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LETTER TO THE EDITOR

Mettl3–Mettl14 methyltransferase complex regulates the quiescence of adult hematopoietic stem cells

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Dear Editor,

N^6-methyladenosine (m^6A) is a common modification of mRNA that is catalyzed by the Mettl3–Mettl14 methyltransferase complex[1], with WTAP as its regulatory subunit[2]. The physiological importance of m^6A has been evidenced by its pivotal roles in tissue development and differentiation[3–9]. Hematopoietic stem cells (HSCs) are self-renewable multipotent progenitor cells that sustain all blood cell lineages throughout life. It has been well recognized that epigenetic modifications, such as histone or DNA modifications, are implicated in HSC self-renewal[10]. Recent studies have shown that the mRNA modification m^6A also functions in the hematopoietic system. In human, Mettl3–Mettl14-mediated m^6A was found to promote the development of acute myeloid leukemia and maintain leukemia-initiating cells[5,6,11]. In zebrafish and mouse, knockdown of *Mettl3* blocked the endothelial-to-hematopoietic transition during embryonic development, thereby repressing the generation of the earliest HSCs[7]. Surprisingly, deletion of *Mettl3* in *Vav1-cre; Mettl3*$^{fl/fl}$ mouse embryos did not have a significant effect on the number or function of E10.5 fetal hematopoietic stem and progenitor cells (HSPCs)[4], suggesting that cell-autonomous m^6A is dispensable for HSC self-renewal during early development. Conditional deletion of *Mettl14* in adult mice caused a mild but significant reduction of the hematopoietic repopulation ability in recipient mice[6]. The repopulation defect observed in *Mettl14*-deficient bone marrow cells could be interpreted as a myeloid differentiation defect or a defect in HSC self-renewal. However, until now, there is a lack of evidence that m^6A plays a role in HSC self-renewal in the bone marrow. In this study, we took genetic approaches to investigate the physiological roles of Mettl3 and Mettl14 in the regulation of HSC self-renewal in adult mouse bone marrow.

By quantitative real-time PCR, we detected the expression of both *Mettl3* and *Mettl14* in many hematopoietic cell populations, including HSCs (Fig. 1a, b and Supplementary information, Figure S1a-c, Table S1). To address the potential roles of m^6A in HSC self-renewal in adult bone marrow, we crossed *Mx1-cre* mice with *Mettl3*$^{fl/+}$ and *Mettl14*$^{fl/+}$ mice[3], respectively, to generate *Mx1-cre; Mettl3*$^{fl/fl}$ and *Mx1-cre; Mettl14*$^{fl/fl}$ mice. By administering polyinosine–polycytosine (plpC) to these mice at 6 weeks of age every other day for 10 days (Fig. 1c), we were able to achieve 100% deletion of *Mettl3* or *Mettl14* from $CD48^-CD150^+Sca\text{-}1^+c\text{-}kit^+Lineage^-$ HSCs at 6 weeks after the treatment (Supplementary information, Figure S2a, b). Western blot demonstrated a profound reduction of Mettl3 or Mettl14 protein level in $Lineage^-$ hematopoietic cells from these conditional mutants (Supplementary information, Figure S2c, d). Consistent with this, the m^6A levels of $Lineage^-$ hematopoietic cells were significantly reduced in *Mx1-cre; Mettl3*$^{fl/fl}$ and *Mx1-cre; Mettl14*$^{fl/fl}$ mice (Fig. 1d, e). Notably, the m^6A level in *Mx1-cre; Mettl3*$^{fl/fl}$ mice was significantly lower than that in *Mettl14*$^{fl/fl}$ mice (Fig. 1e). Thus we confirmed that the Mettl3–Mettl14 complex catalyzes m^6A formation in HSPCs.

Next, we utilized the conditional mutant mice to investigate the specific roles of Mettl3 and/or Mettl14 in regulating hematopoiesis and HSC self-renewal in adult bone marrow (see Supplementary data S1 for detail methods). At 6 weeks after plpC treatment, the bone marrow cellularity of either *Mx1-cre; Mettl3*$^{fl/fl}$ or *Mx1-cre; Mettl14*$^{fl/fl}$ mice was indistinguishable from their littermate controls (Fig. 1f). When analyzing the bone marrow cells by flow cytometry, we observed an approximately ten-fold increase of the frequency of $CD48^-CD150^+Sca\text{-}1^+c\text{-}kit^+Lineage^-$ HSCs in the bone marrow from *Mx1-cre; Mettl3*$^{fl/fl}$ mice relative to controls (Fig. 1g, h). In contrast, *Mx1-cre; Mettl14*$^{fl/fl}$ mice did not show significant changes of the frequency of bone marrow HSCs as compared to control mice (Fig. 1g, h). Thus conditional deletion of *Mettl3*, but not *Mettl14*, expands the phenotypic HSCs in adult bone marrow.

