# m$^6$A reader Ythdf3 protects hematopoietic stem cell integrity under stress by promoting the translation of *Foxm1* and *Asxl1* transcripts

N$^6$-methyladenosine (m$^6$A), which is the most abundant internal modification of eukaryotic mRNA, is dynamically regulated by RNA methyltransferases (writers) and demethylases (erasers).[1] Many RNA binding proteins, including the YT521-B homology (YTH) domain family of proteins (YTHDF1, 2, 3, YTHDC1, 2) and insulin-like growth factor 2 mRNA-binding proteins (IGF2BP1, 2, 3), which can recognize unique m$^6$A-modified RNA and mediate its function, are characterized as "readers".[1] The fate of m$^6$A-marked RNA is determined by different readers through binding to alternative locations of m$^6$A modifications. m$^6$A methylation adds another layer of post-transcriptional regulation of gene expression during normal and malignant hematopoiesis.[2] YTHDF family proteins display high similarity in protein structure with a YTH domain near the N-terminus and contain a low complexity structure that includes a prion-like domain.[3] Previous studies showed that YTHDF proteins have unique functions in RNA metabolism with some overlapping functions. YTHDF1 facilitates the translation of target transcripts by interacting with translation initiation factor complex3 (elF3), while YTHDF2 modulates mRNA decay by recruiting the CCR4-NOT deadenylase complex, and YTHDF3 cooperates with YTHDF1 and YTHDF2 to facilitate the translation and decay of target mRNA.[1,3] However, there are conflicting data. Two recent reports described a different model of YTHDF function, in which YTHDF1, 2, and 3 bind the same m$^6$A-modified mRNA and function together to regulate mRNA stability in HeLa and mouse embryonic stem cells.[4,5] Analysis of publicly available RNA-sequencing data of human CD34$^+$ stem/progenitor cells[6] revealed that YTHDF2 has a relatively higher level of expression compared with YTHDF1 and YTHDF3, which have comparable levels of expression (Figure 1A). Knockout of *Ythdf2* results in the increase of functional hematopoietic stem cells (HSC) in young mice,[7,8] but reduces HSC self-renewal upon aging by stimulating pro-inflammatory pathways.[9] However, the role of Ythdf3 in hematopoiesis remains undetermined.

To elucidate the function of Ythdf3 *in vivo*, we generated and characterized a *Ythdf3* knockout mouse model, in which exon 3 of *Ythdf3* was deleted using CRISPR-Cas9 mediated genome editing technology (Figure 1B). The deletion of the *Ythdf3* allele was validated by polymerase chain reaction (PCR) analysis of genomic DNA from mouse tails (Figure 1C). *Ythdf3* deletion led to the absence of Ythdf3 expression but did not affect the expression of Ythdf1 and Ythdf2 in c-Kit$^+$ mouse bone marrow cells (*Online Supplementary Figure S1A*). Ythdf3-deficient mice were viable and displayed normal hematopoiesis, as evidenced by normal white blood cell, platelet, and red blood cell counts and hemoglobin level in the peripheral blood compared with those of control littermates at 2-3 months of age (*Online Supplementary Figure S1B*). Both *Ythdf3* wildtype and knockout mice had comparable bone marrow cellularity (*Online Supplementary Figure S1C*). As determined by flow cytometric analysis, the lineage distribution of mature myeloid cells, B cells, T cells, and red cells was comparable in bone marrow, spleen, or thymus in a cohort of *Ythdf3*-knockout mice and control mice (*Online Supplementary Figure S1D, E*). We further characterized the hematopoietic stem and progenitor cell compartments in these mice. As shown in *Online Supplementary Figure S1F* and Figure 1D, there were no differences in the frequency and the absolute number of the HSC-enriched populations LSK (Lin$^-$Sca1$^+$c-Kit$^+$), multipotent progenitor (MPP), short-term HSC (ST-HSC), and long-term HSC (LT-HSC, CD150$^+$CD48$^-$Lin$^-$Sca1$^+$c-Kit$^+$) in bone marrow between 2- to 3-month-old *Ythdf3* knockout and control mice. Additionally, we found that apoptosis and quiescence of HSC were not disturbed by *Ythdf3* loss (Figure 1E, F). The frequency and number of hematopoietic progenitor cells (HPC) and subpopulations of myeloid progenitors, including common myeloid progenitors, megakaryocyte and erythroid progenitors, and granulocyte and macrophage progenitors, were not changed as a consequence of Ythdf3 depletion (*Online Supplementary Figure S1G*). These results suggest that Ythdf3 is dispensable for normal hematopoiesis and the maintenance of HSC/HPC during steady-state conditions.

