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Supplemental Information

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

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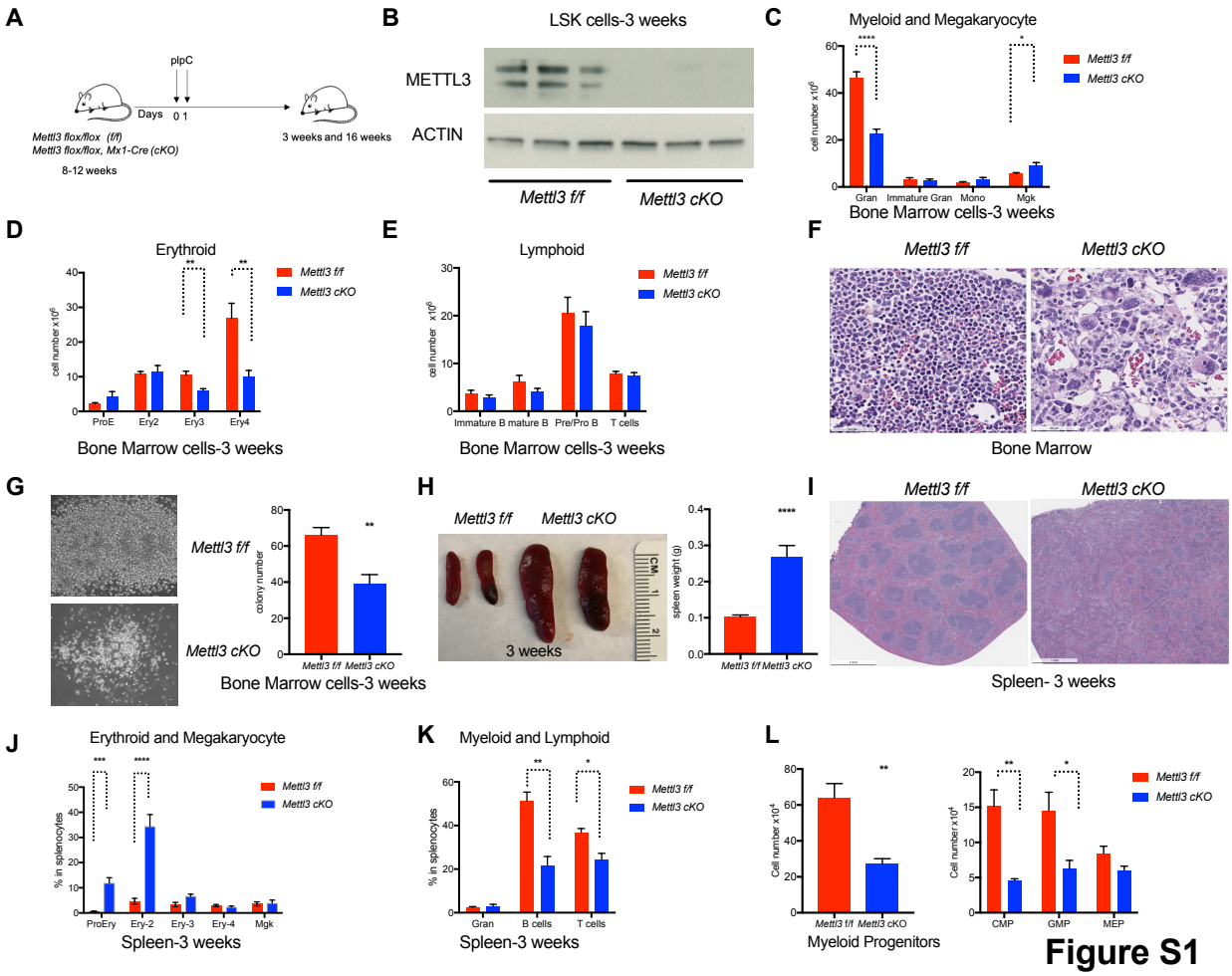
A
pIpC
Days 0 1
Mettl3 flox/flox (f/f)
Mettl3 flox/flox, Mx1-Cre (cKO)
8-12 weeks
3 weeks and 16 weeks
B
LSK cells-3 weeks
METTL3
ACTIN
Mettl3 f/f
Mettl3 cKO
C
Myeloid and Megakaryocyte
cell number x10^6
Gran
Immature Gran
Mono
Mgk
Mettl3 f/f
Mettl3 cKO
Bone Marrow cells-3 weeks
D
Erythroid
cell number x10^6
ProE
Ery2
Ery3
Ery4
Mettl3 f/f
Mettl3 cKO
Bone Marrow cells-3 weeks
E
Lymphoid
cell number x10^6
Immature B
mature B
Pre/Pro B
T cells
Mettl3 f/f
Mettl3 cKO
Bone Marrow cells-3 weeks
F
Mettl3 f/f
Mettl3 cKO
Bone Marrow
G
Mettl3 f/f
Mettl3 cKO
colony number
Mettl3 f/f
Mettl3 cKO
Bone Marrow cells-3 weeks
H
Mettl3 f/f
Mettl3 cKO
3 weeks
spleen weight (g)
Mettl3 f/f
Mettl3 cKO
I
Mettl3 f/f
Mettl3 cKO
Spleen- 3 weeks
J
Erythroid and Megakaryocyte
% in splenocytes
ProEry
Ery-2
Ery-3
Ery-4
Mgk
Mettl3 f/f
Mettl3 cKO
Spleen-3 weeks
K
Myeloid and Lymphoid
% in splenocytes
Gran
B cells
T cells
Mettl3 f/f
Mettl3 cKO
Spleen-3 weeks
L
Cell number x10^4
Mettl3 f/f
Mettl3 cKO
Myeloid Progenitors
Cell number x10^4
CMP
GMP
MEP
Mettl3 f/f
Mettl3 cKO

