergistic response when the two factors are coordinately elevated.

Reciprocal knockdown experiments with shRNAs targeting USF2 and PLAG1 resulted in a 40% and 50% decrease in endogenous MSI2 transcription, respectively, in comparison to control cells expressing non-targeting shRNA (shLuc) (Figure 2.3E-G). In addition, we measured the effects of PLAG1 loss of function using a dominant negative approach wherein we introduced short PLAG1 forms missing their transactivation domains

(PLAG1-B DN and PLAG1-S-DN, respectively) (Figure 2.3H). The introduction of either form led to the attenuation of MSI2 transcription by approximately 1.4 fold as compared to controls (Figure 2.3H). These results confirm that PLAG1 transactivation activity is a necessary requirement for maintenance of physiological levels of MSI2 transcription. In summary, these gain- and loss-of-function assays demonstrate that USF2 and PLAG1 constitute critical components of the regulatory circuitry controlling MSI2 transcription and are critical for maintaining physiological MSI2 expression levels.

If the synergistic effects of USF2 and PLAG1 is indeed a key component in driving MSI2 expression in primary hematopoietic cells, it would be expected that maximal MSI2 expression would be observed in cells where the combined levels of these two factors peak. Indeed, this is the case, as USF2 is ubiquitously expressed across the human hematopoietic hierarchy, while PLAG1 is at its highest levels in the most primitive HSC subset and declines rapidly in expression with differentiation (Figure 2.4A), a profile that is also consistent with MSI2 expression (Rentas et al., 2016). Moreover, we confirmed PLAG1-B isoform expression follows the same pattern of being more highly expressed in HSC enriched CB populations (Figure 2.4B). To prove that this maximal presence of PLAG1 in combination with USF2 in more primitive hematopoietic cells translates to an increase in actual MSI2 promoter binding we performed ChIP to examine PLAG1 and USF2 binding to the -588 bp region of Lin⁻CD34⁺ HSPCs and Lin⁻CD34⁻ restricted progenitors. Importantly, ChIP demonstrated that PLAG1 and USF2 do indeed directly bind to the MSI2 promoter with more than 4.5- and 2.5-fold enrichment, respectively, in Lin⁻CD34⁺ as compared to Lin⁻CD34⁻ cells (Figure 2.4C-E). Furthermore, selective

knockdown of PLAG1 in Lin⁻CD34⁺ cells results ~60% down-regulation of MSI2 transcript after 72hours of transduction further indicating the specificity and reproducibility of regulation by these transcription factors in primitive HSPCs (Figure 2.4F-G).

Co-overexpression of USF2 and PLAG1 phenocopies expression of ectopic MSI2 and enhances primitive cell output

We have previously reported that MSI2 enforces HSPC expansion ex vivo in part through the post-transcriptional repression of the AHR effector CYP1B1 (Rentas et al., 2016). As would be expected if a major effect of their co-operative function is MSI2 upregulation, co-ordinate overexpression of USF2 and PLAG1-S in Lin⁻CD34⁺ cells resulted not only in a significant 4.5-fold elevation in MSI2 transcription at day 7 of ex vivo culture (D7) but also in the selective repression of CYP1B1 protein (and not its mRNA levels) (Figure 2.5A-C). Most importantly, relative to controls, significant 5- and 6-fold enhancements in the proportion and total numbers of primitive CD34⁺ cells respectively was observed following a 7-day culture of CB cells jointly overexpressing USF2 and PLAG1-S (Figure 2.5D-F). Together, these results are consistent with USF2 and PLAG1-S acting in combination to enforce MSI2 expression, thereby promoting primitive hematopoietic cell maintenance.

To date, no endogenous regulators of MSI2 transcription in the human HSPC compartment have been described. Given the powerful role MSI2 plays in promoting HSPC self-renewal, identifying MSI2 activators and their mechanism of action is required before their regenerative potential can be fully harnessed. Our identification of USF2 and

PLAG1 as being both necessary, and together, sufficient for significant transcriptional activation of MSI2, represents an important advance in our understanding of how these factors regulate MSI2. Interestingly USF2, while known to regulate a number of diverse biological processes, has also previously been shown to regulate HOXB4 in human hematopoietic cells (Giannola et al., 2000; Zhu et al., 2003). As such, USF2's involvement in MSI2's transcriptional regulation transactivation hints at a larger potential for this TF to function in combination with others in the regulation of “stemness” genes. PLAG1 was initially discovered in human pleomorphic adenomas of the salivary gland as a proto-oncogene (Debiec-Rychter et al., 2001; Kas et al., 1997; Voz et al., 1998). Importantly, we have shown that it is the selective expression of PLAG1 within the most primitive cells of the blood system (Gazit et al., 2013) that enables it to function in synergy with the ubiquitously expressed USF2, ensuring appropriate, high level expression of MSI2 in these cells. Our findings thus identify PLAG1 as a novel transcriptional regulator of HSPC in its own right (independent of MSI2). As a result, Chapter 3 of this thesis will be dedicated to deciphering the functional relevance of *PLAG1* in CB HSPCs.

Experimental Procedures

MSI2 promoter cloning and screening for associated transcription factors

A 3238 bp 5'- flanking sequence upstream of the translation start site of human MSI2 (transcript variants 1 and 4) was PCR amplified from BAC clone RP11-784M23 (Roswell Park Cancer Institute) and cloned upstream of firefly luciferase in pGL3-basic (Promega). The transcriptional start site is predicted to be 173 nucleotides upstream of the

translational start site. 5'-serial truncations were generated using primers listed in Supplemental Table 2.1. TFs predicted by MatInspector (Quandt et al., 1995) to bind the human 385 bp minimal MSI2 promoter were retained for comparison against publicly available databases of global gene expression profiles of diverse hematopoietic subpopulations to facilitate a final selection of genes that are expressed in primitive mouse and human hematopoietic stem cells (Bagger et al., 2016; Heng and Painter, 2008; Jojic et al., 2013b; Novershtern et al., 2011). For subsequent promoter mutagenesis screening we prioritized TFs that a) had commercially available antibodies, b) are expressed in K562s, c) are known to be expressed in other stem cell types and/or to regulate their homeostasis and d) exhibit binding sites that are conserved in the MSI2 promoter region shared between human and mouse. Predicted TF binding sites were mutated by rolling circle site-directed mutagenesis (Zheng et al., 2004). Primers and nucleotide substitutions are provided in Supplementary Table 2.2. Heatmap depicting putative MSI2 regulating transcription factors and their expression in the mouse hematopoietic hierarchy was generated using IMMGEN's online My GeneSet tool.

Promoter clone transfections and reporter assays

500ng of each clone were transiently co-transfected with 25ng of pRL-TK (internal transfection control expressing *Renilla* luciferase) in K562 cells in triplicate. Luminescence was measured 24-36 hrs after transfection using Dual-Luciferase-Assay Kit (Promega) in a FLUOstar Omega luminometer (BMG Labtech). Firefly luciferase activity was normalized to *Renilla* luciferase activity to adjust for transfection efficiency.

Western blots

Total protein was extracted from K562 cells using lysis buffer (50mM Tris HCl pH 8, 150 mM NaCl, 1% NP-40, 0.5% sodium Deoxycholate, 0.1% SDS, EDTA (2mM) supplemented with protease inhibitor cocktail (Roche diagnostics). Lysate was spun at 14000 RPM for 20 minutes on refrigerated tabletop centrifuge. Total protein concentration was determined by Bradford assay using Bovine Serum Albumin (BSA) as standard (Bio-rad laboratories, Inc). Ten microgram total protein was resolved on 10% Bis-Tris PAGE. Resolved protein was transferred onto immobilon-P PVDF membrane (EMD Millipore) on transfer buffer (25mM Tris, 250mM Glycine and 15% methanol) using Trans-blot Turbo apparatus (Bio-rad laboratories, Inc). Membrane was blocked for 1hr using LI-COR blocking buffer (LI-COR Biosciences). Primary antibodies against MSI2 (#ab7614, Abcam) β -Actin (Cat# A5441 clone AC15, Sigma-Aldrich), were diluted in LI-COR blocking buffer (1:1000) and incubated for 1hr at room temperature, washed 3 times at 15 min interval by using 1x TBST buffer (50 mM Tris, 150 mM NaCl, 0.05% Tween-20 pH 7.6). IRDye 680 Goat Anti-Rabbit (cat# 926-32221, LI-COR Biosciences) and IRDye 800CW Goat anti-Mouse (cat# 926-32210, LI-COR Biosciences) secondary antibodies were diluted (1:15,000) in LI-COR blocking buffer and incubated for 1hr at room temperature. Membrane was washed three times at 15 minutes interval using 1x TBST buffer. Blots were imaged using LI-COR Odyssey imaging station (LI-COR Biosciences).

Quantitative Real Time PCR (qRT-PCR)

Total RNA was isolated using TRIzol-LS reagent (Life Technologies). cDNA synthesis and qPCR was done using the same protocol as Rentas et al. (Rentas et al., 2016) using Roche UPL primer and probe sets specific to each of PLAG1, β -ACTIN, MSI2 and CYP1B1 (Supplemental Table S2.4). PLAG1 isoform specific qPCR assays were purchased from IDT (PrimeTime Mini qPCR Assay, PLAG1-A assay ID, Hs.PT.58.26984634; PLAG1-B assay ID, Hs.PT.58.22864822). Fold change in transcript level was calculated according to the $2^{-\Delta\Delta C_t}$ method.

Generation of PLAG1 and USF2 independent overexpression

Open reading frame (ORF) cDNA clones for PLAG1 (Clone Id: 30915361) and USF2 (Clone Id: 5483523) were obtained from Mammalian Gene Collections (GE Healthcare). cDNAs were PCR amplified using PrimeSTAR Max enzyme and subcloned into EcoRI/XhoI (NEB) digested MSCV-PGK/GFP vector. Primers used were: USF2-sense, CAT GCA TGG AAT TCG CCA CCA TGG ACA TGC TGG ACC, USF2-antisense, CAT GCA TGC TCG AGT CAC TGC CGG GTG CC; (EcoRI and XhoI sites are underlined). To generate 3xFLAG epitope-tagged versions of the predominantly expressed variants of PLAG1-B and PLAG1-S, the respective PCR products were generated and cloned into the shuttle vector p3xFLAG-CMV10 (Sigma-Aldrich) at EcoRI/KpnI (NEB) sites. FLAG-tagged clones were PCR amplified and subcloned into MSCV-PGK/GFP vector at EcoRI/XhoI sites using MfeI/SalI (NEB) compatible end ligation. Primers used were: FLAG-PLAG1-sense, GAT CGA TCC AAT TGA TGG ACT ACA AAG ACC, FLAG-PLAG1-antisense, CAT GCA TGG TCG ACC TAC TGA

AAA GCT TGA (MfeI and SalI sites are underlined).

Generation of PLAG1 and USF2 co-overexpression and knockdown constructs

PLAG1 isoforms and USF2 were cloned into the pSMALB vector also encoding mtagBFP (van Galen et al., 2014a). For co-overexpression 3xFLAG-PLAG1-100 and USF2 were cloned consecutively into pSMALB and separated by a P2A site. shRNAs against PLAG1 and USF2 were designed using the RNAi Central tool and ligated downstream of the minCMV promoter in the modified cppt-PGK-EGFP-IRES-PAC-WPRE lentiviral expression vector (Hope et al., 2010a). Lentivirus production and transduction of K562 or primary Lin- CB cells was done as previously reported (Rentas et al., 2016). Stable lines were propagated with biological replicate lines created for every condition from 3 or more distinct transfections.

Chromatin Immunoprecipitation (ChIP) assay

ChIP was done as per Carey et al with slight modifications (Carey et al., 2009). Briefly, cells were cross-linked, lysed and nuclei subjected to M220 focused-ultra sonicator (Covaris) to generate sheared chromatin fragments of ~500 bp. 5µg anti-USF2 (SantaCruz, #sc-862), anti-PLAG1 (GenTex, # GTX124217) or anti-rabbit IgG (SantaCruz) were added per 1000µL of IP volume followed by precipitation of chromatin-antibody complex by Protein G dynabeads (Invitrogen). Immunoprecipitated DNA was extracted using phenol/chloroform, quantified and subjected to qPCR. Primers and probes were designed by the Universal Probe Library (UPL) probe finder tool (Roche) using input sequence comprising 588 bp upstream of the MSI2 TSS. For the promoter region we used UPL probe #63 and forward and reverse primers as follows:

CGCTCGCAGAGAGATTCTG; GAGATCTCCGCTCCCTCCT. ChIP fold enrichment was calculated using the $2^{-\Delta\Delta C_t}$ method (Schmittgen and Livak, 2008).

Cord blood CD34⁺ cell isolation, lentiviral transduction and *in vitro* culture

FACS isolated Lin⁻ CD34⁺ cells were prestimulated for 8-12 hours in StemSpan medium (StemCell Technologies) supplemented with growth factors Interleukin 6 (IL-6; 20 ng/ml, Peprotech), Stem cell factor (SCF; 100 ng/ml, R&D Systems), Flt3 ligand (FLT3-L; 100 ng/ml, R&D Systems) and Thrombopoietin (TPO; 20 ng/ml, Peprotech). Cells were transduced at an MOI of 50-100 with pSMALB lentiviral particles encoding 3xFLAG-PLAG1-p2A-USF2 (PLAG1+USF2) or firefly luciferase (Vector). BFP⁺ cells were purified using a MoFlo XDP (Beckman Coulter) 72hrs post-transduction. For *in vitro* culture, cells were counted and analyzed by flow cytometry for CD34 positivity at day 3 and 7. Flow cytometry data was analyzed by Flowjo (Tree star). Immunocytochemistry staining, imaging and analysis for MSI2 and CYP1B1 protein levels was done as previously reported (Rentas et al., 2016).

Statistical analysis

All statistical analyses were performed using PRISM (Graphpad software, Inc). Where appropriate statistical significance was estimated using non-parametric two-tailed t-test for pairwise comparisons or 1-way ANOVA multiple comparisons of control and test transfections/transductions. Data shown as mean \pm SEM. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

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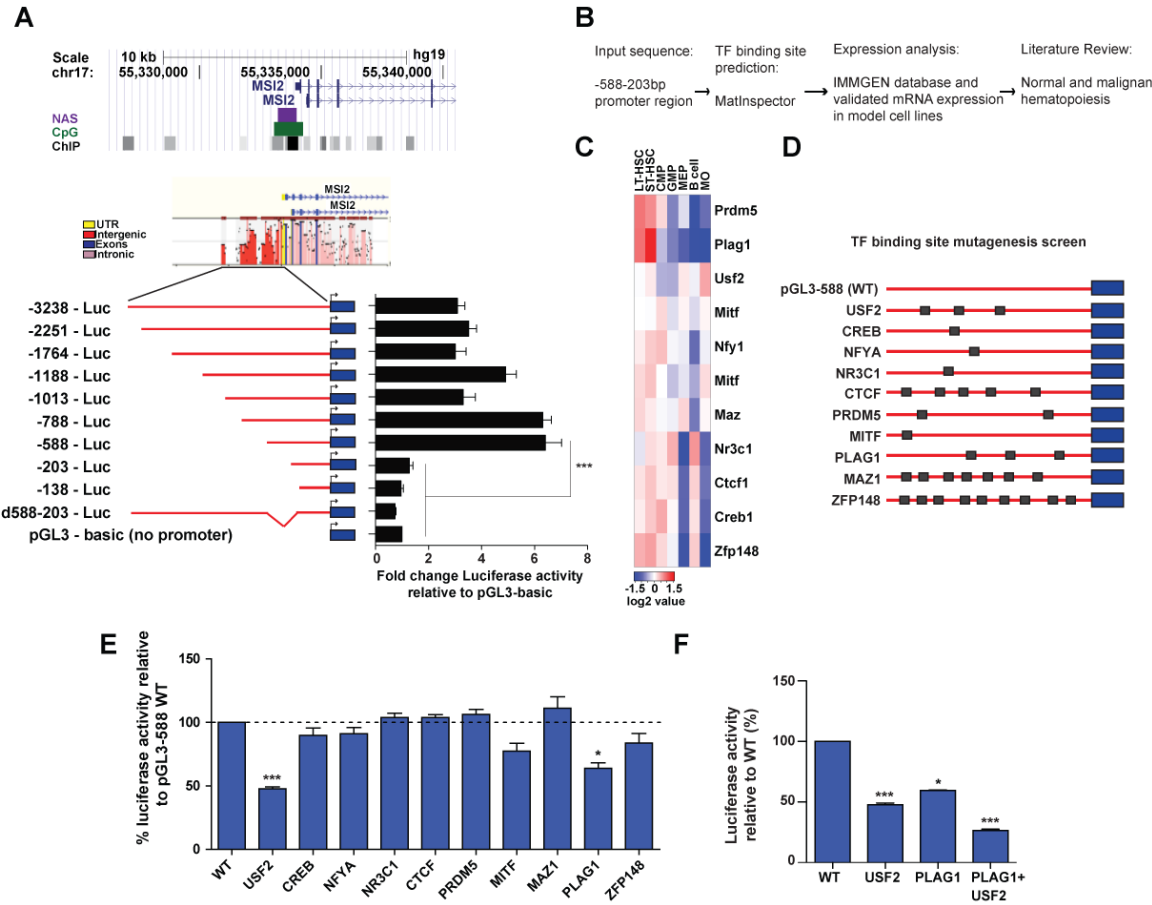


Figure 2.1. *In silico* mapping and mutagenesis screening identifies the MSI2 promoter in hematopoietic cells with dependence on USF2 and PLAG1 binding sites for activity. (A) UCSC genome browser annotation of features within the region directly 5' upstream of MSI2 (top panel) including ChIP-validated transcription factor binding sites, a CpG Island and nuclease accessible site (NAS). Middle panel depicts MSI2 genomic sequence alignment and homology between mouse and human species as analyzed by MULAN. Bottom panel shows a schematic representation of the serial 5'-promoter truncations (red) placed upstream of the firefly luciferase (Luc) reporter gene (blue) and their corresponding luciferase reporter activity. (B) Workflow of transcription factor selections for binding site mutagenesis screen. (C) Heatmap demonstrating the relative expression across the hematopoietic hierarchy of a prioritized subset of transcription factors predicted *in silico* to bind the MSI2 promoter. (D) Schematic depicting the binding sites mutated for each of the 10 candidate MSI2-regulating transcription factors in independent reporter constructs. (E) Percentage changes in luciferase activity after specific transcription factor binding site mutagenesis within the minimal promoter sequence as compared to the wild type (WT) promoter. (F) Reporter activity upon combinatorial binding site mutagenesis for USF2 and PLAG1 within the MSI2 minimal promoter region. Data for all reporter assays was generated from n=3-6 independent experiments

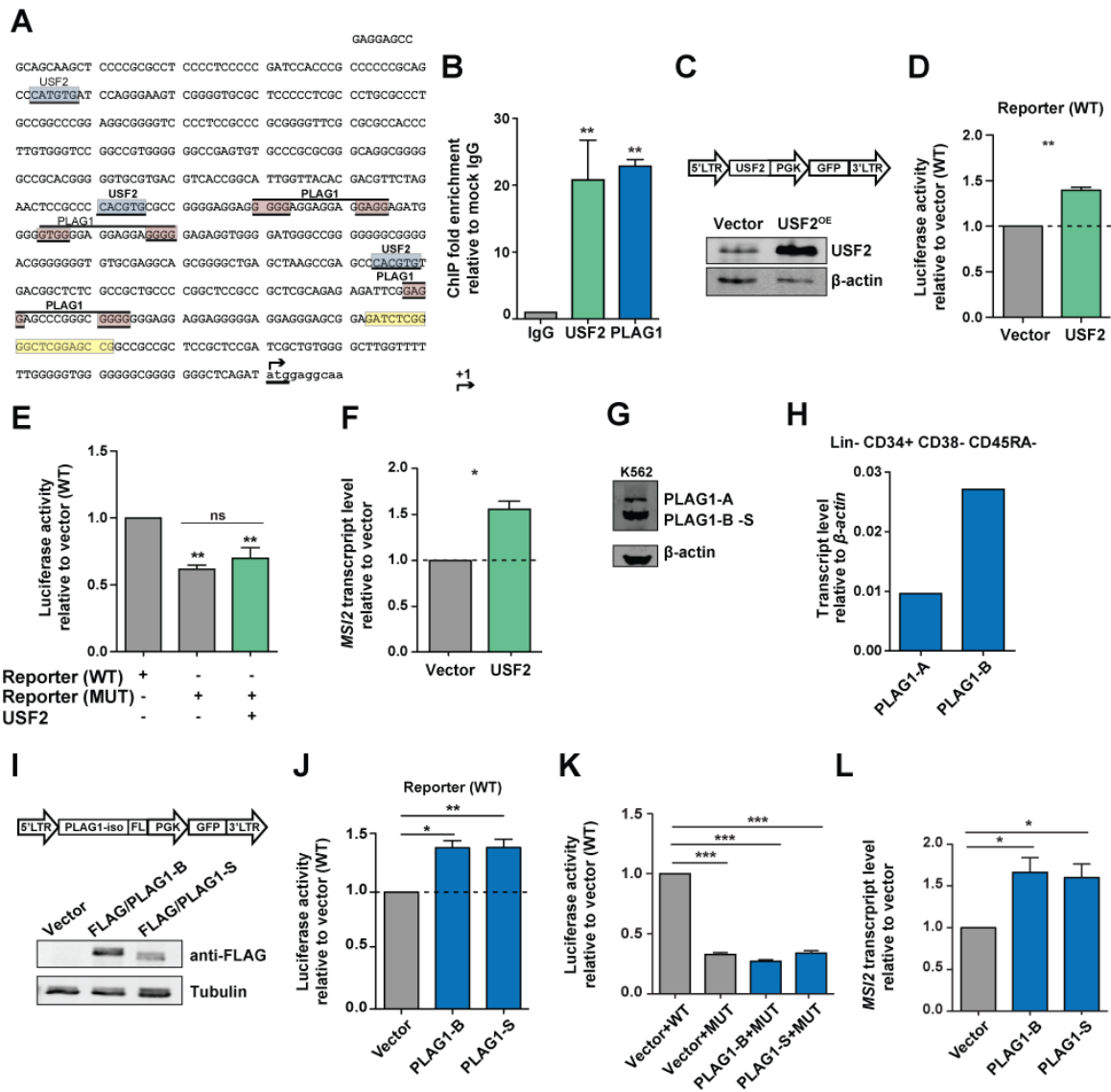


Figure 2.2. Ectopic PLAG1 and USF2 bind the MSI2 promoter and enhance its transcription in K562 cells. (A) DNA sequence of the minimal promoter for MSI2 with predicted (MatInspector) USF2 (blue) and PLAG1 (red) binding sites shown. Predicted (MatInspector) transcription start site is highlighted in yellow box. The translation start site of MSI2 is identified by the forward arrow from lower case nucleotides and the initiation codon is underlined. (B) ChIP-qPCR for PLAG1 and USF2 binding of the minimal MSI2 promoter (n=4 experiments). (C) USF2-overexpressing lentiviral construct schematic (top) and western blot quantification of its enhanced protein-levels in K562 cells (bottom). (D) Wild type (WT) MSI2 minimal promoter reporter construct luciferase activity upon USF2 overexpression (n=3 experiments). (E) Luciferase activity of USF2 binding site-mutated (MUT) reporter with and without USF2 overexpression as compared to WT (n=4 experiments). (F) Endogenous MSI2 transcript levels following USF2 overexpression in K562 cells (n=3 experiments). (G) Characterization of PLAG1 isoform expression by western blot in K562 cells. (H) Comparison of PLAG1-A and PLAG1-B expression in flow-sorted CB HSPCs (performed once from pooled CB samples). (I) FLAG-tagged PLAG1-short-isoform overexpressing lentiviral construct schematic and western blot quantification of enhanced protein-levels in K562 cells (bottom). (J) WT reporter construct luciferase activity upon PLAG1-B or PLAG1-S overexpression (n=4 experiments). (K) Luciferase activity of PLAG1-B and PLAG1-S binding site-mutated (MUT) reporters with PLAG1-B or PLAG1-S overexpression as compared to WT (n=4 experiments). (L) Endogenous MSI2 transcript levels following PLAG1-B or PLAG1-S overexpression in K562 cells (n=3 experiments).

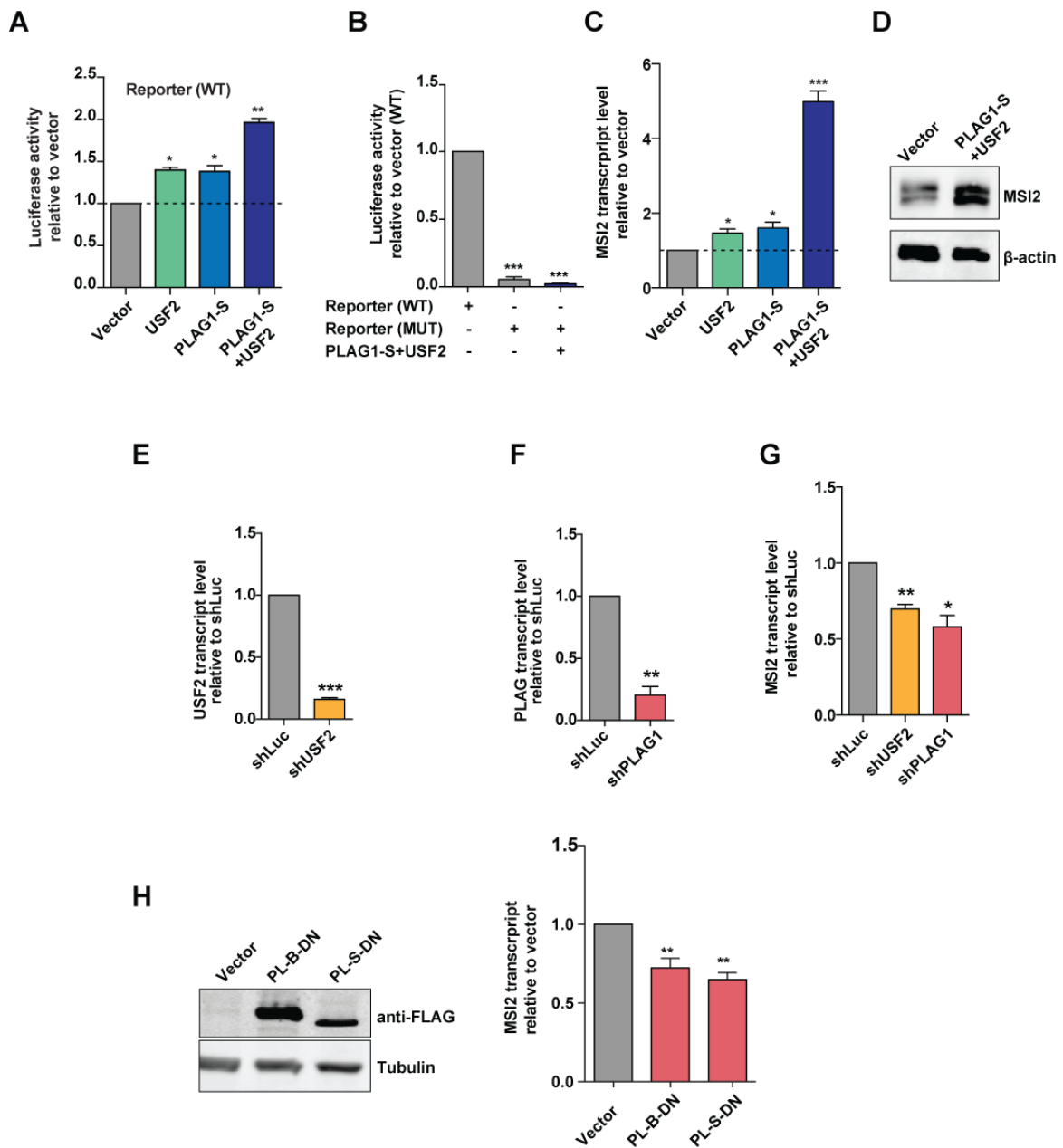


Figure 2.3. PLAG1-S and USF2 synergistically regulate and are required for MSI2 promoter activity and MSI2 expression.

(A) Reporter construct luciferase activity upon independent or coincident overexpression of USF2 and PLAG1-S (n=3 experiments). (B) Luciferase activity of PLAG1 and USF2 binding site-mutated (MUT) reporters upon PLAG1-S and USF2 co-overexpression (n=3 experiments). (C, D) Endogenous MSI2 transcript (C) (n=3 experiments) and protein levels (D) following overexpression of PLAG1-S and USF2 in K562 cells as compared to vector control. (E-F) Knockdown efficiencies of shUSF2 (E) and shPLAG1 (F) as measured post-infection by q-RT-PCR in K562 cells (n=3 experiments for each hairpin). (G) MSI2 transcript level reductions following USF2 and PLAG1 knockdown (n=3 experiments). (H) Left panel shows Western blot validation of overexpression of the dominant negative forms of PLAG1-B (PL-B-DN) and PLAG1-S (PL-S-DN). Right panel depicts MSI2 transcript levels following introduction of PL-B-DN or PL-S-DN into K562 cells. (N=3 experiments).

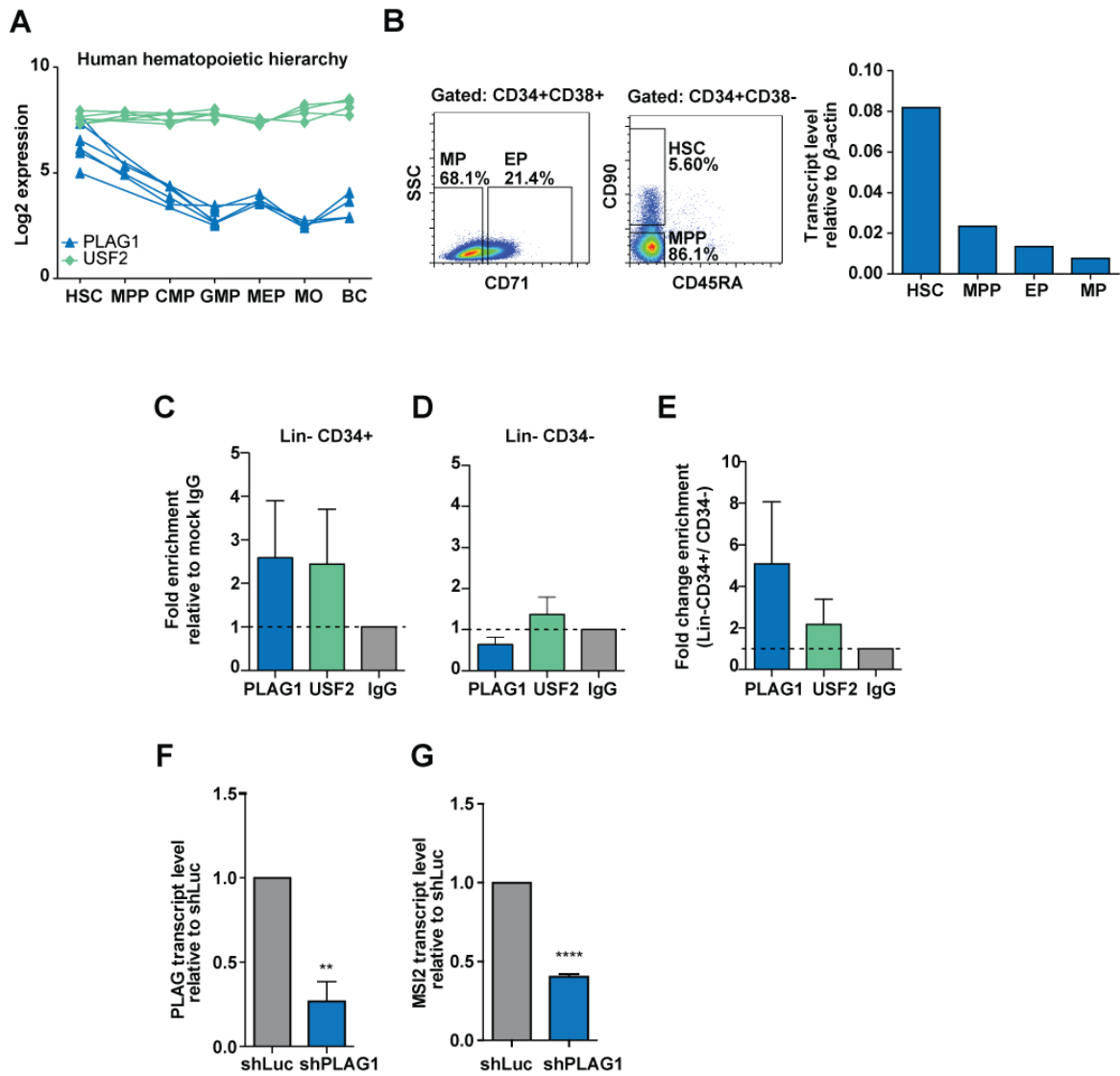


Figure 2.4. PLAG1-S and USF2 stoichiometry and joint binding of the MSI2 promoter is maximal in primitive hematopoietic cells.

