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Research Paper

Zebrafish Lbh-like Is Required for *Otx2*-mediated Photoreceptor Differentiation

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Abstract

The homeobox transcription factor *orthodenticle homolog 2* (*otx2*) is supposed as an organizer that orchestrates a transcription factor network during photoreceptor development. However, its regulation in the process remains unclear. In this study, we have identified a zebrafish limb bud and heart-like gene (*lbh-like*), which is expressed initially at 30 hours post fertilization (hpf) in the developing brain and eyes. Lbh-like knockdown by morpholinos specifically inhibits expression of multiple photoreceptor-specific genes, such as *opsins*, *gnat1*, *gnat2* and *irbp*. Interestingly, *otx2* expression in the morphants is not significantly reduced until 32 hpf when *lbh-like* begins to express, but its expression level in 72 hpf morphants is higher than that in wild type embryos. Co-injection of *otx2* and its downstream target *neuroD* mRNAs can rescue the faults in eyes of Lbh-like morphants. Combined with the results of promoter-reporter assay, we suggest that *lbh-like* is a new regulator of photoreceptor differentiation directly through affecting *otx2* expression in zebrafish. Furthermore, knockdown of *lbh-like* increases the activity of Notch pathway and perturbs the balance among proliferation, differentiation and survival of photoreceptor precursors.

Key words: Lbh-like, retina development, photoreceptor differentiation, *otx2*, Notch

Introduction

The vertebrate retina, a highly organized neural structure with three major cellular layers, is an excellent model for studying cell fate decision [1-3]. During retinogenesis, multipotent retinal progenitor cells (RPCs) give rise to six types of neurons and one type of glial cells in a precise and conserved order [4]. Rod and cone photoreceptor cells are light-sensitive neurons in vertebrates [5] and account for over 70% of the cells in mammalian retina. Early in retinogenesis, RPCs proliferate and produce additional multipotent progenitors or lineage-restricted progenitors. After exiting from final mitosis, some of lineage-restricted progenitors are directed to become photoreceptor precursors. Although numerous studies on regulatory networks in which photoreceptor precursors differentiate into rod or cone photoreceptors have been made, there is only a limited knowledge about the decision how RPCs become photoreceptor precursors [6]. To date, about 20 transcription factors had been identified as intrinsic factors involved in the cell fate determination [6]. Among these factors, a homeobox transcription factor orthodenticle homolog 2 (Otx2) was found to act in the very first step of ocular development and to direct RPCs to differentiate into photoreceptor precursors [6, 7], and the Otx2 conditional knockout mouse was observed to lose photoreceptor, bipolar and horizontal cells [8]. Furthermore, Otx2 was revealed to induce ciliary- and iris-derived cells to differentiate into photoreceptor-like cells [9]. These findings suggest that Otx2 should be a key director in photoreceptor lineage commitment. However, how Otx2 responds to the induced signals is still unclear.

Limb bud and heart (LBH) is a novel high-conserved transcription cofactor in vertebrates involved in embryonic development [10]. It was firstly identified as a novel mouse gene and named by its unique spatiotemporal expression pattern in the

developing limb bud and heart [11]. LBH family proteins contain a conserved acidic glutamate-rich transcriptional activation domain, but lack the ability to bind DNA directly [11]. Recently, LBH was identified as a regulator of mammary stem cell expansion/maintenance [12], synoviocyte growth [13], and cranial neural crest cell migration [14]. However, the functions remain largely unknown. In this study, we have identified and characterized a novel zebrafish LBH gene, named *lbh-like*. Furthermore, we have performed a series of experimental investigations, and demonstrated that *lbh-like* is required for retina development and photoreceptor differentiation through affecting the expression of *otx2*.

Materials and Methods

Zebrafish husbandry

Wild type zebrafish (AB strain) was raised and maintained at 28.5℃ as described previously [15]. The developmental stages were staged in hours or days post-fertilization according to morphological criteria described by Kimmel et al. [16]. The animal protocol for this research was approved by the Institute of Hydrobiology Institutional Animal Care and Use Committee (Approval ID: keshuizhuan 0829).

Body length and eye size measurement

The linear distance from the head epiphysis to the tail, along the anterior-posterior axis, was determined as embryo body length. Embryo eye length was quantified by measuring the diameter of eyes. Scales at the same magnification were employed in the measurement.

RNA isolation, semi-quantitative RT-PCR and real-time PCR

Total RNAs were isolated from zebrafish embryos or adult tissues by using SV Total RNA Isolation System (Promega). The quantity and quality were determined by agarose electrophoresis and spectrophotometer [17]. The isolated RNAs were then reverse-transcribed by using the Goldscript cDNA Synthesis Kit (Invitrogen) following the manufacturer's recommendation. *β-actin* was detected as an internal control. Semi-quantitative RT-PCR and real-time PCR analyses were performed as described previously [18], and the sequences of primers were given in Supplementary Table S1.

Whole mount *in situ* hybridization

The procedure of whole mount *in situ* hybridization was performed as described previously [19]. The primers with T7 promoter for probe synthesis were shown in the Supplementary Table S1.

Morpholino and mRNA synthesis

Morpholino oligonucleotides were purchased from GeneTools, LLC (Philomath, OR, USA). Sequences of MOs were listed below: *lbh-like*-MO1 5′-ATCTCCACGCTGCCCATGCCTGGTA-3′ (translational blocking morpholino), *lbh-like*-MO2 5′-AGC ACTGCCCCTCCACAGGCCCCAT-3′ (translational blocking morpholino), Control MO 5′-CCTCTT ACCTCAGTTACAATTTATA-3′ (standard control morpholino), *otx2*-MO 5' - GTTGCTTGAGATACGAC ATCATGCT - 3' [20], *p53* MO: 5'-GCGCCATTGCTT TGCAAGAATTG-3' [21].The primers for rescue experiments were shown in the Supplementary Table S1. Capped RNAs were prepared with the mMESSAGE mMACHINE kit (Ambion). All MOs or mRNAs were injected at the one-cell stage as described previously [21].

Antibody production and Western blot

A peptide (CVSEVESGELRWPPE) was synthesized and conjugated to the KLH peptide (GenScript Company). The production of rabbit polyclonal antibody against zebrafish Lbh-like and analysis of Western blot were performed as described previously [22, 23].

Immunohistochemistry

Section immunohistochemistry and whole-mount immunohistochemistry were performed as described previously [24]. Embryos were sectioned at 10 μm in thickness with frozen microtomy (Leica). The following primary antibodies were used: mouse monoclonal antibodies specific for Rhodopsin (1:500 dilution, Abcam), Arrestin3 (1:500 dilution, Abcam), phospho-histone H3 (1:1000 dilution, Cell Signaling), and active caspase3 (1:1000 dilution, BD Biosciences). Fluorescein conjugated goat anti-rabbit/mouse (Thermo) secondary antibodies were used at 1:200 dilution. Nuclei were stained with propidium iodide (PI, 10μg/ml, Sigma). Images were acquired by confocal microscopy (NOL-LSM 710, Carl Zeiss, Germany).

Luciferase assay

A 1.8kb *otx2* promoter, containing a 1.2-kb promoter proximal to Otx2 translation start site and a 0.6 kb FM enhancer [25, 26], was subcloned into pGL3-basic luciferase reporter vector (Promega). The fertilized eggs were firstly co-injected with pRL-SV40 and Otx2pro-Luc at a ratio of 1:10. Then, the fertilized eggs were injected with ddH$_2$O, *in vitro* transcribed *lbh-like* mRNA or *gfp* mRNA [27]. The luciferase assays in zebrafish embryos were performed as described previously at 36 hpf [28]. The primers for luciferase assay were shown in the Supplementary Ta-

ble S1.

