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LSD1/KDM1A promotes hematopoietic commitment of hemangioblast through downregulation of 
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Abstract

The hemangioblast is a progenitor cell with the capacity to give rise to both hematopoietic and endothelial progenitors. Currently, the regulatory mechanisms underlying the hemangioblast formation are being elucidated, while those for the selection of hematopoietic or endothelial fates still remain a mystery. To answer these questions, we screened for zebrafish mutants that have defects in the hemangioblast expression of Gata1, which is never expressed in endothelial progenitors. One of the isolated mutants, it627, showed not only downregulation of hematopoietic genes, but also upregulation of endothelial genes. We identified the gene responsible for the it627 mutant as the zebrafish homolog of lysine specific demethylase 1 (LSD1/KDM1A). Surprisingly, the hematopoietic defects in lsd1it627 embryos were rescued by the gene knockdown of etv2, an essential regulator for vasculogenesis. Our results suggest that the LSD1-dependent shutdown of the Etv2 gene expression may be a significant event required for hemangioblasts to initiate hematopoietic differentiation.

Significance Statement

Epigenetic regulation of cell fate determination is one of the hottest topics today. In this paper, we isolated and characterized a zebrafish mutant in histone lysine demethylase LSD1/KDM1A, and found that LSD1 plays a role in the initiation of hematopoietic differentiation in the hemangioblast, a bipotent cell that can give rise to hematopoietic or endothelial progenitors. In addition, we identified the major function of LSD1 in hemangioblasts to be the downregulation of Etv2, a critical regulator of hemangioblast development. Our results suggest that the LSD1-dependent shutdown of the Etv2 gene is the significant event required for hemangioblasts to initiate hematopoietic differentiation.

Introduction

It has been proposed that hematopoietic and endothelial cells share a common progenitor, termed hemangioblast, based largely on the intimate association of these lineages in the blood islands of the developing yolk sac (1-3). The sharing of markers between blood and blood vessel cells, and the impairment of both tissues in mutants, such as the mouse Flk1 knockout (4) and zebrafish cloche (5), are consistent with a common origin. Evidence for the presence of hemangioblasts has been provided by clonal studies using in vitro differentiating mouse and human embryonic stem (ES) cells (6-8) and in vivo analyses.
of both mouse and zebrafish embryos (9-11).

Precisely how blood cells are being formed from the hemangioblasts has been a topic of much interest. Although several transcription factors have been suggested to be required for hemangioblast formation, it is largely unknown how presumptive hematopoietic cells are selected from hemangioblasts to differentiate into the individual hematopoietic lineages. The transcription factor Scl/Tal1 has been demonstrated to play a critical role in the generation of the hemogenic endothelium, an endothelial intermediate between hemangioblasts and hematopoietic cells, but not in the development of hemangioblasts (12). Another transcription factor, Runx1/AML1, is dispensable for the generation of the hemogenic endothelium, but is required for the production of definitive hematopoietic precursors from the endothelium (12, 13). In this way, the molecular mechanisms of hematopoietic development from the hemangioblast are beginning to be understood, but many pieces of this puzzle are still missing.

To identify these missing pieces, we focused our attention on the regulation of the Gata1 gene expression. Gata1 encodes a transcription factor that transactivates a variety of erythropoietic genes. Gata1 gene knockout mice and mutant zebrafish have been demonstrated to be lethally anemic because of drastic defects in erythropoiesis, and Gata1 has been considered to be a key factor required for erythropoiesis (14-16). Interestingly, the expression of Gata1 appears to define the hematopoietic commitment stage, because Gata1-positive cells display hematopoietic, but not endothelial, differentiation potential (17). We hypothesized that key factors involved in the commitment to the blood cell lineage are related to the factor(s) regulating Gata1 expression. The regulators of the Gata1 expression have been studied for two decades, but it is still unknown how the Gata1 expression is regulated in the early developmental stage, such as in hemangioblasts (18).

In this paper, we tried to identify the factors that regulate the Gata1 gene in the early developmental stage through a genetic approach using zebrafish. We isolated a zebrafish mutant whose gata1 expression is reduced, and identified the responsible gene as the zebrafish homolog of lysine specific demethylase 1 (LSD1/KDM1A). LSD1 belongs to the flavin adenine dinucleotide (FAD)-dependent amine oxidases which demethylates mono- and di-methylated lysine 4 (K4) and/or 9 (K9) within histone H3 through an oxidative process (19) and has been suggested to play key roles in multiple aspects of cell differentiation through epigenetic regulation (20).

