ARTICLE
Journal of Cellular Biochemistry 116:2980–2991 (2015)

Journal of Cellular Biochemistry

Zdhhc15b Regulates Differentiation of Diencephalic Dopaminergic Neurons in Zebrafish

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ABSTRACT

The aspartate-histidine-histidine-cysteine (DHHC) protein family shares a 50-amino acid cysteine-rich domain with a conserved DHHC signature motif. DHHC proteins play a critical role in several biological processes. Several DHHC family members have been implicated in neuronal differentiation and synaptic plasticity. And disruptions to their function can lead to disease in the nervous system. Here, we investigate the role of Zdhhc15b, a DHHC family member, in neuro development in zebrafish. Whole-mount in situ hybridization (WISH) revealed that *zdhhc15b*, an ortholog to human ZDHHC15, is abundant in zebrafish (*Danio rerio*) forebrain, especially in the diencephalon. Downregulation of *zdhhc15b* resulted in a smaller diencephalon and a reduction in mature dopaminergic neurons (DA neurons). In the meanshile, mutant *zdhhc15b* zebrafish was associated with poor learning behavior as detected by T-maze testing. The expression of *zdhhc15b* was upregulated during DA neuronal differentiation whereas knock-down of *zdhhc15b* diminished DA neuronal differentiation. Tyrosine hydroxylase (TH) immunofluorescence of cultured DA neurons in vitro also showed that DA neurons were immature following *zdhhc15b* knock-down. Consistent with the decreased number of DA neurons following knock-down of *zdhhc15b*, the expression of fate determination-related transcription factors such as *nurr1, foxA2,* and *lmx1a* were also reduced in morphant zebrafish. Our results reveal that *zdhhc15b* controls DA neuronal fate decisions by regulating differentiation but not progenitor cell proliferation or DA neuronal survival. J. Cell. Biochem. 116: 2980–2991, 2015.

KEY WORDS: Zdhhc15b; DHHC PROTEIN; DIENCEPHALON; DOPAMINERGIC NEURON; NEURODEVELOPMENT; FATE DETERMINATION; RELATED TRANSCRIPTION FACTORS

Posttranslational modifications markedly affect protein function [Huang and El-Husseini, 2005; Linder and Deschenes, 2007]. As a post-translational modification, palmitoylation is important for protein trafficking, stability, and activity of numerous. So far, many proteins involved in neural development, neurotransmission, and synaptic plasticity have been known to be palmitoylated. Signaling proteins, ion channels, G protein-coupled receptors, cell adhesion molecules, transporters, adaptors, and

Conflict of interest :None.

Grant sponsor: National Natural Science Foundation of China; Grant numbers: 81071057, 31271289; Grant sponsor: Ministry of Education of China; Grant number: 20110131110036; Grant sponsor: Natural Science Foundation of Shandong Province; Grant numbers: 2012GSF11842, ZR2010HQ022; Grant sponsor: Shandong University; Grant number: 2012JC006.

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Manuscript Received: 8 February 2015; Manuscript Accepted: 9 June 2015

Accepted manuscript online in Wiley Online Library (wileyonlinelibrary.com): 11 June 2015

DOI 10.1002/jcb.25256 • © 2015 Wiley Periodicals, Inc.

scaffolding proteins are included [Fukata and Fukata, ; Resh, 2006; Greaves et al., 2008].

Palmitoylation is executed by a family of proteins that contain a unique cysteine-rich domain (cRD) with a conserved aspartate-histidine-histidine-cysteine (DHHC) signature motif [el-Husseini Ael and Bredt, 2002; Smotrys and Linder, 2004; Resh, 2006]. Forward genetic screens have identified Erf2/Erf4 and ARK1 as DHHC proteins in yeast, and they are responsible for RAS2 and yeast casein kinase two modifications [Lobo et al., 2002; Roth et al., 2002; Linder and Deschenes, 2004]. In addition to the seven DHHC proteins that have been identified in yeast, 23 DHHC members classified into several subfamilies have been predicted in mammals [Linder and Deschenes, 2004; Ohno et al., 2006] and several of these proteins have since been shown to have palmitoyl transferase activity[Greaves et al., 2008].

Genetic studies reveal that dysfunction of DHHC proteins contributes to a number of neurological disorders [Young et al.]. For example, DHHC8 is associated with a higher risk to develop schizophrenia and other neuropsychiatric disorders [Chen et al., 2004; Mukai et al., 2004]. ZDHHC12 has been linked to regulation of amyloid precursor protein (APP) trafficking and metabolism. DHHC17 was originally identified as a huntingtin-interacting protein [Singaraja et al., 2002]. A balanced reciprocal translocation between chromosomes X and the ZDHHC15 gene resulted in an absence of ZDHHC15 transcripts [Mansouri et al., 2005], which is associated with X-linked mental retardation (XLMR) and mutations in ZDHHC9 on the X-chromosome were also involved in XLMR [Raymond et al., 2007]. Moreover, Increasing evidence shows that DHHC3 is involved in synapse function [Oku et al.], while DHHC5 participates in neuronal differentiation in cultured cells [Li et al.], and DHHC17 deficiency is associated with a decreased striatal volume [Singaraja et al.]. The aforementioned studies clearly implicate DHHC proteins in neuronal development, likely through their ability to regulate gene expression in cell growth, fate, and function.

ZDHHC15 is conserved from *Homo sapiens* to *Danio rerio* and dysfunction of ZDHHC15 induced defects in cell fate differentiation and cell function in vitro [Leong et al., 2009]. As a candidate for XLMR, ZDHHC15 could induce impaired learning-memory ability. Nevertheless, the role(s) of DHHC15 in DA neuron which is regard as the executor of learning-memory is unknown. Zebrafish share most human genes and gene functions are well conserved. Together with the advantage of their fast development and a transparent body, zebrafish have been used widely in neurodevelopmental studies [Lieschke and Currie, 2007]. We used zebrafish to explore the functional role of DHHC15 in neuronal development in vivo. In the present study, ZDHHC*15b* was identified homologous to human ZDHHC15. Transcripts of *zdhhc15b* were expressed in the developing forebrain, and particularly in diencephalon. Moreover, we extended our research and provided the evidence that ZDHHC15b regulates differentiation of DA neurons in developing zebrafish.

METHODS

ZEBRAFISH MAINTENANCE

Zebrafish strains AB were maintained and bred according to standard procedures [Talbot et al., 1995]. All embryos were maintained at 28.5°C, under a 14 h light/10 h dark cycle. To inhibit pigmentation, 0.03% phenylthiourea (PTU, Sigma) was added to water.

MORPHOLINO OLIGONUCLEOTIDE AND *Zdhhc15b* mRNA INJECTIONS

zdhhc15b morpholino (MO) that targeted the translational start site as well as control MO were synthesized (Gene Tools LLC, Philomath, OR). MOs were injected at the 1-cell stage and the embryos were allowed to develop at 28.5°C. The sequence of *zdhhc15b* MO is: 5′-*AGTGCTCTCGACAGAGAGCCATGATCA*-3′, and the control MO sequence is: 5′-*CCTCTTACCTCAGTT*-3′. We cloned the full-length *zdhhc15b* sequence into the pCS2^{+} vector with a GFP sequence downstream and *zdhhc15b* mRNA was synthesized in vitro. The primer for the full-length *zdhhc15b* sequence: sense primer:5′-*CGCGGATCCATGGCTCTGTCGAGAGCACTCAGAT*-3′ and anti-sense primer:5′-*GATCAGATCTTGATTCAGTCCTAATAAGGAGT*-3′. A modified *zdhhc15b* mRNA (△*zdhhc15b*mRNA) was also generated by deleting the 25 bp sequence that complementary with the MO for the rescue assay.