Statistical analyses

Statistical data are presented as Mean ± standard deviation (SD). The body/eye length, numbers of photoreceptor per section, numbers of active caspase3-positive cells and pH3-positive cells in Lbh-like morphants and control groups were statistically analyzed by Independent-Samples T Test with SPSS 13.0 software.

Results

Molecular and expressional characterization of *lbh-like* in zebrafish

To identify the LBH proteins in zebrafish, the amino acid sequence of LBH domain in mouse LBH was used to search against zebrafish RefSeq DNA database by tblastn with default parameters (NCBI). A significant uncharacterized LOC100000094 (XM_001336435) sequence was observed in zebrafish database. According to the sequence, we cloned the full-length cDNA through RACE PCR. Its full length is 1920 bp, and has an ORF of 633 bp that encodes a protein of 211 amino acids (Supplementary Fig. S1). The encoded protein contains an acidic glutamate-rich region at the carboxy terminus (Fig. 1A), and has 47%, 47% and 43% identities to LBHL of cichlids, tilapia, and guppy, while it only shares 18% to 20% identities to zebrafish Lbh and other mammal LBH (Fig. 1A).

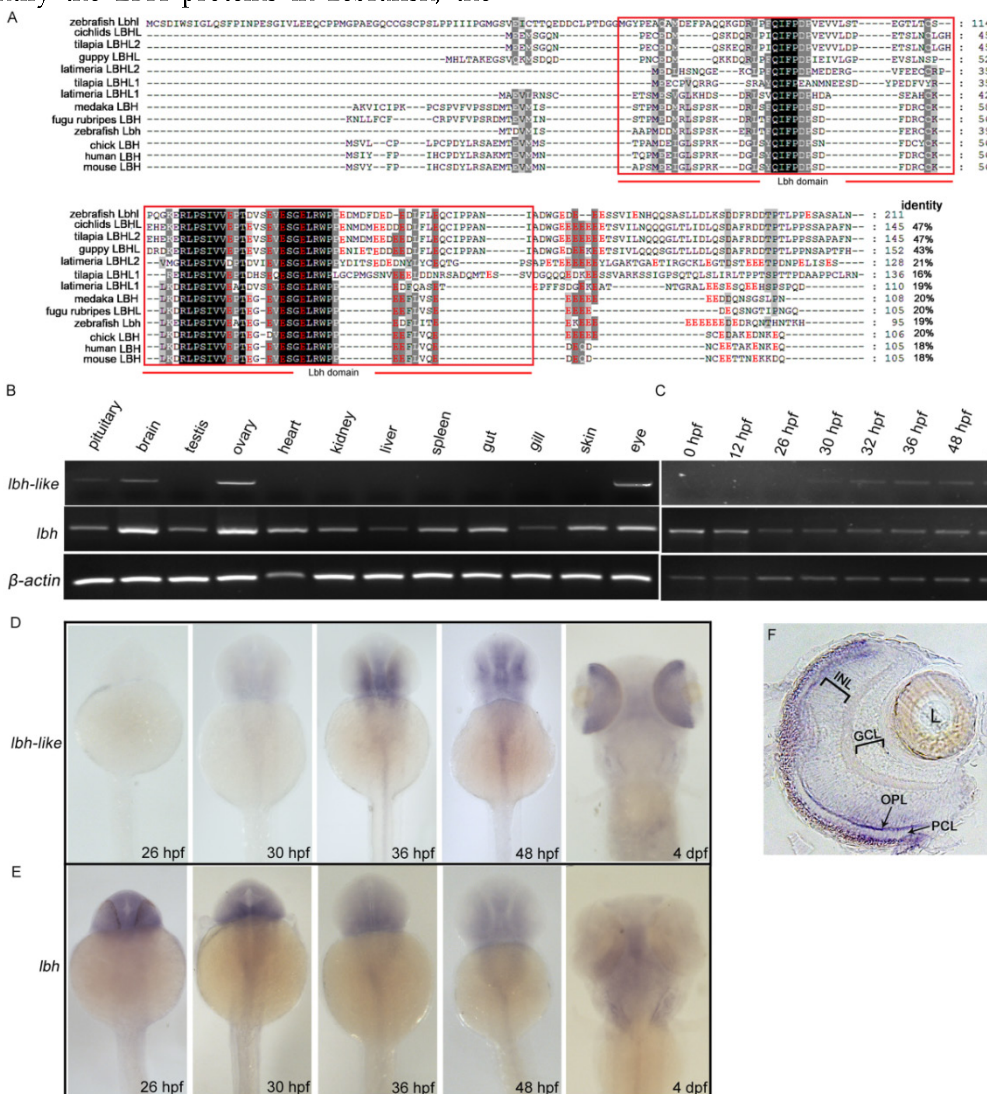

Figure 1. Molecular and expressional characterization of *lbh-like* (*lbhl*) and *lbh* in zebrafish. (A) Multiple amino acid sequence alignment of zebrafish Lbh-like (Lbhl) and Lbh as well as other vertebrate LBHL and LBH. The black and dark shadings indicate identical sequences. The C-terminal glutamate-rich (red color) conserved LBH domain is labeled by red box. The GenBank IDs for these proteins used in this study are as follows: zebrafish (*Danio rerio*) Lbhl, XP_001336471; cichlids (*Haplochromis burtoni*) LBHL, XP_005927379; tilapia (*Oreochromis niloticus*) LBHL2, XP_003441738; guppy (*Poecilia Formosa*) LBHL, XP_007575999; latimeria (*Latimeria chalumnae*) LBHL2, XP_006000505; tilapia (*Oreochromis niloticus*) LBHL1, XP_005464744; latimeria (*Latimeria chalumnae*) LBHL1, XP_005988936; medaka (*Oryzias latipes*) LBH, XP_004082531; fugu rubripes (*Takifugu rubripes*) LBHL, XP_003962799; zebrafish (*Danio rerio*) Lbh, NP_956814; chick (*Gallus gallus*) LBH, NP_001026209; human (*Homo sapiens*) LBH, NP_112177; mouse (*Mus musculus*) LBH, NP_084275. (B) Semi-quantitative RT-PCR detection of *lbh-like* and *lbh* expression in adult tissues. (C) Semi-quantitative RT-PCR detection of *lbh-like* and *lbh* expression during embryogenesis. The *β-actin* was used as internal control. (D, E) Whole-mount *in situ* hybridization with *lbh-like* antisense probe (D) or *lbh* antisense probe (E). (F) Cryosection of eye from embryo hybridized with *lbh-like* antisense probe at 4 dpf stage. INL, inner nuclear layer; GCL, ganglion cell layer; OPL, outer plexiform layer; PCL, photoreceptor cell layer; L, lens. Dorsal views.

We further examined the expression patterns of *lbh-like* and *lbh* in zebrafish by semi-quantitative RT-PCR analysis and whole-mount *in situ* hybridization (WISH). As shown in Fig. 1B-E, *lbh-like* transcript is expressed in pituitary, brain, ovary and eye, whereas *lbh* is more widely expressed in the analyzed tissues. During embryogenesis, *lbh-like* message is initially detected in retina and brain at 30 hpf, which increases and maintains a relatively high level at the following stages (Fig. 1C and 1D). At 4 dpf, *lbh-like* is highly transcribed in retina (Fig. 1D) and mainly distributed in the photoreceptor cell layer (PCL) and outer plexiform layer (OPL) (Fig. 1F). In contrast to *lbh-like*, *lbh* is maternally expressed, and zygotic *lbh* is predominantly expressed in the developing brain and craniofacial structures (Fig. 1C and 1E), as previously shown [14]. Therefore, *lbh-like* and *lbh* have different expression patterns, suggesting that they might play different functional roles.