The expression analyses of a variety of marker genes in lsd1 mutant embryos revealed that hematopoietic progenitors were reduced, while the number of endothelial progenitors were increased. Among a variety of hemangioblast markers examined, we found that the expression of etv2, which is only expressed transiently in early progenitors in the mesoderm (21), was upregulated in this mutant, suggesting that the hemangioblasts in the mutant embryos were generated but failed to initiate hematopoietic differentiation in the early step. It is noteworthy that the gene knockdown of etv2 could rescue the hematopoietic phenotypes
of lsd1 mutants, suggesting that the upregulation of etv2 was a principal cause of their hematopoietic defects. Based on these results, we hypothesized that the shutdown of the Etv2 gene expression is a critical event required for hemangioblasts to initiate hematopoietic differentiation, and that LSD1 regulates this event.

Results

LSD1 is a responsible gene for the hematopoietic mutant *it627*. A genetic screen was conducted to isolate zebrafish mutants that showed decreased *gata1* expression in presumptive erythroid progenitors. *it627* is one of the mutants isolated from this screen. The expression of the zebrafish Gata1 gene (*gata1*) is first observed in the posterior lateral plate mesoderm (LPM) at 11 hours post-fertilization (hpf) and is maintained after the formation of the intermediate cell mass (ICM)(22). The *it627* mutant showed decreased expression of *gata1* in both the LPM and ICM (Fig. 1A). The *it627* mutation mapped by positional cloning to the zebrafish homolog gene for LSD1 (*lsd1*), which is a founding member of histone demethylase family (23). Zebrafish *lsd1* encodes a protein of 830 amino acids that shares 85% homology with human LSD1. LSD1 has been demonstrated to demethylate mono- and di-methylated lysine 4 (K4) and/or 9 (K9) within histone H3 (19). Fig. S1A shows a phylogenetic tree, which indicates the high conservation of LSD1 proteins among vertebrates (24).

We found a C to T nonsense mutation in exon 17 only in the DNA isolated from the mutants. As shown in Fig. 1B, LSD1 contains several structural domains, including AOD-N and AOD-C (the N- and C-terminal parts of the amine oxidase domain, respectively). The mutation replaces Gln609 with a stop codon that leads to an approximately 200 amino acid truncation of AOD-C. AOD-C contains the FAD- and substrate-binding subdomains, suggesting that the truncation eliminates its enzymatic activity. The demethylase activity of the *it627*-type LSD1ΔC609 protein was investigated to test this assumption (Fig. 1C). Wild-type LSD1 protein demethylated mono- and di-methylated histone H3-K4 but not K9, consistent with the human and *Drosophila* LSD1 proteins (25, 26). In contrast, the LSD1ΔC609 protein was unable to demethylate either methylated-histone H3-K4 or K9, suggesting that the LSD1 protein in *lsd1*mutants has lost its enzymatic activity.

The effects of *lsd1* knockdown in zebrafish embryos were analyzed using morpholino techniques (Fig. S1B). The injection of morpholino antisense oligonucleotides against *lsd1* (1 pmole) resulted in a marked reduction of *gata1* expression, but the effect was eliminated when five-base mismatches were introduced. Moreover, the *gata1* expression was reduced in wild-type embryos treated with tranylcypromine (Fig. S1C), a well-known LSD inhibitor (27, 28). We further examined whether an LSD1 overexpression could rescue *gata1* expression in *lsd1*mutants or not (Fig. S1D). Mutant embryos overexpressing wild-type LSD1
showed a complete rescue of the *gata1* expression, while an overexpression of LSD1ΔC609 failed to restore it. Since overexpressed LSD1ΔC609 protein was unstable compared to the wild-type LSD1 (Fig. S1E), this may be another reason, in addition to its enzymatic inactivity, why it could not rescue the phenotype in *lsd1*it627 mutants.

