NEURAL REGENERATION RESEARCH
Volume 7, Issue 34, December 2012

www.nrronline.org

doi:10.3969/j.issn.1673-5374.2012.34.010 [http://www.crter.org/nrr-2012-qkquanwen.html]
Li AH, Sun Y, Dou CM, Chen JX, Zhang J. Lysine-specific demethylase 1 expression in zebrafish during the early stages of neuronal development. Neural Regen Res. 2012;7(34):2719-2726.

Lysine-specific demethylase 1 expression in zebrafish during the early stages of neuronal development★

Aihong Li[1], Yong Sun[2], Changming Dou[3], Jixian Chen[4], Jie Zhang[5]

1 *Department of Internal Neurology, Affiliated Hospital of Nantong University, Nantong 226019, Jiangsu Province, China*
2 *Key Laboratory of Acupuncture Combined with Medication, Nanjing University of Chinese Medicine, Nanjing 210000, Jiangsu Province, China*
3 *Anhui Academy of Environmental Sciences, Hefei 230061, Anhui Province, China*
4 *Department of Cardiothoracic Surgery, Third Affiliated Hospital of Wenzhou Medical College, Wenzhou 325000, Zhejiang Province, China*
5 *Department of Immunology, School of Medicine, Nantong University, Nantong 226019, Jiangsu Province, China*

Abstract

Lysine-specific demethylase 1 (Lsd1) is associated with transcriptional coregulation *via* the modulation of histone methylation. The expression pattern and function of zebrafish Lsd1 has not, however, been studied. Here, we describe the pattern of zebrafish Lsd1 expression during different development stages. In the zebrafish embryo, *lsd1* mRNA was present during the early cleavage stage, indicating that maternally derived Lsd1 protein is involved in embryonic patterning. During embryogenesis from 0 to 48 hours post-fertilization (hpf), the expression of *lsd1* mRNA in the embryo was ubiquitous before 12 hpf and then became restricted to the anterior of the embryo (particularly in the brain) from 24 hpf to 72 hpf. Inhibition of Lsd1 activity (by exposure to tranylcypromine) or knockdown of *lsd1* expression (by morpholino antisense oligonucleotide injection) led to the loss of cells in the brain and to a dramatic downregulation of neural genes, including *gad65*, *gad75*, and *reelin*, but not *hey1*. These findings indicate an important role of Lsd1 during nervous system development in zebrafish.

Key Words

zebrafish; lysine-specific demethylase; morpholino; tranylcypromine; nerve cells; embryonic development; histone methylation; histone demethylase; brain; neural regeneration

Research Highlights

(1) We utilized a zebrafish model to study Lsd1 expression and function.
(2) Inhibition of zebrafish Lsd1 activity led to significant cell loss in the brain.
(3) Inhibition of Lsd1 activity led to the downregulation of neural genes, such as *gad65*, *gad75* and *reelin*, but not *hey1*.

Abbreviations

LSD, lysine specific demethylase; CNS, central nervous system; MO, morpholino antisense oligonucleotides

Aihong Li★, Master, Associate professor, Department of Internal Neurology, Affiliated Hospital of Nantong University, Nantong 226019, Jiangsu Province, China

Aihong Li and Yong Sun contributed equally to this work.

Corresponding author: Jie Zhang, Ph.D., Associate professor, Department of Immunology, School of Medicine, Nantong University, Nantong 226019, Jiangsu Province, China
zhangjie@ntu.edu.cn

Received: 2012-08-15
Accepted: 2012-11-06
(N20120702005/H)