Figure S1

Supplementary Figure 1. m^{6}A 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^{hi}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).

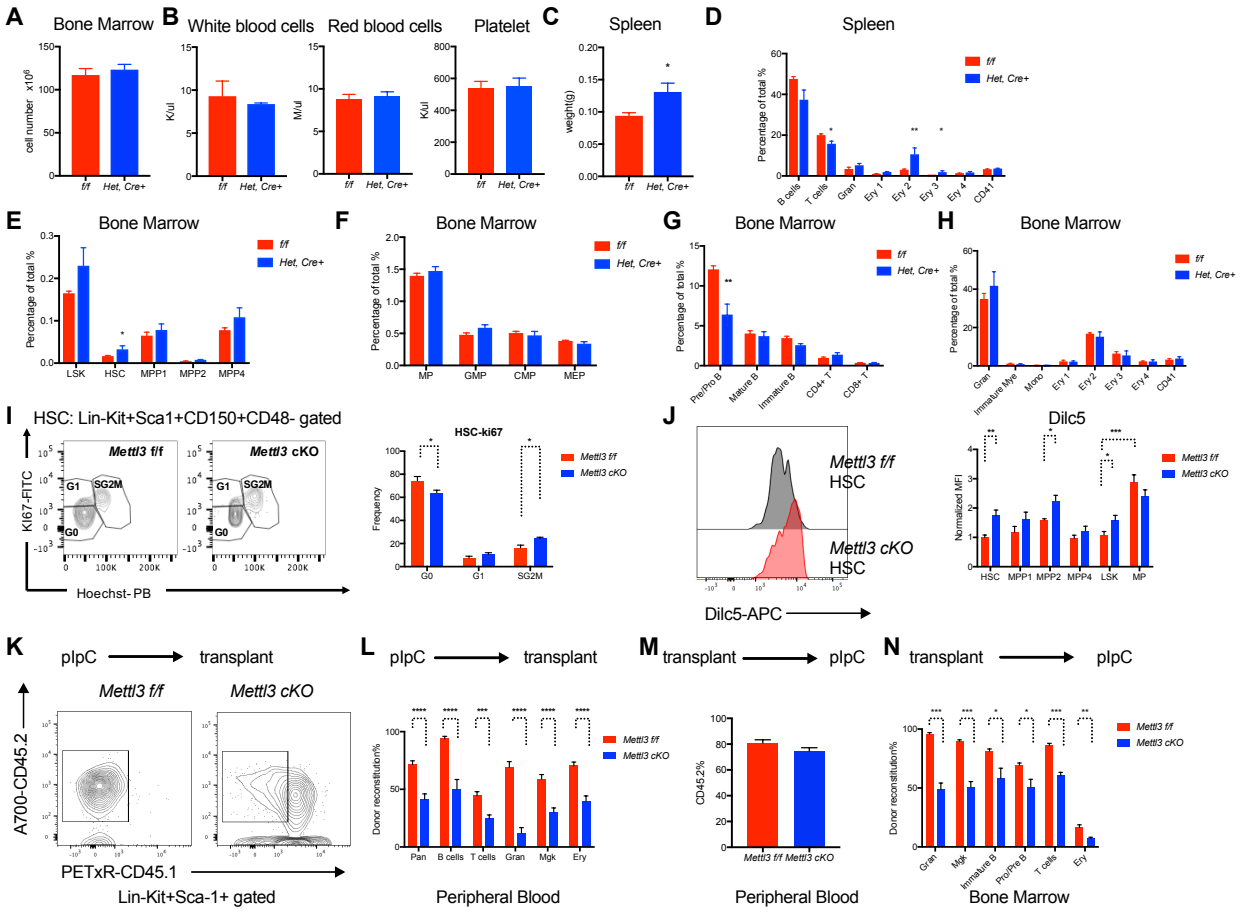
A
Bone Marrow
cell number x10^6
f/f
Het, Cre+
B
White blood cells
K/ul
Red blood cells
M/ul
Platelet
K/ul
C
Spleen
weight(g)
D
Spleen
Percentage of total %
B cells
T cells
Gran
Ery 1
Ery 2
Ery 3
Ery 4
CD41
E
Bone Marrow
LSK
HSC
MPP1
MPP2
MPP4
F
Bone Marrow
MP
GMP
CMP
MEP
G
Bone Marrow
Pre/Pro B
Mature B
Immature B
CD4+ T
CD8+ T
H
Bone Marrow
Gran
Immature Mye
Mono
Ery 1
Ery 2
Ery 3
Ery 4
CD41
I
HSC: Lin-Kit+Sca1+CD150+CD48- gated
Mettl3 f/f
Mettl3 cKO
G1
SG2M
G0
KI67-FITC
Hoechst- PB
HSC-ki67
Frequency
G0
G1
SG2M
J
Mettl3 f/f
HSC
Mettl3 cKO
HSC
Dilc5-APC
Dilc5
Normalized MFI
HSC
MPP1
MPP2
MPP4
LSK
MP
K
pIpC
transplant
Mettl3 f/f
Mettl3 cKO
A700-CD45.2
PETxR-CD45.1
Lin-Kit+Sca-1+ gated
L
pIpC
transplant
Donor reconstitution%
Pan
B cells
T cells
Gran
Mgk
Ery
Peripheral Blood
M
transplant
pIpC
CD45.2%
Mettl3 f/f
Mettl3 cKO
Peripheral Blood
N
transplant
pIpC
Donor reconstitution%
Gran
Mgk
Immature B
Pro/Pre B
T cells
Ery
Bone Marrow

