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Biochemical and Biophysical Research Communications 308 (2003) 184–190
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Zath3, a neural basic helix-loop-helix gene, regulates early neurogenesis in the zebrafish

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Received 2 July 2003

Abstract

We have isolated a basic helix-loop-helix (bHLH) gene homologous to the *Drosophila* proneural gene *atonal*, termed *zath3*, from zebrafish. *zath3* is expressed in neurons of the central nervous system and in subsets of cranial ganglia. Zebrafish *mindbomb* (*mib*) mutants have a higher density of *zath3* expressing cells and *narrowminded* (*nrd*) mutants lack *zath3* expression in a domain corresponding to primary sensory neurons showing that the expression of *zath3* is regulated by both *mib* and *nrd*. Injection of synthetic *zath3* RNA into zebrafish embryos expands the neural plate size, promotes ectopic expression of neuronal markers, and partially rescues the deficit of sensory neurons seen in *nrd* mutants. Interfering with *zath3* function using antisense morpholino oligonucleotides (MO) has no significant effect on early neurogenesis. However, a double knock down of *zath3* and *neurogenin1* (*ngn1*), another *atonal* homologue, with morpholinos (MOs) leads to more severe defects in neurogenesis than are seen with *ngn1* MO alone: a subtle reduction of motor and inter-neurons, and an almost complete loss all cranial ganglia. This study suggests that *zath3* and *ngn1* have partially overlapping roles in early neurogenesis.

Keywords: Basic helix-loop-helix gene; *zath3*; *neurogenin1*; Neural plate; Neuronal determination; Cranial ganglia; Neurogenesis; *mindbomb*; *narrowminded*

Neuronal precursor cells are generated through a series of steps in vertebrates: neural induction, regional patterning, and neurogenesis [1]. After neural induction and patterning, neurogenic regions, the domains in which neurogenesis takes place, are established at the late gastrula or neurula stages. The neurogenic regions are defined by the expression of proneural genes, which function to promote the formation of neurons. Many of these genes are homologues of the *Drosophila achaete-scute* and *atonal* genes, and encode basic helix-loop-helix (bHLH) transcription factors [2].

In zebrafish as well as in *Xenopus*, the proneural gene *neurogenin1* is expressed in three longitudinal domains on either side of the caudal neural plate, where primary motor-, inter-, and sensory neurons, respectively, arise at the late gastrula stage [3,4]. Proneural genes further induce downstream bHLH genes, such as *neuroD*, to elicit the transition from proliferative neural precursor cells to postmitotic neurons, which express neuron-specific markers, such as *huC* [5,6]. In addition to neuronal specification and determination, proneural genes also trigger the process of lateral inhibition. While promoting a cell's ability to become a neuron, proneural gene expression drives the expression of the Notch ligand Delta, which activates Notch signaling in neighboring cells and inhibits them from becoming neurons. As a consequence of Notch activation, neighboring cells express transcription factors belonging to the Hairy-Enhancer of Split related (His/Her) family that inhibit cells from becoming neurons by inhibiting the function of proneural genes. Failure of lateral inhibition mediated by Notch signaling leads to a neurogenic phenotype

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0006-291X/$ - see front matter © 2003 Published by Elsevier Inc.
doi:10.1016/S0006-291X(03)01353-6

where too many cells are permitted to become neurons [7–9].

We report here the isolation and characterization in the zebrafish of a proneural gene *zath3*, a homologue of bHLH gene *atonal*, which is expressed in the developing nervous system and regulates neurogenesis in the neural plate and cranial ganglia.

Materials and methods

Fish maintenance and mutants. Zebrafish were raised and maintained under standard conditions. Embryos were obtained by spontaneous spawning and grown at 28.5 °C in 1/3 Ringer's solution (39 mM NaCl, 0.97 mM KCl, 1.8 mM CaCl2, and 1.7 mM Hepes at pH 7.2) and the developmental stage of the embryos was determined by the time after fertilization (hpf, hours post-fertilization) and by morphological criteria [10]. Zebrafish neurogenesis mutants *mindbomb* [11–13] and *narrowminded* [14] were previously described.

