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PLAG1 dampens protein synthesis to promote human hematopoietic stem cell self-renewal

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Abstract:
Hematopoietic stem cell (HSC) dormancy is understood as supportive of HSC function and their long-term integrity. While regulation of stress responses incurred as a result of HSC activation is recognized as important in maintaining stem cell function, little is understood of the preventative machinery present in human HSCs that may serve to resist their activation and promote HSC self-renewal. We demonstrate that the transcription factor PLAG1 is essential for long-term HSC function and when overexpressed endows a 15.6-fold enhancement in the frequency of functional HSC in stimulatory conditions. Genome-wide measures of chromatin occupancy and PLAG1-directed gene expression changes combined with functional measures reveal that PLAG1 dampens protein synthesis, restrains cell growth and division, and enhances survival, with the primitive cell advantages it imparts being attenuated by addition of the potent translation activator, c-MYC. We find PLAG1 capitalizes on multiple regulatory factors to ensure protective diminished protein synthesis including 4EBP1 and translation-targeting miR-127, and does so independently of stress response signaling. Overall, our study identifies PLAG1 as an enforcer of human HSC dormancy and self-renewal through its highly context-specific regulation of protein biosynthesis, and classifies PLAG1 among a rare set of *bona fide* regulators of mRNA translation in these cells. Our findings showcase the importance of regulated translation control underlying human HSC physiology, its dysregulation under activating demands, and the potential if its targeting for therapeutic benefit.

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PLAG1 dampens protein synthesis to promote human hematopoietic stem cell self-renewal

Short Title: PLAG1 dampens translation to promote human HSC

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Key Points

1) The PLAG1 transcription factor promotes human HSC self-renewal and dormancy.
2) PLAG1 enforces stemness by dampening expression of translation machinery activated in HSC-stimulatory conditions.

Abstract

Hematopoietic stem cell (HSC) dormancy is understood as supportive of HSC function and their long-term integrity. While regulation of stress responses incurred as a result of HSC activation is recognized as important in maintaining stem cell function, little is understood of the preventative machinery present in human HSCs that may serve to resist their activation and promote HSC self-renewal. We demonstrate that the transcription factor PLAG1 is essential for long-term HSC function and when overexpressed endows a 15.6-fold enhancement in the frequency of functional HSC in stimulatory conditions. Genome-wide measures of chromatin occupancy and PLAG1-directed gene expression changes combined with functional measures reveal that PLAG1 dampens protein synthesis, restrains cell growth and division, and enhances survival, with the primitive cell advantages it imparts being attenuated by addition of the potent translation activator, c-MYC. We find PLAG1 capitalizes on multiple regulatory factors to ensure protective diminished protein synthesis including 4EBP1 and translation-targeting miR-127, and does so independently of stress response signaling. Overall, our study identifies PLAG1 as an enforcer of human HSC dormancy and self-renewal through its highly context-specific regulation of protein biosynthesis, and classifies PLAG1 among a rare set of *bona fide* regulators of mRNA translation in these cells. Our findings showcase the importance of regulated translation control underlying human HSC physiology, its dysregulation under activating demands, and the potential if its targeting for therapeutic benefit.

Introduction

Hematopoietic stem cells (HSCs) ensure long-term multilineage blood regeneration through their enduring self-renewal capacity[1,2]. As such transplantation of hematopoietic stem and progenitor cells (HSPCs) sourced from bone marrow (BM) or umbilical cord blood (CB), can be life-saving for patients with myriad malignant and non-malignant disorders[3,4]. Widespread application of HSPC therapies remains limited by disease-causing mutations in autologous HSPC and difficulties sourcing HLA-matched allogenic BM[3,5]. Efforts to bridge these gaps include attempts to achieve therapeutic HSPC genome editing and amplification of less plentiful but immunologically superior CB HSPCs[6-13]. However, these goals remain challenged by our incomplete understanding of the fundamental biology underlying

human HSC identity and fate-decisions, especially in stimulatory regenerative conditions where HSCs are predisposed to depletion[6,14-19].

Despite their extensive regenerative capacity, the most potent HSCs live principally in a state of quiescence and dormancy, wherein cells exhibit reduced anabolic activity which preserves the integrity of the HSC pool[20-23]. Indeed, conditions that cause HSPCs to exit dormancy, such as the regenerative stress of *ex vivo* manipulation or transplantation, compromises the pool of long-term (LT) repopulation-competent HSCs[24-26]. Interventions that restrain mitochondrial metabolism or cell cycle to limit HSC activation in these settings can aid in preservation of mouse and human HSC[21,27-31]. Importantly, however, the process of protein biosynthesis, which in many contexts is co-regulated with metabolism and can fuel cellular growth and division, has not been well explored for its role in human HSC dormancy and transplantation paradigms.

In their native environment murine HSCs, like several other model stem cell types[32-35], require tightly-controlled, low levels of protein synthesis[36,37], understood to limit exhaustive proliferation and safeguard the proteome to evade attrition or death due to proteotoxicity[37-40]. In response to stimulatory conditions the activation of stress effectors in murine HSC serves to rebalance proteostasis by favouring diminished translation rates[41,42]. These studies have begun to elucidate the multifactorial contributions to HSC dormancy and fate, however they raise a number of key questions: under activating conditions what are the dynamics of translation in stem vs mature human hematopoietic cells?; can translation modulation be supportive of human HSCs?; can translation regulation be decoupled from stress response? and what factor(s) control translation in human HSCs?

PLAG1 is a zinc finger transcription factor (TF), first discovered as being rearranged in pleomorphic adenomas of the salivary gland and plays essential regulatory roles in mammalian fetal growth and development[43,44]. We now identify PLAG1 as an essential regulator of human HSC dormancy and self-renewal by acting as a novel negative regulator of protein synthesis independently of triggering intrinsic stress-responsive effectors. Our study supports an emerging paradigm that dysregulation of protein synthesis is a key clinical demand on human HSC and its modulation could be leveraged for therapeutic benefit.

Methods

CB Lin$^-$CD34$^+$ culture and analysis

Lin^-CD34^+ CB cells were isolated, transduced with lentiviral vectors (Supplemental Methods) and cultured in StemSpan Serum Free Expansion Medium with 20ng/mL Thrombopoietin and Interleukin 6 and 100ng/mL SCF and FLT3 ligand at 37°C 5% CO_2. Culture-derived cells were analyzed by xenotransplantation, Extreme limiting dilution analysis (ELDA)[45], colony forming unit (CFU) assay, flow cytometry, immunofluorescence microscopy, qPCR, CUT&RUN[46] or RNA-seq (Supplemental Methods, Tables S7-10).

Mouse Xenotransplantations

Mouse work was done in pathogen-free facilities in compliance with ethical regulations approved by McMaster University's Animal Research Ethics Board. Age- and sex-matched *NSG* mice (Jackson Laboratories) were sub-lethally irradiated (315 cGy) 1 day prior to intra-femoral injection of HSPCs and endpoint tissues were processed by passage through 40uM cell strainer as previously described[47].

Informed consent was received for all of the human AML samples used.

Data Availability

CUT&RUN and RNA-seq data SuperSeries accession number is GSE181992 and can be accessed at https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE181992, reviewer token: qpcduaeknfybvqn

Results

PLAG1 is enriched in and essential to human HSC

We previously showed that when co-overexpressed, short isoforms of PLAG1 (-S and -B) (Figure S1A) and the USF2 TFs can co-operatively transactivate the pro-self-renewal gene MSI2[48]. However, while USF2 expression is stable across the human hematopoietic hierarchy, PLAG1 is specifically elevated in HSC from human CB[48-51], murine[52] and human[53] BM (Figure S1B-E); altogether suggesting that PLAG1 may have important heretofore unexplored functions in the most primitive hematopoietic cells.

To evaluate this, we queried the *in vitro* and *in vivo* potential of CB-derived Lin^-CD34^+ HSPCs expressing PLAG1-targeting shRNAs (Figure 1A, S1F). PLAG1-depleted HSPCs generated fewer colonies, due mainly to reduced erythroid (BFU-E) and primitive granulocyte-erythroid-megakaryocyte-monocyte (CFU-GEMM) colonies (Figure 1B). This was mirrored in suspension culture where shPLAG1 significantly reduced total nucleated cell (TNC) and $CD34^+$ outputs over 7 days (Figure 1C, S1G). Six weeks following NSG mouse xenotransplantation, when engraftment is largely contributed by

progenitors, there was modest but non-significant reduction in the representation of shPLAG1-expressing GFP^+ cells (Figure 1D). However, sixteen weeks following transplant when the graft is sustained by *bona fide* HSCs, engraftment by shPLAG1-expressing cells was significantly impaired 11-14-fold (Figure 1E). Patterns in reduced CFU output and impairment of long-term BM reconstitution were replicated by a second independent PLAG1-targeting hairpin (Figure 1F-G, S1H).