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 base 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 base 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-pIpC. Chimerism of CD45.2 donor cells in peripheral blood from recipient mice was analyzed before pIpC injection. n=15. **(N)** CD45.2 chimerism analysis of myeloid, erythroid and lympoid cells from recipient mice. Cell compartment is determined by cell surface marker as showed previously. n=5. Mean and SEM are shown (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$, ****, $P < 0.0001$).

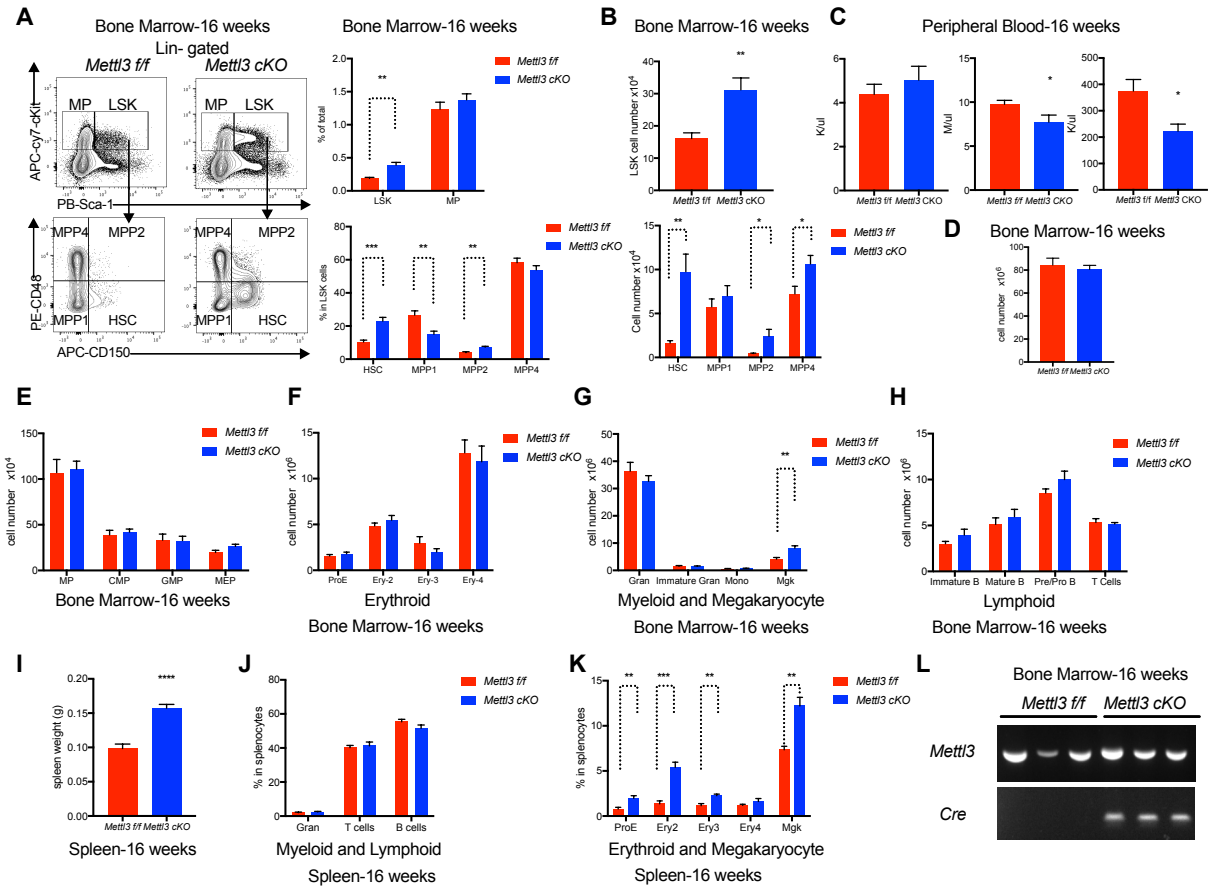
A
Bone Marrow-16 weeks
Lin- gated
Mettl3 f/f
Mettl3 cKO
MP
LSK
APC-cy7-cKit
PB-Sca-1
MPP4
MPP2
MPP1
HSC
PE-CD48
APC-CD150
Bone Marrow-16 weeks
% of total
% in LSK cells
B
Bone Marrow-16 weeks
LSK cell number x10⁴
C
Peripheral Blood-16 weeks
K/ul
M/ul
D
Bone Marrow-16 weeks
cell number x10⁶
E
cell number x10⁴
MP
CMP
GMP
MEP
Bone Marrow-16 weeks
F
cell number x10⁶
ProE
Ery-2
Ery-3
Ery-4
Erythroid
Bone Marrow-16 weeks
G
cell number x10⁶
Gran
Immature Gran
Mono
Mgk
Myeloid and Megakaryocyte
Bone Marrow-16 weeks
H
cell number x10⁶
Immature B
Mature B
Pre/Pro B
T Cells
Lymphoid
Bone Marrow-16 weeks
I
spleen weight (g)
Spleen-16 weeks
J
% in splenocytes
Gran
T cells
B cells
Myeloid and Lymphoid
Spleen-16 weeks
K
% in splenocytes
ProE
Ery2
Ery3
Ery4
Mgk
Erythroid and Megakaryocyte
Spleen-16 weeks
L
Bone Marrow-16 weeks
Mettl3 f/f
Mettl3 cKO
Mettl3
Cre

Figure S3

Supplementary Figure 3. HSPC expansion persists in *Mettl3 cKO* mice 16 weeks post pIpC. Related to Figure 3.

All samples used were from *Mettl3 f/f* and *Mettl3 cKO* mice at 16 weeks post pIpC**. (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 pIpC 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 pIpC. Mean and SEM are shown (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$, ****, $P < 0.0001$).