Isolation of zebrafish zath3 cDNA. To isolate the zebrafish homologues of *Drosophila* proneural genes, we screened the zebrafish expressed sequence tags (ESTs) database (WashU-Zebrafish Genome Resources, http://zfish.wustl.edu/). One of these ESTs (fb17e09) contained a partial coding sequence homologous to *atonal* homologue genes, *Xenopus Xath3* and mouse *MATH3*. To obtain the full-length cDNA, two specific primers (RACE-1, TACTCTCTCTCAGTGAGC GTTAAGG; RACE-2, GAGCAGGAGCGTCTCTGGTGCTC) were designed and used for the rapid amplification of cDNA ends (RACE)-PCR. For DNA sequencing, cycle sequencing reactions were performed and analyzed with an automated sequencing machine. The confirmed *zath3* cDNA sequence was deposited in the database under the Accession No. AF204240.

Whole-mount in situ hybridization. Whole-mount in situ hybridization was performed as described previously [5]. Briefly, digoxigenin (DIG)-labeled antisense RNA probes were produced using DIG-RNA labeling kit (Roche, IN) following the manufacturer's instructions. Hybridization and detection with an anti-DIG antibody coupled to alkaline phosphatase (Vector, CA) was performed with fixed zebrafish embryos. Double in situ hybridization using DIG- and fluorescein-labeled RNA probes and antibody staining was performed as described [15]. Other plasmids that have been used to make in situ probes have been published previously: *huC* [5], *deltaA*, and *deltaB* [16].

Microinjection of synthetic zath3 RNA. To make the Myc epitope-tagged Zath3 expression vector, we amplified a fragment encoding the full-length protein (used primers are 5′-gaattcACCAGGAGATGTGA CTGAGCTGGT-3′ and 5′-ATTCGCTGTAAATGCTGCTCATCTG T-3′) which was subcloned into *Eco*RI site of the CS2 + MT vector [17]. The sense RNAs encoding Myc-Zath3 were in vitro transcribed using *Not*I-linearized plasmid DNA and the SP6 Message Machine (Ambion). Synthesized mRNA was dissolved in distilled water and microinjected into one blastomere of two-cell stage embryos as described previously [3]. Injections were performed with an air injection apparatus. The amount (approximately 100 pg) injected into embryos was estimated by visualizing the injection volume. Unless otherwise indicated, at least 50 embryos were injected for each experiment. At appropriate stages, the embryos were dechlorionated with watchmaker's forceps and fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS). For embryos co-injected with LacZ mRNA, we stained fixed embryos with X-gal prior to in situ hybridization [18].

Antisense morpholino injection. Antisense morpholino oligonucleotides were designed to complement the *zath3* cDNA and synthesized by GeneTools (Corvallis, OR). The sequences of the oligonucleotides used were 5′-CATTGTGATCTGTTATTGAAGCACC-3′ (*zath 3*-MO1), 5′-TTGGTCATCATTGTGATCTGTTATT-3′ (*zath 3*-MO2), and 5′-CGATCTCCATTGTTGATAACCT-3′ (*ngn1*-MO). The *ngn1*-MO oligonucleotide complement to the *neurogenin1* cDNA is identical to that used by Cornell and Eisen [19]. Embryos were injected at the one-cell stage by using a pressure injection apparatus. Approximately 2.5 ng of morpholino oligonucleotides per embryo was injected into more than 50 embryos for each experiment.

Results and discussion

Molecular cloning and structural analysis of zath3 cDNA

To isolate the bHLH transcription factors involved in early zebrafish neurogenesis, we screened the zebrafish ESTs database. One of these positive ESTs contained a partial coding region homologous to *Drosophila atonal* homologue genes, *Xenopus Xath3* and mouse *MATH3* [20], thus we termed it as *zath3*. To obtain its full-length coding sequences, we performed RACE-PCR with zebrafish embryo cDNA library. Sequence analysis of *zath3* full-length cDNA predicted an open reading frame of 347 amino acids (Fig. 1A). Comparison with other bHLH proteins revealed that *zath3* clearly belongs to the group of vertebrate bHLH proteins related to *Drosophila* proneural gene *atonal*. In the bHLH domain, *zath3* has 96% identity with its human, mouse [20], and chick [21] counterparts and 92% with *Xenopus* (Fig. 1B).

