Memory of Divisional History Directs the Continuous Process of Primitive Hematopoietic Lineage Commitment

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https://doi.org/10.1016/j.stemcr.2020.03.005

SUMMARY

Hematopoietic stem cells (HSCs) exist in a dormant state and progressively lose regenerative potency as they undergo successive divisions. Why this functional decline occurs and how this information is encoded is unclear. To better understand how this information is stored, we performed RNA sequencing on HSC populations differing only in their divisional history. Comparative analysis revealed that genes upregulated with divisions are enriched for lineage genes and regulated by cell-cycle-associated transcription factors, suggesting that proliferation itself drives lineage priming. Downregulated genes are, however, associated with an HSC signature and targeted by the Polycomb Repressive Complex 2 (PRC2). The PRC2 catalytic subunits Ezh1 and Ezh2 promote and suppress the HSC state, respectively, and successive divisions cause a switch from Ezh1 to Ezh2 dominance. We propose that cell divisions drive lineage priming and Ezh2 accumulation, which represses HSC signature genes to consolidate information on divisional history into memory.

INTRODUCTION

Hematopoietic stem cells (HSCs) represent a critical source of regenerative cells for curative therapies for a vast array of immunohematological disorders. As such, understanding the mechanisms that regulate their regeneration and lineage commitment are important for the ex vivo production and expansion of cells for therapy. In vivo, adult HSCs reside in a quiescent state and proliferate infrequently in the absence of stress (Bernitz et al., 2016; Qiu et al., 2014; Wilson et al., 2008). When HSCs proliferate in vivo their regenerative potential progressively declines (Qiu et al., 2014). This phenomenon was previously proposed by the Generation-Age Hypothesis (Rosendaal et al., 1976, 1979), which states that HSC regenerative potential is determined by the number of its past divisions. While not explicitly stated, this hypothesis also implies that HSCs must store information about their divisional history as a form of memory, without which past divisions could not exert an effect on HSC function. Presently there is no explanation for how past cell divisions result in HSC functional decline or how memory of these divisions is stored.

The polycomb repressive complex 2 (PRC2) is canonically involved in the silencing of gene expression. It accomplishes this through the addition of di- and trimethylation of the tails of histone 3 at lysine 27 (H3K27me2 and H3K27me3), enabling chromatin compaction and gene silencing (Cao et al., 2002; Czermin et al., 2002; Kuzmichev et al., 2002; Margueron et al., 2008; Muller et al., 2002). Although PRC2 is composed of several proteins, the four major components are EED, SUZ12, EZH1, and EZH2. EZH1, and EZH2 are the catalytic subunits, and while both are capable of methylating H3K27, EZH1 performs this function weakly and mostly in the context of EZH2 loss (Margueron et al., 2008; Shen et al., 2008; Xu et al., 2015). Both require occupation within the PRC2 complex for functional activity, but they rarely interact with each other (Margueron et al., 2008; Shen et al., 2008; Xu et al., 2015), suggesting mutual exclusivity in PRC2 complex occupancy and competition with each other for interaction with other PRC2 complex members. Indeed, Ezh1 and Ezh2 tend to have opposite expression patterns. Ezh2 is frequently associated with a proliferative state (Attwood et al., 2005; Bracken et al., 2003), whereas Ezh1 is found in post-mitotic or quiescent populations including myotubes, aging kidney, and HSCs (Hidalgo et al., 2012; Margueron et al., 2008; Mousavi et al., 2012; Stojic et al., 2011). While EZH1/2 are primarily associated with gene repression, an increasing body of evidence shows that they both play non-canonical roles in gene activation, indicating complex and context-specific functional behavior.
Gene Expression Changes with Divisional History Are Progressive

