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The histone demethylase Jmjd3 regulates zebrafish myeloid development by promoting *spi1* expression

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Abstract

The histone demethylase Jmjd3 plays a critical role in cell lineage specification and differentiation at various stages of development. However, its function during normal myeloid development remains poorly understood. Here, we carried out a systematic *in vivo* screen of epigenetic factors for their function in hematopoiesis and identified Jmjd3 as a new epigenetic factor that regulates myelopoiesis in zebrafish. We demonstrated that *jmjd3* was essential for zebrafish primitive and definitive myelopoiesis, knockdown of *jmjd3* suppressed the myeloid

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T.X.L. designed research. S.H.Y. and P.F.X. performed most of the experiments. S.H.Y. analyzed data and wrote the manuscript. K.Y.Z., F.Z., M. Dong, L.W., X.E.J., L.G., L.T.C., Z.W.D., C.G.R., Y.J. and Y.C. assisted with experiments. J.W. performed statistical analysis. Y.Z., J.Z. and P.F.X. provided important instructions and revised the manuscript. Z.L., H.Y., Q.H.H. and M. Deng critically read the manuscript.

Competing financial interests

The authors declare no competing financial interests.

commitment and enhanced the erythroid commitment. Only overexpression of *spi1* but not the other myeloid regulators rescued the myeloid development in *jmjd3* morphants. Furthermore, preliminary mechanistic studies demonstrated that Jmjd3 could directly bind to the *spi1* regulatory region to alleviate the repressive H3K27me3 modification and activate *spi1* expression. Thus, our studies highlight that Jmjd3 is indispensable for early zebrafish myeloid development by promoting *spi1* expression.

Keywords

histone demethylase; *jmjd3*; myelopoiesis; *spi1*; zebrafish

1. Introduction

Myelopoiesis is the process of producing all types of myeloid cells including granulocytes and monocytes/macrophages from stem and progenitor cells. Myeloid cells are critical effectors and regulators of tissue regeneration, innate and adaptive immune response [1]. Malfunction or dysregulation of myelopoiesis is tightly associated with various human blood diseases including acute myeloid leukemia (AML) [2]. Previous studies have illustrated the essential role of several transcription factors for normal vertebrate myelopoiesis, including *Spi1*, *C/ebpa*, *Gcsfr*, *Irf8*, and *Gfi1* [3–7]. However, the orchestrated regulatory programs, consisting of both transcriptional and epigenetic controls during normal myeloid development, remain unclear.

Recently, zebrafish has emerged as an excellent vertebrate animal model to study the development of myelopoiesis [8–10]. Similar to mammals, zebrafish has most of the myeloid cell types including neutrophil, monocyte, eosinophil, mast cell and dendritic cell [11–15]. In a developing vertebrate embryo, hematopoiesis consists of two successive waves, termed primitive hematopoiesis and definitive hematopoiesis [9]. In zebrafish, the primitive hematopoiesis takes place in the anterior lateral plate mesoderm (ALPM) and intermediate cell mass (ICM), producing primitive myeloid cells and erythrocytes, respectively [16]. On the other hand, definitive hematopoiesis occurs at the ventral wall of dorsal aorta at a site called aorta-gonad-mesonephros (AGM) around 28 hours post-fertilization (hpf) [17]. By 48 hpf, the AGM-derived hematopoietic stem/progenitor cells (HSPCs) migrate to the caudal hematopoietic tissue (CHT, equivalent to the mammalian fetal liver) for rapid lineage expansion and differentiation [18, 19]. Alternatively, the erythromyeloid progenitors (EMPs) that arise autonomously in the posterior blood island (PBI) between 24 and 36 hpf can also generate definitive erythroid and myeloid colonies [20].

The important role that histone methylation plays during transcriptional regulation of gene expression in cell differentiation and proliferation has been long recognized [21]. Trimethylated histone H3 at lysine 4 (H3K4me3) marks transcriptionally active chromatin states, whereas trimethylated histone H3 at lysine 27 (H3K27me3) marks transcriptionally repressive chromatin states. Jumonji domain-containing protein D3 (Jmjd3, also named Kdm6b) is a member of the H3K27me3/2-specific demethylase family that promotes gene

transcription by acting as a rival of the Polycomb repressive complex 2 (PRC2) [22, 23]. Studies using embryonic stem cells suggest that *Jmjd3* is required for the development of all three germ layers [24–26], *Jmjd3* accelerates the specification of pluripotent cells by removing H3K27me3 barriers [27]. It has also been shown that *Jmjd3* function is necessary for the differentiation and proliferation of cells in different tissues, such as neurons, epidermal cells, cardiac cells, M2 macrophages and T cells [23, 25, 28–30]. However, the *in vivo* function of *Jmjd3* in myeloid lineage development remains to be determined.

Here, we show that *Jmjd3*, a zebrafish *Jmjd3* domain-containing protein, possesses H3K27 histone demethylase activity *in vivo*, and down-regulation of *jmd3* leads to significant reduction of primitive and definitive myelopoiesis. Importantly, we have demonstrated that *jmd3* directly upregulates *spi1* expression to promote myeloid commitment in a histone demethylase-dependent manner. Our data, therefore, delineate the biology and the underline mechanism of *jmd3* function during normal myelopoiesis.

2. Materials and methods

2.1. Zebrafish maintenance and breeding

All experimental procedures followed the rules of the Committee on Animal Care of Shanghai, China. All animal experiments were approved by the Institutional Animal Care and Use Committee (IACUC) of Shanghai Jiao Tong University. Zebrafish were raised and maintained at 28.5 °C in an aquatic system equipped with continuously filtration, UV treatment and aeration of fish water in circulation. The embryos were collected in dishes and reared in an incubator at 28.5 °C. The transgenic line Tg (*gata1*: EGFP) [31], Tg (*lyz*: EGFP) [32] and Tg (*spi1*: EGFP) [33] were used in this study.

2.2. Plasmids construction, morpholino and mRNA injections

The zebrafish WT cDNA of the *jmd3*, *spi1*, *c/ebpa*, and *gcsfr* genes were amplified from reverse transcription products and cloned into the pCS2+ vector. Zebrafish morpholino oligonucleotides (MOs) were purchased from Gene Tools. The sequences of MOs used in this studies are following; *Jmjd3* translation-blocking MO: 5'-CCCATCTCGCTGTTACTGTGTTTTTC-3'; *Jmjd3* splice-blocking MO: 5'-ATATTTTGTGTGTGTGGACTGACCT-3'; *Spi1* MO: 5'-GATATACTGATACTCCATTGGTGGT-3'; control MO: 5'-CCTCTTACCTCAGTTACAATTATA-3'. Capped mRNAs were transcribed with mMESSAGE mMACHINE Kit (Ambion). All MOs and mRNAs were injected into embryos at the one-cell stage.

2.3. WISH

Antisense RNA probes were transcribed using linearized constructs with T3 or T7 polymerase (Ambion) in the presence of digoxigenin (DIG, Roche)-labeled UTP using the DIG-RNA Labeling Kit (Roche). DIG-labelled antisense probes for these genes were synthesized with T3 or T7 polymerase (Ambion). WISH was performed as described previously, using 5-nitro-blue tetrazoliumchloride/bromo-4-chloro-3'-indolyl phosphate p-toluidine salt (NBT/BCIP, Vector Laboratories) as substrates [34]. Embryos were mounted in

4% methylcellulose and captured under a Nikon SMZ1500 microscope equipped with a Nikon DXM1200F digital camera and ACT-1 software.

