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The zebrafish forkhead transcription factor Foxi1 specifies epibranchial placode-derived sensory neurons

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SUMMARY

Vertebrate epibranchial placodes give rise to visceral sensory neurons that transmit vital information such as heart rate, blood pressure and visceral distension. Despite the pivotal roles they play, the molecular program underlying their development is not well understood. Here we report that the zebrafish mutation *no soul*, in which epibranchial placodes are defective, disrupts the fork head-related, winged helix domain-containing protein Foxi1. Foxi1 is expressed in lateral placodal progenitor cells. In the absence of *foxi1* activity, progenitor cells fail to express the basic helix-loop-helix gene *neurogenin* that is essential for the formation of neuronal precursors, and the paired homeodomain containing gene *phox2a* that is essential for neuronal differentiation and maintenance. Consequently, increased cell death is detected indicating that the placodal progenitor cells take on an apoptotic pathway. Furthermore, ectopic expression of *foxi1* is sufficient to induce *phox2a*-positive and *neurogenin*-positive cells. Taken together, these findings suggest that Foxi1 is an important determination factor for epibranchial placodal progenitor cells to acquire both neuronal fate and subtype visceral sensory identity.

Key words: epibranchial placodes, *foxi1*, *phox2a*, Neurogenin, Zebrafish, Visceral sensory neurons

INTRODUCTION

One central question in vertebrate development is how multipotent progenitor cells give rise to diverse cell types in an organism. Such a question is arguably more profound in the nervous system (the central and peripheral nervous system, CNS and PNS), where many hundreds of different cell types exist. Whereas CNS originates from the neural tube, PNS has two different origins: the neural crest and cranial ectodermal placodes. The cranial placodes are discrete regions of thickened columnar epithelium formed at the junction of the neural and superficial ectoderm. They give rise to diverse structures such as the anterior pituitary gland, olfactory sensory epithelium, lens, and part of the cranial sensory ganglia (Le Douarin, 1984).

Visceral sensory neurons are derived from such neurogenic placodes, known as the epibranchial placodes. These neurons constitute the distal ganglia of cranial sensory nerves and carry out important functions including transmitting information on heart rate, blood pressure and visceral distension from the periphery to the central nervous system. Visceral sensory neurons are composed of the geniculate, petrosal and nodose ganglia, which are developed in a rostral to caudal sequence. The geniculate ganglion originates from the first epibranchial placode, associates with the VIIth (facial) cranial nerve, and primarily innervates taste buds. The petrosal ganglion originates from the second epibranchial placode, associates with the IXth (glossopharyngeal) cranial nerve, and innervates taste buds, the heart and other visceral organs. The nodose ganglion originates from the third epibranchial placode, associates with the Xth (vagal) cranial nerve, and primarily innervates the heart and other visceral organs (Baker and Bronner-Fraser, 2001). Once the connectivity of visceral sensory neurons is established, their survival and maintenance are dependent upon GDNF, a neurotrophic factor of the TGFβ superfamily (Buj-Bello et al., 1995; Moore et al., 1996; Trupp et al., 1995).

In contrast to the knowledge about their physiological properties and neurotrophic dependency, the molecular program underlying the specification of visceral sensory neurons is just beginning to be unraveled. The bHLH transcription factor Neurogenin 2 (Ngn2), a vertebrate homologue of the *Drosophila* proneural gene *atonal* (Jan and Jan, 1993), is expressed in the epibranchial placodes (Gradwohl et al., 1996; Sommer et al., 1996). Its expression coincides with the timing when neuronal precursors delaminate from these placodes, migrate dorsomedially, and aggregate to form the distal ganglia. *ngn2* expression is confined to the undifferentiated neuronal precursors and absent in the ganglionic anlagen. In contrast to Ngn2, the paired homeodomain transcription factor Phox2a is turned on in differentiating visceral sensory neurons (Tiveron et al., 1996;

Valarche et al., 1993). Targeted disruption of *ngn2* in mice results in a transient loss of neuronal fate in geniculate (VIIth) and pertrosal (IXth) ganglia at early stages, but the development of these ganglia appear to recover at later stages, possibly due to functional compensation by *ngn1* (Fode et al., 1998; Ma et al., 1998; Ma et al., 1999). Nevertheless, the lack of *ngn2* is shown to block the delamination of neuronal precursors from the placodes and abolish their pan-neuronal fate in early stage embryos, but, Phox2a activation is not affected in these embryos (Fode et al., 1998). Conversely, inactivation of *phox2a* in both mice and zebrafish leads to atrophy of visceral sensory neurons (Guo et al., 1999a; Morin et al., 1997), but they are able to undergo general neuronal differentiation, as is evident from their expression of pan-neuronal genes (Morin et al., 1997). These analyses suggest that two distinct subprograms controlled by *ngn* and *phox2a* may operate independently in determining visceral sensory neuronal identity. The genetic mechanisms that act upstream of *ngns* and *phox2a* in the visceral sensory lineage are currently unknown.

In this study, we have analyzed a zebrafish mutant named *no soul*, in which the development of most visceral sensory neurons fails to occur. The positional candidate approach reveals that *no soul* encodes a winged helix domain-containing protein, belonging to the *foxi1* subfamily. Foxi1 expression is detected in the placodal progenitor cells at the neural plate stage, prior to their subdivision into neurogenic placodes and the expression of *ngn*. Subsequently, its expression is maintained during the period of the birth of visceral sensory neurons. Finally, its expression is extinguished upon the differentiation of visceral sensory neurons. The expression of both *ngn* and *phox2a* are defective in the *no soul* mutant, indicating that in the absence of *foxi1* activity, placodal progenitor cells fail to express both neuronal fate and subtype identity of visceral sensory lineage. Shortly thereafter, increased cell death is detected, suggesting that in the absence of their normal developmental program, the placodal progenitor cells undertake an apoptotic pathway. Furthermore, ectopic expression of *foxi1* in zebrafish embryos is sufficient to induce ectopic *phox2a*$^+$ and *ngn*$^+$ cells. Taken together, our analyses indicate that the winged helix transcription factor No soul/Foxi1 is an important determination factor for epibranchial placodal progenitor cells to acquire both neuronal fate and subtype visceral sensory identity.

MATERIALS AND METHODS

Fish stocks and maintenance

Fish breeding and maintaining were performed as previously described (Guo et al., 1999b). Embryos were raised at 28.5°C and staged according to Kimmel et al. (Kimmel et al., 1995).

Mapping and cloning

AB/EK female fish carrying the *no soul* mutation were crossed to wild-type WIK male fish, and F_1 progeny were raised to adulthood. Genomic DNA was extracted from pools of wild-type sibling and mutant embryos, and PCR reactions were performed using microsatellite marker primers (Knapik et al., 1998). The genetic linkage of *no soul* to microsatellite markers was established by detection of differential amplification of wild-type and mutant DNA pools (Knapik et al., 1998), and microsatellite marker Z1400 yielded two recombinants and marker Z13938 yielded 23 recombinants out of 276 individual *no soul* mutant embryos tested.

For mutation detection in *no soul*, primers specific for the *foxi1* cDNA were used to amplify genomic DNA from pools (~10) of the *no soul* mutant and wild-type sibling embryos. PCR products from mutant and wild-type sibling embryos (two independent sets) were directly sequenced by automated cycle sequencers (ABI).

Time lapse confocal analysis

Embryos carrying an *islet*-GFP (green fluorescent protein) transgene were anesthetized with 0.01% ethyl-m-aminobenzoate methanesulphonate (Sigma), and mounted in 3% methyl cellulose. They were viewed and photographed at different stages using a Bio-Rad confocoal microscope. After viewing, they were immediately returned to the 28.5°C incubator. The genotype of photographed embryos was subsequently confirmed by genotyping with tightly linked polymorphic Z markers.