Spatial and temporal distribution of zath3 transcripts during neurogenesis

The spatiotemporal expression of *zath3* was examined by whole-mount in situ hybridization. *zath3* transcripts were first detectable at late gastrula stage (9 hpf) in trigeminal ganglion precursors (data not shown). At tailbud stage (10 hpf), *zath3* was expressed in three longitudinal stripes on both sides of the neural plate (Figs. 2A and B): lateral, intermediate, and medial domain. By their positions, cells in these stripes correspond to primary neuronal precursors, which differentiate into sensory, inter-, and motorneurons, respectively [5]. To determine the character of *zath3*-positive cells, we performed two-color double in situ hybridization with antisense probes that recognize *zath3* and *huC*, one of the earliest neuronal markers in zebrafish [5]. While *huC* expression is not so prominent at the tailbud stage, by the 3-somite stage it reveals differentiating neurons in medial, intermediate, and lateral proneuronal domains. *zath3* expression overlaps with *huC* expression in the intermediate neuronal domain, however, by this stage no *zath3* expression is observed in the lateral sensory neuron domain where cells differentiate as neurons a little earlier than in the intermediate domain [13] (Fig. 2C). The dynamic expression of *zath3* suggests that its expression is transient and is initiated in neurons prior to the onset of *huC* expression. In chick, *zath3* homologue *NeuroM* is expressed after *Neurogenin* but

A

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MTKPHGKPGDVTELVSSLGWMDEDLSSQDGDRTPEIGHYSLHRSNRGPLEIGSEDMDEEE   60
EEEEDEEMGLDGEKAPKRRGPKKKKMTKARQERFRARRIKANARERSRMHGLNDALDNLR  120
RVMPCYSKTQKLSKIETLRLARNYIWALSEVLESGQSPESHGFVEMLCKGLPQPTSNLVA  180
GCLQLGPTTMLKLDEKCGVPGAGVPQGHPISYPSPGLPSPPYGTMEASHLLLHLKGYKGPP  240
YENSSPNECSSGTPPYDGPLTPPLSISGNFALKQEPSPREAERNFTPHPTHYISSHPYPP  300
TSLAGLPAPQGHPLFPGSRYELPLDMAFEPFAPSHIVTSQMSSIYSE               347
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B

	Basic	Helix-1	Loop	Helix-2	
Human	RRVKANARERTR	MHGLNDALDNLRRVMP	CYSKTQKLS	KIETLRLARNYIWALSEV	96%
Mouse	RRVKANARERTR	MHGLNDALDNLRRVMP	CYSKTQKLS	KIETLRLARNYIWALSEV	96%
Chick	RRVKANARERTR	MHGLNDALDNLRRVMP	CYSKTQKLS	KIETLRLARNYIWALSEV	96%
Xenopus	RRVKANARERSR	MHGLNDALENLRRVMP	CYSKTQKLS	KIETLRLARNYIWALSDI	92%
Zebrafish	RRIKANARERSR	MHGLNDALDNLRRVMP	CYSKTQKLS	KIETLRLARNYIWALSEV	
	:*****:*	********:*******	*********	**********:::	

Fig. 1. (A) The *zath3* cDNA isolated from the zebrafish embryos encodes 347 amino acids which belong to the family of basic helix-loop-helix (bHLH) transcription factors. The bHLH domain is underlined. (B) CLUSTAL W multiple sequence alignments of the bHLH domains of vertebrate *atonal* homologue 3 (Ath3) proteins. In this domain, identities to zebrafish Zath3 range from 92% to 96%. Conserved residues are highlighted. The GenBank Accession numbers for human *NEUROD4*, mouse *MATH3*, chick *NeuroM*, *Xenopus Xath3*, and zebrafish *zath3* are NM_021191, D85845, Y09597, D85188, and AF204240, respectively.