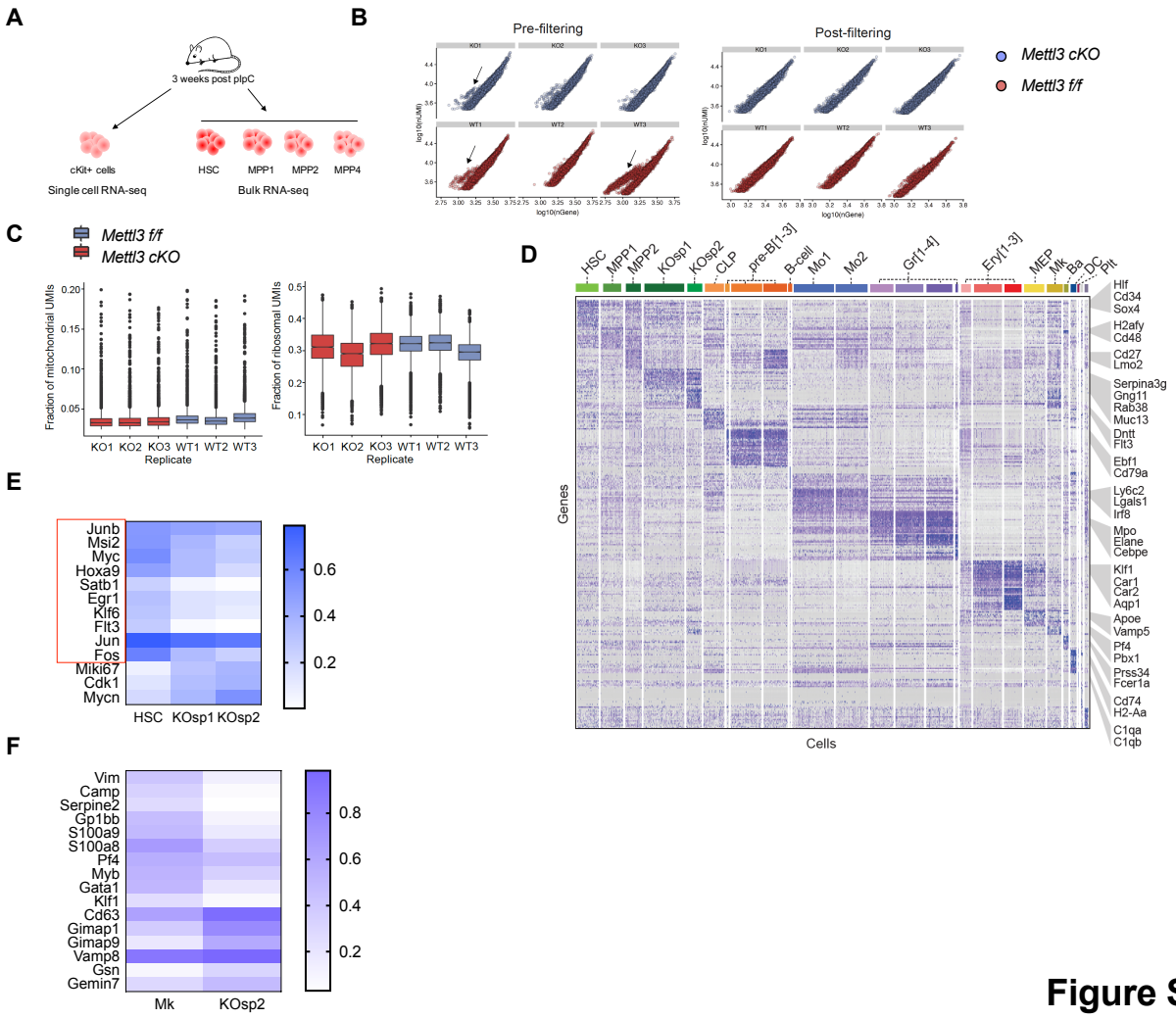
A
3 weeks post plpC
cKit+ cells
Single cell RNA-seq
HSC
MPP1
MPP2
MPP4
Bulk RNA-seq
B
Pre-filtering
Post-filtering
Mettl3 cKO
Mettl3 f/f
C
Mettl3 f/f
Mettl3 cKO
Fraction of mitochondrial UMIs
Fraction of ribosomal UMIs
KO1 KO2 KO3 WT1 WT2 WT3
Replicate
D
HSC
MPP1
MPP2
KOsp1
KOsp2
CLP
pre-B[1-3]
B-cell
Mo1
Mo2
Gr[1-4]
Ery[1-3]
MEP
Mk
Ba
DC
Plt
Genes
Cells
Hlf
Cd34
Sox4
H2afy
Cd48
Cd27
Lmo2
Serpina3g
Gng11
Rab38
Muc13
Dntt
Flt3
Ebf1
Cd79a
Ly6c2
Lgals1
Irf8
Mpo
Elane
Cebpe
Klf1
Car1
Car2
Aqp1
Apoe
Vamp5
Pf4
Pbx1
Prss34
Fcer1a
Cd74
H2-Aa
C1qa
C1qb
E
Junb
Msi2
Myc
Hoxa9
Satb1
Egr1
Klf6
Flt3
Jun
Fos
Miki67
Cdk1
Mycn
HSC KOsp1 KOsp2
F
Vim
Camp
Serpine2
Gp1bb
S100a9
S100a8
Pf4
Myb
Gata1
Klf1
Cd63
Gimap1
Gimap9
Vamp8
Gsn
Gemin7
Mk
KOsp2

Figure S4

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.