PLAG1-S is a positive regulator of human HSPC fitness

Mining expression profile data we next uncovered intriguing differences in the expression profiles of PLAG1 and MSI2 signifying that the role of PLAG1 in human HSC physiology may transcend the PLAG1/USF2-MSI2 regulatory axis. First, in the human hierarchy PLAG1 expression is highly restricted to non-cycling HSCs, whereas MSI2 is strongly expressed in both non-cycling and cycling HSC and $CD34^+$ progenitors (Figure 2A)[54]. Secondly, upon 5-fluoruracil (5-FU) stress-induction of mouse HSC cycling, Msi2 is elevated while Plag1 is repressed (Figure S2A)[55]. Most importantly and unexpectedly, PLAG1-S overexpression without USF2 in Lin^-CD34^+ cells is insufficient to enhance MSI2 protein expression (Figure 2B). Moreover, PLAG1 levels are reduced in human HSPCs activated by transplantation (Figure S2B)[28] or culture (Figure 2C, S2C)[39,56,57]. Therefore, to explore the potential for an independent function of PLAG1 in modulating human HSPC fate decisions in these contexts we assayed Lin^-CD34^+ CB cells *in vitro* and *in vivo* upon gain of PLAG1 (Figure 2D, S2D).

Overexpression of each of the three PLAG1 isoforms (Figure S1A)[43,44,48] increased BFU-E output with only the shorter isoforms (-S and -B) enhancing GEMM replating efficiency (Figure 2E-F) and $CD34^+$ cell expansion in culture over 7 days (peak advantage at day 4) (Figure 2G-I, S2E). In PLAG1-S overexpressing ($PLAG1\text{-}S^{OE}$) cultures enhanced CD34 maintenance occurs concurrently with a reduction of committed $CD33^+$ cells and an elevated frequency of $CD34^+CD71^+$ (BFU-E) progenitors (Figure S2F-G). Altogether, these findings point to the short isoforms of PLAG1 as important positive regulators of human HSPC, and contextualize our past observation that these isoforms are preferentially expressed in the HSC-enriched compartment of human CB[48].

Given the strong and comparable phenotypes between PLAG1-S and PLAG1-B, we prioritized the shortest form, PLAG1-S, for assessment in competitive repopulation assays (Figure 2D). Following a 4 or 6-week repopulation period in NSG mice, $PLAG1\text{-}S^{OE}$ and control short-term progenitors are similarly capable of contributing to engraftment (Figure S2H). After 16 weeks, the proportion and intensity of the BFP transduction marker relative to input levels was however significantly higher in the BM of $PLAG1\text{-}S^{OE}$ recipients compared to control (Figure 2J-K, S2I). As BFP intensity from this bidirectional promoter vector provides a surrogate measure for transgene expression[40,58] this indicates that co-transplanted cells overexpressing PLAG1-S to higher levels outcompete those expressing lower levels.

Importantly, the enhanced fitness of PLAG1-S^{OE} HSCs was neither associated with splenomegaly (Figure S2J), nor elevation of the CD34^{+} compartment; and despite erythroid bias *in vitro*, in the *in vivo* niche PLAG1-S^{OE} grafts displayed balanced multilineage output (Figure S2K, Table S1).

PLAG1-S overexpression promotes self-renewal of human LT-HSC

To evaluate the capacity of PLAG1-S to promote LT-HSC self-renewal we performed serial gold-standard primary xenotransplantation in limiting dilution to quantify HSC frequencies in the culture-derived progeny of Lin^{-}CD34^{+} cells immediately post-induction of ectopic PLAG1-S (Figure 3A). Fourteen weeks following primary xenotransplantation only 33% of mice transplanted with control cells, compared to 66% of mice transplanted with PLAG1-S^{OE} cells met multilineage BM engraftment criteria (Figure 3B,D). ELDA[45] determined HSC frequencies of 1 in 715 PLAG1-S^{OE} vs 1 in 11,157 control cells, representing 15.6-fold enhancement of functionally-validated HSC (Figure 3C-D). A similar analysis of splenic grafts revealed 75-fold enhancement in primitive cells capable of repopulating this environment (Figure S3A-B). Notably, HSC renewal achieved by ectopic MSI2 does not occur at this early timepoint post-transduction[47], highlighting a unique functional capacity of PLAG1-S enacted via MSI2-independent means.

At the secondary transplant endpoint we confirmed that recipients of PLAG1-S^{OE} cells exhibited enduring multilineage reconstitution and heightened engraftment due to a 4.8-fold higher HSC frequency relative to controls (Figure 3E-G, S3C). Accounting for initial HSC input into primary recipients we find that enhanced engraftment in secondary recipients is not the result of enhanced renewal *in vivo* (Figure 3G) and is primarily attributed to the initial *ex vivo* promotion of the HSC compartment. These results, quantitively demonstrate that PLAG1-S^{OE} does not impart excessive HSC self-renewal characteristic of clonal hematopoiesis or pre-malignancy; and that the potent promotion of HSCs in a stimulatory setting is not associated with detrimental exhaustion of the LT-HSC compartment.

To test for the effect of PLAG1-S on HSC renewal over longer periods *in vitro,* cells cultured for 7 additional days were subjected to limiting dilution xenotransplantation (Figure S3D). Relative to immediately post-induction of ectopic PLAG1-S, the number of functional HSCs continue to increase 1.6-fold and sustain 4.3-fold higher frequencies relative to control (Figure S3E-G). Thus, the potent stem cell advantage endowed by PLAG1-S, though sustained in culture, can be maximally achieved shortly after PLAG1-S induction, underscoring PLAG1-S as an early-actor in promoting HSC function to improve hematopoietic repopulation.

PLAG1-S enforces a pro-HSC transcriptional state

Genomic binding and transcriptomic profiles were next used to uncover molecular targets underpinning the positive regulation of HSC by PLAG1-S. CUT&RUN performed in PLAG-1S^{OE} Lin^{-} CD34^{+} cells identified 9788 reproducible genomic binding sites[46,59] (Figure S4A, Table S2). Consistent with our published ChIP-seq in K562 cells[48] and its known role as a TF, PLAG1-S sites are principally located in promoter regions (58.4%) (Figure 4A). *De novo* motif discovery revealed that PLAG1-S is predominantly (35%) bound to G-rich core consensus sequences expected for PLAG family members, likewise supporting the specificity of the CUT&RUN profile generated (Figure 4B). To a lesser extent PLAG1-S is also bound to non-canonical motifs, including those associated with other zinc-finger, GATA or RUNX TFs (Figure 4B, Table S3).

RNA-seq of HSPCs directly following up- or down- modulation of PLAG1 levels (Table S4-5) corroborates the respective immunophenotypic and functional outcomes both through the expression of surface markers (Figure S4B) and by global alignment to transcriptional states of 20 human hematopoietic cell subpopulations[49], which show positive associations to primitive and erythroid signatures and negative associations to myeloid signatures correlated with high PLAG1 levels *in vitro* (Figure 4D).

Ectopic PLAG1-S significantly altered the expression of 543 genes (Table S4, Figure 4C). Consistent with its understood function as a transcription activator, 60% of the 291 upregulated genes are proximally bound by PLAG1-S. Thirty percent of downregulated genes are also directly bound, which similarly to other recent publications[60], suggests an underappreciated role for PLAG1-S in negative regulation of gene expression. Gene set enrichment analysis (GSEA) revealed that coordinated pathway-level changes are dominated by negative enrichments (Table S6). The top-most negatively enriched gene sets coalesce in the largest cluster of altered signatures and point to a synchronized attenuation of mRNA translation machinery in PLAG1-S^{OE} HSPC (Figure 4E, S4C). These negative enrichments are driven largely by reduced expression of genes encoding ribosomal proteins (RPs). (Figure S4D). Moreover, there is a significant over-representation and overlap of genes belonging to the cluster of protein synthesis gene sets in the repertoire of genes directly bound by PLAG1-S (Figure 4E orange edges, 4F, Table S7). Altogether this speaks to an unexpected ability of PLAG1-S to intersect with the regulation of protein synthesis machinery to regulate fate decisions in human HSPC.

PLAG1-S dampens protein synthesis and promotes dormancy in stimulated human HSPC

The state of attenuated translation machinery in PLAG1-S^{OE} HSPC is intriguing given that tightly controlled protein synthesis is a hallmark of stem cell biology[32-35,37]. While others have demonstrated that culturing human HSPC, like their murine counterparts, promotes exit from dormancy, loss of quiescence, and differentiation[24,25,28,39], little is known of their translation dynamics within immediate and prolonged timeframes, and in comparison to more committed cells[42]. To gain these insights and contextualize the

HSPC-specific PLAG1-S regulon we measured O-propargyl-puromycin (OP-Puro) incorporation in CB cells upon culture-induced stimulation[37,61,62]. As early as 4-hours after being placed in culture Lin^-CD34^+ cells activate protein synthesis, and these levels progressively increase peaking at 9.5-fold after 48-hours. Subsequently translation rates drastically decline between 48 and 72-hours and plateau between 3-10 days, but do not return to base-line levels (Figure 5A, S5A). In contrast, more mature Lin^-CD34^- cells experience only a modest elevation of translation after 24-hours that diminishes by 48-hours (Figure 5B), indicating that human HSPCs selectively undergo an immediate but transient pro-translation response when placed into culture. Evaluating PLAG1-S^{OE} HSPCs we observed that transcriptional reprogramming of protein biosynthetic processes indeed supports a 13% reduction in global translation rates in Lin^-CD34^+ cells *ex vivo* (Figure 5C, S5B-C). This appears selective to primitive hematopoietic cells as comparably handled PLAG1-S^{OE} K562 and Lin^-CD34^- cells display unchanged and heighted translation levels, respectively (Figure S5D).