To gain molecular insight into how memories of divisional history are stored, we examined gene expression changes that occur as HSCs divide within a young HSC population. For RNA-seq analysis, we used an H2BGFP Tet-off hematopoietic stem and progenitor (HSPC)-specific label-retaining system (Bernitz et al., 2016; Qiu et al., 2014) to sort the HSC compartment (Lin–SCA-1–C–Kit–CD48–FLK2–CD150+) into four subpopulations based on GFP-label retention, which represent incremental increases with respect to divisional history (Figures 1A and S1). In this experimental setup, cells labeled GFP4 are considered dormant and retain the greatest regenerative potential, whereas the GFP0–GFP1 population has the greatest divisional history and the smallest regenerative potential. Our analysis identified 2,715 differentially expressed genes (DEGs) across the four populations (false discovery rate <0.05 with fold change >1.5, Table S1). Hierarchical clustering revealed that nearly all DEGs grouped into two categories that either increase or decrease in expression as HSCs divide (hereafter referred to as divisional history upregulated/downregulated [DH-up and DH-down] genes, Figure 1B and Table S2). These changes are progressive and indicate a continuum of gene expression changes that occur with divisions, rather than discrete stages of differentiation. We compared these two gene sets with our previously published microarray expression data from Lin–SCA-1–C–Kit+ (LSK) cells also differing in divisional history (Qiu et al., 2014), and found that the DH-up and DH-down genes were largely consistent with the previous analysis (Figures S2A and S2B).

Next, we computed enriched gene ontology (GO) terms for the genes that change with divisional history. As anticipated, DH-up genes are enriched for terms associated with the cell cycle (Figure 1C). To better understand changes in processes other than the cell cycle, we excluded genes associated with the GO terms “mitotic cell cycle,” “nuclear division,” “DNA replication,” and “cell cycle phase transition” (GO:0000278, GO:0000280, GO:0006260, and GO:0044770, respectively) from the list of DH-up genes to generate a cell-cycle-depleted gene list. This list included terms associated with mRNA processing, protein translation, metabolic, and biosynthetic processes (data not shown), consistent with published literature on HSC exit from dormancy (Cabezas-Wallscheid et al., 2017). However, we also found enrichment in GO terms associated with cell activation and diverse mature lineage fates (Figure 1D). We then examined the expression of genes associated with lineage development and maturation. Many of these genes increase their expression with cell divisions, albeit at low levels (Figures 2A and 2B). Transcription factor (TF) chromatin immunoprecipitation (ChIP)-x enrichment analysis (ChEA) identifies E2F1, E2F4, FOXM1, and MYC as significantly enriched near the promoters of the genes from the cell-cycle-depleted gene list (Figure 1E). E2Fs, FOXM1, and MYC are proliferation-associated TFs critical for cell-cycle progression (Bretones et al., 2015; Trimarchi and Lees, 2002; Wierstra and Alves, 2007; Wu et al., 2001), and cMyc is known to be essential for HSC differentiation (Wilson et al., 2004). Importantly, all of these TFs increase their expression progressively with divisional history (Figure 1F). Cumulatively, these data indicate that proliferation-associated TFs drive HSC activation and lineage priming, suggesting that early lineage priming of HSCs is a function of cell divisions.
Next, we examined the expression of DH-down genes. As DH-up genes were associated with cell activation and lineage priming, we hypothesized that DH-down genes were associated with an HSC “state.” We compared both gene sets with a published gene signature for HSCs (Cabezas-Wallscheid et al., 2014) and found a significant overlap between...
DH-down genes and HSC signature genes (Figure 1G). Gene set enrichment analysis (GSEA) of the molecular overlap stem cell signature (Wilson et al., 2015) also shows a significant enrichment toward genes highly expressed in the GFP4 population that decrease with divisions (Figure S2C). Additionally, both cell surface markers used to enrich for stem cell identity and HSC-specific reporters (Acar et al., 2015; Cabezas-Wallscheid et al., 2017; Chen et al., 2016; Gazit et al., 2014; Sawai et al., 2016) are highly expressed in the dormant GFP4 population, and progressively decrease with divisions (Figure S2D) with the exception of Ctnnal1, which has no clear expression pattern across HSC divisions (Figure S2D).

