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Her4 is necessary for establishing peripheral projections of the trigeminal ganglia in zebrafish

Ju-Hoon So [a,1], Hang-Suk Chun [b,1], Yong-Ki Bae [c,1], Hyun-Seo Kim [d], Yeoll-Mae Park [d], Tae-Lin Huh [b], Ajay B. Chitnis [e], Cheol-Hee Kim [a], Sang-Yeob Yeo [d,*]

[a] Department of Biology, Chungnam National University, Daejeon 305-764, Republic of Korea
[b] Department of Genetic Engineering, Kyungpook National University, Daegu 702-701, Republic of Korea
[c] Cancer Experimental Resources Branch, Division of Basic & Applied Sciences, Research Institute, National Cancer Center, Gyeonggi-do 410-769, Republic of Korea
[d] Department of Biotechnology, Division of Applied Chemistry & Biotechnology, Hanbat National University, Daejeon 305-719, Republic of Korea
[e] Section on Neural Developmental Dynamics, Laboratory of Molecular Genetics, NICHD, NIH, Bethesda, MD 20892-2785, USA

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ABSTRACT

Transcripts of *notch* and its target genes have been detected in some differentiating neurons. However, the role of Notch in neuronal differentiation remains poorly defined. Here, we show that a subset of differentiating sensory neurons in the trigeminal ganglia express *her4*. Expression of *her4* requires Notch signaling during neurogenesis but not during differentiation, when peripheral projections of the trigeminal ganglia are established. These projections develop poorly in *her4* morphants. While many components of the canonical Notch signaling pathway are not required for late *her4* expression or peripheral axon outgrowth in trigeminal neurons, simultaneous knock-down of Notch receptors prevents establishment of these peripheral projections. These observations suggest that Her4 and Notch play a role in peripheral outgrowth of sensory neurons.

In *Drosophila*, Notch activation drives expression of basic helix–loop–helix (bHLH) *Enhancer-of-split* [*E(spl)*] transcription factors that suppress the transcription of proneural genes and prevent neighboring cells from adopting a neuronal fate [1], a process called lateral inhibition [2,3]. Proneural genes in zebrafish, for example, *neurogenin1* (*ngn1*) and *zath3*, help define neurogenic domains in the neural plate, where cells have the potential to become neurons [4]. Expression of the Notch ligand Delta is driven by these proneural factors [5]. The interaction between Delta and Notch stimulates proteolysis of the Notch receptor, which results in the release of the Notch intracellular domain (NICD) [6]. Upon translocation into the nucleus, NICD forms a transcriptional activator complex with Suppressor-of-Hairless [*Su(H)*] [7] to drive *E(spl)* expression [1].

Vertebrate homologs of the *E(spl)* genes, the HER or HES genes, are members of the bHLH-Orange (bHLH-O) superfamily because they contain a conserved Orange domain [8]. In zebrafish, the bHLH-O superfamily has been subdivided into three groups based on whether Notch signaling activates (Group I, *her1* and *her4*), inhibits (Group II, *her3* and *her5*), or does not alter (Group III, *her6*) transcription [9]. The Group I genes are regulated in a manner similar to the Drosophila *E(spl)* complex genes: they are up-regulated by Notch activation and down-regulated by reductions in Notch signaling during the development of the central nervous system (CNS) 10–12. Previous studies have suggested that while the genes in Groups I and II inhibit neurogenesis, the genes in Group III promote neurogenesis [9]. However, other studies report that, in zebrafish, Her6, which is thought to be a homolog of mammalian Hes1, may also inhibit neurogenesis [19].

Previous studies have shown that while Notch signaling helps maintain proliferating neural progenitors, one of its target genes, *her4*, is also expressed in some differentiating neurons [12,13]. However, the role of Notch activation and/or Her4 in differentiating neurons is largely unknown. Our goal was to define the function of Her4 by examining loss-of-function mutants using the *Tg[her4:dRFP]* transgenic line which can be used to visualize Notch activation during late neural development [12].