Anabolic processes such as protein biosynthesis are generally correlated with cellular enlargement and division[63-67], both of which can predict HSC exhaustion[24,68]. To this point the diameter of untransduced cultured HSPC is significantly enlarged coincident with the peak in translation rates, which notably precedes when the first cellular division is expected[28] (Figure S5A). Likewise, we find that depressed translation in PLAG1-S^{OE} HSPCs is associated with restraint in size (Figure 5D). GSEA did not uncover consensus control over cell cycle progression by PLAG1-S (Table S6, Figure 4E). However Hoechst/Ki-67 staining indicated that PLAG1-S^{OE} HSPCs are restrained in cell cycle progression (Figure 5E), paralleling the correlation between PLAG1 expression and dormant HSCs (Figure 2A-C). While this may likely be secondary to translation regulation, we also noticed that expression of CDKN1C, an essential regulator of murine HSC quiescence and renewal[69,70], appears directly activated 2.5-fold by PLAG1-S in HSPCs (Table S2,4), possibly contributing to the cell cycle profile of these cells.

In vitro survival, which could contribute to amplifying a stem cell pool experiencing infrequent and/or slow division dynamics, is enhanced in PLAG1-S^{OE} cells, as measured by Annexin V (Figure 5F). Given that translation dynamics are highly interconnected with stress signaling that can dictate survival decisions, we profiled stress effectors in PLAG1-S^{OE} HSPCs. Firstly, pro-apoptotic p53 targets, which can be induced by imbalances in ribosome components, are not activated within the PLAG1-S^{OE} transcriptome (Figure S5E)[71,72]. Low levels of EIF2 complex restrains global translation while dichotomously promoting translation of the intregrated stress response effector (ISR) ATF4 in CB HSPCs[73] and muscle stem cells to support regeneration[35]. However neither EIF2 subunits nor ATF4 targets[73,74] are differentially regulated in PLAG1-S^{OE} HSPCs suggesting this pathway is not significantly at play (Figure 5G-H). Lastly, CB HSPC are selectively sensitive to stress associated with misfolded proteins[40] and low translation rates in murine HSCs is a mechanism that protects their proteostatic

integrity[38]. To this point, reduced translation in PLAG1-S^{OE} HSPC is associated with depressed expression of unfolded protein response (UPR) signatures (Figure 5I). Altogether this suggests that dampened translation rates imparted by PLAG1-S is protective and forestalls stress responses.

In sum, we show that human HSPC selectively experience an immediate and transient pro-translation response when exposed to stimulatory conditions; and through transcriptional programming PLAG1-S limits translation in human HSPC to mitigate the impact of culture-induced protein stress and HSC activation. This manifests as PLAG1-S-induced reductions in cell enlargement, division, differentiation and death in human HSPC, altogether significantly enhancing HSC fitness and output *in vivo*.

PLAG1-S activates imprinted loci to support human HSPC

A notable finding of the PLAG1-S regulatory network is its direct binding and activation of DLK1/MEG3 and IGF2/H19 (Figure 4C, 6A, S6A), affirming that as in mouse tissues[75], these imprinted loci are targets of PLAG1-S in primitive human hematopoietic cells. PLAG1-induced activation of IGF2 can stimulate mitogenic and PI3K-AKT-mTOR signaling to promote tumorigenic growth and division[75-77]. However, H19 and MEG3 act in opposition to PI3K-AKT-mTOR signaling in support of fetal murine HSC quiescence and function[78,79]. When activated this pathway can stimulate protein synthesis dually through phospho-dependent activation of RPS6K and inhibition of translation initiation-regulating 4EBPs (Figure S6B). At the transcript level PI3K-AKT-mTOR signaling signatures are both up- and down-regulated in PLAG1-S^{OE} HSPC (Figure S6C). Definitive characterization of the pathway flux by intracellular flow cytometry (IFC) reveals subtle reductions in AKT and 4EBP1 phosphorylation, while RPS6 phospho-status was unchanged (Figure 6B, S6D), suggesting selective repression of 4EBP1-regulated translation initiation could partially contribute to reduced protein synthesis in PLAG1-S^{OE} HSPC. Human HSPC fitness can be enhanced by pharmacological inhibition of AKT (AKTi) which promotes quiescence[80] or addition of the mTOR inhibitor, rapamycin[81]. Given the pleiotropism of these signaling factors we investigated whether combining PLAG1-S^{OE} with these inhibitors could produce combinatorial effects on human HSPC output. As demonstrated previously in human Lin$^-$CD34$^+$ cells treated with AKTi[80] and murine Lin$^-$Sca$^+$ cells treated with rapamycin[82], either inhibitor reduced total cells in both control and PLAG1-S^{OE} cultures (Figure 6C) while enhancing the proportion of primitive CD34$^+$ cells (Figure 6D), resulting in maintenance of similar total CD34$^+$ cell outputs (Figure 6C). Only with rapamycin were these growth dynamics associated with a reduction in translation rates in PLAG1-S^{OE} cells (Figure 6E) while neither treatment altered apoptosis in control or PLAG1-S^{OE} cultures (Figure S6E).

The DLK1/MEG3 locus also encodes the largest miRNA mega-cluster in the mammalian genome, with possible roles transcending PI3K-AKT-mTOR regulation[83,84] (Figure 6F). RNA-seq read counts identified 4 microRNAs from within this locus (miR-770, miR-433, miR-127 and miR-370) reproducibly over-represented in PLAG1-S^{OE} cells (Figure 6G). Comparison of experimentally-supported miRNA targets to transcripts down-regulated in PLAG1-S^{OE} HSPC found the highest overlap for miR-127 (Figure S6F)[85,86], specifically including genes involved in complex cap-dependent translation and RNA and peptide metabolic processing (Figure 6H), providing impetus to test its role downstream of PLAG1-S. Simultaneous ectopic expression of PLAG1-S and an inhibitory miR-127-5p sponge[87,88] netted significantly reduced CD34$^+$ output (Figure 6I, S6G-H), and in the two CBs where sample amounts supported testing OP-Puro incorporation this was associated with an increase in protein synthesis (Figure S6I). Finally, overexpression of miR-127 enhanced CD34$^+$ output while imparting reduced levels of protein synthesis (Figure 6J, Figure S6J-L). Together these results suggest that miR-127 partially contributes to the effects of PLAG1-S in promoting a specific translational state and primitive cell maintenance.

MYC-induced translation impairs PLAG1-S-mediated stemness in human HSPC

MYC is a potent tissue non-specific transcriptional activator of cytoplasmic translation and nuclear ribosome biogenesis[89]. In the murine context Myc deletion impairs hematopoietic differentiation[90-92], and Myc expression is concomitantly activated with translation machinery when murine HSC exit dormancy[93]. Recent findings in cultured human HSPC also establish that MYC drives their *ex vivo* activation via promotion of anabolic programs[56]. Therefore, we next investigated whether PLAG1-S acts through repression of MYC, and whether MYC-mediated activation of translation could influence the ability of PLAG1-S to promote stemness.

Neither c-MYC protein levels, post-translational regulation nor the expression of MYC target genes are reduced in PLAG1-S^{OE} HSPCs (See Supplemental Results, Figure S7A-D). In fact, in contrast to diminished cytosolic ribosome expression MYC-regulated nuclear ribosome assembly targets trend upwards in PLAG1-S^{OE} HSPCs (Figure 7A), altogether suggesting PLAG1-S acts autonomously of MYC repression. As such, modulation of MYC expression could serve as a tool to independently activate translation rates as a means to query the dependency of PLAG1-S-enforced stemness on its attenuation of protein synthesis. Indeed modest c-MYC overexpression via the PGK promoter endowed a 25% increase in OP-Puro incorporation by Lin$^-$CD34$^+$ cells (Figure S7E-F). Next, BFP$^+$GFP$^+$ PLAG1-S and c-MYC co-overexpressing Lin$^-$CD34$^+$ cells and their control counterparts were assessed for primitive cell maintenance *ex vivo* (Figure 7B-C). Consistent with other reports[56,90-92], relative to control ectopic c-MYC independently promotes hematopoietic differentiation, as measured by loss of CD34$^+$ and gain of CD33$^+$

cells (Figure 7D-E, blue vs. red). Over culture c-MYC^{OE} cells also become enlarged relative to control (Figure 7F, blue vs. red) and display the highest rates of active translation (Figure 7G), We observed that PLAG1-S^{OE} significantly reduced protein production rates in c-MYC^{OE} cells (Figure 7G), and concomitantly countered c-MYC-induced cellular enlargement and pro-differentiative phenotypes (Figure 7D-E, F top, blue vs. green). Finally, after 7 days we observed that cells co-overexpressing PLAG1-S and MYC have significantly reduced primitive cell output relative to cells solely overexpressing PLAG1-S (Figure 7D-E, bottom, green vs. grey). Together these findings provide important proof of principle that dampened translation is key to the HSC-supportive programming imparted by PLAG1-S.