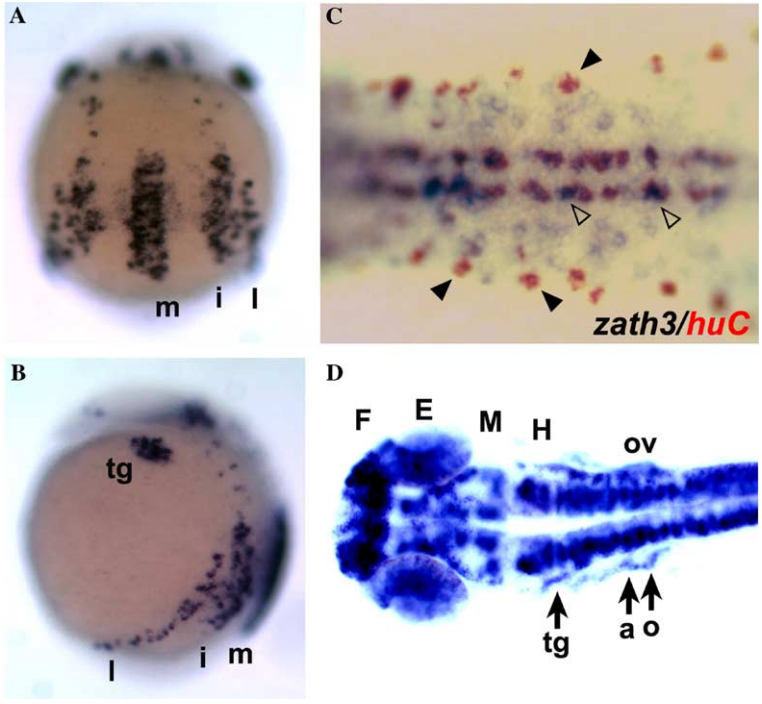

Fig. 2. Spatiotemporal expression of *zath3* in zebrafish embryos. Expression of *zath3* was detected by whole-mount in situ hybridization with antisense RNA probe. (A,B) Tailbud stage embryo (10 hpf), anterior to the up. (A) Dorsal view. (B) Lateral view. *zath3* is expressed in three longitudinal stripes on either side of the midline of the neural plate; lateral stripe (*l*) containing sensory neuron precursors, intermediate stripe (*i*) containing interneuron precursors, and medial stripe (*m*) containing motoneuron precursors. tg, trigeminal ganglion precursors. (C) Double in situ hybridization with *zath3* (purple) and pan-neuronal marker *huC* (red) in 3-somite stage (11 hpf) embryo. Dorsal view, anterior to the left. Some of the neuronal cells expressing exclusively *huC* are indicated by closed triangles, whereas cells expressing both *zath3* and *huC* are marked by open triangles. *zath3*-expressing neural precursor cells are also seen between those indicated cells. (D) *zath3* expression in most of the nervous systems of 24 hpf embryo. Dorsal view, anterior to the left. F, forebrain; E, eye; M, midbrain; H, hindbrain, ov, otic vesicle; tg, trigeminal ganglion; a, anterior lateral line ganglion; and o, octaval ganglion in the otic vesicle.

before *NeuroD*, defining a transition stage between the undifferentiated, proliferating precursors cells and postmitotic, differentiated neurons [21]. Later, at 24 hpf, *zath3* was expressed broadly in the nervous system, including the eye, forebrain, and cranial ganglions (Fig. 2D).