INTRODUCTION

Lysine specific demethylase 1 (Lsd1, also known as BHC110 or AOF2)[1-2] is a flavin-dependent histone demethylase that specifically removes methyl groups from Lys4 of histone H3 (H3K4), which is important for a wide range of biological processes, such as development, differentiation, cancer, and neurological disorders[3-5]. The structure and function of Lsd1 is conserved from yeast to humans. Shi[5] has described the discovery of a new family member – Lsd2 (AOF1). Lsd1 is comprised of an N-terminal SWIRM (Swi3p, Rsc8p and Moira) domain and a C-terminal flavin domain, which shows homology to members of the amine oxidase family. Lsd1 has been found in association with CoREST and histone deacetylases 1 and 2[3, 6]. In the central nervous system (CNS), chromatin integrates a number of converging signaling pathways, leading to short- and long-term changes in gene expression that are crucial for neuronal commitment and terminal maturation, as well as for neuroplasticity throughout the life[7]. Although a variety of ubiquitously expressed chromatin-remodeling complexes support tissue-specific transcription factors, neurorestricted chromatin-remodeling factors have only recently been identified[8]. Using standard gene targeting technology in mouse embryonic stem cells, *lsd1* knockout causes death in prenatal and perinatal stages. Despite the sudden cardiac death phenotype, the *lsd1* knockout strategy resulted in a major effect on the development of the pituitary and neural systems at a very early stage[9].

Over the past decade, the zebrafish model has come to the forefront of biological research; this model system has allowed the elucidation of a plethora of fundamental developmental processes[10]. Apart from being a vertebrate with organs and tissues, such as a brain and spinal cord, that have conserved organization, the zebrafish system bridges the gap between fruit fly/worm and mouse/human genetics, making it feasible to address issues of early development, organ formation, integrative physiology, pharmacology, and complex disease[8, 11-13]. In the absence of blood circulation, zebrafish receive some oxygen by passive diffusion and are able to survive and develop in a relatively normal fashion for several days, thereby allowing a detailed analysis of animals with severe cardiovascular defects.

Although an increasing number of histone demethylases have been identified and biochemically characterized, their biological functions, particularly in the context of an animal model, are not well understood[14]. There is some information regarding the expression and function of Lsd1 in the mouse brain; however, only limited studies have been performed on zebrafish[15]. Thus, we used zebrafish as a model to study the functions and related mechanisms of Lsd1. Here, we report on the Lsd1 expression pattern during the early developmental stages and further demonstrate a neuronal phenotype. Our results also suggest that zebrafish is a promising model for the detection of nerve disorders.

RESULTS

lsd1 mRNA expression during zebrafish development

To localize *lsd1* transcripts during zebrafish development, we performed whole-mount RNA *in situ* hybridization. At early stages (12 hpf, hours post-fertilization), *lsd1* mRNA was localized throughout the whole embryo. At late somitogenesis (24 hpf), *lsd1* expression was observed in the head and spinal cord (Figure 1A). By the late pharyngula stages (48 hpf), *lsd1* expression was restricted to the anterior region. At the early larval stages (72 hpf), *lsd1* was expressed in a more refined region, but in a similar pattern to that at 48 hpf. Expression of *lsd1* was present in the brain (such as in the neural fields in the diencephalon and dorsal hindbrain; Figures 1A, C) and spinal cord. Spinal cord expression occupied three planes along the dorsoventral axis, presumably the motor neurons and interneurons.

Furthermore, *lsd1* transcripts showed strong expression during very early stages. At the one cell stage, strong *lsd1* expression was distributed throughout the embryo (Figure 1A).

To determine if the transcript levels observed by whole-mount RNA *in situ* hybridization on staged zebrafish embryos were consistent with the hypothesized expression during neurogenesis, we performed semi-quantitative reverse transcription (RT)-PCR analysis on 12 to 72 hpf embryos (Figure 1B) and found that transcripts were present at each stage investigated.

Exposure to PCPA (tranylcypromine) or morpholino antisense oligonucleotide (MO) injection inhibited Lsd1 histone demethylase activity

The zebrafish *lsd1* gene contains 21 exons, encodes 833 amino acids, and is located on chromosome 17. To study the effects of *lsd1* downregulation on embryonic development, morpholino antisense oligonucleotides (*lsd1* MO) were designed to block splicing of exon 11 of *lsd1*, which plays a crucial role in histone demethylation.