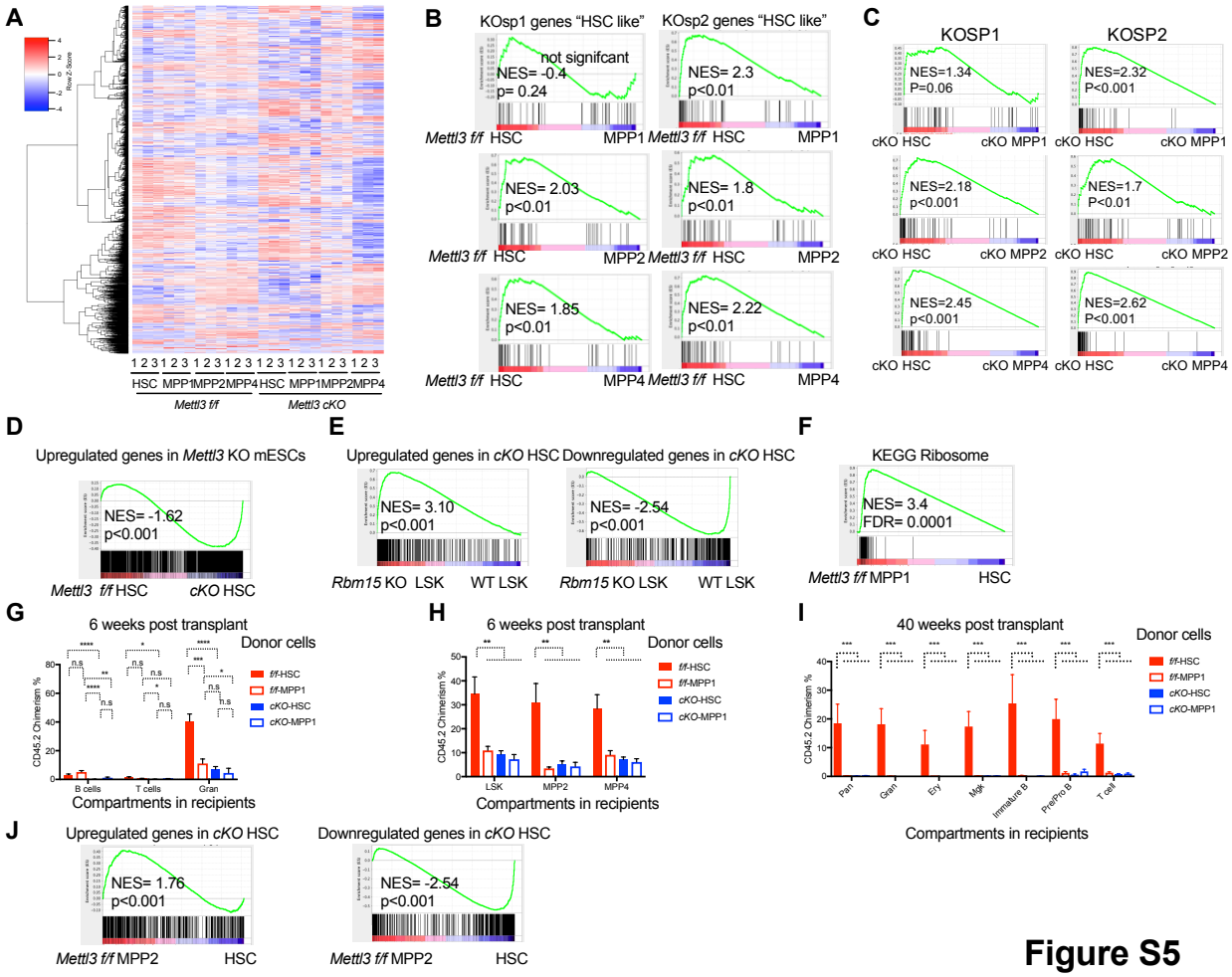
A
Row Z-Score
1 2 3 1 2 3 1 2 3 1 2 3 1 2 3 1 2 3 1 2 3 1 2 3 1 2 3
HSC MPP1 MPP2 MPP4 HSC MPP1 MPP2 MPP4
Mettl3 f/f
Mettl3 cKO
B
KOsp1 genes "HSC like"
KOsp2 genes "HSC like"
not signifcant
NES= -0.4
p= 0.24
NES= 2.3
p<0.01
Mettl3 f/f HSC
MPP1
NES= 2.03
p<0.01
NES= 1.8
p<0.01
MPP2
NES= 1.85
p<0.01
NES= 2.22
p<0.01
MPP4
C
KOSP1
KOSP2
NES=1.34
P=0.06
NES=2.32
P<0.001
cKO HSC
cKO MPP1
NES=2.18
p<0.001
NES=1.7
P<0.01
cKO MPP2
NES=2.45
p<0.001
NES=2.62
P<0.001
cKO MPP4
D
Upregulated genes in Mettl3 KO mESCs
NES= -1.62
p<0.001
Mettl3 f/f HSC
cKO HSC
E
Upregulated genes in cKO HSC
Downregulated genes in cKO HSC
NES= 3.10
p<0.001
NES= -2.54
p<0.001
Rbm15 KO LSK
WT LSK
F
KEGG Ribosome
NES= 3.4
FDR= 0.0001
Mettl3 f/f MPP1
HSC
G
6 weeks post transplant
Donor cells
f/f-HSC
f/f-MPP1
cKO-HSC
cKO-MPP1
CD45.2 Chimerism %
B cells
T cells
Gran
Compartments in recipients
H
6 weeks post transplant
Donor cells
LSK
MPP2
MPP4
Compartments in recipients
I
40 weeks post transplant
Donor cells
Pan
Gran
Ery
Mgk
Immature B
PreProB
T cell
Compartments in recipients
J
Upregulated genes in cKO HSC
NES= 1.76
p<0.001
Mettl3 f/f MPP2
HSC
Downregulated genes in cKO HSC
NES= -2.54
p<0.001