Lbh-like knockdown inhibits eye development

To reveal function of Lbh-like during embryogenesis in zebrafish, a translation-blocking morpholino (MO1) was designed to knockdown Lbh-like expression. The *lbh-like* MO1 effectiveness was firstly evaluated by co-injecting MO1 and plasmid pTag-RFP-N-Lbh-like containing the morpholino binding site. As shown in Supplementary Fig. S2A, MO1 is highly efficient and its activity lasts through 3 dpf. And, Western blot detection verified the down-regulated expression of endogenous Lbh-like in the *lbh-like* MO1 embryos at 48 hpf (Supplementary Fig. S2B). Moreover, the Lbh-like antibody specificity was also confirmed through Western blot detection of EPC cell extracts with Lbh-like-GST overexpression by pre-immune serum as negative control (NC) and anti-Lbh-like antiserum as positive control (PC) (Supplementary Fig. S2C).

Subsequently, the loss-of-function experiments were performed by MO1 in zebrafish embryos. As shown in Fig. 2, in comparison with wild type embryos or control embryos (CON) injected with control MO (Fig. 2A), 68% embryos (n=31) injected with MO1 show a similar morphant phenotype with smaller eyes at 48 hpf (Fig. 2B). And, the smaller eye defect can be rescued by co-injecting with *in vitro* transcribed *lbh-like* mRNA lacking the morpholino binding site (rcRNA) (Fig. 2C). Moreover, we measured their body length and eye length, and calculated the ratio of eye length to body length. As shown in Fig. 2D-F, a significant (9.96%) reduction of eye length/body length is found in the Lbh-like morphants. Another MO (MO2), targeted to a different region around the translational start site, was also tested to be highly efficient and produced similar phenotypes when injected alone or mixed with MO1 (data no shown).

Morpholino would probably induce development delay that causes small eyes in zebrafish [29]. To exclude this possibility, we tested the expression of thymus-specific *rag1*, since thymus size was usually used as one of morphological markers to distinguish developmental stages of zebrafish larva [30, 31]. As shown in Supplementary Fig. S3, the thymus size does not differ between Lbh-like morphants and wild-type embryos at 3 dpf. Therefore, the data indicate that the microphthalmic phenotype in the morphants is specifically resulted from Lbh-like knockdown.

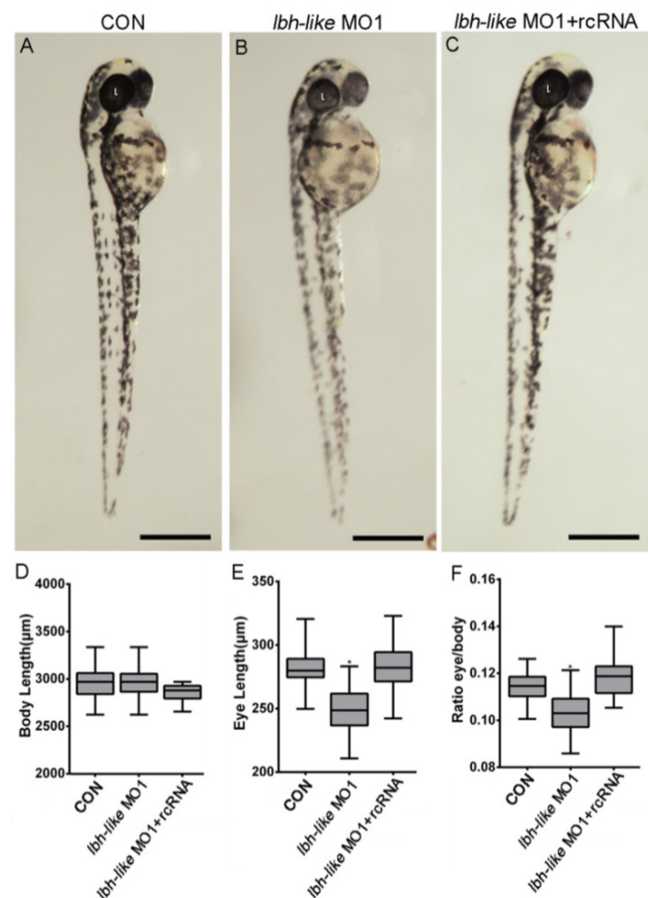

Figure 2. Smaller eye defect in Lbh-like-deficient embryos. (A-C) Lateral views of control embryos (A), *lbh-like* deficient embryos (B) and co-injected embryos with *lbh-like* morpholino and rcRNA (C) at 48 hpf. (D-F) Graphic analysis of body length (D), eye length (E) and the ratio of eye length to body length (F) of embryos shown in A-C. Scale bar=500μm; *p<0.001, *t*-test; n>20; L, lens.

Lbh-like knockdown influences expression of marker genes specific to Müller cells and bipolar cells in retina

To further understand the effect of *lbh-like* in eye development, we investigated the expression of retina markers both by WISH and real-time PCR. Compared with that in control embryos, significant expression increases of Müller cell maturation markers *glutamine*

synthetase (*gs*) and *carbonic anhydrase* (*cahz*) [32] were observed in the Lbh-like morphants at 72 hpf (Fig. 3A,B and Supplementary Fig. S4A-B). In contrast, *visual system homeobox 1* (*vsx1*), essential for the proper development of bipolar cells [33-35], was markedly down-regulated in the morphants at 48 hpf (Fig. 3C and Supplementary Fig. S4C). In addition, the expression levels of other type cell marker genes, such as *alcama* involved in retinal ganglion cell differentiation [36], *ptf1a* expressed in horizontal and amacrine differentiating cells [37], and amacrine cell's marker gene *tyrosine hydroxylase* (*th*) [38], were similar to those in control embryos (Fig. 3D-F and Supplementary Fig. S4D-F). Identical expression patterns between the morphants and control embryos (Fig. 3G, H and Supplementary Fig. S4G, H) were also found in *atonal homologue 5* (*ath5*), a retinal primordium marker involved in RGC specification [39, 40], and *insulinoma-associated 1a* (*insm1a*), a developing retina marker required for photoreceptor differentiation [41]. Therefore, the data suggest that Lbh-like knockdown might specifically influence expression of marker genes specific to Müller cells and bipolar cells in retina.

Lbh-like knockdown inhibits photoreceptor differentiation

We next tested whether Lbh-like knockdown altered photoreceptor-specific gene expression and photoreceptor development in the embryonic retina. As shown in Fig. 4, all extensively expressed *opsins* including *opn1sw2*(*blue*), *opn1mw1*(*green*), *opn1lw1*(*red*), *opn1sw1*(*UV*), and *rho* in the ONL and ventral patch of control embryos are remarkably reduced in the Lbh-like morphants at 3 dpf (Fig. 4 and Supplementary Fig. S5), and the reduction can be rescued by co-injection with *lbh-like* rcRNA (Fig. 4), but not with *lbh* mRNA (Supplementary Fig. S6). And, *lbh* mRNA co-injection further aggravated the Lbh-like morphant's eye defect rather than rescuing it (Supplementary Fig. S6). This indicates that the defect is specific to the Lbh-like knockdown.