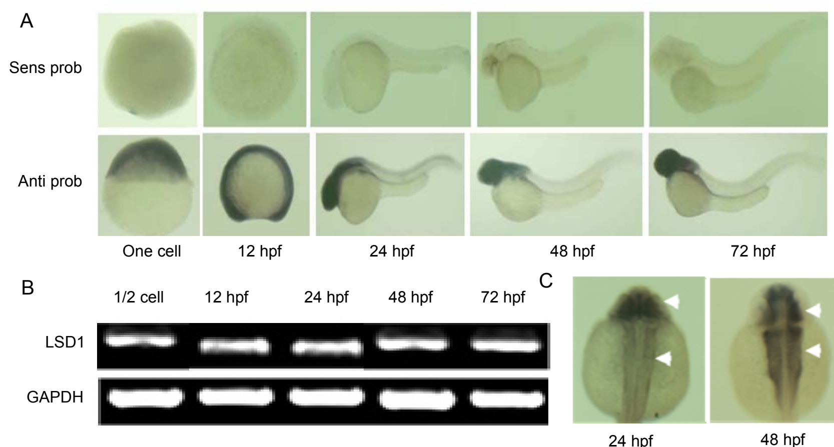

Figure 1 Expression pattern of lsd1 during zebrafish embryogenesis.

(A) Expression of *lsd1* in wild-type embryos from the one cell stage to 72 hpf (left lateral view). Embryos were treated with 1-phenyl-2-thiourea to prevent the production of melanin to facilitate the identification of stained structures; expression of *lsd1* in wild-type embryos at the one cell stage shows that *lsd1* is maternally derived.

(B) Reverse transcription-PCR analysis was performed with whole embryo RNA samples isolated from one cell stage to 72 hpf embryos. *lsd1* specific products were amplified from RNA isolated from whole embryos. The GAPDH primers amplified a single fragment in all the samples.

(C) Expression of *lsd1* in the head at 48 hpf and 72 hpf (dorsal view). Arrows in C show expression of *lsd1* in the central nervous system (brain and spinal cord).

Lsd: Lysine specific demethylase; hpf: hours post-fertilization.

Using RT-PCR, we found that injection of the *lsd1* MO into early zebrafish embryos resulted in the production of aberrantly spliced messages (Figure 2A). Sequence analysis of the corresponding cDNAs revealed that deletion of the targeted exon (exon 11) was caused by the *lsd1* MO, and that normal *lsd1* was partially downregulated. The effect of morpholinos was also tested by western blot analysis (Figures 2B, C). Western blotting showed that the H3K4 demethylation level was increased significantly in the MO group as well as in the PCPA group compared to the controls. Therefore, a loss-of-function lsd1 zebrafish can be obtained by lsd1 MO or PCPA treatment.

lsd1 was expressed in the CNS region in early developmental stages. Therefore, we addressed the question of whether a neuronal phenotype occurs when Lsd1 activity was inhibited.

The expression of *gad65*, *gad75*, and *reelin* decreased when Lsd1 activity was inhibited

After injection of *lsd1* MO, histological examination revealed that a mass of cells were absent in the brain CNS region (particularly in the tectum) and the structure of the trabecula was dramatically altered (Figure 3B). Further examination by RT-PCR showed the expression of several neuronal markers, including *gad65*, *gad75*, *reelin*, and *hey1*. Strikingly, we found that injection of LSD1 MO or exposure to PCPA significantly inhibited *gad65*, *gad75*, and *reelin* expression ($P < 0.05$) at 24 hpf. However, the expression of *hey1* showed a slight increase in the MO group (Figure 3A). These data indicate that the downregulated expression of *gad65*, *gad75*, and *reelin* may be connected with impaired nerve cells.

