Received: 25 November 2024 | Accepted: 2 April 2025

DOI: 10.1002/hem3.70140

ARTICLE

HemaSphere eha

TET3 regulates hematopoietic stem cell homeostasis during embryonic and adult hematopoiesis

Harmony C. Ketchum[1,2,3,^] | **Claudia Morganti**[1,4,^] | **Chie Yanase**[1,4] | **Blake Ebert**[1,2,3] | **Keisuke Ito**[1,4] | **Meelad M. Dawlaty**[1,2,3]

Correspondence: Keisuke Ito (keisuke.ito@einsteinmed.edu) and Meelad M. Dawlaty (meelad.dawlaty@einsteinmed.edu)

ABSTRACT

The ten-eleven translocation family of enzymes (TET1/2/3) promotes DNA demethylation and is essential for hematopoiesis. While the roles of TET1 and TET2 are well-studied in hematopoiesis, the requirement of TET3 in embryonic and adult hematopoiesis is less investigated. In this study, by characterizing embryonic and adult hematopoiesis in *Tie2*$^{+/cre}$; *Tet3*$^{f/f}$ mice, we have established a requirement for TET3 in regulating hematopoietic stem cells (HSCs; CD150^{+}CD48^{-}). We found that loss of TET3 in the fetal liver and adult bone marrow causes a reduction in the percent of long-term HSCs (LT-HSCs; CD150^{+}CD48^{-}CD34^{-}). This was accompanied by reduced colony forming capacity of TET3-deficient HSCs in vitro and reduced contribution of HSCs after a competitive bone marrow transplantation in vivo. TET3 deficiency increased DNA methylation at several cell cycle regulator genes leading to their down regulation. This is consistent with, and likely underpins, the reduced number of quiescent HSCs in TET3-deficient bone marrow. These findings uncover a new role for TET3 in HSC homeostasis during embryonic and adult hematopoiesis.

INTRODUCTION

The ten-eleven translocation family of proteins (TET1/2/3) is composed of DNA dioxygenases that are essential for embryonic development and hematopoiesis.[1,2] They regulate gene expression by promoting DNA demethylation through oxidation of 5-methylcytosine to 5-hydroxymethylcytosine (5hmC) and other oxidized derivatives.[3–5] These bases are excised by thymine DNA glycosylase (TDG) and replaced by unmodified cytosines completing active DNA demethylation.[5–7] 5hmC also inhibits recruitment of the maintenance DNA methyltransferase DNMT1 during replication and thereby promotes passive DNA demethylation.[8,9] TET enzymes can also regulate gene expression independent of their enzymatic functions[10–15] by partnering with chromatin activating and repressive complexes.[11,14,16,17] TET enzymes are dynamically expressed during development and are essential for regulating various developmental programs.[1,18] Combined deletion of all three TET enzymes arrests gastrulation resulting in early embryonic lethality.[19] *Tet3* is uniquely expressed in the oocyte and is required for demethylation of the paternal genome upon fertilization.[20–24] Oocyte specific deletion of TET3 results in mid-gestational lethality.[20] *Tet3* is not expressed in the blastocyst, but its levels increase during post-implantation development. Global loss of TET3 leads to perinatal lethality with no known cause of death[20] and the requirement of TET3 in lineage specification is less investigated.

TET enzymes play critical roles in regulating embryonic and adult hematopoiesis. They are required for specification of hematopoietic stem cells (HSCs) from endothelial cells during embryonic hematopoiesis. Global inducible deletion of *Tet1*, *Tet2*, and *Tet3* at E7.5 using Rosa26-CreER or endothelial specific deletion of all three *Tet* genes using Tie2-cre leads to embryonic lethality by E12.5 with severe impairment of embryonic hematopoiesis.[2] In adult mice, loss of TET enzymes leads to severe bone marrow failure and onset of leukemogenesis.[25] While all three TET enzymes are deregulated in hematologic disorders and cancers,[26] *TET2* is the most frequently mutated *TET* gene in clonal hematopoiesis (CH), myelodysplastic syndrome (MDS) and various forms of leukemias.[27–29] Therefore, TET2 has been extensively studied in hematopoiesis.[13,30–32] TET2 loss in mice causes aberrant differentiation of HSCs and leads to myeloid and lymphoid malignancies by 1 year of age and

[1]Ruth L. and David S. Gottesman Institute for Stem Cell and Regenerative Medicine Research, Albert Einstein College of Medicine, Bronx, New York, USA
[2]Department of Genetics, Albert Einstein College of Medicine, Bronx, New York, USA
[3]Department of Developmental and Molecular Biology, Albert Einstein College of Medicine, Bronx, New York, USA
[4]Departments of Cell Biology, Medicine and Oncology, Albert Einstein College of Medicine, Bronx, New York, USA
^These authors contributed equally to this study.

recapitulates onset of CMML in patients.[13,33,34] TET1 has a less severe impact on mouse bone marrow than TET2 but its loss leads to onset of B cell malignancies later in life.[35,36] *Tet3* is expressed in hematopoietic cells during development and in adults;[37] however, there are limited studies investigating the role of TET3 in hematopoiesis. Most studies investigating the role of TET3 were conducted in mature blood cells and often in conjunction with TET2.[38–41] TET2 and TET3 are the critical DNA demethylases in B cells where their deletion results in hypermethylation of essential enhancer regions and impaired B cell formation.[39] In mouse regulatory T cells, deletion of TET2 and TET3 causes inflammatory disease and aberrant expression of cell cycle genes.[40] Additionally, *Tet3* was shown to be upregulated in patients with acute myeloid leukemia (AML), and *Tet3* overexpression promoted the growth of AML cell lines and perturbed myeloid differentiation.[38] These studies support a role for TET3 in regulating hematopoiesis that warrants further investigation. In this study, we have characterized embryonic and adult hematopoiesis in $Tie2^{+/cre}$; $Tet3^{F/F}$ mice. We have found a requirement for TET3 in regulating HSCs ($CD150^+$ $CD48^-$ $CD135^-$ Lin^- $Sca1^+$ $cKit^+$ hereafter referred to as $CD150^+CD48^-$), especially long-term HSCs (LT-HSC; $CD150^+CD48^-CD34^-$). We also found that TET3-mediated DNA demethylation drives proper expression of hematopoietic transcription factors and cell cycle regulators in hematopoietic cells. These findings uncover a new role for TET3 in HSC homeostasis during embryonic and adult development.

MATERIALS AND METHODS

Generation of $Tie2^{+/Cre}$; $Tet3^{f/f}$ mice and $Tet3^{-/-}$ embryos

Male $Tie2^{+/Cre}$ mouse (Jax lab, stock no. 004128) were bred to our previously published $Tet3^{f/f}$ female mice[2] to generate $Tie2^{+/Cre}$; $Tet3^{+/f}$ male mice, which were then bred to female $Tet3^{f/f}$ mice to obtain $Tie2^{+/Cre}$; $Tet3^{f/f}$ mice. Genotype of mice and excision of floxed exon 4 of *Tet3* in BM-MNC were confirmed by PCR using our previously published primers and protocols.[2] To generate $Tet3^{-/-}$ embryos, our previously published $Tet3^{+/-}$ mice[1] were time-mated, and embryos were harvested at embryonic (E) day 9.5, E13.5, and E18.5. All embryonic hematopoietic analyses were performed using E13.5 fetal liver.

Flow cytometry and cell sorting

BM-MNCs were isolated from femurs and tibias of mice using the flushing technique as described previously.[42] For quantification of HSCs ($CD150^+$ $CD48^-$), after isolation, BM-MNCs were incubated with the following mix of monoclonal antibodies against lineage committed cells: Ly6G/Ly6C (Gr-1)-biotin from Biolegend; CD11b-biotin, CD19-biotin, CD45R/B220-biotin, CD4-biotin, NK-1.1-biotin, and TER-119-biotin from BD Bioscience; CD135 (Flt3)-biotin, CD127-biotin, CD3e-biotin, CD8a-biotin, Mouse IgM-biotin from Life Technologies, and then resuspended in 2% FBS-PBS and incubated for 30 min on ice. Lineage antibody staining was followed by incubation with an antibody mix for the following markers: CD117 (c-kit)-APC/CY7, CD150 (SLAM)-PerCp/Cy5.5 from Biolegend; CD34-FITC, CD48-APC or CD48-biotin, Ly-6A/E (Sca-1)-PE/Cy7 or Ly-6A/E (Sca-1)-FITC, Streptavidin-Pacific Blue or Streptavidin-PE from Life Technologies; resuspended in 2% FBS-PBS and incubated for 30 min on ice. For multipotent progenitor cells, lineage antibody staining was followed by incubation with an antibody mix of the following markers: CD117 (c-kit)-APC/CY7, Ly-6A/E (Sca-1)-FITC, CD16/32-PE from Biolegend; CD34-APC, Streptavidin-Pacific Blue from Life Technologies; resuspended in 2% FBS-PBS for 30 min in ice. Myeloid and lymphoid cells were stained with antibodies against CD3e-APC (for T cell, Life Technologies), B220-PacificBlue (BD Bioscience), CD11b-FITC, and Gr-1-PE (for myeloid cells, Life Technologies and Biolegend, respectively). All antibodies were used at a 1:100 dilution. Samples were acquired on an LSR II flow cytometer (Becton Dickinson), and the data were analyzed using FlowJo 10 (Becton Dickinson). For cell sorting, the BM-MNCs were prepared as mentioned previously. In addition, cells were incubated with Anti-Biotin MicroBeads (Mylteni Biotech) for 10 min at room temperature and then subjected to an MACS LS column (Mylteni Biotech), and the flow threw was collected for lineage depletion. Cells were sorted directly into StemSPAN SFEM (STEMCELL Technologies) through a BD FACS ARIA II (Becton Dickinson). For quantification of quiescent and active HSCs ($CD150^+$ $CD48^-$), BM-MNCs were prepared as mentioned previously. In addition, after the antibody staining, cells were fixed by Cyto-Fast™ Fix/Perm Buffer Set (Biolegend) according to manufacturer's instructions. Then, cells were incubated with ki67-Alexa660 Ab (Invitrogen, eBioscience) at a 1:50 dilution over night at 4°C. After washing, Hoechst 33342 (Invitrogen) was added at a concentration of 2 μg/mL, incubated for 15 min, and then analyzed by flow cytometry.

In vitro colony formation and in vitro limiting dilution assays

Colony formation assays were performed using MethoCult (Stem Cell Technologies, Vancouver, BC, Canada) according to manufacturer's instructions. Briefly, 1500 LSKs were plated per well. After 7 days, the number of erythroid (E), macrophage (M), granulocyte macrophage (GM), and granulocyte, erythrocyte, macrophage, megakaryocyte (GEMM) colonies were quantified. In vitro limiting dilution assay was performed by sorting 8, 4, 2, and 1 HSC (LSK $CD150^+CD48^-$) into single wells of 96 well plates and cultured in StemSPAN SFEM (StemCell Technologies) supplemented with 50 ng/mL SCF (Peprotech), and 50 ng/mL TPO (Peprotec) for 6 weeks. Replicates for the control group included: for dilution of 8 cells/well ($n = 24$ replicates), for dilution of 4 cells/well ($n = 24$ replicates), for dilution of 2 cells/well ($n = 24$ replicates), and for dilution of 1 cell/well ($n = 48$ replicates). Replicates for the cKO group included: for dilution of 8 cells/well ($n = 36$ replicates), for dilution of 4 cells/well ($n = 36$ replicates), for dilution of 2 cells/well ($n = 36$ replicates), and for dilution of 1 cell/well ($n = 60$ replicates). Half of medium was replaced once a week. Frequencies of cells with colony-forming capacity after long-term in vitro culture were calculated using extreme limiting dilution analysis (ELDA).[43] L-CalcTM Software (StemCell Technologies) was used to assess the difference between the two conditions.