**LSD1 is essential for zebrafish development.** No obvious morphological defects, except for blood circulation, were observed in *lsd1*it627 embryos before hatching [2.5 days post-fertilization (dpf)]. It was surprising to find that the obvious phenotype of *lsd1*it627 embryos was the only defect in hematopoiesis. This is in contrast to LSD1 knockout mice, which die around 6.5-7.5 days postcoitum, corresponding to the developmental stage of about 10 hpf in zebrafish (29-31). One possible explanation for this discrepancy between zebrafish and mice may be the differences in the expression profiles of LSD1 genes. To test this possibility, the expression of *lsd1* was analyzed using wild-type and *lsd1*it627 mutant embryos (Fig. S2A). We found that *lsd1* was expressed maternally in the early stage embryos, which may be the reason for the phenotypic differences of LSD1-deficient animals between zebrafish and mice, because it was later ubiquitously expressed in every stage. Interestingly, the zygotic expression of *lsd1* was greatly downregulated in *lsd1*it627 mutants, suggesting that *lsd1* positively regulates itself. Taking this observation into account along with the low enzymatic activity and protein instability of the *it627*-type LSD1ΔC609 protein, *lsd1*it627, it was deduced to be a null mutation of *lsd1* in zebrafish.

Since the dispensability of LSD1 in zebrafish embryonic stages could be explained by its maternal expression, the viability of *lsd1*it627 mutants was next investigated in later larval stages (Fig. S2B). Homozygous mutants died around 10 dpf, indicating that *lsd1* is an essential gene required for zebrafish development. Interestingly, the swim bladder of *lsd1*it627 larvae failed to inflate, while that of a blood-less mutant, *vlad tepes* (*gata1*m651), was normal, as observed in wild-type larvae (Fig. S2C), thus suggesting that there are other non-hematopoietic functions of LSD1 in the larval stage.

**Erythropoiesis and ectopic myelopoiesis are impaired in LSD1 mutants.** We next analyzed the detailed *gata1* expression in *lsd1*it627 embryos (Fig. S3A). In wild-type embryos, the expression of *gata1* in the posterior LPM was initiated around 11 hpf, and its level was upregulated until 15 hpf. In *lsd1*it627 embryos, the *gata1* expression at 12 hpf was similar to that in wild-type siblings, but its upregulation after 13 hpf was impaired, suggesting that LSD1 is not required for the initiation of *gata1*, but is required for its upregulation. To examine whether defects of the *gata1* upregulation by the *lsd1* mutation affect the expression of Gata1 downstream genes (16, 32, 33), the expression of *hbbe1*, *alas2* and *klfd* was analyzed and demonstrated to be greatly reduced in *lsd1*it627 embryos (Fig. 2A). Cell staining with *o*-dianisidine, which reflects hemoglobin production, revealed that there were fewer *o*-dianisidine-positive cells in *lsd1*it627 embryos in comparison to the wild-type siblings (Fig. 2A).

In wild-type embryos, blood circulation initiates around 27 hpf, while in *lsd1*it627 it began after 32 hpf.
The number of circulating blood cells was dramatically lower in \textit{lsd1}\textsuperscript{it627} embryos at 2 dpf in comparison to wild-type embryos (Movies S1 and S2). The difference in cell numbers became less apparent at 4 dpf, while the shapes of blood cells seem to be distinct (Movies S3 and S4). To examine the morphology of the blood cells, we isolated circulating blood cells (Fig. 2B). In 4 dpf larvae, 40\% of the wild-type cells were large immature erythroblasts and 60\% were small mature erythrocytes, while almost all of them were large in the \textit{lsd1}\textsuperscript{it627} larvae. Later at 6 dpf, 40\% of the cells isolated from \textit{lsd1}\textsuperscript{it627} larvae became mature erythrocytes, but the rest were still immature (Movies S5 and S6, Fig. 2B). All these results indicate that the larvae with the LSD1 mutation have defects in the differentiation of erythroid cells.

In contrast to erythropoietic markers, no differences were observed between the wild-type and \textit{lsd1}\textsuperscript{it627} embryos in the expression of myelopoietic markers, such as \textit{pu.1} (Fig. 2C, left panels). In zebrafish, it has been demonstrated that the downregulation of \textit{gata1} by gene mutations or gene knockdown, reprograms the fate of the erythroid lineage to a myeloid lineage via the upregulation of \textit{pu.1} (33, 34). Since \textit{gata1} was significantly downregulated in \textit{lsd1}\textsuperscript{it627} embryos, ectopic \textit{pu.1} expression in ICM can be induced, but this did not happen (Fig. 2C, middle panels).

