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**Abstract:**
**KDM4/JMJD2 are H3K9- and H3K36- specific demethylases, which are considered promising therapeutic targets for the treatment of acute myeloid leukemia (AML) harboring MLL-translocations. Here, we investigate the long-term effects of depleting KDM4 activity on normal hematopoiesis to probe potential side effects of continuous inhibition of these enzymes. Utilizing conditional *Kdm4a/Kdm4b|Kdm4c* triple-knockout mice we show that KDM4 activity is required for hematopoietic stem cell (HSC) maintenance *in vivo*. The knockout of the KDM4 demethylases leads to accumulation of H3K9me3 on transcription start sites and the corresponding downregulation of expression of several genes in hematopoietic stem cells. We show that two of these genes, *Taf1b* and *Nom1*, are essential for the maintenance of hematopoietic cells. Taken together, our results show that the KDM4 demethylases are required for the expression of genes essential for the long-term maintenance of normal hematopoiesis.**

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**COI notes:**

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**Clinical trial registration information (if any):**

**Brief Report**



# The KDM4/JMJD2 histone demethylases are required for hematopoietic stem cell maintenance

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**Key Points**

- Combined knockout of *Kdm4a*, *Kdm4b* and *Kdm4c* results in hematopoietic stem cell defects
- KDM4 demethylases are required for sustained expression of genes important for survival of hematopoietic stem cells

## Abstract

**KDM4/JMJD2 are H3K9- and H3K36- specific demethylases, which are considered promising therapeutic targets for the treatment of acute myeloid leukemia (AML) harboring MLL-translocations. Here, we investigate the long-term effects of depleting KDM4 activity on normal hematopoiesis to probe potential side effects of continuous inhibition of these enzymes. Utilizing conditional *Kdm4a/Kdm4b/Kdm4c* triple-knockout mice we show that KDM4 activity is required for hematopoietic stem cell (HSC) maintenance *in vivo*. The knockout of the KDM4 demethylases leads to accumulation of H3K9me3 on transcription start sites and the corresponding downregulation of expression of several genes in hematopoietic stem cells. We show that two of these genes, *Taf1b* and *Nom1*, are essential for the maintenance of hematopoietic cells. Taken together, our results show that the KDM4 demethylases are required for the expression of genes essential for the long-term maintenance of normal hematopoiesis.**

## Introduction

Chromatin-modifying enzymes are important during development of leukemia where they sustain the erroneous expression pattern of oncogenes and tumor suppressor genes. Importantly, their catalytic activities are often required for growth of leukemic cells making them potential therapeutic targets[1,2]. The H3K9me3/me2 and H3K36me3/me2 specific histone lysine demethylase family KDM4/JMJD2 have four members: KDM4A-D[3-6]. While KDM4A-C are expressed in many tissues, expression of KDM4D is confined to testis[7]. Mice single knockout for *Kdm4a-c are* viable, whereas the combined deletion of *Kdm4a* and *Kdm4c* or *Kdm4a*, *Kdm4b* and *Kdm4c* is embryonic lethal[8,9]. KDM4 enzymes localize to H3K4me3 positive promoters preventing accumulation of H3K9me3 and H3K36me3[9]. KDM4 activity is required for the growth of MLL-AF9 translocated acute myeloid leukemia (AML) cells, and considered promising therapeutic targets[10,11]. Here we addressed the role of the KDM4 enzymes in normal hematopoiesis.

## Methods

### Animal studies

Mouse lines and tamoxifen injection procedures have been described[9,11]. BM and PB cells were isolated and stained as described[12]. All animal studies were approved by the Danish Animal Ethical Committee.

### RNA-seq

RNA from 10.000 cells was converted to cDNA using Nugen Ovation RNA-seq System V2 and sequenced on a Nextseq500 (Illumina). Reads where mapped using RNA STAR[13] (Galaxy Version 2.4.0d-2) and counted using htseq-count[14] (Galaxy Version 0.6.1galaxy1). Differentially expressed genes were identified using DESeq2[15] (Galaxy Version 2.1.8.3).

### ChIP-seq

30.000 LSK cells were fixed, sonicated and subjected to immunoprecipitation as described[12]. Mapping and peak calling were done using Bowtie2 (Galaxy Version 2.2.6.2) and EaSeq[16]. RNA-seq and ChIP-seq data are desposited under GSE129137, GSE129156 and GSE129157.