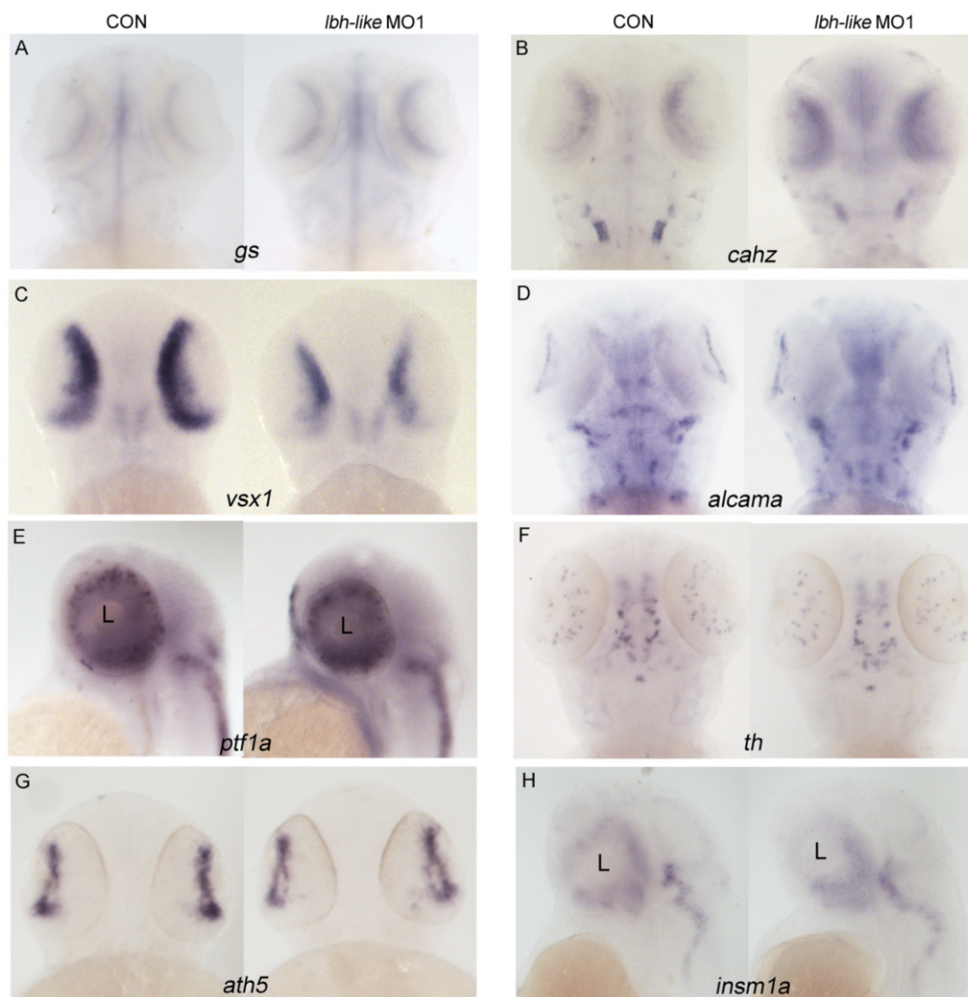

Figure 3. Effects of Lbh-like knockdown on retina marker genes in zebrafish. Gene names are marked in the bottom of panel. In each panel, the left embryo was injected with 4 ng control morpholino and the right one with 4 ng *lbh-like* MO1. (A, B, D and F) embryos at 72 hpf stage; (C, E, G and H) embryos at 48 hpf stage; (A-D and F, G) Dorsal views; (E and H) Lateral views.

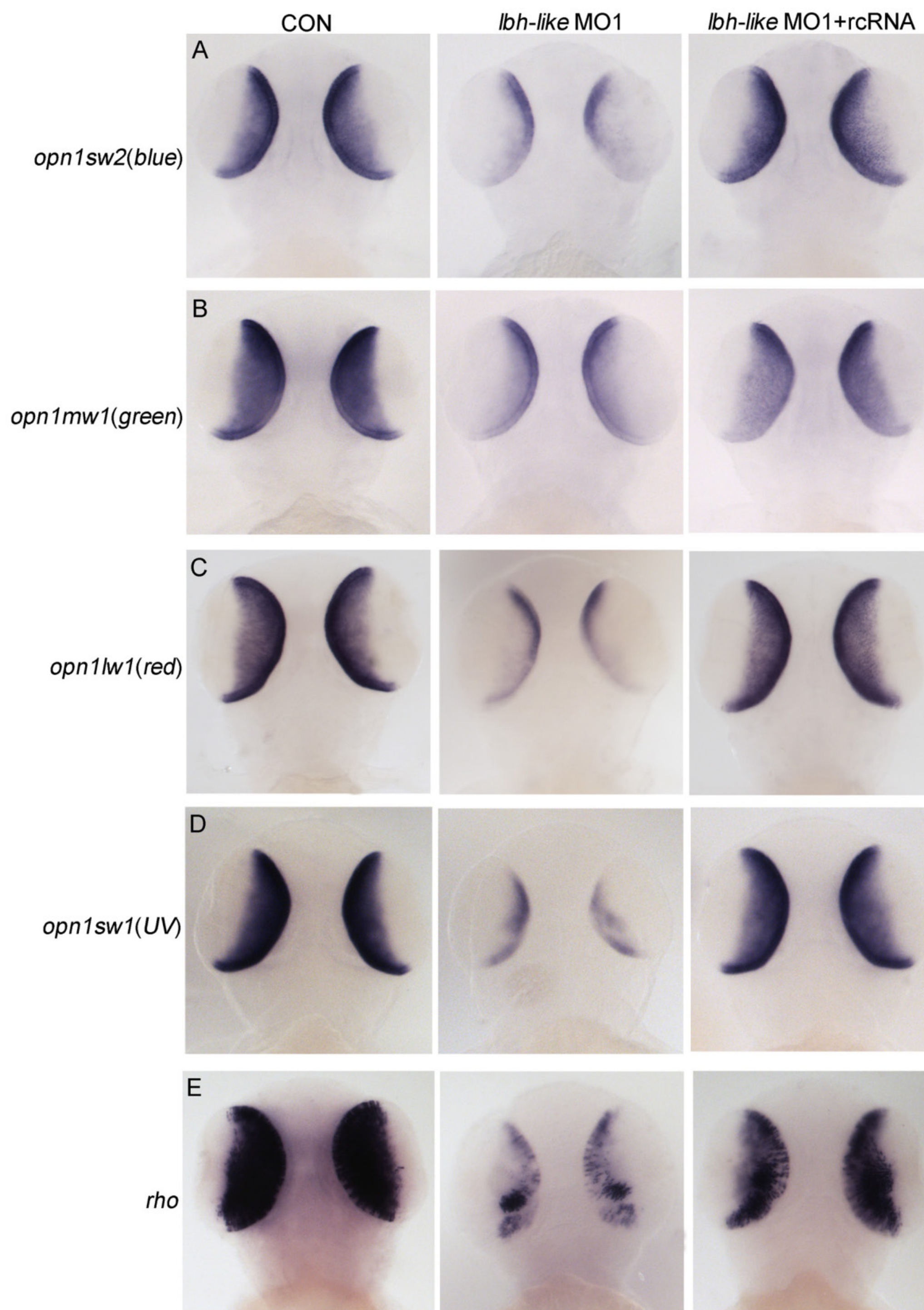

Figure 4. Effects of Lbh-like knockdown on cone and rod photoreceptor marker genes. Four *opsins* (A-D) and *rho* (E) were analyzed by WISH in the embryos at 3 dpf. The left embryos were injected with 4 ng control morpholino, the middle embryos with 4 ng *lbh-like* MO1, and the right embryos with 4 ng *lbh-like* MO1 and 0.1ng rcRNA. Dorsal views.

We further examined the expression of several other photoreceptor-specific genes including rod cell-specific gene *gnat1* (*guanine nucleotide binding protein, alpha transducing activity polypeptide 1*), cone cell-specific gene *gnat2* [42], and *interphotoreceptor retinoid-binding protein* gene (*irbp*) expressed in all photoreceptor cells [43]. *Gnat1* was found to express in the retina and pineal (arrows) of wild type embryos at 72 hpf (Supplementary Fig. S7A). Injection of *lbh-like* MO1 almost eliminated *gnat1* expression in the retina with only a few *gnat1*-expressing photoreceptors at the initial site of retinal differentiation (arrowhead), while its expression in the pineal was not changed (Supplementary Fig. S7A). Similar to *gnat1*,

the retina expression of *gnat2* and *irbp* was also inhibited in Lbh-like morphants, but the pineal expression remained (Supplementary Fig. S7A, B, C). And, the reduced expression of *gnat1*, *gnat2* and *irbp* in retina could be recovered by co-injection with *lbh-like* rcRNA.