Discussion

We identify PLAG1-S as a novel positive regulator of human HSC dormancy and self-renewal. We demonstrate that PLAG1-S enacts multifaceted and combinatorial programs to endow HSPCs with an *in situ*-like rate of protein production and simultaneously restrain growth, proliferation, differentiation and death to ultimately enhance human HSC preservation and function in stimulatory culture and transplantation settings.

The role of PLAG1 in supporting healthy HSC self-renewal is strikingly at odds with its reported functions in oncogenic contexts. In a murine cbfß-translocated AML model ectopic PLAG1 promotes proliferation and its elevation via lentiviral insertions was one of several co-occurring molecular abnormalities associated with the onset and progression of primate myelodysplastic clonal hematopoiesis [94,95], phenotypes notably absent upon PLAG1-S overexpression in the background of healthy human HSPCs. Additionally, effectors downstream of PLAG1-IGF2 reported to support solid tumors[75-77,96] appear inert or repressed in PLAG1-S^{OE} HSPCs, implicating context-specific counter-acting mechanisms. It is particularly interesting that 4EBP1 displayed reduced activation in this setting, given that phospho-4EBP1/2 is currently the most predictive indicator of translation levels in murine HSC[97]. 4EBP1 phosphorylation is unlikely however to fully account for the dampened translation observed in PLAG1-S^{OE} HSPC, where together with multiple functional nodes, including binding and modulation of RP genes and miR-127, PLAG1-S consolidates the restraint of translation to enhance HSC function. Interestingly, rapamycin treatment of PLAG1-S^{OE} HSPC has an additive effect on diminished protein production, possibly due to the preferential inhibition of mTOR-RPS6K1 over mTOR-4EBP1[98]. However, the similar CD34^{+} cell growth dynamics upon AKT inhibition without further reduction of translation also suggests the possible contribution of other dormancy-promoting processes[99]. Although neither AKTi nor rapamycin enhanced total CD34^{+} cell output with PLAG1-S^{OE}, the elevated proportion of CD34^{+} cells from these cultures could suggest their functional nature differs from vehicle-treated cells. As such an interesting question for future investigation is whether pharmacological inhibition of AKT, mTOR or

other pathways in PLAG1-S^{OE} HSPCs could further enhance their *in vivo* repopulating fitness, as has been shown for untransduced CB[10,12,80,81].

Insights garnered from murine models have demonstrated that HSCs strictly control their protein production[37]. Hyperactivation of protein biosynthetic processes can lead to murine HSC impairment or depletion by driving their dormancy and quiescence exit, differentiation, and compromising their proteome integrity[38,42,93]. We now address these phenomena from a human perspective, adding activation of protein synthesis as a selective and robust feature of the molecular summary of compromised human HSC function upon culture-induced stimulation that is commonly required for clinical applications. By preceding cell division kinetics the proteostatic response likely also acts as an early determinant of cell fate[100], and is thus an important but underappreciated checkpoint for therapeutic procedures. The intersection of translation, dormancy and stemness being elucidated in model stem cells is mirrored in PLAG1-S^{OE} human HSPC where concurrent with diminished translation we observe reduced differentiation, enlargement, division, death and enhanced self-renewal; and by toggling c-MYC-driven protein production as a molecular tool we provide proof-of-principal that diminished translation is an essential modality by which PLAG1-S enhances HSPC output. Together our results forward the notion that the physiological importance of low translation rates in homeostatic niche-associated HSC can be harnessed for therapeutic benefit. To this point, rebalancing proteostasis in murine satellite cells and HSCs by enacting stress-responsive effectors can improve their long-term regenerative capacities[35,41,42]. Importantly however, ectopic PLAG1-S does not enact the ISR and depresses UPR signatures suggesting that by directly tuning the translation machinery PLAG1-S pre-empts and averts pro-apoptotic branches of stress signaling[40]. Others have also demonstrated that translation inhibitors can effectively eliminate primitive leukemic cells while sparing healthy HSC[101,102]. Together with our findings, this highlights the possibility of achieving a stem cell advantage by optimizing the timing and dosage of such compounds. The recent success of transient mRNA delivery systems[103] also provides an exciting opportunity through which the rapid pro-stem effect of PLAG1-S could be realized to its full advantage. Our identification of ectopic PLAG1-S as a highly context-selective regulator of protein synthesis also provides impetus for its future investigation in physiological contexts as a regulator of HSC translation, and as a modulator of translation and/or stemness in other primitive cell settings where its expression appears enriched[83,104-106]. Lastly, our findings underscore that addressing the current deficit in our understanding of translation dynamics and its regulators in human HSCs *in vivo* when subject to demands of disease or injury could substantively inform future HSC-focused regenerative therapies.

Altogether our discovery and characterization of PLAG1-S as a novel regulator of human HSC dormancy and self-renewal has derived insights germane to the appreciation of translation control in

determining human HSC fate and function, and highlights the promise of exploiting regulators of this fundamental feature of stem cell physiology to enhance regenerative therapies.

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Authorship Contributions

A.K.C and M.S.B. designed and performed experiments, analyzed and interpreted results. J.X., H.T.T.C, and S.R. assisted with cord blood experiments. A.K.C, H.T.T.C. and V.V. under supervision of G.D.B. performed RNA-seq data analysis. G.K. under supervision of E.L. and J.E.D. assisted with CUT&RUN and microRNA experiments. S.A.M. under supervision of D.D. performed CUT&RUN data analysis. A.K.C. and K.J.H. wrote the manuscript and all authors reviewed the manuscript. K.J.H. conceived and supervised the project, and designed and interpreted results.

Conflict of Interest Disclosures

The authors declare no competing financial interests.

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Figure Legends

Figure 1: PLAG1 is enriched and essential in human HSC. (A) Schematic of Lin^-CD34^+ CB HSPC *in vitro* and *in vivo* functional assay timelines and lentivectors used for PLAG1 knockdown. **(B)** Primary CFU output by Lin^-CD34^+ HSPC expressing control or PLAG1-targeting hairpins. (**C)** Cumulative *in vitro* $CD34^+$ cell fold change of cultured of Lin^-CD34^+ HSPC expressing 1shPLAG1 or control hairpins. **(D)** GFP^+ engraftment in the uninjected femur of primary NSG mice 6 weeks after xenotransplantation of Lin^-CD34^+ cells expressing either 1shPLAG1 (n=4) or control (n=6) hairpins normalized to input % GFP^+ levels. **(E)** GFP^+ engraftment in the injected femur and bone marrow of primary NSG mice 16 weeks after xenotransplantation of Lin^-CD34^+ cells expressing either 1shPLAG1 (n=4) or control (n=5) hairpins normalized to input % GFP^+ levels. **(F)** Primary CFU output by Lin^-CD34^+ HSPC expressing control or a second (2sh) PLAG1-targeting hairpin. **(G)** GFP^+ engraftment in the injected femur and bone marrow of primary NSG mice 16 weeks after xenotransplantation of Lin^-CD34^+ cells expressing either 2shPLAG1 (n=3) or control (n=6) hairpins normalized to input % GFP^+ levels.
Data is presented as average +/- SEM unless otherwise indicated. Each point represents one mouse or an individual CB unit. *** $p<0.005$, ** $p<0.01$, * $p<0.05$. See also Supplemental Figure 1.

Figure 2: PLAG1-S is a positive regulator of human HSPC fitness. (A) PLAG1 and MSI2 transcript expression in human bone marrow cell populations determined by single-cell RNA-seq[54]. **(B)** MSI2

protein expression measured by immunofluorescence microscopy in PLAG1-S overexpressing Lin^-CD34^+ cells. **(C)** Change in variance-stabilizing transformed (vst) PLAG1 transcript expression in Lin- cord blood cells cultured for 2 or 4 days showing the p value from one-tailed Student's t-test[39] and in 72 hour-cultured long-term ($Lin^-CD34^+CD38^-CD45RA^-CD90^+CD49f^+$) CB HSC showing the p value from one-tailed Student's t-test and differential expression from DEseq analysis[56]. **(D)** Schematic of Lin^-CD34^+ CB HSPC *in vitro* and *in vivo* functional assay timelines and lentivectors used for overexpression of PLAG1 protein isoforms. **(E)** Primary CFU output by BFP^+ Lin^-CD34^+ cells overexpressing PLAG1-A, B or S or Luciferase control (n=3). **(F)** Secondary CFU replating efficiency (for each condition 12 GEMMs from three distinct CB units were replated into new wells. Negative indicates no secondary colonies were derived from the primary GEMM, Positive indicates at least one secondary colony was derived from the primary GEMM) and the total number of secondary colonies on positive plates with images of representative primary GEMM colonies used. **(G)** Cumulative *in vitro* total nucleated cell (TNC) and **(H)** $CD34^+$ cell fold change of cultured of Lin^-CD34^+ cells overexpressing PLAG1-A (n=3), B, or S or Luciferase control (n=6). **(I)** Frequency of CD34 positivity in PLAG1-A (n=3), B, or S or Luciferase control (n=6) overexpressing cultures after 4 and 7 days *ex vivo*. **(J)** Representative flow plots and quantification relative to input proportions of BFP representation in $CD45^+$ human grafts in bone marrow of primary NSG mice 16 weeks after receiving Lin^-CD34^+ cells overexpressing either PLAG1-S or Luciferase control (n=6). **(K)** Representative flow plots of input and output BFP fluorescence intensity and quantification of output/input BFP median fluorescence intensity in bone marrow of primary NSG mice 16 weeks after receiving Lin^-CD34^+ cells overexpressing either PLAG1-S or Luciferase control (n=6).

