Supplemental Methods

Vectors
Previously used shRNA against the PLAG1 coding sequence (1shPLAG1) or Luciferase\(^1\) had been designed using RNAi Central Design Tool as described in Hope et al. (2010)\(^2\) and were cloned downstream of CMV in a miRE30 scaffold\(^3\) in pZIP-CMV-ZsGreen lentiviral expression vector via EcoRI and XhoI for use in vitro. For use in vivo the same 1shPLAG1 was cloned downstream of U6 via AgeI and EcoRI in PLKO.1-TRC (Addgene Plasmid 10878\(^4\)) in which Puro was replaced with GFP (PLKO.1-TRC-GFP) and compared to shRNA against scramble sequence. A second PLAG1 shRNA targeting the 3’UTR (2shPLAG1) was designed by the Broad Institute GPP Web Portal [https://portals.broadinstitute.org/gpp/public/gene/search](https://portals.broadinstitute.org/gpp/public/gene/search) and cloned downstream of U6 via AgeI and EcoRI in PLKO.1-TRC-GFP. Human PLAG1-A, B and S and firefly Luciferase control were previously cloned into pSMALB downstream of the SFFV promoter and bidirectionally to minimal CMV driving BFP expression\(^1\). Human c-MYC and truncated-NGFR control were expressed in MA1 downstream of hPGK promoter and bidirectionally to minimal CMV driving GFP expression as previously described in Rentas et al. (2016)\(^5\). The microRNA-127 sequence was purchased from Abmgood in the pLenti-GIII-EF1a vector and ZsGreen was cloned downstream of the microRNA sequence or control empty vector via XbaI. The inhibitory sponge against miR-127-5p consisted of eight consecutive bulged 26-mer target sequences separated by 4-mers which was purchased from ThermoFisher Scientific and cloned downstream of SFFV and GFP with XbaI and EcoRI as described previously in Gentner et al. (2009)\(^6\) and Lechman et al. (2012)\(^7\). Refer also to Supplemental Table 8.

Cord blood CD34\(^+\) cell isolation, lentiviral transduction and ex vivo culture
Lin\(^-\) cord blood cells were isolated by density gradient centrifugation with Ficoll-Paque and magnetically enriched with EasySep Human Progenitor Cell Enrichment Kit according to the manufacturer’s protocols and viably frozen in FBS-10% DMSO. Lin\(^-\)CD34\(^+\) cells were isolated by fluorescence-activated cell sorting (FACS) and cultured in StemSpan Serum Free Expansion Medium supplemented with 20ng/mL Thrombopoietin and Interleukin 6 and 100ng/mL Stem Cell Factor and Flt3 ligand at 37°C 5% CO\(_2\) for 6-12 hours prior to lentiviral introduction at multiplicity of infection of 50-100 lentiviral particles per cell. Following a 72 hours transduction period when transgene expression is maximal, transduction-marker-positive cells were isolated by FACS. For cultures treated with 1uM AKTi\(^8\) or 50nM rapamycin\(^9\), inhibitors were purchased from MedChemExpress, dissolved in DMSO as 500x stocks, and added to cultures following FACS-isolation of BFP\(^+\) transduced cells.

Clonogenic progenitor assays
Clonogenic progenitor cell (colony forming unit, CFU) assays were done in complete semi-solid methylcellulose medium (ColonyGEL™1102) with FACS-purified transduced cells. 300 cells/mL were mixed with ColonyGEL™1102 using a blunt end needle and syringe and plated in technical duplicate per biological replicate in 35 mm tissue culture dishes and incubated at 37°C 5% CO₂. Manual colony enumeration and scoring as BFU-E, CFU-G, CFU-M, CFU-GM or CFU-GEMM was performed 10-14 days after plating. For secondary clonogenic assays, single primary CFU-GEMMs were plucked and dissociated by vortexing in IMDM, resuspended in 1mL ColonyGEL™1102 and plated in single wells of a 24-well plate. Primary CFU-GEMMs were imaged with a Q-Colour3 digital camera mounted to an Olympus IX5 microscope with a 40X objective lens. Image-Pro imaging software was used to acquire images and subsequent image processing was performed with ImageJ software. Refer also to Supplemental Tables 9-11.

**Cell line culture and lentiviral production**

To generate lentivirus LentiX cells cultured in DMEM with 10% FBS and 1mM sodium pyruvate at 37°C 5% CO₂ were transiently transfected with Lipofectamine 2000 (ThermoFisher) following manufacturer’s protocol with pMD2.G and psPAX2 packaging plasmids (Addgene) to create VSV-G pseudotyped lentiviral particles. Lentivirus was harvested 72-hours post-transfection by ultracentrifugation (25,000g, 2-hours, 4°C) and resuspended in StemSpan Serum Free Expansion Medium. Lentiviral preparations were titrated on HeLa cultured in DMEM with 10% FBS at 37°C 5% CO₂. K562 cells were cultured in IMDM with 10% FBS at 37°C 5% CO₂.

**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 facility. Six to twelve-week-old, age- and sex-matched NSG mice (Jackson Laboratories) were sub-lethally irradiated (315 cGy) 1 day prior to intra-femoral injection of CB HSPC.