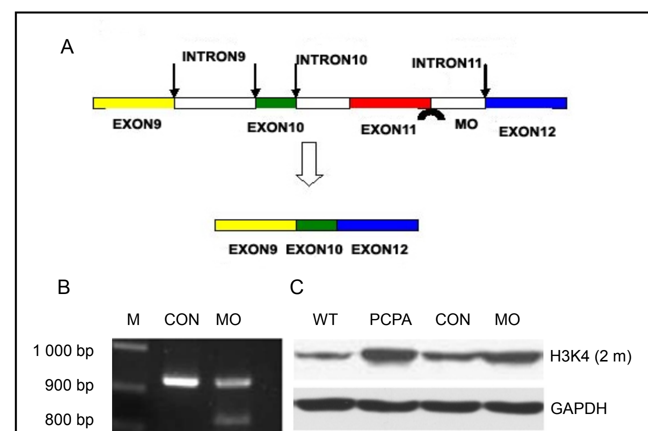

Figure 2 Splice-site-targeted morpholino oligonucleotides can alter lsd1 expression in zebrafish at 24 hpf.

(A) The splice site of Lsd1-MO. (B) Reverse transcription-PCR analysis of *lsd1* mRNA in Lsd1 CON and Lsd1 MO embryos at 24 hpf. cDNA sequence comparison reveals that the MO splice variant lacks exon 11. M: 100 bp DNA Marker.

(C) Lsd1 demethylase was inhibited in Lsd1 MO embryos at 24 hpf. Western blot analysis of histone demethylation using rabbit polyclonal anti-human H3K4 (2 m; demethylation) and GAPDH antibody. H3K4 (2 m) means H3K4 demethylation.

Lsd: Lysine specific demethylase; hpf: hours post-fertilization; CON: control; PCPA: tranylcypromine; MO: morpholino antisense oligonucleotide; WT: wild type; hpf: hours post-fertilization.