We thought that the level of the \textit{gata1} downregulation in \textit{lsd1}\textsuperscript{it627} embryos might have been insufficient to induce ectopic \textit{pu.1} expression, and injected a \textit{gata1}-specific antisense morpholino oligonucleotide (\textit{gata1MO}, 0.5 pmole) into the embryos to further reduce the \textit{gata1} expression (Fig. 2C, right panels). Unexpectedly, \textit{gata1MO}-induced ectopic \textit{pu.1} expression in the ICM was only observed in wild-type siblings (wild-type 98\% n=41, heterozygous mutant 97\% n=74), and not in \textit{lsd1}\textsuperscript{it627} embryos (homozygous 6.7\% n=45), thus suggesting that ectopic myelopoiesis was impaired by the LSD1 mutation. To confirm this observation, we used \textit{gata1} mutant embryos (\textit{gata1m621}) and an LSD1 inhibitor, tranylcypromine, instead of the \textit{gata1MO} injection and \textit{lsd1}\textsuperscript{it627} mutation, respectively, and compared the effects (Fig. S3B). Again, the ectopic \textit{pu.1} expression was induced in \textit{gata1}\textsuperscript{m621} embryos, while the induction was abrogated when the embryos were treated with tranylcypromine. These results suggest that hematopoietic defects brought on by the \textit{lsd1}\textsuperscript{it627} mutation could not be fully explained by the downregulation of \textit{gata1}.

**Upregulation of \textit{etv2} and endothelial markers in LSD1 mutants.** The presence of defects in both erythropoiesis and myelopoiesis suggests that the generation of common hematopoietic progenitors, such as hemangioblasts, may be impaired by the LSD1 mutation. We therefore analyzed the expression profiles of two hemangioblast markers, \textit{scl} and \textit{lmo2}, but no differences were observed between wild-type and \textit{lsd1}\textsuperscript{it627} embryos (Fig. S4). This result implies that the LSD1 mutation has no effect on the hemangioblast formation. To confirm this assumption, the expression of a variety of hemangioblast markers was analyzed using embryos at 12 hpf when hemangioblasts were forming (Fig. S5A). The \textit{etv2} expression was slightly upregulated in \textit{lsd1}\textsuperscript{it627} embryos compared to wild-type embryos, while none of the other markers (\textit{fli1a, scl, lmo2, gata2, cdx4, draculin, ets1 and hhex}) was affected. The upregulation of \textit{etv2} was not observed in
11.5-hpf embryos, suggesting that LSD1 is not involved in the initiation of the etv2 expression (Fig. 3A). Interestingly, the upregulation was only observed in the posterior, not anterior, LPM (Fig. 3A).

We also analyzed expression of hemangioblast markers in 15-hpf embryos and observed enhanced expression of fli1a in addition to etv2, in lsd1<sup>it627</sup> embryos, while there were again no differences in the other markers (Fig. S5B). Since etv2 has been shown to positively regulate the expression of endothelial genes (35-37), the expression of fli1a and other endothelial markers (flk1 and tie1) was analyzed at later developmental stages (Figs. 3B, 3C, and S6). As expected, the expression levels of all three endothelial markers were upregulated in lsd1<sup>it627</sup> embryos. It was noted that the expression of these markers in the hematopoietic region at 24 hpf was decreased in wild-type embryos, but not in lsd1<sup>it627</sup> mutants (Fig. 3C, blue arrows). Instead, hematopoietic marker biklf, was downregulated in this subregion (red arrow).

To answer the question whether excess endothelialization occurs in LSD1 mutants, we crossed lsd1<sup>it627</sup> mutant with Tg(kdrl:EGFP)<sup>843</sup> line, which can visualize vasculogenesis (38), and analyzed the GFP expression in progeny lines. No obvious difference in the GFP expression was observed between lsd1<sup>it627</sup> mutants and wild-type embryos (Fig. S7), suggesting that the upregulation of endothelial markers did not lead to excess endothelialization. Considering these results together with the previous finding that the zebrafish Etv2 was a positive regulator of endothelial and anterior hematopoietic development (35, 39, 40), we speculated that the hematopoietic cells in the ICM region of lsd1<sup>it627</sup> mutants were maintained in an undifferentiated state with endothelial characteristics.