(A) Expression profile of PLAG1 and USF2 in the human hematopoietic hierarchy. (B) Flow cytometry gating strategy to isolate erythroid progenitors (EP), myeloid progenitors (MP), multipotent progenitors (MPP) and hematopoietic stem cells (HSC) from cord blood (left) and the q-RT-PCR validated expression of PLAG1-B within these populations (performed once from pooled CB samples). (C-E) ChIP-qPCR-defined fold enrichment of PLAG1 and USF2 at the MSI2 promoter in purified Lin⁻CD34⁺ (C) and Lin⁻CD34⁻ (D) cord blood populations. (n=3 experiments). (E) Fold change in enrichment in Lin⁻CD34⁺ as compared to Lin⁻CD34⁻ cord blood subsets. (F) shPLAG1 knockdown efficiency in Lin⁻CD34⁺ cells. (G) Level of MSI2 transcript after 72hrs of shPLAG1 transduction in Lin⁻CD34⁺ Cord blood cells.

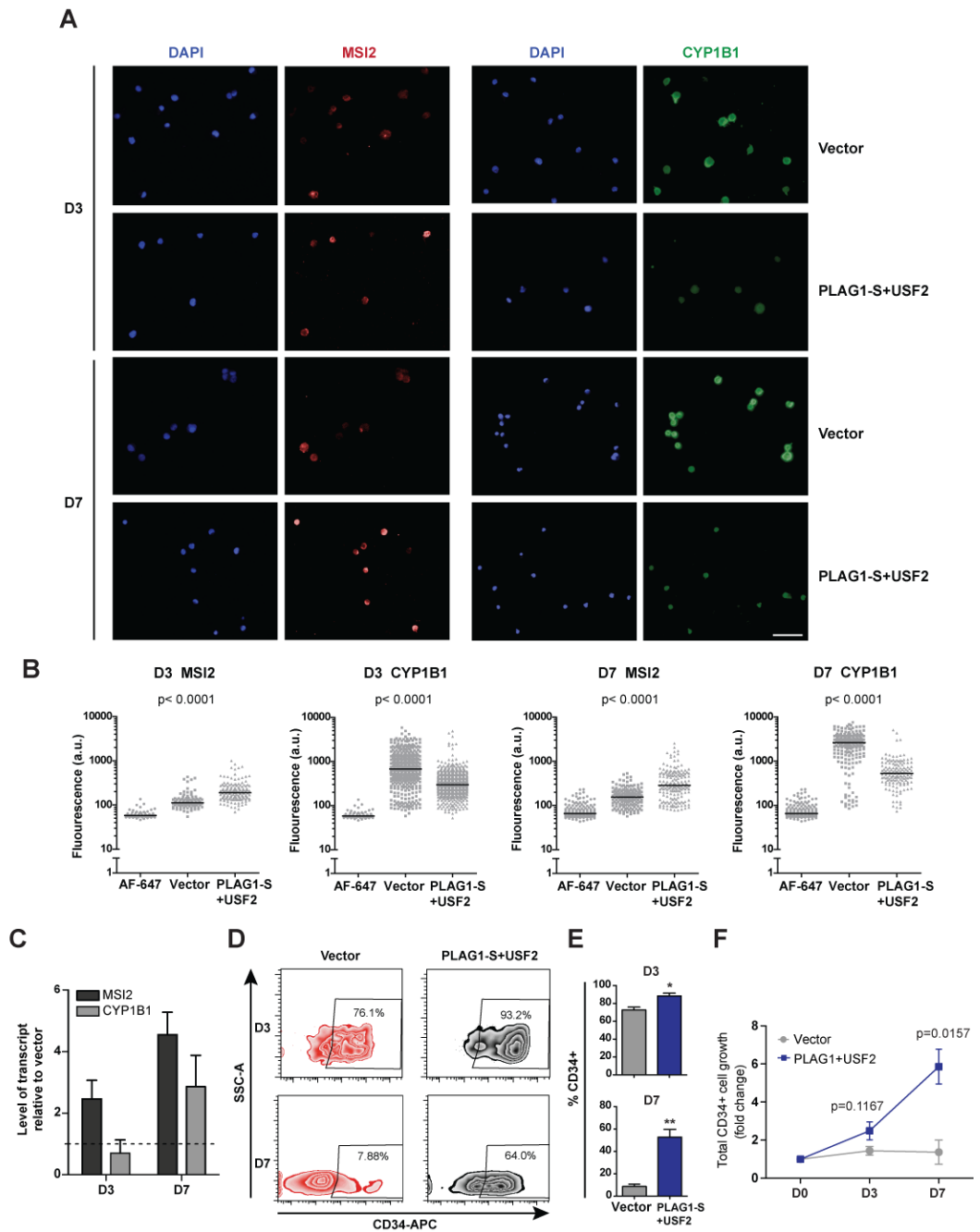
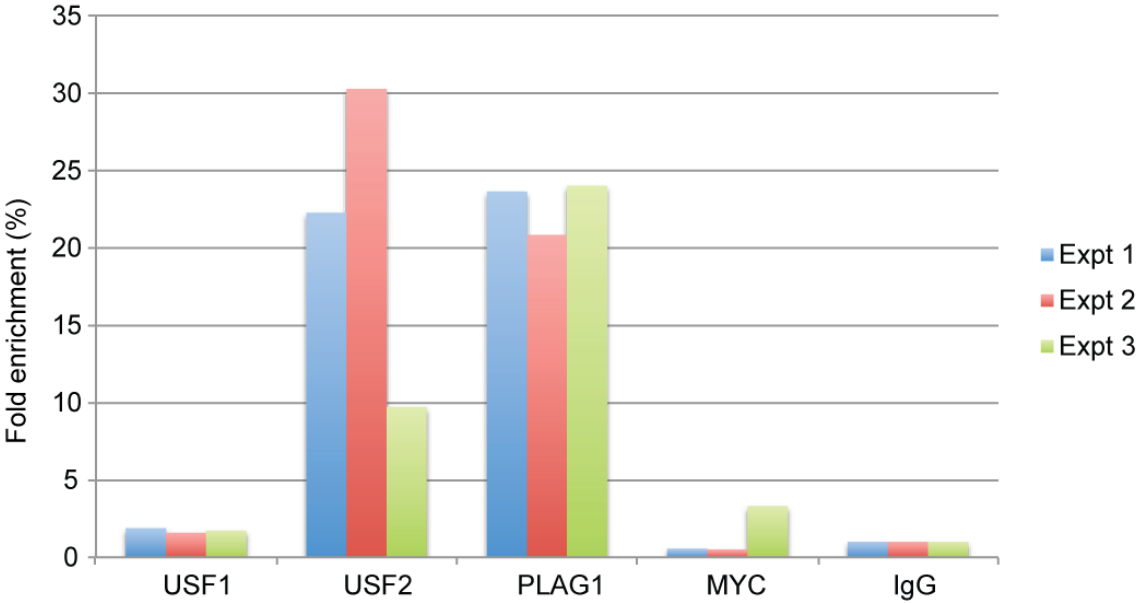


Figure 2.5. PLAG1-S and USF2 co-regulate MSI2 expression and its downstream functions in Lin-CD34⁺ CB cells.

(A) Representative immunofluorescence images of MSI2 and CYP1B1 proteins in Lin⁻CD34⁺ CB cells expressing control vector or co-overexpressing PLAG1-S and USF2 and cultured for 3 (D3) and 7 (D7) days. (B) Quantification of MSI2 and CYP1B1 expression on a per cell basis. Each point in the graph represents an individual stained cell and the solid line denotes the mean fluorescence intensity of each dataset. Cells stained with Alexa-fluor-647 (AF-647) conjugated secondary antibody alone were considered as background fluorescence controls. (>100 cells analyzed per condition for each experiment, plots summarize n= 3 experiments). (C) Fold change in the transcript level of MSI2 and CYP1B1 upon co-expression of PLAG1-S and USF2 at day 3 (D3) and day 7 (D7) of in vitro culture relative to vector control. (D) Representative flow cytometry plots showing the frequency of CD34⁺ cells at the D3 and D7 in vitro culture points with the co-overexpression of PLAG1-S and USF2. (E) Quantification of CD34⁺ cell frequency (n=3 experiments). (F) Fold change in total CD34⁺ cells over the 7 day culture period.



Supplemental Figure 2.1. ChIP-qPCR fold enrichment for putative transcription factors. Each column represents individual ChIP-qPCR fold enrichment data for USF1, USF2, PLAG1 and MYC binding to the *MSI2* minimal promoter in K562 cells relative to mock IgG (generated from three independent experiments) (related to Figure 2.2).

PLAG1-A_NM_002655_1-500aa	1 MATV I PGDL SEVRDTQKVP SGKRKRGETKPRKN	1	FPCQLCDKAFNSVEKLKVHSYSHTGERPYKCIQQDCTKA	72
PLAG1-B_NM_001114635_1-418aa	-----		-----	
PLAG1-S_1-401aa	-----		-----	
PLAG1-A_NM_002655_1-500aa	73 FVSKYKLQRHMATHSPEKTHKCNCEKMFHRKDHLKNHLHTHDPNKETF	3	KCEECGKNYNTKLGFKRHLALHA	144
PLAG1-B_NM_001114635_1-418aa	1 -----MATHSPEKTHKCNCEKMFHRKDHLKNHLHTHDPNKETF		KCEECGKNYNTKLGFKRHLALHA	62
PLAG1-S_1-401aa	1 -----MATHSPEKTHKCNCEKMFHRKDHLKNHLHTHDPNKETF		KCEECGKNYNTKLGFKRHLALHA	45
PLAG1-A_NM_002655_1-500aa	145 ATSGDLTCKVCLQTFESTGVLLLEHLKSHAGKSSGGVKEKKHQCEHC	5	DRRFYTRKDVRHRMVVHTGRKDFLCQ	216
PLAG1-B_NM_001114635_1-418aa	63 ATSGDLTCKVCLQTFESTGVLLLEHLKSHAGKSSGGVKEKKHQCEHC		DRRFYTRKDVRHRMVVHTGRKDFLCQ	134
PLAG1-S_1-401aa	46 ATSGDLTCKVCLQTFESTGVLLLEHLKSHAGKSSGGVKEKKHQCEHC		DRRFYTRKDVRHRMVVHTGRKDFLCQ	117
PLAG1-A_NM_002655_1-500aa	217 YCAQRFGRKDHLTRHMKKSHNQELLKVKTEPVDFLDPFTCNVSVPIKDELLPVM	7	SLPSSSELLSKPFTNTLQL	288
PLAG1-B_NM_001114635_1-418aa	135 YCAQRFGRKDHLTRHMKKSHNQELLKVKTEPVDFLDPFTCNVSVPIKDELLPVM		SLPSSSELLSKPFTNTLQL	206
PLAG1-S_1-401aa	118 YCAQRFGRKDHLTRHMKKSHNQELLKVKTEPVDFLDPFTCNVSVPIKDELLPVM		SLPSSSELLSKPFTNTLQL	189
PLAG1-A_NM_002655_1-500aa	289 NLYNTPFQSMQSSGSAHQMITTLPLGMTCPIDMDTVHPSHLSFKYPFSSTSYAIS		IPKEQPLKGEIESYL	360
PLAG1-B_NM_001114635_1-418aa	207 NLYNTPFQSMQSSGSAHQMITTLPLGMTCPIDMDTVHPSHLSFKYPFSSTSYAIS		IPKEQPLKGEIESYL	278
PLAG1-S_1-401aa	190 NLYNTPFQSMQSSGSAHQMITTLPLGMTCPIDMDTVHPSHLSFKYPFSSTSYAIS		IPKEQPLKGEIESYL	261
PLAG1-A_NM_002655_1-500aa	361 MELQGGVPSSSQDSQASSSSKLG	Transactivation Domain		
PLAG1-B_NM_001114635_1-418aa	279 MELQGGVPSSSQDSQASSSSKLG	DPQIGSLDDGAGDLSLSKSSISISDPLNTPALDFSQLFNFIPLNGPPY		
PLAG1-S_1-401aa	262 MELQGGVPSSSQDSQASSSSKLG	DPQIGSLDDGAGDLSLSKSSISISDPLNTPALDFSQLFNFIPLNGPPY		
PLAG1-A_NM_002655_1-500aa	433 NPLSVGSLGMSYSQEEAHSSVSQ	PPQTQDLQDPANTIGLGLHSLSAAFTSSLSTSTTLPRFHQAFQ		
PLAG1-B_NM_001114635_1-418aa	351 NPLSVGSLGMSYSQEEAHSSVSQ	PPQTQDLQDPANTIGLGLHSLSAAFTSSLSTSTTLPRFHQAFQ		
PLAG1-S_1-401aa	334 NPLSVGSLGMSYSQEEAHSSVSQ	PPQTQDLQDPANTIGLGLHSLSAAFTSSLSTSTTLPRFHQAFQ		

Supplemental Figure 2.2. Sequence alignment of human PLAG1 isoforms.

Shown is the alignment of the three PLAG1 isoforms and the location and number of Zn finger domains (blue boxes), transactivation domain (green box) and alternative start sites for PLAG1 isoforms (red).

Supplemental Table S2.1: Promoter clones truncation and deletion primers

Promoter clone	Forward primer sequence(5'-3')	Reverse primer sequence (5'- 3')
-3238-Luc	CTAGCTAG <u>CTCGAG</u> ATGGAGAGCAGAAAGGCACG	
-2251-Luc	CTAGCTAG <u>CTCGAG</u> GTGAGTTTGGCACCTCTGAG	
-1764-Luc	CTAGCTAG <u>CTCGAG</u> GTGAGTTTGGCACCTCTGAG	
-1188-Luc	CTAGCTAG <u>CTCGAG</u> AGGGGTTTCCTGAAATAACC	CTAGCTAG <u>AAGCTT</u> ATCTGAGCCCCCGCCCCC
-1013-Luc	CTAGCTAG <u>CTCGAG</u> CTTCTCGCCACTGGAATCG	
-788-Luc	CTAGCTAG <u>CTCGAG</u> CGGGGGCCGCCAAAGGAG	
-588-Luc	CTAGCTAG <u>CTCGAG</u> GAGGAGCCGCAGCAAGC	
-203-Luc	CTAGCTAG <u>CTCGAG</u> TGAGCTAAGCCGAGCCC	
-138-Luc	CTAGCTAG <u>CTCGAG</u> ATTCGGAGGAGCCCGG	
d588-203-Luc	TGAGCTAAGCCGAGCCC	ACCCGGACCTGGGAGAAG

The same reverse primer was used in combinations with the respective forward primer sequence to generate full length and truncation clones.

Underlined are the XhoI and HindIII restriction sites in the forward and reverse sequences respectively

Supplemental Table S2.2: Transcription factor binding site mutagenesis primers

Transcription factor	Predicted binding sites	Consensus binding site nucleotide substitutions	Forward primer sequence(5'-3')	Reverse primer sequence(5'-3')
USF2	3	CANNTG > GTNNGA	CCCGCCCCCGCAGCCTCTGGAATCCAGGGAAGTCGGG TCTAGAACTCCGCCCTCCGGACGCCGGGAGGAGGG GAGCTAAGCCGAGCCTCCGGATGACGGCTCTCGCCG	CCCGACTTCCCTGGATTCCAGAGGCTGCGGGGGGCGG CCCTCTCCCGCGCTCCGGAGGGCGGAGTTCTAGA CGGCGAGAGCCGTCATCCGGAGGCTCGGCTTAGCTC
NFYA	1	GGCATTGGTT > TCAGCTGTAT	GGTGCGTGACGTACCTCAGCTGTATACACGACGTTCTAGAAC	GTTCTAGAACGTCGTGTATACAGCTGAGGTGACGTACGCACC
CREB	1	TGACGT > TTAAGT	CGCACGGGGGTGCGTTAATTACCGGCATTGGTTACA	TGTAACCAATGCCGGTAAATTAACGCACCCCGTGCG
NR3C1/GRE	1	GTTC > GCCC	CATTGGTTACACGACGCCCTAGAATCCGCCCA	TGGGGCGGAGTTCTAGGGCGTCGTGTAACCAATG
MITF	1	CATGTG > GATATA	CCCGCCCCCGCAGCCGATATAATCCAGGGAAGTCGGG	CCCGACTTCCCTGGATTATATCGGCTGCGGGGGGCGG
CTCF	5	GGGG > ATATT	CCCCCGATCCACCCGAATATCGCAGCCCATGTGAT GTGGGTCCGGCCGTGATATCCGAGTGTGCGCGCG GTGCGCGCGCGGGCAGGCATATCCGCACATATTGCGTGAC- GTCACCGGC GCCGGGGGGCGGGGAATATTGGGTGTGCGAGGCAGC	ATCACATGGGCTGCGATATTCGGGTGGATCGGGGG CGCGCGCACACTCGGAATATCACGGCCGGACCCAC GCCGGTGACGTACGCAAATATGTGCGGAATATGCTGCCCCG- GCGCGCAC GCTGCCTCGCACACCCAATATTCGCCGCCCCCGGC
PRDM5	2	AGGG > TCTT	CGTGCGCGGGGAGGTCTTGGAGGAGGAGGAGGA GCGGGGGGAGGAGGTCTTGGAGGAGGAGCGGA	TCCTCCTCCTCTCCAAGACCTCCCGGCGCACG TCCGCTCCCTCTCCAAGACCTCCTCCCCCGC
ZFP148/ZBP89	10	C-rich: CCCC > CGGT G-rich: GGGG > GCCA	CGCCTCCCCTCGGTGATCCACCCGCGGTCCGCAGCCCAT GTCGGGGTGCCTCGGTCTCGCCCTGCGCCC CCGGGGAGGAGGCCAAGGAGGAGGAGG GGAGGAGATGCCATGGGGAGGAGGAGGCCAAGAGGTGGGGAT GGGATGGGCCGGGGCCACGGGACGGGG CGGGGACGGGGCCATGTGCGAGGCAGC CCGGGCGGGGCCAAGGAGGAGGCCAAGGAGGAGGCG	ATGGGCTGCGGACCGCGGGTGGATCGACCGAGGGGAGGCG GGGCGCAGGGCGAGACCGAGCGACCCCGAC CCTCCTCCTCTTGGCCTCCTCCCGG ATCCCCACCTCTTGGCCTCCTCCTCCCATGGCCATCTCCTCC CCCCGTCCCCGTGGCCCCGGCCCATCCC GCTGCCTCGCACATGGCCCCGTCCCCG CGTCCCTCCTTGGCCTCCTCCTTGGCCCCCGCG
PLAG1	3	GGGG > TGAT	GTGCGCCGGGAGGATGATGAGTAATAGGAGGAGATGG GGAGATGGGGGTATGGAGGAGGAATGAGAGAGGTGGGG CAGAGAGATTGTAATAGCCCGGGCGTATGGAGGAGGA- GGGGAGGAGGAGCG	CCATCTCCTCTATTACTCATCATCTCCCCGGCGCAC CCCCACCTCTCTCATTCTCTCCATACCCCATCTCC CGTCCCTCCTCATCATCTCTCCATCACGCCCGGGCTATTACGA- ATCTCTCTG
MAZI	7	GGGAGGGNNNN / CCCTCCNNNN > TGTATGTNTNT / ACATACANANA	GGGGTGCCTCCACATAGACATGCGCCCTGCC GGAGGCGGGGTACATACACCCGCGGGGTTC CCACGTGCGCGTGTATGATGGGAGGAGGAG GGGAGGAGGAGGTGTATATGTGGGGATGGGC GGGAGGAGGAGGTGTATGATGGAGCGGAGATC TTTTGGGGTGGGGTGTCTGTGGGCTCAGAT	GGCAGGGCGCATGTCTATGTGGAGCGACCCCC GAACCCCGCGGGTGTATGTGACCCCGCCTCC CTCCTCCTCCCATATACACGGCGCACGTGG GCCATCCCCACATATACCTCCTCTCTCC GATCTCCGCTCCATACACCTCCTCTCTCC ATCTGAGCCCCACAGACACCCACCCCAAAA

Supplemental Table S2.3: Chip-qPCR primers for the MSI2 minimal promoter

Primer name	Forward primer sequence(5'-3')	Reverse primer sequence (5'- 3')	UPL
ChIP #1	CGCTCGCAGAGAGATTCG	GAGATCTCCGCTCCCTCCT	63

Supplemental Table S2.4: q-RT-PCR primers for MSI2 and CYP1B1

Gene	Gene bank	UPL	Primer name	Primer sequence(5'-3')
MSI2	NM_138962.2	26	UPL26-huMSI2-I-F	GGCAGCAAGAGGATCAGG
			UPL26-huMSI2-I-R	CCGTAGAGATCGGCGACA
CYP1B1	NM_000104.3	20	UPL26-huCYP1B1-I-F	ACGTACCGGCCACTATCACT
			UPL26-huCYP1B1-I-R	CTCGAGTCTGCACATCAGGA

CHAPTER 3

PLAG1 dampens proteostasis pathways to enforce primitive hematopoietic cell functions

Preamble

1. This Chapter is an original article to be submitted for publication. It is presented in its pre-submission form.

“Belew MS, Rentas, S, Voisin, V, Bader, GD and Hope KJ. PLAG1 dampens proteostasis pathways to enforce primitive hematopoietic cell functions”

2. The findings presented in this Chapter are also incorporated into the provisional patent filing:

“Belew MS and Hope KJ: Methods and compositions for expansion of hematopoietic stem and/or progenitor cells” Filed July 7, 2017; US62/529,694

MSB designed and performed experiments, interpreted results and wrote the manuscript. SR assisted with limiting dilution transplant experiments. VV and GDB performed and supervised RNA-seq bioinformatics and pathway analysis. KJH supervised the project, interpreted results and wrote the manuscript.

In Chapter 2, I identified PLAG1 as a promising, previously uncharacterized TF in the context of HSPC biology. Here I have coupled functional genomics with RNA-sequencing to decipher its specific regulatory role in HSPCs isolated from umbilical CB. Overexpression of PLAG1 (PLAG1^{OE}) imparts a sustained pro-survival advantage to progenitors, enhancing TNC and CD34⁺ frequency and CFU output. Complementary shRNA-mediated knockdown (PLAG1^{KD}) impaired CFU output, increased apoptosis and significantly reduced TNCs and CD34⁺ counts. *In vivo*, using LDA in sub-lethally irradiated NSG mice, PLAG1^{OE} robustly enhanced the number of long-term HSCs with multilineage reconstitution capacity compared to control. RNA-sequencing and transcriptome-wide gene-set enrichment and network analysis revealed that proteostasis pathways are significantly attenuated with PLAG1^{OE}. PLAG1^{OE} up-regulates the H19 and MEG3 imprinted lncRNAs and their embedded miRNA species miR-127 and miR-675 respectively to post-transcriptionally repress protein synthesis pathways. In concert, PLAG1 also represses the pan-ribosome biogenesis transcriptional regulator MYC thereby adding another layer of protein level restraint through the attenuation of protein synthesis. Taken together, functional genomics coupled with global transcriptional profiling in the primitive CB setting revealed PLAG1 as master HSPC regulator that achieves its pro-self-renewal effects by coordinately dampening ribosome biogenesis and protein synthesis.

CHAPTER 3

PLAG1 dampens proteostasis pathways to enforce primitive hematopoietic cell functions

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Abstract

Discovery and characterization of hematopoietic stem cell (HSC) self-renewal regulatory factors is central for harnessing their inherent regenerative capacity and lifesaving potential. In this study, using *in vitro* and *in vivo* functional genomics approaches in human umbilical CB HSPCs we have characterized the TF PLAG1 as a novel enforcer of self-renewal. During *in vitro* culture, overexpression of PLAG1 (PLAG1^{OE}) imparted a sustained pro-survival advantage to progenitors and produced significantly more primitive CD34⁺ cells compared to controls. Furthermore, CFU assays demonstrated that PLAG1^{OE} enhanced total progenitor output and replating potential. Complementary *in vitro* assays using shRNA-mediated knockdown (PLAG1^{KD}) demonstrated PLAG1 repression impaired CFU output, increased apoptosis and significantly reduced CD34⁺ cell counts in culture. PLAG1 upregulation induced significant effects at the stem cell level, increasing the frequency and absolute number of serially transplantable HSCs by a magnitude of 24.1-fold and enhancing their competitiveness *in vivo* compared to control. RNA-sequencing of PLAG1^{OE} CD34⁺ cells indicated a striking upregulation of imprinted genes while transcriptome-wide gene-set enrichment and network analyses revealed that ribosome biogenesis and global protein synthesis signaling pathways were significantly attenuated. We find PLAG1^{OE} up-regulates most significantly the H19 and MEG3 imprinted lncRNAs that act as natural reservoirs for miR-675 and miR-127 respectively. *In silico* analysis of PLAG1^{OE}-down-regulated gene networks with predicted and experimentally validated targets of both miR-127 and miR-675 highlighted substantial overlap in components of proteins synthesis and ribosome biogenesis pathway

components. We found that, at least in part due to these miRNA intermediates, components of the IGF1R-PI3K-AKT-mTOR signaling pathway known to regulate protein synthesis were repressed upon ectopic PLAG1 and signaling flux through the pathway reduced. Consistent with the significant global repression of ribosomal protein coding genes enforced by PLAG1^{OE} we show that the TF MYC, a pan-ribosome biogenesis transcriptional regulator in primitive hematopoietic cells, is suppressed by PLAG1. Conversely, PLAG1^{KD} downregulated H19 and MEG3 and reversed the global gene expression signature observed by PLAG1^{OE}. Taken together, our work reveals PLAG1 as master regulator of ribosome biogenesis and protein synthesis, a function that is essential to its HSPC-promoting activity.

Introduction

To maintain homeostasis HSCs undergo carefully orchestrated cellular decision-making to ensure the replenishment of the stem cell pool and simultaneous production of all mature blood components in the appropriate proportions. In the fetal liver early HSCs make use of mechanisms that allow for elevated self-renewal in order to rapidly develop the large complement of HSCs necessary for seeding adult hematopoiesis in the bone marrow. Despite the importance of highly regulated self-renewal in both early and adult human hematopoiesis our understanding of this process and the major players that enforce it are unclear. Not only does this lack of insight impede our understanding of fundamental mechanisms that may underlie hematopoietic disease establishment, it also limits our capacity to engineer desired HSC decision making for the purposes of augmenting current regenerative therapeutics.

We and others have reported restrictively enhanced expression of the TF PLAG1 within the most primitive mouse and human HSCs, implicating it as having a role in regulating these cells (Gazit et al., 2013). Indeed, we have shown that while insufficient when upregulated in isolation, PLAG1 overexpression in combination with that of USF-2 can achieve significantly enhanced expression of the pro-self-renewal regulator MSI2 (Chapter 2). Nonetheless, no functional evaluations have been performed to date to explore either the requirement of PLAG1 for HSC function or its potential to enforce HSC self-renewal independently.

PLAG1 is a developmentally regulated C₂H₂ zinc finger transcription factor recognizing a bi-partite DNA consensus sequence (Voz et al., 2004). It was initially discovered in

human pleomorphic adenomas of the salivary glands as a proto-oncogene frequently rearranged and activated by chromosomal translocations with *CTNNB1* (Debiec-Rychter et al., 2001; Kas et al., 1997) and *LIFR* (Voz et al., 1998). As a result, the ‘promoter swapping’ events maintain the coding exons of *PLAG1* down stream of *CTNNB1* and *LIFR* genes. In an attempt to identify target genes for *PLAG1* in these adenomas, Voz et al performed microarray analysis after inducible overexpression of *PLAG1* (Voz et al., 2004) in HEK293 cells and found that growth factors and the imprinted gene *IGF-2* in particular are strongly upregulated. In salivary adenomas *IGF-2* is a genuine target of *PLAG1* and understood in this setting to contribute to its oncogenic role (Akhtar et al., 2012). In a transgenic mouse model constitutively overexpressing *PLAG1*, other imprinted gene clusters (*H19*, *Dlk1* and *Gtl2*) were also observed to be strongly upregulated (Declercq et al., 2005). The potential for *PLAG1* in other cell contexts to impact IG expression is intriguing in light of recent demonstrations of the required role of the *Igf-2/H19* and *Dlk1/Gtl2* IG clusters in maintaining long-term self-renewal and reconstitution ability of murine HSCs (Qian et al., 2016; Venkatraman et al., 2013). In combination with its selective HSC-specific expression profile in the human hematopoietic hierarchy and the fact that the contribution of IG in human HSCs has not been explored to date, these findings provide the impetus to explore *PLAG1*’s function in human HSC biology.

In this study, we used a functional genomics approach coupled with high throughput RNA-sequencing and integrated pathway analysis to decipher the biological functions and mechanisms of action of *PLAG1* in CB HSPCs. We report that *PLAG1*^{OE} in HSPCs

imparts sustained pro-survival advantage to progenitors and significantly enhances HSC self-renewal. Mechanistically, we find PLAG1 impinges on ribosome biogenesis and global protein synthesis signaling pathways to impose its functional gains on HSPCs. We find that to enforce this state of dampened protein synthesis PLAG1^{OE} up-regulates among others, the H19 and MEG3 imprinted lncRNAs, which are natural reservoirs for the miRNAs miR-675 and miR-127 that themselves repress a network of targets involved in a variety of cellular pathways whose normal function is to promote ribosome biogenesis and protein synthesis. Importantly we also show through reciprocal knockdown studies that the imprinted gene network enforced by ectopic PLAG1 is reversed upon its repression indicating its requirement in steady state HSCs for maintenance of key imprinted gene expression. All together our study demonstrates PLAG1's capacity to promote strict control on global protein synthesis from multiple levels in order to enforce the human HSC state and thus has important implications for advancing regenerative applications with these clinically relevant cells.

Results

PLAG1 promotes the survival and self-renewal of HSPCs *in vitro*

To decipher the significance of PLAG1^{OE} on HSPC activity *in vitro*, Lin⁻CD34⁺ cells were sorted and infected with lentiviral particles co-expressing blue fluorescent protein (BFP) (Figure 3.1A). BFP⁺ cells were sorted and subjected to CFU, CD34⁺ cell output, cell cycle and survival *in vitro* assays (Figure 3.1A). We observed that PLAG1^{OE} imparts significantly more colony proliferation compared to vector control, specifically enhancing the number of erythroid colonies (BFU-E) (Figure 3.1B). Although the number of

primitive granulocyte-erythroid-megakaryocyte-monocyte (GEMM) colonies was unaltered their replating indicated that PLAG1^{OE} produced significantly more secondary CFUs compared to control (Figure 3.1C). To evaluate the effect on CD34⁺ cell proliferation, BFP⁺ cells were plated in cytokine supplemented serum-free growth media for 7 days (D7). The cumulative fold-change of TNCs and CD34⁺ cells was elevated with PLAG1^{OE} compared to vector control over the culture period which correlated with a trend towards an increase in the CD34⁺ frequency in comparison to controls at both the sampled time points (Figure 3.1D, E). Peak CD34⁺ proliferation was observed at D4 (2.1-fold) with progressively diminished counts at D7.

To assess the effect of knockdown of PLAG1 on HSPC activity *in vitro*, Lin⁻CD34⁺ cells were sorted and transduced with lentiviruses encoding shRNAs against PLAG1 also expressing ZsGreen as a marker of transduction (shPLAG1, also identified as PLAG1^{KD}) (Figure 3.1F, G). In progenitor assays PLAG1^{KD} impairs total CFU output, proliferation of BFU-E and most importantly GEMM colonies indicating its critical role in the output of progenitor cells (Figure 3.1H). When subjected to *in vitro* suspension culture ZsGreen⁺ cumulative TNCs and CD34⁺ cell numbers were significantly diminished compared to those in the vector control culture which correlated with a modest decrease in CD34⁺ cell frequency at D4 (Figure 3.1I, J). Taken together, these observations indicate that PLAG1 is a positive regulator of HSPC proliferation *in vitro*.

As enhanced output of CD34⁺ cells *in vitro* could, in addition to enhanced self-renewal, be the result of increased cycling and/or survival we assessed both possibilities. When subjected to cell cycle analysis at both cell culture time points we found that PLAG1^{OE}

exerts a transient increase in the proportion of G1 cells and a reduction of cells in S/G2/M at D4 which resolves by D7 (Figure 3.2A). Hence, mechanistically, PLAG1's role on influencing cell cycle dynamics to the effected TNC and CD34⁺ proliferation and clonogenicity is negligible. To address whether the enhanced primitive cell output promoted by PLAG1^{OE} is achieved via pro-survival mechanisms cells from D4 and D7 time points were co-stained with 7-AAD and Annexin V to assess apoptosis levels. Indeed, PLAG1^{OE} does exert a sustained pro-survival effect on HSPCs over the *in vitro* culture period as demonstrated by progressively reduced frequency of Annexin V⁺ cells which becomes significant at D7 (1.6-fold) (Figure 3.2B). Our results are thus consistent with PLAG1 overexpression selectively promoting cell survival capacity as opposed to driving cell cycling. Conversely, we find that PLAG1^{KD} does not significantly perturb the cell cycle status of HSPCs compared to the shluciferase control at both D4 and D7 time points nor does it significantly influence progenitor survival (Figure 3.2C, D) which suggests that PLAG1 repression negatively impacts on progenitor output by impairing the more primitive stem and/or multipotent cells that generate them.