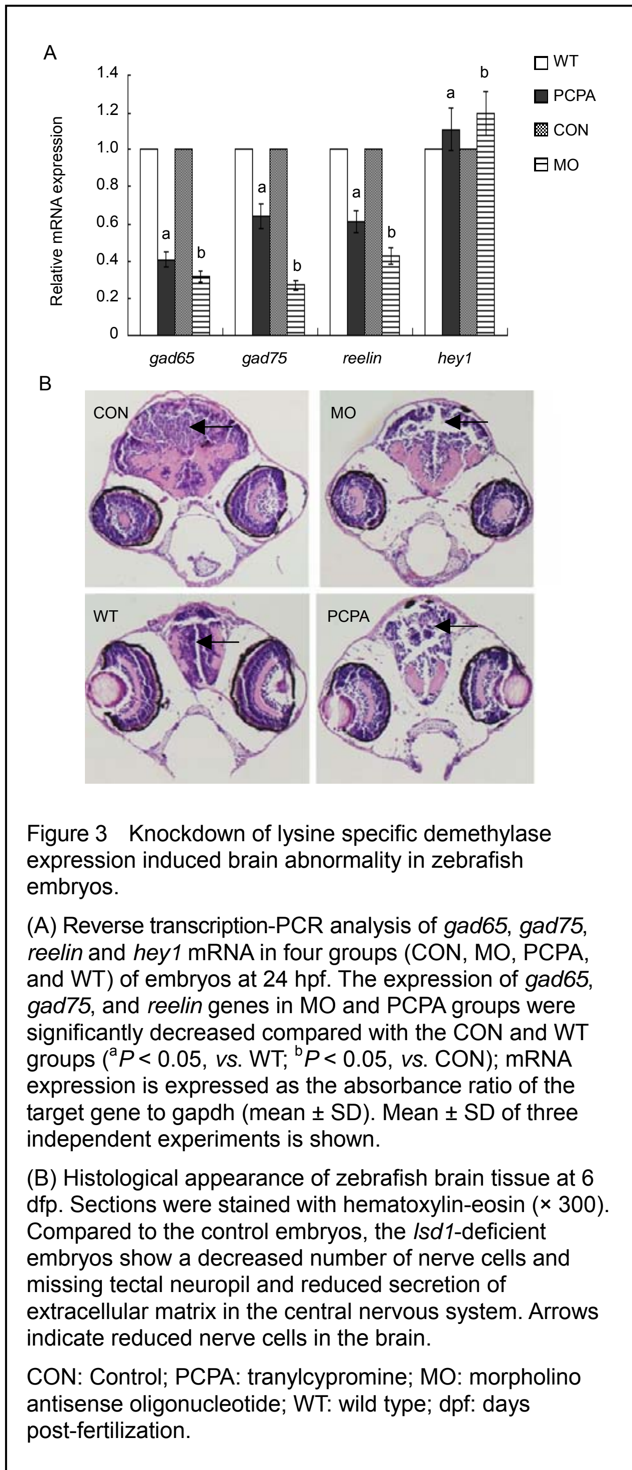

Figure 3 Knockdown of lysine specific demethylase expression induced brain abnormality in zebrafish embryos.

(A) Reverse transcription-PCR analysis of *gad65*, *gad75*, *reelin* and *hey1* mRNA in four groups (CON, MO, PCPA, and WT) of embryos at 24 hpf. The expression of *gad65*, *gad75*, and *reelin* genes in MO and PCPA groups were significantly decreased compared with the CON and WT groups ($^aP < 0.05$, *vs.* WT; $^bP < 0.05$, *vs.* CON); mRNA expression is expressed as the absorbance ratio of the target gene to gapdh (mean ± SD). Mean ± SD of three independent experiments is shown.

(B) Histological appearance of zebrafish brain tissue at 6 dfp. Sections were stained with hematoxylin-eosin (× 300). Compared to the control embryos, the *lsd1*-deficient embryos show a decreased number of nerve cells and missing tectal neuropil and reduced secretion of extracellular matrix in the central nervous system. Arrows indicate reduced nerve cells in the brain.

CON: Control; PCPA: tranylcypromine; MO: morpholino antisense oligonucleotide; WT: wild type; dpf: days post-fertilization.

DISCUSSION

Zebrafish models for the study of neurogenetic disorders

In recent years, zebrafish have been used to study neurodegenerative disorders, such as Alzheimer's disease, Parkinson's disease, and Huntington's disease, with an emphasis on studies of degenerative neurological diseases[8, 16]. More recently, studies have examined the tau protein in zebrafish, which may contribute to understanding Alzheimer's disease in human[16]. Research into Parkinson's disease has also used zebrafish as a model[17-19]. Another CNS disorder that has been studied recently using zebrafish is epilepsy as clonus-like seizures can be induced by convulsant agents[14, 20-21]. This has permitted forward mutagenesis screens and the isolation of seizure-resistant mutants[22].

These recent developments with the new model (especially with transgenic zebrafish) open the possibility of further screens to dissect the molecular mechanisms and discover novel therapeutics for neurodegenerative disorders[23-24].

The characteristics of Lsd1 expression in zebrafish and mouse

The Lsd1 expression pattern was first described in mouse; the processing of the *lsd1* transcript undergoes a dynamic histone demethylase activity modulation within the perinatal window when fundamental processes take place, such as phylopodia formation, neurite sprouting, and the expression of the first synaptogenic markers. Transcriptional profiling of the developing rat brain revealed that the most dramatic changes in gene expression occur post-partum[25], underscoring the relevance of early postnatal life as a critical phase of neural organization and differentiation. Mouse *lsd1* transcripts are seen from embryonic day 10.5 until 2 weeks post-partum[8], but our data show that maternal transcripts are present in zebrafish. Transcriptional profiling of the developing rat brain reveals that the most dramatic regional changes in gene expression occur post-partum[8]. However, *lsd1*$^{-/-}$ mice die within 22 days and more extensive work cannot be done with this model. Thus, other *in vivo* models should be employed.