Mice were euthanized by cervical dislocation 10-20 weeks post-transplant. Injected right femur was separated from other hind limb bones (left femur, tibiae, iliac crests; “bone marrow (BM)”) and mononucleated cells (MNCs) were isolated by crushing in IMDM-2% FBS and passage through 40uM cell strainer. Splenocytes were harvested by passage through 40uM cell strainer. Red blood cells in samples were lysed with ammonium chloride buffer prior to flow cytometric analysis or secondary xenotransplantation. Refer also to Supplemental Tables 9-11.
Extreme Limiting Dilution Analysis

Following xenotransplantation (Supplemental Methods) positive engraftment was considered >0.5% human chimerism including both myeloid (CD45^+BFP^+CD33^+) and lymphoid (CD45^+BFP^+CD19^+) lineages in either the injected femur and/or BM 14 weeks post-primary transplant and 10 weeks post-secondary transplant. Secondary recipient input cells were sourced from pooled BM and injected femur of engrafted primary mice that received equivalent total PLAG1-S^OE or control CD34^+ dosages. HSC frequencies were calculated using ELDA software^10. Total BFP^+ cells within whole-body BM of primary mice was extrapolated based on femur and hind limb counts and proportional accounting from Colvin et al. (2004)^5,11 to derive the fold change of HSC expansion in vivo.

Flow Cytometry

Immunophenotyping: Fresh or cultured CB cells were blocked with human IgG (200ug/ml), and mouse-derived grafts were blocked with mouse Fc and human IgG before staining in PEF (PBS, 2% FBS, 1mM EDTA) with antibodies against human CD45, CD33, CD15, CD14, CD19, CD71, CD235a (GlyA), CD41a, and/or CD34.

Apoptosis: Annexin V and 7-AAD co-staining was done in Annexin Binding Buffer.

Intracellular staining: Cells were fixed and permeabilized with BD Cytofix/Cytoperm plus 1x Halt Protease/Phosphatase inhibitor cocktail as per manufacturer’s instructions. Fixed/permeabilized cells were blocked with human IgG and 10% donkey serum for 10 minutes at 4°C. Primary antibodies were diluted in blocking buffer and incubated with cells for 45 minutes at 4°C, washed with PBS and secondary antibodies were added for 45 minutes at 4°C and washed with PBS prior to analysis.

Cell cycle: Cells were fixed and permeabilized with BD Cytofix/Cytoperm and stained with Ki67 and Hoechst 33342 (10ug/ml).

Protein synthesis measurements: Cells were incubated with 50uM O-propargyl-puromycin (OP-Puro) for 1 hour prior to fixation/permeabilization. Click-iT® cycloaddition reaction was performed using Click-iT® Plus OP-Puro kit following manufacturer’s protocol.

Refer to Supplemental Table 9 for details of reagents and antibodies.

Flow cytometry data acquisitions were done on BD LSR II using FACS Diva software or Beckman Coulter Cytoflex LX using CytExpert software. Data analysis was done on FlowJo software. FACS was done with
Beckman Coulter MoFlow XDP using Summit software or BD Aria II using FACS Diva software. Refer also to Supplemental Tables 9 and 10.

**Immunofluorescence Microscopy**

Immunofluorescence microscopy of MSI2 or cytoplasmic CYP1B1 proteins was done following methods in Rentas et al. (2016)\(^5\). Cell staining buffers were made in PBS. Cells were fixed in 2% paraformaldehyde for 10 minutes, washed and cytospun on to glass slides. Cells were then permeabilized (0.2% Triton X-100) for 20 minutes, blocked (0.1% saponin, 10% donkey serum) for 30 minutes and stained with anti-MSI2 or anti-CYP1B1 antibodies for 1 hour at room temperature. Primary antibody was washed twice by dunking in PBS chamber and secondary antibody staining was performed in 10% donkey serum with Alexafluor-647 donkey anti-rabbit antibody for 45 minutes and washed twice with PBS prior to mounting a coverslip with Prolong™ Gold Antifade containing DAPI. Several images (100-1000 cells total) were captured per slide at 20X magnification using an Operetta HCS Reader with epifluorescence illumination and standard filter sets. Columbus image analysis software was used to automatically identify cytoplasm boundaries and quantify cell area in square micrometer units. Refer also to Supplemental Tables 9-11.

**CUT&RUN**

Lin-CD34\(^+\) cells were sorted 72 hours post-transduction to obtain 6-7x10\(^5\) BFP\(^+\) cells per biological replicate and subsequently 2x10\(^5\) cells were used per antibody condition in CUT&RUN assay. Cells were washed in PBS and CUT&RUN assay was performed as previously described Skene et al. (2018)\(^12\). Protein A-Micrococcal nuclease (pA-MNase) fusion protein was kindly provided by Dr. Steven Henikoff. Briefly, cells were washed twice with a wash buffer and activated BioMag®Plus Concanavalin A Beads were added dropwise while vortexing the samples. Wash buffer was removed by separating the samples on a magnet and antibody buffer containing 0.0125% digitonin and mouse anti-FLAG M2 or mouse IgG were added to the cell nuclei-beads mixture. Samples were incubated on a rotator overnight at 4°C, followed by incubation with rabbit anti-mouse secondary antibody for 1 hour at room temperature. Addition and activation of pA-Mnase and isolation of soluble DNA was performed as previously described\(^12\). DNA was extracted with the MinElute PCR Purification kit and DNA libraries were prepared with ThruPLEX® DNA-Seq and DNA Unique Dual Index Kits according to manufacturer’s instructions. The number of PCR cycles for each library preparation was determined based on the Ct values from qPCR using Power SYBR Green PCR Master Mix. For each sample, 1 ul of DNA, 5 ul of PCR Master Mix, 10uM (0.4 ul) of each forward and reverse primer, and 3.2 uL of water were combined and qPCR was performed according to manufacturer’s instruction. Library purification and size selection was performed with AMPure XP beads. Subsequently,
libraries were sequenced on Novaseq6000 using 50bp paired-end reads to achieve sequencing depth of approximately 40M reads/samples. Refer also to Supplemental Tables 9 and 10.