Finally, comparison of gene signatures associated with HSC and multipotent progenitor (MPP) populations shows that MPP signatures are associated with high divisional histories, further indicating that differentiation is associated with progressive cell divisions (Figure S2E). Interestingly, MPP4s, reported as lymphoid-primed MPPs, are enriched for genes high in dormant cells. This suggests that fewer divisions are needed to generate MPP4 from HSCs, consistent with studies showing limited lineage priming in this population (Rodriguez-Fraticelli et al., 2018).
We next performed ChEA to identify factors regulating DH-down genes. Interestingly, many DH-down genes are targets of PRC2 members (Figure 1H). PRC2 is classically known to repress target gene expression, and the expression of several PRC2 components is enhanced during cell proliferation due to binding of their promoter regions by E2F proteins (Bracken et al., 2003). We also found that many PRC2 complex members increase their expression with divisional history (Figure 1I). These data suggest that cell divisions result in the progressive activation of PRC2 complex gene expression, which may play a role in silencing HSC signature genes that decrease in expression with each division.

Chromatin Marks Are Dynamic with Divisional History
To better understand the role of PRC2 in the regulation of HSC signature genes, we examined gene expression changes of PRC2 target genes with progressive HSC divisions. We extracted information from HSCs on the genome-wide distribution of the repressive mark H3K27me3, a mark specifically deposited by PRC2 (Sun et al., 2014). An examination of all H3K27me3 target genes, on average, showed no significant changes in their expression with divisional history (Figure 3B). However, clustering the H3K27me3 targets by expression patterns identified two groups that either increase or decrease in expression with progressive divisions (Figures 3A and 3B). Interestingly, this type of analysis based on divisional history reveals increasing expression of genes that are targets of PRC2-mediated repression in HSCs (Table S3). This trend is also observable with the active marks H3K4me3 and H3K27Ac (Figure 3 and Table S3). It is unlikely that these H3K27me3 targets are continuously methylated throughout divisional history while simultaneously changing their expression. Rather, it is more likely that different genes are targets for repression at specific divisional history stages. Collectively, this analysis suggests that chromatin mark deposition and removal may exhibit dynamic patterns with cell divisions.

We then took the reverse approach and looked for enrichment of activating and repressive chromatin marks on DH-up and DH-down genes. We found that DH-up genes are largely depleted of the repressive H3K27me3 mark but are enriched for the activating mark H3K27Ac (Figures 3C and 3D). In contrast, DH-down genes are enriched for H3K27me3 and depleted of H3K27Ac. These data corroborate the ChEA analysis identifying PRC2 complex members as potential regulators of DH-down genes (Figure 1H). H3K4me3 was not differentially regulated between DH-up and DH-down genes (Figures 3C and 3D),
consistent with the notion that H3K4me3 is an indicator rather than an instructor of transcription (Howe et al., 2017). Together, these data show that DH-down genes are enriched for targets of PRC2 H3K27me3 in HSCs.

The PRC2 Catalytic Subunit Ezh1 Activates while Ezh2 Represses HSC Signature Genes

Analysis of H3K27me3 in HSCs showed that this histone mark is dynamic with divisional history and that DH-down genes are enriched for targets of PRC2 repression. To better understand the dynamics of this process, we began by examining expression of the PRC2 catalytic subunits Ezh1 and Ezh2. Ezh1 and Ezh2 exhibit opposite expression patterns, with Ezh1 highest in dormant fractions and Ezh2 expression increasing with progressive cell divisions (Figure 1B). This is consistent with data indicating Ezh1 and Ezh2 correlate with quiescent and proliferative states, respectively (Margueron et al., 2008). To gain insight into how these different catalytic subunits regulate H3K27me3 deposition during different divisional history stages, we examined Ezh1−/− HSC microarray gene expression data (Hidalgo et al., 2012). Genes that increased in expression with Ezh1 knockout (hereafter referred to as “Ezh1 Repressed”) are associated with the GFP0–1 HSC fraction (Figures 4A and S4A) and are enriched for cell-cycle genes (Figure S4B). ChEA also revealed these genes to be targets of cell-cycle TFs FOXM1, E2F4, and P53, as well as of PRC1 and PRC2 members (Figure S4C). In contrast, genes with dampened expression in Ezh1−/− HSCs (hereafter referred to as “Ezh1 Activated”) were enriched in dormant HSCs (Figure 4A). Indeed, we found a significant overlap of Ezh1-Activated and DH-down genes (Figure 4B), many of which are important for HSC function (Cabezas-Wallscheid et al., 2014, 2017; Frelin et al., 2013; Ku et al., 2012; Miyamoto et al., 2007; Riddell et al., 2014; Wilson et al., 2015; Yalcin et al., 2008). The transcript of SCA-1, Ly6a, which highly correlates with stem cell function (Wilson et al., 2015), is included among Ezh1 Activated genes, and also shows stepwise decreases with progressive HSC divisions at the protein level (Figure S5).