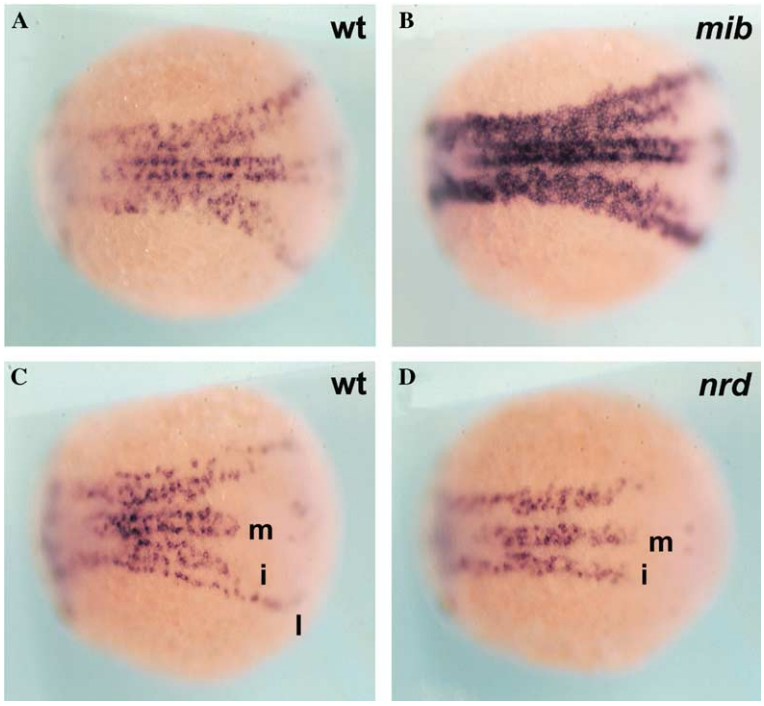

Fig. 3. Expression of *zath3* in the zebrafish neurogenesis mutant embryos at neural plate stage (3-somite stage, 11 hpf). Dorsal views with anterior to the left. (A,C) Wild-type siblings. (B) Neurogenic *mindbomb* (*mib*) mutant embryo, showing a dramatic increase of *zath3*-expressing cells in all three major stripes. (D) *narrowminded* (*nrd*) mutant embryo which lacks specifically sensory neurons. *zath3*-expressing neural precursor cells in the lateral stripe (*l*) are completely missing in mutant embryo, compared to its sibling wild-type embryo (C).

We also examined *zath3* expression in two neurogenesis mutants *mindbomb* (*mib*) and *narrowminded* (*nrd*). The *mib* mutant is characterized by a neurogenic phenotype with overproduction of differentiating neurons that results from deficits in Notch signaling [13]. In contrast, *nrd* mutant embryos lack Rohon–Beard sensory neurons [14]. The number of *zath3*-expressing cells was increased dramatically in *mib* mutants (Fig. 3B), while *zath3* expression is absent completely in the sensory neuron precursors in the neural plate of *nrd* mutant embryos (Fig. 3D). These results suggest that *zath3* expression is directly or indirectly regulated by *mib* and *nrd* during early neurogenesis in zebrafish.

Ectopic induction of neuronal markers by zath3 overexpression

To analyze the effects of *zath3* on neural development, we examined the expression of several neural/neuronal markers in *zath3*-injected embryos (Fig. 4). When approximately 100 pg of synthetic *zath3* RNA was injected into one blastomere of two-cell stage zebrafish embryos, the most notable and frequent phenotype was neural plate expansion on the injected side (Figs. 4B and D). Co-injection of *LacZ* RNA allowed us to confirm the injected side by X-gal staining (Fig. 4F). This *zath3* phenotype of neural plate expansion seems quite similar to that of *Xash3* [17] or *X-ngnr-1* [22]. In addition to the neural plate expansion, ectopic *deltaB* or *huC* expression was detected in the injected side of embryo (Figs. 4D and F). This type of ectopic neurogenesis is similar to that of *NeuroD*, which converts epidermal cells into neurons [23]. In *Xenopus*, when ectopically expressed in neurula embryos, *Xath3* is able to induce cells which have characteristics of sensory neurons [24]. Thus, we tested the activity of *zath3* in the zebrafish neural mutant *nrd*, which specifically lacks sensory neurons (Fig. 3D). Only in the *zath3*-injected side of *nrd* mutant embryo, neuronal cells were rescued in the place where primary sensory neurons are located in normal embryos (Fig. 4F). In this study, we showed that *zath3* not only expands the neural plate but also induces ectopic neurogenesis. It has been proposed that there are at least two separate developmental steps for generation of neurons in vertebrates; the first step involves the initial decision between neural and epidermal fates in the ectoderm by proneural genes [17], while the second step is the subsequent process of neuronal determination/differentiation. Thus, *zath3* is capable of promoting both neural and neuronal fate.