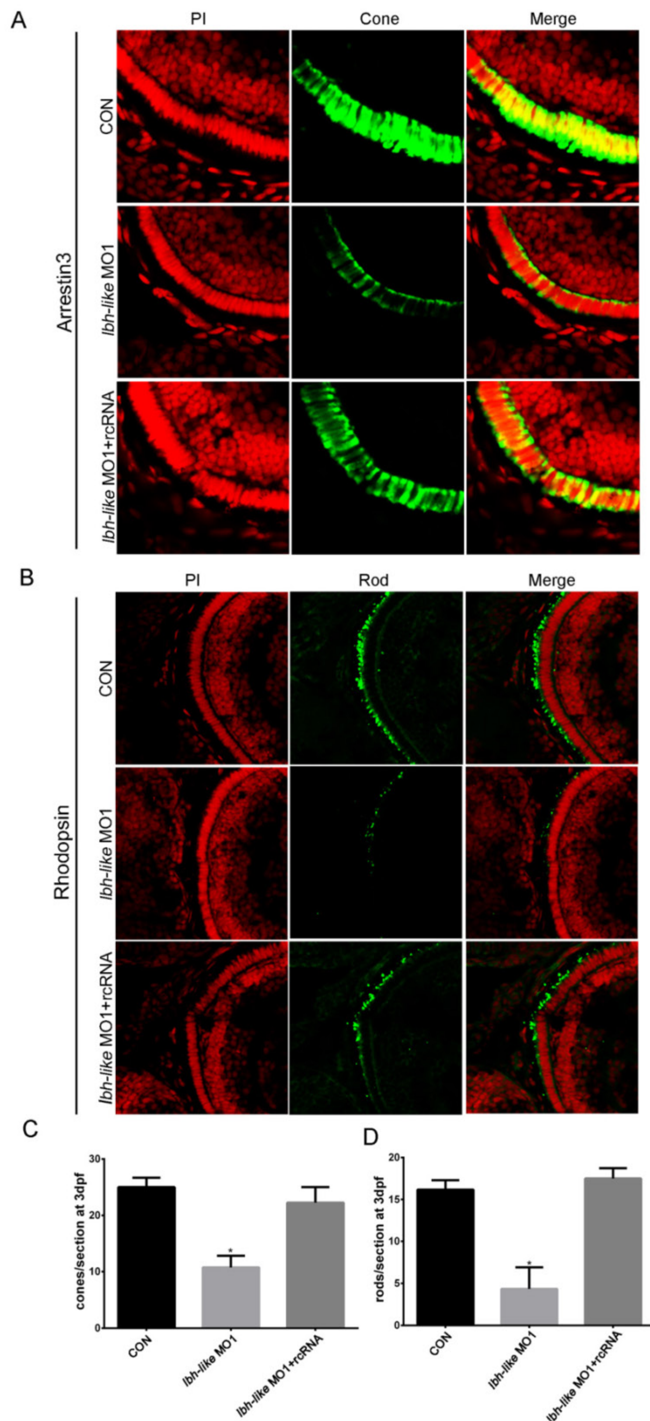

Figure 5. Reduction of cone or rod photoreceptor cells in Lbh-like morphants. (A-B) Immunofluorescence of zebrafish eye sections. Green fluorescence was stained by the anti-Arrestin 3 antibody (A) or anti-Rhodopsin antibody (B). Red fluorescence was stained by PI. Merge was the overlap of green fluorescence and red fluorescence. (C-D) The numbers of cone (C) or rod (D) photoreceptor cells per section counted by immunofluorescence for anti-Arrestin 3 antibody (A) or anti-Rhodopsin antibody (B), respectively. Cone photoreceptor cells per section, **p*<0.01, *t*-test; n=4 eyes for each treatment; Rod photoreceptor cells per section, **p*<0.01, *t*-test; n=6 eyes for each treatment.

To quantify the decrease of opsin expression, we counted the numbers of cone or rod photoreceptors per section by using immunofluorescence with cone photoreceptor-specific antibody (anti-Arrestin 3) and rod photoreceptor-specific antibody (anti-Rhodopsin). At 3 dpf, an average of 25 cones per section was detected in control embryos (Fig.5A, C). However, the Lbh-like morphants displayed greatly reduced numbers of cone photoreceptors (57% reduction) with an average of 10.75 cones per section (Fig. 5A, C). Significantly, the embryos co-injected with *lbh-like* MO1 and rcRNA recovered the cone photoreceptor number to an average of 22.25 cones per section (Fig. 5A, C). Similar phenotype of lacking rod photoreceptors (73.2% reduction) was also observed in Lbh-like morphants compared with controls (Fig. 5B, D). Taken together, these results suggest that Lbh-like is required for the formation of photoreceptor cells.

Lbh-like knockdown leads to cell apoptosis and proliferation in retina

The reduced numbers of photoreceptor cells might be due to the balance change between cell apoptosis and proliferation in the absence of Lbh-like. To address this question, we firstly examined the cell apoptosis in retina by immunostaining assay with antibody against active caspase-3, an apoptosis marker [44]. As shown in Fig. 6A, the number of apoptotic cells in retina is much higher in Lbh-like morphants (122.6±55.59 cells) than that in control embryos (3.67±1.63 cells) at 48 hpf, and it can be rescued by co-injecting with *lbh-like* MO1 and rcRNA (4.43±2.94) (p<0.01; Fig. 6A, C). To avoid unspecific apoptosis due to morpholinos activated by *p53* [45], we also co-injected *lbh-like* MO and *p53* MO into the fertilized eggs (Fig. 6C). The total number of active caspase-3-positive cells (144.2±51.73) is comparable with that in Lbh-like morphants (Fig. 6C), indicating that the abnormal apoptosis in retina is specifically induced by Lbh-like knockdown. These results suggest that the increased apoptosis might lead to small eye in Lbh-like morphants. On the other hand, we examined cell proliferation through observing PH3-positive cells resided in the retina edge [8, 44] by using anti-phospho-histone H3 antibody. Quantitative counting of positive cells showed that the proliferating cells in retina of the Lbh-like morphants (162.57±28.81 cells) was mildly higher than that in control embryos (91.83±30.72 cells) (Fig. 6B, D), and the increased number of proliferating cells could be reduced by co-injecting with *lbh-like* MO1 and rcRNA (111.2±20.33). Previous studies have shown that high level of Notch signaling activity preserves a pool of undifferentiated proliferative RPCs and inhibits cell

differentiation during retinal development [6, 46, 47]. Consistently, Lbh-like morphants also displayed increased expression level of *notch1a* and its downstream target gene *hes5* (Fig. 6E and Supplementary Fig. S8). Therefore, the above results suggest that Lbh-like might have protection against cell apoptosis and balance cell proliferation in retina, and the dual functions might be performed by suppressing Notch signaling.