We then examined the chromatin landscape of genes within this overlap. By simultaneously comparing Ezh1 Activated and DH-down genes with identified targets of Ezh1- and Ezh2-mediated H3K27me3 in HSPCs (Mochizuki-Kashio et al., 2015), we were able to dissect the specific roles of Ezh1 and Ezh2 in activating and repressing HSC signature genes. This comparison identified several overlapping groups, including three of particular interest: DH-down genes activated by Ezh1 that are not targets for H3K27me3 repression (group I), DH-down genes activated by Ezh1 that are targets for Ezh2-mediated H3K27me3 repression (group II), and DH-down genes that are Ezh2 H3k27me3 targets not regulated by Ezh1 (group III). Roughly half of the overlap between Ezh1 Activated and DH-down genes are targets of EZH2-specific H3K27me3 repression (group II, Figures 4C and 4D), suggesting that the switch from Ezh1 to Ezh2 with progressive divisions causes these genes to switch from an activated to repressed state (Figures 4C and 4D). Furthermore, EZH2-specific H3K27me3 targets include 19.8% of DH-down genes (groups II and III), many of which are important for HSC identity and function (Figure 4D; Cabezas-Wallscheid et al., 2014; Chen et al., 2016; Klaue et al., 2013; Qiu et al., 2014; Wilson et al., 2015). Analysis of the chromatin profiles of these three groups in total HSCs (HSCs not subdivided by divisional history) revealed that group II and group III genes have H3K27me3 peaks centered at their transcriptional start site, indicative of transcriptional repression (Figure 4E). In contrast, group I genes show no H3K27me3 enrichment above background levels (Figures 4E and 4F). Consistent with these data, genes in groups II and III have lower average expression levels in total HSCs than those in group I (Figure 4F). Cumulatively, these data suggest that EZH1 promotes the expression of genes important for the stem cell state, and EZH2 targets HSC signature genes for repression, a subset of which are activated by Ezh1. Thus, a divisional history-dependent Ezh1-to-Ezh2 switch could be involved in the suppression of HSC function as cells divide.

Inhibiting Ezh2 Prevents the Loss of Self-Renewal Potential with Progressive Divisions

Our data predict a genetic switch whereby Ezh1 represses cell divisions and activates key genes associated with HSC identity, while Ezh2 accumulates with progressive divisions to repress these HSC genes. To test these predictions, we inhibited Ezh2 activity during HSC growth in culture to find out whether regenerative loss with divisions could be prevented. We sorted either HSCs, gating on the highest 30% of SCA-1 expression to enrich for dormant cells (Figure S5A), or GFP4 HSCs from the 34/H2BGFP mice, and plated them in long-term culture (LTC) on AFT024 stroma with or without media containing the Ezh2 inhibitor (Figure S5A). AFT024 cells maintain HSCs in culture and enable their proliferation (Kokkaliaris et al., 2016; Moore et al., 1997), allowing us to test the effect of Ezh2 inhibition on regenerative potential after several rounds of division in LTC. The Ezh2 inhibitor GSK126 is over 150-fold more specific to Ezh2 than Ezh1 (McCabe et al., 2012), limiting the probability that an effect would be due to Ezh1 inhibition. After 4 weeks of primary culture, hematopoietic cells were harvested and replated on fresh stroma at limiting dilution without inhibitor, and the presence of cobblestone area-forming cells (CAFCs) was scored a week later. Similar cells were also plated into a colony-forming unit (CFU) assay to determine LTC-initiating cell numbers.
Adding GSK126 increased the frequency of regenerative, clonogenic cells that were present after 4 weeks in LTCs (Figures 5B and 5C), suggesting EZH2 inhibition prevents regenerative decline with progressive divisions.