Whole-mount in situ hybridization and immunostaining

Digoxigenin- or fluorescein-labeled antisense RNA probes were prepared from linearized templates using RNA labeling reagents (Boehringer Mannheim). Hybridization and detection with anti-digoxigenin or anti-fluorescein antibodies was done as previously described (Guo et al., 1999b). For two-color in situ hybridization, two RNA probes (digoxigenin- or fluorescein-labeled) were hybridized simultaneously, and developed sequentially with purple and red substrates (Roche). After staining, embryos were cleared with glycerol, either whole-mounted or sectioned for viewing. Immunostaining was performed as previously described (Guo et al., 1999b). TUNEL staining was carried out using the ApopTag kit according to the manufacturer's instructions.

DNA and RNA injection

Capped sense RNA was synthesized by in vitro transcription from linearized pCS2 plasmids. DNA plasmids or in vitro transcribed RNA for either *foxi1* or *lacZ* were microinjected at the 1- to 8-cell stage. At appropriate stages, they were fixed with 4% paraformaldehyde and processed for staining. Morpholino antisense oligonucleotide specific for foxi1 was provided by A. Fritz, the sequence is: 5′-TAATCC-GCTCTCCCTCCAGAAACAT-3′. Gene Tools, LLC standard control oligo was used. Oligos were injected at a concentration of 2-5 μg/μl.

Protein structure modeling

A comparative model of the winged helix domain was constructed by MODELLER (Sali and Blundell, 1993), relying on its high (41-62%) sequence identities to the template structures of genesis (Marsden et al., 1997), FREAC-11 (van Dongen et al., 2000), and AFX (Weigelt et al., 2000) determined by NMR spectroscopy. The native and mutant 7-residue segments (57-63) were modeled de novo in the context of the rest of the comparative model (Fiser et al., 2000).

RESULTS

Defects of the epibranchial placode-derived visceral sensory neurons in the *no soul* mutant

In a molecular marker-based genetic screen for mutations that affect the development of dopaminergic and noradrenergic neurons, we have recovered a mutant named *no soul*, which selectively disrupts one group of noradrenergic cells, the arch-associated CA (AAC) cells (Guo et al., 1999b). Since AAC cells are thought to originate from epibranchial placodes, we examined if the epibranchial placode-derived visceral sensory neurons are affected by the mutation. We crossed the GFP, under the control of the *islet 1* promoter/enhancer, into the *no*

soul mutant background. The *islet 1*-GFP transgene allowed direct visualization of the epibranchial placode-derived sensory neurons, cranial motor neurons and their associated nerves, in living zebrafish (Higashijima et al., 2000). Time-lapse confocal analysis detected the emerging VIIth (geniculate) ganglia in the 26-hour postfertilization (hpf) wild-type sibling embryo (Fig. 1A,C). In the *no soul* mutant embryo, the VIIth ganglia were not detectable (Fig. 1B,D). By 48 hpf, while all three epibranchial-placode derived ganglia were observed in the wild-type embryo (Fig. 1E,G), all were missing in the *no soul* mutant embryo except the most distal division of the Xth ganglia, which appeared to be reduced (Fig. 1F,H). The defects persisted to later stages, suggesting that the *no soul* mutant suffers permanent deficits of epibranchial placode-derived visceral sensory neurons (Fig. 1I,J). In contrast to visceral sensory neurons, the cranial motor neurons appeared to develop normally in the *no soul* mutant (Fig. 1B,D,F,H,J). Taken together, these analyses indicate that the *no soul* mutation causes an eradication of the VIIth and IXth and a reduction of the Xth epibranchial placode-derived visceral sensory neurons.

In addition to Islet-GFP analysis, we also stained embryos with the monoclonal antibody mAb16 A11, which recognizes a specific epitope of the pan-neuronally expressed Hu proteins and labels cranial sensory ganglia in vertebrates (Marusich et al., 1994; Wakamatsu and Weston, 1997). We found that consistent with the analysis with Islet-GFP, nascent neurons of the VIIth (geniculate) and IXth (petrosal) ganglia are absent, and the Xth (nodose) ganglia are reduced in the *no soul* mutant (Fig. 1K,L). In addition to epibranchial placode-derived ganglia, the antibody also labels other cranial ganglia including the trigeminal and lateral line ganglia (Raible and Kruse, 2000). The trigeminal ganglia (V), which are composed of cutaneous sensory neurons that innervate the face and jaws and other parts of the nervous system, appeared normal in the *no soul* mutant (Fig. 1K,L). The lateral line ganglia, which are involved in sensing water movement, however, are differentially affected in the *no soul* mutant: those that are in close association with the VIIth and IXth cranial nerves appeared to be absent, those in association with the Xth cranial nerve appeared to be reduced, whereas those that are near the Vth (trigeminal) ganglia appeared to be preserved (Fig. 1K,L). In addition to cranial sensory neuronal deficits, the *no soul* mutant is defective in subsets of pharyngeal arches (Guo et al., 1999b) and show variable defects of the otic vesicles (data not shown). They do not develop swim bladders and die around 6 dpf. Taken together, these analyses indicate that the *no soul* gene is required for the development of subsets of cranial sensory neurons and craniofacial structures. For the rest of the manuscript, we focused our analysis on the epibranchial placode-derived visceral sensory neurons to elucidate the mechanism of action of the *no soul* gene.

no soul encodes a winged helix domain-containing protein of the Foxi1 subfamily

To identify the gene that is disrupted by the *no soul* mutation, we genetically mapped the mutation to linkage group (LG) 12, between polymorphic microsatellite markers Z1400 and Z13938. Primers for both markers amplified two distinct fragments using genomic DNA prepared from both parents and wild-type sibling embryos, while amplified only one of