In this study, we confirm that *her4* transcripts are detected in sensory neurons. Although *her4* expression requires Notch activation during early neurogenesis [12], during late development of cranial sensory neurons in the peripheral nervous system (PNS) *her4* expression is independent of Notch signaling. An antisense morpholino (MO) complementary to the 5′-untranslated region of the *her4* mRNA effectively blocks translation of the *her4:dRFP*

* Corresponding author. Fax: +82 42 821 1552.
E-mail address: yeosy@hanbat.ac.kr (S.-Y. Yeo).
[1] These authors contributed equally to this work.

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reporter in transgenic embryos. Our analysis shows that peripheral axons of cranial sensory neurons develop poorly in *her4-MO* injected zebrafish embryos. These observations imply that Her4 plays a role in formation of peripheral axons, suggesting that this bHLH-O superfamily member may be required for completion of differentiation in these neurons.

Material and methods

Fish maintenance and strain. Zebrafish were maintained as described by Westfield [14]. The *mind bomb* mutant allele *mib*^{ta52b}, in which Notch signaling is defective, has been previously described [15]. Reporter lines were *Tg[her4:dRFP]*^{knu2}, *Tg[her4:EGFP]*^{y83} [12], and *Tg[ngn1:GFP]* [16].

Whole-mount in situ hybridization and immunohistochemistry. Whole-mount *in situ* hybridization and immunohistochemistry were performed as described previously [17,18]. Antisense riboprobes were synthesized from *her4* cDNA as described previously [12]. We used the following primary antibodies: mouse anti-Islet1/2 (1:50, Developmental Studies Hybridoma Bank (DSHB)) and mouse anti-zn12 (1:250, DSHB). For fluorescent detection, we used Alexa Fluor 488 conjugated goat anti-mouse (1:2500, Molecular Probes).

Antisense morpholino injection. Microinjection was performed at the two-cell stage as described [17]. Morpholinos (MOs) (Gene Tools, LLC) were stored at a stock concentration of 10 mg/ml at −20 °C. The sequences of the MOs used were: 5′-ATT GCT GTG TGT CTT GTG TTC AGT T-3′ for *her4* (*her4-MO*), 5′-tTT GgT GTG TGT CTT cTG TaC AGT T-3′ for the *her4* control *MO* (*con-MO*), 5′-CTT CTC TTT TCG CCG ACT GAT TCA T-3′ for *deltaA* (*deltaA-MO*), and 5′-AAA CAG CTA TCA TTA GTC GTC CCA T-3′ for *deltaD* (*deltaD-MO*). *notch1a-*, *notch1b-*, *notch5-*, *ngn1-*, *zath3-*, *Su(H)-*, and *hdac1-MOs* were injected into transgenic or wild-type embryos [4,12,19].

TUNEL assay. The terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay was performed according to the manufacturer's protocol (Roche). Zebrafish embryos were fixed with 4% paraformaldehyde in PBS overnight. Embryos were dehydrated with −20 °C methanol. Embryos were rehydrated through a series of solutions of decreasing concentrations of methanol. Immunohistochemistry using the anti-Islet1/2 antibody was performed as described previously [18]. The embryos were then partially digested with proteinase K (10 μg/ml; Roche) in Tris/HCl buffer (pH 8.0) containing EDTA (5 mM) at room temperature (RT) for up to 10 min. Digestion was stopped with glycine and the embryos were washed four times in distilled water. Thereafter, embryos were incubated for 90 min at 37 °C with terminal deoxynucleotidyl transferase (75 U/ml) and digoxigenin (DIG)-11-dUTP (5 mM). After 90 min, embryos were washed with PBST buffer (phosphate buffered saline, 0.9% NaCl, 0.1% Tween 20, pH 7.4), followed by Tris/HCl (10 mM, pH 8.0) in 150 mM NaCl. Non-specific binding was blocked with the blocking reagent for nucleic acid hybridization and detection (Roche) for 30 min at RT. DIG-labeled nicked ends of DNA strands were visualized using alkaline phosphatase conjugated anti-DIG Fab fragments and BCIP/NBT as a chromogenic substrate (Roche). The reaction was stopped by washing with PBST. Embryos were mounted in glycerol.