To further test our predictions, we established a single-cell divisional history CFU assay. This assay tests the self-renewal capacity of single HSCs by examining the CFU potential of its progeny as a function of previous division in culture (Figure 5D). After sorting single HSCs and culturing for 8 days, colony diameter was measured as a surrogate for divisional history. Colonies of roughly 0.45 mm diameter contained approximately 600–700 cells, indicating that the initially sorted HSC divided at least nine times to produce colonies of this size. The entire colony was then harvested and transferred to methylcellulose medium and allowed to grow for another 7 days. At the end of the second round of culture, granulocyte, erythroid, macrophage, megakaryocyte (GEMM) colonies were quantified and compared with colon...
the size of the primary colony, giving a measure of regenerative potential as a consequence of proliferation in primary culture. We found that GSK126 did not alter clonogenicity or average colony size (Figures 5E and 5F) of colonies in primary culture. As expected, in secondary cultures colony-forming potential declined after a threshold of primary colony size of 0.5 mm (Figures 5G and 5H). Importantly, inhibiting EZH2 catalytic activity delayed the loss of secondary CFU potential with increased primary colony size (Figures 5G and 5H). Cumulatively, data from both the long-term cultures and single-cell colony assays indicate that EZH2 inhibition delays the loss of regenerative potential with successive divisions.

**DISCUSSION**

Understanding how to preserve stem cell function has been at the heart of HSC research for decades. Despite the wealth of information on individual genes, niches, or processes that act to preserve HSC function, little progress has been made in implementing this understanding to maintain and expand HSCs without causing regenerative decline. Studies focusing on the relationship between divisional history and function have revealed that increasing divisional history correlates with and causes decreased HSC regeneration (Beerman et al., 2013; Bernitz et al., 2016; Qiu et al., 2014; Walter et al., 2015), cumulatively
indicating that HSCs must store information about their past divisions. Thus, robust HSC expansion is difficult to achieve because enforcing HSC divisions results in the loss of their stem cell properties. However, to date no mechanistic link has been provided to explain how cell divisions negatively influence HSC regeneration. Here, we performed RNA-seq on HSC populations that differ only in their divisional history to help elucidate this link.

Our analyses indicate that increasing divisional history results in the progressive loss of HSC signature genes and progressive activation of genes broadly associated with proliferation and cellular activation. A model of progressive change over divisions is consistent with emerging notions of a differentiation continuum that occurs within the stem and progenitor compartment as cells make commitment decisions (Cabezas-Wallscheid et al., 2017; Velten et al., 2017), and contrasts with models in which cells transit through discrete states. Our data also support the body of work on HSC exit from dormancy that highlights the requirement of metabolic activation and cMyc expression to begin differentiation out of the stem cell compartment, indicating the importance of cell divisions in this process (Cabezas-Wallscheid et al., 2017; Signer et al., 2014; Wilson et al., 2004). Surprisingly, we find that even within the highly purified HSC compartment, genes associated with mature lineage identity increase in expression with progressive divisions. Indeed, recent work using single-cell RNA-seq indicates that lineage commitment is a progressive process whereby HSCs steadily acquire lineage fates along the continuum of low-primed undifferentiated states (Laurenti and Gottgens, 2018; Rodriguez-Fraticelli et al., 2018; Velten et al., 2017). Taken together, this suggests that cell divisions themselves begin the process of lineage priming at the level of the HSC and that divisional history is a major factor contributing to transcriptional heterogeneity.