We co-injected *zdhhc15b*-GFP fused mRNA (25 pg) with MO into 1-cell-stage embryos to confirm that MO actually represses the translation of mRNA, the efficiency and specificity of MO oligonucleotides was confirmed by western blot. For the rescue assay, 8ng of *zdhhc15b* MO and 100 pg of modified mRNA were injected.

ANTISENSE PROBE AND *Zdhhc15b* MUTATION SYNTHESIS

Total RNA was extracted by using Trizol Reagent (Invitrogen, Cergy-Pontoise, France) according to the instructions provided by the manufacturer. For developmental expression analysis, about 30 pooled embryos were collected at different stages (8-cell, 1 k, 30% epiboly, shield, 75% epiboly, bud, 18 hpf, 24 hpf, 48 hpf, 72 hpf), quickly frozen on ice and homogenized in 1 ml extraction reagent. Then total RNA of every stage was reverse-transcribed into cDNA using reverse transcriptase according to the manufacturer's instructions. Probes amplified DNA fragments that were cloned into P-easy T vector. Digoxigenin (DIG) labeled antisense probes were synthesized in vitro by Sp6 or T7 RNA polymerase. Primers used are shown in Table I.

zdhhc15b mutation was generated by changing C^{564} to A and was synthesized by Genetimes Technology. Inc.

WHOLE-MOUNT IN SITU HYBRIDIZATION (WISH)

WISH was carried out as described previously [Macdonald et al., 1994; Shanmugalingam et al., 2000]. Zebrafish embryos at the stages less than 3 dpf were fixed with 4% (v/v) paraformaldehyde, whereas embryos more than 2 dpf were pretreated with 0.03% phenylthiourea. Antisense and DIG-labeled RNA probes were generated using the DIG RNA labeling kit (Roche, Switzerland). The WISH procedure followed a standard protocol and required 72 h for completion. The fixed embryos at 24, 48, and 72 hpf were pre-treated with protease K (10 μg/ml) for 10, 30, and 40 min, respectively. Washed by PBST T × 4/5 min, the egg membrane was removed. The embryos were then prehybridized in Hyb^{-} for 5 min and Hyb^{+} for 4 h at 65°C and then incubated in antisense

TABLE I. Primers Used in RT-PCR and in Situ Hybridization for the Following Genes

Zdhhc15b	F.5′CAGATGTTGTCAAAGGAT3′ R.5′TGTAGTTGGAGAACCCGAC3′	476bp
TH	F.5′TCCACCATCTTGAAACCAGACCCA3′ R.5′GCCTCAACTGAAATCCTGTGCGTT3′	537 bp
DAT	F.5′AGACATCTGGGAAGGTGGTG3′ R.5′CTCTCGATGTTTGTGCAGGA3′	560 bp
Nurr1	F. 5′TGTCTCGGTTATTCTCGT3′ R.5′TATCCTGCGGACTTTTAG3′	534 bp
Ngn1	F.5′GGATACTTGCAAACCGACGTA3′ R.5′TCGGACAGATGAGGGTTTCT3′	508 bp
Pitx3	F.5′GCGAGAGGAGATTGCGGTGT3′ R.5′GAGATGAAGGGAGTTTATGCG3′	552 bp
Lmx1a	F.5′GCGTGTGTGAGGGGTGTAAT3′ R.5′CATCTTGGCTCTTTGGTTCT3′	650 bp
Foxa	F.5′CCTTTTCCCTTTTTACCGA3′ R.5′CAGACCCTGACCCGACTTC3′	355 bp
Otx2	F.5′AAAACCGAAGGGCAAAGTGTC3′ R.5′AAGTAGGAAGTGGAACCAGCATAG3′	328 bp
Pax2.1	F.5′GTCAGGCAAAGAATCGTG3′ R.5′TCAGGTGCTTCCGTAAACT3′	622 bp
Nk2.2	F.5′CGAGGAAGATACGGAGGGTT3′ R.5′AGGAGAAGGGAGATGGGTC3′	499 bp
Krox20	F.5′GGTTGTTCCCTATAATCCCG R.5′TCTCTTTCTTTCGTCGCTCC	518 bp
β-actin	F.5′GACCGTATGCAGAAGGAAATCA3′ R.5′TGTGAGGAGGGCAAAGTGGTAA3′	439 bp
Eng1a	F.5′TCTCCACCTTTGCTCCCT3′ R.5′CGTCGCTTTCGGTATTGT3′	370 bp
Eng2a	F.5′ACGAGTCCAACAGTGCCATAC3′ R.5′CTTCTTTACTTGCGGCTTTCT3′	474 bp
Ebf3	F.5′CACGGTGGAATGATGGGAGT3′ R.5′GGAGGTGGAGATGCTTGGGG3′	314 bp

DHHC domain containing 15 (Zdhhc15b), dopamine transporter (DAT), tyrosine hydroxylase. (TH), nuclear receptor1 (nurr1), neurogenin1 (Neurog1), paired-like homeodomain transcription. factor 3 (Pitx3), LIM homeobox transcription factor 1 alpha (Lmx1a), orkhead box A sequence. (Foxa), orthodenticle homolog 2 (Otx2), paired box gene 2a (Pax2a), NK2 transcription factor. related 2a (Nkx2.2a), early growth response 2b (Krox20), engrailed 1a (eng1a), engrailed 2a. (eng2a), early B-cell factor 3 (ebf3).

RNA probes (1 ng/μl) for hybridization overnight. On the second day, we washed the embryos by washing liquid I, II, III step by step; secondly, blocking solution was used to block the non-specific binding sites. Finally, the embryos were incubated in anti-DIG-AP (dilution 1:2,500; Roche) at 4°C overnight. On the last day, INT/BCIP (orange) was used as color detection.

WESTERN BLOT

Western blotting was carried out following standard methods. Primary antibodies were: β-catenin (rabbit; 1:1,000; Abcam) and tyrosine hydroxylase (mouse; 1:1,000; Millipore). Anti-mouse and anti-rabbit IgG (1:1,000; Roche) secondary antibodies were used following the primary antibodies. Results were normalized by running β-actin (rabbit; 1:1,000; Abcam) as a control in parallel.

DA NEURONAL CULTURE AND IMMUNOFLUORESCENCE

DA neurons for primary culture were prepared from 24 hpf embryos. First, the embryos' diencephalons were isolated and dissociated. Then, cells were plated onto poly-L-lysine coated dishes at a density of 30 heads per 35 mm dish and were left to develop at optimal temperature after plating with DMEM-F12 medium added with 20 ng/ml B27 (Invitrogen), 50 ml/L FBS and 7 μmol/L Ara-c [Andersen, 2001; Chen et al.].

TH-immunofluorescence was carried out on the third day. Briefly, cells plated on coverslips were first washed three times with PBS and were then fixed in 4% PFA for 20 min at room temperature. Subsequently, cells were permeabilized with 0.3% Triton X-100 for 30–60 min at room temperature and then cells were incubated with tyrosine hydroxylase primary antibody (mouse, 1:1,000; Millipore). TRITC-conjugated goat anti-mouse IgG (1:200; Millipore) was used as the secondary antibody and DAPI (1:1,000; Sigma) was used as a nuclear counterstain.