The present study also demonstrates an important role for *lsd1* in embryonic neuronal development[26-27]. In zebrafish, *lsd1* is expressed throughout the developing stages: from the one cell embryo the expression pattern becomes restricted to the anterior of the embryo and is particularly enriched at the neural structures as embryonic development progresses. Zebrafish neurogenesis begins at the early tail bud stages (9 hpf), reaches high levels 24–48 hpf, and progresses 72 hpf. The timing of peak *insm1a* and *insm1b* expression coincides precisely with periods of zebrafish neurogenesis. The *lsd1* expression in one cell and two cells stages suggests that *lsd1* transcripts appear to be maternally contributed and are important for the original

polarization.

Maternally derived Lsd1 and *lsd1* mRNA are present during early cleavage stages in the zebrafish embryo. *lsd1* mRNA is detectable in the 1–4 cell-stage embryo, before the start of zygotic gene transcription. Later during embryogenesis, *lsd1* mRNA is expressed in the majority of the embryo (especially the CNS). Maternally derived *lsd1* is most likely the substrate for the *lsd1* transporter at these stages of development because liver, which can synthesize Lsd1 endogenously, does not become functional in zebrafish embryos until 2 days after fertilization. In our study, splice morpholinos are used to block translation, but almost all new zygotic transcription occurs only after the midblastula transition, and the splice morpholino is very unlikely to affect demethylation in 1–2 cells. Thus, we used PCPA to inhibit the H3K4 (2m) demethylase action of Lsd1.

Lsd1 functions in neurogenetic development

Lsd1 downregulation causes a variety of disorders in many tissues, especially the nervous system[22, 25, 28]. The functional characteristics of zebrafish Lsd1 are very similar to those of mammalian Lsd1s, including histone demethylase activity. In zebrafish development, the spatial and temporal patterns of neural induction and axonal tract formation were assessed before 48 hpf. To test whether a special phenotype was induced by neural damage at an early stage, we examined the expression of several neuronal markers, including *gad65*, *gad75*, *reelin*, and *hey1*, following knockdown of *lsd1* expression at 24 hpf.

Reelin is an extracellular matrix protein that is synthesized and secreted from cortical GABAergic interneurons[29-30]; Reelin surrounds apical and basal dendritic spines of pyramidal cortical neurons[31]. Reelin plays a defined role not only in prenatal CNS development[32-33], but also in the adult brain by modulating cortical pyramidal neuron dendritic spine expression density, the branching of dendrites, and the expression of long-term potentiation[31, 34]. It is likely that reelin has a role in regulating the event-related increase in protein synthesis that is mediated by the dendritic translation of cytosolic mRNAs[33]. Gad65 exists primarily as an apoenzyme under normal conditions, whereas Gad67 exists as a holoenzyme. Despite extensive study, little is known about Gad65 and Gad67.

Interestingly, the downregulation of Gad65, as well as Gad67, is likely to be responsible for working memory impairment, and the downregulation of reelin expression in neocortex and hippocampus may be important in mediating the downregulation of pyramidal neuron dendritic branching and spine expression and in the neuropil hypoplasticity typical of schizophrenia patients[35-37]. The histone demethylase activity of Lsd1 may have a regulatory role in the expression of these three target genes (*reelin*, *gad65* and *gad67*). Therefore, Lsd1 deficiency in the CNS may be involved in human schizophrenia.

Hey1 is implicated as a potential regulator of cell differentiation and proliferation through the Notch pathway[38-39]. However, the expression of *hey1* remains unchanged in the MO group in our study. Therefore, our result suggests that the Notch signaling pathway is not likely to be involved in the regulation of the loss-of-function Lsd1 zebrafish.