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

Figure 3: PLAG1-S overexpression promotes self-renewal of long-term human HSC. (A) Schematic of primary and secondary xenotransplantation in limiting dilution format. **(B)** Representative flow plots of human $CD45^+BFP^+$ multilineage ($CD33^+$, $CD19^+$) engraftment of primary recipient mice in injected femur. Percent human $CD45^+BFP^+$ engraftment in injected femur 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. **(C,D)** Quantification of HSC frequency by ELDA[45] of injected femur of primary recipient mice. Shaded area under the curve represents 95% confidence interval of HSC frequency. **(E)** Percent human $CD45^+BFP^+$ engraftment in injected femur of secondary recipient mice across multiple cell input doses. Dashed line indicates cut off for calling engraftment, which was the same as for primary mice. **(F,G)**

Quantification of HSC frequency by ELDA of injected femur or uninjected bone marrow of secondary recipient mice and of *in vivo* expansion. Shaded area under the curve represents 95% confidence interval of HSC frequency. 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)[47,107], and *in vivo* expansion is measured as the fold difference of total BFP^+ HSC in donor mice relative to total day 0 HSCs initially transplanted into the 6 donor mice.

Data is presented as average +/- SEM unless otherwise indicated. Each point represents one mouse. *** $p<0.005$, ** $p<0.01$, * $p<0.05$. See also Supplemental Figure 3.

Figure 4: PLAG1-S enforces a pro-HSC transcriptional state. (A) Loci annotations and distribution of PLAG1-S binding sites in the Lin^-CD34^+ genome identified by CUT&RUN. **(B)** Enriched motifs among PLAG1-S genomic binding sites determined by HOMER indicating the % of PLAG1-S targets bound to the consensus and p-value of the enrichment relative to genome-wide background occurrence of the consensus. **(C)** Volcano plot of differential gene expression in PLAG1-S overexpressing Lin^-CD34^+ cells. Red- or blue-coloured genes are significantly changed by adjusted p value< 0.05 and shaded dark or light based on PLAG1-S binding status. **(D)** PLAG1-S overexpression and shPLAG1 transcriptomic alignment to DMAP signatures of hematopoietic compartments[49]. Numbers above or below the bars indicate the empirical p value determined based on the percentage of times for which the observed value (set of up- or down-regulated genes) was as large or larger in that population than random values (equal number of randomly selected genes) based on 1,000 trials. **(E)** Enrichment map of significantly enriched gene sets (FDR<0.1) in PLAG1-S^{OE} Lin^-CD34^+ cells compared to control. Genes bound by PLAG1-S in Lin-CD34+ cells (CUT&RUN q-value cutoff of 0.05) are intersected to gene sets by Mann-Whitney U test ($p<0.05$) and the width of orange edges correlates with increasing statistical significance of the overlap. Node size reflects the number of genes in the gene set. **(F)** 41 out of 46 gene sets from the "Establishment Protein Localization Translation" cluster that are over-represented among PLAG1-S genomic binding sites (g:Profiler FDR <0.1), and the list of bound leading-edge genes driving negative enrichments in this cluster.

See also Supplemental Figure 4.

Figure 5: PLAG1-S dampens protein synthesis and promotes dormancy in stimulated human HSPC. (A) OP-Puro incorporation dynamics measured as median fluorescence intensity (MFI) in cultured Lin^-CD34^+ cells with representative flow cytometry plots (n=5 for 0 and 24h; n=3 for 4, 48 and 72h). Black and blue asterisks denote statistical significance relative to previous timepoint or T0, respectively. **(B)** Fold difference of OP-Puro MFI relative to T0 in cultured Lin^-CD34^+ compared to Lin^-

$CD34^-$ CB fractions (n=4 for 24 hours, n=2 for 48 hours). Blue statistics are relative to 1x levels at T0 and black statistics are between cell types at matched time points. **(C)** OP-Puro incorporation by PLAG1-S^{OE} and control Lin^-CD34^+ cells on day 4 of *ex vivo* culture (n=8). Data from three experiments normalized to the average MFI in control cells per experiment. **(D)** Reduced size of PLAG1-S^{OE} Lin^-CD34^+ cells on day 4 of *ex vivo* culture determined by flow cytometric MFI of FSC-H profiles (n=7, top) and immunofluorescence microscopy (bottom, each point is a single cell; Scale bar = 25um). **(E)** Cell cycle analysis by Hoechst and Ki67 staining of PLAG1-S^{OE} and control Lin^-CD34^+ cells on days 4 and 7 of *ex vivo* culture (n=3). **(F)** Representative flow plots for PLAG1-S^{OE} and control Lin^-CD34^+ cells stained for 7-AAD and Annexin V with apoptosis measurements of surface positivity of Annexin V on day 4 (n=5) and day 7 (n=4) of *ex vivo* culture. **(G)** Heatmap of log2FC of transcripts coding EIF2 subunits (left) and intracellular flow cytometric measures of EIF2S1 protein expression (n=4) in PLAG1-S^{OE} relative to control Lin^-CD34^+ cells on day 4 of *ex vivo* culture (right). **(H)** GSEA of the PLAG1-S^{OE} transcriptome to curated targets of ATF4 generated by Han et al. (2012)[74] and used by Van Galen et al. (2018)[73] and FPKM heatmap of ATF4 targets differentially expressed in PLAG1-S^{OE} HSPC (p.adj<0.1). **(I)** Negative enrichment of gene sets related to unfolded protein response (p<0.05).
Data is presented as average +/- SEM unless otherwise indicated. Each point represents an individual CB unit otherwise indicated. *** p<0.005, ** p<0.01, * p<0.05. See also Supplemental Figure 5.

Figure 6: PLAG1-S activates imprinted loci to support human HSPC. (A) Heatmap of top 10 differentially expressed transcripts in the transcriptome of PLAG1-S^{OE} Lin^-CD34^+ HSPC. **(B)** Intracellular flow cytometry of components of the PI3K signaling pathway, including phospho-S473 AKT, phospho-S2448 mTOR and phospho-Thr37/46 4EBP1, 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 (n=3, ph-4EBP1 n=5). **(C)** Total nucleated cell (top) and $CD34^+$ cell (bottom) fold change in $Lin^-CD34^+BFP^+$ cultures overexpressing either PLAG1-S or Luciferase control and treated with 50nM rapamycin (RAPA), 1uM AKT inhibitor (AKTi) or vehicle (DMSO) (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. **(D)** CD34 positivity in PLAG1-S^{OE} or control cells following 4 and 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. **(E)** OP-Puro incorporation by PLAG1-S^{OE} cells cultured in the presence of RAPA, AKTi or vehicle on day 4 of culture (n=4). **(F)** Schematic of the imprinted human DLK1/MEG3 locus which encodes miRNA mega-clusters miR127/136 (7 miRNAs) and miR-379/410 (39 miRNAs). **(G)** RNA-seq read tracks for microRNA transcripts from this locus detected in PLAG1-S^{OE} HSPC. **(H)** Overlap of the PLAG1-S overexpression gene set enrichment map (p<0.025) to signatures of miR-127-5p and miR-127-3p

validated targets (Mann-Whitney U test, $p<0.05$). **(I, J)** Schematic of lentivectors used for dual PLAG1-S overexpression and miR127-5p inhibition via a sponge consisting of multiple bulged 26-mer target sequences (miR127TB), or miR127 overexpression. **(I)** $CD34^+$ cell fold change *ex vivo* when PLAG1-S and the miR127-5p inhibitor are co-expressed in Lin^-CD34^+ cells (n=3). **(J)** $CD34^+$ cell fold change *ex vivo* when miR127 is overexpressed in Lin^-CD34^+ cells (n=3); and OP-Puro incorporation Lin^-CD34^+ cells overexpressing miR127 (n=3).