**CUT&RUN analysis**

CUT&RUN sequencing was trimmed using fastp v.0.19.5\(^{13}\) and aligned to GRCh38 and S. cerevisiae yeast genome (sacCer3) for the spike-in normalization using bowtie2 v.2.3.5\(^{14}\) with the same alignment setting as described previously\(^{12}\). Bam files were sorted and indexed based on the genomic coordinates using samtools v.1.9\(^{15}\). Bam files of the replicates were pooled, and peaks were called from the pooled replicates by MACS2 v.2.2.5\(^{16}\) based on FLAG vs IgG with the spike-in control normalization and q-value cutoff <0.05. Peaks that overlap with the Encode black list have been removed. Peaks were annotated with the genomic features to find the overlap with promoter, intron, exon, intergenic, 5'UTR or 3'UTR loci. Promoters were defined as 2Kb upstream to 500bp downstream of the TSS. Motif analysis was performed using HOMER v.4.8\(^{17}\), where normalized enrichment score for each motif is the fold change of the target percentage to the background percentage. If the target percentage is less than 5, 1 was added to the target and the background percentages before calculating the fold change to attenuate the FC of motifs with low target percentages. Bigwig files for the whole genome track signal have been created for the replicates and for the pooled replicates using the bamCoverage command from the deepTools package v.3.5.0\(^{18}\) based on the spike-in control normalization. Heatmaps for the called peaks were plotted from bigwig files of the replicates using computeMatrix and the plotHeatmap commands in the deepTools package.

**RNA isolation, library preparation and sequencing**

Total RNA was isolated from FACS purified transduced BFP\(^+\) Lin'CD34\(^+\) using TRIzol-LS following manufacturer's protocol, and then further purified using Rneasy Micro columns and quantified by QuBit. Quality of total RNA was assessed with BioAnalyzer Nano and all samples had a RIN above 8. 250 ng of total RNA was used for library preparation. Library preparation was done with the KAPA mRNAseq stranded kit. 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. 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. Samples were sequenced at 25-45 million paired-end depth. Refer also to Supplemental Tables 9 and 10.

**RNA sequencing Analysis**
For the read alignments, sequences were trimmed for sequencing adapters and low quality 3’ bases using Trimmomatic v.0.35 and aligned to the reference human genome version GRCh38 (gene annotation from Gencode version 24) using STAR v.2.5.1b. DESeq2 v.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. 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).

**DMAP population comparisons**
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), batch corrected using the ComBat() function of the sva package (sva_3.6.0) and scaled using the standard score. Bar graphs were created by calculating for significantly differentially expressed genes the number of scaled data that were above (>0) or below (<0) the mean for each population. Empirical p values were derived from the percentage of times the observed value (set of up or downregulated genes) was better represented in that population than random values tested for 1000 permutations.

**Pathway analysis and Enrichment mapping**
Pre-ranked GSEA was performed using GSEA software and rank formula (-log10(p-value) * sign(log2FC)). Overrepresentation analysis in unranked gene lists was performed using g:Profiler19. The gene sets used for GSEA and g:Profiler were obtained from MsigDB-c2 and c3, NCI, IOB, Netpath, HumanCyc, Reactome, Panther and the Gene Ontology (GO) MP and BP databases, updated December 11 2020 (http://baderlab.org/GeneSets). Enrichment maps and cluster labels were generated using Cytoscape software (v.3.8.2) and EnrichmentMap (v3.0 and 3.3.1) and AutoAnnotate (v1.3.4) apps (FDR<0.1 and p value <0.05, unless otherwise indicated). Statistically significant overlaps between signature gene sets (PLAG1-S bound or miR-127 target genes) were performed using the EnrichmentMap app in Cytoscape and tested for Mann Whitney U p<0.05 and hypergeometric p<0.05. Refer also to Supplemental Table 11.

**cDNA Synthesis and qPCR for microRNA**
cDNA synthesis from Trizol-isolated RNA was performed with qScript® microRNA cDNA Synthesis Kit with microRNA-specific and oligo dT adapter primers designed as described by manufacturer (Supplemental Table 9). SensiFast SYBR Lo-ROX qPCR master mix was used with cDNA from 20ng total RNA per reaction, universal miRNA reverse primer compatible with qScript® microRNA cDNA Synthesis
oligo dT adapter, and miRNA-specific primers (Supplemental Table 8). Measurements taken on QuantStudio 3 Real-Time PCR System (Supplemental Table 10).

**Myc ChIP-seq analysis**

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 overexpression. 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.

Cytoscape and iRegulon app (v1.3) was used 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 overexpression 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 these 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/).

**Supplemental Results**

**MYC regulation in PLAG1-SOE HSPCs; related to MYC-induced translation impairs PLAG1-S-mediated stemness in human HSPC**

In PLAG1-S\textsuperscript{OE} HSPCs MYC transcripts are modestly repressed however protein levels are not significantly reduced (Figure S7A-B, Table S4). c-MYC activity is also tightly governed by phospho-dependent repression at Ser62, which primes for phosphorylation on Thr58 by the AKT substrate GSK3 and promotes MYC turnover\textsuperscript{20,21}. However, we do not observe changes in total or phosphorylated GSKB levels, nor in MYC phosphorylation at Ser62 or Thr58 (Figure S7C). As expected with no changes in protein regulation, the expression of MYC target genes are also not significantly reduced in PLAG1-S\textsuperscript{OE} HSPCs (Figure S7D)\textsuperscript{22,23}. In fact, in contrast to repression of components of cytosolic ribosomes the class of MYC targets involved in nuclear ribosome assembly trend upwards in their regulation in PLAG1-S\textsuperscript{OE} HSPCs (Figure 7A). Therefore, negative RP gene regulation upon PLAG1-S overexpression appears independent of a PLAG1-S-MYC repressive signaling axis.
Supplemental Tables

Supplemental Table 1: Immunophenotypes within PLAG1-S overexpressing and control human CD45+BFP+ grafts in primary NSG mice 16 weeks post xenotransplantation
Refer to .xlsx file.