In summary, in the zebrafish embryo, *lsd1* mRNA is present during early cleavage stages, indicating that maternally derived Lsd1 protein is involved with embryonic patterning. During embryogenesis (from 0 to 48 hpf), the expression of *lsd1* mRNA in the embryo is ubiquitous before 12 hpf and is more restricted to anterior regions (particularly the brain) from 24 to 72 hpf, which is consistent with neuronal differentiation. Inhibition of Lsd1 activity (using tranylcypromine, PCPA) or knockdown of *lsd1* expression (using siRNA morpholino) leads to a loss of cells in the brain region and dramatically downregulated neural genes, including *gad65*, *gad75*, and *reelin*, but not *hey1*. These results provide insight into the expression pattern and molecular function of the lower vertebrate *lsd1* gene.

MATERIALS AND METHODS

Design

A controlled, observational, *in vivo* study.

Time and setting

Experiments were conducted at the Immunology Laboratory, Nantong University, China from January to July 2011.

Materials

Animals

Wild-type (AB* strain) zebrafish stocks were obtained from the International Zebrafish Research Center (WT group). Embryos were obtained from natural spawning of wild-type adults. Zebrafish were raised, maintained, and staged as previously described[9].

Drugs

Lsd1 inhibitors, trans-2-phenylcyclopropylamine (referred to as PCPA hereafter; also known as tranylcypromine and parnate; Sigma, USA), were dissolved in ddH_2O at stock concentrations of 100 mM, and then diluted in embryo media to a final concentration of 40 μM. Control embryos were wild-type zebrafish. All embryos were incubated at 28.5°C[15].

Methods

Whole-mount RNA in situ hybridization

Plasmids encoding zebrafish *lsd1* (3'-UTR region) were kindly provided by Nantong University, China. Whole-mount RNA *in situ* hybridization using digoxigenin-labeled antisense RNA probes was performed using standard methods as previously described[14]. Briefly, digoxigenin-labeled antisense RNA probes were produced using a digoxigenin-RNA labeling kit (Roche, Mannheim, Germany) following the manufacturers' instructions. Hybridization and detection with an anti-digoxigenin antibody coupled to alkaline phosphatase was performed with fixed zebrafish embryos.

Western blot assay

Embryos (24 hpf) were completely deyolked in Ginzburg Fish Ringer buffer (55 mM NaCl, 1.8 mM KCl, 1.25 mM $NaHCO_3$) and washed with PBS. The embryos were collected by centrifugation and lysed with 2 × Laemmli's buffer for 30 minutes followed by centrifugation to remove the debris. After separation by 12.5% sodium dodecylsulfate polyacrylamide gel electrophoresis, the proteins were blotted onto a nitrocellulose filter and hybridized with rabbit polyclonal anti-H3K4 demethylation (H3K4 (2m)) antibody (1:1 000; Abcam, Cambridge, MA, USA).

Morpholino oligonucleotide injection

Morpholinos were obtained from Gene Tools (Philomath, OR, USA). For *lsd1*, the splice morpholino (exon 11) contained 25 bases. For control, a *lsd1* mismatch morpholino (control MO, CON group) that included five mispaired bases was used. The sequences of *lsd1* MO and CON (mis-match MO) were GGG ACC TTT GGC AAA GTT ACA AAC A and GGc ACC TTT cGC AAA cTT AgA AAg A, respectively. 0.05 μL *lsd1* MO at 15.0 mg/mL (1 ng per embryo) was injected into the blastomere of one-cell or two-cell stage embryos (microinjector from Narishige, Japan). Control MO was injected at the same volume.