ETHOLOGY DETECTION

Learning ability was tested with the T-maze as described previously [Darland and Dowling, 2001]. On the first trial, 5 min were given to the fish to fully explore the maze, and the latency to the first encounter with the reservoir (and remaining in the reservoir for at least 20 s) was recorded. The fish were then given a second trial 3 h later and a final trial 24 h later.

IMAGE AND STATISTICAL ANALYSIS

Images of in situ hybridizations were acquired with a stereo microscope (Olympus SZX16), and a fluorescence microscope (Olympus IX71) for immunostaining. The data are presented as means ± SD. Significance between control and MO groups was tested using Student's t test. Statistical significance was set at $^{*}P < 0.05$ and $^{**}P < 0.01$.

RESULTS

Zdhhc15b IS EXPRESSED DURING NEURONAL DEVELOPMENT IN ZEBRAFISH

Basic local alignment search tool (BLAST) was used to search the zebrafish genome database and *zdhhc15b* was identified to be homologous to the human *ZDHHC15*. *zdhhc15b* localizes to chromosome 14 in zebrafish, and codes for a 331-amino acid protein that has four transmembrane domains (Fig.1A). This zebrafish ortholog showed a high degree of conservation and homology with the human ZDHHC15 as revealed by protein sequence analysis (85% identity and 66% similarity, Fig. 1B, C).

By whole-mount in situ hybridization (WISH), we detected that the *zdhhc15b* transcript was maternally deposited and that ubiquitous zygotic expression was visible from the 1k-cell stage (Fig.1D–a and Fig. 1D–b). From the stage of tailbud, the expression of *zdhhc15b* progressively concentrated in the anterior neural plate (Fig. 1D–c). From stage 18 hpf through stage 48 hpf, *zdhhc15b* was abundant in the forebrain, especially in the diencephalon (Fig. 1Dd–f). Results of RT-PCR were also in agreement with those of WISH (Fig. 1E). The expression of *zdhhc15b* increased from 75% epiboly and endured the crest value from the stage 18 hpf to the stage

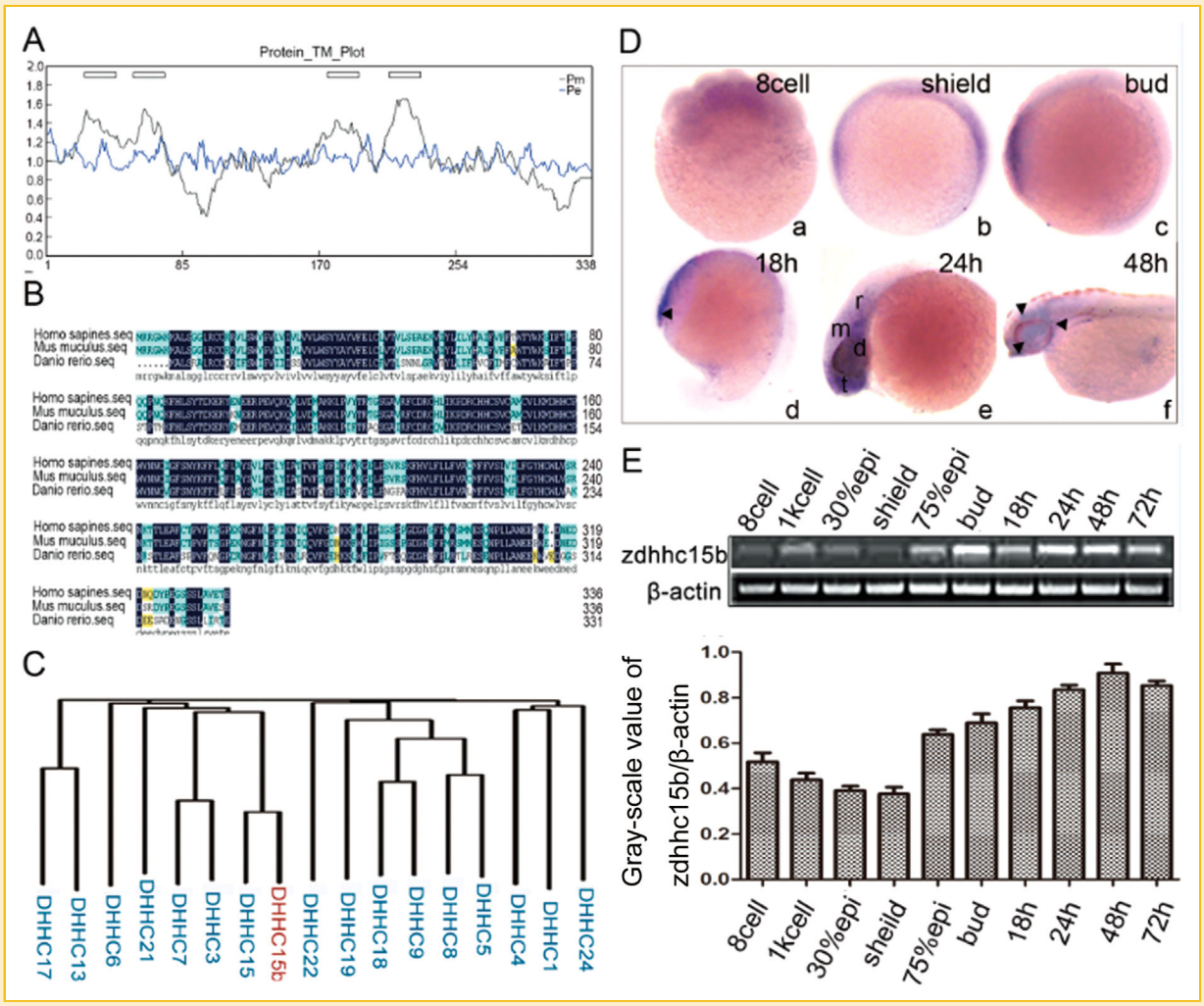

Fig. 1. Homology and phylogeny analysis and expression of ZDHHC15 during zebrafish development. (A) ZDHHC15 is a protein with four transmembrane domains. (B) A high sequence identity exists from Homo sapiens to Danio rerio and the DHHC domain is highly conserved. (C) Phylogenetic tree showed high homology of DHHC15 between *Homo sapiens* and *Danio rerio*. (D) Spatial distribution of *zdhhc15b* revealed by whole-mount in situ hybridization during zebrafish developmental stages. *zdhhc15b* was initially distributed ubiquitously, but became progressively more intense in anterior structures at the 10-somite stages and later; during stages 24 hpf–48 hpf, it was expressed specifically in zebrafish basal forebrain. (E) *zdhhc15b* mRNA expression increased gradually from tailbud in 48 hpf and slightly decreased at the stage of 72 hpf. t, telencephalon; d, diencephalon; m, mesencephalon; r, rhombencephalon.

72 hpf. This expression pattern suggests a potential role of *zdhhc15b* in early neuronal development of zebrafish.

KNOCK-DOWN OF *zdhhc*15b CAUSES DEFICITS IN THE DEVELOPMENT OF THE DIENCEPHALON

To elucidate the function of Zdhhc15b in developing zebrafish, we disrupted its translation with antisense morpholino oligonucleotides (MO) before the 8-cell period in zebrafish embryos.

The embryos injected with *zdhhc15b* MO developed normally till 18 hpf, but at 24 hpf, the embryos exhibited abnormal phenotypes. The morphants showed indistinguishable boundaries among different regions in the forebrain, with a slightly reduced size of diencephalon in comparison with controls (Fig. 2A) at the final concentration 7ng according to the dose analysis (Fig. 2B).