To determine the role of *Ythdf3* in HSC under stress, we performed a bone marrow transplantation assay. More than 90% of donor-derived peripheral blood cells and bone marrow cells were detected in both Ythdf3-deficient and control recipient mice in the first and fourth months after transplantation, respectively (*Online Supplementary Figure S1H*), suggesting a comparable engraftment ability of Ythdf3-deficient and control HSC/HPC. Further characterization of Ythdf3-deficient and control recipient mice revealed there was a decrease of bone marrow cellularity and frequency of LSK (*Online Supplementary Figure S1I*) while the frequencies of MPP, ST-HSC and LT-HSC in the LSK population (*Online Supplementary Figure S1J*) were

comparable in Ythdf3-deficient recipient mice and control recipients. The total numbers of LSK, MPP, and ST-HSC but not LT-HSC were decreased in Ythdf3-deficient recipient mice compared with the numbers in control recipients (Figure 1G). However, there were no differences in mature cell lineage distribution (*Online Supplementary Figure S1K, L*) between Ythdf3-deficient and control recipient mice. The frequency of apoptosis was significantly increased in Ythdf3-deficient HSC and LSK compared with control cells (Figure 1H). To determine the effects of Ythdf3 depletion on hematopoietic reconstitution *in situ*, a cohort of Ythdf3-deficient and control recipient mice were injected