2.4. Real-Time qPCR

Total RNAs from embryos or cells were extracted using TRIzol reagent (Ambion). RNA was reverse transcribed using random hexamers and oligodT primers (Invitrogen). A 2XPCR Mix (Toyobo) was used for the real-time qPCR analysis, with an ABIPrism 7900HT Sequence Detector (Applied Biosystems). The relative expression values were normalized against the internal control gapdh. Primer sequences are available in Table S2.

2.5. Western blotting

Cells were homogenized in lysis buffer (20mM Tris-HCl pH 7.4, 150mM NaCl, 5mM ethylenediaminetetraacetic acid, 10% glycerol, and 0.1% Triton X-100) containing protease inhibitor cocktail and phosphatase inhibitor (Roche Diagnostics). Proteins were separated on a 10% to 12% SDS-polyacrylamide gel and transferred electrophoretically to a nitrocellulose membrane (Schleicher & Schuell). The membrane was blocked and then incubated with the indicated antibodies followed by incubation with an appropriate horseradish peroxidase-conjugated secondary antibody (1:10 000). Band densities were quantified using the Image-Pro Plus software.

2.6. Chromatin Immunoprecipitation (ChIP)

ChIP assays were performed according to the standard cross-linking ChIP protocol (Abcam) with minor modifications. For ChIP using H3K27me3 antibody, 200 each wild-type embryos; control morphants; *jmjd3* morphants and HA-*jmjd3*-overexpression embryos at 22 hpf were used. For ChIP using H3K4me3 antibody, 200 each control morphants and *jmjd3* morphants at 22 hpf were used. For ChIP using HA antibody, 200 each wild-type embryos and HA-*jmjd3*-overexpression embryos at 22 hpf were used. Zebrafish embryos were enzymatically dechorionated, and cells were harvested and crosslinked with 1% formaldehyde for 10 minutes at room temperature. After sonication, the soluble chromatin was incubated with 5 ug antibodies specific for H3K27me3 (ab85392 Abcam) or HA (ab9110, Abcam). Chromatin immunocomplexes were then precipitated with Protein A (Millipore, 16-661) or Protein G (Millipore, 16-662). The immunoprecipitated complex was washed, and DNA was extracted and purified by QIAquick PCR Purification Kit (Qiagen). ChIP DNA was analyzed by qPCR using specific primers against genomic regions of interest, and the data were normalized by input DNA. The results were derived from three independent experiments. The primer sequences are available in Table S2.

2.7. TUNEL assay and pH3 (phospho-histone 3) staining

TUNEL assays were performed using the *In Situ* Cell Death Detection Kit and TMR Red (Roche Diagnostics) according to the manufacturer's recommendations. PH3 labeling of fixed embryos was performed by an overnight incubation with a rabbit anti-phospho-histone H3 antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) at 4 °C, followed by an incubation with the Alexa Fluor 594 donkey anti-rabbit secondary antibody (Invitrogen, Carlsbad, CA, USA). The embryos were mounted in 4% methylcellulose and images

captured under a 270 Nikon SMZ1500 microscope equipped with a Nikon DXM1200F digital camera and ACT-1 software.

2.8. Flow cytometry

Transgenic Tg(*spi1*:EGFP) and Tg(*gata1*:EGFP) zebrafish embryos at 22 hpf were rinsed in phosphate buffered saline (PBS), disassociated in 0.25% trypsin/EDTA (Invitrogen) for 20 min at 37 °C, and filtered through a 40 µm cell strainer (BD Falcon). EGFP positive cells were sorted with a MoFlo FACS sorter (Dako Cytomation).

2.9. Statistical Analysis

Microsoft Excel and GraphPad Prism software were used for statistical analysis. Two-tailed Student's t test was used for statistical analysis. A *p* value of less than 0.05 was considered statistically significant. Data are presented as mean ± SEM.

3. Results

3.1. *Jmjd3* is expressed during hematopoietic development and mediates H3K27 histone demethylase activity in zebrafish

JmjC domain-containing proteins represent an evolutionarily conserved family of epigenetic regulators, which are responsible for most histone lysine demethylation [35, 36]. Given the critical function of JmjC genes in transcriptional regulation, we bioinformatically surveyed the zebrafish reference and identified 39 non-redundant zebrafish JmjC genes. These genes were then cDNA cloned or identified from existing EST libraries and their expression during early development were analyzed by whole-mount *in situ* hybridization (WISH) analyses (Table S1). Among the 39 genes, we found that *jmd3*, *jmd2b*, *utx-a* and *uty* were preferentially expressed in the hematopoietic system at specific developmental stages (Fig. 1A and Fig. S1A). *Jmjd3* was not expressed maternally, and the earliest zygotic transcription of *jmd3* could only be detected ubiquitously from 12 to 22 hpf (Fig. 1A, B). Although *jmd3* was ubiquitously expressed at 22 hpf shown by WISH, our qPCR results showed that *jmd3* was highly expressed in primitive myeloid and erythroid progenitors. These progenitor cells marked by Tg (*spi1*: EGFP) and Tg (*gata1*: EGFP) at 22 hpf were isolated, respectively, via fluorescence-activated cell sorting, and *jmd3* expression in developing hematopoietic site was validated using qPCR assays (Fig. 1B), suggesting that *jmd3* may play a role in early myeloid-erythroid precursor development. Of note, *jmd3* expression was detected in the definitive hematopoietic site PBI at 32 hpf and then CHT at 48 hpf (Fig. 1A), indicating *jmd3* may also play a role in definitive hematopoiesis.

Zebrafish *jmd3* open-reading frame (ORF) extends a length of 4,065 nucleotides encoding a polypeptide of 1,354 amino acids, both the coiled-coil motif and JmjC domain are conserved from zebrafish to human (Fig. S2A). Nevertheless, a majority of the similarity in Jmjd3 orthologs among these species is restricted to the JmjC domain (91%, Fig. S2B). Two human genes (*TMEM88* and *PER1*), along with *JMJD3*, define an approximately 300-kb genomic region on human chromosome 17, which is syntenic to the zebrafish *jmd3* genomic locus on chromosome 10 (Fig. S2C). Phylogenetic tree analysis also indicates that

the JmjC domain of zebrafish *Jmjd3* is closely related to its mammalian orthologs (Fig. S2D).