To test whether deletion of *Mettl3* and *Mettl14* has synergistic effect on hematopoiesis and/or HSC frequency in the bone marrow, we analyzed the *Mx1-cre; Mettl3*$^{fl/fl}$*; Mettl14*$^{fl/fl}$ compound mutant mice at 6 weeks after plpC treatment. Western blot demonstrated a profound reduction of both Mettl3 and Mettl14 protein levels in $Lineage^-$ hematopoietic cells from these compound mutants (Supplementary information, Figure S2e). The m^6A level of $Lineage^-$ hematopoietic cells from *Mx1-cre; Mettl3*$^{fl/fl}$*; Mettl14*$^{fl/fl}$ mice was significantly lower than that from control or *Mx1-cre; Mettl14*$^{fl/fl}$ mice but was similar as that from *Mx1-cre; Mettl3*$^{fl/fl}$ mice (Fig. 1d, e). The bone marrow cellularity of *Mx1-cre; Mettl3*$^{fl/fl}$*; Mettl14*$^{fl/fl}$ mice did not differ from that of control or other single mutant mice (Fig. 1f). The HSC frequency in the bone marrow from *Mx1-cre; Mettl3*$^{fl/fl}$*; Mettl14*$^{fl/fl}$ was significantly higher than that from control or *Mx1-cre; Mettl14*$^{fl/fl}$ mice but was comparable with that from *Mx1-cre; Mettl3*$^{fl/fl}$ mice. (Fig. 1g, h). These data suggest that the functions of Mettl14 in m^6A formation and HSC regulation are dependent on Mettl3.

Next, we performed long-term competitive reconstitution assay to assess the effect of *Mettl3* or *Mettl14* deletion on HSC self-renewal in recipient mice. 300,000 bone marrow cells from control or *Mx1-cre; Mettl3*$^{fl/fl}$ mice, together with 300,000 recipient-typed bone marrow cells, were transplanted into lethally irradiated mice. At 4 months after bone marrow transplantation, we detected a significant reduction of donor-derived myeloid, B and T cells in the peripheral blood of recipient mice that were transplanted with bone marrow cells from *Mx1-cre; Mettl3*$^{fl/fl}$ mice, demonstrating that HSCs from *Mx1-cre; Mettl3*$^{fl/fl}$ mice had significantly lower long-term reconstituting activity in all hematopoietic lineages than controls (Fig. 1i, j). Notably, bone marrow cells from *Mx1-cre; Mettl14*$^{fl/fl}$ mice also conferred lower long-term reconstitution activity in myeloid lineages than controls, but their B and T cell lineage-reconstituting activity was indistinguishable from control