Cooperative function of zath3 and neurogenin1 in the specification of cranial ganglia

To further test the function of *zath3*, we introduced morpholino antisense oligonucleotides complementary

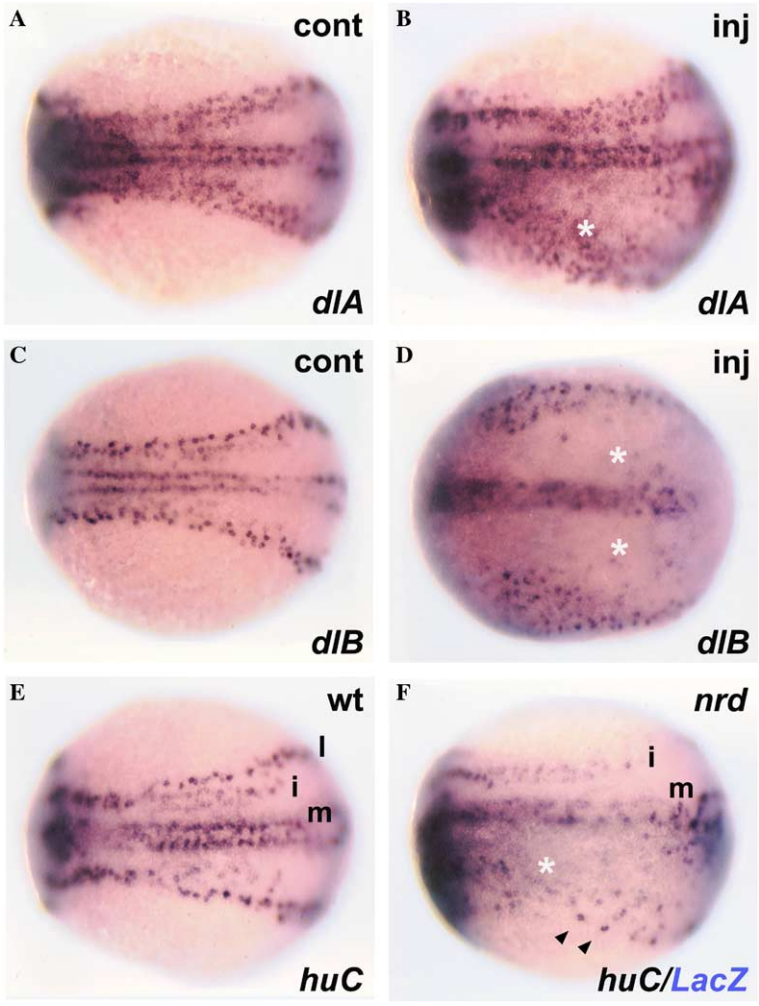

Fig. 4. Overexpression of *zath3* mRNA affects primary neurogenesis in the neural plate of injected embryos. (A, C, and E) Normal expression of neuronal markers in control embryos; *deltaA* (A,B), *deltaB* (C,D), and *huC* (E,F). (B) Expansion of neural plate size in the injected side (indicated by asterisk) of embryo stained with *deltaA* probe. (D) Neural expansion and increased number of neuronal precursor cells in injected embryo stained with *deltaB*. (F) Rescue of primary sensory neurons (arrowheads) in the injected side of the *nrd* mutant embryo double-stained with *huC* and X-gal (*LacZ*).

to the 5′ end of the *zath3* mRNA. Morpholino oligonucleotides (MO) efficiently block translation of mRNAs to which they bind and have been used successfully to disrupt function in various vertebrates [25–27]. After injection of the *zath3*-specific morpholino oligonucleotide (*zath3*-MO), no significant morphological differences occur and embryos are indistinguishable from controls at all stages examined (data not shown). We then examined the effect of *zath3*-MO on neurogenesis in detail with the neuronal marker *huC*. After *zath3*-MO injection, *huC* expression is not changed significantly both in the central nervous system and cranial ganglia in comparison to controls (Fig. 5B).