To determine whether the *Jmjd3* protein act as a functional histone demethylase in zebrafish, we examined whether the histone methylation levels were affected by the *jmd3* knockdown. We designed translation-blocking morpholino oligonucleotides (MO) to target the sequences near and around the ATG of *jmd3* (Fig. S2A). To test the knockdown efficiency and specificity of the MO, we co-injected either 10 ng *jmd3* MO or control MO with 100 pg of EGFP mRNA reporter containing MO-targeting sequences into embryos at the one-cell stage [37]. The *jmd3* morpholino was able to efficiently and specifically block the translation of the co-injected EGFP reporter mRNA (Fig. S3A–C). As expected, western blot assays of protein lysates extracted from 22 hpf embryos injected with either *jmd3* MO or control MO revealed that knockdown of *jmd3* markedly increased the level of H3K27me3 (by 2.89-fold) and an moderately increased amount of H3K27me2 (by 1.76-fold), to be accompanied by a mildly decreased level of H3K4me3 (by 0.70-fold, Fig. 1C). No changes in levels were observed for H3K27me1 and H3K9me3 (Fig. 1C). These results suggest that like mammals *Jmjd3* [38, 39], zebrafish *jmd3* encodes a histone demethylase that specifically mediates the removal of methyl groups from H3K27me3/me2 *in vivo*.

3.2. Knockdown of *jmd3* impairs primitive and definitive myelopoiesis in zebrafish

The expression pattern analysis of *jmd3* indicates that it potentially functions in the hematopoietic process. We further characterize its function in hematopoiesis by WISH examination of expression of a variety of stage- or lineage-specific key transcription factors and hematopoietic markers in control and *jmd3* morphants. The levels of the neutrophil markers *lyz*, *mpx* [40], *nephrosin* [41] and pan-leukocyte marker *l-plastin* [42] were markedly decreased at 22 hpf (Fig. 2A–H). Consistent with our WISH data, the qPCR signals of these myeloid markers were also significantly reduced in *jmd3*-deficient embryos compared to the controls (Fig. S4A). Moreover, in line with detectable expression of *jmd3* in key definitive hematopoietic sites, the PBI at 32 hpf and CHT at 48 hpf (Fig. 1A), *lyz* and *mpx* expression were consistently down-regulated in PBI at 36 hpf (Fig. 2I–L), as well as in CHT at 72 hpf (Fig. S4B, C). These data suggest that *jmd3* deficiency led to a decrease in both primitive and definitive myelopoiesis. To make sure the specificity of the *jmd3* ATG-MO, we designed a splice-blocking MO (*jmd3* Sp MO) to disrupt the coding of the JmjC domain of the zebrafish *jmd3* (Fig. S4D, E), the down-regulation of *jmd3* using this MO had also disrupted myeloid commitment (Fig. S4F–I), similar to what had been observed in ATG MO.

To further confirm the drastic decrease of myeloid development as a result of *jmd3* deficiency, a stable zebrafish transgenic line expressing EGFP under the control of the *lyz* promoter was used to analyze both control and *jmd3* morphants. Consistent with our WISH results, EGFP expression in the embryos injected with the *jmd3* MO was severely impaired during primitive and definitive hematopoietic waves (Fig. S4J–O).

3.3. *Jmjd3* promotes myeloid commitment of erythromyeloid progenitors

To determine the role of *Jmjd3* within the hematopoietic regulating hierarchy, we further examined the expression of several key hematopoietic factors at different developmental stages. No significant changes in the expression of primitive HSC markers *scl* or *lmo2* were found in the *jmjd3* morphants at 22 hpf (Fig. S5A, D). However, the expression of *c-myb* in AGM at 32 hpf, which marks the definitive HSPCs and myeloid lineage cells, was mildly decreased (Fig. S5B, D), while the level of endothelial marker *flk1* expression was essentially unchanged (Fig. S5C–D), indicating that zebrafish *jmjd3* may play a minor regulatory role in the generation of definitive HSCs and in primitive and definitive myeloid differentiation.

Interestingly, *jmjd3* knockdown dramatically reduced the expression of the myeloid precursor marker *spi1* from 20 to 22 hpf (Fig. 3A–D). Of note, in line with the expression of *jmjd3* in PBI (Fig. 1A), knockdown of *jmjd3* resulted in a significant reduction of *spi1*⁺ cells in this region at 32hpf (Fig. 3E). Consistent with these results, qPCR analyses revealed that *spi1* expression was strongly downregulated from 20 to 32 hpf in the *jmjd3* morphants as compared to control (Fig. 3F). It has been reported that the interplay of *spi1* and *gata1* are important for the balance of myeloid and erythroid lineages in zebrafish [43, 44], we wondered whether *jmjd3* deficiency had any influence on erythropoiesis. Our results showed that the expression of erythroid precursor marker *gata1* was slightly increased in ICM at 20 hpf (Fig. 3G). Along with this, the mature erythrocyte markers β -E3 and α -E1 globin were ectopically expressed, especially when observed from the dorsal view at 22hpf (Fig. 3H), and α -E1 globin also showed an increased expression level in PBI at 32hpf (Fig. 3I). Consistently, the qPCR analysis of the expression of these erythroid markers showed a significant increase in *jmjd3*-deficient embryos compared to the controls (Fig. 3J). Taken together, these results indicate a role of *jmjd3* is to enhance the myeloid commitment and to suppress the erythroid commitment during the differentiation of erythromyeloid precursors.

3.4. Myeloid cells in *jmjd3*-deficient embryos display decreased proliferation and increased apoptosis

Previous reports had showed that *Jmjd3* knockout or knockdown could result in either cell proliferation inhibition or cell apoptosis activation [45, 46]. The *lyz*-EGFP⁺ cells in the CHT at 36 hpf represent primitive myeloid cells and are more abundant with higher transgene expression. We chose the *lyz*: EGFP transgenic zebrafish to examine lineage-specific cell death and proliferation in the control and *jmjd3* morphants using pH3 (phospho-histone 3) and TUNEL (terminal deoxynucleo-tidyl transferase dUTP nick end labeling) incorporation assay, respectively. Our results showed that, the percentage of proliferative EGFP⁺ cells in the CHT region was significantly decreased in *jmjd3*-deficient embryos as compared to that in the controls at 36 hpf (Fig. 4A–G), while the total number of EGFP⁺ cells were dramatically decreased (Fig. 4A,D and Fig.S4L–M). Besides, the TUNEL assay also showed that the percentage of apoptotic EGFP⁺ myeloid cells in CHT region was significantly increased in *jmjd3* morphants as compared to that in controls (Fig. 4H–N). These data indicated that the decrease proliferation and increased apoptosis may partially contribute to the decrease of myelopoiesis in *jmjd3*-deficient embryos.

3.5. Jmjd3 is evolutionarily conserved, and its regulation of myelopoiesis requires an intact histone demethylase domain

The observed down-regulation of *spi1*, *lyz* and *mpx* by the knockdown of *jmjd3* could be rescued by co-injection of zebrafish wild-type *jmjd3* mRNA (Fig. 5A–I, Fig. 5P–R). These results confirmed the level of *jmjd3* expression is critical for the commitment of myelopoiesis and also demonstrated the displayed myeloid phenotypes in *jmjd3* morphants were specific to the down-regulation of *jmjd3* gene expression.

The previous study showed that a mutant of JMJD3 (a loss of function mutation in H1390A in the catalytic domain of the demethylase), impaired its histone demethylase activity [47]. The forced expression of human *JMJD3* but not catalytically inactive *H1390A* mutant potentially rescue the myeloid differentiation (Fig. 5A–F, Fig. 5J–R). These data suggest that function of JMJD3 is conserved between zebrafish and human and its regulation of myelopoiesis is dependent on its intact histone demethylase activity.