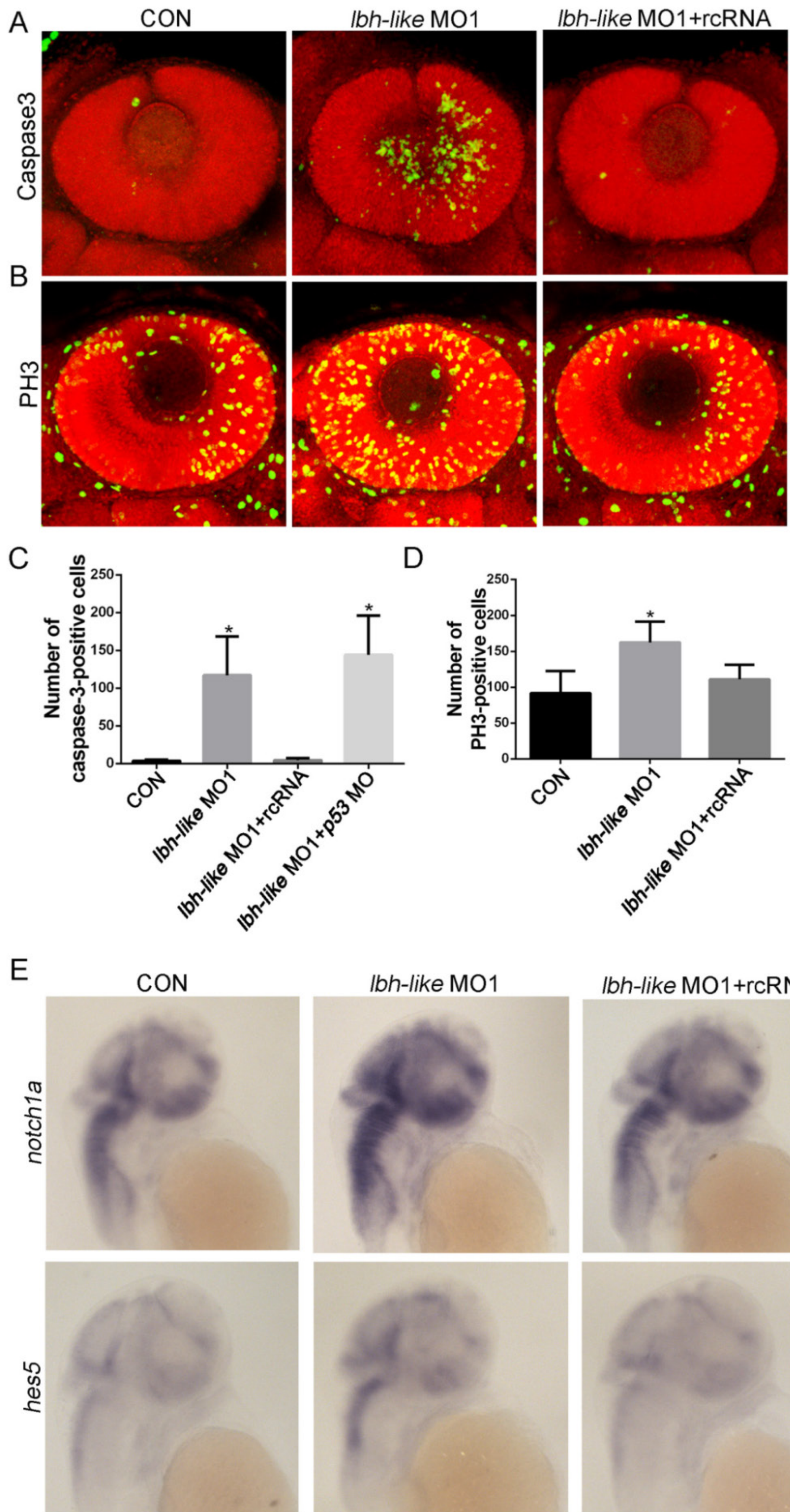

Figure 6. Immunostaining assay of cell apoptosis and proliferation as well as WISH detection of Notch pathway in Lbh-like morphants. (A-B) Confocal z-stacks of apoptotic cells (A) or mitotic cells (B) assessed by immunostaining of Caspase-3 or PH3 antibody in embryos at 2 dpf, respectively. Nuclei were visualized by staining with PI. (C-D) Quantification of numbers of Caspase-3-positive cells (C) or PH3-positive cells (D) in embryos, *$p<0.01$, *t*-test; n=6 eyes for each treatment. (E) *Notch 1* and its downstream target gene *hes5* were analyzed by WISH at 2 dpf. Gene names are marked in the left. The left embryos were injected with 4 ng control morpholino, the middle embryos with 4 ng *lbh-like* MO1, and the right embryos with 4 ng *lbh-like* MO1 and 0.1ng rcRNA. Lateral views.

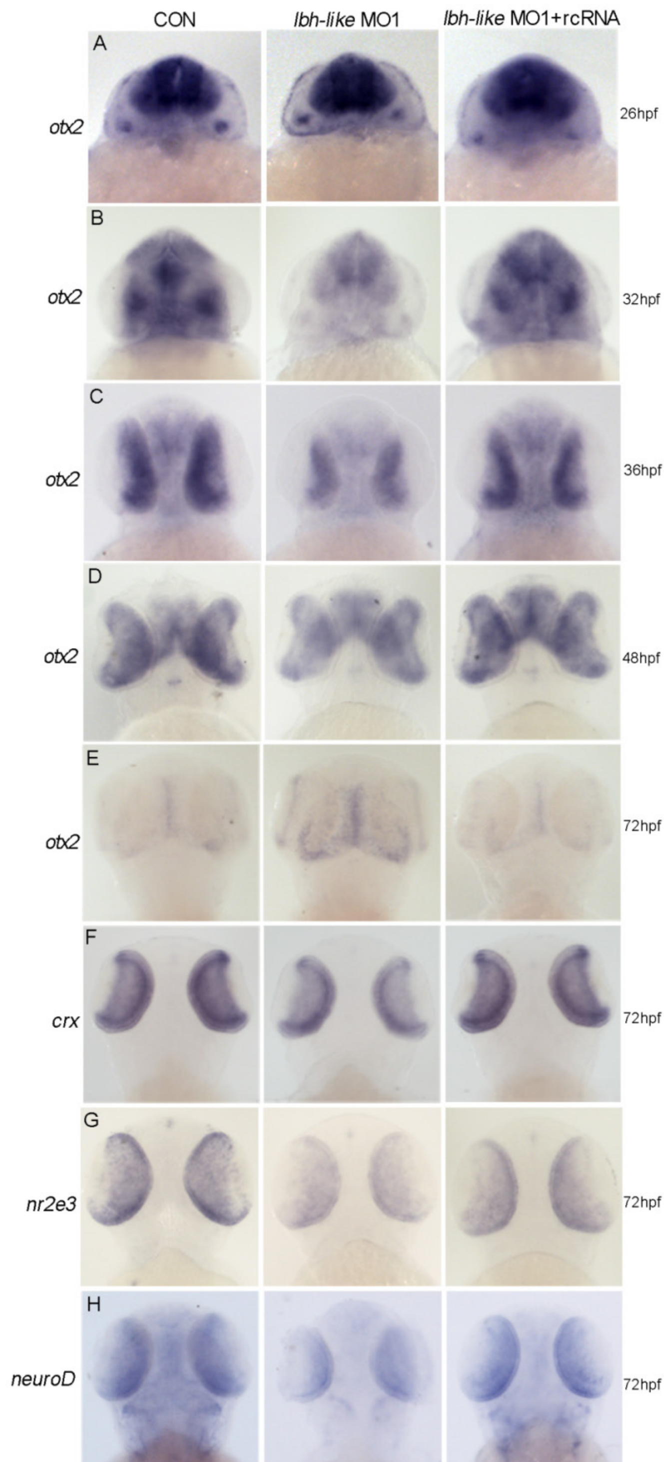

Figure 7. Lbh-like knockdown disrupts *otx2* expression. *otx2* (A-E) and its downstream target genes (F-H) were detected by WISH. Gene names are marked in the left and the stages of embryos are marked in the right. In each panel, the left embryos were injected with 4 ng control morpholino, the middle embryos with 4 ng *lbh-like* MO1, and the right embryos with 4 ng *lbh-like* MO1 and 0.1 ng rcRNA. Dorsal views.