To confirm the efficiency and specificity of MO oligonucleotides, we co-injected *zdhhc15b*-GFP fused mRNA with MO into 1-cell-stage embryos and western blot confirmed that MO actually represses the translation of *zdhhc15b* mRNA (Fig. 2C). Importantly, co-injection of zdhhc15b morpholino with mRNA encoding zdhhc15b which lacks the paired sequence with morpholino (△*zdhhc15b*mRNA) was able to rescue the morphant phenotype (Fig. 2D).

In addition, result of WISH also showed developmental deficits in the diencephalon (otx2, nk2.2) (Fig. 3A), while hindbrain (krox20) and midbrain-hindbrain boundary (MHB) (pax2.1) markers were unaffected after *zdhhc15b* suppression (Fig. 3B). These results indicate that Zdhhc15b might be required for diencephalon development in zebrafish.

The forebrain, especially the diencephalon, is the cognitive learning center in zebrafish. The morphological defects in the diencephalon in *zdhhc15b* morphants led us to investigate learning behavior with a T-maze. We found that *zdhhc15b* mutation zebrafish

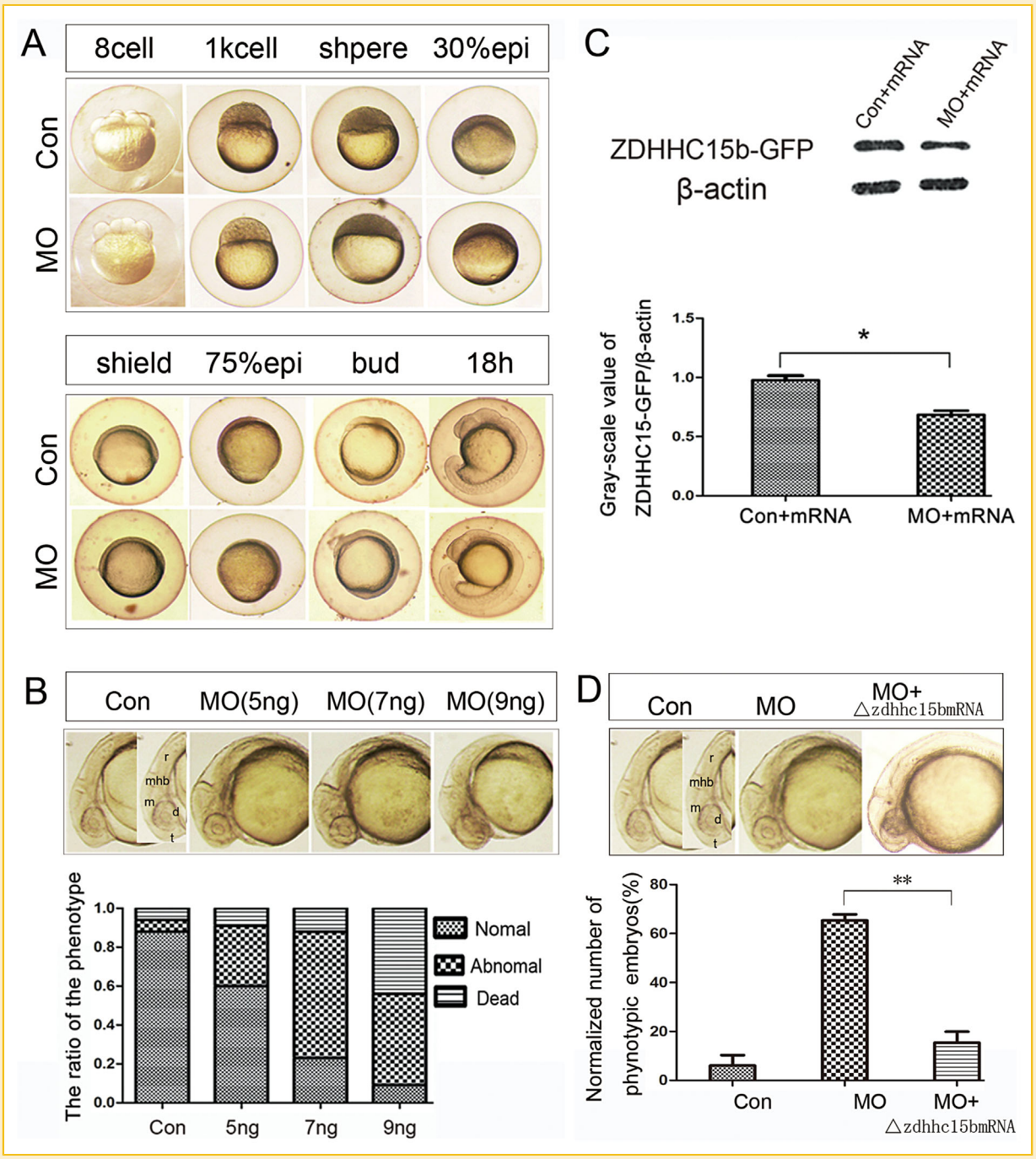

Fig. 2. Phenotypes of *zdhhc15b*-deficient embryos. (A) Embryos developed normally from the stage of 18 hpf after *zdhhc15b* MO injection, forebrain defects appeared from then on and the phenotype was dose-dependent. (B) With the gradual increase in the concentration of *zdhhc15b* MO, forebrain defects became more severe and the mortality rate went up. Therefore, we chose 7 ng as the final concentration. (C) We co-injected *zdhhc15b*-GFP fused mRNA with MO into 1-cell-stage embryos to confirm that MO actually represses the translation of mRNA, by western blot we confirmed MO oligonucleotides could interfere *zdhhc15b* mRNA translation with efficiency and specificity. (D) Rescue assay. Co-injection of *zdhhc15b* morpholino with mRNA encoding *zdhhc15b* which lacks the paired sequence with morpholino was able to rescue the morphant phenotype. It was a supplement to efficiency and specificity of *zdhhc15b* MO. t, telencephalon; d, diencephalon; m, mesencephalon; mhb, mesencephalon-hindbrain boundary; r, rhombencephalon. * $P < 0.05$, ** $P < 0.01$.