Competitive bone marrow reconstitution assay

4×10^5 BM-MNCs from CD45.2 donor mice ($Tie2^{+/+}$; $Tet3^{F/F}$ or $Tie2^{+/cre}$; $Tet3^{F/F}$) were transplanted into sub-lethally irradiated CD45.1 mice in competition with 4×10^5 BM-MNC from CD45.1 mice. Reconstitution of donor cells and repopulation of donor myeloid and lymphoid cells were monitored by staining peripheral blood cells every 4 weeks for up to 16 weeks with antibodies against CD45.1-PE (Life Technologies), CD45.2-APC-Cy7 (Life Technologies), CD3e-APC (for T cell, Biolegend), B220-PacificBlue (Biolegend), CD11b-FITC, and Gr-1-FITC (for myeloid cells, Life Technologies). Chimerism in HSCS and differentiated hematopoietic cells in the bone marrow was analyzed at 16 weeks after BMT. A secondary BMT was performed using BM-MNC isolated from primary

transplanted mice 16 weeks post-BMT. Reconstitution of donor cells and repopulation of donor myeloid and lymphoid cells were monitored by staining peripheral blood cells every 4 weeks post-transplantation. BM of transplanted mice were analyzed at 12 weeks. Isolation, staining, and analysis of BM-MNC at this stage was obtained as described in the "Flow cytometry and cell sorting" section with the addition of CD45.2-PE (Life Technologies) to mark donor cells. For peripheral blood smears, the collected peripheral blood was smeared for May-Grunwald-Giemsa staining (Sigma). Briefly, the samples were stained in May-Grunwald solution for 5 min, washed in PBS for 90 s, stained in Giemsa solution for 15 min, finally washed in running tap water, and let them dry.

RNA-Seq and data analysis

RNA-seq was performed as previously described.[12] Lin^- cells from 2- to 3-month $Tet3^{F/F}$ and $Tie2^{+/cre}$; $Tet3^{F/F}$ mice were sorted and collected by FACs. Total RNA was extracted using the RNeasy micro kit (Qiagen #74004), following manufacturer's instructions. Library preparation and mRNA sequencing were performed by Novogene using their Illumina Novoseq 6000 platform. Adaptors were removed by Trim galore (v0.6.7) and trimmed reads were mapped to the mouse genome (mm10) using STAR (v2.7.9a) with default parameters. Gene counts were obtained from mapped reads using featureCounts with largestOverlap parameter. DEGs were identified from the raw counts by DESeq2 (false discovery rate-adjusted $p < 0.05$ and fold-change >1.5), following the package documentation. Gene ontology (GO) analyses of DEGs was performed using DAVID (https://david.ncifcrf.gov/). All plots were generated in R (https://www.r-project.org/) using custom scripts.

Enzymatic methylation sequencing (EM-seq) and enzymatic 5-hydroxymethylation sequencing (E5hmC-seq)

Lin^- cells from 2- to 3-month $Tet3^{F/F}$ and $Tie2^{+/cre}$; $Tet3^{F/F}$ mice were sorted and collected by FACs. Genomic DNA was extracted with the Quick-DNA Miniprep Kit (Zymo, D3024) according to the manufacturer's instructions. Two biological replicates per genotype were subjected to EM-seq or E5hmC-seq. For EM-seq, 200 ng DNA was oxidized, deaminated, and amplified using the NEBNext EM-seq kit (NEB E7120). For E5hmC-seq, 200 ng DNA was glucosylated, deaminated, and amplified using the NEBNext E5hmC-seq kit (NEB E3350). Both libraries were subjected to 300-bp paired-end sequencing at Novogene using their Novoseq 6000 platform. EM-seq libraries were sequenced to a depth of 100× and E5hmC-seq libraries were sequenced to a depth of 500×. Spike-in lambda (negative control), pUC19 (5mC positive control), and T4 (5hmC positive control) DNA were used to confirm complete enzymatic reactions and were mapped to their respective genomes using Bismark (v0.24.2).[44] In all samples, the negative control lambda DNA mapping rate was <1%; in EM-seq samples, pUC19 mapping rate was >80%; and in E5hmC-seq, T4 mapping rate was >80% confirming reaction efficiency. Data analysis was performed as previously described.[12] Methylation and hydroxymethylation levels were identified by methcounts from the package methpipe. DMRs were defined as regions with >5 CpGs, methylation difference > 20% and $p < 0.05$. DhMRs were defined as regions with >3 CpGs, methylation difference > 20% and $p < 0.05$. The R package ChIPseeker (v1.36.0) was used to annotate DMRs and DhMRs to genomic features. Line plots were generated with the plotProfile function of deepTools (v3.5.1). For visualization on Integrative Genome Browser (IGV), bedGraph files were converted to bigwigs using bedtools bedGraphToBigWig (v2.30.0). Bed files were intersected using bedtools intersect with default parameters. H3K27ac and H3K4me1 ChIPseq data were downloaded from ENCODE (experiments: ENCSR000CCL and ENCSR000CAG). Both experiments were performed in 2-month-old bone marrow cells. The processed data were downloaded and intersected using Bedtools to identify enhancers in bone marrow cells (ENCFF061JZQ.bed and ENCFF826DES.bigWig for H3K27ac; ENCFF699MDE.bed and ENCFF557LTZ.bigWig for H3K4me1).

RESULTS

TET3 regulates long-term hematopoietic stem cells (LT-HSCs; $CD150^+CD48^-CD34^-$) during embryogenesis

To investigate the requirements of TET3 in hematopoiesis, we intercrossed our previously published $Tet3^{+/-}$ mice[1] to obtain $Tet3^{-/-}$ embryos. Consistent with previous reports,[20] $Tet3^{-/-}$ mice die perinatally (Figure S1A). However, $Tet3^{-/-}$ embryos were comparable to their littermate controls and had similar somite counts albeit with a subtle decrease in weight at mid-gestation (Figure S1B–D). Analyses of $Tet3^{-/-}$ embryos at early (E9.5), mid (E13.5), and late (E18.5) gestation revealed no gross anatomical defects, and all embryos analyzed were alive with a beating heart (Figure S1E). Likewise, the development of organs such as the eyes, fetal liver, and placenta in $Tet3^{-/-}$ embryos were unaffected. Next, we quantified hematopoietic stem and progenitor cells (HSPCs) in $Tet3^{-/-}$ E13.5 fetal livers by flow cytometry (Figure 1A) using a defined gating strategy (Figure S2). We found that $Tet3^{-/-}$ fetal livers had an increased percentage of LSK cells compared to their littermate controls (Figure 1B). While the total percentage of HSCs ($CD150^+CD48^-$) was not affected in $Tet3^{-/-}$ fetal livers (Figure 1C), LT-HSCs ($CD150^+CD48^-CD34^-$) were significantly reduced (Figure 1D). This was accompanied by a subtle increase in short-term (ST) HSCs ($CD150^+CD48^-CD34^+$), but it did not reach statistical significance (Figure S1F). These findings suggest that TET3 regulates LT-HSCs during embryogenesis.

Next, we wanted to assess how the deficiency of TET3 only in the endothelial lineage, where HSCs emerge from, impacts embryonic hematopoiesis, and whether TET3 loss in the endothelial compartment is responsible for perinatal lethality of mice. To this end, we conditionally deleted *Tet3* by crossing our previously published $Tet3^{F/F}$ mouse[2] to Tie2-Cre mouse, a strain that specifically expresses Cre in the endothelial cells during embryogenesis. $Tie2^{+/cre}$; $Tet3^{F/F}$ mice were born and weaned at expected mendelian ratios (Figure S3A). Genotyping of bone marrow mononuclear cells (BM-MNCs) isolated from $Tie2^{+/cre}$; $Tet3^{F/F}$ mice confirmed complete excision of the floxed *Tet3* exon 4 (Figure S3B). $Tie2^{+/cre}$; $Tet3^{F/F}$ mice survived to adulthood and had normal weight at 2 months of age (Figure S3C). We analyzed and quantified hematopoietic cells in the fetal liver of E13.5 $Tie2^{+/cre}$; $Tet3^{F/F}$ embryos (Figure 2A). We found an increase in the percentage of LSKs in $Tie2^{+/cre}$; $Tet3^{F/F}$ embryos compared to their littermate controls (Figure 2B). While there was no deficit in the percentage of total HSCs ($CD150^+CD48^-$) (Figure 2C), there was a significant reduction of LT-HSC ($CD150^+CD48^-CD34^-$) in $Tie2^{+/cre}$; $Tet3^{F/F}$ embryos (Figure 2D). These observations are consistent with our findings in $Tet3^{-/-}$ E13.5 embryos (Figure 1) and further supports that TET3 regulates the development of LT-HSCs during embryonic hematopoiesis.

Loss of TET3 leads to a reduction in HSCs ($CD150^+CD48^-$) in the adult bone marrow

The viability of $Tie2^{+/cre}$; $Tet3^{F/F}$ mice suggests that the reduction in LT-HSC observed in TET3-deficient embryos is not responsible for the perinatal lethality of $Tet3^{-/-}$ mice. This led us to investigate the