**Knockdown of the Etv2 gene can rescue the hematopoietic phenotypes of LSD1 mutants.** The earliest difference between wild-type and lsd1<sup>it627</sup> mutants in the expression of hematopoietic/endothelial markers was etv2 at 12 hpf (Fig. S5A) and next gata1 at 13 hpf (Fig. S3A). The fli1a upregulation in lsd1<sup>it627</sup> mutants became obvious at 15 hpf, which was later compared to these genes (Figs. S5 and S6). To elucidate the relationship between these three genes, the expression of etv2 and fli1a was first analyzed in gata1<sup>it624</sup> embryos (Fig. S5B). No effect by the gata1 mutation on the expression of either genes was observed, suggesting that the upregulation of etv2 and fli1a in lsd1<sup>it627</sup> embryos was not due to the gata1 reduction. Second, we examined the effects of the etv2 knockdown on the expression of gata1 and fli1a in lsd1<sup>it627</sup> embryos. The reduced expression of gata1 in lsd1<sup>it627</sup> embryos was rescued by injecting antisense morpholino oligonucleotides against etv2 (etv2MO, 0.5 pmole)[Figs. 4A, 96% n=30 (n: homozygous)], while this effect was eliminated when five-base mismatches were introduced into the etv2MO (0.5 pmole, 0% n=5). Importantly, the etv2 knockdown upregulated only the reduced gata1 expression in lsd1<sup>it627</sup> embryos, not its normal expression in wild-type embryos. In contrast, the expression of fli1a was not altered by the etv2 knockdown (Fig. S8A, 0% reduced n=15), suggesting that etv2 is not the reason for the fli1a upregulation, consistent with a previous report showing etv2-independent regulation of fli1a in the posterior LPM (41).
To elucidate the effects of the etv2 knockdown on erythropoiesis, we analyzed the expression of alas2 and biklf and found that the reduced expression of these genes in lsd1\textsuperscript{it627} embryos was rescued by etv2MO injection (Figs. S8B and C, alas2 93\% n=14, biklf 83\% n=12). To clarify the effects of the etv2 knockdown on myelopoiesis, the ectopic pu.1 expression in gata1-etv2 double knockdown lsd1\textsuperscript{it627} embryos was analyzed (Fig. 4B). The defects of ectopic pu.1 expression in lsd1\textsuperscript{it627} embryos (open arrowhead) were rescued by injecting etv2MO (closed arrowhead, 76\% positive n=21). We also tested the effects of the etv2 knockdown on the endothelial markers, flk1 and tie1 (Figs. S8D and E), and showed that the upregulated expression of these genes in lsd1\textsuperscript{it627} embryos was reduced by the etv2 knockdown (flk1 100\% reduced n=13, tie1 100\% n=6). All of these results suggest that the upregulation of etv2 is the principal cause of the hematopoietic defects and the upregulation of endothelial markers in lsd1\textsuperscript{it627} mutants.

To examine whether LSD1 modifies the in vivo methylation status of histone H3-K4 at the etv2 locus, chromatin immunoprecipitation (ChIP) analysis was carried out using LSD1 morphants (Fig. 4C). We analyzed histone methylation at three etv2 enhancers (up1, int2 and the 35 bp enhancer in the promoter), identified by Veldman and Lin (42), and at exon 8 (gene body). The results demonstrated that mono- and/or di-methylated histone H3-K4 was increased by LSD1 knockdown at the int2 and 35 bp enhancers, suggesting that LSD1 directly downregulates etv2 by histone modification.

Discussion

In this paper, we isolated a zebrafish mutant that shows impaired hematopoiesis, and identified that lsd1 was the responsible gene. Not only erythropoiesis, but also ectopic myelopoiesis, was diminished in the lsd1\textsuperscript{it627} mutants. The earliest hematopoietic/endothelial abnormality detected in the lsd1\textsuperscript{it627} embryos was the upregulation of etv2 in hemangioblasts. The gene knockdown of etv2 rescued both the erythropoietic and myelopoietic defects in lsd1\textsuperscript{it627} embryos.