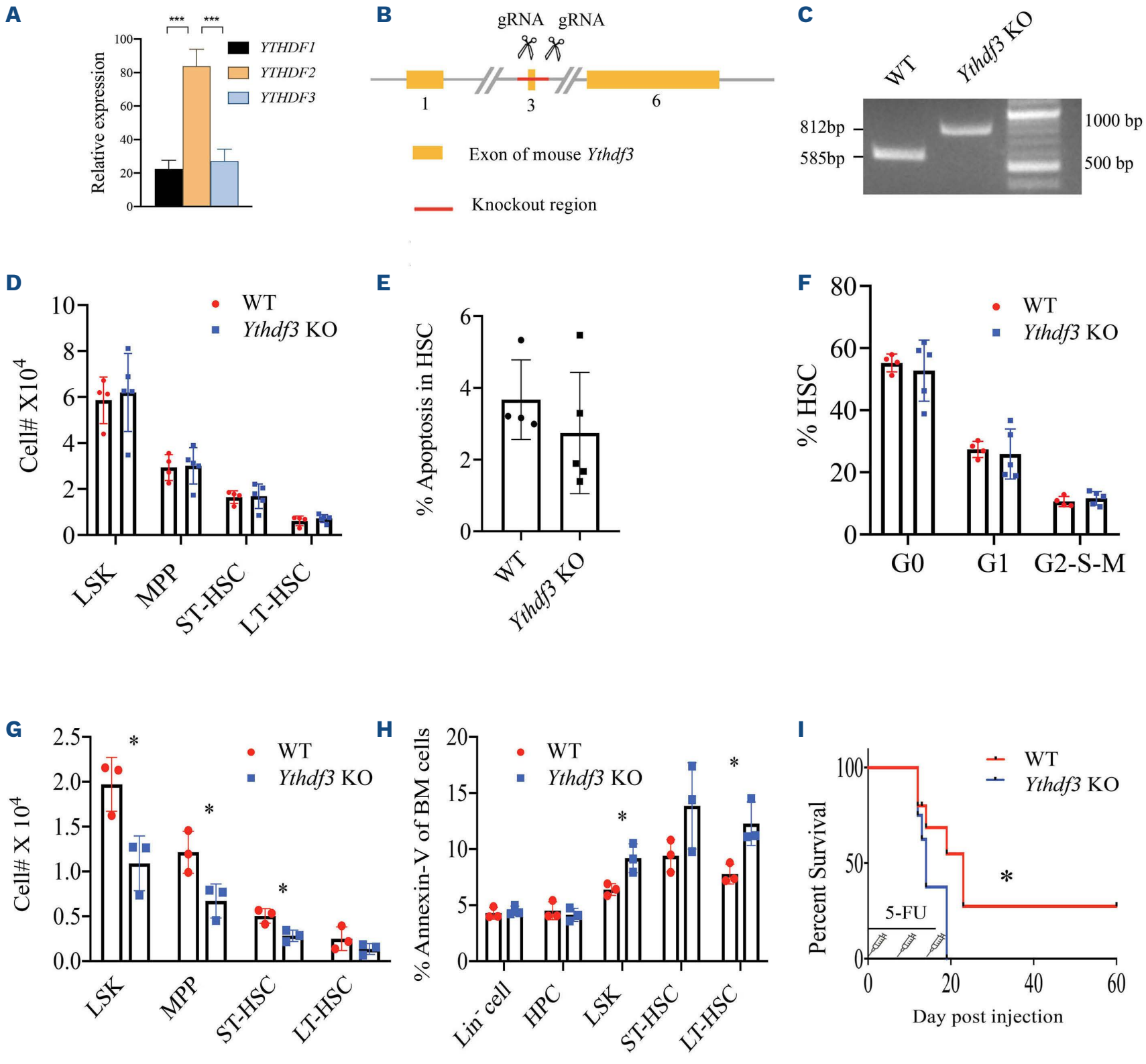


**Figure 1. *Ythdf3* knockout reduces hematopoietic stem cell self-renewal and survival under regenerative stress. (A)** Expression of *YTHDF1*, *YTHDF2*, and *YTHDF3* mRNA in CD34$^+$ cells from healthy individuals. (B) Diagram showing the strategy for generating a mouse with the knockout (KO) of *Ythdf3*. (C) Polymerase chain reaction genotyping of tail genomic DNA from mice generated by heterozygote (Het) crosses. (D) The absolute number of Lin$^-$Sca1$^+$c-Kit$^+$ cells (LSK), multipotent progenitors (MPP), short-term (ST)-hematopoietic stem cells (HSC), and long-term (LT)-HSC in bone marrow (BM) from wild-type (WT) and *Ythdf3* KO mice. (E) Flow cytometric analysis of the frequency of apoptotic cells in the HSC population from 8-week-old WT and *Ythdf3* KO mice (n=4-5). (F) Flow cytometric analysis of cell cycle in the HSC population from 8-week-old WT and *Ythdf3* KO mice (n=4-5). (G) Total LSK, MPP, ST-HSC, and LT-HSC in BM from WT and *Ythdf3* KO recipient mice. The mice were analyzed 4 months after the first round of transplantation (mean ± standard deviation). (H) Frequency of apoptosis in gated Lin$^-$ cells, HPC, LSK, ST-HSC and LT-HSC stained with annexin V and DAPI from *Ythdf3* WT or KO recipient mice. (I) Kaplan-Meier curve representing percent survival of *Ythdf3* WT (n=7) and KO (n=8) mice over time after weekly injections of 5-fluorouracil (5-FU) for three times. **P*<0.05, ***P*<0.01, ****P*<0.001.