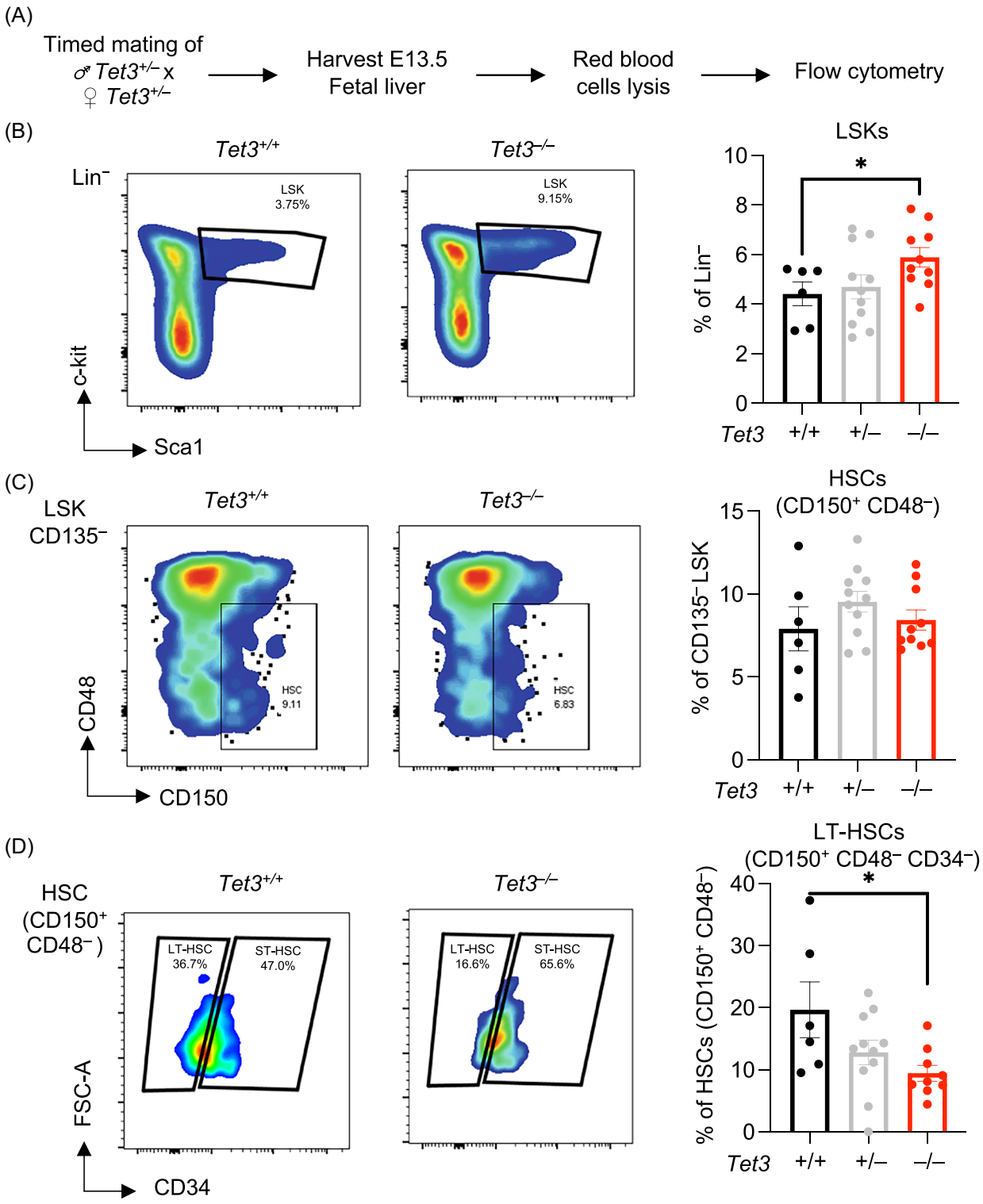

FIGURE 1 ***Tet3*$^{-/-}$ E13.5 fetal livers have a reduction in LT-HSCs (CD150^{+} CD48^{-} CD34^{-}). (A)** Strategy for analyzing hematopoietic cells in E13.5 fetal livers of *Tet3*$^{-/-}$, *Tet3*$^{+/-}$, and *Tet3*$^{+/+}$ embryos. **(B)** Quantification of LSK cells [Lin^{-}Sca1^{+}cKit^{+}] cells in E13.5 fetal livers by flow cytometry. Representative flow plots (left) and LSK cells plotted as percentage of Lin^{-} cells (right). **(C)** Quantification of HSCs [CD150^{+}CD48^{-}] cells in E13.5 fetal livers by flow cytometry. Representative flow plots (left) and HSCs plotted as percentage of CD135^{-}LSK (right). **(D)** Quantification of LT-HSCs [CD150^{+}CD48^{-}CD34^{-}] in E13.5 fetal livers by flow cytometry. Representative flow plots (left) and LT-HSCs plotted as percentage of HSCs (right). For all panels, error bars represent SD, and * indicates statistical significance ($p < 0.05$) using *t*-test.

impact of TET3 deficiency on adult hematopoiesis in *Tie2*$^{+/cre}$; *Tet3*$^{F/F}$ mice. We analyzed BM-MNCs isolated from femurs and tibias of 2-month-old *Tie2*$^{+/cre}$; *Tet3*$^{F/F}$ mice and their littermate controls by flow cytometry. We found that the percentage of LSKs and Lin^{-}Sca1^{-}cKit^{+} cells (Figure 3A) as well as the multipotent progenitor cells, GMPs, CMPs, and MEPs (Figure S4A,B) were unchanged in *Tie2*$^{+/cre}$; *Tet3*$^{F/F}$ bone marrow compared to controls. Consistently, colony forming unit (CFU) capacity of *Tie2*$^{+/cre}$; *Tet3*$^{F/F}$ LSKs was

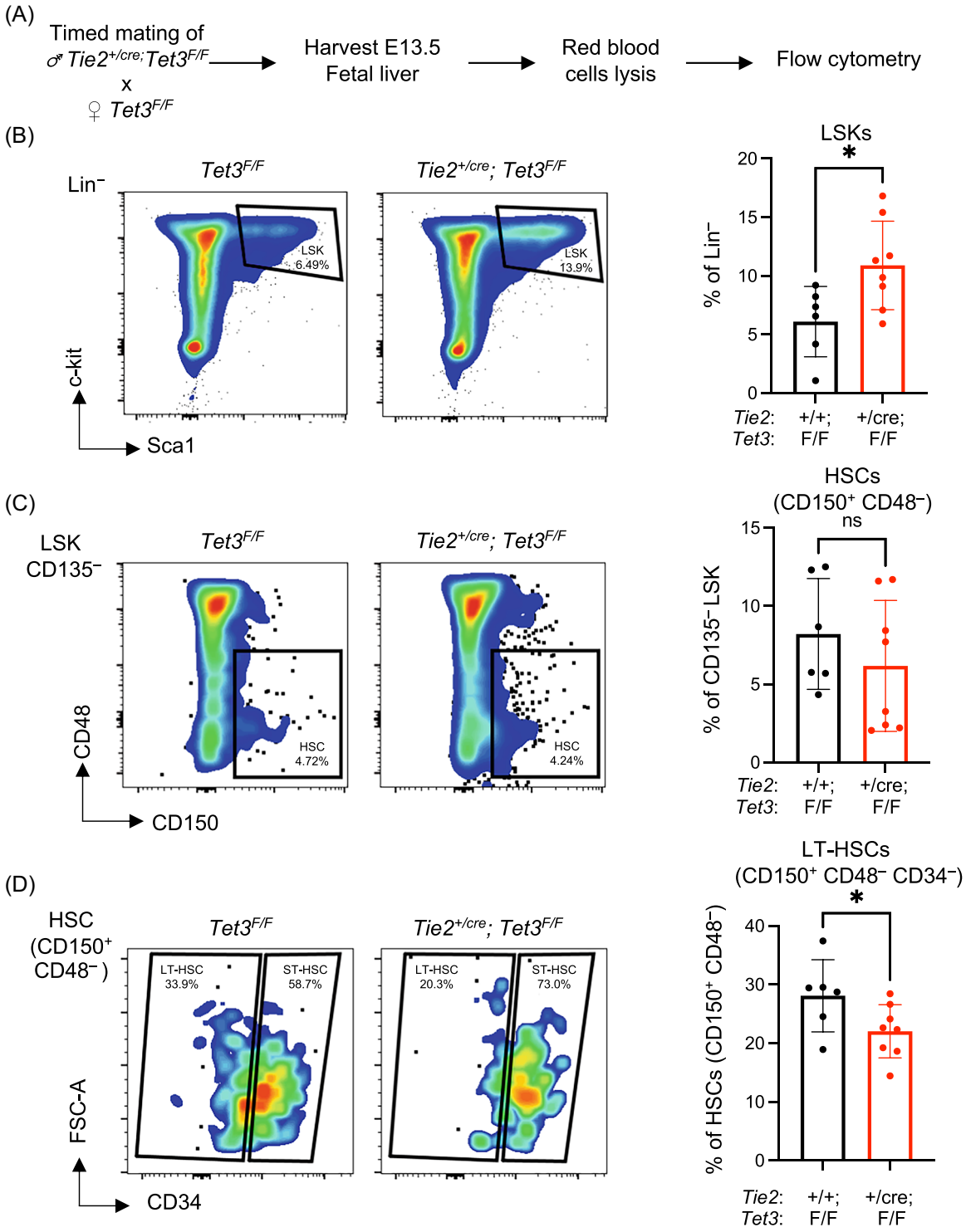

FIGURE 2 E13.5 *Tie2*$^{+/Cre}$; *Tet3*$^{f/f}$ fetal livers have reduced LT-HSCs (CD150^{+} CD48^{-} CD34^{-}). **(A)** Strategy for analyzing hematopoietic cells in E13.5 fetal livers of *Tet3*$^{F/F}$ and *Tie2*$^{+/cre}$; *Tet3*$^{F/F}$ embryos. **(B)** Quantification of LSK cells [Lin^{-}Sca1^{+}cKit^{+}] in E13.5 fetal livers by flow cytometry. Representative flow plots (left) and LSK cells plotted as percentage of Lin^{-} cells (right). **(C)** Quantification of HSCs [CD150^{+}CD48^{-}] in E13.5 fetal livers by flow cytometry. Representative flow plots (left) and HSCs plotted as percentage of CD135^{-}LSK (right). **(D)** Quantification of LT-HSCs [CD150^{+}CD48^{-}CD34^{-}] in E13.5 fetal livers by flow cytometry. Representative flow plots (left) and LT-HSCs plotted as percentage of HSCs (CD150^{+}CD48^{-}) (right). For all panels, error bars represent SD, and * indicates statistical significance ($p < 0.05$) using *t*-test.

unaffected (Figure S4C). The quantification of mature myeloid and lymphoid cells in 2-months-old *Tie2*$^{+/cre}$; *Tet3*$^{F/F}$ and control bone marrow showed no differences in the percentage of myeloid cells (Figure S5A) or T and B cells (Figure S5B). Interestingly, the percentage of HSCs (CD150^{+}CD48^{-}) was significantly reduced in *Tie2*$^{+/cre}$; *Tet3*$^{F/F}$ mice (Figure 3B). This was accompanied by an increase in the LSK CD135^{-}CD150^{-}CD48^{+} cell population (Figure 3B). Notably, we found that *Tie2*$^{+/cre}$; *Tet3*$^{F/F}$ mice, like *Tet3*$^{-/-}$embryos, had a significant reduction in LT-HSCs (CD150^{+}CD48^{-}CD34^{-}) as well as a significant increase in ST-HSCs (CD150^{+}CD48^{-}CD34^{+}) (Figure 3C).