In vivo imaging. Transgenic zebrafish were imaged at various times using the following protocol. Zebrafish were anesthetized in 0.1 mg/ml tricaine (Sigma), placed on a 60-mm diameter Petri dish with 4 ml of 1% agarose and embedded in low-melting 0.7% agarose (FMC) containing 0.1 mg/ml tricaine. After the agarose solidified, zebrafish were imaged using a LSM510 META confocal microscope (Zeiss).

Results and discussion

Transcripts of her4 are detected in a subset of trigeminal ganglion cells

To investigate the expression of *her4* during vertebrate development, we performed whole-mount *in situ* hybridization with a *her4* riboprobe using embryos stained with an anti-Islet1/2 antibody at the 5-somite stage or at 24 hours post-fertilization (hpf) (Fig. 1). Islet1 is expressed in the polster, as well as motor neurons and sensory neurons including those in the trigeminal ganglia. Although *her4* was not expressed in the trigeminal ganglia during primary neurogenesis (data not shown), *her4* was expressed not only in the forebrain, midbrain, and hindbrain, but also in the domain where trigeminal sensory neurons differentiate at the 5-somite stage (Fig. 1A). At 24 hpf, a subset of Islet1/2-positive sensory neurons in the peripheral nervous system (PNS) expressed *her4* transcripts (Fig. 1B). In *Tg[her4:EGFP]* transgenic zebrafish, which express enhanced green fluorescent protein (EGFP) under the control of the *her4* promoter [12], EGFP-positive cells in the trigeminal ganglion extend a central process toward the CNS and a peripheral process to innervate the skin (Fig. 1C). Examination of *Tg[her4:dRFP]* and *Tg[ngn1:GFP]* double transgenic embryos showed that destabilized red fluorescent protein (dRFP) expressed under the control of the *her4* promoter is present in the trigeminal ganglia, in which GFP under the control of the *ngn1* promoter was also expressed at 24 hpf (Fig. 1D and D″) [12,16]. At 29 hpf, the central axon of the trigeminal ganglion extends into the hindbrain.