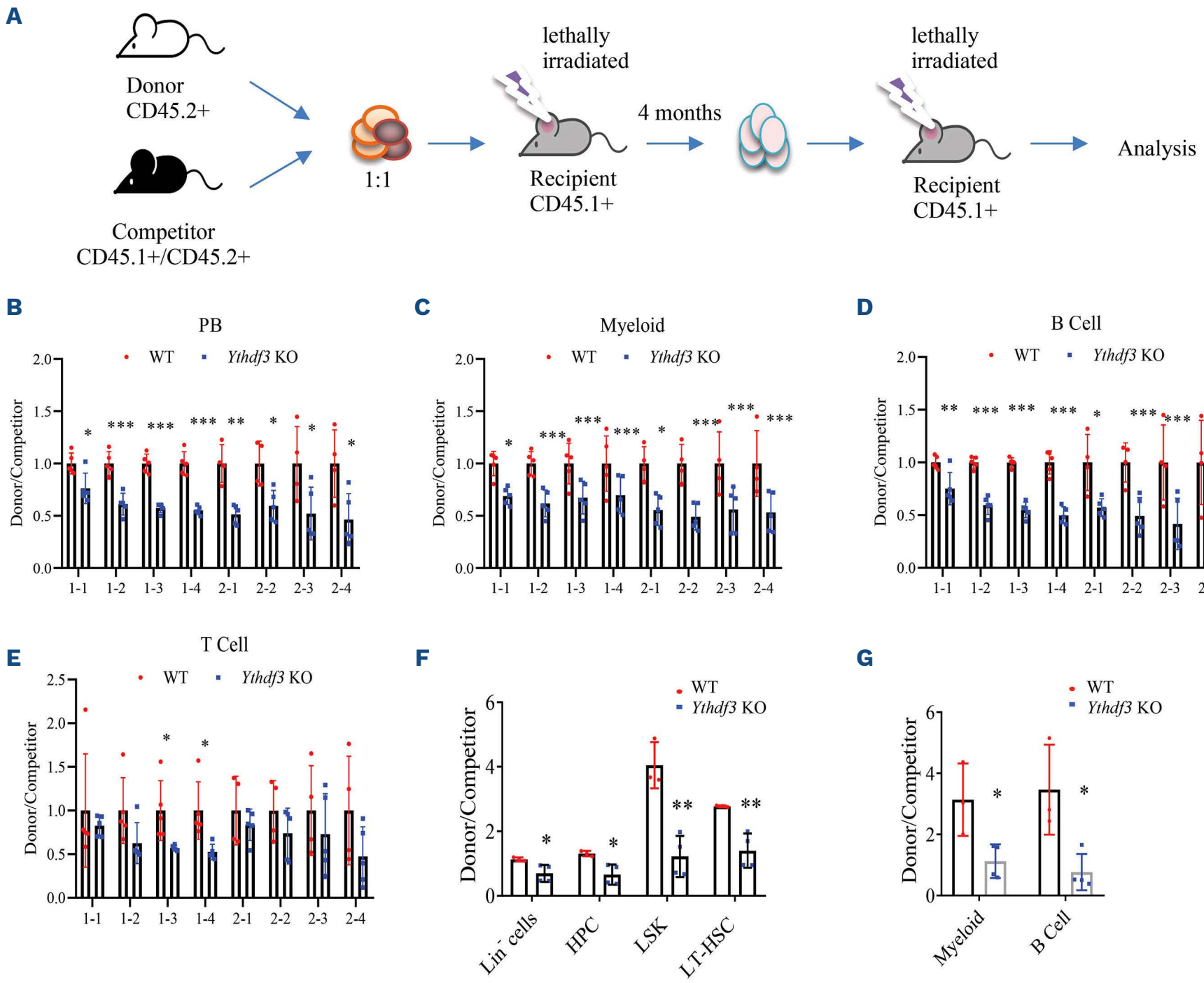


**Figure 2. *Ythdf3* loss reduces the reconstitution ability of hematopoietic stem and progenitor cells.** (A) Schematic diagram of the experimental strategy. (B) Ratio of donor-derived CD45.2$^+$CD45.1$^-$ peripheral blood cells (Donor/Competitor) in chimeric CD45.1$^+$ C57BL/6 wild-type (WT) mice reconstituted with bone marrow cells from *Ythdf3* WT or knockout (KO) mice, assessed every month for 4 months after primary transplantation (1-1, 1-2, 1-2, 1-4) or secondary transplantation (2-1, 2-2, 2-3, 2-4). (C-E) Evaluation of the ratio of donor-derived *vs*. competitor-derived mature lineage cells in peripheral blood after transplantation. (F, G) Ratio of donor-derived Lin$^-$ cells, hematopoietic progenitor cells (HPC), Lin$^-$Sca1$^+$c-Kit$^+$ (LSK), and hematopoietic stem cells (HSC) as well as myeloid and B cells in bone marrow from indicated mice. **P*<0.05, ***P*<0.01, ****P*<0.001.