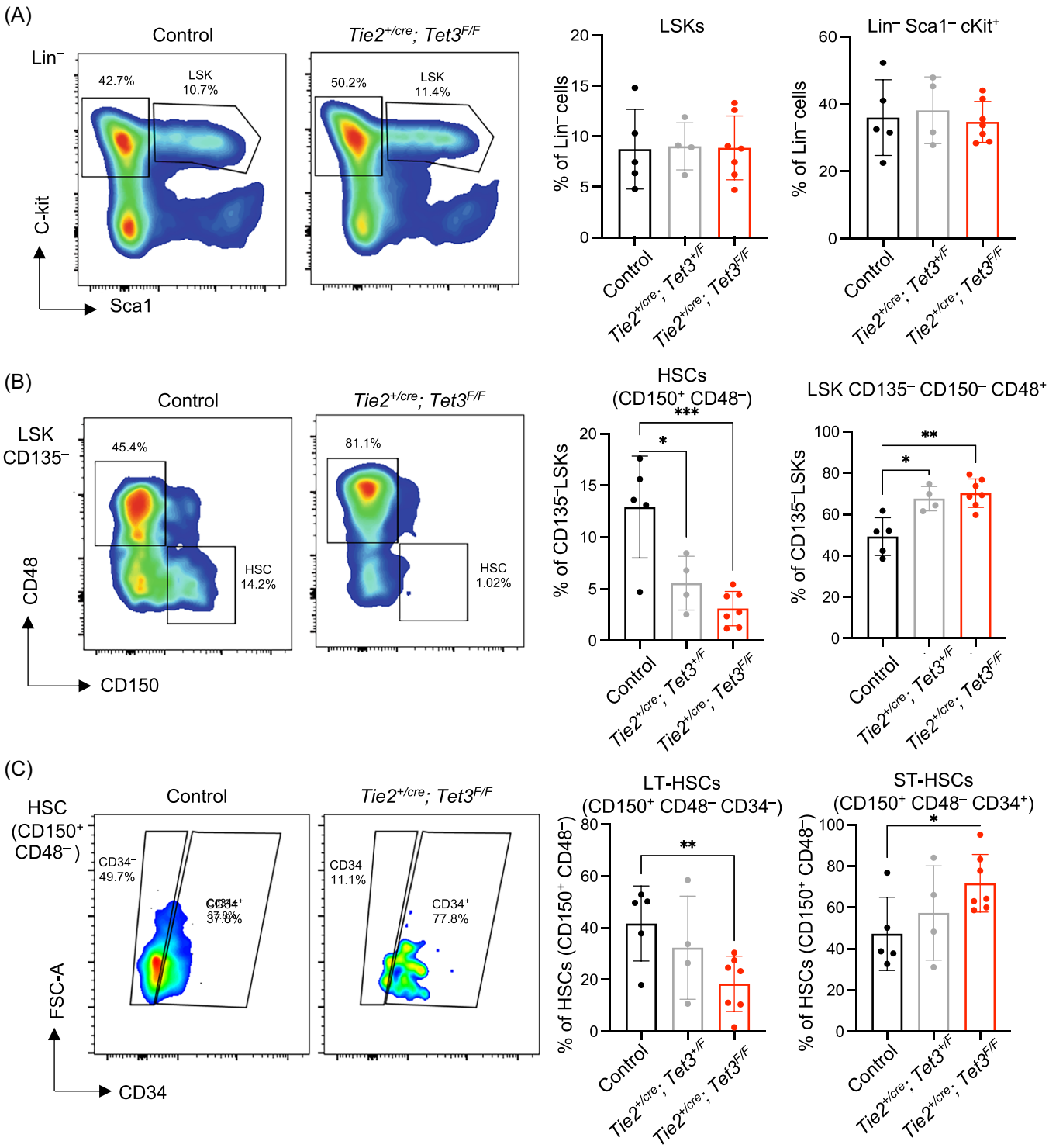

FIGURE 3 **Adult 2-month-old *Tie2*$^{+/Cre}$; *Tet3*$^{f/f}$ mice have reduced HSCs (CD150^{+}CD48^{-}) in the bone marrow. (A)** Quantification of LSK [Lin^{-}Sca1^{+}cKit^{+}] cells and Lin^{-}Sca1^{-} cKit^{+} cells in bone marrow by flow cytometry. Representative flow plots (left) and cells plotted as percentage of Lin^{-} cells (right). **(B)** Quantification of HSCs [CD150^{+}CD48^{-}] cells and LSK CD135^{-}CD150^{-}CD48^{-} in bone marrow by flow cytometry. Representative flow plots (left) and cells plotted as percentage of CD135^{-}LSK (right). **(C)** Quantification of LT-HSC [CD150^{+}CD48^{-}CD34^{-}] and ST-HSC [CD150^{+}CD48^{-}CD34^{+}] by flow cytometry. Representative flow plots (left) and LT-HSCs plotted as percentage of HSCs [CD150^{+}CD48^{-}] (right). In all panels, control samples are either *Tie2*$^{+/+}$; *Tet3*$^{+/F}$ or *Tie2*$^{+/+}$; *Tet3*$^{F/F}$. For all panels, error bars represent SD, and * indicates statistical significance ($p < 0.05$), **$p < 0.01$, and ***$p < 0.001$ using *t*-test.

These findings suggest that TET3 regulates HSC homeostasis and may influence HSC self-renewal without impacting multipotency.

To test the multilineage engraftment potential of *Tie2*$^{+/cre}$; *Tet3*$^{F/F}$ HSCs, we performed a competitive bone marrow transplantation (BMT) in which 50% CD45.1 competitor BM-MNCs were mixed with either 50% *Tie2*$^{+/+}$; *Tet3*$^{F/F}$ or *Tie2*$^{+/cre}$; *Tet3*$^{F/F}$ donor CD45.2 BM-MNCs and transplanted in sub-lethally irradiated CD45.1 recipient mice (Figure 4A). The analysis of peripheral blood every

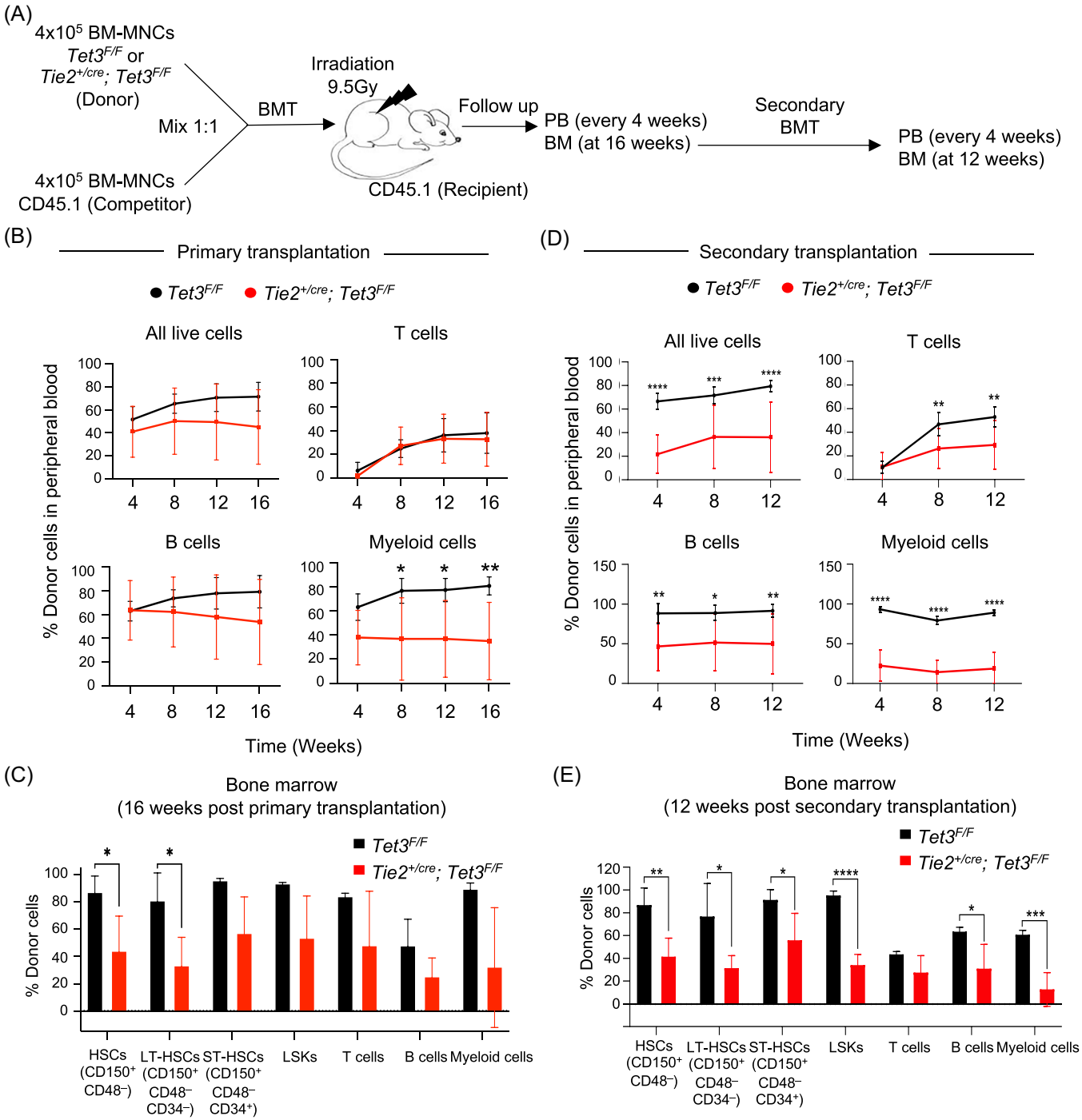

FIGURE 4 **Competitive bone marrow transplantation of *Tie2*$^{+/cre}$; *Tet3*$^{F/F}$ BM-MNCs. (A)** Schematic of competitive bone marrow transplantation (BMT) with either control or *Tie2*$^{+/cre}$; *Tet3*$^{F/F}$ donor BM-MNCs. BM from 2 *Tet3*$^{F/F}$ or 2 *Tie2*$^{+/cre}$; *Tet3*$^{F/F}$ was transplanted into 16 mice (FOUR mice per BM sample). **(B)** Percentage of donor cells in the peripheral blood of transplanted mice analyzed every 4 weeks after primary competitive BMT for 16 weeks. $n = 8$ transplanted mouse per genotype. **(C)** Percentage of donor cells within the bone marrow of transplanted mice 16 weeks after primary competitive BMT. $n = 4$ transplanted mice per genotype. **(D)** Percentage of donor cells in the peripheral blood of transplanted mice analyzed every 4 weeks after secondary BMT for 12 weeks. $n = 8$ transplanted mouse per genotype. **(E)** Percentage of donor cells within the bone marrow of secondary transplanted mice 12 weeks after secondary BMT. $n = 4$ transplanted mice per genotype. For all panels, error bars represent SD, and * indicates statistical significance ($p < 0.05$) and **$p < 0.01$ using *t*-test.

4 weeks post-transplantation revealed an overall reduced contribution of *Tie2*$^{+/cre}$; *Tet3*$^{F/F}$ cells, especially a significantly reduced contribution to the myeloid population (Figure 4B). The morphology of myeloid cells was investigated in the PB smear by May-Grünwald-Giemsa staining. This revealed that no myeloid blasts were present suggesting that the loss of TET3 does not lead to myeloid disease in BMT context (Figure S5C). The analysis of bone marrow at 16 weeks post-transplantation revealed that while contribution *Tie2*$^{+/cre}$; *Tet3*$^{F/F}$ BM-MNCs to mature cell types was largely unaffected, contribution to HSCs ($CD150^+CD48^-$), specifically LT-HSCs ($CD150^+CD48^-CD34^-$),

was significantly reduced (Figure 4C). To further investigate the engraftment and multilineage potential of *Tie2*$^{+/cre}$; *Tet3*$^{F/F}$ HSCs, we performed a secondary BMT 16 weeks post-primary BMT. The analysis of peripheral blood every 4 weeks post-secondary BMT revealed an overall reduced contribution of *Tie2*$^{+/cre}$; *Tet3*$^{F/F}$ cells to B, T, and myeloid cells (Figure 4D). The analysis of bone marrow at 12 weeks post-secondary BMT revealed that contribution of *Tie2*$^{+/cre}$; *Tet3*$^{F/F}$ BM-MNCs to mature cell types (except T cells) and to HSCs (CD150^{+}CD48^{-}), including LT-HSCs (CD150^{+}CD48^{-}CD34^{-}), was significantly reduced (Figure 4E).

We also assessed the functional capacity of *Tie2*$^{+/cre}$; *Tet3*$^{F/F}$ HSCs using an in vitro limiting dilution assay, where we sorted 8, 4, 2, or 1 *Tie2*$^{+/cre}$; *Tet3*$^{F/F}$ or control HSCs into a single well and cultured them for 6 weeks. The assessment of colony formation revealed a reduction of the colony forming capacity of *Tie2*$^{+/cre}$; *Tet3*$^{F/F}$ HSCs in vitro by 2.5 fold (Figure 5A). Finally, we investigated if TET3 influenced HSC quiescence. We found that loss of TET3 caused a reduction in the percentage of quiescent (ki67^{-}) HSCs and an increase in the percentage of active (ki67^{+}) HSCs compared to *Tet3*$^{F/F}$ controls (Figure 5B). Altogether, these findings show that TET3 plays important roles in HSC homeostasis and suggest that TET3 could maintain HSC self-renewal ability in vivo and in vitro by regulating the HSC quiescent state.