PLAG1 qualitatively and quantitatively enhances HSCs *ex vivo*

Having shown its capacity to promote progenitor cells upon overexpression we next explored PLAG1's potential for similarly influencing HSCs by performing LDA reconstitution assays. Lin⁻CD34⁺ cells were transduced with PLAG1^{OE} lentivirus co-expressing BFP and cultured for a total of 3 days. Next, BFP⁺ cells were injected at limiting doses into immunodeficient NSG mice whose resultant human CD45⁺ grafts were assessed at 14 weeks post-transplant (Figure 3.3A). Engraftment was considered the

result of stem cell-based repopulation when recipients displayed >0.1% human chimerism of both the myeloid (BFP⁺CD45⁺CD33⁺) and lymphoid (BFP⁺CD45⁺CD19⁺) lineages detected in either the injected femur and/or bone marrow (Figure 3.3B). With this criteria, ~30% of the mice engrafted with cells transduced with vector control while more than 70% for PLAG1^{OE} (Figure 3.3B-D). Subsequent extreme limiting dilution analysis (ELDA) to quantify HSC inputs based on these results (Hu and Smyth, 2009) indicated an HSC frequency of 1 in every 461 for PLAG1^{OE} versus 1 in 11,157 for the control (Figure 3.3C). Factoring the calculated HSC frequency into the total number of cells injected indicates that PLAG1^{OE} enhanced HSC numbers by a striking 24.1-fold (Figure 3.3C, D). When evaluating all engrafted mice qualitatively, PLAG1^{OE} imparts more than 2-fold human chimerism and enhanced CD19⁺ lineage output both in the injected femur and spleen indicating increased fitness compared to control (Figure 3.3E, F). The lentiviral vector employed in these assays consists of a bidirectional promoter driving equimolar co-expression of BFP in the opposite orientation to PLAG1^{OE}. Therefore, median fluorescent intensity (MFI) of BFP⁺ can be used as a proxy measure of the expression level of PLAG1 in each of the transduced cells. While the MFI of BFP⁺ cells stays the same both for control and PLAG1^{OE} before transplantation, the MFI for huCD45⁺BFP⁺ cells 14 weeks post-transplant was significantly enhanced for PLAG1^{OE} compared to the control (Figure 3.3G)- suggesting preferential long-term maintenance of cells PLAG1^{OE} and/or their enhanced competitiveness *in vivo*.

In order to determine the long-term self-renewal and serial transplantation capacity of HSCs, we pooled all engrafted bone marrow from the control primary mice while an

equivalent number of bone marrow cells from matched primary PLAG1^{OE} mice was also pooled. Both pools were subjected to serial transplantation at several input doses into secondary NSG mice assessed for human engraftment at 10 weeks post-transplant (Figure 3.4A). PLAG1^{OE} cells harvested from primary mice robustly reconstituted secondary recipients. Despite the fact that the BFP⁺ MFI of both transplanted primary bone marrow pools was the same when transplanted, we observed an enhanced fitness of HSCs from PLAG1^{OE} as manifested by 11.3-fold more average level of huCD45⁺BFP⁺ chimerism (Figure 3.4B, C) and sustainably higher MFI (net 3.4-fold) of these cells in the engrafted bone marrow (Figure 3.4D, E). Importantly, secondary mice were reconstituted with normal proportions of both lymphoid (CD19⁺) and myeloid (CD33⁺) lineages when transplanted with primary PLAG1^{OE} cells indicating PLAG1's modulation does not lead to any appreciable lineage skewing during long-term hematopoiesis (Figure 3.4F) in both the injected femur and the rest of the bone marrow.

PLAG1^{KD} does not influence multilineage engraftment of primitive HSCs *in vivo*

To assess the consequence of PLAG1 repression in primitive HSCs, an shRNA mediated knockdown experiment was designed. Lin⁻CD34⁺CD38⁻ cells were sorted and infected with lentiviral particles co-expressing shRNA against PLAG1 (shPLAG1) and red fluorescent protein (RFP) as a marker of transduction (Figure 3.5A). Post-transduction and without RFP⁺ purification, 18, 500 (shPLAG1) and 20, 000 (shLuc) cells from each culture were injected into NSG mice (INPUT human cells) per hairpin per mouse. Engraftment and lineage reconstitution of the resultant grafts (OUTPUT human cells, Figure 3.5A) was assessed by flow cytometry 12 weeks post-transplant and the RFP⁺

frequency within the human graft compared to the input RFP⁺ frequency (Figure 3.5B). Analysis of huCD45⁺RFP⁺ chimerism in both the injected femur and bone marrow showed no significant difference between the control shLuc and shPLAG1 (Figure 3.5C). Furthermore, we did not see any significant differences in the lympho-myeloid lineage reconstitution ability of shPLAG1 in either of the sampled bone marrow sites (Figure 3.5D). In contrast to our results indicating PLAG1 repression compromised progenitor output, these results are intriguing in that they suggest alternate dependencies may exist at the stem cell level with respect to PLAG1 levels.

PLAG1 dampens ribosome biogenesis and global proteostasis pathways

To understand the molecular pathway(s) through which PLAG1^{OE} imparts the aforementioned *in vitro* and *in vivo* functional phenotypes, we performed transcriptome-wide analysis by RNA-sequencing on Lin⁻CD34⁺ cells immediately following PLAG1 overexpression (Figure 3.6A). Interestingly, up-regulated genes did not significantly enrich to a particular pathway (s) (FDR > 0.05). However, gene-set enrichment and network analysis revealed highly significant (FDR < 0.05) clustering of down regulated genes associated with ribosome biogenesis and localized protein synthesis and indicated a large proportion of all down regulated genes were included in this node (Figure 3.6B, red box). Other notable repressed nodes include those that encapsulate genes regulating components of the PI3K signaling pathway (blue box), targets of c-MYC (purple box) and a host of processes that are associated with mature innate and adaptive immune cells (ie. Chemotaxis and bacterial defense) (Figure 3.6B). Given its prominence as a downregulated node in the PLAG1^{OE} signature we further explored by gene set

enrichment analysis (GSEA) the constituent pathways that comprised the ribosome biogenesis/protein synthesis cluster. In particular we find highly significant downregulations of genes involved in 3'-UTR-mediated translation regulation, translation initiation and protein targeting to the endoplasmic reticulum (Figure 3.6C). Intriguingly, the list of leading-edge genes driving the highly significant negative enrichment score of these pathways is comprised largely of genes coding for ribosomal proteins and components of cap-dependent protein synthesis machinery (Figure 3.6D). Ribosome biogenesis and protein synthesis is known to positively correlate with cell size (Kress et al., 2015; Ruvinsky et al., 2005; Thoreen et al., 2012). Consistent with their transcriptional signature indicating repression of both of these pathways, we find that PLAG1^{OE} cells are indeed smaller in size as quantified from their forward-scatter (FSC) flow cytometry profile (Figure 3.7A). In addition, measurements of total cell area after immuno-histochemical (IHC) staining with a cytoplasmic protein also demonstrated cells from PLAG1^{OE} are significantly smaller in size (Figure 3.7B). In order to further validate the repression of ribosome biogenesis and/or protein synthesis, we used a published assay based on O-propargyl-puromycin (O-p-puro) incorporation *in vitro* (Blanco et al., 2016; Cai et al., 2015; Liu et al., 2012; Sanchez et al., 2016; Signer et al., 2014). O-p-puro is incorporated into nascent peptides upon mRNA translation and leads to premature termination (Liu et al., 2012). The rate of O-p-puro incorporation is detected through a cycloaddition reaction with fluorescently-conjugated picolyl azide (Azide-AF488) where elevated fluorescence intensity correlates with heightened translation (Figure 3.7C). PLAG1^{OE} and control transduced CD34⁺ cells were cultured for 4 days *in vitro* (D4) at

which time O-p-puro was included in our standard culture medium for 1 hour. Flow cytometric measurements at the culture endpoint indicated that the AF488 MFI of cells with PLAG1^{OE} was significantly lower than control cells validating the expected functional outcome of a repressed ribosome biogenesis/protein translation transcriptional signature (Figure 3.7D).

PLAG1 enhances the expression of imprinted genes and lncRNA embedded microRNAs

In defining potential direct targets of PLAG1 that could represent upstream regulators enforcing the global repression of ribosome biogenesis/protein synthesis we observed that the 10 most profoundly upregulated genes upon PLAG1 overexpression include 4 imprinted genes (IGs) H19, IGF2, DLK1, MEG3. Of importance, both H19 and MEG3 represent LncRNAs whose expression normally only occurs from their respective maternal loci (Figure 3.8A, B). The H19 lncRNA is a known reservoir of miRNA-675 (Keniry et al., 2012; Venkatraman et al., 2013) while MEG3 is a miRNA mega-reservoir of the miRNA-127/-136 and miRNA-379/-410 clusters (Figure 3.8B) (Qian et al., 2016; Seitz et al., 2004). Importantly, miRNA-qPCR validated a robust 3- and 10-fold level of expression of miR-127 and miR-675 respectively in PLAG1^{OE} compared to control cells (Figure 3.8C, Supplemental figure 3.1A, B; Supplemental figure 3.2). miRNAs have been demonstrated to mediate global repression of gene networks and thus impact significantly on a multitude of biological functions (Gangaraju and Lin, 2009; Lim et al., 2005; Nilsen, 2007). Although miRNA-mediated post-transcriptional regulation can occur via-miRNA binding-induced translational repression, transcript destabilization and degradation upon

miRNA binding is a major mode by which miRNAs ensure the repression of their targets (Bagga et al., 2005; Lim et al., 2005; Nilsen, 2007). We thus proceeded to determine whether the miRNAs embedded in H19 and MEG3 are potentially targeting the gene networks significantly repressed by PLAG1^{OE}. Overlap analysis with experimentally validated targets of miR-127 revealed a very significant enrichment of gene-sets and biological functions also attenuated by PLAG1^{OE} (Figure 3.8D, E). These include ribosome biogenesis, the UPR and differentiated hematopoietic cell functions (Figure 3.8D, E). In the same token, similar analysis for miR-675 showed gene-sets involved in biological processes such as establishment of protein localization to membrane and functions of innate and adaptive immune functions were among the targets (Figure 3.8F). mir-675 is also known to target IGF1R through which PI3K signaling is well known to occur. We find that in addition to IGF1R other components of PI3K signaling, namely PIK3CB, PIK3CD, PIK3CG, PIK3R1, PIK3R5, AKT2, MTOR, RPS6K1 and RHEB were repressed with PLAG1^{OE} (Figure 3.9A, B) PI3K-AKT-mTOR signaling regulates a range of downstream cellular processes including the promotion of protein synthesis (Cai et al., 2015; Laplante and Sabatini, 2013; Thoreen et al., 2012). As such, we interrogated the expression and phosphorylation status of key components of IGF1R-PI3K-AKT-mTOR signaling pathway in PLAG1 overexpressing cells (Figure 3.9C, D) (Chan et al., 2011a). Protein level expression of IGF1R, AKT and mTOR were significantly down regulated with PLAG1^{OE} as determined by respective cell surface or intracellular flow cytometry which correlated with a hypo- phosphorylated status for both protein (Figure 3.9C, D).

When considered together, our data thus suggest that through processing of upregulated imprinted genes, the miRNA-mediated coordinated post-transcriptional repression of multiple mRNAs involved in ribosome biogenesis and protein synthesis programs constitutes a likely mechanism for the observed enrichment of down regulated genes enforced by PLAG1^{OE}.

PLAG1 represses MYC-driven expression of ribosomal proteins

The transcription factor MYC is a known regulator of ribosomal proteins (Oskarsson and Trumpf, 2005; van Riggelen et al., 2010; Wu et al., 2008). We observed that its expression was significantly repressed with PLAG1^{OE} ($P < 0.05$) (Figure 3.10A). To define the complement of ribosomal proteins that represent implicated MYC targets in primitive human hematopoietic cells we mined publicly available Myc ChIP-seq data from murine HPC7 cells (Wilson et al., 2016). We retrieved all genes having Myc peaks up to 5kb upstream and 1Kb downstream of the transcription start site (TSS) and defined their human orthologs (Durinck et al., 2005; Durinck et al., 2009). Overlap analysis between predicted MYC targets and the down regulated gene-set enrichment network induced by PLAG1^{OE} demonstrated significant ($P < 0.05$) representation by components of protein synthesis pathways including predominantly ribosomal proteins and eukaryotic translation initiation and elongation factors (Figure 3.10B). We next made use of iRegulon data to incorporate additional known and predicted human MYC target genes to the list of ChIP-seq validated targets. Analysis of this list provided validation that the significantly downregulated genes upon PLAG1^{OE} are indeed high probability ($P < 0.05$) MYC targets but also revealed that some MYC targets were enriched within the

upregulated gene sets induced by PLAG1^{OE} (Figure 3.10C). Using *in silico* tools including GeneMANIA and g:Profiler we next demonstrated that within their clusters, the upregulated and down regulated MYC targets physically interact within each other and have been implicated in mediating distinct phases of ribosome biogenesis (Figure 3.10D). Specifically, cluster analysis using MCODE algorithm indicated that the upregulated MYC targets code for elements of the pre-ribosomal processing machinery active in the nucleolus while the repressed targets are almost exclusively ribosomal protein coding genes. This dichotomy of expression is intriguing and suggestive that there may be unique transcriptional controls in addition to MYC that are utilized to tune expression of these distinct classes of ribosome biogenesis genes. In this scenario in order to counter the global repression of ribosome biogenesis and MYC dependent ribosomal protein transcription enforced by PLAG1^{OE} these other factors could respond in a negative feedback manner to attempt to enhance processing of the reduced amount of pre-ribosome substrate. Our results, which require further validation of a MYC-specific functional contribution to the PLAG1^{OE} phenotype, are nonetheless suggestive that MYC promotion of ribosomal protein transcription contributes to the overall repression of protein synthesis that defines PLAG1 overexpressing HSPC.

PLAG1^{KD} transcriptome identifies upregulation of proteostasis pathways

To complement the PLAG1^{OE} transcriptome profile, we performed RNA-sequencing for PLAG1^{KD} in Lin⁻CD34⁺ cells (Figure 3.11A). Gene-set enrichment and pathway analysis revealed a dramatic reversal in expression of the biological functions repressed with PLAG1^{OE}. Biological processes related to intracellular protein transport and localization,

protein processing and maturation and components of the ER UPR proteostasis pathways are significantly upregulated (Figure 3.11B). In addition, functions associated with innate and adaptive immune functions and pro-apoptotic signaling pathways are among the enhanced processes following PLAG1^{KD} (Figure 3.11B). Further, comparisons of the global gene-set enrichment and expression signatures showed that pathways /genes activated or upregulated with PLAG1^{KD} were down regulated with PLAG1^{OE} and vice versa (Figure 3.11C, D, Supplemental figure 3.3A). Interestingly, among the genes that showed a reciprocal expression pattern are the maternally expressed H19 and MEG3 lncRNAs and not the paternally expressed IGF2 and DLK1 imprinted genes (Supplemental figure 3.3B). This observation suggests that the paternally expressed IGs could be artifacts of PLAG1 over expression in the context of HSPCs. Noteworthy was also the reciprocal expression pattern of PLAGL2 between PLAG1^{KD} and PLAG1^{OE} gene expression signatures with implications on functional redundancy. Functional redundancy and likely compensatory roles between PLAG1 and its closely related family member PLAGL2 (Hensen et al., 2002) could provide a potential explanation for the lack of significant effect of PLAG1^{KD} on HSPCs survival *in vitro* (Figure 3.2D) and HSC-driven multilineage engraftment *in vivo* (Figure 3.5C, D).

PLAG1 represses global differentiation programs

Differentially expressed genes from PLAG1^{OE} and PLAG1^{KD} were compared to the existing DMAP database which includes global gene expression profiles for a large subset of human hematopoietic subpopulations spanning from primitive to mature cell types (Novershtern et al., 2011). This was done in order to discern the specific hematopoietic

subpopulation with which signatures from each of our PLAG1 modulated conditions most closely associated with. Performing this comparative analysis we observe that expression signatures from PLAG1^{OE} are generally associated with expression profiles from the stem cell compartment and megakaryocyte-erythroid lineages (Figure 3.12A). On the other hand, upregulated genes in the mature myeloid compartment (e.g. neutrophils, basophils, monocyte/macrophages) are substantially down regulated with PLAG1^{OE}. On the contrary, upregulated genes from PLAG1^{KD} were highly expressed within differentiated subpopulations and downregulated genes enriched within stem cell compartment (Figure 3.12B). Together this data echoes our functional data in supporting further the concept that PLAG1 is a critical requirement for and enforcer of the stemness state in the human blood system.

Discussion

PLAG1 is key for progenitor cell proliferation and viability *in vitro*

In defining the functional effects of the HSPC-enriched PLAG1 TF we find that PLAG1^{OE} promotes extensive CFU progenitor cell proliferation while complementary PLAG1^{KD} significantly impairs their output indicating its critical role in regulating progenitor functions. Of note, megakaryocyte/erythroid cells were recently demonstrated to originate early in the hematopoietic hierarchy (Notta et al., 2016). It is known that GEMM progenitors produce the respective granulocyte, erythroid, megakaryocyte and monocyte colonies upon replating in secondary CFU assays (Carow et al., 1993). Using this assay we demonstrated both the ability of PLAG1 to enforce a positive regulatory

role on the short-term self-renewal potential of these progenitor cells as well as its necessity for this capacity.

We interrogated the cell cycle and apoptosis status of CD34⁺ cells in culture upon PLAG1 modulation to explain the observed enhanced progenitor cell output. Studies in pleomorphic adenomas identified PLAG1 as a *de facto* transcriptional regulator of the pro-proliferative IGF2 cytokine enhancing cell cycle progression of tumor cells (Voz et al., 2000). However, in a context dependent manner and contrary to how it might be expected to function in light of these studies, in hematopoietic CD34⁺ cells, PLAG1 delays G1 to S/G2/M phase cell cycle progression at early time points of the culture period and a resolution to control level cell cycle status at later time points. In the tumor setting, some of PLAG1's contribution to cell growth has also been attributed to its anti-apoptotic effects (Declercq et al., 2003). We find that similarly, in the context of CD34⁺ HSPCs, PLAG1 also exerts sustained pro-survival benefits. The subtle cell cycle stalling of primitive cells may in addition, allow these cells to better maintain their identity while committed cells continue to undergo more rapid differentiation/death decisions. Thus we find that PLAG1 capitalizes on a state of enhanced survival and decreased division to maintain primitive hematopoietic cell features.

PLAG1 enhances HSC output *ex vivo* and augments engraftment *in vivo*

Discovery of self-renewal transcriptional networks has the potential to provide opportunities for the *ex vivo* expansion of HSCs or their improved maintenance in culture for gene therapy applications with the ultimate goal of increasing the number of therapeutic bone marrow reconstituting cells that can be transplanted into patients. Using

a pre-clinical model of human HSC-mediated *in vivo* reconstitution, lentivirally-mediated overexpression of PLAG1 in CD34⁺ cells imparts a significant 24.1-fold *ex vivo* expansion of NSG engrafting HSCs. Furthermore secondary transplantations validated that these expanded HSCs maintains the long-term self-renewal and lympho/myeloid reconstitution capacity of normal, healthy HSCs. It is likely that the pro-survival effects of PLAG1 play a significant role in the long-term maintenance of HSCs in addition to the repression of differentiation programs. Practically, increased cell fitness *in vivo* could be inferred from flow cytometry fluorescent intensity measurements of an enhanced expression level of PLAG1 transgene in the engrafted HSCs or their progeny (van Galen et al., 2014b) however commercially available PLAG1 antibodies are not suitable for intracellular staining. Therefore, MFI of BFP⁺ cells was used as a proxy measurement of PLAG1 levels since it is expressed at equimolar levels from the same bidirectional promoter. Of interest, the MFI of BFP⁺ cells stay the same before transplantation both for the control and PLAG1^{OE}. However, MFI of huCD45⁺BFP⁺ cells post-transplant showed a significant increase suggesting that PLAG1^{OE} provided long-term pro-survival and maintenance advantages to the cells.

Somewhat surprisingly, complementary PLAG1^{KD} in CD34⁺CD38⁻ HSCs yielded no significant difference on either engraftment and/or lineage reconstitution compared to control. A likely explanation could be functional compensation by PLAG1 family members, namely PLAGL2 as both PLAG1 and PLAGL2 have been reported to demonstrate similar DNA sequence binding affinity for a target gene's promoter and hence could provide compensatory effects for one another (Hensen et al., 2002). Indeed,

as discussed, RNA-seq demonstrated that in the active presence of one, the other family member is down regulated and vice versa suggesting redundancy of function. Thus, although we speculate that upon PLAG1^{KD}, PLAGL2 could compensate for the loss in HSC maintenance *in vivo*, further experimental validation of this hypothesis is required.

PLAG1 impinges on ribosome biogenesis pathways to effect HSC maintenance

We performed RNA-sequencing to decipher the molecular underpinnings through which PLAG1 functions. First, PLAG1^{OE} significantly repressed gene-sets characteristic of mature cells of the innate and adaptive immune system. In this regard, our findings are reminiscent of a recent study which achieves HSC expansion using the small molecule antagonist UM171 by Fares et al (2014). In this study, transcriptome profiling of CD34⁺ cells treated with UM171 revealed global repression of differentiation programs compared to untreated control (Fares et al., 2014). Most importantly, our RNA-sequencing data revealed a global repression of ribosome biogenesis and localized protein synthesis pathways. Protein synthesis has emerged as a process whose fine-tuned regulation is essential for the long-term self-maintenance capacity of adult stem cell systems including murine HSCs (Goncalves et al., 2016; Signer et al., 2014; Signer et al., 2016), muscle satellite cells (Zismanov et al., 2016), germline stem cells (Sanchez et al., 2016) and skin stem cells (Blanco et al., 2016). In the murine system, attenuated ribosome biogenesis was recently shown to offer a competitive advantage and resistance to chemically-induced and genotoxic stress during engraftment (Cai et al., 2015). It is also interesting to note that the IRE1-mediated UPR is repressed with PLAG1^{OE}. A recent report indicated that hyperactivation of the UPR leads to HSC exhaustion and decreased

fitness (van Galen et al., 2014a). Hence our finding seems to demonstrate multilayered proteostasis checkpoints induced by PLAG1^{OE}. Protein synthesis is known to be directly proportional to cell size (Kress et al., 2015; Ruvinsky et al., 2005; Thoreen et al., 2012). Indeed, measurements by flow cytometry or immunohistochemistry demonstrated reduction in cell size in PLAG1^{OE} compared to control. Further the O-propargyl-puromycin incorporation assay validated a reduced rate of protein synthesis with PLAG1^{OE} and GSEA demonstrated that the leading edge responsible for the enrichment of the repressed protein synthesis signature is largely ribosomal proteins and cap-dependent translation initiation and elongation factors.

The IGF1R-PI3K-AKT-mTOR signaling pathway is an established upstream regulator of protein synthesis (Laplane and Sabatini, 2009, 2013; Thoreen et al., 2012). Indeed components of this pathway were identified by the gene-set enrichment analysis to be repressed with PLAG1^{OE}. Cell surface/intracellular staining for total protein and phosphorylation status of IGF1R, AKT and mTOR validated the pathway is attenuated providing another contributing mechanism underlying the reduced protein translation with PLAG1^{OE}. Qian et al recently reported that the Gtl2 (mouse orthologue of MEG3) lncRNA acts as mega reservoir of miRNAs, which invariably repress all the components of IGF1R-PI3K-AKT-mTOR signaling pathway (Qian et al., 2016). Interestingly, MEG3 is upregulated with PLAG1^{OE} and amongst its constituent miRNAs, miR-127 was significantly upregulated. Experimentally validated targets of miR-127 overlap with down regulated genes from the PLAG1^{OE} enrichment map with a particularly significant overlap for components of cap-dependent protein translation machinery. Hence, our study

highlights for the first time that miRNAs derived from the MEG3 locus globally repress protein synthesis at multiple levels in human HSPCs. Adding to the complexity of PLAG1's master regulatory role, PLAG1^{OE} also upregulated the imprinted lncRNA H19. H19 is a natural reservoir of miR-675 (Keniry et al., 2012) which has been demonstrated to repress IGF1R and to thus be necessary for long-term self-maintenance of murine HSCs (Venkatraman et al., 2013). Importantly, miR-675 is also processed and highly expressed from H19 with PLAG1^{OE}. Similar overlap analysis with validated targets for miR-675 also identified repression of differentiated hematopoietic cell functions and localized protein translation. Taken together, we pioneer in demonstrating H19 and MEG3-derived miR-675 and miR-127 post-transcriptionally repress protein synthesis pathways. Finally, PLAG1^{OE} also imparts significant down regulation of the TF MYC. In a non-tissue specific manner MYC is a known and validated TF driving expression of ribosomal proteins and rRNA and their processing enzymes and is thus involved in several aspects of ribosome biogenesis (Chan et al., 2011a; Iritani and Eisenman, 1999; Kress et al., 2015; Oskarsson and Trumpp, 2005; van Riggelen et al., 2010; Wu et al., 2008). Analysis of available ChIP-seq data generated from progenitor-like hematopoietic cells verified that in this context MYC also directly bound ribosomal genes. When extrapolated to conserved ribosomal proteins in the human setting and considering other human-specific common targets of MYC we noted that the same ribosomal protein genes bound by MYC were also downregulated upon PLAG1 overexpression. The intriguing upregulation of a distinct class of MYC-target genes involved discretely in pre-ribosome processing could be due to alternate mechanisms at play to recover ribosome biogenesis

or to different Myc co-dependencies for regulating these two elements of the larger pathway. Nonetheless, the generalized repression of ribosomal subunits is expected to limit the assembly of complete ribosomes in PLAG1 overexpressing cells and thus suggests that MYC repression offers another layer of regulation PLAG1 imposes on to gain strict control on protein synthesis. In the mouse system it has been shown previously that Myc levels are restricted in HSC and that enforcing elevated MYC promotes their differentiation at the expense of self-renewal however nothing is known of MYC's function in regulating human HSC function (Wilson et al., 2004). It is tempting to predict that miRNAs upregulated through the heightened expression of imprinted lncRNAs via PLAG1 could potentially target MYC however experimental validation of this possibility remains to be done. Should such results be provided this would provide a “missing link” for connecting MYC-mediated transcriptional regulation of ribosomal proteins to PLAG1. In exploring HSC dependencies on PLAG1 we find via RNA-seq from PLAG1^{KD} a reversion of the transcriptional regulations of biological functions that characterized PLAG1^{OE}. In particular PLAG1^{KD} upregulates genes characteristic of differentiated immune cells, proteostasis pathways including protein synthesis and UPR and pro-apoptotic genes implicated in the context of normal HSC homeostasis (e.g. NFkB, FAS, AKT, PRKC among others). Interestingly, among the genes that showed reciprocal expression pattern are the maternally expressed H19 and MEG3 imprinted lncRNAs.. Hence, at physiological levels of expression, PLAG1's master regulation is dictated by coordinate targeting of the maternal allelic components of the two loci.

Altogether our findings unify previous findings from diverse stem cell types and provide for the first time the understanding that the integration of dampened ribosome biogenesis and protein synthesis is an essential avenue for the promotion of human HSCs (Figure 3.13). These findings offer important pre-clinical support for the testing of therapeutically targeting these axes for *ex vivo* expansion of HSCs. Our findings also provide, as far as we are aware, the first uncovering of a human HSC-specific master regulator of imprinted genes that facilitates this coordinated repression of proteostasis to enforce HSC self-renewal and survival. To this point, the discovery of the H19 and MEG3 lncRNAs involvement in PLAG1's functions could have clinical implications for expanding HSCs upon their direct overexpression or the introduction of their constituent microRNAs miR-675 and miR-127. Indeed, this principle of non-coding RNA-mediated expansion has garnered some success in recent reports which have demonstrated the potential of activating and/or repressing lncRNAs (Luo et al., 2015) and miRNAs to achieve this purpose (Lechman et al., 2012; Wojtowicz et al., 2016). Optimization, validation and functional characterization of existing small molecules impairing ribosome biogenesis and protein synthesis also now present another potential avenue for expanding HSCs *ex vivo* which our findings present the impetus for exploring. To that end, such candidate molecules targeting cap-dependent mRNA translation (Ribavirin) (Kentsis et al., 2004), (4EGI-1) (Hoeffler et al., 2011; Moerke et al., 2007) and pre-ribosome assembly (Rbin-1) (Kawashima et al., 2016) could be of interest. Lastly, identification of the regulators of PLAG1 itself and/or the means by which it achieves its pronounced enhancement of imprinted gene expression will be important in further elucidating HSC homeostatic

regulatory circuitry and nodes that could be manipulated for clinical regenerative purposes.

Materials and Methods

Generation of PLAG1 overexpression and knockdown lentiviral constructs

There are 3 isoforms of PLAG1 as annotated in Chapter 2. Only the smaller isoforms PLAG1-B and PLAG1-S are predominantly and invariably expressed in HSCs. For practical reasons, we chose PLAG1-S for the extensive *in vitro* and *in vivo* functional studies. 3xFLAG-PLAG1-S isoform was cloned into the pSMALB destination vector also encoding mtagBFP from bidirectional minCMV/SFFV hybrid promoter (van Galen et al., 2014a) using Gateway[®] cloning kit (Life technologies). A control pSMALB vector containing humanized firefly luciferase was generated using the same cloning protocol. For convenience the control vector is designated as SFFV. shRNAs against PLAG1 or luciferase control were designed using the RNAi Central tool as previous (Hope et al., 2010a). The core sequence: 12-shPLAG1-5'-TTGCAGTTAAACCTCTACAACA-3'. RNAi Central tool generated miR-30 adapted shPLAG1 sequence which was subsequently ligated downstream of the CMV promoter in the pZIP-CMV-Zsgreen or pZIP-CMV-mRFP lentiviral expression vector at the EcoRI/XhoI restriction sites.

Production and titration of lentiviral particles

pSMALB lentiviral overexpression or control vectors were co-transfected with pMD2.G and psPAX2 packaging vectors into 293FT cell line using Lipofectamine LTX (Life technologies). 293FT cells were seeded at density of 8×10^6 per 18mL growth media (DMEM 10% fetal bovine serum (FBS)) in 150cm² tissue culture flask (Corning) 24

hours before transfection. On the day of transfection, cells were replenished with fresh growth media. Transfection is setup as per manufacturer's recommendation for lipofectamine LTX. Specifically, we used 23µg of lentiviral expression vector with 9µg of pMD2.G and 13µg of psPAX2 packaging plasmids (Addgene) per 150cm² flask. Similar transfection protocol format was used for generating pZip-CMV-shPLAG1/Zsgreen lentivirus. Lentiviral supernatant was harvested and filtered through 0.22µm low-protein binding filter to remove cell debris (EMD Millipore). Next, lentivirus was concentrated by ultracentrifugation at 25,000RPM for 2 hours and 30 minutes at 4°C (Sorvall, ThermoFisher Scientific). Lentivirus was resuspended in serum-free media (StemCell Technologies) and stored at -80°C until further use. For titration, 50,000 293FT cells were seeded per well in 24-well tissue culture plate format (Corning) on the same day of lentivirus titration. Lentivirus dilutions were made in 293FT growth media. Cells were incubated for 24 hours in tissue culture incubator (37 °C, 5% CO₂). Cells were topped up with fresh growth media and incubated for additional 48 hours. Transduction efficiencies were determined by flow cytometry and used for calculating the number of infectious viral particles in the stock volume.

Cord blood CD34⁺ cell isolation and lentiviral transduction

Cord blood (CB) samples were obtained voluntarily from full-term neonatal deliveries according to procedures approved by the institutional review boards of McMaster University and McMaster Children's and/or Toronto Trillium Hospital. Mononuclear cells (MNCs) were obtained by density gradient centrifugation on Ficoll-Paque (GE Healthcare). Stem and progenitor cells were enriched by lineage depletion using the

StemSep Human Progenitor Cell Enrichment Kit according to the manufacturer's protocol (StemCell Technologies). Lineage-negative CB were stored at -150°C in vapour phase liquid nitrogen. FACS isolated $\text{Lin}^{-}\text{CD34}^{+}$ cells were prestimulated for 8-12 hours in StemSpan medium (Stem Cell Technologies) supplemented with TSF6 growth factors Thrombopoietin (TPO; 20 ng/ml, Peprotech), Stem cell factor (SCF; 100 ng/ml, R&D Systems), Flt3 ligand (FLT3-L; 100 ng/ml, R&D Systems) and Interleukin 6 (IL-6; 20 ng/ml, Peprotech). Cells were transduced at multiplicity of infection (MOI) of 50-100 with pSMALB lentiviral particles encoding 3xFLAG-PLAG1-S or humanized firefly luciferase control (designated as SFFV) for 24 hours. Cells were washed off the lentivirus and replenished with fresh TSF6 media and incubated for additional 48 hours. BFP^{+} cells were FACS purified using a MoFlo XDP (Beckman Coulter) 72hrs post-transduction and used for subsequent assays. Similarly, $\text{Lin}^{-}\text{CD34}^{+}$ cells were transduced at MOI of 50-100 with pZip-CMV-shPLAG1 or shLuciferase lentivirus. ZsGreen-positive cells were FACS purified using a MoFlo XDP (Beckman Coulter) 72hrs post-transduction and used for subsequent assays. FACS purity was consistently checked to be more than 97% at all times.

