m^6A RNA Methylation Maintains Hematopoietic Stem Cell Identity and Symmetric Commitment

Graphical Abstract

Highlights
- Single-cell RNA-seq uncovers an HSC-MPP intermediate population after m^6A depletion
- m^6A-depleted HSC-MPPs molecularly and functionally resemble normal MPPs
- m^6A controls HSC symmetric commitment and identity through MYC mRNA stability
- Asymmetric segregation of MYC during HSC division provides a marker for commitment

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In Brief
Cheng et al. uncover RNA methylation as a guardian in hematopoietic stem cell (HSC) fate decisions. m^6A maintains hematopoietic stem cell symmetric commitment and identity. This study may provide a general mechanism for how RNA methylation controls cellular fate.

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m\textsuperscript{6}A RNA Methylation Maintains Hematopoietic Stem Cell Identity and Symmetric Commitment

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INTRODUCTION

Hematopoietic stem cells (HSCs) balance their long-lived regenerative capacity with the ability to maintain myeloid, lymphoid, and erythroid lineage output in the blood. This balance is mediated through cell fate decisions that occur during cellular division. When they divide, HSCs either self-renew or undergo differentiation toward a multipotent progenitor cell (MPP) fate, where the cells are metabolically more active than HSCs and retain multi-lineage potency but lack HSC-long-term engrainment activity. The choice between these distinct cellular outcomes is controlled by the ability to alternate between a symmetric or asymmetric fate choice (Knoblich, 2008; Morrison and Kimble, 2006). It remains unclear what signals can determine whether a cellular division leads to cellular commitment (differentiation) or self-renewal. Mechanistic insights into the regulation of cell fate decisions may inform approaches to bone marrow failure syndromes, differentiation therapy of hematopoietic malignancies, and stem cell expansion for therapeutic benefits.

A key controller of cellular fate is mRNA methylation. The most common reversible posttranscriptional mRNA modification on mRNA is N\textsuperscript{6}-methyladenosine (m\textsuperscript{6}A). The presence of m\textsuperscript{6}A alters mRNA metabolism, including mRNA stability, mRNA splicing, and translation efficiency (Coots et al., 2017; Meyer and Jaffrey, 2017; Meyer et al., 2015; Ping et al., 2014; Roundtree et al., 2017; Schwartz et al., 2014; Slobodin et al., 2017; Wang et al., 2015; Xiao et al., 2016). The deposition of transcriptome-wide m\textsuperscript{6}A marks depends on the METTL3-METTL14-WTAP-VIRMA-ZC3H13-RBM15 protein complex, in which METTL3 serves as the catalytic subunit. m\textsuperscript{6}A controls cell state maintenance in a cellular-context-dependent manner. Embryonic stem cells (ESCs) with Mettl3 deficiency remain naive and fail to differentiate into primed ESCs (Batista et al., 2014; Geula et al., 2015) and specification of hematopoietic stem and progenitor cells (HSPCs) requires METTL3 in zebrafish and mouse embryos (Lv et al., 2018; Zhang et al., 2017).

A number of recent studies showed that m\textsuperscript{6}A and METTL3 are important for survival and maintenance of the undifferentiated stages of myeloid leukemia cells (Barbieri et al., 2017; Vu et al., 2017a; Weng et al., 2018). However, as therapeutics toward METTL3 are being developed to target myeloid leukemia...
(Boriack-Sjodin et al., 2018), it is important to how low loss of m\(^6\)A affects normal blood development. Several studies have reported that disruption of m\(^6\)A regulators impacts normal HSC function. Depletion of YTHDF2, a m\(^6\)A reader protein, results in increased HSCs that are capable of normal engraftment, while loss of writer protein METTL3 leads to an accumulation of HSCs with impaired differentiation capacity and normal self-renewal (Lee et al., 2019; Li et al., 2018; Yao et al., 2018). However, the mechanism by which m\(^6\)A affects HSC expansion remains unknown. Additionally, MYC was reported as a major target of m\(^6\)A that contributes to the effects of m\(^6\)A in myeloid leukemia and in HSCs (Lee et al., 2019; Vu et al., 2017a). However, it remains unclear if m\(^6\)A simply alters MYC expression, or if m\(^6\)A has other regulatory roles that mediate MYC’s effects in HSC accumulation.

To understand how m\(^6\)A shapes the early differentiation decisions during hematopoietic differentiation, we performed single-cell RNA sequencing (RNA-seq) in wild-type (WT) and METTL3 knockout hematopoietic progenitor cells. In contrast to the HSC accumulation phenotype that has been described upon METTL3 depletion previously, we report here that HSCs are instead depleted. We show that the expanded population is not in the HSC pool but, instead, comprises a HSC-like intermediate state that molecularly and functionally resembles multipotent progenitors. Mechanistically, we show that m\(^6\)A is required for HSCs’ symmetric commitment step in hematopoietic differentiation, with normal asymmetric commitment upon METTL3 depletion. We find that m\(^6\)A controls Myc mRNA stability and this m\(^6\)A-regulated expression of Myc controls HSC symmetric commitment. The HSC-like intermediate population that is metabolically activated but fails to symmetrically commit has uncoupled the role for MYC in HSC activation and cellular commitment. Our data advance the concepts that m\(^6\)A is essential for HSC identity maintenance and it tightly controls HSCs entry toward commitment. Overall, we find that the major role for m\(^6\)A in hematopoietic differentiation is due to its ability to regulate symmetric commitment via controlling Myc mRNA stability.

**RESULTS**

**Mettl3 Is Required for Normal Hematopoiesis**

To study the role of m\(^6\)A in normal hematopoiesis and cellular fate, we crossed the Mettl3 flox/flox (Mettl3 cKO) mice to generate Mettl3 cKO, Mx1-Cre+ conditional knockout (cKO) mice (Figure 1A). Mettl3 depletion was then induced by injection with polyinosinic-polycytidylic acid (pIpC) (Figure S1A). Analysis of pIpC-treated Mettl3 cKO, Mx1-Cre+ (Mettl3 cKO) and control Mettl3 cKO, Mx1-Cre− (Mettl3 cKO) confirmed efficient Mettl3 genomic deletion (Figure 1B) and ablation of METTL3 protein in HSPCs (LSK, Lineage− cKit+Sca−1) (Figure S1B). We further confirmed that the loss of METTL3 reduced global m\(^6\)A levels in bone marrow cells (Figure 1C).

We then evaluated the hematopoietic system upon depletion of Mettl3 and global m\(^6\)A. We observed in Mettl3 cKO mice a 40% reduction in bone marrow cellularity and pancytopenia (Figures 1D and 1E). Flow analysis of bone marrow cells from Mettl3 cKO mice revealed a marked reduction in Gr1+Mac1+ mature granulocyte and Ter119+CD71med /Ter119+CD71low (Ery3/Ery4) erythrocyte cells (Figures S1C and S1D), as well as an increase in CD41+ megakaryocytes (Figure S1C) without major defects in lymphoid lineages compared to control mice (Figure S1E). Analysis of bone marrow sections confirmed that Mettl3 cKO mice had reduced mature granulocytes and increased immature myeloid cells (Figure S1F). Histopathological analysis in Mettl3 cKO mice further showed dysplasia in megakaryocytes characterized by abnormal micromegakaryocytes with a large fraction characterized with monolobated megakaryocytes, a feature associated with altered differentiation (Figure S1F). Moreover, METTL3-depleted bone marrow cells demonstrated a significant reduction in the ability to proliferate and differentiate to form myeloid colonies in vitro (Figure S1G). Additionally, Mettl3 cKO mice exhibited enlarged spleens with extensively infiltrated red pulp zones and perturbed splenic architecture, which is associated with an accumulation of immature erythroblasts at the expense of B and T cells (Figures S1H–S1K). Taken together, these data indicated an impairment in maturation and terminal differentiation of myeloid and erythroid lineages upon METTL3 ablation and reduction of m\(^6\)A.

Given the broad, multi-lineage defects of METTL3 loss in the hematopoietic system, we hypothesized that the defects originate from the hematopoietic stem and progenitor compartments. The HSPC compartment can be further defined using cell surface markers (i.e., SLAM/CD150 and CD48) into HSCs (LSK, CD150+CD48−) and MPPs. MPPs, which all have short-term stem cell activity, can be subdivided into three populations: MPP1s, which are considered unbiased (LSK, CD150+CD48−); MPP2s, which have a bias toward megakaryocytic and erythroid lineages (LSK, CD150−CD48+); and MPP4s, with lymphoid lineage bias (LSK, CD150−CD48+). (Kiel et al., 2005; Oguro et al., 2013; Rodriguez-Fraticelli et al., 2018). We examined all four populations and observed a 5-fold increase in the frequency of HSPCs (LSKs) and a nearly 10-fold expansion in immunophenotypic HSCs, as well as MPP2s and MPP4s in the bone marrow of Mettl3 cKO mice as compared to control Mettl3 cKO mice (Figures 1F and 1G). Notably, the MPP1 subset of MPPs was not increased in Mettl3 cKO mice. However, the downstream myeloid progenitors (MPS, Lin− cKit+Sca−1) comprising common myeloid progenitors (CMP; Lin− cKit+Sca−1 CD34+ FcR−) and granulocyte myeloid progenitors (GMP; Lin− cKit+Sca−1 CD34+ FcR−) were significantly reduced in Mettl3 cKO mice (Figure S1L). Overall, these data suggested that loss of m\(^6\)A imposed a blockage in transition of HSCs to MPs, thereby hindering commitment of HSCs toward a myeloid fate.