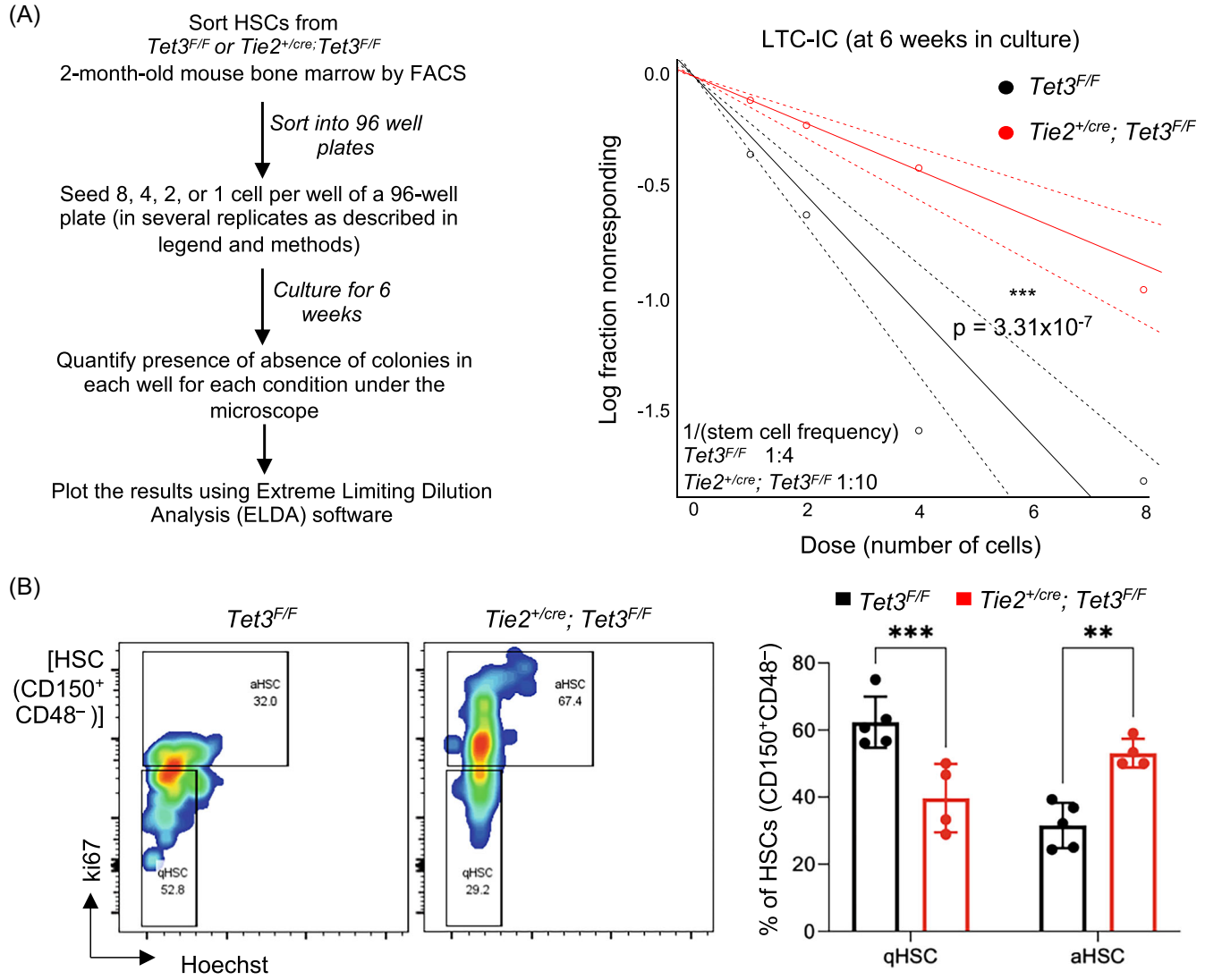

FIGURE 5 **In vitro long-term culture-initiating cell (LTC-IC) assay of *Tie2*$^{+/cre}$; *Tet3*$^{F/F}$ HSCs and quantification of quiescent and active HSCs in the bone marrow. (A)** Long term culture-initiating cell (LTC-IC) capacity of *Tet3*$^{F/F}$ and *Tie2*$^{+/cre}$; *Tet3*$^{F/F}$ HSCs isolated from 2-month-old mouse bone marrow. Overall test for differences in stem cell frequencies between groups calculated by the chi-square test. The slope of the line is the log-active cell fraction. The dashed lines give the 95% confidence interval. $n \geq 24$ wells per condition per genotype. Note that there is a significant reduction in the ability of *Tie2*$^{+/cre}$; *Tet3*$^{F/F}$ HSCs to form colonies. There is a 2.5-fold change between the control (1:4) and experimental (1:10) groups. Replicates for control *Tet3*$^{F/F}$ group: for dilution of 8 cells/well ($n = 24$ replicates), for dilution of 4 cells/well ($n = 24$ replicates), for dilution of 2 cells/well ($n = 24$ replicates), and for dilution of 1 cell/well ($n = 48$ replicates). Replicates for *Tie2*$^{+/cre}$; *Tet3*$^{F/F}$ group: for dilution of 8 cells/well ($n = 36$ replicates), for dilution of 4 cells/well ($n = 36$ replicates), for dilution of 2 cells/well ($n = 36$ replicates), and for dilution of 1 cell/well ($n = 60$ replicates). **(B)** Quantification of quiescent HSCs (qHSC) and active HSCs (aHSC) in *Tet3*$^{F/F}$ and *Tie2*$^{+/cre}$; *Tet3*$^{F/F}$ bone morrow. Representative flow cytometry plots showing percentage of aHSCs or qHSC in each box (left) and cells plotted as percentage of HSCs (CD150^{+}CD48^{-}) (right). *Tet3*$^{F/F}$ group ($n = 5$) and *Tie2*$^{+/cre}$; *Tet3*$^{F/F}$ group ($n = 4$). For all panels, error bars represent SD, and * indicates statistical significance ($p < 0.05$) and **$p < 0.01$ using *t*-test.

Loss of TET3 leads to hyper-methylation and downregulation of cell cycle genes

Since TET3 regulates DNA methylation levels by converting 5mC to 5hmC, we mapped 5mC and 5hmC in Lin$^-$ cells isolated from *Tie2*$^{+/cre}$; *Tet3*$^{F/F}$ and control 2-month-old mice. We found that methylated CpG sites were increased and hydroxymethylated CpG sites were reduced in *Tie2*$^{+/cre}$; *Tet3*$^{F/F}$ cells genome-wide (Figure 6A,B). Differential methylation and hydroxymethylation analyses revealed that the majority of genomic regions were hyper-methylated or

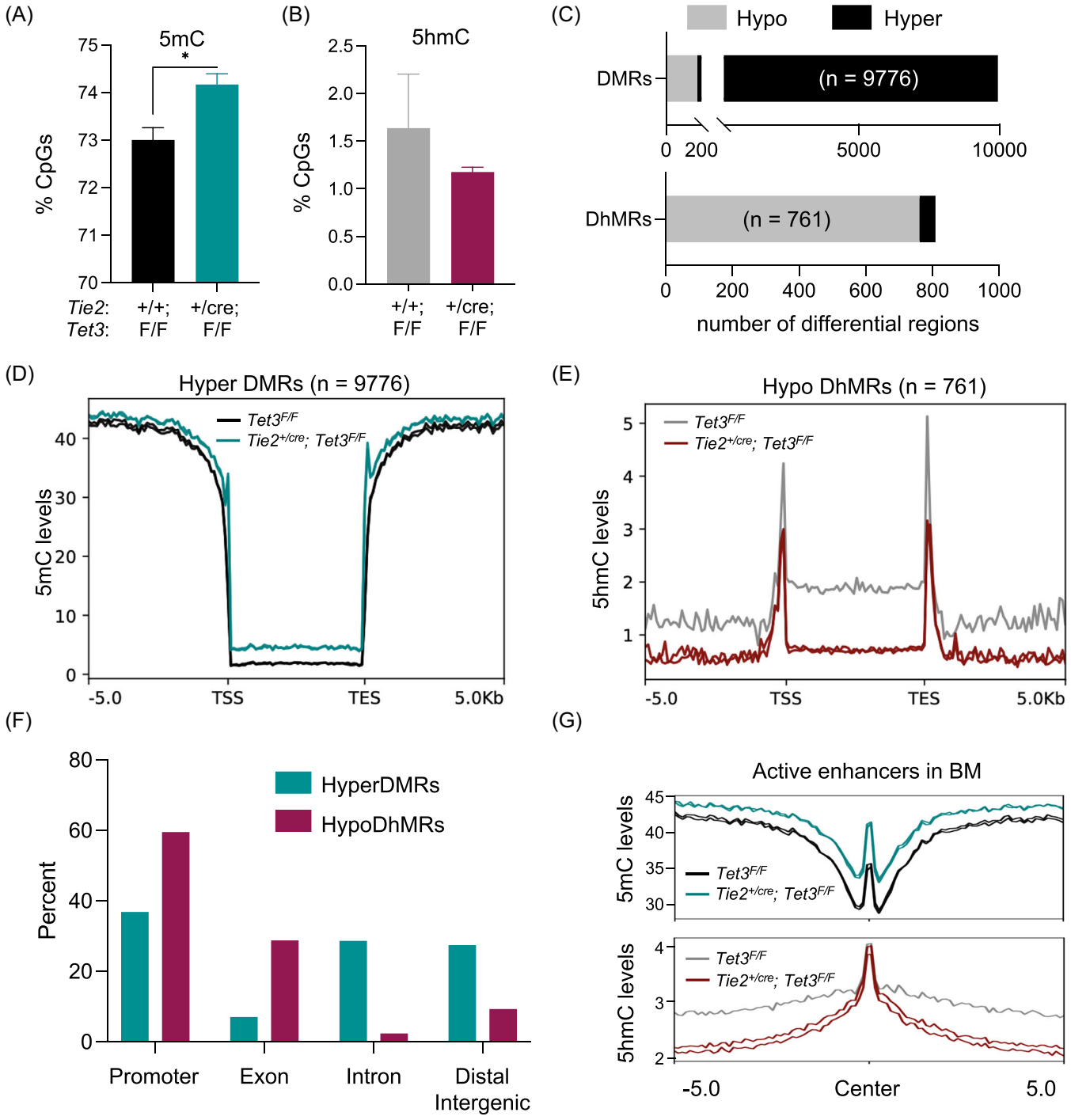

FIGURE 6 **Analysis of DNA methylation and hydroxymethylation levels in *Tie2*$^{+/cre}$; *Tet3*$^{F/F}$ Lin$^-$ cells by EM-seq and E5hmC-seq, respectively. (A)** Percentage of methylated CpG sites (5mC) genome wide. **(B)** Percentage of hydroxymethylated CpG sites (5hmC) genome wide. **(C)** Number of differentially methylated regions (DMRs, top) and differentially hydroxymethylated regions (DhMRs, bottom). **(D)** Profile plot showing 5mC levels at all hyper-DMRs across genes from 5 kb upstream of TSS (transcriptional start site) to 5 kb downstream of TES (transcriptional end site). **(E)** Profile plot showing 5hmC levels at all hypo-DhMRs across genes from 5 kb upstream of TSS to 5 kb downstream of TES. **(F)** Percentage of hyper-DMRs and hypo-DhMRs across genes and gene regulatory regions. **(G)** Profile plots showing 5mC and 5hmC levels at ±5 kb of active enhancers in bone marrow cells. Enhancer coordinates were identified using H3K27ac ChIP-seq data from ENCODE. For panels A and B, error bars represent SD, and * indicates statistical significance ($p < 0.05$) using *t*-test.