PLAG1^{OE} primary and secondary mouse Xenotransplantations

All mouse work was carried out in compliance with the ethical regulations approved by the animal research ethics board (AREB), McMaster University, in pathogen-free Central Animal Facility. Mouse xenografts were performed as described previously (Rentas et al., 2016). Briefly, 6-12 weeks age and sex matched NSG mice were sub-lethally irradiated (315 cGy) 1 day prior to intra-femoral injection. Limiting doses of transduced

CD34⁺BFP⁺ cells were injected with 30 μ l IMDM 1% FBS into the right femur of recipient mice. Doses range from 50 – 25,000 cells per mouse for each of the SFFV control and PLAG1^{OE}. A minimum of 3 and 5 mice were used for highest and lowest doses respectively. For secondary transplantations, frozen vials of bone marrow from primary transplantation mice were thawed in 37°C water bath. For the SFFV control, bone marrow from all engrafted primary mice was pooled together. Cells were washed with PEF media to remove DMSO from freezing media and counted on hemocytometer using Trypan blue dye (Invitrogen). Dose-matched bone marrow from PLAG1^{OE} primary mice were thawed, washed and counted and resuspended in IMDM 1%FBS media. Varying doses ranging from 121,000 – 1,524,800 (SFFV) and 238,000 – 2,998,800(PLAG1^{OE}) were transplanted into sub-lethally irradiated recipient NSG mice.

Analysis of human chimerism and HSC frequency post-xenotransplantation

Unless mice died from radiation sickness or other unexplainable cause, all transplanted mice were included for analysis without randomization or blinding. Mice were euthanized by cervical dislocation 14 weeks post-transplant. Right femur was isolated and crushed separately in 2 ml IMDM 3%FBS. The rest of hind limb bones (femurs, tibiae and iliac crests) were pooled and crushed separately for each mouse. Spleen was also harvested and manually pulverized separately. Red blood cells were lysed with ammonium chloride solution (StemCell Technologies). Bone marrow and spleen were filtered into single cell suspension in PBS 1mM EDTA 2%FBS media (PEF). 25 μ l bone marrow and spleen cells were blocked with mouse Fc block (BD Biosciences) and human IgG (200ug/ml) (Sigma) and then stained with fluorochrome-conjugated antibodies to surface markers specific to

human hematopoietic cells. For multilineage engraftment analysis, cells from mice were stained with CD45-FITC (HI30) (Invitrogen), CD33-PE (P67.6), CD15-APC (HI98), CD14-PE-Cy7(MφP9), CD19-APC (HIB19) and CD34-APC (581) (BD Biosciences). A primary and secondary mouse was scored as positive if it had >0.1% human bone marrow engraftment 14 (primary) and 10 (secondary) weeks post-transplantation. HSC frequency in the primary LDA was estimated using ELDA (<http://bioinf.wehi.edu.au/software/elda/>) (Hu and Smyth, 2009). All multilineage flow cytometry data acquisitions were done on LSR II using FACS Diva software (BD Biosciences). Data analysis was done on FlowJo software (Tree Star)

PLAG1^{KD} mouse Xenotransplantations

For PLAG1^{KD} primary transplantations, CB Lin⁻CD34⁺CD38⁻ HSCs were sorted and prestimulated in TSF6 StemSpan media (StemCell Technologies) for 8-12 hours. Next, cells were transduced with shPLAG1 or shLuciferase control lentivirus for 24 hours after which the virus was washed off with fresh TSF6-supplemented media. Cells were put back in culture and incubated for additional 48 hours. On the day of transplantation (day 0), gene transfer efficiency was assessed by flow cytometer on LSR II (BD Biosciences) using the red fluorescent protein (RFP) marker of transduction. TNCs were counted and 18,500 (shPLAG1) and 20,000 (shLuc) cells were used per short-hairpin transduction per mouse. Cells were injected into the right femur of 6-12 weeks old age and sex matched NSG mice. Mice were euthanized 14 weeks post-transplant. Bone marrow and spleen isolation and processing is done as discussed above. Human chimerism was assessed using CD45-Pacifibblue (HI30) and RFP. For multilineage engraftment analysis, cells from

mice were stained with CD45-Pacific blue (HI30) (Invitrogen), CD33-FITC (P67.6), CD19-APC (HIB19)

PLAG1^{OE} and PLAG1^{KD} clonogenic progenitor assays

Clonogenic progenitor cell (colony forming unit) assays were done in complete semi-solid methylcellulose medium (ColonyGEL™ 1102; Reachbio) with FACS purified transduced cells. For PLAG1^{OE}, BFP⁺ cells were flow-sorted post transduction. Cells (500 cells/ml) were mixed with ColonyGel using a blunt end needle (StemCell Technologies) and syringe and plated on replicate 35 mm tissue culture dishes and incubated at 37°C. Manual colony counts were carried out after 14 days of incubation. Colonies were scored and enumerated as BFU-E, CFU-G, CFU-M, CFU-GM and CFU-GEMM. For secondary clonogenic assays, single CFU-GEMMs were plucked (using inverted microscope inside biosafety cabinet) and dissociated by vortexing in IMDM. Cells were spun and resuspended in fresh ColonyGel and then plated into single wells of a 24-well plate. GEMM colonies were imaged with a Q-Colour3 digital camera (Olympus) mounted to an Olympus IX5 microscope with a 40X objective lens. Image-Pro imaging software (Media Cybernetics) was used to acquire pictures and subsequent image processing was performed with ImageJ software.

For PLAG1^{KD}, Zsgreen⁺ cells were FACS purified post transduction. Cells (300 cells/ml) were mixed with ColonyGel using a blunt end needle and syringe and plated on replicate 35 mm tissue culture dishes and incubated at 37°C. Manual colony counts were carried out after 14 days of incubation. Colonies were scored and enumerated as BFU-E, CFU-G, CFU-M, CFU-GM and CFU-GEMM.

Lin⁻CD34⁺ suspension *in vitro* cultures

For the overexpression *in vitro* culture, Lin⁻CD34⁺ cells were transduced with PLAG1^{OE} and SFFV control lentivirus. FACS purified transduced BFP⁺ cells from PLAG1^{OE} and SFFV control transductions were seeded in serum-free StemSpan media (StemCell technologies) with human growth factors IL-6 (20 ng/ml), SCF (100 ng/ml), FLT3-L (100 ng/ml), and TPO (20 ng/ml) for 7 days. Similarly, Lin⁻CD34⁺ cells were independently transduced with shPLAG1 and shluciferase control. Zsgreen⁺ from shPLAG1 and shLuciferase transductions were FACS purified and seeded for *in vitro* culture for 7 days. Cells from each of PLAG1^{OE} and SFFV, PLAG1^{KD} and shLuciferase conditions were counted on day 4 and 7 time points. At each time points, total nucleated cells (TNCs) were counted, analyzed for CD34 expression to generate growth curves. Additionally, aliquots of cells were used for cell cycle and apoptosis assays from each time point (see below). All *in vitro* assays were done from at least 3 independent biological replicates of cord blood samples.

Cell cycle and apoptosis assays

For the overexpression experiments, Lin⁻CD34⁺ cells were transduced with PLAG1^{OE} or SFFV control lentivirus. FACS purified transduced BFP⁺ cells from PLAG1^{OE} and SFFV control transductions were seeded in serum-free StemSpan media (StemCell technologies) with human growth factors IL-6 (20 ng/ml), SCF (100 ng/ml), FLT3-L (100 ng/ml), and TPO (20 ng/ml) for 7 days. Similarly, Lin⁻CD34⁺ cells were independently transduced with shPLAG1 or shluciferase control. For knockdown experiment, Zsgreen⁺ from shPLAG1 and shLuciferase transductions were FACS purified and seeded for *in*

vitro culture for 7 days (D7). At D4 and D7 time points, cells were used for cell cycle and apoptosis assays. Cell cycle was assessed by co-staining with Ki67-PE-Cy7 (BD Bioscience) and Hoechst 33342 (10ug/ml) (Sigma) on day 4 and 7 time points of the suspension culture. When ever necessary cells were pre-stained with CD34-APC antibody before fixing and permeabilization with the Cytofix/Cytoperm kit (BD Biosciences). For apoptosis assay, Annexin V Alexafluor-350 conjugate (Invitrogen) and 7-AAD (BD Bioscience) co-staining was done on cells from the day 4 and day 7 time points of the suspension cultures according to the manufacture's protocol. All *in vitro* assays were done for at least 3 independent biological replicate cord blood samples.

Cell size measurement with Immunofluorescence

Established protocol for immunofluorescence staining of cytoplasmic CYP1B1 protein was used (Rentas et al., 2016). Briefly, Lin⁻CD34⁺ cells were transduced with PLAG1^{OE} or SFFV lentivirus. Transduced BFP⁺ cells were sorted and cultured in TSF6-supplemented StemSpan medium for 7 days. At day 3 and 7 time points, minimum of 5,000 cells were used for immunostaining. Cells were fixed in 2% PFA for 10 minutes, washed with PBS and then cytospun on to glass slides. Cells were then permeabilized (PBS 0.2 % Triton X-100) for 20 minutes, blocked (PBS 0.1% saponin 10% donkey serum) for 30 minutes and stained with anti-CYP1B1 primary antibody (Abcam EPR14972) for 1 hour at room temperature. Slides were dunk twice into PBS containing washing chamber. Secondary antibody staining was performed in PBS 10% donkey serum (Jackson Immuno research laboratories) with Alexafluor-647 (AF-647) donkey anti-rabbit antibody (Invitrogen) for 45 minutes and washed with PBS. Slides were

mounted with Prolong Gold Antifade containing DAPI (Invitrogen) until ready for imaging. Several images (100-1000 cells total) were captured per slide at 20X magnification using an Operetta HCS Reader (Perkin Elmer) with epifluorescence illumination and standard filter sets. Columbus image analysis software (Perkin Elmer) was used to automatically identify cytoplasm boundaries and quantify cell area in square micrometer units.

Intracellular (Phospho) flow cytometry

Lin⁻CD34⁺ cells were transduced with PLAG1^{OE} or SFFV control lentivirus. FACS purified transduced BFP⁺ cells from PLAG1^{OE} were seeded in TSF6-containing serum-free StemSpan media (StemCell technologies) along with BFP⁺ from the SFFV control. 4 days later, cells from each condition were fixed/permeabilized with Cytofix/Cytoperm reagent (BD Biosciences) supplemented with Halt Protease/Phosphatase inhibitor cocktail (1x) (ThermoFisher Scientific) as per manufacturer's instructions. Fixed/permeabilized cells were blocked with huIgG (200ug/ml) 10% donkey serum for 10 minutes at 4°C. Primary antibodies against phospho-Akt^{S473} (rabbit) (3787), phospho-mTOR^{S2448} (rabbit) (#2971), pan-Akt (rabbit) (C67E7) (#4691) and total mTOR (L27D4) mouse (4517) were incubated in 1x Permashield buffer (BD Biosciences) for 45 minute at 4°C. All the primary antibodies were purchased from Cell Signaling Technologies. Secondary antibody detection was done using AF-647 donkey anti-rabbit or anti-mouse (Invitrogen) for 45 minute at 4°C. IGF1R-APC (1H7) (ThermoFisher Scientific) cell surface staining was done at the same time on aliquots of unfixed cells. Flow cytometry data was acquired on

LSRII (BD Biosciences) and analyzed using Flowjo (Tree star). MFI of AF-647 was determined to calculate expression/phosphorylation levels.

Quantitative Real Time PCR for microRNAs

Total RNA was isolated using TRIzol-LS reagent as per manufacturer's protocol (Life Technologies). miRNA qRT-PCR was done using qScript cDNA synthesis and PerfeCTa SYBR Green kit (Quanta Biosciences). Briefly, 250ng of total RNA was used for poly(A) tailing reaction. Next, poly (A)-tailed RNA is used for cDNA synthesis. Assay specific PerfeCTa primers were designed for miR-127(HSMIR-0127-3P:TCGGATCCGTCTGAGCTTGGCT;HSMIR-0127-5P:CTGAAGCTCAGAGGGCTCTGAT),miR-675(hsa-miR-675-3p:CTGTATGCCCTCACCGCTCA;hsa-miR-675-5p:TGGTGCGGAGAGGGCC CACAGTG),and endogenous control mRNA(HSRNU6:CTGCG CAAGGATGACACG CAAATTCGTGAAGCGTTCCATATTTTT;HS-SNORD48:AGTGATGATGACCCCA GGTA ACTCTGAGTGTGTCGCTGATGCCATCACCGCAGCGCTCTGACC) as recommended. SYBR Green qRT-PCR is performed using 200 nM of each of miRNA specific primers and PerfeCTa Universal PCR Primer along with the appropriate PerfeCTa SYBR Green SuperMix. PCR parameters were pre-incubation/activation at 95°C for 2 minutes followed by 40 cycles of denaturation at 95°C for 5 second and annealing/acquisition of fluorescent signal at 60°C for 20 seconds. Fold change in transcript level was calculated according to the $2^{-\Delta\Delta C_t}$ method.

Measurement of global protein synthesis rate *in vitro*

Protein synthesis assay is determined by using Click-iT[®] Plus O-propargyl-puromycin (O-p-puro) kit (ThermoFisher, Catalogue# C10456). Lin⁺CD34⁺ cells were transduced with

PLAG1^{OE} or SFFV control lentivirus. FACS purified transduced BFP⁺ cells from PLAG1^{OE} were seeded in TSF6-containing serum-free StemSpan media (StemCell technologies) along with BFP⁺ from the SFFV control counter parts for 4 days. For O-p-puro incorporation assay, $1.0 - 2.0 \times 10^4$ cells were incubated in TSF6-supplemented StemSpan media with 50uM O-p-puro for 1 hour in 100ul volume in 96-well round bottom low-binding tissue culture plate (Corning). Concurrently, the same number of cells from PLAG1^{OE} or SFFV control were incubated with Cyclohexamide (100ug/ml) for 30 minutes, followed by O-p-Puro incorporation assay. After 1 hour of O-p-puro incorporation, cells were washed twice with PBS. Next, cells were fixed with 1.5% paraformaldehyde (PFA) in PBS for 15 minutes on ice, and washed twice with PBS. Next, cells were permeabilized with 3% FBS 0.1% saponin for 5 min at room temperature. Click-iT[®] cycloaddition reaction was performed by using Alexa-Fluor[®]488-conjugated (AF-488) picolyl azide as per manufacturer's instructions (ThermoFisher Scientific). Briefly, fixed/permeabilized cells were incubated with reaction cocktail containing Click-iT[®] O-p-Puro reaction buffer (1x), Click-iT[®] reaction buffer additive, Copper protectant and Alexa-Fluor[®]488-conjugated picolyl azide (1.25 μ M) for 30 minutes at room temperature protected from direct light. Cells were washed once with PEF and analyzed by flow cytometry on LSRII (BD Biosciences). Data was analyzed using Flowjo (Tree star). MFI of AF-488 was determined to calculate protein synthesis rate. 3 - 4 independent cord blood biological samples were used for measurement of protein synthesis.

RNA sequencing library preparation and processing for PLAG1 over expression

RNA extraction was done using >100,000 FACS purified transduced Lin⁻CD34⁺ cells. Total RNA was isolated using TRIzol LS as recommended (ThermoFisher Scientific), and then further purified using RNeasy Micro columns (Qiagen) and quantified by QuBit (ABI). Quality of total RNA was assessed with the BioAnalyzer Nano (Agilent) and all samples had a RIN above 8. Next, 250 ng of total RNA was used for library preparation. Library preparation was done with the KAPA mRNAseq stranded kit (KAPA, Cat no. KK8420). Ligation was made with 9 nM final concentration of Illumina index and 10 PCR cycles was required to amplify cDNA libraries. Libraries were quantified by QuBit and BioAnalyzer. All libraries were diluted to 10 nM and normalized by qPCR using the KAPA library quantification kit (KAPA; Cat no. KK4973). Libraries were pooled to equimolar concentration. Sequencing was performed with the Illumina Hiseq2000 using the Hiseq Reagent Kit v3 (200 cycles, paired-end) using 1.7 nM of the pooled library. Around 45 million paired-end PF reads (except PLAG1_2; 25M PF reads) was generated per sample. There are 2 biological replicates comparing PLAG1 overexpression (PLAG1) and control (SSFV). For the read alignments, sequences were trimmed for sequencing adapters and low quality 3' bases using Trimmomatic version 0.35 and aligned to the reference human genome version GRCh38 (gene annotation from Gencode version 24) using STAR version 2.5.1b. DESeq2 version 1.6.2 was then used to identify differentially expressed genes between the 2 groups with base Mean (mean of count values) of 8.552 used to eliminate low count genes. Accordingly, 544 significantly DE features under FDR 0.05 and 290 of those are associated with a positive logFC. RNA library preparation,

sequencing, alignment and differential expression (DE) analysis was done at Institute for Research in Immunology and Cancer's (IRIC) Genomics Platform (Montreal, Canada).

RNA sequencing library preparation and processing for PLAG1 knockdown

RNA extraction was done using >50, 000 FACS purified transduced Lin⁻CD34⁺ cells. Total RNA was isolated using TRIzol LS as recommended (ThermoFisher Scientific), and further purified using RNeasy Micro columns (Qiagen) and quantified by QuBit (ABI). Quality of total RNA was assessed with the BioAnalyzer Pico (Agilent) and all samples had a RIN above 8. Next, 50 ng of ShLuc2_1 and 12-ShPLAG1_1; 400 ng of total RNA was used for library preparation. Library preparation was done with the KAPA mRNAseq stranded kit (KAPA, Cat no. KK8420). Ligation was made with 1.8 nM final concentration of Illumina index and 16 PCR cycles (except 12-ShPLAG1_3 and ShLuc2__3; 9nM index; 11 PCR cycles) was required to amplify cDNA libraries. Libraries were quantified by QuBit and BioAnalyzer. All libraries were diluted to 10 nM and normalized by qPCR using the KAPA library quantification kit (KAPA; Cat no. KK4973). Libraries were pooled to equimolar concentration. Sequencing was performed with the Illumina Hiseq2000 using the Hiseq Reagent Kit v3 (200 cycles, paired-end) using 1.7 nM of the pooled library. Around 50-70 million paired-end PF reads was generated per sample. Library preparation and sequencing was made at the Institute for Research in Immunology and Cancer's (IRIC) Genomics Platform (Montreal, Canada).

Pathway analysis and Enrichment map for PLAG1 over expression

In order to perform pathway analysis using GSEA tool (Gene Set Enrichment Analysis, <http://software.broadinstitute.org/gsea/index.jsp>), genes are ranked from top up-regulated

(higher expression values in PLAG1 compared to SFFV) to top down-regulated (lower expression values in PLAG1 compared to SFFV). In order to rank the genes, the formula $(-\log_{10}(\text{p-value}) * \text{sign}(\log\text{FC}))$ is used to calculate a score for each gene. GSEA will then calculate an enrichment score for each pathway stored in the pathway database in order to determine whether genes in each tested pathway are enriched at the very top or very end of this list. PLAG1 overexpression data were analyzed using GSEA with parameters set to 2000 gene-set permutations and results were analyzed using Cytoscape EnrichmentMap. The gene-sets included in the GSEA analyses were obtained from MsigDB-c2 and c3, NCI, IOB, Netpath, HumanCyc, Reactome, Panther and the Gene Ontology (GO) MP and BP databases, updated September 2016 (<http://baderlab.org/GeneSets>). For genes up-regulated in PLAG1, no pathway was enriched under a false discovery rate (FDR) threshold of 0.05. However, for genes down-regulated in PLAG1, 80 pathways (gene-sets) were enriched under a FDR threshold of 0.05. EnrichmentMap organizes large gene-sets, such as pathways and gene ontology (GO) terms, into a network where closely related gene-sets are clustered together, allowing for an easier visualization and interpretation of the data. The blue nodes (circles) represent pathways enriched in genes down-regulated in PLAG1. For example, enrichment map contains only blue nodes because at GSEA FDR of 0.05, no pathways were significantly enriched in genes up regulated. The green edges represent genes that are common between two nodes. Therefore, pathways are grouped in clusters if they share a lot of genes in common and form modules of biological functions. The app AutoAnnotate was used in Cytoscape to draw the clusters (determined by the MCL-

cluster method and delimited on the figure by black rectangles unless deliberately highlighted by different color) and automatically annotate each module by using most frequent gene-set label words.

Pathway analysis and Enrichment map for PLAG1 knockdown

The ultimate purpose of the shPLAG1 experiment was to validate the significantly downregulated pathways from PLAG1 over expression. Similar to the DEseq ranking for PLAG1 over expression, a score calculated using the formula $(-\log_{10}(\text{p-value}) * \text{sign}(\log\text{FC}))$ was used to rank the genes from top up-regulated in shPLAG1 compared to top down-regulated to shLuciferase control. GSEA was applied on the shPLAG1 ranking using the same method and parameters as for PLAG1 overexpression and results were visualized using EnrichmentMap in Cytoscape using the same parameters. In the next step, enrichment scores of shPLAG1 were compared to the ones of PLAG1 overexpression. The motivation was to restrict the gene-sets to those only which have an enrichment score going in the opposite direction: a gene-set enriched in genes up-regulated in PLAG1 over expression should also be enriched in gene down-regulated in shPLAG1 and vice versa (as expected from the rationale) and the selected gene-sets would have to be significant under a $p\text{-value} < 0.05$ in at least one condition. From 1223 gene-sets that were significant in at least one condition, 720 gene-sets (59%) had an enrichment score going in the opposite direction. An enrichment map was created using these 720 gene-sets.

Heat map of differentially expressed genes that are up-regulated in PLAG1 over expression and also down-regulated in shPLAG1 (related to Supplemental figure 3.3)

Normalized CPM count data of PLAG1 over expression and shPLAG1 were centered and scaled separately such that the scaled values (z scores) are indicative of a higher or lower expression of treated condition compared to the control of each dataset. Genes were selected only if they were up regulated (z score > 0) in PLAG1 OE and also down regulated (z score < 0) in shPLAG1 and for both biological replicates in each dataset. Heat maps using the scaled values were created using the heatmap.2 function in R3.3.3. All selected genes or the top30 genes ranked based on highest sum of absolute deviation to the mean were used to create the heat maps (Supplemental Figure 3.3).

DMAP global differentiation program analysis

The selected genes that are up-regulated in PLAG1 over expression and also down-regulated in shPLAG1 were mapped to the Gene Expression Omnibus dataset GSE24759 (DMAP), containing Affymetrix GeneChip HT-HG_U133A Early Access Array gene expression data of 20 distinct haematopoietic cell states. GSE24759 data were background corrected using Robust Multi-Array Average (RMA), quantile normalized using the `expresso` function of the `affy` Bioconductor package (`affy_1.38.1`, R 3.0.1), array batch corrected using the `ComBat` function of the `sva` package (`sva_3.6.0`), and standardized using a z score for each gene across samples. Bar graphs were created by selecting genes that were up- and down-regulated genes, and summing the number of standardized data points that were above (>0) or below (<0) the mean for each DMAP

cell population, corrected by the number of samples per population and results obtained from 1,000 random selection of DMAP genes.

Target validations for miR-675

Predicted and experimentally validated targets of miR-675 are retrieved and mapped to the PLAG1^{OE} enrichment map. To identify predicted targets, mirDIP (mirDIP-All-Data-Version1.0.txt, 2012) database was downloaded from the mirDIP website and genes predicted to be target of miR-675 were retrieved without further filtering (4000 entries, includes all databases). Enrichment map displays all gene-sets that have a significant overlap (Mann-Whitney two-sided, p-value cut-off 0.05, orange edges) with the miR-675 predicted targets. The gene-sets have a GSEA p-value < 0.05. To identify, experimentally validated targets of miR-675, we used DIANA Tarbase v7.0 database(Vlachos et al., 2015). TarBase hosts data derived from CLIP-Seq and Degradome-Seq studies, significantly more than any other available database. 101 mir-675 experimentally validated targets have been downloaded from TarBase and 93 out of these 101 were present in the original list of predicted targets (4000 entries).

Target validation for miR-127

BAM files contain the RNA-seq reads aligned to the reference genome. These files have been used with the integrated genome viewer (IGV) 2.3 software (<http://software.broadinstitute.org/software/igv/>) to look at the reads in the MEG3 locus using the assembly genome hg38. Reads were more abundant for the coding genes and counts were equal to 0 for many of the miRNAs and snoRNAs except 4 miRNA species

(miR-127, 370, 433, 770). These miRNAs had adequate coverage as can be seen with a higher number of reads in PLAG1 compared to the control SFFV samples and this difference in expression was confirmed by the DEseq analysis with a $\log_{2}FC > 0$ and $FDR < 0.02$. These miRNAs were selected for further study. Experimentally validated targets of these miRNAs were retrieved in the DIANA Tarbase v7.0 database (Vlachos et al., 2015). Overlap with the pathway map and values for differential expression were studied for these targets. Mann and Whitney post-test analysis identified the significantly downregulated enriched pathways. In particular, miR-127 was identified of highest interest due to its targeting of down regulated gene-sets from PLAG1 over expression.

Myc ChIP-seq analysis

We used the publicly available CODEX Myc ChIP-Seq data generated in HPC7 cell line to find the targets and determine how many of these are in the ribosome biogenesis pathway repressed by PLAG1 over expression. The ChIP-Seq data BED file were downloaded from ArrayExpress (accession number: E-MTAB-3954). The BED file contains the chromosomal positions of the binding peaks. Genes nearby the peaks were retrieved using GREAT (Stanford, <http://bejerano.stanford.edu/great/public/html/>) using a 5kb rule (peaks up to 5kb upstream and 1kb downstream the TSS). The mouse mm9 is the reference genome used for the ChIP-Seq reads alignment. Human orthologs to these genes were retrieved using bioMart - Ensembl (<http://www.ensembl.org/biomart/>) with gene symbols as key values. 1747 regions (peaks) were contained in the BED file. 1511 human genes were retrieved using the 5kb rule. .

Validation of the 5kb rule genes as MYC targets using iRegulon and ENCODE**ChIP-Seq data**

We used the iRegulon Cytoscape app version 1.3 (<http://iregulon.aertslab.org/index.html>) to validate the CODEX MYC targets and to visualize the size of the overlap between the CODEX ChIP-Seq targets and the iRegulon prediction. We used the 5kb rule Myc targets list filtered by PLAG over expression at a p-value cutoff of 0.05 which consists of 153 genes as input list for iRegulon. iRegulon was run using default parameters. 104 genes out of this 153 genes also present in a ChIP-Seq track coming from MYC ChIP-seq on human K562 produced by the Snyder lab and available in the iRegulon database (<https://www.encodeproject.org/targets/MYC-human/>).

GeneMANIA network to add physical interactions between Myc targets

Myc targets were half up-regulated and half down-regulated genes in PLAG1 overexpression, further investigations were performed to check whether the up or down regulated genes were involved in different biological functions. These 5kb rule Myc targets were used as input to the GeneMANIA 3.4.1 Cytoscape app to add known physical interactions with 0 added related genes. GeneMANIA network identified that the red and blue nodes (up and down regulated in PLAG over expression respectively) are bound by separate cluster of physical interactions. MCODE algorithm in the Cytoscape application ClusterMaker2 1.1.0 was used to further cluster this network using default parameters. GO Cellular Compartment gene-set and Biological Process analysis of these 2 clusters were run using g:Profiler (<http://biit.cs.ut.ee/gprofiler/>).

Statistical analysis

All statistical analyses (unless specifically noted for the RNA-seq analysis) were performed using PRISM (Graphpad software, Inc). Where appropriate statistical significance was estimated using two-tailed t-test for pairwise comparisons with the respective control experiments. Data shown as mean \pm SEM. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$

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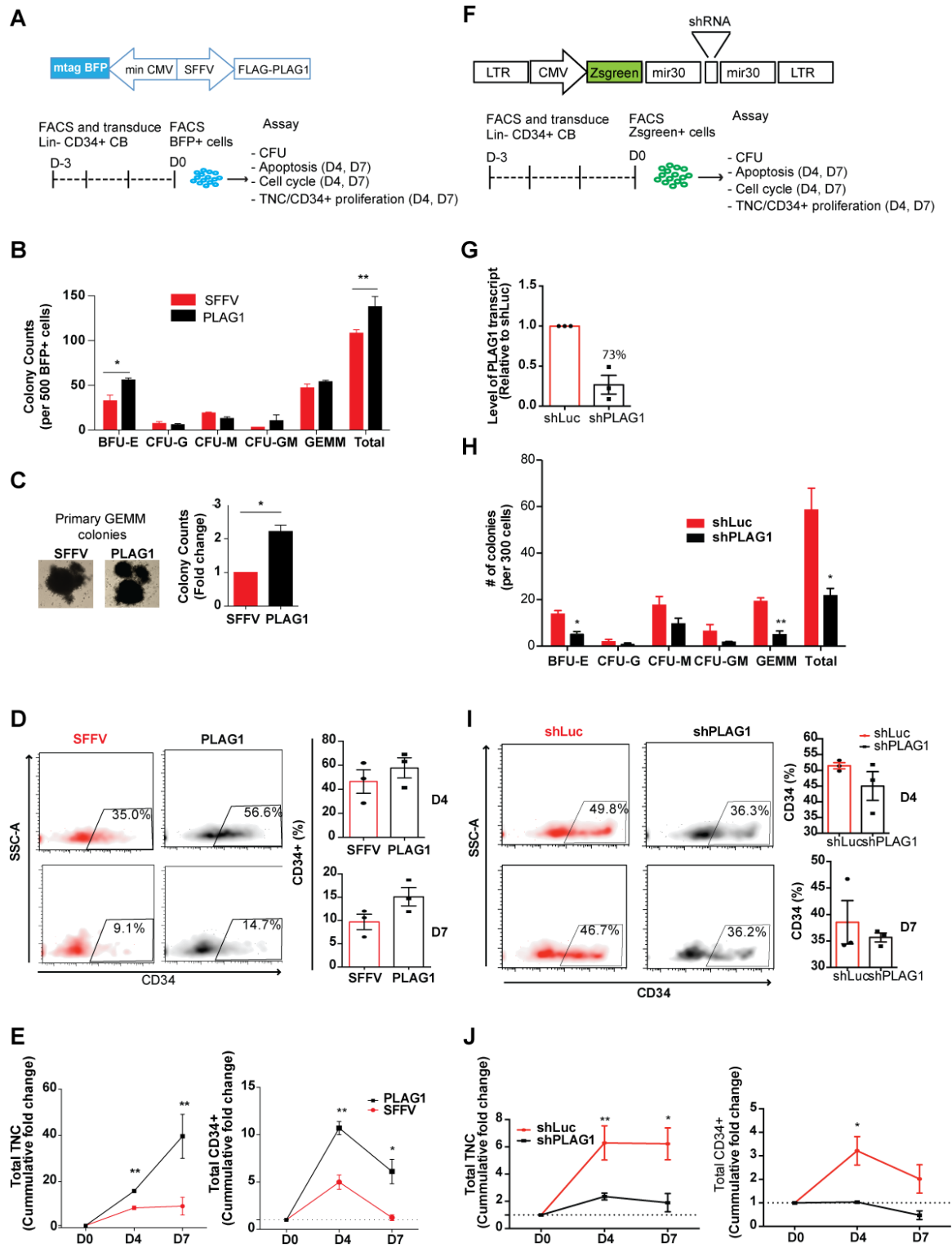


Figure 3.1. PLAG1 modulates progenitor cell activity *in vitro*.