## Results and Discussion

To investigate the role of the KDM4 enzymes in normal hematopoiesis, we performed a series of competitive bone marrow transplantations (BMTs). We used bone marrow (BM) from mice expressing tamoxifen inducible Cre from the Rosa26 locus *(CreER)* in combination with conditional alleles of *Kdm4* (*CreER:Kdm4c*$^{fl/f}$*, CreER:Kdm4ac*$^{f/fl}$ and *CreER:Kdm4abc*$^{fl/fl}$*)* (Figure 1A). Four weeks after transplantation, we measured the percentage of CD45.2$^{+}$ donor derived cells in peripheral blood (PB) and induced knockout through intraperitoneal injection of tamoxifen (Supplementary Figure 1A and 1B). We followed the percentage of CD45.2$^{+}$ cells in myeloid, B- and T-cell populations in PB 1, 3 and 5 months after tamoxifen injection (Figure 1B). Deletion of *Kdm4c* alone did not affect the production of either Myeloid, T- or B-cells (Figure 1B), whereas *Kdm4ac* or *Kdm4abc* deletion resulted in a significant reduction of all three lineages six months after transplantation (Figure 1B). Single knockout or the combined deletion of *Kdm4b* and *Kdm4c* did

not have any gross effect on hematopoiesis (Supplementary Figure 2A-F). These data indicate that KDM4A, KDM4C and to a lesser extend KDM4B play functionally redundant roles in hematopoiesis.

Since *Kdm4abc* triple knockout mice have reduced number of myeloid, B- and T- cells, we hypothesized that the loss of KDM4 activity resulted in defects in a common progenitor. We quantified CD45.2$^+$ cells in CD34$^-$ Lin$^-$ Sca-1$^+$ c-Kit$^+$ (CD34$^-$LSK) HSC, multipotent progenitor (CD34$^+$LSK), and granulocyte/macrophage progenitor (GMP) compartments of the bone marrow six months after transplantation. We found that *Kdm4abc* deletion resulted in a significant reduction in cell numbers for all three cell types (Figure 1C). To understand why the cells were lost, mice treated with tamoxifen for 10 days were subsequently injected with BrdU, and cells were harvested 72 hours after. As shown in Figure 1D, LSK cells showed an increase in apoptosis/S-phase and a reduction of cells in G0/G1, supporting the notion that KDM4A-C exert important functions in HSCs and early progenitors. To investigate this possibility, we generated *in vitro* cultures of CD34$^-$LSK cells FACS sorted from *CreER:Kdm4c*$^{fl/fl}$ and *CreER:Kdm4abc*$^{fl/fl}$ two weeks after tamoxifen injections. These experiments showed that KDM4A-C are required for the proliferation of CD34$^-$LSK cells (Figure 1E). An effect confirmed in methocult replating experiments (Figure 1F). Taken together, we conclude that the KDM4 histone demethylases play functionally redundant, but essential, roles in maintaining HSCs and multipotent progenitors and for the long-term maintenance of B-, T- and Myeloid cells in peripheral blood.

We speculated that KDM4 enzymes are required for proper expression of genes essential for HSCs. To identify such genes, we performed ChIP-seq. We were unable to perform KDM4A and KDM4C ChIPs in LSK cells, however previous results have shown that the KDM4 proteins associate with H3K4me3 positive transcription start sites (TSS)[8,9,11]. We expected that direct target genes of KDM4 would accumulate H3K9me3 at TSSs after KDM4 depletion. Thus, we performed H3K9me3 ChIP-seq on chromatin prepared from LSK cells of *CreER:Kdm4abc*$^{fl/fl}$ and *CreER* mice two weeks after injection of tamoxifen. We quantified the H3K9me3 reads in knockout and control cells in a region of +/- 1000bp around the 9072 H3K4me3 positive TSSs present in LSK cells[12] (Figure 2A-D). We filtered the data to identify TSS regions where H3K9me3 levels changed $\geq$ 2-fold in response to tamoxifen treatment. This led to the identification of 1381 TSSs (Figure 2A and 2D).

To identify genes where an increase in H3K9me3 resulted in a transcriptional change we FACS sorted long-term (LT) HSCs (Lin-Sca-1+, c-Kit+, CD48-, CD150+) from *CreER:Kdm4abc*$^{fl/fl}$ and

*CreER* mice 2 weeks after tamoxifen injection and performed RNA-seq. This led to the identification of 164 downregulated genes and 157 upregulated genes in knockout LT-HSCs (Figure 2E). Comparing ChIP-seq and RNA-seq analysis we identified six genes that are both repressed and have increased H3K9me3 levels (Figure 2E and 2F).