To explore the dosage effect of Mettl3 loss on hematopoiesis, we evaluated heterozygous Mettl3 +/− (Het, Cre+) mice. Similar to the control mice, heterozygous Mettl3 deletion had equivalent bone marrow (BM) cellularity and peripheral blood counts (white blood cells [WBCs], red blood cells [RBCs], and platelets) 3 weeks post-deletion (Figures S2A and S2B). However, we found that the Mettl3 het mice also exhibited splenomegaly, which was associated with an accumulation of erythroid blasts and reduction of T cells (Figures S2C and S2D). Moreover, HSCs were also expanded in the Mettl3 het mice while the downstream lymphoid, myeloid, and erythroid populations were equivalent to controls except for the reduction in the pre-pro B cell population in the Mettl3 het mice (Figures S2E–S2H). These data suggest an intermediate dose requirement for Mettl3 in normal hematopoiesis.
**Mettl3-Depleted HSCs Are More Activated and Functionally Defective in Reconstitution**

As we observed defects in progenitor pools and mature lineages with an expansion of HSCs upon METTL3 depletion, we hypothesized that m^6^A is critical to maintain HSC function. We first examined whether loss of m^6^A results in the exit of quiescence in HSCs. Under normal homeostatic conditions, a majority of HSCs remain dormant in G\(_0\) while a fraction of HSCs enter cell cycle to divide and generate progenitor cells (Attar and Scadden, 2004). Loss of Mettl3 results in an increased frequency of cells that have exited G\(_0\) and increased percentage of cells that are actively dividing (Figures 2A, 2B, and S2I). These proliferative HSCs with depleted Mettl3 are also metabolically activated, with increased mitochondrial mass and activity (Figures 2C, 2D, and S2J). Consistent with previous findings that loss of quiescence in HSCs is attributed to reduced engrafting potential.
Mettl3 cKO bone marrow cells exhibited a significant defect in in vivo repopulating capacity, characterized by reduced chimerism of all HSPC compartments and mature lineage populations in recipient mice (Figures 2E, 2F, S2K, and S2L). These results indicated that m6A is functionally required for maintaining HSC quiescence and self-renewal potential. The data also suggested that the expansion of HSCs is possibly due to the accumulation of dividing HSCs, incapable of properly differentiating.

To determine if the expansion of Mettl3-depleted HSCs and their defective ability to reconstitute the hematopoietic system was cell autonomous and not due to loss of RNA methylation in the heterogeneous niche environment, we engrafted bone marrow cells from both Mettl3 f/f, Mx1-Cre+/C0 and Mettl3 f/f, Mx1-Cre+ bone marrow cells into CD45.1 congenic recipient mice. At 6 weeks post-transplantation, CD45.1 congenic recipient mice were then injected with pIpC to deplete METTL3.

Figure 2. Mettl3 cKO HSCs Are Less Quiescent and Functionally Defective
(A) Mettl3 cKO HSCs are less quiescent. Representative flow cytometry plots for assessing cell cycle status of Mettl3 f/f and Mettl3 cKO HSC by Pyronin Y staining.
(B) Quantification of cell cycle analysis. n = 5.
(C) Increased mitochondrial mass in Mettl3 cKO HSCs. Representative histograms of Mitotracker Green staining in Mettl3 f/f and Mettl3 cKO HSCs.
(D) Mitochondrial mass of different HSPC population was evaluated by Mitotracker Green staining quantified by flow cytometry. n = 5.
(E) Scheme of transplant strategy. Non-competitive reconstitution assay in which 10⁶ donor BM cells from Mettl3 f/f or Mettl3 cKO mice at 3 weeks post-pIpC were transplanted into CD45.1 congenic recipient mice.
(F) Mettl3 cKO bone marrow fails to reconstitute hematopoietic compartments in recipient mice. CD45.2 chimerisms of HSCs, MPPs and progenitor compartments from (E) were analyzed by flow cytometry at 16 weeks post-transplant. n = 10.
(G) Scheme of the experimental procedure in (H) and (I). CD45.1 recipient mice were transplanted with Mettl3 flox/flox Cre− or Cre+ bone marrow cells (pre-pIpC). At 6 weeks post-transplantation, CD45.1 congenic recipient mice were then injected with pIpC to deplete METTL3.
(H) The reconstitution defect in Mettl3 cKO bone marrow is cell-autonomous. CD45.2 chimerism analysis of HSCs, MPPs, and progenitor compartments from experiment (G) at 3 weeks post-pIpC. n = 5.
(I) Frequencies of HSCs and MPPs in the donor CD45.2 population from experiment (G). n = 5. Mean and SEM are shown (*p < 0.05, **p < 0.01, ***p < 0.001, and ****p < 0.0001). n represents number of mice.
Mx1-Cre\(^+\) mice (Figure 2G), and then depleted Mettl3 following engraftment through pIpC administration. Donor reconstitution was comparable between Mettl3 \(^{f/f}\), Mx1-Cre\(^+\)/C0 and Mettl3 \(^{f/f}\), Mx1-Cre\(^+\) mice before pIpC treatment (Figure S2M). However, the chimerism of donor engraftment was significantly decreased in the HSPC compartments and in mature lineages 3 weeks after METTL3 depletion (Figures 2H and S2N). Importantly, the expansion of HSCs following METTL3 depletion was recapitulated within the donor population (Figure 2I). These results strongly supported a cell-intrinsic role of METTL3 and m6A in regulating HSC numbers and function.

Next, we sought to determine if alteration in function of Mettl3 cKO HSCs is sustained after long-term engraftment (16 weeks post-pIpC), in which MPPs and other differentiated cell types are replenished from their precursors. Similar to acute depletion, the METTL3-depleted HSPCs remained expanded (HSC, MPP2, and MPP4) and demonstrated reduced RBCs in the primary Mettl3 cKO mice (Figures S3A–S3C). However, defects in downstream lineages were normalized in Mettl3 cKO mice, as characterized by normal bone marrow cellularity, WBCs, and mature lineages (Figures S3D–S3H). Splenomegaly was also observed in Mettl3 cKO mice with increased immature erythroblasts, even though the reductions of B and T cells were recovered (Figures S3I–S3K). These data were explained by genomic PCR analysis of bone marrow cells, demonstrating an emergence of a WT Mettl3 population (Figure S3L). Furthermore, these data suggest a strong selective pressure against the loss of RNA methylation during myeloid and lymphoid lineage restriction and the sustained increase in the numbers of METTL3-depleted HSCs suggested a continuous block in the ability of HSCs to differentiate to more committed lineage restricted cells.

Acute Depletion of Mettl3 Leads to a Reversible Defect in HSC Repopulating Capacity

Targeting METTL3 is a potential therapeutic strategy in acute myeloid leukemia (AML) (Barbieri et al., 2017; Vu et al., 2017a). Prolonged genetic ablation of METTL3 disrupts adult hematopoiesis in vivo in a dose-dependent manner. However, it remains unclear whether the effects of METTL3 inhibition on normal HSCs are reversible. Thus, we examined the effect of acute Mettl3 inhibition on hematopoiesis by transient small interfering RNA (siRNA) knockdown (Figure 3A). Sorted LSK cells were transfected with control or Mettl3 siRNA and then injected into recipient mice (Figure 3B). Pan hematopoietic donor reconstitution was significantly but modestly reduced in the Mettl3 knockdown group at a short-term time point (4 weeks) (Figure 3C). However, the engraftment defect induced by acute Mettl3 loss was normalized in the HSPC compartments at 12 weeks post-transplant (Figure 3D). The chimerism of donor engraftment of the Mettl3 knockdown group was also comparable to the control group in myeloid, T cell, and megakaryocytes lineages with modest reductions on the erythroid and B cell compartment (Figure 3E). These data suggest that acute inhibition of Mettl3 could induce a reversible defect in normal hematopoiesis.
with the modest hematopoietic defect observed in Mettl3 heterozygous mice, these results highlight a wide therapeutic window to target METTL3 in AML.

**Single-Cell Profiling Identifies Additional Cell Populations in Mettl3 cKO Mice**

To decipher the effect of Mettl3 deletion on lineage commitment, identity, and priming of the hematopoietic hierarchy, we performed single-cell RNA-seq (scRNA-seq) using 6000 sorted LK cells (lineage−/cKit+ cells) from three independent control Mettl3 f/f and Mettl3 cKO mice (Figures S4A–S4C), at 3 weeks post-deletion. Single-cell t-Distributed Stochastic Neighbor Embedding (tSNE) analysis of Mettl3 f/f LK cells revealed the expected stem and progenitor clusters, including HSC, MPP, and erythroid, myeloid, and lymphoid progenitors, described previously in scRNA-seq studies (Giladi et al., 2018; Jaitin et al., 2014; Paul et al., 2015) (Figures 4A and S4D; Table S1). However, in Mettl3 cKO, the frequencies of basophils (Ba), megakaryocyte-erythroid progenitors (MEPs), megakaryocyte primed populations, and HSC clusters were significantly reduced (Figures 4A–4C). These data suggest that the reduction in myeloid and erythroid mature lineages is already manifested in lineage primed progenitors.

Importantly, projection of distinct cell types by tSNE revealed two populations in the Mettl3 cKO samples that were not present in control samples. The two Mettl3 cKO-specific populations (KOsp1 and KOsp2) accounted for nearly 30% of all immature LK cells in Mettl3 cKO bone marrow (Figures 4A–4C). To interrogate the identity of these cKO-specific clusters, we performed a
maximum likelihood projection of KOsp1 and KOsp2 onto the annotated HSPC clusters in control mice. KOsp1 cells predominately clustered with HSCs (50%) and to a lesser degree with MPPs (25%). In contrast, the expression profile of KOsp2 cells was most similar to megakaryocyte progenitors (70%) and had a 20% similarity to HSCs (Figure 4D). A differential expression analysis comparing KOsp1 and KOsp2 cells to WT control HSCs demonstrated a higher expression of cell cycle genes, including K67 and Cdk1, and lower expression of key HSC self-renewal regulators and transcription factors, including Msi2, Hoxa9, Satb1, and Fos (Figures 4E and S4E). KOsp2 differed from normal megakaryocytic progenitors by their low expression of megakaryocyte lineage-regulating genes, including Plf4, Gp1bb, Myb, and Gata1, and increased expression of myeloid and lymphoid genes, including C6d3, Gimap1, and Gimap9 (Figure S4F). These gene expression changes are in line with the defects in HSC self-renewal and megakaryocyte maturation that are observed in these cells. These data indicated that the loss of METTL3 resulted in the emergence of actively dividing “HSC-like” populations: one (KOsp1) with reduced self-renewal and the other (cKOsp2) acquiring lineage priming toward a megakaryocyte fate.