with 5-fluorouracil, which kills proliferating hematopoietic progenitors, thus promoting HSC to enter the cell cycle and reconstitute the hematopoietic system. As shown in Figure 1I, all Ythdf3-deficient mice died within 20 days, while 25% of control mice survived after three weekly treatments of 5-fluorouracil, indicating that Ythdf3-deficient HSC lost the ability to replenish the hematopoietic system more quickly than wildtype HSC did. To further evaluate the long-term self-renewal capacity of Ythdf3-deficient HSC in a competitive situation, we performed competitive serial transplantation assays (Figure 2A). An equal number of bone marrow cells (CD45.2) from Ythdf3-deficient or control mice along with competitor wildtype bone marrow cells (CD45.2/CD45.1) were transplanted into lethally irradiated syngeneic recipients (CD45.1). Thereafter, we performed secondary transplants with mixed bone marrow cells from two or three mice in each group. The frequency of donor-derived peripheral blood cells was monitored monthly after transplantation. The ratio of *Ythdf3*$^{-/-}$derived *vs*. competitor-derived total peripheral blood cells was gradually decreased significantly by approximately 2-fold compared with the ratio of *Ythdf3*$^{+/+}$ derived *vs*. competitor-derived peripheral blood cells after secondary transplantation (Figure 2B, *Online Supplementary Figure S2A*). Of note, *Ythdf3*$^{-/-}$ hematopoietic stem/progenitor cells (HSPC) produced a lower number of all mature cells, including myeloid cells, B cells, and T cells, compared with *Ythdf3*$^{+/+}$ HSPC (Figure 2C-E, *Online*

*Supplementary Figure S2B-D*). Consistent with the results from peripheral blood, *Ythdf3*$^{-/-}$ donor-derived Lin$^{-}$ cells, HPC, LSK, and HSC as well as myeloid and B cells in bone marrow were significantly lower compared with *Ythdf3*$^{+/+}$ donor-derived cells 4 months after secondary transplantation (Figure 2F, G). Collectively, these results indicate