The importance of LSD1 in hematopoiesis has previously been shown using cultured cells (43-45), knockdown mice (46) and conditional knockout mice (47). In addition, the contribution of LSD1 in leukemogenesis has been demonstrated, and the use of LSD1 inhibitors is being investigated as a new therapeutic strategy (48, 49). The roles of LSD1 in hematopoiesis are various, and include the differentiation switch between erythropoiesis and myelopoiesis (43), the terminal differentiation of some hematopoietic lineages (46, 47), maintenance of the undifferentiated state of plasma cells (45), globin switching (50-52), and the self-renewal of hematopoietic stem cells (47). Here, we unveiled new roles of LSD1, as downregulator of the endothelial characteristics and promoter of the hematopoietic commitment in hemangioblasts. Intriguingly, LSD1 seems to play many roles in hematopoiesis, probably binding to gene regulatory regions of key hematopoietic factors throughout the hematopoietic period, and regulating them.
positively and/or negatively depending on the differentiation states. For its multifunctional roles LSD1 may have a variety of hematopoietic partners (43-45, 51). Future studies will be needed to identify the LSD1 partners in hemangioblasts.

Another important finding of the present study is that the main role of LSD1 in hemangioblasts is the shutdown of the Etv2 expression (Fig. S9). LSD1 may not directly transactivate the Gata1 gene, but rather, regulate the differentiation of weakly Gata1-positive hemangioblasts to generate strongly Gata1-positive hematopoietic progenitors. It is not easy to understand why the knockdown of *etv2*, which is essential for hematopoiesis rather than just endothelial development, can rescue the hematopoietic phenotypes of *lsd1*\textsuperscript{it627} embryos. An important observation is that the LSD1 mutation reduced only posterior, not anterior, hematopoiesis, and the upregulation of *etv2* was only detected in the posterior LPM (see Fig. 3A). Previous reports demonstrated that *etv2* plays no role in erythroid and posterior myeloid differentiation (39, 40), while it is strongly required for posterior endothelial development (36, 39). These, combined with our findings indicate that the importance of the LSD1-Etv2 paradigm in hemangioblast differentiation may be restricted in the posterior hemangioblasts. We hypothesize that the Etv2 shutdown in hemangioblasts is an important process for hematopoiesis.

It is interesting that the expression of mouse Etv2 was transiently detected in the beginning of hematopoiesis, and was barely detectable in hematopoietic tissues at later stages, such as the aorta-gonad-mesonephros and fetal liver in embryos and in the bone marrow and spleen in adults (53-55). A dramatic downregulation of Etv2 during hematopoietic differentiation is also observed in zebrafish (35) and *Xenopus* (56). The importance of the Etv2 shutdown in hematopoiesis has been experimentally demonstrated in mice (57). When Etv2 was persistently expressed in hematopoietic tissues using transgenic mouse techniques, abnormal hematopoiesis, especially erythropoiesis, occurred in both primitive and definitive hematopoiesis. Hayashi et al. have proposed a model where persistent endothelialization due to forced-Etv2 expression inhibited hematopoiesis (57). Their model fits well with our current results in zebrafish. It is well known that the Etv2 overexpression also results in endothelialization in zebrafish (35, 37, 58, 59) and *Xenopus* (56). All these observations suggest that Etv2 may play a conserved role in maintaining the endothelial characteristics of hemangioblasts in the early developmental periods.

Hematopoietic cells in *lsd1*\textsuperscript{it627} mutants may change their fate to become endothelial cells. However, this is considered to be unlikely, because no ectopic nor thickened blood vessels were detected in this mutant (see Fig. S7). Instead, we believe that the differentiation of hemangioblasts to hematopoietic cells may be suspended, and as a result, immature hemangioblasts which possess endothelial characteristics are accumulated. LSD1 has been proposed to be involved in shutting down programs of ES cells through enhancer decommission and to promote cell differentiation (60). A similar situation may occur in hemangioblasts. As observed for Sox2 or Fbox15 in the case of ES cells, LSD1 may also shut down Etv2 in
hemangioblasts.

**Materials and Methods**

**Fish and chemicals.** AB and TL strains were used as wild-type zebrafish. The Tg(kdrl:EGFP)<sup>6843</sup> transgenic line was described previously (38). For genotyping lsd1<sup>it627</sup>, genomic DNA at the mutation sites was amplified by PCR using the primers: 5’-ctggcagaggagggacaaactgcacggccggcggcagct and 5’-gcagtcatctgactgtgcg and were digested with PvuII. Genotyping of gata1<sup>m651</sup> was carried out as described previously (16). Tranlycypromine was purchased from Enzo Life Sciences.