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 (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$, ****, $P < 0.0001$).

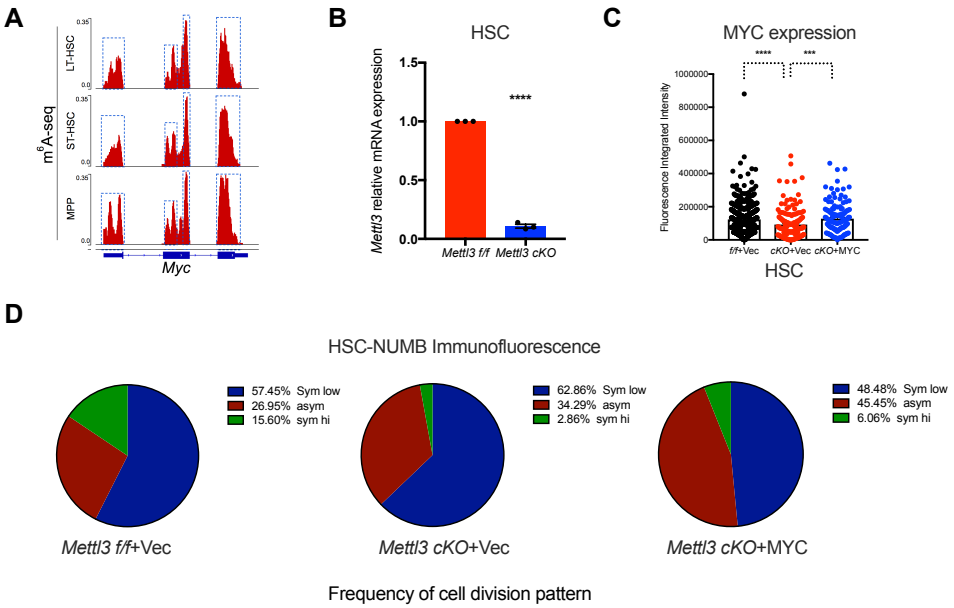
A
LT-HSC
ST-HSC
MPP
m6A-seq
Myc
B
HSC
Mettl3 relative mRNA expression
****
Mettl3 f/f
Mettl3 cKO
C
MYC expression
****
***
Fluorescence Integrated Intensity
f/f+Vec
cKO+Vec
cKO+MYC
HSC
D
HSC-NUMB Immunofluorescence
57.45% Sym low
26.95% asym
15.60% sym hi
Mettl3 f/f+Vec
62.86% Sym low
34.29% asym
2.86% sym hi
Mettl3 cKO+Vec
48.48% Sym low
45.45% asym
6.06% sym hi
Mettl3 cKO+MYC
Frequency of cell division pattern