3.6. Co-injection of *spi1* mRNA rescues the impaired myeloid commitment caused by *jmjd3* deficiency

It is noticeable that the impaired myelopoiesis observed in *jmjd3* morphants at least partially phenocopied which was observed previously in *Spi1*-knockout mice or *spi1*-deficient zebrafish (Fig. S6A–F) [48–50], raising the possibility that Jmjd3 might regulate myeloid commitment through the regulation of *spi1* expression. To test this, we performed a rescue experiment of *jmjd3* morphants by *spi1* co-injection. As expected, the supplementation of 100 pg *spi1* mRNA in *jmjd3* morphants restored the expression levels of *lyz*⁺ (Fig. 6A–B) and *mpx*⁺ (Fig. 6C–D) neutrophils in a high percentage of embryos at 22 hpf and 36 hpf, whereas the co-injection of mRNA of other myeloid regulators such as *c/ebpa* or *gcsfr* failed to do so (Fig. S6G). Taken together, these data indicated that *spi1* might be one of the key downstream targets of *jmjd3* during myeloid development.

3.7. Jmjd3 regulates dynamic H3K27me3 and H3K4me3 modifications in the promoter regions of myeloid genes including *spi1*

To understand how Jmjd3 regulates *spi1* expression, we analyzed sequence similarity between mouse and zebrafish in the 20-kb regulatory region upstream of the *spi1* transcriptional start site (TSS) and found an evolutionarily conserved region (region II) located between –4873 to –4731 to *spi1* TSS in zebrafish (Figure S7A). This region shares 55% nucleotide acid identity with the upstream regulatory region (URE) at –14-kb upstream of the murine *Spi1* TSS (Figure S7B, red dots). This URE region has been shown to regulate *Spi1* gene expression by previous studies [51, 52]. We decided to examine the function of this zebrafish orthologous region in the regulation of *spi1* expression in zebrafish.

As no antibodies are available to detect endogenous zebrafish Jmjd3, we constructed an N-terminal HA-tagged *jmjd3* construct (HA-*jmjd3*). This fusion construct was overexpressed in zebrafish embryos, and its expression was checked by western blotting (Fig. S7C) before the tagged construct was used in the following chromatin immunoprecipitation experiments. Chromatin fragments were extracted from the wild-type embryos, control morphant, *jmjd3* morphant and HA-*jmjd3*-expressing embryos at 22 hpf, the time point at which the dramatic

downregulation of *spi1* transcripts was readily detected in the *jmjd3* morphant (Fig. 3C, D). ChIP analysis of the HA-*jmjd3*-expressing embryos demonstrated that HA-*jmjd3* specifically interacts with the region II but not regions I and III (Fig. S7D). Similarly, the H3K27me3 was predominantly enriched in the region II but not in the other two regions. The knockdown of *jmjd3* resulted in dramatically increase in H3K27me3, whereas the overexpression of HA-*jmjd3* was associated with a moderately decrease in H3K27me3 in region II (Fig. S7E). Previous study showed that chromatin regions containing both H3K4me3 and H3K27me3 termed “bivalent domains” at promoters of genes encoding key developmental transcription factors [53]. We next wondered whether the H3K4me3 altered in these regions in *jmjd3* morphants. ChIP-qPCR assay showed that the H3K4me3 level was significantly decreased in the region II in the *jmjd3* morphants (Fig. S7F). Taken together, these preliminary analyses indicate that *spi1* is a *bona fide* downstream target of Jmjd3 during zebrafish myelopoiesis whereby Jmjd3 occupies specifically the *spi1* regulatory region to reduce the local H3K27me3 level.

We then performed ChIP-qPCR assays to quantify the possible differences in H3K27me3 and H3K4me3 levels of at the promoter regions of the down-regulated myeloid genes and the up-regulated erythroid genes in the control and *jmjd3*-deficient embryos. As expected, the down-regulated myeloid genes such as *lyz*, *mpx*, *nephrosin* and *l-plastin* were associated with an increased H3K27me3 level but a reduced H3K4me3 level around the promoter in *jmjd3* morphants (Fig. S8A–L). However, the up-regulated erythroid genes such as *gata1*, β -*E3* and α -*E1* did not exhibit statistical differences in H3K27me3 and H3K4me3 levels around their promoter regions between control and *jmjd3* morphants (Fig. S8M–U). In agree with the predicted role of Jmjd3 as a transcriptional activator by removing H3K27me3 barriers, our results indicated that a number of myeloid regulators may be the direct targets of Jmjd3 activity during zebrafish myeloid development.

4. Discussion

Hematopoiesis is a complex process of producing all types of blood cells including granulocytes and monocytes/macrophages. The differentiation and activation of myeloid cells require a timely regulation of gene expression, which depends on the interplay between transcription factors and epigenetic regulation, including histone modifications [54]. With this in mind, we screened systematically for both histone methyltransferases [54] and demethylases in zebrafish as epigenetic regulators of hematopoiesis during early development. Several genes with enriched expression in hematopoietic sites were identified, one of them is Jmjd3, an H3K27me3/2-specific demethylase that promotes gene transcription in many biological processes such as senescence and differentiation. In the hematopoietic system, Jmjd3 has been reported to be involved in M2 macrophage differentiation and terminal thymocyte development [29, 30]. However, the roles of Jmjd3 in normal myelopoiesis are poorly understood. In this study, our results suggest that *jmjd3* participates in the induction of myelopoiesis. Deficiency of *Jmjd3* expression resulted in a decrease of myeloid development, at the same time, it resulted in an expansion of erythroid development as measured by the primitive erythroid progenitor marker *gata1* at 20 hpf, the mature erythrocyte markers β -*E3* at 22 hpf, and α -*E1* globin at 22 hpf and 36 hpf. Similarly, previous work of a large-scale reverse genetic screen found that knockdown of *jmjd3*

promotes mature erythrocyte markers β -E3 expression at 17 hpf [55]. Consistently, Peripheral blood CD34+ progenitor cells in patients with lower-risk MDS (myelodysplastic syndromes) have increased expression of JMJD3 and an increased ability to form erythroid colonies upon inhibition of JMJD3 [56]. This suggests that Jmjd3 is intimately involved in erythroid lineage determination. Furthermore, *Jmjd3* conditional knockout mice also exhibit a decrease of myeloid progenitors including granulocyte/monocyte progenitors (GMPs) in bone marrow (unpublished data) [57].

Specification of primitive hematopoietic progenitors into either myeloid or erythroid lineages is controlled by orchestrating the expression of a series of key transcription factors. It is well documented that *spi1* and *gata1* negatively regulate each other and the interplay between these two factors determines myeloid versus erythroid cell fate [43, 44]. Also, *tif1 γ* has been shown to regulate erythroid versus myeloid cell fates by controlling the expression levels of *spi1* and *gata1* [58]. *Gfi1.1* is another myeloid transcriptional factor that governs the balance of primitive erythroid and myeloid lineage determination. Blocking *gfi1.1* function by morpholino knockdown leads to a reduced expression of *gata1* and increased expression of *spi1* [59]. In addition to the transcriptional regulation, post-translational modifications are also important for myeloid-erythroid lineage choice. It has been reported that hypoSUMOylation of C/EBP α leads to expansion of myeloid lineage at the expense of erythroid lineage, suggesting that SUMOylation of C/EBP α is important for the differentiation control of erythroid-myeloid progenitors [60]. In this study, we demonstrated that *jmd3* bias the myeloid commitment at the expense of the erythroid commitment. Thus, cross-talk between transcription factors and epigenetic regulators may have synergistic effects in myeloid fate decisions.