(A) Schematics of lentiviral vector used for PLAG1^{OE} is depicted in the top panel. Experiment plan for sorting and transducing Lin⁻CD34⁺ cells from CB samples for *in vitro* culture. (B) PLAG1^{OE} enhances BFU-E and total CFU output compared to SFFV control. (C) Representative GEMM colonies from SFFV and PLAG1^{OE} (40X magnification) (left panel) and respective secondary CFU outputs after replating (right panel). (D) Representative flow plots showing frequency of CD34⁺ cells from D4 and D7 *in vitro* culture. Right panel is quantifications of CD34⁺ frequencies. (E) Cumulative fold expansion in TNCs (left panel) and CD34⁺ cell counts from D4 and D7 *in vitro* culture. (F) Schematics of lentiviral vector used for shRNA-mediated PLAG1 (shPLAG1, or PLAG1^{KD}) or Luciferase (shLuc) knockdown is depicted in the top panel. Experiment plan for sorting and transducing Lin⁻CD34⁺ cells from CB samples for 7 days *in vitro* culture. (G) Knockdown efficiency of shRNA against PLAG1 (12-shPLAG1) relative to shLuc control. (H) PLAG1^{KD} impairs BFU-E, GEMM and total CFU output compared to shLuc control. (I) PLAG1^{KD} decreases the frequency of CD34⁺ HSPCs over *in vitro* culture. Representative flow plots depicting the frequency of CD34⁺ cells. Right panel is quantification of CD34⁺ frequency. (J) Cumulative fold change in TNCs (left panel) and CD34⁺ cell counts (right panel) from D4 and D7 *in vitro* culture. All experiments were done from at least 3 independent CB samples.

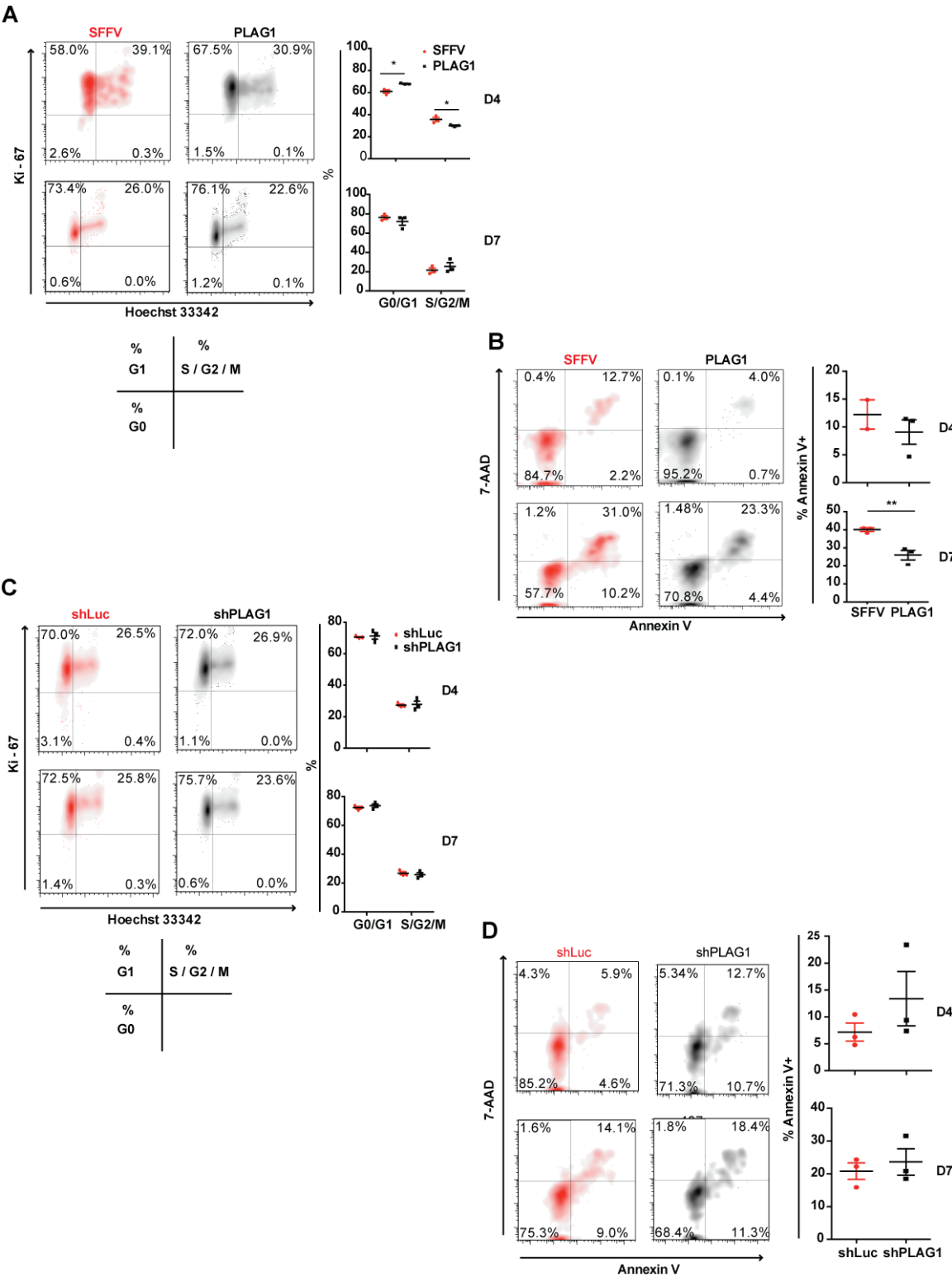


Figure 3.2. Ectopic PLAG1 offers sustained survival and induces transient cell cycle stalling in HSPCs. (A) PLAG1^{OE} transiently increased percentage of cells in G0/G1 and decreased percentage of cells in S/G2/M stage at D4 time point. Left panel shows representative flow plot and right panel depicts quantifications from D4 and D7 time points (N=2 CB samples for D4 SFFV culture). (B) PLAG1^{OE} offers a sustained pro-survival advantage to HSPCs over *in vitro* culture period (indicated by reduced percentage of Annexin V⁺ cells). Left panel shows representative flow plots and right panel depicts quantifications from D4 and D7 time points. (C) shPLAG1 does not significantly modulate cell cycle progression of HSPCs *in vitro*. Left panel shows representative flow plot and right panel depicts quantifications from D4 and D7 time points. (D) shPLAG1 tends towards inducing a sustained pro-apoptotic signal to HPSCs over *in vitro* culture. Representative flow plots (left panel) and quantifications of D4 and D7 time points. Unless specified otherwise, all experiments were done from at least 3 independent CB samples.

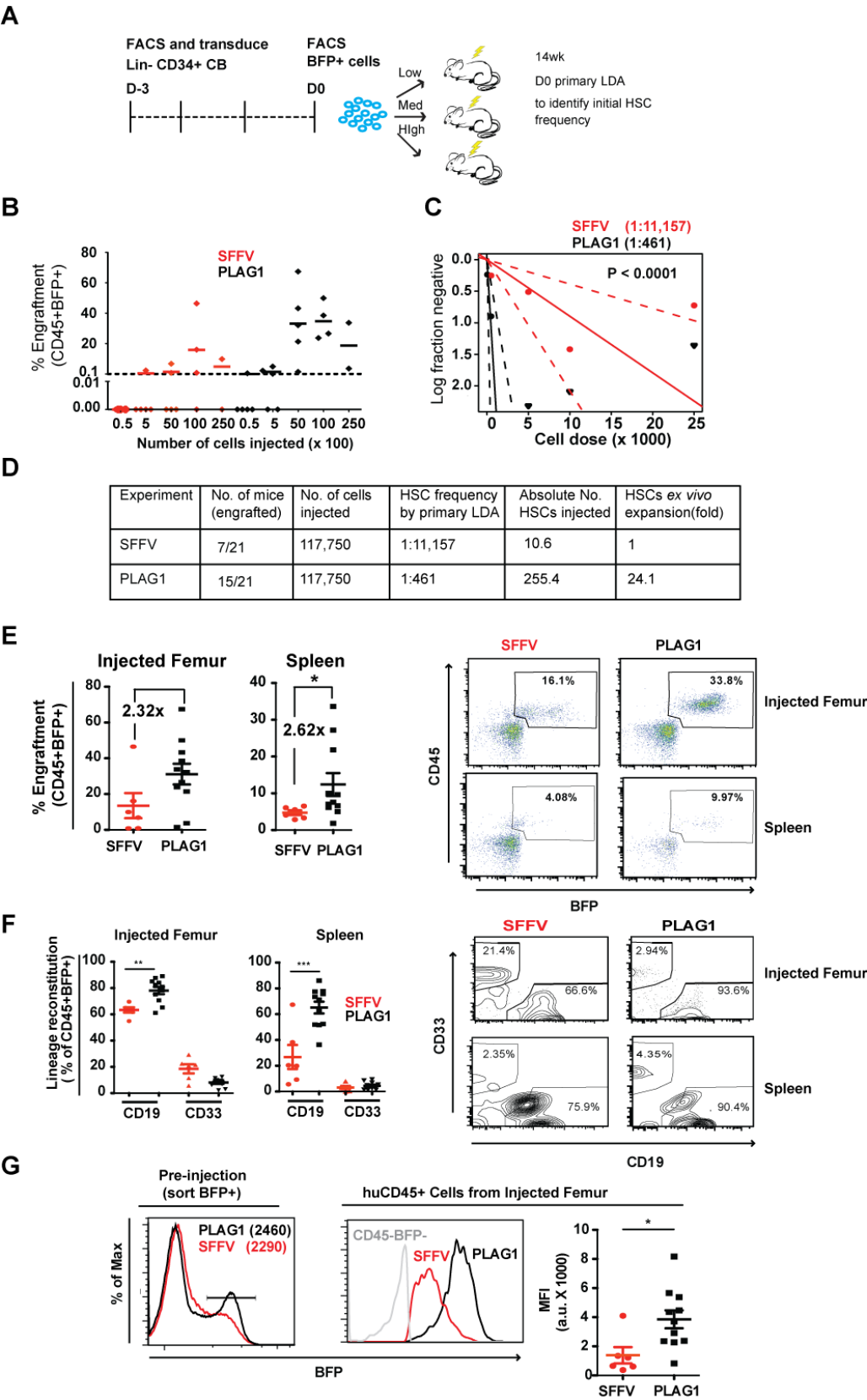


Figure 3.3. Ectopic PLAG1 robustly enhances the frequency and absolute number of HSCs *ex vivo*. (A) Experiment plan for sorting and transducing Lin⁻CD34⁺ cells for intra-femoral injections into sub-lethally irradiated NSG mice for primary LDA. (B) Number of engrafted mice at each dose of primary LDA. The plot depicts level of engraftment from injected femur from all doses. Engraftment is considered positive with >0.1% human CD45⁺BFP⁺ double positive cells and also capable of multi-lineage reconstitution for lymphoid cells (CD19⁺) and myeloid cells (CD33⁺). (C) ELDA plot to determine the HSC frequency in primary LDA. Calculated HSC frequencies both for the SFFV control and PLAG1^{OE} are indicated in parenthesis. (D) Summary table of the primary LDA analysis to determine the fold change HSC *ex vivo* expansion as a result of PLAG1^{OE}. (E) Quantifications of engraftment levels in the injected femur and spleen (left panel) from matching LDA doses of all engrafted mice. Representative flow plots of engraftment levels are depicted in the right panel. (F) Quantifications of lymphoid and myeloid engraftment levels from matching LDA doses of all engrafted mice and representative flow plots from injected femur and spleen (G) MFI values of BFP⁺ cells pre-injection at D0 time point (left panel, values are in inset) . MFI of human CD45⁺BFP⁺ from injected femur of engrafted mice at 14 weeks post-transplant as compared to CD45⁻BFP⁻ (host mouse) cells (middle panel, representative histogram). Quantification of BFP MFI is depicted in the right panel.

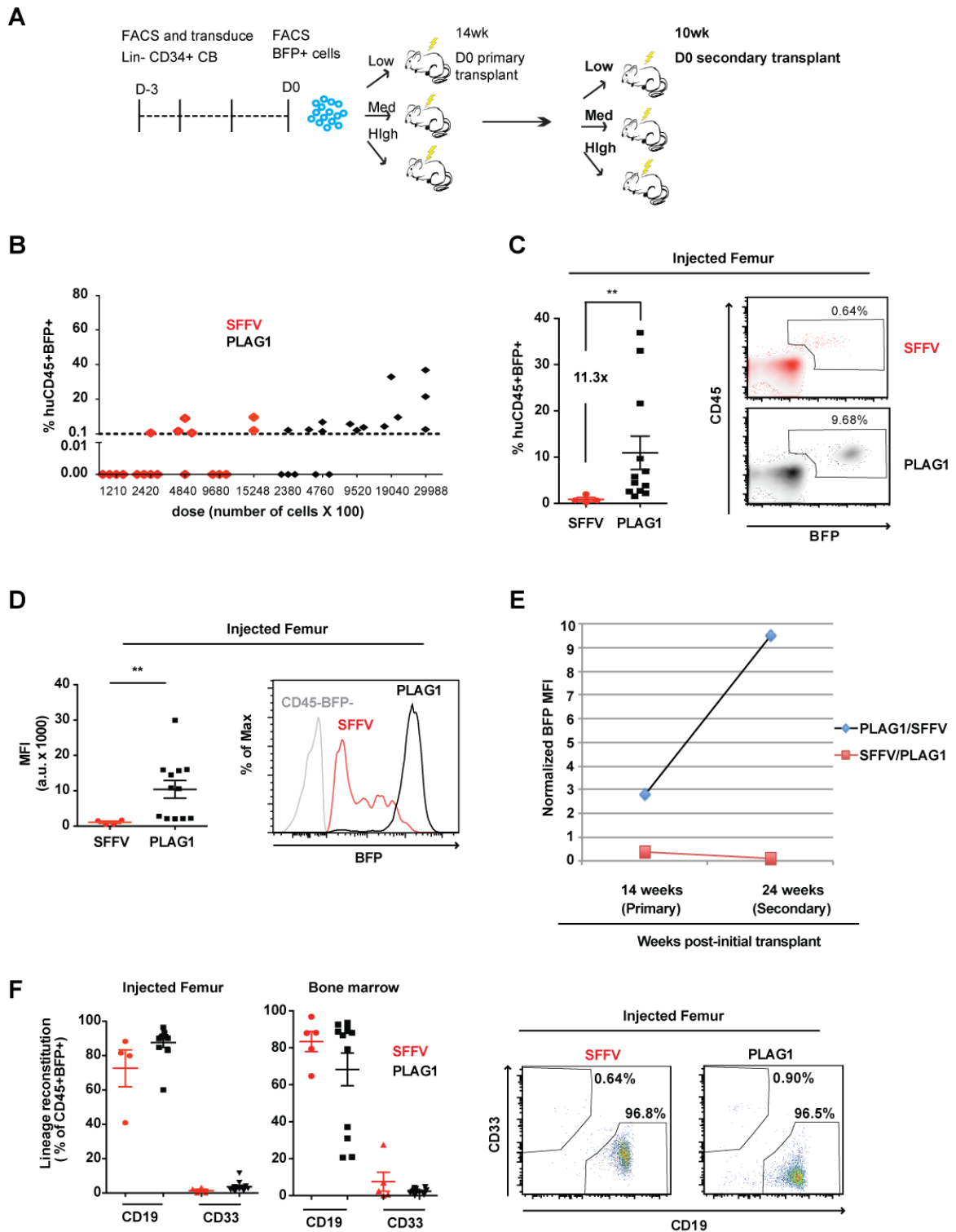


Figure 3.4. Ectopic PLAG1 enhances long-term HSC self-renewal and engraftment in serial transplantations. (A) Experiment plan for intra-femoral injections of BM from primary mice into sub-lethally irradiated NSG mice for secondary transplantation. (B) Number of engrafted mice at varying doses of secondary transplant. Engraftment is considered positive with $>0.1\%$ human $CD45^+BFP^+$ double positive cells. (C) Quantifications of engraftment levels in the injected femur from matching doses of all engrafted mice. Representative flow plots of engraftment levels in injected femur are depicted in the right panel. (D) MFI values of BFP^+ cells from secondary mice (left panel) as compared to $CD45^-BFP^-$ (host mouse) cells. Representative histogram plots from injected femur are depicted. (E) Normalized MFI values from injected femur of engrafted mice at primary (14 weeks) and secondary (24 weeks) post-initial transplant. (F) Quantifications and representative flow plots of lymphoid ($CD19$) and myeloid ($CD33$) engraftment levels in injected femur and spleen from matching doses of all engrafted mice.

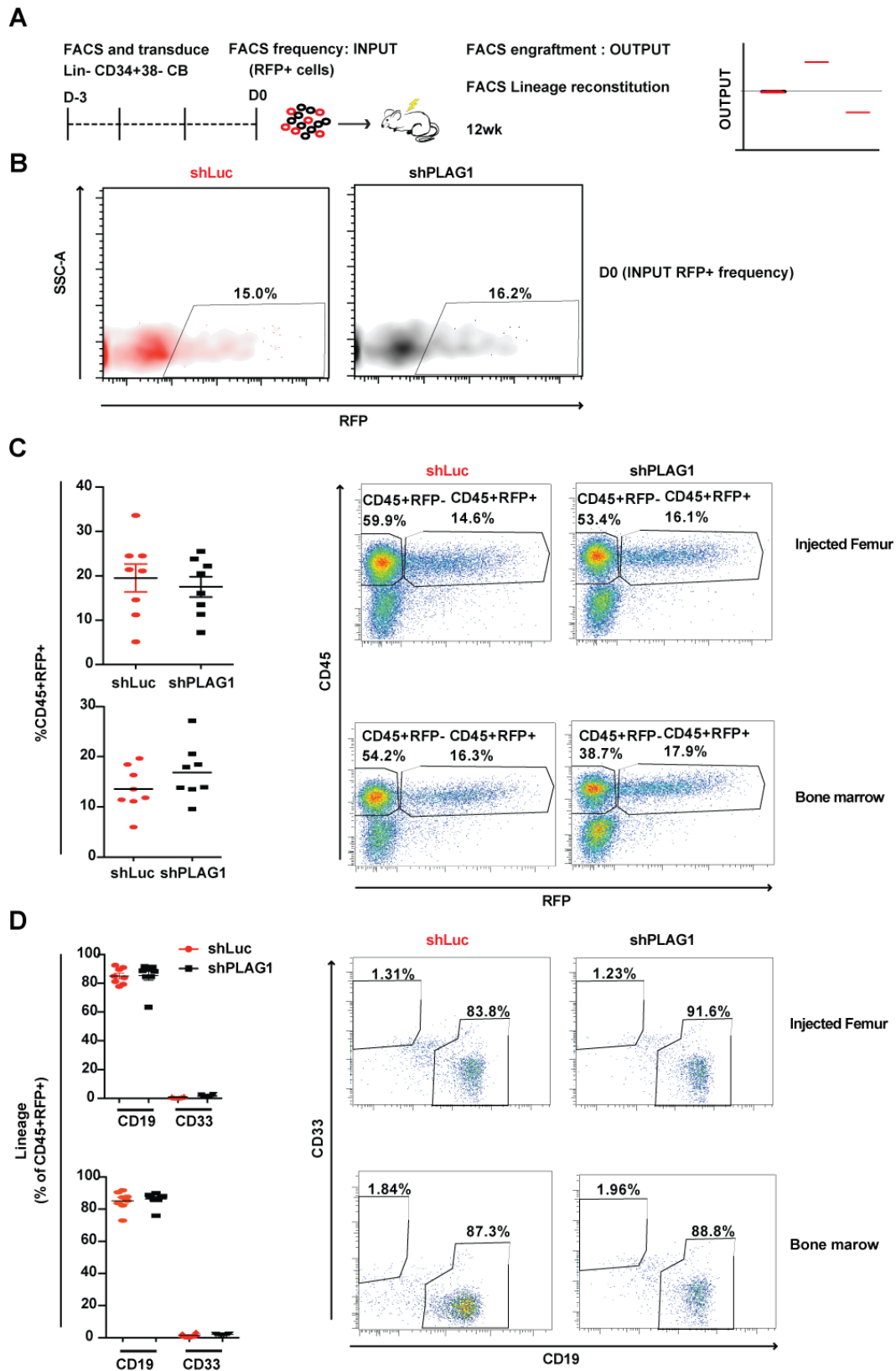


Figure 3.5. PLAG1 knockdown does not influence engraftment and lineage output of Lin⁻CD34⁺38⁻ HSCs *in vivo*. (A) Experiment plan for sorting and transducing Lin⁻CD34⁺38⁻ cells from CB samples for transplantation assay in sub-lethally irradiated NSG mice. Schematic on the right panel depicts the possible outcomes of engraftment (red lines) after 12 weeks. The line crossing the Y-axis marks no change in engraftment (i.e. the same as INPUT frequency). (B) Pre-transplant (D0) frequency of transduced cells as marked by the red fluorescent protein (RFP). No difference on the transduction efficiencies between shLuc and shPLAG1 at D0 time point (INPUT). (C) Quantifications of engraftment levels (frequency of human CD45⁺RFP⁺ cells) in the injected femur and rest of bone marrow (let panels) from all engrafted mice. Representative flow plots of engraftment levels are depicted in the right panel. (D) Frequency of lymphoid (CD19) and myeloid (CD33) engraftment levels (left panels). Right panel depicts representative flow plots from injected femur and rest of bone marrow.

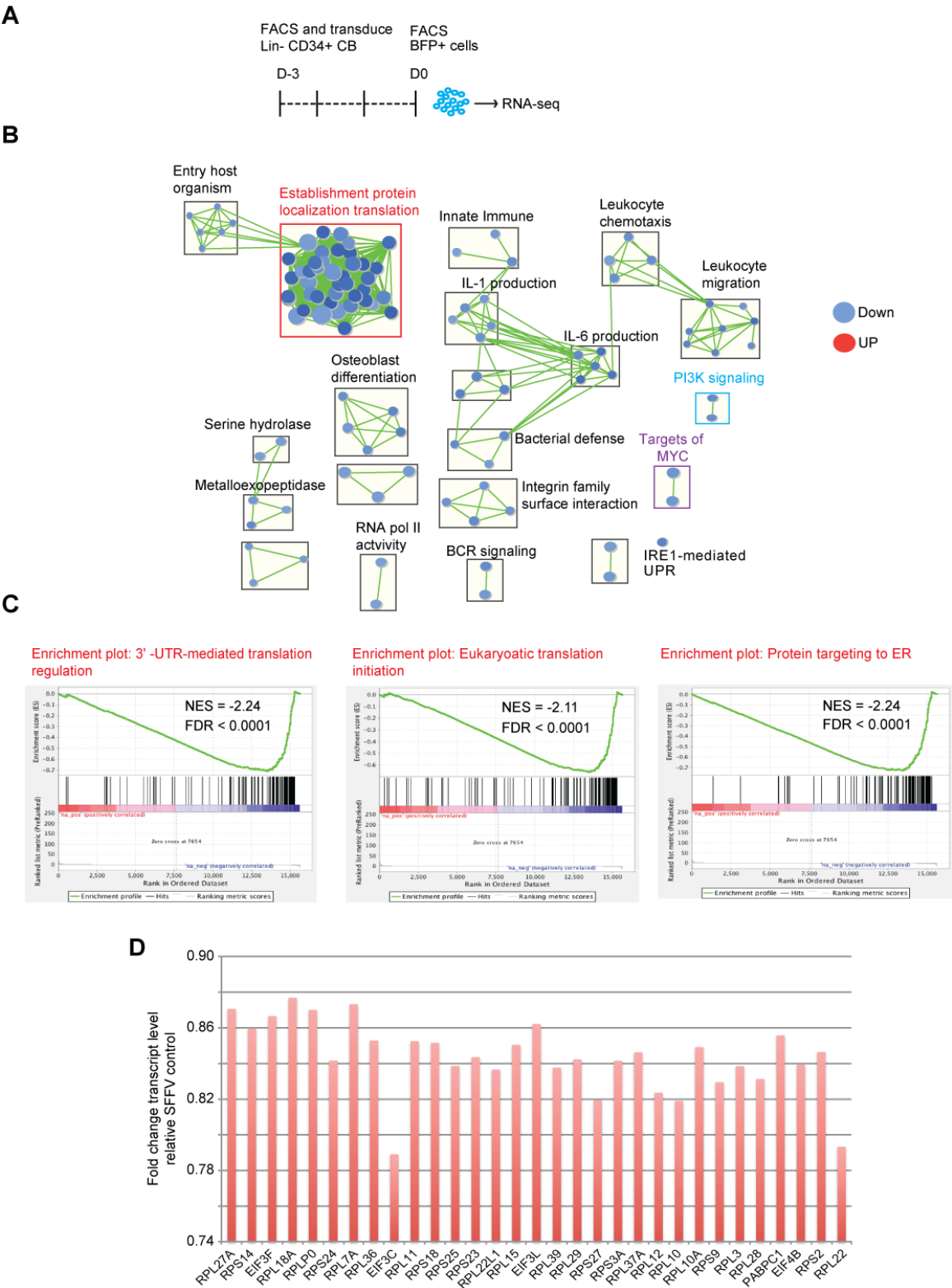


Figure 3.6. RNA-seq identifies global repression of ribosome biogenesis and protein synthesis pathways with ectopic PLAG1 in Lin⁻CD34⁺. (A) Experiment plan for sorting and transducing Lin⁻CD34⁺ cells from CB samples for RNA-seq analysis. (B) Network map of gene-set enrichment analysis demonstrates significant (FDR < 0.05) impairment of genes associated with ribosome biogenesis and localized protein translation (red box). Genes associated with matured hematopoietic cell functions (e.g. leukocyte chemotaxis) are attenuated. Components of PI3K signaling pathways (blue rectangle) are also repressed. Transcriptional targets of the TF MYC (purple rectangle) and the UPR are also down regulated. Green edges represent significant overlap between gene-sets (nodes). (C) Representative GSEA plots of down regulated ribosome biogenesis and protein synthesis functions such as 3'-UTR mediated translational regulation, eukaryotic translation initiation and protein targeting to ER. (D) Bar graph depicting partial list of the most significantly repressed leading edge genes constituting the eukaryotic translation initiation GSE. Fold change transcript levels are transformed from DE-seq logfoldchange values.

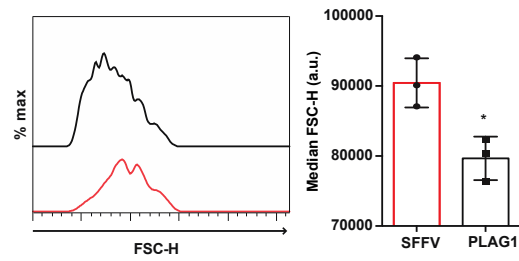
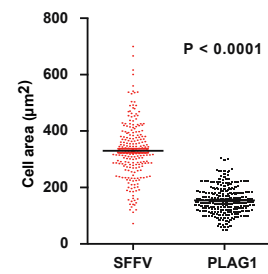
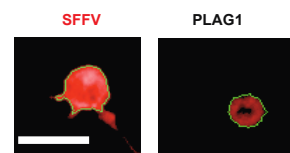
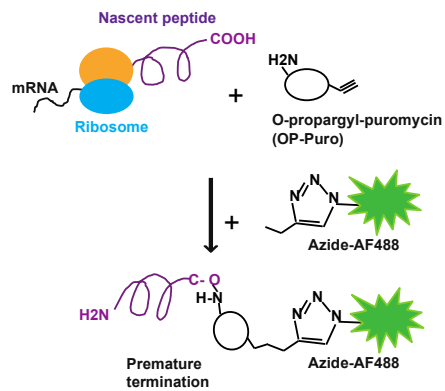
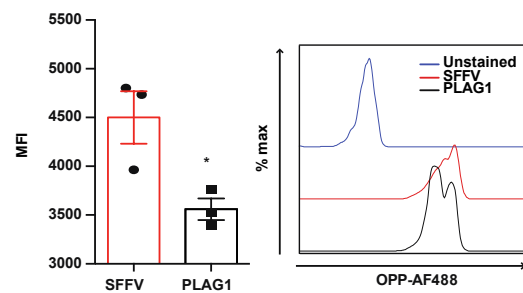
A**B****C****D**

Figure 3.7. Ectopic PLAG1 induces diminished cell size and dampens rate of global protein synthesis. (A) Cell size determination from forward-scatter flow cytometry profiles (FSC-H). Representative histogram depicting the leftward shift in FSC-H for PLAG1^{OE} (left panel). Bar graph on the right panel depicts quantification of FSC-H. (B) Representative IHC images of cells stained with CYP1B1 protein from SFFV and PLAG1^{OE}. Cell boundary (green) was identified by image analysis software Columbus (v2.4.2) (Scale bar = 25um). Bottom panel is quantification of cell area (N > 100 cells). (C) Schematics of O-propargyl-puromycin (O-p-puro) assay to validate rate of protein synthesis *in vitro* culture of Lin⁻CD34⁺ cells. (F) MFI of O-p-puro-AF488 in PLAG1^{OE} cells is significantly lower than control (left panel). Representative histograms are depicted in the right panel. Data is depicted from at least 3 independent CB samples.

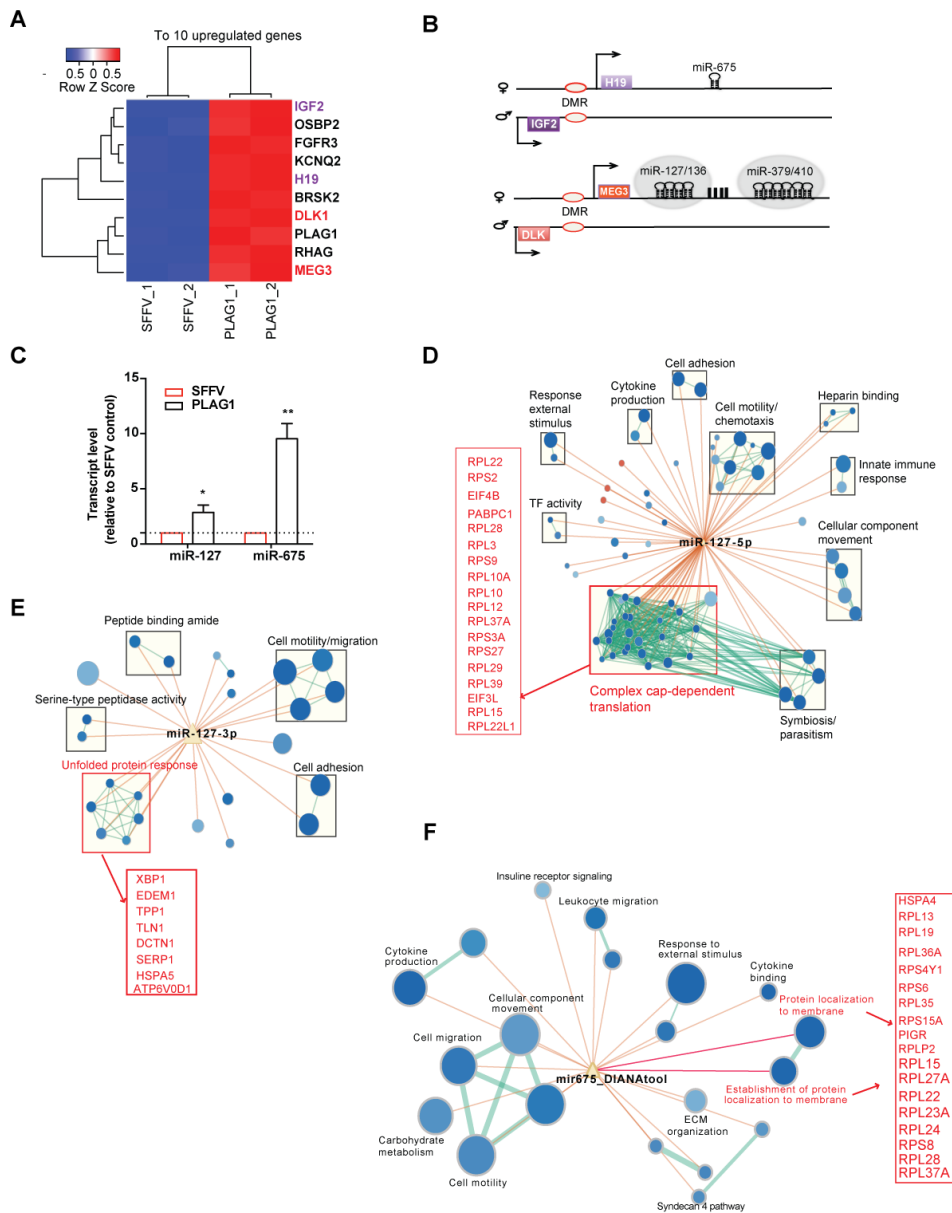


Figure 3.8. Down regulated genes from PLAG1^{OE} overlap with validated targets of miR-127 and miR-675. (A) Heat map of top 10 selected up-regulated genes upon PLAG1^{OE}. Imprinted genes clusters are color coded as purple (H19/IGF2) and red (DLK1/MEG3). (B) Schematics of maternal and paternal gene expression pattern from H19-IGF2 (top panel) and DLK1-MEG3 (bottom panel) loci depicting the respective embedded miRNA species. The H19 locus is reservoir for miR-675. MEG3 locus contains the largest mega reservoir of miR-127/136 and miR-379/410 clusters. (C) miRNA-qPCR validation of miR-127 and miR-675 from Lin⁻CD34⁺ cells with PLAG1^{OE} (data generated from N=3 independent CB samples). (D) Network map of down regulated gene-sets from PLAG1^{OE} overlapping with validated targets of miR-127-5p. Ribosome biogenesis/complex cap dependent protein translation (red box) and matured hematopoietic cell functions are among the major network targets. Targeted genes from representative gene-sets are listed on the left panel (boxed). (E) Network map of down regulated gene-sets from PLAG1^{OE} overlapping with validated targets of miR-127-3p. UPR (red box) and matured hematopoietic cell functions are the major targets. (F) Network map of down regulated genes from PLAG1^{OE} overlapping with validated targets of miR-675. Gene-sets within protein localization to membrane are indicated (red box). Immune cell functions are also targeted by miR-675.