Supplemental Table 2: CUT&RUN peaks for overexpressed FLAG-PLAG1-S in Lin^-CD34+ cord blood.
Refer to .xlsx file.

Supplemental Table 3: HOMER motif enrichment results for CUT&RUN peaks for overexpressed FLAG-PLAG1-S in Lin^-CD34+ cord blood.
Refer to .xlsx file.

Supplemental Table 4: DESeq of PLAG1-S overexpression vs control in Lin^-CD34+ cord blood.
Refer to .xlsx file.

Supplemental Table 5: DESeq of PLAG1 knockdown vs control in Lin^-CD34+ cord blood.
Refer to .xlsx file.

Supplemental Table 6: GSEA and EM for RNA-seq and CUT&RUN of PLAG1-S overexpressing Lin^-CD34+ cord blood.
Refer to .xlsx file.

Supplemental Table 7: Pathway overlap analysis for RNA-seq and CUT&RUN of PLAG1-S overexpressing Lin^-CD34+ cord blood.
Refer to .xlsx file.

Supplemental Table 8: Oligonucleotide Sequences
Refer to .xlsx file.

Supplemental Table 9: Reagents
Refer to .xlsx file.
Supplemental Table 10: Equipment
Refer to .xlsx file.

Supplemental Table 11: Software
Refer to .xlsx file.

Supplemental Figure Legends

Supplemental Figure 1: PLAG1 is enriched and essential in human HSC. (A) Schematic of three PLAG1 isoforms A, B and S from top to bottom. Alternative splicing of the full-length PLAG1-A produces the 82 amino acid N-terminal truncated product, PLAG1-B, and alternative translation start at Met 99 encodes PLAG1-S, the shortest isoform. (B,C) PLAG1 and USF2 transcript expression from RNA-seq of in fractionated human cord blood24,25. Box plot is of exclusive median, whiskers show variability outside upper and lower quartile, and X marks the mean. (D) Plag1 transcript expression from fractionated murine bone marrow26. (E) Mean PLAG1 transcript expression from single cell RNA-seq of CD34+ human bone marrow27. (F) PLAG1 knockdown validation for 1shPLAG1 by qPCR in wild type HeLa cells and western blot with anti-FLAG antibody targeted against overexpressed FLAG-PLAG1-S in HeLa cells. (G) Total nucleated cell (TNC) fold change of cultured Lin-CD34+ cells expressing shPLAG1 or control hairpins. (H) PLAG1 knockdown validation for 2shPLAG1 by qPCR in wild type HeLa cells.

Data is presented as average +/- SEM unless otherwise indicated. Each point represents an individual CB unit or one biological replicate.

*** p<0.005, ** p<0.01, * p<0.05. Related to Figure 1.

Supplemental Figure 2: PLAG1-S is a positive regulator of human HSPC fitness. (A) Changes in transcript expression of Plag1, Msi2, and stem-marker c-Kit in murine LinCd150-Cd48 EPre+ HSCs after treatment with 5-FU28. (B) PLAG1 expression in long-term CB HSC prior to and 4 weeks following xenotransplantation in conditioned NSG mice29. (C) PLAG1 (probe TC08001231.hg.1) expression in long-term (Lin-Cd34-Cd38-Cd71-Cd45RA-) CB HSC co-cultured with stromal cells for 4 days30. (D) Validation of PLAG1-S OE by western blot in K562 and RNA-sequencing in CB Lin-CD34+BFP+ HSPCs. (E) Representative flow plots for CD34 positivity in PLAG1-A, B, S or Luciferase control overexpressing cultures after 4 days ex vivo. (F) Representative flow plots and overall CD33 frequency in PLAG1-SOE Lin-CD34+ HSPC after 4 days of ex vivo culture (n=3). (G) Frequency of markers of primitive erythroid progenitors PLAG1-SOE Lin’CD34+ cultures after 4 days (n=3). (H) Engraftment in the injected femur and
reciprocal bone marrow by Lin-CD34+ cells overexpressing PLAG1-S or Luciferase control (n=5) 4 weeks after xenotransplantation and engraftment in the reciprocal bone marrow by Lin-CD34+ cells overexpressing PLAG1-S (n=6) or Luciferase control (n=5) 6 weeks after xenotransplantation. (I) Engraftment and median fluorescence intensity of the BFP transduction marker in injected femurs and spleens of primary NSG mice 16 weeks after receiving Lin CD34+ CB cells expressing either PLAG1-S (n=6) or control (n=6) normalized to input levels. (J) Spleens from primary NSG mice xenotransplanted with either PLAG1-SOE or LuciferaseOE control Lin-CD34+ cells. (K) Representative flow plots of lineage markers in endpoint primary NSG grafts and endpoint frequencies of lineage markers in PLAG1-SOE or control grafts in primary NSG mice.

Data is presented as average +/- SEM unless otherwise indicated. Each point represents one mouse or an individual CB unit. *** p<0.005, ** p<0.01, * p<0.05. Related to Figure 2.