To identify genes that could explain the phenotype of the KDM4 knockout mice, we analyzed the six genes using the DepMap dataset[17,18]. Here we found that *Nom1* and *Taf1b* are classified as common essential genes. Both genes are expressed at similar levels in both HSCs and AML cells[19] and the H3K9me3 levels on the TSS of none of them changed significantly in KDM4A or KDM4C single knockouts (Supplementary Figure 1B and 1C). We speculated that the decreased expression of *Nom1* and *Taf1b* in HSCs could explain the requirement of the KDM4 histone demethylases for the maintenance of HSCs. To test this more directly, we depleted *Nom1* or *Taf1b* using CRISPR/Cas9 in LSK cells. We cloned and validated lentiviral constructs expressing GFP tagged gRNAs against *Nom1* and *Taf1b* in conjunction with Cas9 (Supplementary Figure 1E and 1F). WT LSK cells were transduced with these constructs and the percentage of GFP positive cells were followed over a period of 12 days of *in vitro* growth. We conclude that CRISPR/Cas9 mediated depletion of NOM1 and TAF1B attenuates the growth of LSK cells (Figure 2G and supplemental Figure 1G). This supports the hypothesis that loss of KDM4 activity results in transcriptional repression of *Nom1* and *Taf1b,* which in turn causes a growth defect in LSK cells.

Previously, we and others have demonstrated that deletion of *Kdm4a-c* had a strong effect on proliferation of MLL-AF9 translocated AML cells whereas the effect on untransformed progenitors was less severe[10,11]. Specifically, we found that KDM4A-C activity is required for the expression of *Il3ra,* a gene essential for the survival of AML cells, but dispensable for normal hematopoiesis[20]. Using non-competitive BMT we also showed that the hematopoietic output of KDM4A-C knockout stem cells was sufficient to confer survival of recipient mice for up to three months[11]. This differential requirement for KDM4 for the proliferation of AML and maintenance of HSCs provides a potential therapeutic window of opportunity. However, the results from this study show that KDM4 is required for normal long-term hematopoiesis, which is critical to take into consideration when KDM4 inhibitors move into a clinical setting.

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**Authorship**

Contribution: K.A., K.N., S.M., J.-E.M., K.D.R., and K.H. designed the experiments and analysed the data; K.A., K.N., S.M., J.-E.M., and K.D.R. performed the experiments; and K.A. and K.H. wrote the manuscript. K.D.R., K.N., S.M. and J.-E.M. provided comments on the manuscript.