The strict control of *Spi1* expression is critical for proper myeloid cell fate determination, and genetic or epigenetic changes in the *Spi1* gene frequently contribute to the leukemogenesis in mice and human. It has been shown that *Spi1* expression is frequently downregulated in human AML patients [61, 62]. Likewise, a graded reduction of *Spi1* to 20% of the wild type level induces AML in mice [63]. Moreover, *Spi1* mutations are found in about 7% of the patients in a survey involving 126 AML patients [64]. In this respect, the H3K4 methyltransferase Mll1 has recently been shown to be able to occupy the regulatory region to activate the transcription of *Spi1* [65]. We provide evidence that *jmd3* play a critical role in controlling *spi1* expression in zebrafish. Using a whole embryo ChIP-PCR analysis, we found that endogenous H3K27me3 modification was predominantly enriched at the *spi1* regulatory region, and Jmjd3 activity was required to prevent a hyper H3K27me3 level at the region. A confirmation by a ChIP-seq analysis in myeloid cell lines and/or purified myeloid cells is necessary and such a genome-wide analysis may also shed light on the regulation of other myeloid factors by the observed JMJD3 activity. However, the mechanism of how *jmd3* is recruited to *spi1* upstream regulator region needs further investigation and remains unknown.

Previous studies and our research showed that Jmjd3 is required for the activation of mammalian developmental programs through a demethylation-activity-dependent mechanism [22, 27–29]. Interestingly, other groups have demonstrated that Jmjd3 also functions independently of its enzymatic activity [66, 67]. Nonetheless, whether the

enzymatic activity of Jmjd3 is required for a proper expression of a target gene needs specific experimental verification.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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References

1. Geissmann F, Manz MG, Jung S, Sieweke MH, Merad M, Ley K. Development of monocytes, macrophages, and dendritic cells. *SCIENCE*. 2010; 327:656–661. [PubMed: 20133564]
2. Herauld A, Binnewies M, Leong S, Calero-Nieto FJ, Zhang SY, Kang YA, Wang X, Pietras EM, Chu SH, Barry-Holson K, Armstrong S, Gottgens B, Passegue E. Myeloid progenitor cluster formation drives emergency and leukaemic myelopoiesis. *NATURE*. 2017; 544:53–58. [PubMed: 28355185]
3. McKercher SR, Torbett BE, Anderson KL, Henkel GW, Vestal DJ, Baribault H, Klemsz M, Feeney AJ, Wu GE, Paige CJ, Maki RA. Targeted disruption of the PU.1 gene results in multiple hematopoietic abnormalities. *EMBO J*. 1996; 15:5647–5658. [PubMed: 8896458]
4. Zhang DE, Zhang P, Wang ND, Hetherington CJ, Darlington GJ, Tenen DG. Absence of granulocyte colony-stimulating factor signaling and neutrophil development in CCAAT enhancer binding protein alpha-deficient mice. *Proc Natl Acad Sci U S A*. 1997; 94:569–574. [PubMed: 9012825]
5. Liu F, Wu HY, Wesselschmidt R, Kornaga T, Link DC. Impaired production and increased apoptosis of neutrophils in granulocyte colony-stimulating factor receptor-deficient mice. *IMMUNITY*. 1996; 5:491–501. [PubMed: 8934575]
6. Becker AM, Michael DG, Satpathy AT, Sciammas R, Singh H, Bhattacharya D. IRF-8 extinguishes neutrophil production and promotes dendritic cell lineage commitment in both myeloid and lymphoid mouse progenitors. *BLOOD*. 2012; 119:2003–2012. [PubMed: 22238324]
7. Karsunky H, Zeng H, Schmidt T, Zevnik B, Kluge R, Schmid KW, Duhrsen U, Moroy T. Inflammatory reactions and severe neutropenia in mice lacking the transcriptional repressor Gfi1. *NAT GENET*. 2002; 30:295–300. [PubMed: 11810106]
8. Jagannathan-Bogdan M, Zon LI. Hematopoiesis. *DEVELOPMENT*. 2013; 140:2463–2467. [PubMed: 23715539]
9. Orkin SH, Zon LI. Hematopoiesis: an evolving paradigm for stem cell biology. *CELL*. 2008; 132:631–644. [PubMed: 18295580]
10. Xu J, Du L, Wen Z. Myelopoiesis during zebrafish early development. *J GENET GENOMICS*. 2012; 39:435–442. [PubMed: 23021543]
11. Lieschke GJ, Oates AC, Crowhurst MO, Ward AC, Layton JE. Morphologic and functional characterization of granulocytes and macrophages in embryonic and adult zebrafish. *BLOOD*. 2001; 98:3087–3096. [PubMed: 11698295]
12. Herbomel P, Thisse B, Thisse C. Ontogeny and behaviour of early macrophages in the zebrafish embryo. *DEVELOPMENT*. 1999; 126:3735–3745. [PubMed: 10433904]
13. Balla KM, Lugo-Villarino G, Spitsbergen JM, Stachura DL, Hu Y, Banuelos K, Romo-Fewell O, Aroian RV, Traver D. Eosinophils in the zebrafish: prospective isolation, characterization, and eosinophilia induction by helminth determinants. *BLOOD*. 2010; 116:3944–3954. [PubMed: 20713961]