RT-PCR

Total RNA was extracted from 25–30 embryos using TRIzol Reagent (Invitrogen, Carlsbad, CA, USA) according to manufacturer's instructions. One microgram of total RNA was used as a template in a 20 mL RT-PCR reaction mixture using a one-step RT-PCR kit (Qiagen, Hilden, Germany). Quantitative analysis of RT-PCR expression prouction by REST soft (Stratagene, La Jolla, CA, USA). The RT-PCR conditions are as previously described[15], except for a change in annealing temperature, which depended on the Tm value of the primers. PCR primers used to detect targeted exon 11 skipping are listed in Table 1. Product concentration was quantified using gapdh as an internal reference.

Table 1 Primer sequences for RT-PCR

Gene	Primer sequence
lsd1 11 exon	
	Upstream: 5'- GAA GGA GAC GGT GGT GAG-3'
	Downstream: 5'-GCT ACA AAG TGA GGG ACA -3'
gapdh	Upstream: 5'-ACC ACA GTC CAT CAC-3'
	Downstream: 5'-TCC ACC ACC CTG TTG CTG TA-3'
gad65	Upstream: 5'-TCT CCA ATC CAG CCG CTA CCT-3'
	Downstream: 5'-GCA ATC CAT CCA GGG ACA CG-3'
gad67	Upstream: 5'-TAG TCC ATT GGG ATA ACA GCC-3'
	Downstream: 5'-CAA GAC CTA CGA CAA GGG AAG-3'
reelin	Upstream: 5'-AGC TGT AAC ACT GCG CTA GAT-3'
	Downstream: 5'-TGT AGG ACA CTC TCT GGG CTT-3'
hey1	Upstream: 5'-GAA CGC CTC CCA GTC ACC-3'
	Downstream: 5'-CTT AGC GGT CCC GTC TGC-3'

Histological examinations

At 6 dpf, 20 fish from each treatment were pooled and euthanized with Tricaine (Sigma). Bodies were fixed in Dietrich's fixative[40] and then embedded in paraffin and sectioned longitudinally along the entire dorsal-ventral axis at 4mm. Sections were stained with hematoxylin-eosin and examined under a light microscope (Olympus, Tokyo, Japan).

Photography

Stained embryos were examined with Olympus BX61 and SZX12 microscopes (Olympus, Japan), and photographed with a DP70 digital camera (Olympus). Images were processed using IMAGE PRO software (Media Cybernetics, Silver Spring, MD, USA).

Statistical analysis

All the results are expressed as mean ± SD and analyzed by SPSS 17.0 statistical software (SPSS, Chicago, IL, USA). Differences among groups were analyzed by one-way analysis of variance, and an independent sample *t*-test was used to analyze the comparisons of MO/PCPA and WT/control zebrafish in western blot. A P value < 0.05 was considered statistically significant.

Acknowledgments: We thank Dr. B.A. Oostra from the Cellular Biology and Genetics Research Center, Erasmus University, Holland for providing the experimental animals, and the staff of the Institute of Neuroscience of the Second Affiliated Hospital of Guangzhou Medical University for technology and material support; as well as Dong Liu, Nantong University, for kindly providing plasmids encoding zebrafish *lsd1* 3'-UTR.

Funding: The study was supported by the National Natural Science Foundation of China, No. 81102643; the Natural Science Foundation of the Higher Education Institutions of Jiangsu Province, No. 10KJB310010; the Science Foundation of Zhejiang Province, No. Y2100917; and the Science Foundation of Anhui Province, No. 1208085MB26.

Author contributions: Jie Zhang conceived and designed the study, and acquired funding; Aihong Li and Yong Sun conducted experiments, designed figures, and wrote the manuscript; Changming Dou provided guidance, advice, and expertise; Jixian Chen contributed to technical assistance. All authors approved the final manuscript.

Conflicts of interest: None declared.

Ethical approval: This study received permission from the Animal Ethics Committee of Nantong University, China.

Author statements: The manuscript is original, has not been submitted to or is not under consideration by another publication, has not been previously published in any language or any form, including electronic, and contains no disclosure of confidential information or authorship/patent application/funding source disputations.

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(Edited by Lin JT, Ma R/Su LL/Song LP)