To better understand the origin of the cKO-specific clusters, we performed pseudo-time analysis and hierarchy reconstruction to capture early commitment decisions in HSCs. Both the KOsp clusters and normal MPP clusters were connected to the HSC cluster (Figure 4F). These data suggested that KOsp clusters represent an intermediate state of HSC that branches away from the normal HSC to MPP transition. While many Mettl3 cKO HSCs were still capable of normal HSC-to-MPP transition, KOsp1 and KOsp2 ascended from HSCs but the lack of m^A prevented them from properly committing to normal MPPs. These data strongly suggest that RNA methylation plays a key role in dictating cell identity and lineage commitment in HSCs.

**Mettl3 cKO HSCs Lose HSC Identity and Function as MPPs**

Since the single-cell sequencing data identified the HSC-like intermediate populations, we next want to delineate the relationship between immunophenotypic HSCs and MPPs and the molecularly defined HSC-like populations. We performed RNA-seq transcriptome profiling of classically defined HSCs and progenitor cells, i.e., HSCs, MPP1s, MPP2s, and MPP4s, whose frequencies were previously found to be altered (Figure S4A). Unsupervised clustering of the differentially expressed genes across all samples distinguished the Mettl3 cKO HSPC populations from the controls (by ANOVA test; padj < 0.01) (Figure S5A). The data confirmed that HSPCs from Mettl3 cKO mice are distinct from their Mettl3 /ff counterparts with a different transcriptome profile. We then examined enrichment of KOs cluster-specific genes from scRNA-seq with the transcriptional profiles of Mettl3 /ff and Mettl3 cKO HSCs and MPPs in bulk RNA-seq. Gene set enrichment analysis (GSEA) analysis using the top expressed genes from each spleen (SP) cluster in scRNA-seq (KOsp1/KOsp2 gene sets) revealed that KOsp1 and KOsp2 cells were enriched with Mettl3 /ff immunophenotypic HSC signatures rather than MPPs (Figure S5B). Additionally, both KOsp1 and KOsp2 gene sets were enriched in Mettl3 cKO HSC cells compared to Mettl3 cKO MPPs (Figure S5C). These results indicated that despite the lineage priming features, the scRNA-seq cKO-specific populations still resemble the phenotypic HSCs. It further supports that ablation of m^A initially alters the cell fate decision of HSCs, and not downstream MPPs.

Next, we further evaluated the altered gene expression program within the HSPC compartment. We performed GSEA of 866 genes that are significantly upregulated in Mettl3 cKO HSCs (false discovery rate [FDR] < 0.05), compared with WT HSCs (Table S2). A gene set obtained from the Mettl3 KO mouse embryonic stem cells (mESCs) (Figures 5A, 5B, and S5D; http://amp.pharm.mssm.edu/Enrichr/) was the top-ranked gene set. These results suggest that loss of METTL3 in HSCs induces a gene expression program that is found in METTL3-deficient naive pluripotent ESCs. Interestingly, this was specific to HSCs, as this analysis in MPPs failed to match with METTL3-deficient ESCs. Thus, the analysis supported that Mettl3-deleted HSCs have a lineage priming defect that traps them in a more primitive state. The Rbm15 KO HSPC signature was also highly enriched in the Mettl3 cKO HSC gene expression program (Figures 5B and S5E). Rbm15 is an RNA binding protein that recruits the m^A methyltransferase complex to target mRNAs (Patil et al., 2016). Moreover, the defects observed in Rbm15 KO mice closely match with many of the phenotypes observed post-METTL3 depletion, suggesting that the phenotypes that have been observed with Rbm15 KO mice may be in part attributable to alteration in the m^A-dependent differentiation program (Patil et al., 2016). These data demonstrate an upregulation of a core m^A-dependent transcriptional program upon Mettl3 depletion in HSCs that is shared among models of RNA methylation depletion.

We next performed unbiased GSEA analysis on all 4,733 curated gene sets in the Molecular Signature Database (MSigDB), combined with 92 relevant gene sets from our experimentally derived or published hematopoietic self-renewal and differentiation signatures using HSC and MPP transcriptome profiles from Mettl3 /ff and cKO mice. We observed a negative enrichment of HSC self-renewal signature and a positive enrichment of ribosomal and protein translation gene sets in the Mettl3 cKO HSC gene expression profile (Figure 5C). Moreover, protein translation-related ribosome signatures were also enriched in Mettl3 /ff MPPs compared to Mettl3 /ff HSCs (Figure S5F). We confirmed the increased protein synthesis in Mettl3 cKO HSCs based on O-Propargyl-puromycin (OP-Puro) (Figure 5D). These data suggest that there is an alteration in identity in Mettl3 cKO HSCs.

As our molecular profiling strongly suggests alterations in cell identity and commitment in METTL3-depleted HSCs, we examined whether these HSCs are functionally defective. To test whether there is an altered identity in Mettl3 cKO HSCs, we performed competitive transplantation using sorted HSCs and MPP1s from Mettl3 /ff and cKO mice (Figure 5E). As observed previously with total bone marrow cells, Mettl3 cKO HSCs and MPP1s had low reconstitution within all peripheral cellular compartments (Figure S5G). Within the bone marrow, the control HSCs engrafted normally and chimerism could be measured in all HSPC compartments: HSCs, MPP1s, MPP2s, and MPP4s. The control MPP1s also correctly reconstituted...
the MPP1 compartment, thus validating our sorting strategy in this functional assay (Figures 5F and S5H). However, the Mettl3 cKO HSCs demonstrated chimerism within the phenotypic MPP1 compartment but not within the HSC compartment (Figure 5F). Additionally, these transplanted HSCs in the MPP compartment behaved as MPP1s, as they were no longer detected at long-term analysis (40 weeks post-transplant) (Figures 5G and S5I). This altered fate was also validated by Spearman correlation analysis using gene expression profiles of Mettl3 f/f and Mettl3 cKO HSCs and MPP1s. Gene expressions between cKO HSCs and control HSCs were less correlated, and the cKO HSCs’ gene profile correlated similarly to control HSCs or MPP1s (Figure 5H). Furthermore, GSEA analysis also showed that Mettl3 cKO HSCs were indeed enriched for Mettl3 f/f

Figure 5. Mettl3-Deleted HSCs Are More Functionally and Molecularly MPP-like
(A) Significantly differentially expressed genes (padj < 0.05) in cKO HSCs compared to Mettl3 f/f were shown as heatmap.
(B) Differentially expressed genes in Mettl3 cKO HSCs were enriched with Mettl3 KO ESC and RBM15 KO LSK expression signatures. Enrichr analysis using upregulated genes in METTL3-depleted HSCs from bulk RNA-seq.
(C) Unbiased gene set enrichment analysis using 4,733 curated gene sets against the rank list of differential expressed genes between Mettl3 f/f and cKO HSCs. Self-renewal signature was negatively enriched in Mettl3 cKO HSCs. Translation and ribosome gene sets were positively enriched in Mettl3 cKO HSCs.
(D) Increased global translation in Mettl3 cKO HSCs. Representative histograms (top) and quantification (bottom) of OP-Puro incorporation into sorted Mettl3 f/f and cKO HSCs.
(E) Scheme of transplant strategy in (F). Sorted HSCs and MPP1s from Mettl3 f/f and Mettl3 cKO mice were injected into CD45.1 recipient mice with CD45.1 competitor BM cells.
(F) Mettl3 cKO HSCs function as MPP1s in competitive transplant assays. Engraftment of CD45.2 donor cells was analyzed in HSPCs from recipient mice at 6 weeks. n = 10.
(G) Engraftment of CD45.2 donor cells was analyzed in HSPCs from recipient mice at 40 weeks. n = 10.
(H) Spearman correlation among HSC and MPP1 populations from Mettl3 f/f and Mettl3 cKO mice.
(I) Gene set enrichment analysis of up- or downregulated genes in Mettl3 cKO HSC identified by bulk RNA-seq, against the rank list of differentially expressed genes between Mettl3 f/f HSC and Mettl3 f/f MPP1. Mean and SEM are shown (*p < 0.05, **p < 0.01, ***p < 0.001, and ****p < 0.0001). n represents number of mice.
m⁶A Regulates HSC Asymmetric and Symmetric Division by Modulating Myc mRNA

We next sought to understand the molecular basis for abnormal cell fate decision and differentiation upon loss of m⁶A in HSCs. Given that m⁶A ablation traps HSCs in a more primitive state during HSC-to-MPP transition, we hypothesized that genes essential for this transition would differentially increase their m⁶A abundance in MPPs compared to HSCs. Thus, we reanalyzed previously obtained m⁶A profiles in HSCs and MPPs and selected genes with higher m⁶A marks in MPPs (Li et al., 2018). To identify m⁶A and METTL3 direct targets, we then intersected the list of increased m⁶A methylated mRNAs in MPPs with the differentially expressed genes in HSCs upon Mettl3 deletion, resulting in eight genes (Figures 6A and S6A; Table S3). MYC was among these genes known to be a critical target of m⁶A methylation (Vu et al., 2017a). Moreover, Mettl3 deletion partially phenocopied the expanded HSPC compartment found in the Myc-deficient mice (Bahr et al., 2018; Wilson et al., 2004). To further determine if MYC is a direct functional target of m⁶A in HSCs, we performed methylated RNA immunoprecipitation (MeRIP)-qPCR and found reduced enrichment of m⁶A in Myc transcripts in Mettl3 cKO HSPCs (Figure 6B). Overall, these data suggest that Myc could be a functional downstream target of m⁶A and METTL3 in HSCs.