14. Dobson JT, Seibert J, Teh EM, Da'As S, Fraser RB, Paw BH, Lin TJ, Berman JN. Carboxypeptidase A5 identifies a novel mast cell lineage in the zebrafish providing new insight into mast cell fate determination. *BLOOD*. 2008; 112:2969–2972. [PubMed: 18635811]
15. Wittamer V, Bertrand JY, Gutschow PW, Traver D. Characterization of the mononuclear phagocyte system in zebrafish. *BLOOD*. 2011; 117:7126–7135. [PubMed: 21406720]
16. Carradice D, Lieschke GJ. Zebrafish in hematology: sushi or science? *BLOOD*. 2008; 111:3331–3342. [PubMed: 18182572]
17. Kissa K, Herbomel P. Blood stem cells emerge from aortic endothelium by a novel type of cell transition. *NATURE*. 2010; 464:112–115. [PubMed: 20154732]
18. Murayama E, Kissa K, Zapata A, Mordelet E, Briolat V, Lin HF, Handin RI, Herbomel P. Tracing hematopoietic precursor migration to successive hematopoietic organs during zebrafish development. *IMMUNITY*. 2006; 25:963–975. [PubMed: 17157041]
19. Jin H, Xu J, Wen Z. Migratory path of definitive hematopoietic stem/progenitor cells during zebrafish development. *BLOOD*. 2007; 109:5208–5214. [PubMed: 17327398]
20. Bertrand JY, Kim AD, Violette EP, Stachura DL, Cisson JL, Traver D. Definitive hematopoiesis initiates through a committed erythromyeloid progenitor in the zebrafish embryo. *DEVELOPMENT*. 2007; 134:4147–4156. [PubMed: 17959717]
21. Ho L, Crabtree GR. Chromatin remodelling during development. *NATURE*. 2010; 463:474–484. [PubMed: 20110991]
22. De Santa F, Totaro MG, Prosperini E, Notarbartolo S, Testa G, Natoli G. The histone H3 lysine-27 demethylase Jmjd3 links inflammation to inhibition of polycomb-mediated gene silencing. *CELL*. 2007; 130:1083–1094. [PubMed: 17825402]
23. Jepsen K, Solum D, Zhou T, McEvilly RJ, Kim HJ, Glass CK, Hermanson O, Rosenfeld MG. SMRT-mediated repression of an H3K27 demethylase in progression from neural stem cell to neuron. *NATURE*. 2007; 450:415–419. [PubMed: 17928865]
24. Kim SW, Yoon SJ, Chuong E, Oyulu C, Wills AE, Gupta R, Baker J. Chromatin and transcriptional signatures for Nodal signaling during endoderm formation in hESCs. *DEV BIOL*. 2011; 357:492–504. [PubMed: 21741376]
25. Ohtani K, Zhao C, Dobrev G, Manavski Y, Kluge B, Braun T, Rieger MA, Zeiher AM, Dimmeler S. Jmjd3 controls mesodermal and cardiovascular differentiation of embryonic stem cells. *CIRC RES*. 2013; 113:856–862. [PubMed: 23856522]
26. Burgold T, Spreafico F, De Santa F, Totaro MG, Prosperini E, Natoli G, Testa G. The histone H3 lysine 27-specific demethylase Jmjd3 is required for neural commitment. *PLOS ONE*. 2008; 3:e3034. [PubMed: 18716661]
27. Akiyama T, Wakabayashi S, Soma A, Sato S, Nakatake Y, Oda M, Murakami M, Sakota M, Chikazawa-Nohtomi N, Ko SB, Ko MS. Transient ectopic expression of the histone demethylase JMJD3 accelerates the differentiation of human pluripotent stem cells. *DEVELOPMENT*. 2016; 143:3674–3685. [PubMed: 27802135]
28. Sen GL, Webster DE, Barragan DI, Chang HY, Khavari PA. Control of differentiation in a self-renewing mammalian tissue by the histone demethylase JMJD3. *Genes Dev*. 2008; 22:1865–1870. [PubMed: 18628393]
29. Satoh T, Takeuchi O, Vandenbon A, Yasuda K, Tanaka Y, Kumagai Y, Miyake T, Matsushita K, Okazaki T, Saitoh T, Honma K, Matsuyama T, Yui K, Tsujimura T, Standley DM, Nakanishi K, Nakai K, Akira S. The Jmjd3-Irf4 axis regulates M2 macrophage polarization and host responses against helminth infection. *NAT IMMUNOL*. 2010; 11:936–944. [PubMed: 20729857]
30. Li Q, Zou J, Wang M, Ding X, Chepelev I, Zhou X, Zhao W, Wei G, Cui J, Zhao K, Wang HY, Wang RF. Critical role of histone demethylase Jmjd3 in the regulation of CD4+ T-cell differentiation. *NAT COMMUN*. 2014; 5:5780. [PubMed: 25531312]
31. Fu YF, Du TT, Dong M, Zhu KY, Jing CB, Zhang Y, Wang L, Fan HB, Chen Y, Jin Y, Yue GP, Chen SJ, Chen Z, Huang QH, Jing Q, Deng M, Liu TX. Mir-144 selectively regulates embryonic alpha-hemoglobin synthesis during primitive erythropoiesis. *BLOOD*. 2009; 113:1340–1349. [PubMed: 18941117]
32. Zhang Y, Bai XT, Zhu KY, Jin Y, Deng M, Le HY, Fu YF, Chen Y, Zhu J, Look AT, Kanki J, Chen Z, Chen SJ, Liu TX. In vivo interstitial migration of primitive macrophages mediated by JNK-

- matrix metalloproteinase 13 signaling in response to acute injury. *J IMMUNOL*. 2008; 181:2155–2164. [PubMed: 18641354]
33. Hsu K, Traver D, Kutok JL, Hagen A, Liu TX, Paw BH, Rhodes J, Berman JN, Zon LI, Kanki JP, Look AT. The pu.1 promoter drives myeloid gene expression in zebrafish. *BLOOD*. 2004; 104:1291–1297. [PubMed: 14996705]
 34. Thisse C, Thisse B. High-resolution in situ hybridization to whole-mount zebrafish embryos. *NAT PROTOC*. 2008; 3:59–69. [PubMed: 18193022]
 35. Klose RJ, Kallin EM, Zhang Y. JmJC-domain-containing proteins and histone demethylation. *NAT REV GENET*. 2006; 7:715–727. [PubMed: 16983801]
 36. Shi Y, Whetstine JR. Dynamic regulation of histone lysine methylation by demethylases. *MOL CELL*. 2007; 25:1–14. [PubMed: 17218267]
 37. Xu PF, Zhu KY, Jin Y, Chen Y, Sun XJ, Deng M, Chen SJ, Chen Z, Liu TX. Setdb2 restricts dorsal organizer territory and regulates left-right asymmetry through suppressing fgf8 activity. *Proc Natl Acad Sci U S A*. 2010; 107:2521–2526. [PubMed: 20133783]
 38. Agger K, Cloos PA, Christensen J, Pasini D, Rose S, Rappsilber J, Issaeva I, Canaani E, Salcini AE, Helin K. UTX and JMJD3 are histone H3K27 demethylases involved in HOX gene regulation and development. *NATURE*. 2007; 449:731–734. [PubMed: 17713478]
 39. Lan F, Bayliss PE, Rinn JL, Whetstine JR, Wang JK, Chen S, Iwase S, Alpatov R, Issaeva I, Canaani E, Roberts TM, Chang HY, Shi Y. A histone H3 lysine 27 demethylase regulates animal posterior development. *NATURE*. 2007; 449:689–694. [PubMed: 17851529]
 40. Lieschke GJ, Oates AC, Crowhurst MO, Ward AC, Layton JE. Morphologic and functional characterization of granulocytes and macrophages in embryonic and adult zebrafish. *BLOOD*. 2001; 98:3087–3096. [PubMed: 11698295]
 41. Song HD, Sun XJ, Deng M, Zhang GW, Zhou Y, Wu XY, Sheng Y, Chen Y, Ruan Z, Jiang CL, Fan HY, Zon LI, Kanki JP, Liu TX, Look AT, Chen Z. Hematopoietic gene expression profile in zebrafish kidney marrow. *Proc Natl Acad Sci U S A*. 2004; 101:16240–16245. [PubMed: 15520368]
 42. Meijer AH, van der Sar AM, Cunha C, Lamers GE, Laplante MA, Kikuta H, Bitter W, Becker TS, Spaik HP. Identification and real-time imaging of a myc-expressing neutrophil population involved in inflammation and mycobacterial granuloma formation in zebrafish. *DEV COMP IMMUNOL*. 2008; 32:36–49. [PubMed: 17553562]
 43. Rhodes J, Hagen A, Hsu K, Deng M, Liu TX, Look AT, Kanki JP. Interplay of pu.1 and gata1 determines myelo-erythroid progenitor cell fate in zebrafish. *DEV CELL*. 2005; 8:97–108. [PubMed: 15621533]
 44. Galloway JL, Wingert RA, Thisse C, Thisse B, Zon LI. Loss of gata1 but not gata2 converts erythropoiesis to myelopoiesis in zebrafish embryos. *DEV CELL*. 2005; 8:109–116. [PubMed: 15621534]
 45. Yang D, Okamura H, Teramachi J, Haneji T. Histone demethylase Jmjd3 regulates osteoblast apoptosis through targeting anti-apoptotic protein Bcl-2 and pro-apoptotic protein Bim. *Biochim Biophys Acta*. 2016; 1863:650–659. [PubMed: 26795455]
 46. Zhang F, Xu L, Xu L, Xu Q, Li D, Yang Y, Karsenty G, Chen CD. JMJD3 promotes chondrocyte proliferation and hypertrophy during endochondral bone formation in mice. *J MOL CELL BIOL*. 2015; 7:23–34. [PubMed: 25587042]
 47. Xiang Y, Zhu Z, Han G, Lin H, Xu L, Chen CD. JMJD3 is a histone H3K27 demethylase. *CELL RES*. 2007; 17:850–857. [PubMed: 17923864]
 48. Bennett CM, Kanki JP, Rhodes J, Liu TX, Paw BH, Kieran MW, Langenau DM, Delahaye-Brown A, Zon LI, Fleming MD, Look AT. Myelopoiesis in the zebrafish, *Danio rerio*. *BLOOD*. 2001; 98:643–651. [PubMed: 11468162]
 49. Scott EW, Simon MC, Anastasi J, Singh H. Requirement of transcription factor PU.1 in the development of multiple hematopoietic lineages. *SCIENCE*. 1994; 265:1573–1577. [PubMed: 8079170]
 50. McKercher SR, Torbett BE, Anderson KL, Henkel GW, Vestal DJ, Baribault H, Klemsz M, Feeney AJ, Wu GE, Paige CJ, Maki RA. Targeted disruption of the PU.1 gene results in multiple hematopoietic abnormalities. *EMBO J*. 1996; 15:5647–5658. [PubMed: 8896458]