**N-ethyl-N-nitrosourea mutagenesis.** N-ethyl-N-nitrosourea mutagenesis was carried out as described previously (61). Seven hundred and sixteen F2 families were screened, and four mutants with reduced gata1 expression were isolated. The gene locus of the it627 mutant was roughly mapped by a bulked-segregant analysis using 333 polymorphic Z-markers (62). Fine mapping with additional polymorphic markers was carried out using 1,656 homozygous mutant embryos in the AB/TL hybrid F1 generation. Polymorphic markers were designed utilizing the database of the zebrafish genome project (http://www.ensembl.org/Danio_rerio/Info/Index).

**cDNA cloning.** Full length cDNA of lsd1 was synthesized by RT-PCR and subcloned into the pBluescript II KS, pET15b and pCS2FL (63) vectors. The constructs pET15lsd1 and pCS2FL<sup>Δ</sup>lsd1 were made by introducing a Gln-to-Stop point mutation at amino acid 609. The nucleotide sequence data of zebrafish lsd1 have been deposited in the DDBJ/EMBL/GenBank databases with the accession number AB494456.

**Staining and imaging analysis.** Whole-mount in situ hybridization was performed using digoxigenin-labelled RNA probes as described previously (64). The o-dianisidine staining was performed as described previously (22). Blood cells for Wright-Giemsa staining were collected from embryos after cardiac puncture, followed by cytospin concentration at 700 rpm for three minutes using a Cytospin 4 (Thermo Scientific). Fluorescent images were taken with Leica SP5 confocal or Leica AF6000 epifluorescence microscopes. Genotyping of the stained embryos was carried out after taking photos.

**Demethylation assay.** A histone demethylation assay was carried out basically as described previously (23). His-tagged LSD1 proteins were expressed in *E. coli* strain BL21(DE3) and purified using a Ni-NTA affinity column (Qiagen). Purified proteins were incubated in a reaction buffer (100 mM Tris pH 8.5, 40 mM KCl, 20 mM MgCl<sub>2</sub>, 20 mM mercaptoethanol, 5% glycerol) with bulk histones from calf thymus.
(Sigma-Aldrich) at 37°C for 12 hours, and were assessed by an immunoblot analysis using specific antibodies: anti-monoMeK4H3 (ab8895, Abcam), anti-diMeK4H3 (UP07-030, Upstate), anti-monoMeK9H3 (UP07-450, Upstate), anti-diMeK9H3 (UP07-441, Upstate) and anti-histone H3 (ab1791, Abcam).

**Microinjection.** Morpholino oligonucleotides or capped mRNAs were injected into one-cell-stage embryos using an IM300 microinjector (Narishige) as described previously (65). The nucleotide sequences of the morpholino oligonucleotides (Gene Tools) were 5’-gttattcacctgttgagatttc (lsd1MO), 5’-gttattgacacgttcttcagatatc (5mis-lsd1MO), 5’-ctgcaagtgtagtattgaagatgtc (gata1MO)(33), 5’-ttggtacatttccatatcttaaagt (etv2MO)(35) and 5’-ttgctagatttcgatatgttaaact (5mis-etv2MO). The lsd1 mRNA was transcribed from linearized pCS2 plasmids using the mMESSAGE mMACHINE kit (Ambion). Overexpressed proteins were analyzed by immunoblotting using anti-FLAG antibody (Sigma-Aldrich) as described previously (64).

**ChIP.** ChIP analysis was basically carried out as described previously (66, 67) using specific antibodies: anti-monoMeK4H3 (ab8895, Abcam), anti-diMeK4H3 (UP07-030, Upstate) and anti-triMeK4H3 (ab8580, Abcam). Two-hundreds and fifty embryos were used for each assay and cells were sonicated by a Branson Sonifier 250. Quantitative PCR was performed using a 7500 Fast Real-Time PCR System (Life Technologies), FastStart Universal SYBR Green Master (Roche) and specific primers listed in Table S1.

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**References**


**Figure legends**

**Fig. 1.** Isolation of LSD1 mutant. (A) The *gata1* expression in wild-type sibling (WT-sib) and *it627* embryos. The arrows indicate hematopoietic tissues: posterior LPM (14 hpf) and ICM (22 hpf). (B) The structures of the LSD1 proteins. SWIRM, Swi3p, Rsc8p and Moira domain; Tower, tower domain. (C) The results of the demethylation assay using bacterially-expressed LSD1 proteins and bulk histones from calf thymus as substrates. Methylated proteins were detected by immunoblotting using specific antibodies.