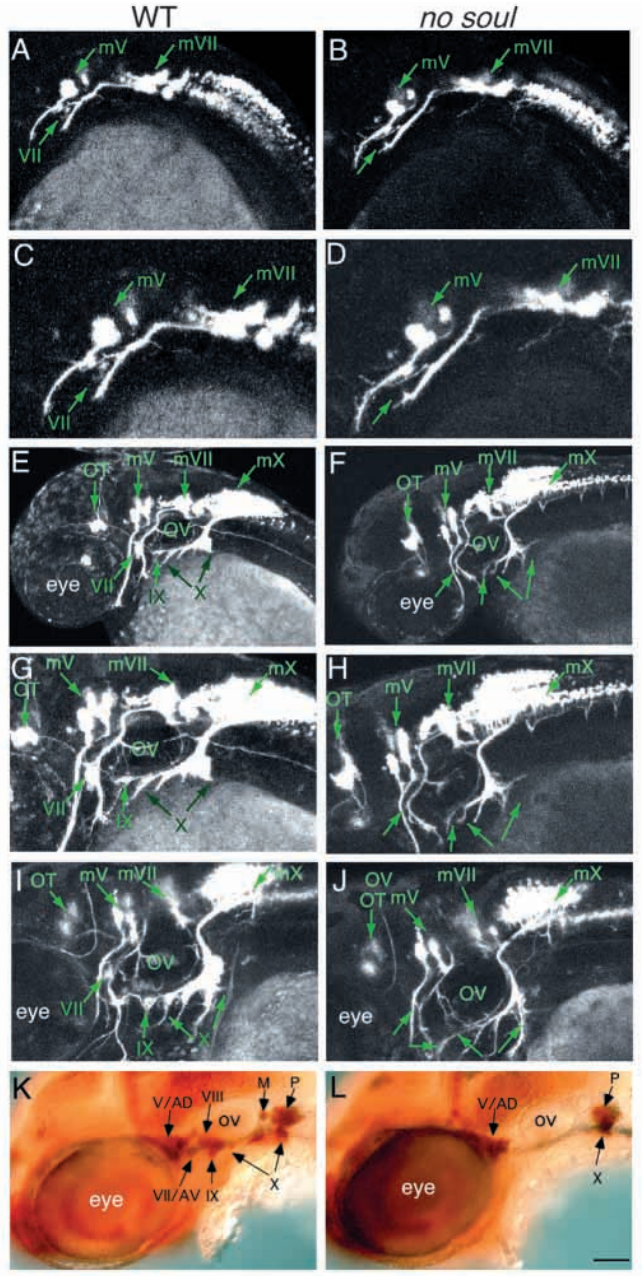

Fig. 1. The *no soul* embryos are defective in visceral sensory neurons. Anterior is to the left. Lateral views of wild-type (left) and *no soul* (right) embryos expressing *islet*-GFP (A-J) or labeled with the monoclonal antibody mAb16A11 (K-L). (A-D) 26 hpf embryos showing that the VIIth ganglia are not generated in the mutant, whereas cranial motor neurons appear normal. (E-H) 48 hpf embryos showing that the VIIth and IXth ganglia are absent while the Xth ganglia are reduced. (I,J) ~3-day old embryos showing persistent defects in visceral sensory neurons of the mutant. (K,L) Anti-Hu antibody labeling of 50 hpf embryos showing that the trigeminal ganglia appear normal, while both epibranchial and lateral line ganglia are defective in the *no soul* mutant. The identity of the *no soul* mutant embryos shown in all figures is confirmed by genotyping with closely linked polymorphic markers Z1400 and Z13938. AD, anterior dorsal ganglia; AV, anterior ventral ganglia; M, middle lateral line; mV/mVII/mX, the Vth, VIIth, or Xth motor neurons; ov, otic vesicle; OT, ocular and trochlear motor nuclei; P, posterior lateral line; V, trigeminal ganglion; VII, geniculate ganglion; IX, petrosal ganglion; X, nodose ganglion. Scale bar: 100 μm (A-B, E-F, K-L), 70 μm (C-D, G-J).

two fragments in DNA from *no soul* mutant embryos, indicating genetic linkage (data not shown). A winged helix domain-containing gene of the Foxi1 subfamily, which was identified in an expression cDNA screen (Kudoh et al., 2001), has been located at this interval by radiation hybrid mapping, and found to be essential for otic development (Solomon et al., 2003). Given the otic phenotypes we observed in the *no soul* mutant and the closeness in genetic distance between *no soul* and *foxi1*, we sought to determine if *foxi1* is disrupted in the *no soul* mutant. We cloned and sequenced *foxi1* from the *no soul* mutant embryos, as well as from their wild-type siblings. This analysis revealed that the *no soul* mutant carries a single substitution from serine to proline at amino acid 194 (Fig. 2A,C). This substitution was never found in wild-type siblings or in *foxi1* from other species (Fig. 2C). Furthermore, serine-194 is an absolutely conserved residue in helix H3, the recognition helix of the winged helix DNA binding domain (Gajiwala and Burley, 2000). Secondary structure prediction and comparative protein structure modeling (Sali and Blundell, 1993; Fiser et al., 2000) indicated that the change of the serine-194 to the helix-breaking proline residue shortens the critical recognition helix of the winged helix domain, which in turn is likely to destroy its ability to bind to DNA (Fig. 2B). It is probable that approximately one half of the crucial residues in the H3 recognition helix are not able to interact with DNA: the number of atomic contacts at less than 4.5 angstroms between the ab initio-modeled 7-residue segment and DNA in the *no soul* mutant is less than half of that in the wild type. In summary, both the experimental evidence and theoretical considerations provide strong evidence that the *no soul* mutation disrupts *foxi1*.

Scanning of sequence databases revealed that the *no soul/foxi1* sequences are most similar to the *Xenopus foxi1c* gene (47.3% overall identity), human *HFH3* (46.9% overall identity) and mouse *fkh10* (46.6% overall identity). The greatest similarity lies in the winged helix domain, whereas only limited similarity is detected outside this region (Fig. 2C). To verify that disruption

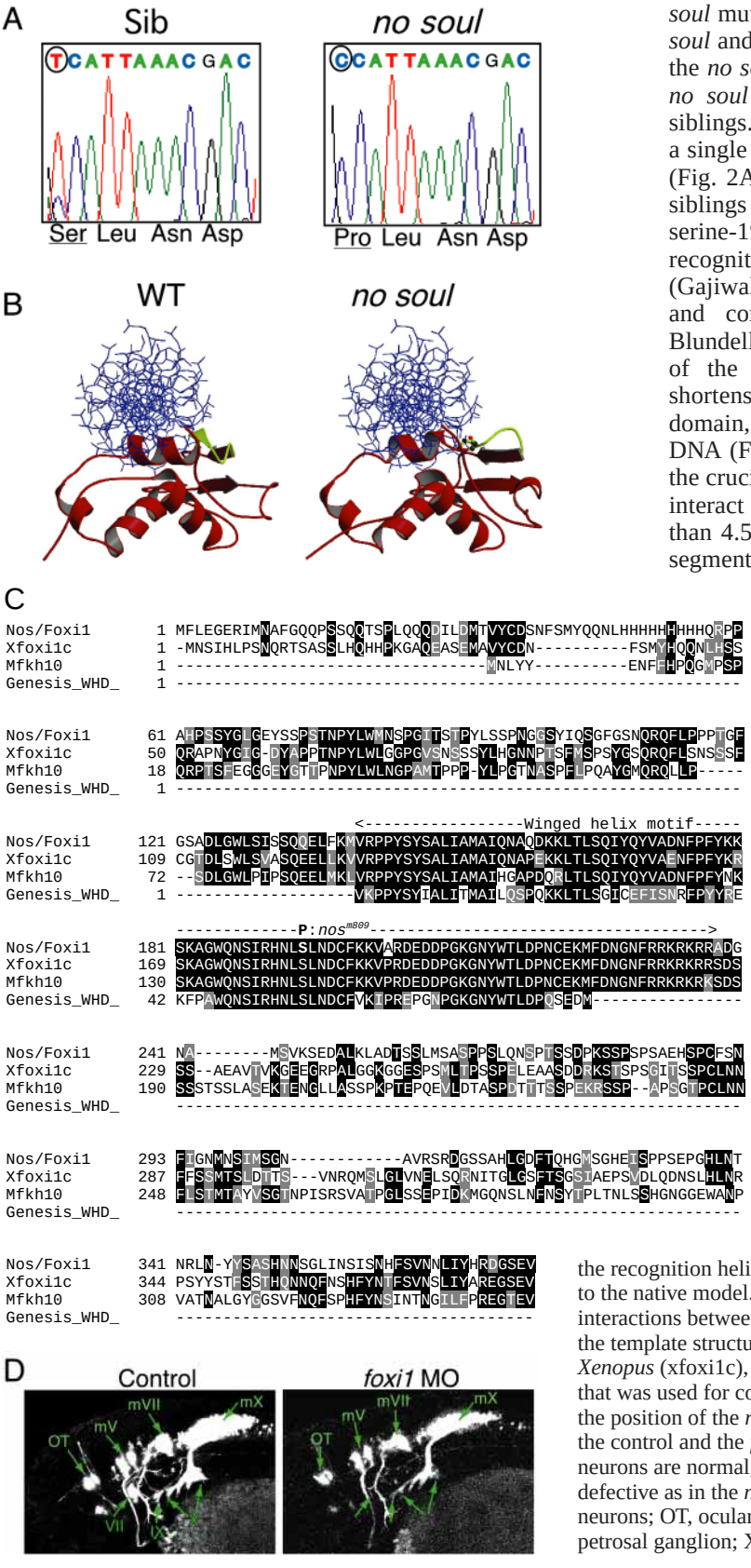

Fig. 2. The *no soul* gene encodes a winged helix domain-containing protein that belongs to the *foxi1* subfamily. (A) ABI automated sequencer-produced chromatographs showing that the Ser^{194} is mutated to Pro in the *no soul* mutant. (B) Comparative model showing that the predicted conformations of the native and mutated segment are different. The ab initio modeled segment is in green (the rest of the protein is shown in brown), the proline mutation is shown in the ball-and-stick style (red), and the bound DNA is blue. In the mutant model, the C terminus of the recognition helix is shortened by one turn and the loop connecting the recognition helix to the subsequent β strand is shifted significantly relative to the native model. These conformational changes decrease the extent of interactions between the protein and DNA, assuming DNA is positioned as in the template structures. (C) Sequence alignment between *no soul/foxi1*, *Xenopus* (xfoxi1c), mouse (Mfkh10) and the winged helix domain of *genesis* that was used for comparative structure modeling. The winged helix motif and the position of the *no soul* mutation are marked. (D) Confocal image showing the control and the *foxi1* morpholino-injected embryos. Although motor neurons are normal, epibranchial placode-derived visceral sensory neurons are defective as in the *no soul* mutant. mV/mVII/mX, the Vth, VIIth and Xth motor neurons; OT, ocular and trochlear motor nuclei; VII, geniculate ganglion; IX, petrosal ganglion; X, nodose ganglion.