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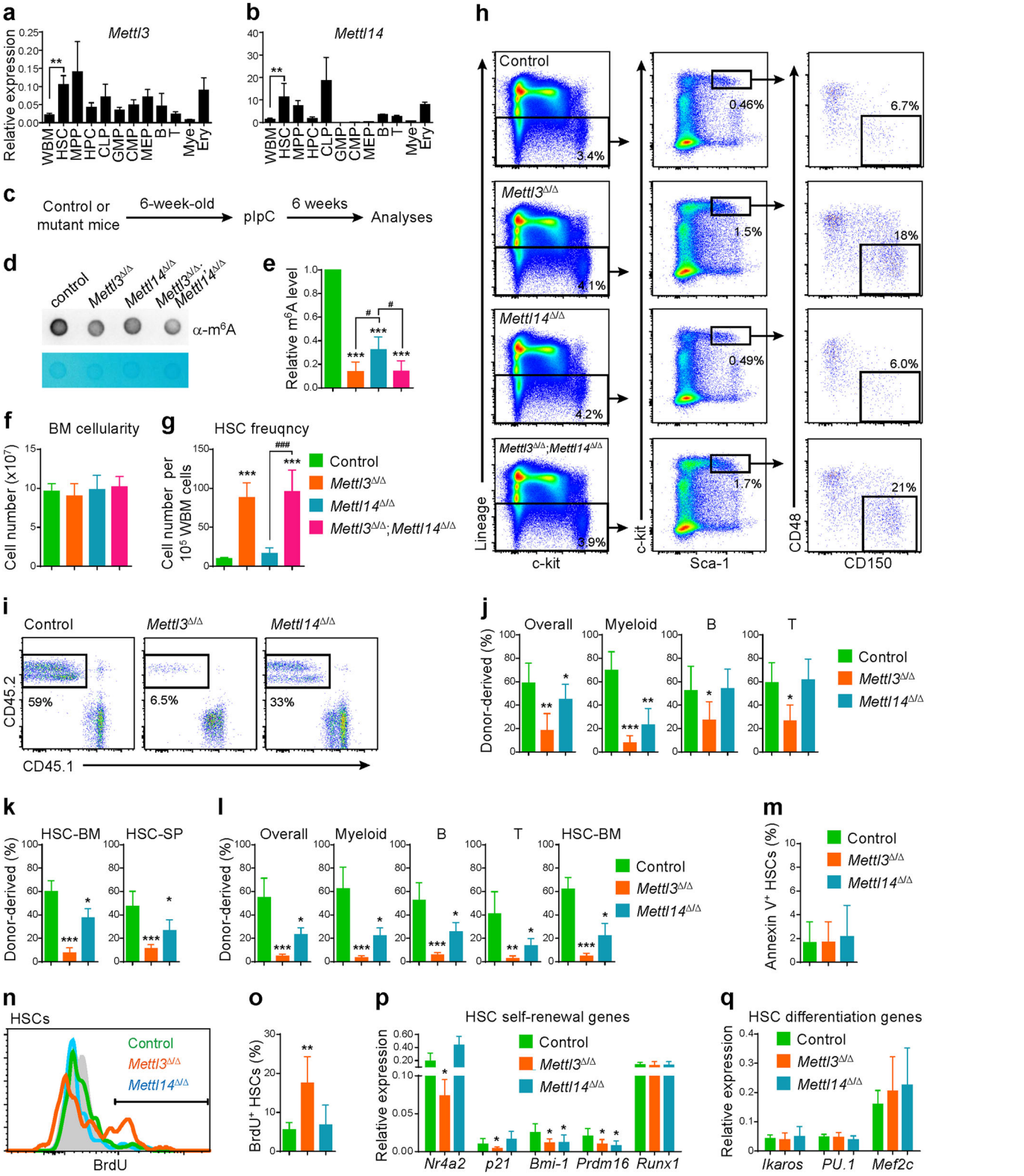

bone marrow cells (Fig. 1i, j). Consistent with this, bone marrows from *Mx1-cre; Mettl3*$^{fl/fl}$ and *Mx1-cre; Mettl14*$^{fl/fl}$ donor mice resulted in significantly lower HSC chimerisms in the bone marrow and spleen in recipients than controls (Fig. 1k). We then transplanted bone marrow cells from primary into secondary recipient mice. At 4 months after the transplantation, myeloid, B, T cells, and HSCs from *Mx1-cre; Mettl3*$^{fl/fl}$ or *Mx1-cre; Mettl14*$^{fl/fl}$ mice were mostly depleted from the recipients (Fig. 1l). Thus Mettl3 and Mettl14 are both required for the self-renewal and hematopoietic reconstitution of HSCs upon transplantation into recipient mice.

The increased cell number but reduced self-renewing capacity of *Mettl3*-null HSCs raised the possibility that Mettl3 maintains HSCs in a quiescent state. Cell apoptosis seemed not a relevant