To directly assess HSC fate commitment and to evaluate MYC expression after Mettl3 deletion, we performed paired daughter cell division assays based on intracellular staining of NUMB and MYC proteins. Previous studies showed that HSCs can undergo three fate choices: symmetric renewal, symmetric commitment, and asymmetric cell division. Cellular commitment toward a differentiation cell fate is measured by increased NUMB staining (MeRIP)-qPCR and found reduced enrichment of m⁶A in Myc transcripts in Mettl3 cKO HSPCs (Figure 6B). Overall, these data suggest that Myc could be a functional downstream target of m⁶A and METTL3 in HSCs.

To determine if asymmetric segregation of MYC was already determined at the level of mRNA, we performed fluorescence in situ hybridization (FISH) of Myc mRNA and fluorescence immuno-nostaining of NUMB. Interestingly, Myc mRNA also can be segregated to daughter cells asymmetrically, symmetrically “low,” and asymmetrically “high” in a manner correlating to NUMB and MYC (Figure 6F). Furthermore, consistent with our scRNA-seq and RNA-seq results, the Myc mRNA was dramatically reduced in Mettl3 cKO HSCs (Figures 6G and 6H). These data suggest that m⁶A RNA methylation controls Myc mRNA abundance that is required for HSC commitment. Given that m⁶A modification affects mRNA stability, we hypothesized that the reduced Myc mRNA level in Mettl3 cKO HSCs is due to decreased Myc mRNA stability. Indeed, the half-time of Myc mRNA was significantly decreased in Mettl3 cKO HSCs (Figures 6I and S6B). Re-introduction of MYC in Mettl3 cKO HSCs partially rescued the HSC commitment defect (Figure S6C and S6D). Overall, these data suggest that m⁶A controls HSC symmetric commitment through MYC regulation.

Next, to determine whether the failure of HSCs to properly differentiate is functionally driven by reduced MYC abundance during cell division, we overexpressed MYC in sorted Mettl3 cKO LSK cells and performed competitive bone marrow transplants. MYC overexpression resulted in a rescue in the peripheral blood and bone marrow HSPC compartment chimerism in mice transplanted with Mettl3 cKO HSPCs (Figures 7A–7C, S7A, and S7B). Furthermore, this increased chimerism could be observed within the HSC compartments in 5 out of the 10 mice (Figure S7B). A lethal myeloproliferative and AML-like disease was observed in the MYC overexpression group as previously reported (Figure S7C) (Luo et al., 2005), suggesting that highly expressed MYC could bypass the requirement for METTL3 in myeloid leukemia. These in vitro and in vivo data suggest that restoring MYC can at least partially override defects in HSC commitment and drive normal HSC differentiation.

Altogether, these data suggest that MYC mRNA and protein is a marker for commitment and m⁶A-deficient HSCs are partially blocked in their ability to differentiate.

METT3 Catalytic Function Controls MYC Abundance and HSC Commitment in Vivo

As METTL3 may have additional functions beyond its methyltransferase activity, we then assessed if METTL3’s catalytic function is directly responsible for alterations in MYC abundance and HSC commitment. To this end, we overexpressed METTL3 or a catalytically dead METTL3 mutant (METTL3-CD) and found that it rescued the reduced overall MYC abundance in either Mettl3-siRNA transfected HSCs or in cKO HSCs (Figures 7D and S7D). Additionally, overexpression of METTL3, but not METTL3-CD, partially rescued HSC symmetric commitment in the paired daughter cell division assay and Mettl3 cKO HSC engraftment (Figures 7E, 7F, and S7E). Moreover, METTL3, but not METTL3-CD, significantly enhanced normal HSPC reconstitution capacity in vivo, suggesting that increased methyltransferase activity can enhance HSPC engraftment (Figure S7F). Overall, these data suggest that METTL3 catalytic activity is essential for MYC regulation, HSC commitment, and HSC function.

DISCUSSION

Here, by integrating transcriptome profiling with single-cell RNA sequencing and single-cell assays, we show that m⁶A is required for the early cell fate decision in adult HSCs. Our studies find that...
Figure 6. Mettl3 Is Required for HSC Symmetric Commitment by Regulating mRNA Stability of Myc

(A) Overlap of the 52 genes that have increased m^6^A abundance in MPPs than HSCs with differentially expressed genes between Mettl3 f/f and cKO HSCs, as depicted in a Venn diagram. Myc is highlighted in red.

(B) m^6^A enrichment in the Myc transcript in Mettl3 f/f and cKO HSPCs was assessed by MeRIP-qPCR. Two primers were used to indicate different m^6^A sites on Myc RNA.

(C) MYC protein level is reduced in Mettl3 cKO HSCs upon cell division. MYC expression in Mettl3 cKO HSCs compared to Mettl3 f/f HSCs as quantified by immunofluorescence.

(D) Mettl3 cKO HSCs fail to undergo symmetric commitment compared to control HSCs. Left: representative immunofluorescence images of paired daughter cells stained with DAPI (blue), NUMB (green), MYC (red), and brightfield. Right: percentages of doublet cells in each type of cell division. Number of daughter pairs assessed: Mettl3 f/f n = 223; cKO n = 245. n represents number of paired HSCs measured.

(E) MYC expression correlates with NUMB expression during HSC division.

(F) Myc mRNA can be segregated into daughter cells asymmetrically, symmetrically high, and symmetrically low. Representative images of fluorescence in situ hybridization (FISH) of Myc mRNA (green) and fluorescence immunostaining of NUMB (red) and DAPI (blue) in Mettl3 f/f and Mettl3 cKO HSCs.

(G) Myc mRNA is significantly decreased in Mettl3 cKO HSCs. Representative images of FISH of Myc mRNA (green) and DAPI (blue) in Mettl3 f/f and Mettl3 cKO HSCs.

(H) Quantification of Myc mRNA in HSCs from Mettl3 f/f and Mettl3 cKO mice.

(I) The mRNA half-life (t_1/2) of Myc transcripts in Mettl3 f/f and Mettl3 cKO HSCs. Mean and SEM are shown (*p < 0.05, **p < 0.01, ***p < 0.001, and ****p < 0.0001).
m6A controls HSC identity by affecting the symmetric commitment step, while asymmetric divisions are less affected. m6A is a known regulator of Myc expression in HSCs. We show that Myc functions to promote HSC symmetric commitment, and its loss in m6A-deficient cells results in the formation of a cell type with MPP-like identity, rather than an accumulation in the HSC population, as is currently thought in the field (Lee et al., 2019; Yao et al., 2018). The current thinking about m6A in normal hematopoiesis is that m6A controls HSC differentiation without affecting self-renewal or proliferation. Here, we show that the accumulated immunophenotypic “HSCs” that were identified in previous studies are instead a blocked HSC-MPP-like cell population. These cells behave as MPP-like cells as they exhaust and thus have reduced self-renewal in vivo. These HSC-like intermediate cells are also metabolically active and highly proliferative, but they fail to symmetrically commit and differentiate. Our study shows that m6A controls cell identity by regulating the symmetric commitment of HSCs during division at the early cell fate determination point. These intermediate populations could also be used to further understand the relationship between HSC expansion and commitment toward MPPs.

Interestingly, while m6A reader YTHDF2-deficient mice also exhibited increased HSCs number, Ythdf2 knockout HSCs demonstrated improved functional and regenerative capacity in contrast to what we observed in Mettl3-deficient HSCs (Li et al., 2018). These data suggest that YTHDF2 depletion may promote HSC self-renewal without compromising commitment, resulting in functional HSC expansion.

Myc was reported as a target of m6A in regulating myeloid leukemia and HSCs (Lee et al., 2019; Vu et al., 2017a). However, it is unclear how m6A controls Myc and in which step Myc affects HSCs. We identified Myc as a functional target of m6A that facilitates the transition from HSCs to MPPs during the early steps of cell fate determination and its RNA stability is controlled by m6A. Surprisingly, we demonstrated that Myc mRNA and protein could be segregated either asymmetrically or symmetrically...
during HSC division. An asymmetric partition of MYC was observed during T cell activation (Pollizzi et al., 2016; Verbist et al., 2016). Thus, the insight of m\textsuperscript{6}A in controlling symmetric commitment by modulating MYC could be a general mechanism for m\textsuperscript{6}A regulation of cell fate. Several known m\textsuperscript{6}A-dependent cell fate regulations are consistent with our model. For example, in mouse ESCs and in naive T cells, loss of m\textsuperscript{6}A locks the cells in its naive state and prevents differentiation (Geula et al., 2015; Li et al., 2017).

We previously reported that depletion of METTL3 in human AML cells results in increased MYC mRNA abundance and reduced translational efficiency, which finally leads to decreased MYC protein (Vu et al., 2017a). In contrast, METTL3 depletion in normal murine HSCs results in a decrease in M\textsuperscript{yc} mRNA and protein levels, suggesting a cellular-context-dependent regulation of m\textsuperscript{6}A targets or differential effects after transient versus prolonged depletion of m\textsuperscript{6}A.

Previous studies revealed that m\textsuperscript{6}A is required for leukemogenesis and AML cell survival, prompting the search for inhibitors to block this pathway as a potential therapeutic strategy in myeloid malignancies and cancer (Barbieri et al., 2017; Vu et al., 2017a; Weng et al., 2018). Our work describes the consequences of METTL3 depletion in the blood system and indicates the importance of this pathway in normal hematopoietic development at various stages including in more mature lineages. Importantly, our data also demonstrate that transient siRNA depletion of METTL3 results in reversible and modest effects on hematopoiesis. However, the true therapeutic window for METTL3 inhibitors will only be known once potent and selective inhibitors are developed.

Overall, our findings demonstrate that m\textsuperscript{6}A is essential for the maintenance of HSC identity and symmetric commitment. As the control of symmetric commitment in adult stem cells is critical for rapidly replenishing tissue compartments after stress or damage, our study highlights the role of RNA methylation in adult stem cell commitment and fate determination.

**STAR METHODS**

Detailed methods are provided in the online version of this paper and include the following:

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**SUPPLEMENTAL INFORMATION**

Supplemental Information can be found online at https://doi.org/10.1016/j.celrep.2019.07.032.