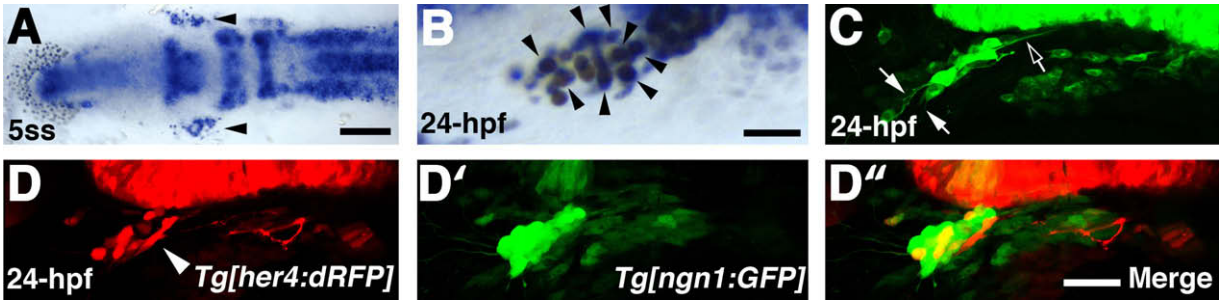

Fig. 1. Zebrafish *her4* is expressed in a subset of trigeminal ganglion neurons. (A,B) Double staining with anti-Islet1/2 (brown) and *in situ* hybridization for *her4* mRNA (purple). Dorsal (A) and lateral (B) views; anterior to the left. Arrowheads indicate the *her4* transcripts detected in the trigeminal ganglion at the 5-somite stage (5ss) (A) and at 24 hpf (B). (C,D) Reporter expression in trigeminal ganglia. Lateral views. Confocal image of *Tg[her4:EGFP]* shows the central (open white arrow) and peripheral projections (solid white arrows) of EGFP-positive cells (C). Confocal images of *Tg[her4:dRFP];Tg[ngn1:GFP]* embryos (D) identified *her4* promoter-driven dRFP-expressing cells (white arrowheads) (D) and GFP-expressing trigeminal ganglion neurons (D′) at 24 hpf (D,D′). D″ is a merged image of D and D′ showing *her4* reporter expression in the trigeminal ganglion. Scale bars: 100 μm (A), 25 μm (B), and 50 μm (C). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this paper.)

At this stage, dRFP-positive cells were still detected in this region (Supplementary Fig. 1A and A″). These data demonstrate that *her4* transcripts and reporters are expressed in a subset of trigeminal ganglion neurons during axonogenesis, suggesting that *her4* may be involved in the process of neuronal maturation.

Expression of her4 is dependent on proneural genes but independent of Notch signaling during late development of the trigeminal ganglion

Previous studies have shown that the simultaneous knock-down of *ngn1* and *zath3*, as well as the simultaneous knock-down of *notch1a* and *notch5*, results in a dramatic reduction of *her4* expression at the 5-somite stage [12]. The expression of *her4* could not be detected in the trigeminal ganglion at 26 hpf following simultaneous knock-down of *ngn1* and *zath3*, and expression was reduced in the CNS (Fig. 2B and Supplementary Fig. 2B). These results suggest that the expression of *her4* is dependent on the proneural genes or, alternatively, that the proneural genes are required for establishment and/or maintenance of cells that express her4.

To validate the expression of *her4* in the trigeminal ganglion, we performed double staining in which *her4* transcripts were visualized by whole-mount *in situ* hybridization and Islet1/2 protein by immunohistochemistry in embryos in which Notch function had been knocked-down with various morpholinos. Expression of *her4* in *mind bomb* (*mib*) mutant embryos at 26 hpf was dramatically reduced in the CNS, but was detected in a larger number of cells in the trigeminal ganglia. These results are consistent with the reduced Notch signaling in the *mib* mutant allowing a larger number of sensory neurons to differentiate (Fig. 2C and Supplementary Fig. 2C). Simultaneous knock-down of *notch1a* and *notch5* by injection of MOs caused a reduction of *her4* expression (Supplementary Fig. 2D). An additional *notch* homolog, *notch1b*, is expressed in the CNS at 26 hpf. The expression of *her4* in *notch1b* morphants was similar to that of *her4* in morphants in which *notch1a* and *notch5* were simultaneously knocked-down (Supplementary Fig. 2E). However, when *notch1a*, *notch1b*, and *notch5* were simultaneously knocked-down, *her4* was expressed in a larger number of cells in the trigeminal ganglia at 26 hpf (Fig. 2D), similar to the expression pattern observed in the *mib* mutants (Fig. 2C), and the *deltaA*- and *deltaD-MO* injected embryos (Fig. 2E) or *Su(H)1* morphants (Supplementary Fig. 2H). Together, these observations suggest that the pattern of *her4* expression in the trigeminal ganglia does not correlate with the amount of Notch signaling but rather with the number of differentiating sensory neurons in the ganglia. These observations are consistent with the previous suggestion that *her4* expression may be independent of Notch signaling during late development of sensory neurons [12].