of *foxi1* is the cause of visceral sensory neuronal defects observed in the *no soul* mutant, we injected wild-type embryos with a morpholino (MO) antisense oligonucleotide (Nasevicius and Ekker, 2000) specific to *foxi1*. This *foxi1*-MO oligo but not the control oligo produced a phenotype that is indistinguishable from that of *no soul*: *foxi1*-MO-injected embryos had variable otic defects and were defective in subsets of pharyngeal arches (data not shown). Furthermore, the visceral sensory neuronal phenotype was also indistinguishable from that of the *no soul* mutant (compare Fig. 2D with Fig. 1F) in all the injected embryos (n=120), thus confirming that *no soul* is indeed *foxi1*. This result also suggests that the *no soul* mutation is likely to be a strong to complete loss-of-function allele of *foxi1*.

Expression of *no soul/foxi1*

Embryological experiments have mapped the origin of cranial placodes to the border of neural plate and future epidermis (Baker and Bronner-Fraser, 2001). At the tailbud stage (~10 hpf), *foxi1* expression is found in bilaterally symmetric domains at the boundary of lateral neural plate/epidermis (Fig. 3A), suggesting that it is expressed in lateral cranial placodal progenitor cells. As the neural tube forms, the *foxi1*-expressing domains become elongated and by 24 hpf, the expression of *foxi1* is somewhat reduced but still detectable (Fig. 3D). By 36 hpf, its expression is still present lateral to the neural tube (Fig. 3G). Cross sectioning showed *foxi1* expression in the ectodermal layer. Weak expression was also detected in a few internal cells, which most likely represent delaminating neuronal precursors (Fig. 3G′). By 48 hpf, *foxi1* expression domain has moved ventrolaterally, and appears to be in the pharyngeal arch primordia (Fig. 3M). Cross sectioning detected *foxi1* expression again in the ectodermal layer (Fig. 3M′). Thus, the expression pattern of *foxi1* is consistent with its role in cranial sensory neuron and pharyngeal arch development.

As mentioned earlier, visceral sensory neuronal lineage expresses two transcription regulators: *ngn* and *phox2a*. We compared their expression to that of *foxi1* to determine their spatial and temporal relationships. A single zebrafish *ngn* gene, the *ngn 1* is expressed in cranial sensory ganglia (Andermann et al., 2002; Blader et al., 1997; Cornell and Eisen,