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**AUTHOR CONTRIBUTIONS**

Y.C. led this project, designed and performed experiments, analyzed data, and wrote the manuscript. H.L. designed and performed experiments, analyzed data, and wrote the manuscript. F.I. analyzed data, and wrote the manuscript. B.F.P. performed the global m\textsuperscript{6}A assay, analyzed m\textsuperscript{6}A-seq, and edited the manuscript. A.S. and S.G. provided experimental supports. L.P.V. provided help with the experimental design and wrote the manuscript. S.R.J. and D.A.L. supervised the project and edited the manuscript. M.G.K. directed the project, analyzed data, and wrote the manuscript.

**DECLARATION OF INTERESTS**

D.A.L. received consulting honoraria from Pharmacyclics. S.R.J. is a scientific founder of Gotham Therapeutics and has equity in this company.

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# STAR★METHODS

## KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| **Antibodies**      |        |            |
| METTL3 Ab           | Proteintech | Cat# 150731-1-AP; RRID: AB_2142033 |
| MYC Ab              | Cell signaling Technology | Cat# 6605S; RRID: AB_1903938 |
| Actin Ab            | Sigma Aldrich | Cat# A3854; RRID: AB_262011 |
| Gr1-APC cloneRB6-8C5 | Thermo Fisher Scientific | Cat# 17-5931-82; RRID: AB_469476 |
| Mac1-PB clone M1/70 | Biolegend | Cat# 101224; RRID: AB_755986 |
| c-Kit APCCy7 clone2B8 | Biolegend | Cat# 105826; RRID: AB_1626278 |
| B220-PeCy7 clone RA3-6B2 | BD Biosciences | Cat# 552772; RRID: AB_394458 |
| CD19-PeCy5 clone eBio1D3 | Thermo Fisher Scientific | Cat# 15-0193-83; RRID: AB_657673 |
| IgM APC clone RMM-1 | Biolegend | Cat# 406509; RRID: AB_315059 |
| CD3e-PB clone 145-2C11 | Thermo Fisher Scientific | Cat# 48-0031-80; RRID: AB_10733280 |
| CD4 PE clone GK1.5 | BD Biosciences | Cat# 557308; RRID: AB_396634 |
| CD8 FITC clone 53-6.7 | BD Biosciences | Cat# 553031; RRID: AB_394569 |
| CD34-FITC clone RAM34 | eBioscience | Cat# 11-0341-85; RRID: AB_465022 |
| Sca1-PB clone E13-161.7 | Biolegend | Cat# 122520; RRID: AB_2143327 |
| CD150-APC clone TC15-12F12.2 | Biolegend | Cat# 115910; RRID: AB_493460 |
| CD48-PE clone HM48-1 | BD Biosciences | Cat# 557485; RRID: AB_396725 |
| FcγRIIb-PeCy7 clone 2.4G2 | BD Biosciences | Cat# 560829; RRID: AB_10563207 |
| CD41-PE clone MWRreg30 | BD Biosciences | Cat# 558040; RRID: AB_397004 |
| Ter119-PeCy5 clone TER-119 | Thermo Fisher Scientific | Cat# 15-5921-82 RRID: AB_468810 |
| CD71-FITC clone R17217 | Thermo Fisher Scientific | Cat# 11-0711-81 RRID: AB_465123 |
| CD45.1 Pe-CF594 clone A20 | BD Biosciences | Cat# 562452; RRID: AB_1115295 |
| CD45.2 Alexa 700 clone 104 | Thermo Fisher Scientific | Cat# 56-0454-82 RRID: AB_657752 |
| CD3-PeCy5 | Invitrogen | Cat# 15-0031-83 RRID: AB_468691 |
| CD4-PeCy5 | Invitrogen | Cat# 15-0041-83 RRID: AB_468696 |
| CD8-PeCy5 | Invitrogen | Cat# 15-0081-83 RRID: AB_468767 |
| Gr1-PeCy5 | Invitrogen | Cat# 15-5931-82 RRID: AB_468813 |
| B220- PeCy5 | Invitrogen | Cat# 15-0452-83 RRID: AB_468756 |
| CD19- PeCy5 | Invitrogen | Cat# 15-0193-83 RRID: AB_657673 |
| Ki67 –FITC | Biolegend | Cat# 652410 RRID: AB_2562141 |
| NUMB Ab | Abcam | Cat# Ab4147 RRID: AB_304320 |
| m6A Ab | Abcam | Cat# Ab151230 RRID: AB_2753144 |

| Chemicals, Peptides, and Recombinant Proteins | | |
|-----------------------------------------------|--------|------------|
| pIpC HMW                          | InvVivogen | Cat# Vac-Pic |
| MitoTracker Green                  | Invitrogen | Cat# M7514 |
| Pyrionin Y                        | Sigma | Cat# 213519 |
| Murine SCF                        | Peprotech | Cat# 250-03 |
| Murine IL-3                       | Peprotech | Cat# 213-13 |
| Murine IL-6                       | Peprotech | Cat# 216-16 |
| Murine TPO                        | Peprotech | Cat# 315-14 |
| Murine FLT3L                      | Peprotech | Cat# 250-31L |
| MethoCult M3434                    | Stem Cell Technologies | Cat# 03434 |
| SFEM medium                       | Stem Cell Technologies | Cat# NC9753895 |

(Continued on next page)
LEAD CONTACT AND MATERIALS AVAILABILITY

Further information and requests for reagents may be directed to, and will be fulfilled by the corresponding author Michael G Kharas (kharasm@mskcc.org).

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Mice
Mettl3 flox/flox mice were generated by inserting loxP sites spanning the fourth exon of Mettl3 via homologous recombination base on construction taken from Knockout Mouse Project Repository (KOMP). We next crossed Mettl3 flox/flox mice with Mx1-Cre mice to

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| O-Propargyl-puromycin (OP-Puro) | Jena Bioscience | Cat# NU-931-05 |
| Actinomycin D | Sigma | Cat# 50-76-0 |

Critical commercial assays

| Reagent or Resource | Source | Identifier |
|---------------------|--------|------------|
| RNaseq Plus Micro Kit | Qiagen | Cat# 74034 |
| Murine CD117 Microbeads Kit | Miltenyi Biotec | Cat# 130091224 |
| Verso cDNA Synthesis Kit | Thermo Fisher Scientific | Cat# A81453-B |
| Power SYBR Green PCR Master Mix | Thermo Fisher Scientific | Cat# 4367659 |
| Click-IT Flow Cytometry Assay kit | Thermo Fisher Scientific | Cat# C10418 |
| RNaseq multiplex fluorescent detection kit | Advanced Cell Diagnostics | Cat# 320851 |

Deposited Data

| Single cell RNA-sequencing data and bulk RNA-sequencing data | National Center for Biotechnology Information Gene Expression Omnibus (GEO) | GSE132357 |

Experimental Models: Strains

| Mouse: Mettl3 f/f Mx1-Cre mice | This paper | N/A |
| Mouse: C57BL/6J | The Jackson Laboratory | Stock No: 000664 |
| B6.SJL-Ptprc < a > /Boy | Taconic Biosciences | Stock No: 4007 |

Oligonucleotides

| Genotyping primers-Forward | ACCACAACAGCGCAAGGAACA | N/A |
| Genotyping primers-Reverse | CCGGAGCCTGAAACCTTGT | N/A |
| Myc-Forward | GCTGTTTGAGGCTGGATTC | N/A |
| Myc-Reverse | GATGAAATAGGGCTGTACGGAG | N/A |
| Mettl3-Forward | AAGGAGCCGGCTAAGAAGTC | N/A |
| Mettl3-Reverse | TCACGTGGTCTCATGCCTTC | N/A |
| Actin-Forward | ACCAACTGGGACGACATGGGAG | N/A |
| Actin-Reverse | TACGACCAGAGGCATACAGGGACA | N/A |

Recombinant DNA

| MSCV-MYC-ires-GFP | This paper | N/A |
| MSCV-FLAG-METTL3-ires-GFP | This paper | N/A |
| MSCV-FLAG-METTL3-CD-ires-GFP | This paper | N/A |

Software and Algorithms

| ImageJ version 2.0.0 | ImageJ | N/A |
| FlowJo software (version10.2) | FlowJo | N/A |
| GraphPad Prism 7 | GraphPad Software | N/A |
| R version 3.5.2 (2018-12-20) | R development core team, 2018 | N/A |
| Rstudio 1.1.456 | Rstudio, Inc. | N/A |
| cellrangerRkit 2.0.0 | 10X Genomics | https://www.r-project.org |
| Seurat 2.3.4 | Raoul Satija laboratory at New York Genome Center | https://www.rstudio.com/ |
| dplyr 0.8.1 | Romain François, Lionel Henry and Kirill Müller | software@10xgenomics.com |
obtain induced hematopoietic conditional knockout mice. Deletion of Mettl3 was initiated using plpC HMW (InVivogen, Vac-Pic) by intraperitoneal injections at a dose of 10 mg/kg on 2 consecutive days as showed previously (Liu et al., 2015). Mice used for this experiment were 8 to 12 weeks of age. Both male and female mice were used. All mice were housed on a 12:12 hr light:dark cycle at 25°C and received water and chow ad libitum. All of the animal experiments were approved under the Institutional Animal Care and Use Committee.

**METHOD DETAILS**

**Noncompetitive Transplant**

Noncompetitive transplants were performed as previously described (Park et al., 2014). Briefly, in the primary transplant, 10^6 whole bone marrow cells from Mettl3 cKO mice or littermate Mettl3 f/f mice (3 weeks post plpC) were injected into lethally irradiated B6SJL congenic CD45.1 recipients. In the cell-autonomous transplants, we transplant 10^5 whole BONE MARROW cells from 8-12 weeks old Mettl3 flox/flox, Mx1-Cre+ or Mx1-Cre- mice into lethally irradiated B6SJL congenic CD45.1 recipients. After 6 weeks of engraftment, these transplanted mice were treated with plpC as shown previously. All recipient mice used were 6-8 weeks old. All animals were randomly assigned to the experimental groups. For chimerism, either peripheral blood or bone marrow cells was extracted and subjected to flow cytometry at different time points.