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

Figure 7: MYC-induced translation impairs PLAG1-S-mediated stemness in human HSPC. (A) Up- (red) or down- (blue) regulation of MYC ribosome biogenesis targets in PLAG1-S^{OE} HSPC. **(B)** Schematic of PLAG1-S and c-MYC overexpression lentivectors. **(C)** Representative sorting gates for dual-overexpression of PLAG1-S and c-MYC or controls. **(D)** CD34 (n=4) and **(E)** CD33 (n=3) positivity over 4 and 7 days of *ex vivo* culture. **(F)** Cell size determined by flow cytometric MFI of FSC-H (n=3-4) on day 4 and 7 of *ex vivo* culture. **(G)** OP-Puro incorporation on day 4 of *ex vivo* culture (n=4).

Data is presented as average +/- SEM unless otherwise indicated. Each point represents one mouse or an individual CB unit. Blue line is t-test relative to MYC alone, Black line is t-test relative to PLAG1-S alone *** $p<0.005$, ** $p<0.01$, * $p<0.05$. See also Supplemental Figure 7.

Figure 1

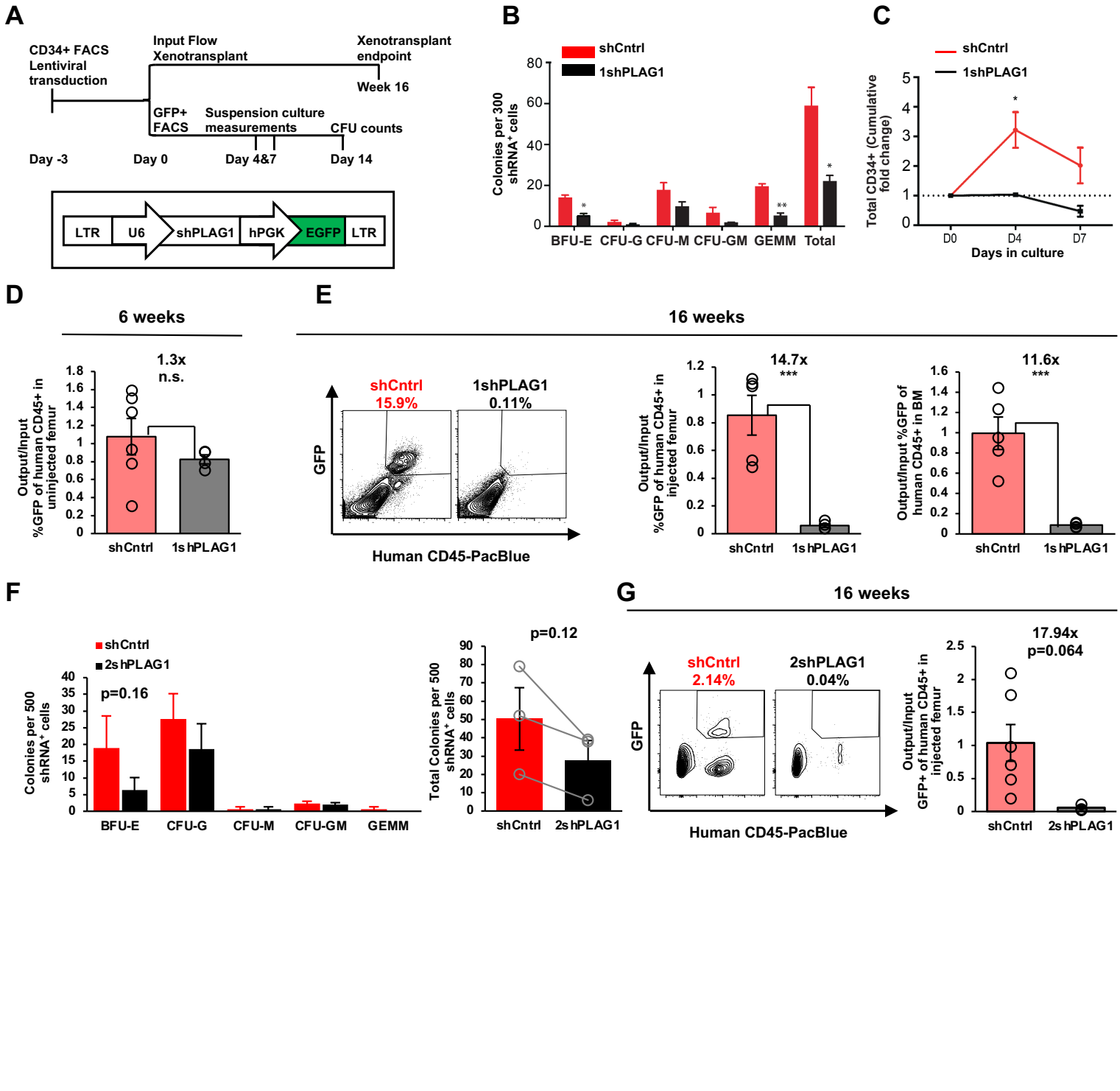


Figure 2

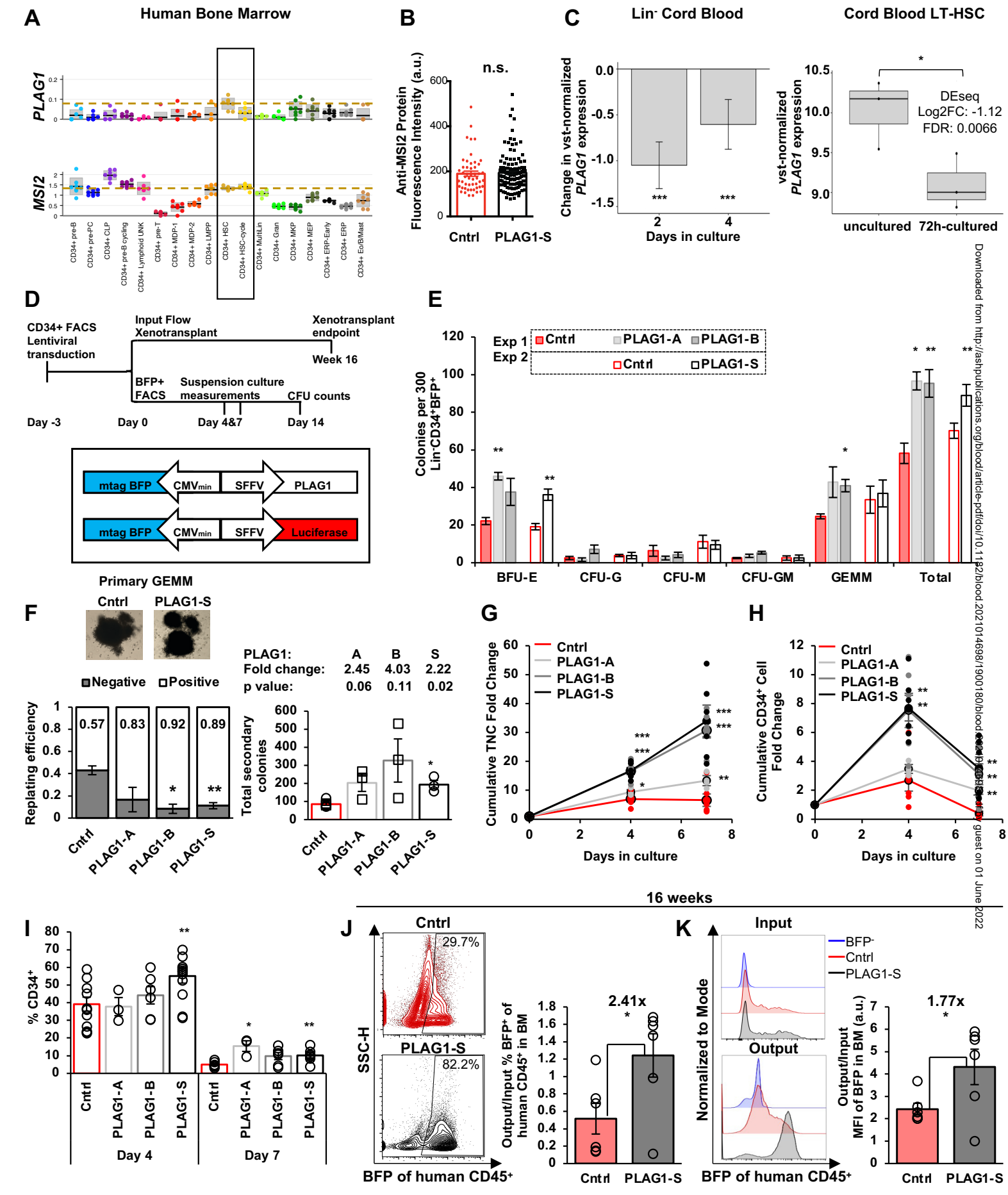
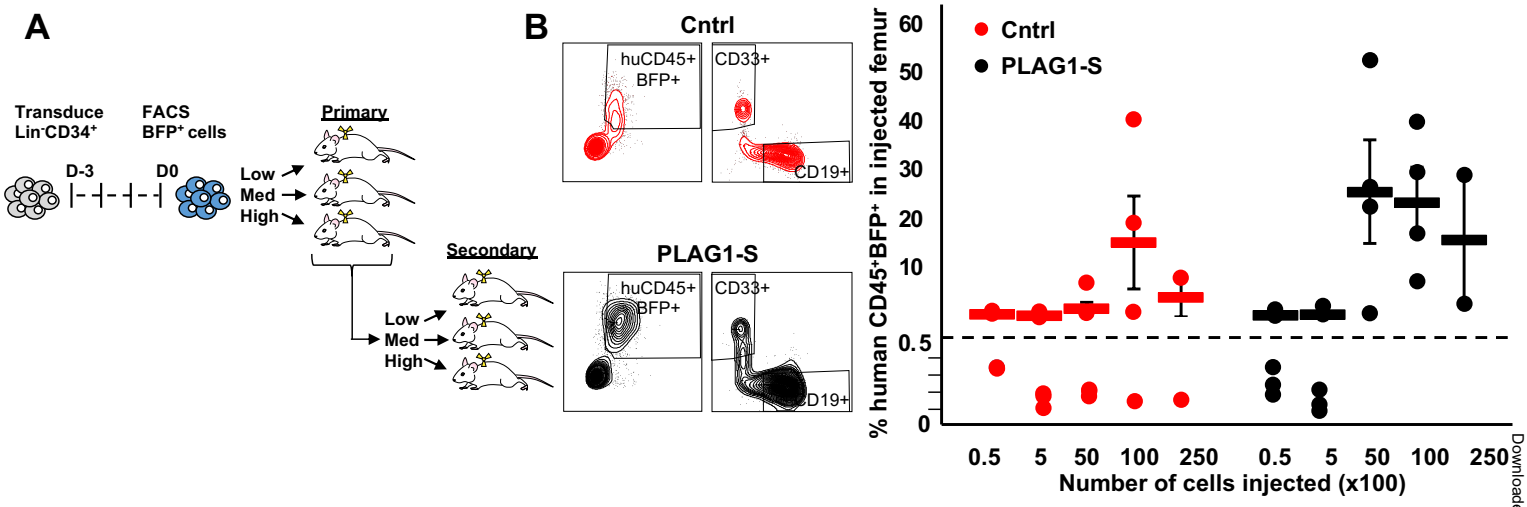
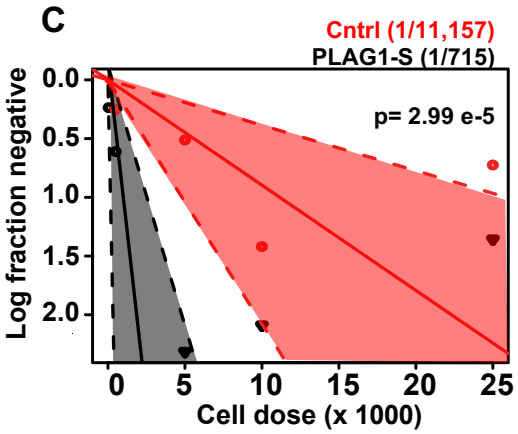