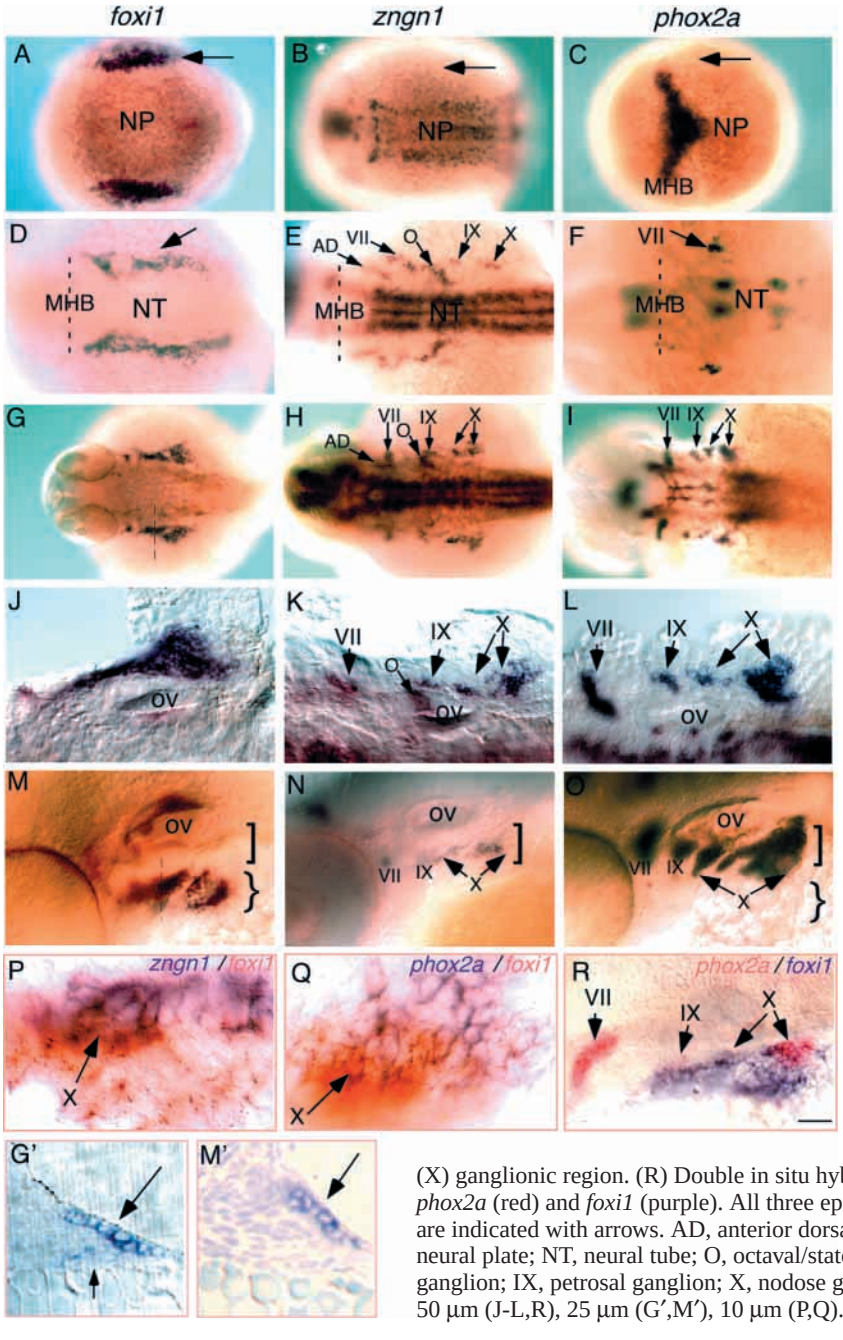

Fig. 3. Spatial and temporal expression of *no soul/foxi1*, *ngn* and *phox2a*. In situ hybridization with antisense *no soul/foxi*, *ngn*, *or phox2a* RNA probe. Anterior is to the left, except in G and 3M inset, where dorsal is at the top. (A-C) Dorsal view of tailbud stage embryo showing *no soul/foxi1* expression in the lateral cranial placodal domain (epibranchial and otic placodes; arrows), while *ngn* and *phox2a* are not yet detectable in this domain. (D-F) Dorsal views of 24 hpf embryos showing persistent expression of *foxi1* (arrow) in the lateral cranial placodal region, while *ngn* and *phox2a* expression are detected in the vicinity of *foxi1*. (G-L) Dorsal views of 36 hpf embryos showing that all three genes are expressed in close proximity to one another. (G′; below) A cross section through the *foxi1*-expressing domain: the large arrow points to *foxi1*-expressing cells in the ectodermal layer, while the small arrow points to cells weakly expressing *foxi1* that are likely delaminating neural precursors. (M-O) Lateral views of 48 hpf embryos. (M′; below) A cross section through the *foxi1*-expressing domain: the arrow points to the *foxi1*-expressing cells. (P,Q) Double in situ hybridization showing the overlapping expression of *foxi1* (red) and *ngn* (P) or *phox2a* (Q) (purple). The arrow indicates the nodose (X) ganglionic region. (R) Double in situ hybridization showing the overlapping expression of *phox2a* (red) and *foxi1* (purple). All three epibranchial placode-derived sensory ganglionic regions are indicated with arrows. AD, anterior dorsal lateral line; MHB, mid-hindbrain boundary; NP, neural plate; NT, neural tube; O, octaval/statoacoustic ganglion; ov, otic vesicle; VII, geniculate ganglion; IX, petrosal ganglion; X, nodose ganglion. Scale bar: 150 μm (A-F, G-I), 100 μm (M-O), 50 μm (J-L,R), 25 μm (G′,M′), 10 μm (P,Q).

2002; Korzh et al., 1998). Zebrafish *phox2a/soulless* was originally discovered in the same genetic screen that identified the *no soul* mutation, and is expressed in the epibranchial placode-derived visceral sensory ganglia (Guo et al., 1999a). In tailbud stage embryos, where *foxi1* expression is detected in the lateral cranial placode progenitor cells, neither *neurogenin* nor *phox2a* is detected in this region (Fig. 3B,C), indicating that *foxi1* expression precedes that of both *ngn* and *phox2a*. At 24 hpf, expression of both *ngn* and *phox2a* was initiated in the visceral sensory neuronal lineage (Fig. 3E,F) and such expression was more pronounced by 36 hpf (Fig. 3H,I). At this stage, *foxi1*, *ngn*, and *phox2a* are expressed in the vicinity of one another (Fig. 3J-L). The expression of *ngn* is also detected in other cranial sensory ganglia (Fig. 3E,H). By 48 hpf, *ngn* is weakly detected in a few cells and *phox2a* is expressed in many differentiating visceral sensory neurons (Fig. 3N,O). Double in situ hybridization with *foxi1* and *ngn* (Fig. 3P) or *foxi1* and *phox2a* (Fig. 3Q,R) confirmed that the *foxi1*-expressing domain overlaps with those of *ngn* and *phox2a*.

Epibranchial placodal progenitor cells fail to initiate *ngn* and *phox2a* expression and subsequently disappear in the *no soul* mutant

To further look into the relationship between *foxi1*, *ngn* and *phox2a*, we examined their expression in the *no soul* mutant. Since *foxi1* expression precedes that of both *ngn* and *phox2a* *foxi1* may be required to initiate their expression in the visceral sensory neuronal lineage. Alternatively, *foxi1* may be required to maintain the integrity of progenitor cells, that is, in the absence of *foxi1* activity, the progenitor cells disappear or are transformed to other cell types, thus, precluding their expression of *ngn* and *phox2a* and subsequent acquisition of visceral sensory neuronal identity. To look into these possibilities, we compared the state of progenitor cells and their expression of *ngn* and *phox2a*. Since the mutated *no soul/foxi1* gene still gave rise to stable transcript, we used the *foxi1* RNA to mark the presence of progenitor cells. At 24 hpf, the *foxi1*-expressing progenitor cells are present in the *no soul* mutant (Fig. 4B). In fact, they appeared to express *foxi1* at a higher level compared to their wild-type siblings, suggesting that *foxi1* negatively regulates its own expression (Fig. 4A-D). While the progenitor cells are still present in the *no soul* mutant at this stage, we detected no *ngn* or *phox2a* expression in the epibranchial placodes (Fig. 4E-H). Double in situ hybridization with *foxi1* and *ngn* or *foxi1* and *phox2a* further confirmed that while *foxi1*-expressing progenitor cells are still present, they fail to initiate the expression of *ngn* and *phox2a* (Fig. 4I-L).

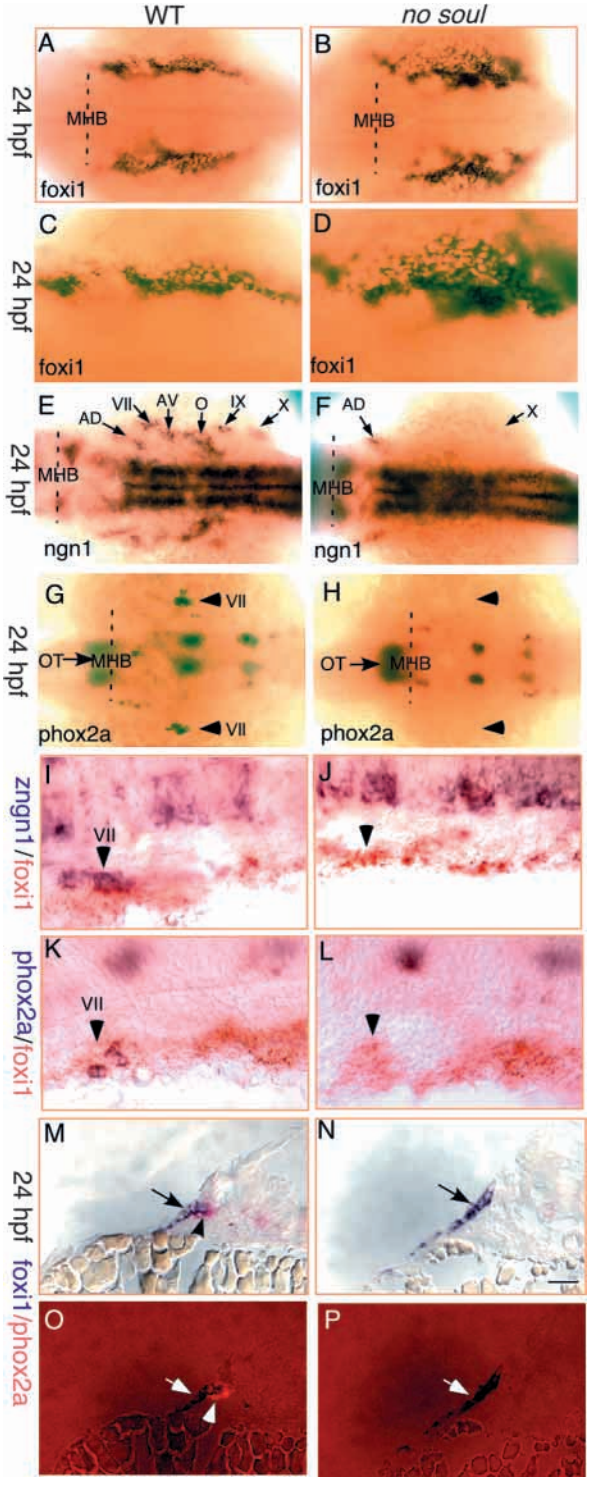

Fig. 4. Foxi1-expressing progenitor cells are present at 24 hpf but fail to initiate *ngn* and *phox2a* expression in the *no soul* mutant. Anterior is to the left in A-L, and dorsal is to the top in M-N. (A-D) 24 hpf embryos showing that the *foxi1*-expressing domain is still intact in the *no soul* mutant and is apparently upregulated. (E-H) 24 hpf embryos showing that *ngn* and *phox2a* expression in the epibranchial placodes fail to be initiated in the *no soul* mutant. (I-L) Double in situ showing that while *foxi1*-expressing cells (red) are still present, they do not express *ngn* or *phox2a* (purple) in the *no soul* mutant. (M-P) Cross sectioning through the *foxi1* (purple)- and *phox2a* (red)-expressing domain in 24 hpf embryos, showing that *foxi1*-expressing cells do not appear to delaminate and no *phox2a*$^{+}$ sensory neurons are generated in the *no soul* mutant. (M,N) Nomarski views, (O,P) rhodamine fluorescence. The arrows point to the *foxi1*-expressing cells in the ectodermal layer, while the arrowhead points to cells that weakly express *foxi1*, are likely to be delaminating neural precursors, and overlapping *phox2a*+ epibranchial sensory neurons. AD, anterior dorsal lateral line; MHB, mid-hindbrain boundary; NP, neural plate; NT, neural tube; O, octaval/statoacoustic ganglion; ov, otic vesicle; OT, ocular and trochlear motor nuclei; VII, geniculate ganglion; IX, petrosal ganglion; X, nodose ganglion. Scale bar: 100 μm (A-B, E-H), 50 μm (C-D, I-L), 40 μm (M-P) 25 μm.