Fig. 1 Mettl3/Mettl14 methyltransferase complex regulates HSC self-renewal in adult bone marrow. **a** Quantitative real-time PCR analyses of the transcript levels (normalized to *β-actin*) of *Mettl3* (**a**) and *Mettl14* (**b**) in the hematopoietic hierarchy. ($n = 3$ mice from 3 independent experiments). **c** Schematic diagram of the experimental procedure for analyzing *Mx1-cre; Mettl3*$^{fl/fl}$, *Mx1-cre; Mettl14*$^{fl/fl}$, and control mice. **d**, **e** Dot blot of 500,000 Lineage-depleted hematopoietic cells showed a reduction of the overall m^{6}A level in *Mx1-cre;Mettl3*$^{fl/fl}$, *Mx1-cre; Mettl14*$^{fl/fl}$, and *Mx1-cre; Mettl3*$^{fl/fl}$*;Mettl14*$^{fl/fl}$ mice after plpC treatment (**d**). The signal intensity was quantified and statistically analyzed in (**e**) ($n = 6$ mice/genotype from 3 independent experiments). **f**–**h** The bone marrow cellularity (two tibias+two femurs, **f**) and CD48$^-$CD150$^+$Sca-1$^+$c-kit$^+$Lineage$^-$ HSC frequency (**g**) in *Mx1-cre; Mettl3*$^{fl/fl}$, *Mx1-cre; Mettl14*$^{fl/fl}$ mice, *Mx1-cre; Mettl3*$^{fl/fl}$, *Mettl14*$^{fl/fl}$, and control mice. Trypan blue staining was used to exclude dead cells in **f**. **h** Representative flow cytometric plots of each genotype ($n = 6$ mice/genotype from 3 independent experiments). **i**–**k** Competitive reconstitution assay in which 300,000 of donor-derived bone marrow cells were transplanted along with 300,000 recipient-type competitor cells into irradiated recipient mice. Percentages of donor-derived cells were analyzed by flow cytometry (**i**). The percentages of donor-derived myeloid, B and T cells in the peripheral blood (**j**), and HSCs in the bone marrow (**k**, left panel) and spleen (**k**, right panel) were assessed at 4 months after transplantation ($n = 11$–12 recipient mice/genotype from 3 independent experiments). **l** Secondary transplantation in which 1000,000 of bone marrow cells from primary recipients in (**i**) were transplanted into secondary recipients ($n = 11$–12 recipient mice/genotype from 3 independent experiments). **m** Analysis of the frequency of apoptotic HSCs by Annexin V staining ($n = 6$ mice/genotype from 3 independent experiments). **n**, **o** BrdU incorporation ratio of bone marrow HSCs at 3 days after BrdU treatment ($n = 6$ mice/genotype from 3 independent experiments). **p**, **q** Quantitative real-time PCR analyses of the transcript levels (normalized to *β-actin*) of genes that regulate HSC self-renewal (**p**) and genes that regulate HSC differentiation (**q**) ($n = 6$ mice/genotype from 3 independent experiments). All data reflect mean ± SD. The statistical significance of differences among genotypes was assessed using a repeated-measures one-way ANOVA with Greenhouse–Geisser correction along with Tukey's multiple comparison tests with individual variances. (*, $^{\#}P < 0.05$, $^{**}P < 0.01$, ***, $^{\#\#\#}P < 0.001$)

factor as *Mettl3*- or *Mettl14*-deficient HSCs had similar frequency of Annexin V$^+$ cells as control HSCs (Fig. 1m and Supplementary information, Figure S3). We then measured the bromodeoxyuridine (BrdU) incorporation ratio of HSCs, which indicates the cycling of HSCs in a defined time period. At 3 days after BrdU injection, 17 ± 6.7% of all bone marrow HSCs became BrdU$^+$ in *Mx1-cre; Mettl3*$^{fl/fl}$ mice, significantly higher than 5.5 ± 1.9% in control mice (Fig. 1n, o), suggesting that *Mettl3* deletion drives HSCs into cell cycle. In contrast, *Mx1-cre; Mettl14*$^{fl/fl}$ mice did not show significant changes to the ratio of BrdU$^+$ HSCs (Fig. 1n, o). Consistent with the increased HSC cycling in *Mx1-cre; Mettl3*$^{fl/fl}$ mice, the expression of many genes that regulate HSC self-renewal were significantly reduced in *Mettl3*-null HSCs, including *Nr4a2*, *p21*, *Bmi-1*, and *Prdm16* (Fig. 1p). *Mettl14* deletion also reduced the expression of *Bmi-1* and *Prdm16* in bone marrow HSCs (Fig. 1p). When the expression of HSC differentiation genes, including *Ikaros*, *PU.1*, and *Mef2c*, were analyzed, we did not detect any significant difference among *Mx1-cre; Mettl3*$^{fl/fl}$, *Mx1-cre; Mettl14*$^{fl/fl}$, and control mice (Fig. 1q).

Taken together, in this study, we provided the evidence that Mettl3 is a pivotal regulator of HSC self-renewal in adult bone marrow. It functions through promoting the expression of genes that maintain HSC quiescence. The accumulation of phenotypic HSCs in *Mettl3*-deficient mice (Fig. 1g) is most likely due to a loss of HSC quiescence (Fig. 1n, o), although it may be also attributed to an alteration of surface marker expression on HSCs. By studying the single and compound knockouts of *Mettl3* and *Mettl14* in HSC regulation, our work supported the idea that Mettl3 dominates the function of m^{6}A methyltransferase complex in catalyzing m^{6}A formation and regulating tissue functions. These findings supported previous structural and biochemical studies demonstrating that Mettl3 is the unique catalytic subunit of the complex[12,13].

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AUTHOR CONTRIBUTIONS

Q.J.Y. and M.L. characterized the hematopoietic phenotypes of all mutant mice. L.S. maintained the mouse colonies and performed all western blot and dot blot experiments. X.Y. and W.D. assisted in flow cytometric experiments. Y.G. and B.O.Z. designed the experiment, analyzed the data, and wrote the manuscript.

ADDITIONAL INFORMATION

Supplementary information accompanies this paper at https://doi.org/10.1038/s41422-018-0062-2.

Competing interests: The authors declare no competing interests.

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