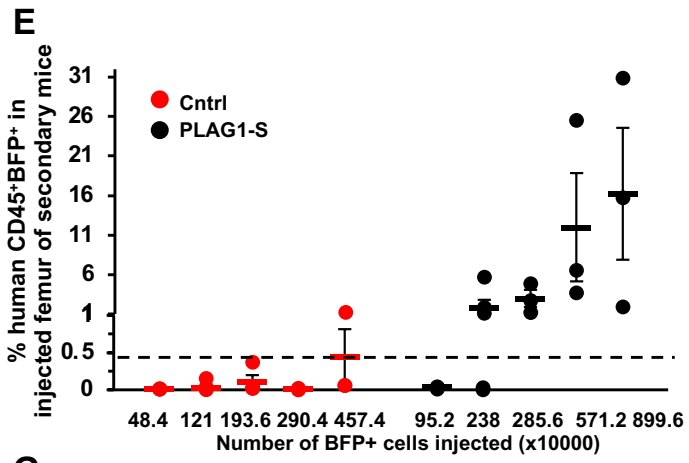
Figure 3

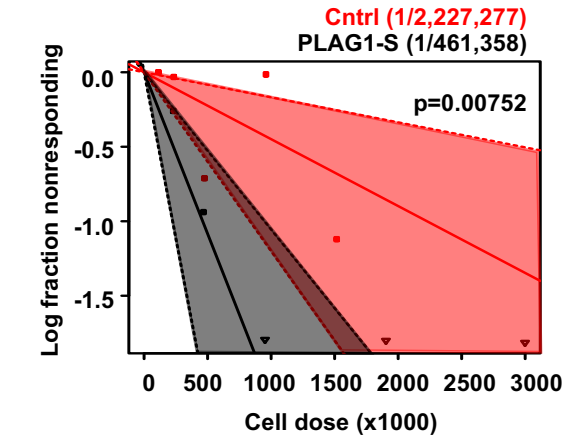




D

Vector	Total BFP+ cells	BFP+ cell dose/mouse	Number of mice transplanted	Number of mice with multilineage engraftment	BFP+ HSC frequency (0.95 confidence interval)	Number of BFP+ HSC
Cntrl	117750	25000	2	1	Lower: 1/28570	10.55
		10000	4	3	**Estimate: 1/11157**	
		5000	5	2	Upper: 1/4811	
		500	5	1		
		50	5	0		
PLAG1-S	117750	25000	2	2	Lower: 1/2225	164.69
		10000	4	4	**Estimate: 1/715**	
		5000	5	5	Upper: 1/230	
		500	5	2		
		50	5	1		





G

Vector	Total BM from 6 mice	Total BM pooled from 6 donor primary mice	% BFP+	Total BFP+ BM pooled from 6 donor primary mice	BFP+ cell dose	Number of secondary mice transplanted	Number of secondary mice with multilineage engraftment	BFP+ HSC frequency (0.95 confidence interval)	Number of BFP+ HSC transplanted into secondary mice	Total BFP+ HSC in 6 donor primary mice	# BFP+ HSC transplanted into 6 donor primary mice	Total BFP+ HSC Fold Change *in vivo*
Cntrl	810,000,000	91,800,000	12.1	11,107,800	4573800	3	2	Lower: 1/5952539	4.98	44.00	5.87	7.50
					2904000	3	0	**Estimate: 1/2227277**				
					1936000	4	2	Upper: 1/833386				
					1210000	5	0					
					484000	4	0					
PLAG1-S	810,000,000	87,800,000	23.8	20,896,400	8996400	3	3	Lower: 1/947791	45.29	417.85	91.60	4.56
					5712000	3	3	**Estimate: 1/461358**				
					2856000	3	3	Upper: 1/224577				
					2380000	5	3					
					952000	4	1					