**PCR and Quantitative RT-PCR**

Genomic DNA from Mettl3 f/f and Mettl3 cKO whole bone marrow cells was isolated by using genomic DNA extraction kit (QIAGEN) and used as template for PCR to genotype. Primers are Forward: ACCACACAGCCAAGGAAAC ; Reverse: CCGGAGCTCT GAAACCTTGT. For qRT-PCR, total RNA was extracted from cells using TRIZOL (Life Technologies) following the standard manual. Equal amount of RNA from samples was reverse transcribed into cDNA with Verso cDNA Synthesis Kit (Thermo), and qPCR was performed using an ABI 7500 sequence detection system using primers together with SYBR green master mix (ABI systems). Primers are listed below:

- **Myc:** Forward: GCTGTGTGAGCGTCGATTTC
  Reverse: GATGAAATAGGGCTGTACGGAG
- **Mettl3:** Forward: AAGGAGCCGGCTAAGAAGTC
  Reverse: TCACTGGCTTTCATGCACTC
- **Actin:** Forward: ACCAACTGGGACGACATGGAGAAG
  Reverse: TACGACCAGGGGATAACGAGGAAC

**Flow Cytometry and Cell Sorting**

Bone marrow or spleen cells were harvested and subjected to red blood cell lysis. To measure HSPC compartments, cells were stained with following cocktail: Linage marker (CD3 (15-0031-83), CD4(15-0041-83), CD8(15-0081-83), Gr1(15-5931-82), B220(15-0452-83), CD19(15-0193-83) and Ter119(15-5921-82))- PE-Cy5(Invitrogen, eBioscience), cKit-APC-Cy7 (BioLegend, 105826), Sca-1-Pacific Blue (BioLegend, 122520), CD150-APC (BioLegend, 115910), CD48-PE (BD Bioscience, 557485), CD34-FITC (eBioscience, 11-0341-85), CD16/32-PE-Cy7 (Invitrogen, 560829). To monitor lineage cells differentiation cells were stained with cocktail including Gr1-APC (Invitrogen, 17-5931-82), Mac1-Pacific Blue (BioLegend, 101224), Ter119-PE-Cy5 (Invitrogen, 15-5921-82), CD71-FITC (Invitrogen, 11-0711-81), CD41-PE (BD Bioscience, 558040) or cocktail containing CD3-Pacific Blue (eBioscience, 48-0031-80), CD4-PE (BD Bioscience, 557308), B220-PE-Cy7 (BD Bioscience, 552772), CD19-PE-Cy5 (Invitrogen, 15-0193-83), IgM-APC (BioLegend, 406509), CD43-FITC (eBioscience, 11-0711-81), CD19-PE-Cy5 (Invitrogen, 15-0193-83). To monitor lineage cells differentiation cells were stained with cocktail containing Gr1-APC (Invitrogen, 17-5931-82), Mac1-Pacific Blue (BioLegend, 101224), Ter119-PE-Cy5 (Invitrogen, 15-5921-82), CD71-FITC (Invitrogen, 11-0711-81), CD41-PE (BD Bioscience, 558040) or cocktail containing CD3-Pacific Blue (eBioscience, 48-0031-80), CD4-PE (BD Bioscience, 557308), B220-PE-Cy7 (BD Bioscience, 552772), CD19-PE-Cy5 (Invitrogen, 15-0193-83), IgM-APC (BioLegend, 406509), CD43-FITC (BD Bioscience, 553270). For transplanted mice, we added CD45.1-PE-Txase Red (BD Bioscience, 562452) and CD45.2-A700 (Invitrogen, 56-0454-82) to distinguish donor and recipient cells. For cell cycle analyses, Ki67/Hoechst staining was performed according to manufacturer’s recommendation (BD Biosciences). For PY/Hoechst staining, bone marrow cells stained with HSC surface markers (Linage marker (CD3, CD4, CD8, Gr1, B220, CD19 and Ter119)-PE-Cy5, cKit-APC-Cy7, Sca-1-PECy7 (BioLegend, 122514), CD150-APC, CD48-FITC (BioLegend, 103404) were resuspended in 500ul NASS buffer (1 phosphate citrate tablet (sigma), 0.9g EDTA, 0.45 g NaCl, 0.25 g BSA in 50ml ddH2O), 3.3ul diluted Hoechst (1:10 in NASS) was added followed by 30mins in room temperature. Samples were then incubated on ice for 5 mins to stop reaction. 2.5ul of diluted PY (1:100 of 10mg/ml PY stock in NASS) was added to samples. Incubate for 10mins at 4 degree. Wash the samples before analysis. For mitochondrial mass and activity, BMCs were incubated with 100nM MitoTracker Green (Invitrogen) or 50nM DilC5 (Thermo Fisher) for 30 min at 37°C in the dark after cell surface staining (Linage marker (CD3, CD4, CD8, Gr1, B220, CD19 and Ter119)-PE-Cy5, cKit-APC-Cy7, Sca-1-Pacific Blue, CD150-APC/CD150 FITC, CD48-PE). Cells were analyzed on a BD FACS LSR or Fortessa instrument. For HSPC(LSK) or HSC (LSK, CD150+CD48-) cell sorting, bone marrow cells were harvested and incubated with MACS beads (CD117, Miltenyi Biotec, 130-901-224). Then enriched c-Kit+ cells were collected by running AutoMACS (Miltenyi Biotec) according to the manufacturer’s instructions. The cells were then stained with cocktail: Linage marker (CD3, CD4, CD8, Gr1, B220, CD19 and Ter119)-PE-Cy5, cKit-APC-Cy7, Sca-1-Pacific Blue, CD150-APC, CD48-PE. Specific cell population was sorted on BD Aria machine.
Peripheral Blood Analysis
Peripheral blood was collected from the retroorbital cavity using a heparinized glass capillary tube. Complete peripheral blood count analysis including a differential blood count was obtained by using Hemavet (Drew Scientific). For flow cytometry, peripheral blood was treated by red blood cell lysis to remove red blood cells and then applied to flow as showed earlier.

Western-Blot
LSK cells were sorted from Mettl3 f/f and Mettl3 cKO bone marrow cells following protocol described before. 50,000 cells were then lysed in 30 μL x Laemml protein loading buffer and boiled for 5 min. Whole-cell lysates were run on a 4%–15% gradient SDS–PAGE and transferred to a nitrocellulose membrane. Membranes were probed with the METTL3 (Proteintech, 150731-1-AP) and ACTIN (Sigma, A3854) antibody.

Determination of Relative m⁶A Levels
Relative levels of global m⁶A was determined by thin-layer chromatography (TLC), as described previously (Vu et al., 2017a). Briefly, poly(A)-purified RNA was digested with 2 U ribonuclease T1 for 2 h at 37°C in the presence of RNaseOUT (Invitrogen) first. Next, we labeled digested RNA at 5’ with [γ-32P] ATP. After removal of γ phosphate of ATP by apyrase (NEB), labeled RNA was purified by phenol-chloroform extraction and ethanol precipitation and digested to single nucleotides nuclease. 1 mL of released 5’ monophosphates was analyzed on glass-backed PEI-cellulose plates (Merck-Millipore) and exposed to a storage phosphor screen (GE Healthcare Life Sciences), as described previously. The relative m⁶A was calculated as a percentage of the total of A, C and U spots which is quantified with ImageJ, as described previously (Mauer et al., 2017).

Colony Assay
10,000 whole Mettl3 f/f or Mettl3 cKO bone marrow cells were plated in in M3434 methylcellulose media (STEMCELL Technologies, 03434), and colonies were scored at 10 days post plating, respectively.

Competitive Transplant
HSC and MPP1 cells from Mettl3 f/f or Mettl3 cKO mice were sorted following protocol described above. 500 sorted HSC and MPP1 cells plus 500K CD45.1 whole bone marrow cells were injected into lethally irradiated B6SJL congenic CD45.1 recipients directly after cell sorting. For chimerism, either peripheral blood or bone marrow cells was extracted and subjected to flow cytometry at different time points.

HSPC Cell Sorting, Cell Culture, Retroviral Infection, and Transplant
HSPC (LSK) cells from Mettl3 f/f or Mettl3 cKO mice were sorted following protocol described above. Sorted LSK cells were cultured in the SFEM medium (STEMCELL Technologies, NC9753895) supplemented with murine cytokines (50 ng/ml SCF, 10 ng/ml IL-3, and 10 ng/ml IL-6, 10ng/ml TPO and 20 ng/ml FLT3L, PeproTec). Cells were then transduced with high-titer concentrated retroviral suspensions in the presence of 4 μg/ml polybrene and followed with spin infection for 1.5 hr. Next day, second round of transduction was performed as described. For transplant, 10,000 LSK cells plus 500K CD45.1 whole bone marrow cells were injected into lethally irradiated B6SJL congenic CD45.1 recipients. For chimerism, either peripheral blood or bone marrow cells was extracted and subjected to flow cytometry at different time points.

O-Propargyl-puromycin (OP-Puro) Flow Analysis
Protein synthesis rate was assessed by O-Propargyl-puromycin (OP-Puro) as described previously (Vu et al., 2017b). Mettl3 f/f and Mettl3 cKO HSC cells were sorted and treated with 50 μM O-propargyl-puromycin (OP-Puro; NU-931-05, Jena Bioscience) for 1 hour. Cells were washed once, collected, and then subjected to processing using the Click-iT Flow Cytometry Assay kit (C10418, Invitrogen) following the manufacturer’s instructions. Labeled cells were analyzed using a BD Fortessa instrument.