Our analyses also implicate components of PRC2 as key molecular factors in the loss of HSC function with divisions. Different PRC2 components play both enhancing and repressive roles at different times during development and at different gene doses (Hidalgo et al., 2012; Kamminga et al., 2006; Lee et al., 2015; Lessard et al., 1999; Mochizuki-Kashio et al., 2011, 2015; Vo et al., 2018; Xie et al., 2014; Xu et al., 2015). Through compartmentalizing HSCs by divisional history, however, we are able to help clarify these complex phenomena. The expressions of many PRC2 components are activated by cell divisions (Bracken et al., 2003) and are classically associated with the repression of target gene expression. Consistent with these canonical patterns, we find increased expression of core PRC2 components Ezh2, Suz12, and Eed with HSC divisions. This is then correlated with a decline in expression of HSC signature genes, many of which are targets of EZH2-mediated H3K27me3 deposition. These results support work showing that Ezh2, Suz12, and Eed function to restrict HSC self-renewal and promote differentiation (Mochizuki-Kashio et al., 2011, 2015; Xie et al., 2014), whereby these genes would accumulate with divisions and suppress HSC signature genes. Indeed, by inhibiting the histone methyltransferase property of EZH2, we found we could delay the loss of secondary colony formation in a cell-division-dependent manner (Figure 5).

In contrast to these PRC2 members, Ezh1 expression exhibits the opposite pattern of expression and declines with divisions. This is consistent with findings indicating preferential Ezh1 expression in quiescent cell populations (Hidalgo et al., 2012; Margueron et al., 2008; Mousavi et al., 2012). Additionally, through cross-comparison of our data with gene expression data from Ezh1 knockout HSCs, our work implicates EZH1 as an important driver of genes promoting a primitive HSC state (Hidalgo et al., 2012). While gene activation is a less commonly described feature of PRC2 components, it is consistent with an increasing body of work indicating non-canonical roles of EZH1 in gene activation (Mousavi et al., 2012; Vo et al., 2018; Xu et al., 2015). In particular, we found that several genes important in retinoic acid signaling, recently shown to be critical to HSC maintenance (Cabezas-Wallscheid et al., 2017), are putative targets of EZH1 activation. This notion that EZH1 may activate HSC signature genes while EZH2 accumulates to suppress them also partially explains complex results with EZH2 loss in cancer. EZH2 loss of function is frequently associated with hematological transformation and clonal hematopoiesis (Bejar et al., 2011; Emst et al., 2010; Mochizuki-Kashio et al., 2015; Nikoloski et al., 2010). This suggests that EZH2 loss prevents the silencing of many potent HSC genes upon progressive divisions, many of which are associated with cancer progression and leukemia. This is consistent with data showing that leukemic transformation in Ezh2−/− cells is dependent on EZH1 functional activity (Mochizuki-Kashio et al., 2015), and with reports in early T cell precursor acute lymphoblastic leukemia showing that EZH2 is important for suppressing stem cell-associated genes (Danis et al., 2016). In light of our results, the data suggest that EZH2 accumulation with progressive divisions functions as a fail-safe to restrict the potential of stem cell-associated genes.

Recent work in both mouse and human HSCs found Ezh1-repressed multilineage potential during the emergence of HSC during development via direct occupancy and H3K27me3-associated repression of multipotency and lymphoid genes (Vo et al., 2018). While this appears to contradict our findings, it is known that embryonic and adult HSCs switch from a fetal to an adult program at 4 weeks after birth. This switch corresponds to a shift from proliferation to quiescence (Bowie et al., 2006),
SOX17 dependence to SOX17 independence (Kim et al., 2007), and Ezh2 dominance to Ezh1 dominance (Vo et al., 2018). Ezh2 can be phosphorylated to both modify its histone methyltransferase activity and switch its role as a transcriptional repressor to a transcriptional activator in a context-specific manner (Xu et al., 2012; Yan et al., 2016). It is conceivable that EZH1 could undergo similar changes in activity as HSCs transition from an active to a quiescent state during postnatal life, given the largely conserved amino acid sequence between the two proteins, with a known canonical to non-canonical EZH2 phosphorylation site S21 being preserved in EZH1 (Xu et al., 2012).