hypo-hydroxymethylated. We identified 9776 hyper differentially methylated regions (DMRs) and 761 hypo-differentially hydroxymethylated regions (DhMRs) (Figure 6C–E). The high number of DMRs compared to DhMRs is consistent with the high abundance of 5mC (~73% of all CpGs) compared to 5hmC (~1.5% of all CpGs). Hyper-DMRs were evenly dispersed throughout the genome at promoters, gene bodies, and distal intergenic regions while hypo-DhMRs were most commonly observed at promoters and exons (Figure 6F). Because TET enzymes have been implicated in demethylation of enhancers,[2,10,32,45,46] we wanted to assess the methylation and hydroxymethylation status of enhancers. To this end, we used H3K27ac and H3K4me1 ChIP-seq data in BM-MNCs from ENCODE[10] and identified 18,536 active enhancers (i.e., regions positive for both marks) (Figure S6A). DNA methylation levels were increased at and around active enhancers, while DNA hydroxymethylation levels were decreased around active enhancers in *Tie2*$^{+/cre}$; *Tet3*$^{F/F}$ Lin$^-$ cells (Figure 6G). Of the ~18,000 active enhancers in BM-MNCs, 564 active enhancers were associated to a hyper-DMR (Figure S6B). Together, these findings suggest that TET3 may influence the expression of genes through DNA demethylation in hematopoietic cells. We found that hyper-DMRs were associated to ~5000 genes and hypo-DhMRs to ~500 genes (Figure 7A). Gene ontology analysis of hyper-DMR-associated genes revealed processes related to cell proliferation, cell cycle, and transcriptional regulation (Figure 7B). Our transcriptomic analysis of *Tie2*$^{+/cre}$; *Tet3*$^{F/F}$ Lin$^-$ cells by RNA-seq identified 498 differentially expressed genes (DEGs) (Figure S6C) with many downregulated genes associated to hyper-DMRs (Figure 7C). Several hematopoietic transcription factors and cell cycle regulators were hypermethylated and downregulated (Figures 7C,D and S6D). Hypermethylation and downregulation of the cyclin-dependent kinase inhibitors *Cdkn2c* and *Cdkn2d*, which are negative regulators of the cell cycle, may underpin the decrease in the number of quiescence HSCs in TET3-deficient bone marrow, leading to a reduction in the HSC population. Together, our findings establish a requirement for TET3 in regulating DNA methylation levels for proper hematopoietic gene expression and HSC function.

Because the hematopoietic phenotypes of *Tie2*$^{+/cre}$; *Tet3*$^{F/F}$ mice (i.e., reduction in percentage of LT-HSCs) are less severe and different than the hematopoietic phenotypes of *Tet2*$^{-/-}$ mice (i.e., block in differentiation and onset of myeloid disease and malignancy[13,30,47]), we compared the gene expression and methylome of our *Tie2*$^{+/cre}$; *Tet3*$^{F/F}$ mice to those of previously published *Tet2*$^{-/-}$ mice.[30] Comparison of DEGs in *Tie2*$^{+/cre}$; *Tet3*$^{F/F}$ Lin$^-$ cells to DEGs in *Tet2*$^{-/-}$ Lin$^-$ cells revealed only 21 common genes and they were not considered main drivers of hematopoietic defects in *Tet2*$^{-/-}$ mice (Figure S7A). In contrast, 477 DEGs were unique to *Tie2*$^{+/cre}$; *Tet3*$^{F/F}$ and 208 DEGs were unique to *Tet2*$^{-/-}$ Lin$^-$ cells. This suggests that the gene expression profile changes of *Tie2*$^{+/cre}$; *Tet3*$^{F/F}$ and *Tet2*$^{-/-}$ mouse models are different, which is consistent with their distinct hematopoietic phenotypes. Similarly, comparison of ~9800 hyper-DMRs in *Tie2*$^{+/cre}$; *Tet3*$^{F/F}$ Lin$^-$ cells to the ~3700 hyper-DMRs present in *Tet2*$^{-/-}$ Lin$^-$ cells[30] revealed only 435 common hyper-DMRs (Figure S7B) none of which were associated to any major hematopoiesis genes. This suggests that TET3 and TET2 largely regulate distinctive genomic regions and genes in Lin$^-$ cells and that is why loss of each have different impacts on hematopoiesis. Finally, comparison of DEGs in our *Tie2*$^{+/cre}$; *Tet3*$^{F/F}$ mouse to that of a published *Tet2*$^{-/-}$; *Tet3*$^{fl/fl}$ *Mx1-Cre*$^+$ double knockout (DKO) mouse[41] identified only 30 common DEGs, while 468 DEGs were unique to *Tie2*$^{+/cre}$; *Tet3*$^{F/F}$ and 684 DEGs were unique to DKO cells (Figure S7C). This analysis suggests that loss of TET3 in the absence of TET2 leads to deregulation of more genes consistent with the stronger phenotypes observed in TET2/3-DKO mice compared to *Tet2*$^{-/-}$ mice alone.

DISCUSSION

Tet3 is expressed in hematopoietic cells but its requirements in hematopoiesis is less studied. Here, we provide four lines of evidence that implicate TET3 in HSC homeostasis during embryonic and adult hematopoiesis. (1) TET3 deficiency reduces the percentage of LT-HSCs in the fetal liver and adult bone marrow. (2) Consistently TET3-deficient bone marrow has less quiescent and more active HSCs. (3) TET3-deficient HSCs have reduced CFU capacity, and TET3-deficient BM-MNCs have decreased contribution to the HSC compartment, especially LT-HSCs, in transplantation assays. (4) Loss of TET3 leads to DNA hypermethylation at key cell cycle genes and hematopoietic lineage driver genes leading to their downregulation. Together these findings suggest that TET3 maintains HSC homeostasis in part by regulating self-renewal and proliferation.

Global loss of TET3 is not compatible with viability and leads to perinatal lethality in mice.[20] Although *Tet3*$^{-/-}$ embryos have reduced LT-HSCs, the viability of our *Tie2*$^{+/cre}$; *Tet3*$^{F/F}$ mice, which deletes TET3 in endothelial cells and hence the hematopoietic lineage, suggests that the perinatal lethality of *Tet3*$^{-/-}$ mice is unlikely due to hematopoiesis-related disorders. Interestingly, the reduction in percentage of LT-HSCs in *Tie2*$^{+/cre}$; *Tet3*$^{F/F}$ E13.5 fetal livers was consistent with findings in *Tet3*$^{-/-}$ E13.5 fetal livers supporting a hematopoietic specific role for TET3 in regulating LT-HSCs. Our findings indicate that TET3 regulates HSC homeostasis by regulating HSC proliferation. One explanation for the reduction of HSCs, particularly LT-HSCs, in adult *Tie2*$^{+/cre}$; *Tet3*$^{F/F}$ mice could be increased proliferation and exhaustion of HSCs. This would be in agreement with the presence of less quiescent and more active HSCs observed in *Tie2*$^{+/cre}$; *Tet3*$^{F/F}$ mice. The downregulation of negative regulators of cell cycle in TET3-deficient bone marrow, in particular the cyclin-dependent kinase inhibitor genes *Cdkn2c/d*, suggests that TET3 likely serves as a negative regulator of HSC proliferation. This is consistent with increased DNA methylation and reduced hydroxymethylation at these genes, supporting that they might be direct targets of TET3-mediated DNA demethylation. In agreement with our findings, CDK inhibitors have been implicated in regulating the quiescent state of HSCs and deletion of CDK inhibitors such as *Cdkn2c (p18)* in HSCs results in increased HSC activity.[48,49] TET enzymes have been implicated in regulating the cell cycle and proliferation of other stem cell types.[12,50] In embryonic stem cells (ESCs), TET1 negatively regulates *p21* levels to control G1/S transition and its loss reduces proliferation of ESCs.[50] In neuroectoderm cells, TET3 loss increases the percentage of cells in S phase, and cell cycle genes were identified as direct targets of TET3.[12] Our findings expand on these findings and implicate TET3 in regulating HSC proliferation by demethylation and activation of cell cycle genes.

The deficits in *Tie2*$^{+/cre}$; *Tet3*$^{F/F}$ HSCs are reflected in both their self-renewal potential, where they performed poorly in limiting dilution assay forming fewer colonies, as well as in competitive BMT, where they contributed poorly to HSCs and in particular LT-HSCs. Interestingly, contribution to more mature cell types was not significantly affected in the bone marrow, and only myeloid cells were reduced in the peripheral blood after primary BMT, while myeloid and B and T cells were reduced in PB after secondary BMT. Since the percentage of myeloid cells was unaffected in the bone marrow of *Tie2*$^{+/cre}$; *Tet3*$^{F/F}$ mice, the reduction in myeloid cell in peripheral blood of transplanted mice may be due to stress of BMT. Interestingly, TET3 has been implicated in myeloid malignancies such as AML.[38] Although we do not see the presence of myeloid blasts in young *Tie2*$^{+/cre}$; *Tet3*$^{F/F}$ mice or in transplanted mice, it is possible that mature blood cells such as myeloid cells are affected with age. Future studies aimed at characterizing the hematopoietic