**Disclosure of conflicts of interest**

The authors declare no competing financial interests

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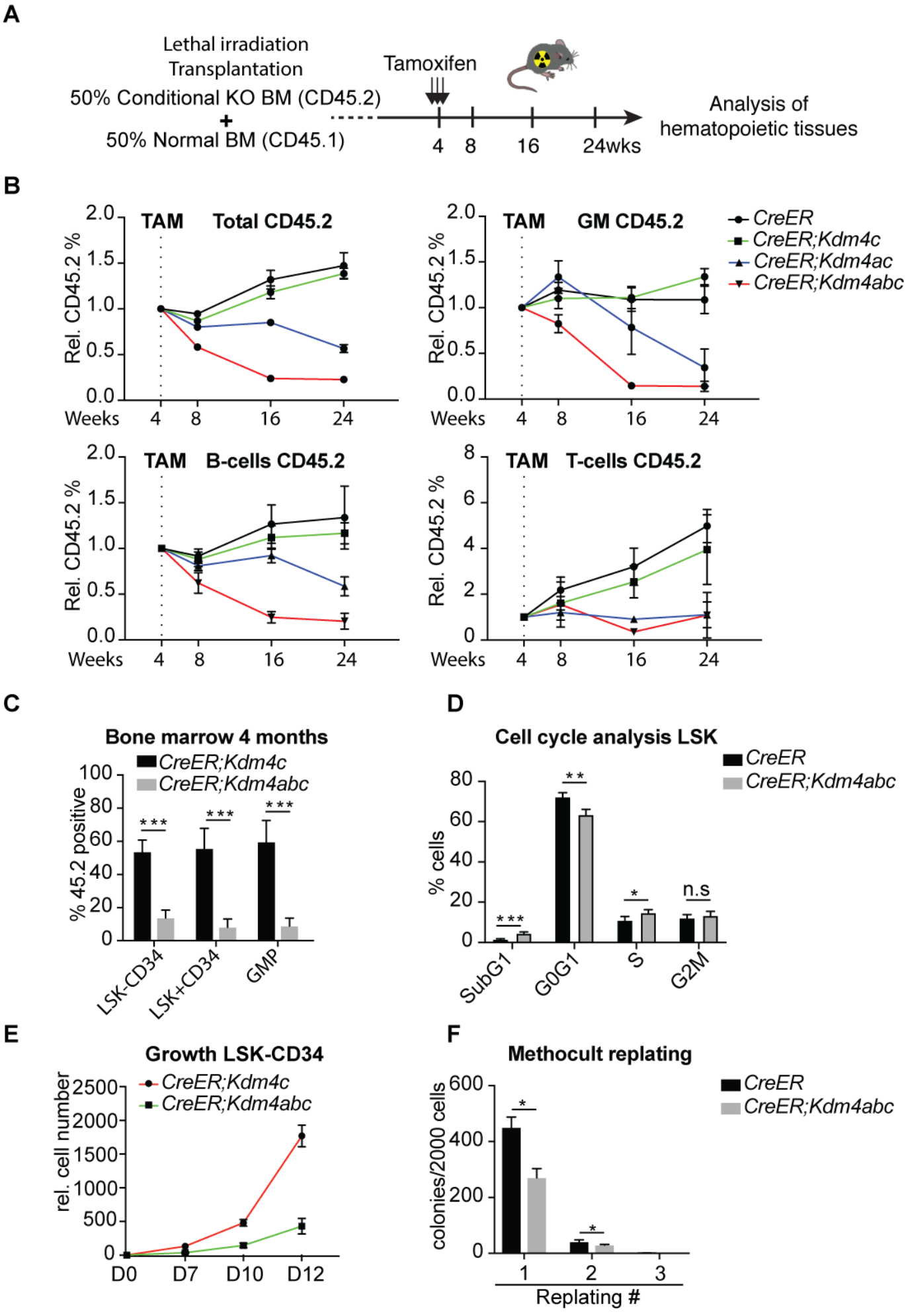
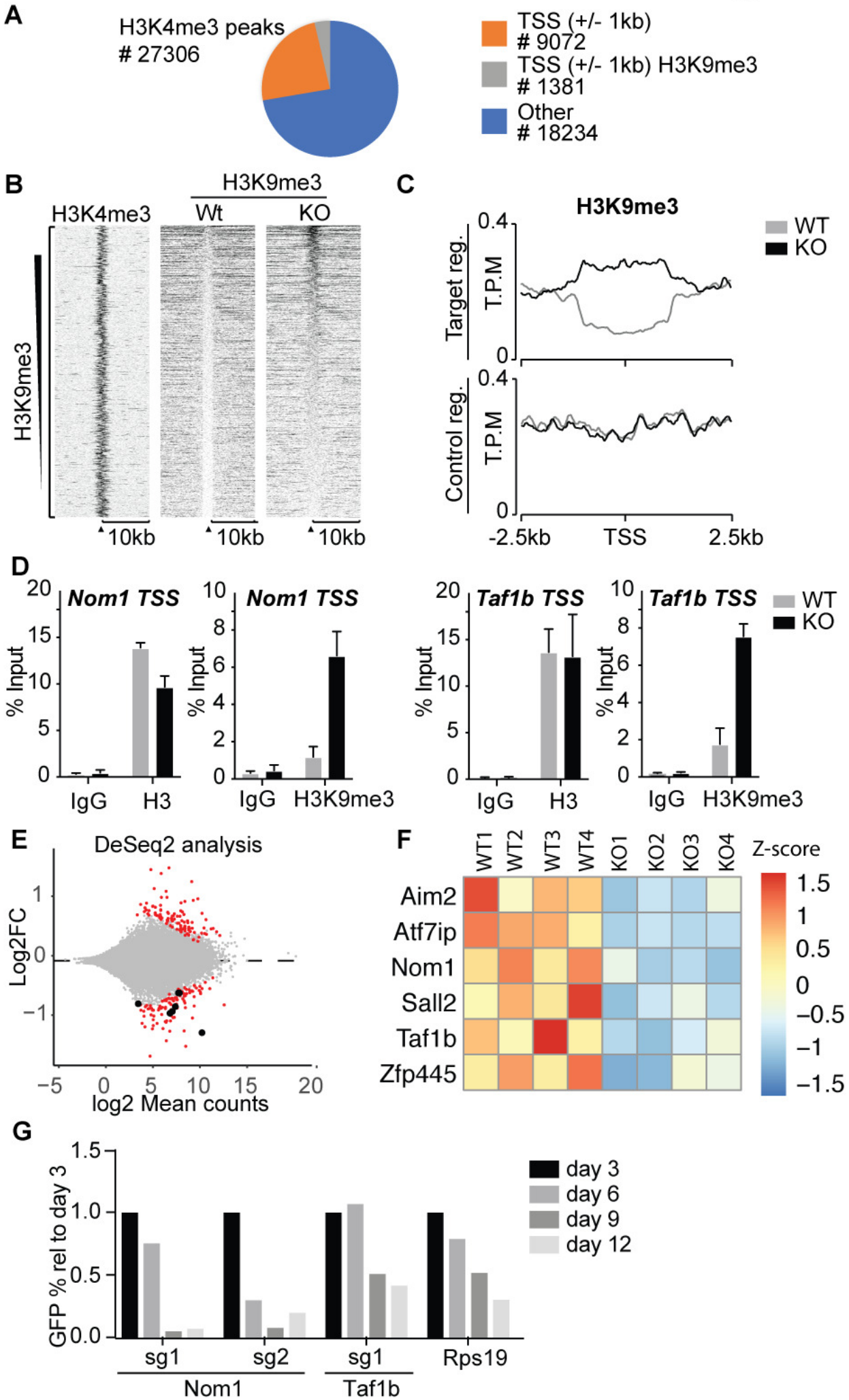
## Figure legends