Consistent with the lack of *ngn* and *phox2a* expression, sectioning analysis of *foxi1* and *phox2a* double-labeled embryos revealed that *foxi1*-expressing epibranchial placode progenitor cells fail to delaminate and subsequently fail to express *phox2a* (Fig. 4M-P). Taken together, this analysis suggests that in the absence of *foxi1* activity, visceral sensory progenitor cells fail to initiate the expression of *ngn* and *phox2a*. In addition, the *ngn* expression in lateral line placodes was also absent in the *no soul* mutant (Fig. 4F), suggesting that *foxi1* is also required to regulate the development of these placodes.

While *foxi1*-expressing progenitor cells are present in the 24 hpf *no soul* mutant embryos, by 36 hpf, it is clear that the anteriorly located *foxi1*-expressing progenitor cells were no longer present in the mutant (Fig. 5A,B). At this and later stages, the *ngn*- and *phox2a*-expressing ganglia were absent in the VIIth and IXth ganglionic regions, and much reduced in the Xth (Fig. 5C-F,I,J). The expression of *phox2b*, a downstream target gene of *phox2a* in some cell types and a regulator of *phox2a* in other cell types (Pattyn et al., 1997) is abolished in the VIIth and IXth ganglia. However, its expression in the Xth ganglion is largely unaffected by the mutation (Fig. 5G,H), suggesting that *phox2b* may be independent of *foxi1* and *phox2a* in at least subsets of the nodose ganglion. Taken together, these analyses favor the hypothesis that *foxi1* is required to initiate *ngn* and *phox2a* expression in the epibranchial placodes, and the failure to do so leads to the disappearance of progenitor cells.

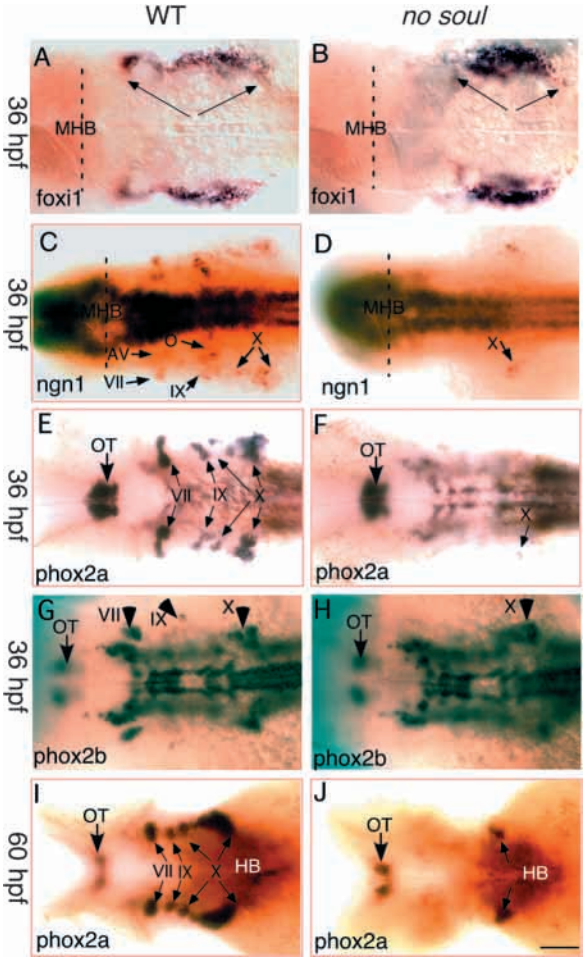

Fig. 5. *no soul/foxi1*-expressing cells later disappear in the *no soul* mutant. Anterior is to the left. (A-B) 36 hpf embryos labeled with *foxi1* showing that the anteriorly located *foxi1*-expressing cells are absent in the *no soul* mutant. (C-F) 36 hpf embryos labeled with *ngn* (C,D) and *phox2a* (E-F) showing their expression in the visceral sensory neurons are largely absent. (G,H) 36 hpf embryos labeled with *phox2b* showing that its expression in the VIIth and IXth ganglia is absent, while its expression in the Xth ganglia appear normal in the mutant. (I,J) 60 hpf embryos labeled with *phox2a* showing that the deficits continue in the *no soul* mutant. AV, anterior ventral lateral line; MHB, mid-hindbrain boundary; O, octaval/statoacoustic ganglion; OT, oculomotor and troclear motor nuclei; VII, geniculate ganglion; IX, petrosal ganglion; X, nodose ganglion. Scale bar: 100 μm.

Increased cell death in the lateral cranial placodal region of the *no soul* mutant

To look into the disappearance of *foxi1*-expressing progenitor cells in the *no soul* mutant, we carried out TUNEL labeling to determine if these cells undergo apoptosis. As described above, the *foxi1*-expressing progenitor cells are still present in the *no soul* mutant at 24 hpf (Fig. 4B). At this stage, the pattern of cell death appeared comparable in the mutants and their wild-type siblings (Fig. 6A-D). However, 4 hours later, at 28 hpf, we observed an increased number of TUNEL$^+$ cells in the vicinity of the VIIth and IXth ganglionic regions in the *no soul* mutant (Fig. 6E-H). Some TUNEL$^+$ cells posterior to the otic vesicle and in the vicinity of the Xth ganglia were also detected. By 36 hpf, when the *foxi1*-expressing progenitor cells are absent in the region of the VIIth and IXth ganglionic regions, TUNEL$^+$ cells were no longer detected in these regions. This is consistent with the observation that dead cell are cleared quite rapidly in zebrafish (Cole and Ross, 2001). Instead, we saw increased TUNEL$^+$ cells in the Xth ganglionic region (Fig. 6I-L). This analysis suggests that upon the failure to initiate their normal developmental program, the progenitor cells undergo apoptosis.