Supplemental Figure 3: PLAG1-S overexpression promotes self-renewal of long-term human HSC. (A) Percent engraftment in spleen of primary recipient mice across multiple cell input doses. Dashed line indicates cut off for calling engraftment, which was >0.5% human chimerism including both myeloid (CD45+BFP+CD33+) and lymphoid (CD45+BFP+CD19+) lineages. (B) Quantification of HSC frequency by ELDA10 of splenic engraftment in primary recipient mice. Shaded area under the curve represents 95% confidence interval of HSC frequency. (C) Frequency of lineage reconstitution in human CD45+BFP+ grafts in injected femur of secondary recipient mice that received either PLAG1-SOE primary bone marrow (n=9) or LuciferaSOE control (n=5). (D) Schematic of xenotransplantation of transduced Lin-CD34+ cells that were cultured 7 days ex vivo. (E) Percent engraftment in injected femur of primary mice that received multiple cell input doses of Lin-CD34+ cells overexpressing PLAG1-S or Luciferase control that were cultured ex vivo for 7 days. (F) Quantification of HSC frequency by ELDA for PLAG1-SOE and LuciferaOE control Lin-CD34+ cells that were cultured 7 days ex vivo prior to xenotransplantation. Shaded area under the curve represents 95% confidence interval of HSC frequency. (G) Summary of HSC frequencies and expansion of PLAG1-SOE and control Lin-CD34+ cells on day 0 and day 7 of ex vivo culture. Data is presented as average +/- SEM unless otherwise indicated. Each point represents one mouse. *** p<0.005, ** p<0.01, * p<0.05. Related to Figure 3.

Supplemental Figure 4: PLAG1-S enforces a pro-HSC transcriptional state. (A) Heatmap of PLAG1-S binding sites identified by CUT&RUN. (B) Heatmap of Log2 expression fold change for common stem and progenitor markers in PLAG1-SOE and shPLAG1 HSPC relative to their respective controls. (C) Normalized enrichment scores of the top 15 lowest-FDR gene sets in the PLAG1-S overexpression transcriptome. (D) Heatmap of Log2 expression fold change of ribosome protein coding genes in PLAG1-
S^OE^ LinCD34^+^ cells relative to control, which are collectively reduced 9.7%+/−5.7% and the top twenty-five are reproducibly repressed (p<0.05) on average 15.7+/−1.8%. (E) Gene sets from RNA-seq enrichment map that are over-represented among PLAG1-S genomic binding sites (gProfiler <0.1).

Related to Figure 4.

Supplemental Figure 5: PLAG1-S dampens protein synthesis and promotes dormancy in stimulated human HSPC. (A) Dynamics of OP-Puro incorporation and flow cytometric median fluorescent intensities of FSC-H profiles of cultured LinCD34^+^ CB cells relative to when freshly isolated. Time interval shaded in grey denotes the expected timing of first cell division for long-term and short-term HSCs. For FSC-H n= 3, 4, 6, 3, 3, 2, 2 for each timepoint respectively. OP-Puro is showing the same 3-5 CB units used in Figure 5A with additional n=3 for 48 and 96 hours, and n=2 for 192 and 240 hours. (B) Three independent measures of OP-Puro incorporation by PLAG1-S^OE^ and control Lin-CD34^+^ cells on day 4 of ex vivo culture that were amalgamated and normalized for Figure 5B. (n= 3, 3, 2 for Experiment 1, 2 and 3, respectively).

(C) OP-Puro incorporation by PLAG1-S^OE^ and control Lin-CD34^+^ cells on day 7 of ex vivo culture (n=3). (D) OP-Puro incorporation by K562 and LinCD34^+^ cells overexpressing either PLAG1-S or Luciferase control (n=4). (E) GSEA of the PLAG1-S^OE^ transcriptome relative to control to a gene set of p53-regulated apoptosis targets. Data is presented as average +/- SEM unless otherwise indicated. Each point represents an individual CB unit. *** p<0.005, ** p<0.01, * p<0.05. Related to Figure 5.

Supplemental Figure 6: PLAG1-S activates imprinted loci to support human HSPC. (A) CUT&RUN Peak tracks of PLAG1-S bound to IGF2/H19 and DLK1/MEG3 loci in LinCD34^+^ cells. (B) Simplified summary of PI3K-AKT-mTOR signaling to positively regulate protein biosynthesis. (C) Subset of the PLAG1-S overexpression enrichment map (p<0.05) showing nodes related to the PI3K signaling pathway. (D) Intracellular flow cytometry of total (n=3) and phosphorylated S240/244 (n=5) RPS6 protein in PLAG1-S^OE^ LinCD34^+^ cells on day 4 of culture. Numbers above PLAG1-S^OE^ bars show the paired Student’s t-test p value relative to control. (E) Annexin V positivity in PLAG1-S^OE^ or control cells following 8 days of ex vivo culture with RAPA, AKTi or vehicle (n=4). Student’s t-test p values in red are relative to Cntrl-DMSO and in black are relative to PLAG1-S^OE^-DMSO. (F) Summary of overlap between genes downregulated in the PLAG1-S^OE^ transcriptome and microRNA target genes. (G) Total nucleated cell fold change ex vivo when PLAG1-S and miR127-5p inhibitor are co-expressed in LinCD34^+^ cells (n=3). (H) CD34^+^ frequency ex vivo when PLAG1-S and miR127-5p inhibitor are co-expressed in LinCD34^+^ cells (n=3). (I) OP-Puro incorporation by LinCD34^+^ cells when PLAG1-S and 5p-miR127 inhibitor are co-expressed (n=2). (J) qPCR measurements of miR127-5p expression in cells transduced with the
EF1alpha-miR-127 overexpression relative to control lentivector. (K) Total nucleated cell fold change ex vivo when miR127 is overexpressed in Lin'CD34' cells (n=3). (L) CD34' frequency ex vivo when miR127 is overexpressed in Lin'CD34' cells (n=3).

Data is presented as average +/- SEM unless otherwise indicated. Each point represents an individual CB unit. *** p<0.005, ** p<0.01, * p<0.05. Related to Figure 6.