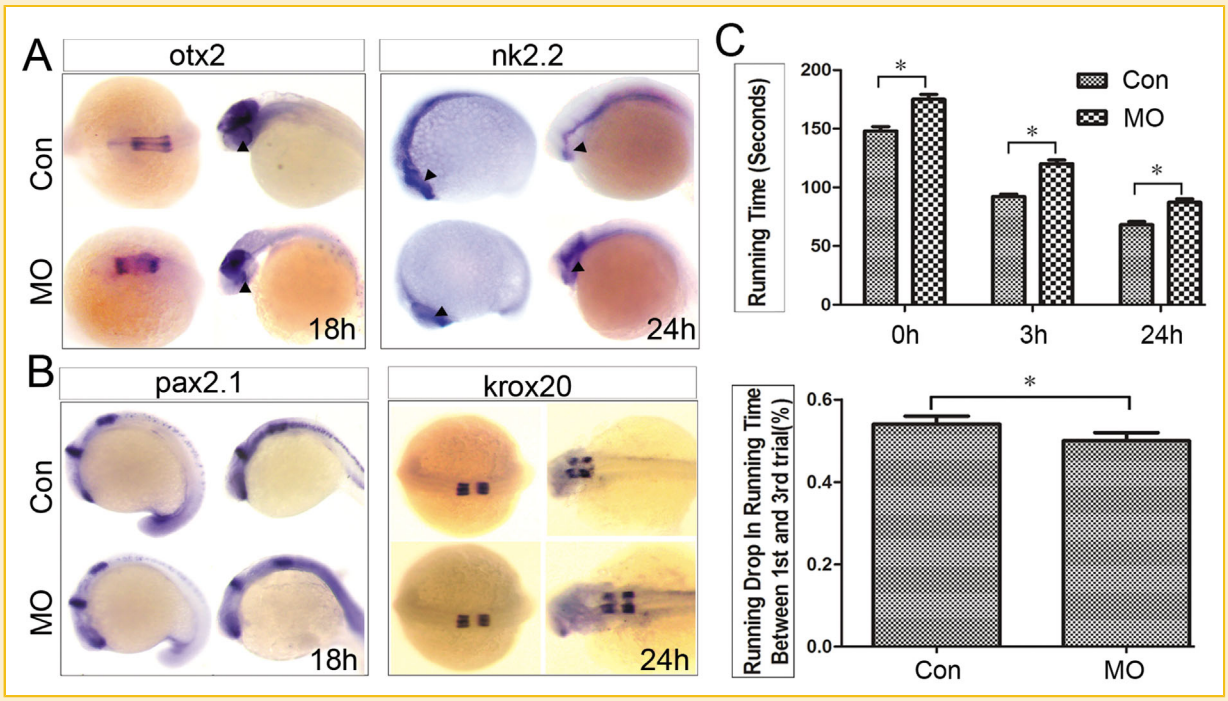

Fig. 3. Changes of diencephalon of zebrafish brain after *zdhhc15b* knock-down and poor learning behavior in mutant *zdhhc15b* zebrafish. (A) Hybridization signals of zebrafish forebrain marker (otx2) and the marker specific for the diencephalon (nk2.2) were weak in *zdhhc15b* morphants. (B) No significant changes were observed in the expression of krox20 for hindbrain and pax2.1 for MHB before or after *zdhhc15b* knock-down.Triangle, zebrafish diencephalon. (C) The learning ability of zebrafish was tested using the T-maze. The time taken for *zdhhc15b* morphants zebrafish ($n = 10$) on the first trial was 175 ± 4.27 s while control ($n = 10$) zebrafish took 148.1 ± 3.57 s ($P < 0.05$). After two trials of learning, the time taken by *zdhhc15b* morphants was 87.2 ± 2.94 s, while the time of control zebrafish was 68.10 ± 2.60 s ($P < 0.05$). (B) The fish reduced their swimming time after learning. Significant difference was found in the running drop between the 1st and 3rd trial. They were ($54\% \pm 0.02$) for control zebrafish and ($50\% \pm 0.01$) for *zdhhc15b* morphants embryos, respectively. * $P < 0.05$.

resulted in learning and memory deficits. On the first trial, the time taken by the control larvae to find the reservoir was 148.1 ± 3.57 s, while the *zdhhc15b* mutation larvae took 175 ± 4.27 s, and this latency difference was significant ($P < 0.05$, Fig. 3C). After two learning trials, the time taken by *zdhhc15b* morphants dropped by $50 \pm 0.01\%$ (Fig. 3C)–87.2 ± 2.94 s, while the time of the control zebrafish dropped by $54 \pm 0.02\%$ (Fig. 3C)–68.10 ± 2.60 s ($P < 0.05$, Fig. 3C). Our results suggest that the defects in diencephalon induced by *zdhhc15b* mutation results in learning impairment.

DOWNREGULATION OF *Zdhhc15b* RESULTS IN A REDUCTION OF DA NEURONS IN ZEBRAFISH DIENCEPHALON

DA neurons, as important components of the vertebrate diencephalon, play crucial roles in cognition, movement control, and endocrine modulation. The defects found in the diencephalon of *zdhhc15b* morphants that were associated with poor learning behavior prompted us to investigate the potential role of Zdhhc15b in DA neuronal development. To investigate DA neuronal development, we first extracted mRNA from the forebrain. With PCR, we discovered that the expression of two reliable markers of DA neurons, tyrosine hydroxylase (TH) and dopamine transporter (DAT), was significantly reduced at 24 and 48 h (Fig. 4A), while no significant changes were found in the expression of the astrocytic marker glial fibrillary acidic protein (GFAP) or the motoneuron marker znp1 (Fig. 4A). This result suggests Zdhhc15b may have a specific role in DA neurons.

To confirm our findings, immunofluorescence of TH was carried out and the result showed TH was expressed in *zdhhc15b* domain (Fig. 4B). Subsequently, we performed in situ hybridization of TH and DAT. We found that both TH/DAT-positive clusters were found in the diencephalon with fewer DA neurons in the *zdhhc15b* morphants. Also the number of embryos with defects was quantified (Fig. 5A, B). Western blot (WB) and immunofluorescence of TH exhibited a similar pattern after *zdhhc15b* knock-down, as shown by a down-regulation in protein TH expression and a decrease in TH-positive DA neurons (Fig. 5B, C). Co-injection of MO with $\triangle$*zdhhc15b* mRNA was able to rescue the morphant phenotype and largely resumed the number of TH/DAT-positive cells (Fig. 5D). Taken together, we propose that *zdhhc15b* regulates the development of diencephalic DA neurons.

To further verify the role of *zdhhc15b* in the development of DA neurons, we turned to DA neurons grown in culture. Diencephalic neurons in both control and *zdhhc15b* knock-down zebrafish were isolated at the stage of 24 hpf and cultured in vitro (Fig. 6A). TH immunofluorescence was used to detect the number of TH-positive cells (Fig. 6B). As shown in Fig. 6C, of the total number of cells labeled with the nuclear dye DAPI, we found that while $14.17 \pm 2.31\%$ of the cells were TH-positive in control