Figure S6

Supplementary Figure 6. m^{6}A-mediated *Myc* regulation is essential for HSC asymmetric and symmetric cell division. **Related to Figure 6.**

(A) m^{6}A marks on *Myc* transcripts in HSCs and MPPs. m^{6}A 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.

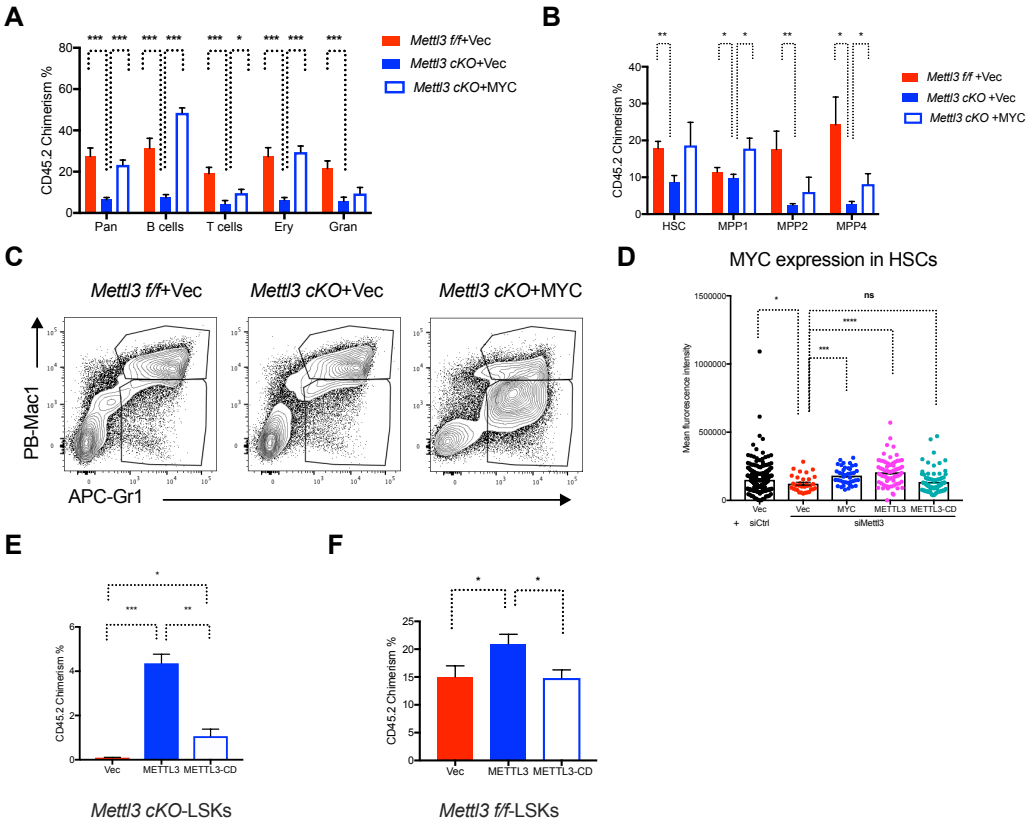
A
CD45.2 Chimerism %
Pan
B cells
T cells
Ery
Gran
Mettl3 f/f+Vec
Mettl3 cKO+Vec
Mettl3 cKO+MYC
B
CD45.2 Chimerism %
HSC
MPP1
MPP2
MPP4
Mettl3 f/f +Vec
Mettl3 cKO +Vec
Mettl3 cKO +MYC
C
Mettl3 f/f+Vec
Mettl3 cKO+Vec
Mettl3 cKO+MYC
PB-Mac1
APC-Gr1
D
MYC expression in HSCs
Mean fluorescence intensity
Vec
MYC
METTL3
METTL3-CD
siCtrl
siMettl3
E
CD45.2 Chimerism %
Vec
METTL3
METTL3-CD
Mettl3 cKO-LSKs
F
CD45.2 Chimerism %
Vec
METTL3
METTL3-CD
Mettl3 f/f-LSKs

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 (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$, ****, $P < 0.0001$).