Lbh-like affects *otx2* expression level during photoreceptor differentiation

To reveal the potential mechanism of Lbh-like function during photoreceptor neurogenesis and differentiation, we screened its effects on several key genes for the development of retinal photoreceptor cells. Of these, Otx2 expressed in all photoreceptor cells [8] was revealed to play a critical role in cell fate determination of retinal photoreceptor cells. Since *lbh-like* message was first detected in the developing brain and eyes at 30 hpf (Fig. 1D), we widely examined the expression of *otx2* at 26, 32, 36, 48 and 72 hpf. At 26 hpf, *otx2* was expressed at high level throughout the midbrain and retina in *lbh-like* morphants, equally to that in control embryos (Fig. 7A). Interestingly, *otx2* expression was significantly down-regulated in Lbh-like morphants from 32 hpf to 48 hpf, and was basically coincident with the expression pattern of *lbh-like.* And, co-injection with rcRNA could rescue this defect (Fig. 7B-D). At 72 hpf, when *otx2* expression level rapidly decreased in control embryos, however, the *otx2* transcript still kept a higher level in Lbh-like morphants (Fig. 7E). The above data implies that Lbh-like might tend to keep a certain expression level of *otx2* during retina development, and the effects of Lbh-like seemed similar to a previous finding about *cone-rod homeobox* gene (*crx*) [48], a direct downstream target gene of *otx2* [8].

Moreover, we analyzed the expression characterization of *crx* and the interacted genes *nr2e3* [49] and *neuroD* [50, 51] in Lbh-like morphants. Consistent with the expression reduction of cone and rod photoreceptor marker *opsins* (Fig. 4), all of the three genes displayed the reduced expression in Lbh-like morphants at 72 hpf (Fig. 7F-H and Supplementary Fig. S9), indicating that the photoreceptor differentiation is blocked. Perhaps, it is the delayed withdrawal of cone and rod progenitors from cell cycle that delays the down-regulation of *otx2* accompanying with retinal differentiation, and leads to the unexpected stronger *otx2* signal in Lbh-like morphants at 72 hpf (Fig. 7E). Therefore, Lbh-like is required for correct expression of *otx2* during retinal development.

Lbh-like regulates photoreceptor development via Otx2

Since Otx2 is required for differentiation of photoreceptor cells, one would expect that Otx2 might be the effector of Lbh-like to regulate photoreceptor development. To address this, cone *opsins* and *rho* expression were examined in Lbh-like morphants at 3 dpf through introducing *otx2*-related treatments. Knockdown of Lbh-like inhibited the expressions of 4 cone *opsins* and *rho* (Fig. 8A and 4), and the inhibition could be rescued by co-injecting with *otx2* mRNA or *otx2* downstream transcription factor *neuroD* mRNA (Fig. 8A and Supplementary Fig. S10). In addition, the rescued effect of *otx2* mRNA was compromised by co-injection with *otx2* morpholino, which blocked the

production of Otx2 protein (Fig. 8A and S10). Finally, we performed a luciferase assay to investigate the proposed mechanism by co-injection with *lbh-like* rcRNA and reporter vector Otx2pro-Luc. Overexpression of Lbh-like gave a high activation of Otx2pro-Luc (112.39±41.57) by up to 4.39-folds against that of control embryos co-injected with ddH_2O (26.54±16.62) or *gfp* (24.60±14.64) mRNA (Fig. 8B). Therefore, our results indicate that Lbh-like is required for normal *otx2* expression to regulate photoreceptor differentiation.

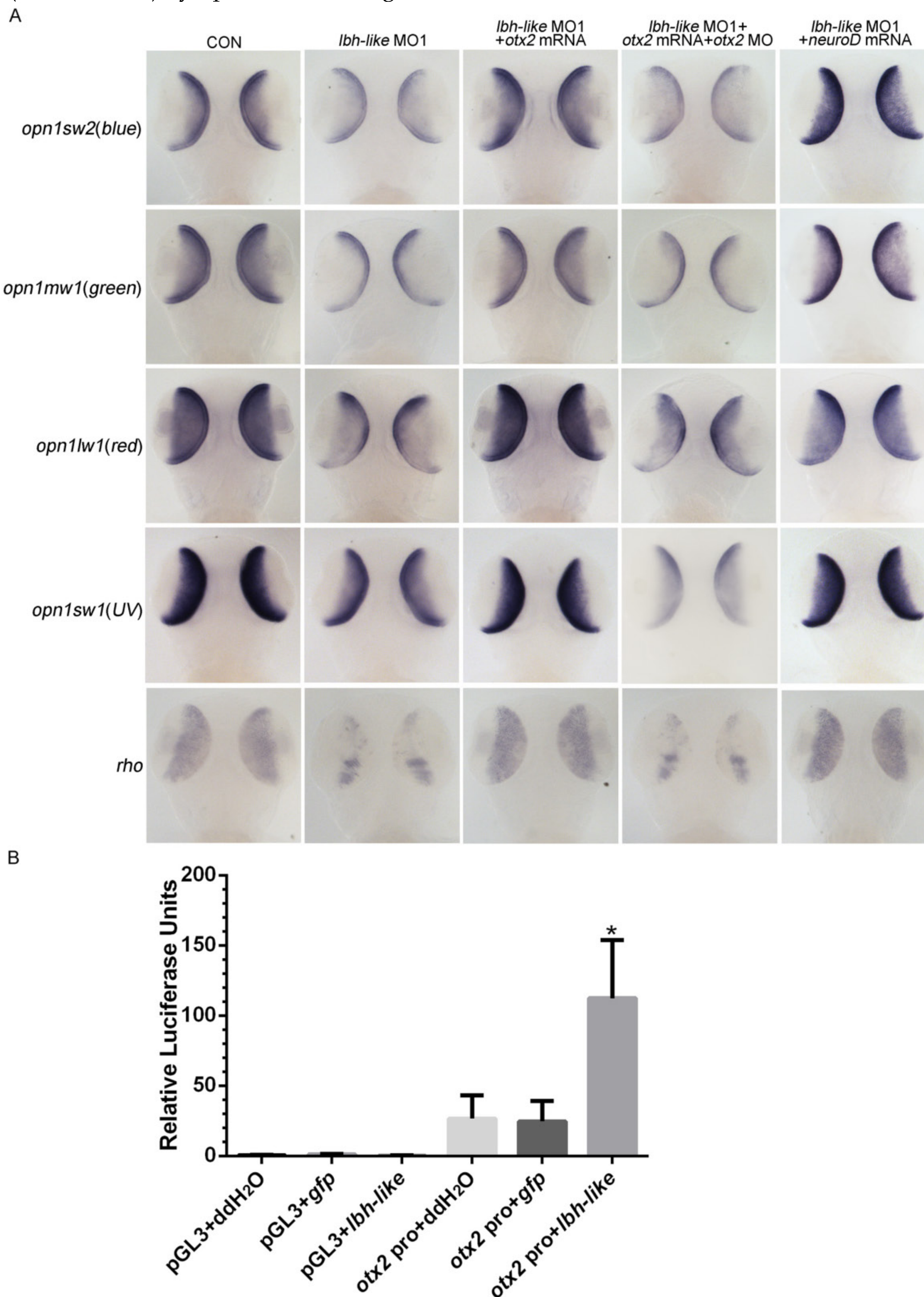

Figure 8. *lbh-like* affects photoreceptor development via *otx2*. (A) WISH analysis of four *opsins* and *rho* in the embryos at 3 dpf. Gene names are marked in the left. From left panel to right panel, embryos were injected with 4 ng control morpholino; 4 ng *lbh-like* MO1; 4 ng *lbh-like* MO1 and 35 pg *otx2* mRNA; 4 ng *lbh-like* MO1, 35 pg *otx2* mRNA and 2.5 ng *otx2* MO; 4 ng *lbh-like* MO1 and 40 pg *neuroD* mRNA. Dorsal views. (B) Relative activities of luciferase driven by a 1.8kb region of the *otx2* promoter (*otx2* pro) in the embryos co-injected with ddH_2O, *lbh-like* or *gfp* mRNA. The pGL3 vector was used as mock. The data was presented as Mean ± SD (3 independent experiments performed in quadruple,*$p<0.05$).