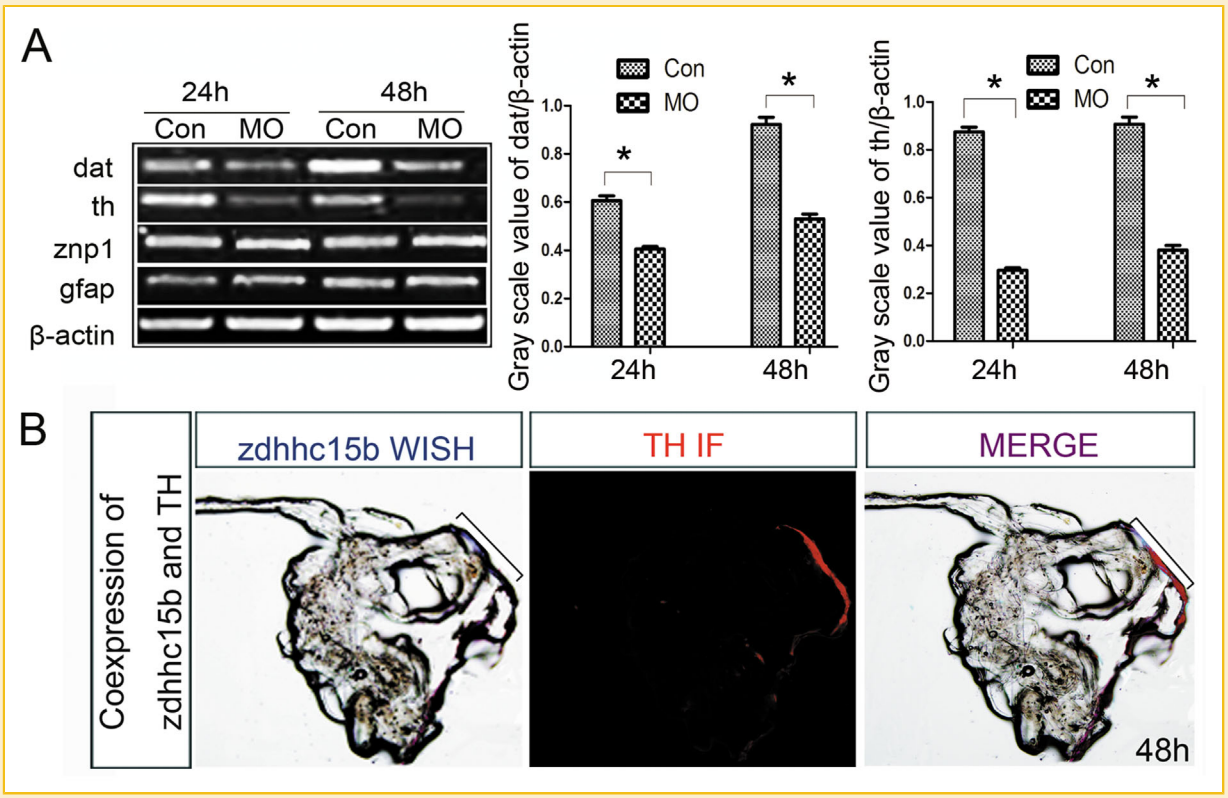

Fig. 4. Reduced expression of th and dat in DA neurons (A) th and dat are two representative markers of DA neurons. RT-PCR showed that the expression of both genes was reduced, while no significant changes were found in the expression of gfap and znp1, markers for astrocytes and motoneurons, respectively. (B) TH was expressed in *zdhhc15b* domain. Immunofluorescence of TH in in situ hybridization of *zdhhc15b* embryos showed TH expression (red) in close proximity to *zdhhc15b* neurons (blue). * $P < 0.05$.

cultures, only $7.83 \pm 1.47\%$ were TH-positive in *zdhhc15b* knock-down cultures ($P < 0.05$). Therefore, knock-down of *zdhhc15b* reduces the number of DA neurons, likely leading to a smaller, less developed diencephalon that results in learning deficits.

KNOCK-DOWN OF *Zdhhc15b* IMPEDES THE DIFFERENTIATION OF DA NEURONS BUT NOT PROGENITOR CELL PROLIFERATION OR DA NEURONAL SURVIVAL

During development, DA progenitor cells undergo an arrest in cell division and acquire differentiated features in response to differentiation signals to eventually mature into DA neurons. In the present study, although the diencephalon as a whole was slightly smaller in *zdhhc15b* morphants, PCNA, and BrdU analyses demonstrated that *zdhhc15b* morphant diencephalons where the DA neurons exist have no obvious changes in the cell cycle as compared to controls (Fig. 7A, B). Therefore, Zdhhc15b may be not necessary for DA progenitor proliferation. Next, we found that *ngn1*, a DA progenitor marker, was comparably expressed in *zdhhc15b* morphants and controls (Fig. 7C), indicating that Zdhhc15b is unlikely to be required for the specification of DA precursor cell identity.

The weak hybridization signals of TH and DAT in *zdhhc15b* morphants raised the question of why knock-down of *zdhhc15b* led to fewer DA neurons in the diencephalon. There are two possibilities. First, the number of DA progenitors and DA neurons may be balanced by increased cell death in *zdhhc15b* morphants. Second, *zdhhc15b* knock-down may have impaired the fate differentiation of DA neurons. To test the first prediction, acridine orange apoptosis staining was performed at 24 hpf. As shown in Fig. 8A, no increase in apoptotic cell death was found at this time point. To determine whether *zdhhc15b* is required for the fate differentiation of DA neurons, WISH and RT-PCR were performed to examine the expression of DA neuronal differentiation factors. *nurr1*, which is expressed in immature DA neurons, decreased in *zdhhc15b* morphants (Fig. 8B). RT-PCR revealed that *lmx1a* and *foxA*, which sequentially regulate the specification and differentiation of neuroepithelial cells toward mature DA neurons, were reduced after *zdhhc15b* knockdown (Fig. 8C). The gene *pitx3* that can induce the expression of immature *DA* neurons showed a similar decrease. In contrast, expression of *enl/2* and *Ebf3* showed no significant changes between control and *zdhhc15b* morphants. Our findings indicate that Zdhhc15b acts as an intrinsic factor that specifically regulates DA neuron differentiation by recruiting the necessary series of transcription factors.

DISCUSSION

In the present study, we used zebrafish to characterize the in vivo spatial and temporal expression pattern of *zdhhc15b*. Results of