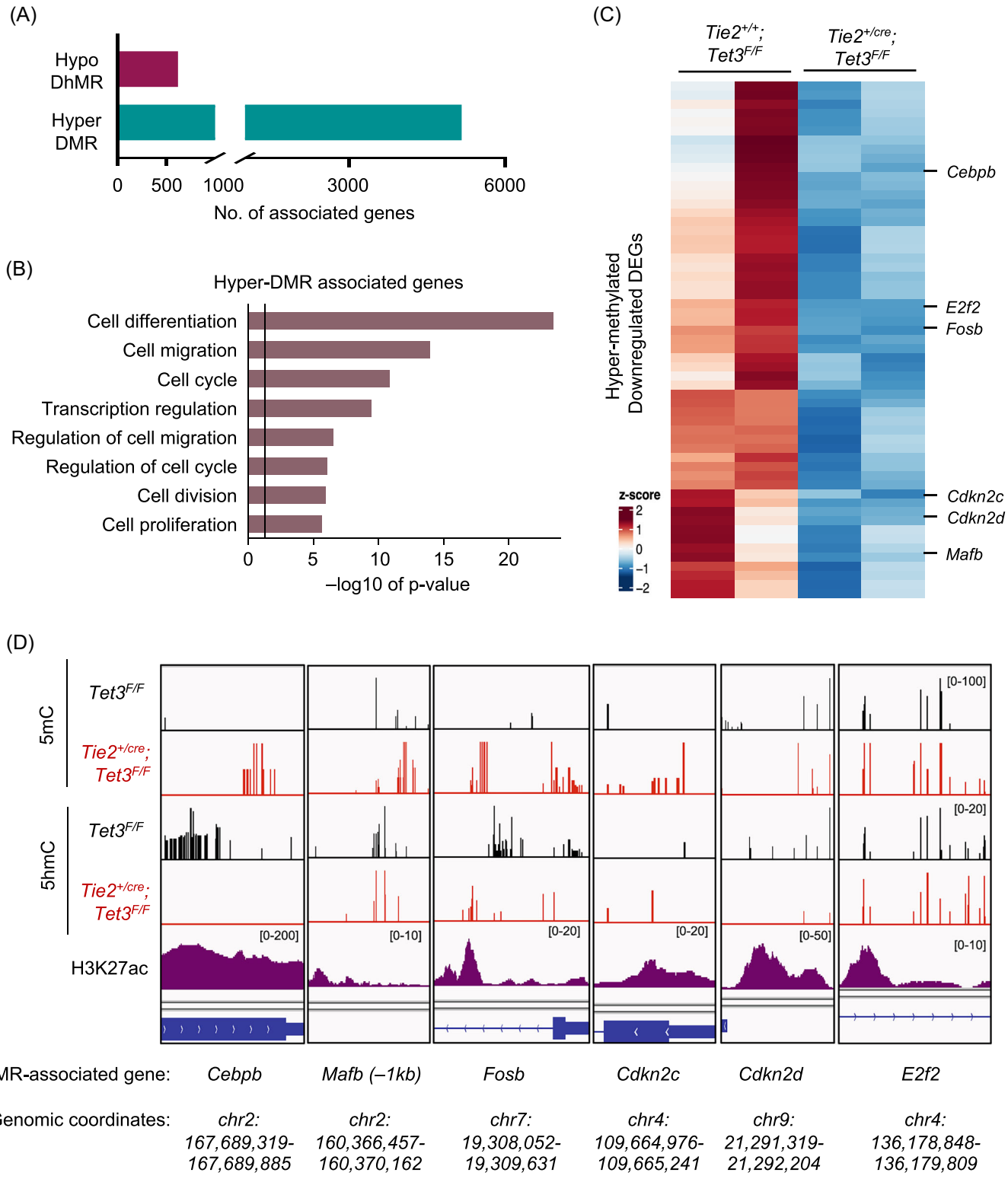

FIGURE 7 **Downregulation and hypermethylation of hematopoietic and cell cycle regulator genes in *Tie3*$^{+/cre}$; *Tet3*$^{F/F}$ Lin$^-$ cells. (A)** Number of genes associated to hyper-DMRs and hypo-DhMRs. **(B)** Gene ontology (GO) analysis of hyper-DMR associated genes. –log of *p* values for top GO terms are plotted. Vertical line indicates significance ($p < 0.05$). **(C)** Heatmap of downregulated differentially expressed genes (DEGs) in *Tie3*$^{+/cre}$; *Tet3*$^{F/F}$ Lin$^-$ cells that are associated to hyper-DMRs. DEGs were identified by global transcriptomic analysis of *Tie3*$^{+/cre}$; *Tet3*$^{F/F}$ and *Tie3*$^{+/+}$; *Tet3*$^{F/F}$ Lin$^-$ cells. Note that several hematopoietic and cell cycle regulator genes are downregulated in *Tie3*$^{+/cre}$; *Tet3*$^{F/F}$ Lin$^-$ cells. **(D)** IGV tracks showing 5mC and 5hmC levels as well as H3K27ac levels (using data from ENCODE) at hyper-DMRs of selected hematopoietic and cell cycle genes.

phenotypes of aged *Tie2*$^{+/cre}$; *Tet3*$^{F/F}$ mice will elaborate more on functions of TET3 in the myeloid lineage. Downregulation of several mature lineage drivers, such as *Cebpb* and *Mafb*, in 2-month-old *Tie2*$^{+/cre}$; *Tet3*$^{F/F}$ mice, may underlie any potential dysregulation of mature blood cell types with age. We found *Mafb* to be downregulated and hypermethylated in *Tie2*$^{+/cre}$; *Tet3*$^{F/F}$ hematopoietic cells. *Mafb* is expressed in the myeloid lineage and regulates monocyte and macrophage differentiation.[51,52] Inhibition of *Mafb* blocks myeloid colony formation and formation of macrophages.[52] While we do not see a reduction in myeloid cells in 2-month-old *Tie2*$^{+/cre}$; *Tet3*$^{F/F}$ mice, it is possible that a reduction in *Mafb* may affect myeloid cells in older mice or upon BMT as we have observed.

TET enzymes are critical regulators of hematopoiesis and are implicated in hematopoietic disorders.[26,53,54] While TET2 is most commonly studied in the hematopoietic lineage due to the prevalence of *Tet2* mutations in blood malignancies, TET1 and TET3 have also been implicated in hematopoiesis.[35,39,40] Despite TET3-regulating LT-HSCs, both embryonic and adult hematopoiesis in *Tie2*$^{+/cre}$; *Tet3*$^{F/F}$ mice allow the formation of mature blood cell types suggesting there may be compensatory mechanisms that allow for contribution of TET3-deficient HSCs to form mature hematopoietic cell types. It is possible that TET1 or TET2 compensates for TET3 loss in HSCs. While we do not see the induction of *Tet1/2* in *Tie2*$^{+/cre}$; *Tet3*$^{F/F}$ bone marrow, it is possible that their redistribution or alternative parallel mechanism ameliorates the impact of TET3 deficiency.

Tet3 is expressed at much lower levels than *Tet2* in HSCs, and it is not surprising that TET3 deficiency has by far milder effects on HSCs compared to TET2 deficiency.[29] However, it has been shown that in the context of TET2 loss, TET3 deficiency has a major impact on hematopoiesis.[41] While TET2 loss blocks differentiation of HSPCs leading to CMML by 1 year of age, combined loss of TET2 and TET3 leads to a much earlier onset of leukemia within 2 months.[41] Similarly, while TET2 loss does not have a significant impact on embryonic hematopoiesis and TET2 null embryos are viable, TET2 and TET3 combined loss leads to embryonic hematopoiesis defects in zebrafish[53] and embryonic lethality in mouse.[55] This suggests that the deficits caused by TET3 deficiency exacerbates the effects of TET2 loss leading to more profound phenotypes during embryonic and adult hematopoiesis. We found no major overlap in the gene expression profiles and methylome of TET3 and TET2 knockout hematopoietic cells. This suggests that TET2 and TET3 regulate distinct genomic regions and genes and that is why TET2 and TET3 knockout models have such distinct phenotypes. It is likely that the combined deregulation of their target genes exacerbates the phenotypes of TET2 knockout cells. For example, loss of TET3 impacting cell cycle programs and loss of TET2 affecting key hematopoietic differentiation programs together lead to a severe phenotype in TET2/3 DKO mice. Consistently, we have shown that more genes are deregulated in TET2/3 DKO mice compared to TET3 knockout mice, albeit with less overlap between them. While these comparisons provide interesting insights, there are developmental differences between our TET3 KO and published TET2/3DKO mice which can complicate interpretations. In our *Tie2*$^{+/cre}$; *Tet3*$^{F/F}$ mouse, *Tet3* is deleted in the entire hematopoietic system early in embryogenesis via an early embryonic expression of Cre in the endothelial compartment. In contrast, in the TET2/3 DKO mouse model (*Tet2*$^{-/-}$; *Tet3*$^{fl/fl}$ *Mx1-Cre*$^{+}$) Mx1-cre is induced upon pIpC injections in adult mice[41] and exclusively studies the impact of TET2/3 loss in adult hematopoiesis. Future studies involving direct comparison of models with similar spatiotemporal deletion of TET2 and TET3 will be essential to accurately compare their requirements in hematopoiesis. In conclusion, TET3 has impactful roles in hematopoiesis and our findings have implications for understanding normal and abnormal hematopoiesis where TET3 alone or in conjunction with other TET enzymes are dysregulated.

Limitations of the study: While this study investigates the effects of TET3 loss in embryonic and adult HSCs and identifies key deficits in HSC homeostasis, the impact of this defect in long-term survival of mice or manifestation of any hematopoietic abnormalities/disease is not covered as part of this study. Future work aimed at aging *Tie2*$^{+/cre}$; *Tet3*$^{F/F}$ mice will help better define long-term consequences of TET3 deficiency during hematopoiesis. Likewise, disruption of TET3 in adult HSCs, using inducible systems such as Mx1-Cre with pIpC, would elaborate on how loss of TET3 at various ages impacts hematopoiesis. Finally, at the molecular level, while we identify several cell cycle and hematopoietic regulators to be perturbed, it is likely that other regulators of hematopoiesis or proliferation are affected as well but detection of those changes were masked in bulk RNA-seq. Future single-cell transcriptomic and epigenomic approaches may identify more discrete targets of TET3 in HSCs. Likewise, challenges in mapping TET3 genomic occupancy due to unavailability of suitable antibodies limit our ability to establish direct targets and regions that TET3 regulates. Improvements in these technologies would provide better mechanistic insights into how TET3 regulates hematopoiesis at the molecular level.

QUANTIFICATION AND STATISTICAL ANALYSES

We used one-way ANOVA test or unpaired *t*-test in GraphPad Prism 8 to calculate statistical significance. Statistical methods used for bioinformatics analyses are explained in detail under the respective methods subsections.

ACKNOWLEDGMENTS

We thank all members of the Dawlaty and Ito lab members for reading the manuscript.

AUTHOR CONTRIBUTIONS

Harmony C. Ketchum: Conceptualization; methodology; data curation; investigation; validation; formal analysis; visualization; writing—original draft; writing—review and editing. **Claudia Morganti**: Conceptualization; methodology; formal analysis; data curation; validation; visualization; writing—review and editing; writing—original draft. **Chie Yanase**: Methodology; investigation. **Blake Ebert**: Methodology; investigation. **Keisuke Ito**: Conceptualization; supervision; resources; project administration; writing—review and editing; funding acquisition; investigation. **Meelad M. Dawlaty**: Conceptualization; supervision; resources; project administration; funding acquisition; writing—review and editing; investigation.

CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

The RNA-seq, EM-seq, and E5hmC-seq data have been deposited in the Gene Expression Omnibus (GEO) database under accession number GSE282720.

FUNDING

This work was supported by funds from the National Institute of Health R01HL172730 to M. M. D., R01DK098263 to K. I., R01HL148852 to K. I. and M. M. D., and LLS Discovery Grant (8040-

24) to K. I. H. C. K. was supported by NIH T32 GM007491 training grant in cellular and molecular biology and genetics and the Paul S. Frenette Scholar Awards Program of the Ruth L. and David S. Gottesman Institute for Stem Cell Research and Regenerative Medicine at Albert Einstein College of Medicine. B. E. was supported by NIH predoctoral fellowship F30HD113183 and the Medical Scientist Training Program T32GM149364 training grant.

ORCID

Meelad M. Dawlaty http://orcid.org/0000-0003-3121-2500

SUPPORTING INFORMATION

Additional supporting information can be found in the online version of this article.