In summary, our data suggest a model in which HSCs record their divisional history in part through an Ezh1-to-Ezh2 switch (Figure 6). Ezh1 is highly expressed in dormant HSCs and plays a role in activating HSC signature genes and suppressing cell divisions. As HSCs divide, cell-cycle-associated TFs are activated. These TFs appear to drive the expression of lineage-commitment genes and Ezh2, increasing their expression with divisions over time. With progressive divisions, EZH1 and EZH2 compete for occupation within the PRC2 complex. As EZH2 outcompetes EZH1, EZH2 targets genes associated with an HSC signature for H3K27me3 suppression. This results in a feedforward loop. Every division contributes to Ezh2 activation, which increases the probability of HSC signature gene suppression. This reduces the ability of HSCs to maintain quiescence, increasing the probability of more divisions (Figure 6). Thus, information about divisional history is stored as H3K27me3 on HSC signature genes. These marks are propagated and maintained across time by persistent Ezh2 expression, consolidating divisional history into memory.

Recent work in the field has found the presence of lineage-restricted self-renewing progenitor populations within the HSC compartment (Carrelha et al., 2018; Yamamoto et al., 2013). Given the hierarchical loss of lineage potential described in these populations, it would be of interest to test whether this loss is associated with cells of greater divisional history. In the homeostatic contexts we describe here, the consequences of divisional history appear irreversible, permanently restricting HSC regeneration. How this record changes during stress and regeneration that occur in a transplantation setting or in culture reported to expand HSCs (Wilkinson et al., 2019) will play a crucial role in further unlocking the mechanistic differences between homeostasis and stress as well as mechanisms of HSC self-renewal.

**EXPERIMENTAL PROCEDURES**

**Contact for Reagent and Resource Sharing**
Information and requests for reagents may be directed to the corresponding author, K.A.M. (kateri.moore@mssm.edu).

**Experimental Model and Subject Details**
TetO-H2BGFP and hCD34-tTA were maintained as previously described (Qiu et al., 2014). C57BL/6-Tg(UBC-GFP)30Scha/J (UBC-GFP) were acquired and maintained as previously described (Bernitz et al., 2017). Mice were maintained on doxycycline for 12–16 weeks prior to analysis to reveal various divisional histories. Both male and female mice were used. Animal experiments were approved by the Institutional Animal Care and Use Committee and conducted in accordance with the Animal Welfare Act.

**Figure 6. HSCs Remember Their Cell Divisions via a Divisional History-Dependent Ezh1-to-Ezh2 Switch**
HSCs with the lowest divisional history express high levels of Ezh1. EZH1 associates with the promoters of HSC signature genes to ensure their expression. With each cell division, E2Fs and Foxm1—typical cell-cycle-associated transcription factors—bind to and activate Ezh2’s promoter to induce Ezh2 expression. With progressive divisions, the expression of Ezh2 increases, allowing it to outcompete EZH1 for PRC2 occupancy and mark the promoters of HSC signature genes with H3K27me3. This progressively restricts HSC regenerative potential and initiates lineage commitment. Progressive divisions are visualized by dilution of the H2BGFP from the nucleus, and lineage commitment is indicated by the accumulation of red in the cytoplasm.
Method Details
Sample Preparation and Flow Cytometry
BM cells were harvested from tibias, femurs, and pelvic bones and prepared for sorting as previously described (Bernitz et al., 2016). Cells were analyzed on an LSRII (Becton Dickenson) flow cytometer and sorted on an Influx (Becton Dickenson). HSCs were sorted using the Lin−3CA-1°-cKit−CD48−FLK2−CD150− phenotype. Label retention was defined by gating above the background GFP levels found in heterozygous single transgenic TetO-H2B-GFP HSCs.