No soul/Foxi1 is sufficient to induce *phox2a*$^+$ and *ngn*$^+$ cells at ectopic locations

Given that *no soul/foxi1* is required to initiate the normal development of visceral sensory neurons, we next asked if it is sufficient to do so. We injected wild-type *foxi1* RNA or DNA into one- to eight-cell stage zebrafish embryos and analyzed the expression of *ngn* and *phox2a* by in situ hybridization. At low concentrations (~10 ng/μl for injected DNA constructs or ~50 ng/μl for injected RNA), the injected embryos appeared morphologically normal, and we did not observe ectopic expression of either *ngn* or *phox2a* (data not shown; n=100). However, when we increased the concentration of *foxi1* (~30 ng/μl for injected DNA constructs or 200 ng/μl for RNA), ectopic *phox2a*$^+$ (~50%, n=50) and *ngn*$^+$ (~40%, n=50) cells were detected (Fig. 7C,D). In addition, the injected embryos showed morphological deformity. To determine whether the ectopic *phox2a* and *ngn* expression is due to *foxi1* or is secondary to morphological deformity, we carried out double in situ hybridization with *foxi1* and *phox2a* or *foxi1* and *ngn*. Indeed, ectopic *phox2a*$^+$ and *ngn*$^+$ cells were located in the region that ectopically expressed *foxi1* (Fig. 7E,F). These data suggest that *no*

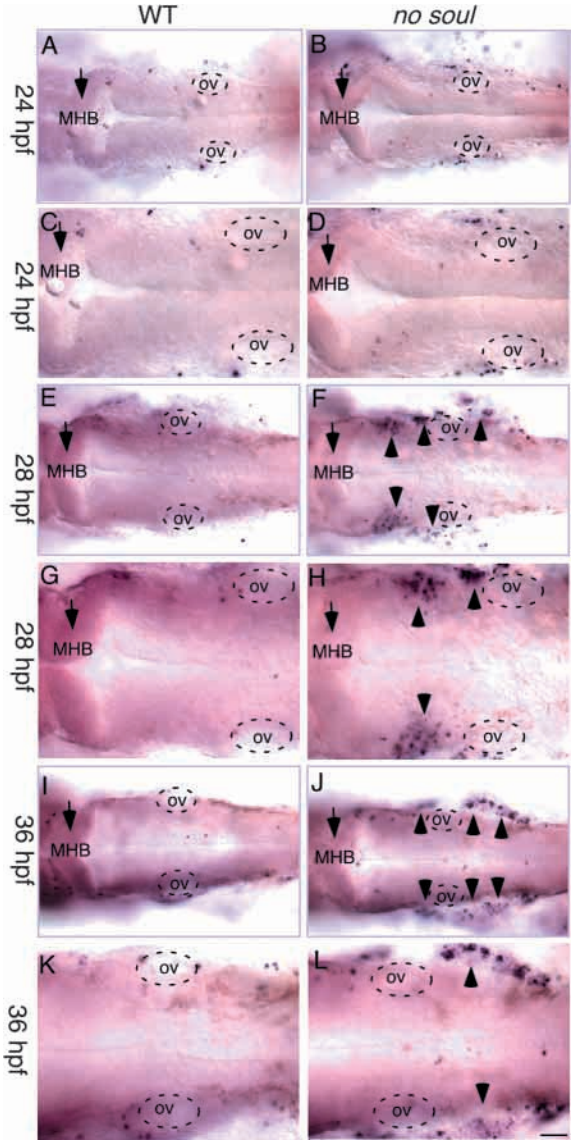

Fig. 6. TUNEL staining of whole-mount wild-type (left) and *no soul* mutant (right) embryos. C,D,G,H,K,L are higher magnification views of A,B,E,F,I,J, respectively. (A-D) 24 hpf embryos showing comparable TUNEL staining in wild-type and *no soul* mutant embryos. (E-H) 28 hpf embryos showing increased TUNEL staining in the anterior lateral cranial placodal region. (I-L) 36 hpf embryos showing increased TUNEL staining in the posterior cranial placodal region. MHB, mid-hindbrain boundary; ov, otic vesicle. Scale bar: 100 μm (A,B,E,F,I,J), 50 μm (C,D,G,H,K,L).

soul/foxi1 is sufficient to induce *phox2a*$^+$ and *ngn*$^+$ cells at ectopic locations. It is worth noting that the ectopic *phox2a*$^+$ and *ngn*$^+$ cells were largely confined to the yolk surface (non-neural ectoderm), and only a small number of cells became *phox2a*$^+$ or *ngn*$^+$ within the ectopic *foxi1*-expressing domains (Fig. 7C-F). These observations are consistent with the fact that not all endogenous *foxi1*-expressing cells became *ngn*$^+$ and *phox2a*$^+$, and further suggest that *foxi1* needs to cooperate with additional signals to determine visceral sensory neurons.

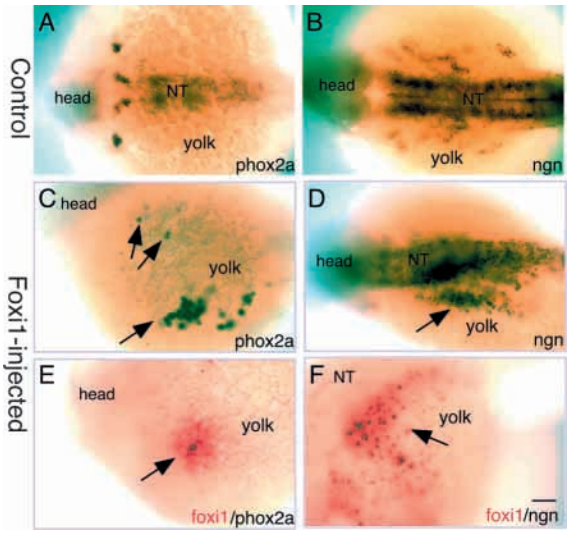

Fig. 7. Ectopic expression of *foxi1*. Anterior is to the left. (A,B) Dorsal views of control embryos labeled with *phox2a* and *ngn* antisense RNA probes, respectively. (C-F) 24 hpf *foxi1*-injected embryos, labeled with (C) *phox2a*, showing ectopic *phox2a*$^+$ cells on the yolk surface (arrows); (D) *ngn*, showing ectopic *ngn*$^+$ cells on the yolk surface (arrow); (E) *foxi1* (red) and *phox2a* (purple), showing that *phox2a*$^+$ cells do express ectopic *foxi1*; (F) *foxi1* (red) and *ngn* (purple), showing that *ngn*$^+$ cells do express ectopic *foxi1*. Scale bar, 100 μm.

DISCUSSION

The development of visceral sensory neurons represents a simple model system to examine the molecular mechanisms that control progenitor cell commitment and differentiation. Here we report that through a systematic genetic screen, we have identified a mutation named *no soul*, in which the development of visceral sensory neurons is severely disrupted. We further show that *no soul* encodes Foxi1, a winged helix domain-containing transcription regulator that is expressed in the placodal progenitor cells. Our analyses suggest that Foxi1 activity is required for the coordinated expression of both neuronal fate and subtype identity in the visceral sensory lineage: in the absence of *foxi1* activity, the placodal progenitor cells fail to express both *ngn* and *phox2a*, and subsequently undergo apoptosis. Furthermore, ectopic *foxi1* expression induces ectopic *phox2a*$^+$ and *ngn*$^+$ cells, suggesting that at least in some cellular contexts, *foxi1* is sufficient to promote visceral sensory neuronal development (Fig. 7). Taken together, our work identifies an important upstream regulatory molecule that controls both pan-neuronal character and subtype identity in visceral sensory neurons (Fig. 8) and supports the emerging view that multi-faceted transcriptional cascades are crucial for the generation of diverse neuronal types from initially multipotent neural progenitor cells (Anderson and Jan, 1997; Edlund and Jessell, 1999).