Although the overexpression study indicated that the *Xenopus Xath3* (and *zath3* in this study) has a neuronal determination activity, disruption of *Math3* in mice failed to demonstrate that it functions at the neural fate determination [28]. This loss-of-function study indicated that *Math3* regulates only the late development of already committed neurons. The discrepancy between the gain- and loss-of-function studies may be due to genetic redundancy among various proneural genes expressed at same time and space. Supporting this idea, in the mice double mutant for the *achaete-scute* complex (*as-c*) homologue *Mash1* and *Math3*, specific neurons in hindbrain and retinal bipolar cells are missing and, instead, those cells that normally differentiate into neurons adopt the glial fate, suggesting the essential role of these genes in neuronal fate determination [28]. In addition, *Math3* and *NeuroD* double mutant mice have defects in specification of amacrine cells in the retina [29]. Thus, we examined the effects of simultaneously knocking down *zath3* and *neurogenin1* (*ngn1*), the earliest known proneural genes expressed at the early neural plate stage in zebrafish.

Although the *zath3*-MO had no significant effect on the normal neurogenesis in the injected embryos

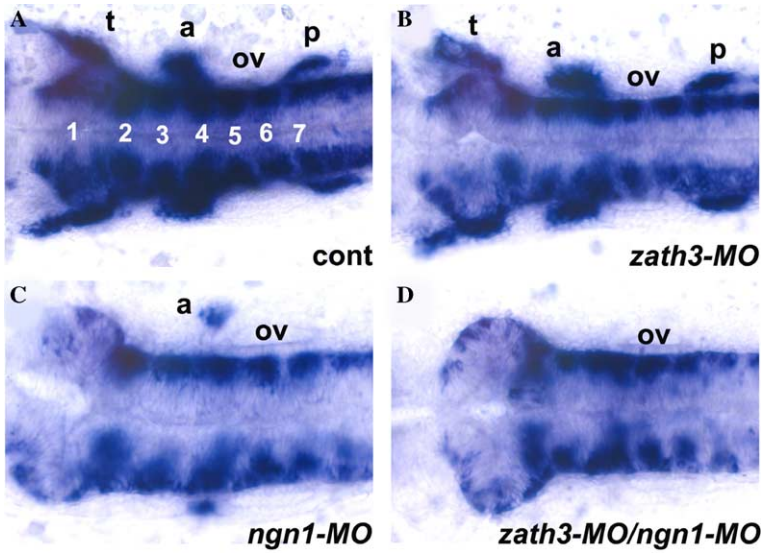

Fig. 5. Zath3 and Neurogenin1 have cooperative but distinct role in the cranial ganglion neurogenesis. Dorsal view of embryos stained with the pan-neuronal marker *huC* at 20 hpf, anterior to the left. (A) Normal expression of *huC* in control embryo. In addition to its strong expression in the central nervous system, *huC* is expressed in all cranial ganglia: trigeminal ganglion (t), anterior lateral line ganglion (a), and posterior lateral line ganglion (p). Each rhombomere in the hindbrain is numbered. ov, otic vesicle. (B) *zath3* morpholino oligonucleotides (MO)-injected embryo, showing relatively normal development of central nervous system and cranial ganglions. (C) *neurogenin1* (*ngn1*)-MO blocks neurogenesis of the most of cranial ganglia, except for a subset of the anterior lateral line ganglion (a). (D) Complete missing of all cranial ganglia in embryo co-injected with *zath3*-MO1 and *ngn1*-MO.

(Fig. 5B), *ngn1*-MO blocked generation of most cranial ganglia, except for a subset of neurons in the anterior lateral line ganglion (Fig. 5C). In addition, the double knock out with *zath3*-MO and *ngn1*-MO inhibited completely the generation of all cranial ganglia in 150/160 (94%) injected embryos (Fig. 5D). The effect of *ngn1*-MO alone on cranial ganglia development was similar to what has previously been reported [19,30]. In mice, targeted inactivation of *neurogenin-1* and *neurogenin-2* has indicated that they are required for the generation of different subsets of cranial sensory neurons [31]. Together, these data suggest that *zath3* and *neurogenin1* cooperate to specify neuronal identity in a subset of CNS neurons and cranial ganglia.

Acknowledgments

This work was supported by grant from Molecular Medicine Research Group Program (M1-0106-01-0002) from the Ministry of Science and Technology and a Vascular System Research Center grant from Korea Science and Engineering Foundation, Korea.

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