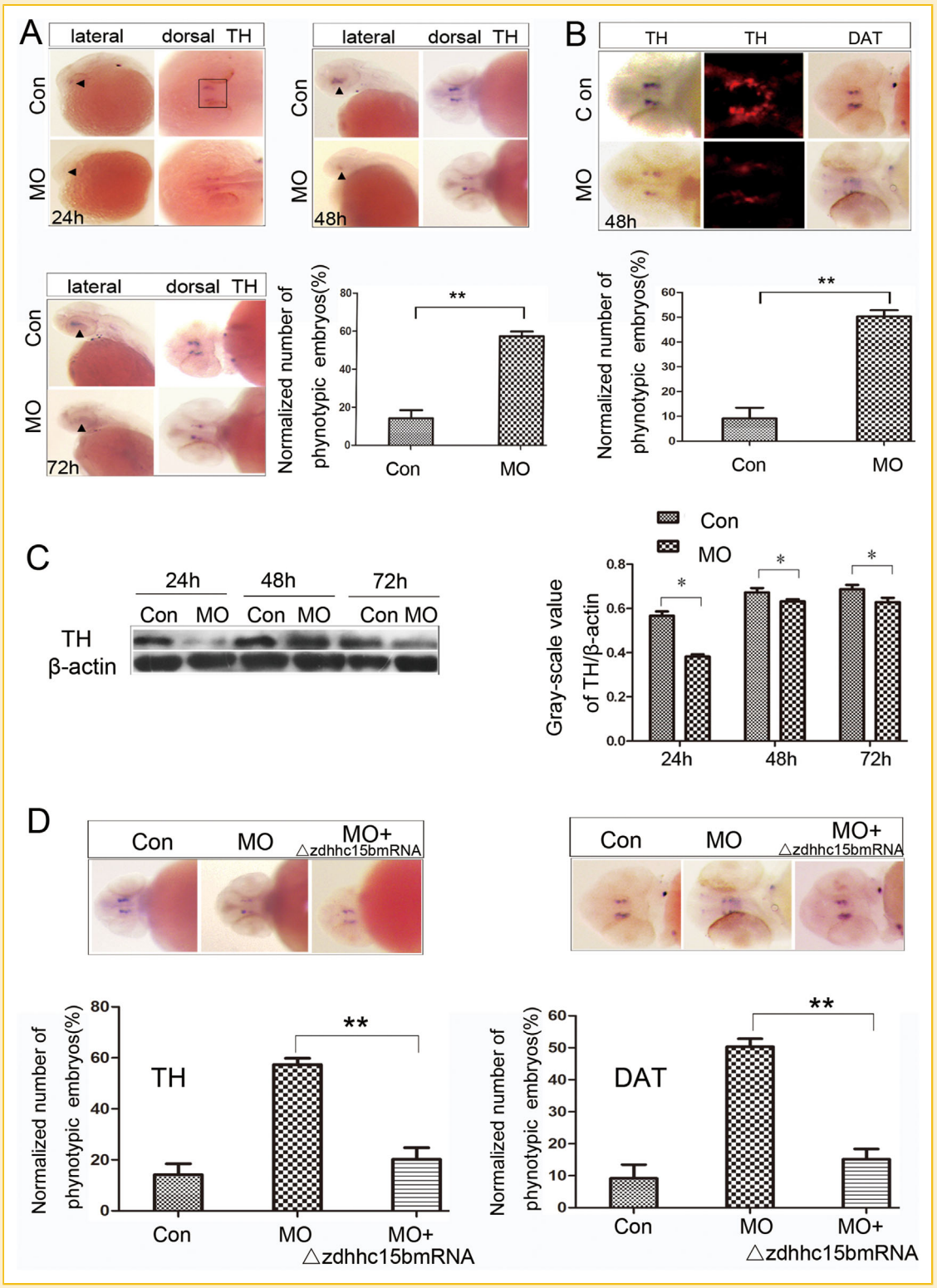

Fig. 5. *zdhhc15b* MO injection resulted in decreased DA neurons. (A) TH WISH revealed that DA neurons decreased in *zdhhc15b*-deficient embryos from 24 hpf to 72 hpf. Rectangle, zebrafish diencephalon. Triangle, DA neurons. Also the number of embryos with defects was quantified. (B) In situ hybridization of DAT in embryos of 48 h postfertilization (hpf). The signals of DAT are decreased in *zdhhc15b* MO-injected embryos. The number of embryos with defects was quantified as shown by the graph. Immunofluorescence of TH also showed reduced expression of TH when *zdhhc15b* was downregulated. (C) Western blot (WB) revealed lower expression of TH protein levels in MO injected-embryos compared with control. Rectangle, zebrafish diencephalon. Triangle, DA neurons. (D) Co-injection of *zdhhc15b* MO with △zdhhc15b mRNA can rescue the defects. Error bars indicate SD, * $P < 0.05$, ** $P < 0.01$.

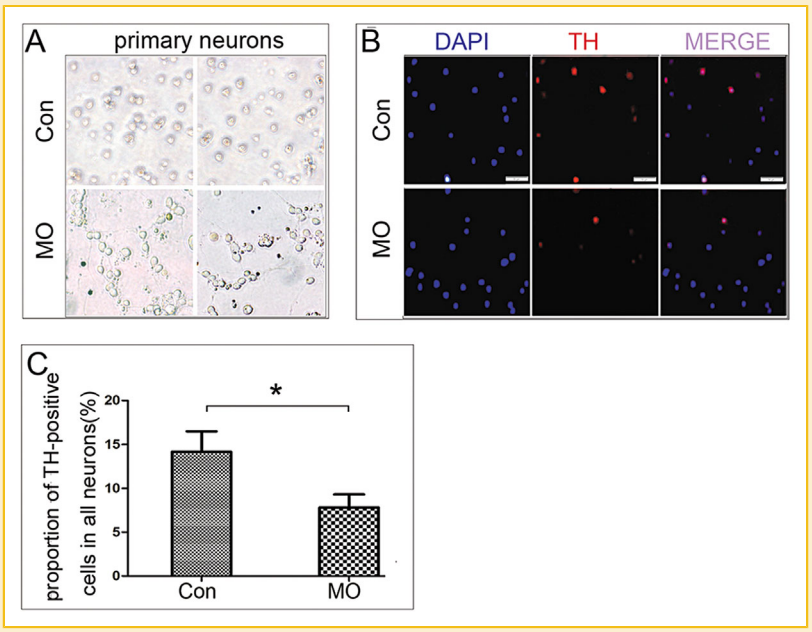

Fig. 6. DA neurons cultured in vitro. (A) Morphous of DA neurons cultured in vitro. (B) TH immunofluorescence showed less TH-positive DA neurons in *zdhhc15b* MO group compared with control. (C) The proportion of TH-positive DA neurons in the MO group was significantly less than control. Scale bar indicates 50 μm. Error bars indicate SD, $^{*}P < 0.05$.

WISH showed that *zdhhc15b* was strongly expressed in the zebrafish forebrain. Down-regulating *zdhhc15b* in developing zebrafish resulted in a fewer mature DA neurons, in turn yielding a smaller diencephalon with learning deficits.

Since the discovery of the DHHC protein family, attention has focused on its role in neural development. Through regulation of neuronal protein trafficking and function, DHHCs have been known to affect neurogenesis, cell growth, fate, and function [Chen et al., ; Ponimaskin et al., 2008]. Neurogenesis requires three general stages: proliferation of neural stem cells, symmetric and asymmetric divisions of neural stem cells into committed progenitor cells, and progenitor cells to establish mature neurons that undergo a series of plasticity changes to form synaptic connections. These processes involve various mechanisms that require intrinsic as well as extrinsic factors such as the bHLH transcription factor Hes family, as well as transient expression of Fgf10 [Paridaen and Huttner].

DA neuronal development may be divided into three stages: the specification of DA progenitors; the differentiation of DA progenitors into immature DA neurons; and the terminal maturation of DA neurons [Li et al., ; Rawal et al., 2006]. The first dopaminergic neurons differentiate at about 18 hpf in the prospective posterior tuberculum (basal plate area of prosomere three). Successively, additional groups of DA neurons are specified to build the full complement of early larval DA groups by 4 days post fertilization (dpf). So far, three main classes of genes have been identified for DA neuronal development: (i) transcription factors exclusively involved in DA specification and differentiation, including *nurr1*, *ngn2*, *lmx1a*, *foxA*, *pitx3*, *enl/2*, and *Ebf3* [Nunes et al., 2003; Smidt et al., 2000; Andersson et al., 2006]; (ii) signaling pathways and transcription factors involved in patterning of brain regions in

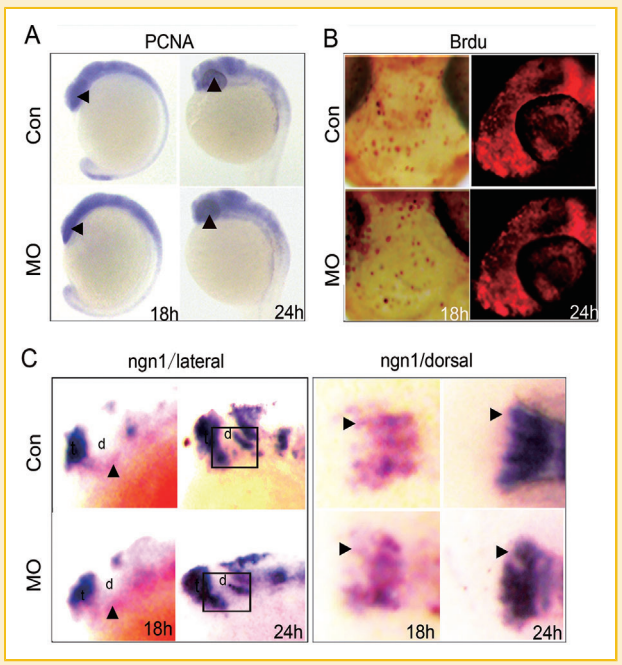

Fig. 7. Proliferation and formation of DA precursors. (A) Cell proliferation in zebrafish was assessed by in situ hybridization of PCNA and a Brdcorporation assay. No significant changes were observed between zebrafish with downregulated *zdhhc15b* and control zebrafish. (B) WISH of ngn1 revealed DA precursors were properly\generated in both control and *zdhhc15b*-deficient embryos. Rectangle, zebrafish diencephalon. Triangle, DA neurons.