51. Li Y, Okuno Y, Zhang P, Radomska HS, Chen H, Iwasaki H, Akashi K, Klemsz MJ, McKercher SR, Maki RA, Tenen DG. Regulation of the PU.1 gene by distal elements. *BLOOD*. 2001; 98:2958–2965. [PubMed: 11698277]
52. Huang G, Zhang P, Hirai H, Elf S, Yan X, Chen Z, Koschmieder S, Okuno Y, Dayaram T, Gowney JD, Shivdasani RA, Gilliland DG, Speck NA, Nimer SD, Tenen DG. PU.1 is a major downstream target of AML1 (RUNX1) in adult mouse hematopoiesis. *NAT GENET*. 2008; 40:51–60. [PubMed: 17994017]
53. Mikkelsen TS, Ku M, Jaffe DB, Issac B, Lieberman E, Giannoukos G, Alvarez P, Brockman W, Kim TK, Koche RP, Lee W, Mendenhall E, O'Donovan A, Presser A, Russ C, Xie X, Meissner A, Wernig M, Jaenisch R, Nusbaum C, Lander ES, Bernstein BE. Genome-wide maps of chromatin state in pluripotent and lineage-committed cells. *NATURE*. 2007; 448:553–560. [PubMed: 17603471]
54. Alvarez-Errico D, Vento-Tormo R, Sieweke M, Ballestar E. Epigenetic control of myeloid cell differentiation, identity and function. *NAT REV IMMUNOL*. 2015; 15:7–17. [PubMed: 25534619]
55. Huang HT, Kathrein KL, Barton A, Gitlin Z, Huang YH, Ward TP, Hofmann O, Dibiasi A, Song A, Tyekucheva S, Hide W, Zhou Y, Zon LI. A network of epigenetic regulators guides developmental haematopoiesis in vivo. *NAT CELL BIOL*. 2013; 15:1516–1525. [PubMed: 24240475]
56. Wei Y, Chen R, Dimicoli S, Bueso-Ramos C, Neuberg D, Pierce S, Wang H, Yang H, Jia Y, Zheng H, Fang Z, Nguyen M, Ganan-Gomez I, Ebert B, Levine R, Kantarjian H, Garcia-Manero G. Global H3K4me3 genome mapping reveals alterations of innate immunity signaling and overexpression of JMJD3 in human myelodysplastic syndrome CD34+ cells. *LEUKEMIA*. 2013; 27:2177–2186. [PubMed: 23538751]
57. Nakata Y, Yamasaki N, Ueda T, Ikeda K, Nagamachi A, Inaba T, Honda H. JMJD3 Plays Essential Roles in the Maintenance of Hematopoietic Stem Cells and Leukemic Stem Cells through the Regulation of p16INK4a. *Blood*. 2016; 128:2653–2653. [Accessed August 27, 2017] Retrieved from <http://www.bloodjournal.org/content/128/22/2653>.
58. Monteiro R, Pouget C, Patient R. The gata1/pu.1 lineage fate paradigm varies between blood populations and is modulated by tlf1gamma. *EMBO J*. 2011; 30:1093–1103. [PubMed: 21336259]
59. Wei W, Wen L, Huang P, Zhang Z, Chen Y, Xiao A, Huang H, Zhu Z, Zhang B, Lin S. Gfi1.1 regulates hematopoietic lineage differentiation during zebrafish embryogenesis. *CELL RES*. 2008; 18:677–685. [PubMed: 18504458]
60. Yuan H, Zhou J, Deng M, Zhang Y, Chen Y, Jin Y, Zhu J, Chen SJ, de The H, Chen Z, Liu TX, Zhu J. Sumoylation of CCAAT/enhancer-binding protein alpha promotes the biased primitive hematopoiesis of zebrafish. *BLOOD*. 2011; 117:7014–7020. [PubMed: 21596856]
61. Steidl U, Rosenbauer F, Verhaak RG, Gu X, Ebralidze A, Otu HH, Klippel S, Steidl C, Bruns I, Costa DB, Wagner K, Aivado M, Kobbe G, Valk PJ, Passegue E, Libermann TA, Delwel R, Tenen DG. Essential role of Jun family transcription factors in PU.1 knockdown-induced leukemic stem cells. *NAT GENET*. 2006; 38:1269–1277. [PubMed: 17041602]
62. Zhu X, Zhang H, Qian M, Zhao X, Yang W, Wang P, Zhang J, Wang K. The significance of low PU.1 expression in patients with acute promyelocytic leukemia. *J HEMATOL ONCOL*. 2012; 5:22. [PubMed: 22569057]
63. Rosenbauer F, Wagner K, Kutok JL, Iwasaki H, Le Beau MM, Okuno Y, Akashi K, Fiering S, Tenen DG. Acute myeloid leukemia induced by graded reduction of a lineage-specific transcription factor, PU.1. *NAT GENET*. 2004; 36:624–630. [PubMed: 15146183]
64. Mueller BU, Pabst T, Osato M, Asou N, Johansen LM, Minden MD, Behre G, Hiddemann W, Ito Y, Tenen DG. Heterozygous PU.1 mutations are associated with acute myeloid leukemia. *BLOOD*. 2002; 100:998–1007. [PubMed: 12130514]
65. Huang G, Zhao X, Wang L, Elf S, Xu H, Zhao X, Sashida G, Zhang Y, Liu Y, Lee J, Menendez S, Yang Y, Yan X, Zhang P, Tenen DG, Osato M, Hsieh JJ, Nimer SD. The ability of MLL to bind RUNX1 and methylate H3K4 at PU.1 regulatory regions is impaired by MDS/AML-associated RUNX1/AML1 mutations. *BLOOD*. 2011; 118:6544–6552. [PubMed: 22012064]