Supplemental Figure 7: MYC-induced translation impairs PLAG1-S-mediated stemness. (A) Log2 expression fold change of N-, C- and L- MYC transcripts from RNA-seq of PLAG1-SOE HSPC relative to control. (B) Intracellular flow cytometric measurements of MYC protein expression in PLAG1-SOE Lin'CD34' cells (n=4). (C) Intracellular flow cytometric measures of total and phospho-Ser9 GSK3β (n=2 for Cntrl, n=3 for PLAG1-SOE) and phospho-Ser62 or Thr58 c-MYC (n=5). (D) GSEA of the PLAG1-S transcriptome to signatures of MYC target genes22,23 (E) Western blot validation of MYC overexpression by the MA1-PGK-c-MYC lentivector. (F) OP-Puro incorporation in Lin'CD34' cells overexpressing c-MYC or control (n=4).

Data is presented as average +/- SEM unless otherwise indicated. Each point represents an individual CB unit. *** p<0.005, ** p<0.01, * p<0.05. Related to Figure 7.

Supplemental Figure 8: Visual Abstract. Stimulation of CB CD34' HSPC activates protein synthesis promoting loss of dormancy and stemness. Overexpression of PLAG1-S defends HSPC dormancy and HSC stemness by directly modulating the translation machinery via a combination of functional nodes.

Supplemental References

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Supplemental Figure 1

A

PLAG1-A
PLAG1-B
PLAG1-S

B

Human Cord Blood

C

Human Cord Blood

D

Mouse Bone Marrow

E

Human Bone Marrow

F

PLAG1 expression
HeLa

G

shCntr1 1shPLAG1

H

PLAG1 expression
HeLa

Days in culture

Days in culture

Fold change relative to beta-actin

Fold change relative to beta-actin

1shPLAG1 1shPLAG1
Supplemental Figure 1: PLAG1 is enriched and essential in human HSC. (A) Schematic of three PLAG1 isoforms A, B and S from top to bottom. Alternative splicing of the full-length PLAG1-A produces the 82 amino acid N-terminal truncated product, PLAG1-B, and alternative translation start at Met 99 encodes PLAG1-S, the shortest isoform. (B,C) PLAG1 and USF2 transcript expression from RNA-seq of in fractionated human cord blood. Box plot is of exclusive median, whiskers show variability outside upper and lower quartile, and X marks the mean. (D) Plag1 transcript expression from fractionated murine bone marrow. (E) Mean PLAG1 transcript expression from single cell RNA-seq of CD34+ human bone marrow. (F) PLAG1 knockdown validation for 1shPLAG1 by qPCR in wild type HeLa cells and western blot with anti-FLAG antibody targeted against overexpressed FLAG-PLAG1-S in HeLa cells. (G) Total nucleated cell (TNC) fold change of cultured Lin−CD34+ cells expressing shPLAG1 or control hairpins. (H) PLAG1 knockdown validation for 2shPLAG1 by qPCR in wild type HeLa cells. Data is presented as average +/- SEM unless otherwise indicated. Each point represents an individual CB unit or one biological replicate. *** p<0.005, ** p<0.01, * p<0.05. Related to Figure 1.
Supplemental Figure 2

A: Plag1, C-Kit ("stem"), Msi2_1, Msi2_2

B: Lin-CD34-CD38-CD45RA-CD90-CD49f-

Cord Blood LT-HSC

C: Cord Blood LT-HSC

D: K562

E: Cntrl, PLAG1-A

F: Cntrl

G: CD34+CD71+

H: 4 weeks

I: 16 weeks

J: PLAG1-S

K: PLAG1-S, Cntrl

L: CD34, CD33, CD14, CD15, CD19, CD71, GlyA, CD41
Supplemental Figure 2: PLAG1-S is a positive regulator of human HSPC fitness. (A) Changes in transcript expression of Plag1, Msi2, and stem-marker c-Kit in murine Lin\(^{-}\)Cd150\(^{-}\)Cd48\(^{-}\)Eprc\(^{+}\) HSCs after treatment with 5-FU\(^{24}\). (B) PLAG1 expression in long-term CB HSC prior to and 4 weeks following xenotransplantation in conditioned NSG mice\(^{25}\). (C) PLAG1 (probe TC08001231.hg.1) expression in long-term (Lin\(^{-}\)CD34\(^{+}\)CD38 CD71\(^{-}\)CD45RA\(^{+}\)) CB HSC co-cultured with stromal cells for 4 days\(^{26}\). (D) Validation of PLAG1-S OE by western blot in K562 and RNA-sequencing in CB Lin-CD34+BFP+ HSPCs. (E) Representative flow plots for CD34 positivity in PLAG1-A, B, S or Luciferase control overexpressing cultures after 4 days ex vivo. (F) Representative flow plots and overall CD33 frequency in PLAG1-S\(^{OE}\) Lin-CD34\(^{+}\) HSPC after 4 days of ex vivo culture (n=3). (G) Frequency of markers of primitive erythroid progenitors PLAG1-S\(^{OE}\) Lin-CD34\(^{+}\) cultures after 4 days (n=3). (H) Engraftment in the injected femur and reciprocal bone marrow by Lin-CD34\(^{+}\) cells overexpressing PLAG1-S or Luciferase control (n=5) 4 weeks after xenotransplantation and engraftment in the reciprocal bone marrow by Lin-CD34\(^{+}\) cells overexpressing PLAG1-S (n=6) or Luciferase control (n=5) 6 weeks after xenotransplantation. (I) Engraftment and median fluorescence intensity of the BFP transduction marker in injected femurs and spleens of primary NSG mice 16 weeks after receiving Lin-CD34\(^{+}\) CB cells expressing either PLAG1-S (n=6) or control (n=6) normalized to input levels. (J) Spleens from primary NSG mice xenotransplanted with either PLAG1-S\(^{OE}\) or Luciferase\(^{OE}\) control Lin-CD34\(^{+}\) cells. (K) Representative flow plots of lineage markers in endpoint primary NSG grafts and endpoint frequencies of lineage markers in PLAG1-S\(^{OE}\) or control grafts in primary NSG mice.