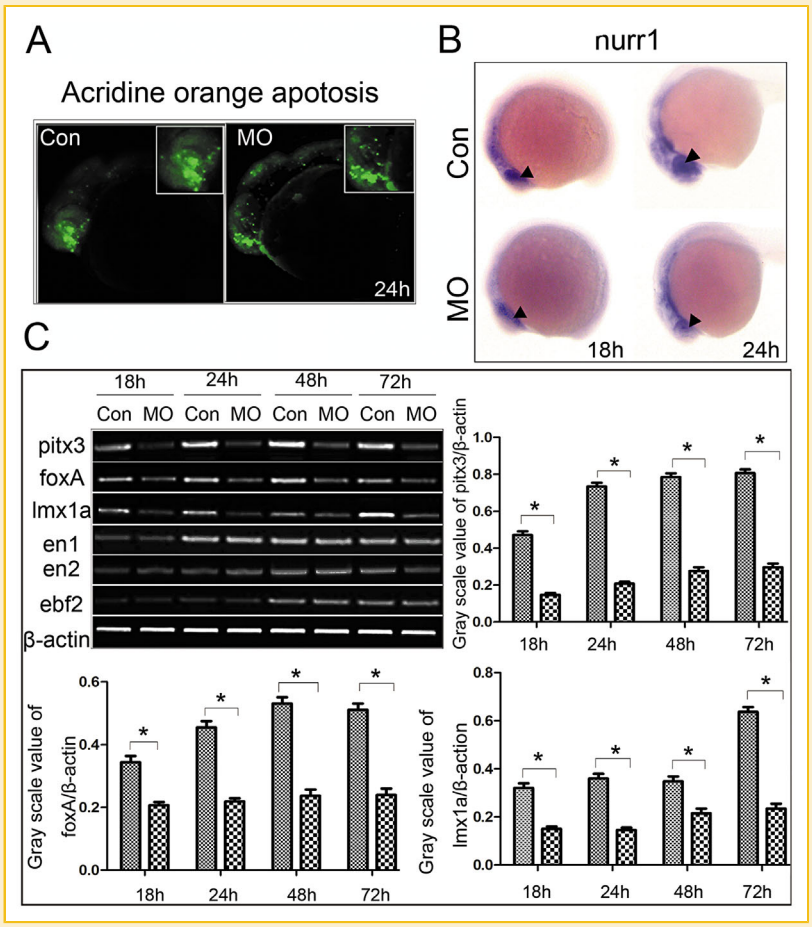

Fig. 8. Survival and differentiation of DA neurons. (A) Acridine orange apoptosis staining showed no significant differences between *zdhhc15b*-deficient embryos and controls. (B) Reduced differentiation of DA neurons in *zdhhc15b*-deficient embryos was revealed by the decreased expression of the immature DA neuronal marker nurr1. (C) The expression of DA neuronal differentiation related transcription factors pitx3, lmx1a, foxA2 were downregulated while no significant changes was seen in en1/2 or ebf3 expression. $^{*}P < 0.05$.

which DA neurons differentiate; (iii) components of the transcription machinery which may have a differential requirement during specific aspects of development, but are not selectively involved in DA neuron specification, including SptS, Med12, and Med27 [Schweitzer and Driever, 2009]. The results of PCNA, BrdU immunofluorescence, and immunohistochemistry suggested Zdhhc15b maybe unnecessary for DA progenitor proliferation. Moreover, the same expression of *ngn1* in control and morphant zebrafish revealed that Zdhhc15b is unlikely to be required for the specification of DA precursor cell identity. Therefore, we propose that Zdhhc15b play a role in the differentiation of DA neurons.

DA neurons in diverse areas of the diencephalon and midbrain play important roles in movement control, endocrine modulation, and many other important physiological processes, and they are also involved in human neurological diseases [Ota et al.], including Parkinson′s disease [Chen et al., ; Conti et al., ; Goldstein, ; Tronci et al.], addiction, depression [Hensler et al.], and a variety of neuronal disorders. For these reasons, a lot of interest and therapeutic hope arose from a better understanding of the molecular mechanisms that govern the differentiation of DA neurons. Moreover, DA diencephalon neurons in zebrafish, although less well studied, form ascending and descending projections, and are believed to be homologous to mammalian mesencephalic DA neurons [Schweitzer and Driever, 2009]. Therefore, zebrafish are a good model to study dopaminergic neurons.

The neuronal gene Olig2 and the transcriptional regulator Sim1 were reported to be co-expressed in a subset of diencephalic progenitors destined towards the DA neuronal fate [Borodovsky et al., 2009]. However, the mechanisms responsible for the differentiation from DA progenitors to mature DA linage in the diencephalon remain elusive. In the present study, we elucidated that *nurr1*, which is mainly expressed in immature DA neurons and responsible for the terminal differentiation of DA neurons, would be downregulated in Zdhhc15b morphants. Nurr1 has been shown to induce TH expression by binding to a NRBE (NGFI-B response element) sequence in the TH promoter, causing the dissociation of transcriptional co-repressors and recruitment of transcriptional co-activators [Blin et al., 2008; Borodovsky et al., 2009] . In addition, RT-PCR revealed that other genes involved in DA neuronal differentiation like *foxA2*, *lmx1a*, and *pitx3* were downregulated after *zdhhc15b* knockdown, leading us to conclude that Zdhhc15b

might affect DA neuronal differentiation by recruiting the required series of transcription factors.

Among DHHC proteins, 6 in yeast (Akr1, Erf2, Swf1, Pfa3, Pfa4, and Pfa5) and 16 in mammals (DHHC2–9, 12, 15, 17–21, and 22) have already been proven to be protein acyl transferases (PATs) [Ohno et al.]. A previous study showed that ZDHHC13 regulates the fate specification of ectodermal and mesodermal cell lineages by modulating BMP activity [Linder and Deschenes, 2004]. In our study, foxA2 and lmx1b, that could directly induce the expression of nurr1-DA neurons, were downregulated as downstream genes of Shh. The downstream genes of SHH like ptc/nk2.2 were changed which was proved by our preliminary study. In short, we speculate that Zdhhc15b regulates DA neuronal differentiation by modulating the Shh signaling pathway as a PAT.

In summary, our work defined a novel mechanism controlling DA neuronal development and suggested further links between dysregulated DHHC proteins and neuro developmental disorders.

CONCLUSIONS

Altogether, the most likely interpretation of our results is that by regulating differentiation of DA neurons, Zdhhc15b acts as the determinant factor mediating the transition of late DA progenitors towards the TH-positive fate. Whilst this study provides new insight into the development of DA neurons and advances our knowledge of the function of the DHHC family, many aspects of this family's functions remain mysterious and require further study.

AUTHORS' CONTRIBUTIONS

FW performed the majority of experiments, analysis of data and writing of themanuscript; XRC, WS, LLY, MG, and YY contributed to the analysis of some data.AJH was involved in study design, data interpretation, and manuscript editing.

ACKNOWLEDGMENTS

This work was supported by funding from National Natural Science Foundation of China (No. 31271289 and 31440056), Ministry of Education of China (No. 20110131110036), Natural Science Foundation of Shandong Province (No. 2012GSF11842 and ZR2010HQ022) and Shandong University (No. 2012JC006).

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