Transcriptional control and the role of *foxi1* in determining epibranchial placode-derived visceral sensory neurons

Differential regulation of gene expression is essential to the control of development. In the case of epibranchial placodes,

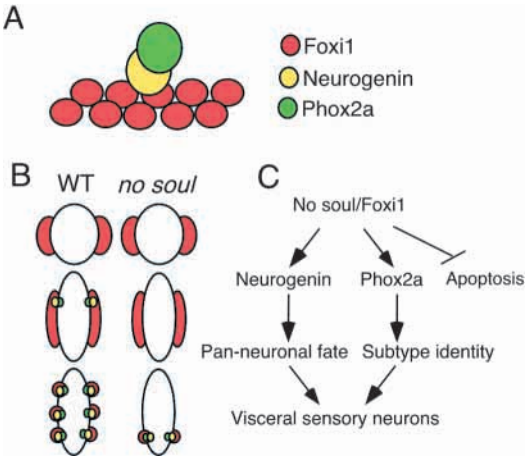

Fig. 8. Schemata depicting the role of *foxi1* in visceral sensory neuron development. (A) Among *foxi1*-expressing cells (red), neuronal precursors delaminate and express *ngn* (yellow) and *phox2a* (green) to adopt epibranchial sensory identity. Foxi1 expression in the placodal progenitor cells coordinate the expression of *ngn* and *phox2a*, which regulate neuronal fate and subtype specification, respectively. (B) Dorsal views of the *foxi1*-expressing domain (red), *ngn*-expressing domain (yellow), and *phox2a*-expressing domain (green). the *foxi1*-expressing domain in the tail bud stage embryos (top) defines the epibranchial (and otic) placodal progenitor cells, from which ngn^+ and $phox2a^+$ neuronal precursors are specified in a rostral to caudal sequence (middle, 24 hpf; bottom, 32 hpf). In the *no soul* mutant, epibranchial placodal progenitors cells are born but their neuronal specification fails to occur, resulting in the subsequent loss of placodal progenitor cells by apoptosis and absence of visceral sensory neurons at later stages. The nodose ganglion may be formed by *foxi1*-independent mechanisms or by compensation from other cell lineages. (C) Hierarchical regulation in visceral sensory neuronal determination.

a few transcription factors have been identified, including the bHLH factor *ngn*, the paired homeodomain factor *phox2a*, and the homeodomain protein *tlx* (Andermann and Weinberg, 2001; Qian et al., 2001) that appears to act later in the differentiation of these neurons. Studies of *ngn* and *phox2a* reveal that *ngn* is required to determine generic neuronal identity (e.g. the ability to outgrow axons and dendrites) but does not affect the subtype identity (e.g. neurotransmitter phenotype and connectivity pattern) in the epibranchial placode-derived sensory neurons, whereas *phox2a* is essential for the subtype differentiation of epibranchial placode-derived visceral sensory neurons but not their pan-neuronal identity. These studies suggest that generic neuronal fate and neuronal subtype identity may be controlled by different subprograms in vertebrates (Anderson, 1999; Bertrand et al., 2002). They also raise the question as to what the upstream regulatory factors are and whether they would be required for the coordinated expression of these subprograms.

Whereas factors like *olig2* (Mizuguchi et al., 2001; Novitch et al., 2001; Zhou et al., 2001), *Mash1* and *phox2b* (Pattyn et al., 1999) have been identified that coordinate pan-neuronal fate and subtype identity of motor neurons and autonomic ganglia respectively, molecules that carry out such function in the cranial sensory neuronal lineage have not been identified. Our study suggests that *foxi1* is one such factor. In the absence of *foxi1* activity, while *foxi1*-expressing progenitor cells are still present in the *no soul* mutant, they express neither *ngn* nor *phox2a*. Thus, *foxi1* is required to coordinate *ngn*-mediated pan neuronal fate and *phox2a*-mediated subtype identity in placodal progenitor cells. Since *foxi1* encodes a putative transcription regulator, a simple mechanism would be that *foxi1* could directly activate the transcription of *ngn* and *phox2a*. The fact that the *foxi1*-expressing domain is much broader than that of *ngn* and *phox2a* suggests that *foxi1* needs to cooperate with yet unidentified factors to turn on the expression of *ngn* and *phox2a*. Bmp7 from the pharyngeal endoderm has been shown to be an inducing factor for epibranchial placodes (Begbie et al., 1999). It will be important in the future to determine if *foxi1* is regulated by Bmp7 in zebrafish.

It is worth noting that in the absence of *foxi1* activity, placodal progenitor cells apparently undergo apoptosis. This observation suggests that *foxi1* is involved in suppressing the apoptotic pathway in these progenitor cells. Since not all *foxi1*-expressing cells undergo apoptosis in the *no soul* mutant, it appears unlikely that *foxi1* directly represses pro-apoptotic genes. Rather, apoptosis may be an alternative pathway when the normal developmental program is not initiated. Whereas it is not well understood how progenitor cells choose to differentiate or undergo apoptosis in vertebrates, it has been reported that progenitor cells can either undergo autonomic neurogenesis or apoptosis in culture in response to different concentrations of TGFβ (Hagedorn et al., 2000), suggesting that progenitor cells can adopt distinct fates in response to different extrinsic signals.

Foxi1 as a forkhead related winged helix domain-containing transcription factor

Winged helix domain (also known as the forkhead domain) was originally found in the *Drosophila forkhead* gene and the rat *hepatocyte nuclear factor 3* (*HNF-3*), which function in determining terminal structures in the *Drosophila* embryo (Weigel et al., 1989), and in controlling gene expression in the liver in mice (Lai et al., 1990), respectively. Subsequently more than 100 winged helix domain-containing genes have been identified in species ranging from yeast to human, which serve such diverse functions as early patterning, differentiation and survival (refer to http://www.biology.pomona.edu/fox.html). X-ray and solution NMR structures of a number of winged helix proteins have been determined. These studies identify the H3 recognition helix as the most important DNA-binding portion of a winged helix (Gajiwala and Burley, 2000). Mutations in this region completely abolish DNA binding (Clevidence et al., 1993). Here we show that the *no soul* mutation lies in the H3 helix, changing a conserved serine to proline. Our comparative modeling analysis strongly suggests that the *no soul* mutation would disrupt DNA binding and thus the regulation of downstream target genes. Furthermore, the morpholino experiment phenocopied the *no soul* mutant phenotype. Taken together, we believe that the *no soul* mutation probably represents a severe to complete loss of function allele of *foxi1*.

Based on the similarity within the winged helix domain, the winged helix domain-containing proteins are further classified into more than 15 subfamilies. Relatively little is known about the expression pattern and function of the *foxi1* subfamily. Our

study with *no soul* demonstrates that *foxi1* is required for the development of zebrafish epibranchial placode-derived visceral sensory neurons. The expression of *Xenopus foxi1c* has been recently reported (Pohl et al., 2002). Similar to our findings, the *Xenopus* Foxi1c is initially expressed in an epidermal ring around the neural field, and subsequently, is exclusively localized to placodal precursor cells. The targeted disruption of a probable homologue, *Fkh10* (*Foxi1*), in mice leads to defects in inner ear development (Hulander et al., 1998). Otic defects were also observed in the *no soul* mutant (our unpublished observation). Furthermore, otic defects were well characterized in the *hearsay* mutation, which is another allele of *foxi1* (Solomon et al., 2003). It will be interesting to determine whether *Fkh10* or a related forkhead gene is required for the development of visceral sensory neurons in mice.

Differential effects of *no soul* on subgroups of visceral sensory neurons

Visceral sensory neurons include the geniculate, petrosal and nodose ganglia, which have distinct but also overlapping connectivity patterns. Fate mapping experiments show that they all derive from the epibranchial placodes (Baker and Bronner-Fraser, 2001). However, in the *no soul* mutant, we saw a differential effect on these neurons: whereas the geniculate and petrosal neurons fail to develop, the nodose ganglia are partially spared. Interestingly, nodose ganglia were also less affected in mice with targeted disruption of *ngn* 2 (Fode et al., 1998) and *phox2a* (Morin et al., 1997). These analyses suggest that although the three distal ganglia share a developmental origin, different mechanisms may operate in their determination. Interestingly, we observed that unlike geniculate and petrosal ganglia, which express *phox2a* prior to *phox2b*, nodose ganglion initiates *phox2b* expression prior to that of *phox2a*. Therefore, it is possible that the commitment and differentiation of at least subsets of nodose ganglion is under the control of yet unidentified regulatory hierarchies. Alternatively, other neural progenitor populations are able to compensate for the loss of epibranchial placode-derived nodose progenitor cells. For example, compensation from cardiac neural crest cells has been previously reported in animals whose nodose precursors have been ablated (Harrison et al., 1995).

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