**Figure 1: The combined knockout of *Kdm4a*, *Kdm4b* and *Kdm4c* leads to reduction of myeloid and lymphoid cells.** (A) Schematic drawing of the experimental setup. Lethally irradiated mice were transplanted with bone marrow from mice with the indicated genotypes (CD45.2) mixed 1:1 with bone marrow from B6-SJL mice. (B) CD45.2 chimerism in peripheral blood at the indicated times after injection of tamoxifen. GM (granulocyte/macrophage population). Data represented as mean +/-SD (n=6 in each group). (C) Histogram depicting the CD45.2 percentage in the indicated cell populations within the bone marrow four months after tamoxifen injection. Data represented as mean +/-SD (n=6 in each group). HSC ($Lin^-Sca^-c\text{-}Kit^+CD34^-$), LSK ($Lin^-Sca^-c\text{-}Kit^+$) and GMP Granulocyte-monocyte progenitor population. (D) Cell cycle profile of LSK cells sorted from the bone marrow of mice that were treated 10 days with tamoxifen and additional 72 hours with BrdU. The percentage of BrdU positive cells in the different populations is indicated. Data is represented as mean +/- SD (n=4 in control group and n=3 in the *Kdm4abc* knockout group). (E) *In vitro* growth curve of HSCs sorted from bone marrow of mice with indicated genotypes two weeks after injection of tamoxifen. Data is represented as mean +/-SD (n=4 in each group). (F) Methocult replating assay using LSK cells sorted from the bone marrow of mice with the indicated genotypes 2 weeks after injection of tamoxifen. 1000 cells per plate were plated in the first round and 5000 in the subsequent rounds of replating. Data is represented as mean +/-SD (n=3 in each group)

**Figure 2: Loss of KDM4A-C leads to accumulation of H3K9me3 at TSS on a subset of genes in LSK cells.** (A) Pie chart indicating the position of H3K4me3 peaks in LSK ($Lin^-Sca^-c\text{-}Kit^+$) cells from C57BL6 mice[12]. Orange and gray represent peaks localized +/- 1kb of TSS, peaks falling outside these regions are represented with blue. H3K4me3 positive regions +/-1kb of TSS that experience a significant change in H3K9me3 levels are indicated with gray. (B) Heat map of H3K9me3 ChIP-seq read counts +- 10kb of TSS. Data has been filtered to only include regions where a significant change in H3K9me3 occurs. (C) Summary of the data in (A) zoomed in to +- 2.5kb of TSS. (D) Direct ChIP-qPCR validation on selected target genes using cells sorted in an independent experiment. (E) RNA-seq analysis of HSCs sorted from *CreER:Kdm4abc*$^{fl/fl}$ *or CreER* mice two weeks after tamoxifen injection (n=4 in each group). (F) Heat map showing the distribution of normalized counts for the

listed genes in the RNA-seq dataset. (G) LSK cells were sorted from C57BL6 mice and transduced with lentiviruses expressing GFP, Cas9 and sgRNA against *Taf1b* and *Nom1* as well as positive (*Rps19*) and negative controls (non-targeting sgRNA). The percentage of GFP positive cells was followed over time by FACS. The percentages have been normalized to the negative control and plotted relative to the value at day 3.





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