Intriguingly, the expression of *her6*, a bHLH-O domain-containing gene, is derepressed in *histone deacetylase 1 (hdac1)*-deficient embryos, independently of Notch signaling [19]. Since *her4* is also a bHLH-O domain-containing gene, we hypothesized that *her4* expression might be derepressed in *hdac1*-deficient embryos. In *hdac1-MO* injected embryos, *her4* was expressed in fewer cells of the trigeminal ganglia. However, expression of *her4* was dramatically reduced in the CNS of the *hdac1-MO* injected embryos (Fig. 2F and Supplementary Fig. 2I). In *hdac1* mutant embryos, *her6* is ectopically expressed at distinct sites within the CNS and proneural genes such as *ngn1* and *ash1b*, are reduced or eliminated [19]. In contrast, *her4* expression was reduced in the CNS of *hdac1-MO* injected embryos, suggesting that the reduction or elimination of proneural genes may suppress *her4* expression. Taken together with our observation that *her4* expression is independent of Notch signaling during late neuronal development, these data suggest that in sensory neurons Her4 is not involved in canonical Notch signaling.

Her4 affects the formation of peripheral nerves in the trigeminal ganglion

To assess the function of Her4, we conducted a loss-of-function study using an MO directed against *her4*. Injection of 2 pg of the *her4-MO* led to a specific reduction in the synthesis of the dRFP reporter in *Tg[her4:dRFP]* embryos (Fig. 3B), whereas a four base pair-mismatched MO (*Con-MO*) did not suppress the synthesis of dRFP (Fig. 3A). The specific ability of the *her4-MO* to suppress translation of dRFP from a 3.4 kb *her4* promoter that includes the 5′-untranslated region of the *her4* cDNA and the ATG start codon suggested that the *her4-MO* would also effectively suppress the synthesis of endogenous Her4. The number of trigeminal ganglion neurons in *Con-MO* injected embryos (Fig. 3C), was similar to the number of trigeminal ganglion neurons in *her4-MO* injected embryos, at 26 hpf (Fig. 3D), consistent with the hypothesis that Her4 is not involved in canonical Notch signaling.

Since programmed cell death and/or necrosis may affect normal development of differentiated cells, we performed TUNEL assays to measure apoptosis related DNA damage. Apoptotic cells were ob-

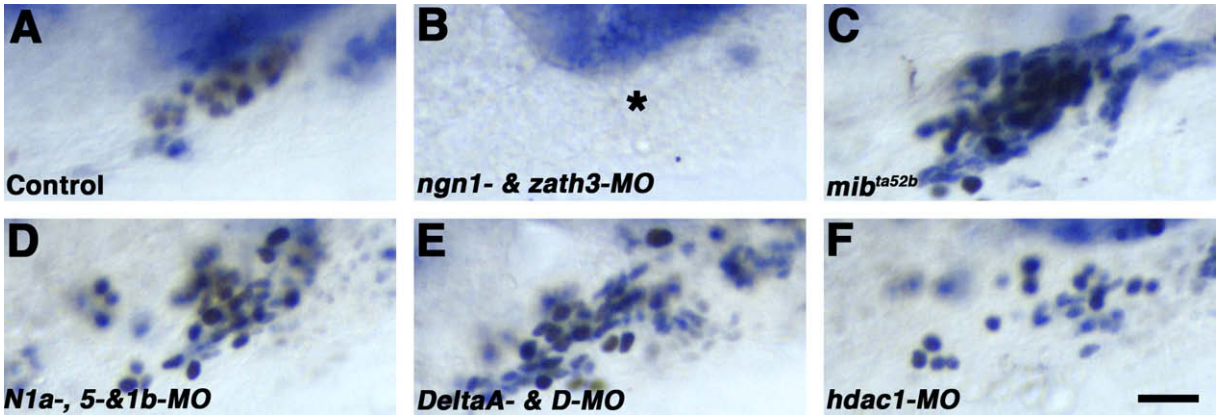

Fig. 2. Inhibition of Notch signaling increases the number of *her4* expressing cells in the trigeminal ganglion at 26 hpf. Double staining with anti-Islet1/2 (brown) and *her4* riboprobes (purple). Lateral views; anterior to the left. Expression *of her4* in control (A), *ngn1*- and *zath3-MOs* (B), *mib*^{ta52b} mutant (C), *N1a*-, *N1b*-, and *N5-MOs* (D), *DeltaA*- and *DeltaD-MOs* (E), and *hdac1-MO* (F) embryos at 26 hpf. Expression of *her4* in the trigeminal ganglion is dramatically reduced in the *ngn1*- and *zath3-MO* injected embryos (asterisks). However, *her4* expression is not significantly decreased in the embryos with reduced Notch signaling. Scale bar, 25 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this paper.)