Immunofluorescence
HSCs were sorted from Mettl3 f/f and Mettl3 cKO mice following protocol shown before. We cultured the sorted HSCs with SFEM media (STEMCELL Technologies, NC9753895) containing 10 ng/ml heparin, 10 ng/ml SCF, 20 ng/ml TPO, 20 ng/ml IGFII, and 10 ng/ml FGF, PeproTec) in 96 round-bottom wells for 16 h and then treated cells with 10 nM Nocodazole for 24 h. After incubation, cells were fixed with 4% paraformaldehyde 15 mins at room temperature and permeabilized with cold methanol. Fixed HSCs were then cytospun onto glass slides and were blocked for 1h followed with staining on slides with anti-NUMB (Abcam, ab4147), anti-MYC (Cell Signaling, 5605) and secondary Ab (donkey anti–goat Alexa fluor 488, Invitrogen, A11055; donkey anti–rabbit Alexa fluor 568, Invitrogen, A10042; Molecular Probes) with DAPI counterstaining. We evaluate symmetric and asymmetric percentages based on the fluorescence signal intensity of each cell acquired by Axio Imager M2 microscope (Carl Zeiss) and quantified by FIJI. Thresholds to determine NUMB high/low/asymmetric were set for experimental replicates. Briefly, both of daughter cells with high NUMB staining or average staining intensity is above NUMB high threshold and there is less than 2-fold difference in the daughter pairs, this condition was counted as symmetric commitment. Daughter pair cells were scored as a symmetric renewal
division when both of them were low or no staining or average staining intensity is below NUMB high threshold and there is less than 2-fold difference in the daughter pairs. If else, the division was considered an asymmetric division (Kharas et al., 2010).

**RNA-FISH in Conjugation with Fluorescent Immunostaining**

Sorted HSCs were cultured in SFEM media and treated with Nocodazole as shown above. HSC cells were then fixed with 4% paraformaldehyde and permeabilized with cold methanol. Fixed HSCs were then cytopsin onto glass slides. RNA in situ hybridization was performed using RNAscope multiplex fluorescent detection kit according to the manufacturer’s instructions (Advanced Cell Diagnostics). RNAscope probes targeting mouse Myc was designed and produced by ACDBio. After the in situ hybridization was completed, slides were washed twice and subjected to immunostaining as shown above.

**RNA Stability Assay**

Sorted HSCs were cultured in SFEM media supplemented with cytokines as shown above. After overnight culture, HSCs were treated with 5 μM actinomycin D (Sigma) for inhibition of mRNA transcription. Cells were collected at 0 min, 10 min, 20 min or 40 min post treatment, and total RNA was extracted and used for qRT-PCR.

**Electroporation Transfection**

Neon nucleofection of HSPCs was performed as described (Gundry et al., 2016). HSCs were sorted and cultured in the SFEM medium for 1-3 hours before transfection. 1 μg Plasmids or 100pmol siRNAs were electroporated into 100,000 HSCs or LSKs by using Neon transfection system (Thermo Fisher Scientific) under a condition: 1700V, 20ms, 1 pulse. If higher numbers of cells were needed, multiple electroporations were performed (e.g., 6 electroporations of 100,000 LSKs for 600,000 total cells).

**m6A-seq Peak Enrichment Analysis**

Previously published data of m6A-seq and input RNA-seq from mouse LT-HSCs, ST-HSCs, and MPPs were downloaded from NCBI GEO (GSE107957). PCR duplicates were removed using the pyDuplicateRemover.py script of the pyCRAC tool suite (version 1.2.2.3). Deducuplicated sequences were then aligned to the mm10 mouse genome build using bowtie2 (version 2.3.4.2) (bowtie2 -x mm10 -N 0 –trim5 3). Significantly enriched m6A peaks were determined using the R/Bioconductor package exomePeak.

**MeRIP-qPCR**

MeRIP-qPCR was performed as described previously (Vu et al., 2017a). Briefly, total RNA was isolated from sorted LSKs cells (from 3 Mettl3 cKO or control mice) by Trizol and poly(A)+ RNA was isolated using Dynabeads Oligo-(dT)25 magnetic beads (ThermoFisher Scientific). 5 μg anti-m6A antibody (Abcam, ab151230) was pre-bound to Protein A/G magnetic beads (Pierce) in IP buffer (20-mM Tris pH 7.5, 140-mM NaCl, 1% NP-40, 2-mM EDTA) for 1h. 1 μg Poly(A)+ RNA was mixed with 400 μl of IP buffer and added to the Protein A/G beads for 2 hours at 4°C. Samples were washed twice in low-salt-wash buffer (10-mM Tris pH 7.5, 5 mM EDTA), twice with high-salt-wash buffer (20-mM Tris pH 7.5, 1-M NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 1-mM EDTA) and twice with RIPA buffer (20-mM Tris pH 7.5, 150-mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 1-mM EDTA). RNA was eluted from the beads by incubating with 200 μl 0.5 mg/ml N6-methyladenosine 5-monophosphate sodium salt (Sigma Aldrich) for 1h at 4°C. Following ethanol precipitation, input and eluted poly(A)+ RNA was reverse-transcribed using Superscript III with random hexamers, and enrichment of m6A-containing transcripts was determined by quantitative PCR.

**RNA-Seq**

3 replicates (from 3 different mice) of HSC, MPP1, MPP2 and MPP4 cells were sorted from Mettl3 f/f and Mettl3 cKO mice base on cell surface marker. RNA was extracted from bulk cells using the SMARTer RNA extraction method for minimal cells RNA extraction. After amplification, cDNA was subjected to automated Illumina paired-end library construction. Libraries were sequenced on Illumina HiSeq2000 instruments with paired reads of 50 bp in length per sample. Sequence data were aligned using the tophat software to the GRCh37-lite human reference genome. Fragments Per Kilobase of transcript per Million mapped reads were calculated using the cufflink software. Differentially expressed genes were identified as those with FPKM greater than 1 for Mettl3 f/f or Mettl3 cKO showing differential expression greater than twofold (up or down) with a Benjamini–Hochberg corrected p value less than 0.05.

**Single-Cell RNA-Seq**

Single-cell RNA data processing, alignment, cell type classification and clustering: 10x data were processed using Cell Ranger 2.1.0 with default parameters. Reads were aligned to the mouse reference mm10. The Seurat package (Butler et al., 2018) was used to perform unbiased clustering of the cells bone marrow Lin-, c-Kit+ sorted hematopoietic progenitors. Briefly, cells with < 200 detected genes or percentage of mitochondrial reads > 20% were filtered out in addition, cells that showed lower number of genes than expected from the number of UMIs detected (Figure S4B) were filtered out as low-quality cells. The data were log normalized using a scale factor of 10,000. Potential confounders (e.g., number of UMI per cell, the proportion of mitochondrial genes and proportion of ribosomal genes) were regressed out of the data before principle component analysis (PCA) was performed using variable genes. JackStraw method was used to determine the statistically significant PCs to be used for graph-based clustering. t-SNE was used to visualize the clusters. Cell types were classified according to expression of previously reported marker genes (Paul et al., 2015).
Pseudotime ordering was performed using Monocle (Trapnell et al., 2014), setting the origin of pseudotime in the cell state containing the highest fraction of cells from the HSC cluster.

QUANTIFICATION AND STATISTICAL ANALYSIS

Data were processed using GraphPad Prism v.7. All analyses were performed using two-tailed Student’s t tests, except where stated otherwise. Graphs and error bars reflect means ± s.e.m., except where stated otherwise. In all corresponding figures, * represents p < 0.05. ** represents p < 0.01. *** represents p < 0.001. ns represents p > 0.05. Replicate information is indicated in the figures.

DATA AND CODE AVAILABILITY

The single cell RNA-seq and bulk RNA-seq data have been deposited in the Gene Expression Omnibus with GEO: GSE132357.
m\(^6\)A RNA Methylation Maintains Hematopoietic Stem Cell Identity and Symmetric Commitment

Yuanming Cheng, Hanzhi Luo, Franco Izzo, Brian F. Pickering, Diu Nguyen, Robert Myers, Alexandra Schurer, Saroj Gourkanti, Jens C. Brüning, Ly P. Vu, Samie R. Jaffrey, Dan A. Landau, and Michael G. Kharas
Figure S1
Supplementary Figure 1. m^6A is essential for normal hematopoiesis. Related to Figure 1.

All samples were collected from mice at 3 weeks post pIpC administration. (A) Schematic diagram of the experimental procedure for analyzing Mettl3 f/f and Mettl3 cKO mice. (B) Immunoblot to determine METTL3 expression using sorted LSK cells. (C-E) Myeloid, megakaryocytes, erythroid, and lymphoid lineage differentiation in BM was determined by flow cytometry base on different cell surface markers. Erythroblast (ProE, Ter119-CD71^+; Ery2, Ter119+CD71^+; Ery3, Ter119+CD71_mid^+; Ery4, Ter119+CD71^-), Gran (Granulocyte, Gr1+Mac1^+), Immature Granulocyte(Mac1+Gr1_mid^+), Monocyte(Mac1+Gr1^-), Megakaryocytes (Mgk, CD41^+), Immature B cells(B220+IgM^+), Pre/Pro B cells (B220+IgM^-), Mature B cells (B220^-IgM^+), T cells (CD3^+). n=11. (F) Representative images show H&E-stained cross sections of BM isolated from the Mettl3 f/f and Mettl3 cKO mice. (G) Mettl3 depleted BM formed fewer and smaller colonies. Mettl3 f/f and METTL3 depleted BM cells were plated in methylcellulose cultures, supplemented with cytokines. Left: Representative images of flox/flox and cKO colony. Right: Colonies were scored 10 days after plating. n =9,7. (H) Splenomegaly is observed in cKO mice three weeks post pIpC administration. Left: representative image of spleens from indicated mice. Right: measurement of spleen weight from Mettl3 f/f and Mettl3 cKO mice. n=11. (I) Representative images show H&E-stained cross sections of spleens isolated from the Mettl3 f/f and Mettl3 cKO mice. (J, K) Erythroid, myeloid and lymphoid cells were determined by flow cytometry in Mettl3 f/f and Mettl3 cKO spleens. Cell population was determined by cell surface marker. n=8. (L) Absolute numbers of myeloid progenitor (MP, Lin-ckit+Sca1^-), CMP(Lin-ckit+Sca1-CD34+FcrR^-), GMP (Lin-ckit+Sca1-CD34+FcrR^+) and MEP(Lin-ckit+Sca1-CD34-FcrR^-) in Mettl3 f/f and Mettl3 cKO BM. n=10, 6. Mean and SEM are shown (*, P < 0.05; **, P < 0.01; ***, P < 0.001, ****, P < 0.0001).
Figure S2
Supplementary Figure 2. *Mettl3* required for HSC reconstitution capacity. Related to Figure 2.