Discussion

An understanding of the photoreceptor differentiation can greatly promote the development of therapies to treat inherited retina diseases because most of these diseases due to dysfunction or loss of photoreceptor cells [52]. In this study, we have characterized a novel member (*lbh-like*) of *lbh* gene family distributed in PCL and OPL of embryonic retina, and have demonstrated that Lbh-like acts as a novel intrinsic factor that regulates retinal development in zebrafish. Based on these findings, we propose a functional pathway of zebrafish *lbh-like* in retinal development, in which Lbh-like is required for correct expression of *otx2* and *notch1*, and under the control of *otx2*, *crx* interacts with *neuroD* and regulates the expression of *opsins*, *gnat1*, *gnat2*, and *irbp*. Thereby, retinal progenitor differentiates into cone and rod photoreceptor (Fig. 9).

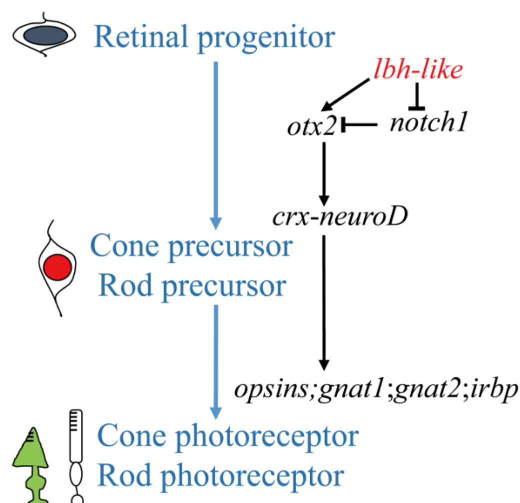

Figure 9. A proposed pathway of zebrafish *lbh-like* in retinal development. The left shows the differentiation of cone and rod photoreceptor, and the right is the regulated pathway.

LBH family members are highly conserved small acidic nuclear proteins in vertebrates [11]. As a novel member of Lbh family, Lbh-like possesses several different characters from Lbh: 1) Zebrafish Lbh contains an N-terminal hydrophobic stretch, a putative nuclear localization signal (NLS) and a C-terminal glutamate-rich acidic domain, same as its orthologous gene in human and mouse [53, 54]. However, Lbh-like shares only 19% amino acid identity with Lbh and contains only a C-terminal glutamate-rich acidic domain (Fig. 1A). 2) Zebrafish *lbh* and *lbh-like* show differential expression patterns (Fig. 1B-E). 3) Zebrafish Lbh mediates migration of cranial neural crest cells [14]. Our result indicates that *lbh-like* is also expressed in the neural crest. However, the function of *lbh-like* gene during neurogenesis needs more exploration.

Otx2 is expressed in retinal progenitors during final mitosis and guides the RPCs into photoreceptor precursor fate [6]. In zebrafish, retinal neurogenesis is initiated around 27 hpf when ganglion cell precursors become postmitotic in a small patch of ventrally located cells [55, 56]. Suzuki and his colleagues [57] traced cone genesis in transgenic zebrafish and observed mitotic division of L-cone progenitors occurred at about 30 hpf. Zebrafish *otx2* is localized in dorsal diencephalon, ventral midbrain and RPE at 26 hpf [58, 59]. At this stage, *otx2* expression is unaffected in Lbh-like morphants (Fig. 7A). Coincidently after the expression of *lbh-like*, *otx2* expression is significantly down-regulated in Lbh-like morphants at 32 hpf (Fig. 7B). Furthermore, the results of rescue experiments (Fig. 8A and S10) and promoter-reporter assay (Fig.8B) indicate that *lbh-like* regulates photoreceptor differentiation directly through affecting the expression of *otx2*. Interestingly, similar to the results in Crx zebrafish morphants [48] and in *Crx*$^{-/-}$ mice [60], *otx2* expression level increases in Lbh-like morphants compared to controls at 72 hpf (Fig. 7E). The delayed withdrawal of cone and rod progenitors from the cell cycle in Crx or Lbh-like morphants might result in delaying the down-regulation of *otx2* [48]. Although the underlined mechanism is unknown, *lbh-like* is an important mediator of *otx2* concerned with photoreceptor development.

Previous studies have shown that LBH proteins regulate cell proliferation and differentiation in mammalian and avian [61, 62]. In our case, smaller eye phenotype and reduced numbers of photoreceptor cells in Lbh-like morphants can be caused by changing the balance between proliferation and lineage differentiation of multipotent RPCs, or increasing the cell death. Interestingly, the increased occurrence of phospho-histone H3 positive cells and active caspase-3 positive cells were both observed in the retina of Lbh-like morphants (Fig. 6A-D). The same phenotypes were observed in zebrafish *hmx1* or *col 15a1* morphant retina [63, 64]. Despite the increased proliferation in the retina (Fig. 6B, D), the mild smaller eye phenotype was most likely due to the high amount of retinal cell apoptosis observed in the morphant (Fig. 6A, C). The microphthalmia in the *Otx2* conditional knockout mouse was also due to increased apoptosis which suggest *Otx2* may play a role in supporting the survival of photoreceptor precursors [8].

Notch1 is required to maintain the progenitor state and inhibit the photoreceptor fate in the developing mammalian retina through the bHLH transcription factors Hes1 and Hes5 [46, 47, 65, 66]. In mice, retinal progenitors lacking Notch1 initiate the photoreceptor transcriptional program and upregu-

late the expression of *otx2* and *crx* (Fig.9) [46, 47]. In *col 15a1* morphants, *notch1a* showed upregulated and broader expression in retina [64]. Consistently, knockdown of zebrafish Lbh-like increased the activity of Notch pathway in eyes (Fig. 6E), which might down-regulate the expression of *otx2* and perturb the balance among proliferation, lineage differentiation and survival of photoreceptor precursors.

Otx2 is also involved in survival and terminal differentiation of retinal bipolar cells. In *Xenopus*, Otx2 is found in bipolar cells but not in photoreceptors [67, 68]. Overexpression of *XOtx2* in developing retinal cells increases the number of bipolar cells [69]. In human, polymorphisms in Otx2 may be a risk factor for bipolar disorder [70, 71]. Koike and his colleagues [70] generated a postnatal bipolar-cell specific *Otx2* conditional-knockout mouse line and observed impaired maturation of the bipolar cells. In our case, the expression of *vsx1*, a marker of bipolar cells, is significantly down-regulated in the retina of Lbh-like morphants (Fig. 3C), which might result from the reduction of *otx2*.

Müller glia functions as radial-glial-like neural stem cells for generation or regeneration of retinal neurons in teleost fish [72]. In developing fish retina, proliferating Müller glia is the source of rod progenitors [73]. When retinal neurons are destroyed, Müller glia partially and transiently dedifferentiates and increases the expression of *BLBP*, *Vsx2* and other stem cell or progenitor markers [74, 75]. In Lbh-like morphants, the Müller cell maturation markers *gs* and *cahz* [32] are up-regulated at 72 hpf (Fig. 3A-B). It seems that Müller glia may respond to the injury signals (the reduction of photoreceptor and bipolar cells) and generate multipotent retinal progenitors to repair the mistakes caused by Lbh-like knockdown.

We have noticed that knockdown of Lbh-like cannot completely eliminate the formation of photoreceptors and the expression of *otx2* and *opsins*. This suggests a complex parallel regulating network in regulating the development of retina. *Insm1a* has been identified as a novel factor to regulate photoreceptor differentiation in the zebrafish retina [41]. Since no any expression change of *insm1a* (Fig. 3H) and its downstream target gene *ath5* (Fig. 3G) occurs in the Lbh-like morphants at 48 hpf, *lbh-like* for regulating photoreceptor cell differentiation should be independent from *insm1a-ath5* pathway. Therefore, our results reveal a new mediator that regulates photoreceptors development through directing the precise expression of *otx2*.

Supplementary Material

Supplementary Figures S1–S10. Supplementary Table S1. http://www.ijbs.com/v11p0688s1.pdf

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Competing Interests

The authors have declared that no competing interest exists.

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