**Fig. 2.** Hematopoietic defects in *lsd1*it627 embryos. (A) Expression of the Gata1 downstream genes at 20 hpf, and o-dianisidine staining in the blood islands at 36 hpf (arrows). (B) Wright-Giemsa staining of cytospin preparations of circulating blood cells. Green (L) and red (S) bars indicate large immature erythroblasts and small mature erythrocytes, respectively. Scale bars, 5 μm. (C) The *pu.1* expression at 18 hpf (anterior-dorsal views) or 24 hpf (lateral views) in wild-type or *lsd1*it627 embryos injected or not with 0.5 pmole *gata1MO*. The *pu.1* expression at 18 hpf in wild-type or *lsd1*it627 embryos. The arrows and arrowheads indicate the anterior LPM and ICM, respectively. The ectopic *pu.1* expression in the ICM (closed arrowhead) induced by *gata1MO* injection was downregulated in *lsd1*it627 embryos (open arrowhead).

**Fig. 3.** Upregulation of endothelial markers in *lsd1*it627 embryos. (A) The *etv2* expression at the indicated developmental stages in wild-type or *lsd1*it627 embryos. The arrowhead indicates the upregulation of *etv2* in the posterior LPM. (B) The *fli1a* expression at 21 hpf in wild-type or *lsd1*it627 embryos. The arrowhead indicates the upregulation of *fli1a* in the ICM. (C) The expression of *fli1a*, *flk1*, *tie1*, and *biklf* at 26 hpf in the trunk region. The right panel shows a diagram of the hematopoietic and endothelial regions in wild-type embryos. The blue and red arrows indicate the upregulation of endothelial markers and downregulation of a hematopoietic marker, respectively, in the hematopoietic region.

**Fig. 4.** Rescue of the hematopoietic defects in *lsd1*it627 embryos by the *etv2* knockdown. (A) The *gata1* expression in wild-type or *lsd1*it627 embryos injected or not with 0.5 pmole each of the indicated morpholino oligonucleotides. The downregulation of *gata1* in *lsd1*it627 embryos (open arrowheads) was rescued by the *etv2MO* injection (closed arrowheads). (B) The *pu.1* expression at 24 hpf in wild-type or *lsd1*it627 embryos.
injected or not with 0.5 pmole each of the indicated morpholino oligonucleotides. The *gata1*MO-induced ectopic *pu.1* expression in the ICM (arrows), which was lost in *lsd1*<sup>627</sup> embryos (open arrowhead), and was recovered by the co-injection of *etv2*MO (closed arrowhead). (C) Methylation status of histone H3-K4 on the *etv2* gene in 15-hpf embryos injected (gray bars) or not (white bars) with 1 pmole *lsd1*MO. Genomic regions (up1, int2, 35 bp, ex8) examined by ChIP are shown as black bars. The results are means and standard errors of three independent experiments.
Fig. 1: Imaging of embryos at 14 and 22 hpf with probe gata1. WT-sib and it627 show different expression patterns. (B) Bar chart showing the expression levels of SWIRM, Tower, AOD-N, and AOD-C. LSD1 and it627 expression in AOD-N and AOD-C is indicated. (C) Western blot analysis of His-tagged proteins H3K4me1, H3K4me2, H3K9me1, H3K9me2, and H3 with LSD1, LSD1 ΔC609, and (-) controls.
Fig. 2

Panel A: Images showing the expression of different genes (hbbe1, alas2, klf4) and o-dianisidine stain in WT and lsd1it627 embryos at 6 dpf.

Panel B: Bar graph showing the ratios of L (left) and S (second) of WT and lsd1it627 embryos at 4 and 6 dpf.

Panel C: Images showing the expression of gata1MO in WT and lsd1it627 embryos at 18 and 24 hpf.
**Fig. 3**

(A) WT vs. *lsd1^{lt627}* at 11.5 hpf and 12.5 hpf.

(B) WT vs. *lsd1^{lt627}* at 21 hpf.

(C) Gene expression analysis:

- **fli1a**
- **flk1**
- **tie1**
- **biklf**

* Probe: *etv2*

Dorsal aorta, Axial vein, Hematopoietic region
Fig. 4