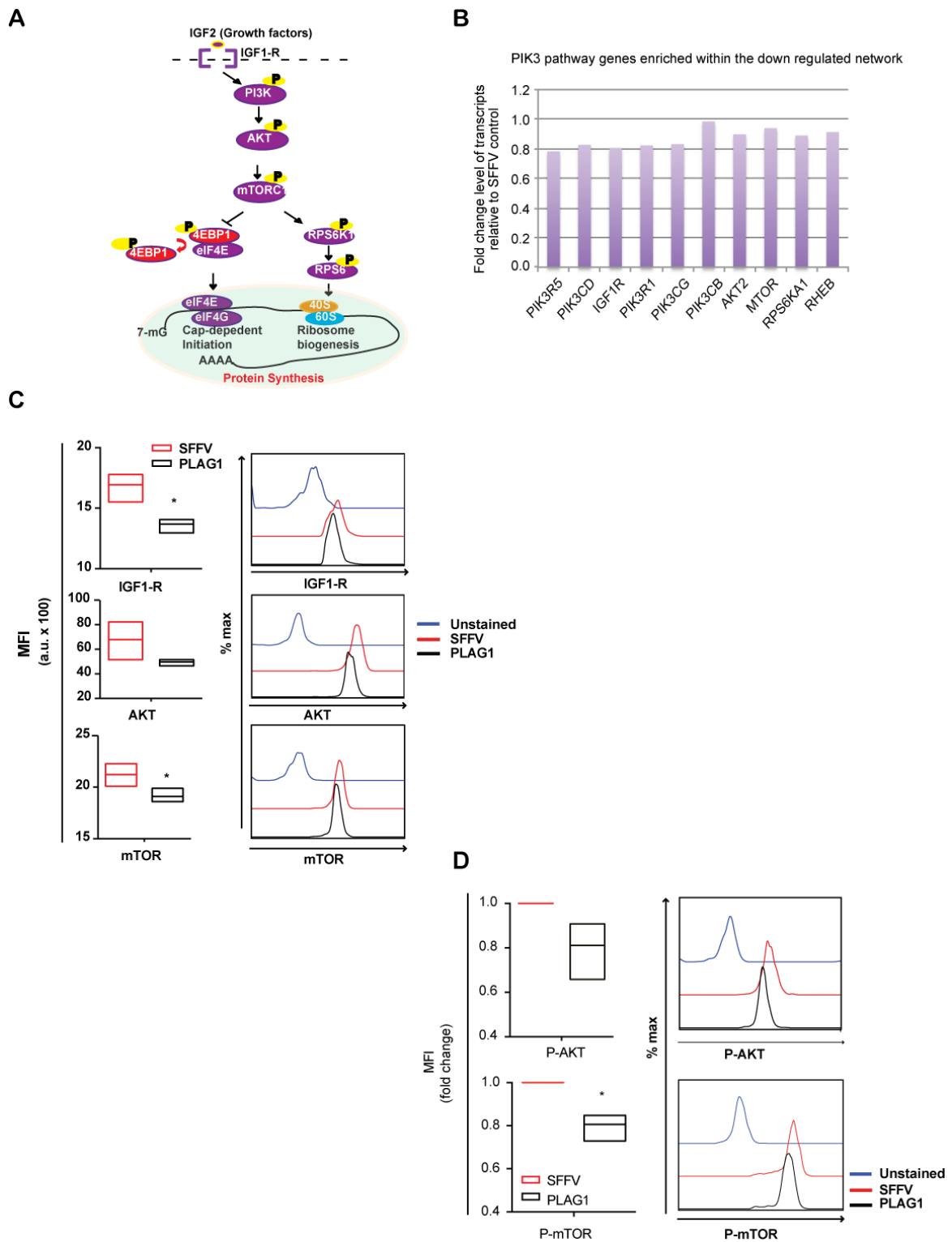


Figure 3.9. PLAG1^{OE} dampens mTOR signaling

(A) Cartoon depiction of IGF1R-PI3K-AKT-mTOR signaling pathway as related to protein synthesis and ribosome biogenesis. Pathway is activated by growth factor (e.g. IGF2) binding to IGF1R. (B) Fold change expression of components of IGF1R-PI3K-AKT-mTOR signaling down regulated with PLAG1^{OE}. Fold change transcript levels are transformed from DE-seq logfoldchange values. (C) Quantifications of total protein expression by surface staining for IGF1R and intracellular staining for pan-AKT and mTOR. Representative histograms for each of IGF1R, pan-AKT and mTOR are shown in the right panel. (D) PLAG1^{OE} hypophosphorylates AKT and mTOR compared to SFFV control. Representative phospho-flow histograms are shown in the right panel.

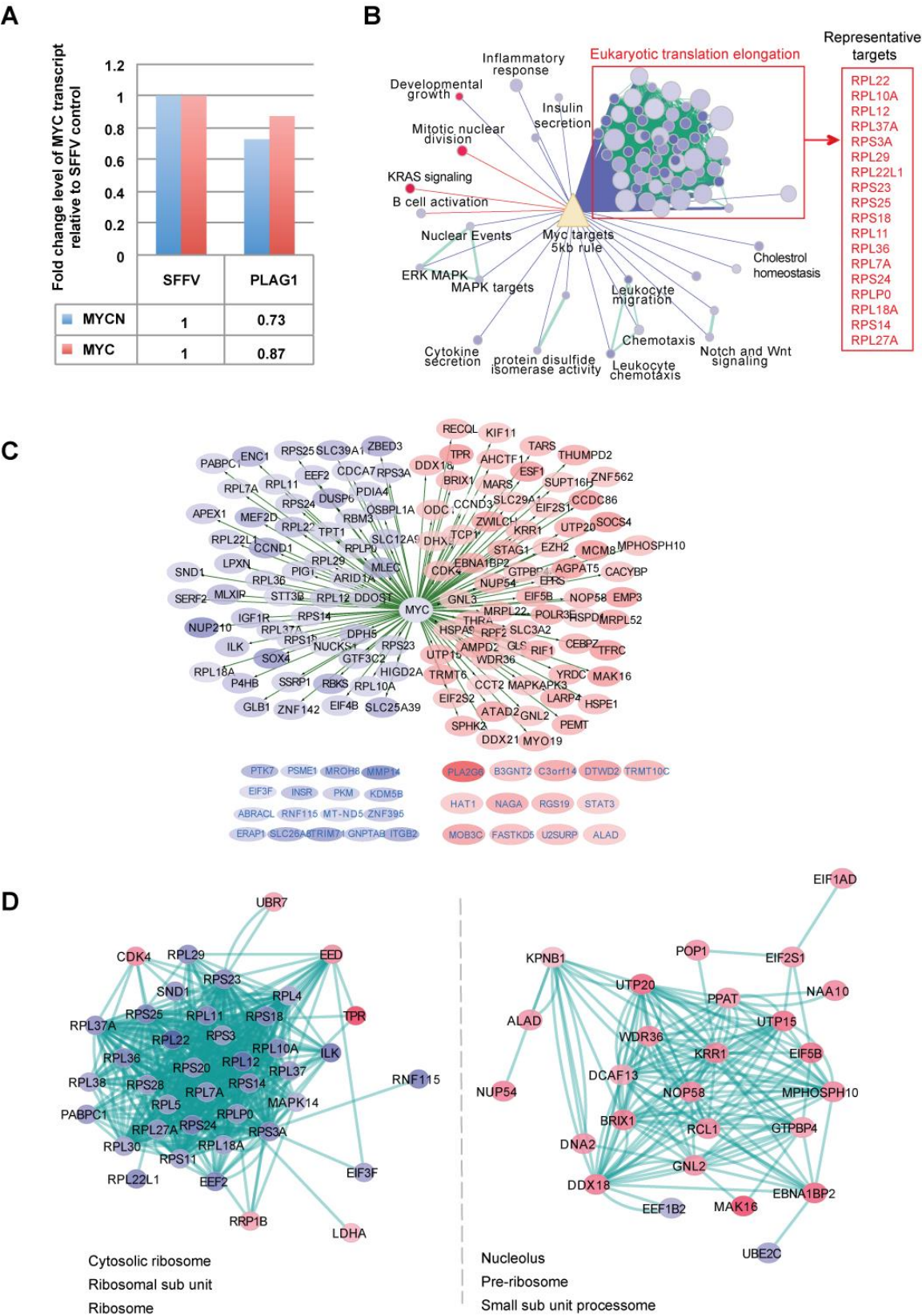


Figure 3.10. Myc ChIP-seq target analysis.

(A) Fold change transcript level of MYC and MYCN with PLAG1 over expression (PLAG1) compared to SFFV control. Fold change transcript levels are transformed from DE-seq logfold change values relative to SFFV control. (B) Overlap analysis of MYC targets using the 5Kb rule with enrichment gene-sets from PLAG1 over expression. Purple edges correspond to an overlap enriched in genes down-regulated and red edges correspond to an overlap enriched in genes up-regulated. Genes present in the most significant overlap are indicated by thick purple edges. Partial list of the genes in the overlap of a representative gene-set called ‘eukaryotic translation elongation’ are presented in the right panel. Green edges represent significant overlap between gene-sets (nodes). Significance of overlap between the list of MYC targets and pathways from the PLAG1 over expression enrichment map was tested using a Mann-Whitney two-sided test ($p < 0.05$). (C) iRegulon analysis validate that the majority of MYC targets using the 5Kb rule using DNA binding motif overrepresentation (Green edges). All nodes represent ChIP-seq 5kb rule MYC targets down regulated genes (blue nodes) and up regulated (Red nodes) from PLAG1 over expression. (D) MCODE analysis demonstrates repressed ribosomal proteins are involved in the cytosolic protein synthesis phase (left panel). Right panel depicts genes involved in pre-ribosome assembly processes in the nucleolus. Cyan edges represent interactions. GO terms for both clusters are shown as inset.

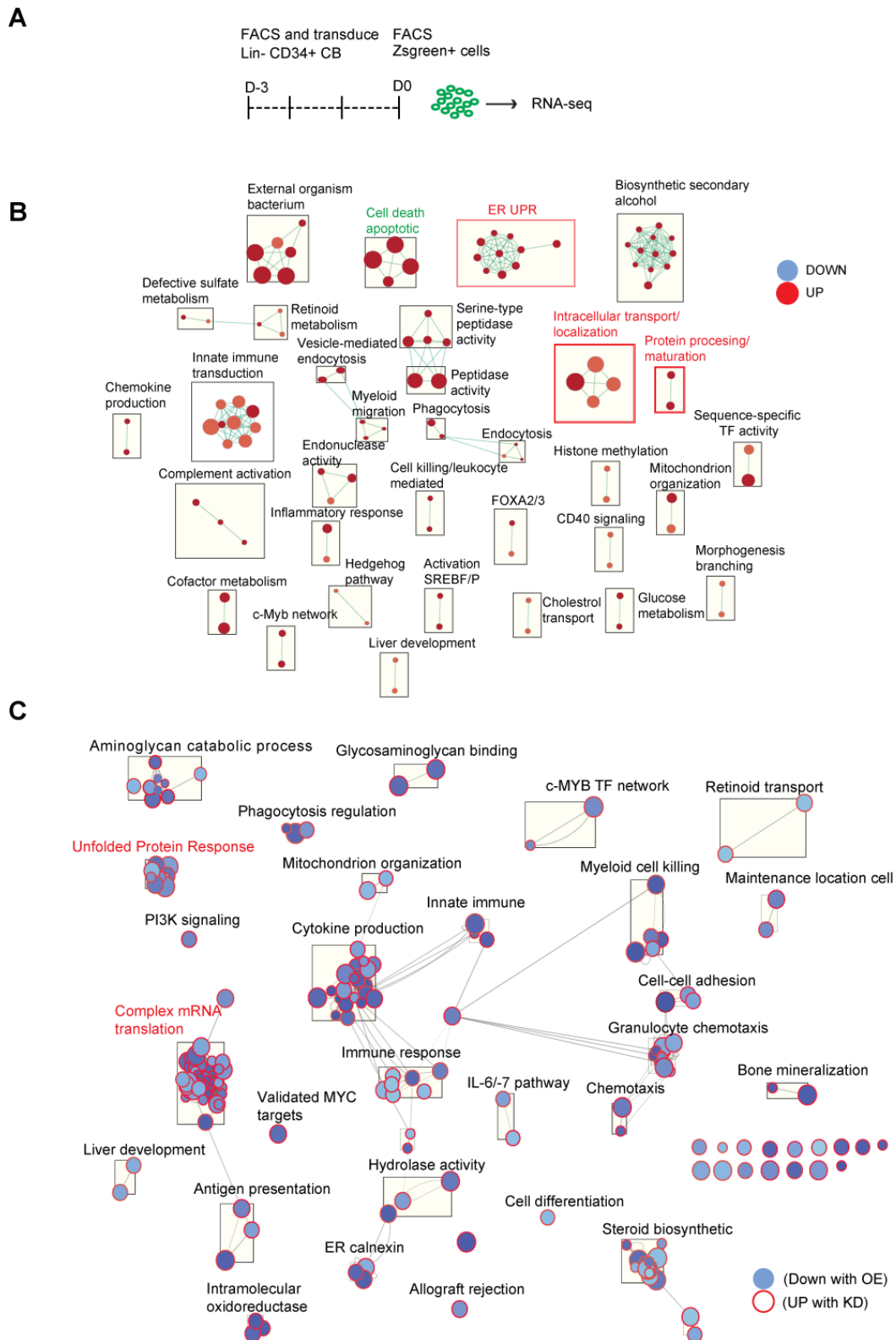
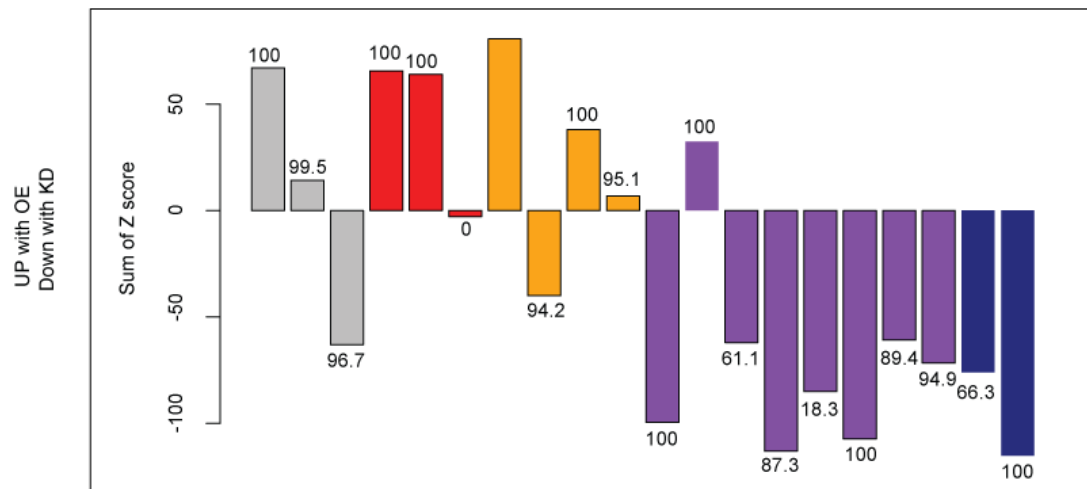
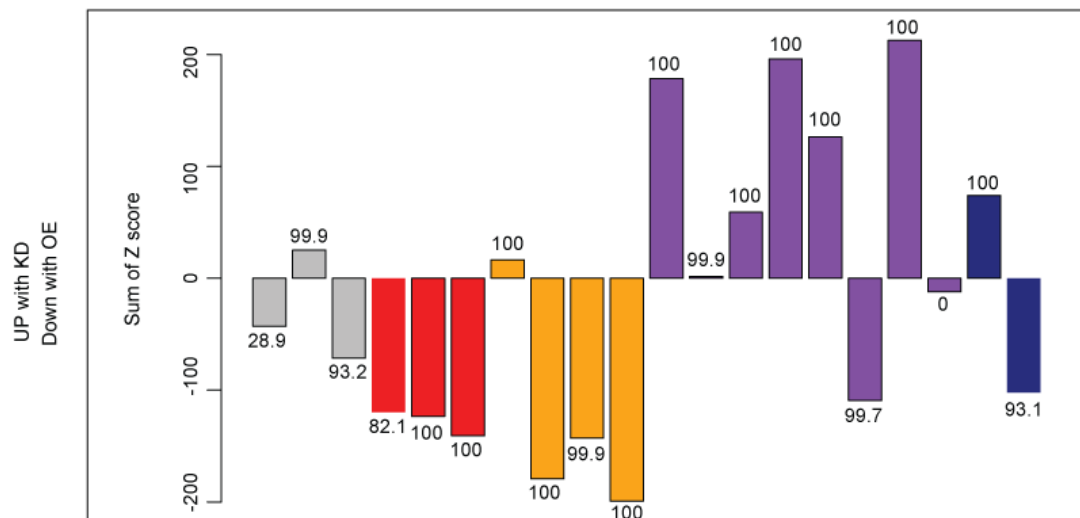


Figure 3.11. RNA-sequencing identifies upregulation of ribosome biogenesis and proteostasis pathways with shPLAG1 in Lin⁻CD34⁺.

(A) Experiment plan for sorting and transducing Lin⁻CD34⁺ cells from CB samples for shPLAG1 RNA-seq analysis. (B) Network map of gene-set enrichment analysis demonstrating significant (FDR <0.05) upregulation of genes associated with ribosome biogenesis and localized protein translation and UPR^{ER} (red box). Matured hematopoietic cell functions, and apoptotic pathways are also up regulated. (C) Network map of gene-sets reciprocally enriched between knockdown and over expression. Node color (blue) denotes down regulated gene-sets from PLAG1 over expression. Node border (red) denotes upregulated genes from PLAG1 knockdown.

A**B**

Hematopoietic sub-population

HSC CD34dim CD133+
 HSC CD34+ CD38-
 Common myeloid progenitor
 Megakaryocyte/Erythroid progenitor
 CFU_Megakaryocyte
 Megakaryocyte
 Erythroid_CD34+ CD71+ GlyA-
 Erythroid_CD34- CD71+ GlyA-
 Erythroid_CD34- CD71lo GlyA+
 Erythroid_CD34- CD71- GlyA+
 Basophils
 Eosinophil
 Granulocyte/Myeloid progenitors
 CFU_granulocyte
 Granulocyte (Neutrophilic Metamyelocyte)
 Granulocyte (Neutrophil)
 CFU_Monocyte
 Monocyte
 Plasmacytoid dendritic cells
 Myeloid dendritic cells

Color code



Figure 3.12. Differentially expressed genes from PLAG1^{OE} and PLAG1^{KD} are reciprocally enriched within DMAP.

(A) Genes from PLAG1^{OE} are preferentially enriched within stem and progenitor cell and erythroid/megakaryocyte compartments compared to differentiated myeloid cells. (B) Genes from PLAG1^{KD} are preferentially enriched within differentiated myeloid cells compared to stem cell and progenitor and erythroid/ megakaryocyte compartments. Numbers on top of each column represent permutations of occurrence of the respective hematopoietic phenotypes from PLAG1^{OE} and PLAG1^{KD} compared to random gene sets. Bottom panel denotes legend for color codes used for the columns in (A) and (B).

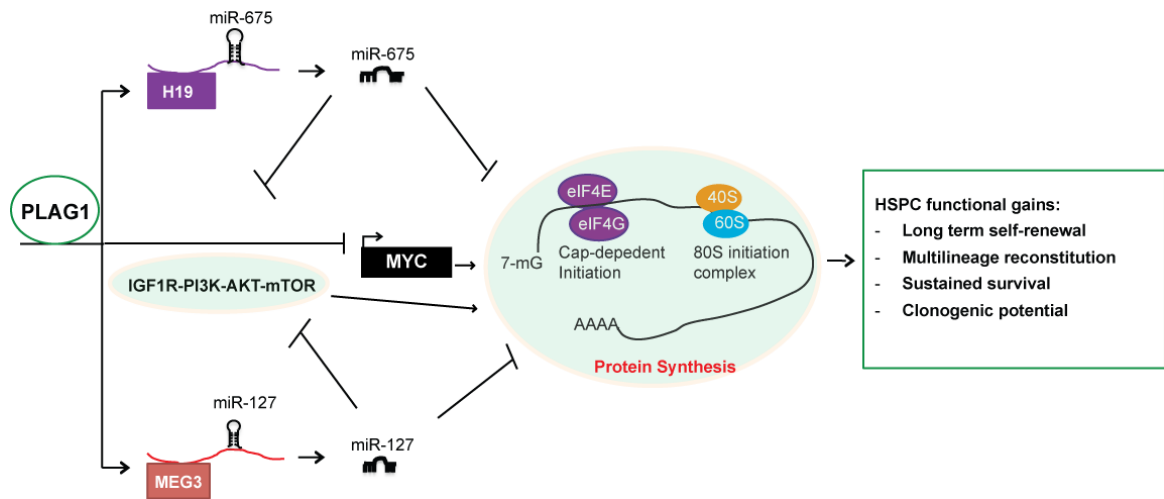
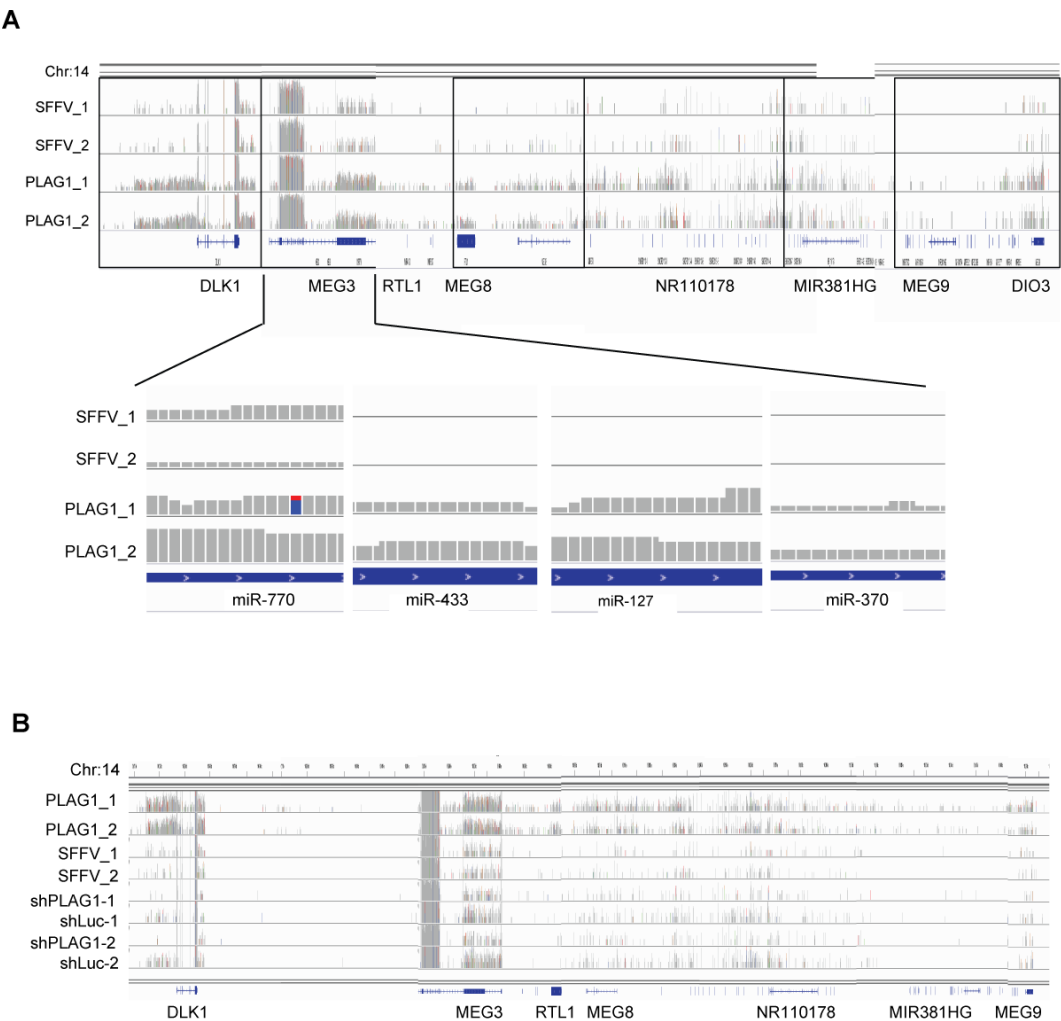
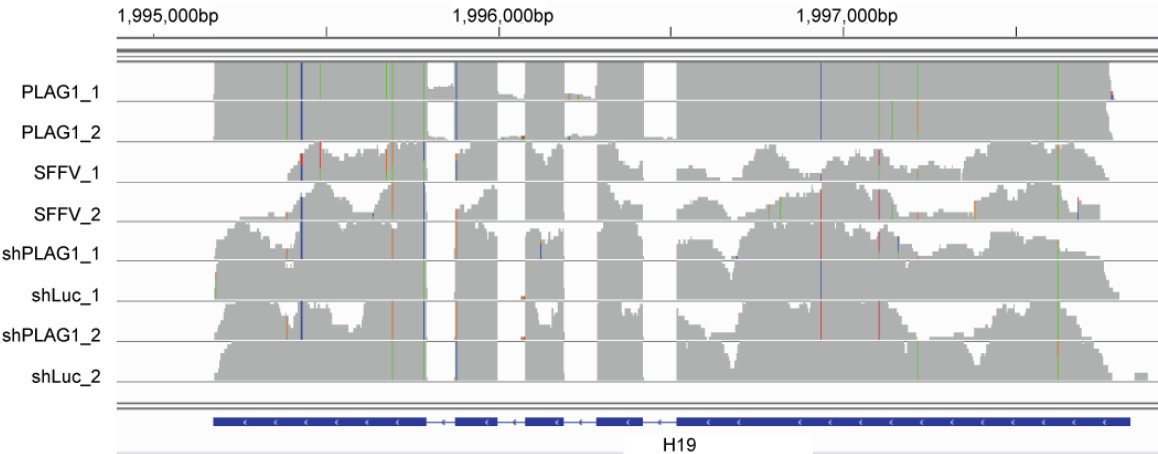


Figure 3.13. Working model of PLAG1-induced global repression of protein synthesis to impart HPSC functional gains. PLAG1 orchestrates biogenesis of miR-127 and miR-675 species from MEG3 and H19 lncRNAs respectively. These miRNA species repress ribosome biogenesis and protein synthesis machinery directly and/or through attenuation of mTOR signaling pathway. PLAG1 also attenuates transcription of the pan-ribosome biogenesis regulator MYC.



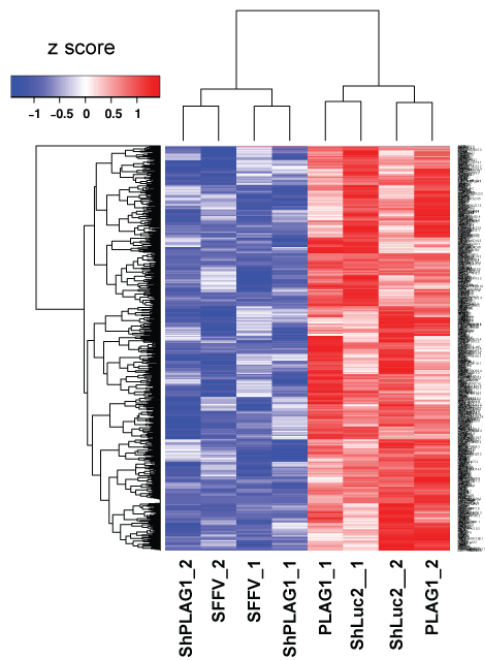
Supplemental figure 3.1. Integrated Genomic Viewer (IGV) of the DLK1-MEG3 locus from replicate over expression and knockdown RNA sequencing reads. (A)

Four miRNAs (miR-770, -433, -127 and -370) from MEG3 alleles that are significantly enriched from PLAG1 over expression compared to SFFV control. (B) MEG3 is depleted with shPLAG1 compared to shluc negative control and PLAG1 over expression.

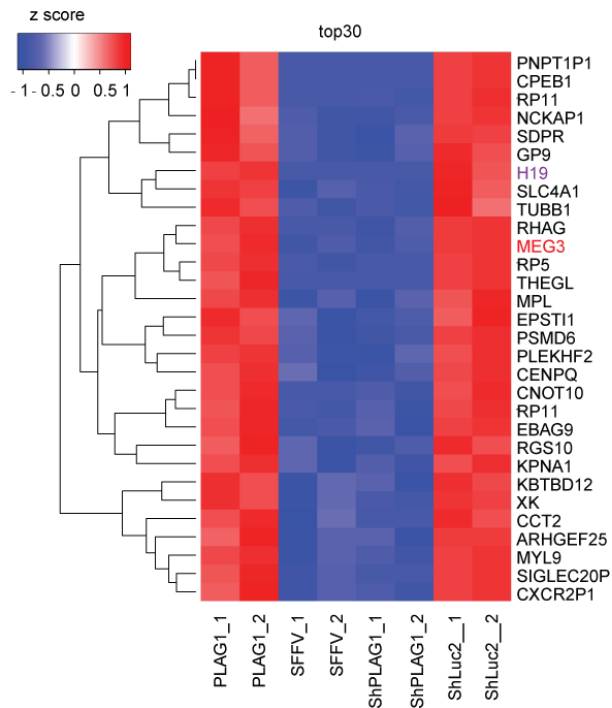


Supplemental figure 3.2. Integrated Genomic Viewer (IGV) of the H19 locus from replicate over expression and knockdown RNA sequencing reads. (A) H19 is strongly enriched with PLAG1 over expression compared to SFFV control. shPLAG1 depletes H19 compared to shLuc and PLAG1 over expression.

A



B



Supplemental figure 3.3. RNA sequencing from PLAG1^{KD} Lin⁻CD34⁺ identifies reversal of gene expression signature. (A) Heat map showing reversal of gene expression patterns between PLAG1 knockdown (shPLAG1) and over expression (PLAG1). Genes up regulated with overexpression are down regulated with knockdown. Normalized CPM count data of PLAG1 over expression and shPLAG1 were centered and scaled (z scores) separately. Expression scores of genes were clustered based on the directionality of expression between experimental groups as outlined in the material and methods section. (B) H19 and MEG3 are among the top 30 genes down regulated with PLAG1^{KD} and up regulated with PLAG1^{OE}.

CHAPTER 4

DISCUSSION

4.0 Preamble

The central themes of this thesis began with appreciation of the novel conserved functions of MSI2 in HSPCs. Subsequently *I proposed MSI2's upstream transcriptional regulation could also reveal other novel, previously uncharacterized HSC-enriched transcription factors (TFs)*. I believed that the transcriptional circuitry of MSI2 regulation could also offer opportunities for the discovery of candidate TFs with similar or superior functions in human HSPCs. To that end, I performed initial investigations to identify a conserved minimal promoter region bound by TFs upstream of the translation start site of human MSI2 (**Chapter 2**). *In silico* analysis of the minimal promoter region and a series of TF binding-site mutagenesis biochemical assays identified a TF duo of USF2 and PLAG1 as key regulators of MSI2 expression in our model K562 cell line and primary cord blood (CB) HSPCs. Through this approach I was able to identify PLAG1 as a candidate TF whose function in HSPCs was not previously reported. I coupled *in vitro* and xenotransplantation functional genomics assays with RNA-sequencing and downstream pathway analyses to characterize PLAG1's novel roles in these cells (**Chapter 3**). This study revealed PLAG1 as a master transcriptional orchestrator of an imprinted gene network including miRNAs that together promote a concerted repression of multiple proteostasis-impacting pathways to drive stemness in the blood system. In this section, I will discuss the significance, limitations and future directions of my findings. Implications of these findings to myeloid leukemia initiation will also be highlighted.