RNA Sequencing
Total RNA was isolated from sorted HSC populations using TRIzol Reagent (Life Technologies) with the addition of 15 µg (15 µg/mL) Glycolblue (Life Technologies) prior to RNA precipitation. Isolated RNA integrity was assessed via RNA 6000 pico chip on an Agilent 2100 Bioanalyzer (Agilent Technologies). Up to 1 ng of total RNA from each sample was taken for library preparation using a SMART-Seq v4 Ultra Low Input RNA Kit for sequencing (Clontech), the cDNA size and integrity was assessed by Bioanalyzer, and 100–300 pg of cDNA was used to prepare libraries for mRNA sequencing using a Nextera XT DNA Sample Preparation Kit (Illumina). Barcoded library concentration and quality were quantified using the Quant-iT PicoGreen dsDNA Assay Kit (Invitrogen) with the addition of 15 mg/mL (15 µg/mL) Glycolblue (Life Technologies) prior to RNA precipitation. Isolated RNA integrity was assessed via RNA 6000 pico chip on an Agilent 2100 Bioanalyzer (Agilent Technologies). Up to 1 ng of total RNA from each sample was taken for library preparation using a SMART-Seq v4 Ultra Low Input RNA Kit for sequencing (Clontech) following the manufacturer’s instructions. The resulting cDNA was quantified using the Quant-iT PicoGreen dsDNA Assay Kit (Invitrogen), the cDNA size and integrity was assessed by Bioanalyzer, and 100–300 pg of cDNA was used to prepare libraries for mRNA sequencing using a Nextera XT DNA Sample Preparation Kit (Illumina). Barcoded library concentration and quality were quantified using Qbit (Invitrogen) and Bioanalyzer (Agilent), and the libraries were pooled together and sequenced on the Illumina HiSeq 2000 sequencer platform using a 75-nt single-read setting.

RNA-Seq, ChIP-Seq, and ATAC-Seq Data Analysis
Details are described in Supplemental Experimental Procedures. In Vitro Cultures
Details are described in Supplemental Experimental Procedures.

ACCESSION NUMBERS
RNA-seq datasets have been deposited to the Gene Expression Omnibus database. The accession number for the RNA-seq data reported in this paper is GEO: GSE145772.

SUPPLEMENTAL INFORMATION
Supplemental Information can be found online at https://doi.org/10.1016/j.stemcr.2020.03.005.

AUTHOR CONTRIBUTIONS
Conceptualization, J.M.B. and K.A.M.; Methodology, J.M.B. and K.A.M.; Software, J.M.B., D.S., Y.Y., A.G., R.B., and A.L.; Validation, M.G.D. and K.R.; Formal Analysis, J.M.B., D.S., Y.Y., A.G., R.B., and A.L.; Investigation, J.M.B., M.G.D., A.G., A.W., and K.R.; Resources, A.M., D.P., and K.M.; Data Curation, J.M.B., D.S., and A.L.; Writing – Original Draft, J.M.B. and K.A.M.; Writing – Review & Editing, J.M.B., M.D., K.R., and K.A.M.; Visualization; J.M.B.; Supervision, A.M., D.P., and K.A.M.; Funding Acquisition, K.A.M.

ACKNOWLEDGMENTS
The authors would like to thank V.J. Valdes and members of the Lemischka lab for discussions on the data, as well as Drs. P. Flora, C. Schaniel, E. Ezhkova, and S. Ghaffari for their critical reading of the manuscript. K.A.M. was supported by National Institutes of Health grant 1R01HL128739, as well as the New York Department of Health grant C32597GG. J.M.B. was supported by National Institutes of Health grants T32HD075735 and F31HL131290, and A.M. was supported by National Institutes of Health grants U54HL127624 and U24CA224260.

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Received: February 12, 2020
Revised: March 3, 2020
Accepted: March 3, 2020
Published: April 2, 2020

Stem Cell Reports | Vol. 14 | 561–574 | April 14, 2020 | 571
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