(A) Whole bone marrow cellularity in *Mettl3* f/f and *Het,Cre*+ (*Mettl3* f/-, *Mx1-Cre*) mice. n=4. (B) Whole blood counts of white blood cell (WBCs), red blood cells (RBCs), platelets (PLT) of *Mettl3* f/f and *Het,Cre*+ mice. n=4. (C) Spleen weight of *Mettl3* f/f and *Het,Cre*+ mice. n=4. (D) Mature lineage cells were determined by flow cytometry in *Mettl3* f/f and *Het,Cre*+ spleens based on cell surface marker as shown in Supplementary Figure1. n=4. (E, F) Frequency of hematopoietic stem and progenitor compartments (LSK, HSC, MPP1, MPP2 and MPP4) and myeloid progenitors (MP, CMP, GMP and MEP) in *Mettl3* f/f and *Het,Cre*+ mice bone marrow. n=4. (G, H) Erythroid, myeloid, megakaryocytes and lymphoid lineage differentiation in *Mettl3* f/f and *Het,Cre*+ mice were assessed by flow cytometry based on cell surface markers. n=4. (I) *Mettl3* cKO HSC cells are more proliferative. Representative flow cytometry plots and quantification of *Mettl3* f/f and *Mettl3* cKO HSC cell cycle by Ki67 staining. n=5. (J) Increased mitochondrial activity in *Mettl3* cKO HSCs. Left: representative histograms of Dilc5 staining in *Mettl3* f/f and *Mettl3* cKO HSCs. Right: Mitochondrial membrane potential in HSPC compartments was assessed by Dilc5 staining quantified by flow cytometry. n=5. (K) Representative flow cytometry of surface markers to indicate engraftment of CD45.2 donor cells in LSK population from non-competitive transplanted recipient mice. (L) *Mettl3* cKO HSCs have impaired reconstitution capacity. Chimerism of CD45.2 in different lineage cell populations in peripheral blood from non-competitive transplanted recipient mice. n=10. (M, N) Cell autonomous transplant experiments. (M) Comparable engraftment of *Mettl3* flox/flox, *Mx1-Cre*- and *Mx1-Cre*+ recipient pre-plpC. Chimerism of CD45.2 donor cells in peripheral blood from recipient mice was analyzed before plpC injection. n=15. (N) CD45.2 chimerism analysis of myeloid, erythroid and lymphoid cells from recipient mice. Cell compartment is determined by cell surface marker as showed previously. n=5. Mean and SEM are shown (*, P < 0.05; **, P < 0.01; ***, P < 0.001, ****, P < 0.0001).
Figure S3
Supplementary Figure 3. HSPC expansion persists in Mettl3 cKO mice 16 weeks post plpC. Related to Figure 3.

All samples used were from Mettl3 f/f and Mettl3 cKO mice at 16 weeks post plpC. (A) Left: Representative flow cytometry plots of surface markers to indicate the stem and progenitor cell compartments in BM. Right: Frequency of LSK and MP in BM cells and percentage of HSC, MPP1, MPP2 and MPP4 in LSK population. n=6. (B) Absolute cell numbers of LSK, HSC and MPPs, from indicated mice at 16 weeks post plpC as assessed by flow cytometry. n=6. (C) Whole blood counts of white blood cell (WBCs), red blood cells (RBCs), platelets (PLT) of Mettl3 f/f and Mettl3 cKO mice. (D) Bone marrow cellularity of Mettl3 f/f and Mettl3 cKO mice. n=6. (E) Absolute cell numbers of progenitor, CMP, GMP and MEP in BM cells. n=6. (F-H) Erythroid, myeloid, megakaryocytes and lymphoid lineage differentiation in long-term METTL3 deleted mice were assessed by flow cytometry base on cell surface markers as shown in Supplementary Figure1. n=6. (I) Measurement of spleen weight from Mettl3 f/f and Mettl3 cKO mice. n=6. (J,K) Erythroid, myeloid and lymphoid cells were evaluated by flow cytometry in splenocytes. n=6. (L) Genotyping PCR using genomic DNA from Mettl3 f/f and Mettl3 cKO mice bone marrow cells at16 weeks post plpC. Mean and SEM are shown (*, P < 0.05; **, P < 0.01; ***, P < 0.001, ****, P < 0.0001).
Supplementary Figure 4. Loss of Mettl3 results in new HSC-like clusters base on scRNA-seq. Related to Figure 4.

(A) Scheme of experiment strategy for single cell RNA-seq and bulk RNA-seq. (B) Filter strategy in three replicates of single cell RNA-seq. (C) Number of ribosomal or mitochondrial reads detected for each cell in each replicate. (D) Single-cell transcriptome landscape defined by gene expression. Different cell cluster was labeled. (E) Heatmap to show differentially expressed genes in Mettl3 cKO specific clusters compared to HSC cluster. (F) Representative differentially expressed genes between KOsp2 cluster and Megakaryocytes cluster were shown as heat map.
Figure S5
Supplementary Figure 5. HSC identity is altered in *Mettl3* cKO mice. Related to Figure 5.

(A) Unsupervised clustering of differentially expressed genes in three replicates of *Mettl3* f/f and *Mettl3* cKO HSC, MPP1, MPP2 and MPP4 cells were shown. (B) *Mettl3* cKO specific clusters are cKO HSC-like based on gene expression. GSEA analysis of cKO specific cell clusters gene sets (top 50 expressed genes from scRNA-seq) against rank lists of differentially expressed genes between *Mettl3* cKO HSCs and cKO MPPs. (C) GSEA analysis with top expressed genes in cKO specific cell clusters from scRNA-seq showing the signature of genes enriched in *Mettl3* f/f HSCs. (D) *Mettl3* KO ESC signature is enriched in *Mettl3* cKO HSCs. Gene Set Enrichment Analysis of upregulated genes in *Mettl3* KO mESCs against the ranklist of differentially expressed genes between *Mettl3* f/f and *Mettl3* cKO HSCs. Normalized enrichment score and p value are shown. (E) Gene-set enrichment for up or down regulated genes in *Mettl3* cKO HSCs, as compared to differentially expressed genes in RBM15 KO LSK cells. Normalized enrichment score and FDR are shown. (F) *Mettl3* f/f MPP1 was enriched in KEGG ribosome gene set in regulating translation compared to *Mettl3* f/f HSCs. (G-I) HSCs and MPP1s from cKO mice fail to engraft in recipient mice in a competitive manner. Sorted HSCs and MPP1s from *Mettl3* f/f and *Mettl3* cKO mice were injected into CD45.1 recipient mice competitively with normal CD45.1 BM cells. (G) CD45.2 chimerism was shown in peripheral blood at 4 weeks post transplantation. n=10. (H) CD45.2 chimerism analysis to show donor engraftment in LSK, MPP2 and MPP4 compartments. n=10. (I) Engraftment of CD45.2 donor cells was analyzed in lineage compartments from recipient mice at 40 weeks. n=10. (J) Gene-set enrichment analysis plots of up or down regulated genes in *Mettl3* cKO HSC identified by bulk RNA-seq, against the rank list of differentially expressed genes between *Mettl3* f/f HSC and *Mettl3* f/f MPP2. Mean and SEM are shown (*, P < 0.05; **, P < 0.01; ***, P < 0.001, ****, P < 0.0001).
Figure S6
Supplementary Figure 6. m⁶A-mediated Myc regulation is essential for HSC asymmetric and symmetric cell division. Related to Figure 6.

(A) m⁶A marks on Myc transcripts in HSCs and MPPs. m⁶A peaks were indicated in dot line square. (B) qRT-PCR of Mettl3 in Mettl3 f/f and Mettl3 cKO HSCs. (C,D) Overexpression of MYC in Mettl3 cKO HSCs partially rescued HSC symmetric commitment defect in pair daughter assay as quantified by immunofluorescence. Number of daughter pairs assessed: Mettl3 f/f +Vec, n=141; cKO+Vec, n=35; cKO+MYC, n=33.
Figure S7
Supplementary Figure 7. METTL3 and MYC partially rescue defect in Mettl3 cKO HSCs. Related to Figure 7.

(A-C) Sorted control or Mettl3 cKO LSK cells transduced with control or MYC overexpression retrovirus and then transplanted into recipient mice. (A) Engraftment of donor-derived CD45.2 cells in different lineage cells including B cells (B220+), T cells (CD3+), Erythroblast (Ter119+) and Granulocyte (Gr1+). n=10. (B) Engraftment of donor-derived CD45.2 cells in HSC and MPP compartments in recipient mice BM. n=10. (C) Representative flow cytometry plots to show abnormal myeloblast in recipient mice transplanted with MYC overexpressed Mettl3 cKO LSKs. (D) Sorted HSCs were co-transfected with control or Mettl3 siRNA together with empty vector or MYC, METTL3, METTL3-CD constructs as indicated in the figure. Overall MYC expression is quantified by immunofluorescence. (E) LSK cells were sorted from Mettl3 cKO mice and transduced with control or wild-type METTL3 or catalytically dead METTL3 (METTL3-CD) overexpression retrovirus. Donor cells were then transplanted into CD45.1 recipient mice with CD45.1 competitor BM cells. CD45.2 chimerism was shown in peripheral blood at 4 weeks post transplantation. n=5. (F) Sorted Mettl3 f/f LSK cells were transduced with a retrovirus expressing GFP together with an empty vector as control, or a retrovirus expressing METTL3 or METTL3-CD and then transplanted into recipient mice. CD45.2 donor engraftment was analyzed at 4 weeks post-transplant. n=10. Mean and SEM are shown (*, P < 0.05; **, P < 0.01; ***, P < 0.001, ****, P < 0.0001).