4.1 Scanning promoter sequences to discover novel transcription factors regulating HSPC functions

Traditionally, discovery of TF functions begins with identification of an active promoter region upstream of the transcription and /or translation start site of a target gene of interest. To that end, promoter clones are generated driving reporter luciferase or fluorescent gene expression followed by biochemical assays measuring capacity of the cloned sequence elements. Such approach has for example enabled the discovery of TFs regulating key hematopoietic stem and progenitor cell specific genes such as HOXB4 (Giannola et al., 2000; Zhu et al., 2003; Zhu et al., 2005). As discussed in **Chapter 2**, I followed a similar approach to identify TFs regulating MSI2 expression and function. Of note, the advent of the ENCODE project significantly facilitated the identifications of genomic regulatory regions such as promoters and enhancers for any gene of interest. I benefited immensely from the ENCODE genome browser in the process of identifying genomic marks of active promoter regions such as a CpG island and nuclease accessible sites within region upstream of MSI2. Subsequently, I identified ~ 3.2kb promoter region conserved between human and mouse MSI2 and cloned it upstream of firefly luciferase reporter gene. Subsequent truncations were done to identify a region faithfully representing the full-length promoter activity. Characteristically, the identified minimal promoter is TATA-less and CCAAT-less but GC-rich. To my knowledge no similar prior attempt was reported to identify functional regulatory region for MSI2. Understandably however, the reductionist and minimalistic nature of the approach could preclude the identification of potentially important regulatory promoter elements. Given MSI2's

conserved expression across different adult somatic stem cells, the approach I took could potentially be utilized in the discovery of TFs regulating its expression in tissue and cell-type specific manners. I envision that the identification of the minimal promoter for MSI2 could provide a stepping-stone for more detailed and comprehensive exploration of additional regulatory elements tailoring its expression in the stem cell context. For example, by tracking upstream and downstream H3K27 acetylation annotations of the MSI2 locus in the ENCODE data and subsequent linking of these regions to the identified minimal promoter reporter could facilitate the discovery of important enhancer elements (**Chapter 2**). Kawase et al for instance utilized similar approach for murine Msi1 to discover novel enhancer elements uniquely employed to tailor Msi1 expression in neural stem and progenitor cells (Kawase et al., 2011; Kawase et al., 2014). Once the full-fledged promoter reporter is constructed and validated, such a system could also be used for drug screening as a tool to discover novel therapeutics that would either enhance MSI2 protein expression and hence expand normal HSC fraction or decrease its expression and thus potentially impair LSCs in acute myeloid leukemia (AML). In either case, MSI2 promoter activity, as measured by the reporter activity, could provide a novel molecular marker to accurately quantify the amount of HSCs and LSCs in both clinical and experimental settings.

My study (**Chapter 2**) emphasized less on the comprehensive analysis of the promoter and enhancer regions of MSI2 but more in the identification of TFs functioning within the auspices of its minimal promoter. I believe that my identification and characterization of PLAG1 with its extremely important functions in the regulation and potential clinical

application of HSPCs achieved that goal very well. Of interest is also the elucidation of transcriptional co-regulation by USF2 and PLAG1 in maintaining the stem cell-restricted expression of MSI2. In this regard, our study further cements the principle that transcription in HSPCs is achieved not only by stem cell restricted TFs but also through ‘re-usage’ of TFs that play roles in other lineages or cell types of the blood system (Novershtern et al., 2011). Such ‘re-usage’ of TFs was comprehensively reported recently for vast arrays of TF networks across the hematopoietic hierarchy (Novershtern et al., 2011). In retrospect, earlier studies also hinted at such TF ‘re-usage’ with regards to HSPC specific expression of HOXB4 which was shown to occur via co-regulation by the TFs USF1/2 and NFY-A both having ubiquitous and stem/progenitor cell-enriched expression profiles respectively (Zhu et al., 2003; Zhu et al., 2005).

In the course of the study, I discovered functionally distinct smaller isoforms of PLAG1 with respect to MSI2 regulation in the context of hematopoietic cells (**Chapter 2**). Full-length PLAG1 is not abundantly expressed in the model K562 cell line and HSPCs suggesting its negligible relevance. Conversely, the small isoforms are abundantly expressed hinting that they may be the more functionally relevant of the isoform types in the hematopoietic system. Indeed, I found that introduction of only the smaller isoforms (in conjunction with ectopic USF2) enable upregulated expression of MSI2 in HSPCs. Of interest it's the fact that no prior attempt has been reported to demonstrate the functional relevance of these isoforms in any tissue type (Debiec-Rychter et al., 2001). Isoform specific expression and differential regulation of hematopoietic function has been observed for other HSPC TFs such as IKAROS (Beer et al., 2014; Klug et al., 1998) and

C/EBP (Bedi et al., 2009; Yamanaka et al., 1997). To date, there is no empirical evidence as to whether the PLAG1 isoforms are generated by alternative promoter usage or post-transcriptional splicing mechanisms. Of note, both mechanisms have been shown to be utilized in the generation of C/EBP isoforms in the hematopoietic system (Yamanaka et al., 1997). Given their potentially unique function in primitive hematopoietic cells, the elucidation of the mechanisms that ensure specific output of the small PLAG1 isoforms will be of interest in advancing our understanding of their functions in the hematopoietic system. The bulk of knowledge regarding PLAG1 is derived from studies on the full-length isoform in salivary gland adenomas (Van Dyck et al., 2007; Voz et al., 2000). Thus in addition, my findings pose questions for future reconsiderations of tissue and isoform-specific explorations of PLAG1.

4.2 Towards addressing the unmet demand for *ex vivo* expanded HSCs for regenerative therapeutics

To date, HSCs have been the sole approved and clinically utilized stem cell type to treat hematologic malignancies through bone marrow transplant (BMT) (Daley, 2012; Walasek et al., 2012). BMT has also been practiced to treat inadvertent high dose chemo and radiotherapy-induced bone marrow failure in breast cancer patients (McNiece et al., 2000; Vavrova et al., 1999). Such promising regenerative therapeutics are however critically impaired by the paucity of HLA-matched donor HSCs needed to ensure sustained recovery of the blood system and to avoid potential graft rejection - two common problems that need to be addressed for successful life-saving BMT. In lieu of allogeneic bone marrow (BM) or mobilized peripheral blood (PB), umbilical CB has become an

important source of HSCs because of the relaxed requirement for HLA-matching. However, there is only finite number of HSPCs in a unit of CB. *Ex vivo* expansion of allogeneic CB HSCs has been considered as a viable alternative but is challenging as culture conditions are inherently differentiation-promoting. To that end, discovery of novel HSC-self-renewal regulators could offer the harnessing of cellular pathways that would permit enhanced *ex vivo* HSC production. Recently, elegant studies have identified bioactive molecules for expanding CB HSCs (Fares et al., 2015; Lund et al., 2015). These include dmPGE2 (Goessling et al., 2011), SR1 (Boitano et al., 2010 Wagner et al., 2016), UM171 (Fares et al., 2014), LY2228820 (Ly)(Baudet et al., 2012) and VPA (Chaurasia et al., 2014). Ultimately, all of these molecules achieve expansion of CB HSC albeit at varying levels. Intriguingly, the mechanisms engaged also vary among the different bioactive molecules utilized for CB HSC expansion. For example, SR1 enforces HSC self-renewal (and expansion) through repression of the AHR signalling pathway. On the other hand, UM171 does so by directly blocking differentiation programs in an AHR-independent and still unknown molecular mechanism. Yet, both lead to extensive HSC expansion. These findings illustrate well that our integrated understanding of the elusive process of HSC self-renewal is still inadequate.

Attempts towards deciphering detailed molecular mechanisms regulating HSCs self-renewal could reveal important “missing links”. For instance, through integrated RNA-seq and CLIP-seq, we have demonstrated that MSI2 attenuates the AHR pathway through post-transcriptional repression of CYP1B1 (Rentas et al., 2016). Wojtowicz et al elucidated that miR-125a targets p38MAPK to enhance HSC self-renewal - the same

target identified through genetic screen by Baudet et al (Baudet et al., 2012; Wojtowicz et al., 2016). Therefore, critical is our understanding of how the different regulatory networks are orchestrated to enforce HSC long-term self-maintenance. One avenue to achieve this could be through, discovery of novel upstream master regulators of miRNAs, HDACs, RBPs, and other molecules with broad target specificity. This approach could help us develop a comprehensive understanding of different HSC self-renewal pathways amenable for *ex vivo* expansion strategies. In **Chapter 2**, I proposed to identify TFs that could not only regulate MSI2 transcription but also its downstream function - namely repression of CYP1B1. In this regard, I demonstrated master regulation of the AHR pathway through the combined effects of USF2 and PLAG1 while also offering the opportunity to fine-tune the expression of other potential downstream targets for the purpose of *ex vivo* expansion. Co-overexpression of USF2 and PLAG1 in CB CD34⁺ cells robustly increases TNCs and absolute number of CD34⁺ cells over 7 days of an *in vitro* culture period (Chapter 2). At the phenotypic level, this enhancement in CD34⁺ output is actually superior to what is achieved via the reported direct ectopic overexpression of MSI2 in the same cells (Rentas et al., 2016). The corollary of our finding is that MSI2 is part and parcel of an extensive network of HSC self-renewal machinery co-regulated by these two TFs. To that end, a number of experiments need to be performed to address outstanding questions. First, an *in vivo* repopulating assay of NSG mice to measure the *in vivo* capacity of CB CD34⁺ cells overexpressing USF2 and PLAG1 and having been propagated for short-term (3 days) and long-term (7-12 days) periods needs to be performed to validate expansion of functional HSC. An LDA assay in particular would

allow quantification of the effect of the USF2/PLAG1 duo on the frequency and number of HSCs maintained/produced. Moreover, serial transplantation assays would in combination reveal the effect of USF2/PLAG1 on the long-term self-propagation capacity of HSCs. Secondly, the use of high throughput next generation RNA-sequencing would provide an understanding of the comprehensive transcriptome changes imparted upon co-expression of USF2 and PLAG1. Bioinformatics pathway enrichment and gene ontology annotation analyses could then be applied in attempt to reveal the networks and mechanisms underlying the effects on HSC self-renewal. Beyond this, ChIP-sequencing for USF2 and PLAG1 could be incorporated and coupled with RNA-sequencing to identify direct targets within the global transcriptome profile. Cumulatively, I envision that such pathway analysis could integrate multiple downstream previously known and novel targets of USF2 and PLAG1 with implications for HSC *ex vivo* expansion purposes. For example, HOXB4 is known target of USF2 (Giannola et al., 2000; Zhu et al., 2003). Interaction of USF2 and HOXB4 is also known to co-regulate the hypoxia related gene HIF2alpha in a feed-forward mechanism (Pawlus et al., 2012) and HIF2alpha is relevant to maintaining long-term HSCs in the bone marrow niche (Fatrai et al., 2011). Taken together, bioinformatics integration of MSI2's findings from Rentas et al (2016) with other known and novel targets of USF2 could reveal network maps linking multiple HSC regulatory circuitries. By the same token, as discussed below, we have identified novel functions for PLAG1 as a master regulator of ribosome biogenesis and enforcer of a stem cell gene signature (**Chapter 3**). Lastly, lentivirally-mediated constitutive overexpression and stable genomic integration of TFs has known safety concerns (Neschadim et al.,

2007; Schambach et al., 2013; White et al., 2017). Therefore, identification of non-integrating viral (such as adeno-associated viruses, AAV)- or bioactive molecule-based strategies to induce the expression of PLAG1 and USF2 could be an important pre-clinical step to expanding HSCs *ex vivo*.

The bottleneck to most *ex vivo* expansion approaches is the spontaneous differentiation and commitment of HSCs to matured cells - a critical impediment to full-fledged clinical application of *ex vivo* expanded HSCs for regenerative therapeutic purposes. One approach has been the successful overexpression of HSC specific TFs which in the mouse system has had success, with a number of factors demonstrated to expand functional murine HSCs *ex vivo* and *in vivo*. Despite these findings, many of these factors elicit insignificant effects on the human HSC counterparts (Aguila et al., 2011; Antonchuk et al., 2002; Ohta et al., 2007; Watts et al., 2012). In this regard, our studies on PLAG1/USF2 (**Chapter 2**) and PLAG1 alone (**Chapter 3**) offer a practical option to expanding HSCs and enforcing a stem cell gene signature. Ectopic overexpression of PLAG1 expands HSCs with short term (3 days) *ex vivo* culture. However, long term *ex vivo* culture (7-12 days) leads to HSC exhaustion in culture (Appendix figure 1). It is certainly possible that a mid-term (4-5 days) *ex vivo* culture growth period and subsequent LDA assay could uncover an even greater PLAG1-induced number of HSCs as this is when we observed simultaneous peak CD34⁺ counts and stalled cell cycle progression *in vitro*. It is intriguing that extended long-term *ex vivo* culture with PLAG1 overexpression leads to loss of HSC fitness. A plausible explanation could lie within the IGF2/H19 imprinted gene cluster upregulated by PLAG1 overexpression. As discussed in

chapter 3, H19-derived miR-675 antagonizes IGF1-R (the receptor for the pro-proliferative cytokine IGF2) (Keniry et al., 2012). I presume that in short/mid-term *ex vivo* cultures the “antagonism and pro-survival principles” govern enhanced HSC expansion and survival. However, with long-term *ex vivo* culture the “pro-proliferative principles” may dominate leading to extensive proliferation and HSC differentiation to matured cells. Regardless, with optimized timing, PLAG1 overexpression is able to impart significant *ex vivo* expansion by targeting previously unknown regulators of HSC homeostasis in clinically relevant human HSCs.

Strikingly, PLAG1 overexpression transcriptome-wide profiling with short-term *ex vivo* culture revealed ribosome biogenesis as a novel, previously unknown “druggable” target amenable for HSC expansion. There are available candidate drugs inhibiting ribosome biogenesis for various applications (Bhat et al., 2015; Brighenti et al., 2015). I anticipate that rational selection and optimized use of such inhibitors dampening ribosome biogenesis, could offer a previously unexplored opportunity to expand HSCs *ex vivo*. Our study also revealed significant repression of IGF1R-PI3K-AKT-mTOR signaling pathway (Chapter 3). Interestingly, previous studies have suggested *ex vivo* expansion of HSC using rapamycin in combination or as an independent agent (Huang et al., 2012; Rohrabough et al., 2011). It could therefore be of interest to combine rapamycin with other optimized ribosome biogenesis inhibitors to determine the potential for their additive and/or synergistic effect on HSC expansion promotion.

HSC *ex vivo* expansion using miRNA overexpression has emerged as alternative post-transcriptional approach (Morhayim et al., 2016; Wojtowicz et al., 2016). Our study

identified robust miR-675 and miR-127 biogenesis with PLAG1 over expression and in the case of mir-675 the potential for repression of PI3K signaling repression due in part to this miRNA is apparent from its known capacity to target Igf1r a receptor through which PI3K is initiated. Through detailed bioinformatics analysis we have performed, miR-675 and miR-127 also target many transcripts coding for proteins essential for ribosome biogenesis (**Chapter 3**). An important validation of the functional contribution of these two miRNAs to PLAG1's effects that remains to be performed is the co-overexpression of both miRNAs in CB CD34⁺ and the determination of the extent to which this can achieve *ex vivo* expansion at the level induced by ectopic PLAG1 overexpression. Overall, through overexpression of PLAG1 in CB CD34⁺ cells, we have uncovered the induction of multiple miRNAs capable of post-transcriptionally attenuating proliferative signaling.

4.3 Orchestration of imprinted gene and proteostasis-regulating networks in HSCs

The concurrent upregulation of multiple imprinted genes with PLAG1 overexpression in CB CD34⁺ cells was a striking finding. Among these, the H19-IGF2 (chr11) and MEG3-DLK1 (chr14) loci are the most significantly upregulated. Noteworthy is the fact that in adenomas of the salivary gland IGF-2 is a genuine target of PLAG1 (Akhtar et al., 2012; Voz et al., 2000). Moreover, Declercq et al observed upregulation of H19, Dlk1 and Gtl2 (murine ortholog of MEG3) in a transgenic mouse model constitutively expressing PLAG1 (Declercq et al., 2005). In this regard our observations validate these previous findings and support that PLAG1 also regulates imprinted genes in human CD34⁺ cells. However, unlike the conventional studies employing the full-length isoform, we used the

PLAG1-S shorter isoform abundantly expressed in hematopoietic cells (Chapter 2). Out of curiosity, ectopic expression was performed for the full-length PLAG1 in CD34⁺ cells to explore its relevance for progenitor activity and inducing expression of H19 and MEG3. Interestingly, the full-length isoform still induces both transcripts albeit only 1.5- and 5.5-fold levels for MEG3 and H19 respectively compared to 27.5- and 1051-fold respective inductions by the shorter-isoforms (Appendix figure 2). However concurrent progenitor activity functional assays demonstrated insignificant effects of the full-length isoform on CD34⁺ proliferation and progenitor self-renewal. Strikingly the full-length isoform dramatically induced sustained apoptosis *in vitro* in contrast to the small isoforms (Appendix figure 2). To my knowledge, this study is the first of its kind to utilize the shorter isoform and identify the same target gene expression. The corollary of these results is that the N-terminal domain and the Zn fingers F1 and F2 present only in the full-length isoform are irrelevant in dictating its target specificity and function, contrary to previous reports by Hensen et al (Hensen et al., 2002).

By definition, imprinted gene clusters are reciprocally, monoallelically expressed based on the parent of origin. In the case of the H19-IGF2 cluster for example the paternal allele expresses IGF2 while the H19 is transcribed from the maternal allele (Hark et al., 2000; Holmgren et al., 2001; Kurukuti et al., 2006). Nonetheless, the synchronized expression of both H19 and IGF2 in CD34⁺ cells with PLAG1 overexpression does not definitively inform on their parent allele of origin. Normally, CTCF binding of the imprinted control region (also called the differentially methylated region) at the maternal locus shields transactivating enhancers responsible for expression of IGF2 (Hark et al., 2000;

Holmgren et al., 2001; Kurukuti et al., 2006). Conversely, DNMT3-mediated methylation of the DMR in the paternal locus prevents expression of H19. Future work is required to decipher the precise molecular mechanism through which PLAG1 regulates H19 and IGF2 transcription and whether PLAG1 is perturbing the gene dosage equilibrium by indiscriminately modulating CTCF binding in the maternal locus and/ or the status of DNA methylation at the DMRs. Given the complexity of regulation of imprinting it is indeed very likely that PLAG1 needs to directly or indirectly interact with other *cis*- and *trans*- acting regulatory factors to alter this strict control. Therefore, global proteome profiling approaches using mass spectrometry could elucidate a PLAG1 interactome that might provide greater insights into its mechanism of action with respect to gene expression alterations. It is important to note that the IGF2 transcript level was not reduced in CD34⁺ cells with complementary PLAG1 knockdown experiments (Chapter 3). These results are consistent with the concept that physiological PLAG1 imprinting regulation is restricted to the H19 allele only and that emphasis should be given to proximal or distal epigenetic factors regulating H19 imprinting.

In terms of other dysregulated imprinted loci it has been described that an IG-DMR regulates the asymmetric expression of the maternal and paternal alleles in the Dlk1-Gtl2 locus in mice (Lin et al., 2003). Unlike the DMR for the H19-IGF2 locus, the exact mechanism of control by IG-DMR at the Dlk1-Gtl2 locus is not known. Nonetheless, Lin et al postulated that maternally coded non-coding RNAs such as miRNAs and snoRNAs from the Gtl2 locus could function as *cis*-acting negative regulators of protein coding counterparts from the paternal allele (Lin et al., 2003). A similar mechanism of regulation

was reported for the *Air/Igf2r* cluster of imprinted genes (Sleutels et al., 2002). In our study using CD34⁺ cells, PLAG1 overexpression induces both DLK1 and MEG3. However, by the same token as with the IGF2, RNA sequencing with complementary PLAG1 knock down represses MEG3 but not DLK1. Taken together, the maternal alleles seem to be the target of PLAG1 transactivation. It will now be interesting to explore whether PLAG1 works through a mechanism reminiscent of that utilized by the *Air/Igf2r* cluster. To this point, the Gtl2 locus consists of the largest mega cluster of miRNAs and snoRNAs in the entire genome (Qian et al., 2016; Seitz et al., 2004). Of note, our library preparation and subsequent RNA-sequencing protocol was not aimed at enriching for smaller RNA molecules such as miRNAs as it was meant to capture larger mRNA molecules for transcriptome analysis. Nonetheless, we did capture reliable sequence information validating the expression of 4 miRNAs from the MEG3 locus (**Chapter 3**). In this regard, it would be revealing to perform dedicated small RNA-seq to annotate the entire map of miRNAs generated from the PLAG1-enforced expression of the MEG3 lncRNA. In future, it remains to be determined whether the non-coding RNAs from the maternal locus are reciprocally repressing transcription and expression of the paternal counterparts in the DLK1 locus. In summary, the exact mechanism through which PLAG1 exerts control on maternally expressed imprinted genes remains to be deciphered. Among their diverse functions, lncRNAs have been shown to recruit TFs to active promoter regions acting as molecular guides and scaffolds (Mercer and Mattick, 2013; Wang and Chang, 2011). It will now be interesting to explore whether similar mechanisms are employed by H19 and MEG3 lncRNAs to recruit PLAG1 and other

transcriptional co-activators to their respective regulatory regions in a possible positive feed-forward mechanism.

The PLAG family of TFs also includes PLAGL-1 and PLAGL-2. While the reported DNA binding specificity PLAG1 (full-length) and PLAGL-2 are very similar, that of PLAGL-1 is slightly different suggesting some developmentally regulated functional redundancy within the family (Hensen et al., 2002). Our RNA sequencing transcriptome analysis with the short isoform of PLAG1 exposed another strong similarity to the network of imprinted genes regulated by murine Zac1 (ortholog of PLAGL-1) (Varrault et al., 2006) however it remains to be determined whether PLAGL-1 functions similarly in the context of human CD34⁺ cells. In this regard, our evidences envisage PLAG1 to be part of the network of non-imprinted genes regulating genomic imprinting in the context of hematopoietic cells (Al Adhami et al., 2015).

Another key finding of my study is the global dampening of protein homeostasis machinery including ribosome biogenesis and UPR pathways (**Chapter 3**). Contrary to what has been previously thought, compelling evidence has emerged indicating the uniquely highly choreographed process of global protein synthesis in various adult stem cells to enforces long-term self-maintenance (Blanco et al., 2016; Cai et al., 2015; Sanchez et al., 2016; Signer et al., 2014; Zismanov et al., 2016). However, it is not clear as to how ribosome biogenesis is orchestrated in adult stem cells in general and HSCs in particular. Critical is also identification of transcriptional effectors mediating reduced ribosome biogenesis and protein synthesis in response to intrinsic and extrinsic cues. In this regard, our discovery uncovered a master regulation of protein synthesis occurring

through multiple arms of control in HSPCs. We present novel evidence for post-transcriptional attenuation of ribosomal genes via H19 and MEG3 imprinted lncRNA-derived miR-675 and miR-127 respectively. Through repression of MYC, we also reported the likely transcriptional obstruction of ribosomal gene expression. However, it remains to be elucidated as to how MYC is repressed with PLAG1 ectopic expression in CD34⁺ HSPCs.

The functional relevance of H19 and Gtl2 imprinted genes in murine HSC self-renewal was presented by two independent studies from Linheng Li's laboratory (Qian et al., 2016; Venkatraman et al., 2013). However, our study presents single TF-mediated co-regulation of both H19 and MEG3 in human HSCs. The H19 lncRNA-derived miR-675 is known to repress the pro-proliferative effect of IGF1-R signaling (Keniry et al., 2012; Venkatraman et al., 2013). IGF1-R is membrane tyrosine kinase surface receptor for cytokine-mediated activation of PI3K-AKT-mTOR signaling (Laplane and Sabatini, 2009). In this regard, it could be expected that the concomitant up regulation of H19 in CD34⁺ cells would nullify the immediate activation of mTOR signaling. Indeed, we reported repression of components of the mTOR signaling pathway upon PLAG1 overexpression. Additionally, miRNAs from the MEG3 locus also repress mTOR signaling (Qian et al., 2016). Collectively, attenuated mTOR signaling provides another layer of repression of the protein synthesis pathway

A recent report indicated that activated UPR pathway is detrimental to HSC homeostasis. In this regard our study presented possible evidence in support of this through ectopic expression of the PLAG1 TF wherein we also see pro-UPR components being repressed.

Taken together, we presented evidence in support of a multi-layered and synchronized regulation of protein homeostasis in HSCs through ectopic expression of a single TF i.e. PLAG1 (Figure 4.1).

4.4 Aberrations in expression of the PLAG family: new insights for myeloid leukemogenesis

Homeostatic signaling pathways regulating HSCs dictate decisions between sustained self-renewal to maintain broad developmental potential and ability to differentiate to each respective lineage to meet the body's needs (Luis et al., 2012). Molecular determinants of HSC self-renewal have been shown to be hijacked by transformation events leading to leukemia (Eppert et al., 2011; Kennedy and Barabe, 2008). Similar to normal hematopoiesis, leukemias also show hierarchical organization whereby the excessively self-renewing leukemic stem cells (LSCs) reside at the apex producing progenitors that have potent proliferative potential (Bonnet and Dick, 1997; Eppert et al., 2011; Hope et al., 2004; Lapidot et al., 1994). Unlike healthy progenitor cells that differentiate into matured and functionally differentiated blood cells, progenitor cells in leukemia have a blockage in differentiation and are thus capable of only producing immature blood cells (blasts). Importantly, steps taken to understand the cell intrinsic regulators of LSCs could also provide an understanding of their role in normal HSC homeostasis and vice versa. Recent genome-scale exome sequencing studies have revealed somatic recurring silent mutations in non-leukemic HSCs and progenitors as pre-leukemic events (Corces-Zimmerman et al., 2014; Jan et al., 2012; Shlush et al., 2014). These discoveries identify pre-LSC mutations as the evolutionary predecessors of eventual leukemia development

(Shlush et al., 2017). Given their importance in disease establishment and progression, further identification and characterization of novel pre-LSC and frank LSC mutational events could be important in facilitating improved treatment regimens.

In mouse AMLs initiated by the oncogenic translocations CALM-AF10 and CBFb-MYH11 retroviral mutagenesis identified as co-operative events the elevation of PLAG1 and PLAGL-2 (Castilla et al., 2004; Caudell et al., 2010; Landrette et al., 2005). It is noteworthy that Landrette et al analyzed AML patient samples for PLAG1 and PLAGL-2 expression and noted that PLAGL-2 is overexpressed in a larger percentage of the samples than PLAG1. Given their presumed functional redundancy, PLAGL-2 could function as the dominant of the two in these AML samples. Interestingly, in normal hematopoiesis, PLAGL-2 showed no appreciable expression in the stem and progenitor compartment thereby ruling out its role in regulating normal HSC function and differentiating it from the highly stem cell specific PLAG1 (Bagger et al., 2016; Jojic et al., 2013a). It is therefore logical to presume that while PLAG1 regulates normal HSCs, PLAGL-2 could be largely responsible for maintaining LSCs in certain subtypes of leukemia (Figure 4.2).

It is intriguing also, that among the PLAG family members, only PLAGL-1 positively correlates with disease progression from the chronic phase (CP) to blast crisis (BC) stage of CML induced by BCR/ABL (Philadelphia (Ph) chromosome) translocation. This suggests a developmentally regulated switch between PLAG1 and PLAGL-1 in regulating normal versus CML stem cells (Figure 4.2). Interestingly, Oncomine database analysis revealed a significant upregulation of PLAGL-1 with severity of disease progression of

CML (oncomine.org). At the same time, differential expression analysis between LSC⁺ and LSC⁻ fractions from a total of 23 AML patients indicated significant up regulation within cytogenetically Ph⁺ LSC⁺ fraction of AML samples (Eppert et al., 2011). In order to further characterize PLAGL-1 for its functional relevance in LSCs, based on published protocols I generated mouse models of chronic and blast crisis phases of CML (Ashton et al., 2012; Mayotte et al., 2002; Neering et al., 2007)(Appendix figure 3A and B). These models recapitulate the cellular and histo-pathological features observed in patients (Appendix figure 3C-F). Interestingly, Plagl-1 expression was significantly enriched within CML blast crisis LSCs (Appendix figure 3G-K). Taken together, in the context of Ph⁺ CML, PLAGL-1 seems to be the dominant PLAG family member- adding complexity to the reconciliation effort within “the family feud”. Intriguingly, these observations are contrary to the popularly appreciated tumor suppressor function of PLAGL-1 in other cell contexts (Abdollahi, 2007; Valleley et al., 2007). PLAGL-1 is localized on human chromosome region 6q24-25 that is frequently deleted in many types of human cancers. Of interest, PLAGL-1 is highly expressed in fetal HSCs compared to adult HSCs (Bagger et al., 2016; Jojic et al., 2013a). Hence, it is tempting to hypothesize that its expression in LSCs implicates PLAGL-1 as an oncofetal gene. There may also be LSC-specific functions induced by PLAGL-1 and PLAGL-2 that are dependent on cooperating oncogenic translocations. Correlative expression studies from large cohorts of AML/CML samples coupled with functional characterization using mouse models could provide better insights into both of these outstanding questions.

Regardless, the cellular and molecular mechanisms reported for PLAG1 in normal HSCs could also open avenues for understanding cellular and molecular events potentially dysregulated in the transformation process leading to leukemia where PLAGL-1/PLAGL-2 is also activated. For example, Cai et al demonstrated that attenuated ribosome biogenesis due to Runx1 mutation confers a pre-leukemic phenotype to normal HSCs including elevating their long-term survival and enhancing their fitness under chemo and genotoxic stress (Cai et al., 2015). RUNX1 mutations are recurrent in myeloid leukemia and leukemogenic fusions involving the principal RUNX1 cofactor, CBF β , such as CBF β -SMMHC (also called inversion16) negatively regulate RUNX-dependent ribosomal gene expression (Cordonnier et al., 2017; Lopez-Camacho et al., 2014). In this context, PLAGL-2 could potentially cooperate with inversion16 (discussed above, Castilla et al and others) and repress ribosome biogenesis and proteostasis pathways. Our data also indicated that repression of protein synthesis increases long-term survival of normal HSCs. It remains to be seen if ectopic PLAGL-1/-2 expression induces pre-LSC or LSC phenotypes on their own or in cooperation with other leukemogenic oncogenes through repression of ribosome biogenesis. Along these lines it would be interesting to explore whether repressed ribosome biogenesis potentially enforced via the members of PLAG family offers LSCs resistance to chemotherapeutic insults as has been shown previously for Runx1 repression (Cai et al., 2015). Such observations could pose challenges to conventional leukemia therapeutic approaches aimed at reigning in the bulk highly proliferative/metabolically active leukemia cells by targeting ribosome biogenesis or pathways positively regulating protein synthesis such as mTOR (Bhat et al., 2015;

Janes et al., 2010; Kentsis et al., 2004). Indeed, previous reports hinted that negatively modulating mTOR signaling using for example miR-126 could predispose HSCs to leukemic phenotypes (Lechman et al., 2016; Lechman et al., 2012).

We presented H19 and MEG3 lncRNA-derived miRNAs mediate the global repression of proteostasis pathways to enforce normal HSC homeostasis. I envision similar RNA sequencing experiments on LSCs from AML samples to determine conserved expression of H19 and MEG3 lncRNAs and the respective miRNAs derived thereof as a potential mechanism for self-maintenance. Prior studies have highlighted the role of H19 in the oncogenesis of BCR/ABL-induced myeloid leukemia (Guo et al., 2014). Other studies have shown that H19 lncRNA sequesters tumor suppressor let-7 microRNA family to enhance tumorigenesis (Kallen et al., 2013). In addition, a positive correlation has been reported between the RNA binding protein Lin28B (a negative regulator of let-7 miRNA) and H19 in paediatric myeloid leukemia (Helsmoortel et al., 2016). H19-derived miR-675 has been reported as an oncogene or negative regulator of tumor suppressor genes in breast cancer (Vennin et al., 2015), hepatocarcinoma (Hernandez et al., 2013), gastric cancer (Zhuang et al., 2014) and colorectal cancer (Tsang et al., 2010). However, it is not known whether H19-derived miR-675 upregulation is involved in the initiation and/or progression of myeloid leukemia. IGF1-R is genuine target of miR-675 (Keniry et al., 2012) and it is established that IGF2 and IGF1 drive leukemic progenitor proliferation through autocrine and paracrine IGF1-R activated PI3K/AKT/mTOR signalling (Chapuis et al., 2010; Doepfner et al., 2007; Pollak, 2012). IGF1-R activity in leukemic blasts suggests absence of its post-transcriptional repression by miR-675. Also, the biogenesis

of miR-675 in leukemic blasts could be negatively modulated by a mechanism that is yet to be identified. The expression and activity of IGF1-R in prospectively isolated LSC fractions is not clearly known however I would predict that if PLAG family members are elevated preferentially in LSC that enhanced miR-675 could follow and thus post-transcriptionally attenuate IGF1-R expression to enhance LSC self-renewal.