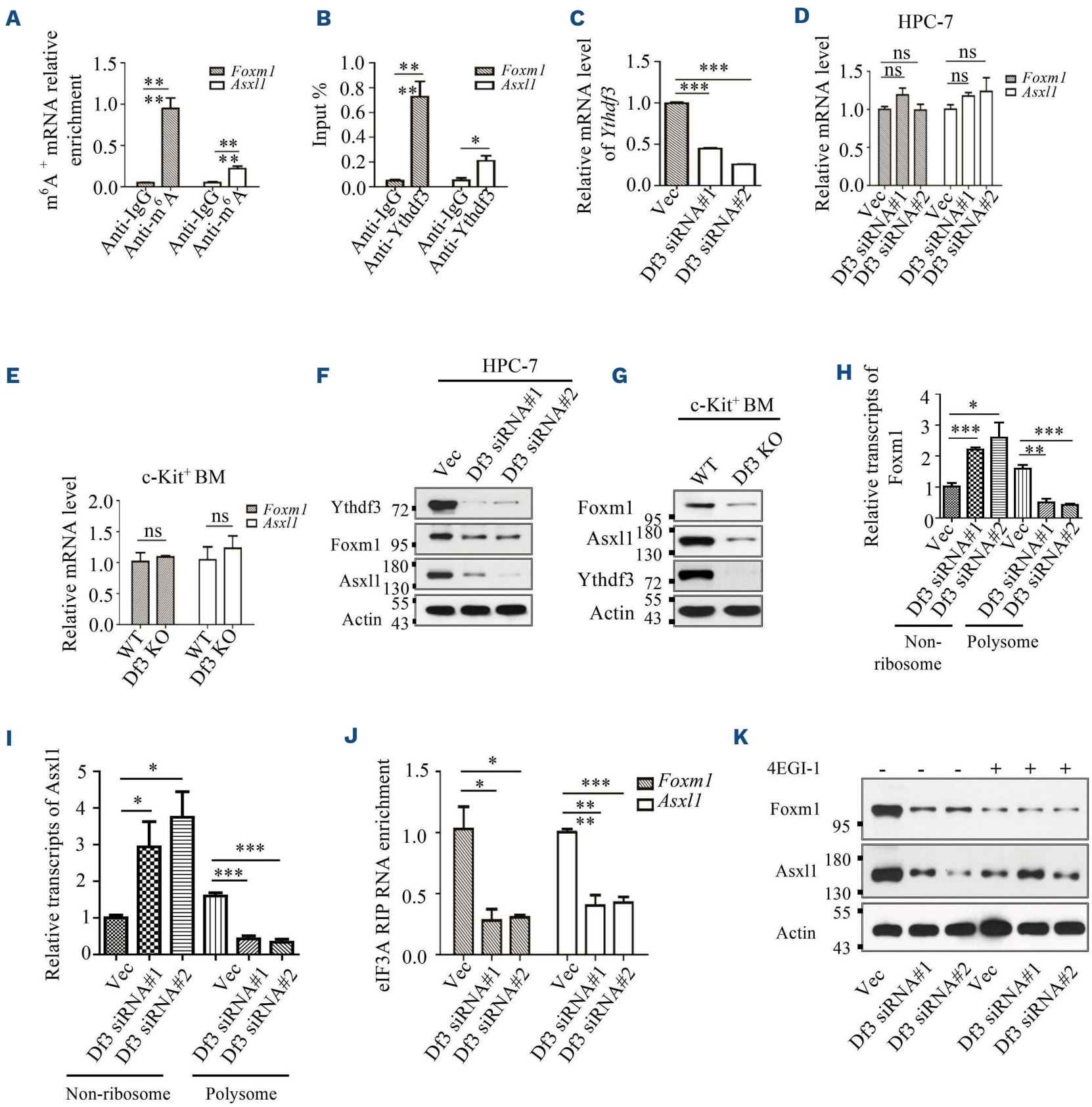


**Figure 3. Ythdf3 facilitates mRNA translation of *Foxm1* and *Asxl1* in hematopoietic stem and progenitor cells.** (A) m$^6$A RNA immunoprecipitation (MeRIP) analysis of mRNA m$^6$A methylation of *Foxm1* and *Asxl1* in mouse hematopoietic precursor cell-7 (HPC-7) cells. (B) Ythdf3 RIP analysis suggesting that Ythdf3 binds directly to mRNA of *Foxm1* and *Asxl1* in HPC-7 cells. (C) Reverse transcriptase quantitative polymerase chain reaction (RT-qPCR) analysis showing the knockdown efficiency of *Ythdf3* in HPC-7 cells. (D) RT-qPCR analysis indicating that levels of *Foxm1* and *Asxl1* transcription are not affected by *Ythdf3* knockdown in HPC-7 cells. (E) RT-qPCR analysis showing transcription levels of *Foxm1* and *Asxl1* in wild-type and *Ythdf3* knockout c-Kit$^{+}$ mouse HSPC. (F, G) Western blot analysis showing the effect of *Ythdf3* knockdown (F), or knockout (G) on protein levels of Foxm1 and Asxl1 in HPC-7 cells (F), or c-Kit$^{+}$ mouse HSPC (G), respectively. (H, I) Polysome profiling analysis indicating that Ythdf3 depletion significantly inhibits mRNA translation of *Foxm1* and *Asxl1*. (J) eIF3A RIP analysis showing the effect of *Ythdf3* knockdown on eIF3A enrichment at mRNA of *Foxm1* and *Asxl1* in HPC-7 cells. (K) Western blot analysis showing protein levels of Foxm1 and Asxl1 in wild-type or Ythdf3 depleted HPC-7 cells with or without 4EGI-1 treatment. All the statistical analyses were performed by GraphPad Prism 5. *$P$<0.05; **$P$<0.01; ***$P$<0.001; ns: no significant difference.

that Ythdf3-deficient HSC had decreased self-renewal capacity and survival under stress conditions.