Data is presented as average +/- SEM unless otherwise indicated. Each point represents one mouse or an individual CB unit. *** p<0.005, ** p<0.01, * p<0.05. Related to Figure 2.
**Supplemental Figure 3**

**A**

![Graph A](image)

**B**

![Graph B](image)

**C**

![Graph C](image)

**D**

![Graph D](image)

**E**

![Graph E](image)

**F**

![Graph F](image)

**G**

![Graph G](image)
Supplemental Figure 3: PLAG1-S overexpression promotes self-renewal of long-term human HSC. (A) Percent engraftment in spleen of primary recipient mice across multiple cell input doses. Dashed line indicates cut off for calling engraftment, which was >0.5% human chimerism including both myeloid (CD45+BFP+CD33+) and lymphoid (CD45+BFP+CD19+) lineages. (B) Quantification of HSC frequency by ELDA\(^10\) of splenic engraftment in primary recipient mice. Shaded area under the curve represents 95% confidence interval of HSC frequency. (C) Frequency of lineage reconstitution in human CD45+BFP+ grafts in injected femur of secondary recipient mice that received either PLAG1-S\(^{OE}\) primary bone marrow (n=9) or Luciferase\(^{OE}\) control (n=5). (D) Schematic of xenotransplantation of transduced Lin−CD34+ cells that were cultured 7 days \textit{ex vivo}. (E) Percent engraftment in injected femur of primary mice that received multiple cell input doses of Lin−CD34+ cells overexpressing PLAG1-S or Luciferase control that were cultured \textit{ex vivo} for 7 days. (F) Quantification of HSC frequency by ELDA for PLAG1-S\(^{OE}\) and Luciferase\(^{OE}\) control Lin−CD34+ cells that were cultured 7 days \textit{ex vivo} prior to xenotransplantation. Shaded area under the curve represents 95% confidence interval of HSC frequency. (G) Summary of HSC frequencies and expansion of PLAG1-S\(^{OE}\) and control Lin−CD34+ cells on day 0 and day 7 of \textit{ex vivo} culture. Data is presented as average +/- SEM unless otherwise indicated. Each point represents one mouse. *** p<0.005, ** p<0.01, * p<0.05. Related to Figure 3.
Supplemental Figure 4: PLAG1-S enforces a pro-HSC transcriptional state. (A) Heatmap of PLAG1-S binding sites identified by CUT&RUN. (B) Heatmap of Log2 expression fold change for common stem and progenitor markers in PLAG1-S\textsuperscript{OE} and shPLAG1 HSPC relative to their respective controls. (C) Normalized enrichment scores of the top 15 lowest-FDR gene sets in the PLAG1-S overexpression transcriptome. (D) Heatmap of Log2 expression fold change of ribosome protein coding genes in PLAG1-S\textsuperscript{OE} Lin-CD34\textsuperscript{+} cells relative to control, which are collectively reduced 9.7%+/−5.7% and the top twenty-five are reproducibly repressed (p<0.05) on average 15.7%+/−1.8%. (E) Gene sets from RNA-seq enrichment map that are over-represented among PLAG1-S genomic binding sites (gProfiler <0.1).

Related to Figure 4.
Supplemental Figure 5

A

B

C

D

E

GO Intrinsic Apoptosis Signaling Pathway by P53 Class Mediator

NES: -1.04
FDR: 0.934

BCL3
IFI16
MIF
PDK2
KDM1A
BAG6
TP53
PYCARD
PML
FHIT
RPL11
BCL2
CD74
PTTG1P
CD44
Supplemental Figure 5: PLAG1-S dampens protein synthesis and promotes dormancy in stimulated human HSPC. (A) Dynamics of OP-Puro incorporation and flow cytometric median fluorescent intensities of FSC-H profiles of cultured Lin-CD34+ CB cells relative to when freshly isolated. Time interval shaded in grey denotes the expected timing of first cell division for long-term and short-term HSCs. For FSC-H n= 3, 4, 6, 3, 3, 2, 2 for each timepoint respectively. OP-Puro is showing the same 3-5 CB units used in Figure 5A with additional n=3 for 48 and 96 hours, and n=2 for 192 and 240 hours. (B) Three independent measures of OP-Puro incorporation by PLAG1-SOE and control Lin-CD34+ cells on day 4 of ex vivo culture that were amalgamated and normalized for Figure 5B. (n= 3, 3, 2 for Experiment 1, 2 and 3, respectively). (C) OP-Puro incorporation by PLAG1-SOE and control Lin-CD34+ cells on day 7 of ex vivo culture (n=3). (D) OP-Puro incorporation by K562 and Lin-CD34+ cells overexpressing either PLAG1-S or Luciferase control (n=4). (E) GSEA of the PLAG1-SOE transcriptome relative to control to a gene set of p53-regulated apoptosis targets.