Figure 4

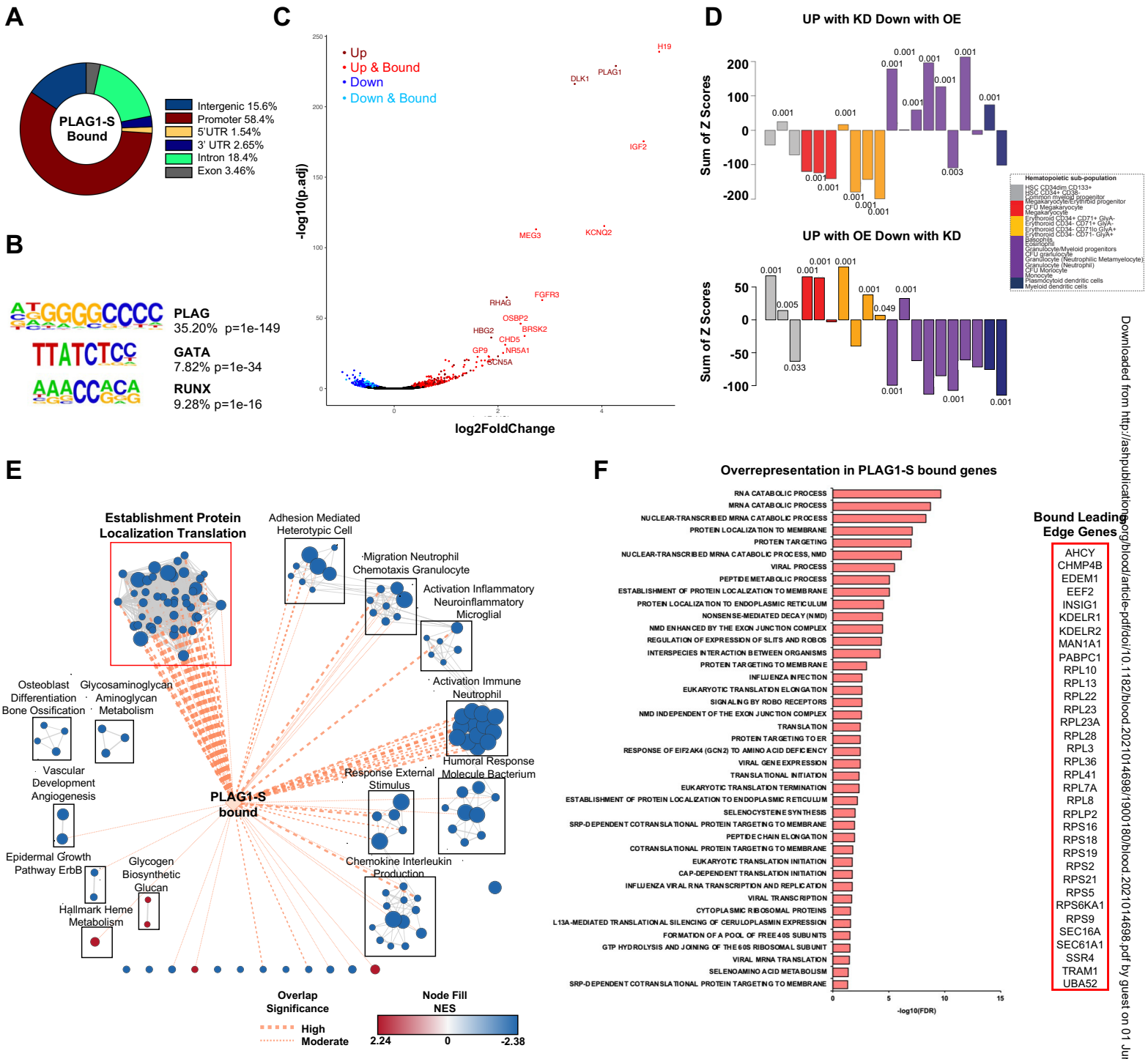


Figure 5

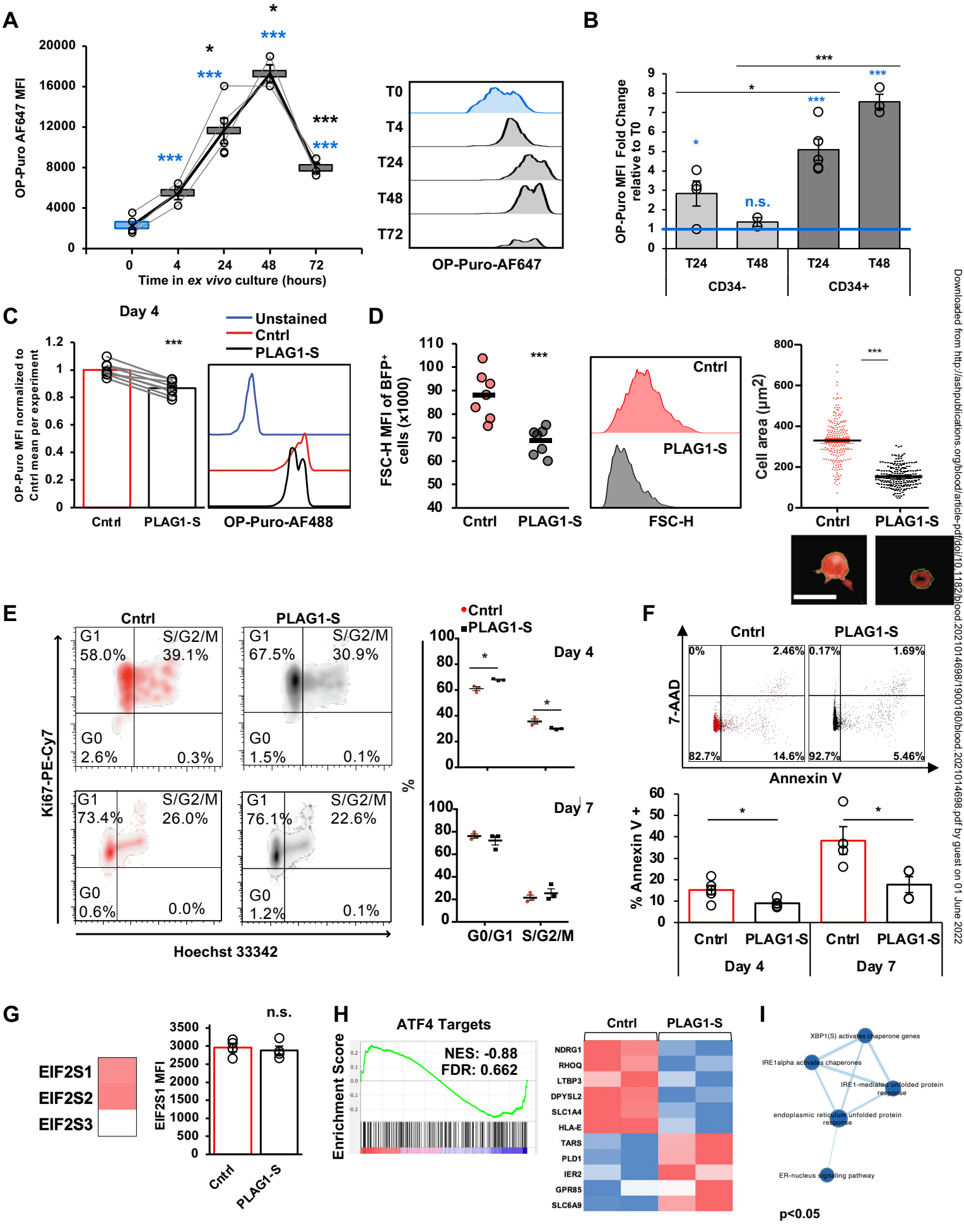


Figure 6

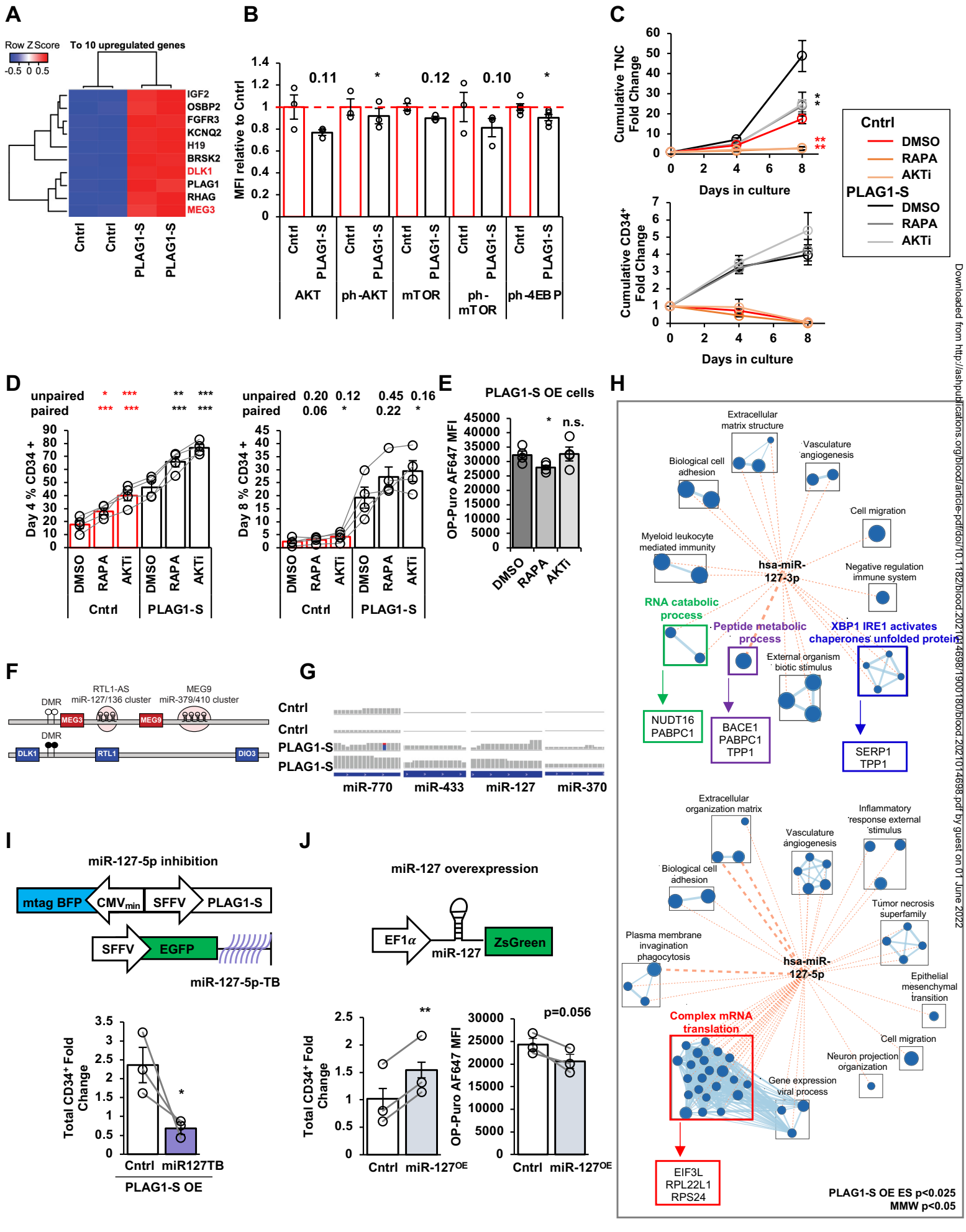


Figure 7