To investigate the molecular mechanisms underlying the function of Ythdf3 in HSPC, we analyzed the publicly available YTHDF3 cross-linking immunoprecipitation-sequencing data in human cell lines.[10] Both Foxm1 and Asxl1, which play important roles in HSC maintenance,[11,12] are potential direct binding targets of Ythdf3. We performed m$^6$A RNA immunoprecipitation (Me-RIP)-PCR analysis and Ythdf3 RIP-PCR analysis of Foxm1 and Asxl1 in HPC-7 cells, multipotent hematopoietic precursors.[13] The results showed significant enrichment of m$^6$A antibody and Ythdf3 binding sites in both *Foxm1* and *Asxl1* transcripts (Figure 3A, B). More importantly, only the wildtype Ythdf3 but not the m$^6$A-binding-deficient Ythdf3-W495A binds to transcripts of *Foxm1* and *Asxl1*, suggesting that Ythdf3 regulates Foxm1 and Asxl1 expression by direct binding in an m$^6$A-dependent manner (*Online Supplementary Figure S3A, B*). We employed a recently reported FAMSi system,[14] in which siRNA oligos against the target genes are expressed in a retroviral backbone vector, to express *Ythdf3*-specific siRNA and scramble siRNA in HPC-7 cells. Quantitative real-time PCR analysis revealed that both *Ythdf3*-specific siRNA significantly knocked down *Ythdf3* expression (Figure 3C) but did not affect the expression of *Foxm1* and *Asxl1* transcripts in HPC-7 cells (Figure 3D). Consistently, *Ythdf3* knockout did not affect *Foxm1* and *Asxl1* mRNA expression in primary c-Kit$^+$ HSPC from mice (Figure 3E). Of note, as determined by western blot analysis, *Ythdf3* knockdown or knockout significantly inhibited Foxm1 and Asxl1 protein expression in HPC-7 cells and primary c-Kit$^+$ HSPC from mice, respectively (Figure 3F, G). Notably, Ythdf3-depletion-mediated downregulation of Foxm1 and Asxl1 was rescued by wild-type but not the m$^6$A-binding-deficient Ythdf3-W495A, suggesting that Ythdf3-mediated regulation of Foxm1 and Asxl1 expression is dependent on its m$^6$A RNA binding activity (*Online Supplementary Figure S3C*). Protein abundance is determined by a balance between protein turnover and protein synthesis, namely mRNA translation. The half-lives of Foxm1 and Asxl1 proteins were not affected by Ythdf3 depletion (*Online Supplementary Figure S3D-I*), indicating that Ythdf3 depletion-mediated downregulation of Foxm1 and Asxl1 protein is not attributed to protein turnover. Meanwhile, we performed polysome profiling analysis and observed that *Ythdf3* deletion led to a significant decrease of *Foxm1* and *Asxl1* transcripts in polysome fractions and an increase of these transcripts in the non-ribosome fraction (Figure 3H, I), suggesting that Ythdf3 regulates *Foxm1* and *Asxl1* mRNA translation. elF3a facilitates the initiation of translation by directly binding to m$^6$A in the 5'-untranslated region.[15] elF3A RIP-PCR analysis showed that *Ythdf3* knockdown markedly reduced the binding of elf3a to *Foxm1* and *Asxl1* transcripts (Figure 3J). We treated HPC-7 cells stably expressing vector, or Ythdf3-specific siRNA with or without 4EGI-1, an inhibitor of Cap-dependent translation initiation by disruption of the eIF4E/eIF4G complex formation. Both *Ythdf3* deletion and 4EGI-1 treatment led to downregulation of Foxm1 and Asxl1 (Figure 3K). More importantly, protein levels of Foxm1 and Asxl1 were not further decreased after 4EGI-1 treatment in Ythdf3-depleted cells (Figure 3K). Thus, we concluded that Ythdf3 regulates the Cap-dependent translation initiation of Foxm1 and Asxl1. In contrast, *Ythdf2* knockdown did not affect either transcriptional or translational regulation of *Foxm1* and *Asxl1* expression in HPC-7 cells (*Online Supplementary Figure S3J-N*).

In conclusion, our study provides the first evidence that Ythdf3 is required for HSC maintenance under stress, but not normal hematopoiesis in homeostatic conditions. We show that Ythdf3 but not Ythdf2 promotes translation of *Foxm1* and *Asxl1*, critical regulators of HSC maintenance in HSPC. However, whether Ythdf1 has a role in HSC maintenance and is involved in Ythdf3-mediated regulation of translation of *Foxm1* and *Asxl1* remains to be determined. Our data suggest that the role of Ythdf1, 2, and 3 in gene regulation is likely cell context-dependent.

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**Disclosures**

No conflicts of interest to disclose.

**Contributions**

ZQ: study design; QD, QW, FY, YS, CY, GS, KP, JL, and ZQ: acquisition, analysis, and interpretation of data; ZQ, QD, QW, FY, and YS: manuscript preparation.

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