Data is presented as average +/- SEM unless otherwise indicated. Each point represents an individual CB unit. *** p<0.005, ** p<0.01, * p<0.05. Related to Figure 5.
**Supplemental Figure 6**

### A

Chromosome 11 - H19

Chromosome 11 - IGF2

Chromosome 14 - MEG3

Chromosome 14 - DLK1

### B

- **PDHK1** → **PI3K** → **mTORC2** → **Rictor**
- **Raptor** → **PI3K** → **mTORC1** → **Raptor**
- **4EBP1** → **S6K1** → **S6**
- **eIF4E** → **40S** → **60S**

**C**

- **FGFR1** Receptor Activation
- **Superpathway** Inositol Process
- **Phosphatidylinositol 3 Kinase**

### D

- MFI relative to Cntrl
- **DMSO** vs **RAPA**
- **AKT1** vs **mTORC2**

### E

- % Annexin V+ in BFP cells
- **Cntrl** vs **miR127**

### F

| miRNA name | # total targets from Diana tools tarBase v7.0 | # targets on EM (p<0.05) | # targets with DEseq p<0.05 | # targets with p<0.05 and down |
|------------|---------------------------------------------|--------------------------|-----------------------------|-------------------------------|
| miR-370-5p  | 0                                           | 0                        | 0                           | 0                             |
| miR-370-3p  | 1,12                                        | 60                       | 20                          | 9                             |
| miR-127-5p  | 172                                         | 99                       | 30                          | 7                             |
| miR-127-3p  | 43                                          | 27                       | 8                           | 5                             |
| miR-770-5p  | 38                                          | 18                       | 3                           | 3                             |
| miR-770-3p  | 0                                           | 0                        | 0                           | 0                             |
| miR-433-5p  | 0                                           | 0                        | 0                           | 0                             |
| miR-433-3p  | 1                                           | 0                        | 0                           | 0                             |

### G

- TNC Fold Change
- **Cntrl** vs **miR127**

### H

- % CD34+
- **Cntrl** vs **miR127**

### I

- OP-Puro AF647 MFI
- **Cntrl** vs **miR127**

### J

- EF1α-miR-127 Overexpression
- Fold Change Relative to SNORD48

### K

- TNC Fold Change
- **Cntrl** vs **miR-127**

### L

- % CD34+
- **Cntrl** vs **miR-127**
Supplemental Figure 6: PLAG1-S activates imprinted loci to support human HSPC. (A) CUT&RUN Peak tracks of PLAG1-S bound to IGF2/H19 and DLK1/MEG3 loci in Lin\(^-\)CD34\(^+\) cells. (B) Simplified summary of PI3K-AKT-mTOR signaling to positively regulate protein biosynthesis. (C) Subset of the PLAG1-S overexpression enrichment map (p<0.05) showing nodes related to the PI3K signaling pathway. (D) Intracellular flow cytometry of total (n=3) and phosphorylated S240/244 (n=5) RPS6 protein in PLAG1-S\(^{OE}\) Lin\(^-\)CD34\(^+\) cells on day 4 of culture. Numbers above PLAG1-S\(^{OE}\) bars show the paired Student’s t-test p value relative to control. (E) Annexin V positivity in PLAG1-S\(^{OE}\) or control cells following 8 days of ex vivo culture with RAPA, AKTi or vehicle (n=4). Student’s t-test p values in red are relative to Cntrl-DMSO and in black are relative to PLAG1-S\(^{OE}\)-DMSO. (F) Summary of overlap between genes downregulated in the PLAG1-S\(^{OE}\) transcriptome and microRNA target genes. (G) Total nucleated cell fold change ex vivo when PLAG1-S and miR127-5p inhibitor are co-expressed in Lin\(^-\)CD34\(^+\) cells (n=3). (H) CD34\(^+\) frequency ex vivo when PLAG1-S and miR127-5p inhibitor are co-expressed in Lin\(^-\)CD34\(^+\) cells (n=3). (I) OP-Puro incorporation by Lin\(^-\)CD34\(^+\) cells when PLAG1-S and 5p-miR127 inhibitor are co-expressed (n=2). (J) qPCR measurements of miR127-5p expression in cells transduced with the EF1alpha-miR-127 overexpression relative to control lentivector. (K) Total nucleated cell fold change ex vivo when miR127 is overexpressed in Lin\(^-\)CD34\(^+\) cells (n=3). (L) CD34\(^+\) frequency ex vivo when miR127 is overexpressed in Lin\(^-\)CD34\(^+\) cells (n=3).

Data is presented as average +/- SEM unless otherwise indicated. Each point represents an individual CB unit. *** p<0.005, ** p<0.01, * p<0.05. Related to Figure 6.
Supplemental Figure 7

**A**

Bar graph showing transcript fold change in PLAG1-S relative to Ctrl.

**B**

Graphs showing MFI relative to Ctrl for different conditions.

**C**

Bar graphs comparing % positive in different conditions.

**D**

Card showing enrichment scores (ES) for Hallmark MYC targets and MYC_UP.V1 UP in Murine HSC for Down in Myc-KO and Up in Myc-KO.

**E**

Western blot images for K562 Cntrl cMYC∞ and α-Tubulin.

**F**

Graph showing OP-Puro MFI relative to average Cntrl per experiment.
Supplemental Figure 7: MYC-induced translation impairs PLAG1-S-mediated stemness. (A) Log2 expression fold change of N-, C- and L- MYC transcripts from RNA-seq of PLAG1-S\textsuperscript{OE} HSPC relative to control. (B) Intracellular flow cytometric measurements of MYC protein expression in PLAG1-S\textsuperscript{OE} Lin\textsuperscript{−}CD34\textsuperscript{+} cells (n=4). (C) Intracellular flow cytometric measures of total and phospho-Ser9 GSK3\textbeta (n=2 for Cntrl, n=3 for PLAG1-S\textsuperscript{OE}) and phospho-Ser62 or Thr58 c-MYC (n=5). (D) GSEA of the PLAG1-S transcriptome to signatures of MYC target genes\textsuperscript{27,28} (E) Western blot validation of MYC overexpression by the MA1-PGK-c-MYC lentivector. (F) OP-Puro incorporation in Lin\textsuperscript{−}CD34\textsuperscript{+} cells overexpressing c-MYC or control (n=4).