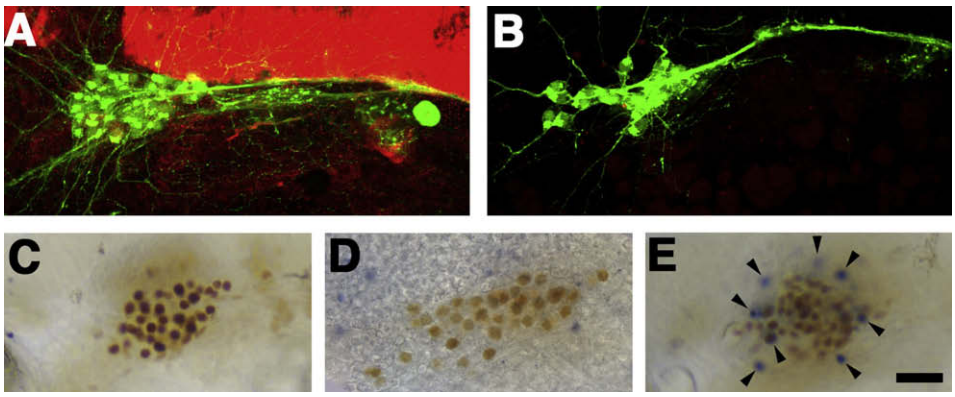

Fig. 3. The number of trigeminal ganglion and apoptotic cells are not affected by the reduction of Her4. (A,B) Specificity of *her4* morpholino (MO). Lateral views; anterior to the left. *Tg[her4:dRFP]* embryos were injected with *con-MO* (A) or *her4-MO* (B), fixed at 26 hpf, immunostained with an anti-zn12 antibody and analyzed by confocal microscopy. (C–E) Apoptosis in the trigeminal ganglion. Double staining using anti-Islet1/2 (brown) and TUNEL assay (purple) in control (C), *her4-MO* injected embryos (D) and mib^{ta52b} mutants (E) shows the emergence of apoptotic cells in the mib^{ta52b} mutant (arrowheads). The number of trigeminal ganglion neurons undergoing apoptosis at 26 hpf increased in the mib^{ta52b} mutants (E), but not in the *her4* morphants (D) compared to control embryos. Scale bar, 25 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this paper.)

served in the trigeminal ganglion of the *mib* mutant (Fig. 3E), but not in *her4* morphants (Fig. 3D) or control embryos (Fig. 3C).

A previous study suggested a model in which expression of Delta on the surface of cells provides a track to direct the growth of motor neurons and that the axons of the motor neurons recognize this track by interactions between Notch on the axons with Delta [20]. Nevertheless, whether this guidance requires downstream effectors of the Notch–Delta interaction is unclear. We observed peripheral and central axons of the trigeminal ganglion in *her4* morphants at 26 hpf and noted that the peripheral axons were poorly developed, in contrast with normal extension of the central axons (Fig. 3B).

To confirm the effect of the *her4-MO* on the formation of peripheral axons, we performed immunohistochemistry at 26 hpf using an anti-zn12 antibody on embryos injected with the *her4-MO* (Fig. 4B), the *notch1a-*, *notch1b-*, and *notch5-MOs* (*notch-MOs*) (Fig. 4C), or the *Su(H)1-MO* (Fig. 4D). Peripheral axons developed poorly in the *her4-MO* and *notch-MOs* injected embryos (Fig. 4B′ and 4C′), whereas development of the peripheral projections was only slightly affected by the injection of the *Su(H)1-MO* (Fig. 4D′). In contrast, *her*, *notch*, and *Su(H)1* morphants all showed normal extension of the central axons of the trigeminal ganglia (data not shown).