66. Miller SA, Mohn SE, Weinmann AS. Jmjd3 and UTX play a demethylase-independent role in chromatin remodeling to regulate T-box family member-dependent gene expression. *MOL CELL*. 2010; 40:594–605. [PubMed: 21095589]
67. Zhao W, Li Q, Ayers S, Gu Y, Shi Z, Zhu Q, Chen Y, Wang HY, Wang RF. Jmjd3 inhibits reprogramming by upregulating expression of INK4a/Arf and targeting PHF20 for ubiquitination. *CELL*. 2013; 152:1037–1050. [PubMed: 23452852]

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Fig.1. *Jmjd3* is expressed in the hematopoietic site and mediates H3K27 histone demethylase activity *in vivo*

(A) WISH analysis of *jmjd3* expression at the embryonic stages indicated. PBI indicates posterior blood island, CHT indicates caudal hematopoietic tissue. The arrows showed a higher magnification of region in the PBI or CHT as defined by the rectangle. All lateral views, anterior to the left. (B) qRT-PCR expression analysis of *jmjd3* as a linear ratio to *gapdh* expression at the embryonic stages indicated. In the two far-right columns, *jmjd3* expression was probed in *spi1*: EGFP and *gata1*: EGFP sorted cells from transgenic zebrafish embryos at 22 hpf. (C) Western blot analyses of histone methylation levels in the control or the *jmjd3* morphants at 22 hpf. The Arabic numbers at the Bottom denote the extent of methylation modification compared with histone H3 level by densitometry. Data are shown as the mean $\pm$ SEM of at least three independent experiments. * $p < 0.05$, ** $p < 0.01$ versus the corresponding control.

Fig.2. Knockdown of *jmd3* perturbs primitive and definitive myelopoiesis in zebrafish
 (A–H) WISH analyses of *lyz* (A), *mpx* (C) *nephrosin* (E) or *I-plastin* (G) expression in control or *jmd3* morphants at 22hpf and quantification of *lyz*⁺ cells (B), *mpx*⁺ cells, (D) *nephrosin*⁺ cells (F) or *I-plastin*⁺ cells (H) within individual larvae. (I–L) WISH analyses of *lyz* (I) or *mpx* (K) expression in control or *jmd3* morphants at 36hpf and quantification of *lyz*⁺ cells (J) or *mpx*⁺ cells (L) within individual larvae. All lateral views, anterior to the left. Data are shown as the mean ± SEM of at least three independent experiments. *** $p < 0.001$ versus the corresponding control.

Fig.3. *Jmjd3* promotes myeloid commitment of erythromyeloid progenitors

(A–D) WISH analyses of *spi1* expression in the control or *jmjd3* morphants at 20hpf (A) or 22hpf (C) and quantification of *spi1*⁺ cells at 20hpf (B) or 22hpf (D) within individual larvae. (E) WISH analyses of *spi1* expression in the control or *jmjd3* morphants at 32hpf. Black arrows indicate PBI region. (F) qRT-PCR assay on the *spi1* mRNA level in the control or *jmjd3* morphants at the embryonic stages indicated. (G) WISH analyses of *gata1* expression in the control or *jmjd3* morphants at 20hpf. (H) WISH analyses of β -E3 or α -E1 expression in the control or *jmjd3* morphants at 22 hpf. (I) WISH analyses of α -E1 expression in the control or *jmjd3* morphants at 36 hpf. (J) qRT-PCR assay on the *gata1*, β -

E3 and *α-E1* mRNA levels in the control and *jmjd3* morphants at the embryonic stages indicated. A, C, E, G, I, lateral views, anterior to the left. H, top left corner, lateral views; H, lower right corner, dorsal views; all are anterior to the left. Data are shown as the mean ± SEM of at least three independent experiments. Data are shown as the mean ± SEM of at least three independent experiments. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ versus the corresponding control.

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Fig.4. Myeloid cells in *jmd3* morphants exhibit reduced proliferation and evaluated apoptosis (A–F) Double staining of *lyz*-EGFP and pH3 (Green: EGFP, Red: pH3) showing pH3 incorporation of CHT *lyz*-EGFP⁺ cells in the control or *jmd3* morphants at 36 hpf. (G) Statistical data showing the percentage of *lyz*-EGFP⁺ myeloid cells for pH3. (H–M) Double staining of *lyz*-EGFP and TUNEL (Green: EGFP, Red: TUNEL) showing TUNEL incorporation of CHT *lyz*-EGFP⁺ cells in the control or *jmd3* morphants at 36 hpf. (N) Statistical data showing the percentage of *lyz*-EGFP⁺ myeloid cells for TUNEL. Arrows indicate *lyz*-EGFP/pH3 or *lyz*-EGFP/TUNEL double positive cell. Data are shown as the mean $\pm$ SEM, at least 6 embryos were analysis for each experimental group. * $p < 0.05$ versus the corresponding control.

Fig.5. Jmjd3 promotes myelopoiesis in a demethylase-dependent manner

(A–O) WISH analyses of *spi1* (A, D, G, J, M), *lyz* (B, E, H, K, N) and *mpx* (C, F, I, L, O) expression in the control morphants, *jmd3* morphants, embryos coinjected with *jmd3* MO and zebrafish *jmd3* mRNA, embryos coinjected with *jmd3* MO and human *Jmjd3* mRNA or embryos coinjected with *jmd3* MO and human *H1390A* mRNA at 22 hpf and quantification of *spi1*⁺ (P) cells, *lyz*⁺ (Q) cells or *mpx*⁺ (R) within individual larvae. All lateral views, anterior to the left. Data are shown as the mean $\pm$ SEM of at least three independent experiments. *** $p < 0.001$ versus the corresponding control.

Fig.6. Co-injection of *spi1* mRNA rescues the impaired myeloid commitment caused by *jmd3* deficiency

(A–B) WISH analyses of *lyz* (A) expression in the control morphants, *jmd3* morphants or embryos coinjected with *jmd3* MO and *spi1* mRNA at the embryonic stages indicated and quantification of *lyz*⁺ (B) cells within individual larvae. (C–D) WISH analyses of *mpx* (C) expression in the control morphants, *jmd3* morphants or embryos coinjected with *jmd3* MO and *spi1* mRNA at the embryonic stages indicated and quantification of *mpx*⁺ (D) cells within individual larvae. All lateral views, anterior to the left. Data are shown as the mean ±

SEM of at least three independent experiments. *** $p < 0.001$ versus the corresponding control.

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