REFERENCES

1. Dawlaty MM, Breiling A, Le T, et al. Loss of Tet enzymes compromises proper differentiation of embryonic stem cells. *Dev Cell*. 2014;29:102-111. doi:10.1016/j.devcel.2014.03.003
2. Ma L, Tang Q, Gao X, et al. Tet-mediated DNA demethylation regulates specification of hematopoietic stem and progenitor cells during mammalian embryogenesis. *Sci Adv*. 2022;8:eabm3470. doi:10.1126/sciadv.abm3470
3. Tahiliani M, Koh KP, Shen Y, et al. Conversion of 5-methylcytosine to 5-hydroxymethylcytosine in mammalian DNA by MLL partner TET1. *Science*. 2009;324:930-935. doi:10.1126/science.1170116
4. Ito S, Shen L, Dai Q, et al. Tet proteins can convert 5-methylcytosine to 5-formylcytosine and 5-carboxylcytosine. *Science*. 2011;333:1300-1303. doi:10.1126/science.1210597
5. He YF, Li BZ, Li Z, et al. Tet-mediated formation of 5-carboxylcytosine and its excision by TDG in mammalian DNA. *Science*. 2011;333:1303-1307. doi:10.1126/science.1210944
6. Maiti A, Drohat AC. Thymine DNA glycosylase can rapidly excise 5-formylcytosine and 5-carboxylcytosine. *J Biol Chem*. 2011;286:35334-35338. doi:10.1074/jbc.C111.284620
7. Pastor WA, Aravind L, Rao A. TETonic shift: biological roles of TET proteins in DNA demethylation and transcription. *Nat Rev Mol Cell Biol*. 2013;14:341-356. doi:10.1038/nrm3589
8. Hashimoto H, Liu Y, Upadhyay AK, et al. Recognition and potential mechanisms for replication and erasure of cytosine hydroxymethylation. *Nucleic Acids Res*. 2012;40:4841-4849. doi:10.1093/nar/gks155
9. Bachman M, Uribe-Lewis S, Yang X, Williams M, Murrell A, Balasubramanian S. 5-Hydroxymethylcytosine is a predominantly stable DNA modification. *Nat Chem*. 2014;6:1049-1055. doi:10.1038/nchem.2064
10. Flores JC, Sidoli S, Dawlaty MM. Tet2 regulates Sin3a recruitment at active enhancers in embryonic stem cells. *iScience*. 2023;26:107170. doi:10.1016/j.isci.2023.107170
11. Chrysanthou S, Tang Q, Lee J, et al. The DNA dioxygenase Tet1 regulates H3K27 modification and embryonic stem cell biology independent of its catalytic activity. *Nucleic Acids Res*. 2022;50:3169-3189. doi:10.1093/nar/gkac089
12. Ketchum HC, Suzuki M, Dawlaty MM. Catalytic-dependent and -independent roles of TET3 in the regulation of specific genetic programs during neuroectoderm specification. *Commun Biol*. 2024;7:415. doi:10.1038/s42003-024-06120-w
13. Ito K, Lee J, Chrysanthou S, et al. Non-catalytic roles of Tet2 are essential to regulate hematopoietic stem and progenitor cell homeostasis. *Cell Rep*. 2019;28:2480-2490.e4. doi:10.1016/j.celrep.2019.07.094.
14. Xue S, Liu C, Sun X, et al. TET3 inhibits type I IFN production independent of DNA demethylation. *Cell Rep*. 2016;16:1096-1105. doi:10.1016/j.celrep.2016.06.068
15. Montalbán-Loro R, Lozano-Ureña A, Ito M, et al. TET3 prevents terminal differentiation of adult NSCs by a non-catalytic action at Snrpn. *Nat Commun*. 2019;10:1726. doi:10.1038/s41467-019-09665-1
16. Deplus R, Delatte B, Schwinn MK, et al. TET2 and TET3 regulate GlcNAcylation and H3K4 methylation through OGT and SET1/COMPASS. *EMBO J*. 2013;32:645-655. doi:10.1038/emboj.2012.357
17. Williams K, Christensen J, Pedersen MT, et al. TET1 and hydroxymethylcytosine in transcription and DNA methylation fidelity. *Nature*. 2011;473:343-348. doi:10.1038/nature10066
18. Dawlaty MM, Breiling A, Le T, et al. Combined deficiency of Tet1 and Tet2 causes epigenetic abnormalities but is compatible with postnatal development. *Dev Cell*. 2013;24:310-323. doi:10.1016/j.devcel.2012.12.015
19. Dai H-Q, Wang BA, Yang L, et al. TET-mediated DNA demethylation controls gastrulation by regulating Lefty–Nodal signalling. *Nature*. 2016;538:528-532. doi:10.1038/nature20095
20. Gu T-P, Guo F, Yang H, et al. The role of Tet3 DNA dioxygenase in epigenetic reprogramming by oocytes. *Nature*. 2011;477:606-610. doi:10.1038/nature10443
21. Yan R, Cheng X, Gu C, et al. Dynamics of DNA hydroxymethylation and methylation during mouse embryonic and germline development. *Nat Genet*. 2023;55:130-143. doi:10.1038/s41588-022-01258-x
22. Kang J, Kalantry S, Rao A. PGC7, H3K9me2 and Tet3: regulators of DNA methylation in zygotes. *Cell Res*. 2013;23:6-9. doi:10.1038/cr.2012.117
23. Iqbal K, Jin SG, Pfeifer GP, Szabó PE. Reprogramming of the paternal genome upon fertilization involves genome-wide oxidation of 5-methylcytosine. *Proc Natl Acad Sci*. 2011;108:3642-3647. doi:10.1073/pnas.1014033108
24. Peat JR, Dean W, Clark SJ, et al. Genome-wide bisulfite sequencing in zygotes identifies demethylation targets and maps the contribution of TET3 oxidation. *Cell Rep*. 2014;9:1990-2000. doi:10.1016/j.celrep.2014.11.034
25. Yuita H, López-Moyado IF, Jeong H, et al. Inducible disruption of Tet genes results in myeloid malignancy, readthrough transcription, and a heterochromatin-to-euchromatin switch. *Proc Natl Acad Sci*. 2023;120:e2214824120. doi:10.1073/pnas.2214824120
26. Joshi K, Zhang L, Breslin S.J. P, Kini AR, Zhang J. Role of TET dioxygenases in the regulation of both normal and pathological hematopoiesis. *J Exp Clin Cancer Res*. 2022;41:294. doi:10.1186/s13046-022-02496-x
27. Sato H, Wheat JC, Steidl U, Ito K. DNMT3A and TET2 in the preleukemic phase of hematopoietic disorders. *Front Oncol*. 2016;6:187. doi:10.3389/fonc.2016.00187
28. Hosono N. Genetic abnormalities and pathophysiology of MDS. *Int J Clin Oncol*. 2019;24:885-892. doi:10.1007/s10147-019-01462-6
29. Langemeijer SMC, Kuiper RP, Berends M, et al. Acquired mutations in TET2 are common in myelodysplastic syndromes. *Nat Genet*. 2009;41:838-842. doi:10.1038/ng.391
30. Flores JC, Ito K, Huang CY, et al. Comparative analysis of Tet2 catalytic-deficient and knockout bone marrow over time. *Exp Hematol*. 2023;124:45-55.e2. doi:10.1016/j.exphem.2023.05.004.
31. Moran-Crusio K, Reavie L, Shih A, et al. Tet2 loss leads to increased hematopoietic stem cell self-renewal and myeloid transformation. *Cancer Cell*. 2011;20:11-24. doi:10.1016/j.ccr.2011.06.001
32. Rasmussen KD, Berest I, Keβler S, et al. TET2 binding to enhancers facilitates transcription factor recruitment in hematopoietic cells. *Genome Res*. 2019;29:564-575. doi:10.1101/gr.239277.118
33. Ko M, Bandukwala HS, An J, et al. Ten-eleven-translocation 2 (TET2) negatively regulates homeostasis and differentiation of hematopoietic stem cells in mice. *Proc Natl Acad Sci*. 2011;108:14566-14571. doi:10.1073/pnas.1112317108
34. Yeaton A, Cayanan G, Loghavi S, et al. The impact of inflammation-induced tumor plasticity during myeloid transformation. *Cancer Discov*. 2022;12:2392-2413. doi:10.1158/2159-8290.Cd-21-1146

35. Cimmino L, Dawlaty MM, Ndiaye-Lobry D, et al. TET1 is a tumor suppressor of hematopoietic malignancy. *Nat Immunol*. 2015;16:653-662. doi:10.1038/ni.3148
36. Huang H, Jiang X, Li Z, et al. TET1 plays an essential oncogenic role in MLL-rearranged leukemia. *Proc Natl Acad Sci*. 2013;110:11994-11999. doi:10.1073/pnas.1310656110
37. Pijuan-Sala B, Griffiths JA, Guibentif C, et al. A single-cell molecular map of mouse gastrulation and early organogenesis. *Nature*. 2019;566:490-495. doi:10.1038/s41586-019-0933-9
38. Pulikkottil AJ, Bamezai S, Ammer T, et al. TET3 promotes AML growth and epigenetically regulates glucose metabolism and leukemic stem cell associated pathways. *Leukemia*. 2022;36:416-425. doi:10.1038/s41375-021-01390-3
39. Tanaka S, Ise W, Inoue T, et al. Tet2 and Tet3 in B cells are required to repress CD86 and prevent autoimmunity. *Nat Immunol*. 2020;21:950-961. doi:10.1038/s41590-020-0700-y
40. Yue X, Lio C-WJ, Samaniego-Castruita D, Li X, Rao A. Loss of TET2 and TET3 in regulatory T cells unleashes effector function. *Nat Commun*. 2019;10:2011. doi:10.1038/s41467-019-09541-y
41. An J, González-Avalos E, Chawla A, et al. Acute loss of TET function results in aggressive myeloid cancer in mice. *Nat Commun*. 2015;6:10071. doi:10.1038/ncomms10071
42. MacArthur IC, Ma L, Huang CY, Bhavsar H, Suzuki M, Dawlaty MM. Developmental DNA demethylation is a determinant of neural stem cell identity and gliogenic competence. *Sci Adv*. 2024;10:eado5424. doi:10.1126/sciadv.ado5424
43. Tian H, Luan P, Liu Y, Li G. Tet-mediated DNA methylation dynamics affect chromosome organization. *Nucleic Acids Res*. 2024;52:3654-3666. doi:10.1093/nar/gkae054
44. Li Z, Cai X, Cai CL, et al. Deletion of Tet2 in mice leads to dysregulated hematopoietic stem cells and subsequent development of myeloid malignancies. *Blood*. 2011;118:4509-4518. doi:10.1182/blood-2010-12-325241
45. Tesio M, Trumpp A. Breaking the cell cycle of HSCs by p57 and friends. *Cell Stem Cell*. 2011;9:187-192. doi:10.1016/j.stem.2011.08.005
46. Yu H, Yuan Y, Shen H, Cheng T. Hematopoietic stem cell exhaustion impacted by p18 INK4C and p21 Cip1/Waf1 in opposite manners. *Blood*. 2006;107:1200-1206. doi:10.1182/blood-2005-02-0685
47. Chrysanthou S, Flores JC, Dawlaty MM. Tet1 suppresses p21 to ensure proper cell cycle progression in embryonic stem cells. *Cells*. 2022;11:1366. doi:10.3390/cells11081366
48. Hamada M, Tsunakawa Y, Jeon H, Yadav MK, Takahashi S. Role of MafB in macrophages. *Exp Anim*. 2020;69:1-10. doi:10.1538/expanim.19-0076
49. Kelly LM. MafB is an inducer of monocytic differentiation. *EMBO J*. 2000;19:1987-1997. doi:10.1093/emboj/19.9.1987
50. Li C, Lan Y, Schwartz-Orbach L, et al. Overlapping requirements for Tet2 and Tet3 in normal development and hematopoietic stem cell emergence. *Cell Rep*. 2015;12:1133-1143. doi:10.1016/j.celrep.2015.07.025
51. Shrestha R, Sakata-Yanagimoto M, Maie K, et al. Molecular pathogenesis of progression to myeloid leukemia from TET-insufficient status. *Blood Adv*. 2020;4:845-854. doi:10.1182/bloodadvances.2019001324
52. Fang S, Li J, Xiao Y, et al. Tet inactivation disrupts YY1 binding and long-range chromatin interactions during embryonic heart development. *Nat Commun*. 2019;10:4297. doi:10.1038/s41467-019-12325-z
53. Ito K, Turcotte R, Cui J, et al. Self-renewal of a purified Tie2+ hematopoietic stem cell population relies on mitochondrial clearance. *Science*. 2016;354:1156-1160. doi:10.1126/science.aaf5530
54. Hu Y, Smyth GK. ELDA: extreme limiting dilution analysis for comparing depleted and enriched populations in stem cell and other assays. *J Immunol Methods*. 2009;347:70-78. doi:10.1016/j.jim.2009.06.008
55. Krueger F, Andrews SR. Bismark: a flexible aligner and methylation caller for Bisulfite-Seq applications. *Bioinformatics*. 2011;27:1571-1572. doi:10.1093/bioinformatics/btr167