Why the *Su(H)* and Notch morphants differ in the development of peripheral axons is unclear. One possible explanation might come from our previous report that knock-down of *Su(H)* results in either no obvious change or an increase in the number of *her4* expressing cells, whereas simultaneous knock-down of the Notch isoforms and proneural genes reduced *her4* expression in the trigeminal ganglia at the 5-somite stage [12]. Consequently, we propose that zebrafish trigeminal sensory neurons require the function of Notch and/or Her4 for axon outgrowth.

Why are the effects of *her4-MO* and *notch-MOs* on the development of the peripheral axons comparable? Given that *her4* expression may be tightly regulated by Notch during early neurogenesis, at least up to the 5-somite stage, but not during later neuronal development, specification of the peripheral axons may be determined before or at the 5-somite stage. Apparently, *her4* expression is dramatically reduced in *notch1a-* and *notch5-MO* injected embryos at the 5-somite stage, but not in *Su(H)1-MO* injected em-

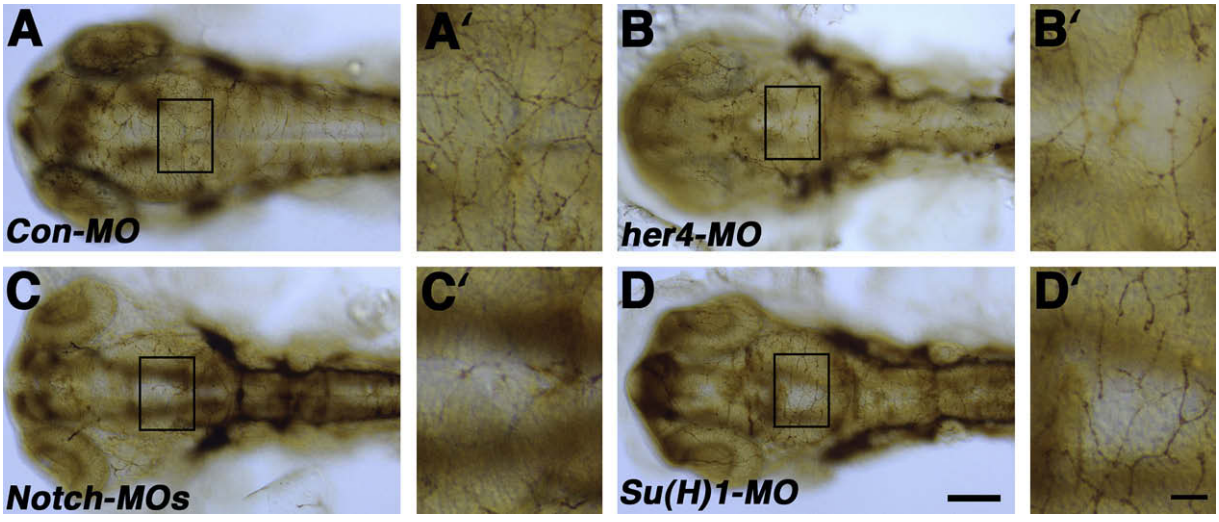

Fig. 4. Reduction in peripheral projections of motor neurons in *her4* morphants. Dorsal views; anterior to the left. Immunostaining with anti-zn12 in *control-MO* (A), *her4-MO* (B), combined MOs against N1a, N1b, and N5 (*Notch-MOs*) (C), and *Su(H)1-MO* (D) injected embryos. High magnification views of the boxed regions in (A–D) are shown in (A′–D′), respectively. Scale bars: 100 μm (A–D) and 20 μm (A′–D′).

bryos [12]. This hypothesis could be verified by pinpointing when peripheral axons of the trigeminal ganglion emerge and/or by generating transgenic fish in which overexpression of *her4* and/or *notch* are temporally regulated. However, Notch signaling and Her4 expression may regulate the development of the peripheral axons through different mechanisms. We are currently investigating these possibilities.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2008.11.149.

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