To date, extensive functional studies have not been reported for the MEG3 lncRNA or constituent miRNAs in the etiology of AML. Studies on acute promyelocytic leukemia (APL) caused by the PML-RAR α fusion have however demonstrated loss of imprinting due to hypermethylation at the MEG3-DMR (Manodoro et al., 2014). Other studies including one by Manodoro et al subsequently showed amplification of MEG3-driven miRNAs including miR-127 (Jongen-Lavrencic et al., 2008; Manodoro et al., 2014; Valleron et al., 2012). Future functional studies could validate if the PML-RAR α translocation induces a repressed protein synthesis pathway to sustain LSC self-renewal. Given the above findings it would be intriguing to see if PLAGL-1 and/or PLAGL-2 could induce MEG3-derived miRNAs including miR-127 in the context of PML-RAR α leukemic fusion. Of interest, loss of imprinting as a result of hypermethylation of distal CpG rich regions in the DLK1 locus has been reported in more than 76% of AML samples overexpressing DLK1 protein (Khoury et al., 2010). However, Khoury et al noted that MEG3 was inversely expressed as monoallelic suggesting a different mechanism of imprinting control in leukemia. At the same time Benetatos et al analyzed the promoter methylation status of MEG3 in AML and MDS patient samples and found that MEG3 promoter hypermethylation occurred in 34.9% MDS and 47.6% of

AML samples regardless of karyotype, subtype or other confounding factors (Benetatos et al., 2010). This study however falls short of correlating the promoter hypermethylation with MEG3 expression levels or that of their embedded miRNAs. It is also not known if MEG3 promoter hypermethylation is associated with loss of imprinting as implied by Khoury et al and Manodoro et al.

Taken together, ultimate targeting of the Achilles heel of leukemia requires an integrated understanding of master regulators of normal HSC homeostasis that are hijacked by LSCs. To that end future efforts should be directed towards deciphering the effect of PLAGL-1/-2 modulations on pre-leukemic HSC functions with and without cooperating oncogenes/translocations.

4.5 Concluding remarks

In this thesis, I have attempted to shed light on the mechanism of regulation of MSI2 in the context of HSPCs. I presented the first attempt to identify a functional minimal promoter region amenable for the discovery of TFs binding and physiologically regulating the expression of MSI2. Future works should focus on further comprehensive characterization of other regulatory distal and proximal genomic sequences such as enhancers. Such an approach will potentially facilitate the utilization of an MSI2 reporter as a biomarker for the prospective isolation of HSCs and LSCs. I believe that the discovery of PLAG1 and USF2 as cooperative regulators of MSI2 enriches our understanding of transcriptional networks in the context of HSPCs. Importantly, the observation that HSPC specific expression of MSI2 is the outcome of a ubiquitous (USF2) and stem cell specific (PLAG1) TF solidifies the long-held TF “re-usage”

principle. The discovery of smaller isoforms of PLAG1 functionally active in HSPCs now provide the impetus to understand their functions in other somatic stem cells and in hematopoietic malignancies.

The identification of PLAG1 as important robust enhancer of *ex vivo* expansion of HSCs paves the way for future discovery of molecules capable of ameliorating its expression for the same purpose. I strongly benefited from RNA sequencing for acquiring the understanding of PLAG1 as a synchronized master regulator of several important biological processes: gene imprinting, miRNA biogenesis and ribosome biogenesis. Recent high profile reports from the Li laboratory presented H19 and MEG3 imprinted loci as important regulators of murine HSCs. My study validates those findings in clinically relevant human HSCs. Importantly, I discovered master regulation of both loci by a single TF, PLAG1. I believe that the discovery of attenuated ribosome biogenesis to enhance long-term HSC self-maintenance makes the pathway the “low hanging fruit” for future targeting in *ex vivo* expansion strategies.

Lastly, the molecular mechanism for PLAG1 in normal HSCs could offer important insight into the understanding of leukemic initiation and recurrence. To that end, future work should focus on detailed and careful functional characterization of other family members known to be targeted by leukemic transformations: PLAGL-1 and PLAGL-2.



Figure 4.1. Model of PLAG1-orchestrated synchronized repression of ribosome biogenesis and protein synthesis pathways. PLAG1 induces H19 and MEG3-mediated biogenesis of miR-675 and miR-127 species respectively. miR-675 and miR-127 globally represses ribosomal proteins and components of protein synthesis machinery including mTOR signaling pathway. PLAG1 also attenuates the transcription of the pan-ribosome biogenesis transcriptional regulator MYC.

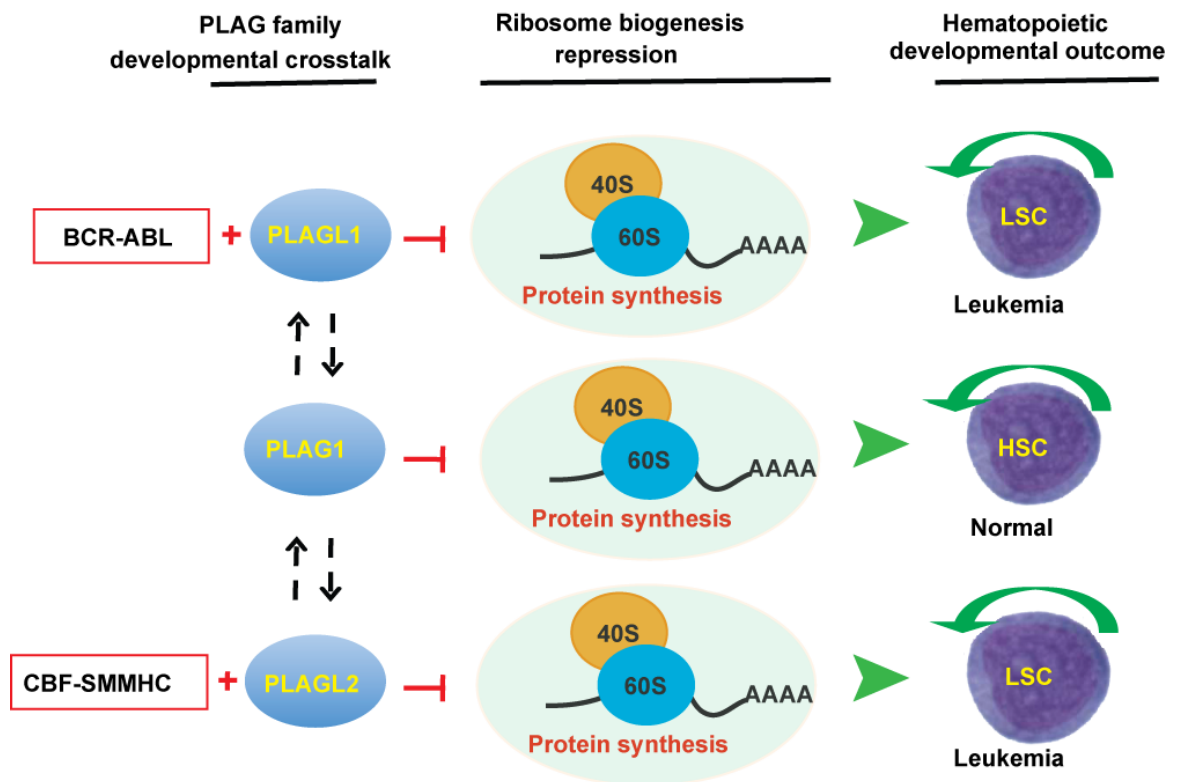
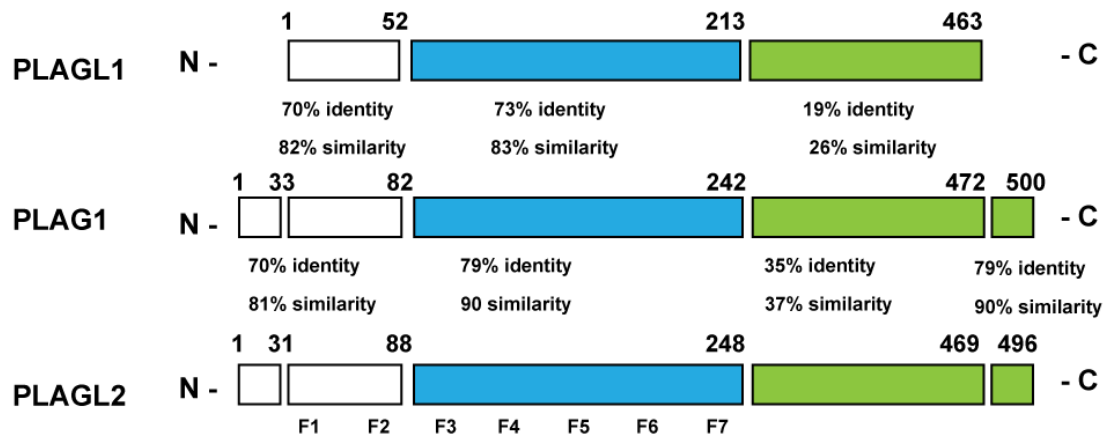


Figure 4.2. Postulated model of developmental crosstalk between PLAG family members in normal hematopoiesis and myeloid leukemogenesis. (Top panel) Primary sequence comparison of the PLAG family Zinc finger proteins showing transactivation C-terminal domain (green) and Zinc fingers (F1-F7) DNA binding N-terminal (white and blue) domain. (Adapted from Voz et al 1998). (Bottom panel) Attenuation of protein synthesis by PLAG1 enhances self-renewal capacity of normal HSCs. I postulate PLAGL-1 and PLAGL-2 repress global protein synthesis in cooperation with oncogenic translocations to offer unlimited self-maintenance capacity to leukemic stem cells (LSCs).

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APPENDIX I: LIST OF SCIENTIFIC PUBLICATIONS AND PATENTS**PUBLISHED REFREED PAPERS**

Rentas S., Holzapfel NT*, **Belew MS***, Pratt G., Voisin V., Wilhelm BT, Bader GT., Yeo GW, Hope KJ. Musashi-2 Attenuates AHR Signaling to Expand Human Hematopoietic Stem Cells. **Nature**. April 28, 2016, 532, 508–511, doi:10.1038/nature17665. Copyright© Nature Publishing Group. **Authors contributed equally*

MANUSCRIPT UNDER PEER REVIEW

Muluken S. Belew, Stefan Rentas and Kristin J. Hope. PLAG1 and USF2 Co-regulate Expression of MSI2 in Human Hematopoietic Stem and Progenitor Cells. *Currently in revision for publication at the journal **Stem Cell Reports***

MANUSCRIPT IN PREPARATION FOR SUBMISSION

Muluken S. Belew, Stefan Rentas, Veronique Voisin, Gary D. Bader and Kristin J. Hope. PLAG1 dampens proteostasis pathways to enforce primitive hematopoietic cell functions. *Currently in preparation for submission at the journal **Cell Stem Cell***

SUBMITTED PATENT PENDING

Belew MS and Hope KJ: Methods and compositions for expansion of hematopoietic stem and/or progenitor cells. Filed July 7, 2017; US62/529,694

APPENDIX II: METHODS FOR CML MOUSE MODEL DEVELOPMENT

Modeling chronic phase and blast crisis stage of chronic myeloid leukemia

CML progresses from benign CP to a more aggressive and fatal BC stage. The stepwise evolution of the disease makes it ideal model to study cooperating genetic lesions such as overexpression of oncogenes or down regulation of tumor suppressors. Identification of such genetic lesions offers opportunities for targeted molecular therapeutics. BCR/ABL translocation (Philadelphia chromosome) is the primary hit and founding mutation initiating the CP(Shtivelman et al., 1985). NUP98-HOXA9 fusion is known cooperating mutations driving BC progression. Subsequently, mouse models of CP and BC will help decipher the underlying molecular mechanisms in the kinetics of CML. To that end, I synthesized and adapted previous protocols by Mayotte et al and Neering et al for the transduction and transplantation of Lin⁻Sca-1⁺c-Kit⁺ (LSK) cells to generate the CP and BC stages of CML(Mayotte et al., 2002; Neering et al., 2007).

Generation of Ecotropic GP+E-86 feeder cell lines

Respective Amphotropic VSV-G pseudotyped retroviruses were generated by transfecting MSCV-BCR/ABL-GFP or MSCV-Nup98/HoxA9-BFP vectors into 293GPG cell line using Lipofectamine reagent following manufacturer's protocol(Ory et al., 1996). Similarly, vector control MSCV-GFP-only and MSCV-BFP-only retroviruses were generated using similar protocol. Retrovirus was harvested after 5 days of transfection, filtered through 0.2 μ m low-protein binding filter and stored at -80°C until further use. Maintenance and transfection growth media supplements for 293-GPG cells were performed as per Ory et al (1996). GP+E-86 cells were transduced with BCR/ABL-GFP

or Nup98/HoxA9-BFP retroviruses. Concurrently, GP+E-86 cells were transduced with GFP or BFP retroviruses for vector only control experiments. Ecotropic viruses producing GP+E-86 cells were maintained using previous protocol (Hope et al., 2010a). MSCV vectors were kind gift from Dr. Guy Sauvageau.

LSK transduction by co-culturing with GP+E-86 feeder cells

MSCV-BCR/ABL-GFP and MSCV-BFP-only Ecotropic virus producing GP+E-86 cells were mixed at 1:1 ratio and plated 24 hours before transduction in 24-well tissue culture format. Concomitantly, MSCV-BCR/ABL-GFP and MSCV-Nup98/HoxA9-BFP Ecotropic virus producing GP+E-86 cells were mixed at 1:1 ratio and plated in the same format. Similarly, as negative control, MSCV-BFP-only and MSCV-GFP-only Ecotropic virus producing GP+E-86 cells were mixed at 1:1 ratio and plated in the same format. Next, on the day of transduction, Lin⁻Sca-1⁺c-Kit⁺ (LSK) cells were co-cultured with the respective Ecotropic virus producing GP+E-86 lines for CP and BC modeling. For CP modeling, LSK cells were co-cultured with BCR-ABL-GFP and BFP-only GP+E-86 mixed culture. For BC modeling, LSKs were co-cultured with BCR-ABL-GFP and Nup98-HoxA9-BFP GP+E-86 mixed culture. As negative control, LSK cells were co-cultured with GFP- and BFP-only GP+E-86 mixed culture. 72 hours later, LSK cells were gently washed off the feeder layer with PBS. Cells were resuspended in 150 μ l DMEM 0.5% FBS.

LSK transplantation and analysis of leukemic mice

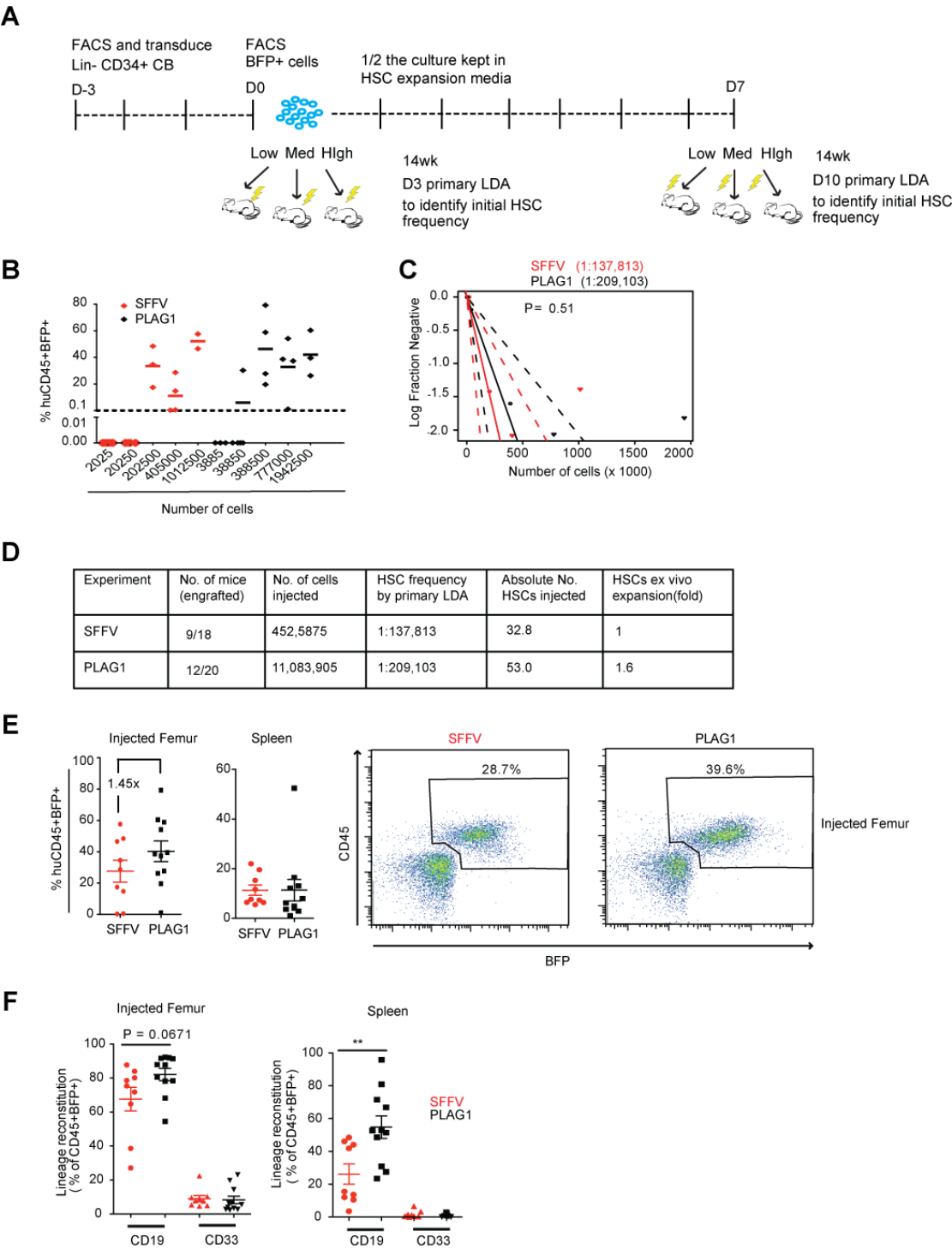
A day before transplantation, recipient C57B6/SJL (Ly5.1) mice were irradiated lethally (1100 cGy) or sub-lethally (580cGy) for CP and BC respectively. Negative control mice

were also lethally irradiated. Subsequently, transduced LSK cells from the respective co-cultures were injected into the lateral tail vein. Mice were considered moribund when they manifest ruffled hair, lethargy and inactivity with squinted eyes among other symptoms for both CP and BC stages. Peripheral blood was drawn from tail vein before the mice were killed. Bone marrow (BM) was isolated from tibia, femur and iliac crest by crushing on mortar and pestle. Spleen size was measured by metric ruler before pulverization for single cells suspension. Giemsa-Wright staining of BM and spleen cells was done using Shandonn Kwik kit (ThermoFisher Scientific). BM and spleen cells were stained with PE-conjugated cocktail of lineage markers (Biolegend, #133303) for flow cytometer analysis. Also, BM and spleen cells were co-stained with CD11b-PE and Gr-1-APC-Cy7 cocktail of myeloid markers.

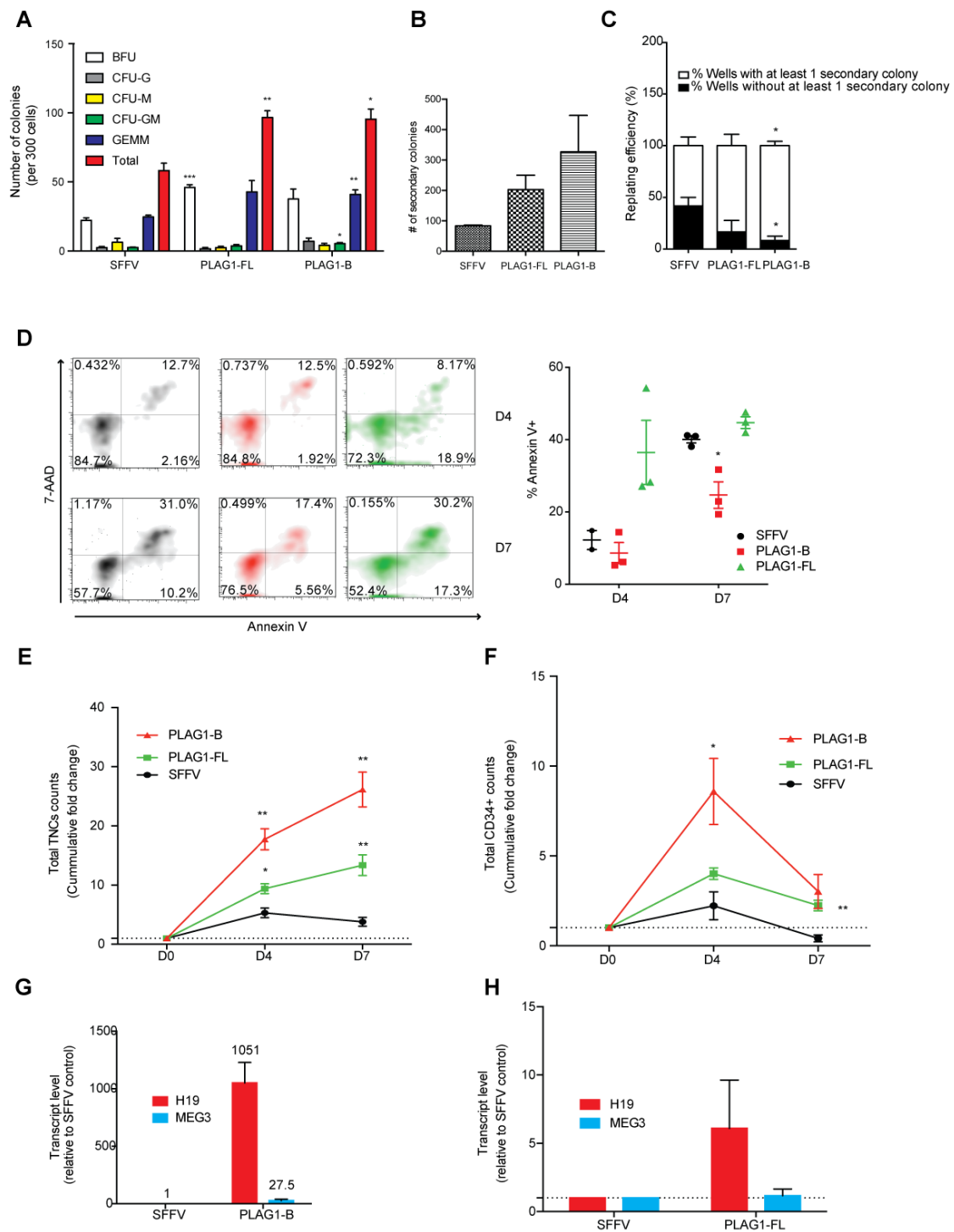
FACS and Q-RT-PCR analysis of the Plag family in CP and BC

Total bone marrow (TBM) cells were FACS purified based on GFP and BFP co-expression from CP and BC mice. Lineage negative and positive fractions were gated on the TBM. Leukemic stem cells ($\text{Lin}^- \text{Sca-1}^{\text{c}}\text{-Kit}^+$) and progenitors ($\text{Lin}^- \text{Sca-1}^{\text{c}}\text{-Kit}^+$) (LK) were gated on the lineage negative fractions and isolated to purity (>97%) using MoFlo XDP (Beckman Coulter). RNA was isolated using TRIzol LS reagent (ThermoFisher Scientific). cDNA was synthesized using qScript Supermix (Quanta Biosciences). Q-RT-PCR was done on PerfeCTa low ROX reagent (Quanta Biosciences) using Universal Probe Library (UPL) designed primers and probes (Roche). Gene specific probe used were: UPL-20 (Msi2; NM_054043.3), UPL-108 (Plag1; NM_019969.3), UPL-76 (Plagl1; NM_009538.2) and UPL-73 (Plagl-1; NM_018807.5). Msi2 expression

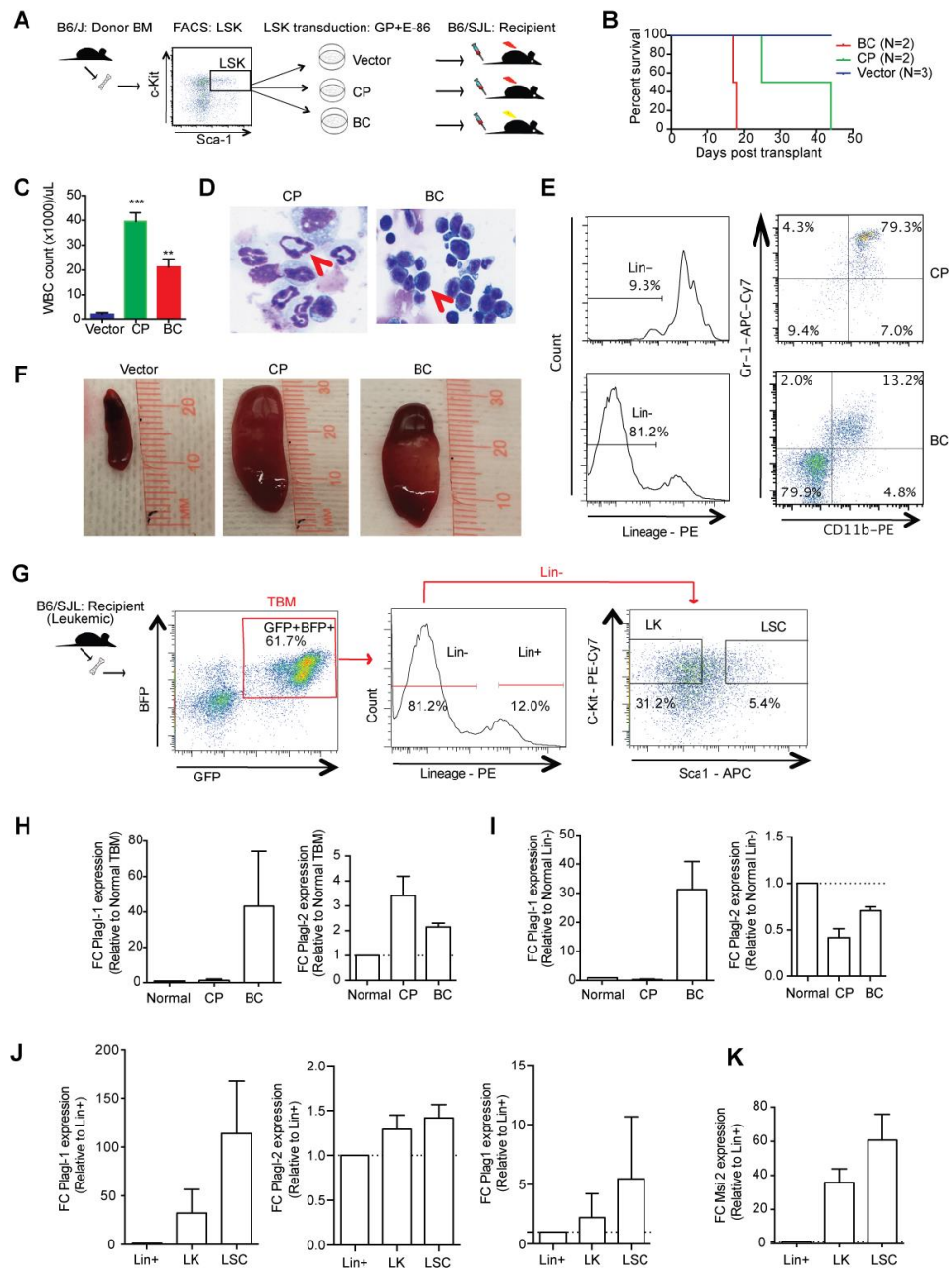
was included as positive control of BC progression. Relative fold change in expression was calculated using the $2^{-\Delta\Delta C_t}$ method.



Appendix figure 1. Long-term ex vivo culture of PLAG1OE does not impart significant HSC expansion. (A) Experiment plan for sorting and transducing Lin⁻CD34⁺ cells for intra-femoral injections into sub-lethally irradiated NSG mice for primary LDA after 7 days *ex vivo* expansion (D7). Half of transduced cells were previously injected into NSG mice as D0 LDA (see Chapter 3 Figure 3). The remaining half of cells from the SFFV control and over expression were kept in culture for additional 7 days. D0 equivalent doses were calculated and used for injections. (B) Graph depicting number of engrafted mice and level of engraftment from injected femur at each dose of primary LDA from D7 *ex vivo* culture. Engraftment is considered positive with >0.1% human CD45⁺BFP⁺ double positive cells and also capable of multi-lineage reconstitution for lymphoid cells (CD19⁺) and myeloid cells (CD33⁺). (C) ELDA plot to determine the HSC frequency in primary LDA. Calculated HSC frequencies both for the SFFV control and PLAG1^{OE} are indicated in parenthesis. (D) Summary table of the primary LDA analysis to determine the fold change in HSC *ex vivo* expansion as a result of PLAG1^{OE}. (E) Quantifications of engraftment level in the injected femur and spleen (left panel) from LDA doses of all engrafted mice. Representative flow plots of engraftment levels in injected femur are depicted in the right panel. (F) Quantifications and representative flow plots of lymphoid (CD19) and myeloid (CD33) engraftment levels in injected femur and spleen from matching LDA doses of all engrafted mice.



Appendix figure 2: Ectopic PLAG1-FL and PLAG1-B expression in CB CD34⁺ cells and assessment of *in vitro* progenitor activity and H19 and MEG3 expression (A) PLAG1-FL increases total CFU and BFU-E output compared to SFFV control. PLAG1-B increases primitive GEMM colonies and enhances total CFU yield. (B) Secondary CFU output from primary GEMM replating in PLAG1-FL is insignificant compared to PLAG1-B and SFFV control. (C) Replating efficiency of primary GEMM colonies from PLAG1-B is significantly robust compared to SFFV control. The replating efficiency of GEMM colonies from PLAG1-FL is statistically insignificant. (D) Representative flow plots for 7-AAD and Annexin V co-staining (left panel). Ectopic PLAG1-FL expression significantly increases the frequency of Annexin V⁺ cells. Conversely, PLAG1-B significantly decreases the frequency of Annexin V⁺ cells (right panel). (E) Both PLAG1-FL and PLAG1-B significantly increases total nucleated cell counts over 7 days *in vitro* culture. However, only PLAG1-B robustly maintains total number of CD34⁺ cells in culture. (F) Relative transcript levels of H19 and MEG3 after ectopic PLAG1-B (G) and PLAG1-FL (H) expression. Numbers on top of columns denote fold change level of H19 and MEG3 transcripts relative to SFFV control. PLAG1-FL only modestly increases the expression of both transcripts compared to PLAG1-B.



Appendix figure 3. Plagl-1 expression positively correlates with disease progression from chronic phase (CP) to blast crisis (BC) stage in mouse model of CML. (A)

Development of CP and BC stages of CML. Lin⁻Sca-1⁺c-Kit⁺ (LSK) cells were FACS purified from donor C57B6/J bone marrow (BM). Ecotropic GP+E-86 lines constitutively expressing BCR/ABL-GFP or Nup98-HoxA9-BFP were used for transduction of LSKs. Transduction with BCR/ABL-GFP generates CP of CML. Co-transduction LSKs with BCR/ABL-GFP and NUP98/HOXA9-BFP models BC stage. Co-culturing of LSKs with GFP and BFP-only vector expressing GP+E-86 cells was used as negative control. Transduced LSK cells were injected into the lateral tail vein of recipient lethally (vector and CP mice) or sub-lethally (BC mice) irradiated C57B6/SJL recipient mice. (B) Kaplan-Meier survival curves for CP and BC mice compared to normal mice. (C) Peripheral blood total white blood cell counts per microliter volume. (D). Giemsa-Wright staining of BM cells from CP and BC stages. BM from CP is overwhelmingly polymorphonuclear cells (red arrow). BM from BC is dominated by immature blasts (red arrow). (E) Flow cytometer characterization of CP and BC BM cells for myeloid lineage composition. CP is predominantly comprised of lineage positive cells. BC is predominantly lineage negative. (F) Splenomegaly is characteristic of both CP and BC phases of CML. (G) FACS gating strategy from CP and BC mice BM for qRT-PCR expression analysis. Transformed leukemic cell from bone marrow were gated on GFP/BFP double positivity for total bone marrow (TBM) FACS (left panel). Lineage negative and positive fractions were FACS purified within TBM (middle panel). LSCs and Lin⁻c-Kit⁺Sca-1⁻ (LK) were gated within Lin⁻ compartment (right panel).

(H) Relative fold change (FC) Plagl-1 (left panel) and Plagl-2 (right panel) transcript levels in TBM from CP and BC compared to normal mice. (I). FC Plagl-1 (left panel) and Plagl-2 (right panel) transcript levels within Lin⁻ from CP and BC compared to normal mice. (J) Relative FC Plagl-1 (left panel), Plagl-2 (middle panel) and Plagl1 (right panel) transcript levels from LSC and LK in BC phase compared to Lin⁺ from same mice. (K) Relative FC Msi2 transcript levels from LSC and LK in BC phase compared to Lin⁺ from same mice. Msi2 expression is used as a positive control for BC stage of CML.